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Review Paper

Advances in consolidated bioprocessing systems for bioethanol and butanol production from biomass: a comprehensive review

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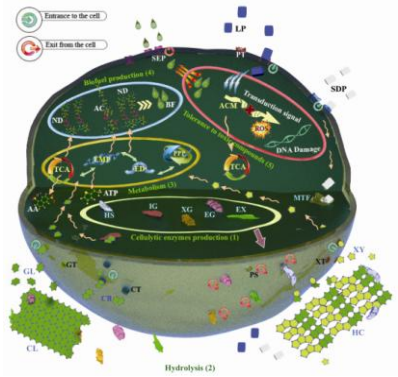
HIGHLIGHTS

- Various CBP strategies have been discussed.
- High-throughput techniques to explore novel microorganisms and powerful enzymes for CBP have been explained.
- Recent advances and challenges faced in CBP for efficient bioalcohols production from biomass have been comprehensively reviewed.

GRAPHICAL ABSTRACT



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ABSTRACT

Recently, lignocellulosic biomass as the most abundant renewable resource has been widely considered for bioalcohols production. However, the complex structure of lignocelluloses requires a multi-step process which is costly and time consuming. Although, several bioprocessing approaches have been developed for pretreatment, saccharification and fermentation, bioalcohols production from lignocelluloses is still limited because of the economic infeasibility of these technologies. This cost constraint could be overcome by designing and constructing robust cellulolytic and bioalcohols producing microbes and by using them in a consolidated bioprocessing (CBP) system. This paper comprehensively reviews potentials, recent advances and challenges faced in CBP systems for efficient bioalcohols (ethanol and butanol) production from lignocellulosic and starchy biomass. The CBP strategies include using native single strains with cellulytic and alcohol production activities, microbial co-cultures containing both cellulytic and ethanologenic microorganisms, and genetic engineering of cellulytic microorganisms to be alcohol-producing or alcohol producing microorganisms to be cellulytic. Moreover, high-throughput techniques, such as metagenomics, metatranscriptomics, next generation sequencing and synthetic biology developed to explore novel microorganisms and powerful enzymes with high activity, thermostability and pH stability are also discussed. Currently, the CBP technology is in its infant stage, and ideal microorganisms and/or conditions at industrial scale are yet to be introduced. So, it is essential to bring into attention all barriers faced and take advantage of all the experiences gained to achieve a high-yield and low-cost CBP process.

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Abbreviations

ABE	Acetone, butanol and ethanol	Lac	Laccase
ACE	Allele-coupled exchange technology	Ldh	Lactate dehydrogenase
ACS	Acetyl-CoA synthetase	NADH	Nicotinamide adenine dinucleotide
ADH	Alcohol dehydrogenase	NSSF	Non-isothermal simultaneous saccharification and fermentation
AFEX	Ammonia fiber expansion pretreatment	OCC	Old corrugated containers
AFEX™-CS	Corn stover pretreated via ammonia fiber expansion	OPEFB	Oil palm empty fruit bunch
ATF	*Agave tequilana* fructans	PASC	Phosphoric acid swollen cellulose
ALD	Acetaldehyde dehydrogenase	Pdc	Pyruvate decarboxylase
ATMT	*A. tumefaciens* -mediated transformation	PEG	Polyethylene glycol
BGL	β-glucosidase	Pfl	Pyruvate formate lyase
BMR (bmr)	Brown mid-rib	Pta	Phosphotransacetylase
CBH	Cellobiohydrolase	SHCF	Separate hydrolysis and co-fermentation
CBP	Consolidated bioprocessing	SHF	Separate hydrolysis and fermentation
Cel	Cellulase	SMW	Spent mushroom waste
Cep	Cellobiose phosphorylase	SSCF	Simultaneous saccharification and co-fermentation
CHE	Combined hydrogen and ethanol production	SSF	Simultaneous saccharification and fermentation
CipC	Noncatalytic cellulosome integrating protein	SSFF	Simultaneous saccharification, filtration and fermentation
CMC	Carboxymethyl cellulose	w/v	Wight/Volume
COMT	Caffeic acid 3-O-methyltransferase	WT	Wild-type
Da	Dalton	XDH	Xylitol dehydrogenase
EG	Endoglucanase	XI	Xylose isomerase
EMP	Embden-Meyerhof-Parnas pathway	*Xk*	Xylulose kinase
ER	endoplasmic reticulum	Xln	β-xylosidase
FB-SSF	Fed-batch simultaneous saccharification and fermentation	XR	Xylose reductase
FP	Filter paper	Xyn	Endoxylanase
g/l	Gram/liter	$Y_{E/s}$	Yield coefficient
g/lh	Gram/liter per hour	Δldh	Mutated lactate dehydrogenase
g/g	Gram/gram	Δpta	Mutated phosphotransacetylase
GlcNAc	N-Acetylglucosamine		

1. Introduction: Bioalcohols from lignocellulosic biomass, challenges and problems

The recent rapid increase in overall awareness concerning environmental threats, global demands for energy and the depleting energy resources have pushed researchers toward finding new alternative, cleaner, renewable and sustainable energy resources, such as solar energy, hydroelectric energy, wind energy, and biomass-derived energy (Chu and Majumdar, 2012). Plant lignocellulosic biomass is the most abundant renewable resource on the earth which is produced at an approximate rate of 150–170 × 10^9 tons annually (Pauly and Keegstra 2008). Commonly, lignocellulosic biomass is obtained from four major sources, including agricultural residues (corn stover, rice straw, etc.), forest residues (woods, branches, foliage, etc.), energy crops (switch grass, yellow poplar, etc.), and cellulosic waste, such as municipal solid waste and food waste (Parisuthan et al., 2014; Sims et al., 2010). It has therefore been an attractive substrate for sustainable production of second-generation bioalcohols such as ethanol, butanol. It is worth quoting that the first-generation biofuels, produced from food crops such as cereals, sugar crops, and oil seeds have been seriously criticized for triggering food vs. fuel competition and the consequent increases in food price.

Lignocellulosic biomass typically contains 50-80% complex carbohydrates consisting of C_5 and C_6 sugar units (**Fig. 1**). This complexity is a challenge for bioalcohols production from lignocelluloses. In addition, the presence of lignin and the crystalline structure of cellulose result in biomass recalcitrance which requires effective pretreatment methods to open up the structure making it more accessible to the enzymes. Therefore, this robust and complex structure in order to be converted into bioalcohols requires a multi-step process, including pretreatment, enzymatic hydrolysis, and fermentation and that increases the cost of biofuels production significantly (Mosier et al., 2005; Klein-Marcuschamer et al., 2012; Kumagai et al., 2014). Although several bioprocessing approaches have been proposed, the main technological hurdle in conversion of this valuable renewable resource into bioalcohols is still the lack of cost effective technologies for pretreatment, saccharification and fermentation to overcome the biomass recalcitrance. The most costly process during alcohol production from biomass is probably enzymatic hydrolysis of lignocelluloses. To degrade them to fermentable glucose, cooperative and synergistic activities of at least three cellulases, including endoglucanase, exoglucanase and β-glucosidase are required (Lynd et al., 2002; Ho et al., 2012).

Moreover, lignocelluloses are obtained from highly diverse environments and hence their composition varies from place to place and over time, so, it is necessary to develop and integrate efficient pretreatment, hydrolysis and fermentation processes, and also to utilize efficient microbes capable of handling effective conversion of different kinds of biomass (Balat et al., 2009; Rumbold et al., 2010; Favaro et al., 2013; Parisuthan et al., 2014; Ragauskas et al., 2014).

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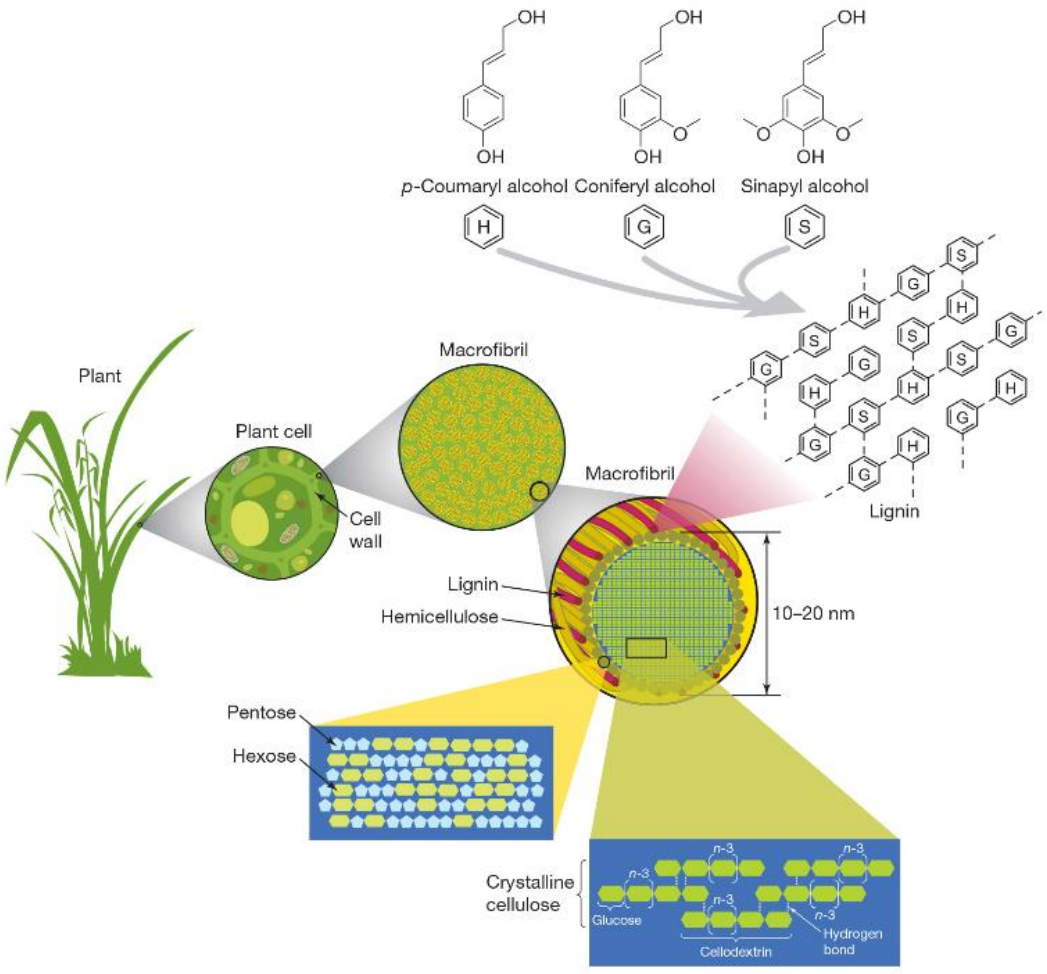

Fig.1. Structure of lignocellulosic biomass containing cellulose (composed of a β-1,4-linked chain of glucose molecules), hemicellulose (composed of various 5- and 6-carbon sugars such as arabinose, galactose, glucose, mannose and xylose) and lignin (composed of three major phenolic components, namely p-coumaryl alcohol (H), coniferyl alcohol (G) and sinapyl alcohol (S)) (Rubin, 2008), Copyright (2015), with permission from Elsevier.

2. New strategies to overcome the problems of conversion of lignocelluloses to bioalcohols

Commonly, the process for bioalcohols production from lignocellulose starts with a thermo-chemical or physical pretreatment to hydrolyze the hemicellulose fraction of biomass, and is then continued by an enzymatic hydrolysis of the cellulose fraction, and finally fermentation of the resulting sugars is performed by an alcohol-producing microorganism. All these steps are costly and time consuming, and so new developing technologies are focused on ways to increase the efficiency of these three steps while striving to reduce their corresponding costs.

2.1. New pretreatment technologies

It is estimated that about 18–20% of the total projected cost for biofuel production from lignocellulosic materials can be attributed to pretreatment. This step is carried out to overcome chemical and physical obstacles in their complex structure and to enhance enzyme accessibility, which finally results in increased fermentable sugars yields (Yang and Wyman, 2008). During a pretreatment procedure, hydrogen bonds in cellulose are disrupted, cross-linked matrix of hemicelluloses and lignin are broken down, and finally, the porosity and surface area of cellulose are increased for subsequent enzymatic hydrolysis (Taherzadeh and Karimi, 2008; Li et al., 2010; Haghighi Mood et al., 2013, 2014; Rajendran and Taherzadeh, 2014). Previously, several pretreatment technologies, such as chemical pretreatment (alkali, acid, organosolv, ozonolysis and ionic liquids), physical pretreatment (grinding and milling, microwave and extrusion), physico-chemical pretreatment (steam explosion, liquid hot water, ammonia fiber explosion, wet oxidation and CO_2 explosion) and biological pretreatment (using microorganisms) have been developed (Palmqvist et al., 2000; Isroi et al., 2011; Haghighi Mood et al., 2013, 2014; Rajendran and Taherzadeh, 2014).

Although each method has some advantages, none could be recommended for all types of biomass. In another word, each pretreatment method has some drawbacks limiting its application. Recently, different combined methodologies have also been developed to overcome the problems associated with individual methods. Utilization of these methods resulted in enhanced efficiency of sugar production, decreased formation of inhibitors and finally shortened process time. These combined pretreatment strategies include combination of alkaline and dilute acid pretreatments (Lu et al., 2009), alkaline and ionic liquid pretreatments (Nguyen et al., 2010), dilute acid and steam explosion pretreatments (Chen et al., 2011), supercritical CO_2 and steam explosion pretreatments (Alinia et al., 2010), organosolv and biological pretreatments (Monrroy et al., 2010), biological and dilute acid pretreatments (Zhang et al., 2007 a,b), biological and steam explosion pretreatments (Taniguchi et al., 2010), microwave-assisted alkali pretreatment (Zhu et al., 2006), microwave-assisted dilute acid pretreatments (Chen et al.,

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2011) and ionic liquids and ultrasonic pretreatments (Ninomiya et al., 2010).

In addition, various recently developed "omics" tools, such as synthetic biology, high throughput sequencing and genetic engineering are also considered as promising tools to enhance the efficiency of pretreatment and hydrolysis procedures for economic biofuel production from biomass. These new promising strategies include (1) increasing cellulose composition (Coleman et al., 2009), (2) reducing plant cell wall recalcitrance and cellulose crystallinity (Fry et al., 2008), (3) producing cellulases and other protein modules which are necessary for disruption of plant cell wall substrates (Vaaje-Kolstad et al., 2005; Biswas et al., 2006; Cosgrove et al., 2007; Mei et al., 2009; Park et al., 2011), and (4) reducing lignin content in plants by down-regulation of lignin biosynthesis (Ralph et al., 2006) or diverting lignin biosynthesis towards cellulose synthesis (Chen and Dixon, 2007; Haghighi Mood et al., 2013).

It is expected that by development of these new pretreatment technologies, pretreatment problems, as one of the costliest steps of lignocelluloses conversion to bioalcohols, will be overcome in the near future. Finally, it can be concluded that for efficient conversion of lignocellulosic biomass to biofuels, achieving an in-depth understanding of the concepts of pretreatment technologies (single or combined) and also the types and composition of available biomass feedstock is essential.

2.2. Integrating enzymatic saccharification and fermentation processes

During the bioalcohol production from lignocellulosic biomass, in addition to pretreatment, both enzymatic saccharification (hydrolysis) and fermentation processes are also the key determinants. In the conventional bioalcohol production process, saccharification and fermentation processes are separately performed (separate hydrolysis and fermentation (SHF) which is a time consuming and costly processes) (**Fig. 2**). The major advantage of SHF is that both hydrolysis and fermentation could be performed at their own optimum conditions. While, the main drawback of this process is the inhibition of cellulase activity by the sugars released in the hydrolysis stage (Tengborg et al., 2001; Goshadrou et al., 2013; Ishola et al., 2013).

2.2.1. Simultaneous saccharification and fermentation (SSF)

Simultaneous saccharification and fermentation (SSF) (**Fig. 2**) is a strategy for increasing cellulose conversion to bioalcohol, in which the enzymatic hydrolysis and fermentation of sugars are combined. In this strategy, the enzyme consumption is maximized since the soluble sugar levels do not reach levels that might inhibit the fermentation microorganisms (Brethauer and Wyman, 2010). Previously, it has been confirmed that the overall ethanol yield in SSF is generally higher than that of the SHF (Wingren et al., 2003). The advantage of the SSF is that the hydrolysis products such as glucose and short cellulose oligomers do not inhibit cellulase activities due to immediate and simultaneous fermentation (Lin and Tanaka, 2006). Some studies have reported successful application of the SSF technology for simultaneous saccharification and fermentation of different energy crops, non edible plants and lignocellulosic biomass by different microorganisms, and confirmed its efficiency. For the first time, South et al. (1993) developed continuous conversion of pretreated hardwood flour to ethanol using *Saccharomyces cerevisiae* in combination with cellulase enzymes and direct microbial conversion with the cellulose-fermenting strain *C. thermocellum*. Fan et al. (2003) developed a semi-continuous SSF system for efficient conversion of paper sludge to ethanol. They managed to achieve an average conversion of 92% and 42 g/l ethanol when 82 g/l cellulose and 20 FPU g/l enzymes were loaded. Moshi et al. (2014) established a fed-batch simultaneous saccharification and fermentation (FB-SSF) approach in order to overcome the inhibition of *S. cerevisiae* by the high sugar concentrations produced after an efficient enzymatic hydrolysis of starch-rich wild cassava. Also, Kumagai et al. (2014) reported the development of an efficient SSF process for production of ethanol from steam-pretreated and subsequently wet-disk milled *Hinoki cypress* and Eucalyptus. In another study, Li et al. (2013) using SSF system for bioethanol production from a brown macroalgae (*Saccharina japonica*) whose carbohydrates contained up to 55% laminarin and mannitol, could achieve a bioethanol concentration of 6.65 g/l and a yield of 67.41% based on the total available glucan in the pretreated *S. japonica*.

The main disadvantage of SSF is that the optimum temperature for cellulases activity (45–60 °C) is commonly higher that the temperatures suitable for the activity of yeast and many bacterial biofuel fermentations (Brethauer and Wyman, 2010; Bhalla et al., 2013; Kumagai et al., 2014).

2.2.2. Non-isothermal simultaneous saccharification and fermentation (NSSF)

In the SSF process, the resultant glucose by the hydrolyzing enzymes is immediately consumed by the bioalcohol-producing microorganisms thus, the inhibition effects of cellubiose and glucose are minimized. As mentioned earlier, the major problem associated with the SSF process is the difference between the optimum temperatures of hydrolyzing enzymes and fermenting microorganisms (Wyman, 1996; Goshadrou et al., 2013). In this system, the enzymatic hydrolysis is performed at a temperature lower than the optimum temperature, which could significantly affect enzyme activity, and therefore, could cause increased enzyme consumption (Taherzadeh and Karimi, 2007).

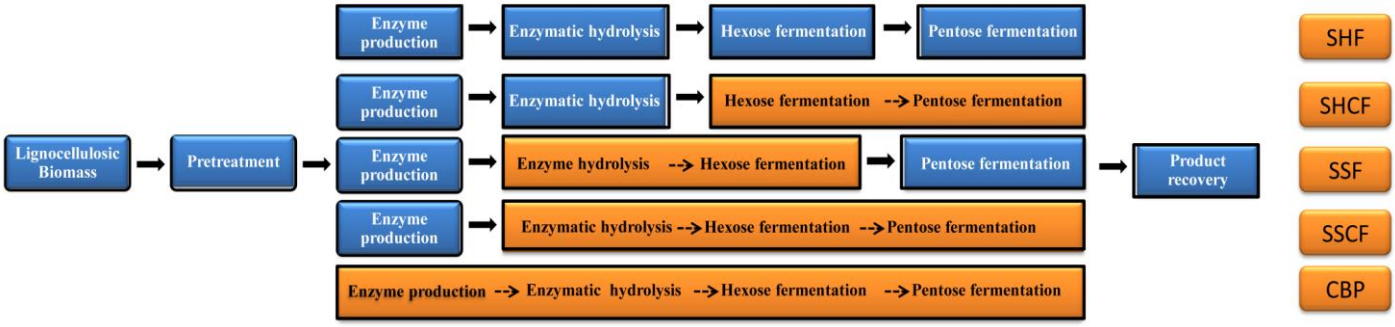

Fig.2. Different bioprocessing strategies available for the conversion of lignocellulosic biomass to bioalcohols. Abbreviations: SHF, separate hydrolysis and fermentation; SHCF, separate hydrolysis and co-fermentation; SSF, simultaneous saccharification and fermentation; SSCF, simultaneous saccharification and co-fermentation; CBP, consolidated bioprocessing.

To overcome this problem, non-isothermal simultaneous saccharification and fermentation (NSSF) has been suggested, in which enzymatic hydrolysis process is incompletely carried out at optimum temperature and after inoculating the media, temperature is set to optimum value for the microorganisms (Wu and Lee, 1998; Goshadrou et al., 2013). In this system, saccharification and fermentation are performed simultaneously but in two separate reactors at different temperatures. The pretreated lignocellulosic materials are transferred to a hydrolysis reactor in which the enzymatic hydrolysis is carried out at the optimum temperature (e.g. 50°C), then the hydrolyzed effluent is recirculated through a fermentor running at optimum temperature for alcohol-producing microorganisms (e.g. 30°C). It has been confirmed that cellulase activity would increase by up to 2-3 times when the hydrolysis temperature was raised from 30 to 50°C, and the total enzyme requirements were reduced by 30-40% compared to the conditions employed in the SSF (Taherzadeh and Karimi, 2007).

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NSSF has been efficiently used for bioethanol production from different lignocellulosic biomass, including corn stover (Varga et al. 2004), eucalyptus (Tamayo et al., 2014), spen wood (Goshadrou et al., 2013), Solka Floc® powdered cellulose, old corrugated cardboard (OCC), and paper sludge (Kadar et al., 2004), softwood spruce and hardwood oak (Shafiei et al., 2011), untreated and fungal-pretreated oil palm empty fruit bunch (OPEFB), untreated and fungal-pretreated oat straw (Millati et al., 2014). The majority of these studies showed that the NSSF is advantageous compared to the SSF.

2.2.3. Simultaneous saccharification, filtration, and fermentation (SSFF)

Recently, one new technology named "Simultaneous Saccharification, Filtration and Fermentation (SSFF)" has been developed for lignocellulosic ethanol production (Ishola et al., 2013). In SSFF, pretreated lignocellulosic slurry is exposed to the enzymes and hydrolyzed in a reactor, while the sugar rich suspension is continuously pumped through a cross-flow membrane to the fermentation system. Fermented liquid is pumped back to the hydrolysis vessel, while a clear sugar-rich filtrate continuously perfuses through the fermentation vessel before it is pumped back to the hydrolysis vessel, and a culture of a flocculating strain of *S. cerevisiae* is retained by settling. By using this system, an ethanol yield of 85% of the theoretical yield was obtained and a flocculating strain of *S. cerevisiae* was successfully reused for 5 cultivations. SSFF seems to be an advantageous alternative to both SHF and SSF since the problem of enzyme inhibition can be avoided. In addition, both the enzyme cocktail and the fermenting organism in SSFF can be used at their different optimal conditions. Furthermore, it would be possible to reuse the fermenting organism several times (Ishola et al., 2013).

2.2.4. Simultaneous saccharification and co-fermentation (SSCF)

During the process of bioalcohol production from lignocelluloses, in addition to the yield, the alcohol concentration is also very important, as the distillation costs decrease when the final alcohol concentration increases (Sassner et al., 2008). One disadvantage associated with the SSF is that the fermentation is performed using only hexoses, and pentose sugars are not used. To increase the ethanol concentration, a high concentration of water-insoluble substrates should be added to the fermentation system, which leads to a high viscosity of the medium. High viscosity may cause mixing problems during the fermentation process, and also may lead to a high concentration of fermentation inhibitors (Ameida et al., 2007). One of the methodologies used to increase ethanol concentration is fed-batch SSF process. In this methodology, the medium`s viscosity is maintained low, by gradually feeding new substrates to the reactor. Furthermore, the effect of the hydrolysate toxicity is decreased because of yeast adaptation and gradual biological detoxification. This system may also have positive effects on xylose uptake owing to the significant changes in xylose to glucose concentration ratio in the medium (Hodge et al., 2009; Olofsson et al., 2009, 2010; Zhang et al., 2009).

Generally, glucose concentration negatively affects xylose uptake in *S. cerevisiae*, and therefore, must be kpt low for achieving an efficient xylose uptake. Low concentration of glucose increases xylose to glucose ratio which is desirable for co-fermentation of glucose and xylose (Kotter et al., 1993; Meiamder et al., 1999). Clearlly, in lignocellulosic ethanol production, in addition to hexose fermentation, pentose fermentation is also an unavoidable part of the process due to the high xylan content of the lignocellulosic materials. However, ethanol concentration in pentose fermentation process is usually too low (<10 g/l), and therefore, is not economic to be distilled (Yasuda et al., 2014). Thus, co-fermentation of hexose and pentose has been performed using a variety of wild type and recombinant microorganisms for ethanol production from different lignocellulosic materials. Simultaneous saccharification and co-fermentation (SSCF) (**Fig. 2**) is a process similar to SSF except that the hexose and pentose fermentations occur in one step and pot. SSCF system is a promising technology to reduce total costs of alcohol production, as pentoses are also consumed during the process, and also the inhibitory effects of xylose are reduced (Zhang et al., 2010). The main advantage of SSCF in comparison with the separate hydrolysis and co-fermentation (SHCF) is that the released glucose is simultaneously fermented, resulting in a low glucose concentration in the medium. This can minimize the end product inhibition during enzymatic hydrolysis, and also increase the xylose- to glucose concentration ratio diverting fermenting microorganism to consume xylose (Olofsson et al., 2010a,b). Previously, this technology has been used for production of both ethanol (e.g. Teixeira et al., 1999, 2000; Zhang et al., 2009; Kang et al., 2010) and lactic acid (e.g. Patel et al., 2005; Zhu et al., 2007). **Table 1** shows a brief history of SSCF for bioethanol production from different lignocellulosic materials by different wild type or genetically-engineered microorganisms.

2.3. Consolidated bioprocessing(CBP)

Depolymerizing lignocellulosic biomass to simple sugars is a complex multi-step process, including loosening the structural complexity of lignocelluloses, pectin hydrolysis, lignin degradation, hemicellulose depolymerization and cellulose hydrolysis (Mazzoli et al., 2012), and the cost of feedstock, enzyme, and pretreatment would account for about two-third of the total production cost, of which the enzyme cost is the largest (Himmel et al., 2007). This cost constraint could be overcome by designing and constructing robust cellulolytic and bioalcohols-producing microbes and by using them in a consolidated bioprocessing (CBP) system (Parisutham et al., 2014). CBP has been known as the most promising fermentation approach for bioethanol production from lignocellulosic biomass and has been investigated increasingly in recent years (**Fig. 2**). Nonetheless, CBP is still in its early stage of establishment, and hence, is the main focus of this review. In CBP, all the processes, including enzyme production, enzymatic saccharification, and fermentation of the resulting sugars to bioethanol or other valuable products proceed simultaneously (Lynd et al., 2005; Olson et al., 2012; Kumagai et al., 2014; Parisutham et al., 2014). During the last decade, several wild-type and genetically-engineered bacteria, fungi and yeasts have been proposed for application in CBP (Schuster and Chinn, 2013). In the next sections, the potential microorganisms and their suitable characteristics for CBP alcohol production will be discussed.

2.3.1. Advantages of CBP

It is suggested that CBP as a promising approach will circumvent the cost and restrictions of conventional workflow for biofuel production from lignocellulosic biomass. CBP technologies using a single organism or consortium of microorganisms combine the enzyme production, hydrolysis, and fermentation stages into a single step. This may enhance processing efficiencies, eliminating the need for added exogenous hydrolytic enzymes and reducing the sugar inhibition of cellulases (Lynd et al., 2005; Olson et al., 2012). CBP is a promising technology which can reduce the number of unit operations, and also reduce the overall capital cost of the process (Olson et al., 2010; 2012). Commonly, the final simple sugars inhibit saccharification process in the conventional systems, whereas in CBP, fermentation transforms these products to biofuel before they become inhibitive to hydrolysis (Dashtban et al., 2009).CBP microorganisms do not need to exogenous saccharifying enzymes, as they produce their own cellulolytic and hemicellulolytic enzymes for lignocellulose decomposition, which result in large cost savings (Lu et al., 2006). CBP systems significantly reduce the number of unit operations (i.e., fewer reactor vessels), and therefore reduce maintenance and capital costs (Xu et al., 2009; den Haan et al., 2015). In addition, if the effective microorganisms will be found, pretreatment step could be avoided partially or entirely (Olson et al., 2012; Schuster and Chinn, 2013).

