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# **Biochemical Processes for Generating Fuels and Commodity Chemicals from Lignocellulosic Biomass**

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Additional information is available at the end of the chapter

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## **1. Introduction**

Fuels and chemicals derived from biomass are regarded as an environmentally friendly alternative to petroleum based products. The concept of using plant material as a source for fuels and commodity chemicals has been embraced by governments to alleviate dependence on the volatile petroleum market. This trend is driven not only by economics but also by social and political factors. Global warming has been associated with CO<sub>2</sub> emissions largely originating from the combustion of fossil fuels.[1] This, together with depleting and finite carbon fossil fuel resources, and insecurity of petroleum supplies has prompted a shift towards biofuels and biomaterials.[1] The use of biomass as an economically competitive source of transport fuel was initiated by the fuel crisis in 1970 and its commercialization was led by the USA and Brazil.[2] In 2010, the USA and Brazil processing corn and sugarcane, respectively, produced 90% of the world's bioethanol. In 2008, the "food for fuel" debate emerged sparked by concerns that the use of arable land for bioethanol and biodiesel crops was placing pressure on food demand for a growing world population.[3] In June 2011, the World Bank and nine other international agencies produced a report advising governments to cease biofuel subsidies as the use of food stock for fuel production was linked to increasing food prices.[4] Subsidies were thus ended in the USA when their Senate voted overwhelmingly to end billions of dollars in bioethanol subsidies.[5] This reform resulted in USA bioethanol plants recording losses in the first quarter of 2012[6] and is foreseen as the end of bioethanol production from corn at least in the USA.

Emerging from the "food for fuel" debate, the concept of commercializing second generation biofuels was embraced by governments as a route to produce biofuels without diminishing global food supplies.[7]. Second generation biofuels address concerns over designating arable land to grow food crops for fuel production as lignocellulosic biomass may consist of waste

materials such as plant residues.[8] In many proposed biorefinery setups, the food portion of the crop is to be used for human consumption and the waste residues, for example, the leaves and stalks, are to be processed for biofuels and chemicals.[8] An illustration of the processes for 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> generation biofuel production is shown in Figure 1. Third generation biofuel production, the generation of biodiesel from algae, is included in the diagram for completeness.

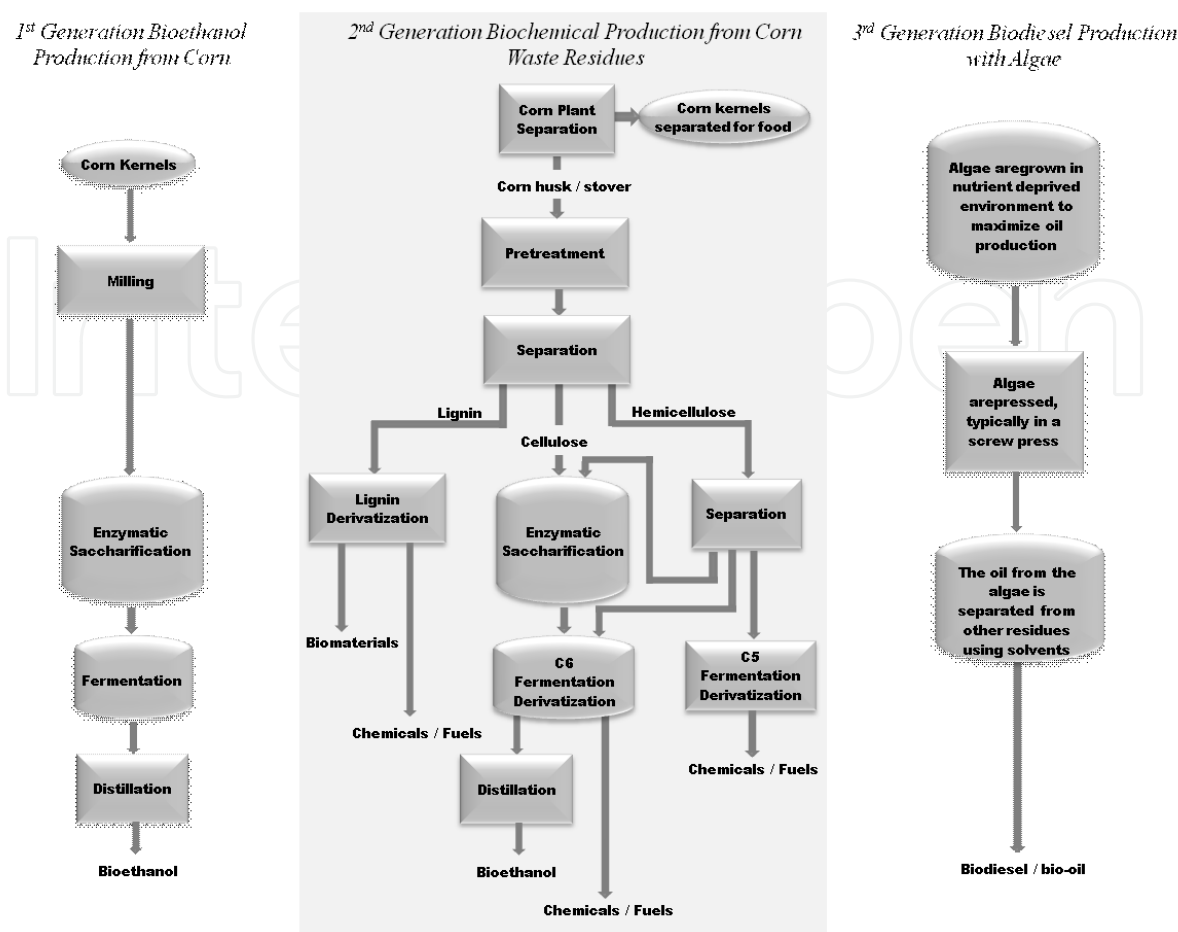
All three of the processes outlined in Figure 1 rely on biotechnology for the conversion of biomass to fuels. First generation bioethanol production traditionally incorporates two biological transformations. The first stage uses commercialized saccharification biotechnology which depolymerises starch into fermentable glucose units. The second stage is the fermentation of sugar units to ethanol and again uses commercialized biotechnology generally with yeast extracts.[1] Although the use of lignocellulosic biomass is socially widely supported, the processes for its conversion are more complex and therefore more costly. The major cost-adding component of 2<sup>nd</sup> generation bioethanol production compared to the 1<sup>st</sup> is the pretreatment step as the removal of the lignin is required for cellulose accessibility.[9] Whilst 1<sup>st</sup> generation bioethanol production converts substrates high in starch (mainly corn, sugarbeet and sugarcane), the effective utilization of lignocellulosic biomass requires at least separation, if not complete conversion, of all plant components. The composition of plant material includes lignin, cellulose and hemicelluloses and a diagram illustrating how these components relate is shown in Figure 2. The percentage of these three plant components varies with species (Figure 2) further complicating the processing of such biomass.

It has been reported that the separation and use of all plant components is required for environmentally and economically viable biorefineries.[1, 8, 9] The application of biotechnology for all aspects of biomass conversion avoids toxic by-products and high energy inputs encountered with chemical, thermal and mechanical processes often used. It is due to these energy and environmental concerns that biochemical methods are feverously being investigated, as enzymatic processes are largely environmentally benign and low in energy demand. Processes for the transformation of biomass need to be carbon efficient, otherwise the environmental objectives of biomass utilization are negated. It is with this in mind that the current chapter is focused on advances using environmentally benign biocatalysts.

The use of biochemical techniques for processing of lignocellulosic biomass is covered herein. This includes the bioprocessing of the plant components, lignin, cellulose and hemicellulose and is focused on progress made in their biochemical conversion not only to ethanol but also to value-added chemicals according to biomass fraction. The review of the literature is concentrated on biocatalytic advances in the past decade and is delineated by the plant-derived substrate. Strategies for the commercialization of 2<sup>nd</sup> generation biofuels and commodity chemicals are discussed.

## 2. Biochemical pretreatment

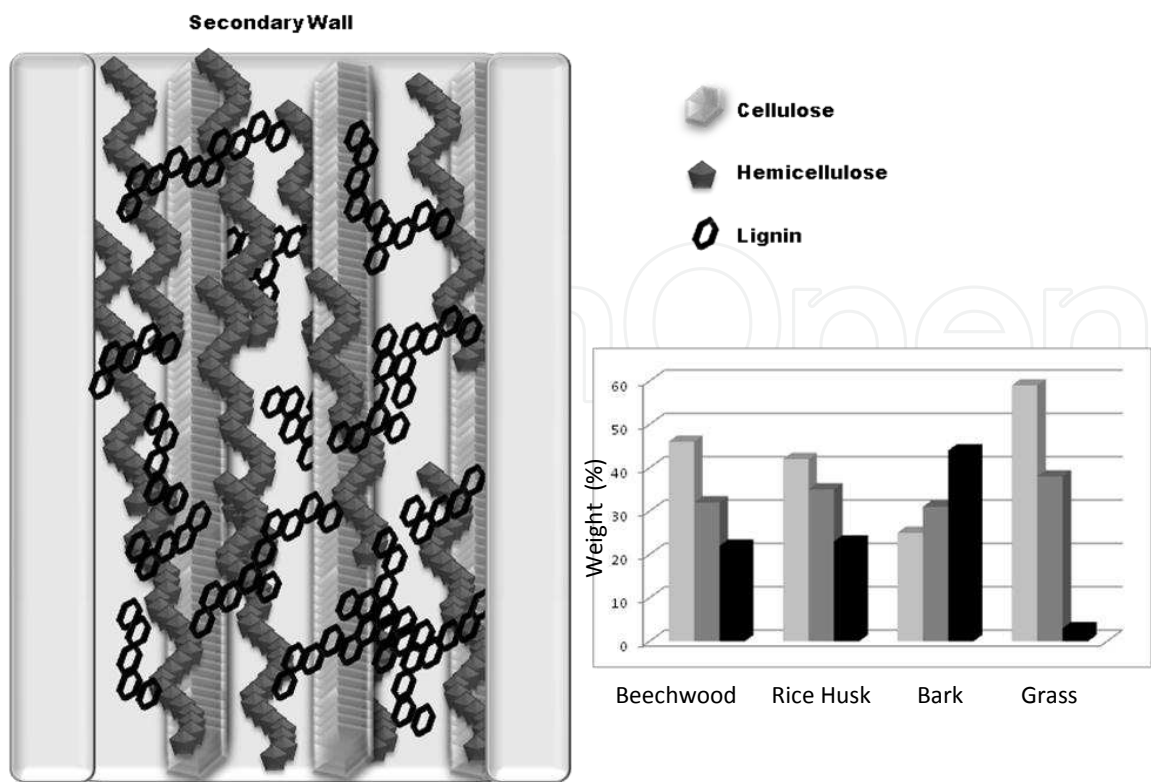
Pretreatment of lignocellulosic biomass is required to increase holocellulose (cellulose and hemicellulose) accessibility for its hydrolysis into fermentable sugars and only 20% of the



**Figure 1.** Examples of processes to produce 1st generation bioethanol from corn, 2nd generation bioethanol from corn waste residues and 3rd generation biodiesel from algae.

theoretical sugar yield can be obtained from lignocellulose without pretreatment.[10, 11] Currently, the biomass pretreatment step for producing 2<sup>nd</sup> generation bioethanol is the most expensive component of the process after the raw material.[12] Thermal and mechanical methods are energy intensive and therefore carbon costly as they indirectly produce CO<sub>2</sub>. Chemical techniques result in contamination of the biomass producing biochemical inhibitors as by-products[13, 14] and require costly neutralization processes.[15] Removal of the lignin fraction using microorganisms has several advantages compared to other pretreatment methods. Firstly, microorganisms function under ambient conditions thus eliminating thermal and electrical energy inputs. When compared to chemical pretreatment methods, biochemical pretreatment does not result in chemical by-products that often inhibit cellulose hydrolysis.

