Transformation of biomass into commodity chemicals using enzymes or cells

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CONTENTS

1. INTRODUCTION

1.1. Bio-based and biochemical production

Biomass can be classified broadly as all the matter on the earth's surface of recent biological origin.[1] Biomass includes plant materials such as trees, grasses, agricultural crops, algal biomass, and organic waste such as animal manure. Recently, the interest in using biomass as a source for energy and chemicals has increased because fossil resources are assumed to become scarce and expensive. Also, this use of renewable resources might reduce greenhouse gas emission. Besides, establishing new value chains from biomass to energy and chemicals should create more economic activity, especially in agriculture, thus leading to jobs.

This "bio-based production" is defined here as chemical or biochemical production using a renewable biomass feedstock. Biochemical production will be discussed, including the chemical steps relevant to such routes, because hybrid routes frequently occur.

A biochemical route involves enzymatic or fermentative conversions. These are not by definition bio-based, because such conversions are also used within a petrochemical context. An example is the enzymatic hydration of acrylonitrile to acrylamide.[2] Such fossil feedstock based conversions are not discussed here, unless they can become part of an alternative renewable route. Biochemical routes using photosynthesis are not treated either, because they convert CO_2 rather than biomass.

Enzymes are nature's main catalysts and have a wide range of technical applications. Ribozymes[3] also perform biocatalysis but have much less synthetic power and value. When one or a few enzymes (or microorganisms containing these enzymes) perform a specific modification of a precursor to a defined product with structural similarity, this is called a biotransformation or bioconversion.[4,5] In longer pathways, involving many enzymatic steps, the product may no longer structurally resemble the substrate; and the probability increases that cofactor recycling is required for one or more of the enzymatic steps. Cofactor recycling will require additional enzymatic reactions. Besides, the cofactors are required. Complicated multi-enzyme systems have been successfully used on laboratory scale. Cell-free synthetic enzymatic pathways, for which the enzymes are obtained from a single microorganism, are also advocated.[6,7,8]

The established technology for longer enzymatic pathways, however, is to use a metabolic pathway of a living microbial cell, either a wild type or an engineered type. These cells are usually cultured in a fermentation vessel, to prevent infection by undesired microorganisms, while controlling O_2 level and pH. Since often many sequential and parallel enzymatic reactions occur in fermentative conversions, the product of metabolic activity usually bears no structural resemblance to the compounds supplied to the microorganism. The cells may grow, and synthesize new enzymes and cofactors.

Whereas in chemistry each conversion may imply that a factory is required for industrial production, in fermentation technology each conversion may simply imply an enzymatic step within a microbial cell.

In-vitro single-enzyme or multi enzyme systems may achieve higher volume-specific productivity than *in-vivo* cell systems, because cells will require membrane transport and contain many macromolecules and regulation mechanisms not required for the actual conversion.[6] Higher concentrations of enzymes can be used *in-vitro* than *in-vivo*, and in many cases they are more resistant than living cells to extreme conditions of temperature, pH, organic solvent, and substrate and product concentrations. However, production of enzymes requires fermentation; isolation from plant or animal tissue is rarely used nowadays.

1.2. Scope of the review

Due to the natural metabolic diversity and the methods to engineer the substrate spectrum of enzymes, the number of chemical compounds that can be produced from biomass using enzymes or cells is huge. From the perspective of replacing large amounts of fossil feedstocks by renewable feedstocks, most potential products are not so relevant. The focus of this review will be on compounds with a large scale current production from fossil or renewable carbon sources, and on compounds that might enter that category in the future because of their assumed potential to be produced from biomass using enzymes or cells at large scale. To exclude fine chemicals, "large scale" is set at approximately 50,000 t/a. Compounds that will be produced by follow up chemistry from precursors that are treated in this review, will be excluded. For example, ethene will be treated because fermentative routes from biomass to ethene are known, but chloroethane production from renewable ethene is not treated because only chemical ethene hydrochlorination is known.

The biochemical formation of commodity chemicals from biomass has been reviewed before,[9,10,11,12] but the current review covers a wider range of chemicals and the most recent literature. The field that is covered shows significant progress each month.

Biopolymers are kept outside the scope because the large number of options that could lead to commodity-scale polymers, with some options already being applied commercially. Otherwise, several relevant manners to convert biomass into biopolymers would be described in detail: (i) Biomass components such as starch can be converted in many ways into useful derivatives, by chemical but also by biochemical reactions.[13] (ii) Commodity chemicals such as hydroxyacids that can be produced from biomass as described in this review can be polymerized using enzymes.[14] (iii) Microbial cells can convert sugars into storage polymers such as poly-3-hydroxybutyrate[15] or into excreted polymers such as xanthan.[16]

1.3. Organization of the review

The reviewed literature has been organized on the basis of the reaction products, ordered along increasing complexity, thus starting with alkanes, such as in some traditional organic chemistry handbooks. The Figures summarize most conversions.

Per commodity product, the status of biochemical production is shortly described, with references to the best literature found concerning product yield, productivity, and achieved product concentration. Actual industrial performance is usually not disclosed, and may be lower than the best published performance because of additional constraints such as impure (cheaper) biomass feedstock.

Productivities will be given in g/(L h), thus indicating space-time yields, which are simply the achieved product concentration divided by the time used for the conversion. This leaves room to boost productivities by starting production with high enzyme or cell concentrations, or not counting the time of a cell growth stage preceding a production stage. Still, such high productivities might best represent what is achievable in an optimized process.

Unless otherwise indicated, product yields that are given are overall yields on the basis of converted rather than of supplied mass of feedstock. When reporting yields, authors may or may not take into account feedstock used for growing cells prior the production stage. The mass of cells grown per mass of glucose may be about 0.5 g/g,[17] but this is highly dependent on cell type and growth conditions. Besides, the mass of cells produced per mass of product produced may range from almost zero in optimized conversions to several g/g in early research phases.

1.4. Biomass degradation

The most abundant type of biomass is lignocellulose, containing the polymers cellulose, hemicellulose,[18] and lignin.[19] These polymers can be used for chemicals production instead of starch, sucrose, proteins, and triglycerides, which may rather serve as human food source. Many other useful biomass components are available at specific locations, for example pectin,[20] which is found in waste from sugar beet, fruits, and vegetables production.

Biomass components can be degraded in many ways. Table 1 shows degradation processes that lead to commodity chemicals or to intermediates that can be used as feedstock for commodity chemicals production. Enzymatic hydrolysis and anaerobic digestion are the processes that involve biochemistry and are most relevant to this review.

Table 1. Biomass degradation processes for commodity chemicals synthesis

Process	Products
Enzymatic hydrolysis	*See Table 2*
Chemical hydrolysis	*See Table 2*
Acid dehydration of monosaccharides	*Furans, levulinic acid, formic acid*
Pyrolysis of polysaccharides	*Methanol, aldehydes, carboxylic acids, anhydrosugars, …*
Gasification	*Syngas*
Anaerobic digestion	*Methane, acetic acid, … (see section 2.1)*

Anaerobic digestion is mainly used for producing methane and is treated together with methane in section 2.1. Enzymatic hydrolysis can lead to a large variety of products (see Table 2), and many of these products are separately described in this review. Enzymatic hydrolysis of biomass components can be performed in a process step separate from the subsequent use of the hydrolysis product(s); or simultaneous. Besides, the hydrolysis reaction can be catalyzed by enzyme(s) from a microorganism that subsequently converts the hydrolysis product(s), or by enzyme(s) from one of more other microorganisms; or chemical hydrolysis can be used. Treatment of all the different possibilities is outside of the scope of the present review, although it is a major focus of current research on bio-based chemicals. Also, the multitude of enzymes required for hydrolysis, despite being of large commercial significance,[21] is only partly treated.

Table 2. Hydrolysable biomass

Biomass component	Hydrolysis product (with section no.)
Starch	*D-Glucose (5.3.1)*
Cellulose	*D-Glucose (5.3.1)*
Hemicellulose	*D-Xylose (5.3.3), L-arabinose, D-glucose (5.3.1), L-fucose, D-galactose, D-mannose, L-rhamnose, D-glucuronic acid, acetic acid (9.1.2), ferulic acid*
Pectin	*D-Galacturonic acid, L-rhamnose, D-galactose, L-arabinose, D-xylose (5.3.3), acetic acid (9.1.2), methanol (5.1.1)*
Protein	*Proteinogenic amino acids (13.1)*
Triglyceride	*Fatty acids (9.5), glycerol*[22] *(5.2.4)*
Sucrose	*D-Glucose (5.3.1), D-fructose (5.3.2)*

For all hydrolysis products that are mentioned in Table 2 there are metabolic routes that will allow their conversion into central intermediates such as pyruvate of acetyl-CoA and then further into other products. Microorganisms that are able to form a desired product from glucose are usually not naturally able to convert all other biomass hydrolysate components into such a desired product. Therefore, for all available hydrolysate components conversion pathways are engineered into recombinant microorganisms to achieve high yields on biomass. The extensive literature on this topic will also not be treated here. One of the challenges associated with utilization of biomass-derived sugars, particularly those produced by chemical hydrolysis, is the formation of degradation products that may be inhibitory during fermentation.[23]

Generally, microbial cells are less productive when consuming crude mixtures of hydrolyzed monosaccharides rather than glucose. With few exceptions, this review will only give data for glucose, because glucose can be used as benchmark substrate for many metabolic routes. The main pathway for glucose conversion is the glycolysis, and Figure 1 shows how this merges with pathways for conversion of some other biomass monomers.

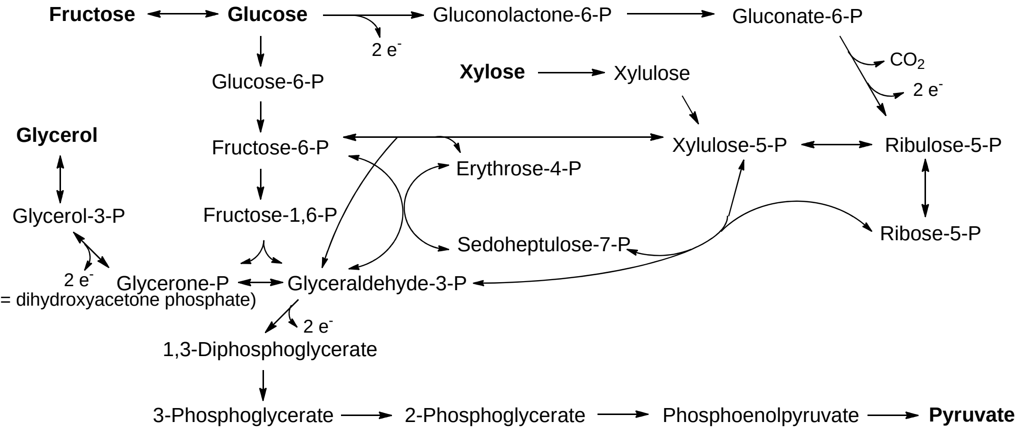

Figure 1. Metabolic pathways of some major biomass monomers up to pyruvate. Glycolysis is the direct route from glucose to pyruvate. Formation and consumption of ADP, ATP, phosphate, and water have not been indicated. Electrons (e^-) are taken up by NAD^+ or $NADP^+$. Components in boldface are treated explicitly in the text.

Table 2 includes all biomass components that are highly relevant for renewable chemicals synthesis, except lignin, because lignin is not considered to be enzymatically hydrolysable. Lignin biosynthesis occurs by radical polymerization of guaiacyl, syringyl, and p-hydroxyphenyl units from the precursors coniferyl, sinapyl, and *p*-coumaryl alcohol, respectively, all containing phenylpropenoid building blocks.[19] The obtained network of aromatic groups can be degraded by some fungi and bacteria using enzymes such as peroxidases and laccase. In the presence of O_2, these enzymes generate oxidative radicals, leading to substituted aromatic compounds that can be further metabolized.[19] In the presence of H_2O_2, lipases have been used to form peroxycarboxylic acids *in situ* that cause lignin degradation.[24] The lignin catabolism of the proteobacterium *Sphingobium* sp. *SYK6*, was recently proposed to include a glutathione-dependent cleavage of a β-O-4 aryl ether linkage bond by an etherase.[25] With the aid of enzymes, some components useful as fine chemicals have been obtained from lignin, and a quest has begun to identify metabolic routes from lignin to commodity chemicals.[26]

Chemical degradation of the lignin fraction of biomass is more straightforward, and the degradation products may be further converted using biochemical methods. In this context, gasification of lignin (and other biomass) to syngas, such as mentioned in Table 1, is particularly relevant. This bio-syngas, a mixture of CO and H_2, like syngas of fossil origin, can be converted into commodity chemicals not only by using chemical methods but also by some microorganisms.[27] Amongst different syngas converting pathways, the Wood-Ljungdahl pathway can be used to produce acetyl-CoA from syngas.[28,29] In short, one CO is reduced to a methyl group that is linked to another CO. Subsequently, all kinds of metabolic routes can lead from acetyl-CoA to commodity chemicals. The companies LanzaTech, Coskata, and INEOS have produced ethanol by fermentation of syngas on pilot scale and are moving toward commercialization.[29] Syngas fermentation performance does not yet seem to be at the level of carbohydrate fermentation performance, but has the advantage that it also uses the noncarbohydrate (lignin) portion of lignocellulosic biomass.

2. ALKANES

2.1. Methane

Methane from fossil sources is an important raw material for the industrial production of acetylene, synthesis gas, H_2, methanol, and many other compounds. Biological methane production is also well-known, and has been applied since 1881.[30]

Methane can be produced from acetic acid by methanogenic bacteria (Figure 2). This occurs spontaneously during biomethanation, a type of anaerobic digestion of biomass. Mixed cultures of microorganisms first hydrolyze complex organic molecules into simple sugars, amino acids, and fatty acids. In the subsequent acidogenesis stage, these are converted into carboxylic acids such as acetic, propionic, butyric, and valeric acid, along with ammonia, carbon dioxide, and hydrogen sulfide. In the third stage, acetogenesis, products from acidogenesis are further digested to produce carbon dioxide, hydrogen, and organic acids, mainly acetic acid. Finally, acetic acid is converted into methane and carbon dioxide during methanogenesis. This final conversion involves several intermediates with enzyme-catalyzed methyl transfers to coenzymes M and B.[31] The maximum yield of methane per glucose equivalent is 0.27 g/g, if the rest becomes CO_2. However, in practice the feedstock is heterogeneous, leading to a different yield. Usually a part of the CO_2 will dissolve in the liquid effluent, leading to biogas containing relatively much methane.

Advantages of anaerobic digestion for converting biomass include the absence of requirement to work under sterile conditions, and automatic product recovery by gas formation. This leads to relatively simple equipment and operations. A disadvantage of the process is the low productivity. Typical values are below 0.03 g/(L h).[32]

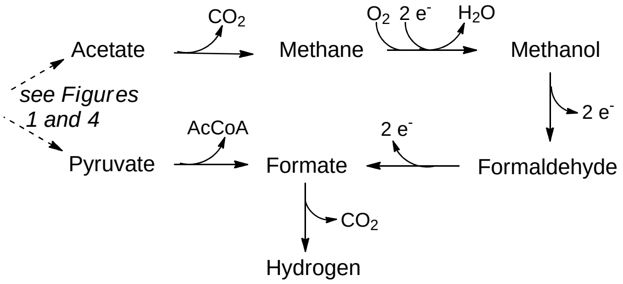

Figure 2. Metabolic pathways to H_2 and some C_1 compounds.

2.2. Long chain alkanes

C_{10}-C_{15} alkanes obtained from fossil carbon sources are used on large scale as main components of diesel fuel, but they are also used in other fuels, as lubricants, and they are catalytically dehydrogenated to alkenes, which serve as intermediate to various other compounds.

Many organisms are able to produce long chain alkanes. This requires biosynthesis of fatty aldehydes, which is described in section 5.1.10. The key reaction is conversion of fatty aldehydes into alkanes (Figure 3). Decarbonylases, which release CO, have been shown in vertebrates, insects, plants, and algae.[33] Ferritin-like nonheme dimetal-carboxylate enzymes catalyze alkane formation from aldehyde in many cyanobacteria. Their coproduct was also thought to be carbon monoxide, but O_2 consumption and formate coproduction have recently been observed, suggesting they are aldehyde-deformylating oxygenases.[34,35]

Incorporating an alkane biosynthesis pathway from cyanobacteria in *Escherichia coli* has led to long chain alkanes.[36] The pathway coexpresses genes for acyl-ACP (acyl carrier protein) reductase and an enzyme from the cyanobacterium *Synechococcus elongatus* converting aldehyde to alkane. This has led to the secretion of 0.3 g/L C_{13} to C_{17} mixtures. The process is currently under optimization, and pilot-plant fermentations (1000 L scale) have already been performed.[37]

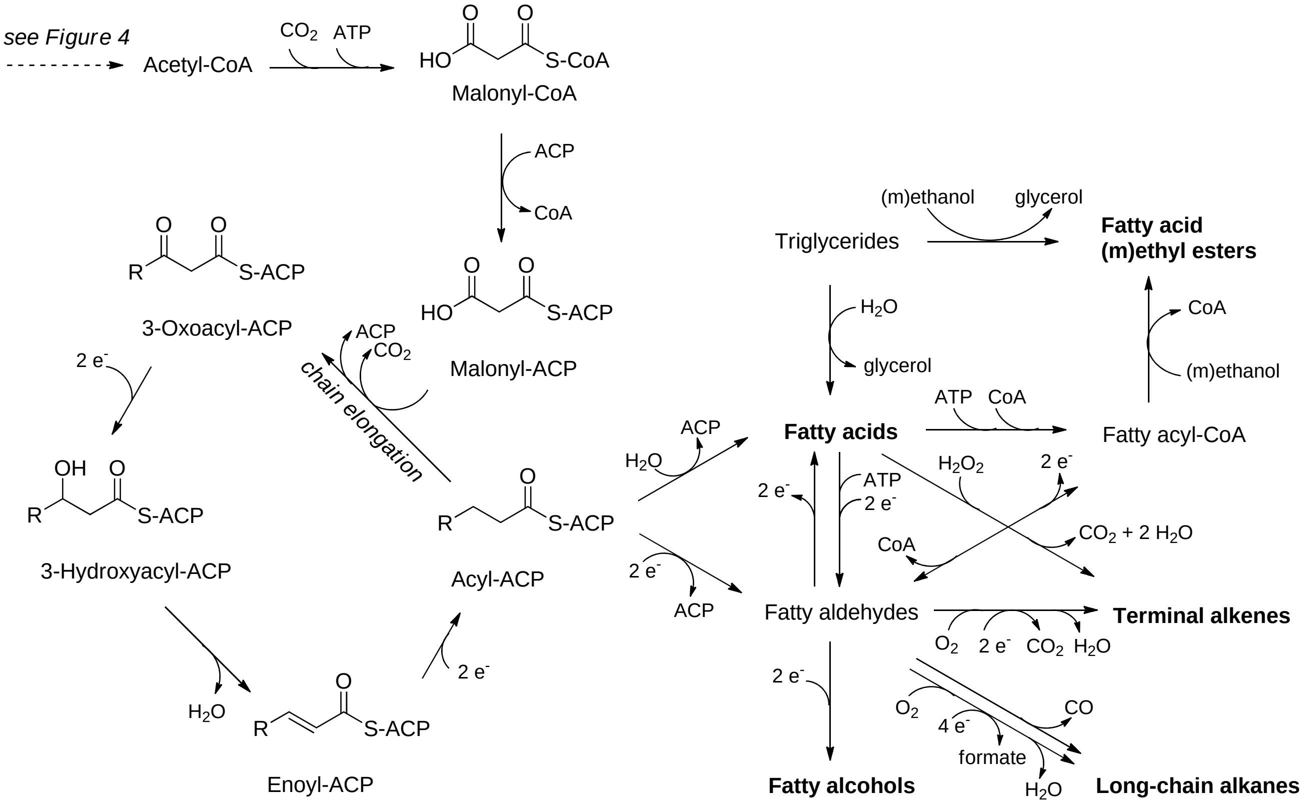

Figure 3. Pathways to fatty acid and derivatives. Compounds in boldface are treated in the text.

3. ALKENES

3.1. Ethene

Ethene (ethylene) is a chemical intermediate used to produce many different products, such as polyethylene, ethylene oxide, vinyl chloride, and styrene. In 2009 its production capacity has been estimated at around 115 million t/a, mainly (>98%) through petrochemical routes.[38]

Due to the importance of ethene as bulk chemical, the possibilities to produce it from biomass have received considerable interest.

Ethene is secreted in small amounts by plants for signaling functions such as stimulation of fruit-ripening.[39] The best-known biosynthesis relies on the enzyme 1-aminocyclopropane-1-carboxylate oxygenase, which also oxidizes L-ascorbate and forms cyanide.[40] The co-produced L-dehydroascorbate can easily be recycled into the consumed L-ascorbate. However, the 1-aminocyclopropane-1-carboxylate synthesis consumes *S*-adenosyl-L-methionine (SAM). This is a coenzyme used for transmethylation, transsulfuration, and aminopropylation. For ethene biosynthesis, SAM must be recycled from its side product 5'-methylthioadenosine by a large metabolic effort.

In a second pathway, occurring in an engineered *E. coli* strain, L-methionine is deaminated by a transaminase to form *(S)*-2-oxo-4-thiomethylbutyric acid. This is decomposed into ethene, methanethiol and CO_2, in the presence of an NADH-Fe(III) oxidoreductase, which activates O_2.[41]

In a third pathway, ethene is produced from 2-oxoglutarate by an enzyme that occurs in *Penicillium digitatum* and *Pseudomonas syringae*. The overall enzymatic reaction is a combination of two ethene-forming reactions and one succinate-forming reaction:[42]

3 oxoglutarate + L-arginine + 3 O_2 $\rightarrow$ 2 ethene + succinate + guanidine + L-Δ^1-pyrroline-5-carboxylate + 7 CO_2

This ethene forming enzyme has been expressed in various other organisms such as *E. coli*,[43] *Saccharomyces cerevisiae*[44] and *Pseudomonas putida*.[45] However, the three aforementioned pathways will lead to yields of ethene on glucose below 0.12 g/g,[44] which is unattractive for large-scale ethene synthesis.

