Developing Organisms for Consolidated Bioprocessing of Biomass to Ethanol

Willem H. van Zyl, Riaan den Haan and Daniel C. la Grange
Department of Microbiology, University of Stellenbosch, Stellenbosch
South Africa

1. Introduction

Lignocellulosic biomass is an abundant, renewable feedstock for sustainable production of biofuels and chemicals. The main technological barrier that impedes widespread utilization of this resource for production of fuels and other commodity products is the lack of low-cost technologies to overcome the recalcitrance of lignocellulose. Organisms that hydrolyse the cellulose and hemicelluloses in biomass and produce a commodity product such as ethanol at a high rate and titre would significantly reduce the costs of biomass conversion. This would allow steps that are currently accomplished in different reactors, often by different organisms, to be combined in a consolidated bioprocess (CBP). While there is still no ideal organism to use in one-step biomass conversion, several candidates have been identified that are in various stages of development for establishment of a cellulolytic system and/or improvement of product-forming attributes. This chapter assesses the *status quo* for CBP organismal development either by enabling non-cellulolytic organisms to grow on cellulosic substrates or by improving product forming abilities of native cellulose utilizing organisms. The authors also discuss feedstocks that are available for the production of biofuels using CBP and assess how process integrations can make CBP economically feasible in the near future.

The increasing demand for oil coupled to the premium many governments place on greater energy security and environmental concerns have led to the development of an active biofuels industry (Van Zyl et al., 2011). First generation biofuels such as ethanol from starch or sugar already contribute considerable amounts of liquid fuels in several countries. However these technologies suffer from a shortage in the availability of feedstock in order to displace a more significant amount of petroleum based fuels. Lignocellulose represents the most widespread and abundant source of carbon in nature and is the only source that could provide a sufficient amount of feedstock to satisfy the world's energy and chemicals needs in a renewable manner (Hill et al., 2006; Van Zyl et al., 2011). Second generation biofuels such as ethanol form cellulosic biomass therefore seeks to overcome the problem of feedstock supply shortage by utilizing the energy contained in total plant biomass. Current technologies for conversion of biomass to ethanol commences with a pretreatment step during which physical and/or chemical processes are used to render the polymeric sugar fractions more accessible to conversion by enzymatic processes (Stephanopoulos, 2007). The type of feedstock will predetermine the optimal type of pretreatment which in turn defines the optimal enzyme mixture to be used in subsequent hydrolysis steps and the composition of the hydrolysis products. Four biologically mediated events occur during conversion of

pretreated lignocellulose to ethanol via processes featuring enzymatic hydrolysis namely: (i) production of depolymerising enzymes (cellulases and hemicellulases), (ii) hydrolysis of the polysaccharide constituents of pretreated biomass, (iii) fermentation of the hexose sugars present, and (iv) fermentation of pentose sugars present (Lynd et al., 2002). Improvements of biomass conversion technology generally entail the consolidation of two or more of these steps. Hydrolysis and fermentation steps can be combined in simultaneous saccharification and fermentation (SSF) of hexoses or simultaneous saccharification and co-fermentation (SSCF) of both hexoses and pentoses. The ultimate objective would be a one-step "consolidated" bioprocessing (CBP) of lignocellulose to bioethanol, where all four of these steps occur in a single reactor and a single microorganism or microbial consortium converts pretreated biomass to a commodity product such as ethanol without added saccharolytic enzymes. CBP would represent a breakthrough for low-cost biomass processing, due to economic benefits of process integration (Galbe et al., 2005; Hahn-Hägerdal et al., 2007; Hamelinck et al., 2005; Robinson, 2006) and avoiding the high costs of enzymes that make the biochemical conversion route unattractive (Anex et al., 2010; Kazi et al., 2010).

Lignocellulosic plant biomass represents the largest source of renewable carbon on earth and consists of 40–55% cellulose, 25–50% hemicellulose and 10–40% lignin, depending on whether the source is hardwood, softwood, or grasses (Sun & Cheng, 2002). The main polysaccharide present is water-insoluble cellulose that represents the major fraction of fermentable sugars. Full enzymatic hydrolysis of crystalline cellulose requires synergistic action of three major types of enzymatic activity (i) endoglucanases, (ii) exoglucanases, including cellodextrinases and cellobiohydrolases, and (iii) β-glucosidases (Zhang & Lynd, 2004). Endoglucanases are active on the non-crystalline or amorphous regions of cellulose and their activities yield cellobiose and cellooligosaccharides as hydrolysis products (Figure 1). Cellobiohydrolases are processive enzymes that are active on the crystalline regions of cellulose and most yield almost exclusively cellobiose as their main hydrolysis product. In turn, β-glucosidases convert cellobiose and some cello-oligosaccharides to glucose. Hemicellulose refers to a number of heterogeneous structures, such as (arabino)xylan, galacto(gluco)mannan, and xyloglucan (Sun & Cheng, 2002). These chemically diverse polymers are linked together through covalent and hydrogen bonds, as well as being intertwined and can be chemically bound to the lignin fraction. Although many pretreatment protocols remove variable amounts of hemicelluloses, it remains imperative from an economic perspective that sugars contained in the hemicellulose fraction of lignocellulose are also converted to ethanol (Hahn-Hägerdal et al., 2001). The compositions of the major and minor types of hemicelluloses present in lignocellulosic feedstocks and the enzymes required to hydrolyze them are reviewed elsewhere (Girio et al., 2010; Van Zyl et al., 2007)

2. Feedstocks and conversion technologies

Many countries are embarking on ambitious biofuels policies resulting in a rapid global increase in the demand and supply of biofuels (Sastri et al., 2008). In recent years significant progress has been made towards the development of different technologies for the production of particularly bioethanol, but also butanol, alkanes and terpenes from lignocellulosic material (Fortman et al., 2008). Sugar-based ethanol is the least expensive biofuel and its production is mainly constrained by the availability of feedstock (Somerville et al., 2010). Grain-based ethanol is hampered by high feedstock prices and competition
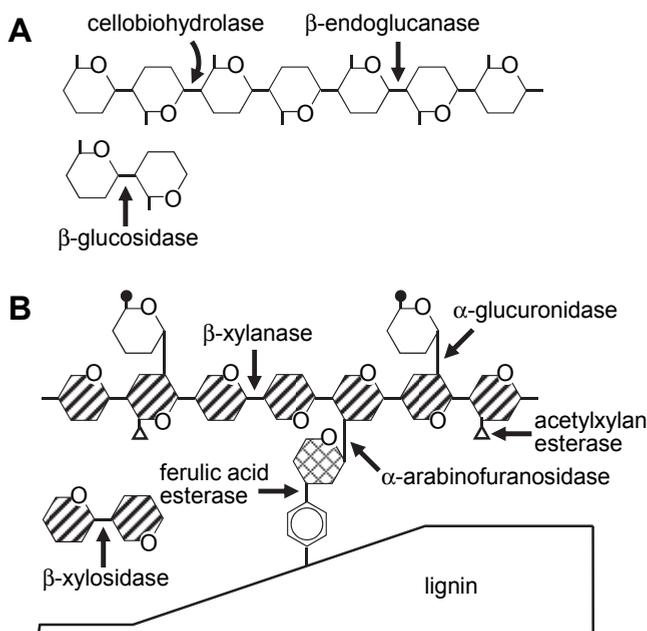
Fig. 1. Illustration of the complexity of cellulose (A) and arabinoxylan (B) as major polysaccharides in lignocellulose and the enzymes involved in their degradation. Hexoses are distinguished from pentoses by the presence of a protruding line from the cyclic hexagon (pyranose ring), depicting the CH_2OH group. Hydrolase enzymes and the bonds targeted for cleavage in the polysaccharides structures are indicated by arrows (Van Zyl et al., 2007).

with food markets. Cellulosic biofuels hold great promise, but the necessary technology advances to overcome the recalcitrance of lignocellulose are needed to enable profitable production of biofuels like ethanol, (Fortman et al., 2008). Potential cellulosic feedstock are numerous and widespread and include woody biomass, perennial grasses, and agricultural and forest residues (Table 1).

Woody biomass can and has been harvested sustainably for lumber and paper for many years (Somerville et al., 2010). Furthermore, the electronic media and paper recycling help to reduce the demand for paper pulp. All this biomass is therefore potentially available for energy. The potential energy available in this biomass source is enormous. It is estimated that biomass harvested in the Northern Hemisphere from wood products has an energy content equal to 107% of the liquid fuel consumption of the United States (Goodale et al., 2002).

Perennial plants such as switchgrass, *Miscanthus* and Napier grass have high photosynthetic capacity, as well as water and nitrogen use efficiency (Somerville et al., 2010; Ansah et al., 2010). They are fast growing and have efficient root systems allowing them to reach deep into the soil for water. The root produces a network of stems and roots that holds onto soil to prevent erosion. These and other perennial grasses are capable of averaging around 30 metric tons of dry matter per hectare per year.

Maize is the largest crop in the world in terms of grain production at around 820 million metric tons per annum (Somerville et al., 2010). A more or less equal amount of stems and stripped cobs (stover) is potentially available for the production of fuel. If half of the stover

can be converted to ethanol it would double the amount of ethanol produced from maize. However, the removal of this much stover would lead to significant losses of carbon from the soil and would aggravate erosion. It would also increase the amount of fertilizer needed to maintain good crop yields.

Crop	Growth cycle (months)	Water needs (mm/season)	Average productivity (dry t/ha/year)	Ethanol yield (l/ha)
Perennial grass				
Switchgrass	12	700	15	5000
Miscanthus	12	750	25	7500
Napier grass	3	1500	40	12500
Wood				
Poplar	36	900	8	2000
Agricultural crop				
Sugarcane	15	2000	21	10000
Sweet sorghum	4	600	20	6000
Corn	4	750	10	3800
Drought resistant crop				
Agave	60	400	20	7500

Ethanol yield refers to the total amount produced from a feedstock, including grain and stover or sugar and bagasse.

Table 1. Summary of biofuels feedstock (adapted from La Grange (2007) and Summerville et al., 2010)

Ethanol from sugarcane constitutes one of the largest sources of biofuels in the world (Somerville et al., 2010). Currently only 4.6 million hectares of sugar cane are used for bioethanol production in Brazil. In a recent announcement the Brazilian government stated that the area used for sugarcane cultivation would be increased substantially, but it would be limited to 63.5 million hectares (Decree No.6.961 2009). Approximately 60 million hectares of this allocated land would be available for biofuels production. Currently only the sugar component is used for the production of bioethanol. Once the technology becomes available, the cellulosic component in bagasse could also be used for the production of ethanol or other liquid fuels (Fortman et al., 2008). Estimates, based on the expected increase in sugarcane crops and cellulosic fuel from the 60 million hectares available land, are that Brazil could produce 14% of the current world transportation fuel demand of 4900 Gl by the year 2030. (Somerville et al., 2010). Currently, South Africa produces about 20 million tonnes of cane (about 50% of Africa's production) on 325 000 hectares of land. If the full potential of the estimated 6 million hectares of land suitable for sugarcane production in Angola, Malawi, Mozambique, Tanzania, Zambia and Zimbabwe are also realised, about 400 million tonnes of cane can be produced, which could yield 49 Gl ethanol, about 20% of Africa's current total petroleum consumption (Somerville et al., 2010; Watson, 2011).

Almost a fifth of the terrestrial surface on earth is semi-arid and prone to droughts with a rainfall of between 200 and 800 mm per year. If this is combined with agricultural land that has fallen out of production, the amount of land available for the production of biomass using drought resistant species such as *Agave* is vast. *Agave* spp. thrive under these conditions and produce between 1 and 34 dry tons of biomass per hectare per year (Davis et

al., 2011). Obtaining biomass in sufficient quantities to merit the construction of commercial scale facilities will be a major concern in future, fortunately there are a number of different crops suitable for different environmental conditions that could enable sustainable production of sufficient quantities of biomass.

