Chapter 3

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_2 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 1st, 2nd and 3rd 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 2nd generation bioethanol production compared to the 1st is the pretreatment step as the removal of the lignin is required for cellulose accessibility.[9] Whilst 1st 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 2nd 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

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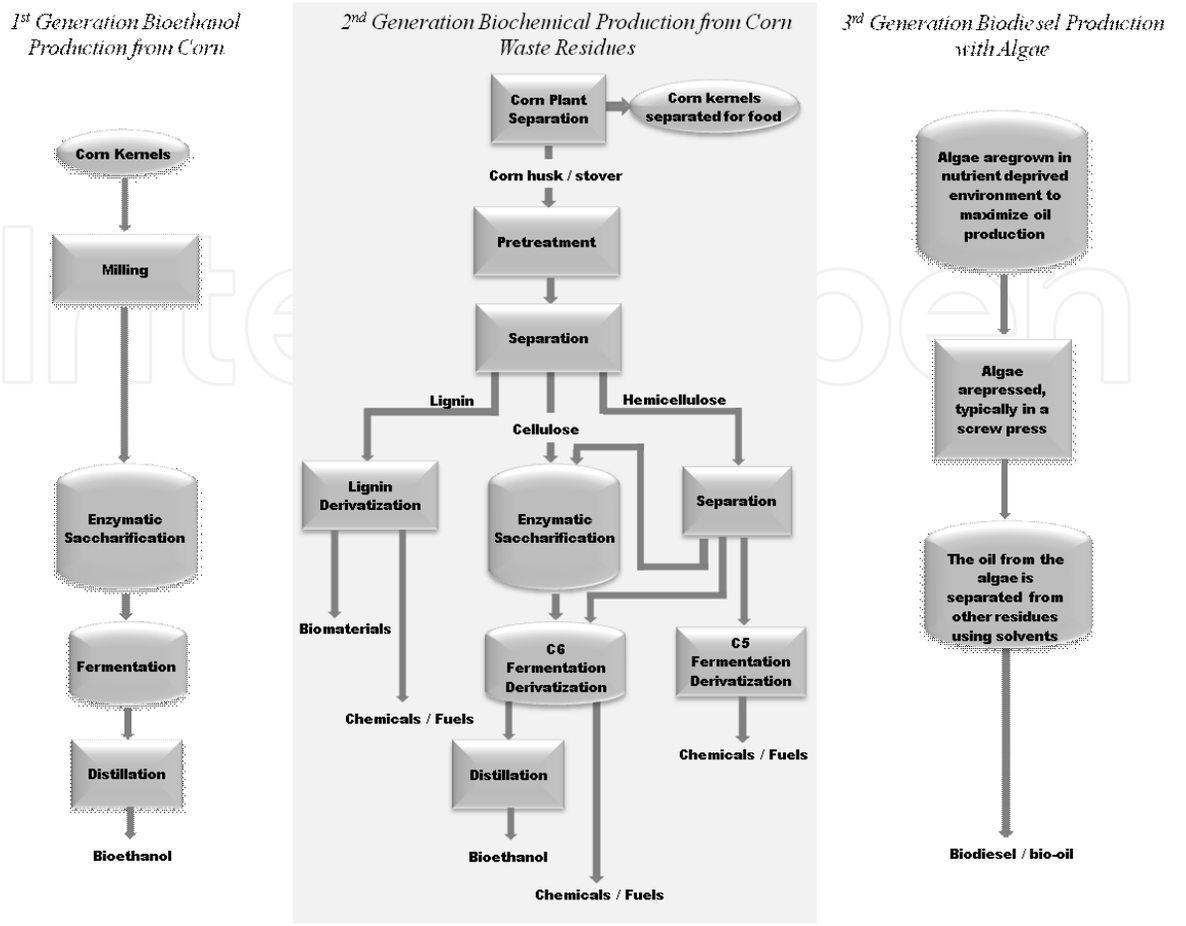

Figure 1. Examples of processes to produce 1st generation bioethanol from corn, 2nd generation bioethanol from corn waste residues and 3rd generation biodiesel from algae.

theoretical sugar yield can be obtained from lignocellulose without pretreatment.[10, 11] Currently, the biomass pretreatment step for producing 2nd 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_2. 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