Enzymatic decarboxylation of acrylic acid to ethene has been suggested to occur in some organisms due to a side activity of pyruvate decarboxylase,[46] but the proof for this is considered to be weak.[47]

Currently, the key option to approach a theoretical yield of ethene on glucose of 2 mol/mol (0.31 g/g) is by using acid-catalyzed dehydration of fermentation-derived ethanol to ethene. This is commercially applied as bio-based route.[38] It would be attractive if enzymatic dehydration would be possible, but such activity is only described in patent applications.[48,49]

3.2. Propene

Propene (propylene) is one of the most important chemicals. In 2002, about 53 million t was obtained from petrochemical processes for conversion into polymers and numerous derivatives.[50]

Biological formation of traces of propene has been observed in aerobic cultures of many different types of organisms, in particular *Rhizopus* strains, but the source was not elucidated.[51,52] In other studies, rabbit cytochrome P-450-catalyzed formation of propene from isobutyraldehyde has been demonstrated, with an activity of 98 mmol/min of product per mol of P-450.[53] It is assumed that by mono-oxygenase action, O_2 is activated using NADPH as an electron source. One oxygen atom is then reduced to water and the other atom is transferred to the isobutyraldehyde, leading to propene and formic acid.[54] Using such a reaction for bio-based propene production does not seem to be pursued, although the required isobutyraldehyde can be produce from glucose (see section 7.4). It will be easier to use a route involving acid-catalyzed dehydration of isopropanol (see section 0) into propene.[55] Besides, patent applications claim that this dehydration can be enzyme-catalyzed.[48,49]

Commercial bio-based propene production is pursued by Braskem, using fermentative ethanol production, followed by chemical conversion into ethene, dimerization and metathesis. [56]

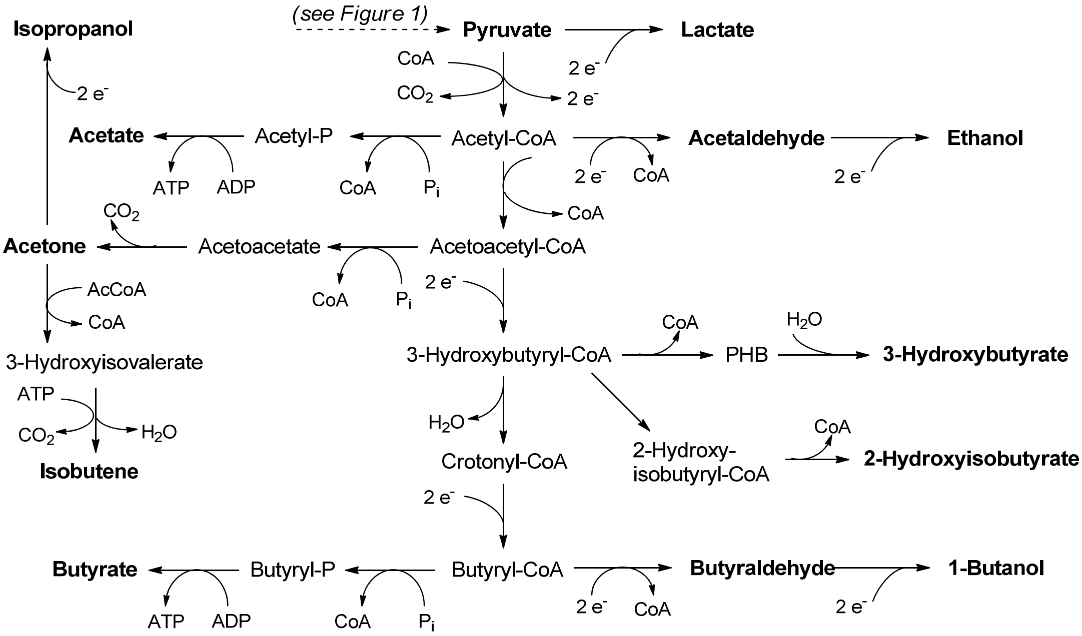

Figure 4. Pathways from pyruvate to a range of compounds, indicated in boldface.

3.3. Isobutene

Currently, more than 10 million t/a isobutene (2-methylpropene) is produced via petrochemical routes. Therefore, production from biomass via biocatalytic pathways is of considerable interest.[57]

Biological isobutene formation is known since the 1970s. The highest production rate, merely 0.45 mg/(L h), was found for a strain of the yeast *Rhodotorula minuta*.[58] Isobutene was formed by reductive decarboxylation of isovalerate, which is produced in the catabolic pathway of L-leucine.[59,60] The reaction seems to be a side reaction of a cytochrome P450 monooxygenase that is involved in hydroxylating benzoate.[61] Besides the low rate of isobutene formation, the used pathway has no obvious potential to approach a theoretical stoichiometry of 1 isobutene + 2 CO_2 + 2 H_2O per mol of glucose.

Two recent developments may enable much higher yields, although they do not describe reasonable isobutene production levels yet. It has been shown that 3-hydroxyisovalerate can be converted to isobutene as a side-activity of mevalonate diphosphate decarboxylase (Figure 4).[62-63] Also, patent applications describe isobutanol dehydration as a side activity of engineered oleate hydratase and other hydratases.[48-49] Fermentative production of isobutanol is described in section 0. If a one-pot biochemical conversion of carbohydrate to isobutene would become possible, isobutene gas would be emitted from a fermentor, and isobutanol recovery and its chemocatalytic dehydration could be bypassed.[57]

3.4. Butadiene

1,3-Butadiene is a very important monomer for synthetic rubbers but also for some plastics. Besides, it is an intermediate in the production of some important chemicals. Production and of butadiene in 2009 was about 9.2 million t.[64]

Several metabolic pathways to 1,3-butadiene have been claimed in a patent application of Genomatica,[65] but without any information on actual formation of the compound. The final reaction should be an elimination that resembles the final reaction of isoprene pathways:

2-butenyl-4-diphosphate → 1,3-butadiene + diphosphate

However, this reaction is hypothetical. It will be very challenging to achieve commercially viable results regarding biosynthetic 1,3-butadiene production.

Other patent applications claim butadiene formation by dehydration reactions from but-3-en-1-ol and but-3-en-2-ol using homologues of known hydratases.[48,49] Furthermore, *E. coli* bacteria are engineered to produce these precursors from glucose (in a way not explained) and subsequently engineered to convert the butenol isomers into butadiene. A range of enzymatic butadiene formation reactions, including the aforementioned ones and pathways to the required precursors, has been described in another patent application.[66] However, data on actual butadiene production are not yet available.

3.5. Isoprene

Isoprene, or 2-methyl-1,3-butadiene, is suitable for addition polymerization. The polymer has traditionally been extracted from rubber trees, but nowadays more than 800,000 t/a isoprene is produced via petrochemistry for polymerization to synthetic rubber and elastomer.[67,68]

In nature, isoprene is formed by various microbial, plant, and animal species. Plants emit it in massive amounts, estimated at 600 million t/a, into the atmosphere.[69] The known metabolic pathways are the mevalonate (MEV) and the methylerythritol phosphate (MEP) pathway. In plants, both pathways exist. The MEV pathway, shown in Figure 5, is used by archaea, some bacteria and most eukaryotes (including the yeast *S. cerevisiae*), while the MEP pathway is used in most bacteria (including *E. coli*) and green algae. In either pathway, isoprene is formed by elimination of pyrophosphate from 3,3-dimethylallyl pyrophosphate by the key enzyme isoprene synthase. Since the yield of isoprene from naturally-occurring organisms is commercially unattractive, Genencor (now DuPont) and Goodyear use genetically engineered *E. coli* for the production of isoprene through fermentation of glucose.[70,68] Although the MEP pathway might yield up to 0.30 g/g isoprene on glucose, they focused on the better known MEV pathway, which has a maximum yield of 0.25 g/g according to the theoretical net overall reaction:[68]

$$1.5\ \text{Glucose} + 2\ O_2 \rightarrow \text{Isoprene} + 4\ CO_2 + 5\ H_2O$$

This work resulted in the emission of isoprene with the fermentor off-gas, from which it was collected with high purity; the boiling point of isoprene is 34 °C and it is hardly water soluble. The isoprene was recovered from the off-gas and polymerized to rubber. The collected amount of isoprene corresponded to 60 g/L in the fermentor broth. The productivity was 2 g/(h L) and the yield on glucose 0.11 g/g.[68] This has been used in the production of prototype tires.

3.6. Farnesene

Isoprenoids are hydrocarbons consisting of several isoprene units. Their biosynthesis resembles that of isoprene. The C_{15} hydrocarbon farnesene is the most relevant isoprenoid with respect to large scale bio-based production.

Farnesene is the name of a group of natural sesquiterpene isomers, including β-farnesene (7,11-dimethyl-3-methylene-1,6,10-dodecatriene). Their catalytic hydrogenation leads to farnesane, which is being evaluated as diesel fuel, using farnesene produced by Amyris in a demonstration plant.[71] Heterologous *E. coli* and *S. cerevisiae* strains have been developed. In *S. cerevisiae*, the mevalonate pathway enzymes, converting acetyl-CoA into farnesyl diphosphate, are overexpressed, and the latter intermediate is converted into *(E)*-β-farnesene and diphosphate (see Figure 5). This final reaction is catalyzed by a farnesene synthase, due to expression of the corresponding gene sequence from *Artesemia annua*. Upon improvement of the *S. cerevisiae* strain and the fermentation conditions, farnesene has been produced at a yield on glucose of 0.12 g/g. The product has low water solubility which should facilitate separation from the culture broth.[72] Production of up to 1.1 g/L farnesene at a productivity of 0.01 g/(L h) has been disclosed.[73]

The potential of engineering the mevalonate pathway for sesquiterpene biosynthesis is more evident from results published for amorpha-4,11-diene, a precursor of the antimalarial agent artemisinin: up to 41 g/L was produced at a productivity of 0.35 g/(L h) using engineered *S. cerevisiae* in aerobic fermentation with ethanol feeding.[74]

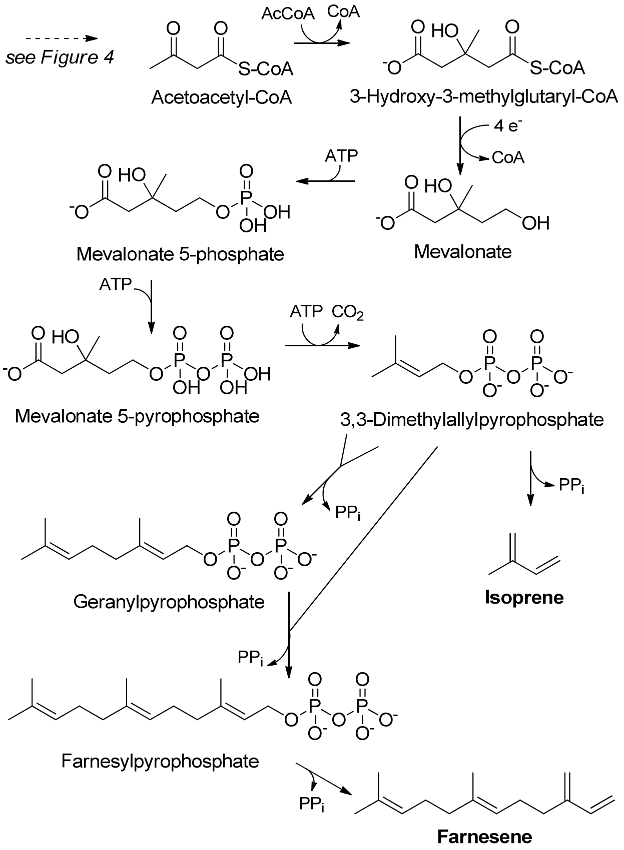

Figure 5. Mevalonate pathway to isoprene and farnesene.

3.7. Terminal long chain alkenes

Linear C_6-C_{20} hydrocarbons with a terminal unsaturation (α-olefins) are very useful as precursor for various surface-active agents. Petrochemical production of terminal long chain alkenes amounted to 2.15 million t in 1994.[75]

Natural linear alkene production such as occurring in many bacteria proceeds by Claisen condensation and yields nonterminal alkenes.[76,77] However, in some eukaryotes and some bacteria, *Jeotgalicoccus* for example, fatty acids are converted into terminal alkenes and CO_2. The responsible enzyme, OleT, is a cytochrome P450, which is assumed to consume H_2O_2 and to form two equivalents of H_2O when abstracting hydrogens from the α and β positions of the fatty acid.[78]

In insects, terminal alkenes are formed from fatty aldehydes using cytochrome P450 enzymes that consume NADPH and O_2, and release $NADP^+$, CO_2 and water.[79]

4. AROMATIC HYDROCARBONS

4.1. Toluene

Toluene is one of the best known petrochemicals, consumed in amounts of about 20 million t/a for use in fuel and for conversion into benzene and other chemicals.[80]

However, low concentrations of biogenic toluene have been detected in freshwater lakes. This toluene originates from anaerobic degradation of phenylalanine by bacteria such as *Tolumonas auensis*.[81] The pathway is assumed to involve oxidation of phenylalanine to phenylacetate, which is then decarboxylated (cf. Figure 6).[82] The responsible enzymes are not known, and there are no publications on production of toluene from glucose using such enzymes.

4.2. Styrene

Styrene, also known as phenylethene, is petrochemically produced at about 26 million t/a, especially as a monomer for the synthesis of many useful polymers.[83] It has been demonstrated that styrene can also be formed by microorganisms from renewable substrates such as glucose.[84] The conversion was achieved by the co-expression of phenylalanine ammonia lyase from *Arabidopsis thaliana* and *trans*-cinnamate decarboxylase from *S. cerevisiae* in an L-phenylalanine over-producing *E. coli* host (Figure 6). This led to the accumulation of up to 0.26 g/L in shake flask cultures. This is close to the styrene toxicity threshold (determined as 0.3 g/L). While genetic engineering approaches will be required to obtain commercially attractive productivities and yields, other approaches will be required to address the toxicity threshold. Organic-solvent resistant bacteria such as *Pseudomonas putida* S12 could be used as host, like in the biosynthesis of *p*-hydroxybenzoate[85] and *p*-hydroxystyrene,[86] and combined with *in-situ* extraction of the product.[86]

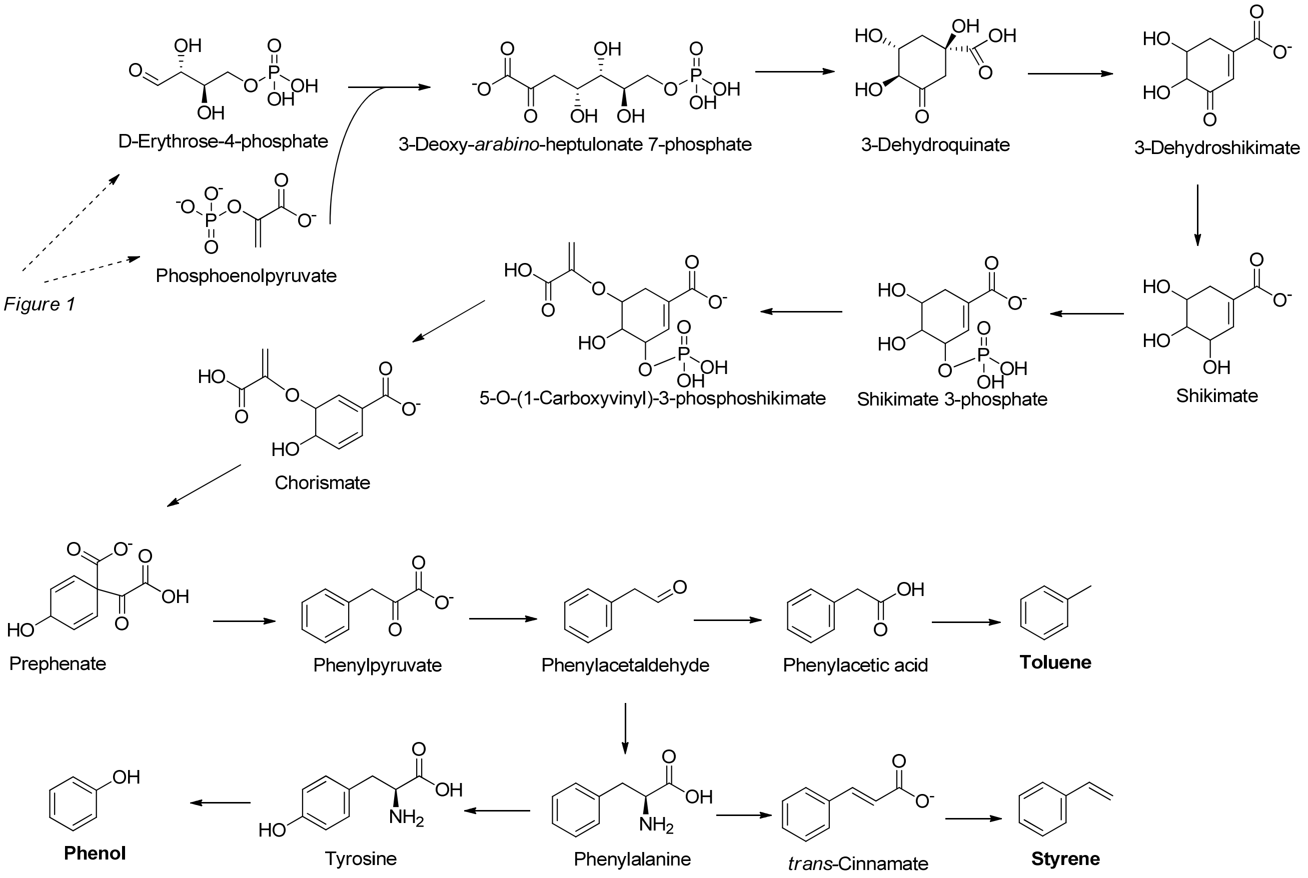

Figure 6. Pathways to toluene, phenol, and styrene. Only the key compounds are shown.

4.3. Naphthalene

Naphthalene was produced at about one million t in 1987, for use as precursor of various chemicals.[87] The current production level might be very different but will still be at commodity scale level. Besides this fossil carbon based production, biosynthesis might be possible. Naphthalene has been found, for example, in termite nests, where it is used by termites as fumigant.[88] The endophytic fungus *Muscodor vitigenus* emits traces of naphthalene when grown of agar plates with glucose.[89] The origin of this naphthalene is not clear yet. Similarly, there is some evidence for biogenic emission of traces of many other volatile organic compounds, including benzene, toluene, and *o*- and *m*-xylene, by endophytic fungi[90,91] and plants.[92] The lack of identification of any biosynthetic pathway precludes that they might be engineered into microorganisms for bio-based production of these aromatic compounds. On the other hand, it also becomes very obvious that nature's biocatalytic potential could be explored much further. For example, the number of different species of fungal endophytes has been estimated at at least one million, and their biosynthetic capability is still a field very open for exploration.[93]

5. ALCOHOLS

5.1. Aliphatic monoalcohols

5.1.1. Methanol

Methanol has historically been obtained by distillation of wood. Currently, it is produced from fossil carbon sources such as methane gas by chemical processes, at a scale of 46 million t/a in 2010.[94] About 85% of the methanol produced is used as a starting material or solvent for synthesis. The remainder is used in the fuel and energy sector; this use is increasing.

Bio-based methanol can be obtained from bio-based methane using chemical catalysis, but biocatalysis is an alternative. In microbial ecology, oxidation of methane to methanol is widely known as part of the global carbon cycle.[95] Many bacteria perform such oxidation, using in some cases soluble methane monooxygenase containing a di-iron center but in most cases a membrane-bound enzyme believed to contain copper and iron. The soluble enzyme has been studied best. It consists of three components: a hydroxylase that houses the active site, a reductase that shuttles electrons from NADH to the active site, and a regulatory protein.[96]

The reaction stoichiometry is:

$$\text{Methane} + \text{NAD(P)H} + O_2 \rightarrow \text{methanol} + \text{NAD(P)}^+ + H_2O$$

So despite the oxidation, NAD(P)H has to be added as reducing agent (see Figure 2) and this requires a regeneration reaction. Formate and formate dehydrogenase have been used in case of immobilized cells of *Methylosinus trichosporium* that displayed methane monooxygenase activity in batch and continuous reactors during some days.[97] Only small amounts of methanol were produced, and not much recent work has been done in this field.

5.1.2. Ethanol

Ethanol fermentation is known since ancient times, and currently it is the most important industrial fermentation process. For several decades, petrochemical production of ethanol from ethene has been important, but nowadays the reverse reaction, ethene production by dehydration of fermentative ethanol, is gaining importance.[38] Ethanol production has been estimated at 99 million t/a in 2010.[98] It is mostly used as fuel,[99] but also as solvent, as chemical intermediate, and in beverages.

During anaerobic fermentation, ethanol is produced from glucose or other carbohydrates via pyruvate (Figure 4). Pyruvate decarboxylase converts pyruvate to acetaldehyde, and alcohol dehydrogenase reduces the acetaldehyde to ethanol, consuming the NADH that had been produced during pyruvate formation. This simple pathway is commonly exploited in the yeasts such as *S. cerevisiae*, which is the default organism, but also in bacteria such as *Zymomonas mobilis* or recombinant *E. coli*. The overall reaction with glucose is:

$$C_6H_{12}O_6 \rightarrow 2\ C_2H_6O + 2\ CO_2$$

This reaction also generates ATP, and cells will multiply if ATP, glucose, and nutrients are available in sufficient amounts. Cell formation is usually accompanied by formation of small amounts of side product such as glycerol. This reduces the ethanol yield, but can be minimized by glucose feeding, and retaining cells in the fermentor using centrifugation, flocculation, membranes, or immobilization.[100] The resulting high cell concentrations favor high productivities. When also removing ethanol in-situ using vacuum, 82 g/(L h) has been achieved using *S. cerevisiae*.[101] At high levels of inoculation and nutrient supply, up to 21.5 % (v/v) ethanol (~170 g/L) has been produced.[102] Ethanol yields on glucose often approach the theoretical maximum of 0.51 g/g.

The current focus of ethanol research is on fermenting all kinds of nonfood carbohydrates[103,104] and even bio-based syngas.[29,27] The cells are selected for or adapted to conditions of high stress due to high ethanol concentration,[105,106] high temperature,[107,108] and presence of toxic components in the carbohydrate feed.[109,110]

5.1.3. 1-Propanol

Industrially, 1-propanol is obtained via propanal from ethene, to be used as solvent or chemical intermediate.[111]

Small quantities of 1-propanol are formed during traditional ethanolic fermentation if the starch source contains protein. The biodegradation of threonine leads to this 1-propanol.[112] The metabolic pathway runs via 2-oxobutyrate (Figure 7).

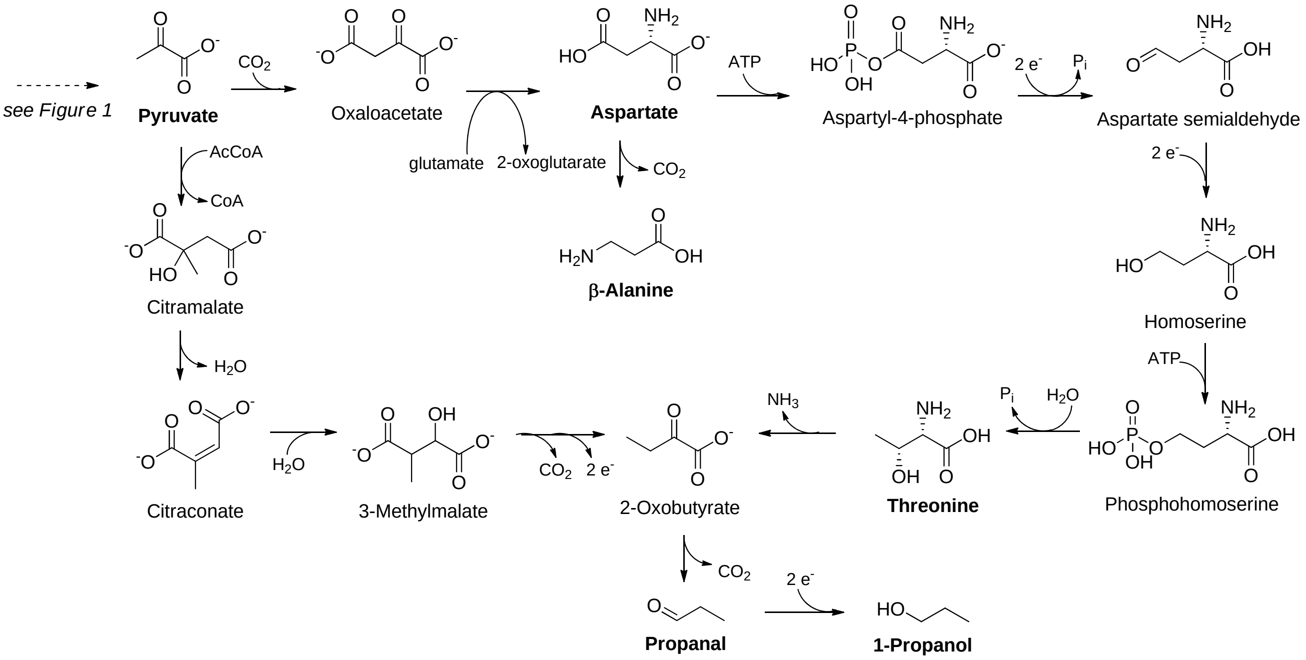

Figure 7. Pathways to 1-propanol, propanal and some amino acids.

