Chapter 2
Chemicals from Metabolic Pathways

For the past 80–90 years petroleum and natural gas have served as raw materials for the majority of the finished products of our daily lives. After World War II these raw materials decisively substituted coal, and they have been the foundation of an enormous increase in material wealth and welfare throughout the World.

A few basic raw materials, petroleum, natural gas, +S from oil or natural gas, and $O_2 + N_2$ from air, generate first primary (or platform) chemicals, next secondary (commodity) chemicals, then intermediates, and finally the finished products of virtually all industries that provide consumer goods.

The aromatic fraction of petroleum delivers *platform chemicals*, such as propylene, ethyl benzene, cyclohexane, and cumene. These are used to synthesize *secondary chemicals* such as styrene, adipic acid, caprolactam, acetone, and terephthalic acid; and these in turn are raw materials for the *polymer industry* that produces textiles, packaging for food products, appliances, and communication equipment (pencils, inks, computer casings, optical fiber). The aliphatic fraction of petroleum contains other platform chemicals (iso-butylene, butadiene, etc.) that supplement the aromatic fraction to produce intermediates for the abovementioned industries. The *transportation sector* directly receives consumer goods from C_5 to C_{14} aliphatic compounds, while products such as antifreeze and gasoline additives are derived by chemical processing of petroleum platform chemicals.

Natural gas and cracked naphtha deliver other platform chemicals (ethylene, propylene, CO/H_2, NH_3) for the *solvent industry* (methanol, ethanol, ethylene glycol, etc.), for the polymer industry (formaldehyde, polyethylene, polypropylene, PVC), and for *fertilizers*.

Together the platform chemicals from petroleum and natural gas are combined to give most of the products of the *health and hygiene industry*, the *housing industry*, and the exploding *recreation industry*. Except for the input from the mining and the forest industry, and the inorganic platform chemicals (such as cement and phosphates) it is, indeed, hard to imagine the modern world without the crucial input from oil and gas.

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It is, however, known to every observant citizen that we have to prepare for a different world, a future in which petroleum can only be used as a raw material for a few platform chemicals (mostly aromatics), and where even natural gas will run scarce. The use of the calorific value of oil and gas for heating and cooling, or for producing electricity will need to be sharply reduced, if not banned. The transportation sector will have to find solutions where gasoline is substituted by other means of vehicle propulsion.

This is the challenge for all modern societies, and at the same time it is likely to be the most brilliant opportunity for science in the 21st century.

In the following, the special role of the **biorefinery** in this quest for new solutions to the raw material challenge will be outlined.

2.1 The Biorefinery

By analogy with the role of the oil refinery, where the raw materials, such as petroleum, natural gas, S, O_2 and N_2, are converted in a series of chemical steps to consumer goods, the role of the biorefinery is to convert raw materials originating in the agricultural sector into *the same* final consumer goods.

Food and feed products are, and will remain the primary products of agriculture. Nevertheless, for many years to come a certain fraction of the primary products from agriculture, such as sugar from beets or cane and starch from grain, potatoes and other storage compounds of plants will be processed into chemicals and transportation fuels – a striking example is the conversion of almost 40% of the enormous cane sugar production of Brazil into bioethanol. The main driver for this development has previously been surplus production of primary agricultural products in the Americas and in Europe. This has resulted in very costly programs where farmers were sometimes subsidized to reduce production. As long as in some parts of the world the production capacity for primary agricultural products remains much higher than the market can absorb, nonfood utilization, also for biofuels, will have a considerable and positive socioeconomic benefit.

The future lies in additional utilization of the huge quantities of waste products from agriculture and forestry – straw, corn cobs, sugarcane bagasse, and forest industry residues – supplemented by household and other waste products of modern society. Typically, one ton of straw is produced per ton of grain.

Hence, *cellulose*, *hemicelluloses, and lignin*, collectively known as *lignocellulosic biomass*, will become an important raw material for the biorefinery.

Cellulose is an unbranched, crystalline microfibril constructed from 7,000 to 15,000 α-D glucose molecules. Embedded in hemicelluloses it acts as reinforcement like iron rods in concrete to give strength to cell walls. Hemicelluloses are composite, branched polysaccharides, polymerized from 500 to 3,000 D-C_5 sugar units to an amorphous structure of xylan, arabinoxylan, glucomannan, and others. In contrast to cellulose, hemicelluloses are relatively easily hydrolyzed by acid treatment or by enzymes to form C_5 monomers, with the C_5 sugar D-xylose being the most abundant pentose derived from many materials.

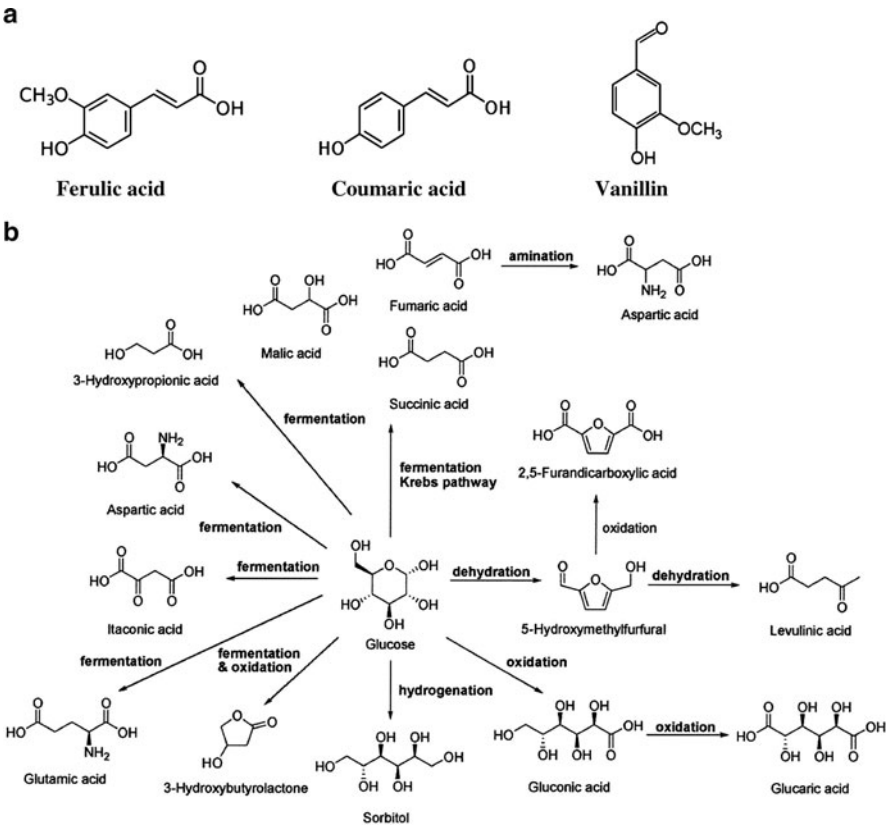


Fig. 2.1 (**a**) Chemicals from lignin. The three compounds all have antioxidant properties that may have possible health effects. They can be used to synthesize other flavor compounds and pharmaceuticals. (**b**) The 12 building blocks derived from the sugars that result by hydrolysis and saccharification of lignocellulusic biomass, exemplified by glucose (Murzin and Leino (2008))

Lignin is an amorphous hydrophobic polymer into which the cellulose microfibrils are embedded. It adds chemical resistance to the network of hemicelluloses and cellulose, but has a number of other functions such as regulating the flow of liquid in the living plant. The building blocks of the very complex lignin polymer change from one lignocellulosic biomass to another, but generally they are of aromatic character, dominated by derivatives of phenyl propane. Figure 2.1a shows three commercially valuable products that can be obtained by hydrolysis of lignin.

2.1.1 Ethanol Production

Given the massive political interest in substituting part of the petroleum-based transportation fuels with fuels derived from cheap and plentiful renewable

resources – about 10^{10} tons/year of lignocellulosic biomass appears to be relatively readily available worldwide – the subject of ethanol production from biomass has been treated in numerous journal papers, books, and pamphlets published by national and international organizations. The three recent reviews (Lin and Tanaka 2006; Hahn-Hägerdahl et al. 2006; Lynd et al. 2008) give short, but well-balanced accounts of the prospects of ethanol production, technological as well as economic, from Japan, Sweden, and the USA.

In March 2009, the price of natural gas was 4.0 US \$/10^6 BTU (=3.7 US \$/10^9 J, or 0.19 US \$/kg based on a heating value of 22200 BTU/lb (ethane)), but the prices fluctuate wildly – the average for 2008 was 0.40 US \$/kg – while the price of cellulosic crops ("energy crops") was about 0.050 US \$ kg or about 2.3 US \$ 10^9 J based on a heat of combustion of 0.023×10^9 J kg^{-1}.

Thus, from the point of view of calorific value, energy crops, even at a much lower cost per J, might seem to have little to offer compared with the user friendly natural gas. Neither is an ethanol price (March 2009) of 1.72 US \$/gallon (0.63 US \$/kg or 21 US \$/10^9 J) very encouraging compared with the relative heating value of either natural gas or energy crops, especially since a yield of ethanol by total conversion of the sugars in lignocellulosic biomass is at most 0.5 kg ethanol/kg biomass. Therefore, when considering ethanol production from biomass one must definitely put a value on the residue in order to get a reasonable economy in the overall process. As will be discussed shortly this is done for all the integrated biomass-to-ethanol processes that are currently examined.

As could be expected, in the heated debate on the socioeconomic benefits of converting biomass to ethanol, the fiscal incentives that subsidize bioethanol are said to be a major incentive for the industry. The raw material is usually considered as waste with no value – or a negative value when considering the environmental costs of burning it on site. Still, the net cost of collecting agricultural waste and transporting it to a central processing facility, added to the capital and running costs of the industrial installation must, at the present, seem discouraging, unless some incentive is given to the entrepreneurs.

For example, using agricultural waste to produce a *liquid fuel* by pyrolysis in an oven directly attached to the equipment that harvests the crop is an appealing, low cost alternative. When the pyrolysis is controlled at about 500°C, and the vapors from the pyrolysis oven are rapidly quenched, 70–80% of the heating value of straw or bagasse can be retrieved. The raw *bio-oil* has a low sulfur content, but it contains about 30% water, is highly oxygenated (10–40% O), and is much too acidic. New research (Bridgewater and Peacocke 2000; Holmgren et al. 2008; Bech 2008) is building up a technology for refining the raw pyrolysis oil, and in numerous patents (e.g., Bech and Dam-Johansen 2006) mobile units for collecting and pyrolysing the biomass are described. According to Holmgren et al. (2008) the raw pyrolysis oil can be refined in a one- or two-step hydrogenation process into a perfectly suitable naphtha or diesel range fuel. The carbon recovery in the liquid fuel is about 45% compared to 25–30% by conversion of wood or corn stover to ethanol with the hemicelluloses as a byproduct. The production cost of refined pyrolysis oil is in the range of 1.7–2.1 US \$/gallon fuel. This is still much too high compared to the

price of raw pyrolysis oil (≈0.35 $/gallon), but the technology is in its infancy, and no large-scale production of pyrolysis oil has as yet been realistically evaluated.

Production of ethanol from lignocellulosic biomass involves a number of steps. Pretreatment of milled or finely cut biomass to open up the structure for further hydrolysis, saccharification of the biomass, and separation of C6/C5 sugars from lignin (Hahn-Hägerdahl et al. 2006). Finally, part or all of the sugars is converted into ethanol. Estimates of the ethanol production cost depend on the nature of the biomass feed (softwood, perennial grass crops, straw, bagasse, etc.), on the process, and on the final product distribution. In some processes, the goal is to ferment all the sugars, either in one or in several steps, and the wastewater is treated in an anaerobic bacterial fermentation to yield biogas. Lignin may be used in further processes in the biorefinery, or it may be burnt to provide heat for the heavy evaporation duties involved in producing almost water-free ethanol based on the 4–8 wt% effluent from the fermentation, and perhaps to turn all the residual in the wastewater into a combustible fuel. The cost of fermenting the C5 sugars is higher than for the C6 sugars, and a good alternative to total conversion of the sugars is to use the C5 and mineral-rich residue from a C6 fermentation with yeast as an excellent cattle feed.

The IOGEN demonstration unit in Ottawa converts 1.5 tons of wheat straw per hour and utilizes both the C5 and the C6 sugars to make a rather weak (4–5 wt%) ethanol solution while the Danish IBUS concept, DONG-Inbicon, produces an 8 wt % ethanol solution from the C6 sugars of steam-treated wheat straw, and with a co-production of a mineral-rich C5 molasses for cattle feed. After pilot scale experiments from 2004, DONG-Inbicon inaugurated in 2009 a demonstration plant for conversion of 4 ton h^{-1} wheat straw to ethanol and C5 molasses. Similar demonstration units have sprung up in many countries. Of particular interest is the large number of straw-based Indian units, inspired and promoted since the 1980s by the "father" of Indian biotechnology, T.K. Ghose. Common to all these envisaged designs for full scale plants is that they incorporate projected advances in both biological and physical process steps. The interest is centered on the hydrolysis step which is at present dreadfully slow compared with the fermentation step (5–7 days in the IOGEN process + 3 days in a separate fermentation step). Hydrolysis of the polymers to C6/C5 sugars is done with cocktails of enzymes with at least three kinds of cellulase activities (endogluconase, exogluconase, and cellobiose), but unless the biomass is properly pretreated large quantities of enzymes must be added. In the Inbicon process (in which only the C6 sugars are converted to ethanol), the enzymatic prehydrolysis liquefaction takes only 5–7 h, and is followed by a *simultaneous saccharification* and *fermentation* (SSF) process to give a *total* hydrolysis + fermentation time of 6 days.

Pretreatment of the raw biomass by hot water, perhaps with the addition of O_2 in "Wet Oxidation" which removes most of the lignin, by steam, by dilute acid, or with a concentrated NH_3 solution has been used to open up the polymer matrix, and to make it more accessible to cellulases. Especially the Ammonium Fiber Expansion (AFEX) method, originally developed in the 1980s with liquid NH_3 soaking the dry biomass, followed by a sudden pressure release, appears to have a high

potential for significantly reducing the hydrolysis time for straw materials. Sendich et al. (2008) summarizes recent progress in the AFEX method. A strong NH_3–H_2O solution is used, and effective recovery of the NH_3 is described. The water/dry biomass ratio is as low as 0.4 with significant implications for the downstream processes.

SSF of pretreated biomass appears to be the correct procedure, despite the difference between optimal hydrolysis temperature (≈50°C) and fermentation temperature (≈33°C) in current processes. The inhibition of the enzymes by the products of the depolymerization process, e.g., glucose and cellobiose (two glucose molecules linked by a $\beta(1 \rightarrow 4)$ bond), is avoided when the sugar is immediately converted to ethanol (Gauss et al. 1976; Olofsson et al. 2008). The ethanol can probably be removed continuously by a moderate stripping of the medium with CO_2, especially at hypobaric fermentor pressure, say 0.5 bar. These low temperature ethanol separation methods could possibly open up for recovery of intact enzymes from the stillage.

The progress of large biotech companies such as Novozymes and Genencor in the construction of new and more potent enzyme cocktails for saccharification, and the selection of robust, ethanol tolerant strains of the yeast *Saccharomyces cerevisiae* will undoubtedly lead to a several fold reduction of the dosage of hydrolytic enzymes. Together with a strong R&D investment to optimize the process design one may hope that the hydrolysis time will be significantly reduced, and finally be able to match the fermentation time of the C6 sugars.

Fermentation of the C5 sugars with yeast presents a problem, and consortia of different bacteria have been used to convert the C5 sugars to ethanol in a second fermentation step. Many bacteria are able to ferment all lignocellulose-derived sugars to ethanol. In Problem 2.3, and in Chap. 5, butanol and acetone are seen to be lucrative products of another bacterial fermentation where the production organism is *Clostridium acetobutylicum*. But bacteria tend to produce mixed acids rather than ethanol (see Fig. 2.5), and they work best at neutral pH (6–7), where they are more susceptible to infection than yeast which works at mildly acidic pH (4.8–6). Their major disadvantage is that they are not nearly as tolerant to ethanol as *S. cerevisiae*. Thus, even the excellent bacterium *Zymomonas mobilis* which is able to produce ethanol in nearly stoichiometric amounts from glucose tends to be severely inhibited by ethanol concentrations above 3 wt%, while new industrial strains of *S. cerevisiae* tolerate more than 15 wt% ethanol.

If one decides to sell the residue from the C6 fermentation as animal feed, the currently slow and incomplete fermentation of C5 sugars in conventional yeasts is not a problem. The value of the cattle feed can be greatly improved if the C5 sugars are converted to biomass with about 70% protein. The company Microbiogen (http://www.microbiogen.com) in Sydney, Australia, has in a long screening program developed an industrial yeast, that by aerobic cultivation quantitatively converts the C5 sugars to yeast biomass, also in the harsh environment of hydrolyzed lignocellulose.

If the goal is to utilize all the sugars for ethanol production then new yeast strains must be engineered. Strong and persistent research efforts toward this goal have

been made by many research groups, see e.g., Hahn-Hägerdahl et al. (2007) and Wisselink et al. (2009). One important breakthrough has been the expression of a fungal xylose isomerase into yeast. This enzyme permits direct isomerization of xylose into xylulose, and an almost simultaneous fermentation of xylose and glucose is obtained (van Maris et al. 2006). In the 2009 paper by Wisselink et al., it is shown that long-term stable co-fermentation of several C5 sugars and glucose can be ensured by *evolutionary engineering*. This is the same strategy used by, e.g., Microbiogen to convert C5 sugars to high value animal feed: A sustained evolutionary pressure is applied to the culture to change the phenotype of the original strain in the desired direction. Problem 2.2 discusses how C5 and C6 sugars are directed to the main metabolic pathways of *S. cerevisiae* through the same feed-line.

When all the evidence from scientific journals[1] is considered together with news releases from reputable biotech companies, it does seem that the goal of producing ethanol from lignocellulosic biomass at a price comparable with gasoline from oil is within reach.

It will be prudent to complement the massive bioscience research effort toward an optimal conversion of lignocellulosic biomass into ethanol with an effort to optimize the engineering design of units that produce ethanol from sugar and starch, the "first generation ethanol processes." Neither the fermentors nor the downstream processes are yet fully optimized, and process intensification as well as energy savings in the up-concentration of dilute ethanol solutions (e.g., using heat pumps) could well lead to a substantial reduction of the production price of ethanol from starch.

With proper foresight these technical improvements will be ready to implement when corn stover, straw, wood chips, or bagasse are included as raw materials for ethanol production.

