Learning Materials in Biosciences

Aydin Berenjian *Editor*

Essentials in Fermentation Technology

Springer

Learning Materials in Biosciences

Learning Materials in Biosciences textbooks compactly and concisely discuss a specific biological, biomedical, biochemical, bioengineering or cell biologic topic. The textbooks in this series are based on lectures for upper-level undergraduates, master's and graduate students, presented and written by authoritative figures in the field at leading universities around the globe.

The titles are organized to guide the reader to a deeper understanding of the concepts covered.

Each textbook provides readers with fundamental insights into the subject and prepares them to independently pursue further thinking and research on the topic. Colored figures, step-by-step protocols and take-home messages offer an accessible approach to learning and understanding.

In addition to being designed to benefit students, Learning Materials textbooks represent a valuable tool for lecturers and teachers, helping them to prepare their own respective coursework.

More information about this series at http://www.springer.com/series/15430

Aydin Berenjian
Editor

Essentials in Fermentation Technology

Springer

Editor
Aydin Berenjian
School of Engineering
The University of Waikato
Hamilton, Waikato, New Zealand

ISSN 2509-6125 ISSN 2509-6133 (electronic)
Learning Materials in Biosciences
ISBN 978-3-030-16229-0 ISBN 978-3-030-16230-6 (eBook)
https://doi.org/10.1007/978-3-030-16230-6

Preface

Traditionally, fermentation has been defined as the use of microorganisms, typically grown at a large scale, to carry out important chemical transformations in the absence of oxygen to produce alcohol from sugar. However, in the present-day context, fermentation can be defined as the breakdown of organic compounds by microorganisms in either the presence (aerobic) or absence of oxygen (anaerobic). Such processes result in the production of valuable commercial products or important chemical transformations. A fermentation process can be used for the production of microbial biomass as well as a range of microbial primary and secondary metabolites. A typical fermentation process consists of two major components known as "upstream" and "downstream." The upstream stage includes, but is not limited to, selection of the organism used in production and development, formulation and selection of the culturing media, sterilization of the media, the fermenter type, all required equipment, and finally growing the organism under appropriate (optimum) conditions in the fermenter, while the downstream stage includes the microbial cell separation from the culture broth, locating and extracting the target product, and its purification.

The subsequent chapters in this book consider the basic principles in developing a typical fermentation process. Chapter 1, by Behera et al., considers the role and selection of a microorganism for a fermentation process. The role of fermentation media and the varying ingredients will be covered by Allikian et al. in Chapter 2. The sterilization of the media, input and output air, and the fermenting vessel are discussed by Vaghari et al. in Chapter 3. A fermentation process can be conducted in a solid-state or liquid-state system. In Chapter 4, Mitchell et al. consider the design aspect and application of a variety of solid-state bioreactors, while in Chapter 5, Yatmaz et al. discuss the liquid-state fermentation, bioreactor systems, and their applications. Chapter 6 will be covered by Rosa et al. and considers the bioreactors' operating conditions and their key role in the success of a fermentation process. In Chapter 7, bioreactor scale-up strategies for industrial-scale application are provided by Mahdinia et al. The downstream steps are covered in Chapters 8. In this chapter, Lee et al. discuss the cell separation and disruption protocols along with the recovery of fermentation products and methods to purify them. Process economics is a key element of the fermentation process and is used to determine the profitability of a process. In Chapter 9, Petrides et al. cover the principles and considerations used to understand the bioprocess economics. Finally, in an attempt to combine the information presented in previous chapters and utilize it in the context of a real-world case study, Chapter 10, by Shoaf et al., will go through a case study for the production of green fluorescent protein.

Essentials in Fermentation Technology aims to teach students who are interested in fermentation technology the fundamentals of a fermentation process. All the chapters in this book are written by world-renowned scientists who are regarded as leaders in their area of expertise. Throughout the book, many real examples are used to enhance the learning of the reader and to illustrate the real-world applica-

tions. I truly hope the approach and methods adopted throughout this book will give the reader an understanding of the basic principles that underlie a majority of the fermentation industry.

Aydin Berenjian
Hamilton, New Zealand

Contents

Contributors

Kathrine Allikian
Integrated Bioactive Technologies (IBT),
Callaghan Innovation
Lower Hutt, New Zealand
kat@letterboxes.org

Navideh Anarjan
Department of Chemical Engineering,
Faculty of Engineering, Tabriz Branch
Islamic Azad University
Tabriz, Iran
anarjan@iaut.ac.ir

Sudhanshu S. Behera
Department of Fisheries and Animal Resource
Development, Government of Odisha,
Bhubaneswar, Odisha, India

Aydin Berenjian
School of Engineering,
The University of Waikato
Hamilton, Waikato, New Zealand
aydin.berenjian@waikato.ac.nz

Doug Carmichael
Intelligen Inc., Scotch Plains
NJ, USA
dcarmichael@intelligen.com

Deniz Cekmecelioglu
Department of Food Engineering
Middle East Technical University
Ankara, Turkey
denizc@metu.edu.tr

Kit Wayne Chew
Department of Chemical and Environmental
Engineering, Faculty of Engineering
University of Nottingham Malaysia Campus
Semenyih, Selangor, Malaysia
Kitwayne.chew@nottingham.edu.my

Urmimala Das
Centre for Food Biology and
Environment Studies
Bhubaneswar, Odisha, India

Saulo Varela Della Giustina
Water Quality
Catalan Institute of Water Research (ICRA)
Girona, Spain
svarela@icra.cat

Ali Demirci
Department of Agricultural and Biological
Engineering
Pennsylvania State University
University Park, PA, USA
demirci@psu.edu

Rebecca Edgar
Integrated Bioactive Technologies (IBT),
Callaghan Innovation
Lower Hutt, New Zealand
rebecca.Edgar@callaghaninnovation.govt.nz

Hoda Jafarizadeh-Malmiri
Faculty of Chemical Engineering, Sahand
University of Technology
Tabriz, Iran
h_jafarizadeh@sut.ac.ir

Daniela Maria Koerich
Chemical Engineering Department
Federal University of São Carlos (UFSCar)
São Carlos, Brazil

Alexandros Koulouris
Alexander Technological Education Institute
Thessaloniki, Greece
akoul@food.teithe.gr

Nadia Krieger
Department of Chemistry
Federal University of Paraná
Curitiba, Paraná, Brazil
nkrieger@ufpr.br

Sze Ying Lee
Department of Chemical Engineering,
Faculty of Engineering and Science,
Universiti Tunku Abdul Rahman
Kajang, Selangor, Malaysia
leeszey@utar.edu.my

Ehsan Mahdinia
Department of Food Science
Pennsylvania State University
University Park, PA, USA
mahdinia@psu.edu

David Alexander Mitchell
Department of Biochemistry and Molecular Biology, Federal University of Paraná, Curitiba, Paraná, Brazil
davidmitchell@ufpr.br

Yahya Najian
Najian Herbal Group, Research and Development Department
Tabriz, Iran

Sandeep K. Panda
School of Biotechnology, KIIT University, Bhubaneswar
Odisha, India
sandeep.panda@kiitbiotech.ac.in

Demetri Petrides
Intelligen Inc.
Scotch Plains, NJ, USA
dpetrides@intelligen.com

Ramesh C. Ray
Centre for Food Biology and Environment Studies
Bhubaneswar, Odisha, India

Leonardo Machado da Rosa
Chemical Engineering Department
University of Blumenau (FURB)
Blumenau, Brazil

P. Saranraj
Department of Microbiology
Sacred Heart College (Autonomous)
Tirupattur, Tamil Nadu, India
saranraj@shctpt.edu

Vernon Shoaf
Technology Services & Media Development, Bionetwork, Charlotte, NC, USA
vshoaf@ncbionetwork.org

Pau Loke Show
Department of Chemical and Environmental Engineering
Faculty of Engineering, University of Nottingham Malaysia Campus
Semenyih, Selangor, Malaysia
pauloke.show@nottingham.edu.my

Charles Siletti
Intelligen Inc.
Scotch Plains, NJ, USA
casiletti@intelligen.com

Rashad Syed
Integrated Bioactive Technologies (IBT), Callaghan Innovation
Lower Hutt, New Zealand
rashad.Syed@callaghaninnovation.govt.nz

Irfan Turhan
Akdeniz University, Göynük Culinary Arts Vocational School
Antalya, Turkey
iturhan@akdeniz.edu.tr

Hamideh Vaghari
Faculty of Chemical Engineering, Sahand University of Technology
Tabriz, Iran
h_vaghari@sut.ac.ir

Ercan Yatmaz
Akdeniz University, Göynük Culinary Arts Vocational School
Antalya, Turkey

Akdeniz University, Faculty of Engineering, Department of Food Engineering
Antalya, Turkey
ercanyatmaz@akdeniz.edu.tr

Shuguang Zhang
Integrated Bioactive Technologies (IBT), Callaghan Innovation
Lower Hutt, New Zealand
shuguang.zhang@callaghaninnovation.govt.nz

Microorganisms in Fermentation

Sudhanshu S. Behera, Ramesh C. Ray, Urmimala Das, Sandeep K. Panda, and P. Saranraj

A. Berenjian (ed.), *Essentials in Fermentation Technology*, Learning Materials in Biosciences,
https://doi.org/10.1007/978-3-030-16230-6_1

What You Will Learn in This Chapter

Microorganisms [bacteria, fungi (yeasts and mold)] have been adopted successfully in a wide range of industries, from food and beverage processing industries to pharmaceutical operations. Additionally, microorganisms offer tremendous unexploited potential for value-added products such as amino acids, nucleotides and nucleosides, vitamins, organic acids, alcohols, exopolysaccharides, antibiotics, antitumor agents, etc., through various fermentation processes and parameters. This chapter reviews the involvement of various groups of microorganisms in fermentation. The measurement of microbial biomass, growth and kinetics, and factors affecting fermentation processes are also explained. The roles of microorganisms (bacteria and yeasts) involved in fermentation processes [solid-state fermentation (SSF) and submerged fermentation (SmF)] mostly related in processing industries are discussed.

1.1 Introduction

The term "fermentation" is borrowed from the Latin word *fevere* which means "to boil." According to Louis Pasteur, fermentation was defined as "La vie sans l'air", i.e., life without air, and the science of fermentation is also known as zymology or zymurgy. However, fermentation is one of the oldest food storage/preservation methods throughout the world dating back to the Neolithic period (10,000 years BC) [39, 41]. In biochemical sense, it is the metabolic measure in which complex organic compounds (particularly carbohydrates) are broken down into simpler compounds without the involvement of oxygen (exogenous oxidizing agent) along with the generation of energy (ATP molecules).

The end products of fermentation differ depending on the microorganism. The scientific motivation behind fermentation started with the naming and identification of microorganisms (in 1665) by Leeuwenhoek and Hooke [15]. Thereafter, Pasteur dismantled the "spontaneous generation theory" in 1859 by ideally designed experimentation. However, the performance of an exclusive bacterium *Lactococcus lactis* in fermented milk was searched out by Sir Joseph Lister in the year 1877 [44]. Generally, microbes in the form of distinct cell or group of cells, usually bacteria, sometimes fungi, algae, or cells of animal or plant origin, are involved in the process of fermentation. Several parameters are associated with the fermentation process, such as concentration of microbial cells and their constituents (enzymes), and conditions like pH, temperature, and fermented medium (aerobic/anaerobic). In general, the microbial fermentation is divided into four categories, viz., (i) generation of biomass (viable cellular material); (ii) production of metabolites; (iii) synthesis of enzymes, vitamins, and proteins; and (iv) transformation/conversion of substrate into value-added products [56]. The current chapter provides an overview of the microorganisms involved in fermentation, growth and kinetics, factors affecting fermentation, bio-products (primary and secondary metabolites) derived from fermentation processes, and uses of probiotics in food fermentation.

1.2 Microbial Diversity: an Overview

There is no universal accord on how to categorize/classify microorganism and such classification is rather arbitrary. However, there are two basic/primary cell types: *prokaryotic* and *eukaryotic*. The fundamental difference/characteristic between them is the presence or absence of membrane around the cell's genetic information.

Prokaryotes are unicellular organism and have simple structure with a single chromosome. *Prokaryotic* cell lacks membrane-bound organelles (i.e., mitochondria and endoplasmic reticulum) and also lacks nuclear membrane.

1

Eukaryotes have a more complex internal structure, with more than one chromosome (DNA molecule) in the nucleus. *Eukaryotic* cells have a true nuclear membrane and hold membrane-bound organelles (i.e., golgi apparatus, mitochondria, endoplasmic reticulum) and a variety of functional/specialized organelles. A detailed contrast/comparison of *prokaryotes* and *eukaryotes* is presented in Table 1.1. Evidence/data recommends

Table 1.1 Comparison between *Prokaryotes* and *Eukaryotes* [48]

Characteristics	Prokaryotes	Eukaryotes
Genome		
Number of DNA molecules	One	More than one
Genetic (DNA) organization	DNA is circular, without protein	DNA is linear and associated with proteins to form chromatin
	Chromosome found freely in a cytoplasomic region called the *nucleiod*	Chromosome found in a membrane bound nucleus
	Naked i.e., not bound with protein and therefore doesn't form chromatin	Bound with histone proteins
DNA in organelles	No	Yes; (chloroplast and mitochondrial genome)
Plasmid	Contain extra-chromosomal plasmids	No (but organelle like mitochondria may contain their own chromosome)
Nuclear membrane	No	Yes
Cell division	Binary fission/budding	Mitosis or meiosis
Organelles		
Mitochondria	No	Yes
Endoplasmic reticulum	No	Yes
Golgi apparatus	No	Yes
Photosynthetic apparatus	Chlorosomes	Chloroplasts (plants)
Ribosome	70S	80S
Flagella	Single protein, simple structure	Complex structure, with microtubules
Spores		
Types	Endospores	Endo- and exospores
Heat resistance	High	Low
Example		
	Bacteria and archaea	Fungi, protozoa, algae and helminths

Table 1.2 Primary subdivision of cellular organisms

Group	Cell structure	Properties	Constituent groups
Eukaryotes	Eukaryotic	Multicellular	Plants (i.e., seed plants, ferns, mosses)
Eubacteria	Prokaryotic	Similar to *eukaryotes*	Most of bacteria
Archaebacteria	Prokaryotic	Distinctive properties	Methanogens, thermophiles,halophiles

that a universal/common ancestor (family history) gave rise to three distinctive/inherent branches of life: *Eukaryotes*, *Eubacteria* (or "true bacteria"), and Archaebacteria. Table 1.2 complies some of the inherent aspects/features of these groups.

1.2.1 Prokaryotes

The sizes of maximum or most prokaryotes differ from 0.5 to 3 micrometers (μm) in length (or equivalent radius). Different species have different shapes such as spiral or spirillum (e.g., *Rhodospirillum*), spherical or coccus (e.g., staphylococci), or cylindrical or bacillus (e.g., *Escherichia coli*). Prokaryotic cells grow rapidly, with usual doubling times of one-half hour to several hours. Moreover, prokaryotes can use/take advantage of a variety of nutrients as carbon source, including hydrocarbons, carbohydrates, proteins, and carbon dioxide [48].

Eubacteria

Eubacteria (genus of gram-positive bacteria in the family *Eubacteriaceae*) are microscopic single-celled organisms, characterized by rigid cell wall, and grow in diverse environments. The *Eubacteria* can be divided into several groups. One differentiation is established on the gram stain—the name comes from Danish bacteriologist Hans Christian Gram who developed the technique in 1884. The staining process first requires fixing/mending the cells (by heating), and then crystal violet (basic dye) is added; all bacteria will stain purple. Next, iodine is added (binds crystal violet), followed by the rapid decolorization/addition of ethanol (or acetone). ***Gram-positive*** cells stay purple, while ***gram-negative*** cells become colorless. Finally, counterstaining with safranin or fuchsine leaves *gram-positive* cells purple, while *gram-negative* cells are red. This ability to react with the gram stain differentiates bacteria by the chemical and physical properties of their cell wall (or structure of the cell envelope) [48].

A typical/ideal *gram-negative* cell is *E. coli* (Fig. 1.1). It has an outer membrane supported by a thin peptidoglycan (also known as *murein*) layer. Peptidoglycan is a polymer of sugars (polysaccharide) and amino acids that forms a structure similar to a mesh/chain-link fence/layer. Peptidoglycan provides structural strength as well as resists (opposing action) the osmotic pressure of the cytoplasm. A second membrane (the inner or *cytoplasmic membrane*) prevails and is detached from the outer membrane by the *periplasmic space*. The *cytoplasmic membrane* (bacterial *plasma membrane*) is composed of phospholipid bilayer

1

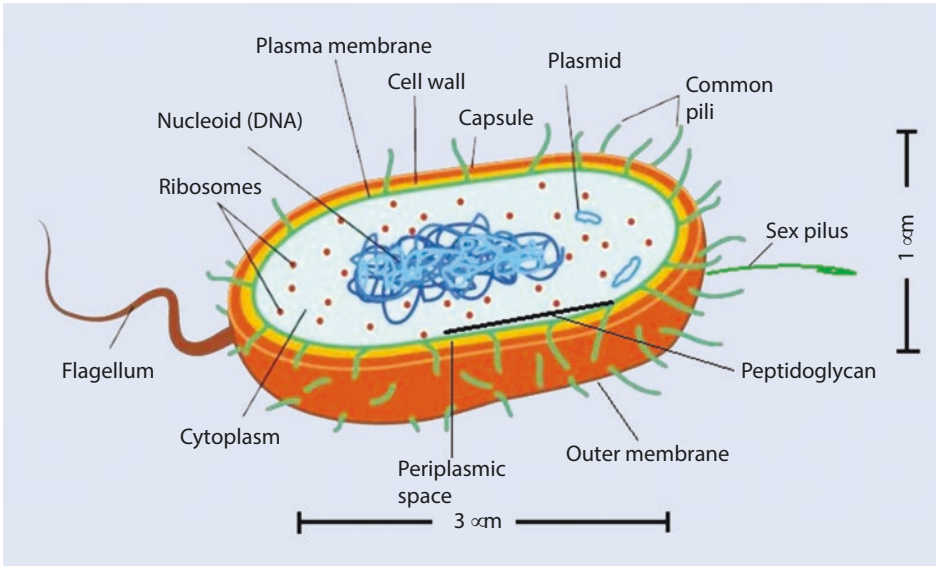


Fig. 1.1 A typical/ideal *gram-negative* cell (*E. coli*)

(50% protein, 30% lipids, and 20% carbohydrates). The cell envelope (capsule of polysaccharide) serves to protect, reserve/retain important cellular compounds, and in turn exclude/prevent undesirable compounds in to the cell environment. It provides structural/membrane integrity and loss of membrane integrity that leads to *cell lysis* and cell death. The cell envelope is essential to the transport of preferred material in and out of the cell [30].

A typical *gram-positive* cell is *Bacillus subtilis*. *Gram-positive* cells do not have an outer membrane. Rather they have a very thick, rigid cell wall with multiple layers of peptidoglycan and are made up of glycan strands that are cross-linked by peptide side chains. *Gram-positive* cells also contain *teichoic acids* covalently bonded to the peptidoglycan (wall *teichoic acids*) or are attached to the lipid membrane (lipo *teichoic acids*). Because *gram-positive* bacteria have a single cytoplasmic membrane, they are often much better suited/appropriate to extraction of proteins. Such extraction can be technologically advantageous when the protein is a desired product. Spore-forming *gram-positive* bacteria (*B. subtilis*) produce morphologically specific daughter cells by asymmetric cell division. The cell wall of spores varies from that of mother cells and has specific sets of proteins [36]. Unlike eukaryotic DNA, which is neatly packed into a cellular compartment called the *nucleus* (within the *nucleus*), bacterial DNA floats freely in the cytoplasm, in a twisted thread-like mass called the *nucleoid* [48].

Actinomycetes are bacteria (generally *gram-positive*, anaerobic), but, morphologically, they resemble molds with their long filaments and highly branched *hyphae*. However, the lack of a nuclear membrane and the composition of the cell wall require/appropriate classification as bacteria. *Actinomyces*, *Thermomonospora*, *Streptomyces*, *Microbacterium*, *Bifidobacterium*, *Brevibacterium*, *Corynebacterium*, *Micrococcus*, and *Brachybacterium* are examples of the genera convenient to this group [62]. *Actinomycetes* are essential sources of antibiotics. Over 500 species of *Streptomyces* bacteria have been recognized as the builders of abundant bioactive metabolites, such as antibacterials, antivirals, antifungals, and

enzyme inhibitors. Certain *actinomycetes* possess amylolytic [23, 26], pectinolytic [25], and cellulolytic [6] enzymes and are useful in enzymatic hydrolysis of starch, pectin, and cellulose, respectively.

Other/alternative characteristics within the *Eubacteria* can be made based on cellular nutrition and energy metabolism. One important example is photosynthesis. The ***cyanobacteria*** (*cyanophyta* or blue green algae) obtain their energy through photosynthesis and have chlorophyll, fix CO_2 into sugars, and are able to produce oxygen. Some of the *cyanobacteria* are nitrogen-fixing (fix atmospheric nitrogen in anaerobic condition) by means of specialized cells called *heterocysts*.

Prokaryotes may have other visible structures/organizations when observed/viewed under the microscope, such as *ribosomes*, *storage granules*, *spores*, and *volutins*. *Ribosomes* are the site of biological protein synthesis (translation). Prokaryotes have 70S *ribosomes*, are made of 50S and 30S subunits, and are highly complex cellular structures [48].

Storage granules (which are not present in every bacterium) act as reservoir—nutrients can be stored in the cytoplasm. *Storage granules* are source of basic/key metabolites in the form of polysaccharides (glycogen), polyphosphates, lipids, nitrogen, and sulfur granules. *Volutins* (polyphosphate bodies) are another granular intracytoplasmic/intracellular energy-rich compound, composed of inorganic polymetaphosphates, present in some species (e.g., A*grobacterium tumefaciens* and *Rhodospirillum rubrum*) [47]. It acts as a reserve store of energy and of phosphate, and, in some cases, nucleic acid, protein, and lipids are associated with these granules. Some photosynthetic bacteria (e.g., *Rhodospirillum rubrum*) have *chromatophores* which are large inclusion bodies (pigment-containing and light-reflecting cells) utilized in photosynthesis for the absorption of light [30].

Some bacteria make intracellular spores or *endospores*. Bacterial *endospores* (seed like) are dormant, tough, and nonreproductive structures and are produced as a resistance to adverse conditions (i.e., high temperature, radiation, and toxic chemicals). Spores can germinate under favorable growth conditions to yield actively growing bacteria. Certain bacteria (e.g., *Streptococcus, Bacillus antracis*) have a coating or outside cell wall called *capsule*, which is usually a polysaccharide (lipopolysaccharides) or polypeptide (lipoproteins). Many bacterial cells secrete some extracellular material in the form of *slime layer* which protects the bacterial cells from environmental changes (e.g., antibiotics and desiccation). Unlike bacterial *capsule*, which is attached tightly to the bacterium and has definite boundaries, the *slime layer* is loosely associated and can be easily washed off [48].
Table 1.3 summarizes the architecture of most bacteria.

Archaebacteria

Archaebacteria are similar to *eukaryotes*, but these cells differ greatly at the molecular level. Archaebacteria and *Eubacteria* or "true" bacteria both are considered to be part of same kingdom of "single-celled" organisms. However, sophisticated genetic and molecular studies allowed the major biochemical differences between Archaebacteria and *Eubacteria*. The differences between Archaebacteria and *Eubacteria* are as follows:

1. Archaebacteria are called ancient bacteria, whereas the *Eubacteria* are called "true" bacteria.
2. *Eubacteria* are usually found in soil and water and living in and on of large organism, whereas Archaebacteria are found in extreme environments and possess unusual metabolism (e.g., salt brines, ocean depths, and hot springs).

1

Table 1.3 Characterization of various components in bacteria [48]

Component	Size/diameter	Characteristics/composition
Extracellular materials		
Microcapsule	5–10 nm	Protein-polysaccharides-lipid complex responsible for the specific antigens of enteric bacteria and other species.
Capsule	0.5–2.0 μm	Mainly polysaccharides (e.g., *Streptococcus*); sometimes polypeptides (e.g., *Bacillus antracis*).
Slime layer	–	Mainly polysaccharides (e.g., *Leuconostoc*); sometimes polypeptides (e.g., *Bacillus subtilis*).
Cell wall		
Gram-positive species	10–20 nm	Confer shape and rigidity to the cell; consists mainly mixed polymers of N-acetyl muramic peptide, teichoic acids, and polysaccharides.
Gram-negative species	10–20 nm	Consists mostly of a protein-polysaccharides-lipid complex with small amount of muramic polymer.
Cell membrane		
	5–10 nm	Semipermeable barrier to nutrients; consisting of 50% proteins, 28% lipid and 15–20% carbohydrate in double layered membrane.
Flagellum		
	10–20 nm by 4–12 μm	Long and helical filament located either ends. Protein of the myosin-keratin-fibrinogen; arises from cell membrane and is responsible for motility.
Pilus		
Fimbria	5–10 nm by 0.5–2.0 μm	Rigid protein projections from the cell. Especially long ones are formed by *E. coli*.
Inclusions		
Spore	1.0–1.5 μm by 1.6–2.0 μm	One spore is formed per cell intracellularly. Spores show great resistance to dryness, heat and antibacterial agents.
Storage granule	0.5–2.0 μm	Glycogen like, sulfur, or lipid granules may be found in some species.
Chromato-phore	50–100 nm	Organelles in photosynthetic species (e.g., *Rhodospirillum rubrum*).
Ribosome (70S)	10–30 nm (approx. 20 nm or 200 A°)	Organelles for synthesis of proteins; about 10,000 ribosomes per cell; contain 63% rRNA and 37% ribosomal protein.
Volutin (polyphos-phate bodies)	0.5–1.0 μm	Inorganic metaphosphates
Nuclear material		
		Composed of DNA that functions genetically as if the genes and arranged linearly on a single endless chromosome.

S Svedberg unit/rate of sedimentation, *rRNA* ribosomal ribonucleic acid, *DNA* deoxy-ribonucleic acid

3. *Eubacteria* are divided into two groups known as *gram-positive* and *gram-negative*; whereas three types of Archaebacteria are found: methanogens (methane-producing bacteria), halophiles (grow at very strong salt solutions), and thermophiles (grow at high temperatures and low pH values).
4. Archaebacteria have no peptidoglycan.
5. The nucleotide sequences in the rRNA are similar within the Archaebacteria and distinctly different from *Eubacteria*.
6. The lipid composition of the cytoplasmic membrane is very different for the two groups [48].

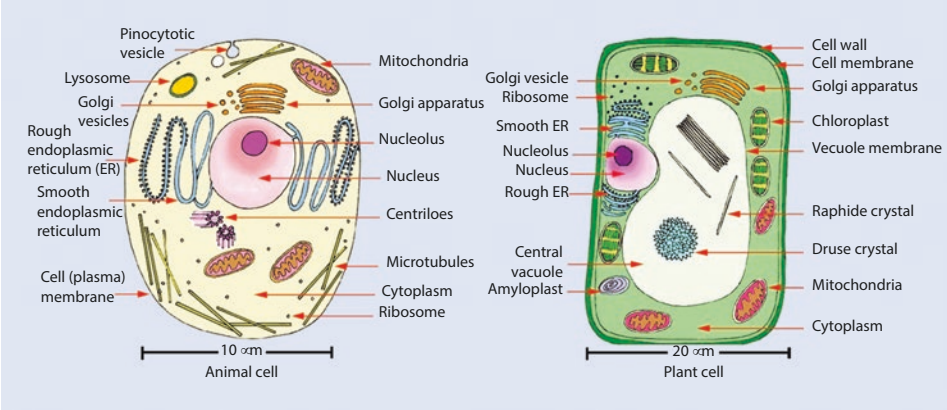
Proteobacteria

Proteobacteria is a major phylum of gram-negative bacteria. Under this phylum, *Acetobacter* sp. and *Gluconacetobacter* sp. are the main bacterial groups consisted with nine and eight species, respectively. They are chiefly employed in the making of vinegar but also of significance in the fermentation of coffee and cocoa [46].

1.2.2 Eukaryotes

Fungi (yeasts and molds), algae, protozoa, and animal and plant cells comprise the eukaryotes. Eukaryotes are five to ten times larger than prokaryotes in diameter (e.g., yeast about 5 μm, animal cell about 10 μm, and plant cell about 20 μm). Eukaryotes have a true nucleus (enclosed within membrane) and a number of cellular organelles (e.g., mitochondria, golgi apparatus etc.) inside the cytoplasm [48]. ◘ Figure 1.2 is a schematic representation of two typical eukaryotic cells.

In ***cell wall*** and ***cell membrane*** structure, eukaryotes are similar to prokaryotes. The plasma membrane is made of proteins and phospholipids that form a bilayer structure (lipid bilayer with embedded protein). Major proteins for the membrane are hydrophobic and are embedded in the phospholipids (50% of all lipids) matrix. Sterols are essential in all eukaryotic cell/cytoplasmic membranes. One major difference is in the presence of sterols (third major class of membrane lipid after phospholipids and glycolipids) in the plasma/cytoplasmic (plasmalemma) membrane of the eukaryotes. For instance,



◘ **Fig. 1.2** Schematic representation of two typical eukaryotic cells

cholesterol is the major sterol in animal plasma membrane. Sterols increase rigidity and strengthen the membrane structure (reduce membrane fluidity and permeability) and make the membrane less flexible [48].

The ***cell wall*** of eukaryotic cells (e.g., algae, plant, fungi) shows considerable variations. Some eukaryotes (e.g., algae) have polysaccharides and cellulose in their cell wall, while others have chitin and other polysaccharides (e.g., fungi). The true fungi do not have cellulose in their cell wall. The plant cell wall is composed of cellulose fibers and is linked via hemicellulose adhere/edge to form the network which is embedded in pectin aggregates. The cellulose-hemicellulose-pectin network imparts strength to the cell wall. Animal cells do not have a cell wall but only a cell/plasma/cytoplasmic membrane. For this logic, animal cells are very shear-sensitive and fragile (delicate) [48]. This aspect significantly complicates/upsets the design of large-scale bioreactor for animal cells [30].

The ***nucleus*** of the eukaryotic cells (except mammalian red blood cells) contains chromosomes as nuclear material (DNA-associated proteins), surrounded by a membrane. The nuclear membrane/envelope, a double membrane, consists of concentric and porous (nuclear pores) membranes. The nuclear envelope completely encloses the nucleus and separates the cell's genetic material from the surrounding cytoplasm. The nuclear pores are required to regulate nuclear transport of the molecules across the envelope. The nucleolus is an area in the nucleus that stains differently and is the site of ribosome and rRNA synthesis. It is not surrounded by a membrane. Nevertheless, many chromosomes have small amounts of RNA and basic proteins called histones adhered to the DNA. Each chromosome contains a single linear DNA molecule on which the histones are attached [48].

The ***mitochondria*** are double membrane-bound organelle and are the powerhouses (generate most of the cell's supply of adenosine triphosphate) of a eukaryotic cell, where respiration and oxidative phosphorylation occur. The mitochondria have a nearly cylindrical (vary considerably in size and structure) shape 1 μm in diameter and 2–3 μm in length. The regular structure of mitochondria is shown in Fig. 1.3. The external membrane is made of a phospholipid bilayer with proteins embedded in the lipid matrix. It has a protein-to-phospholipids ratio very much alike to that of eukaryotic plasma membrane (approx. 1:1). The mitochondria contain complex system of inner membranes (compartmentalized) called *cristae*. A gel-like matrix containing large amounts of protein (mixtures of enzymes) fills the space inside the *cristae*. Some enzymes of oxidative

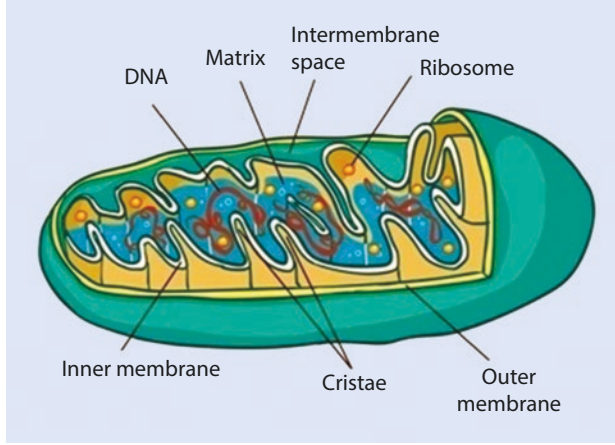


Fig. 1.3 Regular structure of mitochondria

respiration (oxidation of pyruvate, fatty acids, and citric acid cycle) are bound to the *cristae*. Mitochondria have its own DNA which is organized as several copies of single, usually circular chromosome and have protein-synthesizing system and reproduces independently [48].

The ***endoplasmic reticulum*** (ER) is a complex, convoluted/tangled membrane system of interconnected network of flattened, sac- or tubelike structure known as *cisternae*. There are two types of ER, rough *endoplasmic reticulum* (RER) and smooth *endoplasmic reticulum* (SER). The RER (granular) contains ribosomes on the inner surfaces (giving a rough appearance) and is the site of protein synthesis and modifications/corrections of protein structure after synthesis. The SER (agranular) is more engaged with lipid, phospholipids, and steroids synthesis.

Lysosomes are very small membrane-bound organelle that contain and release digestive/hydrolytic enzymes. *Lysosomes* add to the digestion of nutrients and invading substances/biomolecules (e.g., peptides, nucleic acids, carbohydrates, and lipids).

Peroxisomes (also known as *microbody*) are similar to lysosomes in their structure, but not in function. *Peroxisomes* contain oxidative enzymes (carry out oxidative reactions) that produce hydrogen peroxide.

Glyoxysomes (specialized *peroxisomes* found in plants) are also very small membrane-bound particles that contain the enzymes of glyoxylate cycle.

Golgi bodies (*golgi apparatus* or *golgi complex*) are endomembrane system in the cytoplasm and are composed of membrane aggregates/combinations engaged for the secretion of certain proteins. *Golgi bodies* are sites/centers where proteins (*glycosylation* enzymes) are modified by the addition of various sugars monomers in a process called *glycosylation*. Such modification/conversion is essential to protein function (responsibility) in the body.

Vacuoles are membrane-bound organelles (filled with cell sap) of low density (have no basic shape or size) and are enclosed by a membrane called *tonoplast*. *Vacuoles* are responsible for food digestion, osmotic regulation (hydrostatic pressure or turgor within the cell), and waste product storage. *Vacuoles* may occupy a large fraction of cell volume (up to 90% in plant cells) and maintain an acidic internal pH.

Chloroplasts (known as *plastid*) are comparatively large, chlorophyll-containing organelles that are responsible for photosynthesis in photosynthetic eukaryotes (algae and plant cells). Every *chloroplast* is characterized by its two membranes, an outer membrane and a large number of inner membranes called *thylakoids*. Chlorophyll molecules are combined with *thylakoids*, which contain a regular membrane structure with lipid bilayers. Chloroplasts are autonomous units consisting of their own DNA (ctDNA) and protein-synthesizing machinery.

Certain prokaryotes and eukaryotic organisms contain flagella—long, filamentous structures that are connected to one end of the cell and are responsible for the motion of the cell. Eukaryotic ***flagella*** contain two central fibers surrounded by 18 (nine fused pairs of microtubule) peripheral fibers, which exist in doublets (so-called "9 + 2" arrangement). Fibers are in a tube structure called *microtubule* and are composed of proteins called *tubulin*. The whole fiber assembly is installed/embedded in an organic matrix and is surrounded by a membrane. ***Cilia*** are flagella-like structures but are numerous and shorter. Only one group of protozoa, called *ciliates* (e.g., *Paramecium*), contains cilia. Eukaryotic cilia are structurally identical to eukaryotic flagella, although differentiations are made according to length and/or functions. Ciliated organisms move much faster than flagellated ones. The ***cytoskeleton*** (in eukaryotic cells) refers to filaments that provide an internal framework to organize the cell's internal activities and control its shape. These filaments are essential

1

in cell movement and separation of chromosomes into the two daughter cells during cell division and transduction of mechanical forces into biological response. The types of fibers present are *actin filaments*, *intermediate filaments*, and *microtubules*.

Microscopic Eukaryotes

Fungi are heterotrophs (obtain their food by absorbing dissolved molecules) that are widespread in distribution and grow in a wide range of habitats. Fungal cells (fungal *mycelia*) are larger than bacterial cells, and their typical internal structures, such as nucleus and vacuoles. The fungal *mycelia* can become visible to naked eye and can also be seen easily with a light microscope. Two major/dominant groups of fungi are yeasts and molds.

Yeasts are eukaryotic and single-celled microorganism of 5–10 μm size. Yeast cells are usually cylindrical, spherical, or oval. Yeasts can reproduce by asexual or sexual modes. Asexual reproduction (vegetative growth) is by either *budding* or *fission*. In *budding*, a small bud (known as "bleb" or daughter cell) forms on the parent/mother cell; this progressively enlarges/swells and detaches from the mother cell. Asexual reproduction by *fission* is identical to that of bacteria. Only a few species of yeast (e.g., *Saccharomyces pombe*) can reproduce by *fission*. In *fission*, the cell grows to a definite size and divides into two equal cells. Sexual reproduction of yeasts concerns with the formation of a zygote (a diploid cell) from fusion of two haploid cells, each having a single set of chromosomes. The nucleus of the diploid cells divides several times to form *ascospores*. Each *ascopspore* finally becomes a new haploid cell and may reproduce by *budding* or *fission*. The life cycle/life process of typical yeast cell is pictured in Fig. 1.4. The classification (analysis) of *yeast* is based on reproductive means (e.g., *budding* or *fission*) and the nutritional requirement/demand of cells. The most widely

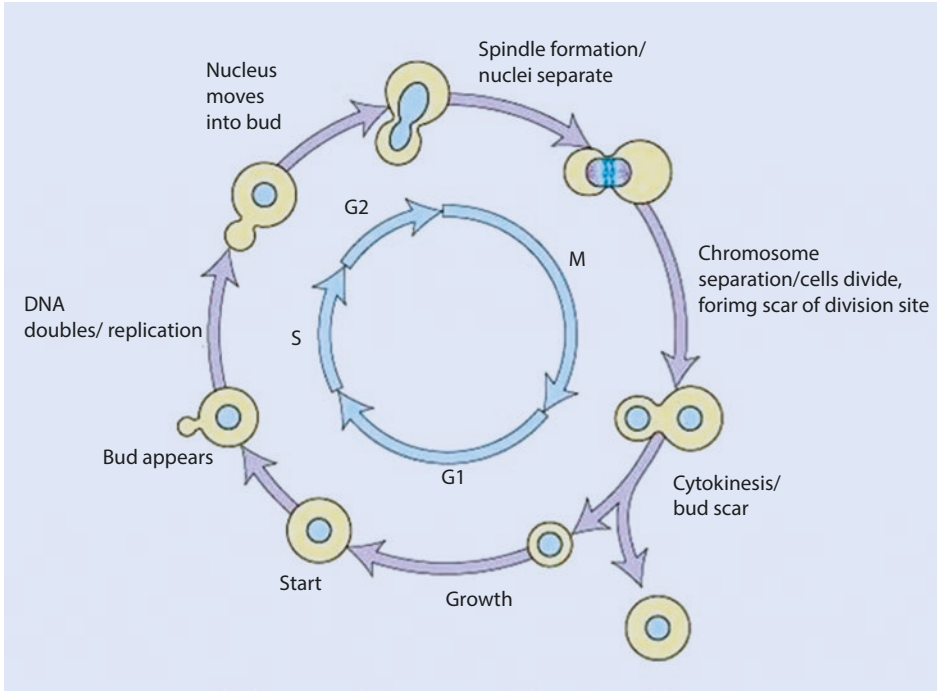


Fig. 1.4 Life cycle/life process of typical yeast cell

used yeast *Saccharomyces cerevisiae* is one of the main microorganisms of industrial interest such as baking, distilling, and wine making. *S. cerevisiae* is used in alcohol fermentation/formation under anaerobic or low-oxygen conditions (e.g. in beer, wine, and whisky making) and also for baker's yeast (leavening agent) production under aerobic conditions, where it converts fermentable sugar (present in dough) into carbon dioxide [48].

Molds are filamentous fungi and have a *mycelial* structure (i.e., network of tubular branching), the highly branched system (*mycelium*) of tubes that contains cytoplasm with many nuclei. Long and thin multicellular filaments on the mycelium are called *hyphae*. Certain branches of mycelium may grow in the air, and the asexual spores (or differentiation at the ends of *hyphae*) formed on these aerial branches are called *conidia*. *Conidia* are nearly spherical in structure and are often colored/pigmented. Some molds reproduce by sexual modes and form sexual spores. These spores provide resistance against heat, drying, freezing, and some chemical agents. Both sexual and asexual spores of molds can germinate and form *hyphae*. Figure 1.5 describes the structure and asexual reproduction of molds. ***Molds*** usually form long, highly branched cells, and easily grow on moist, solid nutrient surface. The growth of molds in the form of pellets can be an alternative in industrial fermentation processes. The growth of mold in submerged culture often forms cell aggregates and pellets. The pellets reach a diameter of 1.0–2.0 mm at the end of fermentation. Pellet formation can cause some nutrient transfer (mainly oxygen) problems inside the pellet. However, pellet formation reduces broth viscosity, which can improve bulk oxygen transfer. ***Molds*** are used for the production of citric acid (*Aspergillus niger*), and the pellet growth is being widely used in the production of citric acid. Several groups of antibiotics such as penicillin are produced by mold (*Penicillium notatum-chrysogenum* group) [48].

Algae are usually unicellular (eukaryotic) organisms (e.g., chlorella, diatoms). The size of a typical unicellular alga is 10–30 μm. However, multicellular algae (e.g., giant kelp, spirogyra, and stonewort) sometimes form a branched or unbranched filamentous structure. Some algae with multicellular structure are present in marine water (e.g., seaweeds).

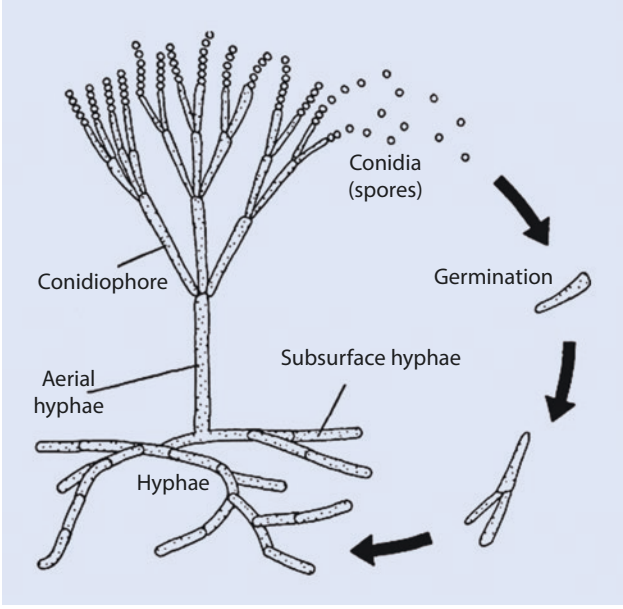


Fig. 1.5 Structure and asexual reproduction of molds

All algae are photosynthetic and contain chloroplasts (similar in structure to cyanobacteria), which commonly impart/transmit a green color to the organisms. The chloroplasts are the sites/centers of chlorophyll pigments and are important for photosynthesis. Some algae contain calcium carbonate or silica in their cell wall. Diatoms containing silica in their cell wall are used as filter aids/supports in industry. Some algae, such as *Chlorella*, *Spirulina*, *Scenedesmus*, and *Dunaliella*, are used for wastewater treatment with simultaneous single-cell protein production. Certain gelling agents (e.g., *alginic acid* or *alginate*) are obtained from marine/brown algae and seaweeds. Some algae are brown or red due to the occupancy of other pigments [30, 48].

Protozoa are unicellular, motile, either free-living or parasitic, and comparably large (1–50 mm) eukaryotic cells that lack cell walls. *Protozoa* are normally heterotrophic and obtain food by ingesting other small organisms or organic particles. *Protozoa* are usually uninucleate and reproduce by sexual or asexual modes. They are classified on the basis of their motion. The *amoeba* move by amoeboid motion, whereby the cytoplasm of the cell flows forward to form a pseudopodium (false foot), and the rest of the cell flows toward this lobe. The *flagellates* move using their flagella. *Trypanosomes* move by flagella and cause a number of diseases in humans. The *ciliates* move by motion of a large number of small appendages on the cell surface called cilia. These *protozoa* do not engulf/imbibe food particles, but absorb dissolved food contents through their membranes. The *sporozoans* are nonmotile and contain members that are human and animal parasites. *Protozoa* cause some diseases (human pathogens), such as malaria, giardiasis, and amoebic dysentery. Some *protozoa* are helpful in removing/eliminating bacteria from wastewater in biological wastewater treatment methods and obtaining clean effluent [48].

1.3 Methods of Fermentation

Fermentation has been classified into liquid fermentation (LF) or submerged fermentation (SmF) and solid-state fermentation (SSF) mainly based on the level of water used during the fermentation (Table 1.4).

1.3.1 Submerged Fermentation (SmF) or Liquid Fermentation (LF)

SmF exploits/utilizes free-flowing liquid substrates, broths, and molasses. The bioactive compounds are secreted into the fermentation broth. The substrates are utilized quite rapidly and hence need to be constantly replaced/supplemented with nutrients. This fermentation method is suitable for microorganisms such as bacteria that need high moisture content (Table 1.5). An additional choice of this technique/method is that purification/refining of products is easier. SmF is mainly used in the extraction of secondary metabolites that necessitate to be used in liquid form.

1.3.2 Solid-State Fermentation

SSF utilizes solid substrates, like bran, bagasse, and paper pulp. The main interest/advantage of using these substrates is that nutrient-rich waste materials can be easily/efficiently recycled as substrates. In this fermentation method/technique, the same substrate

Table 1.4 Comparison between liquid and solid substrate fermentations

Factor	Liquid substrate fermentation	Solid substrate fermentation
Substrates	Soluble Substrates (sugars)	Polymer Insoluble Substrates: Starch Cellulose, Pectines, Lignin
Aseptic conditions	Heat sterilization and aseptic control	Vapor treatment, non sterile conditions
Water	High volumes of water consumed and effluents discarded	Limited Consumption of Water; low A_w. No effluent
Metabolic heating	Easy control of temperature	Low heat transfer capacity
Aeration	Limitation of soluble oxygen. High level of air required	Easy aeration and high surface exchange air/substrate
pH control	Easy pH control	Buffered solid substrates
Mechanical agitation	Good homogenization	Static conditions preferred
Scale up	Industrial equipments available	Need for Engineering & new design Equipment
Inoculation	Easy inoculation, continuous process	Spore inoculation, batch
Contamination	Risks of contamination for single strain bacteria	Risk of contamination for low rate growth fungi
Energetic consideration	High energy consuming	Low energy consuming
Volume of equipment	High volumes and high cost technology	Low volumes and low costs of equipments
Effluent and pollution	High volumes of polluting effluents	No effluents, less pollution
Concentration of Substrate/Products	30–80 g/L	100/300 g/L

Source: Mienda et al. (2011) [32]

can be used for long fermentation periods and can be utilized very slowly and steadily. Henceforth, this technique supports controlled release of nutrients. SSF is best suited/adapted for fermentation techniques including fungi and microorganisms that depend on limited moisture content [6]. Nevertheless, it cannot be used in fermentation processes involving organisms that require high a_w (water activity), such as bacteria [4].

Bacteria and yeasts are equally involved in SmF and SSF, whereas fungi are mostly concerned with SSF processes. The roles of bacteria and yeasts in SmF are mostly related to food and beverage processing industries and will be discussed in detail in the subsequent chapters. Filamentous fungi are best suited for SSF owing to their physiological, biochemical, and enzymological properties and dominate in oriental foods, ensiling and composting processes [24].

1

Table 1.5 Examples of main groups of microorganisms involved in SSF and SmF processes

Microorganisms	SSF	SmF
Bacteria		
Bacillus sp.	Composting, Natto, α-amylase	Enzymes (α-amylase, polygalcturonase, phytase, etc)
Clostridium sp.	Ensiling, food	Pesticide degradation
Lactic acid bacteria	Ensiling, food	Fermented foods (yogurt, lacto-pickle, sausage etc)
Pseudomonas sp.	Composting	Xenobiotic degradation
Serratia sp.	Composting	
Fungi		
Altemaria sp.	Composting	
Penicilium notatum, roquefortii	Cheese	Penicillin
Lentinus edodes	Shiitake mushroom	–
Pleurotus oestreatus, sajor-caju	Mushroom	–
Aspergillus niger, A. oryzae	Food, enzymes (glucoamylase	Food, enzymes (glucoamylase, amylopullulanase)
Amylomyces rouxii	Cassava tape	
Beauveria sp., *Metharizium* sp.	Bioinsecticide	Bioinsecticide
Phanerochaete chrysosporium	Composting, lignin degradation	–
Rhizopus spp.	Composting, food, enzymes, organic acid	Food, enzymes, organic acid
Trichoderma sp.	Composting Biological control, Bioinsecticide	Cellulase
Yeasts		
Endomicopsis burtonii, Schwanniomyces castelli	Cassava tape	–
Saccharomyces cerevisiae	Alcoholic beverages, ethanol	Alcoholic beverages, ethanol

The hyphal means/modes of fungal growth and their good tolerance to low a_w and high osmotic pressure conditions make fungi more efficient for bioconversion of solid substrates. *Koji* (in Japan) and *Tempeh* (in Indonesia) are the two most successful applications of SSF using filamentous fungi. In *Koji* production, the fungus *Aspergillus oryzae* is grown on soybean and wheat bran, which is the first step of soy sauce and citric acid production. Koji is an unpurified form of several hydrolytic enzymes required in further steps of the fermentation process. Similarly, *Tempeh* is a fermented food produced by the growth of *Rhizopus oligosporus* on soybeans. In *Tempeh* fermentation, the hydrolytic

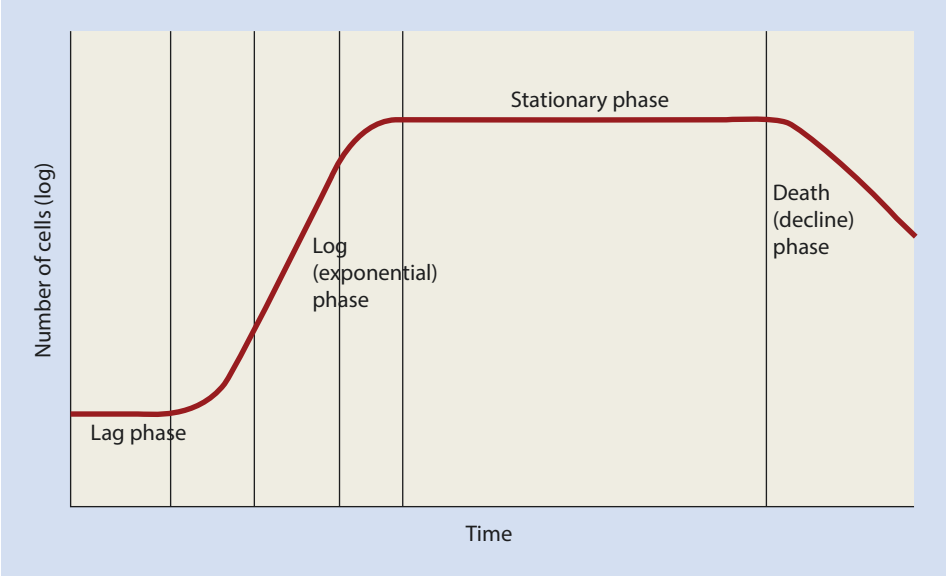
enzymes released by *R. oligosporus* degrade the anti-nutrients such as trypsin inhibitors and hemagglutinins (lectins) in crude soybean and improve taste, flavor, and nutritive quality. Further, the hyphal mode of growth has an added advantage to filamentous fungi over unicellular microorganisms in colonization of solid substrate and better utilization of nutrients [5]. The basic mode of fungal growth is a combination of apical extension of hyphal tips and the mycelium ensures a firm and solid structure. The hydrolytic enzymes are excreted at the hyphal tip, without large dilution like in the case of SmF.

1.4 Growth Measurement During Fermentation Process

In order to grow successfully, microorganisms must have a supply of water as well as numerous other substances including mineral elements, growth factors, and gas, such as oxygen. Virtually all chemical substances in microorganisms contain *carbon* in some form, whether they are proteins, fats, carbohydrates, or lipids.

1.4.1 Growth in SmF

Microbial growth is defined as the increase in all chemical components in the presence of suitable medium and the culture environment. Growth of the cell mass or cell number can be described quantitatively as a doubling of cell number per unit time for bacteria and yeast, or a doubling of biomass per unit time for filamentous organisms such as fungi. After the inoculation of a sterile nutrient solution with microorganisms and cultivation under physiological conditions, four typical phases of growth are observed as indicated in the ◘ Fig. 1.6. Growth is the result of consumption of nutrients.



◘ **Fig. 1.6** Four typical phases of microbial growth

The cells consume a part of the substrate/reactant and essential nutrients from the medium of fermentation. The cells initially multiply and grow. Depending on the type of cells, whether they are unicellular or molds, the growth pattern varies. For example, the unicellular organisms, which divide when they grow, will increase the number of cells or increase the biomass. As they increase in number, they consume more and more of substrate. The other types of cell (e.g., mold) will not increase in number, but they increase in size which also results in increase in viscosity of the broth.

Numerous procedures in biology require cells to be counted. By counting the cells in a known volume of a culture, the concentration can be assessed. For example, the concentration of microorganisms/cell number (e.g., bacteria, virus, and other pathogens) in blood or body fluids can reveal about the progress of an infectious disease and/or about a person's immune system. Knowing the cell concentration is important in molecular biology experiments in order to adjust the amount of reagents and chemicals applied to the experiment.

The various methods of measuring microbial growth are discussed below.

Plate Count

A viable cell count (number of colony-forming bacteria) allows one to identify the number of actively dividing/growing cells in a liquid sample. In this method, fixed amount of inoculum (generally 1 ml) from a broth/sample is placed in the center of sterile Petri dish (containing nutrient medium) using a sterile pipette (Fig. 1.7). The bacteria grow as a colony (i.e., cluster of cells or clones which arise from single bacterium by asexual reproduction) on a nutrient medium. When sample is plated, each colony that grows represents a single cell or spore in the original sample. The colony becomes visible to the naked eye and the number of colonies (colony count multiplied by the dilution factor) on a plate can be counted.

Serial Dilution

A serial dilution is the series of sequential dilution of a substance in solution. Each dilution will reduce the concentration of sample by a specific amount to a more suitable (usable) concentration. The first step in making a serial dilution is to take a known volume (usually 1 ml) of stock and place it into a known volume of distilled water (usually 9 ml) to make a ten-fold (0.1 M) serial dilution (Fig. 1.8). The progressive tenfold serial dilution could be 0.01 M, 0.001 M, 0.0001 M, etc. Usually the dilution factor at each step is constant, resulting in a geometric progression of the concentration in a logarithmic fashion.

A tenfold dilution reduces the concentration of a solution or a suspension by a factor of 10, that is, one-tenth the original concentration. A series of tenfold dilutions is described as tenfold serial dilutions. They are carried out in small sterile test tubes and are usually made of glass, and it is preferable if they have fitted lids to minimize the risk of contamination during the dilution.

The dilution factor is the number which multiplies the final concentration to get actual concentration (cell/ml) and thus decreases the final concentration of stock solution (Fig. 1.9).

Most Probable Number Method

The MPN method is a method used to estimate the concentration of viable microorganism in a sample by means of replicating liquid broth growth in tenfold or twofold (10× or 2×) dilutions. MPN analysis is a statistical method based on random dispersion

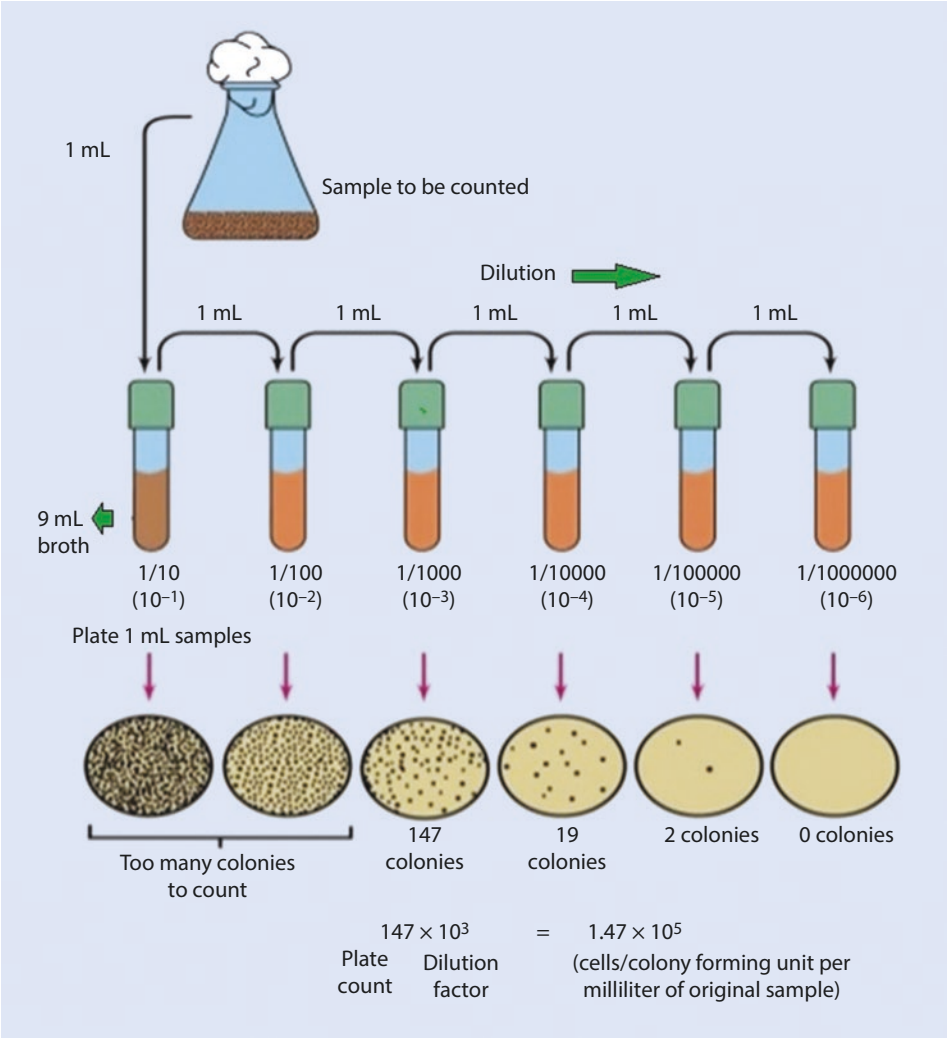


Fig. 1.7 Plate count of viable colony-forming bacteria

of microorganisms per volume in the given sample (Fig. 1.10). In this method, measured volumes of water are added to a series of tube containing a liquid indicator growth medium. The media receiving one or more indicator bacteria show growth and characteristic color change. The color change is absent in those receiving an inoculums of water without indicator bacteria. It is commonly used in estimating microbial populations in soils, waters, agricultural products and is particularly useful with samples that contain particulate material that interferes with plate count enumeration methods.

Optical Density

The optical density/spectrophotometer is usually taken to measure the concentration of growing bacteria. This method is completely based on Lambert–Beer's law. Optical density is directly proportional to the biomass in the cell suspension in a given range that is

1

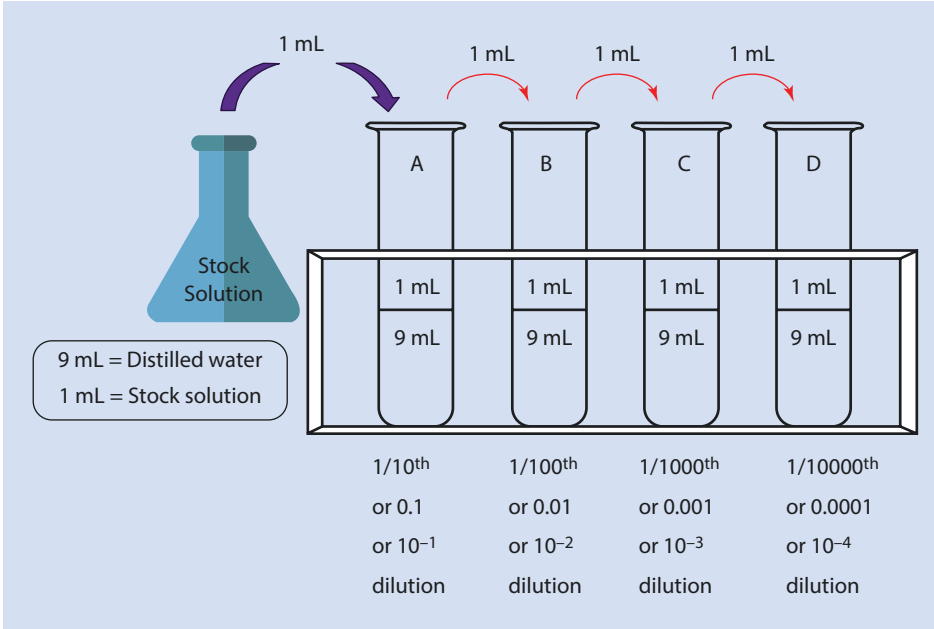
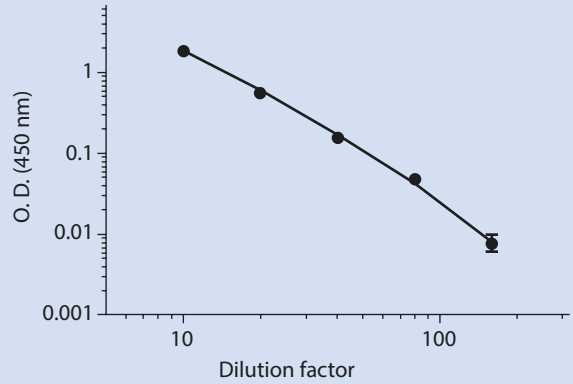


Fig. 1.8 Serial dilution/series of sequential dilution of stock solution

Fig. 1.9 Graph representing optical density (O.D.) with dilution factor



specific to the cell type. Cell suspensions/cell concentrations are turbid and absorb and scatter the intensity of light. The higher the cell concentration, the higher is the turbidity. The cell culture is placed in a transparent cuvette and the absorbance is measured relative to medium alone.

The Lambert–Beer Law

When a ray of monochromatic light of initial intensity (I_o) passes through a solution in a transparent vessel, some of the light is absorbed so that intensity of transmitted light "I" is less than "I_o". There is some loss of light intensity from scattering by particles in the solution and refection at the interfaces.

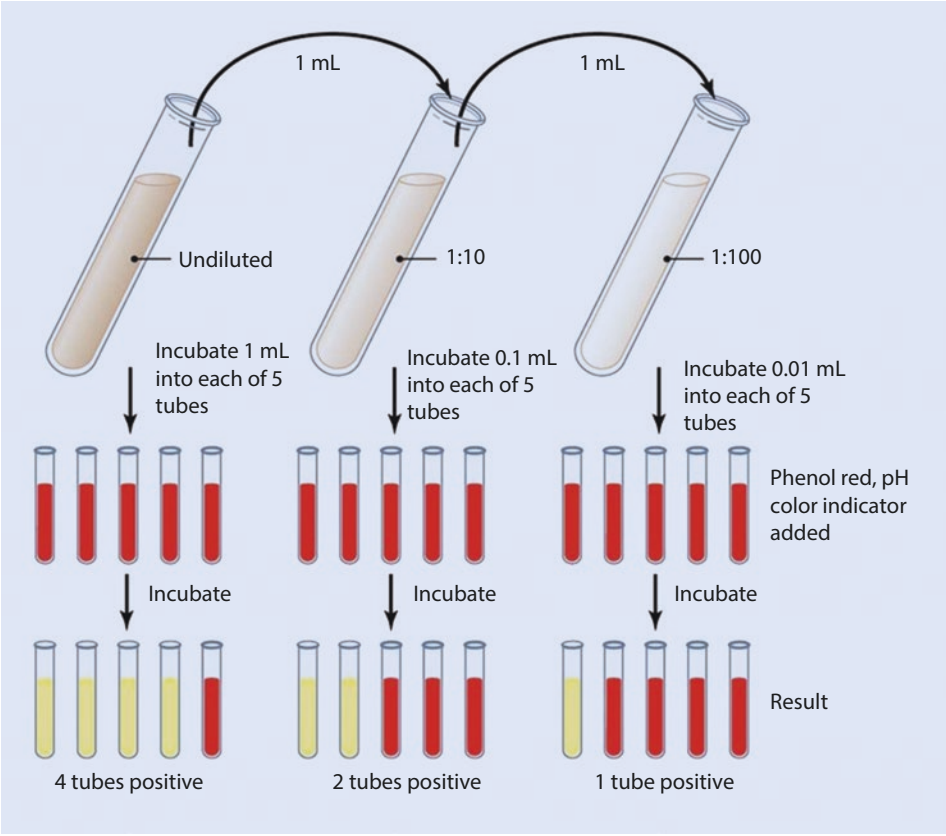


Fig. 1.10 Most probable number (MPN) method of microorganisms per volume of sample

The absorbance of an electronic transition depends on two external factors:

1. The absorbance is directly proportional to the concentration (C) of the solution of the sample used in the experiment.

$$A \propto C$$

2. The absorbance is directly proportional to the length of light path (l), which is equal to the width of the cuvette.

$$A \propto l$$

Combing the two relationships,

$$A = \varepsilon C l$$

This proportionality can be converted into an equation by including a constant.

This formula is known as the Beer–Lambert law (Fig. 1.11), and the constant ε is called molar absorptivity or molar extinction coefficient and is a measure of the probability of the electronic transition. The larger the molar absorptivity, the more probable is the electronic transition. In UV spectroscopy, the concentration of the sample solution is

1

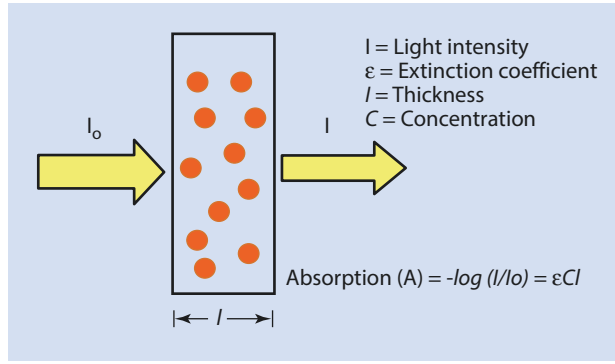


Fig. 1.11 The Beer-Lambert law represents $A = \varepsilon C l$; *A*, absorbance; constant ε is called molar absorptivity or molar extinction coefficient; *C*, concentration of the solution; *l*, light path (*l*), which is equal to the width of the cuvette

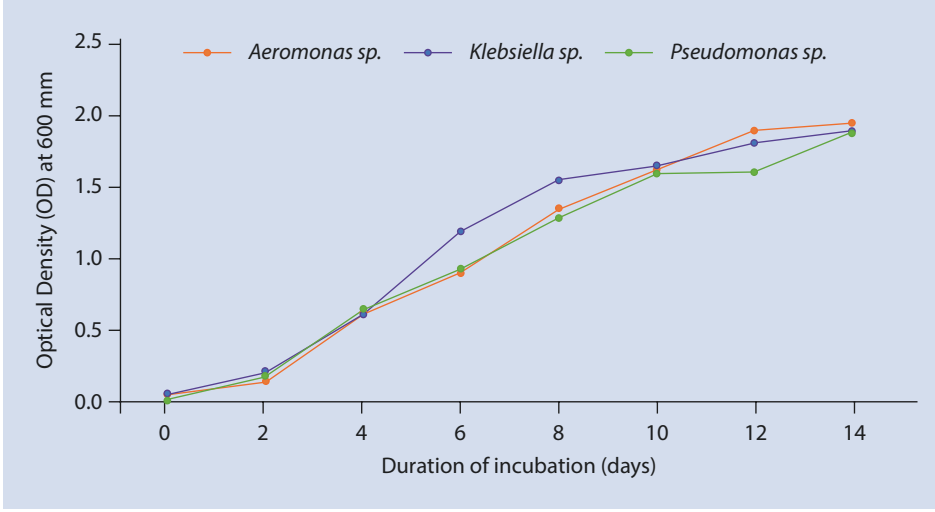


Fig. 1.12 Turbidimetry method (optical density with duration of incubation) of bacterial growth (*Aeromonas* sp., *Klebsiella* sp., *Pseudomonas* sp.,)

measured in mol/L and the light path in cm. Thus, given that absorbance is unit-less, the units of molar absorptivity are L/mol/cm.

Turbidimetry is an established method used to study bacterial growth (Fig. 1.12) since optical density measurements make it possible to follow bacterial population growth in real time. The electronic counting chambers or Coulter counter numbers are used to measure size distribution of cells. Turbidity measurement is done by estimations of large number of bacteria in clear liquid media and broth.

Kinetic Models for Cell Growth

It is generally believed that the growth rate of the cells at any time in the growth phase is proportional to the number of cells present at the time, which is popularly described by Malthus' law [63].

$$\frac{dx}{dt} = \mu x, \qquad x = x_o \quad at \quad t = o \tag{1.1}$$

where x is the mass of the cells per unit volume, μ is the proportionality constant known as specific growth rate in $(\text{hour})^{-1}$, and t is the time in hours.

On integration, we get

$$In\frac{x}{x_o} = \mu t \text{ or } x = x_o e^{\mu t} \tag{1.2}$$

$$In\, x = In\, x_o + \mu t \tag{1.3}$$

The time required to double the microbial mass is given by Eq. (1.2). The exponential growth is characterized by a straight line on a semi-logarithm plot of $In\ x$ versus time.

$$\tau_d = In\frac{2}{\mu} = \frac{0.693}{\mu} \tag{1.4}$$

Where τ_d is the doubling time of cell mass [40].

Monod Model

The growth phase passes through various phases, viz., high growth phase, low growth phase, and finally cessation. In other words, the specific growth rate varies with the residual concentration of the limiting substrate. The relation is well explained by an empirical equation proposed by Monod (1949) [34].

$$\mu = \frac{\mu_m S}{k_s + S} \tag{1.5}$$

where μ_m is the maximum growth rate achievable and k_s is the limiting substrate concentration when specific growth rate is equal to half the maximum specific growth rate, i.e., $\mu = \mu_m/2$.

This is evident by substituting $\mu_m/2$ for μ in Eq. (1.5), i.e.,

$$\frac{\mu_m}{2} = \frac{\mu_m S}{k_s + S} \tag{1.6}$$

By cancelling μ_m on both sides and on arrangement, $k_s = S$.

In Eq. (1.5), if $S >> k_s$, the denominator may be equated to simply S.

$$\mu = \frac{\mu_m S}{k_s + S} = \frac{\mu_m S}{S} = \mu_m \tag{1.7}$$

In other words, the above equation predicts the percentage in μ_m as compared to μ, when S is very large [40].

Example 1.1

A particular organism follows kinetics growth equation (Monod 1949). The kinetic parameter $\mu_m = 0.5\ h^{-1}$, $k_s = 2$ g/L and $S = 3.5$ g/L. Determine the value of μ.

Ans: $\mu = \frac{0.5 \times 3.5}{2 + 3.5} = \frac{1.75}{5.5} = 0.318\ h^{-1}$

1

1.4.2 Growth in Solid-State Fermentation

Direct determination of biomass in SSF is very difficult due to the problem of separating the microbial biomass from the substrate. This is especially true for SSF processes involving fungi, because the fungal hyphae penetrate into and bind tightly to the substrate. On the other hand, for the calculation of growth rates and yields, it is the absolute amount of microbial biomass which is important. However, in the case of bacteria and yeasts, the microbial population (colony-forming units) can be calculated by serially diluting the fermented mash and pour plating on specific culture medium. Desgranges et al. [13] and Terebiznik and Pilosof [55] have outlined the following methods for biomass estimation of fungi (also applicable to bacteria) in SSF.

Metabolic Measurement of Biomass

Respiratory Metabolism

Oxygen (O_2) consumption and carbon dioxide (CO_2) evolution resulting from respiration are the metabolic processes by which aerobic microorganisms derive most of their energy for growth. Carbon compounds within the substrate are metabolized, which are converted into microbial biomass and carbon dioxide. Production of CO_2 causes the weight of fermenting substrate to decrease during growth, and the amount of weight lost can be correlated to the amount of growth that has occurred. The measurement of either CO_2 evolution or O_2 consumption is more powerful when coupled with the use of a correlation model. If both the monitoring and computational equipment are available then these correlation models provide a powerful means of biomass estimation since continuous on-line measurements can be made. Other advantages of monitoring effluent gas concentrations with paramagnetic and infrared analyzers include the ability to monitor the respiratory quotient to ensure optimal substrate oxidation, the ability to incorporate automated feedback control over the aeration rate, and the nondestructive nature of the measurement procedure.

Production of Primary Metabolites

Usually, a growing cell will use its primary metabolites for growth and not export them to the medium. However, in fermentation processes designed for production of primary metabolites, i.e., amino acids, enzymes, organic acids, or nucleotides, their production is often growth-related. It has been frequently observed that there is a good correlation between mycelial growth and organic acid production, which can be measured by the pH analysis or a posteriori correlated by HPLC analysis on extracts. In the case of *Rhizopus*, Sauer et al. [45] demonstrated a close correlation between fungal protein (biomass) and organic acids (citric, fumaric, lactic, or acetic). However, the ratio of product formed per unit cell mass is not necessarily constant and as a consequence correlation with cell mass is poor.

Biomass Components

Protein Content

The most readily measured biomass component is protein. The [*] *biuret method* is easy, with good reproducibility since it measures peptide bonds, but it is not very sensitive. [$] *Kjeldahl nitrogen* when multiplied by 6.25 to obtain crude protein is subject to error from

nonprotein nitrogen. The *Folin–Ciocalteu method* (**Lowry's method*) is more sensitive, but since it responds strongly to the aromatic amino acids, it can give an erroneous value unless samples and reference protein are similar in composition [20].

[$***Kjeldahl method or Kjeldahl digestion*** in analytical chemistry is a method for the quantitative determination of nitrogen contained in organic substances plus the nitrogen contained in the inorganic compounds ammonia and ammonium (NH_3/NH_4^+)].

[**Biuret method*: The chemical text used to assess the concentration of protein (i.e., the presence of peptide bonds). The intensity of color or absorbance measured at 540 nm is directly proportional to the protein concentration (according to the Lambert–Beer law).]

[***Lowry's method*: The biochemical assay for determining the total level of protein in a solution. The intensity of color or absorbance measured at 600 nm is directly proportional to the protein concentration.]

Nucleic Acid

Nucleic acid methods based on DNA or RNA determination are reliable only if there is little nucleic acid in the substrate and if no interfering chemicals are present.

Glucosamine

A useful method for the estimation of fungal biomass in SSF is the glucosamine method. This method takes advantage of the presence of chitin in the cell walls of many fungi. Chitin is a poly-N-acetylglucosamine. Interference with this method may occur with growth on complex agricultural substrates containing glucosamine in glycoproteins.

Ergosterol

Ergosterol is the predominant sterol in fungi. Glucosamine estimation was therefore compared with the estimation of ergosterol for determination of the growth of *Agaricus bisporus*.

1.5 Factors Affecting Microbial Growth in Fermentation

The growth of microbes is influenced by various internal and external factors, viz., pH, temperature, composition of the media, etc.

1.5.1 Temperature

Microorganisms need optimum temperature for growth. If grown at a temperature below the optimum, growth occurs slowly resulting in a reduced rate of cellular production (Fig. 1.13). The bacteria can be divided into following groups based on their optimum growth temperature.

Thermophiles are heat-loving bacteria (i.e., *Thermus aquaticus*, *Thermococcus litoralis, and Bacillus stearothermophilus*). The optimum growth temperature is between 45 and 70 °C and is commonly found in hot springs and in compost heaps. ***Hyperthermophiles*** are bacteria that grow at very high temperatures (i.e., genus *Sulfolobus*). Their optimum temperature is between 70 and110 °C. They are usually members of the *Archaea* and are found growing near hydrothermal vents of great depths in the ocean (Fig. 1.14b, d).

Mesophiles are bacteria that grow best at moderate temperatures (i.e., *Pesudomonas maltophilia, Thiobacillus novellus, Streptococcus pyrogenes, Streptococcus pneumoniae,*

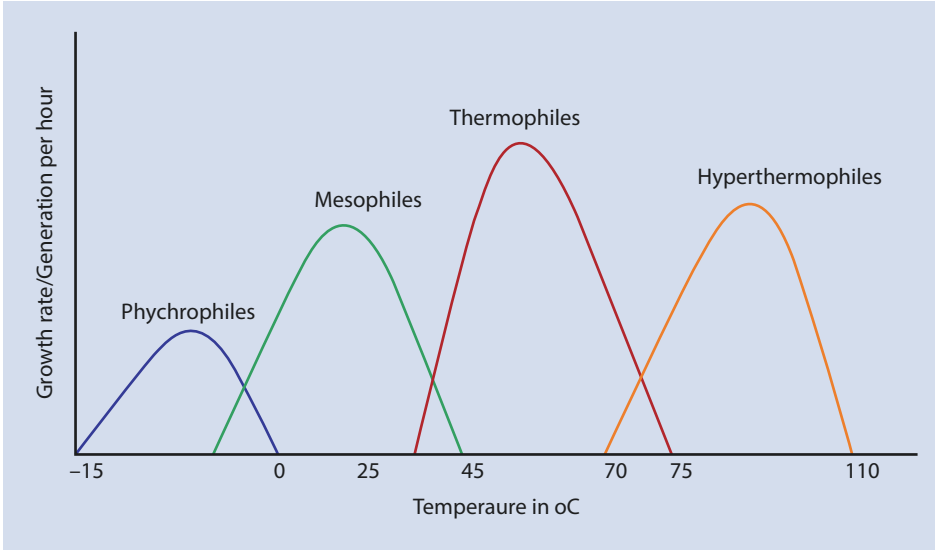


Fig. 1.13 Bacterial growth (growth rate vs. temperature in °C) based on temperature (thermophiles, hyperthermophiles, mesophiles, and psychrophile)

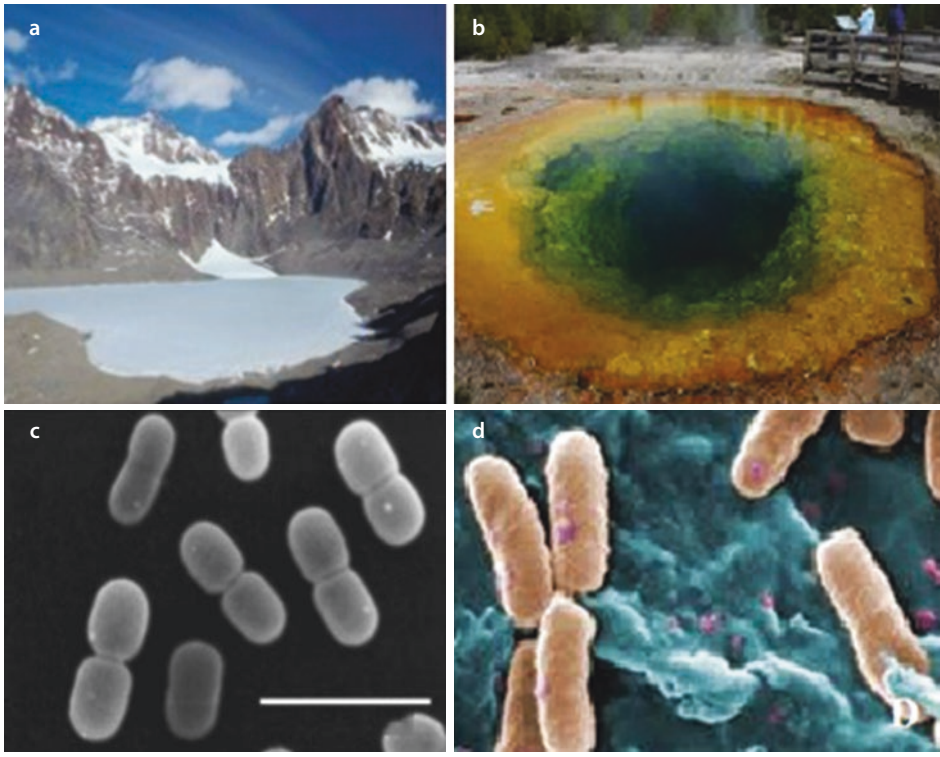


Fig. 1.14 Members of the *Archae* found near hydrothermal vents of great depths in the ocean (**b**, **d**) and Arctic and Antarctic regions (**a**, **c**) in the streams fed by glaciers

Clostridium kluyveri etc). Their optimum growth temperature is between 25 and 45 °C. Most bacteria are mesophilic and include common soil bacteria and bacteria that live in and on the body.

Phychrophiles are cold-loving bacteria (i.e., *Arthrobacter* sp., *Psychrobacter* sp.). Their optimum growth temperature is between −5 to 15 °C. They are usually found in the Arctic (Fig. 1.14a, c) and Antarctic regions and in the streams fed by glaciers. These bacteria are of little importance in fermentation.

1.5.2 pH

Most microorganisms grow optimally between pH 5 and 7 (fungi and yeast grow in acidic conditions) (Fig. 1.15). Microorganisms can be placed in one of the following groups based on their optimum pH requirements. ***Neutrophiles*** grow best at a pH range of 5 and 8. ***Acidophiles*** grow best at a pH below 5.5. ***Alkaliphiles*** grow best at a pH above 8.5.

1.5.3 Oxygen

Oxygen (aeration) is an important factor for aerobic organisms and is very strongly related to growth rate. Optimum growth of many microorganisms usually requires large amounts of dissolved oxygen. As oxygen is sparingly soluble in water (8.4 mg/L at 25 °C), it needs

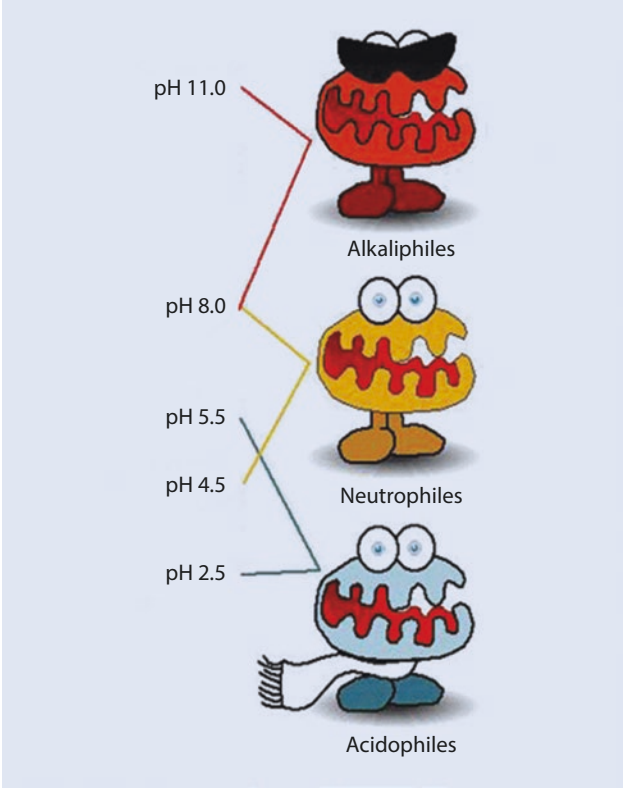


Fig. 1.15 Microorganisms growing in different pH range

1

to be supplied continuously (generally in the form of sterilized air) to a growing culture. The air produces bubbles and the stirrer is used to break up the bubbles and mix content in fermentation. If airflow is inadequate or the air bubbles are too large, the rate of transfer of oxygen to the cells is low and is insufficient to meet oxygen demand.

1.5.4 Constituents of Growth Medium

Macro- and Microelements

All microorganisms require certain elements for growth and metabolism. Macro-elements are needed in concentrations larger than 10^{-4} M. Carbon, nitrogen, oxygen, hydrogen, sulfur, phosphorus, Mg^{2+}, and K^{+} are major elements. Carbon compounds in growth medium are major sources of cellular carbon and energy. However, micro-elements are needed in concentration less than 10^{-4} M. Trace elements such as Mo^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+}, Ca^{2+}, Na^{+} and vitamins, growth hormones, and metabolic precursors are micro-elements.

C:N Ratio of Growth Medium

Carbon-to-nitrogen ratio (C/N ratio or C:N ratio) is a ratio of the mass of carbon to the mass of nitrogen in a substrate. The microorganisms decomposing organic matter with a higher C:N ratio are confronted with a surplus of C in relation to N and microorganisms confronted with a lower C:N ratio are facing a lack of C in relation to N [16]. The most common carbon sources in industrial fermentations are molasses (sucrose), starch (glucose, dextrin), corn syrup, and waste sulfite liquor (glucose). In laboratory fermentation, glucose, sucrose, and fructose are the most common carbon sources. Methanol, ethanol, and methane also constitute cheap carbon sources for the fermentation. Most industrially used microorganisms can utilize inorganic or organic sources of nitrogen. Inorganic nitrogen may be supplied as ammonia gas, ammonium salts, or nitrates. Ammonia has been used for pH control and as the major nitrogen source in a defined medium [52]. Ammonium salts such as ammonium sulfate usually produce acid conditions in the growth medium. However, ammonium nitrates normally cause an alkaline drift (provide basic environment) as they are metabolized.

Growth Factors

Some microorganisms cannot synthesize a full complement of cell components and therefore require preformed compounds called *growth factors*. Growth factors stimulate the growth and synthesis of some metabolites. Vitamins, hormones, and amino acids are the major growth factors. Some commonly used vitamins are thiamine (B_1), riboflavin (B_2), pyridoxine (B_6), biotin, cyanocobalamine (B_{12}), folic acid, lipoic acid, p-amino benzoic acid, and vitamin K. The growth factors most commonly required are vitamins, but there may also be a need for specific amino acids, fatty acids, or sterols. Depending on the organism, some or all of the amino acid may need to be supplied externally. Some fatty acids, such as oleic acids and sterols, are also needed in small quantities by some organisms. Many natural media formulations contain all or some of the required growth factors. For example, in processes used for the production of glutamic acid, limited concentrations of biotin must be present in the medium. Some production strains may also require thiamine (B_1).

1.6 Starter Culture(s)

1.6.1 Concept of Starter Cultures

A starter culture may be defined as a preparation containing large numbers of desired microorganisms, used for accelerating the fermentation process. The preparations may contain some unavoidable residues from the culture substrates and additives (such as antifreeze or antioxidant compounds), which support the vitality and technological functionality of the microorganisms. A typical starter after being adapted to the substrate facilitates improved control of a fermentation process and predictability of its products [18, 41]. Basically there are three categories of starter cultures: (1) single-strain cultures, contain only one strain of a species; (2) multi-strain cultures, contain more than one strain of a single species; and (3) multi-strain mixed cultures, contain different strains from different species [58].

1.6.2 History and Subsequent Development of Starter Culture

Microorganisms are naturally omnipresent and hence observed in raw substrates. This was the basis of the idea of spontaneous fermentation. Backslopping was the important technological phenomenon used in spontaneous fermentation by inoculating the raw material with a small quantity of a previously performed successful fermentation. Hence, the dominance of the best adapted strains resulted in backslopping. This technology is still used for production of foods and beverages where the ecology and concrete knowledge about microbial population and role are not clearly known. This is also an economical and reliable method of production of fermented foods.

Recombinant Starter Culture

Recombinant technology and genetic manipulation offer extensive possibilities to incorporate new traits into organisms and have positive applications in food and pharmaceutical industries. The recombinant *Lactococcus* is starter strains to produced peptidases from *Lactobacillus helveticus* 53/7 for potential use as an additional enzyme supply for accelerated proteolysis during cheese ripening [22]. It is possible to increase the production of expolysaccharides (EPS) by *Streptococcus thermophilus* strains through genetic engineering of galactose metabolism. In situ production of EPS by starter sutures (*S. thermophilus*) is an alternative to the addition of biothickeners of fat-free yogurts [42]. Bohmer et al. [8] reported both LABs (*Lactobacillus plantarum* NC8 and *Lactobacillus casei*) as potential expression hosts for recombinant enzyme [hyperthermophilic β-glycosidase (CelB)] production.

1.7 Facets of Fermentation

Fermentation process is used by human beings for various processes from industrial applications to food and agriculture, bioremediation of organic wastes to biovalorization of food and agricultural wastes, etc.

1

1.7.1 Industrial Fermentation

Industrial fermentation is the intentional use of fermentation by microorganisms such as bacteria and fungi to make a product useful to humans.

Production of Biomass

Microbial cells or biomass is sometimes the intended product of fermentation, for example, single-cell protein (SCP), baker's yeast, lactobacillus, etc. SCP refers to edible unicellular microorganism. The biomass (protein extract) from pure or mixed cultures of algae, yeasts, fungi, or bacteria may be used as an ingredient or a substitute for protein-rich foods and is suitable for human consumption or as animal feeds. Baker's yeast (*Saccharomyces cerevisiae*) is commonly used as a leavening agent in baking bread and bakery products, where it converts the fermentable sugars present in the dough into carbon dioxide and ethanol.

Production of Extracellular Metabolites

Metabolites can be divided into two groups: primary metabolites (ethanol, citric acid, glutamic acid, lysine, vitamins, and polysaccharides) and secondary metabolites (penicillin, cyclosporin-A, gibberellins, and lovastatin) (*described earlier*).

Production of Intracellular Components

The intracellular components are several microbial enzymes, such as catalase, amylase, protease, pectinase, glucose isomerase, cellulase, hemicelluase, lipase, lactase, streptokinase, etc. Moreover, the industrially synthesized recombinant proteins, such as insulin, hepatitis B vaccine, interferon, and granulocyte colony-stimulating factor, play an important role in health care.

1.7.2 Food Fermentation

Fermentation of food is the ancient process of making bread, curds, idli, dosa, cheese, etc., from thousand years ago. However, similar process is employed specifically to the conversion of sugars into ethanol producing alcoholic drinks such as wine, beer, and cider and in the preservation of sour foods with the production of lactic acid such as in sauerkraut and yogurt. More localized foods prepared by fermentation may also be based on beans, grain, vegetables, fruit, honey, dairy products, fish, meat, or tea [7].

1.7.3 Biofuels

Fermentation is the main source of ethanol in the production of ethanol fuel (i.e., ethyl alcohol) and butanol (butyl alcohol). Common crops such as sugarcane, potato, cassava, and corn are fermented by yeast to produce ethanol and are further processed to produce motor fuel or biofuel additive to gasoline.

1.7.4 Sewage Treatment

Sewage treatment (also called "wastewater treatment") is the process of removing contaminants from municipal wastewater (containing mainly household sewage plus some

industrial wastewater). In the process of sewage treatment, sewage is digested by enzymes secreted by bacteria.

1.7.5 Animal Feeds

A wide variety of agricultural waste products can be fermented to use as food for animals, especially ruminants. Fungi have been employed to break down cellulosic wastes to increase protein content and improve in vitro digestibility.

1.8 Representative Metabolites Produced by Microorganisms

Microbial metabolites represent an incredibly diverse array of chemistry. Microbes can make molecules that synthetic chemists cannot access. While over 25,000 microbial metabolites have been reported in the scientific literature, fewer than 2% of these have ever been readily available to the wider research community. Most metabolites have only ever existed in small quantities in the research laboratory in which they were discovered, and their biological activity has never been fully investigated. Representative metabolites produced by various microbial species are shown in ◘ Table 1.6.

1.8.1 Primary Metabolites

Primary metabolites are vital molecules (e.g., amino acids, vitamins, nucleotides, solvents, or organic acids) of all living cells and are the end product of metabolic pathways or their intermediate products or are domicile of essential macromolecules or are converted into coenzymes.

Amino Acids

The amino acid market is over $6 billion and has been growing at 5–10% for each year [9]. For instance, monosodium glutamate, an effective flavor enhancer, is a leading amino acid in terms of tonnage. Various species of the genera *Corynebacterium* and *Brevibacterium* (e.g., *Corynebacterium glutamicum*, *Brevibacterium flavum*, and *Brevibacterium lactofermentum*) are claimed to be producers of glutamate.

Nucleotides and Nucleosides

The nucleotide/nucleoside fermentations are due to the activity of two purine ribonucleoside 5′-monophosphates, [i.e, guanylic acid (5′-GMP) and inosinic acid (5′-IMP)]. Three primary processes are involved: (i) hydrolysis and enzymatic deamination of yeast RNA to IMP, (ii) synthesis and phosphorylation of the nucleoside (inosine and guanosine) by *Bacillus subtilis* mutants, and (iii) direct fermentation of sugar to IMP (by *Corynebacterium glutamicum* mutants) and conversion of guanine to GMP (using intact cells of *Brevibacterium ammoniagenes*). The intracellular AMP and GMP are limited for effective accumulation of purine nucleotides and are mostly explained by control feeding of purine auxotrophs [35], for instance, adenine-needing mutants deficient of nucleotide degrading enzymes (e.g., adenylosuccinate synthetase), which caused accumulation of inosine or hypoxanthine, which resulted from breakdown of intracellular accumulated IMP.

1

Table 1.6 Representative metabolites produced by different groups of microorganisms

Primarty Metabolites	Microorganism involved	Description and usage	References
Amino acids			
Monosodiumglutamate	*Corynebacterium glutamicum, Brevibacterium flavum* and*Brevibacterium lactofermentum*	Effective flavor enhancer	Burkoski and Kramer [9]
Nucleotides and Nucleosides			
Guanylic acid (5′-GMP) and Inosinic acid (5′-IMP)	*Bacillus subtilis, Corynebacterium glutamicum, Brevibacterium ammoniagenes*	ASS; auxotrophs	Nakayama et al. [35]
Inosine	*Bacillus subtilis*	IMP dehydrogenase; 35 g/L; auxotrophs	Miyagawa et al. [33]
Inosine monophosphate/guanosine	*Bacillus subtilis*	IMP gene; 7–20 g/L; auxotrophs	Miyagawa et al. [33]
Cytidine	*Bacillus subtilis*	HSD; 30 g/L; auxotrophs	Asahi et al. [3]
Vitamins			
Riboflavin (vitamin B2)	*Eremothecium ashbyii* and *Ashbyagossypii*	20 g/L; nutrition of animals and humans	Demain [10]
Vitamin B12 (Cyanocobalamin)	*Propionibacterium shermanii* and *Pseudomonas denitrificans*	206 mg/L; nutrition of animals and humans	Spalla et al. [49]
Biotin antimetabolites	*Serratiamarcescens*	600 mg/L; nutrition of animals and humans	Masuda et al. [31]
Vitamin C (L – Ascorbic acid)	*Erwinia herbicola* with *Corynebacterium* sp.	130 g/L; Potent antioxidant	Saito et al. [43]
Organic acids			
Oxalic and gluconic acids	*Aspergillusniger*	IDH; pH 3.0	Kubicek and Rohr [27]
gluconic acid	*Aspergillus niger*	150 g/L	Znad et al. [65]
Gluconic acid	*Aspergillusniger*	GO; pH 1.7–2.0	Kubicek and Rohr [27]
Citric acid	*Candida oleophila*	80% sugar; 100 g/L	Anastassiadis et al. [2]
Citric acid	*Aspergillusniger*	Batch or Fed-batch fermentations; 6–10 days; 150–180 g/L	Anastassiadis et al. [2]
Isocitric acid	*Candida* sp.	Aconitase; 225 g/L	Deppenmeier et al. [12]

Table 1.6 (continued)

Primarty Metabolites	Microorganism involved	Description and usage	References
Vinegar[a]	*Acetobacter polyoxogenes* withplasmid vector, *Acetobacter aceti* subsp. *xylinum*.	AldDH; 68–97 g/L	Fukaya et al. [14]
Lactic acid	*Lactobacilli*	100 g/L	Znad et al. [65]
Itaconic acid	*Candida* sp.	–	Wilke and Verlop [61]
Succinic acid	*Actinobacillus succinogenes*	110 g/L	Zeikus et al. [64]
Pyruvic acid	*Torulopsis glabrata*	69 g/L	Li et al. [29]
Alcohols			
Ethanol	*Escherichiacoli*	ADH II and PDC; 46 g/L	Ingram et al. [19]
Glycerol	*Candida glycerinogenes*	130 g/L	Taherzadeh et al. [54]
Mannitol	*Candida magnoliae*	213 g/L	Lee et al. [28]
Secondary Metabolites			
Antibiotics			
Penicillin	*Penicillium chrysogenum*	70 g/L	Jiang et al. [21]
Cephalosporin-C	*Acremonium chrysogenum*	30 g/L	Jiang et al. [21]
Antitumor agents			
Mitomycin-C, bleomycin, daunorubicin, doxorubicin, etoposide and calicheamicin	*Actinomycetes*	Effective agent against cancer	Strobel et al. [53]
Camptothecin (CPT)	Endophytic fungi	Used against cancer	Amna et al. [1]
Pharmacological agents			
Lovastatin	*Aspergillus terreus*	Cholesterol – lowering agents	Stabb et al. [50]
Cyclosporin-A	*Tolypocladium nivenum*	Immunosuppressants	Omura and Crump [37]

Abbreviation: *5′-IMP* Inosine 5′-monophosphates, *5′-GMP* guanosine 5′-monophosphates, *ASS* adenylosuccinate synthetase, *IMP* inosine monophosphate, *HSD* Homoserine dehydrogenase, *IDH* isocitrate dehydrogenase, *GO* glucose oxidase, *AldDH* aldehyde dehydrogenase, *ADH* alcohol dehydrogenase, *PDC* pyruvate decarboxylase

[a]5–20% acetic acid

1

Vitamins

Several microbial strains are responsible for the production of vitamins or vitamin-like compounds (e.g., β-carotene, vitamin B12, riboflavin, vitamin C, linolenic acid, vitamin F, and ergosterol) [51]. Riboflavin (vitamin B2) has been produced commercially for many years by both fermentation and chemical synthesis; however, fermentation is the major route today. For instance, two yeastlike molds, *Eremothecium ashbyii* and *Ashbya gossypii*, were found to synthesize riboflavin in concentrations more than 20 g/L. Vitamin B12 (Cyanocobalamin) is formed industrially with *Propionibacterium shermanii* and *Pseudomonas denitrificans* [49]. The overproduction of vitamin B12 is absolutely dependent upon addition of betaine (the mechanism of control is unknown). The *P. freudenreicheii* can produce 206 mg/L of vitamin B12 and is not still a dominant industrial producing organism.

Organic Acids

Microbes have been widely used for the commercial production of organic acids (e.g., citric acid, acetic acid, lactic acid, gluconic acid, pyruvic acid, malic acid, tartaric acid, succinic acid, and itaconic acid). Among different types of organic acids, citric acid (CA) is easily palatable, assimilated, and has low toxicity. Therefore, it is extensively used (about15%) in the pharmaceutical and food industry (as an antioxidant, acidifying and flavor-enhancing agent). The CA is produced via the Embden–Meyerhof pathway (EMP) and the first step of the tricarboxylic acid (TCA) cycle. Harmsen et al. [17] reported that a high level of CA production is also linked with an increased level of fructose 2,6-biphosphate (an activator of glycolysis).

Vinegar production is best carried out with species of *Gluconacetobacter* sp. and *Acetobacter* sp. [61]. However, vinegar is consisting of about 5–20% acetic acid (AA). Fermentation has virtually waived chemical synthesis of lactic acid (LA). The LA is produced anaerobically with a 95% (w/w) yield based on carbohydrate, a titer of over 100 g/L, and a productivity of over 2 g/L. This is comparable to processes employing LA bacteria. *Lactobacilli* produce mixed isomers, whereas *Rhizopus* forms L -(+)- LA exclusively. *Rhizopus oryzae* is favored for formation since it makes only the stereochemically pure L -(+)-LA. Itaconic acid is used as a comonomer in resins and synthetic fibers and is produced from the selective fungal (i.e., *Candida*) sp. [61]. Although microbial processes exist for the other acids, they have not been exploited commercially on a large scale. Succinic acid can be made from the rumen organism *Actinobacillus succinogenes* at 110 g/L [64]. Pyruvic acid is formed at 69 g/L at 56 h with a yield of 0.62 g/g glucose using *Torulopsis glabrata* [29].

Alcohols

Ethyl alcohol (C_2H_5OH) is a primary metabolite that can be formed from fermentation of a carbohydrate/sugar or a polysaccharide that can be depolymerized to a fermentable sugar. Yeasts are preferred for these fermentations, but the species used depends on the substrate employed. *Saccharomyces cerevisiae* is used for the fermentation of hexose, whereas *Candida* sp. or *Kluyveromyces fragilis* may be employed if pentose or lactose, respectively, is the substrate. Ethanol is produced in Brazil from cane sugar at 12.5 billion liters/year and is used as a 25% fuel blend or as a pure fuel. With regard to beverage ethanol, some 60 million tons of beer and 30 million tons of wine are produced each year. Production of glycerol is usually done by chemical synthesis from petroleum feedstocks, but better fermentations processes are available [60]. Osmotolerant yeast strains (*Candida glycerinogenes*) can produce up to 130 g/L of glycerol. Six hundred thousand tons of glycerol

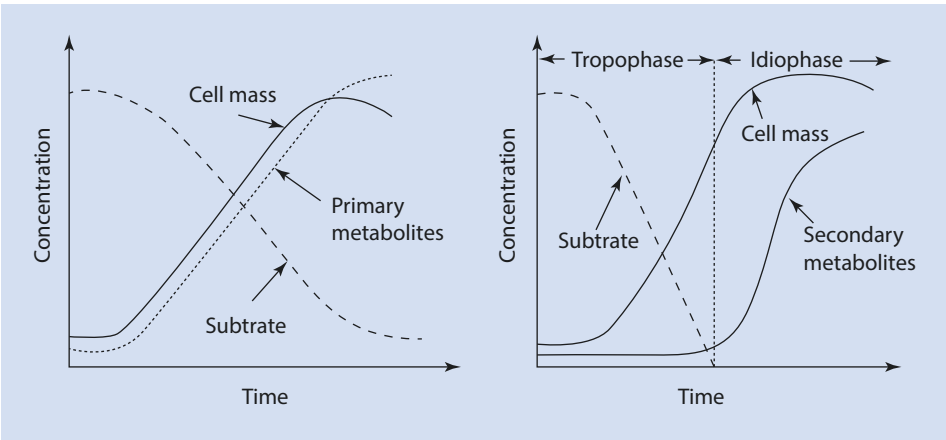
are produced annually by recovery as a by-product of the fat and oil industries, by synthesis from propylene, and, to a small extent, by glucose fermentation using *Saccharomyces cerevisiae* [54].

1.8.2 Secondary Metabolites

Microbial secondary metabolites are compounds produced by strains of certain microbial species (mainly by *actinomycetes* and *fungi*), usually late in the growth cycle (idiophase). The production of secondary metabolites starts when growth is limited with exhaustion of one key nutrient source (i.e., carbon, nitrogen, or phosphate). For example, penicillin biosynthesis by *Penicillium chryosgenum* starts when glucose is exhausted from the culture medium and the fungus starts consuming lactose, a less readily utilized glucose [57]. A characteristic of secondary metabolites is that they are usually not produced during the phase of rapid growth (trophophase) but are synthesized during a subsequent production stage (idiophase). The difference/comparison between primary and secondary metabolites is given in ◘ Fig. 1.16 and ◘ Table 1.7. The secondary metabolites have an enormous range of biological activities and are extremely important to our health and nutrition [10]. A group that includes antibiotics, toxins, pesticides, animal and plant growth factors, and other medicines has tremendous economic importance [11].

Antibiotics

The best known/recognized secondary metabolites are antibiotics (antimicrobial agent). The antibiotic revolutionized medicine in twentieth century and its market involves about 160 antibiotics and derivatives, (e.g., β-lactam peptide antibiotics, the macrolide polyketides and polyketides, tetracyclines, aminoglycosides, and others). The anti-infective market is framed of 62% antibacterials, 13% sera immunoglobulins and vaccines, 12% anti-HIV antivirals, 7% antifungals, and 6% non-HIV antivirals. Titers of *penicillin* with *Penicillium chrysogenum* have reached 70 g/L, whereas those of *Cephalosporin*-C by *Acremonium chrysogenum* are over 30 g/L. Published data on clavulanic acid production by *Streptomyces clavuligerus* indicate the titer to be above 3 g/L [21, 38].



◘ **Fig. 1.16** The difference/comparison between primary and secondary metabolites

Table 1.7 Differences between primary and secondary metabolites

Primary metabolites	Secondary metabolites
Produced during growth phase of cell	Produced during non-growth phase (near completion) of cell
Accumulated large quantities	Accumulated very small quantities
Growth phase where primary metabolites produced is called "Tropophase"	phase where secondary metabolites produced is called "Idiophase"

Antitumor Agents

Ever since the discovery/origination of the *Actinomycins* by Waksman and Woodruff [59] and the use of *Actinomycin*-D against the Wilms tumor in children, microbes have served as a prime source of anticancer agents. The essential microbial molecules are mitomycin-C, bleomycin, daunorubicin, doxorubicin, etoposide, and calicheamicin, all are produced by *Actinomycetes*. Taxol (paclitaxel) is a very productive compound against ovarian and breast cancer, produced by the endophytic fungi [1]. Another plant product is *camptothecin* (CPT), produced by certain angiosperms, which is a modified monoterpene indole alkaloid and is active against type I DNA topoisomerase. Its water soluble derivatives *irinotecan* and *topotecan* are used against cancer. It also can be made by endophytic fungi.

1.9 Concluding Remarks

A wide variety of organic chemicals like enzymes, amino acids, and antibiotics can be formed by fermentation. Fermentation is a metabolic process that converts organic substrates (mainly carbohydrates) into useful products (organic acids, gases, or alcohol) using diverse groups of microbes [bacteria, fungi (yeasts and mold)]. Most microorganisms used in commercial fermentation need disaccharides or six-carbon sugars as substrates, even though the microbial world contains organism that can break down essentially any organic compound. There has been a significant improvement in industrial products by strain selection/manipulation when related with the processes that were used in the first half of the twentieth century. However, further studies should aim to gather knowledge concerning several beneficial/microbial strains and processes involved in the formation of high-value fermented products.

Take-Home Messages

- Presence (in the case of eukaryotes) or absence (in the case of prokaryotes) of membrane around the cell's genetic materials is the fundamental difference between eukaryotes and prokaryotes.
- Gram staining differentiates bacteria into two groups, Gram +ve or Gram –ve, based on chemical and physical properties of their cell walls.
- Water level or water activity is the main difference between solid and submerged fermentation.

- Microorganisms have a supply of water as well as numerous other substances including mineral elements, growth factors, and gas such as oxygen to grow.
- Various methods of measuring microbial growth are plate count, serial dilution, most probable number (MPN), and optical density/spectrophotometer method.
- Monod equation can be used to measure microbial growth of microorganisms.
- Metabolic measurement of biomass includes respiratory metabolism, production of primary metabolites, biomass components of protein content, DNA or RNA components, and glucosamine method for the estimation of fungal biomass in solid- state fermentation.
- The growth of microbes is influenced by various internal and external factors, viz., pH, temperature, composition of the media, etc.
- In spontaneous fermentation, there is no control over the fermentation process and is mediated by natural microflora present on the surface of the substrate(s), whereas in controlled fermentation, the fermentation process is optimized for pH, temperature, incubation time and is mediated by specific microorganism or a group of microorganisms (starter culture).