Upon introduction of the promiscuous 2-oxoacid decarboxylase from *Lactococcus lactis* and alcohol dehydrogenase 2 from *S. cerevisiae* into *E. coli*, 2-oxobutyrate was converted via propanal into 1-propanol.[113-114] This approach was improved when starting form a threonine hyperproducing *E. coli* strain.[115] In aerobic fed-batch culture, glucose was converted into 10.8 g/L of 1-propanol with 0.11 g/g yield and 0.14 g/(L h) productivity. Using glycerol, 10.3 g/L of 1-propanol was obtained with 0.26 g/g yield and 0.083 g/(L h) productivity.

The most direct route to 2-oxobutyrate does not involve transamination followed by deamination of threonine, but runs via *(R)*-citramalate, a C_5 dicarboxylate that can be formed from pyruvate and acetyl-CoA by citramalate synthase. Citramalate is dehydrated to citraconate, which is rehydrated to *(2R,3S)*-3-methylmalate. An oxidative decarboxylation yields 2-oxobutyrate (Figure 7). These last three steps are performed by native enzymes involved in leucine biosynthesis, which can run via this pathway. To take advantage of the pathway via citramalate, the specific activity of *(R)*-citramalate synthase from *Methanococcus jannaschii* was improved using directed evolution and the improved enzyme was expressed in *E. coli*.[113,116] After knocking out competing pathways and evolutionary engineering, the best strain produced more than 3.5 g/L 1-propanol in 92 h with a yield on glucose of about 0.18 g/g.

Another approach for the biosynthesis of 1-propanol extended the well-known 1,2-propanediol pathway, as described in section 5.2.2. The dehydration of 1,2-propanediol into propanal was achieved upon introducing in *E. coli* also a coenzyme B-12 dependent propanediol dehydratase from *K. oxytoca*. Dehydrogenases native to *E. coli* will reduce propanal into 1-propanol. After 48 h fermentation using 20 g/L glucose, 1-propanol was produced at 0.25 g/L, with 0.46 g/L 1,2-propanediol remaining unconverted and with L-lactate as major product.[117]

5.1.4. Isopropanol

Isopropanol, also called isopropyl alcohol or 2-propanol, is industrially produced from propylene, at a scale of more than 2.3 million t/a in 2003.[111] It is used primarily as a solvent in inks and surfactants, but also as chemical intermediate.

Isopropanol is a fermentation product of many species of *Clostridium beijerinckii*. A screen of 52 strains of *C. beijerinckii* showed a maximum production of isopropanol of 1.8 g/L.[118] Although such isopropanol production has been improved using immobilized cells in continuous systems, the formation of acetone and 1-butanol via related pathways (see sections 8.1 and 5.1.5) complicates the process.[119] To prevent such co-production, the isopropanol pathway (Figure 4) has been introduced in *E. coli*.[120,121] Two molecules of acetyl-CoA, obtained via glycolysis, are condensed by acetyl-CoA acetyltransferase to one molecule of acetoacetyl-CoA. Acetoacetyl-CoA transferase transfers CoA from acetoacetyl-CoA to acetate or to butyrate, forming acetoacetate, which is then converted to acetone and CO_2 by an acetoacetate decarboxylase. Finally, an NADPH dependent alcohol dehydrogenase reduces acetone to isopropanol. Isopropanol production of 4.9 g/L has been achieved in 30 h using an *E. coli* strain with *C. acetobutylicum* acetyl-CoA acetyltransferase, *E. coli* acetoacetyl-

CoA transferase, *C. acetobutylicum* acetoacetate decarboxylase, and *C. beijerinckii* secondary alcohol dehydrogenase.[121]

Using a pH-controlled fed-batch culture with the intermittent addition of glucose, a recombinant *E. coli* strain formed 40 g/L isopropanol with a productivity of 0.67 g/(L h) and a yield on glucose of 0.24 g/g. To overcome the toxicity of isopropanol for *E. coli*, a gas stripping recovery method was incorporated into the fed-batch culture system. Using this approach an amount of isopropanol was produced equivalent to 143 g/L in the fermentor, with only a slightly lower productivity and yield.[122]

The theoretical yield of the used pathway is 1 mol propanol per glucose, equal to 0.33 g/g and indicating room for improvement.

5.1.5. 1-Butanol

Already in 1861, Pasteur observed 1-butanol production by anaerobic fermentation.[123] The so-called ABE (acetone-butanol-ethanol) fermentation using *Clostridium* bacteria gives three products in a molar ratio of about 3:6:1 using starch or another glucose source. An industrial process was developed in the beginning of the 20th century, which became the main source of 1-butanol and acetone. These were used as solvent and chemical building block.[123]

In the early 1960s, petrochemical production methods led to the decline of the ABE fermentation industry. The petrochemical production has been estimated at 2.8 million t/a in 2008.[98]

Nowadays, there is a renewed attention for ABE fermentation because of interest in sustainability, and economic opportunities due to increasing oil prices. On a mass basis 1-butanol has a 31 % higher combustion value than ethanol, and it can be used as "drop-in" fuel.

ABE fermentation is performed by a large variety of Clostridia strains. When only butanol is desired, the ideal stoichiometry from hexose sugars is:

$$C_6H_{12}O_6 \rightarrow C_4H_{10}O + 2\ CO_2 + H_2O$$

This overall reaction can be coupled to the formation of ATP, thus enabling cell growth and maintenance.[123] Briefly, the pathway from sugars involves formation of acetyl-CoA via pyruvate. Acetyl-CoA is dimerized to acetoacetyl-CoA, which is reduced to butyryl-CoA, and then further via butanal to 1-butanol (Figure 4). The actual biosynthesis in Clostridia involves some deviations. Initially, acetate and butyrate are released from their CoA derivatives, but when this leads to a too low pH these acids are assimilated again and ABE formation starts. The cells can gain some more ATP from this conversion, until toxic butanol levels are reached.

Metabolic engineering of Clostridia and many other microorganisms is used to adapt the original butanol fermentation in order to:[124,125]

- maximize butanol yield on carbohydrate (by minimizing acetate, butyrate, acetone, and ethanol formation, and minimizing the need for cell disposal and re-growth)
- ferment lignocellulosic sugars and other feedstocks into butanol
- increase the rate of butanol production by the used microorganism
- increase the tolerance of the used microorganism to butanol.

Using engineered *E. coli*, achieved yields on glucose are up to 0.36 g/g.[126] A very high ABE productivity, more 16 g/(L h), has been achieved upon immobilizing *C. beijerinckii*.[127] The most challenging issue remains the butanol concentrations achieved in fermentors, which remain limited to 14-20 g/L.[125,127,128,126,129] The low final butanol concentration leads to relatively short batch fermentations and relatively long downtimes after each batch, thus inefficient fermentor use and high investments. Moreover, for both batch and continuous fermentation, the recovery of butanol from dilute aqueous solution is energy intensive, which is expensive and also energetically unfavorable, in particular if the butanol is to be used as fuel.[130] In-situ stripping can prolong production and lead to higher butanol concentration in the condensed vapor than in the fermentor.[131,126]

Because of the more difficult fermentation, 1-butanol does not yet seem to be competitive with ethanol as biofuel, despite it much better properties. Nevertheless, commercial fermentative 1-butanol production has started again, especially in China. It uses Clostridia and may amount about 500,000 t/a by now.[129] This butanol is probably used as chemical rather than as fuel.

An alternative to the Clostridial pathway from glucose to 1-butanol proceeds via citramalate and 2-oxobutyrate, which are shown in Figure 7. 2-Oxobutyrate can be reduced to 1-butanol. Upon introducing this pathway in *E. coli*, 0.5 g/L 1-butanol has been obtained.[113] Finally, the potential of the hyperthermophile *Pyrococcus furiosus* to reduce butyric acid (see section 9.3.1) to 1-butanol at 5 bar H_2 has been demonstrated.[132]

5.1.6. 2-Butanol

Presently 2-butanol is produced at about 5 million t/a by petrochemical methods.[133] It can be used as solvent and as precursor of amines, esters, and other valuable derivatives.

The occurrence of small amounts of 2-butanol in some fermented products is well known. Distillates of fermented wine residues can contain up to 3 g per liter ethanol.[134] The background is the well-known pathway to 2,3-butanediol (see section 5.2.6), which involves decarboxylative dimerization of pyruvate to 2-acetolactate, and which is performed by *S. cerevisiae*, for example. Certain strains of *Lactobacillus* can dehydrate 2,3-butanediol to an enol, which isomerizes spontaneously to 2-butanone. Subsequent enzymatic reduction by NAD-dependent alcohol dehydrogenase yields 2-butanol (Figure 8).[135,136]

Patents of DuPont describe *E. coli* strains which have been metabolically engineered for 2-butanol production according to the aforementioned pathway.[133,137] The highest reported concentration of 2-butanol is only 0.034 g/L, with a yield on glucose below 0.01 g/g and a productivity of 1.4 mg/(L h). This demonstrates the concept but also suggests that industrial application is still very remote. The potential of the pathway, however, is indicated by the theoretical stoichiometry of 0.41 g/g, identical to the one given for 1-butanol. A modified pathway involving phosphorylated intermediates instead of 2,3-butanediol can also be used.[133,137]

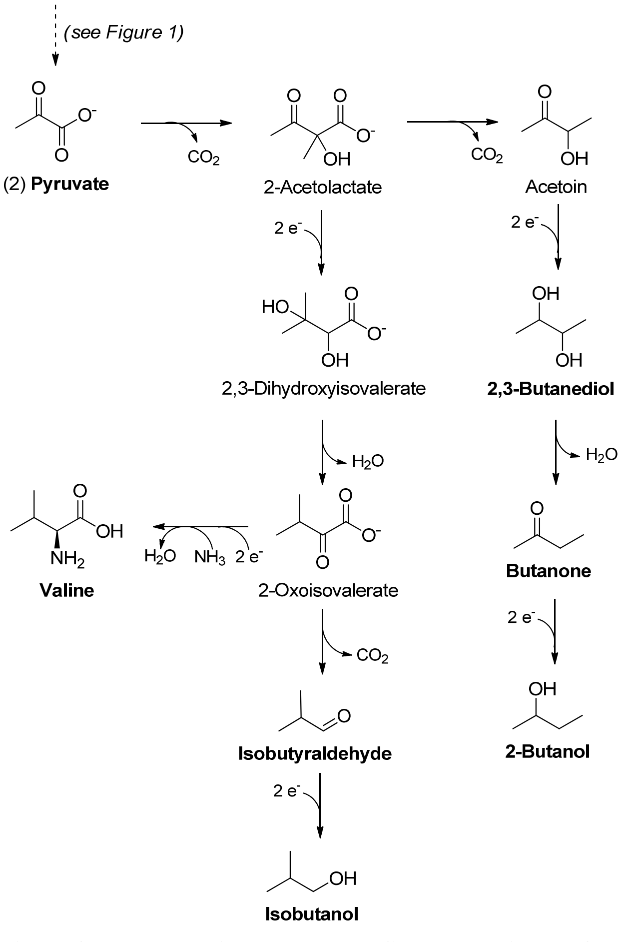

Figure 8. Pathways via acetolactate. Compounds in boldface are treated in the text.

A very different route to 2-butanol has been described in a patent application of Toyota.[138] An *E. coli* strain in which the pathway to isopropanol was introduced (see section 0), formed up to 0.23 g/L 2-butanol after 48 h. The patent application suggests promiscuity of enzymes involved in the pathway from acetyl-CoA to isopropanol but does not clarify this. A patent application of Genomatica[139] describes a comparable route but focuses on microbial butanone formation (see section 8.2).

5.1.7. Isobutanol

The fields of application of isobutanol (2-methyl-1-propanol) closely resemble those of 1-butanol. Its industrial production from fossil carbon sources has been estimated at 500,000 t/a.[98]

Following 1-butanol, isobutanol production from biomass using recombinant microorganisms is approaching large scale commercialization. Plans of Gevo to build a 55,000 t/a isobutanol plant[140] were halted shortly later, however.

E. coli is the main organism that has been described for isobutanol production.[113,141,142,143] Many alternatives are currently being studied because of their robustness or ability to deal with lignocellulosic sugars, for example *S. cerevisiae*,[144,145] *C. glutamicum*,[146,147] *B. subtilis*,[148] and *Clostridium cellulyticum*.[149] The metabolic pathway generally used is a modification of the pathway to L-valine (see Figure 8). After conversion of carbohydrates to pyruvate, two molecules of pyruvate are coupled to acetolactate by acetolactate synthase, releasing CO_2. Using NAD(P)H, this is reduced to 2,3-dihydroxyisovalerate, which is then dehydrated to 2-oxoisovalerate. A subsequent decarboxylation yields isobutyraldehyde, which is reduced to isobutanol using NAD(P)H. Thus, a maximum of 1 mol isobutanol per mol of glucose (0.41 g/g) can be achieved if competing reactions are blocked. Another requirement is that the type of reduced cofactor (NADH or NADPH) formed during pyruvate formation corresponds to the type required for the subsequent reduction steps. By choosing suitable enzymes, the pathway's dependency on NADPH was removed and the maximum yield was achieved, using anaerobic conditions.[142]

The highest published isobutanol concentrations are about 22 g/L, achieved at yield on glucose of about 0.35 g/g and with a productivity of about 0.2 g/(L h).[113,143] The toxicity of isobutanol to *E. coli* limits the production. Using in-situ stripping with gas at nontoxic isobutanol concentrations, a productivity of 0.69 g/(L h) was maintained during 72 h.[150] This approach also facilitates product recovery.

5.1.8. Pentanols

Besides stereoisomers, there are eight alcohol isomers with the formula $C_5H_{12}O$. The individual alcohols and also their mixtures are also known as amyl alcohol and can be used as solvent, fuel component, and ester precursor. Amyl alcohol production was estimated at 50,000 t/a in 2008.[98]

Amyl alcohol is the main component of fusel alcohol, a byproduct of industrial ethanol fermentation, and is formed through three enzymatic steps: Amino acids are transaminated; the formed 2-oxoacids are decarboxylated;

and the resulting aldehydes are reduced to alcohols using NADH-dependent enzymes.[151]

By genetically modifying amino acid metabolic pathways in *E. coli*, pentanol production has been increased. Broad-range 2-oxoacid decarboxylase from *Lactococcus lactis* and alcohol dehydrogenase from *S. cerevisiae* were introduced, besides upregulating the pathway for production of the 2-oxoacid precursor and blocking alternative pathways. Using the isoleucine pathway, 1.25 g/L 2-methyl-1-butanol was obtained at a yield on glucose of 0.17 g/g and a productivity of 0.05 g/(L h).[152] Using the valine pathway, 1.28 g/L 3-methyl-1-butanol (isopentanol) was obtained at a yield on glucose of 0.11 g/g.[153] Further engineering in combination with in-situ extraction by 50 % (v/v) oleylalcohol prolonged the production of 3-methyl-1-butanol while maintaining the yield of 0.11 g/g.[154] The aqueous concentration reached 0.8 g/L but the organic phase concentration became 8.8 g/L, so the overall concentration was 4.8 g/L, and the productivity based on aqueous fermentation volume was 0.16 g/(L h).

2-Oxohexanoate, the 2-oxoacid that would be converted to 1-pentanol, is not normally found in microorganisms. However, an enzyme involved in 2-oxoisovalerate 3-step chain elongation to 2-oxoisocaproate was engineered and became sufficiently promiscuous to also elongate 2-oxopentanoate to 2-oxohexanoate.[155] Besides, the enzyme for subsequent decarboxylation was engineered. This allowed production of 0.75 g/L of 1-pentanol by *E. coli* at a yield on glucose of 0.04 g/g and a productivity of 0.02 g/(L h).

The aforementioned production levels have not been optimized and are not yet commercially interesting, but they demonstrate the principle.

5.1.9. 1-Hexanol

1-Hexanol is used as a solvent, as a basic material for the perfume industry, and for the production of plasticizers. It is prepared at modest scale from ethene or from natural products.[98]

Biosynthesis of small amounts of 1-hexanol from glucose has been achieved in *E. coli*,[113,155] for example by extending the 2-oxoacid pathway that was previously described for 1-butanol production.[113] In this pathway, one acetyl-CoA was used to extend the chain length by one carbon unit. An alternative pathway should be able to achieve a higher carbon yield.[156] As discussed before, Clostridial 1-butanol biosynthesis proceeds via coupling of two acetyl-CoA to acetoacetyl-CoA, which is reduced to butyryl-CoA (see Figure 4). Addition of a third acetyl-CoA can lead to hexanoyl-CoA and subsequently to 1-hexanol, using enzymes with the proper selectivity. The feasibility of 1-hexanol synthesis using *E. coli* has been demonstrated by incorporating such a pathway. Several optimizations including directed evolution by random mutagenesis improved 1-hexanol production to almost 0.5 g/L.[156-157] Interestingly, hexanoic acid (see section 9.4.2) is completely hydrogenated to 1-hexanol by the hyperthermophile *Pyrococcus furiosus* at 5 bar H_2.[132]

5.1.10. Fatty alcohols

Fatty alcohols are aliphatic alcohols with chain lengths between C_6 and C_{22}. They can be unsaturated. Natural fatty alcohols are derived from renewable resources such as fats, oils, and waxes of plant, animal or microbial origin, whereas synthetic fatty alcohols are produced from petrochemicals such as olefins and paraffins. The production capacity of fatty alcohols was estimated to be 2.8 million t/a in 2007, nearly equally distributed between petrochemical and natural feedstocks. The production of natural fatty alcohols proceeds by catalytic hydrogenation of fatty acids or their methyl esters.[158]

Recently, biochemical alternatives have been shown to be possible (Figure 3). In engineered *E. coli*, glucose was converted to fatty acid, from which the CoA derivative was formed. Subsequently, a fatty acyl-CoA reductase and a native aldehyde reductase led to 0.06 g/L fatty alcohol.[159] The intermediate fatty aldehydes can also be formed from fatty acids using a broad specificity carboxylic acid reductase.[160] Such an enzyme was expressed in *E. coli* using a putative carboxylic acid reductase sequence that had been seen in the genome of *Mycobacterium marinum*. The enzyme consumes ATP and NADPH and has as prosthetic group covalently linked 4′-phosphopantetheine. In this case fatty alcohols were formed from fatty aldehydes using aldehyde reductases such as obtained from the cyanobacterium *Synechocystis* species PCC 680.[161] The achieved fatty alcohol concentration was 0.35 g/L. Since fatty acyl CoA reductase from *Marinobacter aquaeolei* has been found to catalyze NADPH-dependent four-electron reduction of fatty acyl-CoA to fatty alcohol, the intermediate free fatty aldehyde might be bypassed.[162]

5.2. Aliphatic diols and triols

5.2.1. Ethylene glycol

Ethylene glycol or 1,2-ethanediol is used mainly as antifreeze agent and as a raw material for the manufacture of polyester. Global demand was estimated at ca. 18 million t/a.[163] It is produced via thermal hydrolysis of ethylene oxide.[164] Biocatalytic hydrolysis is possible as well when using a suitable epoxide hydrolase,[165] but this would pose no clear advantage and is not pursued.

Besides using ethylene oxide such as described in Section 6.1, bio-based routes to ethylene glycol might use direct fermentation of carbohydrates. Figure 9 shows that ethylene glycol formation is linked to the central metabolism via glyoxylate, which could be reduced in three steps to ethylene glycol. The final reduction is catalyzed by lactaldehyde reductase. Its natural reaction is the oxidation of *(S)*-propane-1,2-diol with NAD^+ to *(S)*-lactaldehyde. The *E. coli* enzyme will at almost the same rate also convert ethylene glycol to glycolaldehyde and the other way around.[166] Recently, it has been claimed that mutants of the NADP dependent alcohol dehydrogenase YqhD can also be used for ethylene glycol production.[167] Also, a metabolic route to ethylene glycol has been assembled in *E. coli*.[163] It relies on D-xylose, which is oxidized to D-xylonate by D-xylonate dehydrogenase; then a dehydratase forms 2-dehydro-3-deoxy-D-xylonate, which is split by an aldolase into pyruvate and glycolaldehyde (Figure 9). While glycolaldehyde is reduced to ethylene glycol, pyruvate is converted into side products, which limits the maximum yield on xylose of the pathway to 0.41 g/g. The authors of this first attempt achieved 0.29 g/g, and a final ethylene glycol concentration of 12 g/L at a productivity of 0.24 g/(L h). These promising results should stimulate follow up work.

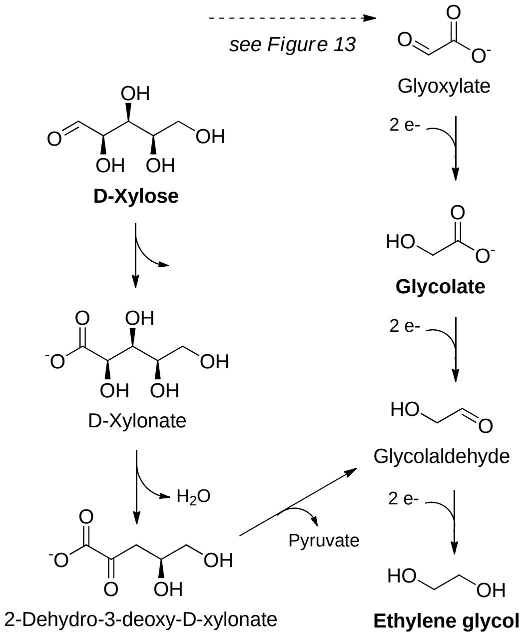

Figure 9. Pathways to ethylene glycol and glycolate.

5.2.2. 1,2-Propanediol

1,2-Propanediol is a commodity chemical with global demand estimated around 1.4 million t/a. It is produced via petrochemistry and has a major role in applications such as antifreeze and heat-transfer fluids, plasticizers and thermoset plastics, and cosmetics.[168]

In wild-type *Clostridium thermosaccharolyticum*, glycerone phosphate (dihydroxyacetone phosphate) is converted to methylglyoxal, which is then reduced to *(R)*-1,2-propanediol via hydroxyacetone (Figure 10). A fraction of the methylglyoxal is also converted to D-lactate. In an anaerobic batch fermentation at 60 °C this led to 9.05 g/L *(R)*-1,2-propanediol, with a yield on glucose of 0.20 g/g and a productivity of 0.36 g/L.[169] More recently, pathways from glucose to *(R)*-1,2-propanediol have been introduced in *E. coli*[170,171] and *S. cerevisiae*,[172,173] but so far not with improved results. Also, glycerol has been used as starting compound. Glycerol is more reduced than glucose and can potentially lead to a higher yield, but achieved yields are still modest.[168]

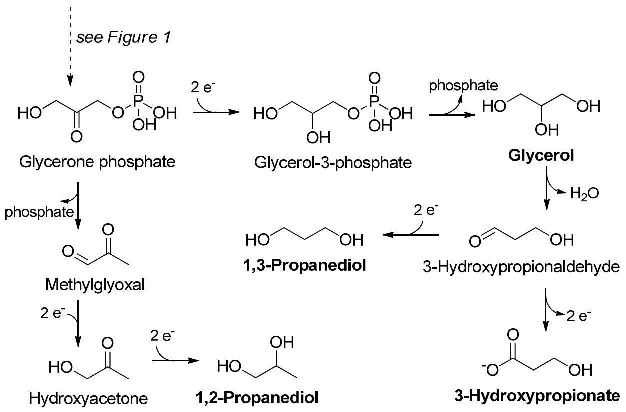

Figure 10. Pathways to glycerol, propanediols and 3-hydroxypropionate. If glycerol is used as starting compound, reactions in reverse direction can be used.

