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Microorganisms in Biorefineries

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Microorganisms in Biorefineries

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Dedicated to Michael Kamm, founder of biorefinery.de GmbH

Preface

Although the chemical industry today still works with fossil raw materials such as petrol and natural gas, even this sector will have a stronger focus on the use of renewable feedstock: biomass from plants. A particular advantage of biorefineries will be effective in this development for exploiting biomass perfectly: the generation of a high number of products and material for further processing in the chemical industry. The development of microbial processes both for the digestion of biomass and for the synthesis of platform chemicals and secondary products is an important object of research in this context.

This monograph delivers a selective outlook on developments regarding microorganisms and their use in several product lines of the biorefinery. Microorganisms in lignocellulosic feedstock biorefineries (chapters by Arkady P. Sinitsyn and Alexandra M. Rozhkova; Alessandro Luis Venega Coradini et al.; M. Teresa F. Cesário and M. Catarina M. Dias de Almeida; and Dženan Hozić), particularly concerning the production of polyhydroxyalkanoates and lipids, alcohol fuels, and hydrocarbons, microorganisms in the green biorefinery focused on organic acids (chapter by Petra Schönicke et al.; Mette Hedegaard Thomsen et al.); and microorganisms for the synthesis of defined platform chemicals and specialty chemicals containing heteroatoms (chapters by Qiang LI and Jianmin Xing; Nick Wierckx et al.; Christine Idler, Joachim Venus, and Birgit Kamm; Robert Kourist and Lutz Hilterhaus). Furthermore, microorganisms for the generation of isoprenoids and methane from biomass are part of the biorefining observations (chapters by Claudia E. Vickers et al.; Vladimir V. Zverlov, Daniela E. Köck, and Wolfgang H. Schwarz)."

Teltow, Germany — Birgit Kamm

Contents

Penicillium canescens Host as the Platform for Development of a New Recombinant Strain Producers of Carbohydrases

Arkady P. Sinitsyn and Alexandra M. Rozhkova

Contents

Abstract The filamentous fungi strain *Penicillium canescens* has been developed as a host for the production of heterologous proteins and enzymes for biorefinery. There are several features of this strain which make it an attractive option as a host expression system. First, *P. canescens* has a high growth rate and the developed system of biosynthesis of extracellular enzymes; second, strain needs inexpensive fermentation medium using sugar beet pulp as a main substrate; third, the fermentation process can be easily scaled up; and fourth, there is auxotrophic strain

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P. canescens which can be transformed by plasmid DNA with exogenous genes. All these factors make possible to create new efficient recombinant strains and enzyme preparations (different endo-glucanases and cellobiohydrolases, β-glucosidase, pectin lyase, inulinases) that are in demand by various sectors of industry and biorefinery.

1 Introduction

Many enzymes used in biorefinery are fungal; in their natural habitat fungi secrete cellulases, hemicellulases, pectinases, amylases, chitinases, other carbohydrases, as well as esterases, ligninases, and related enzymes taking places in renewable biomass degradation. Filamentous fungi also can be efficient in protein secretion and production; besides that, fungi can be relatively easily cultured on the relatively cheap substrates. These circumstances make fungi as an important tool for production of enzymes for the needs of biorefinery. At the same time the secretion level of many fungal enzymes is not high enough, and a number of fungal hosts for fungal gene expression and methods of transformation have been disclosed for improvement of secretion level of target enzymes and enzymatic mixtures. *Aspergillus* (Lubertoz and Keasling 2009; Punt et al. 2002) and *Trichoderma* (Nevalainen et al. 2005; Keranen and Pentilla 1995) are currently the main fungal genera applied as expression system to produce enzymes for biorefinery. Recently *Myceliophthora thermophila* (former *Chrysosporium lucknowense*) was suggested to use as a host system for expression of biomass hydrolyzing enzymes (Visser et al. 2011). But the search for efficient fungal host system is still continued to fulfill the demand of biorefinery area for the source of cheap and efficient enzymes.

The general demands to the host are the following: the host must be readily fermented using inexpensive medium and easy to scale up, should be capable of efficient secretion of the protein, must process the desired protein such that it is produced in an active form not requiring additional activation or modification steps, should be readily transformed, should allow a wide range of expression regulatory elements to be used thus ensuring ease of application and versatility, should allow use of easily selectable markers that are cheap to use, and should produce stable transformants.

We have developed the filamentous fungi strain *Penicillium canescens* as a host for the production of heterologous proteins (enzymes) with many demands to the host listed above: first, *P. canescens* has a high growth rate and the developed system of biosynthesis of extracellular enzymes; second, strain needs inexpensive fermentation medium using sugar beet pulp as a main substrate; third, the fermentation process can be easily scaled up; and fourth, there is auxotrophic strain *P. canescens* which can be transformed by plasmid DNA with exogenous genes. All these factors make possible to create new efficient recombinant strains and enzyme preparations (different endo-glucanases and cellobiohydrolases,

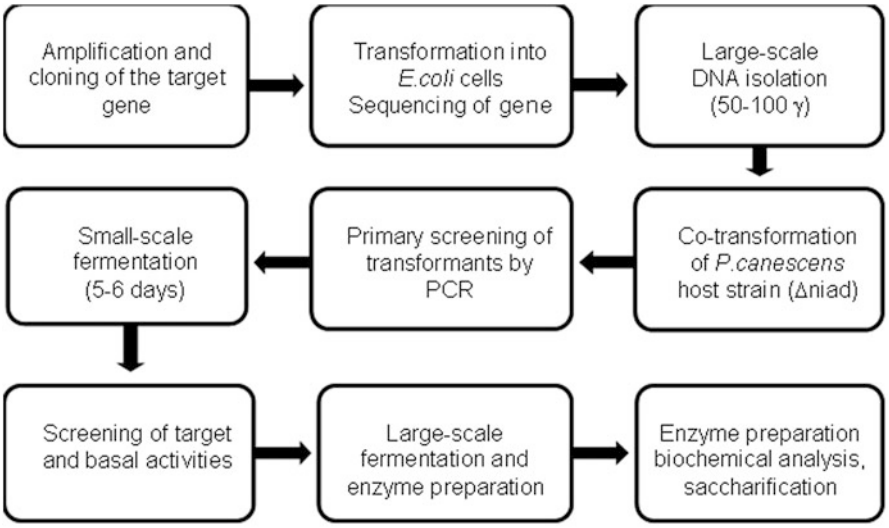

Fig. 1 Scheme of enzyme preparation obtaining in filamentous fungi hosts

β-glucosidase, pectin lyase, inulinases) that are in demand by various sectors of industry and biorefinery.

The general scheme of enzyme preparation obtained in filamentous fungi hosts applying genetic engineering approaches is presented in Fig. 1. Briefly, the first step is amplification and cloning of the target gene into suitable expression vectors. Obtained shuttle expression plasmid transforms into *E. coli* cells to determine sequence of cloned gene (to exclude mismatch, deletions, mutations, and insertions). Then, large-scale DNA isolation is carried out, because large DNA amount (around 10γ) is necessary for fungal transformation. Then expression plasmid together with transformation plasmid containing selective gene to separate recombinant strains is transformed to fungal protoplasts. The next step is primary screening of recombinant fungal clones by PCR to find chromosomal integration of target genes. Then small-scale fermentation of new recombinant strains in shaking flasks is carried out to determine basal and target enzyme activities and level of new recombinant strain productivity. And final step is concluded in a large-scale fermentation for production of enzyme preparation for testing in application trials.

2 Development of *Penicillium canescens* Genetic Tools

It is difficult to imagine modern biotechnology and, in particular, modified strains that produce commercially important enzymes, without the use of genetic engineering methods. Advantages of genetic engineering approaches consist of the (1) possibility of multienzymatic complexes obtained with specified ratio of constituent carbohydrases, (2) reproducible low time for creation of new recombinant strains, (3) possibility to obtain (mono)producers of individual commercially important enzymes, and (4) stable integration of gene(s) of interest to the fungal chromosome.

In the early 1980s, numerous fungal isolates were screened for their natural ability to produce new hemicellulases. This screening resulted in the isolation of a fungal strain from soil capable of secreting xylanases, β-galactosidases, and arabinofuranosidases, and this strain was characterized as a haploid filamentous fungus (USSR Patent 1982, 1984). The fungus showed broad pH (4.5–6.0) and temperature (25–35 °C) ranges for growth. Based on morphological characteristics, the isolate was classified as *P. canescens* (deposited at the Russian Collection of Microorganisms (VKM) of the Russian Academy of Sciences, Accession No. VKM F-175). The *P. canescens* strain was developed by the State Research Institute of Genetics and Selection of Industrial Microorganisms ("Genetika") as a platform for recombinant strain producers of biotechnologically relevant multienzymatic complexes.

In 1994 it was found that the arabinose is the main inductor for biosynthesis of β-galactosidase (Nikolaev and Vinetski 1998).

In 1995 plasmid transformation was developed for *P. canescens* (Aleksenko et al. 1995).

During 1994–1997 multicopy producers of β-galactosidase were obtained. The level of β-galactosidase expression was 200 and 600 U/ml in fermentation broth (Patent RU 1997, 1999).

As a result of application of genetic engineering approaches, the productivity of β-galactosidase was increased 12 times. The specific activity and other properties of the enzyme obtained by the multicopy strain did not change compared to those of the native enzyme.

2.1 Penicillium canescens *Selection Marker Based on Auxotrophic or Nutritionally Deficient* Penicillium canescens *Strains*

Random mutagenesis procedures using UV light or the mutagenic agent N-methyl-N′-nitro-N-nitrosoguanidine (NTG) resulted in a primary strain lineage (Fig. 2). The selection marker was developed for *P. canescens* strain F178 based on complementation of *niaD* mutants lacking nitrate reductase activity, using the

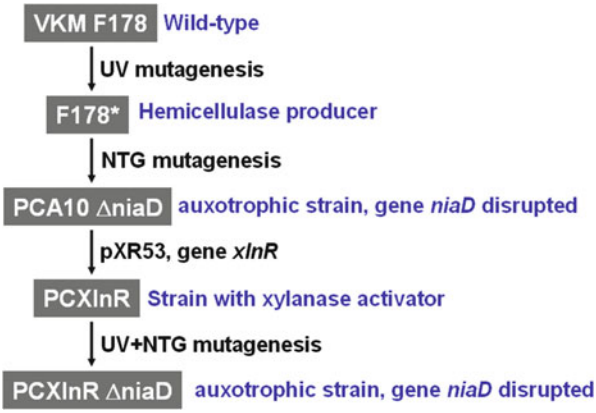

Fig. 2 Partial *Penicillium canescens* strain lineage. The wild-type *Penicillium canescens* F 178 strain was modified by random mutagenesis technology, yielding auxotrophic strain (PCA10 Δ*niaD*). Using recombinant DNA technology, PCA10 Δ*niaD* was transformed with plasmid pXR53 encoding homologous xylanase activator (XlnR). Δ*niaD* refers to gene disruption of nitrate reductase. NTG, N-methyl-N′-nitro-N-nitrosoguanidine

homologous nitrate reductase structural gene *niaD*. Spontaneous *niaD* mutants were isolated after selection for chlorate resistance and characterized further by growth tests and subsequent complementation with the *niaD* gene. The fungus *P. canescens* strain F178 and its niaD^{-} mutant exhibited an increased capability of synthesizing enzymes β-galactosidase (70–80 U/ml) and endo-1,4-β-xylanase (100 U/ml) (Vavilova et al. 2003). The induction of biosynthesis of secreted enzymes endo-1,4-β-xylanase and β-galactosidase in the wild *P. canescens* F178 and mutated *P. canescens* PCA10 strains was investigated. The biosynthesis of these enzymes in both producer strains was mostly induced by arabinose and arabitol, the product of arabinose catabolism. But the difference in the induction of the enzyme biosynthesis was also found out: maximum level of β-galactosidase and xylanase expression was observed at concentrations of arabinose 1 and 10 mM, respectively. Also, it was shown that xylanase expression can be initiated by 1 mM of xylose (Vavilova and Vinetsky 2003). It is assumed that the inductor interacts with the transcriptional activator through the kinase. Transcriptional xylanase activators are important regulatory proteins for the mechanism of transcription start in fungi of the genera *Aspergillus* (van Peij et al. 1998a, b) and *Trichoderma* (Mach and Zeilinger 2003). Therefore, the homologous gene of transcriptional xylanase activator *P. canescens (xlnR)* has been cloned and sequenced; plasmid pXR53 was derived and transformed into the recipient strain *P. canescens* PCA10 ΔniaD. As a result the strain *P. canescens* PCXlnR expressing high level of xylanases in media containing the sugar beet pulp and soybean husks (or oats husks) has been developed. This recombinant strain has been a platform for creation of auxotrophic strain *P. canescens* PCXlnR ΔniaD which was used as a main host strain for a number of recombinant strains and enzyme preparations.

Fig. 3 *Penicillium canescens* host and recombinant strains after transformation (5 days incubation at 30 °C, minimal media supplemented with 10 mM $NaNO_3$, 3×10^7 protoplasts on the both plates)

The enzyme nitrate reductase promotes utilization of nitrate as a sole nitrogen source and probably simultaneously controls transport of amino acids into the cell. Systems of genetical transformation with the nitrate reductase gene (*niaD*) are widely used in *Aspergillus* and related filamentous fungi, because they make it possible to apply direct selection for both mutant and wild-type phenotypes. And although the genetical and biochemical basis of this system were developed for *A. nidulans*, the experimental techniques were easily adapted for *P. canescens* F178 and its derivates (Nikolaev and Vinetski 1998). Results of transformation of host *P. canescens* PCXlnR ΔniaD strain are presented in Fig. 3. Routinely a cotransformation approach is applied where mixtures of the transformation vectors and homologous auxotrophic selection markers in ratio (mkg of DNA) 10:1 are used. Transformation efficiencies typically reach hundreds of transformants per μg of transforming DNA, with cotransformation frequencies of 80 % and higher.

2.2 Identification and Isolation of Strong Promoters for Gene Expression

Strong gene promoters can ensure high-level expression of a gene of interest, which in general leads to high-level biosynthesis of the corresponding gene product. The major extracellular proteins secreted by *P. canescens* strain are β-galactosidase (BGAS, 120 kDa), endo-1,4-β-xylanase (XYL, 30 kDA), and arabinoxylan-furano-hydrolase A (arabinofuranosidase A, ABFA, 70 kDa), constituting up to approximately 10, 20, and 25 % of the secreted enzyme mixture, respectively (Vavilova et al. 2003; Sinitsyna 2002; Patent RU 2001).

The nucleotide sequence of secreted β-galactosidase gene (*bgas) P. canescens* fungus was obtained. The analysis of the nucleotide sequence of the promoter region showed the presence of several potential catabolite repression protein (CREA)-binding sites. The transformants with the increased copy number of the β-galactosidase gene were obtained. The β-galactosidase activity of transformants

grew linearly up with the growth of the copies of the gene until there were 12 per genome (Nikolaev et al. 1999).

The complete gene *xylA* encoding endo-1,4-β-xylanase was also cloned and sequenced. Nucleotide sequences for binding CREA and XlnR were detected in promoter region. Also a set of recombinant strains *P. canescens* PCXlnR displaying seven- to eightfold increase in xylanase activity were created. The fraction of xylanase in most productive strains amounted to 30–50 % of the total secreted protein (Serebryanyi et al. 2002).

Recently, the complete gene *abfA* encoding arabinoxylanfuranohydrolase A was cloned and sequenced. Analysis of nucleotide sequence showed absence of any binding sites for CREA protein. But production of ABFA in fermentation broth of *P. canescens* strain is weaker than the level of XYL and BGAS secretion under the same fermentation conditions. Therefore, the expression of target genes based on *abfA* promoter can be exploited in the case when a minor enzyme(s) needs to be added to the secreted recombinant multienzymatic complexes (Volkov et al. 2010; Volkov 2012).

To be mentioned, inverse PCR method was applied for the cloning of *abfA* full gene. Briefly, the inverse PCR method involves a series of restriction digests and ligation, resulting in a looped fragment that can be primed for PCR from a single section of known sequence. Then, like other Polymerase Chain Reaction processes, the DNA is amplified by the temperature-sensitive DNA polymerase. The process of cloning includes next steps: (1) a target region with an internal section of known sequence and unknown flanking regions is identified; (2) genomic *P. canescens* DNA is digested into fragments of a few kilobases by a usually low-moderate frequency (6–8 kb) cutting restriction enzyme (e.g., HindIII, BamHI, EcoRI, etc.); (3) self-ligation is induced to give a circular DNA product under low DNA concentrations; and (4) PCR is carried out as usual, with primers complementary to sections of the known internal sequence (Siebert et al. 1995; Ochman et al. 1988).

Thus, *xylA*, *bgaS*, and *abfA* promoter-based expression vectors were designed and are now commonly used to drive recombinant gene expression in *P. canescens* host.

2.3 Construction of Expression Vectors and Cloning of Target Genes

Non-replicating vectors PC1, PC2, and PC3 based on three different promoters (Fig. 4) integrate randomly into the *P. canescens* genome. The number of observed integrated gene copies of exogenous DNA per transformant generally varied between 1 and 20 after one transformation round (Nikolaev et al. 1999). As a result of random integration and variation in copy numbers, the expression levels of the target gene varied greatly within a pool of transformants, as was observed before in *A. niger* and *T. reesei* expression systems (Verdoes et al. 1995).

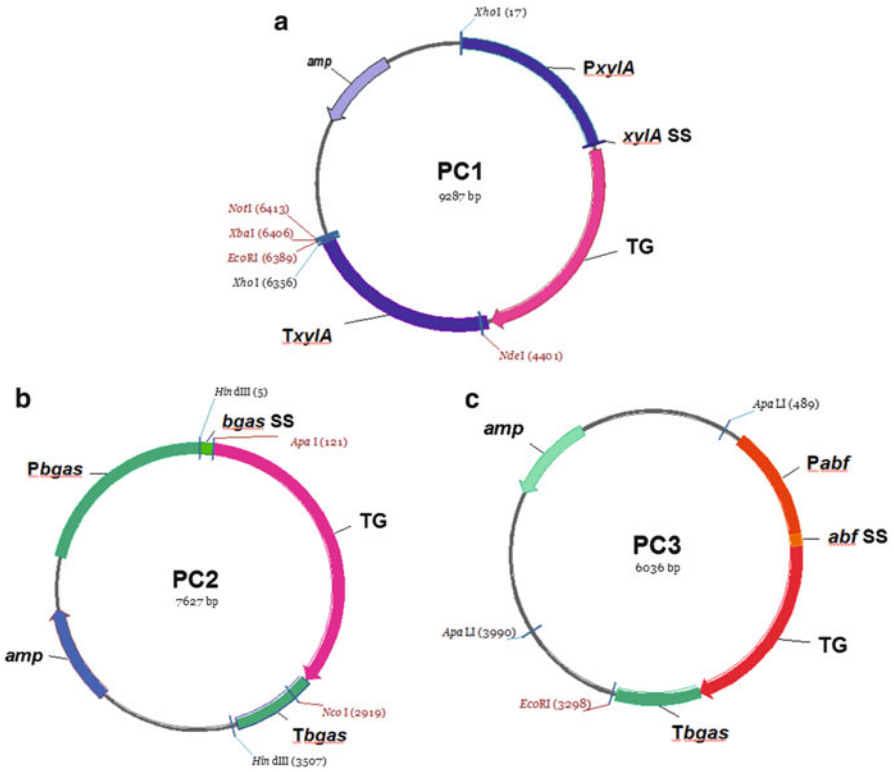

Fig. 4 Schematic representation of the multicopy expression vectors: (**a**) PC1, P*xylA*-promoter region of the *xylA* gene; T*xylA*, terminator region of the *xylA* gene; (**b**) PC2, P*bgas*-promoter region of the *bgaS* gene; T*bgas*, terminator region of the *bgaS* gene; (**c**) PC3, P*abf*-promoter region of the *abfA* gene; T*bgas*, terminator region of the *bgaS* gene; *amp* ampicillin resistance gene, *TG* target gene

In contrast to the traditional method of target genes subcloning by using endonuclease restrictions, ligation-independent cloning (LIC) method was adapted for directional cloning of PCR products to vectors without any endonuclease digestion or ligation reactions (Aslanidis and de Jong 1990). The LIC method takes advantage of the $3' \rightarrow 5'$ exonuclease activity of T4 DNA polymerase to create very specific 12–18 nucleotide single-stranded overhangs in the vector and the insert, so that the vast majority of annealed products consist of the desired molecules. The annealed LIC vector and insert are transformed into competent *E. coli* cells, and covalent bonds are formed at the vector-insert junctions within the cell to yield circular plasmid. Directional cloning of the insert is achieved with minimal nonrecombinant background, and cloning is efficient.

PCR products with complementary overhangs are created by building appropriate 5′ extensions into the primers. The purified PCR products are treated with LIC-qualified T4 DNA polymerase in the presence of the appropriate dNTP to

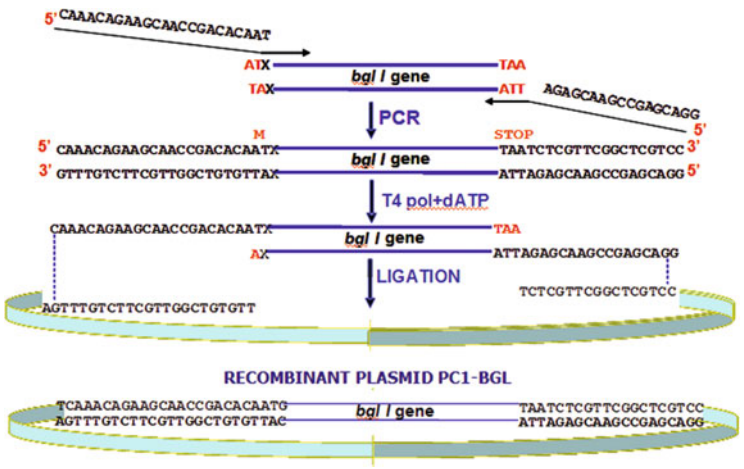

Fig. 5 Scheme of cloning *bglI* gene, encoding *Aspergillus niger* β-glucosidase, to pPC1 vector

generate the specific vector-compatible overhangs. As an example scheme of cloning *bglI* gene, encoding *A. niger* β-glucosidase, to pPC1 vector is presented in Fig. 5. As a result the recombinant plasmid pPC1-BGL was created.

3 *Penicillium canescens* as a Producer of Cellulases

One of the approaches of converting renewable plant biomass into useful products is to produce C6 and C5 sugars by enzymatic hydrolysis followed by their bioconversion to organic acids and derivatives, amino acids, esters, biofuels, and other value added products (Kumar et al. 2008). Effective bioconversion of the plant materials into sugars is affected by multienzyme complex carbohydrases including endoglucanase, cellobiohydrolase, and β-glucosidase (cellobiase). Qualitative and quantitative compositions of the enzymatic complex and the activity of each enzyme determine the effectiveness of its action in the process of hydrolysis of cellulosic substrates. There is an optimum ratio of the abovementioned key enzymes for each particular type of plant material. Optimal composition of enzymatic complex allows to reach the deepest conversion of plant feedstocks and a maximum yield of sugars (Banerjee et al. 2010a, b).

As examples to illustrate the possibilities of fungal *P. canescens* host for the production of high valued heterologous proteins, we investigated the expression of the *cbhI*, *cbhII*, and *eglII* genes from *P. verruculosum* and *bglI* gene from *A. niger* in *P. canescens* PCXlnR ΔniaD host. Obtained enzyme preparations produced by recombinant strains of *P. canescens* possessed heterologous activities of the cellobiohydrolase I (CBHI), cellobiohydrolase II (CBHII), endo-1,4-β-glucanase (EGII), and β-glucosidase (BGL) (Table 1). It is shown that for the most efficient

Table 1 Activities of dry enzyme preparations PC-CBHI, PC-CBHII, and PC-EGII

Enzyme preparation	Activity (units/g preparation)			
	CMC	Avicel	Xylan	PNPG
PC-CBHI	1,287	168	16,005	56
PC-CBHII	819	175	21,489	117
PC-EGII	15,390	220	6,686	38
PC-BGL	184	12	637	1,421
PC-HOST	971	34	23,520	40

PC-BGL and PC-HOST (as a control) toward different substrates—CMC (Na-salt of carboxymethyl cellulose), Avicel (microcrystalline cellulose), xylan (birch wood xylan), and PNPG (p-nitrophenyl-β-D-glucopyranoside)

hydrolysis of microcrystalline cellulose, the optimal ratio of recombinant enzyme preparations in the reaction mixture was of 4:1 of CBHI (or CBHII) to EGII at the total loading of combined enzyme preparation as 10 mg of protein per 1 g of dry mass of a substrate, Fig. 6. The same optimal ratio of recombinant enzyme preparations was demonstrated for the hydrolysis of milling aspen wood—the most common wood feedstock in Russian Federation. It was also proved that a necessary component of the enzyme complex for the hydrolysis of aspen wood hemicellulose matrix was homologous xylanase secreted by the fungus *P. canescens* PCXlnR ΔniaD host (Volkov et al. 2012a)—xylanase activities of recombinant enzyme preparations is given in Table 1.

The hydrolysate of the milled aspen wood obtained using the most efficient enzyme mixture containing 8 mg/g of PC-CBH I (or PC-CBH II) and 2 mg/g of PC-EGII was assayed with HPLC (Table 2). Glucose (35.2–38.5 g/l), xylose (8.5–9.2 g/l), and cellobiose (2.6–3.1 g/l) were found as main products in the reaction mixture (maximal concentration of RS achieved was 62.1 g/l when initial concentration of substrate in the reaction mixture was 100 g/l).

The data in Table 2 completely correlates with the composition of the main polysaccharides of aspen wood (Kumar et al. 2008). It should be noted that the presence of a minor amount of cellobiose in the medium (2.5–3.0 g/l) probably indicates the insufficient amount of PC-BGL preparation (40 units/g of dry substrate) in the reaction mixture.

The example of cellulase application given above is dealing with their ability to aggressive destruction of different renewable feedstock and with the conversion of insoluble cellulose-containing substrates to soluble C6 and C5 sugars (which could be defined as "saccharolytic" activity).

We can give here an example of "topolytic" activity of cellulases (which means the capability of the enzyme to run reactions on the surface of insoluble substrate without deep destruction of cellulose structure), particularly of endo-β-1,4-glucanases (EGs). These enzymes have hydrolytic activities toward polyglucans containing β-1,4-glycosidic bonds, which include cotton and wood cellulose, different soluble cellulose derivatives, β-glucans of oat and barley, and other polysaccharides. Hydrolysis of cellulose by EGs occurs by endo-depolymerization mechanism.

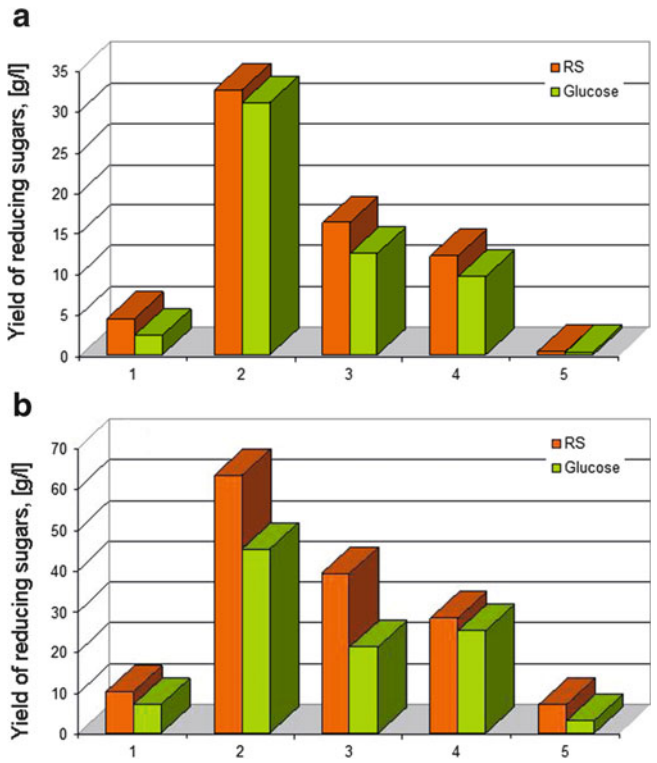

Fig. 6 Yield of reducing sugars (RS) and glucose in the hydrolysis of microcrystalline cellulose (**a**) and milled aspen wood (**b**). Hydrolysis conditions: 50 °C, pH = 5.0, stirring at 250 rpm, and [S] = 100 g/l (dry weight); hydrolysis time was 48 h. The dosage of enzymatic preparations was 10 mg of total protein per 1 g of a dry substrate; the reaction mixture was supplemented with cellobiase in an amount equivalent to 40 CBU per 1 g of dry substrate. (1) PC-HOST; (2) mixture of PC-CBHI and PC-EGII, in ratio 8:2 (mg, loaded by protein), respectively; (3) PC-CBHI; (4) PC-EGII, (5) PC-BGL

Table 2 Sugar composition of aspen wood hydrolysate after 48 h of hydrolysis at the total loading of mixed enzyme preparations as 10 mg of protein per 1 g of dry substrate

		Sugar concentration (g/l)		
	RS	Glucose	Cellobiose	Xylose
PC-CBHI + PC-EGII (4:1) + PC-BGL (40 units/g)	62.1 ± 3.1	38.5 ± 1.3	3.10 ± 0.03	9.2 ± 0.1
PC-CBHII + PC-EGII (4:1) + PC-BGL (40 units/g)	56.3 ± 2.3	35.2 ± 1.1	2.60 ± 0.15	8.40 ± 0.08
PC-HOST + PC-BGL (40 units/g)	26.1 ± 1.1	17.8 ± 0.5	2.5 ± 0.2	8.40 ± 0.08

EG3 from *P. verruculosum* relates to glycosyl hydrolase 12 family (GH12) and shows quite high denim washing (biostoning) capability—among other EGs this shows the highest washing performance (the ability to remove indigo from cellulose fibers of denim), and, at the same time, since this enzyme has no cellulose-binding module (CBM) and because of that could not bind strongly to cellulose, EG3 does not damage cellulosic fibers and does not decrease the mechanical firmness of fabrics. At the same time EG3 has hydrophobic clusters on the surface of the molecule capable to bind indigo, which along with low adsorbability of this enzyme prevent redeposition of indigo on denim and lead to low backstaining (Gusakov et al. 2000). So from the point of view of biotechnological importance, EG3 seems to be a useful enzyme for biostoning processes—it provides high washing performance and abrasive activity but low backstaining without significant damaging of textile matrix. In addition to biostoning capability, EG3 possesses biopolishing activity and is able to remove pills and fuses from textile surface.

Gene *egl3* encoding *P. verruculosum* EG3 was cloned under the control of *bgas* and *xylA* promoters (Patent RU 2001). Expression plasmids were cotransformed to *P. canescens* PCA10 ΔniaD host separately. Panels of enzyme preparations PCB-EG3 and PCX-EG3 were analyzed, and increasing of CMCase activity up to 200 U/mg protein was detected in PCX-EG3-2 enzyme preparation (pH = 4.5, T = 50 °C, Somogyi-Nelson). Results of textile treatment, biopolishing of cotton fabrics (Fig. 7a, b), and biostoning of denim (Fig. 7c, d) by enzyme preparation PCX-EG3-2 in comparison to untreated fabrics are presented.

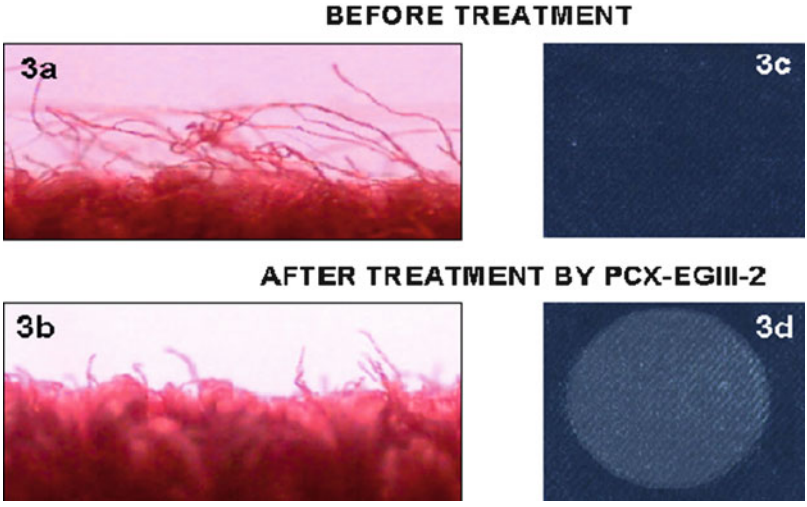

Fig. 7 Treatment of cotton fabric (**b**) and denim (**d**) swatches by PCX-EG3 (treatment conditions: dosage—5 CMCase units per 1 g of fabric, 1 h, pH = 4.5, T = 50 °C). (**a**) and (**c**) untreated fabrics

4 *Penicillium canescens* as a Producer of Other Carbohydrases

One of the approaches to the creation of multienzyme preparations possessing multiple heterologous activities could be cotransformation of the host strain in several expression plasmids simultaneously. Choose the optimal ratio of target and transforming DNA can produce recombinant strains with desired properties and, thus, eliminate the economically inefficient step of enzyme preparation mixing.

It is known that the main technological problem of fruit-berry industry is a low yield of juice and its clarification. Processes of filtration and pressing are often hindered in case of using of fruits and berries because of high content of pectic substances and other polysaccharides. These problems can be solved by using technology of the preprocessing of berry-fruit mash using new multienzyme complexes which converts plant cell wall polysaccharides (such as cellulose, hemicellulose, and pectin) to shorter oligosaccharides decreasing viscosity of juices and increasing the yield of final products (Volchok et al. 2012).

Secreted protein profiles of the culture fluids of the host strain *P. canescens* PCXlnR ΔniaD and recombinant strain PC-PEB-9 are shown in Fig. 8. Briefly, three separate plasmids with gene *pelA*, encoding homologous pectin lyase A (PEL) from *P. canescens*; gene *bgl1*, encoding β-glucosidase (BGL) from *A. niger*; and

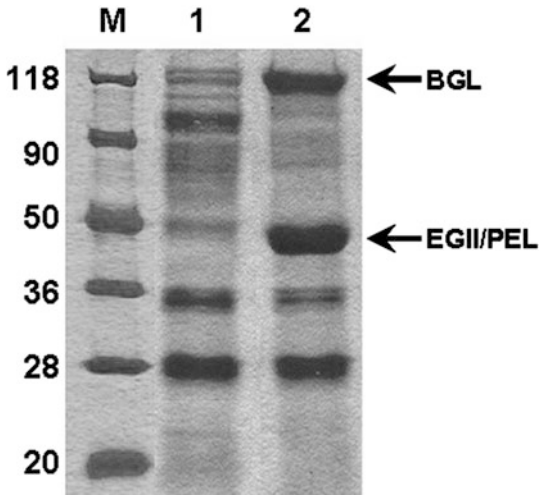

Fig. 8 Secreted protein profiles of *P. canescens* PCXlnR ΔniaD host strain and recombinant of *P. canescens* PEB-9 strain. Spores were used to inoculate 750 mL shake flasks containing 100 mL production medium with 2 % sugar beet pulp and 4.5 % soy husks. Mycelia were grown for 120 h at 30 °C at 250 rpm. Samples were withdrawn from the culture medium of the following strains and analyzed on SDS-PAGE gel. Lane 1, Control, *P. canescens* PCXlnR host strain; Lane 2, *P. canescens* PEB-9 with BGL (120 kDa), EG2 (40 kDa), and PEL (40 kDa); Lane M, molecular mass standard (Da)

Table 3 Specific activities of dry enzyme preparations PC-PEB-9 and PC-HOST (as a control) toward different substrates—CMC (Na-salt of carboxymethyl cellulose), xylan (birch wood xylan), PNPG (p-nitrophenyl-β-D-glucopyranoside), and citrus pectin

Enzyme preparation	Activity (units/g preparation)			
	CMC	Xylan	PNPG	Citrus pectin
PC-PEB-9	5,550 ± 298	13,660 ± 864	960 ± 57	2,600 ± 127
PC-HOST	820 ± 25	27,280 ± 1,564	35 ± 3	25 ± 2

gene *eglII*, encoding EG2 from *P. verruculosum* were created and cotransformed to *P. canescens* PCXlnR ΔniaD host strain in ratio 3:3:3 (mkg of each plasmid). Also cotransforming plasmid pSTA10 (1 mkg of DNA) with homologous selective marker gene *niaD* was added to target plasmid cocktail. As a result of primary screening, recombinant strain PC-PEB-9 was chosen for further experiments (Bushina 2012).

The composition of multienzyme preparation PC-PEB-9 and enzyme preparation PC-HOST based on *P. canescens* PCXlnR host strain was determined using Fast Protein Liquid Chromatography (FPLC) and data of enzymatic activities of BGL, PEL, xylanase, and EG2 toward PNPG, citrus pectin, birch wood xylan, and CMC, respectively (Table 3). Enzyme preparation PC-HOST contained 30 % of xylanases. Enzyme preparation PC-PEB-9 consisted of 15 % xylanase, 11 % PEL, 18 % EG2, and 12 % BGL (Bushina et al. 2012).

Enzyme preparation PC-PEB-9 was applied to different raw fruit and berry materials in dosage 0.05 % from mass of the wet substrates. Enzymatic treatment was carried out for 3 and 6 h at 40 °C. Yield of pressed juices, its viscosity, yield of dry substances, ascorbic acid, and polyphenolic substances content as well as antioxidant activity were analyzed in pressed juices. High efficiency of processing of such hard-to-process raw materials as berries and strawberry was demonstrated during experiments. The yield of briar outcoming juice was increased by about 60–200 % as a result of enzymatic treatment (by PC-PEB-9) compared to control samples without enzymatic treatment. Yield of hawthorn outcoming juice was increased by 20–30 %; antioxidant capacity of juice was by 4.3 times higher compared to samples obtained without enzymatic treatment (Volchok et al. 2014).

5 *Penicillium canescens* as a Producer of Inulinases

The unique chemical composition of Jerusalem artichoke (topinambour) makes it valuable food for diabetics and gourmets, as feed stuff, e.g., for piglet breeding, and as a medicinal plant. The content of the polysaccharide inulin reaches up to

Table 4 Protein content (Cp) and specific activities of dry enzyme preparations PC-EXIN, PC-ENIN, and PC-HOST (as a control) toward IT (inulin from topinambour) and xylan (birch wood xylan)

Enzyme preparation	Cp (mg/g preparation)	Activity (units/g preparation)	
		IT	Xylan
PC-ENIN	237 ± 19	2,844 ± 159	2,844 ± 191
PC-EXIN	245 ± 21	21,315 ± 1,500	19,110 ± 1,400
PC-HOST	336 ± 29	16.7 ± 1.5	26,880 ± 1,500

20 % on wet weight and more than 70 % in terms of dry matter in mature tubers of vegetable forms of topinambour.

The existing technology for the processing of topinambour tubers yields in syrups and powders with a high content of inulin and high molecular weight (>1,800 Da) fructooligosaccharides (FOS). The use of inulinase enzymes for high fructose syrups from inulin-containing material has several advantages over acid-catalyzed hydrolysis. Enzymatic hydrolysis occurs at sufficiently low temperatures, while unwanted (toxic) by-products of sugar decomposition are not produced. A complete enzymatic hydrolysis of topinambour tubers results in high concentrations of monosaccharides, mostly fructose, for dietary products. The cleavage of inner glycosidic bonds of inulin with specific endo-inulinases results in a high FOS content to be used in prebiotic products and functional foods.

Efficient host-vector systems based on filamentous *P. canescens* fungi were exploited for cloning of *inu1* and *inuA* genes from *Aspergillus* sp. encoding exo- and endo-inulinases, respectively. As a result of primary screening, a series of recombinant PC-EXIN and PC-ENIN strains were selected as high-level producers of target exo- and endo-inulinase activities, respectively. In fact, there were obtained producer strains capable to secrete 80 % of total extracellular protein pool as heterologous exo-inulinase and 15–20 % as endo-inulinase. Data of enzymatic activities and protein content of the recombinant enzyme preparations PC-EXIN and PC-ENIN and PC-HOST are presented in Table 4 (Volkov et al. 2012b).

Hydrolysis of inulin was carried out through enzyme preparation PC-ENIN in 1 mg dosage per 1 g of inulin, isolated from Jerusalem artichoke. Samples of hydrolysates were taken in 30 min and 3 h after start of reaction and subjected to HPLC analysis. Results of experiment characterizing the sugar composition in samples are shown in Fig. 9. Based on these results it is evident that FOS with varying degrees of polymerization (DP) are obtained and maximum concentration of FOS is reached after 3 h of the hydrolysis reaction.

Hydrolysis of Jerusalem artichoke syrup by enzyme preparation PC-EXIN was carried out, and sugar composition was also analyzed by HPLC. Chromatographic profile of FOS contained in the syrup from the tubers of Jerusalem artichoke is

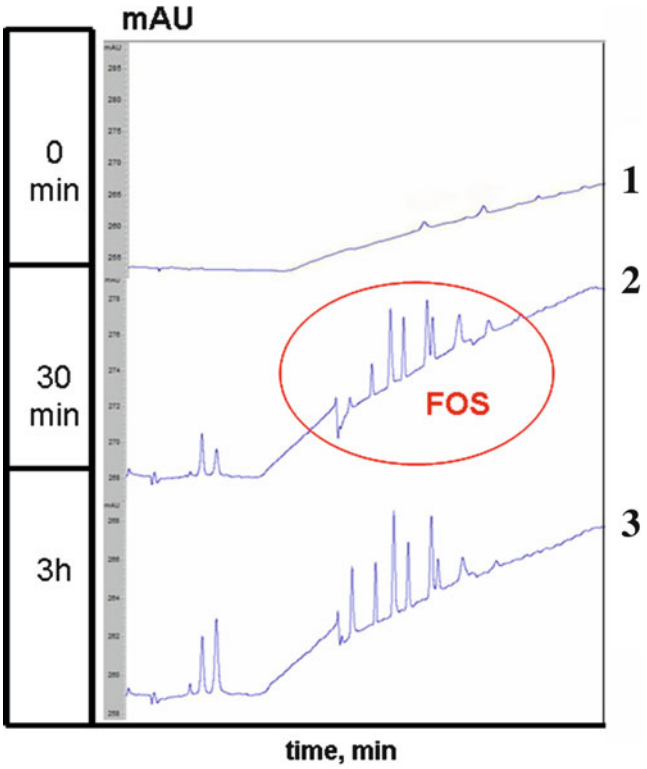

Fig. 9 The composition of the hydrolysis products IT by PC-ENIN using HPLC method. Hydrolysis conditions: 50 °C, pH = 5.0, stirring at 250 rpm, and [S] = 100 g/l (dry weight); hydrolysis time was 3 h. The dosage of enzymatic preparations was 1 mg of total protein per 1 g of a dry substrate. (1) *line* responds to sugar composition of untreated inulin; (2) *line* responds to sugar composition after 30 min; (3) *line* responds to sugar composition after 3 h

presented in Fig. 10. Observed chromatographic peaks corresponds to FOS (DP of 3–42), Fig. 10a. The content of glucose and fructose is sufficiently small. As shown in Fig. 10b, full bioconversion of FOS to fructose (~95 %) and glucose (~5 %) occurs after 3 h.

Finally, hydrolysis of topinambour tubers by new enzyme preparations PC-ENIN, PC-EXIN, and PC-HOST (as a control) was carried out. Enzyme preparations were taken in dosage 5 mg per 1 g of dry mass of tubers. It was shown that maximum yield of reducing sugars was observed in the case of PC-EXIN preparation after 3 h, and the main product of hydrolysis was fructose (Fig. 11).

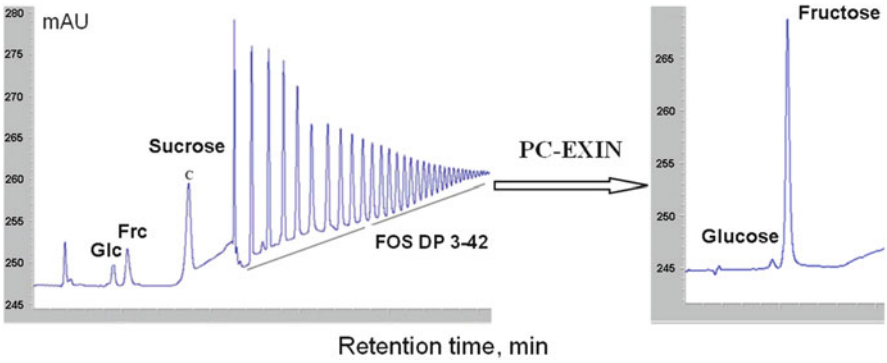

Fig. 10 Conversion of Jerusalem artichoke syrup by the enzyme preparation PC-EXIN. (**a**) Chromatogram corresponds to FOS composition in the intact Jerusalem artichoke syrup; (**b**) chromatogram corresponds to sugar composition after 3 h hydrolysis. Hydrolysis condition: 50 °C, pH = 5.0, stirring at 250 rpm, PC-EXIN dosage—0.5 mg per 1 g of syrup (dry weight); hydrolysis time was 3 h

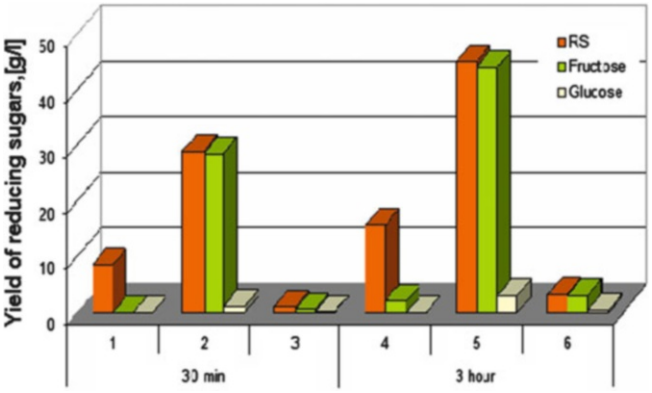

Fig. 11 Yield of reducing sugars (RS), fructose and glucose in the hydrolysis of Jerusalem artichoke tubers. Hydrolysis conditions: 50 °C, pH = 5.0, stirring at 250 rpm, and [S] = 100 g/l (dry weight); hydrolysis time was 30 min and 3 h. The dosage of enzymatic preparations was 5 mg of total protein per 1 g of a dry substrate; 1 and 4 PC-ENIN after 30 min (1) and 3 h (4); 2 and 5 PC-EXIN after 30 min (2) and 3 h (5); 3 and 6 PC-HOST after 30 min (3) and 3 h (6)

Conclusions

The filamentous fungus *Penicillium canescens* was developed into a mature protein production platform. Expression and transformation systems of *Penicillium canescens* allow to apply host strains for production of industrially relevant enzyme preparations with useful properties. *Penicillium canescens* strain characteristics have been a proven starting point for the development of

(continued)

different recombinant strains producing enzymes and enzyme mixtures for various cost-effective industrial applications. The properties of *Penicillium canescens* provide an alternative for traditional fungal protein production hosts, such as *Aspergillus* and *Trichoderma*.

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Microbial Life on Green Biomass and Their Use for Production of Platform Chemicals

Petra Schönicke, Robert Shahab, Rebekka Hamann, and Birgit Kamm

Contents

Abstract This chapter describes the basics for the development of future biotechnology processes for the production of platform chemicals. Microbial life on green plants and harvested plants is very dynamic. Identified microorganisms on green plants and in silage as described in literature are listed in tables. But almost weekly new microorganisms are discovered, which constitute the site of a great variety of so far unknown metabolic pathways. Some microorganisms and their metabolic pathways to six organic acids used as platform chemicals and applications currently and in future are described.

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Abbreviations

C Carbon
MOs Microorganisms

1 Introduction

All aerial parts of plants which perform photosynthesis and are usually in the growth phase can be called green biomass. Green biomass contains mainly carbohydrates, proteins, fibres, flavourings, colourings, vitamins, hormones, amino acids and enzymes, but less starch and lignin. The primary production of photosynthesis in green plants, such as C3 species in temperate climates, can yield up to 20 t dry matter and 4 t of proteins per ha per year, while C4 species in tropical climates can produce 80 t of dry matter and 6 t of proteins (Carlsson 1985). Economically, interesting are mainly alfalfa, clover and grass from permanent grassland and immature crops, but also green parts of plants, such as leaves as a by-product of the harvest of ripe crops (Kamm et al. 2006).

The area of green cropland cultivation in Europe (basis: 15 member states without new member states since a comprehensive European database on grassland areas is not available) amounts to 45 million ha and therewith to 35 % of the agricultural cropland. Based on an average yield of 10 t dry matter per hectare and year, 450 million tons of dry matter is produced annually by the 15 EU member states (FAO 2012). In Europe, the most important forage crop is alfalfa (Lucerne) due to its ability to absorb nitrogen from air and to enrich it in the soil. Alfalfa is cultivated on about 32 million hectares in the mentioned 15 EU member states. In the USA, intensive research in the field of biorefineries has been going on over the past 10 years. The Alfalfa New Products Initiative (ANPI), to which belong five of the states, aims at the intensification of the cultivation and use of Alfalfa. Thereby, known technologies, implemented at large scale only in France, like dehydration and fractionation are utilised. The high protein content and the favourable amino acid pattern make alfalfa exceptionally interesting for feedstuff production and research and development efforts on water-soluble proteins that are about 15 % of the average protein content (Lamsal 2004). The fraction of water-soluble carbohydrates form an important C-source among the nonstructural carbohydrates for microbial use by plant-associated bacteria (Seyfarth and Müller 1997). In harvested green biomass, one can find all microorganisms which already colonised the plant during growth and also those who ended up in the biomass during harvesting.

2 Microorganisms on Green Plants

The aerial parts of plants like leaves, caulis, buds, blossoms and fruits which are a habitat for microorganisms are called 'phyllosphere' (Whipps et al. 2008). Besides the soil, they offer a large habitat for bacteria, yeasts, fungi and protists, called 'epiphytic microorganisms', whereas the bacteria form the biggest group of these microorganisms (Lindow and Brandl 2003; Ruppel and Müller 2012). The phyllosphere is a very dynamic habitat because of strong fluctuating biotic and abiotic conditions of space and time (Kinkel 1997). A very high selective pressure exists because of the limited nutrient supply in many areas on the leaves, the UV-radiation during day time and often a prevalent dryness during the main growing period (in Central Europe) (Thompson et al. 1995; Andrews and Harris 2000). Additional population dynamics are generated during the ageing of the plants because of different survival strategies of the epiphytic microorganisms (Kinkel 1997). Bacteria like to live, for example, in cell agglomerations and are therefore more resistant against dryness and UV-radiation (Lindow and Brandl 2003).

In an analysis about quantity and biodiversity on leaves in a studied area at night, a higher individual number was determined, given that at night there is no UV-radiation and moisture is increasing because of dew production (Thompson et al. 1995). Also the point of time and the frequency of the harvest influence the composition of microorganism populations (Kinkel 1997). Late cut grass has a higher population concentration of heterotrophic bacteria as well as filamentous fungi, while the frequency of yeast and bacteria in the family of Micrococcaceae was always varying strongly (Behrendt et al. 2004).

Under lab conditions, only a few of the existing microorganism can be cultivated (Whipps et al. 2008; Müller and Ruppel 2014). With modern methods of the gene sequencing, which are culture independent, a significantly higher number of microorganism species can be determined, which are mostly unknown so far (Yang et al. 2001; Whipps et al. 2008). In Table 1, some of the identified microorganisms in the phyllosphere are listed alphabetically.

3 Microorganisms and Chemical Compounds in Silage

The production of silage for conservation of green fodder for the feeding of livestock during winter or biogas production has a long tradition in the agriculture of many countries. Extensive knowledge exists about techniques which contribute to a preferably optimal silage fermentation (Dogi et al. 2013). The requirements are a moisture content of the harvested plants of approximately 35 %, a sufficient content of fermentable sugars for lactic acid bacteria and solid compression for the reduction of trapped air (Driehuis and Oude Elferink 2000; Duniére et al. 2013). A good fodder quality can be obtained, if a strong pH-value reduction is achieved

Table 1 Some of the identified MOs of the phyllosphere are listed alphabetically

Microorganism	Plants, parts of plants	References
Acidobacteria	*Thlaspi geosingense*	Idris et al. (2004)
Acinetobacter haemolyticus	Citrus Valencia leaf	Yang et al. (2001)
Acinetobacter sp.	Citrus Valencia leaves	Yang et al. (2001)
Acremonium	Long-lived tropical leaves	Thompson et al. (1993), Inacio et al. (2002)
Actinobacteria	*Thlapsi geosingense*, *Campomanesia xanthocarpa*, *Capsicum annum*, *Solanum tuberosum*, *Crocus albiflorus*	Idris et al. (2004), Lambais et al. (2006), Rasche et al. (2006a, b), Reiter and Sessitsch (2006)
Agrobacterium rubi	Sugar beet, short-term dynamics	Thompson et al. (1995)
Alternaria	Long-lived tropical leaves	Thompson et al. (1993), Inacio et al. (2002)
Alternaria	Dormant spores, growth on healthy, intact, non-senescent leaves is relatively rare	Andrews et al. (1987), Dickinson (1967, 1976), Wildman and Parkinson (1979)
α-Proteobacteria	*Thlapsi geosingense*, *Trichilia catigua*, *Trichilia claussenii*, *Campomanesia xanthocarpa*, *Zea mays*, *Capsicum annum*, *Solanum tuberosum*, *Crocus albiflorus*	Idris et al. (2004), Lambais et al. (2006), Kadivar and Stapleton (2003), Rasche et al. (2006a, b), Reiter and Sessitsch (2006)
Arthrobacter atrocyaneus	Sugar beet, short-term dynamics	Thompson et al. (1995)
Arthrobacter globiformis	Sugar beet, short-term dynamics	Thompson et al. (1995)
Arthrobacter oxydans	Sugar beet, short-term dynamics	Thompson et al. (1995)
Arthrobacter protophormiae	Sugar beet, short-term dynamics	Thompson et al. (1995)
Aspergillus	Long-lived tropical leaves	Thompson et al. (1993), Inacio et al. (2002)
Aureobacterium saperdae	Sugar beets, short-term dynamics	Thompson et al. (1995)
Aureobasidium pullulans	Citrus Valencia leaf	Yang et al. (2001)
Aureobasidium	Dormant spores, growth on healthy, intact, non-senescent leaves is relatively rare	Andrews et al. (1987), Dickinson (1967, 1976), Wildman and Parkinson (1979)
Aureobasidium pullulans	*Acer platanoides*, *Hippophae rhamnoides*	Breeze and Dix (1981)
Bacillus	Heterotrophic	Wipat and Harwood (1999)
Bacillus pumilus	Valencia orange leaf, citrus Valencia leaf	Yang et al. (2001)
Bacillus subtilis		Kong et al. (1997)
Bacillus thuringiensis	Leaves, grass foliage	Hansen et al. (1998), Damgaard et al. (1998)

(continued)

Table 1 (continued)

Microorganism	Plants, parts of plants	References
Bacterial groups		Thompson et al. (1993), Inacio et al. (2002)
Bacteroidetes	*Thlapsi geosingense*, *Trichilia catigua*, *Trichilia claussenii*, *Campomanesia xanthocarpa*, *Zea mays*, *Solanum tuberosum*	Idris et al. (2004), Lambais et al. (2006), Kadivar and Stapleton (2003), Rasche et al. (2006a)
Burkholderia cepacia	Pathogen	Balandreau et al. (2001), Govan et al. (1996)
Cladosporium	Long-lived tropical leaves	Thompson et al. (1993), Inacio et al. (2002)
Cladosporium	Dormant spores, growth on healthy, intact, non-senescent leaves is relatively rare	Andrews et al. (1987), Dickinson (1967, 1976), Wildman and Parkinson (1979)
Clostridia	*Miscanthus sinensis*	Miyamoto et al. (2004)
Clostridium bifermentans	Citrus Valencia leaf	Yang et al. (2001)
Cryptococcus	Active coloniser, growth on healthy, intact, non-senescent leaves is relatively rare	Thompson et al. (1993), Inacio et al. (2002), Glushakova and Chernov (2004), Fokkema et al. (1979)
Cyanobacteria	*Campomanesia xanthocarpa*, *Crocus albiflorus*	Lambais et al. (2006), Reiter and Sessitsch (2006)
Cyanobacteria Nostoc	Autotrophic	Andrews and Harris (2000)
Cytospora	Dormant spores, growth on healthy, intact, non-senescent leaves is relatively rare	Andrews et al. (1987), Dickinson (1967, 1976), Wildman and Parkinson (1979)
Dendrophoma	Dormant spores, growth on healthy, intact, non-senescent leaves is relatively rare	Andrews et al. (1987), Dickinson (1967, 1976), Wildman and Parkinson (1979)
Desulfurominas choroethenica	Citrus Valencia leaf	Yang et al. (2001)
Enterobacter agglomerans	Citrus Valencia leaf	Yang et al. (2001)
Enterobacter asburiae	Citrus Valencia leaf	Yang et al. (2001)
Enterococcus faecalis *Ent. mundtii* *Ent. casseliflavus* *Ent. faecium* *Ent. sulfureus*	Bowel pathogen, bacteriocins on grass	Ott et al. (2001)
Epicoccum	Dormant spores, growth on healthy, intact, non-senescent leaves is relatively rare	Andrews et al. (1987), Dickinson (1967, 1976), Wildman and Parkinson (1979)
Erwinia (Pantoea) ssp.		Lindow and Brandl 2003
Erwinia amylovora	Flowers, causer of fire blight	Johnson and Stockwell (1998), Lindow et al. (1996)
Erwinia amylovora	Citrus Valencia leaf	Yang et al. (2001)
Erwinia herbicola	Sugar beets, short-term dynamics	Thompson et al. (1995)

(continued)

Table 1 (continued)

Microorganism	Plants, parts of plants	References
Erwinia herbicola	Citrus Valencia leaf	Yang et al. (2001)
Erwinia rhapontici	Sugar beets, short-term dynamics	Thompson et al. (1995)
Erwinia rhapontici	Citrus Valencia leaf	Yang et al. (2001)
Escherichia coli	Corn, beans, coriander	Brandl and Mandrell (2002), O'Brien and Lindow (1989)
Firmicutes	*Thlapsi geosingense*, *Campomanesia xanthocarpa*, *Zea mays*, *Capsicum annum*, *Solanum tuberosum*, *Crocus albiflorus*	Idris et al. (2004), Lambais et al. (2006), Kadivar and Stapleton (2003), Rasche et al. (2006a, b), Reiter and Sessitsch (2006)
Fluorescent pseudomonads	Heterotrophic	Andrews and Harris (2000)
γ-Proteobacteria	*Thlapsi geosingense*, *Trichilia catigua, Trichilia claussenii*, *Campomanesia xanthocarpa*, *Zea mays*, *Capsicum annum*, *Solanum tuberosum*, *Crocus albiflorus*	Idris et al. (2004), Lambais et al. (2006), Kadivar and Stapleton (2003), Rasche et al. (2006a, b), Reiter and Sessitsch (2006)
Hydrogenophaga pseudoflora	Sugar beet, short-term dynamics	Thompson et al. (1995)
Lewia infectoria	Citrus Valencia leaf	Yang et al. (2001)
Marinobacter hydrocarbonoclasticus	Citrus Valencia leaf	Yang et al. (2001)
Methylobacterium mesophilicum A47	*Lolium perenne*	Austin and Goodfellow (1979), Green and Bousfield (1983)
Methylobacterium phyllosphaerae CBMB27	*Oryza sativa* 'DongJin'	Madhaiyan et al. (2009)
Methylobacterium platani PMB02	*Platanus orientalis*	Kang et al. (2007)
Methylobacterium spp.	Cytokinins	Holland et al. (2002)
Microbacterium lacticum	Sugar beet, short-term dynamics	Thompson et al. (1995)
Micrococcus kristinae	Sugar beet, short-term dynamics	Thompson et al. (1995)
Micrococcus roseus	Sugar beet, short-term dynamics	Thompson et al. (1995)
Microsphaeropsis	Dormant spores, growth on healthy, intact, non-senescent leaves is relatively rare	Andrews et al. (1987), Dickinson (1967, 1976), Wildman and Parkinson (1979)
Morchella esculenta	Citrus Valencia leaf	Yang et al. (2001)
Mucor	Long-lived tropical leaves	Thompson et al. (1993), Inacio et al. (2002)
Pantoea agglomerans	*Gypsophila paniculata*	Manulis et al. (1998), Brandl and Mandrell (2002)

(continued)

Table 1 (continued)

Microorganism	Plants, parts of plants	References
Penicillium	*Hippophae rhamnoides*, long-lived tropical leaves	Thompson et al. (1993), Inacio et al. (2002)
Pseudomonas aeruginosa	Pathogen	Cho et al. (1975)
Pseudomonas chlororaphis	Cilantro (coriander)	Brandl and Mandrell (2002)
Pseudomonas fluorescens A, B, C, F, G	Sugar beet, short-term dynamics	Thompson et al. (1995)
Pseudomonas fluorescens	Presence of a functional type III secretion pathway	Preston et al. (2001)
Pseudomonas oleovorans	Citrus Valencia leaf	Yang et al. (2001)
Pseudomonas putida	Sugar beet, presence of a functional type III secretion pathway	Preston et al. (2001), Thompson et al. (1995)
Pseudomonas putida	Citrus Valencia leaf	Yang et al. (2001)
P. syringae pathovars atrofaciens, glyciniae, lachrymans, morsprunorum, savastanoi fraxinus, savastanoi oleae, syringae, tabaci, targetes	Sugar beet, short-term dynamics	Thompson et al. (1995)
Pseudomonas spp.	Surfactants (increase the wettability of leaf surfaces)	Bunster et al. (1989)
Pseudomonas syringae	Bean leaves, sugar beet leaves, alginate, ice activity	Brandl and Mandrell (2002), O'Brien and Lindow (1989), Kinkel (1997)
Pseudomonas syringae pv. syringae	Snap bean, syringomycin (toxin, cell lysis)	Hutchison et al. (1995), Uppe et al. (2003), Quigley and Gross (1994)
Pseudomonas tolaasii	Tolaasin	Hutchison and Johnstone (1993)
Rhodosporidium	Active coloniser, growth on healthy, intact, non-senescent leaves is relatively rare	Fokkema et al. (1979)
Rhodotorula		Thompson et al. (1993), Inacio et al. (2002), Glushakova and Chernov (2004)
Salmonella enterica	Corn, beans, cilantro (coriander)	Brandl and Mandrell (2002), O'Brien and Lindow (1989)
Salmonella enterica serovars	Lettuce	Klerks et al. (2007)
Serratia plymuthica	Sugar beets, short-term dynamics	Thompson et al. (1995)
Sphingomonas adhaesiva	Citrus Valencia leaf	Yang et al. (2001)
Sporobolomyces		Thompson et al. (1993), Inacio et al. (2002), Glushakova and Chernov (2004)

(continued)

Table 1 (continued)

Microorganism	Plants, parts of plants	References
Sporobolomyces		Fokkema et al. (1979)
Staphylococcus haemolyticus	Sugar beet, short-term dynamics	Thompson et al. (1995)
Staphylococcus simulans	Sugar beet, short-term dynamics	Thompson et al. (1995)
Unclassified organism	Citrus Valencia leaf	Yang et al. (2001)
Uncultured bacterium	Citrus Valencia leaf	Yang et al. (2001)
Uncultured delta proteobacterium	Citrus Valencia leaf	Yang et al. (2001)
Unidentified Cytophagales	Valencia orange leaf, citrus Valencia leaf	Yang et al. (2001)
Vibrio parahaemolyticus	Polar flagellum, which is surface induced, acts as a sensory tactile device for the microbe	McCarter et al. (1992), McCarter and Silverman (1989)
Xanthomonas campestris pv. campestris	*Brassica campestris* leaves	Kuan et al. (1986)
Xanthomonas campestris pv. undulosa	Wheat leaves	Duveiller (1994)
Xanthomonas maltophilia	Sugar beet, short-term dynamics	Thompson et al. (1995)
Xanthomonas phaseoli	Bean leaves	Weller and Saettler (1980)
Xanthomonas phaseoli fuscans	Bean leaves	Weller and Saettler (1980)
β-Proteobacteria	*Thlapsi geosingense*, *Trichilia catigua*, *Trichilia claussenii*, *Campomanesia xanthocarpa*, *Zea mays*, *Capsicum annum*, *Solanum tuberosum*, *Crocus albiflorus*	Idris et al. (2004), Lambais et al. (2006), Kadivar and Stapleton (2003), Rasche et al. (2006a, b), Reiter and Sessitsch (2006)

through fast lactic acid fermentation during the first 2 days of the ensilage and if the silage also stays under complete air exclusion during further storing (Driehuis and Oude Elferink 2000; Shao et al. 2005). In case the silage is produced without inoculation, the ensiling is a natural fermentation process, in which competition takes place between epiphytic microorganisms (Li and Nishino 2013).

A compilation of identified microorganisms in silage in alphabetical order is included in Table 2.

The most famous microorganism groups in silage are lactic acid bacteria Enterobacteria, Clostridia and some Bacillus species (Hafner et al. 2013) (see also chapter Microorganisms for Production of Lactic Acid and Organic Lactates). Pre-ensiled crop is an excellent start material for the production of lysine (see chapter Microorganisms for Biorefining of Green Biomass).

Besides lactic acid in silages, other organic acids can be found, larger quantities of acetic acid but also propionic acid, butyric acid, isobutyric acid and isovaleric

Table 2 Identified MOs in silage are listed alphabetically

Microorganism	Products and impacts	References
Absidia		Driehuis and Oude Elferink (2000)
Acetobacter		Duniére et al. (2013)
Acetobacter pasterianus		Wang et al. (2014)
Acinetobacter sp.	Pre-ensiled crop	Li and Nishino (2013)
Arthrinium		Driehuis and Oude Elferink (2000)
Aspergillus		Driehuis and Oude Elferink (2000)
Aspergillus fumigatus	Mycotoxin	Driehuis and Oude Elferink (2000)
Aspergillus ochraceus	Ochratoxin A	Duniére et al. (2013)
Aspergillus sp.		Duniére et al. (2013)
Bacillus cereus	Can lead to food poisoning	Duniére et al. (2013), Driehuis and Oude Elferink (2000)
Bacillus firmus		Driehuis and Oude Elferink (2000)
Bacillus lentus		Driehuis and Oude Elferink (2000)
Bacillus licheniformis		Driehuis and Oude Elferink (2000)
Bacillus polymyxa		Driehuis and Oude Elferink (2000)
Bacillus smithii		Wang et al. (2014)
Bacillus sphaericus		Driehuis and Oude Elferink (2000)
Byssochlamys		Driehuis and Oude Elferink (2000)
Byssochlamys nivea	Mycotoxin	Driehuis and Oude Elferink (2000)
Candida		Driehuis and Oude Elferink (2000)
Cladosporium sp.		Duniére et al. (2013)
Clostridium acidisoli		Wang et al. (2014)
Clostridium bifermentas	Highly proteolytic	Driehuis and Oude Elferink (2000)
Clostridium botulinum	Pathogenic toxin	Duniére et al. (2013)
Clostridium butyricum	Weakly proteolytic	Duniére et al. (2013), Driehuis and Oude Elferink (2000)

(continued)

Table 2 (continued)

Microorganism	Products and impacts	References
Clostridium sporogenes	Highly proteolytic	Driehuis and Oude Elferink (2000)
Clostridium tyrobutyricum	Weakly proteolytic	Duniére et al. (2013), Driehuis and Oude Elferink (2000)
E. coli (STEC)	Shiga toxin	Duniére et al. (2013)
E. coli O157		Duniére et al. (2013)
E. coli O157:H7		Duniére et al. (2013)
E. coli O26		Duniére et al. (2013)
Enterobacter aerogenes		McGarvey et al. (2013)
Enterobacter cloacae		Wang et al. (2014)
Enterobacter hormaechei		McGarvey et al. (2013)
Enterobacter ludwigii		McGarvey et al. (2013)
Enterobacter sp.	2,3-butanediol, pre-ensiled crop	Li and Nishino (2013)
Enterobacter sp. FMB-1		McGarvey et al. (2013)
Enterobacter sp. J33		McGarvey et al. (2013)
Enterobacter sp. MPR16		McGarvey et al. (2013)
Enterobacteria	2,3-butanediol	Li and Nishino (2013)
Enterococcus durans		McGarvey et al. (2013)
Epiphytic yeast	CO_2; alcohols	Duniére et al. (2013)
Erwinia amylovora		McGarvey et al. (2013)
Erwinia herbicola		Duniére et al. (2013)
Erwinia persicina		McGarvey et al. (2013)
Fusarium		Driehuis and Oude Elferink (2000)
Fusarium sp.	More than 20 mycotoxins, deoxynivalenol (DON), zearalenone (ZEN), fumonisin (FB)	Duniére et al. (2013)
Geobacillus pallidus		Wang et al. (2014)
Geotrichum		Driehuis and Oude Elferink (2000)
Hafnia alvei		Duniére et al. (2013)
Hansenula		Driehuis and Oude Elferink (2000)
Klebsiella pneumoniae		Duniére et al. (2013)

(continued)

Table 2 (continued)

Microorganism	Products and impacts	References
Klebsiella sp.	2,3-butanediol	Li and Nishino (2013)
Kurthia sp.		Wang et al. (2014)
Lactobacillus acetotolerans		Wang et al. (2014)
Lactobacillus buchneri		Wang et al. (2014), McGarvey et al. (2013)
Lactobacillus diolivorans		Wang et al. (2014)
Lactobacillus diolivorans		McGarvey et al. (2013)
Lactobacillus lindneri		McGarvey et al. (2013)
Lactobacillus plantarum		McGarvey et al. (2013)
Lactobacillus sp. TS4		McGarvey et al. (2013)
Lactococcus garvieae		McGarvey et al. (2013)
Listeria innocua	Cause animal disease	Duniére et al. (2013)
Listeria ivanovii	Cause animal disease	Duniére et al. (2013)
Listeria monocytogenes	Listeriosis	Duniére et al. (2013), Driehuis and Oude Elferink (2000)
Listeria sp.	Pathogenic	Duniére et al. (2013)
Monascus		Driehuis and Oude Elferink (2000)
Morganella morganii	2,3-butanediol	Li and Nishino (2013)
Mucor		Driehuis and Oude Elferink (2000)
Mycobacterium bovis	Causes bovine tuberculosis	Duniére et al. (2013)
Mycobacterium tuberculosis	Causes human tuberculosis	Duniére et al. (2013)
Paenibacillus barengoltzii		Wang et al. (2014)
Pantoea brenneri		McGarvey et al. (2013)
Pantoea agglomerans	Pre-ensiled crop	Li and Nishino (2013)
Pantoea sp.	2,3-butanediol, pre-ensiled crop	Li and Nishino (2013)
Pediococcus pentosaceus		McGarvey et al. (2013)
Penicillium		Driehuis and Oude Elferink (2000)

(continued)

Table 2 (continued)

Microorganism	Products and impacts	References
Penicillium roqueforti	Mycotoxin	Driehuis and Oude Elferink (2000)
Penicillium verrucosum	Ochratoxin A	Duniére et al. (2013)
Penicillium sp.		Duniére et al. (2013)
Pseudomonas oleovorans		McGarvey et al. (2013)
Pseudomonas oryzihabitans		McGarvey et al. (2013)
Pseudomonas sp.		Wang et al. (2014)
Pseudomonas syringae		Duniére et al. (2013)
Rahnella aquatilis		Duniére et al. (2013)
Rahnella aquatilis		Li and Nishino (2013)
Saccharomyces		Driehuis and Oude Elferink (2000)
Scopulariopsis		Driehuis and Oude Elferink (2000)
Serratia fonticola		Duniére et al. (2013)
Torulopsis		Driehuis and Oude Elferink (2000)
Trichoderma		Driehuis and Oude Elferink (2000)
Trichosporon sp.		Duniére et al. (2013)
Uncultured bacterium (band 1)	Pre-ensiled crop	Li and Nishino (2013)
Uncultured bacterium (band 3)	Pre-ensiled crop	Li and Nishino (2013)
Uncultured bacterium (band 31)		Li and Nishino (2013)
Uncultured bacterium (band 4)	Pre-ensiled crop	Li and Nishino (2013)
Weissella kandleri		McGarvey et al. (2013)
Yersinia enterocolitica	Causes yersiniosis	Duniére et al. (2013)

acid. Alcohols (mainly methanol and ethanol), ketones, ester and aldehydes can be determined as other volatile components in silages. While abiotic reactions can be responsible for the production of methanol and esters, the most important acids, alcohols and aldehydes, are caused by microbial activity (Hafner et al. 2013). Apart

Table 3 Identified chemical compounds in silage (Hafner et al. 2013)

Functional group	Chemicals
Alcohol	Methanol, ethanol, 1-propanol, 2-propanol, 2-propenol, 2-methyl-1-propanol, 1-butanol, 2-butanol, 3-methyl-1-butanol, 2-methyl-1-butanol, 1-pentanol, 1-hexanol, phenylmethanol, 2-phenylethanol
Ketone	Acetone, 2-butanone, 3-hydroxy-2-butanone
Aldehyde	Acetaldehyde, propionaldehyde, 2-methylpropanal, butyraldehyde, 2-methylbutanal, 3-methylbutanal, valeraldehyde, hexanal, heptanal

from lactic acid, 1,2-propanediol, propylene glycol and many esters, the following volatile organic compounds from silage were measured (Table 3).

The utilisation of green biomass for the production of platform chemicals in form of silage has two advantages. Firstly, green biomass as a feedstock would be available throughout the whole year, and secondly, by the acid impact, a soft pretreatment happens on the fibres. Simultaneously, the degradation of the proteins to amino acids continues.

For the application in biorefineries, a part of the so far undesired microorganisms from the food and forage production (e.g. silage) and their products can be newly evaluated. It is the case when undesirable by-products become desirable products, e.g. for the chemical industry. For that, the metabolic pathways are specifically utilised for these products and will be modified at time. A few examples of organic acids which could attain greater meaning and their microbial producers are described in the following section.

4 Organic Acid-Forming Bacteria

4.1 Acetic Acid (C2)

The worldwide production of acetic acid exceeds 7 million metric tons per year (Cheung et al. 2005) whereof approximately 2 million metric tons produced using biotechnological processes and renewable resources. There are two different big-scale production ways established. On the one hand, the chemical high-pressure Monsanto process with the catalytic conversion of methanol and carbon monoxide to acetic acid. On the other hand, a widely spread process is used which is known since ancient times. This process contains the biotechnological conversion of ethanol to acetic acid using aerobic acetic forming bacteria of the genus *Acetobacter*.

Under anaerobic conditions, e.g. *Clostridium aceticum* is able to use the homoacetic acid fermentation pathway to produce acetic acid at an optimum pH value of 8.3 and 30 °C. This pathway is divided into two parts. First, the digestible carbohydrates pass through the glycolysis and end up as pyruvate which is

Table 4 Characteristics of *Acetobacter aceti*

Acetobacter aceti		Gillis and de Ley (1980), Leisinger (1965)
Systematic classification	Kingdom	Bacteria
	Phylum	Proteobacteria
	Class	Alpha proteobacteria
	Order	Rhodospirillales
	Family	Acetobacteraceae
	Genus	Acetobacter
	Species	*A. aceti*
Synonyms	ATCC 15973, *Acetobacter aceti subsp. aceti*	
Source or first isolation	From alcohol turned to vinegar	
Characteristics	Gram-negative, peritrichously flagellated, anaerobic	
Morphology	Rod shaped	
Growth conditions	26 °C, medium 1: mannitol agar/broth, aerobic	
Pathogenicity	Class 1	
DNA GC content	55.9	

oxidatively decarboxylated. Acetyl-CoA is formed which will be further converted to acetate and secreted. The released carbon dioxide is converted within the Wood–Ljungdahl pathway (reductive-acetyl-CoA pathway) to acetate as well. This leads to a high overall yield of three molecules acetate per molecule glucose.

Because of the fact that green biomass is used as raw material, a complete conversion of the present carbohydrates should be intended. Especially, cellulose has to be converted to fermentable sugars within the process setup. In the future, it will be desirable to set up an economic process using acetogenic bacteria which are able to utilise a wide variety of carbohydrates including cellulose (Table 4).

Because of the low substrate costs compared to the fossil fuel-based methanol and the higher product yield compared to the ethanol oxidation, experts expect that this approach will be more successful and economic. The increment of the pH tolerance of the acetogenic bacteria will be an essential fact as well (Table 5).

4.2 Propionic Acid (C3)

Currently, propionic acid is produced by a petrochemical production way. The process is called oxo process. Ethylene reacts with synthesis gas (CO/H_2) to propionaldehyde which reacts with oxygen to propionic acid. Because of the raising oil price and the pursuit for oil independency and sustainable granting of industry appreciable chemicals, the public demand for biotechnological production of propionic acid is raised continuously for the last few years.

The worldwide annual production quantity in 2006 was estimated to 377,000 metric tons. BASF qualifies as the biggest manufacturer with 150,000 metric tons per annum.

Table 5 Characteristics of *Clostridium aceticum*

Clostridium aceticum		Wieringa (1936), Gottschalk and Braun (1981), Skerman et al. (1980), Braun and Gottschalk (1981), Karlsson et al. (1948)
Systematic classification	Kingdom	Bacteria
	Phylum	Firmicutes
	Class	Clostridia
	Order	Clostridiales
	Family	Clostridiaceae
	Genus	Clostridium
	Species	*C. aceticum*
Synonyms	ATCC 35044	
Source of first isolation	Mud	
Characteristics	Gram-positive, peritrichously flagellated, anaerobic	
Morphology	Rod shaped	
Growth conditions	30 °C, medium 1612: acetobacterium medium, anaerobic	
Pathogenicity	Class 1	

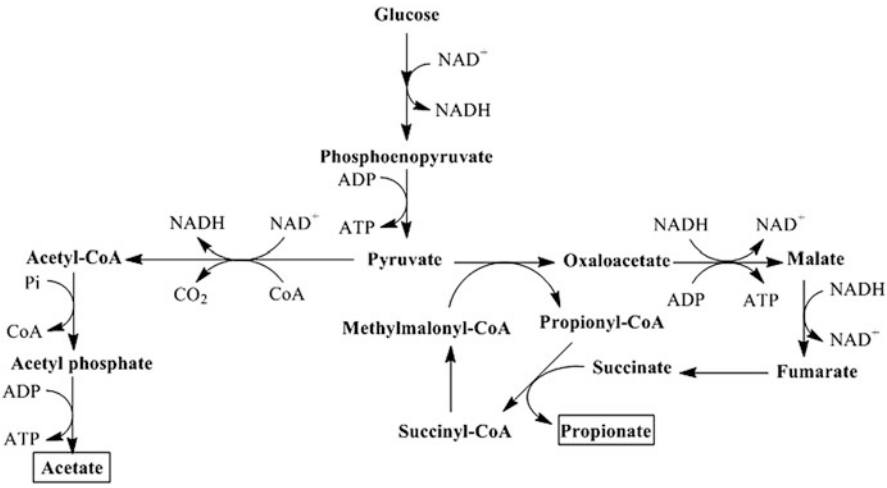

Fig. 1 Dicarboxylic acid pathway

Various bacteria own the ability to produce propionic acid within their metabolic pathways. Present-day research is focused on strains of Propionibacteriaceae and Clostridiaceae. Propionibacteria are using the dicarboxylic acid pathway (methylmalonyl coenzyme A-pathway) to produce the desired product. These gram-positive, anaerobic bacteria are able to use glucose, sucrose, lactate, lactose and glycerol as carbon source. The metabolic end products are propionate, succinate, carbon dioxide and acetate. Professionals acknowledge *Propionibacterium*

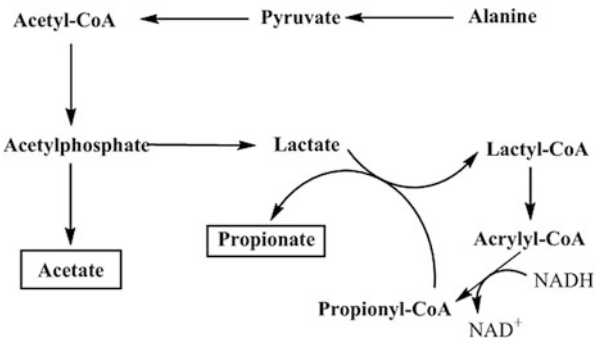

Fig. 2 Acrylic acid pathway

acidipropionici, *P. shermanii* and *P. freudenreichii* the highest potential to achieve an economic, big-scale process. The optimal pH value is between 6.5 and 7.0, and the best growth temperature lies at 30–32 °C (Fig. 1).

Another highly potential production species is the gram-positive bacteria *Clostridium propionicum*. This microorganism is able to utilise lactate, glycerol and alanine as substrate. Propionate, acetate, formate, n-propanol and succinate are produced. The optimal pH value is 6.8 and the best temperature is 30 °C. *C. propionicum* uses the acrylic acid pathway to produce the desired product (Fig. 2).

Lactate is able to start both pathways. If three molecules of lactate enter the pathway, two will be reduced to propionate and one will be oxidised to acetate and carbon dioxide. Because of the fact that these microorganisms ferment the product of an earlier fermentation process, they are called secondary fermenters (Tables 6 and 7).

The biotechnological production of propionic acid has never passed the pilot plant level (Abbas and Adolfo 2000; Balamurugan et al. 1999). Feasible reasons are the fastidious fermentation process, the long cultivation time, the end-product inhibition, the low final titer, the product purity and especially the high costs of fermentation and product recovery processes (Colomban et al. 1993; Liang et al. 2012). In order to be competitive, the biotechnological production process needs to have higher productivity and reduced production costs (Sabra et al. 2013) (Table 8).

The most important step will be the decrease of substrate costs. Green biomass depicts a common and cheap source of raw materials. Because of the broad experiences within the silage process including the formation of high amounts of lactic acids, green biomass would be highly suitable. Within a co-cultivation, there are no costs of lactate recovery which is an advantage.

Table 6 Characteristics of *Propionibacterium acidipropionici*

Propionibacterium acidipropionici		Johnson and Cummins (1972), Skerman et al. (1980)
Systematic classification	Kingdom	Bacteria
	Phylum	Actinobacteria
	Class	Actinobacteria
	Order	Actinomycetales
	Family	Propionibacteriaceae
	Genus	Propionibacterium
	Species	*P. acidipropionici*
Synonyms	*Propionibacterium pentosaceum*, *P. arabinosum*, *P. acidipropionici*, Bacillus acidipropionici, ATCC 25562	
Source of first isolation	Dairy products	
Characteristics	Gram-positive, non-spore forming	
Morphology	Rod shaped	
Growth conditions	37 °C, medium 602: E medium for anaerobes, anaerobic	
Pathogenicity	Class 1	
DNA GC content	68.8	
Special characteristics	High GC content	

Table 7 Characteristics of *Propionibacterium freudenreichii subsp. shermanii*

Propionibacterium freudenreichii subsp. Shermanii		van Niel (1928), Moore and Holdeman (1970), Skerman et al. (1980)
Systematic classification	Kingdom	Bacteria
	Phylum	Actinobacteria
	Class	Actinobacteria
	Order	Actinomycetales
	Family	Propionibacteriaceae
	Genus	Propionibacterium
	Species	*Propionibacterium freudenreichii*
	Subspecies	*P. freudenreichii subsp. shermanii*
Synonyms	*Propionibacterium shermanii*, *Propionibacterium freudenreichii shermanii*, ATCC 9614	
Source of first isolation	Cheese, Weihenstephan, Germany	
Characteristics	Gram-positive, nonmotile, forming	
Morphology	Rod shaped	
Growth conditions	30 °C, medium 593: chopped meat medium, anaerobic	
Pathogenicity	Class 1	
DNA GC content	67.3	
Special characteristics	High GC content	

Table 8 Characteristics of *Clostridium propionicum*

Clostridium propionicum		Cardon and Barker (1946), Ludwig et al. (2009), Janssen (1991)
Systematic classification	Kingdom	Bacteria
	Phylum	Firmicutes
	Class	Clostridia
	Order	Clostridiales
	Family	Clostridiaceae
	Genus	Clostridium
	Species	*C. propionicum*
Synonyms	ATCC 25522	
Source of first isolation	Black mud, San Francisco Bay, USA	
Characteristics	Gram-positive, obligate anaerobic	
Morphology	Rod shaped	
Growth conditions	37 °C, medium 2210: enriched anaerobe medium, anaerobic	
Pathogenicity	Class 1	
DNA GC content		
Special characteristics		

4.3 n-Butyric Acid (C4)

Butyric acid can be conventionally produced by oxidation of butyraldehyde. A variety of anaerobic bacteria are able to produce butyric acid as the major end product during fermentation process. Nevertheless, *Clostridium* species have been used preferentially for butyric acid production because of their plain medium requirements and comparatively high product yields. The major focus in current research is on *C. butyricum* with a pH range between 5.0 and 7.0. But optimisation of *C. tyrobutyricum* within green biomass fermentation could be very worthwhile because of the fact that *C. tyrobutyricum* is able to ferment lactate produced by Lactobacteriaceae. Furthermore, *C. tyrobutyricum* tolerates low pH values down to 4.2 which could be an advantage in big-scale implementation.

The biochemical pathway used by *Clostridia* to produce butyric acid is shown in Fig. 3. The pathway starts with the glycolytic cleavage of glucose to two molecules of pyruvate. After that, pyruvate is oxidised into acetyl coenzyme A. The enzyme which catalyses this step is called pyruvate-ferredoxin oxidoreductase. Two molecules of carbon dioxide and two molecules of hydrogen are released. Starting at acetyl coenzyme A, three possible end products can be formed: ethanol, acetate and butyrate. With the aid of genetic engineering, the formation of side products can be disabled by gene knockout or optimised process control (Tables 9 and 10).

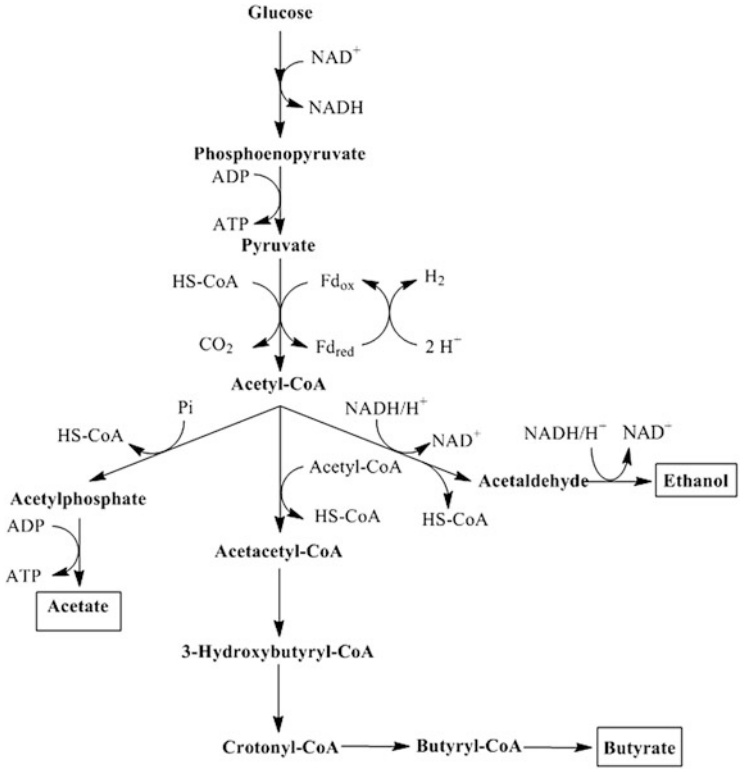

Fig. 3 Butyric acid pathway

Table 9 Characteristics of *Clostridium butyricum*

Clostridium butyricum		Schink and Zeikus (1980), Schink et al. (1981)
Systematic classification	Kingdom	Bacteria
	Phylum	Firmicutes
	Class	Clostridia
	Order	Clostridiales
	Family	Clostridiaceae
	Genus	Clostridium
	Species	*C. butyricum*
Synonyms	ATCC 19398	
Source of first isolation	Intestine of pig	
Characteristics	Gram-positive, strictly anaerobic endospore-forming bacteria	
Morphology	Rods	
Growth conditions	37 °C, medium 1053: reinforced clostridial medium, anaerobic	
Pathogenicity	Class 2	
DNA GC content	28.8	

Table 10 Characteristics of *Clostridium tyrobutyricum*

Clostridium tyrobutyricum		Skerman et al. (1980)
Systematic classification	Kingdom	Bacteria
	Phylum	Firmicutes
	Class	Clostridia
	Order	Clostridiales
	Family	Clostridiaceae
	Genus	Clostridium
	Species	*C. tyrobutyricum*
Synonyms	ATCC 25755	
Source of first isolation	Raw milk, Germany	
Characteristics	Gram-positive, spore forming, anaerobe	
Morphology	Rod shaped	
Growth conditions	37 °C, medium 2107: modified reinforced clostridial agar/broth medium, anaerobic	
Pathogenicity	Class 1	
DNA GC content	30.8	
Special characteristics	Heat-resistant spores	

4.4 *Isobutyric Acid (C4), Isovaleric Acid (C5), Isocaproic Acid (C6)*

Isobutyric acid is used in the production of artificial fibres, plastics and herbicides. It is also used as an intermediate in the production of cosmetics and food additives and in the pharmaceutical industry. There are industrialised chemical syntheses to produce isobutyric acid which does require fossil fuels and harmful chemicals. A biotechnological process based on renewable feedstock is more environmentally friendly and ensures in the long view a cost-effective supply of isobutyric acid.