In nature, fungi are responsible for the biodegradation of lignin, thus the majority of research into biochemical pretreatments has focused on fungi for the delignification of biomass. Early research in the area was led by the pulp and paper industry and focussed on fungal treatment as a method for removing the lignin fraction from wood to facilitate cellulose accessibility and to lower pulping energy costs. In 1982, Eriksson and Vallander were able to achieve a 23% reduction in refining energy by incubating wood chips with the white-rot fungus



**Figure 2.** Diagram of plant components cellulose, hemicelluloses and lignin and a graphical representation of their weight percentage according to biomass source[10].

*Phanerochaete chrysosporium* for 2 weeks.[16] Messner and Srebotnik[17] studied the same species of fungus and reported similar results. Later studies by Akhtar et al.[18] also found substantial energy savings in pulping when the wood was treated with *Ceriporiopsis subvermispora*. Others[19] studied soft and hard wood processes and reported a reduction in refining energy from incubation with strains of white-rot fungi of 33% for soft-wood pulp and more than 50% for hardwood. More recently, Liew et al.[20] reported a lignin loss of 26.9% in biopulping studies with *Acacia mangium* wood chips when incubated with the white-rot fungi *Trametes versicolour*.

The amount of lignin present in the biomass directly affects enzymatic digestion of the holocellulose fraction. For example, a decrease from 22% to 17% lignin in biomass samples doubles the sugar yield and samples with 26% lignin result in virtually no sugar.[21] However, the effectiveness of pretreatment is not only measured by the decrease in lignin content but also by holocellulose recovery and ultimately the saccharification percentage. Table 1 summarizes recent studies conducted on the pretreatment of different biomass substrates. As stated earlier and depicted in Figure 2, the amount of lignin, cellulose and hemicellulose varies greatly with biomass source and it is therefore logical to assess the effectiveness of fungal pretreatment according to substrate. It is important to note that direct comparisons are not always possible as the experimental techniques and measurements vary within many of the cited studies. For example, the fungal incubation times listed in Table 1 vary from 2 to 120 days.



Different species of white-rot fungi, *Echinodontium taxodii*, [22, 23], *Coriolus versicolor* [24] and *Trametes versicolor* [23], have been studied for their ability to degrade lignin to promote cellulose digestibility in bamboo residues. Zeng, Yang et al. [22] recently reported a 29% decrease in lignin content in bamboo treated with *Echinodontium taxodii* however the aim of the work was to improve the thermal decomposition of the bamboo and not to recovery and utilize the holocellulose. Zhang, Xu et al. [24] reported an increase in saccharification rate of 37% when bamboo residues were incubated with *Coriolus versicolor*. Zhang, Yu et al. [23] compared two species of white-rot fungus for their effectiveness in increasing sugar yields and found that incubation with both *Trametes versicolor* and *Echinodontium taxodii* improved sugar yields 5.15 and 8.75 times respectively. Cornstalk and corn stover have been pretreated with different fungal strains for delignification. Impressive results were reported by Wan and Li [25] who pretreated corn stover with *Ceriporiopsis subvermispora* and measured a 31.59% reduction in lignin with only a 6% loss in cellulose. In 2010, Dias et al. [26] reported a nearly 4-fold increase in saccharification of wheat straw treated with basidiomycetous fungi *Euc-1* and *Irpex lacteus*. *Dichomitius squalens*, [27] *Pleurotus ostreatus* [28] and *Phaerochaete chrysosporium* [28] were applied to rice straw with varying effects (Table 1), with the most notable reported by Bak et al., [27] being a 58.1% theoretical glucose yield of rice straw treated with *Dichomitius squalens*. The biochemical pretreatment of cotton stalks was studied by Shi et al. who reported 33.9% lignin reduction [29] using submerged fungus cultivation and 27.6% lignin reduction [30] using solid state cultivation of the same fungus, *Phanerochaete chrysosporium*. Hiden et al. [31] applied *Grifola frondosa* for the pretreatment of sawdust matrix and reported a 21% reduction in lignin with 90% cellulose recovery.

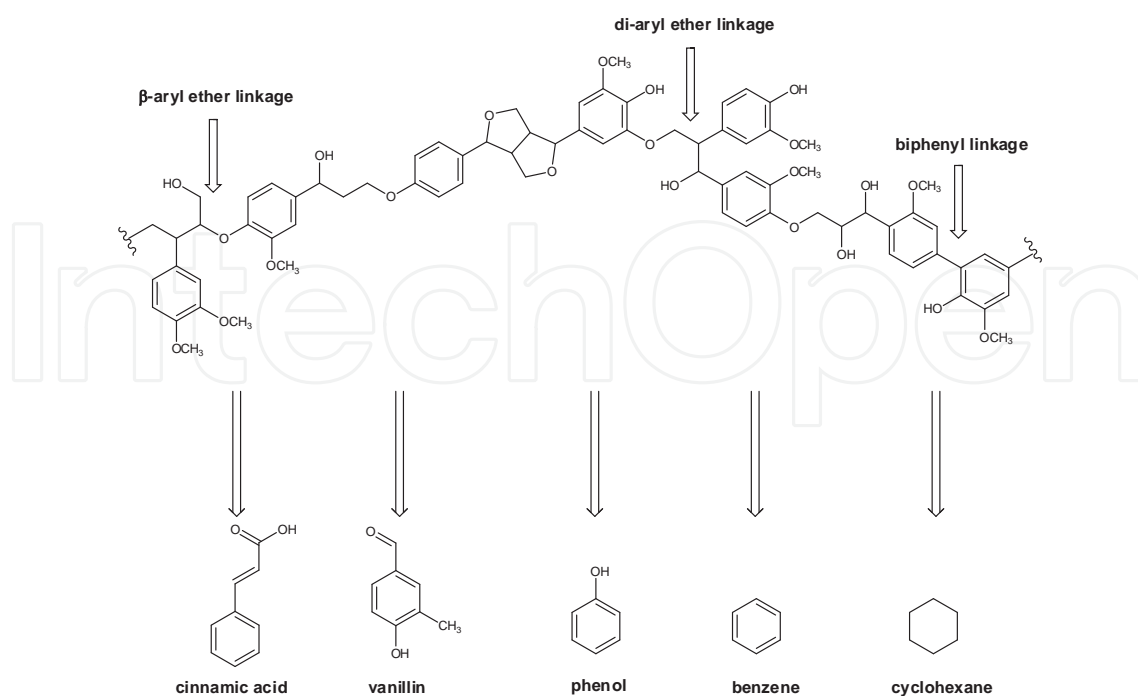
Substrate	Species	Findings	Duration	Ref
Bamboo	<i>Echinodontium taxodi</i>	29% reduction in lignin	30 days	[22]
Bamboo residues	<i>Coriolus versicolor</i>	Enhanced saccharification rate of 37%	2 days	[24]
Bamboo culms	<i>Echinodontium taxodii</i>	5.15-fold increase in sugar yields	120 days	[23]
Bamboo culms	<i>Trametes versicolor</i>	8.75-fold increase in sugar yields	120 days	[23]
pCornstalk	<i>Phanerochaete chrysosporium</i>	34.3% reduction in lignin with a maximum enzyme saccharification of 47.3%	15 days	[32]
Corn Stover	<i>Ceriporiopsis subvermispora</i>	Lignin degradation reached 45%	30 days	[33]
Corn Stover	<i>Irpex lacteus</i> CD2	66.4% saccharification ratio	25 days	[34]
Corn Stover	<i>Ceriporiopsis subvermispora</i>	31.59% lignin degradation with less than 6% cellulose loss	18 days	[25]
Corn Stover	<i>Cyanthus stercoreus</i>	3- to 5-fold improvement in enzymatic digestibility	29 days	[12]
Wheat straw	Basidiomycetous fungi Euc-1	4-fold increase in saccharification	46 days	[26]
Wheatstraw	<i>Irpex lacteus</i>	3-fold increase in saccharification	46 days	[26]
Rice straw	<i>Dichomitius squalens</i>	58% theoretical glucose yield for remaining glucan	15 days	[27]
Rice straw	<i>Pleurotus ostreatus</i>	39% degradation of lignin with 79% cellulose retention	48 days	[35]
Rice straw	<i>Phaerochaete chrysosporium</i>	64.9% of maximum glucose yield from recovered glucan	15 days	[28]
Cotton stalks	<i>Phaerochaete chrysosporium</i>	33.9% lignin degradation with 18.4% carbohydrate availability	14 days	[29]
Cotton stalks	<i>Phaerochaete chrysosporium</i>	27.6% lignin degradation	14 days	[30]
Sawdust matrix	<i>Grifola frondosa</i>	21% reduction in lignin and 90% recovery of cellulose	2 days	[31]