One of the major challenges of biomass harvesting and delivery to conversion facilities remains yield and density, which determines the volume of the biomass. The density of grassy feedstock to woody feedstocks can vary between ~70 kg/m^3 to ~300 kg/m^3. For a 200 – 1 000 million liter per annum cellulosic ethanol plant, 0.8 – 4.0 million tonnes of dry biomass are required, which would require 50 – 250 trucks per day to deliver the biomass (Richard, 2010). Innovative ways of harvesting and delivering biomass to conversion facilities have to be developed to ensure cost-effective production of cellulosic ethanol at significant quantities. These could include dedicated production of biomass (e.g. as found in the sugar and paper-and-pulp industries) or the development of biomass commodity markets, parallel to agricultural commodity markets, such as grain and livestock. Defining of strict specifications for biomass delivered will be crucial to ensure a uniform feedstock for take-off by biomass conversion industries.

3. CBP organismal development

While several microorganisms can be found in nature with the ability to produce the required enzymes to hydrolyse all the polysaccharides found in lignocellulose, there is no organism with the ability to directly hydrolyze these polysaccharides and ferment the liberated sugar to a desired product such as ethanol at rates and titers required for economic feasibility (Hahn-Hägerdal et al., 2006; Lynd et al., 2005). Strain development is therefore the most important technical obstacle towards the conversion of lignocellulose to commodity products in a CBP configuration (Bothast et al., 1999; Alfenore et al., 2002). Organisms with broad substrate ranges and cellulolytic and/or hemicellulolytic abilities generally suffer from poor growth characteristics or poor product producing characteristics. These include poor yield, titer and rate or producing mixtures of products where desirable products are produced along with undesirables. In comparison, organisms with desirable product producing qualities often suffer from limited substrate range including lack of cellulolytic ability, poor fermentation qualities, and sensitivity to the inhibitors present in pretreated lignocellulosic biomass. The ideal CBP organism should be robust with regards to inhibitor tolerance, able to degrade lignocellulose and utilize hexose and pentose sugars at high efficiency. Furthermore characteristics such as the ability to simultaneously utilize sugars, GRAS (Generally Regarded as Safe) status, minimal nutrient supplementation and tolerance of low pH and high temperature would also be desirable in a CBP organism (Zaldivar et al., 2001). Four different approaches have been followed to develop such an organism, these are summarized in Figure 2. Due to the variety of feedstocks likely to be used, the diversity in pretreatment methods and the difference in desired products produced; there is scope for development of organisms with a range of different traits (La Grange et al., 2010).

3.1 Eukaryotic cellulolytic organisms for CBP

Several species of cellulolytic fungi, such as *Trichoderma reesei*, naturally produce a large repertoire of saccharolytic enzymes to digest lignocellulose efficiently, assimilate all lignocellulosic sugars, and convert these sugars to ethanol, showing that they naturally
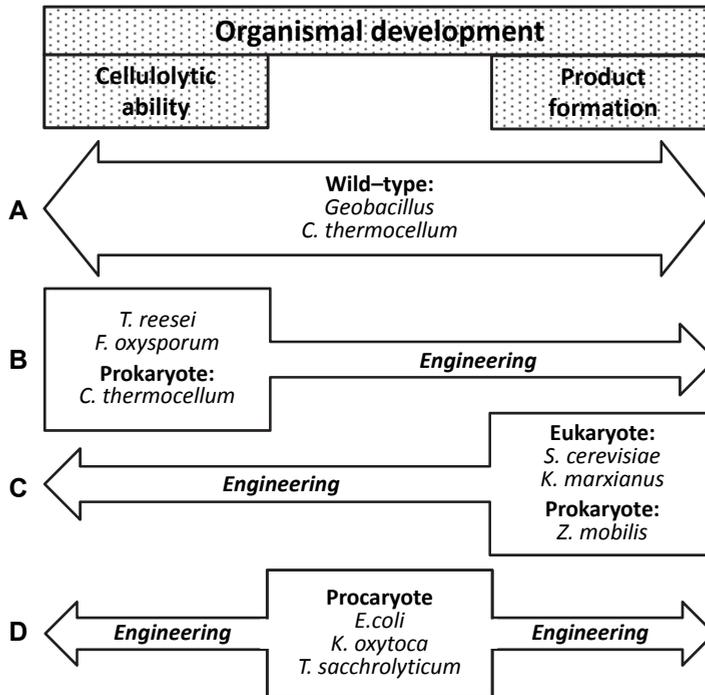
Fig. 2. Different recombinant strategies to engineer cellulolytic and product formation organisms for the CBP process. The four strategies are; (A) isolating microbes with both cellulolytic and product formation properties, (B) engineering superior cellulolytic microbes to produce desired products, (C) engineering cellulolytic activity into superior product forming organisms and (D) select organisms with special features for fermenting lignocellulosics and engineering both cellulolytic and product formation properties.

possess all pathways for conversion of lignocellulose to bioethanol (Chambergo et al., 2002; Lynd et al., 2002). Despite recent advances in engineering cellulases to be more efficient and less costly, the complete saccharification of pretreated lignocellulose still requires a long time for digestion and high loadings of enzyme (30–50 mg enzyme per g of crystalline cellulose) (Xu et al., 2009). Therefore, a biorefinery consuming thousands of tonnes of biomass per day will require many tonnes of cellulase preparation to operate. Currently, only fungi naturally produce the required amounts of cellulase to meet this need. Some strains of *T. reesei* are reportedly able to produce more than 100 g cellulase enzyme per liter of culture broth (Cherry & Fidantsef, 2003). The primary advantages of *T. reesei* as a CBP organism are: (i) the production of cellulases in sufficient quantities and at reasonable cost (ii) that it is already established commercially, and specific mutants are available that can be grown at a low cost and in quantities needed for the emerging biorefinery industry and (iii) that it has all the metabolic pathways necessary to utilize all lignocellulose sugars for production of ethanol (Xu et al., 2009). However, there are significant challenges to overcome before *T. reesei* can be considered as a CBP organism such as (i) ethanol yield and rate of production are low, (ii) ethanol tolerance is low, and (iii) mixing during fermentation may require more energy owing to its filamentous cell morphology.

T. reesei is an obligate aerobe making its survival for long periods without oxygen difficult (Rautio et al., 2006). The foremost reason for this is that the genes encoding glycolytic enzymes are strongly repressed in the absence of oxygen. However, preliminary studies indicated that *T. reesei*, could produce cellulases when grown aerobically on cellulose that continued to degrade cellulose to sugars and ferment these sugars to ethanol when cultures were rendered anaerobic, although acetic acid was produced as a major by-product (Xu et al., 2009). It was also shown that *T. reesei* could convert the five primary lignocellulosic sugars to ethanol but the ethanol yields and production rates were low. Therefore the major limitation for efficient ethanol production by *T. reesei* do not lie in the absence of the relevant genes and pathways but are more likely related to the low expression of these genes or the activity of the enzymes encoded. Approaches to solving these problems are to enhance the expression of the relevant genes at the transcriptional level and/or to introduce heterologous genes that encode enzymes with higher activities. It is expected that ethanol formation and tolerance could be improved using the following strategies: (i) identification and modification of genes involved in ethanol tolerance; (ii) introduction of heterologous genes, such as *S. cerevisiae* pyruvate decarboxylase and alcohol dehydrogenase to enhance the classical ethanol synthetic pathway and (iii) knockout of *T. reesei* genes responsible for the production of byproducts. Furthermore, *T. reesei* is multi-cellular with a growth mode that results in an extended cellular chain forming hyphae. This requires more energy input for mixing and handing in fermentation tanks, compared to unicellular yeast. Another challenge for the application of *T. reesei* as a CBP organism is the modification of its growth into a compact pelleted form rather than as extended hyphae.

Another filamentous fungus*, Fusarium oxysporum*, also produces the enzymes required to break down cellulose and hemicellulose while simultaneously fermenting the corresponding hexoses and pentoses to ethanol albeit at relatively low yields (Anasontzis et al., 2011; Panagiotou et al., 2005). In SSF of cellulose a *Fusarium oxysporum* wild type strain F3 was able to grow at a maximum specific growth rate of 0.023 h^{-1} on cellulose in aerobic conditions and produced ethanol with a yield of 0.35 g/g cellulose under anaerobic conditions. The cellulase system in *F. oxysporum* is well balanced as no cellobiose accumulated during growth on cellulose. The strain was further shown to effectively produce a complete system of hydrolytic enzymes when grown on various agro-industrial lignocellulose by-products, such as dry citrus peels, corn cob and brewer's spent grain and simultaneously ferment the corresponding oligosaccharides to ethanol (Anasontzis et al., 2011; Xiros et al., 2008). In these studies, the hydrolysis of the lignocellulosic material was shown to be the major bottleneck on the productivity of the overall bioconversion process. The corresponding hydrolases, mainly cellulases and xylanases are inducible enzymes and their efficient production in the fermentation medium is a time consuming step. Homologous overexpression of these enzymes under constitutive control, could provide a higher breakdown rate of the (hemi-)cellulosic biomass and thus increase the supply of sugars to the ethanol production pathway. To this end the endo-xylanase 2 of *F. oxysporum*, was overproduced in the F3 strain under control to the constitutive *Aspergillus nidulans gpdA* promoter (Anasontzis et al., 2011). The fermentative performance of the transformants were evaluated and compared to that of the wild type in simple CBP systems using corn cob or wheat bran as sole carbon sources. Transformants produced approximately 60% more ethanol compared to the wild type on corn cob and wheat bran likely due to the high extracellular xylanase activities in the transformants' fermentation broths that were maintained 2–2.5-fold higher compared to the wild type.

3.2 Prokaryotic cellulolytic organisms for CBP

Thermophilic bacteria as a group show great potential as CBP organisms (Xu et al., 2010). These organisms are capable of cellulose hydrolysis and ethanol production under thermophilic conditions. High temperature hydrolysis and fermentation potentially provide a significant energy saving since reactors would not have to be cooled to 30 or 37^0C before inoculation and then heated again for distillation. Furthermore, it has been shown that a 10^0C increase in temperature approximately doubles the reaction rate, which in turn decreases the amount of enzyme needed (Ibrahim & El-diwany, 2007). Because thermostable enzymes are able to tolerate higher temperatures they generally have longer half-lives. The use of higher reaction and fermentation temperatures (above 60^0C) also minimizes the risk of bacterial contamination. Since cellulose hydrolysis and sugar release is in most cases the rate limiting step in a typical CBP process, high temperature hydrolysis will be advantageous.

The thermophilic gram-positive anaerobic bacterium *Clostridium thermocellum* is regarded as a potential CBP-organism (Lynd et al., 2002). It is very efficient at hydrolysing crystalline cellulose, however growth of wild type strains are inhibited in the presence of ethanol concentrations above 2% (v/v) (Xu et al., 2010). Laboratory strains have been evolved that remained viable at ethanol concentrations of up to 8% (v/v). At the heart of *C. thermocellum*'s cellulose hydrolyzing ability lays the cellulosome.