Isovaleric acid is mainly used for perfumery production and within intensive-care medicine. Valerian is a natural source of isovaleric acid which can be extracted. Mainly, proteolytic bacteria can produce different carboxylic acids during the protein degradation. Several members of the family Clostridiae are proficient to use the Stickland fermentation, for example, *Clostridium bifermentans, C. sporogenes* and *C. acetobutylicum* (Brooks and Epps 1958).

Clostridium bifermentans is able to produce a broad range of metabolites such as butyric, acetic and formic acids (Wu and Yang 2003), ethanol, butanol, aceton (Khanal 2003), carbon dioxide, hydrogen and nitrogen (Levin et al. 2006). However, the metabolic pathway of *C. bifermentans* has not been investigated in detail so far (Leja et al. 2013).

C. sporogenes can produce acetic, propionic, butyric, isovaleric, isobutyric and isocaproic acid, hydrogen and carbon dioxide. By the current state of scientific knowledge, *C. sporogenes* produces the carboxylic acids applying the Stickland reaction. This reaction is a particular kind of fermentation of amino acids which is characterised by simultaneous oxidation of one amino acid and reduction of another

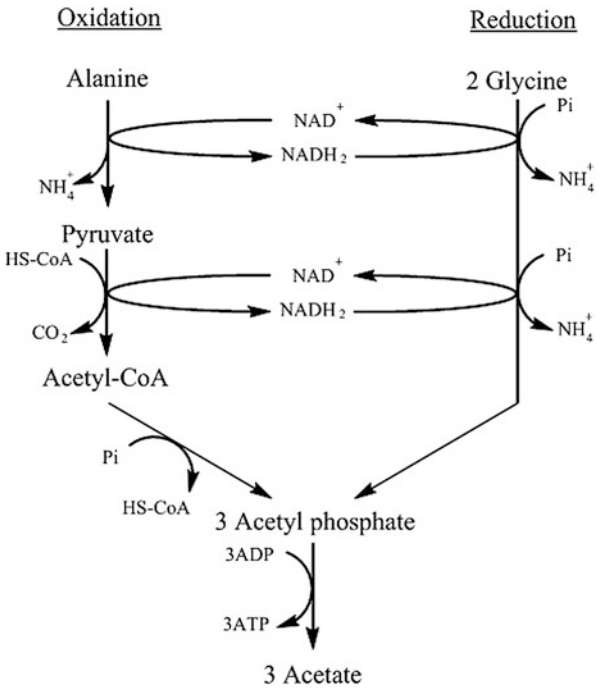

Fig. 4 Stickland reaction scheme (Stickland 1935; Nisman 1954)

Table 11 Characteristics of *Clostridium sporogenes*

Clostridium sporogenes		Bradbury et al. (2012)
Systematic classification	Kingdom	Bacteria
	Phylum	Firmicutes
	Class	Clostridia
	Order	Clostridiales
	Family	Clostridiaceae
	Genus	Clostridium
	Species	*C. sporogenes*
Synonyms	ATCC 3584	
Source of first isolation	Cotton, gas gangrene and silage	
Characteristics	Gram-positive, spore forming	
Morphology	Rod shaped	
Growth conditions	37 °C, medium 2107: modified reinforced clostridial agar/broth medium, anaerobic	
Pathogenicity	Class 2	
DNA GC content	28	

amino acid (Nisman 1954). The utilisation of just one amino acid is not possible (Stickland 1935). Valine is the starting substance for isobutyric acid; leucine is converted to isovaleric acid.

Table 12 Characteristics of *Clostridium bifermentans obligat anaerobes*

Clostridium bifermentans obligat anaerobes		Ludwig et al. (2009)
Systematic classification	Kingdom	Bacteria
	Phylum	Firmicutes
	Class	Clostridia
	Order	Clostridiales
	Family	Peptostreptococcaceae
	Genus	Clostridium
	Species	*C. bifermentans*
Synonyms	Bacillus bifermentans sporogenes, Bacillus biferm., ATCC 638	
Source or first isolation	Silage, H.J. Kutzner, Darmstadt, Germany	
Characteristics	Gram-positive, spore forming, anaerobe	
Morphology	Rods, spores are cylindrical to oval, centrally to subterminally located, which do not noticeably swell the rods	
Growth conditions	37 °C, medium 2107: modified reinforced clostridial agar/broth medium, anaerobic, microaerophilic	
Pathogenicity	Class 2	
DNA GC content	28.4	

Due to the fact that the initial concentration of valine and leucine is crucial for the successful, high product yield fermentation, the engaged green biomass has to be optimised in the future. This represents an important milestone on the way to big-scale implementation (Fig. 4).

The biological high-yield production of isobutyric, isovaleric and isocaproic acid is not commercially established yet (Tables 11 and 12).

5 Sequence Chemical Products and Applications from Organic Acids

The application of biotechnological methods will be highly important with the development of biorefineries for the production of platform chemicals, intermediate chemicals, speciality chemicals and polymers.

The two-carbon short-chain acetic acid is start material for the production of vinyl acetate monomer (VAM). This application consumes one third of the world's production of acetic acid (Cheung et al. 2005). The product of the condensation of two molecules of acetic acid is acetic anhydride. The worldwide production of acetic anhydride is a further major application and uses approximately 25–30 % of the global production of acetic acid. The main process involves dehydration of acetic acid to give ketene at the temperature of 700–750 °C. Ketene is thereafter reacted with acetic acid to obtain the anhydride (Held et al. 2005). Acetic anhydride is an acetylation agent. As such, its major application is for cellulose acetate, a

synthetic textile also used for photographic film. Acetic acid owns a wide field of application. Also acetic acid and various corresponding salts are authorised food additives and increasingly used as preservatives.

The tri-carbon short-chain propionic acid is an important building block chemical and finds a variety of applications in organic synthesis for the production of polymers, such as cellulose acetate propionate, plastic dispersions, textile and rubber auxiliaries, dye intermediates as well as flavours and fragrances. This acid can be used for the synthesis of propionic ether and benzyl propionate, which can be used as additives in cosmetics. Furthermore, sodium 2,2-dichloropropionate is applied as herbicide. Propionic acid anhydride serves as a pharmaceutical intermediate (Kumar and Babu 2006). Also, propionic acid is applied in animal feed and as a grain preservative. The application possibilities cover the affordable preservation of animal feed (especially in cattle husbandry) and foodstuffs for human consumption. The US Food and Drug Administration (FDA) lists the acid and the Na^+, Ca^+ and K^+ salt of it as preservatives in their summary of generally recognised as safe (GRAS) (Colomban et al. 1993).

The four-carbon short-chain n-butyric acid and its derivatives have numerous potential applications in chemical, textile, plastic, food, beverage, dairy and pharmaceutical industries. They are used as solvents, diluents, drugs, plasticisers, perfumes, fibres and additives (Jha et al. 2014). The main field of application of butyric acid is the food industry. The dairy industry is using the pure acids; the esters are used as food additives to amplify the fragrance of tropic fruits (Centeno et al. 2002; He et al. 2005; Watson 2002).

The four-carbon short-chain isobutyric acid is applied as esters for solvents, polymers, flavour and fragrances. Isobutyric acid is suitable as polar solvent for different chemical reactions. Two molecules of isobutyric acid form isobutyric anhydride. The pyrolysis of isobutyric anhydride produces dimethylketene. Dimethylketene is absorbed into certain carboxylate ester solvents which function as process solvents for subsequent dimerisation of the dimethylketene to 2,2,4,4-tetramethylcyclobutandione followed by the catalytic hydrogenation of the dione to the diol products (Sumner et al. 1992). This four-ring diol is a valuable comonomer for the production of a new family of copolyesters (Kelsey et al. 2000).

The five-carbon short-chain isovaleric acid is applied for the production of flavours and perfumes. Also, this acid is broadly used as intermediate for synthesis of insecticides, fungicides and depressants. The esters serve as plasticisers (Römpp 2005).

6 Future Perspectives

Currently, only few industrial products are produced from green biomass as carbon source, such as lactic acid and ammonium lactates (see chapter Microorganisms for Production of Lactic Acid and Organic Lactates), lysine (see chapter Microorganisms for Biorefining of Green Biomass) or 2,3 butanediol. The use of green biomass

could have advantages, if more platform chemicals would be developed. These advantages include using of a variety of microorganisms and potential use of carbon, nitrogen and inorganics, which contain in green biomass. In the case of the production of carboxylic acids, the fermentative use of organic nitrogen compounds like amino acids from proteins by appropriate *Clostridia* would be a rewarding approach.

By the conservation step silage, the green biomass would be available throughout the whole year. Furthermore, by the acid impact, a soft pretreatment happens on the celluloses. Simultaneously, the degradation of the proteins to amino acids continues.

The utilisation of green biomass could open new perspectives for the development of ecologically better adapted biorefineries. For this, it is necessary to work in research and development in order to improve the following areas:

- Improvement of fractionation/hydrolysis/separation methods of green raw feedstock in order to increase the amount of useful and fermentable substances, decrease the amount of inhibitors and lower process costs.
- DNA sequencing of microorganisms and their genome analysis—identify key genes responsible for the expression of useful properties.
- Metabolic engineering of microorganisms in order to broaden their substrate range, increase product tolerance/maximal concentration and increase yield and product specificity. Alternatively, target genes can be transferred into well-known, robust and user-friendly microorganisms (e.g. *E. coli*, *S. cerevisiae*).
- Large-scale screening of microbial genomes for genes or products with market potential.
- Appropriate optimisation of fermentation processes according to the properties of individual microorganisms and products.

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Microorganism for Bioconversion of Sugar Hydrolysates into Lipids

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Contents

Abstract Metabolic and genetic engineering and further other genomics, transcriptomics, and metabolomics tools still need to be further developed to provide more useful information and new ways on enhancing lipid production in oleaginous microorganisms, optimizing fatty acid (FA) profiles, enhancing lipid accumulation, and improving the use of low-cost raw materials as lignocellulosic hydrolysates. Several oleaginous organisms have been described as good lipid producers, being the fast ones the yeasts. However the kinetics for this production is much slower than that required for industrial processes, unless the products are

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sold at high competitive prices (fine chemicals, cosmetics, and food), covering the costs for the long residential bioreactor times.

Microalgal cultivation in heterotrophic systems is able to use organic carbon sources, sugars, or organic acids, and this cultivation mode offers some advantages over autotrophic cultivation including increased lipid productivity, besides good control of the cultivation process and low cost for harvesting the biomass, since higher cell density is obtained. However, the feasibility of large-scale cultures of microalgae in heterotrophic conditions is still limited by, among other things, the high cost of organic substrates used in this type of cultivation, unless urban/agricultural/industrial wastes are used as lignocellulose and wastewater and sewage are used as carbon or mineral sources.

1 Introduction

Lignocellulosic biomass is the most abundant raw materials on Earth and is a promising alternative energy source due to their abundance and renewable characteristics. Agro-industrial residues such as sugarcane bagasse (SCB), wheat straw, rice straw, and corn stover represent feedstocks for their conversion into value-added products (Chandel et al. 2012).

Microbial oils, also called single cell oils (SCO), are produced by some oleaginous microorganisms, such as yeast, fungi, bacteria, and microalgae, which are able to accumulate more than 20 % lipids (dry mass) (Ageitos et al. 2011; Karatay and Dönmez 2011). Different lignocellulosic biomasses have been used for microbial oil production, such as wheat straw, corncobs, rice straw, and SCB. SCB seems to be the most promising and competitive in sugarcane-producing lands, due to the availability of large amounts stored in the sugarcane mills after crushing/pressing (Matos 2012). SCB may be an attractive and promising feedstock for microbial oil production to reduce the costs of culture medium, which conventionally hampers the economic feasibility of SCO as alternative feedstock for biodiesel production (Anschau et al. 2014; Tsigie et al. 2011).

Two problems influence SCO production on lignocellulosic hydrolysates. Few oleaginous microorganisms are able to use xylose for SCO production, and as xylose is the second most abundant component of lignocellulose hydrolysates, substantial amounts of feedstock can be lost. Several strains of oleaginous microorganisms have their growth inhibited by small molecules from lignocellulose hydrolysates. Fortunately, it has been observed that some oleaginous strains are able to metabolize xylose for SCO production, without being so much inhibited by the growth inhibitors (Anschau et al. 2014).

Zhao (2005) described the possibility of SCO production from lignocellulose hydrolysates, the biomass-to-biodiesel three-step plan: lignocellulose biomass depolymerization into fermentable sugars, their conversion into microbial lipids by oleaginous microorganisms, and the chemical transformation of their lipids into biodiesel.

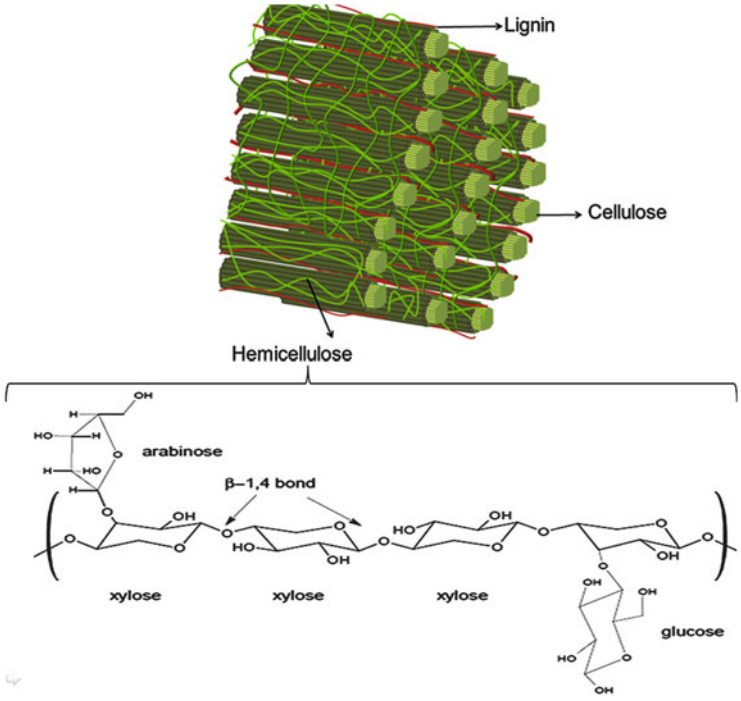

Fig. 1 Lignocellulosic biomass and hemicellulosic structures

2 The Lignocellulosic Biomass

Lignocellulose is a complex polymer of cellulose (40–80 %), hemicellulose (10–40 %), and lignin (5–25 %) (Chandel et al. 2012, Fig. 1). Cellulose, the main fraction of plant cell wall, is linear and crystalline and is a homopolymer of repeating units of glucose linked by β(1-4) glycosidic bonds. Hemicellulose is a highly branched heteropolymer composed of D-xylose, D-arabinose, D-glucose, D-galactose, and D-mannose (Fig. 2). Lignin, formed by polymerization of phenolic compounds, is hydrophobic in nature and is tightly bound to the cellulose and hemicellulose protecting them from microbial and chemical (Sarkar et al. 2012) and enzymatic action (Meng and Ragauskas 2014).

Xylose is the main carbohydrate present in the hemicellulose fraction (second most abundant component of lignocellulose, representing about 80 % of total sugars) (Girio et al. 2010). Potentially, the released carbohydrates, mono- and dimeric sugars, are substrates for the production of value-added products such as ethanol, xylitol, n-butanol, 2,3-butanediol, and lactic acid via microbial fermentation processes (Chandel et al. 2010, 2011, 2012).

The goal of pretreating vegetal biomasses is to remove or modify the lignin (delignification) and to reduce the crystallinity of cellulose (increasing the

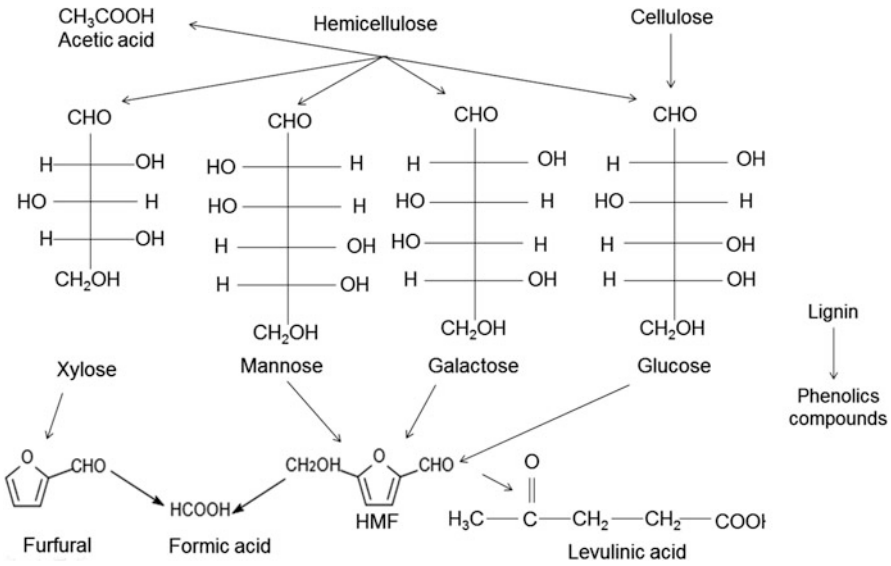

Fig. 2 Structural profile of inhibitors derived from lignocellulosic biomass (Chandel et al. 2011)

accessibility to enzymes, which convert carbohydrates into fermentable sugars). The pretreatment of lignocellulose is usually able to hydrolyze the hemicellulose fraction yielding xylose (mostly) and arabinose, mannose, galactose, and glucose in smaller relative concentrations, in addition to the small molecules, also known as microbial growth inhibitors (Meng and Ragauskas 2014; Girio et al. 2010).

There are different types of pretreatment methods such as steam explosion, gamma radiation, acid or alkali treatment, hydrothermal, ammonia, urea, hydrogen peroxide, solvents, milling, and fungal degradation (Girio et al. 2010; Chandel et al. 2010; Kumar et al. 2009; Moretti et al. 2014), being exclusively physical, chemical, physicochemical, biological, or a combination of mechanisms.

The choice of pretreatment depends on the biomass to be used, its composition, and by-products produced. Furthermore, pretreatments need to avoid sugar degradation and minimize the consequent formation of growth inhibitor molecules (Moretti et al. 2014) and significantly affect their costs.

Hydrochloric acid (HCl), phosphoric acid (H_3PO_4), and sulfuric acid (H_2SO_4) catalyze the hydrolysis of lignocellulosic materials and are used in two different combinations with the reactor's temperature, high concentrations of acid and low temperatures or diluted acid concentrations and high temperatures. In our laboratory, we hydrolyzed the SCB with 1.5 % (w/v) H_2SO_4, solid-to-liquid ratio (1:10) at 120 °C, for 20 min (Fig. 3). Xylose was the major product released (14.3 g/L) by SCB hemicellulosic hydrolysate (H–H) due to its high content of xylan. In addition, glucose and arabinose were found in low concentrations (1.6 and 2.3 g/L, respectively). Glucose was released from the cellulose and hemicellulose fractions, and the arabinose was from the depolymerization of arabino-xylan (a hemicellulosic

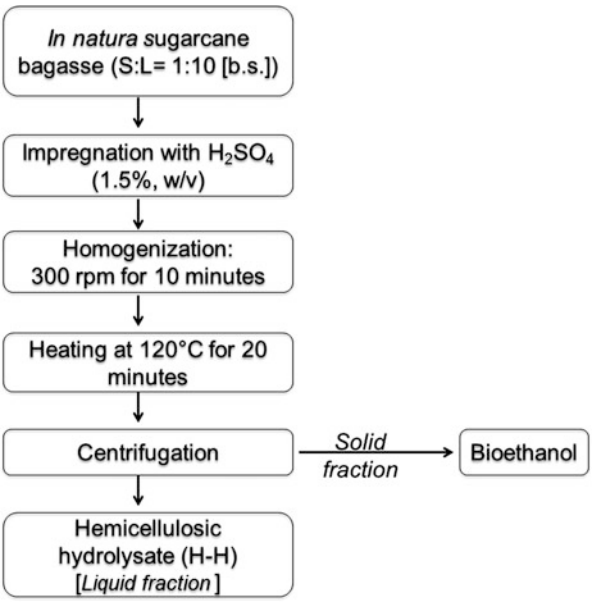

Fig. 3 Obtaining process of hemicellulosic hydrolysate (H–H). S: L = solid:liquid

heteropolymer that contains more xylose than arabinose). Usually, hexose fermenter microorganisms are unable to metabolize pentose sugars and do not tolerate the growth inhibitors generated from the biomass pretreatment (Carvalheiro et al. 2008). The full use of H–H must be understood as a way to reduce waste and to increase the efficiency of conversion of raw material into final products, mainly when value-added products that are obtained improve the economics of the process.

During acid hydrolysis of lignocellulosics, toxic inhibitors to the fermenting microorganism are generated in addition to the sugar monomers. These inhibitors include phenolic compounds, furans (furfural and 5-hydroxymethylfurfural (5-HMF)), and weak acids (acetic acid, formic, levulinic) (Fig. 2). Furfural and 5-HMF are formed by degradation of pentoses and hexoses, respectively. However, the HMF concentration tends to be smaller than furfural due to limited degradation of hexoses in acid saccharification. Acetic acid is formed by hydrolysis of acetyl groups from lignocellulose, as a result of deacetylation of acetylated pentosans, and its generation is mainly dependent on the temperature and residence time of pretreatment with diluted acid (Chandel et al. 2012; Kumar et al. 2009). The formation of the growth inhibitors during the acid hydrolysis depends on multiple factors, including the nature and composition of the lignocellulose, cell wall composition, thermochemical conditions, and reaction time of the hydrolysis (Stoutenburg et al. 2011; Palmqvist and Hahn-Hägerdal 2000a, b). These toxic compounds may affect microbial metabolism hindering the fermentation process (Sangeeta et al. 2014). Their toxicity is the major limiting factor to the bioconversion of lignocellulose, and often they need to be removed from the H–H prior to the

fermentation (Stoutenburg et al. 2011). Detoxification methods have been used to reduce the presence of growth inhibitors or to remove them in order to improve their fermentability and increase their biotechnological applications (Chandel et al. 2010; Palmqvist and Hahn-Hägerdal 2000a).

There are several detoxification methods such as physical, chemical, or biological and may be used individually or in combined form. These methods include activated charcoal treatment, neutralization, calcium hydroxide overliming, extraction with solvents, ion exchange resins, evaporation, membrane-mediated detoxification, enzymatic mediated using laccase, lignin peroxidase, in situ microbial detoxification, etc. (Ge et al. 2011; Chandel et al. 2012; Moretti et al. 2014), which will vary depending on the H–H, the type of microorganisms used in the fermentation, and their degree of tolerance to inhibitors. It is important to compare the different methods of treatment in order to select those that provide greater removal of inhibitors and lower reduction of sugar concentrations. For example, activated charcoal and alkali treatment with calcium oxide are chemical methods used to detoxify acid hydrolysates. Calcium oxide treatment causes precipitation of toxic compounds and instability of some inhibitors in high pH, removing furans and phenolic compounds efficiently with a marginal loss of sugars (Chandel et al. 2010). Activated carbon treatment by adsorption is capable of removing acetic acid, furans, phenolic and aromatic compounds, furfural, and HMF with high efficiency coupled with low cost (Chandel et al. 2010, 2011; Stoutenburg et al. 2011).

In our laboratory, we obtained a H–H with 3.6 g/L of acetic acid, 0.02 g/L of HMF, and 0.04 g/L of furfural. The pH was adjusted with CaO without marginal loss of sugars and slight reduction of inhibitors; therefore, the H–H detoxification was considered as unnecessary. The non-detoxified hydrolysate was used for lipid production by *Lipomyces starkeyi* without appreciable inhibition by the toxic compounds. Furthermore, the yeast consumed or bioreduced the inhibitors during fermentation suggesting that the strain could use these compounds as c Carbon and energy source. High tolerance of *L. starkeyi* is very interesting for the use of H–H since the detoxification step would not be required in the conditions tested.

Therefore, proper pretreatment methods can increase the relative concentrations of fermentable sugars after enzymatic saccharification, thereby improving the efficiency of the whole process. Enzymatic hydrolysis uses enzyme complexes to depolymerize complex carbohydrates into simple monomers and requires less energy and milder environmental conditions than acid hydrolysis. Enzymatic hydrolysis is carried out by cellulase and hemicellulase enzymes, which cleave the bonds of cellulose and hemicellulose, respectively. Enzymatic hydrolysis shows some advantages such as low toxicity and low corrosion compared to acid or alkaline hydrolysis and does not produce inhibitors (Sarkar et al. 2012). On the other hand, enzymes are costly, require longer time of hydrolysis, and have high loads which limit the economical feasibility of technological processes related to the lignocellulosic biomass. Therefore, an efficient pretreatment is to be selected to decrease cellulose crystallinity and to remove lignin to the maximum extent, so that hydrolysis time as well as cellulase loading will be minimized (Sarkar et al. 2012).

3 Microbial Oil Production from Lignocellulosic Biomass

3.1 Microbial Oil Production by Microalgae

Within the broad microbial diversity, microalgae have potential for lipid production, since many species can be induced to accumulate substantial quantities of lipids within the cell (Malcata 2011). The average lipid content of algal cells varies between 1 and 50 % of dry weight under certain conditions (Meng et al. 2009; Spolaore et al. 2006). Also already it has been known that microalgae could accumulate more lipids with stress treatments such as nitrogen deficiency (Illman et al. 2000). Coelho et al. (2014) working at fed-batch culture with *Chlorella*, during nitrogen-limited stage, obtained an increase of 38 % in lipid content (from 21 to 29 %) and 26 % in overall lipid productivity (from 1.25 to 1.58 g/L/day).

Microalgae are able to get energy from different metabolisms (Richmond and Qian 2004). In autotrophic metabolism, in the presence of light, organisms are able to convert CO_2 and water into biomass. Already heterotrophic systems are a different approach to microalgal biomass production with elimination of light requirement and CO_2 absorption.

In heterotrophic cultivation organic carbon sources are used, such as sugars or organic acids, as a source of carbon and energy. This cultivation mode offers several advantages over autotrophic cultivation including increased lipid productivity, besides good control of the cultivation process and low cost for harvesting the biomass because of higher cell density obtained in heterotrophic culture of microalgae. Cell densities as high as 80 g/L (Coelho and Franco 2013) and until 100 g/L (Zheng et al. 2013) have been achieved in heterotrophic systems using glucose as organic carbon source and different feeding strategies. The feasibility of large-scale cultures of microalgae in heterotrophic conditions is still limited by, among other things, the high cost of organic substrates used in this type of cultivation, unless wastes are used as carbon or mineral sources.

In heterotrophic culture, the biosynthesis of products is significantly influenced by medium nutrients, and carbon sources are the most important element for heterotrophic culture of microalgae in the production of lipids. Glucose is most commonly used as carbon source for heterotrophic cultures of microalgae, and far higher rates of growth are obtained with this substrate than with any other. Usually, glucose accounts for most of the medium cost of microalgal cultivation (it was estimated up to 80 %) (Li et al. 2007).

Corn powder hydrolysate has been used to replace glucose for developing a cheap medium for heterotrophic *Chlorella protothecoides* (Xu et al. 2006). Many additional options of organic carbon sources were tested for feasibility instead of glucose (Table 1).

Molasses, a by-product from sugar refinery, also can be a good alternative to glucose in promoting microalgal lipid accumulation. Vidotti et al. (2014) tested the hydrolyzed sugarcane molasses with two microalgal strains, and this substrate

Table 1 Substrates used for lipid production by *C. protothecoides strains*

Substrate	Lipids (%, w/w)	Lipid productivity (g/L/day)	References
Corn powder hydrolysate	55.3	NI	Xu et al. (2006)
Sugarcane juice hydrolysate	53	NI	Cheng et al. (2009a)
Sweet sorghum juice hydrolysate	52.5	0.5	Gao et al. (2010)
Jerusalem artichoke tuber hydrolysate	44	1.6	Cheng et al. (2009b)
Cassava hydrolysate	53	NI	Lu et al. (2009)
	26.5	0.4	Wei et al. (2009)
Waste molasses hydrolysate	57.6	5.5	Yan et al. (2011)

NI not informed

provided a similar cell growth as that observed when glucose was used in the cultivation of *Chlorella vulgaris* and *Scenedesmus bijugatus*.

Sugars released from lignocellulosic materials also are able to provide cheaper and sustainable carbon source for heterotrophic microalgal cultivation. However, besides the physical and chemical barriers already mentioned in this chapter, caused by the close association of the main components of lignocellulosic biomass, a variety of potential inhibitory degradation products are produced during pretreatment of lignocellulosic biomass, and all of these chemicals can potentially cause inhibition to algal growth (Liang 2013).

How microalgal species survive the presence of these toxic degradation by-products is unknown. In the literature, only the effect of acetate on microalgal growth has been evaluated (Perez-Garcia et al. 2011; Heredia-Arroyo et al. 2010, 2011; Yeh et al. 2012; Vidotti et al. 2013). Another important factor is the sugar composition present in the feedstock. As mentioned earlier, glucose is the most commonly used carbon source for heterotrophic cultures of microalgae, but microalgal species that can utilize xylose are few. At present, only one paper in the literature reported the xylose utilization by *Chlorella* strain, but only in the light or when glucose was present in the media (Hawkins 1999). On the other hand, Vidotti et al. (2014) tested xylose as carbon source for *Chlorella vulgaris* and *Scenedesmus bijugatus*; however none of the two strains were able to utilize this sugar.

To solve the xylose utilization issue, some directions can be taken: screening microalgal species that can grow on xylose as well as on glucose and genetically engineering strains to metabolize xylose. Moreover Liang (2013) mentions the possibility of coupling with other xylose utilizing microbial species, either yeast or bacteria in the same reactor or sequentially. In this way, glucose and xylose will both be consumed by different microorganisms, and different products can be resulted from the biomass hydrolysates. Recent research efforts have concentrated on the identification of microalgal strains capable of converting cellulosic feedstocks into oil (Chua and Somanchi 2012; Pourmir and Johannes 2011), but in light of the complexity involved in this application, few studies have been reported in the literature.

3.2 Microbial Oil Production by Bacteria

The advantages of using bacteria for lipid production are obvious since these microorganisms show usually higher growth rates than yeast and fungi and can be more easily genetically manipulated. Prokaryotes have fewer genes allocated all in one chromosome, and the lower complexity of genome demands less complexity of control mechanisms. However, while triacylglycerols (TAG) can be found in most eukaryotic organisms, they are rarely found in bacteria. Extractable lipids in bacteria are usually in the form of specialized lipids such as poly(3-hydroxybutiric acid) or other poly-hydroxyalkanoates. Only a few species of actinomycetes group such as *Rhodococcus* and *Mycobacterium* can synthesize and accumulate TAGs (Alvarez and Steinbuchel 2002). Among these, the genus *Rhodococcus* is frequently studied for its high capacity of accumulating lipids while growing in different substrates. *Rhodococcus opacus* PD630 is usually selected as the model microorganism for bacterial lipid accumulation. It was found to accumulate over 70 % of its cell dry weight (CDW) in FAs under nitrogen limitation condition (Alvarez et al. 2000) and grow at specific growth rates of 0.1–0.2 h^{-1} with glucose as carbon source (Xiong et al. 2012; Baboshin and Golovleva 2005).

Rhodococcus opacus PD630 was shown to grow well in the presence of lignocellulosic biomass pretreatment by-products such as furfural, 5-hydroxymethyl furfural (5-HMF), vanillin, vanillic acids, and *trans*-p-coumaric acid (TPCA), which are known for inhibiting microbial growth. In addition, the strain could use vanillin, vanillic acid, and TPCA for growth and lipid accumulation in the absence of other carbon sources (Table 2). Furfural and 5-HMF were also fully degraded but not as sole carbon sources. The strain could grow in corn stover, sorghum, and grass hydrolysates while accumulating TAGs (Wang et al. 2014).

Xylose is one of the substrates that cannot be metabolized by *Rhodococcus* species. However the metabolic pathway of D-xylose utilization was introduced into strains *Rhodococcus opacus* PD630 and *Rhodococcus jostii* RHA1. The heterogeneous expression of genes xylA (xylose isomerase) and xylB (xylulokinase) from *Streptomyces lividans* TK23 allowed the recombinant *R. opacus* PD630 and *R. jostii* RHA1 to grow on xylose and accumulate 68.3 and 52.5 % of lipids, respectively. However, the specific growth rate on xylose (0.07 h^{-1}) was 63 % lower than the observed for glucose (0.19 h^{-1}) (Xiong et al. 2012).

Regarding cellulose direct utilization, *Rhodococcus opacus* PD630 was engineered to express six cellulase genes from *Cellulomonas fimi* ATCC 484 (cenABC, cex, and cbhA) and *Thermobifida fusca* DSM43792 (cel6A) which enabled the strain to degrade cellulose into cellobiose. The modified strain was able to hydrolyze 9.3 % of cellulose provided, and a second strain engineered to utilize cellobiose could accumulate 15 % of lipids from the hydrolyzed cellulose (Hetzler et al. 2013).

Lignin is usually considered to be a bottleneck in lignocellulosic biomass bioconversion. It represents 10–25 % of lignocellulosic feedstocks, and few microorganisms are capable of metabolizing it, thus limiting its utilization as fuel for

Table 2 Oleaginous bacteria used for lipid production from different substrates

Microorganism	Substrate	Lipids (%)	Major FA (%, w/w)	References
Recombinant *R. opacus* PD630	Xylose	68.3	C16:0 (21.54 %), C17:0 (19.62 %)	Xiong et al. (2012)
R. opacus PD630	Corn stover, sorghum, grass hydrolysates	36	N.A.	Wang et al. (2014)
Recombinant *R. jostii* RHA1	Xylose	52.5	C17:0 (22.39 %), C17:1 (19.37 %)	Xiong et al. (2012)
R. opacus PD630	Lignin model compounds	18.2	C16:0 (27.9 %), C18:1 (14.2 %)	Kosa and Ragauskas (2012)
R. opacus DSM 1069	Lignin model compounds	11.4	C16:0 (22.9 %), C17:1 (14.5 %)	Kosa and Ragauskas (2012)
R. opacus PD630	Glucose	38	C16:0 (27.7 %), C18:1 (24.7 %)	Kurosawa et al. (2010)
Gordonia sp. DG	Orange waste	50	C22:0 (>30 %), C18:3 (>10 %)	Gouda et al. (2008)

Major *FA* the concentration of only the two major fatty acids is shown. *NA* not available data

energy cogeneration or bioconversion through pyrolysis. Recently lignin model compounds (4-HBA, VanA, and SyrA) were found to be directly metabolized by *R. opacus* DSM1069 and PD630 to lipid production (11.44 and 18.22 % of dry cell weight, respectively) (Kosa and Ragauskas 2012).

3.3 Microbial Oil Production by Fungi

Filamentous fungi usually grow at lower growth rates, yields, and productivities than yeasts. However some of these organisms are capable of producing lipids containing more than 70 % of polyunsaturated fatty acids (PUFAs) such as arachidonic acid and γ-linolenic acid. The composition of the lipids produced depends directly on the culture medium composition and may change during cultivation.

It is well known that lipid synthesis and accumulation occur under nitrogen limitation conditions. Interestingly arachidonic acid synthesis seems to occur after carbon source starvation (Nie et al. 2013). However, under carbon-depleted conditions, the concentration of total FAs remains constant, but a rapid increase in arachidonic acid content is observed while at the same time other FAs such as oleic and stearic acids are consumed. It was suggested that saturated FAs and oleic acid are converted to linolenic and γ-linolenic acids and subsequently to arachidonic acid (Eroshin et al. 2002). However if no carbon limitation is induced,

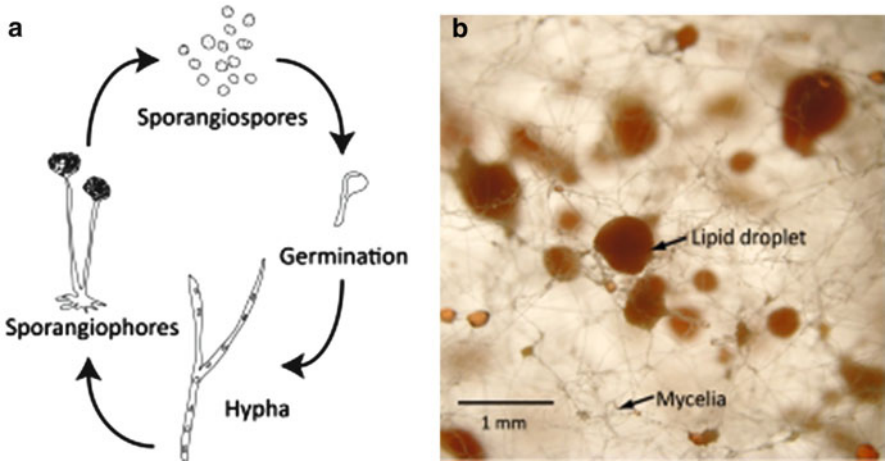

Fig. 4 *Mortierella alpina* (**a**) asexual lifecycle of the fungus. Haploid cells form sporangiophores, and sporangiospores germinate to hypha. (**b**) Fungal culture grown on PDA plate stained with 0.5 % triphenyl tetrazolium chloride. Lipid droplets are stained *brown* (Wang et al. 2011)

lipid composition is mainly composed by saturated and monounsaturated FAs as observed by Stressler et al. (2013) and Nie et al. (2013).

Strains of the genus *Mortierella* (Fig. 4) are regarded as some of the most productive organism for PUFA synthesis, with the capacity of accumulating 20–70 % of total lipids (Table 3) containing mostly PUFA (Sakuradani 2010). These fungi can grow at high growth rates of up to 0.566 h^{-1} when grown in non-limiting conditions and yield lipid productivities as high as 0.785 g/(L h) (Economou et al. 2011a).

Arachidonic acid is already commercially produced from *Mortierella* strains by Cargill Alking Bioengineering in Wuhan, China; by Nippon Suisan Kaisha in Tokyo, Japan; and by Martek Biosciences (DSM) in Columbia, USA (Tyburczy et al. 2011). These fungi are also one of the few highly productive oleaginous microorganisms that can grow well using xylose as sole carbon source (Huang et al. 2013).

Mortierella isabellina could grow on both C5 (arabinose, ribose, xylose, and mannose) and disaccharides (sucrose and cellobiose). When grown in the presence of model lignocellulosic inhibitor compounds, phenolic compounds were highly inhibitory, while furfural, 5-HMF, acetic acid, formic acid, and levulinic acid showed limited inhibition. Surprisingly acetic and formic acid improved lipid production by twofold as compared with that observed for the control. When grown on wheat straw, hydrolysate up to 53 % total lipids was obtained with a lipid yield of 16.8 % from the carbon sources (Zeng et al. 2013).

The oleaginous fungi *Mucor circinelloides* was grown on corn ethanol stillage resulting in 52 % total lipids. When the culture was supplemented with glycerol at the stationary phase, an increase of 46–61 % in lipid content was observed.

Table 3 Oleaginous fungi used for lipid production from different substrates

Microorganism	Substrate	Lipids (%, w/w)	Major FA (%)	References
Mortierella isabellina	Wheat straw hydrolysate	53	C18:1 53 %, 11.6 % PUFA	Zeng et al. (2013)
Mortierella alpina	Glucose	49	C20:4 38 %, 69 % PUFA	Stressler et al. (2013)
Mortierella isabellina	Corn stover, switchgrass, *Miscanthus,* and giant reed hydrolysates	35	N.A.	Ruan et al. (2013)
Mortierella alpina	Glucose (three stage fermentation)	64	C20:4 44.3 %, C18:0 16.5 %	Nie et al. (2013)
Mortierella alpina	Wheat straw hydrolysate	16.8	C18:1 49.9 %, C16:0 26.19 %	Zeng et al. (2013)
Aspergillus oryzae	Potato processing wastewater	40	C18:1 30.3 %, C18:19.3 %	Muniraj et al. (2013)
Mortierella isabellina	Xylose	64.3	C18:1 56.2 %, C16:0 24.9 %	Gao et al. (2013)
Mortierella isabellina	Rice hull hydrolysate	64.3	C18:1 50.7 %, C16:0 22.6 %	Economou et al. (2011b)
Mucor circinelloides	Ethanol corn stillage	61	C18:2 50 %, C18:1 29.6 %	Mitra et al. (2012)
Mucor circinelloides	Glucose	19.9	C18:1 37 %, 18.5 %	Vicente et al. (2009)
Cunninghamella echinulata	Xylose	65.5	C18:3 17 %	Fakas et al. (2009)

Major *FA* the concentration of only the two major fatty acids is shown
NA data not available

3.4 Microbial Oil Production by Yeast

Among fungi, oleaginous yeasts are distinguished by their capacity to accumulate high concentrations of lipids. Species such as *Rhodosporidium toruloides* and *Lipomyces starkeyi* have been found to accumulate lipids at around 60 and 70 % of dry cell weight (Shi et al. 2011). Microbial fermentation for producing biodiesel from lignocellulosic hydrolysates is receiving increasing attention; however, oleaginous yeasts that can utilize lignocellulosic hydrolysates are few (especially for acid lignocellulosic hydrolysates to accumulate lipids) and still at its initial stages (Table 4). Therefore, it is necessary to discover oleaginous strains that have high lipid yield on lignocellulosic biomass hydrolysates.

To date, SCO production from lignocellulosic biomass was usually carried out through a batch fermentation mode. It is possible that using a fed-batch or continuous fermentation mode could fulfill the high-cell-density cultivation on lignocellulosic hydrolysates, and this is undoubtedly beneficial for the industrialization of SCO production (Huang et al. 2013). Anschau et al. (2014) indicated the possibility

Table 4 Microbial oil production on lignocellulosic hydrolysates

Lignocellulosic biomass	Strains	Lipids (%)	References
Sugarcane bagasse	*Lipomyces starkeyi*	26.7	Anschau et al. (2014)
	Lipomyces starkeyi	18.5	Aristizabal et al. (2012)
	Yarrowia lipolytica	58.5	Tsigie et al. (2011)
	Trichosporon fermentans	15.8 g/L 59.5	Huang et al. (2012b) Huang et al. (2011)
Sweet sorghum bagasse	*Cryptococcus curvatus*	64	Liang et al. (2012)
Corncob	*Trichosporon cutaneum*	45.4	Chen et al. (2013)
	Trichosporon dermatis	40.1	Huang et al. (2012a)
Rice straw	*Trichosporon fermentans*	40.1	Huang et al. (2009)
Wheat straw	*Cryptococcus curvatus* *Rhodotorula glutinis* *Rhodosporidium toruloides* *Lipomyces starkeyi* *Yarrowia lipolytica*	33.5 25.0 24.6 31.2 4.6	Yu et al. (2011)

of the transformation of lignocellulosic materials into biodiesel studying the lipid production by *L. starkeyi* DSM 70296 using different feeding strategies. Glucose and xylose were used for batch, fed-batch, repeated fed-batch, and continuous cultures, and hemicellulose hydrolysate (H–H) was tested at continuous culture. High cell mass (~85 g/L) and lipid (~40 g/L) concentrations were achieved through both fed-batch and repeated fed-batch cultivations using glucose:xylose (30:70), with a maximum specific growth rate of 0.07 h^{-1}. The kinetic profile obtained from a continuous cultivation with H–H was similar to that obtained from cultivation in a synthetic medium. This may be explained by the fact that the hydrolysate contained some protein and microelements, which could promote the yeast growth and lipid accumulation. At a dilution rate of 0.03 h^{-1} with H–H, the cultivation yielded cell mass and lipid concentrations of 13.9 g/L and 3.7 g/L (26.7 %), with a maximum specific growth rate at batch step of 0.089 h^{-1}. Arabinose was not consumed throughout the cultivation. The inhibitor concentrations (acetic acid, furfural, and HMF) were reduced during the batch stage and the first residence times of the continuous cultivation, indicating that this yeast can use these compounds as carbon sources. This is of particular interest because it is possible that *L. starkeyi* is highly tolerant to inhibitors, which may allow a broader study of H–H from SCB in fermentation processes. Continuous cultivations at 0.03 h^{-1} using glucose:xylose (30:70, w/w) and H–H from SCB presented the highest yields and productivities, indicating that this approach is the most appropriate for scale-up (Fig. 5).

The same strain (*L. starkeyi* DSM 70296) was adapted by evolutionary engineering in culture medium containing SCBH (H–H) (Aristizabal et al. 2012). The H–H was obtained after seven sequential extraction steps of bagasse previously pretreated by steam explosion. The yeast was adapted by evolutionary engineering in culture medium containing increasing concentrations of H–H. As a result, a yeast adapted to culture medium containing 30 % of H–H was obtained, which showed higher cell concentration (9.79 g/L) with lower lipid content (18.5 %) compared to

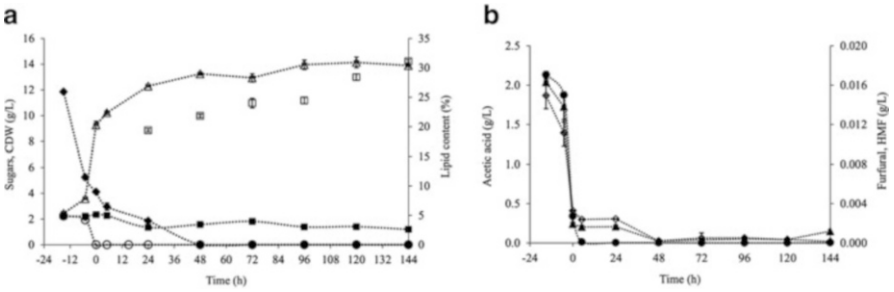

Fig. 5 (**a**) CDW (△), lipid content (□), xylose (♦), glucose (○), arabinose (■), (**b**) acetic acid (●), furfural (▲), and HMF (◊) concentrations during continuous cultivation at 0.03 h^{-1} with H–H

not adapted strain (5.21 g/L of cells with 31.5 % of lipids) with a maximum specific growth rate of 0.039 h^{-1}.

The possibility of utilizing detoxified sugarcane bagasse hydrolysate (DSCBH) was investigated as an alternative carbon source to culture *Y. lipolytica* Po1g for microbial oil and biodiesel production (Tsigie et al. 2011). Compared with (DSCBH) medium, growth in the non-detoxified sugarcane bagasse hydrolysate (NDSCBH) medium was limited because of the presence of inhibitors (furfural and HMF).

Cryptococcus curvatus has great potential in fermenting unconditioned hydrolysates of sweet sorghum bagasse (Liang et al. 2012). During yeast fermentation, glucose and xylose were consumed simultaneously, while cellobiose was released from the residual bagasse. Surprisingly, the cellobiose utilization rates were much higher than those on glucose and xylose. It seems that *C. curvatus* could engulf cellobiose rapidly, but then it had to spend more energy on digesting this disaccharide, which resulted in the lowest cell yield among all of the sugars tested.

Trichosporon fermentans was proved to be able to use sulfuric acid-treated sugarcane bagasse hydrolysate as substrate to grow and accumulate lipid (Huang et al. 2012b). Although the volumetric productivity of *T. fermentans* on bagasse hydrolysate is not as high as that of *Y. lipolytica* on industrial fats, the higher lipid concentration and moderate volumetric productivity make oleaginous yeast *T. fermentans* very promising for lipid production from abundant and inexpensive lignocellulosic materials. The effects of five representative aldehydes in lignocellulosic hydrolysates on the growth and the lipid accumulation of oleaginous yeast *T. fermentans* were also investigated (Huang et al. 2011). There was no relationship between the hydrophobicity and the toxicity of aldehyde, and 5-hydroxymethylfurfural was less toxic than aromatic aldehydes and furfural. Binary combination of aromatic aldehydes caused a synergistic inhibitory effect, but a combination of furan and aromatic aldehydes reduced the inhibition instead. Furthermore, the inhibition of aldehydes on cell growth was more dependent on inoculum size, temperature, and initial pH than that on lipid content.

The inhibitory effect of lignocellulose degradation products on the oleaginous yeast fermentation was carefully investigated by Chen et al. (2009). Preliminary

screening was carried out in the minimum nutritious medium without adding any expensive complex ingredients and then was carried out in the lignocellulosic hydrolysate pretreated by dilute sulfuric acid. Seven typical lignocellulose degradation products formed in various pretreatments and hydrolysis processing were selected as the model inhibitors, including three organic acids, two furan compounds, and two phenol derivatives. The inhibition of the degradation compounds on the cell growth and the lipid productivity of the selected oleaginous yeasts were examined. Acetic acid, formic acid, furfural, and vanillin were found to be the strong inhibitors for the fermentation of oleaginous yeasts, while levulinic acid, 5-hydroxymethylfurfural, and hydroxybenzaldehyde were relatively weak inhibitors. *T. cutaneum* 2.1374 was found to be the most adopted strain to the lignocellulose degradation compounds.

In summary, these works showed the great potential of SCO production from lignocellulosic biomass. The use of lignocellulosic hydrolysates as substrates could serve as the basis for the industrialization of SCO production. However, many problems in this process still exist that must be solved.

4 Fatty Acid Composition in Microbial Lipids

Microbial oils may have great potential for biodiesel production due to FA composition. Microorganisms also produce edible oils rich in PUFA for dietary supplements and infant nutrition applications (Ratledge 2004; Cahoon and Schmid 2008). It is possible to observe that the FA profile produced by microorganisms is quite similar to vegetable oils (Table 5) and can thus be used as a substitute for biofuels.

According to Table 5, most PUFAs produced by fungi, algae, and bacteria have been proposed as producers of certain PUFAs. Yeasts are not potential producers of PUFAs. The number of unsaturations does not have an effect only on the values of viscosity and density of biodiesel but also on the oxidative stability (Lobo et al. 2009). Most biodiesel fuels present significant amounts of esters of oleic, linoleic, or linolenic acids, influencing the oxidative stability of the fuel (Knothe 2005). Biodiesel presents portability, greater renewability, higher combustion efficiency, lower sulfur and aromatic content, and higher cetane number than diesel oil (Balat 2011). It is recommended that these fuels meet the biodiesel standards of US ASTM D 6751 and European EN 14214 (Ashraful et al. 2014).

When the extraction process is used for oils, it seeks to achieve the following objectives: (1) to obtain a fat or oil uninjured and free from undesirable impurities, (2) obtain the best possible performance without changing the cost of litigation, and (3) produce a minimum of waste with the greatest possible value. The choice of the optimal extraction method for large-scale oil must meet certain criteria: the ease with which the cell breaks down, the cost of the method, rate of cell disruption, and so on (Hulteberg et al. 2008).

Table 5 Fatty acid composition of some microorganisms

Microorganism	References	Relative average fatty acids (%, w/w)					
		C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
Microalgae	Meng et al. (2009)	12–21	55–57	1–2	58–60	4–20	14–30
C. vulgaris	Wynn and Ratledge (2005)	16	11.9	2	58	9	14
Yeast	Meng et al. (2009)	11–37	1–6	1–10	28–66	3–24	1–3
Lipomyces starkeyi	Anschau et al. (2014)	33.2	2.6	8	54.4	1.3	0.5
Rhodotorula glutinis	Alvarez and Steinbuchel (2002)	36	1	3	47	8	–
Fungi	Meng et al. (2009)	7–23	1–6	2–6	19–81	8–40	4–42
M. circinelloides	Vicente et al. (2009)	20	2.3	2	37	14.3	18.5
Bacteria	Meng et al. (2009)	8–10	10–11	11–12	25–28	14–17	–
R. opacus	Alvarez and Steinbuchel (2002)	25.7	9.5	3.5	22.0	–	–
Vegetable oils							
Palm oil	Liu and Zhao (2007)	33.0	0.1	4.7	55.1	1.6	–
Soybean	Balat (2011)	11.9	0.3	4.1	23.2	54.2	6.3
Canola	Haagenson et al. (2010)	3.9	–	1.1	64.4	20.4	9.6

Tables 6 contains, respectively, the properties of FAMES collected from the literature and the results of the prediction of quality parameters of oleaginous yeast and microalgae (Lacerda et al. 2013) compared with the American, European, and Brazilian standards. The values of heat of combustion (HC), index of oxidative stability (OSI) and iodine value (II) are out of limits imposed by these standards, however, the values of density (ρ), viscosity (u) and cetane number (CN) are within it. Even though the indexes are slightly out of bounds, the biodiesel obtained from Chlorella vulgaris and *Lipomyces starkeyi* may still be used as fuel.

Biodiesel produced by *Lipomyces starkeyi* has the advantage of being deficient in of C18:3, which is limited to 12 % by EN 14214. *Chlorella vulgaris* has an advantage if compared to mitigation of carbon dioxide associated with the production of biofuel. In both cases, the FA profile showed compounds commonly found in biodiesel. The results of the prediction quality are classified into three available standards, demonstrating the potential of *C. vulgaris* and *L. starkeyi* oil as raw materials for biodiesel production (Lacerda et al. 2013).

Yeasts have several difficulties for lipid extraction, including the presence of a thick cell wall that renders the yeast cells resistant to many solvents, as well as the possible presence of lipases in their cell extracts, and most of the neutral lipids are intracellularly stored in lipid bodies. However, lipid bodies also contain other lipophilic compounds, in particular aromatic compounds, which are difficult to remove during lipid purification (Ageitos et al. 2011). Table 7 shows the profile of TAG for some lipid extraction methods from *L. starkeyi*.

Table 6 Comparison of the results with the limits set by the rules

	ρ (kg m^{-3})	υ (mm^2 s)	ΔHC (kJ g^{-1})	OSI (h)	CN	II (gI2/100 g)	% C18:3
Chlorella vulgaris[a]	863	3.9	39.2	13.8	53	97	12.72
Lipomyces starkeyi[a]	864	4.4	39.7	12.5	63	49	0.00
ASTM D6751	–	1.9–6.0	–	–	Min 47	–	–
EN 14214/ EN14213	860–900	3.5–5.0	35	Min 6 e 4	Min 51	Max 120/130	12
ANP 255/2003	–	–	–	–	Min 45	–	–

ρ density, υ viscosity, ΔHC heat of combustion, *OSI* index of oxidative stability, *CN* cetane number, II iodine value
[a]Results from Lacerda et al. (2013)

Table 7 Lipid extraction methods from *L. starkeyi*

Solvents	Polarity	Extraction efficiency (%)	TAG (%)	DAG (%)	MAG (%)
Soxhlet					
Hexane	0.1	5.06	64.14	20.64	15.20
Chloroform:Methanol (2:1)	4.4	95.41	60.71	20.14	19.13
Ethanol	4.3	65.05	35.93	18.66	45.39
Butt					
Hexane	0.1	56.06	58.47	14.93	26.58
Chloroform:Methanol (2:1)	4.4	90.32	47.50	10.03	42.46
Ethanol	4.3	66.40	45.97	19.46	34.56
Cell hydrolysis					
Hexane	0.1	89.32	81.03	10.10	8.9

TAG triacylglycerols, *DAG* diacylglycerol, *MAG* monoacylglycerol. Adapted from Reis et al. (2012)

Glycerides are desirable for biodiesel production mainly for alkaline transesterifications (Chisti 2007). Acylglycerols generally have a lower degree of unsaturation than other lipid fractions (i.e., polar lipids) and produce FAME with higher oxidation stability (Halim et al. 2012).

From a technological point of view, the TAG profile represents a key to the understanding of the several physical properties of an oil or fat. The ability to change the physical properties of fats has attracted great interest in recent years. Information on the TAG profile is needed to control these processes (Buchgraber et al. 2004).

The microbial oil production process requires four main steps: cell disruption, oil extraction, separation of value-added compounds (PUFAs), and esterification/

cracking of lipids. The improvement of each of these steps demonstrates the use and application of these lipids for biofuels, food and cosmetic industries, jet fuels, health supplements, and PUFA.

5 Genetic and Metabolic Engineering to Enhance Microbial Lipids

In the last years metabolic and genetic engineering has established itself as an enabling technology for biofuel development. Those tools are able to modify microorganisms aiming to improve their natural features and even introduce new characteristics. Metabolic engineering emerged approximately 15 years ago as a distinct field that is differentiated from genetic engineering by its focus on the properties of biosynthetic and metabolic pathways in their entirety, instead of single genes and enzymes (Liang and Jiang 2013). Oleaginous microorganisms able to use lignocellulosic hydrolysates as some yeasts, molds, bacteria, and microalgae can be engineered to be a lipid platform production.

As was presented in this chapter, only few microorganisms are able to convert sugars, from lignocellulosic materials, into lipids. However, these native microorganisms, which are derived from environmental isolates, are often distinct from the microorganisms that are traditionally used and that have been proved to be effective in industrial processes. Furthermore, these isolated strains typically suffer from a lack of genetic and molecular biology traditional tools and therefore require much scientific investment to transform them into modifiable production platforms. Nevertheless, the innate capacity and potential of these cells—which may be able to use recalcitrant substrates or withstand toxic products—is immense (Alper and Stephanopoulos 2009).

To overcome this lack of traditional genetic and metabolic tools, that can be used to genetically manipulate those strains, other genetic approaches as site-direct and random mutagenesis, as made for yeast *Lipomyces starkeyi* (Tapia et al. 2012), which presented a productivity increase of 15.1 % in biomass and 30.7 % in lipid productivity, and microalga *Isochrysis affinis galbana* (Bougaran et al. 2012) resulted in a 1.8-fold increase for neutral lipid productivity, could be important to enhance their natural capacity to storage lipids. However, it is necessary to understand the metabolic pathways linked to lipid material metabolism, in order to select the genes and the best approach to metabolic engineering.

Oleaginous microorganisms do not have high oil levels under balanced nutrient conditions; however, when a given substrate is limiting—typically nitrogen—they channel carbon sources into lipid formation. It has been shown that phosphorus limitation has some advantages; for example, the nitrogen content of the substrate biomass does not influence lipid accumulation. FA synthesis is the first step of lipid accumulation. Subsequently, phospholipid (PL) and triacylglycerol (TAG) (also known as “neutral lipid”) synthesis results in membrane and reserve lipid accumulation, respectively. Conversion of these lipids into FA short-chain alcohol esters

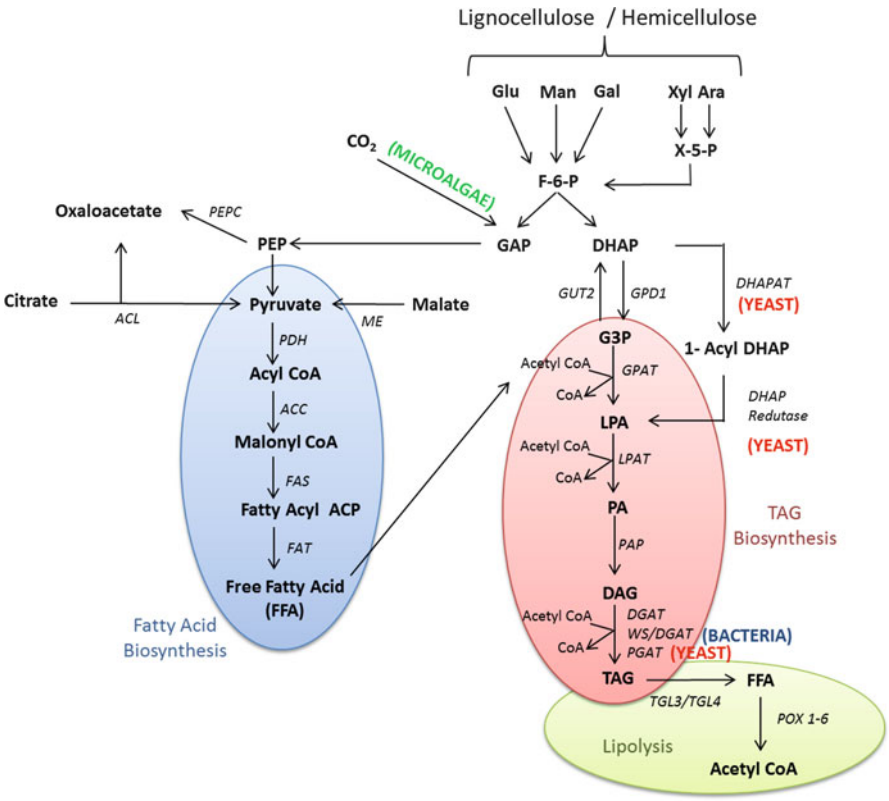

Fig. 6 The fatty acid and TAG biosynthesis pathway in microorganisms. For microalgae, both inorganic carbon (CO_2) and organic carbon sources (glucose) can be utilized for lipid production. For yeasts, de novo formation of LPA can occur either through the G3P or DHAP pathways. In yeasts, the DGAT and PDAT catalyze TAG formation. In *Acinetobacter calcoaceticus* ADP1 (bacteria), WS/DGAT exhibits the DGAT activity. *GAP* glyceraldehyde 3-phosphate, *DHAP* dihydroxyacetone phosphate, *PEP* phosphoenolpyruvate, *ACP* acyl-carrier protein, *FFA* free fatty acid, *G3P* glycerol-3-phosphate, *LPA* lysophosphatidate, *PA* phosphatidate, *DAG* diacylglycerol, *CDPDAG* CDP-diacylglycerol, *TAG* triacylglycerol, *PDH* pyruvate dehydrogenase, *PEPC* phosphoenolpyruvate carboxylase, *ME* malic enzyme, *ACL* ATP citratelyase, *ACC* acetyl-CoA carboxylase, *MAT* malonyl-CoA: ACP transacetylase, *FAS* fatty acid synthetase, *FAT* acyl-ACP-thioesterase, *GPAT* glycerol-3-phosphate acyltransferase, *LPAT* lysophosphatidate acyltransferase, *PAP* phosphatidic acid phosphatase, *DGAT* diacylglycerol acyltransferase, *WS/DGAT* wax ester synthase/acyl-CoA-to-diacylglycerol acyltransferase, *PDAT* phospholipid: diacylglycerol acyltransferase, *DHAPAT* DHAP acyltransferase, *GPD1* and *GUT2* encoding glycerol 3-phosphate dehydrogenase, *TGL3* and *TGL4* encoding triacylglycerol lipases, *POX1–6* encoding the six acyl-CoA oxidases [Adapted from Liang and Jiang (2013)]

[mainly methanol (FAME) and ethanol (FAEE)] is the final step in biodiesel production (Kosa and Ragauskas 2011). The enzymes and steps that lead to TAG accumulation in eukaryotes and prokaryotes microorganism, using as carbon sources sugars from lignocellulosic biomass, are shown in Fig. 6.

The genetic engineering of oleaginous microorganism is focused in enhancing the FFA and TAG biosynthesis by overexpressing main enzymes linked to those metabolic routes, as ACC1, FAT, ME, and ACL (free FA biosynthesis) and GPAT, LPAT, GPD1, and GUT2 (TAG biosynthesis), or partially blocking competing pathways as lipolysis. Furthermore a multigene transgenic approach can be used in order overexpressing more than one key enzyme in the TAG pathway to enhance lipid biosynthesis as showed in Table 8.

Efficient fermentation of hemicellulosic sugars is critical for the bioconversion of lignocellulosics to lipids. While most hexoses are readily phosphorylated as soon as they enter the cell, hemicellulosic sugars must go through several biochemical steps before phosphorylation. Eukarya and bacteria use two distinct pathways each for the assimilation of D-xylose and L-arabinose (Van Vleet and Jeffries 2009) as showed in Fig. 7.

6 Future Perspectives

Only few studies about oleaginous microorganisms have used metabolic and genetic engineering tools to improve metabolism of sugars: oleaginous fungi (*Mortierella isabellina*) and yeasts (as *Lipomyces starkeyi* and *Rhodotorula glutinis*) have a good capacity to assimilate those sugars, and also, majority of autotrophic microalgae are used to accumulate lipids. The lack of traditional genetic and metabolic tools for those nonconventional species, as discussed before, also contributes to the small number of works in this area. However some studies have used heterologous pathways to introduce xylose assimilation capacity in wild strains that accumulate good amounts of lipids but are not able to metabolize xylose. Bacteria *Rhodococcus* sp., which is known as high lipid production strain, was engineered to express heterogeneously two well-selected genes, xylA, encoding xylose isomerase, and xylB, encoding xylulokinase from *Streptomyces lividans* TK23, under the control of the tac promoter with an *Escherichia coli-Rhodococcus* shuttle vector. Lipid produced from xylose by recombinants of *R. jostii* RHA1 and *R. opacus* PD630 carrying xylA and xylB represented up to 52.5 and 68.3 % of the CDW, respectively (Xiong et al. 2012). Microalgae are also engineered to metabolize xylose as described by Solazyme patent (Chua and Somanchi 2012).

Metabolic and genetic engineering and further other genomics, transcriptomics, and metabolomics tools provide more information and new ways on enhancing lipid production in oleaginous microorganisms, optimizing FA profiles, enhancing lipid accumulation, and improving the use of low-cost raw materials as lignocellulosic hydrolysates.

Table 8 Metabolic engineering strategies employed to enhance lipid biosynthesis in oleaginous organisms

Genes (enzymes)	Source—species	Receiver—species	Note	References
FFA biosynthesis				
accA, accB, accC, accD, (ACC), tesA (thioesterase I)	*E. coli* (BL21) (bacteria)	*E. coli* (BL21) (bacteria)	6× fatty acid synthesis	Davis et al. (2000)
Acc1 (ACC)	*Cyclotella cryptica* (algae)	*Cyclotella cryptica* (algae)	2–3× ACC activity, no change in lipid content	Dunahay et al. (1996), Dunahay et al. (1995)
Acc1 (ACC)	*Yarrowia lipolytica* (yeast)	*Yarrowia lipolytica* (yeast)	2× lipid content	Tai and Stephanopoulos (2013)
Acc1 (ACC)	*Mucor rouxii* (fungi)	*Hansenula polymorpha* (yeast)	+40 % fatty acid content	Ruenwai et al. (2009)
FAT	*Ricinus communis* (plant)	*E. coli* ML103 (bacteria)	>2.0 g/L fatty acid content	Zhang et al. (2011)
Malic enzyme (ME)	*Mortierella alpina* and *Mucor circinelloides* (fungi)	*Mucor circinelloides* (fungi)	2.5× lipid accumulation	Zhang et al. (2007)
ACL	*Aspergillus oryzae* (fungi)	*Aspergillus oryzae* (fungi)	1.7× fatty acid content	Tamano et al. (2013)
TAG biosynthesis				
ΔGUT2	*Y. lipolytica* (yeast)	*Y. lipolytica* (yeast)	3× lipid content	Beopoulos et al. (2008)
GPD1	*Y. lipolytica* (yeast)	*Y. lipolytica* (yeast)	1.5× TAG content	Dulermo and Nicaud (2011)
ΔGUT2,GPD1	*Y. lipolytica* (yeast)	*Y. lipolytica* (yeast)	5.6× TAG content	Dulermo and Nicaud (2011)
DGAT	*Arabidopsis* (plant)	Yeast	200–600× DGAT activity	Jako et al. (2001)
Blocking competing pathways				
DAGPase	*Chlamydomonas* (microalgae)	*Chlamydomonas* (microalgae)	10× TAG content	Li et al. (2010)
ΔTGL3, ΔTGL4 (TAG lipases)	*Y. lipolytica* (yeast)	*Y. lipolytica* (yeast)	+ Lipid production	Dulermo and Nicaud (2011)
Multigene approach				
ACP, KAS, FAT	*Haematococcus pluvialis* (microalgae)	*Haematococcus pluvialis* (microalgae)	+ fat acid synthesis	Lei et al. (2012)
POX1-6 (AOXs), MFE1, GPD1, DGUT2	*Y. lipolytica* (yeast)	*Y. lipolytica* (yeast)	+ lipid accumulation	Dulermo and Nicaud (2011)

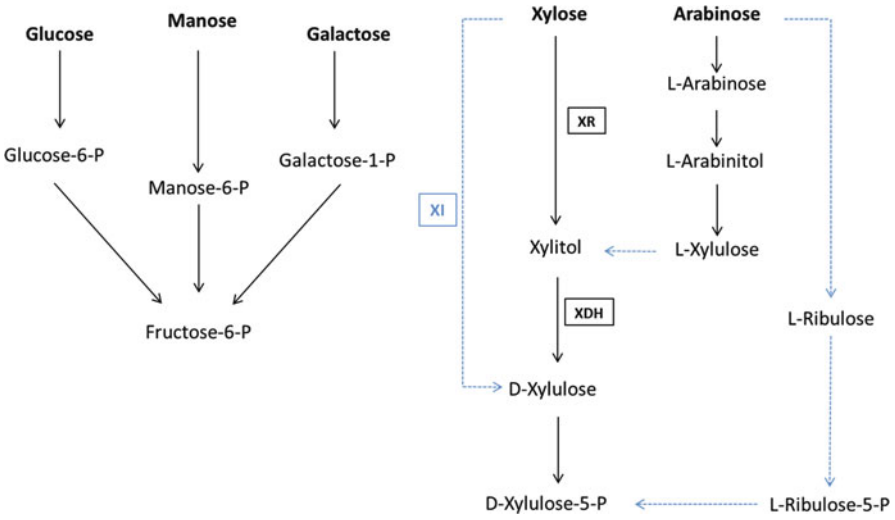

Fig. 7 Hexose and pentose pathways for Eukarya and Bacteria. Mannose, glucose, and galactose are quickly phosphorylated after uptake in the cell. Pentoses are assimilated by yeast (*solid lines*) through an oxidoreductase pathway, whose bottleneck to xylose assimilation is the imbalance redox generated by xylose reductase (XR) and xylitol dehydrogenase (XDH) distinct cofactor preference. The same does not occur in bacteria, once single step xylose assimilation is done by enzyme xylose isomerase (XI). *Blue dotted lines* represent pentose assimilation pathways for Bacteria

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Lignocellulosic Hydrolysates for the Production of Polyhydroxyalkanoates

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Contents

Abstract Lignocellulosic biomass, the worldwide most abundant renewable raw material, comprises different fractions such as carbohydrates, proteins, and fats that can be converted to value-added products, fuels, and chemicals through the implementation of the *Biorefinery* concept.

Upgrading of the biomass carbohydrate fraction to various building blocks and end-materials can take place either through chemical or biochemical processes. This chapter provides an overview of the current research focusing on the bioproduction of polyhydroxyalkanoates (PHAs) using processed lignocellulosic materials as main carbon source. The type of biomass, as well as the treatment applied, originates lignocellulosic hydrolysates with a wide range of sugar compositions and different titers of organic acids and inhibitory compounds. This fact influences not only the overall PHA productivity but also the extraction yield,

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which is very sensitive to the polymer cell content. So far, the majority of the reports in the literature are still at a *proof of concept* stage, even though a few studies already show a successful contribution towards a *Biorefinery* approach.

1 Introduction

Polyhydroxyalkanoates (PHAs) are biodegradable and biocompatible polyesters, synthesized by several microbial strains as carbon and energy storage under adverse conditions (i.e., excess carbon source and limiting concentrations of an essential nutrient as nitrogen, phosphorous, magnesium, or oxygen) (Steinbüchel 1996). These bioplastics are biodegraded to CO_2 and H_2O in aerobic environments or to CH_4 and CO_2 in anaerobic conditions. The most common, the homopolymer poly-3-hydroxybutyrate (P(3HB)), is a brittle and stiff material. However, PHA polymeric chains composed by different monomers at different molar fractions originate materials with a wide spectrum of mechanical, chemical, and thermal properties, suitable for a broad range of applications (Martin and Williams 2003; Sudesh et al. 2000; Chen 2009; Zinn et al. 2001; Chen and Wu 2005; Brigham and Sinskey 2012).

An efficient choice of the downstream extraction steps is crucial, as PHA is accumulated within the cells. Several methods for PHA recovery and purification are described in the literature, depending on the organism used for production and on the final application of this biomaterial (Jacquel et al. 2008). In general, higher purities are achieved when using solvents, although some procedures (e.g., using chloroform) are not feasible at industrial scale. Even though sequences of solvent-free aqueous methods were optimized for high-scale production and are already used for PHA extraction in commercial production, intensive research is still needed to insure lower process costs with high polymer recovery yields and purities.

P(3HB) and other PHAs are currently being produced by several companies [*PHB industrial*/*copersucar* in Brazil; *Biomatera* and *PolyFerm* in Canada; *Tianan Biopolymer*, *Tianjin GreenBio Materials*, and *DSM* and *Tianzhu* in China; *Biomer* in Germany; *Bio-On* in Italy; *Kaneka* in Japan; *DaniMer*/*Meredian* in the USA (Avérous and Pollet 2012)], yet at costs considerably higher than those of petrochemical plastics. The main carbon source for growth and polymer accumulation could account for 70–80 % of the total cost of raw materials and over 40 % of the total production costs (Choi and Lee 1997). The choice of the carbon source is thus a key factor to produce PHAs at competitive prices for industrial scale. Industrial and agricultural by-products such as molasses, whey, lignocellulosic raw materials, fats, oils, waste cooking oil, glycerol, and wastewater have already been successfully tested as main carbon source for PHA production (Du et al. 2012; Albuquerque et al. 2007; Mothes et al. 2007; Cavalheiro et al. 2009; Pais et al. 2009;

Kulpreecha et al. 2009; Bengtsson et al. 2008; Koller et al. 2005; 2010). Among these, lignocellulosic materials are a promising choice due to:

(1) The high quantities available worldwide
(2) The fact that most agricultural and forestry processes generate considerable amounts of lignocellulosic waste
(3) Its renewable nature
(4) Being a carbon source which is a nonedible feedstock

Most efforts are currently being directed to increasing the concentration of monomeric sugars as glucose, xylose, arabinose, mannose, galactose, and rhamnose and to decreasing the amounts of inhibitors in the final hydrolysate by optimizing the hydrolysis step and adopting adequate pretreatments.

2 Lignocellulosic Hydrolysates

Biological conversion of lignocellulosic materials to value-added building block chemicals and fuels usually involves several sequential steps: lignocellulose pretreatment/fractionation, enzymatic cellulose hydrolysis to fermentable sugars (e.g., glucose and xylose), and fermentation. Besides ethanol, many bio-based chemicals such as lactic acid, succinic acid, itaconic acid, and levulinic acid could be produced based on lignocellulosic materials (Lucia et al. 2006; Abdel-Rahman et al. 2011; Maas et al. 2008; Neureiter et al. 2004; Ragauskas et al. 2006; Yang and Wyman 2008; Zeikus et al. 1999; Mussatto and Teixeira 2010).

2.1 Nature of the Biomass

Generally, *Biorefinery* systems can be classified into whole-crop biorefineries (using raw materials such as cereals or maize), green biorefineries (using naturally wet biomass, such as green grass, lucerne, clover, or immature cereal), and the lignocellulosic feedstock biorefineries (using naturally dry raw materials such as cellulose-containing biomass and wastes) (Kamm and Kamm 2004). In this chapter, only the last type will be considered.

Lignocellulosic materials are mainly comprised of cellulose, hemicellulose, and lignin. Cellulose is a linear polymer of repeating β-D-glucopyranose units, joined together in long chains by β-1,4 glycosidic bonds (Mussatto and Teixeira 2010). These long chains are linked together through inter- and intramolecular hydrogen bonds and van der Waals forces and, for this reason, cellulose is highly crystalline. Hemicellulose is a branched polysaccharide that consists of pentoses, mainly xylose and arabinose, and hexoses such as glucose, galactose, and mannose. Cellulose and hemicellulose are embedded in a complex lignin matrix which is a polymer of phenolic units. Lignin acts as a binder of the lignocellulosic constituents, giving the

Table 1 Composition of some lignocellulosic materials

Compound	Unit (dry basis)	Softwood		Hardwood		Agricultural residues		
		Spruce	Pine	Birch	Willow	Wheat straw	Rice straw	Corn stover
Cellulose Glucan[a]	%	43.4	46.4	38.2	43.0	38.2	34.2	35.6
Hemicellulose	%	18.0	22.9	19.7	29.3	24.0	24.5	22.1
Xylan (C5)	%	4.9	8.8	18.5	24.9	21.2	24.5	18.9
Arabinan (C5)	%	1.1	2.4	ND	1.2	2.5	Nd	2.9
Mannan (C6)	%	12.0	11.7	1.2	3.2	0.3	Nd	0.3
Lignin	%	28.1	29.4	22.8	24.2	23.4	11.9	12.3

Adapted from Jørgensen et al. (2007)
[a]Mainly from cellulose
ND Not detected, *Nd* Not determined

plant structural support, impermeability, and resistance against microbial attack and oxidative stress.

Lignocellulosic hydrolysates can either be a result of the processing of wood such as hard wood (e.g., willow, aspen, and oak) or soft wood (e.g., spruce and pine), agricultural residues like corn stover, and different types of straw (rice and wheat straw) and municipal solid waste.

The composition of the different lignocellulosic materials may vary substantially among the various sources (Jørgensen et al. 2007; Jeffries 1983). Wood hydrolysates are mostly derived from forest residues and differ in composition, depending if the wood belongs to the group of the softwoods or hardwoods. Softwood hemicellulose has a higher proportion of C6 monomeric sugars like glucose and mannose than hardwood hemicellulose, which usually contains a higher proportion of xylose units (C5 monomeric sugar). Agricultural waste products like wheat straw, rice straw, and corn stover are also rich in xylose and in general have a lower content of lignin comparing to softwoods which have the highest lignin content (Table 1).

2.2 *Production of Lignocellulosic Hydrolysates*

Cellulose and hemicellulose are natural polymers that constitute an excellent source of carbon to be used in different biological processes after hydrolysis to monomeric sugar units. Due to its composition, cellulose polymer chains are highly crystalline structures that are water insoluble and resistant to depolymerization (Mosier et al. 2005). Unlike cellulose, hemicellulose is not crystalline. The branched organization of hemicellulose confers it a random, amorphous structure and makes it easily hydrolyzed by dilute acids, bases, and hemicellulases (Palmqvist and Hahn-Hagerdal 2000). Both polysaccharides are surrounded by lignin which is covalently linked to hemicellulose, conferring mechanical strength to the whole plant structure (Chabannes et al. 2001). Due to its recalcitrant nature, the disruption

of the lignocellulosic structure and the degradation of the carbohydrate polymers to fermentable sugars often involve two steps: first, the pretreatment step in which the hemicellulose structure is broken down to mono and oligosaccharides and second, the hydrolysis of the cellulose fraction. Lignin remains as a solid by-product. Hydrolysis of cellulose can be achieved either by weak acids or enzymatically (Olsson and Hahn-Hagerdal 1996).

2.2.1 Pretreatment Methods

Different traditional pretreatment methods have been used to disrupt the structure of the cellulosic biomass. They are either physical, physicochemical, chemical, or biological (Mosier et al. 2005). Physical pretreatment methods include comminution (mechanical reduction in biomass particulate size), steam explosion, and hydrothermolysis. Among the chemical methods diluted or concentrated acids or bases (H_2SO_4, NaOH, and lime being the most common), ozone, H_2O_2 (oxidative delignification), and organosolv—a technique that solubilizes lignin and hemicelluloses—have been used. Organosolv uses an organic or aqueous/organic solvent mixed with an inorganic acid catalyst at temperatures ranging from 140 to 220 °C causing lignin to break down into fragments that are soluble in the solvent system (Mosier et al. 2005; Fitzpatrick et al. 2010). This technique yields three separate fractions: dry lignin, an aqueous hemicellulose stream, and a relatively pure cellulose fraction (Duff and Murray 1996).

Physicochemical pretreatment combines chemical and physical treatment processes such as liquid hot water or steam explosion with chemical addition of acids (H_2SO_4) or alkalis (NH_4OH). Often, milder chemical conditions are used, but under more extreme operational conditions such as high pressures and temperatures (Fitzpatrick et al. 2010). AFEX or ammonia fiber/freeze explosion pretreatment yields optimal hydrolysis rates at low enzyme loadings and is particularly suited for herbaceous and agricultural residues (Mosier et al. 2005; Dale et al. 1996). This method works only moderately well on hardwoods and is not attractive for softwoods (McMillan 1994). The AFEX pretreatment simultaneously reduces lignin content and removes some hemicellulose while decrystallizing cellulose. The moderate temperatures (<90 °C) and pH values (<12.0) of the AFEX treatment minimize the formation of sugar degradation products (Mosier et al. 2005).

Finally, the biological pretreatment uses microorganisms to treat the lignocellulosic biomass and enhance enzymatic hydrolysis. The applied microorganisms usually degrade lignin and hemicellulose. Cellulose is degraded to a lesser extent since it is more recalcitrant to the biological attack. Several fungi such as brown-, white-, and soft-rot fungi have been used for this purpose. White-rot fungi are among the most effective microorganisms for biological pretreatment of lignocelluloses. Lignin degradation by white-rot fungi occurs through the action of lignin-degrading enzymes such as peroxidases and laccase (Taherzadeh and Karimi 2008; Kumar et al. 2009). Although the advantages of biological pretreatment include low-energy requirements and mild environmental conditions, the rate of hydrolysis in most biological pretreatment processes is very low (Kumar et al. 2009).

In addition to the traditional pretreatment techniques mentioned above, fractionation, a newer class of pretreatment, aims not only to improve hydrolysis but also to separate the core constituents of lignocellulosic biomass: cellulose, hemicellulose, and lignin (Fitzpatrick et al. 2010). This strategy has advantages since each component may be upgraded separately, improving the economy of a *Biorefinery* process substantially.

Zhang et al. (2007) used a mix of solvents: nonvolatile cellulose solvent (concentrated phosphoric acid), a highly volatile organic solvent (acetone), and water to fractionate different lignocellulosic materials such as corn stover (agricultural residue), switchgrass (herbaceous plant), hybrid poplar (hardwood), and Douglas fir (soft wood) to amorphous cellulose, hemicellulose, lignin, and acetic acid under modest reaction conditions (50 °C and atmospheric pressure) (Zhang et al. 2007). Nearly theoretical sugar yields and high enzymatic hydrolysis rates were achieved due to the low sugar degradation and the high digestibility of cellulose in the amorphous state obtained during fractionation. Although solvent recycling is possible, this type of pretreatment involves a high number of unit operations and is thus very costly.

Another technology for fractionation is using ionic liquids. Ionic liquids (ILs) are organic salts that exist as liquids at temperatures below 100 °C. Recent studies have indicated that both cellulose and lignin can be dissolved in a variety of ILs and easily be regenerated from these solutions by means of addition of a non-solvent (Swatloski et al. 2002; Dadi et al. 2006; Kim et al. 2010; Stark 2011). Dadi et al. (2006) used 1-n-butyl-3-methylimidazolium chloride to dissolve cellulose. The regenerated cellulose had an amorphous structure allowing a greater number of sites for enzyme adsorption and improving the enzymatic hydrolysis rate by 50-fold.

2.2.2 Hydrolysis of Cellulose

The hydrolysis of cellulose is usually performed by acids or by enzymes. When concentrated acids such as H_2SO_4 or HCl are used during the pretreatment, aiming at the disruption of the lignocellulosic structure, hydrolysis of cellulose occurs concomitantly. In this case, pretreatment and hydrolysis are carried out in one step (Olsson and Hahn-Hagerdal 1996). The acid concentration used in the concentrated acid hydrolysis process is in the range of 10–30 %. High hydrolysis yields of cellulose, reaching up to 90 % of theoretical glucose yield, are attained when the process occurs at low temperatures (Verardi et al. 2012). This strategy has the disadvantage of causing corrosion problems in the equipment. When a dilute acid hydrolysis is chosen (2–5 %), high temperatures are needed to achieve good rates of cellulose conversion. In this case, generally, due to the harsh conditions (high temperature under acidic conditions), many inhibiting by-products are formed from the degradation of hemicellulose and lignin. Acetic acid is released from the acetyl groups of hemicellulose, while furfural and 5-hydroxymethylfurfural (HMF) are formed from the degradation of sugars (xylose and glucose, respectively). On the other hand, subsequent degradation of these aldehydes leads to the formation of formic acid and levulinic acid (Palmqvist and Hahn-Hagerdal 2000). The degradation

of lignin generates phenolic compounds such as vanillic acid, vanillin, syringic acid, or syringaldehyde depending on the type of the lignin present in the biomass. These are very toxic compounds to the microorganisms which will be using the hydrolysates as carbon source in a subsequent step. In order to remove these inhibitors, the hydrolysates will have to suffer a number of detoxifying steps (Pan et al. 2012). Examples of detoxification methods are overliming, activated charcoal, membrane filtration, ion exchange resins, and biological treatments (Nilvebrant et al. 2001; Palmqvist and Hahn-Hagerdal 2000; Ranatunga et al. 2000; Wickramasinghe and Grzenia 2008; Mussatto and Roberto 2001).

The use of enzymes in the hydrolysis of cellulose is more effective than the use of inorganic catalysts, because enzymes are highly specific and can work at mild process conditions. The cellulase enzyme system is a mixture of *endo*-β-1,4-glucanglucanhydrolases, *exo*-β-1,4-glucancellobiohydrolases (cellulases), and β-glucosidase (Parisi 1989). The cellulases break down cellulose to cellobiose and β-glucosidase makes subsequently the cleavage to glucose. In addition to the three major groups of cellulose enzymes, there are also a number of other enzymes that degrade hemicellulose, such as glucuronide, acetylesterase, xylanase, β-xylosidase, galactomannase, and glucomannase. These enzymes work together synergistically to break down both cellulose and hemicellulose. Cellulases are produced by various bacteria and fungi. The use of enzymes in industrial processes is still limited by several factors: most enzymes are relatively unstable at high temperatures, the costs of enzyme isolation and purification are high, and the separation from the bulk is difficult. Currently, extensive research is being carried out on cellulases with improved thermostability since high temperatures could speed up the hydrolysis reaction time (Verardi et al. 2012).

3 Biological Production of Polyhydroxyalkanoates from Lignocellulosic Hydrolysates

Despite the many advantages of biodegradable plastics, the commercialization of PHAs still has limited success. One of the reasons is the cost of the substrate for the PHA production via fermentation. Hence, the choice of a suitable, renewable, and inexpensive carbon source is a critical factor that determines the economic feasibility of a PHA producing process.

Lignocellulosic hydrolysates containing different types of sugars are potentially inexpensive carbon substrates for microbial growth and PHA production. PHA productivity is however very dependent on factors such as the hydrolysate composition and the existence of microbial strains able to efficiently produce PHAs based on those hydrolysates. Since an important fraction of the sugars present in the lignocellulosic hydroysates are pentoses such as D-xylose and L-arabinose, their conversion to value-added fermentation products is of the upmost importance to achieve an economically feasible process. Moreover, depending on the

pretreatment applied, those strains have to be able to withstand the presence of inhibitors in the hydrolysate.

3.1 Strains and Substrates

A literature search has revealed a few examples of the use of lignocellulosic hydrolysates as carbon sources for the biological production of PHAs.

3.1.1 PHA: Accumulating Strains from Commercial Pentoses

Since lignocellulosic hydrolysates are rich in C5 sugars such as xylose and arabinose, strains able to metabolize these sugars and to convert them into PHAs are preferred, both to increase the total carbon uptake by the cells and to avoid pentose accumulation in the broth in fed-batch assays, resulting in high, inhibitory concentrations. Only few strains have been reported in literature as being able to metabolize pentoses and accumulate PHAs. An overview of those strains is presented in Table 2. *Pseudomonas pseudoflava* (aka *Hydrogenomonas pseudoflava*) was the first strain reported to be able to use pentoses to produce PHAs (Bertrand et al. 1990). Using glucose, xylose, or arabinose in a 2 L STR, the maximum specific production rate of the homopolymer P(3HB) were, respectively, 0.11 g/(g h), 0.03 g/(g h), and 0.02 g/(g h). In assays with propionic acid addition as co-substrate, the copolymer P(3HB-co-3HV) was also produced. However, when using a hydrolysate from the hemicellulosic fraction of poplar wood as sole carbon source, the growth was completely inhibited (Bertrand et al. 1990).

Burkholderia cepacia (formerly *Pseudomonas cepacia*), a Gram-negative bacterium, is able to utilize a wide variety of carbon sources and accumulate PHAs under nutrient-limiting conditions and excess carbon (Young et al. 1994; Ramsay et al. 1995). Ramsay et al. (1995) have shown its ability to utilize xylose on an ammonium-limited medium and produce P(3HB). Batch fermentation data showed that when xylose was the single substrate, the maximum specific P(3HB) production rate, the yield of P(3HB) produced from substrate consumed ($Y_{PHB/S}$), and the percentage of P(3HB) accumulated in the cells were 0.072 g/(g h), 0.11 g g^{-1}, and 45 % (w/w), respectively. These results were very similar to the ones published for this strain on fructose.