First generation ethanol processes are typical *mature* processes, just as most processes in the petroleum/gas-based refinery, and a 5% reduction of production costs would be significant. Conversion of biomass to ethanol or to pyrolysis oils must at present (2010) be regarded as *immature* technical processes, defined as processes where the processing costs to final consumer goods are at least equal to the raw material costs. In the oil refinery, the processing cost is only on the order of 20–30% of the raw material cost. The challenges of the production process is the reason why the final price of ethanol from biomass is at present higher than the price of starch-based ethanol, despite the very low price of raw material delivered at the processing site.

The obvious immaturity of the biomass-based processes is, of course the incentive for the massive research commitment by industry and by governments. Without indulging in wishful thinking it is predictable that the cost of enzymes for hydrolysis of biomass will be much reduced if microorganisms such as the fungus *Trichoderma reesei* can be seeded on an optimally pretreated biomass to produce the cellulases for saccharification. The simultaneous fermentation of C5 and C6 sugars at a large scale will eventually also become economically feasible, also in fermentation media that

[1]Older references such as Maiorella et al. (1984) should, however, not be neglected. This seminal paper has recently been honored by being reprinted in *Biotechnol Bioeng* (2009).

contain inhibitors released from the pretreatment of the raw material. Finally, the whole engineering of the production system will be subjected to intensive studies by industry to achieve lower capital costs, smaller energy expenditure, and cheap recycling of the process water.

These improvements of Second Generation ethanol processes will be necessary if the political will to support bio-fuels is to be sustained.

The commitment of land to cultivation of energy crops will be decided on the "land use yield." At present, this parameter is stated to be 135 GJ ha^{-1} for lignocellulosic biomass, 85 GJ ha^{-1} for corn kernels, and only 18 GJ ha^{-1} for soy beans. Considerable efforts are, however, made to improve the utilization of the *whole* biomass from energy crops. Also the introduction of new crops for biodiesel production, and the – rather speculative – development of processes where microorganisms with high lipoid content are cultured in large scale, may make also biodiesel production feasible from a socioeconomic perspective. In the mean time, the use of waste materials such as yellow grease, lard, and tallow may quite soon develop into a cheap, large-scale source of biodiesel.

The predictions are, that by a skillful combination of first and second generation bioethanol processes, the production cost of ethanol will decrease to 1.4–0.8 US $/gallon (Sendich et al. 2008).

In the long run, the burgeoning Brazilian production of ethanol from cane sugar will increase dramatically when the 80% of the plant that is not sugar is utilized for ethanol production. Then, undoubtedly, Brazil will develop a large chemical industry where most of the sugar is used to produce chemicals, while maintaining a large export of ethanol. Very recently (October 2010), a large, well-funded project has been initiated with this end goal in mind. Characteristically, the project partners are Petrobras, the national Brazilian oil company, and Novozymes, a major producer of industrial enzymes.

In fact, as discussed in the next section, pure sugar is much more valuable as a raw material for chemical production rather than when converted to a biofuel. Exactly the same arguments are used to motivate a substantial change in the final use of the oil and gas raw materials, from transportation fuels and for heating purposes to an almost exclusive use in the chemical industry.

2.1.2 Production of Platform Chemicals in the Biorefinery

By analogy with the raw materials such as petroleum and natural gas in the oil refinery, the biorefinery operates with cellulose, hemicellulose, lignin, starch, sucrose, vegetable oils, and fats as raw materials. The sugar polymers are converted to mono- or dimeric sugars: glucose, fructose, sucrose, lactose, galactose, xylose, and arabinose, which serve as platform chemicals together with starch.

The triglycerides from oils and fats deliver other platform chemicals, glycerol and fatty acids. After esterification with aliphatic alcohols the fatty acids are used as biodiesel while the byproduct, glycerol has a great potential as a building block.

Glycerol will become an attractive byproduct from biodiesel production. Among the many promising processes in which glycerol is the raw material one can mention a process in which two enzymes are used. In a suitable membrane reactor construction which allows the two enzymes to be separated, 2 mol of glycerol can be converted to 1 mol of 1,3-propane diol and 1 mol of dihydroxy acetone ($CH_2OH–CO–CH_2OH$). One glycerol molecule is oxidized, and the other is reduced. The result is two compounds, each of which has a much higher value than glycerol.

When the considerable difficulties associated with downstream processing of the monomers derived from lignin have been solved this raw material can be used to synthesize a number of aromatic compounds as exemplified by Fig. 1.1a. In the near future, lignin is likely to be combusted to provide process heat. All the biopolymers can also be pyrolyzed to SynGas ($H_2 + CO$) or to pyrolysis oil as discussed in Sect. 2.1.1. In the following only glycerol and the sugars derived from biopolymers will be considered as building blocks.

In 2004, a group of researchers at Pacific Northwest National Laboratory (PNNL) and National Renewable Energy Laboratory (NREL) in the USA made a detailed screening of sugars as potential candidates for building blocks, secondary chemicals, and intermediates to produce final consumer goods in the industry sectors which have traditionally been served by the petroleum industry.[2] Starting with more than 300 candidates the group arrived at a final set of 12 building blocks, Table 2.1. As argued by Werpy and Petersen (2004), all 12 building blocks have a high potential for substituting building blocks derived from oil and gas.

The first three building blocks are made from sugars by simple chemical processes. *Glucaric acid* is a representative of the many, so-called aldaric acids $COOH–(CHOH)_n–COOH$ which are produced from sugar by mild oxidation. Thus, glucaric acid is obtained from D-glucose or D-gulose by oxidation with dilute

Table 2.1 Twelve sugar-based building blocks suggested by Werpy and Petersen (2004)

Glucaric acid
2,5 Furan dicarboxylic acid
Levulinic acid
Itaconic acid
1,4 Diacids (succinic, fumaric and malic)
3 Hydroxy propionic acid
Glycerol
3-Hydroxybutyrolactone
Xylitol and arabinitol
Sorbitol
Aspartic acid
Glutamic acid

[2]Another (comprehensive) report is from OECD ("The application of Biotechnology to Industrial sustainability – a Primer") (1998:ISBN 92-64-16102-3) and 2001. Newer reviews are Haveren et al. (2008), Clark (2007), and Kamm and Kamm (2007).

nitric acid. *Levulinic acid* (CH_3–CO–$(CH_2)_2$–COOH) results when starch is boiled in dilute sulfuric acid. *2,5 Furan dicarboxylic acid* is obtained by oxidative dehydration of C6 sugars, e.g., glucose and *3-hydroxybutyrolactone* by oxidative degradation of starch.

Sugar alcohols, *sorbitol* (CH_2OH–$(CHOH)_4$–CH_2OH) and the corresponding C5 alcohols *xylitol* and *arabinitol* can be produced by chemical hydrogenation of, respectively, glucose, xylose, and arabinose.

1,4 Di-carboxylic acids (succinic acid, fumaric acid and malic acid), *3 hydroxy propionic acid*, and the two amino acids *aspartic acid* and *glutamic acid* are all metabolites from metabolic pathways, and they are more easily produced from sugars by fermentation than by chemical routes.

Finally, *itaconic acid* (methylene succinic acid, COOH–CH_2–C (=CH_2)–COOH) is produced either by distillation of citric acid (COOH–CH_2–C(COOH) OH–CH_2–COOH), a fermentation product with a yearly production volume of about one million tons, or it can result directly from certain fungal fermentations.

The list of 12 building blocks in Table 2.1 can, if desired, be reduced further since several of the compounds can be synthesized from other compounds on the list. Thus, glutamic acid is the starting point of the biosynthesis of a family of amino acids, including aspartic acid, and the fermentation can be conducted such that aspartic acid or lysine is the final amino acid product. In Sect. 2.5, it will be shown that the amino acids can also be used as intermediates in the production of polymers.

Figure 2.1b summarizes the description of the synthesis pathways from lignocellulosic biomass to the 12 building blocks. Glucose is shown as the starting point for the synthesis, but other sugars resulting from enzymatic hydrolysis and saccharification of biomass could also have been used.

Directly aimed toward *polymers* is the Cargill-Dow lactic acid to polylactide facility (180,000 annual ton production) inaugurated in 2002 in Blair (Nebraska).

Since 2006 (at Loudon, TN) DuPont and Tate & Lyle produce annually 45,000 ton 1,3-propane diol by fermentation of hydrolyzed corn starch. In 2008 Novozymes and Cargill initiated a cooperation to produce 3-hydroxy propionic acid (CH_2OH–CH_2–COOH) directly by fermentation from glucose. The goal is to produce acrylic acid (propenoic acid, CH_2=CHCOOH) by fermentation, rather than chemically from propylene – see Problem 2.7.

The production of polymers from biobased raw materials will be further discussed, in this chapter, and in Chaps. 5, 6, and 9.

It is interesting that together the building blocks in Table 2.1 offer a collection of reactive functional groups, –OH, –CHO, –CO, –COOH, and –NH_2. With at least two of these present in a particular building block this is an attractive platform for building other compounds with a C3, C4, C5, or C6 carbon backbone. This property is of course also found in the raw materials, the sugars, whereas ethanol with only one reactive group has much less value as a platform chemical.

The reactivity of the building blocks makes it possible, mostly by chemical routes, to synthesize, e.g., a whole array of secondary chemicals which in their turn can also substitute oil-based building blocks for almost all conventional, and many new, biodegradable polymers, such as polylactides. Environmentally friendly

solvents such as esters of lactic acid (COOH–CHOH–CH_3) and aliphatic alcohols, as well as conventional solvents (butanol, acetone, etc.) are readily available as end products of central metabolic pathways. Whole families of pharmaceuticals, exemplified by penicillins and cephalosporins, are produced by fermentation. No chemical route is competitive for these bulk chemicals. Finally, specialty drugs, produced in small quantities but at high unit price, are synthesized by microorganisms. Therapeutic proteins and polyketides are examples of products that can only be produced by bioroutes.

In the following, we shall examine the metabolic pathways that lead from the raw material, sugar, to the desired end products, and a few examples of complete synthesis paths will be discussed. The goal is to understand the general rules of carbon flow in the main arteries of the immensely complicated metabolic network of any living organism. Nothing in the network structure was developed by nature without serving a purpose. Biochemistry and biology has given us an understanding of the purpose of the major pathways, but we are still a long way from understanding the whole, tightly regulated network. Learning from the pathways and guessing their interconnections are the way forward to design new organisms that serve our needs better. It is through systematic research where new sets of experiments are devised to obtain a better understanding of the rationale for the observed phenomena that we shall eventually reap the full benefit of what living cells offer to us. *Physiological Engineering, Metabolic Engineering, Systems Biology* – new names are constantly coined for this scientific endeavor – is a happy marriage between the biosciences and the engineering sciences.

In the perspective of this textbook all the tools discussed in the following chapters serve the purpose of understanding how to exploit as fully as possible the flux of carbon through the pathways and to engineer the fluxes by quantitative means. Ultimately, we shall have the perfect organism, optimized with respect to its performance in the reactor as well as in the subsequent down-stream processes toward the final product.

2.2 The Chemistry of Metabolic Pathways

In a metabolic pathway a substrate (input reactant) is converted by a series of enzymatic reactions to a final product. All the carbon in the substrate can end up in the final product or it may partly be lost through a number of *diverging* pathways which branch off from the main pathway.

Carbon can also be fed into the pathway by *converging* pathways.

Any of the intermediates of the pathway may be *excreted* from the cell and can be recovered from the *medium* (sometimes called the *fermentation broth*) from which the cell receives its substrates. Both processes occur by *transport* through the cell membrane.

The final product of the pathway can serve as a substrate for one or more pathways, and the notion of a separate pathway is more or less fictitious in the overall picture of a highly connected *metabolic network*. Several pathways have been named after

the persons who discovered and studied the set of reactions leading from the substrate to the "final" product of the pathway: EMP pathway (Embden, Meyerhof, Parnas + several other contributors), Krebs cycle, Entner–Duodoroff pathway, but other names are also used such as *Glycolysis* (*lysis* = degradation (of glucose)) for the EMP pathway and *TCA cycle* (Tri Carboxylic Acid) for the Krebs cycle. We shall largely use the latter names.

In the first pathway to be discussed (Sect. 2.2.2), the glycolysis pathway, the substrate is *glucose*, but through pathways which converge into glycolysis other sugars, galactose, lactose, mannose, and C5 sugars such as xylose can also serve as a carbon source.

The product is the keto acid, *pyruvic acid* (CH_3–CO–COOH). No carbon is lost in the form of CO_2[3] When furthermore no metabolic intermediates are drained away from the pathway as substrates in other reactions, one obtains two molecules of pyruvic acid[4] from one glucose molecule. Since the net formula for glucose is $C_6H_{12}O_6$ the overall stoichiometry – with respect to carbon – is:

$$C_6H_{12}O_6 \rightarrow 2C_3H_4O_3 \tag{2.1}$$

It is obvious that two H_2 have been lost in the conversion from glucose to pyruvic acid, i.e., the net result of the pathway is an oxidation of the glucose, the first step toward the ultimate combustion of glucose to six molecules of CO_2 in the *catabolic metabolism*.

In the following Sect. 2.2.1, the *purpose* of glycolysis as seen from the point of view of the microorganism will be discussed, and in Sect. 2.2.3 it will be shown how the organism compensates for the loss of *reducing power* associated with the oxidation of glucose to pyruvic acid in glycolysis.

2.2.1 The Currencies of Gibbs Free Energy and of Reducing Power

The conversion of a substrate to a final product through a sequence of chemical reactions will only take place if all the reactions in the sequence are thermodynamically favored in the desired direction. The change in free energy ΔG_R for each reaction must be *negative*. As will be discussed in Chap. 4, a reaction A → B which is thermodynamically unfavorable may still proceed in the indicated direction if it runs

[3]Although CO_2 is obviously a metabolic product we shall use "lost carbon" ("lost" for further metabolism) for this compound.

[4]In figures we usually show the non-dissociated metabolites. At the pH at which most fermentations take place we have the anions: pyruvate, acetate, glutamate, CH_3COCOO^-, CH_3COO^-, etc. rather than the free acids. NH_3 occurs as NH_4^+. When we write stoichiometries (e.g., (2.1)) it is simpler to use the non-dissociated molecules. The need to add acid or base to keep the desired pH is implicitly assumed. In Chap. 4 the ionic forms will have to be used.

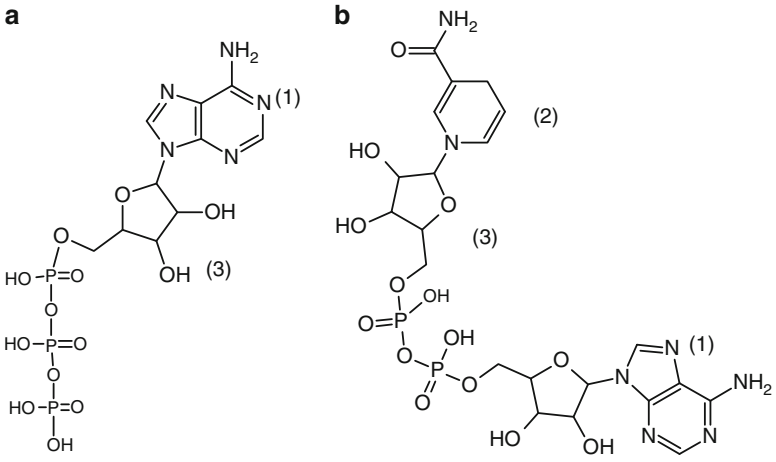


Fig. 2.2 (**a**) The "currency" of free energy, ATP. (**b**) The currency of catabolic reductive power, NADH. In ATP a purine derivative (six amino purine = adenine) (*1*) is attached to the 1′ carbon of the C5 sugar, ribose (*3*). The tri-ester with H_3PO_4 is formed at the 5′ carbon of the sugar. The ribophosphate structure of ATP is also found in NADH, but one of the phosphate groups is substituted with another nucleotide where nicotine amide (*2*) is attached to the 1′ carbon of the C5 sugar. In NAD^+ the 1′ nitrogen in the pyridine ring is positively charged while at the 4′ carbon one H is removed (i.e., the net effect is the removal of "H_2")

in parallel with another reaction C → D that is accompanied by a sufficient loss of free energy to make the sum of the two reactions occur with a negative ΔG_R.

In cell reactions, the reaction C → D is in most cases the hydrolysis of an energy-rich compound adenosine triphosphate (ATP), Fig. 2.2a.

Hydrolysis of ATP removes one molecule of phosphoric acid (or rather one HPO_4^{2-}), and the diphosphate ADP is formed. At the same time, a free energy "package" worth about 30.5 kJ mol^{-1} (at "standard conditions" which will be discussed in Chap. 4) is made available for A → B to proceed against its thermodynamically preferred direction as long as $\Delta G_{A\rightarrow B} < 30.5$ kJ mol^{-1}.

Within the cell, the ratio ATP/ADP is tightly regulated as long as the environment is kept stable, i.e., when the culture grows at *steady state*, the so-called *balanced growth*. Any use of ATP to "help" an energetically unfavorable reaction A → B must be balanced by other reactions $A_i \rightarrow B_i$ which have as sufficiently large, negative ΔG_R ($<<-30.5$ kJ mol^{-1}), and which will still proceed even when ATP is formed besides the main product B_i. The picture of ATP as an energy "currency," continuously exchanged between the thousands of metabolic reactions, is quite suggestive.

Nicotinamide adenine (NADH) dinucleotide, Fig. 2.2b, is the corresponding reductive power currency in the many reactions of the network of (mostly) *catabolic* reactions. The loss of reductive power from a metabolite is counteracted by an increase in NADH:

$$RH_2 + NAD^+ \rightarrow R + NADH + H^+ \qquad (2.2)$$

Generally speaking, the redox carrier serves as a *cofactor* for the enzyme that converts RH_2 to R.

As an example the *redox balanced* overall pathway reaction (2.1) from glucose to pyruvic acid should read

$$C_6H_{12}O_6 + 2NAD^+ \rightarrow 2C_3H_4O_3 + 2NADH + H^+ \tag{2.3}$$

In one of the reactions in glycolysis (reaction (6) in Fig. 2.4: glyceraldehyde-3 phosphate → 1,3 diphosphoglycerate), NAD serves as a cofactor, picking up the H_2 when the triose is oxidized.

On the level of the cell, the ratio NADH/NAD is tightly regulated for a cell culture in balanced growth. Consequently, the sum of all the rates of network reactions that produce NADH is matched with the sum of rates of reactions which consume NADH, just as the sum of rates of ATP producing reactions is matched with the sum of rates of ATP consuming reactions. These two constraints on the rates of pathway reactions in the (necessarily limited) network, with which we decide to represent the totality of the metabolic network of a cell, will be used in Chap. 5 in *Metabolic Flux Analysis* to obtain relationships between the rates of cellular reactions.

