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TOWARDS BIOETHANOL: AN OVERVIEW OF WHOLE LIGNOCELLULOSE PROCESSING

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Abstract

Bioethanol production from lignocellulosic biomass presents great potential regarding fossil fuel dependence mitigation. In fact, extensive research has been performed in order to make biomass-to-bioethanol industry economically sustainable. However, many aspects related to pretreatment of

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lignocellulose, hydrolysis of complex carbohydrates into simple sugars and fermentation of thereof obtained sugars still constitute a limitation and consequently need to be improved. In the present work, a review of the most currently used pretreatment methods is presented as well as detoxification methods to mitigate the effect of toxic compounds formed. The most promising hydrolysis and fermentation technologies, leading to the highest hydrolysis rates as well as ethanol yield, are also reviewed. Lastly, the advantages of "in situ" recovery of ethanol are highlighted and the methods presenting the highest potential to achieve this goal are identified.

1. Introduction

Despite the worldwide petroleum dependence, fossil fuels represent a scarce non-renewable resource. The exploitation of renewable and environmental sustainable energy sources, capable of replacing fossil fuels, at least partially, with competitive price is nowaday a global priority. However, in order to achieve this goal, the alternative energy sources must respond to market demands, be economically sustainable and competitive with conventional fuels. Moreover, it is imperative to meet the standard requirement and specifications of fuel quality and have comparable or better performance than fossil fuels since it is not realistic to aim for the full replacement of all the engines and equipments, operating at the present, in a short period of time. Biomass-to-biofuel industry presents great potential. Lignocellulosic biomass is a strong candidate to be used as feedstock for production of bioethanol that can be used blended with gasoline at any ratio [1]. Lignocellulose is an abundant raw material and it is composed mainly by cellulose, hemicelluloses, and lignin [2], [3]. Cellulose and hemicellulose can be used to produce ethanol by mean of microbial ethanologenic fermentation after their hydrolysis into monosaccharides. Several strains of yeasts are used to carry out ethanol fermentation as well as certain bacterial strains [4], [5], [6]. Examples of lignocellulosic materials that have been researched for bioethanol production are wood (hardwoods and softwood), herbaceous crops (alfalfa, switchgrass), forestry waste and agricultural residues (corn stover, wheat straw) [7], [8]. One of the great advantages

of using lignocellulosic material as feedstock for bioethanol production lies in the fact that it does not compete directly with food chain crops. Moreover, agriculture waste resources can be used and land not suitable for food crops production can be rentabilized. Regarding feedstock supply, cooperative approach established between farmers and ethanol producers can bring advantages to both parties. In addition, those productions can generate employment opportunities in the rural regions. In fact, feedstock collection, handling, and transportation to the ethanol plant can be performed by local farmers. This ensures, in one hand, continuous supply of feedstock to the ethanol producer and, on the other hand, provides the farmers with the possibility to get revenue and valorize the raw material that otherwise would go to waste [9]. In the present review, an approach of the most currently used methods for pretreatment of lignocellulosic material and detoxification of pretreatment generated toxic compounds is made. Furthermore, hydrolysis and fermentation strategies, as well as strategies for ethanol recovery are approached in this overview.

2. Pretreatment

Lignocellulose is mainly composed of three polymers: cellulose, hemicellulose, and lignin. In addition to these three components, lignocellulose may also contain, in smaller amounts, proteins, lipids (waxes, oils, fats), and ash [9]. Cellulose and hemicellulose represent, respectively, the most and the second most abundant polysaccharides in nature [10], [11]. Lignin is a complex polymer of phenolic units [8], [9], [12]. In order to obtain simple sugars from lignocellulosic material, hydrolysis of cellulose and hemicellulose is required [11]. However, without pretreatment of lignocellulose, the hydrolysis is extremely slow due to cellulose recalcitrance [12]. Pretreatment is, therefore, an essential step in order to disrupt the cell wall macromolecular complex [13] and make cellulose more accessible to subsequent hydrolysis [14]. Cellulose crystallinity is decreased as well as the degree of

polymerization [2], [9]. As a consequence of pretreatment, porosity increases and, consequently, the available surface area for enzyme attachment to cellulose and hemicellulose also increases [2], [9].

There is no ideal pretreatment method that is suitable for every feedstock type. Each pretreatment method has its specific effects on the polymers present in the lignocellulosic feedstock. A good pretreatment method should prevent degradation or loss of carbohydrates [9], [12], [15], [16]. Formation of toxic compounds due to severe pretreatment conditions affecting the subsequent hydrolysis and fermentation must be avoided [9], [12], [15], [16]. Cost-effective pretreatment techniques must, also, be chosen since pretreatment step cost represents one of the most relevant fractions of overall cost [9]. Low demand of post-pretreatment processes such as washing, neutralization, and detoxification as well as high treatment rates, relatively low energy input and capital cost are also aspects desirable in a pretreatment method [17], [18]. Alkalinity is an important parameter to consider during pretreatment. Keeping the pH value within the range from 4 to 7 during the pretreatment helps reducing the formation of inhibitory compounds. At these conditions, hemicellulosic sugars are retained in oligomeric form and consequently the formation of degradation product is lower [2]. Furthermore, different methods can be combined in order to increase cellulose and hemicellulose conversion.

Several pretreatment methods can be distinguished and classified as physical, chemical, physicochemical, and biological [9], [12].

2.1. Physical methods

Physical treatments are used to break the lignocellulosic structure, decrease cellulose crystallinity and degree of polymerization and increase accessible surface to enzyme action [19]. Chemical agents are not used in this type of pretreatment [20]. Examples of physical pretreatment techniques include mechanical comminution [2], extrusion [2], and ultrasound pretreatment [2], [21].

2.1.1. Comminution

Comminution consists in reducing the size of lignocellulosic material in order to disrupt cellulose crystallinity and decrease the degree of polymerization [20]. This can be achieved by a combination of grinding, milling and/or chipping depending on lignocellulosic material and on the final particle size intended [15], [22], [23]. After grinding or milling, particle size is reduced to 0.2-2mm. Particles with 10-30mm are obtained after chipping [2], [15]. Hammer- [14], [24], knife- [25], ball- [26] and compression mills are examples of equipments used in mechanical size reduction of lignocellulosic materials [20], [27], [28].

2.1.2. Extrusion

Extrusion involves passing the biomass through an extruder suffering heating, mixing and shearing and allowing physical and chemical modification. The lignocellulose structure is disrupted and the accessibility of carbohydrates to enzymatic attack is increased [2], [29].

2.1.3. Ultrasound

Ultrasound pretreatment consists in the opening of substrate structure by collapsing the bubble formed due to cavitation effect [2], [30]. Cavitation occurs at 50ºC, an optimal temperature for many enzymes enhancing the diffusion of enzyme toward the substrate surface [2].

2.2. Chemical methods

Chemical pretreatments are characterized by a high degree of selectivity for their target component in the biomass and normally involve harsh conditions [21]. Examples of chemical pretreatments are dilute and concentrated acid, alkaline pretreatments and organosolv [13], [21].

2.2.1. Acid pretreatment

Acid pretreatment is one of the most used methods for lignocellulosic biomass treatment [9]. Dilute and concentrated acid treatments can be distinguished. In the former, an acid such as H_2SO_4, HCl, H_3PO_4 or HNO_3 is used at low concentrations (0.7-3.0% (w/w)). Temperatures around 150ºC are used for periods of time of 15-20 minutes followed by temperatures of around 240ºC for 1-5 minutes. This method leads to hydrolysis of hemicellulose and part of lignin [19]. Formation of a large number and types of inhibitory compounds such as furans, organic acids, and phenolics is associated to this method [2], [31]. Concentrated acid pretreatment is not economically feasible since it requires high amounts of acid, which makes it an expensive method [31].

2.2.2. Alkaline pretreatment

Alkaline pretreatment leads to swelling of the biomaterial pores increasing surface area and decreasing the degree of polymerization and crystallinity. In this pretreatment method, biomass is soaked in an alkaline solvent such as NaOH and then heated for a certain time [2], [15], [22]. Since it acts by delignification, it is more effective on agricultural residues and herbaceous crops compared to wood materials that contain less lignin that the two residues first mentioned [22]. $Ca(OH)_2$ is also used for alkaline pretreatment and it is suitable for agriculture residues such as corn stover and hardwood materials such as poplar [2], [22]. A combination of acid and alkaline pretreatment for corncob treatment showed no detectable furfural and HMF [13].

2.2.3. Organosolv

Organosolv pretreatment consist in using an organic or aqueous-organic solvent mixture in order to remove lignin from lignocellulosic material [28], [32]. This occurs at temperatures between 100-250ºC in order to dissolve lignin and it can be performed either in the presence or absence of catalyst [32]. In fact, when high temperatures (above 160ºC)

are used, the need for catalyst is reduced [23]. Notwithstanding, H_2SO_4 or HCl are examples of inorganic catalyst used [12], [22] and organic acids such as oxalic, acetylsalicylic, and salicylic can also be used as catalysts [15]. Methanol, ethanol, acetone, ethylene glycol, and tetrahydrofurfuryl alcohol are examples of organic solvents used in this process.

2.3. Physicochemical methods

Physicochemical treatments such as liquid hot water, steam expansion, SO_2 expansion, ammonia fiber expansion (AFEX), CO_2 expansion, and ionic liquid pretreatment are also examples of methods used for lignocellulosic biomass pretreatment [13], [21].

2.3.1. Steam expansion

Steam pretreatment, also called autohydrolysis [20] is also one of the most common methods used for lignocellulose pretreatment [9]. It removes a major fraction of the hemicellulose and makes the cellulose more susceptible to enzymatic digestion. In this method, only steam water is used [20] and the material is subjected to high pressured (between 6 and 34 bar) [22] for a period varying from seconds to a few minutes and then the pressure is suddenly reduced to atmospheric pressure, which makes the material undergo an explosive decomposition [8], [12]. The temperature used in this process range between 160ºC and 260ºC [12], [20]. It performance can be improved by using H_2SO_4 or SO_2 as acid catalyst [22].