2.3.2. Strategies to design ideal microorganisms for CBP

An ideal microorganism for CBP should simultaneously and with high efficiency produce required hydrolases and transform simple sugars to target alcohol (**Fig. 3**). These microorganisms should have specific traits, including expression and secretion of several glycoside hydrolase enzymes for rapid depolymerization of lignocellulose, simultaneous utilization and conversion of multiple sugars like cellobiose, glucose and xylose to biofuels, and tolerance both to toxic compounds derived from lignin and the final end product (**Fig. 3**) (Vinuselvi et al., 2011; Hasunuma and Kondo, 2012; Kricka et al., 2014; Kumagai et al., 2014; Parisutham et al., 2014). The enzymes needed to make the complete cellulolytic cocktail for the saccharification of lignocellulosic biomass are cellulases (cellobiohydrolase, endoglucanase, β-glucosidase and phospho-β-glucosidase), hemicellulases (endoxylanase,

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Table 1.
A brief history of SSCF for bioethanol production from different lignocellulosic materials by different microorganisms.

Type of Biomass	Pretreatment	Microorganism	References
Bermuda grass and napiergrass	Pressurized batch hot water	*E. coli*	Brandon et al. 2011
Kraft paper mill sludges	-		Kang et al., 2010, 2011
Barley hull	Aqueous ammonia		Kim et al., 2008
Sugarcane bagasse	Phosphoric Acid		Geddes et al., 2011; Nieves et al., 2011
Eucalyptus	Steam		Mullinnix, 2014
Taiwanese chenopod	Sulfite pretreatment and acid explosion		Yang et al., 2014
Kelp slag	Diluted acid		Jin et al., 2014
Corn stover	AFEX™	*S. cerevisiae*	Jin et al. 2012a, 2013
Corn stover	Steam		Ohgren et al. 2006
Birch	Steam		Wang et al., 2014a
Napiegrass	Low-moisture anhydrous ammonia		Yasuda et al., 2014
Corn stover	Aqueous ammonia		Zhu et al., 2014
Wheat straw	Diluted acid		Fonseca et al., 2011
Sugar cane Bagasse	Peracetic acid		Teixeira et al., 1999
Switch grass	Ammonia Fiber Expansion		Jin et al., 2010
Furfural residues and corn kernels	-		Tang et al., 2011
Waste paper sludge	-		Zhang (J) et al., 2009, 2010
Corn stover	Aqueous ammonia		Kim and Lee, 2005, 2007
Rice straw	Butanone and acetaldehyde		Zhang et al., 2012b
Sweet sorghum bagasse	NaOH		Yu et al., 2014
Wheat straw	Laccase and steam-exploding		Moreno et al., 2013
Wheat straw	Steam		Erdei et al., 2013a,b; Ballesteros et al., 2013
Sugar cane and Sorghum bagasse	Dilute acid		Geddes et al., 2013
Wheat straw	Microfluidizer and solid loading		Turhan et al., 2014
Lodgepole pine	Sulfite		Lan et al., 2013
Kappaphycus alvarezii	Diluted acid		Hargreaves et al., 2013
Wheat straw	Steam		Alvira et al., 2011
Wheat straw	Diluted Acid		Olofsson et al., 2010a,b
Corn cob	NaoH	*Zymomonas mobilis*	Su et al. 2013
Yellow poplar	Dilute-acid		McMillan et al., 1999
Waste paper sludge	-		Zhang et al., 2010
Rice straw	-	*S. cerevisiae* and *Scheffersomyces stipitis*	Suriyachai et al., 2013
Rice straw	Diluted acid	*Candida tropicalis*	Oberoi et al., 2010
Rice straw	Steam or butanone exploding	*S. cerevisiae* and *Candida shehatae*	Zhang et al., 2014
Poplar wood	Steam	*E. coli* and *S. cerevisiae*	Chena et al., 2011
Wild sugar cane	Aqueous ammonia	*S. cerevisiae* and *Pichia stipitis*	Chandel et al., 2010
Microcrystaline cellulose and cellobiose	-	*Klebsiella oxytoca* with *S. pastorianus*, *Kluyveromyces marxianus* or *Z. mobilis*	Golias et al., 2002
Rice hull	Diluted acid	*S. cerevisiae* and *Spathaspora arborariae*	Hickert et al., 2013

b-xylosidase, acetyl xylan esterase, glucuronyl esterase, arabinofuranosidase, galactosidase, glucuronidase, mannanase and xyloglucan hydrolase), pectinolytic enzymes (polygalacturonases, pectin/pectate lyases and pectin methyl esterase), lignin degradation (lignin peroxidase, aryl-alcohol oxidase, laccase, glyoxal oxidase and cellobiose dehydrogenase) and cell wall loosening enzymes (expansin expansion, swollenin, loosinin and cellulose induced protein). Finding or designing a single strain or microbial consortium producing all these enzymes could be very effective to enhance CBP efficiency (Parisutham et al., 2014).

Up to now, no wild type microorganism capable of CBP with high efficiency for industrial bioethanol production has been identified. So, designing a single microbe or microbial consortium with desired efficiency for this purpose is necessary (Kricka et al., 2014). Recently, two major strategies (native and recombinant) have been proposed to generate ideal microorganisms for CBP. The native strategy focuses on studying natural cellulolytic microbes with an aim to improve biofuel yield. Some native cellulolytic microorganisms, such as *Clostridium sp.*, *Bacillus subtilis* and *Trichoderma reesei* are capable of producing only simple secondary metabolites such as ethanol or hydrogen (Kricka et al., 2014; Parisutham et al., 2014).Some methodologies, including isolation and characterization of new strains with CBP capacities, adaptive evolution using natural selection to specific environmental conditions (Gefen et al., 2012) or development of bioprocessing and fermentation systems to enhance CBP capabilities (Elkins et al., 2010; Liang et al., 2011; Wang et al., 2012a; Kricka et al., 2014) have also been used. In addition, recent studies have confirmed the feasibility to manipulate some cellulytic microorganisms e.g. *Clostridium* (Brown et al., 2011) and *Thermobifida spp.* (Deng and Fong, 2011). The recombinant technology focuses on directed mutagenesis, genetic and metabolic engineering of cellulolytic microbes to be alcohologenic or alcohologenic microbes to be cellulolytic (Lynd et al., 2005; Anbar et al., 2012; Parisutham et al., 2014). Industrial bioethanol producing microbes do not commonly possess cellulolytic ability, and do not metabolize other sugars in the presence of glucose due to carbon catabolite repression either. These disadvantages result in low biofuel production efficiency when they are used with heterogeneous substrates including lignocellulosic biomass. So recently, research focuses have been directed to genetic engineering of yeast sand *E. coli* to co-metabolize several combinations of hexoses and pentoses e.g. glucose, xylose, cellobiose, galactose or mannose (Ha et al., 2011; Vinuselvi and Lee, 2012). Co-cultures or microbial consortia may also be utilized in the CBP systems as the third methodology.

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In co-culture systems, saccharolytic and ethanologenic microorganisms are co-cultured to enhance efficient saccharification and fermentation in one pot. In addition, it is possible to use two ethanologenic microorganisms, each providing different key saccharifying enzymes (Schuster and. Chinn, 2013). In the next sections, different native and recombinant strategies used for CBP ethanol and butanol production from lignocellulosic and starchy biomass by various microorganisms will be discussed.

2.3.3. CBP in biobutanol production

Butanol is an important chemical with many applications in the production of solvents, plasticizers, butylamines, amino resins, butyl acetates, etc. It has several advantages over ethanol as a fuel extender or fuel substitute. It has an energy content that is similar to gasoline, so, less volume is required than ethanol to achieve the same energy output. Butanol has a lower vapor pressure compared to ethanol, and is therefore safer during transport and use in car engines (Ezeti and Blaschek, 2007; Ni and Sun, 2009; Quershi et al., 2013; Wen et al., 2014a).

Butanol is currently industrially produced from petroleum or fermentation of corn, cassava or molasses as substrate. By increasing the prices of these substrate materials, it has been proposed to produce butanol by fermentation of lignocellulosic biomass. By use of lignocelluloses as substrate, three components, including acetone, butanol and ethanol (ABE) are simultaneously produced, in which butanol is the major product (Ezeji et al., 2012; Jurgens et al., 2012; Wen et al., 2014a,b). Different biomass such as wheat straw (Quershi et al., 2007; Wang et al., 2013; Nanda et al., 2014), rice straw (Gottumukkala et al., 2013, 2014), barley straw (Quershi et al., 2010a), corn stover (Parekh et al., 1988; Quershi et al., 2010b), corn cob and fibers (Marshal et al., 1992; Guo et al., 2013), palm kernel cake (Shukor et al., 2014), cassava starch (Li et al., 2014a,b), pinewood and timothy grass (Nanda et al., 2014), switch grass (Quershi et al., 2010b; Gao et al., 2014), sag pith (Linggang et al., 2013) and dried distillers' grains (Ezeji and Blaschek, 2008) have been used as substrates for ABE fermentation by numerous *Clostridium* strains such as *C. acetobutylicum*, *C. aurantibutyricum*, *C. beijerinckii* , *C. cadaveris*, *C. pasteurianum*, *C. saccharoperbutylacetonicum*, *C. saccharobutylicum*, *C. sporogenes* and *C. tetanomorphum* (Inui et al., 2008; Quershi et al., 2013). This process commonly occurs in two phases, including acidogenic phase where acetic and butyric acids are produced, and solventogenic phase where acids are used and ABE are generated. The major problem in the butanol production from lignocellulosic biomass is that butanol producing cultures cannot tolerate or produce more than 20–30 g/l of ABE in batch reactors due to toxicity of butanol to the culture, which results in low final butanol titer levels (Quershi et al., 2008, 2010, 2013; Ezeji et al., 2012, Nanda et al., 2014). In addition, the cost of exogenous cellulase utilization has made these attempts economically uncompetitive, because the solventogenic clostridia are not able to utilize lignocellulose as a raw material directly (Bayer et al., 2007; Jurgens et al., 2102; Bellido et al., 2013; Wen et al., 2014a,b).

In order to overcome challenge of butanol toxicity to microorganisms, a huge amount of studies on the alternative fermentation and product recovery technologies have been carried out. These studies have applied two kinds of approaches:

(1) Employing process engineering approaches to simultaneously recover butanol from the fermentation broth and thus not allowing butanol concentrations in the reactor to accumulate beyond culture's tolerance. These methodologies include the use of immobilized and continuous bioreactors with cell recycle, adsorption, gas stripping, separation using ionic liquids, liquid-liquid extraction, pervaporation, aqueous two phase separation, supercritical extraction and perstraction, which have allowed the use of concentrated sugar solutions (up to 500 g/l) for the production of a highly concentrated butanol product stream (Ezeji et al., 2010, 2012; Quershi et al., 2013). By application of these strategies, the amount of ABE production has was reportedly increased up to 461 g/l.

(2) Developing more butanol tolerant and cellulase producing strains by finding wild type strains, co-culture systems or using genetic engineering techniques (Quershi et al., 2013; Wen et al., 2014a,b).

CBP has been suggested as an efficient and economical method for butanol production from low-cost renewable feedstock. To materialize the true potentials of CBP, a single wild type microorganism, microbial co-culture or consortium system or genetically-engineered single microorganisms must be developed to utilize lignocellulose at a high conversion rate and produce solvents such as ethanol and butanol at high yields and titers (Lynd et al., 2005; Wen et al., 2014a,b). **Table 2** summarizes some recent reports on CBP strategies used for butanol production from lignocellulosic biomass.

Table 2.
List of microorganisms and CBP strategies used for butanol production from lignocellulosic biomass.

Biomass	Microorganism	CBP strategy	Pretreatment	Butanol concentration/yield	References
Xylan and xylose	*Clostridium* strain BOH3	Single native strain	-	16 g/l	Rajagopalan et al., 2013, 2014
Cellulose	*C. thermocellum* and *C. saccharoperbutylacetonicum*	Co-culture	-	7.9 g/l	Nakayama et al., 2011, 2013
Cellulose	*C. acetobutylicum* and *C. cellulolyticum*	Co-culture	-	350 mg/l	Salimi and Mahadevan, 2013
Birch wood xylan	*Clostridium* strain BOH3 and *Kluyvera* strain OM3	Co-culture	-	1.2 g/l	Xin and He, 2013
Corn cobs	*C. cellulovorans* strain 743B and *C. beijerinckii* strain NCIMB 8052	Co-culture	Alkali	8.30 g/l	Wen et al., 2014a
Cassava starch	*C. acetobutylicum*	Mutant	-		Li et al., 2014a
Cassava starch	*C. beijerinckii* and *C. tyrobutyricum*	Continuous co culture in fibrous bed reactor	-	6.66 g/l (yield: 0.18 g/g)	Li et al., 2013
Cellulose	*C. cellulolyticum*	Recombinant technology: expressing enzymes that direct the conversion of pyruvate to isobutanol	-	660 mg/l	Higashide et al., 2011
Cellulose and lichenan	*C. beijerinckii* NCIMB 8052	Recombinant technology: transferring genes encoding glycoside hydrolases (*celA* and *celD*)	-	4.9 g solvents /l	Lopez-Contreras et al., 2001
Cellobiose	*C. thermosaccharolyticum*	Recombinant technology: overexpression of *bcs* operon	-	5.1 mM	Bhandiwad et al., 2013
Switch grass	*E. coli*	Recombinant technology: expression of hydrolase and butanol pathway genes	Ionic liquid-pretreatment	28 mg/l	Bokinsky et al., 2011
Corn stover	*C. cellulolyticum*	Metabolic engineering: sporulation abolishment and carbon overload alleviation	-	0.42 g/l	Li et al., 2014
Corn stover	*T. reesei and E. coli*	Co-culture	AFEX	1.88 g/l and 62% theoretical	Minty et al., 2013
Cellulose	*Klebsiella oxytoca* mutant , ME-UD-3	Mutation	-	7.8% more 2,3-butanediol than wild type	Ji et al., 2007

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2.3.3.1. The native strategy: single wild type strains for butanol production

The main advantage of butanol-producing *Clostridium* strains over *S. cerevisiae* is their efficient metabolism of both pentose and hexose sugars. *Clostridium* strains produce xylose isomerase which converts xylose into xylulose; a utilizable form of xylose for these strains (Quershi and Ezeji, 2008; Nanda et al., 2014). Xylan is the second most abundant polysaccharides on the Earth. A common problem in the direct fermentation of xylan by solventogenic Clostridial strains is insufficient expression of xylanase. The majority of *Clostridium spp.* strains that naturally produce butanol are non-cellulolytic (Gheshlaghi et al., 2009). Therefore, it is desirable to identify a new solventogenic *Clostridium* strain with the capability of expressing xylanase and utilizing un-pretreated xylan as a substrate for fermentation. Previously, a solventogenic *Clostridium* strain BOH3 with high xylanase activity (21.89 ± 0.1 U/mg) was reported to effectively utilize xylan at low concentrations (10 g/l), and produce both butanol and hydrogen. This strain was suggested to be used in CBP systems for butanol production (Bramono et al., 2011; Rajagopalan et al., 2013). In another study, by optimizing the culture medium contents, the expression level of xylanase in this strain was increased and up to 1.6 times more xylan was hydrolyzed leading to higher butanol and hydrogen concentrations (Rajagopalan et al., 2014). This was the first report dealing with production of xylanase enzyme by using a solventogenic *Clostridium* strain and a CBP for effective utilization of xylan for the production of butanol and hydrogen (Rajagopalan et al., 2014).

2.3.3.2. The native strategy: co-culture systems for butanol production

As the majority of naturally occurring *Clostridium spp.* strains that produce butanol are non-cellulolytic, there have been a series of studies utilizing co-cultures of different microorganisms to produce butanol and other solvents at high levels in a CBP system (**Table 2**). Clostridial co-culture systems containing cellulolytic and solventogenic species are known as potential CBP approaches for producing biochemicals and biofuels from lignocellulosic biomass (Akinosho et al., 2014; Salimi et al., 2013). As the costly enzymatic hydrolysis step is eliminated, co-culture systems have been considered as potential systems for cost-effective CBP (Lynd et al. 2005). Following, some co-culture systems used for butanol production in the format of CBP are reported.

2.3.3.2.1. *C. thermocellum* and *C. saccharoperbutylacetonicum*

It has recently been reported that a co-culture of *C. thermocellum* and *C. saccharoperbutylacetonicum* N1-4 can produce up to 7.9 g/l butanol in 9 d using Avicel cellulose as a carbon source (Nakayama et al., 2011, 2013). This co-culture system caused a significant decrease of hydrogen and acetone production, and butanol was selectively produced.

2.3.3.2.2. *C. acetobutylicum* and *C. cellulolyticum*

It was previously reported that *C. acetobutylicum* strains have an effective capability to ferment sugars derived from cellulose and hemicellulose (such as cellobiose, mannose, arabinose, xylose, glucose, and galactose), and convert them to ABE (Yu et al., 1984; Fond et al., 1986; Ali et al., 2004, Lee et al., 2012; Salimi and Mahadevan, 2013). Co-culture of these bacteria with a mesophilic cellulose degrading bacteria can be an efficient approach for butanol or ethanol production in a CBP system. *C. cellulolyticum* is a cellulolytic mesophilic bacterium with a high ability to solublize crystalline cellulose in pre-treated lignocellulosic biomass (Demain et al., 2005). It has been shown that the efficiency of cellulose utilization in the co-culture of *C. acetobutylicum* and *C. cellulolyticum* was increased compared to the mono-culture of *C. cellulolyticum* (Salimi et al., 2010; Salimi and Mahadevan, 2013). Salimi and Mahadevan (2013) confirmed that these two species showed synergism and that the metabolic activity of *C. acetobutylicum* improved the cellulolytic activity of *C. cellulolyticum* in the co-culture via exchange of metabolites such as pyruvate. In fact, these metabolites supported *C. cellulolyticum* to grow and metabolize cellulose under harsh co-culture conditions. The final concentration of butanol was up to 350 mg/l.

2.3.3.2.3. *Kluyvera* and *Clostridium*

Xin and He (2013) improved a co-culture system using a xylanase producing anaerobic bacteria *Kluyvera* strain OM3 and a biofuel producing *Clostridium* strain BOH3 for butanol production from birch wood xylan in a CBP format. The xylanase of *Kluyvera* was able to release reducing sugars from birch wood xylan, and these sugars were further fermented by the solventogenic *Clostridium sp.* strain BOH3 to biofuel. This co-culture system resulted in 1.2 g/l butanol from birch wood xylan, which was comparable to the amount of butanol (1.7 g/l) produced by the SHF system (separate hydrolysis by the exogenous xylanase application and following fermentation by *Clostridium sp*. strain BOH3).

2.3.3.2.4. C. beijerinckii and C. cellulovorans

Wen et al., (2014a) successfully used a co-culture system for CBP production of butanol from alkali-pretreated deshelled corn cobs. They used a cellulolytic, anaerobic, butyrate-producing mesophilic *C. cellulovorans* strain 743B to hydrolyze pre-treated corn cob and produce butyric acid. Then, the generated reducing sugars and butyric acid were consumed by a non-cellulolytic solventogenic *C. beijerinckii* strain NCIMB 8052 to produce butanol in one pot reaction. After optimization of fermentation conditions, the developed co-culture system could degrade 68.6 g/l alkali-pretreated deshelled corn cobs and produced11.8 g/l solvents (2.64 g/l acetone, 8.30 g/l butanol and 0.87 g/l ethanol) in less than 80 h. The growth kinetics and analytical studies showed that there were mechanisms of cooperation and competition between the two strains during the co-culture process.

2.3.3.2.5. Trichoderma reesei and E. coli

Minty et al. (2013) recently developed a co-culture system for CBP isobutanol production from lignocellulosic material, in which they used *T. reesei*, as cellulolytic and *E. coli*, as butanol producing microorganisms. By designing a modeling system and experimental studies, they showed that this co-culture could result in 1.88 g/l butanol form corn stover pre-treated by ammonia fiber explosion (AFEX) and yielded up to 62%.

2.3.3.2.6. C. beijerinckii and C. tyrobutyricum

Li et al. (2013) used a continuous co-culture system containing *C. beijerinckii* and *C. tyrobutyricum* in free-cell and immobilized-cell fermentation modes in a fibrous-bed bioreactor for butanol production from cassava starch and cane molasses. This system could significantly enhance butanol production, and the maximum butanol production (6.66 g/l), yield (0.18 g/g), and productivity (0.96 g/l/h) were obtained when cassava starch was used as the substrate (the maximum yield of ABE was at about 0.36 g/g).

2.3.3.3. The recombinant strategy for butanol production

Clostridia are known as the most important butanol producers and are widely employed in the industrial-scale production of solvents. Difficulty of performing genetic manipulations and complexity of the acidogenesis and solventogenesis metabolic pathways are the major obstacles during development of engineered *clostridia* for efficient and selective butanol-production capabilities. Recently, metabolic pathways of butanol production and also metabolic engineering approaches have been characterized in clostridia, and have opened new doors to scientists to develop new engineered strains with efficient butanol production capabilities (Gheshlaghi et al., 2009; Jang et al., 2012; Wen et al., 2014a, b). In the following sub-sections, the recent recombinant strategies used for enhancing CBP butanol production are discussed.

2.3.3.3.1. Genetic and metabolic engineering of butanol production pathways

As mentioned earlier, the main obstacles in butanol production by clostridia are endproduct cytotoxicity, formation of byproducts, requirement for strictly anaerobic conditions, low yield and needs for hydrolases (Bayer et al., 2007; Ezeji et al., 2012; Jurgens et al., 2102; Nanda et al., 2014; Wen et al., 2014a,b). These difficulties have driven various research efforts to reconstruct the butanol production pathway in more commonly-used industrial microorganisms. This includes engineering of various bacteria or yeasts for butanol production either by introduction of the Clostridial butanol

pathway (Inuei et al., 2008; Shen and Liao, 2008; Nielson et al., 2009; Shen et al., 2011) or intermediate genes of amino acid pathways (Lee et al., 2008).

During the last years, in some investigations, the butanol biosynthetic pathway of *C. acetobutylicum* has been successfully re-constructed in different heterologous microorganisms, such as *S. cerevisiae* (Steen et al., 2008) and *E. coli* (Atsumi et al., 2008a, b; Inui et al., 2008). Meanwhile, butanol titers in *E. coli* engineered to express the *Clostridium* butanol pathway have been reported as high as 500-1000 mg/l (Atsumi et al., 2008a; Shen et al., 2008, 2011). Shen and Liao (2008) confirmed that butanol could be produced in excess of 800 mg/l as a co-product with n-propanol.

Some wild type bacteria, such as *Rhodococcus*, *Bacillus*, and *Pseudomonas* are naturally tolerant to solvents (de Bont, 1998; Nielsen et al., 2009). For instance, de Carvalho et al. (2004) previously reported that *Pseudomonas putida* S12 had a moderate tolerance to butanol, and subsequently, it was used as a host strain engineered for biosynthesis of different solvents, such as phenol (Wierckx et al., 2005) and cinnamic acid (Nijkamp et al., 2005). Neilsen et al., (2009) by overexpressing the enzymes involved in glycolytic flux or regenerating NADH, engineered biobutanol synthesis in *P. putida* (120 mg/l), *B. subtilis (*24 mg/l) and *E.coli*.

The recent development of a transformation system for *C. thermocellum* has led to engineering *C. thermocellum* with new pathways to produce butanol and ethanol (Bombe, 2014). The expression of *Zymomonas mobilis* pyruvate decarboxylase and alcohol dehydrogenase in mesophilic bacterium *C. cellulolyticum* resulted in increase of ethanol yield by about 60%. Incorporation of the same combination of metabolic enzymes as well as other metabolic enzymes (such as a ketoacid decarboxylase) could increase the production of ethanol and butanol in the thermophilic bacterium *C. thermocellum* (Bombe, 2014).

Despite high titers obtained in some of the studies in which genetically engineered bacteria were used, several major drawbacks exist with the use of such bacteria for industrial biofuel production. These include a complex separation process from the fermentation media, narrow and neutral pH growth rate (Huang et al., 2010), and susceptibility to phage infections when grown at large scale (Huffer et al., 2012). The use of the yeast *S. cerevisiae* as a cell factory for biofuel production could overcome these limitations. *S. cerevisiae* is a robust industrial organism that can grow under various industrial conditions, including low pH, and less stringent nutritional requirements. Moreover, *S. cerevisiae* is well genetically and physiologically characterized. In addition, the larger size (as well as higher mass) of *S. cerevisiae* makes it easier to separate from the fermentation media than bacteria, reducing process costs (Hasunuma and Kondo, 2012; Krivoruchko et al., 2013). The first attempt to engineer *S. cerevisiae* for 1-butanol production involved the introduction of butanol-pathway genes together with overexpression of the native thiolase gene to obtain butanol titers of 2.5 mg/l (Steen et al., 2008). Krivoruchko et al., (2013) in addition to introducing heterologous enzymes for butanol production, engineered yeast in order to increase the flux toward cytosolic acetyl-CoA, the precursor metabolite for1-butanol biosynthesis. They transferred the genes encoding alcohol dehydrogenase (ADH2), acetaldehyde dehydrogenase (ALD6), acetyl-CoA synthetase (ACS) and acetyl-CoA acetyltransferase (ERG10). This resulted in an increase in butanol yield up to 6.5 folds compared to the previous work (Steen et al., 2008).

2.3.3.3.2. Genetic engineering for CBP butanol production in Clostridia

The main challenge for producing different solvents such as ABE from lignocellulosic materials especially celluloses using Clostridia is that these microorganisms are commonly not capable of utilizing cellulosic substrates. To overcome this problem, many efforts have been devoted to enhance cellulase activity to different Clostridial species (Fierobe et al., 2001; Tracy et al., 2012). Lopez-Contreras et al. (2001) cloned two genes encoding glycoside hydrolases (*celA* and *celD*) obtained from the anaerobic fungus *Neocallimastix patriciarum*, and transferred them into *C. beijerinckii* NCIMB 8052 to increase cellulase activity and subsequent solvents (ABE) production. Although the recombinant strains showed cellulase activity, they did not grow individually or in cocultures on microcrystalline cellulose or CMC as a sole carbon source. This might be ascribed to the fact that more proteins were needed to enhance efficient cellulose degradation and to support the growth of the bacteria. The recombinant bacteria harboring *celA* and/or *celD* showed significantly more solvent production (4.9 g/l) during growth on lichenan and more extensive degradation of this polymer than what achieved by the wild-type strain (Lopez et al., 2001).

C. acetobutylicum is able to convert different sugars and polysaccharides into acids and solvents, while is not able to utilize cellulose. To enhance efficient consumption of cellulose, in another study, a heterologous minicellulosome containing two different cellulases bound to a miniscaffoldin (truncated CipC from *C. cellulolyticum* and the hybrid scaffoldin Scaf 3 containing an additional cohesin domain derived from CipA from *C. thermocellum*) was expressed in *C. acetobutylicum* strain ATCC 824. The results showed that the proteins were correctly and functionally matured and secreted in the medium (Perret et al., 2004). The same research group in another study cloned and transferred the gene *man5K* encoding the mannanase Man5K and the gene *cipC1* encoding a truncated scaffoldin (miniCipC1) from *C. cellulolyticum* as operon in the solventogenic *C. acetobutylicum*. It was shown that the secreted heterologous hybrid protein by the recombinant strain was functional, and it could bind to crystalline cellulose via the miniscaffoldin, and the complexed mannanase was active towards galactomannan (Mingardon et al., 2005). Six year later, Mingardon et al. (2011) in another research investigation could successfully transfer 3 genes encoding cellulosomal cellulases (Cel5A, Cel8C and Cel9M) of *C. cellulolyticum* into the *C. acetobutylicum* to introduce high cellulolytic activity to this bacterium. These successes were taken into account as starting point for development of a CBP process to convert cellulose directly into solvents.