References

1. Amna T, Puri SC, Verma V, Sharma JP, Khajuria RK, Musarrat J, Spiteller M, Qazi GN. Bioreactor studies on the endophytic fungus *Entrophospora infrequens* for the production of an anticancer alkaloid camptothecin. Can J Microbiol. 2016;52:189–96.
2. Anastassiadis S, Aivasidis A, Wandrey C. Citric acid production by *Candida* strains under intracellular nitrogen limitation. Appl Microbiol Biotechnol. 2012;60:81–7.
3. Asahi S, Izawa M, Doi M. Effects of homoserine dehydrogenase deficiency on production of cytidine by mutants of *Bacillus subtilis*. Biosci Biotech Biochem. 2016;60:353–4.
4. Babu KR, Satyanarayana T. Production of bacterial enzymes by solid state fermentation. J Sci Ind Res. 1996;55:464–7.
5. Barragán LP, Figueroa JJB, Durán LR, González CA, Hennigs C. Fermentative production methods. In: Biotransformation of agricultural waste and by-products. Netherlands: Elsevier; 2016. p. 189–217.
6. Behera SS, Ray RC. Solid state fermentation for production of microbial cellulases: recent advances and improvement strategies. Int J Biol Macromol. 2016;86:656–69.
7. Behera SS, Ray RC, Zdolec N. Lactobacillus plantarum with functional properties: an approach to increase safety and shelf-life of fermented foods. BioMed Res Int. 2018;2018:9361614. https://doi.org/10.1155/2018/9361614.
8. Bohmer N, Lutz-Wahl S, Fischer L. Recombinant production of hyperthermostable CelB from Pyrococcus furiosus in Lactobacillus sp. Appl Microbiol Biotechnol. 2012;96:903–12.
9. Burkovski A, Kramer R. Bacterial amino acid transport proteins: occurrence, functions, and significance for biotechnological applications. Appl Microbiol Biotechnol. 2002;58:265–74.
10. Demain AL. Fungal secondary metabolism: regulation and functions. In: Sutton B, editor. A century of mycology. Cambridge, UK: Cambridge University Press; 2016. p. 233–54.
11. Demain AL, Fang A. Emerging concepts of secondary metabolism in actinomycetes. Actinomyceto. 2015;9:98–117.
12. Deppenmeier U, Hoffmeister M, Prust C. Biochemistry and biotechnological applications of *Gluconobacter* strains. Appl Microbiol Biotechnol. 2002;60:233–42.
13. Desgranges C, Vergoignan C, Georges M, Durand A. Biomass estimation in solid state fermentation I. Manual biochemical methods. Appl Microbiol Biotechnol. 1991;35(2):200–5.
14. Fukaya M, Tayama K, Tamaki T, Tagami H, Okumura H, Kawamura Y, Beppu T. Cloning of the membrane bound Aldehyde dehydrogenase gene of *Acetobacter polyoxogenes* and improvement of acetic acid production by use of the cloned gene. Appl Environ Microbiol. 2009;55:171–6.

15. Gest H. The discovery of microorganisms by Robert Hooke and Antoni van Leeuwenhoek, Fellows of The Royal Society. Notes Rec R Soc Lond. 2004;58(2):187–201.
16. Grunert O, Reheul D, Van Labeke MC, Perneel M, Hernandez-Sanabria E, Vlaeminck SE, Boon N. Growing media constituents determine the microbial nitrogen conversions in organic growing media for horticulture. MicrobBiotechnol. 2016;9:389–99.
17. Harmsen HJM, Kubicek–Pranz EM, Rohr M, Visser J, Kubicek CP. Regulation of phosphofructokinase from the citric acid accumulating fungus *Aspergillus niger*. Appl Microbiol Biotechnol. 2012;37:784–8.
18. Holzapfel W. Use of starter cultures in fermentation on a household scale. Food Control. 1997;8: 241–58.
19. Ingram LO, Conway E, Clark DP, Sewell GW, Preston JF. Genetic engineering of ethanol production in *Escherichia coli*. Appl Environ Microbiol. 2017;53:2420–5.
20. Jernejc K, Cimerma A, Perdih A. Comparison of different methods for protein determination in Aspergillus niger mycelium. Appl Microbiol Biotechnol. 1986;23:445–8.
21. Jiang SJ, Yang YY, Wang HQ. Optimization of clavulanic acid fermentation. Chi J Antibiot. 2004;6: 335–7.
22. Joutsjoki V, Luoma S, Tamminen M, Kilpi M, Johansen E, Palva A. Recombinant Lactococcus starters as a potential source of additional peptidolytic activity in cheese ripening. J Appl Microbiol. 2002;92:1159–66.
23. Kar S, Ray RC. Partial characterization and optimization of extracellular thermostable Ca^{2+} inhibited αDOUBLEHYPHEN amylase production by *Streptomyces erumpens* MTCC 7317. J Sci Ind Res India. 2008;67:58–64.
24. Kar S, Ray RC. Optimization of thermostable α- amylase production by *Streptomyces erumpens* MTCC 7317 in solid state fermentation using cassava fibrous residue. Braz Arch Biol Technol. 2010;53:301–9.
25. Kar S, Ray RC. Purification, characterization and application of thermostable exo-polygalacturonase from *Streptomyces erumpens* MTCC 7317. J Food Biochem. 2011;35:133–42.
26. Kar S, Ray RC, Mohapatra UB. Alpha-amylase production by *Streptomyces erumpens* in solid state fermentation using response surface methodology. Polish J Microbiol. 2008;57:289–96.
27. Kubicek CP, Rohr M. Citric acid fermentation. CRC Crit Rev Biotechnol. 2016;3:331–73.
28. Lee JK, Song JY, Kim SY. Controlling substrate concentration in fedbatch *Candida magnoliae* culture increases mannitol production. Biotechnol Prog. 2013;19:768–75.
29. Li Y, Chen J, Lun SY, Rui XS. Efficient pyruvate production by a multi-vitamin. Int J Sci Tech. 2011;12: 229–35.
30. Liu S. Bioprocess engineering: kinetics, sustainability, and reactor design. San Diego: Elsevier; 2016.
31. Masuda M, Takahashi K, Sakurai N, Yanagiya K, Komatsubara S, Tosa T. Further improvement of Biotin production by a recombinant strain of *Serratia marcescens*. Process Biochem. 2015;30:553–62.
32. Mienda BS, Idi A, Umar A. Microbiological features of solid state fermentation and its applications-An overview. Res Biotechnol. 2011;2:465–89.
33. Miyagawa K, Kimura H, Nakahama K, Kikuchi M, Doi M, Akiyama S, Nakao Y. Cloning of the *Bacillus subtilis* IMP dehydrogenase gene and its application to increased production of guanosine. Biotechnol. 2016;4:225–8.
34. Monod J. The growth of bacterial cultures. Annu Rev Microbiol. 1949;3:371–94.
35. Nakayama K, Suzuki T, Sato Z, Kinoshita S. Production of nucleic acid - related substances by fermentative processes. J Gen Appl Microbiol. 2014;10:133–42.
36. Navarre WW, Schneewind O. Surface proteins of gram-positive bacteria and mechanisms of their targeting to the cell wall envelope. Microbiol Mol Biol Rev. 1999;63:174–229.
37. Omura S, Crump A. The life and times of Ivermectin - A success story. Nat Rev Microbiol. 2014;2:984–9.
38. Petkovic H, Cullum J, Hranueli D, Hunter IS, Peric Concha N, Pigac J, Thamchaipenet A, Vujaklija D, Long PF. Genetics of *Streptomyces rimosus*, the oxytetracycline producer. Microbiol Mol Biol Rev. 2006;70:704–28.
39. Prajapati JB, Nair BM. The history of fermented foods. In: Farnworth ER, editor. Fermented functional foods. Boca Raton, New York, London, Washington DC: CRC Press; 2003. p. 1–25.
40. Rao DG. Introduction to biochemical engineering. New Delhi: Tata McGraw-Hill Education; 2010.
41. Ray RC, Joshi VK. Fermented foods;: past, present and future scenario. In: Ray RC, Montet D, editors. Microorganisms and fermentation of traditional foods. Boca Raton, Florida: CRC Press; 2014. p. 1–36.
42. Robitaille G, Tremblay A, Moineau S, St-Gelais D, Vadeboncoeur C, Britten M. Fat-free yogurt made using a galactose-positive exopolysaccharide-producing recombinant strain of Streptococcus thermophilus. J Dairy Sci. 2009;92:477–82.

43. Saito Y, Ishii Y, Hayashi H, Imao Y, Akashi T, Yoshikawa K, Noguchi Y, Soeda S, Yoshida M, Niwa M, Hosoda J, Shimomura K. Cloning of genes coding for L - sorbose and L - sorbosone dehydrogenases from *Gluconobacter oxydans* and microbial production of 2-keto-L-gulonate, a precursor of L - Ascorbic acid, in a recombinant *G. oxydans* strain. Appl Environ Microbiol. 2017;63:454–60.
44. Santer M. Joseph Lister: first use of a bacterium as a 'model organism' to illustrate the cause of infectious disease of humans. Notes Rec R Soc Lond. 2010;64:59–65.
45. Sauer M, Porro D, Mattanovich D, Branduardi P. Microbial production of organic acids: expanding the markets. Trends Biotechnol. 2008;26(2):100–8.
46. Sengun IY, Karabiyikli S. Importance of acetic acid bacteria in food industry. Food Control. 2011;22:647–56.
47. Seufferheld MJ, Kim KM, Whitfield J, Valerio A, Caetano-Anollés G. Evolution of vacuolar proton pyrophosphatase domains and volutin granules: clues into the early evolutionary origin of the acidocalcisome. Biol Direct. 2011;6:50–5.
48. Shuler ML, Kargi F. Bioprocess engineering: basic concepts. 2nd ed. Upper SaddleRiver: Prentice Hall; 2002.
49. Spalla C, Grein A, Garofano L, Ferni G. Microbial production of Vitamin B12. In: Vandamme EJ, editor. Biotechnology of vitamins, pigments and growth factors. New York: Elsevier Appl. Sci; 2009. p. 257–84.
50. Stabb EV, Jacobson LM, Handelsman J. Zwittermicin A-producing strains of *Bacillus cereus* from diverse soils. Appl Environ Microbiol. 2014;60:4404–12.
51. Stahmann KP. Vitamins. In: Osiewacz HD, editor. The Mycota X. Industrial applications. Berlin: Springer; 2002. p. 231–46.
52. Stanbury PF, Whitaker A, Hall SJ. Principles of fermentation technology. Netherlands: Elsevier; 2013.
53. Strobel GA, Hess WM, Ford E, Sidhu RS, Yang X. Taxol from fungal endophytes and the issue of biodiversity. J Ind Microbiol. 2016;17:417–23.
54. Taherzadeh MJ, Adler L, Liden G. Strategies for enhancing fermentative production of Glycerol - A review. Enzyme Microb Technol. 2012;31:53–66.
55. Terebiznik MR, Pilosof AMR. Biomass estimation in solid state fermentation by modeling dry matter weight loss. Biotechnol Tech. 1999;13(3):215–9.
56. Thakur SA, Nemade SN, Sharanappa A. Solid state fermentation of overheated soybean meal (Waste) for production of Protease using *Aspergillusoryzae*. Int J Res Sci Eng Tech. 2015;50:228–35.
57. Thomas L, Larroche C, Pandey A. Current developments in solid-state fermentation. Biochem Eng J. 2013;81:146–61.
58. Vogel RF, Hammes WP, Habermeyer M, Engel KH, Knorr D, Eisenbrand G. Microbial food cultures–opinion of the Senate Commission on Food Safety (SKLM) of the German Research Foundation (DFG). Mol Nutr Food Res. 2011;55:654–62.
59. Waksman SA, Woodruff HB. *Actinomyces antibioticus*, a new soil organism antagonistic to pathogenic and non-pathogenic bacteria. J Bacteriol. 1941;42:231–49.
60. Wang ZX, Zhuge J, Fang H, Prior BA. Glycerol production by microbial fermentation: a review. Biotechnol Adv. 2011;19:201–23.
61. Willke T, Verlop KD. Biotechnological production of Itaconic acid. Appl Microbiol Biotechnol. 2011;56:289–95.
62. Xiao JZ, Takahashi S, Nishimoto M, Odamaki T, Yaeshima T, Iwatsuki K, Kitaoka M. Distribution of in vitro fermentation ability of lacto-N-biose I, a major building block of human milk oligosaccharides, in *Bifidobacteria* strain. Appl Environ Microbiol. 2010;76:54–9.
63. Zakaria Z, Chong SF, Zahari AR, Fauzi NA, Shayuti SAM. Growth kinetic of fresh and freeze-dried Pleurotus sajor-caju (Oyster Mushroom) mycelium for preservation study. Key Eng Mat. 2014;594:196–202.
64. Zeikus JG, Jain MK, Elankovan P. Biotechnology of Succinic acid production and markets for derived industrial products. Appl Microbiol Biotechnol. 2009;51:545–52.
65. Znad H, Markos J, Bales V. Production of gluconic acid from glucose by *Aspergillus niger*: growth and non-growth conditions. Process Biochem. 2014;39:1341–5.

Fundamentals of Fermentation Media

Kathrine Allikian, Rebecca Edgar, Rashad Syed, and Shuguang Zhang

A. Berenjian (ed.), *Essentials in Fermentation Technology*, Learning Materials in Biosciences,
https://doi.org/10.1007/978-3-030-16230-6_2

What You Will Learn in This Chapter

A well-designed growth medium is one of the key elements of a successful microbial fermentation. In this chapter, the roles and sources of individual components of submerged and solid-state fermentation media are described. These components include C and N sources, water, minerals, growth factors, precursors, and antifoams. The use of low-value by-products and waste streams as fermentation substrates is also discussed. The design and optimization of fermentation media and considerations for scale-up are critical to the ultimate success of industrial fermentation processes.

2.1 Introduction and Essentials of Media Composition

» We're all just ingredients. What matters is the grace with which you cook the meal. – Erica Bauermeister, *The Lost Art of Mixing*

Whether one is speaking of the skill of a Michelin-starred chef preparing an exquisite dish for the most discerning food connoisseur, or of a fermentation scientist designing an optimized growth medium for a specific microbial strain, Bauermeister's observation holds true: all living things are merely the re-organization of ingredients, be they supplied in the form of a delicate soufflé or in a finely balanced concoction of defined chemicals in a microbial growth medium. However, simply because an organism can grow and maintain itself on one combination of ingredients does not mean that that particular set of ingredients is ideal or optimized. Although growth and maintenance of life on chocolate cake alone is possible (and perhaps even delightful), few will argue that a diet comprising solely of rich desserts is optimal for the function and well-being of the human body. Similarly, microorganisms may grow in certain media, but the media may not be optimal for overall cell health, performance, or productivity [103].

Microorganisms used in lab- and industrial-scale fermentation are as diverse as the ingredients that can be used to support their growth. The challenge for fermentation scientists is to understand the requirements of the microorganism at hand and to design and develop the most suitable growth environment while minding such considerations as the product to be generated, the economics and suitability of the medium ingredients for scale-up, and the ultimate market of the product.

Industrial microorganisms fall into four primary categories based on their mode of energy utilization [59]:

1. Photoautotrophs: light as energy source, CO_2 as carbon (C) source; higher plants, most eukaryotic algae, some photosynthetic bacteria
2. Photoheterotrophs: light as energy source, organic C source; purple non-S photosynthetic bacteria, a few eukaryotic algae
3. Chemoautotrophs: chemical energy sources, CO_2 as C source (reduction of inorganic compounds as energy sources); only bacteria
4. Chemoheterotrophs: chemical energy sources, organic C source; most diverse—fungi, bacteria, some algae

There is growing interest in and commercial viability of processes designed around phototrophy (e.g., photosynthetic algae for the production of biofuel, bioactive fatty acids, or biologically relevant pigments) and chemoautotrophy (e.g., methanogenic bacteria for effluent treatment). These processes require specialized bioreactor systems and cultivation

2

conditions and currently represent a minority of fermentation processes in biotechnology. For the purpose of this chapter, the focus will be primarily on chemoheterotrophs, which are by far the most commercially important microorganisms in the field of industrial fermentation biotechnology. Chemoheterotrophic microorganisms span multiple classifications: bacteria, archaea, fungi (including yeast), and microalgae. Plant and animal cell cultures also fall under the chemoheterotrophic umbrella, but they are out of the scope of this chapter. Some organisms have the ability to employ more than one type of metabolism. For example, microalgae such as *Haematococcus pluvialis* [140] and *Chlorella zofingiensis* [128] can grow under strict heterotrophic, mixotrophic, or strict phototrophic conditions. Most industrially relevant microorganisms, however, are strict chemoheterotrophs.

Many chemoheterotrophs can grow well under unoptimized conditions. Most people have encountered this phenomenon in their own homes. Leave a container of milk too long in the refrigerator and soon the milk sours and curdles because of the microorganisms that have grown in it. Fail to clean a shower stall and an invisible biofilm soon forms that supports visible mold growth. Particularly old and sweaty shoes can lead to a bloom of odiferous microbial communities. Microorganisms take advantage of these environments to survive, but it is rare that they grow optimally under these ambient conditions. However, when the mold from the shower stall is transferred into a flask containing nutrient-rich liquid medium such as potato dextrose broth (PDB) and grown in a shaking incubator, the amount of biomass will be orders of magnitude greater. More favorable environmental conditions, such as temperature, are undoubtedly a factor. But the primary reason is the more suitable growth medium, providing an abundance of easily accessible nutrients.

This is where proper development of fermentation media becomes critical. Industrial fermentation is usually a commercial endeavor, and optimal growth and production conditions ultimately support the economic feasibility of the process. Even in the absence of economic pressures, a carefully developed growth medium can benefit the bench researcher studying a particular microorganism or molecule.

Fermentation media can be crude or refined, complex or defined, and costly or inexpensive. No matter the process, the fundamental building blocks of biological molecules include C, nitrogen (N), oxygen (O), hydrogen (H), phosphorus (P), and sulfur (S). Chemotrophic microorganisms must obtain these elements, plus important cofactors like trace minerals, from their surrounding environment. Oxygen is largely accessed from the atmosphere or through supplementation of the growth medium with pure oxygen. The remaining elements (Table 2.1) are supplied by the growth medium.

The exact chemical composition of cells varies from species to species and can be influenced by culture conditions [47]. The polysaccharide content of microalgae, for example, can vary widely depending on environmental conditions [11]. Table 2.2 shows a typical chemical composition of *Escherichia coli* on a dry cell weight basis, with over 70% of the cell's wet weight comprised of water.

The key objective when developing fermentation media is to supply the microorganism with all required elements, in the proper balance and in accessible forms, for optimal growth, function, and production. Because of the wide array of sources and combinations, a rational approach to designing, developing, and optimizing fermentation media is important. The main inputs into fermentation media include sources of C and N, trace metals and cofactors, and other process-specific compounds such as antifoam and inducers. Optimizing medium formulations, with an eye on the final production scale and target market, is an additional step toward developing robust, scalable, and economical fermentation processes.

Table 2.1 Essential elements for microbial growth and metabolism

Element	Function	Example source
C	Energy source, structural backbone element	Sugars, soybean meal, corn steep liquor
H	Structural molecules, respiration, ATP production	Water, protons from acidic environments
O	Organic molecules	Air
N	Proteins, nucleic acids	Yeast extract, peptone, NH_4^+ salts, amino acids
P	Nucleic acids, phospholipids	Phosphates
S	Amino acids, coenzymes	Sulfates
Na	Cofactor, cation	NaCl, Na_2HPO_4
Mg	Cofactor, cation	$MgSO_4$, $MgCl_2$
Ca	Cofactor, cation	$CaCl_2$
K	Cofactor, intracellular cation	K_2HPO_4, KH_2PO_4
Fe	Cytochromes, cofactor	$FeSO_4$, $FeCl_3$
Trace metals	Cofactors	$ZnCl_2$, $NiCl_2$, Na_2MoO_4, $MnCl_2$, $CoCl_2$, $CuSO_4$, etc.

Adapted from Walker and White [139], Kampen [59]

Table 2.2 Average chemical composition of *E. coli*

Component	% total dry weight
Proteins	55.0
Nucleic acids	28.6
Lipids	9.1
Lipopolysaccharides	3.4
Peptidoglycans	2.5
Glycogen	2.5
Polyamines	0.4
Metabolites, cofactors, ions	3.5

Adapted from Neidhardt [91]

2.2 C Sources

2

Microorganisms utilize C for both biosynthesis and energy generation. An adequate supply of C is vital for microbial growth and product formation. The choice of a suitable C source is influenced by various factors like the microorganism's metabolism, biomass yield per unit of substrate, cost, availability of the source, and type of final product (e.g., whole cell, secondary metabolite, or native or recombinant protein).

Defined C sources include simple sugars like glucose, fructose, mannose, galactose, sucrose, and xylose; polysaccharides such as starch; and lipids including oleates and glycerides. Peptones (digested proteins such as casein or soy), skimmed milk powder, molasses, corn steep liquor (CSL), and malt syrups are few of the complex C sources used in industry. The majority of the complex C sources also serve as a source of N and other nutrients essential for microbial growth. Less traditional sources like methane (CH_4), methanol (CH_3OH), and CO_2 are also utilized in some types of microbial fermentations. Fats and oils can also serve as C sources, alone or in combination with defined or complex sources. For example, the medium used for lipase production by *Candida rugosa* contains both glucose and olive oil [137].

2.2.1 Defined C Sources

Glucose and sucrose are two of the most commonly used sources of C in fermentation due to the ability of a large array of microorganisms to utilize these sugars. Defined C sources are particularly useful when the product of interest is produced extracellularly, as the complete utilization of these sugars can make downstream processing (DSP) efficient and less complicated. Glucose is utilized by most heterotrophic bacteria, yeast, and algae. Sucrose is metabolized by the majority of fungi and soil microorganisms like *Azotobacter* and *Azospirillum*. Glucose and sucrose are also used for producing recombinant products using *E. coli* [67]. A combination of both glucose and sucrose has also been used in the fermentation process for erythritol production by *Trichosporonoides* sp. [6]. Fructose, lactose, and xylose are mostly used in ethanol fermentations, and lactose is used for producing starter cultures of *Lactobacillus* sp. for the dairy and meat industries. Lipids like methyl oleate are used as a C source for producing lipases by *Yarrowia lipolytica* [28]. Glycerol is used in recombinant *E. coli* fermentations. Some other uses of glycerol as a C source include the production of succinic acid by *Anaerobiospirillum succiniciproducens* [68] and 1,3-propanediol by *Citrobacter freundii* [9].

Osmotic pressure as a result of sugar concentration can influence microbial growth. The acceptable concentration of C sources in batch fermentation media varies with the microorganism used. For example, bacteria like *Pseudomonas putida* and *Bacillus subtilis* are routinely grown on media containing 10 g/L glucose, while yeast can tolerate up to 200 g/L glucose. In most fermentations, substrate inhibition can be avoided by operating under fed-batch conditions, supplying a C feed to the culture when the concentrations of the substrates fall below threshold levels.

C content of bacteria can range between 50% and 53% (of dry weight), that of yeast between 45% and 50%, and that of filamentous fungi between 40% and 63% (See Table 2.7). Because C is used by a cell to construct cellular structure and to generate intermediates, proteins, enzymes, and cofactors required for life and, in the case of aerobic cultures, is released as CO_2, less than 100% of the C input results in final biomass. In aerobic cultures, 50–55% of C utilized by a microorganism is converted to biomass, while in anaerobic cultures, ca. 10% of C utilized is converted to biomass [59].

2.2.2 Complex C Sources

Complex C sources can be comparatively economical, as many of them are either by-products or waste streams produced from the processing of primary agricultural products. For instance, cane molasses is a by-product from the sugar cane industry; CSL is a by-product of the corn wet-milling industry; whey is the liquid that remains following the precipitation and removal of milk casein during cheese-making; olive mill effluent is the wastewater generated from the olive oil processing industry; and soybean meal/canola/cotton seed cakes are waste streams from the cooking oil industries. They have high nutritional values, and their use in biotechnological processes is well-established. Some of these low-value streams can be treated and refined to produce higher-value materials, some of which are relevant to industrial fermentation. These include tryptones (enzymatic digest of casein) and peptones (enzymatic digest of proteins from meat or plants). Peptones are predominantly used as a source of N in fermentation, but they also contain carbohydrates, as evidenced by the ability of many microorganisms to grow on a solution of peptone alone. A few of the most commonly used complex C sources are presented in ◘ Table 2.3. For additional complex C source examples, see ► Sects. 2.9 and 2.10.

2.2.3 Other C Sources

CH_4 is the second most abundant source of gaseous C, after CO_2, and can be used as a primary C source in some microbial fermentations [130]. CH_4-consuming bacteria, methanotrophs, can utilize CH_4 produced from landfills, coal mines, wastewater treatment plants, dairy effluent ponds, and wetlands. Polyhydroxyalkanoates, single-cell proteins (SCP), and several other metabolites are produced using CH_4 and methanol as C sources [55, 147]. Algae have the capability to use CO_2 as a source of both C and energy. *Spirulina* sp. and *Chlorella* sp. are used for the production of SCP for humans and animals and the production of other metabolites (e.g., pigments such as carotenes and phycocyanin) [134]. Some algal oils and biofuels are other products formed using CO_2 as a C source.

2.3 N Sources

Nitrogen is a critical component in fermentation media, serving as a key constituent of nucleic acids, proteins, and co-enzymes such as vitamins. Some microorganisms, including most photosynthetic organisms and some bacteria and fungi, can access N from oxidized, inorganic sources such as $NaNO_3$. Others, including industrial workhorses such as *E. coli*, require a reduced N source which can be supplied as ammonia salts or through complex sources including yeast extracts and peptones.

2.3.1 Complex N Sources

Complex N sources offer several nutritional advantages. Often they supply C, S, P, cofactors, and trace metals. In addition, they contain components that are less well-defined but may have considerable effects on microbial growth. They can be highly refined and manufactured to a consistent quality, or they can be relatively crude and minimally processed.

Table 2.3 The most commonly used complex C sources used in fermentation media

C source	Carbohydrates present	Other components present	Example of fermentation products	References
Molasses	Sucrose, fructose, and glucose.	Non-fermentable sugars, organic acids, waxes	Bioethanol, probiotic beverages, organic acids, microalgal fermentation, etc.	Ghorbani et al. [40], Yan et al. [143], Quesada-Chanto et al. [101]
Barley/malt extracts	Maltose, glucose, maltotriose, maltodextrins, sucrose, fructose	Non-fermentable sugars, pyrazines, and hop compounds	Fermented beverages, enzymes	Goldammer [43]
Cheese whey	Lactose	Lipids, soluble proteins, NaCl, lactic acid	Single-cell proteins, xanthan gums, flavors, carotenoids, beverages, gibberellic acid, etc.	Siso [123], Ghaly and Kamal [39]
Citric acids CSL	Glucose, other simple carbohydrates (mono to trisaccharides)	High levels of lactic acid, lipids, and other organic compounds	Organic acids, bioethanol, butanol, enzymes, polysaccharides	Liggett and Koffler [72], Hull et al. [52], Gouda et al. [44]
Oil cakes/meals: almond, mustard, soybean	Polysaccharides (10–54%)	Lipids, crude protein	Microbial lipase production	ul Haq et al. [136], Rigo et al. [106]
Peptones	Polysaccharides	Peptides/amino acids, others	Whole-cell products; enzymes; polysaccharides	Bultel-Ponce et al. [14], Levin et al. [70], Gunasekaran and Poorniammal [46]

Common microbial growth media such as lysogeny broth (LB) and tryptic soy broth (TSB) are based on complex N sources.

Refined complex N sources are those most familiar to laboratory researchers. Two of the most common types of refined complex N sources are yeast extracts and peptones. These are typically soluble ingredients that supply high-quality peptides and cofactors.

Yeast extract itself is the result of a fermentation process by which yeast biomass is grown aerobically in bioreactors, recovered by centrifugation, and autolyzed to release cell contents (the extract). While the production process is broadly similar across manufacturers, differences in yeast strains, fermentation conditions, and recovery processes can introduce differences in the final content (Table 2.4).

Table 2.4 Compositions of yeast extract

	"Yeast extract for technical purposes" (Sigma-Aldrich)	"Yeast extract for microbiology" A (Sigma-Aldrich)	"Yeast extract for microbiology" B (Sigma-Aldrich)	Hy-Yest 412 (Kerry)		Hy-Yest 444 (Kerry)		Hy-Yest 504 (Kerry)	
Total solids (%)	92	92	94	94		94		94	
Total N (%)	>9	>10	11	10.2		10.8		10	
Amino N (%)	>3	>4.5	5	4		5.1		4	
pH (2% solution)	6.5–7.5	6.5–7.5	6.8–7.2	5.0–5.6		6.5–7.1		5.0–6.0	
Ash (% max)	16	15	15	15		17		14	
Salt (% max)	N	NR	NR	1		1		NR	
Peptide distribution									
>10 kDa	NR	NR	NR	0.2		0		0	
5–10 kDa	NR	NR	NR	0.1		0.1		0	
2–5 kDa	NR	NR	NR	3.6		3.5		3.2	
1–2 kDa	NR	NR	NR	14.4		13.1		10.2	
500–1000 Da	NR	NR	NR	17.2		16.3		13.7	
<500 Da	NR	NR	NR	64.4		67.1		72.9	
Amino acids									
Ala	NR	NR	NR	49	34	45	30	41	20
Arg	NR	NR	NR	31	10	28	10	27	9
Asn	NR	NR	NR	0	8	0	8	0	5
Asp	NR	NR	NR	0	17	0	21	56	8
Asx	NR	NR	NR	63	0	64	0	0	0
Cys	NR	NR	NR	3	0	4	1	1	1
Gln	NR	NR	NR	0	0	0	1	0	0
Glu	NR	NR	NR	0	42	0	87	104	32
Glx	NR	NR	NR	102	0	138	0	0	0
Gly	NR	NR	NR	27	6	27	6	21	3
His	NR	NR	NR	12	2	12	2	10	2
Ile	NR	NR	NR	29	17	29	17	22	7

(continued)

Table 2.4 (continued)

	"Yeast extract for technical purposes" (Sigma-Aldrich)	"Yeast extract for microbiology" A (Sigma-Aldrich)	"Yeast extract for microbiology" B (Sigma-Aldrich)	Hy-Yest 412 (Kerry)		Hy-Yest 444 (Kerry)		Hy-Yest 504 (Kerry)	
Leu	NR	NR	NR	43	28	42	28	33	12
Lys	NR	NR	NR	46	10	44	11	45	5
Met	NR	NR	NR	9	6	9	6	7	0
Phe	NR	NR	NR	25	19	24	16	22	9
Pro	NR	NR	NR	22	6	23	7	21	4
Ser	NR	NR	NR	28	9	26	9	24	4
Thr	NR	NR	NR	28	11	27	11	23	5
Trp	NR	NR	NR	NR	NR	NR	NR	0	2
Tyr	NR	NR	NR	21	12	18	9	35	6
Val	NR	NR	NR	35	20	33	19	27	8
Other				Kosher		Kosher			

NR not reported

Broadly speaking, a peptone is digested protein. The protein can be animal or vegetable in nature, and the digestion process can be through enzymatic reaction or acid hydrolysis. Tryptones, which are derived from casein, are a type of peptone. Peptones have different physical and nutritional characteristics (Tables 2.5 and 2.6) and are not necessarily interchangeable. When developing a fermentation process, it is important to utilize consistent sources of ingredients to minimize variation due to growth medium.

Crude sources of N include soy flour, skimmed milk powder, CSL, and cottonseed flour (e.g., Pharmamedia®). These can be excellent, inexpensive ingredients that provide a range of nutritional benefits in fermentation media, including provision of simple and complex carbohydrates. Crude ingredients usually contain an insoluble fraction. Insoluble materials may hinder analyses such as optical density or dry weight measurements to evaluate biomass growth. However, they may be beneficial, particularly in fungal fermentations, to support different morphological growth patterns. For example, Tao et al. [131] demonstrated that mycelial morphology of *Grifola frondosa* could be influenced with the inclusion of insoluble components; looser and smaller hyphal fragments were observed as the concentration of insoluble material in the growth medium increased. In the same study, insoluble material was found to affect the content and composition of exopolysaccharides produced by *G. frondosa*.

While crude ingredients offer many financial and scientific benefits, they may also supply compounds that are anti-nutritive to the microorganism or considered undesirable in the final product. CSL contains high concentrations of phytic acid, a phosphorous-storage

Table 2.5 Compositions of soy peptones

	Hy-Soy (Kerry)		Hy-Soy T (Kerry)		Amisoy (Kerry)		HyPep 1510 (Kerry)		Soy peptone (Biotecnica)
Total solids (%)	95		94		95		95		94
Total N (%)	8		7.5		12		8		7
Amino N (%)	0		NR		9		On COA		2.2
Sugars (%)	17.3		17.8		19.5		NR		NR
pH (2% solution)	6.7–7.5		5.5–7.5		5.0–6.5		6.5–7.5		6.5–7.5
Ash (% max)	12		NR		10		15		15
Peptide distribution									
>10 kDa	0		0		0		0		NR
5–10 kDa	0.3		0.1		0		0.2		NR
2–5 kDa	5.3		4.5		0.4		4.6		NR
1–2 kDa	16.4		16.6		4.5		14.3		NR
500–1000 Da	23.5		23.3		18.6		22.8		NR
<500 Da	54.6		55.4		76.5		58.1		NR
Amino acids									
	Total	Free	Total	Free	Total	Free	Total	Free	Total
Ala	23	3.1	25	0.2	51	43	26	2.6	47
Arg	37	8.9	44	44	66	39	41	9.2	43
Asn	NR	3.4	NR	1.1	NR	NR	NR	NR	NR
Asp	7.4	5.1	74	0.4	82	67	73	2.1	59
Cys	1	0.2	1	0.4	NR	NR	4	NR	3.4
Gln	NR	3.3	NR	NR	NR	NR	NR	3.5	NR
Glu	123	5.3	119	2.5	176	88	122	NR	100
Gly	22	2.8	20	1.7	33	26	25	2.5	114
His	13	1.3	13	0.2	15	9	14	1.4	9.4
Ile	20	0.3	21	0.3	36	19	23	0.5	16
Leu	38	8.1	41	0.6	61	39	40	6.3	28
Lys	39	6.8	42	4.8	54	34	38	4.6	31
Met	5	0.3	8	1.7	10	6	7	0.9	7.3
Phe	26	2.4	27	5.6	42	23	26	0.7	18

(continued)

2

Table 2.5 (continued)

	Hy-Soy (Kerry)		Hy-Soy T (Kerry)		Amisoy (Kerry)		HyPep 1510 (Kerry)		Soy peptone (Biotecnica)
Pro	29	NR	28	0.3	45	29	28	NR	69
Ser	32	5.5	29	2.3	22	12	31	4	28
Thr	22	2.5	22	1.5	18	11	24	1.7	18
Trp	NR	2.1	NR	0.9	NR	17	NR	1.5	1.9
Tyr	18	NR	21	2.8	30	3	21	1.1	11
Val	22	1	76	1.1	88	21	25	1	21
Minerals (mg/100 g)									
Ca	98.6		394		2000		100		26
Cl	NR		NR		1200		200		NR
Cu	<1.0		1.4		NR		<0.1		NR
Fe	4.76		6.3		NR		0.4		NR
Mg	200		298		160		30		12
Mn	0.29		3.5		NR		NR		NR
P	228		682		NR		300		NR
Phosphates	NR		NR		120		NR		NR
K	2540		2260		140		3400		1500
Na	2673.3		437		200		3300		3000
Sulfates	NR		NR		6000		NR		NR
Zn	0.33		4.2		NR		<0.1		NR
Other	Not "identity preserved" (GMO soy)		Kosher Pareve, "identity preserved" (non-GMO soy)		Not "identity preserved" (GMO soy)		Not "identity preserved" (GMO soy)		

NR not reported

compound that binds cations of Ca, Fe, Mg, and Mn among others, restricting the availability of the minerals to cells [10]. Gossypol is a compound found in cottonseed flour that has been demonstrated to cause numerous unwanted effects in animals [38] and has been studied as a male contraceptive in humans [25]. Because of these side effects, gossypol is an unwanted contaminant in products intended for human use. The cost of DSP to remove gossypol may negate any financial benefit gained by using cottonseed meal as an inexpensive N source.

Crude ingredients may be heavily affected by regional and seasonal variability. Ravindran et al. [104] reported significant differences in the content of crude protein, sucrose, Ca, and

Table 2.6 Compositions of tryptones

	N-Z Amine A (Kerry)		N-Z Amine AS (Kerry)		N-Z Amine EKC (Kerry)		Hy-Case Amino (Kerry)		Amicase (Kerry)		Casein pep- tone Type I (Biotec- nica)
Total solids (%)	95		95		95		96		96		94
Total N (%)	11		11		12		7.8		12		10
Amino N (%)	6		6		On COA		6		9		3.9
Sugars (%)	NR		NR		NR		10.3		NR		NR
pH (2% solution)	6.4–7.0		6.4–7.0		6.4–7.2		5.0–6.0		6.0–7.0		6.5–7.5
Ash (% max)	7.5		7.5		5.5		40		2		15
Salt (% max)	NR		NR		NR		40		NR		NR
Peptide distribution											
>10 kDa	0		0		0.3		0		0		NR
5–10 kDa	0		0		2		0		0		NR
2–5 kDa	0.7		0.7		NR		0		0.1		NR
1–2 kDa	5.3		6.2		8		3		3.8		NR
500–1000 Da	17.3		20.6		89.7		20.8		22.9		NR
<500 Da	76.7		72.4		NR		76.1		3.3		NR
Amino acids											
	Total	Free	Total	Free	Total	Free	Total	Free	Total	Free	Total
Ala	23	13	25	14	24	3.5	25	22	36	56.8	29.0
Arg	31	29	34	30	32	5.5	22	13	42	27.7	33.0
Asn	NR	11	NR	12	NR	2.1	NR	NR	NR	NR	NR
Asp	58	13	63	12	60	6.6	44	37	35	33.3	70
Cys	NR	0.2	NR	0.5	NR	0.6	NR	NR	NR	NR	4.4
Gln	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Glu	170	38	187	42	176	15	140	91	175	116	187
Gly	13	3.2	14	3.6	13	1.3	11	9	22	17.6	18.6
His	19	10	21	11	20	1.3	14	8	27	13.7	23.8

(continued)

Table 2.6 (continued)

	N-Z Amine A (Kerry)		N-Z Amine AS (Kerry)		N-Z Amine EKC (Kerry)		Hy-Case Amino (Kerry)		Amicase (Kerry)		Casein pep- tone Type I (Biotec- nica)
Ile	40	23	41	21	36	5.1	30	18	57	27.5	44.5
Leu	76	65	68	55	69	20	53	39	88	54.7	76.2
Lys	72	58	80	65	73	6.8	58	45	104	53.9	66.0
Met	17	16	12	15	7	5	15	12	29	18.1	23.2
Phe	37	30	40	30	41	8	28	16	46	29.3	41.1
Pro	122	6.6	95	7.9	94	0.5	63	44	130	93.7	86.5
Ser	40	16	44	17	43	2.7	24	19	21	18.2	50.8
Thr	32	15	35	17	33	4.5	20	15	27	21.3	39.1
Trp	NR	23	NR	2.2	NR	7.1	NR	4	NR	24.8	9.5
Tyr	49	11	50	11	42	3.7	10	2	37	0	18.6
Val	48	27	53	29	48	9	39	24	73	40	55.1
Minerals (%)											
Ca	0.02		0.04		0.04		0.03		0.03		0.02
Cl	0.45		0.99		1.18		23.7		0.53		NR
Mg	0.01		0.02		0.02		0.03		0		0.01
Phosphates	0.96		2.85		1.62		1.67		0.10		NR
K	0.08		0.08		0.22		0.02		0.01		1.30
Na	2.56		2.49		1.69		14.4		0.99		2.10
Sulfates	<0.01		0.06		0.33		0.09		0.50		NR
Other					Available Kosher		Available Kosher		Available Kosher		Available Kosher

NR not reported

Fe in soybean meals sourced from regions across the world. While Ravindran et al.'s [104] work focused on the potential impact on feed conversion rates in poultry, the same types of environmental variations may impact microbial performance in fermentation.

2.3.2 Defined N Sources

An alternative to complex protein sources is to supply N (and other nutrients) through chemically defined media (CDM), also known as synthetic medium. With CDM, the exact composition of the medium is known and can be precisely controlled, which can

be especially useful when determining the effects of specific components (e.g., minerals or amino acids) on fermentation performance. Variability inherent in complex protein sources can be considerably reduced.

The components of CDM are usually simple chemical compounds with known characteristics. Some common laboratory media are classified as chemically defined, including M9 minimal salts for the growth of *E. coli* and other bacteria and Czapek-Dox for the growth of fungi. *Pichia pastoris* fermentations are commonly carried out in a basal salt medium (BSM) [41].

Nitrogen can be obtained by most industrially relevant microorganisms from ammonia-containing salts such as $(NH_4)_2SO_4$, NH_4Cl, and $(NH_4)_2HPO_4$. In pH-controlled fermentations, aqueous or gaseous ammonia can serve the dual purpose of a pH control agent and a N source [100]. Nitrates can be used by microalgae, some fungi, and some anaerobic bacteria as the sole N source. Organic N, including urea and monosodium glutamate (MSG), is also a common N source in CDM.

In the absence of complex protein sources, which contain peptides, amino acids, minerals, vitamins, and cofactors, some CDM require balancing to ensure that nutrient requirements are met. Individual or combinations of amino acids may be required depending on the microorganism's ability to synthesize them de novo. A solution of essential trace minerals (see ▶ Sect. 2.5) is frequently incorporated. Vitamins, most commonly biotin and thiamine, are regularly included to promote growth. While some microorganisms are able to grow well on minimal CDM, such as *P. pastoris* on BSM, some can benefit from supplemented defined media. Matthews et al. [80] developed a "rich" CDM for *P. pastoris* containing amino acids, fatty acids, and vitamins, improving growth and heterologous protein expression over BSM-grown cells by reducing the burden of metabolite synthesis.

Strains within species may require substantially different formulations of CDM, particularly with respect to N. This can be true for natural or engineered strains. Cocaign-Bousquet et al. [24] found that a strain of *Lactococcus lactis* isolated from dairy was auxotrophic for Arg, His, and Thr (presumably obtained from the dairy environment in the strain's natural origin), while another *L. lactis* strain isolated from a plant source was prototrophic for all amino acids. In the same study, Cocaign-Bousquet et al. [24] discovered that amino acids alone, without the addition of ammonium salt, could supply all required N for the *L. lactis* strains examined.

Microorganisms may not grow as rapidly on CDM as they do on complex media, but similar or better biomass densities and product formation may be achieved with additional incubation time. Zhang et al. [145] developed a CDM based on $(NH_4)SO_4$ and MSG for the growth of *Streptomyces griseofuscus*, an actinomycete that produces a potential therapeutic compound called physostigmine. Although the total fermentation time on CDM was 65% longer than that in a complex medium, physostigmine yield improved by 76% and DSP in the absence of tryptone and soy peptone was more efficient.

2.4 The Role of Water in Media

With few exceptions, water comprises the vast majority of any growth medium. The mineral and salt content and pH of the water may influence the fermentation process. For food-grade fermentations, municipal water is routinely used. Some processes may require treatment of water by passing through sediment- and micro-filters and a UV chamber to eliminate physical and microbial contaminants present in the water. Reverse osmosis (RO) water is most frequently used in laboratory-scale fermentations. If a microorganism/

process is sensitive to components present in the water, then deionized water may be used. Upon scale-up, the type of water can vary depending on the capabilities of the production facility. Some facilities may have large-scale RO systems, some may only filter municipal water across a large-bore membrane to remove particulates, and some may not treat city water in any way. In some low-margin processes like fuel ethanol fermentations, water may be recovered from DSP (e.g., distillation) and reused during fermentation.

2.5 Minerals

2.5.1 Mineral Elements and Their Roles in Microbial Growth

Ecologically, minerals and microorganisms interact intimately. For example, a lack of Fe in the animal gut could reduce the amount of propionate and butyrate produced by intestinal bacteria, whereas a high level of Fe could promote the development of pathogenic microflora. The presence of Mg on the other hand could improve the thermotolerance of probiotic bacteria *L. rhamnosus* GG, *L. casei* Zhang, and *L. plantarum* P-8 [124]. Conversely, microorganisms play key geoactive roles in the biosphere, particularly in the areas of element bio-transformations and bio-geochemical cycling, metal and mineral transformations, decomposition, bio-weathering, and soil and sediment formation [37].

Regarding the microorganisms themselves, certain minerals play important and indispensable roles in their metabolism and growth (Table 2.7), and thus, these minerals must be available in the growth medium. For the preparation of fermentation media, it should be determined whether essential minerals are present in sufficient quantities, as is often the case with complex media, or must be individually added, as is the case with CDM. Some trace minerals are frequently present in appropriate quantities in the water supply and as impurities in other media ingredients, reducing or eliminating the need for their specific addition.

2.5.2 Macro Minerals

P, S, K, Ca, Mg, and Fe are the six main macro mineral elements required by microorganisms. P is important for energy transduction and is a component of phospholipids, proteins, and nucleic acids. S is a component of some amino acids (methionine and cysteine) and vitamins and also serves as a cofactor. K, Ca, and Mg exist in the cells as cations. K^+ is needed for the activity of a number of enzymes and for ionic balance in yeast and fungi. Ca^{2+} is a cofactor for enzymes such as proteases, is required for the heat resistance of bacterial endospores, and has a possible messenger role in yeast and fungi. Mg^{2+} is needed as a cofactor for many enzymes, ATP complexes, and ribosome and cell membrane stabilization. Fe^{2+} and Fe^{3+} are components of cytochromes and cofactors for enzymes and electron-carrying proteins. Na^+ and Cl^- are major cations and anions in the cell and are thought to have a role in osmoregulation; however, despite their presence at high concentrations in many media, they are often excluded from the cell, being necessary only in micro-molar concentrations.

Macro minerals are often supplied through complex ingredients. For example, molasses provides a supply of P, K, and S. Common forms of delivery are described in Table 2.8 for adding macro minerals to the media or preparing CDM. S is often supplied as SO_4^{-2} together with another desired metal, e.g., $ZnSO_4$.

Table 2.7 Elemental composition of bacteria, yeast, and fungi (% of dry weight) [127]

Element	Bacteria [1, 48, 74]	Yeast [1, 48]	Fungi [1, 73]
C[a]	50–53	45–50	40–63
H[a]	7	7	7–10
N[a]	12–15	7.5–11	7–10
P[a]	2.0–3.0	0.8–2.6	0.4–4.5
S[a]	0.2–1.0	0.01–0.24	0.1–0.5
K[b]	1.0–4.5	1.0–4.0	0.2–2.5
Na[b]	0.5–1.0	0.01–0.1	0.02–0.5
Ca[b]	0.01–1.1	0.1–0.3	0.1–1.4
Mg[b]	0.1–0.5	0.1–0.5	0.1–0.5
Cl[c]	0.5	–	–
Fe[d]	0.02–0.2	0.01–0.5	0.1–0.2

[a]Major elements
[b]Major cations
[c]Major anion
[d]Major transition metal

Table 2.8 Range of typical concentrations of mineral components (g/dm^3) [127]

Component	Range
KH_2PO_4[a]	1.0–4.0 (may be part of a buffering system)
$MgSO4{\cdot}7H_2O$	0.25–3.0
KCl	0.5–12.0
$CaCO_3$	5.0–17.0
$FeSO_4{\cdot}4H_2O$	0.01–0.1
$ZnSO_4{\cdot}8H_2O$	0.1–1.0
$MnSO_4{\cdot}H_2O$	0.01–0.1
$CuSO_4{\cdot}5H_2O$	0.003–0.01
$Na_2MoO_4{\cdot}2H_2O$	0.01–0.1

[a]Complete media derived from plant and animal materials normally contain considerable concentrations of inorganic phosphate

P is commonly supplied in the form of PO_4^{-3} and may serve a dual purpose along with pH buffering. K is often supplied as a phosphate salt or as KCl. Ca is often supplied as $CaCl_2$ and occasionally as $CaSO_4$ or $CaCO_3$. Macro minerals may be required in higher concentrations when directly required for the synthesis of the desired fermentation product. For example, the synthesis of methionine by certain overproducing strains requires high concentrations of S in the medium.

Bioavailability also needs to be considered for both macro and micro (▶ Sect. 2.5.3) minerals that may be unavailable due to precipitation, chelation, or absorption. Precipitation of media components is the most common limitation of bioavailability, hampering the adequate supply of nutrients or interfering with the fermentation process and the monitoring devices. Precipitates can also affect DSP and purification operations. Precipitation occurs when non-soluble complexes of divalent metal ammonium phosphates, magnesium phosphates, and other metal phosphates are formed [29]. An approach to minimizing the formation of insoluble complexes is to sterilize problematic components separately. For example, a sterile solution of $MgSO_4$ is often added to PO_4^{-3}-containing media post-sterilization to prevent the formation of $NH_4MgPO_4.6H_2O$, which is highly insoluble. It is important to balance the relative concentration of different anions (PO_4^{-3}, SO_4^{-2} [119] and Cl^-) and cations (Na^+, Ca^{2+}, NH_4^+, and Mg^{2+}) present in the medium. Precipitation may also form during the fermentation process due to the production of organic acids and CO_2.

2.5.3 Micro Minerals

Mn, Zn, Co, Mo, Ni, and Cu are often cofactors of enzymes, and they are functionally involved in the catalysis of reactions and maintenance of protein structure. These elements are generally required in the micro-molar range. Mn^{2+} helps many enzymes catalyze the transfer of PO_4^{-3} groups. Zn^{2+} is present at the active site of some enzymes. Co^{2+} is a component of vitamin B_{12}. Mo^{2+} is required for N fixation. Ni^{2+} is necessary for urease activity and is reportedly required for the growth of a bacterium (*Alcaligenes* (*Hydrogenomonas*) *eutrophus*), a cyanobacterium (*Oscillatoria*), and a green alga (*Chlorella vulgaris*), but its exact function is not clear [141]. Cu is used as an electron carrier. When these micro minerals are present in insufficient concentrations in the medium, common forms of delivery are shown in ◘ Table 2.8.

As with macro minerals, the addition of micro minerals becomes more significant when they are required for the synthesis of certain fermentation products. For example, Zn is particularly important in alcohol fermentations, as it is a cofactor of alcohol dehydrogenase [75]. Fermentation medium deficient in Zn may support growth, but the production of alcohol will be impaired. Furthermore, microorganisms from different environments may require special mineral elements that reflect the characteristics of their niches. For the growth of marine microbes, sea salt is frequently added to the media. Trace levels of toxic minerals (e.g., Ag, As, Ba, Cs, Cd, Hg, Li, Pb) which may be present in complex ingredients or even water may adversely affect growth of many microbes.

2.5.4 Regulation by Minerals

The concentrations of minerals as well as other nutrients in media can affect the growth of microbial cultures. When growing *E. coli* to low density, all the required nutrients can be added initially into the basal medium. The commonly used complex medium LB allows

for the growth of *E. coli* in a temperature-, pH-, and oxygen-controlled environment up to a cell density of ca. 1 g/L DCW. To accommodate the nutritional requirements of denser cultures, concentrations of medium components must be increased, including P, S, and micro minerals. Most ingredients required for growth also become inhibitory when added at high concentrations. It is well-established [105] that *E. coli* growth is inhibited by nutrients such as glucose at a concentration of >50 g/L, NH_4^+at >3 g/L, Fe at >1.15 g/L, Mg at >8.7 g/L, P at >10 g/L, and Zn at >0.038 g/L. A CDM that contains the maximum non-inhibitive concentration of nutrients can support growth of *E. coli* to a cell density of ca. 15 g/L DCW in batch culture [66, 119].

A common strategy to identify minerals that need to be changed beyond standard concentrations is to vary the amounts of different minerals, while maintaining constant concentrations of other components and measuring the effects on growth and production. Often a mineral at a certain range of concentrations may elicit a significant increase in production due to regulatory effects. A study of *Streptomyces fradiae* medium composition for neomycin production demonstrated that Ca, Fe, and Zn at elevated concentrations improved production, whereas Mn and Cu had no effect [76]. Furthermore, the optimal concentration of Ca, Fe, and Zn for growth differed from the optimal concentration for neomycin production. In the case of citric acid production by *Aspergillus niger*, divalent metals at growth-limiting concentrations markedly improve yield [45]. At elevated concentrations, these metals directly interfere with citric acid production by inhibiting entrance into the citric acid-producing phase. High PO_4^{-3} concentrations can be inhibitory to secondary metabolite production, and PO_4^{-3} inhibition has frequently been demonstrated in antibiotic production [109].

2.6 Growth Factors

2.6.1 Growth Factors and Their Roles in Microbial Growth

Growth factors are biologically active molecules such as amino acids, vitamins, purines, and pyrimidines. Amino acids are necessary for protein synthesis; purines and pyrimidines are the basis for DNA and RNA synthesis; and vitamins have diverse functions including as enzyme cofactors and precursors. Growth factors are classified as essential or accessory. Essential growth factors must be added to media for microorganisms incapable of synthesizing them; if they are absent, then growth cannot occur. Accessory growth factors are added to stimulate the rate and/or density of growth, despite the microorganisms possessing the synthesis capability. Some microorganisms can synthesize all growth factors such as non-sporiferous bacteria (i.e., *Pseudomonas* and *Mycobacterium*) and many molds (i.e., *Aspergillus* and *Penicillium*), and thus these microorganisms can grow on minimal media. However, the addition of even small amounts of growth factors can exert an effect on certain microorganisms and thus need to be taken into consideration in the design of fermentation media.

For microorganisms commonly used in fermentation, there is a wealth of literature detailing essential and accessory growth factors. Taking *Lactococcus* strains as an example, Van Niel and Hahn-Hägerdal [138] describe in depth the nutritional requirements of these microorganisms. For the growth factors, several B group vitamins are described as essential, while others were stimulatory under certain growth conditions. A number of amino acids are described as essential (e.g., valine, leucine, isoleucine, methionine) or stimulatory for different strains of *Lactococcus*. Nucleotides are not essential; however, their addition in

fermentation media stimulates growth. Acetic and lipoic acids can be essential or stimulatory under certain conditions, particularly in the absence of biotin. It is worth keeping in mind that a microorganism's requirement for a growth factor can change depending on the C and N sources provided and on growth conditions such as aerobic or anaerobic environments.

2.6.2 Media Design with Growth Factors

Vitamins

Specific vitamins are frequently essential to microorganisms and must be present in the fermentation medium. For CDM, any vitamins required by the microorganism that cannot be synthesized de novo must be added at an appropriate concentration. For complex media, the precise composition, if known, should be examined to determine if all essential vitamins are present in sufficient quantities. A list of commonly supplied vitamins for bacterial nutrition is described in Table 2.9. Fungi will frequently require specific B group vitamins.

Table 2.9 Vitamins and vitamin precursors commonly used for bacterial nutrition

Vitamin	Co-enzyme form	Function
p-Aminobenzoic acid (PABA)	–	Precursor for the biosynthesis of folic acid
Folic acid	Tetrahydrofolate	Transfer of one-C unit and required for synthesis of thymine, purine bases, serine, methionine, and pantothenate
Biotin	Biotin	Biosynthetic reactions that require CO_2 fixation
Lipoic acid	Lipoamide	Transfer of acyl groups in oxidation of keto acids
Mercaptoethane-sulfonic acid	Coenzyme M	CH_4 production by methanogens
Nicotinic acid	NAD and NADP	Electron carrier in dehydrogenation reactions
Pantothenic acid	Coenzyme A and the acyl carrier protein	Oxidation of keto acids and acyl group carriers in metabolism
Pyridoxine (B_6)	Pyridoxal phosphate	Transamination, deamination, decarboxylation, and racemation of amino acids
Riboflavin (B_2)	FMN and FAD	Oxidoreduction reactions
Thiamine (B_1)	TPP	Decarboxylation of keto acids and transaminase reactions
Vitamin B_{12}	Cobalamine coupled to adenine nucleoside	Transfer of methyl groups
Vitamin K	Quinones and napthoquinones	Electron transport processes

From ▶ http://textbookofbacteriology.net/nutgro_2.html

Although some microorganisms possess the synthesis machinery for some or all vitamins, the addition of certain vitamins, even in small quantities, can greatly enhance growth rate and/or density by eliminating the energy demands for their production. Further to this, certain fermentation scenarios may require specific vitamins to be present at higher concentrations due to their role as cofactors or precursors for synthesis of the product of interest. For all microorganisms, the final concentration of any vitamin in the medium is very low, and so it is standard practice to prepare a filter-sterilized vitamin stock solution to be diluted in the bulk sterile media. All B vitamins are water soluble; however, vitamin K is fat soluble and should be dissolved in ethanol.

Amino Acids

Amino acids may be supplied in the fermentation medium through complex N sources or as individual amino acids in CDM (▶ Sect. 2.3). As with vitamins, it is necessary to identify which amino acids are essential to ensure they are added to CDM or present in sufficient quantities in complex media. Microorganisms can secrete proteases to break down proteins into individual amino acids for transport into the cell, as well as catabolize amino acids. Products from catabolism of amino acids are important in the wine industry, impacting on the aromas of the final product [49]. Another consideration when designing fermentation media is the possibility of amino acid substitutions. These arise when a tRNA is charged with a non-natural amino acid, such as selenomethionine for methionine. The incorporation of non-natural amino acids can be desirable, giving rise to a diverse range of proteinaceous products with potential for novel activities or functions. Desired substitutions can be stimulated by the addition of a high concentration of the desired amino acid to the medium and reduction of the amino acid to be substituted. Undesired substitutions may arise when using complex media where amino acid content cannot be easily controlled.

Nucleotides

Nucleotides are used by microorganisms in the synthesis of DNA and RNA, as precursors in metabolic pathways, and in signaling pathways. Depending on the array of nucleotide synthesis machinery, microorganisms may acquire nucleotides or nucleotide precursors from the environment or synthesize them de novo. Nucleotides or nucleotide precursors must be added to the medium if certain synthesis genes are absent. Often the addition of nucleotides or nucleotide precursors is stimulatory to growth, with the microbial synthesis genes being downregulated in preference for uptake from the environment, allowing for increased dedication of resources to growth and product synthesis.

2.7 Precursors and Metabolic Regulators

2.7.1 The Role of Precursors in Fermentation

Precursors are substances added prior to or during fermentation that are used in the synthesis of the fermentation product of interest. Precursors stimulate the synthesis of the product, increase the yield, or improve the quality of the product and may be provided in complex media or added as a pure compound. Manipulating microbial machinery through the addition of an alternative precursor is a frequently used technique that can give rise to alternative products. The production of β-lactam antibiotics has been

optimized over many decades, with modern manufacturing delivering precursors continuously throughout fermentation [33]. Natural penicillins are produced by *Penicillum* spp. in the absence of a side-chain precursor. However, a greater variety of penicillins (>100), and better control of a specific penicillin, can be produced by adding a side-chain precursor. The most commonly manufactured penicillins, penicillin G and penicillin V, are produced when phenylacetic acid and phenoxyacetic acid are added to the fermentation medium, respectively.

2.7.2 Metabolic Regulators and Their Roles in Fermentation

Microbial regulation by metabolites is tightly controlled and critical to their efficient use of resources, preventing energy spent on futile production of unnecessary metabolites. These regulatory mechanisms can be manipulated or overcome through media design to enable high yield of desirable products. These metabolic regulators are classified as inhibitors, inducers, or enhancers. Metabolic inhibitors dampen or turn off a metabolic pathway and must be absent from the medium in order for the pathway to be activated. Inhibitors can be useful for turning off alternative pathways and allowing activity to be redirected to the pathway of interest. In addition, inhibitors can be used to avoid production of undesirable metabolites, such as those that impact on flavor of food and drink products.

Inducers act to turn on the desired pathway and thus are essential for production of a compound under the control of the pathway. In certain scenarios, more than one inducer or structural analogue of the metabolite may be available, enabling selection of the most economical metabolite. Distinct from inducers, enhancers work to increase flux through a pathway as opposed to turning a pathway on.

Metabolic regulators are integral to industrial production of pectinases by bacteria and fungi. Pectinases hydrolyze pectin, a significant component of fruits, and are extensively used in the food industry, for example, in the clarification of fruit juice. Numerous publications describe complex media that induce pectinase synthesis due to the presence of pectinaceous substances, e.g., orange peel, wheat bran, and rice husk [51]. In contrast, the use of some simple sugars including arabinose, glucose, and galactose, is reported to inhibit pectinase synthesis, likely through catabolite repression.

2.7.3 Media Design with Precursors and Metabolic Regulators

For the preparation of fermentation medium, particularly on a large scale, cost, time, and yield are critical elements. The design of a production medium that includes precursors or metabolic regulators should be conducted with these factors in mind, balancing the potential improvement in production rate and overall yield with the cost of including the precursor/metabolite in the production medium. Identifying an alternative cheaper metabolite or precursor is one way to reduce fermentation costs. Determining the optimal concentration is an additional way to reduce costs and is also necessary to ensure any negative pressures on microbial growth are limited, such as diversion of resources from essential pathways or osmotic pressure. The stage of the fermentation at which the precursor is available – in the initial growth medium, at a particular phase of growth, or as a continuously fed supply – is another important aspect to consider.

2.8 Antifoams

Submerged fermentation of microorganisms generally starts in flasks, tubes, or microwell plates, where initial process development and strain selection can be accomplished in a relatively high-throughput manner. When growth is scaled to bioreactors, it is important to consider other factors that are absent or insignificant in smaller volumes. Not least of these considerations is foaming. Agitation, particularly in aerobic systems, can be high, with impeller tip speeds exceeding 3 m/s. In combination with aeration delivered through a sparger, considerable turbulence is present in a stirred-tank vessel. In conjunction with the nature of cell growth and many fermentation media, these physical conditions can lead to foaming.

Foaming is challenging in several ways. Uncontrolled foaming during fermentation can lead to physical loss of microbial containment and product. It may also result in dangerous overpressure conditions if the exhaust path is blocked by foam fouling the exhaust filter. Foaming itself can cause denaturation of proteins [23], which is particularly problematic if the product of interest is a secreted protein.

2.8.1 Factors that Contribute to Foaming

There are two primary chemical factors contributing to foaming. One is proteinaceous in nature. Proteins can stabilize foam films [99], and many media contain a protein component such as yeast extract. Protein secretion by the microorganism, whether the product of interest or intrinsic proteins, is another source. A second factor is surfactant-like compounds produced by some microorganisms. For example, some species of *Bacillus*, including industrially relevant species like *B. subtilis* and *B. amyloliquefaciens*, produce the lipopeptide surfactin, which is a powerful biosurfactant with antibiotic properties [3]. Regardless of the source of the foam, it must be controlled during the fermentation process.

2.8.2 Antifoam Agents

Chemical antifoam agents are an integral part of fermentation medium design. Their inclusion is necessary to prevent foaming during fermentation. The modes of action depend on the properties of the antifoam and include bridging-dewetting, spreading fluid entrainment, and bridging-stretching [30]. There are scores of different antifoams spanning several chemical families, including silicone emulsions, glycols, and insoluble oils. Potentially nutritive compounds such as vegetable oils can serve a dual purpose as a C source and an antifoam [65]. Although often non-nutritive, antifoams can have considerable impact on cell growth and productivity. Therefore, including them as a variable during the design stages of medium development is critical to ensuring a robust and viable fermentation process.

Antifoam is often added to the growth medium before sterilization. In some cases, this batched antifoam is sufficient to control foaming during the entire course of the fermentation. In other cases, antifoam must be added on-demand during the fermentation as foaming capacity increases. Inclusion of antifoam can reduce the volumetric mass oxygen transfer coefficient, k_{La} [110]. This phenomenon is particularly noticeable when antifoam is added to an in-process fermentation. The sudden addition of antifoam can result in an immediate decrease in dissolved oxygen (DO) due to reduced surface tension in the culture broth (Fig. 2.1).

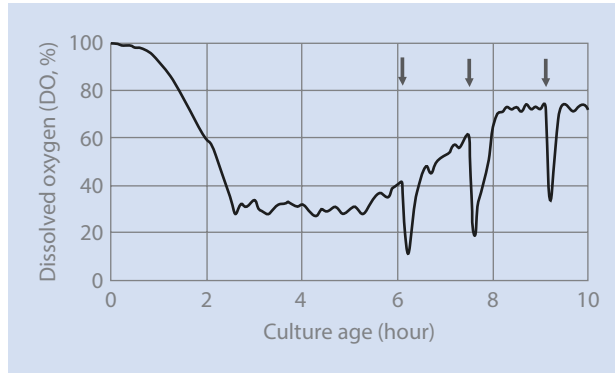


Fig. 2.1 Representative DO profile of an aerobic bacterial fermentation. Arrows indicate the addition of a chemical antifoam agent

Not all antifoams are equal in their defoaming capacity or impact on microbial performance. It is important to screen different types of antifoams to assess the impact on microbial growth, in-process productivity, and DSP. Antifoam 204, a polyether dispersion, is commonly used in recombinant *E. coli* fermentations. However, the endotoxin-free *E. coli* ClearColi® BL21(DE3) host is sensitive to Antifoam 204, with growth stalling in its presence; growth normalizes when a silicone-based emulsion is used in place of Antifoam 204 [4]. Routledge and Bill [111] described an increase in recombinant protein expression from *P. pastoris* upon the inclusion of a range of antifoams in shake flask cultures.

The presence of defoaming agents in whole broth can also complicate DSP. Tangential flow filtration (TFF) membranes are particularly prone to fouling by antifoams [63, 83], which can reduce the ability to separate material by micro- or ultrafiltration. A few recent entries into the market, such as Xiameter 1920-AFE, carry claims that they do not reduce bacterial growth or product formation and do not permanently foul TFF membranes (► www.xiameter.com, also Lecompte 2012 – Dow product information). It is prudent to seek input from DSP scientists when selecting antifoams.

If it is determined that antifoams are incompatible with the fermentation process, whether due to sensitivity of the strain to defoaming agents or incompatibility with DSP, there are some limited alternatives to controlling foam. Increasing back pressure in pressurizable vessels may reduce the foam head to a small degree. Mechanical approaches include the "stirring as foam disruption" technique, which employs strategically placed impellers to minimize the formation of foam [12]. Overall agitation and aeration delivered to the fermentation culture can be manipulated to minimize foam formation, although these actions are likely to have considerable effects on k_{La}.

2.9 Solid State Fermentation Substrates

In the field of biotechnology, fermentation generally means "controlled cultivation of microorganisms" rather than the technical metabolic definition of the term [84]. Solid-state fermentation (SSF) is a "cultivation technique in which microorganisms are grown under controlled conditions on moist solid particles, in beds within which there is a continuous gas phase between the particles, and sufficient moisture is present to maintain microbial growth and metabolism" [61, 78, 85, 102]. SSF as a feasible biotechnology process is still in an evolutionary state and under intensive research [61].

SSF substrates can be anything that is solid and contains nutrients serving as a medium supporting the growth of microorganisms. In practice, these solid particles could be raw cereals or the by-products and wastes of agriculture (e.g., soy cakes). This section will mainly discuss SSF nutrients that themselves have a high value. By-products or wastes with low value used as nutrients for SSF are discussed in ▶ Sect. 2.10.

SSF has been widely practiced in East Asia to manufacture fermented foods such as soya sauce and sake, while the Western world occasionally exploits SSF for the manufacture of antibiotics and enzymes [81]. Compared to submerged liquid fermentation (SLF), SSF is eco-friendly, resource-saving, and high yielding but presents considerable challenges for upscaling and process control [132].

2.9.1 Materials for Making Traditional Fermented Foods

Soy is a rich source of proteins and has been used in Asian countries as a protein source for thousands of years. Soy also has potential effects on health, such as cardiovascular risk reduction or, conversely, on the possible disruption of thyroid function and sex hormones due to isoflavones, which are polyphenols with estrogenic properties [107]. Historically, a variety of fermented food have been made from raw and processed soybeans. Some of the examples of SSF applications in traditional fermented foods are given in ◘ Table 2.10.

Miso is a traditional Japanese food fermented from rice or barley, cooked soybean, and salt. Cooked rice or barley is inoculated with *Aspergillus oryzae* and then incubated to allow for fungal growth, commonly known as koji in Japanese. Koji is the starter for fermentation of cooked soybean, and following this final fermentation, the soybeans are blended to give the product miso [118]. Besides *A. oryzae*, lactic acid bacteria (LAB) and yeast are also involved in the fermentation [20].

Soy sauces are light to dark brown cooking and table condiments widely used in the cuisines of China, Japan, Korea, Thailand, the Philippines, Indonesia, and Malaysia. The fermentation process for making soy sauce includes two stages. The first stage is to prepare koji with cooked soybeans and the filamentous fungus *A. oryzae* or *Aspergillus sojae*, and the second stage is a brine fermentation with LAB and the yeast *Zygosaccharomyces rouxii* [2]. The Korean chungkukjang and Chinese fermented black beans, which is the oldest known soyfood worldwide [120], are fermented in a similar way [54]. After fermentation and during aging, the flavor, color, and bioactive components of these products change [19, 54].

Food-grade cereals are rich in starch and have been used in SSF for brewing alcoholic beverages for thousands of years in East Asia. Sake is one example of Japanese SSF. Dehusked rice is polished, washed and soaked in water, steamed, and inoculated with the koji fungus *A. oryzae*, and then, SSF is carried out at around 30 °C for about 2 days. Following this SSF, the prepared koji is blended with steamed rice and SLF is initiated [53].

In addition to rice, other cereals have also been used in SSF to produce beverages. One example is Chinese liquor (baijiu in Chinese) which varies according to production techniques (SSF and semi-SSF), types of starter cultures, and the dominant flavors of the end products [149]. There are five stages for making Chinese liquor: (1) steaming the cereals, (2) koji (jiuqu in Chinese) preparation; (3) SSF; (4) solid-state distillation; and (5) aging [56, 149]. Technically, the first two stages are similar to sake making. For baijiu, rice, sorghum, wheat, glutinous rice, and maize are used alone or in combinations developed by manufacturers in a long history of tradition. The starter (koji) is made from raw wheat, barley, and/

Table 2.10 Materials used in traditional fermented foods and additives by SSF

Substrate	Organism[a]	Product	References
Soy and cereals			
Soybean	*A. oryzae*, LAB and yeast	Miso	Shibasaki and Hesseltine [118], Chiou et al. [20]
Soybean (usually black soybeans)	*A. oryzae* and LAB	Fermented black bean	Shurtleff and Aoyagi [120], Chen et al. [16]
Soybean	*B. subtilis*	Chungkukjang	Jeong et al. [54]
Soybean	*A. oryzae*, *A. sojae*	Koji for making soy sauce	Aidoo et al. [2]
Soy bean curd (doufu)	*Actinomucor* spp., *Mucor* spp., and *Rhizopus* spp.	Sufu/furu	Cheng et al. [18]
Soybean, cereals, or combinations	Mainly *Rhizopus oligosporus* and LAB	Tempeh	Babu et al. [7]
Rice	*A. oryzae*	Sake	Japan Sake and Shochu Makers Association [53]
Rice, sorghum, wheat, glutinous rice, and maize, alone or in combination	Bacteria, yeast, filamentous fungi and actinomycetes	Chinese liquor	Zheng and Han [149]
Rice	*Monascus purpureus* and other *Monascus* spp.	Red yeast rice (food additive)	Pratoomchai [97], Erdogrul and Azirak [34]
Dairy materials			
Milk	LAB, fungi (e.g., *Penicillium*) and yeast (e.g., *D. hansenii*)	Cheese	Ghosh [42]

[a]*A. oryzae* and *A. sojae* are among the most common fungi used in traditional fermented soy food in East Asia, and these domesticated fungal strains do not produce mycotoxin aflatoxin, a major threat to human health, due to gene deletions and mutation in their aflatoxin gene clusters [15, 69, 148]

or peas by SSF with the microorganisms (bacteria, yeast, and filamentous fungi) naturally inoculated from their environment. For SSF, steamed cereals are combined with koji in earthen jars dug in the ground or in mud pits depending on the tradition of the specific variety of baijiu. SSF is carried out under anaerobic conditions, during which the bacteria naturally present in the walls of the mud pit also contribute to fermentation.

Cheese manufacturing involves two stages of fermentation [42]. In the first stage of cheese manufacturing, pasteurized milk is fermented with *Lactococcus* spp. in SLF to lower the pH for the next step of casein precipitation by rennet. The final stage, which is called the ripening or maturation of the cheese, involves SSF. In this stage, bacteria (mostly LAB or *Propionibacterium* spp.), fungi such as *Penicillium*, or some yeast such as

Debaryomyces hansenii are metabolically active at low levels and contribute greatly to the character and flavor of the cheese.

SSF has also been practiced in the production of food additives. Red yeast rice (known in various regions as RYR, ang-kak, red mold rice, hong qu in Chinese, and red koji and beni-koji in Japanese) is a traditional Chinese food product that has been documented for more than a thousand years. It is used as a flavoring, colorant, and preservative in cooking and medicinally for blood circulation and food digestion properties and continues to be used a dietary staple in numerous Asian countries today [88].

The material for making RYR is cooked nonglutinous whole rice kernel. The production of RYR is achieved by the following steps: the rice is rinsed and soaked in water, drained, steamed, sterilized, fermented with *Monascus* sp., and dried. The optimal cultivation temperature is in the range of 25–30 °C for growth and pigment production for most species, while temperatures above 35 °C inhibit lovastatin production. Optimal aeration, pH, and concentrations of critical elements in the solid substrate can provide a good yield of pigment with low citrinin production [34, 97].

2.9.2 Materials for the Production of Enzymes, Antibiotics, and Biofuels

Actinobacteria (especially actinomycetes) and fungi (especially filamentous fungi) are versatile antibiotic and enzyme producers for industrial applications. With new approaches in microbiology, biochemistry, and biochemical engineering, SSF has been used in the production of antibiotics and enzymes using inexpensive agricultural by-products (for more details, see ▶ Sect. 2.10). These basal substrates may be supplemented with ingredients such as glucose, phosphates, and minerals; SSF production of the lipopeptide antibiotic iturin on okara as a base substrate supplemented with glucose, KH_2PO_4, and $MgSO_4 \cdot 7H_2O$ is a prime example [5]. Food waste and hydrolyzed cellulose-rich agricultural by-products can be used as substrates for microbial growth and the transformation of C sources to ethanol as biofuel. Some examples are listed in ◘ Table 2.11.

2.10 Waste/Alternative Substrates

Raw materials or organic wastes, especially those from agricultural industries (e.g., straw), forestry (e.g., sawdust, wood chips), and urban sources (e.g., food waste), are rich in minerals, and C and N that can be utilized by microorganisms under optimized fermentation conditions (◘ Table 2.12). This opens up great possibilities for producing value-added products such as cellular proteins, organic acids, edible and medicinal mushrooms, biologically important secondary metabolites, enzymes, prebiotic oligosaccharides, and ethanol [79] at lower cost compared with standard fermentation approaches.

2.10.1 Waste Carbon Sources

C wastes can provide energy to heterotrophic organisms and some autotrophic organisms. C wastes are diverse with regard to their forms, nutritional composition, concentrations, and interference from other coexisting materials (see ◘ Table 2.12). Agricultural

Table 2.11 Materials used in production of enzymes, antibiotics, and biofuels by SSF

Substrate	Organism	Product	References
		Enzyme	
Wood chips	*Trichoderma reesei*	(Hemi) cellulase	Xin and Geng [142]
Grape pomace	*Aspergillus awamori*	Xylanase, pectinase	Botella et al. [13]
Lemon peel	*A. oryzae*	Pectin lyase	Koser et al. [60]
Sunflower meal	*A. niger*	Acid protease	Mukhtar and Ikram-Ul-Haq [87]
Coconut oil cake	*R. oligosporus*	Phytase	Sabu et al. [113]
Potato peel	*Pleurotus ostreatus*	Laccase, peroxidases	Ergun and Urek [35]
		Medicinal	
Various (cereal brans, legume husks)	*Amycolatopsis mediterranei*	Rifamycin SV	Nagavalli et al. [89]
Okara	*B. subtilis*	Iturin A	Ano et al. [5]
Wheat bran	*Aspergillus terreus*	Lovastatin	Kamath et al. [58]
Rice	*M. purpureus*	Monacolin K	Priatni et al. [98]
		Biofuel	
Sweet sorghum stalk	*Saccharomyces cerevisiae*, *Issatchenkia orientalis*	Bioethanol	Chen et al. [17], Du et al. [32]
High-starch food wastes (e.g., bread crust)	*Saccharomyces* sp.	Bioethanol	Moukamnerd et al. [86]

and forestry wastes are valuable sources of lignocellulosic materials. Lignocellulose is the main structural constituent of plants and represents a significant source of renewable organic matter for the production of products such as industrial enzymes and ethanol (Table 2.11). C wastes from other industries (e.g., wheat bran which is rich in carbohydrates, proteins, and dietary fibers for human health [8]) have been explored as SSF substrates. Some examples of waste materials used in SSF are shown in Table 2.13.

2.10.2 Waste Nitrogen-Rich Sources

Food-grade protein-rich materials have been applied in fermentation as described in Table 2.10 (e.g., fermented soy products), but in general, low C:N ratio materials are difficult to use as the main substrate for SSF. Nevertheless, it is not surprising that some high-protein wastes have been used in SSF. Soybean wastes, including okara and soy whey from bean curd manufacturing, are rich in proteins, with protein contents of 16–33%

Table 2.12 Composition of some selected raw materials as microbial nutrients

Component	Molasses	Malt wort	Wine must	Cheese whey	CSL
C sources	Sucrose, fructose, glucose, raffinose	Maltose, sucrose, fructose, glucose, maltotriose	Glucose, fructose, sucrose (trace)	Lactose	Glucose, other sugars
N sources	N compounds as unassimilable proteins. N sources need to be supplied	Low-molecular α-amino N compounds, NH_4^+, and a range of amino acids	Variable levels of NH_4^+, which may be limiting. Range of amino acids	Unassimilable globulin and albumin proteins. Low level of ammonium and urea nitrogen	Amino acids, protein
Minerals	Supply of P, K, and S available. High K levels may be inhibitory	Supply of P, K, Mg, and S available	Supply of P, K, Mg, and S available. High levels of SO_3^{2-} often present	Supply of P, K, Mg, and S	Supply of P, K, Mg, and S
Vitamins	Small, but generally adequate supplies. Biotin is deficient in beet molasses	Supply of vitamins is usually adequate. High adjunct sugar wort may be deficient in biotin	Vitamin supply generally sufficient	Wide range of vitamins present	Biotin, pyridoxine, thiamin
Trace elements	Range of trace metals present, although Mn^{2+} may be limiting	All supplied, although Zn^{2+} may be limiting	Sufficient quantities available	Fe, Zn, Mn, Ca, and Cu present	Range of trace elements present
Other components	Unfermentable sugars (2–4%), organic acids, waxes, pigments, silica, pesticide residues, caramelized compounds, betaine	Unfermentable maltodextrins, pyrazines, hop compounds	Unfermentable pentose, tartaric and malic acids. Decanoic and octanoic acids may be inhibitory. May be deficient in sterols and unsaturated fatty acids	Lipids, NaCl, lactic and citric acids	High levels of lactic acid present. Fat and fiber also present

Adapted from Walker and White [139]

Table 2.13 Agricultural, dairy, and other industrial wastes, properties, and applications

Waste	Properties	Application/ product	References
Agricultural and dairy waste			
Molasses	*B. subtilis*	Surfactin	Makkar and Cameotra [77]
Cassava flour wastewater	*B. subtilis* ATCC 21332; *B. subtilis* LB5	Lipopeptide	Nitschke and Pastore [92, 93]
Potato waste	*B. subtilis*	Surfactin	Noah et al. [94], Thompson et al. [133], Fox and Bala [36]
Peat hydrolysate	*B. subtilis*	Surfactin	Sheppard and Mulligan [117]
Wheat bran	*B. subtilis* NB22 (recombinant)	Lipopetide-surfactin	Ohno et al. [96]
Soy molasses-based medium	*Candida bombicola*	Sophorolipids	Solaiman et al. [125, 126]
Animal fat	*C. bombicola*	Sophorolipid	Deshpande and Daniels [31]
Whey and rapeseed oil	*C. bombicola*	Sophorolipids	Daniel et al. [26, 27]
Okara, peanut press cake, etc.	*Neurospora sitophila*, *R. oligosporus*	Oncom	Surono [129], Sastraatmadja et al. [115]
Other industrial waste			
Soap stock oil	*Acinetobacter calcoaceticus*	Expolysaccharide	Shabtai [116]
Lubricating oil	*Bacillus* sp.	Lipopeptide	Mercade et al. [82]
Oil refinery waste	*Candida antarctica, Candida apicola*	Glycolipids	Deshpande and Daniels [31]
Industrial residue	*C. lipolytica*	Biosurfactant	Rufino et al. [112]
Rubber (e.g., used tyres)	Fungi (e.g., *Penicillium chrysogenum*), bacteria (e.g., *Streptomyces labedae*)	Isoprene derivatives	Nayanashree and Thippeswamy [90], Hesham et al. [50]
Plastics (e.g., polyethylene)	Fungi (e.g., *Fusarium* spp.), bacteria (e.g., *Bacillus* sp.)	Carbon metabolites	Yang et al. [144], Kale et al. [57]

(of dry weight) and 3% (w/v), respectively [64, 71]. Okara has been fermented with *Actinomucor elegans* (meitauza), *A. oryzae* (koji), *Neurospora intermedia* (ontjom), and *R. oligosporus* (tempe) to make cholesterol-reducing functional food, and with *B. subtilis* and *Penicillium simplicissimum* to produce bioactive metabolites including surfactin; iturin A (fungicidal); okaramines A, B, D, and F (D is insecticidal); oleanane triterpene; and two dihydroquinolinones [95]. Okara and peanut pressed cakes, after the oil has been expressed, have also been used to produce the traditional Indonesian food oncom by SSF

(Table 2.13). Soy whey can be fermented to soy alcoholic beverage using commercial *S. cerevisiae* strains [22]. Similarly, high-protein content cheese whey has been fermented to produce high-protein beverages [21]. Protein-rich wastes have also been used to produce biogas [62, 114].

2.11 Medium Optimization

While raw materials comprising a fermentation medium contribute to the overall expense of a fermentation process, they are but a fraction of the overall cost. The collective costs of capital equipment, utilities, and skilled labor are far greater than all but the most expensive media. Nevertheless, an optimized medium can have tremendous influence on product yield and fermentation productivity, which ultimately determines the economic viability of a fermentation process.

Medium optimization can be conducted at various scales, with benefits and detractors for each. High-throughput, low-volume platforms using microwell plates and automated analysis (e.g., OmniLog®) are particularly useful for identifying metabolic profiles by screening the effects of a wide variety of nutrients and conditions. These systems are expensive and may not be available to most researchers. A less automated approach using 96-well plates and a standard plate reader to assess biomass growth is more achievable in the average lab. Environmental conditions, such as mixing and oxygen transfer rate, may not be reflective of bioreactor conditions, and these platforms lack pH and DO control. How a microorganism responds to a growth medium may be highly influenced by DO levels or changes in pH.

Tubes and flasks are the most common starting point for fermentation experiments, including medium optimization, and can be considered a medium-throughput, medium-volume platform. No specialized equipment is required. These can easily be run in duplicate for more valid analysis. A primary disadvantage of tubes and flasks is the inability to control critical parameters such as pH and DO in a consistent, simple manner.

Over the past decade, microbioreactors capable of independently controlling pH, DO, temperature, aeration, and agitation have been developed. Examples of these systems include ambr15® (10–15 mL working volume) and DASbox® (60–250 mL working volume). Microbioreactors mimic most features of standard bioreactors and can serve as excellent platforms for media design and optimization in the context of real-life bioreactor conditions. However, the cost of these systems may be prohibitive.

Conducting medium optimization in bench-scale bioreactors (0.5–10.0 L) can reflect the most true-to-life conditions a microorganism may encounter in a scaled fermentation. Multiple bench-scale bioreactors can be run in parallel, allowing for duplicates. As with microbioreactors, agitation, temperature, pH, and DO can be independently controlled and monitored in bench-scale bioreactors. It is common to use a combination of platforms during media optimization, starting with small-volume experiments to narrow the variables and finishing with refinement in bioreactors.

A basic medium with a limited number of factors may be relatively simple to optimize without complex experiments. As the number of factors increases, it becomes impractical to assess the effects of each variable individually. There are also possible interactions between factors that may be critical to process performance. Using proper design of experiments (DOE) to determine critical factors and optimize a process is an important component of medium optimization.

Table 2.14 Common DOE methods for medium optimization

Method	Description	Comments
Full factorial	Design in which all combinations of factors are independently varied at two or more levels	Can require a prohibitively large number of experiments
Fractional factorial	Design in which well-understood factors and combinations are varied	Can maximize efficiency. Multiple designs available (see below). Often requires software or complex mathematics to evaluate
Plackett-Burman (PBD)	Two-level design to evaluate *n* factors in *n* + 1 experiments (*n* + 1 must be a factor of 4)	Good for determining the relative importance of factors. Can mask interactions between factors
Central composite (CCD)	Full or fractional factorial design center and axial points that can fit a quadratic model	Useful for response surface methodology (RSM). Interactions between factors can be determined. Various designs possible
Box-Behnken (BBD)	Alternative design to CCD without full or fractional factorials, without axial points	Useful for RSM. For ≤3 factors, fewer experiments required over CCD

Adapted from Singh et al. [122] and ► https://support.minitab.com

Entire books (e.g., Rodrigues and Iemma [108]) have been written on the topic of statistical approaches to process optimization. There are scores of statistically valid methods and designs that have been developed over the years. Some of the most common DOE methods used in fermentation medium optimization are outlined in Table 2.14. Which DOE method to use will depend on the complexity of the medium, the available time and resources, and the goals of the project. Most of the commercially available statistical software packages (e.g., JMP® or Minitab®) include DOE assistance to select the most appropriate method for the factors to be tested in a given number of experiments.

Medium optimization is frequently a multistage endeavor, starting with standard media compositions and knowledge gleaned from literature. A screening study, often based on the Plackett-Burman design (PBD), can determine the relative importance of various factors. Refinement of the formulation can be done with response surface methodology (RSM) using the central composite design (CCD) or the Box-Behnken design (BBD). The resulting predicted optimal formulation can be confirmed in a final set of studies.

A prime example in which DOE can be used effectively can be found in the work of Zhang et al. [146]. They studied 57 individual components described in various CDM formulations for the growth of *L. lactis*, with the goal of developing an optimized CDM supporting superior biomass growth over existing CDM and complex media. A full-factorial approach examining only two levels of each component would necessitate 2^{57}, or well over one hundred quadrillion, individual cultures. Even with the most sophisticated high-throughput system and unlimited funds, this is an impractical approach.

Instead, Zhang et al. [146] took a three-step approach toward media optimization. To identify the importance of each component, they employed a single-omission technique in 96-well plates, leaving out one component at a time from the 57-component CDM. From this first step, they narrowed the factors to 19 critical component groups, plus two environmental conditions (temperature and pH). They created a two-level fractional factorial design that was conducted across three batches of experiments in 96-well plates. Each batch consisted of 32 experimental conditions and five center points.

In the final optimization step, Zhang et al. [146] selected the five most significant variables identified in the second step and conducted a central composite response surface set of experiments. A polynomial curve was fitted to the resulting data, which was used to generate a predicted optimum formulation. When tested in 25-mL tube cultures, the optimized conditions supported *L. lactis* growth that was over five times better than an earlier CDM. These optimized conditions were developed across 161 experiments, a manageable number and a far cry from 2^{57}, demonstrating the power of DOE for the fermentation scientist.

2.12 Optimized Media for Recombinant Proteins

2.12.1 Strategies for the Expression of Recombinant Proteins

Despite the rise in use of mammalian, plant, and insect systems for recombinant protein expression, yeast and bacterial expression systems still remain a simple, high-yield, and cost-effective choice. Of the biopharmaceuticals approved for use 2004–2013, 24% were produced in *E. coli* and 13% in *S. cerevisiae* [135]. Recombinantly expressed proteins in *E. coli* can represent greater than 30% of the total cell protein [121], with medium optimization playing an important role in attaining high yields. Developing fermentation media for recombinant protein expression requires understanding of any induction and gene maintenance requirements and the risks of proteolytic activity which may compromise the expressed protein.

Recombinant proteins can be expressed constitutively or they can be induced. Inducing expression of a recombinant protein offers several advantages. It enables greater control of the growth stage during which the protein is not synthesized, reduces the burden on cells carrying a plasmid, enables yield optimization, and limits any toxicity of high concentrations of the product to the host. Induction requirements vary depending on the expression system, and where an inducer is added to the medium, the concentration needs to be optimized to balance low yield with potential toxicity arising from high intracellular concentrations. Gene maintenance is ensured in the form of a selection pressure such as the addition of an antibiotic or other medium component. Induction and selective medium requirements are described further in ► Sects. 2.12.2 and 2.12.3.

For recombinant protein expression, and indeed any fermentation process for the production of a proteinaceous material, proteases can have a role in reducing overall yield. Medium optimization can limit their effects, either through the addition of a protease inhibitor, which is often an expensive approach, or by introducing unfavorable conditions for protease synthesis or activity. For example, pH-versatile microorganisms could be grown at a pH that is unfavorable for a problematic protease.

2.12.2 Gene Maintenance and Induction of Protein Synthesis in Bacteria

2

E. coli is the workhorse for bacterial recombinant protein expression and can be used with a variety of well-established plasmid systems. Media requirements for induced or auto-induced expression are dependent on the promoter, with those described in Table 2.15 routinely used. Lactose or the non-hydrolyzable analog iso-propyl β-D-thiogalactopyranoside (IPTG) induces expression from *lac*, *tac*, *trc,* and T7 promoters. Glucose inhibits *lac* operon-based promoters, so either glucose concentrations during expression must be kept low or an alternative primary C source can be used, although glucose-insensitive *lac*-based promoters are available. Furthermore, low glucose concentrations can be useful for preventing leaky expression prior to induction. Auto-induction medium involves providing a limited amount of glucose together with lactose. Glucose is preferentially used and once depleted a switch to lactose occurs, inducing expression. Auto-induction enables the bacteria to reach a suitable density prior to induction to facilitate high product yields. As an aside, glucose as a C source must be carefully controlled for recombinant protein production as acetate and lactate readily accumulate in the presence of excess glucose, which limit cell growth and recombinant protein synthesis.

The *araBAD* and *rhaBAD* promoters are arabinose- and rhamnose-inducible, respectively. Similar to *lac*-based promoters, expression can be auto-induced by incorporating low concentrations of glucose together with the inducer. In contrast, the *phoA* promoter is repressed in the presence of phosphate and induced during phosphate starvation; thus, phosphate needs to be balanced at concentrations to allow sufficient growth prior to depletion. The *pL* phage lambda promoters respond to temperature change and are useful for avoiding the addition of costly inducers. However, this must be balanced with associated stress responses due to increased temperature, and differences in temperature transfer should be considered when scaling up. The cold-inducible promoter *cspA* has the added benefit of not inducing a stress response and can be favorable for promoting proper protein folding.

Antibiotic selection markers are routinely used for maintenance of plasmids in bacteria; however, antibiotics represent a significant cost in large-scale production. Furthermore, for antibiotic resistance conferred through degradation, the antibiotic may need to be fed repeatedly throughout the fermentation. Tetracycline is often favorable for high-density

Table 2.15 Routinely used inducers for recombinant protein expression in *E. coli*

Promoter	Inducer	Conditions
lac, *tac*, *trc*, T5*lac*, T7*lac*	Lactose, IPTG	0.05–2 mM; auto-induction
araBAD	Arabinose	0.5–20 mM; auto-induction
rhaBAD	Rhamnose	0.025–4 mM; auto-induction
phoA	Phosphate starvation	Auto-induction
pL	Temperature change	From 37 °C to 42 °C
cspA	Temperature change	From 37 °C to 15 °C

and/or large-scale production because resistance is through an export pump and thus does not result in antibiotic degradation. Antibiotic-free plasmid selection systems, such as those that encode an essential gene deleted from the chromosome (auxotrophic selection), can be a cheaper alternative. Stably integrating the gene expression cassette into the genome also avoids the need for selection; however, this approach must be weighed against the inability to incorporate multiple copies of the gene that plasmid-based expression provides.

2.12.3 Gene Maintenance and Induction of Protein Synthesis in Yeast

As a simple eukaryote system, yeast expression systems are frequently used for recombinant protein synthesis and are particularly useful when proteins require post-translational modifications. Common yeast strains for recombinant protein expression are *S. cerevisiae*, *P. pastoris*, and *Hansenula polymorpha*. Inducible gene expression, to separate growth phase from production phase, frequently involves changes in a catabolite or an environmental condition. Induction through changes in the C source is often the simplest and most common method, and thus media formulation must be carefully considered. As with expression in *E. coli*, consideration needs to be given to the system used and the cost of the associated inducer. Optimization studies to identify the optimal concentration or delivery time become important when producing recombinant protein on an industrial scale. For example, it may be possible to reduce the length of the induction phase significantly by growing the yeast to a higher density prior to induction. Alternatively, a constitutive promoter requires no inducer and the yeast can often be grown on a variety of C sources; however, this approach must be balanced with potential cytotoxicity from continuous protein production.

A common gene expression system in *P. pastoris* exploits the two alcohol oxidase genes (*AOX1* and *AOX2*) that allow this yeast to grow on methanol as a sole C source. The *AOX1* recombinant gene expression cassette inserted into the genome enables expression to be induced through adjustments to the C source. The changes in the C source depend on whether the host has been made Mut^+, Mut^s, or Mut^- as a result of inserting the expression cassette. Initially, a glycerol batch phase is followed by a glycerol-fed phase releasing limited glycerol to repress the *AOX1* promoter which allows for accumulation of biomass. To induce expression, the C source is switched to methanol (Mut^+, Mut^s), or methanol is delivered in combination with glycerol (Mut^-). The glutathione-dependent formaldehyde dehydrogenase (*FLD1*) promoter can also be induced by methanol alone or by methylamine in the presence of glucose or glycerol.

Similar to *P. pastoris*, *H. polymorpha* is methylotrophic, and the promoters for two genes for methanol utilization, formate dehydrogenase (*FMD*) and methanol oxidase (*MOX*), are used in recombinant gene expression cassettes. Expression from *FMD* or *MOX* is repressed by glucose and induced by glycerol or glycerol and methanol, with methanol generating stronger induction. Being highly flammable, methanol presents an added risk in fermentation, and so for regular production, it may be beneficial to use non-methanol inducible or constitutive systems.

In *S. cerevisiae*, the *GAL* expression system (*GAL1* and *GAL10*) is frequently used, with a growth phase in the presence of glucose that represses the *GAL* promoters, followed by an induction phase with galactose upon glucose depletion. In contrast, the *MET25* promoter

is repressed by L-methionine, requiring a limited initial concentration of L-methionine so that it may be completely metabolized for auto-induction of protein expression.

Recombinant gene expression cassettes in yeast can be selected for with an antifungal resistance gene or more commonly an auxotrophic marker. Auxotrophic selection markers, such as nucleotide or amino acid synthesis genes, require that the final formulation of the growth medium does not contain the nutrient. The use of stably integrated recombinant genes avoids the need for including costly antifungal compounds in the medium or modifications to the medium to accommodate auxotrophic selection.

2.13 Considerations for Scale-Up

Fermentation process development starts in the lab. Cost, optimal productivity, and process consistency are frequently secondary to proof-of-concept or experimental goals. When designing a fermentation process for commercial-scale production, a different approach is warranted. With specific respect to fermentation media development, it is best to work from the beginning with an understanding of the types of ingredients that will be permitted, economical, and available at the ultimate production scale.

It is important to know the limitations of the production facility before developing media. Depending on the facility, there may be restrictions on the types of ingredients permitted in a process. It is inefficient to dedicate time and resources to developing an optimized medium only to learn that one ingredient in the medium is not permitted. For example, facilities that are certified as kosher or halal will not accept ingredients derived from porcine material. This restriction extends to the source of the enzyme used to hydrolyze an otherwise permitted protein. Some tryptones fall into this category, where the protein being digested (casein) is permitted, but the enzyme used to digest it (trypsin) may be sourced from porcine pancreas. Another common restriction is on bovine-sourced materials to prevent the possible contamination of the facility with the causative agent of bovine spongiform encephalitis. Some facilities do not accept any animal-sourced materials as a safeguard.

Many standard microbiological media can be purchased in prepared, dehydrated form, including LB, nutrient broth, PDB, and TSB. At lab-scale, these commercial preparations are common and convenient. When using one of these formulations as the foundation of a fermentation process, it is useful to consider the target scale and determine whether preparing the medium in-house with bulk, industrial ingredients is more cost-efficient. An additional advantage of preparing such formulations in-house is the ability to substitute more appropriate components (e.g., vegetable peptones for animal-derived peptones) and to optimize individual sources (e.g., a specific type of yeast extract).

When taking a fermentation process to production-scale, it is important to understand whether and how the grades, sources, and brands of ingredients impact the overall process. The most common chemical grades, from highest to lowest purity, are American Chemical Society, reagent, United States Pharmacopeia/National Formulary, laboratory, purified, and technical. Lower-grade materials may contain considerable levels of impurities, including flow agents and anticaking agents, which may impact the growth and productivity of specific microorganisms. They are also largely unsuitable for food or pharmaceutical use. If the product is for use in animals, ensure that the ingredients used in fermentation are acceptable for use in the target animal(s).

With few exceptions, fermentation media ingredients are available through multiple suppliers. Yeast extracts, for example, can be purchased from local laboratory supply

houses and multinational distributors or directly from manufacturers. As noted in ▶ Sect. 2.3, although all yeast extracts have similarities, there are differences that may impact fermentation performance. Once a fermentation medium has been designed, it can be worthwhile to test ingredients sourced from multiple suppliers. Not only can this exercise highlight the robustness of the process, it can give the researcher peace of mind knowing whether an ingredient sourced from different suppliers can be substituted should a supply chain fail during production. A good rule of thumb is to qualify no fewer than two suppliers for each ingredient.

Simplicity is a good goal when developing fermentation media. While it may be practical and relatively easy to filter-sterilize multiple components of a medium separately and aseptically combine them in a small bioreactor in the lab, to follow the same procedure at large scale, especially in a cost-sensitive process, may prove to be unfeasible. Inclusion of heat-labile compounds may be unavoidable, but only those compounds that absolutely cannot be heat-sterilized should be filter-sterilized. Sterilizing filters can be costly, and post-sterilization additions contribute to the risk of contamination.

Ingredients that are routinely used at laboratory scale may prove to be prohibitively expensive at production scale. The use of antibiotics is a good example. As noted in ▶ Sect. 2.12.2, antibiotics are useful tools to exert selective pressure on cultures, to maintain plasmid stability in the culture, and to help control possible contamination. At small scale, the cost of antibiotics may be manageable. However, especially in cost-sensitive processes, antibiotics become a considerable raw material cost at large scale. It may be practical to include antibiotic pressure in the seed train but not in the production medium but only after removal of the antibiotic selective pressure in the production culture is tested at laboratory -scale.

One type of sterilization process commonly found in large-scale fermentation production facilities is called continuous sterilization. Continuous sterilization differs from batch sterilization (akin to autoclave sterilization of medium in laboratory settings) in that the medium components are exposed to high temperature (e.g., 140 °C) for a shorter period of time (e.g., 30 seconds) followed by rapid cooling through the use of a series of heat exchangers. A key advantage of continuous sterilization is the reduced time at which the medium is exposed to high temperature, resulting in less damage (e.g., scorching) of the medium. Another advantage is the ability to heat-sterilize individual medium components as separate solutions, allowing for otherwise incompatible ingredients to be treated within the same sterilization cycle. For example, a concentrated solution containing proteins and phosphates can be sent through the continuous sterilizer, chased by a bolus of water to flush the system, followed by a concentrated solution of sugar and Mg^{2+} salts. In this manner, there is no interaction of sugars and proteins or phosphates and Mg^{2+} until the sterilized ingredients are at a safe, nonreactive temperature. Continuous sterilization can be difficult to mimic at laboratory scale, so evaluating the effects of high-temperature, short-duration heat treatment on fermentation performance may not be possible prior to scale up.

Other potential issues previously mentioned in this chapter become particularly important upon scale-up. Fermentation whole broth that could be easily centrifuged in a bottle in the lab may require a completely different biomass removal process at large scale, depending on the facility and equipment. A fermentation process that requires relatively large quantities of antifoam to control foaming may cause great challenges with DSP at production scale if the only available biomass separation process is TFF. Insoluble components in the fermentation broth may cause similar challenges with TFF. These considerations must be taken into account when developing fermentation media for large-scale production.

Take-Home Messages

- The design and development of industrial fermentation media require a balance of macronutrients (C, N, P, S); micronutrients (trace metals and co-factors); appropriate water; suitable selective pressures and inducers, if required; and antifoam, if required.
- Sources of fermentation media ingredients are wide and varied, from highly refined chemicals to crude waste streams.
- Consideration of the ultimate scale, market, and regulatory environment is paramount prior to developing an industrial fermentation medium.
- Different microbial species, strains within species, and recombinant products from within strains may have different nutritional requirements for optimal performance.
- Growth medium can have a considerable influence on downstream processing and product recovery, which should be factored into any design and development.
- Looking beyond, commercially available, prepared media can be a more economical approach when scaling fermentation processes to large volumes while simultaneously providing flexibility with respect to suppliers and specific ingredient characteristics.
- Statistical design of experiments (DOE) is an efficient tool to identify significant factors of media components in a relatively small number of experiments.

Acknowledgments The authors would like to acknowledge and thank Callaghan Innovation (New Zealand's Innovation Agency) for funding.

References

1. Aiba S, Humphrey AE, Millis NF, editors. Scale-up. Biochemical engineering. 2nd ed. New York: Academic Press; 1973. p. 195–217.
2. Aidoo KE, Nout MJR, Sarkar PK. Occurrence and function of yeasts in Asian indigenous fermented foods. FEMS Yeast Res. 2006;6(1):30–9. https://doi.org/10.1111/j.1567-1364.2005.00015.x.
3. Al-Ajlani M, Sheikh M, Ahmad Z, et al. Production of surfactin from *Bacillus subtilis* MZ-7 grown on pharmamedia commercial medium. Microb Cell Fact. 2007;6:17. https://doi.org/10.1186/1475-2859-6-17.
4. Allikian K, Rasiah I, Zhang S. Effects of common defoaming agents on the growth of the endotoxin-free ClearColi® *Escherichia coli* host. In: Abstracts of the Society for Industrial Microbiology and Biotechnology Annual Meeting, New Orleans, LA, USA, 24-28 July 2016; 2016.
5. Ano T, Jin G, Mizumoto S, et al. Solid state fermentation of lipopeptide antibiotic iturin A by using a novel solid state fermentation reactor system. J Environ Sci. 2009;21:S162–5. https://doi.org/10.1016/s1001-0742(09)60064-4.
6. Aoki MA, Pastore GM, Park YK. Microbial transformation of sucrose and glucose to erythritol. Biotechnol Lett. 1993;15(4):383–8. https://doi.org/10.1007/BF00128281.
7. Babu PD, Bhakyaraj R, Vidhyalakshmi R, et al. A low cost nutritious food "tempeh" – a review. World J Dairy Food Sci. 2009;4(1):22–7.
8. Babu CR, Ketanapalli H, Beebi SK, et al. Wheat bran-composition and nutritional quality: a review. Adv Biotech & Micro. 2018;9(1):MS.ID.555754. https://doi.org/10.19080/AIBM.2018.09.555754.
9. Boenigk R, Bowien S, Gottschalk G. Fermentation of glycerol to 1, 3-propanediol in continuous cultures of *Citrobacter freundii*. Appl Microbiol Biotechnol. 1993;38(4):453–7. https://doi.org/10.1007/BF00242936.
10. Bohn L, Meyer AS, Rasmussen SK. Phytate: impact on environment and human nutrition. A challenge for molecular breeding. J Zhejiang Univ Sci B. 2008;9(3):165–91. https://doi.org/10.1631/jzus.B0710640.

11. Boni L, Cangini M, Grifoni A, et al. Polysaccharide production by microalgae from the Adriatic Sea. In: Faranda FM, Guglielmo L, Sperle G, editors. Mediterranean ecosystems: structures and processes. Milan: Springer-Verlag; 2001. p. 61–4. https://doi.org/10.1007/978-88-470-2105-1_7.
12. Boon L, Hoeks F, van der Lans R, et al. Comparing a range of impellers for "stirring as foam disruption". Biochem Eng J. 2002;10(3):183–95. https://doi.org/10.1016/s1369-703x(01)00180-2.
13. Botella C, Diaz A, de Ory I, et al. Xylanase and pectinase production by *Aspergillus awamori* on grape pomace in solid state fermentation. Process Biochem. 2007;42(1):98–101. https://doi.org/10.1016/j.procbio.2006.06.025.
14. Bultel-Ponce V, Debitus C, Berge JP, et al. Metabolites from the sponge-associated bacterium *Micrococcus luteus*. J Mar Biotechnol. 1998;6:233–6.
15. Chang PK, Matsushima K, Takahashi T, et al. Understanding nonaflatoxigenicity of *Aspergillus sojae*: a windfall of aflatoxin biosynthesis research. Appl Microbiol Biotechnol. 2007;76(5):977–84. https://doi.org/10.1007/s00253-007-1116-4.
16. Chen YS, Yanagida F, Hsu JS. Isolation and characterization of lactic acid bacteria from Dochi (fermented black beans), a traditional fermented food in Taiwan. Lett Appl Microbiol. 2006;43(2):229–35. https://doi.org/10.1111/j.1472-765x.2006.01922.x.
17. Chen HZ, Liu ZH, Dai SH. A novel solid state fermentation coupled with gas stripping enhancing the sweet sorghum stalk conversion performance for bioethanol. Biotechnol Biofuels. 2014;7(1):53. https://doi.org/10.1186/1754-6834-7-53.
18. Cheng YQ, Hu Q, Li LT, et al. Production of Sufu, a traditional Chinese fermented soybean food, by fermentation with *Mucor flavus* at low temperature. Food Sci Technol Res. 2009;15(4):347–52. https://doi.org/10.3136/fstr.15.347.
19. Chiou RYY, Cheng SL. Isoflavone transformation during soybean Koji preparation and subsequent Miso fermentation supplemented with ethanol and NaCl. J Agric Food Chem. 2001;49(8):3656–60. https://doi.org/10.1021/jf001524l.
20. Chiou RY, Ferng S, Beuchat L. Fermentation of low-salt Miso as affected by supplementation with ethanol. Int J Food Microbiol. 1999;48(1):11–20. https://doi.org/10.1016/s0168-1605(99)00033-1.
21. Cho YH, Shin IS, Hong SM, et al. Production of functional high-protein beverage fermented with lactic acid bacteria isolated from Korean traditional fermented food. Korean J Food Sci Anim Resour. 2015;35:189–96. https://doi.org/10.5851/kosfa.2015.35.2.189.
22. Chua JY, Lu Y, Liu SQ. Biotransformation of soy whey into soy alcoholic beverage by four commercial strains of *Saccharomyces cerevisiae*. Int J Food Microbiol. 2017;262:14–22. https://doi.org/10.1016/j.ijfoodmicro.2017.09.007.
23. Clarkson J, Cui Z, Darton R. Protein denaturation in foam. J Colloid Interface Sci. 1999;215(2):323–32. https://doi.org/10.1006/jcis.1999.6255.
24. Cocaign-Bousquet M, Garrigues C, Novak L, et al. Rational development of a simple synthetic medium for the sustained growth of *Lactococcus lactis*. J Appl Bacteriol. 1995;79(1):108–16. https://doi.org/10.1111/j.1365-2672.1995.tb03131.x.
25. Coutinho EM. Gossypol: a contraceptive for men. Contraception. 2002;65(4):259–63. https://doi.org/10.1016/s0010-7824(02)00294-9.
26. Daniel HJ, Otto RT, Reuss M, et al. Sophorolipid production with high yields on whey concentrate and rapeseed oil without consumption of lactose. Biotechnol Lett. 1998a;20(8):805–7. https://doi.org/10.1023/B:BILE.0000015927.29348.1a.
27. Daniel HJ, Reuss M, Syldatk C. Production of sophorolipids in high concentration from deproteinized whey and rapeseed oil in a two stage fed batch process using *Candida bombicola* ATCC 22214 and *Cryptococcus curvatus* ATCC 20509. Biotechnol Lett. 1998b;20(12):1153–6. https://doi.org/10.1023/A:1005332605003.
28. Darvishi F, Destain J, Nahvi I, et al. High-level production of extracellular lipase by *Yarrowia lipolytica* mutants from methyl oleate. New Biotechnol. 2011;28(6):756–60. https://doi.org/10.1016/j.nbt.2011.02.002.
29. Dean JA, editor. Lange's handbook of chemistry. 12th ed. McGraw-Hill: New York; 1979.
30. Denkov ND, Marinova KG, Tcholakova SS. Mechanistic understanding of the modes of action of foam control agents. Adv Colloid Interface Sci. 2014;206:57–67. https://doi.org/10.1016/j.cis.2013.08.004.
31. Deshpande M, Daniels L. Evaluation of sophorolipid biosurfactant production by *Candida bombicola* using animal fat. Bioresour Technol. 1995;54(2):143–50. https://doi.org/10.1016/0960-8524(95)00116-6.
32. Du R, Yan J, Feng Q, et al. A novel wild-type *Saccharomyces cerevisiae* strain TSH1 in scaling-up of solid-state fermentation of ethanol from sweet sorghum stalks. PLOS One. 2014;9(4):e94480. https://doi.org/10.1371/journal.pone.0094480.

