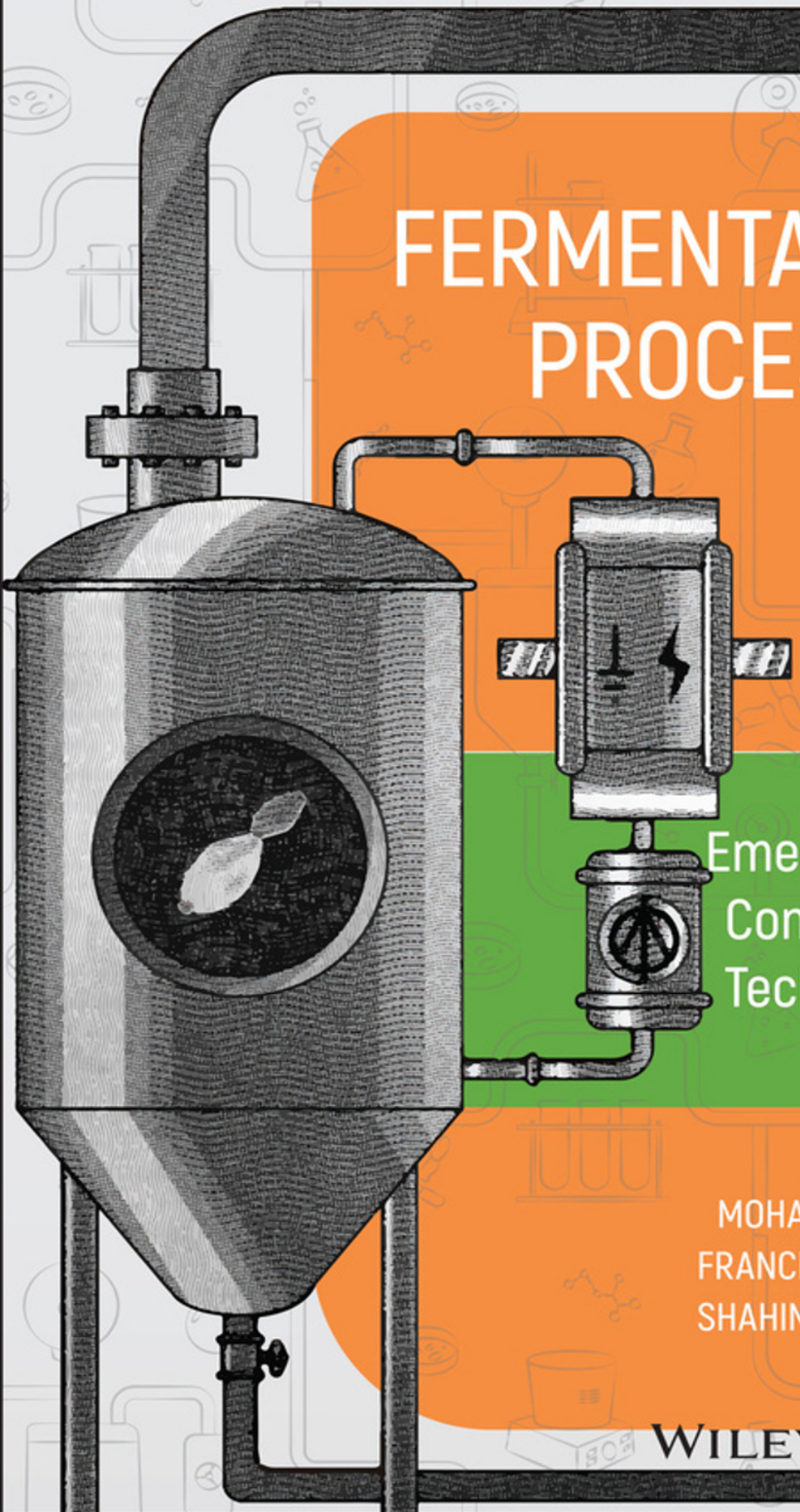
FERMENTATION PROCESSES



Emerging and Conventional Technologies

Edited by
MOHAMED KOUBAA
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WILEY Blackwell