5.2.3. 1,3-Propanediol

The main use of 1,3-propanediol is as monomer, for example to obtain a polyester with terephthalic acid. It has traditionally been produced via petrochemical routes, at a scale much smaller than for 1,2-propanediol, but recently 1,3-propanediol production by fermentation of glucose and glycerol has been developed.

Glycerol fermentation to 1,3-propanediol is performed by many natural organisms. The pathway involves merely two steps, and requires anaerobic conditions (Figure 10). Firstly, coenzyme B12-dependent glycerol dehydratase is used to obtain 3-hydroxypropionaldehyde, which is then reduced to 1,3-propanediol by an NADH-dependent oxidoreductase. This pathway is used, for example, by *Clostridium butyricum* AKR102a.[174] In fed-batch fermentations on 1-L and 200-L scale the strain reached 94 g/L 1,3-propanediol with an overall productivity of 3.3 g/(L h). The yield was 0.52 g/g, because the requirement for NADH implies that part of glycerol is oxidized to side products such as carboxylic acids and CO_2. Using a metabolically engineered *C. acetobutylicum* strain, 0.54 g/g has been achieved.[175]

Glucose fermentation to 1,3-propanediol requires a pathway that directs glucose towards glycerol, which is then further converted as mentioned before. For this, the glycolysis is performed up to glycerone phosphate (dihydroxyacetone phosphate), which is then reduced and dephosphorylated to glycerol (Figure 10).[176] Such a pathway has been introduced in *E. coli*. However, *E. coli* lacks coenzyme B12, which is required by glycerol dehydratase. Therefore, the pathway to synthesize coenzyme B12 was introduced in *E. coli*, including a reactivation enzyme.[176,177] In a patent of DuPont,[177] the highest final 1,3-propanediol concentration is 141 g/L, with a yield on glucose of 0.44 g/g. A higher yield, 0.51 g/g, is mentioned for a fermentation that led to 135 g/L 1,3-propanediol and a productivity of 3.5 g/(L h).[176]

The fermentative production of 1,3-propanediol is performed at 45,000 t/a scale by a joint venture of DuPont and Tate & Lyle. Metabolic Explorer is currently building a plant for 1,3-propanediol based on glycerol.

5.2.4. Glycerol

Glycerol is the trivial name of propane-1,2,3-triol. For some time it has been produced from petrochemical sources, but currently it is produced from natural fats and oils. These consist of fatty acid esters of glycerol, which are converted into biodiesel, leading to glycerol as co-product, as mentioned in Table 2. On the basis of the proportionality to biodiesel production (section 10.1), about 2 million t/a glycerol is produced. Many new processes, including fermentations,[22] are in development to use this currently so abundant and cheap glycerol as carbon source. Considering Figure 1, it requires only a few metabolic steps to form the same products as from glucose.

Consequently, glycerol might also easily be produced by fermentation of carbohydrates, although this is economically unattractive. For example, 302 g/L glycerol has been obtained at a yield on glucose of 0.70 g/g using engineered *E. coli*.[177] The pathway is described in Section 5.2.3.

5.2.5. 1,4-Butanediol

1,4-Butanediol is produced via petrochemistry at a scale of about 1.3 million t/a.[178] It is converted into polyesters, polyurethanes, and other polymers. Recently, a fermentation route to 1,4-butanediol has been developed by engineering *E. coli*.[179] The metabolic route selected branches off from the citrate cycle at 2-oxoglutarate (Figure 11). This is decarboxylated by 2-oxoglutarate decarboxylase to succinate semialdehyde, which is then reduced by 4-hydroxybutyrate dehydrogenase. The next reduction, of the carboxylic acid group, is thermodynamically difficult and was therefore done after CoA activation by 4-hydroxybutyryl-CoA reductase. In the final step, 4-hydroxybutyraldehyde is reduced using an alcohol dehydrogenase.

This led to production of 18 g/L 1,4-butanediol from glucose in 5 days.[179] Side products were still at too high levels, and the theoretical yield of the pathway of 0.5 g/g was not approached. However, this is a good basis for the further development that is currently being carried out to achieve commercially attractive levels. Genomatica researchers used evolutionary engineering methods to increase the tolerance of *E. coli* to 1,4-butanediol to more than 100 g/L, thus overcoming a potential major hurdle to commercialization.[178] Upon engineering the final three enzymes of the pathway, 115 g/L 1,4-butanediol was achieved with a productivity of 3.3 g/(L h). Using lignocellulosic feedstock somewhat lower numbers are achieved, and production has been tested already on a scale of thousands of tonnes.

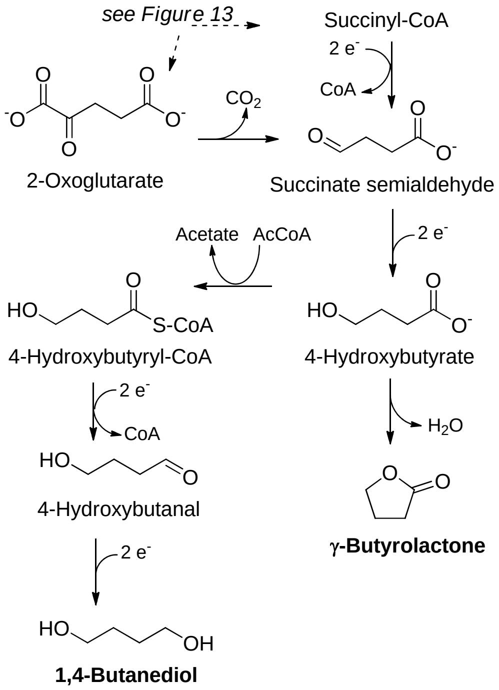

Figure 11. Pathways from intermediates of the citrate cycle to 1,4-butanediol and γ-butyrolactone.

5.2.6. 2,3-Butanediol

This diol has no history as petrochemical platform chemical, but its biochemical production is relatively easy and might become important. The dehydration of 2,3-butanediol yields the industrial solvent 2-butanone, and further dehydration produces 1,3-butadiene for the manufacture of synthetic rubber.[180] For polymerization it is much less desired than 1,4-butanediol, but its microbial production is relatively easy because it is a natural fermentation product of several bacteria. In the 1940s, pilot scale operation was performed, and also now industrial fermentation is considered.[181]

The used pathway is shown in Figure 8, and has a maximum yield on glucose to 1 mol/mol (0.50 g/g). The pathway needs some O_2, or leads to more oxidized fermentation byproducts such as acetoin, to close the redox balance. The dehydrogenases responsible for reduction of diacetyl determine which enantiomer of 2,3-butanediol is obtained, but chirality is not a major research issue if the main anticipated destination of 2,3-butanediol is chemical conversion into butadiene or butanone.

The highest fermentation concentration obtained for 2,3-butanediol is 152 g/L, using aerobic fed-batch fermentation with a mutant of *Serratia marcescens*.[182] The yield on sucrose was 0.49 g/g, whereas the theoretical yield on sucrose is 2 mol/mol, corresponding to 0.53 g/g. The productivity was 2.7 g/(L h). Almost the same titer (150 g/L) has been achieved from glucose with *Klebsiella pneumonia*, but with a relatively higher yield (0.48 g/g as compared with the maximum of 0.50 g/g on glucose) and higher productivity (4.2 g/(L h)).[183]

Recovery of 2,3-butanediol from aqueous solution is relatively difficult because of its hydrophilicity and low volatility.

5.3. Carbohydrates

5.3.1. Glucose

D-Glucose is the hydrolysis product of many polysaccharides. To obtain it in pure form, homopolymers of glucose are desired. The β-1,4 polymer cellulose is the most abundant one, but difficult to hydrolyze.[21] Starch, which contains the α-(1,4) polymers amylose and amylopectin, with amylopectin having α-(1,6) branches, are relatively easy to convert into glucose. First an α-amylase is used for hydrolysis at ~100 °C into shorter chains and subsequently glucoamylase hydrolyses the remaining oligosaccharides at more modest temperatures. Additional enzymes can be added with specificity for debranching amylopectin, for example.[184] Considering merely the US production of ethanol by fermentation of hydrolyzed starch, the capacity of glucose production by starch hydrolysis will exceed 60 million t/a. World-wide production of glucose for various fermentation or food related purposes will be significantly larger.

5.3.2. Fructose

In 2005, D-fructose was produced on a scale of about 6.5 million t in mixtures with D-glucose.[185] The advantage of fructose is that it is sweeter. Packed beds of immobilized glucose isomerase are used to continuously isomerize the more available glucose into a mixture containing 42% fructose (~200 g/L), close to the equilibrium composition of about 50%. Chromatographic enrichment to higher fructose contents is used. The productivity is very high, about 800 g/(L h)[185,186]

Instead, the disaccharide sucrose can also be hydrolyzed into 1:1 mixtures of glucose and fructose, using the enzyme invertase, which is excreted by *S. cerevisiae*, for example. During sucrose fermentation to ethanol by this yeast, the hydrolyzed monosaccharides are directly taken up by the cells for further conversion.

Fructose homopolymers such as inulin are, upon enzymatic hydrolysis, also a source of fructose, but this process does not have much economic significance yet.[185]

5.3.3. Xylose

The C_5 monosaccharide D-xylose is one of the most important constituents of hemicellulose, which makes up 20-35% of lignocellulosic materials such as wood. Hemicellulose is relatively easily hydrolyzed by chemical methods, and this is the main method for industrial production of xylose. However, enzymatic methods are also considered because they lead to less degradation products. The xylanases that cleave β-(1,4) glycosidic bonds of a xylan backbone are blended with arabinofuranosidases, mannanases, hemicellulolytic esterases, and glucuronidases, for example to synergistically hydrolyze hemicellulose completely.[21,187]

5.3.4. Isomaltulose

Isomaltulose, also called palatinose and 6-*O*-α-D-glucopyranosylfructose, is an isomer of sucrose. Isomaltulose and its hydrogenated derivative replace sucrose as sweetener in some applications. In 2011, the production of isomaltulose has been estimated at 120,000 t/a.[188] This occurs by transglucosidation of sucrose catalyzed by immobilized *Protaminobacter rubrum* cells that contain an α-glucosyl transferase. Productivities of 40 g/(L h) have been achieved in continuous reactors, leading to about 200 g/L isomaltulose. Yields on sucrose can reach 99%.[189]

5.4. Sugar alcohols

5.4.1. Erythritol

Erythritol is a polyol ((2*R*,3*R*)-butane-1,2,3,4-tetraol) and widely distributed in nature. It can be safely used as a noncariogenic sweetener in foods as it cannot be fermented by the bacteria that cause dental caries. In 2006, the world market was 20,000 t.[190]

Erythritol is formed from glucose by yeasts and other fungal species via the pentose phosphate pathway, which has D-erytrose-4-phosphate as intermediate (see Figure 1). This is dephosphorylated by a kinase and subsequently reduced by erythrose reductase using NAD(P)H.[190] Such a pathway can lead theoretically to 1 mol of erythritol per mol of glucose (0.68 g/g), and requires aeration for NAD(P)H regeneration. Some bacteria use a slightly different pathway. However, osmophilic yeasts are preferred for production, and these have been subjected to classical mutation to improve their performance.

Some of the best results have been obtained using *Pseudozyma tsukubaensis* KN75.[191] In a fed-batch culture, an erythritol yield on glucose of 0.60 g/g was obtained at a final erythritol concentration of 241 g/L and at a productivity of 2.8 g/(L h). Such good results were not only obtained at laboratory scale but also at 50 m^3 scale. For industrial production, other strains have been described. For example, using *Trichosporonoides megachiliensis*, the yield on glucose, product titer, and productivity reached 0.47 g/g, 192 g/L, and 2.0 g/(L h), respectively, in a 100 m^3 fermentation tank.[191]

5.4.2. Xylitol

Xylitol is an optically inactive C5 polyol. It is industrially produced by reducing pure D-xylose, obtained from hardwood hydrolysate, using H_2 and a nickel catalyst.[187] The annual production has been estimated in 2006 to lie between 20,000 and 40,000 t/a.[192] It has sweetening power as high as sucrose but prevents caries.

To be able to use impure D-xylose, mixed with other sugars obtained from wood hemicellulose hydrolysate, biotechnologicial production has been pursued. This involves NAD(P)H dependent xylose reductase. For example, xylitol has been produced in the presence of significant amounts of yeast extract during cell recycling of *Candida tropicalis* ATCC 13803. The yield of xylitol on xylose was 0.83 g/g, at a productivity of 4.0 g/(L h) and final xylitol concentration of 244 g/L.[193] Using recombinant *S. cerevisiae*, almost quantitative yields on xylose have been obtained while using glucose as co-substrate.[194,195]

5.4.3. Sorbitol

Sorbitol is a C6-sugar alcohol obtained by reduction of the aldehyde group of L-sorbose or D-glucose, and is therefore also called D-glucitol. The reduction of glucose can efficiently be done with H_2 using nickel catalysts. This has led to industrial production amounting to 500,000 t/a in 1994, for use of sorbitol in food products, as polymeric building block, and in the classical vitamin C synthesis.[196] Recently, the production has been estimated at 700,000 t/a.[197]

Nevertheless, biochemical production has also received considerable interest. Most research has focused on using the potential of the bacterium *Zymomonas mobilis* to co-produce sorbitol and D-gluconic acid from sucrose or 1:1 glucose-fructose mixtures. A single enzyme, glucose-fructose oxidoreductase, a tetrameric protein with tightly coupled NADP, is responsible for both the reduction of fructose to sorbitol and the dehydrogenation of glucose to glucono-δ-lactone. Additionally, glucono-δ-lactonase is present to speed up the (otherwise spontaneous) hydrolysis of glucono-δ-lactone to gluconic acid.[198,199]

Although systems with isolated enzymes and with immobilized cells have been developed,[196] the best results seem to have been obtained with free, untreated, *Z. mobilis* ATCC 29191 in a batch system. The use of up to 650 g/L of an equimolar mixture of glucose and fructose resulted in an almost complete conversion to sorbitol and gluconic acid, with final concentrations of 300 and 320 g/L, respectively, without ethanol formation. Yields were 0.91 g/g for both products, in 8 h of operation.[200] However, commercial operation was not achieved.[196]

5.4.4. D-Mannitol

Mannitol is a C6-sugar alcohol naturally produced by many organisms to protect cells and proteins against heat and osmotic changes. It is used as low-caloric and low-cariogenic sweetener in food for diabetic patients, and is widely used as filler in pharmaceutical applications.[201] Industrial production of mannitol has been performed by reduction of glucose–fructose mixtures with hydrogen using nickel as a catalyst. However, this mainly leads to sorbitol.[202]

Therefore biochemical alternatives have been developed.[203] These rely on NAD(P)H dependent reduction of fructose, obtained by isomerization of glucose. Alternatively, glucose is phosphorylated to glucose 1-phosphate, which is isomerized to fructose 1-phosphate before reduction to mannitol 1-phosphate and dephosphorylation. Consequently, the yield on glucose can be 1 mol/mol (1.01 g/g) if a reducing agent would be available. Wild-type lactic acid bacteria use 1/3 of the glucose for NAD(P)H generation, limiting the yield to 0.67 g/g.[201] Theoretically, 0.93 g/g might be obtained if the glucose carbons used for NAD(P)H generation would all end up as CO_2. Such approaches have been studied with yeasts, for example. Using classical mutagenesis, mutants of the yeast *Candida magnoliae* NCIM 3470 were generated that produced in a two-stage fermentation mannitol up to 240 g/L, at a yield of 0.81 g/g and a productivity of 4 g/(L h) without formation of any by-product.[201] The first stage was aerobic growth and the second stage anaerobic conversion of fructose rather than glucose.

Similar as for sorbitol, an elegant enzymatic cofactor regeneration system has been developed to produce mannitol simultaneously with gluconic acid (a compound treated in section 9.4.6) at 50-60 g/L.[204,205] This starts with a glucose/fructose mixture (1:1) such as obtained from glucose using glucose isomerase or sucrose using invertase. A glucose dehydrogenase converts glucose to gluconic acid, using NAD^+ and generating NADH. Mannitol dehydrogenase uses NADH to convert fructose to mannitol and regenerates NAD^+.

5.5. Phenol

Production of phenol from fossil resources amounts to about 9 million t/a, mostly for the production of polycarbonates and resins.[206]

Using a solvent-tolerant *Pseudomonas putida* S12 strain, glucose has been converted into phenol with a yield of 0.035 g/g. The pathway (Figure 6) follows formation of L-tyrosine, and its conversion with water into phenol, pyruvate and ammonia, using a tyrosine–phenol lyase encoded by a gene from *Pantoea agglomerans*. The *P. putida* strain produced 0.14 g/L of phenol in 24 h in a shake flask using mineral medium supplemented with glucose and some salicylate. In a fed-batch culture, 0.47 g/L phenol was achieved. To overcome the product toxicity, 1-octanol was used *in-situ* as extractant. The phenol concentration in the octanol phase reached 5.5 g/L and the productivity was doubled as compared to the single phase fed-batch.[207] Further improvements will be required to achieve commercial significance.

6. ETHERS

6.1. Epoxyethane

Epoxyethane, also known as ethylene oxide and as oxirane, is a simple cyclic ether. World production of epoxyethane was ca. 15 million t/a in 2000. The most important application is production of ethylene glycol.[208]

Bio-based epoxyethane will require bio-based ethene (see section 3.1). Although epoxidation of ethene is conveniently carried out on industrial scale using chemical catalysis, biocatalytic epoxidation has been studied as well.[209] The reason for this was probably that production of epoxyethane was a model system for production of other epoxides, such as styrene oxide, where the biocatalysis can be used to obtain enantiopure products. Epoxidations have been performed using immobilized *Mycobacterium* Py1. An NAD(P)H-consuming monooxygenase used O_2 for the epoxidation, thus allowing gas phase supply of reactants if this gas phase also contained propanal as cosubstrate for NAD(P)H regeneration.[209]

The biocatalytic epoxidation of ethene is a very slow reaction compared to the chemocatalytic epoxidation and does not offer a clear advantage. Introducing the enzyme for epoxidation in a hypothetical ethene-emitting microorganism would require aerobic conditions for epoxidation activity. This might easily conflict with the conditions required for biochemical ethene formation.

6.2. Epoxypropane

Epoxypropane, also called propylene oxide or methyloxirane, is produced at about 9 million t/a. It is the starting material for a broad spectrum of polymers (polyurethanes, polyesters) and liquid chemicals (propylene glycol, polyglycols, propylene glycol ethers).[210]

The chemistry and biochemistry of epoxypropane resemble those of epoxyethane. An important difference is that in case of propene epoxidation, enantioselectivity may play a role. The biocatalytic approach is usually *R*-selective.[211] Bio-based *(R)*-epoxypropane production will require bio-based propene (see section 3.2), which is not yet available.

6.3. Other ethers

For other ethers that are currently produced at significant scale by petrochemistry, such as methyl-*tert*-butyl ether, ethyl-*tert*-butyl ether, and tetrahydrofuran, biocatalytic routes have not yet been proposed. Biosynthetic ether formation is not a focal area in bio-based production. However, an analysis of the occurrence of ethers in natural products showed that there are many types of enzymatic ether formation known, so this is actually a rich field of biochemistry.[212]

7. ALDEHYDES

7.1. Formaldehyde

Formaldehyde has numerous applications, in particular for producing resins. In 1996, the production capacity of formaldehyde was about 9 million t/a.[213] Besides this fossil-carbon based production, biochemical production has received some interest. It is based on oxidation of methanol, so it would require bio-based methanol to become bio-based (cf. Figure 2). Using an alcohol oxidase-enhanced mutant strain of the methylotrophic yeast *Candida boidinii*, 35 g/L formaldehyde has been produced at aerobic conditions. The productivity was high, about 5 g/(L h), but the yield on methanol was only 0.36 g/g, probably due to further oxidation. Under appropriate culture conditions, alcohol oxidase comprised nearly 50% of the cells' total soluble proteins. [214,215]

7.2. Acetaldehyde

Acetaldehyde is produced from fossil resources at a scale of about 1 million t/a, although Brazilian production is bio-based, using catalytic bioethanol oxidation.[216] Acetaldehyde is an important intermediate in the production of many chemicals.

Acetaldehyde is the precursor of ethanol in fermentations (see Figure 4), and usually its formation is minimized. *S. cerevisiae* strains that were used for wine production formed 0.01-0.09 g/L of acetaldehyde, although certain wines contain 0.3 g/L.[217] The toxicity of acetaldehyde to the producing microorganisms, in combination with the ease of petrochemical production, has limited the development of dedicated fermentation processes for acetaldehyde. Heat-treated (nonviable) cells of *Candida boidinii*, however, have produced 75 g/L acetaldehyde by aerobic oxidation of ethanol.[215] The yield on ethanol was almost quantitative, 0.98 mol/mol, corresponding to 0.94 g/g, whereas the productivity was 4.2 g/(L h).

Engineered *E. coli* has been used for the following overall reaction:[218]

$$\text{glucose} \rightarrow 2\ \text{acetaldehyde} + 2\ H_2 + 2\ CO_2$$

This was done by deleting some native pathways, and introducing an exogenous acetyl-CoA reductase, which converts acetyl-CoA with NADH into acetaldehyde. The achieved molar yield was 86%, corresponding to a mass yield of acetaldehyde on glucose of 0.42 g/g, with a productivity of 0.03 g/(L h). In addition, the process is anaerobic and produces H_2 as a valuable coproduct. The achieved acetaldehyde concentration was only 0.7 g/L, because it is toxic to the cells, but its high volatility will enable in-situ removal by stripping.

7.3. Propanal

Propanal, often called propionaldehyde, is used as intermediate in chemical synthesis of a variety of compounds. World production was estimated at 154000 t/a in 1988.[219] Production uses fossil resources.

Fermentative 1-propanol production proceeds via propanal (see section 5.1.3 and Figure 7). On the other hand, propanal has also been produced from 1-propanol using heat-treated (nonviable) cells of *Candida boidinii* containing alcohol oxidase.[215] In some cases the yield was quantitative, but it dropped to 0.82 g/g at conditions leading to the highest propanol concentration (16 g/L). The productivity was 0.9 – 3.2 g/(L h).

7.4. Butanal and isobutyraldehyde

These C_4 aldehydes are jointly produced by catalytic conversion of propene, at a scale of about 6 and 1 million t/a, respectively. They are used for production of a range of other chemicals, such as their alcohols.[220]

Also in metabolic routes, formation of butanal and isobutyraldehyde precedes the formation of the corresponding alcohols, as shown in Figure 4 and Figure 8, respectively. Small amounts are excreted by several microorganisms.[221] A mutant of *C. acetobutylicum* was found to produce up to 1.7 g/L butanal during ABE fermentation, and it was mentioned that due to its low boiling point (75 °C) recovery should be easier than for 1-butanol (b.p 117 °C).[222]

A recent study focused on maximizing aldehyde production. Deleting all genes expected to be involved in isobutyraldehyde reduction to isobutanol from a isobutanol producing *E. coli* strain led to formation on mainly isobutyraldehyde from glucose, with a yield of 0.18 g/g.[223] The productivity was about 0.29 g/L, and this was maintained during 120 h using in-situ product removal by gas stripping. Although the dissolved concentration was kept at about 1 g/L, the amount produced in this way corresponded to about 35 g/L in the fermentor, which otherwise would have been toxic to the cells.