33. Elander R. Industrial production of β-lactam antibiotics. Appl Microbiol Biotechnol. 2003;61(5-6):385–92. https://doi.org/10.1007/s00253-003-1274-y.
34. Erdogrul O, Azirak S. Review of the studies on the red yeast rice (*Monascus purpureus*). Turk Electr J Biotech. 2004;2:37–49.
35. Ergun SO, Urek RO. Production of ligninolytic enzymes by solid state fermentation using *Pleurotus ostreatus*. Ann Agrar Sci. 2017;15(2):273–7. https://doi.org/10.1016/j.aasci.2017.04.003.
36. Fox SL, Bala GA. Production of surfactant from *Bacillus subtilis* ATCC 21332 using potato substrates. Bioresour Technol. 2000;75(3):235–40. https://doi.org/10.1016/s0960-8524(00)00059-6.
37. Gadd GM. Metals, minerals and microbes: geomicrobiology and bioremediation. Microbiology. 2010;156(3):609–43. https://doi.org/10.1099/mic.0.037143-0.
38. Gadelha ICN, Fonseca NBS, Oloris SCS, et al. Gossypol toxicity from cottonseed products. Sci World J. 2014;2014:231635. https://doi.org/10.1155/2014/231635.
39. Ghaly A, Kamal M. Submerged yeast fermentation of acid cheese whey for protein production and pollution potential reduction. Water Res. 2004;38(3):631–44. https://doi.org/10.1016/j.watres.2003.10.019.
40. Ghorbani F, Younesi H, Sari AE, et al. Cane molasses fermentation for continuous ethanol production in an immobilized cells reactor by *Saccharomyces cerevisiae*. Renew Energy. 2011;36(2):503–9. https://doi.org/10.1016/j.renene.2010.07.016.
41. Ghosalkar A, Sahai V, Srivastava A. Optimization of chemically defined medium for recombinant *Pichia pastoris* for biomass production. Bioresour Technol. 2008;99(16):7906–10. https://doi.org/10.1016/j.biortech.2008.01.059.
42. Ghosh JS. Solid state fermentation and food processing: a short review. J Nutr Food Sci. 2016;06(01):1000453. https://doi.org/10.4172/2155-9600.1000453.
43. Goldammer T. The Brewer's handbook. Clifton: KVP Publishers; 1999.
44. Gouda MK, Swellam AE, Omar SH. Production of PHB by a *Bacillus megaterium* strain using sugarcane molasses and corn steep liquor as sole carbon and nitrogen sources. Microbiol Res. 2001;156(3):201–7. https://doi.org/10.1078/0944-5013-00104.
45. Grewal H, Kalra K. Fungal production of citric acid. Biotechnol Adv. 1995;13(2):209–34. https://doi.org/10.1016/0734-9750(95)00002-8.
46. Gunasekaran S, Poorniammal R. Optimization of fermentation conditions for red pigment production from *Penicillium* sp. under submerged cultivation. Afr J Biotechnol. 2008;7(12):1894–8. https://doi.org/10.4314/ajb.v7i12.58846.
47. Heldal M, Norland S, Fagerbakke KM, et al. The elemental composition of bacteria: a signature of growth conditions? Mar Pollut Bull. 1996;33(1-6):3–9. https://doi.org/10.1016/s0025-326x(97)00007-6.
48. Herbert D. Stoichiometric aspects of microbial growth. In: Dean CR, Ellwood DC, Evans CGT, et al., editors. Continuous culture 6: applications and new fields. Chichester: Ellis Horwood; 1976. p. 1–30.
49. Hernandez-Orte P, Ibarz M, Cacho J, et al. Addition of amino acids to grape juice of the Merlot variety: effect on amino acid uptake and aroma generation during alcoholic fermentation. Food Chem. 2006;98(2):300–10. https://doi.org/10.1016/j.foodchem.2005.05.073.
50. Hesham AEL, Mohamed NH, Ismail MA, et al. Degradation of natural rubber latex by new *Streptomyces labedae* strain ASU-03 isolated from Egyptian soil. Microbiology. 2015;84(3):351–8. https://doi.org/10.1134/S0026261715030078.
51. Hoondal G, Tiwari R, Tewari R, et al. Microbial alkaline pectinases and their industrial applications: a review. Appl Microbiol Biotechnol. 2002;59(4-5):409–18. https://doi.org/10.1007/s00253-002-1061-1.
52. Hull SR, Yang BY, Venzke D, et al. Composition of corn steep water during steeping. J Agric Food Chem. 1996;44(7):1857–63. https://doi.org/10.1021/jf950353v.
53. Japan Sake and Shochu Makers Association. A comprehensive guide to Japanese sake. Tokyo: Japan Sake and Shochu Makers Association; 2011.
54. Jeong H, Park S, Pak V, et al. Fermented soybean products and their bioactive compounds. In: El-Shemy H, editor. Soybean and health. InTechOpen; 2011. https://doi.org/10.5772/10670.
55. Jiang H, Chen Y, Jiang P, et al. Methanotrophs: multifunctional bacteria with promising applications in environmental bioengineering. Biochem Eng J. 2010;49(3):277–88. https://doi.org/10.1016/j.bej.2010.01.003.
56. Jin G, Zhu Y, Xu Y. Mystery behind Chinese liquor fermentation. Trends Food Sci Technol. 2017;63:18–28. https://doi.org/10.1016/j.tifs.2017.02.016.
57. Kale SK, Deshmukh AG, Dudhare MS, et al. Microbial degradation of plastic: a review. J Biochem Tech. 2015;6(2):952–61.

58. Kamath PV, Dwarakanath BS, Chaudhary A, et al. Optimization of culture conditions for maximal lovastatin production by *Aspergillus terreus* (KM017963) under solid state fermentation. HAYATI J Biosci. 2015;22(4):174–80. https://doi.org/10.1016/j.hjb.2015.11.001.
59. Kampen WH. Nutritional requirements in fermentation processes. In: Vogel HC, Todaro CM, editors. Fermentation and biochemical engineering handbook: principles, process design and equipment. 3rd ed. Westwood: Elsevier; 2014. p. 37–57.
60. Koser S, Anwar Z, Iqbal Z, et al. Utilization of *Aspergillus oryzae* to produce pectin lyase from various agro-industrial residues. J Radiat Res Appl Sci. 2014;7(3):327–32. https://doi.org/10.1016/j.jrras.2014.05.001.
61. Kosseva MR. Recovery of commodities from food wastes using solid-state fermentation. In: Kosseva M, Webb C, editors. Food industry wastes. 1st ed: Elsevier; 2013. p. 77–102. https://doi.org/10.1016/B978-0-12-391921-2.00005-6.
62. Kovacs E, Wirth R, Maroti G, et al. Biogas production from protein-rich biomass: fed-batch anaerobic fermentation of casein and of pig blood and associated changes in microbial community comosition. PLOS One. 2013;8(10):e77265. https://doi.org/10.1371/journal.pone.0077265.
63. Kroner K, Hummel W, Volkel J, et al. Effects of antifoams on cross-flow filtration of microbial suspensions. In: Drioli E, Nakagaki M, editors. Membranes and membrane processes. New York: Springer; 1986. p. 223–32.
64. Kumar Y, Dhingra MD. Inventive utilization of soybean whey as beverage. Int J Multidiscip Approach Stud. 2014;1(2):100–7.
65. Lakshmi BS, Kangueane P, Abraham B, et al. Effect of vegetable oils in the secretion of lipase from *Candida rugosa* (DSM 2031). Lett Appl Microbiol. 1999;29(1):66–70. https://doi.org/10.1046/j.1365-2672.1999.00578.x.
66. Lee SY. Bacterial polyhydroxyalkanoates. Biotechnol Bioeng. 1996a;49(1):1–14. https://doi.org/10.1002/(SICI)1097-0290(19960105)49:1<1::AID-BIT1>3.0.CO;2-P.
67. Lee SY. High cell-density culture of *Escherichia coli*. Trends Biotechnol. 1996b;14(3):98–105. https://doi.org/10.1016/0167-7799(96)80930-9.
68. Lee PC, Lee WG, Lee SY, et al. Succinic acid production with reduced by-product formation in the fermentation of *Anaerobiospirillum succiniciproducens* using glycerol as a carbon source. Biotechnol Bioeng. 2001;72:41–8. https://doi.org/10.1002/1097-0290(20010105)72:1<41::AID-BIT6>3.0.CO;2-N.
69. Lee YH, Tominaga M, Hayashi R, et al. *Aspergillus oryzae* strains with a large deletion of the aflatoxin biosynthetic homologous gene cluster differentiated by chromosomal breakage. Appl Microbiol Biotechnol. 2006;72(2):339–45. https://doi.org/10.1007/s00253-005-0282-5.
70. Levin L, Herrmann C, Papinutti VL. Optimization of lignocellulolytic enzyme production by the white-rot fungus *Trametes trogii* in solid-state fermentation using response surface methodology. Biochem Eng J. 2008;39(1):207–14. https://doi.org/10.1016/j.bej.2007.09.004.
71. Li S, Zhu D, Li K, et al. Soybean curd residue: Composition, utilization, and related limiting factors. ISRN Ind Eng. 2013;2013:1–8. https://doi.org/10.1155/2013/423590.
72. Liggett RW, Koffler H. Corn steep liquor in microbiology. Bacteriol Rev. 1948;12:297–311.
73. Lilly VG. The chemical environment for growth. 1. media, macro and micronutrients. In: Ainsworth GC, Sussman AS, editors. The fungi, vol. 1. New York: Academic Press; 1965. p. 465–78.
74. Luria SE. The bacterial protoplasm: composition and organisation. In: Gunsalus IC, Stanier RY, editors. The bacteria, vol. 1. New York: Academic Press; 1960. p. 1–34.
75. Magonet E, Hayen P, Delforge D, et al. Importance of the structural zinc atom for the stability of yeast alcohol dehydrogenase. Biochem J. 1992;287(2):361–5. https://doi.org/10.1042/bj2870361.
76. Majumdar M, Majumdar S. Effects of minerals on neomycin production by *Streptomyces fradiae*. Appl Microbiol. 1965;13(2):190–3.
77. Makkar RS, Cameotra SS. Utilization of molasses for biosurfactant production by two *Bacillus* strains at thermophilic conditions. J Am Oil Chem Soc. 1997;74(7):887–9. https://doi.org/10.1007/s11746-997-0233-7.
78. Manan MA, Webb C. Modern microbial solid state fermentation technology for future biorefineries for the production of added-value products. Biofuel Res J. 2017;4(4):730–40. https://doi.org/10.18331/brj2017.4.4.5.
79. Martins DAB, do Prado HFA, Leite RSR, et al. Agroindustrial wastes as substrates for microbial enzymes production and source of sugar for bioethanol production. In: Kumar S, editor. Integrated waste management, vol. II. Rijeka: InTechOpen; 2011. p. 319–60.
80. Matthews CB, Kuo A, Love KR, et al. Development of a general defined medium for *Pichia pastoris*. Biotechnol Bioeng. 2017;115(1):103–13. https://doi.org/10.1002/bit.26440.

81. Mazumdar-Shaw K, Suryanarayan S. Commercialization of a novel fermentation concept. Adv Biochem Eng/Biotechnol. 2003;85:29–42. https://doi.org/10.1007/3-540-36466-8_2.
82. Mercade M, Monleon L, de Andres C, et al. Screening and selection of surfactant-producing bacteria from waste lubricating oil. J Appl Bacteriol. 1996;81(2):161–6. https://doi.org/10.1111/j.1365-2672.1996.tb04494.x.
83. Minier M, Fessier P, Colinart P, et al. Study of the fouling effect of antifoam compounds on the crossflow filtration of yeast suspensions. Sep Sci Technol. 1995;30(5):731–50. https://doi.org/10.1080/01496399508013889.
84. Mitchell DA, Krieger N, Berovic M, editors. Solid-state fermentation bioreactors: fundamentals of design and operation. Berlin: Springer; 2006.
85. Mitchell DA, De Lima Luz LF, Krieger N. Bioreactors for solid-state fermentation. In: Moo-Yong M, editor. Comprehensive biotechnology. 2nd ed. Manchester: Elsevier; 2011. p. 347–60.
86. Moukamnerd C, Kawahara H, Katakura Y. Feasibility study of ethanol production from food wastes by consolidated continuous solid-state fermentation. J Sustain Bioenergy Syst. 2013;3(02):143–8. https://doi.org/10.4236/jsbs.2013.32020.
87. Mukhtar H, Ikram-Ul-Haq. Production of acid protease by *Aspergillus niger* using solid state fermentation. Pakistan J. Zool. 2009;41(4):253–60.
88. Musselman ME, Pettit RS, Derenski KL. A review and up-date of red yeast rice. J Evid Based Complementary Altern Med. 2011;17(1):33–9. https://doi.org/10.1177/2156587211429703.
89. Nagavalli M, Ponamgi S, Girijashankar V, et al. Solid state fermentation and production of rifamycin SV using *Amycolatopsis mediterranei*. Lett Appl Microbiol. 2014;60(1):44–51. https://doi.org/10.1111/lam.12332.
90. Nayanashree G, Thippeswamy B. Natural rubber degradation by laccase and manganese peroxidase enzymes of *Penicillium chrysogenum*. Int J Environ Sci Technol. 2014;12(8):2665–72. https://doi.org/10.1007/s13762-014-0636-6.
91. Neidhardt FC, editor. *Escherichia coli* and *Salmonella*: cellular and molecular biology (2 Volumes). 2nd ed. Washington, D.C.: ASM Press; 1996.
92. Nitschke M, Pastore GM. Biosurfactant production by *Bacillus subtilis* using cassava-processing effluent. Appl Biochem Biotechnol. 2004;112(3):163–72. https://doi.org/10.1385/ABAB:112:3:163.
93. Nitschke M, Pastore GM. Production and properties of a surfactant obtained from *Bacillus subtilis* grown on cassava wastewater. Bioresour Technol. 2006;97(2):336–41. https://doi.org/10.1016/j.biortech.2005.02.044.
94. Noah KS, Bruhn DF, Bala GA. Surfactin production from potato process effluent by Bacillus subtilis in a chemostat. In: Davison BH, editor. Twenty-sixth symposium on biotechnology for fuels and chemicals. New York: Springer; 2005. p. 465–73.
95. O'Toole DK. Characteristics and use of Okara, the soybean residue from soy milk production – a review. J Agric Food Chem. 1999;47(2):363–71. https://doi.org/10.1021/jf980754l.
96. Ohno A, Ano T, Shoda M. Production of a lipopeptide antibiotic surfactin with recombinant *Bacillus subtilis*. Biotechnol Lett. 1992;14(12):1165–8. https://doi.org/10.1007/BF01027022.
97. Pratoomchai R. Increasing the value of rice by transformation into red yeast rice. J Sci Technol MSU. 2015;34(5):503–12.
98. Priatni S, Damayanti S, Saraswaty V, et al. The utilization of solid substrates on *Monascus* fermentation for anticholesterol agent production. Procedia Chem. 2014;9:34–9. https://doi.org/10.1016/j.proche.2014.05.005.
99. Prins A, van't Riet K. Proteins and surface effects in fermentation: foam, antifoam and mass transfer. Trends Biotechnol. 1987;5(11):296–301. https://doi.org/10.1016/0167-7799(87)90080-1.
100. Pritchett J, Baldwin SA. The effect of nitrogen source on yield and glycosylation of a human cystatin C mutant expressed in *Pichia pastoris*. J Ind Microbiol Biotechnol. 2004;31(12):553–8. https://doi.org/10.1007/s10295-004-0181-2.
101. Quesada-Chanto A, Afschar AS, Wagner F. Microbial production of propionic acid and vitamin B_{12} using molasses or sugar. Appl Microbiol Biotechnol. 1994;41:378–83. https://doi.org/10.1007/BF01982523.
102. Rahardjo YS, Tramper J, Rinzema A. Modeling conversion and transport phenomena in solid-state fermentation: a review and perspectives. Biotechnol Adv. 2006;24(2):161–79. https://doi.org/10.1016/j.biotechadv.2005.09.002.
103. Ratzke C, Denk J, Gore J. Ecological suicide in microbes. Nat Ecol Evol. 2018;2(5):867–72. https://doi.org/10.1038/s41559-018-0535-1.

104. Ravindran V, Abdollahi MR, Bootwalla SM. Nutrient analysis, metabolizable energy, and digestible amino acids of soybean meals of different origins for broilers. Poult Sci. 2014;93(10):2567–77. https://doi.org/10.3382/ps.2014-04068.
105. Riesenberg D, Schulz V, Knorre W, et al. High cell density cultivation of *Escherichia coli* at controlled specific growth rate. J Biotechnol. 1991;20(1):17–27. https://doi.org/10.1016/0168-1656(91)90032-q.
106. Rigo E, Ninow JL, Luccio MD, et al. Lipase production by solid fermentation of soybean meal with different supplements. LWT-Food Sci Technol. 2010;43(7):1132–7. https://doi.org/10.1016/j.lwt.2010.03.002.
107. Rizzo G, Baroni L. Soy, soy foods and their role in vegetarian diets. Nutrients. 2018;10(1):43. https://doi.org/10.3390/nu10010043.
108. Rodrigues MI, Iemma AF. Experimental design and process optimization. Boca Raton: CRC Press; 2014.
109. Rokem JS, Lantz AE, Nielsen J. Systems biology of antibiotic production by microorganisms. Nat Prod Rep. 2007;24(6):1262–87. https://doi.org/10.1039/b617765b.
110. Routledge SJ. Antifoams: the overlooked additive? Pharm Bioprocess. 2014;2(2):103–6. https://doi.org/10.2217/PBP.14.5.
111. Routledge SJ, Bill RM. The effect of antifoam addition on protein production yields. In: Bill RM, editor. Recombinant protein production in yeast: methods and protocols. Totowa: Humana Press; 2012. p. 87–97.
112. Rufino RD, Sarubbo LA, Campos-Takaki GM. Enhancement of stability of biosurfactant produced by *Candida lipolytica* using industrial residue as substrate. World J Microbiol Biotechnol. 2006;23(5):729–34. https://doi.org/10.1007/s11274-006-9278-2.
113. Sabu A, Sarita S, Pandey A, et al. Solid-state fermentation for production of phytase by *Rhizopus oligosporus*. Appl Biochem Biotechnol. 2002;102(1):251–60. https://doi.org/10.1385/ABAB:102-103:1-6:251.
114. Sakiewicz P, Piotrowski K, Cebula J, et al. Alternative utilization of protein-rich waste by its conversion into biogas in co-fermentation conditions. Pol J Environ Stud. 2017;26(3):1225–31. https://doi.org/10.15244/pjoes/68189.
115. Sastraatmadja DD, Tomita F, Kasai T. Production of high-quality Oncom, a traditional Indonesian fermented food, by the inoculation with selected mold strains in the form of pure culture and solid inoculum. J Grad Sch Agr Hokkaido Univ. 2002;70(2):111–27.
116. Shabtai Y. Production of exopolysaccharides by *Acinetobacter* strains in a controlled fed-batch fermentation process using soap stock oil (SSO) as carbon source. Int J Biol Macromol. 1990;12(2):145–52. https://doi.org/10.1016/0141-8130(90)90066-j.
117. Sheppard JD, Mulligan CN. The production of surfactin by *Bacillus subtilis* grown on peat hydrolysate. Appl Microbiol Biotechnol. 1987;27(2):110–6. https://doi.org/10.1007/BF00251931.
118. Shibasaki K, Hesseltine CW. Miso – I. Preparation of soybeans for fermentation. J Biochem Microbiol Technol Eng. 1961;3(2):161–74. https://doi.org/10.1002/jbmte.390030206.
119. Shiloach J, Fass R. Growing *E. coli* to high cell density – a historical perspective on method development. Biotechnol Adv. 2005;23(5):345–57. https://doi.org/10.1016/j.biotechadv.2005.04.004.
120. Shurtleff W, Aoyagi A. History of fermented black soybeans (165 BC to 2011): extensively annotated bibliography and sourcebook. Lafayette: Soyinfo Center; 2011.
121. Singh SM, Panda AK. Solubilization and refolding of bacterial inclusion body proteins. J Biosci Bioeng. 2005;99(4):303–10. https://doi.org/10.1263/jbb.99.303.
122. Singh V, Haque S, Niwas R, et al. Strategies for fermentation medium optimization: an in-depth review. Front Microbiol. 2017;7:2087. https://doi.org/10.3389/fmicb.2016.02087.
123. Siso M. The biotechnological utilization of cheese whey: a review. Bioresour Technol. 1996;57(1):1–11. https://doi.org/10.1016/0960-8524(96)00036-3.
124. Skrypnik K, Suliburska J. Association between the gut microbiota and mineral metabolism. J Sci Food Agric. 2018;98(7):2449–60. https://doi.org/10.1002/jsfa.8724.
125. Solaiman DK, Ashby RD, Nunez A, et al. Production of sophorolipids by *Candida bombicola* grown on soy molasses as substrate. Biotechnol Lett. 2004;26(15):1241–5. https://doi.org/10.1023/B:BILE.0000036605.80577.30.
126. Solaiman DKY, Ashby RD, Zerkowski JA, et al. Simplified soy molasses-based medium for reduced-cost production of sophorolipids by *Candida bombicola*. Biotechnol Lett. 2007;29(9):1341–7. https://doi.org/10.1007/s10529-007-9407-5.
127. Stanbury PF, Whitaker A, Hall SJ. Principles of fermentation technology. 2nd ed. Tarrytown: Pergamon; 1995.

128. Sun N, Wang Y, Li YT, et al. Sugar-based growth, astaxanthin accumulation and carotenogenic transcription of heterotrophic *Chlorella zofingiensis* (chlorophyta). Process Biochem. 2008;43(11):1288–92. https://doi.org/10.1016/j.procbio.2008.07.014.
129. Surono IS. Ethnic fermented foods and beverages of Indonesia. In: Tamang YP, editor. Ethnic fermented foods and alcoholic beverages of Asia. Delhi: Springer; 2016. p. 341–82.
130. Syed R, Saggar S, Tate K, et al. Assessing the performance of floating biofilters for oxidation of methane from dairy effluent ponds. J Environ Qual. 2017;46(2):272–80. https://doi.org/10.2134/jeq2016.08.0310.
131. Tao TL, Cui FJ, Chen XX, et al. Improved mycelia and polysaccharide production of *Grifola frondosa* by controlling morphology with microparticle Talc. Microb Cell Fact. 2018;17(1) https://doi.org/10.1186/s12934-017-0850-2.
132. Thomas L, Larroche C, Pandey A. Current developments in solid-state fermentation. Biochem Eng J. 2013;81:146–61. https://doi.org/10.1016/j.bej.2013.10.013.
133. Thompson DN, Fox SL, Bala GA. The effect of pretreatments on surfactin production from potato process effluent by Bacillus subtilis. In: Davison BH, McMillan JD, Finkelstein M, editors. Twenty-second symposium on biotechnology for fuels and chemicals. New York: Springer; 2001. p. 487–501.
134. Torzillo G, Pushparaj B, Masojidek J, et al. Biological constraints in algal biotechnology. Biotechnol Bioprocess Eng. 2003;8(6):338–48. https://doi.org/10.1007/BF02949277.
135. Tripathi NK. Production and purification of recombinant proteins from *Escherichia coli*. ChemBioEng Rev. 2016;3(3):116–33. https://doi.org/10.1002/cben.201600002.
136. ul Haq I, Idrees S, Rajoka M. Production of lipases by *Rhizopus oligosporous* by solid-state fermentation. Process Biochem. 2002;37(6):637–41. https://doi.org/10.1016/s0032-9592(01)00252-7.
137. Valero F, del Rio JL, Poch M, et al. Fermentation behaviour of lipase production by *Candida rugosa* growing on different mixtures of glucose and olive oil. J Ferment Bioeng. 1991;72(5):399–401. https://doi.org/10.1016/0922-338x(91)90095-x.
138. Van Niel E, Hahn-Hägerdal B. Nutrient requirements of lactococci in defined growth media. Appl Microbiol Biotechnol. 1999;52(5):617–27. https://doi.org/10.1007/s002530051569.
139. Walker GM, White NA. Introduction to fungal physiology. In: Kavanagh K, editor. Fungi, biology and applications. 3rd ed. Hoboken: Wiley-Blackwell; 2017. p. 1–34.
140. Wan M, Zhang Z, Wang J, et al. Sequential heterotrophy-dilution-photoinduction cultivation of *Haematococcus pluvialis* for efficient production of astaxanthin. Bioresour Technol. 2015;198:557–63. https://doi.org/10.1016/j.biortech.2015.09.031.
141. Welch RM. The biological significance of nickel. J Plant Nutr. 1981;3(1-4):345–56. https://doi.org/10.1080/01904168109362843.
142. Xin F, Geng A. Horticultural waste as the substrate for cellulase and hemicellulase production by *Trichoderma reesei* under solid-state fermentation. Appl Biochem Biotechnol. 2009;162(1):295–306. https://doi.org/10.1007/s12010-009-8745-2.
143. Yan D, Lu Y, Chen YF, et al. Waste molasses alone displaces glucose-based medium for microalgal fermentation towards cost-saving biodiesel production. Bioresour Technol. 2011;102(11):6487–93. https://doi.org/10.1016/j.biortech.2011.03.036.
144. Yang J, Yang Y, Wu WM, et al. Evidence of polyethylene biodegradation by bacterial strains from the guts of plastic-eating waxworms. Environ Sci Technol. 2014;48(23):13776–84. https://doi.org/10.1021/es504038a.
145. Zhang J, Martin C, Shifflet M, et al. Development of a defined medium fermentation process for physostigmine production by *Streptomyces griseofuscus*. Appl Microbiol Biotechnol. 1996;44(5):568–75. https://doi.org/10.1007/BF00172487.
146. Zhang G, Mills DA, Block DE. Development of chemically defined media supporting high-cell-density growth of lactococci, enterococci, and streptococci. Appl Environ Microbiol. 2008a;75(4):1080–7. https://doi.org/10.1128/aem.01416-08.
147. Zhang Y, Xin J, Chen L, et al. Biosynthesis of poly-3-hydroxybutyrate with a high molecular weight by methanotroph from methane and methanol. J Nat Gas Chem. 2008b;17(1):103–9. https://doi.org/10.1016/s1003-9953(08)60034-1.
148. Zhao G, Yao Y, Wang C, et al. Comparative genomic analysis of *Aspergillus oryzae* strains 3.042 and RIB40 for soy sauce fermentation. Int J Food Microbiol. 2013;164(2-3):148–54. https://doi.org/10.1016/j.ijfoodmicro.2013.03.027.
149. Zheng XW, Han BZ. Baijiu, Chinese liquor: history, classification and manufacture. J Ethnic Foods. 2016;3(1):19–25. https://doi.org/10.1016/j.jef.2016.03.001.

Sterilization Process

Hamideh Vaghari, Navideh Anarjan, Yahya Najian, and Hoda Jafarizadeh-Malmiri

A. Berenjian (ed.), *Essentials in Fermentation Technology*, Learning Materials in Biosciences,
https://doi.org/10.1007/978-3-030-16230-6_3

3

What You Will Learn in This Chapter

In all fermentation processes, the equipment, raw materials, and air to be used must be provided free from contaminating organisms at all stages. Since most of the targeted biological products are conducted in pure culture, sterilization should be done prior to any operation. In sterilization process all living organisms, spores, and viruses are destroyed and it is performed by either chemical and /or physical agents. Although a number of chemical disinfectants are known, they cannot be used to sterilize nutrient media. Physical sterilization is performed through any physical process such as heating, filtration, radiation, and sonication to eliminate microorganisms. Although chemical treatment and physical sterilization methods are occasionally used in the fermentation industry, in industrial fermentation sterilization is normally carried out by heat or filtration processes. Heat sterilization is the most useful method for the sterilization of nutrient media and equipment and can be conducted in a batch or continuous process. Due to rapid advances in filtration technology, filtration is getting much more common for sterilization of fermentation media and is often used for components of nutrient solutions which are heat sensitive and would thus be denatured through the steam sterilization process.

3.1 Introduction

The most common meaning of fermentation is to use microorganisms such as bacteria, yeast, and fungi to make products useful to humans (biomass, enzymes, primary and secondary metabolites, recombinant products, and products of biotransformation) on an industrial scale [8]. Commercial fermentations typically require thousands of liters of liquid medium and millions of liters of air. For processes operated with axenic cultures, the raw materials and air to be used must be provided free from contaminating organisms [3]. In virtually all fermentation processes, it is mandatory for a cost-effective operation to have contamination-free seed cultures at all stages, from the preliminary culture to the production fermenter. If the fermentation is invaded by the foreign microorganism, then the following consequences may occur:

1. The medium would have to support the growth of both the production organism and the contaminant, resulting in a loss of productivity.
2. If the fermentation is a continuous process, then the contaminant outgrow the production organism and displace it from the fermentation.
3. The foreign organism may contaminate the final product, e.g., single-cell protein where the cells, separated from the broth, constitute the product.
4. The contaminant may produce compounds which make subsequent extraction of the final product difficult.
5. The contaminant may degrade the desired product, e.g., the degradation of ß-lactam antibiotics by ß-lactamase-producing bacteria.
6. Contamination of a bacterial fermentation with phage could result in the lysis of culture. Avoidance of contamination may be achieved by using a pure inoculum to start the fermentation, sterilizing the fermentation medium, sterilizing of air, sterilizing the fermenter vessel, sterilizing all materials to be added during the fermentation process, and maintaining aseptic conditions during the fermentation [13].

Since most of the targeted biological products are conducted in pure culture, sterilization should be done prior to any operation [7]. In sterilization process all living organisms,

spores, and viruses are destroyed and it is performed by either chemical and /or physical agents. Although a number of chemical disinfectants are known, they cannot be used to sterilize nutrient media because there is a risk of the fermentation organism inhibition by the residual chemicals. Any physical process, such as heating, filtration, radiation (gamma, UV, X-Rays, or Y-Rays), and sonication, which can eliminate organisms without a chemical reaction is called physical sterilization. Heating is one of the methods of action at high temperatures. Although chemical treatment and the mentioned physical sterilization methods are occasionally used in the fermentation industry, using heat and filtration are the common sterilization methods at industrial scale. Heat sterilization is the most useful method for the sterilization of nutrient media and equipment and can be carried out in a batch or continuous process. Filtration is much more common for fermentation media than previously, due to rapid advances in filtration technology and the advantages of the on-thermal sterilization route [6]. Filter sterilization is often used for components of nutrient solutions which are heat sensitive and would thus be denatured through the steam sterilization process.

The objective of the present chapter is to discuss the approaches to avoid contamination in fermentation processes. Moreover, heat sterilization and filter sterilization of fermentation medium and their kinetics will be discussed in more details.

3.2 Heat Sterilization

Physical agents, such as high temperature or heating, osmotic pressure, radiation, and surface tension and filtration, may cause the reduction of a microbial population. Heat sterilization is one of the most effective methods and has been widely used in the sterilization of nutrient media and equipment. Heat sterilization is defined as the process which can provide near-complete inactivation of microorganisms by applying a high-intensity heat [5].

In a wide-reaching review of aseptic operation, Pollard [9] summarized the microbial taxa causing contamination of fermentations and their likely origin. The most common contaminants are Gram-positive spore-forming rods (*Bacillus* spp.). Applying high temperature for a long duration may kill all living microorganisms, spores, and viruses, probably through denaturing the protein content of the contaminant cells. However, overheating the prepared media in a long duration sometimes may have a negative impact causing inhibition on the growth of desired organisms. Furthermore, even at high temperatures, the fungal spores may survive if only heat is used. Therefore, heat sterilization is usually performed at high pressures. At the laboratory scale, huge steel vessels with live steams are autoclaved at 121 °C and 105 kPa (15 psig) commonly for 20–30 min [7]. In the high-pressure membrane damage, denaturation of protein and decrease of intracellular pH are responsible for the inactivation of microorganisms [11]. However, the mechanism of inactivation of spores has not been elucidated. The resistances of spores are different even among the same species [14]. It is assumed that spores will germinate under moderate pressure condition (the germination pressure depends on the types of spores), then the germinated spores will be thermally inactivated [5].

Heat sterilization mainly used for sterilization of medium and equipment and can be performed in batch or continuous process. Basic principles and kinetics of the batch and continuous sterilization processes will be discussed in the coming subsections.

3.2.1 Heat Sterilization of Fermentation Medium

Sterilization of nutrient media is an operation essential to all industrial fermentation processes requiring pure culture maintenance. Nutrient media contains a variety of different vegetative cells and spores, derived from the constituents of the culture medium, the water, and the vessel. These must be eliminated by a suitable means before inoculation. Culture medium can be sterilized by a number of means such as sonic vibration, chemical, irradiation, and heating. Mechanical procedure such as centrifugation, adsorption to ion exchange, adsorption to activated carbon, or filtration are also possible for culture medium sterilization. But in practice for large-scale installations, heat and filtration are the main mechanisms to be used. Heat under pressure is the most common method of sterilization of liquid, as well as for solid media. For liquid media in flasks and tubes, this is usually done at 120 °C for 20 min. The design of heat sterilization in large scales may be applied in batch or continuous sterilization systems.

Batch Heat Sterilization of Fermentation Medium

Batch thermal sterilization processes tend to be used for liquid and equipment in fermentation systems, as these tend to be reliable and cost-effective options for both small- and large-scale systems, and there is a degree of confidence in being able to assure sterility in a batch operation. In this process, the liquid medium and equipment are heated to sterilization temperature by (1) electrical heating of vessel, (2) introducing steam directly into the medium, and (3) introducing steam into the coils or jacket of the vessel (Fig. 3.1). If direct steam injection is used, allowance must be made for dilution of the medium by condensate which typically adds 10–20% to the liquid volume; quality of the steam must also be sufficiently high to avoid contamination of the medium by metal ions or organics.

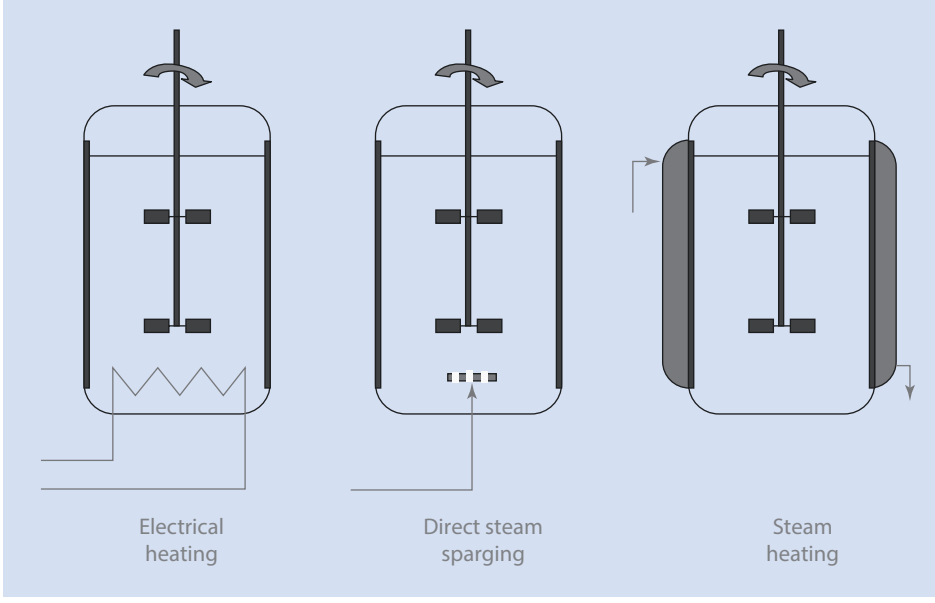


Fig. 3.1 Batch sterilization method

Kinetics of Batch Heat Sterilization of Fermentation Medium

A typical temperature-time profile for batch sterilization is shown in Fig. 3.2a. Depending on the rate of heat transfer from the steam or electrical element, raising the temperature of the medium in large fermenters can take a significant period of time. Once the sterilization temperature is reached, the temperature is held constant for a period of time t_{hd} (holding time). Cooling water in the coils or jacket of the fermenter is then used to reduce the medium temperature to the required value. Contaminant cell destruction occurs at all times during batch sterilization, including the heating up and cooling down periods. For operation of batch sterilization systems, we must be able to estimate the holding time required to achieve the desired level of cell destruction. As well as destroying contaminant organisms, heat sterilization also denatures some of the nutrients in the medium. To minimize this loss, holding times at the highest sterilization temperature should be kept as short as possible.

Let us denote the number of contaminants presented in the raw medium N_0. As indicated in Fig. 3.2b, during the heating period, this number is reduced to N_1; at the end of the holding period, the cell number is N_2; and the final number after cooling is N_t [3].

If N_0 and N_t are known, we can determine the holding time required to reduce the number of cells from N_1 to N_2 by considering the kinetics of cell death. For first-order death kinetics, in a batch vessel where cell death is the only process affecting the number of viable cells

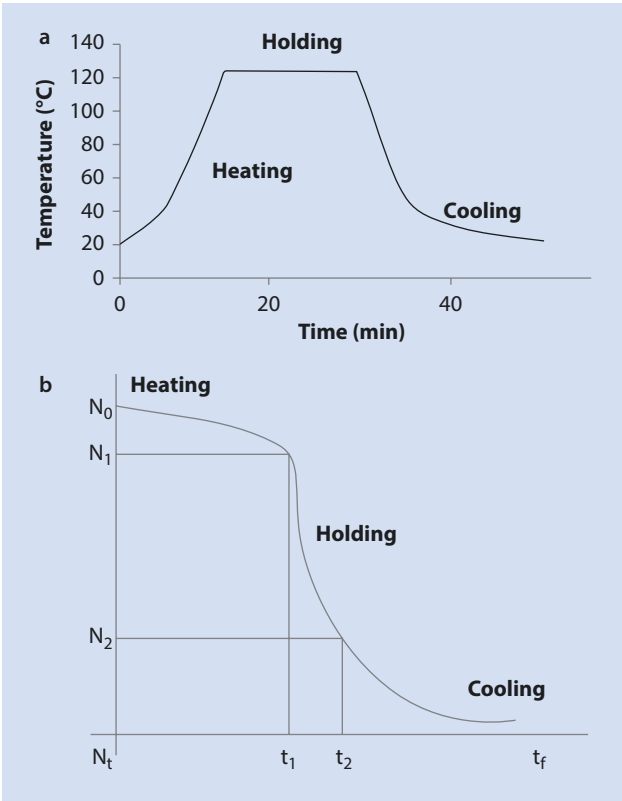


Fig. 3.2 **a** Batch sterilization temperature – time profiles. **b** Reduction cell population during batch sterilization

$$\frac{dN}{dt} = -kN \tag{3.1}$$

where N is number of viable organisms presented at the sterilization treatment, t is the time of sterilization treatment, and k is the reaction rates' constant or specific death rate.

Equation (3.1) applies to each stage of the batch sterilization cycle including heating, holding, and cooling. However, because k is a strong function of temperature, direct integration of Eq. (3.1) is valid only when the temperature is constant.

On integration of Eq. (3.1), the following expression is obtained

$$\frac{N_t}{N_0} = e^{-kt} \tag{3.2}$$

where N_0 is the number of viable organisms presented at the start of sterilization treatment and N_t is the number of viable organisms presented after a treatment period, t.

On taking natural logarithms, Eq. (3.2) is reduced to:

$$\ln\left(\frac{N_t}{N_0}\right) = -kt \tag{3.3}$$

The graphical representations of Eqs. (3.1) and (3.3) are illustrated in Fig. 3.3, from which it may be seen that viable organism number declines exponentially over the treatment period. A plot of natural logarithm of N_t/N_0 against time yields a straight line, the slope of which equals $-k$ [13].

Ideally, N_t is 0, and there is no contaminants present at the end of sterilization. However, because absolute sterility would require an infinite sterilization time, it is theoretically impossible. Normally, the target level of contamination is expressed as a fraction of a cell value of 10^{-3}, which is related to the probability of contamination [13].

As with any first-order reaction, the reaction rate increases with increase in temperature due to an increase in the reaction rate constant, which, in the case of the destruction of microorganisms, is the specific death rate. Thus, k is a true constant only under constant temperature conditions.

Reaction rate constant, k, is evaluated as a function of temperature as demonstrated by the Arrhenius and may be represented by Eq. 3.4:

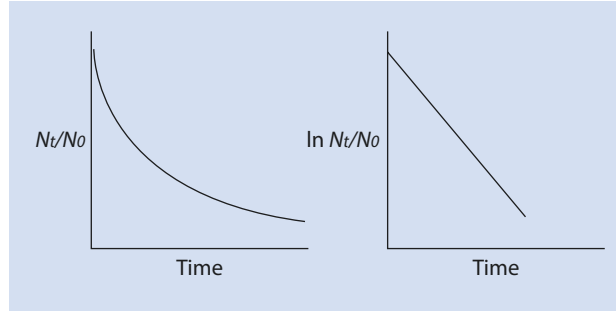


Fig. 3.3 Plots of the proportion of survivors and the natural logarithm of the proportion of survivors in a population of microorganisms subjected to a lethal temperature over a time period

$$d\frac{\ln k}{dt} = \frac{E}{RT^2} \tag{3.4}$$

On integration, Eq. (3.4) gives:

$$k = A\mathrm{e}^{\frac{-E}{RT}} \tag{3.5}$$

where A is the Arrhenius constant or frequency factor, E is the activation energy for the thermal cell death, R is the ideal gas constant, and T is absolute temperature.

On taking natural logarithms, Eq. (3.5) becomes:

$$\ln k = \ln A - \frac{E}{RT}. \tag{3.6}$$

From Eq. (3.6), it may be seen that a plot of ln k against the reciprocal of absolute temperature will give a straight line. Such a plot is termed an Arrhenius plot and enables the calculation of the activation energy and prediction of the reaction rate for any temperature. By combining together Eqs. (3.3) and (3.5), the following expression may be derived for the heat sterilization of a pure culture of a constant temperature:

$$\ln\frac{N_0}{N_t} = A.t.\mathrm{e}^{\frac{-E}{RT.}} \tag{3.7}$$

Deindoerfer and Humphrey [2] used the term $\ln\frac{N_0}{N_t}$ as a design criterion for sterilization, which has been variously called the Del factor, Nabla factor, or sterilization criterion represented by the term∇. Thus, the Del factor is a measure of the fractional reduction in viable organism count produced by a certain heat and time regime.

$$\nabla = \ln\frac{N_0}{N_t} = A.t.\mathrm{e}^{\frac{-E}{RT}} \tag{3.8}$$

On rearranging, Eq. (3.7) becomes:

$$\ln t = \frac{E}{RT} + \ln\frac{\nabla}{A}. \tag{3.9}$$

Calculation of the Del Factor During Heating, Holding, and Cooling Time

The relationship between Del factor, the temperature, and time is given by Eq. (3.7).

$$\nabla = \ln\frac{N_0}{Nt} = A.t.\mathrm{e}^{\frac{-E}{RT}}$$

However, during the heating and cooling period, the temperature is not constant, and, therefore, the calculation of ∇ would require the integration of Eq. (3.7) for the time-temperature regime observed.

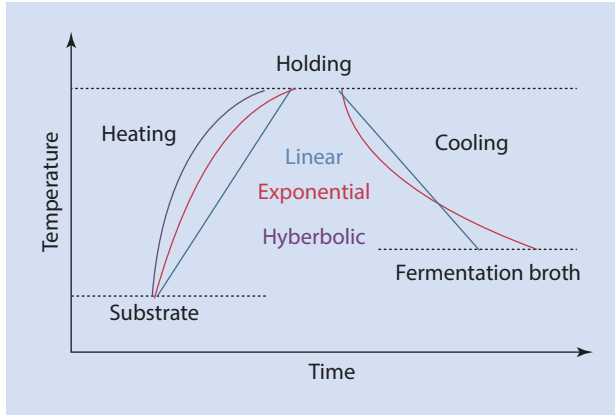


Fig. 3.4 Generalized temperature-time profiles for the heating and cooling stages of a batch sterilization cycle

$$\ln\frac{N_0}{N_1} = \int_0^{t_1} A\mathrm{e}^{\frac{-E}{RT}}\,dt \tag{3.8}$$

$$\ln\frac{N_1}{N_t} = \int_{t_1}^{t_t} A\mathrm{e}^{\frac{-E}{RT}}\,dt \tag{3.9}$$

where t_1 is the time at the end of heating, t_2 is the time at the end of holding, and t_t is the time at the end of cooling. We cannot complete integration of these Equations until we know how the temperature varies with time during heating and cooling. Deindoerfer and Humphrey [2] produced integrated forms of Equation for the time-temperature profile including linear, exponential, and hyperbolic. The general form of these Equations is shown in Fig. 3.4 and Table 3.1.

Applying an appropriate expression for T in Eq. (3.8) allows us to evaluate the cell number N_1 at the start of the holding period. Similarly, substituting for T in Eq. (3.9) for cooling gives N_2 at the end of the holding period. Use of the resulting values for N_1 and N_2 in Eq. (3.3) completes the holding-time calculation.

However, the regime observed in practice is frequently difficult to classify, making the application of these complex equations problematical. Richards [10] demonstrated the use of a graphical method of integration, and this is illustrated in Fig. 3.5. The time axis is divided into a number of equal increments, t_1, t_2, t_3, etc. Richards suggests 30 as a reasonable number. For each increment, the temperature corresponding to the midpoint time is recorded. It may now be approximated that the total Del factor of the heating up period is equivalent to the sum of the Del factors of the midpoint temperature for each time increment.

The value of the Del factor corresponding to each time increment may be calculated from the equation:

$$\nabla_1 = k_1 t$$

$$\nabla_2 = k_2 t$$

$$\nabla_3 = k_3 t$$

etc.

Spores of *Bacillus stearothermophilus* are the most heat-resistant organisms. Therefore, they are used as assay for testing the various procedures used to sterilize equipment. The

Table 3.1 General temperature-time profiles during the heating and cooling periods of batch sterilization

Heat transfer method		Temperature-time profile
Heating		
Direct sparging with stem	Hyperbolic	$T = T_0\left(1+\dfrac{\dfrac{h\,M_s\,t}{M_m\,C_p T_0}}{1+\dfrac{M_s}{M_m}t}\right)$
Electrical heating	Linear	$T = T_0\left(1+\dfrac{Q\,t}{M_m C_p T_0}\right)$
Heat transfer from isothermal steam	Exponential	$T = T_s\left[1+\dfrac{T_0-T_s}{T_s}\,e^{\left(\frac{-UA}{M_m\,C_p}\right)t}\right]$
Cooling		
Heat transfer from non-isothermal steam	Exponential	$T = T_{ci}\left\{1+\dfrac{T_0-T_{ci}}{T_{ci}}\,e^{-\left[\left(\frac{-M_w\,C_{pw}\,t}{M_m\,C_p}\right)\left(1-e^{\left(\frac{-UA}{M_w\,C_{pw}}\right)}\right)\right]}\right\}$

A surface area for heat transfer, C_p specific heat capacity of medium, *Cpw* specific heat capacity of cooling water, *h* specific enthalpy difference between steam and raw medium, M_m initial mass of medium, M_s mass flow rate of steam, M_w mass flow rate of cooling water, *Q* rate of heat transfer, *T* temperature, T_0 initial medium temperature, T_{ci} inlet temperature of cooling water, T_s steam temperature, *t* time, and *U* overall heat-transfer coefficient

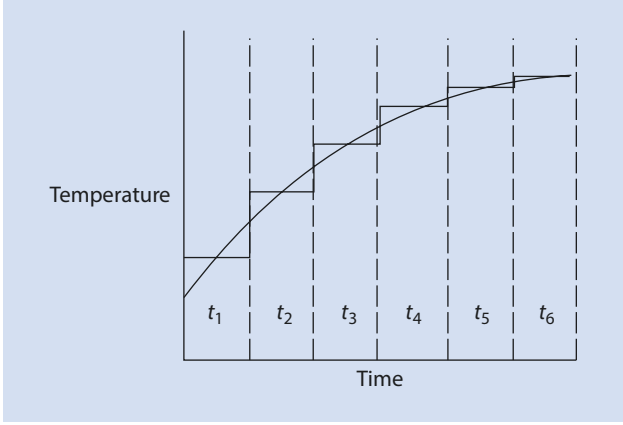


Fig. 3.5 The graphical integration method applied to the increase in temperature over a time period t_1, t_2, t_3, etc. represent equal time intervals

value of rate constant (specific death rate) of *B. stearothermophilus* spores at each midpoint temperature may be deduced from the Arrhenius equation using the thermal death characteristic published by Deindoerfer and Humphrey [2].

The sum of the Del factors for all the increments will then be equal to the Del factor for the heating-up period. The cooling-down period and overall Del factor may be calculated in a similar way. Therefore, the Del factor to be achieved during the holding time may be calculated by differences as shown in Eq. (3.10).

$$\nabla_{\text{holding}} = \nabla_{\text{overall}} - \nabla_{\text{heating}} - \nabla_{\text{cooling}} \tag{3.10}$$

Example

Del factor is 32.2 and if it is taken that the heating Del factor was 9.8 and the cooling Del factor 10.1, (1) calculate the holding Del factor. The specific death rate of *B. stearothermophilus* spores at 121°C is 2.54 min^{-1}; based on that (2) calculate the holding time. If the contribution made by the heating and cooling parts of the cycle were ignored, then (3) calculate the exposure time.

- **Solution**

(i) $\nabla_{\text{holding}} = 32.2 - 9.8 - 10.1$
$\nabla_{\text{holding}} = 12.3$

(ii) $\nabla = k\,t$
$t = \nabla / k$
$t = 12.3/2.54 = 4.84$ min

(iii) $\nabla_{\text{overall}} = k\,t$
$t = \nabla_{\text{overall}} / k$
$t = 32.2/2.54 = 12.68$ min

Scale Up of Batch Sterilization Process

As scale increases, autoclaves become impractical for sterilizing liquid medium. Typically, fermenters greater than 5 L in volume are sterilized in situ using live steam injection [13]. When heat sterilization is scaled up to larger volumes, longer treatment times are needed to achieve the same sterilization result at the same holding temperature. For a given raw medium, the initial number of organisms N_0 is directly proportional to the liquid volume. Therefore, to obtain the same final N_t, a greater number of cells must be destroyed. Scale-up also affects the temperature-time profiles for heating and cooling. Heat-transfer characteristics depend on the equipment used, and heating and cooling of larger volumes usually take more time. Sustained elevated temperatures during heating and cooling could damage vitamins, proteins, and sugars in nutrient solutions and consequently reduce the quality of the medium [1]. Because it is necessary to hold large volumes of medium for longer periods of time, this problem is exacerbated with scale-up [3].

3.2.2 Continuous Heat Sterilization of Fermentation Medium

The design of continuous sterilization cycles could be achievable in the same way as for batch sterilization systems. The continuous system includes a time period during which the medium is heated to the sterilization temperature, a holding time at desired temperature, and cooling period to restore the medium to the desirable fermentation temperature [13].

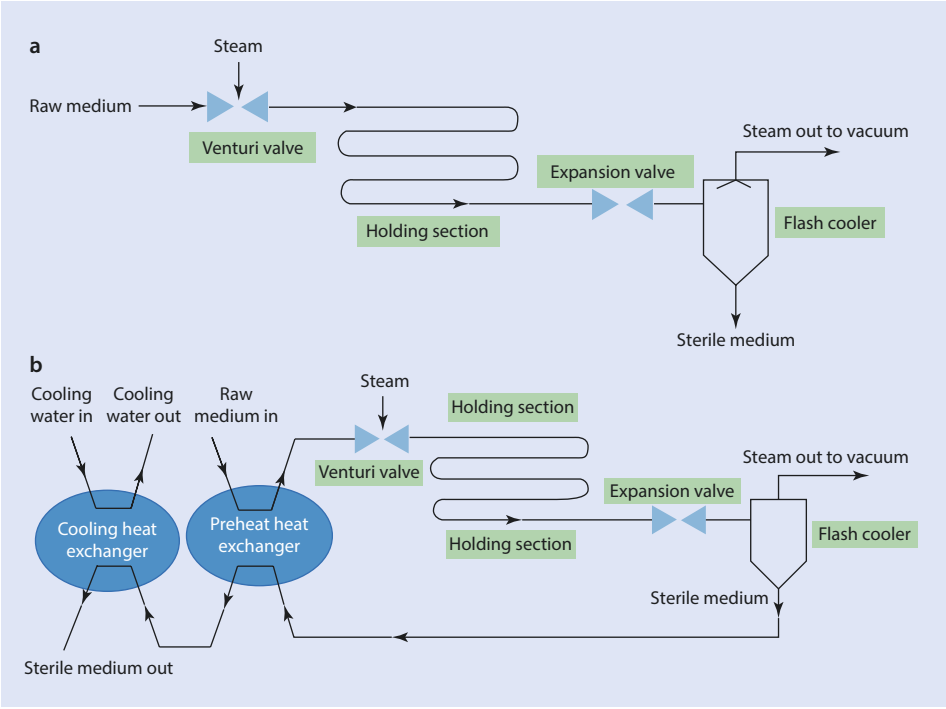


Fig. 3.6 Continuous sterilization: **a** with flash cooling, **b** with flash cooling and heat exchanger

Basically, a simple heat exchange system comprises concentric stainless steel tubes carrying high-pressure steam in one direction and a stream of medium in the other [6]. Another method for continuous sterilization of medium for fermentation is the direct use of live steam by injection of steam into the medium. The medium stays in a loop for a predetermined holding time until the entire medium is sterile. After sterilization, the medium is cooled instantly by passing through an expansion valve into a flash cooler. The problem with directly injecting steam is dilution of medium as it is initially cold. Furthermore, foaming from direct steam injection can also cause problems with operation of the flash cooler [3].

However, it has better heat economy because it comes from substituting heat exchangers for direct steam injection. In another typical configuration for continuous sterilization, raw medium entering the system is first preheated by a heat exchanger. Steam is then injected directly into the medium as it flows through a pipe. The time of exposure to this temperature depends on the length of pipe in the holding section of the sterilizer. The medium cooling is instantly carried out by passing through an expansion valve into a vacuum chamber. Further cooling takes place in the heat exchanger where residual heat is used to preheat incoming medium.

Continuous sterilization with flash cooling and heat exchanger are illustrated in Fig. 3.6.

A continuous sterilizer can be used to deliver high-quality medium to the bioreactor. Continuous sterilizers have the advantage of a very high temperature and a short time exposure. Thus, this sterilization method can significantly reduce damage to medium ingredients, while achieving high levels of cell destruction [13]. Rates of heating and cooling in continuous sterilization are much more rapid than a batch process. Accordingly, in design of continuous

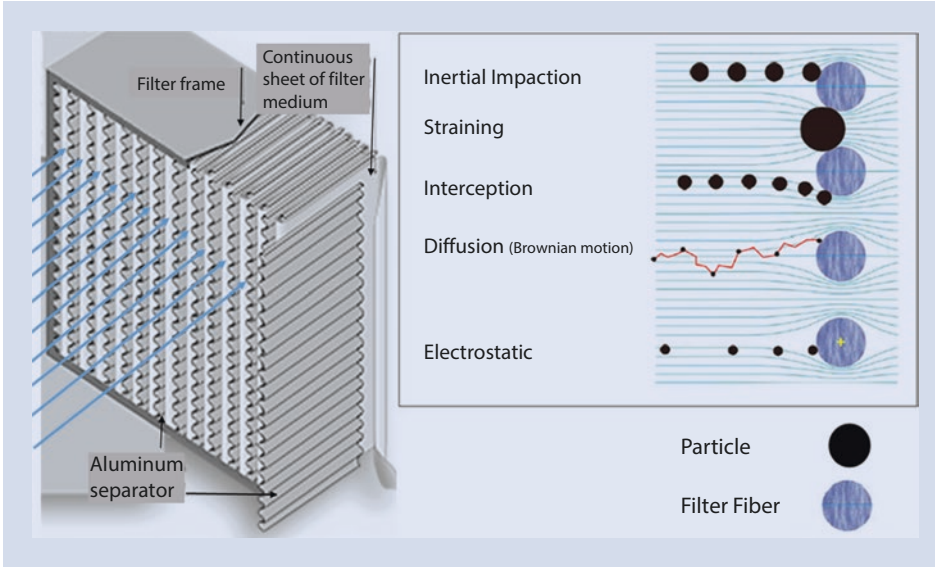


Fig. 3.7 Suspended solids filtration mechanisms

sterilizers, contributions to cell death outside of the holding period are generally ignored. Other advantages include improved steam economy and more reliable scale-up. The amount of steam needed for continuous sterilization is 20–25% that is used in batch processes. The time required is also significantly reduced because heating and cooling are virtually instantaneous [3]. Continuous sterilization also saves both capital and running costs in the design of the fermenter. If medium is sterilized batchwise in the fermenter, it must be agitated with agitators without aeration, and thus the highest power draw occurs during batch sterilization [4, 12].

3.3 Filter Sterilization

Filtration is extremely useful in the production of media for many bioprocesses. Although it can be used for all processes, filtration dominates the cell culture industry, where components often cannot be heat sterilized.

Suspended solids may be separated from a fluid using filtration by the mechanisms, including inertial impaction, straining, interception, diffusion, and electrostatic attraction (Fig. 3.7).

1. Inertial impaction

Suspended particles in a fluid have momentum. The fluid containing suspended particles will flow through the filter by the route of least resistance. Particle may therefore become impacted upon the fibers where they may remain. Inertial impaction is more significant in the filtration of gases than in liquids.

2. Straining

Similar to sieving, i.e., particles of large sizes cannot pass through smaller pore size of filter medium.

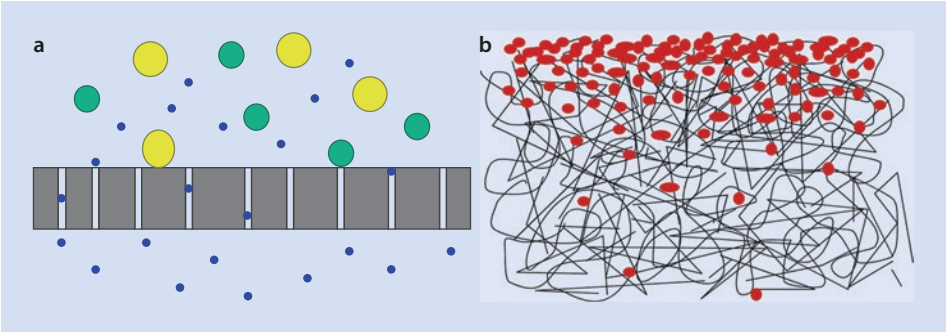


Fig. 3.8 Types of filters: **a** fixed pore filters (absolute filters) and **b** non-fixed pore filters (depth filters)

3. Interception
The fibers comprise a filter with various pore sizes. Particles which are larger than the filter pores are removed by direct interception. However, a significant number of particles which are smaller than the fiber pores are also retained by interception. This may occur by several mechanisms, for example, more than one particle accumulate at a pore, an irregularly shaped particle may bridge a pore, and particle may be trapped by other mechanisms. Interception is important in filtration of gases and liquids.

4. Diffusion
Extremely small particles suspended in a fluid are subjected to Brownian motion which is random movement due to collisions with fluid molecules. Thus, such small particle may become impacted upon the filter fibers. Diffusion is more significant in the filtration of gases than in the filtration of liquids.

5. Electrostatic attraction
Charged particles may be attracted by opposite charges on the surface of the filtration medium.

Filters as illustrated in Fig. 3.8 have been classified into two main types: fixed pore filters (absolute filters) and non-fixed pore filters (depth filters). Pore size of fixed pore filters is controlled during manufacturing so that an absolute rating can be quoted for filter. For example, the removal of particles above a certain size can be guaranteed. Thus, interception is the major mechanism by which particles are removed. They are also capable of removing particles smaller than the pores because they have depth. The mechanisms of inertial impaction, diffusion, and attraction play significant roles in this action. Fixed pore filters are superior for most purposes as they have absolute ratings, are less susceptible to change in pressure, and are less likely to release trapped particles. The major disadvantage associated with absolute filters is the resistance to flow and consequently the large pressure drop across the filters which results in a major operational cost. However, pleated membranes with large surface areas minimize the pressure drop across the filter.

Non-fixed pore filters consist of compacted beds or pads of fibrous material such as glass wool. These filters have been used widely in the fermentation industry. Non-fixed pore filters rely on the removal of particles by inertial impaction, diffusion, and electrostatic attraction rather than interception. In theory, the removal of microorganisms by a fibrous filter cannot be absolute, as there is always the possibility of an organism passing through the filter, regardless of the filter's depth. Also, because the fibers are not cemented

into position, an increase in the pressure may result in movement of the material. This may result in producing larger channels through the filter. Increased pressure may also result in displacement of previously trapped particles.

It is important to realize that filters should be steam sterilized before and after operation. Thus, the material must be stable at high temperatures, and the steam must be free of particulate matter because the filter modules are particularly vulnerable to damage at high temperature. Thus, the steam itself is filtered through stainless steel mesh filters with 1 μm size [13].

Filtration is generally not as effective or reliable as heat sterilization. Viruses and mycoplasma are able to pass through membrane filters. Care must also be taken to prevent holes or tears in the membrane.

3.3.1 Filter Sterilization of Fermentation Medium

Sometimes, fermentation media or selected ingredients are sterilized by filtration rather than heat. For example, media containing heat-labile components such as vitamins, enzymes, serum, and some growth factors can be easily destroyed by heat and must be sterilized by other means. In microbial systems, the filtration step may involve the simple filtration of one medium component which can be added after the bulk medium has been sterilized by heat, or it may be necessary to filter sterilize the whole medium.

Usual applications of filtration include medium, additive and buffer sterilization, cell debris and endotoxin removal, cell culture, and serum and plasma clarification. Typically, membranes used for filter sterilization of liquids are made of cellulose esters or other polymers and have pores between 0.2 and 0.45 μm in diameter. The level of sterility required is important. If a system requires bacterial removal, then a filter pore size of 0.22 μm is required; if viruses are to be removed, then a pore size of 20 nm is needed. As medium is passed through the filter, bacteria and other particles with dimensions greater than the pore size are screened out and collect on the surface of the membrane. The small pore sizes used in liquid filtration mean that the membranes are readily blocked unless the medium is prefiltered to remove any large particles. To achieve high flow rates, large surface areas are required to minimize the pressure drop across the filter.

Filtration systems either can or cannot be disposable, as per operational requirements. Use of disposable filter units eliminates the need for cleaning validation, which is both timely and costly. The companies supplying the filters will validate the filter system, again reducing the need for this to be carried out in-house. Housings for the filters again are varied and include vacuum cups, syringe filters, bench-top filters, and a range of housings that vary in size and complexity, depending on the application [3, 6].

3.3.2 Filter Sterilization of Fermentation Air

Aerobic fermentations require the continuous addition of desirable quantities of sterile air. Although it is possible to sterilize air by heat treatment, especially in large-scale bioprocesses, heat sterilization of air is impractical, and filtration is the most common method for this purpose [6].

Depth filters which have an absolute rating have been used widely in the fermentation industry. Distances between the fibers in depth filters are typically 2–10 μm, about ten times greater than the dimensions of the bacteria and spores to be removed. Airborne particles penetrate the bed to various depths before their passage through the filter is arrested; the

depth of the filter medium required to produce air of sufficient quality depends on the operating flow rate and the incoming level of contamination. Cells are collected in depth filters by a combination of impaction, interception, and electrostatic effects. For particles smaller than 1.0 μm, diffusion to the fibers is the main mechanism of filtration. Depth filters do not perform well if there are large fluctuations in flow rate or if the air is wet. Liquid condensing in the filter increases the pressure drop, causes channeling of the gas flow, and provides a pathway for organisms to grow through the bed. Increasingly, depth filters are being replaced for industrial applications by membrane cartridge filters. These filters use steam sterilizable polymeric membranes which act as surface filters trapping contaminants as on a sieve [3]. These systems, like those for the sterilization of liquids, are designed to be accommodated in stainless steel modules. They consist of pleated and hydrophobic membrane filter cartridges with small and uniformly sized pores of 0.45 μm or less in diameter. The hydrophobic nature of the surface minimizes problems with filter wetting, while the pleated configuration allows a high filtration area to be packed into a small cartridge volume.

Prefilters built into the cartridge or upstream reduce fouling of the membrane by removing large particles such as dust, oil, carbon (from the compressor), water droplets, and foam (from the incoming air).

The most common construction material used for the pleated membranes for air sterilization is polytetrafluoroethylene (PTFE), which is naturally hydrophobic with excellent chemical resistance. PTFE-made filters are the ideal choice for sterile venting of gases. It is essential that a prefilter is incorporated upstream of the absolute filter [13].

3.3.3 Filter Sterilization of Fermentation Exhaust Air

Filters are also used to sterilize effluent gases leaving fermenters. In filter sterilization of fermentation exhaust air, the objective is to prevent microbial release into the atmosphere from the fermenter. The concentration of cells in fermenter off-gas is several times greater than in incoming air. Containment is particularly important when organisms used in fermentation are potentially harmful to plant personnel or the environment. Companies operating fermentations with pathogenic or recombinant strains are required by regulatory authorities to prevent escape of the cells [3].

Depth filters and fixed pore membrane modules may be used for effluent gas sterilization. However, the system must be able to cope with the sterilization of water-saturated air at a relatively high temperature. Also, foam may overflow from the fermenter into the air exhaust line. Thus, some forms of pretreatment of exhaust gas are necessary before it enters the absolute filter. This pretreatment may be a hydrophobic prefilter or a mechanical separator to remove water, aerosol particles, and foam. The pretreated exhaust air is then fed to a 0.2 μm hydrophobic filter.

3.3.4 Basic Principles of Filter Design

Several equations have been developed relating the collection efficiency of a filter bed based on various characteristics of the filter and its components. However, a simple description may be used to illustrate the basic principles of filter design.

It is assumed that if a particle touches a fiber, it remains attached to it and that there is a uniform concentration of particles at any given depth in the filter. Consequently, each

filter layer should reduce the population entering it by the same proportion. This may be expressed mathematically as:

$$\frac{dN}{dx} = -KN \tag{3.11}$$

where N is the concentration of particles in the air at depth x in the filter and K is a constant. K is function of the air velocity, filter density, fiber size, and size and density of the organism to be removed.

On integration of Eq. (3.11) over the length of the filter, it becomes

$$\frac{N}{N_0} = e^{-Kx} \tag{3.12}$$

where N_0 is the number of particles entering the filter and N is the number of particles leaving the filter.

On taking natural logarithms, Eq. (3.11) becomes

$$\ln \frac{N}{N_0} = -Kx \tag{3.13}$$

Equation (3.13) is termed the log-penetration relationship.

The efficiency of the filter is given by the ratio of the number of particles removed to the original number present, thus

$$E = \frac{N_0 - N}{N_0} \tag{3.14}$$

where E is the efficiency of the filter.

But:

$$\frac{N_0 - N}{N_0} = 1 - \frac{N}{N_0} \tag{3.15}$$

Substituting

$$\frac{N}{N_0} = e^{-Kx}$$

Thus:

$$\frac{N_0 - N}{N_0} = 1 - e^{-Kx} \tag{3.16}$$

$$E = 1 - e^{-Kx}$$

Log penetration relationship (Eq. 3.1) has been used in filter design. By using the concept X_{90}, the depth of filter that requires to remove 90% of the total particles entering the filter can be calculated.

If N_0 were 10 and x were X_{90}, then N would be 1.

$$\ln \frac{1}{10} = -KX_{90}$$

or

$$2.303 \log_{10} \frac{1}{10} = -KX_{90}$$

$$2.303(-1) = -KX_{90}$$

Therefore,

$$K = 2.303 / K$$

Example

It is required to provide a 20 m^3 fermenter with air at a rate of 10 m^3 min^{-1} for a fermentation lasting 100 h. From an investigation of the filter material to be used, the optimum linear air velocity was shown to be 0.15 m sec^{-1}, at which the value of K was 1.535 cm^{-1}. Calculate the dimensions of the filter. (The air in the fermentation plant contained approximately 200 microorganisms per 1 m^3.)

Solution

The log penetration relationship states that

$$\ln \frac{N}{N_0} = -Kx$$

N_0 = total amount of air provided × 200,
$N_0 = 10 \times 60 \times 100 \times 200$
$= 12 \times 10^6$ microorganisms
The acceptable final microbial count in the medium after sterilization is one in thousand (10^{-3} Viable cells), therefore,
$N = 10^{-3}$

$$\ln \frac{10^{-3}}{12 \times 10^6} = -1.535\, x$$

$x = 15.12$ cm

Therefore, the filter to be used should be 15.12 cm long.

Cross-sectional area of the filter is given by the volumetric air flow rate divided by the linear air velocity:

$$A = \pi r^2 = \frac{\text{volumetric air velocity}}{\text{linear air velocity}}$$

$$\pi r^2 = \frac{10}{0.15 \times 60}$$

where r is radius of filter

$$R = 0.59\,\text{m}$$

Thus, the filter to be employed should be 15.12 cm in length and 1.18 m in diameter.

3.4 Summary

Sterilization is a key factor in a successful fermentation process. Obtaining the correct sterilization method, in the correct conditions, is not easy, and considerable research has to be done in order to obtain the required information to optimize the process. Avoidance of contamination may be achieved by using a pure inoculum to start the fermentation, sterilizing fermentation medium, sterilizing air, sterilizing the fermenter vessel, sterilizing all materials to be added during the fermentation process, and maintaining aseptic conditions during the fermentation process. Proper handling of the sterilization will ensure that the constituents are free from contaminants and all growth factors are available to the desired cells to achieve the maximum productivity.

Take-Home Messages

- Commercial fermentations typically require thousands of liters of liquid medium and millions of liters of air.
- Heat sterilization is one of the most effective methods and has been widely used in the sterilization of nutrient media and equipment.
- Batch thermal sterilization processes tend to be used for liquid and equipment in fermentation systems, as these tend to be reliable and cost-effective options for both small- and large-scale systems.
- Depending on the rate of heat transfer from the steam or electrical element, raising the temperature of the medium in large fermenters can take a significant period of time.
- Spores of *Bacillus stearothermophilus* are the most heat-resistant organisms.
- The continuous system includes a time period during which the medium is heated to the sterilization temperature, a holding time at desired temperature, and cooling period to restore the medium to the desirable fermentation temperature.
- Filtration is extremely useful in the production of media for many bioprocesses. Although it can be used for all processes, filtration dominates the cell culture industry, where components often cannot be heat sterilized.
- Suspended solids may be separated from a fluid using filtration by the mechanisms, including inertial impaction, straining, interception, diffusion, and electrostatic attraction.
- Typically, membranes used for filter sterilization of liquids are made of cellulose esters or other polymers and have pores between 0.2 and 0.45 μm in diameter.
- Aerobic fermentations require the continuous addition of desirable quantities of sterile air. Although it is possible to sterilize air by heat treatment, especially in large-scale bioprocesses, heat sterilization of air is impractical, and filtration is the most common method for this purpose.
- Filters are also used to sterilize effluent gases leaving fermenters.

References

1. Chopra CL, Qazi GN, Chaturvedi SK, Gaind CN, Atal CK. Production of citric acid by submerged fermentation. Effect of medium sterilisation at pilot-plant level. J Chem Technol Biotechnol. 1981;31(1):122–6. https://doi.org/10.1002/jctb.503310117.
2. Deindoerfer FH, Humphrey AE. Analytical method for calculating heat sterilization times. J Appl Microbiol. 1959;7(4):256.
3. Doran PM. Bioprocess engineering principles. London: Academic Press; 1995.
4. Junker BH, Lester M, Brix T, Wong D, Nuechterlein J. A next generation, pilot-scale continuous sterilization system for fermentation media. Bioprocess Biosyst Eng. 2006;28:351–78. https://doi.org/10.1007/s00449-005-0041-0.
5. Li X, Farid M. A review on recent development in non-conventional food sterilization technologies. J Food Eng. 2016;182:33–45. https://doi.org/10.1016/j.jfoodeng.2016.02.026.
6. McNeil B, Harvey L, editors. Practical fermentation technology. Chichester, England: Wiley; 2008.
7. Najafpour G. Biochemical engineering and biotechnology. Amsterdam, Netherlands: Elsevier; 2015.
8. Paulová L, Patáková P, Brányik T. Advanced fermentation processes. In: Vicente AA, Teixeira JA, editors. Engineering aspects of food biotechnology. Boca Raton: CRC Press; 2013. p. 97–123.
9. Pollard D. Aseptic operations. In: Webb C, editor. Comprehensive biotechnology, vol. 2. 2nd ed: Pergamon, Turkey: Elsevier; 2011. p. 933–56.
10. Richards JW. Introduction to industrial sterilization. London/New York: Academic Press; 1968.
11. Smelt JPPM. Recent advances in the microbiology of high pressure processing. Trends Food Sci Technol. 1998;9(4):152–8. https://doi.org/10.1016/S0924-2244(98)00030-2.
12. Soderberg AC. Fermentation design. In: Vogel HC, Todaro CM, editors. Fermentation and biochemical engineering handbook, principles, process design and equipment. Waltham: Elsevier; 2014. p. 85–108.
13. Stanbury PF, Whitaker A, Hall SJ. Principles of fermentation technology. Oxford, United Kingdom: Elsevier; 2013.
14. Wilson DR, Dabrowski L, Stringer S, Moezelaar R, Brocklehurst TF. High pressure in combination with elevated temperature as a method for the sterilisation of food. Trends Food Sci Technol. 2008;19(6):289–99. https://doi.org/10.1016/j.tifs.2008.01.005.

Solid-State Cultivation Bioreactors

David Alexander Mitchell and Nadia Krieger

A. Berenjian (ed.), *Essentials in Fermentation Technology*, Learning Materials in Biosciences,
https://doi.org/10.1007/978-3-030-16230-6_4

What You Will Learn in This Chapter

Solid-state cultivation (SSC) involves the cultivation of microorganisms on moist solid particles surrounded by a continuous gas phase. Although SSC has been used for centuries in the production of traditional fermented foods, most biotechnological products are currently produced by submerged culture. However, SSC will become of increasing importance for processing solid residues in biorefineries, especially when filamentous fungi are involved. This chapter presents the basic design and operating principles of the four bioreactor types available for SSC processes: trays, packed beds, rotating (or stirred) drums, and forcefully aerated agitated bioreactors. Control of bed temperature is the main challenge, with the selection of an appropriate bioreactor depending on the specific growth rate of the process organism and also its tolerance of agitation. The bioreactors differ with respect to whether or not the substrate bed is agitated and whether or not the substrate bed is forcefully aerated, offering different combinations of conductive, convective, and evaporative cooling. The best tools for guiding the scale-up of SSC bioreactors are mathematical models that integrate growth kinetics with energy and water balances and which recognize and describe the gradients that occur across the bed during periods of static operation. Due to the complexity of the microscopic-scale phenomena involved in growth of the microorganism on the substrate particles, the kinetic equations used in these models are usually simple empirical equations, such as the logistic equation.

4.1 Solid-State Cultivation: Its Key Characteristics and When You Would Use It

Microorganisms can be cultivated in systems that have quite different characteristics. The two extremes are "submerged liquid cultivation" with a soluble nutrient and "solid-state cultivation." The system in submerged liquid cultivation has been described elsewhere in this book (cite section when book is finalized). Solid-state cultivation (SSC) involves the cultivation of a microorganism on moist solid particles in a situation in which the void spaces between the particles are filled with a continuous gas phase (Fig. 4.1). This cultivation method is also commonly referred to as solid-state fermentation (SSF), with the word fermentation being used as a synonym for cultivation; it does not imply fermentation in the metabolic sense of the word. Of course, there is a range of other cultivation processes in which microorganisms grow on solid surfaces or use nutrients originating from solid particles, such as trickling filters, suspensions of solid particles in a liquid medium, and slurry cultivation.

Submerged liquid cultivation (SLC), is used industrially to produce the majority of microbial products. However, there are some cases in which it is necessary or advantageous to use SSC processes such as those described in this chapter. For example:

- When the product has a solid form, consisting of the microbial biomass and residual solid substrate. This is the case with various "fermented foods," such as tempeh.
- When the product is only produced in SSC or is produced in much higher levels or with desirable characteristics in SSC. For example, fungal conidia can be used as biopesticides. In some cases, these conidia are only produced in SSC. In other cases, the conidia are produced in both SLC and SSC, but those produced in SSC are more robust and therefore survive better when applied in the field. As another example, many filamentous fungi produce extracellular enzymes more efficiently in SSC than in SLC.

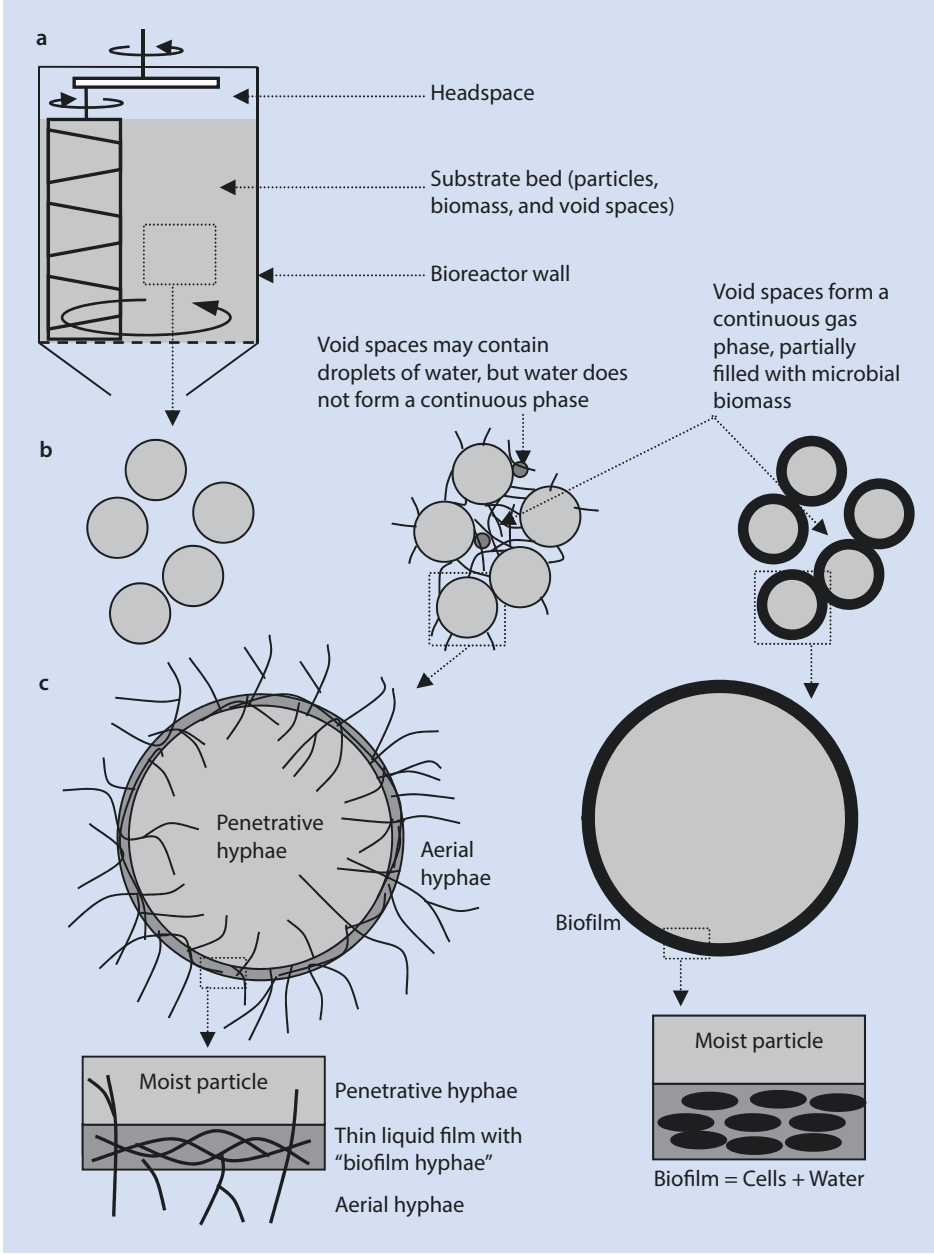


Fig. 4.1 The phases present within an SSC bioreactor and the distribution of biomass within the system. **a** Macroscale view. **b** Microscale view. From left to right the diagrams represent uninoculated substrate, the growth of a filamentous fungus and the growth of a unicellular organism, such as a yeast or bacterium. **c** Greater detail of the microscale, showing a transverse section through the particles. (Adapted from Mitchell et al. [6] with kind permission from Springer Science and Business Media)

- When there is a specific desire to use solid residues, in cases where the addition of large volumes of water is not desirable. For example, there is a growing interest in developing biorefineries to take advantage of agricultural, forestry, and food processing residues. SSC uses significantly less water than SLC and also produces less liquid effluent.

The solid substrate particles used in SSC are typically derived from low-value solid organic residues originating from agriculture, forestry, or food processing. Higher-value materials, such as whole grains, are sometimes used. These substrates typically contain complex mixtures of carbon sources, including lignocellulose, hemicellulose, starch, and soluble sugars. They may need thermal, chemical, or mechanical pretreatment to increase their susceptibility to microbial attack and to produce particles of an appropriate size. Some processes use an inert solid matrix, such as polyurethane foam, impregnated with a nutrient solution.

The restricted amount of water is a key feature of SSC systems. The greater part of the water in the system is held within the substrate particles; therefore water contents must be below the maximum water-holding capacity of the substrate. The maximum water-holding capacity of organic solid materials can vary significantly. At one extreme, sugarcane bagasse can hold a weight of water more than threefold its dry weight; this corresponds to a moisture content of over 75% (wet basis). In practice, moisture contents are below the maximum water-holding capacity and, consequently, water activities are relatively low, with values of between 0.9 and 0.95 being common.

Most SSC processes involve filamentous organisms, such as filamentous fungi or actinomycetes, many of which grow well on solid surfaces with relatively low water activities. However, there are also processes involving unicellular bacteria and yeasts. The mode of growth affects the distribution of biomass in the system: Unicellular organisms grow as a biofilm, while filamentous organisms produce a mycelium (Fig. 4.1). In the case of a fungal mycelium, there will be aerial and penetrative hyphae. If the layer of hyphae at the surface is thick, then water can move by capillary action from the substrate, converting this layer into a moist biofilm (Fig. 4.1). A biofilm can also be formed if the bed is mixed, since mixing will squash aerial hyphae onto the surface of the particle.

This chapter deals with aerobic SSC processes that involve a single microorganism and for which the temperature needs to be controlled as near as possible to the optimum temperature for growth of that microorganism. It does not address composting, in which the temperature reaches high values during the process, leading to a succession of microbial communities. It also does not address dry anaerobic digestion.

4.2 Batch, Fed-Batch, and Continuous Operation in SSC

Before addressing bioreactor designs themselves, it is useful to consider the mode of operation. The great majority of SSC processes are batch processes, not continuous. In fact, although well researched in SLC, the continuous mode of operation has received little attention in SSC and will not be covered in this chapter. However, it is still useful to consider, briefly, the potential for using the "continuous stirred-tank reactor" (CSTR) and the "continuous plug-flow reactor" modes of operation in SSC.

CSTR-type operation is not appropriate in the case of SSC. In SLC, it is not necessary to inoculate new medium as it is added to the CSTR; when new medium is added, it is immediately distributed throughout the cultivation medium and, therefore, brought into contact with the microorganism. In the case of SSC, if new uninoculated particles were added to a CSTR, then it would be necessary for them to be colonized by the microorganism growing on the older particles already present in the CSTR. This transfer of biomass (or spores) is likely to be a slow process. Even if the new particles were inoculated upon addition, there would be a lag phase before the start of growth. A further difference is that, in SLC, the medium leaving a CSTR is uniform. On the contrary, in SSC, the medium leaving a CSTR would contain a mixture of particles that have been in the bioreactor for vastly different times (from a few seconds to several average residence times).

Plug-flow-type continuous operation might be appropriate in the case of SSC. In this type of continuous operation, the substrate is inoculated as it enters the bioreactor and the microorganism grows as the substrate moves through the bioreactor. The residence time in the bioreactor is equal to the total cultivation time in batch culture. In fact, this type of operation is analogous to batch culture, with distance traveled along the bioreactor being the independent variable, instead of time.

Note that fed-batch operation is possible in the case of SSC. It would not be the case to add new substrate particles, but it is possible to spray nutrients onto the substrate bed in the form of a fine mist.

4.3 Overview of Bioreactor Classifications and Choice of Bioreactor Type

Many different designs have been proposed for SSC bioreactors for use in batch processes. It is useful to classify these diverse bioreactors into four groups, based on how they are aerated and agitated, as these two factors are crucial in determining bioreactor performance (Fig. 4.2). With respect to agitation, the extremes are a completely static bed and a continuously agitated bed. Infrequently agitated beds perform like static beds for most of the time. If agitation is intermittent but frequent, then the bed will perform similarly to a continuously agitated bed. The distinctions between different groups of bioreactors are not absolute. For example, there is not a sharp division between "infrequent mixing" and "frequent mixing." Likewise, some bioreactors may have forced aeration, but the air may not be forced to flow uniformly through all the bed, such that some regions of the bed are better aerated than others.

The next four subsections present the principles of design and operation of these four bioreactor types. However, before this, it is appropriate to consider the factors that influence the choice of bioreactor type. There are three key questions:

1. How fast does the microorganism grow?
2. How sensitive is the process microorganism or the desired final product to damage by agitation?
3. How valuable is the product?

Figure 4.3 shows how these questions might be used in the selection of a bioreactor. The rate of growth of the microorganism determines the peak rate of metabolic heat production and, therefore, the rate at which heat must be removed from the bed. For a

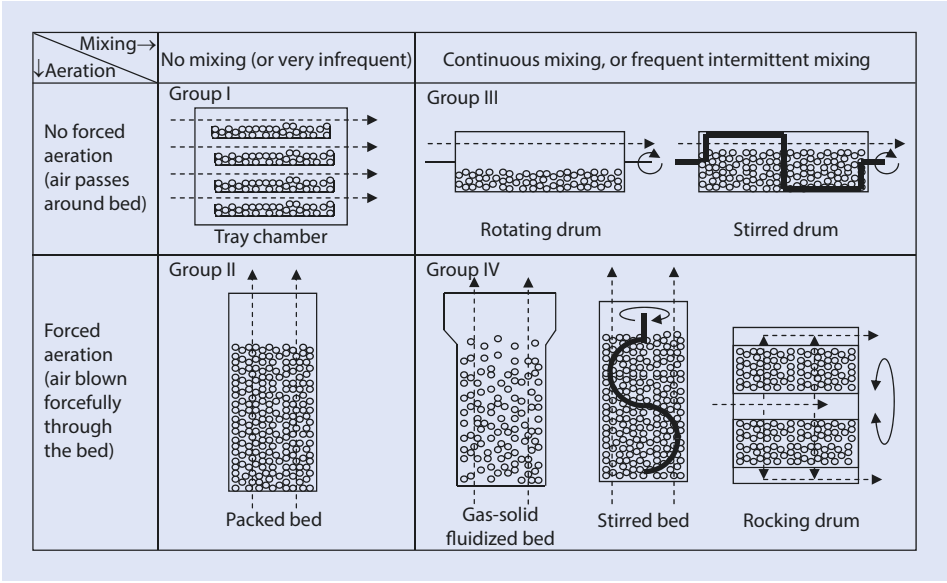


Fig. 4.2 Basic design features of the various bioreactors used in solid-state cultivation, showing how they can be classified into four groups based on how they are mixed and aerated. (Adapted from Mitchell et al. [5] with kind permission from Springer Science and Business Media)

fast-growing microorganism, it is probably necessary to use forced aeration. Agitation of the bed can help to promote heat removal, but it can only be used to the degree that it is tolerated by the microorganism. Depending on the microorganism, it might be possible to agitate the bed continuously or frequently. In other cases, the bed can only be agitated infrequently, for example, once or twice per day. At the extreme, the substrate bed must be left completely static. The characteristics of the final product can also be important, for example, if it is necessary for the substrate bed to be bound into a tight mass by a fungal mycelium (such as is the case in tempeh production), then the bed must remain static throughout the process. The value of the product determines whether it will be economically viable to incorporate bed agitators and blowers, or to use intricate designs with multiple small substrate beds.

Table 4.1 shows a selection of pilot-scale and large-scale bioreactors that have been used to produce a range of products by SSC. More details about the various types of bioreactors are given in the following sections.

4.4 Design and Operation of Tray-Type Bioreactors

4.4.1 Basic Features of Tray Bioreactors

A tray bioreactor is characterized by a tray chamber containing several trays (Fig. 4.4). Each tray contains a static substrate bed, although it may be agitated once or twice per day, usually manually. The air may be circulated around the trays; it is not forced to flow through the substrate bed itself.

Fig. 4.3 A general guide to the selection of an appropriate bioreactor for solid-state cultivation

Table 4.1 A selection of processes carried out in pilot-scale and large-scale solid-state cultivation bioreactors

Product	Microorganism and substrate	Key bioreactor details	More information
Soy sauce *koji* (a step in the manufacture of soy sauce)	*Aspergillus oryzae* on soybeans	Packed bed with capacity for 1000 kg Rotating drum with capacity for 1000 kg Forcefully aerated intermittently agitated bioreactor with capacity for 15,000 kg	Sato and Sudo [11]
Tempeh	*Rhizopus oligosporus* on soybeans	Produced domestically and in small industries in tray-type bioreactors with substrate layers up to 5 cm high	Nout and Rombouts [7], Dinesh Babu et al. [1]
Penicillin (during the early 1940s)	*Penicillium notatum* on wheat bran	40 rotating-drum bioreactors, each 11.3 m long and 1.2 in diameter (i.e., each has a volume of 13 m^3)	Ziffer [14]
Cyclosporin	*Fusarium solanii* on wheat bran	A "multiple-packed-bed" system denominated Plafractor™ with 20 kg substrate on a total plate area of 2.26 m^2	Suryanarayanand Mazumdar [13]
Conidia of a biocontrol fungus	*Beauveria brongniartii* on crushed barley-corn	A modular packed-bed system. The bed occupies a volume of 30 L, in seven 6 cm high layers	Lüth and Eiben [4]
Phytase	*Aspergillus niger* on wheat bran	Tray system – facility initially built for 10,000 trays	Filer [3]
Protein enrichment	*Trichoderma viride* on sugar beet pulp	Forcefully aerated intermittently agitated bioreactor of 1.6 m^3 capacity, holding 1000 kg of wet substrate (~200 kg dry matter)	Durand [2]
Cellulase	*Trichoderma harzianum* on an 80:20 (m/m) mixture of sugarcane bagasse and wheat bran	Packed bed with vertical heat transfer plates within the bed. Base of 0.2 m^2, height of 0.65 m, working volume of 100 L. Contained up to 40 kg wet medium	Roussos et al. [10]
Gibberellic acid	*Gibberella fujikoroi* on wheat bran	Two forcefully aerated intermittently agitated bioreactors: 50 kg capacity and 200 kg capacity	Pérez-Correa and Agosin [8]
Pectinases	*Aspergillus niger* on a 90:10 (m/m) mixture of wheat bran and sugarcane bagasse	Forcefully aerated intermittently agitated bioreactor with bed volume of 200 L, capacity for 30 kg dry substrate (~80 kg moist substrate)	Pitol et al. [9]

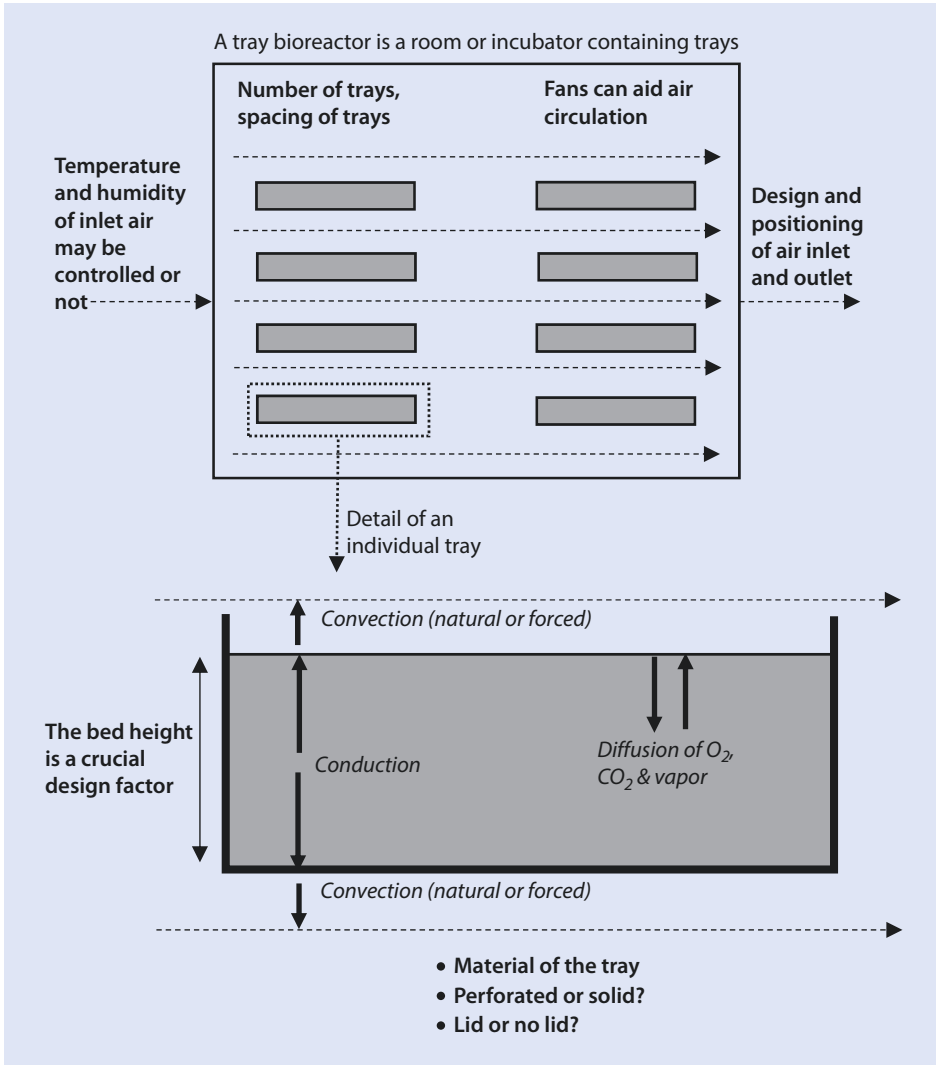


Fig. 4.4 Key design and operating variables and heat and mass transfer processes in tray bioreactors. **Bold text** indicates design and operating variables; *Italic text* indicates heat and mass transfer mechanisms. Dashed arrows represent air flow; solid **thick** arrows represent key heat and mass transfer mechanisms

The nature of the tray chamber depends on the scale of production. If hundreds of kilograms or several tonnes of substrate are to be processed in each batch, then the trays will be housed in incubation rooms. If only several kilograms are processed at any one time, then it may be appropriate to put the trays in an incubator. If the process needs to be completely aseptic, then it may be necessary to use a closed, autoclavable reactor chamber, to which sterile air can be supplied.

The aeration system can have different levels of sophistication. In traditional processes, the tray chamber is often simply left closed to minimize temperature fluctuations and

maintain a humid atmosphere, although fans may be used to circulate the air within the tray chamber in order to avoid stagnant zones. In more sophisticated processes, an external air preparation system is used to circulate air of controlled temperature and humidity through the chamber, from an inlet to an outlet, with care being taken to ensure that air is circulated past all trays. The efficiency of air circulation will depend on the positioning of trays in the tray chamber: the smaller the spacing between the trays, the more substrate can be processed per cubic meter of chamber volume; however, the more difficult it will be to ensure effective circulation of air past the tray.

The tray itself can be constructed of different materials. In traditional processes, bamboo or wooden trays are often used. Glass bottles have been used at small scale. Metal trays are durable and resist autoclaving; the metal might be unperforated or perforated, with the perforations facilitating gas exchange. The body of the tray can even be made of wire mesh, maximizing gas exchange. One interesting alternative is to use microporous plastic bags. This plastic allows the exchange of gases (including water vapor) over the whole bed surface, but prevents the entry of contaminants.

It is possible for the tray to have a lid. A lid can help to prevent water that condenses on the surfaces in the tray chamber from dripping onto the substrate bed. It can also help to reduce evaporation of water from the bed. However, even if the lid is loose fitting, it will restrict the exchange of O_2 and CO_2 between the bed and the bulk air phase in the tray chamber, creating a headspace within the tray that can have a quite different gas composition from that of the bulk air phase. When glass bottles or impermeable plastic bags are used, the mouth can be plugged with cotton wool.

4.4.2 Key Heat and Mass Transfer Processes in Trays and How Design and Operation Can Maximize Performance

The fact that air is circulated around the substrate bed in the tray but not forced to flow through the bed itself means that, within the bed, mass transfer is limited to diffusion and heat transfer is limited to conduction. The height of the substrate bed in the tray is therefore a critical variable. A greater bed height means that fewer trays are required to hold the same overall mass of substrate. However, with greater bed heights, higher temperatures will be reached in the middle of the bed and the more likely it is that O_2 will become limiting within the bed.

Circulating air past the surface of the bed improves the bed-to-headspace heat transfer. However, although this reduces the temperature at the surface of the bed, it is relatively ineffective at controlling the temperatures within the bed itself. Also, using dry air in the tray chamber to promote evaporation of water from the bed surface is not effective. The evaporation is limited to the exposed bed surface, and this surface will quickly dry out.

One variation of the tray bioreactor is the pressure pulsation bioreactor. In this bioreactor, the pressure within the chamber is varied during the cultivation, from atmospheric pressure to several atmospheres. The variation in pressure forces some air to flow into and out of the bed, which does improve heat and mass transfer to some degree. However, this type of bioreactor is significantly more expensive to build and operate: the tray chamber must be a pressure vessel and the pressure is continuously cycled over a wide range.

4.4.3 Scale-Up Strategies for Tray Bioreactors

An appropriate bed height (typically of a few cm) will be established during laboratory-scale studies. Increase in scale may be achieved by increasing the horizontal dimensions of the tray (i.e., longer and wider trays). However, the tray dimensions cannot be increased indefinitely, as the tray will become too difficult to manipulate. The main strategy for scale-up of tray bioreactors is, therefore, to build larger tray chambers and increase the number of trays.

4.5 Design and Operation of Packed-Bed Bioreactors

4.5.1 Basic Features of Packed-Bed Bioreactors

The basic characteristic of a packed bed is that air is forced to pass uniformly through a static bed. There are various ways in which this can be achieved, but the most basic design is a vertical column, with the substrate bed being supported on a perforated base plate through which air is forcefully blown. The temperature, humidity, and flow rate of this air are controlled by an air preparation system.

There are two basic designs for packed beds, the traditional packed bed and the Zymotis-type packed bed (Fig. 4.5). Large-scale, traditional packed beds are usually a meter or more in diameter and a water jacket is not used because it would only make a minimal contribution to cooling the bed. However, it may be appropriate to use a water jacket (or to immerse the packed bed in a water bath) for laboratory-scale studies with thin packed beds (of 2–5 cm diameter). This allows reasonably good control of bed temperature for studies of growth kinetics. At large scale, it may be appropriate to have a system that allows intermittent agitation of the bed. In the so-called Zymotis design, the bed is divided into compartments by closely spaced heat exchange plates. In this case, agitation of the bed is not feasible.

4.5.2 Key Heat and Mass Transfer Processes in Traditional Packed-Bed Bioreactors and How Design and Operation Can Maximize Performance

In traditional packed-bed bioreactors, convective cooling with evaporation is the key heat removal method (Fig. 4.6a). However, it is usually not appropriate to aerate the bed with dry air in an attempt to maximize evaporative cooling, as this will cause the bed to dry out quickly. In fact, evaporation occurs even if saturated air is supplied to a traditional packed-bed bioreactor. This happens because the convective removal of waste metabolic heat increases the air temperature and therefore increases its water carrying capacity, creating a driving force for evaporation.

Another consequence of the removal of metabolic heat from the solids to the air is that the air becomes increasingly warmer as it passes through the bed and does not cool the remaining parts of the bed as effectively. This results in a rise in bed temperature between the air inlet and air outlet. This phenomenon has consequences for the design and operation of traditional packed-bed bioreactors: there is an interplay between peak heat generation

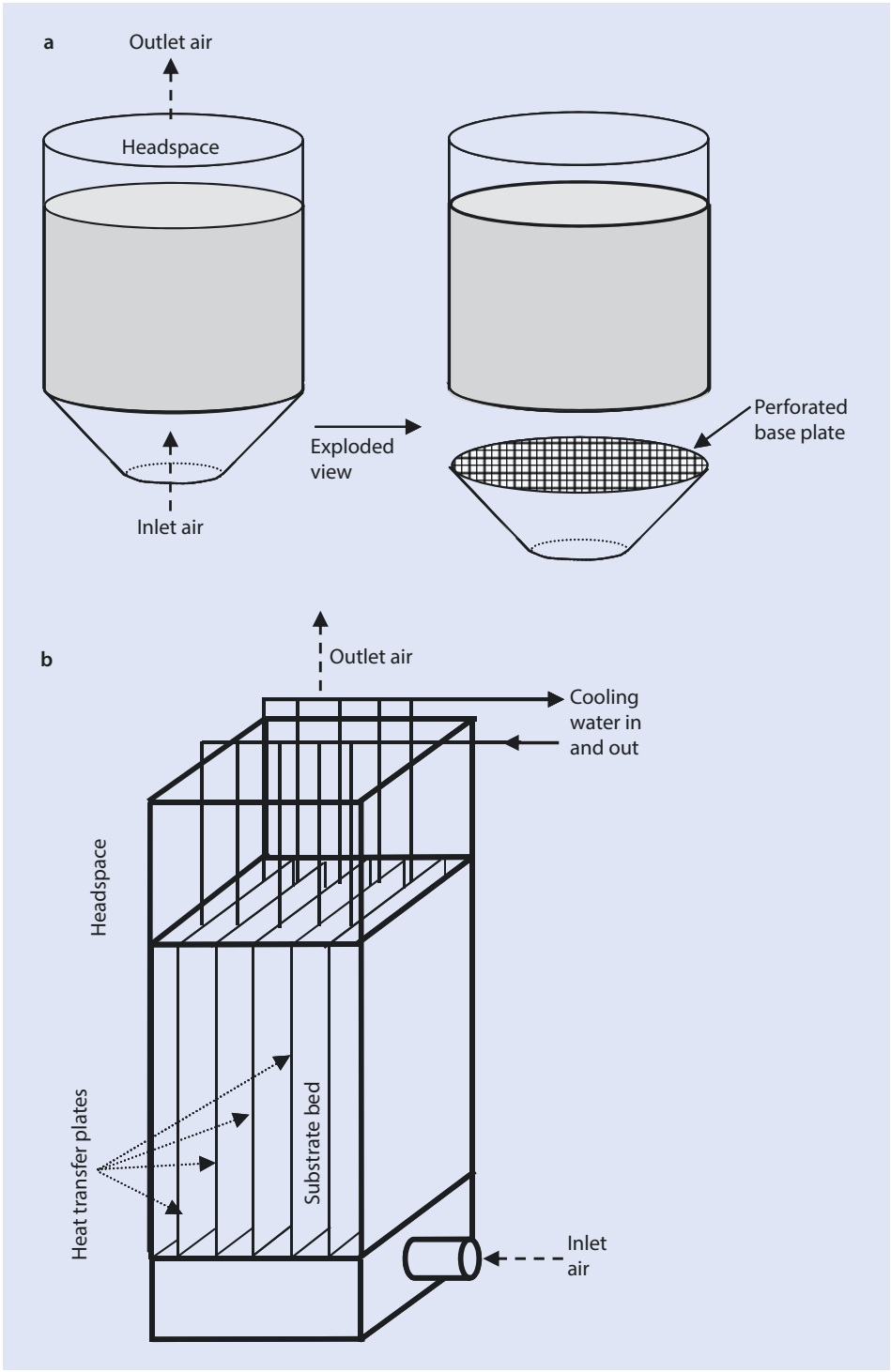


Fig. 4.5 Basic features of packed-bed bioreactors. **a** A traditional packed-bed bioreactor. **b** A Zymotis-type packed-bed bioreactor. (Adapted from Mitchell et al. [6] with kind permission from Springer Science and Business Media)

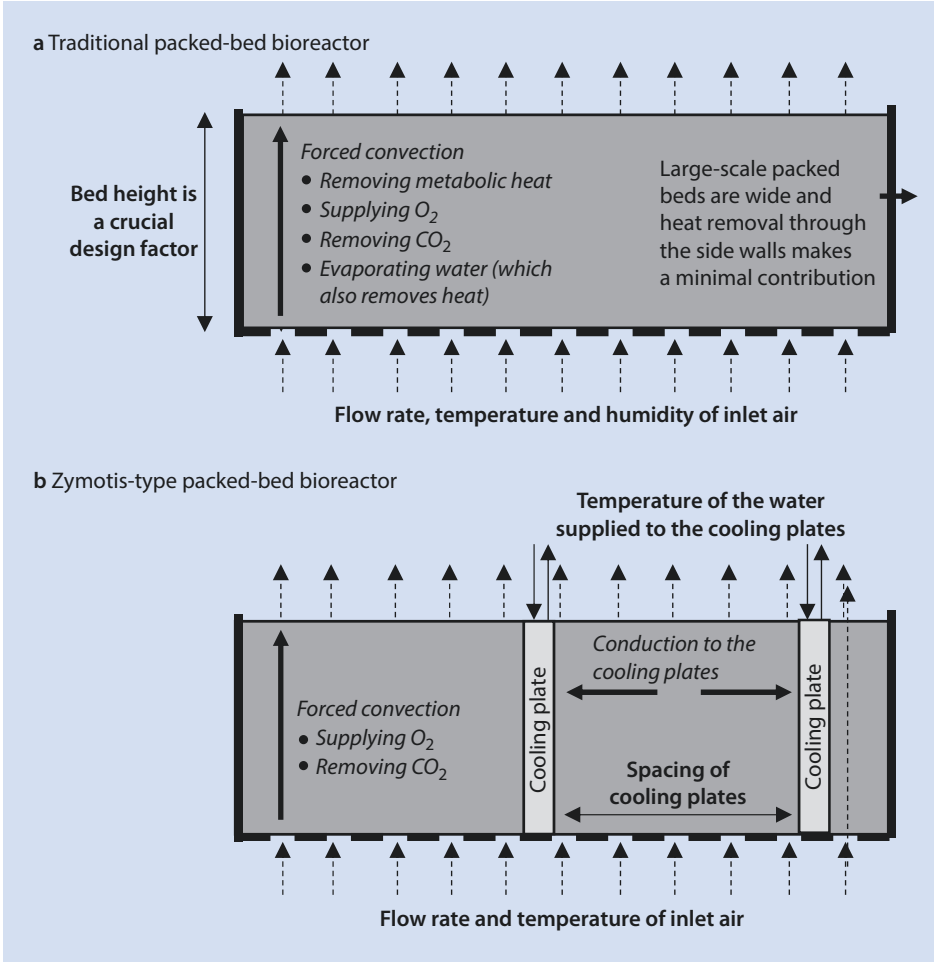


Fig. 4.6 Key design and operating variables and heat and mass transfer processes in packed-bed bioreactors. **a** Traditional packed-bed bioreactors. **b** Zymotis-type packed-bed bioreactors. **Bold text** indicates design and operating variables; *Italic text* indicates heat and mass transfer mechanisms. Dashed arrows represent air flow; solid **thick** arrows represent key heat and mass transfer mechanisms

rate (which depends on the peak growth rate of the microorganism), bed height, superficial air velocity, and the maximum temperature reached in the bed. This interplay is summarized in a simple manner by what has been called the modified Damköhler number (Da_M):

$$Da_M = \frac{0.25\rho_s(1-\varepsilon)Y_Q\mu_{opt}X_{max}}{\rho_a(C_{pa}+f\lambda)V_Z(T_{out}-T_{in})/H}$$

where

- ρ_a is the density of the air (kg m^{-3});
- ρ_s is the density of the solid substrate particles (kg-initial-wet-substrate m^{-3});
- λ is the enthalpy of evaporation of water (J kg^{-1});
- Y_Q is the yield of metabolic heat (J kg-dry-biomass^{-1});

- C_{Pa} is the heat capacity of dry air (J kg-dry-air^{-1} °C^{-1});
- μ_{opt} is the value of the specific growth rate constant in the logistic equation for optimal growth conditions (h^{-1});
- X_{max} is the maximum biomass content (kg-dry-biomass kg-initial-wet-substrate^{-1});
- f is the slope of a linear approximation to the humidity curve as a function of temperature;
- ε is the porosity of the bed (m^3-air m^{-3});
- H is the height of the substrate bed (m);
- T_{in} is the temperature of the air at the inlet (°C), with the bottom of the bed being maintained at this temperature;
- T_{out} is the temperature of the air at the air outlet (°C), which is equal to the temperature at the very top of the bed;
- V_Z is the apparent superficial velocity of the air (m s^{-1}), given by the volumetric flow rate of the air (m^3 s^{-1}) divided by the overall cross-sectional area of the bioreactor (m^2).