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This edition first published 2021

Registered Offices
John Wiley & Sons, Inc., 111 River Street, Hoboken, NJ 07030, USA
John Wiley & Sons Ltd, The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ, UK

Editorial Office
The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ, UK

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Library of Congress Cataloging-in-Publication Data

Names: Koubaa, Mohamed, editor. | Barba, Francisco (Francisco J.), editor. | Roohinejad, Shahin, editor.
Title: Fermentation processes : emerging and conventional technologies / edited by Mohamed Koubaa, Francisco J. Barba, Shahin Roohinejad.
Description: Hoboken, NJ : Wiley-Blackwell, [2020] | Includes bibliographical references and index.
Identifiers: LCCN 2020031691 (print) | LCCN 2020031692 (ebook) | ISBN 9781119505853 (cloth) | ISBN 9781119505846 (adobe pdf) | ISBN 9781119505839 (epub)
Subjects: LCSH: Fermentation.
Classification: LCC QR151 .F449 2020 (print) | LCC QR151 (ebook) | DDC 572/.49–dc23
LC record available at https://lccn.loc.gov/2020031691
LC ebook record available at https://lccn.loc.gov/2020031692

Cover Design: Wiley
Cover Image: Courtesy of Mohamed Messaoud

Set in 9.5/12.5pt STIXTwoText by SPi Global, Pondicherry, India

10 9 8 7 6 5 4 3 2 1

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Preface

Bioprocesses find many traditional or new applications in the agri-food, chemical, pharmaceutical, and environmental industries. Enhancing these processes for better production of microbial biomass and/or products has interested many scientists in the last two decades. One of the strategies consists of changing the medium composition or the fermentation parameters (e.g. oxygenation, agitation, temperature, etc.), most of the time via a design of experiment approach. Besides, some emerging technologies (e.g. pulsed electric fields, ultrasounds, high hydrostatic pressure, microwaves, etc.) have shown their efficiency to enhance the fermentation processes. These technologies when applied at high intensities cause cell disintegration and find their applications in bioprocesses, for example, to produce sugar monomers from lignocellulosic biomass. Nonetheless, their application at sublethal levels may induce stress of microorganisms and affect the microbial growth and the formation of the products during fermentation. The beneficial effects of microbial stimulation by emerging technologies include mainly the shortening of the fermentation time, the acceleration of the substrate consumption, and the increase of the microbial biomass.

This book covers the principles of conventional fermentation processes, the major microorganisms used in bioprocesses, their implementation in industrial fermentation processes, the medium condition changes, and the use of emerging technologies for enhancing the fermentation processes. Besides, the mechanisms of action of the above-mentioned emerging technologies are discussed.

This book is designed to assist scientists working on fermentation processes as well as those working in the food, nutraceutical, pharmaceutical, and beverage industries. The topics covered in this book are suitable for teaching in courses such as bioprocess technology, microbiology, new product development, and food processing.

We gratefully acknowledge the contribution of colleagues from all around the world, the cover designer Mohamed Messaoud, and the professional assistance provided by the staff of Wiley.

Mohamed Koubaa, Francisco J. Barba, and Shahin Roohinejad

1

Introduction to Conventional Fermentation Processes

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1.1 Bioprocesses

Bioprocesses represent all the methods and techniques that use microbial, plant, or animal cells or their components (e.g. enzymes, proteins, genes, etc.) for the production of goods and services (Sindhu et al. 2017). Bioprocess technology is, in fact, an extension of the ancestral techniques used at the time to develop useful products (Kalaichelvan and Pandi 2019). Nowadays, microbial cells are not only used in common processes, such as for the production of alcoholic beverages (e.g. wine, beer, etc.) or dairy products (e.g. yogurt, cheese, etc.), but also to produce a wide diversity of complex molecules. In this sense, bioprocesses find many traditional or new applications in the following industries:

- Agri-food industry: production of animal proteins, amino acids, fermented foods and beverages, vitamins, enzymes, etc.
- Chemical industry: production of organic acids, ethanol, solvents, polymers, biogas, etc.
- Pharmaceutical industry: production of antibodies, vaccines, hormones, plasmids, steroids, etc.
- Environmental industry: decontamination of wastewater, air, and soil; development of agricultural and industrial by-products, etc.

In this respect, bioprocesses are exploited in three specific fields: fermentation processes, animal and plant cell cultures, and environmental bioprocesses. This chapter will mainly focus on conventional fermentation processes.

Most of the methods and techniques used in bioprocesses are based on fermentation technology. This is not surprising since the first ancestral processes were based on microbial fermentation. For most people, fermentation simply refers to the production of alcohol (beer and wine) or the deterioration of food by microorganisms (curd). Nevertheless, the word fermentation takes on a broader common industrial meaning. It is any process for producing a substance or biomass of cells on a large scale by using the culture of a microorganism, in aerobic or anaerobic conditions.

Fermentation Processes: Emerging and Conventional Technologies, First Edition.
Edited by Mohamed Koubaa, Francisco J. Barba, and Shahin Roohinejad.

To be able to carry out these fermentations, it is imperative to cultivate microorganisms in tanks equipped with a certain number of more or less sophisticated systems; these tanks are called *fermenters* or *bioreactors*. Their role is to provide a controlled environment for optimal growth of microbial cells throughout the culture by constantly stirring the medium, infusing sterile air – in the case of aerobic fermentation – and controlling the temperature and pH of the fermentation broth. By using these tanks, contamination by other microorganisms is avoided by constantly maintaining asepsis conditions.

The first modern fermenters were designed in the 1950s to support the industrial production of penicillin and other newly discovered antibiotics. Since then, they have been able to control several other types of crops and to substantially increase the quantity of products marketed in each of the three fields of application of industrial bioprocesses mentioned above. Six major groups of products could then be obtained by fermentative processes, namely the production of (i) microbial biomass, (ii) microbial metabolites, (iii) microbial enzymes, (iv) recombinant proteins, (v) microbial plasmids, and (vi) bioconversion.

1.1.1 Production of Microbial Biomass

Commercial production of microbial biomass can be divided into two major processes: the production of viable microorganisms used primarily for fermentative applications (Vitorino and Bessa 2017) and the production of microbial cells, usually dead, that can serve as protein-rich supplements (Matassa et al. 2016).

In the first case, we can cite several examples: the production of bakery yeasts for the production of bread, the production of yeasts to perform alcoholic fermentation (e.g. beers, wines, spirits, etc.), and the production of lactic acid bacteria for the manufacturing of cheese, yogurt, fermented meats (i.e. sausages), or fermented vegetables (e.g. sauerkraut, marinated pickles, etc.). Some food supplements composed of live lactic acid bacteria, also called *probiotics*, are produced by fermentation. They can be defined as live microorganisms, and the adequate amounts of them supply a health benefit to the host (Otles and Ozyurt 2019). Their role is to exert a beneficial effect by improving the quality of the intestinal flora. These microorganisms are usually supplied as a lyophilized powder in hermetically sealed sterile bags or containers. Generally, the name of ferments is given to microorganisms that serve to start a fermentation process (Koutinas 2017). Some microbial strains such as the bacterium *Bacillus thuringiensis*, whose spores produce a very effective toxin against pest larvae (biological insecticide), are also grown.