As one may guess, the picture of energy transfer and of redox transfer in metabolic networks is not as simple as described here. There are other "currencies" of energy transfer, such as guanosine triphosphate (GTP) which structurally resembles ATP, except that the purine derivative guanosine is used instead of adenine. In certain catabolic reactions, GTP is produced rather than ATP (e.g., in one of the TCA reactions, Fig. 2.7), and GTP is primarily used in protein synthesis. The energy content of the two molecules is the same, and they are easily converted to each other.

Also there are several currencies of redox power besides NADH. Of particular interest is NADPH. The structure of NADPH is the same as that of NADH in Fig. 2.2b, except that a third phosphate ester is attached to the 2′ carbon of the ribose next to the adenine.

Whereas NADH is produced and consumed in cell reactions connected with energy (ATP) production, *the catabolic reactions*, NADPH is (mainly) produced in reactions which lead to synthesis of building blocks for production of more cell mass, and consumed in the reactions which eventually lead to production of proteins and genetic material, RNA and DNA, the *anabolic reactions*.

Many organisms (e.g., *Escherichia coli*, the preferred workhorse of many industrial companies) are able to convert NADH to NADPH and vice versa

$$NADH + NADP^+ \rightarrow NADPH + NAD^+ \tag{2.4}$$

The reaction is catalyzed by an oxido-reductase, *NADPH: NAD transhydrogenase*.

Occasionally, it may be desirable to convert NADH to NADPH, especially in anaerobic fermentations, and reaction (2.4) may become useful. A typical case is the production of fuel-ethanol, where glycerol is an undesired byproduct that must

be produced by the yeast cell in order to satisfy the NADH balance (see Sect. 3.3). In a study by Nissen et al. (2001), the *E. coli* gene for transhydrogenase was inserted in *S. cerevisiae* in order to introduce an alternative NADH sink, and hereby eliminate glycerol production. A potential 5% increase of ethanol yield was expected. However, the strategy did not work, since the NADPH/NADP ratio is higher than the ratio of NADH/NAD, and consequently the reaction runs in the opposite direction, and this resulted in an increased NADH production in the cell, leading to a larger glycerol production.

Another important redox carrying dinucleotide is $FADH_2$, flavin adenine dinucleotide, which plays a role as a cofactor for the enzyme *ATP synthase* in *oxidative phosphorylation*. $FADH_2$ is mainly produced in the TCA cycle, Fig. 2.7, reaction (7).

Other redox carriers such as PQQ, pyrroloquinoline quinine, are used as cofactors in specific reactions, e.g., in the oxidation of methanol to formaldehyde by the enzyme methanol dehydrogenase. It is a key player in the production of single cell protein (SCP) from natural gas or methanol (Matsushita et al. 2002).

Besides their role as redox carriers in metabolic networks the redox cofactors play a part as anti-oxidant vitamins, e.g., in the human body.

All these complex organic molecules function either as donors of H_2 or acceptors of H_2, and one molecule of the cofactor can be interpreted as "H_2." Only in a few cases the H_2 will actually be liberated as molecular hydrogen.

Some reactions use one cofactor while others use a different cofactor. Structurally related cofactors can be converted between each other, e.g., NADH to NADPH (2.4). Although the cofactors are different and have different properties it is often possible in simple calculations to operate with only one common representative for all of them. Thus, if in *Metabolic Flux Analysis* all the anabolic reactions are lumped into one reaction there is, as will be discussed in Chap. 5, a small net-production of redox power. This is written as "NADH" in the stoichiometry for biomass formation, but the small positive production of redox is the result of a large consumption of NADPH and an approximately equal production of NADH. Since both NADH and NADPH are produced from their oxidized counterpart by the same consumption of substrate (sugar), the overall rate of substrate consumption is the same. In more sophisticated analyses, e.g., in the quantitative study of amino acid production by fermentation, the distinction between different cofactors needs to be maintained.

Whereas the net consumption, or production, of redox cofactors in a pathway can always be deduced from the level of reduction of the substrate and the product from the pathway, this is not so for the energy production.

Thus, the net reaction (2.1) does not reveal how much free energy in the form of ATP has been produced, although as will be seen in Chap. 5 oxidative pathways will generally produce free energy. Whether a pathway reaction will produce ATP by hydrolysis of high energy phosphate bonds depends on the enzyme used in the reaction.

Thus *kinases* will produce free energy, ATP, while *hydrolases* will produce enthalpy, which is transferred to the medium as heat.

The only way to find out whether a particular reaction produces ATP (or any other product) is to consult one of the metabolic network encyclopedias which are

now available on the Internet, e.g., EXPASY-Molecular Biology Server, subsection Biological Pathways, see http://www.expasy.ch., KEGG (Kyoto Encyclopedia of Genes and Genome, http://www.genome.jp/kegg/), and http://www.brenda-enzymes.org/, an excellent database for enzyme properties.

2.2.2 *Glycolysis*

In order to produce more biomass the necessary nutrients (or substrates) must be available. Nutrients can roughly be divided into (1) carbon source, (2) energy source, (3) nitrogen source, (4) minerals, and (5) vitamins. The energy source ensures supply of the necessary Gibbs free energy for cell growth, and often the carbon and energy sources are identical. The most common carbon and energy source is glucose, but carbohydrates such as maltose, sucrose, dextrins, or starch are frequently used. Many cells can also use organic acids, e.g., acetic acid or lactic acid, alcohols or polyols, such as glycerol, as carbon and energy source. A few microorganisms grow on hydrocarbons and on CO_2 (as do plant cells), but using H_2 as an energy source.

In Sect. 2.2.7, we shall return to the subject of cellular requirements for nutrients, and in Table 2.5 typical media used in industrial fermentation are listed (Fig. 2.3).

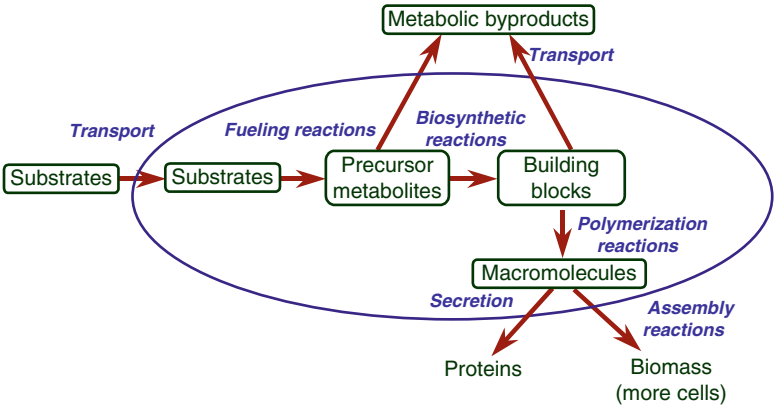


Fig. 2.3 Overview of reactions and processes involved in cellular growth and product formation. Substrates are taken up by the cells by transport processes and converted into precursor metabolites via fueling reactions. The precursor metabolites are converted to building blocks, and these are polymerized to macromolecules. Finally, macromolecules are assembled into cellular structures such as membranes, organelles, etc. that make up the functioning cell. Precursor metabolites and building blocks may be secreted to the extracellular medium as metabolites, or they may serve as precursors for synthesis of metabolic end products. The cell may also secrete certain macromolecules – primarily proteins that can act as hydrolytic enzymes, and some cells may also secrete polysaccharides

We shall first study the generation of energy from sugars, the most common carbon and energy source for microorganisms. The "standard" sugar substrate is *glucose*. This is taken up by the cell either by *passive diffusion* or by various forms of *active transport*, using free energy in the form of ATP to mediate the transport process. In *group translocation*, the glucose molecule is phosphorylated to an activated glucose, *glucose-6 phosphate*, at its passage from the medium through the cell membrane to the *cytosol*, the fluid inside the cell. The activation of the glucose molecule occurs by transfer of phosphate from an activated molecule further down in the path of glucose metabolism. The donor is *phosphoenolpyruvate* (PEP), see Fig. 2.4b, and the process is used for fast glucose transfer by many bacteria such as *lactic acid bacteria* and *E. coli*.

The metabolic pathway by which most living cells create energy from growth on sugars is the EMP-pathway, Figure 2.4a, b, and the end product of the pathway is pyruvate (2.1).

Using yeast as an example, the first reaction of the pathway (1) is the phosphorylation of intracellular glucose to glucose-6 phosphate (G6P) using one ATP (i.e., an energy package of 30.5 kJ) per mole of glucose. Next follows a reversible conversion (2) of G6P to fructose-6 phosphate (F6P), and a further activation of the sugar (3) is achieved by donation of one ATP to F6P to form fructose-1,6 diphosphate (F1,6P).

F1,6P is highly energized and splits by reaction (4) into two *trioses*, dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3 phosphate (GAP). Since DHAP is a symmetrical molecule the position of the phosphate molecule is not specifically indicated, whereas the GAP that results from G6P is phosphorylated in the 3-position, i.e., furthest away from the aldehyde group, see Fig. 2.4a(f).

The entry point to the pentose phosphate pathway (PP), Fig. 2.8, is from G6P (b). F6P and GAP formed in the PP pathway join the two pools (d) and (f) in Fig. 2.4a and are metabolized further in the EMP pathway.

G6P and F6P are used for the production of storage carbohydrates (glycogen and trehalose), and of cell wall components (chitin and glucans). GAP is used for the production of lipids.

The two trioses are connected by a fast reversible reaction (5). When we are looking at the energy production by the EMP pathway we assume that consumption of sugar in the pathway that leads from the DHAP is blocked. Hence all the carbon ends up in GAP and is further metabolized from this intermediate. In a real fermentation the ratio between the pools of GAP and the DHAP is, of course, controlled by the relative rates of consumption of the two compounds.

An interesting observation, of relevance to the discussion of labeled carbon atoms in Chap. 5, is that a glucose molecule labeled in position 1 (i.e., $^{13}C_1$-glucose) will lose 50% of its labeling after the split at F1,6P. GAP is obtained from carbon atoms 1–3, while DHAP stems from carbons 4–6. The GAP obtained from DHAP by the fast reaction (5) is unlabeled, and the resulting concentration of labeled molecules (in the C_3-position of GAP) is only half of what it was in the glucose. This topic is discussed further in Sect. 5.3.3.

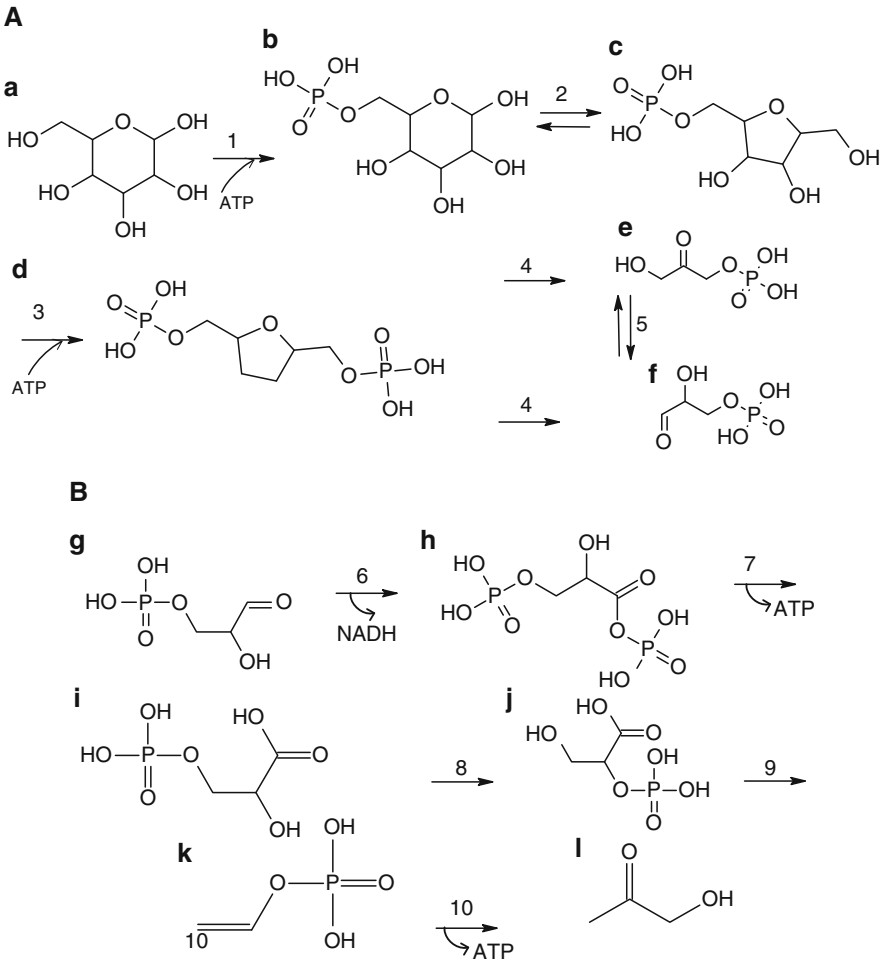


Fig. 2.4 (**A**) The first part of the EMP pathway, until the hexose is split into two trioses. (a) Glucose, (b) glucose 6 phosphate (G6P), (c) fructose 6 phosphate (F6P), (d) fructose 1,6 diphosphate (F1,6P), (e) dihydroxyacetone phosphate (DHAP), and (f) glyceraldehyde 3-phosphate (GAP). The reactions are catalyzed by (*1*) hexokinase, (*2*) phosphohexose isomerase, (*3*) phosphofructokinase, (*4*) aldolase, (*5*) triosephosphate isomerase. (**B**) Reactions of the EMP pathway from GAP (g). (h) 1,3 Diphosphoglycerate (1,3 DPG), (i) 3-phosphoglycerate (3 PG), (j) 2-phosphoglycerate (2 PG), (k) phosphoenolpyruvate (PEP), and (l) pyruvate (PYR). The respective enzymes are (*6*) 3-phosphoglyceralde-hydehydrogenase, (*7*) 3-phosphoglycerate kinase, (*8*) phosphoglycerate mutase, (*9*) enolase, and (*10*) pyruvate kinase. Carbon is drained off from the 3PG and PEP pools (in both cases for amino acids)

The pathway from G6P to pyruvate can also work in the opposite direction. This is called *gluconeogenesis* ("new glucose synthesis"). When small molecules such as a primary alcohol (ethanol) or a carboxylic acid (lactic acid (HLac), formic acid or acetic acid), all products from glycolysis, are used as carbon source, the reactions of Fig. 2.4a, b from G6P to pyruvate must be traced in the opposite direction in order to

synthesize the precursor metabolites that are drained away from the EMP pathway when it operates in its normal direction from glucose to pyruvate. These branch points away from the EMP pathway are indicated in the caption to Fig. 2.4a, b.

The *purpose* of the EMP pathway is, as mentioned previously, to create energy for biosynthesis reactions. When Fig. 2.4a, b is studied from this perspective the result is *that 2 mol ATP is produced for each mole glucose that is converted to 2 mol pyruvate*. In reactions 1–3 the glucose molecule is energized to provide free energy for the split of F1,6P in reaction (4). When calculating the maximum ATP yield in the pathway all DHAP is funneled back into the glycolysis pathway, and each GAP produces two ATP when metabolized to pyruvate. The net result is consequently two ATP, when no carbon is lost to produce metabolites other than pyruvate.

As discussed in Sect. 2.2.1, an overall redox balance on the pathway from glucose to pyruvate reveals that two redox packages (NADH) are synthesized by reduction of the oxidized form of the cofactor when glucose is metabolized to pyruvate (2.3). In Fig. 2.4b, we identify reaction (6) where each of the two aldehydes, GAP, is oxidized to diphosphoglycerate (1,3 DPG), a carboxylic acid, as the reaction in which NADH is synthesized.

Whereas the redox production in the pathway can be deduced by considering the substrate and the final product of the pathway, there is no easy way to find the amount of ATP produced or consumed in the pathway. As indicated at the end of Sect. 2.2.1, one must consult the pathway diagrams, either in textbooks or on the net. For the EMP pathway ATP is produced in reactions (7) and (10) which are both catalyzed by *kinases*. When sufficient energy is stored in an activated metabolic intermediate, kinases release an energy package, generally as ATP. Other hydrolyzing enzymes, *hydrolases*, release energy as *heat*, and this happens when the free energy change of the reaction is insufficient to allow an ATP to be synthesized – see Fig. 5.1 and also the discussion in Chap. 4, where glycerol 3-phosphate hydrolase is used as an example. Generation of heat instead of ATP is certainly practical in many circumstances (in humans to maintain the body temperature), but it is of little use when the organism needs free energy for growth.

The gluconeogenetic path from pyruvate to G6P requires ATP. If a waste product from glycolysis, e.g., ethanol, is used as a substrate rather than glucose, then the precursors from the EMP pathway can only be synthesized if the substrate is also consumed in ATP generating reactions. This is where *respiration* (Sect. 2.2.5) plays a crucial role.

Only reactions (1), (3), and (10) in glycolysis are, as will be seen in Table 4.2, accompanied by large losses of free energy. At physiologically relevant conditions, reactions (6)–(9) have a ΔG that varies between -2.7 and $+2.7$ kJ mol^{-1}. Hence they can easily be reversed, when NADH is provided for reaction (6).

Many of the glycolytic reactions are reversible, and they are thermodynamically favored when the energy status (ATP and NADH) of the cell is high. Reactions (1), (3), and (10) are, however, not reversible. Other enzymes can carry out reactions similar to reactions (1) and (3), but without production of ATP. Reversal of the almost irreversible reaction (10) follows a circuitous route that involves metabolites from the TCA cycle.

In a similar fashion, the reactions from DHAP to glycerol can be reversed at high ATP and NAD level. It is noteworthy that the conversion of glycerol to glycerol 3-phosphate (G3P) is catalyzed by a kinase that requires ATP while as indicated above the hydrolysis of glycerol phosphate by a hydrolase liberates no ATP.

G6P, the final product of gluconeogenesis is used either as a substrate for the PP pathway or it is stored as poly-carbohydrates, e.g., *glycogen* $(\alpha\text{-D glucose})_n$, in yeast. This serves as a final energy source if glucose or degraded macromolecules are unavailable.

The reversal of the metabolic pathways when carbon compounds of the right structure, e.g., proteins and lipids are degraded and serve as alternate sources of building blocks and energy is, of course of preeminent importance in nutrition. But in the context of the present text, where the objective is to present ways of producing chemicals from renewable resources, the pathways that depend on sugars as substrate are in focus. Hence gluconeogenesis will not be given much attention in the following.

