

Edited by David B. Wilson, Hermann Sahm,
Klaus-Peter Stahmann, and Mattheos Koffas

Industrial Microbiology



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This book is dedicated to
Nancy, Allison, Ashley, and Laurie
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Contents

Preface *xvii*

1	Historical Overview and Future Perspective	1
	<i>Bernhard Eikmanns, Marcella Eikmanns, and Christopher J. Paddon</i>	
1.1	Use of Fermentation Procedures Before the Discovery of Microorganisms (Neolithic Era = New Stone Age Until 1850)	1
1.2	Investigation of Microorganisms and Beginning of Industrial Microbiology (1850 Until 1940)	7
1.3	Development of New Products and Procedures: Antibiotics and Other Biomolecules (From 1940)	11
1.4	Genetic Engineering Is Introduced into Industrial Microbiology (From Roughly 1980)	15
1.5	Future Perspectives: Synthetic Microbiology	18
	References	20
	Further Reading	21
2	Bioprocess Engineering	23
	<i>Michael R. Ladisch, Eduardo Ximenes, Nathan Mosier, Abigail S. Engelberth, Kevin Solomon, and Robert Binkley</i>	
2.1	Introduction	23
2.1.1	Role of Bioreactors	25
2.1.2	Basic Bioreactor Configurations	26
2.1.3	Types of Growth Media	27
2.2	Nonstructured Models	28
2.2.1	Nonstructured Growth Models	28
2.2.1.1	Unstructured Models	29
2.2.1.2	Biotechnical Processes	30
2.2.2	Modeling Fermentations	32
2.2.3	Metabolic Pathways	39
2.2.4	Manipulation of Metabolic Pathways	40
2.2.5	Future of Pathway Design	42
2.3	Oxygen Transport	43
2.3.1	Aerobic versus Anaerobic Conditions	43
2.3.2	$k_L a$ – Volumetric Mass Transfer Coefficient	44
2.4	Heat Generating Aerobic Processes	46

2.5	Product Recovery	49
2.5.1	Basics	49
2.5.2	<i>In Situ</i> Product Recovery (ISPR)	49
2.6	Modeling and Simulation of Reactor Behavior	51
2.6.1	Basic Approaches and Software	51
2.6.2	Numerical Simulation of Bioreactor Function	51
2.6.3	Contamination of Bioreactors	52
2.7	Scale-up	53
	References	54
	Further Reading	57
3	Food	59
	<i>Gülhan Ünlü and Barbara Nielsen</i>	
3.1	Fermented Foods	59
3.1.1	Food Preservation	59
3.1.2	Flavor and Texture	60
3.1.3	Health Benefits	60
3.1.4	Economic Impact	62
3.2	Microorganisms and Metabolism	62
3.2.1	Fermentation Processes	64
3.2.2	Starter Cultures	65
3.3	Yeast Fermentations – Industrial Application of <i>Saccharomyces</i> Species	65
3.3.1	Grain Fermentation for Ethanol Production – Beer	66
3.3.2	Grain Fermentation for CO ₂ Production – Bread	69
3.3.2.1	Yeast Preparation	69
3.3.3	Fruit Fermentation – Wines and Ciders	71
3.4	Vinegar – Incomplete Ethanol Oxidation by Acetic Acid Bacteria Such as <i>Gluconobacter oxydans</i>	75
3.4.1	Substrates: Wine, Cider, and Malt	75
3.4.2	Distilled (White) Vinegar	77
3.4.3	Balsamic and Other Specialty Vinegars	77
3.5	Bacterial and Mixed Fermentations – Industrial Application of Lactic Acid Bacteria, With or Without Yeast or Molds	78
3.5.1	Milk – Cultured Milks – Buttermilk, Yogurt, Kefir, and Cheese	78
3.5.1.1	Bacteriophage Contamination – Death of a Culture	81
3.5.2	Meats – Sausages, Fish Sauces, and Pastes	82
3.5.3	Vegetables – Sauerkrauts and Pickles, Olives	83
3.5.4	Grains and Legumes – Soy Sauce, Miso, Natto, and Tempeh	86
3.5.5	Cocoa and Coffee	87
3.6	Fungi as Food	88
3.6.1	Mushrooms	88
3.6.2	Single-Cell Protein – <i>Fusarium venenatum</i>	90
3.7	Conclusions and Outlook	91
	References	92
	Further Reading	92

4	Technical Alcohols and Ketones	95
	<i>Peter Dürre</i>	
4.1	Introduction	95
4.2	Ethanol Synthesis by <i>Saccharomyces cerevisiae</i> and <i>Clostridium autoethanogenum</i>	97
4.2.1	Application	97
4.2.2	Metabolic Pathways and Regulation	97
4.2.3	Production Strains	98
4.2.4	Production Processes	98
4.2.5	Ethanol – Fuel of the Future?	100
4.2.6	Alternative Substrates for Ethanol Fermentation by Cellulolytic Bacteria and <i>Clostridium autoethanogenum</i>	100
4.3	1,3-Propanediol Synthesis by <i>Escherichia coli</i>	101
4.3.1	Application	101
4.3.2	Metabolic Pathways and Regulation	102
4.3.3	Production Strains	102
4.3.4	Production Processes	104
4.4	Butanol and Isobutanol Synthesis by Clostridia and Yeast	105
4.4.1	History of Acetone–Butanol–Ethanol (ABE) Fermentation by <i>Clostridium acetobutylicum</i> and <i>C. beijerinckii</i>	105
4.4.2	Application	106
4.4.3	Metabolic Pathways and Regulation	107
4.4.4	Production Strains	110
4.4.5	Production Processes	110
4.4.6	Product Toxicity	113
4.5	Acetone Synthesis by Solventogenic Clostridia	113
4.5.1	Application	113
4.5.2	Metabolic Pathways and Regulation	113
4.5.3	Production Strains	114
4.5.4	Production Processes	114
4.6	Outlook	115
	Further Reading	115
5	Organic Acids	117
	<i>Michael Sauer and Diethard Mattanovich</i>	
5.1	Introduction	117
5.2	Citric Acid	119
5.2.1	Economic Impact and Applications	120
5.2.2	Biochemistry of Citric Acid Accumulation	120
5.2.3	Industrial Production by the Filamentous Fungus <i>Aspergillus niger</i>	122
5.2.4	<i>Yarrowia lipolytica</i> : A Yeast as an Alternative Production Platform	123
5.3	Lactic Acid	124
5.3.1	Economic Impact and Applications	124
5.3.2	Anaerobic Bacterial Metabolism Generating Lactic Acid	125
5.3.3	Lactic Acid Production by Bacteria	125