In the second case, it is a question of producing microbial biomass to exploit the nutritional potential of the proteins that it produces (Matassa et al. 2016). This biomass is incorporated into prepared foods to increase their protein content without significant fat intake, which improves their nutritional quality. The yeast *Candida utilis* is mostly used as a dietary supplement because of its exceptionally high protein content (50–55% of dry weight). This yeast can be used as a valuable raw material to produce various preparations enriched with valuable bioelements (e.g. selenium, magnesium, etc.). The use of such preparations in the human diet provides an interesting alternative to classical, pharmacological supplementation and prevents deficits of important elements, while their addition to feedstock significantly improves the results of animal production (Kieliszek et al. 2017).

1.1.2 Production of Microbial Metabolites

Microorganisms are characterized by a variety of metabolic pathways that allow them to synthesize a host of organic compounds, called *metabolites*, many of which are potentially useful. In this type of fermentation, it is sought to produce by the metabolic activity of a microorganism a substance that is too complex to be chemically synthesized at a reasonable cost (Jeandet et al. 2013).

Metabolites are generally divided into two categories depending on whether they are produced in relation to growth or not. The first ones are called *primary metabolites* and are produced in large enough quantities during the exponential growth phase by essential metabolic pathways that are common to many microorganisms. Several primary metabolites produced by fermentation are the residues of the energetic catabolism of microorganisms. These are mainly alcohols, solvents, and organic acids used in food or the chemical industry. Others are derived from cellular anabolism. These are mostly amino acids and vitamins produced for food or pharmaceutical purposes (Sanchez and Demain 2009).

The second ones are called *secondary metabolites* and are usually produced in small quantities during the stationary phase, and sometimes even during the decline phase, by particular metabolic pathways that are exclusive to a few species and usually give them a survival advantage in the wild. Secondary metabolites form an extremely heterogeneous group of compounds, derived from anabolism, whose main uses are in pharmaceuticals (e.g. antibiotics, growth factors, enzyme inhibitors, etc.). Although not essential for microbial growth, secondary metabolites are very important for health, nutrition, and the economics of our societies (Bérdy 2005).

1.1.3 Production of Microbial Enzymes

Enzymes are proteins that act as catalysts in the biochemical reactions of metabolism (Cooper 2000). When purified, they make it possible to carry out these reactions under controlled conditions. They can be produced from animal, plant, and microbial cells. Nevertheless, microbial enzymes stand out in large quantities and often at low cost by fermentation processes (Raveendran et al. 2018). Most enzymes produced by fermentation are associated with primary metabolism and are primarily used in the agri-food industry to process many foods; however, more and more enzymes associated with secondary metabolism are produced for pharmaceutical purposes.

1.1.4 Production of Recombinant Proteins

Nowadays, the advances in genetic engineering techniques allow introducing genes from animal and plant cells into microorganisms. These genetically modified cells will produce the so-called *recombinant proteins* because their synthesis relies on the recombination of microbial DNA with foreign DNA (Griffiths et al. 2000). Several microbial species have been selected as hosts for such productions (e.g. *Escherichia coli*, *Saccharomyces cerevisiae*, *Yarrowia lipolytica*, etc.). The research and development effort required to develop such strains is, however, colossal, and the recombinant proteins produced by fermentation are therefore almost all dedicated to pharmaceutical uses (e.g. human insulin, human growth hormone, etc.).

1.1.5 Production of Microbial Plasmids

There has been a marked interest in the production of plasmids by fermentation (Carnes and Williams 2014; Carnes et al. 2006). Plasmids are self-replicating extrachromosomal DNA molecules found in Gram-negative and Gram-positive bacteria as well as in some yeast and other fungi (Actis et al. 1999). To produce appreciable amounts, it is first necessary to introduce a plasmid of interest into microbial cells such as *E. coli* or *S. cerevisiae*. Subsequently, culturing these microorganisms in a bioreactor develops significant biomass. The plasmids then replicate independently in the new cells produced, and at the end of the fermentation, they are recovered and purified (Carnes and Williams 2014).

