Guillermo Gosset *Editor*

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Preface

The fossil fuel era started in the 1890s; it is characterized by the widespread utilization of coal, natural gas, and petroleum. As a result, modern industrial civilization has become highly dependent on petroleum for the manufacture of fuels and chemicals. During the past one hundred years, energy and materials derived from this fossil feedstock have enabled the rapid development of several regions of the world, including sustained economic, industrial, and population growth. However, despite the usefulness of petroleum, its extraction, processing, and the combustion of fuels has resulted in severe negative consequences to the environment. Furthermore, fossil fuels are non-renewable, and their demand is constantly increasing. Therefore, it is clear that alternatives to the use of petroleum are highly desirable.

An ideal replacement for petroleum would be a raw material that is renewable, non-polluting, and does not compete with human food supplies. Most of these characteristics can be found in plant-derived biomass. Lignocellulosic agricultural residues, non-food crops, and by-products, such as glycerol, can be considered partial replacements for petroleum. One approach for generating useful products from these renewable feedstocks involves their chemical transformation by microbes. This is a major challenge since microbes do not have the natural capacities for efficiently utilizing the carbon sources from biomass and transforming them into the many products currently obtained from petroleum. Furthermore, the chemical and thermal treatments usually required to facilitate the utilization of plant-derived biomass also generates by-products that are toxic to the microbes. Therefore, the development of commercially viable processes for the transformation of renewable resources into useful products requires the genetic modification of microbes to improve their production capacity and enable them to resist toxic conditions, among other useful traits.

The objective of this book is to provide reviews on the current knowledge regarding strategies for the generation and improvement of microbial strains designed for the transformation of renewable raw materials into useful products. This book aims to become a source of reference for researchers and students working in this field. Leading experts wrote the chapters and included up-to-date

information as well as the in-depth analysis of current issues and challenges in this field. Key topics in this book include specific approaches for the engineering of thermophilic bacteria, *Saccharomyces cerevisiae*, *Escherichia coli*, and *Zymomonas mobilis*. These microbes possess particular advantages as production strains and are currently employed for the synthesis of biofuels and chemicals. The improvement of sugars and glycerol catabolism, as well as the issue of lignocellulosic hydrolysate toxicity, is addressed in several chapters, where genetic engineering and adaptive laboratory evolution strategies are discussed.

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Cuernavaca, Mexico Guillermo Gosset
October 2016

Contents

Recent Advances in Genetic Engineering of Thermophilic Ethanol Producing Bacteria

Sean Michael Scully and Johann Orlygsson

Abstract Thermophilic bacteria have gained increased interest as bioprocessing platforms for bioethanol production from second generation biomass with a particular emphasis on thermophilic clostridia species. Although thermophilic bacteria possess many advantages such as broad substrate spectra, fast growth rates, and high tolerance for environmental factors, they usually tolerate less ethanol than yeasts and produce various by-products apart from ethanol. These two factors have been addressed for several thermophilic bacteria through genetic engineering, to increase ethanol tolerance or cut off branching fermentation pathways and direct end product formation towards ethanol only. The best wild type ethanol producers belong to clostridia, particularly *Thermoanaerobacter*, *Thermoanaerobacterium*, and *Clostridium*. Additionally, non-native ethanol producers exhibiting cellulolytic properties have been genetically modified to insert genes for ethanol production pathways, as of *Caldicellulosiruptor bescii*. The scope of this chapter is on recent genetic engineering of thermophilic ethanol-producing bacteria.

Keywords Lignocellulose · Bioethanol · Extremophiles · Combined bioprocessing · Anaerobes

1 Introduction

The genetic modification of ethanol producing microorganisms is one strategy to meet increased global mandates for highly-renewable and environmentally benign liquid energy carriers. An ideal bioethanol producing organism would meet the demands of combined bioprocessing (CBP) in which the degradation of lignocellulose and the subsequent fermentation of the liberated sugars is carried out by the

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same organism in one reaction vessel (Jouzani and Taherzadeh 2015; Scully and Orlygsson 2014). As of yet, no single organism solidly meets the criteria of being a good CBP candidate although a number of thermophilic anaerobes show great potential as either being strongly ethanologenic and/or capable of degrading cellulose, starch, and hemicellulose such as xylan. While a number of cellulases are commercially available, studies have demonstrated that they do not function well under anaerobic conditions or at suboptimal temperatures (Herring et al. 2016). Genetic engineering approaches offer potential solutions to improve already ethanologenic and cellulolytic strains. While the introduction of novel genetic elements or the modification of existing elements is facile in principle, in practice, alterations of genes can disrupt other metabolic pathways having unintended results such as poor growth, low ethanol yield, or the production of unwanted side products. In this work, the genetic engineering of natively ethanologenic thermophilic anaerobes is reviewed.

Beyond the utilization of renewable substrates, achieving a high ethanol titer is critical. Historically, the wild type strains of many thermoanaerobes cannot directly compete with the ethanol titers achieved by yeasts due to poor ethanol tolerance, substrate inhibition, or lower yields because of mixed end product formation thus making these shortcomings attractive targets for genetic improvement. In order for a given fermentative organism to become economically and industrially feasible, ethanol production must be at least 40 g/L (Dien et al. 2003). Additionally, achieving a strong expression of hydrolytic enzymes is necessary.

The commercial production of bioethanol produced from biomass for many decades, has primarily been from plants rich in easily fermentable carbohydrates including starch from corn and sucrose from sugarcane. This production methodology is controversial due to the environmental issues associated with using arable land for fuel production and the fact this first generation biomass is directed to fuel production that directly competes with feed and food production. Thus, second generation bioethanol production from lignocellulosic biomass has been named as a future biomass for ethanol production. Lignocellulose originates mainly from four sources; (a) energy crops (e.g. switch grass, yellow poplar), (b) agricultural residues (e.g. corn stover, wheat straw, rice straw) as summarized in Fig. 1, (c) forest residues (woods, foliage, branches) and (d) waste (municipal solid waste, food waste) (Sánchez and Cardona 2008). However, lignocellulosic biomass is much

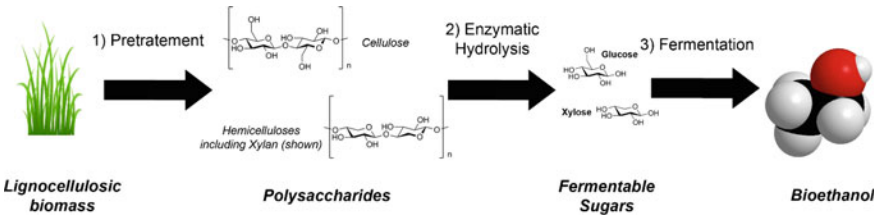

Fig. 1 Conversion of biomass to bioethanol

more complex than starch and sugars. It is composed of cellulose (glucose units linked with β-1,4-*O* glycosidic bonds), hemicelluloses (several heteropolymers composed of a variety of hexoses and pentoses including glucose, galactose, mannose, xylose, arabinose, glucuronic acid, galacturonic acid, and L-rhamnose) and lignin which is composed of randomly connected aromatic structural units (Hahn-Hägerdal et al. 2006; Sánchez and Cardona 2008). These structures are all embedded in the complex matrix of plant cells which needs to be disrupted to make polysaccharides more accessible to enzymatic degradation. Thus, for releasing the sugars present in lignocellulose and lignin removal, which cannot be used for ethanol production, the biomass needs to be pretreated and enzymatically hydrolyzed (Kumagai et al. 2014; Mosier et al. 2005). The costliest step in ethanol production from lignocellulose is the enzymatic hydrolysis (Lynd et al. 2002) making native cellulolytic ethanologens particularly attractive candidates for bioethanol production.

Ethanol production from simple substrates, such as starch and sucrose-rich crops, have traditionally been fermented by yeasts of which the best known is *Saccharomyces cerevisiae*. The main reason for using *S. cerevisiae* is that it is homoethanologenic, produces high ethanol yields (>95% of the theoretical yield) from sugars, possesses high ethanol tolerance, its cells can be recycled with ease and it has a long history in industrial production. The reader is directed to a number of recent reviews covering traditional mesophilic ethanologens (such as yeasts) and process considerations (Jouzani and Taherzadeh 2015).

Degradation of more complex biomass such as lignocellulose, however, requires microorganisms that have broader substrate spectrum due to the increased variety of sugars present. A number of thermophilic clostridia are noteworthy for their broad substrate spectra often degrading most of the hexoses, pentoses, and disaccharides liberated from complex lignocellulose biomass (Carreira et al. 1983; Herring et al. 2016; Jessen and Orlygsson 2012). The earliest applications of thermophilic anaerobes for bioethanol production date back to the late 1970s (Ben-Bassat et al. 1981; Lamed and Zeikus 1980a, b) including ethanol production directly from lignocellulosic biomass (Carreira et al. 1983; Lamed et al. 1988). High growth rates, and high ethanol titers make these bacteria attractive candidates for large-scale industrial bioethanol production. Furthermore, their broad substrate spectra make them of particular interest for the fermentation of lignocellulosic hydrolysates. However, thermophilic bacteria are not homoethanologenic and produce many other end products which lower ethanol yields, they tolerate only very low initial substrate concentrations and display relatively low ethanol tolerance. Recent work has focused on the genetic modification of ethanologenic thermophilic anaerobes to decrease carbon flow to side-products and improve the biological robustness to make these organisms powerful platforms for bioethanol production.

2 Thermophilic Ethanol Producing Bacteria

Thermophiles are classified according to their optimum growth temperature with the "lower" thermophilic boundary being widely recognized as 50 °C (Sundaram 1986). Moderately thermophilic bacteria have temperature optima between 45 and 55 °C whereas "true" thermophiles have optima between 55 and 75 °C and extreme or ("hyper") thermophiles with optimum temperature above 80 °C (Burgess et al. 2007; Wagner and Wiegel 2008). While geothermal areas often offer stability in heat and are thus favorable habitats for thermophilic bacteria (Brock 1986), they are ubiquitous and have been isolated from a wide range of habitats in which a complex community of organisms are responsible for the solubilization, degradation, and fermentation of various carbon sources to many types of end products. While it is true that there seems to be less diversity amongst strict anaerobes in geothermal habitats (Wagner and Wiegel 2008), and that many ethanologenic thermoanaerobes have broad substrate spectra, some degree of specialization does occur. Generally, most known thermophilic species are obligate or facultative anaerobes since geothermal areas have low oxygen concentrations due to the limited solubility of oxygen at elevated temperatures.

Several genera of thermoanaerobes are notable for their highly ethanologenic species within of *Clostridium*, *Thermoanaerobacter*, and *Thermoanaerobacterium* although work with the cellulolytic genera of *Caldicellulosiruptor* has been recently reported. The amount of available information regarding biofuel production from individual species varies often with only basic physiological properties being available. Furthermore, studies on lignocellulosic biomass and hydrolysates and the effect of inhibitory compounds produced as a part of the pretreatment process has not been widely reported. Selected properties of the wild types of several well-known cellulolytic or ethanologenic thermoanaerobes with potential as platforms for CBP ethanol production that have been selected for genetic modification or are outstanding ethanologens in their own right are presented in Table 1.

One of the barriers to the adoption of thermoanaerobes is the limited availability of phenotypic and physiological data. Below is a description of the best known thermophilic ethanol producing bacteria.

2.1 Clostridium

The genus *Clostridium* is a large, polyphyletic genus currently containing 211 species according to list of prokaryotes names with standing in nomenclature (LPSN) (Parte 2014). Cellulolytic and ethanologenic *Clostridium* species have been extensively investigated, particularly those which produce cellulosomes due to their potential to degrade cellulose and hemicellulose (Canganella and Wiegel 1993; Carreira et al. 1983; Demain et al. 2005). Cellulosomes as well as the functionality of specific glycohydrolase genes is reviewed in Doi and Kosugi (2004). Most

Table 1 Comparison of potential saccharolytic and ethanol-producing thermoanaerobes

Characteristic	*Caldicellulosiruptor bescii*	*Clostridium* strain AK1	*Clostridium thermocellum*	*Thermoanaerobacterium saccharolyticum*	*Thermoanaerobacter pseudoethanolicus*	*Thermoanaerobacter ethanolicus*
$T_{min}/T_{opt}/T_{max}$	40/78–80/90	45/55/60	X/65/X	45/60/70	ND/65/ND	37/69/78
Substrate spectra	Broad	Broad	Medium	Broad	Broad	Broad
Cellulose	+	–	+	–	–	–
Pectin	+	+	NR	NR	NR	NR
Starch	+	+	NR	+	+	+
Xylan	+	+	+	+	+	NR
Ethanol production	–	+	+	+	+	+
Ethanol tolerance	NR	NR	Low	NR	NR	NR
Inhibitory compound resistance	NR	NR	Low	NR	NR	NR
Commercial acceptance	No	No	No	No	No	No
Whole genome sequenced	Yes	IP	Yes	Yes	Yes	Yes
Ease of genetic modification	High	NR	Poor	High	NR	NR
Cellulase genes	+	NR	+	–	NR	–
Xylanase genes	+	+	+	+	+	+
References	Yang et al. (2010)	Orlygsson (2012)	Freier et al. (1988)	Lee et al. (1993)	Lamed and Zeikus (1980a)	Wiegel and Ljungdahl (1981)

NR not reported

Clostridium species are mesophilic and only about 15 thermophilic strains have been isolated and characterized of which most are moderately thermophilic. The most widely studied ethanologenic and thermophilic member of *Clostridium* has been *Cl. thermocellum* (Ben-Bassat et al. 1981; Lamed and Zeikus 1980a, b). Major works on *Cl. thermocellum* are reviewed in Akinosho et al. (2014). Recent studies on ethanol production from cellulose and lignocellulosic biomass were done with *Cl. thermocellum* SS21 and SS22 with yields ranging from 4.60 (corn stubs) to 8.10 mM/g hexose equivalent (sorghum stover) (Sudha Rani et al. 1998). The carbon balances for *C. thermocellum* strains are often low due to the production of other end products such as pyruvate and free amino acids (Ellis et al. 2012). Strain of *Clostridium* strain AK1, a moderate thermophile, has demonstrated ethanol yields up to 1.5 mol ethanol/mol hexose (Orlygsson 2012).

2.2 Thermoanaerobacterium *and* Caldanaerobium

Thermoanaerobacterium was first described as a new genus in 1993 when two thermophilic, xylan degrading strains were isolated from Frying Pan Springs in Yellowstone National Park in Wyoming (Cann et al. 2001). Today, the genus of *Thermoanaerobacterium* consists of 7 species while *Caldanaerobium* contains 2 species isolated from various environments (Parte 2014). These bacteria degrade a variety of sugars present in lignocellulosic biomass and produce a variety of end-products. Some species have shown promising ethanol and hydrogen production capacity, but production of mixed end products limit their use (Ren et al. 2008, 2009; Romano et al. 2010; Sveinsdottir et al. 2009). *Thermoanaerobacterium saccharolyticum* has, however, been genetically engineered and both acetate and lactate formation has been knocked out (Shaw et al. 2008). *Thermoanaerobacterium* AK_{17}, isolated from Icelandic hot spring, has been extensively studied for ethanol production (Almarsdottir et al. 2012; Koskinen et al. 2008; Sveinsdottir et al. 2009). This strain produces 1.5 and 1.1 mol ethanol from one mole of glucose and xylose, respectively. The strain has been genetically modified to knock out acetate and lactate formation (unpublished results).

2.3 Thermoanaerobacter *and* Caldanaerobacter

The genus *Thermoanaerobacter* and *Caldanaerobacter* are closely related to *Thermoanaerobacterium*. The genus was first described in 1981 when Wiegel and Ljungdahl described *T. ethanolicus*, the type species of the genus. The genus *Thermoanaerobacter* contains 15 species and 5 subspecies while *Caldanaerobacter* contains 2 species and 4 subspecies (Parte 2014). Most species within these genera have a very broad substrate spectrum but produce a mixture of end products. Earlier investigations on their ethanol production property include work on *T. brockii* and

T. thermohydrosulfuricus (Ben-Bassat et al. 1981; Lamed and Zeikus 1980a, b; Lovitt et al. 1984). Ethanol yields by *T. brockii* were only moderate or between 0.38 (Lamed and Zeikus 1980b) and 0.44 mol ethanol/mol glucose equivalents (Ben-Bassat et al. 1981). Higher yields were later observed by *T. thermohydrosulfuricus*, or 0.9–1.9 mol ethanol/mol glucose (Lovitt et al. 1984, 1988). *T. ethanolicus* showed yields ranging from 1.1 to 1.9 mol ethanol/mol glucose (Wiegel and Ljungdahl 1981). Later this strain has been extensively studied by Lacis and Lawford more than 20 years ago (Lacis and Lawford 1988a, b; 1989; Lacis et al. 1991). *T. ethanolicus* JW200 showed also very good ethanol yields from xylose and glucose at low (10 g/L) substrate concentrations, or 1.45 and 1.95 mol/mol, respectively (Carreira et al. 1982). A mutant strain was later developed [JW200Fe(4)] that showed similar yields but at higher (30 g/L) substrate concentrations (Carreira et al. 1983). Other investigations on this species on sucrose showed between 1.76 and 3.60 mol ethanol/mol sucrose with high substrate concentrations (15–30 g/L) (Avci and Dönmez 2006). A study of bacteria isolated from Icelandic hot spring shows that a *Thermoanaerobacter* strain AK33 showed good ethanol yields on monosugars (Sveinsdottir et al. 2009). Glucose and xylose fermentations resulted in 1.5 and 0.8 mol ethanol from one mole of glucose and xylose, respectively. Later studies on *Thermoanaerobacter* species isolated from Icelandic hot springs show that *Thermoanaerobacter* strain J1, was a good ethanol producer, producing 1.7 and 1.5 mol ethanol from one mole of glucose and xylose, respectively (Jessen and Orlygsson 2012). Recent studies of *T. pentosaceus* showed ethanol yields of 1.36 mol ethanol/mol consumed sugars in batch study. Immobilization in different support material in continuous culture gave better yields or 1.5 mol ethanol/mol glucose (Sittijunda et al. 2013).

2.4 Caldicellulosiruptor

The genus *Caldicellulosiruptor* contains nine species and are mostly known for their high hydrogen production capacity (De Vrije et al. 2007; Kádár et al. 2004; Parte 2014; Zeidan and van Niel 2010). All species have been isolated from geothermal environments and have relatively broad substrate spectrum capable of utilizing cellulose, cellobiose, xylan, and xylose among other substrates (Rainey et al. 1994; Yang et al. 2010). Members of the genus have emerged as candidates for CBP since they are capable of growing on biomass without conventional pretreatment (Ivanova et al. 2009). A comparative analysis of the genus revealed that there is a high degree of variability in the ability of type strain to degrade cellulose due to a diversity of glycohydrolase genes present (Zurawski et al. 2015). Ethanol production from wild type *Caldicellulosiruptor* is generally very low but *C. bescii* has been genetically modified to produce high ethanol yields (Chung et al. 2015a). Additionally, all nine of the type strains have been whole genome sequenced.

2.5 Other

Other well know thermophilic bacteria that have been investigated for ethanol production are e.g. *Caloramator boliviensis* (Crespo et al. 2012a, b) and *Geobacillus thermoglucosidasius* (Barnard et al. 2010) who has also been genetically manipulated to enhance ethanol production (Cripps et al. 2009).

3 Systems Biology for Better Understanding the Physiology of Ethanol Production

While the fermentative pathways for the degradation of carbohydrates and their subsequent fermentation to end products such as ethanol are well known, the complex interactions between other pathways are not well understood. The use of a systems biology approach including genomics, metabolomics, proteomics, and transcriptomics have been applied in recent years for understanding solvent production by thermoanaerobes. While these approaches are useful, understanding the substrate and cofactors preferences of specific gene products is dependent upon the use of old-fashioned enzymological techniques. Over the past few years, a large number of whole genome sequences for thermophilic clostridia species have become available which greatly aids our understanding of the interactions of specific pathways leading to the formation of different end product ratios. Early studies of the systems biology of anaerobes have been restricted to mesophilic bacteria, particularly *Clostridium acetobutylicum* (Durre 2009 and references therein) although several more recent studies on thermophilic anaerobes are highlighted here in the context of the physiology of thermoanaerobes.

A simple model of the production of ethanol involves the degradation of polysaccharides leading to the formation of oligo-, di-, and monosaccharides, the import of carbon into the cell, followed by the fermentation of sugars to end products as summarized in Fig. 2.

3.1 Degradation of Polysaccharides

Among thermophilic clostridia, the degradation of biopolymers such as cellulose, xylan, pectin, and starch is performed by systems of extracellular enzymes. The degradation of biopolymers by clostridia species is reviewed in Leschine (2005). Among the best-studied system is the cellulosome produced by *Cl. thermocellum* (Demain et al. 2005; Doi and Kosugi 2004). The degradation of crystalline cellulose by *Caldicellulosiruptor* species is recently reviewed in Zurawski et al. (2015). Work on *Cl. thermocellum* has revealed the presence of many glucosidases (Lagaert et al. 2014; Sizova et al. 2011). Much work has been done on the production of

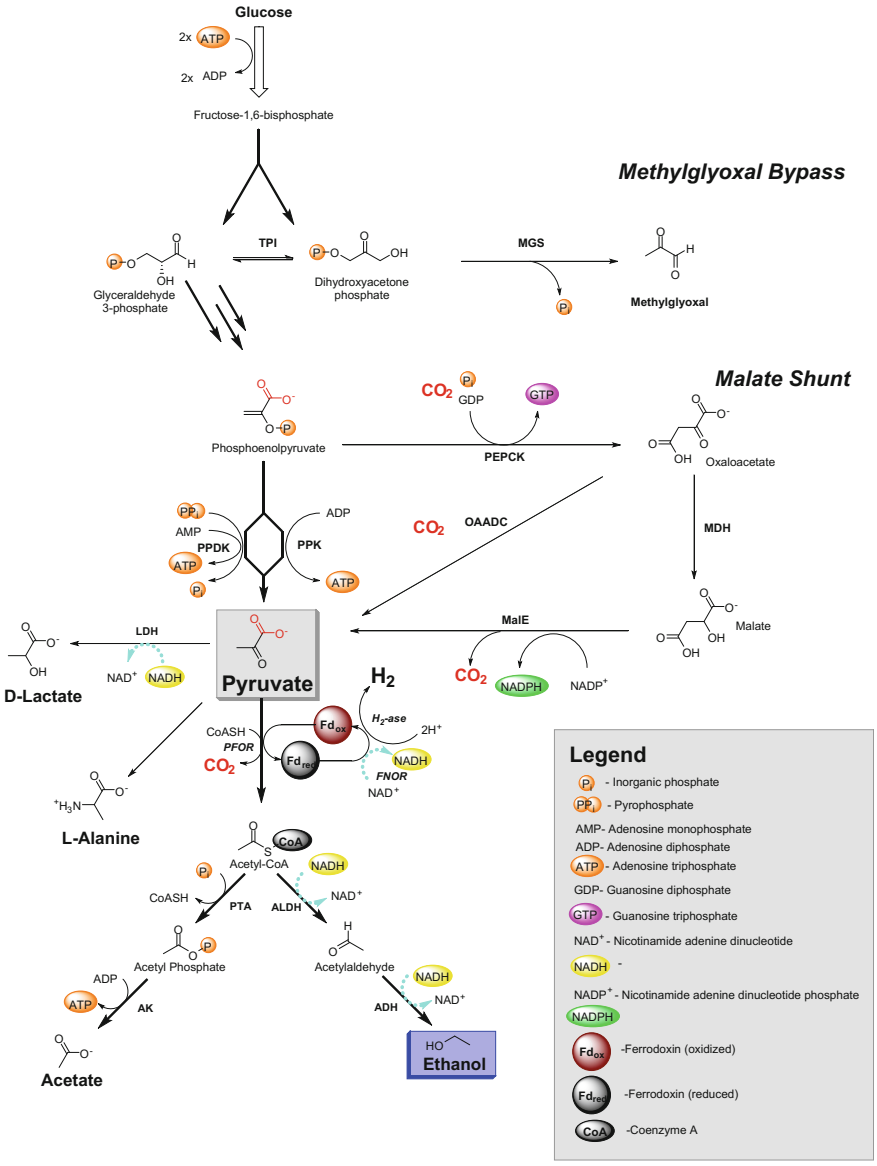

Fig. 2 Embden-Meyerhof and related pathways involved in fermentation of carbohydrates to ethanol and other end products. *TPI* triose phosphate isomerase; *MGS* methylglyoxal synthase; *PEPCK* Phosphoenolpyruvate carboxykinase; *OAADC* oxaloacetate decarboxylase; *MDH* malate dehydrogenase; *MalE* malic enzyme; *LDH* lactate dehydrogenase; *PFOR* pyruvate-ferredoxin oxidoreductase; *H_2-ase* hydrogenase; *FNOR* formate NADH ferredoxin oxidoreductase; *PTA* phosphate acetyltransferase; *ALDH* aldehyde dehydrogenase; *ADH* alcohol dehydrogenase; *AK* acetate kinase

designer cellulosomes which is beyond the scope of this review but is covered in the following references (Barak et al. 2012; Doi and Kosugi 2004; Stern et al. 2015, 2016; Vazana et al. 2013). Interestingly, a study revealed that *Cl. thermocellum* directly imports small oligosaccharide products of cellulose hydrolysis thus making *Cl. thermocellum* highly efficient at cellulose degradation despite the need to produce specialized enzymes (Zhang and Lynd 2005). While it is known that *Caldicellulosiruptor* and *Thermoanaerobacter* species often grow on cellobiose, their ability to import oligosaccharides has not been explored.

3.2 Fermentative Pathways

As mentioned above thermophilic bacteria can degrade many carbohydrates and produce various end products, among them ethanol. There is tremendous metabolic diversity among thermoanaerobes, particularly thermophilic clostridia. Central to bioethanol production is the degradation of glucose to end products. Figure 2 shows the carbon flow from glucose by fermentation by the use of Embden-Meyerhof pathway (EMP). The degradation of glucose via EMP generates two moles of NADH and pyruvate, the key intermediate in most organisms.

Several alternatives to the flow of carbon through glycolysis have been described that are relevance to ethanol production. The methylglyoxal bypass of glycolysis provides an alternative route for carbon through glycolysis; the interconversion of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate is a bottleneck particularly when glucose is rapidly catabolized or phosphate becomes limiting. Thus the conversion of DHAP to methylglyoxal enables the recovery of inorganic phosphate although generates highly cytotoxic methylglyoxal. The malate shunt can generate reducing potential as NADPH via transhydrogenation which can influence the flow of electrons to end products (Carere et al. 2012). *Clostridium thermocellum* has some atypical features in glycolysis particularly the flow of carbon through the malate shunt (Zhou et al. 2013).

Pyruvate can be converted to a variety of volatile fatty acids, predominately acetate with traces of propionate and butyrate occasionally reported, and reduced end products including hydrogen, ethanol, lactate, and alanine (Fig. 2). The distribution of end products is highly dependent upon the microorganism and the carbon source as well as the cultivation conditions. It should be noted that while alanine production has been reported among some *Thermoanaerobacter* and *Thermoanaerobacterium* species, its has not been universally reported. The presence of other reduced end products, such as 1,2-propanediol, have also been reported for *Thermoanaerobacterium saccharolyticum* (Lee et al. 1993) but specific end product concentrations were not described.

The conversion of acetyl CoA to ethanol is facilitated by alcohol dehydrogenase (ADH) or the bi-functional alcohol and aldehyde dehydrogenase (ADHE). The consumption of one mole of NADH for the reduction of pyruvate regenerates the NAD^+ required for the first steps of glycolysis thus highlighting the critical nature

of intracellular redox balance. Pyruvate can also be reduced to lactate by lactate dehydrogenase (LDH).

Understanding the correlation between the presence or absence of specific genes and observed end product distributions is critical. A meta-genomic analysis of selected Firmicutes including several thermoanaerobes, found that organisms that are regarded as good hydrogen producers (*Caldicellulosiruptor*, *Thermococcus*, *Thermotoga*, and *Pyrococcus*) lack genes coding for acetaldehyde dehydrogenases and bifunctional acetaldehyde/alcohol dehydrogenases while organisms lacking hydrogenases have higher lactate production (Carere et al. 2012).

3.3 Importance of Hydrogen Synthesis to Ethanol Production

The most thermodynamically favorable pathway for anaerobic bacteria is to oxidize pyruvate to acetyl-CoA and CO_2 by using pyruvate:ferredoxin oxidoreductase (PFOR); acetyl-CoA is then converted to acetate with the production of ATP from the acetyl-phosphate intermediate. The primary advantage for an organism to produce acetate is the formation of ATP. The electrons are transported to reduced ferredoxin which acts as an electron donor for hydrogenases and H_2 is produced as the reduced product. There are mainly two types of hydrogenases: NiFe hydrogenases and the FeFe hydrogenases. Recent overview articles have been published on the subject (Chou et al. 2008; Kengen et al. 2009). Acetyl coenzyme A can also be converted to acetaldehyde by acetaldehyde dehydrogenase (ACDH) and further to ethanol by alcohol dehydrogenase.

Strictly anaerobic bacteria generate hydrogen using either pyruvate ferredoxin oxidoreductase or NADH ferredoxin oxidoreductase while facultative anaerobes also rely upon pyruvate-formate lyase (PFL). Firstly, from a NAD(P)H by GAPDH and from pyruvate ferredoxin oxidoreductase (PFOR) (Jones 2008). The principal H_2 production pathway is through PFOR because of thermodynamics hindrance of re-oxidizing NADH (Jones 2008). It is a well-known phenomenon that the low hydrogen yields observed by mesophilic and moderate thermophilic bacteria are due to the fact that hydrogen production from either ferredoxin or NAD(P)H are thermodynamically unfavorable (Hallenbeck 2009; Jones 2008). The redox potential of Fd_{red}/Fe_{ox} couple depends on the microorganism and temperature involved; the redox potentials for relevant reactions involved in fermentations are shown in Table 2.

In nature, high partial pressures of H_2 are relatively uncommon because of the activity of H_2 scavenging microbes, e.g. methanogens or sulfate reducing bacteria (Cord-Ruwisch et al. 1988). This results in a low partial pressure of H_2 which is favorable for a complete oxidation of glucose to acetate and CO_2. At high temperatures, the influence of the partial pressure of H_2 is less on the key enzymes

Table 2 Selected redox potentials for some electron transfer reactions

Redox couple	Enzyme system	$E^{0'}$ (mV)
CO_2/formate	Pyruvate-formate lyase	−432
H^+/H_2	Hydrogenase	−414
Fd_{ox}/Fd_{red}	Ferredoxin oxidoreductase	−398
$NAD(P)^+$/NAD(P)H	Hydrogen:NAD^+ oxidoreductase	−320
FAD/$FADH_2$	Hydrogen:FAD^+ oxidoreductase	−220

Modified from Plugge and Stams (2005)

responsible for hydrogen production. This is the main reason why extremophilic bacteria have been reported to produce up to 4 mol of H_2 together with 2 mol of acetate in pure cultures and also for the fact that microorganisms growing at lower temperatures direct their end product formation to other reduced products. At lower temperatures, the oxidoreductases that convert NADH to Fd_{red} is strongly inhibited by hydrogen. The E° is −398 mV for Fd_{red}/Fd_{ox} couple but −320 mV for the NADH/NAD^+ couple (Hallenbeck 2009; Jones 2008). Therefore, at low temperatures, elevated H_2 concentrations inhibits H_2 evolution at much lower concentrations as compared to extreme temperatures.

3.4 Other Aspects of Ethanol Production

Detoxification of hydrolysates prior to fermentation is an obstacle for some organisms that can potentially be overcome with genetic engineering. 2-Furfuraldehyde (2-FF) and 5-hydroxymethylfurfuraldyde (5-HMF), produced from xylose and glucose under acidic conditions and high temperatures, respectively, are highly inhibitory for bacterial growth. A recent proteomic analysis of *Thermoanaerobacter pseudoethanolicus* 39E revealed that the addition of 2-FF and 5-HMF to the growth medium upregulated enzymes that catalyzed the reduction to their corresponding alcohols (Clarkson et al. 2014). It is worth noting that the identified gene products with this aldehyde reducing activity also showed activity towards acetaldehyde, butyraldehyde, and isobutyraldehyde.

Other factors of importance for thermophilic ethanologenic bacteria is their pH and temperature growth optimum, and their need for trace elements and vitamins often originating from complex medium supplements like yeast extract. Also, initial substrate concentrations are of importance, in general yeasts tolerate much higher (often more than 50 times) initial substrate concentration compared with thermophilic bacteria. Some thermophiles, such as *Thermoanaerobacter* strain J1 and *Thermoanaerobacterium saccharolyticum* have though been shown to tolerate quite a high substrate concentration (Altaras et al. 2001; Jessen and Orlygsson 2012). Finally, ethanol tolerance is of great importance, from an economic point of view

since low ethanol titers obtained will lead to more water usage and more power need for distillation processes. In general, thermophiles have low ethanol tolerance (often below 3% v/v). Several attempts have been made to improve ethanol tolerance in thermophilic bacteria and can be addressed in Scully and Orlygsson (2014).

4 Evolutionary Adaptation and Genetic Engineering in Thermophilic Bacteria for Ethanol Production

Recent reviews have covered the genetic engineering of thermoanaerobes for bioethanol production (Olson et al. 2015). An overview of the use of systems biology tools is covered in Mielenz and Hogsett (2010). While the authors are not aware of any specific overviews on the modification of thermoanaerobes, several relevant overviews on *Clostridium* might be of relevance (Bradshaw and Johnson 2010; Davia et al. 2005).

4.1 Evolutionary Adaptation

Classical evolutionary adaptation methods, such as non-specific mutagenesis and artificial selection, have been applied to thermophilic anaerobes on a limited basis to improve their ethanol production capacity. A major drawback of these approaches is a lack of control over which genetic changes take place. One of the major drawbacks associated with thermophilic anaerobes that must be overcome is their low substrate and ethanol tolerance compared to their mesophilic counterparts for which one solution is to adapt the strains by slowly increasing ethanol concentrations over the course of multiple fermentations or in continuous culture.

Three novel *Thermoanaerobacter ethanolicus* mutants were obtained by pyruvate and iron deprivation (He et al. 2009) leading to enhanced ethanol tolerance (10% v/v) at substrate concentrations above 10 g/L. *Clostridium thermocellum* also showed increased ethanol tolerance (up to 5% v/v) by transferring cultures stepwise to increased ethanol concentrations (Shao et al. 2011) as has *Thermoanaerobacter pentosaceus* which has been gradually adapted to higher substrate concentrations and demonstrated higher ethanol tolerance and substrate utilization (Sittijunda et al. 2013). Thus, evolutionary adaptation may still be used for evolving wild type strains with enhanced ethanol, substrate and inhibiting compound tolerance of 5-HMF and 2-furfuraldehyde. In general, less work has been done on thermophiles compared with mesophiles in this aspect.

4.2 Genetic Engineering

Genome sequencing technology and gene transfer systems have recently resulted in a new generation of engineered thermophilic ethanologens (Shaw et al. 2010, 2012). Traditional tools for genetic modification rely extensively on the use of plasmids to facilitate the introduction of genes, antibiotic markers or other reporter genes for the selection of transformants, or the natural competence of target strains.

Genetic engineering of thermophiles has mainly focused on two aspects. Firstly, on knocking out metabolic pathways leading to undesired end products (primarily lactate and hydrogen), and secondly on either inserting cellulolytic genes into non-cellulolytic microorganisms, or inserting genes for increased ethanol titers (Shaw et al. 2010). The majority of work appearing in the literature focuses on the latter approach. The first approach involves increasing ethanol yields by eliminating other fermentation products and improving ethanol tolerance whereas the second approach involves the addition of cellulolytic genes to the genome of a good ethanol producing bacterium.

It should be noted that Shaw et al. (2010) demonstrated that several strains of *Thermoanaerobacter* and *Thermoanaerobacterium* are naturally competent for genetic alteration. *Caldicellulosiruptor*, on the other hand, extensively methylates its genome making and using a system of restriction enzymes making it resistant to transformation (Chung et al. 2013a, b and references therein). This requires an improved understanding of its restriction-modification system which seems to differentiate between species (Pawar 2014).

4.3 Case Studies

Among thermoanaerobes, relatively few have been targeted for genetic modification/strain improvement. All of the strains that have been selected for genetic modification are all good ethanol producers with the exception of *Caldicellulosiruptor bescii* for which the wild type is a cellulose degrading acetate and hydrogen producer. Recent advances made with modified *Thermoanaerobacterium* and *Thermoanaerobacter strains* highlight the potential improvements that can be made to thermophilic anaerobes that are highly ethanologenic while attempts to modify *Caldicellulosiruptor bescii* provide some promise for engineering cellulolytic bacteria to produce higher ethanol titers. A summary of the ethanol yields for genetically engineered thermoanaerobes discussed below is presented in Table 3.

Table 3 Ethanol yields of genetically engineered thermophilic bacteria from different substrates and fermentation conditions

Strain	Genotype	Substrate	Substrate concentration (g/L)	Culture mode	Ethanol yields (mol EtOH/mol substrate)	Reference
Cl. thermocellum	ΔpyrF, Δpta:: gapDHp-cat	Celo	5.0	Batch	0.59	Tripathi et al. (2010)
Cl. thermocellum	ΔpyrF, Δpta:: gapDHp-cat	Av	5.0	Batch	0.71	Tripathi et al. (2010)
Cl. thermocellum	adhE*(EA), Δhpt, Δldh	Celo	5.0	Batch	0.37	Biswas et al. (2014)
Cl. thermocellum	Δhpt, Δldh, Δpta (evolved)	Av	19.5	Batch	1.08	Biswas et al. (2014)
Cl. thermocellum/*T. saccharolyticum*	Δhpt, Δldh, Δpta (evolved) and Δpta Δack Δldh	Av	19.5	Batch	1.26	Biswas et al. (2014)
T. saccharolyticum TD1	Δldh	Xyl	5.0	Batch	0.98	Biswas et al. (2014)
T. saccharolyticum ALK2	Δpta, Δack, Δldh	Celo	70.0	Cont	ND	Shaw et al. (2008)
T. saccharolyticum HK07	Δldh, Δhfs	Celo	1.8	Batch	0.86	Shaw et al. (2009)
T. saccharolyticum M0355	Δldh, Δack Δpta	Celo	50	Batch	0.44 g/g glu eq	Shaw et al. (2011)
T. saccharolyticum M0355	Δldh, Δack Δpta	Celo	50.0	Batch	1.73	Argyros et al. (2011)
T. saccharolyticum M1051	Δldh, Δack Δpta, ure	Celo	27.5	Batch	1.73	Shaw et al. (2009)
T. saccharolyticum ALK2	Δpta, Δack, Δldh	Mock HL	16.5[s1]	SSCF	1.81 glu eq	Herring et al. (2016)
T. saccharolyticum M1442	Δpta, Δack, Δldh; ΔTsac_0795, +ure, +metE	Mock HL	16.5[s1]	SSCF	1.83 glu eq	Herring et al. (2016)
T. saccharolyticum M1442	Δpta, Δack, Δldh	Mock HL	16.5[s1]	SSCF	1.72 glu eq	Herring et al. (2016)

(continued)

Table 3 (continued)

Strain	Genotype	Substrate	Substrate concentration (g/L)	Culture mode	Ethanol yields (mol EtOH/mol substrate)	Reference
T. saccharolyticum ALK2	Δpta, Δack, Δldh	PT Hardwood	12.0[s2]	SSCF	1.52 glu eq	Herring et al. (2016)
T. saccharolyticum M2886	+pta/ach-KanR into mgs gene	PT Hardwood	12.0[s2]	SSCF	1.62 glu eq	Herring et al. (2016)
T. saccharolyticum M2886	+pta/ach-KanR into mgs gene	PT Hardwood	12.0[s3]	SSCF	1.57 glu eq	Herring et al. (2016)
T. saccharolyticum M2886	+pta/ach-KanR into mgs gene	Hardwood HL	118.1[s4]	SHF	1.80 glu eq	Herring et al. (2016)
G. thermoglucosidasius TM242	Δldh^{-}, pdh up, $pflB^{-}$	Glu	34.0	Batch	1.73	Cripps et al. (2009)
G. thermoglucosidasius TM242	Δldh^{-}, pdh up, $\Delta pflB^{-}$	Glu	34.0	Batch	1.84	Cripps et al. (2009)
G. thermoglucosidasius TM242	Δldh^{-}, Δpdh up, $\Delta pflB^{-}$	Xyl	29.0	Batch	1.37	Cripps et al. (2009)
T. mathranii BG1L1	Δldh	Wheat straw	30–120	Cont.	1.53–1.67	Georgieva et al. (2008)
T. mathranii BG1G1	Δldh, GldA	Glu + gly	5.0	Batch	1.68	Yao and Mikkelsen (2010b)
T. mathranii BG1G1	Δldh, GldA	Xyl + gly	5.0	Batch	1.57	Yao and Mikkelsen (2010b)
T. mathranii BG1G1	Δldh, GldA	Xyl + gly	12.8 and 7.2	Cont.	1.53	Yao and Mikkelsen (2010b)
Thermoanaerobacter Pentocrobe 411	Δldh, Δack Δpta	Wheat straw	65[x]	Cont.	1.84 sug eq	Andersen et al. (2015)

(continued)

Table 3 (continued)

Strain	Genotype	Substrate	Substrate concentration (g/L)	Culture mode	Ethanol yields (mol EtOH/mol substrate)	Reference
Thermoanaerobacterobacter Pentocrobe 411	Δldh, Δack Δpta	Wheat Straw	137[x]	Cont	1.88 sug eq	Andersen et al. (2015)
Thermoanaerobacter Pentocrobe 411	Δldh, Δack Δpta	Wheat straw	77[x]	Cont	1.84 sug eq	Andersen et al. (2015)
Thermoanaerobacter Pentocrobe 411	Δldh, Δack Δpta	Birch	86[x]	Cont	1.84 sug eq	Andersen et al. (2015)
Thermoanaerobacter Pentocrobe 411	Δldh, Δack Δpta	Corn cob	134[x]	Cont	1.84 sug eq	Andersen et al. (2015)
Thermoanaerobacter Pentocrobe 411	Δldh, Δack Δpta	Cane bagasse	436[x]	Cont	1.88 sug eq	Andersen et al. (2015)
Thermoanaerobacter Pentocrobe 411	Δldh, Δack Δpta	Cardboard	56[x]	Cont	1.84 sug eq	Andersen et al. (2015)
Thermoanaerobacter Pentocrobe 411	Δldh, Δack Δpta	Biowaste	75[x]	Cont	1.88 sug eq	Andersen et al. (2015)
Thermoanaerobacter Pentocrobe 411	Δldh, Δack Δpta	Oil palm EFB	190[x]	Cont	1.84 sug eq	Andersen et al. (2015)
Thermoanaerobacter Pentocrobe 411	Δldh, Δack Δpta	Oil palm frond	130[x]	Cont	1.92 sug eq	Andersen et al. (2015)
Thermoanaerobacter Pentocrobe 411	Δldh, Δack Δpta	Cane syrup	868[x]	Cont	1.84 sug eq	Andersen et al. (2015)
Thermoanaerobacter Pentocrobe 411	Δldh, Δack Δpta	Cane molasses	878[x]	Cont	1.88 sug eq	Andersen et al. (2015)

(continued)

Table 3 (continued)

Strain	Genotype	Substrate	Substrate concentration (g/L)	Culture mode	Ethanol yields (mol EtOH/mol substrate)	Reference
C. bescii JWCB018	Δldh^-	Celo	10	Batch	0	Chung et al. (2014)
C. bescii JWCB032	Δldh^-, $adhE^+$	Celo	10	Batch	0.66	Chung et al. (2014)
C. bescii JWCB049	ΔpyrFA, Δldh^-	Celo	10	Batch	0.54[a]	Chung et al. (2015a)
C. bescii JWCB054	ΔpyrFA, Δldh^-	Celo	10	Batch	0.28	Chung et al. (2015a)

ack acetate kinase; *GldA* glycerol dehydrogenase A; *hfs* hydrogenase; *hpt* hypoxanthine phosphoribosyl transferase; *ldh* lactate dehydrogenase; *pdh* pyruvate decarboxylase; *pyrF* orotidine-5-phoshate decarboxylase; *pta* phosphotransacetylase; *pfl* pyruvate formate lyase; *ure* urease; *ΔTsac_0795* possible helicase or protein kinase, *metE* methionine synthase; *mgs* methylglyoxal synthase; *celo* cellobiose; *Av* avicel; *Xyl* xylose; *Glu* glucose; *Gly* glycerol; *SSCF* simultaneous saccharification and co-fermentation; *SHF* simultaneous saccharification and fermenation; *Batch* batch culture; *Cont* continuous culture

[a]Calculated per mole cellobiose; [s1]on a % solids basis (100 g/L cellulose, 20 g/L glucose, 35 g/L xylose); [s2]on a # solids basis (65.5 g/L cellulose, 1.0 g/L glucose, 13.9 g/L xylose, 3.8 g/L other sugars); [s3]on a # solids basis (65.5 g/L cellulose, 1.0 g/L glucose, 13.9 g/L xylose, 3.8 g/L other sugars; [s4]total sugar basis (88.6 g/L glu, 24.3 g/L xyl, 5.2 g/L other sugars); [x]on a total sugar basis

4.3.1 *Thermoanaerobacterium saccharolyticum*

The first thermophilic bacterium to be genetically engineered to enhance ethanol production was *Thermoanaerobacterium saccharolyticum* in 2004 (Desai et al. 2004). The species has a very broad substrate spectrum, degrading for example starch, xylan, glucose, cellobiose, xylose, arabinose, mannose and galactose. As for other members of *Thermoanaerobacterium* they cannot degrade cellulose (Lee et al. 1993). The sugars are degraded to a wide array of end products, including ethanol, acetate, lactate, carbon dioxide, and hydrogen.

From the early work on *T. saccharolyticum* using electroporation and shuttle vectors (Rachek et al. 1997), this strain has been further modified by inserting a cellobiohydrolase gene from *Clostridium thermocellum* into its genome (Biswas et al. 2014). Later work with *T. saccharolyticum* involved an *ldh* gene knock out (Desai et al. 2004; Shaw et al. 2008). Elimination of acetate production was then established by knocking out genes responsible for phosphotransacetylase (PTA) and acetate kinase (AK) and the resulting strain (ALK1) produced ethanol with 90–100% of the theoretical yield. Knocking out acetate formation leads to less available energy and thus less cell biomass and increased ethanol yields, both from glucose and xylose. Strain ALK2 was obtained by cultivating strain ALK1 in continuous culture for almost four months with increasing substrate loadings. This strain produced up to 33 g/L of ethanol corresponding to 92% of the theoretical yields and ethanol productivity rate of 2.2 g/L/h. Another double knock-out of *T. saccharolyticum* focused on the electron transfer system of the bacterium (Mai and Wiegel 2000). The authors deleted the *hfs* gene cluster and *ldh* gene which encode for a hydrogenase and LDH, respectively. Again, a considerable increase in ethanol (44%) production was obtained as compared with the wild type.

Shaw et al. (2011) reported the development of a markerless strategy using haloacetic acid as the selective agent to generate strain M0355 which lacked *ldh*, *pta*, and *ack* genes and produced high ethanol titers on cellobiose, up to 1.72 mol ethanol/mol glucose. The use of a markerless strategy is advantageous because it does not rely upon antibiotic resistance as a selective agent and enables multiple modifications for further strain improvement.

Finally, in a recent study of *T. saccharolyticum* strain M2886, the genes for exopolysaccharide synthesis were removed, the regulatory gene *perR*, and re-introducing phosphotransacetylase and acetate kinase into the methylglyoxal synthase gene (Herring et al. 2016). Also, this strain was subjected to multiple rounds of adaptation and selection, resulting in mutations later identified by resequencing. This strain produced 70 g/L of ethanol in batch on cellobiose and maltodextrin. These are the highest titers obtained by a thermophilic bacterium. Ethanol concentration on hardwood obtained by this strain was 26 g/L.

4.3.2 *Clostridium thermocellum*

Clostridium thermocellum is a cellulolytic bacterium producing a mixture of ethanol, acetate, lactate, hydrogen and carbon dioxide (Lynd et al. 1989). Its capability of hydrolyzing cellulose and producing ethanol has led to intensive investigations of this bacterium. The first successful transformation of the species was performed in 2006 (Tyurin et al. 2006), later on leading to the development of genetic systems to knock out the *pta* gene and thus acetate formation (Argyros et al. 2011). This strain, however, grew abnormally but retained its cellulase activity. At first, this strain did not produce more ethanol compared with the wild type but subsequent serial transfer resulted in 5.6 g/L of ethanol (52% of theoretical yields). Later work on *Cl. thermocellum* showed improved ethanol yields in a Δ*hpt*, Δ*ldh*, Δ*pta* evolved strain as well as the successful use of co-culture of this strain with *Thermoanaerobacterium saccharolyticum* (Argyros et al. 2011). Another approach to increase ethanol production was made by disrupting the sporulation pathway gene (*spo0A*) and then deletion of both *ldh* and *pta* genes (van der Veen et al. 2013). Thereafter the strain was adapted in continuous culture resulting in 29% of the theoretical yield.

4.3.3 *Thermoanaerobacter mathranii*

Thermoanaerobacter mathranii was isolated from an Icelandic hot spring (Larsen et al. 1997) and has later been modified and used in several investigations. The wild type produces from 62 to 90% of ethanol theoretical maximum (Georgieva et al. 2008; Yao and Mikkelsen 2010a, b) depending on the carbon source given. The first mutant generated was BG1L1 by knocking the LDH gene out of the wild type. This strain showed more than two-fold increase in ethanol production as compared to the wild type, up to 1.52 mol ethanol/mol xylose (Yao and Mikkelsen 2010b). The strains have also been shown to have similar ethanol yields from undetoxified pretreated corn stover and wheat straw (Georgieva and Ahring 2007; Georgieva et al. 2007, 2008). Further manipulation of this strain involves overexpression of NAD(P)H-dependent bi-functional aldehyde/ADH, resulting in the strain BG1E1. Clearly, this enzyme is of great importance for ethanol production and its over-expression resulted in higher ethanol yields (Crespo et al. 2012a, b). The electron balance for sugar degradation was also examined using mannitol, which is more reduced than glucose and xylose, as a substrate (Yao and Mikkelsen 2010a) leading to more ethanol production. Another *Thermoanaerobacter mathranii* strain was developed, BG1G1 where the gene encoding for NAD^+-dependent glycerol dehydrogenase was inserted. This increased ethanol production by 40% as compared to the wild type. Additionally, the strain utilized the highly reduced glycerol and co-metabolism of glycerol and sugars.

4.3.4 *Thermoanaerobacter italicus*

A recent paper using a lactate dehydrogenase, phosphotransacetylase and acetate kinase knockout strain of *Thermoanaerobacter* BG1, dubbed Pentocrobe 411 (DSM 23015), was recently described by Andersen et al. (2015). Pentocrobe 411 achieved impressive ethanol titers (1.84–1.92 mol ethanol/mol sugar equivalent) nearing the maximum theoretical yield from hexoses and pentoses on various pretreated biomass in continuous culture.