The numerator of the above equation is an estimate of the maximum rate of metabolic heat production (in J h^{-1} m^{-3}). In this case, it is assumed that metabolic heat production is directly growth associated (i.e., there is no maintenance component) and growth follows logistic kinetics. However, the numerator could be any estimate of the maximum heat production rate. For example, it could be an estimate based on the maximum O_2 uptake rate.

The denominator is an estimate of the removal of heat from the bed. It assumes that there is no heat transfer through the side walls of the bed. In other words, all the heat removed from the bed is removed through convective and evaporative cooling caused by the air passing through the bed. For simplicity of calculation, it is assumed that the air is saturated (effectively, $f\lambda$ is an apparent heat capacity of the air due to water evaporating to maintain the air saturated).

This equation can be used to guide the selection of an appropriate bed height. To do this, Da_M is set as equal to 1 (i.e., the rate of heat removal is equal to the rate of metabolic heat production) and the expression is rearranged to give

$$H = \frac{\rho_a \left(C_{pa} + f\lambda\right) V_Z \left(T_{out} - T_{in}\right)}{0.25\rho_s \left(1-\varepsilon\right) Y_Q \mu_{opt} X_{max}}$$

It is first necessary to decide what is an acceptable maximum temperature difference over the bed (i.e., what range of temperatures from T_{in} to T_{out} will give acceptably good growth of the microorganism). One can then see that the bed height that can be used is directly proportional to the superficial velocity of the air, but inversely proportional to the maximum rate of growth of the microorganism. It should be noted that the superficial velocity cannot be increased indefinitely. At very high superficial velocities, the air may fluidize the particles in the bed (fluidized beds are considered in the section that deals with forcefully aerated agitated beds).

Example 4.1

How do you calculate the maximum bed height that you can use in a packed-bed bioreactor while ensuring that the temperature at the top of the bed (i.e., at the air outlet) does not exceed a given value?

Let us assume that (i) air will be supplied at the optimum temperature for growth and that the temperature at the top of the bed must not be more than 5 °C higher than this optimum temperature (i.e., $T_{out} - T_{in} = 5$ °C); (ii) the air system can supply air at an apparent

superficial velocity (volumetric flow rate divided by the total cross-sectional area of the bed) of 0.1 m s^{-1} (i.e., 10 cm s^{-1}, which is equivalent to 360 m h^{-1}); and (iii) the organism follows logistic growth kinetics.

We will use the values below:

- Density of the air: $\rho_a = 1.14$ kg m^{-3}
- Density of the solid substrate particles: $\rho_s = 700$ kg-initial-wet-substrate m^{-3}
- Enthalpy of evaporation of water: $\lambda = 2{,}414{,}300$ J kg^{-1}
- f (for the range of 35–52 °C): 0.00246 kg-water-vapor kg-dry-air^{-1} $°C^{-1}$
- Metabolic heat yield: $Y_Q = 8.4 \times 10^6$ J kg-dry-$biomass^{-1}$
- Heat capacity of dry air: $C_{Pa} = 1180$ J kg-dry-air^{-1} $°C^{-1}$
- Specific growth rate constant in the logistic equation: $\mu_{opt} = 0.1$ h^{-1}
- Maximum biomass content: $X_{max} = 0.3$ kg-dry-biomass kg-initial-wet-$substrate^{-1}$
- Bed porosity: $\varepsilon = 0.35$ m^3-air m^{-3}-total-bed-volume

What is the maximum rate of metabolic heat production (Q_{met})? It is estimated by the denominator of the equation for H above:

$$Q_{met} = 0.25\rho_s(1-\varepsilon)Y_Q\mu_{opt}X_{max}$$

Substituting the given values in this equation gives

$$Q_{met} = 0.25 \times 700\frac{kg}{m^3} \times (1-0.35)\frac{m^3}{m^3} \times 8.4 \times 10^6 \frac{J}{kg} \times 0.1\,h^{-1} \times 0.3\frac{kg}{kg}$$

This gives a value of 28.7 MJ per h per m^3 of total bed volume.

What is the heat removal capacity of this bioreactor (Q_{rem})? It is estimated by the numerator of the equation for H above:

$$Q_{rem} = \rho_a\left(C_{pa} + f\lambda\right)V_Z\left(T_{out} - T_{in}\right)$$

$$Q_{rem} = 1.14\frac{kg}{m^3}\left(1180\frac{J}{kg\,^\circ C} + 0.00246\frac{kg}{kg\,^\circ C} \times 2\,414\,300\frac{J}{kg}\right) \times 360\frac{m}{h} \times 5^\circ C$$

This gives a value of 14.6 MJ per h per m^2 of cross-sectional area of the bed. It must be remembered that this calculation assumes that the air remains saturated with water vapor as it heats up while passing through the bed. If it does not, then heat removal will be less effective.

Dividing Q_{rem} by Q_{rem} gives a value of 1.96 m. In other words, for the given conditions, a bed of about 2 m high will give a 5 °C temperature rise between the air inlet and the air outlet at the peak of metabolic heat generation if an apparent superficial velocity of 0.1 m s^{-1} is used. Note that this calculated value is directly proportional to the apparent superficial velocity: if the apparent superficial velocity is reduced to 0.01 m s^{-1} (i.e., 1 cm s^{-1}), then the bed height that is calculated will be only 20 cm.

As pointed out above, even if saturated air is supplied to a traditional packed-bed bioreactor in an effort to minimize drying, evaporation will still occur. Evaporation can dry the bed to water activities that are low enough to slow growth considerably. It is not possible to add water uniformly to a static bed, meaning that if water is to be added during the process to bring the water activity back up to values that are favorable for growth, then it is necessary to mix the bed (with a fine mist of water being sprayed on the bed surface during this mixing). If the microorganism can tolerate being agitated several times per day, then this strategy can be used to maintain the water activity within an interval that allows reasonably good growth.

If the microorganism cannot tolerate mixing, then efforts must be made to avoid the need to add water. One possibility is to use a substrate with a very high water-carrying capacity, such that the water activity falls only slowly over a very wide range of water contents. Another possibility is to use a Zymotis-type bioreactor, which is discussed in the next subsection.

4.5.3 Design and Operation of Zymotis-Type Packed-Bed-Type Bioreactors

The Zymotis-type packed-bed bioreactor has internal heat transfer plates (Fig. 4.6b). Most of the metabolic heat is removed by these plates, rather than by the process air. As a result, the air increases in temperature only very slightly as it passes through the substrate bed and therefore its water-carrying capacity does not increase significantly. In this case, if near-saturated air is used at the air inlet, then the rate of evaporation in the bed can be maintained at a very low value.

For the removal of heat by conduction through the bed to the heat transfer plates to be efficient, the plates must be close together. Depending on the growth rate of the microorganism, the plates may need to be spaced only 5–10 cm apart. As mentioned above, these closely spaced heat transfer plates make it impractical to agitate the bed. It would therefore be problematic if the bed were to shrink and pull away from the walls, leading to "channeling," since it would not be a simple matter to agitate the bed in order to reseat it (see the next subsection). When using the Zymotis design, it is therefore essential to use a substrate that maintains its structure during the process.

4.5.4 Porosity, Pressure Drop, Bed Shrinkage, and Channeling in Packed Beds

In packed-bed bioreactors, one of the key properties of the bed is its porosity, which is defined as the volume of void spaces (air spaces between the particles) divided by the overall volume occupied by the bed. The porosity of the bed affects the pressure drop across the bed. The pressure drop across the bed, in turn, affects the aeration system, since the pressure at the air inlet must be maintained at a value greater than the sum of the pressure at the outlet and the pressure drop through the bed.

The porosity at the start of the process will depend on the size and shape of the substrate particles and on how the packing of the bed is done (for example, whether the bed is compressed or not during packing). The bed porosity can change significantly during the process: the degradation of the substrate particle by the microorganism will tend to increase porosity, while, in processes involving filamentous fungi, the filling up of the interparticle spaces by aerial hyphae will tend to decrease the porosity. During the process, it may be necessary to agitate the bed in order to disrupt these aerial hyphae, with the intention of preventing the pressure drop from becoming too high. The first agitation must be done before the fungus has bound the particles together tightly, otherwise agglomerates of particles might be able to resist breakage during agitation of the bed.

In some situations, agitation is not an option. This is the case when the process organism does not tolerate agitation at all and also with the Zymotis bioreactor, in which agitation is simply problematic. In these cases, it may be appropriate to incorporate a "bed porosity modifier," which is an inert material that tends to pack loosely. For example,

sugarcane bagasse can be used for this purpose with microorganisms that are not capable of degrading lignocellulosic substrates. When added in sufficient amounts (contributing from 10% to 50% of the weight of the bed), it prevents filamentous fungi from filling up the pores and binding the substrate into a tight mass.

Considerations of pressure drop have implications for the geometry of the bed: The bed should have straight sides and a uniform height (see Fig. 4.6). If the bed does not have a uniform height, then the air will tend to flow through the shallower regions of the bed (where the resistance to flow is lower). The regions where the bed is deeper will then not receive adequate aeration.

The bed held in a packed-bed bioreactor can shrink if it dries out, if the particle size decreases during growth and if the particles are bound together by fungal mycelium. This can lead to the phenomenon of channeling, with cracks appearing in the bed or the bed pulling away from the walls. The air will flow preferentially through these cracks and the bed itself will not be aerated. Therefore, if cracks do appear, it will be necessary to mix the bed in order to "reseat it." It should be noted that a bed porosity modifier, in addition to minimizing the increase in pressure drop, can help the bed to maintain its structure, minimizing shrinkage. In fact, if it is essential to avoid shrinkage, it is possible to use a substrate that is entirely composed of such an inert material, impregnated with an appropriate nutrient solution.

4.6 Design and Operation of Rotating-Drum and Stirred-Drum Bioreactors

4.6.1 Basic Features of Rotating-Drum and Stirred-Drum Bioreactors

The defining feature of rotating-drum and stirred-drum bioreactors is that the air is blown through the headspace as the bed is mixed either continuously or frequently. In the rotating drum, the mixing is attained by rotation of the whole bioreactor body; in the stirred drum, the bioreactor body remains static and the bed is mixed by paddles that rotate around a central axis (Fig. 4.7). For both types of designs, the drum should not be too full. If it is too full, it will be difficult to mix efficiently. In other applications of rotating drums, considerations about mixing lead to percentage fillings of around 25%.

4.6.2 Design and Operation of Rotating-Drum Bioreactors

A rotating-drum bioreactor will be designed as a horizontal or inclined cylinder with lifters (Fig. 4.7). The lifters ensure that the bed is mixed as the drum rotates. In the absence of lifters, the bed will tend to "slump" at the slow speeds of only a few revolutions per minute that are usually used with rotating drums. When slumping occurs, the whole substrate bed moves as a single mass, rising with the rotating wall, but then sliding back down.

The drum axis may be horizontal and the lifters flat, such that portions of the substrate are lifted out of the bed; the substrate particles then fall back down like a curtain and tumble down the surface of the bed (Fig. 4.7). Alternatively, the drum can be designed to promote end-to-end mixing of the bed. In this case, the drum axis is inclined and curved lifters are designed to push the substrate particles up the slope, from where they tumble back down (Fig. 4.7b).

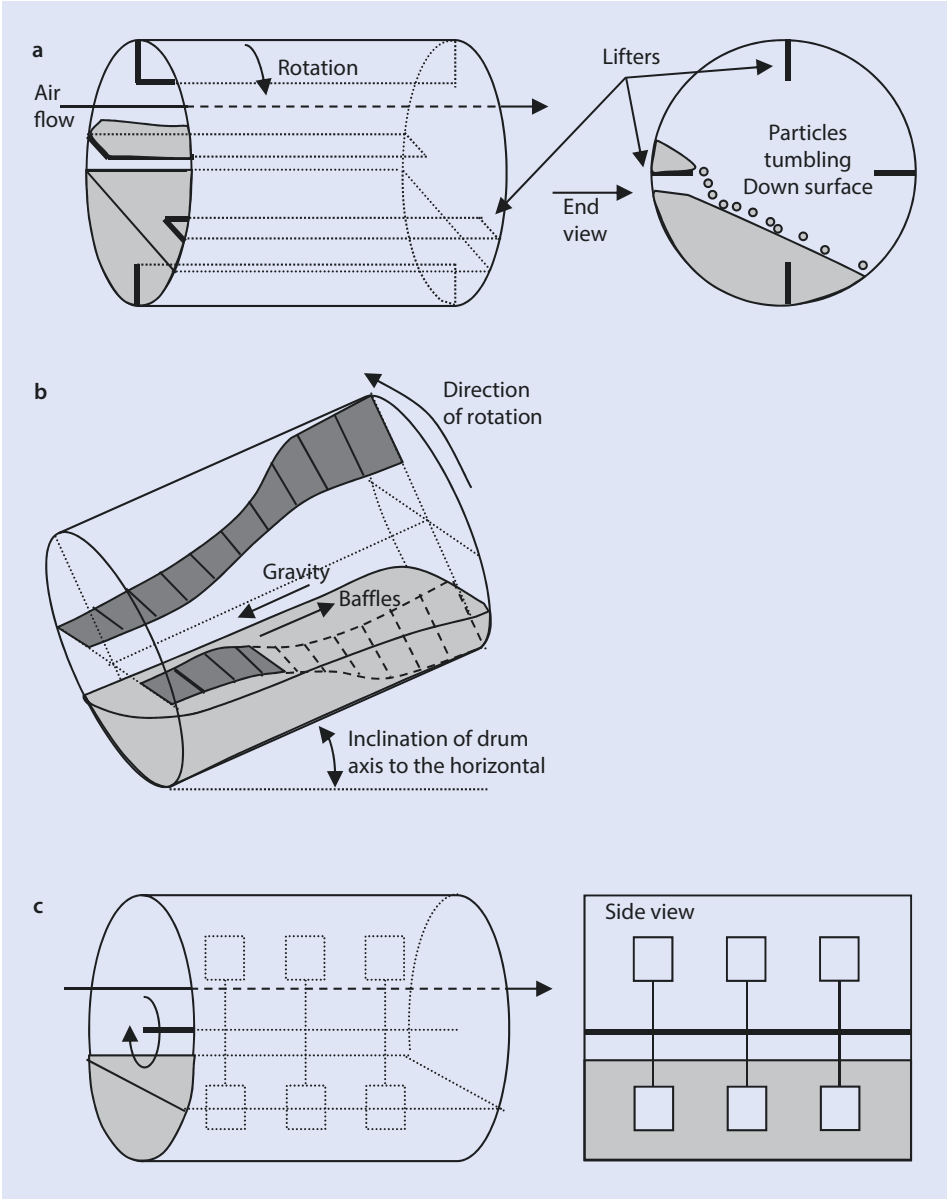


Fig. 4.7 Basic design features of rotating-drum bioreactors and stirred-drum bioreactors. **a** A traditional horizontal rotating-drum bioreactor. **b** A rotating-drum bioreactor with an inclined axis and curved lifters, designed to promote axial mixing. **c** A horizontal stirred-drum bioreactor. (Adapted from Mitchell et al. [6] with kind permission from Springer Science and Business Media)

4.6.3 Design and Operation of Stirred-Drum Bioreactors

The key issue for stirred drums is the design of the agitator, which must ensure good radial mixing and can also be designed to aid in end-to-end mixing. V-shaped paddles are common. Stirrer speeds do not need to be high; they are typically of the order of a few revolutions per

minute. In some cases, air is introduced into the bed through the ends of the paddles themselves or through a perforated tube within the bed, but this does not give uniform aeration of the bed (such that the bioreactor does not fit fully into the classification of a forcefully aerated bed).

4.6.4 Key Heat and Mass Transfer Processes in Rotating- and Stirred-Drum Bioreactors and How Design and Operation Can Maximize Performance

Since the air is blown through the headspace and not forcefully through the bed itself, the heat transfer across the bed-headspace interface is of critical importance in rotating and stirred drums (Fig. 4.8). This transfer is not as effective as the heat transfer in forcefully aerated bioreactors (such as in packed-bed bioreactors and forcefully aerated agitated bioreactors): in those bioreactors, the individual substrate particles are in contact with the free-flowing air passing through the void spaces of the bed; in the case of rotating and stirred drums, only the particles at the surface of the substrate bed are in contact with free-flowing air.

Strategies for promoting bed-to-headspace transfer include the following:

- Ensuring good mixing within the bed, such that substrate particles are constantly being circulated past the bed surface.
- In rotating drums, using flat lifters that lift a portion of substrate that then falls like a curtain, through flowing headspace gases.
- Increasing the air flow rate through the headspace, while ensuring that the air in the headspace is not stagnant near the bed surface. Flow patterns in the headspace can be complex; if care is not taken with the design of the air inlet and outlet, there can be plug flow of air along the central axis of the bioreactor, but relatively stagnant regions near the bed surface.
- Supplying unsaturated air to the bioreactor to promote evaporation; since the substrate bed is agitated, a mist of water can be sprayed onto the surface of the bed, when necessary, to prevent it from drying out.

Agitation also means that the substrate is circulated past the drum wall, so heat removal through the wall can be promoted to aid in cooling of the bed. However, rotating drums typically do not have water jackets, for two reasons. First, it is more complicated to supply water to a rotating jacket. Secondly, extra energy is required to rotate the mass of water in the jacket. On the other hand, it is feasible to use a water jacket with a stirred drum, the body of which remains stationary.

A "dimensionless design factor" (DDF), based on a simplifying assumption that the bed and headspace are in thermal and moisture equilibrium, has been proposed as a guide to obtaining adequate heat removal for this type of bioreactor:

$$\mathrm{DDF} = \frac{R_Q}{F_a C_{pa}\left(T_{OUT} - T_{IN}\right) + F_a\left(H_{OUT} - H_{IN}\right)\lambda + hA\left(T_B - T_{SURR}\right)}$$

where:

- R_Q is the maximum rate of production of metabolic heat in the bed (J h^{-1}) (it could be calculated in a manner similar to that of the numerator of the modified Damköhler number presented above for packed beds, where that numerator would be multiplied by the volume occupied by the bed).

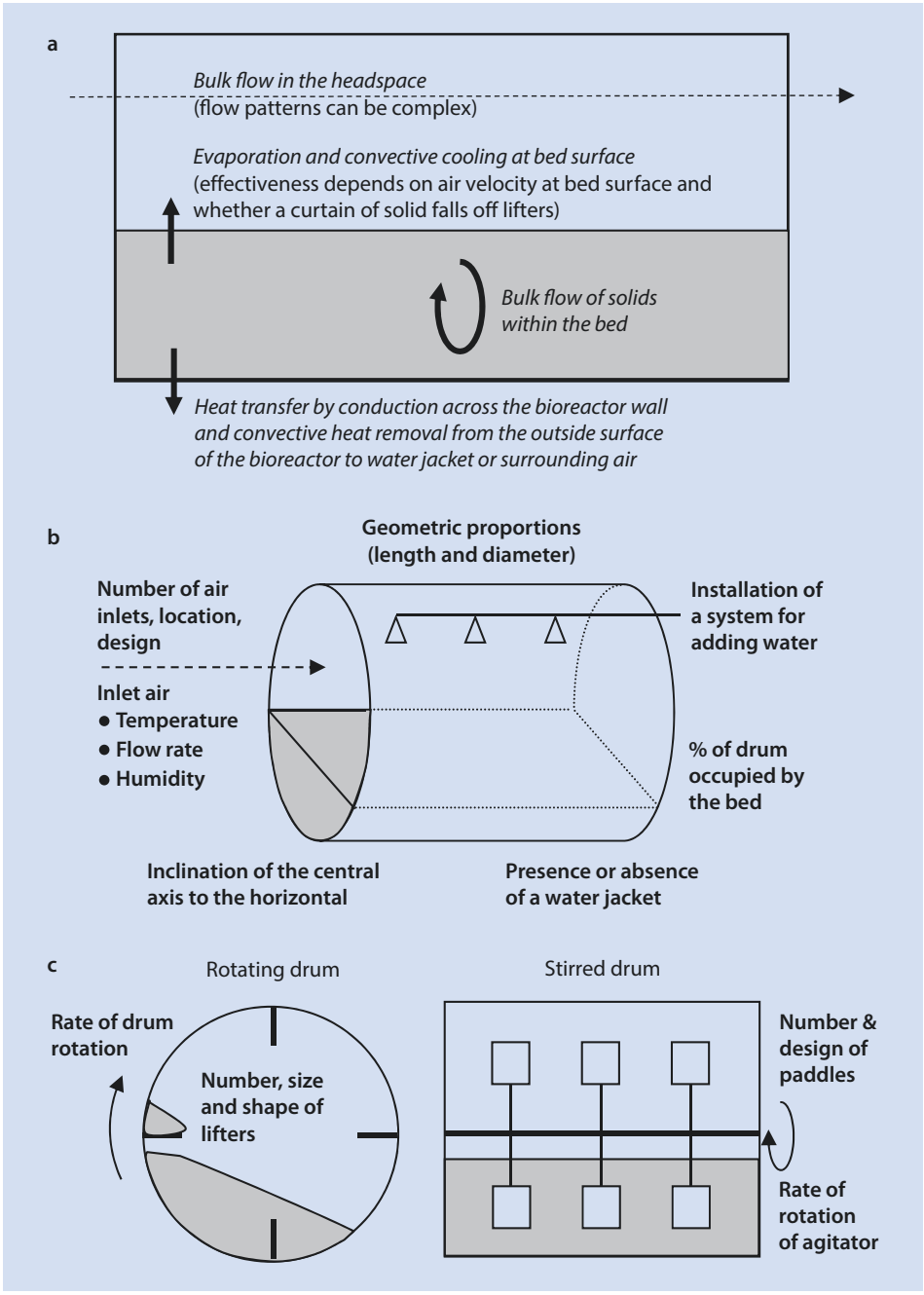


Fig. 4.8 Key heat and mass transfer processes and design and operating variables for rotating-drum and stirred-drum bioreactors. **a** Key heat and mass transfer processes in both types. **b** Key design and operating variables that are the same for both types. **c** Design and operating variables that are specific for each bioreactor type. **Bold text** indicates design and operating variables; *Italic text* indicates heat and mass transfer mechanisms. Dashed arrows represent air flow; solid **thick** arrows represent key heat and mass transfer mechanisms. (Adapted from Mitchell et al. [6] with kind permission from Springer Science and Business Media)

- The first term in the denominator is an estimate of energy removed by convection to the process air, with T_{IN} and T_{OUT} being the air temperatures at the air inlet and the air outlet, respectively.
- The second term in the denominator is an estimate of energy removal by evaporation, where F_a is the flow rate of air (kg-dry-air h^{-1}), λ is the enthalpy of vaporization of water (J kg-vapor^{-1}), and H_{OUT} and H_{IN} are the humidities of the air (kg-vapor kg-dry-air^{-1}) at the air outlet and the air inlet, respectively.
- The third term in the denominator is an estimate of energy removal by heat exchange across the bioreactor wall to the surroundings (which could be to water in a water jacket), where h is the overall heat transfer coefficient across the bioreactor wall (J h^{-1} m^{-2} °C^{-1}), A is the surface area of the bioreactor (m^2), T_B is the temperature of the bed, and T_{SURR} is the temperature of the surroundings (which might either be air or water in a water jacket).

If the air at the outlet is assumed to be saturated at T_B, and a linear approximation is made for the dependence of saturation humidity on temperature (as was done for the modified Damköhler number above), then all terms in the denominator can be expressed as functions of T_B. The DDF can be set to 1, meaning that the rate of heat removal is equal to the rate of metabolic heat generation. The equation can then be reorganized to give

$$T_B = \frac{R_Q + \left(F_a C_{pa} T_{IN} + F_a f T_{IN} \lambda + hAT_{SURR}\right)}{\left(F_a C_{pa} + F_a f \lambda + hA\right)}$$

This rearranged version of the equation can be used to explore the effect of operating variables on the temperature of the bed in the bioreactor. These operating variables include the flow rate of air (F_a) and the heat transfer coefficient across the bioreactor wall (h), which can be affected by promoting forced convection (to air in the surroundings or water in a water jacket).

4.7 Design and Operation of Forcefully Aerated Agitated Bioreactors

4.7.1 Basic Features of Forcefully Aerated Agitated Bioreactors

There are many different designs of forcefully aerated agitated bioreactors.

In the classical design, the substrate bed sits on a perforated plate through which air is blown, but the mixing can be done in several different ways. First, a "stationary" solid mixer (which rotates but does not travel) that mixes the whole of the bed can be used in a "stationary" (non-traveling) bed (Fig. 4.9a). Second, the bed can remain stationary, with the mixer traveling from one end of the bed to the other, back and forth (Fig. 4.9b). Third, the mixer can remain stationary (either fixed, or rotating), while the bed is moved (usually rotated) past it (Fig. 4.9c). In the second and third cases, even if the mixer is operating continuously, the mixing in any particular location within the bed is intermittent, but each region of the substrate will be mixed at least once every hour or so. The fluidized-bed bioreactor is a specialized type of forcefully aerated agitated bioreactor (Fig. 4.9d). In this case, the air passes through the bed at a velocity that is sufficiently high to fluidize the particles. In other words, the particles become buoyant; the bed expands and the particles circulate freely around the bed. In this type of bioreactor, the major challenge is to maintain the

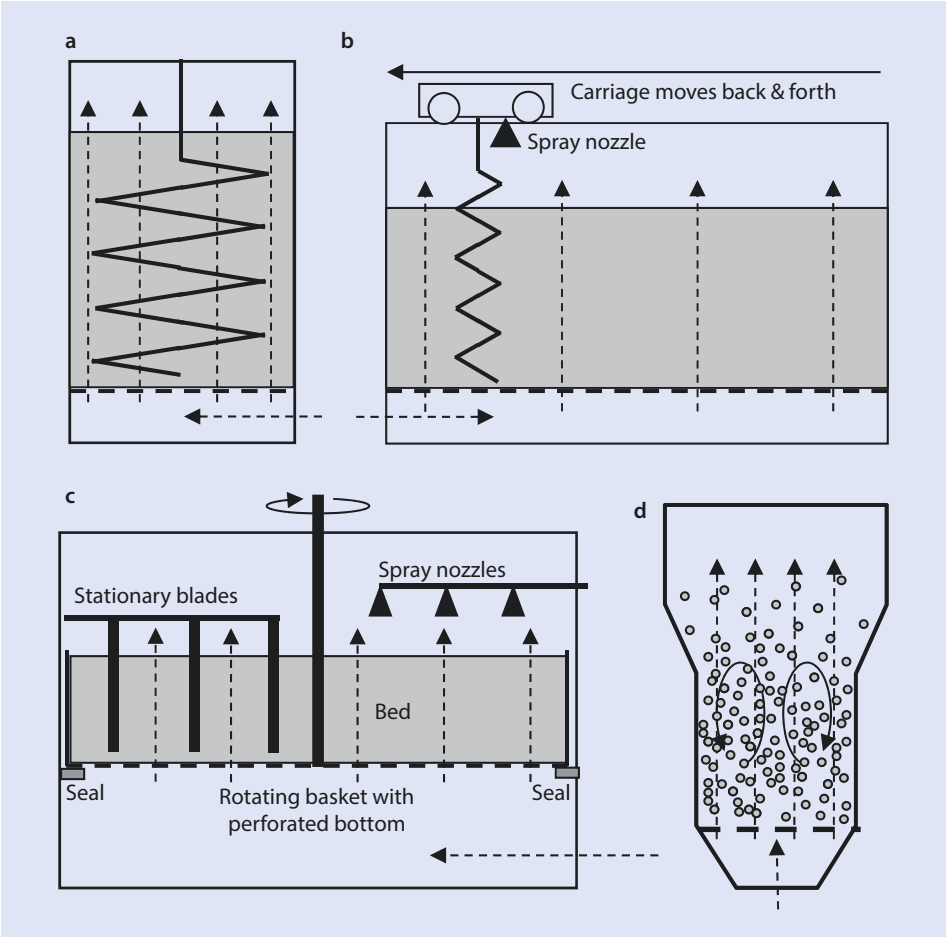


Fig. 4.9 Different ways in which agitation can be provided in forcefully aerated agitated bioreactors. **a** A stationary solid mixer (which rotates but does not travel) in a stationary bed. **b** A stationary bed with a mixer that travels back and forth from one end of the bed to the other. **c** A rotating bed with stationary mixing blades. **d** A fluidized bed, in which the agitation is provided by the air flow itself. (Adapted from Mitchell et al. [6] with kind permission from Springer Science and Business Media)

particles fluidized, since their size, shape, and density can change quite considerably during the fermentation, affecting their fluidization properties. Also, if the particles are sticky, they tend to agglomerate, and it may be necessary to have a "particle breaker" in the bed.

4.7.2 Key Heat and Mass Transfer Processes in Forcefully Aerated Agitated Bioreactors and How Design and Operation Can Maximize Performance

Forcefully aerated agitated bioreactors have the best heat and mass transfer. The forced aeration assures intimate contact of the particles with a flowing gas phase. Mixing prevents fungal mycelia from knitting the substrate particles into compact agglomerates and also ensures that the axial temperature gradients that occur in packed beds do not occur,

so convective cooling is reasonably efficient. Also, since the bed is continuously or frequently mixed, it is easy to add water if necessary, in the form of a fine mist sprayed onto the surface of the bed during agitation. It is therefore possible to promote evaporative heat transfer by supplying unsaturated air to the bed. In fact, in fluidized-bed bioreactors, the heat transfer is so efficient that heat removal is not an issue in the design of this type of bioreactor.

4.8 Monitoring and Sampling of Solid-State Cultivation Bioreactors

SSC systems present particular challenges with online monitoring and sampling that are not faced in SLC.

There are relatively few possibilities to monitor the process online. Typically, it is only practical to measure the temperature in the bed with thermocouples (or thermistors) and the temperatures, gas concentrations (O_2 and CO_2) and relative humidities in the gas phase at the bioreactor inlet and outlet. This is different from SLC, where probes are available for measuring the pH, dissolved gas concentrations, and even the concentrations of various ions. Also, in SLC, automatic sampling systems can be used to remove samples of the culture broth and send them to automated analysis (e.g., by gas chromatography or high-performance liquid chromatography, coupled with mass spectrometers, if appropriate). In SSC, online monitoring by gas chromatography is only practical for headspace gases (e.g., to detect the concentrations of volatile products in the gas phase).

In SSC bioreactors in which the bed remains static (i.e., tray bioreactors and packed-bed bioreactors), the removal of samples for off-line analysis is problematic, for two reasons. First, since the conditions in the bed are not uniform, the biomass, nutrient, and product contents will vary across the bed. It is therefore necessary to "map" this non-uniformity by removing samples from different parts of the bed. Second, in packed-bed bioreactors, the removal of samples from the interior of a static bed can disrupt the bed, creating preferential flow paths (requiring mixing to "reseat the bed," but this is not possible if the bed must remain static). The removal of samples from the top of the bed will not cause these problems, but these samples are not representative of the whole bed.

In any case, the information that is obtained by monitoring off-gases, or removing samples from the bed and analyzing them, is limited. The off-gases reflect the overall performance of the bed, but do not give information as to the performance of different regions of the bed. Likewise, when a sample containing many particles is removed from the bed and homogenized before assaying for a particular compound, the value obtained represents the average content in the sample; no information is obtained about the distribution of that compound within the particles (e.g., whether the compound is near the surface or limited to the interior of the particles).

4.9 Modeling, Design Rules, and Scale-Up of SSC Bioreactors

Although SSC has been practiced for many centuries in the production of fermented food and although large-scale SSC bioreactors have been built and even commercialized (especially for the production of soy sauce *koji*), the design and operation of SSC

bioreactors have received much less attention than have the design and operation of SLC bioreactors. The scale-up of bioreactors for SLC is covered in ▶ Chap. 7 of this book. This section addresses the situation for SSC bioreactors briefly.

The dimensionless numbers mentioned above in relation to packed-bed and drum bioreactors are only rules of thumb. Mathematical models based on mass and energy balances are potentially better tools for guiding the scale-up of SSC bioreactors. Such models have been proposed for all four bioreactor types (trays, packed beds, rotating drums, and forcefully aerated agitated bioreactors). These mathematical models contain differential equations that describe metabolic heat generation and heat removal by convection, conduction, and evaporation. A differential equation is also typically included to describe the water balance. As a result, the models are able to describe the formation of temperature and moisture gradients within the substrate bed, for those bioreactors in which such gradients occur. Kinetic equations are included that describe the consumption of residual substrate and the production of microbial biomass. However, these kinetic equations are usually highly simplified.

Simple kinetic equations are used because the growth of the microorganism in SSC is quite complex, especially when filamentous fungi are used (◘ Fig. 4.10). First, transport within the substrate particle is limited to diffusion. This leads directly to the establishment of concentration gradients of soluble nutrients and O_2 within the particle. Additionally, many substrates contain polymeric carbon sources, such as polysaccharides, and polymeric nitrogen sources, such as proteins. Utilization of these polymers involves the secretion of hydrolytic enzymes and their diffusion through the particle, with the soluble hydrolysis products diffusing back to the microorganism. These phenomena lead to a non-uniform distribution of biomass in space, with the biomass at different locations in the particle experiencing quite different local conditions and therefore having different physiology and different growth rates. Second, many substrates contain complex mixtures of nutrients, such that there are complex patterns of induction and repression of genes, including genes coding for extracellular enzymes. Third, in SSC processes involving filamentous fungi, not all the biomass contributes actively to growth. Rather, growth is limited to the regions near the tips of the hyphae, while hyphae in older regions of the mycelium may have a different physiology (◘ Fig. 4.10). Differentiation may also occur, for example, with the production of reproductive hyphae, such as conidiophores. Additionally, if the bed is agitated during the process, hyphae will be crushed onto the surface of the substrate particle and may also suffer physical damage.

When the aim is to simulate bioreactor performance, it is not practical to propose a model for growth kinetics that takes all these phenomena into account, even though such kinetic models have been developed for research purposes. A mechanistic description of growth kinetics within a bioreactor model would not only require high computational power (due to the need to describe heterogeneity both across the bed and within individual particles) but would also require a substantial amount of experimental work for determination of the model parameters. For example, since the biomass does not experience a single substrate (or O_2) concentration, it is not convenient to use the Monod equation to express the growth rate as a function of substrate (or O_2) concentration. This is different from the situation in SLC, where it is common for the specific growth rate to be expressed as a function of the concentrations of a limiting substrate and of O_2.

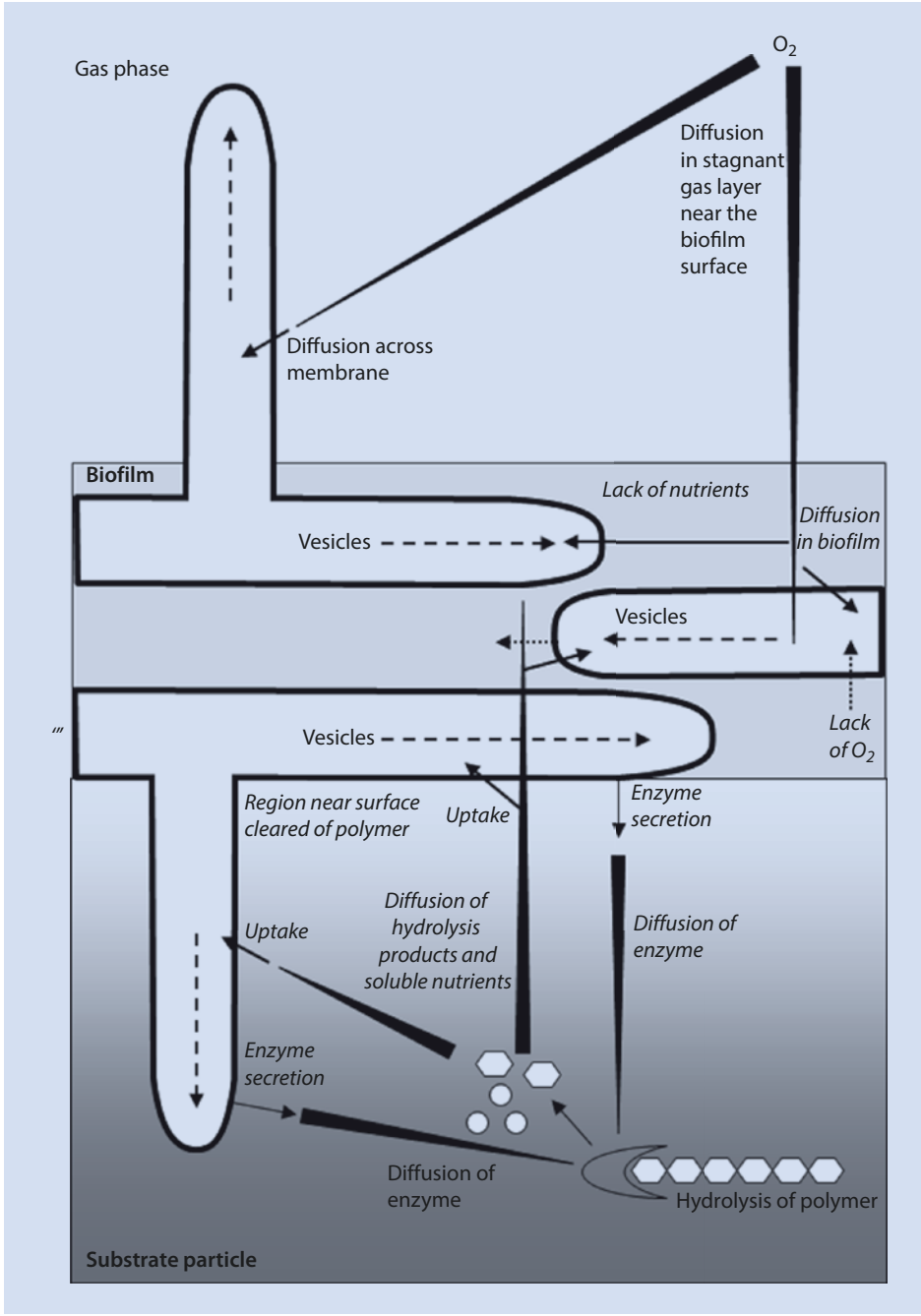


Fig. 4.10 Complexity of microbial growth in SSC, illustrated for the case of a filamentous fungus growing at the surface of a substrate particle. "Long triangles" represent diffusion down concentration gradients. "Dotted arrows" represent flow of water. "Dashed arrows" represent transport of vesicles toward the tips of hyphae. (Adapted from Sugai-Guérios et al. [12] with kind permission from Springer Science and Business Media)

In SSC, the usual strategy is to use a simple empirical equation, such as the logistic equation:

$$\frac{dX}{dt} = \mu_{max} X \left(1 - \frac{X}{X_{max}}\right)$$

where X is a biomass concentration (it could have units of g dry biomass per g initial dry solids), t is time (h), μ_{max} is the maximum specific growth rate constant, and X_{max} (with the same units as X) is the maximum biomass concentration. Any other appropriate empirical equation can be used.

When the logistic equation is used, the parameters μ_{max} and X_{max} can be expressed as functions of the water activity and the temperature of the solid substrate, which are two parameters that affect growth and which can be influenced by how the bioreactor is operated. However, even so, it is a challenge to obtain accurate equations. Due to the difficulties in temperature control within bioreactors, the microorganism will typically experience varying temperatures during the cultivation, with at least some of the biomass being exposed to temperatures significantly above the optimum temperature for growth for some time. The kinetics of growth in this situation is quite complex: the growth rate at any particular time is affected not only by the temperature at that particular time but also by the temperatures experienced by the microorganism in its recent past. It is quite difficult to characterize this phenomenon quantitatively; current models express the growth rate at any particular instant as depending only on the temperature and water activity at that instant.

The significant simplifications that are made in describing the growth kinetics mean that the mathematical models of bioreactors cannot be expected to be highly accurate. However, they are still useful tools. For example, they can be used during scale-up to determine the required capacity of auxiliary equipment, such as air blowers and coolers for water jackets. More details on how these mathematical models can be used to guide the scale-up of bioreactors are given in Mitchell et al. [6].

Learning Questions

These questions are based on the content of the chapter, but, in answering them, you might find it useful to use internet resources to explore the issues.

1. What are the main differences between solid-state cultivation (SSC) systems and submerged liquid cultivation (SLC) systems?
2. SSC has advantages over SLC, such as the possibility of using agro-industrial residues and the production of products in a more concentrated form. So why are most microbial products produced by SLC? As part of your answer, you should address the main difficulties associated with SSC processes.
3. What are the main differences between SSC processes involving bacteria and SSC processes involving filamentous fungi?
4. Continuous stirred-tank bioreactors are used in various SLC processes. Why is this mode of continuous operation not appropriate for SSC processes?
5. In the case of SSC, how would a continuous plug-flow bioreactor work? Explain why this type of continuous operation is feasible in SSC.
6. There are several different types of bioreactors that can be used in batch SSC processes. What are these different bioreactor types and which criteria must be considered in the selection of an appropriate bioreactor for a particular SSC process?

7. What are the main limitations of tray bioreactors? What are the considerations involved in scaling up this type of bioreactor?
8. The properties of the substrate bed have a significant influence on SSC processes. What are the key bed properties that need to be considered? Why are these properties important, in other words, how do they influence the process?
9. What online measurements can be made in SSC bioreactors? What are the main challenges in monitoring SSC processes online? Why is online monitoring more difficult in SSC than in SLC?
10. It is usually necessary to remove samples in order to monitor the performance of SSC processes. What are the key issues to be considered in the development of a sampling protocol?
11. What variables need to be considered in the scale-up of SSC bioreactors? Is the answer different for different types of SSC bioreactors? What tools are helpful in guiding scale-up?
12. Suppose that you work in a biotechnology company that wants to establish a SSC facility. Your boss asks you to suggest a SSC process that would be technically feasible and economically viable. So, what is your suggestion? To answer this question, you should address the following points, taking time to explore various SSC processes on the internet:
    (a) What product would you suggest? Why?
    (b) What solid substrate will be used? Why did you suggest this substrate? How will it be prepared?
    (c) Which microorganism will be used? Why did you suggest this microorganism?
    (d) What type of bioreactor will be used? How large will the production-scale bioreactor be? What design features will it have? What are the key operating variables of this bioreactor?

Take-Home Messages

- Solid-state cultivation (SSC) involves the cultivation of microorganisms on moist solid particles surrounded by a continuous gas phase.
- The majority of industrial processes involve submerged liquid cultivation, but SSC is preferred in specific situations.
- Bioreactors for SSC can be classified into trays, packed beds, rotating (or stirred) drums, and forcefully aerated agitated bioreactors depending on the agitation regime and the aeration method.
- Key factors that determine bioreactor selection are the growth rate of the micro-organism, its sensitivity to agitation, and the value of the final product.
- The removal of waste metabolic heat is the key consideration in most SSC processes, but the supply of O_2 can be limiting when the bed is not forcefully aerated.
- Forced aeration is the most effective way of removing heat: Air flow is affected by bed porosity, which changes during the process; cracks and gaps can form in the bed, leading to the phenomenon of channeling.
- The solid nature of the bed means that monitoring and control is a greater challenge in SSC than it is in submerged liquid cultivation.
- Although the kinetic expressions in SSC bioreactor models are empirical, the models are still useful tools for optimizing the design and operation of SSC bioreactors and guiding scale-up of processes.

References

1. Dinesh Babu P, Bhakyaraj R, Vidhyalakshmi R. A low cost nutritious food "Tempeh"- A review. World J Dairy Food Sci. 2009;4:22–7. https://www.idosi.org/wjdfs/wjdfs4(1)/5.pdf.
2. Durand A. Bioreactor designs for solid state fermentation. BiochemEng J. 2003;13:113–25. https://doi.org/10.1016/S1369-703X(02)00124-9.
3. Filer K. Industrial production of enzymes for the feed industry. In: Roussos S, Soccol CR, Pandey A, Augur C, editors. New horizons in biotechnology. Dordrecht: Springer Science + Business Media; 2003. p. 1–16. https://doi.org/10.1007/978-94-017-0203-4. https://link.springer.com/content/pdf/10.1007%2F978-94-017-0203-4_1.pdf.
4. Lüth P, Eiben U. Solid-state fermenter and method for solid-state fermentation. US Patent 6,620,614 B1, 16 September, 2003. https://patentimages.storage.googleapis.com/33/5a/c5/332f2e9d923201/US6620614.pdf.
5. Mitchell DA, Berovic M, Krieger N. Biochemical engineering aspects of solid state bioprocessing. AdvBiochemEngBiotechnol. 2000;68:61–138. https://doi.org/10.1007/3-540-45564-7_3.
6. Mitchell DA, Krieger N, Berovič M. Solid state fermentation bioreactors: fundamentals of design and operation. Heidelberg: Springer; 2006. ISBN: 3-540-31285-4 3540312854.
7. Nout MJR, Rombouts FM. Recent developments in tempe research. J ApplBacteriol. 1990;69:609–33. https://doi.org/10.1111/j.1365-2672.1990.tb01555.x.
8. Pérez-Correa JR, Agosin E. Solid-substrate fermentation, automation. In: Flickinger MC, Drew SW, editors. The encyclopedia of bioprocess technology: fermentation, biocatalysis and bioseparation, vol. 5. New York: John Wiley; 1999. p. 2429–46. https://onlinelibrary.wiley.com/doi/abs/10.1002/0471250589.ebt200 (online version).
9. Pitol LO, Biz A, Mallmann E, Krieger N, Mitchell DA. Production of pectinases by solid-state fermentation in a pilot-scale packed-bed bioreactor. ChemEng J. 2016;283:1009–18. https://doi.org/10.1016/j.cej.2015.08.046.
10. Roussos S, Raimbault M, Prebois JP, Lonsane BK. Zymotis, a large scale solid state fermenter. Design and evaluation. ApplBiochemBiotechnol. 1993;42:37–51. https://doi.org/10.1007/BF02788900.
11. Sato K, Sudo S. Small-scale solid-state fermentations. In: Demain AL, Davies JE, editors. Manual of industrial microbiology and biotechnology. 2nd ed. Washington DC: ASM Press; 1999. p. 61–79. ISBN 1-55581-128-0.
12. Sugai-Guérios MH, Balmant W, Furigo A Jr, Krieger N, Mitchell DA. Modeling the growth of filamentous fungi at the particle scale in solid-state fermentation systems. AdvBiochemEngBiotechnol. 2015;149:171–221. https://doi.org/10.1007/10_2014_299.
13. Suryanarayan S, Mazumdar K. Solid state fermentation. US Patent 6,197,573 B1, 6 March 2001. https://patentimages.storage.googleapis.com/93/4b/cc/9fc8fc8f8c8ecf/US6197573.pdf.
14. Ziffer J. Wheat bran culture process for fungal amylase and penicillin production. In: Raimbault M, editor. Solid state fermentation in bioconversion of agro-industrial raw materials. Montpellier: ORSTOM; 1988. p. 121–8. http://horizon.documentation.ird.fr/exl-doc/pleins_textes/divers09-06/30605.pdf.

Liquid State Bioreactor

Ercan Yatmaz and Irfan Turhan

A. Berenjian (ed.), *Essentials in Fermentation Technology*, Learning Materials in Biosciences,
https://doi.org/10.1007/978-3-030-16230-6_5

What You Will Learn in This Chapter

Liquid culture and solid culture fermentation are widely used to produce a range of metabolites. Despite the use of solid culture fermentation in some cases, most fermentations are carried out in a liquid broth. This type of production is called liquid state fermentation or submerged fermentation. Most fermentation processes are performed on aseptic conditions, with aeration and agitation, while some fermentations, such as beer and wine production, are carried out with no need of aseptic conditions, aeration, and agitation. This chapter will discuss liquid state fermentation principles.

5

5.1 Introduction

Fermentation is known as a process in which microorganisms grow in aerobic or anaerobic conditions and produce metabolic products in a closed container called a fermenter or bioreactor. For this purpose, it is necessary to provide suitable producer microorganisms that will consistently produce the highest amount of target product. Subsequently, operations such as the development of strain, preservation, inoculation preparation, media optimization, and application of the correct fermentation strategy are required. Important parameters such as production efficiency, productivity, and production economy are taken into account in all these choices. The type and amount of products produced by microorganisms vary according to the stages of microbial growth.

The stage in which microorganism produces the product and the type of growth is very important for the fermentation process success. For this purpose, two methods, liquid culture (Submerged Fermentation; SmF) and solid culture (SSF) fermentation, are widely used. Despite the use of solid culture fermentation, most fermentations are carried out in aerobic or anaerobic conditions in broth. This type of production is called liquid state fermentation (SmF). Most fermentation processes are performed on aseptic conditions, with aeration and agitation, while some fermentations, such as beer and wine production, are carried out with no aseptic conditions, aeration, and agitation. Fermentation processes are designed according to strategies such as suspension of the cell, filming on a support material, trapping in a matrix.

The basic processes required for liquid state fermentation in bioreactors are as follows:

1. Selection of the appropriate microorganism for the target product
2. Determination and preparation of the most suitable medium for microbial growth
3. Preparation and preservation of stock culture and precultures
4. Sterilization of medium and fermenters
5. Inoculation of the fermenter
6. Production of microbial products
7. Monitoring of fermentation
8. Harvesting of products
9. Purification of products and finishing steps
10. Cleaning up the fermenter

All these steps are summarized in Fig. 5.1.

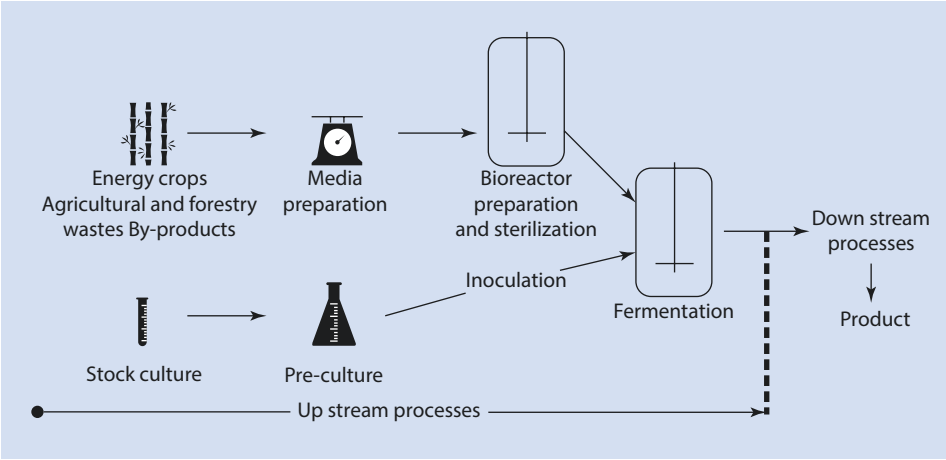


Fig. 5.1 Complete overview of liquid state fermentation processes

5.2 The Basics of Liquid State Bioreactor

SmF is a cultivation method where the microorganisms grow in a liquid medium. The bioreactor which is used for SmF is called liquid state bioreactor (LSB). Bioreactors are the bioprocess vehicles of any microorganism-based processes, be it for amino acids, organic acids, ethanol, drinking alcohols, enzymes, vaccines, etc. Bioreactor systems can be used for both microbial conversions (fermentations) and bioconversions (enzymatic processes) [37]. The most suitable bioreactor design must be organized to enhance microbial growth and metabolic activity of the biocatalyst [48]. The types of LSB systems differ from microorganisms to products.

LSB systems can be modified for microorganism requirements and generally are capable to control the parameters such as temperature, agitation, pH, aeration, and foam to keep the bioreactor conditions between set limits [37]. For the best bioreactor design, a number of points must be considered (revised from [48, 49]):

- The vessel should be glass or stainless steel, be sterilized and operated aseptically for days, have smooth internal surfaces, and be designed as user-friendly (easy operating, harvesting, cleaning, and maintenance).
- Aeration and agitation should be operated at the specific levels of microorganism requirements. Air sparger and stirring impeller should be changeable for adequate aeration and agitation.
- Power consumption should be limited as low as possible or supplied from renewable sources (solar panel, wind turbine, etc.).
- Temperature, pH, and other controls should be provided with the lowest deviation (if possible maximum: ±0.01).
- Temperature, pH, aeration, and other controls should be calibrated at the place of use.
- It should have baffle system for efficient agitation.
- Bioreactor materials should be corrosion resistant to prevent any type of contamination.
- All materials should be nontoxic for microorganisms.

- For in situ sterilization, bioreactor system should have special steam unit and pressure control system (This system is generally used in volumes of 10 L or more). And also, it must have aeration unit for in situ sterilization cooling process.
- Aseptic sampling port should be provided.
- The vessel ports should be re-editable for fermentation requirements.
- Exhaust port should be added, and evaporation loss should be limited.
- Different volumes of vessels should be the similar geometry in the pilot or scale-up plant.
- It should have a good service provision.

5

Fermentations are carried out in different volumes of fermenters depending on the product produced. While products such as ethanol and citric which are widely used in industry are produced in very large volume fermenters (over than 300 m^3), health products like hyaluronic acid and antibody are produced in smaller volume fermenter (less than 25 m^3). For this purpose, there are many fermenters, from simple fermenters with no mixing/mixing to more advanced ones with computer control. The fermenter and all its components must be sterilizable. In this sense, stainless steel which does not interact with products or microorganisms is widely preferred.

In laboratory-type productions, fermenters consist of glass bottles, Erlenmeyers, or vessels made of glass which are generally used according to the required volume. Fermenters reaching several hundred thousand liters of capacity are used in industrial production. Fermenters used for industrial processes should be able to prevent contamination risk. It must be resistant to repeated sterilization and cleaning procedures. It must also be made of corrosion-resistant material. Fermenters for pilot scale are produced from stainless steel, while fermenters for very large volumes are produced from mild steel coated with glass or plastic to reduce production costs. In addition, the air, inoculum and nutrient pipelines, and connection points required for fermentation must be steam sterilizable. The basic rule for a fermentation to occur in aseptic conditions is that there is no contact between sterile and non-sterile parts. Each part of the fermenter must have sterilizable and cleanable valves. There should not be unnecessary horizontal pipes, connections, and dead zones in the fermenter. The inside of the fermenter should be cleaned with special sprayer systems. This type of cleaning process is called cleaning in place, CIP. Small fermenters containing the medium are sterilized in autoclaves suitable for their size. Laboratory and industrial fermentation systems are operated by batch, fed-batch, and continuous. During the fermentation, acid or base addition is carried out to control the pH. Besides, the fermenter is not interfered except air supply, inoculation, and addition of substrate if necessary. In batch fermentations, the process starts and ends. The medium is added to the fermenter, sterilized, and inoculated, and the microorganism begins to work. At the end of fermentation, the product is removed and the fermenter is cleaned. The fermenter is then prepared again for all these operations. This period, in which no action is taken, is called "down time."

These practices are essential for any type of bioreactors. However, bioreactor properties and specifications are varied depending on the microorganisms and target product. In general, bioreactors can be classified into two specific types: high-performance bioreactors in the laboratory studies and industrial bioreactors in the production processes [37]. This classification is very basic and in the following sections LSF systems will be reclassified for specific applications.

5.3 Advantages of Liquid State Bioreactors

The aim of using a liquid medium is to provide an appropriate environment for microorganisms to grow easily and produce specific microbial metabolites or by-products rapidly. Any type of microorganisms (filamentous fungi, yeast, bacteria, algae, etc.) can be used for submerged fermentation. Traditional glass jar fermentation is still used to produce fermented foods all over the world. For the laboratory assays or industrial production, the specially designed bioreactors must be used to get significant and repeatable results, enhance product yield, and prevent contamination.

Using liquid medium and liquid state bioreactors has lots of advantages such as follows (organized from [20, 48]):

- All types of microorganism can be used for fermentation: filamentous fungi, yeast, bacteria, or algae.
- There is no limit for scale-up operation.
- It is easy to control fermentation parameters such as pH, temperature, O_2, CO_2, etc.
- Lots of different bioreactor designs can be derived.
- Microorganisms can grow and pass lag phase faster.
- Different fermentation types can be used easily to enhance product yield: batch fermentation, fed-batch fermentation, continuous fermentation, and their combinations.
- Product limitation can be solved using fed-batch or continuous fermentation techniques, so there is no product limitation.
- It is easy to adjust media composition.
- Lots of different agricultural sources or wastes can be used as carbon or nitrogen sources.
- Online production and recovery systems can be organized to provide unlimited production strategy.
- Analyses can be carried out directly without any extraction process for extracellular products.

5.4 Type of Liquid State Bioreactors

Microbial growth is a complex process because the large number of factors can affect the production. In order to perform successful fermentation, both advanced technological and simple bioreactors can be used. Some properties and factors are important in deciding which bioreactor system is the best. Some of those are the biological constraints of the organism, the scale of production, the technology level, economics, and the range of products [44]. There can't be a single fermentation system that adequately meets the needs of all biological systems. In general laboratory fermenter systems, up to approximately 20 L are made of glass, and larger systems are made of stainless steel. Bulk products are usually produced in single bioreactor systems where the design provides efficient mass transfer and heat removal in order to minimize the costs [44].

The efficiency of a bioreactor's performance depends on the concentration of biomass, aseptic conditions, mass and heat transfer efficiency, and operation under optimum processing conditions. Bioreactors can be divided into three groups [46]:

- Bioreactors without agitation and aeration: This system is used for anaerobic fermentations, e.g., wine and beer production.
- Bioreactors with aeration, but not agitation: This system is used for aerobic liquid state fermentations, e.g., food enzyme production.
- Bioreactors with aeration and agitation: This system is used for aerobic liquid state fermentations, e.g., citric acid and penicillium production.

However, bioreactors are classified with their construction and design properties in the bioprocess industry. The commonly used bioreactor systems and their specifications will be mentioned below.

5

5.4.1 Stirred Tank Bioreactors (STBs)

The most commonly used bioreactor for industrial applications is the conventional stirred tank bioreactor. STBs combine the advantages of high oxygen transfer rates required for high biomass efficiency with low investment and operating costs that form the basis of successful aerobic fermentation process. STBs typically have height/diameter ratios between 1:3 and 1:6 [46]. The basic stirred tank bioreactor system and some control parameters are shown in Fig. 5.2.

Oxygen mass transfer in STBs is affected by many variables, such as liquid physical properties (viscosity, surface tension, etc.), vessel geometry, sparger type, impeller type,

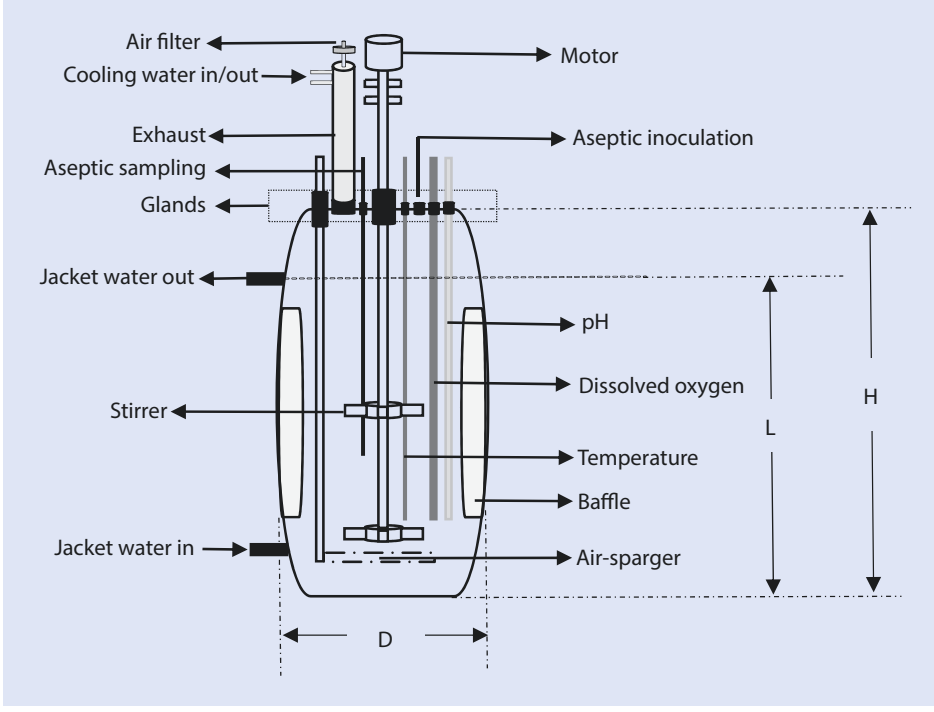


Fig. 5.2 Basic stirred tank bioreactor system. (Revised from [49])

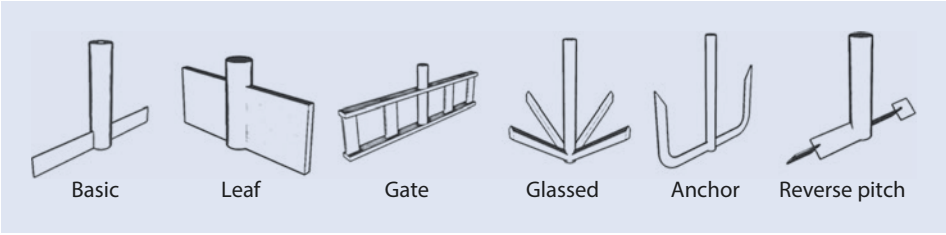


Fig. 5.3 Paddle impeller designs

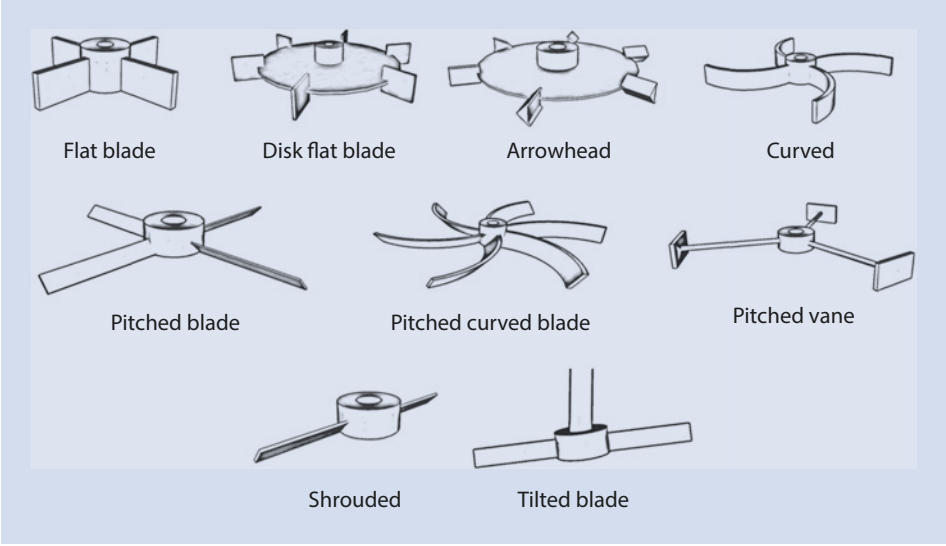


Fig. 5.4 Turbine impeller designs

and operational parameters [24]. Impellers and spargers are the most important parts of the STBs which provide higher oxygen transfer rate and mixing. The impeller types can be classified by providing mixing regimes: laminar or turbulent mixing. The other classifications of the impellers are constructions characteristics: turbine or paddle impellers [18].

Helical ribbons, screws, helical-ribbon screws, anchor, disks, paste rollers, high shear, and gate impellers are used in laminar mixing. Disk-style, flat-blade, and curved-blade impellers are radial turbulent flow impellers; pitched-blade turbines and propellers are axial or mixed turbulent flow impellers (Figs. 5.3 and 5.4) [18]. STBs with multi-impeller systems can be designed with the combination of different impellers, but the design must provide adequate mixing intensity and sufficient mass transfer rate without causing any serious shear force that damage to the microorganism cells [57].

Not only the choice of impeller but also the usage of baffle is important to avoid vortex formation and improve mixing [46]. Baffle systems are generally located close to vessel wall (Fig. 5.2) and produced from stainless steel. Air spargers are also important for good aeration and mixing. Different manufacturers produce micro- or macro-air spargers to provide effective aerating. The porous sparger, the orifice sparger (a perforated pipe), and the nozzle sparger (an open or partially closed pipe) are the three basic air sparger

systems for bioreactors [49]. Also, combined air sparger systems can be used. The most common air sparger type for STBs is a ring-type sparger with perforations as shown in Fig. 5.2. Air spargers are located just below or above the impellers. The other parts of the STBs such as heating system, probes, calibration parameters, stirring motor technology, sealing glands system, baffle, controlling unit, control precision levels, calibration parameters, exhaust system, vessel type, and configurability are depended on manufacturer and user requirements.

STBs can be used for lots of different bio-products such as ethanol, organic acids, antibiotics, enzymes, etc. Because the scale-up methods are fairly well understood, and STBs are easily adapted for multiproduct use, they can be constructed from 1 L laboratory units to 150–200 L or over commercial fermenters by the manufacturers [44].

5.4.2 Airlift Bioreactors (ALBs)

The airlift bioreactors are special systems where the fluid circulation enhances the gas–liquid or gas–liquid–solid phases contact [1]. ALB has a simple design because it has no moving parts or agitator. ALB is generally constructed with a concentric tube which is divided into two parts: riser and downcomer sections that provide for oxygenation and mixing [44, 46] (Fig. 5.5). Internal mixing and aeration are done with pressurized gases. This system provides low shear stress values which is important for shear-sensitive

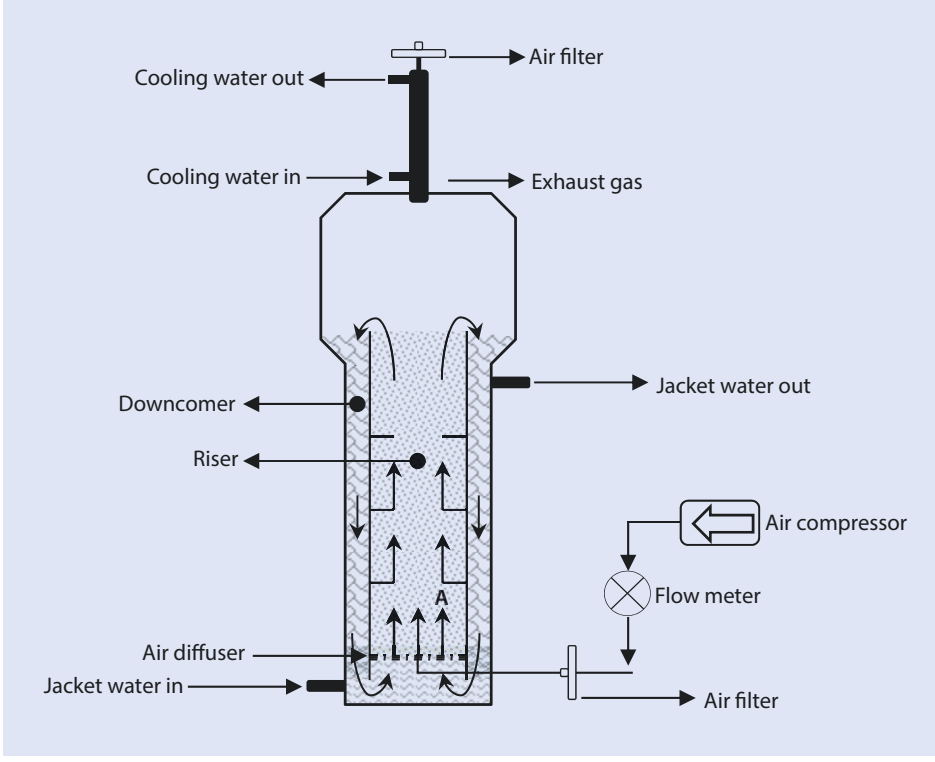


Fig. 5.5 Airlift bioreactor system with inner loop. (Revised from [39, 49])

microorganisms [39]. The care must be given for downcomer part to avoid oxygen limitation during cell growth. ALBs are economical at larger sizes because air compression systems need high energy input for aeration and mass transfer [44].

Some of the other advantages of ALBs are reduced risk of contamination, efficient gas–liquid dispersion, and low power consumption. This system trapped the oxygen bubble into the fluid by circulation which improves the oxygen mass transfer compared to an STB system [1]. But sometimes, ALB systems cannot provide enough oxygen transfer rate due to the nonexistence of any mechanical agitator. So, it cannot be chosen for high cell density aerobic fermentations [5]. ALBs can also be used for highly viscous fermentations with a low volumetric mass transfer coefficient [46]. Different fluid dynamics and shearing forces characteristics of ALBs affect the fungal cells growth positively than STBs if the oxygen transfer rate is enough for cell growth [43]. The temperature or pressure can be changed to provide the oxygen transfer rate by increasing the oxygen dissolution rate in media.

A well-known sample of the ALBs is the ICI pressure cycle fermenter which is used for Single-Cell Protein (SCP) production from methanol by *Methylophilus methylotrophus* [46]. Mechanical stirring systems (i.e., STBs) would be uneconomical because of the low cooling capacity without external cooling. ALBs provide economical SCP production from methanol by providing cooling loops with its outer or inner loops [49].

5.4.3 Bubble Column Bioreactors (BCBs)

Bubble column bioreactor is also one of the aerated bioreactors which is a good gas–liquid contactor for bioprocessing industry [34]. Bubble column bioreactor provides good mixing by aeration, ease of operation, and economic benefits like airlift bioreactor [22]. It has a perfect heat and mass transfer. This bioreactor system has been investigated for gas holding, air bubble features, flow regime and fluid dynamic investigations, and heat and mass transfer studies [26]. BCBs usually provide high cell concentration and productivity values where the cost of the bioreactor is low. It also can be used for continuous flow mode easily by a simple reconstruction [46]. The system consists of very few parts, and one sample of BCBs is shown in Fig. 5.6.

5.4.4 Fluidized Bed Bioreactors (FBBs)

The fluidized bed bioreactor is usually a system of choice for microbial degradation of toxic pollutants in wastewater treatment (especially in continuous mode) with its hydrodynamics and mass transfer phenomena (Fig. 5.7) [4, 49]. The support matrix (sand, anthracite, reticulated foam, etc.) improves the surface area on the biofilm adheres and let the operation of high biomass concentrations. So, small bioreactors could be used for the treatment of biological pollutants [49].

The state of fluid-like solids by its contact with a gas phase or a liquid phase or both in one operation is known as fluidization. This operation is called two-phase or three-phase fluidization depending on the number of phases involved in the reaction. In a three-phase fluidization, the bed of solid-medium particles is suspended in a liquid and gas medium creating an intimate contact between these three phases—solid, liquid, and gas, which can be cocurrent or countercurrent causing a good mass transfer. In recent years, the three-phase fluidized bed

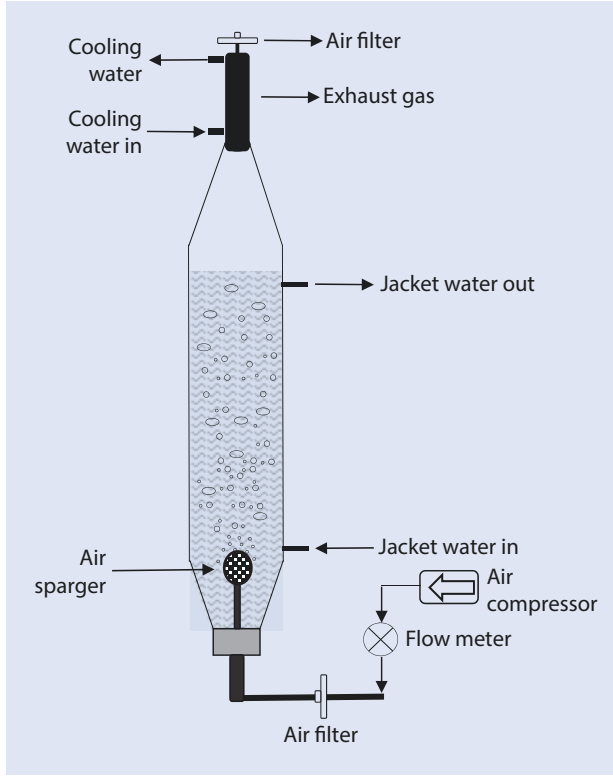


Fig. 5.6 Bubble column bioreactor. (Revised from [22, 40, 41])

system (gas, liquid, and solid) has been applied in particular for biotechnological processes, including wastewater treatment [50]. A typical aerobic fluidized bed bioreactor system is shown in Fig. 5.7. The most important advantages of fluidized bed bioreactor are highly expanded interphase surface, simple construction, high cell concentration with immobilized system, and the possibility of the decreasing of the mean residence time of the liquid phase [47]. Superior mass and heat transfer, good mixing, and low energy requirements with low shear rates are the most important advantages of FBBs for ethanol production from *Saccharomyces cerevisiae*. If the glass or ceramic carrier is used to be a supporting material, it may cause cell leakage because of higher pumping capacity requirement [46].

5.4.5 Packed Bed Bioreactors (PBBs)

PBBs are application of immobilized cells on inert materials such as wood shavings, twigs, aggregates, polythene, etc. where the medium and cells are fed into the top of the bioreactor due to provide thin cell film on the support material (Fig. 5.8) [49].

The best-known sample of the PBBs is ethanol oxidization processes to acetic acid by *Acetobacter* strains [49]. Nowadays, PBBs have been widely used in the gas absorption industry because of their low energy consumption and high gas/liquid contact density and mass transfer rate. A typical PBB is packed a column with solid packing material, and a flow of liquid spread over the bed, moving down with a co-gas flow or against the flow through the bed. The liquid films on the surface of the packaging material are continuously renewed,

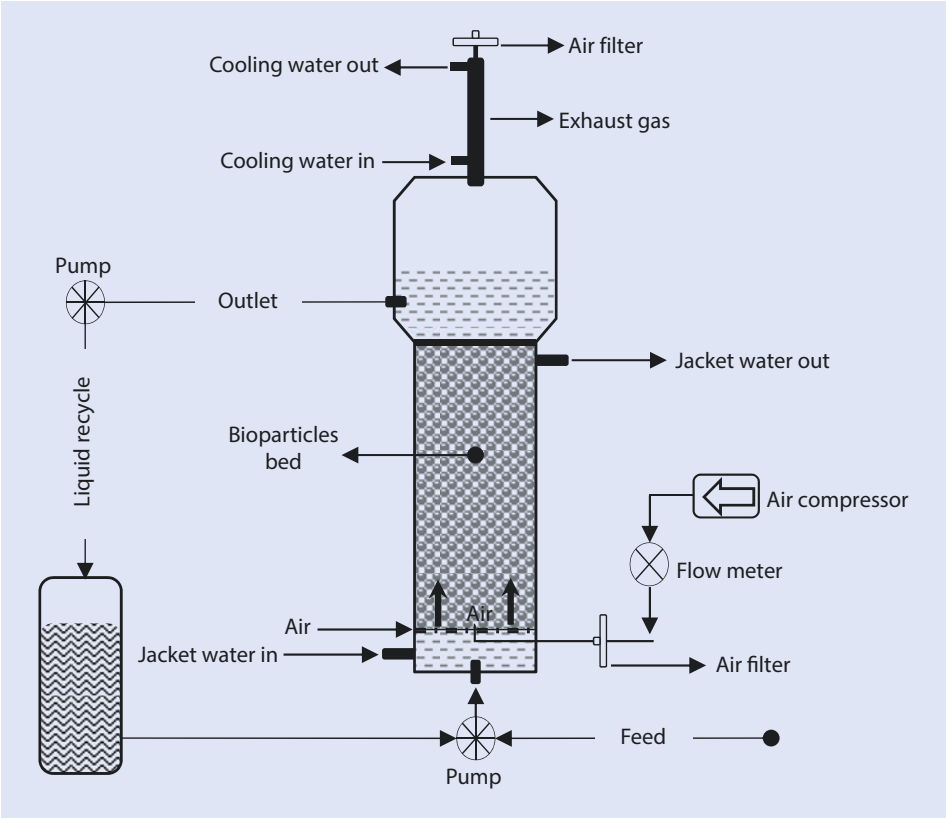


Fig. 5.7 Aerobic fluidized bed bioreactor. (Revised from [2, 4, 19, 36, 51])

which facilitates gas–liquid contact and mass transfer. Different packaging materials with different material properties and geometric structures are available for mattresses with unique properties, including hydrophobicity surface, liquid hold-up, local hydrodynamics, empty space, pressure drop, and the effective area. Bed efficiency and mass transfer rate are also affected by liquid distributions as well as gas and/or liquid velocities. PBBs have been used in the treatment of wastewater or waste gases as bio-filters or bio-scrubbers [29].

The bioreactor evaluation on a laboratory scale is a necessary step before scale-up. It has a static bed above a perforated plate through which the air conditioning system is blown. They are easy to handle and operate continuously, allowing the extraction of enzymes in situ [38].

5.4.6 Photobioreactors (PBs)

In recent decades, microalgae bioreactors in biological processes which are called as Photobioreactors (PBs), particularly for wastewater treatment applications, are interested which is commonly performed with photobioreactors. In this system, microalgae consume CO_2 and produce oxygen. Consequently, aerobic bacteria biodegrade pollutants using this oxygen [58]. An example of tubular PB for algae study is shown in Fig. 5.9.

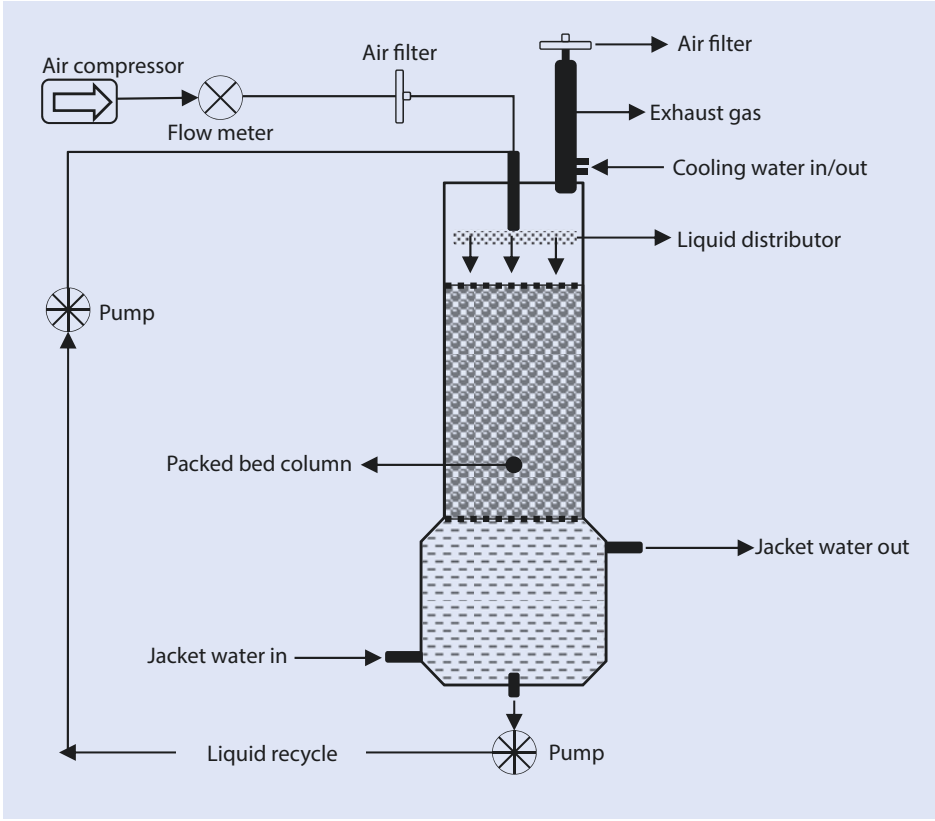


Fig. 5.8 Packed bed bioreactor. (Revised from [16, 28, 29]

PB was first used for microalgae culture in the 1940s. A tight structure, good mixing performance, high heat transfer rate, and appropriate detection and control unit provide the excellent photobioreactor performance. Proposed for a better PB, the most important factors which affect the performance are light distribution, biomass concentration, stirring force, temperature control, transfer rate liquid mass, etc. Intermittent illumination was found to be more favorable for improving the efficiency of photosynthesis, and mixing was beneficial for enhancing mass transfer inside the photobioreactor [59].

The benefits of the photobioreactor system are simple control of internal environmental parameters, increasing productivity using denser cell concentrations, more efficient use of light absorption, and more efficient use of the surface compared to the pond system. Sunlight should be the main source of illumination for power generation system instead of artificial lamps such as fluorescent lamps and light-emitting diodes which require additional energy resources. Internal mixing is also the most important factor for delivering light evenly to microalgae cells for scale-up processes [45]. The various types of photobioreactors are tubular PBs, stirred tank PBs, flat plate PBs, hollow fiber membrane PBs, and airlift PBs [42]. Closed photobioreactor systems are designed not only to provide good mixing, higher mass and heat transfer, and light to algal cultures but also prevent contamination, evaporation, and weather effects to get efficient results [23].

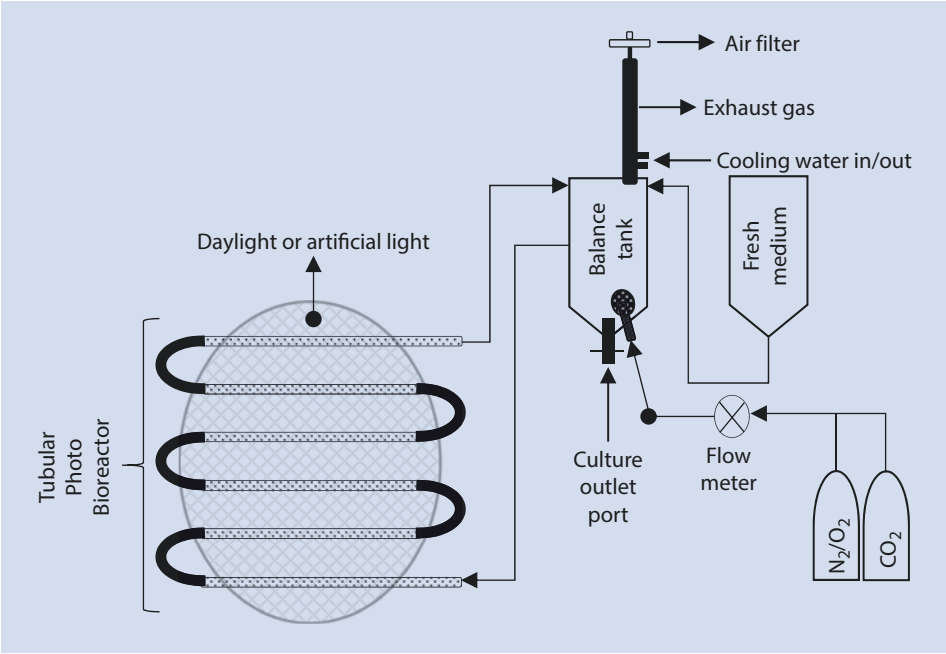


Fig. 5.9 Tubular photobioreactor for algae study. (Revised from [21, 42, 59])

5.4.7 Membrane Bioreactors (MBs)

Membrane Bioreactors (MBs) are hybrid systems which are composed of bioconversion and separation part [15]. Membrane bioreactors with the inclusion of hollow fiber systems have been used to improve the growth of the mammalian and plant cells, and immobilization of bacteria, yeast, and enzymes for different biotechnological applications [46]. This system is relied on the membrane separation of biomass or product from effluent and used for wastewater treatment carefully because of a long-term sludge retention time and high sludge concentration. MBs make the removal of nitrogen and phosphorus and the degradation of organic pollutants easier than other bioreactors systems with its high cell community [60] (Fig. 5.10).

Cellulose acetate and acrylic copolymers or polysulphone fibers are used to produce uniform and asymmetric wall matrix, respectively [46]. High density of cell growth, simultaneous separation of product and biomass, and biocatalyst regeneration are the advantages of MBs. However, difficulties in monitoring and controlling the growth and metabolism of the culture, low oxygen transfer rate at high cell density, and accumulation of toxic products are the disadvantages of MBs [46].

5.4.8 Microcarrier Bioreactors (MCBs)

Microcarriers are generally used for mammalian cells to provide the necessary surface for attachment where it is a problem for growing anchorage-dependent cultures. Growth of the cells on microcarriers is directly dependent on the area available for growth to the point where the microcarrier particles reach a concentration sufficient to inhibit cells and thus

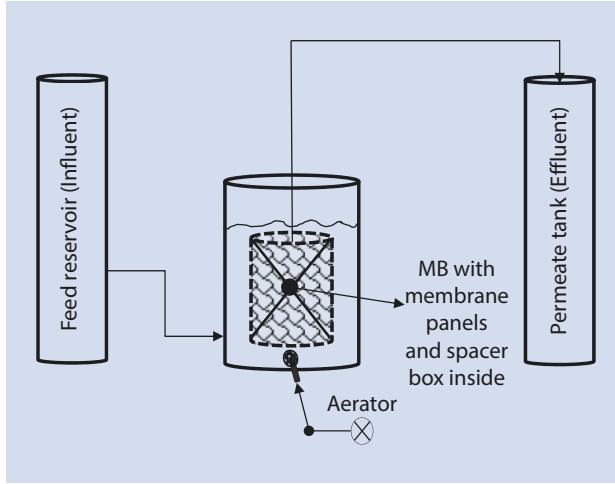


Fig. 5.10 Membrane bioreactor system. (Revised from [15, 31, 55, 60])

reduce cell yield [46, 49]. Microcarriers are generally added into STBs to produce special cells with high surface area. Different materials (e.g., polystyrene, dextran) can be used to be microcarriers for human pluripotent stem cells where the commercial microcarriers have different functional groups such as positively charged groups (e.g., DEAE) or non-ionic materials from biological origin (e.g., collagen) [3]. The support material toxicity may have resulted in lag phase time, early death phase, and limited cell yield. The most important advantages of the MCBs usage are the high surface area and low shear conditions, but the type of microcarrier and impeller collisions may cause reduced cell viability [46, 49].

5.4.9 Innovative Fermentation Strategies and Bioreactor Modifications for Liquid State Bioreactors

It is important to understand the development mechanisms of microorganisms to run a successful fermentation, because in some cases, the traditional bioreactor systems are not capable to meet the fermentation requirements. For this reason, researchers are trying to enhance or control the microbial growth for getting the best production rates' values by combining or modifying fermentation techniques or bioreactors. This section will focus on common innovative fermentation strategies and bioreactor modifications for LSBs.

Biofilm Formation Biofilm is a microbial community system where the microorganisms attached on biofilm material surfaces and embedment in an extracellular matrix (Fig. 5.11). In biofilms, the bacteria gain more viability than suspended cells to desiccation, grazing, and antimicrobial agents. Bacterial multispecies consortia in biofilm structure trigger the syntrophic interactions, horizontal gene transfer, and co-metabolism. Biofilms provide either beneficial or negative effects in the industry and natural environment. Biofilm bioreactor systems provide some advantages for LSBs such as working with more biomass concentration (5–10 times), increasing productivity, reducing the risk of washout for continuous fermentations, and eliminating the reinoculation process for repeated-batch fermentation [14]. These biofilm systems can be used for wastewater treatment, alcohol, enzyme, organic acid, and other value-added products because of these advantages.

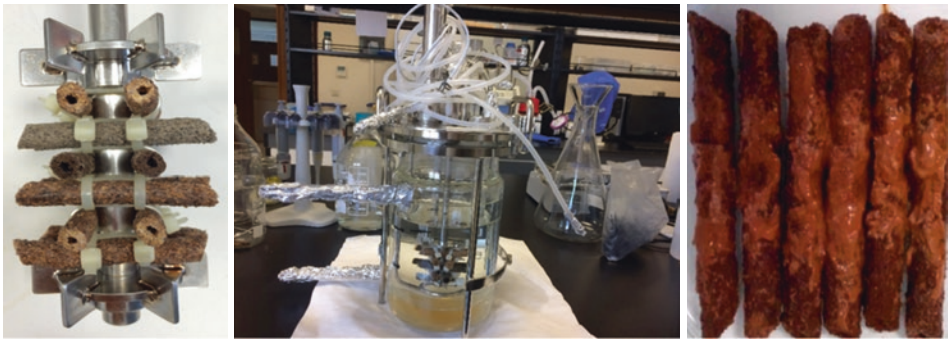


Fig. 5.11 Stages of biofilm (From left to right: Preparation, fermentation, and formed biofilm. Photos are taken from Lab Research Results in Turkey)

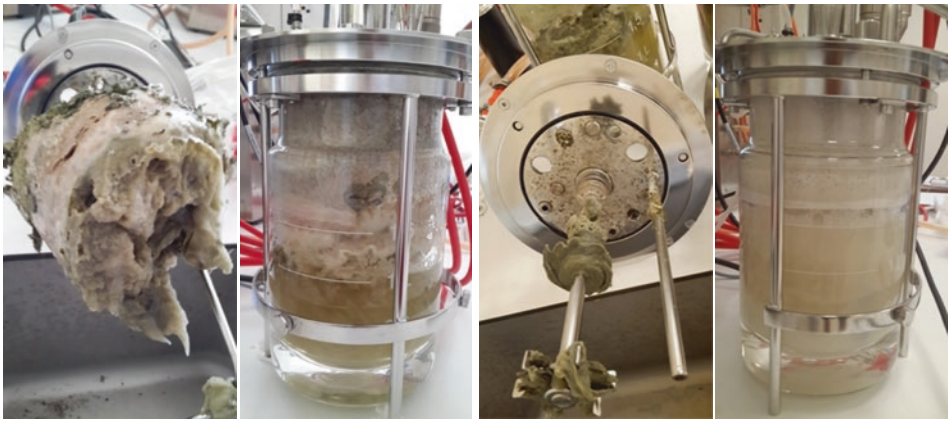


Fig. 5.12 Microparticle usage for filamentous fungi fermentation (Left: Mannanase fermentation without microparticle addition, Right: Mannanase fermentation with talcum addition; Photos are taken from Lab Research Results in Turkey)

Microparticle Usage for Filamentous Fungi Fermentation The hyphae development during filamentous fungi growth is hard to control and limited in medium or LSBs. High cell concentration and hyphae development cause a spore to inosculate another spore, and the hyphae stack is consisted which block the extracellular enzyme to let out of the cell and decrease fungi active zone. To solve all these problems, researchers start to use microparticle agents such as talcum, aluminum oxide, or titanium oxide. Microparticle usage facilitates the control of fungal morphology and enhances productivity values for enzyme production (Fig. 5.12) [11, 12, 25, 56]. Generally, microparticle addition decreases the pellet diameter size of the fungal cells, and this limitation improves the active zone of the fungal cells. Consequently, the appropriate amount of microparticle addition enhances the fermentation results and improves the cell control.

Shifting Strategy Fermentations are generally performed at specific process parameters such as constant temperature, pH, dissolved oxygen, agitation, etc. But some fermentation processes need well-directed shifting strategy (pH, dissolved oxygen, etc.) to provoke physiological changes to improve process performance, thus providing a controllable strategy for

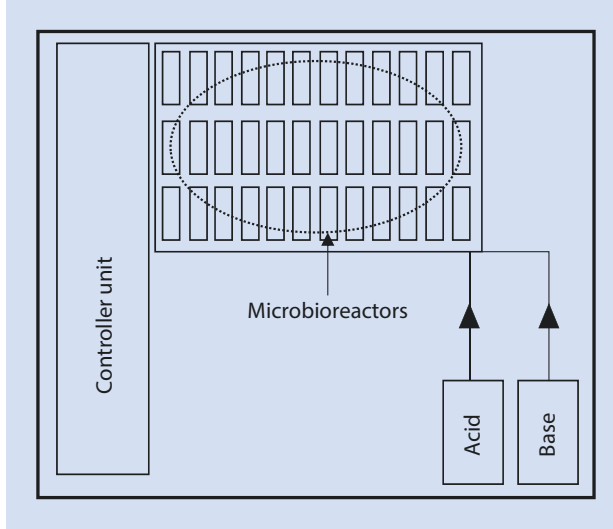


Fig. 5.13 Microbioreactor bioreactor systems

the cell growth and biosynthesis of metabolites [52]. This approach is performed by changing one or more fermentation parameters at different times of the fermentation to enhance productivity or control cell viability and metabolites [6, 52].

Microbioreactors It is hard to determine the new microorganisms' initial substrate requirements and working parameters. It is necessary to make several experiments to determine the requirements of a microorganism, which is very difficult for traditional bioreactors. For this purpose, multi-parallel microbioreactor system, which working volume is smaller than 250 ml, has begun to be used recently (Fig. 5.13). Microbioreactors provide an efficient strategy to determine initial operating parameters with its high throughput screening capacity [54]. These micro-systems also improve early clone selection decisions, increase lab productivity, and reduce the lab cost per experiment for any type of microbial researches. There are different microbioreactor systems for different fermentation requirements such as microbubble column [9], photonic microbioreactors [8], microfluidic reactor [13], etc.

Tissue Bioreactors Ex vivo engineering of living tissues is a new and rapidly developing field with its potential usage on a wide range of medical applications [33]. Tissue bioreactor systems are using to be an agent which provide the in vitro simulation of the in vivo biological, physical, and mechanical properties of growing tissues. Tissue engineering is a multidisciplinary field to develop biological substitutes that restore, maintain, and improve tissue function [10]. A wide variety of tissue bioreactors have been developed such as vocal fold, retina, skin, muscle, ligament, tendon, bone, cartilage, and liver [7]. In the twenty-first century, tissue engineering and regenerative medicine are expected to be powerful tools for medical treatment of tissues and organs [46].

Nuclear Magnetic Resonance (NMR) Compatible Bioreactors Cell population physiological, nutritional, and metabolic states and dynamic behaviors in bioreactor systems can be determined by different analytical techniques such as optical sensors for UV, fluorescence spectroscopy, in situ microscopy, or in vivo NMR [17]. NMR spectroscopy is inimitable in its

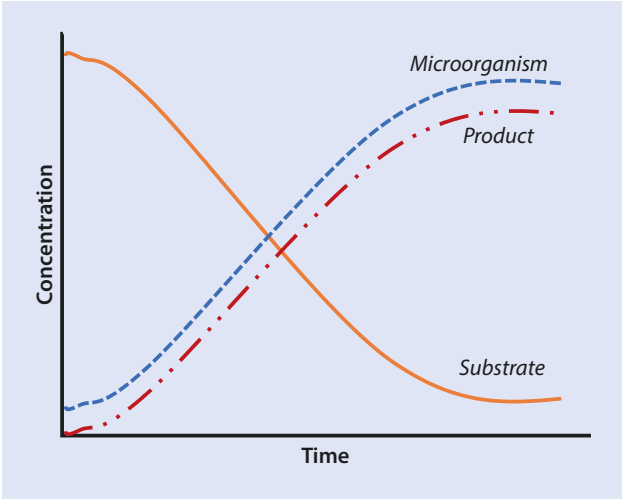


Fig. 5.14 Microbial growth, substrate consumption, and product production for batch fermentation

capability to follow up in situ metabolic flux. Different types of NMR-compatible bioreactors with its magnet designs have been developed to perform NMR analyses in or immediately adjacent to the reaction vessel under controlled environment [32].

5.5 Feeding Strategy for Liquid State Bioreactors

Fermentation technology has been used for many years in the supply of medicine, cosmetics, food, and other industrial needs of people. The biological requirements of microorganisms, the size of production, technological facilities, cost, and many other conditions must be considered in the selection of the bioreactor and the fermentation feeding type. Fermentation technique (feeding strategy) is one of the most useful parameters to produce highly yield final products by bacteria, yeast, fungi, or algae. Due to all these requirements, this section will refer to different feeding strategies for liquid state fermentations.

5.5.1 Batch Fermentation

Batch fermentation is the oldest fermentation technique to produce food, alcohols, pharmaceuticals, antibiotics, etc. The most widely used fermentation technique in lab-scale or industrial processes is batch fermentation because it is simple to operate, where all of the ingredients (carbon source, nitrogen source, and mineral) are added in bulk at the beginning of the process, and then microorganisms grow until carbon source is depleted [35]. The typical microbial growth for batch fermentation is shown in Fig. 5.14.

The batch culture fermentation stages are stock culture, pre-culture, sterilization of medium, inoculation, production, cleaning, and recovery processes. Lag, exponential, stationary, and death phases are observed at batch fermentations. The death phase is generally not achieved for batch fermentation because of the high accumulation of metabolites

and cell lysis. Batch fermentations that are especially used for ethanol fermentation can take several hours or weeks depending on the product and working conditions of the chosen microorganism [46]. The advantages of batch culture are as follows:

- Minimum risk of contamination,
- Operating with minimum system requirements,
- Easy to rearrange for different microorganisms and products,
- Operating with any type of microorganisms, and
- Easy to standardize the operating conditions.

Kinetics in Batch Fermentation

5

When the development of a single microorganism is called, the mass increase of the living cell is understood and can be defined as growth. In microbiology, this means that cells increase in number or mass. In the case of microorganisms that reproduce by dividing into two parts like bacteria, the expression of mass and number increase is the same. In microorganisms containing more than one nucleus in cells or hyphae, such as fungi, cell divisions are not accompanied by repeated cell divisions, and therefore it is impossible to correlate the increase in mass with the increase in cell count. It is the mass increase of the cells (mycelia) which are understood from the fungus developments. When cell growth is monitored in the intermittent fermentation process, the logarithmic phase lasts for a while and a typical growth curve for the cell population is encountered. The growth curve refers to the entire reproductive cycle including the lag phase, the logarithmic phase, the stationary phase, and the death phase [30, 53].

The growth rate of microorganisms is generally expressed by Monod equation:

$$dx/dt = \mu x \tag{5.1}$$

μ : Specific growth rate (h^{-1})

x : Microorganism concentration (g dry weight/L)

dx/dt : Growth rate (g/L/h)

When cells are inoculated into a fresh medium, they cannot develop and replicate immediately. This time slot is called the lag phase, which is necessary for the cells to become adaptive to the fermentation media and conditions, and after that phase cell mass and number of cells start to increase [30, 53]. For the lag phase, μ and dx/dt are equal to 0 (zero). Depending on the sources and other factors present in the microorganisms at the logarithmic phase, each cell divides to form two cells, and this process continues during the logarithmic phase. In the logarithmic phase, the rate of biomass increases, and the concentration of microorganisms is directly proportional:

$$dx/dt = \mu x$$

$$\mu = \frac{1}{x}\frac{dx}{dt}$$

If the integral of equation is taken,

$$\int_{x_0}^{x} \frac{dx}{dt} = \mu \int_{0}^{t} dt$$

If the equation is edited,

$$\ln x = \ln x_0 + \mu t \tag{5.2}$$

where x_0 represents the concentration of the microorganism inoculated to the culture medium at the beginning (at $t = 0$), and x represents the concentration of microorganisms at any t.

The secondary metabolites synthesized by microorganisms are industrial significance, and these metabolites accumulate especially at the log phase in molds or at the beginning of metabolite production phase (stationary phase). Microorganisms in the stationary phase can catabolize added substrates and convert intermediate metabolites that they have synthesized in the preliminary stages of biosynthetic pathways to new products such as antibiotics [30, 53].

In this phase,

$$\frac{dx}{dt} = 0,$$

$\ln\left(\frac{x}{x_0}\right) = \text{constant}$, and in the stationary phase, $x = x_{max}$ is often assumed.

If the incubation continues after the population has reached the stationary stage, the cells will remain alive and continue their metabolic processes, but eventually die. In this case, the population enters the death phase of the growth and in some cases the death occurs by cell disruption. The death phase is also logarithmic, but the cell death rate is much slower than it is in the logarithmic growth phase [30, 53].

In this phase,

$$-\frac{dx}{dt} = \mu_d x_{max} \tag{5.3}$$

μ_d : Specific growth rate constant (h^{-1})

x_{max} : Stationary phase biomass concentration (g dry weight/L)

Analyses are carried out prior to the calculation of kinetic parameters in the samples taken during the fermentation processes. The main ones are residual sugar concentration, product concentration, and biomass. Analyses are performed with the help of the following formulas using the data.