**Table 1.** Fungal strains studied for pretreatment of lignocellulosic biomass.

### 3. Bioconversion of lignin to chemicals and fuels

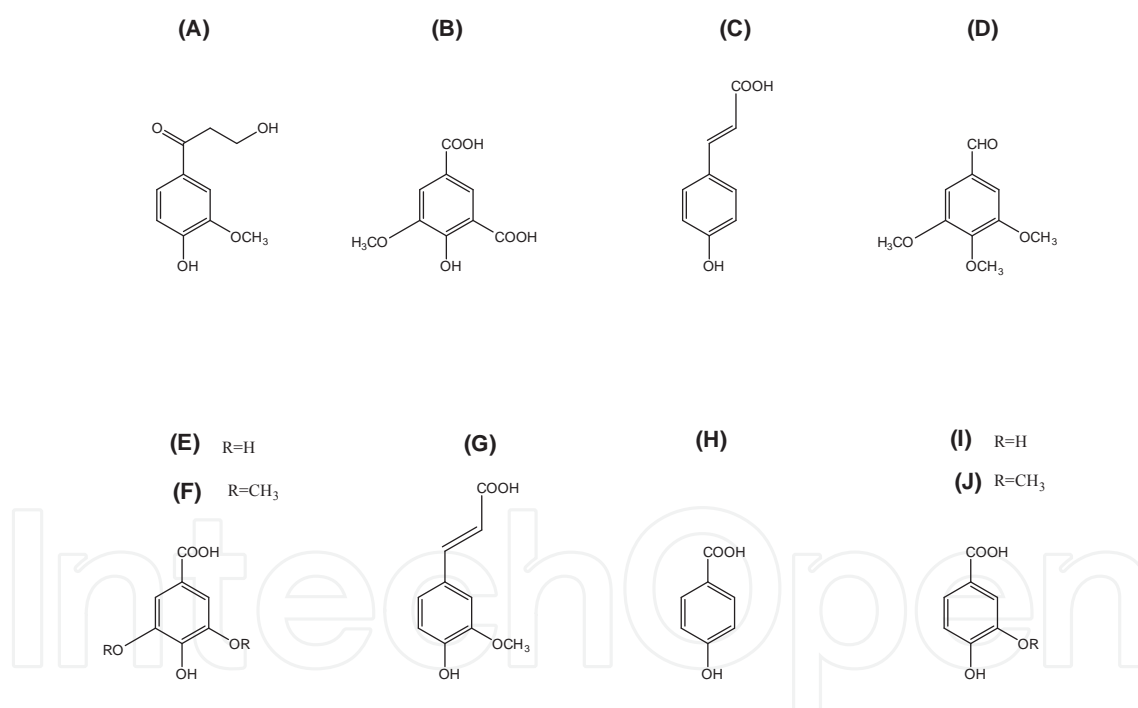
During biochemical pretreatment, the lignin fraction is metabolized by the microorganism. In chemical and thermal pretreatment processes the lignin fraction often remains intact and is thus able to be separated and utilized. After separation, microorganisms could in principle transform the lignin into materials, chemicals and fuels. Despite efforts over a long period of time, research into the bioconversion of lignin into economically viable products is still in its infancy, primarily because of the complex and irregular structure of lignin (Figure 3). However, advancements for the valorization of lignin are actively being pursued, as lignin is the second most abundant carbon source in nature and contains valuable phenolic building blocks within its structure.[36]

Although lignin has traditionally been burned as an inefficient energy source by-product from bioethanol or pulping production, lignin derived value-added products are necessary to improve biomass conversion economics.[37] Lignin has been used in the manufacture of wood adhesives as a component of phenol-formaldehyde resins (LPF resins).[38, 39] Lignin-derived commodity chemicals have been targeted through chemical and biological routes (Figure 3). Vanillin and cinnamic acid are subunits of the complex lignin structure and are used commercially as food sweeteners, as additives for fragrances and as precursors for pharmaceuticals. Phenol is the most widely used starting material in the plastic and resin industry and phenolic monomers have also been targeted from lignin. After the depolymerisation of lignin into monomeric units, the substituted monomers are precursors of a range of products including fuels such as cyclohexane.



**Figure 3.** Examples of chemicals targeted from lignin.

Although biochemical pretreatment methods generally use fungi as the lignin degrading microorganism, it is unlikely that usable lignin-derived materials and chemicals will result from fungal processes as white-rot fungi are known to mineralize lignin.[40] Thus bacterial conversion of lignin into chemicals and fuels constitutes an attractive method for the valorization of lignin. Classes of bacteria capable of degrading lignin have been identified as Actinomycetes,  $\alpha$ -Proteobacteria and  $\gamma$ -Proteobacteria.[41-44] Recently, a range of metabolites (Figure 4) have been isolated from the bacterial degradation of lignocelluloses. [40] Metabolites A and B have been observed from lignocelluloses processed by the bacteria *Pseudomonas putida* mt-2[43], *Rhodococcus jostii* RHA1[43] and *Sphingobium* sp. SYK-6.[42] Furthermore *Sphingobium* sp. metabolizes  $\beta$ -aryl ether linked aromatics to vanillin.[42] Compounds C, D, E and G were identified using GC-MS as bacterial degradation products of Kraft lignin.[45] Ferulic acid as well as compounds F, H, I and J were identified by GC-MS as products of waste paper effluent treated with *Aeromonas formicans*.[46] There are established chemical and biochemical methods for converting lignin derived monomers, like those observed in the bacterial degradation of lignin (Figure 4), into simpler aromatics like phenol (Figure 3) as well as hydrocarbons like cyclohexane (Figure 3).



**Figure 4.** Compounds isolated after bacterial conversion of lignin.[40]

## 4. Biochemical conversion of cellulose

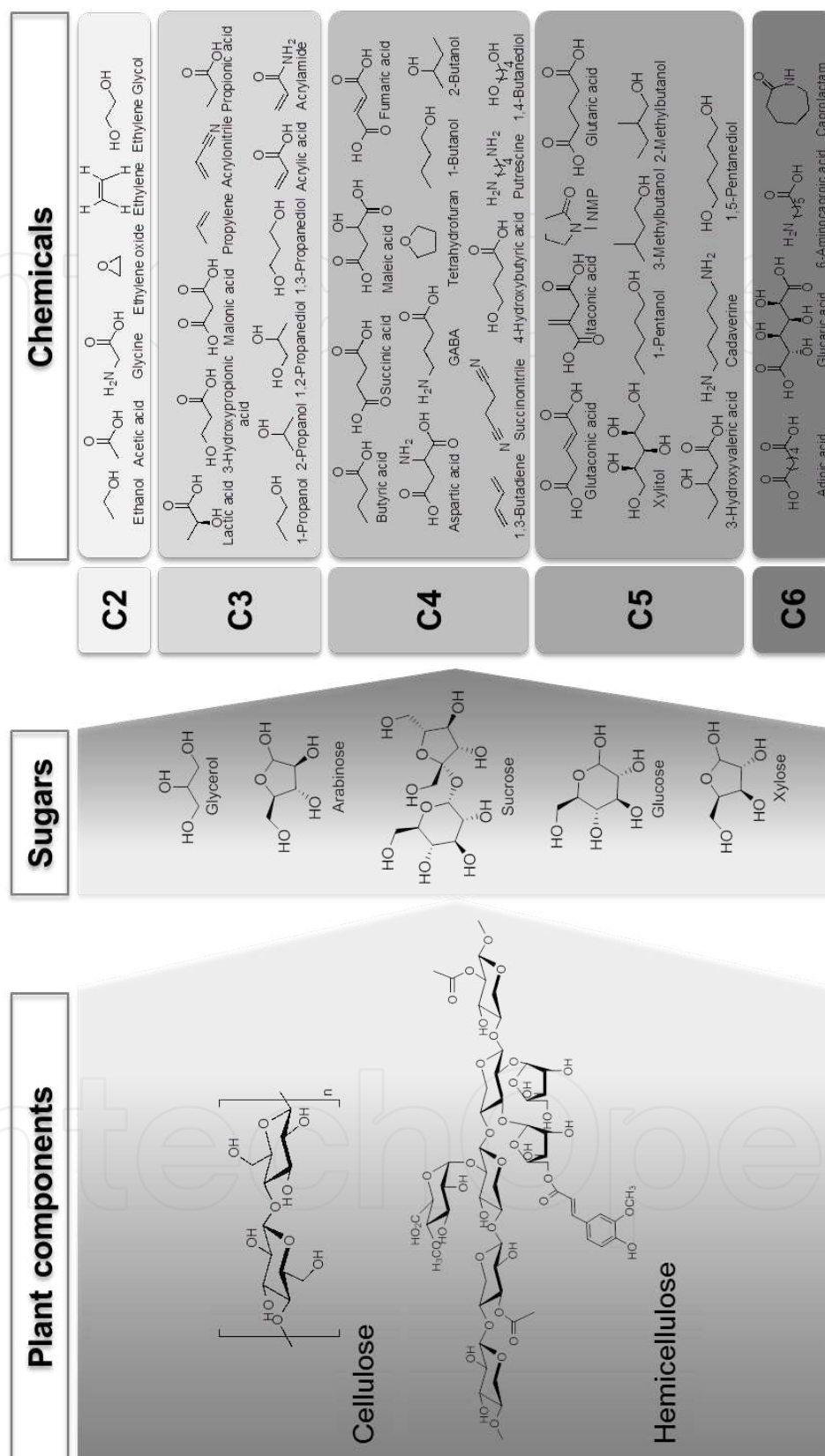
The use of starchy feedstock, such as corn and sugar cane, is problematic in relation to food sustainability and biodiversity. Therefore, as mentioned, the focus in second generation biofuel production processes has been on biomass consisting mainly of cellulose. A high percentage



of cellulose (usually 35-50% dry weight) is consistently found in all plants despite the vast genetic diversity that is observed within the plant kingdom.[47] For the production of ethanol, cellulose is exposed during pretreatment, hydrolysed by either chemical or enzymatic hydrolysis and then fermented into ethanol. The material, once stripped from other biopolymers surrounding it within the plant structure, also appears to have characteristics independent of plant taxa. Cellulose is a linear polymer, composed of glucose monomers held together by  $\beta$ -1,4-glucosidic bonds (Figure 5), in contrast to  $\alpha$ -1,4-bonds found in other common glucans such as starch and glycogen. Through interchain and intrachain hydrogen bonding as well as Van der Waals forces, cellulose chains self-assemble on biosynthesis into protofibrils, then microfibrils, which are in turn packed into fibres with high crystallinity, imparting the material with high tensile strength and water insolubility.[48] These very properties that make it a suitable structural polysaccharide are the cause of the main difficulties associated with the use of biomass rich in cellulose, for the generation of products through fermentation. The additional energy required to break down the rigid structure of cellulose is one of the main obstacles towards commercialization of lignocellulosic biomass processing.

Cellulases, the enzymes responsible for cellulose hydrolysis, differ from other glucoside hydrolases in that they are able to catalyse hydrolysis of  $\beta$ -1,4-glucosidic bonds. Cellulases vary significantly and belong to several glycoside hydrolase families.[49] The main differences between them relate to their mode of action. While endoglucanases are thought to randomly hydrolyse the amorphous fraction of cellulose, exoglucanases process the polysaccharide preferentially from a reducing or non-reducing end, releasing cellobiose (cellobiohydrolases) or glucose (glucanohydrolases).[47] An important feature of the exoglucanase structure is a distinct domain termed the carbohydrate binding module (CBM), which allows the enzyme to remain attached to the cellulose chain during catalytic action. This aids enzymatic action upon crystalline material by bringing the catalytic domain closer to the substrate and has been suggested to also help catalysis by peeling fragments of cellulose from the cellulosic surface.[50]  $\beta$ -Glucosidases are the third general category of cellulolytic enzymes; they act upon bonds in soluble cellobiose or celloextrins formed by the action of the other two types of cellulases. The different types of cellulases act in coordination to efficiently hydrolyse cellulose, displaying synergy and, depending on the host, may or may not form stable complexes of high-molecular weight.[51] These complexes, although beneficial to penetration of cellulosic material *in vivo*, when used in bioprocessing are generally considered problematic.[52]

The microorganism to receive by far the most attention in relation to sourcing of cellulolytic enzymes has been *Trichoderma reesei*. [53] This fungus was identified by E.T. Reese as the culprit for the rapid destruction of allied forces' cotton tents during WWII. Since its isolation, it has been extensively studied in relation to its cellulolytic capability and various cellulase hyper-producing strains have been developed, with RUT C30 currently the benchmark strain for production of cellulases in high yields.[54] One of the problems associated with this fungus has been the low expression of  $\beta$ -glucosidases, the enzymes responsible for liberation of glucose from short oligosaccharides. This, however, has been overcome with genetic engineering and supplementation of commercial preparations with foreign  $\beta$ -glucosidases.[55] Other promising fungal sources for cellulases exist, such as *Acremonium*, *Penicillium* and



**Figure 5.** Cellulose and hemicellulose, their sugar units and some potential chemical targets organized by carbon chain number.