2.3.2. Ammonia fiber expansion

Ammonia fiber expansion is an alkaline method in which lignocellulose is exposed to liquid ammonia at high temperatures (90-100ºC) for a period of time (10-60 minutes) [22] and then the pressure is suddenly reduced [12], [22]. Due to the sudden pressure release and subsequent rapid expansion of the ammonia gas, biomass fibers are disrupted and cellulose partially decrystallized [2], deacetylation of

hemicellulose occurs and biomass digestibility is increased. It is worth-mentioning that ammonia pretreatment does not lead to formation of fermentative inhibitory compounds [2], [15]. Furthermore, more than 90% conversion of cellulose and hemicellulose into fermentable sugars can be achieved [2]. Alvira et al. [2] have suggested that alkaline-based pretreatment methods such as with $Ca(OH)_2$, ammonia fiber expansion (AFEX), and ammonia recycling percolation (ARP), are promising methods reducing lignin content of agricultural residues but are less effective treating recalcitrant substrate as softwoods [2].

2.3.3. SO_2 expansion

SO_2 expansion is a hydrothermal pretreatment in which the biomass, firstly impregnated with SO_2 [33], is subjected to pressurized steam for a period of time ranging from seconds to several minutes and then suddenly depressurized [2]. This leads to fiber separation [2] and leads to high glucose and high xylose yields during enzymatic hydrolysis [33].

2.3.4. CO_2 expansion

Supercritical CO_2 is mostly used for extractive purpose. However, as reported by Alvira et al. [2], supercritical pretreatment appears to be a promising method for lignin removal. Given that CO_2 molecule is small, it can penetrate the pores of lignocellulose in the same way as H_2O molecule, increasing accessible surface area [23]. This pretreatment process, using pressurized CO_2, can be performed at lower temperatures (such as 35ºC [34]) compared to other pretreatment methods, such as steam expansion, contributing for decreasing cost, and sugar degradation [2], [28]. When the pressure is suddenly released, cellulose structure is disrupted leading to increased accessible surface [34].

2.3.5. Liquid hot water

Liquid hot water is a hydrothermal treatment in which pressure is applied to maintain the water at the liquid state at high temperatures (160-240ºC) causing modification in the structure of lignocelluloses [2], [20]. Hemicellulose is solubilized without extensive inhibitory compounds formation and cellulose becomes more accessible [2], [8].

2.3.6. Ionic liquid pretreatment

Apart from the most common used pretreatment process such as dilute acid pretreatment or steam expansion, pretreatment using ionic liquids represents a promising method to be used in lignocellulosic materials [35]. Ionic liquids (IL), salts that exist as liquids at relatively low temperatures (< 100ºC) [36] and that present high thermal and chemical stability, can selectively extract lignin from lignocelluloses [37]. They also allow cellulose crystallinity reduction which may have practical advantages for enzymatic hydrolysis of cellulose [38]. Lee et al. [39] have, in fact, observed lower degree of crystallinity for cellulose reconstituted after being dissolved in 1-allyl-3-methylimidazolium chloride ([Amim][Cl]) and 1-butyl-3-methylimidazolium chloride ([Bmim][Cl]) compared to native cellulose [39]. Pinkert et al. [36] found that regenerated cellulose had higher enzyme-adsorption capacity than untreated one. This fact suggested higher accessible surface area and more binding sites for cellulase. Higher attachment of enzymes to the substrate was expected as well [36]. Studies performed by Zhao et al. [38] revealed better protection of cellulase against thermal denaturation in the presence of ionic liquid. Consequently, hydrolysis at higher temperature was possible [38]. It is worth-mentioning that complete extraction of lignin is not needed in order to achieve high enzymatic hydrolysis yield, which can result in cost reduction [39]. In fact, Lee et al. [39] have been able to obtain cellulose degradability higher than 90% at 40% total lignin extracted from maple wood [39] using the ionic liquid 1-ethyl-3-methylimidazolium acetate ([Emim][CH_3COO]). The regeneration

of cellulose is achieved by adding an excess of polar solvent like water, acetone, dichloromethane, acetonitrile, or mixtures of them [36]. Spronsen et al. [35], have investigated the hydrolysis of pine wood and wheat straw using a combination of [Emim][Cl] and acetic acid. Using acetic acid along with [Emim][Cl] enhanced the rate of hydrolysis and dissolution allowing pretreatment at milder conditions. As a result, formation of inhibitory components is minimized. Lignocellulosic biomass was hydrolyzed into cellulose, hemicellulose, and lignin. Cellulose and then lignin could be isolated and recovered by precipitation with ethanol and water, respectively [35]. Although ionic liquids present great potential for biomass pretreatment, residual IL entrapped by cellulose can lead to cellulase activity reduction. Thus, IL residues should be carefully removed otherwise the final concentration of total reducing sugars and glucose can be affected [39]. Examples of ionic liquids that have been used in lignocellulose pretreatment are presented in Table 1.

Table 1. Ionic liquid application for biomass pretreatment

Substrate	Ionic liquid	Treatment conditions	Extracted lignin (g/Kg biomass)	Cellulose dissolution % (w/w)	Reducing sugars yield (%)	Cellulose crystallinity index reduction (%)	References
Wood flour	[Emim][CH_3COO]	80ºC; 24h	4.4	–	–	–	[39]
	[Amim][Cl]	80ºC; 24h	5.2	–	–	–	
	[Mmim][$MeSO_4$]	80ºC; 24h	–	–	–	–	
	[Bmim][CF_3SO_3]	80ºC; 24h	–	–	–	–	
Dissolved pulp	[Amim] [Cl]	60ºC; stirring	–	–	–	–	[40]
	[Amim] [Cl]	80ºC; 30min	–	5	–	–	
	[Amim] [Cl]	80ºC; > 30min	–	14.5	–	–	
BSP	[Amim][Cl]	80ºC; 8h	–	8	–	–	[41]
NSS	[Bmim][Cl]	110ºC; 8h	–	8	–	–	
NSTP	[Bmim][Cl]	130ºC; 8h	–	7	–	–	
	[Bmim][Cl]	130ºC; 8h	–	7	–	–	
SPTP	[Amim][Cl]	130ºC; 8h	–	5	–	–	
	[Bmim][Cl]	130ºC; 8h	–	5			
	[Bzmim][Cl]	130ºC; 8h	–	5			
	[Bz-ome-mim][Cl]	130ºC; 8h	–	5			

Table 1. (Continued)

Rice straw	[C4mim][Cl]	100°C; 45min	–	–	74	–	
Pine wood	[C4mim][Cl]	30°C; 60min	–	–	85	–	
Bagasse	[C4mim][Cl]	100°C; 60min	–	–	66	–	[42]
Corn stalk	[C4mim][Cl]	100°C; 30min	–	–	66	–	
Corn stalk	[C4mim][Br]	100°C; 60min	–	–	47	–	
Corn stalk	[Amim][Cl]	100°C; 90min	–	–	65	–	
Pine wood	[C4mim][Cl]	100°C; 12h	–	44	–	–	[43]
Oak wood	[C4mim][Cl]	100°C; 12h	–	35	–	–	
Avicel PH-101 cellulose	[Bmim][Cl]	110°C; 15min	–	–	–	~ 38	
	[Emim][OAc]	110°C; 15min	–	–	–	~ 23	
	$[Me(OEt)_2–Et–Im][OAc]$	110°C; 15min	–	–	–	~ 29	
	$[Me(OEt)_3–Et–Im][OAc]$	110°C; 15min	–	–	–	25	[38]
	$[Me(OEt)_4–Et–Im][OAc]$	110°C; 15min	–	–	–	~ 24	
	$[Me(OEt)_3–Et_3N][OAc]$	110°C; 15min	–	–	–	~ 29	
	[Bmim][Cl]	110°C; 15min	–	–	–	~ 38	

The abbreviations mean ball-milled southern pine powder (BSP), Norway spruce sawdust (NSS), Norway spruce thermomechanical pulp (NSTPB) and southern pine thermomechanical pulp (SPTP).

2.4. Biological pretreatment

Biological pretreatment of lignocellulosic biomass involves the use of lignin-degrading microorganisms [2], [15], [22] such as brown, white, and soft-rot fungi applied to degrade lignocellulosic material. While brown-rots are responsible for cellulose conversion, white- and soft-rot convert both cellulose and lignin [28]. Lignin degradation by white-rot fungi is due to the presence of lignin-degrading enzymes such as laccase and peroxidase [2]. Examples of white-rot fungi showing high delignification efficiency are *Phanerochaete chrysosporium, Ceriporia lacerata, Cyathus stercolerus, Ceriporiopsis subvermispora, Pycnoporus cinnarbarinus*, and *Pleurotus ostreatus* [2]. Wheat straw was biologically pretreated using a white-rot fungus, *Irpex lacteus* followed by fermentation with the yeast *Pachysolen tannophilus* leading to a yield of 163mg ethanol per gram of raw wheat straw [44]. *P. ostreatus, Pleurotus* sp. 535, *P. cinnabarinus* 115 (white-rot fungi), and *Ischnoderma benzoinum* 108 were also tested for pretreatment of wheat straw increasing subsequent biomass enzymatic hydrolysis after 5 weeks of pretreatment [45]. Corn stover was pretreated using the white-rot fungus *P. chrysosporium* CGMCC5.776 improving its enzymatic digestibility. The highest enzymatic hydrolysis yield was achieved after 9 days of pretreatment [46]. Results from recent studies have shown that fungal pretreatment of wheat straw for 10 days with a high lignin-degrading and low cellulose-degrading fungus (fungal isolate RCK-1) has increased the release of fermentable sugars and reduced fermentation inhibitors [2]. Although biological pretreatment is characterized by low energy requirements and by application of mild conditions, the reaction rate is very low [15], [36]. Thus, its industrial applicability is limited. Notwithstanding, it can be applied as a first step followed by other types of pretreatment methods [22].

3. Toxic Compounds and Strategies to Overcome their Effect

3.1. Toxic compounds

Although pretreatment is needed to deconstruct the plant cell wall, compounds that are toxic to fermentative microorganisms are normally formed due to the harsh conditions applied [2], [47], [48]. Toxic compounds formed during pretreatment and affecting fermentative microorganism performance can be divided in three main classes: furan derivatives, weak acids, and phenolic compounds [49].

Furan derivatives such as furfural and 5-hydroxymethyl furfural (HMF) are generated from degradation of hemicellulose [2]. Furfural and HMF are formed at high temperatures and pressures [7] due to degradation of pentose and hexose monomers of hemicellulose, respectively, [7], [47], [50]. Furfural affects cell replication [50], cell growth rate and ethanol productivity. Moreover, it induces DNA damage and inhibits several enzymes in glycolysis [51]. Cell growth was suggested to be more sensitive to furfural than the ethanol production [7]. Sainio et al. [52] have compared results obtained by several authors and observed different effects of furfural depending on concentration and strain. HMF, is also a yeast growth inhibitor in a similar manner to furfural, although it is less toxic than furfural [7], [47], [53].