C. cellulolyticum, as a potential CBP organism, similar to *C. thermocellum* can utilize cellulose as well as other sugars released from hemicellulose degradation, including xylose, fructose, galactose, arabinose, mannose, and ribose (Gowen and Fong, 2010; Higashide et al., 2011). Recently, investigations have been focused on engineering of these bacteria to enhance their CBP butanol production. Higashide et al. (2011) by expressing different enzymes (*B. subtilis* α-acetolactate synthase, *E. coli* acetohydroxyacid isomeroreductase, *E. coli* dihydroxy acid dehydratase, *Lactococcus lactis* ketoacid decarboxylase, and *E. coli* and *L. lactis* alcohol dehydrogenases) involved in direct conversion of pyruvate to isobutanol could engineer valine biosynthesis pathway. This metabolic engineering approach resulted in production of up to 660 mg/l isobutanol from cellulose.

Thermoanaerobacterium thermosaccharolyticum (formerly called *C. thermosaccharolyticum*) is another CBP suitable organism for n-butanol production. This microorganism natively has the required genes involved in the n-butanol biosynthetic pathway. Bhandiwad et al. (2013) by overexpression of the natively occurring *bcs* operon containing the genes *thl*, *hbd*, *crt*, *bcd*, *etfA*, and *etfB* responsible for the formation of butyryl CoA could increase the n-butanol production on the cellobiose containing medium by 180% compared to the wild type (from a n-butanol titer of 1.8 mM to 5.1 mM, respectively). One interesting study was carried out by Bokinsky et al. (2011) in which they engineered *E. coli* strains to enhance both cellulolytic activities and hydrocarbon biosynthesis capabilities. Engineered strains expressed cellulase, xylanase, beta-glucosidase, and xylobiosidase enzymes under control of native *E. coli* promoters, and also were further engineered with three biofuel synthesis pathways to demonstrate the production of fuel substitutes. Co-culture of the engineered strains resulted in 28 ± 5 mg/l butanol from ionic liquid-pretreated switch grass.

Recently, *C. cellulovorans*, a cellulolytic and acid-producing anaerobic bacterium, has been used as a suitable host for direct production of n-butanol from cellulosic biomass. An engineered strain of *C. cellulovorans* was constructed by expressing an aldehyde/alcohol dehydrogenase 2 (adhE2) to produce n-butanol and ethanol directly from cellulosic biomass. Then, fermentation conditions were optimized for the engineered strain which enhanced production of n-butanol directly from cellulose (1.6 g/l). This concentration was significantly more than the concentrations achieved by wild-type and engineered cellulolytic strains (Yang, 2014).

In another recent report, to increase cellulose utilization efficiency of *C. cellulolyticum* and to enhance its application in CBP for butanol production, two metabolic engineering strategies, including sporulation abolishment and carbon overload alleviation were used. These changes improved cellulose consumption from 17.6 g/l to 28.7 g/l with a production of 0.42 g/l isobutanol in the 50 g/l cellulose medium (Li et al., 2014a,b). Kovacs et al. (2014) using Allele-Coupled Exchange (ACE) technology could engineer a strain of the butanol producing *C. acetobutylicum* with different synthetic genes encoding *C. thermocellum* cellulosomal scaffoldin proteins and glycoside hydrolases

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(GHs, Cel8A, Cel9B, Cel48S and Cel9K) as well as synthetic cellulosomal operons directing the synthesis of Cel8A, Cel9B and a truncated form of CipA. Their results confirmed the functional self-assembly of cellulosomal subunits and also successful expression and secretion of the recombinant genes by the recombinant *C. acetobutylicum* strains. These novel cellulosomes and recombinant strains could provide a novel platform to enhance CBP production of butanol.

2.3.4. CBP in bioethanol production

2.3.4.1. The native strategy: wild type single microorganisms with CBP capabilities

2.3.4.1.1. Bacteria

2.3.4.1.1.1. Clostridium thermocellum

One of the most popular microorganisms used in the CBP systems is *C. thermocellum* that fits the native strategy (**Table 3**). This bacterium produces an extracellular multi-enzyme complex containing different types of glycosyl hydrolases, such as cellulases, hemicellulases, and carbohydrate esterases (cellulosome) on the surface of cell membranes (Taylor et al., 2009; Kumagai et al., 2014). The high capability of *C. thermocellum* in hydrolysis of different cellulosic materials, such as crystalline cellulose (Hörmeyer et al., 1988; Puls and Wood, 1991; Hall et al., 2010; Shao et al., 2011; Zhao et al., 2012), poplar (*Populous tremuloides*), wheat straw (*Triticum vulgare*) (Hörmeyer et al., 1988), and switch grass (Fu et al., 2011; Yee et al., 2012) has been confirmed.

Kundu et al. (1983) could develop a direct anaerobic bioconversion of cellulosic substances (raw and mild alkali/steam pre-treated bagasse) into ethanol by *C. thermocellum* ATCC 27405. By using mild alkali and steam pretreatment, the conversion rate of raw bagasse increased from 52% (w/w) to 79% (w/w). Yee et al. (2012) used wild type *C. thermocellum*, *Caldicellulosiruptor bescii* and *Caldicellulosiruptor obsidiansis* for CBP ethanol production from dilute acid-pretreated transgenic and wild type switchgrass biomass. In the transgenic plants, the caffeic acid 3-O-methyltransferase (*COMT*) gene, involved in the lignin biosynthesis pathway, was down-regulated to enhance the efficiency of dilute acid pretreatment and enzymatic hydrolysis procedures. The maximum ethanol yield was achieved when hot water-extracted biomass was used for *C. thermocellum* (330 mg/l). In another study, Kumagai et al. (2014) used steam with wet disk milling treated woods of *Hinoki cypress* (softwood) and Eucalyptus (hardwood) for CBP ethanol production by a *C. thermocellum* strain (ATCC 27405). Maximum ethanol production via the CBP was 79.4 mg/g-cellulose from *Hinoki cypress*, and 73.1 mg/g-cellulose from Eucalyptus, respectively, which were about 20-25% of the SSF system by *S. cerevisiae*.

2.3.4.1.1.2. Clostridium phytofermentans

Jin et al. (2011) used *C. phytofermentans* (ATCC 700394) as CBP organism for ethanol production from AFEX-treated corn stover. After optimization of fermentation conditions, the strain could hydrolyze 76% and 88.6% of glucan and of xylan, respectively, and produced 2.8 g/l which was 71.8% of the SSCF yield (3.9 g/l). This group in their next experiments evaluated high solids loading CBP performance on AFEX-treated corn stover. They showed that when AFEX-treated corn stover was used as sole carbon source, no nutrients supplementation was needed, and it showed similar sugar conversions compared to when fermentation was performed with nutrients supplementation. Glucan and xylan conversion were recorded at 48.9% and 77.9%, respectively, and ethanol concentration was measured at 7.0 g/l after 264 h (Jin et al., 2012a).

2.3.4.1.1.3. Thermoanaerobacterium sp.

The thermophilic anaerobe *Thermoanaerobacterium* species are considered for their xylanolytic activities, and ability to ferment xylose, mannose, galactose, and glucose. Thus, they are hemicellulolytic in contrast to the clostridia which are cellulytic (Shaw et al., 2008; Sigurbjornsdottir and Orlygsson, 2011; Schuster and Chinn, 2013). These bacteria grow in the temperatures between 45 and 65°C and pH between 4.0 and 6.5. They commonly produce different hydrolases, including endoxylanase, which breaks down xylan chains to xylobiose and xylotriose, and β-xylosidase, which breaks the oligosaccharides into xylose, and other xylanolytic enzymes with minor roles (Lee et al., 1993; Shaw et al., 2008, 2012). Recent studies have shown that these bacteria could be promising candidates for CBP bioethanol production. For example, Sigurbjornsdottir and Orlygsson (2011) isolated a *T. aciditolerans* strain AK54 with combined bioethanol and biohydrogen production (CHE) capabilities from a hot spring in Iceland. They confirmed that this bacterium was able to utilize various carbon substrates, such as xylose, glucose, fructose, mannose, galactose, sucrose and lactose, and produce ethanol, acetate, lactate, hydrogen and carbon dioxide. They also showed that this strain could ferment cellulose, newspaper, grass (*Phleum pratense*), barley straw, and hemp. The maximum ethanol (24.2 mM) production was observed for cellulose, however it was less for lignocelluloses. Shang et al. (2013) isolated and characterized a novel thermophilic anaerobic *T. calidifontis sp. nov.* strain (RX1) with ethanol production ability from hot sprongs of China. The strain was able to use xylan, starch, glucose and xylose, and produce ethanol (81 and 58% of the theoretical yields for xylose and glucose, respectively, after 48 h), lactate, acetate, CO_2, and H_2.

2.3.4.1.2. Fungi

It has been previously reported that filamentous fungi such as *Trichoderma* sp., *Neurospora* sp., *Aspergillus* sp., *Rhizopus sp., Mucor sp., Paecilomyces* sp. *Fusarium* sp., and many white-rot basidiomycetes are capable of producing a large numbers of lignocellulolytic enzymes due to their co-evolution with plants, and also possess high abilities to convert released plant-derived sugars into ethanol (Lübbehüsen et al., 2004; Dashtban et al., 2009; Fan et al., 2012b; Olson et al., 2012; Hennessy et al., 2013) (**Table 3**). In the following sub-sections, some single wild type fungi and white rot basidiomycetes as CBP organism for ethanol production from different biomass are presented.

2.3.4.1.2.1. Mucor circinelloides

Chitin is also one of the most abundant renewable resources in nature, after cellulose. Inokuma et al. (2013) firstly isolated and characterized some native *M. circinelloides* strains with ability to use of N-Acetylglucosamine (GlcNAc) and chitin substrates as carbon sources for growth due to their chitinolytic and direct ethanol production. The strain NBRC 6746 could produce 18.6 g/l of ethanol from 50 g/l of GlcNAc after 72 h (ethanol production rate of 0.75±0.1 g/l/h). Another strain of this fungus (NBRC4572) could produce 6 and 0.46 g/l of ethanol from 50 g/l of chitin after 12-16 d, respectively. These results confirmed that it was possible to use *Mucor* strains and abundant chitinous wastes for direct CBP ethanol production. However, the chitinolytic activities in these strains is low, and it is necessary to optimize all aspects of fermentation, characterize individual chitinolytic enzymes, screen chitinase for addition, or engineer *Mucor* strains for more chitinolytic activities and ethanol production efficiency.

2.3.4.1.2.2. Fusarium oxysporum

F. oxysporum, a well known crop pathogen, can convert lignocellulosic biomass (straws) to ethanol via CBP. Previously, a few studies have shown the capability of this fungus as CBP organism for ethanol production from various cellulosic substrates, including untreated and pre-treated straw (Christakopoulos et al., 1989, 1991; Ali et al., 2012; Hossain et al., 2012), brewer's spent grain (Xiros and Christakopoulos, 2009) (**Table 3**). Such capability is due to the fact that *F. oxysporum* has an efficient cellulolytic system and is able to produce the enzymes necessary to degrade lignocellulosic biomass to sugars and also ferment both pentose and hexose sugars to ethanol under anaerobic or microaerobic conditions (Schuster and Chinn, 2013; Ali et al., 2014; Anasontzis et al., 2014). These studies showed that based on the strain used and also the methodology of CBP ethanol production, this fungus was able to produce up to 0.35 g ethanol/g cellulose (**Table 3**). This fungus is considered as a promising CBP organism because of its wide host range and high ability to hydrolyze and ferment lignocelluloses to ethanol (Hennessy et al., 2013). Another advantage of *F. oxysporum* is its

Table 3.
List of single native microorganisms used as CBP organisms for ethanol production from lignocellulosic biomass.

Biomass	Microorganism	Pretreatment	Ethanol concentration/yield	References
Bagasse	*C. thermocellum ATCC 27405*	Mild alkali pretreated	21 % maximum theoretical (1.09 g/l)	Kundu et al., 1983
Filter paper	*C. thermocellum*	-	81% maximum theoretical (0.41 g/g)	Balusu et al., 2005
Sugar cane Bagasse	*C. thermocellum ATCC 27405*	Solid	3.5 g/l	Chinn et al., 2008
Paper pulp sludge		Solid	14.1 g/l	Chinn et al., 2008
Crystalline cellulose	*C. thermocellum ATCC 27405*	-	2.66 g/l	Dharmagadda et al., 2010
Transgenic and wild type switch grass	*C. thermocellum*, *Caldicellulosiruptor bescii* and *Caldicellulosiruptor obsidiansis*	Diluted acid and hot water extracted biomass	330 mg/l	Yee et al., 2012
Hinoki cypress and Eucalyptus	*C. thermocellum* strain (ATCC 27405)	Steam with wet disk milling	79.4 mg/g-cellulose from Hinoki cypress, and 73.1 mg/g- cellulose from Eucalyptus	Kumagai et al., 2014
Corn stover	*C. phytofermentans*	AFEX	2.8 g/l	Jin et al. 2011, 2012a,b
Corn cob, *Napiergrass*, Avicel, α-cellulose, purified bamboo, rice straw	*Klebsiella oxytoca THLC0409*	-	0.0623, 0.0475, 0.019, 0.02, 0.018, 0.016 g/ g, respectively	Tran et al., 2011
Xylan	*Thermoanaerobacterium saccharolyticum B6A-RI*	-	1.75 g/l	Lee et al., 1993
Cellulose, newspaper, grass (Phleum pratense), barley straw (Hordeum vulgare), and hemp (Cannabis sativa)	*T. aciditolerans strain AK54*	Acid and Alkali	Ethanol (24.2 mM) and hydrogen (6.7 mol-H_2/g substrate) From cellulose	Sigurbjornsdottir and Orlygsson, 2011
Xylan, starch, glucose and xylose	*T. calidifontis sp. nov. (RX1)*	-	81 and 58 % of the theoretical yields for xylose and glucose, after 48 h)	Shang et al., 2013
Xylose and glucose	*T. aotearoense*	-	32 mM	Cai et al., 2011
GlcNAc and Chitin	*Mucor circinelloides*		18.6 g/l from GlcNAc and 6 g/l from Chitin	Inokuma et al., 2013
D-glucose, sucrose, maltose, cellobiose, cellotriose, and cellotetraose	*Flammulina velutipes-FV1*	-	Conversion rate of 70-91%	Mizuno et al., 2009b
Sorghum	*F. velutipes-FV1*	Grinding with an ultra-fine friction grinder	180-200 g/l	Mizuno et al., 2009b
Sugarcane bagasse cellulose	*F. velutipes- FV1*	-	0.36 g ethanol/g cellulose	Maehara et al., 2013a
Unbleached hardwood kraft pulp	*Phlebia sp. MG 60*	-	8.4-37.3 g/l based on the substrate concentration	Kamei et al., 2012a, b, 2014a
Spent mushroom waste	*Phlebia sp. MG 60*	-	-	Kamei et al., 2014b
Sugarcane bagasse	*Phlebia sp. MG 60*	Diluted acid	44.2 - 64.2 mg ethanol/ gram of pretreated bagasse	Khoung et al., 2014a
Sugarcane bagasse	*Phlebia sp. MG 60*	Alkali	210 mg/g untreated bagasse	Khoung et al., 2014a
Glucose, mannose, fructose, galactose, sucrose, maltose and cellobiose	*Peniophora cinerea*	-	0.40-0.45 g/g hexose	Okamoto et al., 2010
Glucose, mannose, fructose, galactose, sucrose, maltose and cellobiose	*Trametes suaveolens*	-	0.1-0.39 g/g hexose	Okamoto et al., 2010
Wheat straw	*Fusarium oxysporum*	Crystallinity reduction	0.28 g ethanol/g straw	Christakopoulos et al., 1991
Cellulose	*F. oxysporum*	-	0.35 g/g cellulose	Panagiotou et al., 2005
Wheat straw blended with wheat bran (10:1 ratio)	*F. oxysporum*	Non-treated	80 mg/g straw and bran	Ali et al., 2012
Wheat straw	*F. oxysporum*	Untreated, lime pretreated and dilute alkaline peroxide pretreated	0.756 , 0.796 and 0.810 g/g of wheat straw, respectively	Hossain et al., 2012
Brewer's spent grain	*Fusarium oxysporum* *Submerged and microaerobic conditions (0. 01 vvm)*	Alkali-pretreated	109 g ethanol/kg dry bewer's spent grain	Xiros and Christakopoulos, 2009
Sugarcane bagasse (40 g/l)	*Fusarium verticillioides*	Alkali-pretreated	4.6 g/l	de Almeida et al., 2013
Sugarcane bagasse (40 g/l)	*Acremonium zeae*	Alkali-pretreated	3.9 g/l	de Almeida et al., 2013
Corn stover	*Aspergillus oryzae*	Non pretreated and pretreated (Alkali and Diluted acid)	0.762 g/g, 0.799 g/g and 0.819 g/g for non-treated, dilute acid and dilute alkaline peroxide pretreated corn stover, resp.	Hossain, 2013
Glucose and xylose	*Paecilomyces variotii*	-	close to the theoretical maximum	Zerva et al. 2014
Agave tequilana fructans (ATF)	*Kluyveromyces marxianus*	-	20 g/l	Flores et al., 2013
Jerusalem artichoke tubers	*K. marxianus*	-	94.2 g/l	Yuan et al., 2012
Jerusalem artichoke tubers	*K. marxianus*	-	73.6 g/l	Hu et al., 2012
Filter paper, Japanese cedar and Eucalyptus	*Mrakia blollopis*	-	Without Tween 80: up to 12.5 g/l With Tween 80: increased by 1.1. to 1.6 fold	Tsuji et al., 2013, 2014
Xylose-extracted corncob residue (25%)	*Clavispora NRRL Y-50464*	Solids loading	25 g/l	Liu et al., 2012

endogenous ability to tolerate commonly produced inhibitory compounds during CBP ethanol production, including lignocellulosic hydrosylates (e.g. carboxylic acids, phenolic compounds, furan derivatives) and the fermentation by-product acetic acid (Panagiotou et al., 2008; Xiros et al., 2011; Hennessy et al., 2013). However, in order to become an economically suitable CBP organism, a better understanding of the hydrolysing and ethanol production pathways of this fungus as well as the mechanisms involved and bottlenecks faced is required. Fortunately, recently some studies have been devoted to understanding details of ethanol production in *F. oxysporum* which will be useful for development of CBP ethanol production by this fungus (Ali et al., 2012, 2013, 2014; Hennesy et al., 2013; Anasontzis et al., 2014).

2.3.4.1.2.3. Fusarium verticillioides and Acremonium zeae

Recently, de Almeida et al. (2013) has used two corn endophytic fungi, *F. verticillioides* (the causal agent of kernel and ear rot of maize) and *Acremonium zeae* (an antagonist of other fungal pathogens) (Poling et al., 2008), in single and co-culture system for CBP production of ethanol. When glucose, xylose and a mixture of both sugars were used as carbon source, the yields of 0.47, 0.46 and 0.50 g ethanol/g sugar for *F. verticillioides* and 0.37, 0.39 and 0.48 g ethanol/g sugar for *A. zeae* were achieved. When pre-treated sugarcane bagasse (40 g/l) was used, the ethanol concentration was recorded at 4.6 and 3.9 g/l for *F. verticillioides* and *A. zeae*, respectively (0.31 g ethanol/g consumed sugar). Both fungi were able to co-ferment glucose and xylose at high yields. These results confirmed that these fungi could be suitable CBP organisms to produce ethanol directly from lignocellulosic biomass (de Almeida et al., 2013).

2.3.4.1.2.4. Aspergillus oryzae

A. oryzae is a fungus with high potential for the secretory production of various enzymes and is commonly used in traditional Japanese fermentation industries (Machida et al., 2008). Recently, Hossain (2013) reported the optimization of direct ethanol production using *A. oryzae* from non-pretreated and pre-treated corn stover in a continuous stirred batch bioreactor. The maximum ethanol concentration of 0.819 g/g was achieved when dilute alkaline peroxide pre-treated corn stover at optimum fermentation conditions was used as carbon source for the *A. oryzae* strain.

2.3.4.1.2.5. Paecilomyces variotii

P. variotii is an ascomycete fungus commonly found in composts, soils and food products. This fungus is also known from decaying wood and creosote-treated wood utility poles (Houbraken et al., 2008, 2010). Zerva et al. (2014) evaluated this fungus for the first time as a candidate CBP species for the production of bioethanol from lignocellulose. They confirmed that the fungus was able to efficiently ferment both glucose and xylose to ethanol with yields close to the maximum theoretical value, but the efficiency was higher when xylose was used. These results confirmed that this fungus seemingly possessed the necessary enzyme factory for the degradation of lignocellulosic biomass, as it was able to grow and produce ethanol on common agro-industrial derivatives (Zerva et al., 2014).

2.3.4.1.3. White rot basidiomycetes

Basidiomycetes (white-rot fungi) are able to completely breakdown lignin, and are considered as primary agents of plant litter decomposition in terrestrial ecosystems (Thom et al., 1996). This ability is due to the secretion of different extracellular lignin-degrading enzymes, such as manganese peroxidase, lignin peroxidase, versatile peroxidase, and laccase (Lundell et al. 2010; Isroi et al., 2011). In addition, it is clear that a number of basidiomycetes produce alcohol dehydrogenase, and therefore, it is possible to produce wine and alcohols using a mushroom (Okamura et al., 2000, 2001). A number of white-rot basidiomycetes are particularly suited for the biological pretreatment or SSF of lignocellulosic biomass (Shi et al. 2009; Dias et al. 2010). However, some studies showed that a few white-rot basidiomycetes, including *Phanerochaete chrysosporium*, *Flammulina velutipes*, *Peniophora cinerea*, *Trametes versicolor* and *Trametes suaveolens* are capable of producing ethanol from hexose sugars (Kenealy and Dietrich 2004; Mizuno et al. 2009a,b; Okamoto et al. 2010), while *T. Hirsute* can use xylose as carbon source at low level (Okamoto et al. 2011).

2.3.4.1.3.1. Trametes versicolor

Okamoto et al. (2014) characterized a *T. versicolor* that was capable of efficiently converting not only hexose sugars, but also xylose, to ethanol. The CBP efficiency of the strain for direct ethanol production from 20 g/l of non pretreated corn starch, cellulose, xylan, wheat bran and rice straw was evaluated. The strain could effectively produce 9.8, 4.7, 4.4, 5.0 and 4.8 g/l ethanol from the 20 g/l starch, cellulose, xylan, wheat bran and rice straw, respectively. These results revealed that *T. versicolor* was a suitable CBP organism as it could efficiently perform fermentation of xylose-containing lignocellulosic biomass due to its ability for assimilating a broad spectrum of carbon sources. Furthermore, *T. versicolor* would have advantages over *S. cerevisiae* and *Pichia stipitis*, due to its ability to directly convert starch, cellulose, xylan, wheat bran and rice straw into ethanol without the need for costly pre-treatment processing (Okamoto et al., 2014).

2.3.4.1.3.2. Flammulina velutipes

F. velutipes is a basidiomycete mushroom and is well-known for its application in food industry. This mushroom also has fermentative abilities and high ethanol tolerance, which make it a valuable potential CBP organism. More specifically, it has high ability to convert glucose, mannose, sucrose, fructose, maltose, and cellobiose to ethanol. However, the fungus does not ferment galactose and pentose sugars to ethanol, and also fermentation times are relatively long (6 d or more) (Mizuno et al., 2009a; Schuster and Chin, 2013). Mizuno et al. (2009a) evaluated the possibility of CBP ethanol production by *F. velutipes* strain. The fungus could efficiently convert glucose to ethanol with a theoretical recovery rate of 88%, whereas conversion of pentose to ethanol was not observed. These properties of *F. velutipes* are similar to those of *S. cerevisiae*, but the difference is that the mushroom in addition to sucrose, can also efficiently ferment maltose, cellobiose, cellotriose, and cellotetraose to ethanol (Mizuno et al., 2009a). In another study, the same research group evaluated the possibility of using this basidiomycete for CBP ethanol production from two kinds of brown mid-rib (*bmr*) mutated and wild type sorghum. The final concentrations of ethanol produced from the two varieties of sorghum (mutated and wild type) were at about 200 and 180 g/l, respectively. They also managed to significantly increase ethanol yield by addition of cellulase and xylanase to the CBP process (Mizuno et al., 2009b).

In other study, it was shown that *F. velutipes* could produce about 40-60 g/l ethanol from 15% (w/v) d-glucose, d-fructose, d-mannose, sucrose, maltose, and cellobiose. In addition, it was shown that these fungi could directly produce CBP ethanol from sugarcane bagasse cellulose with a hydrolysis rate of 15% (w/v) bagasse, and when a commercial cellulase was partially used (9 mg/g biomass), this value reached 20% with an ethanol yield of 0.36g/g cellulose (Maehara et al., 2013a). These results suggest that genetic or metabolic engineering of these fungi to further enhance their cellulase activities could be very useful to materialize an industrial CBP ethanol production by mushrooms. It was further reported that these kinds of microorganisms were tolerant to up to 120 g/l ethanol further confirming their suitability for CBP compared to *C. thermocellum* (Okamura et al., 2001).

2.3.4.1.3.3. Phlebia sp.

Kamei et al. (2012a) reported that the white rot fungus *Phlebiasp.* (strain MG 60) was able to convert lignocellulose to ethanol under semi-aerobic conditions and that could be used as a suitable CBP organism. When this fungus was cultivated with 20 g/l of unbleached hardwood kraft pulp or waste newspaper, 8.4 and 4.2 g/l ethanol were produced after 168 and 216 h of incubation (ethanol yield of 0.42 and 0.20 g/g lignocellulose), respectively. In addition, it was shown that glucose, mannose, galactose, fructose, and xylose were completely assimilated by this strain to give ethanol yields of 0.44, 0.41, 0.40, 0.41, and 0.33 g/g of sugar, respectively (Kamei et al., 2012a). This white-rot fungus was able to selectively degrade lignin, and directly produced ethanol from delignified oak wood under aerobic solid-state fermentation conditions. This group of researchers in another study (Kamei et al. 2012b)

designed an integrated fungal fermentation process of unified aerobic delignification and anaerobic saccharification and fermentation of wood by this strain. Transition from aerobic conditions (biological delignification pretreatment) to semi-aerobic conditions (saccharification and fermentation) enabled the fermentation of wood solely by biological processes (Kamei et al. 2012b). To optimize using higher concentrations of cellulosic substrates, Kamei et al. (2014a) designed new experiments with high concentrations (high-solid loadings) of unbleached hardwood kraft pulp (2.0, 4.7, 9.1, and 16.5%w/w) for CBP ethanol production. The maximum ethanol concentration was produced (25.9 g/l) in the cultures containing 9.1% substrate. Temporarily removing the silicone plug from Erlenmeyer flasks and a small amount of aeration improved the ethanol yield by up to 37.3 g/l. In another study, these researchers used spent mushroom waste (SMW) produced by *Lentinula edodes* cultivation in CBP fermentation using this strain, and confirmed that the combination of edible mushroom cultivation and CBP fermentation could be potential used as a new cost-effective bioethanol production process with little environmental impacts (Kamei et al., 2014b). Khoung et al. (2014a) by developing an integrated fungal fermentation, involving a unified process for biological delignification and fermentation, could optimize direct ethanol production from sugarcane bagasse by *Phlebia sp*. MG-60. They optimized bagasse moisture content (75%) for selective lignin degradation and subsequent ethanol production. Furthermore, they used some additives, such as basal media, organic compounds, or minerals, and showed that these factors could affect biological delignification of bagasse by the strain. Basal medium and inorganic chemical factors, such as Fe^{2+}, Mn^{2+}, or Cu^{2+} could improve both delignification and ethanol production. This group in another investigation (Khoung et al., 2014b) reported that alkaline pretreatment of sugarcane bagasse for CBP by the *Phlebia sp.* MG-60 could improve direct ethanol production. When the strain was cultured with 20 g/l of alkali-pretreated sugarcane bagasse, 210 mg ethanol/g of the original untreated bagasse (65.7% of the maximum theoretical value) was produced after 240 h fermentation.

