Bio-Products from Sugar-Based Fermentation Processes

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1 Introduction

The transition from oil-based to bio-based economy is on the way, and technologies including microbial conversion processes are taking a significant share in this path, not only to replace products generated by chemical synthesis or extracted directly from natural resources, but also to obtain molecules with new functionalities (IEA Bioenergy 2012).

Among feedstock used for sugar-based fermentation processes, dedicated plant crops (e.g., sugarcane and cereal grains) are the preferred ones, followed by agriculture residues (e.g., sugarcane bagasse, wheat straw, corn stover), forestry residues, agro-industrial by-products, and bio-wastes. This biomass can also be categorized based on their carbohydrate composition as mono-, di-, or oligosaccharides (e.g., glucose and sucrose), polysaccharides (e.g., starch, cellulose, hemicellulose), or a mixture of those.

All sugar-based biomass requires a certain level of upstream processing to make its sugar content available for efficient microbial fermentation processes (Fig. 1). Biomass containing mono- or disaccharides (e.g., sugarcane and sugar beet) needs minimal crushing/extraction upstream processing to obtain a sugar solution (Nag 2008). Starch-based biomass (e.g., cereal grains) usually undergoes milling/grinding and an additional enzymatic hydrolysis step to convert starch into mono- and disaccharides (Nag 2008). In turn, lignocellulosic biomass, composed of cellulose, hemicellulose and lignin, often requires chopping and a thermochemical pretreatment step, which loosens the recalcitrant structure of lignocellulose and, in some cases, partially hydrolyzes its components, also contributing to a better access of enzymes in a subsequent hydrolysis step (Wyman 1996).

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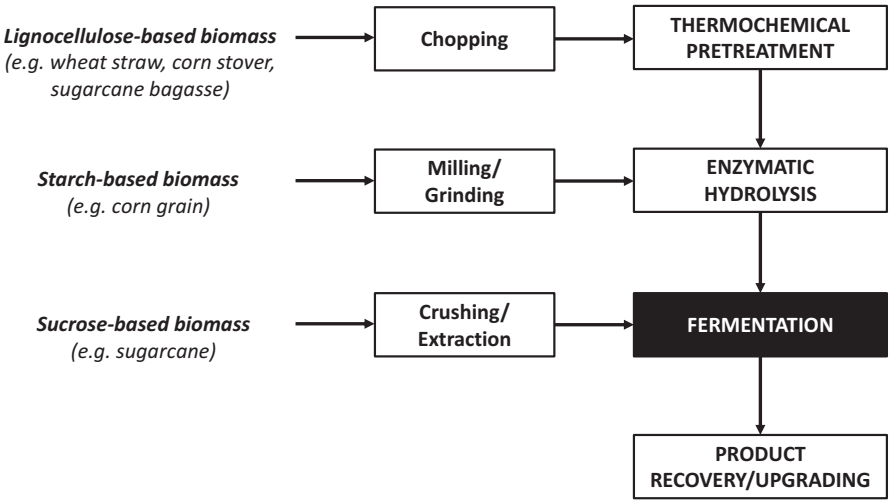


Fig. 1 Biomass processing for sugar-based fermentation processes

The aim of these upstream processes is to achieve maximal carbohydrate recovery yield from biomass in a concentrated and readily fermentable sugar solution—a sugar platform—at the minimal cost. While sucrose- and starch-based biomass has relatively easy upstream processing, the processing of lignocellulosic biomass is more complex, mainly due to the pretreatment step (Wyman and Dale 2015). Whole-crop processing is gaining interest in order to increase process efficiency, namely in terms of energy requirements for the upstream processing, even if it results in a more complex sugar mixture for the microbial fermentation process. The same challenge is faced when complex bio-wastes (e.g., organic fraction of municipal solid wastes) are used for microbial fermentation processes, especially if pure microbial cultures are applied.

Although several microorganisms are able to produce hydrolytic enzymes, most processes include the enzymatic hydrolysis of polysaccharides into a mono- and disaccharides to generate sugar solutions that are compatible with the microorganism used in the fermentation step. Starch, a homopolymer of glucose units linked via linear α-1,4 (amylose) and branched α-1,6 (amylopectin) linkages, is converted into glucose and maltose by the action of α-amylase and glucoamylase. Cellulose is also a homopolymer of glucose units, but linked by β-1,4 glycosidic bonds. Its crystalline structure makes it resistant to hydrolysis under most pretreatment processes. Endo-β-1,4-glucanases and cellobiohydrolases are applied to generate cellobiose, and β-glucosidase hydrolyzes the disaccharide into glucose. Hemicelluloses are branched heteropolysaccharides consisting of the pentose (D-xylose and L-arabinose), hexose (D-mannose, D-glucose, D-galactose), and uronic acid units. Hemicelluloses from agricultural residues are mainly arabinoxylans, i.e., a back-

bone of xylose units linked by β-1,4 glycosidic bonds with branches of several arabinose units (Gírio et al. 2010). The structure and composition of hemicelluloses from wood biomass is distinguished between softwood (e.g., spruce, pine) and hardwood (e.g., willow, aspen, and oak). Softwood hemicellulose has a higher mannose and glucose content, while hardwood contains a higher proportion of xylose and acetyl groups (Gírio et al. 2010). The branched and non-crystalline structure of hemicelluloses makes them more susceptible to hydrolysis during pretreatment and, in the case of agricultural residues and hardwood, hemicellulose hydrolysates rich in xylo-oligosaccharides are obtained. The complete hydrolysis of hemicellulose into monosaccharides may require the use of enzymes like endo-xylanase and β-xylosidase. Nowadays, commercial enzyme cocktails are available for the virtually complete and efficient hydrolysis of different types of biomass.

The sugar platform available from the upstream processing of different biomasses usually contains monosaccharides in the form of hexoses (often referred as C6 sugars, typically glucose) or pentoses (often referred as C5 sugars, as xylose) and/or disaccharides like sucrose or maltose. While sugarcane juice and molasses mainly contain sucrose and the hydrolysate from starch-based biomass contains maltose and glucose, the hydrolysate from lignocellulosic biomass is often composed of a mixture of C6 and C5 sugars.

The benchmarking of microbial conversion processes using sucrose-, starch-, and lignocellulose-based biomass as feedstock is usually the production of ethanol with the yeast *Saccharomyces cerevisiae*, as a long-standing biocatalyst in the food and biofuel industries (Kang and Lee 2015). This microorganism is able to efficiently convert hexoses and respective disaccharides (sucrose and maltose) into ethanol, but lacks the ability to naturally use polysaccharides and pentoses. On the contrary, other microorganisms are able to directly convert polysaccharides into bio-products, like some filamentous fungi, co-producers of enzymes (Jun et al. 2011) and organic acids. Several others are able to use hexoses and pentoses, just requiring an external source of enzymes for the hydrolysis of polysaccharides. Microbial fermentation processes requires the efficient use of all the carbohydrates from biomass and, in the past decades, major efforts have been made to engineer microorganisms towards this goal (Jansen et al. 2017).

The capacity of microorganisms to tolerate industrial settings and process specificities is also a key factor in the success of bioprocesses. The robustness of microorganisms is often challenged by upstream processing of biomass. For example, the chemicals and/or high temperature applied during pretreatment of lignocellulosic biomass often generate degradation products of cellulose, hemicellulose, and lignin. During pretreatment, the hydrolysis of hemicellulose can lead to the formation of furfural and acetic acid while lignin can release phenols, all known as microbial inhibitors (Palmqvist and Hahn-Hägerdal 2000).

Nowadays, one of the main objectives in sustainable biotechnology processes is the development of robust and tailor-made microorganisms—cell factories—for microbial fermentation processes, with efficient conversion of specific sugar

mixtures into specific bio-products (IEA Bioenergy 2012). For ethanol production, the focus of strain improvement has been the substrate conversion, particularly C5 sugars by *S. cerevisiae* (Moysés et al. 2016). Conversely, *Escherichia coli* and *S. cerevisiae* have been the most common microbial chassis for metabolic engineering and synthetic biology approaches for the generation of commercially viable bio-products (Kang and Lee 2015). In fact, these microorganisms are, respectively, the prokaryote and eukaryote model organisms, and the accumulated knowledge on molecular and cell biology and the effective tools available for their genome editing, make them an excellent platform to develop a new generation of cell factories for the production of bio-products through sugar-based fermentation processes.

2 Alcohols

2.1 Ethanol

Ethanol is a two-carbon alcohol and the main liquid biofuel replacing gasoline for road transportation (Wyman 1996; Nag 2008). The research and development on biochemical conversion of biomass relied mostly on ethanol production processes. The so-called second-generation (2G) bioethanol biorefineries use lignocellulose-based substrates, in contrast to the first-generation (1G) bioethanol biorefineries, which uses sucrose- or starch-based substrates. Many microorganisms can produce ethanol as the major fermentation product from sugars, but the yeast *S. cerevisiae* is the preferred cell factory in industrial alcoholic fermentation, due to: (1) high ethanol yield and productivity; (2) robustness to harsh environments, including low pH, tolerance to inhibitors, like acids (acetic and formic) and furans (furfural and HMF); and (3) generally be regarded as safe (GRAS) (Hägerdal et al. 2007).

As the natural and most robust microorganism for ethanol production from hexoses and respective disaccharides (sucrose and maltose), *S. cerevisiae* has been engineered to face the challenges and opportunities of converting lignocellulose hydrolysates into 2G bioethanol. The metabolic and evolutionary engineering approaches included the adaptation to inhibitors generated in the pretreatment step (Almeida et al. 2007; Demeke et al. 2013), the reduction of by-product formation to increase ethanol yield (Medina et al. 2010), the hydrolysis of polysaccharides (van Zyl et al. 2007), and the fermentation of the C5 sugars obtained from hemicellulose (Gírio et al. 2010).