Burkholderia sacchari IPT101 was isolated from the soil of sugarcane plantations in Brazil and selected as a good PHA producing strain able to use sucrose as carbon source (Bramer et al. 2001; Gomez et al. 1996). Silva and coauthors studied P(3HB) accumulation on xylose, on xylose + glucose, and on bagasse hydrolysates (see Sect. 3.1.2) (Silva et al. 2004). *B. sacchari* IPT101 showed promising results on xylose, accumulating 58 % of P(3HB). However, carbon catabolite repression (CCR) was preventing an efficient metabolization of pentoses in sugar mixtures composed of glucose, xylose, and arabinose by this wild-type strain. Through CCR,

Table 2 Summary of the strains reported in literature able to metabolize xylose and to produce P(3HB)

Strain	μ_{max} (h^{-1})	DCW (g L^{-1})	P(3HB) (%)	$Y_{P(3HB)/xyl}$ (g g^{-1})	$q_{P(3HB)\ max}$ (g/g h)	$Prod_{vol}$ (g/L h)	References
Burkholderia cepacia ATCC 17759	0.34	7.5	49	0.11	0.02	[a]	Young et al. (1994)
	[a]	[a]	45	0.11	0.07	[a]	Ramsay et al. (1995)
Burkholderia cepacia IPT 048	[a]	4.1	54	0.20	[a]	[a]	Silva et al. (2004)
Burkholderia sacchari IPT 101	[a]	5.5	58	0.26	[a]	0.07	Lopes et al. (2009)
Burkholderia sacchari LMF828 (mutant PTS^{-} glu+)	0.35	5.3	50	0.17	[a]	0.07	Lopes et al. (2011)
Bacillus cereus CFR06	[a]	1.1	35	[a]	[a]	[a]	Halami (2008)
Bacillus sp. MA3.3 (*Bacillus megaterium*)	[a]	5.5	64	0.24	[a]	0.06	Lopes et al. (2009)
Escherichia coli TG1(pSYL107)	[a]	4.8	36	[a]	[a]	0.028	Lee (1998)
Pseudomonas pseudoflava ATCC 33668	0.13	4.0	22	0.04	0.03	[a]	Bertrand et al. (1990)
Isolated bacterium strain QN271	[a]	4.3	29	[a]	[a]	0.04	Doan and Nguyen (2012)

[a]Unknown

the cells select from a mixture of carbon sources the one that allows for the highest growth rate, by inhibiting cells' transport capacity of other carbon sources, enzymatic activity, and related gene expression. It has been described that CCR is mediated by proteins of the phosphoenolpyruvate: sugar phosphotransferase system (PTS) (Li et al. 2007). Li et al. (2007) were the first to construct an *E. coli* PTS- mutant (*E. coli* LR1010) able to produce PHB (the strain contained the PHB synthesis genes from *C. necator*). In this mutant, the *ptsG* gene is disrupted and the carbon uptake metabolism is not mediated by the PTS. As PTS is blocked, xylose and glucose are utilized by a galactose permease pathway. Hence, this *ptsG* mutant was successful in utilizing glucose and xylose simultaneously and was still able to synthesize PHB, although further work is still needed to increase PHB productivity (Li et al. 2007). Following a similar strategy aiming at alleviating CCR, a *B. sacchari* UV mutant with a different carbon uptake metabolism (non-mediated by PTS) was isolated by Lopes et al. (2011). At shaking flask scale, in a medium supplemented with 1 g L^{-1} of yeast extract, specific growth rate, volumetric productivity, and PHA/carbon yield were 0.43 g/(g h), 0.12 g/(L h),

and 0.23 g g^{-1}, respectively. For the wild strain, these parameters were 0.41 g/(g h), 0.11 g/(L h), and 0.25 g g^{-1}. The mutant LFM828 (PTS- glu+) was indeed able to increase the relative uptake rate of xylose and arabinose in the presence of glucose; however, similar P(3HB) productivities were reached, mainly due to a decrease in specific growth rate and specific sugar consumption rates (Lopes et al. 2011). These authors have studies under way to further overcome CCR in *B. sacchari*.

For medical applications, such as controlled drug delivery devices, polymeric bioresorbable scaffolds for cellular proliferation, stents, and sutures, PHA production using Gram-positive strains is particularly interesting because these bacteria do not produce lipopolysaccharide endotoxins responsible for immunogenic reactions in the patient (Martin and Williams 2003). Hence, when using Gram-positive strains, a lower number of PHA purification steps is needed. *Bacillus sp*. COL1/A6 and CFR06 were reported to be able to produce PHA from xylose, although no data is available on the amounts accumulated by the cells (Santimano et al. 2009; Halami 2008). Lopes and coworkers selected *Bacillus* sp. MA3.3 after screening from a total of 3,152 bacterial isolates for a good PHA producer able to grow on xylose (Lopes et al. 2009). This strain showed the best results for Gram-positive strains, regarding P(3HB) production yields and productivities from glucose and xylose. P(3HB) contents of cell dry weight were, 62 and 64 %, PHB yield on substrate 0.25 and 0.24 g g^{-1} and PHB productivity 0.10 and 0.06 g/(L h) in shake flask assays respectively. The authors studied cell growth and P(3HB) production in mineral medium containing glucose or xylose as single sugars and sugar mixtures of glucose + xylose and glucose + xylose + arabinose and verified that all production parameters were reduced when compared with the production from glucose as sole carbon source, on account of CCR. In fact, glucose and arabinose were preferably consumed by the strains. Comparing P(3HB) produced from glucose and xylose in single sugar experiments, the yields and accumulated polymer are similar, although the productivity is 40 % lower when using xylose. The main reason for this is the energetic balances of the sugars, as the molar ATP/3HB monomer ratio is three from xylose and seven from glucose (Lopes et al. 2009).

A PHA producing bacterium (strain QN271) was isolated from soil samples collected from Quang Ninh mangrove in Thailand (Doan and Nguyen 2012). This strain was able to accumulate high P(3HB) amounts from different carbon sources, namely, glucose, xylose, fructose, and glycerol. Fructose was found to be the most suitable substrate for PHA synthesis, yielding a dry cell weight (DCW) of 6 g L^{-1} with a P(3HB) content of 63.3 % after 32 h of cultivation, while these values were 4.3 g L^{-1} and 29 %, respectively, for xylose.

In another study, recombinant *Escherichia coli* TG1 (pSYL107) harboring the *Alcaligenes eutrophus* PHA biosynthesis genes could efficiently produce P(3HB) from xylose and therefore potentially from low-cost hemicelluloses hydrolysate (Lee 1998). A P(3HB) concentration of 1.7 g L^{-1} was obtained in a defined medium containing 20 g L^{-1} xylose. P(3HB) production could be enhanced by more than twofold if a small amount of cotton seed hydrolysate or soybean hydrolysate was supplemented. The P(3HB) concentration, P(3HB) content, and P(3HB) yield on

xylose obtained by supplementing soybean hydrolysate were 4.4 g L^{-1}, 73.9 %, and 0.23 g P(3HB)/g xylose, respectively.

An interesting approach was successfully used to produce tailor-made medium-chain length (mcl) PHAs by sequential feeding of xylose and octanoate (Le Meur et al. 2012). *xylAB* [the genes encoding xylose isomerase (XylA) and xylulokinase (XylB)] from *E. coli* W3110 were inserted into *Pseudomonas putida* KT2440. The recombinant strain was able to efficiently utilize xylose as carbon source for growth, but the PHA—a polymeric chain containing 87 % (w/w) 3-hydroxyoctanoate monomers—was produced only from octanoic acid. This product resulted from sequential feeding of a low-cost carbohydrate (xylose) and an expensive fatty acid (octanoic acid), showing a promising strategy for reducing mcl-PHA production costs (Le Meur et al. 2012).

3.1.2 PHA Production from Lignocellulosic Materials

Several works aiming at producing PHAs based on lignocellulosic hydrolysates have been reported on literature. Table 3 gives an overview of the strains versus hydrolysates used as well as the results achieved so far.

Recently, it has been reported that *Burkholderia cepacia* ATCC 17759 is able to metabolize lignocellulosic biomass fractions, such as hemicellulose hydrolysates, levulinic acid derived from cellulose, and tall oil fatty acids from kraft pulping, to PHAs (Keenan et al. 2004, 2006). *B. cepacia* ATCC 17759 produced P(3HB-co-3HV) copolymers using xylose and levulinic acid (precursor for 3HV monomers) as carbon sources (Keenan et al. 2004; Jang and Rogers 1996). Studies with the same strain show a P(3HB) accumulation of 51.4 % of DCW when using sugar maple hemicellulosic hydrolysate containing 71.9 g L^{-1} xylose as sole carbon source (Pan et al. 2012). However, this process still needs further optimization, as, even after detoxifying steps to remove inhibitory compounds such as furfural, vanillin, levulinic, and acetic acid, a low productivity of 0.09 g/(L h) (8.72 g L^{-1} after 96 h with average cell content of 51.4 % (g g^{-1}) in a fed-batch 1 L stirred-tank bioreactor) is reached (Pan et al. 2012).

Screening of isolates led to the selection of *B. sacchari* IPT 101 and *B. cepacia* IPT 048 (refer to Sect. 3.1.1) for 10 L bioreactor assays to compare PHA production using bagasse (the cellulosic stem of sugarcane) hydrolysates or a mixture of commercial glucose and xylose as carbon sources (Silva et al. 2004). As acid pretreated bagasse hydrolysates were used, activated charcoal was needed to remove toxics and improve carbon uptake. Higher biomass concentrations, polymer contents, and yields from the carbon source were observed with bagasse hydrolysate than with glucose and xylose. Namely, $Y_{P(3HB)/S}$ increased from 0.11 to 0.39 g g^{-1} with strain *B. sacchari* IPT 101 and from 0.15 to 0.29 g g^{-1} for *B. cepacia* IPT 048. The authors suggested that an unknown carbon source might be present in the hydrolysate and that acetic acid and phenol compounds were possibly metabolized to P(3HB), justifying the higher yields observed when compared with experiments using xylose plus glucose. A productivity of circa 0.5 g/(L h) was reached

Table 3 Overview of studies reporting PHA production from lignocellulosic materials

Microorganism	C-source	PHA				Scale and operation mode	References
		g L^{-1}	g/(L h)	DCW (%)	Type		
Bacillus firmus NII 0830	Rice straw hydrolysate	1.7	0.02	89	P(3HB)	100 mL shake flask B	Sindhu et al. (2014)
Brevundimonas vesicularis LMG P-23615	Hydrolyzed pine saw dust	0.16	[a]	64	P(3HP-3HB-3HV)	250 mL shake flask B	Silva et al. (2007)
Burkholderia cepacia ATCC 17759	Xylose and levulinic acid	4.2	[a]	44	P(3HB-co-3HV)	2.8 L shake flask B	Keenan et al. (2004)
	Sugar maple hemicellulosic hydrolysate	8.7	0.09	51	P(3HB)	1 L STR B	Pan et al. (2012)
Burkholderia sacchari IPT 101	Sugarcane bagasse hydrolysate	2.7	0.11	62	P(3HB)	10 L STR B	Silva et al. (2004)
	Wheat straw hydrolysate	84	1.6	68	P(3HB)	2 L STR FB	Cesário et al. (2013a)
Burkholderia sacchari IPT 048	Sugarcane bagasse hydrolysate	2.3	0.09	53	P(3HB)	10 L STR B	Silva et al. (2004)
Cupriavidus necator (tolerant inoculum)	Bagasse hydrolysate	6.3	[a]	56	P(3HB)	Shake flask B	Yu and Stahl (2008)
Cupriavidus necator MTCC-1472	Water hyacinth hydrolysates	7.0	0.10	58	P(3HB)	V = [a] STR B	Radhika and Murugesan (2012)
	Paddy straw hydrolysates	7.2	[a]	38	P(3HB)	V = [a] shake flask B	Sandhay et al. (2013a)

Haloferax mediterranei	Extruded rice bran and extruded corn starch	77.8		55.6	PHA	5 L jar FB	Huang et al. (2006)
Halomonas boliviensis LC1	Wheat bran	2.8	0.14	43	P(3HB)	2 L STR B	Van-Thuoc et al. (2008)
Saccharophagus degradans ATCC 43961	Cellulosic waste from tequila bagasse	1.5	[a]	[a]	P(3HB)	V = [a] shake flask B	Munoz and Riley (2008)
Sphingopyxis macrogoltabida LMG 17324	Hydrolyzed pine saw dust	0.23	[a]	72	P(3HP-3HB-3HV)	250 mL shake flask B	Silva et al. (2007)

B batch experiments, *FB* fed-batch experiments
[a]Unknown

in high-cell-density cultures using xylose plus glucose under phosphorous limitation in fed-batch operation, both strains attaining about 60 g L^{-1} dry biomass, with 60 % P(3HB). In 10 L bioreactors operated in batch mode with bagasse hydrolysates as carbon source, P(3HB) volumetric productivity was 0.1 g/(L h). High-cell density assays using real bagasse hydrolysates were not tested in this work.

Cesário and coworkers used wheat straw hydrolysates prepared by the German company Biorefinery.de GmbH as carbon source for P(3HB) bioproduction. The biomass was pretreated using the AFEX process, which was followed by an enzymatic hydrolysis and a subsequent concentration step. After a preliminary study, *Burkholderia sacchari* DSM 17165 was selected due to its ability to convert both glucose and xylose (the main sugars in the straw hydrolysate) into remarkably high amounts of P(3HB). Growth and biopolymer production were studied using the real hydrolysate and also a mix of sugars simulating the hydrolysate composition (control) in 2 L fed-batch stirred-tank reactors (STR). A polymer concentration of 84 g L^{-1} was reached corresponding to an accumulation in the cells of 68 %. Polymer yield and productivity was 0.22 g P(3HB)/g sugar and 1.6 g/(L h), respectively. These values were very similar to those obtained in the control cultivations and are the best so far reported for PHA production from lignocellulosic hydrolysates (Cesário et al. 2013a). The same authors designed fed-batch strategies for P(3HB-co-4HB) production from glucose and gamma-butyrolactone (Cesário et al. 2013b). P(3HB-co-4HB) copolymers exhibit attractive thermal and mechanical properties since the presence of the 4HB monomer reduces the melting temperature and the polymer crystallinity and provides higher flexibility, thus facilitating plastic processing. These copolymers find numerous applications in the medical and pharmaceutical fields (Brigham and Sinskey 2012).

Some strains of *Cupriavidus necator* (formerly known as *Ralstonia eutropha*, *Alcaligenes eutropha*, and *Wautersia eutropha*) are also chosen to convert lignocellulosic hydrolysates into PHA (Radhika and Murugesan 2012; Yu and Stahl 2008). This bacterium is a convenient choice when adopting a *Biorefinery* rationale as it can metabolize toxics and convert these molecules into PHAs, allowing for the co-production of biofuels (with other strains and from the sugars) and bioplastics (Yu and Stahl 2008). Yu and Stahl (2008) used *Cupriavidus necator* to remove major organic inhibitors from process water while at the same time PHA polyesters were synthesized. This strain was reported to be able to metabolize furfural from a bagasse hydrolysate. The authors studied the growth of this *C. necator* on individual carbon substrates in a mineral solution and found high metabolic activity on glucose and fructose, poor activity on xylose and arabinose, and no growth on maltose and other disaccharides. Also, although xylose, as other C5 sugars, is not metabolized, furfural seemed to be a good substrate for growth and PHA production. However, when showing the results of furfural consumption, the authors do not distinguish between furfural assimilation by the cells and furfural transfer to the gas phase, which is likely to occur. According to the authors, PHA, mainly P(3HB), was accumulated in the cells when using bagasse hydrolysate, as organic inhibitors (formic acid, acetic acid, furfural, and acid soluble lignin) were degraded and removed to low concentration levels (Yu and Stahl 2008). The same authors

compared the performance of the original strain and a tolerant strain, developed in the laboratory via directed adaption and evolution. PHA production and productivities were significantly improved for higher hydrolysate concentrations.

C. necator MTCC-1472 was able to produce P(3HB) from saccharified water hyacinth hydrolysates (acid and enzyme hydrolysate) (Radhika and Murugesan 2012). The presence of a high phenolic load (which lowered the sugar consumption, microbial growth rate, and PHB storage capacity) justifies the lower P(3HB) concentrations (2 ± 0.1 g L^{-1}) obtained from acid hydrolysates when compared with the ones from enzymatic hydrolysates (4.3 ± 0.4 g L^{-1}). Further fed-batch assays with optimized conditions yielded a 0.09 g/(L.h) PHA volumetric productivity (Radhika and Murugesan 2012). *Cupriavidus basilensis* HMF14 was also used to biologically detoxify lignocellulosic hydrolysates prior to their use as fermentation feedstock while accumulating PHAs (Wierckx et al. 2010; Koopman et al. 2010). This bacteria completely removed furfural, HMF, acetate, and formate, leaving the sugar fraction in the wheat straw hydrolysate intact for bioproduction of other valuable chemicals with other strains.

Cupriavidus necator JMP 134 is known for its ability to degrade chloroaromatic and chemically related compounds, besides being a good PHA producer (Pérez-Pantoja et al. 2008). Although unable to metabolize C5 sugars, this strain is potentially useful when working with acid-hydrolyzed lignocellulosic hydrolysates due to the high amounts of aromatic inhibitors that result from this procedure.

In a recent work, paddy straw was hydrolyzed using a nonenzymatic, chemical method for straw hydrolysis to remove lignin and release the carbon fraction as simple sugars. *C. necator* MTCC 1472 was used for polymer accumulation. A dry cell weight of 19.2 g L^{-1} containing 38 % PHA was achieved (Sandhay et al. 2013b).

Silva et al. (2007) selected two strains to study the production of PHAs from acid-hydrolyzed sawdust: *Brevundimonas vesicularis* LMG P-23615 and *Sphingopyxis macrogoltabida* LMG 17324, both previously reported to be able to accumulate PHAs (Spiekermann et al. 1999; Godoy et al. 2003; Silva et al. 2007). Organic material generated by the acid hydrolysis of wood with H_2SO_4 was evaluated for use in the bacterial synthesis of PHA. Acid-hydrolyzed sawdust was prepared and adjusted to pH 7. After hydrolysis, the authors identified acetyl 2,3,4,6-tetra-o-acetyl-β-D-glucopyrane and α-D-glucopyranose pentaacetate by GC-MS as the two sugar molecules present in the hydrolysate at sugar concentration of 112.5 mg L^{-1} in a total of 601.5 mg L^{-1} organic material. Mineral salts with carbon/nitrogen (C:N) proportions of 100:1, 100:3.5, 100:10, 100:30, or 100:50 and trace elements were added, and these solutions were inoculated with a bacterial strain. Over 96 % of the sugars were consumed, and more than 90 % of the bacterial cells accumulated poly(3-hydroxypropionate-3-hydroxybutyrate-3-hydroxyvalerate) (P(3HP-3HB-3HV)), reaching 64 % of the cell dry weight in *B. vesicularis* and 72 % in *S. macrogoltabida*, when using a 100:3.5 C:N proportion (Silva et al. 2007). The results suggest that acid-hydrolyzed sawdust can be used by bacteria as a carbon source for growth and PHA production.

Halomonas boliviensis LC1 was grown on 1 % (w/v) wheat bran hydrolysate, a complex mixture of hexoses and pentoses, as C-source in a 2 L bioreactor operating

in a batch cultivation mode (Van-Thuoc et al. 2008). Digested potato extract (1 % w/v) was supplemented to the medium as a source of acetic and butyric acids required to achieving higher productivities. In these conditions, maximum cell mass concentrations and polymer contents observed were 6.6 g L^{-1} and 43 % polymer content, respectively, after 20 h of growth.

Haloferax mediterranei ATCC 33500, a member of the Archaea family, was used to produce PHA using a mixture of extruded rice bran (ERB) and extruded cornstarch (ECS) as carbon source (Huang et al. 2006). This strain cannot use native rice bran or cornstarch as C-sources. By employing pH-stat control strategy in a 5-L jar bioreactor using ERB:ECS (1:8 g g^{-1}) as the major carbon source, the authors obtained a cell concentration of 140 g L^{-1}, PHA concentration of 77.8 g L^{-1}, and PHA content of 55.6 wt% in a repeated fed-batch fermentation. Otherwise when ECS was used as the major carbon source, a 62.6 g L^{-1} cell concentration, 24.2 g L^{-1} PHA concentration, and 38.7 wt% PHA content were achieved .

The biomass super degrader *Saccharophagus degradans* was reported to be able to produce PHA from tequila manufacturing bagasse (the rind and fibrovascular bundles dispersed in the Agave tequilana stalk), converting crystalline cellulose into PHA without the need for extensive hydrolysis or fiber reducing procedures (Munoz and Riley 2008). The bacteria used in this study not only produced PHAs but also degraded insoluble cellulose under similar conditions, reducing the amount of required pretreatments. The authors found that PHA accumulation can be induced by nitrogen depletion; however, the process still needs further optimization regarding final biopolymer concentration and productivity.

Sindhu et al. (2014) compared the ability of three bacterial strains (*Bacillus firmus* NII 0830, *B. sphaericus* NII 0838, and *Paracoccus denitrificans*) to accumulate P(3HB) using a rice straw hydrolysate as carbon source. This mild acid pretreated hydrolysate contained 23 g L^{-1} of xylose and 4.9 g L^{-1} of acetic acid, together with much lower concentrations of glucose, arabinose, formic acid, furfural, and hydroxyl-methyl-furfural. They selected *B. firmus* NII 0830 as this strain produced higher amounts of P(3HB) using the hydrolysate without any detoxifying step (Sindhu et al. 2014).

Although most productivities should definitely be increased aiming at industrial operation, all the strains show promising results, and further work must now be addressed at process optimization. In fact, remarkable productivities were reached with *B. sacchari* IPT101 after choosing an improved process for hydrolysate production and high-cell-density fed-batch operation (Cesário et al. 2013a).

3.1.3 Brief Overview of Morphology and Physiological Aspects of Pentose-Degrading PHA-Producing Bacteria

PHA production by bacteria has been described by eight different pathways, depending on the microorganism and chosen carbon source (Chen 2010; Tsuge 2002; Rehm 2003; Steinbüchel and Lütke-Eversloh 2003). As referred in the previous section, a limited number of microorganisms successfully metabolize

pentoses in lignocellulosic hydrolysates and convert them into PHAs. A concise summary of the morphologic characteristics and metabolic pathways for pentose degradation and PHA biosynthesis of two different species, *Burkholderia* and *Bacillus*, is hereby presented. Strains belonging to these species have shown their capability to accumulate high PHA contents based on xylose and arabinose (Table 2).

In bacteria, the metabolism of D-xylose uptake is quite different from the one for D-glucose, which occurs by the Embden-Meyerhof-Parnas pathway (Jeffries 1983). D-xylose is degraded by the pentose phosphate pathway (PPP) (also called hexose monophosphate pathway, pentose phosphate shunt, phosphogluconate pathway) after being transported into the cell. Once inside the cell, it is either isomerized or reduced and then reoxidized to form D-xylulose. Further steps lead to phosphorylated 3-, 4-, 5-, 6-, and 7-carbon sugars. Intermediates can then be used by other metabolic pathways to produce nucleic acids, aromatic amino acids, lipids, and other metabolic end products (Jeffries 1983).

Burkholderia

Burkholderia sacchari was isolated from the soil of a sugar cane plantation in Brazil (Gomez et al. 1996; Bramer et al. 2001). *B. sacchari* strain IPT 101 (=LMG 19450, =CCT 6771, =DSM 17165) cells are Gram-negative, rod-shaped, and motile, presenting polar flagella. When plated on nutrient broth medium (Difco), they form white opaque colonies as the result of PHA accumulation. The optimum growth temperature is 28–30 °C, growing well in the temperature range 27–35 °C (Bramer et al. 2001). A transmission electronic microscope (TEM) image of *B. sacchari* DSM 17165 cells with PHA granules is presented in Fig. 1.

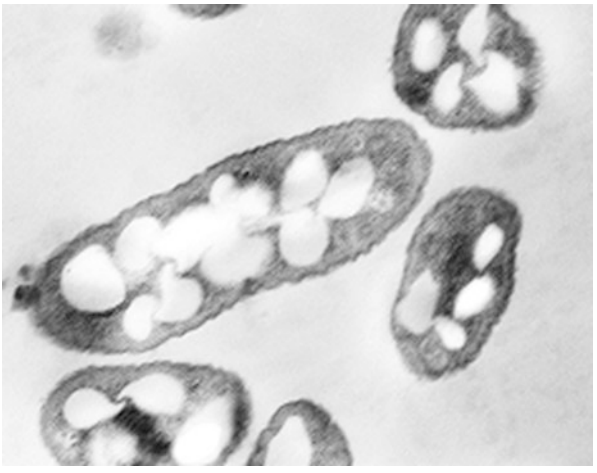

Fig. 1 TEM image of *Burkholderia sacchari* containing 70 % (dry weight) of P(3HB)

According to what has been described by Lopes et al. (2011), and similarly to what is known in *E. coli*, the consumption of sugar mixtures by *B. sacchari* is subjected to CCR, mediated by proteins of the PTS, as described in Sect. 3.1.1.

A schematic representation of glucose and xylose metabolism for PHA production is presented in Fig. 2. The first step for xylose assimilation in bacteria by the PPP is an isomerization to D-xylulose by xylose isomerase, followed by a phosphorylation by xylulokinase that produces D-xylulose 5-phosphate, yielding finally glucose 6-phosphate. This intermediate from the glucose metabolism can be produced by

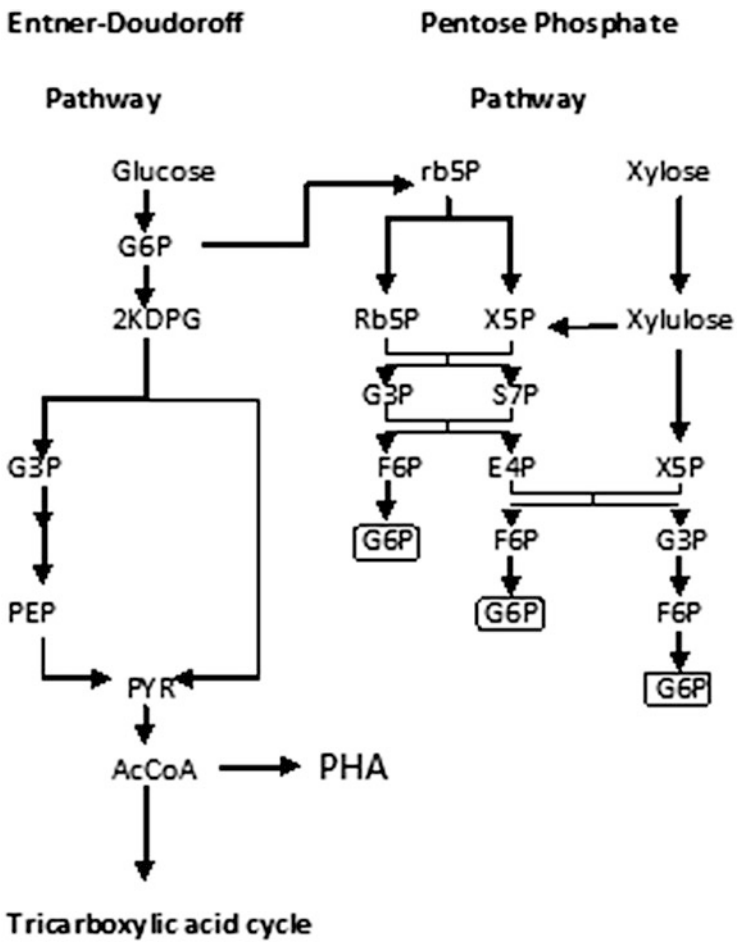

Fig. 2 Representation of the sugar to PHA metabolism in *Burkholderia sacchari*. Adapted from Lopes et al. (2011) and Lopes et al. (2010) (*G6P* glucose 6-phosphate, 2*KDPG* 2-keto-3-deoxy-6-phosphogluconate, *G3P* D-glyceraldehyde 3-phosphate, *PEP* phosphoenolpyruvate, *PYR* pyruvate, *rb5P* ribulose-5-phosphate, *Rb5P* ribose-5-phosphate, *X5P* xylulose 5-phosphate, *S7P* sedoheptulose 7-phosphate, *F6P* fructose 6-phosphate, *E4P* erythrose 4-phosphate, *AcCoA* acetyl-coenzyme A)

different paths, converted into acetyl-coenzyme A and finally to PHA, provided PHA accumulating conditions (e.g., N or P starvation) are imposed.

Besides the homopolymer P(3HB), *B. sacchari* (IPT 101) is able to produce the copolymer P(3HB-co-3HV) containing six molar % of 3HV when propionic acid is used as precursor (Silva et al. 2000). Those authors isolated a UV mutant (IPT 189) that was able to incorporate ca 55 % of this monomer with a yield $Y_{3HV/prop}$ of 0.8 (g g^{-1}) compared to 0.1 (g g^{-1}) for the wild strain. This value reached 1.20 (g g^{-1}) when this mutant was cultivated in a bioreactor under pH-controlled feeding of propionic acid. Their results strongly suggest the existence of at least two pathways involved in the oxidation of propionic acid with rates modulated by the availability of this precursor.

Recently, the authors' laboratory succeeded in producing the copolymer P(3HB-co-4HB) using *B. sacchari* IPT 101 (Cesário et al. 2013b).

Burkholderia cepacia (also known as *Pseudomonas cepacia* and *P. multivorans*) was firstly isolated from diseased onion scales and was subsequently found in soil, water, other infected plants, animals, and humans (Burkholder 1950; Miller et al. 2002). *B. cepacia* cells are Gram-negative and strictly aerobic, non-sporulating, straight rod shaped with multitrichous polar flagella and pili used for attachment. The optimum temperature for growth is about 30 °C (Burkholder 1950).

Rodrigues et al. (1995) described for the first time the incorporation of 3-hydroxy-4-pentenoic acid (3HPE) from unrelated substrates into PHA produced by two *Burkholderia cepacia* strains (IPT64 and IPT17B) isolated from soil samples. The authors refer the production of the copolymer P(3HB-co-3HPE4) from simple carbohydrates and gluconic acid, without the need of a specific precursor (Rodrigues et al. 1995). Further studies revealed that the produced PHA from sucrose or gluconate was not a copolymer; instead two homopolymers were being synthesized (Valentin et al. 1999). The fact that both homopolymers were produced and accumulated in the cell simultaneously cannot be explained by a mechanism similar to the *C. necator* PHA synthetic pathway (Rodrigues et al. 2000b). In fact, studies by Rodrigues and coworkers with *B. cepacia* IPT64 indicated that this bacterium must possess at least two PHA synthase genes (Rodrigues et al. 2000a, b). However, by complementation of a PHA-negative mutant of *C. necator*, only one PHA synthase gene was detected. Beta-ketothiolase and acetoacetyl-CoA reductase genes were identified in a gene cluster together with the PHA synthase gene, with a similar arrangement to the *C. necator* poly-β-hydroxybutyrate biosynthesis operon (Rodrigues et al. 2000a).

B. cepacia metabolizes sugars by the Entner Doudoroff pathway or through the PPP, similarly to *B. sacchari* (Allenza and Lessie 1982).

Bacillus

Bacillus spp. are rod-shaped, Gram-positive, endospore-forming bacteria. The species of this genus, with a wide range of physiologic characteristics, are found in every natural environment (Turnbull 1996). Some *Bacillus spp.*, such as *Bacillus*

megaterium or *Bacillus cereus*, are able to accumulate PHA from pentoses (Halami 2008; Lopes et al. 2009; Valappil et al. 2007a, c; Santimano et al. 2009).

PHB was firstly identified and isolated from *Bacillus sp*. by Lemoigne (1926). A major advantage of using *Bacillus sp*. for PHA production for medical applications is the fact that, being Gram-positive bacteria, they do not possess membrane lipopolysaccharides (LPS) which can co-purify with the extracted PHAs, causing immunogenic reactions (Lee et al. 1999). On the other hand, spores are formed when PHA accumulation conditions are imposed, decreasing yields and productivities. Valappil and coworkers completely suppressed sporulation by using acidic conditions during the PHA accumulation phase, allowing for an increased PHA production (Valappil et al. 2007b).

A putative metabolic pathway for P(3HB-co-3HV)/P(3HB-co-3HV-co-4HB)/P(3HB-co-4HB) synthesis from different carbon sources in *B. cereus* SPV was proposed by Valappil and coauthors for Gram-positive PHA accumulating strains (Valappil et al. 2007a). Work by McCool and Cannon (2001) with *B. megaterium* strains showed that *Bacillus sp*. PHA synthases have subunit compositions that are distinctively different from all known PHA synthases, both in sequence and in arrangement, providing the bases for a separate class of synthases (McCool and Cannon 2001).

Concluding Remarks

The economic feasibility of using lignocellulosic hydrolysates as carbon sources to biologically produce ethanol, bio-based building blocks, or biopolyesters strongly depends on the capacity of microorganisms to consume both the hexoses and the pentoses released from the lignocellulosic biomass and to convert these sugars into products at high conversion yields ($Y_{P/S}$). Moreover, systems featuring high volumetric productivities should be designed. This is achieved both by choosing strains with high specific production rates and by using high cell-density cultivations. The latter can be attained in fed-batch operation, however optimal culture conditions should be maintained, i.e. the build-up of inhibitors should be prevented. This is true either for the inhibitors which might be present in the hydrolysates used as carbon source (released during biomass pretreatment) or the high concentration of pentoses which can accumulate in the medium due to Carbon Catabolite Repression. To overcome catabolite repression, (1) efficient process design strategies based on wild robust strains will have to be developed or (2) genetically modified microorganisms will have to be constructed. Both strategies are already being addressed (Cesério et al. 2013a; Lopes et al. 2011). Alternatively, pentoses will have to be first transformed into more easily assimilated intermediate species prior to the cultivation step.

In order to circumvent the effect of CCR resulting from the simultaneous presence of hexoses and pentoses in the hydrolysate, fractionation of the

(continued)

biomass components by means of mix of solvents (Zhang et al. 2007) or by ILs (Swatloski et al. 2002; Dadi et al. 2006; Stark 2011) are also rather promising ideas, allowing for different polysaccharides to be upgraded separately. Fractionation is, however, still not economically feasible, due to the number of unit operations required and to the high price of the ILs (Fitzpatrick et al. 2010). In the specific case of PHAs production from lignocellulosic materials, some microorganisms, namely, *Saccharophagus degradans* (Munoz and Riley 2008), which are able to directly uptake cellulose from biomass and produce PHAs, are certainly good candidates, as costs associated with biomass pretreatment are decreased. Moreover, recently isolated bacterial strains, including *Brevundimonas vesicularis* LMG P-23615 and *Sphingopyxis macrogoltabida* LMG 17324 (Silva et al. 2007), are able to directly use oligosaccharides as C-sources to produce various PHA co- and terpolymers, which makes them attractive, since the enzymatic hydrolysis to simple sugars is not required. However, processes using these microorganisms need to be optimized so that high productivities are attained.

Despite the difficulties found in transforming lignocellulosic wastes from agriculture, forestry, and industry to second-generation biocommodities in a cost-effective way, brilliant solutions have been disclosed as proven by the vast amount of scientific literature that has been published in the last decades.

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Microbial Research in High-Value Biofuels

Dženan Hozić

Contents

Abstract Biorefineries focus on fermentative production of butanol, isobutanol and isobutene. This paper encompasses the entire development process, such as strain and substrate selection and optimisation, fermentation modes and purification techniques. Special attention is dedicated to various natural or synthetic metabolic pathways for obtaining these compounds.

Great effort has been invested in research on biobutanol production, which is in the last preparation stages for an industrial-scale process, with pilot plants being built around the world. Some of the main issues are still butanol toxicity, low strain productivity, low yield, incomplete substrate usage, inability to use cheap lignocellulosic feedstock and high separation costs. Many of these problems are caused by butanol toxicity.

Fermentative production of isobutene requires a lot more research and optimisation to reach large-scale production and economic viability. Low strain productivity, together with low yield, is the main drawback. Metabolic engineering

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approaches need to be employed to enable versatile carbon source use and increase productivity. An important advantage is the diffusion of gaseous isobutene out of the fermentation broth, thus circumventing the product toxicity problem.

1 Introduction

The main idea behind the production of biofuels is to capture the energy of the Sun in the form of chemical energy contained in various chemical compounds. The plants transform the energy of the Sun into potential (chemical) energy of carbohydrates or fatty acids, which are then further processed to obtain the fuel with desirable properties.

This means that any pathway towards a biofuel chemical starts with photosynthesis. There are three ways to process biomass feedstock into energy-rich molecules:

- Direct synthesis by organisms capable of photosynthesis
- Fermentation of biomass into biofuels by heterotrophic microorganisms
- Chemical conversion of biomass to fuels (Rude and Schirmer 2009)

In the last decade, production of biofuels has gained momentum and grown into a large industry, often heavily subsidised. From 2007 to 2011 worldwide production of all biofuels rose from over 175,000 to over 300,000 m^3/day. In bioethanol production, the USA and Brazil are leading the way, with more than 144,000 and 62,000 m^3/day, respectively, being produced in 2011. Biodiesel is produced most intensively in Europe with over 28,000 m^3/day being made in 2011 (U.S.E.I. Administration 2012).

Furthermore, much effort is being invested into research on improving the production of the existing biofuels, but also discovering ways to new biofuels with superior properties.

Advances in processing of biomass feedstock to fuels are being made principally in two areas:

- Enabling and improving utilisation of different sources of biomass (e.g. use of lignocellulose) (Kamm et al. 2011);
- Creation of fuel molecule(s) with optimal properties (Wackett 2008)

Various biomass sources as feedstock are a great opportunity and a major cost driver at the same time (Rude and Schirmer 2009). This refers mostly to the use of lignocellulosic biomass. In order to achieve economical and environmentally sustainable biofuel production, plant cellulose and hemicellulose will have to be utilised more efficiently. This will require a better understanding of the cellulose-degrading multiprotein complex—the cellulosome (Kamm et al. 2011; Wackett 2008).

Additionally, lignin presents a great barrier to cellulosic biomass utilisation (Kamm et al. 2011; Wackett 2008). The protective layer that lignin forms is an obstacle to use of cellulose and hemicellulose. Since lignin is a heterogeneous polymer, its enzymatic degradation is relatively difficult and slow, and its separation from the raw biomass presents an energy-intensive process.

One more important economic factor is the costs for the recovery of the fuel from the fermentation broth. Additionally, it is possible that the separated molecule is a precursor that needs to be modified to transform it into an engine fuel. Ideally, biomass feedstock would be directly transformed, through microbial conversion, into fuel-grade molecules, which would then be secreted into the fermentation broth and separated relatively easily due to their immiscibility in water (Rude and Schirmer 2009).

Some fuel molecules are toxic to the microorganisms that produce them and can halt their growth and metabolism beyond a certain threshold. For example, this is the case for short-chain alcohols where their intrinsic toxicity combined with their relatively high solubility in water imposes limitations on production (Kamm et al. 2011; Wackett 2008).

In recent years, much research and progress have been made to develop fermentation processes for the production of novel biofuels to overcome disadvantages of ethanol and biodiesel. These biofuels should give advantage over ethanol, for example, higher energy density, lower hygroscopicity and vapour pressure. Also, these biofuels should fit the existing energy infrastructure, such as the option of use in existing car engines without modification and transportation in pipelines (Connor and Liao 2009).

Examples of such fuel molecules are butanol and isobutanol, where so far the largest amount of research has been made on new biofuels. Their production is near commercial level, and there are several pilot plants and demonstration facilities being constructed around the world.

2 Types of Microbial Fuels

Microbial fuels can be sorted into four classes according to the pathway that was taken in their production: fermentative short-chain alcohols, nonfermentative short-chain alcohols, isoprenoid-derived hydrocarbons and fatty acid-derived hydrocarbons (Rude and Schirmer 2009).

It is probable that, over time, one class or one fuel molecule will prevail, but whatever that compound will be, it will probably have the following properties:

- Be relatively cheap
- Have a high energy density (large energy to mass ratio)
- Be highly combustible, but not explosive
- Be stable during long-term storage
- Be in liquid form at room temperature and pressure
- Be suitable for transportation by pipeline (Kamm et al. 2011; Wackett 2008)

The most intensively produced microbial biofuel today is surely ethanol. More than 86 billion liters were produced in 2011 worldwide (U.S.E.I. Administration 2012). However, there are several distinct disadvantages to ethanol as a fuel that the advanced biofuels should overcome.

Ethanol has a relatively low energy density (30 % lower than gasoline) and is incompatible with the existing fuel infrastructure and therefore cannot be used as a drop-in fuel. This primarily refers to the fact that gasoline blends with high percentage of ethanol (10 % and higher) are not suitable for use in the existing petrol engines, but require vehicle modification in the fuel system (Boundy et al. 2011). Additionally, ethanol is relatively hygroscopic—it has a tendency to absorb water (e.g. air moisture), and therefore, it cannot be transported using existing pipeline systems. Due to its high vapour pressure, ethanol has a negative effect on air quality, and its corrosiveness causes further handling complications (Connor and Liao 2009; Atsumi and Liao 2008a).

Due to these issues, it is unlikely that ethanol will be the future biofuel that will replace gasoline on a large scale. This is why more attention is being given to potential advanced biofuels, as they normally consist of more carbon atoms, and this gives them the properties that are believed to bypass these issues (Atsumi and Liao 2008a).

Unfortunately, such advanced biofuel molecules are not naturally produced by microbes in concentrations large enough for an economically feasible fuel production. One exception is *n*-butanol, which is produced by *Clostridium* sp. (Jones and Woods 1986).

Thanks to the expeditious development of synthetic biology information, metabolic engineering, growing genomic information and high-throughput tools, substantial progress is being made in constructing non-native organisms that are capable of production of high-value fuel-grade molecules beyond the capabilities of native organisms. These compounds include C3–C5 alcohols, fatty acid esters and isoprenoids (Atsumi and Liao 2008a).

Even though many chemical compounds are being examined today as next-generation biofuels, alcohols, esters, ethers and hydrocarbons, we will principally focus on an alcohol and a hydrocarbon—butanol and isobutene.

2.1 Alcohol Fuels

A major part of today's biofuel generation consists of the production of bioethanol through fermentation of sugars with yeast *Saccharomyces cerevisiae*. This is perhaps the oldest bioprocess utilised by humans.

However, *S. cerevisiae* has a crucial disadvantage with regard to substrate consumption and sustainability—it can consume only glucose as an energy source. In order to utilise lignocellulosic feedstock (LCF), it is imperative for the microbe to be able to utilise the variety of sugars it contains and transform them into biofuels.

This drawback inspired a novel approach to use a homoacetogenic bacterium that can concurrently metabolise hexoses and pentoses to produce acetic acid (Eggeman and Verser 2006). Further on, acetic acid can be esterified with ethanol directly in the fermentation broth which generates ethyl acetate. Ethyl acetate is relatively water insoluble and therefore can be easily separated. The isolated ethyl acetate can be hydrogenated and will yield 2 mol of ethanol, which means that from 1 mol of hexose, 3 mol of ethanol could be created (Kamm et al. 2011; Wackett 2008).

Still, the disadvantages of ethanol named above make it less attractive to further develop its production. Instead, scientists are trying to generate new fuel molecules with more favourable properties. One of these is butanol.

2.1.1 Butanol

Butanol is a four-carbon, saturated, primary, aliphatic alcohol. It has the molecular formula of C_4H_9OH and molar mass of 74.12 g/mol. Butanol is hydrophobic, hence completely miscible with organic solvents and partly miscible with water. *n*-Butanol and gasoline have a similar energy content with 27 MJ/L and 32 MJ/L, respectively. Additionally, *n*-butanol has roughly 11 times lower vapour pressure (0.533 kPa at 20 °C) than ethanol (6.00 kPa at 20 °C). This makes it less volatile and safer to use as a fuel from the health and environmental perspective. It is also less corrosive than ethanol. This and other properties of butanol make it possible for it to mix with gasoline or diesel at any ratio or to completely replace gasoline and to be transported and stored using the existing infrastructure, unlike ethanol (Atsumi and Liao 2008a; Lee et al. 2008; Raganati 2012). One disadvantage of butanol is the higher boiling temperature and enthalpy of vaporisation than ethanol, which means that more energy is needed for its distillation (Rude and Schirmer 2009).

Fermentative production of butanol by bacteria is known since the second half of the nineteenth century, and industrial production of butanol, based on fermentation with bacteria *Clostridium acetobutylicum*, was the second largest fermentative process in the world after ethanol production, during the first half of the twentieth century (Jones and Woods 1986; Durre 2008). In this process, known as the acetone–butanol–ethanol (ABE) fermentation, *Clostridia* consumes carbohydrates to produce primarily butanol and acetone.

ABE fermentation lost competitiveness in most of the world in the 1960s, due to the increasing demand for butanol and the development of more efficient petrochemical processes. However, in recent years fermentative butanol production has regained interest, due to the rising and unstable crude oil prices and environmental concerns (Lee et al. 2008).

It is estimated that 5–6 billion kilograms of butanol are produced every year (Donaldson et al. 2007), which sums up to a 7–8.4 billion dollar market. This market is estimated to have an annual expansion of around 3 % (Donaldson et al. 2007; Kirschner 2006).

Butanol is an important platform chemical that has various applications in chemical, pharmaceutical and cosmetics industry, besides its possible use as a biofuel. Primary uses of butanol are:

- As an intermediate in chemical synthesis and as a solvent for a wide variety of chemical and textile industry applications
- As a solvent for the production of antibiotics, vitamins and hormones
- As an important chemical precursor for paints, polymers and plastics
- As a precursor for compounds in latex surface coating, enamels and lacquers
- As a diluent for brake fluids
- As a chemical feedstock in the plastic and flavour industries (Lee et al. 2008; Kirschner 2006)

Around half of the produced butanol is used as butyl acrylate and methacrylate esters, for latex surface coating, enamels and lacquers (Kirschner 2006). Further significant derivatives of butanol are butyl glycol ether (a solvent and surfactant in many domestic and industrial products), butyl acetate (solvent in the production of lacquers) and plasticisers. Butanol is also an excellent thinner for brake fluids and solvent used in the production of antibiotics, vitamins and hormones (Lee et al. 2008).

Besides these important applications, butanol is being considered as a fuel supplement or replacement. As discussed above, butanol has enough properties similar to gasoline that it can be used directly in gasoline engines, with no need for their modification (Lee et al. 2008). As a biofuel, butanol has distinct advantages over ethanol. It has a higher energy density and is less hygroscopic, corrosive and volatile. However, one disadvantage of butanol is that its viscosity is two times larger than the viscosity of ethanol and 5–10 times larger than the viscosity of gasoline (Kamm et al. 2011; Wackett 2008; Dürre 2007).

2.1.2 Isobutanol

Isobutanol is a four-carbon, branched-chain, mono-hydroxyl, primary alcohol. It is used both as a solvent and as a feedstock/intermedier for syntheses in chemical, pharmaceutical and cosmetics industry.

Isobutanol is a clear, colourless and neutral liquid with a characteristic, sweet, musty odour. It is miscible with all nonpolar solvents, but its solubility in water is limited to 8.5 % at 20 °C (Company DC 2012; Petronas 2006).

As a biofuel, it has all the advantages of butanol over ethanol like higher energy density, fits existing gasoline engines and infrastructure, lower hygroscopicity and volatility. Additionally, it has the benefit of having a larger octane number. Energy content of isobutanol according to various sources amounts to 82–89 % that of gasoline (Lu et al. 2012; Peralta-Yahya et al. 2012).

At the moment, isobutanol is mostly produced through petrochemical processes, from crude oil as the starting feedstock through Reppe carbonylation and hydroformylation (Hahn et al. 2000). It has been detected that certain microorganisms

produce isobutanol during their metabolism, but none to industrially relevant quantities. Atsumi et al. managed to engineer a strain of *Escherichia coli* to produce isobutanol using its native amino acid pathways to a concentration of around 22 g/L (Atsumi et al. 2008a). Other possible engineered microorganisms include *Cyanobacteria*, *S. cerevisiae*, *Ralstonia eutropha* and *Bacillus subtilis* (Varman et al. 2013; Kondo et al. 2012; Li et al. 2012a, b).

Most of the produced isobutanol is converted into derivatives that are used as solvents in the coating industry. Isobutanol is an exceptionally suitable solvent for acid-curable lacquers and baking finishes. There it is mostly used together with glycol ethers, ethanol or aromatic hydrocarbons (Company DC 2012; Petronas 2006). Besides this, isobutanol is also used as a solvent for printing inks, an extractant in the production of pharmaceuticals, an additive in polishes and cleaners, a solvent in the textile industry, an intermediary for glycol ethers and esters, an additive in de-icing fluids, a mobile phase in paper and thin-layer chromatography, a substrate in the production of wear inhibitors and anticorrosion additives, a dehydrating agent and an intermediate for agricultural chemicals (Company DC 2012; Petronas 2006).

2.2 Hydrocarbons

Hydrocarbons are substances composed of hydrogen and carbon. Due to the ability of carbon to connect into large chains, they can have many different structures.

As motor fuels they have superior properties, such as a high energy density, possibility to be transported by pipelines and compatibility to existing motors and infrastructure (Kamm et al. 2011; Wackett 2008).

More and more attention is being given to bioproduced hydrocarbons as the next renewable fuel type. This is largely due to the fact that existing petroleum-derived gasoline is composed mainly of hydrocarbons.

Conventional gasoline is a refined product of petroleum, obtained through fractional distillation of crude oil, consisting of a mixture of hydrocarbons, additives and blending agents. It is composed out of hundreds of different kinds of hydrocarbons that have between 4 and 12 carbon atoms, but predominantly between 5 and 10 carbon atoms (Dabelstein et al. 2000; IARC et al. 1989).

A variety of microorganisms produce hydrocarbons or their precursors. For example, bacteria, primarily *Bacillus*, produce isoprene (Kuzma et al. 1995). Isoprenoid compounds are common in nature, mostly in plants, and find application in human life in the production of pharmaceuticals, flavours, fragrances and pigments (Walsh 2007). Due to interest in these applications, strains of *E. coli* and *S. cerevisiae* have been established for the overproduction of certain isoprenoids. One instance is artemisinic acid, a precursor to artemisinin and an antimalaria drug

(Ro et al. 2006; Chang and Keasling 2006). This technology could be further developed to produce high-value fuels derived from isoprenoids.

It is possible to produce alkenes through enzymatic decarbonylation of unsaturated fatty acids, through terminal alkene biosynthesis or through long-chain alkene biosynthesis by head-to-head condensation of two fatty acids (Rude and Schirmer 2009).

Fujii et al. (1988) reported a mechanism of isobutene formation by the yeast *Rhodotorula minuta* in a culture medium that contained branched-chain carboxylic or amino acids. It was suggested that the pathway leads over isovalerate that is decarboxylated to isobutene.

For this review we focused principally on the biological production of isobutene (isobutylene, 2-methylpropene) as a high-value, advanced, hydrocarbon-type biofuel.

2.2.1 Isobutylene (Isobutene)

Isobutylene (methylpropene) is a monounsaturated hydrocarbon with 4 carbon atoms. Under normal conditions it is a colourless, highly flammable gas (mixtures with air between 1.8 and 8.8 vol% at 20 °C can lead to explosions), and it has the characteristic alkene, petroleum-like scent. Isobutene is soluble in organic solvents (like ether, alcohol and hydrocarbons), but almost insoluble in water (0.263 g/L at 25°). It has no corrosive properties and has a low level of acute toxicity (still, extensive exposure should be avoided) (Industries E 2010; Liquide A 2013; Obenaus et al. 2000).

Since isobutene has a double bond in its molecular structure, it can undergo different chemical reactions typical for olefins and yield a great variety of products. Some examples of these reactions are hydrogenation, oxidation, electrophilic addition, polymerisation and hydration (van Leeuwen et al. 2012).

Presently, more than 10 million tons of isobutene are produced annually, and its market value is assessed to approximately 18 billion euro (de Guzman 2011). Isobutene is currently produced at industrial scale mostly through various methods of petrochemical cracking of crude oil.

Major methods for isobutene production are from a C4 stream of a steam cracker, from a catalytic cracker butene–butane stream, through dehydration of *tert*-butanol (which is obtained from a propene oxide process) and through isomerisation of *n*-butane to isobutene and subsequent dehydrogenation to isobutene (Obenaus et al. 2000; van Leeuwen et al. 2012; Romanow-Garcia et al. 2007).

Isobutene is primarily used as a precursor for various chemicals. It is used in a polymerisation reaction with isoprene for the production of polybutene (butyl rubber used for the production of tires and other synthetic rubbers). Further products include polyisobutene, diisobutene, methacrolein, methacrylic acid, *tert*-butanol, alkyl-*tert*-butyl ether, additives for fuels and lubricants, vitamins, fragrances, isoprene, mercaptanes, alkylphenols, antioxidants in the food industry,

synthetic resins, adhesive resins and organic acids (Obenaus et al. 2000; Romanow-Garcia et al. 2007).

A reaction widely used in the industry is the electrophilic addition of methanol to obtain methyl *tert*-butyl ether (MTBE). MTBE is an antiknocking agent in automotive fuels (van Leeuwen et al. 2012). Besides that, isobutene can be used to produce isooctane, a high-octane aviation fuel.

It is possible to produce biological isobutene using chemocatalytic or thermochemical methods from ethanol, for example (Sun et al. 2011), but this process does not seem to be economically feasible (Posada et al. 2013).

Biological production of isobutene from microbes is already known since the 1970s. However, meticulous metabolic engineering is essential to achieve economically feasible yields and productivities (van Leeuwen et al. 2012). This contrasts biobutanol production by *Clostridia*, where metabolic engineering to improve butanol tolerance and productivity is desirable, but it is theoretically possible to achieve economic viability by just improving substrate preparation and separation techniques.

On the other hand, a biological route for isobutene production has a distinct advantage over biobutanol fermentation. During the fermentation process not liquid products but instead gaseous isobutene is formed. This means that it can be relatively easily recovered from the fermenter together with CO_2. Furthermore, due to the low solubility of isobutene in water, there are no problems with product toxicity, unlike butanol or isobutanol (Lee et al. 2008; van Leeuwen et al. 2012).

Theoretically, bio-based isobutene is an attractive option. However, there is still relatively little research and gathered knowledge on how to design an economically and technically viable production process from renewable sources of biomass.

3 Lignocellulosic Biomass as a Feedstock

One of the most abundant biological raw materials in nature is the plant cell wall. This is often referred to as the lignocellulosic biomass (LCB). Plant cell walls are constructed out of many microscopic fibres called macrofibrils. Each macrofibril consists of a cellulose core covered with an intertwined hemicellulose/lignin fibre coating.

In order to make biofuel production more sustainable, it is necessary to use the biomass effectively, and for that, lignocellulosic material will need to be utilised extensively.

Cellulosic material is the most abundant biopolymer on the planet, and it represents the biggest portion of biological waste materials (Somerville 2006).

Every year, microorganisms hydrolyse 10^{11} tons of plant biomass, which is mostly composed out of plant cell wall material, meaning cellulose, hemicellulose and lignin. This amount of biomass contains the energy equivalent of 640 billion barrels of crude oil (Ragauskas et al. 2006).

Since cellulose and hemicellulose are polymers with complex and heterogeneous structure, various equally structurally complex hydrolases are needed to effectively degrade them into monosaccharides (Somerville 2006). Therefore, an effective enzymatic hydrolysis of cellulose requires a multicomponent enzyme system, and in most anaerobes, enzymes that degrade the lignocellulosic material are congregated into a macromolecular complex, called cellulosome (Gilbert 2007).

Cellulosomes are multienzyme complexes that contain various cellulose-degrading enzyme modules and a multifunctional subunit called scaffoldin that organises these enzyme subunits appropriately. Scaffoldin includes carbohydrate-binding domains that lock onto the substrate. Cellulosomes also contain dockerins (on each enzyme subunit) and cohesins (on the scaffolding subunit) whose purpose is to attach both the enzyme- and carbohydrate-binding domains to the surface of the cell (Kamm et al. 2011; Wackett 2008; Fontes and Gilbert 2010).

Even though enzymatic hydrolysis of cellulose is in place in industry as an established process, it is still not feasible for use in large-scale fuel production from hydrolysates, where it is necessary to improve enzyme efficiency and reduce process costs (van Zyl et al. 2007; Lu et al. 2006).

As mentioned before cellulose in biological systems does not come alone, and therefore, it is necessary to employ other enzymes to efficiently hydrolase the LCB. This primarily refers to xylanases that degrade hemicellulose to xylose by hydrolysing the β-1,4-xylan linkage (Collins et al. 2005), and it is to be expected that they will be used more extensively in the future either as an added enzyme or as a part of a microbial system.

Another issue is the plant lignin. Lignin is a heterogeneous polymer that encompasses the cellulosic material and can also be covalently linked to it. This gives the plant material greater structural stability and resistance to microbial and enzymatic degradation.

Lignin also makes the use of cellulases more difficult because they are irreversibly adsorbed to the surface of lignin and thus lose their activity. Due to the heterogeneous nature of lignin, it is extremely demanding to degrade. In nature, lignin degradation takes place fairly slowly through the activity of wood rot fungi that produce ligninases (Kamm et al. 2011; Wackett 2008).

Resolution of the problem of lignin in LCF is essential in order to fully utilise cellulose and hemicellulose. So far, appropriate pretreatment in order to release cellulose and hemicellulose is needed.

Some propose to genetically engineer power crops to yield less or no lignin and in that way facilitate degradation of cellulose and hemicellulose. There are already many crops that produce food as well as LCB where this is not acceptable, due to biological, environmental, social or political reasons. This would potentially be an acceptable solution for specialised power crops that are able to grow on agriculturally unusable areas, where the climate conditions or soil composition does now allow agricultural production. Also, growing power crops for fuel production can be environmentally questionable due to deforestation for new surfaces for cultivation and monoculture-type cultivation.

With or without these modified crops, it seems that the question of lignin will in any case has to be resolved in order to fully utilise the LCB available, since most plants used in agriculture also yield cellulosic material that represents an opportunity for cheap feedstock.

4 Fermentative Fuel Production and Metabolic Engineering

4.1 Butanol and Isobutanol

Out of all the fuels described in this review (and probably out of all advanced biofuels being developed at the moment), industrial-scale production of butanol is the most realistic to begin in the near future. There are a number of pilot plants around the world, and several companies have shown interest in this biofuel (Gevo, Butamax, Cobalt Technologies, etc.) with much research being performed and many parents published on the topic.

4.1.1 Microorganisms

Clostridium Genus

So far, the only natural producers of butanol are members of the *Clostridium* family (Branduardi et al. 2014). *Clostridium* are rod-shaped, endospore-forming, Gram-positive bacteria and are normally strict anaerobes that are universally present in the environment (e.g. soil and organic waste) (Lee et al. 2008; Branduardi et al. 2014; Qureshi et al. 2013). Still, they are more commonly linked with living plants instead of decomposing plant material or soil. Isolating clostridia from the environment is relatively simple, since they (as many anaerobes) have relatively modest growth requirements, and cultivation and isolation methods have been well studied and documented. For example, they have been found on root crops and roots of nitrogen-fixing legumes (Jones and Woods 1986; Beesch 1952, 1953; Calam 1980; Underkofler et al. 1954). From many clostridial species, the two that are considered the best butanol producers are *C. acetobutylicum* and *C. beijerinckii*, with *C. saccharobutylicum* and *C. saccharoperbutylacetonicum* also being regarded as productive strains (Qureshi et al. 2013; Dürre et al. 1987).

Butanol is naturally synthesised by clostridia in a process called ABE (acetone–butanol–ethanol) fermentation. Butanol pathway, within ABE fermentation, consists of condensing two acetyl-CoA molecules (catalysed by a thiolase) and then reducing the product to butanol (requiring four reductases and one dehydratase).

Clostridia have an important property of being able to consume a variety of carbon sources like various monosaccharides (both pentoses and hexoses including

glucose, xylose and arabinose) and oligo- and polysaccharides (starch, galactose, cellobiose and mannose) and synthesise solvents (butanol, acetone and ethanol) (Jones and Woods 1986; Ezeji et al. 2007a), acids as well as different chiral substances whose chemical synthesis is challenging and other diverse metabolites (Rogers et al. 1986; Tracy et al. 2012). They can also consume and degrade several toxic compounds (Francis et al. 1994; Spain 1995). This ability to consume various carbon sources (especially xylose) is very important for use of cheap lignocellulosic feedstock like agricultural by-products and wastes, since substrate cost is one of the most important factors affecting the cost of biobutanol production (Qureshi and Blaschek 2000a).

There are several issues with the use of clostridia for butanol production. Butanol toxicity to microorganisms is one of the biggest ones. Due to butanol toxicity, its concentration in the fermentation broth is limited and relatively low, which causes a new set of problems, primarily high separation costs of butanol from dilute solutions, but also low yield and inability to use high sugar concentration in the fermenting medium (Ezeji et al. 2007b). This can be illustrated by the fact that as much as 6–7 t of corn are used to produce 1 t of butanol, but only 3 t of corn are needed to produce 1 t of ethanol (Xue et al. 2013a). Furthermore, clostridia grow relatively slowly, the cultures are unstable (requiring constant re-inoculation unlike *Saccharomyces* ethanol fermentation), and they have a spore-forming life cycle and produce undesirable by-products like butyrate, acetate, acetone and ethanol. Lastly, there are no commercially available microorganisms that can ferment lignocellulosic hydrolysates into butanol at an economically feasible level (Jones and Woods 1986; Qureshi et al. 2013).

Engineered Strains

Even though there are ongoing efforts for screening natural sources for new strains, it is unlikely that new strains that resolve all of the above-named issues will be found in nature. This is why researchers worldwide are employing various techniques for genetic manipulation, like mutagenesis, evolutionary engineering and metabolic engineering (Xue et al. 2013a).

In terms of product tolerance and resistance to phage infections, already significant improvements have been made by methods of classical mutagenesis and selection. Still, with the development of new tools for genetic engineering, more complete genetic sequencing of *Clostridium* and other microorganisms and better understanding of their genomic and metabolic pathways, it is expected that many other improvements will be developed (Branduardi et al. 2014).

Biotechnological production problems like product toxicity have traditionally been addressed by random mutagenesis by physical or chemical methods. One example of a successful application of mutagenesis is the creation of a hyper-butanol-producing strain *C. beijerinckii* BA101. This strain was created from *C. beijerinckii* NCIMB 8052 by chemical mutagenesis using *N*-methyl-*N*-nitro-*N*-nitrosoguanidine. The mutant strain had an increased tolerance to butanol at 19 g/L

as compared to 9 g/L with the parent strain (Qureshi and Blaschek 2001a). However, a drawback of mutagenesis is mutant instability and a low percent of desirable mutants (Xue et al. 2013a).

An approach that could overcome the problem of mutant instability is evolutionary engineering. Evolutionary engineering uses the same principle of evolution in nature—natural variation and selection under artificial conditions that imitate the expected conditions in industrial fermentation processes (Xue et al. 2013a). An efficient evolutionary approach is genome shuffling, a technique that combines benefits of repeated shuffling of DNA from multiple parents with the recombination of entire genomes which are usually associated with conventional breeding (Zhang et al. 2002a; Gao et al. 2012). Still, this approach has its drawbacks, the main ones being that screening for mutants is a slow and mundane task which largely depends on luck (Xue et al. 2013a).

Metabolic engineering, as a rational strategy, could help resolve some of the drawbacks of the methods above. A prerequisite for controlled and efficient use of this technology is full understanding of metabolic pathways for synthesis of butanol and other relevant metabolites.

For example, Jiang et al. (2009) used an approach to increase butanol ratio in the total solvent mixture, by eliminating the pathway for formation of acetone. This was done by disrupting acetoacetate decarboxylase gene (*adc*) in *C. acetobutylicum* EA2018. This technique resulted in increased butanol ratio from 70 % to 80.05 %.

In order to improve butanol yield and increase the pool of precursors for butanol synthesis, Lee et al. (2009) engineered a megaplasmid pSOL1-deficient strain, *C. acetobutylicum* M5 with the *adhE1* and *ctfAB* genes. These genes catalyse the formation of acetoacetate and ethanol/butanol with acid reassimilation in solventogenesis. Acetoacetate is formed by moving the CoA group of acetoacetyl-CoA to acetate and butyrate and forming acetyl-CoA and butyryl-CoA, which are precursors for ethanol and butanol, respectively. The result was an increase in butanol selectivity (a molar ratio of butanol to total solvents) from 0.57 to 0.84. This strategy produces butanol by reassimilation of acetate and butyrate (cold channel); however, Jang et al. (2012a) showed that a better approach is to produce butanol directly from acetyl-CoA through butyryl-CoA (hot channel). In this approach *pta* and *buk* genes (encoding phosphotransacetylase and butyrate kinase) were deleted, and the *adhE1* gene (encoding a mutated aldehyde/alcohol dehydrogenase) was overexpressed. This resulted in the production of 18.9 g/L of butanol in a batch fermentation with a yield of 0.71 mol butanol/mol glucose.

A drawback of clostridial butanol production is endospore formation or sporulation during the solventogenesis. Therefore, options of engineering non-sporulating strains that produce butanol have been investigated as well. Some strategies were successful in eliminating sporulation and reducing or even completely stopping acetone formation, due to which butanol yield was significantly improved (Lee et al. 2009; Sillers et al. 2008, 2009).

Not only clostridial species have been modified but also strains of *E. coli* (Atsumi et al. 2008b; Inui et al. 2008; Nielsen et al. 2009) and *Saccharomyces cerevisiae* (Steen et al. 2008) than butanol-resistant species including *Pseudomonas*

putida, *Bacillus subtilis* (Nielsen et al. 2009), *Lactobacillus brevis* (Berezina et al. 2010), *Lactococcus lactis*, *Lactobacillus buchneri* (Liu et al. 2010), *Corynebacterium glutamicum* (Smith et al. 2010), *C. tyrobutyricum* and *Synechococcus elongatus* (Lan and Liao 2012) and *Cyanobacteria* spp. (Xue et al. 2013a). Every heterologous microorganism used for construction of butanol-producing pathway has its advantages and disadvantages, and the next paragraphs will present some of them.

One of the major advantages of using non-clostridial hosts for butanol production is to avoid creation of by-products. For this purpose, *E. coli* and *S. cerevisiae* represent two obvious microorganisms of choice since they are well-defined, well-established and user-friendly platforms for various modifications (Hong and Nielsen 2012; Xu et al. 2012). Also, Gram-positive bacteria in general have their set of advantages. For example, they can ferment various sugars concurrently and grow at lower pH values, and some strains are mesophilic, i.e. they can grow optimally at a temperature range from 30 to 50 °C, which is the temperature of many fermentation processes (Bothast et al. 1999). From this group, lactic acid bacteria and other nonspore-forming Gram-positive species are considered an attractive alternative to clostridia and should be further researched for butanol production by metabolic engineering (Qureshi et al. 2013).

With *E. coli*, Atsumi et al. (2008b) made the first steps in reconstructing the butanol pathway by expressing six enzymatic steps from *C. acetobutylicum* that transform acetyl-CoA to butanol. Furthermore, moderate oxygen supply increased butanol production, indicating that the NADH pool under anaerobic conditions represented a bottleneck. The researchers also found the *adhE2* from *C. acetobutylicum* to be more effective in butanol production than *adhE* from *E. coli* because *adhE* had a higher affinity towards acetyl-CoA than towards butyryl-CoA (Atsumi et al. 2008b). This and other artificial pathways and metabolic engineering approaches are discussed more in detail in Sect. 4.1.2.2. Recently, Shen et al. (2011) developed a strategy for acquiring high-titre butanol production in *E. coli*. This approach is based on creating NADH driving force by deleting genes for the mixed-acid fermentation reactions (ethanol, lactate and succinate) in *E. coli*. At the same time, the NADH driving force was coupled to the desired 1-butanol pathway by engineering the crotonyl-CoA reduction step to be catalysed by *trans*-enoyl-CoA reductase (Ter) since this enzyme utilises NADH directly as the electron donor.

S. cerevisiae is an attractive organism for butanol production because of its expected tolerance to this alcohol (Knoshaug and Zhang 2009). *S. cerevisiae* was engineered with a butanol-producing pathway using enzymes from different organisms, and 2.5 mg/L butanol was produced (Steen et al. 2008). The main problem with engineering *S. cerevisiae* with butanol production pathway is engineering a eukaryotic organism with enzymes cloned from prokaryotic species, which leads to lower activities of those enzymes (Xue et al. 2013a).

Microorganisms producing butanol are under constant stress due to the simultaneous impact of different toxic substances that can have a synergistically harmful effect on the cellular physiology and metabolism. These are above all solvents,

particularly butanol, and acids like acetate and butyrate. This is why butanol toxicity is one of the major obstacles in the production of this alcohol. Some of the highest reported butanol concentrations in clostridial ABE fermentation are around 20 g/L in laboratory conditions (Ezeji et al. 2004a; Isar and Rangaswamy 2012; Xue et al. 2012), but only around 12 g/L of butanol is produced in commercial/industrial production (Green 2011).

In ABE fermentation, butanol is the most toxic solvent due to its lipophilicity. It affects the physiology of cell membranes by dissolving their phospholipid components (Bowles and Ellefson 1985). As with acids, the molecular mechanism underlying butanol tolerance is still not fully understood.

Some genetic manipulations have been found to affect butanol tolerance of microorganisms. The expression of gene *groESL* from *B. subtilis* in *C. acetobutylicum* resulted in increased butanol tolerance and production (Tomas et al. 2003). A potentially promising approach is to create a genomic library under increased butanol stress and perform a microarray analysis to detect genes responsible for butanol tolerance (Xue et al. 2013a).

4.1.2 Metabolic Pathways for Butanol and Isobutanol Production

Butanol is naturally produced by a number of microorganisms from the *Clostridia* genus. The metabolism of these organisms is regulated by their genetic information, and a number of metabolic manipulations have already been examined, mostly in order to improve butanol tolerance.

However, these microorganisms are more complex and not fully understood or analysed. Other approaches have focused on using well-understood and more user-friendly organisms (*E. coli*, *S. cerevisiae*).

We will first give an overview of the traditional ABE fermentation of clostridia before focusing on different metabolic engineering approaches.

Clostridial ABE Fermentation

So far, the only known natural butanol produced are numerous clostridial species in a process called ABE (acetone–butanol–ethanol) fermentation. Butanol pathway in ABE fermentation consists of condensing two acetyl-CoA molecules (catalysed by a thiolase) and then reducing the product to butanol (requiring four reductases and one dehydratase). Except butanol, acetone and ethanol, clostridia can also synthesise different chiral substances whose classical chemical synthesis would be challenging (Rogers et al. 1986), and they can degrade several toxic compounds (Francis et al. 1994; Spain 1995).

Clostridia have the ability to use many different carbon sources like pentoses and hexoses, but also polysaccharides like starch (Jones and Woods 1986; Ezeji et al. 2007a). This ability is important for the use of low-cost cellulosic waste materials from agriculture as fermentation substrates, since substrate cost is one of

the main factors that determine the cost of biobutanol production (Qureshi and Blaschek 2000a).

Since clostridia are amylolytic, there is no need for starch pretreatment and degradation to glucose as with yeast. Hexoses are metabolised over glycolysis to 2 mol of pyruvate, 2 mol of adenosine triphosphate (ATP) and 2 mol of reduced nicotinamide adenine dinucleotide (NADH) for each mol of hexose (Jones and Woods 1986). Pentoses are degraded using the pentose phosphate pathway to pentose 5-phosphate which is further transformed to fructose 6-phosphate and glyceraldehyde 3-phosphate (Cynkin and Delwiche 1958; Cynkin and Gibbs 1958). These two substances that enter the glycolytic pathway and further on yield 5 mol of ATP and 5 mol of NADH for every 3 mol of pentose. The pyruvate obtained in glycolysis is further on transformed to acetyl-CoA (Fig. 1).

Butanol in the ABE fermentation is synthesised condensing two acetyl-CoA molecules that form the 4 C atom basis of the butanol molecule (initially in the form of acetoacetyl-CoA). In the following enzymatic steps, it is reduced and dehydrated to butyryl-CoA, which is reduced two times again to yield butanol (Branduardi et al. 2014).

The characteristic property of clostridial solvent synthesis is a two-phase fermentation. In a batch process, during the first, acidogenic phase hydrogen, carbon dioxide, acetate and butyrate are produced. This leads to a drop in the pH of the fermentation medium (Jones and Woods 1986). This acidogenic phase typically takes place during the exponential growth phase of the culture (Andersch et al. 1983; Hartmanis and Gatenbeck 1984). When the culture passes in the stationary growth phase, it goes through a metabolism shift to solvent production. In this solventogenic phase acids are consumed and transformed to produce acetone, butanol and ethanol, which results in the rise of pH (Jones and Woods 1986; Dürre et al. 1987). Concurrently with the acid reassimilation, the consumption of carbohydrates continues. There is a close relationship between the pH level and the start of the solventogenic phase. Therefore, low pH is a requirement for solvent production (Jones and Woods 1986; Kim et al. 1984). Solventogenesis is also closely tied to sporulation (Lee et al. 2008).

One characteristic of clostridial butanol production is that it requires a high redox potential in order to produce butanol (and ethanol). That means, if a substrate is enriched with supplementary-reducing substances, this will intensify butanol and ethanol synthesis, but reduce acetone production (Mitchell 1998).

As described above, the clostridial ABE fermentative path leads to synthesis of butanol, together with smaller amounts of acetone, ethanol and acetic and butyric acids, together with carbon dioxide and hydrogen (Branduardi et al. 2014). Normally, the solvent ratio of acetone, butanol and ethanol, respectively, is 3:6:1, and the total solvent concentration is around 20 g/L (Connor and Liao 2009). Many natural clostridial strains have the upper butanol tolerance limit at about 11–12 g/L. However, some mutants and engineered strains can tolerate up to 19 g/L of butanol (Jang et al. 2012a).

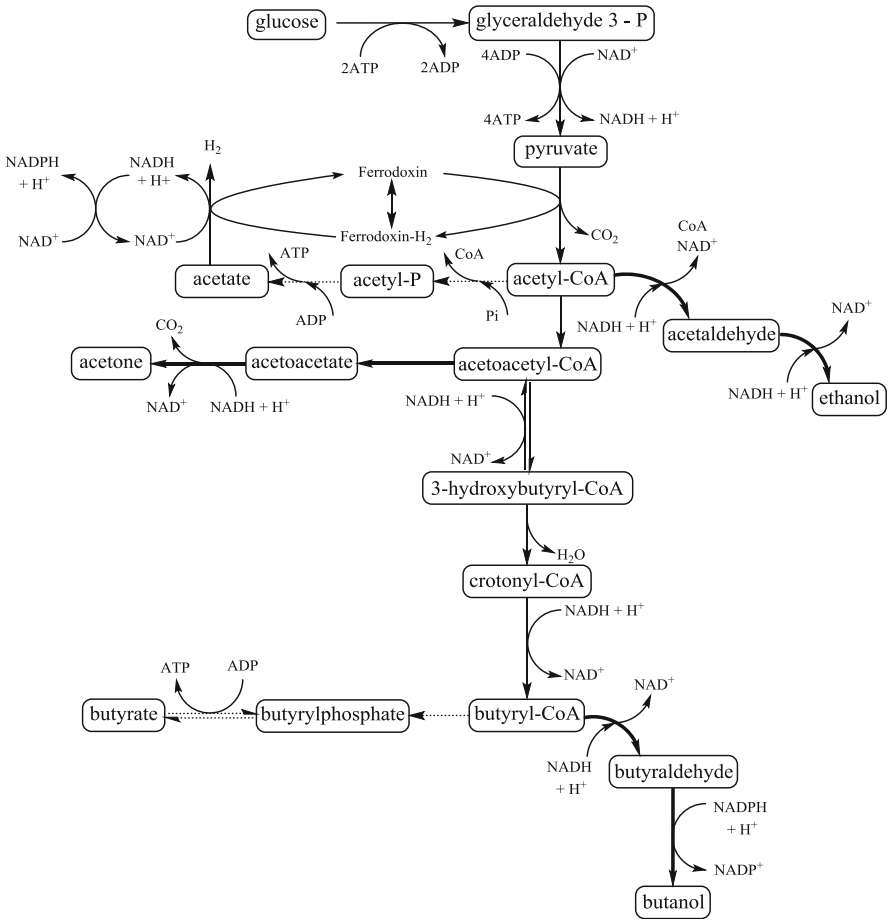

Fig. 1 Biochemical pathways of ABE fermentation in *C. acetobutylicum*. Reactions prevailing during the acidogenic phase of the fermentation are shown with *dotted arrows*, and reactions prevailing during the solventogenic phase are displayed by *thick arrows*

Engineered Metabolic Pathways

There are different tactics in the metabolic engineering of heterotrophic microbes for butanol and isobutanol production. Even though new methods are being examined intensively, engineered metabolic pathways for production of butanol can be classified in three groups:

- Through traditional ABE fermentation pathway (Fig. 2—third from the left)
- Over the amino acid pathways (Fig. 2—left-hand side)
- Over citramalate pathway (Fig. 2—right-hand side)

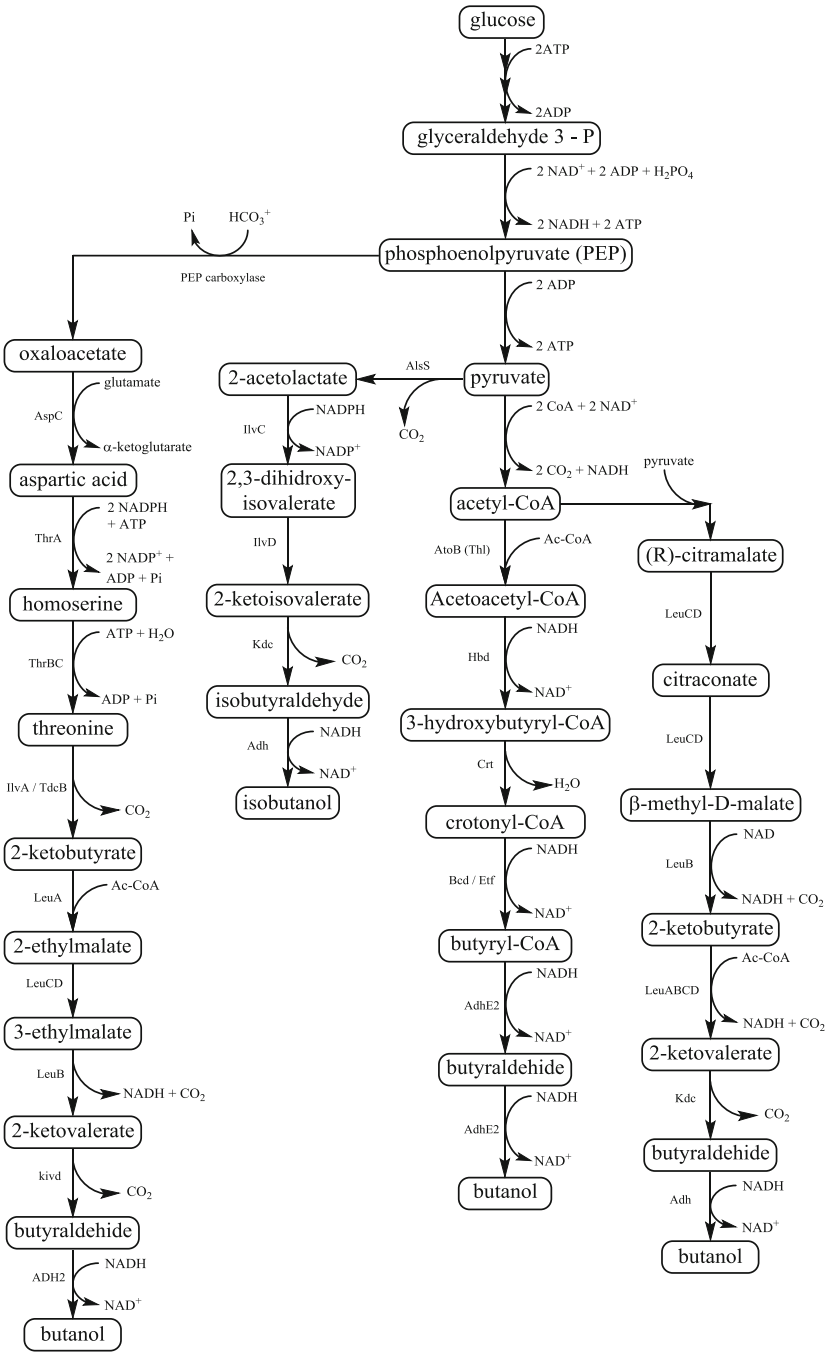

Fig. 2 Engineered metabolic pathways for synthesis of butanol and isobutanol

Traditional Fermentation Pathway in Heterologous Organisms

One approach, first examined by Atsumi et al. (2008b), is the metabolic engineering of the relatively easily manipulated bacteria *E. coli* to make *n*-butanol, not characteristic for this species. The first part of the metabolic pathway used *E. coli* metabolism from glucose to acetyl-CoA, and the second followed the engineered 1-butanol production pathway from *C. acetobutylicum*, consisting of six enzymatic steps from acetyl-CoA to *n*-butanol. In order to achieve the required transformations in these steps, it was necessary to express genes that encode the synthesis of acetoacetyl-CoA, 3-hydroxybutryl-CoA, crotonyl-CoA, butyryl-CoA, butyraldehyde and, lastly, *n*-butanol.

In order to achieve homofermentative production of 1-butanol, only these genes encoding 1-butanol production from the ABE fermentation pathway native to the *C. acetobutylicum* (Atsumi et al. 2008b; Inui et al. 2008; Boynton et al. 1996; Fontaine et al. 2002) were cloned and expressed in *E. coli* using expression plasmids (Atsumi and Liao 2008a).

Even though the activity of two enzymes, butyryl-CoA dehydrogenase (Bcd), and an electron transfer flavoprotein (Etf) was not definitely determined, possibly due to the instability of the enzymes, this method yielded 14 mg/L of 1-butanol under anaerobic conditions (Atsumi and Liao 2008a). Since the expression and activity of the Bcd and Etf were not conclusive, genes encoding homologues and isoenzymes from other microorganisms were expressed. Genes *bcd* and *etfAB* from *Megasphaera elsdenii* and *ccr* (that encodes a crotonyl-CoA reductase) from *Streptomyces coelicolor* were expressed. However, the genes from *M. elsdenii* and *S. coelicolor* led to a lower production of 1-butanol.

Another strategy is to delete the host pathways that compete for carbon and reduce sources, in this case for acetyl-CoA and NADH. When the genes *ldhA* (encoding lactate dehydrogenase) (Jiang et al. 2001), *adhE* (encoding alcohol dehydrogenase) (Leonardo et al. 1996), *frdBC* (encoding fumarate reductase) (Garnier et al. 2003), *pta* (encoding phosphate acetyltransferase) (Presecan-Siedel et al. 1999) and *fnr* (encoding FNR, a transcriptional activator) were deleted from this engineered strain of *E. coli*, *n*-butanol production increased almost threefold, and levels of acetate, lactate, ethanol and succinate produced were significantly reduced (Atsumi and Liao 2008a).

According to Atsumi and Liao (2008a), these combined strategies resulted in a maximal 1-butanol concentration of 500 mg/L, while Inui et al. (2008) achieved a maximum concentration of 1.2 g/L of 1-butanol after 60 h fermentation, through expression of the entire heterologous pathway from a single plasmid.

These butanol titres are by far too small for an economically viable butanol production, but they represent starting points that demonstrate the potential of this technology.

As can be seen from the previous example, simply overexpressing the genes responsible for butanol production is not enough to achieve the desired butanol concentration. In other words, these are just proof-of-concept solutions, but they cannot be applied in industry without obtaining the metabolic switch. Shen

et al. (2011) reviewed high-yield processes and concluded that all of them have some sort of a driving force, for example, in the form of an irreversible reaction, where the release of CO_2 directs the carbon flux in the desired direction. However, the clostridial CoA-dependent pathway engineered in *E. coli* has no such driving force. Therefore, these researchers created a NADH-dependent driving force by deleting the genes encoding enzymes that catalyse mixed-acid fermentation reactions (*adhE*, *ldhA* and *frd* that lead to ethanol, lactate and succinate formation). Since these NADH-consuming pathways were deleted, the strain cannot recycle NADH, which creates a driving force for reactions consuming NADH.

Additionally, the enzyme catalysing crotonyl-CoA reduction, butyryl-CoA dehydrogenase complex (Bcd-EtfAB), was replaced by *trans*-enoyl-CoA reductase (Ter) that catalyses an irreversible reduction. Furthermore, *trans*-enoyl-CoA reductase utilises NADH directly as the electron donor, thus is coupled to the driving force more tightly.

Using this approach, a high butanol concentration of 15 g/L was achieved, and when in situ product removal was coupled with the fermentation system, butanol concentration of 30 g/L was attained.

Similarly, another study (Ohno et al. 2013) confirmed that deletion of the ethanol (*adhE*) and acetate (*pta*) production pathways is essential for high-titre production of *n*-butanol in engineered *E. coli* cells. In this study, constraint-based metabolic flux simulation was used to predict the effect of different triple knockouts on the productivity of butanol and identify knockout candidates. Also, the authors proposed leading the flux through the pentose phosphate pathway to increase the pool of NADPH that could make further NADH available for *n*-butanol production because of transhydrogenase activity (Ohno et al. 2013).

It was mentioned above that the traditional ABE pathway has been expressed in other organisms except *E. coli*: *Saccharomyces cerevisiae* (Steen et al. 2008), *Lactobacillus brevis* (Berezina et al. 2010), *Pseudomonas putida*, *Bacillus subtilis* (Nielsen et al. 2009) and *Synechococcus elongatus* (Lan and Liao 2012).

One of the main reasons for expression of the ABE pathway in heterologous hosts is achieving higher butanol tolerance. Butanol toxicity is an obstacle that causes a whole other set of problems towards economically feasible butanol production—before all, high separation costs.

That is why some of the chosen heterologous hosts are the ones with known solvent tolerance like *P. putida* (Heipieper and de Bont 1994; Poblete-Castro et al. 2012), and *B. subtilis* (van Dijl and Hecker 2013) was then explored for the reconstruction of the *n*-butanol biosynthetic pathway (Nielsen et al. 2009). The Gram-negative *P. putida* was cultured in terrific broth medium under aerobic conditions and produced 50 and 122 mg/L butanol using glucose and glycerol as carbon source, respectively. *B. subtilis* produced 23 and 24 mg/L of *n*-butanol using glucose and glycerol as carbon source, respectively, under anaerobic conditions. This was somewhat a surprise, since *B. subtilis* was expected to be the better producing strain to its greater solvent tolerance and greater phylogenetic similarity with *Clostridium* (Branduardi et al. 2014).

In *L. brevis*, endogenous aldehyde and alcohol dehydrogenases were used acting as butyraldehyde (Bldh) and butanol dehydrogenase (Bdh), respectively, to convert butyryl-CoA into 300 mg/L of butanol. Due to the presence of the endogenous Bldh and Bdh, less heterologous expression was required (Berezina et al. 2010).

Considering alcohol tolerance, *Saccharomyces cerevisiae* comes as an obvious choice. Butanol being more toxic to microorganisms, also yeast has a lower tolerance level for butanol than for ethanol, around 2 % w/v (Knoshaug and Zhang 2009; Fischer et al. 2008). A similar strategy to the one with *E. coli* was attempted by Steen et al. (2008) by using *S. cerevisiae* as platform organism. *S. cerevisiae* was chosen because it is a genetically controllable, well-described organism, it is a current industrial alcohol producer (hence, greater butanol tolerance is expected), and it was already used to produce heterologous metabolites through genetic manipulations.

Several heterologous genes from *S. cerevisiae*, *Streptomyces collinus*, *E. coli*, *Clostridium beijerinckii* and *Ralstonia eutropha* were expressed for construction of the *n*-butanol fermentation pathway. The best result was achieved with the strain ESY7, in which several heterologous genes from *C. beijerinckii* and *S. collinus* were expressed. This strain produced 2.5 mg/L of 1-butanol after 72 h from 2 % w/v solution of galactose as a carbon source (Steen et al. 2008). Even though the metabolite concentrations in each of these strains are low, further research and optimisation could raise the butanol levels produced.

S. cerevisiae is the only eukaryotic organism used for expression of the *n*-butanol pathway. The possible incompatibility of bacterial genes in a eukaryotic organism is likely one of the limitations. Probably the biggest obstacle concerns the poor availability of acetyl-CoA in the cytoplasm (Pronk et al. 1996), which is where the expressed butanol pathway enzymes are active.

Via Amino Acid Pathways

In the previous section, an expression of a heterologous pathway in a user-friendly microorganism was reviewed. However, this expression of non-native metabolic pathways causes certain issues:

- Non-native pathways may compete for precursors with the native metabolism necessary for growth or maintenance.
- Competition for precursors can lead to a metabolic imbalance.
- Accumulation of the heterologous metabolites may cause cytotoxicity.
- Fine-tuning the expression of genes to avoid bottlenecks and optimise target compound production can be complicated (Connor and Liao 2009; Atsumi and Liao 2008a).

In order to achieve high biofuel productivity, the synthetic pathway should be compatible with the host (Atsumi and Liao 2008a).