2.3.4.1.3.4. Peniophora cinerea and Trametes suaveolens

Okamoto et al. (2010) used two white-rot basidiomycetes, *Peniophora cinerea* and *Trametes suaveolens,* for direct ethanol production from hexose. *P. cinerea* produced ethanol under both aerobic and semi-aerobic conditions and assimilated glucose, mannose, fructose, galactose, sucrose, maltose and cellobiose with ethanol yields of 0.41, 0.45, 0.44, 0.19, 0.41, 0.44 and 0.45 g per g hexose, respectively. The ethanol production by *T. suaveolens* in aerobic conditions was low, whereas in the semi-aerobic conditions was at 0.39, 0.3, 0.13, 0.2, 0.37, 0.35 and 0.31 g ethanol/g hexose, respectively.

2.3.4.1.4. Yeasts

2.3.4.1.4.1. *Kluyveromyces marxianus*

It is clear that most ethanol-fermenting microorganisms prefer mesophile conditions (28 °C to 37 °C) for ethanol production, while the maximum activity of cellulases occurs at higher temperatures (~50 °C). This challenge results in a significant decrease in ethanol production efficiency when mesophile ethanol fermenting microorganisms are used, and so introducing thermotolerant microorganisms with high growth and fermentation capacity at elevated temperatures would result in significant increase in ethanol production efficiency (Yanase et al., 2010). One of the thermotolerant yeasts that was recently used for CBP ethanol production is *K. marxianus* (van Zyl et al., 2011; Yuan et al., 2012; Flores et al., 2013). Strains of this thermotolerant yeast can efficiently grow at temperatures up to 52°C, and have a short generation time (Rajoka et al., 2003). They can convert a wide range of substrates, such as xylose to ethanol. Previous studies have shown the high potential of *K. marxianus* for CBP ethanol production using different feedstocks at high temperatures (Fonseca et al., 2007, 2008; Yuan et al., 2012; Flores et al., 2013). Hu et al. (2012) isolated and characterized two *K. marxianus* PT-1 (CGMCC AS2.4515) and *S. cerevisiae* JZ1C (CGMCC AS2.3878) and reported the highest extracellular inulinase activity and ethanol yield of 73.6 and 65.2 g ethanol/l, respectively, in Jerusalem artichoke tuber flour fermentation (200 g /l) at 40 °C. This study confirmed the distinctive superiority of *K. marxianus* as CBP organism for ethanol production from inulin-type oligosaccharides such as Jerusalem artichoke tubers (Hu et al., 2012). Yuan et al. (2012) could successfully produce ethanol from inulin-rich Jerusalem artichoke tubers using the inulinase-producing yeast *K. marxianus* Y179 in a fed-batch operation. In this CBP strategy, all steps, including inulinase production, saccharification of inulin as well as ethanol production were integrated. They showed that the yeast prefered anaerobic conditions for the CBP system, and by medium content optimization, the maximum ethanol concentration reached 93.2 g/l. In another study, Flores et al. (2013) screened some *K. marxianus* strains for their fructanases activity and ethanol production from *Agave tequilana* fructans (ATF) as substrate. They found a few strains with fructanases activity and CBP ethanol production capability (20 g/l ethanol). Kim and Kim (2013) evaluated the effectiveness of chemical pretreatment with dilute acid or alkali in the CBP ethanol production from Jerusalem artichoke (*Helianthus tuberosus* L.) stalks and tubers (whole plant) by a *K. marxianus* strain. They showed that dilute acid-pretreated stalks, 10% (w/v), and tubers, 8% (w/v), resulted in 45.3 g/l ethanol after 30 h.

2.3.4.1.4.2. *Clavispora*

It is well known that *S. cerevisiae* is unable to utilize cellobiose, and therefore, addition of β-glucosidase is required to digest cellobiose into glucose in order to be utilized by the fermentation yeast. Also, optimum temperatures for efficient enzymatic saccharification are significantly higher than that required for microbial growth and fermentation function. Recently, Liu et al. (2012) isolated and characterized a new yeast strain of *Clavispora* with an ability to use cellobiose as sole carbon source, and to produce sufficient amounts of β-glucosidase for cellulosic ethanol production in a SSF system. This strain was tolerant to the major inhibitors produced during pretreatment process. The strain could produce 23 g/l ethanol from 25% xylose-extracted corn cob residue at 37 °C in a SSF system, without addition of any exogenous β-glucosidases (Liu et al., 2012).

2.3.4.1.4.3. Cryophilic yeast Mrakia blollopis

Cryophilic yeasts *Mrakia spp*. and *Mrakiella spp.* are dominant culturable yeasts in different Arctic and Antarctic regions. Tsuji et al. (2013) isolated and characterized *M. blollopis* SK-4 from Nagaike Lake in Skarvsnes ice-free area (East Antarctica), and showed that this strain was able to ferment typical sugars such as glucose, sucrose, maltose, raffinose and fructose at low temperatures. They evaluated the ability of the strain for direct ethanol production from glucose and different lignocellulosic materials in the presence and absence of Tween 80 at 10 °C. When Tween 80 was not used as substrate, the final concentrations of ethanol from glucose, filter paper, Japanese cedar and Eucalyptus were 48.2 g/l, 12.2 g/l, 12.5 g/l and 7.2 g/l, respectively, while the presence of 1% (v/v) Tween80, increased the ethanol concentration by about 1.1–1.6-fold compared to that of without Tween 80 (Tsuji et al., 2013). In another study, this group of researchers showed that the presence of 1% (v/v) Tween 80 and 5 U/g-dry substrate lipase, increased ethanol concentration from 1.4- to 2.4-fold compared to that of without Tween 80 and lipase (Tsuji et al., 2014).

2.3.4.2. Synthetic microbial consortium for consolidated production of bioethanol

It is well known that in the nature microbes rarely live in isolation, and they commonly exist in highly diverse and complex communities (Davey and O'toole, 2000). These consortia often give them the capability of performing complex tasks that are not possible to be performed by any single organisms. The microorganisms living in these consortia interact in different mechanisms, such as symbiosis, cooperation and direct competition. By understanding these interactions, it has been proposed to use synthetic consortia for biotechnological purposes (Prosser et al., 2007; Shong et al., 2012; Zuroff et al., 2012, 2013).

One of the strategies recently proposed for CBP is using microbial consortium containing different microorganisms with different cellulytic and fermentation capabilities as usually occurring in the nature, e.g. in the soil or in the digestive tracts of termites or ruminant animals (**Table 4**). When consortia are constructed, synergies may exist and therefore, may enhance more efficient substrate utilization and increase product yield (Alper and Stephanopoulos, 2009; Zuroff and Curtis, 2012; Brethauer and Studer, 2014).

Obviously, for successful application of microbial consortia for CBP ethanol production from lignocellulosic materials, establishing stable co-culture systems with the necessary functionality, process control and efficiency is required (Zuroff et al., 2013). Xu and Tschirner (2011) improved a co-culture system consisting of *C.thermocellum* and *C. thermolacticum* for CBP ethanol production. They showed that the co-culture shortened the lag time of fermentation (48 h) compared to the mono-cultures, and was able to actively ferment glucose, xylose, cellulose and micro-crystallized cellulose.

Park et al. (2012) developed co-culture system for one-pot bioethanol production, in which *Acidothermus cellulolyticus* C-1 and *S. cerevisiae* were co-cultured in a single reactor. After production of cellulase by *A. cellulolyticus* C-1, subsequently, *S. cerevisiae* was added to produce ethanol. The ethanol concentration and yield based on the initial Solka floc were as 8.7-46.3 g/l and 0.15-0.18 g/g, respectively. Zuroff et al (2013) developed a symbiotic (obligate mutualism) co-culture of two microorganisms, including *C. phytofermentans* (as a cellulolytic mesophilic bacterium) and *Candida*

Table 4.
List of microbial consortia used as CBP system for ethanol production from lignocellulosic biomass.

Biomass	Consortium	Pretreatment	Ethanol concentration/yield	References
Wheat straw	*T. reesei*, *S. cerevisiae* and *Scheffersomyces stipitis*	Dilute acid	10 g/l (67% yield)	Brethauer and Studer, 2014
Cellulose and xylose	*C. thermocellum* and *C. thermolacticum*	-	75% (w/w) theoretical maximum for cellulose and 90% for xylose	Xu and Tschirner, 2011
Cellulose and Filter paper	*C. phytofermentans* and *Candida molischiana* or *S. cerevisiae* cdt-1	-	22 g/l	Zuroff et al., 2013
Corn stover	*Geobacter sulfurreducens* and *Cellulomonas uda*	AFEX	73% (theoretical maximum)	Speers and Reguera, 2012
Filter paper	Microbial Consortium H (*C. thermosuccinogene*, *C. straminisolvens* and *C. isatidis*, …)	-	1.54 g/l	Du et al., 2010
Corn cob	*C. cellulovorans* 743B and *C. beijerinckii* NCIMB 8052	Alkali	11.8 g/l solvents (2.64 g/l acetone, 8.30 g/l butanol and 0.87 g/l ethanol)	Wen et al., 2014a
Corn cob	Susequential co-culture of *C. thermocellum* ATCC 27405 and *C. beijerinckii* NCIMB 8052	Alkali	19.9 g /l (acetone 3.96 g/l, butanol 10.9 g/l and ethanol 5.04 g/l)	Wen et al., 2014b
Avicel and *Napiergrass*	Mixed culture of *Clostridium* strain TCW1, *Bacillus* sp. THLA0409, *Klebsiella pneumoniae* THLB0409, *K. oxytoca* THLC0409, and *Brevibacillus* strain AHPC8120	-	0.108 g/g and 0.040 g/g for Avicel and *Napiergrass*, resp.	Lin et al., 2011
Cellulose	*C. thermocellum* with *Thermoanaerobacter* strains (X514 or 39E)	-	194-440% more than monoculture	He et al., 2011
Solka-Floc (SF)	*Acremonium cellulolyticus* C-1, and *S. cerevisiae*	-	8.7-46.3 g/l and 0.15-0.18 (g ethanol/g Solka Floc)	Park et al., 2012

It was shown that in this fermentation system, ethanol yield was up to two-fold higher than in mono-cultures, and reached 75% and 90% (w/w) of the maximum theoretical value for cellulose and xylose, respectively. In another study, a mixed culture, including *Clostridium* strain TCW1, *Bacillus* sp. THLA0409, *Klebsiella pneumoniae* THLB0409, *K. oxytoca* THLC0409, and *Brevibacillus* strain AHPC8120 previously isolated from compost of *Napiergrass* and sheep dung under anaerobic thermophilic conditions (60 °C) was used for CBP ethanol production from cellulose (Avicel) and *Napiergrass* (Lin et al., 2011). In this system, ethanol yields from Avicel and *Napiergrass* reached up to 0.108 and 0.040 g/g, respectively.

He et al. (2011) developed co-cultures of cellulolytic *C. thermocellum* with non-cellulolytic *Thermoanaerobacter* strains (X514 and 39E), and confirmed that this system could significantly improve ethanol production by 194-440%. The ethanol production was 62% higher when the strain X514 was used in co-cultivation system instead of the strain 39E. This increase was due to the presence of vitamin B_{12} biosynthesis pathway in the strain X514. Ho et al. (2011) identified a functional rumen bacterial consortium, containing different species of *Clostridium* and *Ruminococcus* sp. with high potentials for bio-hydrogen and bio-fuel production from lignocelluloses. They then reconstructed this functional rumen bacterial consortium by coculturing two selected strains, *C. puniceum* strain Ru6 (exhibiting xylanase and pectinase activity and higher hydrogen productivity) and *C. xylanolyticum* Ru15 (showing additional endoglucanase activity), and confirmed that the efficiency of hydrogen and ethanol production was comparable to that of the natural functional rumen bacterial consortium. In another study (Ho et al., 2012), a combined recombinant co-culture system was developed for CBP ethanol production, in which a dual-microbe *Bacillus*/yeast co-culture was used. They used a recombinant cellulosomal *B. subtilis* containing eight cellulosomal genes of *C. thermocellum* and a wild type kefir yeast *K. marxianus* KY3, *K. marxianus* KY3-NpaBGS (containing a β-glucosidase [NpaBGS] gene) or *K. marxianus* KR5 strain (containing endoglucanase (*egIII*), exo-glucanase (*cbhI*) and NpaBGS genes). Their results confirmed that all three *Bacillus*/yeast co-culture systems could achieve the cellulose saccharification and ethanol conversion simultaneously better than KR5 alone, and therefore, this microbial consortium could be of great potentials for integrating into a CBP system (Ho et al., 2012).

molischiana or *S. cerevisiae* cdt-1 (as cellodextrin fermenting yeasts) by controlling oxygen transport rate for CBP ethanol production. In this system, the yeasts provide respiratory protection to the obligate anaerobe *C. phytofermentans*, and this bacterium by hydrolysis of cellulose, releases soluble carbohydrates. The yeast converts these soluble carbohydrates to ethanol. The results showed that the co-cultures were only able to degrade filter paper at 30°C under semi-aerobic conditions. The co-culture of *C. phytofermentans* and *S. cerevisiae* with partially added endoglucanase could produce about 22 g/l ethanol from 100 g/l α-cellulose which was significantly more than that achieved in the mono-culture system of these microorganisms (6 and 9 g/l, respectively).

Recently, Brethauer and Studer (2014) developed a process containing three cellulytic and ethanol producing fungi and yeasts, including *Trichoderma reesei*, *S. cerevisiae* and *P. stipitis*, in which CBP of lignocellulose to ethanol occurred in a single multi-species biofilm membrane reactor. In this system, both aerobic and anaerobic conditions, which are necessary for the simultaneous fungal cellulolytic enzyme production and yeast fermentation for alcohol production from reducing sugars, were present. *T. reesei* grew directly on the membrane in an aerobic condition, and produced the required cellulases. The carbohydrate fraction of the feedstock was hydrolyzed to soluble sugars by the released enzymes. The reducing sugars were then metabolized by both *S. cerevisiae* and *P. stipitis* to ethanol in the anaerobic parts of the reactor. An ethanol production with a 67% yield (10 g/l) from the undetoxified whole slurry dilute acid-pretreated wheat straw was finally achieved using this microbial consortium system (Brethauer and Studer, 2014).

Speers and Reguera (2012) used a microbial electrolysis cell containing the exoelectrogen *Geobacter sulfurreducens* and the CBP bacterium *Cellulomonas uda* on $_{\text{AFEX pre}}$-treated corn stover for CBP ethanol and hydrogen production. By supplying nitrogen, the growth of *C. uda*, corn stover hydrolysis, and ethanologenesis were stimulated. The final substantial energy recovery from the ethanologenesis alone was about 56%, whereas cogeneration of cathodic H_2 increased it to ca. 73%. In another study, by using a microbial consortium containing *C. thermosuccinogene*, *C. straminisolvens* and *C. isatidis*, Du et al. (2010) could completely degrade

0.5 g/l filter paper and produce 1.54 g/l ethanol within 3 d. Wen et al. (2014a) used a microbial consortium containing a cellulolytic, anaerobic, butyrate-producing mesophile (*C. cellulovorans* 743B) to saccharify lignocellulose and produce butyric acid, and a non-cellulolytic, solventogenic bacterium (*C. beijerinckii* NCIMB 8052) to produce butanol and ethanol from alkali extracted deshelled corn cobs in one pot reaction. After optimizing the co-culture conditions, 11.8 g/l solvents (2.64 g/l acetone, 8.30 g/l butanol and 0.87 g/l ethanol) was achieved from 68.6 g/l degraded corn cobs in less than 80 h. In another work, Wen et al. (2014b) by subsequential co-culturing of *C. thermocellum* ATCC 27405 and *C. beijerinckii* NCIMB 8052 and using combinatorial optimal culture parameters for sugars accumulation and ABE production, could improve the yield up to 19.9 g/l (acetone 3.96, butanol 10.9 and ethanol 5.04 g/l) after 200 h.

2.3.4.3. The recombinant technology: mutant and genetic engineered microorganisms for CBP in lignocellulosic biomass

In order to obtain an ideal microorganism for CBP systems, two genetic engineering strategies have been used; (a) Engineering cellulase producers, such as *C. thermocellum, C. cellulolyticum, T. saccharolyticum, Trichoderma reesei* and *F. oxysporum*, to be ethanologenic (**Table 5**); and (b) Engineering ethanologens, such as *Z. mobilis, S. cerevisiae, Klebsiella oxytoca, P. stipitis, Candida shehatae, F. velutipes, Clavispora* and *E. coli*, to be cellulolytic (**Table 6**). For the first strategy, anaerobic cellulolytic bacteria were the first candidates, and their model microorganism was *C. thermocellum*. The main research objectives for the first strategy have been to increase ethanol yield and tolerance to ethanol, eliminating byproducts and introduction of new and desirable metabolic pathways for utilization of most of lignocellulose sugars, whereas in the second strategy, these aims have been to achieve functional expression and secretion of different exoglucanases and endoglucanases, growth on un-hydrolyzed lignocellulosic biomass, and utilization and fermentation of all the reduced sugars from lignocellulose (Xu et al., 2009). In the following sub-sections, these two strategies are comprehensively explained and discussed.

2.3.4.3.1. Engineering cellulase producers to be ethanologenic

2.3.4.3.1.1. Clostridium thermocellum

The anaerobic bacterium *C. thermocellum* is capable of degrading cellulose and hemicelluloses at a fast rate, and using them as carbon source at high temperatures (50-68 °C) via its cellulytic activities. Recent discoveries about the unique and multivariate enzyme cellulosome complex of *C. thermocellum* and role of this complex system in biomass degradation which have resulted from genomic, transcriptomic, proteomic, and metabolomic responses of *C. thermocellum* to varying biomass sources, have opened a new era for application of this species as a suitable CBP organism. The cellulosome of *C. thermocellum* is an extracellular multi-enzyme complex containing more than 20 different enzymes, including housing cellulases, hemicellulases, pectinases, chitinases, glycosidases, and esterases giving it the capability to efficiently breakdown lignocelluloses (Zverlov et al., 2005a,b; Wertz and Bédué, 2013; Ragauskas et al., 2014). This complex is 18 nm in diameter and with a molecular weight greater than 2×10^6 Da (Uversky and Kataeva, 2006). Although these advances have significantly improved *C. thermocellum* amenability to industrial use, several hurdles are still be overcome (Reddy et al., 2010; Ragauskas et al., 2014). One of the main challenges of ethanol or ABE production by this species is that the wild type *C. thermocellum* can only tolerate ethanol up to 5 g/l, and beyond that it is significantly inhibited (Herrero and Gomez, 1980; Ragauskas et al., 2014). Other problem is that it can only utilize C_6 sugars, and during its fermentation, C_5 sugars are maintained useless. So, the objectives of genetic engineering of this species should be directed to improve ethanol production capability, ethanol and inhibitors tolerance and also C_5 utilization.

Recently, mutation breeding systems, such as adapted or directed evolution, were used to engineer *C. thermocellum* strains to increase ethanol titer and tolerance to the minimum value of 40 g/l, that is required for the economic viability of cellulosic ethanol production (Dien et al., 2003; Ragauskas et al., 2014). For instance, Linville et al. (2013) using direct evolution methodology developed a mutant strain of *C. thermocellum* with the etahnol tolerance of up to 17.5% (Linville et al., 2013). In another investigation (Williams et al., 2007), a mutant strain of *C. thermocellum* with an ethanol tolerance of 8% was produced using sequential passaging. Proteomics analysis confirmed that the mutation occurred in membrane-associated proteins (Williams et al., 2007) and also the fatty acid membrane composition was changed. These changes resulted in prevention of fluidity upon ethanol exposure, and also increased membrane rigidity, which reduced the fluidizing effect of ethanol (Timmons et al., 2009). Brown et al. (2011) developed a mutant alcohol dehydrogenase in this species, which improved ethanol tolerance in *C. thermocellum* up to 80 g/l (within consistent and slow growth) and up to 50 g/l (with stable growth). Argyros et al. (2011) by metabolic engineering enhanced ethanol production and tolerance for a *C. thermocellum* strain. They employed counter-selections developed from the native *C. thermocellum hpt* gene and the *T. saccharolyticum tdk* gene to delete the genes for both lactate dehydrogenase (Ldh) and phosphotransacetylase (Pta). The obtained *ldh/pta* mutant showed 40:1 ethanol selectivity and a 4.2-fold increase in ethanol yield over the wild-type strain. Co-culture of both organic acid-deficient engineered *C. thermocellum* and *T. saccharolyticum* strains in 92 g/l Avicel, resulted in 38 g/l ethanol with acetic and lactic acids below detection limits after 146 h.

In another study, using a mobile group II intron, a thermotargetron for gene targeting in thermophiles such as *C. thermocellum* was developed. This system was used to disrupt six different chromosomal genes (*cipA, hfat, hyd, ldh, pta*, and *pyrF*), and it was confirmed that the disruption of either the gene encoding lactate dehydrogenase (ldh) or phosphotransacetylase (pta) *ldh* or *pta* by thermotargetrons in *C. thermocellum* strain DSM 1313, increased ethanol production by 37 and 42%, respectively, by decreasing carbon flux toward lactate and acetate. The double mutant strain showed strong decreases in both lactate and acetate production, but its ethanol production was increased only up to 56% (1.8 g/l) (Mohr et al., 2013).

Although, previous studies showed that strains with mutations in genes associated with production of l-lactate (Δldh) and/or acetate (Δpta) were characterized to gain more ethanol production from cellulose, but van Der Veen et al. (2013) obtained contradictionary results. In their work, the Δldh Δpta double-mutant strain evolved for faster growth had a growth rate and ethanol yield comparable to the parent strain (DSM1313 Δhpt Δspo0A), whereas its biomass accumulation was comparable to Δpta. Deng et al. (2013) engineered the ‘‘malate shunt’’ pathway, including phosphoenolpyruvate carboxykinase, NADH-linked malate dehydrogenase, and NADP-dependent malic enzymes. The engineering included expression of the pyruvate kinase gene from *T. saccharolyticum*, mutation of the phosphoenolpyruvate carboxykinase and deletion of malic enzyme gene in *C. thermocellum*. They showed that the novel strain with heterologous pyruvate kinase activity and diminished phosphoenolpyruvate carboxykinase exhibited 3.25-fold higher ethanol yield than the wild-type strain. Also, another strain with heterologous pyruvate kinase activity whose malic enzyme gene and a part of its malate dehydrogenase genes were deleted showed over 3-fold higher ethanol yield than the wild-type strain. Overall, their novel mutant strains achieved 3-fold higher ethanol yield, increased carbon recovery, increased formate production, increased ethanol tolerance, and decreased amino acid secretion relative to the parent strain (Deng et al. 2013; Ragauskas et al., 2014). These findings confirmed that the malate shunt could be used to convert phosphoenol pyruvate to pyruvate.

Maki et al. (2013) by overexpressing the β-glucosidase A (*bglA*) in the *C. thermocellum* 27405 could increase cellulase activity. The ethanol titer was increased as result of lowering the end product inhibition of cellulase. The β-glucosidase and cellulase activities of the recombinant strain were 1.6 and 2.3 fold higher than those of the wild-type during different phases of growth.

2.3.4.3.1.2. *Clostridium cellulolyticum*

This mesophilic bacterium *C. cellulolyticum* similar to *C. thermocellum* and *C. cellulovorans*, contains cellulosome which could enhance its growth on cellulose, hemicellulose and xylan, but it has weak fermentative abilities. The cellulosome complex of *C. cellulolyticum* contains five endoglucanases (CelA, CelD, CelC, CelG, and CelE), exoglucanase (CelF), scaffolding protein and the noncatalytic cellulosome integrating protein (CipC) (Bélaich et al., 1997; Gal et al., 1997; Schuster and Chinn, 2013). In spite of fermentation of both pentose and hexose sugars, there is a major obstacle for CBP application of *C. cellulolyticum*, as it produces a mixture of ethanol, acetate, lactate, H_2 and CO_2, in which the final ethanol concentration is very low. Therefore, it is necessary to use new genetic and metabolic engineering approaches to improve the ethanol yield (Li et al., 2012).

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Table 5.
List of cellulolytic microorganisms engineered to be ethanologenic (Category I).

Substrate	Microorganism	Engineering technology	New trait	References
Dilute acid pretreated poplar hydrolysate (17.5%)	*C. thermocellum*	Mutation: direct evolution	Ethanol and substrate tolerance-	Linville et al., 2013
Cellulose	*C. thermocellum*	Mutation: sequential passaging	8% ethanol tolerance	Williams et al., 2007
Cellulose	*C. thermocellum*	Mutation: mutant alcohol dehydrogenase	Ethanol tolerance 50-80 g/l	Brown et al., 2011
Avicel (92 g/l)	*C. thermocellum* and *T. saccharolyticum*	Deletion of the genes for both lactate dehydrogenase (*Ldh*) and phosphotransacetylase (*Pta*)	Significant increase of ethanol production (38 g/l ethanol)	Argyros et al. 2011
Cellobiose or Avicel	*C. thermocellum*	Disruption of six different chromosomal genes (*cipA, hfat, hyd, ldh, pta*, and *pyrF*)	Ethanol production was increased up to 56% (1.8 g/l)	Mohr et al., 2013
Avicel or Cellobiose (5 g/l)	*C. thermocellum*	Engineering the ‘‘malate shunt’’ pathway	3.25-fold higher ethanol yield than the wild type	Deng et al. 2013
Cellobiose (1% (w/v))	*C. thermocellum* 27405	Overexpression of β-glucosidase A	1.6 - 2.3 fold greater cellulase activity compared to the wild type	Maki et al., 2013
Cellulose	*C. cellulolyticum*	Expression of pyruvate decarboxylase and alcohol dehydrogenase from *Z. mobilis*	150% increase in cellulose consumption and a 53% increase in ethanol	Guedon et al., 2002
Switch grass, Cellobiose, cellulose	*C. cellulolyticum*	Disrupt of L-lactate dehydrogenase (*ldh*) and L-malate dehydrogenase (*mdh*) genes	Substantial shift toward ethanol production (93%), molar ratio of ethanol to organic acids of 15 *vs.* 0.18 in the wild-type cells, ethanol concentration increased 8.5-times more than the wild type	Li et al., 2012
Avicel (50 g/l)	*Thermoanaerobacterium saccharolyticum*	Knockout of genes involved in organic acid formation	Stable strain with more ethanol productivity (37 g/liter) as the only detectable organic product	Shaw et al., 2008
Urea and ammonium salts	*T. saccharolyticum*	Expression of genes encoding the enzyme urease	Enhanced urease activity, and 54 g/l ethanol production	Shaw et al., 2012
Glucose and xylose	*T. aotearoense*	Deactivation of the lactate dehydrogenase gene (*ldh*)	2.37- (81.67 mM) and 2.1-fold (75 mM) increases in the yield of ethanol (mole/mole substrate)	Cai et al., 2011
Undetoxified wet-exploded wheat straw hydrolysates	*Thermoanaerobacter mathranii*	Deletion of lactate dehydrogenase gene (*ldh*)	Resistance to up to 10 g/1 organic acids and inhibitors, and ethanol yield of 0.39–0.42 g/g (sugar efficiency to ethanol: 68–76%)	Georgieva et al., 2008
Xylose and glycerol	*T. mathranii*	Inactivation of lactate pathway and expression of a heterologous gene *gldA* encoding an NAD^+-dependent glycerol dehydrogenase	Shift of the cells metabolism toward the ethanol production and increase of ethanol yield	Yao and Mikkelsen, 2010
Switch grass (2%) (wt/vol))	*Caldicellulosiruptor bescii*	Deletion of the lactate dehydrogenase gene, and expression of a *C. thermocellum* bifunctional acetaldehyde/alcohol dehydrogenase	70% of the fermentation products were ethanol (12.8 mM ethanol directly from switch grass). Production of acetate was decreased by 38% compared to the wild type	Chung et al.. 2014
Cellobiose and cellulose	*Geobacillus thermoglucosidasius.*	Disruption of lactate dehydrogenase and pyruvate formate lyase genes and up-regulation expression of pyruvate dehydrogenase	Enhanced effective and rapid production of ethanol (90% of theoretical yield)	Cripps et al., 2009
Amorphous cellulose	*K. oxytoca*	Transferring *Z. mobilis* genes for ethanol production and genes encoding endoglucanases from *C. thermocellum*.	Enhanced accumulation of thermostable enzyme and hydrolysis of amorphous cellulose to cellobiose	Wood and Ingram, 1992
Amorphous cellulose	*K. oxytoca*	Integration of genes for ethanol production from *Z. mobilis* (*pdc*, *adhB*) and endoglucanase genes from *Erwinia chrysanthemi*	Enhanced high endoglucanase activity and ability to ferment amorphous cellulose to ethanol (58–76% of theoretical yield)	Zhou and Ingram, 2001
Filter paper and corncob residue	*T. reesei*	Expression of a cellobiase gene from *Aspergillus niger*	Enhanced 5.3 IU/ml cellobiase activity, and the FPase activity and saccharification activity on corncob residue was 44% and 21% higher than that of the host strain	Wang and Xia, 2011
Cellulose and cellubiose	*T. reesei*	T-DNA-tagged mutation: library of *T. reesei* by using *Agrobacterium*-mediated transformation.	31-51% higher cellulolytic activity compared to the parental strain	Zhong et al., 2012
Filter paper, CMC, pretreated corn stover hydrolysates	*T. reesei*	Heterologous expression of *cbh1* from *T. reesei* and *e1*, encoding an endoglucanase from *A. cellulolyticus*	FPase and CMCase were increased 39% and 30%, respectively, and concentration of reduced sugars was significantly increased by 169% at 60 °C	Zou et al., 2012
Cellulose	*T. reesei*	Constitutive expression of *xyr1* combined with down regulation of the negative regulator encoding gene *ace1*	103, 114, and 134 % more secreted protein levels, FPase and CMCase activity, respectively	Wang et al., 2013
Filter paper, CMC, pretreated corn stover	*T. reesei* Rut-C30.	Expression of novel cellulase gene (*exo2b*) and the *cbh1* gene	24% and 18% increase of filter paper activity and CMCase activity, respectively. 19.8% increase of glucose release from pretreated corn stover	Geng et al., 2012
Corn residue	*T. reesei*	Expression of *Trametes* sp. laccase gene *lacA* fused to cellobiohydrolase I signal peptide coding sequence	Enhancing recombinant laccase A activity and secretion which increased 31-72% reducing sugar yields	Zhang et al., 2012a
Corn stover and rice straw	*T. reesei* strain C10	Recombinant strain with enhanced cellobiohydrolase activity	Enhanced cellulase activity compared to the wild type	Fang and Xia, 2014
Glucose, Sugarcane bagasse,	*T. reesei* CICC 40360	Genome shuffling for ethanol production and tolerance	5-folds more ethanol than wild type (9.7 g/l ethanol from 50 g/l glucose, and 3.1 g/l ethanol from 50 g/l sugarcane bagasse	Huang et al., 2014
-	*F. oxysporum*	*A. tumefaciens*-mediated transformation (ATMT) to enhance alcohol tolerance (disruption of sugar transporter)	Enhanced tolerance to ethanol was increased more than 11.74%,	Hennessy et al., 2013
Straw, glucose and xylose	*F. oxysporum*	Overexpression of the sugar transporter (Hxt)	Enhanced the glucose and xylose transport capacity and ethanol yield (39% increase)	Ali et al., 2013
Corn cob and wheat bran	*F. oxysporum*	Expression of the endo-β-1,4-xylanase gene under control of the *gpdA* promoter	Higher extracellular xylanase activities (5–10%) and produced about 60% more ethanol compared to the wild type	Anasontzis et al., 2011

Table 6.
List of ethanologenic microorganisms engineered to be cellulolytic (Category II).