In the past decades, the capacity of *S. cerevisiae* to ferment C5 sugars, mainly xylose, has significantly improved, with the development of many laboratory and industrial pentose-fermenting strains. Two different metabolic pathways for xylose assimilation have been introduced in *S. cerevisiae*: (1) the "redox pathway," using xylose reductase (XR) and xylitol dehydrogenase (XDH) found in natural xylose-fermenting non-conventional yeasts (Eliasson et al. 2000), or (2) the isomerase

pathway, using xylose isomerase (XI) from bacteria or from an anaerobic filamentous fungus (Kuyper et al. 2005a) (Fig. 2). Both pathways produce D-xylulose which is converted to D-xylulose 5-phosphate by xylulokinase (XK). Both the overexpression of endogenous and heterologous XK has proven to improve xylose fermentation (Eliasson et al. 2000; Jin et al. 2002). D-Xylulose 5-phosphate is an intermediate of the pentose phosphate pathway (PPP) and carbon flux can then follow glycolysis towards ethanol production through the common PPP/glycolysis intermediates, fructose 6-phosphate and glyceraldehyde 3-phosphate.

The overexpression of the endogenous PPP enzymes promoted further improvement in pentose fermentation (Karhumaa et al. 2005). Other significant improvements in xylose fermentation by *S. cerevisiae* included the reduction of by-product formation and deregulation of pentose metabolism. For example, the disruption of *GRE3*, coding an unspecific xylose reductase, reduces xylitol accumulation (Träff et al. 2001). In turn, the disruption of *PHO13*, coding a phosphatase, revealed to be

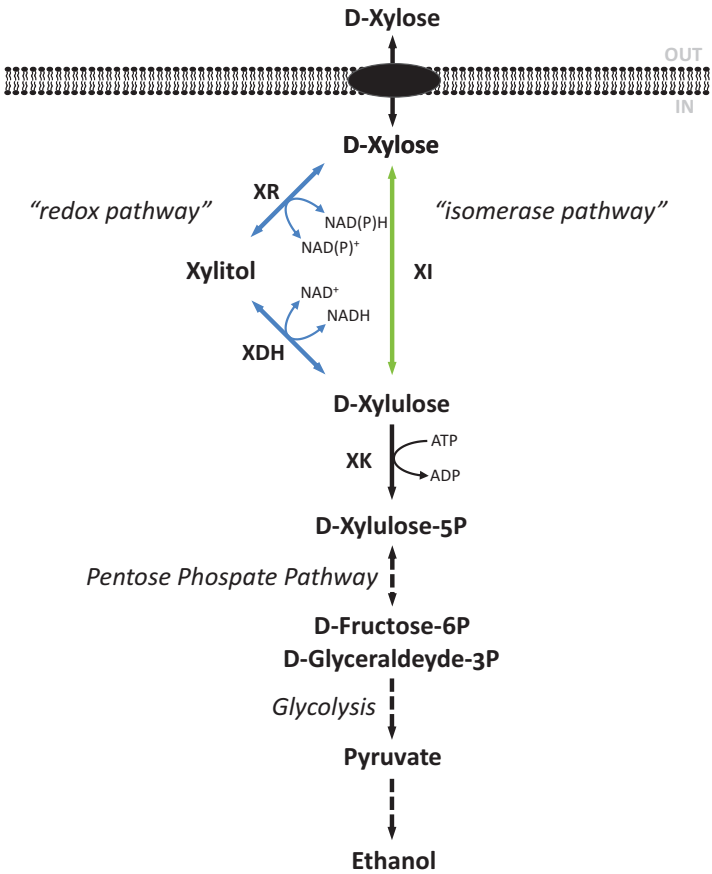


Fig. 2 Pathways for xylose fermentation in recombinant *Saccharomyces cerevisiae* (*XR* xylose reductase, *XDH* xylitol dehydrogenase, *XI* xylose isomerase, *XK* xylulokinase)

relevant to increase xylose consumption and the carbon flux through PPP, with consequent enhancement of ethanol yield and productivity (Xu et al. 2016). The metabolic engineering approaches have been often followed by evolutionary engineering protocols for further strain improvement (Kuyper et al. 2005b; Wisselink et al. 2009; Garcia-Sanchez et al. 2010). This methodology, also known as adaptive evolution, is a slow process based on natural mutations that can rationally be accelerated by appropriate selection pressure during cultivation (Mans et al. 2018). The analysis of improved C5-fermenting strains often revealed that the kinetic properties of pentose transport were altered towards increased sugar uptake fluxes when a mixed-sugar platform was used in the evolutionary engineering protocols (Kuyper et al. 2005b; Garcia-Sanchez et al. 2010). The sugars present in lignocellulose hydrolysates are often consumed by *S. cerevisiae* in a sequential mode, first glucose and then xylose, with consequences at the level of ethanol yield and, mainly, productivity. This fermentation profile is correlated to the biochemistry of sugar uptake in yeasts, which is usually dependent of nonspecific sugar transporters generally preferring glucose. Therefore, attempts have been made to develop specific transporters for xylose to overcome the inhibitory effect of glucose. The heterologous expression of a glucose/xylose transporter from *Candida intermedia* (Leandro et al. 2006) in industrial xylose-fermenting *S. cerevisia*e led to improved D-xylose uptake kinetics and revealed that, under low D-xylose concentration, some strains are limited at the level of xylose transport (Fonseca et al. 2011). Also, glucose-insensitive xylose transporters have been developed from mutated *S. cerevisiae* hexose transporters (Farwick et al. 2014), which can contribute to more efficient glucose/xylose co-consumption.

Several industrial *S. cerevisiae* strains are being used in C6/C5 fermentation in lignocellulosic ethanol demonstration and commercial plants. The providers of industrial C6/C5 yeasts include traditional yeast manufacturers like Lesaffre (CelluX™), Lallemand (C5 FUEL™), and DSM (*Saccharomyces cerevisiae* expressing xylose isomerase from *Piromyces* sp. E2) but also new players like the companies resulting from research and development performed at universities like C5 Ligno Technologies AB (C5LT), GlobalYeast (Excellulor™), and Terranol A/S (cV-110). Most of these strains use the "isomerase pathway" and are able to produce ethanol from glucose and xylose at high yields in the presence of inhibitory compounds. However, glucose/xylose co-consumption is still a challenge to be overcome in currently available commercial strains, with xylose fermentation being particularly compromised by the amount of inhibitory compounds.

2.2 Butanol

Butanol is a four-carbon alcohol with chemical formula $C_4H_{10}O$ which has four isomeric structures (*n*-butanol, isobutanol, 2-butanol, and *tert*-butanol). They differ in physicochemical properties and production methods but the applications are similar in some aspects. Their applications are abundant, such as: chemical

intermediate for fuels and jet fuel and bio-lube oil; chemical intermediate in the production of monomers, polymeric emulsions, esters, plasticizers, glycol ethers, and amines; solvent for paints, coatings, and varnishes; extractant for antibiotics, hormones, and vitamins; perfume and cosmetics ingredient; degreasers and cleaning solutions (Schiel-Bengelsdorf et al. 2013). Compared to ethanol, *n*-butanol, and isobutanol are superior liquid fuels due to their higher energy content and lower volatility. Therefore, they are more gasoline-like and can thus be blended more easily with gasoline or even used directly in conventional internal combustion engines. Furthermore, butanol can also be blended with diesel fuels and used in jet fuels and it does not absorb moisture, so does not cause corrosion (Zhao et al. 2013).

At present, butanol and higher alcohols are mainly produced by thermochemical routes (Ndaba et al. 2015). However, the interest in the production of butanol through microbial fermentation processes has been renewed due to the general trend on the shift to renewable fuels and chemicals and the recent advances in strain and process development. Biological production of *n*-butanol has a long history (Jones and Woods 1986). Butanol fermentation process was the second largest industrial fermentation process in the world during the first part of the twentieth century. Early industrial production of *n*-butanol was based on fermentation of sugar and starch using *Clostridium* spp., typically referred to as acetone-butanol-ethanol (ABE) fermentation (Jones and Woods 1986; Sauer 2016).

ABE process historically relies on *Clostridia* spp., which are natural acetone-butanol-ethanol producers, but also known as able to generate several products which cannot be obtained through chemical synthesis (Ndaba et al. 2015). The metabolism (Fig. 3) is divided into two phases (Jones and Woods 1986; Qureshi and Ezeji 2008).