First developed in research programs in molecular biology and genetic engineering, plasmids are todays used in new applications of high technology such as in gene therapy (Sousa et al. 2009). Indeed, plasmids obtained by fermentation may contain therapeutic genes derived from the recombination of DNA that will be used to produce previously defective or nonexistent proteins to correct a genetic abnormality in a human organ. These plasmids are inserted into synthetic vectors and injected into the target cells of the affected organ by using particular techniques.

1.1.6 Bioconversion

A microbial cell can be used to convert or transform any substance into a value-added product (Garlapati et al. 2016), a bit like conventional conversions of grape must into wine, wine into vinegar, or milk into yogurt. These transformations contribute to producing very valuable compounds in the pharmaceutical industry, such as antibiotics, vitamins, steroids, and prostaglandins. These conversions are based on the biochemical reactions of microorganisms used such as hydroxylation, dehydroxylation, O-methylation, O-demethylation, glycosylation, deglycosylation, dehydrogenation, hydrogenation, C-ring cleavage of the benzo-γ-pyrone system, cyclization, and carbonyl reduction (Cao et al. 2015).

The bioconversion of compounds by microorganisms is much more advantageous than the conventional chemical transformation because the reactions can occur at low temperature and low pressure and without the addition of catalysts. To understand the different steps required to carry out a fermentation process, it is of paramount importance to understand first the microbial metabolism and how a substrate could be transformed by a microorganism to maintain its growth and the production of targeted compounds.

1.2 Energetic Metabolism

Microorganisms need energy and carbon for their metabolism and are classified as autotrophs and heterotrophs depending on the sources of energy and nutrients (Misra 2011). There are only two sources of energy metabolizable by the cells: light energy captured during photosynthesis and energy from the oxidation of organic and inorganic molecules. Nevertheless, cells can be categorized into nutritional categories depending on how they meet these needs. In bioprocesses, three of these classes are potentially exploited on an industrial scale: phototrophs, chemolithotrophs, and chemoorganotrophs (Jurtshuk 1996).

Phototrophs use light as a source of energy and carbon dioxide (CO_2) as a source of carbon. They include photosynthetic bacteria (cyanobacteria), algae, and green plants. Chemolithotrophs rely on electrons from reduced inorganic compounds, such as iron, nitrogen, or sulfur, as a source of energy (oxidation of the inorganic material) and CO_2 as a carbon source. They include several bacterial species that are primarily used in environmental bioprocesses, particularly in aerobic wastewater treatment. The chemoorganotrophs use, as a source of energy, electrons from hydrogen atoms that are part of organic compounds (oxidation of organic matter), which also serve as a carbon source. They are the ones who enter the vast majority of bioprocesses, particularly in fermentation processes (bacteria, yeasts, and molds) and in animal cell cultures. The next sections will discuss the metabolism of this class of cells.

All living organisms need energy to grow and reproduce. In chemoorganotrophs, this energy is obtained during the degradation of organic compounds. Mainly, carbohydrates, lipids, and proteins are oxidized to release the chemical energy they contain. This energy will be then transferred to adenosine diphosphate (ADP) and inorganic phosphate (P_i) molecules before being stored in the form of adenosine triphosphate (ATP) (Figure 1.1). ADP and ATP correspond to molecules of adenosine monophosphate (AMP) plus one and two high-energy phosphates (AMP ~ P and AMP ~ P ~ P, respectively). The energy is stored in these compounds as high-energy phosphate bonds. All living cells must maintain steady-state biochemical reactions for the formation and use of such high-energy compounds.

Biochemical assimilation (anabolism) and dissimilation (catabolism) of nutrients by a chemoorganotroph are mediated by a network of enzymatic reactions perfectly synchronized and regulated. Anabolic pathways consist mainly of the reductive processes that lead to producing new cellular molecules, while catabolic pathways include the oxidative processes involved in removing electrons from substrates or intermediates that are used to generate energy.