5.3.4	Lactic Acid Production by Yeasts	126
5.4	Gluconic Acid	127
5.4.1	Economic Impact and Applications	127
5.4.2	Extracellular Biotransformation of Glucose to Gluconic Acid by <i>Aspergillus niger</i>	128
5.4.3	Production of Gluconic Acid by Bacteria	129
5.5	Succinic Acid	129
5.5.1	Economic Impact and Applications	130
5.5.2	Pilot Plants for Anaerobic or Aerobic Microbes	130
5.6	Itaconic Acid	132
5.6.1	Economic Impact and Applications	132
5.6.2	Decarboxylation as a Driver in Itaconic Acid Accumulation	132
5.6.3	Production Process by <i>Aspergillus terreus</i>	132
5.6.4	Metabolic Engineering for Itaconic Acid Production	132
5.7	Downstream Options for Organic Acids	134
5.8	Perspectives	135
5.8.1	Targeting Acrylic Acid – Microbes Can Replace Chemical Process Engineering	136
5.8.2	Lignocellulose-Based Biorefineries	136
	Further Reading	137
6	Amino Acids	139
	<i>Lothar Eggeling</i>	
6.1	Introduction	139
6.1.1	Importance and Areas of Application	139
6.1.2	Amino Acids in the Feed Industry	140
6.1.3	Economic Significance	141
6.2	Production of Amino Acids	142
6.2.1	Conventional Development of Production Strains	142
6.2.2	Advanced Development of Production Strains	144
6.3	L-Glutamate Synthesis by <i>Corynebacterium glutamicum</i>	145
6.3.1	Synthesis Pathway and Regulation	145
6.3.2	Production Process	148
6.4	L-Lysine	148
6.4.1	Synthesis Pathway and Regulation	148
6.4.2	Production Strains	150
6.4.3	Production Process	152
6.5	L-Threonine Synthesis by <i>Escherichia coli</i>	153
6.5.1	Synthesis Pathway and Regulation	153
6.5.2	Production Strains	154
6.5.3	Production Process	155
6.6	L-Phenylalanine	155
6.6.1	Synthesis Pathway and Regulation	155
6.6.2	Production Strains	156
6.6.3	Production Process	157
6.7	Outlook	158
	Further Reading	159

7	Vitamins, Nucleotides, and Carotenoids	161
	<i>Klaus-Peter Stahmann and Hans-Peter Hohmann</i>	
7.1	Application and Economic Impact	161
7.2	L-Ascorbic Acid (Vitamin C)	163
7.2.1	Biochemical Significance, Application, and Biosynthesis	163
7.2.2	Regioselective Oxidation with Bacteria in the Production Process	164
7.3	Riboflavin (Vitamin B ₂)	166
7.3.1	Significance as a Precursor for Coenzymes and as a Pigment	166
7.3.2	Biosynthesis by Fungi and Bacteria	167
7.3.3	Production by <i>Ashbya gossypii</i>	168
7.3.4	Production by <i>Bacillus subtilis</i>	171
7.3.5	Downstream Processing and Environmental Compatibility	173
7.4	Cobalamin (Vitamin B ₁₂)	174
7.4.1	Physiological Relevance	174
7.4.2	Biosynthesis	176
7.4.3	Production with <i>Pseudomonas denitrificans</i>	176
7.5	Purine Nucleotides	178
7.5.1	Impact as Flavor Enhancer	178
7.5.2	Development of Production Strains	178
7.5.3	Production of Inosine or Guanosine with Subsequent Phosphorylation	179
7.6	β-Carotene	180
7.6.1	Physiological Impact and Application	180
7.6.2	Production with <i>Blakeslea trispora</i>	181
7.7	Perspectives	181
	Further Reading	183
8	Antibiotics and Pharmacologically Active Compounds	185
	<i>Lei Fang, Guojian Zhang, and Blaine A. Pfeifer</i>	
8.1	Microbial Substances Active Against Infectious Disease Agents or Affecting Human Cells	185
8.1.1	Distribution and Impacts	185
8.1.2	Diversity of Antibiotics Produced by Bacteria and Fungi	189
8.2	β-Lactams	190
8.2.1	History, Effect, and Application	190
8.2.2	β-Lactam Biosynthesis	190
8.2.3	Penicillin Production by <i>Penicillium chrysogenum</i>	193
8.2.4	Cephalosporin Production by <i>Acremonium chrysogenum</i>	193
8.3	Lipopeptides	193
8.3.1	History, Effect, and Application	193
8.3.2	Lipopeptide Biosynthesis	194
8.3.3	Daptomycin Production by <i>Streptomyces roseosporus</i>	194
8.3.4	Cyclosporine Production by <i>Tolypocladium inflatum</i>	194
8.4	Macrolides	197
8.4.1	History, Effect, and Application	197
8.4.2	Macrolide Biosynthesis	197
8.4.3	Erythromycin Production by <i>Saccharopolyspora erythraea</i>	197