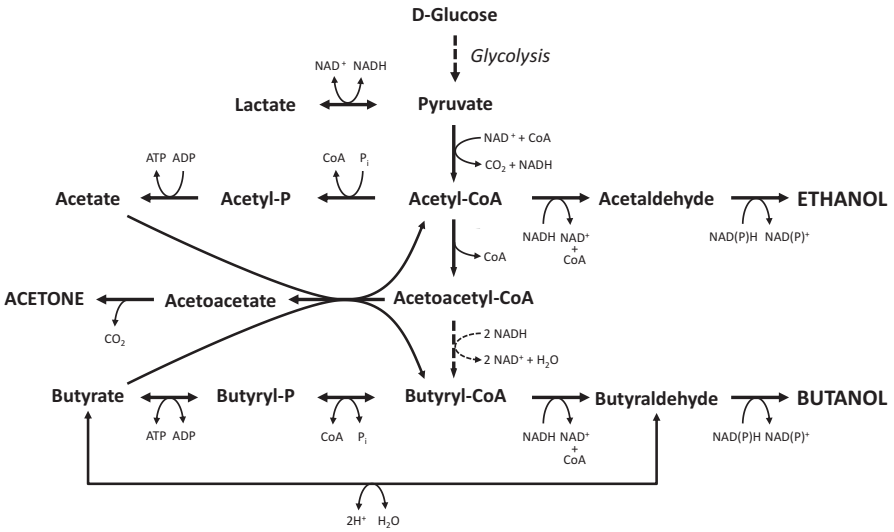


Fig. 3 Acetone Butanol Ethanol (ABE) fermentation pathway in clostridia

In the first, acidogenic phase (acidogenesis), butyrate and acetate are formed in a standard butyric acid pathway. In the second, solventogenic phase (solventogenesis), acids are converted into butanol, acetone, and ethanol. Some strains of *C. beijerinckii* are able to further reduce acetone to isopropanol (Schiel-Bengelsdorf et al. 2013). The fermentation is strictly anaerobic. The produced organic acids and alcohols above a certain titer are toxic to the cells, *n*-butanol being the most toxic (the natural tolerance is about 11–12 g/L) (Branduardi and Porro 2016). Therefore, usually *in situ* product recovery techniques are integrated in ABE fermentation (Schiel-Bengelsdorf et al. 2013).

Even though *n*-butanol is recognized as an alternative fuel, its production is still not considered economical due to several limitations, such as: (1) low *n*-butanol titers (<20 g/L) caused by inhibition during fermentation; (2) low *n*-butanol yield due to hetero-fermentative metabolism (0.28–0.33 g/g); and (3) high cost of *n*-butanol recovery from broths with low product concentration (Ndaba et al. 2015). Hence, strain improvement has been attempted to overcome the bottlenecks of *Clostridia* spp. in this process, aiming at increasing butanol yield and tolerance, but also expanding substrate utilization (e.g., xylose) and air tolerance. Some robust strains were obtained, like *C. beijerinckii BA 101* (Li and Ge 2016) and *C. acetobutylicum ATCC 55025*, reaching approximately 20 g/L *n*-butanol titers (Zhao et al. 2013). Metabolic engineering of these organisms has been challenging and the achievements on improved butanol titer, yield and productivity, enhanced butanol selectivity and increased tolerance to solvents have been mainly achieved with *C. acetobutylicum* (Lee et al. 2016; Li and Ge 2016). Still, one of the main challenges in butanol production with *Clostridia* spp. is acetone production together with butanol and ethanol. Acetone cannot be used as a fuel and reduces the yield of butanol. Therefore, metabolic engineering was also targeting eliminating acetone production pathway, but usually this resulted in reduced solvent production (Li and Ge 2016). Genome shuffling and evolutionary engineering approaches have also been applied (Li and Ge 2016). The recent development of efficient genome editing tools (Lee et al. 2016) offers great potential for further strain improvement.

Many clostridia are able to metabolize several carbohydrates, including hexoses and pentoses (Jones and Wood 1986). However, xylose utilization in mixtures with glucose is poor, due to carbon catabolite repression (Schiel-Bengelsdorf et al. 2013). Some Clostridia species are able to directly convert polysaccharides, like *Clostridium* sp. *strain NUP7*, which is able to produce butanol or isopropanol from hemicellulose (Xin et al. 2017). Some solventogenic Clostridia, such as *Clostridium thermocellum*, *Clostridium cellulolyticum*, and *Clostridium thermopapyrolyticum*, can directly convert lignocellulosic biomass (Lee et al. 2016). The different abilities of Clostridia strains in carbohydrate utilization and product formation prompt the study of mixed-culture fermentation processes in order to improve synergies in the production of lignocellulose-degrading enzymes (Baral et al. 2016).

Other cell factories, such as *S. cerevisiae*, *E. coli*, and *Pseudomonas putida* (Sauer 2016; Li and Ge 2016), have been considered as suitable chassis to introduce the pathways for *n*-butanol production. The latest results on development such strains

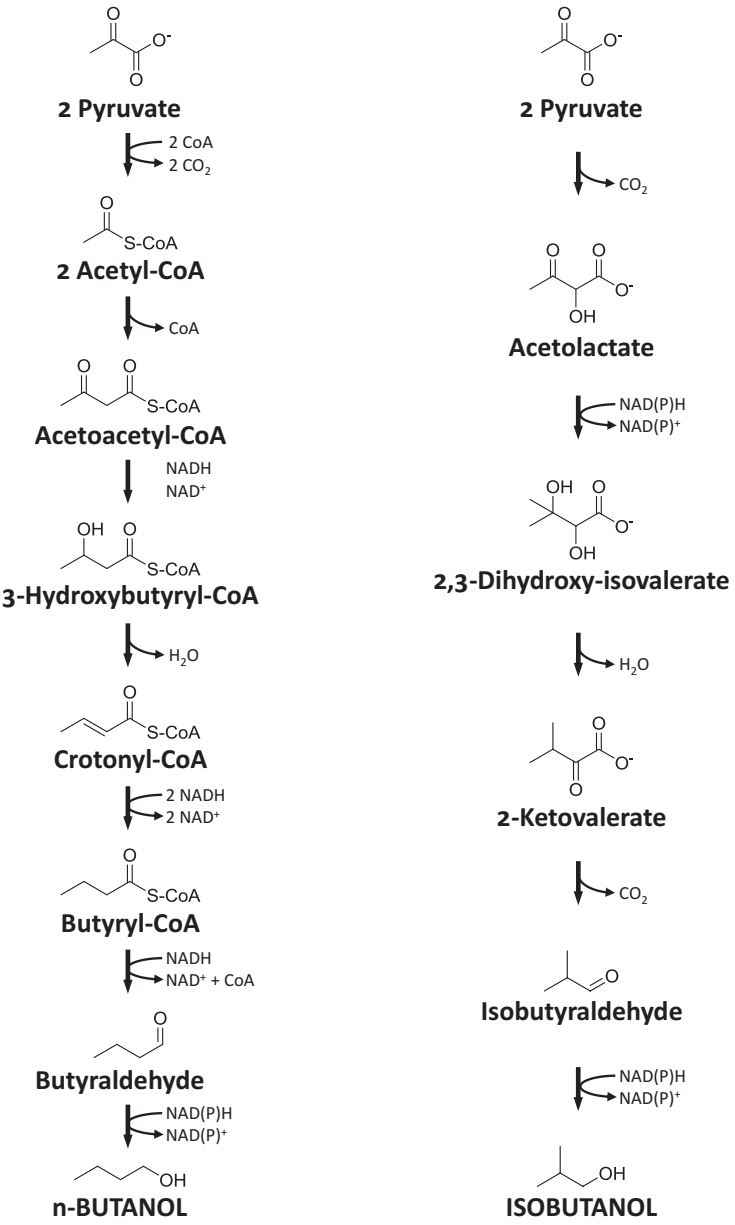


Fig. 4 Butanol production pathways in yeast

have been summarized by Li and Ge (2016). *S. cerevisiae* is not able to produce *n*-butanol, but can produce a central intermediate, acetyl-CoA, and also acetoacetyl-CoA. The "Clostridia" metabolic pathway for *n*-butanol production was introduced in *S. cerevisiae* making use of enzymes from different microorganisms (Fig. 4) (Swidah et al. 2015; Schadeweg and Boles 2016). In this pathway, two molecules of

acetyl-CoA are condensed into acetoacetyl-CoA, which is reduced to 3-hydroxybutyryl-CoA, then dehydrated to crotonyl-CoA. Further reductions generate butyryl-CoA, butyraldehyde, and finally *n*-butanol (Schadeweg and Boles 2016).

An alternative route to produce butanol is the 2-keto-acid or Ehrlich pathway. This pathway involves the decarboxylation of a 2-keto-acid to form the corresponding aldehyde, and the subsequent reduction of the aldehyde to form the alcohol. The 2-keto-acid pathway was successful expressed in different chassis, like *E. coli*, *Corynebacterium glutamicum*, *Bacillus subtilis*, and *S. cerevisiae*, among others, to produce isobutanol (Felpeto-Santero et al. 2015). In this pathway, two molecules of pyruvate are condensed into 2-acetolactate, which is reduced to 2,3-dihydroxyisovalerate, then dehydrated to 2-ketoisovalerate. Then the decarboxylation of the 2-keto-acid to isobutyraldehyde is followed by reduction to isobutanol (Fig. 4).

Companies operating at demonstration and commercial scale, like Gevo Inc. and Butamax, use modified *S. cerevisiae* in their processes. Yeast has preferentially been utilized as host cell factory since it is easy to handle, it is a facultative anaerobe and it tolerates higher alcohol concentrations. Still, continuous product removal during fermentation is part of the industrial process, allowing high yields and productivities (Ryan 2018).

3 Hydrocarbons

3.1 Farnesene

Isoprenoids (such as farnesene) are the largest and most diverse group of natural products, composed of over 50,000 compounds including primary and secondary metabolites (George et al. 2015). Isoprenoids are divided according to the number of carbon atoms: hemiterpenoids (C5), monoterpenoids (C10), sesquiterpenoids (C15), diterpenoids (C20), and triterpenoids (C30). The sesquiterpenoids (C15) are one of the largest groups of isoprenoid natural products (Demain and Martens 2017). Farnesene is a 15 carbon long-chain, branched, unsaturated hydrocarbon, which can be found in nature mainly in the skin of apple and other organic materials. It is also a renewable chemical building block which was unique physical properties and reactivity for new materials with a broad range of applications, from cosmetics to biofuels.