Weak acids, affecting fermentation performance, are derived from hemicellulose and lignin degradation. Acetic acid, for instance, is a weak acid formed from de-acetylation of hemicellulose, for instance, during steam expansion [7], [47]. Other weak acids formed during pretreatment are levulinic and formic acids derived from the degradation of furfural and HMF [2], [7]. It was suggested that weak acids, such as formic, acetic, and levulinic, reduce yeast growth and ethanol yield by decreasing intracellular pH [54]. Undissociated acid enters the cell through diffusion over the cell membrane and its dissociation in the cytosol leads to a decrease of the intracellular pH affecting cell viability [7], [48].

Phenolic compounds are another class of toxic compounds formed during pretreatment [7], [55] due to the lignin breakdown [2], [50]. Examples of such compounds are syringaldehyde, syringic acid, syringone, vanillic acid, vanillone, and vanildehyde causing loss of integrity of cell membranes of microorganisms [7], [50]. Low molecular weight phenolic acids show similar behaviour to weak acids and appear to be even more toxic to the microbial cell than furfural or HMF [50]. As weak acids, phenolic acids are able to affect intracellular pH affecting cell viability. They interfere with the cell membrane influencing its function and changing its protein-to-lipid ratio [48], which affects its ability to serve as selective barrier [7], [50]. A considerable decrease in inhibition was observed by selectively removing phenolic compounds with the enzyme laccase suggesting that phenolic compounds are highly inhibiting compounds [55]. Furthermore, results comparing the inhibitory effects of a dilute-acid hydrolysate of spruce and a model sample containing furfural, HMF and weak acids but not containing phenolic compounds showed that the former presented a more inhibiting effect than the latter [55]. It is worth-mentioning that a negative synergetic interaction effect between different toxic compounds leading to increased inhibition exist [55], [56].

Toxic compounds formed during pretreatment depend on the characteristics of the lignocellulosic biomass used as feedstock as well as on the pretreatment conditions chosen. Similarly, the effect that each toxic compound has on the fermentative microorganism depends on the ability of such organism to resist to the toxicity of the inhibitory compound, nutrient supplementation, cultivation mode and conditions [8], [50], [56], [57]. The tolerance of *Saccharomyces cerevisiae* Lalvin EC1118 to furfural was study by Tofighi et al. [58]. The highest glucose consumption rate after 48h obtained in the absence of furfural was 93% which was decreased to 60%, 51%, and 33% in the presence of 4, 5, and 6g/L furfural, respectively [58]. Ask et al. [51] have studied the impact of HMF and furfural on the anaerobic physiology of a xylose-utilizing *S.*

cerevisiae strain VTT C-10883. Including furfural and HMF in the medium resulted in decreased specific uptake-rates for both glucose and xylose compared to the control. HMF and furfural perturbed the redox system of xylose-utilizing *S. cerevisiae* with consequences for metabolism [51]. The tolerance of yeast isolate of Bekonang (*S. cerevisiae*) against furfural, HMF, and acetic acid was studied by Wikandari et al. [59] under aerobic conditions. Addition of furfural concentrations higher than $0.5 gL^{-1}$ has significantly reduced glucose consumption. Ethanol yield and productivity decreased in 26 and 73%, respectively [59]. The effect of furfural, HMF, and vanillin on ethanol production by *Issatchenkia orientalis* IPE 100 was investigated [60]. Concentration of furfural, HMF, and vanillin above $5.56 gL^{-1}$, $7.81 gL^{-1}$, and $3.17 gL^{-1}$, respectively, inhibited ethanol production and led to extended lag phase [60]. Formic, acetic, levulinic, benzoic, and syringic acid, as well as fufural, HMF, vanillin, and 4-hydroxybenzaldehyde were inhibitory to *S. cerevisiae* CEN.PK113-7D [61].

Although the inhibitory effect of furans, weak acids and phenolics, some compounds such as acetic, formic, and levunilic acids can improve ethanol yield when they are present at low concentrations [48].

Examples of toxic compounds affecting ethanologenic organisms' performance are shown in Table 2.

Table 2. Effect of inhibitory compounds on fermentative microorganisms performance

Microorganism	Inhibitor	Inhibitor concentration (gL^{-1})	Ethanol yield (%)	Ethanol production (gL^{-1})	Ethanol productivity ($gL^{-1}h^{-1}$)	References
Zymomonas mobilis ZM4 and A3	Furfural	4[a]	–	–	–	[53]
I. orientalis IPE 100	Furfural	5.56[b]	–	–	–	[60]
	HMF	7.81[b]	–	–	–	
	vanillin	3.17[b]	–	–	–	
S.cerevisiae CEN.PK113-7D	Furfural	0.06[a]	–	–	–	[61]
S.cerevisiae Lalvin EC1118	Furfural	0	9.4	–	–	[58]
		4	4.8	–	–	
		5	3.7	–	–	
		6	1.4	–	–	

Table 2. (Continued)

Isolate of Bekonang (*S. cerevisae*)	Furfural	0	44	22.90	0.48	[59]
	Furfural	1	33	6.14	0.13	
	Furfural	1.5	28	0.53	0.01	
	HMF	0	44	22.90	0.48	
	HMF	1	36	6.55	0.14	
	HMF	3	3	1.67	0.03	
	Acetic acid	0	44	22.90	0.48	
	Acetic acid	1.5	48	24.48	0.51	
	Acetic acid	3.0	44	22.87	0.48	

[(a)]Growth interruption above the referred concentration.
[(b)]Ethanol production inhibition above referred concentration.

3.2. Physical and chemical detoxification methods

Using the whole slurry obtained after pretreatment and/or chemical hydrolysis implies that inhibitory compounds formed are carried to subsequent hydrolysis and fermentation steps [15], [62]. Washing prior to enzymatic hydrolysis could represent a solution to reduce the effect of inhibitory compounds on fermentative microorganism [62]. However, besides removing inhibitors, washing can also lead to loss of some soluble sugars, mainly generated by hemicellulose hydrolysis [15]. Thus, several detoxification methods have been used in order to remove toxic compounds generated during pretreatment and/or chemical hydrolysis allowing better performance of enzymatic hydrolysis and fermentation.

Membrane separation can be used for the extraction of toxic compounds [8], [48], [50], [52]. In fact, a microporous polypropylene hollow fiber was used to remove sulfuric acid, acetic acid, 5-hydroxymethyl furfural, and furfural from dilute sulfuric acid corn stover hydrolyzed [63]. Nanofiltration has been tested in acetic acid and furfural removal [64], [65], [66].

Overliming represents another detoxification method. This method consists in addition of $Ca(OH)_2$ to adjust the pH to 9-10, leading to precipitation of inhibitory compounds. After filtration, pH is readjusted to 5.5 with dilute H_2SO_4 [8]. Besides calcium hydroxide, the application of other alkalis such as sodium hydroxide, sodium thionite, and sodium disulphite is also possible [48], [50]. A combination of sulphide and overliming has been suggested as an efficient method to detoxify willow hemicellulose hydrolyzate prior to fermentation by recombinant *Escherichia coli* [55]. $Ca(OH)_2$, NH_4OH, or NaOH showed potential for detoxification of a dilute-acid spruce hydrolyzates. Detoxification using any of these three alkalis resulted in low sugar degradation and effective inhibitors removal [48].

Activated charcoal represents another chemical detoxification method and it can lead to selective removal of furfural [48], [50], [53]. Activated charcoal treatment was used to detoxify an enzymatic hydrolyzed steam-exploded corn stover, increasing its fermentability [67]. Detoxification using activated charcoal or anion exchange resins was found to lead to fermentation performance similar to that of an inhibitor-free model substrate [3].

Cation exchange resin (CS16GC), neutral polymer adsorbent (XAD-16), and granulated activated carbon (GAC) were compared by Sainio et al. [52] in order to remove furfural, HMF, and acetic acid from a synthetic concentrated acid hydrolysate containing 20 (wt%) H_2SO_4. The order of increasing detoxification efficiency was CS16GC < XAD-16 < GAC for all species. Hydroxymethyl furfural and furfural were the two most strongly adsorbed species [52]. Weil et al. [68] have investigated the removal of furfural from heat pretreated-acid hydrolyzed corn fiber by polymeric adsorbents, XAD-4 and XAD-7. Effective removal of furfural was observed and hydrophobic interactions are the predominant mechanism of adsorption between furfural and the resin [68].

Certain zeolites can selectively absorb inhibitors such as furfural, HMF, and vanillin from pretreated biomass hydrolysate with minimal loss of sugars [3]. Ranjan et al. [3] have investigated several types of zeolites (MFI, β, faujasite, and FER) as adsorbents of HMF, furfural, and vanillin of auto-hydrolyzed aspen wood chips dilute sulfuric acid pretreated hydrolyzate. Zeolite adsorption improved the ethanol yield during fermentation with *E. coli* FBR5 by removing fermentation inhibitors from the media with almost no loss of fermentable sugar [3].

Polyelectrolyte flocculating agents may provide a feasible detoxification method for removal of inhibitors [56]. Carter et al. [56] have been able to remove furfural and HMF from a dilute acid pretreated Ponderosa pine slurry using the polyelectrolyte polyethyleneimine (PEI).

An ethanol yield of 92.6% of theoretical was achieved with *S. cerevisiae* D5A fermenting the detoxified hydrolysate, while no significant ethanol was produced in the undetoxified hydrolysate [56].

Alriksson et al. [112] have used reducing agents dithionite and sulfite to promote in-situ detoxification of an acid pretreated spruce wood and sugarcane bagasse. Alkali was also used for detoxification. No effect of the addition of dithionite or sulfite was observed on total amount of phenolic compounds, HMF and furfural, acetic acid, formic acid or levulinic acid in separate hydrolysis and fermentation (SHF). However, ethanol yield and productivity were increased when dithionite, sulfite or alkali was used.

3.3. Biological detoxification methods

Biological detoxification is among the most promising detoxification approaches although characterized by slow reaction time [50]. It consists in the application of living microorganisms or enzymes to detoxify inhibitory compounds in lignocellulosic hydrolysates [50].

Biological detoxification can be performed "in situ". In this case, subsequent ethanol production can be carried out in the same vessel [1]. Treatment with the filamentous soft-rot fungus *Trichoderma reesei* is one of the biological methods used for inhibitors degradation in a lignocellulosic hydrolysate [48], [50], [55], [56] increasing ethanol productivity. The fungus *Coniochaeta ligniaria* was used to detoxify dilute acid-pretreated hydrolysates of three perennial herbaceous crops (switchgrass, reed canarygrass, and alfalfa stems) prior to simultaneous saccharification and fermentation with *S. cerevisiae* D5A ATCC200062. The treatment reduced furan and acetic acid concentrations by 33-100% and 32 ± 23%, respectively, and eliminated the extended fermentation lag times [47].