Biomass population at the end of batch culture is calculated by

$$\Delta X = X_{max} - X_0 \tag{5.4}$$

X : Biomass concentration (g dry weight/L)

X_{max} : Maximum biomass concentration (g dry weight/L)

X_0 : Initial biomass concentration (g dry weight/L)

Residual sugar concentration at the end of batch culture is calculated by

$$\Delta S = S_0 - S_1 \tag{5.5}$$

S : Residual sugar concentration (g/L)
S_1 : Minimum or final sugar concentration (g/L)
S_0 : Initial sugar concentration (g/L)

Product concentration at the end of batch culture is calculated by

$$\Delta P = P_1 - P_0 \tag{5.6}$$

P : Product concentration (g/L)
P_1 : Maximum product concentration (g/L)
P_0 : Initial product concentration (g/L)

Yield of substrate to product ($Y_{P/S}$) is calculated by

$$\text{Yield}\left(Y_{P/S}\right) = \frac{\Delta P}{\Delta S} \tag{5.7}$$

Yield of substrate to biomass ($Y_{X/S}$) is calculated by

$$\text{Yield}\left(Y_{X/S}\right) = \frac{\Delta X}{\Delta S} \tag{5.8}$$

Yield of biomass to product ($Y_{P/X}$) is calculated by

$$\text{Yield}\left(Y_{P/X}\right) = \frac{\Delta P}{\Delta X} \tag{5.9}$$

Biomass population at the end of batch culture is also calculated by

$$X = Y_{X/S}\left(S_0 - S\right) \tag{5.10}$$

Calculation of the maximum consumption rate is performed by taking the slope of the steepest part of the sugar consumption graph (Fig. 5.15). For this calculation,

$$\text{max.cons.rate}\left(\text{g / L / h}\right) = -\left(\text{the slope of the steepest part of sugar cons. graph}\right)$$

Calculation of the maximum growth rate is performed by taking the slope of the steepest part of the biomass growth graph (Fig. 5.16). For this calculation,

$$\text{max.growth rate}\left(\text{g / L / h}\right) = \text{the slope of the steepest part of biomass growth graph}$$

Calculation of the maximum production rate is performed by taking the slope of the steepest part of the product production graph (Fig. 5.17). For this calculation,

$$\text{max.prod.rate}\left(\text{g / L / h}\right) = \text{the slope of the steepest part of production graph}$$

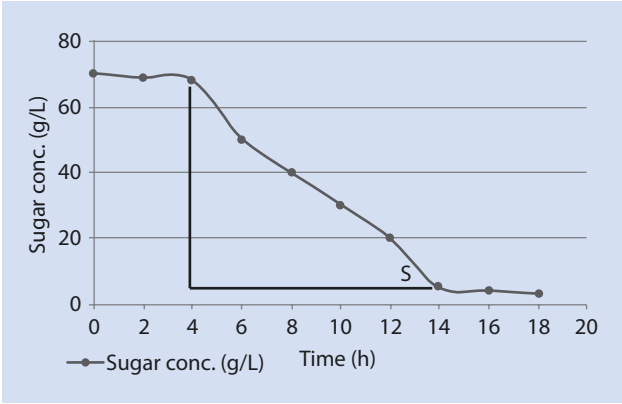


Fig. 5.15 Graph of maximum consumption rate calculation

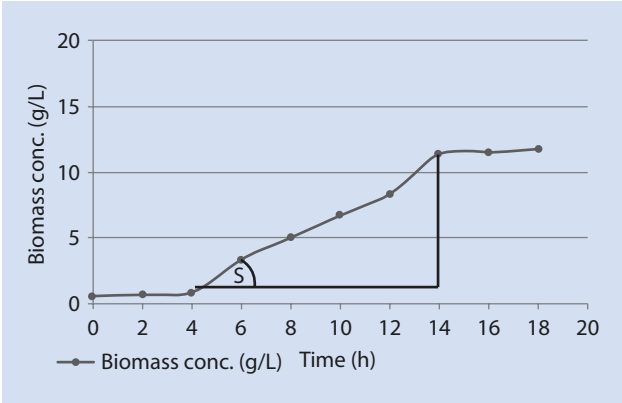


Fig. 5.16 Graph of maximum growth rate calculation

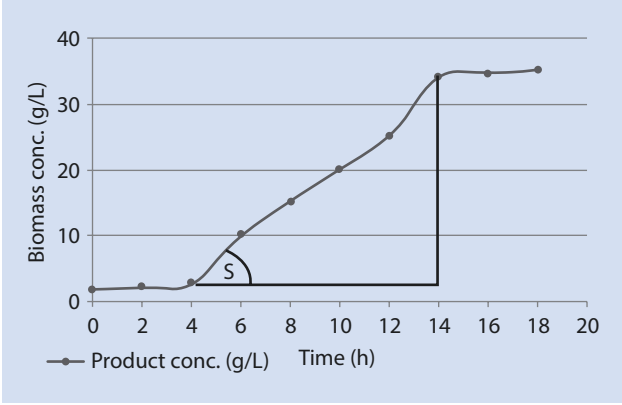


Fig. 5.17 Graph of maximum production rate calculation

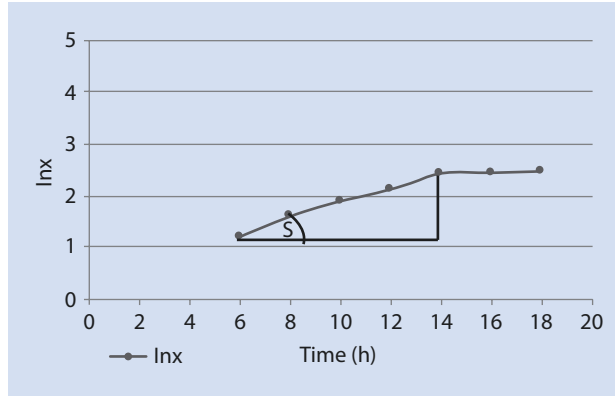


Fig. 5.18 Graph of specific growth rate calculation

Specific growth rate can also be calculated by taking the slope of the steepest part of the ln*x* graph (Fig. 5.18). For this calculation,

$$\text{Specific growth rate}\left(\mu,\text{h}^{-1}\right)=\text{the slope of the steepest part of }\ln\left(x\right)\text{graph}$$

Doubling time can be calculated using specific growth rate using the equation

$$\mu x=\frac{dx}{dt}$$

$$x=x_0\text{e}^{\mu t}$$

For doubling time, x_0 to $2x_0$

$$2x_0=x_0\text{e}^{\mu t_d}$$

$$\ln\left(2\right)=\mu t_d$$

$$t_d\left(h\right)=\frac{\ln\left(2\right)}{\mu} \tag{5.11}$$

Exercise 5.1

One of the most used strains in ethanol production is *Saccharomyces cerevisiae*. The ethanol fermentation results obtained with *Saccharomyces cerevisiae* in a stirred tank bioreactor are as follows in Table 5.1.

Calculate X, S, P, $Y_{P/S}$, $Y_{X/S}$, $Y_{P/X}$, maximum consumption rate, maximum growth rate, maximum production rate, μ, and t_d using these values.

$$\Delta X=X_{\max}-X_0$$

$$\Delta X=6.46-0.01$$

Table 5.1 Fermentation results obtained with *Saccharomyces cerevisiae* in a stirred tank bioreactor

Time (h)	Residual sugar conc. (g/L)	Ethanol conc. (g/L)	Biomass (g/L)	ln (Biomass)
0	107.94	0.06	0.01	−4.89
2	106.38	0.68	0.09	−2.47
4	103.97	6.32	0.79	−0.24
8	87.92	23.13	2.89	1.06
11	76.19	42.65	5.33	1.67
14	46.14	49.10	6.41	1.86
24	19.12	50.10	6.44	1.86
30	4.96	49.70	6.46	1.87

$$\text{Biomass}\left(\frac{\text{g}}{\text{L}}\right) = \Delta X = 6.45\,\text{g/L}$$

$$\Delta S = S_0 - S_1$$

$$\Delta S = 107.94 - 4.96$$

$$\text{Residual Sugar Conc.}\left(\frac{\text{g}}{\text{L}}\right) = \Delta S = 102.98\,\text{g/L}$$

$$\Delta P = P_1 - P_0$$

$$\Delta P = 50.10 - 0.06$$

$$\text{Ethanol Conc.}\left(\frac{\text{g}}{\text{L}}\right) = \Delta P = 50.04\,\text{g/L}$$

$$\text{Yield}\left(Y_{P/S}\right) = \frac{\Delta P}{\Delta S}$$

$$\text{Yield}\left(Y_{P/S}\right) = \frac{50.04}{102.98} = 0.49$$

$$\text{Yield}\left(Y_{X/S}\right) = \frac{\Delta X}{\Delta S}$$

$$\text{Yield}\left(Y_{X/S}\right) = \frac{6.45}{102.98} = 0.06$$

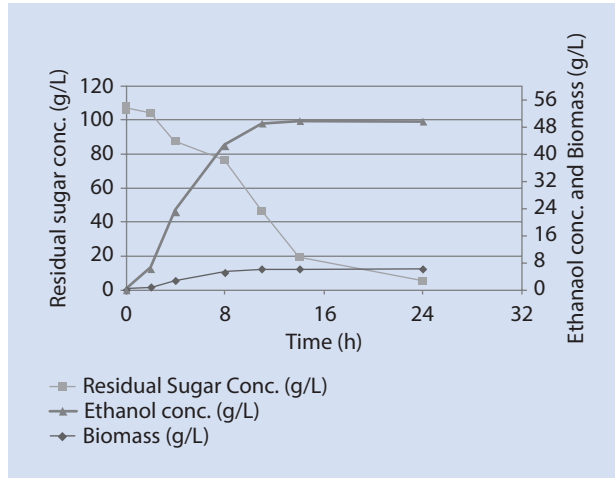


Fig. 5.19 Graphs of residual sugar, ethanol, and biomass concentration

$$\text{Yield}\left(Y_{P/X}\right) = \frac{\Delta P}{\Delta X} \times 100$$

$$\text{Yield}\left(Y_{P/X}\right) = \frac{50.04}{6.45} = 0.78$$

We have to graph the results for the consumption rate, production rate, and growth rate calculations (Fig. 5.19). The graphs are given below.

$$\text{max.cons.rate}\left(\text{g/L/h}\right) = -\left(\text{the slope of the steepest part of sugar cons.graph}\right)$$

$$\text{max.cons.rate}\left(\text{g/L/h}\right) = -\frac{S_{t_y} - S_{t_z}}{t_y - t_z}$$

For this calculation, we can use the values between fourth and 14th hours. So,

$$\text{max.cons.rate}\left(\text{g/L/h}\right) = -\frac{S_4 - S_{14}}{14 - 4}$$

$$\text{max.cons.rate}\left(\text{g/L/h}\right) = -\frac{46.14 - 103.97}{14 - 4} = 5.78\,\text{g/L/h}$$

$$\text{max.grow.rate}\left(\text{g/L/h}\right) = \left(\text{the slope of the steepest part of biomass growth graph}\right)$$

$$\text{max.growth rate}\left(\text{g/L/h}\right) = \frac{X_{t_y} - X_{t_z}}{t_y - t_z}$$

For this calculation, we can use the values between fourth and 14th hours. So,

$$\text{max.growth rate}\left(\text{g/L/h}\right) = \frac{X_{14} - X_4}{14 - 4}$$

$$\text{max.cons.rate}\left(\text{g/L/h}\right) = \frac{6.41 - 0.79}{14 - 4} = 0.56\,\text{g/L/h}$$

$$\text{max.prod.rate}\left(\text{g / L / h}\right) = \left(\text{the slope of the steepest part of production graph}\right)$$

$$\text{max.prod.rate}\left(\text{g / L / h}\right) = \frac{P_{t_y} - P_{t_z}}{t_y - t_z}$$

For this calculation, we can use the values between fourth and 14th hours. So,

$$\text{max.prod.rate}\left(\text{g / L / h}\right) = \frac{P_{14} - P_4}{14 - 4}$$

$$\text{max.prod.rate}\left(\text{g / L / h}\right) = \frac{49.10 - 6.32}{14 - 4} = 4.28\,\text{g / L / h}$$

For the specific growth rate, we have to calculate $\ln(x)$ values and graph the $\ln(x)$ values to time.

$$\text{Specific growth rate}\left(\mu, 1/\text{h}\right) = \left(\text{the slope of the steepest part of } \ln\left(x\right) \text{graph}\right)$$

$$\text{Specific growth rate}\left(1/\text{h}\right) = \frac{\ln\left(x\right)_{t_y} - \ln\left(x\right)_{t_z}}{t_y - t_z}$$

For this calculation, we can use the values between eighth and 14th hours. So,

$$\text{Specific growth rate}\left(1/\text{h}\right) = \frac{\ln\left(x\right)_{14} - \ln\left(x\right)_4}{14 - 4}$$

$$\text{Specific growth rate}\left(1/\text{h}\right) = \frac{1.86 - 1.06}{14 - 4} = 0.081/\text{h}$$

and doubling time is $t_d = \dfrac{\ln(2)}{\mu} = \dfrac{0.693}{0.08} = 8.66\,\text{h}$

Exercise 5.2

Reaching stationary phase time for *E. coli* fermentation is 17.4 h into a 30 L stirred tank bioreactor containing 50 g/L initial glucose concentration by inoculating 9 g cells. 85% of the initial sugar concentration is consumed when the fermentation is completed the log phase. If the biomass yield from glucose is 0.341 g biomass/g glucose, what is the specific growth rate value for this fermentation?

$V = 30$ L

$t = 17.4$ h

$S_0 = 50$ g/L

$S = 50\ \text{g/L} \times 0.15 = 7.5$ g/L

$Y_{X/S} = 0.341\,\text{g biomass / g glucose}$

We can use the equation: $X = X_0 + Y_{X/S}\left(S_0 - S\right)$ and $\ln\dfrac{X}{X_0} = \mu\, t.$

First, we need to calculate X (g/L):

$$X = \frac{9\,\text{g}}{30\,\text{L}} + 0.341\left(50 - 7.5\right) = 0.3 + 14.49 = 17.49\,\text{g / L}$$

$$\ln\frac{X}{X_0}=\mu t;\ln\frac{17.49}{0.3}=\mu 17.4;\ \mu=0.23\,\mathrm{h}^{-1}$$

5.5.2 Continuous Fermentation

Continuous fermentation is an open system in which the growth of microorganisms is tried to be kept in the logarithmic phase by feeding the medium containing the nutrients in the same ratio to the used medium [46]. Continuous fermentation is preferred because the growth of the microorganism in the logarithmic phase shifts to the stationary phase after a few batch fermentations which does not involve the addition of any nutrients or metabolites (Tunail et al. 2009). Two main factors in the transition of microorganism to the stationary phase are (i) reduction in the amount of substrate available in the medium and (ii) accumulation of metabolites in the medium. The microorganism may not reach to the stationary phase at continuous fermentation if the medium is maintained by feeding the fresh fermentation medium continuously to control the substrate concentration and taking the fermented liquid from the medium continuously to control the metabolite accumulation. The typical microbial growth for continuous fermentation is shown in Fig. 5.20.

The most important thing for the continuous fermentation is to determine the optimum dilution rate to prevent cell leakage. It means that the cells that develop in the logarithmic phase at a certain speed in the vessel must be equal to the cells (cell loss) to be removed in the culture medium used in the vessel [53].

Kinetics in Continuous Culture

The kinetic parameters can be calculated by the following equations for continuous fermentation (if X_o and P_o is equal to 0 at steady state):

$$\Delta X = X_o - X_e \tag{5.12}$$

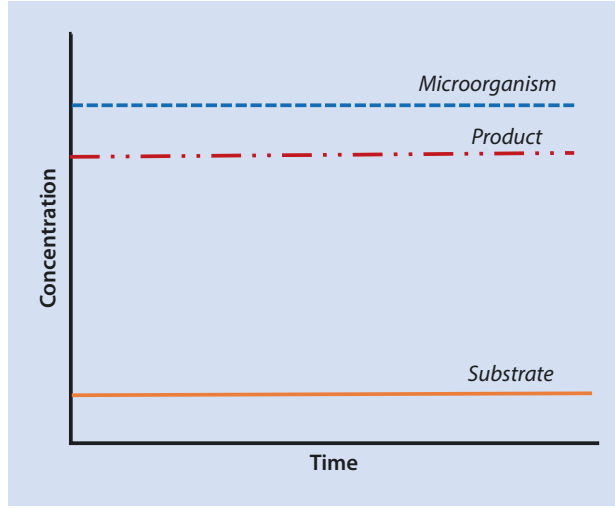


Fig. 5.20 Microbial growth, substrate consumption, and product production for continuous fermentation

ΔX : Total amount of the biomass (g/L)

X_o : Feed biomass concentration (g/L)

X_e : Effluent biomass concentration (g/L)

$$\text{or} \quad X = Y_{X/S}\left(S_0 - S\right) \tag{5.13}$$

or biomass population can be calculated by

$$\frac{dx}{dt} = \text{in} - \text{out} + \text{accumulation} - \text{consumption}$$

$$\frac{dx}{dt} = X_0 \frac{F}{V} - X\frac{F}{V} + \left(\frac{dx}{dt}\right)_A - \left(\frac{dx}{dt}\right)_C \tag{5.14}$$

$$X_0 \frac{F}{V} \text{ and } \left(\frac{dx}{dt}\right)_C \text{ is equal to } 0\left(\text{zero}\right)$$

$$\frac{dx}{dt} = \left(\frac{dx}{dt}\right)_A - X\frac{F}{V}$$

We know that $\left(\frac{dx}{dt}\right)_A = \mu X$ and $\frac{F}{V} = D\left(\text{Dilution rate}, \text{h}^{-1}\right)$;

$$\frac{dx}{dt} = \mu X - X D = 0 \text{ at steady state}$$

Then $\mu X = X D$; $\mu = D$ (For one stage continuous fermentation) (Fig. 5.21).

Residual sugar mass balance is calculated by

$$\Delta S = S_o - S_e \tag{5.15}$$

ΔS : Total amount of the sugar utilized (g/L)

S_o : Feed sugar concentration (g/L)

S_e : Effluent sugar concentration (g/L)

$$\text{or} \quad S = S_0 - X\frac{1}{Y_{X/S}} \tag{5.16}$$

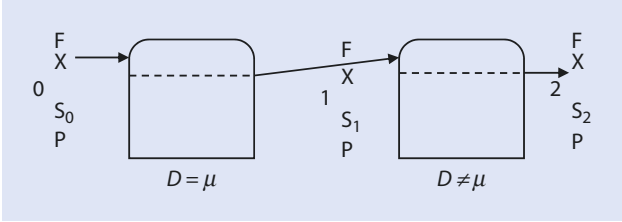


Fig. 5.21 Multistage continuous fermentation (For second-stage fermentation $D \neq \mu$ because X_1 is not equal to zero)

Product mass balance is calculated by

$$\Delta P = P_o - P_e \tag{5.17}$$

ΔP : Total amount of the product produced (g/L)
P_o : Feed product concentration (g/L)
P_e : Effluent product concentration (g/L)

$$\text{or } P = Y_{P/X} X \tag{5.18}$$

5

Productivity is calculated by

$$\text{Productivity}\left(\text{g/L/h}\right) = D X \tag{5.19}$$

Product productivity is calculated by

$$\text{Product productivity}\left(\text{g/L/h}\right) = D P \tag{5.20}$$

Hydraulic residence time (HRT, h) is calculated by

$$\text{HRT}\left(\text{h}\right) = \frac{1}{D} \tag{5.21}$$

Optimum dilution rate is calculated by

$$\frac{d\left(D_x\right)}{dD} = 0 = \frac{d}{dD}\left[DY_{X/S}\left(S_0 - S\right)\right]$$

$$S = \frac{DK_s}{\mu_m - D}$$

$$D_{opt}\left(\text{h}^{-1}\right) = \mu_m\left(1 - \sqrt{\left(\frac{K_s}{K_s + S_0}\right)}\right) \tag{5.22}$$

Optimum X is calculated by

$$X = Y_{X/S}\left(S_0 - S\right) = Y_{X/S}\left(S_0 - \frac{DK_s}{\mu_m - D}\right)$$

$$X_{opt}\left(\text{g/L}\right) = Y_{X/S}\left[S_0 + K_s - \left(K_s^2 + K_s S_0\right)^{1/2}\right] \tag{5.23}$$

Exercise 5.3

Continuous fermentation in a 100 L stirred tank bioreactor system is used to produce lactic acid from whey. The microbial system follows a Monod relationship with the kinetic parameters as below:

$Y_{X/S}$ = 0.36 g biomass/g lactose
Feed rate = 15 L/h
$\text{Feed}_{\text{lactose concentration}}$ = 40 g/L

Effluent$_{\text{lactose concentration}}$ = 3 g/L
Please calculate the biomass production rate in g/L/h at steady state.

We can use the equation: $X = Y_{X/S}\left(S_0 - S\right)$ and Productivity (g/L/h) = $D\,X$

First, we need to calculate X (g/L):

$$X = 0.36\ \text{g biomass / g lactose} \times \left[(40-3)\ \text{g lactose / L}\right]$$

$$X\left(\text{g / L}\right) = 13.32\ \text{g biomass / L}$$

$$D = \frac{F}{V} = \frac{15\ \text{L / h}}{100\ \text{L}} = 0.15\ \text{h}^{-1}$$

$$\text{Biomass productivity}\left(\text{g / L / h}\right) = 13.32 \times 0.15 = 1.998\ \text{g / L / h}$$

5.5.3 Fed-Batch Fermentation

Fed-batch fermentation is a system that includes both batch and continuous fermentation techniques. Batch and continuous systems are not suitable for products which are produced dependent on microorganism growth. It is aimed to primarily provide high cell density environment in the process using fed-batch systems. The precursors, carbon sources, and oxygen are added to fermentation medium to protect cells and produce the product. Production is continued by adding specific level of the components. It means that the semi-cut fermentation consists of two phases: development and production phases [46]. Simple fed-batch control system is shown in Fig. 5.22.

Fed-batch fermentation systems are formed by simple modification of the batch systems. It is also superior to continuous systems by having a lower risk of contamination. Fed-batch fermentation systems are preferred in situations where the amount of substrate inhibits cell growth and where product or cell yield is higher at low substrate concentrations (e.g., antibiotic production) [46]. Penicillin, produced as a secondary metabolite, is one of the most successful examples of fed-batch fermentation techniques. Penicillin production is a two-stage process. The first stage is the process called "rapid growth phase" to promote cell growth. The second stage is called "slow growth phase" or "production phase" where the goal is to produce penicillin, the secondary metabolite, with a substrate concentration that minimizes the death of the cell [49]. In the production of many enzymes, rapid usage of carbon source prevents enzyme synthesis due to catabolic depression. The simplest and most effective way to adjust the carbon source concentration is the use of the fed-batch fermentation technique, which successfully performed for lipase and cellulase production [49].

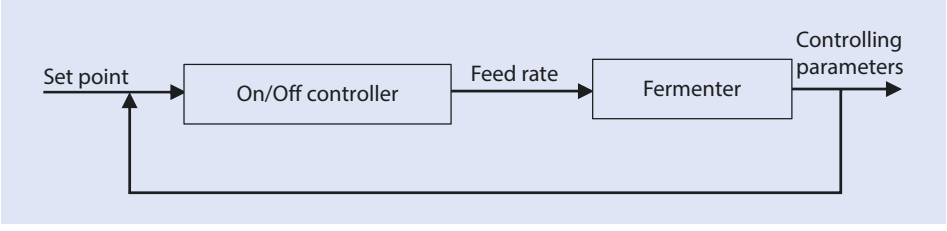


Fig. 5.22 Fed-batch basic control system [27]

Kinetics in Fed-Batch Fermentation

Biomass population for fed-batch culture is calculated by

$$X^t = X_0^t + F\,t\,Y_{X/S}\,S_0 \tag{5.24}$$

X^t : Total Biomass concentration (g dry weight/L)
F : Feed (ml/h)
t : time (h)

Volume for fed-batch culture is calculated by

$$V = V_0 + (Ft) \tag{5.25}$$

V : Total volume at specific time (L)
V_0 : Initial volume (L)

Biomass concentration per liter for fed-batch culture is calculated by

$$\left[X^t\right] = \frac{X^t}{V} \tag{5.26}$$

$[X^t]$: Biomass concentration per liter (g/L)

Biomass concentration per liter for fed-batch culture is calculated by

$$P = \frac{P_0 V_0}{V} + q_p X_m \left(\frac{V_0}{V} + \frac{Dt}{2}\right) t \tag{5.27}$$

q_p : Specific rate of product formation (g/gh)
X_m : Maximum biomass concentration (g/L)

Exercise 5.4 (Data is Generated from Lab Research Results in Turkey)

Mannanase enzyme is produced by *Aspergillus sojae* in fed-batch culture with addition of carob pod extract to medium in laboratory conditions. Initial working volume in steady state is 600 ml, and medium is added with a flow rate of 100 ml/h. Total sugar concentration in feed solution and initial cell concentration are 200 g/L and 8 g/L, respectively.
The kinetic parameters of microorganisms are

$$\mu_m = 0.289\ \mathrm{h}^{-1}, K_s = 0.45\ \mathrm{g/L}, Y_{X/S} = 0.5\ \mathrm{g\ dry\ weight\ /\ g\ total\ sugar.}$$

1. What is the culture volume in bioreactor at *t*:20 h?
2. What is the total sugar concentration at *t*:20 h at steady state?
3. What is the concentration of cells at steady state when *t*:20 h?
4. What is the total cell (maximum) at steady state when *t*:20 h?
5. What is the product concentration in bioreactor at *t*:20 h? (Please consider q_p = 0.02 g product/g cells h, and P_0 = 0.05 g/L)

$$V_0 = 600\ \mathrm{ml} = 0.6\ \mathrm{L}; F = 100\ \mathrm{ml/h} = 0.1\ \mathrm{L/h}; S_0 = 200\ \mathrm{ml}; X_0 = 8\ \mathrm{g/L}$$

1. $V = V_0 + (Ft)$

$$V = 0.6\,\text{L} + \left(0.1\,\text{L/h} \times 20\,\text{h}\right) = 2.6\,\text{L}$$

2. $D = \dfrac{F}{V}$

$$D = \frac{0.1\,\text{L/h}}{2.6\,\text{L}} = 0.038\,\text{h}^{-1}$$

$$D = \mu \qquad S = \frac{K_s \times D}{\mu_m - D} = \frac{0.45\,\text{g/L} \times 0.038\,\text{h}^{-1}}{0.289\,\text{h}^{-1} - 0.038\,\text{h}^{-1}} \qquad S = 0.068\,\text{g/L}$$

3. $X^t = (X_0\,V_0) + (F\,Y_{X/S}S_0\,t)$

$$X^t = \left(8\,\text{g/L} \times 0.6\,\text{L}\right) + \left(0.1\,\text{h}^{-1} \times 0.5\,\text{g/g} \times 200\,\text{g/L} \times 20\,\text{h}\right)$$

$$X^t = 46.8\,\text{g} + 200\,\text{g} = 246.8\,\text{g}$$

4. $\left[X^t\right] = \dfrac{X^t}{V} \left[X^t\right] = \dfrac{246.8\,\text{g}}{2.6\,\text{L}} = 94.92\,\text{g/L}$

5. $P = \dfrac{P_0 V_0}{V} + q_p X_m \left(\dfrac{V_0}{V} + \dfrac{Dt}{2}\right) t$

$$P = \left(\frac{0.05\,\text{g/L} \times 0.6\,\text{L}}{2.6\,\text{L}}\right) + \left(0.02\,\text{g/gh} \times 94.92\,\text{g/L} \times \left(\frac{0.6\,\text{L}}{2.6\,\text{L}} + \frac{0.038\,\text{h}^{-1} \times 20\,\text{h}}{2}\right)\right)$$

$$P = 23.20\,\text{g/L}$$

5.6 Conclusion

Liquid state fermentation is a process for microbial production of value-added products. Different bioreactor systems are investigated in laboratories, and many experiments are carried out to find the best bioreactor systems for microbial strains. The most common bioreactor systems are stirred tank bioreactors, airlift bioreactors, bubble column bioreactors, fluidized bed bioreactors, packed bed bioreactors, photobioreactors, membrane bioreactors, and microcarrier bioreactors. Other bioreactor systems are innovative bioreactor systems which are developed for special fermentation strategies such as biofilm, microparticle usage, shifting strategy, etc. The appropriate bioreactor systems let more efficient fermentation processes. So, the best fermentation strategy and bioreactors system is the most important parameters for large-scale operations.

Take Home Messages

- Processes to operate LSB systems include the growth and preservation of strain, inoculum preparation, media optimization, and choosing the right fermenter and operation strategy.
- LSB systems can be modified for any type of microorganisms to control the parameters such as temperature, agitation, pH, aeration, foam, etc. to provide high productivity.
- The efficiency of a bioreactor's performance depends on the concentration of biomass, aseptic conditions, mass and heat transfer efficiency, and operation under optimum processing conditions.
- All kinetic parameters such as biomass, product, yield, production rate, consumption rate, growth rate, specific growth rate, and doubling time should be calculated for providing optimum conditions in fermentation process.
- Feeding strategy in fermentation is one of the most useful parameters to produce high yield final products by bacteria, yeast, fungi, or algae. Due to all these requirements, batch, fed-batch, and continuous fermentation techniques are widely used in industry.
- The most common bioreactor systems are stirred tank bioreactors, airlift bioreactors, bubble column bioreactors, fluidized bed bioreactors, packed bed bioreactors, photobioreactors, membrane bioreactors, and microcarrier bioreactors.
- Other bioreactor systems are innovative bioreactor systems which are developed for special fermentation strategies such as biofilm, microparticle usage, and shifting strategy.

References

1. Asadi A, Zinatizadeh AA, van Loosdrecht M. Effects of operational models (batch, continuous and CFID modes) on the performance of a single A_2O airlift bioreactor for treatment of milk processing wastewater. Chem Eng Res Des. 2017;125:471–82.
2. Aslam M, McCarty PL, Shin C, Bae J, Kim J. Low energy single-staged anaerobic fluidized bed ceramic membrane bioreactor (AFCMBR) for wastewater treatment. Bioresour Technol. 2017;240:33–41.
3. Badenes SM, Fernandes TG, Rodrigues CAV, Diogo MM, Cabral JMS. Microcarrier-based platforms for in vitro expansion and differentiation of human pluripotent stem cells in bioreactor culture. J Biotechnol. 2016;234:71–82.
4. Banerjee A, Ghoshal AK. Biodegradation of real petroleum wastewater by immobilized hyper phenol-tolerant strains of *Bacillus cereus* in a fluidized bed bioreactor. 3 Biotech. 2016;6:137.
5. Campani G, Gonçalves da Silva G, Zangirolami TC, Perencin de Arruda Ribeiro M. Recombinant *Escherichia coli* cultivation in a pressurized airlift bioreactor: assessment of the influence of temperature on oxygen transfer and uptake rates. Bioprocess Biosyst Eng. 2017;40:1621–33.
6. Chan Z, Zhong T, Yi Z, Xiao J, Zeng R. A pH shift feeding strategy for increased enduracidin production during fed–batch fermentation by a deep–sea, bacterium, *Streptomyces* sp. MC079. Biotechnol Bioprocess E. 2015;20:908–14.
7. Chen H-C, Hu Y-C. Bioreactors for tissue engineering. Biotechnol Lett. 2006;28:1415–23.
8. Demming S, Vila-Planas J, Zadeh SA, Edlich A, Franco-Lara E, Radespiel R, Büttgenbach S, Llobera A. Poly(dimethylsiloxane) photonic microbioreactors based on segmented waveguides for local absorbance measurement. Electrophoresis. 2011;32:431–9.
9. Demming S, Peterat G, Llobera A, Schmolke H, Bruns A, Kohlstedt M, Al-Halhouli A, Klages C-P, Krull R, Büttgenbach S. Vertical microbubble column – a photonic lab-on-chip for cultivation and online analysis of yeast cell cultures. Biomicrofluidics. 2012;6:034106.
10. Dermenoudis S, Missirlis YF. Bioreactors in tissue engineering. Adv Eng Mater. 2010;12(11):592–608.

11. Driouch H, Sommer B, Wittman C. Morphology engineering of *Aspergillus niger* for improved enzyme production. Biotechnol Bioeng. 2010;109(2):462–71.
12. Driouch H, Hänsch R, Wucherpfennig T, Krull R, Wittman C. Improved enzyme production by biopellets of *Aspergillus niger*: targeted morphology engineering using titanate microparticles. Biotechnol Bioeng. 2012;105(6):1058–68.
13. Edlich A, Magdanz V, Rasch D, Demming S, Zadeh SA, Segura R, Radespiel R, Büttgenbach S, Franco-Lara E, Krull R. Microfluidic reactor for continuous cultivation of *Saccharomyces cerevisiae*. Biotechnol Prog. 2010;26:1259–70.
14. Ercan D, Demirci A. Current and future trends for biofilm reactors for fermentation processes. Crit Rev Biotechnol. 2015;35(1):1–14.
15. Fan R, Ebrahimi M, Czermak P. Anaerobic membrane bioreactor for continuous lactic acid fermentation. Membranes. 2017;7:26.
16. Geed SR, Kureel MK, Giri BS, Singh RS, Rai BN. Performance evaluation of Malathion biodegradation in batch and continuous packed bed bioreactor (PBBR). Bioresour Technol. 2017;227:56–65.
17. Gmati D, Chen J, Jolicoeur M. Development of a small-scale bioreactor: application to in vivo NMR measurement. Biotechnol Bioeng. 2005;89(2):138–47.
18. Gogate PR, Beenackers AACM, Pandit AB. Multiple-impeller systems with a special emphasis on bioreactors: a critical review. Biochem Eng J. 2000;6:109–44.
19. Hatzinger PB, Lewis C, Webster TS. Biological treatment of N-nitrosodimethylamine (NDMA) and N-nitrodimethylamine (NTDMA) in a field-scale fluidized bed bioreactor. Water Res. 2017;126:361–71.
20. Hölker U, Höfer M, Lenz J. Biotechnological advantages of laboratory-scale solid-state fermentation with fungi. Appl Microbiol Biotechnol. 2004;64:175–86.
21. Hülsen T, Barry EM, Lu Y, Puyol D, Keller J, Batstone DJ. Domestic wastewater treatment with purple phototrophic bacteria using a novel continuous photo anaerobic membrane bioreactor. Water Res. 2016;100:486–95.
22. Jamshidi N, Mostoufi N. Measurement of bubble size distribution in activated sludge bubble column bioreactor. Biochem Eng J. 2017;125:212–20.
23. Jones SMJ, Brighton MB, Harrison STL. Exploring the tension between energy consumption, light provision and CO_2 mass transfer through varying gas velocity in the airlift bioreactor. Algal Res. 2016;19:381–90.
24. Karimi A, Golbabaei F, Mehrnia MR, Neghab M, Mohammad K, Nikpey A, Pourmand MR. Oxygen mass transfer in a stirred tank bioreactor using different impeller configurations for environmental purposes. Iranian J Environ Health Sci Eng. 2013;10:6.
25. Kaup BA, Ehrich K, Pescheck M, Schrader J. Microparticle-enhanced cultivation of filamentous microorganisms: increased chloroperoxidase formation by *Caldariomyces fumago* as an example. Biotechnol Bioeng. 2008;99(3):491–8.
26. Kumar RN, Vinod AV. Oxygen mass transfer in bubble column bioreactor. Chem Eng. 2014;58(1):21–30.
27. Lee J, Lee SY, Park S, Middelberg APJ. Control of fed-batch fermentations. Biotechnol Adv. 1999;17:29–48.
28. Longanesi L, Frascari D, Spagni C, DeWever H, Pinelli D. Succinic acid production from cheese whey by biofilms of *Actinobacillus succinogenes*: packed bed bioreactor tests. J Chem Technol Biotechnol. 2018;93:246–56.
29. Lu Y, Yu J. Gas mass transfer with microbial CO_2 fixation and poly(3-hydroxybutyrate) synthesis in a packed bed bioreactor. Biochem Eng J. 2017;122:13–21.
30. Madigan MT, Martinko JM. Brock biology of microorganisms. Upper Saddle River: Prentice Hall PTR; 2006.
31. Mahboubi A, Ylitervo P, Doyen W, De Wever H, Molenberghs B, Taherzadeh MJ. Continuous bioethanol fermentation from wheat straw hydrolysate with high suspended solid content using an immersed flat sheet membrane bioreactor. Bioresour Technol. 2017;241:296–308.
32. Majors PD, McLean JS, Scholten JCM. NMR bioreactor development for live in-situ microbial functional analysis. J Magn Reson. 2008;192:159–66.
33. Martin I, Wendt D, Heberer M. The role of bioreactors in tissue engineering. Trends Biotechnol. 2004;22(2):80–6.
34. McClure DD, Kavanagh JM, Fletcher DF, Barton GW. Characterizing bubble column bioreactor performance using computational fluid dynamics. Chem Eng Sci. 2016;144:58–74.
35. Mears L, Stocks SM, Sin G, Gernaey KV. A review of control strategies for manipulating the feed rate in fed-batch fermentation processes. J Biotechnol. 2017;245:34–46.
36. Moreno-Medina CU, Poggi-Varaldo HM, Breton-Deval L, Rinderknecht-Seijas N. Effect of sudden addition of PCE and bioreactor coupling to ZVI filters on performance of fluidized bed bioreactors operated in simultaneous electron acceptor modes. Environ Sci Pollut Res. 2017;24:25534–49.

37. Nielsen J, Villadsen J. Bioreactors: description and modelling. In: Stephanopulos G, editor. Biotechnology, 2nd completely, vol. 3. Revised ed. New York: VCH; 1993. p. 77–104.
38. Oliveira F, Salgado JM, Abrunhosa L, Pérez-Rodríguez N, Domínguez JM, Venâncio A, Belo I. Optimization of lipase production by solid-state fermentation of olive pomace: from flask to laboratory-scale packed-bed bioreactor. Bioprocess Biosyst Eng. 2017;40:1123–32.
39. Patel BP, Kumar A. Biodegradation of 4-chlorophenol in an airlift inner loop bioreactor with mixed consortium: effect of HRT, loading rate and biogenic substrate. 3 Biotech. 2016;6:117.
40. Rahnama F, Vasheghani-Farahani E, Yazdian F, Shojaosadati SA. PHB production by *Methylocystis hirsuta* from natural gas in a bubble column and a vertical loop bioreactor. Biochem Eng J. 2012;65:51–6.
41. Rasoulnia P, Mousavi SM. Maximization of organic acids production by *Aspergillus niger* in a bubble column bioreactor for V and Ni recovery enhancement from power plant residual ash in spent-medium bioleaching experiments. Bioresour Technol. 2016;216:729–36.
42. Rocha AA, Wilde C, Hu Z, Nepotchatykh O, Nazarenko Y, Ariya PA. Development of a hybrid photo-bioreactor and nanoparticle adsorbent system for the removal of CO_2, and selected organic and metal co-pollutants. J Environ Sci. 2017;57:41–53.
43. Rossi MJ, Nascimento FX, Giachini AJ, Oliveira VL, Furigo A Jr. Transfer and consumption of oxygen during the cultivation of the extomycorrhizal fungus *Rhizopogon nigrescens* in an airlift bioreactor. Appl Microbiol Biotechnol. 2017;101:1013–24.
44. Russel PD. Fermenter and bioreactor design. In: King RD, Cheetham PSJ, editors. Food biotechnology-1. New York: Elsevier; 1987. p. 1–48.
45. Seo I, Lee I, Hwang H, Hong S, Bitog JP, Kwon K, Lee C, Kim Z, Cuello JL. Numerical investigation of a bubble-column photo-bioreactor design for microalgae cultivation. Biosyst Eng. 2012;113:229–41.
46. Shetty K, Paliyath G, Pometto A, Levin RE. Food biotechnology second edition. Boca Raton: CRC Press; 2006.
47. Skoneczny S, Stryjewski W, Bizon K, Tabiś B. Three-phase fluidized bed bioreactor modelling and stimulation. Biochem Eng J. 2017;121:118–30.
48. Smith JE. Biotechnology. 4th ed. New York: Cambridge University Press; 2004.
49. Stanbury PF, Whitaker A, Hall SJ. Principles of fermentation technology. 2nd ed. Burlington: Butterworth-Heinemann; 2003.
50. Sur DH, Mukhopadhyay M. Process aspects of three-phase inverse fluidized bed bioreactor: a review. J Environ Chem Eng. 2017a;5:3518–28.
51. Sur DH, Mukhopadhyay M. COD reduction of textile effluent in three-phase fluidized bed bioreactor using *Pseudomonas aureofaciens* and *Escherichia coli*. 3 Biotech. 2017b;7:141.
52. Tang Y-J, Zhang W, Zhong J-J. Performance analyses of a pH-shift and DOT-shift integrated fed-batch fermentation process for the production of ganoderic acid and *Ganoderma* polysaccharides by medicinal mushroom *Ganoderma lucidum*. Bioresour Technol. 2009;1000:1852–9.
53. Tunai N. Mikrobiyoloji. Ankara: Pelin Ofset; 2009. p. 448.
54. Velugula-Yellela SR, Williams A, Trunfio N, Hsu C-J, Chavez B, Yoon S, Agarabi C. Impact of media and antifoam selection on monoclonal antibody production and quality using a high throughput micro-bioreactor system. Biotechnol Prog. 2018;34(1):262–70.
55. Yao P, Xiao Z, Chen C, Li W, Deng Q. Cell growth of *Clostridium acetobutylicum* in a pervaporation membrane bioreactor for butanol fermentation. Biotechnol Appl Biochem. 2016;63(1):101–5.
56. Yatmaz E, Karahalil E, Germec M, Ilgin M, Turhan I. Controlling filamentous fungi morphology with microparticles to enhanced b-mannanase production. Bioprocess Biosyst Eng. 2016;39:1391–9.
57. Xia JY, Wang YH, Zhang SL, Chen N, Yin P, Zhuang YP, Chu J. Fluid dynamics investigation of variant impeller combinations by simulation and fermentation experiment. Biochem Eng J. 2009;43:252–60.
58. Zambrano J, Nehrenheim E. Light and duty cycle optimization of a photo-bioreactor in batch mode. Energy Procedia. 2017;105:773–9.
59. Zhang Z, Zhou X, Hu J, Zhang T, Zhu S, Zhang Q. Photo-bioreactor structure and light-heat-mass transfer properties in photo-fermentative bio-hydrogen production system: a mini review. Int J Hydrog Energy. 2017;42:12143–52.
60. Zhu X, Li M, Zheng W, Liu R, Chen L. Performance and microbial community of a membrane bioreactor system — treating wastewater from ethanol fermentation of food waste. J Environ Sci. 2017;53:284–92.

Bioreactors Operating Conditions

Leonardo Machado da Rosa, Daniela Maria Koerich, and Saulo Varela Della Giustina

A. Berenjian (ed.), *Essentials in Fermentation Technology*, Learning Materials in Biosciences,
https://doi.org/10.1007/978-3-030-16230-6_6

What You Will Learn in This Chapter

Fermentation is a delicate biological process, which is affected by several factors. Among these, engineers should know how physico-chemical factors such as the presence of hydrodynamic stresses, temperature, pH, concentration of microbes and chemical species (oxygen, salt, alcohol) affect the operation. The production of primary or secondary metabolites can also inhibit the biosynthesis of value-added products. Another important aspect is the oxygen transfer, as well as the contact between microorganisms and substrate. They can be enhanced by employing agitation. However, intense agitation may also promote cell damage. The specificities of each process can lead to the adoption of a particular operation mode as well as the use of different configurations of bioreactors. Regardless of the operation mode, fermentative processes require extra-operational considerations such as pretreatment of substrate, sterilization, media preparation and separation of inhibitors. Hence, engineers should also focus on control strategies to be employed in order to keep operational conditions as close to optimal conditions as possible.

6.1 Introduction

Wine production was one of the first areas where fermentation was used. At that time, the term fermentation was related to the conversion of sugar into alcohol and carbon dioxide. Later, the word was related to microorganisms, due to the demonstration of the role of yeasts by Pasteur, and in the following decades became more related to enzymes [39]. The term fermentation is associated with the metabolic process in which an organic substrate undergoes chemical changes due to the activities of enzymes secreted by microorganisms [33]. Nowadays, fermenters are widely used in the most diverse processes, as in the conversion of biomass to biofuels such as ethanol [5, 41], biohydrogen [34, 35], and butanol [18, 25], food additives and supplements [40], animal nutrition [17], industrial enzymes [48], pharmaceutical products [30], and chemicals [6], as well as to treat [59] or provide valorization of industrial waste [24].

The success of fermentation processes depends crucially on the operational conditions. Unlike most chemical processes, in which concentration, temperature, and pressure are controlled basically to guarantee a certain efficiency and/or reaction rate, in biological systems, microorganisms are part of the process. Since they are living beings, each one of them can respond in a particular way to a certain condition. However, the behavior of a set of microorganisms can be predicted with some accuracy. Even so, because they are living organisms, extreme operating conditions lead not only to process inefficiency but also to biomass death and process failure.

In addition, unlike other biological processes (such as those adopted in the wastewater treatment), in which the objective is the simple biodegradation of chemical residues, in general the objective of fermentation is the biosynthesis of value-added products. Thus, operational requirements are more restricted and may involve extra-operational considerations such as pretreatment of substrate, sterilization, media preparation, and separation of inhibitors.

This chapter presents the main parameters that have to be observed in fermentative processes.

6.2 Factors Affecting the Design and Operation of Bioreactors

Numerous factors influence the growth and metabolism of microorganisms. These can be used to control the progress of the fermentation, as well as promoting the growth of certain types of microorganisms over the others in the medium. The design of a bioreactor should take into account several physicochemical conditions: agitation and aeration, rheology, hydrodynamic stresses, temperature, biochemical kinetics, form of feeding, pH, concentration of microbes, and chemical species (oxygen, salt, alcohol). Two fermentation techniques have emerged as a result of the continuous refinement of these conditions. In the submerged fermentation (SmF), liquid substrates flow free and the bioactive compounds are secreted in the fermentation broth. This fermentation technique is best suited for microorganisms that require high moisture content. SmF is also used in the extraction of secondary metabolites in liquid form. On the other hand, solid substrates are utilized in the solid-state fermentation (SSF). Solid substrates are consumed very slowly and steadily, enabling the use of the same media for long fermentation periods. SSF is suitable for fermentation involving microorganisms that require less moisture content. Nonetheless, water activity is an important factor for solid-state fermentation.

The cellular physiology, mechanisms of control in cell metabolism and growth phase in which the products of interest are formed must also be taken into account [11]. These conditions are detailed below.

6.2.1 Agitation and Aeration

In biological processes, mixing can be promoted by pneumatic, hydraulic, or mechanical agitation. Figure 6.1 illustrates the different types of agitation commonly used. Agitation of the fermenting broth is important to provide homogeneity during the fermentation period, blend soluble compounds of medium, prevent aggregate formation, promote gas transfer (both increasing the gas residence time and breaking nearby bubbles into smaller sizes), enhance heat exchange, and distribute spore inoculum homogeneously.

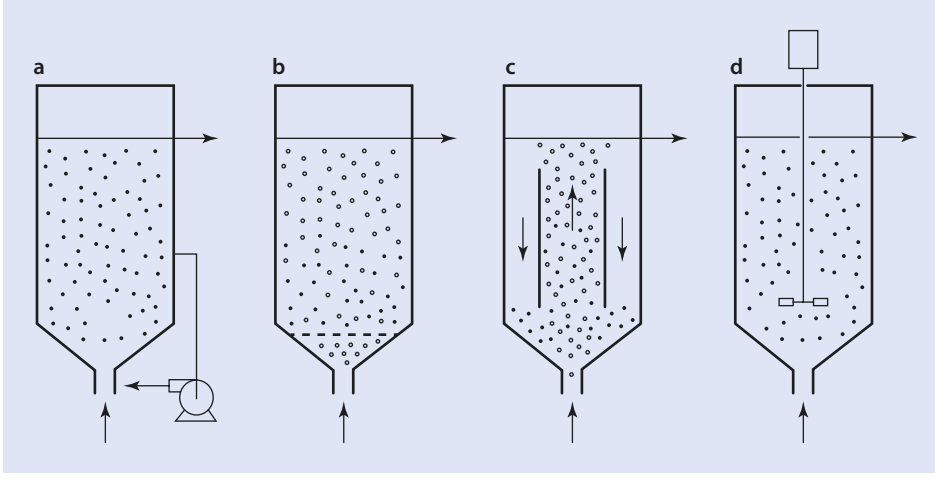


Fig. 6.1 Types of agitation in fermenters: **a** hydraulic recirculation, pneumatic in **b** bubble column and **c** loop configurations, and **d** mechanical stirring

In the hydraulic agitation, mixing is promoted by substrate circulation. It may provide greater or lesser mixture intensity, depending on the ratio between flow rate and volume (which corresponds to the residence time of the fermenter).

When the agitation is mainly promoted by the injection of gas bubbles, there is pneumatic agitation. In several configurations, the flow is separated in an ascending region (riser), whose movement is enhanced by bubbles moving upward, and a descending region (downcomer), in which substrate is recirculated inside the fermenter. These regions form a loop-type (air-lift or gas-lift) system. Air-agitated fermenters are also a viable alternative to mechanically agitated systems [13]. The advantages are the following [52]:

- Improved sterility due to the absence of an entering agitator shaft.
- Very large fermenters are not limited by motor size, shaft length, and its weight.
- Energy requirements are reduced 20–35% (which would be required for mechanical agitation).
- Less structural steel is used, resulting in cheaper fermenter design.
- No maintenance of motors, gear boxes, bearings, or seals.
- Provide a variable mixing power unit.
- Air compressors can be steam-driven to reduce power consumption and to continue operating during power outages in large plants that have minimal power generation for controls.

However, air agitation has the following disadvantages:

- Greater air throughput and higher pressures are needed.
- Inefficient breaking of the foam when foaming occurs.
- Bigger bubble diameter due to the absence of blades that could reduce their size, resulting by consequence the aeration efficiency.
- k_{La} obtained in air-lift reactor will be less than in bubble fermenter due to shorter contact time between bubble and medium.

Mechanical agitation is still the most efficient mixing method in terms of mixing intensity per consumed power unit. It is widely used in wastewater treatment plants because it promotes the necessary contact between the organic material and the microorganisms. A rotating mixer impeller generates a flow velocity profile along the length of its blades, which can be nearly uniform or variable. The flow direction will therefore be either predominantly axial or radial (i.e., parallel or perpendicular to the axis of rotation) depending its geometry. The most commonly used impeller designs are illustrated in Fig. 6.2.

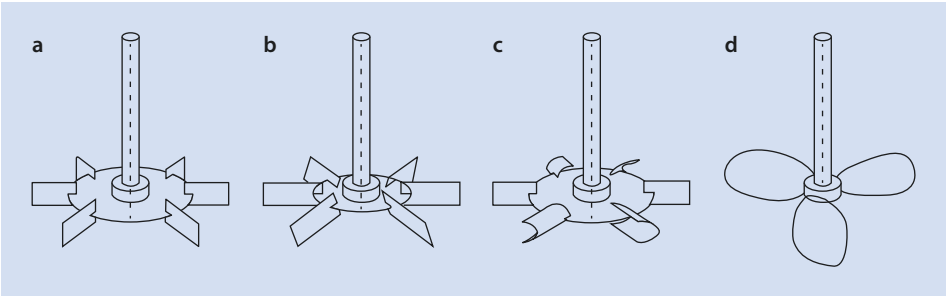


Fig. 6.2 Commonly used stirring devices: **a** Rushton, **b** pitched blade, **c** concave blades, and **d** marine impeller

Radial flow impellers, such as Rushton turbines, impose shear stress to the fluid, and are used, for example, to mix immiscible liquids and to mix very viscous fluids. Conversely, axial flow impellers (pitched blades and marine impellers) impose bulk motion and are used on homogenization processes, in which increased fluid volumetric flow rate is important. Concave blade impellers (Smith impellers) provide higher mass transfer factor (k_{La}) in applications with high energy agitation and high gas rates.

The design of the impeller is based on the mass transfer across the liquid-solid slurry boundary, possible effects of fluid shear on the organisms, and overall blending or bulk mixing of nutrients in the tank. Some fermentation processes require a combination of impellers; thus experimentation with various geometries, number, agitator speed, etc. should be conducted both to optimize mixing and process results and also to obtain initial scale-up parameters. Furthermore, magnetic-driven stirrers may be used when the risk of contamination is high.

Impellers are characterized by their power number, pumping number, shear level, and flow patterns. All the power applied to the mixing system produces a circulating capacity (Q) and a velocity head (H). Head results in shear, which is dissipated by turbulence. The circulating capacity is given by:

$$Q = N_Q N D^3$$

where N_Q is the pumping number, which depends on the impeller type, the impeller diameter to the vessel diameter ratio (D/T), and impeller Reynolds number, defined as

$$\mathrm{Re} = \rho N D^2 / \mu.$$

N_Q values for the commonly used impellers under turbulent conditions are given in Table 6.1.

The power consumed by a mixer can be obtained by multiplying pumping (Q) and head (H) and is given by

$$P = N_p \rho N^3 D^5.$$

The power number (N_p) is also a function of impeller blade width, number of blades, blade angle, D/T, baffle configuration, and impeller elevation. Values of N_p for different impellers are given in Table 6.1. It is important to recognize that for Re < 100, the flow reaches a laminar condition, compromising the mixing quality obtained using these impellers.

Table 6.1 Impellers characteristic numbers under turbulent conditions for various impellers [37]

Impeller type	N_Q	N_p (with four standard baffles)
Disk flat-blade turbine (Rushton)	0.72	4.13–4.75
Pitched blade turbine	0.79	1.27–1.64
Hollow-blade turbine (Smith)	0.76	4.1
Propeller	0.4–0.6	0.34–0.62
Flat-blade turbine	0.7	2.14–2.78

Measuring the slope of the velocity gradient along the blade length gives the shear rate at any point on the profile. The product of shear rate and viscosity gives the fluid shear stress—it is the shear stress that performs the work necessary in the fluid. Although viscosity may have little effect on power consumption in the turbulent (i.e., high Reynolds number) flow regime, it is a direct multiplier on shear rate from the impeller, yielding the shear stress.

Agitation and aeration are generally studied together. Due to the low solubility of oxygen, and consequent low rate of oxygen transfer, large quantities of oxygen are needed, and additional interfaces must be formed. Both agitation and aeration affect directly the state of agitation of the system, interfering in the residence time of the air bubble in the reactor and also in its interfacial area (function of the number and of the size of the bubbles), therefore affecting directly the mass transfer coefficient. The speed of agitation or pump rotation is dictated by the same factors as those governing the rate of aeration. The aeration rates depend on the nature of microorganism used, the degree of oxygen required, the thickness of the substrate layer employed, the degree to which carbon dioxide and other volatile metabolites are to be eliminated, and the degree of air spaces available in the substrate. Oxygen may be transferred directly from the gas phase and also from the oxygen dissolved in the water which keeps the substrate moist, though the contribution of the latter will be negligible [28].

6.2.2 Oxygen Transfer

In general, in fermentation processes with aerobic or facultative anaerobic microorganisms, the transfer of oxygen to the broth is fundamental to obtain good results. In the case of aerobic use, there will always be a need for an efficient air supply so that the microorganism can develop, and form a product, without limitation of oxygen. For this case, aeration is achieved by injecting sterilized and compressed air in the fermenting broth. On the other hand, in processes that use facultative anaerobic microorganisms, a certain pattern of air supply, as defined by the experiment, will allow to obtain good cell growth and appreciable concentrations of the fermentation product. In the air supply for a microbial culture, oxygen should be considered as a nutrient component of the culture medium. Hence, oxygen has to be dissolved in the medium in order for the microorganism to use this nutrient. This requirement is the biggest problem in oxygen transfer, as oxygen has a very low solubility in water. Henry's law states that at a constant temperature, the amount of a given gas that dissolves in a given type and volume of liquid is directly proportional to the partial pressure of that gas in equilibrium with that liquid. An equivalent way of stating the law is that the solubility of a gas in a liquid is directly proportional to the partial pressure of the gas above the liquid:

$$H = C / P_{\text{gas}}$$

where C is the concentration of a gas at a fixed temperature in a particular solvent, K is Henry's solubility constant, and P_{gas} is the partial pressure of the gas. At ambient pressure and at a temperature of 30 °C, typical conditions of a fermentation process, the oxygen concentration at the saturation in distilled water is only 0.23 mmol/L or 7.5 mg/L. With the dissolution of substances in the culture medium, oxygen concentration at saturation is furtherly reduced. Thus, since the dissolved oxygen consumption by an aerobic microor-

Table 6.2 Solubility of oxygen (mg/L) in water exposed to water-saturated air at 760 mmHg pressure [54]

Temp. °C	Chlorinity: 0 Salinity: 0	5.0 ppt 9.0 ppt	10.0 ppt 18.1 ppt	15.0 ppt 27.1 ppt	20.0 ppt 36.1 ppt	25.0 ppt 45.2 ppt
0.0	15	14	13	12.1	11	11
5.0	13	12	11	11	10	9
10.0	11	11	10	9	9	8
15.0	10	10	9	9	8	8
20.0	9	9	8	8	7	7
25.0	8	8	7	7	7	6
30.0	8	7	7	7	6.2	5.9
35.0	7	7	6	6	6	5
40.0	6	6	6	6	5	5
45.0	6	6	5	5	5	5

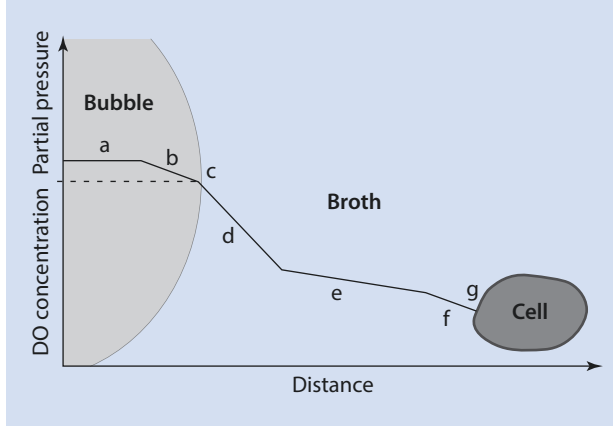


Fig. 6.3 Oxygen transfer across (a) bulk gas, (b) gas film, (c) gas-liquid interface, (d) liquid film, (e) bulk liquid, (f) liquid film, and (g) cell wall. (Adapted from Garcia-Ochoa et al. [14])

ganism is generally high, a permanent aeration of the medium is required. Table 6.2 presents the solubility of oxygen (in mg/L) in water at different salt concentrations.

The oxygen transfer depends on several operational parameters and its efficiency is crucial for systems containing high biomass load. The transport of oxygen from gas bubbles to cells occurs through several steps, depicted in Fig. 6.3. The oxygen diffusion is represented by:

$$r_{O_2} = -D_m \frac{dC}{dx}$$

where r_{O_2} is the rate of mass transfer per unit of time, D_m is the molecular diffusion coefficient (in x direction), C is the oxygen concentration, and x is the direction. Several theories

have been proposed to explain the gas transfer across gas-liquid interfaces. The simplest and most common conceptualization is the two-film theory, which postulates that near both sides of the interface there exists a hypothetical stagnant film, whose velocity profile is unknown. The two films, one liquid and one gas, provide most of the resistance to the passage of gas molecules between the bulk-liquid and the bulk-gaseous phases.

The oxygen transfer can be improved by either increasing the mass transfer coefficient (k_L) or increasing the interfacial area (a), in addition to the use of pure oxygen or of higher rates of air flow (which contains about 21% in volume of oxygen). There is an optimum bubble size for a given biochemical kinetics that promotes maximum overall biological reaction rates, for both gas-liquid and liquid-solid transfer resistances. If bubbles are too small, a larger surface is provided, enhancing the oxygen transfer between gas and liquid. However, too much energy is needed in order to produce small bubbles. On the other hand, the area of contact may be increased, as in the production of alcoholic cider: after it has been inoculated with large amounts of vinegar bacteria, it is drained through a fixed bed, while injecting air upward through them. Depending on the process, the oxygen transfer rate can also be increased by placing a draft tube in the bed, or by using a fine bubble generator [51]. For high-viscosity liquid media such as those involving filamentous organisms or biopolymer-producing organisms, mechanical agitation is required in order to assist in oxygen transfer.

The mass balance for the dissolved oxygen in an assumed well-mixed liquid phase can be written as

$$\frac{dC}{dt} = \mathrm{OTR} - \mathrm{OUR}$$

where dC/dt is the accumulation of oxygen in the liquid phase, OTR is the oxygen transfer rate, and OUR is the oxygen uptake rate. The oxygen transfer rate is estimated by:

$$\mathrm{OTR} = k_L a\left(C^* - C\right)$$

where C is the dissolved oxygen level and C^* is the equilibrium oxygen concentration corresponding to partial pressure in air stream. If a mixer is capable of supplying oxygen faster than the organisms can use it in their growth process, the main effect will be to increase the dissolved oxygen level (C) and to balance out the mass transfer equation so that the dissolved oxygen level may or may not have an effect on the growth process. $k_L a$ values can be established with several methods:

- The *chemical method*, also known as the sulfite oxidation method, involves the determination of the maximum rate of oxidation of sodium sulfite to sodium sulfate in the presence of $CuSO_4$ catalyst, in which there is no back pressure of dissolved oxygen. A plot of

$$\ln\left(C^* - C\right) = -k_L a t + C^*$$

will result in a straight line, whose slope and intercept provide $k_L a$ and C^*, respectively.

- The *dynamic differential gassing-out (DDGO) method* is based on following the dissolved oxygen during a brief interruption of aeration in the fermentation system. The rate of change of dissolved oxygen (DO) concentration is measured, and a plot of

$$C = \frac{-1}{k_L a}\left(\frac{dC}{dt} + rC_X\right) + C$$

will provide a line whose slope represents $1/k_L a$.

- The *dynamic integral gassing-out (DIGO) method* is based on the DDGO method. The experimental procedure is the same as that in the previous method, but in the case of low DO concentration in equilibrium in microbial cultivation, the integral form of the oxygen balance equation provides more accurate values for rC_X and dC/dt and thus for $k_L a$. By rearranging the equation,

$$\ln\left(\frac{C - B'}{C_i - B'}\right) = k_L a\left(t_i - t\right)$$

where C_i is the oxygen concentration at the time $t = t_i$, and B' is the equilibrium concentration of DO in the broth under aerated cultivation. $k_L a$ is, again, obtained by the slope of the straight line observed in a plot of $\ln\left(\frac{C - B'}{C_i - B'}\right)$ versus $(t_i - t)$.

- The *oxygen balance (OB) method* requires less assumptions on the effects of cell, surface active agents, viscosity, and forth. Considering that during fermentation air density at the inlet and outlet does not change appreciably, both air flow rates are equal, and it is possible to write the oxygen balance in the aerobic bioprocessing system as:

$$C = \frac{-\rho_a Q}{k_L a V}\left(f_i - f_o\right) - C$$

where f_i and f_o refer to the proportion of oxygen at the inlet and outlet, respectively. From the slope of the straight line produced, $k_L a$ can be easily obtained.

The oxygen uptake rate is expressed by the product between specific oxygen uptake rate and the cell concentration ($q_{O2}\, C_X$). The main experimental techniques employed to measure the oxygen uptake rate in cultures are [14]:

- The *gas balancing method* is the most reliable and accurate. It requires a precise gaseous oxygen analyzer to measure the oxygen concentration in the gas streams entering and leaving the bioreactor. The OUR can be determined from the difference from OTR:

$$\text{OUR} = \frac{Q}{V}\left(C^{\text{in}} - C^{\text{out}}\right) - \frac{\Delta C_L}{\Delta t}$$

This method may be imprecise when the difference between and C^{in} and C^{out} is very small (e.g., in small bioreactors).

- In the *dynamic technique*, the OUR is determined from the depletion in the dissolved oxygen (DO) concentration after stopping the air flow. The procedure can be repeated several times during the production process. Under these conditions, the oxygen balance is reduced to:

$$\text{OUR}_d = -q_{O2} C_X = \left(\frac{dC}{dt}\right)_d,$$

and OUR_d is obtained from the slope of the plot of DO concentration versus time after stopping air flow.

- The *yield method* is based on the oxygen uptake rate of the organism rather than the rate of depletion of oxygen in the gas or liquid phase. In this method, the OUR is obtained by using a stoichiometric balance of oxygen together with the kinetic model for the growth rate, thus

$$\mathrm{OUR} = \frac{\mu C_X}{Y'_{\mathrm{XO}}},$$

where μ is the specific growth rate of the microorganisms, and Y'_{XO} presents the overall yield of cell on oxygen.

- *Knowing OTR from the oxygen concentration profile data*, experimental OUR_p values can be calculated from OTR and the values of the derivative of oxygen concentration versus time curve measured during the course of fermentation:

$$\mathrm{OUR}_p = k_{\mathrm{L}}a\left(C^* - C\right) - \left(\frac{dC_{\mathrm{O2}}}{dt}\right)_p$$

The air or oxygen supply can be used to stimulate or inhibit certain microorganisms. It must be noted, however, that the amount of oxygen that an organism requires for growth, i.e., for cell multiplication, may differ from that required for fermentative activity. Table 6.3 presents the oxygen consumption for some organisms.

Thus, the rate of air or oxygen supply must be adjusted accordingly. Anaerobic processes require low mass-transfer rates of gas to liquid due to the low oxygen uptake rates.

Table 6.3 Parameter consumption values for some microorganisms [14]

Microorganism	q_{O2} (mol_{O2} kg X^{-1} h^{-1})	m_{O2} (mol_{O2} kg X^{-1} h^{-1})	Y_{O2} (mol_{O2} kg X^{-1})
Xanthomomas campestris	2–15	1.0	0.6
Escherichia coli	0.9–23	2.4–6.4	12.5–520
Bacillus acidocaldarius, subspecies kurstaki	3.1–31.2	2.2–16.6	0.3–43.8
Phaffia rhodozyma	1.9	–	–
Bacillus thuringiensis	2–15.5	0.9	17.2
Rhodococcus erythropolis	02.-4.3	0.8	16.4–20
Pseudomonas putida	2–18	1.9	52.6
Trigonopsis variabilis	2–3	0.03	13–16
Candida bombicola	0.3–1	0.01	4.4
Hansenula anomala	0.8	–	–

As an example, mammalian cell fermentation occurs under anaerobic conditions. Aerobic fermentation processes, on the other hand, require moderate to high oxygen rates between gas and liquid phases. Examples of aerobic fermentations include bacterial fermentation of *Escherichia coli* strains and yeasts and mycelial fermentations to produce antibiotics such as penicillin.

6.2.3 Flow Regime and Rheological Properties

Fermentation processes can be conducted either in laminar or turbulent regime that is determined according to the Reynolds number. An advantage of laminar operation is the lower energy required to promote fluid movement. However, turbulence is crucial to promote mixing, as the whole flow field is dramatically affected by its presence.

In turbulent flows, the fluid motion displays velocity fluctuations in time and in all three directions in space. These fluctuations reflect the complex interactions among flow structures, i.e., eddies that present a wide range of shapes and sizes. Moreover, velocity fluctuations are so intense that inertial forces prevail over the viscous forces, promoting the rapid dispersion of scalar fields, compared to the laminar case [37]. The effect of flow turbulence is often compared to an increase of the viscosity of the fluid, which promotes homogeneity as well as greater contact between the biomass and the substrate.

The Reynolds number used to establish the flow regime is defined as:

$$\mathrm{Re} = \frac{ND^2\rho}{\mu}$$

where N is the rotation of the impeller and D its diameter. Reynolds numbers below 1,000 indicate fully or partially laminar regime in stirred tanks. Fully turbulent flows are achieved for Reynolds numbers above 10,000. For numbers between 1,000 and 10,000, the flow regime is transitional. However, care must be taken with this value: a fermenter with a high Reynolds number, indicating that it will operate with turbulent regime, may still present laminar/dead zones, hindering the efficiency of the process. Moreover, both shear stresses and velocity fluctuations are more intense near the impeller. Currently, the best approach to fully assess the fluid dynamics inside the fermenter is the use of numerical techniques, such as the computational fluid dynamics (CFD).

In addition to the turbulent viscosity, the apparent viscosity of fermentation broths affects the shear stress and, consequently, cell viability. The typical viscosity of broths containing freely suspended microbial species ranges from about 0.01 Pa.s up to 100 Pa.s [11]. Furthermore, the presence of particles with immobilized cells in a fermentation broth can affect the apparent rheological properties, primarily due to the presence of suspended solids. The degree of deviation in the rheological properties depends on the size, density, compressibility, and concentration of the immobilized cell particles. Further fermentation reactions in the cells on the immobilizer support produce high molecular weight metabolites, different from those formed with freely suspended cells, under the same conditions, further altering the rheology of the medium. The rheological behavior of the fermentation broth may cause problems to mixing, heat transfer, and oxygen supply in solid-state fermenters, thus limiting both the maximum metabolites concentration achievable and the quality of the product [47]. Rheological

properties are particularly important in the maintenance of fluidized beds, in which the fluidization quality (and hence the mass transfer efficiency) depends on a delicate balance of forces.

Furthermore, fluids can be classified into Newtonian fluids, whose viscosity is dependent only on temperature, and non-Newtonian fluids, whose viscosity depends on temperature and shear rate (γ). The apparent viscosity of some non-Newtonian fluids may also be dependent on time [53]. A general equation to model the apparent viscosity (η) of Newtonian and non-Newtonian fluid is:

$$\eta = k\gamma^{n-1}$$

where k represents the consistency coefficient. For Newtonian fluids, the flow behavior index n is equal to 1. Under most circumstances, the organic wastes such as wastewater sludge and manure slurry may exhibit non-Newtonian behavior. Viscoelastic materials exhibit both viscous and elastic characteristics when undergoing deformation. Shear-thinning (pseudo-plastic) fluids present values of $0 < n < 1$, thus the apparent viscosity diminishes with the shear rate. On the other hand, dilatant fluids have $n > 1$, and their apparent viscosity increases with the increase of the shear rate.

6.2.4 Hydrodynamic Stresses

Another important aspect to be considered is the ability of the cells to withstand hydrodynamic stresses, especially shear stresses. This cell capacity normally dictates various operating conditions, e.g., liquid and/or gas flow rates, or turbine speed of agitation, and the types of reactors that can be used for cultivation and production. Agitation is commonly adopted to reduce non-uniformity in the fluid, removing gradients of nutrient concentration and temperature [25]. However, intense agitation may lead to high hydrodynamic stresses [22], which can cause changes in morphology, even cell destruction. Yeasts, in particular, are generally considered resistant to physical stress due to their cell form and dimensions but also due to the rigid cell walls [4].

When cells are immobilized on a support surface, they move with the same support average velocity, which may be significantly greater than the velocity of suspended cells in bioreactors. In this case, when the immobilized cells are under the same conditions of flow or agitation, they undergo much greater shear stresses than the free cells. The viability of the cells is also affected by the time of exposure to high stresses.

The shear stress, on the other hand, can be used as a measure to control the thickness of the biofilm. In addition to (turbulent) shear stresses, particle integrity is also governed by particle-particle collisions and abrasion.

There are a few correlations for estimating the average shear rate (γ_{av}) in the literature. For stirred tanks, Metzner et al. [32] defined the average shear rate as a function of the rotational impeller speed (N) only:

$$\gamma_{av} = k \cdot N$$

where the rotational speed is given in rpm and k is a constant particular to the impeller geometry. For Rushton, pitched blades, and marine impellers (illustrated in Fig. 6.2), the reported values of k are 11.4, 13, and 10, respectively. Impellers with smaller k values

generate low values of γ_{av}, which makes them appropriate to be used in systems with high viscous fluids but with low oxygen demand [7].

6.2.5 Temperature

To some extent, the increase in temperature promotes an increase in the reaction rates. However, too high temperatures cause the rapid denaturation of the enzymes (Fig. 6.4) and the death of the cells. Psychrophile organisms are those that tolerate the lowest temperatures, mesophile ones works best at ambient temperatures, and extreme thermophilic bacterium and extreme thermophilic archaeon supports the highest temperatures. Thermal transfer can be used in several stages of fermentation: high temperatures to sterilize the medium, heat to promote biomass development, and heat removal to maintain an exothermal process under the same temperature.

According to the temperature employed, various types of microorganisms may predominate in a mixed fermentation. An example of the way in which optimum temperatures are provided for the type of organism to be favored is in the fermentation for food production. Sauerkraut fermentation is particularly sensitive to temperature, which affects the final concentration of acid in the fermentation and the time required to reach various levels of acidity. In consequence, low temperatures are used at the beginning of the sauerkraut fermentation process, and increased in the later stages.

For the dark biohydrogen fermentation, the ideal temperature range is between 75 and 80 °C, which both favors kinetics and prevents contamination by bacteria that consume hydrogen [9]. However, temperature has many other effects besides its direct effect on growth and microbial activity. In the case of wine fermentation, the ideal temperature for most yeasts is between 22 and 27 °C. Higher temperatures affect the fermentation efficiency, and also promote losses of alcohol and aromatic constituents. Fortunately, yeasts can be acclimatized to ferment at relatively low temperatures [3].

Fermentation is an exothermic process, and maintaining an isothermal operation may require the continuous removal of the heat produced. In addition, supplies should be kept at an ideal temperature before putting them in contact with microorganisms in order to promote optimal fermentation conditions. Often this step is the one that requires the most energy. For example, the cephalosporin C fermentation is a sterile process with complete sterilization of bioreactors and peripheral feed vessels prior to inoculation. The medium is prepared in a stirred media preparation tank and preheated to 90 °C before transfer to the production reactor. Preheating aims at avoiding hydraulic shocks during sterilization. The

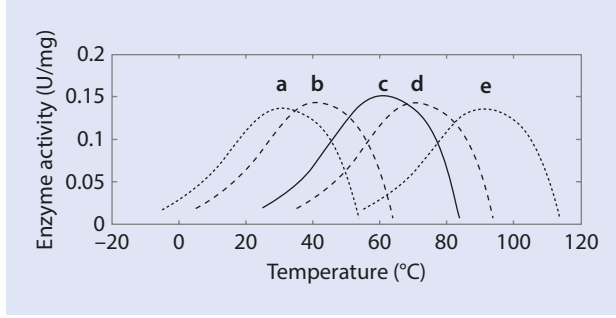


Fig. 6.4 Effect of temperature on enzyme activity on (a) psychrophile, (b) mesophile, (c) moderate thermophile, (d) extreme thermophilic bacterium, and (e) extreme thermophilic archaeon

sterilization is done with steam at 120–122 °C, and the medium kept for 30 min at this temperature. The operating temperature is controlled by means of chilled water at 10 °C through the vessel jacket and cooling coils, which also serve as baffles.

In the brewing industry, the optimum temperature for beer fermentation ranges from 7 to 13 °C for lagers, and from 20 to 22 °C for ales. For Weizen beer production, it is desired to maintain the fermentation temperature at about 20 °C. However, in the previous stage, wort is boiled at 100 °C. The required refrigeration is commonly done in two stages: the first with ambient water (which is heated from 25 to 78 °C and reused in the process), where wort is cooled from 100 to 50 °C, and the second with a mixture of ethylene glycol, in which the final temperature of 20 °C is reached. As an example, for a batch of 1050 kg, the first stage of wort cooling is calculated as:

$$q_{\text{wort}} = m \cdot Cp_{\text{wort}} \cdot \Delta T$$
$$= 1050\,\text{kg} \cdot 3.858\,\text{kJ} / \left(\text{kg.}^{\circ}\text{C}\right).\left(100 - 50\right)^{\circ}\text{C}$$
$$= 202{,}545\ \text{kJ}$$

If this operation takes half an hour,

$$\dot{q} = q / t = 405{,}090\ \text{kJ/h}$$

To determine the thermal exchange,

$$\dot{q} = U \cdot A \cdot \Delta T_{\text{ml}}$$

where

- $\Delta T_a = 100 - 78 = 22$ °C.
- $\Delta T_b = 50 - 25 = 25$ °C
- $\Delta T_{\text{ml}} = \dfrac{\Delta T_a - \Delta T_b}{\ln\left(\Delta T_a / \Delta T_b\right)} = \dfrac{22 - 25}{\ln\left(22/25\right)} = 23.47^{\circ}\text{C}$

If a global average heat-exchange coefficient is adopted (this value must be confirmed with data from the exchanger or the material used) of 4600 kJ/h, the area (A) required is determined:

$$\dot{q} = U \cdot A \cdot \Delta T_{\text{ml}}$$
$$405{,}090\ \text{kJ/h} = 4600\ \text{kJ} / \left(\text{h.m}^2.^{\circ}\text{C}\right) \cdot A \cdot 23 \cdot 47^{\circ}\text{C}$$
$$A = 3.75\ \text{m}^2$$

In order to calculate the amount of water ($\dot{m}_{\text{water}}$) required,

$$\dot{q}_{\text{water}} = \dot{m} \cdot Cp_{\text{water}} \cdot \Delta T$$
$$405{,}090\ \text{kJ/h} = \dot{m}_{\text{water}} \cdot 4.187\ \text{kJ} / \left(\text{kg.}^{\circ}\text{C}\right).\left(78 - 25\right)^{\circ}\text{C}$$
$$\dot{m}_{\text{water}} = 1825.5\ \text{kg/h}$$

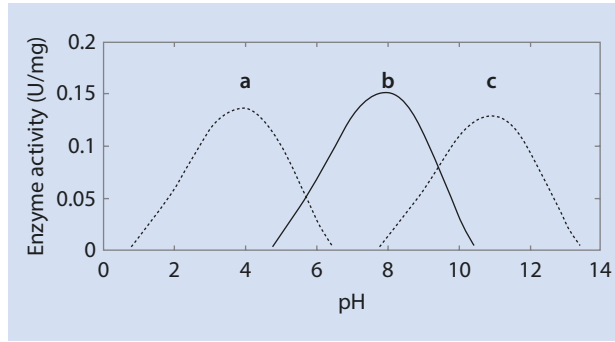


Fig. 6.5 Effect of pH on enzyme activity on (a) acidophile, (b) neutrophile, and (c) alkaliphile organisms

6.2.6 Acidity

The pH of the medium has been recognized as one of the most important factors affecting metabolic pathways [44]. pH is a logarithmic scale used to quantify the acidity of a solution. It is approximately the negative of the base 10 logarithm of the molar concentration of hydrogen ions. Acid solutions, with a pH less than 7, have a higher amount of H^- ions, which may bind to an enzyme and decreases its activity. This can stop a substrate from accessing the active site of the enzyme.

Different enzymes work best at different pH values: acidophile organisms (such as *A. niger* used in the citric acid production) tolerate acid environments, while alkaliphile organisms tolerate alkaline environment (e.g., *B. halodurans*, used in the production of cyclodextrins). Hence, an enzyme in a medium with non-optimum pH value will have its activity decreased. In the example given in Fig. 6.5, the optimum pH is around 8 for a neutrophile organism (such as *S. cerevisie*, used in ethanol production).

Inhibition effects due to the presence of acids in the medium may occur at any stage of production: when it is one of its natural components, when it is produced by fermentation, and/or when it is added directly into the food. The addition of acid in foods aims at inhibiting the multiplication of harmful organisms that can degrade the products formed. In addition, the pH of the fermentative medium must be controlled so that it is suitable for the microorganism employed.

6.2.7 Salt

Microorganisms are also affected by the presence of salts. When salt is found in reasonable quantities, it causes exosmosis in yeast cells, and at high enough concentrations, salt causes excessive water loss which impairs cellular function. Hence, salt tolerance is an important parameter for microorganisms, which can be classified according to this criterion. A concentration of about 15 g/L is sufficient to inhibit by 50% the growth rate of *C. acetobutylicum*, used in the production of acetone, butanol, and ethanol [29]. Lactic acid-generating organisms, which are used to ferment pickled olives, sauerkraut, some meat sausages, and the like, generally tolerate moderate salt concentrations of the order of 10–18%. However, many other types of proteolytic organisms do not tolerate more than 2.5% salt, and especially do not tolerate the combination of salt and acid [39]. In these fermentations, the added salt gives lactic acid-producing organisms the initial advantage over other microor-

ganisms, which may be already present. Once the production of lactic acid by organisms has begun, the acid combines with the salt to strongly inhibit proteolytic organisms.

The same principle applies in the cheese manufacture. The addition of salt to the curd is a common practice, whose purpose is the control of proteolytic organisms during the long periods of maturation—which can last for more than a year for certain types of cheese. In this case, several lactobacilli with a high degree of salt tolerance continue to produce acid, modifying cheese curd during the maturation period.

The presence of salts in fermentation broth had adverse effects on the extraction process of succinic acid [20]. Phosphate, in addition to its specific function, exerts a general depressing effect on the alcoholic fermentation. Salt may be accumulated due to the utilization of bases for pH control during the fermentation, thus limiting the growth of bacterium in fuel ethanol production [43]. Conversely, supplementary doses of inorganic salts have been useful in enhancing ethanol production [27]. Optimum quantities of ammonium and phosphate ions ensure a maximum ethanol yield of about 90% of the theoretical one. Magnesium ions provided a slightly negative effect on the ethanol yield [50]. A 1:10 (v/w) ratio of salt solution to weight of oil cake is also beneficial in the production of L-Asparaginase by *Aspergillus* sp. [45].

6.2.8 Alcohol

In the same way as the acid and salt concentration, alcohol can be a preservative and its effectiveness will depend on its concentration. Similarly to the acid-producing organisms, yeasts cannot tolerate their own alcohol or other fermentation products above a certain level. It is generally recognized that ethanol has three inhibitory effects: inhibition of cell multiplication, inhibition of fermentation, and a lethal effect on cells. In general, the intoxicating potency, lipid solubility, and membrane lipid-disordering potency increase in an exponential manner as the number of carbon atoms in an aliphatic n-alcohol is increased from one to five. For example, butanol produced by *Clostridium acetobutylicum* is a potent inhibitor of the growth of this organism. For many yeasts, the tolerable limit of ethanol is reached within the range of 12–15%. The percentage of ethanol contained in wine, for example, depends in part on the amount of sugars originally contained in the grapes, the type of yeast, the temperature used for fermentation, and the level of oxygen. Natural wines generally are produced with 9–13% of alcohol, which in itself is not sufficient to ensure its complete conservation. Thus, they should undergo a brief pasteurizing treatment. The so-called fortified wines are natural wines in which extra alcohol is added in order to increase the final concentration of alcohol to 20% by volume, reducing the need for pasteurization treatment [39]. In the ethanol production, alcohol inhibition may limit the fermentation yield [19, 56]. Although it is easier to produce ethanol using submerged fermentation, an alternative route is to use solid-state fermenters due to their lower water requirement, smaller volumes of fermentation mash, and mainly prevention of end product inhibition.

6.2.9 Water Activity

The water activity (a_w) of a product is defined as the ratio between the vapor pressure of the product itself, when in a completely undisturbed balance with the surrounding air media, and the vapor pressure of distilled water under identical conditions – e.g., both at

Table 6.4 Water activity of some common foods [12]

Liverwurst	0.96
Cheese spread	0.95
Red bean paste	0.93
Caviar	0.92
Fudge sauce	0.83
Soft moist pet food	0.83
Salami	0.82
Soy sauce	0.80
Peanut butter 15% total moisture	0.70
Dry milk 8% total moisture	0.70

ambient temperature. It is closely related to the water content, but it is not the same. In fact, the water activity represents the water available for the activity of the microorganism. It varies due to the evaporation and metabolism processes and increases with temperature. The moisture condition of a product can be measured as the equilibrium relative humidity expressed in percentage or as the water activity expressed as a decimal. In solid-state fermentation, exact quantity of water is added to the substrate in order to provide suitable water activity. The amount of available moisture can be used to modify the metabolic production or excretion of a microorganism [36]. Sufficient low values of a_w will inhibit the growth of the organisms. The a_w of the medium has been attributed as a fundamental parameter for mass transfer of the water and solutes across the microbial cells.

Most foods have a water activity above 0.95, and that will provide sufficient moisture to support the growth of bacteria, yeasts, and mold. Values of water activity of some common foods are given in Table 6.4.

6.2.10 Biochemical Kinetics

The selection of the operation mode and bioreactor configuration depends on the biochemical kinetics involved. Fermentation reactions may be inhibited by the substrate or by the products; otherwise, they commonly follow a Monod kinetic, and the operation should be designed in such a way as to maintain low concentrations of the inhibitor species in the mixture, promoting as much microbial activity as possible.

Substrate-Inhibited Kinetics

When a reaction is inhibited by substrate, a suitable approach is the use of a stirred continuous flow reactor (CSTR) followed by a plug flow reactor, thus the highest concentration of substrate is isolated in one reactor. In practice, this same approach can also be achieved by using a series of small reactors, or by a stage bioreactor, with a first large stage followed by a small, or a series of small stages in a bioreactor. Thus, in the latter stages, higher conversion is achieved in smaller volume. The use of a set of smaller reactors gives

higher flexibility to the system (in which different conditions may be employed in each reactor). However, this setup also requires higher maintenance costs.

Products-Inhibited Kinetics

This situation occurs when the formation of products causes a decrease in the enzymatic activity of microorganisms. A plug flow reactor is more favorable for high substrate conversion than CSTR, for systems with a kinetic inhibited by products. However, the conversion may be enhanced further by removal in situ of any inhibitory components: this removal can be achieved by the use of several separation methods, such as:

- Adsorption, in which the adsorbent should preferably remove the products (inhibitors) and resist the fixation of free cells. It may also be another liquid phase, as long as it is immiscible to the fermentation broth. Liquid-liquid extraction, in particular, was proved to be an efficient and environment-friendly method for organic acids recovery [21].
- Integrated in situ gas-stripping process with product separation efficiency adjustments under non-strict anaerobic conditions, which has been shown to be an effective method for improving acetone-butanol-ethanol production [26].
- Online recovery considering foam fractionation by bubbling of sterile air and nitrogen was also proposed for recovering nisin during the fermentation of *Lactococcus lactis* [57].
- Use of hollow fiber placed in the reactor to give optimal removal of inhibitors. However, this requires the cells not to stick to the fibers, as this may cause blockage of the permeation pathway of liquids and a higher pressure drop in this removal process. Nonetheless, research has been conducted to investigate separation/recovery of fatty acids such as acetic, propionic, and valeric acids via membrane processes [49].
- Air stripping and chemical precipitation are used for ammonia disinhibition. Both of them are effective at controlling high ammonia concentrations in wastewater treatment [55].
- Immobilizing the microorganisms with different types of inert materials (clay, activated carbon, zeolite, polyvinyl alcohol) has been showing high effect to reduce ammonia inhibition in the anaerobic digestion for biogas production;
- Other techniques applied for the recovery of organic acids from fermentation broths include electrodialysis and ion-exchange [55].

Oriented acclimatization may also be an effective method for the improvement of the specific fermentation performance of yeast, such as its ability to utilize pentose and tolerate high temperatures or high concentrations of product and substrate [56].

Monod Kinetics

The Monod equation is an empirical model for the growth of microorganisms, which relates microbial growth rates in an aqueous environment to the concentration of a limiting nutrient. This model has been successfully used for more than a century to predict the rate of product formation in enzymatic reactions. It states that the rate of an enzymatic reaction will increase as substrate concentration increases. When microorganisms follow this kinetic, there is no substrate inhibition, and bioreactors can be better operated under conditions of high substrate concentration, which promotes faster biomass growth. In a continuous operation, this condition can be achieved with a plug flow standard for the liquid flow. The adoption of fluidized bed reactors is also possible; however, the speed of

the liquid in the bioreactor is usually very small. In most cases, a plug flow pattern for the liquid cannot be approximated under low liquid flow conditions. The use of fixed bed to minimize the axial liquid mixture is also not desired because of plugging problems inherent in the bed when immobilized live cells are used. Further information can be found in ► Chap. 5.

6.2.11 Feeding Strategy

Figure 6.6 shows a typical cell growth pattern in batch fermentation: yeast cells, in contact with substrate, require an amount of time to start to multiply. Substrate fermentation firstly produces primary metabolites, during the log (or exponential) growth phase. These metabolites may be further degraded in order to produce secondary metabolites. The growth phase is followed by a stationary phase in which the cell production is balanced by the death of other cells. When more cells are dying than being produced, the process reaches the death zone.

If biomass or primary metabolites (those produced during the exponential growth phase) are the products of interest in the fermentation process, the medium must be formulated in order to allow the maximum growth potential. In the case of metabolic repression, a vital strategy is the use of intermittent or gradual substrate feeding, or the use of slowly fermenting carbohydrates. When the production of secondary metabolites (which are synthesized late during exponential growth or stationary growth) is desired, maximum initial growth is still required to provide the maximum allowable biomass. However, when a sufficiently high concentration of biomass is obtained, the medium is then switched to a (so-called) "production" medium, which should maximize the production of the desired metabolites, while keeping a slow or standing cell growth. Usually the production medium is deficient in one or more nutrients crucial to the production of primary metabolisms, such as nitrogen, phosphorus, or trace metals [11].

The cell deactivation by age is a common problem in immobilized cell systems, especially those involving the production of secondary metabolites, which causes a decrease in the cell productivity with time of operation. To overcome this issue, the aged cells should be rejuvenated by intermittently feeding the key nutrients required for primary metabolism. It should be noted that differences in the composition of the medium affect the rheological properties and surface tension of the fermentation broth, which in turn influences

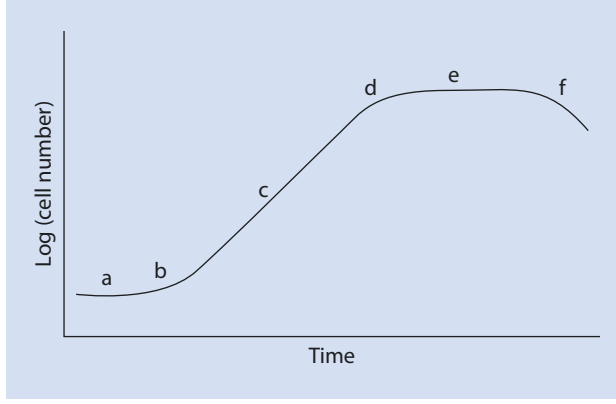


Fig. 6.6 Microbial cell growth pattern in batch fermentation: (a) lag phase, (b) acceleration phase, (c) log or exponential phase, (d) deceleration phase, (e) stationary phase, and (f) death phase

the reactor design. Such properties affect bubble size and rise velocity, and hence the phase holdup distribution, heat transfer, liquid-solid and gas-liquid mass transfer, and phase mixing states.

6.3 Selection of a Fermenter Operation Mode

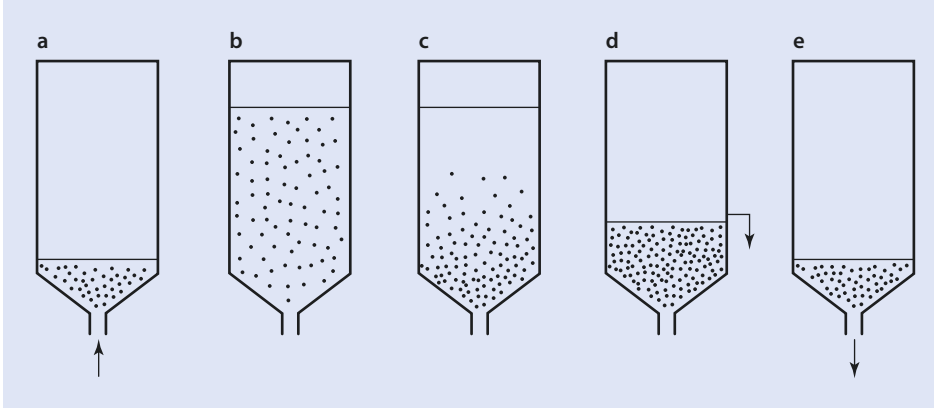
One of the most important steps for proper operation of a fermentation process is the identification of the most appropriate type of real-scale bioreactor. In fact, one of the major difficulties is the upscaling of fermentation processes that are developed and/or optimized on bench scale and pilot scales. Often such studies are performed on flasks, beakers, and other containers with a completely different hydrodynamic behavior than that observed in large reactors. Hydraulic short circuits, mixing problems, dead zones, different retention times along the reactor, scale problems with reagent supply and product withdrawal, and other hydrodynamic problems observed in real reactors are easily bypassed or ignored in bench reactors or laboratory. Not taking into account these factors result in failures in the operation of large-scale reactors.

Many considerations need to be taken into consideration when selecting a fermenter operation mode. A key point is the criterion for comparing different fermentation bioreactors and different operating conditions to achieve the best possible system for the process. At present, the only way to compare the economic performance of different systems consists in building and operating a full-scale version of each device and record its operational and capital costs. This approach is likely to take a long time before it is possible to use economic performance as a criterion to guide choice by one process or another.

Selection is usually guided by answers to several key questions about the factors that affect productivity, for example, the theoretical yields which can be achieved for fermented sugar are 51.1% alcohol and 48.9% carbon dioxide by weight. However, this cannot be obtained biologically, and in practice, it will depend on factors such as the amount of by-products, amount of sugar used by yeasts and other microorganisms, alcohol lost by evaporation or entrainment (which in turn depends partly on the temperature and rate of fermentation), presence of air, and agitation and movement of the fermentative mass, among other factors. In practice, yields can reach 90–95% of the theoretical value [3]. Thus, care must be taken in the operation of the process, so as to ensure that operating conditions lead to the highest possible yield. Several operating modes can be used in fermentation processes, e.g., batch, fed-batch, semi-continuous, and continuous. Each mode offers a set of advantages and disadvantages, and its main characteristics are described below.

6.3.1 Batch

Batch processes are widely used. They are the simplest to operate: all carbon source and media components are added to the bulk at the start of the fermentation, and then the batch runs until carbon source is depleted. Batch fermentations are reliable and able to operate aseptically for the long periods that are generally needed.



6

Fig. 6.7 Batch cycle: **a** feed, **b** fermentation, **c** settling, **d** products withdrawal, and **e** discharge of biomass in excess

A common batch reactor type is the sequencing batch reactor (SBR). The operation of an SBR takes place in a single tank and comprises steps such as sterilization, incubation, settling, and discharge [22, 31]. This sequence is illustrated in Fig. 6.7.

The actual fermentation process is just part of the batch cycle (the incubation phase). A complete fermentation cycle can typically include the following steps (depending on vessel design):

1. Empty (blank) sterilization of vessel and pipework using direct steam.
2. Injection of the fermentation broth.
3. Charging with base medium.
4. Indirect sterilization via steam injected into the vessel jacket.
5. Cooling and jacket drain.
6. Pre-inoculation, where the pre-culture is prepared, and the vessel environment is maintained under suitable conditions.
7. Inoculation, consisting of the injection of a small sample of the monoculture.
8. Incubation, which is the fermentation process itself, in a controlled environment.
9. Harvesting, when the finished products are removed by downstream processes such as centrifugation and filtration.

The conditions are highly dynamic in batch fermentations, with the substrate, biomass, and product concentrations changing over time. The main advantages are the simplicity of this operation, and the low risk of a contamination due to its closed nature. However, the need for sterilization requires a long downtime for batch turnaround. Moreover, it does not allow the control of the growth rate or the product formation rates. Batch processes are used:

- When secondary products are desired.
- To prevent genetic instability, which makes continuous culture less productive.
- When operability and reliability is sought—sterility and equipment reliability are bigger in batch cultures.

Batch processes are flexible; they can produce many products per year. This type of process is employed for small-scale processes, beer and biofuel production. Once a batch is

started, temperature can be used to control the yeast growth. Temperature control is used to maintain isotherm conditions, through the removal of the heat generated by fermentation. Agitation is also a controlled parameter, through the impeller speed, recirculation rate, and/or gas feed rate.

6.3.2 Fed-Batch

It is the process where the inoculum is initially introduced until it takes a fraction of the useful volume of the order of 10–20%. Successively, feeding with the culture medium is started at a suitable flow, without the withdrawal of processed liquid. This operation is carried on until the useful volume of the reactor is filled, and at this point, the process broth is withdrawn to recover the product. Such operations may include the recycling of cells in order to initiate a new feeding period. The feed can be constant or intermittent, with constant flow and composition or not. This process can be seen in Fig. 6.8.

In the fed-batch process, addition of substrate may or may not be controlled by a feedback mechanism. In the feedback control mode, the substrate supply can be controlled as a function of its concentration in the medium (direct control) or other parameters (indirect control), such as optical density, pH, and respiratory ratio. A majority of industrial fermentation processes employ a fed-batch operating mode in a stirred tank, as in the antibiotic production. Fermentation is firstly operated in batch mode to promote biomass accumulation. Once the carbon source is depleted, feeding begins in order to supply the system with feed for product formation, biomass growth, and maintenance, enabling significantly greater biomass and product concentrations than batch operation. Processes can be operated for significantly longer periods of time, reducing the down-time in relation to the process time, thus increasing equipment utilization. Fed-batch processes are useful:

- In antibiotic fermentation
- When the reactor is fed continuously (or intermittently)
- When the purpose is to maintain low substrate concentration
- In overcoming substrate inhibition or catabolic repression

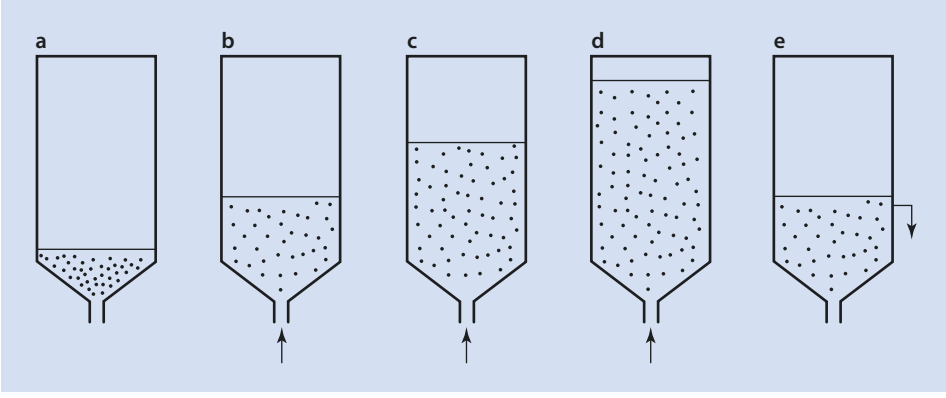


Fig. 6.8 Fed-batch cycle: **a** culture medium feed, **b–d** substrate feed, and **e** products withdrawal

There is a need for improved monitoring and control of the process in order to supply the feed at a suitable rate, and to monitor the tank fill which is continuously increasing over the process time. The common strategies used to control the growth in a fed-batch process are the maintenance of dissolved oxygen concentration, oxygen uptake rate, glucose and acetate concentrations, pH, and ammonia. Temperature may be adjusted according to OUR or pO_2.

6.3.3 Semi-continuous

In the semi-continuous system, the filling of the reactor is made at a very high flow rate. At the end of the new cycle, a given fraction of the volume is again withdrawn, 30–60%, and the reactor is filled instantly. For large-volume bioreactors, this continuous fill does not occur, falling into the fed discontinuous reactor. The same control approach is used for both processes. In any case, it is a distinct technique, in which the operation is carried out with shocks of substrate loading.

6.3.4 Continuous

In the continuous process, continuous flow of liquid is sought through the reactor, or reactors arranged in series. Feed is added at the same rate of the product stream removal. The continuous reactor allows the recycling of cells: the bioprocessed liquid, effluent from a given bioreactor, can be subjected to a system of separation of the microorganisms, which can be returned to the reaction volume, being liquid sent for recovery of the product. Continuous processes can maintain the system at a steady state with high product formation. This is a highly productive process, with a comparably low operational cost. There are several types of continuous processes, and some of them are illustrated in Fig. 6.1. Continuous processes are used in wastewater treatment and biogas digesters. Approximately 20% of the Brazilian refineries adopt continuous process for ethanol production. It is normally operated at steady state, and in the case of CSTR reactors, it is assumed to be perfectly mixed. Consequently, there is no time dependence or position dependence for temperature, concentration, and reaction rate. In systems where mixing is highly non-ideal, the well-mixed model is inadequate, and other modeling techniques such as residence-time distributions are needed to obtain meaningful results. Some basic calculations and design can be found in ▶ Chap. 5.

The hydraulic retention time (HRT) in these systems can be estimated by [1]:

$$\mathrm{HRT} = V / q$$

where V is reactor volume and q is volumetric flow rate of the reactants. For a digester of 400 L volume, fed at the rate of 50 L/day,

$$\mathrm{HRT} = 400 / 50 = 8\,\mathrm{days}$$

There are operational challenges, as it requires tightly controlled conditions and robust monitoring methods, especially at industrial scale. Other challenges include the difficulty to maintain the downstream operated continuously. In addition, the operation can last a long time, which requires genetically stable strains. There is also a higher risk of contamination. On the other hand, the advantages are:

- Growth rate can be controlled and maintained.
- Effect of changes in physical or chemical parameters can be examined.
- Biomass concentration can be maintained by varying the dilution rate.

Control in flowing systems is similar to those used in semi-continuous mode. Regarding the temperature control, different from batch processes, it is not enough to guarantee that the heat removed equals the heat generated in a continuous reactor, as this does not prevent the development of hot spots within the reactor. The simplest solution to eliminate these spots is to relocate the temperature sensor to the point where the hot spots exist. This however leads to overcooling downstream of the temperature sensor. Regarding the flow rate, it is an important parameter for fluidized bed reactors, which require the superficial velocity be lesser than the terminal velocity of (bio)particles, in order to maintain them inside the bioreactor.

6.4 Bioreactors Adopted in Fermentative Processes

Traditionally, enzymatic processes in suspended cells have been intensively applied in the fermentation industry. However, the demand for higher quality food in higher quantities has increased together with the intense population growth, requiring improvements in the processes such as the use of immobilized cells [10]. The term "immobilized cells," also known as "bioparticles" or "biocatalysts," relates to cells (or enzymes) that are physically confined in a given medium. The main purpose of immobilization is to avoid the loss of activity of a valuable biocatalyst. With the retention of their catalytic activity and/or viability, they can be used repeatedly and continuously. The use of immobilized cells presents apparent advantages over the use of immobilized enzymes by the following aspects [11]:

- No enzyme extraction and purification is required.
- In whole cells, enzymes are kept in their natural environment; consequently, its stability is greater and the loss of catalytic activity is highly reduced.
- Cofactors can be regenerated in immobilized living cells.
- Multi-step enzymatic reactions can be conducted using immobilized cells.

The reuse of biocatalysts is essential to maintain a low cost of production, since enzymes and cellular material (primers) represent a significant amount of the production cost.

Different techniques of biocatalyst immobilization are available. Along with the immobilization type, the requirements are different depending on the type of reactor used, thus each technique and support material must be adapted accordingly, in order to promote the highest yield. Stirred reactors are easy to operate and easy to scale-up, while hollow fiber reactors have low cost and low catalyst comminution. The fluidized bed, however, provides better mixing of solids and liquids, greater oxygen transfer, easy cell regeneration, and a more uniform cell population in the reactor than the hollow fiber reactor. Thus, in order to minimize shear effects while maintaining adequate bed expansion, solid mixing, and gas-liquid mass transfer, gas and liquid flow rates in the reactor need to be

Table 6.5 Qualitative comparison among different bioreactors commonly used to conduct fermentative processes [38, 44]

Reactor type/aspect	CSTR	Packed bed	Fibrous bed	Loop	Fluidized bed
Design	Simple	Simple	–	Complex	Complex
Operation	Simple	Simple	Difficult	–	Difficult
Energy requirements	High	Low	Low	Reduced	High
Mixing	Optimal	Poor	Fair	Plugging	Optimal
Products separation	–	Poor	Easy	Easy	Easy
Scale-up	Easy	Easy	Complex	Easy	Complex
Shear	High	Low	Low	Low	Low
Foaming	Yes	No	Yes	Yes	Yes

properly controlled [11]. A qualitative comparison among different bioreactors commonly used to conduct fermentative processes is presented in Table 6.5.
It can be seen that each reactor offers some advantages for microbiological processing using immobilized cells. Some aspects on the operation of the most common bioreactors are given below (more detailed explanation is given in the next chapter).

6.4.1 Continuous Flow Reactor

Continuous stirred tank reactors (CSTR) are widely used in fermentation processes due to their high capacity and ease of control of production parameters. In the first fermentation studies in CSTRs, non-immobilized processes were considered. However, reactor designs adopting biofilms or granules are highly recommended because they retain more active and effective microorganisms [42].

The CSTR bioreactor usually operates in the presence of vigorous mechanical stresses, causing damage to both fixed biomass and support material. On the other hand, the reduction of mechanical agitation generally causes poor mass transfer, reducing fermentation productivity. Such a disadvantage is very apparent with the use of filamentous fungi; however, in this case, the disadvantage can be eliminated by the self-immobilization of the fungi, which form pellet or floc-like structures. Moreover, mixing and aeration should be carefully designed; otherwise, productivity will be unfavorable for fermentation.

In ideal operation, a CSTR would be considered as a perfectly mixed medium. However, the mixture in the reactor will depend on the amount of agitation that can be added to the medium without damaging the microorganisms, and this is limited by the maximum shear supported by the biomass. The agitation will dictate the volume of the reactor, and therefore the flow rate of operation will be limited.

6.4.2 Packed-Bed Bioreactor

Packed-bed bioreactors (PBRs) correspond to the operation in which biocatalysts, immobilized in the form of beads or pellets, are fixed and immobile in a column bioreactor. The fermentation broth is fed in this medium. The design of this bioreactor should be handled with care, in order to avoid nutrient depletion which can occur for excessively long beds (since nutrients are gradually consumed along the height of the bioreactor), and thus the decrease in viable cell population. High pH gradients along the column can also be a critical limitation: under non-optimal pH conditions, microorganisms may not perform the fermentation efficiently.

6.4.3 Fibrous-Bed Bioreactor

Fibrous-bed bioreactors (FBB) are designed to be efficiently integrated with immobilized biocatalysts. These bioreactors have a simple design, which allows the production with high cell density and long-term stability, maintaining sufficient productivity. In this bioreactor configuration, the biomass fixation is performed on a support material (cotton tissue was initially used in a spiral tubular bioreactor), which should be chosen with caution to eliminate diffusion limitations between immobilized microorganisms and fermentation broth, which can result in low cell efficiency [10].

FBB can operate in adapted batch fermentative processes. Compared to free-cell systems, the productivity in FBB is much higher when operated with batch fermentation, and even more using repeated batch. Taking into account repeated batches, special attention must be given to the determination of the initial concentration of substrate, since it influences the final productivity.

6.4.4 Loop Bioreactor

A loop bioreactor is composed of two distinct regions: a riser, in which the flow is upward, and a downcomer, which enables the fluid circulation. The downcomer region may be present inside the bioreactor, separated from the riser region by a cylinder or a plate, or outside.

The upflow in the riser section is promoted by the drag from a gas phase, usually injected at the bottom of the bioreactor. In some configurations, the hydrodynamic movement is aided by a pump. Most of the gas-liquid and liquid-substrate mass transfers occurs in this section. The gas sparger is responsible for injecting bubbles in the bioreactor, which causes the flotation effect in the column. The bubbles generation is the major target for performance enhancement: if nutrients (such as oxygen) are introduced along the gas phase, a higher mass flow rate should lead to greater bioculture activity, and also a lower residence time. Conversely, low gas flow rates could be introduced to save energy consumption while achieving the same oxygen transfer rate, due to the higher oxygen transfer efficiency [58].

6.4.5 Fluidized Bed Bioreactor

The use of fluidized bed reactors (FBR) is a relatively recent innovation. They are useful for processing high concentrations of biomass and high flow rates, or when loadings are variable. In a fluidized bed fermenter, the solid phase is composed of particles with a large surface area, and the immobilized cells (or enzymes) in which the desired biochemical reactions occur. The liquid phase is constituted by the culture medium required for cell growth and maintenance. The gas phase, when present, will have its constitution dependent on the type of the process: for anaerobic fermentation, it is constituted by an inert gas (such as nitrogen), or gases produced during fermentation (carbon dioxide, hydrogen, etc.). For aerobic fermentation, the gas phase is constituted by air or oxygen used for microbial respiration and/or carbon dioxide involved in the metabolism [11].

Immobilized cell particles are typically made from solid particles containing cells attached to their surface or to their internal pore structure. Cells that are physically attached to the outer surface of the solids will grow in a matrix consisting of multiple layers of cells and biopolymers excreted by them—the "biofilm." The composition of carrier particles with a layer of cells is also called "bioparticles." In some cases, when structures are formed by self-flocculation of cells without the support of carrier particles, the immobilized cell particles are called "bioflocs."

Due to the low liquid phase flow rate found in fermentation processes, the particles are mostly supported by the gas phase. However, when the liquid phase is externally recirculated by a pump or impeller, particles are supported by the liquid recirculation.

The design, operation, and control of this type of bioreactor are complex due to the complicated physiological and energetic behavior of microorganisms under process conditions. The good performance of a fluidized bed depends on its quality (degree of expansion), which is only achieved by controlling the feed velocity to be between the minimum fluidization velocity (u_{mf}) and the terminal velocity (u_t) of the bioparticles. Both velocities depend on the size and density, which vary with the accumulation of biomass on the support particle, affecting the fluidized bed [23].

6.5 Process Operation

Bioreactors can operate in either a batch or continuous mode. In batch mode, cell growth proceeds for a set period, necessary for the final product to reach a desired concentration. Subsequently, the contents are harvested, separated, recovered, and purified. Continuous culture production requires a sterile medium continuously entering and exiting the vessel at the same rate. This operation frequently maximizes productivity, although the aseptic steady-state environment is somewhat difficult to maintain.

The process operation is defined by standard operation procedures (SOP). If manual intervention is required, it should be correctly defined and described in the SOP. Depending on the process, unplanned process interventions may be not acceptable, and will trigger a formal investigation under the supervision of quality assurance (QA).

The typical fermenting process setup is described in the following:

6.5.1 Preliminary Tasks

Before use, the fermenter should be verified to check if the glass vessel is not damaged and if all o-rings, tubing, and sample ports are still in good shape. Moreover, probes must be calibrated, so that the control of the operation is performed with precise values:

- pH probes are calibrated using standard solutions of pH = 4 and pH = 7.
- DO electrodes are calibrated using deaerated water (DO = 0%) and saturated water (DO = 100%).
- Temperature probes are calibrated with melting ice (0 °C) and boiling water (100 °C).
- Level probes calibration is only necessary if the 0%-value or the 100%-value should be adjusted to suit specific measurement requirements; in this case, the probe can be calibrated for its full range, i.e., recording its lower level (0% level calibration) and high level (100% level). Other intermediate values can also be performed.
- Probes, electrodes, and all other devices that come into direct contact with internal components need to be sterilized.