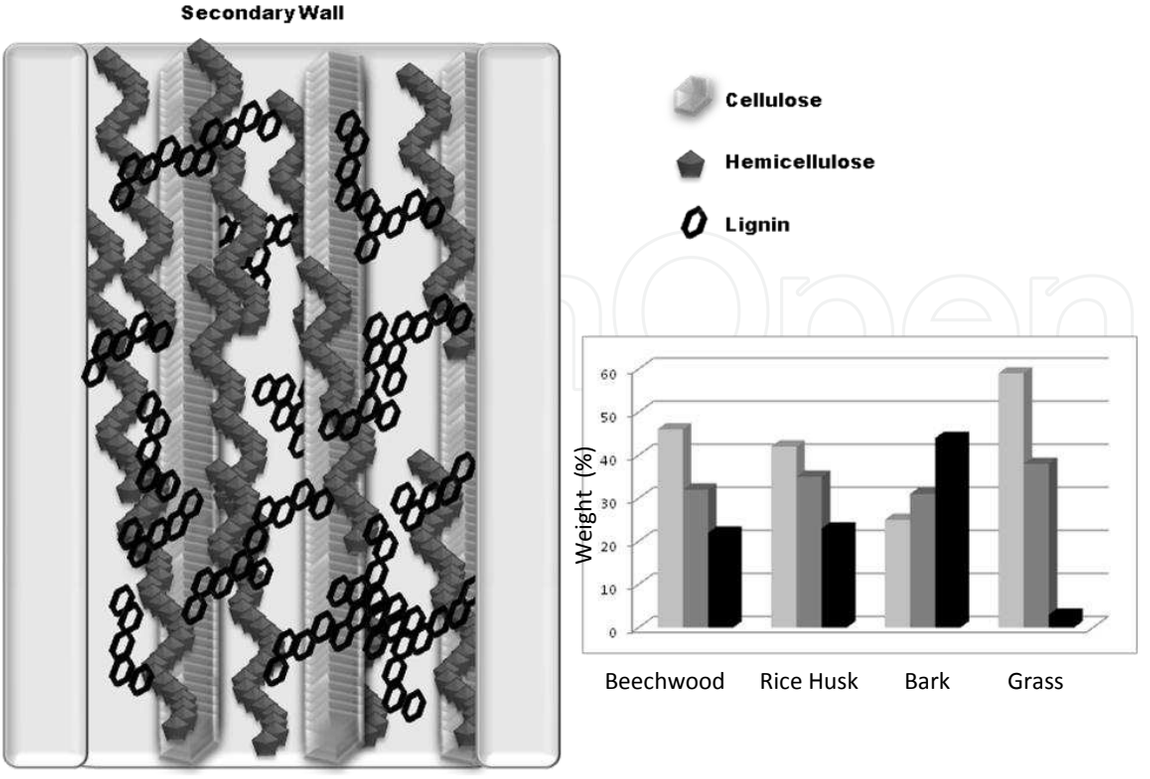

Figure 2. Diagram of plant components cellulose, hemicelluloses and lignin and a graphical representation of their weight percentage according to biomass source[10].

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*. *Dichomitus 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 *Dichomitus 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*. Hideno 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	*Echinodotium taxodi*	29% reduction in lignin	30 days	[22]
Bamboo residues	*Coriolus versicolor*	Enhanced saccharification rate of 37%	2 days	[24]
Bamboo culms	*Echinodontium taxodii*	5.15-fold increase in sugar yields	120 days	[23]
Bamboo culms	*Trametes versicolor*	8.75-fold increase in sugar yields	120 days	[23]
pCornstalk	*Phanerochaete chrysosporium*	34.3% reduction in lignin with a maximum enzyme saccharification of 47.3%	15 days	[32]
Corn Stover	*Ceriporiopsis subvermispora*	Lignin degradation reached 45%	30 days	[33]
Corn Stover	*Irpex lacteus CD2*	66.4% saccharification ratio	25 days	[34]
Corn Stover	*Ceriporiopsis subvermispora*	31.59% lignin degradation with less than 6% cellulose loss	18 days	[25]
Corn Stover	*Cyanthus stercoreus*	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	*Irpex lacteus*	3-fold increase in saccharification	46 days	[26]
Rice straw	*Dichomitus squalens*	58% theoretical glucose yield for remaining glucan	15 days	[27]
Rice straw	*Pleurotus ostreatus*	39% degradation of lignin with 79% cellulose retention	48 days	[35]
Rice straw	*Phaerochaete chrysosporium*	64.9% of maximum glucose yield from recovered glucan	15 days	[28]
Cotton stalks	*Phaerochaete chrysosporium*	33.9% lignin degradation with 18.4% carbohydrate availability	14 days	[29]
Cotton stalks	*Phaerochaete chrysosporium*	27.6% lignin degradation	14 days	[30]
Sawdust matrix	*Grifola frondosa*	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 pretreament 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.