Substrate	Microorganism	Genes	Cellulase/ production system	Ethanol concentration/yield	References
PASC (10 g/l)	*S. cerevisiae*	*T. reesei* EGII, CBHII, *A. aculeatus* BGL1	Tethered (Cell surface display)	2.9 g/l	Fujita et al., 2004
PASC (10 g/l)	*S. cerevisiae*	*T. reesei* EGI, *S. fibuligera bgl*	Secretion	1 g/l	Den Haan et al., 2007a
PASC (10 g/l)	*S. cerevisiae*	*T. reesei* EGII, CBHII, *A. aculeatus* BGL1	Secretion	1.6 g/l	Yanase et al., 2010
PASC (10 g/l)	*S. cerevisiae*	*T. reesei* EGII, CBHII, *A. aculeatus* BGL1	Tethered	2.1 g/l	Yanase et al., 2010
PASC (10 g/l)	Consortium of four recombinant *S. cerevisiae* strains with different ratios	*C. thermocellum* endoglucanase, *C. cellulolyticum* exoglucanase, *T. reesei* CBHII, *T. aurantiacus* BGLI	Minicellulosome	1.25 g/l	Goyal et al. 2011
PASC (10 g/l)	Consortium of four recombinant *S. cerevisiae* strains with different ratios	*C. thermocellum* endoglucanase, *C. cellulolyticum* exoglucanase, *T. reesei* CBHII, *T. aurantiacus* BGLI	Minicellulosome	1.9 g/l	Tsai et al., 2010
PASC (10 g/l)	*S. cerevisiae*	*T. reesei* EGII, CBHII, *A. aculeatus* BGLI, *C. thermocellum* miniscaffoldin	Minicellulosome	1.8 g/l	Wen et al., 2010
PASC (20 g/l)	*S. cerevisiae*	*T. reesei* EGII, CBHII, *A. aculeatus* BGLI	Tethered	7.6 g/l	Yamada et al., 2011
PASC (10 g/l)	*S. cerevisiae*	*C. cellulyticum* celCCA, celCCE, Ccel_2454	Minicellulosome	1.1 g/l	Fan et al., 2012a
PASC (20 g/l)	*S. cerevisiae*	*T. reesei* EGII, CBHII, *A. aculeatus* BGLI *A. oryzae* Aoelpl	Tethered	3.4 g/l	Nakatani et al., 2013
PASC (25 g/l)	*S. pastorianus*	*T. reesei* EGI, CBHII,BGLI	Secretion	16.5 g/l	Fitzpatrick et al., 2014
PASC (10 g/l)	*E. coli*	*C. cellulyticum* Cel5A, Cel9E,BGL	Tethered	3.6 g/l	Ryu and Karim, 2011
Cellobiose (5 g/l)	*S. cerevisiae*	*Saccharomycopsis fibuligera* β-glucosidase gene (*bgl1*)	Tethered	2.3 g/l	van Rooyen et al., 2005
Cellulose (10 g/l)	*S. cerevisiae*	*T. reesei* EG1 (*cel7B*) and *S. fibuligera* β-glucosidase (*cel3A*).	Tethered	1 g/l	den Haan et al., 2007a
Pretreated corn stover (10%)	Industrial *S. cerevisiae* (K1-V1116)	*T. reesei* Endoglucanase, exoglucanase and β-glucosidase genes	Secretion	2.6% v/v (63% of theoretical yield)	Khramtsov et al., 2011
Pretreated corn stover	*S. cerevisiae*	*T. reesei* Endoglucanase, exoglucanase and β-glucosidase genes	Secretion	2.6% (v/v) (63% of theoretical value)	McBride et al., 2010
PASC (10 g/l) and Avicel	*S. cerevisiae*	*C. cellulolyticum* celCCA (endoglucanase), a celCCE (cellobiohydrolase), and a Ccel_2454 (β-glucosidase)	Minicellulosome	1.4 g/l	Fan et al., 2012a
Avicel (10g/l)	Consortium of four recombinant *S. cerevisiae* strains	*C. cellulolyticum* celCCA (endoglucanase), a celCCE (cellobiohydrolase), and a Ccel_2454 (β-glucosidase)	Minicellulosome	1.14 mg/l	Fan et al., 2013
PASC (10 g/l)	Consortium of four recombinant *S. cerevisiae* strains with different ratio	Displaying a scaffoldin (mini CipA) containing three cohesin domains, endoglucanase (CelA), exoglucanase (CBHII) or β-glucosidase (BGLI)	Minicellulosome	1.80 g/l	Kim et al., 2013
Rice straw (high-solid (200 g/l))	*S. cerevisiae*	Cell surface display of fungal endoglucanase, cellobiohydrolase, and β-glucosidase and cell recycle batch fermentation system (CRBF)	Tethered	42.2 g/l (86.3% of theoretical yield)	Matano et al., 2012, 2013
Avicel (8% (w/v))	*S. cerevisiae*	Expression of a cellodextrin transporter and an intracellular β-glucosidase from *N. crassa.*	Secretion	27 g/l	Lee et al., 2013
PASC (10 g/l)	*S. cerevisiae*	Co-expression (Cell surface display) of cellulases and a cellodextrin transporter	Tethered	4.3 g/l	Yamada et al., 2013
Inulin from Jerusalem artichoke tuber flour	*S. cerevisiae*	Expression of an endoinulinase gene from *A. niger*	Secretion	55.3 g/l (89.0% of theoretical yield)	Yuan (B) et al., 2013
Xylose	*S. cerevisiae*	Overexpressing of the genes encoding xylose isomerase, xylulose kinase (*xk*), and the non-oxidative pentose phosphate pathway enzymes	Secretion	Ethanol yields: 0.41 g/g (1.866 g/gh)	Zhou et al., 2012
Birchwood xylan	*S. cerevisiae*	Expression of *β*-xylosidase and xylanase II genes (co-displayed on the cell-surface)	Tethered	0.30 g/g (productivity rate: 0.13 g/lh)	Katahira et al., 2004
Rice straw hydrolysate	*S. cerevisiae*	Expression of endoxylanase from *T. reesei*, β-xylosidase from *A. oryzae*, β-glucosidase from *A. aculeatus*, xylose reductase and xylitol dehydrogenase from *P. stipitis* and xylulokinase from *S. cerevisiae*.	Tethered	0.32 g/g of total sugars consumed (productivity rate 0.37 g/lh)	Sakamoto et al., 2012
Birchwood xylan	*S. cerevisiae*	Miniscaffoldin harboring xylanase II, arabinofuranosidase, and *β*-xylosidase	Mini-hemicellulosome	1 g/l (ethanol yield: 0.31 g ethanol/g)	Sun et al., 2012
Birchwood xylan	*S. cerevisiae*	Assembling five trimeric xylanosome on three dockerin-tagged fungal enzymes, including endoxylanase (XynAc), β-xylosidase (XlnDt) and acetylxylan esterase (AwAXEf).	Xylanosomes	Xylanase activity was increase up to 3.3 times more than free enzymes after 72 h hydrolysis	Srikrishnan et al., 2013
Cellobiose or CMC (100 g/l)	*K. marxianus*	*Thermoascus aurantiacus* cellulase genes, including cellobiohydrolase 1 (*cel7A*), endoglucanase 1 (*cel5A*) and β-glucosidase (*bgl3A*) genes	Secretion	43.4 g/l ethanol	Hong et al. 2007

Table 6. (Continued)

Substrate	Microorganism	Genes	Cellulase/ production system	Ethanol concentration/yield	References
Cellulosic β-glucan (10 g/l)	*K. marxianus*	Displaying *T. reesei* endoglucanase and *A. aculeatus* β-glucosidase on cell surface	Tethered	4.24 g/l (yield: 0.47 g ethanol/g substrate)	Yanase et al., 2010
Cellobiose	*K. marxianus*	β-glucosidase	Secretion	28 g ethanol/100 g cellobiose	Matsuzak et al., 2012
Jerusalem artichoke tubers and inulin	*K. marxianus*	Inulinase gene *INU* under endogenous promoter	Secretion	Ethanol: 96.2 g/l and 1.34 g/l/h from inulin and 69 g/l and 1.44 g/l h from Jerusalem artichoke tubers	Yuan et al., 2013
Avicel (10g/l)	*K. marxianus*	Two cellobiohydrolases, two endo-β-1,4-glucanases and one beta-glucosidase genes from different fungi, and a fungal cellodextrin transporter gene	Secretion	0.42 g/l and 0.6 g/l after 2 and 5 d, respectively.	Chang et al., 2013
Xylose	*H. polymorpha*	Deletion of *XYL1* coding xylose reductase and two paralogs of xylitol dehydrogenase *XYL2A* and *XYL2B*, and expression of *E. coli* or *Streptomyces coelicolor* gene *xylA*	Secretion	0.15 g /l	Voronovsky et al., 2005
Xylose	*H. polymorpha*	Overexpression of *E. coli xylA* with *H. polymorpha XYL3* coding xylulokinase	Secretion	0.6 g/l	Dmytruk et al., 2008a
Xylose	*H. polymorpha*	Overexpression of three xylose reducatse genes, including the modified *XR* (*XYL1m*), native *XDH* (*XYL2*) and *XK* (*XYL3*)	Secretion	1.3 g/l	Dmytruk et al., 2008b
Xylose	*H. polymorpha*	Overexpression of the gene PDC1 coding for pyruvate decarboxylase (PDC) in the mutant strain $2EthOH^-$	Secretion	2.5 g /l	Ishchuk et al., 2008
Starch and Birchwood xylan	*H. polymorpha*	Coexpression of genes *SWA*2 and *GAM*1 from the yeast *Schwanniomyces occidentalis*, encoding α-amylase and glucoamylase or co-expression *T. reesei xyn11B* (endoxylanase) and *A. niger xlnD* (β-xylosidase)	Secretion	Up to 10 g/l	Voronovsky et al., 2009
Xylose	*H. polymorpha*	Overexpression of *H. polymorpha* genes *XYL1m, XYL2, XYL3* and *PDC1* in the strain $2EtOH^-$, and next mutant screening for resistance to the anticancer drug 3-bromopyruvate)	Secretion	Up to 10 g/l	Kurylenko et al., 2014
Sweet potato starch (1.5%)	*Z. mobilis*	Four fused glucoamylase genes from *Aspergillus awamori* more than that in the parental strain.	Secretion	14.73-folds more than wild type strain (92.69% of the theoretical yield)	Ming-xiong et al., 2009
Cellobiose (40 g/l)	*E. coli* strain MS04	Display the β-glucosidase BglC from *Thermobifida fusca*	Tethered	81% of the theoretical maximum.	Muñoz-Gutiérrez et al., 2012
Cellobiose	*E. coli*	Pyruvate decarboxylase gene *pdc*, alcohol dehydrogenase gene *adhB* from *Z. mobilis* and β-glucosidase gene *bglB* from *Bacillus polymyxa*	Secretion	33.99% of theoretical yield.	Lue et al., 2014
Xylose (11.4%)	*P. stipitis*	Mutation	-	4.4%	Watanabe et al., 2011
Xylose from steam-pretreated and enzymatically hydrolyzed poplar hydrolysate,	*P. stipitis*	Genome-shuffling	-	0.39–1.4% (w/v)	Bajwa et al., 2011
Xylose	*P. stipitis*	Genome-shuffling	-	2.6% v/v	Shi et al., 2014

However, as *C. cellulolyticum* prefers mesophilic temperature, in contrast to *C. thermocellum* which prefer thermophilic temperatures, it is possible to use it in co-culture systems with other more robust mesophiles, such as *Z. mobilis* for ethanol production (Schuster and Chinn, 2013). Guedon et al. (2002) could decrease accumulation of pyruvate, which is responsible for the cessation of growth, by heterologous expression of pyruvate decarboxylase and alcohol dehydrogenase from *Z. mobilis* in *C. cellulolyticum*. The recombinant strain showed a 150% increase in cellulose consumption and a 180% increase in cell dry weight after 145 h in comparison with the wild type strain. Lactate production decreased by 48%, whereas the concentrations of acetate and ethanol increased by 93 and 53%, respectively. Li et al. (2012) firstly developed a targeted gene inactivation system for *C. cellulolyticum*, in which they used a markerless targeted mutagenesis system to disrupt both the paralogous L-lactate dehydrogenase (*ldh* (Ccel_2485)) and L-malate dehydrogenase (*mdh* (Ccel_0137)) genes in a single strain. This modification resulted in a substantial shift in fermentation toward ethanol production in which ethanol constituted 93% of the major fermentation products (molar ratio of ethanol to organic acids of 15, *vs.* 0.18 in the wild-type cells) when cellobiose, cellulose and switch grass were used. It also resulted in enhanced ethanol production by 8.5-times compared to the wild-type cells growing on crystalline cellulose. Metabolomic studies showed that in the mutant strain the flux was increased through the oxidative branch of the mutant's tricarboxylic acid pathway.

2.3.4.3.1.3. Thermoanaerobacterium saccharolyticum

In spite of the advantages mentioned before for *Thermoanaerobacterium* species, ethanol production by these bacteria suffers from low productivity, yield, and final product concentration and purity (Lin and Tanaka, 2006). These disadvantages are caused by the many byproducts derived from the branch pathways including various organic acids, such as acetic and lactic acids, which in turn result in salt accumulation during pH-controlled fermentations, and consequent hindrance in ethanol production by these bacteria (Lynd et al., 2001, 2002; Cai et al., 2011). So, engineering *Thermoanaerobacterium* species to overcome these problems could be a promising strategy to commercialize ethanol production by these organisms.

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As *T. saccharolyticum* is capable to effectively hydrolyze xylan, it has been proposed to genetically engineer it to produce higher levels of ethanol. To do this, it was suggested that the genes involved in production of organic acid byproducts during fermentation be knocked out to achieve more ethanol formation as the major product (Shaw et al., 2008; Schuster and Chinn, 2013). Shaw et al. (2008) confirmed that knock-out of genes involved in organic acid formation (acetate kinase, phosphate acetyltransferase, and L-lactate dehydrogenase) resulted in a stable strain with growth rate similar to the wild type parent with more ethanol productivity (37 g/l) as the only detectable organic product and substantial changes in electron flow relative to the wild type. In addition, it was proposed to use this genetically-modified *T. saccharolyticum* in a co-culture system with a cellulolytic ethanologen for maximal biomass conversion in CBP, because of its strong xylose fermentation capabilities (Argyros et al., 2013; Schuster and Chinn, 2013). Shaw et al. (2011) developed a marker removal strategy for *T. saccharolyticum* to select against the *pyrF*, *pta* and *ack* genes. In this system, the *pta*- and *ack*-based haloacetate selective strategy was subsequently used to create a markerless Δldh Δpta Δack strain producing ethanol at a high yield. In another investigation, Shaw et al. (2012) developed a recombinant *T. saccharolyticum* strain containing genes encoding the enzyme urease. The engineered strain showed urease activity, hydrolyzed urea and increased cellular growth when urea was used as minimal medium. Replacement of ammonium salts with urea resulted in production of 54 g/l ethanol by the transgenic strain. They proposed that this increase may be ascribed to reduced pH, salt, and osmolality stresses during fermentation (Shaw et al., 2012).

2.3.4.3.1.4. Thermoanaerobacterium aotearoense

Another thermophilic anaerobic bacterium recently considered as a desirable biological catalyst for the conversion of cellulosic biomass to ethanol is *T. aotearoense*. Similar to *T. saccharolyticum*, ethanol production by this species suffers from low ethanol productivity because of the byproducts produced during the process. Cai et al. (2011) isolated a *T. aotearoense* strain with extant xylan-digesting capability which could ferment a wide spectrum of carbohydrates (xylose and arabinose) from natural hot spring in the south of China. To enhance ethanol production in this species, they deactivated the lactate dehydrogenase gene (*ldh*) involved in lactic acid production *via* homologous recombination in *T. aotearoense*. The obtained mutant strain exhibited 31.0% and 31.4% more microbial biomass and 2.37-(81.67 mM) and 2.1-fold (75 mM) increases in the yield of ethanol (mole/mole substrate) under glucose and xylose cultivation, respectively, compared to the wild type (32 mM ethanol). Moreover, no lactic acid was detected in Δldh mutant fermentation mixtures, whereas it was readily detected in the cultures of the wild type strain (Cai et al., 2011).

2.3.4.3.1.5. Thermoanaerobacter mathranii

T. mathranii is a thermophilic anaerobic and xylanolytic bacterium which can produce ethanol from lignocellulosic biomass at high temperatures. This bacterium has the ability to ferment all sugars contained in the lignocellulosic materials at high temperatures with high productivities, high conversion rates, low risk of contaminations and easy products recovery (Mikkelsen and Ahring, 2007; Yao and Mikkelsen, 2010). Similar to other *Thermoanaerobacter* species, its exploitation for ethanol production will require metabolic engineering to reduce byproducts and to increase its ethanol yield and tolerance. Georgieva et al. (2008) developed a mutant strain of *T. mathranii (BG1L1)* in which the lactate dehydrogenase gene (*ldh*) was deleted to increase ethanol production and tolerance from undetoxified wet-exploded wheat straw hydrolysates. The mutant strain showed an effective resistance to high concentrations of acetic acid and other metabolic inhibitors (up to 10 g/1), and ethanol yield reached 0.42 g/g. In another study, Yao and Mikkelsen (2010) expressed a heterologous gene *gldA* encoding an NAD^+-dependent glycerol dehydrogenase to facilitate NADH regeneration in ethanol formation in *T. mathranii* strain BG1L1 in which the *ldh* gene encoding lactate dehydrogenase was deleted previously to eliminate an NADH oxidation pathway (Georgieva et al., 2008). They produced a new recombinant *T. mathranii* strain BG1G1 (*Δldh*, P_{xyl}GldA) with an inactivated lactate pathway expressing glycerol dehydrogenase. These changes caused a shift in cells metabolism toward the production of ethanol over acetate and restored the redox balance. The recombinant strain showed an increased ethanol yield in the presence of glycerol using xylose as a substrate. Also, the strain was capable to utilize glycerol as an extra carbon source in the presence of xylose, and utilization of more reduced substrate i.e. glycerol resulted in a higher ethanol yield (Yao and Mikkelsen, 2010).

2.3.4.3.1.6. Caldicellulosiruptor bescii

C. bescii which is a thermophilic, anaerobic, cellulolytic bacterium, commonly grows optimally at 80 °C. This bacterium is the most thermophilic cellulolytic bacterium described so far, and is capable of using and fermenting a wide range of substrates, e.g. cellulose, hemicellulose, and lignocellulosic plant biomass without harsh and expensive chemical pretreatment (Blumer-Schuette et al., 2008; Argyros et al., 2011; Chung et al., 2014). Recently, a mutant strain of *C. bescii* (JWCB018) was isolated in which the lactate dehydrogenase gene (*ldh*) was disrupted spontaneously *via* insertion of a native transposon (Cha et al., 2013a; Chung et al., 2013). This research group in another attempt could completely delete the *ldh* gene by genetic engineering, which resulted in diverting metabolic flux to additional acetate and H_2 instead of lactate production (Cha et al., 2013b). Chung et al. (2014) developed a genetically-engineered strain of *C. bescii* with an ability for direct conversion of switch grass, a non-food renewable feedstock, to ethanol without conventional pretreatment of the biomass. They deleted the lactate dehydrogenase gene, and expressed a heterologous *C. thermocellum* bifunctional acetaldehyde/alcohol dehydrogenase in *C. bescii*. As a result of this strategy, 70% of the fermentation products in the engineered strain were ethanol (12.8 mM ethanol directly from 2% (w/v) switch grass), whereas the wild-type *C. bescii* lacked the ability to produce ethanol. Also, the production of acetate was decreased in the engineered strain by 38% compared to the wild type (Chung et al., 2014).

2.3.4.3.1.7. Geobacillus thermoglucosidasius

The *Geobacillus* species are aerobic or facultatively anaerobic, Gram-positive, thermophilic bacilli isolated from a wide range of environments, including temperate soils, as well as natural and artificial hot environments (Nazina et al., 2001; Burgess et al., 2010; Suzuki et al., 2012). These microorganisms have great environmental adaptability, including ethanol tolerance (Fong et al., 2006) and the ability to degrade long-chain alkanes (Wang et al., 2006). These bacteria have been considered as suitable candidates for ethanol production at high temperatures. Performing bioprocesses at high-temperatures results in reduction of contamination risk, low energy consumption for agitation and cooling, and easy removal of volatile products (Wiegel and Ljungdahl, 1983; Cripps et al., 2009; Taylor et al., 2009; Suzuki et al., 2012). Some *G. thermoglucosidasius* strains (e.g. M10EXG) are considered as a potential strain for ethanol production, because they are tolerant to 10% (v/v) ethanol (Fong et al. 2006; Tang et al., 2009), and they also can utilize a wide range of substrates, including pentose (C_5) and hexose (C_6) (Riyanti and Rogers, 2013). Cripps et al. (2009) used a metabolic engineering methodology to divert fermentative carbon flux from a mixed acid pathway to one ethanol production pathway, and therefore, reduced the byproducts quantity and increased ethanol production in *G. thermoglucosidasius.* They disrupted the *ldh* (lactate dehydrogenase) and *pfl*B (pyruvate formate lyase) genes and upregulated the expression of pyruvate dehydrogenase. The mutant strains containing all three modifications could effectively and rapidly produce ethanol (90% of the theoretical yield) at temperatures more than 60 °C. In addition, one of these strains (TM242) efficiently fermented cellobiose and a mixed hexose and pentose feed (Cripps et al., 2009).

2.3.4.3.1.8. Klebsiella oxytoca

The Gram-negative bacterium, *K. oxytoca*, has the native ability to transport and metabolize cellobiose, and therefore, does not need extracellular cellobiase application. Previously, recombinant strains of *K. oxytoca* containing *Z. mobilis pdc* (pyruvate decarboxylase) and *adhB* (alcohol dehydrogenase) genes have been developed. These strains are able to direct the metabolism of pyruvate to ethanol, but for efficient ethanol production they need cellulase to be added to the cellulosic materials, which imposes additional cost on the ethanol production process (Ohta et al., 1991b; Doran and Ingram, 1993; Bothast et al., 1994; Doran et al., 1994; Brooks and

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Ingram, 1995; Golias et al., 2002). In order to overcome this problem, Tran et al. (2013) developed a single-step process for converting lignocelluloses to ethanol using a co-culture of mesophilic *Bacillus sp.* THLA0409 (as a cellulose-degrading bacterium) and the strain *K. oxytoca* THLC0409 (as a sugars-utilizing bacterium). This significantly enhanced the utilization efficiency of hydrolysates from acid-pretreated raw bamboo, napiergrass, rice and straw resulting in ethanol production rates of 145, 276, and 219 g/kg substrate used, respectively. In some other studies, the focus has been on transferring cellulase genes from other microorganisms to give cellulase activities to the recombinant strains of *K. oxytoca.* Wood and Ingram (1992) developed a dual recombinant strain of *K. oxytoca* containing the *Z. mobilis* genes for ethanol production and genes encoding endoglucanases from *C. thermocellum*. The selected recombinant strain (P2) containing *celD* could accumulate the thermostable enzyme and hydrolyze amorphous cellulose to cellobiose, and could also produce ethanol in a two-stage process. The results showed that the recombinant strain needed less commercial cellulase for ethanol production. Zhou and Ingram (2001) developed a recombinant strain *K. oxytoca* containing chromosomally-integrated genes for ethanol production from *Z. mobilis* (*pdc*, *adhB*) and endoglucanase genes from *Erwinia chrysanthemi* (*celY*, *celZ*) with high endoglucanase activity and ability to ferment amorphous cellulose to ethanol (58–76% of the theoretical yield) without the addition of cellulase enzymes from other organisms.

The 2,3-butanediol is a valuable chemical which is usually produced petrochemically, but can be synthesized by bacteria as well. Recently, efficient production of this chemical has been performed in a genetically-improved *K. Oxytoca* strains (Cheng et al., 2010; Kim et al., 2013). To date, *K. pneumonia* and *K. oxytoca* are considered as the most powerful 2,3-butanediol producers as they can consume different substrates, such as cellulose and hemicellulose contained in lignocellulosic biomass (Cheng et al., 2010; Kim et al., 2013; Guo et al., 2014).