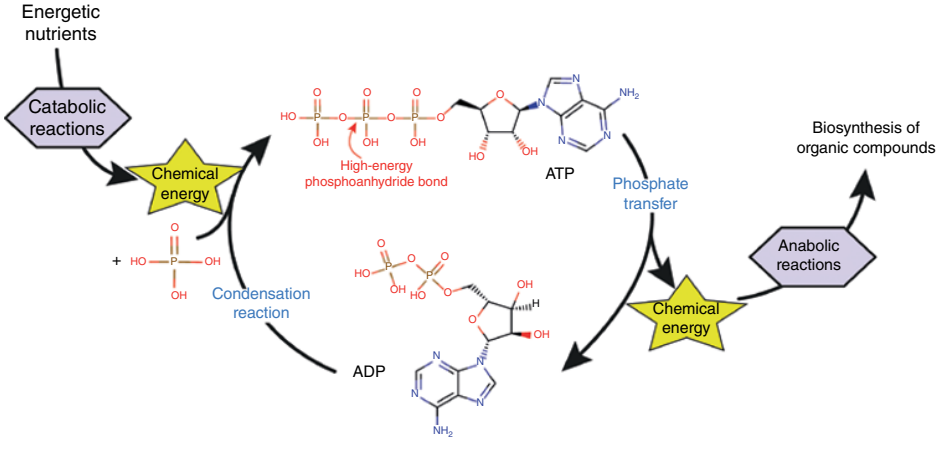

Figure 1.1 Energy coupling and the role of ATP in microbial metabolism. ADP: adenosine diphosphate, ATP: adenosine triphosphate

1.2.1 Energy Transfer and Redox Reactions

The energy released by the catabolic reactions is associated with the electrons of molecules that are degraded during these reactions. This energy is transferred to a molecule of ADP to form ATP. More specifically, a phosphate group is added to the ADP molecule, with an energy investment, to form an ATP molecule (Figure 1.1). The energy contained in organic molecules cannot be released all at once; otherwise, it would be practically all lost in the form of heat, as during combustion. It must, therefore, be gradually transferred to the ATP molecules via the cascades of redox reactions.

First, the energetic nutrients are oxidized, which allow them to behave as electron donors (e^-). During this reaction, two electrons and two protons (H^+) are transferred to a coenzyme known as nicotinamide adenine dinucleotide (NAD^+) to be reduced in the form of NADH + H^+. A part of the chemical energy contained in nutrients is then transferred to the NAD^+ by the electrons, according to the reduction reaction in Eq. (1.1).

$$NAD^+ + 2H^+ + 2e^- \rightarrow NADH + H^+ \tag{1.1}$$

Overall, each time an organic molecule is oxidized (the loss of electrons and H^+ ions), there is simultaneously a reduction of NAD^+ taking place. This is why we talk about "redox reactions." The newly formed NADH will then undergo oxidation, in turn, to release stored energy (Eq. (1.2)), which will eventually be transferred to ATP molecules by various chemical processes.

$$NADH + H^+ \rightarrow NAD^+ + 2H^+ + 2e^- \tag{1.2}$$

At the end of the energy transfer process, the released electrons and protons (H^+) must be picked up by a final acceptor. This acceptor will vary according to the preferred catabolic pathway: aerobic respiration, anaerobic respiration, or fermentation.

1.2.2 Aerobic Respiration

In this catabolic pathway, the final electron acceptor is molecular oxygen (O_2), and the organisms using it are, therefore, dependent on air for their survival. It takes place in three stages, each involving a series of chemical reactions: glycolysis, citric acid cycle, and electron transport chain.