8.5	Tetracyclines	200
8.5.1	History, Effect, and Application	200
8.5.2	Tetracycline Biosynthesis	200
8.5.3	Tetracycline Production by <i>Streptomyces rimosus</i>	201
8.6	Aminoglycosides	201
8.6.1	History, Effect, and Application	201
8.6.2	Aminoglycoside Biosynthesis	201
8.6.3	Tobramycin Production by <i>Streptomyces tenebrarius</i>	203
8.7	Claviceps Alkaloids	203
8.7.1	History, Effect, and Application	203
8.7.2	Alkaloid Biosynthesis	203
8.7.3	Ergotamine Production by <i>Claviceps purpurea</i>	203
8.8	Perspectives	203
8.8.1	Antibiotic Resistance	203
8.8.2	New Research Model for Compound Identification	206
8.8.3	Future Opportunities	207
	Further Reading	211
9	Pharmaceutical Proteins	213
	<i>Heinrich Decker, Susanne Dilsen, and Jan Weber</i>	
9.1	History, Main Areas of Application, and Economic Importance	213
9.2	Industrial Expression Systems, Cultivation and Protein Isolation, and Legal Framework	215
9.2.1	Development of Production Strains	215
9.2.2	Isolation of Pharmaceutical Proteins	221
9.2.3	Regulatory Requirements for the Production of Pharmaceutical Proteins	222
9.3	Insulins	223
9.3.1	Application and Structures	223
9.3.2	Manufacturing Processes by <i>Escherichia coli</i> and <i>Saccharomyces cerevisiae</i>	225
9.3.2.1	Production of a Fusion Protein in <i>E. coli</i>	226
9.3.2.2	Production of a Precursor Protein, the So-Called Mini Proinsulin with the Host Strain <i>S. cerevisiae</i>	228
9.4	Somatropin	230
9.4.1	Application	230
9.4.2	Manufacturing Process	231
9.5	Interferons – Application and Manufacturing	232
9.6	Human Granulocyte Colony-Stimulating Factor	234
9.6.1	Application	234
9.6.2	Manufacturing Process	235
9.7	Vaccines	235
9.7.1	Application	235
9.7.2	Manufacturing Procedure Using the Example of Gardasil™	236
9.7.3	Manufacturing Process Based on the Example of a Hepatitis B Vaccine	237
9.8	Antibody Fragments	238

9.9	Enzymes	239
9.10	Peptides	240
9.11	View – Future Economic Importance	240
	Further Reading	242
10	Enzymes	243
	<i>David B. Wilson, Maxim Kostylev, Karl-Heinz Maurer, Marina Schramm, Wolfgang Kronemeyer, and Klaus-Peter Stahmann</i>	
10.1	Fields of Application and Economic Impacts	243
10.1.1	Enzymes are Biocatalysts	243
10.1.2	Advantages and Limitations of Using Enzymatic Versus Chemical Methods	244
10.1.3	Brief History of Enzyme Used for the Industrial Production of Valuable Products	245
10.1.4	Diverse Ways That Enzymes Are Used in Industry	246
10.2	Enzyme Discovery and Improvement	250
10.2.1	Screening for New Enzymes and Optimization of Enzymes by Protein Engineering	250
10.2.2	Classical Development of Production Strains	251
10.2.3	Genetic Engineering of Producer Strains	253
10.3	Production Process for Bacterial or Fungal Enzymes	255
10.4	Polysaccharide-Hydrolyzing Enzymes	255
10.4.1	Starch-Cleaving Enzymes Produced by <i>Bacillus</i> and <i>Aspergillus</i> Species	257
10.4.2	Cellulose-Cleaving Enzymes: A Domain of <i>Trichoderma reesei</i>	259
10.4.3	Production Strains	261
10.5	Enzymes Used as Cleaning Agents	263
10.5.1	Subtilisin-Like Protease	264
10.5.2	<i>Bacillus</i> sp. Production Strains and Production Process	265
10.6	Feed Supplements – Phytases	266
10.6.1	Fields of Applications of Phytase	267
10.6.2	Phytase in the Animals Intestine	267
10.6.3	Production of a Bacterial Phytase in <i>Aspergillus niger</i>	269
10.7	Enzymes for Chemical and Pharmaceutical Industry	271
10.7.1	Examples for Enzymatic Chemical Production	271
10.7.2	Production of (S)-Profens by Fungal Lipase	271
10.8	Enzymes as Highly Selective Tools for Research and Diagnostics	272
10.8.1	Microbial Enzymes for Analysis and Engineering of Nucleic Acids	272
10.8.2	Specific Enzymes for Quantitative Metabolite Assays	275
10.9	Perspectives	276
10.9.1	L-DOPA by Tyrosine Phenol Lyase	276
10.9.2	Activation of Alkanes	276
10.9.3	Enzyme Cascades	276
	References	277
	Further Reading	277