*Chrysosporium* strains.[52] Their cellulase properties are comparable to those of *T. reesei*, however, they are unlikely to replace it as the standard enzyme source due to the amount of improvement already achieved with the latter. Bacterial cellulases have been the focus of some attention due to the higher robustness observed with some hyperthermophilic enzymes, making them more adaptable to the harsh conditions of industrial processes.[56] However, the production of cellulases as part of complexed systems (cellulosomes) in anaerobes, as well as the much lower protein yields in bacteria, means that interest in these enzymes is mainly restricted to their heterologous expression in fungi and use in consolidated bioprocessing (CBP, see *Consolidated fermentation*).[47, 56]

## 5. Biochemical conversion of hemicellulose

Hemicellulose (Figure 5) is a mixture of several different polysaccharides, the composition of which varies from plant to plant as well as within the same plant.[57] While cellulose is built from a single building block, a number of different monomers compose hemicellulosic heteropolymers including pentoses, hexoses and sugar acids. Commonly xylans, glucomannans, arabinogalactans and different types of glucans are found in hemicellulose. Xylans are comprised of  $\beta$ -1,4-linked xyloses interspersed with arabinose and glucuronic acid, while glucomannans are a mixture of  $\beta$ -1,4-linked glucose with  $\alpha$ -1,6-substituted mannose side chains (Figure 5). The presence of acetyl substitutions on hydroxyl groups of carbohydrates in hemicellulose is not completely understood, but may pose difficulties in hydrolysis due to the generation of acetate which acts as an enzyme and microorganism inhibitor.[58] The network of hemicellulosic chains is highly branched, cross-linking with cellulose microfibrils and lignin, creating a very compact material from which plant cell walls are composed. It is generally agreed that economically viable bioprocessing of lignocellulosic biomass requires efficient extraction and conversion of the hemicellulosic sugars.

As is the case with cellulases, hemicellulases constitute a useful tool for the generation of fermentable sugars from hemicellulose and are sometimes classed as cellulases themselves. Due to the diversity of components and complexity of structure found in this polymer it is only natural that a myriad of enzymes with different catalytic functions have been produced by microorganisms to effectively attack this matrix.[53, 59] Therefore, for example, endoxylanases, exoxylanases and  $\beta$ -xylosidases have been identified to break the linkages between xylose moieties, while esterases releasing acetyl and ferulic acid groups are also found amongst this category of enzymes. These enzymes sometimes display relative promiscuity towards the type of bond they hydrolyse, making it extremely difficult to measure a specific enzymatic activity. They also display significant synergy between themselves as well as with other lignocellulose hydrolysing enzymes.[52] As expected, microorganisms that express cellulose degrading enzymes also possess the ability to degrade hemicellulosic polymers. Accordingly it has been highlighted that *Trichoderma* and *Penicillium* fungi contain efficient hemicellulolytic catalysts.[59] Another group of fungi identified for their important xylan degrading capabilities have been *Aspergillus* spp.[60]

## 6. Fermentation to fuels and chemicals

The vast amount of available know-how, due to the fact that this process is one of man's earliest biotechnological applications, continues to set the use of *Saccharomyces cerevisiae* for the production of ethanol as the benchmark fermentation system employed for second generation biofuel processes. This yeast's properties, particularly in relation to robustness, toxicity, ethanol productivities approaching the theoretical maximum and ease of genetic manipulation make it an extremely suitable microorganism for the fermentation step of lignocellulose conversion.[61, 62] As a result, much has already been accomplished in production of efficient yeast strains for the conversion of hexoses from starchy feedstock in first generation biofuel production. The issues that require addressing for carrying over these microorganisms to second generation biofuel production processes relate to tolerance to by-products of lignocellulose pretreatment and digestion, and the ability to ferment pentoses generated by the hemicellulosic fraction of the biomass. Furthermore, the possibility of combining efficient pentose and hexose utilisation as well as production of lignocellulose hydrolysing enzymes within a single host would allow the combination of hydrolysis and fermentation steps, greatly reducing the overall cost of the production process.

Other types of microorganisms have also been investigated as alternatives, mainly for the coproduction of other compounds. A recent review by Jang et al.[63] lists organisms according to their corresponding C2-C6 platform chemical products (Figure 3 and Table 2). Anaerobic clostridial strains have been of particular interest due to their ability to efficiently generate butanol as well as their tolerance to other common metabolites (acetate, lactate) which, they are able to use as nutrients for the further production of alcohols.[64, 65] As a result the use of microorganisms such as *Clostridium acetobutylicum* has been proposed for acetone-butanol-ethanol (ABE) bioprocesses, since butanol is an attractive alternative to ethanol as a biofuel due to its lower vapour pressure and higher energy density.[66] *Clostridia* are also interesting because of the broad spectrum of chemicals that they are able to produce, as well as recent advances in their genetic manipulation.[67]

Related types of yeast have also been investigated as alternatives in order to produce microorganisms with superior properties. Thermophilic yeasts show increased ability to work at elevated temperatures which may present great advantages, particularly in relation to *in situ* evaporative removal of the product in batch processes, a procedure that is generally considered essential for the reduction of down-stream costs as well as minimising product toxicity issues.[123]

The pretreatment and hydrolysis of the complex mixture of lignocellulose, unlike the simple hydrolysis of starch, yields a number of additional by-products. These may pose problems to the growth of the microorganism fermenting the simple sugars as feedstock for fuel or chemical production. Toxic compounds encountered in lignocellulosic hydrolysates normally consist of phenolic compounds, weak organic acids and furan aldehydes.[61] Complex strategies have been employed to combat the effects of the presence of these compounds. Genetic engineering approaches have aimed at overexpression of pathways which, metabolise the inhibitors.[62]

	Platform chemical	Leading host	Substrates and/or conditions	Titer (g/L)	Yield (g/g)	Productivity (g/L/h)	Ref
C2	Ethanol	<i>S. cerevisiae</i>	Ammonia fiber expansion (AFEX)-corn stover (CS)-hydrolysates, batch fermentation	40	0.46	0.8	[68]
		<i>S. cerevisiae</i>	Cellobiose, xylose, and glucose, batch fermentation	48	0.37	0.8	[69]
		<i>Z. mobilis</i>	Glucose and xylose, batch fermentation	62	0.46	1.29	[70]
	Acetic acid	<i>E. coli</i>	Xylose, batch fermentation	23.5	0.48	n/a	[71]
		<i>A. aceti</i>	Ethanol, batch fermentation	111.7	n/a	0.6	[72]
C3	Propionic acid	<i>P. acidipropionici</i>	Glycerol, fed-batch fermentation in fibrous bed bioreactor	106	0.56	0.035	[73]
	Lactic acid	<i>Sporolactobacillus</i>	Glucose, fed-batch supplemented with 40g/L peanut meal	207	0.93	3.8	[74]
		<i>E. coli</i>	Glucose, fed-batch fermentation	138	0.86	3.5	[75]
	3-Hydroxypropionic acid	<i>K. pneumonia</i>	Glycerol, fed-batch fermentation	16	n/a	0.01	[76]
	Propanol	<i>E. coli</i>	Glycerol, fed-batch fermentation	38.7	0.34	0.53	[77]
		<i>E. coli</i>	Glucose, flask culture	3.9	n/a	0.04	[78]
	Iso-propanol	<i>C. acetobutylicum</i>	Glucose, anaerobic flask culture	5.1	n/a	n/a	[79]
		<i>E. coli</i>	Glucose, batch-fed fermentation	13.6	0.15	0.28	[80]
	1,2 propanediol	<i>C. thermosaccharolyticum</i>	Glucose, anaerobic batch fermentation	9.1	0.20	0.35	[81]
	1,3 propanediol	<i>E. coli</i>	Glycerol, batch fermentation	5.6	0.21	0.077	[82]
		<i>C. acetobutylicum</i>	Glycerol, anaerobic fed-batch fermentation	83.6	0.54	1.70	[83]
		<i>E. coli</i>	Glucose, fed-batch 10L fermentation	135	0.51	3.5	[84]
C4	Butyric acid	<i>C. tyrobutyricum</i>	Glucose fed-batch fermentation	32.5-41.7	0.38-0.42	0.24-0.68	[85, 86]
		<i>C. tyrobutyricum</i>	Glucose, repeated fed-batch fermentation by immobilized cells in a fibrous bed bioreactor	86.9	0.46	1.1	[87]
	Succinic acid	Engineered rumen bacteria	Glucose, anaerobic fed-batch fermentation	52-106	0.76-0.88	1.8-2.8	[88, 89]
		<i>E. coli</i>	Glucose, fed-batch fermentation	73-87	0.8-1.0	0.7-0.9	[90-92]
	Malic acid	<i>C. glutamicum</i>	Glucose, fed-batch fermentation	140-146	0.92-1.1	1.9-2.5	[93, 94]
		<i>Aspergillus flavus</i>	Glucose, batch fermentation	113	0.95	0.59	[95]
		<i>S. cerevisiae</i>	Glucose, fed-batch fermentation	59	0.31	0.19	[96]
		<i>E. coli</i>	Glucose, two-stage fermentation	33.9	0.47	1.06	[97]
	Fumaric acid	<i>R. arrhizus</i> NRRL 2582	Glucose, batch fermentation	97.7	0.81	1.02	[98]
	GABA	<i>L. brevis</i> NCL912	Glucose and glutamate, fed-batch fermentation	103.7	n/a	n/a	[99]
	1-butanol	<i>C. glutamicum</i>	Glucose, batch fermentation	2.2	n/a	0.01	[100]
		<i>C. acetobutylicum</i>	Glucose, anaerobic batch fermentation	16.7	n/a	0.31	[101]
	Isobutanol	<i>E. coli</i>	Glucose, batch cultivation	14-15	0.33-0.36	0.20-0.29	[102, 103]
		<i>E. coli</i>	Glucose, batch cultivation	20	n/a	n/a	[104]
	1,4-butanediol	<i>C. glutamicum</i>	Glucose, fed-batch fermentation	13.0	0.20	0.33	[105]
		<i>E. coli</i>	Glucose, microaerobic fed-batch fermentation	18	n/a	0.15	[106]
	2,3-butanediol	<i>K. pneumonia</i> SDM	Glucose, fed-batch fermentation	150	0.48	4.21	[107]
	Putrescine	<i>S. marcescens</i>	Glucose, fed-batch fermentation	152	0.46	2.67	[108]
		<i>E. coli</i>	Glucose, fed-batch culture	24.2	n/a	0.75	[109]
C5	Itaconic acid	<i>Aspergillus terreus</i> IFO-6365	Glucose and corn steep, flask and 100 L batch fermentation	82-85	0.54	0.57	[110]
		<i>E. coli</i>	Glucose, flask batch culture	6	0.61	0.06	[111]
	3-hydroxyvalerate	<i>P. putida</i>	Glucose and levulinic acid, flask batch cultivation	5.3	n/a	n/a	[112]
		<i>E. coli</i>	Glucose and threonine, flask batch cultivate	1.3	n/a	n/a	[112]
	1-pentanol	<i>E. coli</i>	Glucose, flask batch cultivation	0.81	n/a	n/a	[112]
		<i>E. coli</i>	Glucose	0.5	n/a	n/a	[113]
	2-methyl-1-butanol	<i>E. coli</i>	Glucose	1.25	n/a	0.17	[114]
	3-methyl-1-butanol	<i>E. coli</i>	Glucose	1.28	n/a	0.11	[115]
	Xylitol	<i>C. tropicalis</i>	Xylose, oxygen-limited condition with cell recycling	1.82	0.85	12.0	[116]
	Cadaverine	<i>E. coli</i>	Glucose and xylose, fed-batch fermentation	38	n/a	n/a	[117]
		<i>E. coli</i>	Glucose, fed-batch fermentation	9.61	n/a	0.12	[118]
C6	Glucaric acid	<i>E. coli</i>	Glucose, flask culture	2.5	n/a	n/a	[119]
	Anthranilic acid	<i>E. coli</i>	Glucose, fed-batch cultivation	14	0.20	0.41	[120]
	Phenol	<i>P. putida</i> S12	Glucose, flask batch culture	0.14	3.5	0.006	[121]
	Catechol	<i>P. putida</i> ML2	3-Dehydroshikimate	4.2	n/a	0.12	[122]