4.3.5 *Geobacillus thermoglucosidasius*

Thermophilic bacteria within the genus of *Geobacillus* have also attracted increased interest due to their ethanol production capacity recently. These bacteria are facultative anaerobes and can ferment various sugars to pyruvate by pyruvate dehydrogenase to acetyl-Coenzyme A (Cripps et al. 2009). Under aerobic conditions, however, pyruvate formate lyase is used and a variety of end products are formed. Cripps et al. (2009) manipulated *Geobacillus thermoglucosidasius*, obtaining an upregulated expression of pyruvate dehydrogenase under anaerobic conditions in lactate dehydrogenase-inactivated strain. Several mutants were developed (TM89; *ldh* knockout; TM180; *ldh* knockout and upregulated *pdh*; TM242; *ldh*, *pdh* up, and *pfl*). The TM180 strain produced 1.45 mol ethanol/mol hexose (the wild type produced 0.39 mol ethanol/mol hexose and TM89 0.94 mol ethanol/mol hexose). The triple mutant TM242 produced 1.65 mol ethanol/mol hexose. This mutant also showed good yields on xylose (1.33 mol/mol) and good productivity rates. *Geobacillus thermoglucosidasius* has recently be genetically modified by expressing pyruvate decarboxylase from *Gluconobacter oxydans* (Van Zyl et al. 2014). Ethanol yields obtained were as high as 1.37 mol ethanol/mol glucose.

4.3.6 *Caldicellulosiruptor bescii*

New genetic tools have been used recently for insertion, deletion and heterologous/homologous expression of genes in *C. bescii* with considerable success (Chung et al. 2013a, b, 2014, 2015a, b).

One species, *C. bescii* has been genetically engineered to shift end product formation towards ethanol production (Cha et al. 2013). The wild type produces mainly lactate, acetate and hydrogen as main end products. Some members within the genus produce small amounts of ethanol but *C. bescii* does not. Recently, the species was targeted for gene deletion when the gene coding for lactate dehydrogenase (LDH) was deleted (Cha et al. 2013). The *ldh* gene was deleted by constructing a non-replicating plasmid which was introduced into the *C. bescii* chromosome by marker replacement. The resulting strain did not produce any lactate but instead increased acetate and hydrogen by 21–34% when cultivated on sugars (cellobiose and lactose) and on switch grass. Also, the authors noticed

increase in biomass formation because of the extra ATP produced via acetate production. Later work on this strain was performed to introduce ethanol production pathways (Chung et al. 2014). The authors introduced the NADH-dependent *adhE* gene from *Clostridium thermocellum* to the *ldh* mutant (JWCB018) resulting in strain *C. bescii* JWCB032. The wild type of *C. bescii* is very tolerant for ethanol concentrations and shows no inhibition up to 13.8 g/L. The wild type produced lactate (3.1 mM) and acetate (5.4 mM) from the three tested substrates (cellobiose, avicel, switchgrass). Not surprisingly, the *ldh* mutant produced only acetate and hydrogen from these substrates, but acetate concentrations increased. The ldh^{-} $adhE^{+}$ mutant produced lower yields of acetate (4.3 mM) and redirected the carbon flux to ethanol (14.8 mM from 29.2 mM cellobiose). This strain did however only use 4.4 mM of cellobiose, but ethanol yields were high, or 1.67 mol ethanol/mol glucose. This strain did however not produced ethanol above 65 °C, most likely since the alcohol dehydrogenase genes originate from a moderate thermophile. More recent efforts have therefore been to introduce *adhB* and *adhE* genes from a true thermophile, *Thermoanaerobacter pseudoethanolicus*, to the genome of the *ldh* mutant (Chung et al. 2015a). The two mutant strains obtained produced ethanol at higher temperatures, although yields were considerably lower as compared with the JWCB032 (the ldh^{-} $adhE^{+}$ mutant). The *adhB* mutant produced 1.4 mM ethanol on avicel, and 0.4 mM on switch grass as well as acetate (13.0 mM and 15.7 mM on avicel and switch grass, respectively). The *adhE* mutant produced 2.3 and 1.6 mm of ethanol on avicel and switch grass, respectively and 12.3 and 15.1 mM of acetate on avicel and switchgrass, respectively. The authors speculate that the main limiting factor for ethanol production is the availability of cofactors. The *C. thermocellum* mutant containing *adhE* utilizes NADH as an electron donor for both acetyl-CoA reduction to acetaldehyde (ALDH) and acetaldehyde reduction (ADH) to ethanol. *T. pseodoethanolicus adhB* and *adhE* however both utilize NADPH for ADH activity and adhB also uses NADPH for acetyl-CoA thioesterase activity. The authors hypothesize that low electron flux through NADPH could be the limiting factor for ethanol production since the AdhE pathway needs one NADPH per mole ethanol produced, while the AdhB pathway requires one NADPH. This difference then explains the different ethanol yields between the two *adh* mutants tested.

5 Challenges and Future Directions

Overall, efforts to engineer thermophilic anaerobes to increase ethanol titers has resulted in modest gains in yields while minimizing or eliminating the formation of unwanted end products. Future targets for genetic manipulation might include the inclusion of the cellulolytic machinery of *C. thermocellum* into highly ethanologenic *Thermoanaerobacter* and *Thermoanaerobacterium* strains. The relatively low tolerance of thermoanaerobes to inhibitory compounds formed under high temperatures and acidic conditions remains a major challenge for ethanol production from lignocellulosic biomass. While some work indicates that some strains can

convert inhibitory aldehydes to other less toxic compounds, the inclusion of genes responsible for these enzymes could make a promising tool to improve the already impressive ethanologenic bacteria.

The main reason for the increased interest of using thermophiles for ethanol production is their ability to degrade broad array of substrates present in lignocellulosic biomass. Some of these bacteria, e.g. *Clostridium* and *Caldicellulosiruptor* are cellulolytic whereas others like *Thermoanaerobacter* and *Thermoanaerobacterium* are not. *Clostridium thermocellum* has been investigated and genetically manipulated to increase ethanol titer by eliminating production of acetate. The wild type of *Caldicellulosiruptor bescii* does not produce ethanol, but the insertion of ethanol producing genes has shown promising results. Most work on genetic engineering of thermophilic ethanol producing bacteria has been towards eliminating by-product formation in naturally good ethanol producers like *Thermoanaerobacter* and *Thermoanaerobacterium* that are not cellulolytic. Less work has been on inserting genes to make these bacteria cellulolytic. Often, manipulating fermentative pathways results in undesired consequences, e.g. when cutting off acetate formation the bacteria will get less energy and become less stable. Interestingly, many thermophiles that have been regarded as moderate ethanol producers and can be manipulated to produce more ethanol by simply increasing the partial pressure of hydrogen that will direct the flow of electrons to more reduced end products like ethanol and lactate. This type of microorganism would be ideal by simply cutting out the lactate formation pathway.

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Systems Metabolic Engineering of *Saccharomyces cerevisiae* for Production of Biochemicals from Biomass

Luis Caspeta and Tania Castillo

Abstract In the production of biofuels and chemicals from biomass-derived sugars, the yeast *Saccharomyces cerevisiae* has emerged as a key microbial host. Producing these biochemicals in yields and productivities satisfactory to be useful for establishing a cost-effective production process requires the engineering of the yeast's metabolism. This is a challenging mission since metabolic pathways are intriguingly connected with genetic regulatory circuits, and we are just deciphering these networks. However, global technologies of systems biology in combination with the adequate design capabilities of synthetic biology, and random or rational mutagenesis through adaptive laboratory evolution have emerged to improve our understanding of basic aspects of yeast cellular processes and come up with proper metabolic engineering strategies (the systems metabolic engineering approach). In this chapter, we will review recent advances in systems metabolic engineering of *S. cerevisiae* for production of biofuels and commodity chemicals from lignocellulosic biomass.

Keywords Systems metabolic engineering · *Saccharomyces cerevisiae* · Biochemicals · Biomass

1 Introduction

Matters of pollution and the environment, as well as of petroleum economy play a key role in the increasing interest for the production of fuels and commodity chemicals from lignocellulosic biomass. Another stimulus is the fact that this type of biomass can be renewed at a rate close to 55 Petagrams (Pg) of carbon per year (Field 1998; Barber 2009), which is around twelve times higher than the ~4.5 Pg

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of petroleum carbon currently in use. Of the different methods for biomass conversion, the microbial fermentation of biomass-derived sugars will offer a huge diversity of molecules that can be used as biofuels and commodity chemicals (Rabinovitch-Deere et al. 2013; Straathof 2014). Nevertheless, microbes were not naturally shaped to satisfy practical requirements of commercial industrial applications. In particular, they cannot produce a single molecule with high yield (grams of product produced per gram of biomass) and productivity (grams of product produced per hour and fermentor volume). However, the scientific and technological advances in biosciences and bioengineering, as well as the progress in methods to delete and transfer genes, modulate gene expression and to study biological systems in a holistic manner, have been motivating metabolic engineers to take microbial production to its full capacity (Stephanopoulos 2007; Nielsen and Jewett 2008; Lee et al. 2011; Rabinovitch-Deere et al. 2013).

Of the different microorganisms utilized for the biosynthesis of biofuels and chemicals (Lee et al. 2011; Rabinovitch-Deere et al. 2013), this review focuses on *Saccharomyces cerevisiae* since this one is a repository of many scientific and technological advances allowing for systems metabolic engineering. This yeast has an unprecedented recognition in industrial fermentations, holds the generally recognized as safe (GRAS) designation, and is the repository of a large repertory of recombinant DNA technologies. Furthermore, the scientific community possesses a thorough knowledge of its biochemistry and genetics, and genomic revolution has provided with a new array of methods for studying the yeast system in a holistic manner. The combination of these factors with the advances in metabolic engineering (Bailey 1991; Stephanopoulos 1999; Nielsen et al. 2001) have been used for developing yeast strains for cost-efficient production of diverse biochemicals from biomass.

The basic operations of a biomass-to-biochemicals conversion process are shown in Fig. 1. Once harvested, lignocellulosic biomass is chopped and pretreated to disrupt the tangled structure of cellulose, hemicellulose and lignin. Pretreatment methods include diluted acid, ammonia fiber expansion, and steam explosion (Hahn-Hägerdal et al. 2006). After pretreatment, cellulose and hemicellulose are digested to release six- and five-carbon sugars, primarily glucose and xylose (Caspeta et al. 2014a). Naturally, *S. cerevisiae* cannot metabolize xylose which comprises up to $\sim$30% of total lignocellulosic biomass. Byproducts such as acetic acid, furfural, hydroxyl-methyl-furfural (HMF), and phenolics can also be released during pretreatment. These chemicals impair yeast metabolism, resulting in a reduction of yields and productivities of targeted biochemicals (Palmqvist and Hahn-Hägerdal 2000; Caspeta et al. 2015). Fermentation of biomass-derived sugars to the desired product is the last step before downstream processing. High product titers (grams of product per fermentor volume) are required to reduce efforts in the last step of the process. Ethanol fermentation at high yields, titers and productivities is relatively simple since yeast naturally synthesizes this in response to the presence of glucose or low oxygen concentrations. However, restructuration of yeast metabolic and regulatory networks to overproduce other biochemicals besides ethanol is a key challenge.

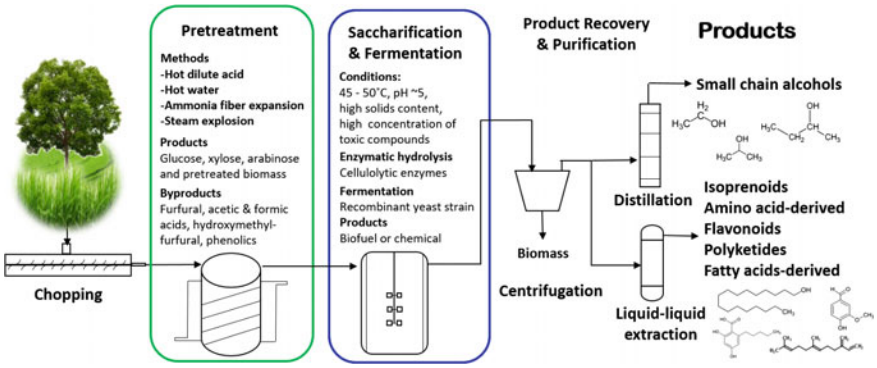

Fig. 1 Representation of the general unit operations for the conversion process of lignocellulosic biomass to biofuels and chemicals

Metabolic engineering is useful for overcoming yeast obstacles in biomass-to-biochemicals conversion processes. This integrates detailed biological information into graphic and mathematical representations of metabolic and regulatory networks and then used these to seek for cellular functions which constrain either, the overproduction of a biochemical, or the development of the desired performance. A standard metabolic engineering strategy is cyclic (Fig. 2), initiating with the analysis of biological information obtained from previous experiments, published data and/or in silico simulation (Park et al. 2008). Mathematical modeling of metabolic networks can be used for calculating the conversion yield of a biochemical, as well as for targeting network elements constraining its overproduction. The detected constrains are then released by modifying genetic networks via recombinant DNA technologies—including the chance to insert heterologous genes and regulatory elements (Bailey 1991; Nielsen 1998; Stephanopoulos 1999). Cycles of these activities are performed while the targeted yield or productivity is not reached. Commonly, constrains releasing tasks in cells are based on the overexpression of genes encoding the rate-limiting enzymes in the biosynthesis pathway; knockout or down-regulation of genes encoding the enzymes of competing metabolic pathways; heterologous expression of genes completing non-natural pathways in the host strain; and engineering of enzyme functions (Bailey 1991; Yang et al. 1998; Nielsen 1998; Stephanopoulos 1999).

Genome sequencing and associated technologies for functional annotation of genes certainly bring the study of *S. cerevisiae* to a systems level. After sequencing its genome (Goffeau et al. 1996), the first generation of DNA microarrays and associated computational algorithms for transcriptomic analysis appeared (DeRisi et al. 1997; Eisen et al. 1998). These allowed for the analysis of genes functions through global gene expression analyses of mutants and wild-type yeast strains under various internal and external changes (Gasch et al. 2000; Boer et al. 2003).

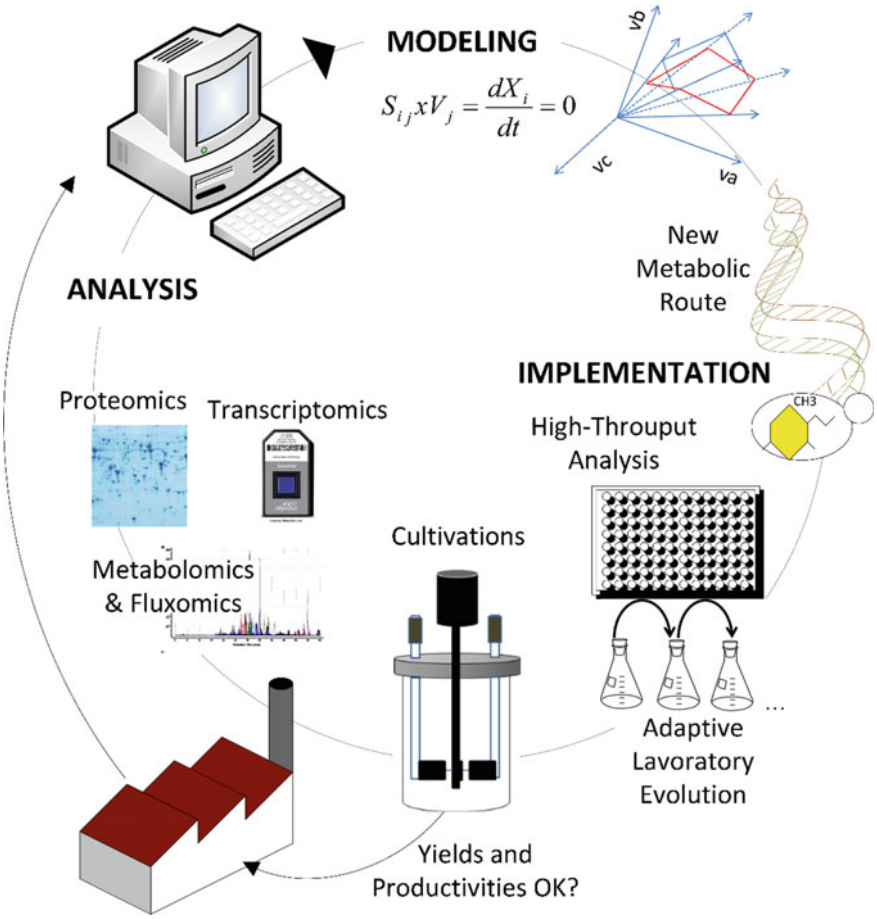

Fig. 2 General procedure of the use of systems metabolic engineering for yeast strains improvement

Also, an extensive analysis of genes functions through gene deletions and proteins localization become available (Winzeler et al. 1999; Giaever et al. 2002; Huh et al. 2003). The abundance of basic biological information accumulated in the pre- and post-genomic era required integrative platforms, thus leading the reconstruction of the first genome-scale metabolic model (GEM) (Förster et al. 2003a). Development of GEMs was accompanied by an expansion of computational algorithms and methods which allow GEMs to perform multiple tasks (Park et al. 2009; Thiele and Palsson 2010; Osterlund et al. 2012), such as: integrating 'omics' data, performing metabolic flux analysis, analysis of signaling and regulatory networks, predicting cell growth and gene essentiality, comparing gene functions among different species, and seeking for target gene functions for metabolic engineering.

Frequently, the complexity of metabolic and molecular interactions in a cellular system cannot be captured in a model. When that happen, the rational application of metabolic engineering through modeling of metabolism and recombinant DNA technologies is not likely. In this case, the application of evolutionary engineering approaches, which follow nature's engineering principles of variation and selection, is used as a complementary strategy for strain development (Sauer 2001; Dragosits and Mattanovich 2013). This method exploits in vivo recombination through evolution of populations aiming that the generated phenotype is coupled with the genotype. One can further know, through 'omics'-based characterization, the genetic changes leading the desired phenotypic response, and transfer them to the desired microbial host (Dettman et al. 2012; Caspeta et al. 2014b). This approach is called inverse metabolic engineering.

In this chapter, we provide a short description of concepts end methods used in the systems metabolic engineering, and what this platform is useful for realizing the full potential of *S. cerevisiae* as a cell factory for converting biomass-derived sugars into biofuels and chemicals. We also give information about the applications of systems metabolic engineering to overproduce natural and non-natural chemicals and biofuels from biomass with this yeast.

2 Systems Metabolic Engineering Tools and Methods

Classical physiological studies and quantitative analyses of metabolism have been supporting traditional methods for targeting gene manipulations, such as: metabolic flux analysis (MFA), metabolic control analysis (MCA), thermodynamic analysis of pathways, and kinetic modeling (Nielsen 1998; Stephanopoulos 1999). The MFA approach is the simplest but very powerful method. This is based on a stoichiometric model constructed with metabolic coefficients of participating reactions. Extracellular metabolic fluxes are used for the calculation of Internal fluxes by applying mass balances in intracellular metabolites. However, the integration of measured internal fluxes with ^{13}C-enriched carbon sources improves MFA predictions (Stephanopoulos 1999). The advent of new concepts and methods molded by the genomic age upgraded traditional MFA to a systems analysis of metabolic fluxes through GEMs.

2.1 Genome-Scale Metabolic Models (GEMs) of S. cerevisiae

As any global reconstruction of cellular metabolism, the GEMs of *S. cerevisiae* were reconstructed with the annotation of its genome sequence, and copious experimental evidence on metabolic reactions, pathways and associated genes

(Osterlund et al. 2012). A protocol for generating high-quality GEMs has been published (Thiele and Palsson 2010). The first model draft is structured with stoichiometric reactions compiled from gene annotation data (e.g. E.C. numbers of enzyme-coding genes). Extensive information published in literature is then used to ensure the validity of the information contained in the model. The curated GEM is then examined on its ability to connect metabolic reactions through the synthesis of biomass and relevant byproducts from typical elements of the culture media. Further introduction of non-native reactions is required to represent heterologous pathways. After checking the connectivity, the GEM is converted into a computational format represented by a matrix S of stoichiometric coefficients arranged in rows and columns, representing N reactions and M metabolites. This representation enables innumerable computational biological studies, such as the valuation of network content and capabilities, testing and generation of hypotheses, phenotypes analyses, and metabolic engineering (Thiele and Palsson 2010).

Today, there are not less than ten GEMs of *S. cerevisiae* (Osterlund et al. 2012; Aung et al. 2013). The very first one, called iFF708 (Förster et al. 2003b), contains 1175 reactions, 584 metabolites, 3 compartments, and 708 genes (comprising $\sim$16% of total yeast genome). Simulations with this model proved its value for predicting experimental values of the specific rates of glucose and oxygen consumption, and biomass, CO_2 and ethanol production, as well as the impact of single gene deletions on cell growth and metabolic shift in anaerobic/aerobic glucose-limited continuous culture; in addition to the correlation between metabolic shift and gene expression (Famili et al. 2003). The iFF708 model was fully compartmentalized (Duarte et al. 2004) and used as a scaffold to generate the iIN800 model, which covers a larger extension of lipid metabolism, thus containing 1446 reactions, 1013 metabolites, and 800 genes (Nookaew et al. 2008). The first model containing regulatory information based on genes interactions with 55 transcription factors (TFs) was the iMH805/775 GEM. This was useful to predict growth and gene expression profiles upon deletions of TFs in different *S. cerevisiae* in silico and experimental strains (Herrgård et al. 2006b). The reconstruction of additional models brought the necessity to make a consensus yeast GEM called Yeast 4.0 (Dobson et al. 2010), which contains 16 compartments and 924 genes. GEM-based analyses and their utility in metabolic engineering projects are described below and in Table 1.

2.2 GEM-Based Analysis of Targets for Genetic Manipulations

Building a comprehensive GEM of a whole organism and using this in simulations has several limitations because we do not know all the biological information (Palsson 2000)—including for *S. cerevisiae*. However, if internal and external environmental constraints limiting particular cell behaviors are known (e.g. systems

Table 1 Examples of systems metabolism engineering tools for developing yeast strains

Metabolic engineering tools	Approach	Reference
iFF708/FBA	Reduce NADH accumulation to decrease glycerol synthesis and increase ethanol and biomass production	Bro et al. (2005)
iTO977/FBA/ALE/genome sequence	Metabolic engineering for the production of 3-Hydroxypropionic acid production based on ALE experiments and genome sequence	Kildegaard et al. (2014)
iMM904/Dynamic FBA	Improve xylose and arabinose metabolism by metabolic engineering of redox potentials	Ghosh et al. (2011)
iND750/MADE	Integration of gene expression data into the GEM to generate models that can predict gene expression through metabolic adjustments. This GEM accurately predicted gene expression upon transition from fermentation to respiration	Jensen and Papin (2011)
iMM904/MOMA	Metabolic engineering for the production of terpenoids through the knockout of genes encoding enzymes of competitive pathways	Sun et al. (2014)
iFF708/MOMA/OptGene	Metabolic engineering for the production of sesquiterpenes via the mevalonate pathway	Asadollahi et al. (2009)
iFF707/MOMA/OptGene/OptKnock	The GEM was implemented with the heterologous reactions for vanillin biosynthesis and used to generate targets for gene manipulation leading vanillin overproduction	Brochado et al. (2010)
iFF708/OptGene/ALE/transcriptomic data	Rounds of GEM simulations and ALE were used in combination with global expression analyses to engineer succinate metabolism (yield increased by 40 times)	Otero et al. (2013)
iFF708/OptKnock/GDLS	Metabolic engineering of L-tyrosine production from chorismate. Prediction of multiple gene manipulations	Cautha et al. (2013)
iND750/iND850/TIGER/transcriptomic data	Evaluation of GEM/transcriptomic inconsistencies within previous models	Jensen and Papin (2011)
iND750/FBA/transcriptomic data	Improving external metabolic fluxes prediction during ethanol production from glucose. Targeting of gene modifications	Guo and Feng (2016)
ALE	Metabolic engineering of xylose metabolism to improve 1-hexadecanol production over 1.2 g/L	Guo et al. (2016)
ALE/iIN800/FAB	Inverse metabolic engineering of yeast thermo-tolerance. Thermotolerance increase until 50 °C	Caspeta et al. (2014b)
ALE	Metabolic engineering of xylose transport through evolutionary engineering of glucose transporters	Farwick et al. (2014)
ALE/genome sequence	Inverse metabolic engineering of yeast tolerance to high concentrations of 3HP and acidic conditions	Kildegaard et al. (2014)
ALE/genome sequencing	Inverse metabolic engineering to generate strains with increased consumption of xylose under anaerobic conditions	Sonderegger et al. (2004)
Proteomic and transcriptomic data	Define molecular changes leading yeast resistance to furfural for targeting gene manipulations	Lin et al. (2009)

stoichiometry, maximum/minimum metabolic fluxes, enzyme kinetics, gene knockouts and knockins, regulation, and molecular diffusion), then it is possible to examine, understand and predict the genotype-phenotype relationships. In silico algorithms to evaluate cells behavior through GEM simulations were generated based on optimization techniques using constraints to improve simulations performance. Some useful methods are described, for a complete reference see Machado and Herrgård (2015).

2.2.1 GEM Analysis Metabolically Constrained

FBA

Flux balance analysis (FBA) is applied to the estimation of optimal states of metabolic fluxes attained for maximizing growth, ATP, and ethanol production using *S. cerevisiae* GEMs (Förster et al. 2003a; Famili et al. 2003). FBA calculations around metabolites in the S matrix is formalized as follow.

$$\frac{dx}{dt} = \sum_{j=1}^{N} S_{ij} v_j = 0; \quad i \in M;\ j \in N$$

where any v that satisfies this equation is a null space of S, and is part of the flux space of solutions (Φ). FBA requires that the objective of GEM simulations is to maximize or minimize a desired linear function ($max/min\ v_j$), such as maximize biomass production (μ, or $v_{biomass}$) or minimize glucose consumption ($v_{glucose\text{-}intake}$). Hence, FBA uses linear programming (LP) to calculate internal and external metabolic fluxes to max/min the objective function, according to $Z = c^T v$ (Orth et al. 2010). Where c is a vector of weights, usually with zeros and a one in the targeted reaction. Some fluxes can be constrained by limiting their upper and lower boundaries, e.g. $\alpha_j \leq v_j \leq \beta_j$. α_j and β_j are intake or uptake fluxes measured experimentally, or forced to zero for irreversible and disabled reactions (e.g. during catabolic repression or after gene knockout). Hence, an important application of FBA is the study of phenotypic effects of gene deletions which allow a specific Φ_{mutant}, which can be compared with an $\Phi_{wild\text{-}type}$ calculated for a wildtype cell strain. Notice that S_{ij}, v_j, N, M, $dx/dt = 0$ and $v_{min} \leq v_j \leq v_{max}$ is the set of constrains required for the calculation of Φ. FBA-based calculations are routinely performed to catch optimum cellular states. For example, these are valuable to predict gene essentially in *S. cerevisiae* (Famili et al. 2003; Duarte et al. 2004); also serve as the initiating point for many calculations using different algorithms.

MOMA

Maximization of growth may not apply to lab mutants, where knockouts may not impose comparable constraints. For this case, the method of minimization of metabolic adjustment (MOMA) establishes that knockout strains undergo minimal changes in metabolic fluxes (x_i) compared to the wild type (w_i) (Segrè et al. 2002). Hence, this method has the following minimization problem.

$$min\|w_i - x_i\| = min\sqrt{\sum_{i=1}^{N}(w_i - x_i)^2}$$

MOMA seeks the minimal distance between two points in Φ of the wild-type and knockout strains subjected to the same set of FBA constraints, but using quadratic (QP) solver instead. Also, MOMA defines another set of constraints, $v_d = 0, \forall\, d \in A$—where d and A are the index and set of deleted reactions. The aim is to find a x in $\Phi_{knockout}$ for which the Euclidean distance from $\Phi_{wild\text{-}type}$ is minimized by $f(x) = \frac{1}{2}x^T Qx + c^T x$. This contains linear and quadratic parts of the objective function (Q and cT).

ROOM

The regulatory on/off minimization algorithm (ROOM) is used for predicting metabolic fluxes at the steady-state after gene knockouts (Shlomi et al. 2005)—similar to FBA. ROOM establishes that cells do not evolve to cope with non-natural knockouts but regulatory mechanisms seem to minimize flux changes of knockout strain.

$$min\sum_{j=1}^{N} y_j$$

where, for each flux j, $y_j = 1\ (1 \le j \le N)$ for a significant flux change in v_j, and $y_j = 0$ otherwise. As $y_j \in \{0, 1\}$ is an integer constrain, ROOM solves this as a mixed-integer LP (MILP) problem. Flux distributions satisfy the same set of constrains as FBA and MOMA, in addition to the following constrains.

$$v_i - y_i\left(v_{max,i} - w_i^u\right) \le w_i^u, \text{ and } v_i - y_i\left(v_{min,i} - w_i^l\right) \le w_i^l;\ y_j \in \{0, 1\}$$

$$w_i^u = w_i + \delta|w_i| + \varepsilon, \quad and \quad w_i^l = w_i + \delta|w_i| - \varepsilon$$

where w^u and w^l are thresholds determining the significance of flux changes. δ and ε are relative and absolute ranges of tolerance with values 0.03 and 0.001 for flux predictions, and 0.1 and 0.01 for lethal predictions.

FBA, MOMA and ROOM were applied to the calculation of external metabolic fluxes in two petite mutants of *S. cerevisiae* under respiratory deficient conditions. The iFF708 GEM was constrained with external fluxes from chemostat cultivations of these mutants (Cakir et al. 2007). Ethanol production was simulated using various objective functions, such as maximizing/minimizing oxygen consumption. GEM predictions resulted more accurate when using FBA, ROOM and MOMA, in this successive order. However, internal metabolic fluxes in central metabolism calculated over a pyruvate-carboxylase mutant were more accurately predicted with MOMA compared to FBA (Segrè et al. 2002).

OptKnock

This is the first, on purpose, framework for targeting gene knockouts which constrain the overproduction of a biochemical (Burgard et al. 2003). Using a bi-level optimization problem, OptKnock establishes one particular cellular objective (e.g. $\max v_{biomass}$), and another consisting on maximizing the metabolic engineering objective (e.g. $max\ v_{biofuel\ or\ chemical}$). Hence, this method is based on the idea that metabolite overproduction is obligatory coupled with a cellular objective, and a combination of gene knockouts which maximize both is found to solve $Sv = 0$. OptKnock is similarly constrained as FBA, with the following distinctive constrains.

$$v_{biomass} \geq v_{biomass}^{min}; v_j^{min} \times y_j \leq v_j \leq v_j^{max} \times y_j, \forall j \in M; \sum_{j \in M} (1 - y_i) \leq K, y_i \in \{1, 0\}$$

where y_j assumes 0 or 1 if a reaction j is non-active (knockout) or active, respectively. K is the number of allowable knockouts. The bi-level formulation of OptKnock is solved through MILP.

Agreement of gene knockouts predicted by OptKnock and results with mutant strains overproducing succinate, lactate, and 1,3-propanediol, confirmed the value of this algorithm for analysis of targets for gene manipulations (Burgard et al. 2003). In *S. cerevisiae*, this framework was used to confirm the value of targeted genes suggested by OptGene (Patil et al. 2005) and MOMA for metabolic engineering of yeast to produce vainillin (Brochado et al. 2010).

OptStrain

Through OptStrain, pathway modifications can be achieved by gene knockouts and knockins (Pharkya et al. 2004). This utilizes a comprehensive database of cellular reactions (the universal database). A combinatorial optimization is used for searching a set of non-native functions, obtained from the universal database, which is added to the GEM host to enable the synthesis of the targeted biochemical. Biochemical reactions can be also removed when they constrain the

overproduction. OptStrain consists of two steps of optimization, one consisting in maximizing the biochemical yield.

$$max\, vj; max\, MW_i \times \sum_{j=1}^{M} S_{ij} v_j, \quad i = P$$

$$\text{Constrained by} \sum_{j=1}^{M} S_{ij} v_j \geq 0, \forall\, i \in N, i \notin \Re; \quad and \quad \sum_{i \in \Re} \left(MW_i \times \sum_{j=1}^{M} S_{ij} v_j \right) = -1$$

where $\Re$ is the set of substrates, MW_i is the molecular weight of metabolite i. In the second optimization problem, OptStrain computes the minimum number of non-native reactions required to reach the maximum yield calculated in the first step. To do that, the following objective function is stablished.

$$min\, v_j y_j; \sum_{j \in M_{non-native}} y_j$$

$$\text{Constrained by} \sum_{j=1}^{M} S_{ij} v_j \geq 0, \forall\, i \in N, i \notin \Re, \forall j \in M, \sum_{i \in \Re} \left(MW_i \times \sum_{j=1}^{M} S_{ij} v_j \right) = -1;$$

$$MW_i \times \sum_{j=1}^{M} S_{ij} v_j \geq Yield^{target}, \quad i = P; v_j \leq v_j^{max} \times y_j, \quad \forall j \in M_{non-native};$$

$$v_j \leq v_j^{min} \times y_j, \forall j \in M_{non-native}; \quad and \quad y_i \in \{0, 1\}, \forall i \in M_{non-native}$$

The elimination of reactions from the augmented network is performed with the OptKnock framework in the last step (Burgard et al. 2003).

This approach uses a set of non-natural reactions that, otherwise, can be added to the GEM manually. That is, whereas OptStrain can be used for targeting gene modifications in *E. coli* for production of vanillin (Pharkya et al. 2004), in *S. cerevisiae,* the heterologous reactions were incorporated in the iFF708 GEM and used OptGene, OptKnock and MOMA to seek for gene deletions (Brochado et al. 2010). The intensive computational time required by OptStrain is probably the main disadvantage of this method.

OMNI

Experimental metabolic fluxes are used in the optimal metabolic flux identification (OMNI) framework to recognize a reaction set which leads the consistency between prediction and experimental (Herrgård et al. 2006a). In order to find the optimal

solution of a metabolic reaction set which match model predictions and experimental data, the problem can be formulated as a bi-level optimization problem. An outer optimization problem that searches for a set of reactions to incorporate in the model, and an inner optimization problem which computes a flux distribution to solve the FBA problem with the following objective function.

$$min \sum_{i \in M} w_i \left| v_i^{opt} - v_i^{exp} \right|$$

$$\text{Constrained by } v^{opt} = max\, v_{biomass}, \sum_{j=1}^{M} S_{ij} v_j = 0, 0 \le v_j \le v_j^{max} j \in F, 0 \le v_k \le v_k^{\max} y_k k \in D;$$

$$v_l = v_l^{exp} l \in E; v_{biomass}^{opt} \ge v_{biomass}^{\min}; \quad \sum_{k \in D} (1 - y_k) = K; \quad y_k \in \{0, 1\}, \forall k \in D$$

It can be seen that the bi-level optimization formulation problem (1, 0) in the OMNI is similar to that used in OptKnock. Here, E represent the exchanged, measured fluxes, F is the set of reactions that cannot be removed from the model, and D the set of reactions which can be removed from the model. The linear programming nature of the inner problem allows for the overall problem to be solved by MILP. The OMNI method can be potentially used for deciphering unnecessary reactions in a GEM which, upon deletion, increases the accuracy of model predictions and experimental fluxes. Therefore, OMNI is also reliable for analyzing evolved strains through the evolution of fluxes.

OptReg

Besides finding gene knockouts and knockins, OptReg also seeks for modulation of gene functions (Pharkya and Maranas 2006). This algorithm requires optimal metabolic fluxes of the wild-type strain calculated with FBA—it is desirable to constrain the GEM with few experimental fluxes. A min/max problem is then solved to maximize $v_{biochemical}$, constrained as following.

$$\sum_j S_{ij} v_j = 0, \forall i \in N, \forall j \in M; v_j = v_j^{exp}, \forall j \in M_{\exp}; v_j \ge 0, \forall j \in M$$

M_{exp} is the set of reactions which fluxes are fixed with experimental values. Minimum and maximum values for each flux through reaction j are denoted $v_{j,L}^0$ and $v_{j,U}^0$. Then, modeling of genetic manipulations based on three sets of binary variables (0, 1) for each reaction j are included to all possible combinations in the model: gene downregulation (y_j^d), upregulation (y_j^U) and knockout (y_j^k). The fluxes are calculated based on the following constrains.

$$\text{Downregulation: } v_j^{\min} \le v_j \le \left[\left(v_{j,L}^0\right) \times (1-C) + \left(v_j^{min}\right) \times C\right] \times \left(1 - v_j^d\right) + v_j^{max} \times y_j^d$$

$$\text{Upregulation: } \left[\left(v_{j,U}^0\right) \times (1-C) + \left(v_j^{max}\right) \times C\right] \times \left(1 - y_j^d\right) + v_j^{min} \times y_j^U \le v_j \le v_j^{max}$$

$$\text{Knockout: } v_j^{min} \times y_j^k \le v_j \le v_j^{max} \times y_j^k$$

where v_j^{min} and v_j^{max} are minimized and maximized fluxes according to some specific considerations. The strength parameter C contains values between 0 and 1. A unique optimum solution value to the inner primal and the dual problem can be solved by MILP—see details at Pharkya and Maranas (2006).

Results from simulations with other algorithms have revealed the existence of synergism between reaction deletions and modulations, implying that the simultaneous application of both types of genetic manipulations produces more promising results. For example, the regulation of phosphoglucomutase activity in conjunction with the deletion of the oxygen uptake rate function and pyruvate formate lyase, yields 99.8% of maximum theoretical ethanol yield in *E. coli*. This yield was higher that when all the enzymes were deleted (Pharkya and Maranas 2006).

As the number of gene modifications increased due to the more objectives to cover in metabolic engineering projects, the necessity of algorithms with the ability to recognize more than three to four gene targets is clear. For example, the artemisinic acid-producing strain of *S. cerevisiae* required around seven gene modifications, including knockins and modulations (Ro et al. 2006).

EMILiO

The enhancing metabolism with iterative linear optimization (EMILiO) algorithm aims to meet increasing demands of the number and variety of genetic manipulations involved in metabolic engineering (Yang et al. 2011). Derived from OptKnock and OptReg, EMILiO uses a successive LP solution to individually optimize reaction fluxes, thus incrementing the scope of strain design. EMILiO identifies the optimal set of modified reactions and their optimal fluxes for overproduction of a target biochemical subjected to two objective functions, a cellular one $max\ v_j = v_{bio} - \varepsilon \times v_{chemical/biofuel}$ and a biochemical-production one $max\ v_{chemical/biofuel}$, which are constrained as following.

$$v_{min} \le v \le v_{max}; w_i^L \mu_i^L + w_i^L \mu_i^L = 0, \forall\, i \in N; Tv^f + \mu^U = v^U; Tv^f - \mu^L = v^L;$$

$$w^U T - w^L T = c^T \times T - \varepsilon \times c_p^T \times T; v_{bio} \ge v_{bio}^{min}; w^L, w^U, \mu^L, \mu^U \ge 0$$

where the product $\varepsilon \times v_{chemical/biofuel}$ is a small weighted minimization—$\varepsilon = 0.001$. This algorithm also couples biochemical production with growth, where v_{bio}^{min} represents the minimum growth required for product formation and c_p the objective vector of the exchange fluxes of the targeted metabolites. Therefore, EMILiO is

formulated as a bi-level optimization problem with additional constrains: w^L and w^U are slack variables for the lower and upper bounds, and μ^L and μ^U dual variables for the lower and upper flux bounds. Compared with OptReg, EMILiO is faster and obtains similar results.

GDLS

The genetic design through local search (GDLS) method also aims to meet increasing demands of the number and variety of genetic manipulations involved in metabolic engineering (Lun et al. 2009). GDLS starts with a user-defined strategy which then uses for searching better ones, limited to a maximum size (M). Best M strategies are used for another round, resulting in k additional manipulations. This approach continues until no better manipulations can be found—see Lun et al. (2009). GDLS initiates with a reduction of an FBA model. Dead-end reactions that do not carry any flux are deleted and reactions with linked metabolites are included in one reaction as following, $S_{ij_1}v_{j_1} + S_{ij_2}v_{j_2} = 0$; thus $v_{j_1} = -S_{ij_2}/S_{ij_1}v_{j_2}$. Then, v_j is maximized or minimized subject to $S_{ij}v_j = 0$, for $v_{min} \le v \le v_{max}$. If v_j^L and v_j^U are the minimizing and maximizing individual fluxes, for any reaction with $v_j^U \le v_j^L$, this is removed from model. Then GDLS looks for genetic manipulation strategies as a bi-level optimization problem and converting them to an optimization MILP problem, having $max\, g_j v_j$ as the objective function, and the following constrains.

$$\sum_{l=1}^{L} y_l \le C, y_l \in \{0,1\}, l \in \{1,\ldots,L\};$$

$$S_{ij}v_j = 0, (1-y)'G_j a_j \le v_j \le (1-y)'G_j b_j, \forall j \in N;$$

$$f'v = \sum_{j=1}^{N} v_j b_j - \mu_j a_j, f_i - \sum_{i=1}^{M} \lambda_i S_{ij} - v_j + \mu_j - \xi_j = 0,$$

$$\forall j \in N; -Dy^l G_j \le \xi_j \le Dy^l G_j, \forall j \in N; \mu, v \ge 0;$$

$$\sum_{l:\overline{y_l}=0} y_l + \sum_{l:\overline{y_l}=1} (1-y_l) \le k, \quad \sum_{l:\overline{y_l}=0} y_l + \quad \sum_{l:\overline{y_l}=1} (1-y_l) \ge 1$$

where G is the $L \times N$ matrix, with L genetic manipulations. g is the synthetic vector, y is the knockout vector, and C is the maximum number of knockouts. Converting the bi-level problem to a MILP problem using dual variables, results in the following constrains: λ is used for equality constrains, υ and μ for the lower and upper bounds, and ξ for $v_j = 0$ if $y_j = 1$, and D a scalar for ensuring that ξ_j is effectively unconstrained. The summaries represent interactions for each knockout strategy until the best set of manipulations is found (Lun et al. 2009). Compared with OptKnock, GDLS is ten times faster when targeting the same number of genes.

SIMUP

This is a bi-level based framework that searches for gene knockouts to allow the utilization of two carbon sources simultaneously (Gawand et al. 2013)—e.g. glucose and xylose from biomass hydrolysates. This algorithm is based on the idea that lethality of a gene knockout depend on the external nutrient conditions, without considering regulatory networks. The framework was formulated as a bi-level optimization problem to maximize the following objective functions.

$$max\, y_j \frac{\mu^1}{\mu^1_{WT}} - \frac{\mu^2}{2\mu^2_{WT}} - \frac{\mu^3}{2\mu^3_{WT}} \quad \text{and} \quad max\, v_{rev},\ v_{irr} \sum_{k=1}^{3} \left(\sum_{i=1}^{N_{rev}} c_{rev} v^k_{rev,j} + \sum_{j=1}^{N_{irr}} c_{irr} v^k_{irr,j} \right)$$

where μ is the specific growth rate of the mutant strain, and μ_{WT} for the wild type strain. Superscripts define the growth condition—e.g. 1 for glucose and xylose, 2 for glucose and 3 for xylose. Calculations with the GEM are performed using the following constrains.

$$\sum_{j=1}^{N_{rev}} S^k_{rev,j} v^k_{rev,j} + \sum_{j=1}^{N_{rev}} S^k_{rev,i,j} v^k_{rev,j} = b^k_j, \quad \forall i \in M, k \in \{1,2,3\};$$

$$\sum_{j=1}^{N_{rev}+N_{irr}} \left(1 - y_j\right) \leq K_{max;} v^{min}_j \times y_j \leq v_j \leq v^{max}_j \times y_j,\ j \in N_{rev}, N_{irr},;\ y_j = \{0,1\}$$

Superscripts L and U define lower and upper bounds and subscrips *rev* and *irr* are for reversible and irreversible. C is the coefficient vector of the objective function. K_{max} is the limit of reactions that can be deleted. Decision variables y_j have a value of 0 or 1. This is converted to a MILP problem.

2.2.2 GEM Analysis Constrained by Metabolism and Gene Expression

Besides invariant constraints that limits possible cellular behavior, such as stoichiometry, capacity, and thermodynamic limits, there are also variant constraints that limit allowable behavior, such as enzyme kinetics and regulation of gene expression which are adjustable through evolutionary processes (Palsson 2000). Both groups of constraints can be applied to narrow the possible space of solutions for the attainable distribution of metabolic fluxes.

Incorporation of gene expression data from microarrays and RNAseq technologies into GEMs can be achieved by Boolean logic equations representing the transcriptional regulatory structure—e.g. 1 for transcriptionally active gene and 0 for the opposite (Covert et al. 2001). This structure was established on the base that mRNA accumulation depends on both, a time interval and a defined environmental condition. In a metabolic reaction, X can be converted in Y, and Y may interact with

the binding site of gene A which catalyzes the conversion of X. Hence, the transcription of A can be expressed as $transformation = IF(A)\,AND\,NOT(Y)$. In reactions conditioned by the presence of both the metabolite and the enzyme, this can be represented as $rxn = IF(X)\,AND(A)$. If the presence of all the regulated enzymes in the metabolic network is determined for a time interval, then one can stablish a set of constrains when the absence of a given enzyme transcript is found during this interval.

$$v_j(t) = 0, \quad \text{when} \quad t_1 \leq t \leq t_2$$

where v_j is the flux through the reaction at a given time point. After, the GEM can be converted in a problem that can be solved by FBA. This strategy is useful for calculating the effects of gene mutations and knockouts as well as for simulating gene expression profiles which can rise with new components and interactions in biological networks (Covert et al. 2001, 2004).

GIMME

The gene inactivity moderated by metabolism and expression (GIMME) algorithm uses quantitative gene expression and various metabolic objectives to calculate metabolic fluxes (Becker and Palsson 2008). This algorithm works in two steps. First, the algorithm finds maximum fluxes through a metabolic network with required metabolic functionalities (RMF), using FBA and typical constraints. The second step involves the calculation of a set of minimum available reactions that best fit the quantitative data ($min \sum c_i \times |v_i|$). Those reactions should operate above some minimum level—a percent of those found in the first step. This can be solved as a linear programing problem subjected to the following constrains.

$$S_{ij}v_j = 0; v_{min} < v_i < v_{max}$$
$$\text{where } C_i = \left\{X_{cutoff} - X_i \text{ where } X_{cutoff} > X_i, 0 \text{ otherwise; for all } i\right\}$$

where X_i is the normalized gene expression data, and X_{cutoff} is the cutoff value set by the user. Since the algorithm provides an inconsistency score indicating the consistency of gene expression data with a particular metabolic objective, this can be used to check biological experiments and as an intuitive approach in adaptive evolution and rational design of metabolic networks. Thus, this algorithm was used for targeting gene modifications to increase lactate production in *E. coli* strains with knockouts in the Phosphate acetyltransferase (*Pta*) and the aldehyde-alcohol dehydrogenase E (*AdhE*) and exposed to an evolution process (Becker and Palsson 2008).

E-Flux

E-Flux (flux-expression method) uses FBA to calculate maximum metabolic fluxes constrained by measured gene expression (Colijn et al. 2009). This approach modifies the typical FAB to the following optimization problem.

$$\max v_j;\ \text{subject to}\ S_{ij}v_j = 0; \quad a_j \leq v_j \leq b_j,$$

Maximum flux (v_j) is constrained by gene expression according to $maxFlux = f(G_1)$, $b_j = f(\textit{expression level of genes associated to reaction } j)$. Expression data is represented by y_{ijkg}, which is the log transformation of the signal measured in the ith channel, jth chip, kth experimental condition, and gth gene, subjected to an error ε_{ijkg}, thus: $y_{ijkg} = \mu_{ij} + G_g + (AG)_{jg} + (DG)_{ig} + \hat{y}_{kg} + \varepsilon_{ijkg}$. Where μ_{ij} is the average for channel j of chip i, G_g the effect of gene g, $(DG)_{jg}$ the effect of chip j and gene g, $(DG)_{ig}$ the effect of channel i and gene g, and $\hat{y}_{kg}$ the effect of variety k and gene g. With E-Flux, it is generated the constraint vectors a and b from control and experimental conditions. Thereby, b is a vector weighing the magnitude of metabolic fluxes and introduce additional constrains for a given FBA-based objective function.

This method was utilized for predicting the metabolic state constrained by gene expression data, and was useful for targeting the impact of different drugs in *Mycobacterium tuberculosis*. This approach can be also used to calculate a consistency correlation among gene transcription and translation. In *S. cerevisiae*, for example, this correlation is 0.61% (Ideker et al. 2001). Therefore, additional care should be put in model predictions when using gene transcription levels.

Random sampling

Transcriptional regulation of key metabolic enzymes can be evaluated by the Random Sampling method (Bordel et al. 2010). This algorithm requires the calculation of the space of feasible solutions (Φ) among a set of different strains or environmental conditions. Random Sampling then defines a set of possible flux distributions by randomly sampling the Φ—particularly in the corners. These values are used for the calculation of an average and standard deviation for every GEM reaction and then used them for the estimation of a significant change in flux.

$$Z_j^{flux} = \frac{E_2(v_j) - E_1(v_j)}{\sqrt{Var_2(v_j) + Var_1(v_j)}}$$

A significant change in gene expression between conditions can be also calculated based on p_i values from transcriptomics analysis $\left(Z_j^{ex} = \pm inverf(1 - p_i/2)\right)$.

Comparison between both values allows the identification of enzymes showing a significant correlation between expression and metabolic flux $\left(P = \phi\left(Z_j^{flux}\right)\right.$ $\left.\phi\left(Z_j^{exp}\right)\right)$. The classification of enzymes according to their regulation can be as follow: enzymes showing transcriptional regulation, posttranscriptional regulation and metabolic regulation.

Transcriptomic data from different experiments consisting in gene knockouts and diauxic growth in *S. cerevisiae* were used along with the iFF708 GEM to analyze gene expression programs under these conditions. This kind of analysis allowed for the identification of a group of genes regulated by certain transcription factors (Bordel et al. 2010; Österlund et al. 2013; Caspeta et al. 2014b).

MADE

The metabolic adjustment by differential expression (MADE) is a method to map expression data onto a metabolic network, using non-arbitrary expression thresholds (Jensen and Papin 2011). Base on the statistical significance of an increase (I; 1), decrease (D; −1) or constancy (C; 0) of gene expression, MADE calculates a vector of binary expression states $(x_1 \ldots x_n)$, $x_i \in \{0, 1\}$, for n conditions. This vector is partitioned into three sets I, D and C, and the optimization objective is the weighted sum:

$$f_{i\to i+1}(x) = \sum_{x\in I} w\left(p_{x_{i\to i+1}}\right)(x_{i+1} - x_i) + \sum_{x\in D} w\left(p_{x_{i\to i+1}}\right)(x_i - x_{i+1}) - \sum_{x\in C} w\left(p_{x_{i\to i+1}}\right)\Delta_{x_i,x_{i+1}}$$

MADE then maximize the sum of the objective function: $max \sum_{i=1}^{n-1} f_{i\to i+1}(x)$

$$\text{Constrained by } S_{ij}v_j = 0; \quad v_{min} \le v_j \le v_{max}; \quad v_{obj} \ge v_{min}; \quad N\begin{pmatrix} v \\ x \end{pmatrix} = b;$$

$$v_{min} = 0.1 - 0.3 \text{ for bacteria;}$$

$$\Delta_{x_i,x_{i+1}} \in \{0, 1\}; \quad w(p) = -\log p; \quad x_i \in \{0, 1\}$$

This results in a mixed inter problem solved by MILP. MADE was used to construct a set of models that better reflect adjustments in the metabolism when the *S. cerevisiae* transits from fermentative to glycerol-respiration, attaining 98.7% of possible changes in expression (Jensen and Papin 2011).