11	Microbial Polysaccharides	279
	<i>Volker Sieber, Jochen Schmid, and Gerd Hublik</i>	
11.1	Introduction	279
11.2	Heteropolysaccharides	282
11.2.1	Xanthan: A Product of the Bacterium <i>Xanthomonas campestris</i>	282
11.2.1.1	Introduction	282
11.2.1.2	Regulatory Status	282
11.2.1.3	Structure	282
11.2.1.4	Biosynthesis	284
11.2.1.5	Industrial Production of Xanthan	286
11.2.1.6	Physicochemical Properties	287
11.2.1.7	Applications	289
11.2.2	Sphingans: Polysaccharides from <i>Sphingomonas</i> sp.	291
11.2.3	Hyaluronic Acid: A High-Value Polysaccharide for Cosmetic Applications	293
11.2.4	Alginate: Alternatives to Plant-Based Products by <i>Pseudomonas</i> and <i>Azotobacter</i> sp.	294
11.2.5	Succinoglycan: Acidic Polysaccharide from <i>Rhizobium</i> sp.	294
11.3	Homopolysaccharides	295
11.3.1	α -Glucans	296
11.3.1.1	Pullulan	296
11.3.1.2	Dextran	296
11.3.2	β -Glucans	297
11.3.2.1	Linear β -glucans like cellulose and curdlan	297
11.3.2.2	Branched β -Glucans Like Scleroglucan and Schizophyllan	297
11.3.3	Fructosylpolymers like Levan	298
11.4	Perspectives	298
	Further Reading	299
12	Steroids	301
	<i>Shuvendu Das and Sridhar Gopishetty</i>	
12.1	Fields of Applications and Economic Importance	301
12.2	Advantages of Biotransformations During Production of Steroids	303
12.3	Development of Production Strains and Production Processes	305
12.4	Applied Types of Biotransformation	307
12.5	Synthesis of Steroids in Organic – Aqueous Biphasic System	310
12.6	Side-chain Degradation of Phytosterols by <i>Mycobacterium</i> to Gain Steroid Intermediates	311
12.7	Biotransformation of Cholesterol to Gain Key Steroid Intermediates	313
12.8	11-Hydroxylation by Fungi During Synthesis of Corticosteroids	313
12.9	Δ^1 -Dehydrogenation by <i>Arthrobacter</i> for the Production of Prednisolone	316
12.10	17-Keto Reduction by <i>Saccharomyces</i> in Testosterone Production	317
12.11	Double-Bond Isomerization of Steroids	318
12.12	Perspectives	319
	References	320
	Further Reading	321

13	Bioleaching	323
	<i>Sören Bellenberg, Mario Vera Véliz, and Wolfgang Sand</i>	
13.1	Acidophilic Microorganisms Dissolve Metals from Sulfide Ores	323
13.1.1	Brief Overview on the Diversity of Acidophilic Mineral-Oxidizing Microorganisms	325
13.1.2	Natural and Man-Made Habitats of Mineral-oxidizing Microorganisms	325
13.1.3	Biological Catalysis of Metal Sulfide Oxidation	328
13.1.4	Importance of Biofilm Formation and Extracellular Polymeric Substances for Bioleaching by <i>Acidithiobacillus ferrooxidans</i> and <i>Leptospirillum ferrooxidans</i>	330
13.2	Bioleaching of Copper, Nickel, Zinc, and Cobalt	334
13.2.1	Economic Impact	334
13.2.2	Heap, Dump, or Stirred-tank Bioleaching of Copper, Nickel, Zinc, and Cobalt	337
13.3	Gold	342
13.3.1	Economic Impact	343
13.3.2	Unlocking Gold by Biooxidation of the Mineral Matrix	343
13.4	Uranium	346
13.4.1	Economic Impact	346
13.4.2	<i>In Situ</i> Biomining of Uranium	346
13.5	Perspectives	347
13.5.1	Urban Mining – Processing of Electronic Waste and Industrial Residues	347
13.5.2	Microbial Iron Reduction for Dissolution of Mineral Oxides	348
13.5.3	Biomining Goes Underground – <i>In Situ</i> Leaching as a Green Mining Technology?	348
	References	351
	Further Reading	351
14	Wastewater Treatment Processes	353
	<i>Claudia Gallert and Josef Winter</i>	
14.1	Introduction	354
14.1.1	Historical Development of Sewage Treatment	354
14.1.2	Resources from Wastewater Treatment	357
14.1.3	Wastewater and Storm Water Drainage	358
14.1.4	Wastewater Characterization and Processes for Effective Wastewater Treatment	358
14.1.5	Suspended or Immobilized Bacteria as Biocatalysts for Effective Sewage Treatment	360
14.2	Biological Basics of Carbon, Nitrogen, and Phosphorus Removal from Sewage	362
14.2.1	Aerobic and Anaerobic Degradation of Carbon Compounds	362
14.2.1.1	Mass and Energy Balance	366
14.2.2	Fundamentals of Nitrification	368
14.2.3	Elimination of Nitrate by Denitrification	371
14.2.4	New Nitrogen Elimination Processes	371
14.2.5	Microbial Phosphate Elimination	372

14.3	Wastewater Treatment Processes	374
14.3.1	Typical Process Sequence in Municipal Sewage Treatment Plants	374
14.3.2	Activated Sludge Process	376
14.3.3	Trickling Filters	378
14.3.4	Technical Options for Denitrification	379
14.3.5	Biological Phosphate Elimination	381
14.3.6	Sewage Sludge Treatment	382
14.3.6.1	Aerobic and Anaerobic Sewage Sludge Treatment	382
14.3.6.2	Sanitation and Quality Assurance of Sewage Sludge	384
14.4	Advanced Wastewater Treatment	384
14.4.1	Elimination of Micropollutants	385
14.4.2	Wastewater Disinfection	385
14.5	Future Perspectives	386
	References	386
	Further Reading	388
	Index	389

Preface

“Nature is by far the best chemist and the best engineer. Nature also has the best engineering process: evolution.”

Dr. Frances H. Arnold,
Linus Pauling Professor of Chemical Engineering at Caltech,
2018 Nobel Laureate in Chemistry.

In the endeavor of the chemical industry to reduce dependence on fossil raw materials, the application of microorganisms contributes to an increasing extent. Not only bacteria and fungi but also archaea are able to explore renewable resources efficiently and environmentally friendly and convert them into sustainable products. As an innovative cross-disciplinary field, the application of industrial microbiology will gain importance not only in the traditionally related areas of food and pharmaceutical industry but also increasingly in the chemical industry. Today, the global market for microbial products is in the order of 10^{11} US\$. In many states, funding programs are running to replace significant proportions of chemical processes with biological ones.