For these reasons, a different approach was examined that uses the amino acid biosynthesis pathways, universal for almost all microorganisms. Atsumi et al. (2008a) employed this strategy by using the native amino acid pathway of

E. coli until 2-keto acid intermediates, which were then transformed to isobutanol or butanol using the last two steps of the Ehrlich degradation [2-keto acid is decarboxylated using 2-keto acid decarboxylases (KDCs) (de la Plaza et al. 2004) to an aldehyde, which is then reduced to an alcohol using alcohol dehydrogenases (ADH) (Sentheshanuganathan 1960)]. For the last two steps, 2-keto acid decarboxylase (Kivd) from *Lactococcus lactis* and the alcohol dehydrogenase (Adh2) from *S. cerevisiae* are examples of appropriate enzymes with broad substrate activity (Rude and Schirmer 2009).

In this way, by only using two non-native steps, the amino acid synthesis pathway was diverted towards alcohol generation. Additionally, unlike native butanol formation pathways in clostridia, the amino acid biosynthesis pathways avoid the requirement of involving CoA-dependent intermediates (Shen and Liao 2008).

Isobutanol Production Via Valine Pathway The above-named approach was first examined on isobutanol biosynthesis. Isobutanol can be converted from 2-ketoisovalerte, which is an intermediate in valine synthesis pathway. Atsumi et al. (2008a) first tested five different KDCs (Pdc6, Aro10, Thi3 from *S. cerevisiae*, Kivd from *L. lactis* and Pdc from *C. acetobutylicum*) by overexpressing them together with alcohol dehydrogenase 2 (Adh2) of *S. cerevisiae*. Subsequently, it was concluded that Kivd is the most active and versatile of the decarboxylases. Furthermore, *ilvIHCD* genes were overexpressed on a plasmid in order to boost biosynthesis of 2-ketoisovalerate, which combined with the alcohol-producing part of the pathway (Kivd and Adh2) resulted in 23 mM (1.70 g/L) of isobutanol being created, i.e. five times more than the strains without *ilvIHCD* overexpression.

In order to reduce competitiveness for precursors (particularly to increase the amount of pyruvate available for isobutene pathway), genes leading to by-product formation, like *adhE*, *ldhA*, *frdAB*, *fnr*, *pta* and *pflB*, were deleted. Lastly, *ilvIH* was replaced with *alsS* gene from *Bacillus subtilis*, since it has high affinity for pyruvate.

Combination of all of these methods and gradual optimisation of the organism resulted in a strain that synthesised ~300 mM (22 g/L) isobutanol, with a yield of 0.35 g isobutanol per g glucose after 112 h, which is 86 % of the theoretical maximum (Atsumi et al. 2008a).

The relatively high-level isobutanol production using the amino acid pathway shows the potential of this strategy and small stepwise optimisation. This versatile approach was used for production of 1-butanol (Atsumi et al. 2008a) over the norvaline biosynthesis pathway, which is a minor side reaction of leucine biosynthesis (Connor and Liao 2009).

1-Butanol Production via Norvaline Pathway Besides isobutanol it is possible to use the amino acid pathways to produce 1-butanol and 1-propanol that share a common precursor—threonine.

As stated above, clostridial species use a coenzyme-A-dependent biosynthesis pathway to produce 1-butanol (Jones and Woods 1986; Atsumi et al. 2008b). On the

other hand, it is possible to produce butanol using the threonine biosynthesis pathway until 2-ketobutirate which can be transformed to 2-ketovalerate using the pathway for a non-proteinogenic amino acid—norvaline (Kisumi et al. 1976) [which is a side reaction of leucine formation pathway (Atsumi and Liao 2008a)]. Lastly, instead of being transaminated into norvaline, 2-ketovalerate is transformed to 1-butanol using the last two steps of the Ehrlich degradation as described above (Shen and Liao 2008). The direct precursor to 1-butanol, 2-ketovalerate, is a rare metabolite, unlike 2-ketobutyrate. This is why it was needed to increase pool of this precursor by overexpressing enzymes leading to its synthesis and deleting competitive pathways. Initially, genes *ilvA* and *leuABCD* (leucine biosynthesis operon) from *E. coli* were overexpressed, the first one to increase the metabolic flux towards 2-ketobutyrate and the second one to intensify the norvaline synthetic pathway towards 2-ketovalerate. Also, Kivd and ADH2 were overexpressed in order to transform the precursors into alcohols (Shen and Liao 2008).

Since threonine production was one of the bottlenecks identified. In order to improve butanol production, the following steps were implemented: overexpression of appropriate genes leading towards key precursors, expression of enzymes resistant to allosteric inhibition and deletion of competing pathways.

By expressing an operon thrA^{fbr}BC containing an allosteric resistant mutant from the threonine hyper-producer *E. coli* ATCC 21277 (Shiio et al. 1971), production levels of 1-propanol and 1-butanol increased three to four times compared to the strain without the mutant. Additionally, genes *metA* and *tdh* (encoding homoserine O-succinyltransferase and threonine dehydrogenase) were deleted to disrupt loss of precursors to methionine and 2-amino-3-ketobutyrate synthesis, respectively. In order to preserve acetyl-CoA and 2-ketobutyrate, routes leading to biosynthetic pathways of valine, leucine and isoleucine are removed by deleting *ilvB* (encoding a catalytic subunit of the acetohydroxyacid synthase) and *ilvI* (encoding a catalytic subunit of acetohydroxyacid synthase III). Lastly, in order to reduce the loss of acetyl-CoA and increase the specificity by lowering ethanol production, *adhE* gene [encoding acetaldehyde–alcohol dehydrogenase (Leonardo et al. 1993)] was deleted.

This approach resulted in a strain that produced a total of 2 g/L total alcohols with nearly equal amounts of butanol and propanol (Shen and Liao 2008). Even though the alcohol titre achieved is nowhere near the level of production of clostridial species, it does demonstrate the potential of the strategy. An approach using amino acid pathways is especially interesting, since threonine hyper-producing strains (Shiio et al. 1971; Miwa et al. 1983) have already been developed and are in use in industry (Atsumi and Liao 2008a).

Some possible points of improvement are increasing butanol tolerance either by developing new methods or using existing ones [e.g. Lee et al. (2011), Borden and Papoutsakis (2007), Yomano et al. (1998)]. Another issue is increasing concentrations of acetic and lactic acid that could be consequences of surplus of acetyl-CoA and NADH, respectively (Shen and Liao 2008). If production of butanol only is desirable, than the transformation of 2-ketovalerate to butanol needs to be given the

advantage over propanol formation. This could potentially be done by improving the *leuABCD* to increase the rate of reaction from 2-ketobutyrate to 2-ketovalerate and by designing a keto acid decarboxylase and alcohol dehydrogenase that have greater specificity towards 2-ketovalerate.

Butanol Production via Citramalate Pathway

Atsumi and Liao (2008b) used another approach to construct a metabolic "shortcut" to 2-ketobutyrate. As described above, 2-ketobutyrate is an important intermedier and a product of threonine deamination, and threonine is produced by six enzymatic steps from oxaloacetate (Connor and Liao 2009). The scientists found a way to circumvent this (thus avoiding transamination and the subsequent deamination) and designed a more direct route towards this important metabolite, by using the growth phenotype linked to 2-keto acid deficit. 2-Ketobutyrate can be synthesised from citramalate, which in turn can be produced directly from pyruvate and acetyl-CoA (Atsumi and Liao 2008b).

Some microorganisms [e.g. *Leptospira interrogans* (Westfall et al. 1983; Xu et al. 2004) and *Methanococcus jannaschii* (Howell et al. 1999)] can convert pyruvate and acetyl-CoA to 2-ketobutyrate, by using citramalate synthase (CimA). Atsumi and Liao used this to construct a propanol and butanol hyper-producer.

First, gene cimA from *M. jannaschii* (encoding citramalate synthase) and leuABCD from *E. coli* were cloned and overexpressed in *E. coli*. In this strain genes *ilvA* and *tdcB* (encoding threonine deaminase and catabolic threonine dehydratase (Riley et al. 2006), respectively) were deleted. Hence, the enzymatic step that converts threonine into 2-ketobutyrate was interrupted, making the strain auxotrophic for isoleucine, thus requiring 2-ketobutyrate for growth (Atsumi and Liao 2008b). In this way, it was possible to differentiate strains with an active citramalate pathway, since these were the strains that exhibited growth.

Overexpression of these genes enabled growth, but in order to improve the activity of citramalate synthase, directed evolution strategy was used (Kuchner and Arnold 1997). Growth phenotype linked to 2-keto acids (as precursors of amino acids) was used as selection in directed evolution (Connor and Liao 2009).

The first round of mutations was performed by error-prone PCR, and strains with increased growth were isolated and used in the second round of evolution performed by DNA shuffling in a second round of evolution (Atsumi and Liao 2008b).

In the next two rounds of selection, an additional introducing an *ilvI* deletion was introduced, to reduce enzyme affinity towards 2-ketobytirate and lower isoleucine synthesis. This was another eliminating criterion for CimA expression, since strain growth is determined by the ability of CimA to provide precursors for L-isoleucine synthesis (Atsumi and Liao 2008b).

Six rounds of directed evolution were performed, and the best strains demonstrated a 9-fold and 22-fold increase in 1-propanol and 1-butanol synthesis,

respectively, over the wild-type strain. This corresponds to a maximum concentration of 3.5 g/L and about 0.50 g/L of 1-propanol and 1-butanol after 92 h, respectively.

4.1.3 Substrate Selection

An advantage that solventogenic clostridia have over, for example, yeast in terms of alcohol production is their ability to ferment various sugars into solvents. They can utilise, except glucose, also cellobiose, galactose, mannose, arabinose and xylose (Ezeji et al. 2007a). When a complex mixture of sugars is fermented by clostridia (like those obtained through hydrolysis of lignocellulosic biomass), the sugars are consumed simultaneously, however at a different rate and preference, where glucose is the preferred monosaccharide (Raganati 2012; Ezeji et al. 2007a; Tracy et al. 2012). Since some of these sugars are components of cellulose and hemicellulose, this ability of clostridia (and some other anaerobic microbes) is essential for use of cheap, non-foodstuff, renewable biomass for butanol production. Especially since substrate cost constitutes the greatest part of total butanol production costs (Lee et al. 2008).

Many different raw materials have been used to ferment butanol, including molasses, whey permeate, spray-dried soy molasses (SDSM) (Qureshi et al. 2001), starch, corn (Jones and Woods 1986; Ezeji et al. 2007c; Ezeji and Blaschek 2008; Qureshi et al. 2008a) and glucose derived from corn (Qureshi and Blaschek 2005), including, more recently, cheap waste materials like starch packing peanuts (Jesse et al. 2002), corn fibre xylan (Qureshi et al. 2006a), corn stover (Ezeji et al. 2007b), corn fibre (Qureshi et al. 2008a), orchard waste (Qureshi and Blaschek 2005), distillers dried grains and solubles (DDGS), wheat straw (Qureshi et al. 2006b) and wheat straw hydrolysate (WSH) (Qureshi et al. 2007), barley and rice straw hydrolysates (Qureshi et al. 2010a), switchgrass (Qureshi et al. 2010b), alfalfa, reed canary grass, sugarcane bagasse, *Miscanthus* and waste paper (Qureshi et al. 2013). From all the reviewed biomass, pretreated softwood shows most resistance to enzymatic hydrolysis (Galbe and Zacchi 2002).

Wheat straw hydrolysate was pretreated with dilute sulphuric acid, enzymatically hydrolysed and fermented in batch fermentation, producing 25 g/L total solvents. The productivity of this system (0.60 g/L h) was more than double than that of an analogue control glucose fermentation (Qureshi et al. 2007). On the other hand, corn stover pretreated and hydrolysed in the same manner did not support fermentation, until it was treated with lime, when it yielded 26.27 g/L total solvents (Qureshi et al. 2010b).

Barley straw hydrolysate was also toxic to clostridia, and several treatments were applied like diluting the hydrolysate with water, mixing with wheat straw hydrolysate and overliming. Overliming gave the best results at 26.64 g/L total solvents (Qureshi et al. 2010a).

A combination of corn stover hydrolysate and corncob hydrolysate (corncobs pretreated with steam expansion and hydrolysed enzymatically) was also tested as a

substrate, through fermentation in large reactor (48 m^3) which produced 20.50 g/L ABE (Marchal et al. 1992).

Switchgrass was also used as a substrate source (Qureshi et al. 2010b). It was pretreated with dilute sulphuric acid and hydrolysed with enzymes, but fermentation with untreated hydrolysate resulted in poor ABE concentration. Hydrolysate was mixed with wheat straw hydrolysate, treated with lime and diluted twofold with water, from which the last method gave the best results of 8.91 g/L total solvents.

Clostridia possess various numerous enzymes that catalyse degradation of polysaccharides (like starch) into their monomers, which are transported into the cell and subsequently metabolised through glycolysis or the pentose phosphate pathway (Lee et al. 2008; Ezeji et al. 2007b). The early ABE fermentation relied largely on corn and other starchy plants that required little pretreatment since clostridia, unlike yeast, are amylolytic and can consume starch without prior hydrolysis. Some clostridial strains are even reported to consume complex polysaccharides like cellulose and hemicellulose (Jang et al. 2012b). It has also been demonstrated that cell cultivation on xylose induced cellulose activities, i.e. production of hydrolytic enzymes (Andersch et al. 1983; Dürre 1998; Lopez-Contreras et al. 2004). Still, direct utilisation of cellulose by clostridia has not yet been achieved.

This is why lignocellulosic biomass has to be pretreated and hydrolysed for fermentation by clostridia to be possible. The purpose of pretreatment is to separate lignin and polysaccharides and make them accessible to enzymes either by removing the protective layers of either hemicelluloses (through acidic processes) or lignin (through alkaline processes) (Mosier et al. 2005a; Bozell 2010). The latter are generally regarded less feasible because of difficult recovery of alkali/ammonia (Mosier et al. 2005a; Wyman et al. 2005).

Examples of some of the pretreatment methods are sulphite pretreatment to overcome recalcitrance of lignocellulose (SPORL) (Zhu et al. 2009), dilute sulphuric acid (Purwadi et al. 2004), hot water-controlled pH (Mosier et al. 2005b), ammonia fibre expansion (AFEX) (Teymouri et al. 2005), steam explosion, SEW fractionation, ammonia recycled percolation (ARP) and lime treatment (Jurgens et al. 2012).

One issue with pretreatment is that it is difficult to predict the final chemical composition of the hydrolysate and the behaviour of pretreated biomass (Kumar et al. 2009). Still, a more serious issue of pretreatment, neutralisation and hydrolysis is the creation of a complex blend of microbial inhibitors (Ezeji et al. 2007a). These inhibitors are created regardless of the method used, but are considered that dilute acid pretreatment generates a relatively large amount of inhibitors, in contrast to relatively small concentrations produced by hot water and AFEX pretreatment methods (Ezeji et al. 2007b).

These inhibitors include compounds such as salts [sodium acetate, sodium chloride and sodium sulphate (Ezeji et al. 2007d)] and chemicals like furfural, 5-hydroxymethylfurfural (HMF) syringaldehyde and dissolved lignin (Ezeji et al. 2007d; Wang and Chen 2011) and acids (acetic, glucuronic, ferulic, formic and r-coumaric) (Qureshi et al. 2013). Even though clostridia have relatively good

resistance to some compounds (like some acids), examinations showed that syringaldehyde, ferulic and *p*-coumaric acids were especially powerful inhibitors of fermentation and cell growth (Green 2011; Ezeji and Blaschek 2008; Patakova et al. 2013).

On the other hand, some products of biomass pretreatment seem to be beneficial for fermentation and microbial growth, like furfural, HMF or acetic acid (Pienkos and Zhang 2009). This goes against the claims above, and it seems that these substances are stimulatory up to a certain concentration.

In order to reduce the concentration of inhibitory compounds, detoxification methods like electrodialysis (Qureshi et al. 2008b), liming (Qureshi et al. 2010b), treatment with ion exchange materials (Qureshi et al. 2007) and activated carbon (Wang and Chen 2011) are used. On the other hand, some authors find the detoxification of hydrolysates not economically viable, but that the more realistic solution lies in developing strains that are tolerant to inhibitors (Qureshi et al. 2013). Obviously every method has its own set of advantages and disadvantages, and a good comparison was made in a review by Jurgens et al. (2012).

One example of a method that could be interesting for industrial practice is SEW (SO_2/ethanol/H_2O) fractionation, which has multiple advantages over other pretreatment methods including it has relatively low energy and liquid requirement per unit biomass, it does not have hemicellulose degradation problems, it does not create sticky lignin precipitates (Iakovlev et al. 2011), it can concurrently process softwoods and hardwoods biomass (Yamamoto et al. 2011), and it only requires evaporation of ethanol and SO_2 to remove these inhibiting chemicals (which can then be reused for the next cycle) from the mixture (Jurgens et al. 2012).

Presently, substrates used for biological butanol production are sugar and starch based, where molasses are most practical due to the simplicity of the production process. At the moment, there is no economically viable microbial butanol production using lignocellulosic biomass, and even with all the treatment of corn for fermentation, it is still more economically competitive than the methods for butanol production from lignocellulosic feedstock (Xue et al. 2013a). The price range of lignocellulosic substrates is between 24 and 60 USD/ton, while the price range of corn is between 153 and 218 USD/ton. The production of corn-based ethanol in the USA is constantly on the rise, and simultaneously there is a noticeable increase in corn prices, even though there is still debate over how much these two trends are connected (Qureshi et al. 2013). Therefore, more effort needs to be invested into research and development of improved pretreatment methods for preparation of fermentation substrates from lignocellulosic biomass.

It seems that the crucial problem of lignocellulosic biomass utilisation is a lack of an economically feasible technology and the relatively high investment cost compared to crude oil derivatives since biomass processing is done in a smaller scale (van Heiningen 2011). This demonstrates the importance of operational demonstration plants (incorporated into existing industrial lignocellulosic production system) to integrate and test new technologies, reduce wastes and utilise by-products. From such facilities it would be possible to obtain data, not only in the context of technology but also finances.

4.1.4 Fermentation Processes

All three modes of reactor operation can be employed for ABE fermentation, each with its set of advantages and disadvantages. The choice is mostly dependant on the production capacity, with batch or fed-batch fermentation being more suitable for small-scale production (Xue et al. 2013a).

Conventionally, batch fermentations were used for butanol production. However, process productivity is limited to a maximum of 0.50 g/L/h, due to low cell concentration, idle time and product toxicity (Ezeji et al. 2006). It takes 2–6 days to complete a batch fermentation depending on the conditions, the substrate employed and the fermenting strain.

The final total concentration of solvents in a batch fermentation can vary from 12 to 20 g/L (Lee et al. 2008). At a butanol concentration of approximately 16 g/L, cell growth is inhibited and the fermentation ends (Ezeji et al. 2007b). However, this is also dependent on the microbial strain and fermenting conditions. The products are then separated from the fermentation broth by distillation.

The main reason why batch reactors are often preferred in the industry is the simple operation and lower contamination risk. Still, batch fermentation has a low productivity due to the lag phase of the culture; product inhibition; downtime for cleaning, sterilising and filling the reactor; low butanol concentration; and low initial substrate concentration. This is why fed-batch and continuous fermentation modes are intensively examined (Lee et al. 2008).

Standard fed-batch operation is not economically viable due to solvent toxicity, so the system can be coupled with an in situ recovery process (Lee et al. 2008; Ezeji et al. 2004a, b). Fed-batch fermentation is used principally in laboratories, but it cannot be applied without in situ product removal due to its strong cell inhibition. Semi-continuous operation is also used in industrial practice in order to avoid "strain degeneracy" (Branduardi et al. 2014; Xue et al. 2013a).

Continuous reactors are most appropriate for large-scale production due to their high productivity and lower labour and maintenance costs (Xue et al. 2013a). They can be used in the form of tank-in-series system (multistage continuous fermentation), with tanks that imitate the biphasic nature of the ABE fermentation, and cell growth, acid phase and solvent production take place consecutively within different tanks (Ni and Sun 2009). Single-stage continuous fermentation would not be feasible. Another approach is to use immobilised cell reactors and cell recycle reactors in order to increase cell concentration and productivity (Lee et al. 2008; Jang et al. 2012c; Tashiro et al. 2005). A continuous reactor with immobilised cells on clay bricks achieved high productivity of 15.8 g/L/h, while a continuous reactor with cells immobilised on a fibrous support had a productivity of 4.6 g/L/h (Qureshi and Blaschek 2005; Huang et al. 2004). A fermentation scheme with cell recycle had a productivity of up to 6.5 g/L/h (Ezeji et al. 2006). However, these systems lack testing on an industrial scale due to the high costs of product recovery (Branduardi et al. 2014).

Strain degeneration is a known problem of ABE fermentation, especially with continuous operation. Unlike ethanol fermentation by yeast, clostridial fermentation demands constant inoculation with viable cells for the forces to proceed without interruption (Xue et al. 2013a).

Various methods of in situ butanol removal have been tested in research, but so far they have not found application in industrial practice, due to the fact that the investment and additional energy costs are simply too high compared to the economic benefit resulting from the increased substrate efficiency and productivity. Also, combining the production system with in situ butanol removal increases the possibility of contamination (Xue et al. 2013a).

One of the biggest problems in ABE fermentation is product toxicity. Out of the produced solvents, butanol is the most toxic to the cellular metabolism, as it disrupts the lipophilic components of the cell membrane, and it is the only solvent in the ABE fermentation produced to the level that becomes toxic to the cells (Jones and Woods 1986; Bowles and Ellefson 1985). Clostridia cease with growth and fermentation when the solvent concentration reaches 20 g/L (Woods 1995). One of the consequences of this is that the concentration of carbon substrate that can be used for fermentation is limited, which means that the final product concentration and productivity will also be low, which further leads to high product separation costs. This demonstrates how butanol toxicity causes a whole chain of further problems and presents one of the main obstacles in making biobutanol production economically less feasible. Butanol toxicity has been so far addressed by strain development through random mutagenesis and process optimisation. Examples of these approaches are the development of more solvent-tolerant *C. beijerinckii* BA101 and integration of fermentation with an in situ solvent recovery process (Lee et al. 2008; Qureshi and Blaschek 2001a). However, random mutagenesis is simply not efficient enough to develop targeted mutations and physiological properties, and in situ product recovery is economically not viable. One of the possible solutions is using metabolic engineering and system biology approaches to develop solvent-tolerant strains.

4.1.5 Separation Methods

Traditionally, butanol was recovered from the fermentation broth by distillation; however, this method has high operation cost caused by the low butanol titre. Various technologies have tested for in situ butanol removal, since they have the potential to solve both solvent toxicity and low butanol concentration problems at the same time. However, they all share the problem of not being able to purify butanol efficiently enough as the final product (Xue et al. 2013a). Also, their benefits are usually not economically justified compared to the high investment and operation costs, and they increase the risk of contamination.

This is why a significant amount of research on biobutanol production has been made on the use of alternative fermentation and product recovery methods (Lee et al. 2008; Ezeji et al. 2007b). Some of these methods include, besides distillation,

pervaporation, adsorption, ionic liquids, liquid–liquid extraction, aqueous two-phase separation, supercritical extraction, gas stripping, perstraction and reverse osmosis (Ezeji et al. 2007b; Dürre 1998). Economically, reverse osmosis is most desirable, but it suffers from membrane clogging or fouling. On the other hand, liquid–liquid extraction has high capacity and selectivity, but it is relatively expensive (Dürre 1998).

Distillation is the separation method used in industry for fractioning of ABE fermentation products. Even though high energy consumption is often cited as one of the main drawbacks of this technique, energy consumption of the distillation system is not as high as would be assumed from the low butanol concentration (Xue et al. 2013a). For example, in a biobutanol production plant, cornstarch makes up 70–79 % of the total production cost, while the total energy costs (including energy required for distillation) contribute 14–20 % of the overall cost (Xue et al. 2013a; Pfromm et al. 2010).

A conventional design can be used to first increase the concentration of solvents up to 40 % (which would consume the biggest amount of energy) and then further fractioned by columns. Also, further optimisation of the beer stripper with more contacting devices could condense solvents to around 90 % and thus save energy in the following columns (Xue et al. 2013a). In large-scale butanol production, it would be possible to recycle latent heat from multistage pressure columns and use it to preheat bottom products from low-pressure columns. However, this setup would require more capital investment (Xue et al. 2013a).

Liquid–liquid extraction is a separation technique that uses the differences in the distribution coefficients of the chemicals in a carrier substance (extractant) to extract butanol from the fermentation broth without removing substrates, water or nutrients at the same time (Lee et al. 2008). In this case the extractant is a water-insoluble organic solvent that is mixed with the fermentation broth. And butanol and other nonpolar solvents are more soluble in the organic extractant than in water, and they concentrate in this organic phase. Since the extractant and the polar aqueous fermentation broth do not mix, the extractant is simply separated from the fermentation broth. Common extractants are decanol and oleyl alcohol (the latter one being promising, due to its relatively low toxicity) (Lee et al. 2008; Ezeji et al. 2006, 2007b; Evans and Wang 1988; Karcher et al. 2005). Drawbacks of liquid–liquid extraction are loss of extractant, formation of emulsions, toxicity of the extractant, etc. These drawbacks can be overcome with a modified method called perstraction, where fermentation broth and the extracting solvent are separated by a membrane (Ezeji et al. 2007b).

As mentioned above **perstraction** is a technique developed in order to solve the issues of liquid–liquid extraction. With this method the fermentation broth and the extractant are separated by a membrane that provides surface area over which the two immiscible liquids can exchange butanol molecules. Since there is no direct contact between the two liquids, this reduces or eliminates the named problems of liquid–liquid extraction. However, the membrane can reduce the rate of butanol extraction (Ezeji et al. 2007b).

Gas stripping is a simple but efficient method that can be used for in situ butanol recovery during the ABE fermentation. CO_2 and H_2 (gases that are formed during ABE fermentation) are bubbled through the fermentation broth, and as they pass through the fermenter, they capture produced solvents. This mixture is then led through a condenser to recover the solvents, and the purified gas is then recycled back to the fermenter (Lee et al. 2008; Ezeji et al. 2005a, b, 2006, 2007b). An advantage of gas stripping is that it enables the use of a concentrated sugar solution in the fermenter (Qureshi and Blaschek 2001b), a reduction in butanol inhibition and greater sugar utilisation (Maddox et al. 1995). Fed-batch fermentation can also be integrated with gas stripping to reduce substrate inhibition and increase cell mass (Ezeji et al. 2004b). Also, a two-stage gas stripping process was recently developed, for in situ butanol recovery, where a highly concentrated product of about 420 g/L butanol was obtained (Xue et al. 2013b).

Pervaporation is a membrane-based separation process that enables selective removal of volatile substances from the fermentation broth. When the membrane is in contact with the fermentation broth, the volatile liquids or solvents diffuse through the membrane as vapour that is subsequently recovered by condensation. This permeate contains all of the fermentation solvents; they still need to be fractioned by distillation. The pervaporation membranes can be both liquid and solid (Lee et al. 2008; Ezeji et al. 2007b). There are two main factors indicating the effectiveness of pervaporation: the selectivity (a measure of the selective removal of volatiles) and flux (the rate at which substances pass through the membrane per unit area) (Ezeji et al. 2007b). It seems that pervaporation does not have a negative effect on microorganisms, and there is a clear increase in reactor productivity due to reduction in product inhibition (Qureshi and Blaschek 2000b).

4.1.6 Current Challenges of Sustainable Biobutanol Production

In order to make ABE fermentation a viable and economically feasible option to butanol production, a number of technical issues need to be resolved. In general, there is a need for cheaper feedstock and improved fermentation performance than butanol toxicity/inhibition to the fermenting microorganisms as well as low final butanol titre need to be resolved or alleviated, and finally more sustainable process operations for solvent recovery and water recycle are required (Qureshi et al. 2013; Ezeji et al. 2007b). Green (2011) gave a concise overview of the different challenges of biobutanol production as well as potential approaches to solving them. They are presented in Table 1.

Above all, feedstock prices influence production cost. In the context of using low-cost lignocellulosic biomass as substrate source, direct cost for raw feedstock is lower, but more energy is needed for the pretreatment, and additional costs arise through consumption of cellulases that are expensive for the production of bulk commodities like fuel. On the other hand, such costs do not arise in starch-based feedstock since clostridia are amylolytic and do not require additional enzymes (Xue et al. 2013a). Research is made on understanding the mechanisms of sugar

Table 1 The challenges and solutions of biobutanol production (Green 2011)

Problem	Solution
High feedstock cost significantly increases operating costs	Transition towards cheaper (and more sustainable) feedstock such as wastes and agricultural residues
Low butanol titres increase recovery costs. Low titres also reduce sugar loadings and increase water usage	Develop improved microbes with improved solvent titres and/or develop methods for in situ product removal to alleviate end product tolerance
Low butanol yield increases feedstock costs	Develop improved microbes with higher butanol yields and/or develop microbes with higher butanol– solvent ratios
Low volumetric solvent productivities increase capital and operating costs	Develop continuous fermentation processes that reduce downtime and increase volumetric productivity
Solvent recovery using conventional distillation is energy intensive and relatively expensive	Develop low-energy methods for solvent recovery and purification. Recovery can also be improved by improving the solvent titre
High water usage is not sustainable and increases the cost of effluent treatment	Recycle process water back through the fermentation

transport, regulation of butanol production, butanol tolerance, utilisation of lignocellulosic biomass hydrolysates and cell inhibition by lignocellulosic degradation products, with the aim of improving butanol productivity, concentration and yield (Ezeji et al. 2007b). Still, first-hand data from demonstration plants are needed to provide a reliable understanding of production costs of butanol from lignocellulosic biomass (Xue et al. 2013a).

Butanol toxicity is an issue that causes a chain of problems as described previously. In order to overcome butanol toxicity, two approaches have been applied:

– Developing more butanol-tolerant strains using genetic engineering techniques
– Employing process engineering approaches to simultaneously recover butanol from the fermentation broth (Qureshi et al. 2013)

Also, advances in integrated fermentation and in situ product removal processes have resulted in a dramatic reduction of process streams, reduced butanol toxicity to the fermenting microorganisms, improved substrate utilisation and overall improved bioreactor performance (Ezeji et al. 2007b). Other limitations include the drawbacks of multiproduct fermentation which lowers the yield of butanol as the target molecule. This is also influenced by simultaneous "nonproductive" spore formation, low cell densities attainable with clostridia during anaerobic fermentation and strain degeneration that leads to production instability (Lee et al. 2008; Green 2011; Jang et al. 2012c; Zheng et al. 2009).

4.2 Isobutylene (Isobutene)

When considering the knowledge base of biobutanol production, enormous amounts of time and effort have already been invested into research and development, there is an abundance of scientific and industrial resources, and there already are production plants in place, while sustainable industrial-scale production is plausible in the foreseeable future. On the other hand, research and know-how on industrial bio-isobutene production is practically still in its infancy.

Isobutene is an important short-chain hydrocarbon and platform chemical used for the synthesis of various compounds like butyl rubber, terephthalic acid (precursor to the polyester PET), alkylate (octane enhancer in gasoline) and other specialty chemicals, and it has been used for the production of diesel substitutes from glycerol (Cheng et al. 2011; Jhung and Chang 2009; Marchionna et al. 2001; Sassmannshausen 2009). It is commercially produced through catalytic or thermal cracking of the C4 section of crude oil (Calamur et al. 2000). Isobutene can also be dimerised and hydrogenated to produce isooctane. Isooctane is an isomer of octane that is used as a standard for determining the octane number. It has a high energy density and could be used as a direct gasoline replacement that fits the existing infrastructure. It is also a promising replacement for the gasoline oxygenate methyl *tert*-butyl ether (MTBE), which is being replaced due to environmental concerns (Marchionna et al. 2001). Its importance is reflected in its price of around 2USD/kg (Martín and Grossmann 2014).

Biological hydrocarbons in general and isobutene in specific are promising next-generation biofuels with favourable physical and chemical properties compared to bioethanol, but the technology needed to economically produce these molecules in bulk amounts is not available at the moment (Rude and Schirmer 2009; Atsumi and Liao 2008a; Alper and Stephanopoulos 2009; Dellomonaco et al. 2010). Even though microbial production of isobutene is known since the 1970s, the lack of highly productive natural strains and appropriate genetic manipulation approaches made this process in a large scale unfeasible. However, this is rapidly changing in the last years with research gaining momentum, first patents being filed and pilot plants planned.

Microbial synthesis of isobutene was first officially identified with yeast *Rhodotorula minuta*, and later it was discovered that a microsome-associated cytochrome—P450rm—was the enzyme system responsible for isobutene formation. The rate of formation of isobutene from isovalerate is 0.18 nmol/nmol$_{P450rm}$/min or 16.4 μL/L/h from a live cell system (Fukuda et al. 1984, 1994). However, this rate is too low for commercial application. Additionally, P450rm is still not a very well understood membrane-bound hemeprotein that is difficult to produce in heterologous hosts (Shiningavamwe et al. 2006).

It was also discovered that some amino acids had a regulatory effect on the microbial synthesis of isobutene. For example, the addition of L-leucine into the fermentation broth stimulated isobutene formation. This was further accentuated by a synergistic effect of aromatic amino acids (L-phenylalanine, L-tryptophane and L-

tyrosine) as well as benzene compounds (benzoic acid, L-phenylglycine, L-phenylpyruvic acid, etc.) (Fukuda et al. 1985). Even though L-phenylalanine was shown not to be a direct precursor itself, it was presumed that it induced the synthesis of some enzymes involved in the formation of isobutene (Fujii et al. 1987, 1988; Ladygina et al. 2006).

There are some obvious advantages of isobutene fermentation compared to liquid fuels. For example, in bioethanol production, distillation accounts for about 9 % of its total cost and 58 % of the total energy use at an ethanol plant, and distillation of 1-butanol would be more expensive due to its higher heat of vaporisation and presence of other solvents that need to be fractioned (Rude and Schirmer 2009; Gogerty and Bobik 2010). On the other hand, since isobutene is a gaseous substance practically insoluble in an aqueous fermentation broth, it can easily be recovered from the bioreactor (van Leeuwen et al. 2012; Martín and Grossmann 2014).

Therefore, microbial isobutene from renewable feedstocks has potential as a next-generation biofuel produced at lower cost (Gogerty and Bobik 2010). Even though microbial synthesis of isobutene seems promising, there is little practical knowledge on how to realise this fermentation process economically (van Leeuwen et al. 2012). The main obstacle to commercial production of bio-isobutene from renewable resources is the lack of an efficient fermentative pathway for its synthesis (Gogerty and Bobik 2010).

As was mentioned and as will be discussed in greater detail in this review, there are several different approaches, and all of them have certain gaps in research that need to be filled before commercial application is possible.

4.2.1 Enzymatic Reactions and Metabolic Pathways of Isobutene Synthesis

Isovalerate Decarboxylation by Cytochrome P450

The first time that biological production of isobutene was documented was by Fukuda et al. among various fungi, yeasts and bacteria that were tested; yeast *Rhodotorula minuta* var. texensis IFO 1102 that produces isobutene at the stationary phase of growth had the highest production rate (Fujii et al. 1988; Fukuda et al. 1984, 1987) (Fig. 3).

Later, it was concluded that isobutene was synthesised through decarboxylation of isovalerate that is produced in the catabolic pathway of L-leucine (Fukuda et al. 1985, 1994). The decarboxylation reaction is catalysed by a microsomal cytochrome P450 (cytochrome P450rm) in the presence of NADPH, O_2 and cytochrome P450, which was an NADPH reductase (Fujii et al. 1988; Fukuda et al. 1994). The initial (and also the rate limiting) step of this reaction is the removal of the hydrogen atom at the β-carbon atom of isovalerate, which is then followed by decarboxylation. P450rm accepts electrons from the reductase and catalyses the formation of isobutene from isovalerate (Fukuda et al. 1994).

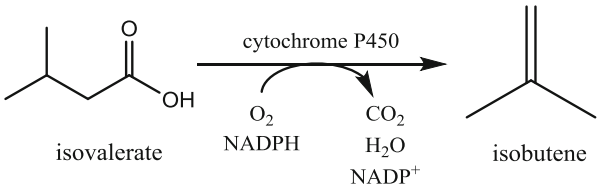

Fig. 3 Isobutanol synthesis using cytochrome P450 (van Leeuwen et al. 2012)

The isobutene metabolic pathway goes along the leucine biosynthesis pathway, from glucose to 2-ketoisocaproate (4-methyl-2-keto-pentanoate). The next two steps lead to isovalerate with a CO_2 unit splitting off, which is then transformed to isobutene with cytochrome P450 as catalyst and additional CO_2 equivalent separating (van Leeuwen et al. 2012).

Production of isobutene is stimulated when L-leucine is added to the medium since it is a precursor of isobutene. L-Phenylalanine has a synergistic effect with L-leucine as an enzyme inducer and is thus effective only in systems with living cells (Fujii et al. 1989a). Isobutene was produced only under aerobic conditions and none under nitrogen gas or with heat-treated reaction mixture; therefore, oxygen was essential for its synthesis (Fujii et al. 1987, 1988). The optimum pH was around 6.0 and optimum temperature between 25 and 27 °C for resting and growing cells, but above 30 °C growth was inhibited and there was no isobutene production. Isobutene formation rate peaked at 0.45 mg/L/h when the concentration of both L-leucine and L-phenylalanine was low; as they were depleted, the rate decreased quickly (Fujii et al. 1987).

A cell-free isobutene-forming system from disrupted *R. minuta* cells was also created (Fujii et al. 1988). Isobutene was produced from α-ketoisocaproate, isovaleryl-CoA and isovalerate where isovalerate gave the best isobutene formation rate of these three with 9.1 nL/L/h. Adding NADPH to the reaction mixture proportionally increased the rate of isobutene production, until a concentration of around 0.1 mM, as did increasing the concentration of isovalerate until 30 mM. EDTA had no inhibitory effect (Fujii et al. 1988). The formation of isobutene was inhibited by some redox reagents and completely eliminated by the presence of carbon monoxide (Fujii et al. 1989b).

Differently than with living cells, the optimal pH for isobutene production was between pH 7.3 and 8.0, while no formation of isobutene occurred above pH 8.5 or below pH 6.5. The optimum temperature was, as in the cell system, cs. 25 °C (Fujii et al. 1988).

In a cell cultivation system with L-phenylalanine, L-leucine yielded the higher production rates of isobutene than α-ketoisocaproate and isovalerate. This is possibly because L-phenylalanine causes the cells to take up organic acids at lower rates than L-leucine. When isobutene was grown in a medium without L-phenylalanine, the best results were achieved with the use of isovalerate, α-ketoisocaproate and L-leucine in that order (Fujii et al. 1988, 1989a).

Except L-phenylalanine, aromatic carboxylic acids and aromatic amino acids (mandelate, protocatechuate, benzoic acid, *p*- and *m*-hydroxybenzoate, *p*-

aminobenzoate, tryptophan and tyrosine, but not LDOPA and *p*-fluorophenylalanine) also enhanced the isobutene production rate synergistically in the presence of L-leucine. Furthermore, hydroxyphenylglycine and phenylglycine had the same effect as well. Cycloheximide, an inhibitor of protein synthesis, inhibited the synergistic effect of L-phenylalanine on the production of isobutene (Fujii et al. 1989a).

R. minuta is a natural producer of isobutene, and in context possibility of its industrial application, there are several important drawbacks that should be taken into account:

- This pathway has a low maximum yield of isobutene from glucose due to the fact that 2 mol pyruvate and 2 mol acetyl-CoA are required per mol isobutene.
- O_2 is required for butanol synthesis, but in industrial practice anaerobic fermentation is preferred due to lower costs (e.g. for reactor cooling) and lower risk of explosion (no air/isobutene mixtures).
- The productivity is low—the highest achieved isobutene production rate is 0.45 mg/L/h, but at least 1 g/L/h is required for commercial production of low-value chemicals (Fujii et al. 1987; Gogerty and Bobik 2010).
- A more robust, reliable and manageable organism, like *E. coli* or *S. cerevisiae*, should be used as heterologous hosts for this pathway.
- Cytochrome P450 requires the presence of heme cofactor, which is difficult to express in bacteria and is not optimal for improvement of enzyme parameters (van Leeuwen et al. 2012).

Decarboxylation of 3-Hydroxyisovalerate by Mevalonate Diphosphate Decarboxylase

Another reaction to isobutene is through decarboxylation and subsequent dehydration of 3-hydroxyisovalerate (3-hydroxy-3-methylbutyrate), catalysed by mevalonate diphosphate decarboxylase (MDD, EC 4.1.1.33) (Gogerty and Bobik 2010; Marliere 2010). This enzyme, from the class of carboxy-lyases, is part of terpenoid or ergosterol biosynthesis, and isobutene formation is its side reaction. Even though the MDD family of enzymes is present in many microorganisms, none of them are known to synthesise isobutene (van Leeuwen et al. 2012; Bloch et al. 1959) (Fig. 4).

There are several pathways that can lead to 3-hydroxyisovalerate and isobutene.

The pathway to the right-hand side of Fig. 5 (Gogerty and Bobik 2010; Marliere 2010) requires acetyl-CoA and acetoacetyl-CoA for the production of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA).

For example, Gogerty and Bobik (2010) used this pathway when they expressed a mevalonate diphosphate decarboxylase of *Saccharomyces cerevisiae* (ScMDD) in *E. coli* strain DG30. This strain formed isobutene at a rate of around 155 pmol/g_{cells}/h.

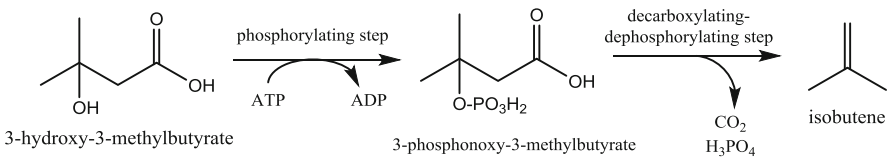

Fig. 4 Mechanism of isobutene synthesis by mevalonate diphosphate decarboxylase (Marlière et al. 2013)

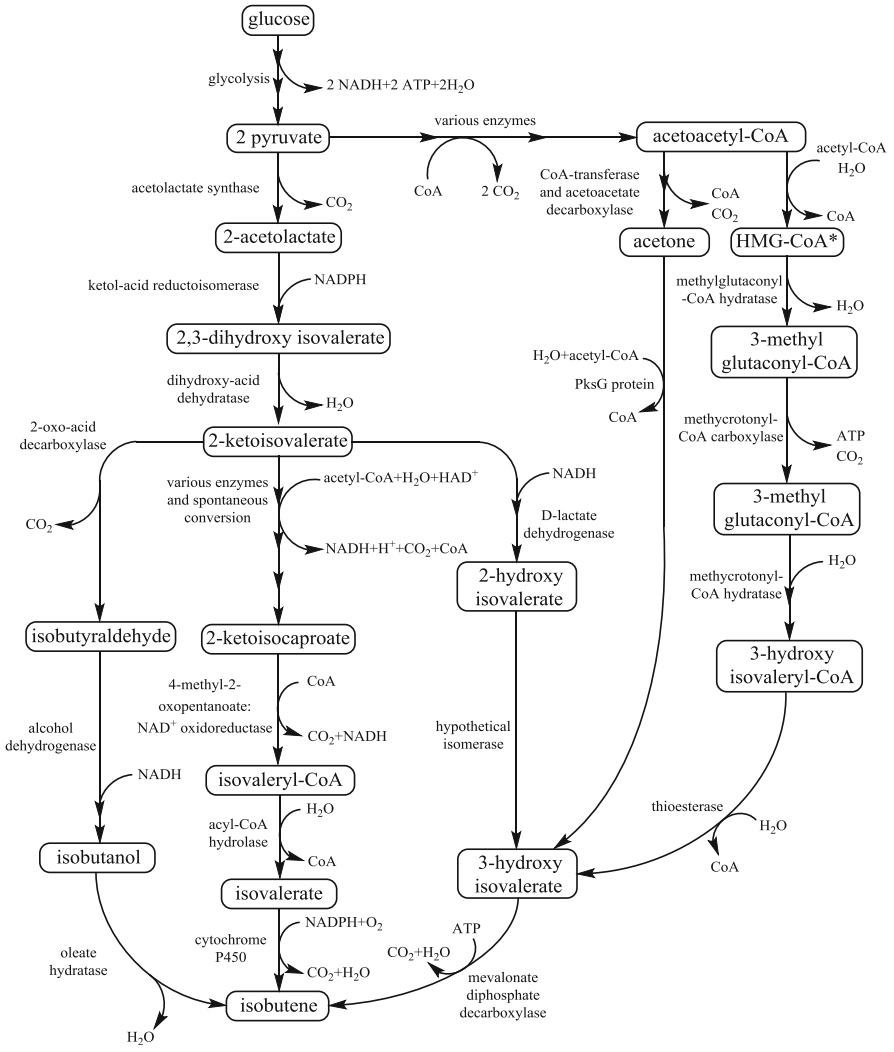

Fig. 5 Metabolic pathways for isobutene synthesis (van Leeuwen et al. 2012)

When the gene was mutagenised using error-prone PCR and expressed again in *E. coli*, the two most productive strains had isobutene formation rates of 3,000 and 5,888 pmol/g_{cells}/h which are about 18-fold and 38-fold increase compared to the initial strain with wild-type ScMDD. Still, the authors estimated that 10^6 times greater productivity is needed for a commercially feasible process. Also, a cell-free system with the purified enzyme of the more productive mutant accomplished a rate of only 4.8 times higher than the system with purified wild-type ScMDD. This could be due to the instability of the mutant enzyme (Gogerty and Bobik 2010).

This pathway was also used in a patent by Marliere (2010), which describes how MDDs from *S. cerevisiae* and 10 other microorganisms were analysed for isobutene synthesis. From the tested genes, MDD from *Picrophilus torridus* (an obligate aerobic archaeon) expressed in *E. coli* achieved the highest isobutene production rate.

In a more recent patent (Marlière et al. 2013), the same author describes a selection among MDDs from an even greater variety of microorganisms. MDDs were evaluated using a complementation assay—P. torridus; MDD was incubated with each one of the tested enzymes. The highest production of isobutene was observed in a system with purified *Streptococcus gordonii* MDD, which showed that the two enzymes were working complementarily (Marlière et al. 2013).

Additional MDDs were assayed using *Th. acidophilum* MDD. Special focus was on *Streptococcus* genus. Enzymes that showed especially greater efficiency in isobutene production are from *S. gallolyticus*, *S.* sp. *M143*, *S. salivarius* and *S. mitis*, achieving approximately two-fold increase in isobutene production than the *Th. acidophilum* MDD alone (Marlière et al. 2013).

This shows us that expressing diverse MDDs in one host can lead to a synergistic effect on the isobutene formation rate, since the two enzymes can perform the two reaction steps complementarily. Unfortunately, the patents did not indicate clear values for productivity or yield.

The pathway has two main issues. First, *E. coli* leucine biosynthesis pathway is unsuitable for this approach, since two molecules of pyruvate and one molecule of acetyl coenzyme A (acetyl-CoA) are required per mol isobutene. This means that a maximal possible yield is 0.21 $g_{isobutene}/g_{glucose}$. Also, aeration is required to regenerate the formed NADH into NAD^+. This problem could be alleviated by transferring the pathway into a homoacetogen that would produce 3 mol (instead of 2 mol) acetyl-CoA and 2 mol ATP per mol glucose (van Leeuwen et al. 2012; Gogerty and Bobik 2010).

The second problem is the low productivity rates of the enzymes. The only realistic solution is improvements through metabolic engineering (Gogerty and Bobik 2010).

Another patent by Marlière (2012) describes an alternative pathway leading also over 3-hydroxyisovalerate that is synthesised from acetyl-CoA and acetone. This approach represents a shortcut to 3-hydroxyisovalerate, but it suffers from the same problem as the previous one. Namely, it requires 3 mol pyruvate or acetyl-CoA for the production of 1 mol 3-hydroxyisovalerate and subsequently isobutene. Similarly, aeration is required (van Leeuwen et al. 2012).

Theoretically, it should be possible to reach 3-hydroxyisovalerate over 2-hydroxyisovalerate. This approach would use only 2 mol of acetyl-CoA per mol of isobutene and use the MDD decarboxylation into isobutene. First, part of this pathway would be similar to the pathways via isovalerate and isobutanol. The reduction of 2-oxoisovalerate to L- or D-2-hydroxyisovalerate requires D-lactate dehydrogenase (van Leeuwen et al. 2012; Chambellon et al. 2009).

The problem with this approach is that the isomerase for the transformation of 2-hydroxyisovalerate to 3-hydroxyisovalerate has yet to be discovered by screening.

With all the drawbacks, fermentative production of isobutene might have two important advantages over other biofuels:

- Due to its gaseous state at fermenting conditions, isobutene diffuses out of the fermentation broth easily, with only trace amounts actually dissolved in the liquid. This reduces the toxicity and inhibition of the fermenting culture, which is one of the main problems with many other fuels (e.g. butanol).
- This also enables simple and cheap isolation of isobutene, while with most liquid biofuels, this can be a major cost (Rude and Schirmer 2009; Zheng et al. 2009; Gogerty and Bobik 2010).

Dehydration of Isobutanol by Oleate Hydratase

Normally, it is difficult to enzymatically dehydrate an alcohol to form an olefin, and a water elimination reaction usually requires a C=O group conjugated to the C=C bond. This reaction is also possible using chemical engineering processes with dehydration catalysts (van Leeuwen et al. 2012; Jin and Hanefeld 2011).

However, oleate hydratase (EC 4.2.1.53), an enzyme from the class of hydrolyases, can catalyse this reaction on oleate creating (R)-10-hydrohystearate and vice versa. The enzyme and its appropriate gene were purified from *Elizabethkingia meningoseptica* and cloned and expressed in *E. coli* (Bevers et al. 2009). The problem is that this enzyme has a relatively narrow specificity and cannot catalyse a conversion from isobutanol to isobutene.

Still, this discovery was used to screen homologues of the *E. meningoseptica* sequence in other organisms for the activity towards isobutanol (Marliere 2011). The genes were expressed in *E. coli*, and enzymes were partially purified, and some of them showed isobutene formation activity from isobutanol or *tert*-butanol. There is no clear information on the productivity of isobutene by the enzyme or strain (van Leeuwen et al. 2012).

There are already microorganisms that can produce isobutanol naturally, but also strains developed for industrial isobutanol production (see Atsumi et al. (2008a) and Sect. 4.1.2.2.2). This high-yield pathway that Atsumi et al. developed for isobutanol production runs over 2-ketoisovalerate, instead of acetoacetyl-CoA (Fig. 5, left-hand side). Also, a relatively high productivity of 1.35 g/L/h was accomplished by Baez et al. (2011).

Therefore, there are already suitable isobutanol producers that could be good candidates for this pathway, with the appropriate optimisations. For example, for the production of 1 mol isobutanol, 1 mol NADPH would be consumed and 1 mol NADH produced; this might lead to NADH/NADPH imbalance inhibiting isobutanol formation. This could be prevented by a transhydrogenase and by NADPH production over pentose phosphate pathway (van Leeuwen et al. 2012; Blombach et al. 2011; Sauer et al. 2004).

An advantage of this approach is that the conversion of toxic isobutanol into gaseous isobutene (that would constantly diffuse out of the reaction broth) would circumvent the solvent toxicity problems.

4.2.2 Substrate and Strain Selection

Unlike biobutanol, microorganisms that are natural isobutene producers have production rates and yields that are nowhere near being commercially feasible. Therefore, genetic manipulations are absolutely essential for the development of industrially applicable strains.

E. coli and *S. cerevisiae* could be used as robust and manageable platforms for the expression of desired metabolic pathways, and they can both grow under aerobic and (the preferred) anaerobic conditions. *E. coli* does not have the problems associated with the expression of prokaryotic genes in a eukaryotic system and can operate at somewhat higher temperatures and with higher production rates. On the other hand, *S. cerevisiae* has the advantage of reduced risk of phage problems, robustness in lignocellulosic hydrolysates and possibility to grow at low pH to avoid contamination. Still, other strains, like the genetically less manageable acetogens, can be considered depending on the process conditions and design (van Leeuwen et al. 2012; Gogerty and Bobik 2010).

Since every platform has its benefits and drawbacks, and with no product toxicity as with butanol, efficient expression of the desired genes and pathways and possibility to utilise lignocellulosic hydrolysates will be the main selection criteria (van Leeuwen et al. 2012).

Substrate selection and raw biomass processing, especially in the context of lignocellulosic biomass utilisation, have already been addressed in detail in the previous chapters. Here, we will focus on specificities in terms of isobutene production. In order to achieve sustainable and economically viable isobutene production, future isobutene processes should also be focused on using lignocellulosic biomass as a substrate.

Since *E. coli* and *S. cerevisiae* cannot naturally utilise the wide variety of pentoses and hexoses as clostridia can, the engineered isobutene-producing strains should also be developed to efficiently utilise these saccharides and to be tolerant to hydrolysate inhibitors such as furanics, phenolics and acetic acid. Similar research is being done on ethanol-producing microorganisms, and analogous methods can be applied to butanol-producing strains. Also, the pretreatment methods should be considered based on the specific characteristics of isobutene-producing strains. In

terms of scale-up possibility, two prospective methods are the dilute acid pretreatment and ammonia fibre explosion (AFEX) (van Leeuwen et al. 2012; Martín and Grossmann 2014; Geddes et al. 2011; Sun and Cheng 2002; Taherzadeh and Karimi 2008; Alvira et al. 2010). Alternatively, it may be feasible to couple two fermentation processes, so that the undigested sugars from isobutene fermentation are reused for a second fermentation (Martín and Grossmann 2014).

Isobutene production could use more dilute hydrolysate substrate than the ones in ethanol fermentation. This is because isobutene concentration in the off-gas will not depend on substrate concentration, and the risk of product separation problems due to contamination is much smaller for isobutene (van Leeuwen et al. 2012).

4.2.3 Fermentation and Product Separation

There are very limited information on this topic, since most of the experiments on bio-isobutene production are lab scale and did not reach the phase of commercial productivity. This is why there is almost no data on isobutene fermentation in different bioreactor operation modes. Still, some considerations can be made theoretically.

Isobutene fermentation so far required aeration, but according to the stoichiometric equations, it can also be performed without aeration (van Leeuwen et al. 2012). Anaerobic fermentation would reduce costs in terms of cooling, simplify the process and equipment needed and reduce the amount of the off-gas, thus reducing separation costs.

If the optimal stoichiometry is achieved, molar ratios in the off-gas would be around 2/3 CO_2 and 1/3 isobutene. Since the solubility of isobutene in water is only 267 mg/L at 30 °C and 1 atm and considering partial vapour pressure, concentration of isobutene dissolved in the fermentation broth will be of the order of magnitude around 0.1 g/L. This is probably not yet toxic to the microorganism (van Leeuwen et al. 2012; Zhang et al. 2002b; Straathof 2003).

We can assume that only <0.5 % of the produced isobutene will remain dissolved in the fermentation broth and therefore separate isobutene only from the off-gas. Off-gas consists of around 2/3 CO_2, 1/3 isobutene, around 1.5 % water vapour and trace amounts of different volatile compounds. Since some impurities are allowed, we can concentrate on separation of water and carbon dioxide (van Leeuwen et al. 2012).

Several methods can be used for gas separation, like stagewise condensation to liquid, pressure swing adsorption, membrane permeation, absorption or combinations of these techniques (Hiller et al. 2000).

Stagewise condensation is one method obvious due to the large difference in boiling points. A drawback is the high cost of electricity. Also, countercurrent condensation–vaporisation could be performed for higher purity of isobutene.

In pressure swing adsorption, stronger adsorbed species push poorly adsorbed species forward through the column. The problem with this method is the high investment costs. Separation of isobutene from CO_2 by membrane technology can

be performed by either adsorption or permeation using DD3R-zeolite. It also has the drawback of high capital investment costs. CO_2 can be removed by absorption in aqueous amine solutions, from which it is subsequently released by heating.

The disadvantage of the adsorption, permeation and absorption methods is that CO_2 is isolated, while isobutene is the minor component of the two. In that respect, condensation to liquid is more favourable (van Leeuwen et al. 2012; Martín and Grossmann 2014).

Conclusion

Fermentative production of butanol and isobutanol is not yet competitive to the petrochemical production route. Yet, intensive research in the last years is slowly but constantly closing the gap between the two processes.

There are several options in regard to choice of substrate, fermenter operation and separation methods, each with their own advantages and drawbacks. Real data, from pilot plants that run over longer time periods, is needed to fully estimate the feasibility of each option.

If low-cost feedstock like lignocellulosic biomass is used, direct cost for feedstock is lower, but this creates more costs for energy in the pretreatment process, cellulases, wastewater treatment and potential substrate loss due to the presence of microbial inhibitors.

Butanol toxicity is another great limitation, causing a whole new set of issues (low butanol titre and yield, lower substrate utilisation, higher separation costs). This problem can be addressed by strain development and in situ separation techniques (Xue et al. 2013a).

Strain development is crucial for viability of fermentative butanol production. Approaches targeting individual genes, enzymes or pathways can be used, but also approaches at global and system levels are more effective for stress tolerance improvement to improve butanol titre. There are three priority directions for strain development: improvement of butanol tolerance, increased butanol production and utilisation of cheap feedstock (utilisation of various monosaccharides and resistance to microbial inhibitors). Clostridia have potential as a commercial strain, but heterologous hosts might prove advantageous, due to the homofermentative pathway (Branduardi et al. 2014; Xue et al. 2013a).

Process engineering is important for process optimisation, to complement the properties of the microorganism. In principle, a continuous fermentation mode is preferred in industry, due to higher productivity. In case clostridia are used, fermentation should be designed as a multistage process to correspond to the biphasic character of butanol synthesis.

The distillation system is not as energy intensive as imagined simply based on the low solvent titres in the fermentation broth. On the other hand, various in situ butanol removal technologies can relieve butanol inhibition and

(continued)

improve productivity and substrate use, but they require more capital investment on facilities and energy consumption (Xue et al. 2013a).

In the recent years there is a number of pilot plants being built or planned that will ensure real production data that will help further asses the feasibility of fermentative butanol production and facilitate further optimisation.

Research on microbial production of isobutene is just starting to gain momentum. Besides aerobic pathways from *Rhodotorula*, pathways over 3-hydroxyisovalerate and isobutanol will enable higher yields and substrate utilisation.

Even though the productivities of isobutene fermentation are too low for commercial application, further research and optimisation can help these processes realise their potential. Also, isobutene-producing strains cannot yet utilise cheap substrates like lignocellulosic biomass hydrolysates. Further strain development will probably give a solution.

Besides these drawbacks, fermentative isobutene production has a clear advantage over butanol, considering the relatively simple separation methods and circumvention of product toxicity. Also, direct fermentative production seems to be more favourable than fermentative production of isobutanol followed by chemical dehydration (van Leeuwen et al. 2012).

In terms of theoretical yields and energy requirements, the production of isobutene from glucose appears to be competitive with other biofuels. Isobutene is at a disadvantage compared to ethanol and 1-butanol in terms of energy yield. However, these costs might be more than offset by the higher energy density of alkylate (Gogerty and Bobik 2010). Generally, isobutene seems to have substantial potential as a renewable chemical.

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Microorganisms for Biorefining of Green Biomass

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Abstract Traditional green crops such as grass, clover, alfalfa as well as new (halophytic) green biomass of *Salicornia* have great potential to be utilised in the concept of the green biorefinery, where the first step is a wet fractionation of the biomass to yield a sugar- and protein-rich juice and a lignocellulosic pulp fraction.

An array of industrially important microorganisms is needed in order to efficiently convert green biomass into useful products such as lactic acid, L-lysine and ethanol using the concept of green biorefining. The first—and vital microorganism used—is lactic acid bacteria, which has the ability to quickly acidify the easy perishable juice fraction and convert it into a storable nutrient-rich medium, e.g. L-lysine fermentation. The acidification also leads to precipitation of the "leaf" protein of the juice which allows for separation of this fraction to yield a value-added protein product. The resulting brown juice can be used as medium for L-lysine fermentation, e.g. using *Corynebacterium glutamicum*. The pulp fraction which is primarily lignocellulose is suggested as a good substrate for ethanol fermentation after physicochemical pretreatment and enzymatic hydrolysis. The most important microbes, given the current state of green biorefining, have been identified in this book chapter as *Lactobacillus salivarius, Corynebacterium glutamicum and Saccharomyces cerevisiae*.

1 Introduction

There is a great potential in utilising green biomass in green biorefineries. Green biomass contains significant amounts of high-value protein (e.g. functional proteins such as RuBisCo), phytochemicals and carbohydrates. These carbohydrates can be converted into bulk chemicals, such as lactic acid and acetic acid, or fuels such as ethanol, butanol and biogas. Traditionally, green biomass has been a waste product from the green crop-drying industry in Europe. The green crop-drying factories would heat and press fresh biomass of grass, clover and alfalfa before drying in order to produce green pellets (animal feed) from the resulting fibre fraction. The waste stream from this process is a juice rich in nutrients, called brown juice. Acidification of brown juice through the use of lactic acid bacteria or addition of an organic acid produces a stable, storable fermentation substrate. Indeed, acidified

brown juice has been demonstrated to possess potential as a universal fermentation medium for the production of various useful fermentation products including organic acids, amino acids, enzymes, protein, peptides, fungus or bacteria (Margrethe Andersen and Kiel 2000). However, many green crop-drying factories in the EU have discontinued the production of green pellets due to a decision by the EU governments to stop supporting the industry, as the energy cost for drying green biomass is too high to be economically (and environmentally) feasible. This has led players in the green biomass biorefinery industry to develop new processes in order to obtain fermentation media. Indeed, more green biomass biorefinery projects are now first wet separating the green biomass into green juice and press cake and extracting proteins as well as other high-value products from the green juice before finally using the residual brown juice as fermentation medium. Furthermore, the types of biomasses to be used in the green biorefinery have been expanded beyond just grasses to include sugar beet top, green leaves and green halophytes such as annual salt-marsh succulent *Salicornia* and even more biomasses. A common feature for all the green biomasses is that they can be fractionated into a fibre fraction and a fresh juice. The fibre (lignocellulosic) fraction can be ensiled for animal feed or combined with dry lignocellulosic materials (such as wheat straw and corn stover) in second-generation biofuel processes. The fresh green juice containing high-value protein, phytochemicals and easily accessible carbohydrates is used for direct lactic acid fermentation, which eliminates some of the challenges associated with pretreatment processes and heat sterilisation such as inhibitor formation and high energy usage (Kamm and Kamm 2004; Kerfai et al. 2011). Another advantage of using fresh biomass in the green biorefinery is that the natural enzymes in the juice are still active after cropping, helping the release of fermentable sugars (Johnston et al. 2013; Dodić et al. 2009).

To convert the fresh green juice into a stable, storable product (acidified brown juice) to be used for microbial fermentation, pH has to be lowered to less than 4.5 and preferably to 4.0. Acidification may be performed by adding an inorganic or organic acid or, preferably, by inoculating the plant juice with lactic acid-producing microorganism. During lactic acid fermentation, carbohydrates are converted into lactic acid, thereby lowering the pH of the green juice (Joglekar et al. 2006) and precipitating most of the soluble proteins. The remaining plant proteins in the acidified brown juice are hydrolysed to smaller (bioavailable) peptides and amino acids when the brown juice is stored (Weinberg 2008). The precipitated protein can be separated from the fermented green juice by sedimentation, filtration or centrifugation (Wang et al. 2009). There are many advantages to lactic acid fermentation of the fresh juice over traditional methods such as heat sterilisation. For example, green crops such as clover and Italian ryegrass contain fructans (polymeric carbohydrates consisting of variable numbers of fructose molecules terminating with a glucose) as storage carbohydrate. Fructan is degraded to free sugars by plant enzymes and by certain strains of microorganisms such as *Lactobacillus plantarum* and *Lactobacillus paracasei* subsp. *paracasei* and *Bacillus subtilis* (Biggs and Hancock 2001; Kaplan and Hutkins 2003). If the green juice is used for fermentation after being sterilised at either 121 °C for 20 min in batch sterilisation or at

140 °C for a few seconds in continuous sterilisation, fructan-degrading enzymes (fructan hydrolases) will be inactivated, resulting in less simple accessible sugars in the brown juice decreasing its value as a universal fermentation media. During lactic acid fermentation of fresh non-heat-treated brown juice, fructans are hydrolysed to mono- and disaccharides by microorganisms and/or enzymes present in the juice and subsequently converted into lactic acid by the lactic acid bacteria.

Another disadvantage of heat sterilising the fresh brown juice is the formation of Maillard reaction products between amino acids and carbohydrates when they are heated to these temperatures. Maillard reactions' products are growth inhibitors and reduce the amount of available amino acids and carbohydrates, again, reducing the value of the brown juice as a universal fermentation media. Furthermore, heat sterilisation significantly adds expenses to the low-price end product.

As the green juice is an extremely good substrate for many microorganisms, it must be treated by lactic acid fermentation immediately and continuously after production in order to prevent uncontrolled fermentation by undesirable contaminating microorganisms. This means that the lactic acid fermentation step should be performed right after wet fractionation in green juice and press cake. To maintain the advantage over heat sterilisation, lactic acid fermentation process must be simple, robust and cost-effective and require low energy consumption.

2 Examples of Green Biomass Biorefineries

2.1 Alfalfa Green Biorefinery

In Denmark alfalfa is used for animal feed, especially for cattle and horses. Most alfalfa is grown separately or mixed with Italian ryegrass and used for production of normal silage or dried to 70 % dry mass in the field and wrapped. Some alfalfa is also dried artificially and made to fodder pellets. Figure 1 shows a concept for an alfalfa biorefinery.

The first step in the utilisation of the alfalfa is wet separation into a green juice and a press cake (pulp). In order to precipitate the proteins and stabilise the juice, the green juice has to be fermented with a suitable *Lactobacillus* strain. Based on our laboratory trials as well as pilot plant trials, we can conclude that 5–11 % of the green juice dry matter can be isolated as native, soluble white protein product with about 75 % protein. This product has a great potential as functional food as alternative to egg white, for example. 33–38 % of the green juice dry matter can be isolated as a feed additive (e.g. for egg production) with 35 % protein and a content of xanthophyll. The resulting brown juice can be used in fermentation processes, such as lactic acid, lysine and ethanol fermentation, or in biogas production. The press cake (pulp) can be used for silage, dried and used for fodder pellets, go directly to the biogas plant or used in a second-generation bioethanol plant. In summary, there are many interesting aspects for both traditional and

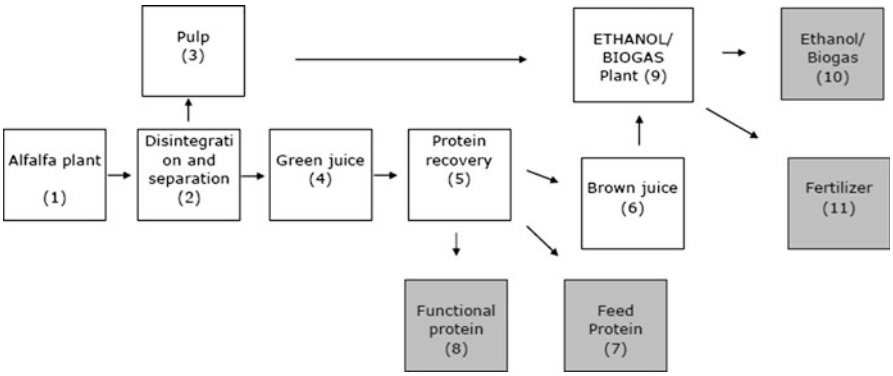

Fig. 1 Alfalfa in green biorefinery

organic farmers in the alfalfa green biorefinery concept: energy production (biogas or bioethanol), high-value protein feed, organic food and feed materials and finally utilisation of the liquid residue as fertiliser.

2.2 Salicornia *Green Biorefinery*

Halophytes such as *Salicornia sinus-persica* are plants that can grow and complete their life cycle in saline habitats. They are interesting biofuel/biochemical crops, especially in arid and coastal areas. *Salicornia bigelovii* is an oilseed crop that will produce oilseeds when grown to maturity (Glenn et al. 1991). Thus, the plant will produce an oil-rich fraction and a dry lignocellulosic fraction. Therefore, most bioenergy researches on *Salicornia* focus on biodiesel/kerosene fuel from the oil-rich seeds (Akbar et al. 2009; Balat 2011; Stratton et al. 2010) and second-generation ethanol and biogas processes for the lignocellulosic residue (Malça and Freire 2006; Abideen et al. 2011; Jeon et al. 2010). However, as the plant has succulent stems, the fresh/green *Salicornia* biomass is high in moisture (65 %) and hence very suitable for juicing. Juicing of the biomass allows for water preservation in the system, which is very important especially when developing biofuel processes in arid area. We are examining the fresh use of a species of *Salicornia* native to the Persian Gulf region (*Salicornia sinus-persica*) in a green biorefinery as described in Fig. 2.

Similar to the alfalfa green biorefinery, the *Salicornia* biomass is fractionated into a juice and a pulp fraction by simple wet separation. Laboratory trials have shown that approximately 25 % of the dry matter (DM) is extracted as juice components and 75 % of the DM is the fibre-rich pulp fraction. Total percentage of DM in fresh biomass was found to be 22.42 ± 0.12 % (w/w), of which 47.08 ± 1.12 % is ash. Donghe Lu et al. studied the nutritional composition of the fresh *Salicornia* biomass, with a lower DM content (11.58 ± 1.36 %); *Salicornia*

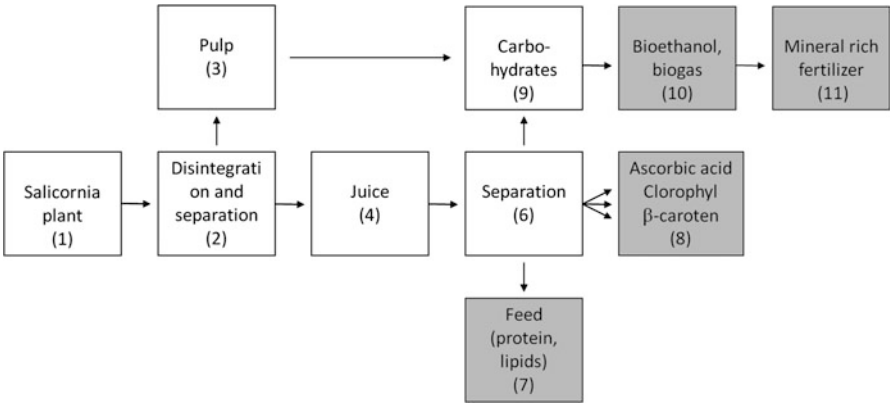

Fig. 2 Fresh *Salicornia* plant in green biorefinery

contains 1.54 % (w/w) protein (13 % of DM), 0.37 % lipids (3 % of DM), 0.83 % crude fibres (7 % of DM), 4.48 % carbohydrate (38 % of DM) and 4.36 % ash (37 %). Furthermore, it contains 569, 159 and 58 mg/kg fresh weight of chlorophyll, β-carotene and ascorbic acid, respectively (Lu et al. 2010).

Preliminary fractionation studies have shown that wet separation yields a juice with a DM content of approximately 13 %, of which approximately 1 % is sugar and about 60 % is ash. The pulp with a DM of circa 40 % contains approx. 10 % glucan, 16 % hemicellulose, 15 % lignin and 19 % ash. The protein fraction of *Salicornia* has been shown to contain substantial amounts of many different essential amino acids, with the most predominately amino acids being asparagine and glutamic acid (Lu et al. 2010). The high nutritional value of the protein and lipids in the juice allows for separation of a high-quality feed/food product. In addition there is the potential to extract active components prior to bioenergy production from the carbohydrate fraction. The pulp fraction can be supplemented with other carbohydrate-rich lignocellulosic materials in order to achieve high biofuel yields. Furthermore, the sugary juice after step 6 can act as a cheap water source in enzymatic hydrolysis and fermentation of the lignocellulosic biomass. In the same way as for the alfalfa biorefinery, the liquid that resides from bioenergy processing can be used as a fertiliser for farmland.

3 Microorganisms in Refining of Green Biomass

3.1 Lactic Acid Fermentation

The fresh juices produced in wet fractionation of green biomass are very perishable and need to be treated right after juicing to avoid contamination with unwanted microbes, which leads to nuisances such as unpleasant odours, gas production, decreased sugar yield and other factors leading to low juice quality. Most green

crops contain natural enzymes such as fructan hydrolases in clover and Italian ryegrass that are activated after harvesting and help improve the digestibility of juice carbohydrates. After removal of the proteins and other high-value products from the green juice, the remaining juice is now brown and named brown juice. Under the following storage of the brown juice, the remaining plant proteins in the juice are hydrolysed to smaller (bioavailable) peptides and amino acids or precipitated. Lysine fermentation process has been demonstrated on brown juice, produced by hot (80 °C) pressing of grass, clover and alfalfa. The produced medium has been tested and shown to be an excellent substrate for production of L-lysine in fermentation with *Corynebacterium glutamicum* (M Andersen and Kiel 1999).

3.2 Lactic Acid Bacteria and Its Preservative Action

Lactic acid bacteria are a group of bacteria which excrete lactic acid as the main fermentation product into the medium if supplied with suitable carbohydrate. Lactic acid bacteria consist of the genera *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* (Stiles and Holzapfel 1997). They are important microorganisms in the body and environment of human beings. They are part of the natural flora of the intestinal tract, mouth and vagina where they take part in preventing infection with pathogens. They are of vital importance in the feed and food industry, where lactic acid is used as an acidulant and preservative, or in nutraceuticals as probiotics. A probiotic is defined as a culture of microorganisms, which when applied to man or animal beneficially affects the host by improving the properties of the indigenous microflora. Figure 3 shows the industrial applications of lactic acid bacteria. Lactic acid bacteria are used as probiotics because of their potential to inhibit pathogens and to detoxify carcinogens (Weinberg and Muck 1996; Lindgren and Dobrogosz 1990). In the environment, lactic acid bacteria are prominent in spontaneous fermentations of organic matter of animal and plant origin, containing sufficient levels of mono- and disaccharides. Their acid tolerance gives them a competitive advantage over other bacteria. Fermented plant materials, such as fodder silage made from grass and maize, are the most abundant products made by spontaneous fermentation with lactic acid bacteria (Treuber 1996).

The antimicrobial mechanisms of lactic acid bacteria exploited in the biopreservation of grass, fodder and food/beverages include the production of organic acids, hydrogen peroxide, carbon dioxide, diacetyl and a broad-spectrum of antimicrobials called bacteriocins (Caplice and Fitzgerald 1999; Paul Ross et al. 2002). Organic acids are generally thought to exert their antimicrobial effect by interfering with the maintenance of cell membrane potential, inhibiting active transport, reducing intracellular pH and inhibiting a variety of metabolic functions. They have a very broad mode of action and inhibit both gram-positive and gram-negative bacteria as well as yeast and moulds (Doores 1993; Lindgren and Dobrogosz 1990).

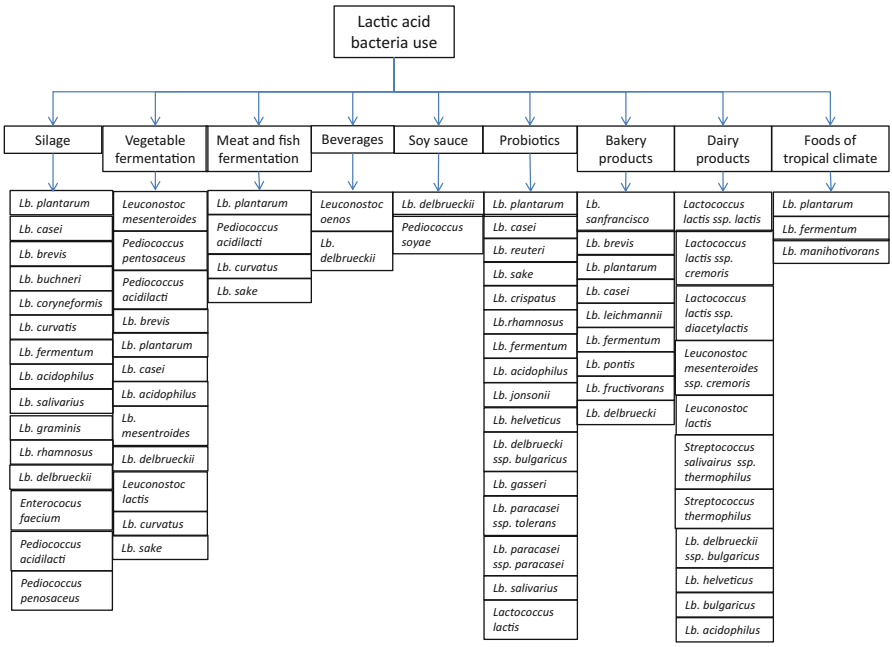

Fig. 3 Lactic acid bacteria and their industrial applications (Thomsen 2005)

Lactic acid bacteria lack true catalase to break down the hydrogen peroxide generated in the presence of oxygen. The inhibitory effect of hydrogen peroxide is mediated through the strong oxidising effect on membrane lipids and cell proteins (Lindgren and Dobrogosz 1990). Carbon dioxide formed by heterofermentative lactic acid bacteria can directly create an anaerobic environment and is toxic to some aerobic food microorganisms, through its action on cell membranes and its ability to reduce internal pH (Caplice and Fitzgerald 1999). Diacetyl is a product of citrate metabolism and is responsible for the aroma and flavour of butter and some other fermented milk products. Many lactic acid bacteria including strains of *Leuconostoc*, *Pediococcus* and *Lactobacillus* may produce diacetyl, although production is repressed by the fermentation of hexoses. Gram-negative bacteria, yeast and moulds are more sensitive to diacetyl than gram-positive bacteria, and its mode of action is believed to be due to interference with the utilisation of arginine (Caplice and Fitzgerald 1999). Bacteriocins produced by lactic acid bacteria are proteinaceous inhibitors that generally act through depolarisation of the target cell membrane or through inhibition of cell wall synthesis, and these range in specificity from a narrow spectrum of activity (lactococcins which only inhibit lactococci) to those having a broad range of activity such as the lantibiotic nisin (Paul Ross et al. 2002). In the context of fermentation, important targets include spoilers such as species of *Clostridium* and heterofermentative lactobacilli and foodborne pathogens including *Listeria monocytogenes*, *Staphylococcus*, *Clostridium*, *Enterococcus and Bacillus* (Caplice and Fitzgerald 1999).

3.3 Characteristics Required of a Potential "Acidifier" for Green Juice

The following criteria have been suggested for microorganisms to be used as an inoculant in silage fermentation (Morlon-Guyot et al. 1998):

1. It must be a vigorously growing organism able to compete with and dominate other organisms.
2. It must be homofermentative (produces L-lactate as the main product).
3. It must be acid tolerant and capable of reducing pH to at least 4.0 as quickly as possible.
4. It must be able to ferment glucose, fructose, sucrose and preferably fructosans and pentoses.
5. It should not produce dextran from sucrose or mannitol from fructose. If mannitol appears, it should be able to ferment it rapidly.
6. It should have no action on organic acids.
7. It should have a growth temperature range extending to 50 °C.
8. It should be able to grow at high dry mass contents.

With the exception of criterion number 8, lactic acid bacteria satisfy the rest of these requirements and can thus be used in the acidification of green and brown juice. Instead of being able to grow at high dry matter contents, lactic acid bacteria used in acidification of green and brown juice should be able to grow at low dry matter, especially in autumn, where the dry matter of the juice can be as low as 3 %. Importantly, based on the criteria, *Leuconostocs* and heterofermentative lactobacilli can immediately be excluded due to their metabolism and streptococci because of acid tolerance. In sugar-rich silages, it has been proposed that using strains of heterofermentative lactic acid bacteria or heterofermentative lactic acid bacteria in combination with homofermentative lactic acid bacteria is preferable (Holzer et al. 2003; Driehuis et al. 2001). In silage made of crops with low content of water-soluble carbohydrates, it is preferred to use homofermentative lactic acid bacteria to obtain a rapid and efficient utilisation of crops-water-soluble carbohydrates resulting in a rapid decrease in pH (Weinberg and Muck 1996; Holzer et al. 2003). Furthermore, homofermentative lactic acid fermentation is preferred in the acidification of green and brown juice, which has low sugar concentrations, because the objective is a fast conversion of sugar to lactic acid. The pH of brown juice should be lowered to 4.0–4.5 and preferably below 4.0 in the acidification process in order to obtain long durability of the brown juice (Whittenbury 1962; Zhang et al. 2000). This limits the choice to pediococci and homofermentative lactobacilli. An important factor to consider when producing a fermentation medium that might eventually end up in a feed product is using a microorganism, which can easily be approved for animal consumption. Since strains of *Lactobacillus* are widely used in food production, a strain of this genus is preferable. It has also been suggested that fructan-degrading strains are better inoculants for silage compared to other strains. Strains capable of fermenting fructan have been identified as

Lb. casei, Lb. plantarum, Lb. brevis and P. pentosaceus (Dunlop and Hammond 1965). It has been observed that fructan is degraded relatively slowly during the early stages of the ensilage process, suggesting that it may make a poor contribution to carbohydrate supply in the primary stages of the silage fermentation. Plant fructan hydrolases are reported to be most active between pH 4.5 and 5.5 (Müller and Lier 1994). It is expected that plant fructan hydrolases and/or fructan-degrading strains present in the non-heat-sterilised green and brown juice take part in the utilisation of the fructans; therefore, fructan utilisation will not be the main criterion for the selection of the "acidifier" strain. Indeed using a strain which is not able to utilise fructan leads to a rapid rate of pH decline, Suggesting that low molecular weight soluble sugar has been efficiently used. (Simpson and Bonnett 1993).

If hot separation is applied, the brown juice will have a temperature of 60–70 °C after the separation process. Hence, lactic acid bacteria that grow fast at 40 °C + are advantageous. A high fermentation temperature will also reduce the risk of contamination with other microorganisms.

Most lactic acid bacteria are facultative anaerobes, but few show poor growth in the presence of oxygen (obligate anaerobes). In a simple industrial process, it can be difficult to keep the fermenter completely free of oxygen, and therefore the microorganism should not be inhibited by oxygen. Immobilisation of the microorganism by natural flocculation or adsorption to bioreactor surfaces would be advantageous, because a higher production rate can be achieved with immobilised cells, and immobilisation techniques such as entrapment in Ca-alginate gels are not robust enough for the industrial process. In conclusion, the most important criterion for choosing a lactic acid bacterium for acidification of brown juice is choosing a robust and fast-growing strain, with high growth temperature and a high yield, in addition to the ability of growing at low pH. Furthermore, the strain should be a facultative anaerobe, and it would be advantageous if the strain can be immobilised by flocculation or adsorption inside the bioreactor.

Table 1 suggests 12 strains of *Lactobacillus* and one yoghurt culture that were tested for the acidification of brown juice (Thomsen 2005). These strains were selected due to their characteristics, availability and current use in the food and feed industry. Both mesophilic and thermophilic *Lactobacillus* were selected because a fast-growing mesophilic strain might be more suitable than a more slowly growing thermophilic strain. The robustness of the strains, with regards to specific growth rates and ability to grow at low pH as well as the ability to flocculate, was tested in fermentation experiments (Thomsen and Kiel 2008).

Of all the strains in Table 1, the 3 strains of *Lactobacillus salivarius* showed the best performance when cultivated in brown juice (Thomsen and Kiel 2008). These strains efficiently lowered the pH in the brown juice in less than 10 h, with specific maximum growth rates in the juice of around 1.0/h and lactic acid productivities of more than 7 g/(l.h) (for *Lactobacillus salivarius* subsp. *salivarius* DSM 20555) (Thomsen and Kiel 2008).

Table 1 Nomenclature and characteristics of strains of lactic acid bacteria that were selected for testing in fermentation experiments

Strains of *Lactobacillus*	Robust	Homofermentative	Acidophilic	Thermophilic	Approved for feed	Facultative anaerobe	Fructan degrading	Flocculates
Lactobacillus salivarius BC 1001	?	X	?	–	X	X	–	?
Lactobacillus plantarum LP1 CHR, MKE 03011 CHR	?	X	?	–	X	X	–	?
Lactobacillus paracasei subsp. *paracasei* P 4126	?	X[a]	?	–	X	X	X	?
Lactobacillus paracasei subsp. *paracasei* P 4155	?	X[a]	?	–	X	X	X	?
Lactobacillus plantarum P 5868	?	X	?	–	X	X	–	?
Lactobacillus plantarum P 6100	?	X	?	–	X	X	–	?
Lactobacillus delbrueckii	?	X	?	–	X	X	–	?
Lactobacillus salivarius BC 1001	?	X	?	X	X	X	–	?
Lactobacillus salivarius subsp. *salivarius* DSM 20492	?	X	?	X	X	X	–	?
Lactobacillus salivarius subsp. *salivarius* DSM 20555	?	X	?	X	X	X	–	?
Lactobacillus paracasei subsp. *tolerans* DSM 20012	?	X[a]	?	X	X	X	?	?
Lactobacillus paracasei subsp. *tolerans* DSM 20258	?	X[a]	?	X	X	X	?	?
Mixed strain culture								
Yoghurt culture (Yoghurt 709, Visbyvac®B50)	X	X	X	X	X	X	?	?

[a]These strains produce a mixture of D- and L-lactic acid. The other strains produce primarily L-lactic acid

Table 2 Lysine, threonine and methionine in plant biomass (digestible amino acids)

	L-Lysine (g/kg)	L-Threonine (g/kg)	L-Methionine (g/kg)
Barley	3.8	3.6	1.7
Wheat bran	6.1	5.0	2.3
Corn germ meal	4.6	4.4	1.9
Peas	17.7	7.9	2.0
Rapeseed meal	11.0	8.6	4.0
Soybean meal	22.5	14.1	5.3

3.4 L-Lysine Fermentation of Acidified Brown Juice

L-lysine is an alkali-amino acid that belongs to the aspartate branch in the biosynthesis of amino acids. It is an essential amino acid in animal nutrition. Many plant products used for livestock feed, such as wheat and corn, are deficient in L-lysine, and thus, it must be added as a supplement in the form of soybean meal. The content of lysine in such products in comparison with soybean meal is shown in Table 2.

The bulk of L-lysine production throughout the world depends on direct fermentation of carbohydrates by auxotrophic and regulated mutants of corynebacteria and brevibacteria species in the batch culture (Winters et al. 1998). L-lysine is added to feed in amounts of 0.2–1.0 % and reduces the livestock's consumption of nitrogen-rich feed such as soybean, fishmeal and flesh and bone meal. Furthermore, L-lysine improves nitrogen's biological availability in the animal, thereby diminishing the agricultural nitrogen emission to the environment (Kiss and Stephanopoulos 1992). Efficiency of the use of lysine is the reason for the dramatic increase of its annual production during the recent decades (Fig. 4). Lysine production has been one of the main platforms in green biorefining, being an important co-product to lactic acid and protein (Mandl 2010; Becker and Wittmann 2012).

3.5 Corynebacterium glutamicum

Although several methods exist for the industrial production of lysine, it is predominantly produced by microbial fermentation of inexpensive carbon sources such as cane molasses with strains of *Corynebacterium glutamicum* (Winters et al. 1998). *C. glutamicum* belongs to a group of organisms, referred to as glutamic acid bacterial, which can all excrete glutamate or lysine. Glutamic acid bacteria are gram-positive, non-sporulating, nonmotile, short rods or cocci, require biotin for growth and all have a propensity to excrete glutamate under biotin limitation. Most strains can utilise acetate or ethanol as a primary carbon source (and *C. glutamicum* can utilise lactate as well). Most strains will grow between pH 6.0 and 9.0 and optimally between 7.0 and 8.0. Their optimum temperature for growth is between 25 and 37 °C, and they are typically cultured at 30 °C. *C. glutamicum* is a

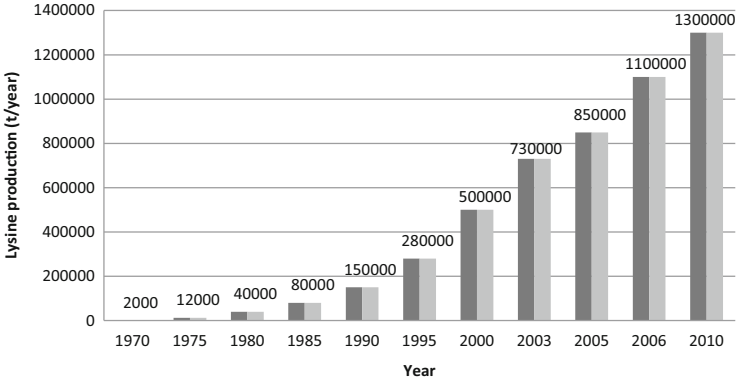

Fig. 4 Annual world lysine production in (t/year)

facultative aerobe. In the absence of oxygen, it converts glucose into lactic acid and other organic acids, whereas in the presence of oxygen, it produces L-lysine and carbon dioxide. The primary pathways in *C. glutamicum* are Embden-Meyerhof-Parnas (EMP) pathway followed by the tricarboxylic acid cycle (TCA). The (EMP) pathway catalyses the conversion of glucose-6-phosphate to pyruvate. Pyruvate is decarboxylated leading to the production of one molecule of NADH and an acetyl moiety coupled to coenzyme A (acetyl-CoA). The TCA cycle is primarily responsible for the generation of NADH through the oxidation of acetyl-CoA, as well as the synthesis of precursor metabolites such as oxaloacetate and α-ketoglutarate. Aspartate is the precursor of lysine, methionine and threonine. Aspartate is formed from oxaloacetate 2. In the branch leading to lysine, pyruvate is the source of carbon atoms added to the skeleton of aspartate β-semialdehyde, and glutamate is the source of the ε-amino group (Neuner et al. 2013). The branched pathway that leads from aspartate to lysine, methionine and threonine is tightly regulated. Aspartate kinase, the enzyme which catalyses the step from aspartate to aspartyl phosphate in *C. glutamicum*, is subject to feedback inhibition by lysine and threonine. Since homoserine auxotrophic strains are unable to synthesise threonine (or methionine), the concerted feedback on aspartate kinase is relieved, and lysine synthesis becomes deregulated. However, if too much threonine or homoserine is added to the medium, the feedback will be re-established, and lysine overproduction will be inhibited. This inhibition, however, can be reduced by the addition of methionine. Although strains that lack any of the enzymes between aspartate β-semialdehyde and threonine will produce lysine, those strains which lack homoserine dehydrogenase (HDH) (first enzyme of the threonine branch) produce the most lysine. Most strains used for industrial production of lysine are mutated to lack HDH (Ruklisha et al. 2002).