**Table 2.** Current status of the production of platform chemicals using microorganisms. Duplicated with permission.[63]



Another approach is adaptation of the microorganism to an inhibitor rich environment through evolutionary processes. It has been observed that the stress imposed stimulates changes in the resulting strains, usually in relation to glycolytic enzyme activity, levels of intracellular materials and expression of inhibitor metabolising enzymes, which impart increased tolerance. The new strains are generally able to grow at higher hydrolysate, and consequently inhibitor, concentrations thus reducing processing time and cost.[124] Cell viability is also threatened by the target products of fermentation, as these may cause damage to cell membranes and interfere with physiological processes. Tolerance to these compounds without decreasing the process yield may be achieved by regulation of membrane transporters such as efflux pumps, modification of the membrane composition or regulation of heat shock proteins that have been found to be linked to stress response in cells.[125] An added benefit to the increase of tolerance in some cases may be an increase in product yield.[126, 127]

Strain	Inhibitor	Approach	Reference
<i>S. cerevisiae</i>	acetate	Deletion of <i>HRK1</i> gene regulating membrane transporter activity	[128]
<i>S. cerevisiae</i> PK113-7D	formate, acetate	Expression of formate dehydrogenase structural gene FAHD2	[129]
<i>S. cerevisiae</i>	vanillin	Overexpression of laccase gene <i>lacA</i> from <i>Trametes sp.</i> AH28-2	[130]
<i>E. coli</i>	biodiesel, biogasoline	RND efflux pumps heterologously expressed	[131]
<i>C. acetobutylicum</i>	butanol	Overexpression of GroESL heat shock protein	[127]
<i>E. coli</i>	isobutanol	Simultaneous disruption of five unrelated genes	[132]
<i>S. cerevisiae</i>	ethanol, glucose	Global transcriptional machinery engineering, also improved ethanol yield by 15%	[126]

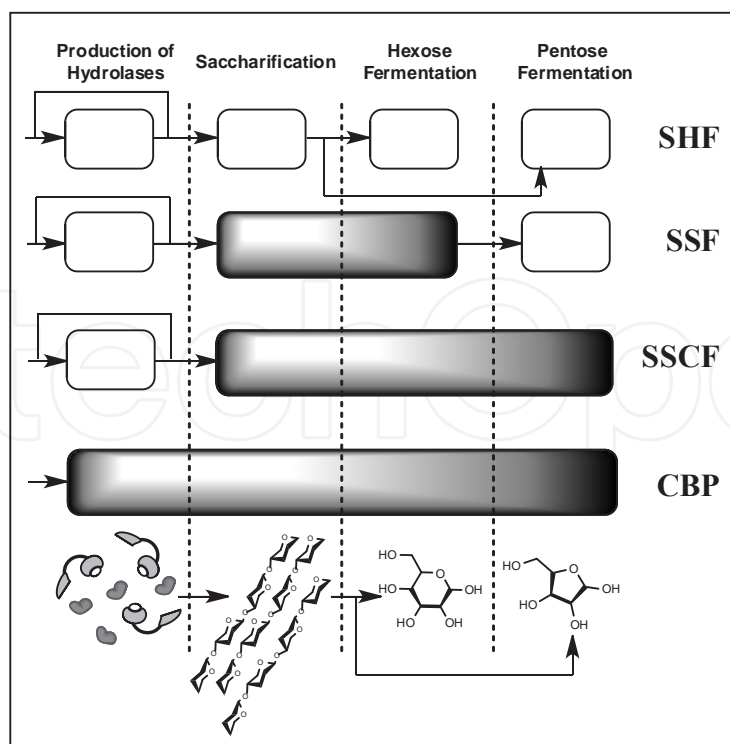
**Table 3.** Examples of engineering microorganisms for improved tolerance to inhibitors in lignocellulosic biomass processing.

Microorganisms naturally capable of fermenting pentoses such as *Pichia stipitis*, *Kluyveromyces marxianus*, *Clostridium saccharolyticum* and *Thermoanaerobacter ethanolicus* exist and may well be employed in processes for the production of ethanol as well as other chemicals.[62] However, considerable effort has been put into engineering pentose fermentation capability into strains traditionally used for ethanol production, such as *S. cerevisiae*, with great success. This yeast is able to take up pentose with hexose transporters, however the ability to metabolise these sugars had to be introduced with expression of bacterial and fungal gene insertion. This has also led towards engineering hexose/pentose efficient cofermentation, something that has not been identified in native microorganisms. The ability to coferment xylose, arabinose and glucose has been successfully introduced to *S. cerevisiae*, however modern approaches to metabolic engineering need to be employed in order to improve on this, concentrating more on non-traditional aspects of cell engineering, such as catabolism repression mechanisms and stress response.[133]



## 7. Consolidated fermentation

One of the great advantages of biochemical methods of biomass conversion is that they all require mild conditions, which makes them relatively compatible, allowing for potential consolidation of processing steps. This has been identified as an area of great potential in relation to process optimization, cost reduction and ultimately biorefinery commercialization. Instead of applying four distinct biochemical processing steps (cellulose production, cellulose hydrolysis, hexose fermentation, pentose hydrolylate fermentation), a setup termed Separate Hydrolysis and Fermentation (SHF), two or more steps may be consolidated leading to alternate process configurations for biomass conversion.[47] This requires generation of biocatalysts with properties suited to the optimum processing conditions, or engineering of microorganisms with more than one processing capability. Simultaneous Saccharification and Fermentation (SSF) involves performing cellulase-catalysed cellulose hydrolysis in the cellulose hydrolylate fermenter, after the enzymes are produced in a separate fermentation. Further consolidation may include cofermentation of the hemicellulose hydrolysate, either by a separate pentose utilising microorganism or by an engineered strain capable of efficient cofermentation of hexoses and pentoses. This configuration is termed Simultaneous Saccharification and Cofermentation (SSCF). The most desirable setup that minimises utility costs is direct fermentation of biomass to the product of choice with the aid of a cellulase expressing, hexose/pentose cofermenting microorganism. This approach was first introduced in 1996 as consolidated bioprocessing (CBP).[53, 134]



**Figure 6.** Consolidated fermentation processes.

## 8. Future outlook for commercialization

Inedible crops are a renewable and sustainable source of fuels and chemicals and it has been estimated that replacing corn with cellulosic stock would result in an 82% increase in bioethanol production. Despite this, 2<sup>nd</sup> generation biofuel and chemical production is yet to be commercialized.

Finding economical pretreatment methods has been recognized as one of the hurdles to commercializing 2<sup>nd</sup> generation biofuels and chemicals. The results listed in Table 1 show that fungal treatment can reduce lignin content of biomass and in most cases improve sugar yields. However, chemical methods are deemed more economical at present, mainly due to the long incubation times as well as the loss of holocellulose during biological pretreatment. With further screening studies, it is to be expected that fungal strains with selectivity for lignin and faster metabolic processing will be discovered, thus reducing the overall process cost. Bacteria present advantages for biotechnological applications in terms of growing times and being more prone to metabolic manipulation. As discussed above, bacterial strains have been identified that convert lignin into valuable phenolic monomers. The conversion of all plant components, and in particular the lignin fraction, is the basis for 2<sup>nd</sup> generation biorefineries, where a vast array of products may be prepared in conjunction with a central fermentation for biofuel production. Therefore integration of bacterial utilization of lignin will greatly contribute to the economic viability of these processes.

The cost of hydrolytic enzyme production greatly influences the overall cost of cellulosic biomass conversion thus hindering commercialization.[135] There have been great strides forward in this respect with the estimated cost being driven down from US\$5.40 to US\$0.20 per gallon of ethanol produced, according to claims of major enzyme producers.[136] The use of such information however in techno-economical analysis of biomass conversion is problematic. These values relate to the production of a specific target, usually ethanol, and depend on a range of variables other than enzyme production. Klein-Marcuschamer et al. prepared a model for the calculation of the cost for the production of cellulases from *T. reesei* that could then be applied to another model for the estimation of its contribution to the cost of ethanol production.[137] The results showed that there is systematic underestimation of the contribution of enzyme costs to biofuels production in the literature, as conservative calculations pointed to around US\$1.00 per gallon ethanol. The authors highlighted that approaches aiming to decrease enzyme loading in the pretreatment steps should become a focus point. It seems that lowering the enzyme production cost will be a significant obstacle towards the commercialization of any process based on lignocellulosic feedstock.

Despite the hurdles that need to be overcome for commercialization, there is much anticipation from federal governments that biofuels and chemicals derived from lignocellulosic biomass will play a central role in overcoming fossil fuel dependence. In October 2012 the EU commission issued a proposal stating that advanced biofuel development has to be encouraged due to their high greenhouse gas savings and lower risk of land use change, and this should be mirrored in post-2020 renewable energy policies.[138] In accordance with this a directive was proposed to limit the allowed contribution of food crop derived biofuels, towards the 10%

2020 renewable transportation fuel objective, to only 5%. This means that a greater contribution from lignocellulosic biomass and especially agricultural waste derived biofuels will be required. Experts including those from Shell Corporation recognized that substantial research and development from industry and academia is still required in order to achieve this target. However, it is generally agreed upon that the rapid advances in enzyme, microbial and plant engineering as well as biocatalyst optimization suggest that biochemical processes are much more likely to provide the necessary breakthroughs that will propel second generation biofuels and chemicals into the marketplace.