As a final note on glycolysis it is worth noting that the two ATP gained by *substrate level phosphorylation*, the reactions that convert glucose to pyruvate, *is generally the only source of free energy obtained in anaerobic fermentations*. Apart from certain anaerobic reactions, such as the conversion of acetyl-phosphate (Acetyl-P) (see Fig. 2.5a, b) to acetic acid, HAc, all further free energy generation must come from respiration, and respiration requires molecular oxygen, i.e., an *aerobic* fermentation process.

2.2.3 *Fermentative Metabolism: Oxidation of NADH in Anaerobic Processes*

At the exit from the EMP pathway 2 mol of ATP and 2 mol of NADH have been produced for each mole of glucose converted to 2 mol of pyruvate.

The ATP is used to drive other, energetically unfavorable processes, but the NAD consumed must be regenerated.

In *anaerobic* fermentation this occurs by reduction of the catabolic product, pyruvate. Figure 2.5a, b shows how *E. coli* and *lactic bacteria* sp. manage this task.

Apart from minor differences (the enzymes of the two bacteria can be slightly different, but they have the same function) the pathways from pyruvate to the final products, lactate, formate (or CO_2), acetate, and ethanol, are identical for most Gram-negative (e.g., *E. coli*) and Gram-positive bacteria (e.g., lactic acid bacteria).

At the first branch point, pyruvate is *either* reduced to lactic acid

$$CH_3COCOOH + NADH(= ''H_2'') \rightarrow CH_3CHOHCOOH(\text{lactic acid},\ HLac), \tag{2.5}$$

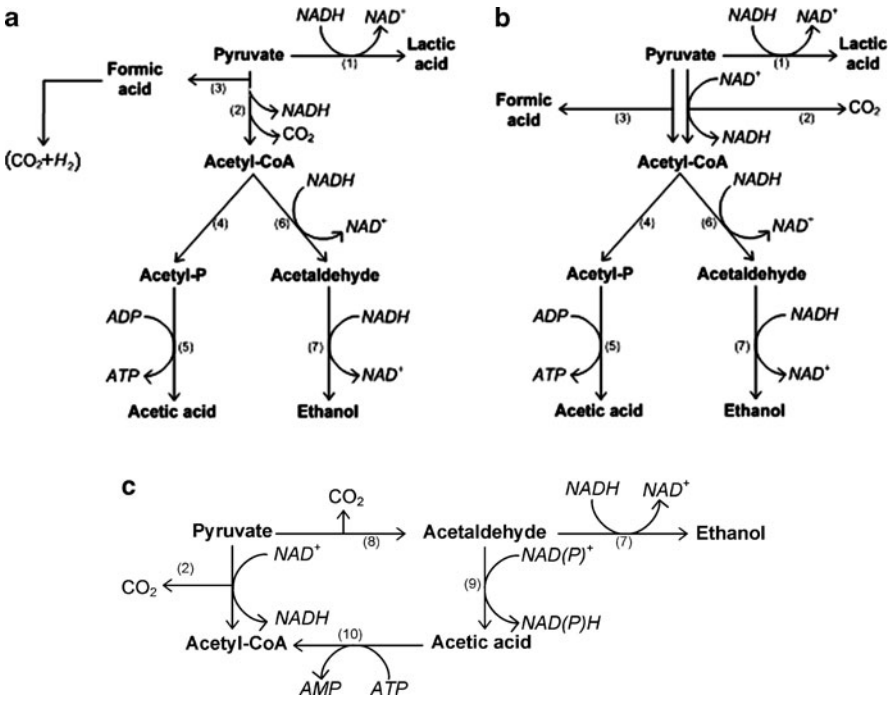


Fig. 2.5 Fermentative pathways for oxidation of NADH in bacteria. Only the main fermentative products are shown. The enzymes are (*1*) lactic acid dehydrogenase; (*2*) pyruvate dehydrogenase; (*3*) pyruvate-formate lyase; (*4*) phosphate acetyltransferase; (*5*) acetate kinase; (*6*) acetaldehyde dehydrogenase, (*7*) alcohol dehydrogenase. **(a)** The fermentative (or mixed acid) metabolism of *Escherichia coli*. (**b**) The fermentative metabolism of lactic acid bacteria. (**c**) Fermentative metabolism in the yeast *Saccharomyces cerevisiae*. (*2*) Pyruvate dehydrogenase, (*7*) alcohol dehydrogease (ADH), (*8*) pyruvate decarboxylase, (*9*) acetaldehyde dehydrogenase, (*10*) acetyl-CoA synthase. The notation NAD(P)H means that acetaldehyde dehydrogenase can use either NAD^+ or $NADP^+$ as cofactor. Note that yeast does not contain lactate dehydrogenase and therefore does not produce lactic acid

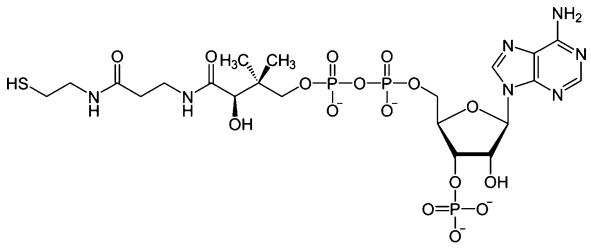
or pyruvate splits into two molecules, acetyl-coenzyme-A, AcCoA, and either formic acid (HCOOH) or CO_2. The cofactor, coenzyme-A, CoA (Fig. 2.6), is used in the synthesis of AcCoA.

$$CH_3COCOOH + CoA \rightarrow HCOOH + AcCoA \tag{2.6}$$

$$CH_3COCOOH + CoA \rightarrow CO_2 + AcCoA + NADH \tag{2.7}$$

When the lower part of the metabolic network is inactive, i.e., lactic acid is the only fermentation product, we have *homo-lactic fermentation*. The overall anaerobic

Fig. 2.6 Coenzyme-A (CoA)



metabolism of glucose to the final product, lactic acid, which is excreted from the cells to the medium is

$$C_6H_{12}O_6 \rightarrow 2C_3H_6O_3(2\,\text{mol of lactic acid}) + 2\text{ATP} \tag{2.8}$$

When both the upper and the lower part of the network are active we have *mixed-acid fermentation*. Lactic acid, together with formic acid or CO_2, acetic acid (HAc) and ethanol, are the normal metabolic products, but under certain conditions both pyruvate and acetaldehyde can be excreted from the cell. CO_2 is produced in lactic acid bacteria by (2.7), and in *E coli* possibly also (together with H_2) by decarboxylation of formic acid.

In mixed acid metabolism of glucose the overall reaction is more complicated than (2.8):

$$C_6H_{12}O_6 \rightarrow \alpha\text{HLac} + \beta\text{HCOOH} + \gamma CO_2 + \delta\text{HAc} + \varepsilon C_2H_5OH + (2+\delta)\text{ATP} \tag{2.9}$$

The stoichiometric coefficients in (2.9) are not fixed quantities as in (2.8), but they change with the environment, i.e., the conditions under which the fermentation is carried out. If the substrate, glucose, is available at high concentration in the medium, then all the coefficients β to ε are practically zero (homo-lactic fermentation), but for very low glucose concentrations in the medium, a large part of the carbon flux from pyruvate is diverted toward the products in the lower part of the network. The term "very low glucose concentration" is quantified in Chap. 7 to be in the mg L^{-1} (ppm) range. In Chaps. 3 and 5, the ratios between the coefficients in (2.9) will be determined by carbon and redox balances.

Coenzyme A, CoA, is a large organic molecule with a structure similar to ATP (Fig. 2.2a). At the end of the long chain on the left is a thiol group that can form thioesters with carboxylic acids. It plays a crucial role, both in catabolic reactions and in anabolic reactions, e.g., in the synthesis of long-chain fatty acids.

Acetyl-CoA, abbreviated to AcCoA, is the ester with acetic acid, HAc.

$$CH_3COOH + \text{CoA} \rightarrow CH_3CO - S - \text{CoA}(\text{Acetyl CoA}) + H_2O \tag{2.10}$$

Acetyl-CoA is converted to Acetyl-P and CoA by reaction with phosphate (P):

$$\text{Acetyl CoA} + P \rightarrow CH_3CO\,O\,PO_3(\text{Acetyl} - P) + \text{CoA} \tag{2.11}$$

Finally, Acetyl-P is hydrolyzed by acetate kinase, to give acetic acid (HAc) + ATP. Thus, if the only metabolic product from anaerobic degradation of glucose could be HAc, the ATP yield per molecule of glucose would double, from two to four ATP per glucose. It is, however, impossible[5] in anaerobic fermentation to have "homo-acetic" metabolism of glucose ($\delta = 2$ in (2.9)) since the other product, HCOOH or CO_2, from the breakdown of pyruvate is less reduced than HAc, and ethanol which is more reduced than HAc must necessarily accompany the formation of HAc. The detailed discussion of this topic is deferred to Chap. 5.

Figure 2.5c shows the, apparently, much simpler looking reduction of pyruvate to final products in the anaerobic fermentation of glucose by the *eukaryote, S. cerevisiae*.

All EMP pathway reactions take place in the cytosol. The decarboxylation (8) of pyruvate to acetaldehyde is followed by reduction (7) to ethanol also takes place in the cytosol. As a side reaction the acetaldehyde can be oxidized to HAc (9), and the final metabolic products, ethanol and HAc are excreted to the medium from the cytosol.

Yeast is a so-called "higher" organism than the bacteria (*prokaryotes)*. The metabolism takes place in different compartments of the cell. One such compartment, separate from the cytosol, is the *mitochondrion* where the TCA cycle reactions (Sect. 2.2.4) occur. In Fig. 2.5c reaction (2) transfers part of the pyruvate to the mitochondria where it is converted to AcCoA with the stoichiometry (2.7).

In reaction (10), HAc is activated to cytosolic AcCoA which may either be used for lipid production in the cytosol, or it may be transferred to mitochondrial AcCoA for further use in mitochondrial processes.

To sum up: *In yeast the anaerobic fermentation of glucose has ethanol as its main product*:

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 + 2ATP \tag{2.12}$$

Other metabolic products of anaerobic yeast fermentation are HAc, acetaldehyde, and TCA cycle metabolites. All these compounds are more oxidized than ethanol. Some NADH must be consumed in a side reaction in order to balance the total NADH production since only the NADH from the EMP pathway is taken care of by reaction (7) in Fig. 2.5c. The pathway in which the extra NADH is consumed is that leading from dihydroxyacetone phosphate, DHAP, to glycerol[6]:

$$(CH_2OP)COCH_2OH + NADH \xrightarrow{(1)} CH_2OP\,CHOH\,CH_2OH \xrightarrow{(2)} (CH_2OH)_2CHOH \tag{2.13}$$

[5]Metabolism is full of surprises: Lengeler et al. (1999, p. 285) describes the anaerobic metabolism of glucose by to three HAc + four ATP. What happens is that CO_2 from decarboxylation of pyruvate is reduced to Acetyl-P using the two NADH created in the EMP pathway.

[6]To save space in writing the stoichiometries only the reduced form of the redox carrier and the activated form (ATP) of the energy carrier are shown in this chapter. H_2O is generally left out. The stoichiometric coefficient for H_2O can be found from an O and an H balance (see Chap. 3).

Reaction (1) is catalyzed by glycerol 3-phosphate dehydrogenase.[7] The hydrolysis reaction (2) is catalyzed by the non-ATP-generating glycerol 3-phosphatase (also called glycerol 1-phosphatase).

The metabolism of glucose to glycerol via the DHAP pathway is summarized as:

$$C_6H_{12}O_6 + 2ATP + 2NADH \rightarrow 2CH_2OH\,CHOH\,CH_2OH \qquad (2.14)$$

As a result of the discussion in Sects. 2.2.2 and 2.2.3, we have learnt how glucose, the preferred substrate of most microorganisms is converted by *anaerobic* fermentation to final metabolic products, some of which are of considerable economic value. Equations (2.8), (2.9), and (2.12) are examples of redox balanced overall pathways, while (2.14) requires balancing by other metabolic pathways. Production of ATP is of course the purpose of the processes when seen from the perspective of the organism.

Other sugars such as fructose, mannose, sucrose, lactose, and xylose can also be used as substrates. The transport processes whereby sugars are taken up to the cell by "facilitated" or by "active" transport will be discussed in Sect. 7.7. Inside the cell disaccharides hydrolyze to monosaccharides (sucrose to glucose and fructose, lactose to glucose and galactose), and phosporylation of one of the hexoses takes place while the other may be excreted. Certain C5 sugars, such as xylose are easily utilized by bacteria while their utilization by yeast is difficult, leading to a very slow fermentation.

The important question of general carbon and energy source utilization is generally outside the scope of this text and it will only be addressed in problems.

2.2.4 *The TCA Cycle: Provider of Building Blocks and NADH/FADH$_2$*

The main entry point to the tri-carboxylic acid cycle (TCA cycle) is via AcCoA which is synthesized by reaction (2.7) from pyruvate, the product of the EMP pathway. To understand the working of the TCA cycle one can imagine that one pyruvate enters through reaction (1) in Fig. 2.7, while another pyruvate enters via

[7]There are actually two cytosolic enzymes coded by different genes, GDPH1 and GDPH2. One enzyme is concerned with redox regulation in the cell whereas the other gives osmotolerance to the yeast when it grows in high salt concentrations (or at very high glucose concentration, e.g., in wine making). The glycerol production at the expense of some ethanol and biomass increases the quality of many wines, and yeast with overexpression of the gene is used by some winemakers (Remize et al. 1999). In the present text we are only concerned with the redox regulation role of GDPH to counteract the production of less reduced metabolic products, especially succinic acid, and the production of NADH when glucose is converted to biomass.

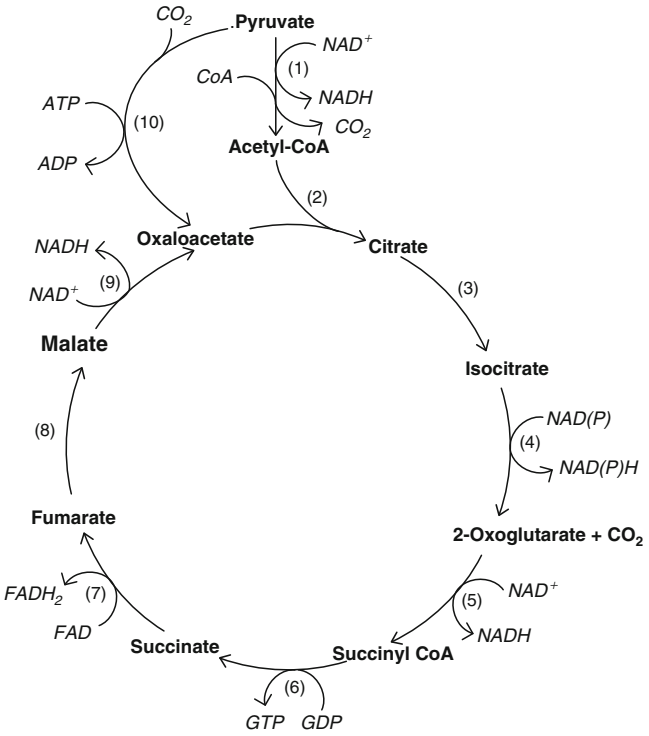


Fig. 2.7 The TCA cycle and the pyruvate carboxylation shunt. To simplify the diagram CoA is not shown. The enzymes are (*1*) pyruvate dehydrogenase; (*2*) citrate synthase; (*3*) aconitase; (*4*) isocitrate dehydrogenase; (*5*) 2-oxoglutarate dehydrogenase; (*6*) succinate thiokinase; (*7*) succinate dehydrogenase; (*8*) fumarase; (*9*) malate dehydrogenase; (*10*) pyruvate carboxylase. In Chap. 5 (Fig. 5.9) the "glyoxylate shunt" will be discussed. The shunt connects isocitrate and malate by the reactions: isocitrate → succinate + glyoxylate, and glyoxylate + AcCoA → malate, using the enzymes (11) isocitrate lyase and (12) malate synthase. Glyoxylic acid is CHO–COOH

the "side door," reaction (10), where it is carboxylated to oxaloacetate. By reaction (2) in Fig. 2.6 citric acid is synthesized, (2.15):

$$\text{Pyruvic acid} + CO_2 \xrightarrow{(10)} \text{Oxaloacetic acid} \xrightarrow{(2)} \text{Citric acid} + \text{pyruvic acid} \qquad (2.15)$$

Following the TCA reactions clockwise from citric acid, the six-carbon tricarboxylic acid is converted by a series of oxidation reactions[8] to the four-carbon

[8]Note in Fig. 2.7 that there are two forms of isocitrate dehydrogenase. One form which is located only in the mitochondria uses NAD^+ as cofactor, the other is located both in the cytosol and in the mitochondria, and it uses $NADP^+$ as cofactor. Equation (2.16) is written for the first form and holds for yeast, while bacteria must operate with the $NADP^+$ requiring cytosolic enzyme. In fact this becomes an advantage when we produce, e.g., amino acids in bacteria, since the NADPH obtained in reaction (4) in Fig. 2.7 is needed in the biosynthesis of the amino acid (see Sect. 2.4).

dicarboxylic acid, oxaloacetic acid. Two carbons are lost to CO_2 if none of the intermediates is drained off the cycle to enter into other pathways, e.g., synthesis of amino acids. The net result is that *one pyruvate is completely ground up to form 3 CO_2*. The oxaloacetate is ready to accept a new pyruvate for the next round of the cycle.

Thus, if no intermediates from the TCA cycle are used for other purposes the net result of one turn of the TCA cycle is:

$$CH_3CO\,COOH \xrightarrow{(+3H_2O)} 3CO_2 + 4NADH + FADH_2 + GTP \tag{2.16}$$

Five redox packages (=5"H_2") and one energy package (GTP, see Sect. 2.2.1) are produced in addition to the "waste product" CO_2.

In *aerobic* processes, and if the microorganism has a functioning *respiration system*, the redox carriers are oxidized by the use of molecular O_2, and large amounts of ATP are produced.

In *anaerobic* processes, the TCA cycle functions exclusively to produce building blocks for cell synthesis. The activity of the TCA cycle will be very low, and the cycle is likely to operate both clockwise and counter-clockwise in order to balance the redox equivalents produced on the right hand side of the cycle with those consumed counter-clockwise on the left hand side.

In the TCA cycle, one finds several of the sugar-based building blocks for the bio-based production of bulk chemicals in Table 2.1 and Fig. 2.1b. Succinic acid is a raw material for the polymer industry, 2-oxoglutarate and oxaloacetic acid are starting points for production of amino acids, and citric acid is one of the most important products of the food industry with an annual production of more than 1 M ton.