PROM

The probabilistic regulation of metabolism (PROM) requires a GEM, a regulatory network structure based on gene-TFs interactions, abundant gene expression data, and information about enzymatic regulation by metabolites (Chandrasekaran and Price 2010). This establishes the possibility to represent gene states and gene-TF interactions—e.g. gene A is active when the regulating TF B is off or $P(A = 1|B = 0)$. This is evaluated with abundant microarray data—e.g. the P of gene A to be ON is 0.8, if this appears 80% of the time in microarray experiments when TF B is knocked out. These gene state values are used to constrain metabolic fluxes in a GEM using FBA method—e.g. v_{max} trough gene A is $0.8xv_{max}$; uperbound $= Pxv_{max}$. When the constrains have been set in the GEM, the optimal solution space for the desired objective function is solved as a LP problem using FBA. Predictions of *E. coli* growth rate upon different knockout strains were accurately predicted with PROM—correlation coefficient of 0.96.

TIGER

The toolbox for integrating genome-scale metabolism, expression, and regulation (TIGER) uses Boolean or multilevel rules to stated arrangements of the relation between gene-TF-metabolite in the form: *TF B* and (not metabolite *M*) then gene *D*, then the constrain set $A = (1, 0, 1)$ is stablished for this state (Jensen et al. 2011). A is converted to an inequality $(-1 \leq 2x + 2y - 4I_1 \leq 3)$. The structure of simulations then gets stablished as following.

$$\min obj'x$$
$$\text{subject to } Ax(\leq|=|\geq)b; lb \leq x \leq ub$$

The inequality constrain A is converted to an indicator of reaction participation (R_i). Thus, $v_j^{min}R_i \leq v_j \leq v_j^{max}R_j$. Boolean rules are converted to a MILP problem.

Variations on GIMME and MADE algorithms can be made for contextualizing specific models with global expression data using TIGER. The flexibility of TIGER for using Boolean or multilevel rules formats allows more accurate descriptions of cellular functions, such as reactions with isozymes and protein quaternary structures, and hence flux control and transcriptional regulation. The authors used these features to identify and solve inconsistencies within existing transcriptional regulatory networks in the GEM iND750 of *S. cerevisiae*.

2.3 *Inverse Metabolic Engineering Through Adaptive Evolution*

Adaptive laboratory evolution (ALE) combined with whole-genome sequencing and global analysis has become a compelling strategy to study the biological basis of evolution (Dettman et al. 2012). Combined with 'Omics' technologies, the evolutionary engineering can lead to a comprehensive understanding of the basis of microbial evolution (Dragosits and Mattanovich 2013). These can serve for a rational application of recombinant DNA to generate the desired phenotype in an anticipated cell host. This approach is called inverse metabolic engineering.

Compared with procedures to generate temporal tolerance, ALE experiments generate heritable tolerance phenotypes. Spontaneous mutations in microbial populations occur at a rate close to 0.0033 per genome (Drake 1991). Thus, microbial evolution can be applied to populations exceeding 10^{11} cells per liter and continuous evolution can be more effective than step-wise approaches (Sauer 2001). Mutations can be appearing because of single-nucleotide polymorphisms, DNA rearrangements and horizontal DNA transfer (Arber 2000). The number of mutations can change with cell type and hardness of environmental condition, for instance, metabolic stress, stationary phase or high temperature (Sauer 2001; Caspeta et al. 2014b). During adaptive laboratory evolution, the fitness can be measured by competition (Sauer 2001).

$$ln\left[x_i(t)/x_j(t)\right] = ln\left[x_i(0)/x_j(0)\right] + S_{ij}t$$

where x_i and x_j are cell densities of two populations, competitive fitness of one strain can be quantified by the selection coefficient S_{ij}.

3 Systens Metabolic Engineering for Biomass-to-Biochemicals Conversion with *S. cerevisiae*

The application of methods described above for metabolic engineering of *S. cerevisiae* for the production of biofuels and chemicals from biomass-based sugars is discussed below.

3.1 *Ethanol Overproduction*

Thus far, one of the most important applications of the systems metabolic engineering is to generate gene modifications to reduce glycerol formation during ethanol production. Glycerol works as the yeast's predominant sink of the NADH

accumulated during aerobic growth, and its formation restores the redox balance in the cytosol under anaerobic conditions, and when the electron transport chain (ETC) is damaged (Van Hoek et al. 1998). Since NADH is used in ETC as a proton donor to move electrons and produces ATP, an important factor in the GEM is to accurately reproduce the amount of ATP produced from the movement of two electrons in the ETC—the P/O ratio. Using the iFF708 GEM, the formation of ATP in the glycolysis pathway and ETC can be distinguished for calculating the amount of ATP produced per NADH during glucose consumption. Using the exometabolome of cultivations in chemostats, it is possible to fix the NADH oxidized during glycerol synthesis and ATP production through ETC. The P/O value was calculated in 1.04 (Famili et al. 2003), which is similar to 0.95 previously reported (Verduyn et al. 1991) ∼12.5 mol of ATP per mole of glucose. Ethanol and glycerol productivities in chemostats were accurately calculated with the constrained iFF708 (Famili et al. 2003). The same model was only able to reproduce ethanol and glycerol yields in batch cultivations when gene expression data constrained GEM calculations (Akesson et al. 2004). Incorporation of transcriptomic data also improved the prediction of internal fluxes and the metabolic adjustments during the transition from glucose to glycerol (Akesson et al. 2004; Jensen and Papin 2011)—e.g. from fermentation to respiration. In the latter case, MADE based analysis of metabolic fluxes matched 83.5% of gene expression transitions during the diauxic growth.

Since the accumulation of NADH stimulates glycerol synthesis, one criterion used for reducing its formation was to use this cofactor in the synthesis of ethanol or biomass (Bro et al. 2006). To evaluate strategies, the iFF708 GEM was constrained with gene knockouts/knockins for activating or inactivating metabolic fluxes of glycerol synthesis or the accumulation of NADH. In silico evaluation of strategies resulted in the elimination of glycerol formation and an increase of 10% in ethanol yield. The best strategy consisted in the insertion of the non-phosphorylating, NADP-dependent glyceraldehyde-3-phosphate dehydrogenase gene (*GapN*). Implementation of this strategy resulted in a decrease of 40% in glycerol accumulation, but ethanol yields just increased by 3%, whereas growth remained unaffected. Reducing the expression of the NAD-dependent glycerol-3-phosphate dehydrogenase (*Gpd1*), a key enzyme of glycerol synthesis, showed a 44–61% reduction of glycerol yield, 2–7% increase of ethanol yield, and 20% reduction of biomass during anaerobic fermentation (Pagliardini et al. 2013). However, *Gpd1* mutants were most susceptible to stress probably because of a reduction in the ATP yield or an inefficient response to cell-wall damage stress.

Experimental data for oxygen and glucose uptake kinetics from fed-batch cultivations, as well as frontiers parameters (e.g. initial and final volume, biomass concentration) were used to constrain the iND750 GEM (Hjersted et al. 2007). The limited model was used for calculating the dynamic of ethanol productivity under various cultivation strategies: switching dissolved oxygen concentrations from 50-to-0% DO at air saturation, cultivation media with glucose, xylose and 50%/50% glucose/xylose, and changing the ethanol inhibition constant. These simulations served for a dynamical screening of gene modifications to increase ethanol

productivity. Interestingly, this strategy predicts the 4% increment in ethanol production with gene modifications used by Bro et al. (2006). Furthermore, new gene insertions were proposed to increase ethanol yield by 8%. In both studies, authors concluded that conversion of NADH to NADPH and its further utilization for ethanol and biomass synthesis can lead to decrease glycerol formation. For instance, cytosolic NADPH consumption for biomass synthesis is ~4.8 mmol/gDW. In another study, the overexpression of the NADPH-dependent, modified *Bdh1* which enzyme product catalyzes the oxidation of (R,R)-2,3-butanediol to (3R)-acetoin decreased glycerol production (Celton et al. 2012). The insertion of an alternative oxidase (AOX) or the overexpression of a NADH oxidase also reduces the accumulation of glycerol, but also the production of ethanol (Vemuri et al. 2007). Higher negative effects were observed with AOX which insertion affected mitochondrial functions including downregulation of the mitochondrial inner membrane ADP/ATP translocator (*AAC1*).

In silico knockout of *Gdh1* (NADP-dependent glutamate dehydrogenase) and overexpression of *Gdh2* (NAD-dependent glutamate dehydrogenases) in the iMM904 GEM was used to calculate external metabolic fluxes. This in silico strain reduced NADPH consumption during ammonium assimilation for xylose fermentation in recombinant yeast strains carrying *Xyl1* and *Xyl2* (Mo et al. 2009). These results showed that ethanol production from glucose and xylose need opposite metabolic engineering strategies when xylose is metabolized via the two-step reduction-oxidation pathway.

Exometabolome of the wild-type and *Gdh1*/*Gdh2* mutant strains was incorporated as constraints in this model and calculations of internal fluxes were performed. Results were consistent with intracellular metabolite levels and fluxes previously reported. In this study, predictions with FBA, MOMA and linear MOMA perform similarly (Mo et al. 2009). However, under similar simulations using exometabolome of nuclear petit yeast mutants, the iFF708 GEM predicted better results with FBA and ROOM than with MOMA (Cakir et al. 2007). The compartmentalization of both GEMs is the only difference in calculations.

3.2 Xylose Utilization

Xylose comprises ~30% of total biomass-based fermentable sugars but is not naturally metabolized by *S. cerevisiae*. There are two distinct approaches to introduce this metabolic function in the yeast. One consisting in the heterologous expression of *Xyl1* and *Xyl2* coding for the NADH-preferring xylose reductase and the NADPH-preferred xylose dehydrogenase from *Pichia stipitis;* and another consisting in the expression of the xylose isomerase (*XylA*) from bacteria (Jeffries 1985). The XYL1/XYL2 strategy is disadvantageous since the yeast cannot easily deal with the redox balance. To find the constraints that limit xylose utilization,

FBA was used to calculate metabolic fluxes with a model of xylose metabolism including central metabolism and a P/O ratio of one (Jin and Jeffries 2004). This study ended with the incorporation of the xylulokinase activity (*Xyl3*) in the *Xyl1/Xyl2* background. FBA based calculations predicted that maximum ethanol production in this strain could be reached under oxygen-limited conditions, a fact that was proved experimentally.

An inverse metabolic engineering strategy was also used to seek for constraints limiting xylose utilization in a XYL1/XYL2 mutant strain. DNA fragments of a genomic library of *P. stipitis* were used to complement this strain (Jin et al. 2005). Serial dilutions of strain populations carrying the complementary gene were used to enrich the population with individuals having the useful gene function. 16 colonies were selected, and their plasmids sequenced. 10 out of the 16 strains harbored plasmids with the *Xyl3* gene, and one with high homology to *S. cerevisiae Tal1* encoding the transaldolase, an enzyme of the non-oxidative pentose phosphate pathway. Yeast strains with *Xyl1*, *Xyl2*, *Xyl3* and *PsTal1* insertions increase xylose consumption and ethanol production by 100% and 70% compared with the parental strain. Similarly, the overexpression of a native xylulokinase *(Xks1)* in a recombinant yeast carrying *Xyl1/Xyl2* increased xylose consumption which was then improved by chemical mutagenesis and adaptive evolution over 60 days (Liu and Hu 2010).

Anaerobic fermentation of xylose to ethanol via the two steps strategy was possible with the insertion of the *Xks1* gene (Eliasson et al. 2000). However, the resulting strain was unable to grow in the absence of oxygen. Adaptive laboratory evolution of this strain over 460 generations on, consecutively, aerobic, microaerobic and anaerobic serial cultivations allowed for the generation of strains able to utilize xylose under anaerobic conditions (Sonderegger and Sauer 2003). Transcriptomics and metabolic flux analyses of these strains cultivated in chemostats with xylose and xylose-glucose under aerobic conditions and xylose-glucose under anaerobic conditions, suggested that cytosolic NADPH formation and NADH consumption enabled a high flux through the two-step oxidoreductase reactions (Sonderegger et al. 2004). Anaerobic fermentations were not improved probably because the absence of a NADH sink or by an increased production of ATP—similar results were found in (Wasylenko and Stephanopoulos 2015). Complementation of the pathway can be achieved by reducing acetate to ethanol through the activity of the acetylating acetaldehyde dehydrogenase (*AadH*) into *S. cerevisiae,* which served as a sink for NADH excess. This strategy also increased ethanol production (Wei et al. 2013).

Adaptive laboratory evolution of a respiratory deficient, *Xyl1/Xyl2* yeast strain, lacking the cytochrome C oxidase subunit IV was used as a strategy to increase growth rate and ethanol production under anaerobic conditions. The specific growth rate, ethanol yield, and xylitol yield of the evolved strain on xylose were 0.06 1/h, 0.39 g/g, and 0.13 g/g, respectively (Peng et al. 2012). An *S. cerevisiae* strain over-expressing genes of the non-reductive pathway of xylose utilization and the non-oxidative PPP, was evolved through a three steps evolution strategy (aerobic, anaerobic and xylose-limited chemostat). This approach allowed for the generation

of a strain with a high xylose consumption (1.86 g/gDCW/h) and ethanol conversion yield (0.41 g/g) (Zhou et al. 2012). Combining different media compositions with mixtures of glucose, xylose and arabinose, the use of adaptive evolution can be also used to generate yeast strains capable of producing ethanol at a yield of 0.43 g/g of total sugars under anaerobic fermentation (Wisselink et al. 2009). The parental yeast strains overexpressed *XylA* from *Piromyces* sp., endogenous genes of the PPP and *Xks1*, and *Lactobacillus plantarum AraA*, *AraB*, and *AraD* genes.

Insertion of *XylA* in *S. cerevisiae* avoids the utilization of pyridine nucleotide cofactors during xylose consumption. According to a ^{13}C-FBA performed in a central metabolism model, yeast strain carrying *XylA* had a low distribution of metabolic fluxes in the non-oxidative PPP and did not show a full carbon catabolic repression typical of glucose fermentation (Wasylenko and Stephanopoulos 2015) —mutation of *Hxt7* and *Gal2* in a *XylA* mutant strains also generated glucose-insensitive phenotypes (Farwick et al. 2014). Higher concentrations of NADH were observed in xylose consumer strains growing under anaerobic conditions, whereas energy charge remained similar (Wasylenko and Stephanopoulos 2015). Lower metabolic fluxes in the last tree reactions of glycolysis seemed to limit the production of ethanol with xylose under anaerobic conditions. Therefore, the productivity and yield of anaerobic conversion of xylose to ethanol were 6% lower compared with 12% increase observed with glucose under anaerobic and aerobic conditions. Compared with aerobic conditions, glycerol accumulation increased in cultivations with glucose and xylose under anaerobic conditions.

3.3 Tolerance to Toxic Byproducts and Temperature

Adaptive laboratory evolution has been successfully used for generating yeast strains tolerant to furfural, HMF and acetate (Liu et al. 2005; Heer and Sauer 2008). Increased tolerance to 30 and 60 mM of furfural and HMF were observed in yeast strains isolated from ALE experiments (Liu et al. 2005). These strains also increased glucose consumption. Two isolated strains efficiently transform HMF to 2,5-bis-hydroxymethylfuran and one transformed furfural into furfuryl alcohol (Liu et al. 2005). Evolved strains reduced the lag phase of growth suggesting that furfural conversion into its alcohol is the mechanism for yeast adaptation to this byproduct (Liu et al. 2005; Heer and Sauer 2008). Evolution of the industrial strain ethanol-red of *S. cerevisiae* in spruce hydrolysate and high temperature resulted in the selection of strains capable of converting spruce hydrolysates into ethanol with high efficiency (Wallace-Salinas and Gorwa-Grauslund 2013). Compared with evolved strains selected with furfural and HMF alone, which increased the conversion of these into their alcohols, these strains tolerance did not rely on higher reductase activities, but rather on a higher thermotolerance.

Microarray analysis of strains with different abilities to tolerate HMF permitted the identification of 15 reductase/dehydrogenase genes, whose overexpression in poor resistance yeast strains generated tolerance to HMF (Petersson et al. 2006).

Among them, the overexpression of NADH/NADPH dependent *Adh6*, which converts HMF into 5-hydroxymethylfurfuryl alcohol, resulted in the highest increment in HMF transformation, and tolerance. Proteomic and transcriptomic analyses of *S. cerevisiae* cultivated with furfural showed the downregulation of genes involved in glycerol synthesis, changed the expression of alcohol dehydrogenases, and reduced the levels of cytosolic NADH (Lin et al. 2009), suggesting an increased demand in redox potential for transformation of furfural into its alcohol. This demand seems to cause the long lag phase of ethanol production in cultivations with furfural (Liu et al. 2005). Besides the changes in redox metabolism, yeast subjected to furfural also change expression levels of genes involved in oxidative and salt stress as well as the TFs, *Msn2*/*Msn4*, *Yap1*, and *Hsf1* which regulate different stress responses (Lin et al. 2009). Overexpression of *Yap1* and *Msn2* highly correlated with the increase in yeast tolerance to furfural and HMF (Lin et al. 2009; Sasano et al. 2012). The former results were generated in cultivations with glucose as a carbon source but were also reproduced in cultivations with xylose as a carbon source (Ask et al. 2013).

The iFF708 GEM was complemented with metabolic equations for oxidative and reductive conversion of furfural into furfuryl alcohol, and constrained with experimental data from fed-batch fermentations in glucose-xylose media containing furfural. Dynamic FBA was carried out with the model and the results from simulations showed increasing fluxes though PPP, TCA cycle and serine-proline synthesis to replenish the extra consumption of NADPH (Pornkamol and Franzen 2015).

Many of the thermo-tolerant phenotypes produced trough metabolic engineering methods, have been generated by inverse metabolic engineering through adaptive evolution and multi 'omics' analyses. Yeast strains with improved high-temperature tolerance can be isolated after several hundreds of generations in serial cultivations at high temperatures (Cakar et al. 2012; Yona et al. 2012; Caspeta et al. 2014b). Genome sequencing and multi-'omics' analyses drove the identification of gene rearrangements responsible for the improved performance (Yona et al. 2012; Caspeta et al. 2014b). Remarkably, deleterious mutations in just one gene (*Erg3*) allowed for the parental strain to obtain 85% of the tolerant phenotype observed in seven evolved strains (Caspeta et al. 2014b). Complete and segmental duplications of chromosome III were also detected in the genome sequence and transcriptomics analyses of evolved strains (Yona et al. 2012; Caspeta et al. 2014b). From genes comprising the chromosome III, *Hcm1* and *Rrt12* encoding a TF involved in chromosome segregation and a probable subtilisin-family protease, partially recovered thermo-tolerance in the parental strain (Yona et al. 2012).

A possible disadvantage of thermotolerance based on *Erg3* mutations is that the related yeast strains are deficient in the electron transport chain and ATP synthesis. Therefore, they displayed their thermal niche to higher temperatures and are inefficient to synthesize biomass under aerobic conditions. In fact, thermotolerant yeast strains (TTs) showed similar behavior to the parental yeast growing under anaerobic conditions (Caspeta and Nielsen 2015). Molecular responses in evolved strains cultivated under optimal (30 °C) and high temperatures (40–50 °C) were analyzed

using the iIN800 GEM constrained by exofluxome and with a thermodynamic model coupling protein folding-unfolding thermodynamics and growth kinetics (Caspeta and Nielsen 2015). TTs displaced their thermal niche while keeping high tolerance to higher temperatures than the wild type strain. Also, TTs showed a preemptive response to high temperature when cultivated at 30 °C (Caspeta et al. 2016). These responses limited the growth of evolved yeasts in cultivations at optimal temperature.

3.4 Production of Biochemicals

Genome-scale metabolic modeling with the iFF708 GEM and FBA method were used to set up metabolic engineering strategies to increase the production of succinic acid (Agren et al. 2013). Objective functions included: growth maximization with limiting glucose uptake, oxygen uptake was unconstrained for aerobic and microaerobic processes, and under anaerobic conditions, this was constrained to the minimum (0.016 mmol/gDCW/h). Glucose uptake rate was constrained with experimental data, and maintenance ATP was set to 1 mmol/gDCW/h. According with model predictions, the top three single gene deletions include *Mdh1*, *Oac1* and *Dic1* coding the mitochondrial malate dehydrogenase, a mitochondrial inner membrane transporter, and a mitochondrial dicarboxylate carrier, respectively. Model simulations also detected that succinate production is sensitive to the oxygen uptake rate, and it is more sensitive for *Mdh1* mutant than the *Mdh1*/*Rip1* (*Rip1* coding the ubiquinol-cytochrome-c reductase) double mutant. Transcriptional analysis of *Dic1* mutant suggested that electron transport chain, ATP synthesis, sterol transport and metabolic processes for energy formation are coupled with succinate formation. Targeting gene modifications for succinate overproduction was also carried out with the OptGene algorithm and iFF708 GEM (Patil et al. 2005). The results guided the modification of a yeast strain with gene knockouts including *Sdh3* (cytochrome b subunit of succinate dehydrogenase complex), *Ser3p*/*Ser33* (3-phosphoglycerate dehydrogenase isoenzymes) (Otero et al. 2013). A maximum yield of 0.14 g/g biomass was obtained. Further evolution in media containing glycine leads the generation a strain which accumulates 0.69 g/g biomass. Transcriptomics analysis of the evolved strain lead the identification of *Icl1* (isocitrate lyase) as a target to increase succinate production in the evolved strain. Incorporation of this mutations increased succinate production to 0.9 g/g biomass (Otero et al. 2013).

The production of the non-natural compound vanillin was evaluated in *S. cerevisiae*. Reactions for vanillin production from protocatechuate and formaldehyde were introduced in the iFF708 GEM. The set of gene knockouts for overproduction of vanillin were predicted with OptGene (Patil et al. 2005)—an algorithm that derives from OptKnock. GEM simulations predicted that deletion of pyruvate decarboxylase and glutamate dehydrogenase activities could improve

vanillin production to 90% of maximum theoretical value. In a complementary study, the iFF708 GEM combined with OptGene, FAB and MOMA served to find five reactions to convert 3-dehydroshikimate, a natural intermediate in aromatic amino acids biosynthesis, into vanillin β-D-glucoside (VG) (Brochado et al. 2010). OptGene was used for predicting metabolic engineering targets. MOMA was used as the biological objective function with wild type flux distributions spanning three modes of yeast physiological responses. The optimality of the targeted genes was verified with OptKnock. Based on this and their previous analysis, two gene candidates, *Pdc1* (Pyruvate decarboxylase) and *Gdh1* (Glutamate dehydrogenase), were selected for strain construction. Compared with the reference strain, *Pdc1/Gdh1* mutant strains produced 40% more vanillin, whereas ethanol yield was similar and protocatechuic acid yield increased.

The production of terpenoids with recombinant *S. cerevisiae* has been also targeted. The iMM904 GEM was used with FBA/MOMA analyses to find metabolic engineering targets for increasing terpenoids production (Sun et al. 2014). A set of single mutations predicted in simulations were tested, showing that mutation of *Alt2*, *Hxk2* and *Sor1* resulted in the highest titer of amorphadiene (55 mg/L)—the artemisinic acid precursor. Amorphadiene production at concentrations >40 g/L was reached with recombinant yeast strains overexpressing every enzyme of mevalonate pathway, using fed-batch cultivations (Westfall et al. 2012). Incorporation of additional three heterologous enzymes was needed to convert amorphadiene into the antimalarial drug artemisinin. Since terpenoids derivatives have applications as biofuels, its overproduction in yeast also became strategic. Terpenoids can serve as drop-in fuels and can substitute gasoline, diesel, and kerosene (Rabinovitch-Deere et al. 2013). Deletion of *Hfd1* together with the expression of an alkane biosynthesis pathway resulted in the production of the alkanes tridecane, pentadecane, and heptadecane (Buijs et al. 2015). Metabolic flux analysis of a reduced model with 69 reactions of central metabolism was used to calculate yields for terpenoids production using the pyruvate-glyceraldehyde-3-phosphate (DXP) pathway and mevalonate pathway (MVA). Although carbon balances favor terpenoids production via DXP, further reduction occurs when redox and energy is considered (Gruchattka and Kayser 2015).

GEM simulations combined with industrial process analysis can be used for the selection of biosynthetic routes which allow the economical synthesis of the desired target molecule. For instance, this approach was useful to identify advanced biofuels as more efficient fuels in terms positive energy balances and production costs (Caspeta and Nielsen 2013). With this approach, it was also identified that the most promising route for 3-Hydropropionic acid (3HP) synthesis is the β-alanine biosynthetic route (Borodina et al. 2015). With this in mind, a yeast strain expressing the heterologous pathway for β-alanine synthesis from *Bacillus cereus* and its subsequent conversion into 3HP was engineered. This strain produced 3HP at a titer of 13.7 ± 0.3 g/L, and 0.14 C-mol/C-mol yield. Adaptive laboratory evolution and genome sequence of 3HP production strains at pH 3.5 revealed that mutations in *Sfa1* gene encoding S-(hydroxymethyl)-glutathione dehydrogenase increased tolerance to 50 g/L of 3HP, suggesting that detoxification of

3-hydroxypropionic aldehyde via glutathione is the main factor (Kildegaard et al. 2014).

Esterification of fatty acids (FA) is another source of biofuels with properties similar to diesel. A strategy to increase the accumulation of FA involves the deactivation of beta-oxidation pathway and the increase of steryl-esters degradation (Valle-Rodríguez et al. 2014). A broader approach also involved the overexpression of TCA enzymes to replenish acetyl-CoA pull and deletion of *Pox1* (encoding the fatty-acyl coenzyme A oxidase). This strategy led the accumulation of FA at concentrations up to 10.4 g/L (Zhou et al. 2016). Lipid metabolism in *S. cerevisiae* plays a key role in many cellular functions. To get insight on lipid metabolism, the measurement of 5636 mRNAs, 50 metabolites, 97 lipids, and 57 ^{13}C-reaction fluxes were performed (Jewett et al. 2013). The results were mapped into the iIN800 GEM, which was used to map network topologies of lipid metabolism and regulation. Results suggested that sterols are mainly regulated at the transcriptional level, whereas FA synthesis at the metabolic level. Using a GEM of *Yarrowia lipolytica* and multi-'omics' analysis, including RNAseq, metabolic profiling and lipidomics, it was found that lipid accumulation does not involve transcriptional regulation, and is associated with regulation of amino acids synthesis (Kerkhoven et al. 2016). Finally, the manipulation of structural and regulatory genes of lipid metabolism, including the overexpression of *Acc1*, the deletion of *Ino1,* and the overexpression of *Rpd3* induced the production of 1-hexadecanol from xylose in a yeast strain carrying *Xyl1*, *Xyl2* and *Xyl3* (Feng et al. 2015). Adaptive evolution of these strains on xylose as a sole carbon source improved 1-hexadecanol production to a final concentration of 1.2 g/L (Guo et al. 2016).

4 Concluding Remarks

The yeast *S. cerevisiae* is a very tractable microorganism with a long record of useful applications in classical and modern industrial fermentations. This is hitherto the workhorse in winemaking, brewing and baking, as well as in the production of different pharmaceuticals and fuel ethanol from sugarcane and starch. The capabilities that highly positioned this yeast in these applications were however not fully appropriate under practical situations of the cost-efficient, biomass-based processes. Most importantly, the yeast did not metabolize pentoses and had an undesired limited tolerance. Also, a key challenge was to find metabolic and genetic regulatory conditions leading to the synthesis of other molecules besides ethanol in the presence of glucose, including non-natural chemicals.

Through metabolic engineering, yeast capabilities have been improved to fit practical applications of the biomass-to-biochemicals conversion processes. The current progress in the procedures for deciphering genomes, transcriptomes, proteomes, fluxomes and metabolomes, along with mathematical and computational tools, synthetic biology and evolutionary engineering, have led to a new set of technology platforms for metabolic engineering of *S. cerevisiae* in a holistic

manner. These platforms have been providing concepts and methods to partially resolve the obstacles for cost-efficient production of chemicals and biofuels from biomass.

Many more examples of how the holistic understanding of *S. cerevisiae*'s biology has impacted metabolic engineering have been seen up today compared with a few years ago (Nielsen and Jewett 2008). During this period, there has been an explosion of new in silico systems biology methods for mapping detailed phenotypes and for targeting gene modifications for metabolic engineering purposes (Machado and Herrgård 2015). Combined with the advances in sequencing and synthesis of whole genomes and high-throughput technologies, these in silico methods have provided a valuable platform for increasing the production of desired chemicals and improving yeast behavior under commercial-process environments. A key challenge is to find or generate ideal metabolic and regulatory networks for supporting both, cell growth and product formation. As systems metabolic engineering becomes more robust regarding better predictions with genome-scale simulations, we will see that this challenge will be overcome in the near future.

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Zymomonas mobilis for the Conversion of Lignocellulosic Biomass to Fuels and Chemicals

Manoj Agrawal, Kori L. Dunn and Christopher V. Rao

Abstract *Zymomonas mobilis* is a promising organism for the production of biofuels from lignocellulosic biomass as it natively produces ethanol at high yields and at rates far greater than other microorganisms, including *Saccharomyces cerevisiae*. This makes *Z. mobilis* attractive not only for ethanol production but potentially other products as well. One limitation of *Z. mobilis* is that it cannot natively ferment the pentose sugars, xylose and arabinose, present in lignocellulosic hydrolysates. Over the past few decades, a number of strains have been engineered that produce ethanol from lignocellulosic sugars. While many advances have been made, many challenges still remain. This chapter reviews the basic physiology of *Z. mobilis* and the numerous efforts devoted to engineering strains capable of producing ethanol and other chemicals from lignocellulosic sugars.

Keywords Zymomonas mobilis · Biofuels · Ethanol · Metabolic engineering

1 Introduction

Microorganisms have historically been used for the production of a variety of value-added products, including pharmaceuticals, foods, and biofuels (Da Silva et al. 2013). The yeast *Saccharomyces cerevisiae* and the bacterium *Escherichia coli* are popular hosts for product synthesis, because they are well-characterized and are highly amenable to genetic manipulation (Leonelli and Ankeny 2013). However, researchers in recent decades have realized the potential of less common,

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non-model organisms for answering key biological questions and, consequently, for producing chemicals and fuels (Armengaud et al. 2014).

One of the most intriguing non-model organisms is the bacterium *Zymomonas mobilis*. *Z. mobilis* is the only known bacterium that uses the Entner-Doudoroff pathway anaerobically in combination with the pyruvate decarboxylase and alcohol dehydrogenase enzymes (Swings and De Ley 1977). Combining this with the fact that *Z. mobilis* displays the "uncoupled growth" phenomenon, which enables it to metabolize sugars rapidly regardless of its requirements for growth, makes *Z. mobilis* an outstanding ethanol producer (Kalnenieks 2006). This capacity for ethanol production has led many to explore *Z. mobilis* for industrial biofuel production since the early 1980s (Baratti and Bu'lock 1986). Around that time, oil embargoes were threatening the United States' fuel supplies. In response, the development of alternative energy sources became a priority, and the Department of Energy created the Office of Alcohol Fuels (OAF). The OAF was charged with accelerating the scale-up of ethanol production by funding research projects aimed at producing the fuel cheaply in an environmentally-conscious manner (Wyman 2001). This research brought *Z. mobilis* into the spotlight as an organism with the potential to bring the United States out of its energy crisis, commencing years of investigation into this organism's interesting physiology.

Despite its many attractive features, several factors have limited the use of *Z. mobilis* for the commercial production of ethanol from lignocellulosic sugars. The greatest one is that it grows on only a few sugars, namely glucose, fructose and sucrose. In particular, *Z. mobilis* cannot natively ferment pentose sugars such as xylose and arabinose, which are significant components of lignocellulosic hydrolysates. Furthermore, the slow growth rate of *Z. mobilis* prevents it from reaching high cell densities during fermentation. *Z. mobilis*, like *E. coli* and *S. cerevisiae*, cannot tolerate the inhibitors present in lignocellulosic hydrolysates, including acetic acid and various phenolic compounds.

Over the past few decades, significant effort has focused on engineering strains of *Z. mobilis* that produce ethanol from lignocellulosic sugars. While many advances have been made, many challenges still remain. In this chapter, we review the unique characteristic of *Z. mobilis* and the work focused on engineering this bacterium for the production of ethanol and other chemicals from lignocellulosic biomass. A number of excellent reviews on *Z. mobilis* have been published (He et al. 2014b; Yanase et al. 1994; Doelle et al. 1993; Rogers et al. 1984, 2007b; Swings and De Ley 1977; Kalnenieks 2006), and the reader is directed towards them for an alternate and more expansive review of this promising microorganism.

2 Physiology and Metabolism

Z. mobilis is a Gram-negative, obligately fermentative, motile, rod-shaped bacterium frequently found in tropical plant saps and spoiled cider (Swings and De Ley 1977). It produces ethanol and carbon dioxide in equimolar, near-theoretical

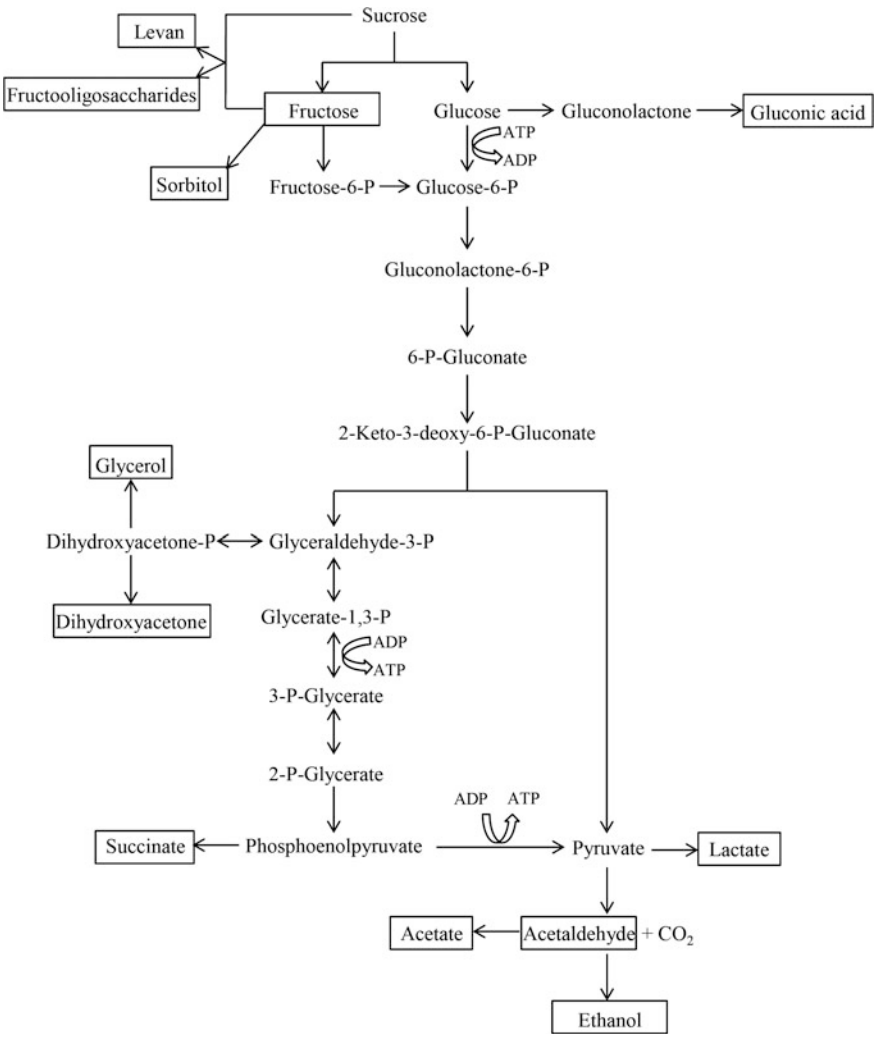

Fig. 1 Pathway for native product formation in *Z. mobilis*

amounts from the sugars glucose, fructose, and sucrose using the Entner-Doudoroff (ED) pathway (Fig. 1), also known as the 2-keto-3-deoxy-6-phosphogluconate (KDPG) pathway, in combination with the pyruvate decarboxylase (Pdc) and alcohol dehydrogenase (Adh) enzymes (Gibbs and Demoss 1954; Dawes et al. 1966). In contrast to other bacteria, *Z. mobilis* uses the ED pathway anaerobically to produce only one mole of ATP per mole of glucose, in place of the Embden-Meyerhof-Parnas (EMP) glycolysis pathway which produces two moles of ATP (Kalnenieks 2006; Conway 1992). *Z. mobilis* is restricted to the ED pathway for the metabolism of sugars because it lacks genes for the 6-phosphofructokinase

enzyme of the EMP pathway and the 2-oxoglutarate dehydrogenase complex and malate dehydrogenase enzymes of the tricarboxylic acid (TCA) cycle (Raps and Demoss 1962; Seo et al. 2005). Interestingly, expression of 6-phosphofructokinase does not activate the EMP pathway in *Z. mobilis* (Chen et al. 2013), suggesting that additional factors such as redox balance prevent activation. Genes for most pentose phosphate pathway (PPP) enzymes are also not present on the *Z. mobilis* chromosome (Seo et al. 2005). The TCA cycle and PPP enzymes present, along with phosphoenolpyruvate carboxylase and malic enzyme, are used for anaplerotic reactions (Sprenger 1996; Bringermeyer and Sahm 1989). Similar to glucose and fructose, sucrose metabolism also proceeds through the ED pathway in *Z. mobilis*, with the sugar first being degraded into glucose and fructose monomers by extracellular levansucrases, frequently with the formation of a levan byproduct (Gunasekaran et al. 1990; Song et al. 1993, 1994; Kannan et al. 1995a, b; Kyono et al. 1995; Goldman et al. 2008; Senthilkumar et al. 2009; Silbir et al. 2014). Roughly 2% of the carbon source by *Z. mobilis* is converted into biomass (McGill and Dawes 1971; Belauich and Senez 1965). This low growth yield is a characteristic of *Z. mobilis* that makes it ideal for product synthesis.

2.1 Uncoupled Growth

A distinguishing characteristic of *Z. mobilis* is that it exhibits uncoupled growth, meaning cells will consume sugars rapidly regardless of their requirements for growth (Belauich and Senez 1965). The catabolic rates in this bacterium are up to 5 times faster than those seen in yeast, reaching up to 1.0 μmol glucose mg dry wt^{-1} min^{-1} during anaerobic exponential growth (Kalnenieks 2006). All ED enzymes are highly expressed, most of them constitutive, and most are not allosteric. The ED proteins comprise up to half of the cells' total protein (Algar and Scopes 1985; An et al. 1991). Although the ATP yield of the ED pathway is the lowest of the fermentative pathways, the high catabolic rate in *Z. mobilis* prevents the organism from suffering from a lack of ATP (Kalnenieks 2006). In fact, due to its low biomass yield, *Z. mobilis* produces excess ATP that must somehow be eliminated. Lazdunski and coworkers provided evidence for a membrane-associated ATPase that eliminates the excess ATP (Lazdunsk and Belaich 1972). Reyes and Scopes later purified what they believed to be the same ATPase, and found that it was of the proton-pumping F_0F_1-type and that it likely accounted for 20% of the total ATP turnover in the cells (Reyes and Scopes 1991). The rest of the ATP turnover is likely due to the action of acid and alkaline phosphatases and a periplasmic 5′-nucleotidase (Reyes and Scopes 1991). The bacterium *Streptococcus bovis* also displays uncoupled growth (Forrest 1967). In this bacterium, ATP spilling by the ATPase is coupled to a cycling of protons across the membrane (Cook and Russell 1994). Presumably *Z. mobilis* employs a similar mechanism, because its ATPase does not actually dissipate energy but instead converts it into proton-motive force (PMF) (Kalnenieks 2006). While a large pH gradient has been

observed across the *Z. mobilis* membrane (Barrow et al. 1984; Kalnenieks et al. 1987; Osman et al. 1987), no accurate determination of the transmembrane electric potential has been made to verify this hypothesis (Kalnenieks et al. 1987; Ruhrmann and Kramer 1992). In addition to the cycling of protons across the membrane, Kalnenieks and coworkers proposed that the PMF could be dissipated through the export of bicarbonate anions from the cell (Kalnenieks 2006). This hypothesis is supported by the presence of the gene for carbonic anhydrase, which converts carbon dioxide into bicarbonate anions, on the *Z. mobilis* chromosome (Seo et al. 2005).

2.2 Respiration

Although obligately fermentative, *Z. mobilis* does possess a constitutive respiratory chain (Belauich and Senez 1965). The respiration rate is higher in *Z. mobilis* than in several well-studied organisms, including *E. coli* and *S. cerevisiae*, but the physiological role of respiration in this organism is still unknown (Kalnenieks 2006). Experiments using inhibitors like cyanide, chlorpromazine, and myxothiazol to quantify and differentiate terminal oxidases support a respiratory chain with a branched structure (Kita et al. 1984a, b; Poole 1994; Kalnenieks et al. 1998). The primary electron donors are NADH and NADPH (Bringer et al. 1984). Glucose and D-lactate are also able to donate electrons to the respiratory chain, but to a much lesser extent—the activity of their dehydrogenases is only about 5% or 10–20% of that of the NADH dehydrogenases, respectively (Strohdeicher et al. 1988, 1989, 1990). NADH donates electrons to either a NADH:ubiquinone oxidoreductase complex or a type II NADH dehydrogenase (ndh) (Seo et al. 2005; Hayashi et al. 2012). The NADH:ubiquinone oxidoreducatse is closely homologous to the genes of an electron transport complex responsible for nitrogen fixation in the bacterium *Rhodobacter capsulatus* (Saez et al. 2001; Schmehl et al. 1993). Evidence of nitrogen fixation in *Z. mobilis* was provided recently, after years of failed attempts (Seo et al. 2005; Kremer et al. 2015). The ability to oxidize NADPH in the respiratory chain of bacteria is rare, but *Z. mobilis* appears to do so using its type II NADH dehydrogenase (Kalnenieks et al. 2008). Glucose and D-lactate donate electrons to membrane-bound glucose dehydrogenase and D-lactate dehydrogenase, respectively (Strohdeicher et al. 1988, 1989; Kalnenieks et al. 1998). All respiratory dehydrogenases then pass their electrons onto the vitamin-like substance ubiquinone, or coenzyme Q_{10}. From Q_{10} the electrons are passed to terminal oxidases that are able to reduce oxygen to water. Researchers are confident that a cytochrome *bd* terminal oxidase is present in *Z. mobilis* (Kalnenieks et al. 1998; Seo et al. 2005), but the identity of the oxidase that terminates the other branch of the *Z. mobilis* respiratory chain is still unclear.

2.3 Oxidative Phosphorylation

The presence of the components of the respiratory chain combined with the presence of the F_0F_1-type ATPase mentioned above would indicate that *Z. mobilis* is able to perform oxidative phosphorylation for energy production, such that its aerobic growth yield would be higher than its anaerobic yield (Kalnenieks 2006). However, this is not the case. Growth yields are typically the same or lower when *Z. mobilis* is grown aerobically versus when it is grown anaerobically (Toh and Doelle 1997; Belauich and Senez 1965). While this implies a non-functional respiratory pathway, Dawes and Large did show that ATP could be produced by starved, aerated *Z. mobilis* from ethanol, presumably through oxidative phosphorylation (Dawes and Large 1970). Kalnenieks and coworkers went on to provide support for this hypothesis by showing that a proton gradient was present in these cells and that the production of ATP was coupled to NADH oxidation. They also showed that the *Z. mobilis* ATPase could directly produce ATP by providing it with an artificial proton gradient (Kalnenieks et al. 1993). In 1997, Zikmanis and coworkers showed that *Z. mobilis* could in fact achieve a higher growth yield during exponential growth in the presence of oxygen than it could without oxygen present, but the effect was only seen under precise culture conditions. In addition to the higher specific growth rate, a lower specific glucose consumption rate and a lower specific ethanol production rate were seen, providing evidence for the importance of aerobic ATP production under these conditions (Zikmanis et al. 1997). Toh and Doelle were also able to achieve higher growth yields in the presence of oxygen, although they were not completely convinced that oxidative phosphorylation was occurring (Toh and Doelle 1997). Regardless, it is clear that if oxidative phosphorylation is functional in *Z. mobilis*, the efficiency of the process is extremely low. The original consensus among researchers was that this is due to the accumulation of acetaldehyde in the culture medium, which is more pronounced in aerobic cultures due to cofactor competition (Tanaka et al. 1990). Acetaldehyde was believed to inhibit *Z. mobilis* growth when present in extremely low amounts (Wecker and Zall 1987). However, it was later determined that under conditions of environmental shock acetaldehyde can actually be beneficial (Stanley et al. 1997), and that reports of its inhibitory effects were inconsistent (Zikmanis et al. 1999; Kalnenieks et al. 2000). It is also known that the inhibition of the oxidative phosphorylation process itself by acetaldehyde is negligible (Kalnenieks et al. 1993). Therefore, the cause of inefficient respiration in *Z. mobilis* is still unclear.

2.4 Tolerance and Nutritional Requirements

While the tolerance of *Z. mobilis* to the ethanol precursor acetaldehyde may be low, its tolerance to ethanol is high. Strains have been reported to tolerate more than 100 g/L, higher than the tolerance of the yeast *S. cerevisiae* (Swings and De Ley

1977; Sprenger 1996). At ethanol concentrations above this amount, leakage of essential cofactors and coenzymes through the plasma membrane occurs (Osman and Ingram 1985). Interestingly. Cho and coworkers have shown that small RNA may regulate the response to high ethanol concentrations (Cho et al. 2014). *Z. mobilis* is also able to tolerate high sugar concentrations of up to 400 g/L (Swings and De Ley 1977). When growing on sucrose, sorbitol is produced by the *Z. mobilis* glucose-fructose oxidoreductase (GFOR), which helps it overcome the osmotic stress of growth in high sugar concentrations. If sucrose is not the carbon source, the addition of sorbitol to the culture medium improves growth at high sugar concentrations and also at high ethanol concentrations (Loos et al. 1994; Sootsuwan et al. 2013). Similarly, salt increases sorbitol production during growth on sucrose, presumably to help overcome osmotic stress (Bekers et al. 2000). However, *Z. mobilis* is not able to grow in media containing more than 20 g/L sodium chloride (Swings and De Ley 1977). The optimum temperature for the growth of *Z. mobilis* is 30 °C, with most strains also able to tolerate temperatures up to 36 °C fairly well (Swings and De Ley 1977; Fieschko and Humphrey 1983). Temperatures above this range cause the lipid-to-protein ratio in the membrane to decline, leading to the leakage of soluble proteins into the surroundings (Benschoter and Ingram 1986). Growth occurs over a large range of pH values, from about 3.5 to 7.5 (Swings and De Ley 1977). Baumler and coworkers found that the expression of a proton-buffering peptide in *Z. mobilis* could improve its survival rate in pH 3 to pH 3.5, but its growth rate at these pH levels was unaffected (Baumler et al. 2006). The need for pantothenate, a precursor of coenzyme A, in *Z. mobilis* minimal medium has been confirmed (Belauich and Senez 1965; Swings and De Ley 1977). In rich medium, yeast extract is not a suitable carbon source and a fermentable sugar must be added (Swings and De Ley 1977). All amino acids, ammonium salts, and peptides can act as nitrogen sources for *Z. mobilis* (Belauich and Senez 1965). Most assimilated sulfur comes from sulfate and methionine (Rogers et al. 1982). The addition of inorganic phosphate and certain metallic ions can also improve the growth of *Z. mobilis* (Dawes and Large 1970; Belauich and Senez 1965).

2.5 Metabolic Models and Systems Biology

Metabolic models provide a deeper understanding of the cellular metabolism, thus paving the way for the rational development of strains with desirable industrial traits. A number of genome-scale metabolic models for *Z. mobilis* have been constructed (Widiastuti et al. 2011; Lee et al. 2010; Motamedian et al. 2016; Tsantili et al. 2007; Pentjuss et al. 2013) following the publication of the *Z. mobilis* ZM4 genome in 2005 (Seo et al. 2005). The models use stoichiometric equations coupled with substrate-uptake and thermodynamic constraints to optimize a particular dependent variable, most commonly growth rate. In one interesting study,

Widiastuti and coworkers investigated gene essentiality using their genome-scale model (Widiastuti et al. 2011). Compared to *E. coli* and *S. cerevisiae*, the fraction of essential genes in *Z. mobilis* is much higher. This indicates that the size of *Z. mobilis* genome, which is more than twofold smaller than *E. coli* and *S. cerevisiae*, has been minimized such that it is left with a much higher fraction of essential genes. Widiastuti and coworkers also found that metabolism is rigid in *Z. mobilis*, which is consistent with the inability of experimentalists to increase glycolytic flux in *Z. mobilis* (Snoep et al. 1996). In addition, they found that alcohol dehydrogenase and pyruvate decarboxylase were the most significant genes for high ethanol productivity of *Z. mobilis*.

Genome-scale models are typically used to investigate steady-state metabolism and growth. More detailed models, which require kinetic rate equations, are needed to understand dynamic changes in metabolism and growth. A few kinetic models of *Z. mobilis* have been reported to date (e.g. Rutkis et al. 2013, 2016). Interestingly, these models predict that the majority of flux control resides outside of the E-D pathway, predominantly in ATP consumption. These predictions are consistent with the lack of increase in glycolytic flux when the glycolytic enzymes are over-expressed (Snoep et al. 1996).

Several industrially desirable strains of *Z. mobilis* have been obtained using evolutionary adaptation. Analysis of these strains provides a mechanistic-level understanding of the factors governing *Z. mobilis* physiology and provides genetic targets for further strain improvement. For example, one study used transcriptomics and proteomics to investigate a strain of *Z. mobilis* adapted for acetate tolerance (Yang et al. 2014). Increased tolerance resulted, in part, from the upregulation of *nhaA*, which encodes for a sodium-proton antiporter. A second study by the same research group compared the transcriptome and metabolome of *Z. mobilis* during aerobic and anaerobic growth. They found that the transcripts for Entner-Doudoroff (ED) pathway genes (*glk*, *zwf*, *pgl*, *pgk*, *eno* and *pdc*) were at least 30-fold more abundant under anaerobic conditions (Yang et al. 2009). This is consistent with the reduced growth rate of *Z. mobilis* under aerobic conditions. Transcriptome profiling has also been used to study the regulation of genes in response to various environmental stresses (He et al. 2012). Transcriptomic profiling of a flocculant mutant identified the downregulation of cellulose synthesis genes and upregulation of flagella-related genes. Both of these changes impart better flocculation (Jeon et al. 2012).