2.3.4.3.1.9. Trichoderma reesei

As mentioned in the previous sections, one of the most challenging barriers in commercialization of ethanol production from biomass is the cost of high enzyme loadings (30–50 mg enzyme/g crystalline cellulose) for complete saccharification of pretreated biomass. The maximum cellulase enzyme production has been reported by fungi, especially *T. reesei* (more than 100 g/l culture broth), whereas the most cellulase productivity in bacteria such as *Clostridia* is only a few grams per liter (Cherry and Fidantsef, 2003; Xu et al., 2009). In addition to the high enzyme concentration productivity, fungi are able to efficiently secrete the produced enzyme because of their robust secretion system (endoplasmic reticulum (ER) and the Golgi complex), whereas this is not possible in most of the candidate bacteria. Hence, *T. reesei* is considered as one of the most promising candidate fungi for CBP ethanol production. Beside the fact that these fungi are able to produce the cellulase enzyme complex in sufficient quantities, they can also be grown at low cost in high quantities. Moreover, all the metabolic pathways for utilization of lignocellulose sugars to produce ethanol exist in these fungi, some new pathway controls are required though.

These fungi are able to saccharify the lignocellulosic materials to simple sugars in aerobic conditions, and then ferment them to ethanol in anaerobic conditions (Xu et al., 2009; Amore et al., 2012). Xu et al. (2009) confirmed the presence of the major metabolic pathways for converting lignocellulosic sugars to ethanol (i.e. glycolysis, pentose phosphate pathway, xylose and arabinose assimilation pathways, and ethanolic fermentation) in *T. reesei*. they also confirmed the presence of all the genes involved in these pathways through the data achieved from the genome sequencing of the fungus. Furthermore, they reported that this fungus was able to survive up to 13 d (Xu et al., 2009). Another advantage of *T. reesei* in CBP is that cellobiose does not accumulate during fermentation owing to the cellobiase activity of the fungus (Rabinovich et al., 2002; Xu et al., 2009; Amore et al., 2012). In spite of these advantages, there are some challenging problems in application of *T. reesei* as CBP organism for ethanol production from biomass. The first one is that ethanol yield, productivity and tolerance are low, and also because of its filamentous cell morphology, mixing and aeration during fermentation is more difficult and may require more energy consumption. These disadvantages are probably related to the low expression or low activity of the enzymes involved in ethanol production pathways (Xu et al., 2009; Amore et al., 2012). Furthermore, the low tolerance of *T. reesei* to ethanol in high ethanol concentration is ascribed to the hindrance of glycolysis resulting in inhibition of cell growth. Moreover, as *T. reesei* is known as an obligate aerobe, the genes encoding the required enzymes for glycolysis are repressed in the absence of oxygen, and therefore, cells growth will be stopped in the anaerobic conditions (Bonaccorsi et al., 2006; Amore et al., 2012).

Up to now, different methodologies such as protoplasting-based transformation (Gruber et al., 1990), *Agrobacterium*-mediated transformation (Zhong et al., 2006) and biolistic transformation (Te et al., 2002) have been developed for efficient genetic manipulation of *T. reesei* strains. In addition, different selectable markers, such as hygromycin (Mach et al., 1994), benomyl resistance (Peterbauer et al., 1992; Schuster et al., 2007), *amdS* gene from *Aspergillus nidulans*, conferring ability to grow on acetamide as sole nitrogen source (Penttila et al., 1987), the auxotrophic markers *pyr4* (Gruber et al., 1990) and *hxk1* (Guangtao et al., 2010) have been successfully used for genetic manipulation of *T. reesei* strains (Amore et al., 2012).

Taking into account the above-mentioned challenges, strategies to be used to overcome these problems are as follows: (a) development of *T. reesei* strains with high ability to grow in anaerobic conditions, (b) improvement of its ethanol production, (c) increasing its ethanol tolerance, and (d) improvement for hyperproduction of cellulase by *T. reesei* strains. These strategies are possible to be performed by different techniques, such as screening wild-type isolates, random and targeted mutagenesis for selection of strains able to grow in the absence of oxygen for longer times, introducing heterologous genes, such as *S. cerevisiae* pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase (*adh*) genes to construct ethanol biosynthesis pathway in the fungus, knockouting genes responsible for byproducts production in *T. reesei*, and/or engineering or increasing the number of sugar transporters to enhance sugar utilization at low concentration and simultaneous use of all biomass sugars. Ethanol tolerance could be improved by identification and engineering of the genes and transcription factors responsible for ethanol tolerance (Xu et al., 2009; Amore et al., 2012).

Recently, some investigations have been devoted to mutation breeding or genetic engineering of *T. reesei* to enhance cellulase and cellobiase activity of the fungus as CBP organism. For example, Wang and Xia (2011) using the modified PEG–$CaCl_2$ method expressed a cellobiase gene from *Aspergillus niger* in *T. reesei*. This resulted in 5.3 IU/ml cellobiase activity after 48 h fermentation, which was 106 times more than that of the host strain. Meanwhile, the filter paper activity and saccharification activity of the recombinant *T. reesei* (on corn cob residue) were recorded 44% and 21% higher than those of the host strain. Zhong et al. (2012) to improve cellulase production developed a T-DNA-tagged mutant library of *T. reesei* using *Agrobacterium*-mediated transformation. Three putative mutants showed 31-51% higher cellulolytic activity compared to the parental strain. Moreover, endoglucanase, cellobiohydrolase and β-glucosidase activities as well as the hydrolysis efficiencies of the mutants were improved. Zou et al. (2012) replaced *cbh1* promoter CREI binding sites of *T. reesei* (which is used by repressor CREI reducing the strength of the promoter) by the binding sites of transcription activator ACEII and the HAP2/3/5 complex to improve the promoter efficiency. They constructed a hybrid heterologous gene, containing *cbh1* gene from *T. reesei* and *e1*, encoding an endoglucanase from *Acidothermus cellulolyticus* linked by a flexible polyglycine linker and a rigid a-helix linker. These modifications resulted in 39% and 30% increases in the filter paper (FPase) and CMCase activities, respectively, compared to the parental strain, and when the crude enzyme mixture obtained from the recombinant strain was used, the concentration of reduced sugars in the pretreated corn stover hydrolysates was significantly increased by 169% at 60°C .

In another research work, constitutive expression of *xyr1* (positive regulator gene) combined with down-regulation of the negative regulator encoding gene *ace1* resulted in 103, 114, and 134% more secreted protein levels, FPase and CMCase activity, respectively (Wang et al., 2013). In a different work, a hybrid fused sequence, containing a novel cellulase gene isolated from metagenomic library (*exo2b*) and the *cbh1* gene, was expressed in *T. reesei* Rut-C30. The recombinant strain showed 24 and 18% increase in filter paper activity and CMCase activity, respectively. When pretreated corn stover was used as carbon source by the transformant, the released glucose concentration was increased by 19.8% compared to the parent (Geng et al., 2012). The expression of *Trametes* sp. AH28-2 laccase gene *lacA* fused to cellobiohydrolase I signal peptide coding sequence in *T. reesei*, resulted in two recombinant strains i.e. L8 and L38 with an ability to secrete recombinant

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laccase A. Reducing sugar yields obtained through corn residue hydrolysis by crude enzyme of the strains L8 and L38 increased by 31.3 and 71.6%, respectively, compared to the wild-type strain (Zhang et al., 2012a). Fang and Xia (2015) developed fed-batch fermentation for a recombinant *T. reesei* strain C10 with enhanced cellobiohydrolase activity. They showed that C10 cellulase showed better yields in the enzymatic hydrolysis of both corn stover and rice straw.

Enhanced ethanol tolerance in *T. reesei* as a candidate for CBP microorganism has also investigated. Huang et al. (2014) could increase ethanol production of *T. reesei* CICC 40360 by genome shuffling while simultaneously enhancing the ethanol resistance. They produced an initial mutant population by nitrosoguanidine treatment of the spores of *T. reesei*, and then constructed an improved population producing more than 5 folds ethanol than the wild type by genome shuffling. The strain HJ48 showed the maximum ethanol productivity of 9.7 g/l ethanol from 50 g/l glucose, and also could efficiently convert lignocellulosic sugars to ethanol (ethanol yields/g sugarcane bagasse: 0.10 g/g) under aerobic conditions and was tolerant to up to 4% (v/v) ethanol stress. This strain also showed the maximum FPase and endoglucanase activity of 0.34 and 3.25 IU/ml, respectively, which were 1.8 and 2.1-fold higher than the parental strain (0.19 and 1.55 IU/ml, respectively).

2.3.4.3.1.10. Fusarium oxysporum

In addition to *Trichoderma*, some other fungi belonging to the genera *Aspergillus*, *Fusarium*, *Monilia*, *Rhizopus*, *Neurospora* and *Paecilomyces* have been reported to hold the ability to directly ferment cellulose to ethanol (Xue et al., 2009; Amore et al., 2012; Ferreira et al., 2013; Zerva et al., 2014). Recently, a few investigations were focused on genetic improvement of these fungi to enhance efficient CBP ethanol production. The crop pathogen *F. oxysporum* is considered as a promising CBP organism due to its innate ability of co-saccharification and fermentation of lignocelluloses to ethanol. Low alcohol tolerance is one of the major challenges for this fungus as a CBP microorganism. Hennessy et al. (2013) used *A. tumefaciens*-mediated transformation (ATMT) to enhance alcohol tolerance in *F. oxysporum* strains. They constructed a random mutagenesis library of gene disruption transformants. By screening the mutant strains, tolerance to 6% ethanol was increased by more than 11%, whereas tolerance to 0.75% n-butanol decreased by 43% compared to the wild–type. Molecular analysis confirmed that a coding region homologous to a putative sugar transporter (FOXG_09625) was disrupted.

Ali et al. (2013) identified a novel hexose transporter (Hxt) in *F. oxysporum* which could positively affect sugar uptake by *F. oxysporum* and would enhance the ethanol yields from lignocellulosic biomass. Overexpression of the sugar transporter (Hxt) in *F. oxysporum* significantly enhanced the glucose and xylose transport capacity and ethanol yield (39% increase), when straw, glucose and xylose were used as carbon source. In another attempt to enhance ethanol productivity, the endo-β-1,4-xylanase 2 gene was incorporated into the *F. oxysporum* genome under control of the *gpdA* promoter using *Agrobacterium*-mediated transformation (Anasontzis et al., 2011). Two transformants showed marginally higher extracellular xylanase activities (5–10%) compared to the wild type, a significant increase in xylanase transcript was observed though. These strains produced about 60% more ethanol compared to the wild type on corn cob, while for wheat bran this increase was observed only for one of the strains (Anasontzis et al., 2011).

2.3.4.3.2. Engineering an ethanologen to be cellulolytic

The majority of research on designing and optimizing CBP candidates has been focused on the recombinant expression of cellulase genes in natural ethanologenic microorganisms (**Table 6**). Commonly, cellulose hydrolysis occurs through the cooperation of three groups of gluconases, including endoglucanases (EGs) with activity on the amorphous regions of cellulose for production of free chain ends, exoglucanases (cellodextrinases and cellobiohydrolases (CBHs)) with activity on crystalline cellulose to release cellobiose from free chain ends, and β-glucosidases (BGLs) which hydrolyze cellobiose and small cello-oligosaccharides to glucose. It is generally accepted that these enzymes act sequentially and synergistically (Lynd et al., 2002; Zhang et al., 2004; den Haan et al., 2013; Kricka et al., 2014). In addition to cellulases, some other recently-described enzymes, such as the copper-requiring polysaccharide monooxygenases which have synergy with the exo- and endoglucanases, and elastin-like proteins which enhance access of the cellulases to the cellulose chains ends, are involved in lignocellulose hydrolysis (Leggio et al., 2012; Kubicek, 2013; Kricka et al., 2014; Nakatani et al., 2013). Cellulase enzymes are naturally produced by a variety of fungi such as *Trichoderma*, *Aspergillus*, *Talaromyces*, and several anaerobic bacteria such as species of the *Clostridium* and *Ruminococcus* genera, which can be used as sources of cellulase genes for enhancing cellulase activity in yeasts (Martinez et al., 2008; Fontes and Gilbert, 2010; Kubicek, 2013; Kricka et al., 2014). To achieve an efficient and complete hydrolysis of lignocelluloses, at least one copy of each of the cellulase genes must be functionally expressed in the ethanologenic host organism (e.g. *S. cerevisiae*) (Kricka et al., 2014). Recently, different ethanologenic microorganisms, such as *S. cerevisiae*, *K. marxianus*, *H. polymorpha*, *Z. mobilis*, *E. coli*, *P. stipitis* and *F. velutipes* have been engineered to be cellulolytic. In the following sub-sections, the recombinant strategies used to enhance cellulase activities in these ethanologenic microorganisms are discussed (**Table 6**).

2.3.4.3.2.1. Saccharomyces cerevisiae

As wild-type *S. cerevisiae* strains do not produce hydrolases with efficient activities on lignocellulosic biomass, so to use this yeast as a suitable CBP organism, genetic engineering for enhancing hydrolase activities is necessary. Different genetic engineering strategies used to design *S. cerevisiae* strains with enhanced hydrolase activities are discussed herein.

- Expression of single or many hydrolase genes in S. cerevisiae

During the last two decades, many genes encoding glycoside hydrolases (i.e. cellulases, hemicellulases, β-D-glucosidases) as well as xylose-utilizing and arabinose-utilizing enzymes from various species have been introduced into *S. cerevisiae*, and as a result, some engineered strains that can grow on biomass containing cellulose, hemicellulose, cellobiose, xylose, or arabinose have been reported (Becker et al., 2003; Fujita et al., 2004; Katahira et al., 2004, 2006; van Rooyen et al., 2005; Karhumaa et al., 2006; Van Zyl et al., 2007; den Haan et al., 2007a,b, 2013; Xu et al., 2009; Tsai et al., 2010; Wen et al., 2010; Yamada et al., 2011; Fan et al., 2012b; Nakatani et al., 2013) (**Table 6**). van Rooyen et al. (2005) designed recombinant strains of *S. cerevisiae* expressing a β-glucosidase gene (*bgl1*) from *Saccharomycopsis fibuligera*. The new strain could grow on and ferment cellobiose at the same rate as on glucose. The final achieved ethanol yield by the recombinant strain was 2.3 g/l from 5 g/l cellobiose, compared to 2.1 g/l when glucose (5.26 g/l) was used as substrate. In another attempt, a recombinant strain of *S. cerevisiae* with *β*-glucosidase and an exo/endocellulase activity was produced, and as a result, external application of cellulase was significantly reduced (Cho et al., 1999). den Haan et al. (2007a) produced a recombinant *S. cerevisiae* strain co-expressing the *T. reesei* EG1 (*cel7B*) and *S. fibuligera* β-glucosidase (*cel3A*) with an ability to grow on and convert 10 g/l cellulose to ethanol up to 1 g/l.

In a different study, Jeon et al. (2009) designed a similar recombinant strain expressing *C. thermocellum* endoglucanase and *S. fibuligera* β-glucosidase genes which showed significantly higher endoglucanase activity and higher cellulose conversion to ethanol. Yamada et al. (2010a) developed a genetic engineering system to integrate a cocktail of cellulase genes through multi-copy *δ*-integration, to obtain a CBP strain with high hydrolase activity. They transferred different expression cassettes containing three main cellulases (endoglucanase, exoglucanase and b-glucosidase) into the yeast chromosomes in one step. The selected strain showed significantly higher and optimum ratio of cellulases activities (64.9 mU g/l-wet cells) compared to the wild type strain (57 mU g/l-wet cells) when grown on phosphoric acid swollen cellulose as carbon source. Khramtsov et al. (2011) engineered industrial *S. cerevisiae* yeast strain (K1-V1116) by transferring genes encoding three main cellulases into the chromosomal ribosomal DNA and delta regions. The engineered cellulolytic yeast produced ethanol in one step through SSF from pretreated corn stover with 63% efficiency and the ethanol titer of 2.6% v/v. In spite of the efforts devoted to designing recombinant *S. cerevisiae* strains containing *T. reesei* cellobiohydrolases (cellobiohydrolase I and cellobiohydrolase II), the expression of these genes in the yeast were generally poor, and the results obtained were unable to demonstrate crucial

levels of crystalline cellulose conversion required to confirm true cellulase activity i.e. activity needed for biomass conversion (Chow et al., 1994; Takada et al., 1998; Hong et al. 2003; Xu et al., 2009).

- *Application of new promoters to increase heterologous expression of cellulase genes in yeast*

As the native promoters of cellulase genes are commonly repressed by glucose, replacing these promoters with other inducible or constitutive promoters would enhance cellulase genes expression in the yeast. For example, inducible promoters such as *S. cerevisiae* GAL1 or CUP1 promoters may be efficient. The most important disadvantage of these promoters is that they need galactose or copper as inducers, respectively, which can be expensive and incompatible with the ethanol production process. For instance, the presence of glucose acts as repressor of the GAL promoters, and therefore, this promoter will not be suitable for industrial CBP ethanol production (Kricka et al., 2014). Recent studies on inducible and constitutive promoters have confirmed that the use of constitutive promoters, such as TEF1 and PGK1, could significantly increase cellulase expression in the yeast by up to two folds, while the yeast reportedly produced the most constant expression profiles (den Haan et al., 2007a,b; Partow et al., 2010; Yamada et al., 2011; Fitzpatricketal.,2014; Kricka et al., 2014).

- *High copy number of cellulase genes*

One of the suggested strategies for increasing cellulase activity in the yeast is by transferring high copy numbers of the cellulase genes. To do this, there are different strategies, such as use of episomal plasmids or integration of the genes into the yeast chromosome (Kricka et al., 2014). Previously, episomal plasmids containing cellulase genes have been extensively used in yeast transformation. In spite of the overexpression of the transformed genes by this systems, unfortunately, plasmids used in this system were lost after many generations (Fujitaetal.,2004; den Haan et al.,2007a,b; Tsai et al., 2010; Wen et al., 2010; Fan et al., 2012b). Recently, Fitzpatrick et al., (2014) using a high copy number of episomal vector (pRSH-series) could significantly increase the expression of *T. reesei* endoglucanase gene (EGI) in yeast (50-folds greater than when an ARS/CEN vector (pGREG-series) was used). To overcome the problem of genetic instability of the episomal plasmids, the strategy of multi-copy integration of cellulase genes cassettes directly into the host chromosome has been proposed (Du Plessis et al., 2010; Yamada et al., 2010 a,b, 2013). For instance, Yamada et al. (2010a) integrated multiple-copies of cellulase genes into the delta (δ) repeat sites of transposable elements (Tn) in the *S. cerevisiae* chromosome, and achieved increased ethanol yield.

It is clear that in addition to endoglucanase, exoglucanase and cellobiohydrolases (CBHs) activity is also required for successful hydrolysis of lignocelluloses. Thus, heterologous expression of these enzymes in the ethanologenic hosts would enhance efficient conversion of cellulose to ethanol (den Haan et al., 2013). Recently, relatively high levels of CBH1 (0.3 g/l) and CBH2 (1 g/l) production in *S. cerevisiae* have been reported (McBride et al., 2010; Ilmen et al., 2011). Ilmen et al. (2011) developed a recombinant strain with a high ability to convert most of the glucan available in paper sludge to ethanol and displace about 60% of the enzymes usually required. Furthermore, McBride et al. (2010) developed a recombinant strain expressing three cellulases with high abilities to convert pre-treated corn stover to 2.6% (v/v) ethanol within 96 h (63% of the theoretical value) in one step without the addition of exogenous enzymes.

Previous studies have confirmed that in addition to copy number of cellulase genes, the ratio of each cellulase is also crucial to ensure efficient cellulose hydrolysis (Tien-Yang et al., 2012; Kricka et al., 2014). So, designing the optimum ratio of each cellulase will guarantee efficient cellulose hydrolysis. The ratios of secreted cellulases in *T. reesei* under inducing conditions is 60% CBHI, 20% CBHII, 10% EG, and 1% BGL (Takashima et al., 1998), however, these may be different in other recombinant organisms.

- *Yeast surface display (non-complexed cellulase systems, cellulosomes and minicellulosomes)*

Cellulosomes are natural exocellular enzymatic complexes with synergistic activities and are derived from some anaerobic microorganisms for efficient hydrolysis of cellulose. This highly ordered complex allows the assembly of multiple enzymes to attach to lignocellulosic materials through their carbohydrate-binding modules or polypeptides which have high affinity for polysaccharides (Fierobe et al., 2005; Tsai et al., 2010; Schuster and Chin, 2013). It has been proposed that engineering a CBP yeast to express multiple components of a cellulolytic system (cellulosomes) from different microorganisms could be the most dominant strategy, and heterologous displaying cellulases and hemicellulases on the yeast cell surface could represent a significant progress toward commercialization of CBP technology (Hasunuma and Kondo, 2012). Recent studies showed that construction of minicellulosomes was possible by simply appending a dockerin domain and up to three cellulase enzymes (either cellulosomal or noncellulosomal) integrated into a chimeric mini-scaffoldin containing different cohesin domains *in vitro* (Wen et al., 2010). The chimeric mini-scaffoldin could be purified (Fierobe et al., 2005; Mingardon et al., 2005; Caspi et al., 2008) or yeast surface-displayed (Tsae et al., 2009, 2010; Wen et al., 2010), and both cases showed efficient hydrolysis of cellulose. These results confirmed that the high-affinity cohesin-dockerin interactions were sufficient to induce formation and assembly of a functional cellulosome, and therefore, this system could also functionally be achieved *in vivo* by heterologous co-expression of the cellulosomal components in a CBP host (Wen et al., 2010). The first works on *in vivo* production of heterologous cellulosomes were dedicated to unifunctional complexes containing only one type of cellulases. None of these recombinant strains could directly utilize cellulose, as complete cellulose hydrolysis required synergistic action of at least three main cellulases (Cho et al., 2004; Mingardon et al., 2005; Arai et al., 2007).

Fujita et al. (2002, 2004) reported co-expression and surface display of cellulases in *S. cerevisiae*. The recombinant strain displaying three EG2 and CBH2 (derived from *T. reesei*), and the β-glucosidase (derived from *Aspergillus aculeatus*) enzymes could directly convert 10 g/l phosphoric acid swollen cellulose to approximately 3g/l ethanol. To enhance the capability of hydrolyzing *β*-glucan, Ito et al. (2009) designed and constructed a chimeric scaffoldin for cell surface display of EG2 (from *T. reesei*) and BGL1 (from *A. aculeatus*) at an optimum ratio. In next studies, successful assembly of trifunctional minicellulosomes in *S. cerevisiae* was reported by Tsai et al., (2009, 2010) and Wen et al. (2010). Tsai et al. (2009) reported the functional assembly of minicellulosomes on the yeast surface. In their work, the functional display of a mini-scaffoldin on the *S. cerevisiae* cell surface consisting of three different cohesin domains was demonstrated. The functional minicellulosome contained an endoglucanase (CelA) fused with a dockerin domain from *C. thermocellum*, an exoglucanase (CelE) from *C. cellulolyticum* fused with a dockerin domain from the same species, and a β-glucosidase (BglA) from *C. thermocellum* tagged with the dockerin from *Ruminococcus flavefaciens*. The recombinant strain displaying the new minicellulosome showed high cellulolytic activity, and could directly produce 3.5 g/l ethanol (ethanol yield of 0.49 g/g carbohydrate consumed and 95% of the theoretical value) from phosphoric acid swollen cellulose, which was 2.6-folds higher than that obtained by exogenous application of purified cellulases. In another study, Wen et al. (2010) constructed trifunctional minicellulosome containing a mini-scaffoldin. The mini-scaffoldin contained a cellulose-binding domain, three cohesion modules, and three cellulases, including an endoglucanase, a cellobiohydrolase, and a β-glucosidase, each bearing a C-terminal dockerin. The recombinant yeast could break down and ferment phosphoric acid-swollen cellulose to ethanol with a titer of 1.8 g/l.

Commonly, co-expression of all components of a minicellulosome in a single strain results in relatively low levels of each cellulase. This is probably due to the heavy metabolic burden and potential jamming of the secretion machinery. So, in other works, surface assembly of functional minicellulosome by using a synthetic yeast consortium has been reported (Tsai et al., 2010; Goyal et al., 2011). Tsai et al. (2010) used a synthetic yeast consortium composed of four engineered strains, including one strain displaying a mini-scaffoldin carrying three different cohesin domains (SC), and three strains secreting dockerin-tagged cellulases (endoglucanase (AT), exoglucanase (CB) and β-glucosidase (BF)).The optimized consortium consisted of a SC:AT:CB:BF ratio of 7:2:4:2 and produced 1.87 g/l ethanol (ethanol yield of 0.475 g/g cellulose consumed, and 93% of the theoretical value). This value was two times more than that when a consortium with an equal ratio of the different populations was used (Tsai et al. 2010).

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To increase the display efficiency of the cellulosomes, Fan et al. (2012a) developed an *in vivo* display of trifunctional minicellulosomes with two individual mini-scaffoldins on the yeast cells. The recombinant yeast showed a significant cellulolytic activity on Avicel, although, the *in vivo* assembly mode caused metabolic burden for the yeast, and some difficulties were observed during the ethanol production. So, this group of researchers in another work, used different *in vitro* strategies for assembly of minicellulosomes with two mini-scaffoldins on the *S. cerevisiae* cell surface. They incubated the yeast cells displaying scaffoldins with *E. coli* lysates containing recombinant cellulases, or using a four-population yeast consortium. The results confirmed that cellulases production in *E. coli* or other yeast cells could significantly increase the display level of mini-scaffoldin, and the metabolic burden imposed on the yeast host was decreased. The *E. coli* lysates-treated yeast with optimized anchoring mini-scaffoldin length was able to produce 1138 mg/l ethanol from cellulose after 4 d which was comparable to the results obtained for the yeast with self-assembled minicellulosomes (Fan et al., 2013). In another attempt, Kim et al. (2013) used a yeast consortium which consisted of four different yeast strains displaying a scaffoldin (mini CipA) containing three cohesin domains, endoglucanase (CelA), exoglucanase (CBHII) or β-glucosidase (BGLI). They showed that the optimized ratio for mini CipA:CelA:CBHII:BGLI was 2:3:3:0.53, and the maximum ethanol production was 1.80 g/l after 94 h, which was 20% more than that when a consortium composed of equal amounts of each cell type was used (1.48 g/l).

Recently, Matano et al. (2012, 2013) developed a combined bioprocessing and genetic engineering system to enhance ethanol production from hydrothermally-pretreated rice straw. By using a recombinant yeast strain displaying cellulases on cell surface and also a drum-type rotary fermentation system (Matano et al., 2012) or cell recycle batch fermentation system (CRBF) (Matano et al., 2013), they could produce up to 42.2 g ethanol/l representing 86.3% of the theoretical yield based on the starting biomass.

- *Expression of other genes in the yeast*

Cellodextrin transporters

Clearly, *S. cerevisiae* in contrast to some other fungi, such as *Neurospora crassa*, cannot take up and use cellooligosaccharides, as it does not contain cellodextrin transporters to facilitate rapid assimilation of cellulose (Tian et al. 2009; Yamada et al., 2013). Hence, in a research investigation, *N. crassa* cellodextrin transporter gene was expressed in *S. cerevisiae* to assimilate cellooligosaccharide. The recombinant strain was able to produce ethanol directly from cellooligosaccharide. Furthermore, addition of cellulase to the SSF process resulted in ethanol production from cellulose with more efficiency compared to the wild-type strain (Galazka et al. 2010; Ha et al., 2013). Lee et al. (2013) demonstrated efficient ethanol production without supplementation of β-glucosidase using an engineered *S. cerevisiae* strain expressing a cellodextrin transporter and an intracellular β-glucosidase from *N. crassa.* The engineered strain did not need an exogenous supplementation of β-glucosidase, and showed better ethanol productivity from 8% (w/v) pure Avicel (27.0 g/l ethanol) than the parental strain which required β-glucosidase supplementation. Yamada et al. (2013) developed a recombinant *S. cerevisiae* strain co-expressing genes for cell surface-displayed cellulases and a cellodextrin transporter to improve the efficiency and yield of direct ethanol production from cellulose. The recombinant strain co-expressing cellulase and cellodextrin transporter showed 1.7-fold more ethanol production (4.3 g/l) than the strain expressing only cellulase (2.5 g/l) from phosphoric acid swollen cellulose after 72 h of fermentation.