Cellulosomes are extracellular multienzyme systems produced by some cellulolytic bacteria to degrade crystalline cellulose. The cellulosome concept was originally defined in *C. thermocellum* in 1983 by Lamed et al. (1983a, 1983b). They were searching for the molecular component on the cell surface of *C. thermocellum* responsible for specific binding to cellulose. Cellulosomes complexes typically consist of a scaffoldin molecule with enzymatic units attached to it. Scaffoldins contain cohesin domains to which enzymatic-units can bind by means of their respective dockerin domains. The cohesin-dockerin interaction is Ca-dependent and species-specific. Cellulases from *C. thermocellum* failed to interact with the scaffoldin protein from *C. cellulolyticum* and vice versa (Fierobe et al., 1999). In cells growing on cellulose, cellulosomes are typically attached to the host cell surface and they also contain at least one cellulose binding domain enabling the whole complex to effectively bind to cellulose. This arrangement enables enzyme proximity synergy as well as enzyme-substrate-microbe synergy. This results in cellulosomes being much more efficient at breaking down cellulose than free enzymes.

Until recently it was not certain what the optimal reaction conditions for cellulosomes were since host cell growth temperatures do not necessarily match those of the enzymes they produce. Furthermore, during pretreatment of lignocellulosic materials side reactions lead to the formation of compounds, which are inhibitors of cell growth. Xu et al (2010) tested the effect of some of these inhibitors on cellulosome activity of *C. thermocellum*. They found that organic acids like formate, acetate and lactate actually promoted cellulolytic activity and that the *C. thermocellum* cellulosome could tolerate certain concentrations of furfural (up to 5 mM), *p*-hydroxybenzoic acid (up to 50 mM) and catecol (up to 1 mM). The *C. thermocellum* cellulosomes were also able to tolerate higher ethanol concentrations and temperatures than the *T. reesei* enzymes used commercially. Using conditions optimal for cellulosomal activity *C. thermocellum* produced 22.6 g/L ethanol, the highest ever reported for *C. thermocellum*.

Not all cellulolytic bacteria produce cellulosomes. *Clostridium phytofermentans* with a genome encoding the highest number of cellulosic enzymes of all sequenced Clostridia, secretes enzymes enabling it to hydrolyze lignocellulose to fermentable sugars (Jin et al.,

2011). However, unlike *C. thermocellum, C phytofermentans* can consume most of the sugars present in lignocellulosic biomass, including xylose and produce ethanol and acetate from it. Jin et al (2011) used AFEX treated corn stover to test *C. phytofermentans* as a potential CBP organism. Under optimal fermentation conditions *C. phytofermentans* hydrolyzed 76% of glucan and 88.6% of xylan in 10 days and yielded 2.8 g/L ethanol as well as 2.6 g/L acetate.

Another group of organisms with great CBP potential is from the genus *Geobacillus*. These are thermophilic bacilli with certain species being able to ferment sugars like glucose, xylose and arabinose at temperatures of between 55 and 70^0C, producing a mixture of lactate, formate, acetate and ethanol (Barnard et al., 2010). Certain species like *Geobacillus* R7 also have the ability to produce lignocellulose-degrading enzymes including cellulases, xylanases and lignases. All the above mentioned attributes make *Geobacillus* a very good candidate for CBP, however the production of lactate and formate is not desirable. Therefore genetic engineering of these strains has been carried out at a British company, TMO Renewables Ltd, to improve ethanol production by *Geobacillus*.

3.3 Engineering cellulolytic ability into eukaryotic process organisms

The yeast *Saccharomyces cerevisiae* has long been employed for the industrial production of ethanol from hexose sugars (Kuyper et al., 2005; Nissen et al., 2000; Van Dijken et al., 2000). *S. cerevisiae* has many positive attributes which makes it suitable for industrial ethanol production such as a high rate of ethanol production from glucose (3.3 g/L/h) and its GRAS status. However this yeast species also has a number of shortcomings in terms of a CBP processing organism such as its inability to hydrolyze cellulose and hemicellulose or utilize xylose or arabinose. A number of research groups around the world have been working on improving the substrate range of *S. cerevisiae* to include the monomeric forms of sugars contained in plant biomass (Hahn-Hägerdal et al., 2001; Hahn-Hägerdal et al., 2007; Karhumaa et al., 2006; Kuyper et al., 2005). A *S. cerevisiae* strain that expressed the xylose isomerase gene from the fungus *Piromyces* sp. E2 was further metabolically engineered to allow anaerobic growth on xylose in synthetic media (Kuyper et al., 2004). Furthermore, laboratory and industrial *S. cerevisiae* strains were also engineered to co-ferment the pentose sugars xylose and arabinose (Karhumaa et al., 2006).

There have been many reports detailing the expression of one or more cellulase encoding gene(s) in *S. cerevisiae* (Van Zyl et al., 2007). Strains of *S. cerevisiae* were created that could grow on and ferment cellobiose, the main product of the action of cellobiohydrolases on cellulosic substrates, at approximately the same rate as on glucose in anaerobic conditions (van Rooyen et al., 2005). Recently the high affinity cellodextrin transport system of the model cellulolytic fungus *Neurospora crassa* was reconstituted into *S. cerevisiae* (Galazka et al., 2010). This led to the efficient growth of a recombinant strain also producing an intracellular β-glucosidase on cellodextrins up to cellotetraose. Furthermore, strains of *S. cerevisiae* were engineered to co-ferment mixtures of xylose and cellobiose, using a xylose fermenting strain that also produced a high affinity cellodextrin transporter and an intracellular β-glucosidase to hydrolyse cellobiose (Ha et al., 2011). It was shown that intracellular hydrolysis of cellobiose minimised glucose repression of xylose fermentation allowing co-consumption of cellobiose and xylose that improved ethanol yields. This was partly due to circumventing the competition between xylose and glucose for transport into the cell. Sadie et al. (2011) recently showed that expression of the gene encoding lactose permease of *Kluyveromyces lactis* (*lac12*) also facilitated transport of cellobiose into a recombinant *S. cerevisiae* strain. This report further showed the successful expression of a

Clostridium stercorarium cellobiose phosphorylase (*cepA*) that hydrolyses cellobiose and simultaneously phosphorylates one of the glucose molecules with an inorganic phosphate group yielding one glucose molecule and one glucose-1-phosphate molecule that are both further metabolised through glycolysis. Strains co-producing the heterologous CepA and Lac12 were able to grow on cellobiose as sole carbohydrate source.

There have also been reports showing co-production of cellulases specifically with the aim of enabling the organism to grow on a polymeric substrate. Cho et al. (1999) showed that for SSF experiments with a strain producing a β-glucosidase and an enzyme with exo- and endocellulase activity, loadings of externally added cellulase could be reduced. Fujita et al. (2002; 2004) reported co-expression and surface display of cellulases in *S. cerevisiae.* High cell density suspensions of a recombinant strain displaying the *Trichoderma reesei* endoglucanase II, cellobiohydrolase II, and the *Aspergillus aculeatus* β-glucosidase were able to directly convert 10 g/L phosphoric acid swollen cellulose (PASC) to approximately 3 g/L ethanol. However, growth of this strain on the cellulosic substrate was not demonstrated. An *S. cerevisiae* strain co-expressing the *T. reesei* endoglucanase 1 (*cel7B*) and the *S. fibuligera* β-glucosidase 1 (*bgl3A*) was able to grow on and convert PASC to ethanol up to 1.0 g/L (Den Haan et al., 2007b). Jeon et al. (2009) constructed a similar strain expressing the *S. fibuligera bgl3A* and the *Clostridium thermocellum cel5E* endoglucanase genes that produced significantly more endoglucanase activity than the strain reported by Den Haan et al. (2007b) and notably improved conversion of PASC to ethanol was achieved. When the processive endoglucanase Cel9A of the moderately thermophilic actinomycete *Thermobifida fusca* was functionally produced in *S. cerevisiae* growth of the strain expressing only this one cellulase encoding gene could be demonstrated on media containing PASC as sole carbohydrate source (van Wyk et al., 2010). Growth by the recombinant strain on amorphous cellulose was possible due to the sufficient amount of glucose cleaved from the cellulose chain as it was shown that the enzyme released cellobiose and glucose from cellulosic substrates in a ratio of approximately 2.5:1. In an effort to construct an engineered yeast with efficient cellulose degradation, Yamada et al. (2010) developed a method to optimize cellulase expression levels, named cocktail delta-integration. Several different cellulase expression cassettes were integrated into yeast chromosomes simultaneously in one step, and strains expressing an optimum ratio of cellulases were selected for by growth on media containing PASC as carbon source. Although the total integrated gene copy numbers of an efficient cocktail delta-integrant strain was about half that of a conventional delta-integrant strain, the PASC degradation activity (64.9 mU/g-wet cell) was higher than that of a conventional strain (57.6 mU/g-wet cell) suggesting that optimization of the cellulase expression ratio improved PASC degradation activity more than overexpression. As exoglucanase activity is required for the successful hydrolysis of crystalline cellulose, it is hypothesized that the addition of successful, high-level expression of a cellobiohydrolases to these strains will enable conversion of crystalline cellulose to ethanol. While there have been reports of successful expression of CBH encoding genes in *S. cerevisiae* the titres achieved were generally too low to allow CBP (Den Haan et al., 2007a). Recently the expression of relatively high levels of exoglucanases in *S. cerevisiae* was reported for the first time (Mcbride et al., 2010). Using these, the authors were able to construct a yeast strain that was able to convert most of the glucan available in paper sludge to ethanol. The strain was also able to displace 60% of the enzymes required to convert the sugars available in pretreated hardwood to ethanol in an SSF configuration.

Several other yeast strains have innate properties that make them attractive as possible CBP organisms (Lynd et al., 2005). The multistress tolerant yeast *Issatchenkia orientalis* was recently engineered to produce *Aspergillus aculeatus* β-glucosidase (Kitagawa et al., 2010). The transformant could convert cellobiose to ethanol under acidic conditions and at temperatures exceeding 40°C. Strains of the yeast *Kluyveromyces marxianus* can grow at temperatures as high as 52°C and have a short generation time and high growth rate at elevated temperatures (Rajoka et al., 2003). *K. marxianus* can convert a wide range of substrates, including xylose, to ethanol and successful SSF with a variety of feedstocks at elevated temperatures was demonstrated with *K. marxianus* (Fonseca et al., 2007; Fonseca et al., 2008). Thermotolerant cellobiohydrolase, endoglucanase and β-glucosidase encoding genes were expressed in combination in a strain of *K. marxianus* (Hong et al., 2007). The resulting strain was able to grow in synthetic media containing cellobiose or carboxymethylcellulose as sole carbon source but the hydrolysis of crystalline cellulose was not shown. Recently, a *K. marxianus* strain was engineered to display *T. reesei* endoglucanase II and *Aspergillus aculeatus* β-glucosidase on the cell surface (Yanase et al., 2010). This strain successfully converted 10 g/l of a cellulosic β-glucan to 4.24 g/l ethanol at 48°C within 12 h.

Some strains of the methylotrophic yeast *Hansenula polymorpha* are also able to grow at elevated temperatures up to 48°C and ferment glucose, cellobiose and xylose to ethanol (Ryabova et al., 2003). Additionally, attributes such as process hardiness and a high capacity for heterologous protein production make this yeast an attractive candidate for CBP. A recent report highlighted the promise of *H. polymorpha* in biomass conversion when strains were constructed that could ferment starch and xylan (Voronovsky et al., 2009). *Pichia stipitis* is one of the best studied xylose-fermenting yeasts and has a substrate range including all the monomeric sugars present in lignocellulose (Jeffries & Shi, 1999). Some *P. stipitis* strains produce low quantities of various cellulases and hemicellulases to break down wood into monomeric sugars although it cannot utilize polymeric cellulose as carbon source (Jeffries et al., 2007). Among the enzymes that are naturally produced are a β-glucosidase that allows the yeast to ferment cellobiose. Endoglucanases were successfully produced in *H. polymorpha* (Papendieck et al., 2002) and *P. stipitis* (Piotek et al., 1998). As these yeasts are capable of growth on cellobiose these recombinant strains should theoretically have the ability to hydrolyse amorphous cellulose although this aspect was not tested. The xylanolytic ability of *P. stipitis* was enhanced by the co-expression of xylanase and xylosidase encoding genes (Den Haan & Van Zyl, 2003). The resulting strains displayed improved biomass production on medium with birchwood glucuronoxylan as sole carbohydrate source. Even though mutant strains of *P. stipitis* with increased ethanol tolerance were recently isolated, *P. stipitis* remains a relatively poor fermentor (Watanabe et al., 2011). However, its ability to consume acetic acid and reduce the furan ring in furfural and hydroxymethylfurfural (HMF) creates an opportunity for this yeast to clean up some of the toxins in cellulosic biomass conversion (Agbogbo & Coward-Kelly, 2008). This could be very beneficial in waste water treatment.