The fully reduced (hydrogenated) form of farnesene (farnesane) is being pursued as an alternative biosynthetic diesel (George et al. 2015), as it has a cetane number that falls within the expected range for diesel (Peralta-Yahya et al. 2012). It can be mixed directly into diesel or jet fuels without requiring any engine modifications and it is also resistance to cold. Farnesene-based renewable diesel and jet fuel is likely to hit price target and prove to be cost-efficient, which will drive its use as a drop-in replacement for synthetic fuels, lower GHG emissions and reduce particulate matter

emissions, decrease pollution near airports and major metropolitan area. Amyris and Total together developed a drop-in jet fuel that contains up to 10% blends of renewable farnesane, which meets the rigorous performance requirements set for Jet A/A-1 fuel used by the global commercial aviation industry (El Takriti et al. 2017).

High-purity farnesene can be used in tire manufacturing as in polymerized form it can easily and completely react with tire rubber and unlike oil additives it can attain strong adhesion of rubber components for improving performance and shape stability. Moreover, it conveys high plasticity, maintains excellent flexibility even at low temperatures, and improves ice grip performance. Commercial tires marketed by Sumitomo Rubber Industries, Ltd. under their Dunlop brand utilizing renewable liquid-farnesene rubber was developed with Kuraray (Japan) and already launched in early 2017 (RJA 2017). Squalene is a C30 molecule formed by either biological or chemical condensation of two farnesene units. Squalane is a hydrogenation product of squalene, is used as an important moisturizing and anti-aging ingredient in the cosmetics (Beller et al. 2015). As cosmetic formulation companies prefer to procure squalane derived from biotechnology route rather than expensive and unsustainable animal sources such as ultra-refined oil or shark liver, it will continue to drive farnesene demand. The production of squalene is robust and reproducible, and along with the availability of feedstock, ensures the reliable and sustainable production of squalene both from a chemical and sensorial (i.e., odor and color) standpoint (McPhee et al. 2014).

According to Global Market Insights, Inc., the farnesene market size was estimated at over 8 kton in 2015 (https://www.gminsights.com). Cosmetics and personal care took up 37.6% of farnesene market share, followed by fuels and lubes (25.6%), while the flavors and fragrances market share was at 23.6% and performance materials (13.2%). Growing trend towards biofuel use in aviation and automobile sector to curb carbon emissions may boost farnesene market growth. Farnesene market is predicted to increase with a forecasted compound annual growth rate (CAGR) at over 40% up to 2023 (https://www.gminsights.com). The global farnesene market share is currently dominated by Amyris Inc. (California, USA).

The common biochemical precursor of all isoprenoids is the 5-carbon intermediate isopentyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP). There are two pathways for the biosynthesis of isoprenoids (Fig. 5): the mevalonate pathway (MVA) and the methylerythritol phosphate (MEP) pathway (George et al. 2015; Beller et al. 2015; Benjamin et al. 2016; Leavell et al. 2016). When IPP and DMAPP are formed, they are used for carbon chain elongation reactions to produce longer prenyl pyrophosphate precursors such as geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP), geranylgeranyl pyrophosphate (GGPP) (Fig. 5).

Both pathways were engineered in *E. coli* and *S. cerevisiae* but the MEP pathway has turned out to be less effective than the MVA pathway (Beller et al. 2015; Benjamin et al. 2016). Amyris's trans-β-farnesene is produced through fermentation

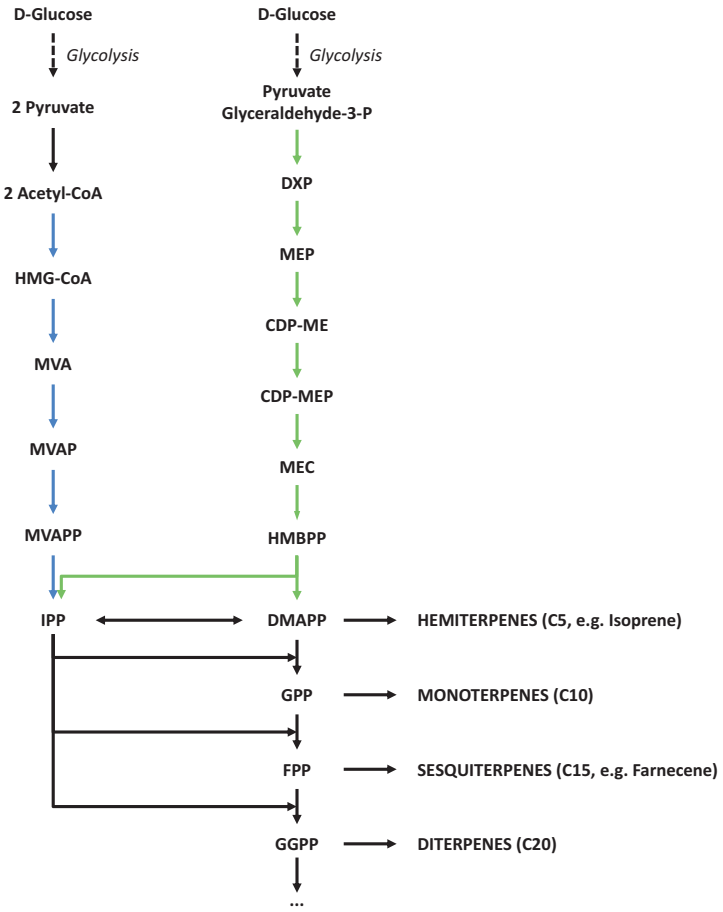


Fig. 5 Biosynthetic pathways for the production of isoprenoids, the Mevalonate pathway (MEV, in blue) and the Methylerythritol phosphate (MEP, in green). *Abbreviations*: *HMG-CoA* 3-hydroxy-3-methylglutaryl-CoA, *MVA* mevalonate, *MVAP* mevalonate-5-phosphate, *MVAPP* mevalonate-5-pyrophosphate, *IPP* isopentyl pyrophosphate, *DXP* 1-deoxy-D-xylulose-5-phosphate, *MEP* 2-C-methyl-D-erythritol-4-phosphate, *CDP-ME* 4-diphosphocytidyl-2-C-methyl-D-erythritol, *CDP-MEP* 4-diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate, MEC 2-C-methyl-D-erythritol 2,4-cyclodiphosphate, *HMBPP* hydroxymethylbutenyl-4-pyrophosphate, *DMAPP* dimethylallyl pyrophosphate, *GPP* geranyl pyrophosphate, *FPP* farnesyl pyrophosphate, *GGPP* geranylgeranyl pyrophosphate

of sugars by yeast. Target genes were selected to shift the carbon flux from ethanol to hydrocarbons. Development of the pathway for production of the antimalarial artemisinin has served as the foundation for building pathways for other terpenes (Beller et al. 2015). The first product that was developed by Amyris Inc. was artemisinin, an anti-malaria drug where the specific enzyme (amorphadiene

synthase) from plant was introduced in *S. cerevisiae* to generate amorphadiene from FPP. Oxidation of amorphadiene to artemisinic acid is accomplished by the action of five plant enzymes expressed in the engineered yeast, and the final conversion of purified artemisinic acid to artemisinin is performed by organic chemistry (Benjamin et al. 2016). The flexibility of the *S. cerevisiae* chassis allowed scientists to rapidly switch from amorphadiene to β-farnesene as final product, by introducing a plant β-farnesene synthase in the MVA pathway (Benjamin et al. 2016; George et al. 2015). Since *S. cerevisiae* uses a chemically inefficient pathway for isoprenoid biosynthesis, the first attempts to produce β-farnesene resulted in low yield and productivity, with titers lower than 1 g/L (Meadows et al. 2016). Amyris Inc. generated an evolved *S. cerevisiae* strain capable of converting sugars into β-farnesene at titers as high as 100 g/L and volumetric productivities of 16.9 g/L/day (Meadows et al. 2016; Demain and Martens 2017). The microbial fermentation product is a high purity single isomer β-farnesene after distillation (Leavell et al. 2016).

Availability and cost of substrates in combination with life cycle assessment led Amyris Inc. to open a full-scale production plant in Brotas, Brazil (2013), to produce β-farnesene from sucrose (Benjamin et al. 2016; Leavell et al. 2016). The product is commercialized under the name Biofene®. Expected demand serving the polymers, nutraceuticals, and solvents markets through 2020 will require new farnesene manufacturing capacity beyond the company's current capacity. Therefore nowadays Amyris Inc. is developing an integrated scalable process with the aim to produce farnesene from cellulosic sugars at 2 USD per liter in the USA in connection to Renmatix's Plantrose® technology for cost-effective production of lignocellulosic sugars from woody feedstock (Mitrovich and Wichmann 2017). This will require further strain development for the efficient conversion of xylose from hemicellulose hydrolysates in the presence of microbial inhibitors generated during biomass processing. Alternatively, purification of sugar streams may reduce the concentration of inhibitors but those processes need to be cost-effective.

4 Organic Acids

4.1 Lactic Acid

Lactic acid (or 2-hydroxypropionic acid) is the simplest and most widely occurring natural hydroxyl acid and, similarly to ethanol, has a long history. It has an asymmetric carbon atom and is present in two optically active forms, L(+)- and D(−)-lactic acid. These isomers have the similar chemical and physical properties, making them difficult to separate with traditional techniques (Komesu et al. 2017). In humans and other mammals, only the L(+)-isomer is present (Ghaffar et al. 2014).