Assessment of the foaming behavior of the selected fermentation system is also carried out, in order to characterize the pattern of foaming during fermentation. Foam stability can be estimated by measuring the foam evolution as a function of time, after a given volume of foam is produced. The effect of different level and type of antifoam can be assessed by comparing the resulting foam level.

6.5.2 Sterilization

Aseptic conditions are critical, since the contamination will decrease productivity, as the medium would have to support the growth of both the production organism and the contaminant. The contaminant organism and their toxic byproducts can contaminate the final product, altering its properties or even degrading it. Fermenters are typically made of noncorrosive and nontoxic material that can be repeatedly sterilized. Pipework should be constructed as simply as possible, avoiding horizontal pipes in which stagnant spaces and accumulation of material occur, leading to ineffective sterilization. Moreover, for long-term aseptic operation, welded joints should be used wherever possible, even though sections may have to be cut out and re-welded during maintenance and repair. Apart from continuous sterilizers, pumps are not a major concern. Inoculum may be transferred from a large laboratory flask to a seed fermenter using a peristaltic pump. Centrifugal pumps are used to pump non-sterile raw materials. These pumps and piping should be cleaned immediately after a transfer has been completed. Contamination may be avoided by using a pure inoculum to start the fermentation and sterilizing the medium, the fermenter, and all materials to be added during the fermentation. If the media is heat-sensitive, it may be sterilized by "cold sterilization" methods, such as filtration, radiation, ultrasonic treatment, and chemical treatment; otherwise, thermal sterilization (employing heat) is used. Growth media must be regulated and controlled through agitation or mixing, temperature, aeration, pH, DO, and antifoam control, as well as through maintaining and controlling other critical parameters. However, the sterilization itself can alter properties of the medium, which is not inert, resulting in a loss of nutritive quality. The loss of nutrient quality during sterilization is due to the interactions between nutrient components of the medium and degradation of heat-

labile components. With this caution in mind, sterilization may be done in batch or continuous mode. The advantages of continuous sterilization over batch sterilization are:

- Superior maintenance of medium quality
- Ease of scale-up
- Easier automatic control
- Reduction of surge capacity for steam
- Reduction of sterilization cycle time
- Reduction of fermenter corrosion

Conversely, the advantages of batch sterilization over continuous sterilization are:

- Lower capital equipment costs
- Lower risk of contamination
- Easier manual control
- Easier to use with media containing a high proportion of solid matter

The production of sterile air in the large volumes for aerobic fermentations is a particular problem. It is important when considering the costs associated with loss of fermentation due to contamination and production downtime due to filter failure. However, sterilization by heating is generally too costly for full-scale operation. Savings may also be made by introducing series of filter media, in which most of the series of filter material in which most of the contaminants present in the air stream are removed in the coarser filters at the first stages of filtration, thereby reducing the cost of renewing the more expensive high-efficiency filter media.

6.5.3 Media Preparation

Once all microorganisms require water, sources of energy, carbon, nitrogen, and mineral elements (and possibly vitamins plus oxygen if aerobic), any medium must provide these basic requirements. A medium should act as a source of nutrients to the biomass development. The elemental composition of the microorganism must be known to establish the elemental balance needed to formulate the medium. Trace elements (such as metals) may also be needed in smaller quantities.

Commonly used carbon sources are carbohydrates, oils and fats, hydrocarbons, and derivatives. Ammonia is the major nitrogen source. Ammonium salts and nitrates may also be used as nitrogen source, as well as to provide acid and alkaline conditions, respectively. Other products which may be added to the medium are inhibitors (to avoid the metabolism of an intermediate metabolic) and antifoams. Some of the most commonly used materials are presented in Table 6.6.

It is desirable that a medium meet as many as possible of the following criteria:

- Maximize the yield of product or biomass per gram of substrate used.
- Maximize the concentration of product or biomass.
- Provide the maximum rate of product formation.
- Minimize the yield of undesired products.
- Cause minimal problems during media making and sterilization.
- Cause minimal problems in other aspects of the production process particularly aeration and agitation, extraction, purification, and waste treatment.
- Have a consistent quality, and their supply should not change due to seasonality.

Table 6.6 Commonly used carbon and nitrogen sources, pH control, and antifoam materials [46]

Carbohydrates	Starch obtained from maize grain, which is the most widely available carbohydrate. Starch from other cereals, potatoes, and cassava. Malt (barley grains partially germinated and heat treated), which contains a variety of sugars besides starch. Sucrose, obtained from sugar cane and sugar beet. Lactose and crude lactose (milk whey powder). Corn steep liquor, which is a by-product after starch extraction from maize, contain lactic acid, small amounts of reducing sugars, and complex polysaccharides. Lignocellulose, which is the most abundant and lowest-cost biomass, can be used as alternative raw materials for fuel ethanol production.
Oils and fats	Vegetable oils (olive, maize, cotton seed, linseed, soya bean, etc.) are interesting carbon substrates due to their content of the fatty acids, oleic, linoleic, and linolenic acid. Glycerol trioleate is a suitable substrate for antibiotic production.
Hydrocarbons and derivatives	Development work has been done using n-alkanes for production of organic acids, amino acids, vitamins and cofactors, nucleic acids, antibiotics, enzymes, and proteins. Methane, methanol, and n-alkanes have all been used as substrates for biomass production.
Nitrogen	Inorganic nitrogen: ammonia gas, ammonium salts, or nitrates. Organic nitrogen may be supplied as amino acid, protein, or urea. A few microorganisms have an absolute requirement for amino acids. Other nitrogen compounds include corn-steep liquor, soya meal, peanut meal, cotton-seed meal, distillers' solubles, and meal and yeast extract.
pH control	Calcium carbonate, phosphates, ammonia, sodium hydroxide, and sulfuric acid.
Antifoams	Alcohols, esters, fatty acids and derivatives, silicones, and sulfonates.

Media are formulated according to the scale of application. Non-idealities present in a large fermenter, such as concentration gradients, could preclude the use of a laboratory medium. A medium with a high viscosity will also require more powerful pumps for effective stirring.

6.5.4 Inoculation

Inoculation is the process of introducing microorganisms to the medium. The addition of fresh inoculum to the culture can be a feasible way to maintain production when the cell is deactivated by age [41]. Moreover, when a certain type of microorganism is represented by a large population and continues to multiply, it generally predominates in its environment, preventing the growth of other microorganism types. This principle can be used to promote the cultivation of some species over others. In the past, wine and cheese makers applied this method without knowing exactly why, by pouring part of a previous batch of wine—the primer—into grape juice or even part of the cheese whey to fresh milk into the next batch.

The culture used to inoculate a fermentation must be in a healthy, active state, developed from selected species of organisms that should have fast and guaranteed production, thus minimizing the length of the lag phase in the subsequent fermentation. It must also be free of contamination. The initial inoculum size used depends on the scale, medium, and cell line used. The quantity of inoculum normally depends on the microorganism type. Examples of typical percentages on the medium volume are:

- *Streptomyces clavuligerus* (bacteria): 3%
- *Acremonium chrysogenum* (mold): 14–18.5%
- *Saccaromyces cerevisiae* (yeast): 10–15%

A relatively large inoculum volume may be used to reduce the length of the lag phase, producing the maximum biomass as fast as possible, thus increasing vessel productivity. However, the use of inoculum with 10% of the size of the production fermenter is an investment that must be justified in terms of productivity. The time of inoculum transfer must be determined experimentally; procedures may also be established, so that inoculation with an optimum culture may be achieved routinely.

6.5.5 Incubation

Once the cells are transferred to the seed fermenter, they are grown to a particular density near the end of their exponential phase. During the incubation, growth media must be regulated through the control of critical parameters such as agitation, temperature, aeration, pH, DO, and antifoam. The agitator is used to keep the mixture of cells and growth media inside the fermenters relatively homogeneous. It also increases oxygen mass transfer by decreasing the size of the oxygen bubbles. The fermenter is operated at a constant growth temperature to achieve the required growth rate. Since cells liberate heat during growth, a constant temperature is maintained using either cooling jackets surrounding the fermenters, coils inside the fermenter, or a combination of both. In addition, the cells may secrete acids as they metabolize, which decrease the pH level within the fermenter. The cells are grown to their mid to late exponential phase. At this point, the depletion of nutrients eventually causes the cells to enter their stationary growth phase. At this point, the cells are no longer capable of producing appreciable amounts of the desired protein and the fermentation is ended.

6.5.6 Harvest and Purification

Once the fermentation process is over, the fermentation broth containing the cells and the extracellular media is removed from the production fermenter. After the cells are harvested, protein needs to be separated from the cells that are produced through the downstream process of purification. The extraction and purification of fermentation products may be difficult and costly. The first stages for the recovery of an extracellular product aim to the separation of solid particles and microbial cells. This is usually done by centrifugation or filtration. Then, ultrafiltration, reverse osmosis, adsorption/ion-exchange/gel filtration or affinity chromatography, liquid-liquid extraction, two-phase aqueous extraction, or precipitation process is used to fractionate the broth into major fractions. Afterward, the product-containing fraction is purified by fractional precipitation and

crystallization to obtain a product which is highly concentrated and essentially free from impurities. Membranes and drying may be also employed to further concentrate the desired products.

6.6 Process Control

Bioreactors are multivariate systems, which require control systems involving many variables. In addition, they also exhibit nonlinear dynamics which is complicated due to the also nonlinear unsteady behavior. Hysteresis (transients during an increase in reactor feed rate far different than when correspondingly equivalent decreases in feed) may be observed, as well as multiple steady states for identical feed conditions, and "exotic dynamics", i.e., limit cycles, oscillatory transients, and longtime lags. Moreover, key variables desirable for monitoring and control are only measurable after large periods of time, or not at all. Parameters such as temperature, pH, demand of oxygen (or redox), agitation, pressure, foam control, auxiliary feed, or a combination of these controllers are of primary importance. The best values for these parameters can be evaluated through the use of chemostats, which are reactors primarily used to do basic physiological studies.

A chemostat (Fig. 6.9a) is a fermenter in which the feed medium containing all the nutrients is continuously fed at a constant rate while the cultured broth is simultaneously removed at the same rate. It is quite useful in the optimization of media formulation, in cultures adaptation, and to investigate the physiological state of microorganisms. When the optimum conditions are already established, adaptive control is of great importance, even on a commercial scale, because in ordinary fermentation there are several interrelated variables regarding culture conditions and raw materials.

Chemostats can operate with recycle of the cells in the effluent back to the reactor, to keep the cell concentration higher than the normal steady-state level (Fig. 6.9b). The advantages of cell recycling are the increase in productivity for biomass production and the increased stability by dampening perturbations of input stream properties.

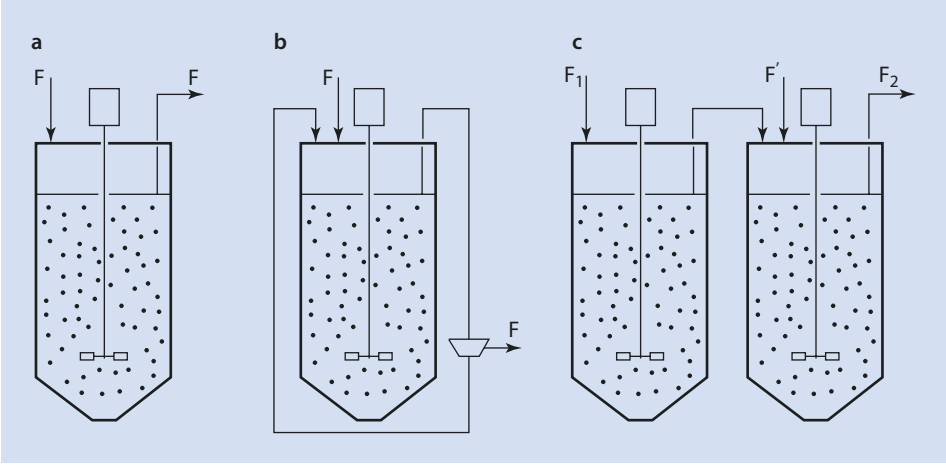


Fig. 6.9 **a** Common, **b** recycle, and **c** multistage chemostats

Multistage chemostats (Fig. 6.9c) are applicable to fermentations where the growth and product formation need to be separated into stages. In the first stage, only cell growth occurs, at the maximum rate, and no inducer is added for product formation. When cell concentrations are high, an inducer is added in the following chemostat to produce product at a higher rate. In the field of genetically engineered cells, this method allows for the production of a desired protein product. It is also useful when genetically engineered cells grow slower than original non-modified strain and when genetic instability causes the culture to lose ability to maintain production quality.

Although the complexity of the process and the number of control parameters make control problems in fermentation very difficult to solve, the solution of adaptive optimization strategies is worthwhile. The critical chemical, physical, and physiological parameters affecting cell cultures are [52]:

- Decrease of general critical nutrients, such as glutamine and glucose
- Increase of inhibitory metabolites, as ammonium ions and acids (pH control)
- Oxidation-reduction potential: chemical gas sparging, e.g., by adding cysteine, ascorbic acid, and sodium thioglycolate
- Decrease of dissolved oxygen: aeration volume, agitation speed, and oxygen contents of gas phase
- Temperature and pressure: optimum condition control
- Osmotic pressure: control of additional ion concentration
- Cell viability: contamination of cytotoxic compounds
- Cell density: increase of inhibitory metabolites and chalone-like substance, ratio of fresh medium, and cell adhesive surface
- Product concentration: cell density and induction conditions

Instrumentation applications have progressed to the point where advanced control strategies using sensors developed specifically for biological systems. Regardless of the measured parameter in commonly used control system hardware platforms, sensors can be:

- Installed inside the vessel
- Operate on continuously withdrawn samples
- Isolated, without contact with either the medium or gases
- In-line sensors, part of the fermentation equipment, in which the measured value is used directly for process control
- Online sensors, part of the fermentation equipment, in which the measured value is entered by an operator into the fermentation system for process control
- Off-line sensors, not part of the fermentation equipment, in which the intervention of an operator is essential for the actual measurement and for entering the measured value into the system for process control

The control of any parameter is most usually carried out in fermenter vessels specifically designed for the purpose and accommodating various working volumes depending on the yield and production requirements. The smallest units may incorporate an electrical heater and the feedstocks (e.g., nutrient and pH control agents) may be fed from flasks via peristaltic pumps. Larger vessels must have an integral jacket for controlling temperature via hot or cold water and allowing indirect sterilization using injected steam. Where larger quantities of feedstock are required, they may be held in separate pressurized tanks and fed via a “thrust pump” arrangement of valves.

A control system must therefore provide flexibility in the way in which accurate and repeatable control of the fermentation environment is achieved and will include the following features:

- Precise loop control with setpoint profile programming
- Recipe management system for easy parametrization
- Sequential control for vessel sterilization and more complex control strategies
- Secure collection of online data from the fermenter system for analysis and evidence

The control strategy for each type of reactor is considered special for the particular production unit, and there is an incredible difference in process dynamics and control strategies, and tuning rules varies with the reactor type. The type of dynamic response depends on the source of the dynamics, which drastically changes with the type of equipment. Reactors can be categorized as the type of dynamic response and relative dead time expected based on the reactor type. There are several automatic control systems suitable for different types of dynamic responses:

- On/off controllers, in which a device (a feed, heater, etc.) is turned on or off according to the difference between the desired value and the measured one
- Proportional controller (linear feedback control system), in which the correction applied to the controlled variable is proportional to the difference between the desired value and the measurement
- Proportional-integral controllers (PI), in which the output signal is determined by the integral of the error over operating time
- Proportional-derivative controllers (PD), which sense the rate of change of an error signal and contribute an output signal component that is proportional to the derivative of the error signal
- Proportional-integral-derivative controllers (PID), which applies a correction based on proportional, integral, and derivative terms
- A combination of PI, PD, and PID controllers

Control strategies to inherently maximize production rate or maintain reaction stoichiometry are based on reactor type. The production rate of plug flow gas reactors can be maximized by the temperature controller manipulating reactant feed rates. Reaction stoichiometry for two phase continuous reactors can be maintained by pressure and level control when the product and reactants are in different phases. When a reactant is recycled, the "snowballing effect" can be prevented by various control strategies depending upon the source and path of the recycle.

When the maximization of the production rate and the maintenance of reaction stoichiometry are not inherent, the necessary feedback control is obtained through control strategies along with PID tuning and the key PID feature. The PID feature is found to provide the output response needed to deal with discontinuous signals, such as those from analyzers and valves. The feature also enables directional move suppression needed to provide a slow gradual optimization and fast recovery for large fast disturbances and abnormal operation.

Control strategies for fed-batch and continuous operations are similar. A PID structure without integral action is used in cases where the batch response is unidirectional. Alternately, the controlled variable may be used to calculate the rate of change of a key process variable

to enable the use of integral action and the control of the batch profile and end point. A batch may be terminated or extended, estimating a future process variable value.

6.6.1 Agitation

Agitators are used in fermenters to provide uniform dispersion of small gas bubbles by shearing the inlet gas with fluid velocity gradients, maximize retention time of the gas in the broth by driving the gas bubbles to the bottom of the tank, and produce good bulk velocity and top-to-bottom turnover. In spite of the advantages promoted by intense agitation, the biological products of fermentation are living organisms composed of cells of limited resistance to fluid shear stress damage. The sensitivity of microbial cells to fluid shear varies greatly, hence the degree of agitation must be carefully designed and controlled in order to maintain satisfactorily yield and productivity of fermented products. Otherwise, the fermenter mixer should be designed and controlled in order to minimize fluid shear. The requirements of different cultures are presented in Table 6.7.

Products of aerobic fermentations are not usually shear sensitive and heat transfer can often be a major consideration in agitator design. In particular, there are very sensitive

Table 6.7 Fermentation and agitation requirements for several processes [16]

Fermentation type	Oxygen transfer rate	Shear sensitivity	Agitation requirements	Impeller types
Anaerobic				
Mammalian cell	Low	High (need low fluid shear)	Mild agitation Little or no gas dispersion Little or no heat transfer Liquid-solid mass transfer controlling Low viscosity Small-scale Shear sensitive	Low-shear propellers or hydrofoils running at slow speed
Aerobic				
Escherichia coli bacteria (yeast)	Moderate to high	Low (need high shear for gas dispersion)	Vigorous agitation Uniform gas dispersion Heat transfer required Gas-liquid mass transferring controlling Low viscosity Large-scale Not shear sensitive	Radial flow turbines or wide-blade hydrofoils (or a combination) at moderate to high speed
Mycelial				
Antibiotics	Moderate to high	Low (need high shear for gas dispersion)	Not shear sensitive but viscosity can be low to moderate	Radial-flow turbines or wide-blade hydrofoils (or a combination) at moderate to high speed

cells made using genetic engineering techniques that have more diverse requirements for cultivation, which has led to the proliferation of many mixing devices other than impeller mixers. This requires more attention in the development of equipment that can be maintained aseptic for long periods of time.

6.6.2 Foam Control

In most microbiological processes, foaming is a problem. It may be due to a component in the medium or some factor produced by the microorganism. Fermentation is often accompanied by foam formation, due to the high foaming tendency of solutions containing biomaterials. The most common cause of foaming is due to proteins in the medium, subjected to agitation. Foam is an agglomeration of gas bubbles separated from each other by a thin liquid film. As a high amount of gas is dispersed in liquid, the bulk density approaches that of the gas. Foaming during a fermentation may result in the loss of broth, cells, and product which are carried out via the air outlet as well as putting the fermentation at risk from contamination [46].

Foam control is costly and nonproductive, but it is a necessary operation, since a ruined batch can often represent hundreds of thousands of dollars in lost product. Filters must then be cleaned or replaced prior to the next batch. If foam gets into the vacuum pump, the pump must be taken apart and sterilized. In some cases, the foam will short-circuit the pump requiring its replacement (Fig. 6.10). Therefore, an effective foam control with reduced costs greatly influences the economics of the process.

The preferred method of foam control in fermentation is by the use of surface active chemical agents (antifoaming agents), although in a few instances, a mechanical foam breaker is used along with the chemical antifoam [15]. Screening of a range of antifoams during complete fermentation batches can help selecting the most appropriate compound for a specific process. Although recommended, this methodology is time demanding and depends heavily on the previous experiences of the operator, due to the great availability of industrial antifoaming compounds, to the specificities of the process and to the usual short time available for testing.

The foam formation is specific for each process, and its origin must be analyzed first. Also, how the foam affects the process and how it is detected will guide the choice of the best method.

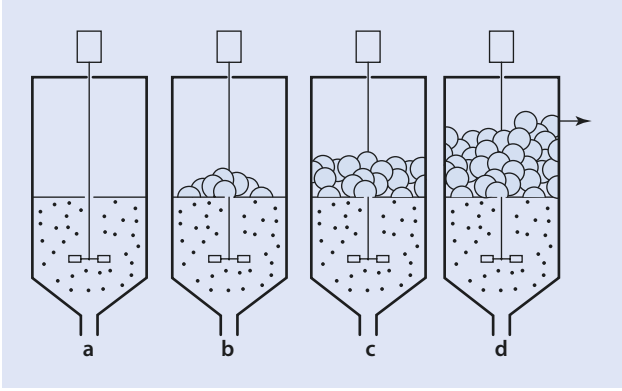


Fig. 6.10 **a**–**c** Foam formation, increasing in volume until **d** it reaches the fermenter outlet

Among the foam control methods, chemical agents are preferred due to the ease of operation and lower costs. However, foam breaking chemicals usually also lowers $k_L a$ values, reducing reactors' capacity to supply oxygen or other gases, and some cases they inhibit the cell growth. In these situations, mechanical foam breakers can be used to assist the process.

Antifoam oils may be synthetic, such as silicones or polyglycols, or natural, such as lard oil or soybean oil [8]. Either will substantially change the physical structure of foam, principally by reducing surface elasticity. While excessive foaming causes loss of material and contamination, excessive oil additions may decrease the product formation. Industrial antifoam systems usually operate automatically from level-sensing devices. The most common technologies are as follows:

- *Laser technology* offers flexibility, ease of setup and alignment, and cost. They are well suited for bulk and liquid, continuous, and switching applications.
- *Microwaves* have the ability to penetrate temperature and vapor layers, which may cause problems for other techniques. Guided microwave is also among the handful of technologies that works well with foam and sticky materials.
- *Tuning fork* is a vibrating-style sensor technology ideal for solid and liquid detection, including sticky substances and foam, as well as bulk powders. However, tuning forks are limited to detection applications only.
- *Ultrasonic devices* measure the duration and intensity of echoes from short bursts of energy. They are ideal for many types of liquids, but performance drops off in applications involving foam.
- *Optical prisms* are inexpensive and simple to set up and operate. However, they work only in clean translucent to transparent liquids.
- *Pressure sensors* measure the hydrostatic pressure of the liquid at the bottom of the tank with respect to atmospheric pressure to determine the level of the liquid. They are highly accurate, but their setup and calibration requirements make them more of a specialty solution in situations where all other options are not viable.
- *Capacitance level sensors* operate with a variety of solids, liquids, and mixed materials. However, it must be noted that not every capacitance sensor works with every type of material or vessel. Moreover, this technology is not always suitable for use with sticky fluids, as the probes are a contact-based measurement system.
- *Floats* are oldest and simplest measuring technology and can still be found in automated manufacturing processes. Being a mechanical device, however, floats offer little other advantage to users for all but the most basic applications.

A qualitative comparison among these sensor types is presented in Table 6.8.

Agitation should be run in a few cases at the superficial gas velocity levels expected in the full-scale plant, in order to simulate the typical foaming conditions. Moreover, the fermenter should be provided with enough head space to make sure the foam levels can be adequately controlled in the pilot plant.

6.6.3 Temperature Control

In the design and construction of a fermenter, there must be adequate provision for temperature control—which will affect the design of the vessel body. Heat will be produced by

Table 6.8 Common level-sensing devices applied to different phases [2]

	Liquids	Solids	Clear	Opaque	Sticky fluids/foam
Laser	Yes	Yes	No	Yes	Material dependent
Microwave	Yes	No	Yes	Yes	Yes
Tuning fork	Yes	Yes	Yes	Yes	Yes
Ultrasonic	Yes	Yes	Yes	Yes	No
Optical prism	Yes	No	Yes	No	No
Pressure	Yes	No	Yes	Yes	No
Capacitance	Yes	Material dependent	Yes	Yes	No
Float	Yes	No	Yes	Yes	Material dependent

microbial activity and mechanical agitation, and if it leads to temperatures not ideal for a particular manufacturing process, then heat may have to be added to or removed from the system. Temperature is one of the more traditional measurements in bioreactors so there is quite a variety of techniques. On laboratory scale, little heat is normally generated and extra heat has to be provided by placing fermenter in a thermostatically controlled bath or by use of internal heating coils or by a heating jacket through which water is circulated or a silicone heating jacket. However, above certain reactor size, the surface area covered by the jacket becomes too small to remove efficiently the heat produced by the fermentation. In this situation, internal coils must be used and cold water is circulated to achieve correct temperature.

Available heating/cooling approaches are:

- Welded to the outside—in the fermenter
- Jacket pillow plates
- External tube coils
- Pillow plate thermo channels
- Tube bundles (vertical calandria)

There are different types of temperature probes. Filled thermal systems take advantage of the thermal expansion coefficient of a sealed fluid to convert temperature into pressure or movement. These probes require essentially no power, but the receiver must be close to the sensor. If the sensor must be remote, thermocouple assemblies are a suitable choice. They are based on the thermoelectric principle, in which a closed circuit of two dissimilar metals generate an electromotive force when the metal junction points are at different temperatures. Thermocouples can measure a wide temperature range depending upon the alloys used but have a lack of sensitivity. Higher sensitivity can be obtained with resistance temperature detectors and thermistors, which are especially useful for measuring small temperature ranges. The principle behind these devices is the change of the resistance to current flow with temperature for the used materials.

6.6.4 pH Control

Certain microorganisms grow in particular pH only. Metabolic processes are typically susceptible to changes in pH, hence it is very essential to control pH in fermentation in order to grow the desired microorganisms for product formation. Deviations of pH as little as 0.2 may adversely affect a batch in some cases. pH control sensors are used in fermenter for periodically checking of pH, and if it differs from a reference value, then acid or alkali is automatically added into the solution to correct its acidity. The pH of the medium affects the ionic states of the components in the medium and on the cellular exterior surface. Shifts in pH probably affect growth by influencing the activity of permease enzymes in the cytoplasmic membrane or enzymes associated with enzymes in the cell wall. The pH range tolerated by most microorganisms ranges from 3 to 5. Rapid growth and/or reaction rates are normally in a much more narrow range, of ≤1. pH probes are packaged in a sterilizable inert casing with permeable electrode facings for direct insertion into the bioreactor. The measurement principle is the oxidation reduction potential of the hydrogen ion and the electrode materials are selected for that purpose [52]. A pH meter is a voltmeter that measures the electrical potential between two electrodes. One electrode is in contact with the solution, and the other is in contact with a reference solution. Most pH meters will only give accurate readings for solutions between −5 and 60 °C and may be damaged in solutions of pH > 12 or in the presence of high sodium ion concentrations. The calibration is made with pH 7, pH 10, and pH 4 standard buffers.

6.6.5 Valves and Steam Traps

Valves attached to a fermenter are used to control the flow of liquids and gases in a variety of ways. A wide range of valves are available, but not all of them are suitable for use in fermenter construction. These also have a significant role in fermenter productivity.

The different valves available are gate, globe, piston, needle, plug, ball, butterfly, pinch, diaphragm, check, pressure control, safety, and steam traps valves. Depending upon fermentation type and requirements, these valves are chosen in the design of a bioreactor with good productivity.

6.6.6 Sampling

The necessity of knowing and maintaining the current growth status and reactor broth conditions are among the more critical bioprocess operations in fermentation and cell culture. In order to control and optimize bioreactor functions, frequent aseptic sampling of these operations is required. The sampling procedure seems to be simple—one can imagine that simply opening a valve in the bioreactor vessel, obtaining the necessary fermentation broth for the sample, and then closing the valve. The primary goal is to maintain asepsis during the sampling process and that the integrity of the sample is compromised. However, many environmental and process factors may interfere with proper biosampling, thus it is not possible to guarantee that infection will not occur. Sampling construction should avoid contamination before and after sampling at all costs. At sites likely to cause contamination, sterilization should be performed immediately after sampling with the use of alcohol or steam.

The biosampling systems, manual or automated, have two major stages or platforms: the sample module, placed directly on the bioreactor; and the sample delivery components, usually at the sample testing destination. Components of a biosampling system may be of reusable or disposable design, or a combination of both. In general, hardware is considered for reusable sampling and where current reactor designs require them. Examples of components include:

- Peristaltic or rotor lobe pumps or pneumatic transport if the vessel is pressurized.
- Probes, which range in scope from reusable to disposable.
- Cleanable ports and valves that minimize hold-up/dead volume, cleanable.
- Components autoclavable and/or for steam sterilization.
- Components for automated sampling systems with single-use bags.
- Bags and tubing sets; they have typically small volumes, e.g., smaller than 100 mL.
- Welders and cutters, which detached sample bag assemble from bioreactor.
- Sample port devices with multiple connectors to bag assemblies.
- Sample bags and tubing sets typically used in manual operations, with application in automated systems.

Equipment and sampling components may be permanently installed as hardware or placed on a movable cart. Regardless of whether the system is manual or automated, the smaller the footprint the more acceptable the design will be. A possible method of sampling involves the use of a bladder made of silicone or a similar material, which is placed in the sampling tube and then clamped. In this way, the bladder is sterilized together with the bioreactor vessel, and remains in such a state until sampling. At the time of sampling, the clamp is removed, releasing the bladder. With the sample properly collected, the tip of the sample tube is immediately washed with alcohol.

There are several equipment hardware choices to be made for automated biosampling systems, from mechanical pumps, multiple bioreactor sampling capacities, tubing/piping, sample containers through to the analyzing instrumentation. The direct and automatic interface with specific instrumentation reduces operator contact and reduces the risk of sample and bioreactor contamination. Among the numerous testing possibilities once the sample has been either manually taken or automatically sampled and then transported for testing and analysis, there are:

- Autosamplers/fraction collector
- Media/nutrient content, metabolites, proteins, glucose biochemical analyzers
- High-performance liquid chromatography (HPLC) and gas chromatography/mass spectroscopy (GC/MS), ultraviolet (UV) detectors
- Level, pH, DO, OD, and temperature biosensors/probes
- Membrane chromatography/biochips—arrays/polymerase chain reactions (PCR)

Automated systems provide feedback control loops and functions and linkage to online monitoring of real-time bioreactor conditions with multiple bioreactor control. These automation attributes and testing capabilities enable more frequent and reproducible sample testing. This closer monitoring of bioreactor conditions can reduce sample volumes, decrease operator manipulation and exposure, leading to lower contamination rates and improved batch yields through better control, and increase of cell viability and density.

It must be noted that all permit effluent limitations, standards and prohibitions will be stated as limitations for all dischargers, including those discarded from samples.

6.7 Concluding Remarks

We have seen that several parameters can influence the efficiency of fermentative processes, which is optimum only for a restricted range of values. The estimation of optimal parameters is made by carrying out experimental assays but may be also obtained through the use of empirical correlations. However, the relationship between process variables among each other is complex and sometimes counterintuitive: higher stirring provides better mixing but also higher shear stresses and foaming. Fortunately, the adoption of adequate reactors, care with the process asepsis, medium culture, allied to an automatic control of the main parameters, favors the maintenance of healthy cultures and thus efficient processes. In addition, the reader should bear in mind that, like other processes, fermentation units may also present gradients of these parameters in their interior: for example, despite the attempt to ensure that a process occurs isothermally, small temperature variations may exist close to the walls. High shear stresses located near impellers are also expected. In this sense, more and more numerical techniques have been adopted to obtain more precise estimates in specific regions of fermenters.

Take Home Messages

- The operational requirements of fermentative processes may involve extra-operational considerations such as pretreatment of substrate, sterilization, media preparation, and separation of inhibitors.
- There are different operation modes, as well as different bioreactors configurations suitable for specific processes.
- Fermentation may be affected by physicochemical factors such as the presence of hydrodynamic stresses, temperature, pH, concentration of microbes, and chemical species (oxygen, salt, alcohol).
- The biosynthesis of value-added products can be inhibited by the production of primary or secondary metabolites.
- Agitation is important to promote oxygen transfer, as well as a greater contact between microorganisms and substrate; however, it may also be responsible for promoting cell damage.
- Control strategies should be employed to maintain operational conditions as close to optimal conditions as possible.
- Sterilization is important to grant aseptic conditions, which are critical since the contamination will decrease productivity and contaminate the final product.

References

1. Abbasi T, Tauseef SM, Abbasi SA. Anaerobic digestion for global warming control and energy generation—an overview. Renew Sustain Energy Rev. 2012;16:3228–48.
2. Allied Electronics & Automation. 8 common level sensing technologies. 2018. https://www.alliedelec.com/mkt/lp/articles/pdfs/8-Common_Level_Sensing-Technologies.pdf. Accessed 17 Apr 2018
3. Amerina MA, Berg HW, Kunkee RE, Ough CS, Singleton VL, Webb AD. Technology of wine making. 4th ed. Westport: AVI Publishing Company Inc.; 1979.
4. Bleoanca I, Bahrim G. Overview on brewing yeast stress factors. Rom Biotechnol Lett. 2013;18:8560.
5. Brennan L, Owende P. Biofuels from microalgae – a review of technologies for production, processing, and extractions of biofuels and co-products. Renew Sust Energ Rev. 2010;14:557–77. https://doi.org/10.1016/j.rser.2009.10.009
6. Brosse N, Dufour A, Meng X, Sun Q, Ragauskas A. Miscanthus: a fast-growing crop for biofuels and chemicals production. Biofuels Bioprod Biorefin. 2012;6(5):580–98.

7. Campesi A, Cerri MO, Hokka MO, Badino AC. Determination of the average shear rate in a stirred and aerated tank bioreactor. Bioprocess Biosyst Eng. 2009;32:241–8. https://doi.org/10.1007/s00449-008-0242-4
8. Chandrashekhar H, Rao JV. An overview of fermenter and the design considerations to enhance its productivity. Pharmacologyonline. 2010;1:261–301.
9. Chong M-L, Sabaratnam V, Shirai Y, Hassan MA. Biohydrogen production from biomass and industrial wastes by dark fermentation. Int J Hydrog Energy. 2009;34:3277–87. https://doi.org/10.1016/j.ijhydene.2009.02.010
10. Es I, Khaneghad AM, Barba FJ, Saraiva JA, Sant'Ana AS, Hashemi SMB. Recent advancements in lactic acid production – a review. Food Res Int. 2018;107:763–70. https://doi.org/10.1016/j.foodres.2018.01.001
11. Fan LS. Gas-liquid-solid fluidization engineering. Boston: Butterworth-Heinemann; 1989.
12. FDA – U.S. Federal Food and Drug Administration. Inspection technical guide: water activity (aw) in foods. 1984. https://www.fda.gov/ICECI/Inspections/InspectionGuides/InspectionTechnicalGuides/ucm072916.htm. Accessed 27 July 2018.
13. Fontana RC, Polidoro TA, Silveira MM. Comparison of stirred tank and airlift bioreactors in the production of polygalacturonases by Aspergillus oryzae. Bioresour Technol. 2009;100(19):4493–8. https://doi.org/10.1016/j.biortech.2008.11.062
14. Garcia-Ochoa F, Gomez E, Santos VE, Merchuk JC. Oxygen uptake rate in microbial processes: an overview. Biochem Eng J. 2010;49(3):289–307. https://doi.org/10.1016/j.bej.2010.01.011
15. Ghildyal NP, Lonsane BK, Karanth NG. Foam control in submerged fermentation: state of the art. Adv Appl Microbiol. 1988;33:173–222. https://doi.org/10.1016/S0065-2164(08)70207-7
16. Goldberg E. Handbook of downstream processing. London: Elsevier; 1997. 720p. ISBN: 978-94-009-1563-3.
17. Graminha EBN, Gonçalves AZL, Pirota RDPB, Balsalobre MAA, da Silva R, Gomes E. Enzyme production by solid-state fermentation: application to animal nutrition. Anim Feed Sci Technol. 2008;144(2008):1–22. https://doi.org/10.1016/j.anifeedsci.2007.09.029
18. Green EM. Fermentative production of butanol – the industrial perspective. Curr Opin Biotechnol. 2011;22:337–43. https://doi.org/10.1016/j.copbio.2011.02.004
19. Jönsson LJ, Alriksson B, Nilvebrant N-O. Bioconversion of lignocellulose: inhibitors and detoxification. Biotechnol Biofuels. 2013;6:16. https://doi.org/10.1186/1754-6834-6-16
20. Jun Y-S, Lee EZ, Huh YS, Hong YK, Hong WH, Lee SY. Kinetic study for the extraction of succinic acid with TOA in fermentation broth; effects of pH, salt and contaminated acid. Biochem Eng J. 2007;36:8–13. https://doi.org/10.1016/j.bej.2006.06.011
21. Kaur G, Srivastava AK, Chand S. Debottlenecking product inhibition in 1,3-propanediol fermentation by in-situ product recovery. Bioresour Technol. 2015;197(2015):451–7. https://doi.org/10.1016/j.biortech.2015.08.101
22. Koerich DM, Rosa LM. Optimization of bioreactor operating conditions using computational fluid dynamics techniques. Can J Chem Eng. 2017;95:199–204. https://doi.org/10.1002/cjce.22635
23. Koerich DM, Lopes GC, Rosa LM. Investigation of phases interactions and modification of drag models for liquid-solid fluidized bed tapered bioreactors. Powder Technol. 2018;339:90–101.
24. Koutinas AA, Vlysidis A, Pleissner D, Kopsahelis N, Garcia IL, Kookos IK, Papanikolaou S, Kwan TH, Lin CSK. Valorization of industrial waste and by-product streams via fermentation for the production of chemicals and biopolymers. Chem Soc Rev. 2014;43(8):2587–627. https://doi.org/10.1039/C3CS60293A
25. Kumar M, Gayen K. Developments in biobutanol production: new insights. Appl Energy. 2011;88:1999–2012. https://doi.org/10.1016/j.apenergy.2010.12.055
26. Lin Z, Liu H, Yan X, Zhou Y, Cheng K, Zhang J. High-efficiency acetone-butanol-ethanol production and recovery in non-strict anaerobic gas stripping fed-batch fermentation. Appl Microbiol Biotechnol. 2017;101:8029–39. https://doi.org/10.1007/s00253-017-8520-1
27. Liu R, Li J, Shen F. Refining bioethanol from stalk juice of sweet sorghum by immobilized yeast fermentation. Renew Energy. 2008;33(5):1130–5. https://doi.org/10.1016/j.renene.2007.05.046
28. Lonsane BK, Ghildyal NP, Budiatman S, Ramakrishna SV. Engineering aspects of solid state fermentation. Enzym Microb Technol. 1985;7:258–65.
29. Maddox IS, Qureshi N, Roberts-Thomson K. Production of acetone-butanol-ethanol from concentrated substrates using clostridium acetobutylicum in an integrated fermentation-product removal process. Process Biochem. 1995;30(3):209–15.
30. Martínez JL, Liu L, Petranovic D, Nielsen J. Pharmaceutical protein production by yeast: towards production of human blood proteins by microbial fermentation. Curr Opin Biotechnol. 2012;23(6):965–71. https://doi.org/10.1016/j.copbio.2012.03.011
31. Maurina GZ, Rosa LM, Beal LL, Baldasso C, Gimenez JR, Torres AP, Sousa MP. Effect of internal recirculation velocity in an anaerobic sequencing batch reactor (ASBR). Braz J Chem Eng. 2014;31(4):895–903. https://doi.org/10.1590/0104-6632.20140314s00002895

32. Metzner AB, Feehs RH, Ramos HL, Otto RE, Tuthill JD. Agitation of viscous Newtonian and non-Newtonian fluids. AICHE J. 1961;7(1):3–9. https://doi.org/10.1002/aic.690070103
33. Naik SN, Goud VV, Rout PK, Dalai AK. Production of first and second generation biofuels: a comprehensive review. Renew Sust Energ Rev. 2010;14:578–97. https://doi.org/10.1016/j.rser.2009.10.003
34. Nath K, Das D. Modeling and optimization of fermentative hydrogen production. Bioresour Technol. 2011;102:8569–81. https://doi.org/10.1016/j.biortech.2011.03.108
35. Ni M, Leung DYC, Leung MKH, Sumathy K. An overview of hydrogen production from biomass. Fuel Process Technol. 2006;87:461–72. https://doi.org/10.1016/j.fuproc.2005.11.003
36. Pandey A. Solid-state fermentation. Biochem Eng J. 2003;13:81–4. https://doi.org/10.1016/S1369-703X(02)00121-3
37. Paul EL, Atiemo-Obeng VA, Kresta SM. Turbulence in mixing applications. In: Handbook of industrial mixing: science and practice. Hoboken: Wiley-Interscience; 2004. p. 43–5.
38. Pilkington PH, Margaritis A, Mensour NA, Russel I. Fundamentals of immobilised yeast cells for continuous beer fermentation: a review. J Inst Brew. 1998;104:19–31. https://doi.org/10.1002/j.2050-0416.1998.tb00970.x
39. Potter NN. La ciencia de los alimentos. Westport: AVI Publishing Company Inc.; 1973.
40. Ravindra P. Value-added food: single cell protein. Biotechnol Adv. 2000;18(6):459–79. https://doi.org/10.1016/S0734-9750(00)00045-8
41. Razzak SA, Hossain MM, Lucky RA, Bassi AS, de Lasa H. Integrated CO_2 capture, wastewater treatment and biofuel production by microalgae culturing – a review. Renew Sust Energ Rev. 2013;27:622–53. https://doi.org/10.1016/j.rser.2013.05.063
42. Ren N, Guo W, Liu B, Cao G, Ding J. Biological hydrogen production by dark fermentation: challenges and prospects towards scaled-up production. Curr Opin Biotechnol. 2011;22:365–70. https://doi.org/10.1016/j.copbio.2011.04.022
43. Sánchez OJ, Cardona CA. Trends in biotechnological production of fuel ethanol from different feedstocks. Bioresour Technol. 2008;99(13):5270–95. https://doi.org/10.1016/j.biortech.2007.11.013
44. Show K-Y, Lee D-J, Chang J-S. Bioreactor and process design for biohydrogen production. Bioresour Technol. 2011;102:8524–33. https://doi.org/10.1016/j.biortech.2011.04.055
45. Sreenivasulu V, Jayaveera KN, Mallikarjuna RP. Solid-state fermentation for the production of L-asparaginase by *Aspergillus* sp. Res J Pharmacogn Phytochem. 2009;1:21–5.
46. Stambury PF, Whitaker A, Hall SJ. Principles of fermentation technology. 2nd ed. Oxford: Butterworth-Heinemann; 1995.
47. Stredansky M, Conti E. Succinoglycan production by solid-state fermentation with agrobacterium tumefaciens. Appl Microbiol Biotechnol. 1999;52(3):332–7. https://doi.org/10.1007/s002530051528
48. Thomas L, Larroche C, Pandey A. Current developments in solid-state fermentation. Biochem Eng J. 2013;81:146–61. https://doi.org/10.1016/j.bej.2013.10.013
49. Tugtas AE. Recovery of volatile fatty acids via membrane contactor using flat membranes: experimental and theoretical analysis. Waste Manag. 2014;34:1171–8.
50. Veljković VB, Lazić ML, Rutić DJ, Stanković MZ. The effects of some inorganic salts on the ethanol fermentation of Juniper berry sugars. Acta Biotechnol. 1989;9(4):379. https://doi.org/10.1002/abio.370090419
51. Vesvikar MS, Al-Dahhan M. Flow pattern visualization in a mimic anaerobic digester using CFD. Biotechnol Bioeng. 2005;89(6):719–32. https://doi.org/10.1002/bit.20388
52. Vogel HC, Todaro CL. Fermentation and biochemical engineering handbook: principles, process design, and equipment. 2nd ed. Westwood: Noyes Publications; 1997.
53. Wu B. Advances in the use of CFD to characterize, design and optimize bioenergy systems. Comput Electron Agric. 2013;93:195–208. https://doi.org/10.1016/j.compag.2012.05.008
54. YSI Inc./Xylem Inc. Oxygen solubility table. 2018. http://www.ysi.com. Accessed 20 Oct 2018.
55. Yuan H, Zhu N. Progress in inhibition mechanisms and process control of intermediates and by-products in sewage sludge anaerobic digestion. Renew Sust Energ Rev. 2016;58:429–38. https://doi.org/10.1016/j.rser.2015.12.261
56. Zhang Q, Wu D, Lin Y, Wang X, Kong H, Tanaka S. Substrate and product inhibition on yeast performance in ethanol fermentation. Energy Fuel. 2015;29(2):1019–27. https://doi.org/10.1021/ef502349v
57. Zheng H, Zhang D, Guo K, Dong K, Xu D, Wu Z. Online recovery of nisin during fermentation coupling with foam fractionation. J Food Eng. 2015;162(2015):25–30. https://doi.org/10.1016/j.jfoodeng.2015.04.006
58. Zimmerman WB, Hewakandamby BN, Tesar V, Bandulasena HCH, Omotowa OA. On the design and simulation of an airlift loop bioreactor with microbubble generation by fluidic oscillation. Food Bioprod Process. 2009;87:215–27. https://doi.org/10.1016/j.fbp.2009.03.006
59. Zupančič GD, Žgajnar Gotvajn A. Anaerobic treatment of pharmaceutical waste fermentation broth. Chem Biochem Eng Q. 2009;23(4):485–92. https://hrcak.srce.hr/45411

Bioreactor Scale-Up

Ehsan Mahdinia, Deniz Cekmecelioglu, and Ali Demirci

The original version of this chapter was revised. The correction to this chapter can be found at https://doi.org/10.1007/978-3-030-16230-6_11

A. Berenjian (ed.), *Essentials in Fermentation Technology*, Learning Materials in Biosciences,
https://doi.org/10.1007/978-3-030-16230-6_7

What You Will Learn in This Chapter

Scale-up is the process of expanding a fermentation process from a smaller-scale fermenter, where operational and production parameters have been studied, to a larger scale. Scale-up is perhaps one of the hardest and most complex steps of any fermentation process for engineers. Engineers must take into account all aspects that affect the integrity of the fermentation during the scale-up process. These aspects include physical (namely, heat and mass transfer phenomena such as oxygen transfer rates and mixing time), biochemical (such as medium compositions and rheology), and process (such as conditions of the pre-culture and inoculum) factors. Usually, engineers focus on the most effective factor and take in one of the common scale-up strategies. A constant height to diameter ratio for the bioreactors is perhaps the simplest and most common strategy. A constant oxygen transfer rate coefficient is usually the path for engineers, where oxygen is crucial for the fermentation and oxygen concentration limits product secretions. Also, a constant mixing time during the scale-up comes into play, where engineers usually face a viscous sophisticated fermentation broth. Yet, there are many other approaches that engineers can take. Often they blend these strategies and improvise a new strategy that serves the fermentation in the larger scale. However, engineers' ultimate goal is to maximize production and efficiency in plant-scale production.

7.1 Introduction

It would be a misconception to think that an ideal bench-top bioreactor in the lab could simply be enlarged to hundreds of thousands of liters in a fermentation plant, to give the same production performances and yields. Surely, every bioprocess engineer wishes it was that simple! But, will a 100,000-liter bioreactor with yeast converting cornstarch to bioethanol give out exactly the same outcome as the 2-liter bench-top glass bioreactor does? In this chapter, main factors and issues surrounding bioreactor scale-up in different types of bioreactors are discussed. Furthermore, we will review mass and heat transfer phenomena with an emphasis on their role in scale-up.

7.2 Scale-Up

A fermentation process development usually starts with *lab* scale with bench-top bioreactors (1–50 L) or even shake flasks (100–1000 mL) and microbial cells (few mL). After optimization has convinced that a bioprocess is feasible, then a *pilot*-scale process (50–10,000 L) is designed and implemented to establish the optimal operating conditions. It is only then, after successful lab- and pilot-scale studies, that the bioprocess is implemented on a *plant* scale (> 10,000 L) for commercial productions. Therefore, the step of setting up a bioprocess from a small to a large scale is called scale-up. The objective of scale-up is to transfer the optimal conditions obtained in small-scale bioreactors to the large-scale bioreactor. Scale-up studies are indispensable for the development of any fermentation process, so that an appropriate criterion for changing the scale can be established without damaging the kinetic behavior of microorganisms and hence the process performance. However, the kinetic behavior of microorganisms is affected by local environmental conditions such as nutrient concentration, pH, temperature, dissolved oxygen, etc. It is well known that microor-

ganisms are more sensitive to these environmental variables in a large scale. Therefore, small-scale trials have the tendency to overpredict the process performance at larger scales unless inconsistencies in scale-up are eliminated. This is where scale-up techniques become crucial. For this purpose, the environmental conditions affecting the bioprocess must be controlled. This is done by considering the physical, biochemical, and bioprocess factors. *Physical factors* include mass and heat transfer conditions, mixing (agitation) conditions, shear stress regimes, power consumption, pH, temperature, dissolved oxygen, etc. *Biochemical factors* mainly are medium components and their concentrations along with their physiochemical properties. Finally, *process factors* including pre-culture conditions, sterilization quality, and inoculation ratio also dictate how successful scale-up is implemented [1].

The traditional method for scale-up of a fermentation process involves determining the reactor geometry, impeller speed, and aeration rate of the large-scale bioreactor on the basis of the experimental results of the lab-scale bioreactor. The most common method of scale-up is based on maintaining geometric similarity of bioreactors. Once the volume of the large-scale bioreactor has been chosen, its geometric parameters, namely, tank height, tank diameter, and stirrer dimension, can be estimated. The typical methods of determining impeller speed and aeration rate are dependent on empirical correlations to keep relevant parameters constant with the change in scale. Evaluation of impeller speed is based on keeping agitation power input per unit volume (P/V), volumetric oxygen mass transfer coefficient ($k_L a$), or impeller tip velocity constant, whereas the aeration rate is estimated by using those criteria such as keeping equal superficial gas velocity, specific gas flow rate, or gas flow number. Engineers often keep one or several parameter(s) constant through scale-up and build their strategy around it.

Typical fermenters are made cylindrical and have a height to diameter ratio (H/D) between 2/1 and 3/1. This ratio can be kept constant as the simplest scale-up strategy. However, even that would not be so simple in reality. If diameter is increased by a factor of 5 and the ratio is kept constant, the vessel volume increases 125-fold, which would undoubtedly make fermentation in the larger scale quite distinct. Also, there are other parameters and ratios that can be considered besides the H/D ratio, which can be solely considered or in combinations. Table 7.1 presents some of these parameters and how the rest of them are affected when each is kept constant in scaling up from an 80-L pilot-scale fermenter to a plant-scale 10,000 L fermenter. As Table 7.1 indicates, if the impeller speed is maintained constant, the energy input of the impeller(s) will be 3,125 times higher in the large-scale fermenter. It is safe to presume that such a significant increase can change markedly the performance of the larger fermenter, e.g., with oxygen transfer rates, temperature gradient, etc.

Example Problem 7.1

Lysozyme is an anti-bacterial and anti-fungal enzyme widely present in animals, plants, and microorganisms. Recently, bioprocess engineers have focused on the production of human lysozyme via genetically modified microorganisms owing to the health concerns associated with egg lysozyme. The bioprocess parameters of the recombinant strain of *Kluyveromyces lactis* were studied in a lab-scale fermenter giving 110 IU/mL of lysozyme within 43 h. Engineers plan to produce annually 3×10^{12} IU of the enzyme. Thus, what should the main and inoculum fermenters look like if a $H/D = 3$ and 20% headspace are assumed and if the fermenters are to be operated for 11 months per year?

Table 7.1 Some common scale-up parameters and their after effects

			Production fermenter: 10,000 L			
Scale-up riterion	**Designation**	**Pilot-scale Fermenter 80 L**	**Constant, P_0/V**	**Constant, N**	**Constant, $N.D_i$**	**Constant, Re**
Energy input	P_0	1.0	125	3125	25.0	0.2
Energy input/ volume	P_0/V	1.0	1.0	25	0.2	0.0016
Impeller rpm	N	1.0	0.34	1.0	0.2	0.4
Impeller diameter	D_i	1.0	5.0	5.0	5.0	5.0
Pump rate of impeller	Q	1.0	42.5	125	25.0	5.0
Pump rate of impeller /volume	Q/V	1.0	0.34	1.0	0.2	0.04
Max. impeller speed (max. shear stress)	$N.D_i$	1.0	1.7	5.0	1.0	0.2
Reynold number	$ND_i^2\rho/\mu$	1.0	8.5	25.0	5.0	1.0

Adapted from Shuler et al. [13]

7

Solution:

The complete batch period is an important parameter used to determine the number of batches that can be processed and hence, the total production per year. This time period includes medium preparation, sterilization time, fermentation time, product harvest, and cleaning time, which in this case can be assumed to be 10 h. Therefore, each batch will take roughly 53 h to complete. Thus we have:

$$\text{The number of units of lysozyme produced per month} = \frac{3\times10^{12}\ \text{IU}}{11\ \text{months}} = 2.7\times10^{11}\ \text{IU / month.}$$

Volume of the broth required to achieve the projected production per month

$$= \frac{2.7\times10^{11}\ \dfrac{\text{IU}}{\text{month}}}{110{,}000\ \text{IU / L}} = 2.5\times10^{6}\ \text{L / month;}$$

the number of batches per month = 30 days per month × 24 hours per day/53 hours per batch ≈ 13 batches per month ≈ 156 batches per year; and

$$\text{volume per batch} = 2.5\times10^{6}\ /\ 13 = 192{,}307\ \text{L / batch}$$

Since the volume is high, two bioreactors will be used to produce the projected amount of lysozyme. Volume of each bioreactor =192,307/2 = 96,154 L

Thus, for a working volume of 96,154 L and assuming 20% headspace, the total volume of the bioreactor is the following:

$$\text{total volume of bioreactor} = 96{,}154 + 96{,}154 \times \frac{20}{100} = 115{,}385\ \text{L or} \sim 116\ \text{m}^3$$

Assuming that the height to diameter ratio is 3:1, the reactor dimensions are calculated as follows:

$$H = 3D$$

$$V = \pi\left(\frac{D^2}{4}\right)H$$

$$115.385 = \pi\left(\frac{D^2}{4}\right)(3D)$$

$$D = 3.66\ \text{m and}\ H = 11\text{m}$$

The width of baffles can be assumed to be 10% of the diameter of the bioreactor. Therefore, the width is

$$W = \frac{10}{100} \times 3.66 = 37\ \text{cm}$$

Impeller diameter is calculated assuming that it is 20% of the tank diameter.

$$D_i = \frac{20}{100} \times 3.66 = 73\ \text{cm}$$

As for the pre-fermenter, since the strain used is yeast with a relatively long lag-phase time, the inoculation percentage is assumed as 5%. The working volume of the pre-fermenter is thus 4808 L, and with 20% headspace and the total prefermenter volume, we have:

$$\text{total volume of bioreactor} = 4808 \times 1.2 = 5770\ \text{L or}\ 5.77\ \text{m}^3$$

$$H = 3D$$

$$V = \pi\left(\frac{D^2}{4}\right)H$$

$$5.77 = \pi\left(\frac{D^2}{4}\right)(3D)$$

$$D = 1.35\text{m and}\ H = 4\text{m}$$

Similarly, the baffle width will be 13.5 cm and impeller diameter will be 27 cm (Fig. 7.1).

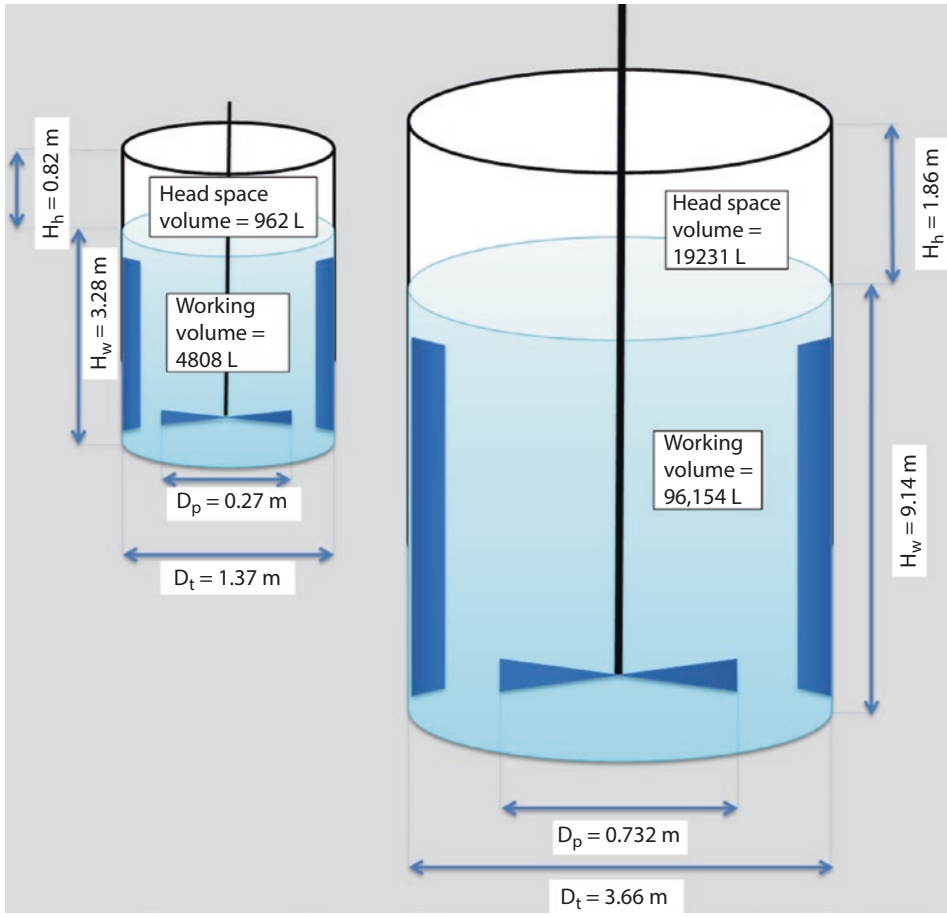


Fig. 7.1 Schematic drawing of prefermenter and main fermenter for ▶ Problem 7.1

7.2.1 Physical Properties

Mass and heat transfer along with mixing conditions (or flow behavior) are the physical properties that affect scale-up strategies. For instance, in most fermentation processes, the heat generated by catabolism is taken into account with heat transfer rates in large-scale bioprocesses. Also, oxygen transfer rate (OTR) that controls oxygen uptake rates (OUR), especially in aerobic fermentations where oxygen is limiting, is another crucial factor in scaling up. Here we will discuss the effects of these mass transfer phenomena on scale-up strategies.

Aeration and Agitation

The oxygen uptake rate (OUR) in a fermentation process is expressed as

$$\mathrm{OUR} = q_{O_2} x = k_L a \left(C_L^{*} - C_L \right) \tag{7.1}$$

Table 7.2 Specific oxygen uptake rates for some microorganism and human cells

Organism	qO_2 (mole O_2 kg X^{-1} h^{-1})
Bacteria	
Escherichia coli	0.9–23
Bacillus acidocaldarius	3.1–31.2
Rhodococcus erythropolis	0.2–4.3
Yeast	
Candida bombicola	0.3–1.0
Saccharomyces cerevisiae	8
Hansenula anómala	0.8
Animal cells (adherent cells)	
Lung To (Human embryonic lung cells	0.24 mmol $h^{-1}(10^9 cells)^{-1}$
Conjunctiva (Human eye cells)	0.28 mmol $h^{-1}(10^9 cells)^{-1}$

Adapted from Shuler et al. [13]

where x is the biomass concentration (g/L), q_{O_2} is the specific oxygen uptake rate (gmol O_2/g. h), C_L^* is the saturation oxygen solubility under the given conditions, and C_L is oxygen concentration at the given time. The combination of these two variables determines the total oxygen demand of the biomass. Table 7.2 depicts typical specific oxygen uptake rates for some common cells.

Note how the uptake rates can be different from strain to strain. *Candida bombicola* and *Saccharomyces cerevisiae* are both yeast strains, but as you can see, *Saccharomyces cerevisiae* can consume over eight times as much oxygen under aerobic conditions. Also, it is interesting to note how bacterial strains can adapt to different conditions and how the uptake rates differ accordingly. See how the uptake rate can be over 23- and 10-folds for *Escherichia coli* and *Bacillus acidocaldarius*, respectively (Table 7.2) [2].

The other side of Eq. 7.1, however, represents the oxygen supply to the fermenter, which, like any other mass transfer phenomenon, is composed of a driving force $(C_L^* - C_L)$ (g/L) combined with a constant value, which is the volumetric oxygen transfer coefficient k_La (h^{-1}). For the cases where aeration is critical, it is most common to monitor and consider a constant oxygen transfer rate (OTR) throughout the scale-up. This is, for instance, mostly the case in novel applications of biofilm reactors for value-added products, where higher cell densities are utilized for higher production rates but at the same time oxygen diffusion into the biofilm matrices becomes critical. Thus, in such aerobic fermentations, k_La becomes the crucial factor in scale-up. As it is obvious from Eq. 7.2, k_La is the dominant factor that reflects the effects of agitation, viscosity, impeller(s) and bubbles' dimensions and shapes, rheological properties of the liquid phase, and even the working volume. This is due to the fact that the term in the parenthesis $(C_L^* - C_L)$ is mainly a function of temperature and oxygen partial pressure only. Of course, nowadays it is possible and even

preferable to directly measure the dissolved oxygen (DO) levels in bioreactors and simply maintain them through the scale-up process; yet, this is not usually the option that bioprocessing engineers prefer. Rather, engineers follow a constant $k_L a$ coefficient, which they can empirically estimate using Eq. 7.2 [8].

$$k_L a = k\left(\frac{P_g}{V_w}\right)^{\alpha}\left(v_s\right)^{\beta}\left(N\right)^{\gamma} \tag{7.2}$$

where k, α, β,and γ are empirical constants, P_g is the gassed power output, V_w is the working volume, v_s is the superficial exit gas velocity, and N is the speed of impeller(s). The constant k in the equation for lab-scale stirred-tank bioreactors with Newtonian fluid regimes is 0.001–0.005 for $k_L a$ expressed in mmol/L-h-atm, depending on the geometry of the vessel and impeller(s). In common small-scale reactors, $\alpha = 0.4$ and $\beta = \gamma = 0.5$ are good assumptions. As it can be seen, besides the aeration rates, the agitation rates also play a direct effect on the oxygen transfer rate and mass transfer phenomena in the process in general. Again, in small-scale Newtonian regimes, the dependency on agitation rates is negligible and therefore the $(N)^{0.5}$ term is eliminated. Then the constant term varies even more from geometry to geometry. The term $\frac{P_g}{V_w}$ can be defined as the volumetric agitation power output. Sometimes, it is more convenient to use a scale-up model based on keeping this parameter constant, since the data obtained in bench-top or pilot-scale fermenters are often available and can be easily used for scale-up. The other reason is that other effective rheological properties such as viscosity are incorporated in the term $\frac{P_g}{V_w}$. When there is a need for a higher DO level, engineers choose either a stronger agitator motor or a smaller working volume. Such a model simplifies the scale-up strategy; however, there is still a need to have an estimate of the required power input. It is usually easier to determine the power input for a similar ungassed fermenter and then empirically transcend it to the aerated vessel. For this purpose, an empirical equation such as the following is used:

$$P_g = K\left(\frac{P_u{}^2 N d^3}{Q^{0.56}}\right)^{0.45} \tag{7.3}$$

where K is a constant, P_u is the power required in the ungassed vessel, d is the impeller(s) diameter, and Q is the volume of air supplied per minute per volume of the liquid in the vessel for which the unit is often referred to as vvm. The constant term in this equation is strictly dependent on the geometry of the tank and impeller(s) and the operational conditions.

Although these empirical correlations give fair estimations on how oxygen uptake rates are affected by scale-up, they are only estimations. For many cases of Newtonian or non-Newtonian systems, these correlations are unable to cope with the significant effects resulting from changes in viscosity that usually occur in fermentation processes or medium compositions. For such effects unfortunately, it is not easy to make such estimations. Also, many times when viscosity increases to high levels as the fermentation process proceeds, engineers simply water down the composition to counter it.

Moreover, the presence of salts and surfactant or antifoam agents in the fermentation broth, which is very common, not only can significantly affect $k_L a$ but also affect oxygen solubility in the broth. Yet, the driving force term in Eq. 7.1 $(C_L^* - C_L)$ is not controllable but providing good mixing to avoid the increasing liquid film resistance around the gas bubbles or keeping the operating temperatures as low as possible will work. The example problems below show how $k_L a$ can be measured in real-time fermenters to help the scale-up or design strategies.

Example Problem 7.2

Assume you have a 2-L bench-top bioreactor for production of L-asparaginase by *Candida utilis* and you would like to scale it up to a pilot scale of 100 L using a conventional medium composition. You have two different impellers. How can you determine which impeller is better for aerobic fermentation?

■ **Solution:**

Candida utilis is a yeast that excretes the enzyme L-asparaginase under highly aerobic conditions. It is safe to presume that oxygen transfer rates are limiting and thus critical in this case. Therefore, whichever impeller that provides higher $k_L a$ values with the same power inputs is the winner. Also we must determine $k_L a$ values in both bioreactors.

There are basically three methods for determining $k_L a$ in a bioreactor: unsteady-state, steady-state, and the sulfite method.

In the *unsteady-state* method, we fill fermenters with the medium (or as an easier estimation with DI water) and accurately measure the C_L^* *and* C_L using a calibrated DO probe. By sparging the medium with nitrogen for an ample period of time, we make sure that there is no oxygen left in it. At this time C_L is zero. Then, we start sparging it with air and measure C_L values over time until we eventually reach C_L^*. If mixing is sufficiently robust, whichever impeller enables us to reach the C^*value faster is essentially better. Nonetheless, we have

$$\frac{dC_L}{dt} = k_L a\left(C_L^* - C_L\right) \tag{7.4a}$$

and so knowing that C_L^* is constant with constant temperature we have:

$$\frac{-d\left(C_L^* - C_L\right)}{\left(C_L^* - C_L\right)} = k_L a\, dt \tag{7.4b}$$

thus

$$\ln\left(C_L^* - C_L\right) = -k_L a\, t \tag{7.5}$$

Therefore, a plot of $\ln(C_L^* - C_L)$ *versus time can be drawn, whose slope is the* k_La term. In other words, the steeper slope means higher $k_L a$ values.

Similarly, in the *steady-state* method, the fermenter is filled with the broth and oxygen is monitored as fermentation takes place. Oxygen is uptaken by the biomass and at the same time it is provided by aeration. Thus, from Eq. 7.1 we have

$$k_L a = \frac{\text{OUR}}{C_L^* - C_L} \tag{7.6}$$

Assuming that the fermentation process is slow enough that the OUR values at the time of measurements are constant, it is possible then to measure the OUR value in the bioreactor or externally in a respirometer, and then C_L^* *and* C_L must be accurately measured at the same time to calculate the respective k_La value. However, this requires exact measurement of OUR and oxygen concentrations. Moreover, if later on we decide to scale up the process to industrial-scale fermenters with tens or hundreds of thousands of liter volumes, turning the huge fermenter into a real-time respirometer is easier said than done! In such large volumes, even oxygen concentration measurements become a challenge when the mixing is never ideal anymore and the liquid depth and hydrostatic pressure are significant at the sparger level at the bottom making the C_L^*values significantly different at the bottom compared to the headspace zones.

As a result of such complications, the more common *sulfite* method comes into play, where the fermenter is filled with the medium along with sulfite anions (SO_3^{2-}). Sulfite anions irreversibly and readily react with dissolved oxygen and are converted to sulfate (SO_4^{2-}) *until* C_L *reaches zero.*

$$2SO_3^{2-} + O_2 \xrightarrow{Cu^{2+}} 2\,SO_4^{2-}$$

Then, we start aerating the mixture and as oxygen is dissolved, it is instantly consumed. As the stoichiometry of the above reaction dictates, the rate of sulfate formation doubles the rate of oxygen consumption and considering Eq. 7.4a we have

$$\frac{dC_{SO_4}}{dt} = 2\ k_L a\, C^* \tag{7.7a}$$

and

$$k_L a = \frac{dC_{SO_4}/dt}{2\,C^*} \tag{7.7b}$$

Thus, by monitoring the sulfate concentration in the mixture over a short period of time and calculating the rate of change we can calculate the k_La value. Note that determining sulfate concentration in the mixture is essentially easier than monitoring dissolved oxygen concentrations or finding OUR values, but, obviously the sulfite method cannot be applied to a real-time fermentation process unlike the steady-state method [5].

Example Problem 7.3

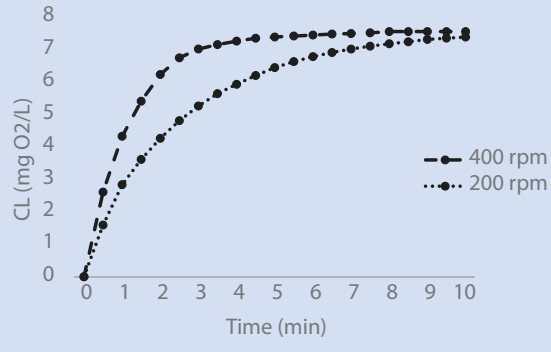
In order to scale up a prototype fermenter design for citric acid fermentation using *Yarrowia lipolytica* from bench-top to pilot scale, we wish to use a constant k_La approach. To obtain information about the coefficient, the bench-top bioreactor was filled with DI water at 30°C. Nitrogen was sparged into the vessel with 500 rpm agitation to take out all the dissolved oxygen. Then, air was introduced instead of nitrogen and agitation was set at 200 rpm and another time at 400 rpm. Percentage of DO saturation was recorded versus time as shown in Table 7.3. Using these findings and oxygen solubility, determine the k_La values for each agitation regime.

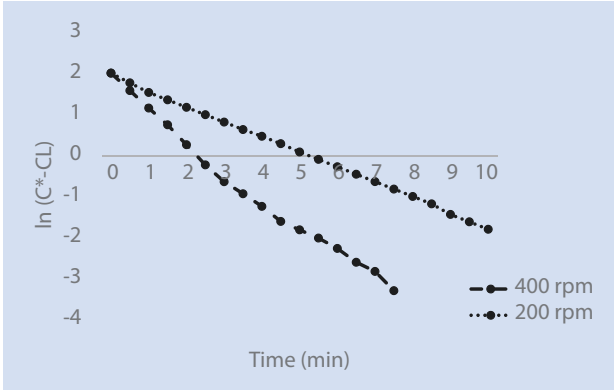
From the oxygen solubility table, we can see that C_L^* = 7.559 mg/L. Therefore, the data for 200 and 400 rpm can be processed to prepare Tables 7.4, 7.5, and 7.6, respectively.

Table 7.3 Diffused oxygen measurements within 10 minutes of aeration with 200 rpm and 400 rpm agitation

Time (min)	%DO (200 rpm)	%DO (400 rpm)
0	0.0	0
0.5	21.1	34.6
1	37.5	57.3
1.5	47.9	71.6
2	56.5	82.5
2.5	63.7	89.3
3	69.7	92.9
3.5	74.7	94.7
4	78.5	96.1
4.5	82.1	97.3
5	85.3	97.8
5.5	87.8	98.2
6	89.8	98.6
6.5	91.5	99.0
7	92.9	99.2
7.5	94.1	99.5
8	95.0	100.0
8.5	95.9	100.0
9	96.8	100.0
9.5	97.3	100.0
10	97.8	100.0

Then, plotting C_L and $\ln(C_L^* - C_L)$ changes versus time at 200 and 400 rpm we have:





and from Eq. 7.4b, for 200 rpm, we have:

$$k_L a = 0.3714 \text{ min}^{-1} \text{ or } 22.28 \text{ h}^{-1}$$

And for 400 rpm we have

$$k_L a = 0.6828 \text{ min}^{-1} \text{ or } 40.96 \text{ h}^{-1}$$

As it can be seen, by doubling the agitation rate from 200 to 400 rpm, the $k_L a$ coefficient and thus OUR is almost doubled as well. This is the simplest example of a bench-top bioreactor with DI water. Things can be much more complex as size increases and complex broth compositions are used. Still, this example clearly shows how complicated operational physical properties can be formed in a bioreactor, and all without exception must be taken into account carefully while designing or scaling up a fermentation process. See how nicely the 200 rpm points in the second plot fall into a straight line and 400 rpm ones don't… why??

Table 7.4 Solubility of oxygen in water exposed to water-saturated air at atmospheric pressure (101.3 kPa)

Temperature (°C)	Oxygen Solubility mg/L					
	Chlorinity: 0	5.0	10.0	15.0	20.0	25.0
0.0	14.621	13.728	12.888	12.097	11.355	10.657
1.0	14.216	13.356	12.545	11.783	11.066	10.392
2.0	13.829	13.000	12.218	11.483	10.790	10.139
3.0	13.460	12.66	11.906	11.195	10.526	9.897
4.0	13.107	12.335	11.607	10.920	10.273	9.664
5.0	12.770	12.024	11.320	10.656	10.031	9.441
6.0	12.447	11.727	11.046	10.404	9.779	9.228
7.0	12.139	11.442	10.783	10.162	9.576	9.023
8.0	11.843	11.169	10.531	9.930	9.362	8.826
9.0	11.559	10.907	10.290	9.707	9.156	8.636

Table 7.4 (continued)

Temperature (°C)	Oxygen Solubility mg/L					
10.0	11.288	10.656	10.058	9.493	8.959	8.454
11.0	11.027	10.415	9.835	9.287	8.769	8.279
12.0	10.777	10.183	6.621	9.089	8.586	8.111
13.0	10.537	9.961	9.416	8.899	8.411	7.949
14.0	10.306	9.747	9.218	8.716	8.242	7.792
15.0	10.084	9.541	9.027	8.540	8.079	7.642
16.0	9.870	9.344	8.844	8.370	7.922	7.496
17.0	9.665	9.153	8.667	8.207	7.770	7.356
18.0	9.467	8.969	8.497	8.049	7.624	7.221
19.0	9.276	8.792	8.333	7.896	7.483	7.090
20.0	9.092	8.621	8.174	7.749	7.346	6.964
21.0	8.915	8.456	8.021	7.607	7.214	6.842
22.0	8.743	8.297	7.873	7.470	7.087	6.723
23.0	8.578	8.143	7.730	7.337	6.963	6.609
24.0	8.418	7.994	7.591	7.208	6.844	6.498
25.0	8.263	7.850	7.457	7.083	6.728	6.390
26.0	8.113	7.711	7.327	6.962	6.615	6.285
27.0	7.968	7.575	7.201	6.845	6.506	6.184
28.0	7.827	7.444	7.079	6.731	6.400	6.085
29.0	8.691	7.317	6.961	6.621	6.297	5.990
30.0	7.559	7.194	6.845	6.513	6.197	5.896
31.0	7.430	7.073	6.733	6.409	6.100	5.806
32.0	7.305	6.957	6.624	6.307	6.005	5.717
33.0	7.183	6.843	6.518	6.208	5.912	5.631
34.0	7.065	6.732	6.415	6.111	5.822	5.546
35.0	6.950	6.624	6.314	6.017	5.734	5.464
36.0	6.837	6.519	6.215	5.925	5.648	5.384
37.0	6.727	6.416	6.119	5.835	5.564	5.305
38.0	6.620	6.316	6.025	5.747	5.481	5.228
39.0	6.515	6.217	5.932	5.660	5.400	5.152
40.0	6.412	6.121	5.842	5.576	5.321	5.078
41.0	6.312	6.026	5.753	5.493	5.243	5.005

(continued)

Table 7.4 (continued)

Temperature (°C)	Oxygen Solubility mg/L					
42.0	6.213	5.934	5.667	5.411	5.167	4.933
43.0	6.116	5.843	5.581	5.331	5.091	4.862
44.0	6.021	5.753	5.497	5.252	5.017	4.793
45.0	5.927	5.665	5.414	5.174	4.944	4.724
46.0	5.835	5.578	5.333	5.097	4.872	4.656
47.0	5.744	5.493	5.252	5.021	4.801	4.589
48.0	5.654	5.408	5.172	4.947	4.730	4.523
49.0	5.565	5.324	5.094	4.872	4.660	4.457
50.0	5.477	5.242	5.016	4.799	4.591	4.392

Table 7.5 Oxygen transfer rate terms for 200 rpm

Time	%DO	C_L	$C_{L^*} - C_L$	$\ln(C_{L^*} - C_L)$
0.0	0.0	0.000	7.559	2.023
0.5	21.1	1.593	5.966	1.786
1	37.5	2.838	4.721	1.552
1.5	47.9	3.618	3.941	1.371
2	56.5	4.269	3.290	1.191
2.5	63.7	4.813	2.746	1.010
3	69.7	5.267	2.292	0.830
3.5	74.7	5.645	1.914	0.649
4	78.5	5.935	1.624	0.485
4.5	82.1	6.203	1.356	0.304
5	85.3	6.451	1.108	0.103
5.5	87.8	6.634	0.925	−0.078
6	89.8	6.787	0.772	−0.258
6.5	91.5	6.914	0.645	−0.439
7	92.9	7.021	0.538	−0.619
7.5	94.1	7.110	0.449	−0.800

Table 7.5 (continued)

Time	%DO	C_L	$C_{L^*} - C_L$	$\ln(C_{L^*} - C_L)$
8	95.0	7.184	0.375	−0.981
8.5	95.9	7.246	0.313	−1.161
9	96.8	7.317	0.242	−1.418
9.5	97.3	7.357	0.202	−1.599
10	97.8	7.390	0.169	−1.779

Table 7.6 Oxygen transfer rate terms for 400 rpm

Time	%DO	C_L	$C_{L^*} - C_L$	$\ln(C_{L^*} - C_L)$
0.0	0	0.000	7.559	2.023
0.5	34.6	2.612	4.947	1.599
1	57.3	4.331	3.228	1.172
1.5	71.6	5.412	2.147	0.764
2	82.5	6.236	1.323	0.280
2.5	89.3	6.750	0.809	−0.212
3	92.9	7.022	0.537	−0.622
3.5	94.7	7.158	0.401	−0.915
4	96.1	7.264	0.295	−1.221
4.5	97.3	7.355	0.204	−1.589
5	97.8	7.393	0.166	−1.794
5.5	98.2	7.423	0.136	−1.995
6	98.6	7.453	0.106	−2.246
6.5	99.0	7.483	0.076	−2.582
7	99.2	7.499	0.060	−2.806
7.5	99.5	7.521	0.038	−3.276
8	100.0	7.559	0.000	–
8.5	100.0	7.559	0.000	–
9	100.0	7.559	0.000	–
9.5	100.0	7.559	0.000	–
10	100.0	7.559	0.000	–

Another common parameter for scale-up is the impeller power consumption per volume P/V. This strategy is perhaps the oldest scale-up strategy that has been used ever since penicillin production revolutionized the early twentieth century. Usually a ratio of 1.0:2.0 KW/m^3 is simply maintained. However, such simplification in massive scale-up operations leads to significant energy inefficiency, which is certainly unacceptable. Therefore, more complex alterations emerge. For instance, instead of a constant P/V ratio, impeller power number (N_p) is defined, measured, and held constant.

$$N_p = \frac{2\pi\left(M - M_d\right)}{\rho N^2 d^5} \tag{7.4}$$

In the above equation, M is torque (with full working volume of DI water) (N·m), M_d is torque (empty vessel) (N·m), ρ is broth density, N is agitation speed (rpm), and d is impeller diameter. Note that as the impeller gets larger, the power number decreases drastically. The only perquisite to this strategy is that the torque must be carefully measured and it is important to measure the net impeller torque without bearing resistance. A constant power number scale-up strategy in some cases may prove more energy efficient than the constant P/V ratio strategy. Nevertheless, the power number can be obtained alternatively to calculate the P/V ratio as:

$$P/V = \frac{N_p \rho N^3 d^5}{V} \tag{7.5}$$

As the flow regimes change with the Reynolds number, the power number also changes empirically for different impeller geometries. Figure 7.2 shows the dependency of power

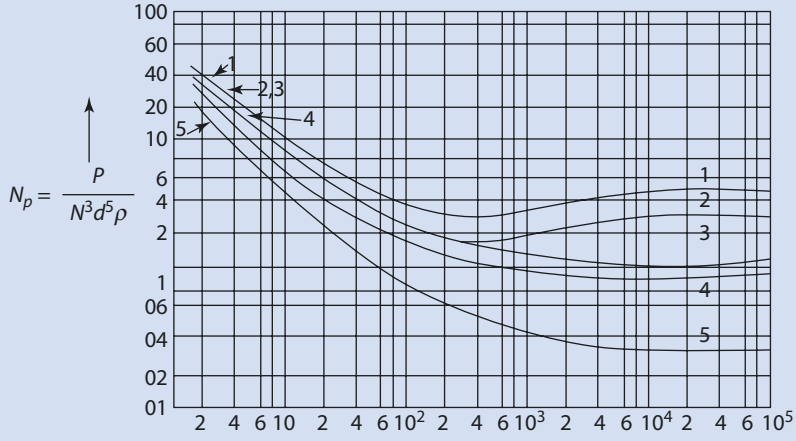


Fig. 7.2 Power number versus Reynolds number in a model bioreactor. (Adapted from Bates et al. [3])

number in model bioreactors with some most common impellers. Such a graph is handy for engineers to estimate the power input needed in the large-scale fermenter based on the Reynolds number.

Case Study 7.1

Xanthan gum is a natural polysaccharide heavily used in food and cosmetic industries for a number of important reasons, including emulsion stabilization, temperature stability, compatibility with food ingredients, and its pseudoplastic rheological properties [6]. Xanthan gum is produced by the bacterium *Xanthomonas campestris* through aerobic fermentation. Shortly after the fermentation starts, the gum production excels and the broth viscosity increases dramatically. Although, the produced gum needs to be dewatered and dried in the downstream processing, engineers have no choice other than adding water to the broth to dilute it so that the heat and oxygen transfer and even agitation are not impaired by the viscosity jump. It is possible to defeat viscosity to some extent, by heating the beer up in the downstream steps, but it is not feasible during the fermentation since the temperatures for optimum production are around 30°C. Now can you imagine how such a viscosity jump that deeply affects production itself may affect your scale up strategy? Which strategy would be the best?

Shear Rate

Shear stress in a fermenter depends on the rheological properties of broth, which are defined for broth viscosity during the fermentation process and shear rate, which is a function of impeller geometry and impeller rotational speed. For Newtonian fluids they are defined as

$$\tau_{ave} = \mu \nu_{ave} \tag{7.6}$$

$$\nu_{ave} = \kappa N \tag{7.7}$$

where τ_{ave} is the average shear stress between impeller blades and fermenter inner wall ($N.\,m^{-2}$), μ is dynamic viscosity ($N.\,s.\,m^{-2}$), ν_{ave} is the average shear rate between impeller blades and fermenter inner wall (s^{-1}), κ is a constant that depends on the system geometry only for Newtonian fluids, and N is the impeller rotational speed (rps). These equations can now estimate the shear rates in agitated systems with viscosities similar to water and with perfect Newtonian behaviors. With deviations from these conditions, which is usually the case in most fermentation broths, complex equations must be used. Most mold strains due to filamentous growth and mammalian cells are very sensitive to shear rates for which there is a threshold. Higher shear rates are simply fatal or reduce product yields. Thus, in these cases, the highest feasible shear rate is calculated and kept constant, which depends on the impeller tip speed and thus on the agitator speed. Since agitation is critical in fermentation, especially in aerobic and/or viscous conditions, increasing the impeller diameter or the number of impellers may provide robust agitation without creating overstress. The case study below shows that overstress may not always be a conspicuous matter of life and death and yet very problematic [14].

Case Study 7.2

Bacillus subtilis natto is a highly aerobic Gram-positive bacteria that excretes menaquinone-7, a potent form of vitamin K, under aerobic conditions [10]. This form of vitamin K2 is the most expensive vitamin. Thus, this bacterium has been used to produce supplementary vitamin K2 for decades. Scientists have discovered that this strain has a high potency to form biofilm matrices

that have a positive effect on vitamin secretion. Furthermore, engineers are working to perform the fermentation in agitated and aerated liquid states, since conventional static solid states are not easy to scale up. Although robust agitation and aeration do not affect growth or metabolism in *B. subtilis* but actually improve them, the shear stress caused by them decimates the biofilm formations and therefore knocks out vitamin secretion. To overcome this dilemma, engineers are working on biofilm reactors where mature biofilm formations are allowed to form on suitable support surfaces, which would be resilient enough to tolerate robust agitation and aeration up to feasible extents. The downside to biofilm reactors, however, is that oxygen molecules need to diffuse all the way into the biofilm to reach the production sites [4]. Would optimization methods be helpful to find optimum conditions for maximum vitamin secretion and solve such trade-off equations? How do these considerations come into play when optimum conditions in lab-scale studies are supposed to be scaled up?

Mixing Time

For highly viscous, non-Newtonian broths, the conventional equations are not valid. In these cases, robust agitation becomes the ultimate goal. For instance, when acid or base solutions, antifoam agents, or fed-batch ingredients are to be added to the broth periodically, it is essential to have robust mixing. Providing robustness with a larger impeller or higher numbers of impellers is easier and yet much less energy efficient. Alternatively, we can opt for a taller fermenter where the impeller diameter does not need to increase for robustness; however, a deep stack of broth can definitely be more troublesome. For example, oxygen solubility at the bottom of the deep fermenter near the sparger will be significantly higher than the surface. This inevitably not only creates an undesirable oxygen gradient, but also significantly increases the gas power input ($P/V\alpha\ d^3$) and thus power requirements become prohibitive in large-scale fermenters. Thus, there is a trade-off. In this case, engineers may choose to scale up based on equal mixing or blending time. Mixing time can be defined as

$$t_m = \frac{V}{Nd^3} \tag{7.8}$$

where V is the working volume (m^3), N is the impeller rotational speed (rpm), and d is the impeller diameter (m).

7.2.2 Biochemical Factors

So far, we have discussed how fermentation at a large scale can be distinct from a lab scale, backing it up through physical and design point of views. But that is not all of it. The media compositions used for plant production scarcely include any pure or lab-grade components. Rather, economy is engineers' first priority and therefore low-grade abundant and natural resources are used. For instance, if scientists find out that glucose is the key nutrient to producing an enzyme, they should undertake a series of lab-scale experiments to figure out how glucose affects enzyme production and what the optimum conditions are and perhaps use pure glucose only for clarity purposes of the results. Yet, it is quite impractical to use pure glucose for plant-scale production, because it would be too expensive. It is replaced with either unrefined molasses or unrefined dextrose extracts from inexpensive sources. Now, the best way to tackle such a situation is to carry out some

lab experiments using the exact plant-scale composition and thus reiterate the conditions. But, even this may not be sufficient as the composition, purity, and physical properties of these natural resources may change from batch to batch or over time. The emergence of a trace toxic or inhibitory component in the resources may halt the process or result in changes in purity, and amounts of the nutrients may take the metabolic paths sideways. Therefore, engineers do keep lab-scale fermentation studies on the side even after the plant starts its work specifically to monitor and tackle such unprecedented changes. The incoming nutrient purity and composition are monitored using analytical chemistry techniques such as high performance liquid chromatography (HPLC) or gas chromatography (GC), and changes in the composition of the working medium are implemented accordingly to keep the fermentation process smooth [7, 11].

7.2.3 Process Conditions

Processes that are undertaken prior to inoculation of a plant-scale fermenter and officially starting the production may affect how well the fermentation continues. Basically, these processes are sterilization of the working medium and pre-culture fermentation to produce the inoculum. Sterilizing the working medium for a large fermenter is quite different from the lab-scale counterpart. For the large-scale medium, a longer sterilization time is required to ensure a complete sterilization, as heat transfer rate into the large fermenter is always lower. Also, as mentioned earlier, the industrial medium may contain complex components. As sterilization temperatures reach 121.1 °C (250 °F), many spontaneous chemical reactions take place between these components (i.e., reducing sugar and amino groups end up with Maillard reactions). These unwanted reactions not only degrade valuable nutrients in the medium, but also may create substances which are harmful to microorganisms and biosynthesis of the product. Thus, efficiency of the fermentation process is lowered. Engineers often sterilize different medium components such as carbon sources, nitrogen sources, and minerals separately to minimize these undesirable side reactions. Usually growth rates (which are very important) in the media that are sterilized as a whole are significantly lower than those that are sterilized separately. However, before deciding to sterilize components separately, studies on the lab scale must prove it to be practical [16].

Another factor that affects the growth and condition of the main fermentation process is the condition of the inoculum. Engineers always keep a keen eye for the integrity and condition of the inoculum. Cell concentration, age, and phase of the inoculum cells even by an hour, morphology of the cells, and the metabolic trait from which the inoculum comes are imperative parameters that can determine the success or failure of a fermentation process [12].

Therefore, it's good to remember the following tips:

- If there are not enough cells in the inoculum despite a constant inoculation volumetric ratio, lag phase of the main fermentation will be prolonged. This not only imposes higher operational costs but also may decrease the product yields drastically as the optimum window for harvest is lost.
- Usually, the inoculum cells are best when they are in their late exponential phases of the growth (e.g., for enzyme production). This is true for all scales of production, and that is when they should be harvested from the pre-cultures.

- The number of pre-cultures may have significant effects on how fast and robust the inoculum is. This is a sensitive effect because the number of pre-cultures needed for a large fermentation process may be several more than a lab-scale one.
- For fermentation of filamentous microorganisms like fungi, in addition to all of the above parameters, it is also important whether the inoculum is in pellet or filamentous form as metabolism in these forms is quite distinct especially for biosynthesis of complex materials such as enzymes and other secondary metabolites. The case study below investigates these effects in an important industrial application of filamentous strains [17].

Case Study 7.3

Citric acid is a weak organic acid that naturally occurs in citrus fruits and gives them the special taste. It has a very vast and diverse range of industrial applications in food and drink, detergent, cosmetic, pharmaceutical, dietary supplement, and even steel industries. For over a century, bioprocess engineers have produced citric acid using filamentous bacterial and fungal strains such as *Aspergillus niger*. They have learned by experience that broth pelleting and morphology in the seed stages strongly influence the outcomes. For instance, it was found that broth morphology influences broth thickness, which affects not only mixing but also aeration resistance and coating of instrument sensors, which can be a huge problem, especially in large-scale fermenters. Thus, they learned that identification and consideration of this phenomenon, which has close correlations to shear in the pre-cultures and the main fermenter, in developing scale-up conditions and interpreting scale-up behavior can be extremely beneficial for better production [9, 15].

7.3 Summary

The ultimate goal in fermentation process development is the large-scale commercial implementation. Plant-scale fermenters give us the opportunity to produce numerous valuable products through microbial fermentation. Before plant-scale fermenters are designed and put to work, bioprocess engineers must closely study and optimize the process in lab-scale and pilot-scale fermenters. An optimized lab-scale process can then be transferred to pilot scale following the established scale-up strategies. In doing so, engineers must consider all factors that make the fermentation process distinct in larger scales. These factors primarily include physical properties of the broth and the fermenter itself such as heat and mass transfer (especially oxygen transfer in aerobic fermentations) that are affected by agitation, aeration, broth rheology and fermenter geometry and design. Secondly, the broth or medium biochemical properties such as deviations from ideal and pure compositions in small-scale fermentations followed by the physical factors are also an aspect to consider. Finally yet importantly are process conditions that include sterilization step(s) and inoculum conditions. Considering these factors, the scale-up strategies can simply be based on a constant height to diameter ratio (H/D) up to constant impeller power input to working volume ratio (P_0/V), power number (N_p), or in most aerobic fermentations a constant volumetric oxygen transfer coefficient (k_La). As process conditions get more complicated and the rheological properties of the medium deviate from ideal Newtonian fluids, a combination of these strategies may be considered. A constant H/D ratio is perhaps the simplest

scale-up strategy and can easily miss some special cases. On the other hand, keeping a constant P_0/V ratio is perhaps the most common scale-up strategy due to good flexibility and simplicity and yet is usually not energy efficient and so following a constant N_p may prove a better option. In aerobic fermentation, where oxygen mass transfer rates prove to be limiting, engineers try to provide the best aeration efficiency by implementing a constant k_La strategy. In some more exclusive cases, where shear rate is crucial, such as fermentation with filamentous microorganisms, engineers focus on agitation rates and impeller designs to carry out a successful scale-up. Of course, there may be more specific combinations and conditions that can be applied to pre-defined cases; yet, in this chapter, the most common and basic scale-up strategies that have been implemented by bioprocessing engineers are covered.

Problems

1. Which of the H/D ratios of a fermenter is better for oxygen transfer efficiency, a tall-narrow or short-squat fermenter? Briefly explain.
2. A stirred tank reactor is to be scaled up from 0.1 m^3 to 10 m^3. The dimensions of the small tank are D_t = 0.64 m, $D_{impeller}$ = 0.106 m, and N = 470 rpm. Thus:
   (a) Determine the dimensions of the large tank (D_L, $D_{impeller}$, H_L) by using geometric similarity.
   (b) What would be the required rotational speed (N) of the impeller in the large tank for a constant impeller speed taken as $N \times D_{impeller}$ = constant?
3. A strain of *Azotobacter vinelandii* is cultured in a 15-m^3-stirred fermenter for the production of alginate. Under current conditions, the mass transfer coefficient, k_La, is 0.18 s^{-1}. Oxygen solubility in the fermentation broth is 8×10^{-3} kg/m^3. The specific oxygen uptake rate is 12.5 mmol/g. h. What is the maximum cell density in the broth?
4. A value of k_La = 30 h^{-1} has been determined for a fermenter at its maximum practical agitator rotational speed with air being sparged at 0.5 L gas/L reactor volume/min. *E. coli* with qO_2 of 10 mmol O_2/g dry wt./h are to be cultured. The critical dissolved oxygen concentration is 0.2 mg/L. The solubility of oxygen from air in the fermentation broth is 7.3 mg/L at 30°C. Thus:
   (a) What maximum concentration of *E. coli* can be sustained in this fermenter under aerobic conditions?
   (b) What concentration could be maintained if pure oxygen was used to sparge the reactor?
5. *E. coli* has a maximum respiration rate, qO_2max, of about 240 mg O_2/g dry wt./h. It is desired to achieve a cell mass of 20 g dry wt./L. The k_La is 120 h^{-1} in a 1000 L reactor (800 L of working volume). A gas stream enriched in oxygen is used (i.e., 80% oxygen) which gives a value of C_L^* = 28 mg/L. If oxygen becomes limiting, growth and respiration become slow. For these conditions it is safe to presume:

$$qO_2 = \frac{qO_2 \max C_L}{0.2\frac{\text{mg}}{\text{L}} + C_L}$$

   where C_L is the dissolved oxygen concentration in the fermenter. What is C_L when the cell mass is at 20 g/L?

6. Calculate the oxygen transfer rate and $k_L a$ of an air-water system in a fermenter, in which the experimental work was carried out with a working volume of 20 L of water at 30°C. The pressure inside the fermenter was kept constant at 5 psig while two agitation rates of 100 and 300 rpm were employed. The results for the two runs are presented in the table below.

Time	%DO		Time	% DO	
	100 rpm	300 rpm		100 rpm	300 rpm
0	3.2	0	5.5	79.2	99.2
0.5	6.2	25.7	6	82.2	99.4
1	16.8	65.3	6.5	85	100
1.5	27.1	83.9	7	87.3	100
2	37	91.8	7.5	89.2	100
2.5	45.5	95	8	90.9	100
3	52.9	96.7	9	93.7	100
3.5	59.3	97.6	10	95.6	100
4	64.8	98.2	11	97.1	100
4.5	71.3	98.6	12	98.2	100
5	75.6	98.9	13	99.1	100

7. Bacterial fermentation was carried out in a bioreactor containing broth with average density of 1200 kg/m^3 and viscosity of 0.02 N.s/m^2. The broth was agitated at 90 rpm and air was introduced through the sparger at a flow rate of 0.4 vvm. The fermenter was equipped with two sets of flat-blade turbine impellers and four baffles. Tank diameter is D_t = 4 m, impeller diameter is D_i = 2 m, baffle width is W_b = 0.4 m, and the liquid depth is H = 6.5 m. Therefore, determine:
   (a) Ungassed power, P
   (b) Gassed power, P_g
   (c) $K_L a$
8. A medium containing 10^5 spores per liter needs to be sterilized before starting the fermentation. By assuming that the death rate for spores (k_d) at 121 °C is 0.903 min^{-1}, determine the sterilization time for 10 L and 10,000 L fermenters for 10^{-3} probability of failure. Ignore effects of heat-up and cool-down periods.
9. Suppose we want to produce vitamin K from *Bacillus subtilis natto* in a fed-batch biofilm reactor using glycerol as a limiting nutrient. The biofilms are placed on straight-blade Rushton turbine rings. Since the vitamin is produced in the biofilm matrices; in order to preserve the biofilm formations from overstress, we need to hold Reynold numbers below 10,000. Since the glycerol medium is quite viscous, engineers have suggested a constant mixing time strategy to scale up from a 2-L bioreactor to a 10,000-L pilot-scale one. If the mixer for the pilot-scale fermenter is 1000 times stronger than the one in the model fermenter, what should be the impeller rotational speed and diameter for maximum mixing robustness?

Take Home Messages

- Every value-added product that comes from fermentation in bioreactors starts with studying the process in small-scale flask fermentations and bench-top bioreactors in labs. Once they know enough about the bioprocess, biological engineers need to expand the process from those lab-scale modules to pilot-scale fermenters and finally plant-scale ones. This is called scale-up.
- The bioprocess behavior in small-scale bench-top bioreactors in labs that can be only a few liters in volume are mostly distinct from the ones in plant-scale fermenters that can be up to hundreds of thousand liters of volume.
- To address complexities during the scale-up process, engineers often follow certain and well-examined scale-up strategies while fully considering physical, biochemical, and process factors that dictate such complexities in the process.
- Some of the most commonly used scale-up strategies include maintaining a key parameter fixed throughout the scale-up process. These key parameters are selected considering the nature of the bioprocess and to minimize distinctions in behavior to keep optimum conditions as much as possible.
- Most common parameters are volumetric oxygen transfer coefficient $k_L a$ (in aerobic fermentations of course), volumetric power consumption of the impeller(s) P/V, impeller power number (N_p), shear stress τ, and mixing time t_m.
- Sometimes engineers are not content with these conventional strategies and end up blending them together or crafting a process-specific strategy, all to ensure best of production conditions on the final scale.

References

1. Bailey JE, Ollis DF. Biochemical engineering fundamentals. Chemical Engineering Education; 1976.
2. Baltz RH, Demain AL, Davies JE, editors. Manual of industrial microbiology and biotechnology. Washington, DC: American Society for Microbiology Press; 2010.
3. Bates RL, Fondy PL, Corpstein RR. Examination of some geometric parameters of impeller power. Industrial & Engineering Chemistry Process Design and Development. 1963;2(4):310–4.
4. Ercan D, Demirci A. Current and future trends for biofilm reactors for fermentation processes. Crit Rev Biotechnol. 2015;35(1):1–14.
5. Garcia-Ochoa F, Gomez E, Santos VE, Merchuk JC. Oxygen uptake rate in microbial processes: an overview. Biochem Eng J. 2010;49(3):289–307.
6. Garcıa-Ochoa F, Santos VE, Casas JA, Gomez E. Xanthan gum: production, recovery, and properties. Biotechnol Adv. 2000;18(7):549–79.
7. Hsu YL, Wu WT. A novel approach for scaling-up a fermentation system. Biochem Eng J. 2002;11:123–30.
8. Islam RS, Tisi D, Levy MS, Lye GJ. Scale-up of Escherichia coli growth and recombinant protein expression conditions from microwell to laboratory and pilot scale based on matched kLa. Biotechnol Bioeng. 2008;99(5):1128–39.
9. Junker BH, Hesse M, Burgess B, Masurekar P, Connors N, Seeley A. Early phase process scale-up challenges for fungal and filamentous bacterial cultures. Appl Biochem Biotechnol. 2004;119(3):241–77.
10. Mahdinia E, Demirci A, Berenjian A. Production and application of menaquinone-7 (vitamin K2): a new perspective. World J Microbiol Biotechnol. 2017;33(1):2.
11. Najafpour GD. Biochemical engineering and biotechnology. Amsterdam: Elsevier; 2007.
12. Oliveira SC, De Castro HF, Visconti AES, Giudici R. Bioprocess Eng. 2000;23:51–5.
13. Shuler ML, Kargi F, DeLisa M. Bioprocess engineering: basic concepts, vol. 576. Englewood Cliffs, NJ: Prentice Hall; 2017.

14. Silva-Santisteban BOY, Maugeri Filho F. Agitation, aeration and shear stress as key factors in inulinase production by *Kluyveromyces marxianus*. Enzym Microb Technol. 2005;36(5–6):717–24.
15. Tang YJ, Zhang W, Liu RS, Zhu LW, Zhong JJ. Scale-up study on the fed-batch fermentation of *Ganoderma lucidum* for the hyperproduction of ganoderic acid and *Ganoderma* polysaccharides. Process Biochem. 2011;46:404–8.
16. Vogel HC, Todaro CL. Fermentation and biochemical engineering handbook, *Principles, Process Design*, and Equipment. 2nd ed. Westwood: Noyes Publications; 1997.
17. Yang H, Allen G. Model-based scale-up strategy for mycelial fermentation processes. Can J Chem Eng. 1999;77:844–54.

Cell Separation and Disruption, Product Recovery, and Purification

Sze Ying Lee, Kit Wayne Chew, and Pau Loke Show

A. Berenjian (ed.), *Essentials in Fermentation Technology*, Learning Materials in Biosciences,
https://doi.org/10.1007/978-3-030-16230-6_8

What You Will Learn in This Chapter

Advances in upstream production (in the bioreactor) have been successful in promoting high product titres, and downstream processing now is important to obtain the final biological product in its desired purity and concentration level. Downstream processing of a biological product consists of multiple steps of individual physicochemical operations. These operations are integrated into a sequence to yield an optimal product recovery scheme. Downstream processing of a biological product that is produced via fermentation process, in general, starts with cell separation step and is followed by cell disruption operation and, finally, product recovery and purification. All the major elements of relevant technologies are presented in this chapter.

After fermentation process, the target product has to be separated from the depleted fermentation broth containing microorganism cells, fermentation ingredients remained, and metabolic waste products generated along the fermentation process. For an extracellular product, a solid-liquid separation operation can be applied directly just after fermentation process to acquire a product-rich suspension. These separation processes might involve filtration and centrifugation steps, which are detailed in this chapter. On the other hand, for a product of interest that is synthesized intracellularly by the producing host cell and is not secreted to surrounding medium, it is necessary to harvest the cells first and subsequently release the target product from the cell compartments by external physical force and/or chemical treatment. Several classical cellular lysis procedures are available and are practically applied for a wide range of biological products. The cell disruption techniques are reviewed in this chapter. After the cells are broken open, the target product is released to surrounding medium. The product-rich suspension is then processed to remove cell debris in order to prepare an extract that is free of cell or cell debris for further product recovery and purification processes.

8.1 General

Many of today's commercial biological products are produced by microorganisms via fermentation processes. Microbial fermentation allows us to scale-up the production, by utilizing a large quantity of microbial cells to generate the product of interest in a controlled fermentation process [111]. Besides, the use of microorganism as a source of biological products facilitates an enhanced efficiency in production [130].

In a culture medium containing nutrients that favorably support the metabolic growth of a specific microorganism, the product of interest is produced by the cells. There are two modes of production, either by extracellular or intracellular. The extracellular products are produced within the microbial cell but are then excreted into the surrounding environment. The examples of extracellular products are amino acids [45], enzymes (lipases [1, 37, 79], amylases [9, 42], hydrolases [68], oxidases [35], xylanases [22], proteases [61], and pullulanases [167]), recombinant proteins [26], and polymeric substances [24, 59, 73]. Intracellular production, on the other hand, refers to the process whereby the product of interest produced by the cells is stored inside the host cell. Some glycoproteins [132] and enzymes like β-fructofuranosidases [47], lipases [123], and glutamate dehydrogenases [141] are produced intracellularly.

At the end of the fermentation process, the final fermentation medium, also known as broth, in either large-scale bioreactor or laboratory-scale flask, contains the target

product stored in the host cells (intracellular) or directly suspended in culture medium (extracellular). The broth invariably has to undergo separation and purification operations to recover the target product in the desired form, purity, and concentration standards. There are many reasons to recover a biological product, probably to precisely characterize it or to mass produce it for commercial purposes [28]. Usually, the product for industrial applications does not have to be of high purity. On the other hand, the product intended for therapeutic use needs to attain exacting and high standards of purity or even up to homogeneity. Depending on the required purity level, the product recovery protocols are developed. Additionally, selection of recovery processes heavily relies on the characteristics of target product and unwanted components (impurities). The separation and purification processes should not affect the nature and structure of target product.

The advances in the recovery processes of a fermentation product were carried out by considering five main heuristic rules that will determine the success of the recovery procedures applied [53], namely:

1. The easiest-to-remove impurities should be removed first.
2. The most abundant impurities should be removed first.
3. The separation processes should be highly selective by making use of the greatest differences in the properties of the target product and impurities. These properties include the physical form at operating temperature (solid or liquid), size, density, solubility (in water or any other specific solvent), ionic charge, hydrophobicity, and ligand specificity.
4. The processes that employ different separation driving forces should be selected and carried out in an optimum sequence.
5. The most demanding and costly purification steps should be performed at last.

Over the years, the recovery processes for a specific product are widely developed according to the aforementioned rules of thumb. Besides, these processing operations can be categorized into four main groups which are applied to bring a product from its natural state, whereby it is produced (i.e., fermentation broth) through progressive improvements in purity and concentration. Downstream processing encompasses four major phases:

1. Removal of insoluble. This first approach aims to separate whole cells (i.e., biomass) and other insoluble components from the broth. In this step, a solid-liquid separation operation occurs, by making use of different physical forms of the biomass (in solid form) and the broth (in liquid form). Filtration and centrifugation are the unit operations frequently involved.

For an intracellular product, biomass that contains target product is collected for further processes, while the depleted broth is discarded, whereas for extracellular production, this step allows the capture of product as a solute in a cell-free liquid suspension by removing biomass.

2. Product extraction and isolation. In this stage, the impurities that have considerable different physicochemical properties from the target product are removed. The common operations performed include ammonium sulfate precipitation, solvent extraction, liquid-liquid extraction, and ultrafiltration.

The extraction of intracellular product involves cell lysis protocols to break open the cell wall for the liberation of intracellular content. Therefore, the recovery of an intracellular

product is relatively difficult compared to an extracellular product due to the complications happened during cell lysis process such as product degradation with the use of chemicals. In addition, the cell debris generated might become process constraints for subsequent purification steps, and hence extra steps are required to remove them. Adding extra steps into downstream processing is definitely unfavorable since every process step might lead to additional loss or degradation of product and at the same time increase the production cost.