When one of the TCA cycle intermediates is desired to be the main product of the fermentation the microorganism is selected or engineered to give a high flux away from the TCA cycle at the right point. Thus, *Asperillus niger*, a filamentous fungus which is the main work-horse for production of citric acid, is operated such that very little carbon is allowed to pass further than to citrate. This means that building blocks for cell synthesis such as 2-oxoglutarate, succinic acid, and oxaloacetate are formed at a minimum rate, just enough to keep the culture alive. The cell functions more or less as a complex catalyst that converts glucose to citric acid.

In Chaps. 3 and 4, it will be shown that the ratios between the production rates of different end products are quantified by *yield coefficients*. Hence, in citric acid production the yield of citric acid on glucose is desired to be as high as possible, at the expense of the yield of biomass on glucose. Some carbon must, as mentioned above be used to maintain vitality of the cell culture, and in *Aspergillus* which produces the main part of its ATP by respiration, even the most efficient citric acid process must allow a certain production of NADH/$FADH_2$ in the TCA cycle.

By the same arguments it is not optimal in an amino acid production process to allow the TCA cycle to use almost all the glucose fed to the organism. In lysine production, it is desirable to have a high flux to 2-oxaloglutarate from where

the carbon is drained off to form glutamate, the primary amino donor for the biosynthesis of most amino acids (see (2.22) and (2.23)).

Some glucose has to be diverted to form the redox carrier NADPH which is used in the amino acid synthesis pathways. The primary source of NADPH is the pentose phosphate (PP) pathway, Sect. 2.2.6, and the ratio between the carbon flow to the PP pathway and to the TCA cycle via pyruvate must be fine-tuned to get the best yield of lysine on glucose.

The main entry to the TCA cycle is, as mentioned earlier, by reaction (2) in Fig. 2.7. Another entry point is by reaction (10), carboxylation of pyruvate, which was used to illustrate how the cycle regenerates oxaloacetate after consumption of one pyruvate molecule. The entry to the reductive part of the TCA cycle via pyruvate (PYR) to oxaloacetate (OOA) is useful when only the metabolites in this branch are needed, e.g., in anaerobic processes.

A similar shunt from OOA to PEP plays a part in gluconeogenesis where the high energy barrier from PYR to PEP must be overcome. OOA, a degradation product from amino acids, is decarboxylated to PEP using one GTP. The reverse reaction PEP to OOA proceeds without the use of ATP.

Besides the cyclic path between oxaloacetate and the last two metabolites of the EMP pathway there are other cycles and shunts in the TCA cycle. The tasks of the TCA cycle are many, and it has to operate properly both for prokaryotes where all TCA cycle reactions occur in the cytosol and for eukaryotes where the TCA cycle reactions occur in the mitochondria, and substrates and products have to be transferred between the cytosol and the mitochondria. Furthermore, the TCA cycle must operate both for conversion of glucose to products, and for synthesis of glucose from the products, i.e., for supplying essential nutrients to fasting animals.

To appreciate the full significance of the many variations in TCA cycle metabolism the reader must consult standard texts in biochemistry. In the present context where synthesis of products from sugars is the main theme, the important duties of the TCA cycle are summarized as follows:

1. Deliver NADH and $FADH_2$ as substrates for respiration.
2. Deliver metabolites that will be used as building blocks for cell synthesis.

2.2.5 *Production of ATP by Oxidative Phosphorylation*

The bacteria and the yeast *Saccharomyces cerevisiae* discussed in Fig. 2.5a–c are all *facultative anaerobes*. This means that *E. coli, lactic acid bacteria* (there are many families), and *S. cerevisiae*, are able to grow both in the presence of molecular O_2 (*oxic growth*) and without O_2 (*anoxic growth*). This is a great advantage in industrial fermentation, since both the aerobic and the anaerobic fermentation paths can be used in the screening for the best production organism.

Other microorganisms (all of them prokaryotes) will die in the presence of O_2. These are called *obligate anaerobes*, and examples are *Clostridium* sp., the soil bacteria *Actinomyces*, and methanogenic bacteria.

The facultative anaerobes can generate free energy (ATP) by the use of O_2 as an electron acceptor. The obligate anaerobes can use other electron acceptors such as SO_4^{2-}, NO_3^-, and metal ions (e.g., Fe^{3+}), or in the case of methanogens, fatty acids, which are reduced to CH_4.

The evolutionary advantage of facultative anaerobes is that (besides the other electron acceptors) they can also use O_2, an abundantly available compound in the atmosphere and in the marine environment. Evolution has provided these organisms with protection agents against the lethal byproducts of oxic growth, e.g., the enzymes *catalase* and *manganese-superoxide dismutase* which are able to scavenge the toxic oxygen radicals that are formed in small quantities in aerobic metabolism.

Once O_2 is available, the facultative anaerobes are able to switch very rapidly from anaerobic to aerobic growth. The changes in metabolism, which are tightly regulated to avoid a dramatic and unhealthy utilization of building blocks from cell synthesis to an "energy feast," are found in many places in the metabolic network. Thus the operation of the TCA cycle will be almost exclusively in the clockwise direction in Fig. 2.7. The fermentative pathways, Fig. 2.5a–c, are down-regulated, and the activity of some enzymes, e.g., pyruvate formate lyase, (3) in Fig. 2.5 a, b, is immediately quenched. In *E. coli*, this has the effect that the pyruvate is degraded, not in the fermentative pathways to give formic acid, ethanol, and acetic acid, but in respiration via the TCA cycle. Lactic acid bacteria which lack an essential part of the electron transfer chain, *cytochrome-c*, produce AcCoA via reaction (2). The total two NADH per glucose which is thus generated can be oxidized in the presence of molecular O_2 using the enzyme *NADH-oxidase*, and as described in Sect. 2.2.3, the lactic acid bacteria can now gain four ATP per mole of glucose and secrete HAc as the only fermentation product. In contrast to *S. cerevisiae* almost all other yeasts are *obligate aerobes*, i.e., they *only* grow in the presence of O_2.

With this introduction to oxic growth we shall now turn to *oxidative phosphorylation* (or *respiration*), the mechanism by which evolutionarily privileged organisms (including humans) are able to reap much more energy from consumption of glucose.

A glucose molecule that has been completely metabolized to CO_2 in glycolysis and through the TCA cycle has obtained a total of 12 reduced redox cofactors:

$$C_6H_{12}O_6 \rightarrow 6CO_2 + 2NADH + (8NADH + 2FADH_2) + 2ATP + GTP \quad (2.17)$$

The oxidized forms of the cofactors are regenerated through a series of steps in the respiratory chain, the so-called *oxidative phosphorylation* process:

$$NADH + 1/2\,O_2 \rightarrow NAD^+ + (P/O)_{NADH}ATP \quad (2.18)$$

$$FADH_2 + 1/2\,O_2 \rightarrow FAD + (P/O)_{FADH_2}ATP \quad (2.19)$$

In Chap. 4, it will be shown that the oxidation of NADH to NAD and H_2O at physiological conditions is accompanied by a change in free energy of about 220 kJ. The activation of 1 mol of ADP to ATP requires 30.5 kJ free energy at "standard conditions," and in Chap. 4 the free energy needed to produce 1 mol of ATP

at physiological conditions will be discussed. At standard conditions and at a thermodynamic efficiency of 100%, about 7 mol of ATP should be synthesized per mole of NADH oxidized. Studies of the electron transfer chain in the respiratory system conducted over the last 70 years have shown that *theoretically* three ATP should be synthesized by reaction (2.18) if all (three) steps in the electron transfer chain are used, while $FADH_2$ which passes through only the last two steps of the chain has a *theoretical* yield of two ATP.

Since the stoichiometric coefficient P/O for ATP (called "the P/O number") is at most 3 the conversion of H_2 to H_2O has a theoretical thermodynamic efficiency of $91.5/220 = 0.42$.

Still, 42% is a remarkable efficiency compared to the efficiency of free energy ("electricity") generation in even the most elaborate modern H_2-based fuel cells. The efficiency is even more remarkable when considering that the $NADH/FADH_2$ originates from "combustion" of glucose to CO_2, a process which human ingenuity cannot hope to replicate with a thermodynamic efficiency that even approaches that of the microbial metabolism.

In actual fermentation practice, the theoretical P/O ratio of (2.18) and (2.19) is not obtained. Experimental studies of aerobic yeast fermentation (to be discussed in Chap. 5) have shown that an overall P/O number of 1.2–1.3 is obtained.

Also with the lower values for P/O, an ATP generation of about $1.25 \times 10 = 12.5$ mol ATP/mol glucose in the respiration of yeast cells is a remarkable improvement on the two ATP gained by glycolysis of glucose to pyruvate and further to ethanol.

In fact, oxidative phosphorylation, whether it is carried out in the space between the cell membranes in *E. coli* or in the mitochondria of eukaryotes, is a remarkable "invention" of nature that permits living organisms with a functioning respiratory chain to consume much less sugar than those organisms which must create all their growth energy by substrate level phosphorylation.

The regulation of the carbon utilization in the cell that was mentioned in the introduction to Sect. 2.2 prevents all the carbon being diverted to an unnecessary and potentially dangerous overproduction of ATP by respiration. Both *E. coli* and yeast have an *overflow metabolism*. At high glucose flux down the EMP pathway (i.e., a high glucose concentration in the medium) some of the pyruvate is directed toward the fermentative pathways. *E. coli* will produce acetate, formate, and ethanol when the glucose concentration in the medium exceeds a certain low level, and at the same time the oxygen consumption reaches an upper, constant level (see Example 7.3).

In yeast fermentation it has been shown that even at low glucose concentration there is a substantial flow of pyruvate into the fermentative pathway in Fig. 2.5c, but up to a critical (also quite low) level of glucose the carbon is shunted back into the mitochondria by reaction (10) in Fig. 2.5c and degraded in the TCA pathway. When the critical glucose level is reached the oxygen uptake rate becomes constant, and an increasing portion of the carbon ends up as ethanol. This is called the *Crabtree effect*, and it sets in also when there is plenty of oxygen available. A corresponding phenomenon, the *Pasteur effect*, is seen when oxygen is scarce, and some of the

carbon *must* be sent toward ethanol. The Pasteur effect is consequently not a metabolic overflow mechanism, but results from an inadequate O_2 supply.

The Crabtree effect will be discussed several times in this text, first in Example 3.5.

The biology and the complicated biochemistry that ensures a highly efficient free energy production in the respiratory system has been the subject of a vast number of theoretical and experimental studies during the last 60 years. In the context of this textbook it is probably enough to be familiar with the few major results outlined above. In Chap. 4, the thermodynamics of the electron transport chain and the reactions by which ATP is generated will be briefly touched upon to give some understanding of the fascinating work of the brilliant scientists who contributed to our present knowledge of the system. There are still some ambiguities in the more or less accepted explanations, and further study of respiration is surely going to reveal new layers of fundamental research problems.

2.2.6 *The Pentose Phosphate Pathway: A Multipurpose Metabolic Network*

The pentose phosphate (PP) pathway shown in Fig. 2.8 is in reality a network of tightly regulated reactions. There is one entry point, G6P, from the EMP pathway and at several points metabolites from the pathway, identical to those formed in the EMP pathway (F6P and three GAP), are sent back to the energy creating EMP pathway. Metabolites are drained off the PP pathway to be used as substrates for production of key products in cellular metabolism. NADPH, a product from the first two reactions of the PP pathway is used to provide reducing power in a number of anabolic reactions. Two ribulose-5 phosphate (Ru5P) molecules react to form ribose-5 phosphate (R5P) and xylulose-5 phosphate (Xu5P). The ten carbons in R5P and Xu5P are "reshuffled" to one 7-carbon sugar, sedoheptulose phosphate (S7P), and the 3-carbon GAP, which may, again through a reshuffling process, produce F6P and a 4-carbon sugar, erythose-4 phosphate (E4P).

In long pathways, and interacting with different amino acids, R5P is converted to adenine and guanine ribonucleotides which we recognize in the structures of the energy and redox carriers of Fig. 2.2. Genetic material (RNA and DNA) are products of polymerization reactions of these compounds. E4P is one of the substrates for synthesis of the aromatic amino acids, phenylalanine, tyrosine, and tryptophan. Hence, in the industrial production of pharmaceuticals (L-DOPA and related products from tyrosine, and artificial sweeteners from phenylalanine) the PP pathway is engineered to give a high yield of E4P compared to the other products of the pathway.

The many different purposes of the PP pathway explain the complexity of the constituent reaction network. In a situation where cellular growth is at a minimum the carbon flux into the PP pathway at G6P is very low, and the full potential for production of energy and of primary metabolites such as lactic acid and ethanol from glucose is realized. When production of amino acids is desired, NADPH is the

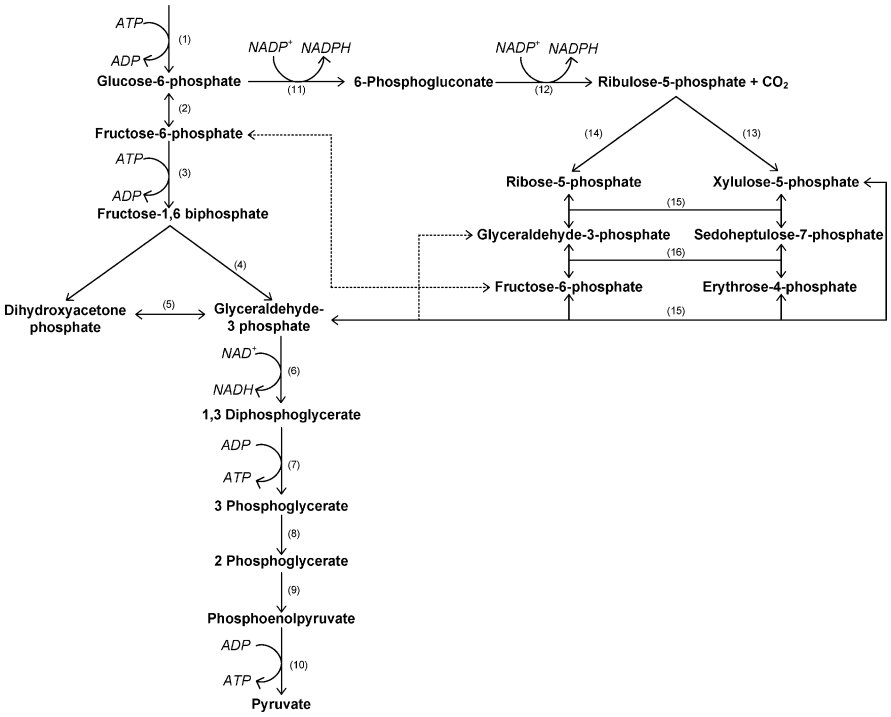


Fig. 2.8 The Pentose Phosphate pathway (PP) seen as an appendix to the EMP pathway (Fig. 2.4a, b, reactions (*1*)–(*10*)). The enzymes in the PP pathway are (*11*) G6P dehydrogenase, (*12*) 6-phosphogluconate dehydrogenase, (*13*) ribulosephosphate 3-empimerase, (*14*) ribosephosphate isomerase, (*15*) transketolase, and (*16*) transaldolase. Transaldolase can catalyze two reactions (**1**) interconversion of xylulose 5-phosphate and ribose 5-phosphate to GAP and sedoheptulose 7-phosphate and (**2**) interconversion of xylulose 5-phosphate and erythrose 4-phosphate to GAP and F6P. Carbon that is not used in the PP pathway reenters the EMP pathway as GAP and F6P

main product of the PP pathway, and the remainder of the carbon is sent back to the EMP pathway as GAP and F6P:

$$3\text{G6P} \rightarrow 6\text{NADPH} + 3\text{CO}_2 + 2\text{F6P} + \text{GAP} \tag{2.20}$$

The F6P can return to G6P via the reversible reaction between F6P and G6P, and more CO_2, GAP, and NADPH are produced in a new round of the *oxidative* part of the PP pathway.

The net result of recycling F6P produced by (2.20) until all F6P is used up is that 3/2 G6P is metabolized to 9 CO_2 and 18 NADPH while 3/2 G6P is metabolized to 3 GAP, i.e., that

$$\text{C}_6\text{H}_{12}\text{O}_6 \xrightarrow{\text{ATP}} 6\text{CO}_2 + 12\text{NADPH}, \text{ and } 2\text{C}_6\text{H}_{12}\text{O}_6 \xrightarrow{\text{ATP}} 4\text{GAP} \tag{2.21}$$

Thus, in circumstances where much NADPH is needed, the PP pathway serves the same purpose as the combined EMP and TCA cycle pathways: One carbon atom

of glucose is combusted to give one CO_2. The free energy in the glucose carbon is preserved in either two NADPH or two NADH.

The application of the two products in cell metabolism is very different. NADPH is used in synthesis reactions, but cannot provide growth energy for the cell by respiration. Only NADH can serve this purpose.

2.2.7 *Summary of the Primary Metabolism of Glucose*

In Sects. 2.2.2–2.2.6, the degradation of glucose through a number of pathways has been followed. All the pathways, except perhaps the PP pathway, serve the purpose of releasing energy for growth of the microorganism, while building blocks for synthesis of cell mass components are drained off the catabolic pathways at rates that depend on the conditions of the fermentation.

In the EMP pathway several intermediate metabolites are diverted to form building blocks, e.g., 3-PG in Fig. 2.4b to form the amino acid *serine*, and serine is used to synthesize other amino acids. Likewise, in anaerobic yeast fermentation it is difficult to avoid a flux via DHAP to glycerol in order to remove NADH. The carbon that leaves the EMP pathway and the TCA cycle is lost for energy production.

The fermentative pathways, Sect. 2.2.3, are necessary in anaerobic fermentations to remove NADH produced in the EMP pathway. Their final products, HLac, ethanol, formic acid, and HAc are not used as building blocks for cell synthesis, and from the perspective of the organism these *primary metabolites* must be considered as waste products.

In many industrial processes, the objective is to maximize the production of primary metabolites and other compounds that are not needed in anabolic catabolism.

Catabolism can be almost decoupled from anabolism, and then the primary metabolic metabolites are the sole sinks for the sugar substrate. For many hours after the biomass concentration has grown to a suitable level and feed of an essential nonsugar substrate has been stopped, the biomass continues to produce the desired product using the ATP that is always obtained as part of the sugar metabolism to "maintain" the organism (see Sect. 5.2) and possibly also to supply ATP for continued production of the metabolite.