1.2.2.1 Glycolysis Pathway

In the glycolytic pathway, occurring in all tissues, glucose is oxidized to provide energy (i.e. ATP) and intermediates for other cellular metabolic pathways. Glycolysis is at the hub of carbohydrate metabolism where glucose is converted to pyruvate following a series of 10 enzymatic reactions (Figure 1.2). This metabolic pathway is known as *aerobic glycolysis*, as the reoxidation of the NADH formed during the oxidation of glyceraldehyde 3-phosphate requires O_2. Aerobic glycolysis sets the stage for the oxidative decarboxylation of pyruvate to acetyl coenzyme A (acetyl-CoA), a major fuel of the tricarboxylic acid cycle (Ferrier 2017).

During glycolysis, two ATP molecules are initially consumed to phosphorylate glucose, which thus receives an essential energy supply to continue the catabolic pathway.

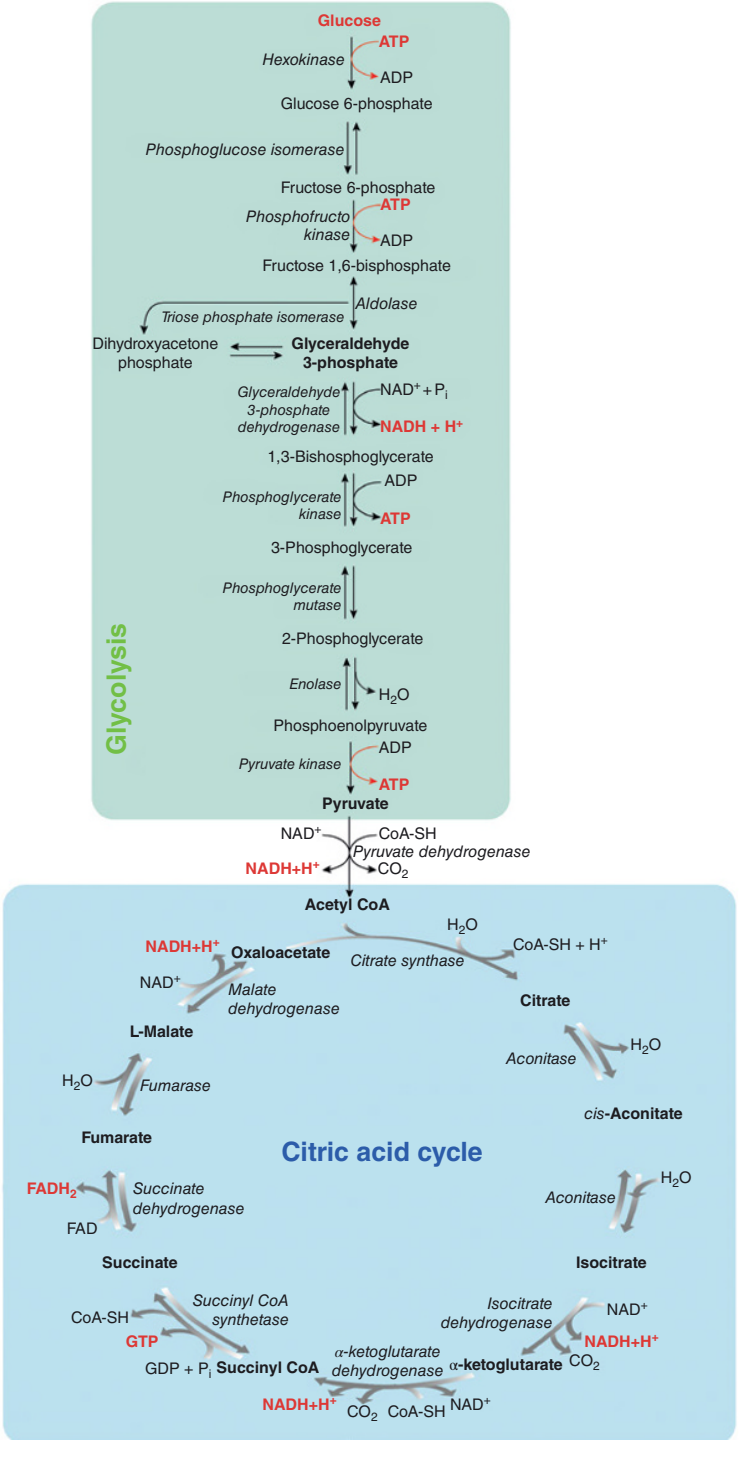

Figure 1.2 Glycolysis and citric acid cycle pathways.