Genetically engineered *C. glutamicum* with overexpressed fructose 1,6-bisphosphatase (fbp) and glyceraldehyde 3-phosphate dehydrogenase (gapX) as well as D-lactate dehydrogenase (dld), pyruvate carboxylase (pyc) and malic enzyme (malE) has been produced to be able to generate lysine using all quantified

low molecular weight substrates, including lactate, glucose, fructose, maltose, quinate, fumarate, glutamate, leucine, isoleucine and alanine. It has been reported that this bacterium can produce lysine independent of the availability of oxygen (Neuner et al. 2013; Neuner and Heinzle 2011). Lysine production can be further improved by regulation of essential nutrients in the fermentation medium. Phosphoenol pyruvate in EMP pathway is the bottleneck; the lower the growth rate of *C. glutamicum*, the more phosphoenol pyruvate is converted into oxaloacetate, and the more lysine is produced. Therefore, the reduction of essential nutrients such as glucose and ammonia is used to control the growth rate of *C. glutamicum* (Winters et al. 1998).

3.6 L-Lysine Fermentation

In the lysine factory, the acidified brown juice is sterilised in a continuous steriliser in order to kill the lactic acid bacteria. At this point, the lactic acid bacteria have converted all the sugar in the brown juice to lactic acid, thereby preventing formation of Maillard reaction products (MRP) between amino acids and carbohydrates. After sterilisation the brown juice is led to a sterile, aerated stirred tank reactor. The initial charge medium is supplemented with necessary minerals, amino acids, vitamins (not sufficiently present in brown juice), sugar and ammonia. The fermenter is inoculated with a culture of *C. glutamicum* and carried out as fed-batch fermentation. A sterile carbohydrate solution is added continuously to the fermentation tank, after the sugar in the initial medium has been utilised, whilst the pH of the medium is controlled by the addition of ammonia. The fermentation continues, until a certain L-lysine concentration is reached. Figure 5 shows lysine fermentation of brown juice medium (160 g/l brown juice, 40.0 g/l $(NH_4)_2SO_4$, 2.0 g/l KH_2PO_4, 0.5 g/l Na_2HPO_4, 0.8 g/l $MgSO_4.7H_2O$, 10.0 mg/l $FeSO_4.7H_2O$, 1.0 mg/l $CuSO_4.5H_2O$, 10.0 mg/l $ZnSO_4.7H_2O$, 10.0 mg/l pantothenic acid, 5.0 mg/l nicotinic acid, 3.0 mg/l thiamine hydrochloride, 1.2 mg/l biotin, 0.2 mg/l citric acid) carried out by *Corynebacterium glutamicum B-036 (NBIMCC 3624)* in 1.5 l lab fermentation. The fermentation was carried out as fed-batch fermentation, where 2 portions of 1,000 ml feed were added during fermentation; the feed medium composition is shown in Table 3.

Brown juice is used as the only protein source in the medium. It insures all needed growth and productive factors for the strain producer. Applying optimal feed rates and profile, in addition to applying optimal physicochemical parameters for carrying out of fermentation process, high cell concentration and high L-lysine concentration were achieved in less than 60 h. As it is shown in Table 3, supplying of the culture with proper growth and productive factors by small amount of brown juice in the second feed portion leads to prolongation of the fermentation process saving the high lysine production rate.

At the end of fermentation, the pH of the culture broth is dropped to 4.0 by adding sulfuric acid, and the final liquid product with 25 % L-lysine is achieved

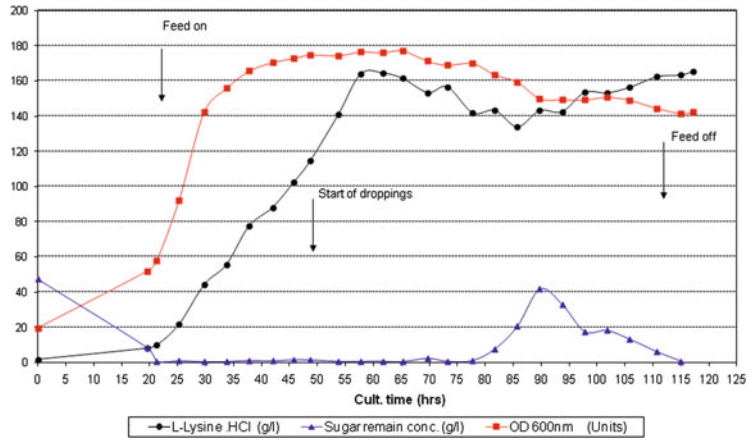

Fig. 5 Time course of fed-batch L-lysine fermentation with droppings of brown juice medium with *Corynebacterium glutamicum B-036 (NBIMCC 3624)*

Table 3 Feed medium for fed-batch fermentation of L-lysine in brown juice medium

Components	Amount	First portion of 1,000 ml	Next portion of 1,000 ml
Sucrose	g/l	600.0	600.0
Brown juice (25 % ds) (as is)	g/l	–	**20.0**
$(NH_4)_2SO_4$	g/l	50.0	50.0
KH_2PO_4	g/l	**1.0**	**1.0**
Nicotinic acid	mg/l	5.0	5.0
Pantothenic acid (as Ca-pantot.)	mg/l	10.0	10.0
Thiamine hydrochloride	mg/l	3.0	3.0
Biotin	mg/l	1.2	1.2

after vacuum evaporation of the whole media containing all remaining nutrients and biomass. The result is production of a new valuable product without formation of new waste streams.

3.7 Ethanol Fermentation in the Green Biorefinery

Ethanol fermentation is often suggested in green biorefinery concepts for utilisation of the lignocellulosic green pulp. Bioethanol from lignocellulosic biomasses has been heavily investigated during the recent years (Lynd et al. 1991; Lin and Tanaka 2006). Biomasses include wheat straw (Talebnia et al. 2010), corn stover (Kadam et al. 2008), sugar cane bagasse (Cardona et al. 2010), switchgrass (Pimentel and Patzek 2005), sorghum (Sheorain et al. 2000; Chuck-Hernandez et al. 2009) and

also woody biomasses (Pimentel and Patzek 2005). The pulp fraction needs physical/chemical pretreatment (such as hydrothermal treatment, wet oxidation, steam explosion or AFEX) followed by enzymatic hydrolysis to convert the carbohydrates (cellulose and hemicelluloses) to fermentable sugars. Fermentation inhibitors are typically formed during the high-temperature pretreatment. Carboxylic acids such as formic acid and acetic acid are the degradation products formed at highest concentration (0.5–5.0 g/l) (Öhgren et al. 2006). Acetic acid is formed by the initial hydrolysis of acetyl groups in hemicellulose but is also the end product from many oxidation reactions (Walton et al. 2010; Mamlouk and Gullo 2013; Foussard et al. 1989; Mishra et al. 1995). Aromatic products from sugar degradation are predominantly furans: 2-furfural (referred to as furfural in the following text) from pentose degradation, 5-hydroxy-2-methylfurfural (5-HMF) from hexose degradation (Fengel and Wegener 1983) and 2-furoic acid from furans degradation (Klinke et al. 2002); these have been found after pretreatment of lignocellulose in the range of 0.007–11.00 g/l (Almeida et al. 2007). Phenolic monomers with different functional groups are produced from solubilisation and hydrolytic or oxidative cleavage of lignin (Klinke et al. 2002). These phenolic compounds include alcohols, aldehydes, ketones and acids (Almeida et al. 2007). The dual challenge of ethanol fermentation of lignocellulosic biomasses is utilisation of pentose sugars (from hemicelluloses) and the presence of the inhibitors. *Saccharomyces cerevisiae* or baker's yeast is the most widely used microorganism for bioethanol fermentation even though this strain lacks the two initial enzymatic steps of the xylose metabolism, xylose reductase and xylose dehydrogenase. It is often used for fermentation of the glucose fraction due to its high inhibition tolerance and unrivalled productivity on the complex lignocellulosic substrates. However, much research has been performed to find or develop microorganisms for bioethanol fermentation of all the sugars in lignocelluloses hydrolysates. Table 4 shows the three main approaches to find a pentose-fermenting ethanol producer.

As illustrated in Table 4, the three main approaches to developing pentose sugar (e.g. xylose and arabinose)-fermenting ethanologenic microorganisms are (1) the search for naturally occurring organisms that satisfy these requirements, (2) the genetic modification of microorganisms that can naturally ferment pentose sugars but cannot produce ethanol and (3) the genetic modification of microorganisms that can produce ethanol very well but cannot ferment pentose sugars. Indeed, scientists have been able to isolate a plethora of diverse microorganisms from various environmental sources and have characterised them for their ability to ferment various sugars including xylose and arabinose into ethanol (Table 4, column 1). Furthermore, substantial efforts have been directed towards the genetic modification of both microorganisms and their enzymes in order to produce efficient xylose-fermenting, ethanol-producing microorganisms. However, *Saccharomyces cerevisiae*, or baker's yeast, remains the undisputed "king of bugs" when it comes to bioethanol production. *S. cerevisiae* has the ability to efficiently detoxify lignocellulosic substrates, as it can metabolise several of the lignocellulosic degradation products, e.g. furans and phenol aldehydes (Palmqvist and Hahn-Hägerdal 2000; Almeida et al. 2007; Torry-Smith et al. 2003). *S. cerevisiae* is also resistant to

Table 4 Main approaches in finding pentose-fermenting ethanol-producing microorganism

Search for natural ethanologenic microorganisms	Mutation/metabolical engineering of acid-producing, C-5-fermenting microorganisms	Genetical engineering of ethanologenic microorganism with expression of xylose-utilising genes
Mucor (Bansal et al. 2011)	*Bacillus stearothermophilus* (Payton and Hartley 1985; Wu et al. 2008)	*Zymomonas mobilis* (Mohagheghi et al. 2002; Deanda et al. 1996)
Fusarium oxysporum (Anasontzis et al. 2011)	*Escherichia coli* (Saha et al. 2011)	*Saccharomyces cerevisiae* (Bettiga et al. 2009; Verho et al. 2003; Johansson and Hahn-Hägerdal 2002)
Pichia stipitis (Xavier et al. 2010)	*Klebsiella oxytoca* (Golias et al. 2002; Karimi et al. 2006; Ohta et al. 1991)	*Clostridium acetobutylicum* (Li et al. 2013)
Candida shehatae (Palnitkar and Lachke 1990)		
Pachysolen tannophilus (Lachke 2002; Matsushika et al. 2009)		
Clostridium thermohydrosulphuricum (Saddler and Chan 1984)		
Clostridium acetobutylicum (Sivagnanam et al. 2011; Qureshi et al. 2008)		
Thermoanaerobacter ethanolicus (Sveinsdottir et al.)		
Erwinia chrysanthemi (Tolan and Finn 1987)		
Candida arabinofermentans (Kurtzman and Dien 1998)		
Thermoanaerobacter yonseiensis (Kim et al. 2001)		
Pestalotiopsis (Pang et al. 2011)		
Kluyveromyces marxianus (Rodrussamee et al. 2011; Kumar et al. 2009)		
Caloramator boliviensis (Crespo et al. 2012)		
Thermoanaerobacter pentosaceus (Iwasaki et al. 1990)		
Caldicellulosiruptor saccharolyticus (Isern et al. 2013)		

higher concentrations of ethanol which means that a more concentrated yield can be achieved when compared to other microorganisms. Additionally, the genome of *Saccharomyces cerevisiae* was among the first to be sequenced, and very powerful genetic manipulation techniques have been developed in the baker's yeast system making it a very attractive organism to work with when genetically modifying and constructing synthetic microorganism strains. The drawback of baker's yeast, of course, is its inability to efficiently ferment pentose sugars like xylose, a major concern for engineers designing second-generation bioreactors which use lignocellulosic biomass as feedstock. For this reason a lot of work has gone into expressing foreign bacterial genes in yeast in order to give them the ability to utilise these sugars. The work also includes the genetic modification of these pathway enzymes in order to make them more active followed by their expression in *S. cerevisiae*. Until these strains are developed further, current biorefinery practices often solve the inability of baker's yeast to ferment the pentose fraction by using the pentose-rich distillate for biogas production (Liu et al. 2006; Kaparaju et al. 2009).

For biomasses with high salinity levels such as *Salicornia* juice (Fig. 2), salt-tolerant ethanologenic microorganisms might be needed for efficient ethanol production. Salt tolerance of baker's yeast (*Saccharomyces cerevisiae*) in model substrates is reported to be around 1 %; at higher concentrations, growth is significantly affected. In lignocellulosic substrates, the tolerance might be lower due to the presence of fermentation inhibitors. Salt-tolerant yeast strains have been isolated from several sources, including soy sauce processing (Driehuis et al. 2001), salt-excreting arid plants (Muck 1993) and hypersaline waters (Danner et al. 2000), and include strains such as *Zygosaccharomyces rouxii* (Driehuis et al. 2001), *Yarrowia lipolytica* (Muck 1993), *Hortaea werneckii* (Danner et al. 2000), *Pichia pastoris* (Hansen 2002) and *Debaryomyces hansenii* (Cogan and Hill 1993).

We tested *S. cerevisiae* on pretreated pulp fraction from wet separation of *Salicornia sinus-persica*. The pulp was pretreated using very low process severity of 121 °C for 30 and 60 min. The fibres were subjected to simultaneous saccharification and fermentation using 15 FPU/g DM of cellulases (Novozymes), with a pre-hydrolysis time of 2 h at 50 °C with intensive shaking (120 rpm) prior to addition of *S. cerevisiae* at 32 °C for 7 days. Final ethanol yields were analysed by HPLC and calculated as percent of theoretical based on carbohydrate analysis of the pretreated biomass fractions. SSF was carried out both on pretreated fibre fraction resuspended in hydrolysate and on pretreated fibres in water. This was done to examine possible effect of fermentation inhibitors (degradation products) in the hydrolysate. Figure 6 shows the SSF results.

The salt concentration in the pulp fraction of *Salicornia sinus-persica* was not too high for fermentation with *S. cerevisiae*. This is probably due to most of the salt being extracted into the juice fraction during wet separation. Both pretreatment conditions showed ethanol yields between 70 and 80 %, and no significant inhibitory effect was observed in pretreated hydrolysates, probably due to the low pretreatment severity used in this study. These results show that the green pulp of *Salicornia sinus-persica* can be pretreated at low severity and the glucan fraction fermented to ethanol by *S. cerevisiae*. However, a significant fraction of the

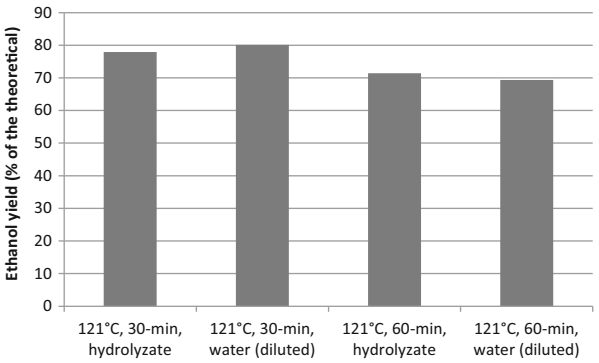

Fig. 6 Ethanol yield in SSF of pretreated pulp from wet fractionation of *Salicornia sinus-persica*

Salicornia biomass is pentose sugars (60–65 %), so for this biomass continued work will focus on finding/developing a pentose-fermenting strain.

Conclusions

Traditional green crops such as grass, clover, alfalfa as well as new (halophytic) green biomass of *Salicornia* have great potential to be utilised in the concept of the green biorefinery, where the first step is a wet fractionation of the biomass to yield a sugar- and protein-rich juice and a lignocellulosic pulp fraction. Microorganisms play a major role in green biorefinery. The first—and vital microorganism used—is lactic acid bacteria, which has the ability to quickly acidify the easy perishable juice fraction and convert it into a storable nutrient-rich medium, e.g. L-lysine fermentation. The acidification also leads to precipitation of the "leaf" protein of the juice which allows for separation of this fraction to yield a value-added protein product. The properties of a good acidifier are fast growing, homofermentative, acidophilic, thermophilic and GRAS (generally recognised as safe). By comparison of many strains, *Lactobacillus salivarius* subsp. *salivarius* DSM 20555 has been identified as a very promising strain for acidification. After separation of the leaf protein, the colour of the juice will change to a clear yellow-brownish colour, brown juice. The brown juice can be utilised as a nutrient-rich medium (protein source) for L-lysine fermentation using *Corynebacterium glutamicum* when a carbohydrate source is added. Most strains used for industrial production of lysine are mutated to lack homoserine dehydrogenase HDH. Furthermore, genetically engineered *C. glutamicum* with overexpressed fructose 1,6-bisphosphatase (fbp) and glyceraldehyde 3-phosphate dehydrogenase (gapX), as well as D-lactate dehydrogenase (dld), pyruvate carboxylase (pyc) and malic enzyme (malE) have been produced to be able to generate lysine using all quantified low molecular weight substrates, including lactate, glucose, fructose, maltose, quinate, fumarate, glutamate, leucine, isoleucine

(continued)

and alanine. Lysine production can be further improved by regulation of essential nutrients in the fermentation medium. The lower the growth rate of *C. glutamicum,* the more lysine is produced. Therefore, the reduction of essential nutrients such as glucose and ammonia is used to control the growth rate of *C. glutamicum* in fed-batch fermentation. The engineered strain *Corynebacterium glutamicum B-036 (NBIMCC 3624)* showed high cell concentration and high L-lysine concentration when cultivated in brown juice medium using fed-batch process.

The pulp fraction which is primarily lignocellulose is suggested as a good substrate for ethanol fermentation after physicochemical pretreatment and enzymatic hydrolysis. The dual challenge of ethanol fermentation of lignocellulosic biomasses is utilisation of pentose sugars (from hemicelluloses) as well as the presence of the inhibitors (degradation product formed during pretreatment). Three main approaches have been taken to develop pentose (e.g. xylose and arabinose)-fermenting ethanologenic microorganisms, the search for naturally occurring organisms that satisfy these requirements, genetic modification of microorganisms that can naturally ferment pentose sugars but cannot produce ethanol and the genetic modification of microorganisms that can produce ethanol very well but cannot ferment pentose sugars. However, so far most processes are using *Saccharomyces cerevisiae* (even though this strain does not ferment pentose) because of its high productivity and high inhibitor tolerance. The genome of *Saccharomyces cerevisiae* was among the first to be sequenced, and very powerful genetic manipulation techniques have been developed in the baker's yeast system making it a very attractive organism to work with when genetically modifying and constructing synthetic microorganism strains. However, for most biorefinery processes, the inability of *S. cerevisiae* to ferment the pentoses is solved by using the pentose-rich distillate for biogas production. *S. cerevisiae* was also shown to perform well, giving ethanol yields of 70–80 % (of theoretical) in hydrolysates of *Salicornia* pulp, showing that the salt content of this halophytic biomass did not inhibit the yeast.

An array of industrially important microorganisms is needed in order to efficiently convert green biomass into useful products such as lactic acid, L-lysine and ethanol using the concept of green biorefining. The most important microbes have been identified in this book chapter as *Lactobacillus salivarius, Corynebacterium glutamicum and Saccharomyces cerevisiae*.

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Microbial Succinic Acid Production Using Different Bacteria Species

Qiang Li and Jianmin Xing

Contents

Abstract Succinic acid (1,4-butanedioic acid) is identified as one of the important building-block chemicals, which can be used for the synthesis of high value-added derivatives, such as 1,4-butanediol (BDO), tetrahydrofuran (THF), g-butyrolactone (GBL), succinimide, and especially the biodegradable material polybutylene succinate (PBS). Confronted with the gradual and inescapable exhaustion of the earth's fossil energy resources, fermentative succinate production from renewable biomass to replace the conventional petrochemical process is receiving an increasing amount of attention. Great efforts have been made to develop biotechnology methods to produce succinic acid using different bacteria which mainly include the following species, *Actinobacillus succinogenes*, *Escherichia coli*, *Saccharomyces cerevisiae*, *Anaerobiospirillum succiniciproducens*, *Corynebacterium glutamicum*, *Mannheimia succiniciproducens*, and *Basfia succiniciproducens*. The fermentative succinic acid production has been made commercially available by the joint efforts of researchers in different fields. In this chapter, bacteria species for succinate production, including natural succinate overproducers and recombinant

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overproducers, are discussed. Besides, latest efforts and experiences devoted to microbial succinic acid production are also summarized, including biomass-based biorefining strategy, in situ product removal process, and novel downstream processing. Finally, the key limitations and challenges faced in recent microbial production systems are also proposed.

1 Introduction

The Nobel Prize winner, Robert Knock (1886), who is the pioneer of modern bacteriology, proved that succinic acid, which is also called 1,4-butanedioic, has a positive influence on human metabolism and there is no risk of its accumulation in the human body. In the 1930s and 1940s, European biochemists discovered that succinic acid is an acid created naturally in every cell of the body capable of aerobic respiration, participating in the tricarboxylic acid (TCA) cycle. Nowadays succinic acid is among the new bio-derived building-block chemicals which could replace the present maleic anhydride C_4 platform (Thakker et al. 2012). It gradually becomes a real potential platform chemical for the production of various high value-added derivatives, as it can be transformed into numerous interesting products: 1,4-butanediol (BDO), g-butyrolactone (GBL), tetrahydrofuran (THF), *N*-methyl-2-pyrrolidone (NMP), 2-pyrrolidone (2-Pyrr), succinimide, succinic esters, maleic acid (M.A.)/maleic anhydride (M.Anh.), and several others as shown in Fig. 1. Succinic acid is also used in foods as a flavoring agent because it contributes a somewhat sour and astringent component to umami taste. It is widely available as the mono- or disodium salt for adding flavor to meats, soups, and so forth. Some succinates are used as counterion in many pharmaceutical formulations. Various derivatives of succinate have found uses for their innocuous properties in food projects. Esterification with various small alcohols produces additives for the food industry too. Octenyl succinate modified starch is used as an emulsifier and thickener in many foods requiring an appropriate texture or reliability. Sodium dioctyl sulfosuccinate is used as a surfactant and wetting agent in textile and printing products and is a constituent of the Corexit dispersant used in the recent Gulf oil-spill cleaning operations.

C_4 dicarboxylic acids are already identified in the US Department of Energy's list (2004) of potential large-scale biomass-derived chemicals (Werpy and Petersen 2004). In the past decade, succinic acid has established itself as a forerunner in biorefinery platform chemicals. Compared with traditional synthetic methods by fossil fuels, the production of succinic acid from natural derived biomass would alleviate our dependence on oil supply for the production of these platform chemicals in future. Recognizing the importance of the biotechnological production of succinic acid, herein we intend to review microorganism producers and

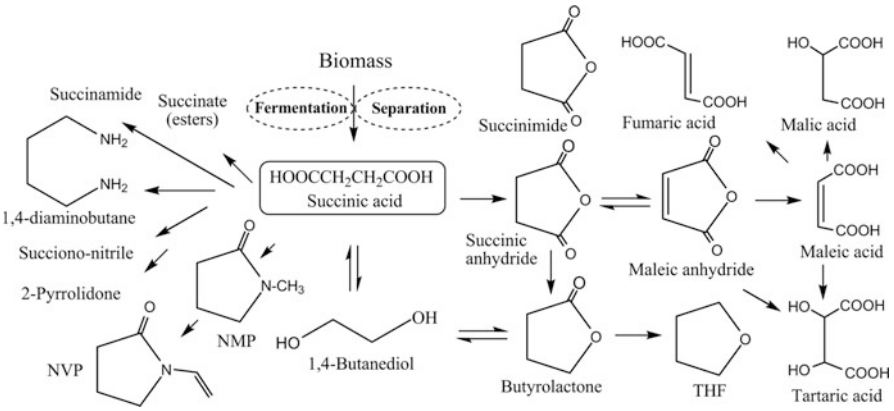

Fig. 1 Derivatives from succinic acid

cultivation and separation technologies as well as propose effective strategies toward the succinic acid bioprocess commercialization.

2 Production of Succinic Acid Using Different Bacteria Species

Succinic acid is an intermediate of the TCA cycle and one of the fermentation end products of anaerobic metabolism. Thus, it can be synthesized in almost all microbe, plants, and animal cells. Those organisms suitable for the efficient production of succinic acid can be categorized into fungi and bacteria. Note that the fact that CO_2 is assimilated during succinic acid fermentation can be considered as an advantage of environmental benefits. Fermentative production of succinic acid is regarded as one of the most attractive options to replace the fossil oil-based production of bulk chemicals by bio-based production.

2.1 Succinic Acid Producers

Up to now, a number of fermentative succinate-producing bacteria have been isolated and characterized to some extent. These microorganisms can be normally classified into two categories: natural producers and engineered producers. Several representative species that have been extensively investigated are listed in Table 1.

Table 1 Succinate production in several typical microorganisms

Type		Species	Cultivation	References
Natural species	Bacteria	*Actinobacillus succinogenes*	Facultative	Guettler et al. (1999)
	Bacteria	*Anaerobiospirillum succiniciproducens*	Obligate anaerobe	Davis et al. (1976)
	Bacteria	*Mannheimia succiniciproducens*	Facultative	Lee et al. (2002)
	Bacteria	*Bacteroides fragilis*	Obligate anaerobe	Isar et al. (2006)
	Bacteria	*Enterococcus flavescens*	Facultative	Agarwal et al. (2007b)
	Bacteria	*Klebsiella pneumoniae*	Facultative	Thakker et al. (2006)
	Bacteria	*Basfia succiniciproducens*	Facultative	Kuhnert et al. (2010)
	Bacteria	*Succinivibrio dextrinosolvens*	Obligate anaerobe	O'herrin and Kenealy (1993)
	Fungi	*Aspergillus niger*	Facultative	David et al. (2003)
	Fungi	*Paecilomyces variotii*	Facultative	Ling et al. (1978)
	Fungi	*Penicillium simplicissimum*	Facultative	Gallmetzer et al. (2002)
Genetically modified strains	Bacteria	*Escherichia coli*	Facultative	Thakker et al. (2012)
	Bacteria	*Corynebacterium glutamicum*	Aerobic	Okino et al. (2005)
	Yeast	*Saccharomyces cerevisiae*	Facultative	Raab et al. (2010)

2.1.1 *Actinobacillus succinogenes*

Figure 2 shows succinic acid production pathway in *A. succinogenes*. Under anaerobic conditions, succinate derives from PEP, via several intermediate compounds of the TCA cycle, including oxaloacetate (OAA), malate, and fumarate, is recognized as the chief pathway. Depending on the microorganism and cultivation condition, other metabolites, such as ethanol, acetate, formate, and lactate, can be produced when pyruvate is further oxidized (McKinlay et al. 2007). The fermentative pathway converts OAA to malate, fumarate, and then succinate, and this pathway requires 2 mol of NADH per mole of succinate produced. One major difficulty to high succinate yield through the fermentative pathway is due to NADH limitation. This is because 1 mol glucose can provide only 2 mol of NADH through the glycolytic pathway; however, the formation of 1 mol succinate through the native fermentative pathway requires 2 mol NADH. Therefore, the maximum molar theoretical yield of succinate from glucose is limited to 1 mol mol^{-1} glucose, assuming that all the carbon flux will flow through the native succinate fermentative pathway. Besides, key enzymes are important in the bacterium *A. succinogenes*. In the glycolysis process and the oxidative pentose phosphate pathway, glucose is

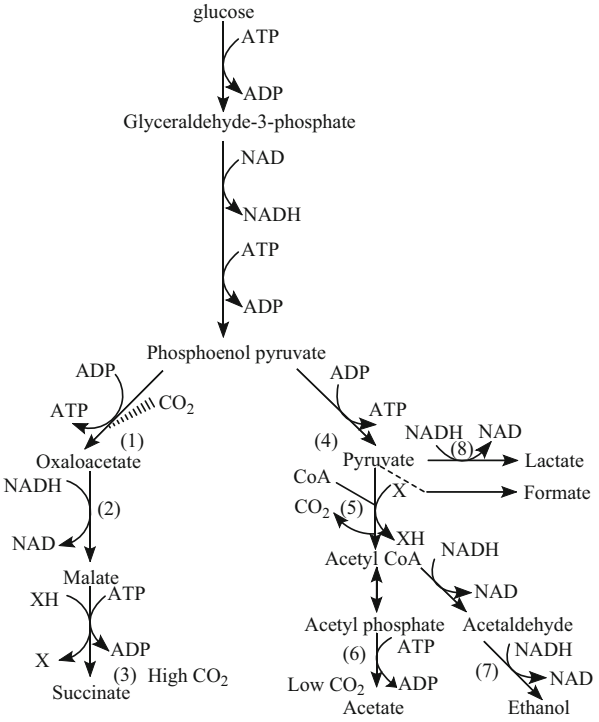

Fig. 2 Succinic acid production pathway of *A. succinogenes*: (1) PEP carboxykinase; (2) malate dehydrogenase; (3) fumarate reductase; (4) pyruvate kinase; (5) pyruvate oxidase/ reductase; (6) acetate kinase; (7) alcohol dehydrogenase; (8) lactate dehydrogenase

metabolized to PEP. In order to further synthesize succinate from PEP, four key enzymes, including phosphoenolpyruvate carboxykinase, malate dehydrogenase, fumarase, and fumarate reductase, are required (Van der Werf et al. 1997). Normally lactate flux is not regarded as one of the major by-products such as formate, acetate, and ethanol; thus, lactate is not listed in the *A. succinogenes* metabolic pathways (McKinlay et al. 2007). However, Li et al. (2010a) find initial aerobic operation is very effective in enhancing LDH activities effectively in a subsequent anaerobic culture. The advantages include a high glucose consumption rate and high lactate productivity in the overall fermentation process. With respect to lactic acid promotion in a subsequent anaerobic phase, most of the carbon flowed through the C_3 metabolic branch rather than C_4 metabolic branch, which would diminish succinate accumulation and enhance the lactate production. Exactly efficient control schemes for fermentation should be taken into account in order to realize a high production of succinate by *A. succinogenes*.

2.1.2 Engineering *E. coli* for Succinic Acid Production

In many cases, production processes are not economically feasible due to a relatively low product yield, low productivity, or difficulties in cultivating the natural

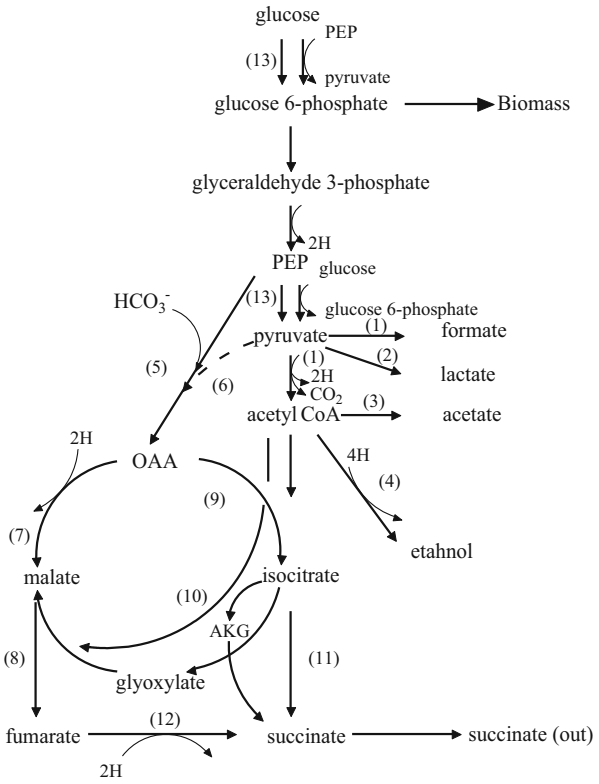

Fig. 3 Succinic acid production pathways of *E. coli*: (1) pyruvate-formate lyase; (2) lactate dehydrogenase; (3) fumarate reductase; (4) alcohol dehydrogenase; (5) phosphoenolpyruvate carboxylase; (6) pyruvate carboxylase; (7) malate dehydrogenase; (8) fumarase; (9) citric synthesis; (10) malic enzyme; (11) isocitric lyase; (12) fumarate reductase; (13) glucose phosphotransferase

producers. Production and productivity are generally affected by a variety of aspects. Reducing equivalents and the metabolic pathway used by *E. coli* for product synthesis are critical; various pathways leading to the identical product can require different precursors and have different reducing equivalents, energy requirements, and theoretical yields.

Succinic acid production pathway in *E. coli* is shown in Fig. 3. In wild-type *E. coli*, the maximum theoretical yield of succinate is 1 mol mol^{-1} glucose in anaerobic conditions as its yield is limited by reducing equivalents availability (NADH). Approaches to promote succinate production in *E. coli* have included the removal of competing pathways, the stimulation of pathways with lower reducing equivalent requirements, and the development of strains with improved succinate-producing capabilities. Several mutant and recombinant strains have been constructed and tested under aerobic (Lin et al. 2005), anaerobic (Jantama et al. 2008), or two-/dual-stage fermentation processes. For the two-/dual-stage fermentation processes, the first stage is aerobic for cell accumulation, and the second stage is anaerobic for succinate production (Vemuri et al. 2002b).

Recombinant *E. coli* strains show many advantages such as fast growth, simple requirements for nutrients and relative higher succinate yields. In McKinlay et al. (2007) research, continued research and development of *E. coli*-based

succinate production should focus on using constitutive expression systems or inexpensive induction methods to overexpress some key genes. One of the significant approaches to produce succinate in *E. coli* is to eliminate competing pathways by inactivating the pyruvate-formate lyase (*pflB*) and lactate dehydrogenase (*ldhA*) genes (Bunch et al. 1997). This strain, named NZN111, only produces minor amounts of succinate and shows growth impairment on glucose in anaerobic conditions. One spontaneous NZN111 mutant strain, named AFP111, can generate succinate as the major product; succinate, acetate, and ethanol are produced in a 2:1:1 ratio and molar yields of 1.0, 0.5, and 0.5 mol mol^{-1} glucose, respectively (Donnelly et al. 1998). From strict carbon balance arising from three carbon precursors, 2 mol of succinate could be produced from 1 mol of glucose consumed, upon appropriate carboxylation and redox availability. Then, the production of succinate using recombinant *E. coli* has been studied by several groups (Song et al. 2007; Kim et al. 2004a, b, c). In these continuous studies, the homologous or heterogenous pyruvate carboxylase (*pyc*), carbonic anhydrase (*ca*), phosphoenolpyruvate carboxykinase (*pck*), phosphoenolpyruvate carboxylase (*ppc*), NADP-dependent malic enzyme (*maeB*), and carbonic anhydrase (*ecaA*) genes cloned under the *lac* promoter or its derivatives *tac* and *trc* are induced by isopropyl-β-D-thiogalactopyranoside (IPTG) to enhance the production of metabolic engineered overproducers (Donovan et al. 1996; Gombert and Kilikian 1998; Wang et al. 2009). Metabolic evolution of energy-conserving pathways can convert the wild-type *E. coli* fermentation pathway for succinate into a functional equivalent of the native pathway that nature evolved in succinate-producing rumen bacteria. In this way, the net ATP yield during succinate production is doubled to 2.0 ATP per glucose (Zhang et al. 2009).

2.1.3 *Saccharomyces cerevisiae*

S. cerevisiae is a robust and important industrial microorganism with a thoroughly researched genetic background. Well-established manipulation tools are also available for this organism. Recently, the use of *S. cerevisiae* in production of biochemical besides the traditional ethanol has been intensively investigated. Compared with prokaryotes, *S. cerevisiae* is highly tolerant of low pH values (3.0–6.0), making it superior for succinic acid production (Raab et al. 2010). Succinic acid is a highly sought after value-added chemical for which there is no native predisposition for production and accumulation in *S. cerevisiae*. The genome-scale metabolic network reconstruction of *S. cerevisiae* enables in silico gene deletion predictions using an evolutionary programming method to couple biomass and succinate production (Otero et al. 2013). Agren et al. (2013) apply a genome-scale metabolic model and flux balance analysis for the prediction of succinic acid overproduction strategies in *S. cerevisiae*. *Δdic1* is tested using knockout strains cultivated anaerobically on glucose, coupled with physiological and DNA microarray characterization. *Δdic1* produced 0.02 C-mol/C-mol glucose, in close agreement with model predictions (0.03 C-mol/C-mol glucose). Transcriptional

profiling suggests that succinate formation is coupled to mitochondrial redox balancing and, more specifically, reductive TCA cycle activity. While far from industrial titers, this proof-of-concept suggests that in silico predictions coupled with experimental validation can be used to identify novel and nonintuitive metabolic engineering strategies (Agren et al. 2013). Using industrial systems biology and metabolic engineering of *S. cerevisiae*, under optimal supplemental CO_2 conditions in a bioreactor, the engineered strain produced 12.97 g L^{-1} succinate with a yield of 0.21 mol mol^{-1} glucose at pH 3.8 by a pyruvate decarboxylase (pdc)-deficient *S. cerevisiae* strain, which might be a novel succinic acid cell factory (Yan et al. 2013).

2.1.4 Other Succinic Acid Producers

Many natural bacterial species have the pathway for converting phosphoenolpyruvate (PEP) to succinate. Besides *A. succinogenes*, *E. coli*, and *S. cerevisiae*, experimental data infer the presence of the succinic acid pathway (in whole or in part) in the following species: *Anaerobiospirillum succiniciproducens*, *Corynebacterium glutamicum*, *Bacteroides amylophilus*, *Bacteroides fragilis*, *Bacteroides succinogenes*, *Clostridium thermosuccinogenes*, *Cytophaga succinicans*, *Fibrobacter succinogenes*, *Klebsiella pneumoniae*, *Mannheimia succiniciproducens*, *Paecilomyces variotii*, *Penicillium simplicissimum*, *Succinivibrio dextrinosolvens*, and *Basfia succiniciproducens*.

M. succiniciproducens, isolated and characterized as a succinic acid-producing bacterium from the rumen of a Korean cow, is able to produce a large amount of succinic acid as a major fermentation product under anaerobic conditions in the presence of CO_2 (Lee et al. 2002; Song et al. 2007). Genome-based metabolic engineering studies have also been performed to reduce the by-products formation (Lee et al. 2006). *C. glutamicum* is well known as workhorse for the industrial production of numerous amino acids. However, latest studies also explore the usefulness of this organism for the production of succinic acid (Okino et al. 2005, 2008; Wieschalka et al. 2012). *C. glutamicum* ATCC 13032 lacking the succinate dehydrogenase complex can produce succinate aerobically with acetate representing the major by-product (Zhu et al. 2013). *B. succiniciproducens* is a new facultative anaerobe of the family *Pasteurellaceae*, naturally secretes substantial amounts of succinic acid, favoring it as future industrial producer (Kuhnert et al. 2010). Systems-wide analysis and engineering of metabolic pathway fluxes in *B. succiniciproducens* has revealed excellent succinate production performance (Becker et al. 2013). It is worth noting that not all species produce succinate as an end product, and sometimes succinate as an intermediate can be further converted to a different product, such as propionate.

2.2 Succinic Acid Fermentation from Biomass

CO_2 fixation is required to produce succinate: one molecule of CO_2 is incorporated into PEP to form OAA catalyzed normally by PPC. In succinate production processes, CO_2 is usually provided in the form of carbonate (e.g., $MgCO_3$, Na_2CO_3) or directly by CO_2 sparging into the bioreactor. CO_2 sparging serves a double purpose: (1) supplying the required CO_2 for OAA formation from PEP and (2) maintaining an anaerobic condition for succinate production. The effect of CO_2 concentrations in the gas phase is evaluated on succinate production by AFP111. To increase metabolic flux to succinate, several enzymes involved in CO_2 fixation are overexpressed, including PPC and PEPCK, which transform PEP into OAA with the incorporation of one molecule of CO_2 and the generation of one inorganic phosphate (Pi) or ATP, respectively (Gokarn et al. 2001; Wang et al. 2009, 2010, 2011a, b).

For each engineered strain, the optimal growth and production conditions need to be examined, and the optimal production window and the effect of parameters such as pH, temperature, metal cations, CO_2 supply, feedstock concentrations, level of aeration if necessary, and batch strategies (Table 2) need to be defined for suitable fermentative scale-up. Process engineering strategies are used to enhance the fermentation efficiency. For example, media components and fermentation process parameters are the most basic and simplest approach to achieve a high production of the desired products. Since the media for succinate fermentation always contain a variety of nutrient components, the effects of an individual component and the interactions between different components are needed to be studied, which needs exact experiment design. Plackett–Burman design (PBD) and central composite design (CCD) could be used for rapid screening of factors to find

Table 2 The succinic acid batch fermentation strategies

Mode	Principles
Constant feeding	Constant feed of nutrients with preset speed, cell-specific growth rate gradually decreased, cell density increases linearly
Speed change feeding	Accelerated rate in the fermentation process (gradient, phase, linearity, etc.), specific growth rate is constantly changing
Index feeding	Feed rate increases exponentially, the value of specific growth rate is constant, the cell density increases exponentially
pH-control feeding	Estimation of the cell growth state based on the changes in the pH value and regulate the glucose feed rate to adjust the pH to a constant value
DO-control feeding	Using the dissolved oxygen as the fed-batch index adjusted the feed rate of the carbon source according to the curves of the dissolved oxygen
Biomass feedback feeding	Adjusted the amount of added carbon source by detecting the cell concentration and fitting the nutrient utilization
CER-control feeding	The nutrition feed rate is controlled by detecting the rate of CO_2 release (CER) and estimation of the carbon source utilization
DO-stat feeding	To maintain a constant dissolved oxygen by controlling the dissolved oxygen, the mixing rate and feeding rate to reduce the production of acids

the most significant factors. Moreover, response surface methodology (RSM) is already employed to optimize the concentration of the important factors.

Most studies of succinic acid fermentation are executed on glucose as the feedstock; however, different carbon sources, such as glucose, sucrose, xylose, galactose, and glycerol, have been also examined by numerous research groups (Table 3). Wang et al. (2011a, b) have engineered an *E. coli* strain capable of fermenting fructose, sucrose, and glucose mixtures. Xylose is examined as an only carbon source for succinate production in their study. Andersson et al. (2007) find that xylose and glucose could be simultaneously consumed when they use a mutant strain AFP184 for succinate fermentation, and they attribute this phenomenon to a glucose phosphotransferase (*ptsG*) mutation in AFP184. In a dual-phase batch fermentation containing 100 g L^{-1} of total initial xylose, this *E. coli* strain produces succinate with a yield of 0.50 g g^{-1}.

Recently, jobs have been done to use renewable agriculture resources with pretreatment to release sugar molecules for succinate production. Much information is accessible on the feasibility of converting the biomass containing rough cellulose into fermentative products. Many agricultural and industrial wastes or residues such as whey, stalk, straw, and wood have been reported as raw carbon materials for the production of succinic acid. Kim et al. (2004a, b, c) use wood hydrolysate-based medium to culture *M. succiniciproducens* MBEL55E, and the final succinic acid concentration of 11.73 g L^{-1} is obtained in batch fermentation, resulting in a succinic acid yield of 0.56 g g^{-1}. Du et al. (2008) carry out two wheat-based biorefining strategies converting wheat to succinic acid in which *A. succinogenes* fermentation using only the wheat-derived feedstock resulted in a succinic acid concentration of 16 g L^{-1} with an overall yield of 0.19 g g^{-1}. In batch fermentation of succinic acid from straw hydrolysates by *A. succinogenes*, 45.5 g L^{-1} succinic acid concentration and 0.81 g g^{-1} yield are attained after 48 h incubation with 58 g L^{-1} of initial sugar from corn straw hydrolysates (Zheng et al. 2009). Economical succinic acid production from cane molasses can yield 50.6 g L^{-1} succinic acid in continuous anaerobic fermentation (Liu et al. 2008). Li et al. (2010d) enzymatically convert crop stalk wastes, corn stalk, and cotton stalk into a carbohydrate-rich feedstock, obtaining glucose concentrations approaching 65–80 % of the total reducing sugar. And for the anaerobic batch cultivation with cotton stalk hydrolysates, the production of succinic acid is 15.8 g L^{-1} with a high yield of 1.23 g g^{-1} glucose.

Typically biorefinery consists of two platforms: a sugar platform and a thermal platform. Nowadays, renewable biomass has been intensively investigated to produce biofuels and chemicals via the sugar platform Kim et al. (2004a, b, c). This process usually includes pretreatment of biomass, obtaining sugars and the final products fermentation. Meanwhile, substantial research is being carried out to produce alternative fuels from biomass to replace the gasoline and diesel via thermal platform (Jae et al. 2010). Wang et al. (2013) find the transgenic *E. coli* strain could grow in modified M9 medium containing 20 v/v% aqueous phase bio-oil (AP-bio-oil) with an increase in OD_{660} value from 0.25 to 1.09. With the presence of 4 g L^{-1} glucose in the media, succinic acid concentration increases

Table 3 Microbial succinic acid fermentation

Strain	Substrates	Fermentation	Titers ($g\ L^{-1}$)	Productivity ($g\ L^{-1}\ h^{-1}$)	Yield ($g\ g^{-1}$)	References
A. succinogenes 130Z	Glucose	Batch	67.2	0.8	0.7	Guettler et al. (1998)
A. succinogenes 130Z	Whey	Batch	70.6	0.7	0.88	Guettler et al. (1998)
A. succinogenes 130Z	Sake lees	Batch	48	0.94	0.75	Chen et al. (2010)
A. succinogenes FZ53	Glucose	Batch	105.8	1.36	0.83	Guettler et al. (1996)
A. succinogenes FZ6	Corn fiber hydrolysate	Batch	70.6	0.7	0.88	Guettler et al. (1996)
A. succinogenes CGMCC 2650	Corn straw	Batch	15.8	0.62	1.23	Li et al. (2010d)
A. succinogenes CGMCC 1593	Cane molasses	Batch	50.6	0.84	0.8	Liu et al. (2008)
A. succinogenes CGMCC1593	Corn stover hydrolysate	Fed-batch	53.2	1.21	0.83	Zheng et al. (2010)
A. succinogenes CIP 106512	Sugarcane bagasse hydrolysate	Batch	22.5	1.01	0.43	Elcio and Nei (2011)
A. succiniciproducens ATCC29305	Whey	Fed-batch	34.7	1.02	0.91	Samuelov et al. (1999)
A. succiniciproducens ATCC29305	Glycerol	Batch	19	0.15	1.6	Lee et al. (2001)
A. succiniciproducens ATCC29305	Wood hydrolysate	Batch	24	0.74	0.88	Lee et al. (2003)
A. succiniciproducens ATCC29305	Galactose	Batch	15.3	1.46	0.87	Lee et al. (2003)
M. succiniciproducens MBEL55E	Whey	Continuous	6.4	3.9	0.69	Lee et al. (2002)

(continued)

Table 3 (continued)

Strain	Substrates	Fermentation	Titers ($g\ L^{-1}$)	Productivity ($g\ L^{-1}\ h^{-1}$)	Yield ($g\ g^{-1}$)	References
M. succiniciproducens MBEL55E	Wood hydrolysate	Continuous	8.2	3.19	0.55	Kim et al. (2004a, b, c)
E. coli AFP111-pyc	Glucose	Dual phase aeration, batch	99.2	1.31	1.1	Ma et al. (2011)
E. coli AFP184	Fructose	Dual phase aeration, batch	30	1.01	0.7	Vemuri et al. (2002a)
E. coli AFP184	Xylose	Dual phase aeration, batch	23	0.78	0.5	Vemuri et al. (2002a)
E. coli AFP184	Softwood dilute acid hydrolysate	Dual phase aeration, batch	42.2	0.78	0.72	Hodge et al. (2009)
E. coli W3110	Sucrose	Dual phase aeration, batch	24	0.81	1.2	Chatterjee et al. (2001)
E. coli W3110	Cane molasses	Dual phase aeration, batch	26	0.87	0.52	Agarwal et al. (2007a)
E. coli MG-PYC	Bio-oil	Dual phase aeration, batch	11.5	–	–	Wang et al. (2013)
C. glutamicum R ΔldhA-pCRA717	Glucose	Fed-batch with cell recycling	146	3.17	0.92	Okino et al. (2008)
B. fragilis MTCC1045	Glucose	Batch	20	0.83	0.57	Isar et al. (2007)
P. ruminocola ATCC 19188	Glucose	Batch	18.9	0.52	–	Guettler et al. (1998)
F. succinogenes S85	Wheat straw	Batch	1.55	0.022	0.05	Li et al. (2010a, b, c, d, e)
F. succinogenes S85	Orange peel	Batch	1.75	0.025	0.044	Li et al. (2010a, b, c, d, e)
B. succiniciproducens	Glucose	Batch	20	–	0.475	Becker et al. (2013)
S. cerevisiae	Glucose	Batch	9.98	–	0.133	Yan et al. (2014)

from 1.4 to 2.4 g L^{-1} by addition of 20 v/v% AP-bio-oil. When enzymatic hydrolysates of corn stalks are used as carbon source, 10.3 g L^{-1} succinic acid is produced. The obtained succinic acid concentration increased to 11.5 g L^{-1} if 12.5 v/v% AP-bio-oil is added.

2.3 *Enhanced Fermentation Process Via In Situ Product Removal Strategy*

The product inhibition is one of the major reasons that made the production of succinic acid via fermentation economical infeasibility. Cell growth curves show microbial cells decline rapidly after the stationary phase as a result of acid accumulation over inhibitory concentrations not only for natural species but also for genetically modified strains (Li et al. 2010c). Many researchers presently report that inhibitory product deteriorates the cells and limits the attainable product accumulation. Systematic investigation of inhibition kinetics for the substrate and succinate is done for the development and improvement of the effective control strategy for succinate production (Song and Lee 2006; Lin et al. 2008). Metabolic and genetic engineering or effective screening methods are used to identify sorts of succinate-tolerant strains (Vemuri et al. 2002a, b; Guettler et al. 1998). Moreover, cell immobilization is used to recycle the cells and increase the yield (Urbance et al. 2004). Immobilization technique cannot overcome inhibition unless it enables a continuous process to be operated with cell recycle. However, these methods do not overcome product inhibition basically.

Although large amount of commercially useless gypsum is accumulated in the traditional succinate fermentation and separation, it is a potential alternative to minimize the product inhibition with the precipitation of succinate. In the process, calcium hydroxide is added to neutralize the acidic fermentation broth and precipitate the succinate. Free acid is then released from the calcium succinate by adding sulfuric acid (see Sect. 3). Another approach to improve the efficiency of the biotechnological process is to remove the inhibitory product from the vicinity of the cells as soon as it is formed (Schügerl and Hubbuch 2005). With this method, the product of the transformation is captured fast in a product recovery phase, which overcomes inhibitory or toxic effects of product to allow continuous fermentation. A given succinic acid production process can be developed and optimized following in situ product removal (ISPR) strategy.

Meynial-Salles et al. (2008) developed an integrated fermentation-membrane separation system, which produces a concentrated succinate solution of 83 g L^{-1} using *A. succiniciproducens*. In this way, much attention should be paid to the pollution and throughput of membranes in the modules. Thus, it is important to select appropriate ISPR techniques according to the physicochemical properties of the target product and the fermentation process. Li et al. (2011) apply ISPR technique to *A. succinogenes* fed-batch fermentation. Expanded bed adsorption

separation with anion exchange resins is used to directly capture succinic acid from bioreactor effectively. Via the ISPR approach, *A. succinogenes* fermented glucose continuously and effectively with the prolonged cell growth cycle from 48 to 126 h and produced succinic acid up to the final titer of 145.2 g L^{-1} with an average yield of 0.52 g g^{-1} and productivity of 1.3 g L^{-1} h^{-1}. However, the cycling adsorption and fermentation process usually produces a large quantity of wastewater. And microbial contamination should be carefully controlled in the coupled processes. Table 4 summarizes several integrated bioprocess for succinic acid production.

3 Downstream Processing of Microbial Succinic Acid

Recovery of succinic acid from the fermentation broth is the last stage for microbial succinate production. The separation and purification of succinate are estimated to make up more than half of the total cost (Luque et al. 2009). To make fermentation-based succinate production competitive with petrochemical processes, the development of optimized producing strains and fermentation processes must be combined with cost-saving and energy-effective downstream processes (Efe et al. 2013). Because succinic acid is hydrophilic diacid with a high boiling point, and the concentration of the succinate in fermentation broth is usually not very high, about 2–14 % in media, it is a big challenge to efficiently separate succinate from the mixture of multiple components, such as succinate, residual sugar, residual N source, or glycerol, some by-products (e.g., ethanol, acetate, lactate, formate, malate, pyruvate, etc.), biomacromolecules (e.g., proteins, nucleic acids, and polysaccharides), salts, and water. Table 5 summarizes the common succinate separation strategies.

$Ca(OH)_2$ or CaO precipitation is the traditional method for the succinic acid isolation from fermentation broth. After adding $Ca(OH)_2$ or CaO, the calcium succinate is separated from the broth by filtration. The obtained calcium succinate reacts with concentrated sulfuric acid, which releases free succinic acid. Succinic acid is further purified by active carbon absorption or ion exchange, and then the product is further concentrated and crystallized by evaporation (Datta et al. 1992; Guettler et al. 1998). During the precipitation process, the dosages of Ca $(OH)_2$, CaO, and H_2SO_4 are very large. Those reagents cannot be used repeatedly, which leads to large operation cost. Moreover, another drawback of precipitation with Ca $(OH)_2$ or CaO is the substantial by-product calcium sulfate (gypsum), which cannot be sold directly as a commodity due to odor and color impurities (Cheng et al. 2012). Ammonia precipitation is investigated by Berglund et al. (1999) and Yedur et al. (2001). Diammonium succinate is obtained by adjusting the pH of the broth using an ammonia-based compound or by replacing the cation in the succinate salt with ammonia. Diammonium succinate in the broth reacts with sulfuric acid or by combining it with ammonium bisulfate at low pH to produce the precipitated succinic acid and ammonium sulfate. The precipitated succinic acid is purified further using methanol recrystallization. The by-product ammonium sulfate can be

Table 4 Integrated bioprocess for succinic acid production

Strain	Titer ($g\ L^{-1}$)	Productivity[a] ($g\ L^{-1}\ h^{-1}$)	Yield[a] ($g\ g^{-1}$)	Time (h)	ISPR Technique	References
E. faecalis	46.2	9.2	0.92	360	Cell immobilized[b]	Wee et al. (2002)
A. succinogenes	40	0.9	0.87	38.5	Cell immobilized[c]	Urbance et al. (2004)
A. succiniciproducens	84	10.4	0.89	350	Electrodialysis	Meynial-Salles et al. (2008)
A. succinogens	145.2	1.3	0.52	126	Expanded bed adsorption	Li et al. (2011)
C. glutamicum	146	3.17	0.92	46	Membrane for cell recycling	Okino et al. (2008)
E. coli	73	–	–	190	Hollow fiber ultrafiltration membrane	Wang et al. (2014)

The fermentation medium is complex, with batch or fed-batch operation. Carbon source is glucose. Nitrogen source is chosen as peptone, tryptone, or yeast extract

[a]The average value calculated with the overall fermentation time

[b]Cell immobilized in an asymmetric hollow-fiber bioreactor (HFBR) with fumarate as carbon source

[c]Continuous biofilm fermentation with using plastic composite support bioreactors

Table 5 Succinate separation strategies

Separation strategies	Advantage	Drawbacks	References
Precipitation	Low technical barriers; low-priced precipitants	Requirement of large quantities of precipitants; useless by-products	Guettler et al. (1996, 1998)
Crystallization	Easy operation; without additional reagents	Low succinate yield and purity; other unit operation is required	Luque et al. (2009)
Extraction	High output and low energy consumption	Requiring broth pretreatment; expensive extraction agents used for reactive extraction	Hong et al. (2000)
Membrane filtration/ electrodialysis	Relatively mild conditions; can be used for continuous separation	High energy consumption; high cost of the membranes; low selectivity for succinate	Wang et al. (2012, 2014)
Resin adsorption	Easy operation and scaling up	Water consumption; regeneration and clean of adsorbents is required	Li et al. (2009)

converted to ammonia and ammonium bisulfate by pyrolysis method. One advantage of ammonia precipitation is the fewer amounts of waste by-products and the possible recycling used reagents. The main disadvantages are the high energy consumption and equipment erosion due to the very low pH and high temperature.

Crystallization is able to recover the desired product (in solid or crystal form) without many process control operations. After removal of cell biomass and organic impurities by centrifugation and activated carbon absorption, respectively, a direct vacuum distillation–crystallization is used for succinic acid recovery from broth by Luque et al. (2009). The pH of the aqueous broth is adjusted to 4.2 by addition of hydrochloric acid before vacuum distillation. Some volatile by-product carboxylic acids, such as acetic, formic acids, in broth are removed under vacuum distillation at 60 °C. The followed crystallization of succinic acid is carried out at 4 °C. When this method is used in a simulated broth, the highest succinic acid yield and purity are 75 % and 97 %, respectively. Another direct crystallization method at normal pressure bases on the principle that carboxylic acids have different distribution with their dissociated and undissociated forms at different pH, and the undissociated carboxylic acid has different solubility (Li et al. 2010b). The solubility of succinic acid is 3 % at 4 °C, pH 2.0, while the other acid by-products, such as lactic acid, acetic acid, and formic acid, are still fully water miscible. Thus, crystallization of succinic acid can be carried out at 4 °C and pH < 2. While acidic by-products remain in the solution, succinic acid could be selectively crystallized. By this one-step recovery technique, succinic acid yield and purity are 70 % and 90 %, respectively. However, much succinate is still residual in the broth, and the product of low purity cannot be used as a monomer for polymerization. To obtain a higher purity of succinic acid, coupled purification processes such as adsorption or membrane separation are needed for broth to remove residual impurities (Wang et al. 2014).

The reactive extraction of succinic acid with amine-based extractants, employing hydrophobic tertiary amines, has been considered as an effective and economical purification method because the process can be operated at normal temperature and pressure (Kim et al. 2004a, b, c). This process is based on reversible reaction between the extractant and the extracted carboxylic acid. The selective separation of specific acid from fermentation broth containing mixed acids can be achieved based on the pK_a values of the acids and pH. Solvent extraction has another advantage, such as high output and low energy consumption. Tri-n-octylamine can be used as an extractant in the recovery of succinic acid from the binary mixture of succinic acid and acetic acid with high selectivity (Hong et al. 2000). Orjuela et al. (2011) present an approach to recover succinic acid from fermentation broth via acidification and esterification in ethanol. The fermentation broth is treated as usual by centrifugation to remove cell biomass and by addition of activated carbon to remove impurities such as protein fragments and pigments. Water is then removed from the clean broth until the succinate salts are present as wet solids. The wet solids are placed in ethanol along with a slight stoichiometric excess of sulfuric acid. Simultaneous acidification and esterification take place, with the inorganic sulfate salt formed precipitating out of the ethanol solution. The succinate is recovered as a solution of free succinic acid, monoethyl succinate, and diethyl succinate in ethanol, a mixture suitable for further esterification via reactive distillation.

Membrane filtration such as microfiltration, ultrafiltration, and nanofiltration, together with electrodialysis, has been tested for the separation and purification of succinic acid (Wang et al. 2012, 2014). Glassner and Datta (1992) develop a succinic acid purification process using combined desalination electrodialysis and bipolar membrane electrodialysis. In the first stage, the desalination electrodialysis is applied to separate the ionic species, such as acids, from an aqueous solution and other uncharged compounds, such as sugars, saccharides, or proteins. In desalination electrodialysis stage, all charged molecules pass through the membrane and a sodium succinate solution is obtained. In the next stage, using a bipolar membrane electrodialysis, the concentrated sodium succinate is converted to succinic acid and NaOH which can be recycled back to the fermentation process for pH control. After two stages of electrodialysis, a total purification yield of 60 % is achieved. Disadvantages of electrodialysis are the cost of device and a low product yield due to loss of succinic acid and membrane pollution. Wang et al. (2012) study the membrane fouling mechanism in treating succinic acid fermentation broth during dead-end ultrafiltration. Membrane with hydrophilic characteristic tends to have a higher flux. Resistance-in-series model analysis shows that membranes with higher flux tend to be fouled by cake layer or concentration polarization. Hermia's model analysis indicates that membrane named RC 10 kDa and PES 30 kDa are controlled by complete blocking, membrane named PES 100 kDa by intermediate blocking, and membrane named PES 10 kDa by cake layer fouling.

Resin adsorption including anion exchange resin, alumina, silica, and zeolite molecular sieve adsorption has been recently reported in some publications (Nam et al. 2011; Straathof et al. 2010; Li et al. 2009). The key desired properties for one

type of ideal adsorbents are high capacity, complete stable regenerability, and selectivity toward the product. Ponnampalam (1999) suggests alkaline-type anion exchange resins to recover succinic acid from broth. Water wash step is used firstly to eliminate the unbonded material from the resin. Then a strong inorganic acid is used to release succinic acid. This method can specifically recover succinic acid over acetic acid and lactic acid. Further purification of succinic acid is carried out by evaporative crystallization, which produces more than 99 % pure succinic acid crystals. Davison et al. (2004) screen the best resin XUS 40285 from 25 adsorbents and then test for successive succinic acid loading and regeneration with hot water. This kind of resin has a stable capacity of about 0.06 g of succinic acid g L^{-1} of resin at moderate concentrations of succinic acid. XUS 40285 also has a good stable isotherm capacity, prefers succinate over glucose, and has good capacities at both acidic and neutral conditions. Using XUS 40285 in a packed column, succinic acid is removed from simulated media containing salts, succinic acid, acetic acid, and sugar. The fermentation by-product, acetate, is completely separated from succinate. A simple hot water regeneration successfully concentrates succinate from 10 g L^{-1} (inlet) to 40–110 g L^{-1} in the effluent. Inci et al. (2011) recover succinic acid from aqueous solutions by alumina adsorption. They find that the recovery yield of succinic acid increases with an increasing amount of alumina. Initial acid concentration does not have a similar effect. For 2 g alumina dose and a 0.45 mol kg^{-1} initial succinic acid concentration at 25 °C, the maximum adsorption is reached and the minimum equilibrium concentration is 0.28 mol kg^{-1}.

4 Industrialization and Future Prospects

Succinate has been included in the US Department of Energy's *top value-added chemicals from biomass* based on its potential to become an important building block for deriving both commodity and specialty chemicals (Werpy and Petersen 2004). Three important cultivation parameters decide the economic viability of a bioprocess: titer, yield, and productivity. The yield relates more to the variable cost of raw feedstock and will be of growing importance with increasing prices of substrate sugar. Productivity and concentration relate more to the fixed cost and total investment. Low rates imply larger energy and labor cost; low concentrations will result in larger energy input for product recovery. Compared with petrochemical-derived succinate, biological succinate production is still not economically competitive. The major drawbacks include high cost of the feedstock, low titers in the fermentation broth, the coproduction of low-value acid by-products, and difficult downstream recovery process. To increase the competitiveness of the microbial succinate production, future work can be addressed as increasing succinate concentration and yield through synthetic biology technique and metabolic engineering, introducing and optimizing the succinate synthetic ways in the species with high succinate tolerance, and integrating production of succinate with other high-value-added products, overcoming the substrate

repression effect and utilizing low-cost nonfood-based feedstock (Cheng et al. 2012; Thakker et al. 2012). For example, lignocellulose is the most abundant renewable resource on Earth. Succinate production from lignocellulose is promising using a consolidated bioprocessing system in which cellulase and hemicellulase production, substrate hydrolysis, and succinate fermentation are coupled or partially coupled by a succinate-producing organism that expresses a cellulolytic or a cellulolytic enzyme gene or engineering cellulolytic organism with succinate production properties. By studying metabolic regulation and synthetic biology, combined with physical and chemical pretreatment technology, it is expected to design novel integrated biorefinery pathway to effectively convert lignocellulosic biomass into succinate.

At present, the microbial production of succinic acid is still at a demonstration scale. A techno-economic analysis of succinic acid production using adsorption from fermentation medium is made by Efe et al. (2013). Their process begins with aerobic fermentation using a hypothetical *S. cerevisiae* strain at pH 4. After centrifugation the broth is sent to adsorption. A ZSM-5 zeolite is used to preferentially adsorb succinic acid. Desorption is performed using hot water. This water is then flashed off, and succinic acid is crystallized and dried. This plant capacity is set to 30 ktons a^{-1} according to the projected demand. Cane sugar is the selected feedstock. The calculated selling price of succinic acid is 2.26 \$ kg^{-1} with the potential to decrease to values as low as 1 \$ kg^{-1}. Another life cycle assessment also suggests that low pH yeast fermentation with direct crystallization is the most beneficial process to bio-based succinic acid from an environmental perspective (Cok et al. 2014). However, the important progress is expected in light of major business joint ventures and research activities. For example, BioAmber, a US company which dedicates to the production of bio-based succinic acid, has constructed a demonstration plant in a French village, Pomacle, with a capacity of 2,000 tons per year. In parallel, BioAmber developed a turn-key technological package available for licensing in 2011. DSM and Roquette are currently building a large-scale plant with a capacity of 10 ktons per year and will begin commercial production since 2012. Myriant, a successor to BioEnergy International, is recently awarded \$50 million by the US Department of Energy to construct a succinic acid plant with an initial capacity of 1.35 ktons per year in Louisiana, USA, in 2012. In addition, BASF, Purac, Mitsubishi Chemicals, and PTT have their individual plans for commercial bio-based production of succinic acid. Therefore, it is no surprise that commercial bio-production of succinic acid by companies such as BioAmber, Reverdia, and Myriant Technologies and a joint venture between BASF and DSM-Purac are either already happening or in the construction phase. These four companies plan to produce microbial succinic acid in excess of 150 ktons per annum by the end of 2015 (Bomgardner 2011; Yuzbashev et al. 2011; Van Heerden and Nicol 2013).

Obviously, biological succinic acid can decrease the use of nonrenewable resource and reduce the greenhouse gas emission. Currently, the demand for succinic acid has been entering a time of rapid increase, bringing both economic and environmental driving force. This chapter gives a slight review of the

technology development and update research progress for the microbial succinate production. For the development of a highly efficient technology, certain challenges such as developing a high-succinate-tolerant strain with the maximum theoretical yield and optimizing the cultivation method to increase productivity need to be considered. Recovery and purification of succinic acid is also a technological obstacle and an economical challenge for a successful commercialized microbial production. The separation methods and techniques so far studied have some drawbacks or limitations. Separation technologies coupled with synthetic biology technology, in situ product removal, and biorefining strategy deserve more attentions in the future.

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Whole-Cell Biocatalytic Production of 2,5-Furandicarboxylic Acid

Nick Wierckx, Tom D. Elink Schuurman, Lars M. Blank, and Harald J. Ruijssenaars

Contents

Abstract 2,5-Furandicarboxylic acid (FDCA) is considered a highly promising bio-based alternative to terephthalic acid for the production of polymers. Whole-cell biotransformation of 5-(hydroxymethyl)furfural (HMF) into FDCA is catalyzed by the HMF/furfural oxidoreductase HmfH from *Cupriavidus basilensis*

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HMF14 in *Pseudomonas putida* S12. The conversion rate was enhanced by the additional expression of *hmfT1* and *adh*, respectively encoding a transporter for HMF and/or its derivatives and an aldehyde dehydrogenase. With this optimized whole-cell biocatalyst, FDCA could be produced at titers exceeding 100 g l^{-1} in a pH-controlled fed-batch process. Purification of FDCA can be achieved by centrifugation, precipitation, and solvent extraction. In all, biocatalytically produced FDCA has great potential as a "green" substitute for terephthalic acid, although further developments are required for industrial-scale production and application.

Glossary

FDCA	2,5-furandicarboxylic acid
HMF	5-(hydroxymethyl)furfural
HMF acid	5-(hydroxymethyl)furoic acid
FFA	5-formylfuroic acid
DFF	2,5-diformylfuran

1 Introduction

The production of renewable chemicals and fuels is widely considered a priority for achieving a sustainable society with minimal impact on the environment. To this end, lignocellulosic biomass is gaining increasing attention as a cheap, abundant, and renewable feedstock (Ragauskas et al. 2006). The sugars contained in lignocellulosic biomass can be liberated by a combination of thermochemical and/or enzymatic treatments, but this often results in the formation of by-products such as resins, weak acids, phenolics, and furanic aldehydes (Almeida et al. 2007; Palmqvist and Hahn-Hagerdal 2000b; Thomas et al. 2002; Klinke et al. 2004). These by-products can strongly inhibit the microorganisms in subsequent fermentation steps and pose a severe hurdle in the development of efficient biotechnological processes based on lignocellulosic hydrolysate. Especially the furanic aldehydes such as furfural and 5-(hydroxymethyl)furfural (HMF) are considered problematic, since they are highly toxic to microorganisms and can occur in relatively high concentrations in lignocellulosic hydrolysates (Heer and Sauer 2008; Wierckx et al. 2011; Zaldivar et al. 1999). Several techniques have been developed to remove these inhibitors from lignocellulosic hydrolysates in a process termed detoxification, including overliming (Mussatto and Roberto 2004), solvent extraction (Palmqvist and Hahn-Hagerdal 2000a), and bioabatement (Nichols et al. 2008; Wierckx et al. 2010).

The latter approach to remove fermentation inhibitors in lignocellulosic hydrolysate using whole cells was also the focus of study of Wierckx et al. (2010). In this study, the bacterium *Cupriavidus basilensis* HMF14 was identified as a promising detoxification bacterium, as it consumes HMF, furfural, acetic acid, and a wide

range of phenolics while at the same time being unable to consume sugars. A subsequent study identified the genes responsible for HMF and furfural degradation and characterized the biochemical pathway by which these toxic compounds are degraded (Koopman et al. 2010b). This characterization unveiled the HmfH oxidoreductase, which showed great promise as a catalyst for the conversion of HMF into 2,5-furandicarboxylic acid (FDCA).

1.1 Potential of Bio-Based 2,5-Furandicarboxylic Acid

In the landmark study by the US Department of Energy (Werpy et al. 2004), FDCA was identified as one of the top value-added chemicals from biomass, which was confirmed in a follow-up paper revising the famous "top 10" list (Bozell and Petersen 2010). FDCA's main potential lies in its structural analogy to terephthalic acid, and it has been heralded as a bio-based alternative to this oil-based bulk chemical (de Jong et al. 2012; Koopman et al. 2010a). It can be used for the production of polyesters such as polyethylene-, polypropylene-, and polybutylene-FDCA, which have similar—or even superior—properties to their terephthalate-based counterparts (de Jong et al. 2012; Gandini 2011; Moreau et al. 2004). Given the large market volume of these currently oil-based plastics [approximately 50 million tonnes per year for PET alone (de Jong et al. 2012)], the possible impact of FDCA can be very high. Especially for PET-based bottles and packaging materials [approximately 15 million tonnes per year (Eerhart et al. 2012)], there is a strong "green" customer demand, as illustrated by press releases by the Coca-Cola company and Danone. These companies have publicly stated the intent to develop and use FDCA-based PEF bottles, e.g., in collaboration with the company Avantium, which is developing a chemical FDCA production and polymerization process (Coca-Cola 2011; Avantium 2012). Besides PEF, numerous other applications have been proposed, including other polyesters, polyamides, and polyurethanes (de Jong et al. 2012; Gandini 2011; Moreau et al. 2004).

1.2 Chemical Production of 5-(Hydroxymethyl)furfural and 2,5-Furandicarboxylic Acid

Several chemical routes for the production of FDCA have been investigated, generally starting from fructose as a renewable substrate. Often, fructose is first converted into HMF by dehydration using mineral acids, metal salts, organic solvents, and high pressure and temperature (Carlini et al. 2005; Casanova et al. 2009). Direct one-pot conversions of fructose to FDCA have also been described (Kroger et al. 2000; Ribeiro and Schuchardt 2003). The rapid formation of humins and other by-products pose a significant hurdle to the development of an

efficient HMF production process, and intensive research effort is ongoing on this subject [for an excellent overview, see (van Putten et al. 2013)]. In fact, HMF is considered a "sleeping giant" in the sense that its economical production would facilitate a great advancement in the bio-based chemicals industry with a very wide range of high-impact applications (Bicker et al. 2003).

Many of the catalytic FDCA production processes involve high temperature, high pressure, metal salts, and organic solvents [reviewed by (van Putten et al. 2013)]. Some of these catalytic conversions show good promise, and the company Avantium has announced the start of a pilot plant for the production of FDCA and its methyl esters from fructose, via ethers of HMF, using a Co/Mn/Br catalyst (Munoz de Diego et al. 2011; van Putten et al. 2013). An energy and greenhouse gas balance study based on this chemical process, assuming a conversion efficiency from fructose to FDCA of 38–47 %, concluded that PEF production has the potential to reduce the nonrenewable energy use by approximately 40–50 %, while greenhouse gas emissions can be reduced by 45–55 % compared to the established PET process (Eerhart et al. 2012).

Despite these promising developments, chemical production of FDCA still often suffers from low selectivity, generating by-products such as 5-(hydroxymethyl) furoic acid (HMF acid), 2,5-formylfuroic acid (FFA), and 2,5-diformylfuran (DFF). This necessitates a greater energy and capital expenditure for product purification, since monocarboxylic acid impurities cause chain termination reactions in the intended polymer applications. Furthermore, the catalytic process is operated at high temperature and pressure, adding to the already significant energy expenditure.

2 Biological Conversion of HMF to FDCA

As an alternative to chemical conversion, bioconversion offers superior chemo-, regio-, and enantioselectivity, using nontoxic biocatalysts at ambient temperature and pressure while producing biodegradable waste (Thomas et al. 2002). However, biocatalysis requires the availability of a specific enzyme for each individual reaction. The same is true for FDCA, where indeed the lack of suitable enzymes was long considered the major technical barrier for the development of an efficient bioprocess (Werpy et al. 2004).

2.1 Biological Oxidation of 5-(Hydroxymethyl)furfural

Conversion of HMF to FDCA, whether chemically or biologically, involves at least three subsequent oxidation steps (Fig. 1). Two of these steps concern the oxidation of an aldehyde moiety, which is in fact a very common phenomenon. Aldehydes in general are highly toxic to living cells, as they cause extensive damage to proteins, nucleic acids, and organelles through the formation of reactive oxygen species

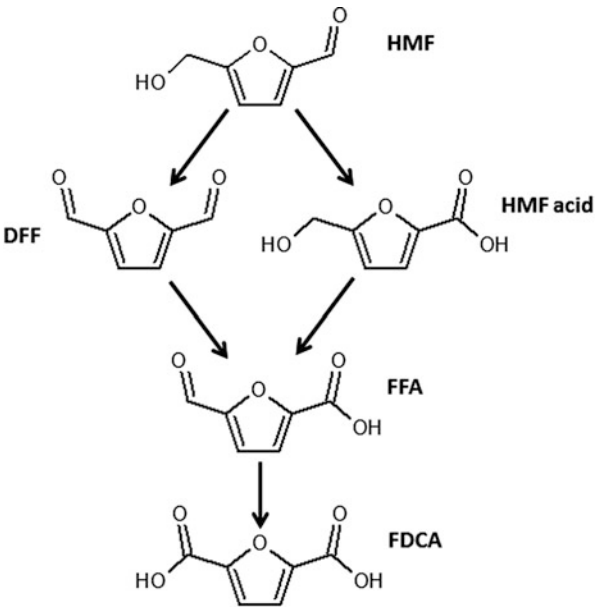

Fig. 1 Theoretical oxidation steps involved in the conversion of 5-(hydroxymethyl)furfural (HMF) into 2,5-furandicarboxylic acid (FDCA). Intermediates include 2,5-diformylfuran (DFF), 5-(hydroxymethyl) furoic acid (HMF acid), and 5-formylfuroic acid (FFA)

(Zaldivar et al. 1999; Feron et al. 1991). Besides this general mode of toxicity, furanic aldehydes also inhibit essential enzymes in primary metabolism (Modig et al. 2002). Because of this, many organisms have evolved 'generic' aldehyde dehydrogenases, which convert these aldehydes to their less toxic alcohol or acid forms (Wierckx et al. 2011). In this context, conversion of HMF to HMF acid has been observed in, among others, *Pseudomonas putida* (Koopman et al. 2010a), *Cupriavidus basilensis* (Wierckx et al. 2010), *Acetobacter rancens*, *Serratia liquefaciens* (Mitsukura et al. 2004), *Aspergillus* sp. (Kimura et al. 2007) and *Ureibacillus thermosphaericus* (Okuda et al. 2008). Small amounts of FDCA or HMF acid can also be detected in the urine of humans and other higher eukaryotes, likely from the ingestion of HMF found in a wide variety of foodstuffs (Flaschenträger and Wahhab 1960; National Toxicology Program 2010).

2.2 *Enzymatic Catalysts for the Production of 2,5-Furandicarboxylic Acid*

In spite of the very common phenomenon of aldehyde detoxification, only a few HMF oxidizing enzymes have been identified and characterized. The first instance of enzymatic conversion of HMF into FDCA, to our knowledge, was described by van Deurzen et al. (1997), Hanke (2009) using a chloroperoxidase from *Caldariomyces fumago*. This enzyme was able to oxidize both the alcohol and aldehyde moieties of HMF, producing a mix of FDCA, HMF acid, and in some cases DFF. The oxidation requires stoichiometric quantities of hydrogen peroxide, which was fed to the

reaction at a limiting rate since even low concentrations of H_2O_2 inactivate the enzyme. This cell-free enzymatic system suffered from low conversion and selectivity, and the requirement for hydrogen peroxide decreased enzyme stability.

More recently, Koopman et al. described the oxidation of HMF to FDCA using a newly identified HMF/furfural oxidoreductase named HmfH (Koopman et al. 2010b). This enzyme was discovered as a key component in the biological degradation pathway of HMF in the Gram-negative bacterium *Cupriavidus basilensis* HMF14 (Wierckx et al. 2010). It is a member of the FAD-containing glucose-methanol-choline (GMC) oxidoreductase family. The HmfH enzyme oxidizes HMF, HMF acid, furfural, and furfuryl alcohol, indicating that it can act on both alcohol and aldehyde moieties. Contrary to the chloroperoxidase mentioned above, HmfH uses molecular oxygen (O_2) as an electron acceptor, producing H_2O_2. Other electron acceptors such as NADH or DCPIP/PMS are not used by the enzyme, indicating that it is a true oxidase.

3 Design and Physiological Aspects of a Whole-Cell Biocatalyst for 2,5-Furandicarboxylic Acid Production

A cell-free biocatalytic process using the HmfH enzyme is difficult to achieve. The stability of the enzyme is likely negatively affected by the reactive aldehyde group of the HMF substrate, the H_2O_2 by-product, and the stress of aeration (Zaldivar et al. 1999; Fernandez-Lafuente et al. 1998). Therefore, Koopman et al. developed a whole-cell biocatalyst for the conversion of HMF into FDCA (Koopman et al. 2010a). *Pseudomonas putida* S12 was chosen as a host because of its innate tolerance to chemical stressors and the presence of endogenous aldehyde dehydrogenases that oxidize HMF to its corresponding carboxylic acid. The membrane of the cell provides a barrier from the bulk of the highly reactive aldehyde substrate, and the H_2O_2 is converted by endogenous catalases. In addition, contrary to, e.g., *E. coli* (Boopathy et al. 1993), reduction of HMF to HMF alcohol is only transiently observed, and the HMF alcohol is quickly oxidized back to HMF acid (Koopman et al. 2010a).

In order to achieve FDCA production in *P. putida*, the *hmfH* gene from *C. basilensis* HMF14 gene was cloned into vector pJT'mcs, which has an RO1600 origin of replication and the constitutive *tac* promoter (Labes et al. 1990; Koopman et al. 2010b; Meijnen et al. 2008). Transformation of this expression vector yielded the initial FDCA production host *P. putida* S12_hmfH (Koopman et al. 2010a).

3.1 Optimization of the Whole-Cell FDCA Biocatalyst

The *P. putida* S12_hmfH biocatalyst was able to convert HMF into FDCA at an apparent yield of 97 %, although the actual yield is likely higher. Virtually no other conversion products were detected and the initial amount of HMF may have been

lowered due to adsorption to cells or formation of humins (Koopman et al. 2010a). The maximum rate of FDCA production (q_{FDCA}) of 0.5 mmol (g CDW)$^{-1}$ h^{-1} (Koopman et al. 2010a) was low compared to other *P. putida* biotransformation processes, such as the oxidation of styrene to (*S*)-styrene oxide at 3.6–6 mmol (g CDW)$^{-1}$ h^{-1} by *Pseudomonas* sp. VLB120ΔC (Park et al. 2007). In addition, the HMF acid formation rate ($q_{HMF\ acid}$) in *P. putida* S12_hmfH was approximately fivefold higher than the q_{FDCA}, indicating that productivity can be increased.

Given these observations and the fact that substrate and product transport across the membrane was excluded as a limiting factor for this strain (Koopman et al. 2010a), the rate-limiting factor likely is the activity of the HmfH enzyme. In order to increase this activity, the *hmfH* expression cassette was transferred to vector pBT'mcs, which has a pBBR1 origin of replication that is believed to lead to a higher plasmid copy number (Wierckx et al. 2012, 2013; Buch et al. 2010). This indeed led to an increased maximum q_{FDCA}, to the point where—presumably—substrate transport across the bacterial membrane became the rate-limiting factor.

This limitation was overcome by the expression of the major facilitator superfamily transporter HmfT1. The gene encoding this transporter (originally named *mfs1*) is associated with the HMF gene cluster of *C. basilensis* HMF14 and is believed to encode a transporter for HMF and/or its derivatives (Koopman et al. 2010b). *P. putida* strain S12_B38, which expresses both HmfH (high-level expression) and HmfT1 (low-level expression), has a greatly increased maximum q_{FDCA}. HMF acid accumulation in this strain is strongly decreased, indicating that the main function of HmfT1 is HMF acid uptake, although transport of other HMF derivatives cannot be excluded. Instead of HMF acid, strain S12_B38 transiently accumulates FFA in batch HMF conversions, suggesting that in this case the activity of the endogenous aldehyde dehydrogenases is falling short.

In order to overcome this bottleneck, the *adh* gene, which is associated with the HMF and/or furfural gene clusters of several HMF-degrading bacteria (Wierckx et al. 2011), was co-expressed with *hmfH* and *hmfT1*. The ADH enzyme likely is an NAD(P)-dependent aldehyde dehydrogenase, as it contains a putative NAD(P)-binding site and shows sequence similarity to known NAD(P) aldehyde dehydrogenases (Marchler-Bauer et al. 2011). The enzyme also shows 63 % sequence identity to PsfA from *P. putida* Fu1, which was associated with furfural metabolism (Nichols and Mertens 2008). *P. putida* strain S12_B97, expressing HmfH, ADH (high-level expression), and HmfT1 (low-level expression), converted HMF into FDCA at a high, constant rate with virtually no accumulation of intermediates (Wierckx et al. 2012). Although the maximum q_{FDCA} of this strain was slightly lower than that of the strain expressing only HmfH and HmfT1, the overall FDCA production rate was higher. This likely relates to different affinity constants of the "generic" aldehyde dehydrogenases for HMF and FFA. It appears that HMF is the preferred substrate, as with strain S12_B38 the conversion from FFA to FDCA proceeds at a low rate until HMF is almost depleted, upon which the q_{FDCA} increases dramatically since FFA is at that point the only remaining substrate (Wierckx et al. 2012).

3.2 Subcellular Localization of Enzymes

The in vitro activity of HmfH measured in the HMF-metabolizing *P. putida* S12_HMF (Koopman et al. 2010b) is relatively low (>0.5 μmol min^{-1} g CDW^{-1}; unpublished data) compared to the activity of "generic" HMF dehydrogenases of *P. putida* S12 (27 μmol min^{-1} g CDW^{-1}). Therefore, it is likely that these "generic" aldehyde dehydrogenases catalyze the majority of the aldehyde oxidations, whereas the HmfH mainly converts HMF acid to FFA. The in vitro activity of the generic aldehyde dehydrogenases was found to be dependent on phenazine methosulfate/2,6-dichlorophenol-indophenol (PMS/DCPIP), suggesting that the in vivo cofactor for these enzymes is pyrroloquinoline quinone (PQQ), although NAD^+ cannot be excluded as a cofactor due to the competing action of alcohol dehydrogenases (Koopman et al. 2010b). PQQ-dependent enzymes are located in the periplasmic space (Matsushita et al. 2002), and indeed a periplasmic aldehyde dehydrogenase would enable *P. putida* to detoxify aldehydes before they enter the cell. However, the heterologous HmfH is assumed to be expressed in the cytoplasm, indicating that different oxidation steps from HMF to FDCA take place in different cellular compartments. In the initial FDCA production strain S12_hmfH, oxidation of the aldehyde moieties of HMF and FFA likely proceeds in the periplasm, while oxidation of the alcohol moiety takes place in the cytoplasm. In the fully optimized *P. putida* S12_B97, co-expressing HmfH, ADH, and HmfT1, more oxidation steps likely occur in the cytoplasm, and transport of HMF and its derivatives across the cytoplasmic membrane is facilitated by HmfT1 (Fig. 2).

3.3 Cofactor Regeneration

The oxidation of one molecule of HMF to one molecule of FDCA has a net yield of two reducing equivalents, either in the form of NADH or PQQH. These can be lumped to NAD(P)H since they yield the same amount of energy in *P. putida* (Wierckx et al. 2009; Hardy et al. 1993). Given this, the maximum NAD(P)H generation rate can be estimated from q_{FDCA}, yielding approximately 1 mmol $(g\ CDW)^{-1}\ h^{-1}$ for *P. putida* S12_hmfH and 3 mmol $(g\ CDW)^{-1}\ h^{-1}$ for *P. putida* S12_B38 during fed-batch FDCA production. The maintenance requirement of *P. putida* is approximately 0.8 mmol NADH $(g\ CDW)^{-1}\ h^{-1}$ in mineral glucose medium (unstressed) and 2.7 mmol NADH $(g\ CDW)^{-1}\ h^{-1}$ under extreme solvent stress in a second phase of toluene (Isken et al. 1999; Ebert et al. 2011). Given the harsh conditions associated with FDCA production (aldehyde toxicity, H_2O_2 formation, osmotic stress) and the fact that the maximum growth rate is greatly reduced (Koopman et al. 2010a), it may be hypothesized that the maintenance demand during FDCA production is near the solvent-stressed value of 2.7 mmol NADH $(g\ CDW)^{-1}\ h^{-1}$. Thus, especially in optimized strains, high-rate FDCA

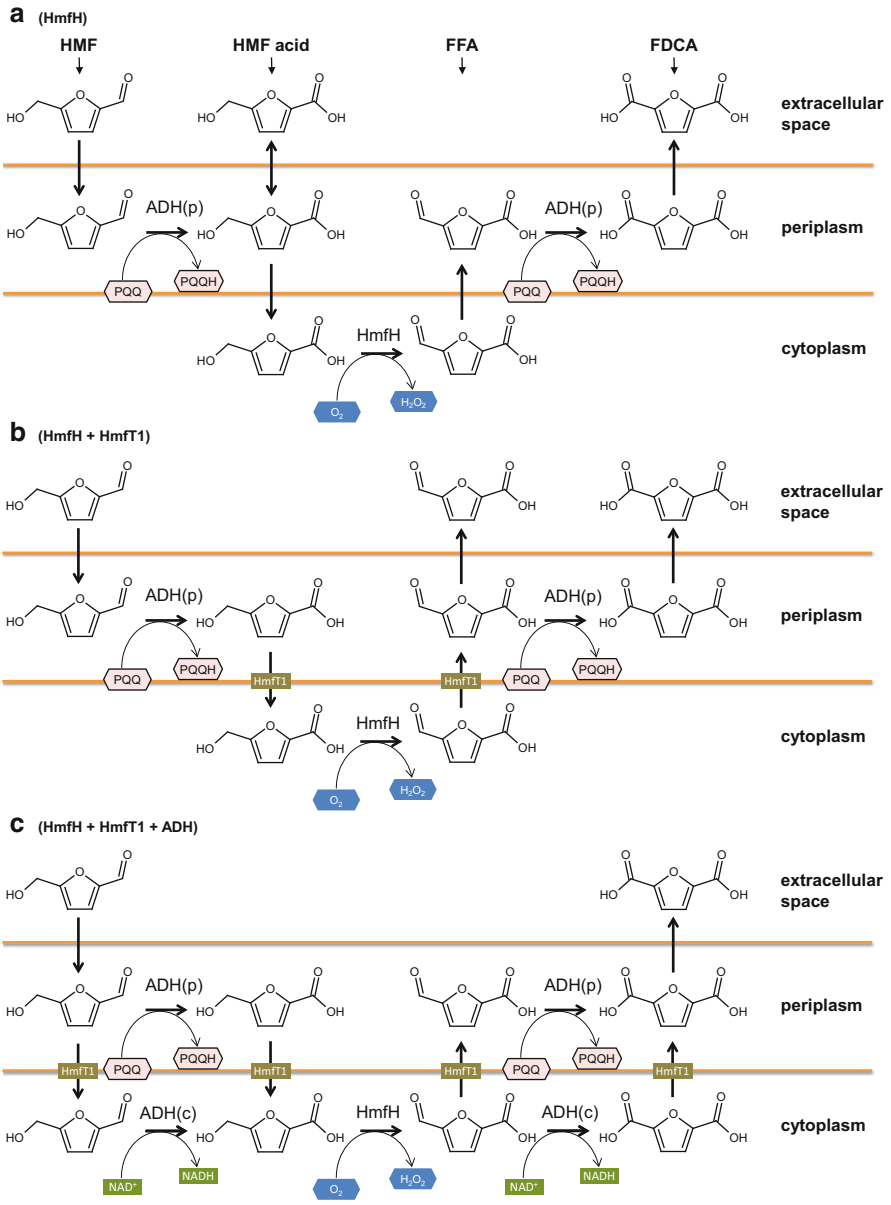

Fig. 2 Putative subcellular localization of HMF oxidizing reactions in *P. putida* strains S12_HmfH (**a**), S12_B38 (**b**), and S12_B97 (**c**). ADH: aldehyde dehydrogenase from either *C. basilensis* HMF14 (**c**) or native to *P. putida* S12 (p). *HMF* 5-(hydroxymethyl)furfural, *HMF acid* 5-(hydroxymethyl)furoic acid, *FFA* 5-formylfuroic acid, *FDCA* 2,5-furandicarboxylic acid

production would result in an excess of NADH and PQQH, which need to be re-oxidized in order to achieve redox homeostasis.