3.4 Engineering prokaryotic organisms to hydrolyze polysaccharides

Although *Escherichia coli* cannot hydrolyze cellulose or produce ethanol at appreciable quantities it has been shown to metabolize all major sugars present in plant biomass, producing a mixture of organic acids and ethanol (Alterthum & Ingram, 1989). Bräu and Sahm (1986) successfully modified *E. coli* metabolism by expressing the *Zymomonas mobilis*

pyruvate decarboxylase at high levels. The resulting strain produced ethanol at levels comparable with *Z. mobilis*. Subsequent work has focused on improving ethanol yields, growth rate, strain stability and ethanol tolerance (Ingram et al., 1987; Ohta et al., 1991a; Ingram et al., 1991; Chen et al., 2009; Da Silva et al., 2005a; Yamano et al., 1998). Wild type *E. coli* strains are incapable of rapid growth on cellobiose (Moniruzzaman et al., 1997). *Klebsiella oxytoca* contains a phosphoenol-dependent phosphotransferase system (PTS) enabling it to utilize cellobiose. The *K. oxytoca casAB* operon coding for an enzyme $II^{cellobiose}$ and a phospho-β-glucosidase was expressed in the ethanol producing strain of *E. coli*. While expression was initially poor, spontaneous mutants were isolated which exhibited over 15-times higher specific activities for cellobiose metabolism. The best mutant produced 45 g/L ethanol - a yield of 94% of the theoretical maximum. Several endoglucanases have been expressed in *E. coli* allowing it to hydrolyze amorphous and soluble cellulose to shorter cello-oligosaccharides (Da Silva et al., 2005b; Seon et al., 2007; Srivastava et al., 1995; Wood et al., 1997; Yoo et al., 2004; Zhou et al., 2001). Among these are Cel5Z and Cel8Y from *Erwinia chrysanthemi*. Zhou et al. (2001) successfully reconstructed the type II secretion system, the predominant secretion system type in Gram negative bacteria, encoded by the *out* genes from *E. chrysanthemi*, in *E. coli*. This enabled *E. coli* to secrete more than 50% of the recombinant Cel5Z it produced. Recently, Shin et al. (2010) demonstrated a binary strategy for CBP of xylan. Two *E. coli* strains were designed to function cooperatively in the process of transforming xylan into ethanol. The first strain was engineered to co-express *axeA*, the acetylxylan esterase gene from *Streptomyces violaceoruber* and *xyl11A*, the xylanase gene from *Bacillus halodurans*. The recombinant enzymes were secreted into the growth medium by a method of *lpp* deletion with over 90% efficiency. Secreted enzymes hydrolyzed xylan into xylo-oligosaccharides, which were taken in by the second strain, designed to use the xylo-oligosaccharides for ethanol production. The second strain was based on the KO11 strain optimized for ethanol production. Into this strains the *KxynB* gene encoding β-xylosidase from *Klebsiella pneumonia* and *KxynT* encoding xyloside permease from *Klebsiella pneumoniae* were introduced. Co-cultivation of the two strains converted xylan to ethanol with a yield of about 55% of the theoretical value.

Klebsiella oxytoca is a hardy prototrophic bacterium with the ability to transport and metabolize cellobiose, cellotriose, xylobiose, xylotriose, sucrose, and all other monomeric sugars present in lignocellulosic biomass (Zhou & Ingram, 1999b). Four fermentation pathways are present in *K. oxytoca* producing formate, acetate, ethanol, lactic acid, succinate and butanediol (Ohta et al., 1991b). Through metabolic engineering and expression of the *Z. mobilis pdc* and *adhB* genes it was possible for a recombinant *K. oxytoca* strain to produce ethanol from soluble sugars at 95% of the maximum theoretical yield (Wood & Ingram, 1992). Unlike most other ethanol producing organisms *K. oxytoca* has the ability to ferment xylose and glucose at equivalent rates (Ohta et al., 1991b). This significantly shortens the time required to ferment the mixtures of glucose and xylose typically present in lignocellulosic hydrolysates. Zhou and Ingram (1999a) constructed a *K. oxytoca* strain expressing the *E. chrysanthemi cel8Y* and *cel5Z* endoglucanase genes. By also introducing the genes that encode the type II secretion system from *E. chrysanthemii*, both Cel8Y and Cel5Z were secreted effectively by *K. oxytoca.* This strain was capable of fermenting amorphous cellulose and producing a small amount of ethanol without the addition of cellulases.

Z. mobilis is a well known fermenting bacterium that produces ethanol at high rates (Zhang et al., 1997). However, *Z. mobilis* cannot ferment or utilize xylose as carbon source and it cannot hydrolyze polysaccharides. Zhang et al. (1997) engineered a *Z. mobilis* strain capable

of fermenting both xylose and arabinose, the major pentose sugars present in plant material. Co-fermentation of 100 g/L sugar (glucose:xylose:arabinose - 40:40:20) yielded a final ethanol concentration of 42 g/L in 48 hours. Brestic-Goachet et al. (1989) expressed the *E. chrysanthemi cel5Z* in *Z. mobilis*. The maximum activity obtained was 1000 IU/L with 89% of the recombinant endo-glucanase secreted to the extracellular medium. Expression of the *Ruminococcus albus* β-glucosidase enabled *Z. mobilis* to ferment cellobiose to ethanol very efficiently in two days and most of the recombinant enzyme was transported across the cytoplasmic membrane (Yanase et al., 2005). Recently, numerous strains of *Z. mobilis* were shown to possess endogenous extracellular activities against carboxymethyl cellulose (Linger et al., 2010). Furthermore, two cellulolytic enzymes, E1 and GH12 from *Acidothermus cellulolyticus*, were heterologously produced as soluble, active enzymes in *Z. mobilis*. While the E1 enzyme was less abundantly expressed, the GH12 enzyme comprised as much as 4.6% of the total cell protein. Additionally, fusing predicted secretion signals native to *Z. mobilis* to the N-termini of E1 and GH12 was found to direct the extracellular secretion of significant levels of active E1 and GH12 enzymes though a significant portion of both resided in the periplasmic space.

The thermophilic anaerobic bacterium *Thermoanaerobacterium saccharolyticum* is also under development for biomass conversion. *T. saccharolyticum* grows in a temperature range of 45 - 65°C and a pH range of 4.0 – 6.5 and is able to ferment a wide range of sugars present in cellulosic biomass including cellobiose, glucose, xylose, mannose, galactose, and arabinose (Shaw et al., 2008a). Unlike most organisms *T. saccharolyticum* metabolizes xylose and glucose essentially at the same rate (Shaw et al., 2008a; Shaw et al., 2008b) but it produces organic acids in addition to ethanol. Knockout mutants were created that produced almost exclusively ethanol from xylose. Furthermore, a strain with *hfs* and *ldh* deletions exhibited an increased ethanol yield from consumed carbohydrates and represents a new strategy for engineering increased ethanol yields in *T. saccharolyticum* (Shaw et al., 2009). *T. saccharolyticum* naturally produces both a xylanase and a β-xylosidase (Lee et al., 1993a; Lee et al., 1993b) enabling it to ferment xylan directly to ethanol. Furthermore, *T. saccharolyticum* was able to produce as much ethanol from Avicel with 4 filter paper units (FPU) of externally added enzyme as *S. cerevisiae* was with 10 FPU in SSF, the result of improved enzyme efficiency at higher temperatures (Shaw et al., 2008b). This shows the potential of this thermophile as CBP organism if a cellulolytic system can be established.

4. Integrating consolidated bioprocessing with existing bio-based industries

Although major advances have been made, the cost of second generation biofuels still remains high. Integrating cellulosic ethanol technologies with first generation bio-based and thermochemical processes helps to minimize the capital investment, maximize energy efficiency and improve overall economics (Van Zyl et al., 2011). Various biological and thermochemical processes will be discussed and their integration in a few bio-based industries highlighted.

Three thermochemical options are available for the conversion of biomass: combustion, pyrolysis, and gasification. Combustion involves burning of biomass in the presence of air, which generates hot gases at temperatures of around 800-1000°C and energy that can be harvested as heat. Pyrolysis is the conversion of biomass to liquid (bio-oil), solid (char) and gaseous fractions by heating the biomass in the absence of air to about 500°C. Bio-oils can be

upgraded to transport fuels, bio-oils and char can be gasified or used to improve soil quality. In contrast, gasification is the conversion of biomass by partial oxidation at higher temperatures (in the range of 800-900°C) to generate syngas that can be used for synthesis of different synthetic fuels (using the Fischer-Tropsch process) or burned for heat production (McKendry, 2002; Bridgwater, 2011).

In the biological process for lignocellulose hydrolysis-fermentation, large amounts of energy remain in the non-fermentable lignin-rich residues from the distillation process. Conversion of these residues through high-efficiency processes, such as a high pressure boiler coupled with a multi-stage steam turbine (Aden & Foust, 2009; Piccolo & Bezzo, 2009) can provide all the heat and electricity needed for cellulosic ethanol production, together with surplus electricity production for sale (Cardona & Sanchez, 2007; Leibbrant 2010; Reith et al., 2002). Energy consumption in the biochemical process can be reduced further by performing enzymatic hydrolysis and/or SSF processes at high substrate loadings (as typically used in high-gravity brewing), together with recycling of the process streams, both of which have substantial benefits in terms of process energy efficiency and economics (Martin et al., 2010; Wingren et al., 2003). Anaerobic digestion for wastewater treatment can be used to lower organic loadings while simultaneously producing methane-rich biogas that can be captured and used to generate electricity and/or process heating (Banerjee et al., 2009). Similarly, the integrated production of synthetic biofuels and electricity from lignocellulose in the gasification-synthesis process route will provide higher energy efficiencies than production of synfuels alone (Leibbrant 2010; Swanson et al., 2010). As an example, heat integration within biological (Aden & Foust, 2009; Kazi et al., 2010) and thermochemical routes for second generation biofuels production have the potential to increase overall energy efficiency by as much as 15% (Leibbrant 2010) and can reduce capital and operational costs substantially (Galbe et al., 2005).

4.1 Integration between lignocellulosic conversion processes and electricity production

Optimum use of cellulosic feedstocks can be achieved by integrating cellulosic ethanol processes with electricity production to achieve economies of scale and reduce capital investment per unit of electricity substantially (Easterly 2002; Hahn-Hägerdal et al., 2006; Laser et al., 2009a; Laser et al., 2009b; Sassner et al., 2008; Sims et al., 2008). Integration and scale-up of electricity and steam production can be achieved by combining feedstocks for electricity generation, such as lignin-rich residues from biological processing and using heat recovery/integration in both biofuel and electricity generation for steam production and distillation (Easterly 2002; Laser et al., 2009b; Sassner et al., 2008). By maximizing electricity production, increased revenue from second generation biofuels production and a reduction of GHG emissions from these processes can be achieved (Eriksson & Kjellström, 2010). Sharing of feedstock supply and handling infrastructure and logistics will bring about further savings.