Despite the obvious utility of computational models and tools from systems biology, their application to *Z. mobilis* is still quite limited. In particular, the synergy between computation and experimental studies is largely missing. Bridging this gap will further our understanding of the complex physiology of *Z. mobilis* and enable rapid development of strains for the industrial production of fuels and chemicals.

3 Genetic Engineering

3.1 Early Progress

After the ethanol-producing potential of *Z. mobilis* was brought to light by the laboratory of P.L. Rogers (Rogers et al. 1979), attempts to modify the strain for industrial use intensified. In 1980, Skotnicki and coworkers showed that plasmids of the IncP and IncFII incompatibility groups could be transferred into the strain by conjugation from *E. coli* and *Pseudomonas aeruginosa* (Skotnicki et al. 1980). Realizing that the substrate range of *Z. mobilis* would need to be expanded in order for it to prosper in the ethanol industry, Carey and coworkers used a conjugable vector containing the tetracycline resistance gene to express the lactose operon in the strain (Carey et al. 1983). In 1984, it was shown that *Z. mobilis* could be chemically transformed with a hybrid vector created by fusing an *E. coli* vector and a *Z. mobilis* native vector (Browne et al. 1984). Additional *Z. mobilis* native vectors have since been characterized and used in shuttle vectors (Reynen et al. 1990; Strzelecki et al. 1987; Afendra et al. 1999; Arvanitis et al., 2000; Scordaki and Drainas 1987, 1990; Misawa and Nakamura 1989; Dally et al. 1982; Drainas et al. 1983). Conway and coworkers were the first to construct a vector designed specifically for protein expression in *Z. mobilis* (Conway et al. 1987a). The vector contained a broad-host-range origin of replication, a chloramphenicol acyltransferase gene, and a sequenced *Z. mobilis* promoter directly upstream of a restriction site that could be used for gene cloning (Conway et al. 1987a). This group also sequenced the promoter of pyruvate decarboxylase, one of the most abundant proteins in *Z. mobilis* (Conway et al. 1987c). This strong promoter has been used frequently for heterologous protein expression in the strain since then. Additionally, Conway and coworkers characterized promoter structure in general in *Z. mobilis* using beta-galactosidase (LacZ) as a reporter (Conway et al. 1987b). They found that the *Z. mobilis* promoter sequences share several features with *E. coli* promoter sequences and contain similar consensus sequences in the −10 region and partial sequence homology in the −35 region. The sequence AGGA was also identified as a possible ribosome binding site in *Z. mobilis*, appearing 8–12 base pairs upstream of the start codon (Conway et al. 1987b). In 1997, Pappas and coworkers were the first to show that transposable elements present on suicide vectors could be used to mutagenize the *Z. mobilis* chromosome (Pappas et al. 1997). The authors successfully used the mini Mu transposon to construct stable *Z. mobilis* auxotrophs, thereby demonstrating the feasibility of the method for chromosomal integrations.

3.2 Challenges Associated with Genetic Engineering

While initial attempts to genetically modify *Z. mobilis* showed promise, several difficulties were later realized. Although gene expression from the lactose operon of

Carey and coworkers was confirmed in *Z. mobilis*, the strain was unable to grow on lactose as its sole carbon source (Carey et al. 1983). Later attempts to expand the substrate range of *Z. mobilis* were also met with difficulty (Liu et al. 1988; Feldmann et al. 1992; Yanase et al. 1988; Byun et al. 1986). While conjugation was a reliable method for introducing plasmid DNA into *Z. mobilis*, chemical transformation and electroporation were not (Skotnicki et al. 1982; Buchholz and Eveleigh 1986; Sprenger 1993). If plasmid DNA was successfully transformed into the cells, it was often unstable and readily lost, especially if it did not contain an origin of replication from a *Z. mobilis* native vector (Afendra and Drainas 1987; Pappas et al. 1997; Strzelecki et al. 1990; Bresticgoachet et al. 1990; Scordaki and Drainas 1987; Dong et al. 2011). Low plasmid stability in the strain is thought to be due to the presence of both type I and type IV restriction-modification systems, which are able to degrade foreign DNA (Kerr et al. 2011; Typas and Galani 1992). Indeed, inactivating these restriction-modification enzymes in *Z. mobilis* increases the transformation efficiency (Kerr et al. 2011). Protocols for chromosomal integrations and gene knockouts have not improved since the suicide vector technique developed by Pappas and coworkers (Pappas et al. 1997), and targeted methods that utilize suicide vectors require hundreds or thousands of base pairs of homology to the target region but still yield only a few transformants (Senthilkumar et al. 2004; Agrawal et al. 2012; Delgado et al. 2002; Kerr et al. 2011). To date, only a few genes have been deleted in *Z. mobilis*, often with great difficulty. Further, an accepted set of promoters and ribosome binding sites for the precise control of gene expression in *Z. mobilis* has not been developed. Studies to date have primarily relied on the use of strong and constitutive promoters for the uncontrolled expression of heterologous genes, further complicating gene expression optimization in the strain (Zhang et al. 1995; Deanda et al. 1996; Jeon et al. 2005). Collectively, the resistance of *Z. mobilis* to genetic modification has led to the emergence of only a few recombinant strains (Kerr et al. 2011). To date, *Z. mobilis* has not been accepted as an industrial organism, despite its unique physiology and rapid ethanol production. The acceptance of *Z. mobilis* for the industrial production of ethanol and other value-added products will require the improvement and standardization of genetic techniques. In the absence of efficient rational genetic engineering tools, researchers employed evolutionary adaption and mutagenesis instead to develop several industrially desirable strains. Some of these strains are discussed in subsequent sections.

4 Production of Lignocellulosic Ethanol

Ethanol is traditionally produced by the yeast *S. cerevisiae* from the six-carbon sugars found in food crops such as corn and sugarcane (Wheals et al. 1999). More recently, focus has shifted to producing biofuels from lignocellulosic feedstocks, such as agricultural and municipal wastes (Schubert 2006). Cellulosic feedstocks

are cheap, abundant, and do not compete with the nation's food supply (Lynd et al. 1991). They are made up of three primary components: cellulose, hemicellulose, and lignin (Demirbas 2009). After mechanical degradation and an acid pretreatment, cellulose and hemicellulose can be broken down enzymatically into a mixture primarily composed of the six-carbon sugar glucose and the five-carbon sugars xylose and arabinose, which can then be fermented into ethanol or other biofuels by microorganisms (Lawford and Rousseau 2002; Carroll and Somerville 2009). The sugar mixture may also contain a variety of growth inhibitors formed during pretreatment, including acetic acid, formic acid, furfural, 5-hydroxymethylfurfural and levulinic acid (Shuai et al. 2010; Yang et al. 2010a, b). In order for the cellulosic ethanol production process to be economical, it is important that the microorganism used have several important characteristics. It must be able to ferment both five and six-carbon sugars rapidly to ethanol, in a process tolerating high concentrations of both the sugars and the ethanol, and it must also have a high tolerance to the inhibitors in the hydrolysate (Dien et al. 2003; Bothast et al. 1999). Further, the organism should be safe, resistant to contamination, and amenable to genetic manipulation (Nevoigt 2008).

Due to its naturally high ethanol productivity, *Z. mobilis* is an obvious contender in the search for an industrial cellulosic ethanologen. This productivity is up to 5 times higher than that observed in *S. cerevisiae*, the traditional host for ethanol production (Rogers et al. 1979; Zhang et al. 1995). Ethanol yields of up to 97% of theoretical are possible with *Z. mobilis*, primarily due to its unique homoethanologenic metabolism and low biomass yield (McGill and Dawes 1971; Belauich and Senez 1965). Furthermore, *Z. mobilis* has an extremely high tolerance to both ethanol and the sugars present in the hydrolysate (Rogers et al. 1979). As an organism frequently used for beverage making, *Z. mobilis* is generally regarded as safe for industrial use (Swings and De Ley 1977). It is resistant to contamination by other microorganisms, and there are no known phage (Sahm et al. 2006). Its ability to grow at an acidic pH further renders it robust to contamination (McGill and Dawes 1971; Belauich and Senez 1965). Finally, *Z. mobilis* is aerotolerant but does not require oxygen for growth, simplifying process design requirements and reducing production costs (Swings and De Ley 1977).

In spite of these attractive advantages, several characteristics of *Z. mobilis* have prevented it from gaining acceptance for lignocellulosic ethanol production. The main one is that *Z. mobilis* has a very narrow substrate range and is unable to natively digest the five carbon sugars, xylose and arabinose, that are present in the lignocellulosic hydrolysate (Swings and De Ley 1977). Additionally, *Z. mobilis* has a low tolerance to some of the inhibitors present in this hydrolysate, and is especially sensitive to acetic acid (Jeon et al. 2002; Kim et al. 2000b; Ranatunga et al. 1997). Finally, as discussed above, researchers have faced several difficulties genetically modifying *Z. mobilis* (Kerr et al. 2011). In order for *Z. mobilis* to be competitive in the lignocellulosic ethanol industry, these difficulties must be overcome.

4.1 *Engineering* Z. mobilis *for the Production of Lignocellulosic Ethanol*

Early attempts to engineer *Z. mobilis* specifically for lignocellulosic ethanol production focused on expanding the organism's substrate range to include xylose and arabinose (Rogers et al. 2007a). In 1987, Liu and coworkers expressed xylose isomerase, xylose permease, and xylulokinase from *Xanthomonas* XA1-1 in *Z. mobilis*. Although enzyme expression was confirmed, the strain was unable to grow on xylose as its sole carbon source (Liu et al. 1988). Similarly, Feldmann and coworkers expressed xylose isomerase and xylulokinase from *E. coli* in *Z. mobilis*, and used this strain to isolate a mutant that produced less xylitol phosphate, a potent growth inhibitor produced when xylose is consumed (Feldmann et al. 1992). They then expressed the *E. coli* transketolase gene in the mutant strain, and again, although enzyme activities were confirmed, the strain was unable to grow on xylose as its sole carbon source. The authors determined that this was likely due to the absence of detectable transaldolase activity in *Z. mobilis*, but since no transaldolase gene had yet been cloned the authors did not attempt to express this protein in the recombinant strain (Feldmann et al. 1992). It was not until 1995, when researchers at the National Renewable Energy Laboratory were able to clone and express the putative *E. coli* transaldolase gene together with the *E. coli* xylose isomerase, xylulokinase, and transketolase genes in *Z. mobilis*, that an engineered strain was able to grow on xylose as its sole carbon source (Zhang et al. 1995) (Fig. 2). Shortly thereafter, the same laboratory was able to engineer *Z. mobilis* to consume arabinose as its sole carbon source by expressing the arabinose isomerase, ribulokinase, ribulose-5-phosphate-4-epimerase, transaldolase, and transketolase genes from *E. coli* in the strain (Deanda et al. 1996).

The rates of xylose and arabinose consumption, however, in these strains were far slower than the rate of glucose consumption, even though the ATP yields are the same for the two sugars on a carbon-content basis (Deanda et al. 1996; Zhang et al. 1995; Agrawal et al. 2011; De Graaf et al. 1999; Joachimsthal and Rogers 2000; Lawford and Rousseau 2000). In addition, this prevents the simultaneous utilization of these sugars. In a manner similar to catabolite repression, these engineered strains of *Z. mobilis* will first consume the glucose before they consume xylose or arabinose. Consequently, later efforts focused on investigating the bottlenecks present in xylose and arabinose metabolism in *Z. mobilis*. The production of xylitol and xylitol phosphate, which was uncovered by Feldmann and coworkers in 1992, was determined to play an important role in limiting xylose fermentations by the strain (Feldmann et al. 1992; Kim et al. 2000a; Akinterinwa and Cirino 2009). At least two pathways contribute to xylitol formation in *Z. mobilis*—one involving the glucose-fructose oxidoreductase of central metabolism, and the other involving an NADPH-dependent xylose reductase (Feldmann et al. 1992; Zhang et al. 2009). Agrawal and Chen later found that a strain of *Z. mobilis* adapted to growth on xylose as its sole carbon source had a mutation in the NADPH-dependent xylose reductase that greatly decreased its activity (Agrawal et al. 2011). They

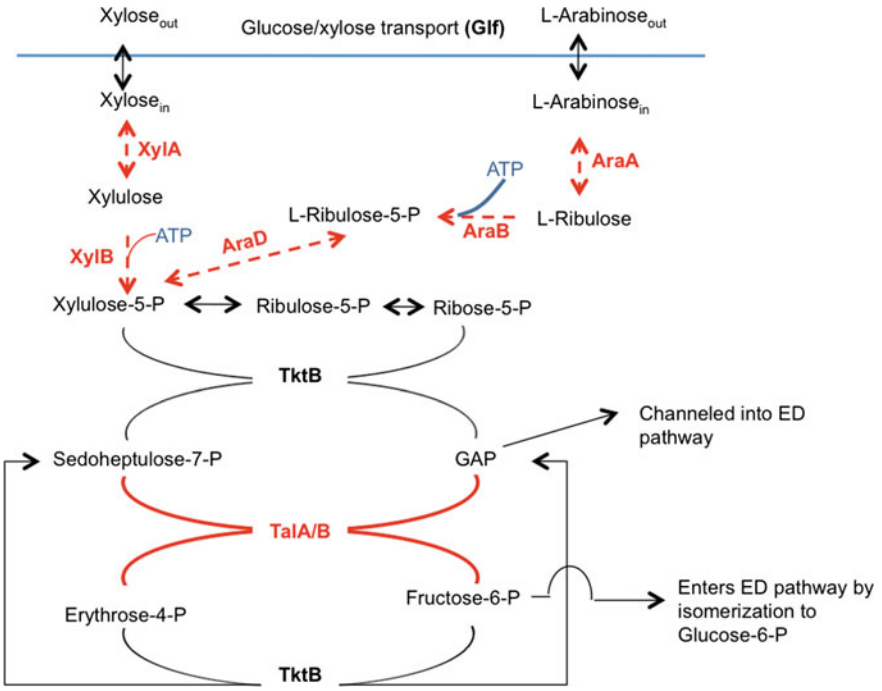

Fig. 2 Engineered pathway for the utilization of pentose sugars in *Z. mobilis*. Heterologous enzymes are denoted in *red*

subsequently deleted this reductase from the chromosome of their xylose-fermenting strain, and found that xylitol production was decreased with a concomitant increase in the rate of xylose metabolism (Agrawal and Chen 2011). Aside from xylitol formation, Kim and coworkers found that *Z. mobilis* grown on xylose was in a less-energized state than *Z. mobilis* grown on glucose, possibly due to the weak activities of the heterologously-expressed xylose isomerase and xylulokinase enzymes (Kim et al. 2000a). Although the studies are unrelated, the adapted strain of Agrawal and coworkers did have a mutation in the promoter region of xylose isomerase that increased its activity and presumably expression by roughly fivefold, revealing the importance of the proper expression level of this enzyme. However, in another separate study, increased expression of xylulokinase was found to not increase the rate of xylose metabolism (Jeon et al. 2005). If anything, it decreased it, possibly due to the associated increase in xylitol production, although no increase in intracellular xylitol-5-phosphate was observed in the strain.

Less work has been done to determine bottlenecks present in arabinose metabolism in *Z. mobilis*. Deanda and coworkers were not able to discern why their strain did not perform as well on arabinose as it did on glucose, but the authors did

propose that inefficient arabinose transport by the *Z. mobilis* native transporter, the glucose facilitated diffusion protein or Glf, may be limiting arabinose fermentations in the strain (Deanda et al. 1996). Dunn and Rao used high-throughput sequencing to identify the mutations that increased growth on xylose and arabinose in separately adapted strains (Dunn and Rao 2015). Among the many mutations identified in the two adapted strains, they found mutations in both Glf, the glucose facilitated diffusion protein involved in sugar transport, and AddB, a double-strand break repair protein involved in plasmid stability (Meima et al. 1995, 1997), for both the xylose and arabinose adapted strains. Regarding AddB, they found that the adapted strains had improved plasmid stability (~2–4 fold), and that loss of AddB severely reduced plasmid stability (~40 fold). These results further demonstrate the need for improved genetic tools, in particular better tools for the stable expression of heterologous proteins. In addition, Dunn and Rao found that expression of the mutated Glf increased the rate of xylose and arabinose metabolism in unadapted strains, suggesting that inefficient transport is indeed a key bottleneck. Along these lines, Dunn and Rao found in a separate study that expression of the low-affinity xylose transporter XlyE from *E. coli* in *Z. mobilis* increased the rate of xylose metabolism, but only at high-xylose concentrations (Dunn and Rao 2014). These results suggest that introducing alternate transporters can be used to increase the rate of arabinose and xylose metabolism. In addition, it may overcome the issue of catabolite repression, as glucose is thought to competitively inhibit the uptake of xylose and arabinose (Deanda et al. 1996; Weisser et al. 1996). Besides glucose, xylose and arabinose, the other minor sugars present in lignocellulosic hydrolysates are mannose, galactose and rhamnose. *Z. mobilis* has been engineered to ferment mannose into ethanol (Weisser et al. 1996). In addition, it has also been engineered to ferment galactose to ethanol (Yanese et al. 1991).

Work has also been done to increase the tolerance of *Z. mobilis* to the inhibitors that are present in lignocellulosic hydrolysates. Ranatunga and coworkers determined that acetic acid had by far the largest detrimental effect on xylose-fermenting *Z. mobilis*, with cells performing at only 9% of maximal in the presence of acetic acid concentrations typically present in cellulosic hydrolysates (Ranatunga et al. 1997). Far behind were caproic acid and furfural, which caused cells to perform at only 57 and 58% of maximal, respectively. Kim and coworkers used nuclear magnetic resonance studies to determine that acetic acid inhibits *Z. mobilis* by acidifying the cytoplasm and decreasing the amounts of nucleotide triphosphates and sugar phosphates present in the cell (Kim et al. 2000b). Yang and coworkers later isolated a strain of *Z. mobilis* with increased acetic acid tolerance using chemical mutagenesis and by gradually increasing the concentration of sodium acetate present in the culture medium. They discovered that their strain had increased expression of the sodium-proton antiporter gene *nhaA* that likely improved the sodium tolerance of their strain (Yang et al. 2010a). Chen and colleagues also isolated a strain of *Z. mobilis* with improved acetic acid tolerance using adaptation, and separately isolated strains with improved tolerance to other

inhibitors present in lignocellulosic hydrolysates, also using adaptation (Chen et al. 2009). Hfq, an RNA-binding proteins, has also been shown to increase tolerance in Z. *mobilis* towards acetate, sodium, vanillin, furfural and HMF (Yang et al. 2010b). Skerker and coworkers took a different approach and constructed a library of *Z. mobilis* mutants using transposons. They then analyzed the library for inhibitor tolerance using chemogenomic profiling and found 44 genes important for hydrolysate tolerance in *Z. mobilis*. The overexpression of one of these genes, ZMO1875, improved the specific ethanol productivity of the strain 2.4-fold in the presence of miscanthus hydrolysate (Skerker et al. 2013). In addition to inhibitors, Tan and coworkers used random mutagenesis, specifically by mutating the sigma factor RpoD, to further improve the tolerance to high ethanol concentrations in *Z. mobilis* (Tan et al. 2016).

While some bottlenecks in xylose and arabinose fermentations by *Z. mobilis* have been identified and its tolerance to important inhibitors has been increased, ethanol production by the strain from lignocellulosic pentose sugars still lags behind ethanol production from hexose sugars. In order for the industrial ethanol production process to be economical, pentose sugar fermentations by *Z. mobilis* must be improved further.

4.2 Consolidated Bioprocessing

Consolidated bioprocessing (CBP) refers to the use of a single microorganism to convert pretreated lignocellulosic biomass to ethanol through the simultaneous production of saccharolytic enzymes and fermentation of the liberated monomeric sugars. A CBP microbe can be created by imparting fermenting capabilities to a cellulolytic microbe or cellulolytic capabilities to an ethanologen such as *Z. mobilis*. To depolymerize cellulose, *Z. mobilis* needs to be capable of secreting at least three enzymes: an endoglucanase, an exoglucanase, and a β-glucosidase. By expression of a native signal peptide attached to a heterologus β-glucosidase, Yanse and coworkers engineered *Z. mobilis* to ferment cellobiose to ethanol with near theoretic yields (Yanase et al. 2005). Two heterologous endo-1,4-β-glucanases, one which was putative, were successfully expressed and secreted by *Z. mobilis* while still retaining activity (Linger et al. 2010). In addition, several *Z. mobilis* strains have endogenous activity against carboxymethyl cellulose and this activity can be detected extracellularly (Linger et al. 2010). This means that *Zymomonas* is amenable to heterologous expression and secretion of cellulolytic enzymes. The Z. *mobilis celA* gene has been shown to possess β-1,4-endocellulolytic activity (Rajnish et al. 2008). Thus, *Z. mobilis* possesses native cellulolytic activity and can be engineered to secrete cellulolytic enzymes, which makes it a promising candidate for consolidated bioprocessing.

5 Production of Alternate Chemicals and Fuels

Z. mobilis has historically received considerable attention for its high ethanol productivity from glucose. However, researchers often overlook the fact that *Z. mobilis* can also be used to produce other valuable metabolites under the correct culture conditions (Johns et al. 1991). When grown on sucrose, *Z. mobilis* produces considerably less ethanol and considerably more byproducts than when grown on glucose, suggesting that glucose availability and concentration affect whether cells channel their carbon source into the ED pathway to produce ethanol or elsewhere to produce byproducts (Johns et al. 1991). *Z. mobilis* is a promising biocatalyst for alternative product synthesis because of its low biomass yield and high sugar uptake rate, which allows carbon to quickly become fuels or chemicals instead of biomass (McGill and Dawes 1971; Belauich and Senez 1965). It also produces a wide variety of growth-linked byproducts that hold commercial value, allowing for these products to all be isolated in a single process (Johns et al. 1991). Further, it is a rich source of many enzymes currently used in diagnostic analysis and research (Rogers et al. 2007a). As with ethanol production, *Z. mobilis* is also a desirable host for alternative product synthesis because it is generally regarded as safe, it is resistant to contamination, and it has relatively simple growth requirements (McGill and Dawes 1971; Belauich and Senez 1965; Swings and De Ley 1977).

Regardless of the potential of *Z. mobilis* for the production of value-added products, the organism will need to be further engineered to make any of the processes economical. Cellular metabolism should be shifted away from ethanol production and toward the production of the desired product—this will require the construction of knockout and overexpression mutants and the precise control of heterologous gene expression in *Z. mobilis* (Rogers et al. 2007a). Therefore, genetic techniques for constructing these recombinant strains clearly need to be improved in order for the potential of *Z. mobilis* for the production of value-added products to be realized.

5.1 Production of Alternative Fuels and Chemicals

Z. mobilis is natively able to produce the byproducts fructose, sorbitol, gluconate, fructooligosaccharides, and levan at concentrations exceeding 1 g/L (Johns et al. 1991). It also produces a variety of byproducts in small amounts, including mannitol, acetaldehyde, glycerol, dihydroxyacetone, and lactic, gluconic, succinic, and acetic acids (Viikari and Gisler 1986; Wecker and Zall 1987; Schmidt and Schugerl 1987; Jobses et al. 1985). *Z. mobilis* has received some attention for the commercial production of fructose from sucrose with the concomitant production of ethanol, both of which are value-added products (Doelle and Greenfield 1985). In order for this to be possible at industrially desirable levels, however, a fructokinase-negative mutant must be used so that the cells cannot consume the fructose that is being

produced by sucrose splitting, and instead only convert the glucose into ethanol (Pankova et al. 1988). This process is desirable over the process currently used to produce the food sweetener high-fructose corn syrup because fructose and ethanol are relatively easy to separate from one another (Johns et al. 1991). Sorbitol, levan, and fructooligosaccharides are also value-added products that can be produced by *Z. mobilis* during growth on sucrose under very specific culture conditions. These products are commonly used in the food and pharmaceutical industries. A high rate of sucrose hydrolysis favors sorbitol production, while a low rate favors levan production (Viikari and Gisler 1986; Doelle and Greenfield 1985). Fructooligosaccharide production occurs as an alternative to levan production when salt concentrations are high (Doelle et al. 1990; Doelle and Doelle 1990). Sorbitol production in *Z. mobilis* has been improved by overexpressing the glucose-fructose oxidoreductase enzyme, responsible for producing sorbitol in a single step from glucose and fructose (Liu et al. 2010). Levan production in *Z. mobilis* has been improved by lowering the rate of sucrose hydrolysis using a strain with the *sacC* gene inactivated (Senthilkumar et al. 2004). *Z. mobilis* can also be used for the isolation of a variety of industrially-relevant enzymes, including fructokinase, glucose-6-phosphate dehydrogenase, pyruvate kinase, pyruvate decarboxylase and glucose-fructose oxidoreductase (Johns et al. 1991). A mutant form of pyruvate decarboxylase was engineered and used in the production process of an important pharmaceutical (Rogers et al. 1997; Pohl 1997; Goetz et al. 2001; Iwan et al. 2001), but little else has been done toward improving enzyme yields for commercial use in *Z. mobilis*.

While *Z. mobilis* is promising for industrial fructose production, the process is tainted by the concomitant production of large amounts of sorbitol. This is detrimental because the amount of sorbitol allowed in food products is often limited, and fructose and sorbitol are difficult to separate. While growth condition optimization can be used to decrease the amount of sorbitol that is produced, careful genetic engineering of the fructokinase-negative mutants can possibly decrease sorbitol production further, making the process more industrially feasible. Similarly, it is possible that overexpressing the *Z. mobilis* sucrases could improve sorbitol production in the strain and improve its industrial outlook.

Aside from sugar production, *Z. mobilis* has been engineered to produce l-alanine and β-carotene (Uhlenbusch et al. 1991; Ruhrmann et al. 1994; Misawa et al. 1991). However, the resulting titers are low. More recently, Yang and coworkers engineered *Z. mobilis* to produce the commodity chemical 2,3-butandiol with a concomitant decrease in ethanol production (Yang et al. 2016). This work is promising as they were able to achieve reasonable titers (>10 g/L). In general, all routes of alternative product synthesis in *Z. mobilis* would benefit from additional engineering of the strains to decrease ethanol production and increase production of the desired products. In working toward this goal, the current consensus in the field is that genetic engineering techniques need to be improved and modernized in order for *Z. mobilis* to be considered as a viable platform for future biorefineries (He et al. 2014a).

6 Conclusions

Z. mobilis is a promising microorganism for the commercial production of the ethanol from lignocellulosic biomass and potentially for the commercial production of other value-added products. In order for these processes to be economical, however, more work needs to be done. The metabolism of the lignocellulosic pentose sugars by this microorganism is still slow and lags far behind the digestion of hexose sugars; the full reason for this is unknown. Ideally, all pentose and hexose sugars would be consumed simultaneously. Furthermore, although *Z. mobilis* is a promising platform for the isolation of other biofuels and chemicals, little progress can be made with the inefficient genetic tools available today. Ideally, modern genetic engineering techniques would be applied for use in *Z. mobilis*. In addition, development of reliable metabolic models will accelerate the development of *Z. mobilis* by guiding strain design.

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Glycerol as Carbon Source for Production of Added-Value Compounds

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Abstract Glycerol and crude glycerol from the biodiesel industry have become large scale and inexpensive products making them very attractive for biotechnological use of these carbon sources. Glycerol catabolic pathways (aerobic and anaerobic) in microorganisms (bacteria such as *Klebsiella*, *Escherichia coli*, Clostridia, or yeast) are discussed in the context of products which can be derived from glycerol with good to excellent productivities. Important products are dihydroxyacetone, 1,3-propanediol, acetol, 3-hydroxypropionic acid, ethanol, succinate, malate, aromatic amino acids, hydrogen, polyhydroxyalkanoates, lipids, various natural products, and novel fine chemicals. Glycerol and crude glycerol have become attractive carbon sources for biotechnological uses.

Keywords Glycerol · Crude glycerol · Biodiesel · 1,3-propanediol · Dihydroxyacetone · 3-hydroxypropionic acid · Hydrogen · Amino acids · Aromatics

1 Introduction

Glycerol (glycerine, propane-1,2,3-triol; $CH_2OH–CHOH–CH_2OH$) is a compound which is found in all living cells as membrane constituent or in storage lipids. In recent years, glycerol and crude or raw glycerol (roughly 80% purity; nowadays mainly from biodiesel production) have received renewed interest in biotechnology as they can be used as inexpensive carbon and energy sources for various microbial productions. Mainly due to the steep decrease in the price of crude glycerol as a consequence of increasing worldwide biodiesel production [about 100 kg of crude glycerol is a byproduct of 1 ton of biodiesel (McCoy 2006)], considerably more

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research is heading for substitution of glucose by glycerol in processes for the production of bulk chemicals such as bioethanol, 1,2- or 1,3-propanediol (1,3-PDO), dihydroxyacetone, hydroxyacetone/acetol, hydrogen, 3-hydroxypropionic acid (3HP), acrylic acid, pyruvic acid, malic acid, succinic acid, amino acids, biopolymers, oils and lipids, and other fine chemicals. The wealth of recent scientific literature in this field already has led to many reviews on glycerol sources and their utilization in chemical and biotechnological processes. Therefore and due to space limitations of the present review, in many cases only recent examples of applications (2013 and onward) can be quoted here; the reader is referred to several excellent reviews for further comprehensive treatments of the whole or partial fields of glycerol conversions, including tabular compilations and further references therein (Cameron and Tong 1993; Deckwer 1995; Wang et al. 2001; Taherzadeh et al. 2002; Da Silva et al. 2009; Saxena et al. 2009; Chatzifragkou et al. 2011; Wendisch et al. 2011; Zeng and Sabra 2011; Dobson et al. 2012; Khanna et al. 2012; Yang et al. 2012; Clomburg and Gonzalez 2013; Li et al. 2013; Tan et al. 2013; Jiang et al. 2016). I apologize to those colleagues whose work cannot be treated adequately herein.

Glycerol as feed additive for animals or as bulk chemical for chemical syntheses is not considered herein (for a review see Behr et al. 2008), in vitro enzymatic conversions of glycerol will be discussed briefly only. Glycerol as carbon source for biomass production only, or for protein expression in microorganisms is not within the frame of this review.

2 Glycerol and Crude Glycerol: Natural Occurrence and Source for Biotechnological Applications

Glycerol is widely found in natural compounds such as lipids and has become a commodity chemical. It is being used for preparation of soaps, lubricants, cosmetics and pharmaceuticals. Moreover, it is added to anti-freeze solutions and can also be used in feed and food (Yang et al. 2012). Glycerol can be found in ester bonds with fatty acids or phosphoric acid. Phosphoglycerolipids consist of esters of glycerol with one phosphoric acid moiety and one or two fatty acids; they are essential constituents in membranes of eukaryotes and bacteria. Esterification of glycerol with up to three fatty acids leads to mono-, di- or triglycerides which can be stored as lipid droplets or waxes in bacteria (*Rhodococcus*), in algae, oleaginous yeasts (*Yarrowia lipolytica*, *Debaryomyces occidentalis* a.o.), in plants and animals (Lamers et al. 2016). The reverse reaction of esterolysis leads to free glycerol and fatty acid(s). Plant oils, nowadays mainly from oil palms, soybeans or rapeseed/canola are rich sources of triglycerides and are of commercial importance. Other technical sources are waste plant oils (from production of deep-fried foods), from fat saponification, or alcohol beverage production (Mironczuk et al. 2015). Over the last decades, demand for plant oils has been steadily increasing due to the

use of plant oils in food, feed, non-food and personal care (soap, cosmetics), technical use (lubricants) and biodiesel production (Ciriminna et al. 2014). Especially with the preparation of fatty acid methyl esters (FAME) from animal fat or plant oils by chemical transesterification (with methanol) or alcoholysis (with ethanol) (Tan et al. 2013; Loaces et al. 2016), biodiesel production has skyrocketed since the early 2000s (Clomburg and Gonzalez 2013; Ciriminna et al. 2014). As a by-product, glycerol is inevitably formed at about 10% (100 kg of crude glycerol for every ton of biodiesel produced) (McCoy 2006). This led to a surplus of crude glycerol on the markets as for many years demand has been lower than the supply. This has led to a severe drop in prices which in turn made crude glycerol a commercially available carbon source for biotechnology which could compete with others like molasses or glucose. For 2015, the US Energy Information Administration reported an annual production of 1268 billion gallons (almost 5 million tons) of biodiesel in the USA (Monthly Biodiesel Production Report 2016) roughly the same number as for 2014.

Prices for crude glycerol have been down to 5 cents per pound (11 cents per kg) making it almost a waste stream rather than a valued by-product (Yang et al. 2012); meanwhile a modest rebound in price has occurred (Pyne et al. 2016). A small special market, which absorbs only a part of the annual production, lies in pharma grade glycerol after costly refining by filtration and vacuum distillation. Crude glycerol is being used in animal feed, for technical applications or as chemical building blocks. Crude glycerol is often composed of glycerol (>80%), methanol, ash, water, and organic matter non glycerol (OMNG) with an alkaline pH (Tan et al. 2013; Loaces et al. 2016).

Formerly, glycerol has also been produced by chemical means (reduction of dihydroxyacetone), or by microbial biotransformations such as the long known fermentative route from sugar in yeast (Neuberg and Reinfurth 1918; Connstein and Lüdecke 1919; Wang et al. 2001; Buchholz and Collins 2013). Apart from the classical bisulfite method or other chemical interventions, yeast mutant strains have been generated with improved glycerol formation. For example on a 400 g/l of glucose a nearly molar yield ($\sim$200 g/l of glycerol) were obtained from a quadruple mutant of yeast lacking genes for triosephosphate isomerase, two NADH-dehydrogenases and a glycerol 3-phosphate (G3P) shuttle transporter (Overkamp et al. 2002). More recently, in recombinant *E. coli* strains expressing yeast GPD and GPP enzymes, glycerol was produced from glucose, as a transient intermediate of the industrially implemented 1,3-PDO process of DuPont Tate & Lyle Bio Products (Nakamura and Whited 2003). The current surplus of glycerol from biodiesel on the world market (Posada et al. 2012), makes glycerol production from glucose or other carbohydrates less interesting (Saxena et al. 2009) unless glycerol is shunted directly into other products like 1,3-PDO (see below) (Ciriminna et al. 2014).

3 Glycerol Metabolic Pathways in Microorganisms

To understand glycerol as carbon source for possible biotechnological uses, a knowledge of glycerol-specific metabolic pathways is necessary. Elucidation of these glycerol-specific metabolic features has already led to a series of new products which are of potential commercial interest. Among others are pathways leading to dihydroxyacetone, 1,3-propanediol, 1,2-propanediol, acetol, and various organic acids. Let us have a look first on the general catabolic pathway and then to the specific issues of glycerol metabolism.

While most microorganisms share similar pathways for glycerol breakdown (Lin 1996), there exist a few noteworthy exemptions. Uptake of glycerol across biological membranes can occur via simple diffusion (if glycerol is present in millimolar concentrations), or by facilitated diffusion making use of aquaporin-like transporters (glycerol facilitator, GlpF in *E. coli*). GlpF is necessary when external concentrations are in micromolar ranges (Richey and Lin 1972; Lin 1996). In yeast, a proton-coupled uptake has been described (Swinnen et al. 2016). As diffusion also could lead to loss of glycerol, chemical transformation either by oxidoreduction to dihydroxyacetone (DHA) by a glycerol dehydrogenase (Gld) or phosphorylation by an ATP-dependent kinase (GlpK) are the main routes found in microorganisms (Lin 1996). GlpK in *E. coli* is both subject to inducer exclusion exerted by non-phosphorylated Enzyme IIA^{Glc} from the PEP-dependent sugar phosphotransferase (PTS) and is allosterically inhibited by fructose-1,6-bisphosphate (see Fig. 1a). This blocks glycerol catabolism as long as glucose or other catabolite-active compounds are present.

Glycerol-3-phosphate (G3P) can be transformed into DHAP by several routes. Apart from L-α-glycerophosphate oxidases (H_2O_2-forming) which occur in some Streptococci, Enterococci and *Mycoplasma* (Parsonage et al. 1998; Colussi et al. 2008; Pilo et al. 2005), most yeasts and bacteria utilized G3P dehydrogenases. This can be either NAD-dependent enzymes or flavin-dependent dehydrogenases that deliver their electrons to membrane-bound quinones of the electron transport chain (Lin 1996). Both reactions lead to 1,3-dihydroxyacetone 3-phosphate (DHAP) which can enter the lower trunk of glycolysis or gluconeogenic pathways isomerization to glyceraldehyde 3-phosphate (Ga3P) via triosephosphate isomerase. The further route to pyruvate utilizes the enzymes of the Embden-Meyerhof-Parnas (EMP) pathway (see Fig. 1a) and the TCA cycle leading to complete oxidation. Through $NADH_2$ oxidation, electron transport chain enzymes and finally oxidative phosphorylation, ATP is yielded. Compared to energy yield per carbon atom, glycerol thus is a richer source than glucose due to its higher degree of reduction which yields more reducing equivalents ($NADH_2$ or other redox equivalents).

For growth on glycerol as sole carbon source, gluconeogenesis is also necessary. At the stage of DHAP and glyceraldehyde 3-phosphate (Ga3P), an aldol reaction leads to fructose-1,6-bisphosphate (Fig. 1a). Many microorganisms have a fructose-1,6-bisphosphatase encoded by a glycerol-inducible isoenzyme (GlpX) which is different to the gluconeogenic Fba enzyme (Gottlieb et al. 2014).

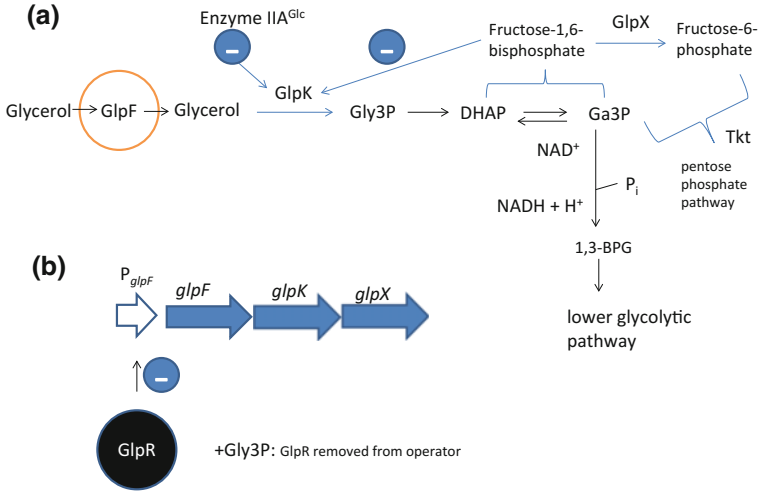

Fig. 1 Enzymatic and genetic regulations in glycerol catabolism of *Escherichia coli.* **a** Schematic depiction of glycerol uptake, catabolism and enzyme inhibition in *E. coli* cells (Lin 1996). Glycerol can either diffuse across the inner membrane (not depicted) or being taken up by facilitated protein-mediated diffusion through the carrier, GlpF (Richey and Lin 1972). Inside the cell, an ATP-dependent kinase, GlpK, phosphorylates glycerol to glycerol-3-phosphate (Gly3P). GlpK is subject to inhibition by non-phosphorylated EnzymeIIA^{Glc} molecules (indicating presence of glucose, *inducer exclusion*) and by the glycolytic intermediate, fructose-1,6-bisphosphate. Oxidation of Gly3P to DHAP is catalyzed aerobically via a dehydrogenase (GlpD) which delivers the electrons via ubiquinone to the electron transport chain. Under anaerobic conditions, a flavin-linked dehydrogenase (GlpABC) is used (Lin 1996). DHAP is isomerized via triosephosphate isomerase to glyceraldehyde-3-phosphate, Ga3P. The lower trunk of the glycolytic pathway then takes care of Ga3P. For gluconeogenesis, DHAP and Ga3P are condensed by the aldolase to fructose-1, 6-bisphosphate. Note that this is an allosteric inhibitor of GlpK. A fructose-1,6-bisphosphatase isozyme, GlpX (encoded in the *glpFKX* operon, see below) enhances formation of fructose-6-phosphate. GlpX is an isoenzyme of the FbaA phosphatase which is active under gluconeogenic conditions (e.g. growth on acetate or lactate). Fructose-6-phosphate and Ga3P are substrates of the transketolase, Tkt, to enter the (non-oxidative) pentose phosphate pathway (fructose-6-phosphate could also be converted to glucose-6-phosphate and then enter the oxidative pentose phosphate, not depicted here). Note that *glpK*-negative mutants of *E. coli* do not grow aerobically on glycerol (Lin 1996) despite the presence of the *gldA* gene for a glycerol dehydrogenase and *dhaKLM* genes for dihydroxyacetone kinase which together could form a bypass reaction (glycerol > dihydroxyacetone > DHAP). GldA activity, however is not induced by the presence of glycerol (Tang et al. 1979; Lin 1996) and the glycerol dehydrogenase does not seem to play a role under aerobic conditions therefore. *glpK* mutants have been selected which display the NAD-dependent glycerol dehydrogenase pathway under aerobic conditions have been selected (Tang et al. 1979). Anaerobic growth on glycerol by recruitment of the GldA pathway has been studied by the group of Gonzalez et al. (2008). **b** Simplified scheme of the *glpFKX* operon and its regulation. The genes for the glycerol facilitator GlpF, the glycerol kinase GlpK, and the fructose-1,6-bisphosphatase GlpX form an operon. The repressor GlpR inhibits transcription from the promoter, P_{glpF}, in the absence of the inducer, Gly3P. Gly3P (formed from glycerol when GlpK is active) inhibits the repressor and leads to transcription. Transcription from the P_{glpF} is also enhanced by the cAMP-CRP complex (not depicted here). Recently, ribose-5-phosphate was proposed an inhibitor of glycerol metabolism by making the DNA-GlpR complex less sensitive to the inducer, Gly3P (Vimala and Harinarayanan 2016)

Biochemistry and regulation of glycerol metabolism natural glycerol utilizing microorganisms of biotechnological interest has mainly been studied in *E. coli* (Lin 1996; Clomburg and Gonzalez 2013), *Klebsiella* sp. and *Clostridium* sp. (Deckwer 1995), and in the yeasts *Saccharomyces cerevisiae* (Swinnen et al. 2016), and *Yarrowia lipolytica* (Dobrowolski et al. 2016). In the best studied microorganism, *E. coli*, glycerol catabolism is subject to catabolite repression both by the non-phosphorylated EnzymeIIA^{Glc} ("inducer exclusion", indicating presence of the preferred carbon source, glucose), and by its dependence on cAMP-CRP for gene activation which is also governed by the presence of glucose as EnzymeIIA$^{Glc\sim P}$ which activates adenylate cyclase (Escalante et al. 2012). In addition the allosteric inhibitor, fructose-1,6-bisphosphate (see Fig. 1a), indicates presence of hexoses as glucose or fructose in the growth medium. Gene expression of the operon *glpF-glpK-glpX* is controlled by the negative regulator, GlpR (see Fig. 1b). Gly3P (the product of glycerol kinase) is the inducer of the system. Presence of Gly3P, and not of glycerol, makes sense as this signals the cell that glycerol kinase is active and not subject to the regulations described above.

When glucose is converted anaerobically to lactic acid, both reducing equivalents which stem from the EMP pathway are re-oxidized at the step of pyruvate reduction to lactate (be it D- or L-lactate). Glycerol however, yields two $NADH_2$ (or other redox equivalents) per mole, but only one mole of pyruvate as electron acceptor is generated. Therefore, in many bacteria additional (external) electron acceptors are necessary to allow strictly anaerobic growth on glycerol. *E. coli* can utilize externally added fumarate, TMAO, or other inorganic electron acceptors of an anaerobic membrane-bound electron transport chain for anaerobic glycerol dissimilation (so-called anaerobic respiration). Under anaerobic conditions in the absence of external electron acceptors, glycerol catabolism leads to an inevitable imbalance as the complete stoichiometric re-oxidation of $NADH_2$ to NAD^+ cannot occur. This leads to a metabolic halt at the step of NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (see Fig. 1a). Whether *E. coli* can grow anaerobically on glycerol as sole carbon source on a defined minimal medium without exogenous electron acceptors (true glycerol fermentation) is still under debate (Lin 1996; Yazdani and Gonzalez 2008; Martinez-Gomez et al. 2012; Clomburg and Gonzalez 2013).

Other Enterobacteria like the facultative anaerobes *Klebsiella pneumoniae* or *Citrobacter freundii*, or some Gram-positive, strictly anaerobic Clostridia display a different anaerobic glycerol catabolism which leads eventually to 1,3-propanediol (1,3-PDO) formation and excretion of this compound as one end product of fermentation (as the gene nomenclature varies between the different strains it will be omitted here). Briefly, one half of the glycerol which is consumed passes through an oxidative branch to yield dihydroxyacetone (DHA) via a NAD^+-dependent enzyme (glycerol dehydrogenase). DHA is then phosphorylated by an ATP-dependent DHA kinase; DHAP then enters the lower glycolysis path (see Fig. 2b). For the purpose of re-oxidation of $NADH_2$ which is formed in the oxidative branch, the other half of glycerol is "sacrificed" in the reductive branch to eventually form 1,3-propanediol (Lin 1996). In the reductive branch, glycerol is

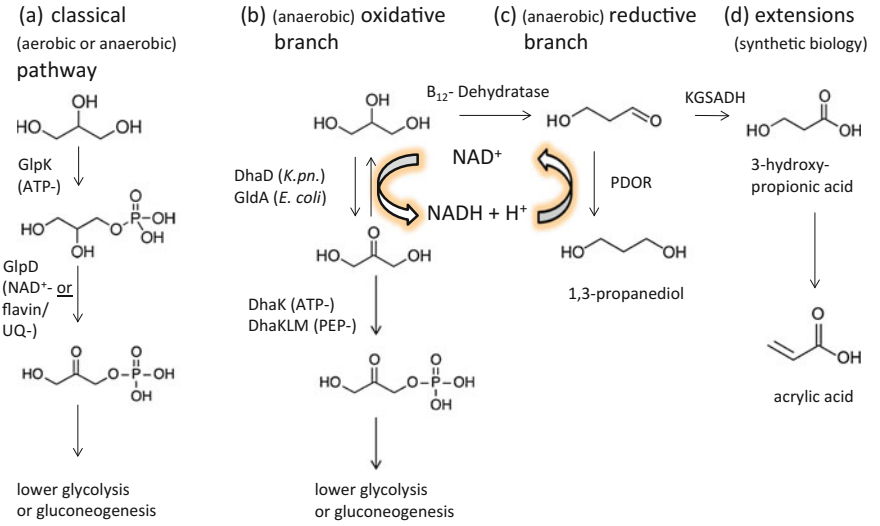

Fig. 2 Main pathways for glycerol dissimilation in microorganisms**. a** The classical pathway starts with uptake of glycerol into cells (either by diffusion or facilitated diffusion) followed by ATP-dependent phosphorylation of glycerol to *sn*-glycerol-3-phosphate (G3P). G3P-dehydrogenase (either NAD-dependent or membrane-bound GlpABC, or linked via ubiquinone to the electron transport chain) oxidizes G3P to dihydroxyacetone-phosphate (DHAP) which can enter the glycolytic or gluconeogenic pathways. **b** oxidative branch: in some bacteria, and mostly under anaerobic conditions, e.g. in *Klebsiella*, *Citrobacter*, or *Clostridium* species, glycerol can be oxidized to dihydroxyacetone (DHA) by a reversible NAD-dependent glycerol dehydrogenase. DHA can be phosphorylated to DHAP either by an ATP-dependent kinase (DhaK in *Klebsiella*), or a PEP-dependent three-component kinase (DhaKLM) in *Escherichia coli* (Erni et al. 2006). **c** Reductive branch: glycerol is converted by glycerol dehydratase into 3-hydroxy-propionaldehyde (HPA). Most glycerol dehydratases are coenzyme B_{12}-dependent (Jiang et al. 2016). HPA is reduced by $NADH_2$-dependent 1,3-propanediol oxidorecuctase (PDOR) to 1,3-propanediol which is excreted. **d** In biotechnology, HPA can be converted to 3-hydroxypropionic acid by various aldehyde dehydrogenase such as α-ketoglutarate semialdehyde dehydrogenase (KGSADH) (Rathnasingh et al. 2009) and further on to acrylic acid

first dehydrated to 3-hydroxypropionaldehyde (3-HPA) and then reduced by an $NADH_2$-dependent oxidoreductase (PDOR) to 1,3-propanediol (Fig. 2c; see also chapter on 1,3-PDO production). Glycerol dehydration in *Klebsiella* and *Citrobacter* strains is catalyzed by a complex, coenzyme B_{12}-dependent and oxygen-sensitive glycerol dehydratase (Lin 1996; Seifert et al. 2001; Jiang et al. 2016) which is formed from three different subunits as hetero-hexamer (α_2,β_2,γ_2; Jiang et al. 2016). Most of these glycerol dehydratases undergo irreversible inactivation by glycerol during catalysis. Moreover, several auxiliary proteins for cofactor insertion/exchange, and a reactivase activity are necessary (Seifert et al. 2001; Jiang et al. 2016). Coenzyme B_{12}-independent glycerol dehydratases have been described meanwhile from some clostridial species (Jiang et al. 2016).

Genes from the *dha* regulon of *Klebsiella pneumoniae* strains have been transferred either by in vivo cloning to *E. coli* mutants with defects in glycerol catabolism (*glpK*-, *gldA*-, *ptsD*-) to restore anaerobic growth on glycerol without added hydrogen acceptors (but glycerol dehydratase activity was not detectable) (Sprenger et al. 1989), or by cosmid cloning to yield 1,3-propanediol from glycerol (Tong et al. 1991). Despite these complexities in the anaerobic glycerol pathway, eventually all necessary genes from either *Klebsiella*, *Citrobacter*, or clostridial sources have been cloned and successfully expressed in *Klebsiella*, *E. coli* or *Clostridium* strains (for review see: Jiang et al. 2016).

4 Biotechnological Products from Glycerol

4.1 Dihydroxyacetone (DHA) as Product of Glycerol Oxidation

Dihydroxyacetone (DHA) is a valuable compound (prices up to 150 $/kg) which is used as a non-toxic sun-less tanning agent in cosmetics (Kumar et al. 2015). DHA is also a versatile building block for the synthesis of fine chemicals. Besides chemical synthesis, microbial enzymes can be used for dihydroxyacetone production from glycerol. For example, NAD-dependent glycerol dehydrogenases are known from various bacteria (*Klebsiella* sp. DhaD, *Escherichia coli* GldA (Kumar et al. 2015), *Thermotoga maritima* (Beauchamp et al. 2014), a.o. see Fig. 2b). Enzymatic production of DHA from glycerol has been described (for recent refs. see Beauchamp et al. 2014; Kumar et al. 2015). As reduced NAD is formed, re-oxidation of the cofactor is necessary, however. By introduction of a water forming NADH oxidase (from *Enterococcus faecalis*), *E. coli* cells which gave DHA titers of up to 2.13 g/g cell dry weight were obtained (Zhou et al. 2013).