Feed of the nitrogen source is typically stopped, and synthesis of new functional biomass becomes impossible. For many hours thereafter, a lactic acid bacterium continues to produce HLac from the sugar source, *Xanthomonas campestris* continues to produce xanthan gum, and an engineered strain of *Bacillus subtilis* synthesizes hyaluronic acid, another moderate molecular weight polysaccharide used in the surgery and in the cosmetics industry.

The processes involved in the "natural life" of an organism, i.e., the anabolic metabolism to produce and maintain a viable cell, and the catabolic metabolism needed to produce energy to sustain the anabolic processes, are together named the *primary metabolism* of the organism. A few of the pathways of primary metabolism have been introduced in the previous sections. Obviously, a standard text in Biochemistry (e.g., Voet and Voet 1995) must be studied to get even a superficial

picture of the whole genome scale primary metabolism. The pathways introduced in this chapter are, however, sufficient to give at least some biochemical background for the quantitative treatment of carbon flux distribution in microorganisms (Chaps. 3–5) and the kinetics of bioreactions (Chaps. 7 and 9).

The literature refers to *secondary metabolism* (or *special metabolism*) along with the primary (or *basic*) metabolism. In secondary metabolism, metabolic products are synthesized in (often very long) pathways which are not absolutely necessary to maintain vitality of a culture *in normal circumstances*. β-Lactam antibiotics (penicillins), pigments, flavors, and the large group of polyketides (including many antibiotics) are filed under the common heading of secondary metabolites. It is difficult to make a clear distinction between metabolites of the primary and secondary metabolism, since some of the "secondary" metabolites and their pathways may play as a yet undiscovered role for the "normal" life of the organism. But definitely, when one of the secondary metabolites is the desired product, the pathways to its formation have to be carefully explored, and key enzymes must be identified and over-expressed in order to get an acceptable yield of the product. This is a major task for Systems Biology, and it requires much experimental and theoretical work.

In Sect. 2.3, a number of primary and secondary pathways are described in somewhat more detail. The goal is not to obtain a hands-on knowledge of how the pathways operate and how they can be optimized, but rather to give a glimpse of the complexity of microbial metabolism.

The discussion of the primary metabolism is supplemented by Tables 2.2–2.4 in which typical building blocks and the requirement for redox and energy carriers are listed.

Table 2.2 Precursor metabolites and some of the building blocks synthesized from the precursors

Precursor metabolite	Building blocks	Amount required (μmol (g DW)$^{-1}$)
Glucose-6-phosphate (G6P)	UDP-glucose, UDP-galactose	205
Fructose-6-phosphate (F6P)	UDP-*N*-acetylglucosamine	71
Ribose-5-phosphate (R5P)	Histidine, tryptophane, nucleotides	898
Erythrose-4-phosphate (E4P)	Phenylalanine, tryptophane, tyrosine	361
Glyceraldehyde-3-phosphate (GAP)	Backbone of phospholipids	129
3-Phosphoglycerate (3GP)	Serine, Cysteine, Glycine, Nucleotides, Choline,	1,496
Phosphoenolpyruvate (PEP)	Phenylalanine, tryptophane, tyrosine	519
Pyruvate (PYR)	Alanine, isoleucine, valine	2,833
Acetyl-CoA (AcCoA)	Lipids	3,747
2-Oxoglutarate (2OG)	Arginine, glutamate, glutamine, proline	1,079
Succinyl-CoA (SuCoA)	Hemes	–
Oxaloacetate (OOA)	Aspartate, asparagine, isoleucine, methionine, threonine, lysine, nucleotides	1,787

A number of important industrial products are found in the list of building blocks. The last column shows the amount of precursor metabolites needed to synthesize 1 g (dry weight = DW) of *Escherichia coli* (Lengeler et al. 1999, p. 115)

Table 2.3 Composition of *E. coli* cells grown at 37 °C on a glucose minimal medium at a specific growth rate $r_x = \mu = 1.04$ g cell formed per gram cell per hour (Ingraham et al. 1983), and the corresponding requirements for ATP and NADPH

Species	Content (g (g DW)$^{-1}$)	ATP (μmol (g DW)$^{-1}$)		NADPH (μmol (g DW)$^{-1}$)	
Protein	0.55	29,257	(21,970)	11,523	(0)
RNA	0.20	6,796	(2,146)	427	(0)
rRNA	0.16				
tRNA	0.03				
mRNA	0.01				
DNA	0.03	1,240	(450)	200	(0)
Lipid	0.09	2,836	(387)	5,270	(0)
Lipopolysaccharide	0.03	470	(125)	564	(0)
Peptidoglycan	0.03	386	(193)	193	(0)
Glycogen	0.03	154	(154)	0	(0)
Building blocks	0.04				
Total	1.00	41,139	(25,425)	18,177	(0)

Numbers in parenthesis for ATP and NADPH are for growth at the same conditions, but on a rich medium that contains all the necessary building blocks (amino acid, nucleotides, fatty acids, etc.). The ratio between NADPH and (NADP + NADPH), the anabolic reduction charge, is usually around 0.5, indicating a need for "preparedness" for the reductive macromolecular synthesis. The corresponding ratio for catabolic reduction charge NADH/(NADH + NAD^+) is small (<0.1) since the oxidized form of the redox carrier must be in ample supply. Data from Ingraham et al. (1983)

Table 2.4 Measured concentrations of AMP, ADP, and ATP in a continuous culture of *Lactococcus lactis*, a common lactic acid bacterium

Specific growth rate (h^{-1})	Substrate	[AMP]	[ADP]	[ATP]	Total adenylate pool	Energy charge
0.03	Glucose	12	17	25	54	0.62
0.48	Glucose	17	23	52	92	0.69
0.69	Glucose	15	27	50	92	0.69
0.15	Maltose	5	8	20	33	0.73
0.32	Maltose	12	23	41	76	0.69
0.58	Maltose	17	26	44	87	0.66

The data are given for different specific growth rates μ (Sjöberg and Hahn-Hägerdahl, 1989). Concentrations are in μmol (g DW)$^{-1}$. The energy charge is defined as $E_c = ([ATP] + (1/2)[ADP])/$total adelynate pool. The value of E_c is about 0.9 in healthy *E. coli* cells (Lengeler et al. 1999, p. 124), which makes the numbers in the table seem rather small, especially at high μ

Table 2.2 shows that the drain of carbon to form building blocks is relatively modest from EMP pathway intermediates, except perhaps from 3GP. Most building blocks are synthesized with pyruvate, AcCoA, and TCA cycle intermediates as precursors. Especially, AcCoA is seen to be an important precursor for the formation of fatty acids and acetyl groups. Pyruvate and the TCA cycle precursors are almost exclusively used to form amino acids as building blocks for protein synthesis.

Table 2.3 shows that protein synthesis requires much ATP, and the difference in ATP consumption between a so-called "minimum medium" and a "complex

medium" is surprisingly small. The reason is that polymerization of amino acids to form protein is very costly in ATP while the production of amino acids from precursor metabolites is relatively cheap.

2.3 Examples of Industrial Production of Chemicals by Bioprocesses

Any medium used as a feed for a fermentation process should satisfy the following requirements:

It should contain a carbon, nitrogen, and energy source.
It should contain all essential minerals and growth factors to ensure rapid growth and a high yield of the desired product.
It should be of a consistent quality and be readily available throughout the year.
It should as far as possible not cause operational problems, neither in the fermentation process (foaming, reduction of mass transfer from a gas phase), nor in the downstream processing.

In the industrial production of biochemicals, cheap media obtained as byproducts from the agricultural sector are used. Sometimes the complex media contain growth factors that promote growth or give the right flavor to the product (such as molasses for bakers' yeast production and for production of rum).

Table 2.5 lists some typical complex media components used in the fermentation industry together with some typical fermentation products. Sugar cane juice is a natural "full" substrate used in fermentations, especially in Brazil.

A defined medium contains all the substances needed for growth and in standardized concentrations following a recipe that may change from organism to organism and depends on whether the fermentation process is aerobic or anaerobic. Lactic acid bacteria which are unable to synthesize certain amino acids are supplied with these in the medium (in Nature the bacteria import the amino acids from degraded proteins in the surroundings). Anaerobically growing yeast needs a supplement of ergosterol (an essential component of cell membranes) and unsaturated fatty acids which can be synthesized by the yeast only at aerobic conditions. A defined medium may also contain defined concentrations of, e.g., an antibiotic for which the producing strain has been made resistant. This serves to ensure that the "wild-type" strain of the organism is kept at bay. The wild-type strain usually grows faster than the "production strain" with its heavy burden of overproducing a desired metabolite or a foreign protein, and it would otherwise out-compete the producing strain in the "fight" for the (limiting) substrate.

The defined media are much more costly than most complex media, but the seasonal variation of the composition of complex media can give quality variations in the product. The purification of the product may become a problem for complex media, and the risk of contamination (e.g., with toxins) is always present.

Table 2.5 Typical complex media used in the fermentation industry

Medium	Contents	Origin	Typical application
Corn steep liquor	Lactate, amino acids, minerals, vitamins	Starch processing from corn	Antibiotics
Corn starch	Starch, glucose	Corn	Ethanol, industrial enzymes
Barley malt	Starch, sucrose	Barley	Beer, whiskey
Molasses	Sucrose, raffinose, glucose, fructose, betain	Sugar cane or sugar beet	Bakers' yeast, ethanol
Pharma media	Carbohydrates, minerals, amino acids, vitamins, fats	Cotton seed	Antibiotics
Serum	Amino acids, growth factors	Serum	Recombinant proteins
Whey	Lactose, proteins	Milk	Lactic acid
Yeast extract	Peptides, amino acids, vitamins	Yeast	Enzymes

Corn steep liquor is a byproduct of the corn wet-milling industry. The shelled and air-cleaned corn is soaked ("steeped") counter-currently in water for about 45 h, whereby the structure is softened and partly broken down. The water leaving the process at the point of entry of the corn contains up to 50 wt% solids. It is combined with gluten and fibers and sold as fermentation medium (Ligget and Koffler, 1948)

In scientific studies, defined media (and often defined *minimal* media) are generally preferred since the quantity of specific components in the medium is known, and this makes it easier to apply mass balances for individual atoms (C or N) and to interpret the results.

2.3.1 Amino Acids

The production of amino acids is one of the oldest industrial bioprocesses. The production scale of these low value added products is huge, and the processes have been optimized both from an engineering standpoint (low energy input, efficient downstream recovery, and purification processes), and from a biological standpoint (detailed analysis of the metabolic pathways, followed by strain modifications, whereby the carbon flux has been directed toward the desired product).

The amino acids of Table 2.6 are traditionally classified into two groups, *essential* and *nonessential*. Mammals, including humans, are unable to synthesize certain amino acids which must therefore be supplied in the diet. These are the *essential* amino acids. In contrast, *nonessential* amino acids are synthesized, also in mammals, either directly from one of the common precursor metabolites of Table 2.2, 3-phosphoglycerate, pyruvate, 2-oxoglutarate and oxaloacetate, or indirectly, e.g., tyrosine can also be synthesized by hydroxylation of phenylalanine.

One may argue that arginine is a nonessential amino acid since it is actually synthesized in human cells, but in too small quantity to satisfy the needs of growing children. Similarly, histidine must be supplemented in diets for children, but is not

Table 2.6 The 20 physiologically important (L-) amino acids and their net-chemical formula (based on the undissociated acids)

Essential	Nonessential
Arginine ($C_6H_{14}N_4O_2$)	Alanine ($C_3H_7NO_2$)
Histidine ($C_6H_9N_3O_2$)	Aspargine ($C_4H_8N_2O_3$)
Isoleucine (C_6H_{13} NO_2)	Aspartate ($C_4H_7NO_4$)
Leucine (C_6H_{13} NO_2)	Cysteine ($C_3H_7NO_2S$)
Lysine ($C_6H_{14}N_2O_2$)	Glutamate ($C_5H_9NO_4$)
Methionine ($C_5H_{11}NO_2S$)	Glutamine ($C_5H_{10}N_2O_3$)
Phenylalanine ($C_9H_{11}NO_2$)	Glycine ($C_2H_5NO_2$)
Threonine ($C_4H_9NO_3$)	Proline ($C_5H_9NO_2$)
Tryptophan ($C_{11}H_{12}N_2O_2$)	Serine ($C_3H_7NO_3$)
Valine ($C_5H_{11}NO_2$)	Tyrosine ($C_9H_{11}NO_3$)

They all contain the elements C, H, N, and O. Cysteine and methionine also contain S. Phenylalanine and tyrosine contain a benzene ring, tryptophan an indol ring, and histidine an imidazol ring. All four aromatic amino acids have the amino acid, serine (2-amino-3-hydroxy propionic acid), in the 1′ position on the ring. The other 16 amino acids are derived from aliphatic carboxylic acids. In the text the name of the amino acid is given without the (L-)

needed for adults. Milk proteins contain all the essential amino acids in an ideal mixture for human diets.

Bacteria have an amino acid profile almost identical to that of salmon, chicken, pigs, and cattle. This is the reason why SCP, actually dried and heat-treated bacterial cells, is able to supply all the essential amino acids to the feed of these animals when the main protein source of the feed is suboptimal in its amino acid profile. SCP is a fine substitute for fish meal, a product that becomes difficult to obtain due to overfishing of the seas. It is also almost free of phosphorous (less environmental burden in aquacultures). Due to its industrial production from simple substrates its availability does not depend on the seasons, and it never contains toxins. SCP production is treated in Chap. 3, Chap. 5, and in problems to Chap. 9.

Glutamic acid and monosodium glutamate (MSG), $COOH–(CH_2)_2–HC(NH_2)–COONa$, is one of the largest volume chemicals produced by fermentation, with an annual production of 1.5 M ton. Glutamic acid is used in many sauces and in cheese, while MSG is used as a general flavor enhancer in the food industry. Glutamic acid is at a main junction in the cell synthesis of amino acids and in the degradation of proteins. The direct, and usually dominant path to glutamate is by reductive amination of 2-oxoglutarate, a TCA cycle metabolite produced from the precursor pyruvate. $NADP^+$ is generally used as the cofactor of reaction (4) in Fig. 2.7, but a variant of the enzyme, isocitrate dehydrogenase, accepts NAD^+ as cofactor (see footnote to Fig. 2.7). This direct path from 2-oxoglutarate to glutamate is called the *GDH-path* after the name of the enzyme, glutamate dehydrogenase (GDH).

The nitrogen required is often present in the medium as NH_4^+, and is taken up by the cell as NH_3. One ATP is used to transport the proton back to the medium. *GDH* has a low affinity for NH_3. This means that the saturation constant K_m for uptake of NH_3 by the enzyme is high (see Chap. 6), and the enzyme is therefore only effective at high intercellular concentration of NH_3.

A second synthesis route to glutamate is via glutamine, (NH_2)–CO–$(CH_2)_2$–HC(NH_2)–COOH, an amide of glutamic acid. Glutamine is formed from glutamate and NH_3 using the enzyme *glutamine synthetase* (GS), which has a much lower K_m for NH_3 uptake than GDH and therefore works also at low $[NH_3]$ in the cell, i.e., at nitrogen limitation in the medium. It does, however, require an extra ATP, and consequently the energy source, glucose, must be available in sufficient amounts.

The second step is the reaction between glutamine and 2-oxoglutarate using the enzyme *glutamine oxoglutarate aminotransferase* (GOGAT) to give two molecules of glutamate. The net effect of the *GS–GOGAT* pathway (2.22), (2.23) is that one glutamate has been synthesized from 2-oxoglutarate at low concentration of NH_4^+ in the medium, but at the expense of an ATP besides that needed to transport NH_4^+ over the cell membrane to form NH_3 inside the cell and transport H^+ back.

$$2-\text{oxoglutarate} + NH_3 + \text{NAD(P)H} \rightarrow (\text{glutamate}),\ \textit{GDH path} \tag{2.22}$$

$$\text{glutamate} + NH_3 + \text{ATP} \rightarrow \text{glutamine}, \textit{GS path} \tag{2.23}$$

$$\text{glutamine} + 2-\text{oxoglutarate} + \text{NADPH} \rightarrow 2\ \text{glutamate}, \textit{GOGAT path}.$$

Other amino acids can be synthesized from the corresponding oxo-acids (e.g., alanine, CH_3–HC(NH_2)–COOH) by amination of pyruvate), but only at high-medium concentrations of NH_4^+ due to high values of the dissociation constant K_m.[9]

A much better method is to use *transamination* of the oxo-acid with glutamate or glutamine as the NH_3 donor since the K_m value for the transamination reaction is much smaller than that for the direct amination. In this way, a large pool of glutamate or glutamine in the cell is used to feed the synthesis of other amino acids.

One example from the many amino acid synthesis paths where transamination via glutamate or glutamine is a key step is that of *Aspartate* from glutamate and the precursor molecule oxaloacetic acid. The enzyme is *aspartate(amino)transferase*:

$$\begin{aligned}\text{Oxaloacetate(OOA)} + \text{Glutamate} \rightarrow \text{Asparate}(&\text{COOH} - \text{CH}_2 \\ &- \text{HC}(\text{NH}_2) - \text{COOH}) + 2-\text{oxoglutarate}\end{aligned} \tag{2.24}$$

From aspartate and following a long path in which one pyruvate is incorporated, one finally arrives at *Lysine*:

$$\begin{aligned}\text{Aspartate} + \text{pyruvate} + 3\text{NADPH} + NH_3 \rightarrow \text{Lysine}(&\text{COOH} - \text{HC}(\text{NH}_2) \\ &- (\text{CH}_2)_3 - \text{CH}_2 - \text{NH}_2) + CO_2\end{aligned} \tag{2.25}$$

[9] In a very interesting process Hols et al. (1999) have used genetic engineering to convert a homo (lactic)fermenting *Lactococcus lactis* to become a homo(alanine) fermenting organism. Since alanine has a much higher sales price than lactic acid this is a splendid idea. The paper does, however, show that very high NH_3 concentrations are needed.

Actually, along the path from aspartate to lysine one encounters a branch point at the metabolite piperidine-2,6 dicarboxylate. From this branch point there are two branches that meet again at the final intermediate, meso-2,6 diaminopimelate, before lysine. One branch, the one summarized in (2.25), is a direct shunt from one branch point to the other. The second branch (the Succinyl CoA branch) has many steps, involves transamination by means of a second glutamate molecule, and interacts with the TCA cycle reaction between succinyl CoA and succinic acid. The direct path gives the highest yield of lysine on glucose, but it works best at high NH_3 level, where the facilitation of uptake of the second NH_3 by means of the glutamate/2-oxoglutarate cycle is not needed.