Subsequently, two molecules of NAD^+ oxidize phosphorylated sugar and are reduced to $NADH + H^+$. During these reactions, which will lead to the synthesis of two molecules of pyruvic acid from a glucose molecule, a part of the energy released allows the direct synthesis of four molecules of ATP. Considering that two molecules of ATP are consumed and four are produced, glycolysis presents a net balance of two molecules of ATP for each molecule of oxidized glucose.

1.2.2.2 Citric Acid Cycle

Pyruvic acid is first decarboxylated (release of CO_2) and then oxidized by a molecule of NAD^+ (reduced to $NADH + H^+$) to form the two carbon molecules of acetyl-CoA. As two molecules of pyruvic acid are produced from a glucose molecule, two molecules of CO_2, $NADH + H^+$, and acetyl-CoA are produced. In prokaryotic cells (e.g. bacteria), the citric acid cycle (or Krebs cycle) occurs in the cytosol, while in eukaryotic organisms (e.g. yeast), it takes place in the mitochondrial matrix.

Overall, two molecules of CO_2 are produced and three molecules of NAD^+ are reduced to $NADH + H^+$ during a turn of the citric acid cycle. Besides, two electrons and two protons released from pyruvic acid are used to reduce a molecule of flavin adenine dinucleotide (FAD) into $FADH_2$, similar to that of NAD^+. Finally, there is a release of chemical energy, which allows the synthesis of an ATP molecule. To summarize, for each molecule of glucose oxidized, four molecules of CO_2, six molecules of $NADH + H^+$, two molecules of $FADH_2$, and two molecules of ATP are generated.

1.2.2.3 Electron Transport Chain and Oxidative Phosphorylation

In a process called *oxidative phosphorylation*, the energy stored in the reduced coenzymes (NADH and $FADH_2$) is released to produce ATP molecules. During this process, each coenzyme is oxidized and gives its two energy-rich electrons to an electron transport chain, or respiratory chain, located in the plasma membrane in prokaryotes and the inner membrane of mitochondria in eukaryotes. The chain consists of complex organic acceptors that transfer electrons through a series of redox reactions (Figure 1.3). As the electrons are transferred in this chain, the energy that they contain is released by stages and is used to activate chemiosmosis. This creates a gradient of H^+ protons whose energy is reinvested, through the enzyme ATP synthetase, in the production of three molecules of ATP per molecule of oxidized NADH and two molecules of ATP per molecule of oxidized $FADH_2$.

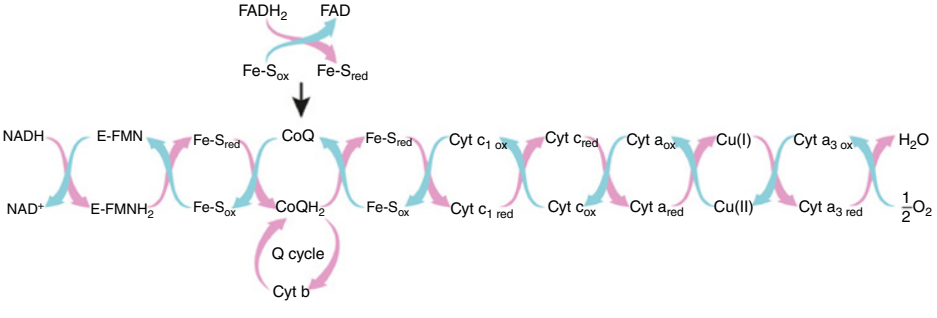

Figure 1.3 The electron transport chain showing the respiratory complexes.