4.2 Integration with biomass processing for pulp or sugar production

Both the sugar and pulp-and-paper industries only process biomass in part. Substantial quantities of residues from both these industries, not suitable or useful in the primary biomass processing, could be an attractive feedstock supply for the production of cellulosic ethanol. The cost of raw material and the capital costs of raw material handling adds substantially to the total production cost of cellulosic ethanol (Aden & Foust, 2009; Anex et al., 2010; Gnansounou et al., 2005; Hahn-Hägerdal et al., 2006; Kazi et al., 2010; Piccolo & Bezzo, 2009). Therefore, integrating cellulosic ethanol production from lignocellulose

residues emanating from with these industries can improve efficient use of the residues and waste streams and savings in feedstock supply and/or energy integration (Goh et al., 2010; Hahn-Hägerdal et al., 2006; Soccol et al., 2010).

Highly efficient sugar mills can liberate up to 50% of the bagasse present in cane supply as surplus (Botha & von Blottnitz, 2006). However, the availability of bagasse can be highly variable and often limiting because many conventional sugar mills are designed to dispose of bagasse residues by inefficient burning. The cost of converting sugarcane bagasse to cellulosic ethanol is therefore inevitabilty coupled to the cost of capital investments required to improve the energy efficiency. However, optimum use of energy generated in both processes for the co-production of electricity and heat could result in economic benefits (Leibbrant 2010). Swedish researchers suggested that such an integration could reduce the cost of cellulosic ethanol production with up to 20 percent in Sweden (Hahn-Hägerdal et al., 2006; von Sivers & Zacchi, 1995).

4.3 Integration of biological first and second generation biofuels production

Integration of second generation cellulosic ethanol production with first generation production from sugars or starches can have multiple benefits, including reduction of capital costs and investor risk, increased economic attractiveness and environmental acceptance (Gnansounou et al., 2005). Such integration can provide joint feedstock supply, fermentation, water and nutrient recycle, distillation, and further opportunities for energy integration (Easterly 2002; Galbe et al., 2007). Sugar-rich crops for first generation ethanol production, such as sugarcane, sweet sorghum and sugarbeet, are particularly attractive for integration with cellulosic ethanol processes that ensured optimum use of the feedstock and its logistics (Gnansounou et al., 2005; Sims et al., 2008). These crops also allow flexibility of switching between the production of crystallized sugar and ethanol, as practiced in some Brazilian sugar mills (Gnansounou et al., 2005). Further benefits of the combined fermentation-distillation processes for ethanol production from lignocellulose and sugar streams could be (i) to replace exogenous nutrient supplements (Banerjee et al., 2009), (ii) mixing of sugars from juice and lignocellulose to increase sugar concentrations and resulting ethanol levels at the end of the cellulose fermentation, and (iii) scale-up of ethanol purification/distillation to achieve economies of scale and improve energy efficiency (Soccol et al., 2010). Similar integration possibilities also exist for starches (small grains, corn, etc.), where ethanol production could be supplemented with sugars from bran (starch fibre) and polysaccharide-rich waste streams such as thin stillage (Cardona & Sanchez, 2007; Linde et al., 2010).

5. Discussion

There are several types of feedstock that can be considered viable options for the production of cellulosic biofuels. The choice of feedstock will vary between geographical areas and depend on the availability of arable land and prevailing climatic conditions and will influence downstream processes such as pre-treatment and the CBP organism used for bioconversion. To date no ideal organism has been developed for CBP conversion of biomass. Bacteria generally have a high growth rate but lack process robustness. Yeasts are often sufficiently robust, but lack substrate range. Filamentous fungi often have a wide substrate range, but grow relatively slowly and do not produce enough of a desirable product. While the advantages of using the yeasts *P. stipitis*, *K. marxianus* and *H. polymorpha*

are well appreciated, the engineered cellulolytic ability of these strains are currently rudimentary. None of the strains are as yet capable of utilizing crystalline cellulose and the high level production of an exocellulase remains a requirement. New information on secretion pathways, chaperones and metabolic engineering should help alleviate this problem in future. The *S. cerevisiae* strain developed by the Mascoma Corporation represents the best CBP organism engineered thus far as this strain could convert several cellulosic substrates to ethanol with addition of minimal exogenous enzymes in an SSF configuration (Mcbride et al., 2010). Compared to *S. cerevisiae*, all of the bacterial species discussed above are relatively sensitive to inhibitors associated with lignocellulosic hydrolysates (Bothast et al., 1999; Yamano et al., 1998; Ohta et al., 1991b). Engineering enhanced protein secretion allowed the successful secretion of endoglucanases in *E. coli* (Ji et al., 2009) and *K. oxytoca* (Zhou & Ingram, 2001). *E. coli* and *K. oxytoca* strains capable of breaking down cellulose could also be modified to produce other commodity products such as lactic acid, succinic acid, acetic acid or 2, 3-butanediol (Ji et al., 2009). The *Geobacillus* strain used by TMO Renewables Ltd. is capable of producing ethanol at appreciable titers from pretreated lignocellulosic feedstock and represents a very promising organism for CBP.

Candidate CBP organisms are in various stages of development for establishment of a cellulolytic system or improvement of product forming attributes. It is likely that more than one organism may eventually be used in various biomass conversion processes and the choice may depend on the sugar composition of the feedstock, the pretreatment method used and the end product required. The cost disadvantage of current second-generation biofuels configurations may be partially addressed through innovative methods of process integration, in order to minimize capital investment and maximize energy efficiency and improve overall economics. Integration of second generation biofuel production processes into existing first generation biofuel production or into other biomass based industries with integration strategies to ensure optimal energy usage and synergy may be the most effective way to bring second generation biofuels to market.

6. References

Aden, A. & Foust, T. (2009). Technoeconomic analysis of the dilute sulfuric acid and enzymatic hydrolysis process for the conversion of corn stover to ethanol. *Cellulose*, Vol.16, (2009), pp. 535-545

Agbogbo, F.K. & Coward-Kelly, G. (2008). Cellulosic ethanol production using the naturally occurring xylose-fermenting yeast, *Pichia stipitis*. *Biotechnology Letters*, Vol.30, No.9, (September 2008), pp. 1515-1524

Alfenore, S.; Molina-Jouve, C.; Guillouet, S. E.; Uribelarrea, J.-L.; Goma, G. & Benbadis, L. (2002). Improving ethanol production and viability of *Saccharomyces cerevisiae* by a vitamin feeding strategy during fed-batch process. *Applied Microbiology and Biotechnology*, Vol.60, (2002), pp. 67-72

Alterthum, F. & Ingram, L. O. (1989). Efficient ethanol production from glucose, lactose, and xylose by recombinant *Escherichia coli*. *Applied and Environmental Microbiology*, Vol.55, (1989), pp. 1943-1948

Anasontzis, G.E.; Zerva, A.; Stathopoulou, P. M.; Haralampidis, K.; Diallinas, G.; Karagouni, A. D. & Hatzinikolaou, D. G. (2011). Homologous overexpression of xylanase in *Fusarium oxysporum* increases ethanol productivity during consolidated

bioprocessing (CBP) of lignocellulosics. *Journal of Biotechnology*, Vol.152, No.1-2, (March 2011), pp. 16-23

Anex, R.P.; Aden, A.; Kazi, F. K.; Fortman, J.; Swanson, R. M.; Wright, M. M.; Satrio, J. A.; Brown, R. C.; Daugaard, D. E.; Platon, A.; Kothandaraman, G.; Hsu, D. D. & Dutta, A. (2010). Techno-economic comparison of biomass-to-transportation fuels via pyrolysis, gasification, and biochemical pathways. *Fuel*, Vol.89, (2010), pp. S29-S35

Ansah, T.; Osafo, E. L. K. & Hasen, H. H. (2010). Herbage yield and chemical composition of four varieties of Napier (*Pennisetum purpureum*) grass harvested at three different days after planting. *Agriculture and Biology Journal of North America*, Vol.1, No.5, (2010), pp. 923-929

Banerjee, S.; Mudliar, S.; Sen, R. & Giri, B. (2009). Commercializing lignocellulosic bioethanol: technology bottlenecks and possible remedies. *Biofuels, Bioproducts and Biorefining*, Vol.4, (2009), pp. 77-93

Barnard, D.; Casanueva, A.; Tuffin, M. & Cowan, D. (2010). Extremophiles in biofuel synthesis. *Environmental Technology*, Vol.31, No.8, (2010), pp. 871-888

Botha, T. & von Blottnitz, H. (2006). A comparison of the environmental benefits of bagasse-derived electricity and fuel ethanol on a life-cycle basis. *Energy Policy*, Vol.34, No.17, (November 2006), pp. 2654-2661

Bothast, R.J.; Nichols, N. N. & Dien, B. S. (1999). Fermentations with New Recombinant Organisms. *Biotechnology Progress*, Vol.15, (1999), pp. 867-875

Bräu, B. & Sahm, H. (1986). Cloning and expression of the structural gene for pyruvate decarboxylase of *Zymomonas mobilis* in *Escherichia coli*. *Archives of Microbiology*, Vol.144, (1986), pp. 296-301

Brestic-Goachet, N.; Gunasekaran, P.; Cami, B. & Baratti, J. C. (1989). Transfer and expression of an *Erwinia chrysanthemi* cellulase gene in *Zymomonas mobilis*. *J.Gen.Microbiol.*, Vol.135, (January 1989), pp. 893-902

Bridgwater, A.V. (2011). Review of fast pyrolysis of biomass and product upgrading. *Biomass and Bioenergy*, [Corrected Proof]

Cardona, C.A. & Sanchez, O. J. (2007). Fuel ethanol production: Process design trends and integration opportunities. *Bioresource Technology*, Vol.98, (2007), pp. 2415-2457

Chambergo, F.S.; Bonaccorsi, E. D.; Ferreira, A. J.; Ramos, A. S.; Ferreira, J., Jr.; Abrahao-Neto, J.; Farah, J. P. & El Dorry, H. (2002). Elucidation of the metabolic fate of glucose in the filamentous fungus *Trichoderma reesei* using expressed sequence tag (EST) analysis and cDNA microarrays. *Journal of Biological Chemistry*, Vol.277, No.16, (April 2002), pp. 13983-13988

Chen, J.; Zhang, W.; Tan, L.; Wang, Y. & He, G. (2009). Optimization of metablolic pathways for bioconversion of lignocellulose to ethanol through genetic engineering. *Biotechnology Advances*, Vol.27, (2009), pp. 593-598

Cherry, J.R. & Fidantsef, A. L. (2003). Directed evolution of industrial enzymes: an update. *Current Opinion in Biotechnology*, Vol.14, No.4, (August 2003), pp. 438-443

Cho, K.M.; Yoo, Y. J. & Kang, H. S. (1999). δ-Integration of endo/exo-glucanase and β-glucosidase genes into the yeast chromosomes for direct conversion of cellulose to ethanol. *Enzyme and Microbial Technology*, Vol.25, No.1-2, (July 1999), pp. 23-30

Da Silva, G.P.; De Araujo, E. F.; Silva, D. & Guimaraes, W. V. (2005a). Ethanolic fermentation of sucrose, sugarcane juice and molasses by *Escherichia coli* strain KO11 and

Klebsiella oxytoca strain P2. *Brazilian Journal of Microbiology*, Vol.36, (2005a), pp. 395-404

Da Silva, G.P.; De Araujo, E. F.; Silva, D. & Guimaraes, W. V. 2005b). Ethanolic fermentation of sucrose, sugarcane juice and molasses by *Escherichia coli* strain KO11 and *Klebsiella oxytoca* strain P2. *Brazilian Journal of Microbiology*, Vol.36, (May 2005b), pp. 395-404