Endoinulinase and inherent invertase

Commonly, *S. cerevisiae* does not contain genes encoding inulinase, and therefore, it has been considered to be inulin negative (Ohta et al., 1993; Yuan et al., 2013). However, a few *S. cerevisiae* strains are able to utilize inulin and convert inulin-type sugars to ethanol (Lim et al., 2011; Hu et al., 2012). It was shown that the invertase SUC2 is a key enzyme responsible for inulin utilization by *S. cerevisiae* due to its exoinulinase activity (Wang and Li, 2013). The critical problem of converting inulin (as fructooligosaccharide) is that the conversion efficiency of inulin to ethanol is below 80% of the theoretical ethanol yield. It was shown that endoinulinase could digest fructooligosaccharides with high degree of polymerization (>20) into short molecules that may be readily hydrolyzed by SUC2 (Lim et al., 2011; Hu et al., 2012; Yuan et al., 2013). Yuan et al. (2013) introduced an endoinulinase gene from *A. niger* into *S. cerevisiae* to improve inulin assimilation and ethanol fermentation through the collaboration between the recombinant endoinulinase and the endogenous invertase SUC2. The resulted recombinant strain (JZ1C-I) could efficiently convert inulin from Jerusalem artichoke tuber flour to ethanol (55.3 g /l and 89.0% of the theoretical yield).

- *Engineering for pentose fermentation by S. cerevisiae*

Unlike cellulose and starch, hemicelluloses are heterogeneous polysaccharides with diverse structural compositions. Depending on the kind of plant species, four major hemicelluloses groups, including xyloglucan, xylans, mixed-linkage glucans and mannans have been characterized (Tester et al., 2004). Hemicelluloses bind to cellulose fibers within lignocellulosic structures and strongly inhibit the activities of cellulase enzymes (Qing et al., 2010; de Haan et al., 2013). The ability to assimilate pentose sugars such as xylose and arabinose which are prevalent in hemicelluloses is very important. During the last 30 years, different studies were devoted to develop recombinant xylose and arabinose utilizing yeast strains (de Haan et al., 2013). In order to enhance xylose utilization in *S. cerevisiae*, heterologous expression of xylose reductase/xylitol dehydrogenase (XR/XDH) or xylose isomerase (XI) encoding genes has been performed (Matsushika et al., 2009; van Vleet and Jeffries, 2009). Traff et al. (2001) confirmed that overexpression of the genes involved in pentose phosphate pathway of *S. cerevisiae* could increase the xylose metabolism. They also showed that deletion of the non-specific aldose reductase (*GRE3*) in *S. cerevisiae* could significantly reduce xylitol formation. In another study, Zhou et al. (2012) overexpressed the genes encoding xylose isomerase, xylulose kinase (*xk*), and the non-oxidative pentose phosphate pathway enzymes from different organisms in *S. cerevisiae*, and developed a recombinant yeast strain with an ability to anaerobically consume xylose with a rate of 1.9 g/g h and ethanol yield of 0.41 g/g.

As xylans are the most abundant hemicellulose found in most biomass materials, so most studies were focused on this substrate to create recombinant *S. cerevisiae* with hemicellulolytic activities against these abundant substrates. The complete hydrolysis of xylan needs actions of six enzymes, including endoxylanase, *β*-xylosidase, *α*-arabinofuranosidase, *α*-glucuronidase, acetylxylanesterase and ferulic acid esterase (Jordan et al., 2012; de Haan et al., 2013). la Grange et al., (2001) expressed *β*-xylosidase and xylanase II genes extracellularly, and the resultant recombinant yeast strain was able to convert birchwood xylan to short-chain xylo-oligomers. In another work, *β*-xylosidase and xylanase II genes were co-displayed on the cell-surface of *S.cerevisiae* (Katahira et al., 2004). In both of these studies, xylose was the major end product. The recombinant strain obtained in the latter study was able to directly convert birchwood xylan to ethanol with a productivity rate of 0.13 g/l h and a yield of 0.30 g/g sugar consumed. In a different study, the expression of the *K. lactis* gene encoding lactose permease in *S. cerevisiae* enhanced cellobiose transport in the recombinant strain. In addition, in this study it was reported that heterologous expression of *C. stercorarium* genes encoding cellobiose phosphorylase (*cepA)* and LAC12 increased yeast ability to grow on cellobiose (Sadie et al., 2011). Aeling et al. (2012) reported that the recombinant *S. cerevisiae* strain expressing the *R. flavefaciens* genes encoding xylose isomerase and cellobiose phosphorylase could uptake and assimilate glucose, xylose, and cellobiose under anaerobic conditions (de Haan et al., 2013).

Sakamoto et al. (2012) developed a recombinant *S. cerevisiae* strain co-displaying three types of hemicellulolytic enzymes, including endoxylanase from *T. reesei*, β-xylosidase from *A. oryzae*, and β-glucosidase from *A. aculeatus* on the cell-surface. This strain could also assimilate xylose through the expression of xylose reductase and xylitol dehydrogenase from *P. stipitis* and xylulokinase from *S. cerevisiae*. The recombinant strain was able to directly produce ethanol from rice straw hydrolysate consisting of hemicellulosic material containing xylan, xylooligosaccharides, and cellooligosaccharides with a productivity rate of 0.37 g/l h and an ethanol yield of 0.32 g/g of total sugars consumed. Sun et al. (2012) could enhance recombinant synergism amongst cellulases and hemicellulases, by development of engineered *S. cerevisiae* strains displaying mini-hemicellulosome's on the cell-surface consisting of a mini-scaffoldin

harboring xylanase II, arabinofuranosidase, and *β*-xylosidase enzymes. The strain displaying a bi-functional (xylanase II and *β*-xylosidase) hemicellulosome could produce 1 g/l ethanol after 80 h (ethanol yield: 0.31 g ethanol/g birchwood xylan consumed). Srikrishnan et al. (2013) successfully assembled five trimeric xylanosomes on the cell surface of *S.cerevisiae*. To enhance synergistic hydrolysis of birchwood xylan, three dockerin-tagged fungal enzymes, including endoxylanase (XynAc), β-xylosidase (XlnDt) and acetylxylan esterase (AwAXEf) were displayed. The scaffoldin-based enzymes with and without xylan binding domain (derived from *T. maritima*) could increase the hydrolytic activity up to 3.3 (with xylose productivity of 105 mg/g substrate) and 1.6 folds over free enzymes after 72 h hydrolysis, respectively. The scaffoldin-based enzymes containing xylan binding domain was also 1.4 fold more effective than the xylanosome containing the cellulose binding module from *C. thermocellum*. Ota et al. (2013) developed a recombinant *S. cerevisiae* strain displaying xylose isomerase derived from *C. cellulovorans* on the cell-surface. The recombinant strain showed an enhanced ability to grow on medium containing solely xylose and directly produced ethanol from xylose under anaerobic conditions.

2.3.4.3.2.2. Kluyveromyces marxianus

Commonly, the pretreatment of lignocellulosic materials involves a heat intensive method, and after that the pretreated biomass needs to be cooled down to a temperature at which the optimum enzymatic activities and fermentation could be occurred. Furthermore, to separate the ethanol produced after fermentation, the mixture then has to be reheated during the distillation process. So, it will be advantageous if these biological processes could occur at an elevated temperature, because it would increase enzyme activity and decrease the amount of cooling required. As mentioned in the previous sections, this could also result in decreasing cost and reducing the risk of contamination (Lynd et al., 2005; Fonseca et al., 2008; la Grange et al., 2010). The main advantages of the yeast *K. marxianus*, as mentioned earlier, are its high growth rate at temperatures up to 52 °C, short generation time (Rajoka et al. 2003), and assimilation of a wide range of substrates, including xylose to produce ethanol (Fonseca et al. 2007, 2008). Previously, three *Thermoascus aurantiacus* cellulase genes, including cellobiohydrolase 1 (*cel7A*), endoglucanase 1 (*cel5A*) and β-glucosidase (*bgl3A*), were expressed in *K. marxianus* strains. The recombinant strain could efficiently grow in synthetic media containing cellobiose or CMC as sole carbon source, and produced 43.4 g/l ethanol from 100 g/l cellobiose in 24 h at 45°C (Hong et al. 2007). Yanase et al. (2010) used cell surface technology to develop a new recombinant strain of *K. marxianus* with cellulytic activities for CBP ethanol production. The strain was genetically-engineered to display *T. reesei* endoglucanase and *A. aculeatus* β-glucosidase on the cell surface. The strain could successfully convert a cellulosic β-glucan to ethanol directly at 48 °C with a yield of 4.24 g/l from 10 g/l within12 h (yield: 0.47 g ethanol/g of β-glucan consumed, 92.2% of the theoretical yield). In another study, Matsuzak et al. (2012) designed ventilation-mediated, simultaneous ethanol fermentation by growing recombinant *K. marxianus* expressing β-glucosidase, and could produce and recover 28 g ethanol/100 g cellobiose.

Yuan et al. (2013) improved CBP ethanol production from Jerusalem artichoke tubers using a recombinant inulinase-producing yeast *K. marxianus*. They overexpressed the inulinase gene *INU* under endogenous promoter of *K. Marxianus* using an integrative cassette. It was shown that the inulinase activity was significantly increased by more than 2 folds (114.9 U/ml compared to 52.3 U/ml for the wild-type strain). Ethanol concentration and productivity of the recombinant yeast (96.2 g/l and 1.34 g/l h) were significantly more than those of the wild type (69 g/l and 1.44 g/lh) when inulin and Jerusalem artichoke tubers were used. Chang et al. (2013) to enhance CBP ethanol production from cellulose, developed a synthetic biology technique, called “promoter-based gene assembly and simultaneous overexpression” in which multiple genes were simultaneously transformed and expressed in *K. marxianus*. They transferred 7 different genes, including 5 cellulase genes (two cellobiohydrolases, two endo-β-1,4-glucanases and one beta-glucosidase genes from different fungi), a fungal cellodextrin transporter gene and a selection marker gene to the yeast. The recombinant strain KR7 could convert Avicel (crystalline cellulose) into ethanol. The ethanol production by the recombinant strain was recorded at 0.42 g/l and 0.6 g/l after 2 and 5 d, respectively.

2.3.4.3.2.3. Hansenula polymorpha

Commonly, the thermotolerant methylotrophic yeast *H. polymorpha* (syn. *Pichia angusta*) strains are able to ferment glucose, cellobiose, and xylose to ethanol (la Grange et al., 2010). This yeast is also able to convert glycerol to ethanol (Suwannarangsee et al., 2010; Kurylenko et al., 2014). Different physiological characteristics such as resistance to heavy metals and oxidative stress, ability to use a wide range of substrates such as soluble sugars present in lignocellulosic biomass, thermotolerance, process hardiness and a high capacity for heterologous protein production, all make this yeast an attractive candidate for CBP organism development (la Grange et al., 2010). The optimal growth temperature of this yeast is 37 °C, but it can grow at temperatures up to 48 °C, whereas at 50°C fermentation is strongly suppressed (Kurylenko et al., 2014). Genetic manipulation of this yeast has resulted in an increase in intracellular trehalose and knock out of acid trehalase gene *ATH1*. In addition, the overexpression of the heat shock proteins Hsp16 and Hsp104 allowed normal xylose fermentation at 50°C (Ishchuk et al., 2009). Ishchuk et al. (2010) in a different investigation confirmed that the ethanol tolerance of *H. polymorpha* could be increased by the overexpression of the heterologous gene *MPR1*. In spite of many advantages of *H. polymorpha* as a CBP organism, ethanol production by this yeast is not free of drawbacks. The most important problem is low ethanol yield and productivity from xylose in wild-type strains, which could be improved by classical selection and metabolic engineering (Kurylenko et al., 2014). It was previously confirmed that this yeast was able to produce high volumetric levels of recombinant endoglucanases derived from other fungi such as *A. aculeatus* and *Humicola insolens* (Müller et al. 1998). A thermostable endoglucanase was also successfully produced in this yeast (Papendieck et al. 2002). Voronovsky et al. (2005) deleted three endogenous genes, including *XYL1* encoding xylose reductase and two paralogs of xylitol dehydrogenase *XYL2A* and *XYL2B* in the *H. polymorpha* strain CBS4732. They also expressed the *E. coli* or *Streptomyces coelicolor* gene *xylA* in the strain CBS4732. These genetic modifications resulted in high growth rate of transformants on xylose as sole carbon source due to high expression of xylose isomerase, the amount of the accumulated ethanol was very low though (0.15 g/l).

Dmytruk et al. (2008a) could increase ethanol production up to 0.6 g/l at 48°C by overexpression of *E. coli xylA* and *H. polymorpha XYL3* encoding xylulokinase. In another work, these researchers overexpressed three xylose reducatse genes, including the modified *XR*(*XYL1m*), native *XDH* (*XYL2*) and *XK* (*XYL3*) in the strain CBS4732 leading to an increased ethanol accumulation by up to 2 folds with the ethanol concentration reaching 1.3 g/l (Dmytruk et al., 2008b). Ishchuk et al. (2008) constructed a *H. polymorpha* mutant strain ($2EthOH^-$) which could ferment xylose more efficiently compared to the parental strain (NCYC495), but it was not able to utilize ethanol as a sole carbon source. The mutant strain showed an increased ethanol accumulation by up to 3 folds. By overexpressing the gene *PDC1* encoding pyruvate decarboxylase (PDC) in the strain $2EthOH^-$, the ethanol production reached 2.5 g/l at 48°C. Voronovsky et al. (2009) confirmed the potentials of this yeast for biomass conversion, and the new strain could ferment starch and xylan. They co-expressed *T. reesei xyn11B* (encoding an endoxylanase) and *A. niger xlnD* (encoding β-xylosidase) in *H. polymorpha* under control of glyceraldehyde-3-phosphate dehydrogenase gene promoter. The recombinant strains could efficiently grow in the minimal medium containing birchwood xylan as a sole carbon source, and produced alcohol at 48 °C. Recently, Kurylenko et al. (2014) constructed more efficient *H. polymorpha* capable of producing ethanol from xylose at high temperatures. They overexpressed *H. polymorpha* genes *XYL1m*, *XYL2*, *XYL3* and *PDC1* in the strain $2EtOH^-$ (without ability of ethanol production). The selected mutant showed a 15 fold increase (up to 10 g/l) in ethanol synthesis from xylose at 45°C compared to the wild-type strain. This is the maximum ethanol concentration produced by *H. polymorpha* (Kurylenko et al., 2014).

2.3.4.3.2.4. Zymomonas mobilis

Z. mobilis is a unique Gram-negative and facultative anaerobic ethanologenic bacterium previously isolated from different sources such as pulque, alcohol, tainted cider, palm and sugarcane juice, ripening honey, and tainted beer. Commonly, these bacteria can grow on raffinose, glucose, fructose and sucrose (Rogers et al., 1982; Schuster and Chinn, 2013). They can metabolize glucose anaerobically through the Entner-Doudoroff (ED)

pathway in contrast to the other Gram-negative organisms (e.g. *E. coli*) that utilize the Embden-Meyerhof-Parnas (EMP) pathway. Over the last three decades, extensive fundamental studies have also made this bacterium a promising ethanologenic organism for large-scale bioethanol production (He et al., 2014).

Z. mobilis has several advantages, including high sugar-uptake ability, a lower cellular biomass yield, a higher ethanol yield/tolerance, as well as unnecessity of controlled addition/depletion of oxygen during fermentation as it can grow microaerobically making it a potential organism for CBP ethanol production (Jung et al., 2012). The optimal growth temperatures of *Z. mobilis* strains are between 25 and 31°C at pH 3.5-7.5, but mutant strains could grow at higher temperatures. This bacterium is susceptible to inhibition by biomass pretreatment byproducts, but some strains are resistant to these toxic compounds. Recombinant technology has been used on *Z. mobilis* and recombinant strains capable of fermenting arabinose and xylose to produce ethanol at 98 and 86% of the theoretical yield have been developed, respectively (Zhang et al., 1995; Deanda et al., 1996).

In order to expand the substrate range, especially to enhance effective utilization and assimilation of lignocelluloses, several cellulase genes have been cloned and expressed in *Z. mobilis*. However, similar to the other Gram-negative bacteria, the presence of an outer membrane results in an inefficient protein secretion, which is a major technical challenge in engineering cellulolytic *Z. mobilis* (Jung et al., 2012). Recently, Jung et al. (2012) and He et al. (2014) reviewed the studies focused on expression of cellulase enzymes in this bacterium. Endoglucanase genes such as *B. subtilis* endo-*β*-1,4-glucanase gene (Yoon et al., 1988), *Enterobacter cloacae* endoglucanase gene (Thirumalai et al., 2011), *eglX* (Lejeune 1988), CMCase (Misawa et al., 1988; Okamoto et al., 1994), and *celZ* (Brestic-Goachet et al., 1989) were reportedly expressed in *Z. mobilis.* Only in the case of *celZ* from *E. chrysanthemi*, approximately 35% of the endoglucanase was released into the medium in the absence of detectable cell lysis (Brestic-Goachet et al., 1989). This probably occurred because of the correct recognition of the secretion signal of *E. chrysanthemi* by *Z. mobilis* (Jung et al., 2012). The *β*-glucosidase gene from *Xanthomonas albilineans* or *Ruminococcus albus* was also expressed in *Z. mobilis* (Su et al., 1989; Yanase et al., 2005). Yanase et al. (2005) found that the produced *β*-glucosidase was secreted into both the periplasmic and extracellular spaces, and the maximum *β*-glucosidase activity of 11.2% was detected in the extracellular space of the recombinant *Z. mobilis.* It is important to note that these genes could not support the growth of recombinant *Z. mobilis* on cellobiose as the sole carbon source.

In the next studies, multiple plant cell wall degrading-enzymes were cloned and expressed in *Z. mobilis*. Linger et al. (2010) fused two *Acidothermus* endo-β-1,4-glucanase genes (*E1* and *GH12*) with secretion signals of two endogenous genes of *Z. mobilis* (*phoC* and *ORFZM0331*), and expressed them in *Z. mobilis.* The expression of *GH12* with phoC secretion signal enhanced the activity observed in the periplasmic space by 26%, extracellular space by 13%, and cytoplasm by 61%. The expression of *E1* with the PhoC secretion signal resulted in an enhanced E1 activity in the extracellular medium contents (20%), periplasmic space (30%), and cytoplasm (50%). Ming-xiong et al. (2009) developed recombinant *Z. mobilis* strains containing four fused glucoamylase genes from *A. awamori* with capability of direct production of ethanol from 1.5% sweet potato starch. One of the recombinant strains showed highest glucoamylase activity (157 U/ml, in which about 80% of that was detected in extracellular medium contents. The ethanol production by this strain was 14.73-fold (92.69% of the theoretical yield) more than that in the parental strain. Wu et al. (2014) used three native signal peptides of *PhoC*, *PhoD*, and *ZMO0331* genes for construction of novel secretion expression systems in *Z. mobilis*. They fused *Bacillus amyloliquefaciens* (BAA) α-amylase gene with these signal peptides, and expressed them in *Z. mobilis.* The genetically engineered strain expressing α-amylase fused with *PhoD*'s signal peptides showed more hydrolysis of starch than the strains expessing the other two signal peptides. Extracellular and intracellular α-amylase activities of the strain containing *PhoD* were also higher.

2.3.4.3.2.5. Escherichia coli

E. coli has extensively been used as a valuable model organism for genetic studies and also a host for production of numerous commodities. By expressing *Z. mobilis* fermentation genes, pyruvate decarboxylase and alcohol dehydrogenases, the sugars fermentation and ethanol production abilities have been given to *E. coli* strains (Schuster and Chinn, 2013). Ingram et al. (1987) first engineered *E. coli* for ethanol production. They transferred the genes from plasmid (pLOI295) to this bacterium which resulted in ethanol production by the new strain as the major fermentation product, and a 10 fold increase in cell density. In another study, ethanol yields were improved by transferring the genes encoding pyruvate decarboxylase and alcohol dehydrogenase II from *Z. mobilis*. Formation of succinate, a competing fermentation product, was eliminated by the deletion of a gene (Ohta et al., 1991a). In the next experiments, Gonzalez et al. (2003) could increase ethanol tolerance of *E. coli* by mutagenesis in the strain LY01, and as a result the new strain produced up to 60 g/l of ethanol. They showed that the improved tolerance was a result of significant changes in the metabolism and alterations to the cell envelope.

Application of *E. coli* as CBP organisms will be feasible if they also could hydrolyze cellulose. Bolshakova et al. (1994) cloned and expressed cellulases and xylanases genes from the anaerobic thermophile, *Anaerocellum thermophilum*, and Fau et al. (1988) expressed cellulases from *C. cellulolyticum* in *E. coli.* In a different investigation, two cellulases from the guts of wood-feeding termite, *Coptotermes formosanus*, were also expressed in *E. coli*, resulting in carboxymethyl cellulose hydrolysis and production of oligosaccharides with some glucose (Zhang et al., 2009).

Shin et al. (2014) developed two engineered *E.coli* strains containing two mechanisms of cellobiose (cellodextrin) assimilation, hydrolysis *vs.* phosphorolysis. They showed that phosphorolysis cells could more effectively tolerate common inhibitors under both anaerobic and aerobic conditions than the cells assimilating cellobiose hydrolytically. In addition, it was shown that these cells directed the favorable energy metabolism to production of recombinant proteins which resulted in a significant increase in recombinant proteins production (up to 500%). Recently, multifunctional cellulolytic enzymes and chimeric cellulases such as the codon-optimized CelEx-BR12 have been investigated to enhance endoglucanase, exoglucanase and xylanase activities of *E. coli* (Ko et al., 2013). Muñoz-Gutiérrez et al. (2012) used the autodisplay secretion system AIDA-I to display the β-glucosidase BglC from *Thermobifida fusca* on the outer membrane of the ethanologenic *E. coli* strain MS04. The recombinant strain showed cellobiase activity (171 U/g) and fermented 40 g/l cellobiose in mineral medium in 60 h with an ethanol yield of 81% of the theoretical maximum. In another work, Lue et al. (2014) developed an ethanologenic *E. coli* ZY81/*bglB* by integrating pyruvate decarboxylase gene *pdc*, alcohol dehydrogenase gene *adhB* from *Z. mobilis* and β-glucosidase gene *bglB* from *Bacillus polymyxa* into the genome of *E. coli*. The recombinant strain showed an obvious activity of β-glucosidase in extracellular soaces with more than half in the periplasmic space, and could utilize cellobiose as sole carbon source for ethanol production (33.99% of theoretical yield).

2.3.4.3.2.6. Pichia stipitis (Scheffersomyces stipitis)

P. stipitis is an ethanologenic yeasts which can ferment mannose, glucose, and galactose, but it stands out in comparison wth *S. cerevisiae* due to its ability to efficiently utilize xylose (Watanabe et al., 2011; Schuster and Chin, 2013). *P. stipitis* is also able to ferment cellobiose, while neither can ferment arabinose. It has been reported that this yeast could produce over 60 g/l ethanol from xylose and could ferment cellobiose almost as rapidly as glucose (Jeffries et al., 2010). It was previously shown that *P. stipitis* was able to use 88% of the available xylitol for growth. This yeast preferentially ferments glucose when other sugars are present (Preez et al., 1986). Nigam et al. (2001) developed a modified strain of *P. stipitis* grown on acid-pretreated wheat straw containing 46.4% cellulose and 31% hemicelluloses, and produced a high yield of ethanol despite the inhibition by toxic compounds resulting from the pretreatment. Watanabe et al. (2011) developed mutant strains of *P. stipitis* with higher ethanol production (4.3%) from 11.4% xylose compared to the parent strain (3.1%). In their next experiments, by sequential cultivation of the mutant strain in the medium containing 2% xylose and 5-7% ethanol, they selected a novel strain with high tolerance to ethanol, and an ability to produce 4.4% ethanol from 11.4% xylose.

Xylose reductase is known as a key enzyme in bioethanol production from lignocelluloses. It was previously shown that intercellular redox imbalance caused by the inclusion of different coenzyme specific to xylose reductase and xylitol dehydrogenase could significantly reduce xylitol production (Khattab et al., 2011). Recently, a novel strictly NADPH-dependent xylose

reductase was constructed in *P. stipitis* using site-directed mutagenesis. This strategy resulted in an effective recycling of cofactors between xylose reductase and xylitol dehydrogenase, which subsequently reduced xylitol accumulation (Khattab et al., 2011). In another attempt, Hughes et al. (2012) using UV-C irradiation could develop some *P. stipitis* mutant strains with high growth rate on xylose/glucose substrates and higher ethanol production rates under anaerobic conditions compared to the industrial *S. cerevisiae* strains used for ethanol production. In an investigation, Wongwisansri et al. (2013) expressed a gene encoding thermotolerant β-xylosidase from *Aspergillus sp.* in methylotrophic yeast *P. pastoris* KM71. The recombinant strain showed high yields of secreted enzyme (K_{cat}/K_m = 198.8 mM^{-1} s^{-1}) at 60 °C and pH 4.0-5.0. The identified β-xylosidase showed clear synergism with xylanase for hydrolysis of xylan at *in vitro* and SSF *in vivo* fermentations by *P. stipitis*.

Genome shuffling is known as one of the most widely used genetic engineering methods for rapid improvement of microbial strains to be more industrially acceptable. Bajwa et al. (2011) produced two genome-shuffled *P. stipitis* strains with improved tolerance to hardwood spent sulphite liquor. These strains could completely utilize glucose and xylose in different hydrolysate, including steam-pretreated enzymatically-hydrolyzed poplar hydrolysate, steam-pretreated poplar hydrolysate and mixed hardwoods pre-hydrolysate, and could produce 0.39-1.4% (w/v) ethanol. Recently, Shi et al. (2014) improved the ethanol productivity of xylose-fermenting *P. stipitis* by genome shuffling. Using this technology, they could achieve a genetically stable and high-ethanol-producing *P. stipitis* strain which could ferment xylose and produce1.5 folds more ethanol (2.6% v/v) than the wild-type strain (1.7%) after 96 h of fermentaion.

2.3.4.3.2.7. Flammulina velutipes

F. velutipes due to its fermentative abilities, high ethanol tolerance, high conversion efficiencies of glucose, mannose, sucrose, fructose, maltose, and cellobiose to ethanol, as well as its ability for lignin degradation is considered as a potential CBP organism. Disadvantages of this fungus are inability to ferment galactose and pentose sugars to ethanol, and long time fermentation process (Schuster and Chin, 2013). To improve the pentose fermentation rate in *F. velutipes*, recently for the first time, the putative xylose isomerase (XI) gene from *Arabidopsis thaliana* was cloned and introduced into *F. velutipes*. The results confirmed that the putative gene was successfully expressed in *F. velutipes* as a xylose isomerase, and the transformants could produce more ethanol from xylose compared to the parental strain (Maehara et al., 2013b).

2.3.5. CBP in starchy biomass (amylolytic yeasts)

As the ethanol production from lignocellulosic materials has not been commercialized yet, currently, bioethanol is being produced from molasses and starchy grains using industrial *S. cerevisiae* strains. In addition to starchy grains (wheat and corn), some starchy feedstocks, such as wasted crops, cereal bran, potato peels and brewery spent grains, have been proposed for low cost production of bioethanol (Bothast et al., 2005; Favaro et al., 2012a, b, 2013; Schuster and Chinn, 2013). The process of starch conversion into ethanol is a costly and time-consuming process involving milling, starch hydrolysis into glucose, yeast fermentation and alcohol distillation steps. Furthermore, to achieve a high ethanol yield, it is necessary to cook starchy materials at high temperatures (Schuster and Chinn, 2013). So, the main bottle neck of this system is that *S. cerevisiae* strains are not commonly capable to produce amylolytic enzymes required for starch utilization, and therefore, cost and energy consuming enzyme addition and cooking steps are needed for ethanol production from starchy biomass. To overcome this problem, it was proposed to express starch-hydrolyzing enzymes in a fermenting yeast to achieve liquefaction, hydrolysis, and fermentation by a single organism. A CBP process for raw starch conversion to ethanol can save on the amylolytic enzyme consumptions and also excess energy needs for cooking, pumping or stirring of the starch slurry (Favaro et al., 2010a, b; van Zyl et al., 2012; Schuster and Chinn, 2013; Görgens et al., 2014). Ideal CBP yeast is a microorganism producing sufficient quantities of amylase to ensure full hydrolysis of high concentrations of starchy grains or feedstocks at moderate temperatures while converting simple sugars to ethanol (**Fig. 3**). Different technologies have been used to achieve CBP ethanol production from starchy materials, including native single microorganisms, co-culture systems, mutation breeding and genetic engineering. **Table 7** presents a summarized list of different studies, in which these technologies were used to achieve ethanol production from starchy feedstocks by CBP systems.