The electron transport chain (ETC) is known to be an important factor for maintaining redox balance, especially for PQQH which transfers its electron directly to intermediate components of the ETC (Blank et al. 2010). *P. putida* possesses a highly flexible ETC with several terminal oxidases of varying proton pumping efficiency (Ugidos et al. 2008), making it likely that a significant part of the reducing equivalents generated by FDCA production are regenerated here. In addition, FDCA production in *P. putida* S12_hmfH was most efficient in growing cells [i.e., in the presence of both a carbon and nitrogen source (Koopman et al. 2010a)], indicating that biomass formation may also be used as a redox sink.

Toward the end of an FDCA production culture, q_{FDCA} decreases. At the same time, the maintenance demand will be highest during this phase, possibly even exceeding values observed during organic solvent stress. This would lead to a situation where FDCA production alone can no longer satisfy the maintenance demand. Thus, co-feeding of an energy supplying carbon source is more critical at this stage in order to maintain cell viability and to establish complete conversion of HMF to FDCA, as was also observed during fed-batch FDCA production with *P. putida* S12_hmfH (Koopman et al. 2010a).

3.4 Carbon and Energy Source Requirement

Although FDCA production should, at least for part of the process, be able to satisfy the cellular maintenance demand, FDCA production cultures using whole-cell *P. putida* biocatalysts are generally co-fed with an additional carbon and energy source. Although glucose can be used for this, it is converted by *P. putida* S12 into gluconate and 2-ketogluconate (Wierckx et al. 2009), which may hamper pH and process control. Therefore glycerol is often used, which simplifies the process and is also a good substrate for *P. putida* S12 (Verhoef 2010). Although co-feeding increased q_{FDCA} and biocatalyst stability, it should be minimized from an economical point of view. In the first-generation biocatalyst *P. putida* S12_hmfH, the rate of glycerol feeding exceeded the HMF feed rate, even though the glycerol feed rate was minimized during the production phase to the point that growth no longer occurred (Koopman et al. 2010a). With the subsequently optimized strains, the applied HMF feed rate can be significantly higher, which leads to a significantly improved process efficiency when the glycerol feed rate is kept in the same range (Fig. 3). However, even with a highly optimized biocatalyst, substantial amounts of glycerol are needed, mainly for the formation of sufficient biomass.

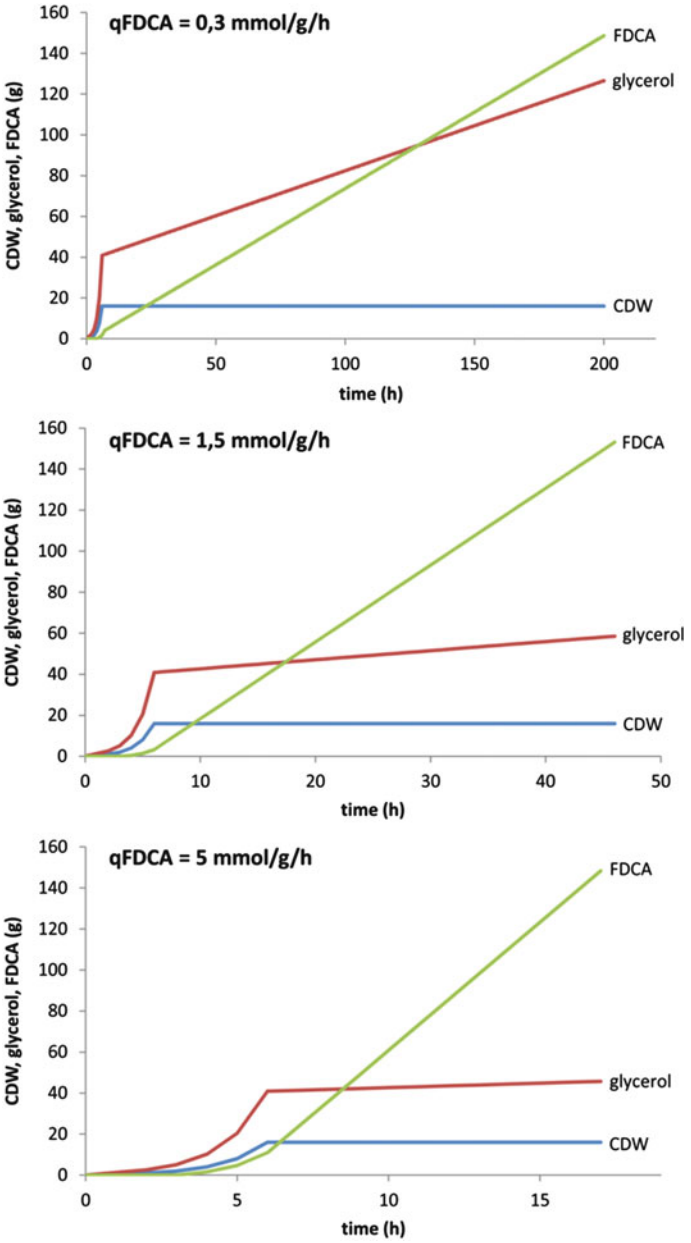

Fig. 3 Theoretical estimation of the effect of improvement of the FDCA production rate (q_{FDCA}) on the relative glycerol consumption. The process is based on an initial biomass growth phase from 0.5 to 16 (g CDW) l^{-1} with a doubling time of 1 h, after which the glycerol feed is reduced to the maintenance requirement. FDCA production is started at 4 (g CDW) l^{-1} and is assumed to proceed at a constant specific rate as indicated in the graphs. Calculations assume a biomass yield of 27.8 g CDW (mmol glycerol)$^{-1}$ and a maintenance requirement of 0.3 mmol glycerol (g CDW)$^{-1}$ h^{-1}

4 FDCA Production from HMF Using Whole-Cell *P. putida* Biocatalysts

4.1 Batch Conversion of HMF into FDCA

The efficiency of FDCA production in *P. putida* S12 biocatalysts was initially assessed in batch cultures. An initial biomass-forming phase was crucial in this setup, as higher biomass concentrations enable the rapid conversion of the toxic HMF aldehyde. This use of high cell densities to overcome toxicity of inhibitors such as HMF is well known (Wierckx et al. 2010; Yu and Stahl 2008; Palmqvist and Hahn-Hagerdal 2000a), and concentrations of up to 50 mM HMF can be completely converted into FDCA using approximately 15 g CDW l^{-1} (Koopman et al. 2010a). However, higher substrate concentrations severely inhibit the biocatalyst and these shake-flask batch conversions are characterized by the transient accumulation of intermediate oxidation products and the requirement for high concentrations of phosphate buffer to prevent acidification of the medium. Although they are excellently suited for physiological characterization of the biocatalyst, for efficient production of higher titers of FDCA using whole-cell *P. putida* biocatalysts, a pH-controlled process with a controlled HMF feed is needed.

4.2 Fed-Batch Conversion of HMF into FDCA

For FDCA production, *P. putida* can be first grown up to a certain density in a batch culture. In the subsequent production phase, both HMF and nutrients (carbon and nitrogen sources) are fed into the fermenter. In the production phase, the culture should be frequently monitored in order to prevent HMF "overfeeding," while the HMF feed is maintained as high as possible to ensure maximum productivity. With the first-generation *P. putida* S12_hmfH strain, HMF could be fed faster than the maximum q_{FDCA}, which would typically lead to HMF acid accumulation. Transient accumulation of HMF acid is of limited concern due to its low toxicity. However, the time needed for complete conversion of this intermediate adds significantly to the overall biotransformation time. The last traces of HMF acid (±5 mM) were converted very slowly with strain S12_hmfH, likely due to transport limitation across the cell membrane (Koopman et al. 2010a; Wierckx et al. 2012).

This problem was solved by co-expressing the HmfT1 transporter, eliminating the accumulation of HMF acid and resulting in rapid metabolism of low concentrations of HMF acid (Wierckx et al. 2012). However, in these strains the risk of HMF "overfeeding" is much more prominent, as this immediately leads to the accumulation of toxic aldehydes. Such problems can be avoided by frequent

sampling and offline HPLC analysis for HMF and its derivatives and adaptation of the HMF feed as required (Koopman et al. 2010a).

With such a fed-batch approach, *P. putida* strain S12_B38, which expresses both HmfH (high expression) and HmfT1 (low expression), was reported to produce over 100 g l^{-1} FDCA in 90 h (Wierckx et al. 2012).

5 Downstream Processing

Since FDCA is mostly intended to be used for polymer applications, the final product must be of extremely high purity (Okada et al. 1999; Storbeck and Ballauff 1993), making efficient separation of the FDCA from the fermentation broth and subsequent purification of high importance. Generally, the whole-cell biocatalyst is first removed from the fermenter broth by centrifugation, filtration, or flocculation followed by settling. Further removal of proteins and other high molecular weight biopolymers can be achieved by boiling and centrifugation. Given the low solubility of FDCA in water at pH 0.5 ($\pm$0.4 g l^{-1} at 4 °C) in relation to the product titer (>30 g l^{-1}), acid precipitation is considered excellently suited to recover the majority of the product from the clarified fermentation broth (Koopman et al. 2010a). When the production process is stopped at a relatively low titer (30 g l^{-1}), the dried precipitate will have a purity of approximately 90 %. However, if the biocatalyst is pushed to very high titers, this purity can drop down to <70 %, likely as a result of the presence of cell lysis products. Further purification of the precipitated FDCA to 99.4 % can be achieved by solvent extraction with tetrahydrofuran (THF), which was found to selectively dissolve FDCA up to 18 g l^{-1} (Koopman et al. 2010a).

6 Outlook

Although whole-cell FDCA production from HMF has quickly developed into an efficient lab-scale process, several hurdles will need to be taken before it can be produced at an industrial scale. The main challenge for an economically viable process will likely be upstream and downstream of the biocatalysis. Currently the prices of kg amounts of HMF and FDCA are at a similar level, making purified HMF an unlikely substrate. Considerable efforts will be needed to awake the "sleeping giant" and integrate the HMF production from biomass-derived substrates into the FDCA process. Significant efficiency gains can be expected from the omission of intermediate purification steps and the microbial utilization of unconverted sugar streams from HMF production. This can greatly reduce the cost of the substrate, especially if the process is based on lignocellulosic

hydrolysate. However, the latter substrate could provide severe difficulties for subsequent product purification.

At the downstream side, the method of THF extraction is unlikely to be applicable on a large scale due to the low solubility of FDCA in this solvent. Well-established methods for the purification of, e.g., bio-based citric, glutamic, and succinic acid may serve as a starting point for optimization, although the specific physical properties of FDCA will eventually require a tailor-made purification process.

Besides these process optimizations, there is also room for improvement of the biocatalyst itself. An obvious step is the further overexpression of the *hmfH* and *adh* genes. Although the use of a vector with a higher copy number did provide an improvement, further enhancement of the expression level of these two key enzymes can be achieved through the use of promoter systems with higher activity. Furthermore, integration of the constructs into the genome of *P. putida* would increase strain stability and abolish the need for antibiotic selection, which is generally undesirable on a large scale. Further bioprospecting of HMF-degrading organisms may also lead to the discovery of new enzymatic tools for more efficient production of FDCA.

It is clear that quite some development is needed to replace oil-based terephthalate with bio-based FDCA. Still, the reduction of fossil reserves needed for packaging materials manufacture and the concurrent potential impact on the environment make such an effort more than worthwhile. We therefore aim to further develop this process to make industrial bioproduction of FDCA a reality in the near future.

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Microorganisms for the Production of Lactic Acid and Organic Lactates

Christine Idler, Joachim Venus, and Birgit Kamm

Contents

Abstract Biorefineries consider lactic acid as one of the most promising platform chemicals which are being extensively used in a wide range of food and nonfood applications. Since lactic acid is produced via biotechnological processes, the microbial strains are in the focus of interest, besides all the other aspects of raw materials, fermentation mode, etc.

Microorganisms, which are able to produce lactic acid and organic lactates, are systematically classified and morphologically and biochemically characterized, and their different metabolic pathways for the formation of various lactic acid

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enantiomers are described in detail. The genera *Lactobacillus* and *Bifidobacterium* are regarded as well as the order of the Bacillales. In addition, the important individual yeasts, moulds and other bacteria were also characterized.

The present review work is summarized on the fermentation systems used for the biotechnological production, the various raw materials and applications of lactic acid and organic lactates. Future developments in this area with respect to the strain selection and modifications, genetic-engineering approaches, carbohydrate sources and their pretreatment, fermentation techniques and the downstream processing options are discussed.

Abbreviation

G + C	Guanine + cytosine
LA	Lactic acid
LAB	Lactic acid bacteria
PLA	Polylactic acid
ssp.	Subspecies

1 Introduction

The biotechnological production of lactic acid (2-hydroxypropionic acid) as an example of a platform chemical for the subsequent processing (e.g. into PLA) is carried out in technical reactors by using a suitable strain. LA can be produced by several microorganisms classified into bacteria, fungi, yeast, cyanobacteria and algae (Abdel-Rahman et al. 2013; Thongchul 2013). Besides the wide group of *Lactobacillus* (Antonio et al. 1996; Hofvendahl and Hahn-Hägerdahl 1997; Berry et al. 1999; Fu and Mathews 1999; Kwon et al. 2001), other bacteria like *Bacillus* (Payot et al. 1999; Danner et al. 2002; Patel et al. 2004), *Enterococcus* (Walczak et al. 2012), *Lactococcus* (Ramchandran et al. 2012), *Pediococcus* (Zhao et al. 2013b), *Streptococcus* (Tang et al. 2013) and filamentous fungi (Martak et al. 2003), especially *Rhizopus oryzae* (Yin et al. 1997; Bai et al. 2004), were also used as production strains. These microorganisms convert easily monosaccharides like glucose or fructose into cell mass and LA. LA formation and cell growth are closely coupled in LA fermentation (Zacharof and Lovitt 2013). An overview about the utilization of different renewable resources for LA fermentation, other microorganisms and yields depending on several process parameters was given by Hofvendahl and Hahn-Hägerdal (2000) and Castillo Martinez et al. (2013).

Whereas the fermentation of glucose can be carried out efficiently, the bioconversion of the pentose fraction out of lignocellulosic feedstocks and residues presents a challenge. A lot of attention has therefore been focused on genetically engineering strains that can efficiently utilize both glucose and pentose and convert them to useful compounds. The metabolic engineering objectives so far have

focused on higher yields, productivities and expanding the substrate and product spectra (Aristidou and Penttilä 2000; Hua et al. 2006; Singh et al. 2006; Ilmen et al. 2007; Adler et al. 2012).

For the industrial production of L-(+)-LA, it is necessary to provide cheap carbon sources that can be easily metabolized by lactic acid bacteria (LAB) and to obtain the optimal conditions of fermentation with higher yields and production rates (John et al. 2007).

The different microbes have achieved one or more improvements over the others, such as a broader substrate range, improved yield and productivity, reduction of nutritional requirements or improved optical purity of LA (Abdel-Rahman et al. 2013). In view of the above-mentioned several complex substrates, also the use of mixed cultures in fermentation may provide useful combinations of metabolic pathways for the utilization of complex raw materials containing a mixture of carbohydrates (Cui et al. 2011; Trontel et al. 2011; Secchi et al. 2012). Several genetic-engineering approaches have been exploited in order to improve performance, LA yield and optical purity by various microbial producers (Nagamori et al. 2013; Wu et al. 2013; Zhao et al. 2013a). An extensive review by Okano et al. (2010) provides a broad collection of genetically engineered microorganisms for LA production including their characteristics and applicability for fermentation processes, respectively.

2 Lactic Acid-Forming Bacteria

Important LA-forming bacteria include the genera *Lactobacillus* and *Bifidobacteria*. Also the genera *Bacillus*, *Lactococcus*, *Streptococcus*, *Pediococcus* and *Enterococcus* are able to produce LA. There are also reports about the LA fermentation by some yeast and fungi.

2.1 The Genus Lactobacillus

Lactobacillus (*L.*) is a very heterogeneous genus, comprising species with a large diversity of phenotypic, biochemical and physiological features. More than 70 species are recognized, and all are able to convert carbohydrates into LA. The most important LA-forming bacteria belong to this genus. These include *L. acidophilus, L. brevis, L. casei, L. delbrueckii, L. fermentum, L. helveticus, L. plantarum, L. paracasei* and *L. rhamnosus*.

Table 1 Characteristics of the genus *Lactobacillus*

Classification[a]	Kingdom	Bacteria
	Phylum	Firmicutes
	Class	Bacilli
	Order	Lactobacillales
	Family	Lactobacillaceae
	Genus	*Lactobacillus*
Type species	*L. delbrueckii*	
Habitats	Dairy products, silage, water, soil, sewage, part of the normal flora of human and many animals	
Characteristics	Gram positive, non-spore forming, catalase negative, motile or immotile, facultative anaerobic	
Morphology	Long and slender or bent rods or coccobacilli common in chains Motile by peritrichous flagella	
Metabolism	Obligately saccharolytic, end products: lactate, acetate, formate, succinate, ethanol, CO_2	
Fermentation types	Obligate homofermentative: *L. acidophilus, L. delbrueckii* Obligate heterofermentative: *L. brevis, L. fermentum* Facultative heterofermentative: *L. casei, L. rhamnosus*	
Growth conditions	$T_{opt.}$: 30–40 °C (2–53 °C) $pH_{opt.}$: 5.5–6.2, tolerant < 4 Requirements: individually various complex nutritional requirements for peptides, amino acids, nucleotides, vitamins and fermentable carbohydrates	
Pathogenicity	No or in rare case, e.g. *L. rhamnosus,* biosafety level 2[b]	
DNA GC content	32.5–55 mol %	
Remarks	Than other LAB more resistant to acid conditions	

[a]Hammes and Hertel (2009)
[b]Directive 89/391/EEC (2000)

2.1.1 Short Characteristics of the Genus *Lactobacillus*

The genus can be divided into three subgroups based on their type of fermentation: obligate homofermentative, obligate heterofermentative and facultative heterofermentative. Important features of this genus are summarized in Table 1.

2.1.2 Carbohydrate Fermentation of Lactobacilli

The metabolism of carbohydrate utilization depends both on the kind of the sugar (e.g. hexoses, pentoses) and from the type of fermentation by the LAB. General, the fermentation types differ in the utilization of hexoses and pentoses.

Fermentation of Hexoses

Obligate Homofermentative LAB

In principle the obligate homofermentative LAB converted hexoses to lactate by the Embden-Meyerhof-Parnas (EMP) glycolytic pathway (Fig. 1) (Wood 1961; Kandler 1983; von Wright and Axelsson 2012).

Glucose is first broken down in glycolysis to pyruvate. Pyruvate is reduced by the enzyme lactate dehydrogenase to lactate, which is present under physiological conditions in dissociated lactate ions and protons. During the glycolysis of glucose or fructose per molecule, two molecules lactate and ATP are formed, so that the sum of the equation homofermentative LA fermentation is

$$C_6H_{12}O_6 + 2\,ADP \rightarrow 2\,C_3H_6O_3 + 2\,ATP$$

Pentoses and gluconate were not fermented by this pathway because of lack of enzyme phosphoketolase. This type of fermentation includes some species of the genus *Lactobacillus*. The important LA producers in this genus are *L. acidophilus* and *L. delbrueckii* (Hofvendahl and Hahn-Hägerdal 2000; Kwon et al. 2001).

Obligate Heterofermentative LAB

The pathway of obligate heterofermentative LA fermentation is formed by LAB, which had a lack of enzyme aldolase. This enzyme is required for the glycolysis of fructose-1,6-bisphosphate into the two phosphotriose dihydroxyacetone and glyceraldehyde. This type of fermentation includes organisms of the genera *Leuconostoc, Weissella* and *Oenococcus* as well as some species of the genus *Lactobacillus* (Hammes et al. 1991; Hammes and Vogel 1995). Important obligate heterofermentative LA producers are *L. brevis, L. fermentum* and *L. reuteri*. These bacteria can degrade hexoses in the phosphogluconate pathway (Fig. 2) to lactate, ethanol and CO_2 or furthermore to acetate.

During the fermentation of glucose per molecule, one molecule lactate and ATP are formed, so that the sum of the equation of heterofermentative LA fermentation is

$$C_6H_{12}O_6 + ADP \rightarrow C_3H_6O_3 + C_2H_5OH + CO_2 + ATP$$

Other hexoses (such as mannose or fructose) enter the pathway as either glucose-6-phosphate or fructose-6-phosphate. Fructose, however, is reduced not only to lactate and CO_2 but also to mannitol and acetate:

$$3\,C_6H_{12}O_6 + H_2O + 2\,ADP \rightarrow C_3H_6O_3 + 2\,C_6H_{14}O_6 + C_2H_4O_2 + CO_2 + 2\,ATP$$

For the fermentation of galactose, there are two different pathways, depending on the form it enters in the cells (von Wright and Axelsson 2012). If galactose enters

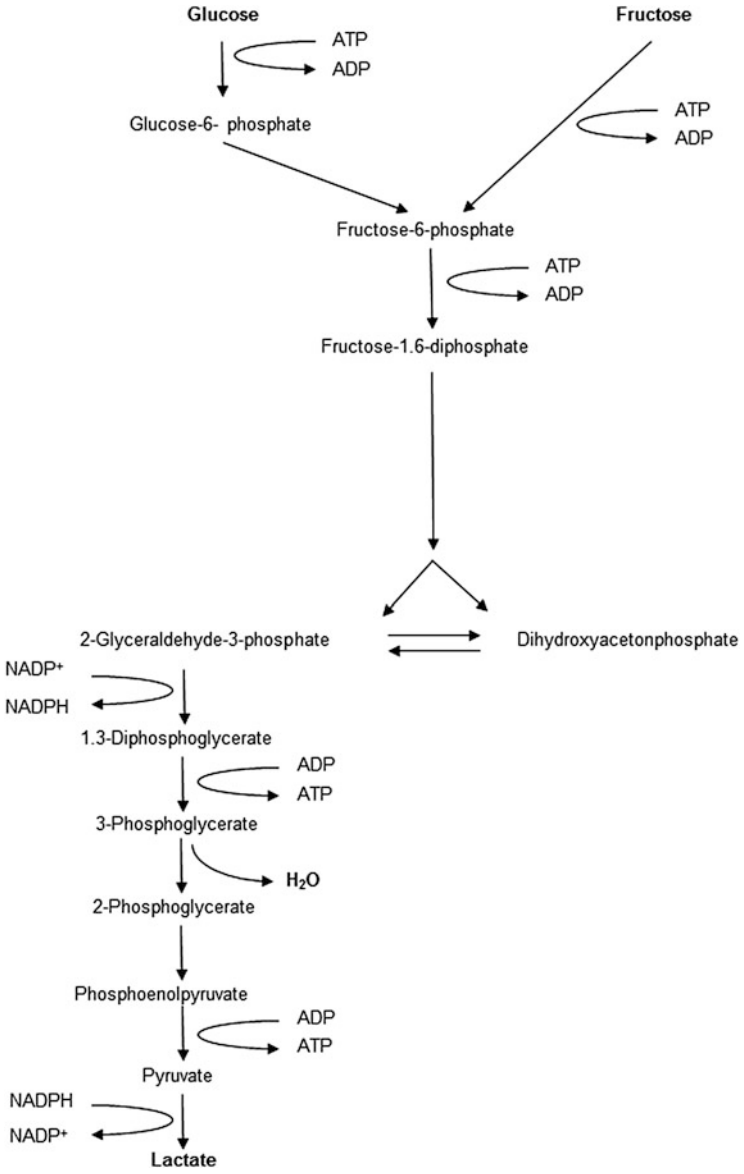

Fig. 1 Fermentation of hexoses in obligate homofermentative LAB, EMP pathway

the cells as galactose-6-phosphate, it will ferment also to pyruvate however via the tagatose-6-phosphate pathway (Bisset and Anderson 1974) (Fig. 3a). As a free galactose, imported in the cells by a specific permease, it will ferment via glycolysis to pyruvate by the so-called Leloir pathway (Kandler 1983) (Fig. 3b).

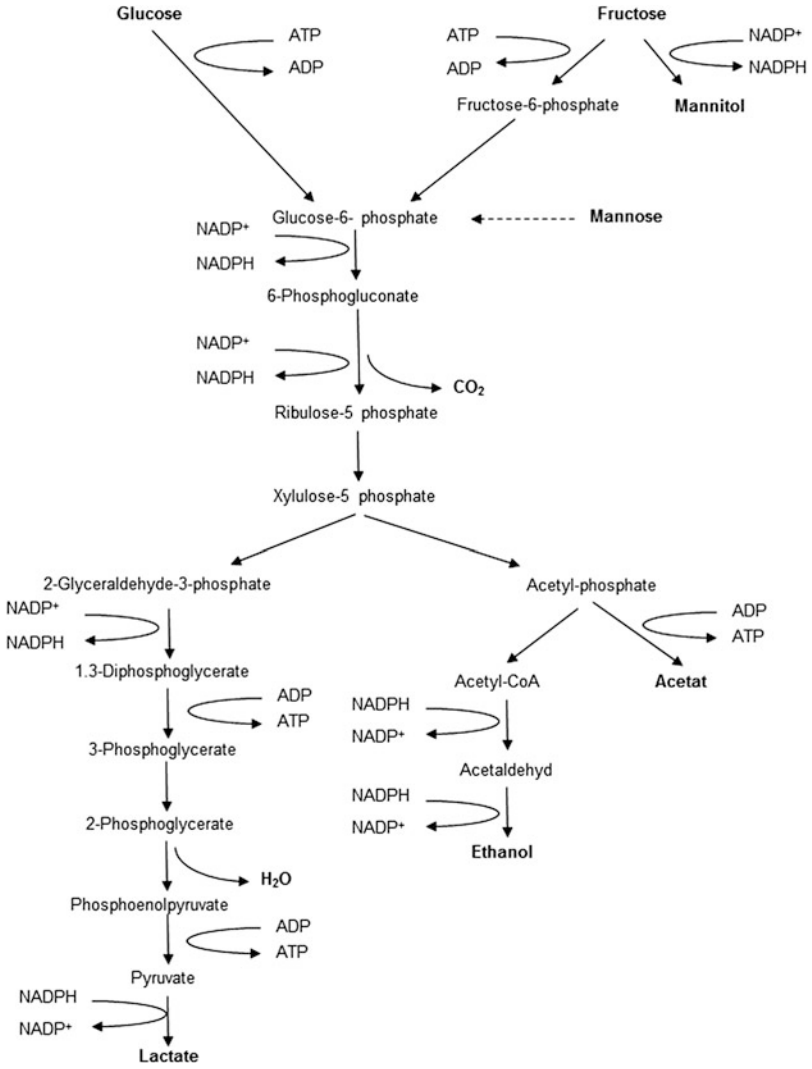

Fig. 2 Fermentation of hexoses in heterofermentative LAB, phosphogluconate pathway

Fermentation of Pentoses

Many LAB are able to ferment pentoses. They can only ferment heterofermentatively by entering the phosphogluconate pathway as either ribulose-5 phosphate or xylulose-5 phosphate (Kandler 1983) (Fig. 4). Pentoses (such as arabinose, ribose, xylose) are converted into lactate and acetate; CO_2 is not produced. The sum of the equation is

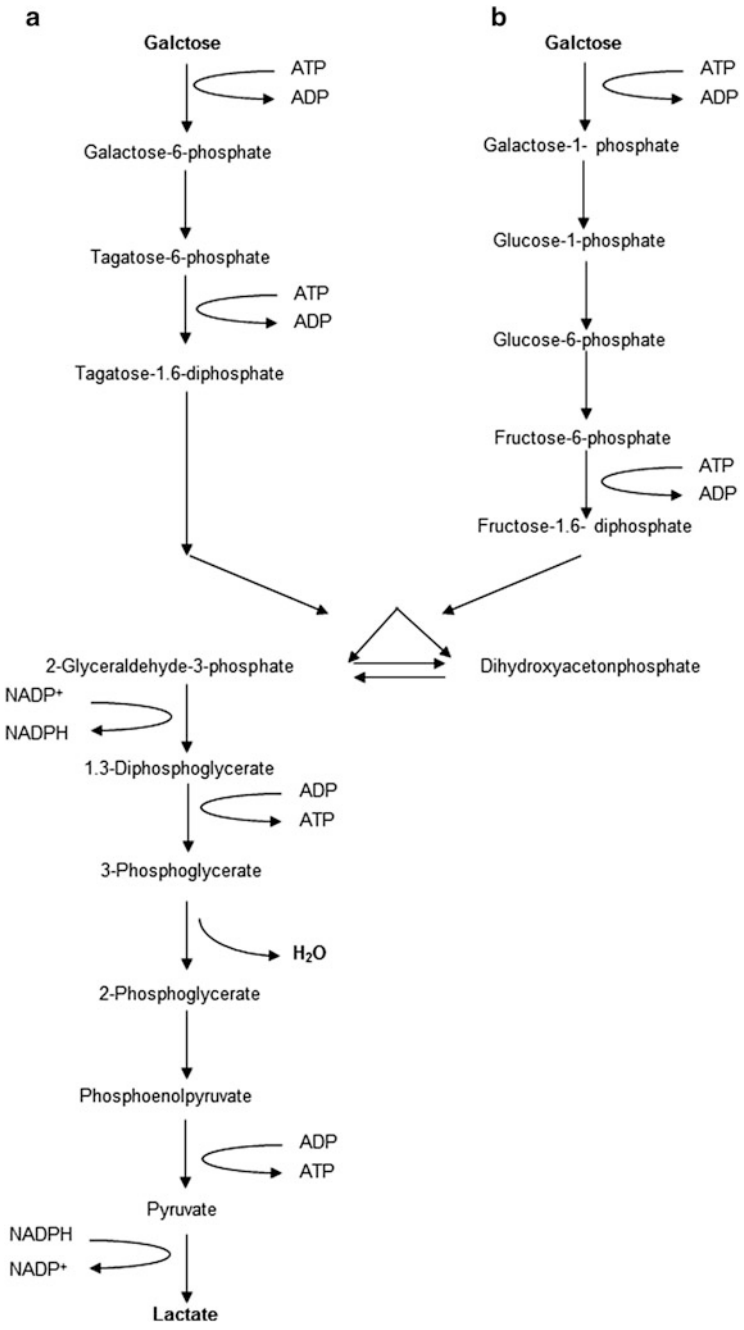

Fig. 3 Fermentation of galactose in LAB, Leloir pathway

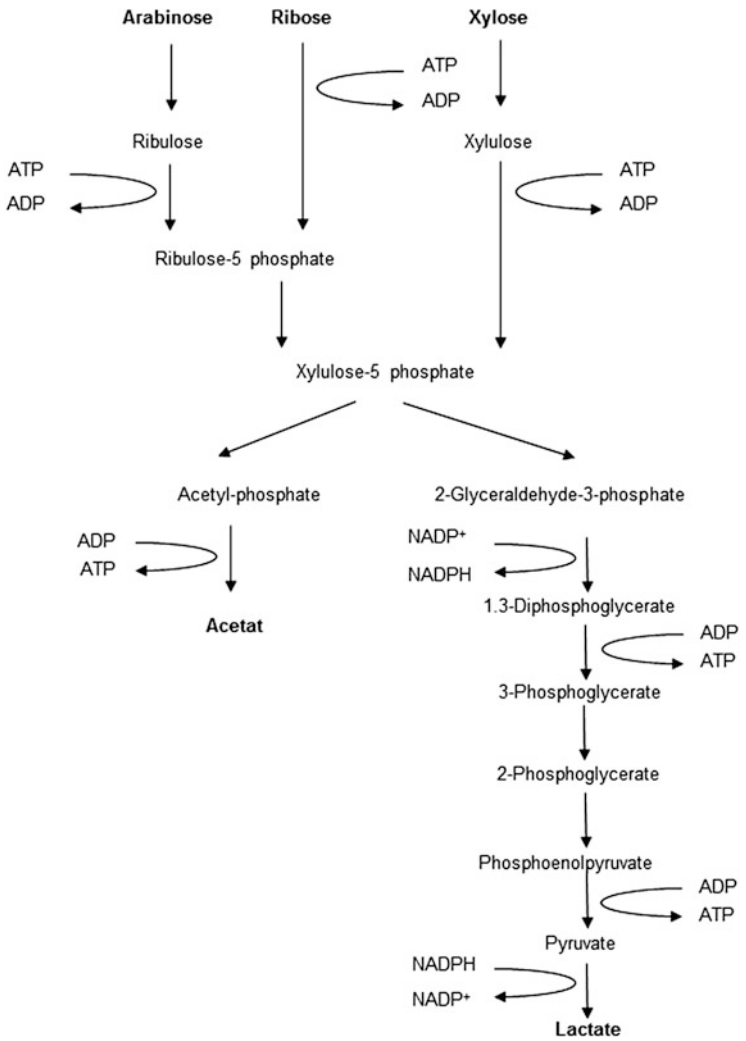

Fig. 4 Fermentation of various pentoses in LAB

$$C_5H_{10}O_5 + 2\,ADP \rightarrow C_3H_6O_3 + C_2H_4O_2 + 2\,ATP$$

However, there are reports of homofermentative fermentation of pentoses by engineered strains of *L. plantarum* (Okano et al. 2009a, b).

Facultative Heterofermentative LAB

LAB, which can ferment hexoses and pentoses, belong to the group of facultative heterofermentative LAB. Hexoses are fermented by the EMP glycolytic pathway to

lactate. Under glucose limitation some species can produce also ethanol, acetic acid and formic acid (Hammes and Hertel 2009). Pentoses enter this pathway and are fermented to LA and acetic acid. The important LA producers belong to this fermentation type, e.g. *L. casei, L. plantarum* and *L. paracasei*.

Fermentation of Other Important Carbohydrates

For the production of LA, the most important carbohydrates are the disaccharides lactose, maltose and sucrose. In principle they are split enzymatically into their monosaccharide, and then they enter the various pathways.

Lactose can enter the cells in two ways, either by means of a specific permease or as lactose-6-phosphate (von Wright and Axelsson 2012). In the first case, lactose is split into glucose and galactose, which can enter the major fermentation pathway (e.g. *Streptococcus thermophilus*). In the second case, lactose-6-phosphate is cleaved to glucose and galactose-6-phosphate. While glucose is processed by the glycolytic pathway, the galactose-6-phosphate enters the tagatose-6-phosphate pathway (e.g. *Lactococcus lactic*). In some cases both systems can coexist (Wood and Holzapfel 1995; von Wright and Axelsson 2012).

The fermentation of maltose is known; in lactococci the permease system is active (Sjöberg and Hahn-Hägerdahl 1989), while in various strains of *L. sanfranciscensis*, maltose is converted to glucose-1-phosphate and glucose (von Wright and Axelsson 2012).

In general, sucrose enters the cells also by a specific permease system, and it is split into glucose and fructose. Also it was reported that lactococci can convert lactose into glucose-6-phosphate and fructose (von Wright and Axelsson 2012).

Starch is fermented only by very few homofermentative species such as *L. amylophilus* (Altaf et al. 2006; Vishnu et al. 2006), *L. manihotivorans* (Morlon-Guyot et al. 1998; Ohkouchi and Inoue 2006) and *L. amylovorus* (Zhang and Cheryan 1991; Hammes and Hertel 2009). However, of these species *L. amylophilus* and *L. manihotivorans* are important for the production of LA (Altaf et al. 2007; Yen and Kang 2010; Son and Kwon 2013).

It is reported that the strain *Lactobacillus plantarum SW14* has a potential for LA production directly from cassava starch under laboratory conditions (Bomrungnok et al. 2012). Also *Enterococcus faecium* was already described for the direct fermentation of starch containing feedstocks (Shibata et al. 2007; Nolasco-Hipolito et al. 2012).

2.1.3 Enantiomers of LA

An asymmetric C-atom LA exists in two enantiomeric forms L (+) and D (−) and in a racemic form (DL). The stereoisomeric composition of the formed LA by the various species of lactobacilli is enzymatically determined. The configuration of the LA L (+) or D (−) depends on the stereospecificity of the lactate dehydrogenase in

the cells. Racemate (DL) formed either when D-(−)- and L-(+)-dehydrogenases are present in the same cells or when an inducible lactate racemate reacts with a constitutive L-(+)-lactate dehydrogenase (Hammes and Hertel 2009).

However Setter and Stetter and Kandler (1973) reported that the D-(−)-lactate formers produce D-(−)-lactate exclusively, whereas all L-(+)-lactate formers always produce a few percent of the other isomer. This is caused by the presence of an NAD-dependent D-lactate dehydrogenase of very low activity.

2.1.4 Characteristics of the Most Important Lactic Acid Producers

For the selection of strains for the LA production, the fermentation type, the fermented carbohydrates and the temperature of growth are very important. These typical facts for the most used LA producers in the genus *Lactobacillus* are summarized separately in Tables 2, 3 and 4 after the formation of the various LA enantiomer.

2.2 The Genus Bifidobacterium

There are many references of the use of bifidobacteria for LA production. Many investigations have shown that bifidobacteria promote health preferably via their application in the food industry (Shene et al. 2005; Popa and Ustunol 2011). From that perspective the interest has been focused more on the fermentation performance in combination with typical carbohydrates containing foodstuff (Buruleanu et al. 2011; Watson et al. 2012) than an industrial LA production (Li et al. 2008). In the genus *Bifidobacterium* (*B.*), *B. adolescentis, B. animalis, B. bifidum, B. breve, B. longum* and *B. thermophilum* are important LA producers.

2.2.1 Short Characteristics of the Genus *Bifidobacterium*

Important features of this genus are summarized in Table 5.

2.2.2 Carbohydrate Fermentation

Bifidobacteria ferment various types of sugars. Lactose, galactose and sucrose are metabolized by a large number of species. The bacteria lack enzyme aldolase (fructose-1,6-bisphosphate-aldolase), like other heterofermentative LAB, but they metabolize sugars via their own complicated pathway so-called *bifid shout* (De Vries and Stouthamer 1967) (Fig. 5). Hexoses are degraded via phosphoric esters of the hexoses, erythrose, glyceraldehyde and pentoses. At two sites acetyl-phosphate is cleaved and 2-glyceraldehyde-3-phosphate is formed. This is

Table 2 Characteristics of *Lactobacillus* species produced L-(+)-LA

Species	Fermentation type	Growth (°C)	Relevant fermented carbohydrates[a]												
		15/45	Arabinose	Cellobiose	Fructose[b]	Galactose[b]	Glucose[b]	Lactose	Maltose[b]	Mannose	Raffinose	Ribose	Sucrose	Starch	Xylose
L. amylophilus	A	+/−	−	−	+	+	+	−	+	+	−	−	−	+	−
L. casei	C	+/−	−	+	+	+	+	d	+	+	−	+	+	ND	−
L. paracasei ssp. *paracasei*	C	+/d	−	+	+	+	+	+	+	+	−	+	+	−	−
L. rhamnosus	C	+/+	d	+	+	+	+	+	+	+	−	+	+	−	−
L. manihotivorans	A	+/+	−	+	+	+	+	+	+	+	+	−	+	+[c]	−
L. salivarius ssp. *salivarius*[d]	A	−/+	−	−	+	+	+	+	+	−	+	−	+	−	−
L. salivarius ssp. *salicinius*[e]	A	−/+	−	−	+	+	+	+	+	−	+	−	+	−	−

A, obligate homofermentative; C, facultative heterofermentative; +, 90 % or more of strains are positive; −, 90 % or more of strains are negative; d, 11–89 % of strains are positive

[a]Hammes and Hertel (2009)

[b]Kandler and Weiss (1986)

[c]Son and Kwon (2013)

[d]Ferments rhamnose but not salicin and esculin

[e]Ferments salicin and esculin but not rhamnose

Table 3 Characteristics of *Lactobacillus* species produced D-(−)-LA

Species	Fermentation type	Growth (°C)	Relevant fermented carbohydrates[a]												
		15/45	Arabinose	Cellobiose	Fructose[b]	Galactose[b]	Glucose[b]	Lactose	Maltose	Mannose	Raffinose	Ribose	Sucrose	Starch	Xylose
L. coryniformis ssp. *coryniformis*	C	+/−	−	−	+	ND	+	+	−	ND	d	−	+	ND	−
L. coryniformis ssp. *torquens*	C	+/−	−	−	+	ND	+	+	−	ND	−	−	+	ND	−
L. delbrueckii ssp. *delbrueckii*	A	−/+	−	−	+	−	+	−	d	+	−	−	+	ND	−
L. delbrueckii ssp. *bulgaricus*	A	−/+	−	d	+	−	+	+	−	−	−	−	−	ND	−
L. delbrueckii ssp. *lactis*	A	−/+	−	d	+	d	+	+	+	+	−	−	+	ND	−

A, obligate homofermentative; C, facultative heterofermentative; +, 90 % or more of strains are positive; −, 90 % or more of strains are negative; d, 11–89 % of strains are positive

ND no data available

[a]Hammes and Hertel (2009)

[b]Kandler and Weiss (1986)

Table 4 Characteristics of *Lactobacillus* species produced DL-LA

Species	Fermentation type	Growth (°C)	Relevant fermented carbohydrates[a]												
		15/45	Arabinose	Cellobiose	Fructose[b]	Galactose[b]	Glucose[b]	Lactose	Maltose	Mannose	Raffinose	Ribose	Sucrose	Starch	Xylose
L. acidophilus	A	−/+	−	+	+	+	+	+	+	+	d	−	+	ND	−
L. amylovorus	A	−/+	−	+	+	+	+	−	+	+	−	−	+	+	−
L. brevis	B	+/−	+	−	+	d	+	ND	+	−	d	+	d	ND	d
L. fermentum	B	−/+	d	d	+	+	+	+	+	d	+	+	+	ND	d
L. helveticus	A	−/+	−	−	+	+	+	+	d	d	−	−	−	ND	−
L. plantarum ssp. *plantarum*	C	+/−	d	+	+	+	+	+	+	+	+	+	+	[c]	d
L. reuteri	B	−/+	+	−	+	+	+	+	+	−	+	+	+	ND	−

A, obligate homofermentative; B, obligate heterofermentative; C, facultative heterofermentative; +, 90 % or more of strains are positive; −, 90 % or more of strains are negative; d, 11–89 % of strains are positive

ND no data available

[a]Hammes and Hertel (2009)

[b]Kandler and Weiss (1986)

[c]Only some strains (Giraud et al. 1994)

Table 5 Characteristics of the genus *Bifidobacterium*

Classification[a]	Kingdom	Bacteria
	Phylum	Actinobacteria
	Class	Actinobacteria
	Subclass	Actinobacteridae
	Order	Bifidobacteriales
	Family	Bifidobacteriaceae
	Genus	*Bifidobacterium*
Synonyms[b]	Before the 1960s, *Bifidobacterium* species were collectively referred to as *Lactobacillus bifidus*	
Habitats	Human, animal and insect intestine, sewage	
Characteristics	Gram positive, non-spore forming, catalase negative, immotile, mainly anaerobe	
Morphology	Rods of various shapes, bifid morphology of the cells that means long cells with slight bends	
Metabolism	Obligately sacchoroclastic, main products: lactate, acetate	
Fermentation types	Carbohydrate processed via a special fructose-6-phosphate phosphoketolase pathway	
Growth conditions	$T_{opt.}$: 37–41 °C (25–47 °C) $pH_{opt.}$: 6.5–7.0, no growth at 4.5–5.0 and 8.0–8.5[c] Requirements: complex biological substances, as casein, bovine serum albumin digest, casein digest, hog gastric mucin or yeast extract	
Pathogenicity	No	
DNA G + C content	55–67 mol%	
Remarks[d]	Species and strains differ in the sensitivity to oxygen, important probiotics and used in the food industry	

[a]Stackebrandt et al. (1997)
[b]Sgorbati et al. (1995)
[c]Scardovi (1986)
[d]De Vries and Stouthamer (1969)

metabolized by the Embden-Meyerhof-Parnas pathway to L-(+)-lactic and acetic acid in the ratio 2:3 (Sgorbati et al. 1995). Gas is not produced. This pathway has a 25 % higher yield of ATP (2.5 moles per mole of glucose) as the homofermentative LA fermentation (2 moles per mole of glucose). The sum of the fermentation of glucose is

$$2\,C_6H_{12}O_6 \rightarrow 2C_3H_6O_3 + 3C_2H_4O_2 + 5\,ATP$$

Galactose is fermented by the Leloir pathway (Fig. 3) because the enzymes for this are basically available in glucose-grown cells. On this pathway gas is formed (Scardovi 1986).

The fermented carbohydrates from the most important species of the genus *Bifidobacterium* are summarized in Table 6.

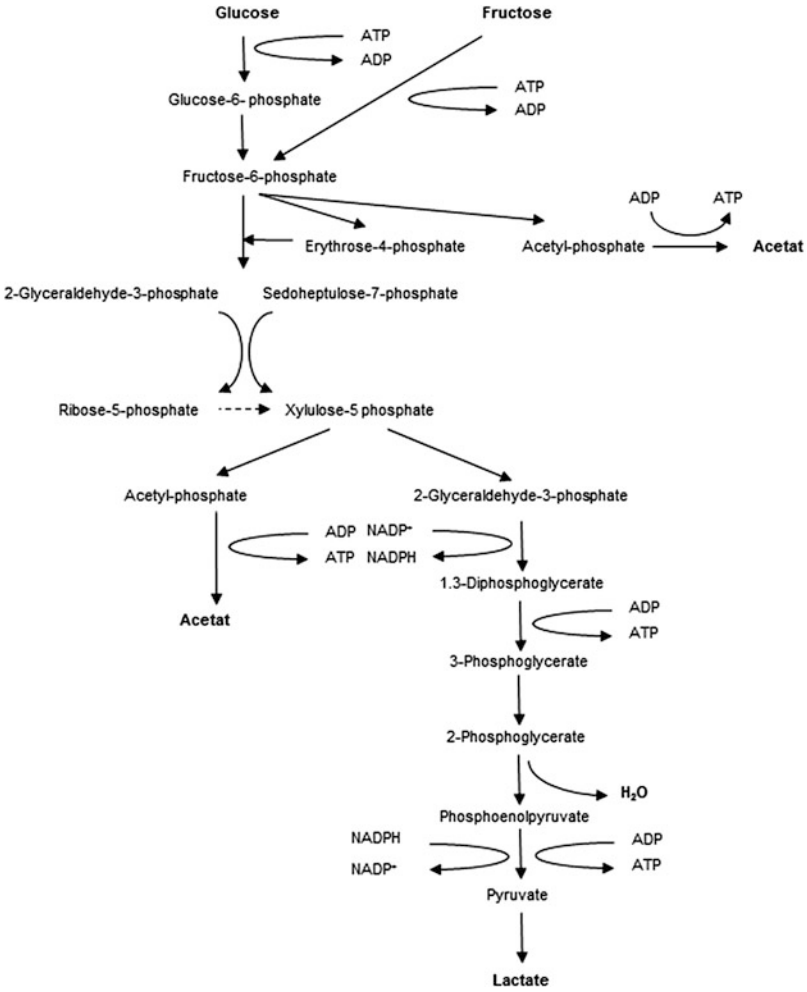

Fig. 5 Fermentation of glucose and fructose by bifidobacteria, *bifid shout*

2.3 The Order Bacillales

LA production is traditionally associated with non-spore-forming bacteria. In addition to these organisms, a number of LA-producing spore-forming bacteria have been described. They are allocated to the genera *Bacillus* and *Sporolactobacillus*. The most important LA producer in the genus *Bacillus* is the species *Bacillus* (*B*.) *coagulans* and in the genus *Sporolactobacillus* (*S*.) the species *S*. *inulinus* and *S*. *laevolacticus*.

Table 6 Fermentation of carbohydrates by important species of the genus *Bifidobacterium*

	Relevant fermented carbohydrates[a]												
Bifidobacterium	Arabinose[b]	Cellobiose[b]	Fructose	Galactose	Glucose	Lactose[b]	Maltose	Mannose	Raffinose[b]	Ribose[b]	Sucrose	Starch	Xylose
B. adolescentis	+	+	+	+	+	+	+	d	+	+	+	+	+
B. animalis	+	d	+	+	+	+	+	d	+	+	+	+	+
B. bifidum	+	–	+[e]	+	+	+	–	–[d]	–	–	d[d]	–	–
B. breve	–	d	+	+	+	+	+	+	+	+	+	–	–
B. longum	+	–	+	+	+	+	+	d	+	+	+	–	d
B. thermophilum	–	d	+	+	+	d	+	–[d]	+	–	+	+	–

+, 90 % or more of strains are positive; –, 90 % or more of strains are negative; d, 11–89 % of strains are positive

[a]Scardovi (1986)

[b]Sgorbati et al. (1995)

[c]Few strains do not ferment this sugar

[d]Few strains ferment this sugar

[e]Some strains are weak fermenters

Table 7 Characteristics of the genus *Sporolactobacillus*

Classification[a]	Kingdom	Bacteria
	Phylum	Firmicutes
	Class	Bacilli
	Order	Bacillales
	Family	Sporolactobacillaceae
	Genus	*Sporolactobacillus*
Type strain	*Sporolactobacillus inulinus*	
Habitats	Soil, canned food, compost, chicken feed, sea mud, plants	
Characteristics	Gram positive, endospore forming, facultative anaerobic or microaerophilic, catalase negative, motile	
Morphology	Straight rods singly, in pairs or rarely in short chains, long peritrichous flagella, oval spore shape	
Growth conditions[a]	$T_{opt.}$: 35 °C (25–40 °C) $pH_{opt.}$: 5.5 Requirements: carbohydrates, no growth in nutrient media	
Fermented carbohydrates[a]	Galactose, lactose, glucose, fructose, mannose, sucrose, maltose, trehalose, starch (Table 8)	
Fermentation type	Homofermentative	
LA enantiomer	D (−) or DL	
Pathogenicity	No	
DNA G + C content[a]	43–50 mol%	
Remarks[b]	Strains of the species may appear gram stain negative when the cells are older, e.g. when they enter the stationary phase	

[a]Ludwig et al. (2009)
[b]Fritze and Claus (1995)

2.3.1 The Genus *Sporolactobacillus*

The species *S. inulinus* and *S. laevolacticus* belong to the genus *Sporolactobacillus*, the only genus of the family of Sporolactobacillaceae. Important features of this genus are summarized in Table 7.

The species *S. inulinus* (Fukushima et al. 2004; Wang et al. 2011; Zheng et al. 2012) and *S. laevolacticus* (Mimitsuka et al. 2012; Li et al. 2013) are important D-(−)-LA producers particularly because of their ability to metabolize starch and inulin (Table 8).

2.3.2 *Bacillus coagulans*

A lot of species of the genus *Bacillus* (*B.*) are known for producing LA such as *B. lentimorbus*, *B. popilliae*, *B. smithii*, *B. stearothermophilus*, *B. licheniformis* and *B. subtilis* (Thomas et al. 1979; Claus and Berkeley (1986) Fritze and Claus 1995; Abdel-Rahman et al. 2013). One of the most important species forming LA is

Table 8 Fermented carbohydrates of important species of the genus *Sporolactobacillus*

Species	Relevant fermented carbohydrates													
	LA enantiomer	Arabinose	Cellobiose	Fructose	Galactose	Glucose	Inulin	Lactose	Maltose	Mannose	Raffinose	Ribose	Starch	Xylose
S. inulinus	D	–	–	ND	–	+	+	–	ND	No	+	–	+	–
S. laevolacticus	D	–	+	ND	+	+	+	+	ND	+	+	–	d	–

+, 90 % or more of strains are positive; –, 90 % or more of strains are negative; d, 11–89 % of strains are positive
ND no data available

Table 9 Characteristics of *B. coagulans*

Classification[a]	Kingdom	Bacteria
	Phylum	Firmicutes
	Class	Bacilli
	Order	Bacillales
	Family	Bacillaceae
	Genus	*Bacillus*
	Species	*B. coagulans*
First isolation	Hammer 1905, spoiled canned milk	
Synonyms[b]	*B. calidolactic, B. thermoacidurans, B. dextrolacticus, B. thermoacidificans, Lb. cereal*	
Habitats[a]	Soil, canned food, tomato juice, gelatin, milk, silage	
Characteristics	Gram positive, spore forming, facultative anaerobic, catalase positive, motile, facultative thermophilic	
Morphology	Rods, spores are oval or cylindrical and terminally or subterminally located, peritrichous flagella	
Growth conditions[a]	$T_{opt.}$: 50 °C (30–55 °C) $pH_{opt.}$: 7.0 (4–11) Requirements: simple, mineral salt medium with few nitrogen sources	
Fermented carbohydrates[a]	+: fructose, glucose, galactose, maltose, mannose, starch, trehalose, xylose d: arabinose, cellobiose, lactose, raffinose, ribose, sucrose	
Fermentation type	Heterofermentative	
LA enantiomer	L (+)	
Pathogenicity	No	
DNA G + C content[a]	44.3–50.3 mol%	
Remarks[c]	Strains of the species may appear gram stain negative when the cells are older, e.g. when they enter the stationary phase	

+, 90 % or more of strains are positive; d, 11–89 % of strains are positive
[a]Logan and de Vos (2009)
[b]Wood and Holzapfel (1995)
[c]Fritze and Claus (1995)

B. coagulans (Ou et al. 2011; Wang et al. 2012; Tashiro et al. 2013; Ma et al. 2014). Important features of this species are summarized in Table 9.

Glucose is mainly fermented to L-(+)-LA and smaller amounts of 2.3-butanediol, acetoin, acetic acid and ethanol (Fritze and Claus 1995). Some strains of *B. coagulans* are able to ferment hexoses and pentoses homolactic to L-(+)-LA (Wang et al. 2012; Ou et al. 2011).

2.4 *Other LA-Forming Microorganisms*

Besides the previously described species of the genera *Lactobacillus, Bifidobacteria, Bacillus* and *Sporolactobacillus*, there are a few bacteria of different systematic positions that are significant for forming LA. The most important bacteria are *Enterococcus faecium*, *Lactococcus lactis*, *Pediococcus acidilactici* and *Streptococcus thermophilus*. Yeasts and fungi are also of increasingly economic importance such as *Saccharomyces cerevisiae* and *Rhizopus oryzae*. Since over 10 years there are reports from LA-producing *E. coli* strains. Most of them are described as metabolically engineered strains for the production of LA (Chang et al. 1999; Dien et al. 2001; Baba et al. 2006; Kim et al. 2013).

2.4.1 *Enterococcus faecium*

Compared to other LA-producing microorganisms, *E. faecium* does not play a major role for the industrial application, but it was already described for the direct fermentation of starch containing feedstocks (Shibata et al. 2007; Nolasco-Hipolito et al. 2012). Important features of *E. faecium* are summarized in Table 10.

2.4.2 *Lactococcus lactis*

From the family of Streptococcaceae, the species *Lactococcus lactis* (*L. lactis*) and *Streptococcus thermophilus* are particularly very important for LA production. The species of the genus *Lactococcus* differ from the other LAB by their pH and by their salt and temperature tolerance for growth (Table 9). Among LAB, *L. lactis* is the most extensively studied regarding its physiology, metabolic pathways and regulatory mechanisms. Its genome was the first LAB genome to be completely sequenced (Oliveira et al. 2005). Important features of *L. lactis* are summarized in Table 11.

Among the subspecies of *L. lactis*, *L. lactis* ssp. *lactis* (John et al. 2007) and *L. lactis* ssp. *cremoris* are most important for the production of LA (Ramchandran et al. 2012; Mukisa et al. 2012). Fermented carbohydrates of the important species are arranged in Table 12.

It is reported in the literature that *L. lactis* produce LA as the sole metabolic product at high dilution rates during continuous cultivations or at high glucose concentrations during batch growth (Benthin 1994; Melchiorsen et al. 2002). In contrast, growth at low dilution rates in continuous conditions or at low concentrations of glucose in batch conditions results in a mixed-acid fermentation, where formate, ethanol and acetate are produced in a molar ratio of 2:1:1 (Melchiorsen et al. 2001).

Table 10 Characteristics of *E. faecium*

Classification[a]	Kingdom	Bacteria
	Phylum	Firmicutes
	Class	Bacilli
	Order	Lactobacillales
	Family	Enterococcaceae
	Genus	*Enterococcus*
	Species	*E. faecium*
First isolation	Orla-Jensen 1919	
Synonyms	*Streptococcus faecium*	
Habitats[b]	Gastrointestinal tract of mammals, birds, reptiles, food (raw milk, milk products), environment (plant, water)	
Characteristics	Gram positive, non-spore forming, facultative anaerobic, catalase negative (on blood agar same strains reveal pseudocatalase), immotile, alpha haemolytic or nonhaemolytic, anaerobic and aerobic metabolism	
Morphology	Ovoid cells, single, in pairs or chains	
Growth conditions	$T_{opt.}$ 35–37 °C (10–40 °C), survive 30 min. temperatures at 60 °C pH 4.6–9 Requirements: complexes such as brain heart infusion, blood, folic acid, some strains the same requirements such as LAB	
Fermented carbohydrates	+: cellobiose, fructose, glucose, galactose, lactose, maltose, mannose, ribose, starch, xylose[b] d: arabinose, raffinose, sucrose[a]	
Fermentation type	Homofermentative	
LA enantiomer	L (+)	
Pathogenicity	Human pathogenic, causing diseases such as neonatal meningitis	
DNA G + C content[a]	37.0–40.0 mol%	
Remarks[a]	Some strains are used as probiotics in animals, can produce enterotoxins (bacteriocin 31, enterotoxins A, B, P and 50)	

+, 90 % or more of strains are positive; d, 11–89 % of strains are positive
[a]Svec and Devriese (2009)
[b]Devriese and Pot (1995)

2.4.3 *Pediococcus acidilactici*

There have been not much applications published for *P. acidilactici* (Hofvendahl and Hahn-Hägerdal (2000), Giurca and Levin (1992, 1993)), but the new strain DQ2 has been recently described, which is characterized by high-temperature tolerance, high lignocellulose-derived inhibitor resistance and high LA production performance in combination with simultaneous saccharification and fermentation at high-solids loading of corn stover (Dao et al. 2013; Zhao et al. 2013b). Important features of *P. acidilactici* are summarized in Table 13.

Table 11 Characteristics of *L. lactis*

Classification[a]	Kingdom	Bacteria
	Phylum	Firmicutes
	Class	Bacilli
	Order	Lactobacillales
	Family	Streptococcaceae
	Genus	*Lactococcus*
	Species	*L. lactis*
	Subspecies	*L. lactis* ssp. *lactis*, *L. lactis* ssp. *cremoris*, *L. lactis* ssp. *hordniae*
First isolation	Joseph Lister (1873)	
Synonyms	*Bacterium lactis, Streptococcus lactis, Streptococcus cremoris*[b]	
Habitats	Raw milk, dairy products	
Characteristics	Gram stain positive, non-spore forming, facultative anaerobic, catalase negative, immotile	
Morphology	Ovoid cells, mostly in pairs, or in short chains	
Growth conditions	$T_{opt.}$: 30–40 °C (10–40 °C), no growth at 45 °C $pH_{opt.}$: 7.0 (tolerate 4.5) Requirements: individually various complex nutritional requirements for peptides, amino acids, vitamins and fermentable carbohydrate, phosphate, potassium, magnesium[c]	
Fermented carbohydrates	s. Table 12	
Fermentation type	Homofermentative	
LA enantiomer	L (+)	
Pathogenicity[d]	Absent or in rare case	
DNA G + C content[c]	33.8–36.8 mol%	
Remarks	Type strain of the genus *Lactococcus,* starter culture in the dairy industry, especially in the cheese manufacture, produced bacteriocin nisin, type of species of the genus	

[a]Teuber (2009)
[b]Teuber (1995)
[c]Oliveira et al. (2005)
[d]Teuber (2006)

2.4.4 *Streptococcus thermophilus*

The classification and nomenclature of streptococci have undergone significant changes over the years. In recent years, based on biochemical characteristics as well as RNA analysis, members of the genus *Streptococcus* have been reclassified into *Lactococcus*, *Vagococcus*, *Enterococcus* and *Streptococcus*. This genus includes both significant human and animal pathogens but also nonpathogenic species, in particular *S. thermophilus*, for the production of food (especially milk, milk products and cheese) and LA, particularly on lactose-rich substrates (Hofvendahl and Hahn-Hägerdal 2000; Pescuma et al. 2008; Secchi et al. 2012;

Table 12 Fermented carbohydrates in *L. lactis* subspecies (Teuber 2009)

Species	Relevant fermented carbohydrates												
	Arabinose	Cellobioe	Fructose	Galactose	Glucose	Lactose	Maltose	Mannose	Raffinose	Ribose	Sucrose	Starch	Xylose
Lactococcus lactis ssp. *lactis*	–	d	+	+	+	+	+	+	–	+	d	d	d
Lactococcus lactis ssp. *cremoris*	–	d	+	+	+	+	–	+	–	–	d	–	d

+, 90 % or more of strains are positive; –, 90 % or more of strains are negative; d, 11–89 % of strains are positive

Table 13 Characteristics of *P. acidilactici*

Classification[a]	Kingdom	Bacteria
	Phylum	Firmicutes
	Class	Bacilli
	Order	Lactobacillales
	Family	Lactobacillaceae
	Genus	*Pediococcus*
	Species	*P. acidilactici*
First isolation	Lindner 1887	
Synonyms	*P. lindneri, P. cerevisiae, Streptococcus lindneri*[b]	
Habitats	Fermenting plant material (silage, cereal mashes, pickles), hey, fruits, salami	
Characteristics	Gram stain positive, non-spore forming, facultative anaerobic with lesser sensitivity to oxygen, oxidase and catalase negative, immotile, alpha-haemolytic	
Morphology	Cocci, often found in pairs or tetrads	
Growth conditions	$T_{opt.}$: 40 °C (35–53 °C) $pH_{opt.}$: 6.0–6.5 (4.2–8.5) Complex requirements: various vitamins, organic acids (nicotinic and pantothenic acid), biotin, riboflavin stimulates the growth[c]	
Fermented carbohydrates[d]	+:,cellobiose[b], fructose, glucose, galactose, mannose[b], ribose, xylose d: arabinose, lactose[b], raffinose, sucrose[b]	
Fermentation type	Homofermentative	
LA enantiomer[c]	DL	
Pathogenicity	No	
DNA G + C content	38–44 mol%	
Remarks	Some strains produce the bacteriocin pediocin	

+, 90 % or more of strains are positive; d, 11–89 % of strains are positive
[a]Holzapfel et al. (2009)
[b]Simpson and Taguchi (1995)
[c]Garvie (1986)
[d]Holzapfel et al. (2009)

Tang et al. 2013). Important features of *S. thermophilus* are summarized in Table 14.

S. thermophilus, like other streptococci that ferment carbohydrates, produce L-(+)-LA as well as minor amounts of acetic and formic acids, ethanol and CO_2 (Whiley and Hardie 2009).

2.4.5 *Saccharomyces cerevisiae*

Recently, there are reports about LA-forming yeasts such as *S. cerevisiae* (Dequin and Barre 1994; Praphailong and Fleet 1997; Bianchi et al. 2001; Lu et al. 2012) and other *Saccharomyces* species, *Zygosaccharomyces, Candida, Pichia and*

Table 14 Characteristics of *S. thermophilus*

Classification[a]	Kingdom	Bacteria
	Phylum	Firmicutes
	Class	Bacilli
	Order	Lactobacillales
	Family	Streptococcaceae
	Genus	*Streptococcus*
	Species	*S. thermophilus*
First isolation	Orla-Jensen 1919	
Synonyms	*S. salivarius* ssp. *thermophilus*	
Habitats	Dairy sources, heated and pasteurized milk, milk products	
Characteristics	Gram stain positive, non-spore forming, facultative anaerobic, oxidase and catalase positive, immotile, alpha-haemolytic, moderate thermophilic	
Morphology	Cells spherical or ovoid, forming pairs or chains	
Fermented carbohydrates[a]	+: fructose, glucose, lactose, mannose, sucrose d: galactose, raffinose, ribose, starch[b]	
LA enantiomer	L (+)	
Growth conditions	$T_{opt.}$: 45 °C, minimum 19–21 °C, maximum 52 °C $pH_{opt.}$: 6.5, no growth at pH 9.6 Requirements: B vitamins, some amino acids	
Pathogenicity	No	
DNA G + C content[c]	37–40 mol%	
Remarks	It is currently discussed whether the species *S. thermophilus* to be renamed in *Lactococcus thermophilus* to avoid confusion with the pathogenic part of the *Streptococcus* genus such as *S. pneumonia and S. pyogenes* Important species in the cheese manufacture	

+, 90 % or more of strains are positive; d, 11–89 % of strains are positive
[a]Whiley and Hardie (2009)
[b]Hardie (1986)
[c]Hardie and Whiley (1995)

Kluyveromyces that have been engineered to produce LA (Abdel-Rahman et al. 2013). Important features of *S. cerevisiae* are summarized in Table 15.

Carbohydrate Utilization

Under aerobic conditions, *S. cerevisiae* respire glucose, producing H_2O and CO_2. Under anaerobic conditions, cells ferment sugars to ethanol and CO_2. Galactose and fructose are shown to be two of the best fermenting carbohydrates. *S. cerevisiae* do not ferment pentoses such as arabinose, ribose and xylose and also disaccharides, e.g. lactose and cellobiose. Glucose oxidation under aerobic conditions provides more energy than fermentation. Therefore, the mass increases rate, and the cell division rate in oxidative decomposition of sugar is much higher than in

Table 15 Characteristics of *S. cerevisiae*

Classification[a]	Kingdom	Fungi
	Phylum	Ascomycota
	Subphylum	Saccharomycotina
	Class	Saccharomycetes
	Order	Saccharomycetales
	Family	Saccharomycetaceae
	Genus	*Saccharomyces*
	Species	*S. cerevisiae*
First isolation	Meyer *ex* Hansen 1883	
Synonyms[b]	Ca. 150 synonyms	
Habitats	Dairy products: wines, beer, fruits and berries, trees, cheese, kefir, sugar cane, man and other mammas	
Characteristics[b]	Facultative anaerobic, reproduction vegetative by budding, can form filaments and none or simple pseudohyphae, can be formed oval or round ascospores	
Morphology	Cells round or oval, 5–10 μm	
Growth conditions	$T_{opt.}$: 25–30 °C $pH_{opt.}$: 6.5–7.0, tolerate pH values as low as 1.5 Requirements: phosphorus, sulphur, amino acids methionine and cysteine, metals (magnesium, iron, calcium, zinc), biotin, pantothenate, prototrophic vitamins	
Fermented carbohydrates[b]	+: fructose, glucose, sucrose, raffinose d: galactose, maltose, melibiose, starch −: lactose, trehalose[c]	
LA enantiomer	D (−)	
Pathogenicity	No	
DNA G + C content[b]	38.8–42.0 mol%	
Remarks	Important in the baking and brewing, model organisms for genetic engineering	

+, 90 % or more of strains are positive; d, 11–89 % of strains are positive; −, 90 % or more of strains are negative
[a]Suh et al. (2006)
[b]Barnett et al. (1990)
[c]De Hoog et al. (2000)

fermentation. The ability of yeasts to use different sugars can differ depending on the oxygen conditions. So some strains cannot grow anaerobically on sucrose and trehalose. D-(−)-lactate formation is described by Stewart et al. (2013) (Fig. 6).

Experiments with *S. cerevisiae* under aerobic conditions demonstrated that during glucose consumption D-(−)-lactate forms as a result of methylglyoxal metabolism. The data suggest that increased glucose uptake by cells grown in a glucose-rich environment results in an increased generation of methylglyoxal with subsequent metabolism to D (−) lactate.

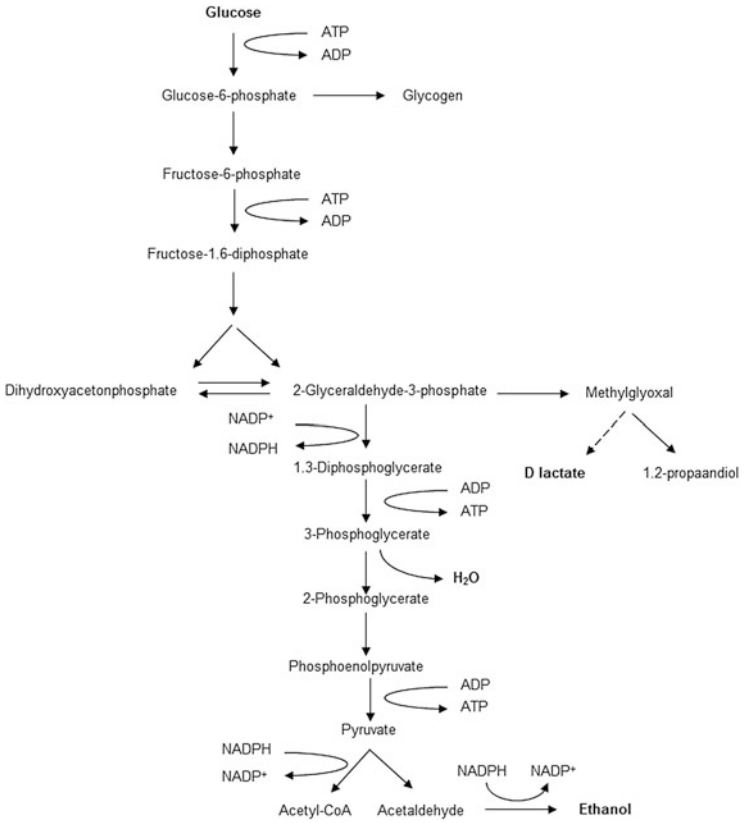

Fig. 6 D-(−)-Lactate production by *S. cerevisiae* (mod. Stewart et al. 2013)

2.4.6 *Rhizopus oryzae*

It is known that the mould in the genera *Rhizopus*, *Mucor* and *Monilia* form LA (Yadav et al. 2011). Currently, there are increasing reports on the production of LA by various species of *Rhizopus*, especially *R. oryzae* but also *R. arrhizus* (Bai et al. 2008; Taskin et al. 2012; Wu et al. 2011). *R. oryzae* produce a wide spectrum of metabolites, in the form of enzymes, esters, organic acids, volatile materials, polymers and bioalcohols. Biodiesel can also be produced. The fungus is a rich source of LA but also fumaric acid and to a better extent malic acid (Ghosh and Ray 2011). *R. oryzae* is a filamentous fungus belonging to the traditional Zygomycota (Hibbett et al. 2007). Its important features are summarized in Table 16.

Table 16 Characteristics of *R. oryzae*

Classification[a]	Kingdom	Fungi
	Subphylum	Mucoromycotina
	Order	Mucorales
	Family	Mucoraceae
	Genus	*Rhizopus*
	Species	*R. oryzae*
First isolation	Went and Prinsen Geerlings 1895	
Synonyms[b]	*R. arrhizus*, about more than 55 synonyms	
Habitats	Cereals, bread waste, compound fodder, vegetables, fruits, soil, polluted water	
Characteristics	Filamentous fungus, obligate aerobe	
Morphology[c]	– Fungus cobweb-like lawn with low slopes – Colonies are typically greyish to brownish – Rhizoid sparingly branched, up to 250 µm long, brownish – Sporangiospores brown, 1–2 mm high, up to 18 µm wide, angular, subglobose to ellipsoidal, with ridges on the surface, single or in tufts – Sporangia spherical, 50–250 µm in diameter, brownish-grey to black – Columella comprising 50–70 % of sporangium – Apophysis short, 3–12 µm high – Sporangiospores greyish green, angular, subspherical to ellipsoidal, longitudinally striate 6–8 × 4.5–5.0 µm – Chlamydospores single or in chains, spherical to ovoidal, 10–35 µm diameter, hyaline, smooth walled	
Growth conditions[d]	$T_{opt.}$: 30–35 °C, (5–7 °C, 44 °C) no growth at 45 °C $pH_{opt.}$: 5.5 (4–7) No special requirements: inorganic nitrogen sources, no amino acids and vitamin supplements	
Carbon sources[e]	Cellobiose, glucose, mannose, fructose, sucrose, starch, xylose, ethanol, glycerol, LA, fatty acids, oil	
Fermentation type	Heterofermentative	
LA enantiomer	L (+)	
Pathogenicity[e]	Generally regarded as safe, but same strains are also plant pathogen, opportunistic human pathogen	
Remarks	Used for food fermentation	

[a]Hibbett et al. (2007)
[b]CBS-Knaw (2014)
[c]De Hoog et al. (2000)
[d]Schmidt (2002)
[e]Meussen et al. (2012)

Formation of LA

In *R. oryzae*, all fermentable carbon sources are metabolized to pyruvate during glycolysis (Fig. 7).

The pyruvate is subsequently channelled to a number of pathways, including the pathways responsible for the formation of ethanol, lactate and fumarate. The dissolved oxygen in the medium influences the flow of pyruvate. Under anaerobic

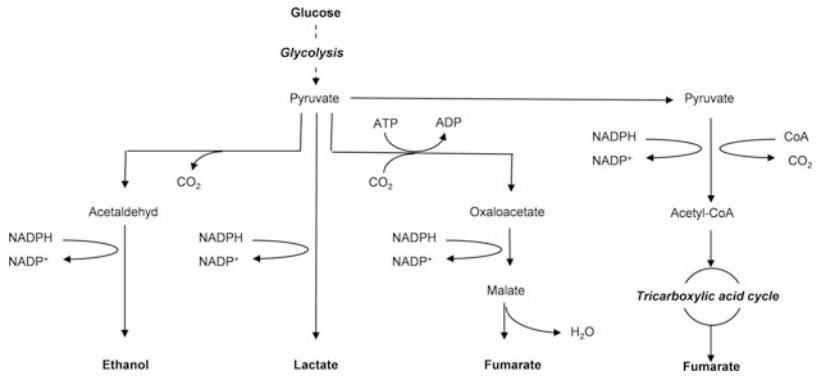

Fig. 7 Fermentation routes of glucose in *R. oryzae* (mod. Meussen et al. 2012)

conditions, the carbon flow is directed towards the formation of ethanol, while under aerobic conditions, with excess of carbon substrate, the flow is directed towards organic acid production (Skory et al. 1998; Meussen et al. 2012). With a mutagenizing strain, they could express almost a tenfold increase in LA production compared to the parental strain. Also the formation of L-(+)-LA is possible by strains of *R. oryzae* or genetically modified strains (Bai et al. 2004; Park et al. 2004). *R. oryzae* is widely studied as a commercially perspective producer of L-(+)-LA (Miura et al. 2003; Yin et al. 1997; Yamane and Tanaka 2013), because the fungus cells possess better resistance to high concentrations of accumulated LA (Hamamci and Ryu 1994; Schepers et al. 2003). Moreover the cells need lower nutrient requirements compared to the commonly used bacterial producers (Hujanen et al. 2001; Kwon et al. 2000). The use of *R. oryzae* in immobilized form is one of the most efficient approaches to improve the LA production process for long-term acid production (Tay and Yang 2002; Efremenko et al. 2006; Yamane and Tanaka 2013).

2.4.7 *Escherichia coli*

There are many challenges for the industrial production of LA, and satisfying all these requirements is very difficult through the traditional use of LAB. Therefore, improving LAB via gene modification and using other microorganisms (e.g. *E. coli*) and yeast for LA production via gene modification have become an essential and interesting research area (Grabar et al. 2006; Okano et al. 2010). Several research activities are directed towards the recombinant improvement of LA yield and the optical purity since *E. coli* produces a mixture of organic acids and other metabolites (Zhou et al. 2012; Mazumdar et al. 2013; Zhao et al. 2013a). Important features of *E. coli* are summarized in Table 17.

Table 17 Characteristics of *E. coli*

Classification[a]	Domain	Bacteria
	Kingdom	Eubacteria
	Phylum	Proteobacteria
	Class	Gammaproteobacteria
	Order	Enterobacteriales
	Family	Enterobacteriaceae
	Genus	*Escherichia*
	Species	*E. coli*
First isolation	Theodor Escherich 1885	
Synonyms	No	
Habitats	Human and warm-blooded animal intestines	
Characteristics	Gram negative, non-spore forming, aerobic and facultative anaerobic, motile or immotile, catalase positive, oxidase negative	
Morphology[a]	Straight, cylindrical rods with rounded end, single or in pairs, diameter 1.1–1.5 µm, length 2.0–6.0 µm, peritrichous flagella	
Growth conditions	$T_{opt.}$: 37 °C (7.5–49 °C) $pH_{opt.}$: 7.0 (5–9) Requirements: no special supplements are necessary	
Fermented carbohydrates	+: arabinose, glucose, lactose, maltose, mannose, xylose[b] d: raffinose, sucrose	
LA enantiomer	D (−)	
Pathogenicity	Most nonpathogenic, but some serotypes or clones are human pathogen (EHEC, STEC, VTEC), animal pathogen	
DNA G + C content	48.5–52.1 mol%	
Remarks	Important host organism in the molecular biology	

+, 90 % or more of strains are positive; d, 11–89 % of strains are positive
[a]Scheutz and Strockbine (2009)
[b]Devriese and Pot (1995)

Lactate Formation

E. coli can grow both under aerobic and anaerobic conditions. Like other species of the family Enterobacteriaceae, *E. coli* ferments glucose anaerobically via pyruvate to various acids and gas. Pyruvate can be formed either via glycolysis or via the EMP or the Entner-Doudoroff (ED) pathway (Fig. 8). However, the ED pathway is by *E. coli* of minor importance, because the enzymes of the ED pathway are only induced at the presence of gluconate, glucuronate, galacturonate or idonate (Eisenberg and Dobrogosz 1967). The resulting pyruvate is converted to D-(−)-lactate, acetate, succinate and formate via so-called mixed-acid fermentation. Part of the formic acid is split into equal amounts of CO_2 and H_2. The sum of the equation is (Schlegel 2007)

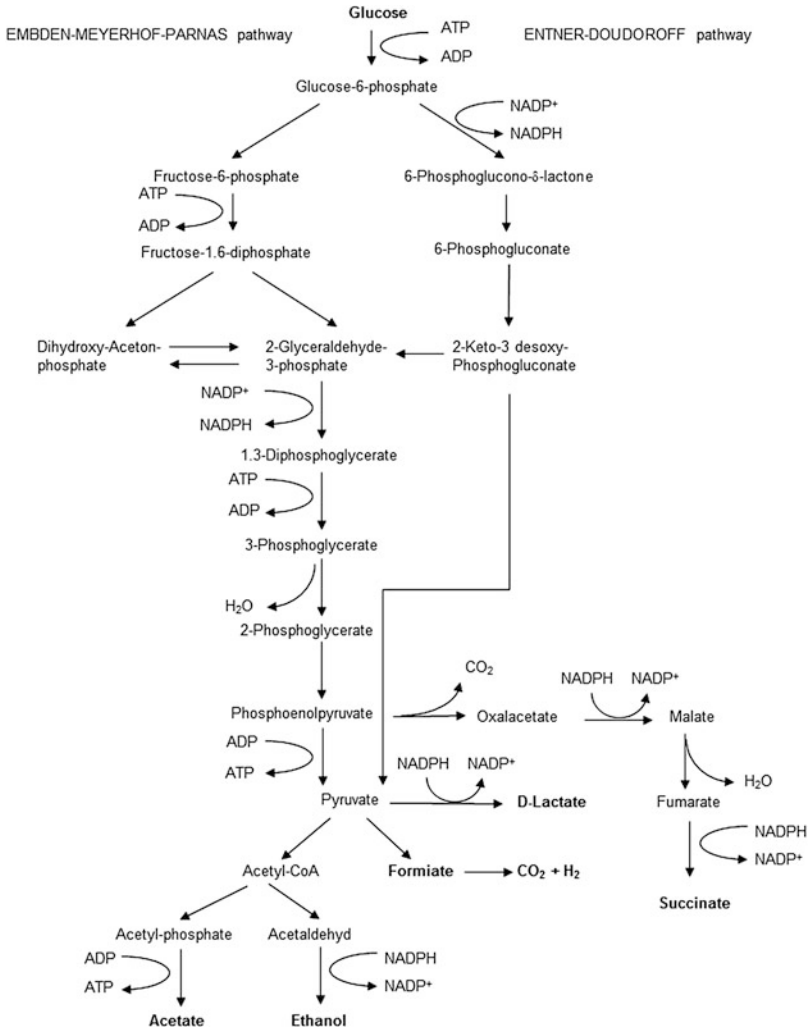

Fig. 8 Anaerobe fermentation of glucose by *E. coli*

$$C_6H_{12}O_6 \rightarrow 0.84\,C_3H_6O_3 + 0.44\,C_2H_4O_2 + 0.42\,C_2H_5O + 0.29\,C_4H_6O_4 + 0.02\,CH_2O_2 + 0.44CO_2 + 0.43\,H_2 + ATP$$

Approximately 80 % of the glucose is fermented by these pathways, and the other 20 % are metabolized by the oxidative pentose phosphate pathway (Fuhrer et al. 2005).

Table 18 Characteristics of *L. paracasei* ssp. *paracasei*

Classification[a]	Kingdom	Bacteria
	Phylum	Firmicutes
	Class	Bacilli
	Order	Lactobacillales
	Family	Lactobacillaceae
	Genus	*Lactobacillus*
	Species	*L. paracasei*
	Subspecies	*L. paracasei* ssp. *paracasei*
First isolation	Collins, Phillips and Zanoni 1989	
Synonyms	*Lactobacillus casei* ssp. *alactosus* Mills and Lessel 1973 *Lactobacillus casei* ssp. *pseudoplantarum* Abo-Elnaga and Kandler 1965	
Habitats	Dairy products, sewage, silage human, and clinical sources	
Characteristics	Gram stain positive, facultative anaerobic, catalase negative, immotile	
Morphology	Rods often with square end, occurring singly or in chains	
Growth conditions	$T_{opt.}$: 30 °C (5–45 °C) $pH_{opt.}$: 6.5 Requirements: individually various complex nutritional requirements for peptides, amino acids, nucleotides, vitamins and fermentable carbohydrates	
Fermented carbohydrates[b]	+: cellobiose, fructose, glucose, galactose, mannose, ribose, xylose d: arabinose, lactose, raffinose, sucrose	
Fermentation type	Facultative heterofermentative	
LA enantiomer	L (+)	
Pathogenicity	No	
DNA G + C content[c]	45–47 mol%	

+, 90 % or more of strains are positive; d, 11–89 % of strains are positive
[a]Hammes and Hertel (2009)
[b]Holzapfel et al. (2009)
[c]Hammes and Vogel (1995)

3 Organic Lactate-Forming Bacteria

Various organic lactates are formed by LAB, such as 1,4-piperazinium-(L,L)-dilactate (Kamm et al. 1997; Richter et al. 2001), imidazole-(L)-lactate (Kamm et al. 1999), hexamethylenediamine-(L,L)-dilactate (Gutmacher 2008) and lysine-(L)-lactate (Leiss et al. 2010). Piperazinium-(L,L)-dilactate, imidazole-(L)-lactate (Kamm et al. 1999) and hexamethylenediamine-(L,L)-dilactate are applied as intermediates for the production of polylactic acid (Kamm et al. 1999, 2000). These lactates are formed by means of strains of *Lactobacillus paracasei* ssp. *paracasei*. *I*mportant features of *L. paracasei* ssp. *paracasei* are summarized in Table 18.

LA is produced through facultative heterofermentation, which means cells of the strain ferment hexoses and pentoses (see Sect. 2.1). Hexoses are fermented by the

EMP glycolytic pathway to L-(+)-lactate. The same strains, which are formerly described as *L. pseudoplantarum*, produce DL-LA (Hammes and Hertel 2009). Pentoses enter this pathway and are fermented to LA and acetic acid.

4 Platform Chemical Lactic Acid

Currently there are criteria for a platform chemical, such as multiple product applicability, high-volume product and potential industrial scaleup (Bozell and Petersen 2010). LA is widely used in the food, cosmetic, pharmaceutical and chemical application, such as lactic acid derivatives as dyeing assistants, and has received increased attention for use as a monomer for the production of biodegradable PLA (Datta et al. 1995; Kamm et al. 1997, 2000; Nampoothiri et al. 2010; Castillo Martinez et al. 2013). LA is one of the most interesting intermediates for the synthesis of industrial relevant bio-based compounds based on carbohydrates, and it was foreseen as a platform chemical for the production of several downstream chemicals. Lactic acid undergoes ready esterification to give lactate esters, of interest as new bio-based 'green' solvents (Kamm et al. 2008). Lactate esters have many potential markets as non-toxic replacements for halogenated and toxic solvents. The use of esters, e.g. ethyl lactate and butyl lactate as solvents for cellulose lacquers or poly(vinyl) compounds, is important, and a great variety of esters have been recommended for use as plasticizers in polymers. Catalytic reduction of lactic acid leads to propylene glycol, which can be further dehydrated to give propylene oxide. Alternatively, lactic acid can be dehydrated to give acrylic acid and esters, but in practice this conversion proceeds in low yield (Walkup 1991).

The global lactic acid demand was estimated to be 714.2 kilo tons in 2013, which is expected to reach 1,960.1 kilo tons by 2020, growing at a compound annual growth rate of 15.5 % from 2014 to 2020 (SpecialChem 2014). Growth in demand for LA and its salts and esters in industrial applications will be driven mainly by LA-based biodegradable polymers and, to a lesser degree, lactate solvents (Abdel-Rahman et al. 2013; Taskila and Ojamo 2013).

5 Intermediate and Speciality Chemicals: Organic Lactates

Aminium lactates, such as piperazinium dilactate, imidazole lactate and hexamethylenediamine dilactate, are applied as intermediates for the production of polylactic acid (Kamm et al. 2000) and for the manufacture of high purity lactic acid (Kamm et al. 1999). Aminium lactates can be applied directly as constituents of pharmaceutical and cosmetic products as well. Piperazine lactate

has anthelmintic activity (Chatterjee et al. 1997). Imidazole lactate is useful as a topical antilipolytic (Carreras Ginjaume 1985) and lysine lactate can be applied as component in skin lotions (Parab 1995). The antimicrobial activities of protic ionic liquids with lactate ion were investigated intensively (Pernak et al. 2004).

6 Biotechnological Production of LA

Biotechnological processes and bio-based products are an interesting alternative compared to classical ones of chemistry. The so-called white biotechnology points to an emerging field in biotechnology with immense potentials via utilization of biocatalysts for the manufacture of industrial products. The goal is to develop a fermentation process based on the substitution of expensive nutrient supplements by cheaper materials from renewable resources due to their main proportion of the whole process costs (Akerberg and Zacchi 2000; Okano et al. 2010). Depending on the further processing of the LA (e.g. for bioplastics), the separation of impurities after fermentation is a major process cost too (Fitzpatrick et al. 2003; Ryu et al. 2012). Therefore an optimization is necessary to find a balance between the substitution of expensive nutrients and the limitation of interfering or undesirable components of natural raw materials, respectively.

The worldwide research is advancing focused on the use of renewable raw materials as carbon substrates as well as nutrient additive resources. In this context, there is a strong interest to reduce costs for raw materials and to use renewable resources.