8. KETONES

8.1. Acetone

Acetone is a chemical intermediate and an excellent solvent for a wide range of industrial materials. Data collected from some major countries indicate a world production capacity in excess of 3 million t/a.[224] As co-product of the more valuable phenol, acetone is not under high demand. Replacement of petrochemical by bio-based production is economically challenging. Technically, however, it would be relatively easy. During the first part of the 20th century, large scale industrial fermentation using *Clostridium* strains was used to produce acetone together with 1-butanol and some ethanol from carbohydrates.[123] This acetone-butanol-ethanol (ABE) fermentation was developed during the First World War because of a high need for acetone, which was solvent in the manufacture of gun powder.

As explained for 1-butanol in section 5.1.5, acetone is formed during the solventogenic phase of the ABE fermentation. Acetic and butyric acid, which are formed during the acidogenic phase, are taken up again in the solventogenic phase by the bacteria and converted into acetyl-CoA or butyryl-CoA. This involves simultaneous conversion of acetoacetyl-CoA into acetoacetic acid (Figure 4). The resulting acetoacetic acid is converted into acetone by acetoacetate decarboxylase, so that the pH of the solution moves toward more neutral levels and the bacteria can continue to grow. According to this route, the molar amount of acetone produced is limited by the molar amount of butyric and acetic acid at the end of the acidogenic phase.[123] However, additional uptake mechanisms recently found for butyric acid complicate this picture.[225]

It has been shown that nonenzymatic decarboxylation of acetoacetate occurs *in vitro*, under conditions similar to *in vivo* ABE fermentation.[226] This explains why knocking out the acetoacetate decarboxylase does not eliminate acetone formation.

Currently, commercial acetone production using the ABE process is mostly in China.[125] The acetone capacity will be about 150,000 t/a. Continuous fermentation is performed using *C. acetobutylicum* mutants obtained via chemical/physical mutagenesis and selection during the fermentation process. According to data from Chinese ABE fermentation industry,[227] the isolated yield of acetone produced from starch is 0.11 g/g. The achieved acetone concentration in the fermentation is about 2 g/L. Probably this could be improved if desired. In the 1990s, overexpressing the acetone-forming enzymes in a *Clostridium acetobutylicum* strain led to 8.7 g/L,[228] and expressing an acetone pathway in *E. coli* led to 8.9 g/L,[229] but recent metabolic engineering studies focus on maximizing 1-butanol formation.

8.2. Butanone

Butanone, also known as 2-butanone and as methyl ethyl ketone, is produced via petrochemical routes at a scale of about 1 million t/a.[139] It is mainly used as solvent for coatings, adhesives, and inks, as well as a chemical building block.

Butanone is an intermediate in the pathway to 2-butanol described in section 5.1.6 and Figure 8. Using the studied recombinant *E.* coli strains, the formation of up 0.032 g/L of butanone from glucose has been claimed. [133,137]

Coupling of acetyl-CoA with propionyl-CoA can give, depending on the enzyme used, 3-oxopentanoyl-CoA or 3-oxo-2-methyl-butanoyl-CoA. Subsequent release of the CoA group and decarboxylation have been suggested by researchers from Genomatica as pathways to butanone.[139] The highest concentration of butanone, 0.14 g/L, was obtained using in an *E. coli* strain a route which combined genes of succinyl-CoA transferase from *Heliobacter pylori*, of thiolase from an *Acinetobacter* strain, and of decarboxylase from *C. acetobutylicum*. The strain had been overproduced for succinate production to improve the carbon flux toward propionyl-CoA.

Bio-based levulinic acid[230] (4-oxopentanoic acid; see Figure 12) is used for another route to butanone. Levulinic acid decarboxylation by a side activity of acetoacetate decarboxylase has been found in *Clostridium acetobutylicum* and expressed in *E. coli*.[231] The molar yield of butanone on initial levulinic acid was 90%, reaching 0.32 g/L butanone without byproduct at a productivity of 0.1 g/L. It is obvious that this interesting route should be developed further.

8.3. Cyclohexanone

Cyclohexanone is produced together with cyclohexanol from fossil carbon sources, as precursor for adipic acid and caprolactam.

A patent application describes metabolic pathway from glucose to cyclohexanone.[232] It involves hypothetical enzymatic reactions. No actual biosynthesis of cyclohexanone has been shown, though.

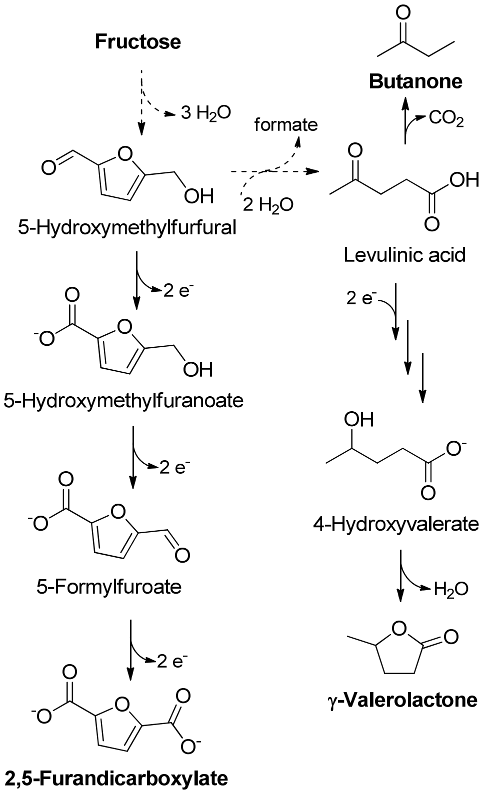

Figure 12. Pathways via 5-hydroxymethylfurfural. Compounds in boldface are treated in the text. Dashed arrows are chemical reactions.

9. CARBOXYLIC ACIDS

As shown subsequently, many carboxylic acids can be produced using biochemical methods. The applications usually require the undissociated acid form, whereas the production methods usually are performed using concomitant titration with base to keep neutral pH, because the pK_a values of the carboxylic acids are typically 3-5. The result is usually a carboxylate solution. To obtain the undissociated acid in reasonable concentration, a pH below about 3 is required. To simplify the overall production process, and in particular the product recovery, acid-tolerant microorganisms such as the yeast *S. cerevisiae* are often developed for carboxylic acid production,[233,234] as alternative to carboxylate-producing bacteria such as *E. coli*.

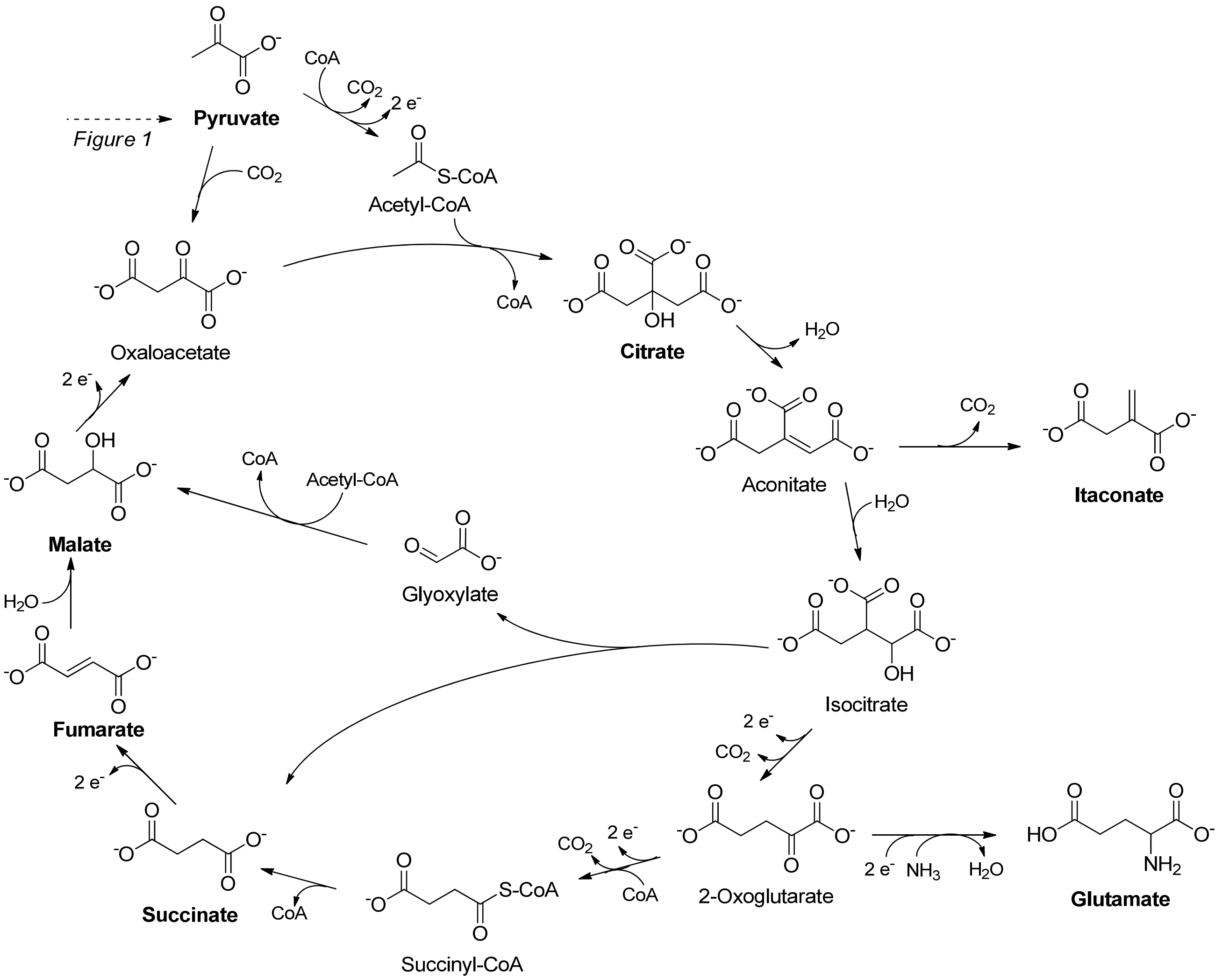

Figure 13. The citrate cycle and some related pathways. The compounds indicted in boldface have individual entries in the text.

9.1. C_1 and C_2 carboxylic acids

9.1.1. Formic acid

Formic acid is industrially produced primarily from methanol and CO via methyl formate, which is then hydrolyzed. In 2009 the formic acid production capacity has been estimated at 720,000 t/a. Among many applications, its use in silaging is prominent.[235,236]

It is well-known that formate is an anaerobic fermentation product of *E. coli* and other bacteria.[237] Usually the research focus is on another fermentation product, and formate formation is minimized.[238] Formate is derived together with acetyl-CoA from pyruvate in a radical-based homolytic cleavage reaction catalyzed by pyruvate formate lyase (Figure 2). Subsequently, formate can be cleaved into H_2 and CO_2, so it is an intermediate in the pathway from glucose to H_2. For fermentative H_2 production from glucose, formate formation and conversion are jointly optimized, and low formate concentrations are desired.[239]

9.1.2. Acetic acid

Acetic acid has a wide variety of uses, including conversion to vinyl acetate, acetic anhydride, acetate salts, acetic esters, and monochloroacetic acid. In 2008, 10.6 million t was produced by petrochemical processes, using mainly methanol carbonylation.[240]

However, acetic acid was historically made and still is made by the fermentation of ethanol, to be used in solution as vinegar. Vinegar production with acetic acid bacteria is very efficient,[241] but the acetic acid thus produced is more expensive than petrochemical acetic acid, due to the ethanol costs. The produced amount of acetic acid in vinegar has been estimated in 2000 at 0.19 million t/a.[242]

Acetic acid bacteria oxidize ethanol to acetate in two consecutive steps using membrane-bound quinoproteins (ethanol dehydrogenase and acetaldehyde dehydrogenase). Molar yields on ethanol can exceed 98%. Using an industrial *Acetobacter* strain in an aerated repeated fed-batch system, optimization of several operation variables using fuzzy control techniques led to up to 203 g/L acetic acid at productivities up to 1.6 g/(L h).[243] Using immobilized *Acetobacter* in continuous fluidized bed reactors, productivities of 9 g/(L h) could be maintained, albeit at 40 g/L acetic acid. The conversion was also done at pilot scale, and aeration at this high productivity required pure O_2.[241]

Instead of ethanol fermentation to acetic acid, carbohydrates can be used. Then, microorganisms will generally produce not only acetic acid. Exceptions are strictly anaerobic homoacetogens such as *Moorella thermoacetica*. Via glycolysis, these organisms convert glucose into two molecules of pyruvate, which are decarboxylated into two acetyl-CoA and two CO_2. Acetyl-CoA is converted into two acetate equivalents. The aforementioned reactions produce eight reducing equivalents, which are utilized to reduce the produced two molecules of CO_2 to an additional acetate equivalent via the Wood–Ljungdahl pathway. Thus, the net product formation reaction would be:

glucose → 3 acetic acid

In this way yields on glucose of ~0.8 g/g have been achieved, leading to acetate concentrations up to 100 g/L at productivities of 0.8 g/(L h).[244] However, such concentrations can only be achieved at neutral pH, whereas the ethanol oxidation proceeds at uncontrolled pH (reaching ~2) and yields the desired acetic acid rather than an acetate salt.

Acetic acid is also formed by the anaerobic digestion of biomass. The four stages of anaerobic fermentation are explained when treating methane in section 2.1. The fourth stage of methane formation can be prevented, for example by adding inhibitors. Then, acetic acid, hydrogen, and carbon dioxide are produced. This production of acetic acid has been advocated as a way to access various commodity chemicals via anaerobic digestion of waste.[245]

Besides all aforementioned microbial conversions, acetate can also be directly liberated from acetylated biomass components such as hemicellulose, by the action of enzymes such as acetyl xylan esterase.[21] However, this will probably lead to relatively low concentrations of acetate and does not seem to be considered as acetic acid production method.

9.1.3. Glycolic acid

Glycolic acid, also known as 2-hydroxyacetic acid, is currently not an important compound despite its simple structure. Annual consumption in the USA of 15000 t has been mentioned[246] but also worldwide production of about 2000 t/a.[247] Some higher plants, eukaryotic algae, and phototrophic bacteria, but also chemolithoautotrophs can synthesize it.[248,249] Oxygenolytic cleavage of D-ribulose 1,5-diphosphate (RuDP) produces phosphoglycolate which is then hydrolyzed to glycolate. The oxygenation reaction is catalyzed by RuDP carboxylase. Using *Alcaligenes eutrophus*, productivities of 16 µg/(h.L) have been obtained from bicarbonate without optimization.[248] No attempt, however, seems to be made to produce bio-based glycolic acid in this way.

However, it is known that ethylene glycol can be efficiently oxidized via glycolaldehyde to glycolic acid using *Gluconobacter oxidans* (cf. Figure 9).[250] Recent patent applications of Roquette Freres describe engineering of *E. coli* to obtain a metabolic route from glucose via pyruvate to glyoxylate and subsequently to glycolic acid.[246,251] The highest yield on glucose was 0.36 g/g, with a glycolic acid concentration of 55 g/L and a productivity of 1.23 g/(L h).

9.2. C_3 carboxylic acids

9.2.1. Propionic acid

The production in 2006 of propionic acid (propanoic acid) was 377,000 t. Food and feed preservation accounted for ca. 78% of the propionic acid consumption in 2009.[252] Commercial production of propionic acid is entirely by petrochemical routes, although a number of pilot plants has been designed and built to produce it by fermentation.[253]

In the 19th century, studies of the anaerobic propionic acid fermentation already resulted in the formulation of the so-called Fitz equation:[253]

3 lactic acid →
2 propionic acid + 1 acetic acid + 1 CO_2 + 1 H_2O

However, dependent on the bacterial species, one out of several different pathways (see Figure 14) is used to achieve this stoichiometry. In the dicarboxylate pathway, glucose is converted into pyruvate or phosphoenolpyruvate, which are carboxylated with CO_2 to oxaloacetate. This is reduced and rearranged in a number of steps to *(S)*-methylmalonate-CoA, which is then decarboxylated to propionyl-CoA, to lead to propionate. Figure 14 shows also the methylcitrate cycle for conversion of pyruvate to propionate. The acrylate pathway is similar until pyruvate. After reduction to lactate, 2-phospholactoyl-CoA is formed to enable dehydration to acrylyl-CoA. Finally this is reduced to propionyl-CoA, which is then hydrolyzed.[253] In each pathway, acetate production leads to NADH required for the reduction in the propionate branch. Obviously, the acetate production constrains the achievable propionic acid yield. According to the given equation, the theoretical propionic acid yield on lactic acid (or glucose) is 0.55 g/g. Elimination of the acetate-forming pathway has been studied,[254] but an alternative NADH supply route will be required instead.

Another problem is the toxicity of propionic acid to the bacteria. However, a high propionic acid-tolerant strain has been developed by adapting a metabolically engineered *Propionibacterium acidipropionici* with gradually increased propionic acid concentration in the fermentation broth via fed-batch fermentation. The adapted mutant grew faster than its parental strain and produced 97 and 104 g/L propionic acid from glucose and lactate, respectively.[255] Productivities were 0.07 and 0.12 g/(L h). The average propionic acid yield on glucose was 0.53 g/g, close to the maximum. Using glycerol, 106 g/L propionic acid has been achieved, but at the expense of yield and productivity.[256]

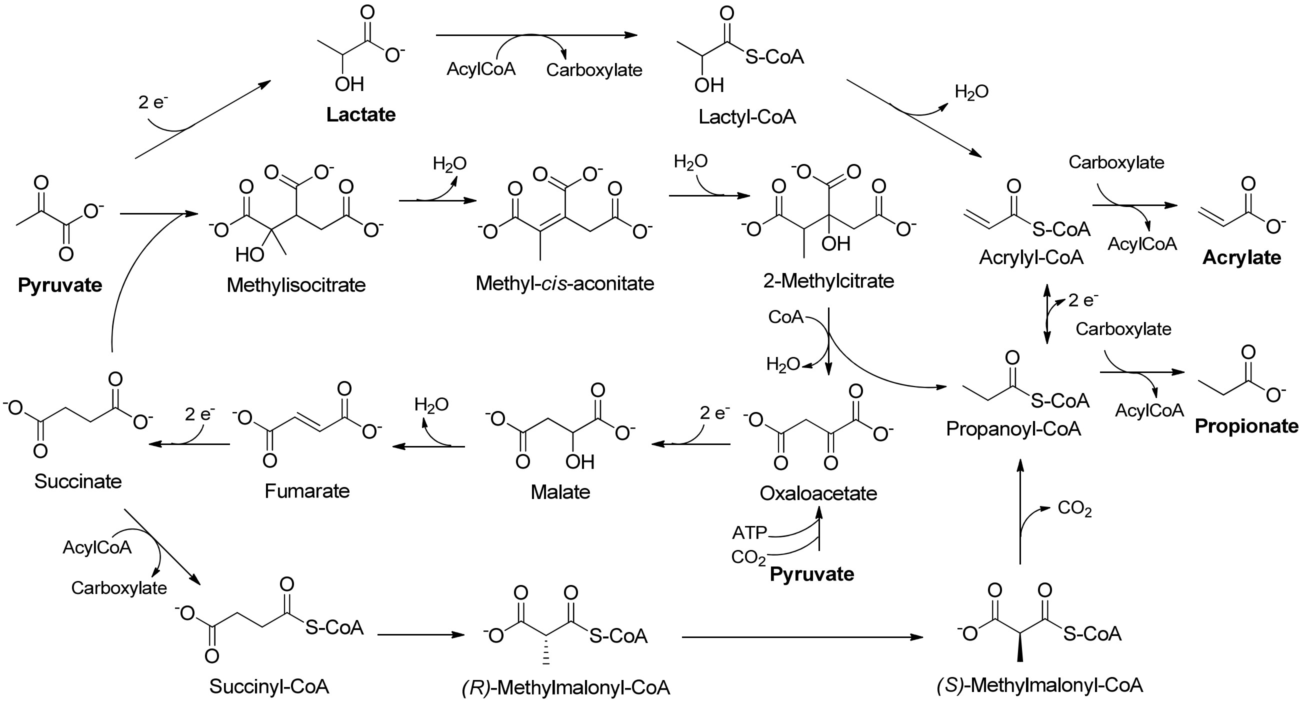

Figure 14. Pathways to lactate, propionate, and acrylate, starting from pyruvate, which is obtained according to Figure 1.

9.2.2. Acrylic acid

Acrylic acid, also known as 2-propenoic acid, is produced at about 4.2 million t/a by petrochemistry. Its major utilization is in polymers.[257]

In nature, free acrylate only seems to play a role in the degradation of dimethylsulfoniopropanoate (DMSP). DMSP is an abundant osmolyte in marine environments. It is made by many single-celled marine phytoplankton, marine macroalgae, and a few angiosperms. DMSP is a potential energy and carbon source for marine bacteria, and for several bacteria the first step in the degradation of DMSP is its cleavage into dimethylsulfide and acrylate by the enzyme DMSP lyase.[258-259] This pathway is not of interest for commercial production of acrylic acid from biomass.[257] More promising routes involve propionic, lactic, and 3-hydroxypropionic acid. The routes to these compounds are explained elsewhere in section 9.2. Enzymatic dehydration of lactic acid or its CoA derivative suffers from a poor equilibrium position at ambient conditions. The equilibrium ratio [acrylyl-CoA]/[lactoyl-CoA] is only about 0.005.[260] The molar ratio [acrylate]/[lactate] was found to be approximately 0.03 at equilibrium.[261]

Equilibrium limitations will also play a role if 3-hydroxypropionate is used for enzymatic acrylic acid production, because cultivation of *R. erythropolis* LG12 with 40 g/L of acrylic acid resulted in 44% conversion into 3-hydroxypropionate.[262]

The metabolic pathway from lactate to propionate as catalyzed by *C. propionicum* proceeds via acrylyl-CoA (Figure 14) and has been reversed for acrylate production. Resting cells of *C. propionicum* converted up to 18.5% of propionate into acrylate when methylene blue was used as an electron acceptor.[263] The acrylate concentration reached up to 2.2 g/L. To obtain renewable propionate from sugars, these authors had fermented lactose into a mixture of propionate, acetate and lactate, using a coculture of *Lactobacillus bulgaricus* and *Propionibacterium shermanii*. This route suffers from some of the same problems as the aforementioned route from lactate, namely the requirement of an external electron acceptor and the formation of several other fermentation products from the sugar.

Thus, so far it is not clear how to obtain a high yield of acrylate on sugars using enzymes.

9.2.3. Lactic acid

Lactic acid (2-hydroxypropanoic acid) and its salts have traditionally been used widely in food, cosmetic, pharmaceutical, and leather industries. The main current application of lactic acid is as building block for polylactic acid (PLA). PLA is considered to be one of the most promising biodegradable polymers that can be applied to textiles, packaging materials, and films, for example. PLA was traditionally produced from optically pure L-lactic acid, and has desirable properties such as good processability, biocompatibility, and biodegradability. Its application is limited by its low melting temperature (180 °C), but poly-L-lactic acid and poly-D-lactic acid can form a racemic complex with a melting point of 230 °C.[264] Therefore, stereocomplexation improves the mechanical performance, thermal resistance, and hydrolysis resistance of PLA-based materials.