In fact, D(−)-lactic acid is harmful to humans since they only have L-lactate dehydrogenase. Therefore, L(+)-lactic acid is the preferred isomer in food and pharmaceutical industries (Reddy et al. 2008).

Currently, lactic acid is used in a wide variety of industrial applications, including chemicals—solvent, emulsifier, plasticizer; pharmaceuticals—implants, drug-release systems, hygiene and aesthetic products; and food—flavoring, preservative, natural product in fermented products. Furthermore, lactic acid is a precursor of several other products, like propylene oxide, acetaldehyde, acrylic acid, among others (Komesu et al. 2017). Due to the demand for bio-products from renewable resources, the biological production of lactic acid as a bulk chemical has been increasing considerably. For example, the polymerization of lactic acid into polylactic acid (PLA) generates an environment-friendly alternative to plastics derived from petrochemicals (Reddy et al. 2008; Komesu et al. 2017). Global lactic acid and PLA demand was estimated to be 714.2 and 360.8 kton in 2013, with expected annual growth of 15.5% and 18.8%, respectively, until 2020 (Abdel-Rahman and Sonomoto 2016).

While chemical synthesis from petrochemicals always generates a racemic mixture, pure lactic acid isomers can be synthetized by microbial fermentation (Komesu et al. 2017). Microbial fermentation processes utilize renewable substrates and require mild production conditions (temperature 30–45 °C, pH 5.5–6.5) and low energy consumption when compared with petroleum-based chemical synthesis (Reddy et al. 2008; Abdel Rahman and Sonomoto 2016).

Biological production of lactic acid is currently driven by lactic acid bacteria (LAB), Gram-positive, facultative anaerobes, with nutritional requirements for amino acids and vitamins (Reddy et al. 2008; Murali et al. 2017). Most common LAB genera include *Lactobacillus, Pediococcus, Aerococcus, Carnobacterium, Enterococcus, Tetragenococcus, Vagococcus, Leuconostoc, Oenococcus, Weissella, Streptococcus, and Lactococcus* (Juturu and Wu 2016). The species of LAB are usually separated into homo- and hetero-fermentative, based on their type of hexose fermentation. The homo-fermentative LAB utilizes glycolysis (Fig. 6) and produces virtually only lactic acid, with a theoretical yield of lactic acid from glucose of 1.0 g/g or 2 mol/mol. The hetero-fermentative LAB utilizes the phosphoketolase pathway and produce lactic and acetic acids, ethanol and carbon dioxide, with the theoretical yield of lactic acid from glucose reaching only 0.5 g/g or 1 mol/mol (Abdel-Rahman et al. 2011; Rooke 2003; Reddy et al. 2008).

Optically, pure lactic acid is synthesized by microbial fermentation of carbohydrates such as glucose, sucrose, lactose, and starch/maltose, which are derived from feedstocks such as sugar beet, sugarcane molasses, whey, and barley malt (Ghaffar et al. 2014). Amylolytic lactic acid bacteria (ALAB) (*Lactobacillus plantarum*, *Lactobacillus manihotivorans*) have been found in different tropical fermented foods (Nwankwo et al. 1989; Morlon-Guyot et al. 1998) and can contribute for the economy of the process by eliminating the two-step process of starch saccharification and lactic acid fermentation (Reddy et al. 2008). Although producing lactic

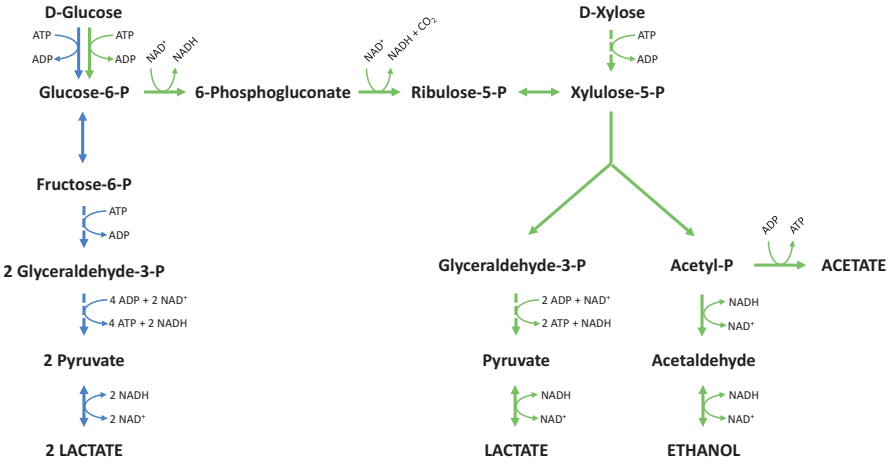


Fig. 6 Typical homolactic (in blue) and heterolactic (in green) fermentation in lactic acid bacteria

acid with high yield and productivity from different sugars, LAB are not particularly fitted to ferment lignocellulosic hydrolysates. For example, pentose fermentation mainly uses the hetero-fermentative pathway (Tan et al. 2017). Strain development for the utilization of lignocellulosic biomass is required to: (1) increase tolerance to inhibitory compounds formed during pretreatment; (2) expand the carbohydrate assimilation capacity (e.g., for direct conversion of cellulose and hemicellulose); and (3) ferment mixed-sugar streams with high lactic acid yield and productivity.

Some filamentous fungi, e.g., *Rhizopus* can also utilize glucose and produce lactic acid (Ghaffar et al. 2014), and *R. oryzae* and *R. arrhizus* can convert starch directly to L(+)-lactic acid due to their amylolytic enzyme activity (Wee et al. 2006). Fermentation by fungi has also other advantages compared to bacterial fermentation, namely in nutrient requirements (Wee et al. 2006; Tan et al. 2017).

Other microorganisms have also been considered as chassis for industrial lactic acid production such as yeasts (*S. cerevisiae, Kluyveromyces lactis, Candida boidinii*), and non-LAB bacteria (*E. coli* and *C. glutamicum*) (Abdel-Rahman et al. 2011). Microalgae and cyanobacteria (photosynthetic microorganisms) also have attracted attention because of their ability to couple CO_2 capture the potential for genetic modification (Tan et al. 2017). Furthermore, *Bacillus* spp. are mostly thermophilic, which enables simultaneous saccharification and fermentation (SSF) and reduces the risk of contamination, have low nutritional requirements and are able to ferment pentoses to lactic acid through a homo-fermentative pathway (Tan et al. 2017).

4.2 Succinic Acid

Succinic acid is a member of the family of C4-dicarboxylic acid, with a molecular formula of $C_4H_6O_4$. Succinic acid is a priority chemical of high value as food and feed ingredient and as platform chemical for replacement of the oil-based building block maleic anhydride. The applications of succinic acid include flavor additives, pharmaceuticals, detergents, and surfactants. In the chemical industry, it is a building block for producing other commodity or specialty chemicals like 1,4-butanediol, gamma-butyrolactone, tetrahydrofuran and polybutylene succinate (PBS), a biodegradable polymer (Vaswani 2010; Song and Lee 2006). Commercial production of succinic acid is deployed by companies like Myriant, BioAmber, BASF-Purac (Succinity), and Reverdia (DSM-Roquette) (Vaswani 2010).

Succinic acid is an intermediate of the tricarboxylic acid (TCA) cycle, thus part of the central metabolism of many organisms, and one of the possible fermentation end products of anaerobic metabolism. Several succinate-producing bacteria (*Actinobacillus succinogenes, Anaerobiospirillum succiniciproducens, Mannheimia succiniciproducens, Bacteroides fragilis, Enterococcus flavescens, Klebsiella pneumoniae, Basfia succiniciproducens, Succinivibrio dextrinosolvens*) and fungi (*Aspergillus niger, Paecilomyces variotii, Penicillium simplicissimum*) have been described (Li and Xing 2015).

Succinate can be generated from PEP (phosphoenolpyruvate), via oxaloacetate (OAA), malate, and fumarate, with incorporation of CO_2 and consumption of NADH. This reductive pathway is also referred as reverse TCA (rTCA) (Fig. 7). Through this pathway, succinate is often produced along with other fermentation products (lactate, acetate, ethanol, and formate), depending on the microorganism and on the cultivation conditions (McKinlay et al. 2007). Aerobically, succinate is often produced through the TCA cycle, an oxidative pathway that generates NADH and the loss of CO_2 (Fig. 7). A third option is the glyoxylate shunt, which eliminates the loss of $2CO_2$ when compared to the TCA cycle, converting isocitrate directly to succinate (Fig. 7). The co-produced glyoxylate can then be converted into malate.

The efficient production of succinate often combines the reductive pathway with the glyoxylate shunt or with the oxidative pathway, due to the requirement for reductive equivalents (in the form of NADH) by the reductive pathway (Nghiem et al. 2017). Therefore, the maximal theoretical yield of succinic acid from 6-carbon sugars, such as glucose, and CO_2 is 1.71 mol/mol sugar, or 1.12 g/g, with 57–71% of the glucose being converted through the reductive pathway and the remaining sugar used through the glyoxylate shunt or the oxidative pathway for the production of the required reducing power (Raab and Lang 2011).

During fermentation, the accumulation of succinic acid lowers the pH of the medium therefore pH control in a range suitable for the microorganism is crucial. However, the choice of the base for pH controlling will determine the produced succinate salt, which will affect its applications, or the required further process steps. Therefore, the interest has been increased significantly to isolate or engineer strains that are able to tolerate low pH.