The future potential of industrial microbiology lies in the fact that it bundles the know-how of biologists, chemists, engineers, and bioinformaticians. This leads to a quality that no specialist can achieve on their own. In recent decades, microbiology, especially by the successful approaches of molecular biologists, has developed fundamentally. The foundation of microbial strain development was and is still random mutagenesis and subsequent selection. However, the modern methods of genetic engineering lead to a targeted change in production strains, down to the position of a single base pair in the DNA, more quickly and accurately. This discipline called Metabolic Engineering is not only suitable to overproduce metabolites, but, in the form of the so-called Synthetic Microbiology, will also help to become independent from secondary metabolism of rare organisms such as plants, fungi, or unculturable bacteria. Not a single one of the dangers of genetic engineering feared in the 1980s become true. On the contrary, drugs produced by Genetically Modified Organisms (GMOs) fill the shelves in pharmacies and are safe and successful.

This textbook is an update of a German edition published by SPRINGER in 2013. Experienced scientists working at universities, research units, or in industry report selected aspects concerning successfully applied processes of industrial microbiology. Representative examples show which processes lead to recyclable

materials of special quality. In the first two chapters, a historical overview is given first (Chapter 1) followed by an introduction to process engineering (Chapter 2). Both chapters are of paramount importance. As food is the most important commodity to the reader as a human being, it will be discussed as the first product group (Chapter 3), allowing the students to rediscover it from a new perspective. In Chapter 4, “Technical Alcohols and Ketones” as well as in Chapter 5 “Organic Acids”, it becomes clear that yields related to sugar as a substrate reach around 100%. In the production of L-enantiomers of amino acids (Chapter 6), the high selectivity of enzymes is most important. The importance of vitamins (Chapter 7) and antibiotics (Chapter 8) is well known. About 10^5 tons of vitamin C per year are produced with the help of bacteria. The penicillins excreted by fungi, cultivated in steel vessels as large as houses, exceed an annual market value of 10^{10} US\$. In Chapter 9, the realization of the great promise of industrial microbiology becomes clear. With the help of genetic engineering pharmaceutical proteins, human-identical insulin and even analogues with improved active profiles can be produced by microorganisms on an industrial scale, so that the needs of more than 10^8 diabetics can be met. This not only means availability in principle but also affordability. Microbially produced enzymes (Chapter 10) are used in a wide range of applications. Today, everyone can use protease-containing detergents at home, e.g. for washing or in tiny amounts to clean contact lenses. Large companies in the United States apply bacterial amylases to hydrolyze more than 10^9 bushels (about 10^8 tons) of corn starch annually, which can then be used in other microbial processes, e.g. by brewer's yeast for the production of 10^{10} gallons (about 10^9 liters) of fuel alcohol. Microorganisms are also used in the production of polysaccharides (Chapter 11). Xanthan for example is added as a thickener to food products such as ketchup. In order to modify steroids for the production of cortisone or contraceptives, microorganisms are used for regioselective biotransformations in multistep processes (Chapter 12). As hydrometallurgy can be accelerated by iron- and sulfur-oxidizing bacteria, both, not only vessel-based but also open pit mining in kilometer scale is increasing to extract copper even from sources where classical techniques are inefficient (Chapter 13). In Chapter 14, highly developed waste water treatment plants are described, where microorganisms not only have a high potential for biosynthesis but also are suitable for degradation. In the future, we will be well advised to not only produce substances but also to consider during the design phase how microorganisms can quickly degrade them in order to prevent their accumulation in any environment. As microorganisms play key roles in nature's material cycle, they might become more important to close cycles urgently needed for human economy.

We are grateful to our colleagues who contributed to this textbook by writing their chapters. It was a pleasure for us to cooperate with internationally recognized scientists. Our colleagues in industry deserve special praise for sacrificing nights or weekends for their contributions. Sometimes, graphically presented relationships had to be simplified in their complexity without getting wrong. We thank Susanne Nieland, MSc, who did not give up until both discussion partners, authors and editors, were satisfied with a recognizable focus of a black and white or a rarely colored graphic. Furthermore, we are grateful to WILEY-VCH,

especially Dr. Frank Weinreich and Dr. Andreas Sendtko, for their help and patience because more than one round was needed to reach the wished quality.

Sadly, during the development of this book, our first editor, David, fell seriously ill and was not able to continue working with us any longer. The idea of an American–German coedition was born after an invited talk David gave at a special meeting of the German Association of General and Applied Microbiology in Senftenberg. David was a very generous and thoughtful colleague, researcher, and teacher who was a pioneer in the study of cellulases and was devoted to the goal of deriving clean fuels from plants. We are very pleased that his efforts helped to bring about this textbook and hope that we helped our authors explain the topics selected in a way that both undergraduate and graduate students can understand. As science and engineering develop at an increasingly rapid pace, causal explanations can only be given for selected topics. We strongly support efforts to discuss open issues in seminars as many outstanding questions remain, e.g. the reason for citrate overproduction of *Aspergillus niger*.

We also hope that this textbook will arise the interest of many students of natural sciences and engineering. We are convinced that industrial microbiology will continue to be a success and hope that our book will help both our teaching colleagues and very young people to make their own contributions, whether at a research or teaching institution or in an industry.

Summer 2019

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Historical Overview and Future Perspective

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CHAPTER MENU

- 1.1 Use of Fermentation Procedures Before the Discovery of Microorganisms (Neolithic Era = New Stone Age Until 1850), 1
- 1.2 Investigation of Microorganisms and Beginning of Industrial Microbiology (1850 Until 1940), 7
- 1.3 Development of New Products and Procedures: Antibiotics and Other Biomolecules (From 1940), 11
- 1.4 Genetic Engineering Is Introduced into Industrial Microbiology (From Roughly 1980), 15
- 1.5 Future Perspectives: Synthetic Microbiology, 18

1.1 Use of Fermentation Procedures Before the Discovery of Microorganisms (Neolithic Era = New Stone Age Until 1850)

The origins of industrial microbiology go back to prehistoric times, as human beings began to learn more about food spoilage, preservation, and storage. Based on their experiences, they developed diverse methods for preserving and refining foods. As we now know, many of these procedures are based on chemical changes brought about by microorganisms. These methods were progressively refined over time and applied in larger scales. Empirical knowledge was initially passed on verbally and later in a written form. Artisans made use of the various fermentation properties of microorganisms, being unaware of the microorganisms involved and the (micro-)biological and biochemical processes taking place. It was not possible to identify the microorganisms or explain the mechanisms of the chemical changes they caused until around 1850. Table 1.1 provides a historical summary of food manufacturing products and procedures as well as microbiological discoveries up to 1850.