Davis, S.C.; Dohleman, F. G. & Long, S. P. (2011). The global potential of *Agave* as a biofuel feedstock. *GCB Bioenergy*, Vol.3, (2011), pp. 68-78

Decree No.6.961. (2009). Zoning agricultural land of sugarcane. Diário Oficial União de 18.9.10, Brazil. (17 September 2009)

Den Haan, R.; McBride, J. E.; La Grange, D. C.; Lynd, L. R. & Van Zyl, W. H. (2007a). Functional expression of cellobiohydrolases in *Saccharomyces cerevisiae* towards one-step conversion of cellulose to ethanol. *Enzyme and Microbial Technology*, Vol.40, (2007a), pp. 1291-1299

Den Haan, R.; Rose, S. H.; Lynd, L. R. & Van Zyl, W. H. (2007b). Hydrolysis and fermentation of amorphous cellulose by recombinant *Saccharomyces cerevisiae*. *Metabolic Engineering*, Vol.9, No.1, (January 2007b), pp. 87-94

Den Haan, R. & Van Zyl, W. H. (2003). Enhanced xylan degradation and utilisation by *Pichia stipitis* overproducing fungal xylanolytic enzymes. *Enzyme and Microbial Technology*, Vol.33, (October 2003), pp. 620-628

Easterly,J. (2002) *AES Greenidge Bioethanol Co-Location Assessment: Final Report*. National Renewable Energy Laboratory Report NREL/SR-510-33001,

Eriksson, G. & Kjellström, B. (2010). Assessment of combined heat and power (CHP) integrated with wood-based ethanol production. *Applied Energy*, Vol.87, (2010), pp. 3632-3641

Fierobe, H.P.; Pages, S.; Belaich, A.; Champ, S.; Lexa, D. & Belaich, J. P. (1999). Cellulosome from *Clostridium cellulolyticum*: Molecular study of the dockerin/cohesin interaction. *Biochemistry*, Vol.38, No.39, (1999), pp. 12822-12832

Fonseca, G.G.; Bombert, A. K.; Heinzle, E. & Wittmann, C. (2007). Physiology of the yeast *Kluyveromyces marxianus* during batch and chemostat cultures with glucose as the sole carbon source. *FEMS Yeast Research*, Vol.7, No.3, (2007), pp. 422-435

Fonseca, G.G.; Heinzle, E.; Wittmann, C. & Gombert, A. K. (2008). The yeast *Kluyveromyces marxianus* and its biotechnological potential. *Applied Microbiology and Biotechnology*, Vol.79, No.3, (June 2008), pp. 339-354

Fortman, J.L.; Chhabra, S.; Mukhopadhyay, A.; Chou, H.; Lee, T. S.; Steen, E. & Keasling, J. D. (2008). Biofuel alternative to ethanol: pumping the microbial well. *Trends in Biotechnology*, Vol.26, No.7, (2008), pp. 375-381

Fujita, Y.; Ito, J.; Ueda, M.; Fukuda, H. & Kondo, A. (2004). Synergistic saccharification, and direct fermentation to ethanol, of amorphous cellulose by use of an engineered yeast strain codisplaying three types of cellulolytic enzyme. *Applied and Environmetal Microbiology*, Vol.70, (February 2004), pp. 1207-1212

Fujita, Y.; Takahashi, S.; Ueda, M.; Tanaka, A.; Okada, H.; Morikawa, Y.; Kawaguchi, T.; Arai, M.; Fukuda, H. & Kondo, A. (2002). Direct and efficient production of ethanol from cellulosic material with a yeast strain displaying cellulolytic enzymes. *Applied and Environmetal Microbiology*, Vol.68, No.10, (October 2002), pp. 5136-5141

Galazka, J.M.; Tian, C.; Beeson, W. T.; Martinez, B.; Glass, N. L. & Cate, J. H. (2010). Cellodextrin transport in yeast for improved biofuel production. *Science*, Vol.330, No.6000, (October 2010), pp. 84-86

Galbe, M.; Liden, G. & Zacchi, G. (2005). Production of ethanol from biomass - research in Sweden. *Journal of Scientific and Industrial Research*, Vol.64, (2005), pp. 905-919

Galbe, M.; Sassner, P.; Wingren, A. & Zacchi, G. (2007). Process Engineering Economics of Bioethanol Production. *Advances in Biochemical Engineering and Biotechnology*, Vol.108, (2007), pp. 303-327

Girio, F.M.; Fonseca, C.; Carvalheiro, F.; Duarte, L. C.; Marques, S. & Bogel-Lukasik, R. (2010). Hemicelluloses for fuel ethanol: A review. *Bioresource Technology*, Vol.101, No.13, (July 2010), pp. 4775-4800

Gnansounou, E.; Dauriat, A. & Wyman, C. E. (2005). Refining sweet sorghum to ethanol and sugar: economic trade-offs in the context of North China. *Bioresource Technology*, Vol.96, (2005), pp. 985-1002

Goh, C.S.; Tan, K. T.; Lee, K. T. & Bhatia, S. (2010). Bio-ethanol from lignocellulose: Status, perspectives and challenges in Malaysia. *Bioresource Technology*, Vol.101, No.13, (July 2010), pp. 4834-4841

Goodale, C.L.; Apps, M. J.; Birdsey, R. A.; Field, C. B.; Heath, L. S.; Houghton, R. A.; Jenkins, J. C.; Kohlmaier, G. H.; Kurz, W.; Liu, S.; Nabuurs, G. J.; Nilsson, S. & hvidenko, A. Z. (2002). Forest carbon sinks in the northern hemisphere. *Ecological Applications*, Vol.12, No.3, (June 2002), pp. 891-899, ISSN 1051-0761

Ha, S.J.; Galazka, J. M.; Rin, K. S.; Choi, J. H.; Yang, X.; Seo, J. H.; Louise, G. N.; Cate, J. H. & Jin, Y. S. (2011). Engineered *Saccharomyces cerevisiae* capable of simultaneous cellobiose and xylose fermentation. *Proceeding of the National Academy of Sciences*, Vol.108, No.2, (January 2011), pp. 504-509

Hahn-Hägerdal, B.; Galbe, M.; Gorwa-Grauslund, M. F.; Lidén, G. & Zacchi, G. (2006). Bio-ethanol - the fuel of tomorrow from the residues of today. *Trends in Biotechnology*, Vol.24, No.12, (December 2006), pp. 549-556

Hahn-Hägerdal, B.; Karhumaa, K.; Fonseca, C.; Spencer-Martins, I. & Gorwa-Grauslund, M. F. (2007). Towards industrial pentose-fermenting yeast strains. *Applied Microbiology and Biotechnology*, Vol.74, No.5, (April 2007), pp. 937-953

Hahn-Hägerdal, B.; Wahlbom, C. F.; Gardonyi, M.; Van Zyl, W. H.; Cordero, O. R. & Jonsson, L. J. (2001). Metabolic engineering of *Saccharomyces cerevisiae* for xylose utilization. *Advances in Biochemical Engineering and Biotechnology*, Vol.73, (2001), pp. 53-84

Hamelinck, C.N.; van Hooijdonk, G. & Faaij, A. P. C. (2005). Ethanol from lignocellulosic biomass: techno-economic performance in short-, middle- and long-term. *Biomass and Bioenergy*, Vol.28, (2005), pp. 384-410

Hill, J.; Nelson, E.; Tilman, D.; Polasky, S. & Tiffany, D. (2006). Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels. *Proceedings of the National Academy of Sciences USA*, Vol.103, No.30, (July 2006), pp. 11206-11210

Hong, J.; Wang, Y.; Kumagai, H. & Tamaki, H. (2007). Construction of thermotolerant yeast expressing thermostable cellulase genes. *Journal of Biotechnology*, Vol.130, No.2, (June 2007), pp. 114-123

Ibrahim, A.S.S. & El-diwany, A. (2007). Isolation and identification of new cellulases producing Thermophilic Bacteria from an Egyptian hot spring and some properties

of the crude enzyme. *Australian Journal of Basic and Applied Sciences*, Vol.1, No.4, (2007), pp. 473-478

Ingram, L. O., Conway, T., and Alterthum, F. Ethanol production by *Escherichia coli* strains co-expressing *Zymomonas* PDC and ADH genes. Filed: 15 May 1989, Published 19 March 1991 [WO 5000000]

Ingram, L.O.; Conway, T.; Clark, D. P.; Sewell, G. W. & Preston, J. F. (1987). Genetic engineering of ethanol production in *Escherichia coli*. *Applied and Environmental Microbiology*, Vol.53, No.10, (1987), pp. 2420-2425

Jeffries, T. & Shi, N. Q. (1999). Genetic Engineering for Improved Xylose Fermentation by Yeasts. *Advances in Biochemical Engineering and Biotechnology*, Vol.65, (1999), pp. 117-161

Jeffries, T.W.; Grigoriev, I. V.; Grimwood, J.; Laplaza, J. M.; Aerts, A.; Salamov, A.; Schmutz, J.; Lindquist, E.; Dehal, P.; Shapiro, H.; Jin, Y. S.; Passoth, V. & Richardson, P. M. (2007). Genome sequence of the lignocellulose-bioconverting and xylose-fermenting yeast *Pichia stipitis*. *Nature Biotechnology*, Vol.25, No.3, (March 2007), pp. 319-326

Jeon, E.; Hyeon, J. E.; Suh, D. J.; Suh, Y. W.; Kim, S. W.; Song, K. H. & Han, S. O. (2009). Production of cellulosic ethanol in *Saccharomyces cerevisiae* heterologous expressing *Clostridium thermocellum* endoglucanase and *Saccharomycopsis fibuligera* beta-glucosidase genes. *Molecular Cells*, Vol.28, No.4, (October 2009), pp. 369-373

Ji, X.J.; Huang, H.; Zhu, J. G.; Ren, L. J.; Nie, Z. K.; Du, J. & Li, S. (2009). Engineering *Klebsiella oxytoca* for efficient 2, 3-butanediol production through insertional inactivation of acetaldehyde dehydrogenase gene. *Applied Microbiology and Biotechnology*, Vol.85, No.6, (2009), pp. 1751-1758

Jin, M.; Balan, V.; Gunawan, C. & Dale, B. E. (2011). Consolidated bioprocessing (CBP) performance of *Clostridium phytofermentans* on AFEX-treated corn stover fro ethanol production. *Biotechnology and Bioengineering*, (2011

Karhumaa, K.; Wiedemann, B.; Hahn-Hägerdal, B.; Boles, E. & Gorwa-Grauslund, M.-F. (2006). Co-utilization of L-arabinose and D-xylose by laboratory and industrial *Saccharomyces cerevisiae* strains. *Microbial Cell Factories*, Vol.5, (2006a), pp. 1-11

Kazi, F.K.; Fortman, J. A.; Anex, R. P.; Hsu, D. D.; Aden, A.; Dutta, A. & Kothandaraman, G. (2010). Techno-economic comparison of process technologies for biochemical ethanol production from corn stover. *Fuel*, Vol.89, (2010), pp. S20-S28

Kitagawa, T.; Tokuhiro, K.; Sugiyama, H.; Kohda, K.; Isono, N.; Hisamatsu, M.; Takahashi, H. & Imaeda, T. (2010). Construction of a beta-glucosidase expression system using the multistress-tolerant yeast *Issatchenkia orientalis*. *Applied Microbiology and Biotechnology*, Vol.87, No.5, (August 2010), pp. 1841-1853