Fermentative lactic acid production from biomass was industrially performed already in 1881. There has also been petrochemical production, but this has disappeared again.[265] In 2011, the fermentative lactic acid production has been estimated at 370,000 t/a.[233]

The metabolic pathway from glucose to lactic acid is straightforward: glycolysis to pyruvate, which is reduced using NADH by D- or L-lactate dehydrogenase (Figure 14). Theoretically, this could overall lead to splitting of 1 mol glucose into 2 mol lactic acid, corresponding to a mass yield of 1 g/g, which has indeed been achieved.[266] Numerous studies has been performed on strain and fermentation development to achieve efficient processes, not only using glucose but also from cheaper carbohydrate sources.[265,233,267] Here only some of the best results using glucose are given.

Using immobilized *Rhizopus oryzae* in a fed-batch culture, 280 g/L partly soluble calcium lactate has been achieved, which corresponds to 231 g/L L-lactic acid.[268] The L-lactic acid yield on glucose was 0.92 g/g and the productivity was 1.83 g/(L h). Fewer studies have been published on D-lactic acid production. Using *Sporolactobacillus* sp. strain CASD, which is a homofermentative D-lactic acid producer, an amount of calcium D-lactate corresponding to 207 g/L lactic acid has been achieved in fed-batch cultivation.[269] The productivity was 4.4 g/(L h) and the yield on glucose was 0.84 g/g; using different feeding the yield was 0.93 g/g, somewhat at the expense of achieved concentration and productivity.

With respect to optimizing the lactate productivity using cell recycling, an impressive 150 g/(L h) has been achieved.[270]

9.2.4. 3-Hydroxypropionic acid

Despite its simple structure, 3-hydroxypropionic acid has never been a significant petrochemical product. Its development as a platform chemical took off when Cargill identified the possibility to produce it efficiently by biochemical methods from biomass, and its potential as polymer building block.[271] 3-Hydroxypropionic acid might be used directly in polyesters, but its potential as precursor of acrylic acid might be more interesting, because acrylic acid has an existing market. This implies that biocatalytic conversion of acrylic acid to 3-hydroxypropionic acid[272] has no clear commercial production value. Dehydration of 3-hydroxypropionic acid may be used to obtain acrylic acid (see section 9.2.2).

3-Hydroxypropionate has several roles in microbial metabolism. This has allowed the formulation of a range of pathways, which could be potentially constructed for 3-hydroxypropionic acid production from glucose or glycerol.[273] The pathway from glycerol is simple, involving only two enzymatic steps (Figure 10). So far, the highest published level of production for this pathway is 39 g/L at 35 % yield using recombinant *E. coli* SH-BGK1.[274] This strain required (expensive) coenzyme B12 supplementation for activity of glycerol dehydratase, in the first step in the pathway. The dehydration leads to 3-hydroxypropanal upon keto-enol tautomerization, and subsequent reduction yields 3-hydroxypropionate. Several enzymes have been used for the reduction, and strains constitutive in coenzyme B12 are advocated.[275]

For starting from glucose, many pathways have been proposed, including pathways with hypothetical enzymatic conversions.[273,276] These pathways have been analyzed with respect to thermodynamic feasibility and achievable yield. One of the pathways involves isomerization of L-alanine to β-alanine (2-aminopropionic acid). Interestingly, this was a hypothetical conversion at the time of the conception of the pathway, but later a gene of *Bacillus subtilis* lysine 2,3-aminomutase was mutated to obtain the desired alanine mutase activity.[277]

Experimental implementation of a metabolic pathway from glucose to 3-hydroxypropionate has been published for an *E. coli* strain.[278] After glycolysis, pyruvate was converted into acetyl-CoA. Overexpression of acetyl-CoA carboxylase led to malonyl-CoA, which was converted into 0.4 g/L 3-hydroxypropionate using NADPH-dependent malonyl-CoA reductase originating from *Chloroflexus aurantiacus*. This leaves much room for further optimization. A recent patent application of OPXBIO[279] describes extensive genetic engineering of *E. coli*. Using the best strain in aerated fed-batch fermentation, a yield on glucose of 0.53 g/g has been achieved with a final 3-hydroxypropionate concentration of 48 g/L. Pilot scale experiments have already been performed.

9.2.5. Pyruvic acid

Pyruvic acid, which has the systematic name 2-oxopropanoic acid, is currently merely a fine-chemical. However, it can be produced from glucose very efficiently, and might become more important.[280,281]

Pyruvate is at important metabolic intermediate between glycolysis and citrate cycle, and a metabolic precursor for ethanol and lactic acid. The pathways to pyruvate are shown in Figure 1. Many pyruvate-overproducing strains have been developed, such as a *S. cerevisiae* strain that produced 135 g/L pyruvate with a productivity of 1.4 g/(L

h) and an overall yield on glucose of 0.54 g/g.[282] Using a recombinant *E. coli*, 0.87 g/g has been achieved at a productivity of 6.0 g/(L h).[283] Prolonged production of pyruvate could be achieved by maintaining the concentration in the reactor below 55 g/L.

Lactic acid is currently available at a much lower price than pyruvic acid, and therefore enzymatic oxidation of D- and L-lactic to pyruvic acid has also been pursued. Efficient production at almost quantitative yields has been obtained.[284,285] Being the metabolic precursor of lactic acid, such pyruvic acid production from lactic acid is not likely to become the prevailing process if pyruvic acid becomes a commodity chemical like lactic acid.

9.3. C_4 carboxylic acids

9.3.1. Butyric acid

Butyric acid, with the systematic name butanoic acid, is produced by petrochemistry at a scale of about 500,000 t/a.[286] It is mainly applied in cellulose acetate butyrate plastics, but also in numerous other products.[287]

Butyric acid is a common metabolite produced under anaerobic conditions by bacteria from various genera. The most important strains studied for industrial application are all Clostridia, namely *Clostridium butyricum*, *C. tyrobutyricum*, and *C. thermobutyricum*.[253-288]

The metabolic pathway from glucose to butyric acid has been described for 1-butanol (see section 5.1.5 and Figure 4). Strains producing butyric acid are not able to reassimilate this butyric acid when the medium becomes too acidic and they do not convert it to 1-butanol. The pathway up to butyric acid also leads to production of H_2:[288]

$$\text{glucose} \rightarrow \text{butyric acid} + 2\ H_2 + 2\ CO_2 + 3\ \text{ATP}$$

The competing pathway to acetic acid, which produces 4 ATP per glucose but leads to more acidification, has to be minimized to maximize butyric acid formation. Since ATP is required to support cell growth, the best results have been obtained using cell retention. Repeated fed-batch fermentation of glucose using an immobilized butyric-acid-tolerant strain *C. tyrobutyricum* increased its tolerance to butyric acid in the course of several months, to reach finally a fermentation with a concentration of 87 g/L.[286] The yield on glucose was 0.46 g/g, which is close to the maximum for the applied pathway (0.49 g/g for the equation shown). A reasonable volume-specific productivity of 1.1 g/(L h) was reached.

In Sweden, production has been tested at 10 m^3 scale using *C. tyrobutyricum*.[289]

9.3.2. 3-Hydroxybutyric acid

3-Hydroxybutyric acid is the monomer of the well-known bacterial storage polymer polyhydroxybutyrate (PHB). Strains of *Cupriavidus necator* (at that time called *Alcaligenes eutrophus*) produced PHB concentrations up to 180 g/L with productivities up to 3.8 g/(L h).[290] Therefore, also biosynthesis of the monomer is relatively simple, and although current industrial production of 3-hydroxybutyric acid is only for fine-chemicals applications, commercial production of the polymer is growing, and the monomer might also become more important.[15]

The metabolic pathway from glucose follows acetyl-CoA formation. Two molecules of acetyl-CoA are condensed by β-ketothiolase to acetoacetyl-CoA. This product is then reduced to 3-hydroxybutyryl-CoA by acetoacetyl-CoA reductase. A synthase catalyzes the polymerization (Figure 4). Accumulated PHB has been intracellularly hydrolyzed by PHB hydrolase leading to excretion of *(R)*-3-hydroxybutyric acid up to 15 g/L using a *Halomonas* strain, for example.[291] A much higher concentration, 118 g/L, was achieved using *Azohydromonas lata*.[292] In this case, cells that had accumulated PHB were transferred to an anaerobic vessel at pH 4, which promoted hydrolysis. This allowed a high overall productivity of *(R)*-3-hydroxybutyric acid of 4.9 g/(L h) with a yield on sucrose of about 0.43 g/g.

In similar ways, *(S)*-3-hydroxybutyric acid was produced to a level of 10 g/L, using recombinant *E. coli* expressing the required *(S)*-enzymes.[293]

9.3.3. Methacrylic acid / 2-hydroxyisobutyric acid

Methacrylic acid (2-methyl-2-propenoic acid) and especially its methyl ester are important precursors for acrylic polymers. In 2007, production of methyl methacrylate was 2.7 million t, with significant extensions planned.[294] This makes methacrylic acid by far the most important C_4 carboxylic acid. Nevertheless, biochemical production has not been published. Only a number of hypothetical pathways has been claimed in a patent application.[295] The focus of bio-based research is on precursors of methacrylic acid, such as itaconic acid (see section 9.4.1), isobutene (see section 3.3) and 2-hydroxyisobutyric acid.[296] The latter compound can be obtained from 3-hydroxybutyryl-CoA (Figure 4) by the action of a cobalamin dependent 2-hydroxyisobutyryl-CoA mutase found in *Aquincola tertiaricarbonis*. This enzyme has been expressed in a recombinant *Cupriavidus necator* strain which otherwise was used for production of PHB (see section 9.3.2). By deleting the gene for PHB synthase, up to 6.4 g/L 2-hydroxyisobutyric acid was obtained from fructose.[297] The productivity was 0.13 g/(L h).

9.3.4. Succinic acid

In the past years, succinic acid, also called butanedioic acid, has been produced from petrochemicals at a scale of about 30,000 t/a.[298] Currently, there is a transition toward a much larger scale using bio-based production. This succinic acid will become cheaper and should not only be used as intermediate for various fine chemicals but especially as building block for polymers.

Succinic acid occurs in most organisms as intermediate of the citrate cycle (Figure 13). It can be excreted as major fermentation end-product by microorganisms like *Actinobacillus succinogenes*, *Anaerobiospirillum succiniciproducens*, *Mannheimia succiniciproducens* and some recombinant *E. coli* and *Corynebacterium glutamicum* strains. Much research has been done on maximizing the yield. Anaerobic conversion is preferred. When using only glucose as electron donor, and supplementing additional carbon using CO_2 or carbonate salt, the achievable yield on glucose would be 1.12 g/g according to:[298]

$$7/6 \text{ glucose} + CO_2 \rightarrow 2 \text{ succinic acid} + H_2O$$

To approach this overall reaction stoichiometry, a pathway consisting of various parallel portions is required. In short, via glycolysis carbohydrates should be converted into pyruvate or phosphoenolpyruvate. These C_3 intermediates should be converted into succinate via the oxidative and reductive branch of the citrate cycle, with the glyoxylate shunt in operation to provide the proper balance of some metabolic intermediates between both branches.[299,300]

The theoretical yield of ~ 1.1 g/g has been achieved using pre-grown engineered *E. coli*.[301] After an aerobic cultivation stage, anaerobic production occurred up to 99 g/L succinic acid with a productivity of 1.3 g/(L h).

The highest final succinic acid concentration published is 146 g/L, using fed-batch production using *C. glutamicum*. A yield of 0.92 g/g was achieved and the productivity was 3.2 g/(L h).[302] Continuous anaerobic production with *Anaerobiospirillum succinoproducens* that was retained by a membrane led to a very high productivity of 15 g/(L h).[303] In this case, integrated succinate removal by electrodialyis was used to keep the succinate concentration low. The yield was 0.76 g/g because the strain formed side products such as acetate.

Commercial fermentative production of succinic acid has been announced by several companies, using different microorganisms: Myriant, 13,600 t/a using *E. coli*; Bioamber with Mitsui, 17,000 t/a; BASF with Purac, 10,000 or 30,000 t/a using *Basfia succiniciproducens*; DSM with Roquette, 10,000 t/a using *S. cerevisiae*.[304] In the latter case the fermentation pH is kept low to facilitate product recovery.

9.3.5. Malic acid

Malic acid is the trivial name of 2-hydroxybutanedioic acid. It is widely used in the food industry as an acidulant. Racemic malic acid is prepared commercially by hydration of maleic anhydride, at 5000 t/a in the USA.[247] Biocatalytic hydration of fumaric using fumarase is used in industry for obtaining *(S)*-malic acid.[305] Neither process uses renewable carbon sources, but bio-based processes are being developed. These involve the citrate cycle to produce malate from glucose (Figure 13). Fermentation using engineered *Aspergillus flavus* led to 113 g/L malate at a productivity of 0.59 g/(L h) and a yield of 0.94 g/g.[306]

9.3.6. Fumaric acid

Fumaric acid, also known as *(E)*-2-butenedioic acid or *trans*-1,2-ethylenedicarboxylic acid, is another key intermediate in the citrate cycle and therefore present in a very wide range of organisms. Some fungi, in particular *Rhizopus oryzae* and *arrhizus* strains, excrete fumaric acid as fermentation product and during the 1940s this has been used at industrial scale to produce fumaric acid.[307] Later this process was discontinued and replaced by petrochemical synthesis. Current production is about 90,000 t/a, mostly for use in polymers and food.[308]

The metabolic pathways to fumaric acid are closely related to those of the aforementioned succinic and L-malic acid (Figure 13). The best fumaric acid fermentations have been obtained using *R. arrhizus*.[309] A concentration of 107 g/L and a yield on glucose of 0.86 g/g were reached after 53 h fermentation in a stirred vessel. The fumaric acid was present as precipitated calcium salt in mixture with fungal pellets, complicating product recovery. Therefore, heterologous production is being developed using recombinant *E. coli* and *S. cerevisiae*.[308]

9.4. C_5 and C_6 carboxylic acids

9.4.1. Itaconic acid

Itaconic acid, an unsaturated C_5 diacid also known as 2-methylenebutanedioic acid and as methylenesuccinic acid, is used worldwide in the industrial synthesis of resins and fine-chemicals.[310,311] Since 1955, commercial fermentation is performed and current production is about 80,000 t/a.[311]

Itaconic acid is a product of *Aspergillus terreus* strains and some other fungi. The key reaction is a decarboxylation of aconitate, an intermediate of the citrate cycle, by *cis*-aconitate decarboxylase (Figure 13). This pathway allows a yield of 1 mol/mol glucose, corresponding to a yield of 0.72 g/g.[312]

Good results have been published for *A. terreus*. In aerated 15-L fermentors, an itaconic acid concentration of 86 g/L was achieved with an overall productivity of 0.51 g/(L h) and a yield on glucose of 0.62 g/g.[313] The recent discovery of the *A. terreus* gene for *cis*-aconitase decarboxylase[314] has led to attempts to achieve itaconic acid production using recombinant strains. For example, there is a report of engineered *Pseudozyma tsukubaensis* producing 113 g/L itaconic acid with a yield of 0.45 g/g and a productivity of 0.18 g/(L h).[315] In the latter case the pH is neutral, whereas *A. terreus* produces at low pH, which is favorable for itaconic acid recovery.

9.4.2. Hexanoic acid

Hexanoic acid (caproic acid) is currently only a fine-chemical and produced from petrochemicals, but bio-based production shows an interesting potential.

Hexanoate (caproate) has been found together with some octanoate (caprylate) as elongation product of acetate, the main intermediate of anaerobic digestion under methanogenesis-suppressed conditions (see section 2.1).[316] Mixed microbial communities produced 8.2 g/L hexanoate in a stable batch reactor run from equimolar ethanol and acetate. The highest hexanoate production rate was 0.15 g/(L h) with a yield of 0.65 g/g. The ethanol is first converted to acetate, while producing NADH and ATP. Acetate is then converted to acetyl-CoA, which is coupled to butyryl-CoA.[317] Another acetyl-CoA is required for further elongation to hexanoyl-CoA, which is converted to hexanoate. Microbial populations were found to be dominated by relatives of *Clostridium kluyveri*.[316]

9.4.3. Adipic acid

Adipic acid, also known as hexanedioic acid, is the most important dicarboxylic acid and currently produced at about 2.6 million t/a using petrochemical methods.[318,234] It is applied in the synthesis of polymers such as Nylon 6,6.

To achieve bio-based adipic acid production, a recombinant *E. coli* strain has been developed that synthesized from glucose 37 g/L of *cis,cis*-muconic acid, an adipic acid precursor having two C=C bonds in the chain. The yield was 0.17 g/g after 48 h of culturing under fed-batch fermentor conditions. Optimization of this synthesis required adaptation of the shikimate pathway (see Figure 6), which is normally used for biosynthesis or aromatic amino acids, by expression of three heterologous enzymes: 3-dehydroshikimate dehydratase, protocatechuic acid decarboxylase, and catechol 1,2-dioxygenase. Hydrogenation of the resulting *cis,cis*-muconic solution with 10% Pt on carbon resulted in a 97% conversion (mol/mol) into adipic acid.[319] There are no enzymes known yet for reducing *cis,cis*-muconic acid to adipic acid.[318]

Alternative pathways to adipic acid have been reviewed recently.[318] After producing D-gluconic acid (see section 9.4.6), further oxidation to D-glucaric acid (a stereoisomer of 2,3,4,5-tetrahydroxyhexanedioic acid) can take place, either chemically or biochemically; or glucaric acid can be produced via alternative routes from glucose. For glucaric acid reduction to adipic acid no enzymes are known yet. Another proposed pathway follows a lysine pathway up to 2-oxoadipate, and then uses a series of five enzymatic steps up to adipate. Alternatively, hexanoic acid (see section 9.4.2) can be ω-oxidized to adipic acid. Finally, benzoate (see section 9.6) might be enzymatically degraded up to *cis,cis*-muconate and this might be hydrogenated as mentioned before. When feeding benzoate to an *Arthrobacter* strain in an aerated 15 L vessel, 44 g/L *cis,cis*-muconate accumulated, with a quantitative yield on benzoate.[320] The productivity was 0.92 g/(L h), but 5.5 g/(L h) has been achieved in a cell recycling system with a *P. putida* strain.[321]

9.4.4. 2,5-Furandicarboxylic acid

Biochemical production of this compound relies on the availability of 5-hydroxymethylfurfural (HMF) by acid-catalyzed dehydration of hexoses.[322] Oxidation of both the aldehyde and hydroxymethyl group of HMF to carboxylic acid groups leads to 2,5-furandicarboxylic acid (Figure 12). This diacid is seen as a bio-based alternative to petrochemically produced terephthalic acid, which is used in formation of polyesters.[323] Oxidation of HMF may be done using heterogeneous or electrochemical catalysis,[324,325] but biochemical oxidation has become an alternative. An HMF/furfural oxidoreductase from a *Cupriavidus basilensis* strain has been introduced into *Pseudomonas putida* S12.[326] The resulting whole-cell biocatalyst produced 30 g/L of 2,5-furandicarboxylic acid from HMF with a yield of 0.97 mol/mol and a productivity of 0.21 g/(L h) under aerobic fed-batch conditions.[327] No residual furan derivatives were found, which is highly beneficial for subsequent purification and polymerization.

To achieve the same oxidation of HMF, the use of chloroperoxidase and *C. antarctica* lipase B has also been explored.[328,329]

9.4.5. Citric acid

Citric acid or 2-hydroxy-1,2,3-propanetricarboxylic acid was first isolated from lemon juice by Scheele in 1784. In the 19th century this led to natural isolation processes, but in the 20th century fermentation of carbohydrates became the dominant industrial process. Alternative chemical routes have been developed, on basis of petroleum conversion, and even yeast fermentation of alkanes has been applied, but such processes have only briefly been in operation.[330] Industrial citric acid production in 2007 was estimated at 1.7 million t, and about 70% is used by the food and beverages industry.[331]

The current industrial production relies on the fungus *Aspergillus niger*. Through the glycolytic pathway, pyruvate is formed from carbohydrates. Pyruvate is converted to acetyl-CoA and CO_2, and an equimolar amount of pyruvate should be carboxylated with CO_2 to oxaloacetate. Then, citrate synthase condenses acetyl-CoA with oxaloacetate to citrate, while releasing CoA (Figure 13). The pathway consumes O_2 to regenerate the NADH that is formed during glycolysis to NAD^+, thus generating ATP. Besides this ATP, the overall pathway reaction is:

$$\text{glucose} + 1.5\ O_2 \rightarrow \text{citric acid} + 2\ H_2O$$

This leads to a maximum yield on glucose of 1.07 g/g, but yields up to 0.88 g/g have been reported.[332] Final concentrations up to 240 g/L has been achieved in aerated fed-batch fermentations using *A. niger*, at a productivity of 1.4 g/(L h).[333] In this case no yield was reported, though.

A major advantage of *A. niger* is that pH 2 can be maintained, where citric acid is mostly undissociated. This facilitates product recovery.

9.4.6. D-Gluconic acid

Oxidation of the aldehyde group of D-glucose yields D-gluconic acid. Gluconic acid and in particular its sodium salt are produced at a scale of about 60,000-90,000 t/a for numerous applications. Usually the capacity of gluconic acid to dissolve multivalent cations such as calcium is used.[334,335,336] Catalytic, electrochemical, enzymatic, and microbial oxidation of D-glucose can be used to produce D-gluconic acid. Large scale production using fungal or bacterial cells is well established. *Gluconobacter suboxydans* uses membrane-bound glucose dehydrogenase with quinoprotein and heme cofactors, and co-produces H_2O from O_2. *Aspergillus niger* has an FAD-dependent glucose oxidase with co-produces hydrogen peroxide. A catalase decomposes the peroxide to water and O_2. Both routes lead to glucono-1,5-lactone, which can spontaneously hydrolyze to gluconic acid, but this hydrolysis is accelerated by a lactonase.[335]

This simple biotransformation of glucose is among the most efficient ones described in this review. The highest product concentration, 504 g/L gluconic acid, has been described for fed-batch fermentation of concentrated glucose solution using wild-type *Aureobasidium pullulans*, a yeast-like fungus.[337] The productivity was 4.5 g/(L h) in this case, but in continuous fermentations with cell retention up to 19 g/(L h) was achieved.[338] To achieve such rates, pure oxygen rather than air was used. Up to 98% of the glucose was converted into gluconic acid, corresponding to a yield of 1.07 g/g.