3. Product purification. This step involves the removal of interfering or contaminating substances that have similar physical and chemical properties as the target product. Therefore, the techniques required for the complete purification are often complex and expensive.

Several chromatographic techniques such as ion-exchange [20, 46], gel filtration [163], affinity [114, 148], and hydrophobic interaction [50, 164] chromatographies are commonly used and combined to achieve the desired purity level [21]. The methods, including column and high-pressure liquid chromatographies, allow high resolution but can handle a low throughput [34, 58]. Therefore, advanced purification strategies such as foam fractionation [91, 94], reversed micellar system [11], aqueous two-phase system (ATPS) [81], and aqueous two-phase flotation [77] have been introduced to overcome the process limitations.

4. Product polishing. This final task involves the preparation of the purified product in a stable form for easy and convenient transportation. Typical unit operations include crystallization, spray drying, and freeze-drying.

Figure 8.1 provides a typical flowsheet for the recovery of a biological product produced via fermentation. It details the different pathways for both the product categories, i.e., intracellular and extracellular production. The skeleton of the recovery processes comprises the sequencing steps appropriately arranged based on the heuristic rules. Refer to Fig. 8.1; downstream processing of a fermentation product can be divided into two sections, namely, primary recovery stage and final recovery phase [75]. Primary recovery stage consists of the first steps of bioprocessing that aim to obtain a well-clarified extract, which is suitable for subsequent high-resolution purification steps. Therefore, the phase involves the removal of insoluble from broth by solid-liquid separation and product isolation where significant broth volume reduction and product extraction occur. For extracellular product, primary stage involves the preparation of cell-free suspension by removing the biomass, whereas for intracellular product, primary section covers the cell harvesting and cell lysis for the extraction of target product and finally the removal of cell debris. The unit operations that are frequently used in each stage are described in detail in this chapter.

8.2 Cell Separation

Cell collection (for intracellular product) or removal (for extracellular product) is the first downstream processing step, which is carried out in accordance with the first general heuristic, i.e., to remove the most plentiful impurities first. By making use of the difference of physical state of target products and fermentation broth, the suspended cells can be easily harvested or eliminated using one or more solid-liquid separation techniques.

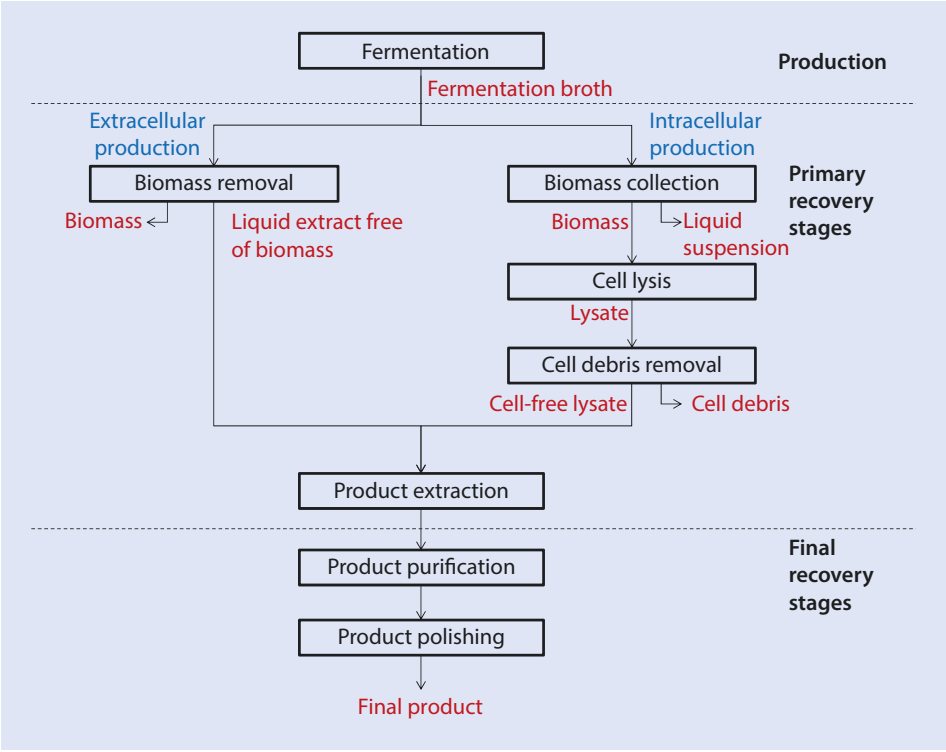


Fig. 8.1 A common recovery scheme of a fermentation product

Centrifugation and filtration are the common techniques used to accomplish the task of biomass separation. These techniques also can be applied to remove the cell debris generated during cell disruption process. Centrifugation is highly efficient for separating large and dense microorganisms and, however, might result in cell loss at around 1–5%. On the other hand, membrane filtration usually works well for separating small and light cells. If the target product is soluble, it can be recovered in the form of supernatant of a centrifuge or alternatively in the permeate stream of a filter (e.g., depth, press, candle, rotary vacuum, and membrane filter). Centrifugation operation is often followed by a polishing filtration step to guarantee the removal of all cell debris particles that might become process constraints in the next purification processes particularly chromatography operation. Likewise, when filtration is used for the removal of cell debris without preceding centrifugation step, some degree of diafiltration is required to achieve acceptable recovery. In case that the product is insoluble, it must be separated from the cell debris particles and then resuspended in a suitable buffered solution for further purification. The details of product purification are discussed in the next section.

8.2.1 Centrifugation

Centrifugation is a method used for the separation of particles from a solution according to their size and density and rotor speed applied. It works using the principle that large

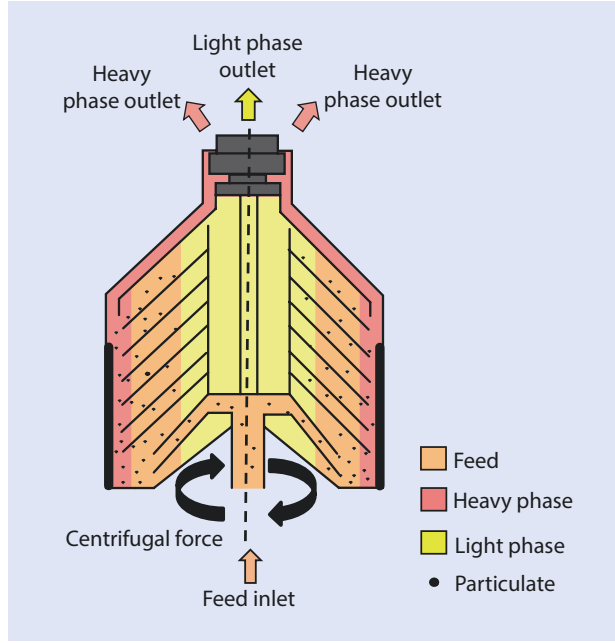


Fig. 8.2 Schematic of a disk stack centrifuge showing the separation of particulate, heavy, and light phases

and dense particles will sediment faster than small and light objects when a centrifugal force is applied. For a laboratory use, the centrifugal acceleration facilitates the settlement of denser particulates at the bottom of the centrifuge tube (in the pellet form) for the collection or removal of certain particles. Batch centrifugation is conveniently used in both laboratory- and large-scales. Industrial-scale continuous flow centrifuges have been introduced to allow continuous flow of feed and collection of clarified supernatant simultaneously, while the solid deposits can be cleaned intermittently. There are many types of centrifuges available commercially, such as tubular bowl, chamber bowl, disk stack (as shown in Fig. 8.2), scroll or decanter, and basket centrifuges.

Centrifugation is the first step applied in any bioprocessing protocols to separate the microorganism cells from the broth. For instances, centrifugation step can be applied for the recovery of an extracellular lipase after lipase production via submerged fermentation, particularly for the removal of *Burkholderia cepacia* cells in pellet form after centrifugation operation at certain centrifugal acceleration and process time [80], as presented in Fig. 8.3. Furthermore, centrifugation is used frequently after cell lysis procedure to remove the cell debris generated [89]. Besides, differential centrifugation, sometimes known as differential pelleting, is a common procedure used to separate particles of different densities in a liquid suspension, since the particles of varying densities have different sedimentation rates [97]. The target product can be separated from pools of contaminants of varying sizes and/or densities using multiple passes of centrifugation operations at different rotor speeds. The technique of differential centrifugation is first introduced for isolating mitochondria from guinea pig liver [12]. In later years, the technique is used to accomplish a complete fractionation of a tissue into nuclei, mitochondria, submicroscopic particles, and other soluble components [72, 131], as illustrated in Fig. 8.4. Another example also demonstrated that *Bacillus thuringiensis* subsp. *tenebrionis* insecticidal protein produced by *Escherichia coli* can be isolated from fragmentized cell debris and some precipitates by means of differential centrifugation step [48].

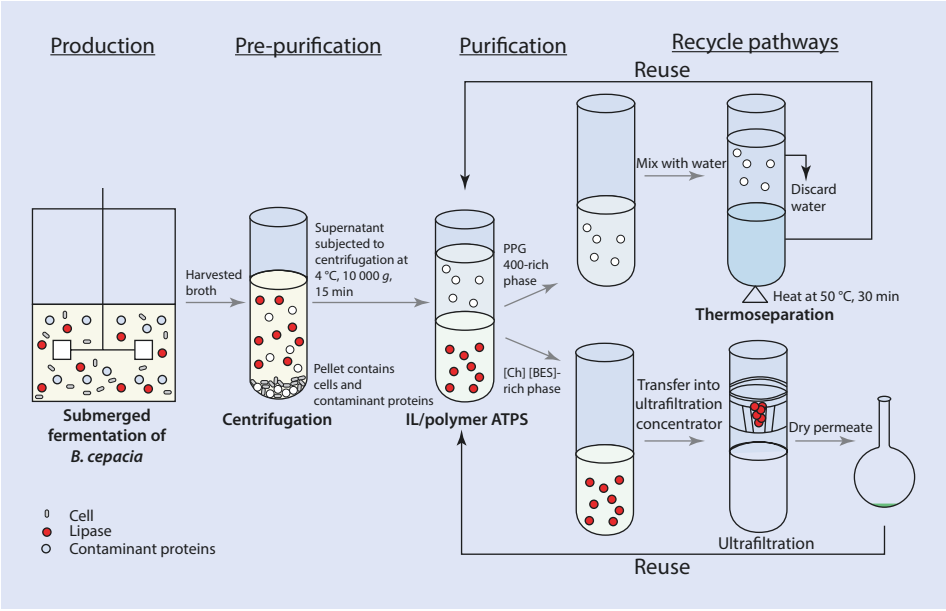


Fig. 8.3 A lipase recovery scheme proposed illustrating the application of centrifugation technique for the separation of cells from broth. ATPS, a type of liquid-liquid extraction technique, was adopted to purify lipase from other contaminant proteins in product-rich suspension [79]

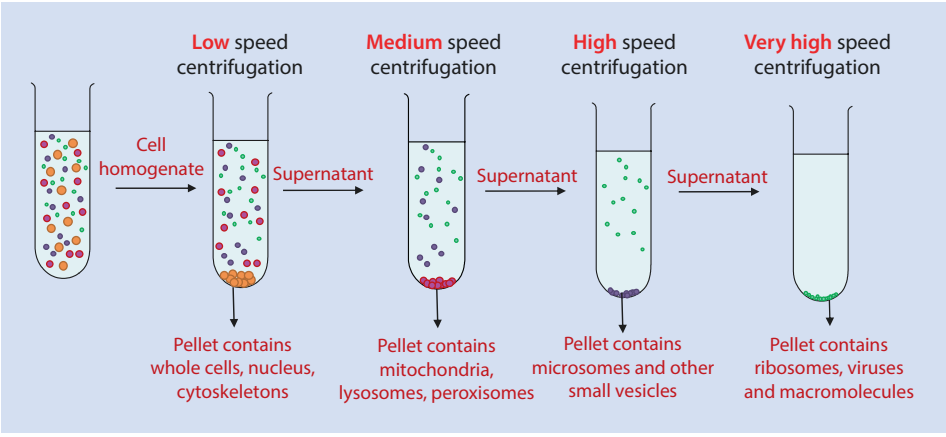


Fig. 8.4 An illustration showing a complete fractionation of a cell homogenate into all its cell components using several centrifugation steps operating at different accelerations that usually expressed as a multiple of the earth gravitational force ($g = 9.81\ ms^{-2}$)

8.2.2 Filtration

Filtration, which competes with centrifugation operation, often occurs in the early stages of downstream processing, aiming to remove the most plentiful impurities first [146]. After fermentation process, the target product usually presents in a large volume of liquid suspension. It is preferable to reduce the broth volume in the early stage of processing not

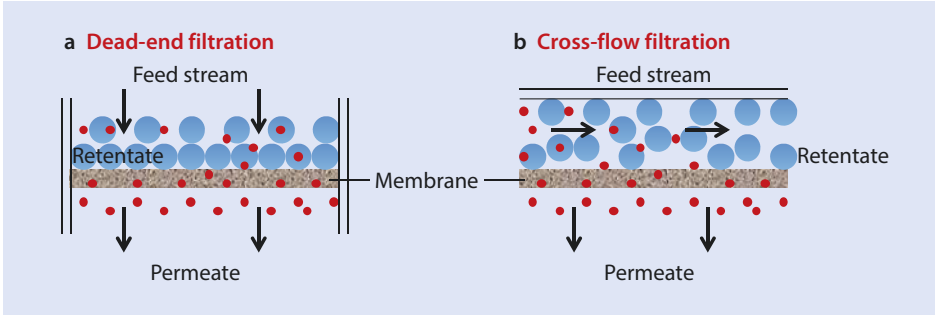


Fig. 8.5 Two types of membrane filtration: **a** dead-end filtration and **b** cross-flow filtration

only to reduce the processing scale but also to cut down the cost of subsequent processing operations. In this sense, filtration is one of the effective means of achieving volume reduction in a single step.

Filtration is used to separate solute components in a fluid solution according to their size by flowing liquid suspension under a pressure differential through a porous medium. There are two main categories of membrane filtration, namely, dead-end filtration and tangential flow filtration, as shown in Fig. 8.5 [129]. In dead-end filtration, the fluid flows perpendicular to the filter membrane. This type of filtration is suffered from the drawbacks such as the deposition of a layer of retentate on the membrane surface, thus limiting the possibility for continuous operation. In tangential flow filtration, or known as cross-flow filtration, the feed flows parallel to the membrane medium allowing the retained components to be swept along the membrane surface and thus minimize buildup of solids on the membrane surface [158]. Selection of the type of filter depends on the task to be accomplished and the product to be acquired at the end of the filtration process, either in the permeate (components that passed through the membrane) or in the retentate (components that are retained by the membrane).

Filtration can be used for a great variety of purposes, including the removal of cells from a target product that has been secreted [102, 152], the elimination of cell debris from lysed cells, the concentration of the product solution [156], the salt exchange in a solution, and also the separation of target product from contaminants [62, 65, 104, 161]. Besides clarification and analytical applications, filtration can be used for sterilization purpose to remove viruses and bacteria in biopharmaceutical production [147] and dairy industry [39]. Table 8.1 lists several investigations addressing the applications of membrane filtration technique in bioprocessing area, which covers from microfiltration (0.1–10 μm) to ultrafiltration (0.01–0.1 μm), nanofiltration (0.001–0.01 μm), and integrated filtration [78]. The process parameters are briefly described based on the types of filtration flow, the membrane material, the specific pore size, and molecular weight cutoffs (MWCO) of the membrane.

8.3 Cell Disruption

With increasing commercial demand of intracellular products, cell disruption unit operation is gaining in importance. Cell disruption serves to break open the host cells allowing the liberation of desired products that are stored inside a cell. Disintegration of microbial

Table. 8.1 Applications of filtration method for the recovery of product of interest from fermentation broth in bioprocessing field

Product	Process parameters	References
Lactic acid	Integrated ultrafiltration and nanofiltration	[78–82]
Cellulase	Microfiltration using attapulgite membranes (0.15, 0.12, and 0.10 μm)	[161]
3-Propanediol	Cross-flow nanofiltration using NF270 membrane with area 150 cm^2 and feed flow rate of 10 dm^3/min	[158]
Lipopeptides	Microfiltration (0.2 μm) and two-step ultrafiltration using 10 kDa to 100 kDa MWCO regenerated cellulose (RC) and polyethersulfone (PES) membranes	[65]
Surfactin	Two-step tangential flow ultrafiltration using 10 kDa MWCO RC and PES membranes	[62]
Therapeutic protein	Microfiltration using membranes (0.1 to 0.45 μm) made of RC, PES, polysulfone (PS), and polyvinylidene fluoride (PVDF)	[152]
Lactic acid	Cross-flow filtration using a tubular ceramic (Al_2O_3, TiO_2) membrane (0.1 μm, 0.8 μm, and 300 kDa MWCO)	[102]

cells is an essential step for the recovery of intracellular products. A significant number of cell disruption technologies have been developed and investigated to obtain biological products at optimum yield and purity, by taking into consideration the nature of microorganism species and properties of target molecules. In all cases, it is important that any potential disruption method adopted should guarantee that the labile target molecules are not degraded or denatured during the process. The subject of microbial cell disruption methods has been reviewed in several excellent articles [10, 25, 30, 44, 52, 63, 101].

Cell disruption techniques are broadly categorized into mechanical, physical, and chemical methods [30]. Mechanical methods refer to the strategies that employ force generated by mechanical devices or objects. Physical methods rely on structural modifications in the cell wall and/or membrane without causing chemical alterations and in the absence of energy application. On the other hand, chemical methods utilize chemical reagents or enzymes to modify the permeability of cell membranes or to digest cell wall components. Figure 8.6 presents major cell disintegration techniques based on these three categories. Cells can be either mechanically lysed by external mechanical compression and shearing forces or disrupted through chemical treatment that dissolves structural constituents of cell wall and/or membrane. Though it is ideal to use a single step of lysis method, two or more methods being performed in conjugation are sometimes necessary to obtain the desired result. The disruption performance would be less than stellar if any one of the steps is omitted. For example, a combination of physical, mechanical, and chemical methods is effective to disrupt the cells that are strongly resistant to disruption such as yeasts [33, 101].

One of the factors affecting selection of cell lysis method is the cell type. Cells of different microbial origins require different force strengths in order to be properly lysed. For example, application of high force of impact is requisite to disrupt yeast that has a hard cell wall. However, if the same degree of force is exerted on *E. coli*, the cell might be

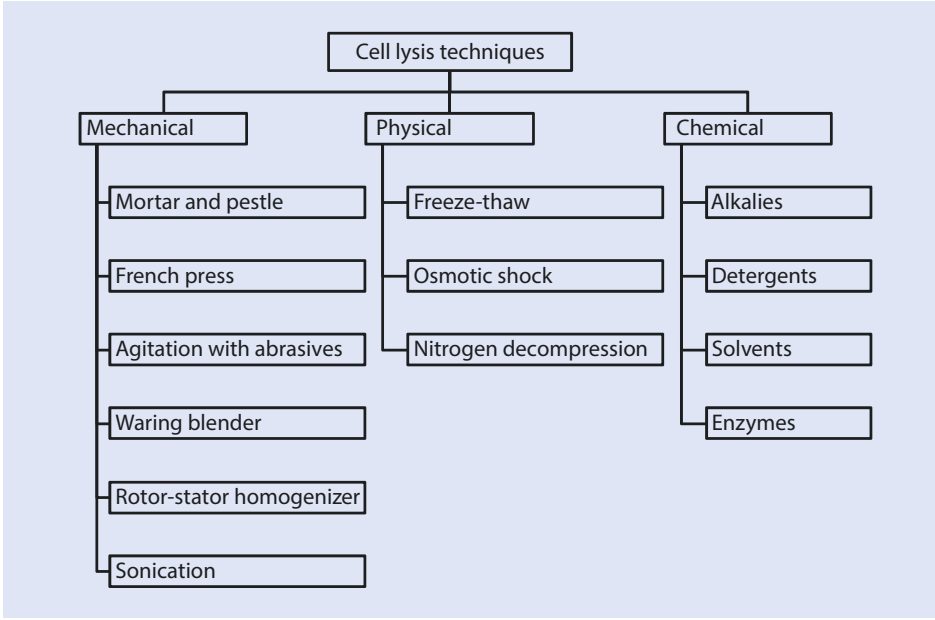


Fig. 8.6 Classification of cell disruption techniques based on mechanical, physical, and chemical groups

destroyed entirely. On the other hand, fungal cells might have different disruption resistances toward some disintegration methods commonly used for yeasts and bacteria [144]. Therefore, it is imperative to custom tailor each disruption protocol to meet the requirements of a specific cell disruption application.

8.3.1 Mechanical Cell Lysis

Mechanical lysis, which relies on grinding, shearing, beating, and compression operation, is a traditional method of choice used for the cell disruption and extraction of intracellular contents. They are not selective, breaking the cells apart and generating a considerable amount of tiny cell debris and releasing concomitantly other unwanted intracellular contents with target product during cell lysis process. It usually requires the use of expensive and cumbersome equipments or shearing devices, e.g., hand-operated or motor-driven pestle homogenizer, high-pressure homogenizer, and bead mill, to produce and exert external force on the cell to tear the cell apart. Figure 8.7 is an illustrative gallery of the commonly used equipments in mechanical procedures. Additionally, the disruption principles, strengths, and limitations of each mechanical method are summarized in Table 8.2.

Mortar and Pestle

Manual grinding using mortar and pestle is the most widely used traditional method for cell lysis. Grinding works by tearing and ripping of the cell samples when sandwiching the samples between two hard surfaces (a pestle as a hard moving object and a mortar as a static sample container) that slide against each other, as illustrated in Fig. 8.7a. Grinding

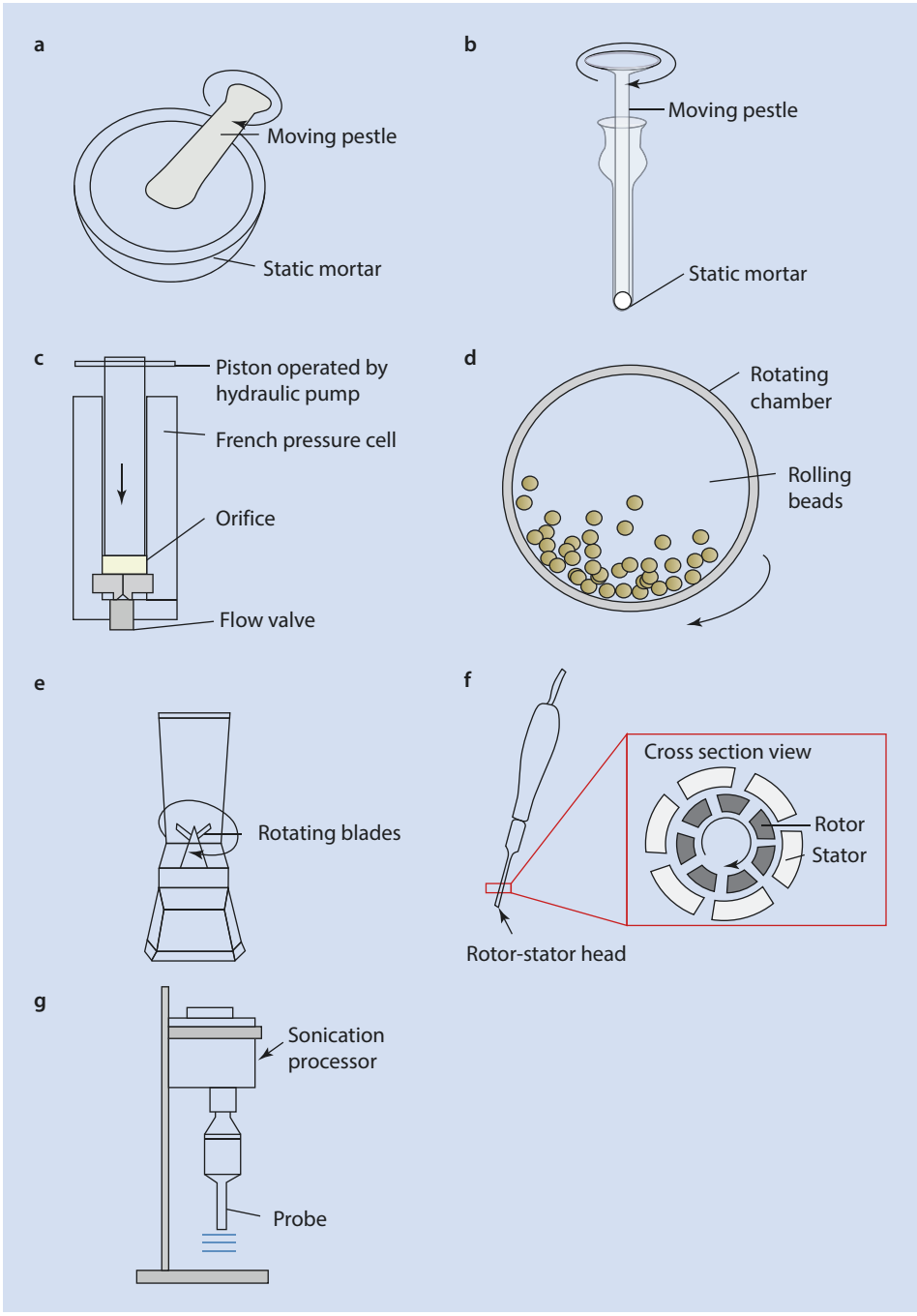


Fig. 8.7 Illustration of mechanical devices or apparatus used for the cell disruption: **a** mortar and pestle, **b** Dounce tissue grinder, **c** French press, **d** bead milling, **e** waring blender, **f** rotor-stator homogenizer, and **g** sonicator

Table. 8.2 The disruption mechanisms, strengths, and limitations of mechanical methods for the cell lysis, based on their apparatus used

Apparatus	Disruption mechanism	Strength	Limitation
Mortar and pestle	Cells are disrupted by shearing between two hard surfaces (and ice crystals in membranes if the cells are frozen in liquid nitrogen prior to grinding)	Inexpensive, easy to use	Low throughput, high possibility of contamination
Dounce or Potter-Elvehjem tissue grinder	Fine grinding by manually reciprocating and rotating a round pestle into a glass tube	Inexpensive, clean, easy to use, effective for soft cell	Fragile, ineffective for solid tissue, low throughput
French press	Cells are broken when they are forced to pass through a narrow valve under high pressure	Effective to generate uniform homogenates	Expensive, high maintenance cost due to clogging, difficult to clean, low throughput
Bead beater	Crushing of cells is caused by collision with agitated beads in liquid suspension	Low possibility of cross-contamination	Sample size limitation, excessive heating
Waring blender	Cells are chopped by rotating sharp blades in a blender	Fast, easy to use, large through-put	Sample foaming, coarse homogenate
Rotor-stator homogenizer	Cells are disrupted by drawing the cell suspension into a long shaft containing a fast-spinning inner rotor and a stationary outer stator	Fast, efficient for single-cell sample	Not suitable for multi-sample, cross-contamination with the use of probe, expensive
Sonicator	Cells are ruptured by cavitation formed by high-frequency sound waves	Fast, effective for hard cell wall	Excessive heating causing product (e.g., protein) denaturation

using mortar and pestle can be applied on wet and dry cell samples. In practice, cells are routinely frozen in liquid nitrogen prior to grinding, not only to increase the brittleness of samples promoting the cell fragmentation but also to chill the samples from friction heat generated during grinding process. With sufficient grinding using a circular motion with downward pressure, the frozen solid samples can be reduced to very fine particles.

Nowadays, motor-driven pestle homogenizer is available and is integrated with liquid nitrogen cooler to allow cryogenic grinding. The rugged pestle has a stainless steel shaft with a hex bit to be easily connected to the motor for motorized operation. Besides, the grinding jar (or mortar) is cooled with liquid nitrogen in a cooling container before and during the grinding process. The extremely low grinding temperature, close to −196 °C (if liquid nitrogen is being used), enhances the brittleness of the sample for better grinding efficiency. At the same time, the sample integrity is preserved, since there is no direct contact of liquid nitrogen with cell sample.

For analytical approaches, Dounce tissue grinder (see Fig. 8.7b), which has similar working principle as mortar and pestle, is routinely used. It is designed to gently homogenize tissues or cells by mechanical shear force and allows a high percentage of cell nuclei and mitochondria remained intact after homogenization step. Each Dounce tissue grinder set contains both a loose and tight-fitting glass pestles and a mortar. The process involves grinding by the use of a loose-fitting pestle in a glass cylinder for initial sample size reduction and then followed by the use of a tightly fitted pestle to form the final homogenate. The process is performed by moving the pestle up and down manually in a twisting motion, and the pestle ball is usually encircled in an excess of an appropriate solvent to avoid heat buildup by friction. Similarly, Potter-Elvehjem tissue grinder works with the same principal mean, but with stainless steel pestle and PTFE tip to allow greater plunger forces without risk of shattering glass. Briefly, both the grinders are relatively inexpensive, easy to use, and clean.

French Press

Another traditional apparatus used for the cell disruption is French press, or known as French pressure cell press. French press is developed by Charles Stacy French in the late 1940s. It is operated by external hydraulic pump and utilizes high pressure (up to 40,000 psi) to force cells passing through a narrow orifice, resulting in the disruption of cells due to shear stress and decompression experienced across the pressure differential (refer to Fig. 8.7c). The samples used are in liquid form with volume from 40 up to 250 mL. French press is considered as an efficient tool to generate uniform homogenates in degree of thoroughness of disruption.

High-pressure homogenizer (including French press) is one of the most widely used equipments for large-scale microbial cell disruption. There are many high-pressure homogenizers commercially available. High-pressure homogenization works well for both the bacteria [70, 127] and yeast [128] cells. Nonetheless, the equipment is associated with some drawbacks such as high maintenance cost due to valve clogging and difficulty to clean. Moreover, thermal degradation of target product always occurs in homogenizer due to excessive heating. Furthermore, fine cellular debris particles formed during the process might interfere with subsequent product purification processes. Therefore, significant efforts are required to investigate optimum operating conditions in order to achieve maximum cell disruption level with minimum negative impacts [70].

Agitation with Abrasives

Homogenization of cells by rapidly agitated beads inside a closed system, as presented in Fig. 8.7d, can be another cell lysis alternative. The working mechanisms behind bead beating operation include the collisions between the sample and the beads, between the sample and the wall of vessel, and between beads. Most of the bead mills utilize external shaking (either side-to-side or up-and-down) or vortexing to agitate the beads in liquid cell suspension. There are a wide range of beads in terms of size (0.1–6 mm in diameter), shape, and material (silica, *zirconium*, *and stainless steel*) available for different applications. Smaller beads work well for grinding of small particles into uniform finer size. On the other hand, large beads are more effective for breaking down large or dense cells. Moreover, denser and irregularly shaped beads aid to break down tougher and harder cells better. Besides factors such as size, density, shape, and material of beads, the process performance is influenced by the quantity of beads, concentration of cell suspension, operating temperature, and speed of agitator.

Bead milling has been reported as a superior technique in comparison with manual grinding using pestle and mortar and enzymatic treatment for the isolation of active enzymes such as catalase and glucose-6-phosphate dehydrogenase from filamentous fungi like *Pleurotus sapidus* and *Lepista irina*, using acid-washed glass beads with 0.25–0.5 mm diameter in a Dyno-Mill equipment [143]. Besides, Ho et al. [57] demonstrated that continuous-recycling bead milling is the most effective method in terms of operating cost and time for the release of intracellular recombinant hepatitis B core antigen from *E. coli*. Moreover, the investigation carried out by Wang and coworkers showed that bead milling achieved the highest efficiency for the extraction of intracellular metabolites from *Bacillus licheniformis* compared to liquid nitrogen grinding and ultrasonication [157].

Bead mill operations are carried out in an enclosed system, and thus there is marginal possibility of cross-contamination. Additionally, bead mills can be used for dry solid or wet sample. However, homogenizing cells by agitated beads generates a significant amount of frictional heat that is created by collision actions. To overcome the drawback, the vortexers and shakers are operated in a pulse mode (alternating on and off) in order to help dissipate heat produced. Moreover, in light of the constraints of heat released, cryogenic mill is tailor-designed to carry out the milling process in liquid nitrogen or at a cryogenic temperature. Cryo-milling has complementary advantages of both the cryogenic temperature and traditional bead milling. The embrittlement of the sample due to low temperature facilitates the bead milling process to obtain finer product in a more rapid way.

Waring Blender

Waring blender (see ◘ Fig. 8.7e), similar to standard household blender, can be used to disrupt cells. Cells are cut and sheared by the fast rotating blades in the blender. Most laboratory blenders are made of stainless steel, avoiding the contamination and making easy the sterilization work. However, the vortexes created by the rotating blades during blending process might result in foaming and protein denaturation. Besides, the output of blending is relatively coarse, and consequently the application of blender approach in microbial cell disruption is significantly limited. This technique is more frequently applied to preliminarily cut down larger size samples such as plant cell [36], algae cell [99], and fat tissue [5].

Rotor-Stator Homogenizer

Rotor-stator homogenizer employs a fast-spinning inner rotor with a stationary outer stator to homogenize samples through mechanical shear fluid forces and/or cavitation (see ◘ Fig. 8.7f). The spinning of rotor produces a vacuum effect which draws the cell suspension into the narrow space between the rotor and stator. The cells experienced high shear forces which resulted in an extreme change in velocity (i.e., high deceleration tangential and radial acceleration forces) when passing and discharged through the narrow gap between the rotor and stator, causing size reduction and homogenization. The shaft of homogenizer has varied sizes to accommodate a large range of volumes from 1 mL and onward for a batch process.

Sonication

Sonication refers to the application of sound energy. It is one of the mechanical disruptions commonly used to break open cells, particularly plant and fungal cells. Since the ultrasonic frequencies used are normally higher than 20 kHz, the process is known as ultrasonication. The technique disrupts tissues and cells through cavitation. Briefly,

high-frequency sound waves are generated using a sonicator with a titanium probe that is immersed in a cell suspension (see Fig. 8.7g). The probe rapidly vibrates in a longitudinal direction, causing the vapor bubbles to form and collapse momentarily, probably in microseconds, in the surrounding solution. The localized cavitation process creates microscopic shear and shock waves to tear apart cells. Sonicator generates a significant amount of heat especially at high power operation. To prevent excessive heating of sample, ultrasonic treatment is always applied in multiple short bursts coupled with the immersion of sample in ice bath.

Ultrasonication is a widely used laboratory-based technique for the disruption of cells. The successful use of ultrasonication for the recovery of cytochrome and proteins from *E. coli* has been reported [126]. In the study, it is observed that the product recovery yield increases with the increase of ultrasonication power and with the decrease of cell concentration [126]. Another study also demonstrated that ultrasonication is a more effective means than enzymatic lysis for the recovery of intracellular hepatitis B core antigen from *E. coli* in functionally active structure [56].

8.3.2 Physical Cell Lysis

There is limited number of physical cell lysis methods available. These methods are comparatively gentle, since they do not require the use of energy-intensive equipments to exert force on the cell nor alter the cell wall constituents chemically.

Freeze-Thaw

Freeze-thaw method can be used for the disruption of soft bacterial and mammalian cells. The protocol involves freezing of a cell suspension in a dry ice or ethanol bath or freezer and subsequently thawing at room temperature or in warm water bath at 37 °C. During freezing process, the ice crystals formed inside the cells result in the swelling of the cells and ultimately breaking the cells. Several cycles of freeze and thaw typically are required to achieve desired disruption, and consequently the process is lengthy. Moreover, freeze-thaw technique is less efficient to lyse cells that have rigid cell walls such as algae [82]. Johnson and Hecht reported that repeated cycles of freezing and thawing are sufficient to facilitate the liberation of highly expressed recombinant proteins from the cellular milieu of *E. coli* [67].

Osmotic Shock

Osmotic shock is often used for the release of periplasmic products that accumulate between the cell membrane and cell wall. In osmotic shock, microbial cells are first immersed and equilibrated in a medium of high osmotic pressure (usually a buffered sucrose solution supplemented with ethylenediaminetetraacetate (EDTA)) [41, 120] and then rapidly shifted to a medium of low osmotic strength (normally cold water). The sudden osmotic transition results in rapid entering of water into the cell and buildup of internal pressure in the cell and, finally, cell bursting. EDTA, a chaotropic agent, is added into buffered sucrose solution to facilitate the release of lipopolysaccharide from microbial cell envelope, thus increasing the permeability of outer cell membrane for the liberation of target periplasmic component [23]. Chen et al. reported that an increase in EDTA concentration (from 0.5 to 5 mM) in combination with cell pretreatment with calcium ion (5 mM for 5 min) significantly enhances the periplasmic release of a recombinant creatinase [23].

The method is gentle and effective to the cells that do not have strong wall or peptidoglycan layer such as mammalian cells. The technique has been used to release intracellular enzymes such as alkaline phosphatase, cyclic phosphodiesterase, and acid phosphatase from *E. coli*, without impairing the viability of the cells or causing enzyme inactivation [105].

Decompression

Cell disruption by decompression from a pressurized vessel is another alternative. The early laboratory-scale cell disruption by decompression has been demonstrated using *E. coli* [43]. In this technique, large quantities of oxygen-free nitrogen (or carbon dioxide is used instead) are dissolved in the cells under high pressure in a pressure vessel. When the pressure is released suddenly, nitrogen bubbles escape out from the cells in point punctures through the cell wall. Nitrogen cavitation can be used for fragile mammalian and bacteria cells, however, is less effective for yeast, fungi, or other cell types that have strong cell walls.

The method has additional advantage than mechanical techniques that usually generate localized heating, since nitrogen cavitation is an adiabatic expansion that cools the cell sample instead. Therefore, the technique is well-suited for the extraction of heat-labile intracellular contents such as proteins and enzymes. Lin et al. [87, 88] reported that the decompression technique using carbon dioxide can be used to disrupt yeast cells while preserving the functional properties of proteins Moreover, nitrogen is inert and has no alteration on the medium's pH, thus preventing oxidation or chemical modification on the cellular components.

8.3.3 Chemical Cell Lysis

The outer cell wall of microorganisms can be disrupted or dissolved by a great variety of chemical agents, such as alkalies, detergents, solvents, and enzymes (through catalytic action). In many cases, chemical techniques are integrated with mechanical methods, in order to achieve better product extraction. Lysis buffer is often added together with the chemical agents to provide suitable cell lysis conditions and, at the same time, prevent degradation of product upon release from the cell.

Alkalies

Alkaline digestion involves the solubilizing of microbial cell membrane through saponification with an alkali [18]. The most commonly used alkali is sodium or potassium hydroxide. The base is dissolved in water or alcohol such as methanol, ethanol, or isopropanol to form an alkaline solution. For instances, Sampathkumar et al. [124] demonstrated that the high alkaline trisodium phosphate solution permeabilizes and disrupts the cytoplasmic and outer membranes of gram-negative bacteria. However, the high pH condition of alkaline treatment (usually above pH 10) might not suitable for the extraction of labile intracellular proteins. On the other hand, López-Abelairas et al. [95] reported that acid treatment is more effective than alkaline digestion for the extraction of intracellular poly(3-hydroxybutyrate) from *Cupriavidus necator* H16 in terms of the recovery efficiency and purity. In the same studies, acid treatment is observed to cause lesser degradation of product compared to the treatment using sodium hydroxide and sodium hypochlorite [95].

Detergents

Detergents, also known as surfactants, are amphiphilic molecules possessing a polar hydrophilic "head" and a nonpolar hydrophobic "tail." Detergents are able to interact with both hydrophilic (e.g., water) and hydrophobic (e.g., lipid) compounds [90]. When they are present above certain concentrations in water (known as critical micelle concentration), they aggregate to form micelles which comprised of nonpolar interior formed by hydrophobic "tails" and polar "heads" group that are oriented outward and interacted with water molecules. According to the ionic character of the polar head group, detergents are classified as ionic (anionic or cationic), nonionic, and zwitterionic. Table 8.3 lists the common detergents according to their classification.

The use of detergents as lysis solvents in cell disruption is not new. Detergent molecules disrupt the cell by solubilizing cell membrane proteins and partitioning into the membrane bilayer [135]. Ionic detergents are denaturing with respect to protein structure. They completely disrupt cell membranes by binding to both the hydrophobic membrane and hydrophilic non-membrane proteins, while nonionic detergents allow the dispersion of hydrophobic parts of membrane proteins into aqueous media, without alternating the structures of water-soluble membrane proteins [52]. Because of their less denaturing nature, nonionic detergents are preferable to obtain the product in its active and stable form.

Table. 8.3 List of common detergents used for the cell lysis

Group	Detergents	Head group	Tail	Dialyzable
Ionic	Sodium dodecyl sulfate (SDS)	Sulfate	Linear hydrocarbon alcohol (C12)	Yes
Nonionic	Triton X-100	Linear PEG	*p*-(2,2,4,4- Tetramethylbutyl) phenol	No
	Triton X-114	Linear PEG	*p*-(2,2,4, 4-Tetramethylbutyl) phenol	No
	Tween 20	Polysorbate	Linear fatty acid (C12)	No
	Tween 80	Polysorbate	Linear fatty acid, unsaturated (C18:1)	No
	β-Octylglucoside	β-Glycosidic glucose	Linear hydrocarbon alcohol (C8)	Yes
	Brij 35	Linear PEG (23×)	Linear hydrocarbon alcohol (C12)	No
Zwitterionic	CHAPS[a]	Dimethylammonium-1-propanesulfonate	Cholesterol derivative	Yes
	CHAPSO[b]	Dimethylammonium-1-propanesulfonate	Cholesterol derivative	Yes

[a]3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
[b]3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate

The choice of detergents for cell lysis depends on the intended application, the cell type, and the properties of target product. If the preservation of native function and structure of protein is essential, milder nonionic (e.g., Triton X-100) or zwitterionic (e.g., CHAPS) detergents are proposed to solubilize membrane proteins. Zhao and Yu [165] reported that the treatment of *E. coli* cells with 2% (w/v) Triton X100 allows the recovery of more than 70% of L-asparaginase, by altering the cell wall surface but without breaking them apart. Besides, product extraction using detergents is specific. Ali et al. [3] reported that Triton X-100 is effective in the selective extraction of recombinant Hepatitis B surface antigens from *Hansenula polymorpha*. On the other hand, the denaturing SDS is frequently used when resolution is most important. The detergents might also couple with the use of chaotropic agents like urea and guanidine hydrochloride for the extraction of intracellular constituents, as demonstrated in the study of extraction of proteins from gram-negative microorganisms such as *E. coli* and *Pseudomonas aeruginosa* [29].

Although detergents offer beneficial advantages like high selectivity in extracting target product, the use of detergents might interfere with subsequent product purification steps. It is therefore crucial to remove all the incompatible detergents prior to purification step. Detergents, which have very high CMCs and small aggregation numbers, can be removed easily by single step of dialysis [151]. For those detergents that have too large micelles to be removed using dialysis (see Table 8.3), gel or ion-exchange chromatographies are the alternatives [55]. Besides, other methods have been proposed for final removal of detergents, including adsorption of detergent to hydrophobic media like resin [8], activated carbon [145], and polystyrene beads [119]; ultrafiltration through a hydrophobic polyethersulfone membrane [40]; the use of ethyl acetate [162], trichloroacetic acid [133], and chlorinated solvents [118]; and precipitation with polyethylene glycol.

Solvents

The use of solvents can be selective. A suitable solvent can act to modify the permeability of cell membrane and then extract target product from cell compartments. For example, ethylene glycol *n*-butyl ether can be used for selective release of a proprietary biopharmaceutical protein produced in the periplasmic space of *Pseudomonas fluorescens* [4]. Faria et al. [38] reported that ethanol is an effective chemical agent to permeabilize cells of yeast *Kluyveromyces lactis* for the release of intracellular β-galactosidase. Besides, Lo and coworkers demonstrated the effectiveness of 1-propanol used for the release of recombinant proteins, i.e., enhanced green fluorescence protein from *E. coli* [93]. Furthermore, certain chlorinated hydrocarbon solvents such as chloroform, 1,2-dichloroethane, and methylene chloride can be used for the recovery of biopolymer, namely, polyhydroxyalkanoates, from bacterial hosts like *Bacillus megaterium* and on *Rhodospirillum rubrum* [64]. Moreover, Park et al. [112] reported that a combination of solvents such as dimethyl sulfoxide, petroleum ether, and acetone improved the extraction of intracellular carotenoid pigments (β-carotene, torulene, and torularhodin) from red yeast *Rhodotorula glutinis* compared with individual solvents.

Chemical cell disruption using solvents brings advantages such as high selectivity in release of target product. Besides, the approach is economically viable considering the wide availability, low cost, and recyclability of solvent. However, the use of water-miscible organic solvents suffers from some limitations mainly about the fire safety hazard due to the flammability of these solvents.

Enzymes

Enzymatic lysis, as reviewed in several articles [7, 122], is a promising process-scale disruption technique for cells of microbial origin. Enzymatic lysis allows the lysis of cells which occurred under mild conditions in a selective manner. Enzymes like lysozyme, cellulose, protease, lysostaphin, zymolyase, or glycanase are valuable tools to recover intracellular products by digesting the microorganism cell wall [122].

Lysozyme, which is derived from hen egg white, is widely used to disrupt the bacteria cell wall owing to its ability to hydrolyze 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in a peptidoglycan (a polymer that confers mechanical resistance to the cell) [115]. Hydrolysis of these bonds in peptidoglycan layer destabilizes the bacterial cell wall creating an osmotic imbalance, which in turn results in cell lysis. A recent study demonstrated the construction of a genetic lysozyme-based lysis system in a polyhydroxyalkanoates-producing strain, where the produced recombinant lysozyme was translocated into the periplasmic space of the cell to disrupt the cells for recovery of intracellular polyhydroxyalkanoates [19]. Besides, zymolyase is used for the degradation of cell wall of yeast and fungi.

8.4 Product Recovery and Purification

Multiple steps of pre-purification and purification are adopted to separate the target product from pools of contaminants after cell separation (for extracellular product) and disruption processes (for intracellular product). These steps normally contribute substantially to the total production cost. The conventional product recovery scheme usually starts with the pre-purification steps such as ammonium sulfate precipitation, solvent extraction, liquid-liquid extraction, and ultrafiltration and is followed by purification techniques based on chromatography and, lastly, the polishing steps like crystallization, spray drying, and freeze-drying.

8.4.1 Product Extraction and Concentration

High-resolution purification techniques often are limited by low process throughput. Additionally, clean process streams free of debris, lipids, and particulates are required in these methods to guarantee their efficiencies. Therefore, product concentration and pre-purification steps are needed.

Ammonium Sulfate Precipitation

Product concentration by ammonium sulfate precipitation is one of the widely used protocols. The target protein and other macromolecules might have markedly different solubilities in concentrated ammonium sulfate solution. At a sufficient ionic strength of surrounding medium, the target protein or other contaminant protein is precipitated out of the solution (the effect is described as “salting out”) and thus is separated from each other [110, 121]. The discrepancies in “salting out” power of proteins allow the fractionation precipitation of proteins obtained from different concentrations of ammonium sulfate solution used.

Ammonium sulfate precipitation method involves the addition of finely ground ammonium sulfate in increasing proportions at 4 °C under continual stirring to raise the

percent saturation of the sample solution from 0% to up to 90% in few process steps. Fractional precipitation allows a degree of purification, by the removal of some contaminants that have different "salting out" abilities from the target product. Besides salts, protein precipitation can be performed using organic solvents (e.g., ethanol and acetone) and polymers (e.g., dextran, polypropylene glycol (PPG), and polyethylene glycol (PEG)).

Liquid-Liquid Extraction

Liquid-liquid extraction is another alternative technique for low-resolution product purification. The liquid-liquid extraction technologies described here are represented by aqueous two-phase systems (ATPSs). In the mid-1950s, Albertsson [2] has first reported the idea of using ATPS as an alternative separation tool for biomolecules. The working principle of product extraction using ATPS approach is based on the selective partitioning of the target product and contaminants in two immiscible aqueous-rich solutions, which are formed when two polymers or one polymer and a salt present beyond particular concentrations in an aqueous-rich medium [54]. The phase-forming constituents investigated include dextran [32]; PEG [96, 109]; PPG [159]; light-, thermo-, and pH-sensitive polymers [85, 106, 153]; ethanol [108, 154]; organic solvents like tetrahydrofuran [137, 138]; and ionic liquids (ILs) [76, 79]. ATPSs have proven their effectiveness for the separation of a wide range of bioproducts from fermentation broth, including amylase [13], protease [116], lysozyme [140], xylanase [107, 160], polyhydroxyalkanoate [83], tetracycline [113], cyclodextrin glycosyltransferase [106], 2,3-butanediol [66], and lipase [149, 166].

Among the phase formers, ILs have soon gained popularity owing to their attractive properties such as non-flammability, low volatility, and high chemical/thermal stability. Aside from traditional imidazolium-based ILs, the ILs studied have been expanded to other families of benign cations and anions. The recent advances of the applications of different ILs for protein separation and purification have been reviewed extensively by Lee and coworkers [81]. The ILs are investigated for their capability to form ATPS with salts and polymers, and most importantly, the partitioning behaviors of the product and contaminants in IL-based ATPS are assessed. It is reported that the partitioning of the product can be made selective by altering the properties of ATPS, including the concentrations and volume ratios of phase-forming components, pH, and temperature, in order to make certain interactions to be dominant [54]. The process condition is viewed to be optimum when all target products are migrated to one of the phases, while the contaminants partition to the opposite phase. Lee and co-workers [81] concluded that majority of bioproducts have strong partition preference toward IL-rich phase in both the IL and salt systems and polymer and IL systems. Figure 8.8 displayed an illustration showing the partitioning behavior of a biomolecule such as bovine serum albumin (BSA), a model protein, in IL-based ATPS with polymer/salt. The partitioning behaviors of biomolecule are driven by the interactions between BSA residues and IL ions in different IL-based ATPSs.

Besides, other advanced alternative has been developed using the basis of ATPS, i.e., aqueous two-phase flotation (ATPF) [14]. ATPF utilizes a combination of the working principles of ATPS and solvent sublation. It makes use of two immiscible aqueous-rich phases as working medium in which the target bioproducts are attached to gas bubbles that are moving upward from the bottom phase to top phase [77]. ATPF has been applied for the separation of biomolecules such as penicillin G [14, 16], puerarin [15], lincomycin [84], baicalin [17], chloramphenicol [51], tetracycline [155], and lipase [134]. Recently, the technique has been integrated with fermentation process for direct recovery and separation of lipase from fermentation broth [125].

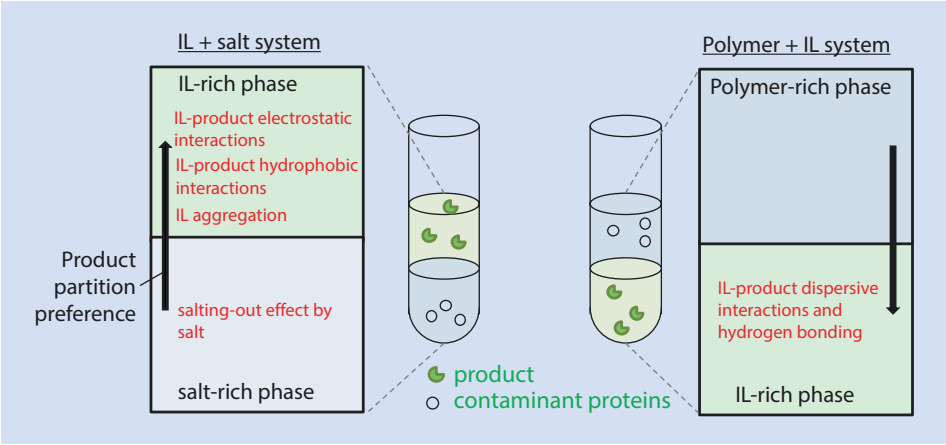


Fig. 8.8 An illustration showing the partitioning behavior of product (such as BSA) and contaminants in IL-based ATPS formed with salt/polymer. The possible interactions between product molecules and IL ions that govern the partitioning preference of product are described. (Adapted from [78–82])

Table. 8.4 Chromatography techniques and their working principles for the purification of bioproducts

Technique	Working principle
Ion-exchange chromatography	Charge
Gel filtration chromatography	Size
Affinity chromatography	Ligand specificity

8.4.2 Product Purification by Chromatographic Techniques

Chromatography is commonly used to achieve the desired purity level of bioproducts. Ion exchange, gel filtration, and affinity are three of the most applied chromatographic techniques. The techniques and their working principles are listed in Table 8.4. Combining two or more chromatographic techniques that utilize different physical-chemical interactions as the basis of separation might be an effective approach. For example, separation scheme incorporating steps of gel filtration chromatography and ion-exchange chromatography in series might be a suitable combination [31].

Ion-Exchange Chromatography

Ion-exchange chromatography separates target product from contaminants based on differences between the overall charges of the compounds in the mixture. It is commonly applied to purify almost all kinds of surface-charged biomolecules, such as large proteins [69], antibody [139], plasmid DNA [117], and hepatitis B core antigen [86]. It is performed in the form of column chromatography. The column materials consist of charged groups that are covalently linked to the surface of an insoluble matrix. There are two main types of ion-exchange chromatography, namely, cation-exchange and anion-exchange

Table. 8.5 Types of ion-exchange chromatography and the functional groups of the common resins used

Ion-exchange chromatography	Resin	Functional group	Matrix	Category
Cation	SP-Sephadex	Sulfopropyl (SP)	Dextran	Strong
	CM-Sephadex	Carboxymethyl (CM)	Dextran	Weak
Anion	Q-Sephadex	Quaternary ammonium (Q)	Dextran	Strong
	QAE-Sephadex	Diethyl-(2-hydroxypropyl) aminoethyl	Dextran	Strong
	DEAE-Sephadex	Diethylaminoethyl (DEAE)	Dextran	Weak

chromatography. Cation-exchange chromatography is applied when the target bioproduct is positively charged. Likewise, negatively charged bioproducts (with isoelectric point below pH 7.0) are processed using anion-exchange chromatography that contains sorbent particle with positively charged groups. Various types of cationic and anionic ion exchangers with different resins and matrices are commercially available, as summarized in Table 8.5, to suit for a wide variety of products with different surface properties.

Ion-exchange chromatography involves two sequential processes, that is, the absorption of the target bioproduct onto an ionic support matrix which has opposite charge to the target bioproducts and the desorption of the target bioproduct from the ionic support matrix by elution operation. The binding of the product to the adsorbent usually takes place under low ionic strength conditions. Their binding strength increases with the size of the charge and charge density of molecules. The bound product molecules are collected using an eluent such as NaCl [98] that is bounded preferably by the support matrix or is used to alter the pH of the column.

Gel Filtration Chromatography

Gel filtration chromatography, also known as size exclusion chromatography, is working based on the molecular size of bioproducts. Protein fractionation can be achieved based on the relative diffusion coefficients of proteins in the gel column, which is depending on molecular size and porosity of the gel matrix. Protein molecules travel through a bed of porous beads with greater or lesser diffusion rates. Smaller biomolecules that can diffuse into the pores of the beads are retained longer and pass through the gel column more slowly. On the other hand, larger molecules which flow through the column's interspaces without entering the pores are eluted rapidly. Therefore, protein fractionation occurs, and the target biomolecules, which may be small, moderate, or large, can be separated from others.

Pore size distribution of the gel matrix network is an important parameter in the design of media for gel filtration chromatography [49]. Several gel filtration media with different porosity behaviors are commercially available, as provided in Table 8.6.

Affinity Chromatography

Product purification by affinity chromatography is principally based on a highly specific binding of the desired product onto the immobilized ligand attached to an inert matrix (i.e., the stationary phase) in a column. The two parties can be receptor and ligand, antigen

Table. 8.6 Types of gel filtration media and their potential applications

Trade name	Matrix	Products in bio-separation application
Hydrophobic/ lipophilic	Hydroxyalkoxypropyl-dextran	Fatty acids, esters, phospholipids, cholesterols, and steroids
Sephacryl	Acrylic (dextran/bisacryl-amide copolymer)	Monoclonal antibodies, peptides, small and large proteins
Sephadex	Dextran (cross-linked with epichlorohydrin)	Poliovirus
Superdex	Dextran (cross-linked with agarose)	Monoclonal antibodies, recombinant DNA products, peptides, oligosaccharides, and small proteins
Superose	Agarose (highly cross-linked)	Proteins, DNA fragments, polysaccharides
Toyopearl HW	Methacrylate (ethylene glycol/methacrylate copolymer)	Glycoproteins, intermediate molecular weight molecules
Ultrogel	Agarose	Globular proteins

and antibody, or enzyme and substrate. They couple with each other through one or more interactions such as ionic interaction, hydrophobic interaction, hydrogen bonding, van der Waals forces, and disulfide bridges. The target product is moved through a bed of polymer or gel matrix in which a specific interaction occurs, and the target product is covalently bound to the matrix. On the other hand, other proteins that have no affinity for the matrix pass through the column rapidly and thus are separated. Similar to ion-exchange chromatography, the bound target product is collected by an elution step that can be achieved by altering the salt concentration and pH.

There are many groups of selective ligands on beaded and porous matrices for binding specific compounds. For instances, a ConA-Sepharose resin with immobilized Concanavalin A is efficient in capturing glycosylated biomolecules including glycoproteins and glycolipids. Hence, ConA-Sepharose resin could be useful for the purification of glycopeptides [103] and lipases that are essentially glycoproteins [27, 60].

8.4.3 Product Polishing

Product polishing is the process steps at final stage to prepare the product in a form that is stable and convenient for transportation and storage. The common strategies are crystallization [100], spray drying, and freeze-drying [92, 142]. Table 8.7 describes the processes, product quality obtained, and constraints of these techniques.

Crystallization process is the formation of solid particles within a homogenous phase. It is based on the principle of solubility of product. It involves the phase change that a solute from a liquid solution is precipitated to a pure solid crystal in supersaturated solution [74]. The impurities remain dissolved in the liquid solution and can be discarded by

Table. 8.7 Product polishing strategies with the descriptions of their processes, product qualities, and constraints

Strategy	Process	Product quality	Constraint
Crystallization	Product is precipitated from a supersaturated solution	Low	Use of solvents or salts to promote crystallization
			High sensitivity of product to temperature, pH, and ionic strength of surrounding medium
			Fragile nature of protein crystal due to irregularly shaped surfaces of proteins
			Filtration step is needed to separate the product crystal from the dissolved impurities in liquid solution
Spray drying	Product-rich liquid feed is dried rapidly with a hot gas to form a dry powder	Low–high	Thermal degradation of product
Freeze-drying	Product is frozen under reduced pressure, and the ice formed is removed by sublimation process	High	Few drying stages are required
			Freeze-dried products can be rehydrated rapidly
			High operation cost

filtration step. Therefore, crystallization offers a practical technique of obtaining pure product in an appropriate form for packaging and storing.

On the other hand, drying involves the removal of moisture and volatile compounds from a product, in order to improve storage life of the product and for ease of handling. Drying aids in the purification of the product by removing water and solvents that are used in the chromatographic purification steps. Spray drying utilizes a hot gas stream to supply heat by convection to vaporize the moisture (free moisture, hygroscopic moisture, or a combination of both) from a liquid feed, as illustrated in Fig. 8.9a [136]. Air is commonly used as hot gas stream, but nitrogen gas is used instead for oxygen-sensitive product such as pharmaceuticals. The flow arrangement of liquid and gas can be concurrent, countercurrent, or a combination of both in the same unit (see Fig. 8.9b) [136].

Because of the high operating temperature of spray drying, products that are vulnerable to thermal degradation should find an alternative. Freeze-drying, also known as lyophilization, is a process for drying heat-sensitive products at temperature below 0 °C. It is suitable for the drying of thermal-sensitive proteins and medicines. In freeze-drying, the water is removed as a vapor by sublimation from the frozen product in a vacuum chamber. Freeze-drying produces high-quality product by preserving the native structure and characteristics of active ingredients.

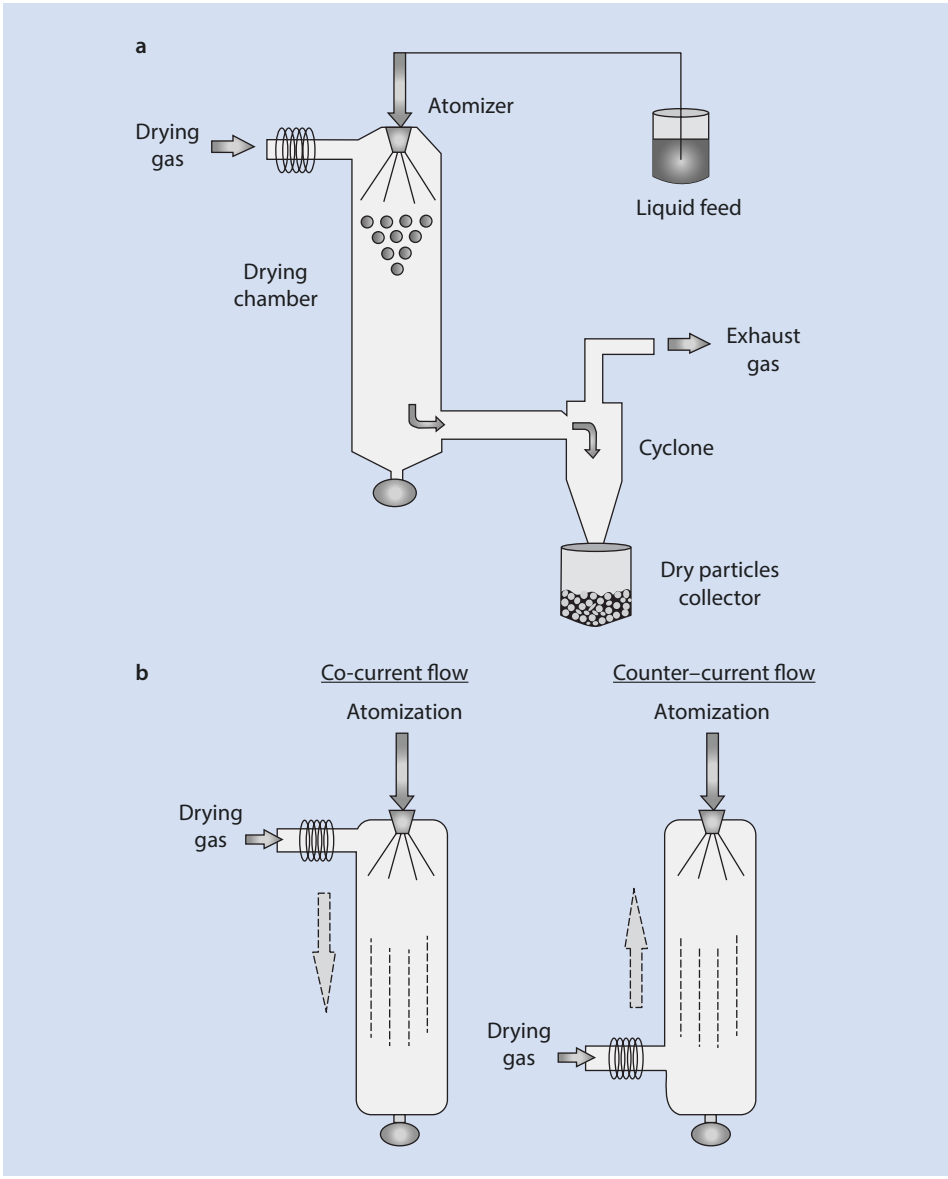


Fig. 8.9 **a** Diagram of the equipment and process of conventional spray drying, **b** scheme of cocurrent and countercurrent flow arrangement [136]

8.5 Conclusions and Future Perspectives

Biological products that are produced via microbial fermentation continue to receive widespread demands. Effective and well-characterized protocols for process-scale product recovery are therefore required. Downstream processing plays a critical role in obtaining final target product in the desired purity and concentration levels. Moreover, it is very important to reduce the production cost, making the products more economically viable for commercial use.

Summing up, primary recovery stages of a biological product involve two main parts, namely, cell separation and cell disruption. Cell separation can be achieved by single or multiple steps of centrifugation and/or filtration operations. Besides, the release of intracellular target product from microbial cell necessitates an additional step, that is, cell disruption. Cell lysis methods can be classified as mechanical, physical, and chemical. Mechanical methods such as high-pressure homogenization and bead agitation are currently preferred for large-scale use. They offer the advantages of continuous operation and high throughput [57] but suffer from the disadvantages such as product degradation due to localized heating and nonselective in their extraction manner, whereas chemical methods have been the method of choice for the cell lysis because of their high selectivity in extracting target product by modifying the permeability of cell membrane or digesting the cell wall.

The methods with different specificities can be synergistically integrated to possess complementary advantages in lysing the cells. A significant number of investigations have been conducted to study the effectiveness of combinations of chemical treatment and mechanical method to enhance the recovery of intracellular product. Vogel et al. [150] reported that a pretreatment of *Bacillus cereus* with a lytic enzyme, namely, cellosyl, improves the efficiency of wet milling and high-pressure homogenization. Likewise, Anand et al. [6] demonstrated that the use of chemical pretreatment prior to high-pressure homogenization step enhances the release of proteins from *E. coli*. The use of chemical agents like EDTA, guanidine hydrochloride, and Triton X-100 weakens and dissolves the cell envelope, allowing a saving of energy input of the subsequent mechanical operation [6]. Regardless of the lysis techniques applied, it is important to retain stable and active nature of target product. Besides, advanced disruption techniques continue to be developed. For instance, electromechanical lysis using electro-convective vortices near ion selective materials has been introduced to recover proteins and nucleic acids from a variety of pathogenic bacteria [71].

As far as possible, the requisite product recovery and purification should be completed with the fewest processing steps. An optimal recovery scheme should be developed based on the aforementioned five main heuristic rules. In conclusion, downstream processes are essential parts of any industrial production of microbial products. Continuous demands for cost-effective and mild downstream processes facilitate an intense effort for the establishment of advanced separation and purification techniques, in order to obtain target products at desirable level of purity and concentration with the least number of steps in downstream processing. The unit operations in primary recovery stages that serve for product isolation and extraction are investigated for its potential to purify the product at least to some degree of purification, in order to reduce the processing steps. Downstream processing, however, remains challenging to increase product quality and at the same time minimize process steps and overall production costs. Recycling and reuse of the chemicals in downstream processing is another area to be explored for the process of sustainability. Besides, the integration of bioreaction stage and parts of downstream processing is studied.

Self-Evaluation

1. What is the first main heuristic rule to design a recovery scheme of a fermentation product? Please give an example of unit operation involved.
2. What are the four main stages of downstream processing of bioproducts?
3. High-speed homogenization is carried out using

(a) French press
(b) Rotor-stator homogenizer
(c) Ultrasonic equipment
(d) Mortar and pestle

4. Column chromatography based on protein sizes is
   (a) Affinity
   (b) Hydrophobic
   (c) Gel filtration
   (d) Ion exchange
5. QAE-Sepharose is
   (a) Strong anion exchanger
   (b) Weak anion exchanger
   (c) Strong cation exchanger
   (d) Weak cation exchanger

Answers

1. The easiest-to-remove impurities should be removed first. The examples are solid-liquid separation operations using centrifugation or filtration.
2. Removal of insoluble, product extraction and isolation, product purification, and product polishing.
3. (b)
4. (c)
5. (a)

Take-Home Messages

- Cell separation involves the separation of whole cells (i.e., biomass) and other insoluble components from the broth. Normally, a solid-liquid separation operation such as filtration or centrifugation occurs.
- Cell disruption serves to break open the host cells allowing the liberation of desired products that are stored inside a cell. It can be categorized into mechanical, physical, and chemical methods.
- Product recovery and purification usually starts with the pre-purification steps such as ammonium sulfate precipitation, solvent extraction, liquid-liquid extraction, and ultrafiltration and is followed by purification techniques based on chromatography and, lastly, the polishing steps like crystallization and drying.

References

1. Adham NZ, Ahmed EM. Extracellular lipase of Aspergillus niger NRRL3; production, partial purification and properties. Indian J Microbiol. 2009;49:77–83. https://doi.org/10.1007/s12088-009-0004-2.
2. Albertsson PÅ. Partition of cell particles and macromolecules. 3rd ed. New York: Wiley; 1986.
3. Ali A, Khan Z, Sahib M, Durrani S, Shaligram U, Raju E, Durrani A. Purification of recombinant subunit vaccine by HbsAg cell lysis using various enrichments of chaotropic and non-ionic detergents. Biosci Biotechnol Res Commun. 2016;9:244–52.
4. Allen JR, Patkar AY, Frank TC, Donate FA, Chiu YC, Shields JE, Gustafson ME. Use of glycol ethers for selective release of periplasmic proteins from gram-negative bacteria. Biotechnol Prog. 2007;23:1163–70.

5. Amirkhani MA, Mohseni R, Soleimani M, Shoae-Hassani A, Nilforoushzadeh MA. A rapid sonication based method for preparation of stromal vascular fraction and mesenchymal stem cells from fat tissue. Bioimpacts. 2016;6:99–104. https://doi.org/10.15171/bi.2016.14.
6. Anand H, Balasundaram B, Pandit AB, Harrison STL. The effect of chemical pretreatment combined with mechanical disruption on the extent of disruption and release of intracellular protein from E-coli. Biochem Eng J. 2007;35:166–73. https://doi.org/10.1016/j.bej.2007.01.01.
7. Andrews BA, Asenjo JA. Enzymatic lysis and disruption of microbial-cells. Trends Biotechnol. 1987;5:273–7. https://doi.org/10.1016/0167-7799(87)90058-8.
8. Antharavally BS, Mallia KA, Rosenblatt MM, Salunkhe AM, Rogers JC, Haney P, Haghdoost N. Efficient removal of detergents from proteins and peptides in a spin column format. Anal Biochem. 2011;416:39–44. https://doi.org/10.1016/j.ab.2011.05.013.
9. Babu K, Satyanarayana T. α-Amylase production by thermophilic *Bacillus coagulans* in solid state fermentation. Process Biochem. 1995;30:305–9.
10. Balasundaram B, Harrison S, Bracewell DG. Advances in product release strategies and impact on bioprocess design. Trends Biotechnol. 2009;27:477–85.
11. Basheer SA, Thenmozhi M. Reverse micellar separation of lipases: a critical review. Int J Chem Sci. 2010;8:57–67.
12. Bensley RR, Hoerr NL. Studies on cell structure by the freezing-drying method VI. The preparation and properties of mitochondria. Anat Rec. 1934;60:449–55.
13. Bezerra RP, Borba FKSL, Moreira KA, Lima-Filho JL, Porto ALF, Chaves AC. Extraction of amylase from fermentation broth in poly (ethylene glycol) salt aqueous two-phase system. Braz Arch Biol Technol. 2006;49:547–55.
14. Bi PY, Li DQ, Dong HR. A novel technique for the separation and concentration of penicillin G from fermentation broth: aqueous two-phase flotation. Sep Purif Technol. 2009;69:205–9. https://doi.org/10.1016/j.seppur.2009.07.019.
15. Bi PY, Dong HR, Yuan YC. Application of aqueous two-phase flotation in the separation and concentration of puerarin from Puerariae extract. Sep Purif Technol. 2010;75:402–6. https://doi.org/10.1016/j.seppur.2010.09.010.
16. Bi PY, Chang L, Dong HR. Separation behavior of penicillin in aqueous two-phase flotation. Chin J Anal Chem. 2011;39:425–8. https://doi.org/10.1016/s1872-2040(10)60428-8.
17. Bi PY, Chang L, Mu YL, Liu JY, Wu Y, Geng X, Wei Y. Separation and concentration of baicalin from Scutellaria Baicalensis Georgi extract by aqueous two-phase flotation. Sep Purif Technol. 2013;116:454–7. https://doi.org/10.1016/j.seppur.2013.06.024.
18. Bimboim H, Doly J. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 1979;7:1513–23.
19. Borrero-de Acuña JM, Hidalgo-Dumont C, Pacheco N, Cabrera A, Poblete-Castro I. A novel programmable lysozyme-based lysis system in Pseudomonas putida for biopolymer production. Sci Rep. 2017;7:4373.
20. Braga ARC, Lemes AC, Kalil SJ. Single chromatographic step for beta-Galactosidase purification: influence of salt and elution parameters. Sep Sci Technol. 2014;49:1817–24. https://doi.org/10.1080/01496395.2014.902960.
21. Carta G, Jungbauer A. Downstream processing of biotechnology products protein chromatography: process development and scale-up. Weinheim: Wiley; 2010. p. 1–55.
22. Chang S, Guo Y, Wu B, He B. Extracellular expression of alkali tolerant xylanase from Bacillus subtilis Lucky9 in *E. coli* and application for xylooligosaccharides production from agro-industrial waste. Int J Biol Macromol. 2017;96:249–56.
23. Chen YC, Chen LA, Chen SJ, Chang MC, Chen TL. Modified osmotic shock for periplasmic release of a recombinant creatinase from *Escherichia coli*. Biochem Eng J. 2004;19:211–5. https://doi.org/10.1016/j.bej.2004.03.001.
24. Chentir I, Hamdi M, Doumandji A, HadjSadok A, Ouada HB, Nasri M, Jridi M. Enhancement of extracellular polymeric substances (EPS) production in Spirulina (Arthrospira sp.) by two-step cultivation process and partial characterization of their polysaccharidic moiety. Int J Biol Macromol. 2017;105:1412–20.
25. Chisti Y, Moo-Young M. Disruption of microbial cells for intracellular products. Enzym Microb Technol. 1986;8:194–204.
26. Choi JH, Lee SY. Secretory and extracellular production of recombinant proteins using *Escherichia coli*. Appl Microbiol Biotechnol. 2004;64:625–35. https://doi.org/10.1007/s00253-004-1559-9.

27. Comménil P, Belingheri L, Sancholle M, Dehorter B. Purification and properties of an extracellular lipase from the fungus Botrytis cinerea. Lipids. 1995;30:351–6.
28. Dako E, Dadie AT, Bernier A-M, Jankowski CK. The problems associated with enzyme purification. United Kingdom: Intech Open Access Publisher; 2012.
29. Danilevich VN, Petrovskaia LE, Grishin EV. Rapid and efficient extraction of soluble proteins from gram-negative microorganisms without disruption of cell walls. Bioorg Khim. 2006;32:579–88.
30. de Carvalho JC, Medeiros ABP, Letti LAJ, Kirnev PCS, Soccol CR. Cell disruption and isolation of intracellular products. Current developments in biotechnology and bioengineering: production. Isolation and Purif Ind Prod. 2017; https://doi.org/10.1016/b978-0-444-63662-1.00035-x.
31. Dedmon RE, Holmes AW, Deinhardt F. Preparation of fluorescein isothiocyanate-labeled γ-globulin by dialysis, gel filtration, and ion-exchange chromatography in combination. J Bacteriol. 1965;89:734–9.
32. Diamond AD, Hsu JT. Protein partitioning in PEG/dextran aqueous two-phase systems. Aiche J. 1990;36:1017–24.
33. Duarte SH, dos Santos P, Michelon M, de Pinho Oliveira SM, Martínez J, Maugeri F. Recovery of yeast lipids using different cell disruption techniques and supercritical CO2 extraction. Biochem Eng J. 2017;125:230–7.
34. Dugo P, Cacciola F, Kumm T, Dugo G, Mondello L. Comprehensive multidimensional liquid chromatography: theory and applications. J Chromatogr A. 2008;1184:353–68. https://doi.org/10.1016/j.chroma.2007.06.074.
35. El-Naggar NE-A, Soliman HM, El-Shweihy NM. Extracellular cholesterol oxidase production by Streptomyces aegyptia, in vitro anticancer activities against rhabdomyosarcoma, breast cancer cell-lines and in vivo apoptosis. Sci Rep. 2018;8:2706.
36. Emek SC, Szilagyi A, Akerlund HE, Albertsson PA, Kohnke R, Holm A, Erlanson-Albertsson C. A large scale method for preparation of plant thylakoids for use in body weight regulation. Prep Biochem Biotechnol. 2010;40:13–27. https://doi.org/10.1080/10826060903413057.
37. Eom GT, Lee SH, Song BK, Chung KW, Kim YW, Song JK. High-level extracellular production and characterization of Candida antarctica lipase B in Pichia pastoris. J Biosci Bioeng. 2013;116:165–70. https://doi.org/10.1016/j.jbiosc.2013.02.016.
38. Faria JTD, Rocha PF, Converti A, Passos FM, Minim LA, Sampaio FC. Statistical investigation of Kluyveromyces lactis cells permeabilization with ethanol by response surface methodology. Braz J Microbiol. 2013;44:1067–74.
39. Fernández García L, Álvarez Blanco S, Riera Rodríguez FA. Microfiltration applied to dairy streams: removal of bacteria. J Sci Food Agric. 2013;93:187–96. https://doi.org/10.1002/jsfa.5935.
40. Fischer I, Franzreb M. Removal of the nonionic surfactant Eumulgin ES from protein solutions by means of adsorption and ultrafiltration. Sep Purif Technol. 2013;118:217–25. https://doi.org/10.1016/j.seppur.2013.07.001.
41. Fonseca LP, Cabral JMS. Penicillin acylase release from *Escherichia coli* cells by mechanical cell disruption and permeabilization. J Chem Technol Biotechnol. 2002;77:159–67. https://doi.org/10.1002/jctb.541.
42. Francis F, Sabu A, Nampoothiri KM, Ramachandran S, Ghosh S, Szakacs G, Pandey A. Use of response surface methodology for optimizing process parameters for the production of α-amylase by Aspergillus oryzae. Biochem Eng J. 2003;15:107–15.
43. Fraser D. Bursting bacteria by release of gas pressure. Nature. 1951;167:33–4. https://doi.org/10.1038/167033b0.
44. Geciova J, Bury D, Jelen P. Methods for disruption of microbial cells for potential use in the dairy industry - a review. Int Dairy J. 2002;12:541–53. https://doi.org/10.1016/s0958-6946(02)00038-9.
45. Granum E, Kirkvold S, Myklestad SM. Cellular and extracellular production of carbohydrates and amino acids by the marine diatom *Skeletonema costatum*: diel variations and effects of N depletion. Mar Ecol Prog Ser. 2002;242:83–94. https://doi.org/10.3354/meps242083.
46. Grodzki AC, Berenstein E. Antibody purification: ion-exchange chromatography. In: Oliver C, Jamur MC, editors. Immunocytochemical methods and protocols, Methods in molecular biology, vol. 588. 3rd ed. Totowa: Humana Press Inc; 2010. p. 27–32. https://doi.org/10.1007/978-1-59745-324-0_4.
47. Guimaraes LHS, Somera AF, Terenzi HF, Polizeli M, Jorge JA. Production of beta-fructofuranosidases by Aspergillus niveus using agroindustrial residues as carbon sources: characterization of an intracellular enzyme accumulated in the presence of glucose. Process Biochem. 2009;44:237–41. https://doi.org/10.1016/j.procbio.2008.10.011.

48. Gustafson ME, Clayton RA, Lavrik PB, Johnson GV, Leimgruber RM, Sims SR, Bartnicki DE. Large-scale production and characterization of *Bacillus thuringiensis subsp. tenebrionis* insecticidal protein from *Escherichia coli*. Appl Microbiol Biotechnol. 1997;47:255–61. https://doi.org/10.1007/s002530050923.
49. Hagel L. Gel-filtration chromatography. Curr Protoc Mol Biol. 1998;44:10.19.11–2.
50. Haimer E, Tscheliessnig A, Hahn R, Jungbauer A. Hydrophobic interaction chromatography of proteins IV - kinetics of protein spreading. J Chromatogr A. 2007;1139:84–94. https://doi.org/10.1016/j.chroma.2006.11.003.
51. Han J, Wang Y, Yu C, Li C, Yan Y, Liu Y, Wang L. Separation, concentration and determination of chloramphenicol in environment and food using an ionic liquid/salt aqueous two-phase flotation system coupled with high-performance liquid chromatography. Anal Chim Acta. 2011;685:138–45. https://doi.org/10.1016/j.aca.2010.11.033.
52. Harrison STL. Bacterial cell disruption: a key unit operation in the recovery of intracellular products. Biotechnol Adv. 1991;9:217–40. https://doi.org/10.1016/0734-9750(91)90005-G.
53. Harrison RG, Todd P, Rudge SR, Petrides DP. Bioseparations science and engineering. Top Chem Eng. 2015;2015:348.
54. Hatti-Kaul R. Aqueous two-phase systems: methods and protocols, vol. 11. Berlin: Springer Science & Business Media; 2000.
55. Hjelmeland LM. [21] removal of detergents from membrane proteins. In: Deutscher MP, editor. Methods in Enzymology, vol. 182. New York: Academic Press; 1990. p. 277–82. https://doi.org/10.1016/0076-6879(90)82023-U.
56. Ho CW, Chew TK, Ling TC, Kamaruddin S, Tan WS, Tey BT. Efficient mechanical cell disruption of *Escherichia coli* by an ultrasonicator and recovery of intracellular hepatitis B core antigen. Process Biochem. 2006;41:1829–34.
57. Ho CW, Tan WS, Yap WB, Ling TC, Tey BT. Comparative evaluation of different cell disruption methods for the release of recombinant hepatitis B Core antigen from *Escherichia coli*. Biotechnol Bioprocess Eng. 2008;13:577–83. https://doi.org/10.1007/s12257-008-0020-9.
58. Hober S, Nord K, Linhult M. Protein a chromatography for antibody purification. J Chromatogr B-Anal Technol Biomed Life Sci. 2007;848:40–7. https://doi.org/10.1016/j.chromb.2006.09.030.
59. Hou J, Miao LZ, Wang C, Wang PF, Ao YH, Lv BW. Effect of CuO nanoparticles on the production and composition of extracellular polymeric substances and physicochemical stability of activated sludge flocs. Bioresour Technol. 2015;176:65–70. https://doi.org/10.1016/j.biortech.2014.11.020.
60. Huge-Jensen B, Galluzzo DR, Jensen RG. Studies on free and immobilized lipases fromMucor miehei. J Am Oil Chem Soc. 1988;65:905–10.
61. Ibrahim ASS, Al-Salamah AA, Elbadawi YB, El-Tayeb MA, Ibrahim SSS. Production of extracellular alkaline protease by new halotolerant alkaliphilic Bacillus sp NPST-AK15 isolated from hyper saline soda lakes. Electron J Biotechnol. 2015;18:236–43. https://doi.org/10.1016/j.ejbt.2015.04.001.
62. Isa MHM, Frazier RA, Jauregi P. A further study of the recovery purification of surfactin from fermentation broth by membrane filtration. Sep Purif Technol. 2008;64:176–82. https://doi.org/10.1016/j.seppur.2008.09.008.
63. Islam MS, Aryasomayajula A, Selvaganapathy PR. A review on macroscale and microscale cell lysis methods. Micromachines. 2017;8 https://doi.org/10.3390/mi8030083.
64. Jacquel N, Lo C-W, Wei Y-H, Wu H-S, Wang SS. Isolation and purification of bacterial poly(3-hydroxyalkanoates). Biochem Eng J. 2008;39:15–27. https://doi.org/10.1016/j.bej.2007.11.029.
65. Jauregi P, Coutte F, Catiau L, Lecouturier D, Jacques P. Micelle size characterization of lipopeptides produced by B. subtilis and their recovery by the two-step ultrafiltration process. Sep Purif Technol. 2013;104:175–82. https://doi.org/10.1016/j.seppur.2012.11.017.
66. Jiang B, Li Z-G, Dai J-Y, Zhang D-J, Xiu Z-L. Aqueous two-phase extraction of 2,3-butanediol from fermentation broths using an ethanol/phosphate system. Process Biochem. 2009;44:112–7.
67. Johnson BH, Hecht MH. Recombinant proteins can be isolated from escherichia-coli-cells by repeated cycles of freezing and thawing. Biotechnology. 1994;12:1357–60. https://doi.org/10.1038/nbt1294-1357.
68. Kasieczka-Burnecka M, Kuc K, Kalinowska H, Knap M, Turkiewicz M. Purification and characterization of two cold-adapted extracellular tannin acyl hydrolases from an Antarctic strain Verticillium sp. P9. Appl Microbiol Biotechnol. 2007;77:77–89.
69. Kaveh-Baghbaderani Y, Blank-Shim SA, Koch T, Berensmeier S. Selective release of overexpressed recombinant proteins from *E. coli* cells facilitates one-step chromatographic purification of peptide-tagged green fluorescent protein variants. Protein Expr Purif. 2018;152:155–60.

70. Kelly WJ, Muske KR. Optimal operation of high-pressure homogenization for intracellular product recovery. Bioprocess Biosyst Eng. 2004;27:25–37.
71. Kim M, Wu LD, Kim B, Hung DT, Han J. Continuous and high-throughput electromechanical lysis of bacterial pathogens using ion concentration polarization. Anal Chem. 2018;90:872–80. https://doi.org/10.1021/acs.analchem.7b03746.
72. Koolman J, Röhm K-H, Wirth J, Robertson M. Color atlas of biochemistry, vol. 2. Stuttgart: Thieme; 2005.
73. Kreyenschulte D, Krull R, Margaritis A. Recent advances in microbial biopolymer production and purification. Crit Rev Biotechnol. 2014;34:1–15.
74. Kumar A, Awasthi A. Bioseparation engineering. India: IK International Pvt Ltd; 2009.
75. Lee S-M. The primary stages of protein recovery. J Biotechnol. 1989;11:103–17. https://doi.org/10.1016/0168-1656(89)90113-2.
76. Lee SY, et al. Evaluating self-buffering ionic liquids for biotechnological applications. ACS Sustain Chem Eng. 2015;3:3420–8.
77. Lee SY, Khoiroh I, Ling TC, Show PL. Aqueous two-phase flotation for the recovery of biomolecules. Sep Purif Rev. 2016;45:81–92.
78. Lee HD, Lee MY, Hwang YS, Cho YH, Kim HW, Park HB. Separation and purification of lactic acid from fermentation broth using membrane-integrated separation processes. Ind Eng Chem Res. 2017a;56:8301–10. https://doi.org/10.1021/acs.iecr.7b02011.
79. Lee SY, Khoiroh I, Coutinho JA, Show PL, Ventura SP. Lipase production and purification by self-buffering ionic liquid-based aqueous biphasic systems. Process Biochem. 2017b;63:221–8.
80. Lee SY, Khoiroh I, Ling TC, Show PL. Enhanced recovery of lipase derived from *Burkholderia cepacia* from fermentation broth using recyclable ionic liquid/polymer-based aqueous two-phase systems. Sep Purif Technol. 2017c;179:152–60.
81. Lee SY, Khoiroh I, Ooi CW, Ling TC, Show PL. Recent advances in protein extraction using ionic liquid-based aqueous two-phase systems. Sep Purif Rev. 2017d;00:1–14. https://doi.org/10.1080/15422119.2017.1279628.
82. Lee SY, Show PL, Ling TC, Chang J-S. Single-step disruption and protein recovery from *Chlorella vulgaris* using ultrasonication and ionic liquid buffer aqueous solutions as extractive solvents. Biochem Eng J. 2017e;124:26–35. https://doi.org/10.1016/j.bej.2017.04.009.
83. Leong YK, Lan JC-W, Loh H-S, Ling TC, Ooi CW, Show PL. Cloud-point extraction of green-polymers from Cupriavidus necator lysate using thermoseparating-based aqueous two-phase extraction. J Biosci Bioeng. 2017;123:370–5. https://doi.org/10.1016/j.jbiosc.2016.09.007.
84. Li M, Dong HR. The investigation on the aqueous two-phase floatation of lincomycin. Sep Purif Technol. 2010;73:208–12. https://doi.org/10.1016/j.seppur.2010.04.002.
85. Li X, Wan J, Cao X. Preliminary application of light-pH sensitive recycling aqueous two-phase systems to purification of lipase. Process Biochem. 2010;45:598–601.
86. Lim SL, et al. Single-step purification of recombinant hepatitis B core antigen Y132A dimer from clarified *Escherichia coli* feedstock using a packed bed anion exchange chromatography. Process Biochem. 2018;69:208–15. https://doi.org/10.1016/j.procbio.2018.03.003.
87. Lin HM, Chan EC, Chen C, Chen LF. Disintegration of yeast-cells by pressurized carbon-dioxide. Biotechnol Prog. 1991;7:201–4. https://doi.org/10.1021/bp00009a001.
88. Lin HM, Yang Z, Chen LF. An improved method for disruption of microbial cells with pressurized carbon dioxide. Biotechnol Prog. 1992;8:165–6. https://doi.org/10.1021/bp00014a012.
89. Ling Y, Williams D, Thomas C, Middelberg A. Recovery of poly-3-hydroxybutyrate from recombinant *Escherichia coli* by homogenization and centrifugation. Biotechnol Tech. 1997;11:409–12.
90. Linke D. Detergents: an overview. In: Methods in enzymology, vol. 463. London: Elsevier; 2009. p. 603–17.
91. Linke D, Berger RG. Foaming of proteins: new prospects for enzyme purification processes. J Biotechnol. 2011;152:125–31. https://doi.org/10.1016/j.jbiotec.2010.07.022.
92. Liu B, Zhou X. Freeze-drying of proteins. In: Cryopreservation and freeze-drying protocols. New York: Springer; 2015. p. 459–76.
93. Lo SC, Ramanan RN, Tey BT, Tan WS, Show PL, Ling TC, Ooi CW. A versatile and economical method for the release of recombinant proteins from *Escherichia coli* by 1-propanol cell disruption. RSC Adv. 2016;6:62291–7.
94. Lockwood CE, Bummer PM, Jay M. Purification of proteins using foam fractionation. Pharm Res. 1997;14:1511–5. https://doi.org/10.1023/a:1012109830424.

95. López-Abelairas M, García-Torreiro M, Lú-Chau T, Lema J, Steinbüchel A. Comparison of several methods for the separation of poly (3-hydroxybutyrate) from Cupriavidus necator H16 cultures. Biochem Eng J. 2015;93:250–9.
96. Lu Y-M, Yang Y-Z, Zhao X-D, Xia C-B. Bovine serum albumin partitioning in polyethylene glycol (PEG)/potassium citrate aqueous two-phase systems. Food Bioprod Process. 2010;88:40–6.
97. Majekodunmi SO. A review on centrifugation in the pharmaceutical industry. Am J Biomed Eng. 2015;5:67–78.
98. Masomian M, Rahman RNZRA, Salleh AB, Basri M. A new thermostable and organic solvent-tolerant lipase from Aneurinibacillus thermoaerophilus strain HZ. Process Biochem. 2013;48:169–75. https://doi.org/10.1016/j.procbio.2012.11.002.
99. McMillan JR, Watson IA, Ali M, Jaafar W. Evaluation and comparison of algal cell disruption methods: microwave, waterbath, blender, ultrasonic and laser treatment. Appl Energy. 2013;103:128–34. https://doi.org/10.1016/j.apenergy.2012.09.020.
100. McPherson A, Gavira JA. Introduction to protein crystallization. Acta Crystallogr Sect F: Struct Biol Commun. 2014;70:2–20.
101. Middelberg APJ. Process-scale disruption of microorganisms. Biotechnol Adv. 1995;13:491–551. https://doi.org/10.1016/0734-9750(95)02007-p.
102. Milcent S, Carrère H. Clarification of lactic acid fermentation broths. Sep Purif Technol. 2001;22(23):393–401. https://doi.org/10.1016/S1383-5866(00)00124-6.
103. Narasimhan S, Wilson JR, Martin E, Schachter H. A structural basis for four distinct elution profiles on concanavalin A–Sepharose affinity chromatography of glycopeptides. Can J Biochem. 1979;57:83–96.
104. Neal G, Francis R, Shamlou PA, Keshavarz-Moore E. Separation of immunoglobulin G precipitate from contaminating proteins using microfiltration. Biotechnol Appl Biochem. 2004;39:241–8. https://doi.org/10.1042/BA20030129.
105. Neu HC, Heppel LA. The release of enzymes from Kwherichia coli by osmotic shock and during the formation of Spheroplasts. J Biol Chem. 1965;240:3685–92.
106. Ng HS, Tan CP, Mokhtar MN, Ibrahim S, Ariff A, Ooi CW, Ling TC. Recovery of *Bacillus cereus* cyclodextrin glycosyltransferase and recycling of phase components in an aqueous two-phase system using thermo-separating polymer. Sep Purif Technol. 2012;89:9–15.
107. Ng HS, Chai CXY, Chow YH, Loh WLC, Yim HS, Tan JS, Lan JC-W. Direct recovery of Bacillus subtilis xylanase from fermentation broth with an alcohol/salt aqueous biphasic system. J Biosci Bioeng. 2018;125:585–9. https://doi.org/10.1016/j.jbiosc.2017.12.010.
108. Ooi CW, Tey BT, Hii SL, Kamal SMM, Lan JCW, Ariff A, Ling TC. Purification of lipase derived from Burkholderia pseudomallei with alcohol/salt-based aqueous two-phase systems. Process Biochem. 2009;44:1083–7. https://doi.org/10.1016/j.procbio.2009.05.008.
109. Padilha GDS, Santana JCC, Alegre RM, Tambourgi EB. Extraction of lipase from *Burkholderia cepacia* by PEG/phosphate ATPS and its biochemical characterization. Braz Arch Biol Technol. 2012;55:7–19.
110. Page M, Thorpe R. Purification of IgG by precipitation with sodium sulfate or ammonium sulfate. In: The protein protocols handbook. New York: Springer; 2009. p. 1749–51.
111. Parekh S, Vinci V, Strobel R. Improvement of microbial strains and fermentation processes. Appl Microbiol Biotechnol. 2000;54:287–301.
112. Park P, Kim E, Chu K. Chemical disruption of yeast cells for the isolation of carotenoid pigments. Sep Purif Technol. 2007;53:148–52.
113. Pereira JFB, Vicente F, Santos-Ebinuma VC, Araújo JM, Pessoa A, Freire MG, Coutinho JAP. Extraction of tetracycline from fermentation broth using aqueous two-phase systems composed of polyethylene glycol and cholinium-based salts. Process Biochem. 2013;48:716–22. https://doi.org/10.1016/j.procbio.2013.02.025.
114. Pessoa A, et al. Purification by affinity chromatography of recombinant L-asparaginase I from *Saccharomyces cerevisiae* expressed in *Escherichia coli*. New Biotechnol. 2014;31:S199–200. https://doi.org/10.1016/j.nbt.2014.05.962.
115. Pierce J, Turner C, Keshavarz-Moore E, Dunnill P. Factors determining more efficient large-scale release of a periplasmic enzyme from *E. coli* using lysozyme. J Biotechnol. 1997;58:1–11.
116. Porto T, et al. Liquid–liquid extraction of proteases from fermented broth by PEG/citrate aqueous two-phase system. Chem Eng Process Process Intensif. 2008;47:716–21.
117. Prazeres DMF, Schluep T, Cooney C. Preparative purification of supercoiled plasmid DNA using anion-exchange chromatography. J Chromatogr A. 1998;806:31–45.

118. Rey M, et al. Effective removal of nonionic detergents in protein mass spectrometry, hydrogen/deuterium exchange, and proteomics. Anal Chem. 2010;82:5107–16. https://doi.org/10.1021/ac100171m.
119. Rigaud JL, Levy D, Mosser G, Lambert O. Detergent removal by non-polar polystyrene beads - applications to membrane protein reconstitution and two-dimensional crystallization. Eur Biophys J Biophys Lett. 1998;27:305–19. https://doi.org/10.1007/s002490050138.
120. Rodriguez M, Güereca L, Valle F, Quintero R, López-Munguia A. Penicillin acylase extraction by osmotic shock. Process Biochem. 1992;27:217–23. https://doi.org/10.1016/0032-9592(92)80021-T.
121. Rothenberg MA, Nachmansohn D. Studies on cholinesterase III. Purification of the enzyme from electric tissue by fractional ammonium sulfate precipitation. J Biol Chem. 1947;168:223–31.
122. Salazar O, Asenjo JA. Enzymatic lysis of microbial cells. Biotechnol Lett. 2007;29:985–94. https://doi.org/10.1007/s10529-007-9345-2.
123. Salleh AB, Musani R, Basri M, Ampon K, Yunus WMZ, Razak CNA. Extracellular and intracellular lipases from a thermophilic rhizopus-oryzae and factors affecting their production. Can J Microbiol. 1993;39:978–81. https://doi.org/10.1139/m93-147.
124. Sampathkumar B, Khachatourians GG, Korber DR. High pH during trisodium phosphate treatment causes membrane damage and destruction of *Salmonella enterica* serovar enteritidis. Appl Environ Microbiol. 2003;69:122–9. https://doi.org/10.1128/aem.69.1.122-129.2003.
125. Sankaran R, Show PL, Lee SY, Yap YJ, Ling TC. Integration process of fermentation and liquid biphasic flotation for lipase separation from *Burkholderia cepacia*. Bioresour Technol. 2018;250:306–16.
126. Santos J, Belo I, Mota M, Cabral J. Freeze/thawing and sonication of *Escherichia coli* TB1 cells for cytochrome b5 recovery. Bioseparation. 1996;6:81–9.
127. Sauer T, Robinson CW, Glick BR. Disruption of native and recombinant escherichia-coli in a high-pressure homogenizer. Biotechnol Bioeng. 1989;33:1330–42. https://doi.org/10.1002/bit.260331016.
128. Save S, Pandit A, Joshi J. Use of hydrodynamic cavitation for large scale microbial cell disruption. Food Bioprod Process. 1997;75:41–9.
129. Saxena A, Tripathi BP, Kumar M, Shahi VK. Membrane-based techniques for the separation and purification of proteins: an overview. Adv Colloid Interf Sci. 2009;145:1–22. https://doi.org/10.1016/j.cis.2008.07.004.
130. Scawen MD, Hammond P. Downstream processing: protein extraction and purification Molocular biology and biotechnology. 4th ed. New York: Royal Society of Chemistry; 2000. p. 461–96.
131. Schneider WC, Hogeboom GH. Cytochemical studies of mammalian tissues; the isolation of cell components by differential centrifugation: a review. Cancer Res. 1951;11:1–22.
132. Scorer CA, Buckholz RG, Clare JJ, Romanos MA. The intracellular production and secretion of HIV-1 envelope protein in the methylotrophic yeast pichia-pastoris. Gene. 1993;136:111–9. https://doi.org/10.1016/0378-1119(93)90454-b.
133. Sharma R, Dill BD, Chourey K, Shah M, VerBerkmoes NC, Hettich RL. Coupling a detergent lysis/cleanup methodology with intact protein fractionation for enhanced proteome characterization. J Proteome Res. 2012;11:6008–18. https://doi.org/10.1021/pr300709k.
134. Show PL, et al. Recovery of lipase derived from Burkholderia cenocepacia ST8 using sustainable aqueous two-phase flotation composed of recycling hydrophilic organic solvent and inorganic salt. Sep Purif Technol. 2013;110:112–8. https://doi.org/10.1016/j.seppur.2013.03.018.
135. Smith SM. Strategies for the purification of membrane proteins. In: Walls D, Loughran ST, editors. Protein chromatography: methods and protocols, Methods in molecular biology, vol. 1485. 2nd ed. Totowa: Humana Press Inc; 2017. p. 389–400. https://doi.org/10.1007/978-1-4939-6412-3_21.
136. Sosnik A, Seremeta KP. Advantages and challenges of the spray-drying technology for the production of pure drug particles and drug-loaded polymeric carriers. Adv Colloid Interf Sci. 2015;223:40–54.
137. Souza RL, Lima RA, Coutinho JAP, Soares CMF, Lima ÁS. Aqueous two-phase systems based on cholinium salts and tetrahydrofuran and their use for lipase purification. Sep Purif Technol. 2015a;155:118–26. https://doi.org/10.1016/j.seppur.2015.05.021.
138. Souza RL, Lima RA, Coutinho JAP, Soares CMF, Lima ÁS. Novel aqueous two-phase systems based on tetrahydrofuran and potassium phosphate buffer for purification of lipase. Process Biochem. 2015b;50:1459–67. https://doi.org/10.1016/j.procbio.2015.05.015.
139. Stein A, Kiesewetter A. Cation exchange chromatography in antibody purification: pH screening for optimised binding and HCP removal. J Chromatogr B. 2007;848:151–8.

140. Su C-K, Chiang BH. Partitioning and purification of lysozyme from chicken egg white using aqueous two-phase system. Process Biochem. 2006;41:257–63.
141. Su ZD, Guo Y, Peng ZY. Production of intracellular enzyme by corynebacterium-glutamicum T6-13 protoplasts immobilized in ca-alginate gels. Enzym Microb Technol. 1993;15:791–5.
142. Tang XC, Pikal MJ. Design of freeze-drying processes for pharmaceuticals: practical advice. Pharm Res. 2004;21:191–200.
143. Taskova RM, Zorn H, Krings U, Bouws H, Berger RG. A comparison of cell wall disruption techniques for the isolation of intracellular metabolites from Pleurotus and Lepista sp. Zeitschrift Fur Naturforschung Sect C-a J Biosci. 2006;61:347–50.
144. Taubert J, Krings U, Berger RG. A comparative study on the disintegration of filamentous fungi. J Microbiol Methods. 2000;42:225–32. https://doi.org/10.1016/s0167-7012(00)00194-9.
145. Tsyntsarski B, et al. Removal of detergents from water by adsorption on activated carbons obtained from various precursors. Desalin Water Treat. 2014;52:3445–52. https://doi.org/10.1080/19443994.2013.801327.
146. Van Reis R, Zydney A. Membrane separations in biotechnology. Curr Opin Biotechnol. 2001;12:208–11.
147. van Reis R, Zydney A. Bioprocess membrane technology. J Membr Sci. 2007;297:16–50.
148. Vandergriff A, Padhye V, Cheng K. Recombinant single chain variable fragment antibody production in Escherichia coli and purification with affinity chromatography using arista slice purification system. Protein Sci. 2016;25:169.
149. Ventura SPM, de Barros RLF, de Pinho Barbosa JM, Soares CMF, Lima ÁS, Coutinho JAP. Production and purification of an extracellular lipolytic enzyme using ionic liquid-based aqueous two-phase systems. Green Chem. 2012;14:734–40. https://doi.org/10.1039/c2gc16428k.
150. Vogels G, Kula MR. Combination of enzymatic and or thermal pretreatment with mechanical cell disintegration. Chem Eng Sci. 1992;47:123–31. https://doi.org/10.1016/0009-2509(92)80206-r.
151. Wacker M, Schubert R. From mixed micelles to liposomes: critical steps during detergent removal by membrane dialysis. Int J Pharm. 1998;162:171–5. https://doi.org/10.1016/s0378-5173(97)00425-0.
152. Wang A, Lewus R, Rathore AS. Comparison of different options for harvest of a therapeutic protein product from high cell density yeast fermentation broth. Biotechnol Bioeng. 2006;94:91–104. https://doi.org/10.1002/bit.20816.
153. Wang W, Wan J, Ning B, Xia J, Cao X. Preparation of a novel light-sensitive copolymer and its application in recycling aqueous two-phase systems. J Chromatogr A. 2008;1205:171–6. https://doi.org/10.1016/j.chroma.2008.07.074.
154. Wang Y, Liu Y, Han J, Hu S. Application of water-miscible alcohol-based aqueous two-phase Systems for Extraction of dyes. Sep Sci Technol. 2011a;46:1283–8. https://doi.org/10.1080/01496395.2010.551168.
155. Wang Y, Xu XH, Han J, Yan YS. Separation/enrichment of trace tetracycline antibiotics in water by [Bmim]BF4–(NH4)2SO4 aqueous two-phase solvent sublation. Desalination. 2011b;266:114–8. https://doi.org/10.1016/j.desal.2010.08.010.
156. Wang FQ, Liang JZ, Wang W, Fu DW, Xiao W. A new and efficient method for purification of poly-gamma-glutamic acid from high-viscosity fermentation broth. Trop J Pharm Res. 2017a;16:1267–75. https://doi.org/10.4314/tjpr.v16i6.9.
157. Wang HB, Feng YR, Gui SQ, Zhang Y, Lu FP. A sample pretreatment method to suit the metabolomic analysis of Bacillus licheniformis based on GC-MS. Anal Methods. 2017b;9:2299–304. https://doi.org/10.1039/c7ay00008a.
158. Waszak M, Markowska-Szczupak A, Gryta M. Application of nanofiltration for production of 1, 3-propanediol in membrane bioreactor. Catal Today. 2016;268:164–70.
159. Wu C, Wang J, Pei Y, Wang H, Li Z. Salting-out effect of ionic liquids on poly(propylene glycol) (PPG): formation of PPG + ionic liquid aqueous two-phase systems. J Chem Eng Data. 2010;55:5004–8. https://doi.org/10.1021/je100604m.
160. Yang S, Huang Z, Jiang Z, Li L. Partition and purification of a thermostable xylanase produced by Paecilomyces thermophila in solid-state fermentation using aqueous two-phase systems. Process Biochem. 2008;43:56–61.
161. Yang XM, Zhou SY, Li MS, Wang R, Zhao YJ. Purification of cellulase fermentation broth via low cost ceramic microfiltration membranes with nanofibers-like attapulgite separation layers. Sep Purif Technol. 2017;175:435–42. https://doi.org/10.1016/j.seppur.2016.11.012.

162. Yeung Y-G, Nieves E, Angeletti RH, Stanley ER. Removal of detergents from protein digests for mass spectrometry analysis. Anal Biochem. 2008;382:135–7. https://doi.org/10.1016/j.ab.2008.07.034.
163. Zhang LL, et al. Purification of transglutaminase from high salt culture by gel and Cation exchange chromatography Chinese. J Anal Chem. 2012;40:1225–30. https://doi.org/10.3724/sp.j.1096.2012.11275.
164. Zhang S, et al. Lactobacillus delbrueckii subsp. bulgaricus proteinase: purification by ion-exchange and hydrophobic interaction chromatography. Int J Food Prop. 2015;18:1560–7. https://doi.org/10.1080/10942912.2014.921199.
165. Zhao F, Yu J. L-Asparaginase release from *Escherichia coli* cells with K2HPO4 and triton X100. Biotechnol Prog. 2001;17:490–4.
166. Zhou YJ, Hu CL, Wang N, Zhang WW, Yu XQ. Purification of porcine pancreatic lipase by aqueous two-phase systems of polyethylene glycol and potassium phosphate. J Chromatogr B Analyt Technol Biomed Life Sci. 2013;926:77–82. https://doi.org/10.1016/j.jchromb.2013.03.005.
167. Zou C, Duan XG, Wu J. Enhanced extracellular production of recombinant Bacillus deramificans pullulanase in *Escherichia coli* through induction mode optimization and a glycine feeding strategy. Bioresour Technol. 2014;172:174–9. https://doi.org/10.1016/j.biortech.2014.09.035.

Bioprocess Simulation and Economics

Demetri Petrides, Doug Carmichael, Charles Siletti, and Alexandros Koulouris

A. Berenjian (ed.), *Essentials in Fermentation Technology*, Learning Materials in Biosciences,
https://doi.org/10.1007/978-3-030-16230-6_9

What You Will Learn in This Chapter

This chapter teaches students and practicing engineers the fundamentals of bioprocess simulation, with an emphasis on economic evaluation of integrated biotech processes. Given a product and a desired annual production rate (process throughput), bioprocess simulation endeavors to answer the following and other related questions: What are the required amounts of raw materials and utilities needed for a single batch? What are the required sizes of process equipment and supporting utilities? What is the total capital investment? What is the manufacturing cost? What is the minimum time between consecutive batches? Which process steps or resources are the likely production bottlenecks? What changes can increase throughput and/or reduce costs? What is the environmental impact of the process? Which design is the "best" (fastest or least expensive) among several plausible alternatives?