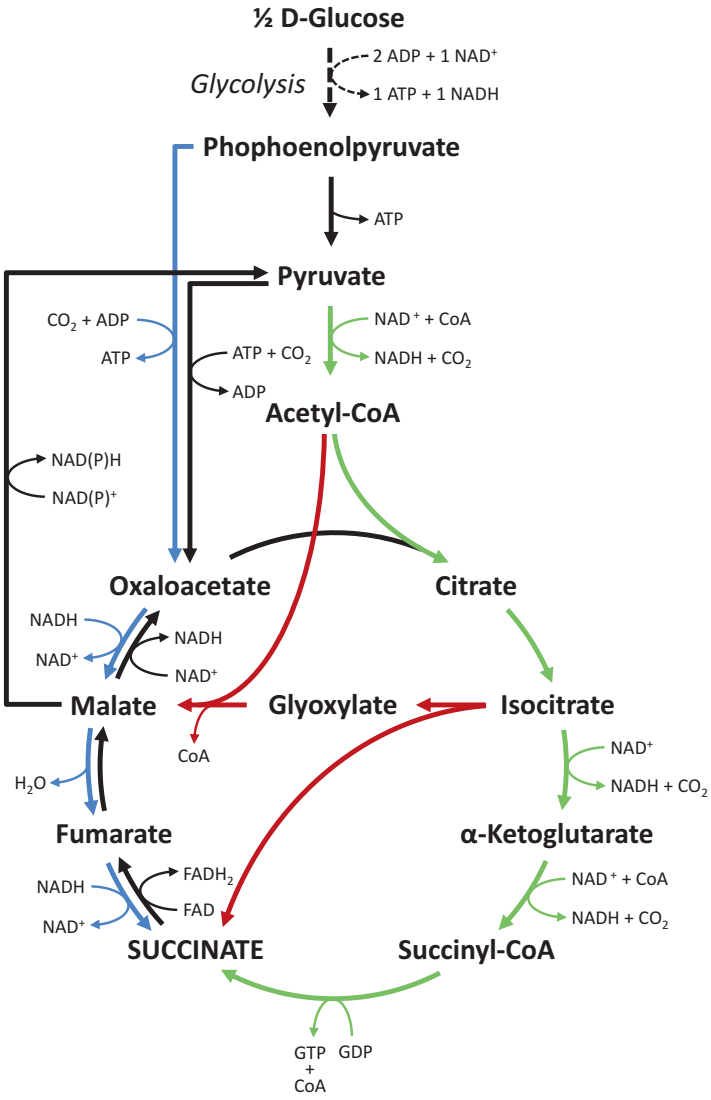


Fig. 7 Metabolic pathways for succinic acid production. Reductive pathway (in blue), a oxidative pathway (in green), and glyoxylate shunt (in red)

The first bacteria described as succinate producer was *Anaerobiospirillum succiniciproducens*, which was engineered and tested under adequate conditions to produce succinate from glucose and wood hydrolysate with yields higher than 70% of the theoretical (Guettler and Jain 1996; Lee et al. 2003). More recently, *Basfia succiniciproducens* another efficient succinate producer was described (Kuhnert et al. 2010) and the efficiency of the process was revealed to be substrate dependent

(Lange et al. 2017). PTS-dependent substrate phosphorylation of sucrose and fructose contributes to an increased pool of pyruvate and the formation of by-products (Lange et al. 2017). The presence of an alternative fructokinase allowed the disruption of the fructose-PTS, which, together with the elimination of by-product pathways, contributed to a succinate yield from sucrose higher than 70% of the theoretical (Lange et al. 2017). Succinity.technology, developed in a joint venture between BASF and Corbion, is using a proprietary strain of *Basfia succiniciproducens* in their process (Nghiem et al. 2017; Vaswani 2010).

E. coli primarily ferments glucose to ethanol, and formic, acetic and lactic acids with only detectable amounts of succinic acid under anaerobic condition (Song and Lee 2006). Approaches to promote succinate production in *E. coli* have included: (1) the removal of competing pathways; (2) overexpression of enzymes involved in the reductive pathway; (3) introduction of heterologous enzymes with superior catalytic efficiency; (4) fine tuning of redox balance for maximal succinate production (Li and Xing 2015; Song and Lee 2006). The main advantage of using recombinant *E. coli* is its fast growth rate, simple requirements for nutrients and easy genetic manipulation for high succinate yield. Recently, *E. coli* was engineered to produce succinate with a combination of the reductive pathway and the glyoxylate shunt or the oxidative pathway (Nghiem et al. 2017). In the BioAmber technology, a modified *E. coli* was used to produce diammonium succinate at ambient temperature and neutral pH, with the use of sugar and CO_2 as a feedstock and NH_3 as a neutralizing agent for the carboxylic acid. In the Myriant technology, a modified *E. coli* is also applied, this capable of utilizing sugars derived from lignocellulosic feedstocks (Ahn et al. 2016).

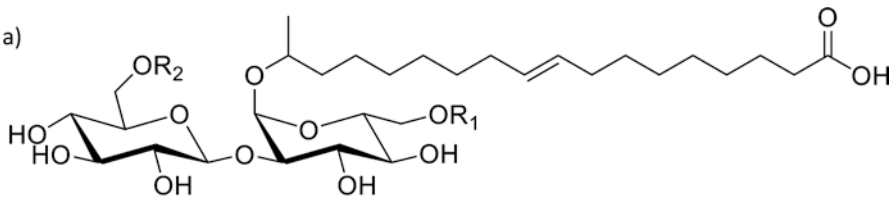
The bacteria described as succinate producers require the neutralization of the fermentation broth to cope with the pH needs of these organisms. The cost associated with the neutralization and consequent requirements in downstream processing for product purification increased the interest of developing cell factories capable of efficient fermentation at low pH. Several fungi can produce organic acids under aerobic conditions, tolerating low pH (Yang et al. 2016). BioAmber, at the scale up phase to commercial, realized that the applied *E. coli* was too sensitive to pH in the fermentation process. Therefore, the pathway for succinate production was re-engineered in the yeast *Pichia kudriavzevii*, which was able to produce succinic acid at a much lower pH than previously used *E. coli* (Alonso et al. 2015). *S. cerevisiae*, as robust and important industrial microorganism, tolerant of low pH values (3.0–6.0) and able to perform anaerobic fermentation, was also considered a suitable chassis for succinic acid production. The Reverdia technology, based on recombinant *S. cerevisiae* developed by DSM, is combining the reductive pathway with the glyoxylate shunt for maximal succinate yield. Moreover, it is also able to operate at low pH, thus less prone to contamination and requiring less chemical processing, equipment, and energy to convert intermediate salts into succinic acid (Nghiem et al. 2017).

5 Others

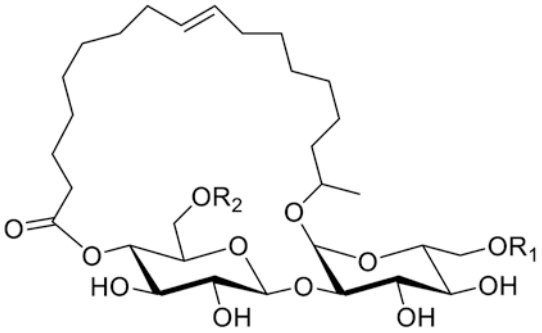
5.1 Biosurfactants

Surfactants are capable of reducing the surface tension and interfacial tension between individual molecule at the surface and interface. Surfactants are widely used in household detergents, industrial and agricultural chemicals (e.g., dispersants), personal care and cosmetics, pharmaceuticals, food, oilfield chemicals, among others (Nitschke and Silva 2017; Vecino et al. 2017; Souza et al. 2014; Sachdev and Cameotra 2013). Traditional surfactants are based on petrochemical resources such as ethylene, benzene, kerosene, and *n*-paraffins. Biosurfactants are produced either by microorganisms or by (bio)chemical conversion of natural products and are seen as potential alternatives to synthetic surfactants due to structural diversity, performance under different conditions and environmental performance (Scott and Jones 2000; Sajna et al. 2015). For some decades, the production of biosurfactants was limited to the use of vegetable oils, as coconut and palm oil, for the hydrophobic part of the molecule. Nevertheless, the production of these so-called first-generation biosurfactants still involves chemical synthesis. Microbial glycolipids (sophoro-, rhamno-, and mannosylerythritol lipids) (Fig. 8) are among the most promising biosurfactants for commercialization due to their technical performance, potential large-scale production through fermentation and recovery as extracellular products. These second-generation biosurfactants were only applied in niche markets until very recently (Brumano et al. 2016). Topics like sustainability and the use of bio-based home care products are getting more popular with consumers and the effect of *Green Premium* (the willingness of consumers to pay an additional price for "green materials") is expected to get more pronounced, not only in developed countries but also in the emerging regions. Also, the production costs are expected to decrease as a result of technological developments. The surfactant market is extremely big with a worldwide annual production of over 13 Mt/y expecting a CAGR (compound annual growth rate) of 5.53% during the forecast period (2018–2023) (Mordor Intelligence 2018). This demands a big input of often petrochemical resources and moreover represents a tremendous ecological load considering the large fraction used in household cleaners (over 50% of total use of surfactants) which end up in wastewater and/or directly in the environment.