Using enzymes able to convert inhibitors in less toxic compounds represents another option to overcome the problem under study. Enzymes would be applied directly to the fermentation broth [7] in order to promote conversion of toxic compounds while they are formed. Enzymes such as laccase and peroxidase from ligninolytic fungus *Trametes versicolor* are used in detoxification processes. These enzymes demonstrated to efficiently remove phenolic compounds from lignocellulosic hydrolysates [8], [48], [50], [55]. Fermentation of willow hemicellulose hydrolysate has been shown to considerably increase with removal of phenolic compounds by detoxification with lignin-oxidizing laccase [7]. Besides phenolic compounds detoxifying enzymes, furfural and 5-HMF detoxifying enzymes may be considered. Gutiérrez et al. [69] have purified and characterized an enzyme catalyzing the reductive detoxification of furfural into furfuryl alcohol. This enzyme, furfural reductase from *E. coli* strain LYO1, maintained 85% of its total activity within a broad range of pH (4-8) and temperature (45-55ºC). However, according to these authors, this enzyme appears to be totally NADPH dependent. Chen et al. [70] have patented methods of reducing toxicity of lignocellulosic hydrolysates. One method reduces the amount of furfural inhibitors and another reduces the amount of xylitol produced during xylose fermentation [70].

It has also been suggested that adapting the yeast cells to pretreatment hydrolysate makes it more resistant to the inhibitors in the fermentation medium [25], [50]. Developing more robust microorganisms, able to resist to higher inhibitors concentrations, can also be a possible solution to overcome inhibitory compounds effect [7], [51]. In fact, strains with increased detoxification ability have been developed. Larsson et al. [31] have developed a Sso2p-overexpressing *S. cerevisiae* transformant strain with increased laccase activity, aiming at fermentation of inhibitory lignocellulosic hydrolysate without previous detoxification. Petersson et al. [71] have developed an overexpressing *ADH6 S. cerevisiae* strain with enhanced capacity to reduce 5-hydroxymethyl

furfural. These strains have increased HMF conversion activity in cell free crude extracts with both NADPH and NADH as co-factors. Nilsson et al. [72] have patented an ethanol producing microbial strain, such as *S. cerevisiae* strain, being able to grow and produce ethanol from lignocellulosic hydrolysates containing furfural and 5-hydroxy-methyl furfural, in a batch, fed-batch or continuous fermentation [72]. The ethanologenic bacterium *E. coli* strain LYO1 is capable of reductive detoxification of furfural to less toxic furfuryl alcohol [8]. Some examples of microorganisms capable of converting furfural and HMF are put together in Table 3.

Table 3. Inhibitory compounds mitigation. Microorganisms capable of converting furfural and HMF

Microorganism	Incubation time (h)	Furfural conversion (%)	5-HMF conversion (%)	References
E. coli ATCC 1175	8 hours	91.5[(a)]	85.0[(b)]	[73]
Enterobacter aerogenes ATCC 13048	8 hours	90.6[(a)]	89.7[(b)]	[73]
Citrobacter freundii	8 hours	89.9[(a)]	82.6[(b)]	[73]
KIebsiella pneumoniae H	8 hours	88.5[(a)]	86.9[(b)]	[73]
Edwardsiella sp.	8 hours	57.8[(a)]	94.4[(b)]	[73]
Proteus vulgaris	8 hours	68.4[(a)]	57.9[(b)]	[73]
Proteus mirabilis	8 hours	51.1[(a)]	16.5[(b)]	[73]
Rhodococcus erythropolis Qla-22	8 – 18 days	30 - 50	–	[74]
Rhodococcus erythropolis N1-43	8 – 18 days	> 50	–	[74]
Hyphozyma *roseoniger*ATCC 20624	8 – 18 days	30 - 50	–	[74]
Pseudomonas putida Ful	8 – 18 days	100	–	[74]

Table 3. (Continued)

E. coli NAR30	8 – 18 days	100	–	[74]
SRB (sulfate-reducing bacterium) isolate	two days	76	–	[74]
SRB (sulfate-reducing bacterium) isolate	2 weeks	> 90	–	[75]
C. ligniaria C8 (NRRL 30616)	5 days	97	78	[76]
S. cerevisiae 307-12H60	–	–	100(c)	[77]
S. cerevisia 307-12H120	–	–	100(c)	[77]
S. cerevisiae 307-12-F40	–	100(a)	-	[77]
*Pichia stipitis*307 10H60	–	–	60(c)	[77]
E. coli KO11	4 – 5h	> 90(a)	–	[78]
E. coli LYO1	4 – 5h	> 90(a)	–	[78]
Klebsiella oxytoca strain P2	4 – 5h	> 90(a)	–	[78]

(a)Furfural conversion into furfuryl alcohol.
(b)5-HMF conversion to 5-hydroxymethyl furfuryl alcohol.
(c)HMF conversion into 2,5bis-hydroxymethylfuran.

4. Enzymatic Hydrolysis and Fermentation

4.1. Enzymatic hydrolysis

4.1.1. Enzymes characterization

Hydrolysis consists in either a chemical reaction, in which acid such as H_2SO_4 or HCl is used to convert cellulose and hemicellulose into theirs monomers [7], or an enzymatic reaction. In this case, cellulose and hemicellulose are converted by naturally occurring cellulases and hemicellulases, respectively.

Cellulose is a homopolyssacharide formed by an unbranched glucan chain of repeating β-(1,4)-D glucose units [79], [80]. Its hydrolysis involves mainly three steps mediated by three different cellulase enzymes: endo-1,4-β-D-glucanases (EG) (EC 3.2.1.4), exo-1,4-β-D-glucanases also called cellobiohydrolases (CBH) (EC 3.2.1.91), and β-glucosidases (BGL) (EC 3.2.1.21), which work synergistically to promote cellulose hydrolysis [81], [82], [83]. The first step corresponds to splitting of linkages between glucan chains by endoglucanases. In the second step, cellobiohydrolases (exoglucanases) promotes the glucan chain hydrolysis and generation of cellobiose. In the third and last step, cellobiose is converted into glucose by β-glucosidases [10], [84]. Hemicellulose, however, presents a random heterogeneous, linear or branched structure and it is composed by different residues such as xylan, xyloglucan, and mannan [85]. The hemicellulose structure and composition may differ depending on lignocellulosic biomass type. For instance, while hardwood hemicellulose contains mainly xylans, softwood contains mainly glucomannan [79]. Regarding hemicellulose composition, xylan is composed by β-1,4-linked D-xylose units, xyloglucan by β-1,4-linked D-glucose backbone substituted mainly by D-xylose, and mannan is composed by a backbone of β-1,4-linked D-mannose (mannans) and D-glucose (glucomannans) residues with D-galactose side chains [85]. L-arabinose, L-rhamnose, and D-glucuronic acid units can also be found

[11]. L-arabinose is present in arabinose-substituted xyloglucan and arabinoxylan [85]. Thus, β-1,4-endoxylanase is required for degradation of xylan, endoglucanases (xyloglucanases) and β-glucosidases for xyloglucan degradation and β-endomannanases (β-mannanases) and β-mannosidases for mannan degradation [83], [85]. Furthermore, arabinases and various esterases may also be involved in hemicellulose degradation [81]. α-arabinofuranosidases and arabinoxylan arabinofuranohydrolases are, in fact, the enzymes responsible for arabinose release [85]. With respect to lignin degradation, this can be performed by means of extracellular enzymes called ligninases such as lignin peroxidases (LiP) (EC 1.11.1.14), manganese peroxidases (MnP) (EC 1.11.1.13), phenol oxidases, laccases (Lac), and β-etherases [85], [86].

Peptidases and proteases are also important in order to enhance the hydrolytic process by debranching polysaccharide attached to plant cell wall protein [10], as well as glycoside hydrolases and polysaccharide lyases for pectin degradation [85]. Cutinase, which degrades cutin, must also be helpful. Other enzymes such as lipases, pectase lyases, in order to achieve efficient hydrolysis, may also be required [87].

4.1.2. Enzyme cocktails

Due to the lignocellulosic material heterogeneity, complex enzyme cocktails should be considered for enzymatic hydrolysis. Normally, more than one type of cellulase is required for efficient cellulose degradation. A synergism between multiple cellulase enzymes is needed in order to achieve better hydrolysis performance. Besides synergism between different cellulases, a combination of cellulases, hemicellulases, and ligninases, is needed for complete degradation of lignocellulose material [86], [88]. Cocktails of different cellulolytic enzymes in which different enzymes work together synergistically to attack cellulose and hemicelluloses [84], [89], [90] are needed. In fact, tailor-made enzymes cocktail, with specific enzymes needed for an efficient enzymatic hydrolysis, can contribute to reduce costs [88]. According to Gao et al.

[88], an optimal enzyme cocktail should include cellulases such as the glycoside hydrolases (GHs) cellobiohydrolase I (CBH I), cellobiohydrolase II (CBH II), endoglucanase I (EG I), and β-glucosidase [88] and hemicellulases such as xylanases (LX1, LX2, LX3, LX4, LX5, and LX6), β-xylosidase (LbX), α-arabinofuranosidase (LArb), and α-glucuronidase (LaGl) [88]. Degradation of hemicellulose along with cellulose is essential for an efficient hydrolysis of lignocellulosic biomass. In fact, co-hydrolysis of xylan can improve cellulose accessibility to the cellulases as a result of xylan solubilization [91]. Hu et al. [91] have observed that co-hydrolysis of xylan enhanced steam pretreated corn stover digestibility, resulting in three time faster cellulose and xylan hydrolysis and decreased cellulase loading in seven times and significantly increased hydrolysis performance of the optimized mixture [91].