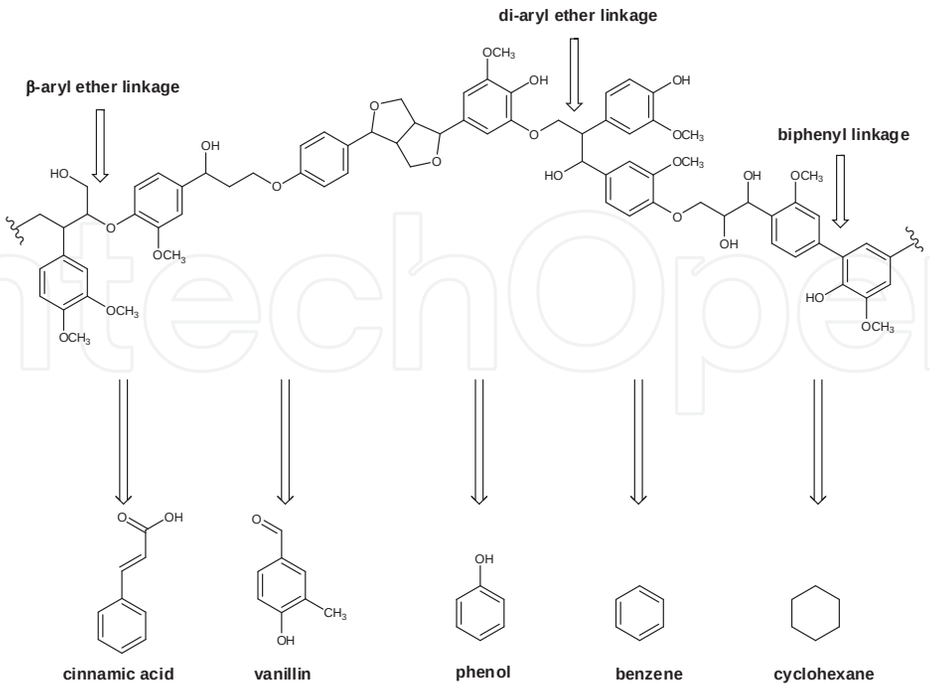

Figure 3. Examples of chemicals targeted from lignin.

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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, α-Proteobacteria and γ-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 *Sphinogobium* sp. SYK-6.[42] Furthermore *Sphinogobium* sp. metabolizes β-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).