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## References

- [1] Kheshgi HS, Prince RC, Marland G. The potential of biomass fuels in the context of global climate change: Focus on transportation fuels. *Annu Rev Energ Env.* 2000;25:199-244.
- [2] MacDonald T. Energy Fuel Use in Brazil. 2007 Jul 27 [cited 2012 Oct 25]. Available from: <http://biomass.ucdavis.edu/newsletters>
- [3] Parker K. Investing in Agriculture: Far-Reaching Challenge, Significant Opportunity. 2009 Jun 25 [cited 2012 Oct 25]. Available from: [http://www.dbcca.com/dbcca/EN/investment-research/investment\\_research\\_1735.jsp](http://www.dbcca.com/dbcca/EN/investment-research/investment_research_1735.jsp)
- [4] Price Volatility in Food and Agricultural Markets: Policy Responses. FAO, IFAD, IMF,OECD, UNCTAD, WFP, the World Bank, the WTO, IFPRI and the UN HLTF 2007.
- [5] Doggett T. Senate vote marks start of end for ethanol subsidies. Reuters [Internet]. 2011 Jun 16 [cited 2012 Oct 25]. Available from: <http://www.reuters.com/article/2011/06/16/us-usa-senate-ethanol-idUSTRE75F5IN20110616>
- [6] Fletcher O. Ethanol Makers' Long Hot Summer. The Wall Street Journal [Internet]. 2012 Jul 26 [cited 2012 Oct 25]. Available from: <http://online.wsj.com/article/SB10001424052702303644004577522962336134368.html>

- [7] Sierra R, Smith A, Granda C, Holtzapple MT. Producing fuels and chemicals from lignocellulosic biomass. *Chem Eng Prog*. 2008 Aug;104(8):S10-S8.
- [8] Sims REH, Mabee W, Saddler JN, Taylor M. An overview of second generation bio-fuel technologies. *Bioresource Technol*. 2010 Mar;101(6):1570-80.
- [9] Yang B, Wyman CE. Pretreatment: the key to unlocking low-cost cellulosic ethanol. *Biofuel Bioprod Bior*. 2008 Jan-Feb;2(1):26-40.
- [10] Koullas DP, Christakopoulos P, Kekos D, Macris BJ, Koukios EG. Correlating the Effect of Pretreatment on the Enzymatic-Hydrolysis of Straw. *Biotechnol Bioeng*. 1992 Jan 5;39(1):113-6.
- [11] Kim TH, Lee YY, Sunwoo C, Kim JS. Pretreatment of corn stover by low-liquid ammonia recycle percolation process. *Appl Biochem Biotech*. 2006 Apr;133(1):41-57.
- [12] Keller FA, Hamilton JE, Nguyen QA. Microbial pretreatment of biomass - Potential for reducing severity of thermochemical biomass pretreatment. *Appl Biochem Biotech*. 2003 Spr;105:27-41.
- [13] Roberto IC, Mussatto SI, Rodrigues RCLB. Dilute-acid hydrolysis for optimization of xylose recovery from rice straw in a semi-pilot reactor. *Ind Crop Prod*. 2003 May;17(3):171-6.
- [14] Klinke HB, Thomsen AB, Ahring BK. Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. *Appl Microbiol Biot*. 2004 Nov;66(1):10-26.
- [15] Sandra T. Merino JC, editor. *Progress and Challenges in Enzyme Development for Biomass Utilization*: Springer Berlin Heidelberg; 2007.
- [16] Eriksson KE, Vallander L. Properties of Pulps from Thermomechanical Pulping of Chips Pretreated with Fungi. *Sven Papperstidn*. 1982;85(6):R33-R8.
- [17] Messner K, Srebotnik E. Biopulping - an Overview of Developments in an Environmentally Safe Paper-Making Technology. *Fems Microbiol Rev*. 1994 Mar;13(2-3):351-64.
- [18] Akhtar M, Attridge MC, Myers GC, Blanchette RA. Biomechanical Pulping of Loblolly-Pine Chips with Selected White-Rot Fungi. *Holzforschung*. 1993;47(1):36-40.
- [19] Kashino Y, Nishida T, Takahara Y, Fujita K, Kondo R, Sakai K. Biomechanical Pulping Using White-Rot Fungi Izu-154. *Tappi J*. 1993 Dec;76(12):167-71.
- [20] Liew CY, Husaini A, Hussain H, Muid S, Liew KC, Roslan HA. Lignin biodegradation and ligninolytic enzyme studies during biopulping of *Acacia mangium* wood chips by tropical white rot fungi. *World J Microb Biot*. 2011 Jun;27(6):1457-68.
- [21] Carroll A, Somerville C. Cellulosic Biofuels. *Annu Rev Plant Biol*. 2009;60:165-82.

- [22] Zeng YL, Yang XW, Yu HB, Zhang XY, Ma FY. The delignification effects of white-rot fungal pretreatment on thermal characteristics of moso bamboo. *Bioresource Technol.* 2012 Jun;114:437-42.
- [23] Zhang XY, Yu HB, Huang HY, Liu YX. Evaluation of biological pretreatment with white rot fungi for the, enzymatic hydrolysis of bamboo culms. *Int Biodeter Biodegr.* 2007 Oct;60(3):159-64.
- [24] Zhang XY, Xu CY, Wang HX. Pretreatment of bamboo residues with *Coriolus versicolor* for enzymatic hydrolysis. *J Biosci Bioeng.* 2007 Aug;104(2):149-51.
- [25] Wan CX, Li YL. Microbial pretreatment of corn stover with *Ceriporiopsis subvermispora* for enzymatic hydrolysis and ethanol production. *Bioresource Technol.* 2010 Aug;101(16):6398-403.
- [26] Dias AA, Freitas GS, Marques GSM, Sampaio A, Fraga IS, Rodrigues MAM, et al. Enzymatic saccharification of biologically pre-treated wheat straw with white-rot fungi. *Bioresource Technol.* 2010 Aug;101(15):6045-50.
- [27] Bak JS, Kim MD, Choi IG, Kim KH. Biological pretreatment of rice straw by fermenting with *Dichomitus squalens*. *New Biotechnol.* 2010 Sep 30;27(4):424-34.
- [28] Bak JS, Ko JK, Choi IG, Park YC, Seo JH, Kim KH. Fungal Pretreatment of Lignocellulose by *Phanerochaete chrysosporium* to Produce Ethanol From Rice Straw. *Biotechnol Bioeng.* 2009 Oct 15;104(3):471-82.
- [29] Shi J, Sharma-Shivappa RR, Chinn MS. Microbial pretreatment of cotton stalks by submerged cultivation of *Phanerochaete chrysosporium*. *Bioresource Technol.* 2009 Oct;100(19):4388-95.
- [30] Shi J, Chinn MS, Sharma-Shivappa RR. Microbial pretreatment of cotton stalks by solid state cultivation of *Phanerochaete chrysosporium*. *Bioresource Technol.* 2008 Sep;99(14):6556-64.
- [31] Hideno A, Aoyagi H, Isobe S, Tanaka H. Utilization of spent sawdust matrix after cultivation of *Grifola frondosa* as substrate for ethanol production by simultaneous saccharification and fermentation. *Food Sci Technol Res.* 2007 May;13(2):111-7.
- [32] Zhao L, Cao GL, Wang AJ, Ren HY, Dong D, Liu ZN, et al. Fungal pretreatment of cornstalk with *Phanerochaete chrysosporium* for enhancing enzymatic saccharification and hydrogen production. *Bioresource Technol.* 2012 Jun;114:365-9.
- [33] Cui ZF, Wan CX, Shi J, Sykes RW, Li YB. Enzymatic Digestibility of Corn Stover Fractions in Response to Fungal Pretreatment. *Ind Eng Chem Res.* 2012 May 30;51(21):7153-9.
- [34] Xu CY, Ma FY, Zhang XY, Chen SL. Biological Pretreatment of Corn Stover by *Irpex lacteus* for Enzymatic Hydrolysis. *J Agr Food Chem.* 2010 Oct 27;58(20):10893-8.



- [35] Taniguchi M, Takahashi D, Watanabe D, Sakai K, Hoshino K, Kouya T, et al. Evaluation of Fungal Pretreatments for Enzymatic Saccharification of Rice Straw. *J Chem Eng Jpn*. 2010;43(4):401-5.
- [36] Zakzeski J, Bruijninx PCA, Jongerius AL, Weckhuysen BM. The Catalytic Valorization of Lignin for the Production of Renewable Chemicals. *Chem Rev*. 2010 Jun;110(6):3552-99.
- [37] Doherty WOS, Mousavioun P, Fellows CM. Value-adding to cellulosic ethanol: Lignin polymers. *Ind Crop Prod*. 2011 Mar;33(2):259-76.
- [38] Tejado A, Pena C, Labidi J, Echeverria JM, Mondragon I. Physico-chemical characterization of lignins from different sources for use in phenol-formaldehyde resin synthesis. *Bioresource Technol*. 2007 May;98(8):1655-63.
- [39] Turunen M, Alvila L, Pakkanen TT, Rainio J. Modification of phenol-formaldehyde resol resins by lignin, starch, and urea. *J Appl Polym Sci*. 2003 Apr 11;88(2):582-8.
- [40] Bugg TDH, Ahmad M, Hardiman EM, Singh R. The emerging role for bacteria in lignin degradation and bio-product formation. *Curr Opin Biotech*. 2011 Jun;22(3):394-400.
- [41] Zimmermann W. Degradation of Lignin by Bacteria. *J Biotechnol*. 1990 Feb;13(2-3):119-30.
- [42] Ramachandra M, Crawford DL, Hertel G. Characterization of an Extracellular Lignin Peroxidase of the Lignocellulolytic Actinomycete *Streptomyces-Viridosporus*. *Appl Environ Microb*. 1988 Dec;54(12):3057-63.
- [43] Ahmad M, Taylor CR, Pink D, Burton K, Eastwood D, Bending GD, et al. Development of novel assays for lignin degradation: comparative analysis of bacterial and fungal lignin degraders. *Mol Biosyst*. 2010 May;6(5):815-21.
- [44] Masai E, Katayama Y, Fukuda M. Genetic and biochemical investigations on bacterial catabolic pathways for lignin-derived aromatic compounds. *Biosci Biotech Bioch*. 2007 Jan;71(1):1-15.
- [45] Raj A, Reddy MMK, Chandra R. Identification of low molecular weight aromatic compounds by gas chromatography-mass spectrometry (GC-MS) from kraft lignin degradation by three *Bacillus* sp. *Int Biodeter Biodegr*. 2007 Jun;59(4):292-6.
- [46] Gupta VK, Minocha AK, Jain N. Batch and continuous studies on treatment of pulp mill wastewater by *Aeromonas formicans*. *J Chem Technol Biot*. 2001 Jun;76(6):547-52.
- [47] Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS. Microbial Cellulose Utilization: Fundamentals and Biotechnology. *Microbiology and Molecular Biology Reviews*. 2002 September 1, 2002;66(3):506-77.