With respect to the above-mentioned cost aspect of bioprocess feedstock, the utilization of residues and waste materials (Pintado et al. 1999; Huang et al. 2005; Bischoff et al. 2010; Ouyang et al. 2013; Tang et al. 2013) and agricultural by-products (Thomsen 2005; John 2009; Alonso et al. 2011; Li et al. 2012) became the focus of public attention.

LA was produced worldwide at first from glucose or pure starch on fermentative ways (Richter and Berthold 1998). First efforts for developing bioconversion processes for the production of LA directly from agricultural starchy feedstock were published by Shamala and Sreekantiah (1987). During the last years, starchy hydrolysates obtained from agricultural resources like corn or barley (Linko and Javanainen 1996; Oh et al. 2005; Venus and Richter 2006), cassava (Xiaodong et al. 1997; Bomrungnok et al. 2012), wheat (Hetenyi et al. 2010), rye (Otlewska et al. 2012), potatoes (Zhang and Jin 2010; Bilanovic et al. 2011) and sago (Nolasco-Hipolito et al. 2002) were also tested on their suitability as substrates for LA fermentation.

Lignocellulosic biomass represents the most abundant global source of biomass, and for this reason it has been largely utilized in many applications (Taherzadeh and Karimi 2007). Lignocellulosic materials can be used to obtain sugar solutions that may be usefully exploited for the production of LA through the following steps: (a) pretreatment to break down the lignocellulosic structure, (b) enzymatic

hydrolysis to depolymerize lignocellulose to fermentative sugars, (c) sugar fermentation to LA by LAB and (d) separation and purification of LA (Moldes et al. 2006, Abdel-Rahman et al. 2011). In recent years, one of the most used processes to obtain LA from lignocellulosic materials is the simultaneous saccharification and fermentation (John et al. 2009; Qi et al. 2011; Zhao et al. 2013b), which is able to prevent enzyme inhibition by the product (Gullon et al. 2007; Castillo Martinez et al. 2013).

LAB need, besides the carbon source, also a source of nitrogen and other nutrients and phosphorus. The latter is available when inorganic phosphate salts are added to the medium. The demand for nitrogen cannot be covered by inorganic salts only. LAB need also a series of nitrogen-containing nutrients (amino acids, peptides, etc.) for growth, and therefore, the medium has to be supplied by complex protein hydrolysates (yeast extract, peptone, etc.). The protein extracts mentioned are very expensive, and their substitution by low-priced nutrient extracts is necessary when a large-scale production is planned. A useful combination of green biomass processing for the production of fodder pellets and the utilization of the pressed juice for the LA fermentation was described by Andersen and Kiel (2000) and Vodnar et al. (2010). The use of date juice together with different nitrogen sources as a substrate for LA production was investigated by Nancib et al. (2001).

7 Future Perspectives

Although LA production by LAB is very efficient, further improvements in the process can help make it more cost competitive with petroleum-based polymers for PLA production. Environmentally friendly, 'green' solvents are another potential area for lactic acid derivatives, particularly lactate esters of low-molecular-weight alcohols such as ethyl, propyl and butyl lactate (John et al. 2007; Delgado et al. 2010). From that perspective the lactate esters have also further applications in order to run alternative downstream technology (Kamble et al. 2012) and PLA polymerization process (Marques et al. 2012).

Yield and purity of the LA produced are currently limited by many factors including the production of both L- and D-LA via L-lactate dehydrogenase and D-lactate dehydrogenase, respectively, low yield due to by-product formation, use of nutritionally rich medium, high risk of bacteriophage infection that results in cell lysis and subsequent cessation of LA production (Abdel-Rahman et al. 2013). In addition to the previously used LAB and LA-producing microorganisms, these organisms are interesting for the production of LA that were until now get low attention such as microalgae and cyanobacteria.

Through different approaches, these defects can be partially remedied today. The usage of mixed strains and/or development of phage-resistant strains can prevent bacteriophage infection (Hassan and Frank 2001).

Various studies have investigated methods to overcome some problems in the field of metabolic engineering of the strains, e.g. improvement of optical purity via

the deletion of either D- or L-LDH genes (Kyla-Nikkila et al. 2000) and increased LA yields through the reduction of by-product levels by the deletion of genes encoding pyruvate formate lyase (formic acid production), alcohol dehydrogenase (ethanol production) and/or acetate kinase (acetic acid production) (Zhou et al. 2003a).

Moreover, the development of bacterial strains producing LA on chemically defined media (Zhou et al. 2003b) and strains improving blocking steps in the phage life cycle (Allison and Klaenhammer 1998; Forde and Fitzgerald 1999) is advanced.

Another way to more effective LA formation is the search for organisms that can tolerate high pH values. Alkaliphilic LAB strains may be promising producers of LA due to their tolerance to high pH levels that would minimize contamination problems during processing. Calabia et al. (2011) isolated an alkaliphilic *Lactobacillus halophilus* from a marine environment that produced 65.8 g/L of L-LA at pH 9.0. By comparison, the strategy of NatureWorks as the main global PLA producer is directed on the yeast fermentation at lower pH, thereby significantly reducing the use of calcium hydroxide and sulphuric acid, in turn resulting in significantly lower quantities of gypsum together with less energy demand for the entire process (Vink et al. 2010).

Also not yet used for the formation of LA are organisms such as microalgae and cyanobacteria. These photosynthetic microorganisms offer an alternative LA production approach and would allow carbohydrate feedstock costs to be eliminated. It has long been known that some microalgae have the ability to convert the starch they accumulated under light and aerobic conditions into organic matter, such as LA, ethanol, acetic acid and formic acid under dark and anaerobic conditions (Hirayama and Ueda 2004; Oost et al. 1989). There are also reports about the LA production by the microalgal species *Nannochlorum* sp. 26A4 from about 26 g/L D-LA production with an optical purity of 99.8 % from their starch (40 % content per dry weight) at yield of 70 % under dark and anaerobic conditions (Hirayama and Ueda 2004).

New strains with new properties alone will not lead to more efficient production of LA, but only in interaction with new raw materials, progress in fermentation technology as well as downstream processing development. Because of the relatively low price of LA, one of the major challenges in its large-scale fermentative production is the cost of the raw material. Lactic acid can be produced from a wide spectrum of carbon sources including starchy materials, many food industry by-products (e.g. molasses, whey), agro-industrial residues and by-products (e.g. lignocellulose hydrolysates, cottonseed hulls, corn cob, corn stalks, wheat bran, brewer's spent grains) and various other renewable resources. Together with the need of low-cost carbon, there is an additional demand of suitable supplements, which should not cause additional costs and problems in view of impurities. Therefore, the kind of nutrients as well as the optimization of their concentration is essential. It is likely that one of the future trends in lactic acid production will end up in mixtures of different low-cost raw materials in order to avoid the use of expensive complex supplements (Taskila and Ojamo 2013; Koutinas et al. 2014).

Besides the strain optimization and alternative raw materials, the transition from traditional batch including repeated batch and fed-batch fermentation to continuous mode fermentation (Dey and Pal 2013; Gao and Ho 2013) with cell recycle (Venus 2009; Wee and Ryu 2009; Lee et al. 2014) as solutions with free cells and the use of immobilized cells in different reactor types (fixed or fluidized bed) could lead to further performance improvement. The number of downstream processing steps strongly influences the quality and the price of the product. Thus the total costs are determined mainly by the purification rather than by LA production using fermentation (Reimann 2006). Open sources provide only limited data about industrial product recovery processes, but the main technology steps are known for large-scale production of carboxylic acids and ongoing research activities are widely discussed (López-Garzón and Straathof 2014). If the disadvantages of traditional fermentation and recovery process are overcome combined with the huge amount of gypsum as a by-product, significant progress for lactic acid production can be expected in the near future.

Furthermore methods for combining the fermentation of lactic acid and production of chemical sequence products derived from lactic acid are required for the development of intermediates and speciality chemicals. A worthwhile approach could be the direct fermentation on organic lactates, such as substituted aminium lactates.

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Microbial Lactone Synthesis Based on Renewable Resources

Robert Kourist and Lutz Hilterhaus

Contents

Abstract Lactones are important flavor compounds and find frequent application in the production of food and as building blocks in fine chemical synthesis. The microbial production of optically pure lactones benefits from the high selectivity of biocatalysts and therefore simplifies downstream processing. A summary of different processes applying microorganisms or whole cells for the synthesis of different lactones is presented, starting at the cell metabolism and analyzing the hurdles in view of process development. Prominent examples are the synthesis of γ-decalactone using fungal strains, the Baeyer–Villiger oxidation using recombinant whole cells, and the γ-valerolactone synthesis.

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1 Introduction: Microbial Lactone Synthesis

Lactones are cyclic esters of hydroxy fatty acids. Applications of this versatile class of compounds span from precursors for the synthesis of polymers to high-value, optically pure fragrance compounds. The possibility to produce lactones by microbial fermentation and biotransformation using biogenic carbohydrates and lipids as starting material makes them promising bio-based commodities. Chemical routes for their synthesis often use petrol-based chemicals. Moreover, the chemical synthesis often leads to undesired racemic mixtures, which require further downstream processing. Lactones produced by microbial cultures need to be isolated afterward for use as additives in food manufacture, too. However, these additional isolation and purification steps circumvent the discrimination of two enantiomers and therefore are easier to achieve. Another advantage of the production in microorganisms lies in the possibility to label the resulting product as "natural," which is an important commercial advantage. Examples for lactones as aroma compounds produced by microbial cultures are δ-decalactone, γ-decalactone (γ-DL), δ-dodecalactone, δ-octadecalactone, or (Z)-6-dodecen-δ-lactone (Asunción Longo and Angeles Sanromán 2006).

Biotransformations of precursors for the production of lactones have been established on industrial large scale to produce chiral building blocks. Green and sustainable aspects of such processes are renewable biocatalysts and generally lower toxicity of processes. Several microorganisms are able to convert hydroxy fatty acids to optically pure compounds. The biotransformation is based on chain-shortening reactions using enzymes from the β-oxidation—a reaction cascade which would be extremely difficult to use in a cell-free system. A highly interesting reaction for biotransformation is the Baeyer–Villiger oxidation. This reaction is mostly carried out with whole-cell systems, because of enzyme stability and efficient cofactor recycling. These processes enable access to a multitude of versatile building blocks for asymmetric synthesis (Mihovilovic 2012).

Very recently, lactones have received increasing attention as potential renewable platform chemicals. Perhaps the most prominent bio-based hydroxy fatty acids lactic acid, whose cyclic ester of two lactate molecules serves precursor for the synthesis of bio-based polymers. Fermentative production of hydroxyl–carboxylic acids from agro-industrial waste is an alternative to the synthesis from dwindling fossil resources (Füchtenbusch et al. 2000). The enzymatic machinery for the production of polyhydroxyalkanoates (PHA) in bacteria offers catalytic pathways for the production of these lactone precursors (Efe et al. 2008). Recent examples include the microbial synthesis of γ-butyrolactone and γ-valerolactone. Particularly γ-valerolactone is of importance and ranks among the top key components of the biomass-based economy. Microbial processes thus offer the perspective of a sustainable fermentative production of optically pure renewable lactones.

Already in 2002, Vandamme and Soetaert summarized the hurdles of the application of microbial strains for the synthesis of flavor compounds. Next to low yields of 100 mg L^{-1} a further challenge was the fact that many flavor

Table 1 γ-Decalactone produced by cultured microorganisms (Figueiredo et al. 2010)

Cultured microorganisms	Substrate	γ-Decalactone concentration	Reference
Yarrowia lipolytica	Castor oil or methyl ester of ricinoleic acid	Up to 12.3 g L^{-1}	Rabenhorst and Gatfield (2002)
Candida sp.	Castor oil hydrolysate	10 g L^{-1}	Farbood and Willis (1985)
Yarrowia lipolytica	10 % v/v castor oil or 0.05 % decanoic acid	Up to 6.9 g L^{-1}	Farbood and Willis (1985)
Rhodotorula aurantiaca	Castor oil	6.6 g L^{-1}	Alchihab et al. (2009)
Sporobolomyces odorus	Hydrolyzed castor oil	5.5 g L^{-1}	Lin et al. (1996)
Aspergillus oryzae	Castor oil	0.86 g L^{-1}	Farbood and Willis (1985)
Trichoderma harzianum	Castor oil	0.260 g L^{-1}	Serrano-Carreon et al. (1997)
Geotrichum klebahnii	Castor oil	0.2 g L^{-1}	Farbood and Willis (1985)
Moniliella suaveolens	Castor oil press cake	0.180 g kg^{-1} dry matter	Laufenberg et al. (2004)
Piptoporus soloniensis	Liquid media of yeast extract and glucose	0.008 g L^{-1}	Kenji et al. (2002)
Sporidiobolus salmonicolor	Vinasse and ricinoleic acid methyl ester		Billerbeck et al. (2003)

compounds or their added precursors are inhibitory or even toxic at high levels to the producer strains. Therefore, strategies like fed-batch fermentation and in situ product removal are technologies which could help to circumvent these limitations (Vandamme and Soetaert 2002). The different hurdles as well as recent publications to overcome them will be discussed.

In case of the microbial synthesis of γ-DL, different strains were identified to synthesize this product in high yields. Depending on the substrate and strain, different product concentrations were achieved and are summarized in Tables 1 and 2.

Next to these fatty acids, hydroxy fatty acids can be used as substrates for δ-lactone synthesis via biotransformation in bacterial cells. *Clostridium tyrobutyricum* uses 5-hydroxy-2-decenoic acid or 5-hydroxy-2-dodecenoic acid to produce δ-decalactone (5.95 g L^{-1}) or δ-dodecalactone (13 g L^{-1}) (Pichersky 1998). *Bacillus subtilis* and *Pseudomonas* sp. transform massoia lactone to δ-decalactone (Gocho et al. 1998). Therefore, in case of γ-DL, δ-decalactone, and δ-dodecalactone, the hurdle to achieve product concentrations far above 100 mg L^{-1} can be overcome by screening and choice of appropriate microorganisms.

The conversion of fatty acids into lactones proceeds via an initial hydroxylation of the fatty acyl chain, shortening of the chain by β-oxidation, and lactonization.

Table 2 γ-Decalactone produced by biotransformations of precursors using microorganisms (Figueiredo et al. 2010)

Biotransformations	Substrate	γ-Decalactone concentration	Reference
Candida boidinii	Ricinoleic acid	40.9 g L^{-1}	Mitsuhashi and Limori (2006)
Mucor circinelloides	Ethyl decanoate, ethyl caprinate	10.5 g L^{-1}	Kümin and Münch (1998)
Sporidiobolus ruinenii	Methyl ricinoleate	5.5 g L^{-1}	Dufossé et al. (1999)
Yarrowia lipolytica	Methyl ricinoleate	5.5 g L^{-1}	Wache et al. (2000, 2001)
Sporidiobolus salmonicolor	Ricinoleic acid	0.132 g L^{-1}	Lee et al. (1998)
Sporidiobolus salmonicolor	Ricinoleic acid	0.040 g L^{-1}	Billerbeck et al. (2003)

These reactions may be performed within one cell or in several distinct process steps. The first important step in the conversion of fatty acids to lactones is synthesis of hydroxy fatty acids. The availability of a certain lactone is limited by the availability of the corresponding hydroxy fatty acids. A prominent source is castor oil, which contains a large amount of ricinoleic acid (Biermann et al. 2011). The hydroxy group is inserted by a non-heme hydroxylase, which is closely related to an oleate-12-desaturase (Broun et al. 1998).

These examples illustrate the variety of species for the production of lactones. Within the following subchapters, the focus is set on microbial processes, the involved enzymes and metabolic pathways, the hurdles for the implementation of bioprocesses, as well as strategies to overcome these. Starting with fermentative routes, also whole-cell synthesis and alternative biocatalytic approaches are discussed.

2 γ-DL Production

γ-DL has a distinct peach flavor and is the most important lactone for food production. Biotransformation in fungi has made the commercial production of the "natural"-labeled compound feasible. While *Yarrowia lipolytica* is the mostly used production organism, other microorganisms such as *Fusarium poae*, *Tyromyces sambuceus*, or *Sporidiobolus salmonicolor* have been described (Alchihab et al. 2009). Usually, yields between a few mg up to 10 g L^{-1} can be achieved. Cells from *Candida sorbophila* produced γ-DL with a titer of 49 g L^{-1} (Mitsuhashi and Iimori 2004). Recently, Alchihab and coworkers identified several psychrophilic strains isolated in Antarctica as possible alternative producers. The best results were achieved with the yeast *Rhodotorula aurantiaca* A19, which led to

a production of 5.8 g L^{-1} in shake flasks at 14 °C (Alchihab et al. 2009). This productivity is already similar to those using genetically engineered *Y. lipolytica* strains.

Starting compound is ricinoleic (12-hydroxy-*cis*-9-ene octadecenoic) acid, which is available from castor oil. The yield of the biotransformation, however, is limited by a consumption of the fatty acid by the organism and side reactions leading to hydroxylated and unsaturated lactones. Within the peroxisomes of the yeast cells, β-oxidation leads to a shortening of the C18 chain to C10 and a reduction of the double bond, which is followed by spontaneous lactonization (Fig. 1). β-Oxidation proceeds via four steps: acyl-CoA oxidation, hydration, oxidation of the OH-group, and thiolysis. The regulation of β-oxidation is very complex and depends on the availability of coenzyme A, NADH, and oxygen. *Y. lipolytica* has five acyl-CoA oxidases with different chain length specificity. By inactivation of acyl-CoA oxidases with short-chain specificity, Waché et al. succeeded in increasing the productivity of γ-DL production to 10 g L^{-1} (Romero-Guido et al. 2011; Wache et al. 2001). Yeast strains with deleted acyl-CoA oxidases also showed a slower γ-decalactone degradation and, surprisingly, showed a much improved ratio of γ-DL formation over the undesired formation of hydroxy lactones. Formation of the latter appears to be connected with the availability of NAD^+, which in turn depends much on aeration. This might be an explanation for upscaling problems in the γ-DL production. Recently, Guo and coworkers combined deletion of short-specific acyl-CoA oxidase POX3 with overexpression of endogenous long-chain-specific POX2 (Guo et al. 2012). While the former was supposed to prevent γ-DL degradation, the latter should accelerate lactone formation. Indeed, formation of γ-DL increased from 0.9 g L^{-1} to 2.75 g L^{-1}. The new strain was still able to grow on fatty acids as carbon source.

In addition to the engineering of the cell metabolism, optimization of the fermentation conditions is still going on. When castor oil is used as substrate, the availability of free ricinoleic acid also plays a role in lactone formation. Recently, Braga et al. showed that induction of the endogenous lipase from *Y. lipolytica* leads to a faster formation of γ-DL. After induction with olive oil, maximal production was found after 140 h of culture, compared to 185 h in the non-induced control (Braga et al. 2012). Methyl ricinoleate or castor oil was supplied as oil-in-water emulsions. Gomes et al. investigated the effect of the washing of the cells and droplet size on γ-DL formation. Interestingly, no γ-DL formation was observed

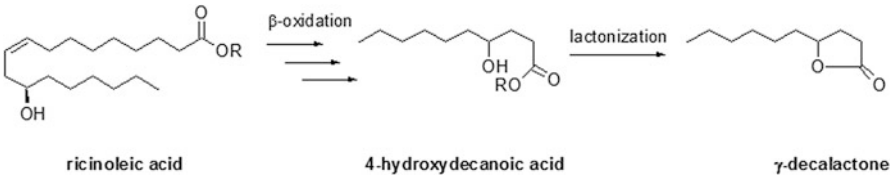

Fig. 1 Biotransformation of ricinoleic acid to γ-decalactone via shortening of the fatty acyl chain by β-oxidation and cyclization (Wache et al. 2001)

with castor oil as substrate and washed cells. Generally, yields where higher using non-washed cells, suggesting that this work-intensive step can be omitted. Larger oil droplets favored aroma production (Gomes et al. 2010, 2011b). Degradation of γ-DL starts when the substrate is consumed. To avoid this, and to minimize inhibitory effects of ricinoleic acid on the cells, Gomes et al. showed that fed-batch cultivation is an interesting alternative. In fed-batch using intermittent feed, they were able to obtain 6.7 g L^{-1}, compared to 1.9 g L^{-1} in batch fermentation. Production of the side product 3-hydroxy-γ-DL increased simultaneously to 10 g L^{-1}. However, due to a longer cultivation time of the fed-batch, the volumetric productivity of the batch (168 mg L^{-1} h^{-1}) was four times higher than that of the fed-batch (43 mg L^{-1} h^{-1}). Accessibility of the substrate is an important issue for the uptake into the cells. Recently, an experimental design study conducted by the same authors optimized the pH values and oxygen concentrations. Response surface methodology analysis showed that these two parameters are indeed critical for the productivity. The best results were achieved with pH 6.17 and 44 % dissolved oxygen concentration (Gomes et al. 2011a).

However, raw material and precursor costs in combination with the removal of unwanted side products and the preparative isolation of the desired products influence the industrial realization of microbial production processes for lactones. Therefore, strategies are asked for keeping the level of side products low. One possibility is the use of a second microorganism metabolizing the side products. In case of the synthesis of γ-DL from ricinoleic acid, the side product 3-hydroxy-γ-decalactone is formed as an intermediate. Because of a dehydration reaction, the 3-decen-4-olide is formed and accumulates during distillation in the distillation residues. The yeast *Saccharomyces cerevisiae* can be used afterward to reduce this compound stereoselectively yielding γ-DL (Gatfield 1995; Vandamme and Soetaert 2002). This strategy allows the conversion of a side product after downstream processing to increase the overall yield of the process (Fig. 2).

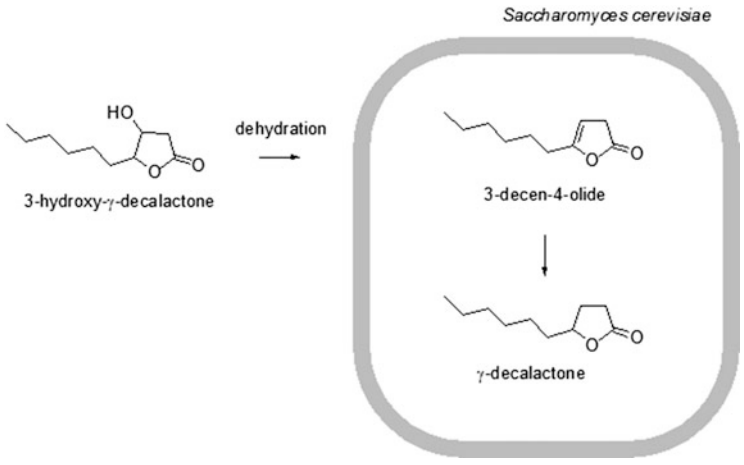

Fig. 2 Conversion of the side product 3-hydroxy-γ-decalactone into γ-decalactone by *Saccharomyces cerevisiae*

A further factor influencing the formation of 3-hydroxy-γ-decalactone is the oxygen transfer rate. The concentration of dissolved oxygen and the oxidative state of the medium influences the pathways of *Yarrowia lipolytica* (Aguedo et al. 2005; Fickers et al. 2005; Kamzolova et al. 2003). In the case of the γ-DL synthesis, the oxygen influence on the β-oxidation is of great importance. From the bioengineer's point of view, the oxygen transfer rate from the gas to the liquid can be improved by increasing the aeration and stirring rates which enhances the k_La value. Alternatively, the air or oxygen pressure of the gas phase directly influences the dissolved oxygen concentration according to Henry's law.

Aguedo et al. investigated the influence of k_La and oxygen pressure on the production of γ-DL (Aguedo et al. 2005). The use of pressure improved the production but increased the concentration of its oxidation compounds. The increase of k_La led to an increase of 3-hydroxy-γ-decalactone and to a smaller extent of decenolides. However, increase of k_La over a certain threshold caused a substantial decrease of all produced compounds, possibly due to the upregulation of the pathway including 3-hydroxy-acyl-CoA dehydrogenase. In summary, the β-oxidation in *Yarrowia lipolytica* is sensitive to the oxidative state of the medium. The dependency on k_La in biphasic medium was also shown by Gomes et al. (2007). Further, the influence of operating conditions like gas flow rate and organic phase composition upon the gas–liquid interfacial area and mass transfer coefficient was analyzed by Gómez-Díaz et al. (2009), illustrating the effect of the reactor setup on the parameter k_La in case of the fermentation of *Yarrowia lipolytica*. A broader study applying the 3^2 level full-factorial design was used to determine the effect of pH in the range of 4.5–6.7 and dissolved oxygen concentration (10–50 %) on the production of γ-DL and 3-hydroxy-γ-decalactone (Gomes et al. 2011a). This study also identified a threshold for dissolved oxygen concentration above which a decrease of 3-hydroxy-γ-decalactone yield was found.

Alternatively, the supply of oxygen can be improved by the use of a second liquid phase of perfluorocarbon, an oxygen-carrying compound, in the culture medium. Here, the availability of oxygen to the microorganisms is increased, and it was found that the specific growth rate of *Y. lipolytica* increases with increasing perfluorocarbon concentration. Most importantly, Amaral et al. (2008) could show that the perfluorocarbon presence lead to an increase of lipase production and not just its secretion to the extracellular medium. Further studies of the same group illustrate that from the bioengineer's point of view the reactor volume, the type of impellers and their position, the organic phase concentration, the aqueous phase composition, and the concentration of inactive biomass are important parameters. In summary, the addition of perfluorodecalin promotes k_La enhancement, while olive oil decreases the overall k_La of the multiphase reactor. Olive oil was used as an inducer for lipase production and also displays very high oxygen solubility (Amaral et al. 2008).

Product recovery is an important step in bioprocesses aiming for products showing a high volatility and low solubility as well as limiting the toxicity of the lactone toward the microorganism. Commonly for the separation from aqueous

media specific membranes or adsorption processes are applied (Edris et al. 2003; Ismadji and Bhatia 2000; Medeiros et al. 2006). The adsorption of γ-DL onto activated carbon and hydrophobic resins by online extraction was reported. However, the presence of these adsorbers in the bioconversion medium also decreased the production of γ-DL (Dufossé et al. 1999; Souchon et al. 1998). Porous organic resins benefit from their hydrophobic nature and their high specific surface area. The adsorption of γ-DL onto Macronet resins (a macroporous polystyrene cross-linked with divinylbenzene) is a method for extracting γ-DL from the culture broth of *R. aurantiaca*, as shown by Alchihab et al. (2010). The studied resins are not chemically reactive with the aroma compounds; they can be easily regenerated and have a low price. Among the tested resins, MN-202 was the most efficient adsorbent for γ-DL.

Fed-batch fermentation is often applied in the production of primary and secondary metabolites, proteins, and biopolymers, because a higher cell density than in batch mode can be achieved (Shiloach and Fass 2005). These high cell densities allow high yields and productivities of desired products. Considering the potential toxicity of the precursors like ricinoleic acid in case of the production of γ-DL, the use of fed-batch fermentation becomes an obvious choice. Different studies were published illustrating the usefulness of fed-batch fermentation for the production of γ-DL (Kapfer et al. 1989; Kümin and Münch 1998; Lee et al. 1995). A recent study of Gomes et al. (2012) illustrates the high potential of fed-batch operation for the *Y. lipolytica* fermentation using methyl ricinoleate as a precursor. The authors compared the constant medium feeding and intermittent fed-batch fermentation with classical fermentation strategies. Although the productivity in view of γ-DL was considerably higher in batch mode (168 mg L^{-1} h^{-1} in comparison to 43 mg mL^{-1} h^{-1}), the substrate conversion to lactone was greater in the intermittent fed-batch yielding 6.8 g L^{-1} instead of 2 g L^{-1} (Gomes et al. 2012).

Another recent study compared batch and fed-batch cultivation for the production of γ-DL using *Yarrowia lipolytica* and castor oil as substrate (Moradi et al. 2013). Also the effect of enhancing oxygen transfer rate by using higher agitation rates or pure oxygen for aeration was investigated. The highest γ-DL concentration (220 mg L^{-1}) was obtained in the fed-batch fermentation using pure oxygen which was three times more compared to the batch cultivation (65–70 mg L^{-1}). Using pure oxygen instead of atmospheric air in the fed-batch fermentation also resulted in 60 % increase of γ-DL production. These studies support the necessity of the control of oxygen as well as precursor concentration in the fermentation medium and the understanding of the involved pathways for the optimized production of lactones.

A highly interesting approach is the use of a lipase to hydrolyze castor oil and yielding ricinoleic acid as a substrate for, e.g., *Yarrowia lipolytica*. Recently Goswami et al. (2013) published a review on the use of lipases for the hydrolysis of castor oil. The activity of lipases of different origins like plants, fungi, and bacteria is compared and clearly illustrates the potential for processes with lower energy consumption, lower temperature, and lower pressure and with higher quality of the final product. Lipases from *Aspergillus niger*, *Aspergillus oryzae*, *Rhizopus*

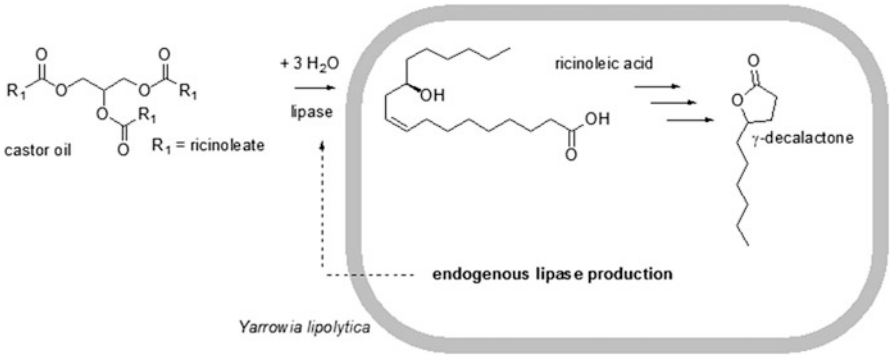

Fig. 3 Conversion of castor oil into ricinoleic acid, the carbon source and precursor for γ-decalactone production by *Yarrowia lipolytica*

delemar, *Humicola lanuginosa*, *Galactomyces geotrichum*, and *Penicillium* sp. were investigated in view of the hydrolysis of castor oil. Different activities were found with reaction times between 1 h and 4 days. Next to these fungal lipases, also bacterial lipases show activity toward this hydrolysis reaction. Neto et al. (2004) showed that the addition of enzymatically hydrolyzed castor oil to the culture medium enhances γ-DL production by *Geotrichum* sp. Braga et al. (2012) published the use of lipase induction in *Yarrowia lipolytica* for castor oil hydrolysis and its effect on γ-DL production (Fig. 3). This approach appears highly interesting, because only one microorganism is involved. The lipase formed by *Yarrowia lipolytica* generates its own carbon source (ricinoleic acid) and the substrate for the bioconversion to yield γ-DL. The goal of this study was to accelerate the hydrolysis of castor oil and in that way to improve the production of γ-DL. The highest values of extracellular lipase activity were obtained with the strain *Y. lipolytica* W29, and with the addition of a lipase preinduction step (using olive oil), it was possible to obtain a gain in the overall process time. Without preinduction of lipase, the lactone production was detected after 120 h, with preinduction after 60 h. However, there might be a limitation because of a possible limitation of the activity of lipase from *Y. lipolytica* toward castor oil.

In summary, different approaches were already realized to overcome hurdles of the application of microbial strains for the synthesis of flavor compounds. Yields of much more than 100 mg L^{-1} can be obtained by choosing the appropriate microorganisms. The inhibitory effect of produced lactone can be overcome by fed-batch fermentation, and effects by added precursors can be minimized by adsorption or extraction. Next to these strategies, the influence of the oxygen concentration has to be considered as well as the availability of the substrate. In all cases, for the bioprocess engineering, a detailed understanding of the regulation of different pathways is of great advantage to achieve maximum yields and product concentrations. Also the coupling of steps as shown with the example of lipase catalyzed hydrolysis of castor oil and subsequent conversion to γ-DL illustrates the

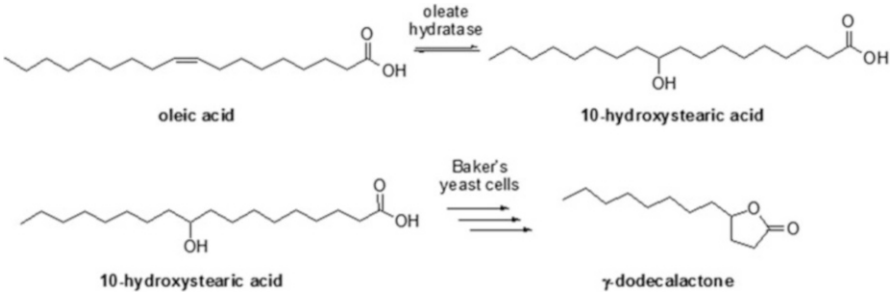

Fig. 4 Enzymatic hydration of unsaturated fatty acids to produce hydroxystearic acid, a precursor of γ-dodecalactone

importance of the mathematical description of enzyme kinetics and growth behavior of microorganisms as well as the microbial transformations. If an accurate adjustment of the kinetics of the different steps is achieved, the long-term stability of the process at maximum productivity can be obtained.

The synthesis of γ-DL is the most prominent example for the microbial production of fragrance lactones. Nevertheless, the biotransformation route via β-oxidation and lactonization (Fig. 1) in yeast can be applied for the production of several lactones provided that a suitable hydroxy fatty acid as starting material is available. For example, ricinoleic acid, the starting compound for the production of γ-DL, can be isolated in large quantities from castor oil. However, other hydroxy fatty acids are more difficult to obtain. For instance, researchers from Takasago International Corporation reported the conversion of 11-hydroxy palmitic acid ethyl ester to δ-decalactone by the yeast *Candida sorbophila* (Mitsuhashi and Iimori 2004). 0.13 g substrate in 30 mL culture medium yielded 0.019 g δ-decalactone in 96 % ee optical purity after 11 days.

γ-Dodecalactone (γ-DoL) is a known whisky flavor. Conversion of 10-hydroxystearic acid (HSA) by Baker's yeast (Fig. 4) formed (*R*)-γ-DoL with a yield of 22 % (Gocho et al. 1995). However, the supply of optically pure (*R*)-10-HSA is difficult. Unlike ricinoleic acid for γ-DL production, HSA does not occur in large quantities in vegetable oils. Very recently, hydratases came into the focus as promising catalysts for a wide range of applications (Jin and Hanefeld 2011). In particular, the hydration of unsaturated fatty acids by enzymatic hydration is a straightforward route to hydroxy fatty acids. Unlike P450-monooxygenases, enzymatic hydration shows excellent product specificity.

Wallen et al. described the hydration of oleic acid to hydroxystearic acid by a *Pseudomonas* strain 3266 already in the 1960s (Davis et al. 1969). Several bacterial strains have been isolated that are able to hydrate oleic acid and in some case catalyze the subsequent oxidation to 10-ketostearic acid (Heo and Kim 2009; Kuo et al. 2000, 2002; Kuo and Levinson 2006). The reaction shows moderate stereospecificity (Gocho et al. 1995). Two groups cloned independently the hydratases from the *Pseudomonas* strain 3266 (Bevers et al. 2009) and *Streptococcus pyogenes*

(Volkov et al. 2010). Since then, several oleate hydratases have been cloned and overexpressed in *E. coli*. A BLAST search of the sequence showed that this enzyme had been previously known as myosin-cross-reactive antigen. The flavin-dependent hydratase may be involved in the production of conjugated linoleic acid. Moreover, there is some evidence that it plays a role in stress tolerance. It could be shown that cells from *Lactococcus* and *Corynebacterium* transformed with the hydratase show an increased viability under heat and solvent stress (Rosberg-Cody et al. 2011). Recently, hydratase was applied in the conversion of ricinoleic acid into a novel dihydroxy fatty acid (Seo et al. 2013). Recombinant oleate hydratases from *Stenotrophomonas maltophilia* and *Lysinibacillus fusiformis* in *E. coli* were successfully applied for the synthesis of 10-hydroxystearic acid with 40 g L^{-1} h^{-1} (Joo et al. 2012; Kim et al. 2012). It can be expected that the efficient supply of 10-HAS by enzymatic hydration will greatly facilitate the microbial production of γ-DoL. Interestingly, δ-DoL was recently isolated from the supernatant of *Lactobacillus* cultures from kimchi, a traditional fermented Korean dish. The authors could show antifungal activity toward *Aspergillus*, *Cladosporium* sp., *Penicillium*, and *Candida*, which might contribute to the conserving properties of the traditional fermentation (Jang and Yang 2011).

3 Baeyer–Villiger Oxidation for Lactone Production

A recently published review summarizes the use of whole-cell biocatalysis for selective and productive C–O functional group introduction and modification (Schrewe et al. 2013). Next to the fermentative routes, such whole-cell processes represent an interesting way to obtain lactones. Within the different host organisms for Baeyer–Villiger monooxygenases (BVMOs) as biocatalyst *E. coli* plays a very prominent role. BVMOs are a group of enzymes that can catalyze the stereoselective oxidation of ketones yielding esters (Kelly et al. 2001). These processes will be discussed in the following. However, one should keep in mind that also various fungal isolates are able to carry out this interesting reaction as a classical microbial biotransformation (Fantin et al. 2006). The authors isolated different microorganisms from environmental samples and found *Fusarium* and *Aspergillus* strains capable to carry out a Baeyer–Villiger oxidation. Also, the discovery of the first Baeyer–Villiger biotransformation was observed in 1948 in fungi (Turfitt 1948). Nevertheless, the bigger part of studies on BVMO processes is using recombinant whole-cell catalysts.

The Baeyer–Villiger oxidation reaction is especially interesting for the oxidation of cyclic ketones into their corresponding lactones. One of the best-characterized BVMO is the cyclohexanone monooxygenase (CHMO) from *Acinetobacter calcoaceticus*. The recombinant expression of this enzyme was successful in *Saccharomyces cerevisiae* (Stewart et al. 1998) and *Escherichia coli* (Doig et al. 2001; Mihovilovic et al. 2001). A major limitation of the industrial implementation of BVMOs is the need for efficient recycling of the cofactor NADPH (Baldwin and Woodley 2006). Therefore, the process development has been

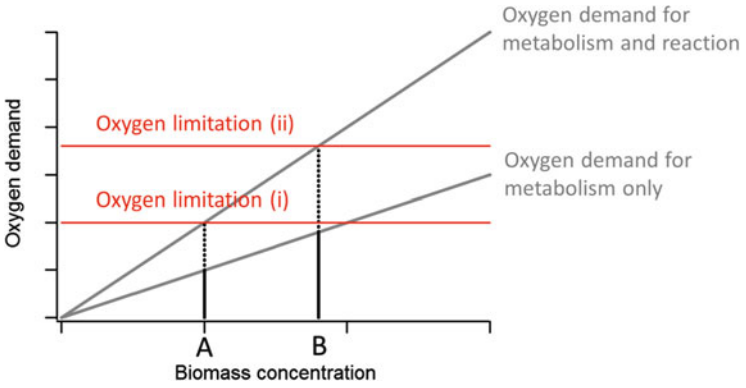

Fig. 5 Conceptual model proposed by Baldwin and Woodley showing the effect of biocatalyst concentration on oxygen demand (Baldwin and Woodley 2006). At a biomass concentration of A, the concentration is optimized for the level of oxygen provided, increasing biomass concentration to B will not increase rate of reaction, unless oxygen supply is increased to a higher value

focused on the use of whole cells, using glycerol or glucose as a reducing equivalent (Walton and Stewart 2002). On the other hand, the lactone production using crude enzyme preparations (CHMO) and applying a second ancillary enzymatic system (glucose-6-phosphate dehydrogenase) was shown to be simple and easy to handle (Ottolina et al. 2005). However, the crude extract contained a dehydrogenase which caused a non-desired reduction. This drawback can be overcome using purified CHMO and illustrates therefore a potential advantage of an enzymatic process.

Further bottlenecks of the whole-cell biocatalytic process are to be addressed such as substrate and product inhibition, pH and temperature intolerance (Doig et al. 2003), as well as oxygen limitations. In the case of the oxidation of, e.g., bicyclo[3.3.0]-hep-2-en-6-one, the process limitations are the oxygen supply which limits the rate of the reaction, the product inhibition which limits the final yield, and the biocatalyst stability which limits the total reaction time (Baldwin and Woodley 2006). Here, with increasing cell concentrations, the dissolved oxygen tension drops to zero, and the initial specific reaction rate is reduced dramatically.

Figure 5 illustrates one basic problem of the BVMO biotransformation: the role of oxygen in limiting the rate of the reaction (Baldwin and Woodley 2006). The model assumes that oxygen is preferentially used by the microorganism for maintenance (oxidative phosphorylation). As soon as this need has been met, any remaining oxygen can be used in the BVMO reaction. For example, at a biomass concentration of A, there is an oxygen demand for the metabolism and an additional demand for the reaction. This demand can be fulfilled by the oxygen concentration present in the reaction medium [oxygen limitation (i)]. Increasing at constant oxygen concentration, the biomass concentration will reduce the amount of oxygen available for the reaction. Only an increase of the oxygen concentration in the reaction medium [oxygen limitation (ii)] will allow an increase of the reaction rate.

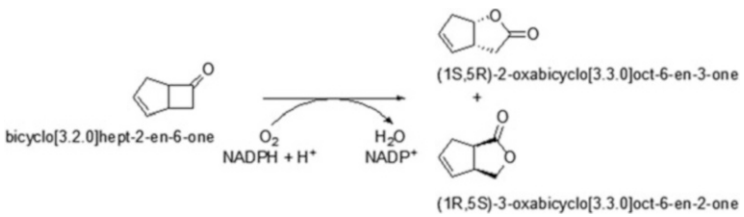

Fig. 6 Cyclohexanone monooxygenase (CHMO)-mediated regiodivergent oxidation of bicyclo [3.2.0]-hept-2-ene-6-one

Hilker et al. (2006) investigated the oxygen transfer rate and its effect on the biotransformation productivity. They could show that an improved mass transfer is beneficial to reach the optimal productivity also at a large scale. Because of the high efficiency of the whole-cell catalyst and the stoichiometric consumption of oxygen in the Baeyer–Villiger oxidation reaction, there is the need for an efficient oxygen supply to the reaction medium. The use of oxygen enriched air is limited because of safety issues. However, Hilker and coworkers achieved good results using a sintered metal sparger, and the oxygen transfer rate increased 40 % in case of the biotransformation of bicyclo[3.2.0]-hept-2-ene-6-one (Fig. 6).

Next to the oxygen supply, the inhibition kinetics of BVMO are of high interest to achieve an economic production of lactones. Substrate inhibition can be addressed using substrate feeding to maintain a low ketone concentration. This attempt aims for a maximal specific activity of the biocatalyst and high final product concentrations. Keeping the feeding rate below the maximum (Doig et al. 2002) could achieve a constant product formation at constant low substrate concentration. However, after 100 min, the product formation rate decreased, and consequently the substrate concentration began to increase. The explanation for the drop in activity was an inhibition by the formed product; because of the similarity of the product and the substrate and the hydrophilic nature of the lactones a selective continuous removal of the product is challenging.

The problem of inhibition, cell toxicity, and substrate solubility as well as substrate and/or product solubility was addressed by (Hilker et al. 2004). They carried out a whole-cell process combined with a “two-in-one” in situ “feeding and product removal” concept (SFPR) using an adsorbent resin (Fig. 7). This approach combines at the same time both in situ substrate feeding and in situ product removal. Thus, substrate and product concentrations in the reaction medium can be controlled to maximize cell productivity and enantioselectivity. The absorbent (macroporous resins) is added to the medium, and desorption of substrates takes place. After diffusion into the cells, the BVMO is oxidizing the substrate. The produced lactones leave the cell and are adsorbed on the resin. Different resins were tested for this reaction system, and Optipore L-493 and Lewatit VPOC 1163 turned out to be the best candidates. They exhibit a high inner surface and small pore diameters. The adjustment of the resin loading in the reactor is of great importance, because the concentrations of substrate and products must be kept under the inhibiting level. Three types of bioreactors were compared (Hilker et al. 2004): a

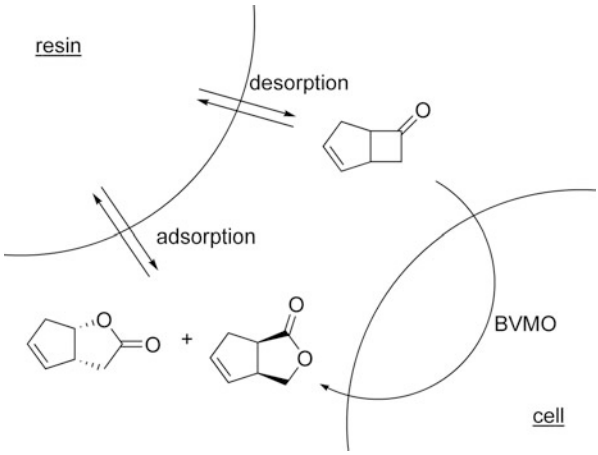

Fig. 7 Resin-based in situ substrate feeding and product removal concept (Hilker et al. 2004)

conventional fermenter, a recycle reactor (the resin is located in a fixed bed in an external loop), and a bubble column reactor. The best one proved to be the bubble column reactor, and both products were obtained in excellent enantiomeric purity (ee $>$ 98 %) and high yield. As an additional benefit, product extraction from the solid adsorbent resin considerably simplified downstream processing (Hilker et al. 2005). Moreover, these authors could show that the adsorbent resin is reusable, which would lower the costs of the entire process.

Another interesting application for adsorbents was shown by Fujii et al. (2007) using the hydrophobic polymer Amberlite XAD-7 to change the reaction selectivity of the microbial conversion of cyclohexyl methyl ketone. The reaction catalyzed by the organism *Dipodascus magnusii* NBRC 4600 in the absence of XAD-7 leads mainly to the reduced product 1-cyclohexylethanol, and the products of the Baeyer–Villiger oxidation reaction are hardly observed (0.2 %). The addition of small amounts of XAD-7 (100 mg XAD-7 per 3 mL) to the reaction system increased the yield of Baeyer–Villiger oxidation products (42 %). Although the cyclohexyl acetate was thought to be the primary Baeyer–Villiger oxidation product, hydrolysis took place and yielded cyclohexanol and acetic acid. Then, cyclohexanol was further oxidized to cyclohexanone (Fig. 8). Possibly, the observed effect is due to the substrate concentration in the aqueous medium. Nevertheless, in comparison to other microorganisms, the reducing power of *Dipodascus magnusii* is higher, and the BVMO reaction can only be observed when adding the resin.

The interest for enantiomerically pure compounds and high product yields led to the development of dynamic kinetic resolution processes. This approach combines an in situ racemization with a kinetic resolution step. Thus, a theoretical product yield of 100 % is possible. Enzymes and whole cells can contribute either to racemization or to kinetic resolution. BVMO were highly efficient to achieve asymmetric Baeyer–Villiger oxidation reactions with high enantioselectivity (Mihovilovic et al. 2002). Gutiérrez et al. (2005) investigated a dynamic kinetic resolution process using an anionic exchange resin to carry out the racemization

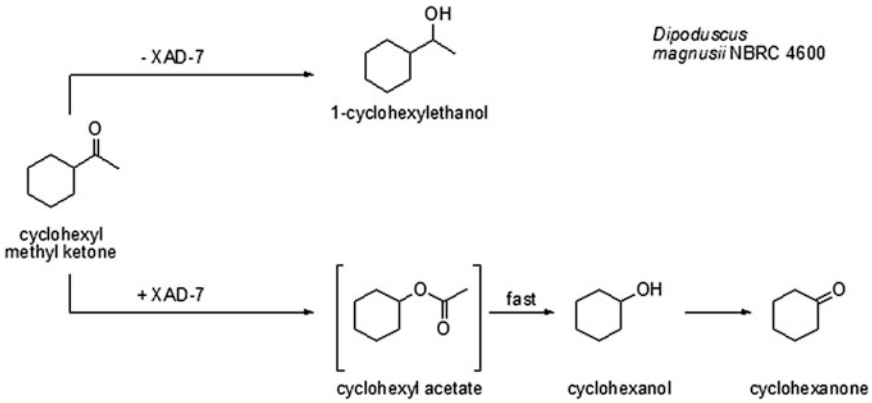

Fig. 8 Reaction of cyclohexyl methyl ketone by *D. magnusii* and effect caused by addition of the adsorbent XAD-7

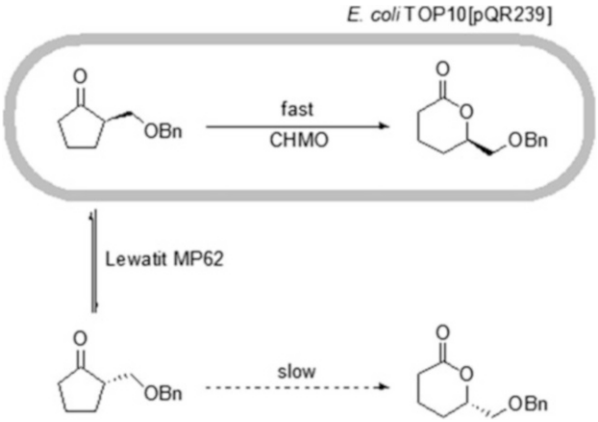

Fig. 9 Dynamic kinetic resolution of benzyloxycyclopentanone catalyzed by whole cells harboring CHMO from *Acinetobacter calcoaceticus* NCIMB 9871 and Lewatit MP62 for racemization

step (Fig. 9). The selective racemization of the substrate is based on the relatively high acidity of the proton on the Cα of the carbonyl group. Insertion of an oxygen atom stabilizes the product against racemization. In comparison to the base-catalyzed variant investigated beforehand, the loss of activity observed at pH 8.5 was reduced when using the resin (Gutiérrez et al. 2005). The authors could show that a satisfactory dynamic kinetic resolution process could be set up combining whole-cell-catalyzed Baeyer–Villiger oxidation and in situ racemization catalyzed by the weakly basic anion exchanger Lewatit MP62. Starting from racemic benzyloxycyclopentanone, nearly enantiopure lactone was obtained (97 % ee) with a yield of 84 %.

4 Lactones from Renewable Feedstocks as Future Platform Chemicals

Lactone production from biomass feedstocks would be a sustainable source of platform chemicals (Efe et al. 2008). Lactic acid is a well-known bifunctional molecule with a hydroxyl and a carboxyl moiety. Therefore, cyclic esters can be synthesized easily from this substance. The esters formed by two molecules of lactic acid are called lactides and can further be converted to polylactide which is a very interesting, biodegradable polymer (Doi and Steinbüchel 2002). The green biorefinery allows the production of lactic acid from green biomass via silage fermentation applying bacteria, e.g., *Lactobacillus plantarum*, *Lactobacillus casei*, *Pediococcus acidilactici*, and *Enterococcus faecium* (Langeveld et al. 2012). This intensively studied example will be used to shortly illustrate benefits and challenges of fermentation to produce small molecules like acids or lactones.

By anaerobic degradation of sugars, lactic acid is formed from silage. Starting from the vegetable carbohydrates, the glycolysis can produce pyruvate which is converted to lactic acid. Next to the carbohydrates, proteins can be degraded via deamination yielding oxoacids, which are converted to lactic acid, too. Using NAD^+ as redox equivalent, ethanol, propionate, butyrate, and lactic acid are formed from pyruvate (Kongo 2013).

The fermentative routes starting at the carbohydrates fructose or glucose can be subdivided into homofermentative and heterofermentative routes. Within the homofermentative process, sugars are exclusively converted to lactic acid. Heterofermentative bacteria produce significant amounts of other metabolites like acetic acid, ethanol, and glycol via a different pathway. Therefore, the industrial processes are desired which are based on the homofermentative route (Chang et al. 1999) to obtain high yields of the desired product from the substrate. Figure 10 illustrates the biochemistry of these two different routes in the case of lactobacilli.

The homofermentative route produces pure lactate from sugar applying the glycolysis pathway. To reduce pyruvate, the formed NADH is directly used yielding lactate, and the energy yield of this route is two molecules of ATP per molecule of glucose. Very small amounts of acetate, diacetyl, and acetoin are formed as side products leading to high yields. However, many homofermentative bacteria can be induced when applying pentoses as an additional substrate resulting in a heterofermentative process.

In comparison to the homofermentative processes, within the heterofermentative route, glucose is not converted by the glycolysis pathway but by the pentose phosphate pathway. Fructose-1,6-bisphosphate-aldolase and triosephosphate isomerase are not involved. The microorganisms are adapted to the utilization of pentoses as carbon source and in addition convert hexoses like glucose via this route. These pentoses are found when degrading plant material and are mainly xylose, ribose, or arabinose. They are converted via xylulose-5-phosphate which is converted to acetyl-phosphate and glycerinaldehyde-3-phosphate. Afterward,

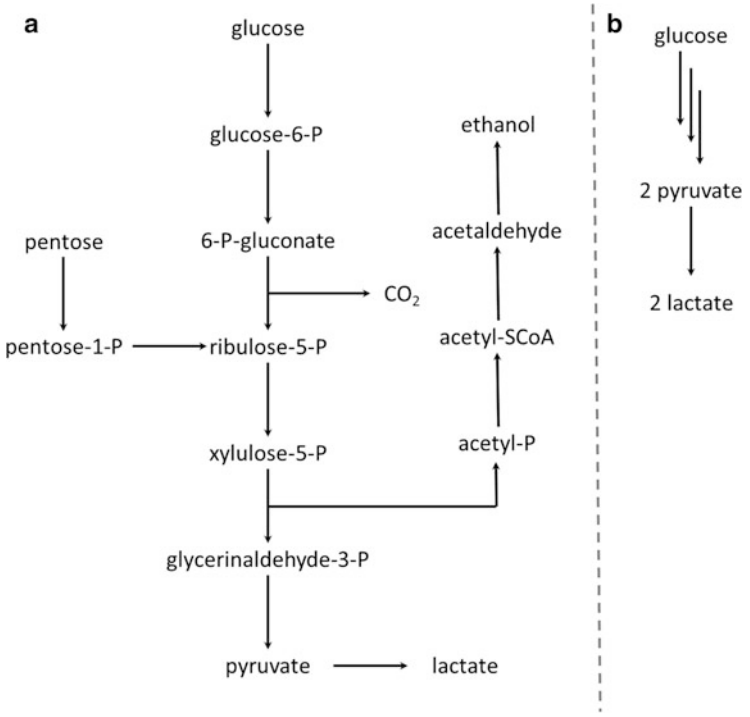

Fig. 10 (**a**) Heterofermentative and (**b**) homofermentative fermentation. The heterofermentative route yields lactate, ethanol, and CO_2 with an energy yield of 2 mol ATP per mole pentose. The homofermentative route produces pyruvate via glycolysis which is reduced to lactate with an energy yield of 2 ATP per mole hexose. For reasons of clarity, cofactors like ATP, NAD^+, and $NADP^+$ are not shown

glycerinaldehyde-3-phosphate is oxidized in the glycolysis yielding pyruvate and lactate.

These very prominent and well-described examples show the versatility of microbial conversions to yield small acids—in this case hydroxy acids. Central challenges of microbial conversions can be derived from this example, which are the carbon efficiency and the formation of side products. Therefore, there is a high interest in the productivity and yield of microbial processes and a detailed understanding of the underlying metabolic pathways. Tools of molecular biology and genetic engineering are used to optimize these pathways next to classical screening approaches identifying promising strains and species to produce small hydroxy acids as well as lactones.

The intramolecular esters of larger hydroxy fatty acids are receiving increasing attention due to their interesting chemical properties and their usefulness for a range of applications, including “green” solvents such as methyl tetrahydrofuran and new biofuels. Recently, the catalytic conversion of microbially produced polyhydroxybutyrates to tetrahydrofuran was suggested (Van et al. 2012). The catalytic process

produces γ-butyrolactone as intermediate, which is further hydrogenated to tetrahydrofuran. Van et al. used a genetically modified strain that increased the content of 4-hydroxybutyrate in the PHA over that of 3-hydroxybutyrate. 4-Hydroxybutyrate is formed via succinic acid semialdehyde (SSA) from the intermediates from the tricarboxylic acid cycle: α-ketoglutarate and/or succinyl-CoA. For the best strain, Van et al. reported a titer of poly-4-hydroxybutanoate of 46 % dry cell weight. Interestingly, overexpression of succinate semialdehyde dehydrogenase, which catalyzes conversion of succinyl-CoA to SSA, was crucial for the success, whereas overexpression of α-ketoglutarate decarboxylase did not lead to increased 4-hydroxybutyrate formation. Fermentative production of γ-butyrolactone would be an alternative to the production from petrol-based chemicals and the catalytic dehydration of pentoses from lignocelluloses, which requires the use of strong acids and high temperature. In conclusion, the investigation of fermentative PHA production as a future synthesis route for sustainable chemicals remains a thrilling field, which leads to expect further findings of this fascinating microbial machinery for polymer synthesis.

γ-Valerolactone (γ-VL) is under consideration as one of the main bases for a future bio-based economy as it is available in large quantities from biomass-derived feedstocks (Bozell 2010; Horvath et al. 2008). Potential applications range from a precursor of valeric biofuels (Palkovits 2010) to green solvents such as 2-methyl tetrahydrofuran (Geilen et al. 2010). It can be produced by chemical reduction from levulinic acid. Levulinic acid is a dehydration product of simple sugars like glucose, and the cellulosic fraction of lignocellulosic biomass can be converted into levulinic acid (Fig. 11). This makes levulinic acid an inexpensive starting material for the production of platform chemicals, green solvents, and even biofuels (Lange et al. 2010).

The first step for a utilization is the reduction of levulinic acid to 4-hydroxyvalerate (4-VL) followed by subsequent cyclization to γ-VL. Several groups have investigated the bioreduction of levulinic acid to 4-hydroxyvaleric acid and subsequent cyclization to γ-VL. A major obstacle lies in the difficulty to find suitable dehydrogenases with high activity for the reduction of levulinic acid, as no wild-type dehydrogenase for this substrate has been reported. Martin et al. used the observation that cells from *Pseudomonas putida* contain polyhydroxyalkanoic acid polymers with 4-HV monomers when grown on media supplemented with levulinic acid.

To redirect hydroxyvalerate production from PHA synthesis, they investigated the use of a *Pseudomonas* strain with systems for a removal of the CoA carriers from the hydroxy acids. Wild-type *Pseudomonas* overexpressing the gene of a recombinant thioesterase from *E. coli* yielded 14 g L^{-1} 4-hydroxyvalerate and 5 g L^{-1} 3-hydroxyvalerate as by-products (Fig. 12). They used minimal medium which was supplemented with glucose and levulinic acid (Martin and Prather 2009). Regarding the production of γ-VL, they faced the problem that the intramolecular cyclization of 4-hydroxyvaleric acid is pH dependent and that the equilibrium at pH 7.5 in the cytosol is unfavorable for lactonization. By using a variant of human paraoxonase I, they achieved a substantial increase in γ-VL production.

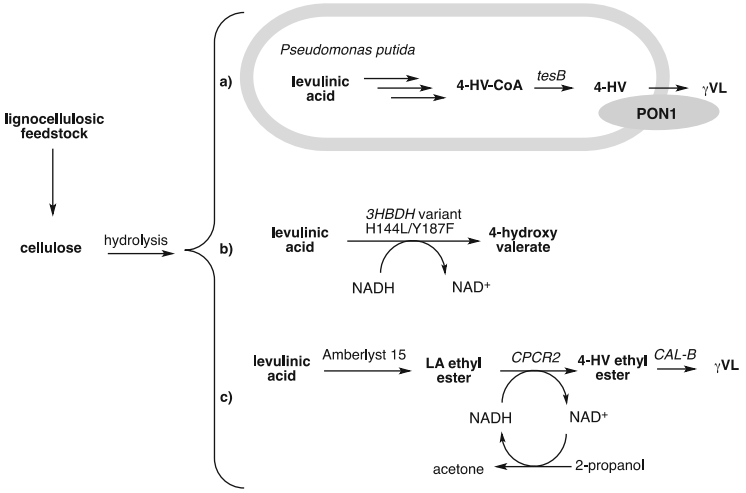

Fig. 11 Biotechnological approaches for the production of γ-valerolactone (γ-VL) from levulinic acid. The key step is the bioreduction of levulinic acid 4-hydroxyvalerate (4-VL), which subsequently can lactonize to γ-VL. (**a**) Biotransformation using *Pseudomonas putida* whole-cell overexpression of thioesterase *tesB* and paraoxonase I *PON1* (Martin et al. 2010). (**b**) A new variant of 3-hydroxybutyrate dehydrogenase *3HBDH* from *Alcaligenes faecalis* catalyzes the bioreduction of levulinic acid to 4-hydroxyvalerate (4-HV) (Yeon et al. 2013). (**c**) Chemoenzymatic route using carbonyl reductase from *Candida parapsilosis CPCR2* and lipase B *CAL-B* from *Candida antarctica* (Götz et al. 2013)

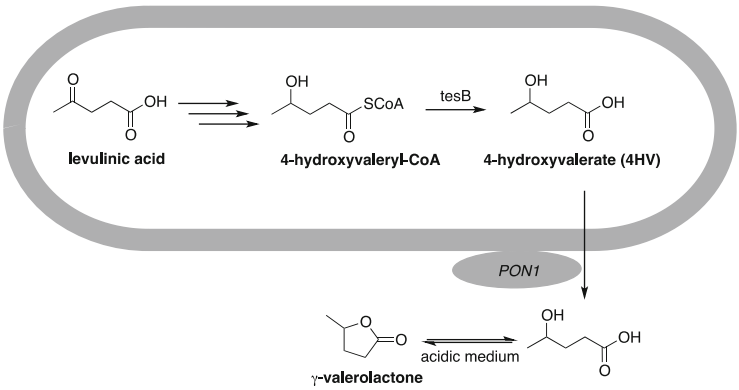

Fig. 12 Biotransformation of levulinic acid to γ-VL using whole cells from *Pseudomonas putida*, a thioesterase from *E. coli* (tesB) and extracytosolically expressed PON1. The cyclization at a low pH value is mediated by the human paraoxonase PON1. From Martin et al. (2010)

The important step was the extracytosolic expression of the paraoxonase I, which allowed to catalyze the pH-dependent lactonization in the acidic medium. Biotransformation and extracytosolic cyclization yielded 27 g L^{-1} 4-hydroxyvalerate and 8 g L^{-1} γ-VL, respectively (Martin et al. 2010). The enzymes involved in the

reduction of 4-VL remain unknown. The increase of 4-HV formation by the expression of a thioesterase suggests, however, that the substrate of the bioreduction is a CoA-ester of levulinic acid.

Use of isolated enzymes would avoid side reactions and thus lead to much higher molar yields. Very recently, Yeon et al. redesigned the active site of 3-hydroxybutyrate dehydrogenase (3HBDH) from *Alcaligenes faecalis* (Yeon et al. 2013). While the wild-type enzyme does not convert levulinic acid, a new variant produced 4-hydroxyvaleric acid with an activity of $k_{cat}/K_M = 578\ min^{-1}\ M^{-1}$, leading to 57 % conversion within 24 h. Based on a docking study of the substrate in the active site of the dehydrogenase, Yeon et al. investigated the potential interaction of levulinic acid with residues in the active site. They focused on the distance between the C4 atom of NADH and the carbonyl group and the distance between the oxygen atom of the active site residue Tyr155 and the carbonyl oxygen atom. 6 Residues with potential impact on the substrate specificity were identified. In an in vitro assay, only mutant H144L showed some activity. This was confirmed by a docking study of the six mutants, in which H144L mutant was the only one where suitable distances were observed. This mutant was used as initial variant for further mutagenesis studies. Double mutant H144L/Y187F showed a further 11-fold increased activity compared to the initial single mutant H144L. This study is an interesting example how protein engineering can create novel biocatalysts for practical applications. Alternatively, Hilterhaus et al. circumvented the lack of suitable dehydrogenases for levulinic acid conversion by using levulinic esters instead of the free acid as substrate. Carbonyl reductase from *Candida parapsilosis* showed excellent activity toward levulinic acid ethyl ester. They developed a chemoenzymatic process for the bioreduction of levulinic acid by using recombinant dehydrogenases and cofactor recycling (Götz et al. 2013)

All the abovementioned approaches depend on the supply of levulinic acid from cellulose. Two recent patents show a perspective for the production of levulinic acid and γ-VL from other feedstocks which would allow utilization of a much wider range of carbohydrates as renewable feedstocks. Kohei et al. have patented the fermentative production of levulinic acid from vanillic acid (Mase et al. 2012). Figure 13 depicts the reaction cascade, which starts with the ring opening by using the dioxygenase PcaHG. 3-Carboxy-cis,cis-muconate cycloisomerase PcaB and 4-carboxymuconolactone decarboxylase PcaC form muconolactone. β-Ketoadipate enol-lactone hydrolase PCaD forms 2-ketoadipic acid, which is subsequently decarboxylated to yield levulinic acid. This fascinating approach is a potential utilization of lignin-derived compounds such a ferulic acid.

Recently, the US company Arzeda has suggested a novel enzymatic pathway for the fermentive production of γ-VL (Fig. 14). The pathway starts with pyruvate, which is derived from the primary metabolism. Pyruvate decarboxylase converts pyruvate into acetaldehyde, which is added by an aldolase to another molecule of pyruvate to 4-hydroxy-2-oxo-pentanoic acid. A specific dehydrogenase reduces this intermediate to 2,4-dihydroxypentanoic acid, and a second catalyzes a selective oxidation of the hydroxyl group on C4 to give 2-hydroxy-4-oxo-pentanoic acid (2-hydroxy-levulinic acid). A hydratase eliminates water and produces 4-oxo-2-

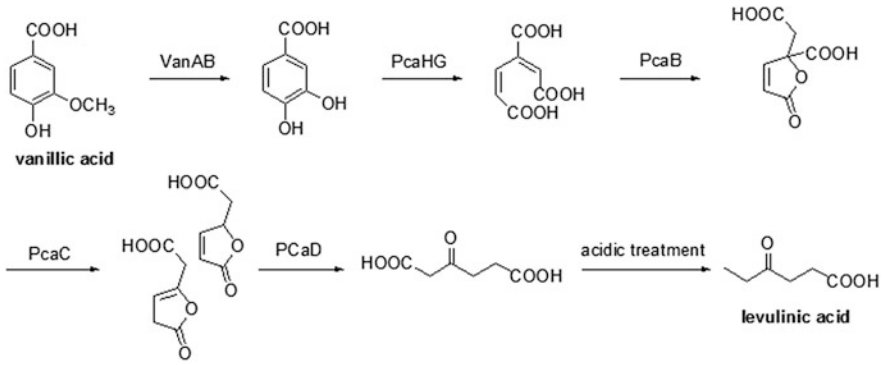

Fig. 13 Reaction sequence for the conversion of vanillic acid in recombinant cells from *Pseudomonas putida*. Abbreviations: *VanAB* vanillate O-demethylase, *PcaHG* dioxygenase, *PcaB* 3-carboxy-cis,cis-muconate cycloisomerase, *PcaC* 4-carboxymuconolactone decarboxylase, *PcaD* β-ketoadipate enol-lactone hydrolase

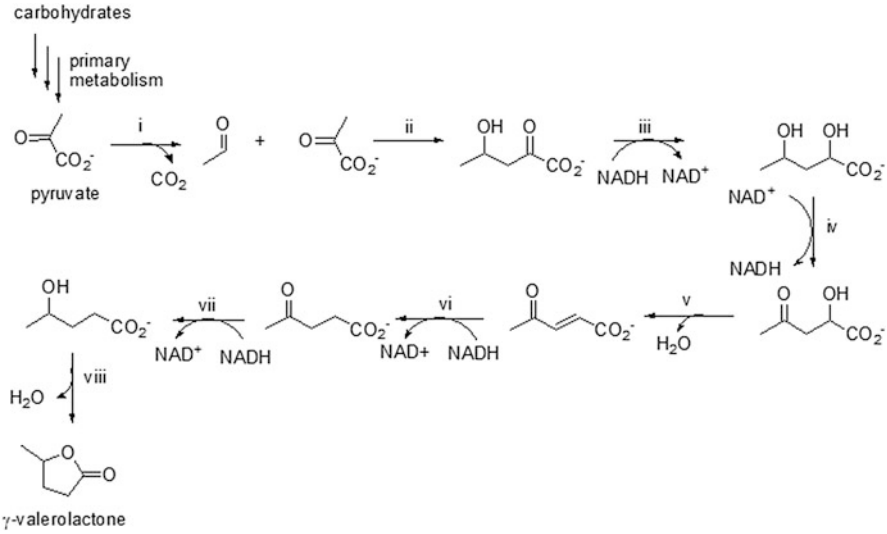

Fig. 14 The US company Arzeda suggested a biosynthetic pathway for the conversion of carbohydrates to γ-valerolactone. i: pyruvate decarboxylase; ii: aldolase; iii, iv, vii: specific dehydrogenases; v: dehydratase; viii: spontaneous lactonization

pentenoic acid, which is again reduced by an enoate reductase to levulinic acid. Levulinic is then reduced by a third dehydrogenase to 4-hydroxypentanoic acid and undergoes spontaneous cyclization to γ-VL. This impressive concept combines enzymes from different sources and pathways to a new metabolic pathway (Zanghellini 2012). It will be thrilling to get to know how all the practical

challenges of the approach will be met, particularly in view of the specificity of dehydrogenases in the presence of several keto acids and acetaldehyde.

Conclusion

Due to the foreseeable depletion of fossil resources, biomass represents a renewable feedstock for the production of different chemicals. Cellulose and hemicellulose can be degraded from vegetable waste by using different thermal processes as well as efficient enzymes to small sugar molecules. These carbohydrates can be used for the production of bioethanol or for fine chemicals in biorefineries. Lignocellulosic biomass typically contains more than 50 % sugar by weight, and valuable platform molecules such as levulinic acid and γ-valerolactone can be obtained therefrom. Next to the potential as platform chemicals to produce different derivatives, lactones represent important flavor compounds and find frequent application in the production of food. They can be produced from fatty acids, which are itself renewable resources. In the last decade, different hurdles of the application of microbial strains for the synthesis of lactones were found and overcome. Yields of more than 10 g L^{-1} were achieved, enabling economically feasible processes. Challenges regarding inhibitory or toxic effects of precursors or products within in microbial conversion were overcome by bioprocess engineering. In future, this story of success must be extended in view of the substrates applied. Here, new origins for precursors must be found, and downstream processing needs to be established. Next to the wild-type microorganisms, the exemplified BVMO processes illustrate the potential of recombinant microorganisms to achieve new products. However, most of these products are formed from substrates which are not originating directly from renewable resources. Here, further research and development needs to be carried out to broaden the range of biomass-derived substrates for lactone synthesis. In conclusion, the high selectivity and the synthesis of optically pure lactones is the outstanding property of microbial production processes. Applications range from potential bulk chemicals to high-value products that have the potential to complement other production processes in the biorefinery. They thus increase the added value and make an important contribution for the transformation of our industry into a truly sustainable, bio-based economy.

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Production of Industrially Relevant Isoprenoid Compounds in Engineered Microbes

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Contents

Abstract Isoprenoids are the largest class of natural compounds and have extremely diverse chemical and functional properties. They are involved in many different cellular processes, including hormonal regulation, signalling, pest/pathogen defence and redox status. They also provide many of the colours, flavours and aromas found in biology. The diversity of isoprenoids lends them to a wide variety

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of biotechnological applications, both by exploiting their myriad natural functions and by using them as industrial chemicals/chemical feedstocks. These applications range from fine chemicals (pharmaceuticals, nutraceuticals, antimicrobials) through mid-volume (flavours, fragrances, colourants, fuel additives) and bulk (fuels, synthetic polymers, agricultural chemicals, etc.) products. However, in their natural context, individual isoprenoids are not usually found at sufficient abundance for industrial use. Moreover, extraction and/or purification may be difficult and/or expensive, or production may be highly variable, making industrial production processes challenging or impossible. Artificial synthesis is often not possible due to complexity, expense or other chemical properties/requirements. Consequently, there is a strong movement towards bioengineering of microbes for production of these valuable compounds in controlled fermentation conditions. Here we consider the requirements for developing economically viable isoprenoid production bioprocesses as well as the current state of the art in engineering production in microbes. We also discuss some of the challenges we face in bringing these technologies to the market.

1 Introduction

With over 70,000 known isoprenoids (dnp.chemnetbase.com/intro/), the isoprenoid (a.k.a. terpenoid) collection of natural products comprises the largest group of natural compounds. They are extremely diverse chemically and functionally and fulfil a host of essential and non-essential roles in all living organisms. They are involved in cell wall biosynthesis, intracellular and extracellular signalling and biotic and abiotic stress responses; they stabilise cell membranes; they are conjugated to tRNAs and proteins to provide targeting functions; and they act as carriers for electron transport, photosynthetic and non-photosynthetic pigments, semiochemicals, hormones, defence molecules, vitamins, virulence factors, antioxidants, etc. (Holstein and Hohl 2004; Lichtenthaler 2010; Gershenzon and Dudareva 2007; Vickers et al. 2009; Loreto and Schnitzler 2010; Sharkey and Singsaas 1995). This multitude of functions provides very great potential for biotechnological exploitation of isoprenoids (Table 1). Many of the functions can provide useful agricultural (e.g. plant hormones to control development, defence against pests and pathogens, protection against abiotic stress) or general industrial applications (food colours, fragrances, flavourings, vitamins and antioxidants); other incidental functions make some members useful as pharmaceuticals (e.g. the antimalarial artemisinin, anticancer taxol) or nutraceuticals (vitamins and provitamins, antioxidants). Moreover, many isoprenoids can be used as industrial chemicals and chemical feedstocks to make fuels, polymers and other valuable petrochemical replacements.

Table 1 Some industrial applications for isoprenoids

Application	Example	Details
Pharmaceutical/ nutraceutical	Limonene, perillyl alcohol	Anticancer agents
	Zerumbone	Anticancer, anti-inflammatory, anti-HIV
	α-Pinene	Bronchodilator, anti-inflammatory, antibiotic
	Geraniol	Cancer chemoprevention agent
	Taxol	Cancer chemotherapeutic
	Artemisinin	Antimalarial agent
Food additive/ supplement	Carotenoids (xanthophylls, carotenes)	Colourants, antioxidants, provitamin A (carotenes)
	Vitamin E (tocopherols/tocotrienols)	Antioxidant, vitamin supplement
	Vitamin K (phylloquinone, menaquinone)	Vitamin supplement
	Vitamin A (retinol, retinal, retinoic acid)	Vitamin supplement
	Coenzyme Q_{10} (ubiquinone)	Antioxidant
Flavour/fragrance/ antimicrobial	Monoterpenes and sesquiterpenes (menthol, eucalyptol, α/β-pinene, limonene, nootkatone, linalool, α/β-farnesene, geraniol)	Food additives, personal care, preservatives, fragrances, cleaning products
Agriculture/ floriculture	Eucalyptol, limonene	Insecticide
	Nootkatone, geraniol	Insecticide/repellent
	Farnesene	Aphid repellent, parasitoid attractant
	Linalool	Insect repellent, predator attractant, pathogen resistance
	(*E*)-β-Caryophyllene	Predator attractant, pathogen resistance
	Isoprene	Abiotic stress protection
	Abscisic acid	Plant propagation
	Gibberellin	Increases life of cut flowers
	Strigolactone	Inhibits branching, promotes growth
Industrial chemical/chemical feedstock	Isoprene	Rubbers, co-block polymers, elastomers, resins, etc.
	α/β-Pinene	Resin precursor, solvent
Fuels	Squalene	Bio-crude
	Isopentenol	Gasoline replacement
	α/β-Farnesene/limonene/*p*-cymene	Jet fuel replacement
	Bisabolene	Diesel replacement
Fuel additives	Isoprenoid alkenes/alkanes/alcohols	Increase octane ratings, anticorrosives, lubricants

Adapted and expanded from Vickers et al (2014)

A limitation for industrial use of biologically sourced isoprenoids is that they are commonly present in low abundance—insufficient for the desired industrial application. Moreover, even when present at useful levels, production rates may be highly variable, or they may be difficult/expensive to extract and/or purify from their natural sources. Chemical synthesis from petrochemical precursors is possible and cost-effective in some cases; but it is too difficult, too expensive, or simply not possible in many others. Correct chirality is often required for industrial application, and synthetic production may not be enantio-specific. In these cases, development of suitable bioprocess is required for cost-effective production. Microbes are typically chosen as bioprocess organisms due to the relative ease of cultivation, amenability to genetic modification (when biological engineering is required), speed of growth and relatively straightforward bioprocess requirements.

There are only a few natural microbial systems where isoprenoids are produced at levels significant enough for industrial applications. One example is the production of carotenoids (isoprenoid pigments in the tetraterpenoid class) in various different microbes. For example, β-carotene, a strong red-orange pigment responsible for the colour of carrots and pumpkins, is produced commercially using the unicellular green alga *Dunaliella* spp. and the fungus *Blakeslea trispora* (Dufossé 2009). It is used as a food colour (E-number: E160a) and as a dietary supplement (it is a precursor to vitamin A and thus has provitamin A activity in humans). However, in general it is rare to find microbes that naturally produce specific isoprenoids at sufficient titres for industrial applications.

Biotransformation is one alternative approach for isoprenoid production. These processes take advantage of relatively inexpensive, readily available precursors to produce higher-value products with desired structural characteristics, including stereo- and regio-specificity. Humankind has exploited the capacity of beer and wine yeasts to biotransform plant isoprenoid precursors into desirable isoprenoid flavour and fragrance compounds for thousands of years (King and Dickinson 2000; Bisson and Karpel 2010; Loscos et al. 2007; Cordente et al. 2012; Abbas 2006; Takoi et al. 2010). More recently, these capacities are being examined for production of bulk isoprenoid biochemicals, particularly flavour and fragrance isoprenoids (de Carvalho and da Fonseca 2006; van der Werf et al. 1997; Badee et al. 2011; Khor and Uzir 2011; Krings and Berger 1998). While development and commercialisation of these processes has proved challenging, advances in bioprocess development are promising (de Carvalho and da Fonseca 2006; Pescheck et al. 2009; Schrader and Berger 2008).

Where natural production is not at sufficient titre and biotransformation is not a viable approach, microbes must be engineered to produce the desired isoprenoid directly from central carbon intermediates. Here we will review engineering approaches for isoprenoid production, focusing on model organisms but also discussing more unusual production strains. We also review some of the economic and practical considerations for isoprenoid bioprocesses, including issues related to feedstock price/availability (particularly important for bulk bioprocesses) and product toxicity (particularly for isoprenoid hydrocarbons with fuel applications).

2 Biosynthesis of Isoprenoids

Isoprenoids are produced by two biochemically distinct metabolic pathways, both of which start at central carbon intermediates. The mevalonic acid (MVA) pathway (Fig. 1) initiates at acetyl-CoA; it has been fully characterised for many decades and is found in most eukaryotes, archaea and some bacteria. Some organisms are now known to use alternate steps that diverge from the canonical MVA pathway (Dellas et al. 2013; Okamura et al. 2010) (see Fig. 1). The methylerythritol phosphate (MEP) pathway (a.k.a. non-mevalonate, deoxyxylulose phosphate [DXP or DOXP] or Rohmer pathway; Fig. 1) initiates from pyruvate and glyceraldehyde-3-phosphate and was only fully characterised just over a decade ago; it is found in plant chloroplasts, most bacteria and some eukaryotic parasites (Eisenreich et al. 2004; Rohmer 2003; Rodríguez-Concepción and Boronat 2002; Zhao et al. 2013b). Both pathways produce the universal five-carbon isoprenoid precursor isomers, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). DMAPP serves as the initial allylic substrate for prenyltransferases, which catalyse sequential condensation of IPP to produce growing allylic polyisoprenoid diphosphate chains (Kellogg and Poulter 1997). The resulting common C_{10}, C_{15} and C_{20} prenyl diphosphate isoprenoid precursors are known as geranyl diphosphate (GPP), farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP), respectively. The isoprenoids that are produced from these precursors are grouped in classes based on their carbon number: hemiterpenes (C_5), monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), triterpenes (C_{30}), tetraterpenes (C_{40}) and longer-chain poly-isoprenoids. Sesquiterpenes and diterpenes are used as precursors to produce shorter-chain C_{16} and C_{11} homoterpenes. The basic isoprenoid structures are subjected to the full range of biochemical modifications and decorations that biology can catalyse, thereby producing the diversity of known isoprenoids; more complex isoprenoids may be produced through the convergence of one or more other metabolic pathways.

3 Economic Considerations for Engineering Isoprenoid Production

Industrial processes convert low-value carbon into high value-added products. To be economically viable, the cost of the overall bioprocess must be significantly less than the selling price of the product. Many variables contribute to bioprocess costs, but for bulk biochemicals such as biofuels and industrial chemicals (which many isoprenoids are used for), the price of the feedstock is the primary cost driver (Rude and Schirmer 2009; Willke and Vorlop 2008). Currently, carbohydrates from plant biomass are the most important carbon feedstock for fermentation. Plants accumulate simple sugars, cellulose or lipids, which bacteria can convert into energy, biomass and products. Popular model organisms exploited in industry (such as

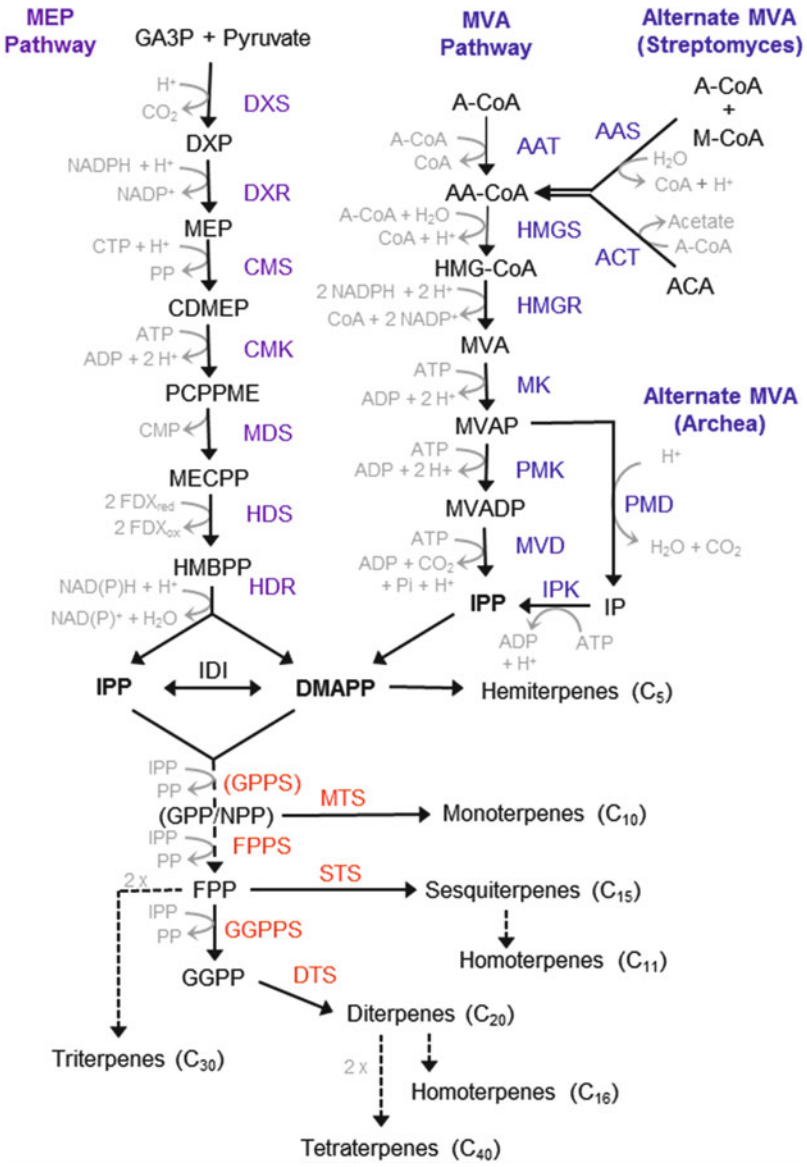

Fig. 1 (continued)

Escherichia coli, *Saccharomyces cerevisiae*, *Pichia pastoris*, etc.) readily metabolise simple sugars, especially glucose. Cellulose and lipids require pretreatment to release fermentable carbon, and generally metabolism of the resulting products is significantly less efficient than for glucose (Bettiga et al. 2009; Fernandes and Murray 2010; Wang et al. 2013; Zhao et al. 2013a).

MVA PATHWAY
Enzymes:
AAT, Acetyl-CoA *C*-acetyltransferase
HMGR, Hydroxymethylglutaryl-CoA reductase
HMGS, Hydroxymethylglutaryl-CoA synthase
MK, Mevalonate kinase
MVD, Diphosphomevalonate decarboxylase
PMK, Phosphomevalonate decarboxylase
PMK, Phosphomevalonate kinase
Metabolites:
ACA, Acetoacetate
A-CoA, Acetyl-CoA
AA-CoA, Acetoacetyl-CoA
HMG-CoA, (S)-3-hydroxy-3-methylglutaryl-CoA
M-CoA, Malonyl-CoA
MVA, (R)-mevalonate
MVAP, Mevalonate-5-phosphate
MVADP, Mevalonate diphosphate

MEP PATHWAY
Enzymes:
DXS, 1-deoxyxylulose-5-phosphate synthase
DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerasease
CMS, 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase
CMK, 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase
MDS, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase
HDS, 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate synthase
HDR, Hydroxymethylbutenyl diphosphate reductase
Metabolites:
GA3P, D-glyceraldehyde 3-phosphate,
DXP, 1-deoxy-D-xylulose-5-phosphate
MEP, 2-C-methyl-D-Erythritol-4-phosphate
CDMEP, 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol
PCPPME, 2-phospho-4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol
MECPP, 2-C-methyl-D-erythritol-2,4-cyclodiphosphate
HMBPP, 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate

PRENYL PHOSPHATE METABOLISM
Enzymes:
IDI, isopentenyl diphosphate isomerase
IPK, isopentenyl kinase
FPPS, farnesyl pyrophosphate synthase
GPPS, geranyl pyrophosphate synthase
Metabolites:
IP, isopentenyl phosphate (C_5)
IPP, isopentenyl pyrophosphate (C_5)
DMAPP, dimethylallyl pyrophosphate (C_5)
GPP, geranyl pyrophosphate (C_{10})
NPP, neryl pyrophosphate (C_{10})
FPP, farnesyl pyrophosphate (C_{15})
GGPP, Geranylgeranyl pyrophosphate (C_{20})

COFACTORS/COFACTOR PRODUCTS
ATP, adenosine triphosphate
ATP, adenosine diphosphate
CTP, cytidine 5' triphosphate
CMP, cytidine 5'monophosphate
CoA, coenzyme A
FDX_{red}, ferrodoxin, oxidised
FDX_{ox}, ferrodoxin, reduced
NADH, Nicotinamide adenine dinucleotide, oxidised
NAD+, Nicotinamide adenine dinucleotide, reduced
NADPH, Nicotinamide adenine dinucleotide phosphate, oxidised
NADP+, Nicotinamide adenine dinucleotide phosphate, reduced

Fig. 1 Isoprenoid pathways in microbes. The mevalonate (MVA) and methylerythritol pyrophosphate (MEP) pathways are biochemically distinct and found in separate organism. They converge figuratively at the five-carbon intermediates isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). Figure modified from Vickers et al. (2014)

Peptides from digestion of proteins (either of animal or vegetal origin) can also be used to support bacterial growth, but their cost is very high.

3.1 Feedstocks and the Cost of Isoprenoids

The cost of isoprenoid production may be estimated based on theoretical production capacity, expected market placement and market size, prospective technological investment, etc. (Bull et al. 2000). A number of analytical tools guide preliminary estimations. Metabolic network analysis helps to delineate potential hosts, theoretical production yields or limiting factors using metabolic models, while techno-economic analysis assesses the technical potential and the economic viability of biobased productions (Vickers et al. 2012). Comprehensive process modelling is rarely required at the planning stage, and often it is sufficient to consider key indicators such as costs deriving from raw materials, process efficiency, potential

production scale together with economic considerations and conceivable technology improvement (Hermann and Patel 2007; Tufvesson et al. 2013). These simple calculations should always be performed when examining the viability of any given process.

A good bulk chemical bioprocess converts sugars to product at a rate greater than 2 g/L/h with a yield exceeding 85 % and a final product titre in excess of 100 g/L. In the best processes, these numbers approach 10 g/L/h, 95 % yield and 200 g/L. At scale, feedstock costs account for 50–80 % of the total costs in good bioprocesses (Van Wegen et al. 1998; Dutta et al. 2011); the highest non-feedstock costs are seen for aerobic processes with complex downstream processing. While isoprenoid production is commonly performed under aerobic conditions, separation can in many cases be achieved through inexpensive phase separation in the presence of an added non-toxic extractant phase, possibly with an additional distillation step.

The yield of isoprenoids (e.g. limonene, farnesene) is 0.27 g/g using the mevalonate pathway, and even at near theoretical yield, it takes 4 kg of sugar to produce 1 kg of isoprenoid. With a typical carbon cost for glucose of $0.20–0.30/kg, the raw material costs amount to $1/kg terpene, suggesting production costs around $1.5–2/kg. Reported productivities and yields for terpenoid production are well below those expected for good bioprocesses (see below). However, in their 2013 financial report, Amyris reported a reduction in farnesene production costs from $12 to $4/L ($5/kg) during 2013 (Amyris 2013), indicating that the gap is closing rapidly.