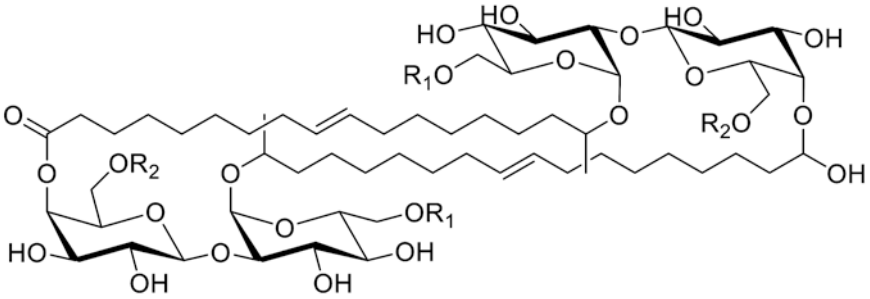
Europe has taken lead in bio-based surfactant consumption and is expected to remain market leader and to enjoy 53.3% of global biosurfactants market revenue share in 2018 (Report of Transparency Market Research 2011). Owing to environmental concerns, this market is expected to grow at a promising rate in Europe in coming years. The glycolipid biosurfactants provide significant opportunities to replace chemical surfactants as sustainable alternatives, in some cases with new functionalities. Sophorolipids are mainly used in household detergents across the globe, with producers, distributers, and applicants such as Soleance (France), Ecover (Belgium), Saraya (Japan), Intobio (Korea), SyntheZyme (USA), and



Sophorolipid (acidic form)



Sophorolipid (lactonic form, monomeric)

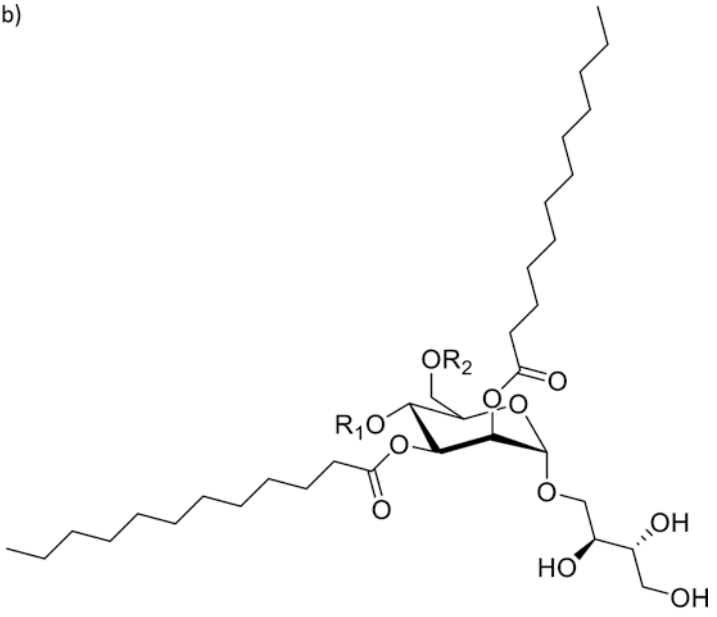


Sophorolipid (lactonic form, dimeric)

Fig. 8 Structure and variants of (**a**) sophorolipids (SL), (**b**) mannosylerythritol lipids (MEL), and (**c**) rhamnolipids (RL) (OR_1 and OR_2 represent positions for acetylation)

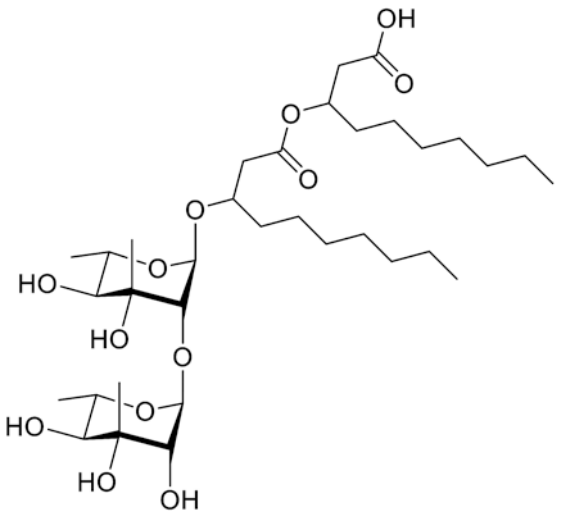
multinationals such as Henkel (Roelants et al. 2016). Mannosylerythritol lipids (MELs) are mainly produced and commercialized in Asia, by Toyobo (Japan) and Biotopia (South Korea), in cosmetics (Morita et al. 2015). Although the sustainability of both first as second-generation biosurfactants was expected to outperform synthetic surfactants based on fossil resources, the impact of their production is still high, mainly due to the use of vegetable oils in their production, either directly in chemical synthesis or as substrate for microbial biosynthesis.

b)



Mannosylerythritol lipid

c)



Rhamnolipid (di-rhamnolipid)

Fig. 8 (continued)

Sophorolipids (SL) are composed of sophorose (a disaccharide of glucose units) as the hydrophilic moiety, usually mono-acylated (typically with fatty acid of 18 carbons) on C-1′ and acetylated on C-6 and/or C-6′. The carboxylic group of fatty acid is either free (acidic or open form) or internally esterified (lactonic form), the later in monomeric or dimeric forms (Fig. 8a). The pathway for SL biosynthesis is described in the yeast *Starmerella bombicola* and typically involves five or six steps: hydroxylation of oleic acid at $\omega - 1$; assembly of a glucolipid by the reaction of the hydroxyl fatty acid with UDP-glucose; formation of the sophorose unit by reaction with another UDP-glucose; mono- or di-acetylation; secretion of the acidic SL; formation of the lactone form in the extracellular space (Roelants et al. 2016). SL are efficiently secreted by *S. bombicola* when produced from vegetable oils, reaching titres of more than 400 g/L (Daniel et al. 1998; Roelants et al. 2013). The SL production from glucose reaches 20 g/L (Konishi et al. 2008). Metabolic engineering of *S. bombicola* led to novel and more effective sophorolipids structures (Roelants et al. 2013, 2016). The effective commercial production still relies on oleaginous feedstock, with high productivity obtained under a fed-batch process combining glucose and rapeseed oil (Baccile et al. 2017).

Mannosylerythritol lipids (MEL) are often produced as major extracellular product by *Moesziomyces*/*Pseudozyma* spp. in a mixture of dozens of analogs composed of a mannosylerythritol hydrophilic moiety, usually diacylated (with fatty acids of 8–12 carbons) and di- (MEL-A), mono- (MEL-B and -C), or non-acetylated (MEL-D) on the mannosyl unit (Fig. 8b) (Morita et al. 2015). The pathway for MEL biosynthesis was first described in the fungus *Ustilago maydis* and later identified in *Moesziomyces/Pseudozyma* spp. It involves five steps: assembly of GDP-mannose and erythritol; acylation on C-2 and C-3 of the mannosyl unit to produce MEL-D; acetylation of the C-4 and/or C-6 (C-6—MEL-B, C-4—MEL-C, C-6 and C-4—MEL-A); and MMF1, for MEL export (Hewald et al. 2006) (Fig. 9). MEL can be produced by *Moesziomyces/Pseudozyma* spp. from vegetable oils at concentrations above 100 g/L (Morita et al. 2015). High production cost, related to the use of soybean oil as substrate and associated solvent-intensive recovery, is impairing their widespread application. *M. antarcticus* (former *Pseudozyma antarctica* and *Candida antarctica*) and *M. bullatus* (former *Moesziomyces aphidis and Pseudozyma aphidis*) are able to produce MEL from glucose, pentoses, glucose/xylose mixtures or directly from xylan (Faria et al. 2014a, 2015). *M. antarcticus* presents equivalent MEL yield from glucose and xylose (Faria et al. 2014a), and a process to produce MEL from cellulosic materials has been developed (Faria et al. 2014b), in which downstream process for MEL recovery is more efficient (>90% recovery with >90% purity in a single-step liquid–liquid extraction with ethyl acetate) than when produced from vegetable oils (multiple liquid–liquid extraction and lower recovery yields for the same purity), but the titers of glycolipid production from sugars are still approx. one order of magnitude lower than from vegetable oils.

Rhamnolipids (RL) are mainly produced by *Pseudomonas aeruginosa*. They are composed of one (mono-rhamnolipids) or two (di-rhamnolipids) rhamnosyl moieties linked to typically one or two beta-hydroxy fatty acids (with 8–16 carbons) (Fig. 8c). The pathway for RL biosynthesis involves the production of

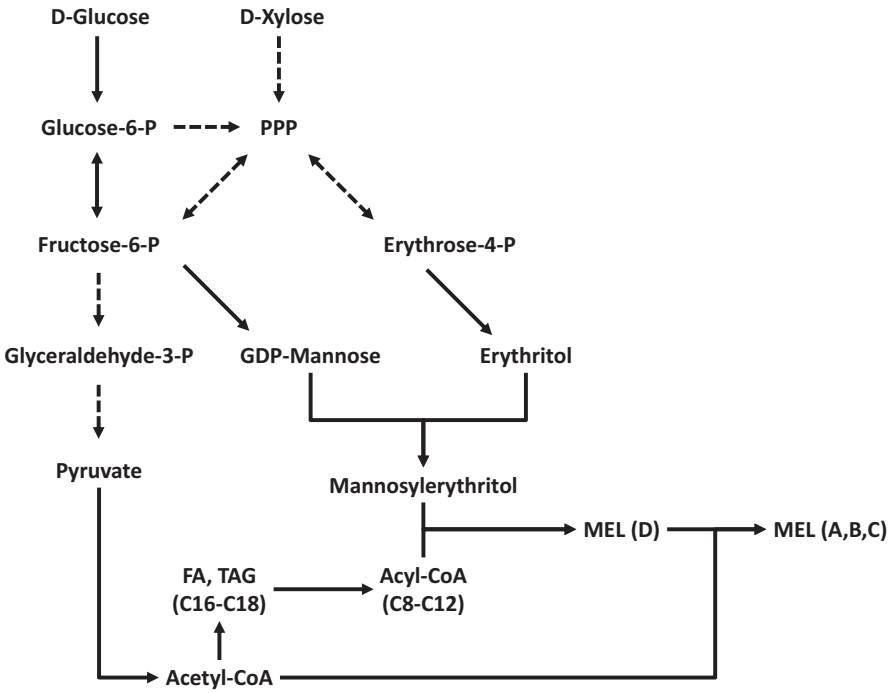


Fig. 9 Metabolic pathways for the biosynthesis of mannosylerythritol lipids (MEL) from glucose and xylose. *PPP* pentose phosphate pathway, *FA* fatty acids, *TAG* triacylglyceride

dTDP-L-rhamnose from D-glucose-1-phosphate and the assembly of beta-hydroxyalkanoyl-beta-hydroxyalkanoic acid units (Chong and Li 2017). RL titers can reach more than 100 g/L from soybean oil (Chong and Li 2017). Metabolic engineering for improved RL production was attempted in *P. aeruginosa* and in other chassis like *Pseudomonas putida*, *E. coli,* and *S. cerevisiae* (Beuker et al. 2016; Cabrera-Valladares et al. 2006; Bahia et al. 2018) but titers are far below those obtained with the natural producers.