9.4.7. L-Ascorbic acid / 2-Keto-L-gulonic acid

L-Ascorbic acid, also known as vitamin C, has always been a bio-based product. The traditional Reichstein-Grüssner synthesis[339] has been performed industrially since the 1930s. It begins with D-glucose and includes an enantioselective microbial dehydrogenation of D-sorbitol to L-sorbose. A *Gluconobacter oxydans* mutant and fermentation protocol have been developed that gave a theoretically maximal productivity of 200 g/L of L-sorbose from 200 g/L of D-sorbitol in 28 h of fermentation.[340] The other steps in the Reichstein-Grüssner synthesis are chemical, and require some protection/deprotection steps. The overall yield is ~50%.[341]

To increase the yield and avoid the protection/deprotection steps, many biocatalytic options have been considered, for each individual step and for the whole synthesis.[341-342] This has led to development of an innovative process for the production of 2-keto-L-gulonic acid (2-KGA). In a first fermentation stage, L-sorbose is produced from D-sorbitol by batch culture of *Acetobacter melanogenum* with a molar yield of about 96%. In a second stage, a mixed culture is used in which *Ketogulonigenium vulgare* is responsible for the conversion of L-sorbose into 2-KGA, while *Bacillus megaterium* turned out to be just a supplier of a growth factor.[343] The 2-KGA is chemically converted into L-ascorbic acid. By now, this process dominates industrial L-ascorbic acid production.[344] The annual production has been estimated at 80,000 t/a.[345,346]

Two-stage continuous fermentation using the aforementioned mixed culture gave an overall 2-KGA productivity of 2.15 g/(L h) at 113 g/L and a molar yield of 90.1%.[343]

9.5. Fatty acids

Fatty acids are C_6-C_{24} linear aliphatic carboxylic acids, either saturated or unsaturated, occurring is esterified form in fats or oils. World production of fatty acids in 2005-2006 was estimated at 6.5-8.1 million t.[347]

Fatty acids are produced by hydrolysis of natural fats and oils. Glycerol is the co-product of this reaction. To simplify recovery, modern industrial processes operate at 210-260 °C and 19-60 bar without catalyst.[347] Thus, immobilized or free lipases are not used as catalyst for bulk fatty acid production, although their mild operation conditions might save energy costs, and might reduce coloration resulting from degradation of fatty acids at high temperature. Therefore, lipases are more likely to be useful for regioselective hydrolysis of triglycerides to obtain specialty fatty acids with heat labile groups, such as polyunsaturated fatty acids.[348] Besides, lipases can be used for producing related products such as fatty acid methyl esters (section 10.1).

As alternative to the aforementioned lipid biomass, carbohydrate biomass can be used as source of fatty acids, after fermentative conversion (Figure 3). The metabolic route involves formation of acetyl-CoA. In *E. coli*, for example, the obtained acetyl groups are used for condensation with a growing acyl chain that is bound as thioester to ACP. After each condensation, the grown chain contains a β-oxo group, which is reduced in three enzymatic steps to a saturated acyl chain. The process is terminated by thioester hydrolysis to obtain free fatty acid. Many recombinant *E. coli* strains have been developed for such conversions.[349] Up to 6.6 g/L fatty acid has been obtained, using shake flask cultures, at a yield on glucose of 0.28 g/g and a productivity of 0.11 g/(L h).[128]

9.6. Benzoic acid

Benzoic acid and its derivatives are widely distributed in nature. For example, gum benzoin contains 12 – 18% benzoic acid in free and esterified forms.[350] Historically, extraction processes have been used to produce benzoic acid, but nowadays oxidation of toluene, originating from fossil carbon sources, is industrially applied. The main destination of benzoic acid is as intermediate for phenol, followed by use of sodium and potassium benzoate as food or beverage ingredient.

Recently, the first fermentative formation of benzoic acid was published.[351] Through a pathway involving L-phenylalanine, cinnamoyl-CoA and benzoyl-CoA, the aerobic bacterium *Streptomyces maritimus* produced benzoic acid (see Figure 15). The highest concentration of benzoate obtained using 3% starch as feedstock was 0.46 g/L after 6 days of cultivation, and the estimated yield was 0.02 g/g.

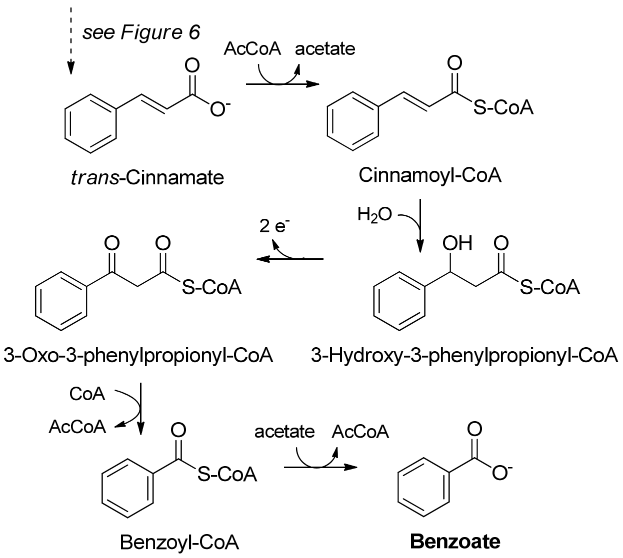

Figure 15. Proposed pathway to benzoic acid.

10. ESTERS AND LACTONES

Obviously, bio-based esters can be obtained by esterification of the alcohols and carboxylic acids that have been treated earlier in this review. This esterification can be catalyzed by mineral acids or by enzymes such as lipases or esterases. Commodity esters that may be enzymatically obtained are ethyl acetate[352] and dimethyl adipate,[353] for example. The latter case was optimized using immobilized *Candida antarctica* Lipase B in hexane with excess methanol to obtain 98% yield. However, acid-catalyzed esterification is usually cheaper. Potential advantages of enzymatic esterification are enantio- and regioselectivity, the decreased risk of substrate and product degradation reactions at the mild reaction conditions used, and the possibility of including the ester formation reaction in metabolic pathways. Some examples are given subsequently.

10.1. Fatty acid (m)ethyl esters

Fatty acid methyl esters (FAMEs) and fatty acid ethyl esters (FAEEs) were used as "biodiesel" at a scale of more than 12 million t/a in 2008,[354] but at about 20 million t/a in 2013.[355] They are made from fatty acid triesters of glycerol, by transesterification using methanol derived from fossil resources or bioethanol from renewable resources. Generally, chemical catalysts are used because enzymes are too costly. However, many studies have been performed to improve this situation.[356,354,357] With combined use of more expensive Lipozyme TL IM (immobilized *Thermomyces lanuginose* lipase) and more active Novozym 435 (immobilized *Candida antarctica* lipase) in mixtures of rape oil, *tert*-butanol, and a slight excess of methanol, a FAME yield of 95% was obtained at a concentration of about 400 g/L and a productivity of about 35 g/(L h). There was no obvious loss in lipase activity during 200 batches of 12 h, because *tert*-butanol eliminated the toxicity of methanol.[358] Such a technology has been applied to achieve industrial biodiesel production in China, at a scale of 20,000 t/a.[356]

FAAEs are naturally produced by some eukaryotes, but this is not well understood.[354] *E. coli* has been engineered to produce FAEE by esterifying exogenously added oleic acid with ethanol endogenously produced from glucose.[359] This involved incorporating an ethanol formation pathway and a low-specificity acyltransferase. Pilot cultivation yielded 11 g/L FAEE with a productivity of 0.24 g/(L h). *E. coli* has also been engineered to produce FAEE directly from glucose via fatty acid and ethanol formation pathways. The achieved FAEE concentration was 0.67 g/L, at 9.4% of the theoretical yield.[159]

Related biochemical routes to FAEE are being developed for commercialization by the company LS9.[360]

10.2. γ-Butyrolactone

γ-Butyrolactone is the cyclic ester of 4-hydroxybutyric acid. It is one of the most valuable alternatives to environmentally harmful chlorinated solvents, and is an important intermediate in fine chemistry.[361] It is commercially produced at a scale of 100,000 t/a via petrochemical processes.[362]

Enzymatic synthesis of γ-butyrolactone has been explored using lactonization of 4-hydroxybutyrate, which itself might obtained by fermentation (Figure 11).[363] *C. antarctica* Lipase B showed good lactonization rates, but this requires a low pH and therefore it was argued that it cannot be used in-vivo. However, the next section shows a solution to this problem for a related compound.

10.3. γ-Valerolactone

γ-Valerolactone is the cyclic ester of 4-hydroxyvaleric acid. The chemical synthesis of γ-valerolactone from bio-based levulinic acid[230] is receiving increased attention because of the potential of γ-valerolactone as transportation fuel and chemical building block.[364,365] Alternatively, biotransformation might be used. *Pseudomonas putida* is capable of producing high titers of 4-hydroxyvalerate from levulinic acid.[366] This proceeds via the CoA derivative of 4-hydroxyvalerate using unknown enzymes (Figure 12). Lactonization would only be achieved at acidic pH due to equilibrium restrictions, and this pH would not be tolerated by the cells. Therefore, human paraoxonase I, a lactonizing enzyme, was expressed extracytosolically in *P. putida*.[367] When using an acidic external pH in a shake flask experiment, lactonization of 4-hydroxyvalerate to γ-valerolactone was enhanced to 2.1 g/L as compared to <0.2 g/L when using an intracellular lactonase. Subsequently, in a 2-liter bioreactor fed with levulinate, 27 g/L 4-hydroxyvalerate and 8.2 g/L γ-valerolactone were obtained after 115 h. Yields on levulinate were below 30%, indicating substantial room for improvement, for example by decreasing the external pH below 6 and by selective *in-situ* removal of the lactone.

11. NITRILES

Several nitriles, are produced at large scale by petrochemical methods. For acrylonitrile, for example, the production capacity is 5 million t/a, for use in a range of fibers and resins.[368] Bio-based alternatives including biochemical steps are under development. Glutamic acid can be oxidatively decarboxylated into 3-cyanopropanoic acid using vanadium chloroperoxidase in the presence of two equivalents of H_2O_2 and a catalytic amount of Br^-.[369] The obtained yield was 1 mol/mol at a final product concentration of 0.5 g/L and a productivity of 0.1 g/(L h). Follow-up chemistry can convert 3-cyanopropanoic acid into the desired acrylonitrile.[370]

If bio-based aldehydes become available, they might be converted into the corresponding nitriles by using an approach that has been demonstrated for 3-phenylpropionitrile, butyronitrile, and others.[371] Aldehydes were chemically converted with hydroxylamine to oximes. The oximes were not isolated but converted, often quantitatively, into nitriles by using *E. coli* containing heterologous phenylacetaldoxime hydratase. Such aldoxime dehydratases are widespread in microorganisms.[372]

12. AMINES

Many amines are metabolites of living organisms. At physiological pH, they usually will be in the ammonium form, whereas it requires pH values above the pK_a (often pH > 10) to have unprotonated amine as prevalent species. For most applications unprotonated amine is required. A high pH and a high free amine concentration will be toxic for the most common microorganisms; finding stable free enzymes may be easier. Otherwise amine formation should be done under conditions of control of pH at neutral values, leading to ammonium salt formation and significant recovery costs to isolate amines. This situation is analogous to the biochemical formation of carboxylic acids as described in the beginning of chapter 9.

12.1. 2-Aminoethanol

2-Aminoethanol is also known as (mono)ethanolamine and is used extensively in gas sweetening, as anticorrosive, detergent and as precursor for the production of ethylene amines. Currently it is industrially produced by reaction of ethylene oxide and ammonia. Therefore it might become bio-based in the future (see section 6.1 on ethylene oxide).

Another approach is also considered[373] (see Figure 16). L-Serine, which may be obtained from sugars by fermentation,[374] can be decarboxylated to 2-aminoethanol. The required decarboxylases are pyridoxal 5'-phosphate dependent and have been found in some plants, for example.[375-376] Expression in *E. coli* has led to small amounts of product from L-serine.[377] Expression in *P. putida* also led to some formation of 2-aminoethanol from glucose, but this was severely constrained by intracellular availability of serine.[378]

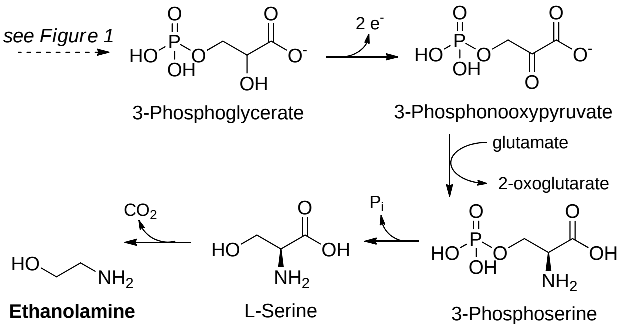

Figure 16. Pathway to ethanolamine.

12.2. 1,4-Diaminobutane

1,4-Diaminobutane, which bears the trivial name putrescine, is being produced via petrochemistry, especially for conversion into nylon-4,6. It is a common metabolite, being the biological degradation product of the amino acid ornithine. The pathway is shown in Figure 17.

Overexpression of this pathway in *E. coli* and deletion of competitive pathways has led to a strain producing 24 g/L putrescine from glucose in a fed-batch culture. The productivity was 0.75 g/(L h) and the yield on glucose was 0.17 g/g.[379] Using a similar approach, fed-batch cultivation with *C. glutamicum* PUT21 was carried out. A putrescine titer of 19 g/L at a volumetric productivity of 0.55 g/(L h) and a yield of 0.16 g/g glucose were achieved.[380]

Assuming the availability of bio-based arginine (see section 13.1.3), arginase-catalyzed hydrolysis to ornithine has been developed,[381] which can be combined with subsequent enzymatic decarboxylation[382] to obtain putrescine (Figure 17). For *B. subtilis* arginase, a remarkably good operational stability (total turnover number, TTN = $1.13 \cdot 10^8$) at the pH of arginine free base (pH 11.0) was observed,[383] which should simplify product recovery.

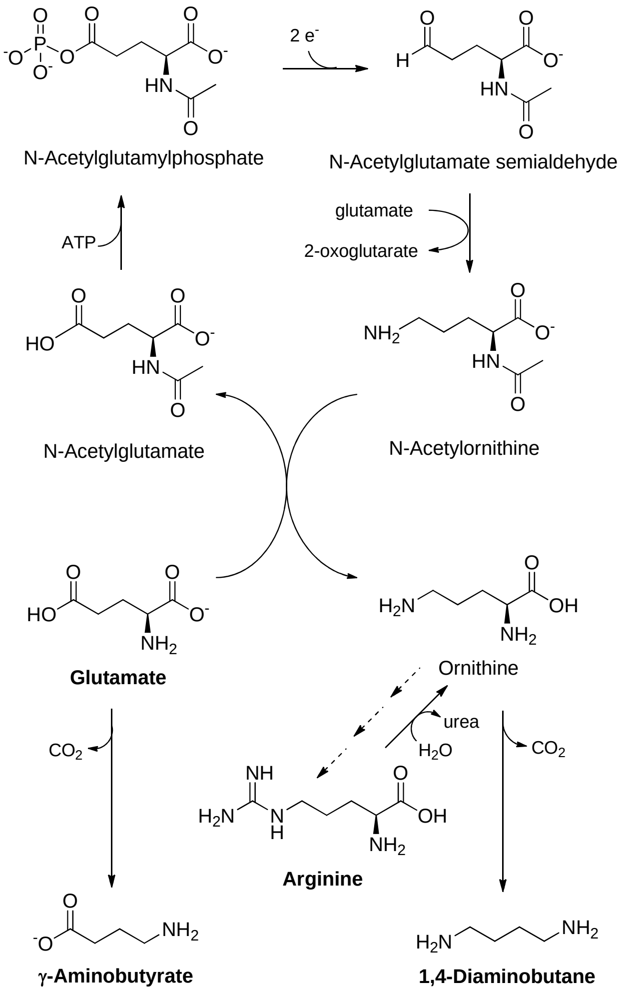

Figure 17. Conversions of glutamate into γ-aminobutyrate and of glutamate and arginine into 1,4-diaminobutane. Glutamate formation is shown in Figure 13. Arginine biosynthesis requires ornithine and carbamoyl phosphate, a. o.

12.3. 1,5-Diaminopentane

Also 1,5-diaminopentane, which is often called cadaverine, can be used in the production of polyamides. In vivo, it is formed in the decarboxylation of L-lysine by lysine decarboxylase (Figure 18). Fermentative lysine production is an established industrial process (see section 13.1.5), and this has triggered interest in bio-based 1,5-diaminopentane production.[384] Formation of 1,5-diaminopentane from crude lysine solutions has been studied using free or immobilized lysine decarboxylase.[385,386] Using immobilized enzyme was calculated to be a basis for economically feasible production.[386] However, to avoid having to produce the (immobilized) enzyme, microbial production from glucose is being pursued using genetically engineered microorganisms such as *C. glutamicum*[387,388,389] or *E. coli*.[390] The latter led to a yield on glucose of 0.12 g/g and a production of 9.6 g/L 1,5-diaminopentane with a productivity of 0.32 g (L h) in a fed-batch fermentation. A higher yield, 0.17 g/g, is described using *C. glutamicum*.[389] Under optimized conditions in a 5 m^3 aerated pilot fermentor, BASF has produced 72 g/L at a productivity of 0.9 g/(L h) with another *C. glutamicum* strain. Byproducts levels were significant: 15 g/L lysine.HCl and 10 g/L acetyl-diaminopentane.

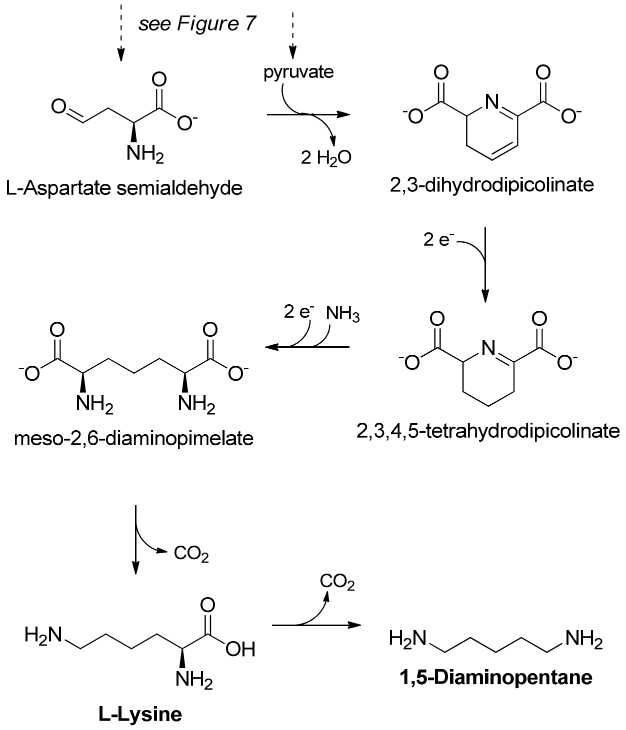

Figure 18. Pathways to L-lysine and 1,5-diaminopentane.

13. AMINO ACIDS

Amino acids are generally used in nutrition and as precursor for a wide range of fine chemicals.

13.1. Proteinogenic amino acids

13.1.1. L-Threonine

In 2012, the production of L-threonine has been estimated at 230,000 t/a.[17] It is produced using randomly mutagenized *E. coli* strains. In the metabolic pathway (Figure 7), biomass is converted into oxaloacetate, which is further converted via aspartate and homoserine, a. o. The final reaction is a conversion of homoserine phosphate into threonine by threonine synthase. This PLP-dependent enzyme eliminates phosphate and adds water to shift the C-O bond from the C_4 to the C_3 position.

In an optimized fed-bed fermentation with *E. coli*, a threonine concentration of 118 g/L was achieved at a yield on glucose of 0.30 g/g and a productivity of 3.1 g/(L h).[391] Higher yields, 0.39 g/g, have been achieved upon metabolic engineering of *E. coli*.[392] The achievable theoretical yield of L-threonine has been calculated to be 0.81 g/g, if there would be no biomass formation. This shows that there is still room for improvement.

13.1.2. L-Valine

About 1100 t of L-valine (2-amino-3-methylbutanoic acid) has been produced in 2005,[393] making it insignificant in the field of commodity chemicals. However, efficient formation of L-valine has been achieved using *C. glutamicum*, which may be a basis for larger scale production in the future.

L-Valine is synthesized in *C. glutamicum* from glucose via pyruvate in a series of reactions that parallel the pathway to isobutanol up to 2-oxoisovalerate, and is described in section 0 and Figure 8. Finally, a reductive amination is required to obtain valine. The theoretical yield on glucose of this pathway is 1 mol/mol, corresponding to 0.65 g/g. For the final step to valine, instead of the natural NADPH dependent reductive transaminase, an NADH-dependent leucine dehydrogenase has been used, in order to eliminate the NADPH dependency of the pathway. In this way a titer of 227 g/L has been achieved with a productivity of 4.7 g/(L h) with yields up to 0.41 g/g.[394] By further eliminating side-reactions, the yield has been increased to 0.57 g/g, at a titer of 150 g/L and a productivity 6.3 g/(L h).[395]

13.1.3. L-Aspartic acid and L-Arginine

The production of L-aspartic acid and L-arginine have been estimated at 15,000 and 3,000 t/a, respectively, in 2005.[393] Potentially, they could be easily produced in the future and become more important. Their structures are shown in Figure 7 and Figure 17, respectively.

L-Aspartic acid is currently industrially produced from ammonia and fumaric acid using immobilized aspartase (L-aspartate ammonia lyase) from *E. coli* or suspended cells of *Brevibacterium bravum.*[396] To become bio-based, renewable rather than fossil-based fumaric acid would have to be used. A fermentative route directly from glucose to aspartic acid cannot simply rely on using aspartase with a fumarate-producing strain, because a favorable equilibrium of the aspartase-catalyzed reaction might require intracellular ammonia and fumaric concentrations that are toxic.

An interesting source for L-aspartic acid and also for L-arginine is the polypeptide cyanophycin. This consists of a poly(L-aspartic acid) backbone with at the β-carboxy group of each L-aspartic acid a branch of one L-arginine, which is bound at its α-amino group. It is a nitrogen storage polymer in cyanobacteria and some other bacteria, but heterologous expression of cyanophycin has been taken up. Using *E. coli* DH1 harboring the cyanophycin synthetase gene from a *Synechocystis* sp., production of cyanophycin at 500 L has been established. Maximum cyanophycin cell content of up to 0.24 g/g of cellular dry matter was obtained. Synthesis of cyanophycin was found to be strongly dependent on the presence of complex organic nutrients such as protein hydrolysate.[397] Subsequent hydrolysis can be done using cyanophycinase, an exopeptidase producing β-Asp-Arg dipeptide, and a peptidase hydrolyzing this peptide to its amino acid monomers.[398]

13.1.4. L-Glutamic acid

The systematic name of glutamic acid is 2-aminopentadioic acid. The industrial production of L-glutamate is by fermentation. In 2012 it has been estimated at 2.5 million t/a.[17] Most is used as monosodium glutamate, a flavoring agent and component of some fermented food. In the mid-1950s, the bacterium *C. glutamicum* was isolated in Japan. It excreted large quantities of L-glutamate into the culture medium. The key precursor is 2-oxoglutarate, which is formed in the citrate cycle. It is converted into L-glutamate by a reductive amination, catalyzed by NADP-dependent glutamate dehydrogenase (Figure 13). Commercial strains have a very low activity for competing pathways.