- [48] Brown RM, Saxena IM. Cellulose biosynthesis: A model for understanding the assembly of biopolymers. *Plant Physiology and Biochemistry*. 2000 Jan-Feb;38(1-2): 57-67.
- [49] Himmel ME, Ruth MF, Wyman CE. Cellulase for commodity products from cellulosic biomass. *Current Opinion in Biotechnology*. 1999;10(4):358-64.
- [50] Teeri TT, Koivula A, Linder M, Wohlfahrt G, Divne C, Jones TA. *Trichoderma reesei* cellobiohydrolases: why so efficient on crystalline cellulose? *Biochemical Society Transactions*. 1998 May;26(2):173-8.
- [51] Din N, Damude HG, Gilkes NR, Miller RC, Warren RAJ, Kilburn DG. C-1-C-X Revisited - Intramolecular Synergism in a Cellulase. *Proceedings of the National Academy of Sciences of the United States of America*. 1994 Nov 22;91(24):11383-7.
- [52] Gusakov AV. Alternatives to *Trichoderma reesei* in biofuel production. *Trends in Biotechnology*. 2011;29(9):419-25.
- [53] Menon V, Rao M. Trends in bioconversion of lignocellulose: Biofuels, platform chemicals & biorefinery concept. *Progress in Energy and Combustion Science*. 2012;38(4):522-50.
- [54] Le Crom S, Schackwitz W, Pennacchio L, Magnuson JK, Culley DE, Collett JR, et al. Tracking the roots of cellulase hyperproduction by the fungus *Trichoderma reesei* using massively parallel DNA sequencing. *Proceedings of the National Academy of Sciences of the United States of America*. 2009 Sep 22;106(38):16151-6.
- [55] Nieves RA, Ehrman CI, Adney WS, Elander RT, Himmel ME. Survey and analysis of commercial cellulase preparations suitable for biomass conversion to ethanol. *World Journal of Microbiology & Biotechnology*. 1998 Mar;14(2):301-4.
- [56] Wilson DB. Cellulases and biofuels. *Current Opinion in Biotechnology*. 2009 Jun; 20(3):295-9.
- [57] Zhang Z, Donaldson AA, Ma X. Advancements and future directions in enzyme technology for biomass conversion. *Biotechnology Advances*. 2012 Jul-Aug;30(4): 913-9.
- [58] Fujitomi K, Sanda T, Hasunuma T, Kondo A. Deletion of the PHO13 gene in *Saccharomyces cerevisiae* improves ethanol production from lignocellulosic hydrolysate in the presence of acetic and formic acids, and furfural. *Bioresource Technology*. 2012;111(0):161-6.
- [59] van Gool MP, Toth K, Schols HA, Szakacs G, Gruppen H. Performance of hemicellulolytic enzymes in culture supernatants from a wide range of fungi on insoluble wheat straw and corn fiber fractions. *Bioresource Technology*. 2012;114(0):523-8.
- [60] Sanchez C. Lignocellulosic residues: Biodegradation and bioconversion by fungi. *Biotechnology Advances*. 2009 Mar-Apr;27(2):185-94.

- [61] Madhavan A, Srivastava A, Kondo A, Bisaria VS. Bioconversion of lignocellulose-derived sugars to ethanol by engineered *Saccharomyces cerevisiae*. *Critical Reviews in Biotechnology*. 2012 Mar;32(1):22-48.
- [62] Lalue C, Schenberg ACG, Gallardo JCM, Coradello LFC, Pombeiro-Sponchiado SR. Advances and Developments in Strategies to Improve Strains of *Saccharomyces cerevisiae* and Processes to Obtain the Lignocellulosic Ethanol-A Review. *Applied Biochemistry and Biotechnology*. 2012 Apr;166(8):1908-26.
- [63] Jang YS, Kim B, Shin JH, Choi YJ, Choi S, Song CW, et al. Bio-based production of C2-C6 platform chemicals. *Biotechnol Bioeng*. 2012 Oct;109(10):2437-59.
- [64] Tracy BP, Jones SW, Fast AG, Indurthi DC, Papoutsakis ET. Clostridia: the importance of their exceptional substrate and metabolite diversity for biofuel and biorefinery applications. *Current Opinion in Biotechnology*. 2012;23(3):364-81.
- [65] Jurgens G, Survase S, Berezina O, Sklavounos E, Linnekoski J, Kurkijarvi A, et al. Butanol production from lignocellulosics. *Biotechnology Letters*. 2012 Aug;34(8):1415-34.
- [66] Green EM. Fermentative production of butanol—the industrial perspective. *Current Opinion in Biotechnology*. 2011;22(3):337-43.
- [67] Hartman AH, Liu H, Melville SB. Construction and Characterization of a Lactose-Inducible Promoter System for Controlled Gene Expression in *Clostridium perfringens*. *Applied and Environmental Microbiology*. 2011 January 15, 2011;77(2):471-8.
- [68] Lau MW, Dale BE. Cellulosic ethanol production from AFEX-treated corn stover using *Saccharomyces cerevisiae* 424A(LNH-ST). *P Natl Acad Sci USA*. 2009 Feb 3;106(5):1368-73.
- [69] Ha SJ, Galazka JM, Kim SR, Choi JH, Yang XM, Seo JH, et al. Engineered *Saccharomyces cerevisiae* capable of simultaneous cellobiose and xylose fermentation. *P Natl Acad Sci USA*. 2011 Jan 11;108(2):504-9.
- [70] Joachimsthal E, Haggett KD, Rogers PL. Evaluation of recombinant strains of *Zymomonas mobilis* for ethanol production from glucose xylose media. *Appl Biochem Biotech*. 1999 Spr;77-9:147-57.
- [71] Wang YZ, Manow R, Finan C, Wang JH, Garza E, Zhou SD. Adaptive evolution of nontransgenic *Escherichia coli* KC01 for improved ethanol tolerance and homoethanol fermentation from xylose. *J Ind Microbiol Biot*. 2011 Sep;38(9):1371-7.
- [72] Nakano S, Fukaya M, Horinouchi S. Putative ABC transporter responsible for acetic acid resistance in *Acetobacter aceti*. *Appl Environ Microb*. 2006 Jan;72(1):497-505.
- [73] Zhang A, Yang ST. Engineering *Propionibacterium acidipropionici* for Enhanced Propionic Acid Tolerance and Fermentation. *Biotechnol Bioeng*. 2009 Nov 1;104(4):766-73.

- [74] Wang LM, Zhao B, Li FS, Xu K, Ma CQ, Tao F, et al. Highly efficient production of D-lactate by *Sporolactobacillus* sp. CASD with simultaneous enzymatic hydrolysis of peanut meal. *Appl Microbiol Biot.* 2011 Feb;89(4):1009-17.
- [75] Zhu Y, Eiteman MA, DeWitt K, Altman E. Homolactate fermentation by metabolically engineered *Escherichia coli* strains. *Appl Environ Microb.* 2007 Jan;73(2):456-64.
- [76] Ashok S, Raj SM, Rathnasingh C, Park S. Development of recombinant *Klebsiella pneumoniae* Delta dhaT strain for the co-production of 3-hydroxypropionic acid and 1,3-propanediol from glycerol. *Appl Microbiol Biot.* 2011 May;90(4):1253-65.
- [77] Rathnasingh C, Raj SM, Jo JE, Park S. Development and Evaluation of Efficient Recombinant *Escherichia coli* Strains for the Production of 3-Hydroxypropionic Acid From Glycerol. *Biotechnol Bioeng.* 2009 Nov 1;104(4):729-39.
- [78] Atsumi S, Cann AF, Connor MR, Shen CR, Smith KM, Brynildsen MP, et al. Metabolic engineering of *Escherichia coli* for 1-butanol production. *Metab Eng.* 2008 Nov;10(6):305-11.
- [79] Lee J, Jang YS, Choi SJ, Im JA, Song H, Cho JH, et al. Metabolic Engineering of *Clostridium acetobutylicum* ATCC 824 for Isopropanol-Butanol-Ethanol Fermentation. *Appl Environ Microb.* 2012 Mar;78(5):1416-23.
- [80] Jojima T, Inui M, Yukawa H. Production of isopropanol by metabolically engineered *Escherichia coli*. *Appl Microbiol Biot.* 2008 Jan;77(6):1219-24.
- [81] Sanchezziera F, Cameron DC, Cooney CL. Influence of Environmental-Factors in the Production of R(-)-1,2-Propanediol by *Clostridium-Thermosaccharolyticum*. *Biotechnol Lett.* 1987 Jul;9(7):449-54.
- [82] Clomburg JM, Gonzalez R. Metabolic Engineering of *Escherichia coli* for the Production of 1,2-Propanediol From Glycerol. *Biotechnol Bioeng.* 2011 Apr;108(4):867-79.
- [83] Gonzalez-Pajuelo M, Meynial-Salles I, Mendes F, Andrade JC, Vasconcelos I, Soucaille P. Metabolic engineering of *Clostridium acetobutylicum* for the industrial production of 1,3-propanediol from glycerol. *Metab Eng.* 2005 Sep-Nov;7(5-6):329-36.
- [84] Nakamura CE, Whited GM. Metabolic engineering for the microbial production of 1,3-propanediol. *Curr Opin Biotech.* 2003 Oct;14(5):454-9.
- [85] Zhu Y, Yang ST. Adaptation of *Clostridium tyrobutyricum* for enhanced tolerance to butyric acid in a fibrous-bed bioreactor. *Biotechnol Progr.* 2003 Mar-Apr;19(2):365-72.
- [86] Liu X, Zhu Y, Yang ST. Construction and characterization of ack deleted mutant of *Clostridium tyrobutyricum* for enhanced butyric acid and hydrogen production. *Biotechnol Progr.* 2006 Oct 6;22(5):1265-75.
- [87] Jiang L, Wang JF, Liang SZ, Cai J, Xu ZN, Cen PL, et al. Enhanced Butyric Acid Tolerance and Bioproduction by *Clostridium tyrobutyricum* Immobilized in a Fibrous Bed Bioreactor. *Biotechnol Bioeng.* 2011 Jan;108(1):31-40.