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Table 1.1 Microbiological procedures and important discoveries from prehistoric times (Neolithic Era) to 1850.

Period/year	Procedures/products/discoveries
Prior to 4000 BC	Finds from Mesopotamia and from regions south of the Alps prove that flatbread was prepared from a grain pulp and then baked. It is likely that yeast dough is already unknowingly used, as it results in lighter, more flavorful bread.
From 4000 BC	The first sources show that the Sumerians in Mesopotamia and shortly thereafter the Egyptians, use grain pulp for beer production and sugary fruit juices for wine production.
From 3000 BC	In Mesopotamia and Egypt, sour dough bread and sour milk products (cheese) are produced. Vinegar is used as a preservative.
From 2000 BC	In Asia (China, Japan), soybeans are fermented with the help of fungi and bacteria (soy sauce) and rice wine is produced. In Egypt, beer brewing is “refined.” Babylon’s King Hammurabi (1728–1686 BC) issues strict beer laws in the “Code of Hammurabi.”
From 1300	Saltpeter production: throughout Europe, excrements were converted to potassium nitrate needed for gunpowder. It was unknown that microorganisms were the catalysts.
Around 1680	Van Leeuwenhoek discovers and describes bacteria and yeasts by use of self-prepared simple microscopes.
1789	Lavoisier identifies the products of alcoholic fermentation.
1837/1838	Cagniard-Latour, Schwann, and Kützing attribute alcoholic fermentation to living yeasts, which divide themselves by means of budding.

The beginning of the New Stone Age (Neolithic Era, Neolithic Revolution) is marked by the transition from a nomadic lifestyle, centered on collecting wild plants and hunting wild game, to a farming lifestyle based on food production (agriculture and livestock breeding) and storage. In the Fertile Crescent (a crescent-shaped region in the Middle East, which includes parts of what is now Israel, Lebanon, Syria, Turkey, Iraq, and Iran), this radical change in subsistence occurred around 9000 BC. Goats, sheep, and cattle were domesticated, and barley, emmer wheat, and one-grain wheat were bred from wild grasses. The wine grape was also cultivated in this area. In other parts of the world, agriculture and livestock breeding practices were not established until later on, and they were often based on different kinds of livestock and crops.

It can be assumed that as soon as human beings adopted a settled lifestyle in the Fertile Crescent, they began to experiment with producing alcoholic beverages. Here, it is important to distinguish between the production of wine and the production of beer. Wine is made from sugary liquids (fruit juices, but also diluted honey). When fruit gets smashed or fruit juice is left standing, fermentation sets in quite quickly. This is due to the fact that sugar-consuming yeasts are naturally found on the skins of sweet fruits. With beer, on the other hand, the raw material is grain. Here, the fermentable sugar must first be released from the starch.

Grain was originally consumed in pulp form, prepared using water and crushed grains. Later, the pulp was formed into a flatbread and then baked. This advancement allowed the bread to be stored longer and made it easier to be transported. Microorganisms (yeast and lactic acid bacteria) were already being used in ancient Egypt (3000 BC – 395 AD) to prepare porous bread. The ancient Egyptians, also known in antiquity as “bread eaters,” observed that bread became lighter and more easily digestible when the bread dough was left standing for some time before it was baked. The process of making beer by means of fermentation of either liquid bread dough or bread that has been baked and then soaked in water has been in practice since approximately 5000 BC. The first records of beer production are about 5500 years old and come from the Sumerians, who resided in Mesopotamia, which is now Iraq, between 5000 and about 1800 BC. Clay tablets were found that show how grain (barley and emmer wheat) was shucked and ground and how the flour was transformed into a flatbread, which was then baked and used to produce beer (called “kasch” or “bread beer”). Clay vessels were used for the fermentation of the flour cakes, which were baked and then kept moist. The success of the fermentation was dependent on the randomly incorporated microorganisms and the conditions at hand for each given attempt. Honey, cinnamon, and other spices were added to the beer; it was therefore generally sweet and could certainly not be stored for a very long period of time.

In Egypt, beer was also being produced out of bread dough by 2500 BC (“henket”; Figure 1.1). It is unclear, however, whether the Egyptians adopted the brewing trade from the Sumerians or developed it themselves. In 1990, a 3300-year-old brewery belonging to King Echnaton (reign: 1351–1334 BC) was excavated. The findings included intact clay vessels, instruments, and ingredients preserved by the dry heat (malt, grain, and dates). From these findings, researchers were able to deduce that the Egyptians had mastered malt preparation (germination and initiation of enzyme formation) and mashing (enzymatic conversion of starch into sugar under optimized conditions) and that they had used these techniques in making beer.

In 1902, a pillar more than 2 m high and consisting of green diorite was found in Susa, in present-day Iraq, which dates back to the Babylonian King Hammurabi (reign 1728 to 1686 BC). Today, the pillar is on display in the Louvre in Paris (see Figure 1.2). The stone stele depicts the King of Shamash, the Babylonian god of law and justice. The text engraved in the pillar, the so-called “Code of

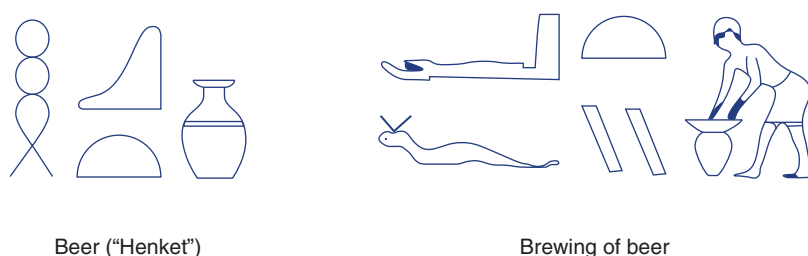


Figure 1.1 More than 3000 year-old Egyptian hieroglyphica, credentials of unconscious use of microorganisms for beer production at ancient times.