9.1 Definitions and Background

When designing a new facility, the technological feasibility, the economic viability, and the environmental impact of the process under design must be assessed before the final decision to proceed with the facility construction is made. The assessment is done at different stages at increasing levels of detail; results from every stage are used to evaluate the feasibility of the entire project and guide the assessment process at the next stage.

As discussed in ▶ Sect. 9.3, capital cost calculation is an important component of the economic analysis. Estimates of the investment required are used to compare alternative designs and for overall project evaluation. Capital cost estimates can be used as an example on how facility design proceeds through different stages of increasing accuracy and complexity. ◘ Table 9.1 presents a classification of capital cost estimates for a new process plant at increasingly detailed levels of design. The accuracy of the estimates increases as the planning moves from Level 1 to Level 5. However, the cost and complexity of generating capital cost estimates also increase from Level 1 to Level 5 because performing more detailed process design and project economic evaluation requires integration of knowledge from many different scientific and engineering disciplines at increasing levels of detail.

◘ **Table 9.1** Types of design estimates and their accuracy for a large capital project [1]

Level	Type of estimate	Error (%)
1	Order-of-magnitude estimate (ratio estimate) based on similar previous projects	≤50
2	Project planning estimate (budget estimation) based on knowledge of major equipment items	≤30
3	Preliminary engineering (scope estimate) based on sufficient data to permit the estimate to be budgeted	≤25
4	Detailed engineering (capital approval stage) based on almost complete process data	≤15
5	Procurement and construction (contractor's estimate) based on complete engineering drawings, specifications and site surveys	≤10

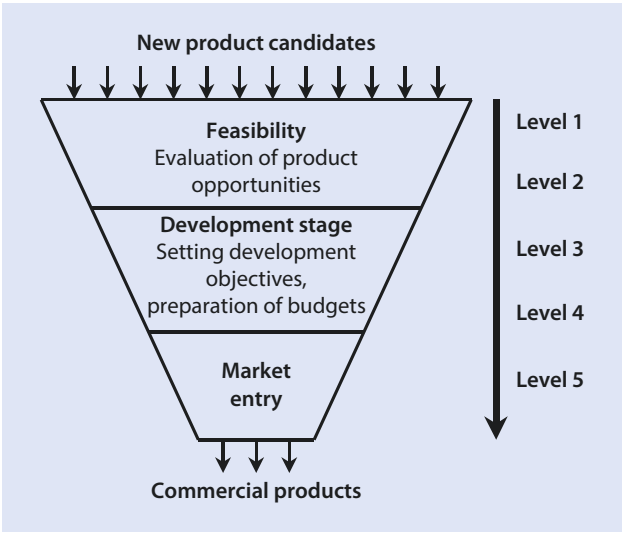


Fig. 9.1 Types of design estimates during the life cycle of a product [2]

Figure 9.1 maps the different types of capital cost estimates to the stages of the product development and commercialization life cycle. The trapezoidal shape of the diagram represents the drastic reduction in product candidates as we move from feasibility studies to commercialization. In fact, the chances of commercialization at the research stage for a new product are only about 1–3%, at the development stage they are about 10–25%, and at the pilot plant stage they are about 40–60% [1].

Order-of-magnitude estimates are usually generated by experienced engineers who have worked on similar projects in the past. These initial estimates only take minutes or hours to complete, but the error in the estimate can be as high as 50%. Table 9.2 presents a sample of historical data that could be used by experienced engineers for order-of-magnitude estimates. The table lists the capital investment for three large-scale facilities built to manufacture therapeutic monoclonal antibodies (mAbs) using cell culture (by growing mammalian cells in stirred-tank bioreactors). Column #2 displays the number of production bioreactors, the working volume of each of them, and the total working volume. For instance, the Genentech facility in Oceanside (former Biogen Idec site) includes six production bioreactors, each having a working volume of 15 m^3. Column #4 displays the total capital investment and column #5 displays the ratio of the total capital investment divided by the total production bioreactor volume. The ratio ranges from 5.0 to 6.2, with an average value of $5.4 million per m^3 of bioreactor volume.

Based on the data in Table 9.2, an engineer may conclude that the capital investment for a new 100 m^3 (total production bioreactor volume) cell culture facility would be in the range of $500–$620 million. Please note, however, that advances in technology (e.g., cell lines that generate higher product titers and the increased usage of single-use systems) and other factors may render such data obsolete and reduce the accuracy of order-of-magnitude estimates based on previous projects. As a result, cost estimates are progressively refined as new product candidates move through the development life cycle shown in Fig. 9.1.

Table 9.2 Capital investments for cell culture facilities

Company	Bioreactor capacity (m^3)	Completion year	Investment ($ millions)	$ million per m^3
Genentech (Oceanside, CA)	6 × 15 = 90	2005	450	5.0
Bristol Myers Squibb (Devens, MA)	6 × 20 = 120	2009	750	6.2
Roche Pharmaceuticals (Switzerland)	6 × 12.5 = 75	2009	375	5.0

Most engineers employed by operating companies usually perform Level 2 and 3 studies. Such studies take weeks or months to complete using appropriate computer aids. The main objective of such studies is to evaluate alternatives and pinpoint the most cost-sensitive areas—the economic "hot spots"—of a complex process. The results of such analyses are used to plan future research and development and to generate project budgets.

Level 4 and 5 studies are usually performed by the engineering and construction companies hired to build new plants for promising new products that are at an advanced stage of development. Such estimates are beyond the scope of this chapter. Instead, the focus of the material in the rest of this chapter will be on Level 1, 2, and 3 studies. It should also be noted that opportunities for creative process design work are usually limited to preliminary studies. By the time detailed engineering work has been initiated, a process is more than 80% fixed. Furthermore, the majority of important decisions for capital expenditures and product commercialization are based on results of preliminary process design and cost analysis. This explains why it is so important for a new engineer to master the skills of preliminary process design and cost estimation.

Environmental impact assessment is an activity closely related to process design and cost estimation. Biochemical plants generate a wide range of liquid, solid, and gaseous waste streams that require treatment prior to discharge. The cost associated with waste treatment and disposal has increased in recent years due to stricter environmental regulations. This cost can be reduced through minimization of waste generation at the source. However, generation of waste from a chemical or biochemical process is dependent on the process design and the manner in which the process is operated. Thus, reducing waste in an industrial process requires intimate knowledge of the process technology. In contrast, waste treatment is essentially an add-on at the end of the process. In addition, minimization of waste generation must be considered by process engineers at the early stages of process development. Once a process has undergone significant development, it is difficult and costly to make major changes. Furthermore, for products in the biopharmaceutical industry, stringent regulatory constraints restrict process modifications after clinical efficacy of the drug has been established. These are only some of the reasons that process modeling and evaluation must be initiated at the early stages of product development.

9.2 Process Modeling and Economic Analysis

A model that is created to represent a bioprocess should be analyzed and compared to alternative processing scenarios in terms of capital investment, manufacturing cost, environmental impact, and other criteria in order to decide which processing setup is superior. Methodologies for estimating capital investment and manufacturing (operating) cost are presented in the next section of this chapter. Both capital and operating cost estimates are based on the results of material and energy balances and equipment sizing. These calculations are typically done using spreadsheets or process simulators, as described below. These tools allow the process design team to characterize a processing scenario and then quickly and accurately redo the entire series of calculations for different sets of assumptions and input data.

9.2.1 Spreadsheets

Spreadsheet applications, such as Microsoft Excel, are commonly used for process calculations and analyses because they are readily available and familiar to the vast majority of scientists, engineers, and other professionals [3]. The user can enter data in different "cells" of the spreadsheet, perform calculations, and generate results. Results from spreadsheets can be easily plotted in a variety of graphs.

9.2.2 Process Simulators

Process simulators are software applications that enable the user to readily represent and analyze integrated processes. They have been in use in the petrochemical industries since the early 1960s. Established simulators for those industries include Aspen Plus and Aspen HYSYS from Aspen Technology (Burlington, MA), ChemCAD from Chemstations (Houston, TX), and PRO/II from Schneider Electric SimSci (Lake Forest, CA).

The simulators mentioned above have been designed to model primarily continuous processes and their transient behavior. Most biological products, however, are produced in batch and semi-continuous mode [4, 5]. Such processes are best modeled with batch process simulators that account for time-dependency and sequencing of events. The first simulator designed specifically for batch processes was called Batches (from Batch Process Technologies in West Lafayette, IN). It was commercialized in the mid-1980s. All of its operation models are dynamic, and simulation always involves integration of differential equations over a period of time. In the mid-1990s, Aspen Technology (Burlington, MA) introduced Batch Plus (now called Aspen Batch Process Developer), a recipe-driven simulator that targeted batch pharmaceutical processes. Around the same time, Intelligen (Scotch Plains, NJ) introduced SuperPro Designer [6, 7]. SuperPro Designer is a flowsheet-driven simulator which handles material and energy balances, equipment sizing and costing, economic evaluation, environmental impact assessment, process scheduling, and debottlenecking of batch and continuous processes.

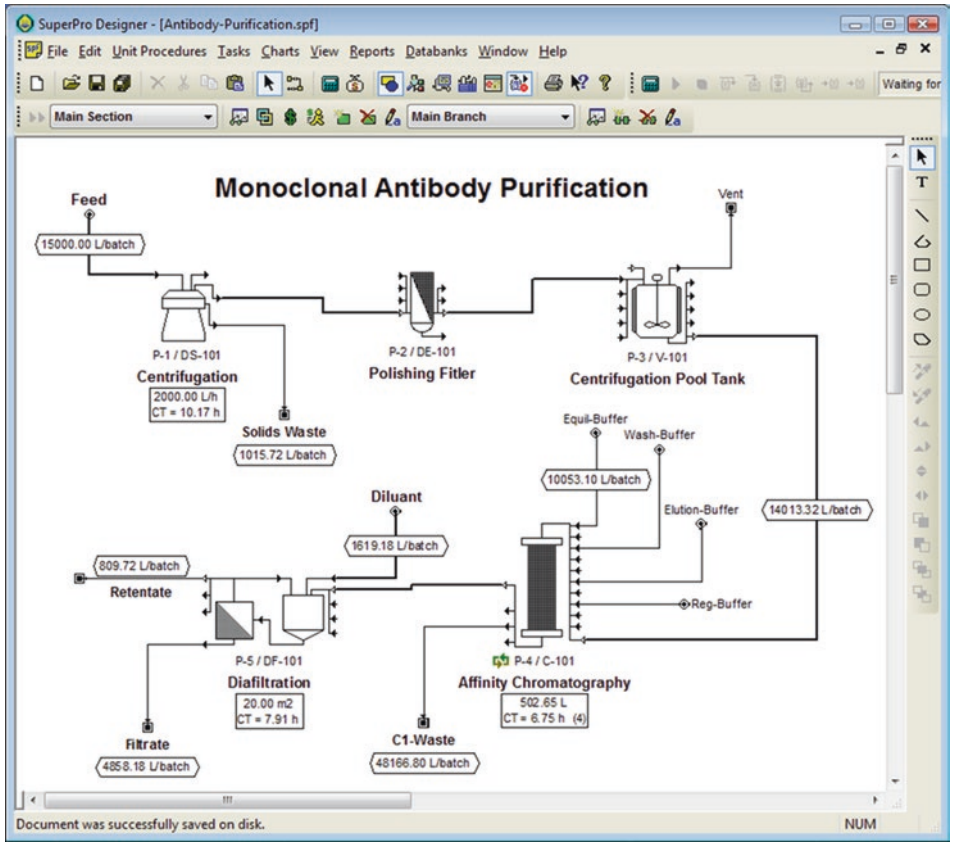
Discrete-event simulators have also found applications in the bioprocessing industries. Established tools of this type include ProModel from ProModel Corporation (Orem, UT), Arena and Witness from Rockwell Automation (Milwaukee, WI), Extend from Imagine That (San Jose, CA), and FlexSim from FlexSim Software Products (Orem, UT). The focus

of models developed with such tools is usually on the minute-by-minute time-dependency of events and the animation of the process. Material balances, equipment sizing, and cost analysis tasks are usually out of the scope of such models.

9.2.3 Using a Biochemical Process Simulator

The minimum requirements for a biochemical process simulator are the ability to handle batch as well as continuous processes and the ability to model the unit operations that are specific to bioprocessing. Because SuperPro Designer (from Intelligen) satisfies these requirements, we will use it to illustrate the role of such tools in bioprocess design. A functional evaluation version of SuperPro Designer and additional information on bioprocess simulation and cost analysis can be obtained at the website ▶ www.intelligen.com. Tutorial videos on the use of SuperPro Designer can be viewed at ▶ www.intelligen.com/videos.

To model an integrated process using a simulator, the user starts by developing a flowsheet that represents the overall process. For instance, ◘ Fig. 9.2 displays the flowsheet of a hypothetical process on the main window of SuperPro Designer. The flowsheet is devel-



◘ **Fig. 9.2** A flowsheet on the main window of SuperPro Designer

oped by putting together the required unit operations (referred to as "unit procedures" in batch processing nomenclature, as explained later in this section) and joining them with material flow streams. Next, the user initializes the flowsheet by registering (selecting from the component database) the various materials that are used in the process, initializing the flow and composition of feed streams, and specifying operating conditions and performance parameters for the various operations.

Most biochemical processes operate in batch or semi-continuous mode. This differs from continuous operation, which is typical in the petrochemical and related industries that handle large throughputs. In continuous operations, a piece of equipment performs the same action all the time, which is consistent with the notion of unit operations. In batch processing, on the other hand, a piece of equipment goes through a cycle of operations. For instance, a typical chromatography cycle includes *equilibration, loading, washing, elution,* and *regeneration*. In SuperPro Designer, the set of operations that comprise a processing step is called a "unit procedure" (as opposed to a "unit operation"). The individual tasks contained within a unit procedure are called operations. A unit procedure is represented on the screen with a single equipment icon (e.g., P-4/C-101 in Fig. 9.2 represents the affinity chromatography procedure). In essence, a unit procedure is the recipe of a processing step that describes the sequence of actions required to complete that step. Figure 9.3 displays the dialog through which the recipe of a chromatography unit procedure is specified. On the left-hand

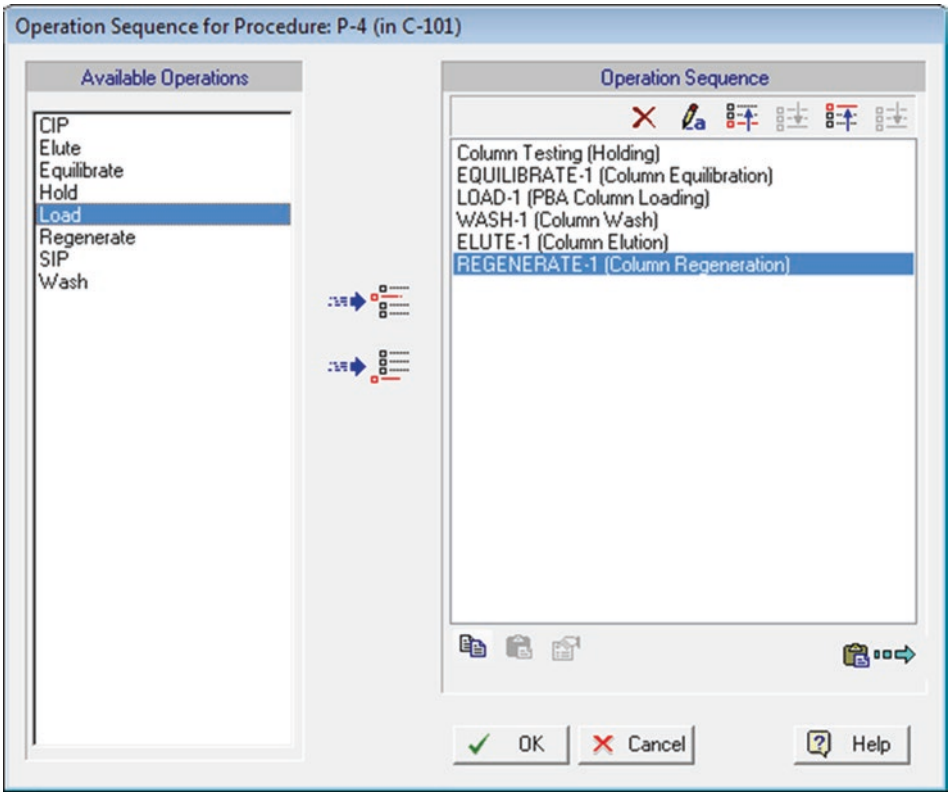


Fig. 9.3 Window for adding operations to a unit procedure using SuperPro Designer

side of that dialog, the program displays the operations that are available in a chromatography procedure; on the right-hand side, it displays the registered operations (i.e., the operations that have been selected for this particular procedure). The significance of the unit procedure is that it enables the user to describe and model the various activities of batch processing steps in detail. Later in this chapter (in the Monoclonal Antibody example), we will see how the execution of these activities can be visualized as a function of time.

For every operation within a unit procedure, SuperPro includes a mathematical model that performs material and energy balance calculations. Based on the results from the material balances, SuperPro also performs equipment-sizing calculations. If multiple operations within a unit procedure dictate different sizes for a certain piece of equipment, the software reconciles the different demands and selects an equipment size that is appropriate for all operations. In other words, the equipment is sized to ensure that it will not be overfilled during any operation but is no larger than necessary (in order to minimize capital costs). In addition, the software checks to ensure that the vessel contents will not fall below a user-specified minimum volume (e.g., a minimum impeller volume) for applicable operations.

Before any simulation calculations can be done, the user must initialize the various operations by specifying operating conditions and performance parameters through appropriate dialog windows. For instance, Fig. 9.4 displays the initialization dialog of a chromatography elution operation. Through this dialog, the user specifies the elution strategy (isocratic or gradient), selects the input and output streams, specifies the eluant volume on a relative (e.g., in terms of bed volumes) or an absolute basis, specifies the fraction of buffer in which the product is recovered, specifies the linear velocity during elution, etc. Through the Labor tab of the same dialog window, the user provides information about labor requirements during this operation. Through the Scheduling tab, the user specifies the sequencing of this operation relative to another operation (either in the same procedure or in a different procedure) or relative to the beginning of the batch. After initialization of the operations, the simulator performs material and energy balances for the entire process and estimates the required sizes of equipment. Based on these calculations, the simulator can perform capital and operating cost estimates and other economic evaluation calculations. The fundamentals of process economics are described in the next section, and a pertinent example is provided later in this chapter.

Having developed a good model using a process simulator or a spreadsheet, the user may conduct "virtual experiments" with alternative process setups and operating conditions. This may potentially reduce costly and time-consuming laboratory and pilot plant effort. One must be aware, however, that the garbage in, garbage out (GIGO) principle applies to all computer models. More specifically, if some assumptions and input data are incorrect, the outcome of the simulation will not be reliable. Consequently, validation of the model is necessary. In its simplest form, a review of the results by an experienced engineer can play the role of validation.

Other tasks that can be handled by process simulators include debottlenecking and throughput analysis (i.e., analysis of the capacity and time utilization of equipment and resources such as utilities, labor, raw materials). Throughput analysis and debottlenecking activities may have a large impact on the economics of a process. As a result, they will be addressed within the example later in this chapter.

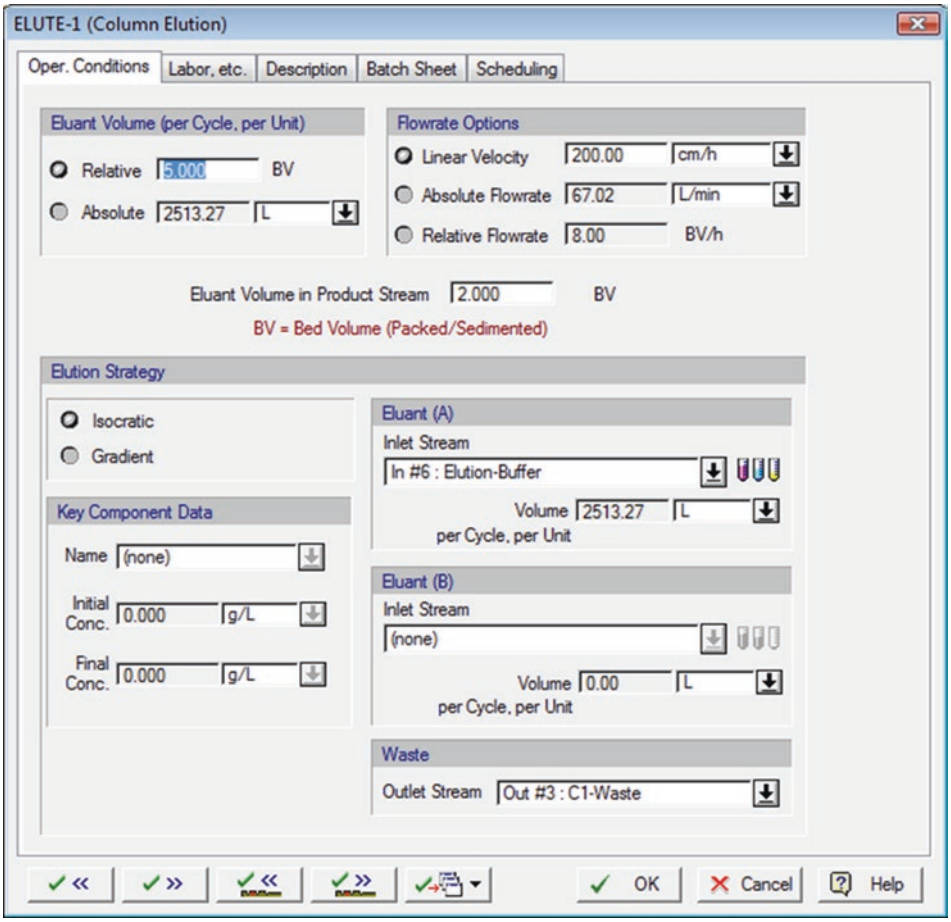


Fig. 9.4 Dialog window of the elution operation

9.3 Process Economics

The preliminary economic evaluation of a project for manufacturing a biological product usually involves the estimation of capital investment, estimation of operating costs, and an analysis of profitability. For biopharmaceuticals, another figure worth considering is the average cost of new drug development, which is estimated to be at least $1.4 billion (2013 dollars) per newly approved drug. If post-approval research and development (R&D) costs are added and the $1.4 billion pre-approval R&D costs are capitalized using a discount rate of 10.5%, the average cost per new drug roughly doubles to $2.87 billion [8]. Note that much of this amount represents research and development (R&D) spending for *un*successful products. This is because the vast majority of new product candidates fail at some stage of development and therefore never reach commercialization. In fact, only about 12% of the compounds which reach clinical testing will eventually be approved [8], and large numbers of compounds fail before they even reach clinical testing. The average R&D costs listed above include spending on the small number of successful product

candidates *as well as* the much larger number of failed product candidates. The massive costs associated with failed product candidates reinforce the need for effective process design tools and methodologies that assist engineers and scientists in efficiently evaluating and eliminating non-promising project ideas at the very early stages of product and process development.

9.3.1 Capital Cost Estimation

The capital investment for a new plant includes three main items: direct fixed capital (DFC), working capital, and start-up and validation cost.

- *Direct Fixed Capital (DFC)*

DFC represents all cost expenditures related to the "fixed" physical assets of a plant site, including its equipment and facilities. The DFC for new biotechnology facilities may range from tens of millions of dollars to well over half a billion dollars, depending on the type of products and the total production capacity. For preliminary design purposes, the various expenses contributing to the DFC may be estimated based on the total equipment purchase cost (PC) and relevant multipliers (sometimes called "Lang Factors"). Table 9.3 provides ranges and average values for the multipliers and a skeleton for the calculations. Detailed definitions of the various cost items and additional information can be found in traditional process design textbooks and the technical literature [1, 9–14].

Based on the multipliers in Table 9.3, it is easy to determine that the total fixed capital investment of a plant may be estimated as a multiple (usually 3–10 times) of its equipment purchase cost. The low end of the range applies to large-scale facilities that produce biofuels and commodity biochemicals, whereas the high end applies to biopharmaceutical facilities. For instance, notice the wide range of multiplier values for estimating the cost of buildings. Plants for commodity biochemicals such as ethanol and citric acid fall on the low end of this range. Conversely, biopharmaceutical facilities fall on the high end due to the expensive heating, ventilation, and air conditioning (HVAC) systems required to ensure high cleanliness and product purity. The average building multiplier value of 0.45 corresponds to relatively large plants that produce medium- to high-value products (e.g., industrial enzymes). It is also important to note that the factors compiled for US-based plants may be inaccurate when applied to other geographic regions.

For more accurate estimation of building costs, it is necessary to estimate the process area required based on the footprint of the equipment and the space required around the equipment for safe and efficient operation and maintenance. Then the building cost is estimated by multiplying the area of the various sections (e.g., process, laboratory, office) of a plant by an appropriate unit cost provided in Table 9.4. This table, which was developed by DPS Engineering (Framingham, MA), also provides information on air circulation rates for the various process areas, which determine the sizing and power requirements of HVAC systems.

Returning to Table 9.3, it is clear that there is also a wide range for the equipment installation cost multipliers. Using multipliers that are specific to individual equipment items leads to the most accurate estimates. In general, equipment delivered mounted on skids has a lower installation cost.

The equipment purchase costs referred to in Table 9.3 can be estimated from vendor quotations, published data, company data compiled from earlier projects, and by using

Table 9.3 Fixed capital cost estimation

Cost item	Average multiplier	Range of multiplier values
Total plant direct cost (TPDC)		
Equipment purchase cost (PC)		
Installation	0.50 × PC	0.2–1.5
Process piping	0.40 × PC	0.3–0.6
Instrumentation	0.35 × PC	0.2–0.6
Insulation	0.03 × PC	0.01–0.05
Electrical	0.15 × PC	0.1–0.2
Buildings	0.45 × PC	0.1–3.0
Yard improvement	0.15 × PC	0.05–0.2
Auxiliary facilities	0.50 × PC	0.2–1.0
Total plant indirect cost (TPIC)		
Engineering	0.25 × TPDC	0.2–0.3
Construction	0.35 × TPDC	0.3–0.4
Total plant cost (TPC)	TPDC + TPIC	
Contractor's fee	0.05 × TPC	0.03–0.08
Contingency	0.10 × TPC	0.07–0.15
Direct fixed capital (DFC)	TPC + contractor's fee and contingency	

Table 9.4 Building cost estimation (Year 2012 prices) [2]

Space function	Unit cost ($/m^2)	Air circulation rates (volume changes/h)
Process areas[a]		
Class 100,000	3000–3750	20
Class 10,000	3750–5200	35–50
Class 1000	6700–9000	100
Class 100	9000–12,000	200–600
Mechanical room (utilities)	450–900	
Laboratory	1500–3000	
Office	750–900	

[a]The class number refers to the maximum allowed number of particles 0.5 μm or larger per cubic foot

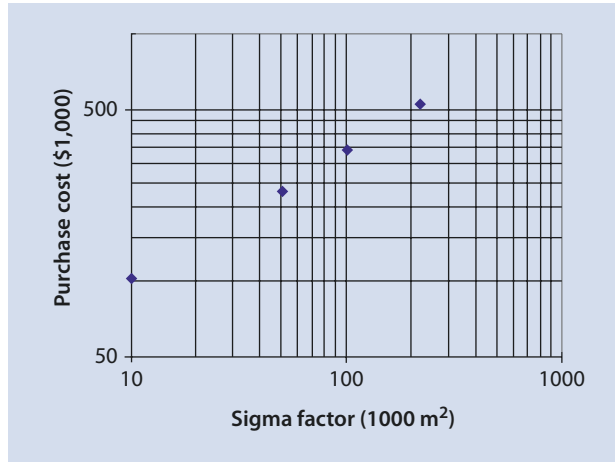


Fig. 9.5 Purchase cost of disk-stack centrifuges vs. sigma factor (2012 prices)

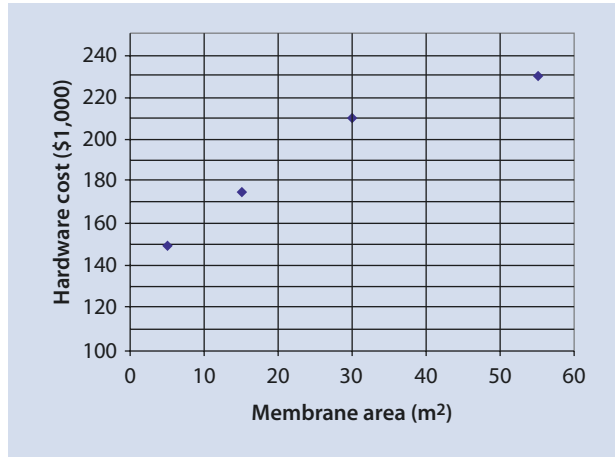


Fig. 9.6 Purchase cost of membrane filtration systems (2012 prices)

process simulators that are equipped with appropriate costing capabilities. Vendor quotations are time-consuming to obtain and are, therefore, usually avoided for preliminary cost estimates. Instead, engineers tend to rely on the other three sources. Figures 9.5, 9.6, 9.7, and 9.8 provide equipment cost data for disk-stack centrifuges, membrane filtration systems, chromatography columns, and vertical agitated tanks that meet the specifications of the biopharmaceutical industry. The cost of the membrane filtration systems in Fig. 9.6 includes the cost of the skid, tank, pumps, and automation hardware and software. The agitated tanks in Fig. 9.8 are appropriate for buffer preparation. They include a low power agitator, but no heating/cooling jacket. The data in the graphs represent average values from several vendors.

It should be noted that equipment purchase cost is a strong function of industrial application and plant location. The data of Figs. 9.5, 9.6, 9.7 and 9.8 are applicable to biopharmaceutical facilities in developed countries. The cost of membrane filtration systems used in the food, biofuel, and water purification industries is more than an order of magnitude lower compared to the biopharmaceutical industry. The much larger equipment scale and the less stringent equipment specifications in these industries

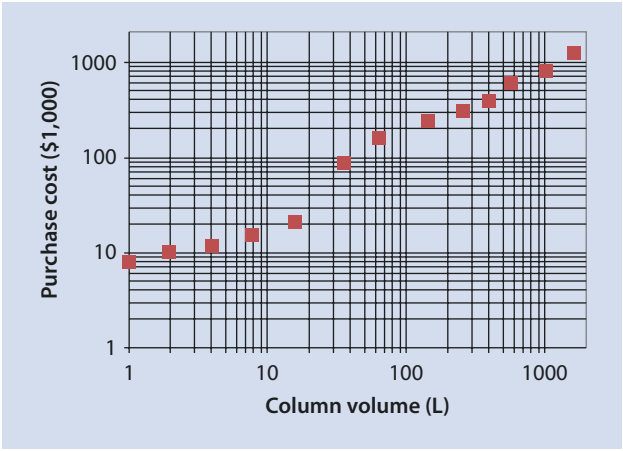


Fig. 9.7 Purchase costs of chromatography columns made of acrylic tube and stainless steel bed supports (2012 prices)

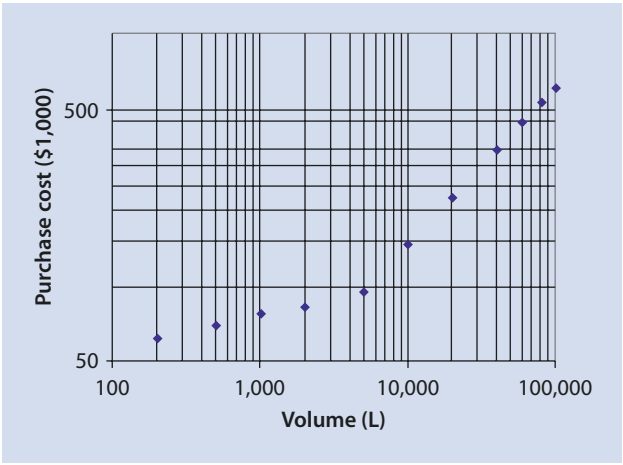


Fig. 9.8 Purchase cost of agitated tanks made of stainless steel (2012 prices)

(relative to biopharmaceuticals) are responsible for the large difference in cost. The same trend applies to the cost of chromatography columns, storage tanks, reactor vessels, and most other equipment items.

Often, cost data for one or two discrete equipment capacities are available, but the cost for equipment with other capacities must be estimated. In such cases, the *scaling law* (expressed by the following equation) can be used:

$$\text{Cost}_2 = \text{cost}_1 \left(\frac{\text{size}_2}{\text{size}_1} \right)^a \tag{9.1}$$

The mathematical form of the scaling law explains why cost-versus-size data graphed on logarithmic coordinates tend to fall on a straight line. The value of the exponent a in Eq. (9.1) ranges between 0.5 and 1.0, with an average value for vessels of around 0.6 (this explains why the scaling law is also known as the "0.6 rule"). According to this rule, when the size of a vessel doubles, its cost will increase by a factor of $(2/1)^{0.6}$, or approximately

52%. This result is a demonstration of what is often referred to as the *economy of scale*. In using the scaling law, it is important to make sure that the piece of equipment whose cost is being estimated has a size that does not exceed the maximum available size for that type of equipment.

The price of equipment changes with time due to inflation and other market conditions. That change in price is captured by cost indices compiled specifically for equipment in the chemical industry such as the Marshall & Swift index or the Chemical Engineering Plant Cost Index (CE Index) that is published monthly by *Chemical Engineering* magazine. The index I is used to update equipment cost data from a reference year "1" to another year "2" according to the following equation:

$$\text{Cost}_2 = \text{cost}_1 \frac{I_2}{I_1} \tag{9.2}$$

Another factor that affects equipment purchase cost is the material of construction. For instance, a tank made of stainless steel costs approximately 2.5–3 times as much as a carbon steel tank of the same size, and a tank made of titanium costs around 15 times as much. Other factors that affect equipment cost include the finishing of the metal surface and the instrumentation that is provided with the equipment.

- *Working Capital*

Working capital accounts for cash that must be available to cover on-going expenses for several months of plant operations. Since expenses always precede revenues, the working capital must be available throughout the lifetime of the plant. Typical working capital expenses include raw materials for 1–2 months, labor for 2–3 months, utilities for a month, waste treatment/disposal for a month, consumables (e.g., filters, chromatography resins, etc.) for several months, and other miscellaneous expenses. The required amount of working capital for a process is usually 10–20% of the DFC.

- *Start-up and Validation*

Start-up and validation costs can also represent a significant capital investment, especially for a biopharmaceutical plant. A value of 20–30% of DFC is quite common for these plants.

9.3.2 Operating Cost Estimation

The operating cost to run a biochemical plant is the sum of all on-going expenses including raw materials, labor, consumables, utilities, waste disposal, and facility overhead. Dividing the annual operating cost by the annual production rate yields the unit production cost (e.g., in dollars per kilogram of product). In general, the unit cost and selling prices of bioproducts are inversely proportional to market size. For instance, commodity biochemicals and biofuels that are produced in large quantities cost around \$1–\$5/kg to make. Specialty biochemicals that are used as food supplements (e.g., vitamins) and flavoring agents have a manufacturing cost of \$5–\$100/kg. The manufacturing cost of therapeutic proteins produced in large quantities is in the range of \$1–\$1000/g. Human serum albumin (HSA), which is extracted from blood plasma and has an annual production volume of more than 500 metric tons, lies close to the low end. The manufacturing cost of

therapeutic proteins with annual production volumes ranging from a few hundreds of kilograms to a few metric tons is in the range of $50–$1000/g. The monoclonal antibody process analyzed later in this chapter represents a product of this type. The manufacturing cost of interferons, erythropoietin (EPO), and other therapeutic proteins with very low annual production volume (from hundreds of grams to a few kilograms) is more than $10,000/g [15].

Table 9.5 displays the various types of operating costs, their direct or indirect nature, and ranges for their values relative to the total operating cost. Sometimes cost items are categorized as either fixed or variable. Fixed costs are those that are incurred regardless of the volume of product output. The most characteristic example of a fixed cost is depreciation, which is part of the equipment-dependent cost. The most characteristic example of a variable cost is the cost of raw materials. Most other costs have a fixed component and a variable component.

It is obvious from the wide range of values in Table 9.5 that industry averages cannot predict the operating cost of a process; a certain level of detailed calculations is required.

Raw Materials

In bioprocessing, the raw materials cost includes the cost of all fermentation/cell culture media, recovery chemicals, and cleaning materials. For commodity bio-chemicals, such as ethanol, the cost of media is the main component. For high-value products, the solutions used for product recovery and equipment cleaning can be a major part of the raw materials cost. Table 9.6 provides a list of commonly used raw materials in the biochemical industries. Note that the price of a raw material can vary widely depending on its required purity. This can be clearly seen for different types of water. Water for injection (WFI), for instance, costs 100–500 times as much as city water. In general, the material costs in Table 9.6 are consistent with large-scale commodity bioprocessing, rather than smaller-scale biopharmaceutical production with higher purity requirements.

Labor

Labor demand for a batch process varies with time. The total labor demand at any given time can be calculated by summing up the labor requirements of the operations which are

Table 9.5 Operating cost items and ranges

Cost item	Type of cost	Range of values (% of total)
Raw materials	Direct	10–80
Labor	Direct	10–50
Consumables	Direct	1–50
Lab/QC/QA	Direct	1–50
Waste disposal	Direct	1–20
Utilities	Direct	1–30
Facility overhead	Indirect	10–70
Miscellaneous	Indirect	0–20

Table 9.6 Common bioprocessing raw materials (Year 2012 prices)

Raw material	Comments	Price ($/kg)
Carbon Source		
Glucose	Solution 70% w/v	0.30–0.40
Corn syrup	95% Dextrose equivalent	0.40–0.50
Molasses	50% Fermentable sugars	0.12–0.20
Soybean oil	Refined	1.10–1.30
Corn oil	Refined	1.30–1.40
Ethanol	USP tax free	0.80–0.90
Methanol	Gulf Coast	0.40–0.45
n-Alkanes		0.75–0.90
Nitrogen Source		
Ammonia	Anhydrous, fertilizer grade	0.30–0.60
Soybean flour	44% protein	0.45–0.50
Cottonseed flour	62% protein	0.50–0.60
Casein	13.5% w/w total N	10.00–12.00
Ammonium sulfate	Technical	0.17–0.25
Ammonium nitrate	Fertilizer grade 33.5% N, bulk	0.20–0.30
Urea	46% N, agricultural grade	0.55–0.65
Yeast	Brewers, debittered	1.25–1.40
Whey	Dried, 4.5% w/w N	1.25–1.40
Salts		
KH_2PO_4	USP, granular	1.65–1.85
K_2SO_4	Granular, purified	2.80–3.00
Na_2HPO_4		1.40–1.80
$MgSO_4 \cdot 7H_2O$		0.45–0.55
$ZnSO_4 \cdot 7H_2O$	Agricultural grade, powder	0.65–0.75
Other		
Process water		0.0001–0.001
RO water		0.005–0.01
Water for injection		0.02–0.5
H_3PO4 (85% w/w)	Food grade	3.5–4.5
NaOH		0.2–0.5
HCl (37% w/w)		0.7–0.8
H_2SO_4 (98% w/w)		0.15–0.25

taking place simultaneously. In a single-product facility, the number of operators in each shift must be based on maximum demand during that shift. In multiproduct facilities, each product line can employ a certain number of dedicated operators and rely on floating operators during periods of peak demand. In general, smaller facilities tend to utilize a larger number of operators per processing step because these plants are less automated. For instance, a small biotech company may utilize two or three operators to set up a fermentor, whereas in a large and highly automated fermentation facility, a single operator may handle the setup of six different fermentors remotely from the control room. In general, a typical biotech company that deals with high-value products will allocate at least one operator to each processing step (centrifugation, membrane filtration, chromatography, etc.) during its operation. The setup of a step may require multiple operators for a short period. The annual cost of an operator (including salary and benefits) varies widely around the globe. It is in the range of $4000–$10,000 in developing nations, but it can exceed $50,000 in developed countries [16].

- *Consumables*

Consumables are items that may be used up, fouled, or otherwise damaged during processing, such as membranes, chromatography resins, activated carbon, etc. These items must be periodically replaced. As the example later in this chapter will illustrate, the high unit cost of chromatography resins and their frequent replacement can make them a major component of the manufacturing cost. The unit cost of typical ion exchange and hydrophobic interaction chromatography resins used for the purification of proteins is in the range of $500–$2000/L of resin. The unit cost of protein-A affinity resins that are commonly used for the purification of monoclonal antibodies is in the range of $5000–$15,000/L of resin. The replacement frequency of such resins is in the range of 50–200 cycles of usage (the high-end resins have a longer useful life). In contrast, the unit cost of polymeric chromatography resins used for the purification of small bio-molecules (e.g., amino acids) is substantially lower (under $100/L of resin) and their life is longer (1000–2000 h of operation). Likewise, the unit cost of silica-based resins used for water demineralization is around $0.5/L and their life is in the range of 2000–6000 h of operation (the life strongly depends on the composition of the treated materials). Regarding membrane filtration operations, the unit cost of MF/UF membranes used in the biopharmaceutical industry (in the form of hollow-fiber cartridges or cassettes) is in the range of $300–$800/m^2. Such membranes typically handle 10–50 filtration cycles before disposal. The unit cost of related membranes used in industrial biotechnology (e.g., for production of industrial enzymes) is considerably lower (under $200/$m^2$) and the expected life is more than 2000 h of operation. The cost of membranes used for large-scale water purification is under $50/$m^2$ and their useful life is at least 6000 h of operation. In general, ceramic membranes cost more than polymeric ones, but they last longer.

The cost of disposable bags or containers, also known as single-use systems, is part of the consumables cost as well. Disposable bags have become popular in biopharmaceutical manufacturing, partially because they substantially reduce the capital and operating costs for a facility. In fact, building a new facility which utilizes single-use systems rather than a conventional stainless steel facility can reduce capital costs by up to 40% [17, 18]. The capital cost reduction is achieved by eliminating the need for various stainless steel tanks, and reducing the facility's piping infrastructure, utility infrastructure, etc. This lowers the facility's operating costs related to maintenance and depreciation as well. The operating cost is reduced further by the elimination of various cleaning and sterilization activities,

Table 9.7 Disposable bags for preparation and storage of solutions (Year 2012 prices)

Volume (L)	Bags for storage ($)	Bags for mixing ($)
50	310	600
100	340	690
200	360	820
500	460	930
1000	650	1180

which decreases raw material costs, utility costs, labor costs, and certain waste costs. These cost savings are partially offset by the increased cost of consumables (e.g., the purchase price and disposal costs of the disposable bags and their associated tubing, connectors, etc.). Nevertheless, the overall economics of the process can be extremely favorable for single-use systems [19, 20]. Other advantages of single-use systems include increased processing flexibility, reduced risk of cross-contamination, and shorter validation, start-up, and commercialization times. Table 9.7 provides information on disposable bags used for the preparation and storage of buffer solutions and fermentation media. Bags with mixing capability are required for solution preparation. Similar bags are used for inoculum preparation in rocking and stirred tank bioreactors. Bags for stirred tank bioreactors are available with working volumes of up to 2000 L. Some biopharmaceuticals are produced exclusively in single-use systems. It should be noted that large disposable bags (larger than 50 L) require appropriate supporting skids. The SuperPro Designer databases provide cost information for such skids as well as for disposable bags and various other types of single-use containers.

- *Laboratory/QC/QA*

Laboratory, quality control (QC), and quality assurance (QA) activities include off-line analysis, quality control (QC), and quality assurance (QA) costs. Chemical and biochemical analysis and physical property characterization, from raw materials to final products, are a vital part of biochemical operations. The Laboratory/QC/QA cost is usually 10–20% of the operating labor cost. However, for certain biopharmaceuticals that require a large number of very expensive assays, this cost can be as high as the operating labor. For such cases, it is important to account for the number and frequency of the various assays in detail, since changes in lot size that can reduce the frequency of analysis can have a major impact on profit margins.

- *Waste Treatment and Disposal*

The treatment of wastewater and the disposal of solid and hazardous materials are other important operating costs. The amount and composition of the various waste streams are derived from the material balances. Multiplying the amount of each waste stream by the appropriate unit cost yields the cost of treatment and disposal. Treatment of low biological oxygen demand (BOD) wastewater (<1000 mg/L) by a municipal wastewater treatment facility usually costs \$0.2–\$2/m^3. This is not a major expense for most biotech facilities

that deal with high-value products. However, disposal of contaminated solvents (typically generated by chromatography steps) and other regulated compounds can become a major expense because their unit disposal cost can be more than $1/kg. Waste disposal may also become a costly problem if very hazardous chemicals are used or produced within a process. Finally, disposal of single-use systems via incineration costs $100–$200 per metric ton of material.

- *Utilities*

Utilities costs include the cost of heating and cooling agents as well as electricity. The required amounts of utilities are calculated as part of the material and energy balances. Aerobic fermentors are major consumers of electricity, but downstream processing equipment generally does not consume much electricity. In terms of unit cost, electricity costs $0.05–$0.15/kWh. The cost of heat removal using cooling water is in the range of $0.002–$0.01 per 1000 kcal of heat removed. The cost of cooling using chilled water and refrigerants is in the range of $0.05–$0.1 per 1000 kcal of heat removed. The cost of producing steam for use as a heating medium in the USA is around $5–$15/1000 kg, depending on pressure (low, medium, high), type of fuel used for its generation, and scale of production. This value varies considerably around the globe. The cost of clean steam (generated utilizing highly purified water) is around $50–$500/1000 kg (depending on the scale of production and level of water purity). Clean steam is used in biopharmaceutical facilities for sterilizing equipment as part of equipment cleaning (e.g., "steam in place" or SIP operations). Note that manufacturers often classify purified water used for buffer preparation and equipment cleaning as a utility and not as a raw material, thus increasing the cost contribution of utilities.

- *Facility Overhead*

Facility overhead costs account for the depreciation of the fixed capital investment, maintenance costs for equipment, insurance, local (property) taxes, and possibly other overhead-type expenses. For preliminary cost estimates, the entire fixed capital investment is usually depreciated linearly over a 10-year period. In the real world, the US government allows corporations to depreciate equipment in 5–7 years and buildings in 25–39 years. The value of land cannot be depreciated. The annual maintenance cost can be estimated as a percentage of the equipment's purchase cost (usually 10%) or as a percentage of the overall fixed capital investment (usually 3–5%). Insurance rates depend to a considerable extent upon the maintenance of a safe plant in good condition. A value for insurance in the range of 0.5–1% of DFC is appropriate for most bioprocessing facilities. The processing of flammable, explosive, or highly toxic materials usually results in higher insurance rates. The local (property) tax is usually 2–5% of DFC. The factory expense represents overhead cost incurred by the operation of non-process-oriented facilities and organizations, such as accounting, payroll, fire protection, security, and cafeteria. A value of 5–10% of DFC is appropriate for these costs.

- *Miscellaneous*

Included in miscellaneous costs are ongoing R&D, process validation, and other overhead-type expenses that may or may not be included in preliminary cost estimates. Other general expenses of a corporation include royalties, advertising, and selling. If any part of the process or any equipment used in the process is covered by a patent not assigned to the corporation undertaking the new project, permission to use the technology covered by

the patent must be negotiated, and some form of royalty or license fee is usually required. Advertising and selling cover expenses associated with the activities of the marketing and sales departments.

9.3.3 Profitability Analysis

Estimates of capital investment, operating cost, and revenues of a project provide the information needed to assess its profitability and attractiveness from an investment point of view. There are various measures for assessing profitability. The simplest ones include gross margin, return on investment (ROI), and payback time (PBT), and they are calculated by using the following equations:

$$\text{Gross margin} = \frac{\text{gross profit}}{\text{revenues}} \tag{9.3}$$

$$\text{Return on investment}\left(\text{ROI}\right) = \frac{\text{net profit per year}}{\text{total investment}} \times 100\% \tag{9.4}$$

$$\text{Payback time}\left(\text{years}\right) = \frac{\text{total investment}}{\text{net profit per year}} \tag{9.5}$$

where gross profit is equal to annual revenues minus the annual operating cost, and net profit is equal to gross profit minus income taxes plus depreciation. All variables are averaged over the lifetime of a project. Gross margin can be used to guide product pricing policies but is not a reliable metric for assessing the viability of investments since it does not take into account the investment cost. ROI and payback time (PBT) represent essentially the same criterion since their values are the inverse of each other (as it can be easily verified by their defining equations.) Evaluating the economic viability of a process based on the metrics above can be quite subjective. For instance, the acceptable lower bound for a project's ROI may range from around 10–20%, depending on the level of uncertainty regarding the project's future profits, the expected duration of those profits, the estimated ROIs of other potential projects, etc.

ROI and PBT assume constant and fixed profits over the project lifetime without considering the time-value of money. Other more sophisticated measures, such as the net present value (NPV) and internal rate of return (IRR), have been developed to take into account the cash flows of a project over its evaluation life and consider the time-value of money.

NPV is defined as the sum of all future cash flows (over the project life time of N years) discounted at the time of investment (present value):

$$\text{NPV} = \sum_{t=0}^{N} \frac{\text{Net Cash Flow on year } t}{\left(1+i\right)^{t}} \tag{9.6}$$

The discount rate, i, is an interest rate that represents the estimated loss of money value with time. Seen differently, it represents a return that could be earned on an investment with similar risk (which, if not realized, corresponds to money lost). In both cases, the discount rate is used to calculate the real value of future cash flows at the present time.

NPV is usually calculated for different values of the discount rate since the latter does not have a fixed value and can only be estimated. Positive NPV represents viable investments; the higher the value, the more favorable the investment. It should be noted that net cash flow values may be negative (especially for the present year, $t = 0$), even for projects with positive net profits, because capital expenditures are also considered in their calculation. A negative NPV indicates that capital investment cannot be recovered through future generated profits and the investment does not add value.

IRR is the value of discount rate that sets NPV to zero (no gain or loss from the investment). Any value of the discount rate that is smaller than the IRR generates positive NPVs. Therefore, the greater the IRR, the more favorable the investment. However, as is the case with the other metrics of economic viability, there is no objective lower bound on IRR for screening investments.

More detailed definitions for NPV and IRR can be found in the literature [9, 11]. The example presented next demonstrates how these measures facilitate the decision-making process.

9.4 Illustrative Example

In this section, SuperPro Designer is used to illustrate the analysis and evaluation of the production of monoclonal antibodies (mAbs) from mammalian cells cultured in stirred tank bioreactors. We will use this example to draw general conclusions on the manufacturing cost of biological products. Note that this example is similar to the "MAB" example which is installed with SuperPro Designer. The MAB example is located within the Pharmaceuticals subfolder of SuperPro's Examples folder. A free evaluation version of SuperPro Designer (including the MAB example) is available at the website ▶ www.intelligen.com/downloads.html. Additional examples and pertinent publications are available at ▶ www.intelligen.com/literature.

■ Therapeutic Monoclonal Antibody Production

Monoclonal antibodies are large protein molecules that consist of two main regions, the Fragment Antigen Binding (Fab) region and the Fragment crystallizable (Fc) region. As of November 2014, 47 monoclonal antibody (mAb) products had been approved in the USA or Europe, for treatment of various types of cancer, rheumatoid arthritis, psoriasis, severe asthma, macular degeneration, multiple sclerosis, and other diseases. Moreover, mAbs are the fastest-growing segment in the biopharmaceutical industry, and it is estimated that approximately 70 mAbs will be on the market by 2020, with combined worldwide sales of roughly $125 billion [21].

The high-dose demand for several mAbs translates into annual production requirements for purified product in the metric ton range. This example illustrates the analysis of a large-scale mAb process. The modeling and calculations are performed with SuperPro Designer.

Figure 9.9 displays the flowsheet of the overall process. The generation of the flowsheet was based on information available in the patent and technical literature combined with the authors' engineering judgment and experience with such processes [22]. The process in this example produces 1550 kg of purified mAb per year. The flow diagram of Fig. 9.9 is a simplified representation of the actual process; it lacks all the buffer preparation and holding activities that a complete diagram would include. In fact, these

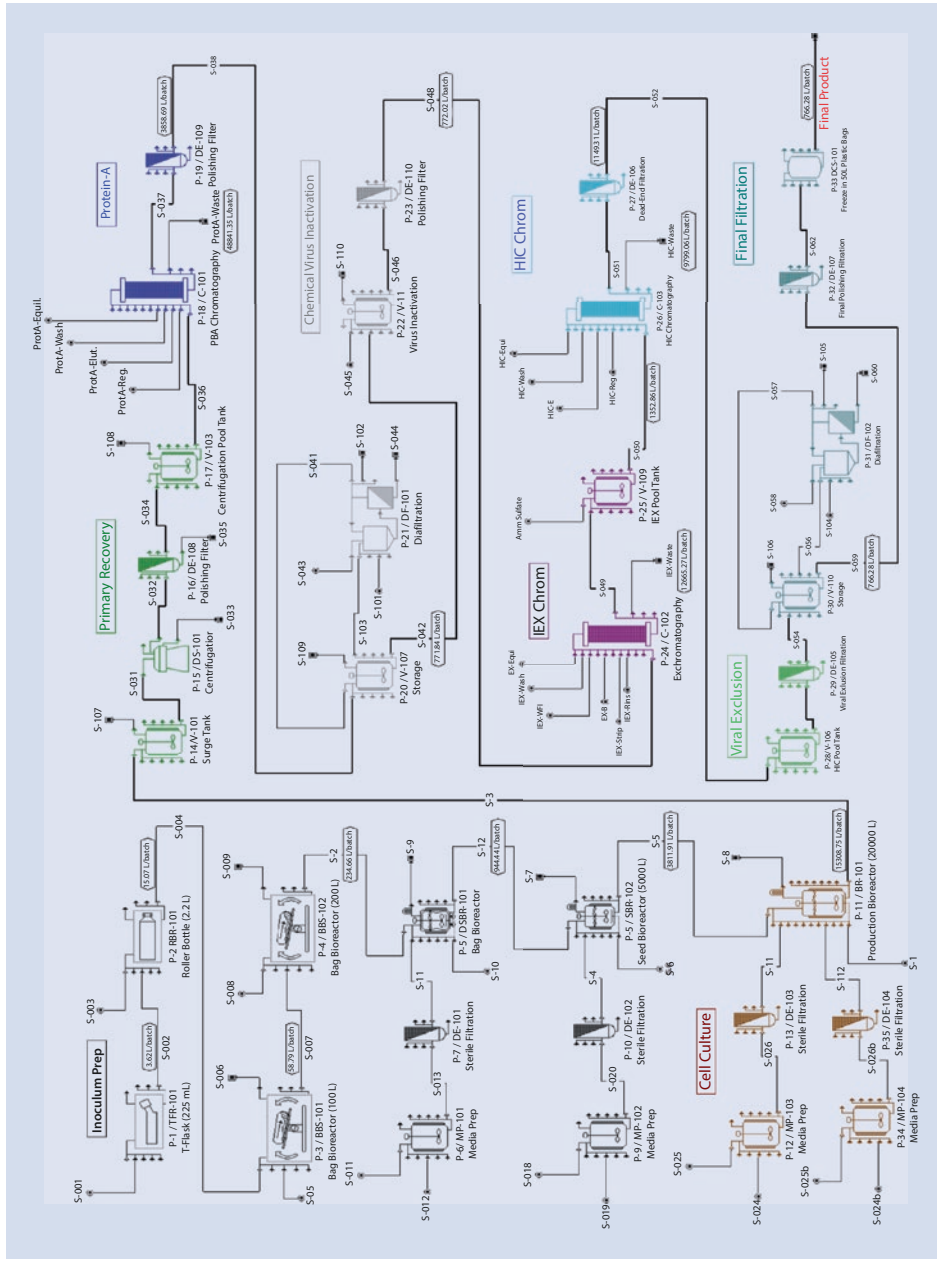


Fig. 9.9 Monoclonal antibody production flowsheet

types of processes require 20–30 cell culture media and buffer solutions. These solutions are prepared in mixing tanks and then stored in holding tanks located close to the units that utilize them. The tanks required for buffer preparation and holding add to the capital investment of the facility, while the required labor adds to the manufacturing cost. The model files for a similar example that is part of the evaluation version of SuperPro Designer (available at ► www.intelligen.com) include the tanks for buffer preparation and holding. In addition, the capital and operating costs associated with buffer preparation activities were considered in the cost analysis results presented in this example.

■ *Process Description*

Upstream Section: The upstream part is split into two sections: Inoculum Preparation and Bioreaction. The inoculum is initially prepared in 225 mL T-flasks (TFR-101). Next, the material from the T-flasks is moved to 2.2 L roller bottles (RBR-101), then to 100 L and subsequently to 200 L rocking bioreactors that utilize disposable bags (BBS-101 and BBS-102). Sterilized media is fed at the appropriate amount in all of these four initial steps (3.6, 11.4, 43.6, 175.4 kg/batch, respectively). The broth is then moved to a 1200 L stirred seed bioreactor (DSBR-101). Following a 6-day cell growth in the first seed bioreactor, the broth is sent to the second seed bioreactor (SBR-102), which is a 5000 L stainless steel vessel. For the two seed bioreactors, the media powder is dissolved in water-for-injection (WFI) in two prep tanks (MP-101 & MP-102) and then sterilized/fed to the reactors through 0.2 μm dead-end filters (DE-101 & DE-102). In the cell culture section, serum-free low-protein media powder is dissolved in WFI in a stainless steel tank (MP-103). The solution is sterilized using a 0.2 μm dead-end polishing filter (DE-103). A 20,000 L stainless stirred-tank bioreactor (BR-101) is used to grow the cells, which produce the therapeutic monoclonal antibody (mAb). The production bioreactor operates in fed-batch mode. High media concentrations are inhibitory to the cells, so half of the media is added at the start of the process and the rest is fed (from MP-104) at a variable rate during fermentation. The concentration of dry media powder in the initial feed solution is 17 g/L. The cell culture time is 12 days. The volume of broth generated per bioreactor batch is approximately 15,000 L, which contains roughly 30.3 kg of product (the product titer is approximately 2 g/L).

Downstream Section: Between the downstream unit procedures there are 0.2 μm dead-end filters to ensure sterility. The generated biomass and other suspended compounds are removed using a Disc-Stack centrifuge (DS-101). During this step, roughly 5% of mAb is lost in the solids waste stream. The bulk of the contaminant proteins are removed using a Protein-A affinity chromatography column (C-101), which processes a batch of material in four cycles. The following operating assumptions were made for each chromatography cycle: (1) resin binding capacity is 15 g of product per L of resin, (2) the eluant or elution buffer is a 0.6% w/w solution of acetic acid and its volume is equal to 5 column volumes (CVs), (3) the product is recovered in 2 CVs of eluant with a recovery yield of 90%, and (4) the total volume of the solution required for column equilibration, wash, and regeneration is 14 CVs. The entire procedure takes approximately 22.6 h and requires a resin volume of 502 L. The protein solution is then concentrated fivefold and diafiltered with two volumes of buffer (in P-21 / DF-101). This step takes approximately 8.3 h and requires a membrane of 21 m^2. The product yield is 97%. The concentrated protein solution is then chemically treated for 1.5 h with Polysorbate 80 to inactivate viruses (in P-22/V-111). The ion exchange (IEX) chromatography step (P-24/C-102) that follows processes one batch of material in three cycles. The following operating assumptions were made for each cycle:

(1) the resin's binding capacity is 40 g of product per L of resin, (2) a gradient elution step is used with a sodium chloride concentration ranging from 0.0 to 0.1 M and a volume of 5 CVs, (3) the product is recovered in 2 CVs of eluant buffer with a mAb yield of 90%, and (4) the total volume of the solutions required for column equilibration, wash, regeneration, and rinse is 16 CVs. The step takes approximately 20.6 h and requires a resin volume of 238 L. Ammonium sulfate is then added to the IEX eluate (in P-25/V-109) to a concentration of 0.75 M. This increases the ionic strength of the eluate in preparation for the hydrophobic interaction chromatography (HIC, P-26 / C-103) step that follows. Like the IEX step which preceded it, the HIC step processes one batch of material in three cycles. The following operating assumptions were made for each cycle of the HIC step: (1) the resin binding capacity is 40 g of product per L of resin, (2) the eluant is a sodium chloride (4% w/w) sodium di-hydrophosphate (0.3% w/w) solution and its volume is equal to 5 CVs, (3) the product is recovered in 2 CVs of eluant buffer with a recovery yield of 90%, and (4) the total volume of the solutions required for column equilibration, wash, and regeneration is 12 CVs. The step takes approximately 20.2 h and requires a resin volume of 196 L. A viral exclusion step (DE-105) follows. It is a dead-end type of filter with a pore size of 0.02 μm. Finally, the HIC elution buffer is exchanged for the product bulk storage (PBS) buffer and concentrated 1.5-fold (in DF-102). This step takes approximately 7.7 h and requires a membrane area of 10 m^2. The approximately 800 L of final protein solution is stored in twenty 50 L disposable storage bags (DCS-101). The overall yield of the downstream operations is 63.1%, and 19.1 kg of mAb are produced per batch.

- *Process Scheduling and Cycle Time Reduction*

Figure 9.10 displays the equipment occupancy chart of the process for four consecutive batches, based on a plant that has a single production train. The clean-in-place (CIP) skids, polishing filters, and media preparation tanks are not displayed on the chart for the sake of simplicity. The batch time is approximately 50 days. This is the time required from the start of inoculum preparation to the final product purification of a single batch. The production bioreactor (BR-101) is the time (scheduling) bottleneck. This unit has the least idle time between consecutive batches, and therefore, it determines the maximum number of batches per year. Its duration is roughly 13.3 days, although this example assumes a small break between consecutive batches, to account for potential delays in the plant. A new batch is therefore initiated every 14 days.

On an annual basis, the plant processes 21 batches and produces approximately 401 kg of purified mAb. It is clear from Fig. 9.10 that under these conditions the downstream train is underutilized and the cycle time of the process (i.e., the time between consecutive batches) is relatively long. The cycle time of the process can be reduced and the plant throughput increased by installing multiple bioreactor trains that operate in staggered mode (out of phase) and feed the same purification train. Figure 9.11 represents a case where four bioreactor trains feed the same purification train. The new cycle time is 3.5 days, which is one-fourth of the original. Under these conditions, the plant processes 81 batches per year and produces 1550 kg of mAb per year. Note that the addition of staggered bioreactor trains reduces the unit production cost (i.e., cost per kg of product) because the throughput of the plant is increased fourfold, but only the upstream equipment units must be replicated; the downstream continues to rely on a single equipment unit for each procedure. In other words, the downstream equipment utilization is much higher than before. Some biopharmaceutical companies have installed more than four bioreactor trains per purification train in order to reduce unit production costs and to achieve cycle times as low as 2 days.

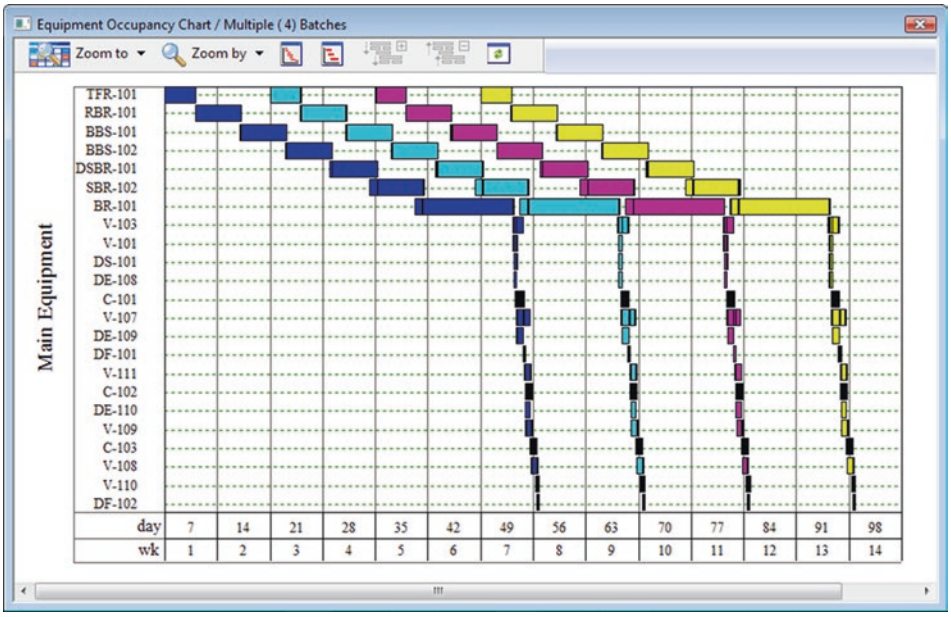


Fig. 9.10 One bioreactor train feeding one purification train

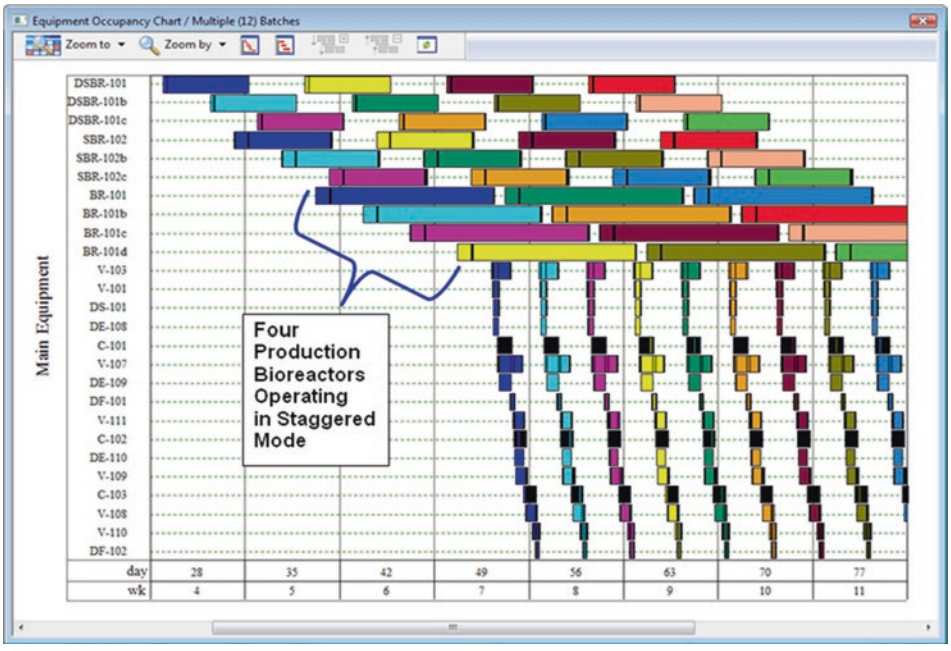


Fig. 9.11 Four bioreactor trains feeding one purification train

Resource Tracking and Utility Sizing

Another characteristic of batch processing is the variable demand for resources (e.g., labor, utilities, and raw materials) as a function of time. For instance, Fig. 9.12 displays the demand for WFI during overlapping batches of the process. The thin red line and thick blue line correspond to the *y*-axis on the left-hand side of the chart, and they represent instantaneous demand (red) and averaged demand for 24-h intervals (blue). The thin black lines, which correspond to the *y*-axis on the right-hand side of the chart, represent the cumulative demand for the same 24-h intervals. These charts are useful for sizing utility systems during the design of new facilities. For instance, a WFI system consists of a still that generates distilled water, a surge tank, and a circulation loop for delivering the material around the plant. The results shown in Fig. 9.12 provide reasonable estimates for the size of the still, the surge tank, and the pumping capacity of the circulation loop. More specifically, the instantaneous demand peak (thin red line) indicates the minimum pumping capacity for the system since this capacity needs to meet the peak demand (72,000 kg/h). The cumulative demand peak (thin black line) can be used to size the surge tank, which must be large enough to maintain capacity during peak cumulative demand (in this case, the highest demand during any 24-h interval is 115,000 kg). Finally, the highest averaged demand (thick blue line) indicates the size of the still (production capacity of 5000 kg/h). The trade-off between still rate and surge capacity can be examined by changing the averaging time interval. For example, selecting an interval of less than 24 h results in a smaller surge tank but a higher still rate (e.g., a larger still) compared to the base case.

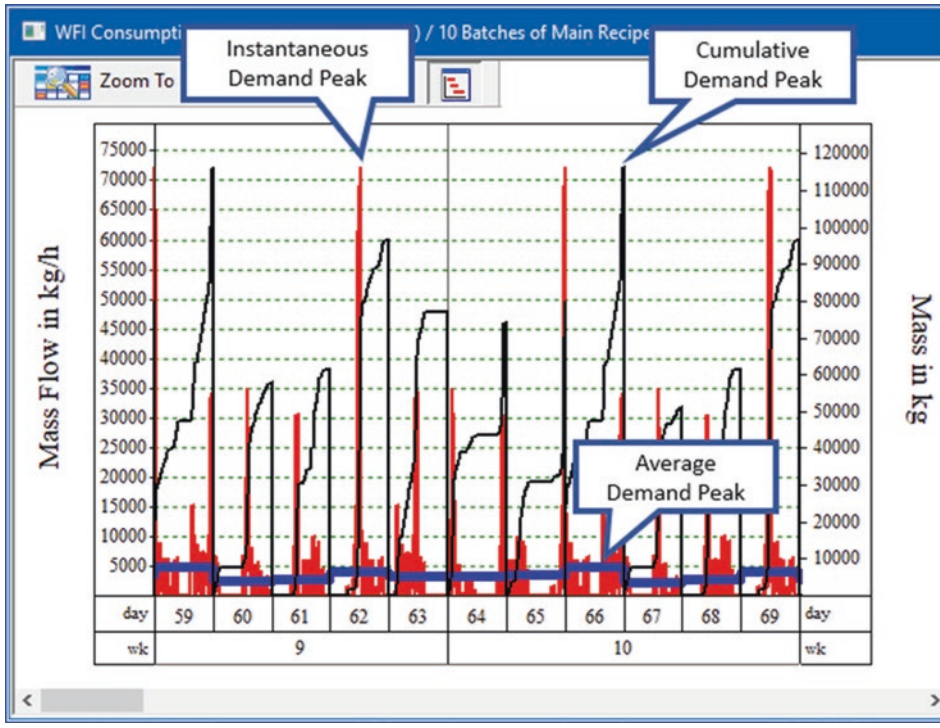


Fig. 9.12 WFI demand versus time

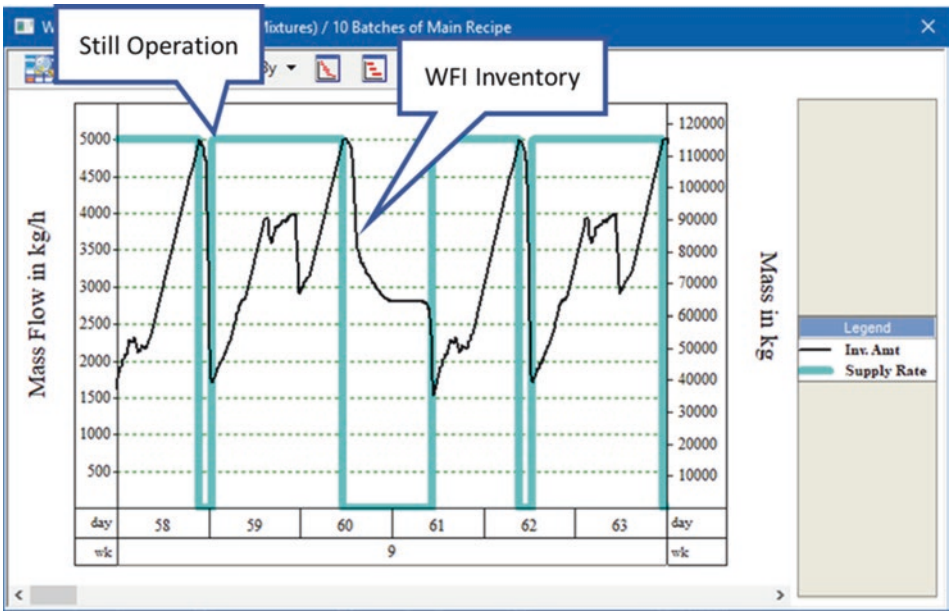


Fig. 9.13 WFI inventory level versus time

Figure 9.13 displays the inventory results that correspond to a tank size of 115,000 kg and a still rate of 5000 kg/h (as indicated by the findings of Fig. 9.12). The still is turned on when the WFI level falls below 35% of the tank's capacity. The still remains on until the tank is full. The operation rate and frequency of the still is depicted by the thick light blue step-function lines, which correspond to the *y*-axis on the left side. The inventory level in the tank is displayed by the thin black line, which corresponds to the *y*-axis on the right side.

Sizing of bio-waste treatment systems can be handled in a similar manner. Such systems typically involve two tanks that alternate in operation periodically (while one is receiving, the other is treating a batch of waste material). The peak cumulative amount for the alternating period indicates the minimum capacity of each tank.

Material Requirements

Table 9.8 provides a summary of the material requirements of the process. Note the large amount of WFI utilized per batch. Most of the WFI is used for cleaning and buffer preparation.

Economic Evaluation

Table 9.9 displays the key economic evaluation results generated using the built-in cost functions of SuperPro Designer. The total capital investment (for the case with the four bioreactor trains) is around $572 million. The total annual operating cost is $149 million, resulting in a unit production cost of around $96/g (based on 1550 kg of purified mAb produced annually). Assuming a selling price of $200/g, the project yields an after-tax internal rate of return (IRR) of 22.4% and a net present value (NPV) of $524 million (assuming a discount interest rate of 7%). Based on these results, this project represents an extremely attractive investment. However, if additional costs are included in the analysis (such as up-front R&D costs, sales and marketing costs, etc.), the profitability metrics above will be reduced.

Table 9.8 Raw material requirements (MP = purified mAb product)

Material	kg/year	kg/batch	kg/kg MP
Inoc Media Sltn	18,858	232.82	12.168
WFI	8,688,648	107,267.26	5606.361
Serum-free media	36,339	448.63	23.448
H_3PO4 (5% w/w)	2,376,420	29,338.52	1533.388
NaOH (0.5 M)	2,121,527	26,191.69	1368.918
NaOH (0.1 M)	7,912,570	97,686.06	5105.596
Amm. Sulfate	12,313	152.01	7.945
Polysorbate 80	7	0.08	0.004
Protein A Equil-Buffer	1,991,715	24,589.08	1285.157
Protein A Elution-Buffer	810,196	10,002.42	522.780
Prot-A Reg Buffer	486,392	6004.84	313.845
NaCl (1 M)	186,484	2302.27	120.329
IEX-El-Buffer	16,335	201.67	10.540
IEX-Eq-Buffer	673,218	8311.33	434.394
HIC-El-Buffer	242,190	2990.00	156.273
HIC-Eq-Buffer	455,253	5620.41	293.753
Concentrated PBS	14,549	179.61	9.388
EtOH (10% w/w)	367,535	4537.47	237.153
Total	**26,410,549**	**326,056**	**17,041**

Table 9.9 Key economic evaluation results for mAb production (2018 costs)

Direct fixed capital	$437 million
Total capital investment	$572 million
Plant throughput	1550 kg of mAb/year
Manufacturing cost	$149 million/year
Unit production cost	$96/g of mAb
Selling price	$200/g of mAb
Revenues	$310 million/year
Gross profit	$161 million/year
IRR (after taxes)	22.4%
NPV (for 7% discount interest rate)	$524 million

Table 9.10 Breakdown of the manufacturing cost for mAb production (2018 costs)

Cost item	$million/year	$/g	%
Raw materials	16.1	10.4	10.8
Facility-dependent	79.5	51.3	53.5
Labor	19.3	12.5	13.0
Consumables	23.4	15.1	15.7
Miscellaneous	10.4	6.7	7.0
Total	**148.7**	**95.9**	**100.0**

Table 9.10 presents a breakdown of the operating cost contributors. The facility-dependent cost is the most important item, accounting for 53.5% of the manufacturing cost or $51.3/g of final product. This is common for high-value products that are produced in small quantities in expensive facilities. Depreciation of the fixed capital investment and maintenance of the facility are the main contributors to this cost. Consumables are the second most important operating cost, accounting for 15.7% of the total or $15.1/g of final product. Consumables include chromatography resins, membrane filters, and disposable bags that need to be replaced on a regular basis. Labor and raw materials costs come third and fourth, accounting for 13.0% and 10.8% of the total cost, respectively. The miscellaneous cost item (7.0% of the total) accounts for lab costs, heating/cooling utilities, electricity, waste treatment, and other costs. The cost of WFI, which is sometimes classified as a utility cost in industry, is accounted for in the cost of raw materials in this example. In terms of cost distribution per section, 65% of the cost is associated with the upstream section and 35% with the downstream.

The economic evaluation relies on the following key assumptions: (1) a new manufacturing facility will be built and dedicated to production of 1550 kg/year of mAb; (2) the entire direct fixed capital is depreciated linearly over a period of 12 years; (3) the project lifetime is 16 years; (4) the unit cost of WFI is $0.15/L; (5) the cost of the serum-free media (in powder form) is $300/kg; (6) all the chemicals used are high purity grade; (7) the unit cost of membranes is $400/m^2; (8) the unit cost of chromatography resins is $6000/L, $1200/L, and $2050/L for columns C-101, C-102, and C-103, respectively; and (9) the chromatography resins are replaced every 60, 50, and 50 cycles for columns C-101, C-102, and C-103, respectively.

Sensitivity Analysis

After a model of the entire process has been developed, the model can be used to readily answer "what if" questions and to carry out sensitivity analyses with respect to key design variables. In this example, we looked at the impact of product titer (varied from 1 to 10 g/L) and bioreactor size (10,000 and 20,000 L) on unit production cost. Figure 9.14 displays the results of the analysis. All points correspond to four production bioreactors feeding a single purification train. For low product titers, the bioreactor volume has a considerable effect on the unit production cost. For instance, for a product titer of 1 g/L, changing the volume of each production bioreactor from 10,000 to 20,000 L reduces the unit cost from $275/g to $172/g. This is due to the "economy of scale" associated with the equipment, as well as the fact that certain costs (such as labor, quality assurance testing, etc.) are essentially constant regardless of whether the process is run in smaller or larger

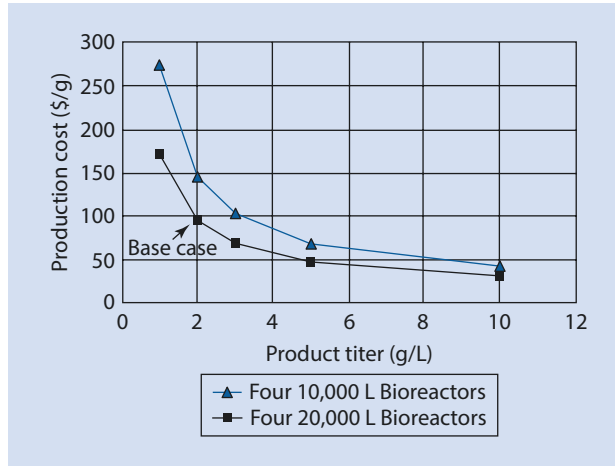


Fig. 9.14 mAb production cost as a function of product titer and production bioreactor volume

equipment. In contrast, for higher product titers (e.g., around 5 g/L), the impact of bioreactor scale is not as important. This can be explained by the fact that at high product titers, the upstream equipment costs become a smaller contributor to the total operating cost, whereas the downstream equipment costs and the cost for consumables (such as chromatography resins) become much more significant since far more product must be purified. In other words, if product titer increases dramatically, the majority of the operating cost shifts from the upstream section to the downstream purification train. It is therefore wise to also shift R&D efforts from cell culture to product purification as the titer increases. Note that a key assumption underlying this sensitivity analysis is that the composition and cost of the cell culture media are independent of product titer.

As noted in ▶ Sect. 9.3.2, the use of disposable bags in biopharmaceutical manufacturing is becoming more common. Given the increasing popularity of these units, another what-if analysis was performed to compare the economics of a conventional stainless steel facility to a facility which makes significant use of disposable bags for solution preparation and storage. However, due to the size limitations of disposable bags, this comparison was performed on a process that was scaled down by a factor of 20 relative to the original Base Case (i.e., the new process uses four 1000 L production bioreactors to produce the new product "mAb-2"). In addition, several units in the seed train were removed from the model since the reduction in processing scale made them unnecessary. Furthermore, it was assumed that the new process had a titer of 5 g/L rather than 2 g/L. These changes result in a production capacity for mAb-2 of 215 kg/year. (it is assumed that there is much less demand for mAb-2 than for the product which was analyzed previously in this section).

Key economic parameters for the new mAb-2 process in stainless steel tanks and the mAb-2 process in disposable bags are compared in Table 9.11. To generate the results for the "disposables" scenario, first all of the stainless steel tanks with volumes below 1000 L were replaced with disposable bags mounted in skids. The disposable bag prices were set equal to the values shown previously (in Table 9.7). The production bioreactor was also replaced with a disposable cell culture bag (with a cost of $6220/bag) mounted in a skid. Finally, cleaning and SIP operations were deleted from the unit procedures which are executed within the new disposable units. Note that the elimination of CIP and SIP operations in the production fermentor results in a slight reduction in its batch cycle time. Since this unit is the cycle time bottleneck, the reduction in cycle time could allow slightly more batches to be produced per year. However, to enable an "apples-to-apples" comparison, it

Table 9.11 Comparison of economic results for stainless steel vs. single-use equipment (2018 costs)

Capital costs	**Stainless steel**	**Disposable bags**
Equipment purchase cost	$21 million	$13 million
Total capital investment	$311 million	$190 million
Annual operating costs:	**Stainless steel**	**Disposable bags**
Facility-dependent cost	$43.2 million	$26.3 million
Materials cost	$1.7 million	$1.2 million
Labor cost	$16.0 million	$15.2 million
Consumables cost	$5.8 million	$8.3 million
Miscellaneous costs	$8.2 million	$7.8 million
Total manufacturing cost	***$74.9 million/year***	***$58.8 million/year***
Production summary:	**Stainless steel**	**Disposable bags**
Annual production (kg/year mAb-2)	215 kg	215 kg
Unit production cost	$348/g of mAb-2	$273/g of mAb-2

was assumed that the required annual production was 215 kg/year for both production scenarios. Therefore, the number of batches per year was held constant, and in both scenarios each new batch was scheduled to start 3.5 days after the start of the previous batch.

As shown in Table 9.11, replacing various vessels with disposable bags caused some very large changes to the economics of the process. In particular, the equipment and the related total capital investment costs were both reduced by approximately 39%. This resulted in a similar reduction in annual facility-dependent costs. There were also reductions in raw material costs (due mainly to reduced use of cleaning solutions that were used for CIP steps), labor (due to elimination of many CIP and SIP operations), and miscellaneous costs. On the other hand, there was a substantial increase in consumables cost. Nevertheless, the project's annual manufacturing cost and unit production cost were reduced by approximately 22% due to the use of disposable vessels. This result highlights the large financial benefits which can be achieved by using disposable units for production of small-volume biopharmaceuticals. It also highlights the benefits of using process modeling to perform what-if analyses in order to improve the efficiency and profitability of a process.

9.5 Summary

In this chapter, the essentials of bioprocess simulation and economic evaluation were presented. In addition, the concepts were explained using a monoclonal antibody production example. Some important points to remember when performing process simulation and cost analysis are:

- Process simulators facilitate the analysis of integrated processes by performing material and energy balances, equipment sizing, economic evaluation, cycle time analysis, environmental impact assessment and other tasks.

- Capital costs are estimated based on direct fixed capital, working capital, and start-up and validation costs.
- The cost of a processing step is the sum of the costs of raw materials, labor, consumables, quality control, waste treatment/disposal, utilities, equipment depreciation, equipment maintenance, and overhead. The cost of the entire process is the sum of the costs for all the steps.
- Profitability analysis consists of the calculation of gross margin, return on investment, payback time, net present value, internal rate of return, and other measures.
- Sensitivity analysis uses a process simulator to investigate the impact of changing a specific process variable, such as product titer or scale of production. This activity can help to focus process design or development resources on areas of the process that are likely to have the greatest economic impact.

The biotechnology industry has only recently begun making significant use of process simulation and scheduling tools. Increasingly, universities are incorporating the use of such tools in their curricula. In the future, we can expect to see increased use of these technologies, resulting in better and more-efficient process design, better project decision-making, and more cost-effective and robust processes.

9

Take-Home Messages

- Bioprocess simulators allow engineers and scientists to create an integrated model of all the important activities involved in a production process.
- Based on the structure of the model (e.g., its specific processing steps) and the parameters specified by the user, the simulator performs material balances, energy balances, and scheduling calculations in order to determine requirements for raw materials, utilities, equipment, and other resources.
- Process simulation can also be used to determine production bottlenecks and evaluate alternative processing configurations in order to improve plant throughput and reduce costs.
- Based on the capacity requirements for each procedure in a process, equipment sizes can be determined and the associated equipment capital costs can be estimated by the simulator.
- Simulators can also estimate capital costs for the entire plant, based on the specific equipment within it.
- The annual facility costs (related to plant maintenance, depreciation, etc.) may then be evaluated alongside other plant operating costs (such as material costs, labor costs, etc.) based on all the resources required for the process.
- Process simulators can also perform a full profitability analysis, including calculation of common financial metrics like gross margin and return on investment.
- Economic analysis based on comprehensive process models can have a large impact on plant profitability because it allows a plant to be run in an efficient and cost-effective manner.
- Two alternative processing scenarios for a monoclonal antibody process (using either stainless steel equipment or disposable units) are described in this chapter in order to demonstrate the methodology and utility of economic analysis from bioprocess simulation.

References

1. Douglas JM. Conceptual design of chemical processes. New York: McGraw-Hill; 1988.
2. Frohlich BT. Considerations for developing in-house capabilities for cost of goods estimation. In: Biopharmaceutical process economics and optimization (conference proceedings). Westborough MA; September 30–October 1, 1999.
3. Burk C. Techno-economic modeling for new technology development, Chemical Engineering Progress (CEP), January 2018, pp. 43–52.
4. Korovessi E, Linningerr A. Batch processes. Boca Raton: Taylor & Francis; 2006.
5. Heinzle E, Biwer A, Cooney C. Development of sustainable bioprocesses. West Sussex: Wiley; 2006.
6. Petrides DP, Koulouris A, Lagonikos PT. The role of process simulation in pharmaceutical process development and product commercialization. Pharm Eng. 2002;22(1):1–8.
7. Toumi A, Jurgens C, Junco C, Maier B, Papavasileiou V, Petrides DP. Design and optimization of a large scale biopharmaceutical facility using process simulation and scheduling tools. Pharm Eng. 2010;20(2):1–9.
8. DiMasi JA, Grabowski HG, Hansen RW. Innovation in the pharmaceutical industry: new estimates of R&D costs. J Health Econ. 2016;47:20–33.
9. Peters MS, Timmerhaus KD. Plant design and economics for chemical engineers. 4th ed. New York: McGraw-Hill; 1991.
10. Ulrich GD. A guide to chemical process design and economics. New York: Wiley; 1984.
11. Valle-Riestra JF. Project evaluation in the chemical process industries. New York: McGraw-Hill; 1983.
12. Garrett DE. Chemical engineering economics. New York: Van Nostrand Reinhold; 1989.
13. Seider WD, Seader JD, Lewin DR. Process design principles - synthesis, analysis, and evaluation. New York: Wiley; 1999.
14. Towler G, Sinnott R. Chemical engineering design; principles, practice and economics of plant and process design. London: Butterworth-Heinemann (Elsevier); 2008.
15. Jagschies G. Where is biopharmaceutical manufacturing heading? BioPharm Int. 2008;21(10):72–82.
16. Pollak P, Badrot A, Dach R. API manufacturing: facts and fiction, contract pharma, January/February 2012, issue: 62–63.
17. Pais-Chanfrau J, et al. The impact of disposables on project economics in a new antibody plant: a case study. BioPharm Int. 2009;22(12):62–74.
18. Minow B, Rogge P, Thompson K. Implementing a fully disposable mAb manufacturing facility. BioProcess Int. 2012;10(6):48–57.
19. Papavasileiou V, Siletti C, Petrides D. Systematic evaluation of single-use systems using process simulation tools. The BioPharm International Guide, November 2008. issue: 16–29.
20. Levine HL, Lilia J, Stock R, Gaasvik A, Hummel H, Ransohoff TC, Jones SD. Single-use technology and modular construction. BioProcess Int. 2013;11(4):40–5.
21. Ecker DM, Jones SD, Levine HL. The therapeutic monoclonal antibody market. MAbs. 2015;7(1):9–14.
22. Kelley B. Very large scale monoclonal antibody purification: the case for conventional unit operations. Biotechnol Prog. 2007;23:995–1008.

Case Study: Green Fluorescent Protein Production Plant

Vernon Shoaf, Aydin Berenjian, and Doug Carmichael

A. Berenjian (ed.), *Essentials in Fermentation Technology*, Learning Materials in Biosciences,
https://doi.org/10.1007/978-3-030-16230-6_10

What You Will Learn in This Chapter

Green fluorescent protein (GFP) is a fluorescent dye that is very well tolerated by most cells and doesn't interfere with cellular function. GFP is broadly used as a biological marker which allows the visualization of where a protein is made or what its movements are within an organism. GFP is becoming a commonplace scientific tool, and its production via fermentation processes is in high demand. In this case study, we will be looking at the production of GFP via one such fermentation process.

10.1 Process Requirements

Our materials for this process will include a bacteria (*E.coli*) which has been genetically enhanced to produce GFP. The basic ingredients for compatible media include nutrients, stabilizers, antibiotics (to prevent contamination during the fermentation by undesirable microorganisms), anti-foaming agents, and isopropyl β-D-1-thiogalactopyranoside (IPTG) which serves as a biochemical inducer to switch on the *GFP* gene.

The equipment that we will be using during the fermentation includes a 300-L bioreactor, UV-Vis spectrophotometer to monitor the optical density (which is a measure of a concentration of cells in a bioreactor), a glucose analyzer to measure glucose concentration (a key nutrient), an offline pH meter to help track the acid/base balance, and a broth tank to receive the fermentation product. The bioreactor is equipped with a water jacket around the vessel to regulate temperature, as well as integrated sensors to monitor key environmental factors including dissolved oxygen levels, pH, internal temperature, water jacket temperature, and vessel pressure. The reactor also has an agitator, dedicated ports for adding seed stock and media ingredients, separate ports for acid and base supplement, air filters for sterile air supply, and outlet ports for samples and for harvesting. Most fermentation and monitoring functions can be managed by the reactor's dedicated process controller.

Before the fermentation process can begin, the area must be prepared. Preparation includes removing the equipment and materials that won't be used in the process, cleaning and sanitizing the area and equipment, and sterilizing the equipment as required by the standard operating procedure (SOP) which is a set of step-by-step instructions compiled by the organization to guide workers carrying out routine operations. Sterilization is used to eliminate unwanted microorganisms which can grow naturally in the fermentation media and process equipment.

Furthermore, all required materials and documentation should be gathered and prepared, and process control software should be loaded and verified. The fermentation batch process will be guided and will be documented with the batch process record (BPR). This record leads the operator through the process step-by-step, with each step requiring a sign-off and separate verification. This record also includes spaces for documenting key times, activities, and instrument readings.

10.2 Process Description (Upstream)

Figure 10.1 shows the process flow diagram of key procedures in the GFP production, starting from P-1 and eventually proceeding to P-17. Note that this figure is a simplified representation which does not include all supporting equipment which might be associated with the process, such as buffer preparation tanks, waste collection tanks, pumps, etc.

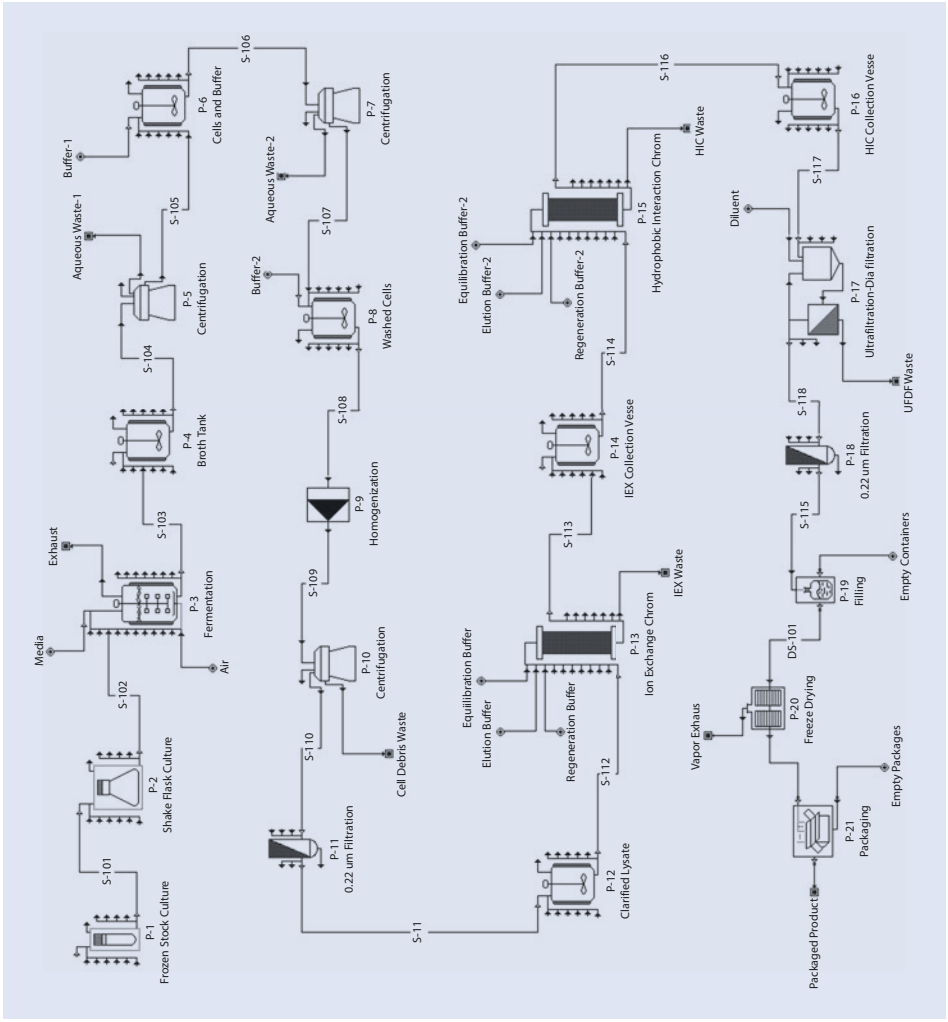


Fig. 10.1 Showing the process flow diagram of the GFP production

The process description below is also simplified, and it omits various details related to specific processing conditions, equipment cleaning/regeneration procedures, etc.

The process begins with the expansion of bacteria seed stock (P-1). After taking out the *E. coli* from the freezer, it is used to inoculate a small amount of fresh media in a shake flask (P-2). After the cells in the flask have reached the target concentration, the thriving cells are ready for fermentation. Meanwhile, in the fermentation area, operators perform a complete check of all critical equipment. Valves, caps, and lines are checked, hoses are tightened, and probes are verified and calibrated. Next, 10 kg of high-purity water is added to the bioreactor (P-3), and it is brought up to normal process pressure and held there in order to check for leaks. The pressure is monitored over a 30-minute period. If a leak is detected, the problem is corrected and the test is run again. Once the reactor passes the test we are ready to mix the media in the bioreactor. The agitator is turned on and the ingredients are added. These include yeast extract, tryptic soy broth, ammonium chloride, sodium biphosphate, monopotassium phosphate, and an anti-foam compound. Once all the initial ingredients are in, another 10 kg of high-purity water is added. Then, all ports and valves are closed and all condensate valves are opened. Next, the bioreactor begins a sterilize-in-place (SIP) cycle. The target for sterilization is 121°C for 30 minutes. As soon as the temperature reaches the targeted temperature, the condensate valves are closed and the SIP cycle completes automatically. Both the vessel and the media are now sterile and we are ready to add the final ingredients to the media. First, the glucose hose is attached to the vessel. Next, the connection is steamed to sterilize it, and the separately sterilized glucose and antibiotic solutions are pumped into the vessel. Then the manual pH reading of the media is taken and the bioreactor is set up for its fermentation cycle. After the inoculation hose is connected to the reactor and steamed for 20 minutes, the expanded seed stock is pumped into the reactor containing the media.

Fermentation now begins; the operator takes zero hour readings and begins to regularly monitor the batch temperature, agitator rpms, dissolved oxygen levels, pH, vessel pressure, optical density, air flow rate, and glucose concentrations. Optical densities and glucose concentrations are of particular interest, so they are graphed as well as documented. When the targeted levels of glucose and optical density are achieved, it's time to add IPTG to the vessel to turn on the expression of GFP in the cells. After allowing enough time for the cells to produce GFP (usually 5 h), more final readings are taken, and a sample is drawn to check the cell count. The fermentation mixture is now referred to as broth. The fermentation is complete when the key nutrient (glucose) is almost entirely consumed and the broth has reached the desired concentration of cells. The batch is then cooled down, pumped into the broth tank, and labelled with the batch number, volume, time, and date.

10.3 Process Description (Downstream)

The downstream purification section includes a disc stack centrifuge (to separate solids from liquids), a homogenizer (to break open the *E. coli* cells), a 0.22 μm filter (to separate any remaining solids in the fermentation broth), several chromatography steps (to eliminate unwanted proteins and other contaminants), additional filtrations (to concentrate the product solution and remove salts), freeze drying, and packaging.

The primary material consumed in this section is high-purity water which has been filtered by reverse osmosis, deionized, and UV sterilized. This water is consumed for several buffer solutions which are used to purify the product in the chromatography steps and

to stabilize the pH of the product in order to keep it in suspension and prevent it from degrading. The recovery process is executed through the use of a batch process record which leads the operator through the process step-by-step, with each step requiring a sign-off and a separate verification by a second operator. Before the process can begin, the recovery area must be cleaned and organized, any unnecessary material or equipment should be removed, and the area must be cleaned and disinfected to eliminate microorganisms. In addition, all the equipment must be cleaned, sanitized, and set up in accordance with the relevant SOPs. All required materials and documentation must be gathered and prepared as well.

The GFP recovery sequence begins with the transfer of broth from the fermentor to the broth tank (P-4). Next, a sterile hose is run from the broth tank to the disc stack centrifuge (P-5). The tank is then pressurized. After the centrifuge has reached a stable running speed, its inlet valve is opened and broth is driven into it from the pressurized broth tank. The centrifugal force of rotation forces the denser material (solids) to the sides of the bowl while liquid flows out of the centrifuge. The liquid leaving the bowl is known as the "clarified stream" because almost all the solids have been removed. The solids include the *E. coli* cells, which contain the product. When the bowl has reached its capacity for retaining solids, the bowl opens and the solids are discharged into an appropriate container for collection. Once the solids are discharged, the centrifugation step can resume. This clarified liquid is sent to waste treatment.

At this point, the cells are in a paste form, and although most of the liquid has been removed, the cell paste still has almost 40% liquid by weight. The remaining liquid contains high levels of metabolites and salts that could complicate downstream processing, so those levels are lowered by washing the cells. To accomplish this, the cell paste is suspended in a buffered solution (P-6) and then recentrifuged using a disc stack centrifuge (P-7). As the clarified liquid leaves the centrifuge, it carries many contaminants from the fermentation step with it. The cells which are removed from the centrifuge are once again in paste form (P-7) and are ready for the next step known as cell disruption or lysing.

The cells are re-suspended in a buffered solution and then pumped at high pressure (900 bar) through a homogenizer (P-9). Inside the homogenizer, they are forced through a tiny orifice, which causes them to rupture and break apart. In order to make sure all the *E.coli* cells are ruptured, the solution is passed through the homogenizer twice. After the second homogenization, the lysed cell solution is pumped through a centrifuge (P-10). Note that all three centrifugations (P-5, P-7, and P-10) could potentially be performed with a single equipment unit, although in many plants different equipment units would be used for these three procedures in order to keep the centrifuge from becoming a cycle time bottleneck which would reduce plant throughput. This time we discard the solid phase (cell debris) and keep the liquid "lysate" which contains the GFP. Although the centrifuge has removed almost all the cell debris, some small particles remain. Next, the lysate from the centrifuge is pumped through a 0.22 μm filter (P-11). This filter's pore size is fine enough that it removes all the remaining solid materials. At this point, the process stream is referred to as "clarified lysate." The clarified lysate is pumped to a vented, temperature-controlled transfer vessel which will be used to feed the next series of purification steps.

The GFP purification begins with ion exchange (IEX) and hydrophobic interaction chromatography (HIC) steps. A typical chromatography apparatus for this type of biological product includes a column filled with resin beads, as well as pumps to move the clarified lysate through the apparatus, a supply hose and port to feed the column, a prefilter to remove any remaining particulates which might have passed through the previous filter, an outlet

port for the product solution and waste streams, and automated valves for directing the column's exit stream to either waste or product collection. To help monitor the chromatography equipment and the solutions flowing through the unit during this step, a number of sensors are located along the product flow path. For instance, there is an electrical conductivity sensor at the column inlet, a pressure sensor just before the prefilter to help determine if the filter becomes clogged, a flowmeter to measure the rate of solution movement through the column, and an air sensor to ensure that no air has entered the flow path. Furthermore, at the outlet of the column, there is a UV sensor that determines the solution's optical density, a second conductivity sensor, and a pH sensor that measures how acidic or basic the solution is. The conductivity sensors identify when a buffered solution has displaced the previous solution within the column. When the conductivity reading from the exit of the column matches the reading from the sensor at the inlet of the column then it is apparent that the new solution has completely displaced the old one. Meanwhile, the UV sensor monitors the concentration of protein at the column outlet by observing the optical density of the passing solution. This sensor works hand in hand with the valves on the outlet of the column. When the optical density leaving the column is below a pre-programmed threshold (corresponding with a lack of GFP product in the stream), the valve directs the flow to waste. When the optical density leaving the column is at or above the threshold, the solution is directed to a collection vessel. The setup for the HIC purification apparatus is similar.

The first chromatography step (IEX) begins with a column equilibration operation, which prepares the column to receive the product stream by feeding an initial buffer solution into it. Next, the clarified lysate tank (P-12) is connected to the inlet pump on the chromatography unit (P-13). At this point of the process, the pH of the clarified lysate is around 8 which means that the protein is negatively charged. Because it is negatively charged, GFP will bind to the positively charged beads. The pump transfers the lysate from its tank, past the first conductivity sensor and the pressure sensor, and through the 0.22 μm prefilter to remove any residual cell debris or any particulates that may have contaminated the solution. The lysate solution then passes through the flowmeter and the air sensor. As the lysate enters the column and passes over the resin beads, the negatively charged protein binds to the positively charged beads. The solution leaving the column passes the UV optical density sensor, the second conductivity sensor, and the pH sensor. The low readings from the optical density sensor confirm that GFP is not in the solution, so the outlet valve sends this solution to waste. When all the lysate has entered the column or when the capacity of the beads to bind the protein has been reached, it is time for elution. Elution in this case means the release of GFP from the beads by feeding a new buffer solution into the column (in this case, a buffer that includes sodium chloride). As the elution buffer is pumped through the beads, the GFP no longer binds to them and it is released into the mobile phase (the flowing buffer). When the UV optical density sensor at the column outlet indicates that GFP product has begun to exit the column, the outlet valves are switched to allow the product stream to be diverted into the collection vessel (P-14). Later, when the UV sensor indicates all the GFP has come off the chromatography resin, the outlet valves are switched back to waste collection. After elution, other buffer solutions may be used to regenerate and store the column resin. Other buffer solutions may also be used during this procedure (such as "wash" buffers) to remove additional impurities.

The next step is hydrophobic interaction chromatography (P-15), which is based on the principle that hydrophobic chemicals on the resin surface will bind to hydrophobic patches on the GFP. In order for this to happen, the resin and protein eluate have to be in a high salt environment to remove the water shielding. In this case, ammonium sulfate is used as the

salt. After the product has been loaded onto the column, and unbound materials (such as impurities) have flowed through to the waste stream, the product must be eluted. To accomplish this, the GFP protein must be detached from the resin by lowering the salt concentration within the column. This causes the water shielding to re-form and allows the GFP protein to enter the elution stream, which is sent to another collection tank (P-16).

The product is now ready for the tangential flow filtration (TFF) step (P-17). TFF will be used to concentrate and diafilter the GFP product stream. Although the eluate from the HIC step is rich in GFP, it is still too dilute and too high in salt to be sent to the subsequent freeze-drying step yet. Therefore, the solution is pumped from the supply tank, past a pressure sensor, and across the filter membrane in the TFF apparatus. Everything that passes through the membrane (including impurities such as dissolved salt) is sent to waste. The GFP protein molecules are larger than the pores of the filter and therefore they are retained. The retained material (called "retentate") is continuously recirculated to the TFF supply tank. Recirculation of the feed continues until the desired concentration of GFP is achieved. Once the concentration operation is complete, a new solution (called "diluent") is added to the feed. This solution eventually replaces the previous solution during an operation called "diafiltration", which takes place in the same unit as the concentration operation (P-17). As the diafiltration proceeds, the new buffer solution that has been added to the feed washes out the buffer solution that GFP was originally in, effectively removing any remaining salts. The old buffer solution, as well as some of the diluent, flows out of the unit as waste. The retentate (including the GFP and the remaining diluent) is then sent through another 0.22 μm filter (P-18) and then collected in appropriate containers (P-19). At this point, the purification process is complete, and the GFP concentrate will be freeze-dried to remove moisture (P-20) and subsequently packaged (P-21).

10.4 GFP Production Plant Videos

Clicking the links below will open videos which describe the production of GFP in a plant that is similar to the one described in this chapter. The videos cover both the upstream and downstream (recovery and purification) portions of the process.

Part 1: Fermentation

► https://www.youtube.com/watch?v=5eKdZ0dVCCo&t=2s

Part 2: Separation/recovery

► https://www.youtube.com/watch?v=VKpthcW1llU

Part 3: Purification

► https://www.youtube.com/watch?v=N7vxq948l-U

Take-Home Messages

- Green fluorescent protein (GFP) is broadly used as a biological marker, and its production via fermentation processes is in high demand.
- Genetically modified *E. coli* has been used for GFP production.
- Upstream and downstream production steps for GFP production have been completely elaborated.
- Process flow diagram and video materials show the key procedures involved in the GFP production.

Correction to: Bioreactor Scale-Up

Aydin Berenjian

Correction to:

Chapter 7 in: A. Berenjian (ed.), *Essentials in Fermentation Technology*, Learning Materials in Biosciences, https://doi.org/10.1007/978-3-030-16230-6_7

On page 224, Chap. 7—Bioreactor Scale-Up, the numbers calculated for kLa were erroneously published. This has been corrected in this version and it should be read as:

"and from Eq. 7.4b, for 200 rpm, we have:

$k_L a = 0.3714\ \text{min}^{-1}$ or $22.28\ \text{h}^{-1}$

And for 400 rpm, we have:

$k_L a = 0.6828\ \text{min}^{-1}$ or $40.96\ \text{h}^{-1}$"

The updated online version of this chapter can be found at
https://doi.org/10.1007/978-3-030-16230-6_7

A. Berenjian (ed.), *Essentials in Fermentation Technology*, Learning Materials in Biosciences,
https://doi.org/10.1007/978-3-030-16230-6_11