The synthesis of the enormously important amino acid lysine with an annual production of 1.1 M ton has of course given rise to an equally impressive scientific literature. A comprehensive reference is Wittmann and Becker (2007). Recently, Kjeldsen and Nielsen (2009) published a *genome scale* reconstruction of the *Corynebacterium glutamicum* metabolic network. In the increasing body of genome scale pathway reconstructions all supposedly relevant pathway reactions are included, but without specifying the finer (and usually unknown) details.

2.3.2 Antibiotics

The biosynthesis of amino acids is used as an example of the numerous chemicals that can be produced from the primary metabolism of microorganisms, either directly or after chemical transformation outside the cell. Many more examples will be discussed in the text or in problems.

Antibiotics are examples of *secondary metabolites*. As was mentioned in the introduction to Sect. 2.3, secondary metabolites do not have a direct function in the normal growth of the cell culture, but the production of any of the antibiotic chemicals is the cellular response to *stress signals*. Organisms competing with, e.g., a penicillin producing microorganism for a limited resource will have their capability to proliferate disrupted by penicillin secreted from the cell of the penicillin producer. Penicillin acts as an inhibitor of formation of peptidoglycan cross links in the cell walls of the competing organism.

Obviously penicillin and other antibiotics with this defense mechanism cannot have any effect on viral infections, since viruses have no cell wall. Likewise they are of little use in combating most fungal infections – e.g., penicillin cannot be used to treat either influenza or ring worm. Its predominant role in human and veterinary medicine is for treatment of bacterial infections.

Although penicillin and other antibiotics have no direct effect on the cells of animals, including humans, all antibiotics can provoke sometimes violent allergic reactions.

Continued use of the first antibiotics on the market has also spawned defensive mechanisms in many bacteria. Penicillin resistance was observed already in the 1950s and has led to the development of a continuous stream of new antibiotics.

Table 2.7 Four classes of antibiotics

Category	Examples	Application	Producer
β-Lactams	Penicillins, cephalosporins	Bacterial infections	Filamentous fungi
Polyketides	Erythromycin, tetracyclines	β-Lactam resistant infections	Actinomycetes (soil bacteria)
Glycopeptides	Teicoplanin	Last resort treatment bacterial infection	Actinomycetes
Antracyclines	Daunorubicin	Chemotherapy	Actinomycetes

The classification of natural products into categories and with respect to applications is difficult, but the listing in the table is at least indicative

Table 2.7 gives a rough classification of antibiotics currently on the market, and Fig. 2.9 shows how two of the early penicillin products, penicillin G and V, are synthesized.

The first step in the biosynthesis of β-lactams is the condensation of L-α amino adipic acid, L-cysteine, and L-valine to form the tripeptide α-aminoadipyl-cysteinyl-valenine, ACV. It is interesting that valine is incorporated as D-valine. The enzyme *ACV Synthase* (ACVS) has several functions: It forms the two peptide bonds and it epimirizes L-valine to the D-valine bound in ACV.

The next step is an oxidative ring closure of LLD-ACV to form isopenicillin N (IPN) which has the characteristic structure of β-lactams. The β-lactam ring is the square with three carbons and one nitrogen in the center of the molecule.

The enzyme *isopenicillin N synthetase* (IPNS) uses free oxygen as electron acceptor. IPN is not in itself a potent antibiotic compound, but based on IPN all the commercial penicillins and cephalosporins can be produced. The side chain (aminoadipic acid) can be cleaved off by the enzymatic action of an *acetylase* to form the "nucleus," 6-APA, which in itself has no antibacterial effect, but is an important commercial product used for the synthesis of other, more active β-lactams than (LLD)-IPN. In the presence of an *acyltransferase* (AT), the commercial products penicillin G and V are formed when benzyl-CoA or phenoxybenzyl-CoA (i.e., the organic acids activated by coupling to CoA) is added. Methicillin and oxacillin are examples of penicillins with other side chain organic acids.

All penicillins are susceptible to the action of β-lactamases secreted by bacteria as a defense mechanism, and over the last 60 years the pharmaceutical industry has struggled to invent new antibiotics that can effectively combat penicillin resistant bacteria. The defense mechanisms of the bacteria have become ever more ingenious as a result of the wholesale application of β-lactams in human and veterinary medicine. The deactivation of penicillin by cleaving off the side chain is supplemented by the gradual appearance of bacterial enzymes that disrupt the β-lactam ring, and the battle to stay ahead of the bacterial defense mechanisms has led to new lines of antibiotics.

The *cephalosporins* are synthesized by filamentous fungi related to *Penicillium crysogenum*, but also in different bacteria. In the last step of the biosynthesis the five member thiazolidine ring next to the β-lactam ring is expanded (using an *expandase* enzyme) to a six member dihydrothiazine ring (Fig. 2.10).

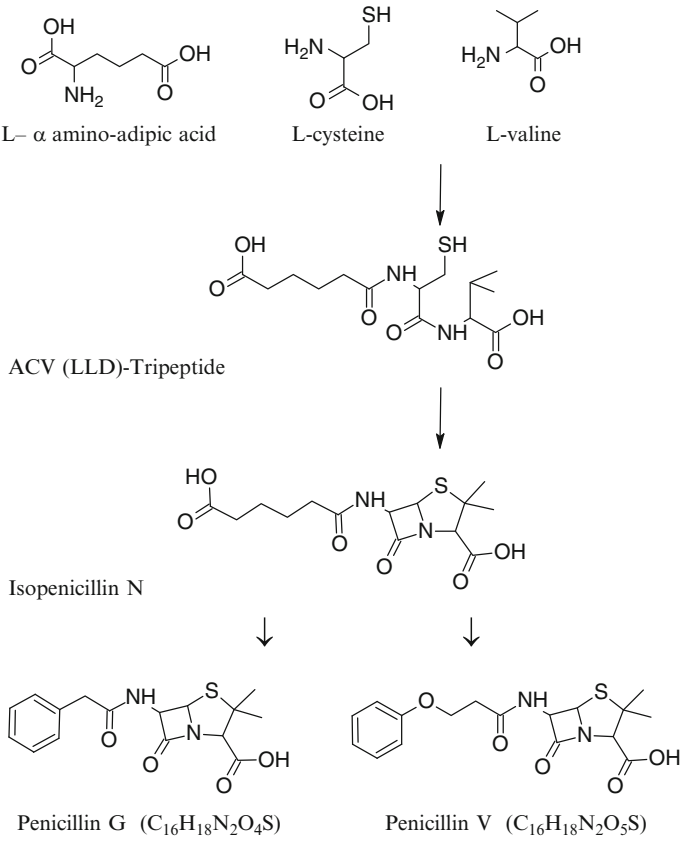


Fig. 2.9 The biosynthetic pathway in *Penicillium crysogenum* from the three amino acid precursors to penicillin. The enzymes involved are ACV synthase, isopenicillin N synthase, and isopenicillin *N* acyltransferase. In the last step adipic acid is exchanged with phenylacetic acid or phenoxy acetic acid to produce, respectively, penicillin G and V

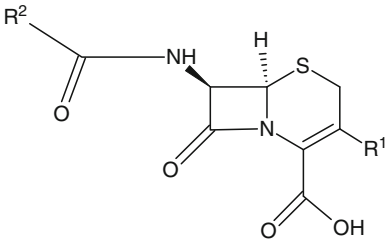


Fig. 2.10 The structure of cephalosporins. In new generations of cephalosporins R^1 and R^2 are very complicated side groups

The cephalosporins are also not immune to the action of β-lactamases, but their broad applicability to fight both Gram-positive and Gram-negative bacteria has won them a strong position in the antibiotics market through the development of several new "generations" of antibiotics with the general structure shown in Fig. 2.10.

The urge to develop new antibiotics that are immune to the very effective defense systems of many microorganisms (e.g., *Staphylococcus aureus*, the infamous organism that causes sometimes life-threatening infections in hospitals – about 500,000 cases each year in US hospitals) has spawned new categories of antibiotics which have a completely different structure from that of β-lactams.

A particularly numerous and varied category is the *microlides* and the *tetracyclines* which belong to the huge group of natural products called *polyketides*. All polyketides are synthesized by very complex *polyketide synthase* enzymes. In a series of chain elongation steps from CoA, activated carboxylic acids are formed, and then condensed to extremely complicated organic compounds. The synthesis mechanism has only been discovered during the last 20 years (Gokhale et al. 1999; Arora et al. 2005), and new polyketides, some with no known origin in nature, are now being synthesized by highly automated biosynthetic processes.

The polyketides have a very broad spectrum of activities: antimicrobial, antifungal, antiparasitic, and antitumor. Still, ever more complex drugs must be invented to combat the inevitable emergence of resistance. Combination of several antibiotics, e.g., glycopeptides in combination with β-lactams, is one way to fight multiresistant organisms (e.g., the use of clavulanic acid, a β-lactamase *inhibitor*, to support the action of β-lactams).

One unfortunate complication is that the new and very effective antibiotics become increasingly toxic to the patients, and they must be administered with great caution. The antracyclines are typical last-resort drugs used to combat life-threatening illnesses such as breast cancer.

The story of antibiotics, from the first observation by Alexander Fleming in 1928 of the antibacterial effect of a *Penicillium* fungus to the multibillion dollar antibiotics industry 80 years later is, indeed, fascinating. Historical accounts of the victory of antibiotics over illnesses and infections that were previously life threatening, followed by the sad aftermath of misuse of antibiotics, must be among the most educating in the whole history of science.

There is no doubt that antibiotics production is synonymous with the story of the development of modern Biochemical Engineering. Fermentation equipment has grown to huge tanks of sizes up to several hundred cubic meters, and many standard down-stream operation processes were developed to capture antibiotics from very dilute solutions and to purify the products to satisfy stringent regulatory requirements.

Antibiotics production has also been a huge money maker for the pharmaceutical industry during the last 60 years. Cephalosporins have gradually replaced penicillins, but as the production of both standard drugs has moved to China and India, prices have declined, and nonbrand antibiotics have put considerable pressure on the original producers. Furthermore, the use of antibiotics in veterinary medicine has been restricted, and the prophylactic use of antibiotics for humans is sternly discouraged.

Still, the value of antibiotics production in 2008 was in the range of 25,000 M US $, just about the level in 1995 (Nielsen 1997). The price of standard penicillins is now as low as 5–8 US $/kg which classifies these pharmaceuticals as commodity chemicals.

2.3.3 Secreted Proteins

Although the production of pharmaceutical proteins by mammalian cells is considered to be outside of what we have defined to be the scope of the present text, many industrial enzymes and some pharmaceutical proteins are produced by standard fermentation technology, using a small selection of microorganisms as producers. These large-scale industrial processes are studied and designed, using the same set of quantitative tools as are applied in the production of simple metabolites with *Lactococcus lactis, E. coli, B. subtilis, S. cerevisiae*, and *A. niger* or *oryzae* as production organisms. Also, it is relevant to introduce the foremost business area of some of the most important biotech companies, Novozymes, Genencor, Genentec, Amgen, Novo Nordisk, Sanofi-Aventis, Merck, Roche, and Eli Lilly.

The most common industrial enzymes are hydrolytic enzymes that degrade macromolecules to monomers that may serve as carbon, energy, and nitrogen sources. They are secreted by a wide variety of microorganisms. Among the most frequently secreted enzymes are *proteases* (for degradation of proteins) and *peptidases* (for degradation of peptides). The biggest production volume is obtained in *amylases* (degradation of starch), *xylanases* (degradation of xylans), and *cellulases* (degradation of cellulose), the three types of enzymes discussed in Sect. 2.1 for the production of ethanol from biomass.

Through the secretion of enzymes some microorganisms are able to grow on very complex nutrients, and the ability of microorganisms to decompose leaves and other plant materials plays an important role in the overall carbon cycle. The ability of microorganisms to secrete enzymes has been exploited for many centuries, particularly in the food and feed industry. Thus, *Aspergillus oryzae*, an efficient producer of the starch degrading enzymes *α-amylase* and *glucoamylase*, has been used for more than thousand years in the Far East for koji-sauce production. Furthermore, the secretion of proteases and peptidases by lactic acid bacteria plays an important role in many dairy processes. Primarily, hydrolysis of proteins and peptides ensures a supply of carbon and energy sources as well as amino acids. Secondly, the hydrolysis of many proteins and peptides is important for proper flavor development. Since the 1950s enzymes have also been used in detergents to improve the washing process, and with the help of enzymes many industrial processes, e.g., the treatment of cotton, have become truly "green" processes, with little impact on the environment and large savings in process energy compared to conventional processes. An overview of the application of industrial enzymes is found at http://www.novozymes.com. The majority of the industrial enzymes are produced, using a small selection of well-studied host cells into which foreign genes are encoded.

The possibility to introduce foreign genes into a microbial host by genetic engineering, and hereby to produce a specific protein in high amounts, also paved the way for a completely new route to pharmaceutical proteins. The first biopharmaceutical proteins (human insulin and human growth hormone) were produced in recombinant *E. coli*, but soon followed the exploitation of other expression systems such as *S. cerevisiae* (used by Novo Nordisk for production of about 50% of the global

market for human insulin), insect cells, and mammalian cells (Chinese hamster ovary cells and hybridoma cells). Today approximately 100 protein drugs, largely recombinant proteins and monoclonal antibodies that are often referred to as biotech drugs or biologics, produce revenues of hundreds of million US $. The biochemical processes underlying the synthesis of a successful *heterologous protein* in a given host cell are quite complex. There are many post-translational modifications, and the secretory pathway may involve many individual steps. Production of a given protein drug is specific for the host system and as mentioned at the start of this section even a brief overview of the subject is far beyond the scope of the present text.

2.4 Design of Biotech Processes: Criteria for Commercial Success

When a microorganism or cell type has been identified to produce an interesting compound there are a number of considerations to be made before an economically viable, industrial process can be realized. In companies with a solid experience in the design and scale-up of fermentation processes, new processes are introduced relatively fast, especially if the product is not a pharmaceutical. Thus, in recent years Novozymes has successfully introduced three to four completely new processes each year, starting in the laboratory, and carrying the R&D work all the way through to industrial scale production.

Development of a fermentation process can roughly be divided into four phases. First the product is identified, and its potential sales value over a prescribed lifetime is closely evaluated. In the case of a pharmaceutical, identification of a potential product may be a result of the random screening for different therapeutic effects by microbial metabolites, e.g., by high throughput screening of secondary metabolites from *Actinomycetes*, or it may be the result of a targeted identification of a novel product, e.g., a peptide hormone with known function. Outside the pharmaceutical sector the product may also be chosen after a random screening procedure, e.g., screening for a novel enzyme to be used in detergents, or it may be chosen in a more rational fashion. With the rapid progress in genomic sequencing programs, it is now possible to search for new target proteins directly in the sequenced genomes by skillful use of *bioinformatics*.

When the product has been identified, the next step is to choose or construct the specific strain to be used for production, and thereafter follows design of the process. This involves choosing an appropriate fermentation medium and the optimal process conditions. In parallel with the design of the production process further research, e.g., clinical tests, is done in order to have the product approved. When the strain has been constructed one of the first aims is therefore to produce sufficient cell material for further research, and this is typically done in pilot plant facilities. For a pharmaceutical compound sufficient material must be produced for clinical trials. For other products it may be necessary to carry out tests of the product and examine any possible toxic effects. The final steps are concerned with

product approval by the proper authorities. Then, perhaps 5 years after starting the process, follows construction of the production facility, sometimes by retrofitting of an existing plant to save on capital costs.

In the next sections, we consider some of the different aspects of process development as an introduction to the more detailed quantitative analysis that will be treated in the remainder of the book.

2.4.1 Strain Design and Selection

A key step in the development of a fermentation process is to choose an appropriate strain. In the past, this choice was normally obvious after the product had been identified, e.g., *Penicillin chrysogenum* was chosen for penicillin production since this was the organism that was first identified to produce penicillin. With the introduction of recombinant DNA technology it is, however, now possible to choose almost any host organism for the industrial production. Thus, strains of *E. coli* have been constructed that can produce ethanol at a high yield, and a recombinant strain of *Penicillin chrysogenum* can now be used directly to produce 7-ADCA (a precursor used for synthesis of cephalosporins) by fermentation. The choice of strain depends a lot on tradition within the company (e.g., Novozymes favors *B. subtilis* over *E. coli*), and most companies use a small set of favorite organisms to produce many different products. For production of a heterologous protein by expression of a foreign gene in a given organism, it is also necessary to consider many other aspects, e.g., whether the protein will be correctly folded and glycosylated.

Table 2.8 gives an overview of the advantages/disadvantages of different cellular systems for the production of recombinant proteins. Although optimization of the process continues even after large-scale production has started, it is important to choose a good host system from the beginning, particularly in the production of pharmaceuticals. The introduction of new strains requires a new approval of the process, and the associated costs may prevent the realization of a new process even though engineering wisdom tells that the over all process economy could be much improved.

As indicated in Table 2.8 the choice of expression system depends on many factors, but the main factors are (1) the desirability of post-translational modification and secretion, (2) the stability of the protein in question, and (3) the projected dose of protein per patient (which determines whether the cost of the drug becomes critical). Thus, for proteins used in large doses, such as human insulin, it is important that the production costs are kept low, which requires an expression system with a high productivity, i.e., *E. coli* or *S. cerevisiae*. For very complex molecules such as tissue plasminogen activator (tPA) and erythropoietin (EPO) it is, however, not possible to obtain sufficiently active compounds in microbial systems, and here a higher (mammalian) eukaryotic expression system is required.

Table 2.8 Pros and cons of different production organisms for recombinant proteins

Host	Advantages	Disadvantages
Bacteria (*E. coli*)	Wide choice of cloning vectors Gene expression easy to control Large yields possible Good protein secretion	Post-translational modifications lacking High endotoxin content Protein aggregation (inclusion bodies)
Yeast (*Saccharomyces cerevisiae*)	Generally regarded as safe (GRAS) No pathogens for humans Large scale production established Some post-translational modifications are possible	Less cloning vectors available Glycosylation not identical to mammalian glycosylation Genetic base is still less solid than is the case for *E. coli*
Filamentous fungi	Experience with large scale production Source of many industrial enzymes Excellent protein secretion	High level of heterologous protein expression is as yet not achieved Genetics only recently characterized
Mammalian cells	Same biological activity as natural protein Expression vectors available	Cells difficult to grow in reactors Expensive Slow growth and low productivity
Cultured insect cells	High level of gene expression possible Post-translational modification possible	Not always 100% active proteins Mechanisms largely unknown

2.4.2 *Criteria for Design and Optimization of a Fermentation Process*

The criteria used for design and optimization of a fermentation process vary with the product to be synthesized. Thus, the criteria used for a high volume/low value added product are normally completely different from the criteria used for a low volume/high value added product. For products belonging to the first category (which includes most whole cell products, all primary metabolites, many secondary metabolites, most industrial enzymes, and most polysaccharides) the three most important design parameters are:

- Yield of desired product on substrate (typical unit: g product per g substrate)
- Productivity (typical unit: g product per L reactor volume per hour)
- Final titer (typical unit: g product per L reactor volume)

Yield of product on the substrate is very important for high volume/low cost products since the raw materials often account for a significant part of the total costs. Thus in penicillin production, the costs of glucose alone may account for up to 15% of the total production costs. In Table 2.5 it was argued that the right choice

of fermentation medium could have a large influence not only on product yield and production cost, but also on product quality.