Figure 1.2 Pillar with the Code of Hammurabi (a) and cutout of the engraved cuneiform scripts (b).

Hammurabi,” represents the oldest set of laws in the world. These provisions address a variety of matters in both the public and private sector, including a series of exceedingly strict laws concerning the production and trading of beer. These laws stipulate quality requirements and establish the maximum prices allowed for about 20 varieties of beer. The Code also details the punishments to be administered to those who violate the laws of beer production. If a brewer was caught watering down his beer, for instance, he was to be drowned in his own beer barrels. The same punishment applied to innkeepers who allowed patrons to pay for their beer with silver rather than grain. Hammurabi’s laws also dealt with the subject of wine. The Code of Hammurabi refers to wine as one of the earth’s most valuable gifts to be handled with love, respect, and esteem. As was the case with beer, these provisions lay out fixed prices and punishments for violating the laws of wine production and trade.

Similar to beer production, the cultivation of grapevines and fermentation of grape juice to produce wine can also be traced back to the early cultures inhabiting the Fertile Crescent. The complicated production process for wine meant that it was considerably more expensive than beer. Wine therefore remained a privilege for the upper class of society until about 1000 BC. In ancient Egypt and Mesopotamia, wine was also processed into vinegar, which was then used as a seasoning or – in diluted form – a beverage. The first fermentation of soybeans dates back to this time period as well. Sake, or rice wine, also falls into the category of beer-like beverages as it is produced from a grain – rice in this case. Records from what is today China, and later from Japan, prove that sake was being produced in Asia by 2000 BC.

As human beings began adopting a settled lifestyle, a long period ensued in which food fermentation processes were refined, expanded, and passed on to other regions. Until far into the Middle Ages, however, there are no reports of new processes emerging for using microorganisms in daily life.

During the late Middle Ages (from roughly 1300), saltpeter manufacturing took root in Europe, delivering potassium nitrate (KNO_3 , saltpeter) for gunpowder production. Nitrate is formed out of organically bound nitrogen in soil, with the help of nitrifying bacteria. To begin with, surfaces saturated with human and animal excrement served as the starting material for saltpeter production. Later, urine and blood were used as direct nitrogen sources in saltpeter huts and the nitrification was kept in progress in well-ventilated beds (Figure 1.3). Microbial production was abandoned in the nineteenth century, when large natural deposits of saltpeter were discovered in Chile.

A significant development in the empirically proven process of microbial production was the Orléans Process, which was established in the fourteenth century. With this method, vinegar was produced in large, open vessels in warm rooms. The large surface area of contact with the air provided the acetic acid bacteria, which were collecting on the surface, with ample amounts of oxygen. It was already evident to scientists at this time that adequate ventilation increased the effectiveness of the process. Since the nineteenth century, acetic acid has been produced either via the “round pump procedure” (a variation on rapid vinegar manufacturing in a trickle bed; Figure 1.4) or the submersion procedure, i.e. in a liquid culture, made from wine, brandy, and fermented fruit, with intensive ventilation.

With the commencement of the modern age (approximately 1500) and the breakthrough of the natural sciences in the seventeenth century, scientists began to examine natural phenomena systematically, by means of observation, experimentation, and measurement. Academies were established with the specific purpose of providing a forum to present and discuss the results of this natural research. The findings were also published in scientific journals. The emerging modern sciences of physics and chemistry provided the tools, along with

Figure 1.3 Saltpeter manufacture toward the end of the middle ages. Heaps of earth, soaked with animal and human excrements and blood were aerated with picks and rakes. The ammonium (and ammonia, respectively) released from the nitrogen-containing organic matter was oxidized by aerobic nitrifying bacteria to nitrate (NO_3^-). Nitrate then was leached out as potassium or sodium nitrate. The depleted soil was recovered and recycled. Source: Reproduced from “Berg- und Probirbuch” by Lazarus Erker (1574).



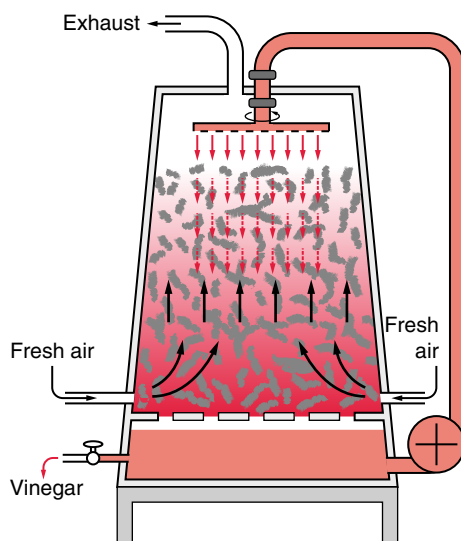


Figure 1.4 Acetic acid production from nineteenth century on. The vessel is filled with beechwood chips, grown over with acetic acid bacteria. 6 – 10% alcohol was trickled from the top of the vessel, at the outlet (bottom) 4 – 10% acetic acid was collected. Aeration was provided by air counterflow to supply the microorganisms with oxygen.

numerous technical inventions and innovations (e.g. lens grinding technology), with which the production processes for bread, wine, beer, and vinegar were scientifically analyzed in the eighteenth and nineteenth centuries. From today's perspective, the works of Antonie van Leeuwenhoek (1632–1723) mark the beginning of microbiology. He was the first to observe various microorganisms, including bacteria, with the aid of a microscope equipped with only one lens. He described the “animalcules,” as he referred to them, in great detail. Although the process of fermentation had already been in practice for a long time, it was not yet recognized that these microorganisms played a significant role.