4.1.3. Enzyme producing microorganisms

The majority of enzymes used in industry are from microbial origin due to the higher stability of such enzymes compared to enzymes obtained from plant or animal origin [92]. Among microorganisms able to produce and secrete hemicellulolytic enzymes aiming biomass degradation, fungi are considered the most important [85]. Regarding fungal species*, Aspergillus niger, P. chrysosporium,* and *T. reesei* are examples of producing lignocellulolytic enzyme producers [88]. *T. reesei* is widely known by its capacity to produce and secret a large quantity of cellulases and hemicellulases [84], [85]. Cellulase system containing cellobiohydrolase, endoglucanase, and β-glucosidase with synergism is produced by the aforementioned microorganism [90]. Several filamentous fungi are able to produce hydrolytic and oxidative extracellular enzymes relevant for lignin degradation [83]. Lignin peroxidase (LiP), manganese-dependent peroxidase (MnP), and laccase (Lac) are examples of extracellular enzymes produced by fungal strain and that are essential for lignin degradation. *Aspergillus tubingensis* KRCF 700 produces lignocellulolytic enzymes such as endoglucanase, β-glucosidase,

mannanase, xylanase, and β-xylosidase [90]. Ball et al. [93] have recovered lignocellulose-degrading enzymes from spent mushroom (*Agaricus bisporus)* compost. The enzymes recovered included: peroxidases, xylan-debranching enzymes acetylesterase, arabinofuranosidase, and the cellulose degrading activities endoglucanase, cellobiohydrase and β-glucosidase [93]. Shimokawa et al. [90] was able to obtain lignocellulose-degrading enzymes from *Trichoderma asperellum* UPM1 and *Aspergillus fumigatus* UPM2. The enzymes recovered were cellulase, xylanases, pectinases or a combination of these enzymes [90]. Obruca et al. [83] have investigated the production of lignocellulolytic enzymes by the fungal strain *Fusarium solani* F-552 on submerged fermentation. The enzymes obtained included hydrolases such as cellulases and xylanases as well as lignin degrading enzymes such as manganese-dependent peroxidase (MnP), lignin peroxidase (LiP), and laccase (Lac) [83].

Certain bacterial strains are also capable of producing lignocellulolytic enzymes. Examples are some strains belonging to *Clostridium, Cellulomonas*, and *Bacillus genus* [88].

Several anaerobic bacteria such as *Acetivibrio cellulolyticus*, *Bacteroides cellulosolvens*, *Ruminococcus albus*, and *Ruminococcus flavefaciens* are known to produce cellulosomal systems. These are arrays of multiple cellulases and hemicellulases attached to the cell surface allowing synergistic breakdown of lignocellulosic substrate [80]. Examples of fungi able to produce cellulosomal systems are some species belonging to the genera *Neocallimastix*, *Piromyces*, and *Orpinomyces* [85].

4.1.4. Enzymatic hydrolysis conditions

Optimal temperature for enzymatic hydrolysis normally ranges between 45 and 50°C and pH between 3 and 7. Milder conditions used in enzymatic hydrolysis compared to acid hydrolysis (120-200°C [7]) lead to less degradation of sugar and consequently higher conversion yields [94],

[95]. Furthermore, due to the same reason, utility cost of enzymatic hydrolysis is low compared to acid hydrolysis [15], [96]. However, the main drawback associated with enzymatic hydrolysis is the low selectivity of commercial enzymes and the high cost of enzyme production [97]. In fact, enzyme cost constitutes a significant fraction of overall ethanol production cost and its reduction must be sought [90]. Moreover, enzymatic hydrolysis is slower than acid hydrolysis. Time required to the hydrolysis is an important feature regarding cellulases activity. Due to long breakdown time, formation of inhibitors takes place. This leads to enzyme deactivation affecting the ability of the microorganism to convert sugars into ethanol [98]. Emerging biotechnology tools provide great potential to develop new enzyme sources with desirable enzyme features, such as higher specific activities, better thermal stability, and improved enzymes combination leading to higher yields [96], [99].

4.1.5. Effects of surfactants on enzymatic hydrolysis

The rate of cellulose conversion is, in part, associated with lignin content. In fact, besides the fact that lignin constitutes a physical barrier to enzymatic digestion, cellulases tend to bind to lignin and decrease the amount of cellulase available for cellulose digestion. Some substances such as Tween 80, polyethylene glycol (PEG), and bovine serum albumin (BSA) are used to reduce the attachment rate of enzymes to lignin and to help improve hydrolysis in other aspects [2], [100]. A number of surfactants were screened for their ability to improve enzymatic hydrolysis of steam-pretreated spruce (SPS) [101]. Non-ionic surfactants were found to be the most effective [101]. Surfactants such as Tween 80 and Tween 20 showed potential to improve hydrolysis of spruce and lodgepole pine pretreated by steam expansion [49]. The potential of non-ionic surfactants to promote enzyme recycle during the enzymatic hydrolysis was shown as well [49]. Surfactants have, in fact, been widely reported as capable of preventing cellulase attachment to lignin [100], by either binding hydrophobically to lignin or to the enzyme [49]. Desorption

of cellulase from lignin through replacement binding has also been suggested as a possible effect of surfactants [49]. Furthermore, previous studies have shown that surfactants can also prevent enzyme denaturation by increasing their stability during hydrolysis. This leads to higher sugar yields [102]. Others assumption is that surfactants are able to change the nature of the substrate increasing available cellulose surface or to stabilize enzymes preventing their denaturation [102]. Kristensen et al. [102] have shown that surfactants are able to increase cellulose conversion up to 70% and consequently enzyme loading can be decreased while keeping the same degree of hydrolysis [102]. The degree of improvement of enzymatic hydrolysis by addition of surfactants depends on several factors, such as enzyme loading, hydrolysis time, and lignin content in the substrates. Surfactants can decrease the enzyme loading and consequently the costs associated with enzymes. Another benefic effect was observed by Tu et al. [49]. In the absence of inhibitors, the addition of 0.2% Tween 80 to simultaneous saccharification and fermentation of steam exploded lodgepole pine, increased the ethanol yield by 58% after 12h. After adding 1.0g/L of furfural and HMF to the pretreated substrates, Tween 80 was still able to improve the ethanol yield by 61% [49]. Although the positive effect of those surfactants appear to have on enzymatic hydrolysis, their application must also take into consideration economic issues. The use of surfactant is only worth it if the cost associated with the enzyme load can be decreased. Furthermore, surfactants must be added in such rate that does not affect the yeast cell growth and ethanol yield.

4.2. Fermentation

Ethanologenic fermentation is carried out by yeasts and also by bacteria capable of converting fermentable sugars such as glucose, xylose, and galactose into ethanol. *S. cerevisiae* is the microorganism most commonly used in commercial ethanol production [103]. It is capable of converting 90 to 93% of sugars into ethanol [103], [104], [105], which

represents an excellent ethanolic yield. Others yeasts such as *Saccharomyces uvarum* [106], *Kluyveromyces marxianus* [106], *Zygosaccharomyces bailii* [89], and *Issatchenkia Orientalis* [59] are also applied in ethanologenic fermentation. Bacteria such as *Z. mobilis* [106], *E. coli* [107], and *K. oxytoca* [108] are also examples of ethanologenic microorganims. Mixed cultures of different ethanologenic strains may, also, be considered in fermentation to improve productivity [109].

One of the main challenges regarding ethanol production from lignocellulosic feedstock is the low yield and high production costs [15], [48]. In order to reduce cost of ethanol production, a more efficient utilization of the raw material must be considered [2], [9], [15]. New strategies, in order to improve currently used technologies, must be established [9], [47].

4.2.1. Simultaneous saccharification and fermentation vs. separate hydrolysis and fermentation

Fermentation is generally carried out subsequently to hydrolysis in a separate vessel, which allows optimum operation conditions for both hydrolysis and fermentation. This process is named separated hydrolysis fermentation (SHF). However, when relatively similar conditions between hydrolysis and fermentation are considered, these two processes can be carried out in the same vessel. This process is known as simultaneous saccharification fermentation (SSF). One of the great advantages of SSF over SHF lies on the fact that sugar produced during hydrolysis of polysaccharides can be immediately taken up by the microorganism. This fact eliminates end-product inhibition of hydrolytic enzymes activity in the broth, improving ethanol yield [15]. Since hydrolysis and fermentation are carried out in the same vessel, SSF allows production and equipment cost reduction and greatly simplifies the process of hydrolysis-fermentation. SSF is also characterized by shorter process times and requires lower enzyme concentrations, when compared to SHF. In SHF, the hydrolysis process is relatively long,

taking one to four days to complete which may increase the risk of contamination [12]. In SSF, the risk of contamination is lower when compared to SHF due to the presence of ethanol in the medium [12]. Although the advantages presented by SSF over SHF, optimal operation conditions for both enzymatic hydrolysis and fermentation cannot be met so easily in SSF. Temperatures between 45ºC and 50ºC are characteristic of enzymatic hydrolysis while the optimal performance temperature of *S. cerevisiae* is around 30ºC. SSF is normally carried out at 38ºC being hydrolysis the rate-limiting process [15]. To enhance SSF performance, commercially available cocktail enzymes, capable of hydrolyzing the substrate at lower temperature, or thermotolerant microorganism, such as *K. marxianus*, should be considered.

4.2.2. Fed-batch simultaneous saccharification and fermentation

Regarding process optimization, inhibitory compounds control and removal represents a topic that arouses great attention of researchers. As mentioned, toxic compounds can be removed by means of chemical, physical or biological detoxification. However, there are other ways to reduce their effect. One solution lies on choosing less recalcitrant feedstock, requiring milder pretreatment conditions, reducing the rate of inhibitors formation [54], [93]. Nevertheless, running fermentation in fed-batch mode ensures that the inhibitory compounds are fed in such small amount so that these can be detoxified by the fermentative microorganism [48], [55], [110] and maximize ethanol production. Some studies have shown that fed-batch SSF can increase the final ethanol concentration and thus decrease the energy consumption of subsequent distillation and minimize the need of detoxification procedure [6].