An alternative and NAD-free production is possible by whole cells of strictly aerobic *Gluconobacter* sp. which oxidize glycerol to DHA by membrane-bound PQQ-dependent dehydrogenases in the process of so-called (sub)oxidation (Hu et al. 2010). As the reaction occurs on the periplasmic side of the inner membrane, DHA as product (up to 161.9 g/l) is released to the medium and not consumed by the microorganism (Hu et al. 2010). *Gluconobacter* thus only uses the electrons for oxidative phosphorylation (see Fig. 3a).

Dihydroxyacetone phosphate (DHAP) as the product of G3P oxidation or phosphorylation of DHA is also an interesting building block for chemo-enzymatic syntheses especially as several DHAP-dependent aldolases can be utilized for the biocatalytic production of rare sugars and sugar phosphates (Clapés et al. 2010). DHAP, however, is rather unstable. Several routes for its enzymatic synthesis have been described (Hettwer et al. 2002; Schümperli et al. 2007).

Glyceraldehyde and glyceric acid: Glycerol could as well be oxidized to D-glyceraldehyde, but this seems to be rarely realized, possibly due to the aggressive

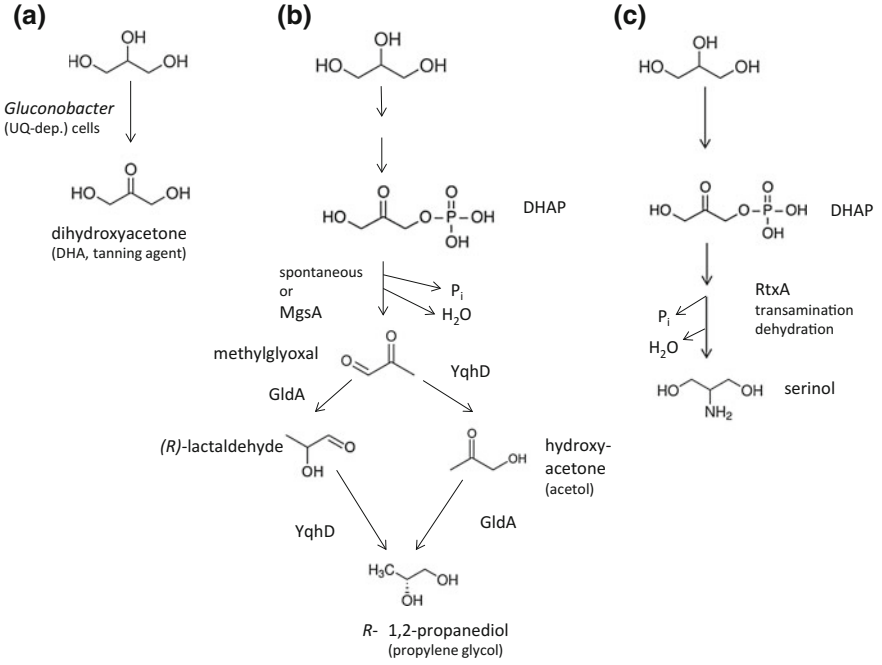

Fig. 3 Pathways from glycerol to valuable products. **a** (Sub)oxidation of glycerol by *Gluconobacter* cells to dihydroxyacetone (DHA) (Hu et al. 2010), electrons from this reaction are funneled via ubiquinone (UQ) into the electron transport chain of the cells. DHA is used, a.o., as tanning agent. **b** 1,2-propanediol formation. Glycerol is first converted to DHAP. If DHAP accumulates (e.g. in triosephosphate isomerase mutant, or under phosphate-limiting conditions which slow down glyceraldehyde 3-phosphate dehydrogenase), DHAP can be spontaneously dehydrated (under phosphate elimination) to methylglyoxal. A methylglyoxal synthase (MgsA, e.g. in *E. coli*) can accelerates this reaction. Methylglyoxal is a highly reactive electrophile and normally needs to be detoxified by glyoxalases via lactoyl-glutathione to lactate [(Cromburg and Gonzalez 2011) not depicted here]. If a NADH-dependent glycerol dehydrogenase (GldA) is expressed, methylglyoxal can be reduced at its keto function to give *R*-lactaldehyde, this in turn can be reduced to *R*-1,2-propanediol by an aldehyde reductase (as example, YqhD of *E. coli* is shown here). An alternative route leads from methylglyoxal to hydroxyacetone (acetol) by reduction of the aldehyde group (here by YqhD). Finally, the *R*-enantiomer of 1,2-propanediol is form by glycerol dehydrogenase, GldA (Altaras and Cameron 1999; Clomburg and Gonzalez 2013). **c** Serinol formation. DHAP can be converted by a specific aminotransferase/dihydrorhizobitoxine synthase (RtxA) from *Bradyrhizobium elkanii* and subsequent dephosphorylation and dehydration to the aminoalcohol, serinol (Andreeßen and Steinbüchel 2012), an interesting building block for organic chemistry

(electrophile) nature of the aldehyde. However, *Gluconobacter frateurii* strains which oxidize glycerol to glyceraldehyde (likely by a membrane-bound alcohol dehydrogenase) and further on to glyceric acid have been reported (Habe et al. 2009) with very high yields (see Table 1). Glyceric acid could be chemically converted to tartronic acid (a dicarboxylic C3 acid) or further to mesoxalic (ketomalonic) acid.

Table 1 Selected examples for glycerol as carbon source for production of high-value products (products, titers, or space-time yields (STY) where available)

Microorganism/system	Product	Titer/productivity	Purpose/comment	References
Yarrowia lipolytica (*LRO1*, *DGA1*, *DGA2*)	Single cell oil (triacylglycerol)	9.9 g/l	Glycerol-based media	Gajdos et al. (2016)
Y. lipolytica A101	Single cell oil	4.72 g/l	Crude glycerol	Dobrowolski et al. (2016)
Candida curvatus ATCC 20509	Lipid	29.5 g/l	Glycerol-based media	Gajdos et al. (2016)
Rhodotorula glutinis	Lipid	16.28 g/l	Glycerol	Karamerou et al. (2016)
Y. lipolytica AMM mutant rec. Yeast Suc2 gene on plasmid	Erythritol	Up to 114 g/l 0.95 g L^{-1} h^{-1}	Molasses + glycerol Two-stage, high-osmotic process	Mironczuk et al. (2015)
Ustilago trichophora (fungus) TZ1	Malic acid	Up to 195 g/l Up to 0.74 g L^{-1} h^{-1}	Crude glycerol, fed-batch $CaCO_3$-buffered	Zambanini et al. (2016)
Rec. *E. coli* (*glpX*+, *tktA*+, *aroFBL*+, *pheA*+)	L-phenylalanine	10.1 g/l 0.37 g L^{-1} h^{-1}	Pure glycerol	Gottlieb et al. (2014)
Rec. *E. coli* (fed-batch)	L-phenylalanine	Up to 13.4 g L^{-1}	Glycerol	Weiner et al. (2014a)
Rec. *E. coli* rec. 4-coumarate ligase, stilbene synthase	Resveratrol	Up to 22 mg/l	MM glycerol (+L-Phe)	Camacho-Zaragoza et al. (2016
Rec. *E. coli* (batch) (metagenomic fosmid DNA)	Ethanol	Up to 75 g/l	Crude glycerol	Loaces et al. (2016)
Escherichia coli (rec. *Rhizobium* Mut*melA* gene), anthranilate dioxygenase	Melanin	1.21 g/l	Complex medium + 40 g/l glycerol	Mejia-Caballero et al. (2016)
Escherichia coli (rec. *futC* from *H. pylori*)	2′-fucosyllactose	20.28 g/l; 0.57 g L^{-1} h^{-1}	Glycerol + lactose feed	Baumgärtner et al. (2013)
Clostridium butyricum DSMZ5431	1,3-propanediol	56 g/l; 2.2 g L^{-1} h^{-1}	Glycerol	Deckwer (1995)
Clostridium pasteurianum DSMZ525	1,3-propanediol *n*-butanol	53.7 g/l 1,3-PDO 39.2 g/l *n*-butanol	Glycerol Gas stripping	Groeger et al. (2016)
Klebsiella pneumoniae (rec. overexpr. *xylAB*+)	1,3-propanediol (also 2,3-butanediol)	Up to 24.4 g/l	Glycerol + xylose cofermentation	Lu et al. (2016)

(continued)

Table 1 (continued)

Microorganism/system	Product	Titer/productivity	Purpose/comment	References
Klebsiella pneumoniae ATCC15380	1,3-propanediol	Up to 56 g/l 0.85 mol/mole glycerol	Crude glycerol from *Jatropha* biodiesel	Hiremath et al. (2011)
E. coli, recombinant	1,3-propanediol	>130 g/l	Glucose > glycerol > 1,3PDO DuPont/Genencor	Nakamura and Whited (2003)
rec. *E. coli*	1,3-propanediol	10.6 g/l; 0.188 g L^{-1} h^{-1}	Crude glycerol (batch)	Kawaguchi et al. (2016)
rec. *E. coli* (*Clostridium* genes *dhaB1B2*)	1,3-propanediol	104.4 g/l; 2.61 g L^{-1} h^{-1}	Glycerol	Tang et al. (2009)
Gluconobacter oxydans mutant strain GM51	dihydroxyacetone	86.3 g/l	From 100 g/l glycerol shake flask	Ma et al. (2010)
G. oxydans ZJB09112 uv-derived mutant	Dihydroxyacetone	161.9 g/l 88.7% conversion rate	Glycerol + yeast extract + mannitol	Hu et al. (2010)
Klebsiella pneumoniae ATCC25955 (*dhaT*-, *yqhD*-negative, pBAD-*ldhA*+)	D-lactate	142 g/l 0.82 g/g glycerol	Microaerobic glycerol	Feng et al. (2014)
Gluconobacter frateurii	Glyceric acid	136.5 g/l	Glycerol	Habe et al. (2009)
Saccharomyces cerevisiae (*GUT1*, *GUT2*, *GUP1*, *E. coli genes mgsA*, *gldA*)	1,2-propanediol	2.19 g/l	YEPD medium + 1% glycerol	Jung et al. (2011)
Klebsiella oxytoca M1 (*pduC*, *ldhA* negative mutant)	2,3-butanediol	131.5 g/l 0.84 g L^{-1} h^{-1}	Crude glycerol	Cho et al. (2015)
Lactobacillus reuteri	3-hydroxypropion-aldehyde (3HPA)	108 g/l 3HPA from 138 g/l glycerol	Immobilized cells	Krauter et al. (2012)
K. pneumoniae/Gluconobacter oxydans	3-hydroxypropionic acid	60.5 g/l 242 g/480 g glycerol	Two-step process from glycerol via 1,3PDO	Zhao et al. (2015)
E. coli i (plasmid P_{ara}-*glpK*, *glpF; dhaB*, *gdr* from *K.pn.*) *ΔackA-pta*, *ΔyqhD ΔglpR*	3-hydroxypropionic acid	42.1 g/l; 0.268 g/g glycerol	Glycerol	Jung et al. (2014)

(continued)

Table 1 (continued)

Microorganism/system	Product	Titer/productivity	Purpose/comment	References
E. coli W3110 (*dhaB1-3*, *gdrAB* from *K. pneumoniae ΔackA-pta*, *ΔyqhD gabD4* variant *C. necator*)	3-hydroxypropionic acid	71.9 g/l; 1.8 g L^{-1} h^{-1}	Glycerol (+glucose)	Chu et al. (2015)
E. col i (optimized *cis*-aconitate decarboxylase)	Itaconic acid	7.23 g/l	Glycerol (+citrate)	Jeon et al. (2016)
Corynebacterium glutamicum (rec. *E. coli* genes *glpF*,*gldA*, *dhaKLM*)	Succinate	38.4 g/l	Glycerol (O_2-deprived)	Wang et al. (2016a)
Rhodopseudomonas palustris	Hydrogen (H_2)	6 mol H_2/mole Gly	Anaerobic photo-fermentation with glycerol	Sabourin-Provost and Hallenbeck (2009)

4.2 1,3-Propanediol (1,3-PDO) as New Commodity Product

1,3-propanediol (1,3-PDO, trimethylene glycol) was reported as bacterial ("schizomycetal") fermentation production on glycerol already in the late 19th century (Freund 1881). As chemical production of 1,3-PDO requires expensive catalysts, high temperature and other non-advantageous conditions (Hiremath et al. 2011), biological fermentation-based production could be safe and based on inexpensive raw materials. 1,3-PDO can be polymerized with terephthalic acid to yield polytrimethylene terephthalate (PTT), an interesting polyester which is further utilized for various products such as fabric and textile applications (carpets, T shirts a.o.). 1,3-PDO based on recombinant *E. coli* has become a hallmark process and has reached an industrial scale and market introduction in 2007 by a joint venture of DuPont and Tate & Lyle companies. Annual production rates of more than 100,000 tons have been reported (Jiang et al. 2016). This success has spurred many attempts to use other bacterial strains and different carbon sources such as glycerol for 1,3-PDO production.

1,3-PDO can be obtained by natural glycerol fermenting bacteria such as *Clostridium* sp., *Klebsiella*, or *Citrobacter* (Ainala et al. 2013) strains. The key enzymes of this pathway have been recently reviewed (Saxena et al. 2009; Zeng and Sabra 2011; Jiang et al. 2016). Formation of 1,3-PDO with glycerol or crude glycerol have been studied by many groups in the last three decades. Natural producers of 1,3-PDO (often also as co-product with *n*-butanol, acetoin or other compounds) are the non-pathogenic *Clostridium butyricum*, or *Cl. pasteurianum* (Groeger et al. 2016) and some lactobacilli. Enterobacteria (with the disadvantage of potential pathogenicity) are from the genera *Klebsiella* (*K. pneumoniae*, *K. oxytoca*, *K. planticola* now reclassified as *Raoultella planticola*), *Enterobacter*, or *Citrobacter*. *Cl. butyricum* and *K. pneumoniae* are considered as best natural 1,3-PDO producers (Saxena et al. 2009; Zeng and Sabra 2011). Also mixed cultures from sludge from full-scale biogas plants have been successfully used for production of 1,3-PDO (up to 70 g/l) in fed-batch cultivations (Dietz and Zeng 2014). The desert plant *Jatropha* forms a rich oil which can be utilized for biodiesel production. Crude glycerol from this process was successfully used for 1,3-PDO with a *K. pneumoniae* strain (see Table 1). Up to 56 g/l with a high molar conversion (0.85 mol/mol of glycerol) was reached. In a downstream process, 99% pure 1,3-PDO was obtained from fermentation broth and subsequently polymerized to fiber grade PTT (Hiremath et al. 2011); this, however, does not yet meet the criteria for an economic process and further research is warranted.

In another approach, 1,3-PDO production from glucose via the intermediate glycerol was followed. No natural microorganism has been known to produce 1,3-PDO directly from glucose. Yeasts have been used to produce glycerol from glucose under certain conditions (Neuberg's and Connstein's findings during and after World War I (Buchholz and Collins 2013), but are unable to make 1,3-PDO. Recombinant non-pathogenic *E. coli* strains for large scale production of 1,3-PDO have been engineered (Nakamura and Whited 2003). A two-step metabolic

engineering to allow such a direct fermentation of glucose via glycerol to 1,3-PDO has been worked out. These strains first produce glycerol first from glucose by importing genes for DHAP reduction to G3P, and dephosphorylation to glycerol (GPP1) from yeast. In a second step, *Klebsiella dha* genes for 1,3-PDO were combined. Further adjustments in the central metabolism and sugar uptake had to be performed. Fine tuning was done by knock-out of triosephosphate isomerase and stabilization of the labile glycerol dehydratase and by exchanging the propanediol-oxidoreductase by an *E. coli* aldehyde reductase, YqhD (Nakamura and Whited 2003).

3-hydroxypropionaldehyde (3HPA, also known as reuterin, Fig. 2c) is the product of glycerol dehydration and an intermediate in the reductive branch leading to 1,3-PDO production. It is a natural product excreted by lactic acid bacteria as *Lactobacillus reuteri* or *K. pneumoniae* (Refs. in Ulmer and Zeng 2007; Sardari et al. 2013). In a fed-batch process at 45 °C, 108 g/l of 3HPA were formed from 138 g/l of glycerol with a *L. reuteri* SD2112 strain. To avoid toxicity, 3HPA was trapped as carbohydrazone (Krauter et al. 2012).

4.3 3-Hydroxypropionic Acid (3HP)

3HPA can be oxidized by aldehyde dehydrogenases [e.g. gene *aldH* of *E. coli*, *puuC* from *K. pneumoniae*, or ketoglutarate semialdehyde dehydrogenase KGSADH from various microorganisms (Kumar et al. 2013)] to form 3-hydroxypropionic acid (3HP; Fig. 2d). 3HP is a structural isomer of lactic acid and is a promising bio-based platform chemical as it can be further converted into bulk chemicals such as acrylic acid, acrylamide, methyl acrylate, propiolactone, malonic acid and others. Among other microbial pathways to 3HP (reviewed in Kumar et al. 2013) the one from glycerol is very attractive and cost competitive. Especially acrylic acid which could be converted from 3HP via dehydration is of interest as production of "bio-acrylic acid" seems to be competitive with estimated 50% lower costs than the petroleum-based process and a 75% reduction in greenhouse gas emissions (Kumar et al. 2013). Recombinant *E. coli* strains with plasmid-borne genes for the *Klebsiella dhaB* plus *gdrAB* gene cluster (glycerol dehydratase complex and reactivase) and the *E. coli aldH* gene were constructed. To reduce competing pathways, chromosomal genes for glycerol consumption (glycerol kinase *glpK*), acetate formation (*pta-ackA*), and the oxidoreductase *yqhD* gene were knocked out, also the repressor gene, *glpR*. The genes *glpK* and *glpF* were put under the strict control of an arabinose promoter. The best strain yielded more than 42 g/l of 3HP from glycerol (Table 1) (Jung et al. 2014). Other good producers of 3HP are recombinant *Klebsiella* strains which have reached up to 49 g/l of 3HP from glycerol (Kumar et al. 2013), often with the by-product, 1,3-PDO. Toxicity of 3HP (and its precursor 3HPA) towards the producer cells are issues which have to be settled to allow even higher product yields (Kumar et al. 2013). To reduce 3HPA toxicity, another aldehyde dehydrogenase (a

protein-engineered GabD4 from *Cupriavidus necator*/*R. eutropha*) was chosen as it showed highest activity towards 3HPA. This resulted in a recombinant *E. coli* strain (see Table 1 for genetic details) with the highest 3HP titer thus far (71.9 g/l) (Chu et al. 2015).

A two-step process for 3HP production (first anaerobic 1,3-PDO production from glycerol by *K. pneumoniae* followed by aerobic oxidation of 1,3-PDO to 3HP by *Gluconobacter oxydans*) came up with about 60 g/l of 3HP from glycerol with about 50% (g g^{-1}) conversion rate. Interestingly, acrylic acid was one of the minor by-products of this process (Zhao et al. 2015). Recently, a recombinant *E. coli* (harboring genes for glycerol dehydratase/reactivase, propionaldehyde dehydrogenase, a enoyl-CoA transferase and a CoA-transferase) strain was reported which produced 37.7 mg/l acryl acid (via 3HPA, 3HPA ~ CoA and acrylyl-CoA) under shaking flask conditions from glycerol (Tong et al. 2016).

Chemical acryl acid formation from 3HP (yield > 95%) via dehydration over titanium dioxide at 230 °C was recently shown for another two-stage process with biodiesel glycerol. Therein, first an anaerobic step with *Lactobacillus reuteri* gave a mixture of 3HP and 1,3PDO which was then aerobically converted by *G. oxydans* cells (Dishisha et al. 2015).

4.4 Methylglyoxal Formation and Derived Products (Lactaldehyde, Hydroxyacetone/Acetol, and 1,2-Propanediol)

It should be noted that *E. coli* also possesses a glycerol dehydrogenase (gene *gldA*), an oxidoreductase which is mainly active under anaerobic conditions and which is not inducible by glycerol (Tang et al. 1979; Clomburg and Gonzalez 2013). GldA acts reversibly and has a preference for the reduction of DHA to glycerol, so some authors proposed that the true role could be detoxification of the reactive DHA (Subedi et al. 2008). *E. coli* lacks an ATP-dependent DHA kinase; instead, a three-enzyme complex (DhaKLM) performs a PEP-dependent phosphorylation (including components of the PTS) of DHA to DHAP; the metabolic role of this enzyme is still enigmatic as dihydroxyacetone is not a growth substrate (Erni et al. 2006) (Fig. 2b).

An interesting bypass of the classical glycerol catabolism is the so-called methylglyoxal pathway (Altaras and Cameron 1999). Under low-phosphate conditions, DHAP may be dephosphorylated and dehydrated to methylglyoxal, a highly reactive electrophile (see Fig. 3b). Detoxification of methylglyoxal can lead to lactyl-glutathione and subsequently to D-lactate (via glyoxalases) (Clomburg and Gonzalez). Depending on the enzyme in use, methylglyoxal can also be reduced to either lactaldehyde or to hydroxyacetone (acetol). Lactaldehyde then can further be reduced to propane-1,2-diol (1,2-PDO). If *R*-lactaldehyde is the precursor, *R*-1,2-propanediol can be formed by glycerol dehydrogenase (Altaras and Cameron 1999; Clomburg and Gonzalez 2011).

Acetol (hydroxyacetone) can be used as skin tanning agent in the cosmetic industry and is used to manufacture reduced dye in the textile industry (Yao et al. 2015). Acetol is chemically synthesized by dehydration of glycerol or by dehydrogenation of propylene glycol at relatively high costs. The route to acetol via DHAP and methylglyoxal (see Fig. 3b) is a biological alternative, where the NADPH-dependent aldehyde reductase YqhD is involved. The obvious demand for NADPH limits acetol formation from glycerol, the best engineered *E. coli* mutant strain thus gave about 0.25 g acetol/g of glycerol (1.82 g/l) in a recent report (Yao et al. 2015).

1,2-propanediol (propylene glycol, 1,2-PDO): 1,2-PDO is a commodity chemical with a global demand of more than 1 million tons/year, with applications as antifreeze, humectant and moisturizer with GRAS status in food, cosmetics and medicines. Biological production by various bacterial and yeast strains has been reported. Recombinant yeast strains which expressed *mgsA* and *gldA* genes from *E. coli* and had the yeast-specific genes encoding glycerol transport (GUP1), glycerol kinase (GUT1), glycerol-3-phosphate dehydrogenase (GUT2) overexpressed, produced 2.19 g/l of 1,2-PDO in a glycerol-containing complex medium (Jung et al. 2011). 1,2-PDO has been obtained by engineered *E. coli* strains growing on glucose (Altaras and Cameron 1999). More recently, the Gonzalez group engineered an *E. coli* mutant which lacked genes of several competing pathways (Δ*ackA-pta* Δ*ldhA*, Δ*dhaK*, Δ*aceEF*, Δ*fdhF*) and overexpressed *mgsA*, *gldA* (alternatively *fucO*), *dhaK* (ATP-dependent DHA kinase from *C. freundii*), and *yqhD* from plasmids. When grown on glycerol, ethanol was a required co-product. The best recombinant strain produced 5.6 g/l of 1,2-PDO (21.3% yield w/w). Crude glycerol could also be used for production (Cromburg and Gonzalez 2011).

4.5 Fermentative Products from Glycerol as Carbon Source

Due to its highly reduced nature, glycerol can be used in (mostly) anaerobic fermentations to yield fermentation products such as ethanol, butanol, methane, lactate, formate, or succinate (via fumarate reduction) (Clomburg and Gonzalez 2013). As well, hydrogen (H_2) can be obtained as (by)product (see below).

Ethanol: Bioethanol production from various carbon sources is certainly not a new approach in biotechnology. Anaerobic or micro-aerobic conversion of by *E. coli* cells was found to deliver mainly ethanol (Yazdani and Gonzalez 2008; Clomburg and Gonzalez 2013); *E. coli* does not have a typical alcohol dehydrogenase but rather a bifunctional acetylCoA/acetaldehyde dehydrogenase (see Fig. 4) (Durnin et al. 2009; Wong et al. 2014). Knocking out competing pathways (mixed acid fermentation products lactate, acetate, succinate) and overexpression of GldA, DhaKLM, and AdhE led to ethanol titers of up to 25.7 g/l on glycerol (Wong et al. 2014). A novel approach was to express a metagenomics library from the sludge of an anaerobic reactor fed with ruminal digesta from an oil-wastewater treatment plant. The fosmid gene bank was transduced to *E. coli* cells; this yielded positive clones with high ethanol productivity from glycerol (see Table 1) (Loaces et al. 2016).

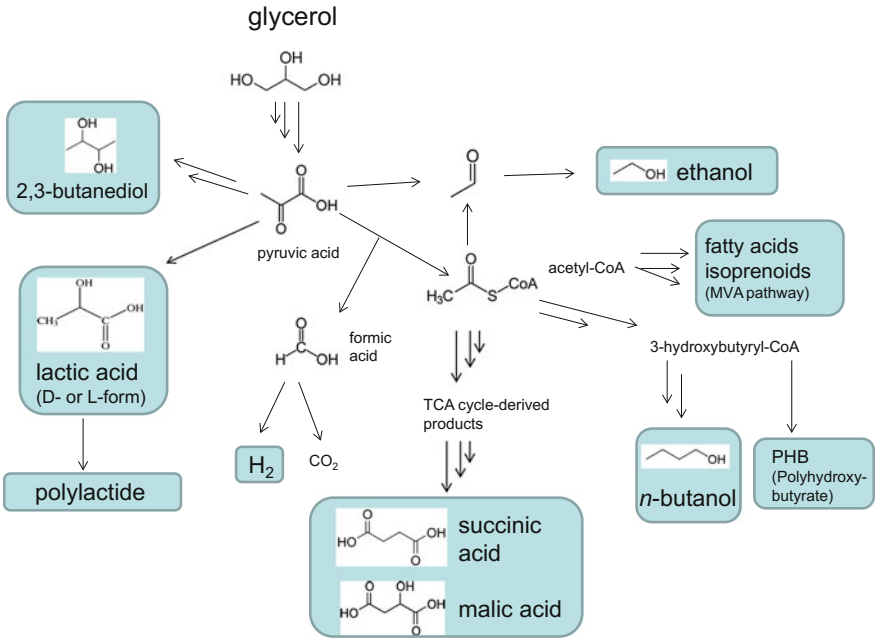

Fig. 4 Some glycerol- derived products from pyruvate and TCA pathway. Schematic description of product formation from glycerol catabolism via pyruvate; products are highlighted by boxes. Most pathways run under anaerobic conditions only, for clarity reasons redox cofactors are not shown. Note that the depicted pathways are from different and sometimes recombinant microorganisms. Pyruvic acid is the central intermediate. Clockwise: pyruvic acid can be decarboxylated to acetaldehyde which is then reduced to ethanol. Alternatively, acetyl-CoA can be converted by a bifunctional enzyme (for example in *E. coli* cells) via acetaldehyde to ethanol (used as biofuel or for other purposes). Two molecules of acetyl-CoA can be condensed to acetoacetyl-CoA (not depicted) and then converted to 3-hydroxybutyryl-CoA (3HBCoA). 3HBCoA can be either converted via crotonyl-CoA, butyryl-CoA and butyraldehyde (not depicted) to *n*-butanol (a third generation biofuel). Otherwise, 3HBCoA can be polymerized by some organisms to polyhydroxybutyrate (PHB), a biodegradable bioplastic. Utilization of the TCA cycle (which is replenished by carboxylation of PEP or pyruvate to yield oxaloacetate, and by acetyl-CoA to yield citrate) can lead to formation of malic acid [*Ustilago*] or succinic acid (from reduction of fumarate by a reverse TCA cycle). Both acids are valuable feedstocks for industry and maybe utilized in C4 chemistry. Anaerobic bacteria which possess a pyruvate-formate lyase can split pyruvic acid into acetyl-CoA and formic acid (formate). Formate is then split by a formate-hydrogen lyase (e.g. in *E. coli* or *Clostridium* cells) to carbon dioxide and molecular hydrogen. Pyruvic acid can be reduced to lactic acid, depending on the respective lactate dehydrogenase, this delivers the D- or L-form. Lactic acid can be chemically condensed to polylactide, a biodegradable plastic. Finally, 2,3-butanediol can be formed from condensation of two molecules of pyruvate via acetolactate and acetoin (not depicted)

2,3-butanediol is the product of various *Klebsiella* or *Bacillus* fermentations. It stems from the condensation of two molecules of pyruvic acid which are then further decarboxylated and reduced to 2,3-butanediol (see Fig. 4). A mutant strain of *K. oxytoca* with defects in the genes for glycerol dehydratase and lactate

dehydrogenase formed up to 131.5 g/l of 2,3-butanediol from crude glycerol, even surpassing pure glycerol as C source (Cho et al. 2015). By improving the NADH availability and by redox recycling with glycerol/glycerol dehydrogenase, recombinant *Bacillus amyliquefaciens* strains achieved 102.3 g/l of 2,3-butanediol on biodiesel-derived glycerol (Yang et al. 2015).

n-butanol: The strict anaerobe, *Cl. pasteurianum* is known to convert waste glycerol into *n*-butanol with the competing product, 1,3-PDO. By inactivation of the *dhaT* gene for the 1,3-PDO dehydrogenase a mutant strain was engineered which showed a 83% decrease in 1,3-PDO (residual 0.58 g/l) and increased formation of *n*-butanol (8.6 g/l). Interestingly, a novel product (1,2-PDO) appeared with about 0.3 g/l as a putative remodeling of redox balances in the cell (Pyne et al. 2016).

Erythritol: is a naturally occurring sweetener used in food and pharmaceutical industries and can be produced by osmophilic yeasts from glucose. *Y. lipolytica* is a GRAS organism and glycerol-positive but cannot grow on sucrose. After introduction of a sucrose gene (SUC2) from *Saccharomyces cerevisiae*, growth on sucrose (molasses) was achieved. In a two-step fermentation process with industrial raw molasses and glycerol up to 114 g/l of Erythritol was produced (see Table 1) (Mirónczuk et al. 2015).

4.6 Hydrogen Production from Glycerol

Although most glycerol-derived products are carbon-based, it should not be forgotten that the high degree of reduction in glycerol has attracted interest to produce molecular hydrogen (H_2) from anaerobic glycerol conversion (Sarma et al. 2012). Fermentation of glycerol with H_2-generating bacteria (*Enterobacter aerogenes*) has led to considerable hydrogen formation (63 m mol h^{-1} L^{-1}) besides ethanol (Ito et al. 2005). A multiple deletion strain of *E. coli* BW25113 (lacking activities of fumarate reductase, lactate dehydrogenase, formate dehydrogenase, nitrate reductasee, pyruvate dehydrogenase, hydrogenase, and methylglyoxal synthase) reached the theoretical maximal yield for a glycerol fermenting strain of 1 mol H_2/mol glycerol after 48 h with little formate and acetate formation (Tran et al. 2014). Even better are anoxygenic phototrophic bacteria (*Rhodopseudomonas palustris*), which showed by photofermentation very high hydrogen yields of 6 mol H_2/mole glycerol which corresponds to 75% of the theoretical yield (Sabourin-Provost and Hallenbeck 2009). These authors showed also the successful use of crude glycerol with low toxicity.

4.7 Compounds from TCA Cycle and Related Pathways

Citric acid production by various *Yarrowia lipolytica* strains (including acetate mutants) has reached up to 139 g/l in fed-batch operation (Refs. in Yang et al.

2012). Lactic acid acid can be utilized for the production of the bioplastic poly (lactic acid). Addition of D-lactate may change physical properties D-lactate. Moreover, D-lactate has many other chemical applications (Posada et al. 2012; Feng et al. 2014). *K. pneumoniae* strains with defects in the competing activities DhaT and YqhD and with enhanced fermentative D-lactate dehydrogenase yielded up to 142 g/l of optically pure D-lactic acid from glycerol (Feng et al. 2014).

Succinic acid production by engineered *E. coli* strains (overexpression of PEP-carboxylase) reached about equal productivities (0.69 g succinate/g glycerol) on glycerol as on glucose (Blankschien et al. 2010). Malic acid: Malic acid could be used as raw material for a novel biodegradable polymer, polymalic acid. Instead of extraction from apple juice (at low yields) an improved fermentation protocol for malic acid reached an enormous productivity (titers up to 195 g/l; see Table 1) with the fungus *Ustilago trichophora* grown on glycerol and $CaCO_3$ as buffer system (Zambanini et al. 2016).

Itaconic acid: (an important raw material for the synthesis of many fibers, paint, resins a.o) up to now has been produced by sugar fermentation with the filamentous fungus, *Aspergillus terreus*. The gene for the crucial enzyme *cis*-aconitate decarboxylase was cloned, optimized and expressed in *E. coli* strains. Fermentation with citric acid and 70 g/l of glycerol yielded up to 7.23 g/l of itaconic acid (Jeon et al. 2016).

Propionic acid can be derived from the TCA cycle intermediate succinyl-CoA via L-methylmalonyl-CoA and propionyl-CoA in Propionibacteria (and some Clostridia). *E. coli* normally does not form propionic acid but has some cryptic genes (sleeping beauty mutase SBM operon) which are similar to those of Propionibacteria. By activation of this SBM pathway, and overexpression of several genes of the glycerol catabolic pathways, propionate producers were obtained which yielded up to 11 g/l (~50% of the converted glycerol), by-products were acetate, succinate and 1-propanol (Akawi et al. 2015). A recombinant strain of *Propionibacterium jensenii* with combined overexpression of the genes for glycerol dehydrogenase and malate dehydrogenase (from *K. pneumoniae*) however was shown to yield about 50% more propionic acid (39.4 g/l) than the wild-type strain (Liu et al. 2015b).

4.8 Products from Aromatic Amino Acid Biosynthesis Pathway and Isoprenoid Formation

The common aromatic amino acid biosynthesis starts from condensation of PEP and erythrose-4-phosphate. At the step of shikimate 3-phosphate, another PEP molecule is introduced and eventually chorismate is formed before the pathway splits to the final products (aromatic amino acids as L-tyrosine, L-phenylalanine or L-tryptophan). Also *p*-hydroxybenzoate, *p*-aminobenzoate and several quinones are based on chorismate (Rodriguez et al. 2014). With glucose as C source, one PEP

molecule is already consumed for glucose uptake via the PTS. Thus, glycerol could theoretically deliver more PEP per mole of substrate for aromatics. This in mind, several groups have studied production of shikimate, aromatic amino acids and other chorismate- or aromatic amino acid-derived compounds using glycerol as C source (Rodriguez et al. 2014).

- shikimate is an important chiral compound and is used in the synthesis of the antiviral drug Tamiflu. While *E. coli* mutants for shikimic acid production were mainly grown with glucose as C source, a recent study looked on the influence of *glpD* and *glpK* genes in strains growing on glycerol. Together with overexpression of transketolase and three *aro* genes, this led to a 5.6 fold increase of shikimic acid compared to the parent strain. Up to 200 mg/l (from 20 g/l of glycerol in a complex medium) were obtained (Yang et al. 2014). These values, however, need to be significantly increased to be able to compete with other C sources (Rodriguez et al. 2014). Dehydroshikimate (DHS) is the immediate precursor of shikimate. In several bacteria it can be catabolized via protocatechuate, catechol and *cis*,*cis*-muconic acid to end up in the TCA cycle. *E. coli* naturally does not show this ability but recombinant strains which have blocks in the aromatic pathway and carry heterologous genes (*aroZ*, *aroY*, *catA*) can metabolize glycerol via DHS to *cis*,*cis*-muconate. The group of Stephanopoulos constructed two mutant strains which shared their work. The first strain converts glycerol to DHS which is excreted from the cells. The second strain takes up DHS via a ShiA transporter and converts DHS via AroZ, AroY, and CatA to *cis*, *cis*-muconate. The best strain which combined all functions in one cell (optimized before for DHS formation) produced about 450 mg/l of *cis*,*cis*-muconate and 600 mg/l of DHS from glycerol. A coculture of two different strains which had the functions separated produced up to 2 g/l of muconic acid from 20 g/l of glycerol with little by-products (Zhang et al. 2015).
- phenylalanine (L-Phe) production with *E. coli* strains has been performed mainly with glucose as carbon source. Successful production of L-Phe from glycerol and crude glycerol has recently been described for recombinant *E. coli* strains (Khamduang et al. 2009). *E. coli* model strains which carried genes for rate-limiting steps of L-phenylalanine on a plasmid produced up to 10 g/l of L-Phe from glycerol or crude glycerol compared to 5.5 g/l L-Phe with glucose. The space-time-yield (STY) was also improved. The STY could be further enhanced when extra chromosomal copies of *glpX* and *tktA* were introduced (see Fig. 1a) (Gottlieb et al. 2014). Working with the same cell line in a fed-batch system, maximal L-Phe concentrations of up to 13.4 g/l were obtained (Weiner et al. 2014a).
- deoxyviolacein is a microbial compound which is active against gram-positive bacteria, some fungal plant pathogens and has antitumor activity. The drug is derived from L-tryptophan. Natural producers like *Chromobacterium violaceum*, however, are potential pathogens. Therefore the *vioABCE* genes were cloned and expressed by the Wittmann group in *E. coli* strains with improved tryptophan productivity. In comparison to growth on L-arabinose (where about

320 mg/l deoxyviolacein were formed), a strain which lacked arabinose catabolism was able to produce up to 1.6 g/l in a fed-batch process based on glycerol (Rodrigues et al. 2014).

- melanin: Novel products from the common shikimate pathway in bacteria can be obtained with the incorporation from genes of other microorganisms or plants (Martinez-Gomez et al. 2012; Rodriguez et al. 2014). As an example, an *E. coli* mutant strain with a block in the synthesis path between anthranilate and tryptophan was used. The tryptophan-auxotroph strain was augmented with an additional plasmid-borne copy of the transketolase gene and a feedback-resistant DAHP-synthase (*aroG*^{fbr}). Genes encoding for an anthranilate dioxygenase (*antABC* from *Pseudomonas aeruginosa*) and a mutated tyrosinase-encoding gene (Mut*melA*) from *Rhizobium etli* were used. In continuation of their work on melanin production with recombinant *E. coli* cells on glucose (3.22 g/l melanin), the group of Gosset reported recently that up to 1.21 g/l of the catechol compound melanin were produced via the anthranilate path from 40 g/l glycerol (Mejia-Caballero et al. 2016).
- resveratrol: this stilbene compound is found in red wine and is being discussed for putative health-improving properties. Its biosynthesis starts from either L-tyrosine, or L-phenylalanine by ammonia lyases which deliver *p*-coumaric acid. Via coumaryl-CoA and a stilbene synthase, trans-resveratrol is formed. The group of Gosset has introduced necessary genes from various microorganisms and from wine. Together with improvements of the aromatic amino biosynthesis pathway, up to 22.6 mg/l of resveratrol were produced from glycerol (Camacho-Zaragoza et al. 2016).

4.9 *Oligosaccharides and Polymers from 3-Hydroxyacids (PHA)*

Human milk oligosaccharides (HMO) can be produced by recombinant *E. coli* mutants which express various heterologous glycosyltransferases. A recent example showed that up to 20.28 g/l of 2′-fucosyllactose (0.57 g l^{-1} h^{-1} STY) could be obtained from a lactose-negative strain which used glycerol as main carbon source for biomass production and provision of nucleotide-activated GDP-L-fucose and which was fed by lactose during a 13 L—scale fed-batch fermentation (Baumgärtner et al. 2013).

Various bacteria are naturally capable to store carbon as intracellular granules as polymers of 3-hydroxy alkanoic acids such as polyhydroxybutyrate (PHB) or polyhydroxyalkanoate (PHA). PHAs can be used to form biodegradable bioplastics with a growing number of applications as substitutes for petrol-based plastics. Many bacteria utilize glycerol or crude glycerol for PHA production although the yields on glycerol may lag behind those obtained on soybean oil or fructose (Fukui et al. 2014). Examples are *Ralstonia eutropha* (synonymous to *Cupriavidus*

necator), *Paracoccus denitrificans*, and various Pseudomonads (Yang et al. 2012; Fukui et al. 2014). Up to 67% of dry cell weight can thus be composed of PHAs (Yang et al. 2012). A newly isolated *Pseudomonas* strain (ASC2) was found to produce medium-chain-length PHAs when grown on refined or crude glycerol, up to 17.5 g/l consisting of 3-hydroxyoctanoate and 3-hydroxy-5-*cis*-dodecanoate (Muangwong et al. 2016).

Poly(3HP): The biopolymer Poly(3-hydroxy-propionate) [poly(3HP)] is an interesting novel compound with promising properties but is not produced by any known wild-type organism (Heinrich et al. 2013). In a first study, using a propionaldehyde-dehydrogenase (PduP from *Salmonella enterica*), HPA was converted to 3-hydroxypropionyl-CoA in recombinant *E. coli*. Then a PHA synthase (PhaC from *Ralstonia eutropha*) that can polymerize this to poly(3HP) was used (Andreeßen et al. 2010). More recently, a different pathway was described for the natural 1,3-propanediol producer, *Shimwellia blattae* (formerly known as *Escherichia blattae*) which carried several recombinant genes from other microorganisms: DhaT (1,3-propanediol dehydrogenase and AldD (aldehyde dehydrogenase) from *Pseudomonas putida* KT2442 to form 3-hydroxypropionate from 1,3-propanediol (itself from glycerol). A propionate-CoA transferase Pct was from *Clostridium propionicum* to form 3-hydroxypropionyl-CoA. Finally PhaC1 from *R. eutropha* was used to form poly(3HP) with a final yield of 9.8% (wt/wt [cell dry weight] poly(3HP) from glycerol as sole carbon source (Heinrich et al. 2013).

4.10 Oils, Lipids, and Isoprenoids

As shown above, glycerol can be yielded from various plant oils in the process of transesterification which leads to fatty acid methyl esters (FAME) and liberates glycerol. Glycerol (or its derivative glycerol-3-phosphate) is necessary for the anabolism of membranes and is a constituent of oils and lipids in many microorganisms. Several fungi and yeasts can thus store up to 70% of their cell dry weight in form of lipids, termed single cell oil (Gajdos et al. 2016; Lamers et al. 2016). So-called oleaginous yeasts as *Yarrowia lipolytica*, *Candida curvatus*, *Rhodotorula glutinis*, *or Rhodosporidium toruloides*, *Schwanniomyces* (*Debaryomyces*) *occidentalis*, and to a lesser extent *Saccharomyces cerevisiae*, have been shown to form single cell oil (triacylglycerol, TAG) or other lipids when grown on pure or crude glycerol, or on glycerol-based media (see Table 1; Yu et al. 2013; Liu et al. 2015a; Dobrowolski et al. 2016; Gajdos et al. 2016; Lamers et al. 2016; Signori et al. 2016; Karamerou et al. 2016). Fatty acids and especially dicarboxylic acids could be further products from oleaginous yeasts growing on glycerol. By overexpression of glycerol kinase and malic enzyme, the oleaginous fungus *Mortierella alpina* showed an improved formation of the polyunsaturated fatty acid arachidonic acid (up to 52 mg/g) (Hao et al. 2015). Genome-metabolic models to optimize lipid

accumulating *Candida tropicalis* strains for these purposes have recently been published (Mishra et al. 2016).

Farnesol: *E. coli* strains with a heterologous mevalonate pathway for isoprenoid formation from acetyl-CoA were equipped with an extra gene for a farnesylpyrophosphate synthase and two phosphatases. This resulted in production of the sesquiterpenoid alcohol farnesol (up to 526 mg/l) from YT medium with 2% glycerol (Wang et al. 2016b).

4.11 Co-utilization of Glycerol with Other Carbon Sources

As lined out above, glucose is the preferred carbon source for many microorganisms and thus prevents the co-utilization of glycerol by various regulatory interventions such as EnzymeIIA^{Glc} of the PTS. In biotechnology this would lead to undesired two-phase growth in production processes as the cells would first utilize glucose to completion before using glycerol and even a prolonged lag (diauxic shift) could occur in between. In order to prevent this, PTS-negative mutants of *E. coli* which show no catabolite repression were successfully used for co-cultures for the production of aromatic compounds (Martinez et al. 2008; Rodriguez et al. 2014). In the same direction, caffeic acid was produced in a culture which utilized glucose + glycerol (Huang et al. 2013). Recombinant *K. pneumoniae* strains which carried extra copies of the *xylAB* genes (from *E. coli*) were grown on a xylose + glycerol medium. The xylose co-substrate helped to increase the NADH/NAD$^+$ ratio and thus 1,3-PDO titers increased up to 24.4 g/l with a molar glycerol conversion to 1,3-PDO of up to 73.8% (Lu et al. 2016). L-Phe production with *E. coli* strains (see Sect. 4.8) that were pyruvate kinase-negative (in order to increase PEP; and thus auxotroph for pyruvate sources such as lactate) were supplied with glycerol + lactate during the growth phase before shifting to glycerol as sole C source during L-Phe production. Interestingly, these strains accumulated trehalose intracellularly as storage product which prohibited better L-Phe productivity (Weiner et al. 2014b).

4.12 Engineering of Glycerol-Negative Microorganisms for Glycerol Conversions

The gram-positive bacterium *Corynebacterium glutamicum* is well known in biotechnology for its production of important food and feed amino acids (L-glutamic acid/monosodium glutamate MSG; L-lysine, L-threonine a.o.). However, this bacterium is not able to utilize glycerol as carbon source. The group of Wendisch introduced *E. coli* genes *glpF*, *glpK*, and *glpD* (as single genes or in cassettes) into a wild-type *C. glutamicum* strain (ATCC13032) and a lysine-producer with 4

essential mutations. Glutamate production was triggered by ethambutol addition and 15 mM glutamate from 20 g/l of glycerol was shown in the wild-type strain carrying *glpFKD* genes. Expression of *glpF* improved growth rate and biomass formation on glycerol as sole carbon source. The lysine producing mutant with the *glp* genes also showed lysine production (26 mM or 0.19 g lysine/g of glycerol) or from glycerol-glucose mixtures (Rittmann et al. 2008). Putrescine (1,4-diaminobutane) is a follow-up product of amino acid production with *C. glutamicum*. Glycerol-converting mutants were able to produce this compound as well (Meiswinkel et al. 2013). In continuation of this work, a similar strain but with mutations in the TCA cycle and in pyruvate/acetyl-CoA metabolism showed an aerobic production of 79 mM of succinate from 375 mM glycerol which was about 42% of the maximal theoretical yield under aerobic conditions (Litsanov et al. 2012). Another group studied glycerol for succinate production under oxygen-deprived conditions. This time, *glpF*, *gldA* and *dhaKLM* genes from *E. coli* were heterologously expressed. Under anaerobic fed-batch fermentation the strain produced 38.4 g/l of succinate (Wang et al. 2016a).

5 Advantages of Glycerol and Problems to Be Solved

As already lined out above, glycerol is higher reduced than glucose. Based on C molarity, therefore a higher energy yield can be obtained. In *E. coli*, G3P is oxidized to DHAP via a dehydrogenase which delivers its electrons to ubiquinone in the electron transport chain (see Fig. 2). Compared to a NAD-dependent dehydrogenase (which exists in other organisms) this leads to a somewhat lower ATP yield (Gottlieb et al. 2014). In total, however, 2 mol of glycerol yield more reducing equivalents than 1 mol of glucose at the same amount of carbon atoms (Yazdani and Gonzalez 2008). Processes with increased demand for reducing equivalents thus may profit from glycerol as carbon and energy source. Glycerol also leads to less overflow metabolism in *E. coli* (Martinez-Gomez et al. 2012).

As outlined above, glycerol and crude glycerol have already been successfully utilized for a wide variety of value-added products. For relatively short conversions such as the ones leading to DHA, 1,2- and 1,3-PDO, acetol, 3-HPA, and 3-hydroxypropionic acid, competitiveness to other carbon sources can be envisaged. The large scale industrial production of 1,3-PDO from glucose (via the intermediate glycerol) shows, however, that industry tends to stick with a conventional carbon source (starch or starch-derived glucose) instead..

For other products (ethanol, butanol, lactic acid, TCA cycle-derived organic acids, aromatics) it has become clear that glycerol and crude glycerol are useful sources but may be less competitive than cheaper starch- or glucose-based carbon sources (Wong et al. 2014). For the production of D-lactic acid with *E. coli* strains grown on crude glycerol, costs have been calculated and found to be attractive (Posada et al. 2012). Large industrial complexes for bioethanol or amino acid productions are nowadays placed in close vicinity to the source of sugar cane (e.g.

Brazil) or corn starch (Midwest USA) and thus take advantage of short transport routes. Pure glycerol needs prior distillation from crude glycerol and is thus less economically attractive. Crude glycerol (from oil palms, soybean or rapeseed), on the other hand usually originates in oil mills which are economically mainly oriented towards their main targets, e.g. the fat and lipid market (cosmetics, soap, margarine, food) or in the transesterification to yield fatty acid methyl ester (biodiesel) market. These oil mills, however, are normally less experienced in fermentative production of bioethanol, organic acids, amino acids a.o. Long transport routes of relatively low-value crude glycerol to other biotechnology companies lead to cost increases. Therefore, crude glycerol may instead end up in nearby regional destinations [such as animal feed (Yang et al. 2012)] or other purposes or even in disposal. Varying batch qualities (residues of methanol, ash, salts, MONG) or seasonal production (rapeseed, soybean) of crude glycerol can constitute further impediments for an application in biotechnology (Chatzifragkou and Papanikolau 2012). The concept of a biorefinery, nowadays mainly discussed as the complete utilization of lignocellulosics, should therefore also encompass the fate of crude glycerol as well. As a consequence, co-utilization of crude glycerol with other C sources (derived from starch or lignocellulosics) should receive more considerations. This however makes it necessary to study co-utilization and diauxic effects in microorganisms in more detail (Martinez et al. 2008). Another problem are by-products which appear to be inevitable in some cases but reduce productivity and demand extra steps in downstream processing which raise costs.

Ecological aspects of biodiesel production include palm oil or soybean production which nowadays are often in conflict with the preservation of tropical rain forests and should not be forgotten, however.

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Metabolic Engineering of *Escherichia coli* for Lactic Acid Production from Renewable Resources

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Abstract Metabolic engineering has been used to develop *Escherichia coli* strains that generate D or L-lactic acid as the predominant fermentation product from different carbon sources, including glucose and xylose, which are present in syrups from lignocellulosic hydrolysates. As an introduction, this review presents the relevance that lactic acid has nowadays in several industrial and commercial applications. It also stresses the relevance of producing D or L-lactic acid as pure optical enantiomers for different applications. The second part reviews the metabolic engineering and adaptive evolution efforts developed with *E. coli* to achieve the production of optically pure D or L-lactic acid using several carbon sources. Furthermore, a set of results using actual mixtures of sugars contained in lignocellulosic hydrolysates is presented and discussed. Even though the efficient conversion of sugars to D or L-lactic acid and high volumetric productivities has been achieved, this review reveals that most work needs to be performed with actual lignocellulosic hydrolysates at the pilot or demonstrative scales to deploy the full potential of this efforts towards industrial production.