Kuyper, M.; Hartog, M. M.; Toirkens, M. J.; Almering, M. J.; Winkler, A. A.; Van Dijken, J. P. & Pronk, J. T. (2005). Metabolic engineering of a xylose-isomerase-expressing *Saccharomyces cerevisiae* strain for rapid anaerobic xylose fermentation. *FEMS Yeast Research*, Vol.5, No.4-5, (February 2005), pp. 399-409

Kuyper, M.; Winkler, A. A.; Van Dijken, J. P. & Pronk, J. T. (2004). Minimal metabolic engineering of *Saccharomyces cerevisiae* for efficient anaerobic xylose fermentation: a proof of principle. *FEMS Yeast Research*, Vol.4, (2004), pp. 655-664

La Grange, D.C. (2007). Bioethanol as renewable transportation fuel for the future. MBA thesis, University of Stellenbosch, Stellenbosch, Available from

http://hdl.handle.net/10019.1/837

La Grange, D.C.; Den Haan, R. & Van Zyl, W. H. (2010). Engineering cellulolytic ability into bioprocessing organisms. *Applied Microbiology and Biotechnology*, Vol.87, No.4, (May 2010), pp. 1195-1208

Lamed, R.; Setter, E. & Bayer, E. A. (1983a). Characterization of a cellulose-binding, cellulase-containing complex in *Clostridium thermocellum*. *Journal of Bacteriology*, Vol.156, (1983a), pp. 828-836

Lamed, R.; Setter, E.; Kenig, R. & Bayer, E. A. (1983b). The cellulosome — a discrete cell surface organelle of *Clostridium thermocellum* which exhibits separate antigenic, cellulose-binding and various cellulolytic activities. *Biotechnology and Bioengineering Symposia*, Vol.13, (1983b), pp. 163-181

Laser, M.; Jin, H.; Jayawardhana, K.; Dale, B. E. & Lynd, L. R. (2009a). Coproduction of ethanol and power from switchgrass. *Biofuels, Bioproducts and Biorefining*, Vol.3, (2009a), pp. 195-218

Laser, M.; Jin, H.; Jayawardhana, K.; Dale, B. E. & Lynd, L. R. (2009b). Projected mature technology scenarios for conversion of cellulosic biomass to ethanol with coproduction thermochemical fuels, power, and/or animal feed protein. *Biofuels, Bioproducts and Biorefining*, Vol.3, (2009b), pp. 231-246

Lee, Y.E.; Lowe, S. E.; Henrissat, B. & Zeikus, J. G. (1993a). Characterization of the active site and thermostability regions of endoxylanse from *Thermoanaerobacterium saccharolyticum* B6A-RI. *Journal of Bacteriology*, Vol.175, No.18, (1993a), pp. 5890-5898

Lee, Y.E.; Lowe, S. E. & Zeikus, J. G. (1993b). Regulation and characterization of xylanolytic enzymes of *Thermoanaerobacterium saccharolyticum* B6A-RI. *Applied and Environmental Microbiology*, Vol.59, (1993b), pp. 763-771

Leibbrant,N. (2010) *Techno-economic study for sugarcane bagasse to liquid biofuels in South Africa: A comparison between biological and thermochemical process routes.* PhD dissertation, Stellenbosch University, South Africa, April 2010

Linde, M.; Galbe, M. & Zacchi, G. (2010). Bioethanol production from non-starch carbohydrate residues in process streams from a dry-mill ethanol plant. *Bioresource Technology*, Vol.99, (2010), pp. 6505-6511

Linger, J.G.; Adney, W. S. & Darzins, A. (2010). Heterologous expression and extracellular secretion of cellulolytic enzymes by *Zymomonas mobilis*. *Applied and Environmetal Microbiology*, Vol.76, No.19, (October 2010), pp. 6360-6369

Lynd, L.R.; Van Zyl, W. H.; McBride, J. E. & Laser, M. (2005). Consolidated bioprocessing of cellulosic biomass: an update. *Current Opinion in Biotechnology*, Vol.16, (2005), pp. 577-583

Lynd, L.R.; Weimer, P. J.; Van Zyl, W. H. & Pretorius, I. S. (2002). Microbial cellulose utilization: fundamentals and biotechnology. *Microbiology and Molecular Biology Reviews*, Vol.66, (September 2002a), pp. 506-577

Martin, M., Ahmetovic, E., and Grossmann, I. E. Optimization of Water Consumption in Second Generation Bioethanol Plants. *Industrial & Engineering Chemistry Research* [In Press]

Mcbride, J., Brevnova, E., Ghandi, C., Mellon, M., Froehlich, A., Deleault, K., Rajgharia, V., Flatt, J, Van Zyl, E, Den Haan, R., La Grange, D., Rose, S., Penttila, M., Ilmen, M., Siika-aho, M., Uusitalo, J., Hau, H., Rice, C., Villari, J., Stonehouse, E., Gilbert, A., Keating, J., Xu, H., Willes, D., Shikhare, I., Thorngren, N., Warner, A., and Murphy,

D. Yeast expressing cellulases for simultaneous saccharification and fermentation using cellulose. Filed :23 November 2009, Published: 27 May 2010. [WO 2010/060056]

McKendry, P. (2002). Energy production from biomass (part 2): conversion technologies. *Bioresource Technology*, Vol.83, No.1, (May 2002), pp. 47-54

Moniruzzaman, M.; Lai, X.; York, S. W. & Ingram, L. O. (1997). Isolation and molecular characterization of high-performance cellobiose-fermenting spontaneous mutants of ethanologenic *Escherichia coli* KO11 containing the *Klebsiella oxytoca casAB* Operon. *Applied and Environmental Microbiology*, Vol.63, No.12, (1997), pp. 4633-4637

Nissen, T.L.; Kielland-Brandt, M. C.; Nielsen, J. & Villadsen, J. (2000). Optimization of ethanol production in *Saccharomyces cerevisiae* by metabolic engineering of the ammonium assimilation. *Metabolic Engineering*, Vol.2, (2000), pp. 69-77

Ohta, K.; Beall, D. S.; Mejia, J. P.; Shanmugam, K. T. & Ingram, L. O. (1991a). Genetic improvement of *Escherichia coli* for ethanol production: Chromosomal integration of *Zymomonas mobilis* genes encoding pyruvate decarboxylase and alcohol dehydrogenase. *Applied and Environmental Microbiology*, Vol.57, No.4, (1991a), pp. 893-900

Ohta, K.; Mejia, J. P.; Shanmugam, K. T. & Ingram, L. O. (1991b). Metabolic engineering of *Klebsiella oxytoca* M5A1 for ethanol production from xylose and glucose. *Applied and Environmental Microbiology*, Vol.57, No.10, (1991b), pp. 2810-2815

Panagiotou, G.; Christakopoulos, P. & Olsson, L. (2005). Simultaneous saccharification and fermentation of cellulose by *Fusarium oxysporum* F3--growth characteristics and metabolite profiling. *Enzyme and Microbial Technology*, Vol.36, No.5-6, (April 2005), pp. 693-699

Papendieck,A., Dahlems, U., & Gellissen, G. (2002) Technical enzyme production and whole-cell biocatalysis: application of *Hansenula polymorpha*. In: *Hansenula polymorpha: biology and applications,* Gellissen,G. (Ed), pp. 255-271. Wiley-VCH, Weinham

Piccolo, C. & Bezzo, F. (2009). A techno-economic comparison between two technologies for bioethanol production from lignocellulose. *Biomass and Bioenergy*, Vol.33, (2009), pp. 478-491

Piotek, M.; Hagedorn, J.; Hollenberg, C. P.; Gellissen, G. & Srasser, A. W. M. (1998). Two novel gene expression systems based on the yeasts *Schwanniomyces occidentalis* and *Pichia stipitis*. *Applied Microbiology and Biotechnology*, Vol.50, (1998), pp. 331-338

Rajoka, M.I.; Khan, S. & Shahid, R. (2003). Kinetics and regulation studies of the production of β-galactosidase from *Kluyveromyces marxianus* grown on different substrates. *Food Technology and Biotechnology*, Vol.41, No.4, (2003), pp. 315-320

Rautio, J.J.; Smit, B. A.; Wiebe, M.; Penttila, M. & Saloheimo, M. (2006). Transcriptional monitoring of steady state and effects of anaerobic phases in chemostat cultures of the filamentous fungus Trichoderma reesei. *BMC Genomics*, Vol.7, (2006), pp. 247

Reith,J.H., den Uil, H., de Laat, W. T. A. M., Niessen, J. J., de Jong, E., Elbersen, H. W., Weusthuis, R., Van Dijken, J. P., & Raamsdonk, L. (2002) *Co-production of bio-ethanol, electricity and heat from biomass residues*. Energy Research Centre, Netherlands, Report ECN-RX-02-030, July 2002.,

Richard, T.L. (2010). Challenges in scaling up biofuels infrastructure. *Science*, Vol.329, No.5993, (August 2010), pp. 793-796

Robinson,J. (2006) *Bio-ethanol as a household cooking fuel: A mini pilot study of the SuperBlu stove in peri-urban Malawi.* Loughborough University, UK

Ryabova, O.B.; Chmil, O. M. & Sibirny, A. A. (2003). Xylose and cellobiose fermentation to ethanol by the thermotolerant methylotrophic yeast *Hansenula polymorpha*. *FEMS Yeast Researh*, Vol.4, No.2, (November 2003), pp. 157-164

Sadie, C.J.; Rose, S. H.; Den Haan, R. & Van Zyl, W. H. (2011). Co-expression of a cellobiose phosphorylase and lactose permease enables intracellular cellobiose utilisation by Saccharomyces cerevisiae. *Applied Microbiology and Biotechnology*, [In press]

Sassner, P.; Galbe, M. & Zacchi, G. (2008). Techno-economic evaluation of bioethanol production from three different lignocellulosic materials. *Biomass Bioenergy*, Vol.32, (2008), pp. 422-430

Sastri, B., Lee, A., Kaarsberg, T., Alfstad, T., Curtis, M., and MacNeil, D. (2008). World biofuels production potential, 02.03.2011,Available from http://www.pi.energy.gov/documents/20080915_WorldBiofuelsProductionPotential.pdf

Seon, P.J.; Russell, J. B. & Wilson, D. B. (2007). Characterization of a family 45 glycosyl hydrolase from *Fibrobacter succinogenes* S85. *Anaerobe*, Vol.13, No.2, (April 2007), pp. 83-88

Shaw, A.J.; Hogsett, D. A. & Lynd, L. R. (2009). Identification of the [FeFe]-hydrogenase responsible for hydrogen generation in *Thermoanaerobacterium saccharolyticum* and demonstration of increased ethanol yield via hydrogenase knockout. *Journal of Bacteriology*, Vol.191, No.20, (October 2009), pp. 6457-6464

Shaw, A.J.; Jenney, F. E.; Adams, M. W. W. & Lynd, L. R. (2008a). End-product pathways in the xylose fermenting bacterium, *Thermoanaerobacterium saccharolyticum*. *Enzyme and Microbial Technology*, Vol.42, (2008a), pp. 453-458

Shaw, A.J.; Podkaminer, K. K.; Desai, S. G.; Bardsley, J. S.; Rogers, S. R.; Thorne, P. G.; Hogsett, D. A. & Lynd, L. R. (2008b). Metabolic engineering of a thermophilic bacterium to produce ethanol at high yield. *Proceedings of the National Academy of Sciences USA*, Vol.105, No.37, (2008b), pp. 13769-13774

Shin, H.D.; McClendon, S.; Vo, T. & Chen, R. R. (2010). *Escherichia coli* binary culture engineered for direct fermentation of hemicellulose to a biofuel. *Applied and Environmetal Microbiology*, Vol.76, No.24, (December 2010), pp. 8150-8159

Sims, R., Taylor, M., Saddler, J., and Mabee, W. (2008). From 1st- to 2nd-generation biofuel technologies - An overview of current industry and RD&D activities. International Energy Agency (IEA Bioenergy).