The potential of fed-batch SSF goes beyond toxic compounds reduction and presents other advantages over batch SSF. One of the disadvantages of batch SSF is the increasing viscosity of fermentation broth due to substrate high dry matter content leading to difficulties in stirring [13], [25] and uneven slurry distribution in the reactor [13]. This

drawback can be overcome by carrying out fermentation in fed-batch mode [25]. In this case, fresh substrate is added when viscosity is decreased and thus viscosity is kept low along the process [13]. Better slurry distribution in the vessel, as well as higher hydrolysis rate and yield are achieved compared to batch SSF [13], [25]. Lower substrate inhibition, which also contributes to higher hydrolysis rate, is also observed [15]. Hoyer et al. [25] have investigated the effects of different hydrolytic enzyme feeding strategies to optimize the SSF process for ethanol production. The enzyme feeding strategy presenting the highest ethanol yield was dependent on substrate and inhibitors concentrations. Addition of the enzymes, together with the substrate feed, is particularly advantageous to mix the enzyme with the substrate before addition to the reactor [25]. Several authors have been studying and exploring the potential of fed-batch SSF using different raw materials. Rudolf et al. [111] have compared batch and fed-batch SSF of steam pretreated spruce with 10% (w/v) dry matter content. In the fed-batch process, the ethanol productivity during the first 24h was considerably higher, suggesting cell inhibition reduction when this approach is used over batch mode [111]. Results obtained by Shengdong et al. [6] have also shown the potential of fed-batch SSF. In their work, microwave/acid/alkali/H_2O_2 pretreated rice straw was used as substrate for both batch and fed-batch SSF and *S. cerevisiae* YC-097 was used as fermentative microorganism. Ethanol concentrations of 29.1g/L and 57.3g/L were obtained for batch and fed-batch approaches, respectively, at 10% (w/v) substrate and 15mg cellulase/g substrate. Ballesteros et al. [4] have studied batch and fed-batch simultaneous saccharification and fermentation processes of paper material using *K. marxianus* CECT 10875 as fermentative yeast. They found a better performance for fed-batch when compared to batch mode fermentation. Comparing both batch and fed-batch procedures at 10% (w/v) substrate concentration and 15 FPU[1]/g enzyme loading, ethanol yields of 56.4% and 79.7% of the theoretical yield were obtained,

[1]FPU-filter paper unit.

respectively. Although the aforementioned examples show the potential of fed-batch SSF, Hoyer et al. [25] have observed that fed-batch SSF does not necessarily leads to higher ethanol yields than batch SSF. According to these authors, the enzyme feeding strategy leading to the highest ethanol yield depended on substrate concentration and inhibitor concentration.

Studies comparing the efficiency of batch SHF and SSF and fed-batch SSF strategies have been carried out by several authors. Several feedstocks and microorganisms have been tested as well as different operation conditions (Table 4).

Table 4. Examples of lignocellulose-to-bioethanol whole process described in the literature. Pretreatment methods and inhibitors formed thereof and hydrolysis and fermentation strategies are presented

Substrate	Pretreatment	Detoxification	Commercial enzymes and hydrolysis conditions	Fermentation conditions and microorganism
Ponderosa pine slurry	Dilute H_2SO_4 (1%(w/w))	polyelectrolyte polyethyleneimine (PEI)	Novozymes NS50010; Novozymes NS50013; 50ºC; pH 5.0	37ºC; pH 5.0; *S. cerevisiae* D5a
Paper material	–	–	Celluclast 1.5L	42ºC; *K. marxianus* CECT 10875
Rice straw	Microwave (300W)/acid (2% H_2SO_4) /alkali (1% $NaOH$)/H_2O_2 (30% H_2O_2)	–	Commercial *T. Reesei* cellulase	*S. cerevisiae* YC-097
Spruce	Milling: 2-10mm; Stream pretreatment: SO_2 (2% (w/w)), 210ºC, 5min	–	Celluclast 1.5 L; Novozyme 188.	37ºC; pH 5.0; Baker's yeast (*S. cerevisiae)*
Spruce wood	Chipping: 2-10mm; Steam pretreatment: SO_2 (3% w/w), 215ºC, 5min	–	Celluclast 1.5 L; Novozyme 188.	30ºC; pH 5; Baker's yeast (*S. cerevisiae*)
Wheat straw	Milling: 1.27mm Dilute acid: 0.5% H_2SO_4 (v/v), 160ºC, 10min	Bioabatement: *C. ligniaria* NRRL 30616; pH 6.5; 30ºC; 15h	Celluclast 1.5 L; Novozym188; 45ºC; pH 5.0; 72h	35ºC; pH 6.5; *E. coli* FBR5
				35ºC; pH 6.0; *E. coli* FBR5
				35ºC; pH 6.0; *E. coli* FBR5
Wheat straw	Milling: 1.27mm Dilute H_2SO_4 (0.75%, (v/v), 21ºC, 1h))	Overliming (calcium hydroxide)	Celluclast 1.5 L; Novozyme 188; 45ºC; pH 5.0; 72h	35ºC, pH 6.5; *E. coli* FBR5
			Celluclast 1.5 L; Novozyme 188;	35ºC, pH 6.0; *E. coli* FBR5
Spruce wood	Dilute H_2SO_4 (4% (w/w))	Dithionite; pH 5.5, 23ºC ("in situ")	Cellulase from *T. reesei* ATCC 26921; Novozyme 188; 50ºC; 70rpm; 48h	30ºC; Baker's yeast (*S. cerevisiae*)
		Sulfite; pH 5.5, 23ºC ("in situ")		
Sugarcane bagasse	Dilute H_2SO_4 (4% (w/w))	Dithionite; pH 5.5, 23ºC	Cellulase from *T. reesei* ATCC 26921; Novozyme 188; 50ºC; 70rpm; 48h	30ºC; Baker's yeast (*S. cerevisiae*)
		Sulfite; pH 5.5, 23ºC		

Table 4. (Continued)

Substrate	Operation mode	Ethanol Concentration [gL^{-1}]	Ethanol yields (% of theoretical yield)	Ethanol productivity [$gL^{-1}h^{-1}$]	Ref.
Ponderosa pine slurry	SHF	–	92.6 (24h)	0.30	[56]
Paper material	Batch SSF	15.6	56.4	–	[4]
	Fed-batch	17.7	79.7		
Rice straw	Batch SSF	–	61.3	4.04	[6]
	Fed-batch SSF	–	60.3	3.98	
Spruce	Batch SSF	–	77.4 (120h)	–	[25]
	Fed-batch	–	68.9 (120h)	–	
Spruce wood	Batch SSF	40-44 (72h)	80-84 (72h)	–	[111]
	Fed-batch SSF	40-44 (72h)	80-84 (72h)	–	
Wheat straw	Batch SHF	21.6 ± 0.3 (96h)	–	0.33	[5]
	Batch SSF	24.9 ± 0.3 (96h)	–	0.26	
	Fed-batch SSF	26.7 ± 0.0 (72h)	–	0.37	
Wheat straw	Batch SHF	14.4 (22h)	–	–	[14]
	Batch SSF	10.9 (22h)	–	–	
Spruce wood	Batch SHF		0.37[(c) (e)]	2.5	[112]
	Batch SHF		0.29[(d) (e)]	1.2	
Sugarcane bagasse	Batch SHF		0.34[(c) (e)]	3.9	[112]
	Batch SHF		0.32[(d) (e)]	2.9	

[(a)]5mM dithionite.

[(b)]5mM sulphite.

[(c)]10mM sulfite.

[(d)]dithionite.

[(e)]g ethanol/g (glucose + mannose).

In conclusion, fed-batch SSF appears to be a potential approach to reduce inhibitory compounds effects. A combination between a pretreatment approach allowing dissociation of lignocellulosic biomass under milder conditions and fed-batch SSF represents an advantageous choice in order to reduce the effect of toxic compounds formed during biomass pretreatment.

4.2.3. Non-isothermal simultaneous saccharification and fermentation

Besides SSF, other worth mentioning approaches have been receiving the attention of researchers. Non-isothermal simultaneous saccharification and fermentation (NSSF) consists in a method in which hydrolysis and fermentation occur simultaneously but in two separate reactors. Comparing NSSF with SSF, in the former, the optimal condition for both hydrolysis and fermentation can be assessed. A reduction of the overall enzyme requirement of 30-40% and higher ethanol yield and productivity compared to SSF has been observed [12], [113]. Furthermore, since the hydrolysis occurs in a different vessel, the incorporation of a rapid detoxification procedure to remove furfural from the hydrolysate can be assessed [134].

4.2.4. Simultaneous saccharification and co-fermentation

Simultaneous saccharification and co-fermentation (SSCF) represents an approach in which hydrolyzed hemicellulose and solid cellulose are not separated after pretreatment, allowing simultaneous conversion of hemicellulose and cellulose into fermentable sugars. Subsequently, simultaneous fermentation of pentoses and hexoses is carried out in the same bioreactor in which hydrolysis was performed, and with a single microorganism [31], [113].

Complete utilization of lignocellulosic material is limited due to lack of microorganisms capable of fermenting a variety of sugars obtained by hydrolysis of lignocellulosic materials [115]. *S. cerevisiae*, for instance, is widely used as fermentative organisms, efficiently ferments hexoses, but

is not naturally able to ferment pentoses such as xylose and arabinose [100], [105], [115], [116], [117], [118]. In fact, xylose represents the second most abundant carbohydrate in the lignocellulosic biomass hydrolysate [116]. Therefore, microorganisms able to ferment not only hexoses but also pentoses are needed. Expanding the substrate fermentation range of certain hexose fermenting microorganisms, by making them able to also ferment pentose is important in order to achieve an economically feasible biomass-to-ethanol fermentation process [105]. Many efforts have been made, regarding genetic engineering in order to construct strains capable of utilizing pentose sugars [119] by cloning pentose utilizing genes into hosts such as *S. cerevisiae*, *Z. mobilis*, and *E. coli* [18], [85]. *K. oxytoca*, and *Candida utilis* are also examples of microorganisms that have been genetically engineered in order to be able to efficiently produce ethanol from hexose and pentose sugars present in hemicelluloses [85], [105].

S. *cerevisiae* strain IBB10B05 was enabled to utilize xylose via introduction of the enzymes xylose reductase (XR) and xylitol dehydrogenase (XDH) [119].

Kumari et al. [115] have attempted the development of a hybrid yeast strain capable of ferment both pentose and hexose sugar present in lignocellulosic substrate. A fusant strains, RPR39, obtained by fusing protoplasts of *S. cerevisiae* (strain NCIM-3090) and xylose-fermenting yeasts *Pachysolen tannophilus* (strain NCIM-3502) showed potential to be used in large-scale fermentation of lignocellulytic sugars [115].

Zhang et al. [116] have developed a method to improve xylose fermentation by S. *cerevisiae*. These authors used a method called recursive DNA shuffling in order to construct recombinant yeast strains with the recombination of entire genome of *P. stipitis* (xylose-fermenting yeast) with that of *S. cerevisiae* [116]. A potential recombinant yeast strain ScF2, capable of producing ethanol more rapidly than *P. stipitis* and with improved ethanol titer and xylose tolerance was obtained [116].

An engineered *S. cerevisiae* strain able to ferment L-arabinose into ethanol have been developed by overexpressing the *Bacillus subtilis* gene araA, *E. coli* genes, araB and araD and the L-arabinose-transporting yeast galactose permease in *S. cerevisiae* CEN.PK2-1C strain [105].