Keywords Lactic acid · Escherichia coli · Metabolic engineering · Glucose · Xylose · Glycerol · Lignocellulosic · Hydrolysates

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1 Introduction: Renewable (Bio)Chemicals

The world's population growth is projected to 9.7 billion in 2050 and 11.2 billion by 2100 (United Nations, Department of Economic and Social Affairs, 2015) generates a demand for larger quantities of food, feed, housing, chemicals, energy and goods that are used in everyday life, including a wide array of plastics. Many of these commodities were made from renewable resources at the beginning of 20th century, afterward, they were substituted by petroleum-derived chemicals (Weber et al. 2002); the main driver was the low price of the raw material. However, manufacturing processes and the plastics produced from petroleum generate wastes that are not biodegradable. One way to turn around this situation is to use renewable biological resources to produce biochemical building blocks, which can then be utilized for the manufacture of biodegradable and renewable bioplastics.

Renewable chemicals are chemical compounds that are generated from renewable feedstocks, which are materials from biological origin, also known as bio-derived chemicals. According to (Vijayendran 2010) there are three ways to obtain bio-derived chemicals: (1) direct production using conventional thermochemical and catalytic processing of bioderived feedstock; (2) biomass biorefining to obtain bioproducts by biochemical conversion technologies; and (3) bioproducts obtained with the aid of genetically engineered organisms, with designed functionality of monomers as building blocks. Direct production and biomass biorefining are already a reality, and probably many of them are derived from the existing technologies from the beginning of the 20th century. Some examples include the production of lactic acid from bacterial fermentation of simple sugars for the food industry (Tsao et al. 1999) or starch (Vishnu et al. 2000) and the production of bioethanol from sugar cane in Brazil or corn starch in the USA. The second wave of bioproducts involve the conversion of bioderived sugars, cellulosic biopolymers and oils trough biochemical routes that require advanced research and development in the biotechnology and bioprocess fields; some of them are now in the early pilot scale phase. Biochemical processing, using metabolically engineered microorganisms, to improve productivities and separation technologies to produce high-value chemicals have made significant advances in the last years. This may allow the second wave of bioproducts to become a reality shortly, yet some technological advances need to be made to turn it profitable.

According to the European Committee for Standardization (CEN 2011), a product wholly or partly derived from biomass is known as a biobased product. From a technical point of view, almost all industrial materials made from fossil resources could be substituted by their biobased counterparts (Carus et al. 2011). Biobased products include all kinds of biobased chemicals, biobased polymers, biobased plastics, and bioadditives, which are biodegradable. Bio-composites, like wood plastics composites and natural fibers, reinforced plastics and insulation materials, and also the traditional products of the timber industry, are considered biobased products.

Table 1 Worldwide production capacities of some bio-based building blocks, feedstocks and polymer production

Feedstock	Bio-based building block	Polymer(s)	Production 2013 (tons/year)	Production forecast 2020 (tons/year)
Glucose	1,3 propanediol	PTT/PU	78,000	120,000
Glucose/isobutanol	*p*-Xylene	PET/PBAT/PTT	1473	201,473
Glycerol	Epichlorohydrin	Epoxy resins	395,000	495,000
Bioethanol	Ethylene	Polypropylene/vinyl chloride	478,000	840,000
Glucose/sorbitol	Isosorbide	PU/PET-like	3000	3,000
Glucose	LA (L-D-L+D)	PLA/PTT	284,000	375,000
Fatty acids	Natural oil polyols	PU	85,000	85,000
Fatty acids	Sebacic acid	PA	22,000	22,000
Glucose	Succinic acid/1,4-butanediol	PBS/PU/PBAT	25,000	250,000
p-Xylene	Teraphtalic acid	PET/PBAT	110	110

Adapted from Dammer et al. (2013) and Aeschelmann and Carus (2015)
PA Polyamides; *PBS* Polybutylene succinate; *PBAT* Poly(butylene adipate-co-terephthalate); *PET* Polyethylene terephthalate; *PLA* Polylactic acid; *PET* Polyethylene terephthalate; *PTT* Polytrimethylene terephthalate; *PU* Polyurethanes
Note Bioethanol used for ethylene production (hence to produce bio-PET) is not indicated, since in the sources of this table is indicated that bioethanol was accounted as biofuel and not for ethylene production

In Table 1 some of the most important renewable (bio)chemicals that are used as biobased building blocks, as well as those that will have a potential development shortly are presented. In this table, glucose and other feedstocks are of biological origin such as corn, sugarcane, and sugar beet, among others. According to Aeschelmann and Carus (2015), the European Bioplastics growth projection of bio-based polymers will range from 1.7 million metric tons in 2014 to 7.8 million tons in 2019. The Projection presented in Table 1 from Nova-Institute was adapted from a report made to the Netherlands government (Dammer et al. 2013), and clearly shows that the biochemical building block market will have a positive growth trend. Part of this expected growth results from drivers that are looking at renewable and sustainable materials. An example is the Coca Cola Company's interest in the "Plant Bottle," which probably deployed the growth of aforementioned bio-building blocks since they are a precursor of bio-PET bottles. This increase in sustainable materials use is followed by biodegradable polyesters such as polybutylene succinate and poly(butylene adipate-co-terephthalate), which are closely followed by polylactic acid (PLA), biobased polyethylene and starch blends. A dynamic development is foreseen for drop-in bio-based polymers because they can use the same technology as those derived from petrochemicals. Drop-in bio-based polymers are chemically identical to their petrochemical counterparts but at least partially derived from biomass. The use of drop-in biobased PET has a projected production capacity of about 7 million tons by 2020, being polybutylene

succinate (PBS) in second place market of drop-in polymers. The biobased polymer market for the biodegradable biopolymers PLA and polyhydroxyalkanoates (PHA) has an impressive growth forecast: between 2014 and 2020, PLA production capacity is expected to almost quadruple, and PHA production capacity is projected to grow tenfold (Aeschelmann and Carus 2015). For a more detailed study of renewable chemicals and markets, the nova-Institute from Germany has launched several reports about this issue (http://www.nova-institut.de).

From the review of various publications about bio-based chemicals and polymers/plastics (de Jong et al. n.d.; Carus et al. 2011) it is clear that one of the main characteristics and advantages of bio-based chemicals is the use of renewable sources as their feedstock. This is relevant in the perspective that the production of many polymers will still be possible even if petroleum depletes in the future. Other social benefits of the manufacture of biobased polymers include the development of rural and forestry activities, the creation of employment and the support for research and development in biotechnology (Golden et al. 2015). However, from the economic point of view, it is necessary to develop stronger policies to support and help the growth of bio-based chemicals. More technological advances are required to make the biobased industry economically viable, the capital risk is high; but there are opportunities to develop new applications in food packaging, promote petroleum independence and generate improved properties to fill new niches or new applications (Aeschelmann and Carus 2015). Furthermore, feedstock availability and the need for large land areas to produce these raw materials are relevant concerns. However, the production of biobased building blocks and polymer derivatives contributes to reducing greenhouse gas emissions, including CO_2, increases sustainability in production processes, and can use a wide array of residual biomasses from water or land sources. Before going to the production at high scale, life cycle analysis for biobased products is a requisite to evaluate the environmental, economic, technological and social impacts, either positive or negative (Lammens et al. 2011).

2 Lactic Acid Production, Applications, Polylactic Acid and Markets

Lactic acid (LA) is one of the oldest biobased chemicals that exist since the very early stages of human history. The first time that LA was isolated as a chemical compound was from sour milk in 1789. Its name comes from the French “acide lactique” as named by Lavoisier; Pasteur discovered that this was not a component of milk, but instead was a product from fermentation (Vijayakumar et al. 2008). The IUPAC nomenclature for LA is 2-hydroxypropionic acid with the formula $CH_3CH(OH)CO_2H$. Since the second carbon is chiral, it may exist in two optical forms: L(+)-LA (L-LA) and D(-)-LA (D-LA). Lactic acid is classified as GRAS (generally recognized as safe) for use as a food additive by the US FDA (Food and Drug Administration). However, D(-)-lactic acid at an elevated concentration is

Table 2 Industrial-commercial uses of lactic acid

Cosmetic	Food	Pharmaceutical	Chemical	Biobased
Moisturizer Skin-lightening agent Skin-rejuvenating agent pH regulator Anti-acne agent Humectant Anti-tartar agent	Acidulant Preservative Flavor pH regulator Bacterial inhibitor Mineral fortification	Parental/intravenous solution Dialysis solution Mineral preparations Tableting Prostheses Surgical sutures Controlled drug delivery systems	Descaling agents pH regulator Neutralizer \chiral intermediate Green solvent Cleaning agent Slow acid releases agent Metal sequestration	Propylene oxide Acetaldehyde Acrylic acid Propanoic acid 2,3-pentanedione Ethyl lactate Dilactide Poly(lactic acid)

Adapted from Wee et al. (2006)

harmful to human metabolism and can result in acidosis and decalcification (Wee et al. 2006; Vijayakumar et al. 2008). The main uses of lactic acid are shown in Table 2.

Humans, animals, plants, and microorganisms produce LA. The first biotechnological production of L-LA was made in 1839 by Fremy, fermenting carbohydrates such as sucrose, lactose, mannitol, starch, and dextrin. The first commercial production was done in the United States of America in 1881 using a microbial process (Vijayakumar et al. 2008).

Probably its first use was related to food, particularly involving natural or processed fermented food preparations, 70–80% of the L-LA produced is used in food processes (John et al. 2009), being the remaining percentage for nonfood applications. The L(+) form of LA is used in the food and pharmaceutical industry because the human body is only adapted to assimilate this isomer (Vijayakumar et al. 2008). L-LA is a valuable chemical in the food industry as a preservative, acidulant, and flavoring agent, it is also used as feedstock for the manufacture of calcium stearoyl-2-lactylates in the baking industry. The water-retaining capacity and the ability to inhibit tyrosinase (the enzyme responsible for melanin formation) of lactic acid makes it suitable for use as moisturizer in cosmetic formulations and as skin lightening and rejuvenation (John et al. 2009). L-LA has numerous uses in medical/pharmaceutical applications, such as electrolytes in many parenteral/intravenous solutions that are intended to replenish bodily fluids. Also, it is used in a wide variety of medical preparations, which include tablets, prostheses, surgical sutures, and controlled drug delivery systems (Wee et al. 2006). In the leather and textile industries, technical-grade LA is extensively used in leather tanning industries as an acidulant for deliming hides and in vegetable tanning (John et al. 2009). Lactic acid as a descaling agent is often used in many decalcification products, such as bathroom cleaners, coffee machines, and toilets.

The chemical synthesis of lactic acid results from the hydrolysis of lactonitrile by strong acids, which produces the racemic mixture of L and D-LA (Wee et al. 2006; Nampoothiri et al. 2010; John et al. 2009). Lactonitrile results from the action of

Table 3 Advantages and disadvantages of chemical and biochemical lactic acid production processes

Advantages	Disadvantages
Chemical synthesis	
High product yield	Produces LA racemic mixtures[a,b] Use high temperatures Generate more contaminant to environment
Biochemical synthesis	
Lower energy consumption[b,c] Lower temperature[b, c] Produces optically pure LA[b,c] High purity[b] Use of low-cost feedstocks[b,c] Environmentally friend	Necessity of pretreatment (starchy/lignocellulosic biomass)[a] Requires enzymatic saccharification[a] Necessity of separation and purification process[a] Use of complex media such as yeast extract and corn steep liquor that hampers not only separation but also purification[a]

[a]Okano et al. (2010); [b]Nampoothiri et al. (2010); [c]John et al. (2009); [d]Mäki-Arvela et al. (2014)

hydrogen cyanide and catalyst on acetaldehyde derived from fossil petroleum (Vijayakumar et al. 2008) and acetaldehyde is obtained from the oxidation of ethylene, also obtained from petroleum (Gupta et al. 2007). The chemical synthesis pathway for commercial LA production was prevalent some decades ago until an inexpensive fermentation process was developed (Gupta et al. 2007). The biochemical pathway produces a desired optically pure L or D-LA (Okano et al. 2010). Advantages and disadvantages of both production pathways are indicated in Table 3. Nowadays, LA comes mainly (90%) from bacterial fermentation of renewable sources (Vijayakumar et al. 2008), it is recyclable, biodegradable and compostable. The polymer obtained from LA, polylactic acid (PLA), has numerous uses in a wide range of applications, such as protective clothing, food packaging, mulch film, trash bags, rigid containers, shrink wrap, and short shelf-life trays, among others (Wee et al. 2006). Besides the uses indicated in Table 2, since it is environmentally friendly, LA is used as a green solvent for epoxy resins. Lactate esters, especially ethyl lactate, are used as green solvents, cleaning agents, and diluents, and as a precursor of herbicides (Vijayakumar et al. 2008; Datta and Henry 2006).

Increasing interest has arised to use LA for the production of the biobased polymer PLA to substitute the poly(ethylene terephthalate) (PET) since PLA can have similar mechanical properties (rigidity and clarity) as PET. Both forms of LA: D and L are employed for the manufacture of PLA. The optical purity of D or L-LA is crucial to the physical properties of PLA, including its thermostability. Hence, the production of pure enantiomeric D- or L-LA is an important goal (Okano et al. 2010). The polymerization of pure forms of LA can produce highly crystalline PLA that are suitable for commercial uses. The use of the racemic form of LA in the production of PLA gives origin to an amorphous structure (Henton et al. 2005). Many commercial

Table 4 Melting and glass transition temperatures for different PLA compositions and structures

PLA structure	Glass transition temperature (°C)	Melting temperature (°C)
PLLA or PDLA[a]	55–65	170–190
Stereocomplexed PLLA/PDLA[a]	65–72	220–230
Stereo block complex PLLA/PDLA[a]	–	179
Random optical copolymers[a]	45–65	130–170
Copolymer ratios (L/D,)-PLA[b]		
100/0	63	178
95/5	59	164
90/10	56	150
85/15	56	140
80/20	56	125

[a]Data from Henton et al. (2005); [b]Data from Lim et al. (2008)

PLAs are copolymers of poly(L-LA) (PLLA) and poly(D, L-LA) (PDLLA), which are produced from L-lactides and D, L-lactides (Lim et al. 2008). Table 4 shows some characteristics of pure PLA and with different proportions of D and L forms. The glass transition temperature (Tg) determines the upper-temperature limit for most commercial applications. The melting point (Tm) is necessary to determine the temperatures used across various processes. "Both of these transitions, Tg and Tm, are strongly affected by overall optical composition, primary structure, thermal history, and molecular weight" (Henton et al. 2005).

As mentioned above the global market for PLA is expected to grow during the next years. The number of companies producing PLA is projected to increase to approximately 27 until the year 2020; the production capacity was 205,000 metric tons in 2014 and is expect to quadruple from 2014 to 2020 (Aeschelmann and Carus 2015). NatureWorks, based in Blair, Nebraska, USA, was established by Cargill Inc. and started producing PLA in 2002 (John et al. 2009; Wee et al. 2006) with a plant capacity in 2010 of 140,000 tons. This company produces polydilactide-based resins (Nature-WorksPLA®), used for packaging applications, and the Ingeo™polydilactide based fibers, which are employed in specialty textiles and fiber applications. Purac, in the Netherlands, produces lactide, as well as medical grade PLA (Schut 2008; Juturu and Wu 2015) for a total of 100,000 tons. Other companies that produce lactic acid and PLA are Pyramid Bioplastics Guben GmBH (60,000 tons, Germany), Archer Daniels Midland Company (USA), Henan Jindan (China) and Galactic (EU, USA).

3 *Escherichia coli* Fermentative Metabolism

The Gram-negative bacteria *Escherichia coli* can grow under aerobic and anaerobic conditions. Under fermentative conditions, *E. coli* transforms carbon substrates into several biochemicals. Using these endogenous organic compounds as terminal

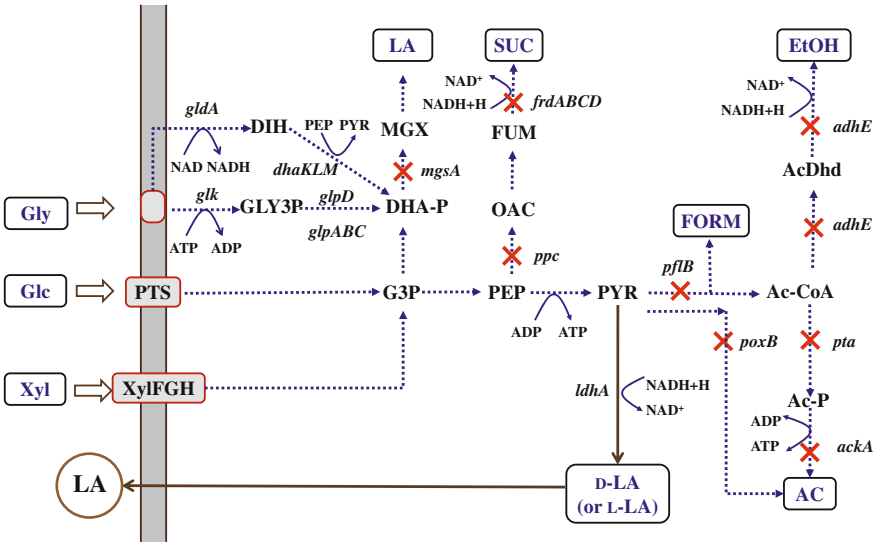

Fig. 1 *Escherichia coli* fermentative metabolism and relevant pathways and modifications for D or L-lactic acid production using metabolic engineering. Nomenclature: **Metabolites**: *Ac* Acetate, *Ac-CoA* Acetyl CoA, *AcDhd* Acetaldehyde, *Ac-P* Acetyl-P, *D-LA* D-lactate, *DHA-P* Dihydroxyacetone phosphate, *DIH* Dihydroxyacetone, *FORM* Formate, *EtOH* Ethanol, *FUM* Fumarate, *Glc* Glucose, *G3P* Glyceraldehyde 3-phosphate, *Gly* Glycerol, *GLY3P*: Glycerol 3-phosphate, *L-LA* L-lactate, *Mgx* Methylglyoxal, *OAC* Oxaloacetate, *PEP* Phosphoenolpyruvate, *PYR* Pyruvate; *SUC*: Succinate, *Xyl* Xylose. **Transporters**: *PTS* phosphotransferase system, mediates uptake of glucose with its concomitant phosphorylation. *XylFGH* Xylose ABC transporter.**Genes**: *ackA* Acetate kinase, *adhE* Alcohol dehydrogenase, *dhaKLM* Dihydroxyacetone kinase, *frdABCD* Fumarate reductase, *glpABC* Anaerobic glycerol 3-phosphate dehydrogenase, *glpD* Aerobic glycerol 3-phosphate dehydrogenase, *glpK* Glycerol kinase, *ldhA* D-Lactate dehydrogenase, *mgsA* Methylglyoxal synthase, *pflB* Pyruvate formate-lyase, *ppc* Phosphoenolpyruvate carboxylase, *poxB* Pyruvate oxidase, *pta* Phosphate acetyltransferase

electron acceptors, rapid growth and redox balance can be achieved by different fermentative processes (Orencio-Trejo et al. 2010). Because the metabolic products generated by *E. coli* have different oxidation states, this microorganism can adjust the metabolic pathways to grow on various carbon sources. Glucose is converted to a mixture of fermentation products consisting primarily of acetate and formate, as well as lower amounts of lactate, succinate and ethanol (Fig. 1) (Clark 1989). The fermentation of hexoses, with the same degree of reduction as glucose, generates four extra reducing equivalents, two ATP molecules by substrate-level phosphorylation, and two pyruvate molecules that are available for the formation of different organic acids and ethanol (Orencio-Trejo et al. 2010). It is known that wild-type *E. coli* strains using glucose as carbon source, under anaerobic conditions, commits only 4.8% of the carbon source to lactate (Yang et al. 1999). If the main acetic acid pathway (AckA-Pta) is deleted in *E. coli*, this bacterium produces mainly lactate

and succinate, with minor amounts of formate, ethanol, and pyruvate (Yang et al. 1999). Moreover, these researchers found that the double mutant Δ*ackA-pta* Δ*ldhA* (*ldhA* is the gene encoding for the stereospecific D-lactate dehydrogenase) increased the carbon flux to formate and ethanol, with a concomitant reduction in the cellular growth and also in the production of succinate and lactate. Furthermore, when *ldhA* was overexpressed in the double mutant (Δ*ackA-pta* and Δ*ldhA*), it was found that 90% of the carbon flux went through the lactate pathway (Yang et al. 1999). These results indicate that, whether redox balance is attained, *ldhA* overexpression can increase the flux to lactate in strains defective in acetate production.

Under fermentative conditions, *E. coli* can obtain redox balance when growing on hexoses or pentoses by reducing pyruvate to lactate. However, it is not able to grow on sorbitol or gluconate, because the redox potential cannot be regenerated by the metabolism of sugar alcohols or acid sugars (Orencio-Trejo et al. 2010). Some pyruvate-consuming pathways must be eliminated to increase the carbon flux to lactate (Fig. 1). The reaction catalyzed by pyruvate formate lyase (Pfl) is the major pyruvate-consuming pathway under anaerobic conditions. Hence, most of the strategies to achieve homolactic fermentation include the elimination of the Pfl activity. The *pfl* knockout in different *E. coli* strains causes abundant production of D-LA, but the LA volumetric productivity is low, reduced amounts of cell mass are formed, and low growth rates are obtained in comparison to the wild-type strains. Some Δ*pfl* strains are unable to grow on glucose under anaerobic conditions without acetate supplementation, because the Pfl reaction is the main supplier of acetyl-CoA under this conditions (Zhou et al. 2003a; Zhu and Shimizu, 2004; Utrilla et al. 2009, 2012).

4 Metabolic Engineering of *E. coli* to Produce D or L Lactate

Wild-type *E. coli* strains have the metabolic pathways to produce lactate (D or L) under anaerobic conditions (Fig. 1). Several strategies have been employed to channel the carbon source mainly to D-lactate. Gupta and Clark (1989) reported that *adh* and *pta* double mutants regained the ability to grow anaerobically on hexoses by LA fermentation. Further modifications, deleting the native D-lactate dehydrogenase (*ldhA*) and expressing heterologous genes encoding L-lactate dehydrogenases, allow the production of optically pure L-LA. The main metabolic engineering strategy has been to increase the pool of pyruvate, through the deletion of genes that consume this metabolite in pathways that channel the carbon source to ethanol, succinate, formate and acetate and increasing the expression of genes with lactate dehydrogenase activity (LDH). One of the first attempts to engineer *E. coli* strains to metabolize glucose to D-LA as the main fermentation product was reported by Chang et al. (1999). The strain produced high amounts of D-LA as result of the deletion of the gene encoding for phosphotransacetylase (*pta*). However, succinate was also produced in significant quantities. The gen *ppc* (encoding

phosphoenolpyruvate carboxylase) was deleted, to reduce the production of succinate and to generate a homofermentative lactogenic *E. coli* (strain RR1), that produced 62 g/L of D-LA from glucose and fed-batch cultivation with a volumetric productivity of 1.03 g/L h. The same research group deleted the *ldhA* gene and overexpressed the *ldh* of *Lactobacillus casei*, producing 45 g/L of optically pure L-lactate and a lower volumetric productivity (0.67 g/L h). Remarkably, these reports showed that when the pathways that compete for pyruvate molecules (succinate, acetate, formate and ethanol) are deleted, lactate, either D or L-LA, with an optical purity that exceed 99% can be produced.

For the sake of comparison, Table 5 shows a summary of results reported for several studies reported below, which are related to the metabolic engineering of *E. coli* and fermentation process conditions to produce D or L-LA. Dien et al. (2001) constructed a non-fermentative strain named ND10 (Δ*pfl* and Δ*ldh*). This strain was transformed with a plasmid that overexpressed the *ldh* gene of *Streptococcus bovis* to produce optically pure L-LA. The strain named FBR11 fermented 100 g/L of glucose in 30 h with a maximum theoretical yield of 93% L-LA. Furthermore, the genes encoding fumarate reductase (*frdABCD*) for succinate production, alcohol/aldehyde dehydrogenase (*adhE*) for ethanol production, and pyruvate formate lyase (*pflB*) for formate and acetyl-CoA production, were deleted in *E. coli* W3110, showing that it was possible to reach 45 g/L of D-LA with a yield of 94% and an optical purity of 99% (Zhou et al. 2003a). The effect in the production of D-LA was studied in a two stage fermentation. The first stage was performed under aerobic growth and second stage under anaerobiosis. Through deletions in single and multiple genes in an *E. coli* this study showed that simple deletions in *ackA*, *pta*, *pflB*, *dld*, *poxB*, and *frdA* genes (Fig. 1) improved yield and productivity of D-LA (*dld* encodes for an aerobic D-lactate dehydrogenase); however, deletions in *ppc* and *adhE* had a negative effect on these parameters. In such strain, with multiple mutations, it was observed that the deletion of *pps-ackA-pta* had not effect in production. However, the strain with deleted *ackA-pta, pps, pflB, dld, poxB* and *adhE* genes, increased the lactate yield, volumetric productivity, and reduced the formation of by-products more than 90%, producing 125 g/L of D-LA from 739.5 g of glucose-mineral medium in a 3 L bioreactor (Zhou et al. 2011).

In the past, most studies for D or L-LA production were focused on combining the deletion of pathways that compete for pyruvate and overexpression of different *ldh* genes; but in recent years the *E. coli* metabolic pathways have been rationally modified. To regulate the rates of D-LA overproduction without decreasing the cellular growth, the chromosomal upstream region of the *ldhA* gene was engineering by predicting possible promoter regions. Sequential shortened chromosomal upstream regions were cloned in a *ldhA* mutant (Zhou et al. 2012). The clones B0013-080C/pTH-rrnB-ldhA6 and B0013-080C/pTH-rrnB-ldhA8 produced D-LA with high efficiency, due to a putative promoter downstream of the −96 site, whose function could be a transcriptional promoter or regulator.

In strains that have been modified by metabolic engineering, the production of optically pure LA has been increased through the expression of heterologous enzymes or modifications in enzymes with LDH activity. The methylglyoxal

Table 5 Summary of results reported for the production of D or L lactate using metabolic engineering *E. coli* strains, different cultivation medium, and sugars from various sources, including some syrups from lignocellulosic hydrolysates

E. coli strain	Genotype	D or L-LA	Medium	Carbon source	LA (g/L)	Q_{LA} (g/Lh)	Yield (g/g)	Reference
FBR11	Δ*pfl*, Δ*ldhA* (*ldh S. bovis*)	L-LA	RM	Glc	73.2	2.2	0.93	Dien et al. (2001)
FBR11	Δ*pfl*, Δ*ldhA* (*ldh S. bovis*)	L-LA	RM	Xyl	63.3	0.73	0.78	Dien et al. (2001)
FBR19	Δ*frdABCD* Δ*ptsG* (*ldh S. bovis*)	L-LA	RM	Xyl-Glc	64.3	1.52	0.77	Dien et al. (2002)
SZ63	Δ*pflB* Δ*frdABCD* Δ*adhE* Δ*ackA*	D-LA	MM	Glc	48	0.53	0.96	Zhou et al. (2003a)
SZ79	Δ*focA-pflB* Δ*frdBC* Δ*adhE* Δ*ackA* Δ*ldhA*:: *ldhL* (*P. acidilactici*)	L-LA	MM	Glc	42.58	0.36	0.86	Zhou et al. (2003b)
$SZ85^{ES}$	Δ*focA-pflB* Δ*frdBC* Δ*adhE* Δ*ackA* Δ*ldhA*:: *ldhL* (*P. acidilactici*)	L-LA	MM	Glc Xyl	44.98 39.10	0.64 0.31	0.94 0.82	Zhou et al. (2003b)
SDU4	*ΔfocA-pflB* Δ*adhE*	L-LA	MM	Glc	100		0.97	Liu et al. (2011)
$XW068^{ES}$	Δ*frdBC* Δ*ldhA* Δ*adhE* Δ*ackA* Δ*rrlE*::(*pdc adhA adhB*)$_{Zm}$ Δ*mgsA*, *yqhD* mutated promoter *ldhL (P. acidilactici)*, *fucO* in pTrc99A	D-LA	MM	Xyl	75.71	1.03	0.85	(Wang et al. 2011a, b)
JU01	Δ*pflB* Δ*adhE* Δ*frdA* Δ*xylFGH*	D-LA	MM	Xyl		0.53	0.95	Utrilla et al. (2012)
$JU15^{ES}$	JU01 Δreg 27.3 kb, *gatC*S184L	D-LA	MM	Xyl		0.79	0.95	Utrilla et al. (2012)
$JU15^{ES}$	JU01 Δreg 27.3 kb, *gatC*S184L	B D-LA	MM	SCBH	55	0.98	1.11	Utrilla et al. (2016)

(continued)

Table 5 (continued)

E. coli strain	Genotype	D or L-LA	Medium	Carbon source	LA (g/L)	Q_{LA} (g/Lh)	Yield (g/g)	Reference
AV03	JU15 Δ*poxB* Δ*ackA-pta*, Δ*mgsA*	D-LA	MM	CSH	58.2	1.21	1.11	Utrilla et al. (2016)
WL204^{ES}	Δ*frdBC* Δ*ldhA* Δ*ackA* Δ*pflB* Δ*pdhR*:: *pflB*p6-acEF-lpd Δ*mgsA* Δ*adhE* *ldhA*::*ldhL* (*P. acidilactici*)	L-LA	MM	Xyl	62	1.63	0.97	Zhao et al. (2013)
JW0886	BW25113, Δ*pflB*	D-LA	MM	Glc	6.5	0.65	73	Zhu and Shimizu (2004)
ALS974	DSM 14335, *Hfr zbi:: Tn10,* Δ*poxB,* Δ *(aceEF),* Δ*rpsL,* Δ*pps,* Δ*pfl,* Δ*frdABCD*	D-LA	MM	Glc	138	6.3	0.99	Zhu et al. (2007)

ES Evolved strain, *Zm Zymomonas mobilis, RM* Rich medium, *MM* Mineral medium, *SCBH* Sugar cane bagasse hydrolysate, *CSH* Corn stover hydrolysate, *Glc* Glucose, *Xyl* Xylose

bypass is a pathway able to produce L and D-LA starting in the dihydroxyacetone phosphate node, and it is induced by low phosphate concentration and an increase in the dihydroxyacetone pool. It is assumed that at a high glycolytic flux this pathway balances ATP production with cellular growth and metabolism by bypassing the ATP production steps in glycolysis. This pathway was inactivated by deleting *mgsA* in an *E. coli* B mutant in *pflB, frd, adhE*, and *ackA* (strain SZ194). Adaptive evolution mutated such strain (evolved) (Grabar et al. 2006) and the resultant strain (TG114) showed a yield of 0.98 g D-LA/g glucose. Co-products and chiral impurities were below <0.1%. The native gene *ldhA*, in strain SZ194, was deleted and replaced with the *ldhL* gene from *Pediococcus acidilactici* encoding an L-lactate dehydrogenase, to obtain a strain that produces L-LA (Zhou et al. 2003b). The strain was evolved to improve growth and productivity; however, 5% of D-LA was present as a contaminant of L-LA. To remove this contaminant, the *mgsA* gene was deleted, obtaining strain TG105, which was able to produce only L-LA. After an additional adaptive evolution process, the strain TG108, derivative of TG105, reached a productivity of 2.7 g/L h of L-LA. This study showed that *mgsA* is a key gen to produce optically pure D or L-LA.

The D-LA isomer has been mainly produced through metabolic modifications and employing the *E. coli* native genes. However, as shown above, it is possible to replace the *ldh* gene to produce optically pure L-LA in metabolic engineered *E. coli*. Furthermore, Zhou et al. (2003b), through a simple adaptive evolution strategy improved the production of L-LA. The strain SZ79 (Zhou et al. 2003b), derivative of *E. coli* W3310 (*focA-pflB, frdBC, adhE, ackA, ldhA*) was constructed, replacing the *ldhA* coding region with the *ldhL* of *P. acidilactici*. However, the production of L-LA was poor. Subsequently, the strain was evolved in mineral medium with 5% of glucose, and before 17 days, samples of the culture were spread in mineral medium plates to select potential mutants. A mutant, named SZ85, exhibited a 30-fold increase in the *ldhL* activity in comparison to the parental strain SZ79. Sequencing revealed mutations in the coding and terminator regions of *ldhL*, which are presumed to be responsible for the increased activity in the L-lactate dehydrogenase enzyme. On the other hand, Wang et al. (2011a) revealed that amino acid changes in the glycerol dehydrogenase enzyme (GlyDH) found in *Bacillus coagulans* generate a protein with the capacity to reduce pyruvate to D-LA. *B. coagulans* produces L-LA as the main fermentation product, at 50 °C and pH 5, conditions that can be used in a simultaneous saccharification and fermentation process of cellulosic materials. A non-fermentative *B. coagulans* strain was obtained by deleting the genes *ldh* and *alsS* (encoding acetolactate synthase), which are essential for 2,3-butanediol production. This strain was unable to growth at pH 5 in anaerobic conditions. The strain was forced to growth in two phases, first in the presence of oxygen and then under oxygen limitation. This adaptive evolution process generated mutants, which were selected growing under fermentative conditions. A mutant strain named QZ19 produced optically pure D-lactate under anaerobic conditions. Through DNA sequencing, the new enzyme responsible for D-lactate dehydrogenase activity was identified as a mutant in glycerol dehydrogenase (*glyDH*; D121 N and F245S). GlyDH, in its native form, did not show detectable

activity with pyruvate; however, when the mutated gene was cloned and expressed in *E. coli*, pyruvate reduction activity was detected, allowing the production of D-LA (Wang et al. 2011a, b).

5 Fermentation of Glucose, Xylose and Mixtures to Lactic Acid Using Metabolic Engineered *E. coli*

In comparison to lactic acid bacteria, *E. coli*, can consume several carbon sources, this advantage can be employed to use sugars from biomass sources such as lignocellulosic hydrolysates, which are rich in hexoses and pentoses. Glucose and xylose are the main sugars contained in syrups from hydrolysates, but they also contain arabinose, mannose, galactose, and galacturonate that *E. coli* can metabolize.

Several studies after the year 2000, related to the production of D or L-LA using alternative carbon sources, have employed xylose as the main sugar. The engineered *E. coli* strain FBR11 (Dien et al. 2001) was grown in xylose, and the L-LA productivity and yield were lower than when the strain was grown in glucose (Table 5), producing 63.3 g/L of L-LA. This work showed that it was possible to produce L-LA from xylose in a complex medium. On the other hand, the evolved strain SZ85, which expresses the *ldhL* from *P. acidilactici* integrated into the chromosome, was evaluated in mineral medium with xylose. The volumetric productivity was half of what was observed with glucose, and a yield of 86% of the theoretical maximum was obtained (Zhou et al. 2003b). The low productivity and yield compared to glucose are due mainly to the ATP balance. When the xylose is transported and the pentoses phosphate pathway is employed to metabolize this carbon source, two molecules of ATP are used for transport and pentose phosphorylation. For glucose and using the phosphotransferase system (PTS), the equivalent of one ATP molecule (as phosphoenol pyruvate) is used for both transport and phosphorylation, increasing the energy available for cellular growth. The PTS system is the main transporter of glucose in *E. coli*, and it participates in catabolic repression. To increase the pool of phosphoenol pyruvate and reduce catabolic repression effects, the strain FBR19 was generated. This strain has deleted the *pfl, ldhA, frdABC* genes and also the *ptsG* component of the PTS system, and, as described above, the *ldhL* from *S. bovis* was overexpressed in a plasmid (Dien et al. 2002). When the strain FBR19 was evaluated in mixtures of glucose-xylose (50–50 g/L), it reached a productivity of 1.52 g/L h of L-LA, consuming the total glucose and 75% of xylose present in the mixture. This study showed that the catabolite repression was eliminated with the *ptsG* deletion, being a precedent to use C5-C6 sugar mixtures to produce L-LA.

Another way to increase the availability of ATP for growth and fermentation when xylose is employed as carbon source was shown by Utrilla et al. (2012). These researchers deleted the XylFGH system, i.e. the main xylose transport system in *E. coli*, which uses one ATP per molecule of transported xylose, and when this

sugar is metabolized trough the pentose phosphates pathway, another ATP is used to phosphorylate the C5 derivative of xylose. The strain JU01 (*E. coli* MG1655 Δ*pflB* Δ*adhE* Δ*frdA* Δ*xylFGH*), a lactogenic strain containing the *xylFGH* deletion was evaluated in mineral medium with xylose (Utrilla et al. 2012). The strain showed a 95% yield of the theoretical maximum and a volumetric productivity of 0.53 g/L h of D-LA. The strain JU01 was subjected to an adaptive evolution process using xylose as selection pressure in mineral medium. After several transfers at two different xylose concentrations (40 and 120 g/L), an evolved strain called JU15 was selected. The D-LA volumetric productivity of JU15 was two-fold higher when compared to JU01 (Table 5). The analysis of the genome sequence of JU15 revealed gene *gatC* (reported as galactitol transporter) as a xylose transporter, and the mutation S184L in the GatC protein improved the capacity of this protein by increasing the uptake rate of xylose without the use one ATP molecule for xylose transport (Utrilla et al. 2012). To improve the growth under anaerobic conditions and the production of L-LA, the strain SZ470 (Δ*frdBC* Δ*ldhA* Δ*ackA* Δ*pflB* Δ*pdhR*::*pflBp6-acEF-lpd* Δ*mgsA*) was evolved through serial transfers employing complex medium supplemented with xylose. The resulting strain after evolution, named WL204, produced 62 g/L of L-LA from 70 g/L of xylose with a yield of 97% of the theoretical maximum, and an L-LA purity of 99.5%.

These studies showed that metabolically engineered *E. coli* strains could metabolize xylose to produce D or L-LA with yields near the theoretical maximum. The modifications to increase yields or productivity consuming xylose, included modifications in specific xylose uptake transporters or disruption of the catabolic repression effect and the use of adaptive evolution process to select strain-specific mutations that favor the transport and rate of xylose metabolism to LA. These characteristics are essential to use lignocellulosic hydrolysates which contain mixtures of hexoses and pentoses.

6 Lactic Acid Production from Lignocellulosic Hydrolysates with Metabolic Engineered *E. coli*

The production of lactate from lignocellulosic hydrolysates have been studied in recent years (Juturu and Wu 2015; Chen et al. 2013). The lignocellulosic hydrolysates contain high concentrations of some toxic compounds such as furfural and 5-hydroxymethyl furfural, and several phenolic compounds, which inhibit growth of *E. coli* (Martinez et al. 2001). Diverse metabolic engineering strategies have been developed to resist toxicity. The strain XW068 was metabolically engineered to produce D-LA from xylose and glucose present in lignocellulosic hydrolysates and to be resistant to toxicity by furfural (Wang et al. 2011a, b). The strain was mutated in the NADPH-dependent oxidoreductase YqhD and gene *fucO* was overexpressed, because previously it was demonstrated that a mutation in *yqhD* and *fucO* overexpression increases the resistance to furfural in other engineered *E. coli* strains. Furthermore, this strain was evolved to improve xylose utilization and productivity;

the resultant strain XW068 was tested using a xylose-mineral medium with increased furfural concentrations. The strain produced 75.71 g/L of D-LA with a productivity of 1.03 g/L h and a yield of 85% of the theoretical maximum in the presence of 15 mM furfural, showing that the mutation in *yqhD* and the overexpression of FucO increased the tolerance of *E. coli* to furfural.

There are two main strategies to improve the fermentation of sugars present in lignocellulosic hydrolysates: (a) reduce the toxic compounds concentration using overliming or another detoxificación method (Martinez et al. 2000); (b) or using non-severe conditions in the pretreatment of the lignocellulosic materials, i.e. avoiding or minimizing the formation of furans and other toxic compounds during the thermochemical pretreatment of biomass (Vargas-Tah et al. 2015; Avci et al. 2013). Diluted acid hemicellulosic hydrolysates from sugar cane bagasse were detoxified using overliming, and D-LA production was tested using strain JU15. From 70 g/L of quantified sugars (xylose, glucose, and arabinose), a productivity of 0.98 g/L h of D-LA was achieved with an apparent yield of 1.11 g D-LA/g of sugars (Utrilla et al. 2016). On the other hand, the strain AV03 (Table 5), a derivative of JU15 (JU15 in Δ*poxB,* Δ*ackA-pta,* Δ*mgsA*) was grown in corn stover hydrolysate that was obtained using non-severe conditions in the pretreatment (Vargas-Tah et al. 2015). Fermentation of these syrups, containing no more than 0.25 g/L of total furans, and using strain AV03 reached 52.2 g/L of D-lactate from a mixture of glucose (42 g/L), xylose (32 g/L) and arabinose (4 g/L), with a productivity of 1.21 g/L h of D-LA and an apparent yield above 100% of the theoretical. In both cases, an apparent high yield was obtained because not all sugars in the hydrolysates were measured, and other carbon sources were also fermented to D-LA. This study showed that it is possible to grow and produce D-lactate from lignocellulosic hydrolysates without a detoxification process. All *E. coli* strains obtained by metabolic engineering, to produce D or L-lactate, showed yields higher than 80% of the theoretical from different sugars. Such results indicate that the redirection of carbon flux to LA, through the deletion of several genes and strain improvement by adaptive evolution allows an efficient production at cultivation conditions that favor *E. coli* growth, 37 °C, pH 7.0 under fermentative conditions.

7 Lactic Acid Production from Glycerol and Sucrose with Metabolic Engineered *E. coli*

Other low-cost substrates that have been used to produce D or L-LA, using metabolically engineered *E. coli* as a biocatalyst include glycerol, sucrose, and molasses. Glycerol has become an attractive carbon source for the production D or L-LA because now it is inexpensive, abundant and has a higher degree of reduction in comparison to sugars, such as glucose and xylose. Glycerol is generated in large amounts as a by-product of the biodiesel and bioethanol industries (Clomburg and Gonzalez 2013). Also, certain microalgae accumulate significant amounts of glycerol (Oren 2005). However, until a decade ago, glycerol has not been

considered as a carbon source for *E. coli*, because the reported inability of this microorganism to ferment glycerol in the absence of external electron acceptors. Moreover, ten years ago a reassessment of glycerol metabolism by *E. coli* revealed key metabolic factors for glycerol metabolism in a fermentative and respiratory manner (Dharmadi et al. 2006; Gonzalez et al. 2008; Durnin et al. 2009). The two pathways involved in glycerol dissimilation to the glycolytic intermediate dihydroxyacetone phosphate by *E. coli* are shown in Fig. 1. Both pathways play a significant role in the conversion of glycerol to the glycolytic intermediate dihydroxyacetone phosphate. Those studies evidenced that microaerobic glycerol metabolism turned out to be very efficiently for ethanol production, hydrogen, and formate in a medium containing mineral salts without rich supplements.

Mazumdar et al. (2010) designed an *E. coli* strain called LA02 Δ*dld* for the efficient production of D-LA by metabolic engineering strategies. The key points for the design of this strain were blocking the production of enzymes leading to the synthesis of competing by-products and overexpressing the enzymes involved in the conversion of glycerol to D-LA (Fig. 1). To minimize the synthesis of succinate, acetate, and ethanol, metabolites that compete with D-LA synthesis from pyruvate, the inactivation of fumarate reductase (*frdA*), phosphate acetyltransferase (*pta*), and alcohol/acetaldehyde dehydrogenase (*adhE*), were carried out. Also, to prevent D-LA from being metabolized, the aerobic D-lactate dehydrogenase (*dld*) was interrupted. The approach described above allowed the accumulation of D-LA. The increase of the respiratory GlpK-GlpD pathway induced by the introduction in *E. coli* LA02 Δ*dld* of the plasmid pZSglpKglpD, resulted in the production, in minimal medium, of 32 g/L of D-LA (with 99.9% of chiral purity) from 40 g/L glycerol. The volumetric and specific rates of D-LA production in *E. coli* LA02 Δ*dld* (pZSglpKglpD) were 1.5 g/L h and 1.25 g/g cell/h, respectively. The redox balance of this pathway also allows the production of 1–2 mol of ATP per mole of D-LA therefore, generating a feasible metabolic pathway.

For the production of D-LA from sucrose, Shukla et al. (2004) engineered *E. coli* W3110 to provide the capability to ferment sucrose. The *E coli* strain KO11 (a metabolically engineered ethanologenic strain that metabolizes sucrose) was used as the source of the sucrose gene cluster. The sucrose gene cluster consists of an operon encoding a repressor protein (*cscR*), an invertase (*cscA*), and a bicistronic operon (*csCKB*), encoding fructokinase and an anion symporter for sucrose. A genomic library was used as a source of genomic DNA, and the genes encoding the sucrose gene cluster were cloned in a plasmid called pLOI3501 and expressed in the strain SZ63. The strain SZ63 (pLOI3501) produced, 568 mM D-LA (in 96 h), from 50 g/L sucrose with a 97% yield of the theoretical yield. Furthermore, a mix of glucose, fructose and sucrose, similar to those encountered in molasses and diluted cane molasses (50 g/L total sugars), was also fermented with SZ63 (pLOI3501), reaching a concentration of 540 mM of D-LA in 24 h, with a yield of 94%. The longer time to ferment the same amount of sugars in the actual diluted molasses can be attributed to inhibitors that can be generated during the molasses sterilization or during the sugar production process. Finally, the metabolic

byproducts synthesized in cultures in diluted molasses were similar to those produced with pure sugars.

8 Concluding Remarks

Several *E. coli* strains for the production of optically pure D-LA or L-LA have been generated by employing metabolic engineering and laboratory adaptive evolution strategies. As shown in Table 5, as part of the strain generation strategies, there is a common set of deletions on genes that encode enzymes that divert pyruvate to pathways different from LA. These modifications can be considered the core of a metabolic engineering strategy for LA production with *E. coli*. Reports with engineered *E. coli* strains indicate that it is possible to produce with high efficiency optically pure D-LA or L-LA (LA yields form carbon source higher that 90% of the theoretical maximum and volumetric productivities higher than 1 g/L h employing mineral salts media). In addition to the rational metabolic engineering strategies employed for production strain generation, the utilization of adaptive evolution schemes has allowed improvement in LA volumetric productivity from a wide range of carbon sources. Such carbon sources can be found in materials derived from residues of several types of industry. Although efforts towards the generation of *E. coli* strains that efficiently ferment several carbon sources have been successful, this review evidences scarce reports where actual industrial residue materials are employed. Therefore, it is clear that further efforts in research and development must be made to evaluate production strain with carbon sources that originate from residual material that has been proposed for the generation of bio-comodities. This research should be performed under conditions similar to those required in an industrial setting and include fermentation processes (upstream) as well as LA purification (downstream) and scale-up.

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Engineering Bacterial Sugar Catabolism and Tolerance Toward Lignocellulose Conversion

Andrew D. Flores, Gavin L. Kurgan and Xuan Wang

Abstract Lignocellulosic biomass represents a renewable domestic feedstock that can support large-scale biochemical production processes for fuels and specialty chemicals. However, cost-effective conversion of lignocellulosic sugars into valuable chemicals still remains a challenge. Biomass recalcitrance to saccharification, microbial sugar co-utilization and toxic chemicals associated with chemical pretreatments are at the center of the bottlenecks limiting further commercialization of lignocellulose conversion. Genetic and metabolic engineering has allowed researchers to manipulate microorganisms to overcome these challenges. In this chapter, a broad overview of the current knowledge and research efforts in two paramount areas is presented: (1) bacterial carbon catabolite repression and sugar co-utilization, and (2) microbial tolerance to inhibitors derived from lignocellulose pretreatments. Lastly, technological gaps and future directions for further improvements are discussed. This chapter will mainly focus on the relevant knowledge and research progress from the perspective of bacterial strain engineering.

Keywords Sugar catabolism · Carbon catabolite repression · Metabolic engineering · Lignocellulose conversion · Furan aldehydes · Alternative feedstock

1 Introduction

Modern society is unsustainably dependent on petroleum. For example, approximately 120 billion gallons of gasoline were consumed in the United States in 2012 (Choi et al. 2013). Besides transportation fuels, many other bulk industrial chem-

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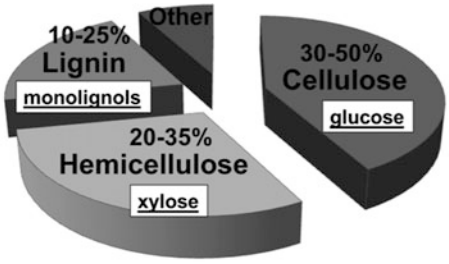
icals, such as solvents, fertilizers, pesticides, and plastics, are also derived from petroleum (Service 2007). Due to escalating global energy consumption, dramatic changes have manifested in the atmosphere and global climate (Karl and Trenberth 2003). To ensure the future advancement of human society, a sustainable supply of energy and chemicals is needed. Production of fuels and chemicals by fermentation-based manufacturing processes using a renewable feedstock is a desirable alternative to petrochemical production.

Lignocelluloses are the most abundant renewable natural organic materials present on the earth (Clark et al. 2006; Saha 2003; Girio et al. 2010). They account for more than 60% of total biomass and are renewable due to carbon-fixing photosynthetic processes of plants, with a net productivity of 155 billion tons per year (Singh and Mishra 1995). Converting lignocellulose into fuels and chemicals does not compete with food sources since economic plants grown for food and other commercial purposes generate millions of tons of lignocellulosic waste. A wide range and variety of agricultural, forestry and industrial wastes are available for value-added microbial conversion. For example, approximately 731 million tons of rice straw is annually produced globally as an agricultural waste (Balat 2011). The stems, leaves and fibers from agricultural crops generally have sugar content higher than 50% of their dry weight, and thus these agricultural waste residues represent abundant carbon feedstocks. Additionally, ample amounts of lignocelluloses are present in forest residues, wood sulfite waste, fruit/vegetable waste, waste paper and municipal solid waste (Saha 2003; Girio et al. 2010; Singh and Mishra 1995). The potential for using such wastes as a renewable carbon source is of great importance.

Lignocellulose is a complex matrix present in plant cell wall structures; composed of many different polysaccharides, phenolic polymers, and proteins. Regardless of



Representative sugar composition (% dry weight) of lignocellulose

Glucose	Xylose	Arabinose + mannose	galactose	total
43.8 ± 1.4	23.4 ± 2.1	5.6 ± 0.7	3.5 ± 0.4	76.3 ± 2.7

Fig. 1 *Composition of lignocellulose*. The approximate lignocellulose composition is given as a percentage of total dry weight. The major carbon monomers of the main polymeric components for typical lignocelluloses are *underlined* in a *white box*. The representative sugar composition shown in the table was obtained from a sugarcane bagasse sample (Geddes et al. 2010). Note that the composition of soft wood materials such as gymnosperm trees is usually different from this graph with higher lignin content and lower xylose content in the hemicellulose portion

source, most lignocellulosic biomass contains cellulose, hemicellulose, and lignin as three major polymeric components as shown in Fig. 1. Unlike starch, lignocellulose has been evolved to resist deconstruction. Cellulose fibers are encased in a covalently-linked mesh of lignin and hemicellulose. Cellulose (30–50% of lignocellulose dry weight) is composed of D-glucose and is highly resistant to deconstruction. Efficient degradation of cellulose generally requires cellulases (Garvey et al. 2013; Hasunuma et al. 2013; Bommarius et al. 2014). Lignin (10–25% lignocellulose dry weight) is a heterogeneous aromatic polymer and is difficult for microbes to use as carbon source (Eudes et al. 2014; Carrott and Carrott 2007). Three basic phenol derivatives (p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol), the so-called monolignols, make up almost all types of lignin found in nature (Eudes et al. 2014; Carrott and Carrott 2007). Hemicellulose (20–35% of lignocellulose dry weight) is composed of a mixture of pentoses and hexoses mostly with D-xylose as the major sugar (Fig. 1) (Saha 2003; Girio et al. 2010; Geddes et al. 2010a). Simple pretreatments such as steam pretreatment with dilute mineral acids (e.g. sulfuric and phosphoric acid) are able to depolymerize hemicellulose into sugar monomers (Saha 2003; Girio et al. 2010; Geddes et al. 2010a).