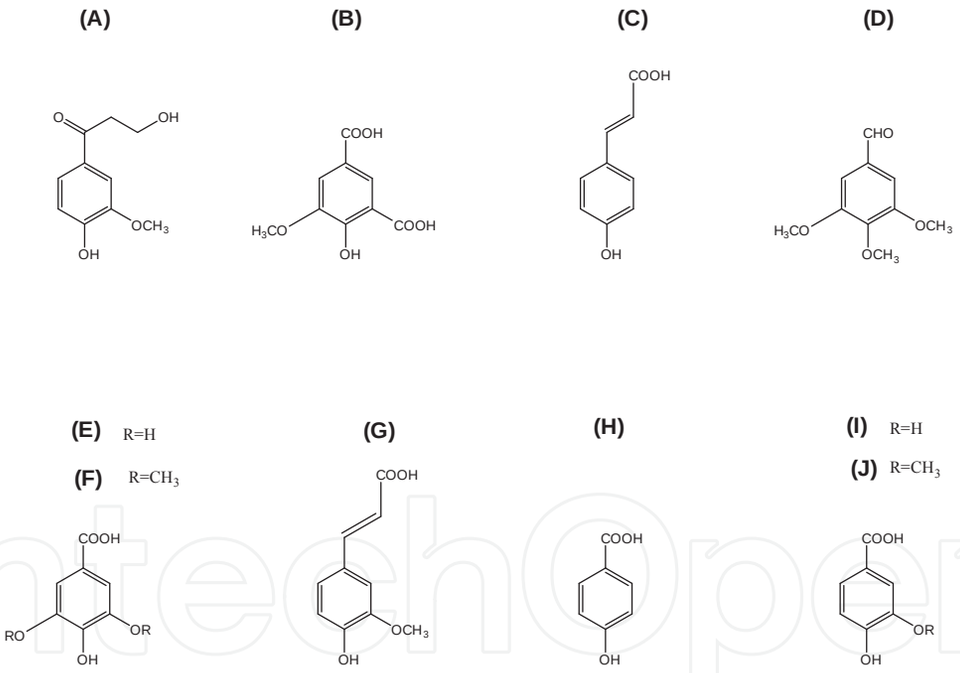

Figure 4. Compounds isolated after bacterial conversion of lignin.[40]

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 β-1,4-glucosidic bonds (Figure 5), in contrast to α-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 β-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] β-Glucosidases are the third general category of cellulolytic enzymes; they act upon bonds in soluble cellobiose or cellodextrins 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 hyperproducing 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 β-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 β-glucosidases.[55] Other promising fungal sources for cellulases exist, such as *Acremonium*, *Penicillium* and

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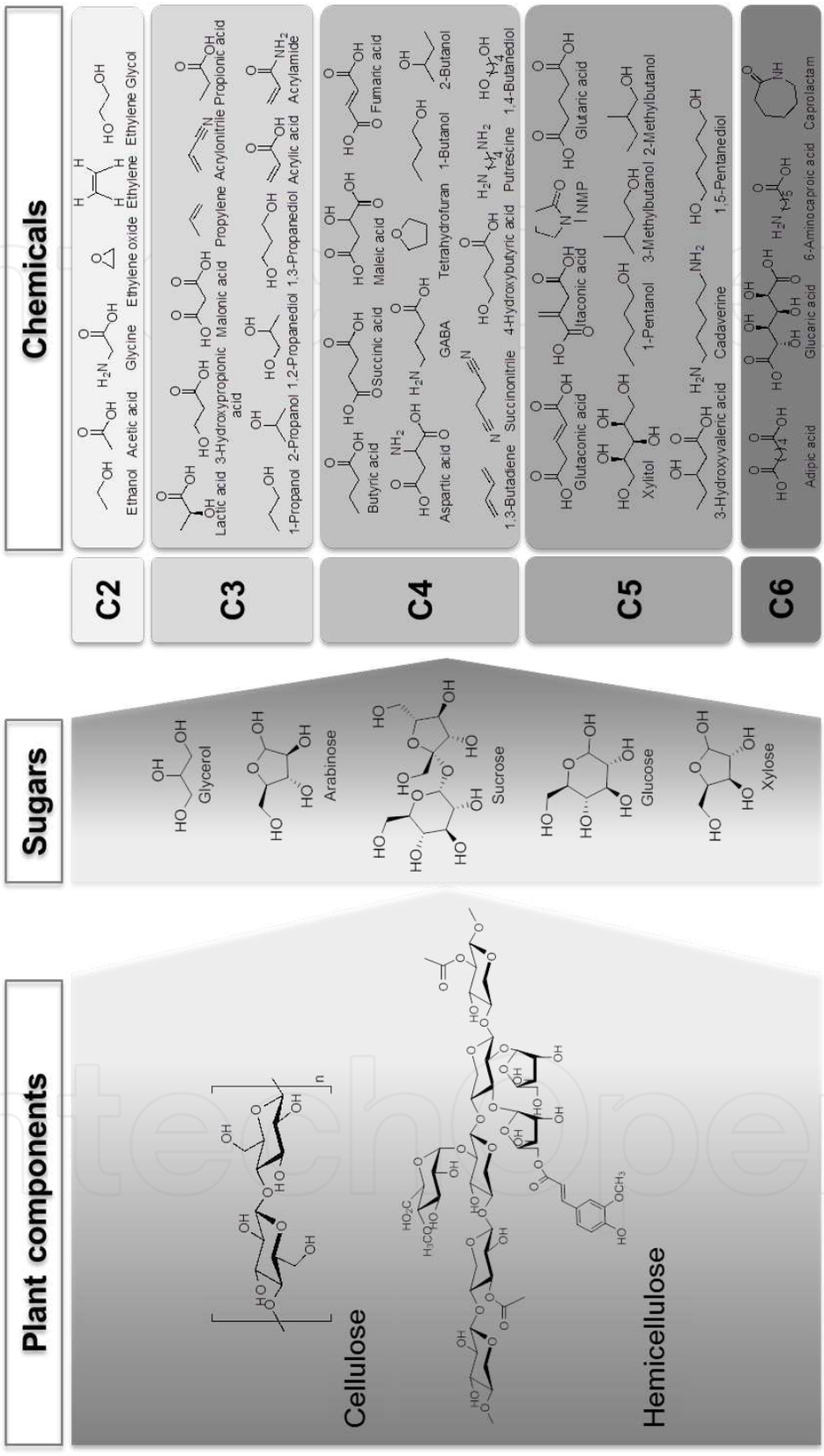