3.2 Glucose and Sucrose

Glucose is the most important simple sugar used in industrial fermentation (Peters 2006). Glucose for industrial microbial fermentation is derived mainly from starch sources such as corn (especially in the USA), wheat, cassava, potato and sorghum. Sucrose is another industrially important simple sugar (Peters 2006). Sucrose is sourced from sugar beet in temperate climates and sugarcane in warm regions. Molasses, a by-product of sugarcane processing, has high sugar content (glucose, fructose and sucrose) and is cheaper than refined sugar, but its high impurity content increases the cost of downstream processing for products that cannot be purified by distillation.

Sucrose from sugarcane has advantages compared to glucose from corn, in terms of monosaccharide yield per unit of land and environmental impact (e.g. water usage, eutrophication potential) (Renouf et al. 2008). Unlike corn processing, no external energy input is required to produce fermentable sugars from sugarcane since plant waste (bagasse) is used to power the extraction process (Panray Beeharry 2001). As a result, sucrose from sugarcane has similar environmental performance to cellulosic glucose when used for microbial fermentation (Renouf et al. 2008). In addition, sucrose is not an essential food and sugarcane can be grown on marginal lands, avoiding the debate on agricultural land use for non-food

applications ('food versus fuel') (King 2010). Although production plant localisation depends on many other issues, the advantages of sugarcane has seen several companies establish production in Brazil and they are considering other large sugarcane producing nations, such as Australia and Thailand.

Surprisingly, only a few (mostly pathogenic) strains of the common industrial bacterium *E. coli* can catabolise sucrose (Reid and Abratt 2005). The paucity of sucrose-utilising industrial *E. coli* strains has driven research into sucrose metabolism in wild-type strains that can utilise sucrose efficiently, such as *E. coli* W (Archer et al. 2011; Sabri et al. 2013a; Steen et al. 2014). Improved sucrose utilisation properties have been engineered in *E. coli* W (Arifin et al. 2011; Sabri et al. 2013a), and using chromosomal engineering tools (Sabri et al. 2013b) or random integration, other lab/industrial strains can be engineered to efficiently utilise sucrose (Bruschi et al. 2011; Sabri et al. 2013a).

3.3 *Lignocellulosic Feedstock*

The importance of feedstock costs, combined with concerns over non-food use of simple sugars, has driven major research efforts into lignocellulosic feedstock for the past four decades. Meeting these needs and deriving value from agricultural residues or dedicated energy crops grown on marginal lands is fundamentally attractive. The obstacles, however, are very significant.

Firstly, marginal lands are marginal for a reason, and no plants grow effectively without sun, water and nutrients; biomass production by even the best energy crops is dwarfed by standard production from sugarcane. At best, output from marginal land will be highly variable (as opposed to low), which is still a problem for a costly biomass processing plant. While agricultural inputs may be low, harvesting and transportation costs for energy crops (as well as agricultural residues such as corn stover) are typically fairly high considering the quality of the feedstock. Even when the residue is already at the plant (e.g. sugarcane bagasse, wood waste), handling costs can be significant and cogeneration may be a far more appropriate option.

Assuming feedstock can be collected in sufficient quantity and at low enough cost, the recalcitrance of lignocellulosic-derived feedstocks remains a major obstacle. Lignocellulose is typically composed of cellulose (an unbranched polymer of glucose which makes highly structured fibre, 40–50 % dry weight), hemicellulose (a branched polymer of five-carbon sugars which constitutes an amorphous matrix, 25–40 % dry weight), lignin (a polymer of aromatic alcohols which fills the space between cellulose and hemicellulose, 15–30 % dry weight) and others (e.g. ash, pectins, etc., up to 16 % dry weight) (Limayem and Ricke 2012). Lignocellulosic carbon cannot be directly fermented by common industrial microorganisms. The harsh processing conditions needed to release fermentable sugars (hexoses and pentoses) dictates substantial capital investments and produces by-products (e.g. furfural) that inhibit and slow down the subsequent fermentation. Moreover,

the final product has to be extracted from a heavily contaminated 'soup' (Kumar et al. 2009), which in many cases will offset any advantage gained.

Engineering of energy crops to reduce recalcitrance, combined with advances in effective deconstruction processes (Blanch et al. 2011), may ultimately make lignocellulosic feedstock viable—at least for fuels where contaminants may be less of a concern.

4 Engineering Isoprenoid Production in Microbes

A variety of hosts have been used to engineer isoprenoid production. Host selection for a given isoprenoid depends on a number of different factors, including (1) genetic tractability ('engineerability'), (2) native capacity for isoprenoid production, (3) potential toxicity of the isoprenoid end product, (4) competition with essential isoprenoid requirements and (5) potential/ability to carry out (desired or detrimental) biotransformations/modifications (Vickers et al. 2014). Genetic tractability is often the driving factor in host selection, and for this reason the model bioprocess organisms *Saccharomyces cerevisiae* (yeast) and *Escherichia coli* are most commonly used. These organisms, however, have relatively limited native isoprenoid production capacity; consequently, significant efforts have been devoted towards engineering them for production of industrially useful isoprenoids. Isoprenoid production capacities are highly variable between strains (Chae et al. 2010; Rodríguez-Villalón et al. 2008; Boghigian et al. 2012; Takahashi et al. 2007), so care should be taken when selecting the host strain. Moreover, different strains demonstrate differences in potential for flux increase upon engineering (Bongers, Behrendorff, Nielsen and Vickers, unpublished data), so it is worthwhile testing several strains before embarking on full-scale engineering.

An enormous number of studies examining many different approaches for engineering isoprenoid production in yeast and *E. coli* have been published; in the interest of brevity, the results of these studies will be summarised in the relevant sections below. In recent years, engineering programmes using other less tractable organisms have been developed. In particular, an interest in the direct conversion of CO_2 to useful biochemicals using photosynthesis has driven research in model photosynthetic microbes, such as cyanobacteria. Early results have demonstrated proof of principle; however, yields/titres still lag far behind *E. coli* and yeast. As there are far fewer studies in these 'non-model' organisms, they will be reviewed in more detail. In addition to examining different organisms, some generic requirements for engineering isoprenoid production in microbes will be discussed.

4.1 Generic Requirements for Engineering Isoprenoid Production

As the organisms used for engineering do not typically produce the desired isoprenoid, genetic capacity for production must be imported from some other source. In comparison with model microbes, plants have a particularly diverse isoprenoid metabolism. For example, of the 500–1,200 Tg carbon equivalents/year of volatile chemicals emitted by plants, the majority belongs to the isoprenoid class (Guenther et al. 2006); there are thousands of different chemicals in this volatile group alone. The diverse isoprenoid metabolism of plants is supported by an equally diverse genetic capacity; consequently, genes for engineering isoprenoid production in microorganisms are often sourced from plants.

Selection of appropriate genes is critical. Enzyme catalytic properties (including product/substrate specificity/affinity, catalytic rates, etc.) should be optimal for the specific metabolic context and be capable of competing with native enzymes for substrate without causing severe growth defects. Ideally, catalytic properties should be tested in vivo rather than in vitro, as substantial variation between properties in the two different systems may be observed (Teusink et al. 2000; Brindle et al. 1993; Houston 1994). High-throughput in vivo screening methods (e.g. Behrendorff et al. 2013; Agresti 2012) can be used to short-list useful genes/enzymes. Enzyme engineering may be required to improve catalytic properties. Once each gene is selected, it must be redesigned for expression in the target organism. To redesign a plant gene for expression in microbes, a number of steps are required: (1) removal of introns to provide an uninterrupted protein coding sequence, (2) removal of subcellular targeting signals if required, (3) codon optimisation to suit the host organism and (4) selection of an appropriate expression system (plasmid/chromosomal/copy number, etc.) (Vickers et al. 2014). Production of the desired metabolite may require one or more genes; if multiple genes are required, multi-gene operons (for prokaryotes) or expression vectors with multiple cassettes (for eukaryotes; e.g. Vickers et al. 2013; Partow et al. 2010) can be used. There is a move towards stable chromosomal integration of genes nowadays to improve stability and minimise requirements for complex media or antibiotics in bioprocesses; a number of integration tools that can handle large DNA sequences are available (Sabri et al. 2013b; Kuhlman and Cox 2010; Ublinskaya et al. 2012; St-Pierre et al. 2013). Each has advantages and disadvantages, and the system most compatible with experimental requirements should be selected.

Commonly (but not always), introduction of an appropriate synthase is sufficient to achieve measurable yields of the desired isoprenoid if the substrate is already available. However, to get reasonable yields, significant engineering of upstream isoprenoid pathways (MVA and MEP) is usually required. As most engineering for MVA and MEP pathway flux has been done in yeast and *E. coli*, respectively, strategies for flux improvement will be reviewed in the relevant sections below for these microbes. There is evidence that these generic strategies for pathway

engineering are transferrable to other microbes (Kiyota et al. 2014; Xue and Ahring 2011; Bentley et al. 2014).

4.2 *Engineering Isoprenoid Production in* E. coli

E. coli is an industrial workhorse and a model prokaryotic organism; by far the most engineering for isoprenoid production has been performed using this organism. The key findings of these studies will be summarised here. *E. coli* uses a MEP pathway for native production of isoprenoids. Its isoprenoid metabolism is relatively simple: it uses prenyl groups derived from IPP/DMAPP for targeting quinones to membranes, tethering of haem A/O in cytochromes, translocation of sugars for cell wall biosynthesis (bactoprenol) and tRNA prenylation (Keseler et al. 2012). While all the MEP pathway genes (except *idi*) are essential for *E. coli*, the actual flux through the pathway is low (Ro et al. 2006; Ajikumar et al. 2008); this is most likely because it has a low native requirement for isoprenoid products.

To engineer isoprenoid production at reasonable levels in *E. coli*, the innate pathway regulation that keeps pathway flux low must be overcome. Our current understanding of MEP pathway regulation in *E. coli* is predominantly derived from attempts to metabolically engineer *E. coli* to produce heterologous isoprenoids, most commonly carotenoids (e.g. lycopene and beta-carotene (Rodríguez-Villalón et al. 2008; Yoon et al. 2007; Jin and Stephanopoulos 2007; Alper et al. 2005a; Farmer and Liao 2000; Farmer and Liao 2001; Kajiwara et al. 1997) and more recently other isoprenoids including isoprene (Zhao et al. 2011; Whited et al. 2010), various mono- and diterpenes (Reiling et al. 2004; Morrone et al. 2010) and taxadiene (Huang et al. 2001; Ajikumar et al. 2010).

The current understanding of MEP pathway regulation in *E. coli* has been reviewed recently (Vickers et al. 2014). While there is much left to learn, it is clear that MEP pathway regulation is extremely complex, with most enzymes contributing to flux regulation at some level. Overlaid on top of this are transcriptional and other post-transcriptional controls. However, despite our growing understanding of MEP pathway regulation, overcoming this regulation to produce high titres of isoprenoids has proven exceedingly difficult. Thus far the best isoprenoid titre achieved in *E. coli* using the MEP pathway is 1 g/L (Ajikumar et al. 2010). This required complex balancing of gene expression through different pathway modules. However, it is far less than the theoretical maximum yield of the pathway (Rude and Schirmer 2009).

To circumvent the regulatory controls on the *E. coli* MEP pathway, a synthetic mevalonate pathway can be imported. Either a partial MVA pathway coupled with mevalonate supplementation (Campos et al. 2001; Rodríguez-Villalón et al. 2008) or a full MVA pathway (Martin et al. 2003) can successfully provide significantly higher flux to isoprenoid precursors than using the MEP pathway (though the former approach is unfeasible for commercial bioprocesses because of the cost of mevalonate addition). Titres in the range of tens to almost a hundred grams per litre

of isoprenoids have been achieved using the latter approach (Beck et al. 2013; Pray 2010; Martin et al. 2003; Paddon et al. 2013; Westfall et al. 2012). Optimisation of the imported MVA pathway is required to achieve maximum flux (reviewed in Vickers et al. 2014).

Regardless of which core isoprenoid pathway is used, an appropriate balance of C_5 precursors (IPP and DMAPP) is required. Longer-chain isoprenoids require increasing IPP:DMAPP ratios, and the isomerisation step becomes rate-limiting under most conditions. Over-expression of isopentenyl diphosphate isomerase (IDI) relieves this bottleneck in *E. coli* (Kajiwara et al. 1997; Alper et al. 2005b; Albrecht et al. 1999; Yuan et al. 2006; Martin et al. 2003; Ohto et al. 2009a; Vadali et al. 2005; Yan et al. 2012).

Currently, the highest titres of C_5 isoprenoids reported are for isoprene, a hydrocarbon that can be polymerised to produce synthetic rubbers, elastomers and a variety of other products (Table 1). Around 80 g/L can be obtained using highly engineered *E. coli* strains coupled with optimised bioprocess conditions (Beck et al. 2013). This titre is the highest reported in scientific literature for any isoprenoid product so far.

The C_{10} monoterpenes are highly attractive as industrial chemicals (see Table 1), so an effective production system is desirable. However, *E. coli* does not naturally produce significant amounts of monoterpenes or the C_{10} precursor GPP (Reiling et al. 2004; Carrau et al. 2005; Oswald et al. 2007), as there is no dedicated GPP synthase encoded on the genome (the FPP synthase, IspA, condenses two IPP moieties with DMAPP to produce a C_{15} with little or no release of C_{10}). Moreover, many monoterpenes are highly toxic (see Sect. 5 below); this will also limit titres unless engineering or process approaches to mitigate toxicity are used. Consequently, production of monoterpenes in *E. coli* is somewhat more challenging. Expression of a heterologous dedicated GPP synthase (GPPS) in *E. coli* increases titres (Carter et al. 2003; Yang et al. 2013; Alonso-Gutierrez et al. 2013), and fusing the GPPS to the monoterpene synthase—an approach first demonstrated effectively in yeast for monoterpene production (Ignea et al. 2013)—also improves titres (Sarria et al. 2014). The maximum titres reported so far are ~2.7 g/L using engineered strains with optimised bioprocess conditions (Willrodt et al. 2014; Zhang et al. 2014).

E. coli naturally produces FPP, but novel sesquiterpenes are produced at only low levels when heterologous sesquiterpene synthases are introduced. To increase production of sesquiterpenes, FPP synthase (*IspA*) can be over-expressed (Ohto et al. 2009a). While *E. coli* does not naturally produce significant amounts of GGPP, heterologous expression of geranylgeranyl pyrophosphate synthase (GGPPS) provides a route for production of higher-order isoprenoids including diterpenes (Reiling et al. 2004; Morrone et al. 2010) and carotenoids (Rodríguez-Villalón et al. 2008; Yoon et al. 2007; Jin and Stephanopoulos 2007; Alper et al. 2005a; Farmer and Liao 2000; Farmer and Liao 2001; Kajiwara et al. 1997). The highest carotenoid yields reported to date in *E. coli* are 20 mg lycopene per g dry cell weight (DCW) in a highly engineered strain (Zhang et al. 2013).

Apart from the above-described core metabolic pathway engineering, production of complex isoprenoids may require introduction and optimisation of a wide number of enzymes downstream of prenyl phosphate precursors. The required downstream modifications are specific to each product, and reviewing these is beyond the scope of this chapter.

4.3 Engineering Isoprenoid Production in S. cerevisiae

Like *E. coli*, the yeast *S. cerevisiae* is an industrial workhorse; it is a model eukaryotic organism, and its more complex metabolism can potentially facilitate production of more complicated isoprenoid products than *E. coli*. *S. cerevisiae* uses a mevalonate pathway for isoprenoid production. Given that yeasts require ergosterol as a membrane component, it seems reasonable that native isoprenoid flux in this organism might be higher than in *E. coli*. Certainly in terms of heterologous isoprenoid production, this appears to be the case. Flux regulation through the MVA pathway is less complex than the MEP pathway; key points have been reviewed recently (Vickers et al. 2014) and will be summarised briefly here.

Hydroxymethyl-CoA reductase (HMGR) is the primary bottleneck in the MVA pathway (Lombard and Moreira 2011). Releasing this flux control point by truncation of the HMGR to alleviate feedback control (Jackson et al. 2003; Ro et al. 2006; Ohto et al. 2009b; Asadollahi et al. 2010; Rico et al. 2010; Westfall et al. 2012; Ignea et al. 2011) or deleting genes that affect its stability (Ignea et al. 2012) has a significant effect on pathway flux. Improving cofactor (NADPH) supply also increases isoprenoid accumulation (Asadollahi et al. 2009). In the absence of engineering at the HMGR node, over-expression of any of the other MVA pathway genes does not improve isoprenoid levels (Ohto et al. 2009b), but over-expression of all pathway genes together is effective (Westfall et al. 2012). Once the HMGR node has been released, over-expression of the global transcriptional regulator upc2-1, which upregulates several genes in the MVA pathway, improves isoprenoid accumulation (Ro et al. 2006; Shiba et al. 2007; Westfall et al. 2012). Engineering a heterologous MEP pathway in yeast in an attempt to circumvent MVA pathway regulation has been unsuccessful (reviewed in Vickers et al 2014).

Once core MVA pathway flux has been engineered, further increases can be achieved by increasing availability of the pathway precursor acetyl-CoA (Shiba et al. 2007). As in *E. coli*, over-expression of IDI downstream of the core pathway also improves isoprenoid production (Ignea et al. 2011).

Only one report of C_5 (isoprene) production in yeast has been made (Hong et al. 2012). However, only very low yields were achieved; this was partially due to extensive bioconversion of the product by native non-specific hydroxylation reactions.

Like the *E. coli* equivalent, the yeast FPPS is a bifunctional enzyme, and no dedicated GPP synthase exists; similar problems are therefore encountered when attempting to produce monoterpenes in yeast. Mutation of the native FPPS to promote the release of GPP can result in production of low levels of monoterpenes

in yeast, even in the absence of a heterologously expressed monoterpene synthase, presumably from the activity of non-specific phosphatases (Chambon et al. 1991; Javelot et al. 1989). Low levels of monoterpenes can also be detected upon over-expression of heterologous monoterpene synthases (MTSs) in yeast (Oswald et al. 2007; Herrero et al. 2008; Fischer et al. 2011; Ignea et al. 2011; Liu et al. 2013), though over-expression of MTSs does not always yield detectable levels of the desired product (Yang et al. 2013). Significantly higher levels can be achieved if the native FPPS is over-expressed (Yang et al. 2013; Ignea et al. 2011) or if mutated (GPP-producing) FPPS is co-expressed with heterologous MTSs (Carter et al. 2003; Oswald et al. 2007; Reiling et al. 2004; Fischer et al. 2011; Ignea et al. 2013). Alternatively, titres can be increased by expression of a heterologous GPPS (Liu et al. 2013; Ignea et al. 2011), though this does not always increase titre (Ignea et al. 2011). As noted above, fusion of the GPPS and MTS can also be used to increase titres (Ignea et al. 2013). Current highest monoterpene titres reported in yeast are 1 g/L (Ignea et al. 2011).

Biotransformation of monoterpene products by endogenous enzymes is a problem likely to be prevalent in yeast, since such biotransformations are well documented in this species. For example, geraniol can be converted into both citronellol and linalool in some yeast strains (King and Dickinson 2000), and limonene can be bioconverted to a variety of products by different organisms (Duetz et al. 2003). Yeast has also been documented to bioconvert the hemiterpene isoprene to oxygenated products (Hong et al. 2012). Avoiding these reactions where necessary will require a thorough knowledge of their biochemistry and genetics.

Yeast isoprenoid metabolism is strongly biased towards native C_{15} production (ergosterol), and production of non-native sesquiterpenes or higher-order products requires alleviation of competition by squalene synthase (ERG9), the first dedicated enzyme in the ergosterol biosynthetic pathway. In the absence of engineering at ERG9, over-expression of FPPS does not significantly increase production of heterologous isoprenoids (Jackson et al. 2003; Ro et al. 2006). However, down-regulation of ERG9 apparently mitigates competition and improves production of heterologous sesquiterpenes (Ro et al. 2006; Asadollahi et al. 2008; Paradise et al. 2008; Babiskin and Smolke 2011; Ignea et al. 2011; Scalcinati et al. 2012; Takahashi et al. 2007; Paddon et al. 2013). The best non-native sesquiterpene titres to date in yeast are ~40 g/L amorpha-4,11-diene, a precursor to the antimalarial drug artemisinin (Westfall et al. 2012).

Over-expression of a GGPPS improves production of diterpenes and higher-order isoprenoids (including carotenoids) (DeJong et al. 2006; Ukibe et al. 2009; Yamano et al. 1994; Verwaal et al. 2007). The diterpene taxadiene, a precursor to the anticancer drug taxol, has been produced in low mg levels in yeast (DeJong et al. 2006). Carotenoid production was initially only achieved at microgram per gram levels (Ukibe et al. 2009; Yamano et al. 1994), but could be increased to ~6 mg/g DCW by a combination of judicious selection of genes, chromosomal integration, engineering at HMGR and increasing copy number of some chromosomally inserted genes (Verwaal et al. 2007). Similar yields were achieved by combining some of these approaches with repression of ergosterol biosynthesis

(Yan et al. 2012). A clever adaptive engineering approach, which exploited the antioxidant capacity of carotenoids, was recently used to further increase yields up to 18 mg/g DCW (Reyes et al. 2014). Carotenoids have also been produced in other yeasts but at lower yields/titres. These include *Pichia pastoris* (74 mg/L lycopene) (Bhataya et al. 2009), *Candida albicans* (0.5–1 mg/g DCW) (Miura et al. 1998) and *Candida utilis* (6–8 mg/g DCW) (Shimada et al. 1998; Miura et al. 1997).

4.4 Engineering Isoprenoid Production in Cyanobacteria

Exploiting photosynthesis for sustainable production of isoprenoids from carbon dioxide is particularly attractive as it avoids the use of food crops as feedstock and can potentially couple chemical production to atmospheric carbon capture. The cyanobacterium *Synechocystis* is increasingly being used to demonstrate the production of isoprenoids via photosynthesis, and there is particular interest in using this concept to produce drop-in fuel substitutes. As a preliminary assessment of whether *Synechocystis* could be a suitable isoprenoid-producing organism, a simple survival assay was conducted in the presence of a selection of isoprenoid compounds with similar chemical properties to fossil diesel (Hellier et al. 2013). The monoterpenes geraniol and geranial were toxic to *Synechocystis* at 0.02 % (v/v), while linalool and the sesquiterpene farnesene were toxic at concentrations of 0.1 % (v/v). Of the compounds tested, only citronellene was tolerated at concentrations as high as 1 % (v/v), but the toxicity of farnesene was alleviated to some extent when two-phase extraction was used.

Early efforts to engineer *Synechocystis* for isoprenoid production have largely been limited to the genomic integration of a single transgene. Different isoprenoid synthase genes have been inserted into the non-essential *psbA2* locus to create strains capable of producing isoprene (Lindberg et al. 2010), the monoterpene β-phellandrene (Bentley et al. 2013) and the sesquiterpene β-caryophyllene (Reinsvold et al. 2011). In each of these cases, the expression of a single heterologous synthase gene was driven by the native *psbA2* promoter, which activates transcription under high light intensity. Under autotrophic conditions, titres of approximately 50 μg isoprene, 25 μg β-phellandrene (using liquid two-phase extraction) or 6 μg β-caryophyllene were achieved on a per litre per day basis. A *Synechocystis* strain has also been engineered for squalene production through inactivation of the squalene-hopene cyclase, accumulating 0.67 mg squalene/$OD_{750\ nm}$/L, or approximately 3 mg squalene/g DCW (Englund et al. 2014).

The development of genetic tools for engineering cyanobacteria is ongoing. A promoter-replacement study demonstrated that the quantity of expressed monoterpene synthase could be increased by using heterologous promoters from *E. coli* or alternative native promoters, improving the titre of β-phellandrene to approximately 45 μg/L/day when the *E. coli trc* promoter was used (Formighieri and Melis 2014). The use of multiple integration events at different loci to recreate a full heterologous pathway for isoprenoid biosynthesis was only recently published

for the first time, demonstrating the integration of the mevalonate pathway into an isoprene synthase-bearing *Synechocystis* (Bentley et al. 2014). Expression of the mevalonate pathway increased the isoprene titre by 2.5-fold, indicating that, as in *E. coli*, the mevalonate pathway was functional and not subject to the same regulatory constraints as the native MEP pathway.

Limonene production in *Synechocystis* was demonstrated by integration of two heterologous operons: one encoding limonene synthase and the other a tricistronic operon encoding *dxs*, *idi* and GPP synthase (Kiyota et al. 2014). Both operons were under the control of the *trc* promoter. In this study limonene was captured by passing the bioreactor off-gas through a chilled solvent trap. Using this method, 99.4 % of the total detected limonene was in the solvent trap and only 0.6 % in cells. No quantifiable amount of limonene was present in the culture supernatant, suggesting that the method of recovering volatile limonene from the off-gas rather than retaining it in a second liquid phase may be an effective way to reduce the burden of monoterpene toxicity. A strain expressing only limonene synthase produced 41 μg limonene/L culture/day at an approximately linear rate for the first 200 h before the production rate began to attenuate. Co-expression of the *dxs*, *idi* and GPP synthase operon increased the production rate to 56 μg limonene/L culture/day for 300 h, attenuating to 19 μg limonene/L culture/day by 712 h. Approximately 1 mg limonene/L was produced over the course of the experiment.

Limonene production has also been demonstrated in the nitrogen-fixing cyanobacterium *Anabaena* using plasmid-based expression systems (Halfmann et al. 2014). Co-expression of *dxs*, *idi* and *GPPS* along with a limonene synthase more than doubled the titre of limonene obtained when limonene synthase was expressed alone, and under optimised autotrophic conditions, the peak titre was 3.6 μg limonene/OD/h. The authors observed that the strain with the greatest limonene titre also exhibited the greatest oxygen evolution, indicating that the addition of limonene synthesis as a carbon sink increased the rate of carbon fixation. The rate of limonene production could also be increased by increasing the light intensity.

To date, the titres of isoprenoids produced under autotrophic conditions are poor compared with heterotrophic organisms engineered to produce isoprenoids from sugars. However, both the means to engineer photosynthetic microorganisms and the understanding of their metabolic regulation are underdeveloped relative to *E. coli* and *Saccharomyces*. Photosynthetic microorganisms may become industrially attractive in the future if mechanisms can be developed to improve the direction of carbon from photosynthesis to isoprenoids. Davies et al. (2014) argued that two of the greatest sources of competition for carbon under autotrophic conditions are glycogen and protein synthesis. The authors sought to examine the effect of these competing pathways on heterologous isoprenoid production by testing limonene and bisabolene biosynthesis in *Synechococcus* sp. PCC 7002 under conditions of nitrogen limitation (to reduce protein synthesis) and in a Δ*glgC* genetic background (preventing glycogen accumulation). *Synechococcus* expressing either limonene synthase or bisabolene synthase under control of the *Synechocystis cpcBA* promoter produced 4 mg limonene/L or 0.5–0.7 mg

bisabolene/L. Production of organic acids increased in the Δ*glgC* genetic background, demonstrating an availability of excess carbon, and nitrogen starvation limited biomass accumulation, but the yields of limonene or bisabolene relative to biomass did not change significantly with any combination of genetic background (*glgC* or Δ*glgC*) or nitrogen starvation/supplementation. Therefore even when excess capacity for flux through the MEP pathway is available, isoprenoid biosynthesis under autotrophic conditions appears tightly regulated and linked to growth.

4.5 Engineering Isoprenoid Production in Other Microorganisms

Beyond *E. coli*, *Saccharomyces* and cyanobacteria, a small number of other microorganisms have been engineered for isoprenoid production, usually in instances where some advantageous phenotype already exists. For example, *Bacillus subtilis* produces isoprene natively to greater concentrations than other known isoprene-emitting bacteria (7–13 nmol/g cells/h) (Kuzma et al. 1995). The gene encoding isoprene synthase activity has not yet been identified in *B. subtilis*, but it was possible to enhance isoprene production by approximately 40 % by over-expression of *dxs* to increase flux through the MEP pathway (Xue and Ahring 2011). Similarly, *Corynebacterium glutamicum* produces high titres of a C_{50} carotenoid, decaprenoxanthin. This was exploited to engineer a lycopene-producing *C. glutamicum* strain by over-expressing geranylgeranyl diphosphate synthase, phytoene synthase and phytoene desaturase (resulting in increased flux to lycopene) and deleting the lycopene elongase (preventing further metabolism of lycopene to decaprenoxanthin). The resulting *C. glutamicum* strain produced 2.4 mg lycopene/g DCW (Heider et al. 2012). For comparison, the greatest reported yield of lycopene in *E. coli* is 20 mg/g DCW in a strain subject to far more comprehensive engineering efforts (Zhang et al. 2013) than the *C. glutamicum* strain described.

Engineering de novo isoprenoid production has also been attempted in *Lactococcus lactis*. Over-expression of the native HMGR with a heterologous plant β-sesquiphellandrene synthase produced a maximum titre of 33 nM (6.5 μg/L) of the sesquiterpene β-sesquiphellandrene (Song et al. 2012). These results are un-encouraging given sesquiterpene titres in other organisms with minimal engineering.

Despite the intense current interest in fermentation of C_1 gases, studies of engineering isoprenoid production from gas fermentation are lacking. The most extensive case study so far is the modification of *Methylomonas* sp. strain 16a, an obligate methanotroph with a native capacity for the production of carotenoids, for the production of the C_{40} astaxanthin using 25 % methane as the sole carbon source. Three papers were published investigating different aspects of astaxanthin engineering in *Methylomonas* sp. (Tao et al. 2007; Sharpe et al. 2007; Ye et al. 2007). A gene cluster for the conversion of FPP to the C_{30} carotenoid canthaxanthin

(a precursor to astaxanthin) was integrated into the *Methylomonas* genome at a non-essential locus. The canthaxanthin biosynthesis gene cluster was expressed constitutively under the control of a native *Methylomonas* promoter, and this strain produced 0.8 mg canthaxanthin/g DCW (Ye et al. 2007). Different combinations of the *crtW* ketolase and *crtZ* hydroxylase genes for conversion of canthaxanthin to astaxanthin were examined by integration at a second locus, and it was determined that the best astaxanthin titre (1.5 mg/g DCW) was achieved by integrating two copies of a full astaxanthin biosynthesis cluster (the initial canthaxanthin cluster plus *crtW* and *crtZ*) into the genome. Transposon-based insertion of a promoterless astaxanthin gene cluster was used to identify highly expressed regions of the genome that could potentially support high-level astaxanthin biosynthesis (Sharpe et al. 2007). Insertion into the relatively active *fliCS* region, encoding cell motility genes, resulted in up to 2 mg astaxanthin/g DCW using a single, promoterless copy of the astaxanthin gene cluster.

Separately, it was observed that the conversion of canthaxanthin to astaxanthin in *Methylomonas* sp. became less efficient with increasing culture volumes, and it was hypothesised that available dissolved oxygen in large reactor volumes may limit crtW activity (Tao et al. 2007). This was addressed by plasmid-based expression of bacterial haemoglobin genes, effectively creating a reservoir of oxygen within the cell. In 5 mL cultures in deep-well blocks, inclusion of bacterial haemoglobins increased the astaxanthin content (as a proportion of total carotenoids) from approximately 15 % to greater than 60 %.

5 Isoprenoid Toxicity in Microorganisms

Chemical stress is routinely encountered in the production of chemicals and fuels from microbial bioprocesses (fermentation, whole-cell biocatalysis, bioremediation). The majority of the literature has focused on how cells respond to chemical stress and only partially focused on exploring and developing tolerant strains through metabolic engineering and systems biology tools (Nicolaou et al. 2010). A fundamental feature in any fermentation process is the inhibitory nature of the desired product. Accumulation of products (e.g. ethanol, butanol, hydrocarbons, organic acids, isoprenoids, etc.), whether they are desirable or not, can be toxic to the biocatalyst, inhibiting cell growth or even causing cell death. This product inhibition typically limits production titres, affects fermentation and operation schemes (continuous, batch, fed-batch) and profoundly impacts the overall process economics (Nicolaou et al. 2010; Stephanopoulos 2007). In the isoprenoids class, isoprenoid hydrocarbons in the monoterpene class are known to be particularly problematic with respect to toxicity (indeed, this is one reason why they are commonly included in household cleaners). Here we will examine some generic

issues around hydrocarbon toxicity and delve more deeply into the mechanisms of monoterpene toxicity and ways of mitigating this problem in a bioprocess setting.

5.1 Hydrocarbon Toxicity in Microbial Bioprocessing

In order to place isoprenoid toxicity in context, it is best to first examine other well-known product toxicity examples. Ethanol toxicity in *S. cerevisiae* is one of the best understood models in bioprocessing. Ethanol comprises 99 % of the total biofuel consumption in the USA (Rao et al. 2007). Ethanol causes an increase in membrane fluidity of *S. cerevisiae* and thus destroys functional membrane structures (Sikkema et al. 1995). In response, yeasts have developed mechanisms to tolerate such alcohol stresses. It is well established that the levels of unsaturated fatty acids and ergosterol production increase while under ethanol stress (Swan and Watson 1998; You et al. 2003). The membrane isoprenoid ergosterol is the most abundant sterol in *S. cerevisiae*, and increasing ergosterol production facilitates membrane rigidity allowing the yeast to maintain membrane functionality (Ding et al. 2009). Unsaturated fatty acids, such as oleic acid, when incorporated into the cell membrane, decrease fluidity, thus compensating for fluidisation effect elicited by ethanol (Ding et al. 2009). A fourfold increase in the ratio of oleic acid to palmitoleic acid was found in an ethanol-tolerant strain (You et al. 2003). Furthermore, whole genome shuffling in *S. cerevisiae* successfully produced a strain (SM-3) with enhanced thermotolerance, ethanol productivity and ethanol tolerance (Shi et al. 2009). They reported strains that can tolerate 25 % v/v ethanol with cell viability up to 60 % compared to the parent strain (Shi et al. 2009). Heat shock proteins, chaperones, transcriptional regulators and other response proteins have also been associated with ethanol tolerance (Nicolaou et al. 2010).

Pseudomonas putida is one of the most solvent-tolerant organisms known. *P. putida* has been shown to tolerate high levels of toluene, butanol, *n*-decane, *n*-octane, *n*-heptane, styrene, xylene and several others (Ramos et al. 1995; Ruhl et al. 2009; Weber et al. 1993). Many factors contribute to the robust nature of this bacterium. In addition to altering the composition of the cell membrane (Heipieper et al. 1994), *P. putida* also utilises cis-to-trans isomerisation to increase the ratio of trans-unsaturated fatty acids (similar to yeast ethanol response) to increase membrane rigidity in the presence of toluene (Weber et al. 1993). Researchers reported that in order to stabilise the membrane under toluene and ethanol stress, the phospholipid headgroup composition is altered to incorporate higher-ordered lipid packing (Weber et al. 1993). Furthermore, *P. putida* has been shown to use energy-dependent membrane-bound efflux pumps to discharge toluene from the cell (Ramos et al. 1998). This organism could be used as a model for understanding successful solvent-tolerant mechanisms and potentially translate these effective response actions into a yeast isoprenoid-producing strain.

5.2 Monoterpene Toxicity in Microbes

Understanding solvent tolerance in microorganisms requires the distinction between solvent toxicity exerted on the microorganism at the molecular level or at the phase level (Bar 1987). Molecular toxicity has been described as compounds that are soluble in the aqueous phase and, due to their hydrophobicity, intercalate within the lipid bilayer and deteriorate membrane function (Sikkema et al. 1995). Increases in membrane fluidity, membrane permeability, denaturation of membrane-bound proteins, loss of transport mechanisms and reduced energy transduction are some of the consequences of a solvent's impact at the molecular level (Sikkema et al. 1994; Inoue and Horikoshi 1991; Vermue et al. 1993; León et al. 1998). Alternatively, phase toxicity is a phenomenological rather than mechanistic definition: toxicity occurs when a solvent has exceeded its aqueous solubility point and a distinct second phase exists. Disruptions of outer cellular components by cell-solvent contact, extraction of nutrients from the medium and limited access to nutrients by cell coating are some proposed mechanisms for phase toxicity (Bar 1987), but a mechanistic understanding of phase-mediated toxicity has yet to be realised.

Recently, the distinction between molecular and phase toxicity has been highlighted in the investigation of monoterpene inhibition in yeast. Monoterpenes have gained considerable attention as precursors for 'drop-in' jet fuel replacements (Lee et al. 2008; Fortman et al. 2008; Rushe 2007; Neales 2013). Monoterpene inhibition has largely been attributed to membrane interference given their lipophilic chemical nature (octanol-water partitioning coefficient log $P = 4.5$) (Uribe et al. 1985; Uribe and Pena 1990). However, recent reports demonstrated that rather than molecular-mediated toxicity in the membrane, monoterpenes cause phase toxicity. For example, limonene inhibited growth at concentrations nearly ten times that of its aqueous solubility point (Brennan et al. 2012). Global transcriptional and physiological studies could not identify perturbations at the membrane. Upregulation of ergosterol synthesis or changes in membrane composition, which are hallmark effects of membrane toxicity due to ethanol in yeast, were not observed in the presence of limonene (Brennan et al. 2013). Conversely, it was found that the surface of the cell was damaged after limonene treatment, further supporting a phase-mediated mechanism rather than molecular toxicity (Brennan et al. 2013). The phase toxicity observed can be mitigated by using a two-phase extractive culture approach, with the non-toxic solvent farnesene as an extractant (Brennan et al. 2012). This approach is particularly attractive, as a limonene/farnesene/*p*-cymene mixture has been proposed as a drop-in jet fuel alternative (Renninger and McPhee 2008). Another possibility is separating biomass accumulation (growth) and production phases to mitigate against toxicity, for example, by using synthetic regulatory circuits (Williams et al. 2013).

Summary, Conclusions and Outlook

While significant advances have been made over the last decade, many technical issues still hinder the development of commercially viable isoprenoid bioprocesses using engineered microorganisms. Engineering enough flux through core isoprenoid pathways to provide sufficient precursors remains the largest challenge. While production of simple isoprenoids using a single terpene synthase has so far yielded the greatest titres, more complex isoprenoids are significantly more difficult—particularly where multiple enzymes are required for biosynthesis and where those enzymes do not work efficiently in their new microbial hosts (Vickers et al. 2014). Non-specific modification is also problematic and may represent significant carbon loss, particularly in yeast where biotransformation capabilities are extensive.

Product toxicity will increasingly become a problem as toxicity thresholds are met and exceeded in de novo production. While our understanding of toxicity in microbes is improving, there is much that is not yet understood. Toxicity is clearly complex, with different levels of toxicity for different compounds in the same organism, different levels of toxicity for the same compound in different microorganisms and different mechanisms of toxicity (Sarria et al. 2014; Brennan et al. 2012; Dunlop et al. 2011; Dunlop 2011; Segura et al. 2012). In addition to two-phase extraction methods, approaches under development to mitigate against toxicity include efflux pumps and stress support mechanisms (heat shock proteins, membrane modifications and media supplementation) and adaptive evolution (Dunlop 2011; Foo and Leong 2013). As new engineering approaches deliver increased titres, co-integration of methods to mitigate toxicity will become more important. In the case of monoterpenes, use of species that tolerate relatively high levels such as *Pseudomonas putida* (Mirata et al. 2009) and *Penicillium digitatum* (Badee et al. 2011) may also provide routes to improved bioprocesses, particularly as our ability to engineer these organisms for de novo production improves.

Production of isoprenoids from inorganic substrates, including CO_2 (via photosynthesis) and COx from industrial exhaust gases, will ultimately become the preferred method. Both approaches, particularly the latter, reduce greenhouse gas production and mitigate against climate change. Technologies for the efficient reduction of oxidised carbon in a form suitable for bacterial metabolism are also under active development. However, these approaches hinge on our ability to develop and apply technologies for engineering appropriate microbial biocatalysts. Eventually, we may be able to tailor ‘chassis cells’ with desired feedstock, product and toxicity mitigation modules, thereby creating designer biocatalysts for specific feedstocks and products (Vickers et al. 2010). Exciting advances in synthetic biology, combined with remarkable increases in isoprenoid titres over the last decade, are moving us towards turning this vision into reality.

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The Role of Cellulose-Hydrolyzing Bacteria in the Production of Biogas from Plant Biomass

Vladimir V. Zverlov, Daniela E. Köck, and Wolfgang H. Schwarz

Contents

Abstract Biological decomposition of biomass, i.e., the abundant and renewably produced whole plant biomass, is the basis for the production of bioenergy and platform chemicals in a biorefinery. Biogas formation is presently the most energy-efficient, versatile, and mature technology of producing energy and (potentially) a number of useful by-products. It can use a wide range of dedicated energy crops and by-products from the biorefinery. Biogas is easily stored and distributed by the existing infrastructure and can be used directly by the end consumers. Although biogas fermentation from plant biomass uses mature technology, the efficiency and yield of biogas plants can however still be increased. Little is, for instance, known about the underlying biology, and the biological basis of the process is not completely understood. This review deals with the first step of biogas fermentation, the hydrolysis of the polysaccharides in plant biomass. It is regarded as one of the

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rate-limiting steps in the process. It also determines the overall efficiency of the process. Cellulose is recalcitrant to enzymatic hydrolysis and needs special enzyme systems which are produced by a limited number of specialized microorganisms. Various bacterial enzyme systems for cellulose degradation are discussed. The bacteria in biogas fermenters are analyzed, and potential key players for cellulose degradation are pointed out. The principles of their enzyme systems could be used for developing new cellulases for cellulosic biomass as a basic substrate in a future biotechnology.

1 Introduction

Biorefinery intends to make comprehensive use of all material and energy streams derived from biomass "by complex systems based on ecological technology" (Kamm and Kamm 2004). It produces a variety of carbon-based products as well as energy—based on renewable material and thus sustainable. However, whereas industrial biotechnology often depends on clean and well-defined biomass (or purified fractions thereof) as substrate for separation and conversion processes, the production of biogas does not necessarily require to grow dedicated biomass (Fig. 1). Waste material and by-products can be used—they do frequently have no further use or are too expensive to reprocess. This material may be worthless in a chemical sense (useless compounds) or bound in a material complex such as a lignin matrix which is recalcitrant to further separation processes. Such material often occurs in too small amounts to set up a special process, and its further use is therefore not economically feasible. This is also often the case for waste material

Fig. 1 A mixture of grass and maize silage fed into a biogas fermenter

such as biological municipal solid waste, garden waste, road cuttings, and other waste material.

For these and other kinds of heterogeneous material, the biogas process may be installed as a channeling reaction to produce a relatively homogeneous, low-value but energy-rich gas consisting mainly of methane (CH_4) and carbon dioxide (CO_2) (Bochiwal et al. 2010). This gas mixture can be used for chemical synthesis of higher value products—and thus for refeeding into the biorefinery process. After purification it can also be used for energetical purposes (by combustion or fuel cell technology), for instance, as process energy for the biorefinery or filled in cars for transportation. On the other hand, it can be fed into the gas grid for a variety of purposes (Antoni et al. 2007). The gas grid needs purification of the biogas; however, it has the additional value of energy storage and utilization at the point of use. The separation of methane gas from the other constituents of biogas such as H_2S or CO_2 is state of the art and even in large scale economically feasible. Other constituents of biogas such as NH_4 or N_2 are avoidable by adapting the process appropriately or are harmless for the intended use. Biogas has advantages over the production of hydrogen gas from biomass, including the versatility of production, the energy content of the gas, and the storability and transportability for which the infrastructure is already existing (Fehrenbach et al. 2008).

Biogas production from waste material and by-products as well as from dedicated energy plants has also the advantage of producing a high-value organic fertilizer, either as a liquid manure, or dried and pelleted, or otherwise formulated. This fertilizer retains from the feed material most of the nitrogen as natural and biologically active ammonium compounds, all of the macro- and microelements contained in (and necessary for the growth of) plant biomass, and, probably most important, all of the phosphorous in natural and biologically accessible form. Whereas lost ammonium/nitrate can be regained by energy-intensive synthetic processes from aerial nitrogen gas, and all macro- and microelements are abundant in minerals, the agriculture of the near future will depend on recycled phosphorous compounds, free (or almost free) of heavy metal contaminants which are unavoidable when natural mineral deposits for phosphor salts will come to exhaustion in the near future (Lebuf et al. 2012).

It is often discussed that biogas fermentation is in competition with the plant material which has to be left on the fields to improve soil quality or at least to avoid soil depletion of humic substances. However, the carbon compounds used for the production of biogas would be lost quickly anyway due to natural aerobic decay when the plant material was distributed to or worked in the soil of fields. The digestate from biogas plants contains all the lignin and a part of other recalcitrant carbon compounds which cannot be used for biogas formation and thus lead at the end to humification and soil improvement when brought back to the fields. The biogas process is thus part of a complete recycling system in agriculture and forestry. The biogas process can therefore become an important stepstone for a biomass-based society built on sustainable energy and material supply; it is recycling as much of its resources as possible (Weiland 2006).

To install such a promising technology on a much broader basis, the technology—which is already mature and economically feasible in its application—still has to be considerably improved. There is potential for improvement on many stages of the process. Its material (substrate) basis should be broadened and its reliability optimized. This will also save the resources and reduce the energy necessary to produce the biomass fed to the process. To achieve these improvements, it will be essential to switch from an empirical approach of optimizing biogas technology toward a knowledge-based biological engineering, including a thorough understanding of the biological processes underlying the system biology of a biogas production plant. Such knowledge is now accumulating by scientifically raveling up the different aspects of substrate decomposition, metabolism, and energy as well as carbon flow within the reactor.

2 The Various Steps in Biogas Production

Biogas is produced by a natural consortium of interacting bacteria and archaea, possibly also involving anaerobic fungi (Fliegerová et al. 2010; Griffith et al. 2010). In contrast to the rumen microbial consortium, ciliates are obviously absent and the role of anaerobic fungi is so far largely unknown. So it seems to be the bacteria which degrade the constituents of the biomass fed into the fermenter vessel, particularly the polysaccharides. They use them for the buildup of cell biomass and the production of enzymes to hydrolyze the polymers in biomass; they release sugars for other bacteria, use up all residual oxygen to make the microenvironment completely anaerobic, and release fermentation products which are in turn utilized by other bacteria and by archaea for biogas production.

Biogas production is a cooperation of basically three types of bacteria, working in a succession of events, to name the most relevant steps: hydrolytic bacteria degrade the polymers in the biomass and produce—together with the saccharolytic bacteria—organic acids, alcohols, CO_2, and H_2 (the hydrolytic and acidogenic step); these products are converted by syntrophic bacteria, the acetogenic bacteria, to acetate; the acetate and the gases CO_2 and H_2 are converted to methane and CO_2 by the acetotrophic and the hydrogenotrophic methanogens, respectively. Other reactions such as acetate oxidation are also involved (Fig. 2).

The low amount of biological energy produced by these processes in the absence of oxygen (and thus in the absence of respiration) leads to massive turnover of substrate with the production of oxidized (such as CO_2) and reduced compounds (such as H_2 and CH_4) through disproportionation. The first step in the biogas process, the hydrolysis, is regarded as crucial for the efficiency—the more of the substrate is utilized, the greater is the amount of methane produced. Moreover, it is regarded as rate limiting because all downstream processes depend completely on the yield and production rate of the initial hydrolysis. The rate of decomposition during the hydrolysis stage depends greatly on the nature of the substrate. The transformation of cellulose and hemicellulose generally takes place more slowly

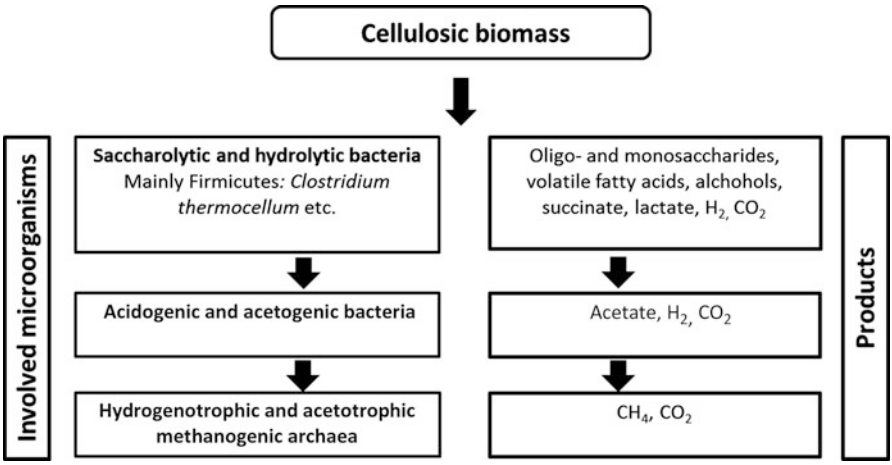

Fig. 2 Biological and metabolic processes during biogas fermentation, based on cellulosic biomass

than the decomposition of proteins (Boone et al. 1993; Lynd et al. 2002; Noike et al. 1985).

The processes downstream from hydrolysis (acidogenesis and acetogenesis) are basically the channeling of all fermentation products into acetate and the gases CO_2 and H_2; these are finally used by the acetotrophic and the hydrogenotrophic archaea which finally produce methane and carbon dioxide, the biogas (Schnürer and Jarvis 2009) (Fig. 2).

The production of biogas from organic materials is widespread in nature and can be found, for instance, in the intestine of plant-feeding animals or insects, in compost formation, in marshes and swamps, or in the debris on the ground of lakes where plant biomass is degraded anaerobically (Görisch and Helm 2006). A number of studies have been carried out on biogas formation, beginning with studies on wastewater and manure, later on plant biomass (Zverlov et al. 2010; Kampmann et al. 2012; Cirne et al. 2007). However, although cellulose is the richest source of organic carbon compounds on earth (Cox et al. 2000), studies on cellulose as substrate and its degradation for biogas production are still insufficient.

Whereas a number of microorganisms are known to be able to degrade natural cellulose, and some bacteria have been studied intensively which degrade cellulose in the rumen of cattle or in the environment, the identity and obvious variety of the cellulose-degrading bacteria in biogas plants is still largely untapped. In general there is a lack of information on the microbiology in various steps of the biogas process, and particularly in the first, the hydrolytic step.

3 The Recalcitrance of Cellulose

The polysaccharides in plant cell walls give the plants structure and sturdiness and strength in stems and stalks. They make up for the greatest part of plant biomass and therefore represent a large potential for biogas production. The intertwined composition of various polymers makes the material resistant to microbiological attack—only a few saprophytic organisms are able to degrade this material relatively efficiently. Some of these microorganisms are degrading it in coexistence or even in symbiosis with plant-feeding animals and insects, such as cattle, camels, roaches, or termites.

There are three major polymeric, interwoven components of biomass: cellulose, hemicellulose/pectin, and lignin. Fundamentally different types of enzyme sets are necessary for their degradation, and no single microorganism is able to degrade all polymers in plant cell walls completely without the help of others.

The slow hydrolysis rate of plant cell wall degradation is mainly related to the presence of lignin, which cannot be substantially degraded under anaerobic conditions. The amount of lignin diminishes degradation rate and degradability, obviously by occluding accession sites for hydrolyzing enzymes (Lynd et al. 2002; Schnürer and Jarvis 2009).

The major cell wall component (in mass) is cellulose. It is an extremely recalcitrant material to degrade and is only degraded slowly. Consistent of long parallel, unbranched, homogeneous chains of β-1,4-linked glucose monomers, cellulose is highly crystalline, interrupted only by short amorphous regions (Fig. 3). Cellulose is thus an extremely tough material for enzymatic degradation, being not hydrated and completely insoluble, with a hydrophobic surface. In addition it is very large compared to the size of an enzyme. To fit into an enzyme substrate pocket for degradation, a cellulose molecule has to be pulled out from the crystalline surface where it is linked with numerous interchain and interlayer hydrogen bonds. Moreover, only a small part of the substrate is "visible" to an enzyme at the surface of the crystal and thus accessible to enzymatic attack; most of the substrate is hidden within the crystal (Schwarz 2004).

These effects make cellulose relatively resistant to degradation; its degradation speed is extremely slow and necessitates the presence of a consortium of different and specialized enzymes which act cooperatively to break up the surface with binding modules (non-catalytic "activity") and a multitude of synergistically acting β-glucanase modules (catalytic activity). These enzymes have different modes of

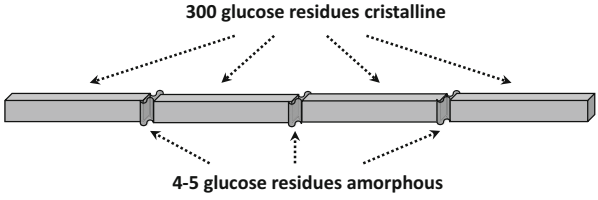

Fig. 3 Approximate structure of a cellulose crystal in ramie wood (according to Nishiyama et al. 2002, 2003a, b)

activity, such as endo-active endoglucanases and processively active exoglucanases (Schwarz et al. 2004). They degrade cellulose to a mixture of cellobiose, cellotetraose, and various other cellodextrins, which are taken up by the cells and further degraded by β-glucosidases/cellobiases and/or cellobiose or cellodextrin phosphorylases.

4 Cellulose-Degrading Bacteria Are Rare

So far only a relatively small number of cellulolytic bacterial species have been isolated and characterized. A list restricted to bacteria which can utilize cellulose in its natural (=crystalline) configuration as sole carbon and energy source is compiled in Table 1. Many more bacteria produce extracellular β-glucanases, usually endoglucanases, which hydrolyze the β-1,4-glucosidic bond only in soluble (mixed linkage) β-glucans or artificial cellulosic compounds such as carboxymethylcellulose (CMC). Although CMC is chemically a cellulose with its typical β-1,4-glucosidic linkages, its degradation is a precondition but not a sufficient indication for degradation of natural (i.e., crystalline) cellulose. The literature was checked carefully for indication of traits such as growth on filter paper or crystalline cellulose powder (such as Avicel) or the like (Fig. 4). Such strains are also called "true cellulolytic" bacteria. Due to their potential importance for biotechnology, the genomes of a number of the species listed in Table 1 are sequenced and deposited in databases.

From the strains listed in Table 1, two general physiological groups of cellulolytic bacteria can be formed: the anaerobic and the aerobic cellulolytic bacteria. The difference is the gain of energy from the sugars obtained from the cellulose. The anaerobic bacteria (e.g., the *Clostridia*) can produce from the same amount of glucose roughly only 1/10 of the Adenosine triphosphate (ATP) by fermentation as aerobic bacteria by respiration. Therefore, the enzymes producing the glucose from cellulose under anaerobic conditions have to be at least ten times as active to compensate for the energy used for their production (Lynd et al. 2002).

It is obvious that the majority of the so far known truly cellulolytic species belong to the bacterial phyla *Firmicutes* and *Actinobacteria*. However, the number of species in an ecosystem does not reflect the prevalence of certain bacteria in a habitat—the number of individual cells of a certain function (such as being truly cellulolytic) in a population is by far more important. This number was estimated for some samples by culture-independent methods such as high-throughput pyrosequencing of 16S rRNA sequences found in a population or by assigning sequence tags to known genes in a database and counting the next known neighbors in a phylogenetic or a similarity tree.

Total DNA or RNA isolated from biogas plant sludge was used for these investigations (Fig. 5). By these methods, *Firmicutes* were found to be the majority of bacteria in saccharolytic bacterial communities such as a biogas fermenter (see

Table 1 List of bacteria reported to grow on crystalline cellulose as carbon and energy source. Utilization of crystalline cellulose t = thermophile (optimum growth above 50 °C), m = mesophile. Material or habitat for isolation and phylogenetic position are indicated

Phylogeny	Species	Temp	Source	Reference
Phylum *Firmicutes*				
Class *Clostridia* Order *Thermoanaerobacterales* Family *incertae sedis*	*Caldicellulosiruptor bescii*	t		Svetlichnyi et al. (1990)
	Caldicellulosiruptor hydrothermalis	t	Hot spring	Miroshnichenko et al. (2008)
	Caldicellulosiruptor kristjanssonii	t	Hot spring	Bredholt et al. (1999)
	Caldicellulosiruptor kronotskyensis	t	Hot spring	Miroshnichenko et al. (2008)
	Caldicellulosiruptor lactoaceticus	t		Mladenovska et al. (1995)
	Caldicellulosiruptor obsidiansis	t	Hot spring	Hamilton-Brehm et al. (2010)
	Caldicellulosiruptor saccharolyticus	t	Hot spring	Rainey et al. (1994)
	Thermoanaerobacter cellulolyticus	t	Hot spring	Bergquist et al. (1999)
Class *Clostridia* Order *Clostridiales* Family *Lachnospiraceae*	*Butyrivibrio fibrisolvens*	m	Rumen	Berger et al. (1990)
	Cellulosilyticum lentocellum	m	Rumen	Cai and Dong (2010)
	Cellulosilyticum ruminicola	m	Rumen	Cai and Dong (2010)
	Ruminococcus succinogenes	m	Rumen	Fields et al. (2000)
Class *Clostridia* Order *Clostridiales* Family *Eubacteriaceae*	*Eubacterium cellulosolvens*	m	Rumen	Anderson and Blair (1996)
Class *Clostridia* Order *Clostridiales* Family *Clostridiaceae*	*Clostridium aldrichii*	m	Wood fermenter	Yang et al. (1990)
	Clostridium alkalicellulosi	m	Soda lake	Zhilina et al. (2005)
	Clostridium caenicola	t	Sludge	Shiratori et al. (2009)
	Clostridium celerecrescens	m	Manure	Palop et al. (1989)
	Clostridium cellobioparum	m	Rumen	Lamed et al. (1987)
	Clostridium cellulofermentans	m	Manure	Yanling et al. (1991)
	Clostridium cellulolyticum	m	Compost	Pagés et al. (1997), Bélaich et al. 1997
	Clostridium cellulosi	t	Manure	Yanling et al. (1991)

(continued)

Table 1 (continued)

Phylogeny	Species	Temp	Source	Reference
	Clostridium cellulovorans	m	Wood	Shoseyov and Doi (1990), Tamaru et al. (2000)
	Clostridium chartatabidum	m	Rumen	Kelly et al. (1987)
	Clostridium clariflavum	t	Sludge	Shiratori et al. (2009)
	Clostridium herbivorans	m	Pig intestine	Varel et al. (1995)
	Clostridium hungatei	m	Soil	Monserrate et al. (2001)
	Clostridium josui	t	Compost	Kakiuchi et al. (1998)
	Clostridium longisporum	m	Rumen	Leschine (1995)
	Clostridium papyrosolvens	m	Paper mill	Pohlschröder et al. (1995)
	Clostridium phytofermentans	m	Soil	Warnick et al. (2002)
	Clostridium populeti	m	Wood fermenter	Leschine (1995)
	Clostridium sp. *C7*	m	Mud	Cavedon et al. (1990)
	Clostridium stercorarium	t	Compost	Schwarz et al. (1995)
	Clostridium straminisolvens	m	Rice straw	Kato et al. (2004)
	Clostridium sufflavum	m	Cattle waste	Nishiyama et al. (2009)
	Clostridium termitidis		Termite	Hethener et al. (1992)
	Clostridium thermocellum	t	Sewage + soil	Lamed et al. (1991)
	Clostridium thermocopriae	t	Hot spring	Jin and Toda (1989)
	Clostridium thermopapyrolyticum	h	Mud	Méndez et al. (1991)
Class *Clostridia* Order *Clostridiales* Family *Ruminococcaceae*	*Acetivibrio cellulolyticus*	m	Sewage	Ding et al. (1999)
	Acetivibrio cellulosolvens	m	Sewage	Khan et al. (1984)
	Ruminococcus albus	m	Rumen	Ohara et al. (2000)
	Ruminococcus flavefaciens	m	Rumen	Aurilia et al. (2000)
Class *Clostridia* Order *Halanaerobiales* Family *Halanaerobiaceae*	*Halocella cellulosilytica*	m	Saline lake	Simankova et al. (1993)

(continued)

Table 1 (continued)

Phylogeny	Species	Temp	Source	Reference
Class *Bacilli* Order *Bacillales* Family *Thermoactinomycetaceae*	*Thermoactinomyces* sp. *YX*	t		Hägerdahl et al. (1979)
Class *Bacilli* Order *Bacillales* Family *Alicyclobacillaceae*	*Caldibacillus cellulovorans*	t		Sunna et al. (2000)
Class *Bacilli* Order *Bacillales* Family *Bacillaceae*	*Bacillus circulans*	m		Kim (1995)
	Bacillus pumilus	m		Ariffin et al. (2006)
Phylum *Actinobacteria*				
Class *Actinobacteria* Subclass *Actinobacteridae* Order *Actinomycetales* Suborder *Frankineae* Family *Acidothermaceae*	*Acidothermus cellulolyticus*	t	Acidic hot spring	Eppard et al. (1996), Maréchal et al. (2000)
Class *Actinobacteria* Subclass *Actinobacteridae* Order *Actinomycetales* Suborder *Micrococcineae*	*Cellulomonas biazotea*	m		Lednicka et al (2000)
	Cellulomonas cartae	m		Thayer et al. (1984)
	Cellulomonas cellasea	m		Lednicka et al. (2000)
	Cellulomonas cellulans	m	Soil	Lednicka et al. (2000)
	Cellulomonas fimi	m	Soil	Lednicka et al. (2000)
	Cellulomonas flavigena	m	Soil	Lednicka et al. (2000)
	Cellulomonas gelida	m		Thayer et al. (1984)
	Cellulomonas iranensis	m	Forest soil	Elberson et al. (2000)
	Cellulomonas persica	m	Forest soil	Elberson et al. (2000)
	Cellulomonas uda	m	Sewage	Thayer et al. (1984)
Class *Actinobacteria* Subclass *Actinobacteridae* Order *Actinomycetales* Suborder *Micrococcineae* Family *Microbacteriaceae*	*Curtobacterium flaccumfaciens*	m	Soil	Lednicka et al. (2000)
Class *Actinobacteria* Subclass *Actinobacteridae* Order *Actinomycetales* Suborder *Micrococcineae* Family *Promicromonosporaceae*	*Xylanimonas cellulosilytica*	m	Decayed tree	Anderson et al. (2012)

(continued)

Table 1 (continued)

Phylogeny	Species	Temp	Source	Reference
Class *Actinobacteria* Subclass *Actinobacteridae* Order *Actinomycetales* Suborder *Micromonosporineae* Family *Micromonosporaceae*	*Actinoplanes aurantiaca*	m	Soil	Coughlan and Mayer (1992)
	Micromonospora melonosporea	m	Compost	Wilson (1992)
	Micromonospora chalcae	m	Soil	Gallagher et al. (1996)
	Micromonospora propionici	m	Termite	Leschine (1995)
	Micromonospora ruminantium	m	Rumen	Leschine (1995)
Class *Actinobacteria* Subclass *Actinobacteridae* Order *Actinomycetales* Suborder *Pseudonocardineae* Family *Pseudonocardiaceae*	*Actinosynnema mirum*	m	Grass blade	Anderson et al. (2012)
Class *Actinobacteria* Subclass *Actinobacteridae* Order *Actinomycetales* Suborder *Streptomycineae* Family *Streptomycetaceae*	*Streptomyces albogriseolus*	m		Van Zyl (1985)
	Streptomyces aureofaciens	m	Compost	EL-Din et al. (2000)
	Streptomyces cellulolyticus	m		Li and Gao (1997)
	Streptomyces flavogriseus	m	Soil	MacKenzie et al. (1984)
	Streptomyces lividans	m		Kluepfel et al. (1986)
	Streptomyces nitrosporeus	m		Van Zyl (1985)
	Streptomyces olivochromogenes	m		Coughlan and Mayer (1992)
	Streptomyces reticuli	m	Soil	Schrempf and Walter (1995)
	Streptomyces rochei	m	Termite gut	Perito et al. (1994)
	Streptomyces thermovulgaris	m		Coughlan and Mayer (1992)
	Streptomyces viridosporus	m		Coughlan and Mayer (1992)
Class *Actinobacteria* Subclass *Actinobacteridae* Order *Actinomycetales* Suborder *Streptosporangineae* Family *Nocardiopsaceae*	*Thermobifida alba*	m		Kukolya, pers. commun.
	Thermobifida cellulolytica	t	Compost	Kukolya et al. (2002)
	Thermobifida fusca	t	Soil	Wilson (1992), Kukolya, pers. commun. (2003)

(continued)

Table 1 (continued)

Phylogeny	Species	Temp	Source	Reference
Class *Actinobacteria* Subclass *Actinobacteridae* Order *Actinomycetales* Suborder *Streptosporangineae* Family *Thermomonosporaceae*	*Thermomonospora curvata*	t		Coughlan and Mayer (1992)
Class *Actinobacteria* Subclass *Actinobacteridae* Order *Actinomycetales* Suborder *Streptosporangineae* Family *Streptosporangiaceae*	*Thermobispora bispora*	t	Soil	Wilson (1992)
	Streptosporangium subroseum	m	Soil	Zhang et al. (2002)
Superphylum *Fibrobacteres*/*Acidobacteria* group, phylum *Fibrobacteres*				
Class *Fibrobacteria* Order *Fibrobacterales* Family *Fibrobacteraceae*	*Fibrobacter succinogenes*	m	Rumen	Schellhorn and Forsberg (1984)
Superphylum *Bacteroidetes*/*Chlorobi* group, phylum *Bacteroidetes*				
Class *Cytophagia* Order *Cytophagales* Family *Cytophagaceae*	*Cytophaga aurantiaca*	m	Soil	Li et al. (1997)
	Cytophaga haloflava	m	Soil	Li et al. (1997)
	Cytophaga hutchinsonii	m	Soil	Li et al. (1997)
	Cytophaga krzemieniewskae	m	Soil	Li et al. (1997)
	Cytophaga rosea	m	Soil	Li et al. (1997)
	Sporocytophaga myxococcoides	m	Soil	Coughlan and Mayer (1992)
Class *Flavobacteriia* Order *Flavobacteriales* Family *Flavobacteriaceae*	*Flavobacterium johnsoniae*	m	Soil	Lednicka et al. (2000)
Class *Bacteroidia* Order *Bacteroidales* Family *Bacteroidaceae*	*Bacteroides cellulosilyticus*	m	Human fecal sample	Robert et al. (2007)
	Bacteroides cellulosolvens	m	Sewage	Lamed et al. (1991)
	Bacteroides sp. P-1	t	Rotting biomass	Ponpium et al. (2000)
Class *Bacteroidia* Order *Bacteroidetes*, order II. *incertae sedis* Family *Rhodothermaceae*	*Rhodothermus marinus*	t	Hot spring	Bergquist et al. (1999)

(continued)

Table 1 (continued)

Phylogeny	Species	Temp	Source	Reference
Phylum *Proteobacteria*				
Class *Betaproteobacteria* Order *Burkholderiales* Family *Alcaligenaceae*	*Achromobacter piechaudii*	m	Soil	Lednicka et al. (2000)
Class *Gammaproteobacteria* Order *Enterobacteriales* Family *Enterobacteriaceae*	*Dickeya dadantii*	m	Plant biomass	Rabinovich et al. (2002)
Class *Gammaproteobacteria* Order *Xanthomonadales* Family *Xanthomonadaceae*	*Xanthomonas* sp.	m	Brack water	Mullings and Parish (1984)
Class *Gammaproteobacteria* Order *Pseudomonadales* Family *Pseudomonadaceae*	*Cellvibrio gilvus*	m	Soil	Coughlan and Mayer (1992)
	Cellvibrio mixtus	m	Soil	Lednicka et al. (2000)
	Cellvibrio vulgaris, *C. fulvus*	m	Soil	Blackall et al. (1985)
	Pseudomonas fluorescens (cellulosa)	m	Plant biomass	Dees et al. (1995)
	Pseudomonas mendocina	m	Soil	Lednicka et al. (2000)
Subphylum *delta/epsilon subdivisions* Class *Deltaproteobacteria* Order *Myxococcales* Family *incertae sedis*	*Myxobacter* sp. AL-1	m	Soil	Avitia et al. (2000), Pedraza-Reyes, pers. commun.
Phylum *Thermotogae*				
Class *Thermotogae* Order *Thermotogales* Family *Thermotogaceae*	*Fervidobacterium islandicum*	t	Hot spring	Huber et al. (1990)
	Thermotoga maritima	t	Hot spring	Bergquist et al. (1999)
	Thermotoga neapolitana	t	Hot spring	Bergquist et al. (1999)

below). It should be kept in mind that the source of the first described isolation is only an indication of the environment in which these bacteria can be found. Most of them have later been isolated from or identified in other environments. For example, *Clostridium thermocellum*, originally isolated from sewage and from soil, has been shown to be a very common environmental bacterium, present in a variety of habitats where plant biomass is degraded, such as cattle manure, garden soil,

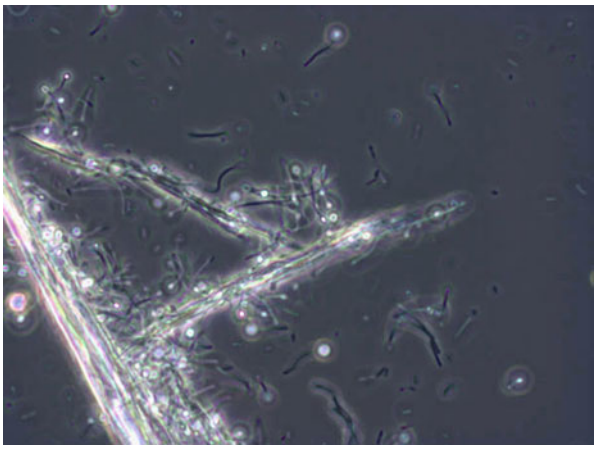

Fig. 4 Cellulose-degrading bacteria on cellulose fibers (phase contrast microscopy, ×1,000 magnification)

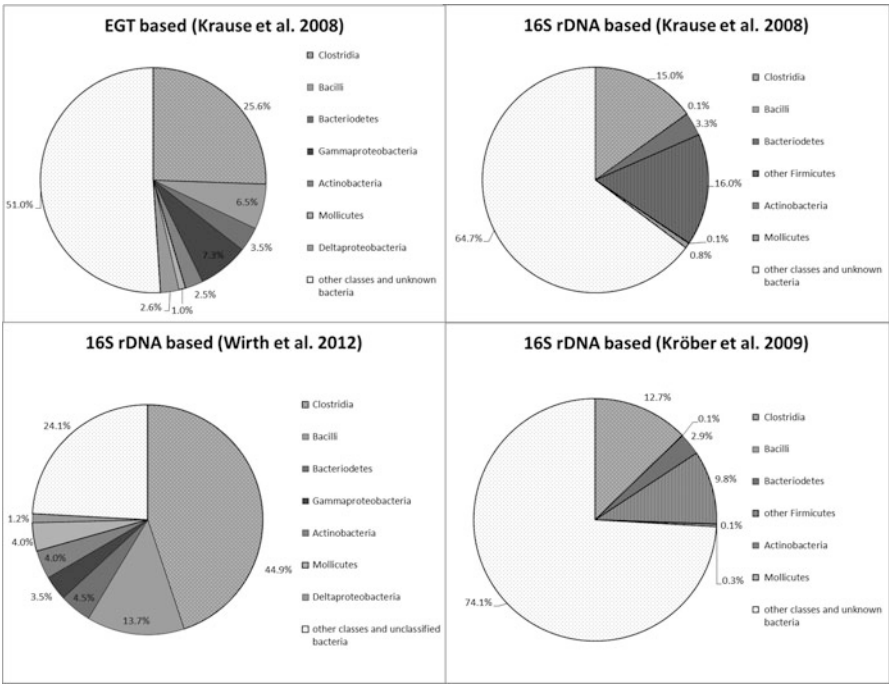

Fig. 5 Taxonomic profiles from the superkingdom bacteria of different biogas microbial communities based on analysis of metagenome sequence reads created by next-generation sequencing techniques. Phylogenetic class is indicated. The authors compared either 16S-rDNA sequences or environmental gene tags (EGTs) with different reference data sets, e.g., Ribosomal Database Project Classifier or ARB Database (modified after Wirth et al. 2012; Kröber et al. 2009; Krause et al. 2008)

bushes, compost, agriculturally used plots, and biogas fermenters run with energy crops (Zverlov et al. 2010; unpublished data).

Most of the cellulolytic species listed in Table 1 are saprophytic, saccharolytic bacteria (especially the thermophilic bacteria); however, some are pathogenic to humans or plants such as *Bacillus circulans* (Leary et al. 1986) or *Pseudomonas mendocina* (Aragone et al. 1992). Whereas cellulolytic activity is apparent for plant pathogenic bacteria, this is not obvious for human pathogens. Most plant pathogens have some cellulolytic activity for loosening the cell wall and getting access to the cell lumen; however, this activity is often not sufficient for providing the sugars from cellulose to support growth. Plant pathogenic bacteria do not seem to play a role in biomass degradation during the biogas process.

5 Taxonomic Composition of Biogas Plants Obtained by Clone Library 16S-rDNA Sequences and Metagenome Sequences Generated by Next-Generation Sequencing

Only a small fraction of the bacterial species present in a biogas fermenter has been isolated in pure culture. Strain purification, however, is a precondition for a thorough characterization of their catabolic and metabolic traits, i.e., the use of substrates and the formation of fermentation products—and hence their role in the biogas process chain in a fermenter. However, culture-independent methods are necessary to analyze the complete composition of the bacterial biogas community. Currently next-generation sequencing methods, like 454 pyrosequencing, are used to analyze bacterial (and archaeal) communities by generation of environmental gene tags (EGTs) and clusters of orthologous groups of proteins (COGs) or 16S-rDNA libraries (Krause et al. 2008; Schlüter et al. 2008; Kröber et al. 2009; Wirth et al. 2012). These methods are however severely hampered by the lack of reference genomes for most of the species involved, often even genera. This is reflected in the high percentage of "unknown bacteria" or "other classes" in Fig. 5.

As shown in Table 1, most of the hitherto known bacteria able to efficiently degrade natural cellulose belong to the phylum *Firmicutes*, particularly to the class *Clostridia*. The overwhelming majority of the identified species in the biogas fermenters were also members of the phylum *Firmicutes*, class *Clostridia* (Fig. 5; Wirth et al. 2012; Krause et al. 2008). Among the EGTs, coding for proteins involved in the hydrolysis of poly- and oligosaccharides, *Firmicutes* are again with over 50 % the dominant phylogenetic group and again mainly represented in the class *Clostridia* (Krause et al. 2008). Consequently, most of the identified species with known cellulolytic members also belong to the class *Clostridia*. Cellulolytic species of the other phyla are only rarely identified.

Although most known cellulolytic bacteria belong to the *Clostridia*, this argument does not work if turned around: most clostridial species do not contain cellulose degraders, and thus the affiliation to the clostridia does not work as an

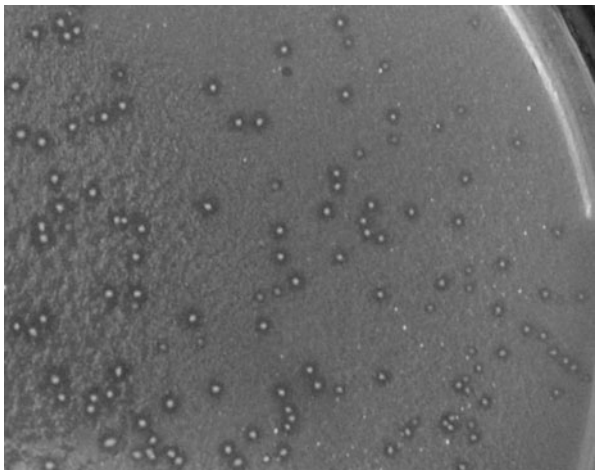

Fig. 6 Colonies of a new isolate of *C. thermocellum* (*white spots*) from biogas plants was plated on an agar plate overlayed with a thin agar layer containing cellulose powder (Avicel). The *hazy background* (from the cellulose fibers) is cleared around the colonies (*darker halo*) which produce cellulases and dissolves the cellulose

argument for a cellulolytic trait—for each single species, it has to be shown that its members are able to utilize crystalline cellulose. Even closely related bacteria (on the 16S-rDNA sequence level) differ in this ability. And the use of genomic sequence data for strain assignment to a functional group can also sometimes be misleading. In the case of *Clostridium acetobutylicum*, all the sequences necessary for expression of cellulosomes (very efficient extracellular cellulase complexes) were present (Sabathé et al. 2002). However, the encoded proteins turned out to be defective in expression and/or activity and did not show sufficient cellulose hydrolysis capability for supporting the bacterium to grow on cellulose. Hence this species is not included in the "cellulolytic" bacterium species listed in Table 1.

Bacteria of the species *Clostridium thermocellum* degrade crystalline cellulose efficiently (Fig. 6). They occur most frequently in metagenome analysis data (Wirth et al. 2012; Krause et al. 2008). Other cellulolytic bacteria with high sequence abundance (within the 40 most frequently found species) were *Clostridium cellulolyticum*, *Ruminococcus albus*, *Clostridium saccharolyticum*, and *Caldicellulosiruptor saccharolyticus* (Wirth et al. 2012). The high abundance of cellulolytic members in the *Clostridia* indicates the important role of these bacteria for degradation of complex substrates in the natural remineralization of biomass.

The metagenomic analysis created a high number of sequences which could not be allocated to any microbial species (Fig. 5: up to 74 % are unclassified bacteria; Kröber et al. 2009). This implies the presence of many still unidentified microorganisms in biogas plants. And it indicates also that probably important bacteria involved in the degradation of cellulose are still unknown and cannot be identified due to the lack of reference sequences. This lack of knowledge can only be overcome by isolating and characterizing new cellulolytic bacteria.

A question still untouched is the cooperation of different bacteria for the degradation of resilient substrates. It was shown that a combination of enzymes of similar but not identical substrate specificity can be more effective in substrate

degradation (Zverlov et al. 2010; see below: cellulosomes). A similar effect could be generated by exoenzymes which work together but are produced by different bacteria (Kato et al. 2005). This is difficult to investigate with the methods at hand and has not been systematically investigated so far. One example is the degradation of cellodextrins to glucose which could be effected by extracellular β-glucosidases. However, most saccharolytic bacteria seem to possess uptake systems for cellodextrins, and these are degraded by intracellular enzymes, either by phosphorolysis leading to glucose-1-phosphate (as was described for *C. thermocellum*) or hydrolytically by β-glucosidases. There is the possibility that free glucose might not be an intermediate in biogas formation from lignocellulosic biomass. Another example is the combination of the cellulose specialist *C. thermocellum* and the hemicellulose specialist *C. stercorarium* which has been shown to degrade maize silage more efficiently than did any of the species alone (Zverlov et al. 2010).

6 The Enzyme Systems Used by Cellulolytic Bacteria in Biogas Plants

The addition of bacteria with superior cellulose-degrading ability could speed up the hydrolytic processes and make biogas production faster and thus more cost-efficient, among others, by utilizing more of the substrate in a shorter time. Addition of external enzyme, majorly consisting of cellulases, has only limited effect and is costly. Added enzymes would only have an effect when they would supplement with types of hydrolytic activity which are not (sufficiently) produced by the bacterial community in the process. The added hydrolytic bacteria would grow on the biomass are adapted to the substrate composition present, and produce sufficient amounts of enzymes of all types to degrade the various polysaccharides in the substrate so that they get at the end (after using up starch, hemicellulose, and pectin) access to the sugars in cellulose. Through the secretion of all necessary enzymes, the sugars released by their activity feed as well the other saccharolytic bacteria which produce the substances needed at the end of the complex biogas process by the archaea for methane production (Fig. 3). The overall methane production has been improved by repeated addition of a selected inoculation culture (Schmack and Reuter 2010). The addition of a selected cellulolytic culture improved the efficiency in a thermophilic technicum scale process (paper in preparation).

There are bacteria with fairly moderate cellulose degradation ability such as *Clostridium stercorarium*, which is more a specialist for the degradation of hemicellulose, but can thrive on filter paper as sole carbon source (Adelsberger et al. 2004). This species produces two cellulolytic enzymes of glycosyl hydrolase family 9 and 48 (GH9 and GH48) (Zverlov and Schwarz 2008). These two separate enzymes, an endoglucanase and a cellobiohydrolase, respectively, act synergistically to degrade the crystalline cellulose, however, slowly and incomplete. It does

this best at moderately thermophilic to thermophilic conditions. This species is found widespread in decaying biomass and has also been identified repeatedly in biogas fermenters (Madden 1983; Schwarz et al. 2004; Zverlov et al. 2010 and unpublished results).

Better cellulose degraders than *C. stercorarium* are thermophilic, anaerobic species within the genus *Caldicellulosiruptor*. The major cellulolytic enzyme of *C. bescii* (former *Anaerocellum thermophilum*) is the extracellular cellulase CelA which consists of a GH9 and a GH48 module combined within one polypeptide (Zverlov et al. 1998). The close neighborhood between the two enzymatic activities seems to enhance the synergistic effect which makes this enzyme system superior to the simple additive mixture of separate activities as is found with *C. stercorarium*.

The best cellulase system so far known is that of *Clostridium thermocellum*. It produces an extracellular, macromolecular complex which is called cellulosome (Schwarz 2001). The cellulosome is also the most elaborate extracellular enzyme system known in bacteria. It was used as a model system for an extracellular bacterial enzyme complex as well as an exemplary cellulase (Arai et al. 2001; Durrant et al. 1991; Kruus et al. 1995; Kurokawa et al. 2002; Zverlov et al. 1998, 2002, 2003, 2005a, b). In this enzyme complex the enzyme components bind via high-affinity and highly specific protein–protein interactions between the dockerins on the enzymatic components which bind to one of eight or nine cohesin modules in a so-called cellulosome-integrating protein, the CipA protein. These complexes stay cell wall bound on the surface of an actively growing bacterial culture (Bayer et al. 1985). The complexes seem to be shed off in later growth stages, where they can be isolated easily for investigation. Besides the cohesin modules, the CipA protein contains a substrate-binding module, a carbohydrate-binding module of type CBM3 which binds the complex (and hence the bacteria) to the surface of crystalline cellulose. This causes on the one hand a high local density of enzymes on the substrate surface (the insoluble cellulose) near the site of binding; this high enzyme density is essential for the high activity on the crystalline substrate. On the other hand, the localization on the cell wall allows direct take up of the degradation products of cellulose, the cellodextrins, by the bacterial cell. Other enzymes in the cellulosome degrade xylan and other hemicellulosic substances, pectin or chitin, and thus make the cellulose accessible for the highly specialized and effective cellulases (Schwarz 2001) (Fig. 7).

Cells of *C. thermocellum* do not use other sugar substrates than cellodextrins—glucose is hardly and other sugars (mono- or oligosaccharides) are not at all metabolized by this bacterium. The degradation products of hemicellulose, cellulose, etc., are left to the other bacteria within the bacterial community. *C. thermocellum* (and probably other similar bacteria) are thus the feeding machines driving the whole biogas process.

Cellulase complexes similar to the cellulosomes of *C. thermocellum* were also identified in other cellulolytic bacteria such as the mesophilic species *C. cellulovorans*, *C. cellulolyticum*, *C. papyrosolvens*, *Bacteroides cellulosolvens*, *Acetivibrio cellulolyticus*, *Ruminococcus albus*, and *Ruminococcus flavefaciens* and the thermophilic species *Clostridium clariflavum* and *Clostridium josui*. They

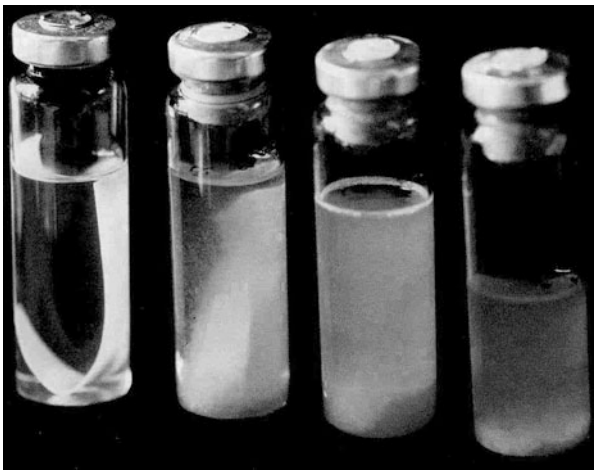

Fig. 7 A culture of *C. thermocellum* is inoculated to an anaerobic flask (rubber stoppered) containing a filter paper strip. The paper is increasingly dissolved after 1, 2, and 3 days (flask 2, 3, and 4 from *left* to *right*). Gas is formed under vigorous growth on day 2 (*bubbles*)

partially have a more or less different cellulosome architecture, and those of, for instance, the ruminococci are even more elaborate than in *C. thermocellum* (Bayer et al. 2013). Of them *C. thermocellum*, *C. cellulolyticum*, and *R. albus* had been detected in biogas plants (Wirth et al. 2012), underscoring their potentially important role for cellulose degradation in the industrial process.

7 The Cellulosome, an Efficient Cellulase Complex

The cellulosomes of *C. thermocellum*, *C. cellulolyticum*, and *C. cellulovorans* have been intensively investigated and still are subject to mechanistic studies including the in vitro reconstitution of complexes (Blouzard et al. 2010; Krauss et al. 2012; Vazana et al. 2012). The potential of complex formation for the degradation of the recalcitrant crystalline cellulose was made obvious by complete abolishment of CipA formation by mutagenesis (Zverlov et al. 2008; Olson et al. 2013). Mutants lacking the non-catalytic CipA protein are defective in cellulose degradation and not able to use filter paper as carbon source for growth anymore, although all the enzyme components of the cellulosome are still produced; i.e., although the cellulases are present in about the same amount, their activity is restricted to the hydrolysis of the soluble glucans CMC and barley β-glucan (Zverlov et al. 2008). Hence, the complex formation is a way of producing a cellulase enzyme system with considerably enhanced activity without having to produce ultra-large proteins as is the case with the enzyme systems of *Caldicellulosiruptor* strains.

The combination of various enzyme types in large protein molecules or protein complexes and with binding modules for the substrate enhances the activity by neighboring effects, a high local concentration of enzymatic activities on the site of binding, and the cooperation between enzymes of different mode of action, such as

endo- and exo-mode. This results in a synergistic effect which has been shown to be as high as a 15-fold activity, when the activity of complexed and comparable noncomplexed system are compared (Zverlov et al. 2008; Krauss et al. 2012).

The production of enzyme systems with enhanced efficiency is a necessity for anaerobic bacteria which can generate only a limited amount of energy in the form of ATP from the glucose produced.[1] This is in line with the general observation that anaerobic organisms use more energy-saving mechanisms than aerobic organisms.

8 The Biogas Process in Biorefinery Context

Although the biogas process, as it is widely established by now, is not a classical part of a biorefinery process chain, it often utilizes by-products or end products which have no further value for other technologies. Biogas formation may produce methane and carbon dioxide from recalcitrant or mixed material too "dirty" (too impure) to be used in the production of clean materials. However, biogas itself can be fed into the production of various chemicals by catalytic technologies using heterogeneous catalysts (Lunsford 2000) and thus making otherwise useless raw materials accessible for biorefinery. On the other hand, the sludge from the biogas process (the digestate) has been extracted for producing considerable amounts of vitamins B2 and B12 (riboflavin and cobalamin) in a complete biorefinery approach of utilizing lignocellulosic agricultural residues via clostridial acetone–butanol fermentation, using the fermentation gas, the biogas sludge for methane and vitamin production, and the biogas digestate as feed for yeast to single-cell protein in husbandry fodder (Zverlov et al. 2006). However, the full chain of biorefinery in this innovative Russian process scheme has not been realized due to economic restrictions and lack of scale.

It can be speculated that advanced membrane technology could separate carbonic acids, higher alcohols, or other intermediate fermentation products from the sludge during biogas fermentation. But none of these processes is so far developed enough to calculate cost-effectiveness, and integration in an economically viable biorefinery process is not foreseeable.

Conclusion
Identification of key players for cellulose degradation in the biogas fermenter is hampered by the limited knowledge on truly cellulolytic bacteria. Some important cellulose-degrading bacteria in nature seem to be still undetected,

(continued)

[1] Only about 1/10 of the amount of ATP can be produced from a glucose molecule by anaerobic metabolism compared to respiration. However, the same amount of energy has to be expended for protein synthesis and secretion.

especially for biogas fermenters. Isolation and thorough characterization of new cellulolytic bacteria from anaerobically decaying plant material will help greatly to develop methods for monitoring the number of cellulolytic bacteria in the fermenters. To know the key players will also help to define the optimal conditions for substrate hydrolysis and to identify the optimal bacteria for inoculating biogas fermenters with the result of an increased space time yield in addition to a better substrate utilization yield.

To identify the mechanisms underlying the extraordinarily effective hydrolysis of recalcitrant substrates such as crystalline cellulose will help to monitor the state of commercial biogas plants and to improve the yield of the process by adjusting to optimized conditions for biomass utilization. Moreover, it will give hints to improve the activity of commercially produced cellulase preparations and thus a crucial leap forward to the biotechnology of the second and third generation which intends to use cellulosic biomass as substrate.

Downstream processes in the biogas formation will have to be improved to take up the increased carbon flow from substrate hydrolysis. This could lead to improved biogas production efficiency and thus a better eco footprint as well as an improved process economy.

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