There are a few studies reporting single native microorganisms with efficient CBP ethanol production capability. For example, Okamoto et al. (2014), recently reported that a naturally-occurring Basidiomycete, *T. versicolor*, was capable to produce ethanol directly from starch, and the ethanol yield was recorded at 0.49 g ethanol/1 g starch which was superior to that of a recombinant starch-utilizing strain of *S. cerevisiae* (0.31 g/g) (Shigechi et al. 2004a). In another study, a native white-rot fungus *Trametes hirsuta* was used as CBP microorganism for direct ethanol production from starch and some other sugars. This fungus was capable of directly fermenting starch, wheat bran and rice straw to 9.1, 4.3 and 3.0 g/l ethanol (89.2%, 78.8% and 57.4% of the theoretical yield) without acid or enzymatic hydrolysis, respectively (Okamoto et al., 2011).

Another methodology for ethanol CBP production from starchy materials is the co-culture system. Tran et al. (2010) used co-culture of a high amylase producing *B. subtilis* and a *C. butylicum* strain to enhance ABE production from cassava starch. After fermentation optimization, the mixed culture of both strains showed an increased amylase activity by 10 folds, and enhanced ABE production rates by upto 5.4 and 6.5 folds from soluble starch and cassava starch, respectively, compared to those of the single culture of *C. butylicum*. Lee et al., (2012) used a co-immobilization system for direct ethanol production from sweet potato. In their work, the saccharification and fermentation conditions were optimized for co-immobilization of saccharolytic molds (*A. oryzae* and *Monascus purpureus*) with *S. cerevisiae*. The maximum bioethanol production achieved was 4.08% (v/v), and a $Y_{E/s}$ of 0.41 after 9 d of fermentation was recorded when the ratio of *A. oryzae* and *M. purpureus* was at 1:2.

The most recent efforts have been devoted to achieve CBP ethanol production from starchy materials by genetic engineering or mutation breeding of yeasts (**Table 7**). Up to now, different amylase genes have been identified and isolated from different sources and transferred to the laboratory and industrial yeast strains to enhance CBP. The transferred genes included the wild type and codon-optimized glucoamylase (GAI) and α amylase genes (Goto et al., 1994, 2004; Nakamura et al., 1997; Murai et al., 1998; Kondo et al., 2002; Eksteen et al., 2003; Shigechi et al. 2004a,b; Khaw et al., 2006a,b; Yamada et al., 2009; Favaro et al., 2010a,b, 2012c). These genetic manipulations critically increased the ethanol production in the CBP system (0.42-0.55 g ethanol/g of consumed sugars).

Ulgen et al. (2002) designed a recombinant *S. cerevisiae* strain YPG/AB expressing *B. subtilis* α-amylase and *A. awamori* glucoamylase as separately-secreted polypeptides. Fermentation of the new strain in a batch and fed-batch fermentor system under controlled conditions in a medium containing 40 g/l initial starch supplemented with 4 g/l glucose resulted in 15.6 and 47.5 g/l ethanol. Khaw et al. (2006a) developed four types of cell-surface-engineered *S. cerevisiae* strains displaying glucoamylase. These included two glucoamylase-displaying non-flocculent yeasts that, could secrete α-amylase into the culture medium, and codisplay α-amylase on the cell surface, and two flocculent yeast counterparts for direct ethanol fermentation from raw corn starch. They showed that glucoamylase-displaying non-flocculent yeast that could secrete α-amylase resulted in the maximum ethanol yield of 0.18 g/g h. In another study, it was shown that by increasing the capability of flocculency of armed yeasts with α-amylase and glucoamylase, the ethanol yield was decreased during the direct ethanol fermentation of raw starch (Khaw et al., 2006b). Nonetheless, one may find it more preferable to use the flocculent yeast because it could be recovered without centrifugation.

Kotaka et al. (2008) could transfer and display 3 different glucoamylases genes on the cell-surface of sake yeast *S. cerevisiae* GRI-117-UK and laboratory yeast *S. cerevisiae* MT8-1. They confirmed that the recombinant strain GRI-117-UK/pUDGAA, displaying *glaA* gene from *A. oryza* produced the maximum ethanol concentration of 18.5g/l after 48h when the liquefied starch was used as substrate. Kusugi et al. (2009) engineered the *S. cerevisiae* Kyokai (strain K7) to display *Rhizopus oryzae* glucoamylase on the cell surface for direct ethanol production from hydrothermally-pretreated and cellulase-hydrolyzed cassava pulp, as an abundant starchy by-product of starch manufacturing. The engineered strain (K7G) could ferment hydrothermally-pretreated cassava pulp starch without the addition of any amylolytic enzymes, and produced ethanol at 91% of the theoretical yield from 5% cassava pulp. Furthermore, Yamada et al.(2009) reported similar efficiency when they constructed a recombinant yeast by mating two

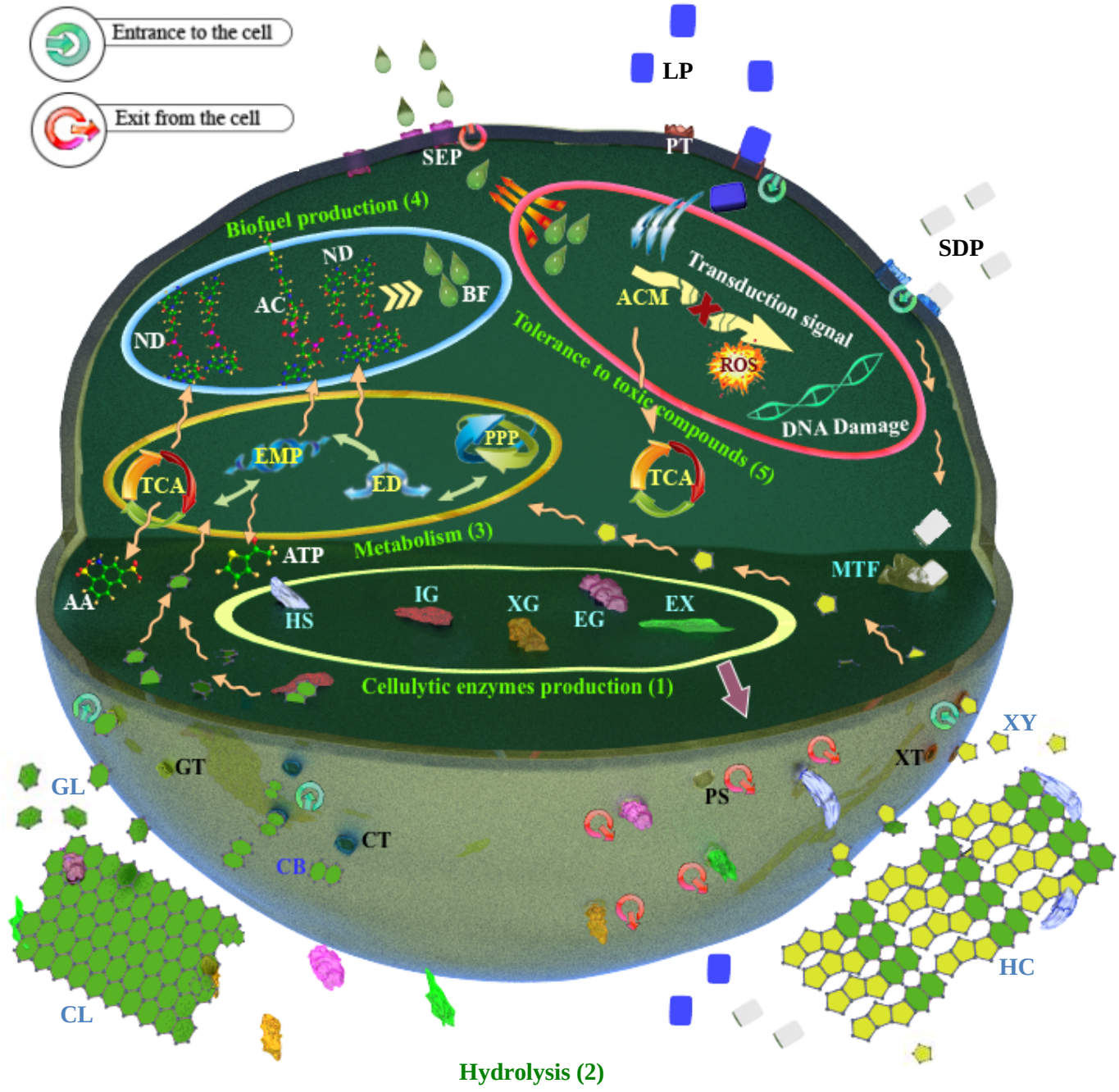

Fig.3. An ideal microorganism with minimum requirements for consolidated bioprocessing (CBP) of lignocellulosic biomass. This ideal CBP microorganism should be capable of expressing and secreting several glycoside hydrolase enzymes (Step 1), hydrolyzing both cellulose and hemicellulose to soluble sugars (Step 2), processively metabolize soluble sugars (Step 3), producing bioalcohols (Step 4), and finally should have a high tolerance against lignin-derived compounds and the biofuels produced (Step 5). Abbreviations: CL, Cellulose; HC, Hemicellulose; CB, Cellobiose; GL, Glucose; XY, Xylose; CT, Cellobiose transporter; GT, Glucose transporter; XT, Xylose transporter; PS, Protein secretion system; IG, Intracellular β-glucosidase; XG, Exoglucanase; EG, Endoglucanase; HS, Hemicellulase; EX, Extracellular β-glucosidase; TCA, Tricarboxylic acids pathway; EMP, Embden-Meyerhof-Parnas metabolic pathway; ED, Entner-Doudoroff metabolic pathway; PPP, Pentose-phosphate pathway; ND, NADH/NADPH; AA, Amino acids; AC, Acetyl coA; BF, Biofuel; PT, Phenolic transporter; LP, Lignin-derived products; ACM: Aromatic compounds metabolism pathway; SEP, Solvent (Biofuel) export pump; SDP, Sugar degradation products; MTF, Mutated transcription factor; and ROS, Reactive oxygen species.

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Table 7.
List of CBP systems for ethanol production from starchy materials.

Biomass	Microorganism	Technology	Ethanol concentration/yield	References
Starch	*T. versicolor*	Native single microorganism	0.49 g ethanol/g starch	Okamoto et al., 2014
Starch and wheat bran	*T. hirsuta*	Native single microorganism	9.1 and 4.3 g/l ethanol (89.2%, 78.8% and 57.4% of the theoretical yield)	Okamoto et al., 2011
Cassava starch	*B. subtilis* WD 161 and *Clostridium butylicum* TISTR 1032	Co-culture of amylase producing and ABE producing microorganisms	ABE production increased by 6.5 folds compared to that of the pure culture of *Clostridium*	Tran et al., 2010
Sweet potato	co-immobilization of *Aspergillus oryzae* and *Monascus purpureus* with *S. cerevisiae*.	Co-immobilization of saccharolytic molds with ethanol producing yeast	4.08% (v/v), and a $Y_{E/s}$ of 0.41 after 9 d	Lee et al., 2012
Starch (50 g/l)	*S. cerevisiae* SR93	Recombinant*:* expressing glucoamylase gene (*STA1*)	Batch culture :14.3 g/l Fed batch culture: 20% more than batch culture	Nakamura et al., 1997
Ground raw corn	*S. cerevisiae* G-1315	Recombinant: displaying glucoamylase on the cell surface	2.34% (w/w) after 7 d	Murai et al., 1998
Starch	Flocculent yeast YF207	Recombinant: cell surface engineering expressing system, glucoamylase/α-agglutinin fusion protein	Fed-batch: 50 g/l ethanol after 120 h	Kondo et al., 2002
Starch (40 g/ l) supplemented with 4 g/l glucose	*S. cerevisiae* strain YPG/AB	Recombinant: expressing *B. subtilis* α-amylase and *Aspergillus awamori* glucoamylase as secreted polypeptides	Batch: 15.6 Fed-batch: 47.5 g/l ethanol	Ulgen et al., 2002
Starch	*S. cerevisiae*	Recombinant*:* expressing *Lipomyces kononenkoae* α-amylase genes (*LKA1* and *LKA2*), or *S. fibuligera* α-amylase (*SFA1*) and glucoamylase (*SFG1*) genes	61 g/l of ethanol after 6 d	Eksteen et al., 2003
Raw corn starch	*S. cerevisiae*	Recombinant: cell surface engineering system, codisplaying *Rhizopus oryzae* glucoamylase and *Streptococcus bovis* α-amylase	61.8 g /l (86.5% of theoretical yield and 0.31 g ethanol/g starch)	Shigechi et al. 2004a
Low-temperature-cooked (80 °C) corn starch (90 g/l)	*S. cerevisiae* Strain YF207/pGA11/pAA12	Recombinant: cell surface engineering system, co-expressing *R. oryzae* glucoamylase and *B. stearothermophilus* α-amylase	30 g/l after 36	Shigechi et al. 2004b
Raw corn starch	Nonflocculent *S. cerevisiae* (YF237)	Recombinant: cell surface engineering system, displaying glucoamylase and secreting α-amylase	Batch: 0.18 g/(g cell) h	Khaw et al., 2006a,b
Liquefied starch	Sake yeast *S. cerevisiae* GRI-117-UK	Recombinant: cell surface engineering system, displaying glaA gene from *A. oryza*	18.5g/l after 48h	Kotaka et al., 2008
Hydrothermal pretreated and cellulase hydrolyzed cassava pulp (5%)	Engineered *S. cerevisiae* Kyokai strain K7 (K7G)	Recombinant: cell surface engineering system, displaying *R. oryzae* glucoamylase	91% of theoretical yield	Kosugi et al., 2009
Potato soluble starch (20 g/l)	*S. cerevisiae* Strain SBCF2	Recombinant: expressing fungal gluco amylase gene (*sgal*)	5 g/l ethanol after 18 h	Favaro et al., 2010a,b
Starch (150 g/l)	Polyploid *S. cerevisiae*	Combining δ-integration and polyploidization: polyploidy yeasts expressing *S. bovis* α-amylase and *R. oryzae* glucoamylase/α-agglutinin fusion protein genes	75 g/ l ethanol after 72 h	Yamada et al., 2010a,b
Raw corn starch (20% (w/v))	Industrial strain *S. cerevisiae*	Expressing *A. awamori* glucoamylase gene (*GA1*), and *D. occidentalis* α-amylase gene (*AMY*)	10.3% (v/v) ethanol (80.9 g/l) after 6 days	Kim et al., 2010, 2011
High-yielding brown rice	*Polyploid S. cerevisiae*	Recombinant and polyploid: amylase expressing yeast without any pretreatment or addition of enzymes or nutrients.	Productivity: 1.2 g/l h (101% yield)	Yamada et al., 2011
Raw starch	*S. cerevisiae (RaGA)*	Recombinant: expressing *R. arrhizus* glucoamylase gene in *S. cerevisiae*	50 g/l ethanol	Yang et al., 2011
Cassava starch and Raw cassava tuber powder (200 g/l)	*K. marxianus* strain YRL 009	Recombinant*: E*xpressing α-amylase from *A. oryzae* as well as α-amylase and glucoamylase from *D. occidentalis*.	79.75 and 69.73 g/l from cassava starch and raw cassava powder, respectively	Wang et al., 2014b
Raw corn starch	*S. cerevisiae* strain Y294	Recombinant: Expressing a native and codon-optimized variant of the *A. awamori* glucoamylase gene.	0.42 (g/g) (75% of the theoretical maximum yield)	Favaro et al., 2012 b
Raw corn starch (100 g/l)	Diploid *S. cerevisiae*	Recombinant: cell surface engineering system, co-displaying glucoamylase and modified α-amylase	Productivity and yield of 1.61 g/l h, and 76.6% of the theoretical yield	Yamakawa et al., 2012
Soluble starch	*Z. mobilis* CICC 10225(p130A)	Recombinant: Two secretion signals zmo130 and zmo331 native to *Z. mobilis* were fused to the N terminal of α-amylase from *B. subtilis* and transformed into *Z. mobilis.*	50% of the theoretical yield	Wang et al., 2012b
Potato starch	Mutant *Candida albicans* strain OMC3E6	Mutation	437 g/Kg potato starch	Aruna et al., 2014
Cassava flour	Mutated *C. acetobutylicum* PW12 (ART18)	Mutation	16.3 g/l Butanol and 0.28 g/l/h ABE	Li et al., 2014 a, b

integrated haploid strains, each expressing either α-amylase or glucoamylase gene. Favaro et al., (2012b) transferred both the native and synthetic glucoamylase genes into the strain Y294. The resultant recombinant strain containing the synthetic glucoamylase gene showed up to 48% amylase activity, and could utilize and ferment raw corn starch as sole carbon source, and effectively produced ethanol (0.42 g/g) with a yield of 75% of the theoretical value.

Recently, Wang et al. (2014b) engineered the thermotolerant yeast *K. marxianus* to enhance expression of α-amylase from *A. oryzae* as well as α-amylase and glucoamylase from *Debaryomyces occidentalis*. The recombinant strain YRL 009 could produce 66.52 g/l ethanol from 200 g/l cassava starch at 42°C, as the optimal fermentation temperature. By increasing the initial yeast inoculum, the efficiency was increased to 79.75 g/l ethanol with a yield of 78.3% of the theoretical value. Moreover, they showed that the recombinant strain could directly ferment 200 g/l non-sterile raw cassava tuber powder (containing 178.52 g/l cassava starch) to produce 69.73 g/l ethanol by consuming 166 g/l sugar without additional nutritional supplements.

Aruna et al. (2014) improved a mutant *Candida albicans* strain for direct hydrolysis of starchy materials and converting to ethanol. The novel strain enhanced the hydrolysis of insoluble starch by 72% and potato starch by 70%, and under optimized conditions, it could produce 437 g ethanol/kg potatoes without any cell immobilizations or exogenous amylase application steps after 48 h of fermentation. Li et al. (2014a) developed a mutated strain of *C. acetobutylicum* (ART18) with more ABE production from cassava flour, compared to the wild type. The novel strain showed 16.3 g/l butanol production, 0.19 g/g butanol productivity, and the total solvent production was measured at 0.28 g/l/h. This research group in another effort developed a mutated *C. acetobutylicum* (SE36) with high butanol and solvent (ABE) production and tolerance (Li et al., 2014b). These studies showed that CBP ethanol and butanol production from starchy biomass could be promising and commercialized in the near future.

2.3.6. Metagenomics and synthetic biology in CBP

New high throughput molecular techniques, such as metagenomics, next generation sequencing, transcriptomics, metatranscriptomics, proteomics, metaproteomics, metabolomics and synthetic biology have opened a new door to enhance commercialization of CBP ethanol and butanol production. Furthermore, recent advances in synthetic biology have helped design artificial microbes as microbial cell factories and better enzymes for bioethanol production from lignocelluloses (Sommer et al., 2010; Parisutham et al., 2014). Multiple cellulases are needed to support the growth of recombinant microbes directly on lignocellulosic biomass. These enzymes should either be secreted extracellular (free cellulases) or bound to the surface (cellulosomes) to achieve an efficient hydrolysis of lignocellulosic biomass (Parisutham et al., 2014). Exploring and designing new cellulases with higher activity, wide substrate specificity, thermotolerance, and chemical tolerance, by using metagenomics or protein engineering techniques is also a very important subject to obtain ideal CBP microorganisms (Pottkamper et al., 2009; Graham et al., 2011; Parisutham et al., 2014). Application of new "omics" technologies especially metagenomics to discover new powerful hydrolases with synergistic activities is of importance.

Newly, many metagenomics studies have been focused on discovering novel cellulases from different sources, such as cow rumen (Hess et al., 2011; Del Pozo et al., 2012; Wang et al., 2013), camel rumen (Gharachaei et al., 2014), and termite digestive system (Warnecke et al., 2007). Surface-display technology, as one of the most successful strategies has been used for designing CBP organisms. This technology has helped in mimicking the cellulosomal structures observed in anaerobic cellulolytic bacteria to be used especially in ethanologenic yeasts (Ueda and Tanaka, 2000; Wen et al., 2010). These designed cellulosomes do not diffuse out into the surrounding media, and are also very close to each other which would enhance the synergy among the different cellulases in hydrolyzing the biomass (Matano et al., 2013). In addition, achieving efficient cellobiose-consuming microbes is known as one of the first steps towards CBP. So, recently, different recombinant microbes were constructed for efficient uptake and intracellular hydrolysis/metabolism of cellobiose and cello-oligosaccharides (Ha et al., 2011; Vinuselvi and Lee, 2012). Another strategy is heterologous expression of thermophilic cellulases in agricultural crops to enhance loosening of the cell wall (via self-destruction) during pretreatment and to ease the microbial hydrolysis (Jung et al., 2012). A combination of modified microbes, plants, and enzymes may be an efficient approach to achieve an ideal CBP system (Parisutham et al., 2014).

An ideal CBP microorganism, in addition to high cellulase activity, should also have a high tolerance to toxic compounds produced during biomass pretreatment that could inhibit microbial growth and ethanol yield. Such capabilities could increase biofuel yield and productivity. Systems biology approaches including disruptome screening, transcriptomics, and metabolomics have been recently exploited to gain insights into the molecular and genetic traits involved in tolerance and adaptation to the fermentation inhibitors (Hasunuma and Kondo, 2012; Schuster and Chin, 2013; Parisutham et al., 2014). Different strategies, such as global transcription factor engineering (Alper et al., 2006) and exporting toxins using a proton motive force, such as specific efflux pumps (Dunlop et al., 2011) have been used to enhance tolerance to biofuels and the toxic compounds. Similarly, metagenomics has been used in searching for genes responsible for enhancing tolerance to lignin in native cellulolytic microbes (Sommer et al., 2010; Hess et al., 2011).

It is worth quoting that the majority of the previous studies to achieve efficient CBP organisms were based on simple forms of cellulose such as Avicel, phosphoric acid-swollen cellulose or ionic liquids-pretreated miscanthus, whereas lignocellulosic materials are more heterogeneous and complex. The hemicellulosic portion of lignocelluloses are more complex and is commonly composed of several backbones like xyloglucan, glucomannan, glucuronoxylan, etc., depending on the type of plant residue (Parisutham et al., 2014). So, an ideal CBP microorganism, similar to native cellulolytic microorganisms, should possess all sets of cellulases and should be able to grow on different available carbon sources (Morisaka et al., 2012). High-throughput screening technologies and metagenomics have been established to screen for better enzymes that can reduce the requirements for a large number of cellulases (Peralta-Yahya et al., 2008).

2.3.7. Challenges in CBP biofuel production toward commercialization

In spite of the above-mentioned advantages and potentials of CBP biofuel production, this process has not been commercialized yet due to some problems. These problems are as follows; a) the ethanol yields and productivity is low and the fermentation process is time-consuming (3 to12 d) (Sarkar et al., 2012), b) optimal hydrolysis rates of cellulases are usually higher than that of ethanol producing microorganisms, however, this disadvantage could be circumvented if thermophilic microbes could be used as a platform host (Lynd et al., 2005; Schuster and Chinn, 2013), c) genetically-modified microorganisms used as CBP organisms generally have problems related to tolerance, genetic stability, and productivity (Lynd et al., 2002; Parisutham et al., 2014), d) adverse effects and possible unwanted consequences of the co-expression of multiple heterologous cellulase genes on cellular performance of the ethanol-producing host, e) it is not clear how many of the cellulase genes should be introduced into a single strain of host organism to convert it into a viable CBP organism, f) modulation of simultaneous co-expression of multiple cellulase genes at the transcription level for their expression at appropriate levels could be difficult, and finally, g) some of the secretory cellulolytic proteins may not fold properly (den Haan et al., 2013). Unfolded secretory proteins are degraded by the cell's endoplasmic reticulum associated protein degradation mechanisms, and also they induce cellular unfolded protein response (UPR) to deal with this stress (Sun et al., 2002; den Haan et al., 2013).

Although the CBP technology encountered many challenges and is still under development, during the last decade a few of the ethanol producing companies have been focused on commercial production of ethanol from lignocelluloses using CBP (Schuster and Chin, 2013). The pioneers of this revolution were Qteros and Mascoma companies which used *C. phytofermentans*, trademarked as the "Q Microbe" (Freeman, 2012) and a mixture of yeasts including *S. cerevisiae* engineered for heterologous expression of termite cellulases (Doran et al., 2012), respectively, to produce ethanol from lignocellulosic materials in a CBP process. However they encountered many challenges on this road. Mascoma is currently the only major industry player utilizing CBP technology, but a number of other companies such as DSM POET (POET, 2009), Abengoa (Anon, 2011) and LanzaTech (Williams, 2012) are striving to produce commercial quantities of

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ethanol from lignocellulosic feedstocks. This successful history indicates that in the near future production of ethanol from lignocellulosic biomass especially using CBP microorganisms will hopefully turn into reality (Schuster and Chin, 2013).

3. Conclusion

Recent advances in biofuel production from lignocellulosic biomass, including new efficient and economic pretreatment technologies, fermentation technologies and CBP have increased hope for a viable solution to the energy crisis. This paper has comprehensively reviewed recent advances in CBP systems for efficient bioalcohols (ethanol and butanol) production from lignocellulosic and starchy biomass. A CBP process would utilize a single substrate and product-tolerant microorganism(s) that perform both hydrolysis and fermentation process at the same time in one pot. CBP microorganism should concurrently hydrolyze lignocellulosic biomass and utilize multiple sugars obtained for fermentation and production of biofuel with metabolic pathways leading to minimal byproducts formation. During the last decade, significant advances have been made in development of CBP biofuel production. Different strategies and a large number of different native and genetically-engineered microorganisms have been used for CBP biofuel production. These strategies include using native single strains with both cellulytic and ethanologenesis activities, microbial co-cultures (co-culture of cellulytic and ethanologenic microorganisms) and genetically-engineered microorganisms. The last strategy includes genetic and metabolic engineering of either single cellulytic microorganisms to be ethanologenic or single ethanologenic microorganisms to be cellulytic. In both cases i.e. native and recombinant strategies, achieving a high performance under industrial conditions is of main importance. The major priority for the native strategy is to reach high fermentation yields and titer from pre-treated lignocellulosic feedstocks while co-expression of multiple proteins allowing the utilization of such pre-treated feedstocks with high hydrolysis yields and reasonable rates of biofuel is the main priority of the recombinant strategy.

An ideal CBP organism should use the synergistic effects of cellulases and hemicellulases together in order to efficiently degrade lignocellulose to a greater extent. Depending on the feedstock used, the ratio of endo- and exoglucanases must be altered in order to maximize polysaccharide degradation and prevent the accumulation of inhibitory intermediate oligosaccharides, such as cellobiose. Cell surface technology and cellulosomes are promising means of achieving stringent specifications for saccharification because through which enzyme ratios could be altered, and substrate-specific carbohydrate-binding modules could be achieved. In addition, bound cellulosomes concentrate sugars near the cells for maximal uptake. High-throughput techniques, such as metagenomics, metatranscriptomics, next generation sequencing and synthetic biology are necessary for exploring novel powerful enzymes with high activity, thermostability and pH stability. Another strategy is to enhance tolerance to stressors, including toxic compounds and final product i.e. biofuels. Different strategies such as engineering of metabolic pathways and also global transcription factors have been employed to enhance tolerance to toxic compounds, and significant successes have been achieved.

Although a few companies have recently started to use CBP technology for biofuel production from lignocelluloses, up to now it has not been fully commercialized. Nevertheless, the valuable findings of the huge amounts of research studies focused on different aspects of CBP signal a bright future for commercial production of biofuels from the widely-abundant lignocellulosic feedstock. A major remaining CBP challenge is to achieve near-optimal conditions within a single bioreactor for all steps in the process: hydrolytic enzyme secretion, saccharification, and fermentation. Overall, to achieve successful commercialization of next-generation lignocellulosic ethanol technology, it is necessary to bring the remaining barriers into focus, and combine a multitude of individual successes into one high-yield, low-cost process.

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