Soccol, C.R.; Vandenberghe, L. P. d. S.; Medeiros, A. B. P.; Karp, S. G.; Buckeridge, M.; Ramos, L. P.; Pitarelo, A. P.; Ferreira-Leitão, V.; Gottschalk, L. M. F.; Ferrara, M. A.; Bon, E. P. d. S.; de Moraes, L. M. P.; Araújo, J. d. A. & Torres, F. A. G. (2010). Bioethanol from lignocelluloses: Status and perspectives in Brazil. *Bioresource Technology*, Vol.101, (2010), pp. 4820-4825

Somerville, C.; Youngs, H.; Taylor, C.; Davis, S. C. & Long, S. P. (2010). Feedstocks for lignocellulosic biofuels. *Science*, Vol.329, (2010), pp. 790-792

Srivastava, R.; Kumar, G. P. & Srivastava, K. K. (1995). Construction of a recombinant cellulolytic *Escherichia coli*. *Gene*, Vol.164, (1995), pp. 185-186

Stephanopoulos, G. (2007). Challenges in engineering microbes for biofuels production. *Science*, Vol.315, (2007), pp. 801-804

Sun, Y. & Cheng, J. (2002). Hydrolysis of lignocellulosic material for ethanol production: a review. *Bioresource Technology*, Vol.83, (2002), pp. 1-11

Swanson, R.M.; Platon, A.; Satrio, J. A. & Brown, R. C. (2010). Techno-economic analysis of biomass-to-liquids production based on gasification. *Fuel*, Vol.89, (2010), pp. S11-S19

Van Dijken, J.P.; Bauer, J.; Brambilla, L.; Duboc, P.; Francois, J. M.; Gancedo, C.; Giuseppin, M. L.; Heijnen, J. J.; Hoare, M.; Lange, H. C.; Madden, E. A.; Niederberger, P.; Nielsen, J.; Parrou, J. L.; Petit, T.; Porro, D.; Reuss, M.; van Riel, N.; Rizzi, M.; Steensma, H. Y.; Verrips, C. T.; Vindelov, J. & Pronk, J. T. (2000). An interlaboratory comparison of physiological and genetic properties of four *Saccharomyces cerevisiae* strains. *Enzyme and Microbial Technology*, Vol.26, (June 2000), pp. 706-714

van Rooyen, R.; Hahn-Hagerdal, B.; La Grange, D. C. & Van Zyl, W. H. (2005). Construction of cellobiose-growing and fermenting *Saccharomyces cerevisiae* strains. *Journal of Biotechnology*, Vol.120, No.3, (November 2005), pp. 284-295

van Wyk, N.; Den Haan, R. & Van Zyl, W. H. (2010). Heterologous co-production of *Thermobifida fusca* Cel9A with other cellulases in *Saccharomyces cerevisiae*. *Applied Microbiology and Biotechnology*, Vol.87, No.5, (May 2010), pp. 1813-1820

Van Zyl, W.H.; Chimphango, A. F. A.; Den Haan, R.; Görgens, J. F. & Chirwa, P. W. C. (2011). Next-generation cellulosic ethanol technologies and their contribution to a sustainable Africa. *Interface Focus*, Vol.1, No.2, (April 2011), pp. 196-211

Van Zyl, W.H.; Lynd, L. R.; Den Haan, R. & McBride, J. E. (2007). Consolidated Bioprocessing for Bioethanol Production Using *Saccharomyces cerevisiae*. *Advances in Biochemical Engineering Biotechnology*, Vol.108, (2007), pp. 205-235

von Sivers, M. & Zacchi, G. (1995). A techno-economical comparison of three processes for the production of ethanol from pine. *Bioresource Technology*, Vol.51, No.1, (1995), pp. 43-52

Voronovsky, A.Y.; Rohulya, O. V.; Abbas, C. A. & Sibirny, A. A. (2009). Development of strains of the thermotolerant yeast *Hansenula polymorpha* capable of alcoholic fermentation of starch and xylan. *Metabolic Engineering*, Vol.11, (2009), pp. 234-242

Watanabe, T.; Watanabe, I.; Yamamoto, M.; Ando, A. & Nakamura, T. (2011). A UV-induced mutant of Pichia stipitis with increased ethanol production from xylose and selection of a spontaneous mutant with increased ethanol tolerance. *Bioresource Technology*, Vol.102, No.2, (January 2011), pp. 1844-1848

Watson, H.K. (2011). Potential to expand sustainable bioenergy from sugarcane in southern Africa. *Energy Policy*, [Corrected Proof]

Wingren, A.; Galbe, M. & Zacchi, G. (2003). Techno-Economic Evaluation of Producing Ethanol from Softwood: Comparison of SSF and SHF and Identification of Bottlenecks. *Biotechnology Progress*, Vol.19, (2003), pp. 1109-1117

Wood, B.E.; Beall, D. S. & Ingram, L. O. (1997). Production of recombinant bacterial endoglucanase as a co-product with ethanol during fermentation using derivitives of Escherichia coli KO11. *Biotechology and Bioengineering*, Vol.55, No.3, (1997), pp. 547-555

Wood, B.E. & Ingram, L. O. (1992). Ethanol production from cellobiose, amorphous cellulose, and crystalline cellulose by recombinant *Klebsiella oxytoca* containing chromosomally integrated *Zymomonas mobilis* genes for ethanol production and

plasmids expressing thermostable cellulase genes from *Clostridium thermocellum*. *Applied and Environmental Microbiology*, Vol.58, No.7, (1992), pp. 2103-2110

Xiros, C.; Topakas, E.; Katapodis, P. & Christakopoulos, P. (2008). Evaluation of *Fusarium oxysporum* as an enzyme factory for the hydrolysis of brewer's spent grain with improved biodegradability for ethanol production. *Industiral Crop Production*, Vol.28, No.2, (September 2008), pp. 213-224

Xu, C.; Qin, Y.; Li, Y.; Ji, Y.; Huang, J. & Song, H. (2010). Factors influencing cellulosome activity in Consolidated Bioprocessing of cellulosic ethanol. *Bioresource Technology,* Vol.101, No.9560, (2010), pp. 9569

Xu, Q.; Singh, A. & Himmel, M. E. (2009). Perspectives and new directions for the production of bioethanol using consolidated bioprocessing of lignocellulose. *Current Opinion in Biotechnology*, Vol.20, No.3, (June 2009), pp. 364-371

Yamada, R.; Taniguchi, N.; Tanaka, T.; Ogino, C.; Fukuda, H. & Kondo, A. (2010). Cocktail δ-integration: A novel method to construct cellulolytic enzyme expression ratio-optimized yeast strains. *Microbial Cell Factories*, Vol.9, (May 2010), pp. 32

Yamano, L.P.; York, S. W. & Ingram, L. O. (1998). Isolation and characterization of ethanol-tolerant mutants of *Escherichia coli* KO11 for fuel ethanol production. *Journal of Industrial Microbiology & Biotechnology*, Vol.20, (1998), pp. 132-138

Yanase, H.; Nozaki, K. & Okamoto, K. (2005). Ethanol production from cellulosic materials by genetically engineered *Zymomonas mobilis*. *Biotechnology Letters*, Vol.27, (2005), pp. 259-263

Yanase, S.; Hasunuma, T.; Yamada, R.; Tanaka, T.; Ogino, C.; Fukuda, H. & Kondo, A. (2010). Direct ethanol production from cellulosic materials at high temperature using the thermotolerant yeast *Kluyveromyces marxianus* displaying cellulolytic enzymes. *Applied Microbiology and Biotechnology*, Vol.88, No.1, (September 2010), pp. 381-388

Yoo, J.S.; Jung, Y. J.; Chung, S. Y.; Lee, Y. C. & Choi, Y. L. (2004). Molecular cloning and characterization of CMCase gene (*celC*) from *Salmonella typhimurium* UR. *Journal Microbiology*, Vol.42, No.3, (September 2004), pp. 205-210

Zaldivar, J.; Nielsen, J. & Olsson, L. (2001). Fuel ethanol production from lignocellulose: a challenge for metabolic engineering and process integration. *Applied Microbiology and Biotechnology*, Vol.56, (2001), pp. 17-34

Zhang, M., Chou, Y. C., Picataggio, S. K., and Finkelstein, M. (1997) Single *Zymomonas mobilis* strain for xylose and arabinose fermentation. Filed:6 May 1997, Published: 1 December 1998 [WO 5843760]

Zhang, Y.H. & Lynd, L. R. (2004). Toward an aggregated understanding of enzymatic hydrolysis of cellulose: noncomplexed cellulase systems. *Biotechnology and Bioengineering*, Vol.88, No.7, (December 2004), pp. 797-824

Zhou, S.; Davis, F. C. & Ingram, L. O. (2001). Gene integration and expression and extracellular secretion of *Erwinia chrysanthemi* endoglucanase CelY (*celY*) and CelZ (*celZ*) in ethanologenic *Klebsiella oxytoca* P2. *Applied and Environmetal Microbiology*, Vol.67, No.1, (January 2001), pp. 6-14

Zhou, S. & Ingram, L. (1999a). Engineering endoglucanase-secreting strains of ethanologenic *Klebsiella oxytoca* P2. *Journal of Industrial Microbiology and Biotechnology*, Vol.22, No.6, (June 1999a), pp. 600-607

Zhou, S. & Ingram, L. O. (1999b). Engineering endoglucanase-secreting strains of ethanologenic *Klebsiella oxytoca* P2. *Journal of Industrial Microbiology and Biotechnology*, Vol.22, (1999b), pp. 600-607

Zhou, S. & Ingram, L. O. (2001). Simultaneous saccharification and fermentation of amorphous cellulose to ethanol by recombinant *Klebsiella oxytoca* SZ21 without supplemental cellulase. *Biotechnology Letters*, Vol.23, (January 2001), pp. 1455-1462
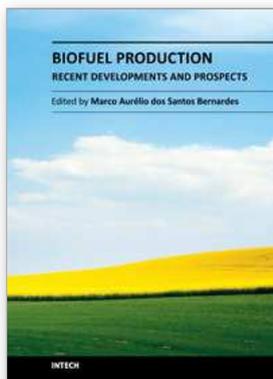
Biofuel Production-Recent Developments and Prospects

Edited by Dr. Marco Aurelio Dos Santos Bernardes

ISBN 978-953-307-478-8

Hard cover, 596 pages

Publisher InTech

Published online 15, September, 2011

Published in print edition September, 2011

This book aspires to be a comprehensive summary of current biofuels issues and thereby contribute to the understanding of this important topic. Readers will find themes including biofuels development efforts, their implications for the food industry, current and future biofuels crops, the successful Brazilian ethanol program, insights of the first, second, third and fourth biofuel generations, advanced biofuel production techniques, related waste treatment, emissions and environmental impacts, water consumption, produced allergens and toxins. Additionally, the biofuel policy discussion is expected to be continuing in the foreseeable future and the reading of the biofuels features dealt with in this book, are recommended for anyone interested in understanding this diverse and developing theme.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Willem H. van Zyl, Riaan den Haan and Daniel C. la Grange (2011). Developing Organisms for Consolidated Bioprocessing of Biomass to Ethanol, Biofuel Production-Recent Developments and Prospects, Dr. Marco Aurelio Dos Santos Bernardes (Ed.), ISBN: 978-953-307-478-8, InTech, Available from: http://www.intechopen.com/books/biofuel-production-recent-developments-and-prospects/developing-organisms-for-consolidated-bioprocessing-of-biomass-to-ethanol

INTECH
open science | open minds