Productivity is important since this ensures an efficient utilization of the production capacity, i.e., the bioreactors. It is of no avail that certain bacteria are able to give a high yield of ethanol on sugar when the production rate of ethanol is very low. Especially in an expanding market, it is important to step up the productivity as has been demonstrated by Novozymes where a huge increase in production has been achieved and many new processes have been developed since 2000 with very little new capital investment.

Final titer is of importance for the further treatment of the fermentation medium, e.g., purification of the product. Thus, if the product is present at a very low concentration at the end of the fermentation it may be very expensive even to extract it from the medium with a satisfactory yield. Again the production of ethanol is a good example. Well designed strains of *S. cerevisiae* are now able to work efficiently at an ethanol concentration that surpasses 10 wt%, a limit that is far from being reached in bacterial fermentations where 4–5 wt% ethanol often stops the fermentation.

Sometimes it is important to consider both an aerobic process and an anaerobic process. The aerobic process may have higher productivity, but it requires more process energy.

In Chap. 3, we shall start the quantitative treatment of reaction rates and yield coefficients, the key design parameters for product yield on substrate and productivity of the process. Qualitatively, these concepts are illustrated in Fig. 2.3 which is a representation of the overall conversion of substrates into metabolic products and biomass components (or total biomass). In Sect. 3.1, the rate of consumption of any substrate is seen to be determined by measurement of the concentration of the substrate in the medium. Similarly, the rates of formation of metabolic products and biomass are determined from measurements of the corresponding concentrations. It is therefore possible to determine the rate of all flows in and out of the total pool of cells. The inflow of a substrate is normally referred to as the substrate uptake rate and the outflow of a metabolic product is normally referred to as the product formation rate. Clearly, the product formation rate is a direct measure of the productivity of the culture. Furthermore, the yield coefficient of any product is defined as the ratio of product formation rate and the substrate uptake rate. The yield coefficient quantifies the efficiency in the overall conversion of the substrate to the product of interest.

In the production of novel pharmaceuticals, which typically belong to the category of low volume/high value added products the abovementioned design parameters are normally not that important. For these processes time-to-market and product quality is generally much more important, and change of the process after implementation is often complicated due to a requirement of FDA approval. In the initial design phase it is, however, still prudent to remember the three design criteria: *Yield, Productivity*, and *Final Titer*. Especially the requirement for high final titer is important since the cost of purification (or "downstream processing") may account for more than 90% of the total production costs.

2.4.3 Strain Improvement

A key issue in process optimization is to improve the properties of the applied strain since the overall conversion of substrates to the product of interest is primarily determined by the properties of the microorganism. The cell may be conceived as a small chemical factory, and through engineering of the pathways it may be possible to redirect the carbon fluxes such that the yield and sometimes also the productivity increases. Engineering of the cellular pathways is done by making changes in the genome of the organism. This may lead to different expressions of the enzymes that catalyze the individual biochemical reactions or processes.

Traditionally, strain improvement through introduction of mutations was done through random mutagenesis and selection of strains with improved properties. This is well illustrated by the industrial penicillin production, where introduction of new strains has resulted in an increase of productivity by a factor of more than 100 between 1962 and 2000. The improvements are the results of large strain development programs, some carried out by the major penicillin producing companies and some by companies dedicated to develop new and better strains for the industry in general. Similar success stories are recorded for improvements in the properties of bakers' yeast, *S. cerevisiae*, and in other microorganisms applied for the production of industrial enzymes or metabolites.

The development of better producing organisms is, unfortunately, accompanied by a decrease in strain stability of the industrial strains. They will all mutate spontaneously to strains with a much reduced productivity, and this, as we shall see in Chap. 9 prevents the use of continuous cultivation in most of the important industrial fermentation processes. A typical example of strain reversion is studied by Christensen et al. (1995). An industrial strain of *P. crysogenum* was subjected to continuous culture for 500 h. For the first 150 h the penicillin V concentration in the exit from the reactor was constant, but over the next 200 h it decreased to a level of only 35% of the initial concentration. Clearly, the high producing strain had reverted to a much poorer strain. The reversion was also visually observed by a gradual change in the color of the spores from green to white. A similar case is studied for the teicoplanin producer *Actinoplanes teichomyceticus* (see Problem 9.7).

Today the genome of many of the favorite industrial "work-horses" has been sequenced, and in combination with the rapid development of recombinant DNA technology it has become possible to apply a rational, directed approach to strain improvement. This is often referred to as *metabolic engineering*, but terms such as molecular breeding or cellular engineering are also used. Several different definitions have been given for metabolic engineering, but they all convey the message that is captured in the definition: *The directed improvement of product formation or cellular properties through modifications of specific biochemical pathways or by introduction of new pathways using recombinant DNA technology*.

Among the applications of metabolic engineering are:

- *Heterologous protein production*. Examples are found in the production of pharmaceutical proteins (hormones, antibodies, vaccines, etc.) and in the

production of novel enzymes. As a first step, the heterologous gene needs to be inserted in the production host. Subsequently, the protein synthesis pathway must often be engineered, e.g., to have an efficient glycosylation or secretion of the protein. In some cases, it may also be necessary to engineer the strain to obtain an improved productivity.

- *Extension of substrate range*. In many industrial processes, it is interesting to extend the substrate range for the applied microorganism in order to use cheaper or more efficient substrates. Initially, the pathway (or enzyme) for uptake and metabolism of the desired substrate is inserted. Subsequently, it is important to ensure that the substrate is metabolized at a reasonable rate, and that the metabolism of the new substrate does not result in the formation of undesirable byproducts. This may in some cases involve extensive pathway engineering.
- *Pathways leading to new products*. It is desirable to use a given, well-studied host for production of many different products. This can be achieved by extending existing pathways from other organisms. An example is the import into *E. coli* of the yeast pathway from DHAP to glycerol, and of the pathway from glycerol to propane 1,3-diol from *Klebsiella pneumonica*. These are key features of the DuPont-Genencor process to produce the diol. Another approach is to generate completely new pathways through gene shuffling or by other methods of directed evolution. In both cases, it is often necessary to further engineer the organism to improve the rate of production and eliminate byproduct formation.
- *Pathways for degradation of xenobiotics*. Many organisms naturally degrade xenobiotics (i.e., compounds that are foreign to the body and to cells), but each organism is usually specific for a given substrate. Thus a consortium of bacteria, collected from an oil field or from a spot where seepage of bitumen from the ground is observed, is likely to be useful for cleaning up oil spills. In bioremediation it is attractive to work with only a few organisms, each of which is able to degrade a wide variety of compounds. This may be achieved either by inserting pathways from other organisms or through engineering of the existing pathways.
- *Engineering of cellular physiology for process improvement*. In the industrial exploitation of microorganisms or higher organisms one may engineer the cellular physiology for process improvement, e.g., make the cells tolerant to low oxygen concentration, less sensitive to high glucose concentrations, improve their morphology, or increase their ability to flocculate. In cases where the underlying mechanisms are known this can be achieved by metabolic engineering. This may involve expression of heterologous genes, disruption of genes, or over-expression of homologous genes.
- *Elimination or reduction of by-product formation*. In many industrial processes byproducts are formed. This constitutes a problem, not only because carbon is lost to formation of the byproducts, but also because the byproducts may be toxic to the organism, or necessitate a major effort to separate the byproduct from the desired product because it is toxic (e.g., oxalic acid from citric acid) or interferes with filtration and other downstream processes. Thus, polysaccharides severely interfere with membrane separation processes. In some cases the byproducts can be eliminated through simple gene disruption, but in other cases the formation of the

byproduct is essential for the overall cellular function, and disruption of the pathway leading to the byproduct may be lethal for the cell, e.g., when the pathway from DHAP to glycerol is knocked out in an attempt to improve the ethanol yield in anaerobic cultivation of yeast. It is often necessary to analyze a large part of the metabolic network in order to devise a safe strategy for reduction of the byproduct formation.

- *Improvement of yield or productivity*. In many industrial processes, especially in the production of low-value added products, it is important to continuously improve the yield and/or productivity. In simple cases this can be achieved by inserting additional gene copies of what is believed to be a key enzyme in the pathway. In other cases the pathways leading to the product of interest have many enzymatic steps which influence each other by feed back and feed forward control mechanisms (see Chap. 6), and a given pathway may even interact with other pathways through the global regulatory system of the organism. Now the single-enzyme manipulation is doomed to failure, and a much more involved analysis of the metabolism is needed to find prospective improvements of the process. Finally, in some cases the limitation is not in the actual pathway, and it may therefore be necessary to engineer the whole central carbon metabolism. This is, however, very difficult to do. For example, it is very difficult to achieve a higher carbon flux through the EMP pathway, due to the tight regulatory structure of the pathway.

Besides application of these tools from the biosciences, the design and proper operation of an industrial plant for low-value added products require a deep technical insight into many of the disciplines that together make up the science of chemical engineering: stoichiometric analysis, modeling of reaction kinetics, transport phenomena, bioreactor design, and unit operations. In this textbook we will, as announced in Chap. 1, highlight the chemical engineering disciplines that are associated with bioreactions and give a quantitative description of a given phenotype of a cell. It will be shown how the organism operates in, and interacts with the technical equipment. A comprehensive treatment of metabolic engineering is given in Stephanopoulos et al. (1998) while the engineering tools of the bioindustry are discussed in quite a few available texts.

2.5 The Prospects of the Biorefinery

In Sect. 2.1.2, the review by Werpy and Petersen (2004) was used to illustrate the enthusiasm that embraces any discussion of the prospects of the biorefinery to substitute chemical routes from oil and gas to commodity chemicals with “green” or “sustainable” routes based on the action of microorganisms or the enzymes produced by these.

Sects. 2.2–2.4 have shown how microorganisms can serve as chemical factories that basically convert solar energy harbored in the raw materials, sugar or starch,

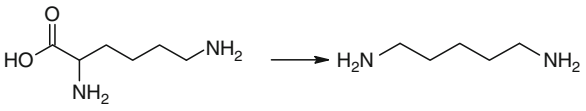


Fig. 2.11 A diamino pentane is the result of lysine production after one gene change

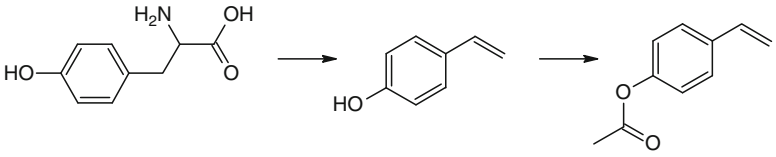


Fig. 2.12 Tyrosine is chemically converted to *p*-hydroxystyrene or *p*-acetoxystyrene

into a multitude of products, both commodity chemicals such as the metabolites of the TCA cycle and amino acids, and the much higher valued pharmaceuticals.

The "natural" products of metabolism are not the only products that can be produced by microorganisms. In Sect. 2.4, it was shown how genetic engineering of "a favorite organism" can make it produce a desired compound, and even synthesize compounds (e.g., certain polyketides) that are not found (or not yet discovered) in nature. These "strange" compounds may have an enormous value in the treatment of human diseases.

Much simpler "tricks" than those used in polyketide chemistry are, however, available and are frequently revealed in the patent literature. Most of these tricks are based on single gene-modifications of standard microorganisms.

Thus, as has been discussed in Sect. 2.3.1 lysine is a huge commodity chemical produced by fermentation. The polymer industry might be more interested in the amines that can be produced by decarboxylation of lysine (Fig. 2.11).

The reason is that the diamine (Cadaverine) can form macromolecules by amide formation with a large number of dicarboxylic acids.

C. glutamicum, the best producer of lysine (partly because it prevents decarboxylation of the product) is modified by inserting the gene for L-lysine decarboxylase from *E. coli*. Now *C. glutamicum* produces L-lysine as before, but as an extra step the acid is decarboxylated and cadaverine is exported to the medium Mimitsouka et al. (2007) and Tateno et al. (2009). Naturally the process is patented (by BASF).

Tyrosine is produced as an intermediate in the neuro-pharma industry. But the biosynthesis toward tyrosine passes through a number of intermediates that could be withdrawn for production of other valuable chemicals.

The DuPont Company has demonstrated that tyrosine can be chemically converted in a two-step process to very valuable monomers for production of adhesives (Fig. 2.12).

The process, described in US patent application 0213569 (2007), comprises the treatment of tyrosine with an aqueous solution of HBr and $NaNO_2$ followed by conversion, using an alkaline catalyst, of the isomeric mixture of brominated tyrosine intermediates to either of the two styrene compounds. The patent is an excellent

illustration of the symbiosis of a fermentation process and organic chemical synthesis which makes a few changes in the metabolite to obtain the desired product. An overview of the possibilities of processes by which fermentation is coupled with classical chemistry is given by Murzin and Leino (2008).

Interestingly, it has recently been shown that *p*-hydroxystyrene can also be produced directly from glucose without ever obtaining the L-tyrosine (Verhoef et al. 2009). A *Pseudomonas putida* strain, originally designed to produce phenol and *p*-coumaric acid from glucose (see Fig. 2.1), was engineered for efficient production of *p*-hydroxystyrene by inserting the genes for the enzymes *L-phenylalanine/L-tyrosine ammonia lyase* and *p-coumaric acid decarboxylase*. Here, we see an example of the combination of metabolic capabilities of two microorganisms to produce a desired product in one synthesis process.

The conclusion from the few examples shown here is that only the imagination of the industrial or academic researcher sets the limit to what can be produced in the biorefinery. Once an interesting organic compound has been identified, the tools of bioinformatics are used to screen for a suitable host organism which from genome-scale models is known to have the capability to produce the compound or can be genetically engineered to do so by gene transfer from other organisms. The steps of Sect. 2.4 are followed to increase the yield and productivity of the selected construct, and suitable cultivation conditions are found through laboratory and pilot plant experiments.

Problems

Problem 2.1. A number of process designs for large-scale production of ethanol are available on the net. IOGEN, DONG-Inbicon, a Danisco-DuPont scheme, supported by U. Tennessee and several others can be found. Make an analysis of the different schemes. What products are made? What is the saccharification process? How well are down-stream processes designed with respect to total energy minimization?

What is the current market price for ethanol in bulk, and how well does it match the production cost of ethanol from lignocellulosic biomass?

References: Lynd et al. (2008), Lin and Tanaka (2006), Sendich et al. (2008), and also material from the biotech companies Novozymes and Genencor.

Problem 2.2. Consult the literature to find profiles for batch cofermentation of glucose and xylose by a "wild-type" yeast strain *S. cerevisiae*, and also by better yeast strains. Observe the *sequential* utilization of the sugars.

1. How has *S. cerevisiae* been engineered in the group of Hahn-Hägerdahl to obtain a more rapid conversion of xylose? Describe the redox reactions used and comment on the difficulties of the process.
2. Compare the process of (1) with that of the Delft group of scientists (Wesselink et al. 2009).

Problem 2.3. Butanol (1-butanol, and perhaps even better iso-butanol) can become an excellent transportation fuel.

On the net you will find many recent proposals for the production of butanol by fermentation (e.g., a huge project by BP and DuPont). Give an account of the advantages of producing butanol rather than ethanol, and also outline some of the disadvantages.

Several papers from 2007/2009 review the biosynthesis of butanol: Lee et al. (2008), Atsumi et al. (2008), Sillers et al. (2008), as well as the history of butanol production by fermentation, Villadsen (2007). Older literature references are given in Chap. 5.

You will notice that the pathway to butanol, acetone, and butyric acid is an extension of the fermentation pathways in Fig. 2.5a, b, starting with dimerization of AcCoA.

1. Write the pathways which lead to the products in the "solvents fermentation process."
2. What are the key issues in the process?
3. How can the formation of butyric acid be (almost) suppressed, and what is the maximum yield of butanol on glucose obtained according to the references?
4. Find the bulk prices of 1-butanol and of technically pure glucose syrup obtained by liquefaction of starch. What is the added value of converting glucose syrup to butanol when the reported yields are used in the calculation?

Problem 2.4. Using the diagrams in a standard text on biochemistry or the much more detailed diagrams on the net you are required to write down the whole pathway from glucose to L-lysine and to L-tyrosine. This will give you an impression of the complexity of the biochemistry, but you will also notice that many parts of the total paths to the two amino acids are similar.

Problem 2.5. In Werpy and Petersen (2004), (Table 2.1), you will find diagrams that show how succinic acid can be used to produce a large number of chemicals.

1. Indicate the chemical processes needed to make these chemicals from succinic acid as a starting material. Could some of the chemicals also have been formed from other building blocks?
2. Look in recent literature for papers from the group of Sang Yup Lee at KAIST for exciting work toward production of succinic acid by fermentation. What are the issues of importance in order to get a high yield on glucose, a high productivity, and a high titer? Is the limited solution of succinic acid in aqueous solutions a problem?

Problem 2.6. In eukaryotes NAD^+ is produced in the mitochondria as a result of respiration. NAD^+ is needed in the cytosol (the EMP pathway). By which process is the NAD^+ transferred from the mitochondria to the cytosol? The answer is found by consultation of standard textbooks on Biochemistry.

Problem 2.7. Since 2008 Cargill and Novozymes have collaborated to develop an entirely bio-based route to acrylic acid (2-propenoic acid), which is the basis for

production of the hugely important acryl-based polymers. Central to their effort is to produce 3-hydroxy propionic acid (see Fig. 2.1b), which by dehydration of the alcohol gives acrylic acid.

Based on two major publications, Straathof et al. (2005) and Henry et al. (2010), you are required to review the research that within less than a decade has led to identification of the best metabolic routes to 3-hydroxy propionic acid, one of the projected platform chemicals in Werpy and Petersen(2004).

A thorough study of this problem will teach you how front-line biotech companies use all the suggestions discussed in Sect. 2.4.3 for strain development, and of pathway engineering in particular.

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