- [88] Guettler MV JM, Rumler D, inventor Methods for making succinic acid, bacterial variants for use in the process, and methods for obtaining variants. USA1996.
- [89] Lee SJ, Song H, Lee SY. Genome-based metabolic engineering of *Mannheimia succiniciproducens* for succinic acid production. *Appl Environ Microb*. 2006 Mar;72(3):1939-48.
- [90] Jantama K, Haupt MJ, Svoronos SA, Zhang XL, Moore JC, Shanmugam KT, et al. Combining metabolic engineering and metabolic evolution to develop nonrecombinant strains of *Escherichia coli* C that produce succinate and malate. *Biotechnol Bioeng*. 2008 Apr 1;99(5):1140-53.
- [91] Jantama K, Zhang X, Moore JC, Shanmugam KT, Svoronos SA, Ingram LO. Eliminating Side Products and Increasing Succinate Yields in Engineered Strains of *Escherichia coli* C. *Biotechnol Bioeng*. 2008 Dec 1;101(5):881-93.
- [92] Thakker C, Martinez I, San KY, Bennett GN. Succinate production in *Escherichia coli*. *Biotechnol J*. 2012 Feb;7(2):213-24.
- [93] Litsanov B, Brocker M, Bott M. Toward Homosuccinate Fermentation: Metabolic Engineering of *Corynebacterium glutamicum* for Anaerobic Production of Succinate from Glucose and Formate. *Appl Environ Microb*. 2012 May;78(9):3325-37.
- [94] Litsanov B, Kabus A, Brocker M, Bott M. Efficient aerobic succinate production from glucose in minimal medium with *Corynebacterium glutamicum*. *Microb Biotechnol*. 2012 Jan;5(1):116-28.
- [95] Battat E, Peleg Y, Bercovitz A, Rokem JS, Goldberg I. Optimization of L-Malic Acid Production by *Aspergillus-Flavus* in a Stirred Fermenter. *Biotechnol Bioeng*. 1991 May;37(11):1108-16.
- [96] Zelle RM, de Hulster E, van Winden WA, de Waard P, Dijkema C, Winkler AA, et al. Malic acid production by *Saccharomyces cerevisiae*: Engineering of pyruvate carboxylation, oxaloacetate reduction, and malate export. *Appl Environ Microb*. 2008 May;74(9):2766-77.
- [97] Zhang X, Wang X, Shanmugam KT, Ingram LO. L-Malate Production by Metabolically Engineered *Escherichia coli*. *Appl Environ Microb*. 2011 Jan;77(2):427-34.
- [98] Kenealy W, Zaady E, Dupreez JC, Stieglitz B, Goldberg I. Biochemical Aspects of Fumaric-Acid Accumulation by *Rhizopus-Arrhizus*. *Appl Environ Microb*. 1986 Jul;52(1):128-33.
- [99] Li HX, Qiu T, Huang GD, Cao YS. Production of gamma-aminobutyric acid by *Lactobacillus brevis* NCL912 using fed-batch fermentation. *Microb Cell Fact*. 2010 Nov 12;9.

- [100] Shi F, Li YX. Synthesis of gamma-aminobutyric acid by expressing *Lactobacillus brevis*-derived glutamate decarboxylase in the *Corynebacterium glutamicum* strain ATCC 13032. *Biotechnol Lett*. 2011 Dec;33(12):2469-74.
- [101] Harris LM, Desai RP, Welker NE, Papoutsakis ET. Characterization of recombinant strains of the *Clostridium acetobutylicum* butyrate kinase inactivation mutant: Need for new phenomenological models for solventogenesis and butanol inhibition? *Biotechnol Bioeng*. 2000 Jan 5;67(1):1-11.
- [102] Dellomonaco C, Clomburg JM, Miller EN, Gonzalez R. Engineered reversal of the beta-oxidation cycle for the synthesis of fuels and chemicals. *Nature*. 2011 Aug 18;476(7360):355-U131.
- [103] Shen CR, Lan EI, Dekishima Y, Baez A, Cho KM, Liao JC. Driving Forces Enable High-Titer Anaerobic 1-Butanol Synthesis in *Escherichia coli*. *Appl Environ Microb*. 2011 May;77(9):2905-15.
- [104] Atsumi S, Hanai T, Liao JC. Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels. *Nature*. 2008 Jan 3;451(7174):86-U13.
- [105] Blombach B, Riester T, Wieschalka S, Ziert C, Youn JW, Wendisch VF, et al. *Corynebacterium glutamicum* Tailored for Efficient Isobutanol Production. *Appl Environ Microb*. 2011 May;77(10):3300-10.
- [106] Yim H, Haselbeck R, Niu W, Pujol-Baxley C, Burgard A, Boldt J, et al. Metabolic engineering of *Escherichia coli* for direct production of 1,4-butanediol. *Nat Chem Biol*. 2011 Jul;7(7):445-52.
- [107] Ma CQ, Wang AL, Qin JY, Li LX, Ai XL, Jiang TY, et al. Enhanced 2,3-butanediol production by *Klebsiella pneumoniae* SDM. *Appl Microbiol Biot*. 2009 Feb;82(1):49-57.
- [108] Zhang LY, Sun JA, Hao YL, Zhu JW, Chu J, Wei DZ, et al. Microbial production of 2,3-butanediol by a surfactant (serrawettin)-deficient mutant of *Serratia marcescens* H30. *J Ind Microbiol Biot*. 2010 Aug;37(8):857-62.
- [109] Qian ZG, Xia XX, Lee SY. Metabolic Engineering of *Escherichia coli* for the Production of Putrescine: A Four Carbon Diamine. *Biotechnol Bioeng*. 2009 Nov 1;104(4):651-62.
- [110] Yahiro K, Takahama T, Park YS, Okabe M. Breeding of *Aspergillus-Terreus* Mutant Tn-484 for Itaconic Acid Production with High-Yield. *J Ferment Bioeng*. 1995;79(5):506-8.
- [111] Liao JCLACA, Chang P-CHC, inventors; Genetically Modified Microorganisms for Producing Itaconic Acid with High Yields. US patent US 2010/0285546 A1. 2010.
- [112] Tseng HC, Harwell CL, Martin CH, Prather KLJ. Biosynthesis of chiral 3-hydroxyvalerate from single propionate-unrelated carbon sources in metabolically engineered *E. coli*. *Microb Cell Fact*. 2010 Nov 27;9.



- [113] Zhang KC, Sawaya MR, Eisenberg DS, Liao JC. Expanding metabolism for biosynthesis of nonnatural alcohols. *P Natl Acad Sci USA*. 2008 Dec 30;105(52):20653-8.
- [114] Cann AF, Liao JC. Production of 2-methyl-1-butanol in engineered *Escherichia coli*. *Appl Microbiol Biot*. 2008 Nov;81(1):89-98.
- [115] Connor MR, Liao JC. Engineering of an *Escherichia coli* strain for the production of 3-methyl-1-butanol. *Appl Environ Microb*. 2008 Sep;74(18):5769-75.
- [116] Granstrom TB, Izumori K, Leisola M. A rare sugar xylitol. Part II: biotechnological production and future applications of xylitol. *Appl Microbiol Biot*. 2007 Feb;74(2):273-6.
- [117] Cirino PC, Chin JW, Ingram LO. Engineering *Escherichia coli* for xylitol production from glucose-xylose mixtures. *Biotechnol Bioeng*. 2006 Dec 20;95(6):1167-76.
- [118] Qian ZG, Xia XX, Lee SY. Metabolic Engineering of *Escherichia coli* for the Production of Cadaverine: A Five Carbon Diamine. *Biotechnol Bioeng*. 2011 Jan;108(1):93-103.
- [119] Moon TS, Dueber JE, Shiue E, Prather KLJ. Use of modular, synthetic scaffolds for improved production of glucaric acid in engineered *E. coli*. *Metab Eng*. 2010 May;12(3):298-305.
- [120] Balderas-Hernandez VE, Sabido-Ramos A, Silva P, Cabrera-Valladares N, Hernandez-Chavez G, Baez-Viveros JL, et al. Metabolic engineering for improving anthranilate synthesis from glucose in *Escherichia coli*. *Microb Cell Fact*. 2009 Apr 2;8.
- [121] Wierckx NJP, Ballerstedt H, de Bont JAM, Wery J. Engineering of solvent-tolerant *Pseudomonas putida* S12 for bioproduction of phenol from glucose. *Appl Environ Microb*. 2005 Dec;71(12):8221-7.
- [122] Wang CL, Takenaka S, Murakami S, Aoki K. Isolation of a benzoate-utilizing *Pseudomonas* strain from soil and production of catechol from benzoate by transpositional mutants. *Microbiol Res*. 2001;156(2):151-8.
- [123] Hasunuma T, Kondo A. Consolidated bioprocessing and simultaneous saccharification and fermentation of lignocellulose to ethanol with thermotolerant yeast strains. *Process Biochemistry*. 2012;47(9):1287-94.
- [124] Heer D, Sauer U. Identification of furfural as a key toxin in lignocellulosic hydrolysates and evolution of a tolerant yeast strain. *Microbial Biotechnology*. 2008;1(6):497-506.
- [125] Dunlop MJ. Engineering microbes for tolerance to next-generation biofuels. *Biotechnology for Biofuels*. 2011 Sep 21;4.
- [126] Alper H, Moxley J, Nevoigt E, Fink GR, Stephanopoulos G. Engineering yeast transcription machinery for improved ethanol tolerance and production. *Science*. 2006 Dec 8;314(5805):1565-8.



- [127] Tomas CA, Welker NE, Papoutsakis ET. Overexpression of groESL in *Clostridium acetobutylicum* results in increased solvent production and tolerance, prolonged metabolism, and changes in the cell's transcriptional program. *Applied and Environmental Microbiology*. 2003 Aug;69(8):4951-65.
- [128] Zhang J-G, Liu X-Y, He X-P, Guo X-N, Lu Y, Zhang B-r. Improvement of acetic acid tolerance and fermentation performance of *Saccharomyces cerevisiae* by disruption of the FPS1 aquaglyceroporin gene. *Biotechnology Letters*. 2011 Feb;33(2):277-84.
- [129] Geertman J-MA, van Dijken JP, Pronk JT. Engineering NADH metabolism in *Saccharomyces cerevisiae*: formate as an electron donor for glycerol production by anaerobic, glucose-limited chemostat cultures. *Fems Yeast Research*. 2006 Dec;6(8):1193-203.
- [130] Ji L, Shen Y, Xu L, Peng B, Xiao Y, Bao X. Enhanced resistance of *Saccharomyces cerevisiae* to vanillin by expression of lacA from *Trametes* sp AH28-2. *Bioresource Technology*. 2011 Sep;102(17):8105-9.
- [131] Dunlop MJ, Dossani ZY, Szmidt HL, Chu HC, Lee TS, Keasling JD, et al. Engineering microbial biofuel tolerance and export using efflux pumps. *Molecular Systems Biology*. 2011 May;7.
- [132] Atsumi S, Wu T-Y, Machado IMP, Huang W-C, Chen P-Y, Pellegrini M, et al. Evolution, genomic analysis, and reconstruction of isobutanol tolerance in *Escherichia coli*. *Molecular Systems Biology*. 2010 Dec;6.
- [133] Young E, Lee S-M, Alper H. Optimizing pentose utilization in yeast: the need for novel tools and approaches. *Biotechnology for Biofuels*. 2010 Nov 16;3.
- [134] Xu Q, Singh A, Himmel ME. Perspectives and new directions for the production of bioethanol using consolidated bioprocessing of lignocellulose. *Current Opinion in Biotechnology*. 2009;20(3):364-71.
- [135] Blanch HW. Bioprocessing for biofuels. *Current Opinion in Biotechnology*. 2012;23(3):390-5.
- [136] Zhang YHP, Himmel ME, Mielenz JR. Outlook for cellulase improvement: Screening and selection strategies. *Biotechnology Advances*. 2006 Sep-Oct;24(5):452-81.
- [137] Klein-Marcuschamer D, Oleskowicz-Popiel P, Simmons BA, Blanch HW. The challenge of enzyme cost in the production of lignocellulosic biofuels. *Biotechnology and Bioengineering*. 2012 Apr;109(4):1083-7.
- [138] COM(2012) 595. Proposal for a directive of the European Parliament and of the Council: amending Directive 98/70/EC relating to the quality of petrol and diesel fuels, and amending Directive 2009/28/EC on the promotion of the use of energy from renewable sources. Brussels: European Commission; 2012.