Chandrakant et al. [120], have attained bioconversion of both glucose and xylose by a *S. cerevisiae* strain, in the presence of xylose isomerase. Simultaneous isomerisation and co-fermentation (SICF) of glucose-xylose mixture was carried out in the presence of xylose isomerase from *Candida boidinii* (DSM-70034). Xylose isomerase was immobilized on a hen egg shell and *S. cerevisiae* NRRL-2358 strain was considered as fermentative yeast [120].

K. marxianus DMKU3-1042, a thermotolerant yeast strain, was shown to have potential to metabolize both hexoses and pentoses derived from hemicelluloses [110].

A mutant *E. coli* strain, capable of fermenting both glucose and xylose, *E. coli* strain SE2378, was developed by Kim et al. [101] only using native enzymes.

4.2.5. Consolidated bioprocessing

Consolidated bioprocessing (CBP) constitutes an approach in which an engineered microorganism or consortium of microorganisms is used to simultaneously produce hydrolytic enzymes and ferment sugars to produce ethanol in a single step [31], [121], [122], [123]. In fact, consolidated bioprocessing, can be seen as an improved simultaneous saccharification and fermentation strategies since, production of saccharifying enzymes, hydrolysis of complex carbohydrate in simple sugars and fermentation of hexose and pentose occurs in a single step [122], [123], [124]. Since CBP is performed in a single step, it shows potential to be an economically feasible bioethanol production strategy due to equipment and operation cost reduction [124]. Moreover, taking into consideration that cellulase cost is one of the bottleneck of

lignocellulose-to-bioethanol technology, CBP shows, once more, potential to reduce costs considering that cellulase is produced by the CBP-microorganism [122], [123], [124].

Due to the lack of naturally occurring microorganisms capable of producing saccharifying enzymes and simultaneously fermenting sugars to ethanol, microorganisms with such capability have to be achieved by genetic engineering. Thus, CBP-microorganisms development implies modification of ethanol producer microorganisms to become also saccharifying enzymes producers or vice-versa [113], [122]. In fact, many microorganisms possess enzymes capable of efficiently degrade lignocellulosic materials but are not capable of producing a large amount of ethanol. Thus, transformation of the lignocellulolytic enzymes into fermentative microorganism shows potential to reach consolidate bioprocessing [89]. Examples of ethanol producers that could be engineered to produce saccharifying enzymes are *S. cerevisiae*, *Z. mobilis, E. coli, K. marxianus*, and *K. oxytoca* [121], [122]. Examples of cellulase producers that can be engineered to produce ethanol are *Clostridium thermocellum*, *Clostridium japonicus*, and *Clostridium cellulolyticum* [122], [123]. An example of CBP approach was presented by Chang et al. [89]. These authors used a technique called "promoter-based gene assembly and simultaneous overexpression" (PGASO) to engineer a yeast to be used in consolidate bioprocessing. Genes were transformed into *K. marxianus* KY3 genome to obtain a new strain, KR7. This should be able to directly convert cellulose to ethanol [89]. *C. japonicus* has shown ability to be used as a CBP microorganism. The bacterium was able to utilize corn stover and switchgrass as sole sources of carbon and have demonstrated efficient cellulase secretion [125]. The major drawback, however, associated to CBP lies on the fact that the process temperature may not be optimal for both saccharification and fermentation [124].

4.2.6. Cell immobilization technology

4.2.6.1. Continuous fermentation and cell immobilization

Ethanologenic fermentation, in batch or fed-batch mode, has been approached previously in this article. However, ethanol production can also be carried out in continuous mode. All these three strategies present advantages and drawbacks regarding achievement of high ethanol productivities and yields. Comparing continuous and batch modes, higher ethanol productivities and yield are achieved when operating in continuous since high cell densities are reached [126]. Moreover, labor costs are lower and the process is easier to control [126], [127], [128].

Cell immobilization can be applied in both batch and continuous fermentation modes [127], [128], [129]. It is useful to help increasing cell density in the fermentation broth [127]. In fact, cell immobilization has been widely applied to continuous fermentation in order to increase volumetric ethanol productivity and yield. Since cell stability is improved, substrate can be better used by the cells, leading to an increase in ethanol productivity. In the other hand, cell immobilization would better protect the cells against inhibitory condition such as high substrate concentration [128], [129], [130] or high final product, ethanol, concentration [105]. Furthermore, cells are also protected against the inhibitory effect of a wide range of toxic compounds originated due to harsh pretreatment conditions [126], [131], [132].

Cell immobilization can be carried out by entrapping the cells within matrix such as calcium alginate, *k*-carrageenan, polyacrylamide or adsorption on to zeolite [128], [130], [133]. Moreover, self-aggregation, in which cells with flocculating properties are used in ethanol production, also constitutes a means to retain cell within the fermenter [133].

Entrapment in calcium alginate beads is one of the most common method used for cell immobilization gel entrapment and the immobilized cell retain their ability to ferment sugar to ethanol for periods that can

achieve months [104], [105], [125]. A drawback associated with cell immobilization using beads is that, since the transport of nutrient to the cells inside the beads is made by diffusion rather than by convection, immobilization can limit the nutrition of the cells located in the core of the beads [132], [134].

Taherzadeh et al. [129], have used yeast *S. cerevisiae* CBS 8066 immobilized in Ca-alginate to ferment acid hydrolyzed wood chips (Swedish wood residues). Inoculum culture was mixed with a sterilized solution of Na-alginate which was subsequently dropped into a sterilized solution of $CaCl_2$ under gentle mixing. 3% of Ca-alginate beads were stable for 4 days in batch and continuous fermentation without additional source of calcium. Better ethanol yield was obtained when immobilized cell were used instead of free cells – 0.47(± 0.01)g/g compared to 0.39g/g (at dilution rate of $0.4h^{-1}$). This was also observed in the presence of inhibitors such as furfural, HMF, and acetic acid [129].

Immobilized recombinant *S. cerevisiae* cells ZU-10 were used in ethanol production from corn stover hydrolyzate detoxified by rotoevaporation and lime neutralization. The immobilized cells could be used in five batches of fermentation. Cells were mixed with 2% (w/v) sodium alginate solution mixed with 1% (w/v) diatomite. Beads of 2-3mm were obtained by dropping the cell-alginate mixture into a 2% (w/v) $CaCl_2$ solution. The strain was able to use both glucose and xylose for ethanol production [131]. An ethanol concentration of 31.1g/L and an ethanol yield on fermentable sugar of 0.406g/g were obtained when 97.1% xylose and 100% glucose in the hydrolysate were utilized [131].

Liu et al. [130] have immobilized *S. cerevisiae* GT4608 cells in magnetic particles and a magnetically stabilized fluidized bed reactor was used. Cells were mixed with sodium alginate (3.0% w/v) containing MnZn ferrite powder (5.0% w/v) and beads of 3mm diameter were prepared by dropping $CaCl_2$ in the previously referred mixture. Beads

with 2% $CaCl_2$ were stable and presented high ethanol fermentation activity. The higher ethanol yield, 95.3% of the theoretical, was achieved at 150g/L of glucose concentration [130]. In this case, ethanol concentration and productivity were, respectively, 66g/L and 26.7g/L.h at a dilution rate of $0.4h^{-1}$ [130].

An immobilized cell reactor (ICR) was applied in ethanol production by *S. cerevisiae*. This consisted in a column packed with beads of calcium alginate in which the cells were entrapped [132]. Ethanol production was stable for 24 hours. Immobilized cell system was compared with batch fermentation. Sugar consumption and ethanol production of 88.2% and 16.7% v/v, respectively, were obtained with 6h retention time for the immobilized cells system while for batch fermentation values of 99.6% and 12.5% v/v after 27h were obtained for sugar consumption and ethanol production, respectively [132].

A study of cell immobilization onto glass microspheres was performed by Zhou et al. [133]. Cell immobilization has increased ethanol tolerance of recombinant *E. coli* B strain KO11 during lignocellulose continuous fermentation of xylose. Cells were immobilized on the glass microspheres and liquid fluidized bed was used [133]. When free cells were used ethanol yield decreased to 60% after 8 days while immobilized cells lead to ethanol yields of 85% and 70% even after 10 and 40 days, respectively [133].

A lyophilization technique was used to immobilize commercial-grade baker's yeast (S. *cerevisiae)* in hydroxyethylcellulose gels containing trimethylammoniumchloride (BBTMAC). The immobilization was efficient (with a value of 0.92) for at least three batches of 72 hours each. For the first, second, and third batch, ethanol yields of 79%, 84%, and 60% of the theoretical were obtained respectively, which in terms of glucose conversion into ethanol corresponded to 0.40, 0.43, and 0.30g ethanol/g glucose, respectively [135].

4.2.6.2. Self-flocculation technology

One of the disadvantages associated with the immobilization of cells using inert carriers is the decrease on their growth rate. Besides, since ethanol is a primary metabolite, decreasing growing rate means consequently decreasing ethanol formation. Moreover, the use of supporting material constitutes an extra cost to the process of fermentation.

In this context, cells self-immobilization represents a potential solution to, in one hand, take advantage of the strengths of cell immobilization, and in the other hand, avoid most of its drawbacks. Some microorganisms, such as certain strains belonging to *Saccharomyces* genus, present the natural ability to self-flocculate. *Schizosaccharomyces pombe* and *K. marxianus* are yeast strains known for their self-flocculation characteristics. Comparatively to free yeasts in the broth, cells are better retained in the reactor during fermentation, improving ethanol yield. Cells flocs can be easily separated from the fermentative broth [136] by filtration rather than by centrifugation, commonly used to recover free cells in ethanologic fermentation. This would save the investment and energy costs associated to centrifuge acquisition and operation, respectively. Moreover, the risk of contamination associated to supporting material utilization can be avoided.

The performance of a self-flocculating yeast, SPSC01 (fusant from a *S. pombe* strain and *S. cerevisiae* strain) and a conventional industrial ethanol fermentation yeast, *S. cerevisiae* K2 were compared for continuous ethanol production. The ethanol productivity of the self-flocculating yeast was the double of the non-flocculating yeast using corn powder as substrate [137].

Different fermenter configurations have been proposed for lab-scale continuous ethanol production using self-flocculating cells, including air-lift reactors, bubble columns, packed and fluidized beds [138]. Most currently available self-flocculation yeast are not suitable for large-scale

ethanol fermentation since they present low ethanol tolerance, degrade a small spectrum of sugar into ethanol and present low conversion yields [137].