Fermentable sugar content of lignocelluloses occupies 50 to 70% of biomass dry weight, which is comparable to corn (Saha 2003). However, these sugars are covalently fixed in polymeric states, and thus require chemical and/or enzymatic pretreatment processes to release them for microbial conversion (Saha 2003; Hendriks and Zeeman 2009). After pretreatment, the resulting syrups contain a mixture of hexoses and pentoses, however, direct use of these carbon sources for microbial conversion is difficult for the following two reasons:

First, utilization efficiency of sugar mixtures by microbes is hindered by a global regulatory mechanism called catabolite repression (Kim et al. 2010, 2012; Doran-Peterson et al. 2009). This regulation is common for most microbes used in bio-based production, if not all, and is tightly controlled at transcriptional and biochemical levels (Kim et al. 2010; Kolb et al. 1993). Glucose is often the preferred substrate by industrial microbes and its presence represses the catabolism of other secondary sugars in lignocellulose such xylose and arabinose (Deutscher 2008; Gorke and Stulke 2008). Under anaerobic or micro-aerobic fermentation conditions, complete consumption of sugar mixtures at high rates is difficult, especially for high sugar concentrations (100 g/L total sugars or higher) (Kim et al. 2010). This results in sugar loss, decreased productivity, and lower product titers for lignocellulose conversion. Another contributing factor to poor sugar mixture utilization is the inability for some common industrial microbes to metabolize the most abundant pentose in hemicelluloses, D-xylose (Fig. 1) (Toivari et al. 2004; Jeffries 1983; Jeffries and Shi 1999). For example, industrial microbes for ethanol production such as *Saccharomyces cerevisiae* and *Zymomonas mobilis* do not natively metabolize xylose, and these catabolic pathways must be integrated into the hosts for xylose utilization (Saha 2003; Jeffries 1983).

Second, toxic side products, such as furan aldehydes, formate, acetate and soluble aromatics, are produced during sugar degradation steps in thermochemical pretreatments (Girio et al. 2010; Hendriks and Zeeman 2009). The aforementioned

toxic side products can hinder or even be detrimental to cell growth (Zaldivar et al. 1999; Liu 2010). Furfural (a dehydration product of pentose sugars) is one of the most important inhibitors due to its cytotoxicity and abundance after lignocellulose pretreatment (Mills et al. 2009; Geddes et al. 2011a). Genetic changes that increased furfural tolerance also increased bacterial tolerance to pretreatment hydrolysate syrups, indicating the importance of furfural for toxicity (Wang et al. 2013). Overliming to pH 10 with $Ca(OH)_2$ or other physical methods such as active carbon filter or vacuum treatment are able to remove inhibitors and thus reduce cytotoxicity of lignocellulosic hydrolysate syrups (Martinez et al. 2000; Geddes et al. 2015). However, these extra steps increase process complexity and operational costs, thus reducing economic viability.

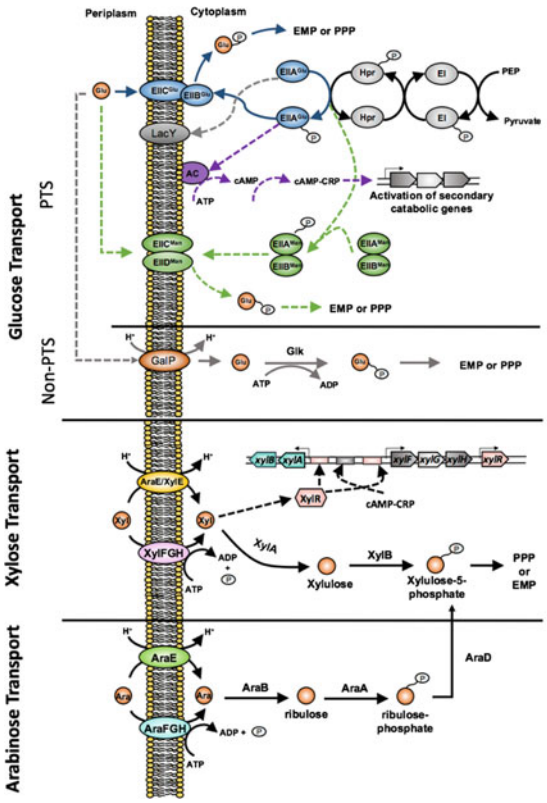
In this book chapter, we will comprehensively review the related knowledge and research progress with respect to these two limiting factors for lignocellulose conversion.

2 Engineering Bacterial Transport and Catabolism of Lignocellulose-Derived Sugars

2.1 Bacterial Transport and Catabolism for Major Lignocellulose-Derived Sugars

In many bacterial species, the phosphoenolpyruvate: sugar phosphotransferase system (PTS) facilitates the transport and concomitant phosphorylation of exogenous carbohydrates across the cytoplasmic membrane (Stulke and Hillen 1999). Using *Escherichia coli* as an example, the PTS of *E. coli* is a multiprotein phosphorelay system consisting of two soluble and non sugar-specific enzymes Enzyme I (EI) and the histidine protein (HPr), encoded by the *ptsI* and *ptsH* genes, respectively, and the sugar-specific enzyme Enzyme II (EII) system (Gorke and Stulke 2008) (Fig. 2). EII is a multicomponent complex composed of two hydrophilic domains, EIIA and EIIB, and one or two carbohydrate-selective transmembrane domains, EIIC and EIID (Kotrba et al. 2001). These mentioned domains of EII may occur as individual proteins or as a combination of subunits in variable order and number (Kotrba et al. 2001) (Fig. 2).

Multiple parallel EII complexes facilitate cellular uptake of different carbohydrates. The *E. coli* genome encodes for more than 20 different EII complexes, thus allowing for the transport and simultaneous phosphorylation of more than 20 different carbohydrates (Tchieu et al. 2001). In the PTS, relay of the phosphoryl group initiates with the autophosphorylation of EI from phosphoenolpyruvate (PEP) and subsequently transfers the phosphoryl group to a histidine residue on the HPr (His-15 in *E. coli*) (Gorke and Stulke 2008; Postma et al. 1993). HPr then phosphorylates various sugar-specific EII complexes. In *E. coli*, the glucose specific EII complex comprises of the soluble enzyme $EIIA^{Glc}$ and the integral membrane permease $EIIBC^{Glc}$, encoded by *crr* and *ptsG*, respectively (Postma et al. 1993).



Enzyme Abbreviation	Enzyme Name	Gene Name
AC	Adenylate Cyclase	*cyaA*
AraE	Arabinose Proton Symporter	*araE*
AraFGH	Arabinose ABC Transporter ATPase	*araFGH*
AraB	Ribulokinase	*araB*
AraA	Arabinose isomerase	*araA*
AraD	Ribulose-5-phosphate 4-epimerase	*araD*
CRP	Catabolite Repression Protein	*crp*
EI	Enzyme I	*ptsI*
$EIIA^{Glc}$	Enzyme IIA Glucose Specific	*crr*
$EIIBC^{Glc}$	Enzyme IIBC Glucose Specific	*ptsG*
$EIIAB^{Man}$	Enzyme IIAB Mannose Specific	*manX*
$EIICD^{Man}$	Enzyme IICD Mannose Specific	*manYZ*
GalP	Galactose Proton Symporter	*galP*
Glk	Glucokinase	*glk*
HPr	Histidine Protein	*ptsH*
LacY	Lactose permease transporter	*lacY*
XylE	Xylose Proton Symporter	*xylE*
XylFGH	Xylose ABC Transporter ATPase	*xylFGH*
XylA	Xylose isomerase	*xylA*
XylB	Xylulose kinase	*xylB*
XylR	Transcriptional activator	*xylR*

Fig. 2 Transport, catabolism and catabolite repression mechanisms of major lignocellulose-derived sugars in *E. coli.* There are three known mechanisms for glucose transport including EII^{Glc}-based PTS, EII^{Man}-based PTS and GalP. EII^{Glc}-based PTS is the predominant mechanism for glucose transport. Glucose induced-repression is mainly caused by low levels of cAMP which leads to nonfunctional CRP. Without CRP activation, the transcriptional activation of most secondary sugar catabolism pathways cannot be achieved. Phosphorylated sugar intermediates from glucose, xylose and arabinose catabolism enter PPP or EMP pathways for full degradation. The details of the important components in *E. coli* are summarized in the table

The reported kinetic activity of EII^{Glc} with glucose as substrate is reported to have a high affinity with K_m and V_{max} values of 3–10 μM and 126 μmol min^{-1} g^{-1}, respectively (Stock et al. 1982; Misset et al. 1983). Lastly, the phosphoryl group is transferred to EII's corresponding sugar during transport across the cytoplasmic membrane (Gorke and Stulke 2008) (Fig. 2). With the monosaccharide phosphorylated it can now be catabolized through the respective pathways. For example, in *E. coli* glucose-6-phospate can be catabolized by the Embden-Meyerhof-Parnas (EMP) pathway or the pentose phosphate pathway (PPP).

Glucose transport by means other than the PTS $EIIBC^{Glc}$ complex has been observed in *E. coli* (Fig. 2). There is compelling evidence that the mannose sugar specific EII complex (EII^{Man}) is a promiscuous component of PTS and is able to transport glucose, fructose and *N*-acetylglucosamine. The EII^{Man} complex comprises the $EIIAB^{Man}$ homodimer enzyme encoded by *manX* and the integral membrane permease $EIICD^{Man}$, encoded by *manY* and *manZ*. The phosphoryl group from PEP is transferred to $EIIAB^{Man}$ and then to $EIICD^{Man}$ which facilitates the transport and concomitant phosphorylation of glucose. EII^{Man} is reported to have a high affinity for glucose with K_m and V_{max} values of 15 μM and 72 μmol min^{-1} g^{-1}, respectively (Stock et al. 1982; Curtis and Epstein 1975; Gosset 2005). Glucose may also be transported via the galactose proton symporter (GalP). Upon transport of glucose via GalP, the enzyme glucokinase, encoded by *glk*, phosphorylates glucose producing glucose-6-phosphate, which can now enter EMP or PPP. The kinetic activity of GalP with glucose as substrate is reported to have K_m and V_{max} values of 10.2 μM and 15.6 μmol min^{-1} g^{-1}, respectively (Gosset 2005; McDonald et al. 1997).

Transport of extracellular lignocellulose-derived pentoses such as xylose and arabinose across the plasma membrane in *E. coli* occurs not through the PTS but through two unique set of transport systems: an ATP-binding cassette (ABC) and a proton symporter. The ABC transporters XylFGH for xylose and AraFGH for arabinose, encoded by *xylFGH* and *araFGH*, respectively, actively transport sugars with the cost of one ATP per sugar, whereas the proton symporters XylE for xylose and AraE for arabinose, encoded by *xylE* and *araE*, respectively, uses a proton gradient to transport the monosaccharide across the plasma membrane (Horazdovsky and Hogg 1987; Sumiya et al. 1995; Jojima et al. 2010; Davis and Henderson 1987; Maiden et al. 1988).

AraE also has affinity towards xylose but its expression is repressed by xylose under normal conditions (Hasona et al. 2004; Koirala et al. 2016). Both ABC transporters XylFGH and AraFGH exhibit a high affinity for their substrates with low K_m values between 0.2–4 and 4.1–6.1 μM, respectively. The proton symporters XylE and AraE possess a relatively low affinity with high K_m values between 63–169 and 150–320 μM, respectively (Sumiya et al. 1995; Henderson 1990; Daruwalla et al. 1981). Immediately following the transport of xylose and arabinose into the cell both substrates are eventually converted to xylose-5-phosphate via the xylose isomerase and arabinose isomerase pathway (Nieves et al. 2015; Schleif 2000) (Fig. 2). In the xylose isomerase pathway, xylose is converted to xylulose through a reversible one-step reaction catalyzed by xylose isomerase, encoded by *xylA*. Xylulose is then converted to xylulose-5-phosphate by the xylulokinase, encoded by *xylB* (Jeffries 1983). Catabolism of arabinose begins by converting arabinose to ribulose by arabinose isomerase, encoded by *araA*. Ribulose is then phosphorylated by ribulokinase, encoded by *araB*, and finally, converted to xylulose-5-phosphate by ribulose-5-phosphate 4-epimerase, encoded by *araD* (Jojima et al. 2010; Schleif 2000). With both xylose and arabinose converted to xylulose-5-phosphate, substrates can now be catabolized eventually by the EMP or PPP pathways. Many bacterial biocatalysts such as *Z. mobilis*,

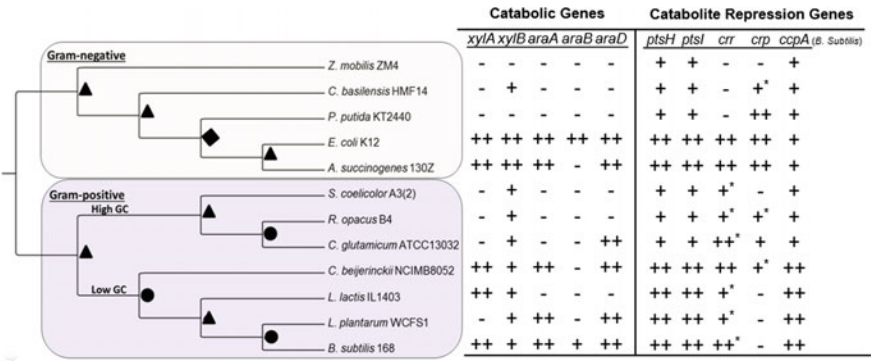


Fig. 3 Bacterial genes for xylose and arabinose catabolism, and major catabolite repression. Midpoint rooted cladogram of some important industrial prokaryotic biocatalysts is constructed using 16s rRNA sequences from the Greengenes database (DeSantis et al. 2006). Sequences were aligned using MAFFT (Katoh et al. 2002), and tree construction was performed using the default PhyML parameters (Guindon et al. 2005). One hundred bootstrap replicates were performed and confidence values of 60–69% (*circles*) 70–79% (*diamonds*) 80–89% (*squares*) and 90–100% (*triangles*) are listed at each respective node. The presence of potential gene homologs were tested in each biocatalyst using blastp (Gish and States 1993). Proteins that exhibited >90% query coverage and identity of 40–100% (++), 25–39% (+) and <25% (−) compared to *E. coli* and *B. subtilis* homologues (only CcpA is from *B. subtilis*) were scored in the table for each respective gene. Proteins that had only 80–89% query coverage are marked with an asterisk (*)

Pseudomonas putida, *Streptomyces coelicolor*, *Corynebacterium glutamicum*, *Rhodococcus opacus* and *Lactococcus lactis,* do not appear to have complete catabolic pathways for either xylose, or arabinose, or both (missing at least two homologues) (Fig. 3). Missing genes need to be integrated into these strains for an ideal lignocellulose conversion to use up all sugar content (Zhang et al. 1995; Kawaguchi et al. 2006).

2.2 *Carbon Catabolite Repression Mechanisms in Bacteria Limit Co-utilization of Lignocellulose-Derived Sugars*

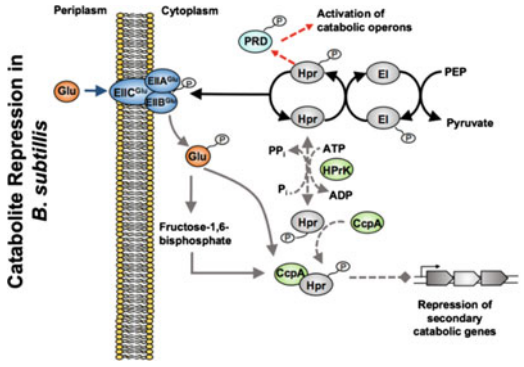
Lignocellulose-derived pentoses and hexoses are transported and preferentially selected for further catabolism based on accessibility and utilization that allows for optimal growth rate by bacteria (Kim et al. 2010; Dien et al. 2002). Glucose commonly represses the catabolism of other secondary sugars such as xylose, arabinose and galactose, which causes hierarchical control of sugar mixture utilization (Liu et al. 2005; Blencke et al. 2003; Moreno et al. 2001; Yoshida et al. 2001). Catabolite repression is a well-studied and classic topic for bacterial global transcriptional regulation. In *E. coli* and many other enteric bacteria, catabolite repression is controlled by two *non-mutually exclusive* mechanisms:

(1) operon-specific regulatory mechanisms, such as inducer exclusion, and (2) global regulatory mechanisms (Gorke and Stulke 2008).

Inducer exclusion is an operon-specific regulatory mechanism that controls the formation or uptake of an operon's inducer. A classic example is the glucose repression of the *lac* operon transcription through lactose permease transporter, LacY, in *E. coli* (Gorke and Stulke 2008; Escalante et al. 2012; Nelson et al. 1983; Osumi and Saier 1982). Expression of the *lac* operon requires lactose to be isomerized by β-galactosidase forming allolactose, which can bind and inactivate the *lac* repressor. In a sugar mixture of glucose and lactose, the preferred sugar glucose at high concentrations causes the PTS glucose specific EIIA ($EIIA^{Glc}$) to be dominantly dephosphorylated and prevents transport of lactose by $EIIA^{Glc}$ binding to LacY (Fig. 2). At low concentrations of glucose, the $EIIA^{Glc}$ is dominantly phosphorylated and cannot bind to LacY, thereby allowing lactose to be transported across the cell membrane, isomerized, and relieve repression of the *lac* operon by binding to the *lac* repressor. The $EIIA^{Glc}$ is an essential component of the PTS not only because is it a major component of catabolite repression, but it has been shown that the same mechanism is applicable to other secondary non-PTS carbohydrates, such as maltose, melibiose and glycerol (Gorke and Stulke 2008; Postma et al. 1993). For abundant secondary sugars in lignocellulose such as xylose and arabinose, the catabolite repression potentially caused by inducer exclusion mechanism remains to be investigated.

The catabolite repression caused by global regulatory mechanisms generally involves global transcriptional regulators to modulate the transcription of catabolic genes for secondary sugars (Figs. 2 and 4). In *E. coli*, the main involved global regulator is CRP (cAMP receptor protein), also called catabolite gene-activator protein (CAP), which is the transcriptional activator for catabolic genes for secondary sugars such as xylose and arabinose when bound by cAMP, an important intracellular signaling molecule employed in many different organisms. The membrane-bound protein adenylate cyclase (AC) and the $EIIA^{Glc}$ component of the PTS are also essential parts of regulation (Gorke and Stulke 2008; Stulke and Hillen 1999; Postma et al. 1993; Gosset 2005). At low glucose concentrations copious amounts of phosphorylated $EIIA^{Glc}$ exist and are able to bind and activate AC, leading to the synthesis of cAMP. As cAMP intracellular concentrations increase, the formation of cAMP-CRP complex activates catabolic operons such as *xylAB*, *xylFGH* and *araBAD* (Fig. 2). The promoters for catabolic operons of secondary sugars are usually weak and require co-activation by both CRP and theirs own sugar-specific activators such as XylR and AraC to enhance binding of the RNA polymerase (Fig. 2). In contrast, at high glucose concentrations, the cAMP level is low due to inactivation of AC, thereby limiting the CRP activity to increase the transcription of catabolic operons for secondary sugars (Gorke and Stulke 2008; Malan et al. 1984; Tagami and Aiba 1998). The global regulator CRP plays an essential role in not only regulating secondary catabolic genes, but also many other important biological processes such as respiratory genes and multidrug resistance, with over 180 genes under its control (Escalante et al. 2012; Geng and Jiang 2015).

The intricate catabolite repression mechanisms in other microorganisms are less understood. The CRP-based catabolite repression mechanism is probably not present in *Z. mobilis*, *P. putida*, *L. plantarum* and *B. subtilis* since there is no CRP homologue in these species (Fig. 3). Another important global regulator CcpA (catabolite control protein A) is employed in Firmicutes such as *B. subtilis*, *Lactobacilli* and *Clostridium* (Figs. 3 and 4). CcpA belongs to the GalR/LacI family and its activity is not regulated by cAMP (Lorca et al. 2005). *E. coli* has a transcriptional regulator, CytR, sharing sequence similarity with CcpA (Fig. 3). However, CytR is involved in transport and utilization of ribonucleosides and deoxyribonucleosides (Jorgensen et al. 1998; Valentinhansen et al. 1986), and does not participate in catabolite repression like *B. subtilis* CcpA. The global regulatory mechanism for catabolite repression in *B. subtilis* also involves PTS components. HPr and EI homologues are found in many industrial bacterial strains (Fig. 3), suggesting the common roles of PTS components with respect to sugar transport and catabolite repression (Gorke and Stulke 2008). Unlike the mechanism employed in *E. coli* where $EIIA^{Glc}$ plays a pivotal role, HPr is the key component used in *B. subtilis* (Gorke and Stulke 2008; Stulke and Hillen 1999). Two glycolytic intermediates, fructose-1,6-bisphosphate and glucose-6-phosphate, and the bifunctional HPr kinase/phosphorylase (HPrK) are also part of catabolite repression system in *B. subtilis* (Fig. 4). Transfer of the phosphoryl group to HPr may occur at two distinct sites: Ser-46 by HPrK and His-15 by EI. At high glucose concentrations, HPr is phosphorylated at Ser-46 by HPrK and binding of HPr-Ser-46-P to CcpA is enhanced by the glycolytic intermediate glucose-6-phosphate and fructose-1,6-bisphosphate, which reflects the presence of preferred sugars. The phosphorylated HPr-CcpA complex is able to bind to catabolite response elements (*cre*) on the chromosome and repress the transcription of catabolic operons for secondary sugars. Under low glucose concentrations, HPr is still phosphorylated at His-15 by EI but not at Ser-46. HPr-His-15-P is used to phosphorylate the PRD



Enzyme Abbreviation	Enzyme Name	Gene Name
CcpA	Catabolite Control Protein A	*ccpA*
EI	Enzyme I	*ptsI*
$EIIABC^{Glu}$	Enzyme IIABC Glucose Specific	*crr*
HPr	Histidine Protein	*ptsH*
HPrK	Histidine Protein Kinase/phosphorylase	hprK
PRD	PTS regulatory domain	

Fig. 4 The mechanism of carbon catabolite repression in *B. subtilis*. Glucose is transported into the cells by PTS and the accumulation of phosphorylated sugar intermediates occurring during glucose catabolism leads to repression of the utilization of secondary sugars through CcpA, the main regulatory component for catabolite repression in *B. subtilis* and other Firmicutes

(PTS regulatory domain) of multiple transcriptional regulators and these phosphorylated regulators activate the catabolic operons of secondary sugars or other carbon sources (Gorke and Stulke 2008; Lorca et al. 2005).

2.3 *Alleviating Bacterial Carbon Catabolite Repression by Genetic Engineering to Enhance Lignocellulose-Derived Sugar Co-utilization*

Sequential sugar utilization is commonly observed in cultivation processes containing sugar mixtures due to catabolite repression. The inability of bacteria to efficiently consume two or more carbon sources hinders commercial use of lignocellulosic biomass due to increased residence time, lower product titer and productivity (Gosset 2005). Although catabolite repression mechanisms have been exhaustively investigated in *E. coli*, understanding the underlying molecular mechanisms of catabolite repression does not simply provide a perfect solution to this problem. In the past few decades, considerable engineering efforts have been made and limited successes have been achieved to engineer bacteria for co-utilization of lignocellulose-derived sugars such as glucose, xylose and arabinose.

The strategies to abolish catabolite repression by inactivating the EIIBC^{Glc} complex, encoded by *ptsG*, have been explored. For example, simultaneous uptake of glucose and pentoses for conversion to lactate was achieved at some degree by expressing the lactic acid dehydrogenase from *Streptococcus bovis* in a *ptsG* mutant yielding the FBR19 strain (Dien et al. 2001, 2002). This strain produced lactic acid at a titer of 64 g/L with a yield (g/g) of 77%. Nichols et al. also showed that EIIBC^{Glc} devoid mutants (*ptsG21*) are able to simultaneously metabolize lignocellulose-derived sugars to produce ethanol by developing a stable plasmid expressing pyruvate decarboxylase and alcohol dehydrogenase genes from *Z. mobilis* (Nichols et al. 2001). However, inactivation of *ptsG* often impairs glucose uptake and thus efforts to compensate this defectiveness are needed. One common strategy is to evolve *ptsG* mutant strains for better growth using glucose as sole carbon source. For example, the work of Hernández-Montalvo et al. showed that subjecting the PTS devoid mutant (PTS$^-$ glucose$^-$-phenotype) NF6 to a continuous culture selective method led to revertants (PTS$^-$ glucose$^+$-phenotype) with a specific growth rates on glucose ($\mu = 0.36\ h^{-1}$) comparable to a PTS$^+$ wild-type *E. coli* strain ($\mu = 0.42\ h^{-1}$) under aerobic conditions (Flores et al. 1996; Hernandez-Montalvo et al. 2001). In these revertant mutants, catabolite repression of arabinose and xylose was alleviated and this allowed for simultaneous mixed sugar uptake (1 g/L glucose, xylose, and arabinose). Although sugar co-utilization was achieved, catabolite repression was only eradicated in a glucose-arabinose sugar mixture. In a glucose-xylose mixture, glucose exerted repression on xylose uptake. In an arabinose-xylose or a glucose-arabinose-xylose mixture, arabinose

repressed xylose utilization (Hernandez-Montalvo et al. 2001). Additionally it was shown that a plasmid overexpressing the non-PTS transport system, the galactose proton symporter and glucokinase, in a PTS-deficient strain (W3110 PTS^-) resulted in growth rates corresponding to 89% of the growth rate for W3110 (Hernandez-Montalvo et al. 2003). Building upon the work of Hernández-Montalvo and other colleagues, a W3110 mutant with a PTS^- $glucose^-$ phenotype was subjected to anaerobic adaptive laboratory evolution to obtain an evolved strain, VH30N4, capable of utilizing glucose (phenotype of PTS^- $glucose^+$). Upon transforming an ethanologenic plasmid pLOI594 into VH30N4, the newly evolved strain was capable of co-utilizing a 5 g/L glucose-xylose sugar mixture with a specific growth rate μ of 0.14 and ethanol titer of 2.1 g/L (Balderas-Hernandez et al. 2011). These adaptive laboratory evolution approaches to obtain PTS^- $glucose^+$ mutants demonstrate that catabolite repression can be alleviated to allow simultaneous uptake of lignocellulose-derived sugar. The further characterization of beneficial genetic changes gained that enabled sugar co-utilization will provide some insights about cellular metabolic flexibility. Similarly, a systematic engineering approach employed in *E. coli* enabled co-utilization of a glucose-xylose mixture. To overcome the glucose transport defectiveness, the glucose facilitator gene *glf* from *Z. mobilis* was introduced into a *ptsG* mutant strain and the pentose phosphate pathway genes *rpiA*, *tktA*, *rpe*, and *talB* were overexpressed. The final plasmid-free strain co-utilized 30 g/L of glucose and xylose within 16 h in mineral salts media with an unusually high starting inoculum at 0.3 g cell dry weight/L (Chiang et al. 2013).

Besides engineering PTS to relieve catabolite repression, the global regulator CRP has been an engineering target to enhance sugar co-utilization for bacteria that use CRP as a catabolite repression mechanism, such as *E. coli* and *Klebsiella oxytoca* (Stulke and Hillen 1999; Kim et al. 1992; Cirino et al. 2006; Ji et al. 2011). Theoretically, a cAMP-independent CRP variant should activate the catabolic operons of secondary sugars even in the presence of glucose. However, CRP globally regulates transcriptional expression of more than 180 genes and such CRP mutants often have slow growth phenotypes because the CRP mutants might alter the transcriptional control for other important genes (Khankal et al. 2009), thereby limiting wide application of this approach and only yielding limited success. Similarly, engineering CcpA (the major global catabolite repression regulator in Firmicutes strains shown in Figs. 3 and 4) or *cre* by either mutagenesis or simple inactivation has yielded successes at some degree (Wu et al. 2015; Ren et al. 2010; Bruder et al. 2015). For example, an engineered *Clostridium acetobutylicum* strain with *ccpA* mutant was able to use more than 90% of the total xylose within 72 h when fermenting a glucose-xylose mixture (30 g/L glucose and 15 g/L xylose) (Wu et al. 2015; Ren et al. 2010). Overexpression of catabolic genes for secondary sugars while relieving catabolite repression was also proven to enhance for sugar co-utilization in different hosts (Chiang et al. 2013; Yu et al. 2015).

Despite extensive research efforts spanning decades developing monocultures for co-utilization of lignocellulose-derived sugars, researchers are now investigating the use of microbial consortia for enhanced sugar co-utilization. Design of synthetic

microbial communities by engineering microbial 'specialists'—having a specific function and/or capability—has been explored. For example, Dr. Eiteman's group engineered two *E. coli* strains for selective-substrate uptake, one that can only metabolize glucose and the other only xylose, by eliminating metabolic pathways for the non-selective substrate (Eiteman et al. 2008). Co-culturing the two 'specialists' together had a synergistic effect by consuming glucose and xylose rapidly under both aerobic and anaerobic conditions. They demonstrated by aerobic fed-batch that the co-culture system has the intrinsic ability to adapt to feed fluctuations. With increased carbon uptake, the *E. coli* 'specialists' were further engineered for parallel conversion of glucose and xylose to lactate and succinate production (Eiteman et al. 2009; Xia et al. 2015). These results show high potential of engineering co-culture systems for lignocellulose bioconversion. However, fermentation of high sugar concentrations needs to be further investigated. The benefit of using microbial consortia of the same species are operating parameters (temperature, pH, oxygen demand and nutritional requirements) and growth rates of each member are similar (Chen 2011). This approach has also been used to simultaneously metabolize glucose, xylose, arabinose and acetate by increasing the number *E. coli* 'specialists' in microbial processes (Lakshmanaswamy et al. 2011; Xia et al. 2012). Although all the above-mentioned strategies have enhanced sugar co-utilization at different levels, the effects of these engineering strategies remain to be validated under more industrially relevant conditions (>100 total sugars g/L, cheap medium, low inoculum, oxygen-limiting conditions, etc.).

3 Engineering Bacterial Resistance to Inhibitors from Lignocellulose Pretreatments

3.1 Growth Inhibitors as Side Products from Lignocellulose Pretreatments

Pretreatment of lignocellulosic materials is considered to be an essential unit process of a lignocellulosic biorefinery, accounting for 16–19% of its total investment (Sousa et al. 2009). In the past few decades, a variety of pretreatment technologies have been developed to overcome the recalcitrance of lignocellulose, increase cellulase efficiency, and improve the yields of monomeric sugars. These different pretreatments yield varied amounts of sugars and degradation products and have varied effects on cellulose digestibility and lignin solubilization, which ultimately affect downstream bioconversion processes.

It is challenging to achieve a high yield of fermentable sugars from lignocellulose while being economically viable. Several physical and chemical pretreatment methods have been developed including dilute acid (Torget et al. 1988, 1990; Lloyd and Wyman 2005; Geddes et al. 2010b), alkaline (Curreli et al. 1997; Gould 1985; Chang et al. 1998), steam explosion (Brownell et al. 1986; Mok and Antal 1992),

organosolv (Connors et al. 1980; Yu et al. 2014; Sun et al. 2011; Zhao et al. 2009; Pan et al. 2005), ozonolysis (Binder and Fiechter 1980; Travaini et al. 2013, 2016; Neely 1984), ammonia fiber expansion (Lau and Dale 2009; Lau et al. 2008; Teymouri et al. 2004; Holtzapple et al. 1991), ionic liquid (Swatloski et al. 2002a, b; Turner et al. 2003; Lee et al. 2009) and other pretreatment technologies. The advantages and disadvantages of these technologies are briefly summarized in Table 1.

Table 1 A summary of representative lignocellulose pretreatment technologies[a]

Methods	Potential inhibitors	Advantages	Disadvantages
Dilute acid	Furan aldehydes; phenolic and small organic acids at low levels	High hemicellulose monosaccharides production; increased cellulase digestibility; relatively low cost (Loow et al. 2016)	Inhibitors; equipment corrosion; low sugar concentration in exit stream (Loow et al. 2016)
Alkaline	Furan aldehydes at low levels	Some lignin degradation; increasing cellulase digestibility (Loow et al. 2016)	Chemical reuse efficiency is low; low hemicellulose monosaccharides production (Loow et al. 2016)
Steam explosion	Furan aldehydes, acetate and other acids	Increasing cellulase digestibility; low environmental impact (Chen and Liu 2015)	Incomplete disruption of lignin-carbohydrate complex; inhibitors (Chen and Liu 2015)
Organosolv	lignin degradation products	High efficiency for lignin degradation (Zhang et al. 2016)	High operational costs (Zhang et al. 2016)
Ozonolysis	Short-chain carboxylic acids	Low furan aldehydes; some lignin degradation; ambient temperature and pressure (Travaini et al. 2016)	Highly reactive and flammable; high energy demand and cost (Travaini et al. 2016)
Ammonia fiber expansion (AFEX)	Small amounts of inhibitors; amide-containing chemicals	Decrystallization of cellulose; lignin removal (Sousa et al. 2009; Mathew et al. 2016)	Low hemicellulose monosaccharide production; high operational cost (Sousa et al. 2009; Mathew et al. 2016)
Ionic liquid	Small amounts of inhibitors	Reducing cellulose crystallinity and lignin content; lignin and hemicellulose hydrolysis; mild processing conditions (Badgujar and Bhanage 2015)	High chemical costs; need for solvent recovery and recycle (Badgujar and Bhanage 2015)

[a]The information of inhibitors was obtained from recent literature (Geddes et al. 2010; Chundawat et al. 2010; van der Pol et al. 2014). The advantages and disadvantages of different pretreatment technologies were summarized in recent excellent reviews (Sousa et al. 2009; Travaini et al. 2016; Loow et al. 2016; Chen and Liu 2015; Zhang et al. 2016; Mathew et al. 2016)

In most pretreatments, three major categories of by-products are produced: furan aldehydes, small organic acids and phenolic compounds (van der Pol et al. 2014). Furan aldehydes include furfural and 5-hydroxymethylfurfural (HMF), which are the dehydration products of pentoses and hexoses, respectively. Most phenolic compounds are degradation products from lignin (Eudes et al. 2014). Pretreated lignocellulosic hydrolysates can also contain large amounts of organic acids, including acetic acid, formic acid, levulinic acid and lactic acid. These acids are only toxic at high concentrations compared to furan aldehydes (Zaldivar et al. 1999; Zaldivar and Ingram 1999). Furan aldehydes, especially furfural, are able to potentiate the toxicity of other side-products and inhibit cell growth at relatively low concentrations (Zaldivar et al. 1999, 2000; Zaldivar and Ingram 1999; Gorsich et al. 2006; Liu et al. 2004). The above-mentioned pretreatments in Table 1 yield different side-product spectra even when treating the same type of biomass. Different types of biomass will also yield different amounts of side-products after the same pretreatment.

Among these techniques, dilute acid pretreatment has widely been regarded as one of the most economically promising methods of biomass pretreatment (van der Pol et al. 2014; Ko et al. 2015). The main advantages of this pretreatment method are that it is effective at hydrolyzing the hemicellulose fraction and increasing enzyme accessibility to the cellulose fraction with a relatively low operational cost. This method employs cooking biomass (5–30 min) at high temperatures (121–210 °C) and low pH, typically using sulfuric acid at a concentration of 0.5–4 %wt (van der Pol et al. 2014). It should be noted that temperature and acid concentration are directly correlated to both inhibitor generation and sugar recovery. As temperature increases, enzymatic digestibility of the solid cellulose fraction is enhanced, but at temperatures above 160 °C pentoses are also more readily dehydrated to furfural (Morikawa et al. 2014). If using phosphoric acid ($\sim$2%) for dilute acid pretreatment and ammonia hydroxide to neutralize the acidic hydrolysate, the resulting phosphates and ammonia salts can be used as nutrient sources for fermentative production (Geddes et al. 2010b). This method yields no waste chemicals from pretreatments. In addition, phosphoric acid is much less corrosive than sulfuric acid, which leads to less equipment corrosion and smaller amounts of furfural and other inhibitors. To mitigate the production of these inhibitors, downstream processes can be performed to decrease the concentration of inhibitors prior to fermentation. These include water washing the pretreated solids (Rajan and Carrier 2014), overliming (Cao et al. 2013; Martinez et al. 2001), vacuum evaporation of volatile inhibitors (Larsson et al. 1999) and reverse osmosis (Grzenia et al. 2012). Although relatively effective, these methods all introduce large additional costs at the industrial scale which are ultimately undesirable.

Similar to dilute acid pretreatments, alkaline pretreatments and steam explosion often generate furan aldehydes as side-products. Advantages of alkaline pretreatments include the partial degradation of lignin, increased enzyme accessibility to the solid fraction, and lower inhibitor concentrations. But similar to acidic pretreatments, increased pretreatment severity increases sugar yield and cellulase

digestibility while increasing the amounts of inhibitors (Zhao et al. 2014). It is challenging to simultaneously achieve high sugar yield and digestibility with a low concentration of inhibitors.

Organosolv, ozonolysis, AFEX and ionic liquid pretreatments generate far less furan aldehydes and other inhibitors, and resulting hydrolysates are less toxic to microbes. However, they also are generally less efficient at liberating sugar monomers from hemicellulose (Chundawat et al. 2010; van der Pol et al. 2014). For example, an excellent detailed comparison study of AFEX and dilute acid pretreatments showed that AFEX yields a reduction of 76% of total carboxylic acids, 97% of furans and 74% sugars compared to amounts of these products produced by a dilute acid pretreatment while generating significantly more phenolic amides and pyrazine derivatives (Chundawat et al. 2010). These pretreatment technologies have also been demonstrated to degrade lignin to some degree. The presence of lignin inhibits cellulase activity, and thus cellulase accessibility is generally increased with lignin degradation. The main caveats of these pretreatments (mostly economic factors and environmental concerns) are summarized in Table 1.

The chosen pretreatment process and specific parameters within have been shown to greatly affect the cost effectiveness, sugar recovery, and inhibitor profile of the resulting slurry from pretreatments. Several key criteria regarding economical, technical and environmental factors need to be considered when applying these pretreatment technologies. Robust biocatalysts capable of tolerating toxic side products will allow more flexibility in the design of a bioconversion process, and ultimately increase economic viability.

3.2 Genetic Engineering of Bacterial Resistance to Toxic Side-Products

Since the toxicity of side-products is an economically limiting factor for at least some important pretreatment technologies, it is desirable to have robust organisms that are capable of tolerating these compounds in fermentation broth. The development of biological solutions for addressing furfural toxicity would not only be cheaper and more sustainable, but also more scalable. Among common toxic side-products derived from lignocellulose pretreatments, furan aldehydes are arguably the most studied due to their abundance, toxicity and the unique property to potentiate the toxicity of other side-products. Engineering the tolerance and detoxification of toxic side-products from lignocellulose pretreatments in fungi and bacteria is an active area of research. In this chapter, we will focus on our understanding and progress about engineering resistance in popular industrial bacterial biocatalysts.

3.2.1 Native Bacterial Detoxification Processes for Furan Aldehydes

Furfural and 5-HMF are formed by the dehydration of sugars (pentoses and hexoses, respectively) during some pretreatment processes with a concentration range between 0 and 5 g/L depending on the severity of the processes (Mills et al. 2009; Almeida et al. 2009). More specifically, furan aldehydes arrest the growth of most microbes when present above a threshold (~0.5–1 g/L furfural and ~3 g/L 5-HMF for *E. coli* in mineral salts medium, respectively). Due to the highly similar structures, furfural and 5-HMF share the same degradation pathways and potential toxicity mechanisms. The genetic traits or methods proven useful for furfural are also effective for 5-HMF (Wang et al. 2012a; Turner et al. 2010; Miller et al. 2009a, 2010). Furfural is a potentially more important inhibitor than 5-HMF for the following reasons: first, dilute acid pretreatments and other pretreatment technologies yield more furfural than 5-HMF in hemicellulose hydrolysates (Saha 2003; Geddes et al. 2010a, 2013). Second, furfural is more toxic than 5-HMF to industrial catalysts including both *E. coli* and *S. cerevisiae*. Therefore, most research about bacterial toxicity of furan aldehydes is focused on furfural.

In spite of the high toxicity of furan aldehydes, a number of microbes have evolved different strategies to deal with furan aldehydes, though with varying degrees of success. Microbes including *S. cerevisiae* and *E. coli*, for example, are capable of transforming furfural to furfuryl alcohol by a reduction reaction catalyzed by oxidoreductases (Fig. 5a) (Liu 2010; Wang et al. 2012a). Furfuryl alcohol is a less toxic compound (Zaldivar et al. 1999, 2000) than furfural. It is excreted into the culture medium and remains in the fermentation broth without further degradation (Wang et al. 2012a). A few bacterial oxidoreductases such as YqhD and FucO in *E. coli* (Turner et al. 2010; Miller et al. 2009a; Wang et al. 2011), FudC in *C. glutamicum* (Tsuge et al. 2016), and ZMO0976 in *Z. mobilis* (Agrawal and Chen 2011) are able to reduce furfural into furfuryl alcohol using reducing factors NADH and/or NADPH (Fig. 5a). This appears to be a common mechanism used by different bacteria to detoxify aldehydes including furfural and 5-HMF. Without further degradation, however, terminal accumulation of furfuryl alcohol (especially in fed-batch operation) can itself eventually lead to growth inhibition. In contrast, certain bacteria (e.g., *Cupriavidus basilensis* HMF14) have evolved a complete biodegradation pathway to fully metabolize furfural (when supplied as a sole carbon and energy source) under aerobic or micro-aerobic conditions (Fig. 5a) (Koopman et al. 2010a, b). Furfural is first oxidized into 2-furoic acid and then further metabolized to α-ketoglutarate that eventually enters TCA cycle to provide energy and biosynthetic building blocks (Fig. 5a). In contrast to biotransformation, biodegradation has the potential to provide an irreversible and absolute solution to the problem of furfural toxicity (Fig. 5). Biotransformation of furan aldehydes seems potentially common for many industrial bacterial hosts since at least one or two oxidoreductase homologs of YqhD, FucO, FudC and ZMO0976 can be found in *Z. mobilis*, *L. plantarum*,

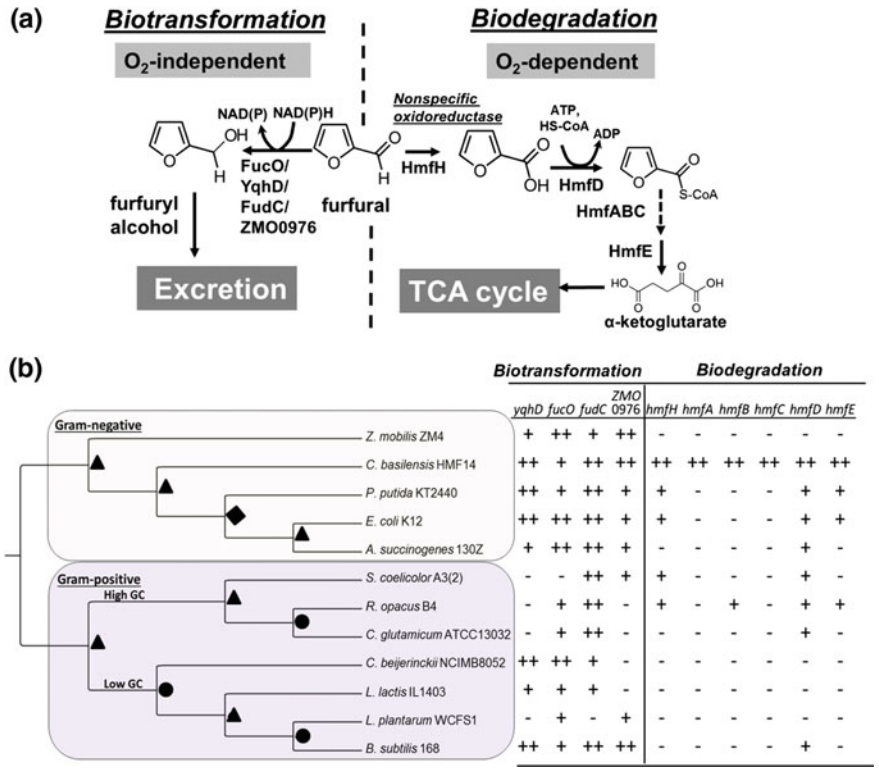


Fig. 5 Naturally evolved mechanisms used by different microbes to detoxify furfural. **a** Two native furfural detoxification mechanisms are used by different bacteria to relieve the toxicity. **b** The presence of potential furfural detoxification protein homologs were tested in each biocatalyst using blastp (Gish and States 1993). Proteins that exhibited >90% query coverage and identity of 40–100% (++), 25–39% (+) and <25% (−) were scored in the table for each respective gene

R. opacus and other important industrial bacteria (Fig. 5b). The complete furfural biodegradation pathway is not present in these bacteria only in *C. basilensis* HMF14 (Fig. 5b).

3.2.2 Engineering Bacterial Tolerance and Potential Toxicity Mechanisms of Furan Aldehydes

The exact mechanism of growth inhibition caused by furan aldehydes is not completely known, but it is thought to be multifaceted (Mills et al. 2009; Nieves et al. 2015). One direct approach for engineering bacterial tolerance to furan aldehydes is to improve microbial native biotransformation or biodegradation abilities. Overexpression of furfural oxidoreductases confers tolerance to a variety of bacteria (Wang et al. 2011; Zheng et al. 2013; Chung et al. 2015; Seo et al. 2016).

However, there are two issues with this approach. First, furfural or 5-HMF is not the most desired substrate for currently identified furfural reductases such as YqhD, FucO, FudC and others, and the K_m and K_{cat} values for furfural and 5-HMF need to be further optimized (Wang et al. 2011; Tsuge et al. 2016; Zheng et al. 2013; Jarboe 2011). There is a space for directed evolution and enzyme engineering to improve the kinetic properties of furfural reductases towards furan aldehydes. With higher furfural reduction activity, the cellular growth lag induced by furan aldehydes will be significantly shortened. Second, the reducing cofactors play an important role for furfural biotransformation and cytotoxicity. Different growth conditions such as medium, aeration levels and carbon source influence the relative abundance of intracellular NADH and NADPH levels. For example, the NADPH source is relatively limited when *E. coli* is growing under anaerobic fermentative conditions using xylose as a sole carbon source. Exposure of furan aldehydes activates the transcription of furfural reductase genes such as *yqhD* (Turner et al. 2010). When *E. coli* cells overexpress the NADPH-dependent furfural reductase YqhD to reduce furfural or 5-HMF, the NADPH intracellular pool is depleted and cell growth is arrested (Turner et al. 2010; Miller et al. 2009a). Actually this depletion of NADPH by YqhD was proposed as the main mechanism for furfural/5-HMF-induced growth inhibition in *E. coli* under xylose fermentative conditions (Turner et al. 2010; Miller et al. 2009a, b). It is plausible that overexpression of *yqhD* can confer furfural tolerance if the growth conditions lead to abundant NADPH such as enough aeration and rich media. It was reported that using rich media or glucose as a carbon source significantly increases bacterial tolerance to furfural (Miller et al. 2009a, b). With respect to the cofactor issue, NADH-dependent furfural reductases such as FucO are a better choice for anaerobic fermentation using xylose as a sole carbon source (Wang et al. 2011).

The complete furfural degradation pathway discovered in *C. basilensis* HMF14 and other species (Koopman et al. 2010a) has not been introduced into common genetically tractable industrial hosts such as *E. coli* and *S. cerevisiae*. One caveat for this pathway is its oxygen requirement which is intrinsically conflicting with the anaerobic/micro-aerobic fermentation condition used in desired bioproduction scenarios for biofuels and bulk chemicals. The complete furfural degradation pathway was also found in fungal isolate *Coniochaeta ligniaria* and *Amorphotheca resinae* ZN1 (Ran et al. 2014; Nichols et al. 2010). Currently, researchers use these microbial whole cells as a biological abatement material to remove lignocellulose inhibitors including furan aldehydes using this native furfural degradation pathway (Koopman et al. 2010b; Ran et al. 2014; Nichols et al. 2010; He et al. 2016). The process is effective, but sugars from lignocellulose hydrolysates will also be consumed. In addition, inclusion of this extra step will increase process complexity and costs.

After NADPH limitation was proposed as one toxicity mechanism for furfural, researchers have identified effective methods to improve NADPH levels and thus to relieve furfural toxicity. Deletion of *yqhD* or increased expression of *pntAB* (a transhydrogenase for interconversion of NADH and NADPH) enhanced tolerance to furan aldehydes in *E. coli* (Miller et al. 2009a, b, 2010). NADH is abundant during xylose fermentation and thereby overexpression of *pntAB* significantly

relieves the NADPH limitation. Nutrient and glycerol supplementation also increased furfural tolerance in *E. coli* and *C. beijerinckii* by potentially increasing NAD(P)H levels or decreasing burden on NAD(P)H intensive pathways (Miller et al. 2009a, b, 2010; Ujor et al. 2014).

To identify more furfural-resistant genetic traits, researchers have performed a variety of genome-scale screening methods. Genomic libraries from three different bacteria and metagenomic libraries have been constructed and screened in separate instances that led to the discovery of *thyA/thyX* as genes which confer furfural tolerance to *E. coli* (Zheng et al. 2012; Forsberg et al. 2016). These genes encode thymidylate synthases, enzymes in dTMP biosynthesis, suggesting that DNA damage is a potential toxicity mechanism of these furans. This is consistent with the early biochemical observation of the damages of plasmid DNA caused by furan aldehydes (Khan et al. 1995; Khan and Hadi 1993). Similar discoveries have been made in Gram positive organisms such as *C. beijerinckii,* where supplementation of an inhibitor of xanthosine dehydrogenase increased furfural tolerance potentially due to the accumulation of purine precursors beneficial for DNA repair (Ujor et al. 2015). This research direction has not been fully explored to further improve bacterial tolerance to furan aldehydes as there are multiple other mechanisms to prevent and repair DNA damages. Theoretically, a highly active DNA repair system may better prepare bacterial hosts to be exposed with high concentrations of furfural or 5-HMF. Besides genetic screens, other transcriptomic and advanced genome-scale analyses have identified more genetic beneficial traits (Wang et al. 2012a; Glebes et al. 2014, 2015). A few genes such as *ucpA*, *lpcA*, *groESL*, *ahpC*, *yhiH*, *rna* and *dicA* are associated with furfural tolerance and the overexpression of these genes individually conferred furfural tolerance at varied degrees (Wang et al. 2012a; Glebes et al. 2014, 2015). However, it is difficult to characterize how these genes are functionally linked in furfural resistance. Therefore, further rational engineering of cellular tolerance using these traits is limited without clear understanding of the resistance mechanism.