5.2 Bioplastics (PHA)

Polyhydroxyalkanoates (PHA) are natural insoluble polyesters accumulated in some bacteria as energy storage. PHA is produced by metabolic transformation of carbon source under nitrogen, phosphorous, and/or sulfur-limiting conditions (Kaur and Roy 2015) although some bacteria are able to produce PHA during growth (Kourmentza et al. 2017). PHAs are composed of R(−)-3-hydroxyalkanoic acid monomers ranging from C3 to C14 carbon atoms with variety of saturated or unsaturated and linear or branched chains containing aliphatic or aromatic side groups (Fig. 10).

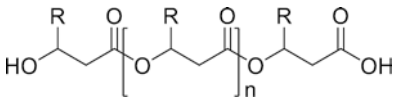


Fig. 10 General structure of PHA. If R = CH_3 – polydroxybutyrate (PHB), if R = C_2H_5 – polyhydroxyvalerate (PHV), if R = C_3H_7 –polyhydroxyhexanoate (PHH); if R = C_4H_9 – polyhydroxyoctanoate (PHO)

PHAs are a group of bioplastics that have a wide range of applications. Based on the carbon atoms comprise their monomeric units they are classified into two groups. Short-chain-length PHA (scl-PHA) consisting of 3–5 carbon atoms, and medium-chain-length PHA (mcl-PHA) consisting of 6–14 carbon atoms. The scl-PHA are mostly used for the production of disposable items and food packaging materials, while mcl-PHA are suitable for high value-added application, such as surgical sutures, implants, biodegradable matrices for drug delivery, among others (Kourmentza et al. 2017; Kootstra et al. 2017; Kaur and Roy 2015; Obruca et al. 2015).

Predictable biodegradability profile, biocompatibility, and the possibility for tailor-made structure and composition makes them attractive substitute for petrochemical plastics owed by its analogous properties (Kaur and Roy 2015). While known biopolymers such as PLA (polylactic acid) and PBS (polybutylene succinate) are produced by chemical polymerization of lactic and succinic acid, respectively, PHA polymerization is naturally performed by bacteria (Kourmentza et al. 2017).

More than 300 microorganisms are known to generate PHA (Endres and Sieber-Rathts 2011). Both native and recombinant strains have been employed in PHA production. Industrial production processes for PHA have generally been developed using Gram-negative bacteria, such as *Cupriavidus necator* and *Alcaligenes latus*, mainly due to the relatively high PHA yield and the ability of some to synthesize PHA under non-limiting nutrient conditions (Jiang et al. 2016; Chen 2010). However, huge efforts have also been directed towards process development based on Gram-positive strains such as *Bacillus* sp. and *Corynebacterium glutamicum*, which can produce ideal PHA for medical applications (Kaur and Roy 2015).

The PHA production process involves a series of steps: (1) biomass growth, (2) polymer accumulation, (3) cell harvesting, (4) polymer extraction, and (5) purification. The microorganism, the respective portfolio of genes and active enzymes, and the growth conditions (medium and operation mode), influence the yield and the polymer structure (composition, molecular weight, and respective physicochemical properties) (Kaur and Roy 2015; Jiang et al. 2016).

Monosaccharides and disaccharides can be used by several microorganisms to produce PHA. The PHA biosynthesis uses acetyl-CoA as intermediate and involves three main steps: production of acetoacetyl-CoA, its reduction to (R)-3-hydroxy-

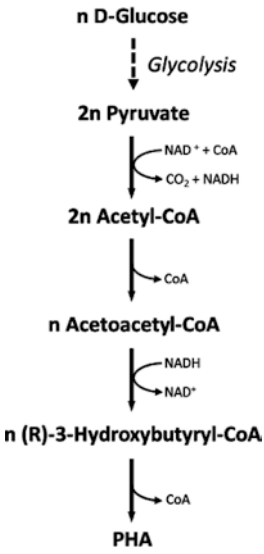


Fig. 11 Metabolic pathway for the biosynthesis of polyhydroxyalkanoates (PHA)

butyryl-CoA, and polymerization of this building block (Fig. 11). Lignocellulosic biomass and other waste materials are abundant and promising substrates for PHA production. Pentoses can be converted into PHA, but their utilization in hydrolysate contains mixtures of different carbohydrates (typically glucose and xylose) is still challenging since, depending on the applied pretreatment, inhibitors can compromise the performance of the microorganism (Jiang et al. 2016; Obruca et al. 2015).

The different composition of waste streams or by-product will significantly influence the choice of the biocatalyst. In cases where the raw material is rich in carbon and nutrients, a growth-associated PHA producer would be selected, such as *A. latus* or *Paracoccus denitrificans*. Conversely, in cases where the feedstock lacks an essential nutrient for growth (e.g., nitrogen), PHA accumulation using non-growth-associated bacteria would be preferred, i.e., *C. necator* (Kourmentza et al. 2017).

Certain bacteria (e.g., *C. necator, Protomonas extorquens, P. oleovorans*) produce PHA only when under nutrient (nitrogen or phosphorous) limitation. In this case, a two-stage process is preferred. In the first stage, growth is promoted, with limited accumulation of PHA, in a nutritionally balanced growth medium. In the second stage, an essential nutrient for growth is limited and the carbon flux is diverted from biomass production to PHA accumulation (Koller and Braunegg 2015). Other bacteria, like *A. latus*, mutant strain of *Azotobacter vinelandii* and recombinant *E. coli,* are able to accumulate PHA during exponential growth phase and are used in a one stage process (Kourmentza et al. 2017).

Industrial scale PHA production uses refined sugars as substrate (sugar beet, sugarcane, or corn) and pure cultures (*A. latus*, *C. necator*, and *P. putida*) (Jiang et al. 2016; Kourmentza et al. 2017). However, economic biotechnological polymer production is set back by (1) substrate cost, which can account for 50% of the total production cost, (2) low polymer titers, and (3) low process yield and productivity (Wang et al. 2014; Kootstra et al. 2017; Kaur and Roy 2015). Therefore, efforts have been made in (1) metabolic engineering to improve product yield and productivity, (2) using inexpensive and renewable carbon (and/or nitrogen) substrates, including waste and by-products from agriculture and industrial sources, and (3) process engineering to improve bioprocess efficiency, for maximum titer, yield and productivity, and for cost-effective product recovery (Wang et al. 2014; Kaur and Roy 2015; Obruca et al. 2015).

6 Conclusions

The European Union has the ambition of replacing at least 30% of the oil-based by bio-based chemicals in Europe by 2030. To achieve this goal and also meet the target on the reduction of greenhouse gases (GHG) emissions, the biological production of fuels and chemicals is mandatory. The deployment of a bio-based economy would not only help to reduce dependence on fossil-based products and lower GHG emissions but would also (1) create value by efficiently using and maximizing the potential of waste and residues; (2) boost the creation of rural and bio-based industrial employment; (3) revitalize industry in rural environment; (4) raise public awareness on the need for bio-based products; (5) decrease the amount of harsh chemicals and by-products.

Biological conversion or fermentation is one of the key processes for the conversion of renewable feedstock into drop-in (or ready to use) bio-products for the chemical industry, in a wide range of industries and a variety of applications. The global fermentation-based industry processes up to 200–250 million tons of carbohydrate equivalents annually from mono- and disaccharide-, starch- and lignocellulosic-based feedstock (Deloitte Report 2014). The economic feasibility of fermentation processes will be depending on the end-use and product value, cost of the feedstock and production cost, which is strongly influenced by the conversion yield and efficiency of product recovery. To increase the conversion yield some challenges remain. Most fermentation processes are still based on refined sugars (glucose or sucrose) and further development on the fermentation of mixed carbon sources (e.g., glucose/xylose) is still required. When processing lignocellulosic biomass, degradation compounds generated during pretreatment are typically highly inhibitory for the fermenting microorganism(s). Therefore, either cost-effective detoxification steps or, more relevant, the development of more robust strains is still required. Those strains should be able to cope with large-scale fermentation processes under non-sterile conditions, and compete effectively against microbial contaminants.

The recent technological advances in bioconversion processes are contributing to the reduction of production costs, making bio-based products more and more competitive against fossil-based alternatives, which, although produced by already well-established technologies, are becoming more and more costly due to the increase of oil prices. Still, the support from stakeholders and policymakers is essential for an effective deployment of fermentation-based processes within biorefineries as a relevant contribute towards a bio-based economy.

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