Figure 5. Cellulose and hemicellulose, their sugar units and some potential chemical targets organized by carbon chain number.

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 β-1,4-linked xyloses interspersed with arabinose and glucuronic acid, while glucomannans are a mixture of β-1,4-linked glucose with α-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 β-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]

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

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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
S. cerevisiae	acetate	Deletion of *HRK*1 gene regulating membrane transporter activity	[128]
S. cerevisiae PK113-7D	formate, acetate	Expression of formate dehydrogenase structural gene FAHD2	[129]
S. cerevisiae	vanillin	Overexpression of laccase gene *lac*A from *Trametes sp.* AH28-2	[130]
E. coli	biodiesel, biogasoline	RND efflux pumps heterologously expressed	[131]
C. acetobutylicum	butanol	Overexpression of GroESL heat shock protein	[127]
E. coli	isobutanol	Simultaneous disruption of five unrelated genes	[132]
S. cerevisiae	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 hydrolysate 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 hydrolysate 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]

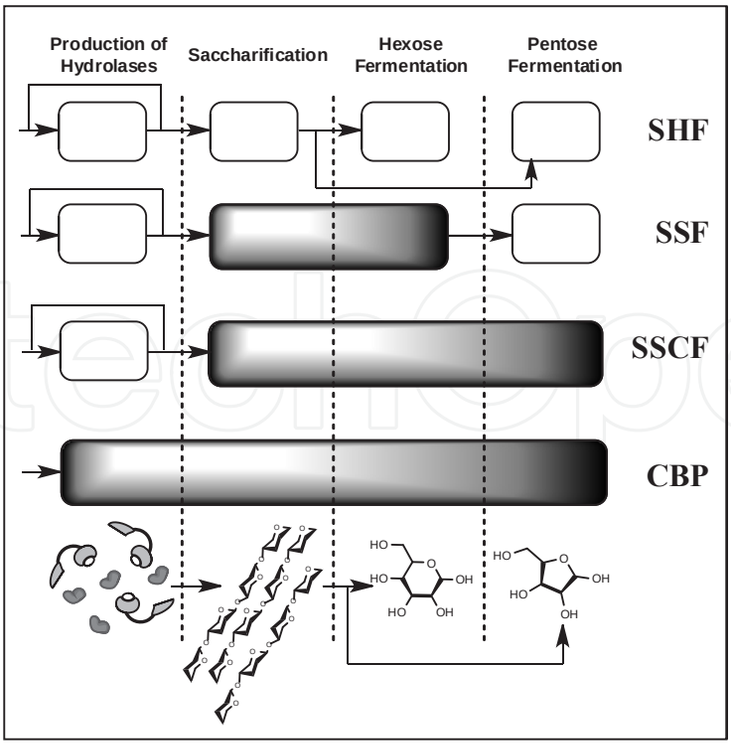

Figure 6. Consolidated fermentation processes.

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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, 2nd generation biofuel and chemical production is yet to be commercialized.

Finding economical pretreatment methods has been recognized as one of the hurdles to commercializing 2nd 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 2nd 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 governements 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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