Because of its huge practical relevance, alcoholic fermentation became the subject of numerous studies. At the beginning of the nineteenth century, scientists were able to describe the process in terms of both phenomenology and quantity. Antoine de Lavoisier (1742–1794) focused on sugar fermentation, identifying two products: alcohol and carbon dioxide. Joseph Gay-Lussac (1778–1850) identified the quantity relation of sugar (two mol each of alcohol and CO_2 per mol of sugar). Charles Cagniard-Latour (1777–1859), Theodor Schwann (1810–1882), and Friedrich Kützing (1807–1893), all supporters of the vitalist philosophy of the 1830s, collected proof that fermentation is sustained by living microorganisms. Independently of each other, they all arrived at the conclusion that yeast was responsible for alcoholic fermentation. One question, however, remained under dispute: did these living microorganisms appear spontaneously by means of abiogenesis or was an inoculum of unknown nature necessary to initiate the process? In opposition to the ideas of the vitalists, chemists Jöns Berzelius (1779–1848), Friedrich Wöhler (1800–1882), and Justus von Liebig (1803–1873) were of the opinion that fermentation involved purely chemical processes of decomposition. It was not until the 1850s that Pasteur's findings were able to settle this debate.

1.2 Investigation of Microorganisms and Beginning of Industrial Microbiology (1850 Until 1940)

Louis Pasteur (1822–1895, Figure 1.5) proved through experimentation that the fermentation processes common during his time were invariably linked to the specific microorganisms present and that the observed chemical changes were based on the physiological abilities of these microorganisms. Between 1856 and 1875, Pasteur studied the life cycle of yeasts and compared how they processed sugar in the presence and absence of oxygen. He also looked at bacterial fermentation (lactic acid and butyric acid fermentation) and the microorganisms responsible for it. He demonstrated that “failed fermentations,” i.e. those that did not result in the formation of the desired product, could be traced back to contamination with other microorganisms (Figure 1.6). With the introduction of sterilization techniques (pasteurization), Pasteur established the necessary conditions for breeding microbial pure cultures.

The founding of modern microbiology is accredited to both Robert Koch (1843–1910), who demonstrated that infectious diseases such as anthrax, typhus, and cholera were caused by bacterial pathogens, and Louis Pasteur. Industrial microbiology also has its roots in Pasteur’s research from 1850. Industrial microbiology refers to the section of microbiology that relates to the microorganisms used by humans to modify and produce substances, as well as the industrial procedures developed for this purpose. Table 1.2 provides an overview of the microbiological procedures between 1850 and 1940, as well as the relevant scientific discoveries in the fields of microbiology and biochemistry from the same period.

Toward the end of the nineteenth century, numerous public and private research institutes sprouted up throughout Europe, focusing on the use of fermentation in food production, food processing, quality control, hygiene, and

Figure 1.5 Portrait of Louis Pasteur at around 1885 (Painting by Albert Edelfeldt 1854–1905).



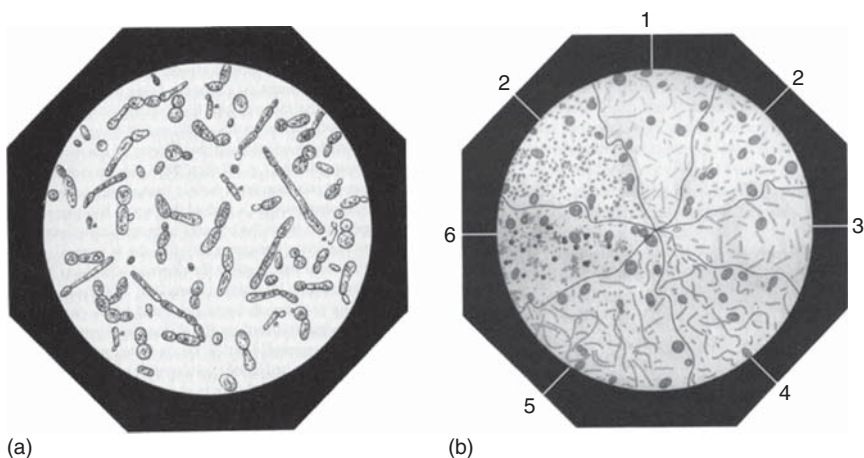


Figure 1.6 Yeast at the beginning of fermentation (a) and from “failed fermentations” (b). Drawing prepared by Pasteur (1876).

Table 1.2 Industrial applications of microbiological procedures and scientific discoveries from 1850 to 1940.

Period/year	Procedures/products/discoveries
1857–1877	Pasteur describes alcoholic fermentation, lactic acid, and butyric acid fermentation and explains the processes of wine and beer production. He introduces sterilization via “pasteurization” and other sterilization techniques for handling microorganisms.
1867	The “Vienna Process” is used for large-scale production of baker’s yeast.
1870	Koch develops procedures for cultivating microorganisms and founds medical microbiology.
1877	Kühne introduces the term “enzyme” for temperature-sensitive, active ferments from living cells.
From 1881	Lactic acid is produced with the help of lactic acid bacteria.
1894	Fischer proves the specificity and stereoselectivity of enzymes.
1896	Buchner proves the existence of fermentation enzymes in yeast cell extract.
From approximately 1900	Municipal wastewater treatment plants are established in larger cities.
1915	Clostridia are used for large-scale production of acetone and butanol. Glycerin is produced with the help of yeasts from molasses.
From 1923	<i>Aspergillus niger</i> is used for large-scale production of citric acid.
1928	Fleming discovers penicillin and its effect on bacteria.
1939–1941	Penicillin is isolated and purified.