Self-flocculation technology appears to be attractive for either continuous operation or for batch and fed-batch, although continuous ethanol fermentations by self-flocculating studies have been more widely reported.

4.2.7. High gravity and very high gravity fermentation

High gravity (HG) and very high gravity (VHG) fermentation are technologies that show good prospective in allowing cost saving and increasing fermentation efficiency [139], [140]. HG and VHG fermentation consist in fermentation technology in which media with sugar concentrations of 180 - 220gl^{-1} and 250gl^{-1} are used to yield final ethanol concentrations of 10-12% (v/v) and 15% (v/v), respectively [104], [141]. Comparing to common fermentation in which final ethanol concentration corresponds to 7-8% (v/v), increased fermentation efficiency is achieved when HG or VHG fermentation are carried out. Regarding cost, these technology allow saving energy consumption during ethanol distillation [141], [142], [143], and reduce water consumption [140].

The industrial strains of *S. cerevisiae*, PE-2 and CA1185 showed potential to be use in VHG fermentation [143]. In fact, *S. cerevisiae* PE-2, was used in VHG of repeated batch fermentation leading to ethanol titers of 21.6 and 19.3% (v/v) at 364g glucose L^{-1} at 27 and 30ºC, respectively [144]. At glucose concentration of 400L^{-1}, the yeast was still tolerant to the osmotic stress and led to ethanol titers of 20.1 and 18.2% (v/v) at 27 and 30ºC, respectively. The VHG system was capable of operating for at least fifteen consecutive batches, with cell recycling, and final ethanol concentration and yield of 17.1 ± 0.2% (v/v) and 80 ± 1%, respectively [144].

In VHG fermentations, yeast cells are exposed to a high osmotic pressure due to high sugar concentrations, which can be harmful for the cells [139], [145]. Furthermore, high ethanol titers that can be obtained in VHG can be harmful to the cells. Thus, addition of osmoprotectant is needed in order to attenuate the osmotic pressure caused by sugar excess, together with high ethanol tolerance microorganisms [5], [145]. Yeast cell flocculation, represents a mean to improve ethanol tolerance of the yeast strain [5]. In fact, VHG fermentation (initial glucose concentration of $300 gL^{-1}$) using a self-flocculating fusant of *S. pombe* and *S. cerevisiae (fusant SPSC)* as fermentative microorganism, showed over 30% increase in final ethanol, 29% decrease in fermentation time and 26% increase in glucose utilization [146]. Immobilization, for instance with Ca-alginate, also represents a strategy to lower the stress of yeast when performing HG and VHG [136] since the microorganism can be better protected against the inhibitory effect of high substrate concentration and even against the inhibitory effect of ethanol [136].

VHG fermentation is potentially feasible with commercial sugars and starch-based materials. Regarding lignocellulose-to-bioethanol technology, fermentation of high concentration of sugar obtained after hydrolysis is, indeed, desirable to achieve high final ethanol concentration and yield. This can be achieved by using the whole slurry obtained after pretreatment of lignocellulosic material for hydrolysis and fermentation, allowing conversion of hemicellulose along with cellulose. The disadvantage of using the whole slurry obtained after pretreatment lies on the fact that this contains high concentration of toxic compounds, which can affect the performance of the fermentative microorganism [147]. Kapu et al. [147] have studied the effect of high cell density and glucose supplementation on inhibitory compounds mitigation. A water soluble fraction rich in hemicellulose, obtained by acid catalyzed steam pretreated Douglas-fir wood chips and containing 2.4g/l HMF and 0.5g/L furfural was used as substrate [147]. The *S. cerevisiae* strains Tembec T1,

T2 and Lallemand LYCC 6469 were used as fermentative microorganisms. At low cell density (6×10^6 cells/ml) no significant amount of ethanol was produced by either of the strains referred above (with or without glucose supplementation). At high cell density (150×10^6 cells/ml), Lallemand LYCC 6469 and Tembec T1 stains led to ~98% ethanol yield without glucose supplementation. With glucose supplementation, the same strains led to 65% ethanol yield (final ethanol concentration of ~72g/L) and 50% ethanol yield (final ethanol concentration of ~62g/L) after 48 hours of fermentation, respectively. Glucose supplementation had a positive effect on inhibitory compounds mitigation. With no supplementation of glucose, 62% reduction in HMF was observed after 48h at high cell density. At glucose concentration of 100g/L, 98% of HMF was metabolized by LYCC 6469 after 24h [147].

5. Ethanol Recovery

Distillation is traditionally used as method for ethanol recovery after its production by fermentative yeasts. However, due to the formation of an azeotrope between ethanol and water at high temperatures, which makes distillation in a single column expensive, alternatives to ordinary distillation are being explored. Moreover, using distillation to recover low concentration of ethanol from fermentation broth (about 5 (w/w)% ethanol) presents high energy requirement [8], [148]. Several technologies such as gas stripping, extraction, adsorption, distillation, reverse osmosis, and pervaporation have been explored for ethanol recovery [114], [141]. Extractive distillation, liquid-liquid extraction and adsorption can be combined with ordinary distillation in order to achieve better results regarding ethanol recovery [8]. Among the technologies presented, pervaporation based on polydimethylsiloxane (PDMS) membranes appears to be a promising method since it allows cost reduction, high productivity and it is non-toxic to microorganisms [150].

5.1. In situ ethanol recovery

Regarding the main focus of the present work, one of the aspects to take into consideration is the inhibitory effect of ethanol on fermentative yeasts. Ethanol that is formed and accumulated in the fermenter as result of sugar breakdown by fermentative microorganism is an inhibitor to the aforementioned microorganism [114]. Taking into consideration that, at a certain level, ethanol inhibits cell growth, ethanol production is automatically affected since it is directly related to cell growth [104]. Furthermore, inhibition of cellulase by ethanol also occurs [12], [113]. As reported by Calinescu et al. [151], it is likely that ethanol affects the performance of important glycolytic pathway enzymes as well as the nutrient uptake by the cell and attack some organelle membranes, changing their membrane permeability. The utilization of yeasts or bacteria species highly tolerant to ethanol is of great importance to ensure high ethanol conversion. "In situ" recovery of ethanol from the broth, in which fermentation and ethanol recovery are integrated, seems to have potential to alleviate ethanol effect on fermentative yeasts. In "in situ" recovery approach, ethanol concentration in the fermentation broth is kept at a level causing the minimal inhibition increasing ethanol volumetric productivity and reducing process costs [31].

Pervaporation constitutes a promising and economically feasible technology for ethanol recover [149], [150], [152], allowing its "in situ" recovery and concentration from the fermentation broth before feeding it to the distillation at a higher concentration and thus reducing the distillation energy cost. Pervaporation is a membrane-based separation technique in which a liquid stream is placed in contact with one side of the membrane while a gas purge is applied to the other side. This process leads to sorption of liquid components onto the membrane, permeation through the membrane and evaporation into the gas phase [8]. Pervaporation using ethanol-selective membranes is a promising approach for recovery of low-concentration solutions from fermentation

broths. Polydimethylsiloxane [PDMS] membrane, often referred to as "silicone rubber" are used as hydrophobic pervaporation membrane material [152], [153]. Sharma et al. [149] have modeled and optimized a three-stage fermentation process which integrates cell recycling and pervaporation. Pervaporation units have been used outside each fermenter for continuous ethanol removal. Better performance of the three-stage fermentation process integrated with pervaporation was obtained compared with the three-stage fermentation process integrated with extraction [149]. Chen et al. [114] have studied ethanol fermentation by *S. cerevisiae* in a continuous and closed circulating fermentation system with a PDMS pervaporation membrane bioreactor. Higher glucose volumetric consumption and ethanol volumetric productivity during the pervaporation were obtained compared to the times pervaporation was shut down in the early days of fermentation. This suggested ethanol as the major inhibitor of fermentation during that period [114]. Gaykawad et al. [148] have been able to recover ethanol from barley straw or willow wood chips hydrolysate broth by pervaporation with a PDMS membrane at 30°C. Ethanol recovery from corn stover hydrolysate fermentation broth was also achieved by pervaporation with a PDMS membrane [150].

6. Conclusion

An overview of the whole lignocellulose-to-ethanol process had been accessed in the present work. Different lignocellulosic biomass pretreatment methods have been described. They should increase cellulose accessibility to hydrolytic enzymes and lead to the lowest amount of inhibitory compounds possible. This is possible by performing pretreatment at milder conditions. Alkaline pretreatment and liquid hot water pretreatment seemed promising preventing inhibitory compounds formation. CO_2 expansion can be performed at milder temperatures preventing sugar degradation. Using ionic liquids to pre-treat the

biomass, high enzymatic hydrolysis yield can be achieved without complete degradation of lignin. Furthermore, using ILs, recovery of cellulose and lignin is possible by precipitation with ethanol and water, respectively and minimal formation of toxic compounds is achieved. Biological pretreatments use milder conditions and are suitable to be used combined with other pretreatment methods as a first pretreatment step. Detoxification of inhibitory compounds affecting fermentation performance can be carried out "in situ" by using suitable enzymes. Enzymes such as laccase and peroxidase can be used for "in situ" detoxification of phenolic compounds. Besides phenolic compounds detoxifying enzymes, furfural and 5-HMF detoxifying enzymes are needed. More robust recombinant microorganisms, able to resist to higher inhibitors concentrations have been also suggested. Regarding hydrolysis and fermentation technologies, simultaneous saccharification and fermentation is presented as a mean to eliminate end product inhibition of hydrolytic enzymes caused by cellobiose and glucose, simplify the process and reduce equipment cost. When performed in fed-batch, simultaneous saccharification and fermentation can also lead to reduction of the inhibitory effect of by-products formed during pretreatment. Non-isothermal simultaneous saccharification and fermentation, in which hydrolysis and fermentation occur simultaneously in two separate reactors, allows performing hydrolysis and fermentation at optimal conditions and in shorter times than SHF. Simultaneous saccharification and co-fermentation enables simultaneous fermentation of pentoses and hexoses in the same bioreactor with a single microorganism. Consolidated bioprocessing in which cellulases production and ethanol fermentation is carried out by the same microorganism is also a promising technology proposed in the literature. Lastly, "in situ" recovery of ethanol from the broth has been suggested in order to reduce ethanol inhibitory effect on fermentative microorganisms. Ethanol can be continuously recovered from the fermentative broth and consequently maintained at a level at which its inhibitory effect to the

fermentative microorganisms is minimal. Pervaporation using ethanol-selective membranes seems an effective approach in order to achieve this goal.

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