The overall reaction for L-glutamic acid production from glucose is:

$$C_6H_{12}O_6 + NH_3 + 1.5\ O_2 \rightarrow C_5H_9O_4N + CO_2 + 3\ H_2O$$

The corresponding maximum yield on glucose is 0.82 g/g. Sufficient aeration and ammonia addition (also used to control pH) are critical factors in industrial production. The yield of L-glutamic acid is up to 0.6 g/g of the sugar supplied, and the final concentration is approximately 100 g/L with a productivity of about 2 g/(L h).[399] Using fed-batch fermentation, 141 g/L glutamate has been obtained with a *C. glutamicum* strain at a productivity of 3.8 g/(L h).[400]

13.1.5. L-Lysine

Lysine is an amino acid required by all organisms, but not all produce it. For animal feed more than 1.4 million t/a of L-lysine.HCl is produced by fermentation processes using strains of *C. glutamicum* and *E. coli* from sugar sources such as molasses, sucrose, or glucose.[318]

The metabolic pathway from glucose to lysine follows that of aspartate. In two steps, this is converted to aspartate semialdehyde, which is condensed with pyruvate to obtain a C_7 compound, L-2,3-dihydrodipicolinate. After two reductions to *meso*-diaminopimelate a final decarboxylation yields lysine (Figure 18). A pathway optimized toward redox equivalents is more complicated though, and leads to a theoretical yield of lysine (not its hydrochloride) on glucose of 0.60 g/g.[401] Using *C. glutamicum*, 112 g/L lysine was produced in fed-batch fermentation with a yield on glucose of 0.44 g/g and a productivity of 3.2 g/(L h). This was achieved by implementation of twelve changes in genes of a wild-type strain. These changes redirected carbon fluxes toward the optimal pathway predicted by metabolic modeling. The introduction of foreign DNA was avoided, so that the obtained strains are considered nongenetically modified organisms by industrial classification.[401]

13.2. Nonproteinogenic amino acids

13.2.1. β-Alanine

β-Alanine is also known as 3-aminopropanoic acid. It has been envisioned as intermediate in the production of nitrogen containing base chemicals such as acrylamide and acrylonitrile.[402] Decarboxylation of the α-carboxylic acid group of L-aspartate leads to β-alanine (Figure 7). *E. coli* L-aspartate α-decarboxylase has successfully been used for this.[402] However, irreversible deactivation caused by transamination of the catalytically essential pyruvoyl group necessitates stabilization by protein engineering before the enzyme can be applied industrially.

13.2.2. γ-Aminobutyric acid

This compound is often abbreviated as GABA, and its systematic name is 4-aminobutanoic acid. Currently, it has fine-chemical applications only. It might be converted to its lactam, 2-pyrrolidone, which has important applications[403] and might also be used to produce Nylon-4, although that is not a polymer of significant current interest.

Nevertheless, GABA is a well-known metabolite,[404] and decarboxylation of L-glutamate gives direct access to bio-based GABA (see Figure 17). Using 40 g/L glucose and 214 g/L glutamate, a wild-type *Lactococcus brevis* strain produced 104 g/L GABA during 72 h fed-batch fermentation.[405] Immobilized glutamate decarboxylase, obtained from recombinant *E. coli* cell lysate, converted glutamate almost quantitatively into 224 g/L GABA in about 1 h, implying a huge productivity.[406] Maintaining the activity of the enzyme's cofactor pyridoxal-5'-phosphate (PLP) is an important issue in research on GABA production.

13.2.3. 6-Aminohexanoic acid / Caprolactam

6-Aminohexanoic acid is also known as 6-aminocaproic acid. Upon ring closure it will lead to caprolactam, which is the monomer for Nylon 6. Caprolactam is currently produced by petrochemical routes at a scale of about 500,000 t/a.[407] Production of 6-aminohexanoic acid by microbial conversion of carbohydrates would lead to a bio-based route.

The first suggested route is based on lysine production from biomass (see section 13.1.5). Lysine can be converted into 6-amino-2-hydroxyhexanoate by chemical methods. 6-Amino-2-hydroxyhexanoate can be dehydrated into 6-aminohex-2-enoate. This has been converted into 6-aminohexanoate using *E. coli* containing a 3-enoate reductase gene from *C. tyrobutyricum* or *Moorella thermoacetica*.[408] However, during the reduction, a severely competing cyclization to β-homoproline occurs, and therefore another pathway is also being patented.[409,410] 2-Oxoheptanedioate can be converted into 6-aminohexanoate via (i) decarboxylation to 5-formylpentanoate, which can be transaminated, or (ii) via transamination to 2-aminoheptanedioate, which can be decarboxylated.

To obtain 2-oxoheptanedionate, it has been suggested to perform twice C_1-elongation, from the C_5 compound 2-oxoglutarate, which is an intermediate of the citrate cycle and can be produced from glucose, via the C_6 compound 2-oxoadipate.[411] However, direct biochemical formation of 6-aminoadipate from glucose was not yet reported.

Although there will be enzymes in nature capable of catalyzing the interconversion between 6-aminohexanoate and caprolactam,[412] the reaction equilibrium under physiological conditions will not lead to ring closure, because 6-aminohexanoate will be zwitterionic at neutral pH. Thus, lactam formation will require a separate conversion step.

14. HALOGEN DERIVATIVES

Enzymatic chlorination and bromination of organic compounds occur widely in nature, and this has inspired numerous studies on the mechanism on such halogenations.[413] Halogenating enzymes have been divided into hydrogen peroxide-requiring haloperoxidases (heme-dependent or vanadium-dependent), O_2-dependent halogenases ($FADH_2$-dependent or nonheme iron-dependent), and nucleophilic halogenases using chloride, for example.[414] Unfortunately, the widely studied haloperoxidases catalyze the formation of hypohalous acid (HOCl or HOBr) but have no control on the regioselectivity of the subsequent halogenation, which occurs outside the enzyme. Chlorination of acetic acid using chloroperoxidase led to dichloroacetic rather than monochloroacetic acid.[415] $FADH_2$-dependent halogenases also form hypohalous acid, but in the best studied cases this does not leave the active site, leading to regioselectivity. The net reaction involves NADH:

$$RH + X^- + NADH + O_2 \rightarrow RX + NAD^+ + 2\ OH^-$$

Modest protein engineering of these enzymes has been done, leading to halogenating activity with compounds closely resembling the natural substrate, but not yet with precursors of commodity chemicals.[416]

The nonheme iron dependent halogenases consume 2-oxoglutarate:

$$RH + X^- + \text{oxoglutarate} + O_2 \rightarrow RX + \text{succinate} + HCO_3^-$$

Nucleophic halogenases have been discovered only recently and seem to be more useful as fluorinases than as chlorinases.[417]

Thus, bio-based commodity chemicals cannot yet be made using the aforementioned enzymes.

14.1. Methyl chloride

Methyl chloride (chloromethane) can be produced from HCl and methanol. The order of magnitude of annual production is 1 million t/a. It is mostly used for production of silicones.[418] The used methanol might be bio-based (see section 5.1.1).

Another possibility to produce bio-based methyl chloride has been demonstrated using methyl halide transferases.[419] These enzymes occur in number of organisms, including marine algae, fungi, and halophytic plants, and are responsible for the natural production of methyl chloride from Cl^-.[420] The methyl group originates from SAM. In a metagenomic screen, methyl halide transferase from *Batis maritima*, a halophytic plant, displayed the highest activity; ten-fold higher for iodide than for chloride, and only methyl iodide activity was checked after expression in *S. cerevisiae*, which was further metabolically engineered. Production of 0.19 g/(L h) of methyl iodide from glucose was achieved at a low yield.[419]

15. INORGANIC COMPOUNDS

15.1. Hydrogen

In industry, most H_2 is produced from natural gas by a combination of steam reforming and water shift reactions, accompanied by net release of CO_2 to the atmosphere. Current production is about 48 million t/a, of which half is used for ammonia synthesis.[421] Reforming of bio-based methane could become an important alternative.

A biochemical alternative using biomass is anaerobic hydrogen fermentation. In the so-called dark fermentation, different anaerobic bacteria use different reactions to release two e^- equivalents to form H_2 using two H^+ equivalents. Strict anaerobes obtain the electrons from pyruvate oxidation. These are then transferred to ferrodoxin and further on to a hydrogenase that catalyzes H_2 formation. Facultative H_2-producers primarily use formate oxidation (Figure 2), which is catalyzed by a formate-hydrogen lyase.[422] The achieved H_2 yield on glucose is 0.022 g/g, corresponding to 2 mol/mol, which is about half of the maximum of the pathway used. Reasonable productivities, about 0.6 g/(L h), are achieved, however, and product recovery is easy because of the gas formation.[422]

To increase the low yield, bioelectrochemically assisted microbial fuel cell reactors are being used. So far these consume relatively much electricity, and require further development.[422]

Cell-free synthetic enzymatic pathways are also studied to produce H_2. Using the enzymes of the oxidative pentose phosphate cycle and hydrogenase purified from the bacterium *Pyrococcus furiosus*, 11.6 mol H_2 was generated per mol glucose-6-phosphate.[423] Polyphosphate can be used as alternative to ATP to obtain glucose-6-phosphate from glucose.[424]

15.2. Carbon monoxide

The final step in alkane biosynthesis involves loss of CO from a fatty aldehyde (Figure 3). This decarbonylation is catalyzed by microsomes from the green alga *Botyrococcus braunii*.[425] Although this could be used for production of CO from biomass, such a pathway would have a much lower mass yield of CO than catalytic conversion of biomass into syngas.

16. SUMMARY AND OUTLOOK

Already 21 of the commodity products treated in this review are commercially produced and at least 9 others have been tested at pilot scale (Figure 19). In particular, carboxylic acids, alcohols, and amino acids are produced. About 5 of the commercial production processes and all 9 pilot tests date back to the past 10 years. It is expected that production of chemicals from biomass will become more important in the near future, and that biochemical methods will play a prominent role in this field. Comparison of the 2nd and 3rd column of Table 3 shows that bio-based biochemical production is already relevant with respect to production tonnage. To get an overall picture, all major commodity products should have been included in the table. Still, that could give a distorted picture. Replacement of petrochemical synthesis by fermentation of renewable resources would decrease total commodity chemicals production in such statistics. For example, fermentative production of 100,000 t/a of 1,4-butanediol would eliminate the requirement of several petrochemistry-based precursors of 1,4-butanediol: ~110,000 t/a maleic anhydride, ~63,000 t/a butane/butene mixture and ~11,000 t/a H_2.

Amongst many issues that play a role before a product can be commercialized,[426] a few will be addressed in the next sections.

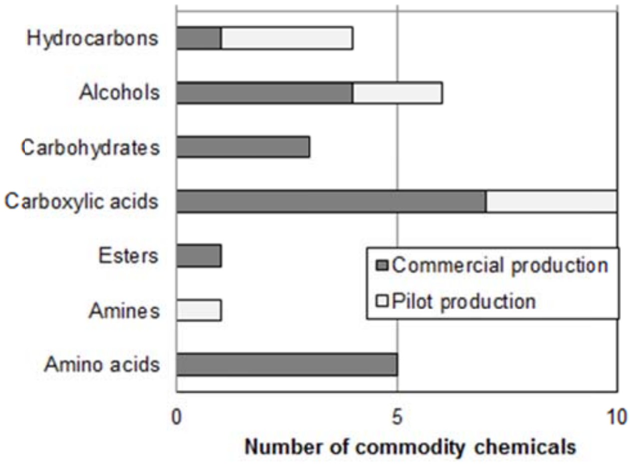

Figure 19. Commerical and pilot production of commodity chemicals from biomass using enzymes or cells according to data in this review.

Table 3. Industrial production and biochemical yield data for formation of some major commodity chemicals from glucose.

Product	Production (millions t/a)	Biochemical production (millions t/a)	x in (Equation 1 (mol/mol)	y in (Equation 1 (mol/mol)	Theoretical yield based on (Equation 1 (g/g)	Theoretical yield of used pathway (g/g)	Achieved yield (g/g)
Methane	>200	>1	3	3	0.27	0.27	0.27
Ethene	115	Research	2	2	0.31	0.12	<0.01
Ethanol	99	99	2	2	0.51	0.51	0.50
Hydrogen	48	Research	6	6	0.13	0.04	0.02
Ethylene glycol	18	Research	2.4	1.2	0.83	0.41	0.27
Acetic acid	11	0.19	3	0	1.00	1.00	0.8
Isobutene	10	Research	1	2	0.31	0.31	<0.01
Phenol	9	Research	0.86	0.86	0.45		0.035
Fatty acids	8	Research	0.26	1.91	0.37		0.28
Fructose	6.5	6.5	1	0	1.00	1.00	1.00
2-Butanol	5	Research	1	2	0.41	0.41	0.01
Acrylic acid	4.5	Research	2	2	0.80	0.80	<0.03
Acetone	3	0.15	1.5	1.5	0.48	0.32	0.11
1-Butanol	2.8	0.5	1	2	0.41	0.41	0.36
Isopropanol	2.3	Research	1.33	2	0.44	0.33	0.24
L-Glutamate	2.5	2.5	1.33	-0.67	1.09	0.82	0.6
Citric acid	1.7	1.7	1.33	-2	1.42	1.07	0.88
L-Lysine	1.4	1.4	0.86	0.86	0.70	0.60	0.44
1,2-Propanediol	1.4	Research	1.5	1.5	0.63	0.51	0.20
1,4-Butanediol	1.3	Pilot	1.09	1.64	0.55	0.5	
Acetaldehyde	1.0	Research	2.4	1.2	0.59	0.49	0.42
Butanone	1.0	Research	1.09	1.64	0.55	0.40	<0.01
Isobutyraldehyde	1	Research	1.09	1.64	0.55	0.40	0.18
Isoprene	0.8	Pilot	0.85	1.71	0.32	0.25	0.11
Isobutanol	0.5	Pilot	1	2	0.41	0.41	0.35
Butyric acid	0.5	Pilot	1.2	1.2	0.59	0.49	0.46
Lactic acid	0.37	0.37	2	0	1.00	1.00	1.00

16.1. Feasible yields

In a bio-based economy, there will be competition between product routes based on their ecologic, social, and economic value. For a fixed raw material and product, the highest yield of product on feedstock is likely to favor all these values. For commodity products that are produced by fermentation, the carbohydrate feedstock is the main cost contributor.[427] So when discussing biochemical routes from carbohydrate to commodity products, it is useful to know which overall reaction yields would be achievable according to stoichiometry calculations in case that no side products are formed.

For simplicity, glucose is taken as C-source and, in case the product contains nitrogen, ammonia as N-source. Side-products considered are CO_2 and H_2O. For each product with the formula $C_aH_bN_cO_d$, the stoichiometric coefficients *w*, *x*, *y*, and *z* can be calculated in a reaction equation from glucose that does not involve other compounds than indicated in (Equation 1:

$$\text{Glucose} + w\,NH_3 \rightarrow x\,C_aH_bN_cO_d + y\,CO_2 + z\,H_2O$$

(Equation 1)

The calculation of the four coefficients can be done using the four elemental balances of C, H, N, and O. For the product and CO_2, the resulting values (*x* and *y*) are shown in Table 3 for a number of products, and the values of *x* are recalculated into mass yields of product on glucose.

The first entry, for example, indicates that 1 mol glucose might lead to 3 mol methane and 3 mol carbon dioxide. No ammonia is consumed or water is produced, so $w = 0$ and $z = 0$, but for brevity w and z values are not included in Table 3. Due to the low molar mass of methane as compared to carbon dioxide, a maximum yield of only 0.27 g per g of glucose is found, and such a yield can indeed be achieved. For some products, a much higher yield can be theoretically achieved, in particular when negative values of z are found, implying carbon dioxide incorporation rather than production. For succinic acid this has indeed been achieved (see section 9.3.4). For other compounds, the yield calculated using Equation 1 is not approached. The first reason is that no pathway has been formulated yet that might lead to such a yield.

For a pathway to a desired product to be feasible, the biochemistry of each enzymatic conversion in the pathway has to be feasible. This review illustrates the large synthetic potential of natural enzymes, and also the potential to engineer enzymes in order to achieve nonnatural reactions using biocatalysis. Reaction databases have been constructed of known enzymatic reactions,[428,429,430] and these databases can be used to generate biochemically feasible candidate pathways from biomass to a desired product. This set of known reactions can be supplemented by hypothetical enzymatic reactions, which are, for example, homologous to known enzymatic reactions with respect to functional groups in substrate and product.[431,432] Obviously, candidate pathways need to include cofactor regeneration, and may require that NAD-dependent enzymes are replaced by NADP-dependent enzymes, for example. Too many candidate high-yield pathways may result, and methods are being developed to determine the likeliness that a known enzyme will catalyze a hypothetical reaction upon protein engineering. These methods include molecular modeling to assess the binding affinity and catalytic efficacy of the hypothetical reaction, and calculating the optimized performance of candidate enzymes if they would be rationally re-engineered.[432] A limitation of such an approach is the lack of structural and mechanistic knowledge of some enzyme types.

If the desired biochemistry (enzymes, cofactors, transport proteins, etc.) is in place and active, the desired product yield will still be unachievable if the thermodynamics of the pathway are unfavorable. Methods are being developed to check this before any experimental work is started.[433] For many enzyme-catalyzed reactions, equilibrium positions have been measured at physiological or near-physiological conditions.[434] Group contribution methods allow the estimation of equilibrium constants of reactions that have not been thermodynamically analyzed.[435] Using corrections of equilibrium constants to a specific physiological pH, temperature, and ionic strength, equilibrium positions of individual reactions and of whole pathways can be determined. Occasionally it is found that part of the available carbohydrate has to be converted by other routes in order to generate sufficient ATP to drive the product pathway and drive reactions required for cell maintenance. This will be at the expense of the achievable product yield. Such additional ATP generating routes lead to undesired side-products or they require aerobic conditions, with part of the carbohydrate being fully oxidized into CO_2 and H_2O.

Once an optimized metabolic route to the product has been formulated that obeys biochemical and thermodynamic constraints, implementation of the pathway and achieving the assumed maximum yield sometimes involves very large efforts on metabolic engineering[436],[437] and protein engineering.[438] This is outside the scope of this review. The last columns of Table 3 indicate that for some products there is still a lot of room for improvement of the yield.

Table 4. High product concentrations achieved according to data in this review.

Product	Concentration (g/L)	(mol/L)	Fermentative or enzymatic conversion
gluconate	*504*	*2.57*	*F*
FAME	*400*	*1.3*	*E*
glycerol	*302*	*3.28*	*F*
sorbitol	*300*	*1.65*	*F*
xylitol	*244*	*1.60*	*F*
erythritol	*241*	*1.97*	*F*
citric acid	*240*	*1.25*	*F*
mannitol	*240*	*1.32*	*F*
L-lactate	*231*	*2.57*	*F*
valine	*227*	*1.94*	*F*
GABA	*224*	*2.17*	*E*
D-lactate	*207*	*2.30*	*F*
acetic acid	*203*	*3.38*	*F*
fructose	*200*	*1.11*	*E*
isomaltulose	*200*	*0.58*	*E*
ethanol	*170*	*3.70*	*F*
2,3-butanediol	*152*	*1.69*	*F*
succinate	*146*	*1.24*	*F*
1,3-propanediol	*141*	*1.85*	*F*
glutamate	*141*	*0.96*	*F*
pyruvate	*135*	*1.53*	*F*
threonine	*118*	*0.99*	*F*
3-hydroxybutyrate	*118*	*1.13*	*F*
1,4-butanediol	*115*	*1.28*	*F*
itaconate	*113*	*0.87*	*F*
malate	*113*	*0.84*	*F*
2-KGA	*113*	*0.41*	*F*
lysine	*112*	*0.76*	*F*
fumarate	*107*	*0.92*	*F*
propionate	*106*	*1.43*	*F*

16.2. Feasible product concentrations

Table 4 convincingly shows that high product concentrations are no exception for enzymatic and fermentative conversions. In some cases not much optimization was performed but in other cases a lot of effort was required to find or develop cells that would tolerate these concentrations. A notorious, so far unsuccessful case is 1-butanol fermentation, where final product concentrations remain below 20 g/L despite all efforts to increase this. In contrast to the fermentative products in Table 4, 1-butanol is only slightly soluble in water and will therefore have relatively much tendency to interact with hydrophobic portions of cell membranes and proteins, thereby destabilizing these structures. By analogy, one cannot expect that it will be easy to achieve high product concentrations for the other butanols, for phenol, and for some undissociated carboxylic acids, for example. Full understanding of stabilizing cells against such toxic products is still lacking, though. Recovering toxic or inhibiting products *in-situ* can partly circumvent the associated problems.[439] *In-situ* product recovery may also improve conversions that are constrained by the occurrence of product degradation or by the enzymatic reaction equilibrium.

Table 5. Highest productivities cited in this review.

Product	Productivity [g/(L h)]	Methods used
fructose	*800*	*immobilized enzyme, packed bed*
lactic acid	*150*	*cell recycling*
ethanol	*82*	*ISPR, cell recycling*
isomaltulose	*40*	*immobilized cells, packed bed*
FAME	*35*	*immobilized enzyme, packed bed*
sorbitol	*38*	-
gluconate	*19*	*cell retention, pure* O_2
succinate	*15*	*cell retention, ISPR*
1-butanol	*~10*	*immobilized cells*
valine	*6.3*	*metabolic engineering*

16.3. Feasible productivities

It is estimated that productivities below 2 g/(L h) are uncommercializable for commodity chemicals because this leads to too high capital costs for bioreactors.[426] Higher values were indeed found, except for citric acid and itaconic acid. These traditional products require aerobic fermentations, but the achievable productivities may easily be limited by oxygen transfer rates in the used fungal pellets.

It is interesting to review the most productive conversions mentioned (Table 5). Some involve simple conversions catalyzed by highly active enzymes. When using the enzyme immobilized in a packed bed reactor, high enzyme concentrations are used. For cells, immobilization but also cell retention by membranes and cell recycling are used to maximize cell concentration and hence productivity. If O_2 is consumed, the productivity can be increased by supplying it as pure O_2 rather than via air. If the product becomes inhibiting, ISPR (in-situ product removal) can be applied. All these process options become important if enzyme and strain development have led to sufficiently fast product pathways. For valine, the last entry of Table 5, rate-limiting metabolic steps were addressed using metabolic engineering.

16.4. Outlook

Transformation of biomass into commodity chemicals using enzymes or cells will be successful if the production process is more attractive than for alternative options to produce these chemicals. Competition is on production costs, but a constraint is that the results of life cycle analysis should not favor the traditional nonrenewable processes.

There are several critical factors:

- Sufficient second generation biomass should be available for a reasonable price; the price will not only be dictated by the biomass production but also by competitive uses of this biomass such as combustion for energy generation.
- All biomass components should be convertible into product, or otherwise into valuable co-product.
- There should be a large margin between cost of biomass and revenues from product, considering the anticipated yield of product on biomass.
- Too high bioreactor investments, due to high O_2 requirements or too low productivities, should be avoided.
- Product recovery should not be too complicated. Prerequisites are high product concentrations and low contaminant concentrations, in particular when the product is nonvolatile and highly soluble in water.
- Biochemical processes compete with chemical processes that aim at similar routes from biomass to product. The biochemical process should be more selective or should avoid production and isolation of intermediate chemicals.
- Although the potential of many biochemical routes is high, approaching this potential for a particular product often requires a larger effort than anticipated. Vision, dedication, and capability to build a team that can develop the whole value chain around the product are key requirements.

Scientific discoveries and method development have been very important to increase the rate of development of biochemical routes; and they will continue to do so. This should be accompanied by increased understanding of the position of biochemical routes within a bio-based economy.

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ABBREVIATIONS

ABE acetone-butanol-ethanol; ACP acyl carrier protein; ATP adenosine triphosphate; CoA coenzyme A; FAEE fatty acid ethyl ester; FAME fatty acid methyl ester; GABA γ-aminobutyric acid; KGA keto-L-gulonic acid; NADH nicotinamide adenine dinucleotide; NADPH nicotinamide adenine dinucleotide phosphate; P_i phosphate; PHB polyhydroxybutyrate; PLA polylactic acid; PLP pyridoxal-5'-phosphate; PP_i pyrophosphate; SAM *S*-adenosyl methionine.

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