Another important and effective approach to improve microbial tolerance for lignocellulose inhibitors is adaptive laboratory evolution (also called "metabolic evolution" or "evolutionary engineering") (Miller et al. 2009a; Shui et al. 2015; Mohagheghi et al. 2015; Geddes et al. 2011b). In this approach, microbes explore genome sequence space to achieve higher fitness, mostly by spontaneous mutations introduced by genome replication errors during continuous propagation (Winkler et al. 2013; Elena and Lenski 2003; Sauer 2001). When beneficial mutations conferring resistance occur, the strain with the mutations will gain growth advantages over its competing peers and eventually the mutations will be fixed after generations of propagation (Elena and Lenski 2003; Barrick and Lenski 2013). This method is able to improve potentially all fitness-related traits including stress tolerance and carbon catabolism. The molecular characterization of underlying mechanisms will advance knowledge about these important phenotypes, and likely stimulate an innovative design above the current paradigm by combining evolution and rational engineering. For example, the mutations that cause *yqhD* inactivation was found in multiple furfural-resistant mutants isolated by adaptive laboratory

evolution, suggesting that NADPH starvation induced by furfural enzymatic reduction is one important toxicity mechanism (Turner et al. 2010). Transcriptomic analysis of some furfural resistant *E. coli* mutants showed that the transcription of multiple polyamine transporters was up-regulated compared to normal *E. coli* due to the gene multiplication at the chromosomal level as indicated by whole genome sequencing (Geddes et al. 2014). Further tests discovered that overexpression of these polyamine transporter genes including *potE*, *puuP*, *plaP* and *potABCD* makes cells more resistant to furfural, suggesting a potential protection role of polyamine for important cellular constituents (Geddes et al. 2014).

Quite a few distinct beneficial genetic traits have been identified due to the complexity of toxicity modes of furan aldehydes (Table 2). It is plausible that multiple pathways need to be co-activated and recruited for optimization of furfural tolerance. One suitable genetic approach called global transcription machinery engineering (gTME) has been developed to improve these kind of complex phenotypes, especially for microbial stress resistance (Alper et al. 2006; Alper and Stephanopoulos 2007). The mechanism behind this approach is to mutagenize global transcriptional machinery such as sigma factors or global regulators for co-activating a few related pathways to achieve the improvement of a complex phenotype (Alper and Stephanopoulos 2007). This research strategy has been used to improve bacterial furfural tolerance. Researchers selected a furfural-tolerant variant of stress-related exogenous regulator IrrE that is able to specifically enhance the furfural tolerance (Wang et al. 2012b). Similarly, the mutant of a sigma factor RpoD is found to increase tolerance to furfural in *Z. mobilis* (Tan et al. 2015). Although it is effective at some degree, the issue of the gTME approach is that the precise resistance mechanism still remains to be characterized even if the beneficial mutations of sigma factors and global regulators are identified.

The beneficial genetic traits mentioned above are summarized in Table 2. However, these individual beneficial genetic traits only have limited effect and these traits may not have a simple cumulative synergy (Wang et al. 2013). To further improve furan aldehyde tolerance, the optimal combination of beneficial traits needs to be identified, but this procedure is tedious and time-consuming since beneficial traits may exhibit negative epistatic effects and all possible combinations need to be carefully evaluated (Wang et al. 2013). How to efficiently identify and integrate optimal beneficial genetic traits remains a great challenge.

3.2.3 Engineering Bacterial Tolerance to Other Side-Products or Lignocellulosic Hydrolysates

Besides furan aldehydes, there are other two groups of toxic side-products: small organic acids and phenolic compounds (van der Pol et al. 2014). Acetic acid and formic acid are the major organic acids after lignocellulose pretreatments, although others may also be present (Mills et al. 2009; van der Pol et al. 2014). Generally lignocellulose hydrolysates need to be neutralized to a certain degree for the following fermentation processes if dilute acid pretreatment is used. Thus, small

Table 2 Summary of beneficial bacterial genetic traits to enhance furan aldehyde tolerance

Potential mechanisms	Beneficial genetic traits	Microbial host	Reference
Accelerated removal			
Reduce furfural to furfuryl alcohol	Increase *fucO* expression	*E. coli*	Wang et al. (2011)
	Directed evolution of FucO	*E. coli*	Zheng et al. (2013)
	Heterologous expression of the NAD(P)H-dependent BdhA enzyme	*C. bescii*	Chung et al. (2015)
	Overexpression of *yqhD* and *fucO*	*E. coli*	Seo et al. (2016)
Complete biodegradation of furan aldehydes	Aerobic HMF degradation	*C. basilensis HMF14*	Koopman et al. (2010)
	Aerobic furfural degradation	*P. putida Fu1*	Koopman et al. (2010), Trudgill (1969)
Relieve toxicity			
Unable to compete NADPH needed for biosynthesis	*yqhD* deletion	*E. coli*	Miller et al. (2009)
Increasing NADPH level	Increase *pntAB* expression	*E. coli*	Miller et al. (2009, 2010)
Increasing the availability of dTMP for DNA repair	Increase *thyA* expression	*E. coli*	Zheng et al. (2012)
	Increase *thyX* expression		Forsberg et al. (2016)
Polyamine binding to negatively charged cellular constituents	Increase *potE, puuP*, *plaP* and *potABCD* expression	*E. coli*	Geddes et al. (2014)
Strengthening cell wall or indirectly increasing NADPH availability	Increase *lpcA* expression	*E. coli*	Glebes et al. (2015)
Possibly similar to solvent stress response	Increase *groESL* expression	*E. coli*	Glebes et al. (2014)
Using gTME approach			
	Mutation of *irrE*	*E. coli*	Wang et al. (2012)
	Mutagenesis of *rpoD*	*Z. mobilis*	Tan et al. (2015)
Unclear mechanism			
Unknown	Increase *ucpA* expression	*E. coli*	Wang et al. (2012)
Unknown	Increase *ahpC*, *yhiH*, *rna* and *dicA* expression	*E. coli*	Glebes et al. (2015)

organic acids will be converted to salt forms after neutralization and the toxicity of these side products is greatly decreased. For example, only lower than 5 g/L acetate and formate were produced after a dilute acid pretreatment of sugarcane bagasse while *E. coli* can tolerate 12 g/L acetate or higher without significant growth defect (Geddes et al. 2010a; Takahashi et al. 1999). Furthermore, it has been shown that an ethanol *E. coli* producer can tolerate up to 40 g/L acetate after adaptive laboratory evolution (Fernandez-Sandoval et al. 2012). Researchers also have identified potential acetate-resistant genetic traits in different microbes such as *E. coli* (Sandoval et al. 2011; Chong et al. 2013) and *Z. mobilis* (Yang et al. 2010). So the toxicity issue of small organic acids is often not significant for industrial bacteria hosts.

A variety of phenolic compounds are produced during some pretreatment processes (Table 1). Most of them are very toxic but the quantity is normally small compared to furan aldehydes and organic acids after pretreatments (van der Pol et al. 2014). Without clear understanding which phenolic compound is the predominant inhibitor, the research of engineering bacterial tolerance to phenolic compounds is limited. A series of studies about the toxicity of side-products derived from lignocellulose suggest that the hydrophobicity of these chemicals is correlated with toxicity (Zaldivar et al. 1999, 2000; Zaldivar and Ingram 1999). The membrane damage caused by aromatic compounds was proposed as an important toxicity mechanism (Zaldivar et al. 1999, 2000; Mills et al. 2009; Zaldivar and Ingram 1999). Recent research in *Z. mobilis* ZM4 showed that phenolic aldehydes were reduced into alcohol forms by oxidoreductases to decrease toxicity, analogous to how cells cope with toxic furan aldehydes by biotransformation (Fig. 5a) (Yi et al. 2015). Conversion of these aldehydes into alcohol form is beneficial due to the reduced toxicity of the functional group and increasing culture inoculum has been shown to enhance tolerance of phenolic aldehydes (Zaldivar et al. 1999, 2000; Zaldivar and Ingram 1999). The research approaches for engineering furfural tolerance, such as enhancing degradation (Yi et al. 2015), addressing toxicity mechanism, random genetic screening (Forsberg et al. 2016) and gTME should also be effective for engineering phenolic tolerance.

Adaptive laboratory evolution is able to improve fitness-related traits such as stress tolerance without knowing the underlying mechanisms (Winkler et al. 2013; Sauer 2001). One *E. coli* ethanol producer has been evolved using hemicellulose hydrolysates of sugarcane bagasse as a media component (Geddes et al. 2011b). Eventually a hydrolysate-resistant mutant was isolated and exhibited higher resistance to furfural, 5-HMF and acetate than its parent strain (Geddes et al. 2011b). This strain is efficient to convert pretreated sugarcane bagasse hydrolysates into ethanol with a high titer (30 g/L ethanol) and yield (0.21 g ethanol/g bagasse dry weight) (Geddes et al. 2011b). Similarly, *Z. mobilis* strain 8b was adapted in the hydrolysate from pretreated corn stover and ethanol productivity using corn stover liquor was significantly improved (Mohagheghi et al. 2015). Current efforts are focusing on understanding the underlying genetic changes by whole genome sequencing and transcriptomic analysis (Shi et al. 2016). It is a great challenge to characterize the precise mechanisms since numerous mutations often occur at

different locations of the chromosome, but new discoveries using this "reverse engineering" strategy may provide unexpected ideas and solutions to this problem.

4 Conclusion and Future Perspectives

A commercially successful lignocellulose conversion requires cost-effectiveness at all steps: liberating sugars, converting sugars into target products at high titer, yield and productivity, and downstream product recovery. There have been a lot of developments in pretreatment technologies and metabolic engineering strategies for sugar co-utilization and microbial robustness in the past few decades. In spite of so many research efforts, challenges such as catabolite repression, degradation recalcitrance, and lignocellulosic toxicity still remain at different degrees, thereby limiting further commercial developments.

Future progress is foreseen in the following aspects regarding the focus of this chapter:

(1) More cost-effective pretreatments with minimal inhibitors will be developed. For example, ionic liquid pretreatment is still a relatively nascent technology that has already drawn great interest due to its thermostability, low volatility, and potential as a "green" chemical to reduce hazardous waste produced in pretreatment (Vancov et al. 2012). Other benefits include a comparable sugar yield to acidic/alkaline pretreatments (Trinh et al. 2015), and a typically negligible inhibitor profile. Although inhibitors are not an issue with this technology, its application is currently cost-prohibitive (Baral and Shah 2016). Another interesting research direction led by the Dr. Lynd's group is to integrate both biodegradation and fermentative production together in order to achieve a cost-effective process (Olson et al. 2012; Lynd et al. 2005). The efficiency of saccharification, sugar co-utilization and fermentative production is a key factor to ultimately determine the success of this approach.

(2) More efficient genetic engineering strategies for xylose fermentation and sugar co-utilization will be developed. One improvement will be further optimization of "monoculture" systems: genetic approaches to further improve xylose catabolism and to relieve catabolite repression. However, monoculture optimization is inherently limited by internal competition of the same metabolic intermediate and co-factors. Alternatively, synthetic communities of microbial "specialists" can be engineered to cooperatively degrade lignocellulose inhibitors and to convert different sugars into value-added chemicals. Each specialist only performs a limited subset (but not all) of the collectively required function. This engineering strategy is suitable for a complex challenge such as lignocellulose conversion due to its modularity and flexibility. On this note, a synthetic fungal-bacterial community was designed to directly convert lignocellulosic biomass into isobutanol (Minty et al. 2013). Although the productivity and titer are still far from commercially practical levels, this research

strategy provides a potentially interesting direction for cost-effective lignocellulose conversion.

(3) More engineering strategies and beneficial genetic traits to increase bacterial tolerance for different lignocellulosic inhibitors will be discovered. For example, more efficient furfural reductases will be discovered to shorten the lag phase of cell growth caused by furan aldehydes. Another promising approach for engineering chemical stress tolerance is to identify and engineer multidrug efflux pumps which have activity towards inhibitors from lignocellulose pretreatments. Researchers are currently engineering different efflux pumps to improve bacterial resistance to biofuel molecules and other hydrophobic molecules (Turner and Dunlop 2015; Foo and Leong 2013; Dunlop et al. 2011; Mukhopadhyay 2015). However, these strategies have not yet been adopted for lignocellulosic inhibitors. In addition, a major challenge for further improvement of bacterial robustness is to integrate different detoxification components into one strain. Most epistatic interactions between beneficial genetic traits are not predictable and the search for the optimal combination of multiple effector genes is time and labor intensive (Sandoval et al. 2012). Negative epistatic interaction between different beneficial traits has already been observed for furfural tolerance (Wang et al. 2013). The development of high throughput chromosome manipulation methods will facilitate the test procedures to identify the best combination of beneficial genetic traits.

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Evolutionary Engineering of Microorganisms to Overcome Toxicity During Lignocellulose Hydrolysates Utilization

Gustavo M. Lastiri-Pancardo and José Utrilla

Abstract Microbial chemicals and fuels production from renewable resources requires the development of biocatalysts that can tolerate toxic chemicals produced during the lignocellulosic hydrolyzation process and also tolerate the end product toxicity. Evolutionary engineering makes use of adaptive strategies and selection procedures to generate and study mutations that will increase tolerance to harmful chemicals and therefore increase productivity and titer in such processes. In this chapter, we will review recent advances in evolutionary engineering strategies, their results and challenges to generate better microorganisms for the production of chemicals and fuels from lignocellulosic hydrolysates.

Keywords Adaptive laboratory evolution · Inhibitors tolerance · Toxicity mechanisms

1 Evolutionary Engineering and Adaptive Laboratory Evolution (ALE) Principles

1.1 Evolutionary Adaptation and Fitness Trade-Offs

Since Darwin's first observations, adaptation has been viewed as a gradual and slow process. The dynamics of evolving populations are often described by the movement through the so-called adaptive landscapes, where peaks and valleys represent states of high and low fitness, respectively. There is considerable interest in the structure of these landscapes (Elena and Lenski 2003) since a significant concept in evolutionary biology is the concept of fitness. Fitness centers in the idea that genotypes with higher fitness will produce more offspring thus increasing the size of their populations over time compared to their less fit competitors (Wiser and Lenski 2015).

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There are many methods to measure bacterial fitness. One commonly employed approach is to calculate the maximum growth rate (μ_{max} or V_{max}) of a culture (Sandegren et al. 2008; Walkiewicz et al. 2012). By measuring the optical density of the bacterial culture over time, this method has the advantage of being simple and fast. Another way to measure fitness consists on performing Minimum Inhibitory Concentration (MIC) assays, where microbes are grown in the presence of stressful compounds such as antibiotics, acids or toxic molecules. Those organisms with higher MICs are the ones that are better adapted to the environments that contain that compound, as more is needed to inhibit their growth (Ripoll et al. 2011). Finally, the third approach for quantifying fitness involves competition assays. The basis of this method is to grow two or more strains together and then determine the differences in growth rate across the lag, exponential and stationary phases (Stewart and Levin 2015) (Fig. 1b).

However, taking in consideration Goethe's Law of Compensation (Lenoir 1984), which states that "in order to spend on one side, nature is forced to economize on the other side", cellular resources allocated to achieve an increase in fitness for one characteristic might be in exchange of a reduced fitness in another feature. This reallocation is defined as a "fitness trade-off"; so strain-to-strain variations would be due to constraints imposed by these trade-offs. There are three cellular processes

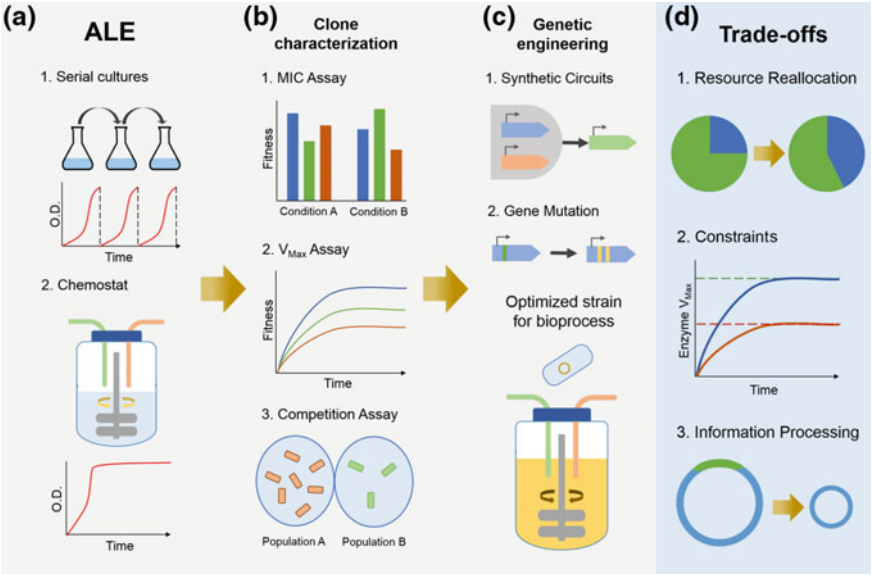

Fig. 1 Adaptive laboratory evolution (ALE). **a** ALE can be performed in sequential serial passages in shake flasks or chemostat cultures. **b** The increase of fitness during ALE experiments is measured by minimum inhibitory concentration assays (MIC), growth rate measurements (V_{max}) or competition assay. **c** The selected clone can be further improved with genetic engineering to be finally used for the desired process. **d** Three types of tradeoffs shape the diversity of phenotypes during ALE experiments: resource reallocation, enzyme constrains and information processing

that result in trade-offs: (a) resource allocation, (b) enzyme constraint and (c) informational processing (Ferenci 2015). Most of the trade-offs are possible through the reallocation of cellular resources between different functions. For example in *Escherichia coli,* mutations in regulatory genes (*rpoB*, *rpoC*, r*poS*) reprogram resource allocation, shifting cellular resources from vegetative to stress-response genes (Ferenci 2008; Conrad et al. 2010; Utrilla et al. 2016). On the other hand, constraints are intrinsic processes found where enzymes are involved, as they usually exhibit an inherent trade-off between speed (turnover rate and processivity) and accuracy (selectivity and specificity). Also, another common trade-off is between enzyme activity and stability (Tawfik 2014). Finally, the accurate storage and processing of biological information also has a cost for the cell, as a result of the resources needed for maintaining the genome. Therefore, genome reduction is a possibility when biosynthetic genes are not needed. However, the trade-off, in this case, is decreased genetic flexibility, and potential nutritional auxotrophy (Souza et al. 2014) (Fig. 1d).

1.2 Adaptive Laboratory Evolution

The fast growing whole-genome sequencing technologies combined with high-throughput data analysis has enabled the growth and improvement of the field defined as Adaptive Laboratory Evolution (ALE).

The main objective of ALE is to generate random mutations in a microbial population, which involves its continuous culturing over multiple generations in selective conditions. With time, selection pressure fixes the beneficial mutations leading to adaptation in a specific environment; these genetic changes provide the organism with an increase in fitness under those conditions. The propagated population (or isolated clones) are compared to the ancestral clone regarding fitness, genome sequence, expression profiling and phenotypic trade-offs (Elena and Lenski 2003; Dragosits and Mattanovich 2013).

ALE is an important tool for biotechnology, its main application in has been to improve the tolerance of an organism to a particular compound of interest (acids and alcohols) (Atsumi et al. 2010; Reyes et al. 2012); also, ALE can be applied to study the dynamics of antibiotic resistance, which is important for clinical research (Jansen et al. 2013).

When performing ALE experiments, there are two main methods used, batch and chemostat or continuous cultures. Each technique has advantages and disadvantages, but independent from the selected culture type, the methodology to follow should be carefully designed according to the objective of the experiment. A critical variable of the ALE experiments is the timescale, as it is often very long, on the order of weeks or months. Consequently, it requires daily attention (Charusanti et al. 2010; Dragosits et al. 2013). An example is the Long Term Evolution Experiment (LTEE) that began in 1988 and still going to the present and which has

now surpassed 60,000 bacterial generations (Lenski 2016). The experiment uses minimal salts medium with 25 μg/mL glucose and every day, the population is diluted into fresh media (Lenski et al. 1991) (Fig. 1a).

Another important variable in these experiments is the amount of cells that are transferred to each new culture. This process has an impact on the number of potentially beneficial mutations on the population that will be carried to the next culture flask, therefore, accelerating or slowing the rate of evolution. Also, the specific growth stage of the culture at the point when cells are transferred is crucial, as different selection pressures and physiological states are present along the full grow cycle (Vasi et al. 1994).

Next-generation sequencing has made it easier to detect mutations in ALE studies (Fig. 1c). However, despite the advances of today's genome engineering tools (Herring et al. 2003; Wang et al. 2009), correlating specific changes from genotype to phenotype is still difficult and time-consuming, and besides the genetic changes, it is also crucial to understand modifications at the system level. Recently, the implementation of "-omics" studies together with in silico modeling has provided a context in which all data can be integrated and interpreted as a whole system. The process termed as constraint-based modeling, has been shown to be a useful tool for integrating all kinds of data generated in -omics studies (Feist et al. 2008; Schmidt et al. 2013). This workflow has already been used in *E. coli* MG1655 for comparing experimental data and in silico predictions of ALE experiments (LaCroix et al. 2015).

2 Challenges to Overcome: Chemical Toxicity

The use of lignocellulose as a feedstock for bioprocesses with microorganisms aimed to produce biofuels and chemicals presents many challenges. In this section, we will review those related to chemical toxicity. To utilize lignocellulose from plant material it first needs to be hydrolyzed to release fermentable sugars by employing any of the many methods available (Sun and Cheng 2002). Lignocellulosic hydrolysates (LcH) contain several types of chemicals derived from the hydrolysis process. It is important to point out that each kind of treatment and each raw material will produce a specific set of inhibitors (Pienkos and Zhang 2009). Among the main inhibitors produced by the hydrolysis processes are aliphatic acids (i.e. acetic, formic, octanoic, levulinic acids), furfurals (Furfural and 5-Hydroxymethyl Furfural HMF), aromatic compounds (phenolics, coumaric acid, etc.), among others (Fig. 2). Furthermore, some treatments leave traces of the chemicals used for the (pre) treatment, which can be toxic for the microbial catalysts (Ouellet et al. 2011). The details of the specific advantages and disadvantages of each treatment on the generation of toxic compounds have been reviewed by several authors (Jönsson et al. 2013). There are several possible treatments aimed at reducing the inhibitors produced in the hydrolysis (detoxification). However, the detoxification of LcH may also result in undesirable substantial sugar loss (9–26%)

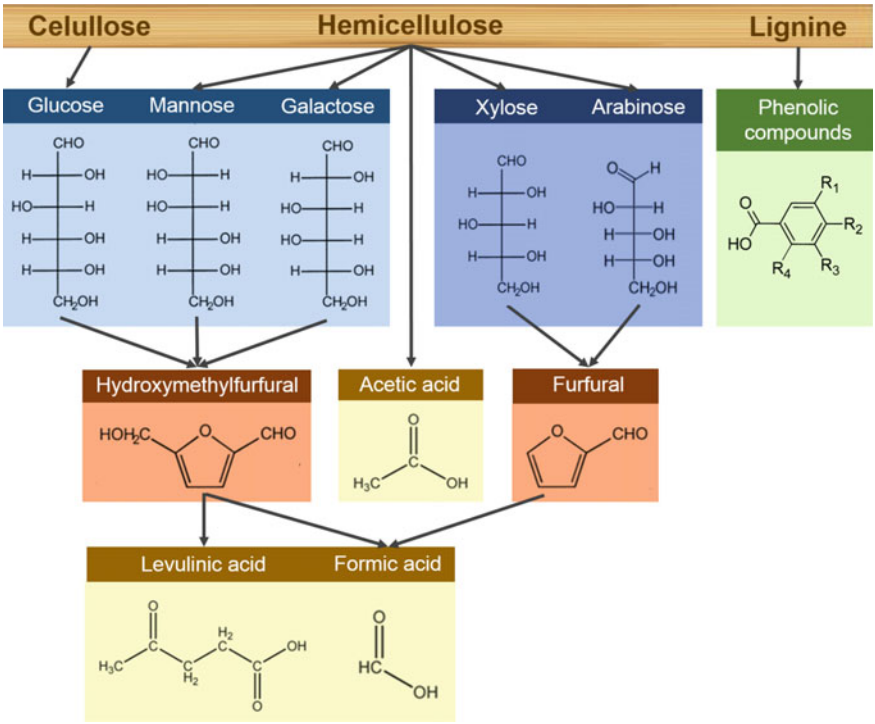

Fig. 2 Hydrolysis of lignocellulose and inhibitor formation. Furan aldehydes (*orange*) and aliphatic acids (*yellow*) are carbohydrates (*light blue*—hexoses; *dark blue*—pentoses) degradation products, while lignin is the main source of phenolic compounds (*green*)

(Larsson et al. 1999b; Martinez et al. 2001). Therefore, it is desirable to generate tolerant microbial strains; here we will review the evolutionary strategies used for this purpose.

Another challenge related to chemical toxicity is imposed by the toxic nature of the end products generated by the microbial production strain. Most of the fermentation end products such as alcohols; organic and carboxylic acids are toxic to their production host. Therefore, the economic viability of fermentation processes, that aim to use lignocellulosic feedstock, may depend on the development of tolerant microbial biocatalyst to LcH inhibitors and also to the inhibitory action of the end products.

Tolerance mechanisms have been extensively studied, however, there are many components partially unknown. Resistance to toxic compounds is a complex trait, often the result of the coordinated action of hundreds of genes. Furthermore, each stressful condition requires a specific set of defense mechanism (Nicolaou et al. 2010). LcH inhibitors and fermentation end products span a wide variety of chemical structures. Thus, they exert toxicity through a wide range of mechanisms.

Many of the tolerance and detoxification mechanisms such as efflux pumps and metabolic transformation for inactivation of compounds (i.e., furfural reduction), compete for the energy required by other cellular processes. Since detoxification mechanisms necessitate energy and cellular resources, such deployment will reduce process productivity. Metabolic detoxification is needed for coping with hydrolysate inhibitors, such as furfural, but not for end products, since this would degrade the desired compounds. Therefore, specifically for fermentation end products, tolerance is the preferred mechanism when compared to detoxification.

The main challenges for understanding and engineering tolerance mechanisms arise from the wide array of toxic effects of such molecules. Furthermore, some combinations of inhibitory chemicals have been shown to interact synergistically to inhibit growth (Palmqvist et al. 1999). Tolerance or detoxification mechanisms are difficult to understand and engineer in a system-wide basis. General stress response generated by the exposure of the cells to a LcH or toxic end product may drain proteomic resources and energy, which are essential for cell growth or high-level expression of native or heterologous production pathways. It has been shown that genome-wide transcriptional responses, such as general stress, to metabolic detoxification of inhibitory compounds, can drain cellular resources and have a negative system-wide effect on cellular physiology (Goodarzi et al. 2010; Utrilla et al. 2016).

A high degree of transcriptional overlap between stress responses has been reported (Dragosits et al. 2013). It has also been proposed that the mechanisms selected during ALE for tolerance or stress response, may be specific when the organism faces toxic compounds and that it may be general when the inhibitor is not usually encountered in the natural environment of the microorganism (Nicolaou et al. 2010). Restorative shifts have been observed in ALE experiments, meaning that upon exposure to a specific stress, cells display a change in global gene expression. After a few generations growing under the same condition, adaptive mutations arise and the general expression profile returns to a pre-perturbed state (Sandberg et al. 2014). Then, as discussed above, in terms of energy costs, the expression of toxic-specific responses should be more effective to counteract an inhibitor than a general stress response. Therefore, a great challenge in years to come will be to engineer those specific responses to generate cells specifically reprogramed for those purposes, we may call those cells “semi-synthetic” cells.

As discussed in the previous section of this chapter, ALE is a powerful tool to improve tolerance mechanisms and to study short-term adaptation to stress response (Dragosits and Mattanovich 2013). The adaptation to a specific stress condition may reduce general stress responses and therefore optimize the cellular resources deployed to alleviate the specific inhibition. Therefore, ALE has proven to be an effective tool to select strains with specific mutations that enable tolerance to toxic compounds. The genome sequencing of evolved tolerant clones combined with allelic replacement into ancestral clones will help to identify causality and key mechanisms of general and specific tolerance.

3 ALE Applied to Increase Tolerance to Hydrolysis Byproducts

Lignocellulosic resources comprise wood, grass, forestry waste and agricultural residues. These materials are formed mostly by polymerized sugars (up to 70% by weight unit), of this, 35–45% is cellulose and 20–35% are hemicellulose residues (Miller et al. 2009a). Cellulose is a linear polymer with a high molecular weight composed of D-glucose units with β-1,4 bonds. Hemicelluloses are branched polysaccharides containing hexoses (D-mannose, D-glucose, D-galactose), pentoses (D-xylose and L-arabinose) and uronic acids residues (Fan et al. 1982; Palmqvist and Hahn-Hägerdal 2000a). Lignocellulosic biomass represents a potential source of sugars for microbial conversion to renewable fuels, plastics and other chemicals (Miller et al. 2010). All the sugar residues in biomass can be released using acids, enzymes, or of both treatments (Um et al. 2003; Wyman et al. 2005; Cheng et al. 2008), which can be later used as carbon sources by microorganisms. As discussed in the previous section of this chapter, during the process of lignocellulose breakdown, growth inhibiting by-products are formed (Fig. 2). These toxic compounds include weak organic acids (acetic, formic and levulinic acid) and the furan derivatives furfural and 5-hydroxymethyl furfural (5-HMF) (Palmqvist and Hahn-Hägerdal 2000a).The ability of a biocatalyst to tolerate toxic compounds from hemicellulose hydrolysates is important for the industrial exploitation of these renewable resources.

3.1 Furan Derivatives

Furfural and 5-HMF are two minor, but toxic byproducts resulting from the dehydration of pentose and hexose sugars, respectively (Martinez et al. 2000; Almeida et al. 2009). They are present in inhibitory concentrations in LcH and it has been shown that these two molecules hinder growth and fermentation performance of ethanologenic *E. coli* and *Saccharomyces cerevisiae* strains (Palmqvist and Hahn-Hägerdal 2000b; Almeida et al. 2008). However, the moderate addition of furfural to the growth medium was found to lead to increased ethanol yields for recombinant xylose-utilizing *S. cerevisiae* (Wahlbom and Hahn-Hägerdal 2002).

Several methods have been used to study the toxic mechanism of these compounds, which are still not completely understood. It has been proposed that they inhibit growth by damaging DNA (Barciszewski et al. 1997), inhibiting glycolytic enzymes (Modig et al. 2002; Hristozova et al. 2006), and chemically reacting with various cellular constituents (Palmqvist and Hahn-Hägerdal 2000a; Horváth et al. 2001). The inhibition of growth caused by furfural and 5-HMF has also been linked to the reduction of these compounds to furfuryl and 5-hydroxymethyl furfuryl alcohols respectively by NADPH-dependent oxidoreductases (Gutiérrez et al. 2006; Glebes et al. 2015). This process causes a decrease of available NADPH necessary

for biosynthetic processes such as sulfur assimilation (Miller et al. 2009a) and pyrimidine synthesis necessary for DNA repair (Zheng et al. 2012). The mechanisms for conferring tolerance to these compounds remain elusive. However, advances have been made by alleviating NADPH starvation by silencing NADPH-dependent oxidoreductases (Miller et al. 2009), increasing NADH dependent reductase expression and activity (Wang et al. 2011a; Zheng et al. 2013) and overexpressing the NADPH-restoring transhydrogenase PntAB (Miller et al. 2009). It has also been reported that overexpression of the protein chaperone GroES-EL or the LpcA isomerase (involved in lipopolysaccharide construction) confers increased tolerance to furfural (Glebes et al. 2014).

The toxic compounds from LcH can be eliminated from by over-liming to pH 10 at elevated temperatures (Martinez et al. 2001). However, this process requires much effort, it generates waste and it may reduce sugar concentration; therefore, the development of furan-resistant microbes could make this process unnecessary. The development of a strain tolerant to furfural and 5-HMF is difficult if direct genetic engineering approaches are employed due to the poor understanding of their toxic molecular mechanisms. Therefore, ALE represents a viable strategy to generate tolerant strains, since this method allows the selection of non-intuitive beneficial mutations that can occur in several different genes (Dragosits and Mattanovich 2013). Adaptation of *S. cerevisiae* to furfural and HMF has been reported in batch culture. Evolving the cells using sugar cane molasses, as well as other crude extracts (peptone and yeast lysate) as grow media makes them resistant enough to withstand the inhibitory effect of these compounds, apparently due to the presence of additional cofactors and vitamins in the molasses, from which the cells benefit (Banerjee et al. 1981; Villa et al. 1992). It has also been reported that in continuous culture when using a high cell load inoculum (10^8 initial cells/mL), 50% of the yeast cells survived the initial cell death period during which furfural and HMF were depleted. The fermentation then proceeded to completion by cell regrowth (Chung and Lee 1985). By performing carbon-flux and global array-based transcript analysis in glucose limited chemostat cultures, Herr et al. 2009 found that in an evolved strain of *S. cerevisiae* the metabolic flux through the pentose phosphate pathway was increased to provide sufficient NADPH for the reduction of furfural. Additionally, they observed a very strong overexpression of the NADPH-dependent oxidoreductase ADH7 and the uncharacterized ORF YKL071 W along with high levels of four additional oxidoreductases (Heer et al. 2009). Using a long-term adaptation strategy, an industrial strain of *S. cerevisiae* (Ethanol Red) was evolved (ISO12). Contrary to the parental strain, ISO12 is capable of growing and fermenting the liquid fraction of non-detoxified spruce hydrolysate at 39 °C with an ethanol yield of 0.38 g ethanol/g hexoses. In contrast with previous studies, the superior phenotype of the evolved strain does not rely on higher reductase activities for furaldehyde inhibitor conversion, but rather on a higher thermotolerance. Evolved strain ISO12 shows a higher capacity to ferment hydrolysate at 39 °C and higher viability during heat-shock at 52 °C (Wallace-Salinas and Gorwa-Grauslund 2013).

The potential of ALE to increase furan derivatives tolerance have been also explored with other microorganisms. In a recent study, *Zymomonas mobilis* was evolved by gradually increasing furfural concentration employing serial cultures. The newly adapted strain showed 94.84% theoretical ethanol yield under 3 g/L furfural stress condition. Unfortunately, clear details about the mechanisms of tolerance are not described (Shui et al. 2015).

3.2 Aliphatic Acids

During lignocellulose hydrolysis, aliphatic acids are released. Formic acid is produced from furfural, while 5-HMF is degraded to levulinic and formic acid (Fig. 2). Furthermore, at high temperatures acetate is released (Fan et al. 1982; Vargas-Tah et al. 2015). These compounds inhibit cell growth because undissociated weak acids are liposoluble and can diffuse across the plasma membrane into the cytosol. Once inside the cell, dissociation of the acid takes place due to the neutral intracellular pH, consequently decreasing the cytosolic pH (Pampulha and Loureiro-Dias 1989). The drop in pH is neutralized by the action of the plasma membrane ATPase, which pumps the excess protons out of the cell at the cost of ATP. This response can lead to the exhaustion of the proton pumping capacity of the cell and possible cell death (Jönsson et al. 2013). In the case of *S. cerevisiae*, the toxic effect of acids is attributed to their undissociated form. A clear difference in toxicity among acetic, levulinic and formic acid has been reported (acetic acid < levulinic acid < formic acid) (Larsson et al. 1999a; Shui et al. 2015). This may be due to differences in their membrane permeability.

It was found that inhibition of yeast growth became apparent at concentrations exceeding 100 mM of aliphatic acids. However, with concentrations lower than 100 mM the ethanol yields were higher than fermentations with no aliphatic acids included (Larsson et al. 1999a). However, the increased yield is at the expense of biomass formation as a consequence of the cell's attempt to maintain a constant intracellular pH by pumping out protons through the plasma membrane ATPase (Verduyn et al. 1990).

Several efforts have been made to engineer strains which can tolerate high concentrations of acids. A recent ALE study revealed that a loss of a 27.3 Kb genome region that harbors genes that code for nitrate respiration, *omp*C, cytochromes, thiamine and colonic acid resulted in tolerance up to 40 g/L of acetate in an *E. coli* ethanologenic strain. The authors did not describe the role of each gene loss on the mechanism of acetate tolerance phenotype (Fernández-Sandoval et al. 2012). Another ALE experiment performed on *Z. mobilis* showed that an evolved strain displayed higher growth under a stress condition of 7 g/L acetic acid by gradually increasing the acetic acid concentration in serial cultures. Using genome sequence analysis techniques the authors identified some key genes responsible for the acetic acid tolerance (a host integration factor: *himA* (ZMO1122); a RNA

binding protein: *hfq* (ZMO0347); and an hydroxylamine reductase: *nhaA* (ZMO0117)) (Shui et al. 2015).

Lennen and Hergard performed transposon mutagenesis followed by sequencing (Tn-seq) to find gene interruptions that could lead to improved stress tolerance for low pH, high salinity and acetate concentrations. They found that combining three clean gene deletions obtained by Tn-seq and selection (Δ*ptsP*, Δ*yobF* and Δ*yciW*), they were able to increase tolerance to 15 g/L NaAc, low pH (5.5) and salinity (0.4M NaCl). PtsP (EI^{Ntr}) a component of the nitrogen phosphotransferase system, its participation in tolerance increase was suspected by the authors to be mainly regulatory. The other two mutations are in genes of unknown function which may be interesting candidates for designing acetate tolerant strains (Lennen and Herrgård 2014). Despite the advances, a greater understanding of the inhibitory mechanisms of these compounds and their effects at a system level is still needed in order to obtain a fully resistant strain for biotechnological uses.

3.3 Phenolic and Aromatic Compounds

Phenolic compounds are mainly generated from the breakdown of lignin, but also have been reported to be formed during carbohydrate degradation (Palmqvist and Hahn-Hägerdal 2000b). The negative effects of phenolics and other aromatic compounds in microbial growth and product yield are very variable, and is related to specific functional groups (Larsson et al. 2000). However, in many cases, the mechanism of toxicity has not been elucidated due to a lack of accurate qualitative and quantitative analyses. One possible mechanism is that phenolics interfere with the cell by changing its protein to lipid ratio, consequently affecting their ability to serve as selective barriers and enzyme matrices (Keweloh et al. 1990). The low molecular weight phenolic compounds are the most toxic, 4-Hydroxybenzoic acid, vanillin, and catechol were major constituents in the untreated hydrolysate (Büchert et al. 1989). 4-Hydroxybenzoic acid has been used as a model compound to study the influence of phenolic compounds on fermentation and it has been reported that in *S. cerevisiae* 1 g/L cause a 30% decrease in ethanol yield. Vanillin also constitutes a considerable part of the phenolic compounds in hydrolysates. Vanillin has been found to be less toxic than 4-hydroxybenzoic acid (1 g/L caused a 25% decrease in the ethanol yield) (Ando et al. 1986).

3.4 Non Organic Compounds

Inorganic ions are also present in lignocellulose hydrolysates; they originate from chemicals added during pretreatment (Palmqvist and Hahn-Hägerdal 2000b). High salts concentrations means a higher osmotic pressure in the environment, which may result in inhibitory effects (Helle et al. 2003). It is proposed that the mechanism

of action is an increased demand of ATP due to increased transport over the plasma membrane (Jönsson et al. 2013).

S. cerevisiae is somewhat salt tolerant compared to other yeasts, and is capable of growing in a 1.5M solution of sodium chloride in glucose based medium (Helle et al. 2003). It has also been investigated the effects of different salts on *S. cerevisiae*, the inhibition decreases as follows: $CaCl_2$ > $(NH_4)_2SO_4$ > NaCl > NH_4Cl > KH_2PO_4 > $MgCl_2$ > $MgSO_4$ > KCl (Maiorella et al. 1984).

4 ALE Applied to Increase Tolerance to Fuels and Chemical Products

4.1 Ethanol and Other Alcohols

Alcohols are very attractive as products mainly for their use as biofuels. Alcohols are toxic to microorganisms and their main effects are on the fluidity of the membranes, protein denaturalization (affecting enzyme activities) and proton flux across the membrane (Cray et al. 2015).

Many ALE experiments have been performed with the aim of improving ethanol tolerance. Horinouchi et al. (2010) reported ALE experiments in six parallel evolution cultures using *E. coli* W3110 as a starting point. First, they evolved the starting strain in M9 media to determine the phenotypic and genetic changes in media without ethanol. They used 5% ethanol comparing it to the evolved strain in media without ethanol; they obtained six tolerant strains displaying a two-fold increase in growth rate with a stable phenotype even when cultivated for more than 100 generations without ethanol. They showed that evolved strains can grow in 6.5% of ethanol, whereas the parental strain cannot grow in the same condition. Microarray analysis of the parental and 6 tolerant strains revealed the overexpression of amino acids biosynthetic pathways (tryptophan, histidine and branched-chain amino acids). Also, they found upregulation in 42 genes related to iron ion metabolism ("iron ion transport," "enterobactin biosynthetic process" and "iron–sulfur cluster assembly" functional categories) (Horinouchi et al. 2010). In a follow-up study, Horinouchi and co-workers sequenced the evolved tolerant strains, performed time-series transcriptomic and metabolomics analyses to provide a quantitative understanding of evolutionary constraints. Mutations appearing in several strains were in genes: *hns, iscR, relA cspC*. They measured time-series transcriptome and metabolome from frozen stocks of the 6 ALE experiments. They found that transcriptomic and metabolomics changes were similar among independently evolved strains with a gradual up-regulation of genes involved in amino acid biosynthesis pathways, possibly caused by a decrease of the amino acid pools in the tolerant strains. The reintroduced mutations of one of the tolerant strains (*relA, iscR, wzxC, cspC, miaB*) into the parental strain could not reproduce totally the ethanol tolerance phenotype. They attribute the result to a probable

transgenerational phenotype plasticity, possibly exerted by epigenetic mechanisms (Horinouchi et al. 2015). In another study, using ALE with a gradual ethanol concentration increase in rich LB medium with ethanologenic strain KC01 *E. coli* resulted in a twofold improvement in ethanol tolerance (measured as ID_{50}: 50% cell growth inhibition) in 30–35 g/L of ethanol when compared to the ID_{50} of 10–15 g/L in the parental strain. The resulting evolved strain showed 48% increased ethanol productivity with a titer of 23.5 g/L (Wang et al. 2011b). To determine the genetic modifications that lead to ethanol tolerance, Goodarzi and co-workers performed a comparison of ALE and single-locus perturbations with high-coverage mutant libraries. ALE resulted in strains able to grow in 7% ethanol in rich media. They discovered intracellular ethanol degradation as a potential adaptive mechanism to ethanol tolerance. They also found that stress responses and osmotolerance are main processes contributing to ethanol tolerance. Unfortunately, ethanol degradation is not a desired characteristic of a biofuel producer organism (Goodarzi et al. 2010).

Reyes et al. (2012) developed an ALE method based on using different fluorescently marked cells. The method allows Visualizing Evolution in Real Time (VERT), and was applied in an n-butanol tolerance study. Using transcriptome profiling and genome resequencing they found a reduced activity of the ferric uptake regulator (Fur) that changes membrane composition by increasing siderophore biosynthesis. However, this tolerance mechanism was specific to one lineage of evolved populations. In a second study, (Reyes et al. 2013) they found that differentially tagged strains show different behavior regarding cross-stress protection against other similar stressors. One lineage showed general solvent tolerance to n-butanol, isobutanol and ethanol; while the other strain showed a more specialist tolerance mechanism only to n-butanol (Reyes et al. 2013).

Atsumi et al. 2010, evolved the isobutanol *E. coli* producer strain JCL260 (Atsumi et al. 2010) by serial transfer in LB media with increasing isobutanol concentration up to 8 g/L. They isolated a strain being able to grow at 8 g/L and two-fold increased cell viability after 24 h exposure to 6 g/L of isobutanol compared to the non-evolved strain. They performed genome sequencing and studied the mutations responsible for the observed phenotype. First, by repairing the mutations in the evolved strain and also by introducing them into the parental strain. They found five key mutations of the isobutanol tolerant phenotype (*acrA, gatY*, *tnaA*, *yhbJ* and *marCRAB*). Presumably, the reduction of the expression of the AcrB-TolC multidrug efflux system, normally induced by isobutanol, increased the tolerance. The deletion of *yhbJ* may change the LPS constituent of the outer membrane. In a similar study, Minty et al. 2011, evolved a non isobutanol producer strain. They observed an adaptation mechanism based on the remodeling of the cell envelope and an attenuation of the stress response mediated by *rpoS*. They reported similar mutations (*arcAB* and *marC*) as Atsumi et al. 2010, showing conserved mechanisms of isobutanol tolerance trough ALE (Minty et al. 2011).

4.2 Organic Acids

Carboxylic acids are widely produced during the sugar metabolism of bacteria. Several organic acids are of interest to be produced from LcH, since they are widely used as chemical building blocks for many applications (Sauer et al. 2008). In this section, we will discuss the toxicity of organic acids in bacteria and the evolutionary strategies that have been employed to deal with them. Many undissociated organic acids can diffuse trough the cell membrane; some others may be transported through channels. One of the main toxicity effects are the change of cytosolic pH and disruption of the proton motive force, thus being strongly pH dependent. Also, export mechanisms through molecular pumps will consume energy, thus competing with other cellular processes (Warnecke and Gill 2005).

Acetic acid is a byproduct of metabolism and also a LcH derived inhibitor. Even in well-aerated conditions, many fast-growing cells produce metabolic byproducts. In bacteria, this is known as overflow metabolism, also known as Warburg effect for cancer cells. The bacterium *E. coli* produces acetic acid as a byproduct during overflow metabolism as a result of an efficient proteome allocation (Basan et al. 2015). Acetic acid can affect growth in concentrations as low as 0.5 g/L. The toxicity of acetic acid is evident in high-density cell cultures where it accumulates as a metabolic byproduct until becomes toxic for *E. coli* cells (Nakano et al. 1997). Evolutionary strategies to overcome acetic acid toxicity have been reviewed in the LcH inhibitors section of this chapter.

Lactic acid is a chiral organic acid with wide applications in the bioplastics, chemical and cosmetic industries (Castillo Martinez et al. 2013). Most bacteria used for lactic acid production are mildly tolerant, and the main toxicity problems arise from lowering of the pH by the acid production or the anion of the alkali used for pH control. In a recent study, ALE was employed to adapt *E. coli* for 73 days to progressively greater sodium concentrations and they applied this strategy to improve lactic acid production. Wild-type and evolved strains showed a roughly linear decrease in maximum specific growth rate as the concentration of sodium ion increased; however, the evolved strains shifted this relationship (growth rate to Na+) by 0.10–0.15 h^{-1} compared to the wild type strain. Genome sequencing of the isolated strain ALS1187 showed that it has mutations in five genes that could impact global cellular functions (*emrR*, *hfq*, *kil*, *rpsG* and *sspA*), while no mutations occurred in genes directly related to Na+ transport (Wu et al. 2014). ALE has also been used as a strategy to optimize lactate production strains, Zhou et al. (2006) performed ALE with an engineered *E. coli* strain to improve its capacity to reach higher lactate titers in mineral salts medium. Using growth-based selection in pH-controlled fermentations, they evolved the producer strains first, in 10% sucrose mineral media. After the isolation of a colony and deletion of by-product formation pathways, they performed ALE experiment in 10% glucose mineral media. The resulting strain can produce a titer of 1.2 M lactate with 95% optical purity. This strain was not sequenced nor the mechanisms increasing productivity studied (Zhou et al. 2006). In another ALE experiment, *Corynebacterium glutamicum* was

adapted to grow at pH 5.7 with lactic acid in a turbidostat. DNA microarray analyses were used to determine the expression response using neutral conditions as a reference. Results showed a significant change of 206 genes of which 115 were upregulated. The induction of iron responsive genes, transport systems, DNA repair, and several regulatory proteins was reported as the main responses to lactic acid stress (Jakob et al. 2007). Wild-type *E. coli* W3110 was evolved in a continuous culture for 268 days with increasing succinate concentration up to 0.592 M (70 g/L). DNA microarray and real-time PCR were used to investigate the succinate tolerance mechanisms. Results showed the upregulation of genes controlling active transport and biosynthesis of osmoprotectants (Kwon et al. 2011).

ALE has also been used to increase tolerance for other types of stress such as temperature, high hydrostatic pressure and ionizing radiation. Using ALE in rich media Rudolph and coworkers (2010) isolated a strain that was able to grow at 48.5 °C. By analyzing heat shock genes and proteins, they found that the observed phenotype was dependent on the expression of high levels of GroEL/GroES chaperones and the lysyl-tRNA-synthetase (LysU) (Rudolph et al. 2010). Sandberg et al. (2014) evolved ten parallel *E. coli* populations at 42 °C under tightly controlled experimental conditions. Adapted strains grew 20% faster on average and showed as few as 6 mutations and as many as 55. They reintroduced some of the mutations and identified 9 that had the largest effect on the phenotype (*pyrE/rph*, *rpoC*, *pykF*, *hfq*, *nagA*, *rne, mlaE, ygaH/mprA*) (Sandberg et al. 2014). In a high hydrostatic pressure ALE study, *E. coli* was evolved during 505 generations. They isolated a strain with the ability to grow at 62 MPa (the unevolved strain grows up to 50 MPa). They found that the isolated strain had a mutation in gene *acpP* (acyl carrier protein P) that allows the alteration of the membrane fatty acid composition in response to pressure (Marietou et al. 2014). ALE has also been employed to study the tolerance to ionizing radiation. By doing an ALE experiment in an increasing amount of irradiation (exposure from 2000 to 10,000 Gy), they isolated a strain able to survive exposure to high doses of radiation values close to what *Deinococcous radiodurans* can tolerate. By sequencing the genomes of 9 purified strains from the evolved populations, they were able to show that the enhanced recombinatiorial DNA repair capacity makes the best contribution to the resistance mechanisms. Main mutations were found in *recA* and the e14 prophage. (Harris et al. 2009).

5 Concluding Remarks

In this chapter, we have reviewed the fundamentals of evolutionary engineering for improved tolerance to inhibitors in lignocellulosic feedstock utilization. The release of fermentable sugars from lignocellulosic residues is accompanied by the production of inhibitory compounds. The development of organisms being able to tolerate the toxic effects of hydrolysates by-products and the inhibitory effects of products may be one of the defining facts of the economic viability of a process to

produce fuels and chemicals from renewable feedstocks such as lignocellulose. There are many challenges to engineer chemical tolerance; one of them is the complex multi-scale response that microbes display to overcome the toxicity of a compound. As reviewed here, ALE has provided an effective tool to obtain and study such responses. The integration of genome-scale data with proper analysis tools and genome editing technologies should aid in the advance of the engineering of complex phenotypes as required.

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