



The second generation ethanol production

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1. Abstract

The shortage of fossil energy sources, the alarm over greenhouse gas emissions (GHGs) and global warming have caused an increasing interest in alternative renewable sources of energy, boosting the worldwide trend to produce and use biofuels in substitution of petroleum-based fuels. Bioethanol represents one of the most promising biofuels. The potential yield of first generation bioethanol, produced from traditional agricultural crops, is not sufficient in many parts of the world. Moreover, first generation ethanol production systems pose a concern about competition with food and feed supplies. To overcome these bottlenecks, second generation bioethanol production from non edible renewable lignocellulosic biomass is attracting keen interest. The present manuscript describes the main steps of the second generation bioethanol production process.

2. Introduction

The shortage of energy fossil sources, the alarm over greenhouse gas emissions (GHGs) and global warming [1] have prompted an increasing worldwide interest in alternative renewable sources of energy. The European Parliament with the DIRECTIVE 2009/28/CE — also known as “Climate–Energy package 20–20–20” — has promoted the

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production and use of biofuels in substitution of petroleum-based fuels, establishing a 10% reduction of conventional fuels as the target for 2020.

Bioethanol represents one of the most promising biofuels, exhibiting several advantages, such as high octane number, low cetane number and high heat of vaporization [2]. A variety of biomass feedstock have been explored for ethanol production and can be classified into three types:

- a) lignocellulosic materials such as woody biomass, herbaceous perennials and various wastes;
- b) starch-rich crops such as maize and grain sorghum;
- c) sucrose-rich crops such as sugarcane and sugar beet [3].

First generation bioethanol is produced from traditional agricultural crops, mainly represented by corn, sugarcane and sugar beet. Brazil and USA are the leaders of ethanol production, together accounting for about 90% of the world production. During 2012, the USA has produced 13.3 billion gallons of ethanol [4]. More than 95% of the ethanol produced in the USA for transportation is currently obtained from corn starch and represents 10% of the fuel supply and 25% of all the motor fuel produced [5]. Nowadays, Brazil is the only country that uses ethanol, produced exclusively from sugarcane, as a full substitute for gasoline [6].

The current production of first generation ethanol in the European Union is less than its consumption. Given that the potential yield is not sufficient to accomplish the prefixed European goals, an increase in ethanol importations can be foreseen. Moreover, first generation ethanol production systems pose a concern about the competition with food and feed supplies.

To overcome these bottlenecks, second generation bioethanol production from non edible renewable lignocellulosic biomass is attracting keen interest. Lignocellulosic biomasses are the most abundant renewable resources on Earth. Thus, their utilization for second generation ethanol production would minimize the conflict between land use for food (and feed) and energy production. Moreover, these raw materials are less expensive and present a more even geographical distribution than the conventional agricultural feedstock. A large

fraction of lignocelluloses is represented by residual biomass such as agro-industrial wastes, agricultural and forest crop residues and the organic and paper fractions of municipal solid waste (MSW) that would represent the key response to the need of increasing renewable energy production particularly in the Mediterranean basin [7]. It is worth noting that only small amounts of cellulose, hemicellulose and lignin composing agricultural residues are currently exploited, as the majority is considered waste. Moreover, second generation ethanol production and use has lower green-house gas emissions (<86%) than the first generation fuels, reducing environmental impact, particularly on climate change.

Lignocellulose consists of three types of polymers — cellulose, hemicellulose and lignin — bonded by both non-covalent and covalent cross linkages [8, 9]. Cellulose is a highly crystalline linear polymer composed of D-glucose units linked by β -1,4 glycosidic bonds [8]. Hemicellulose is likewise a polysaccharide, accounting for 25–35% of dry wood [3]. It is a very heterogeneous and ramified polymer, consisting of a mixture of various monosaccharides, such as xylose and arabinose (both 5-carbon sugars) and glucose, mannose and galactose (all 6-carbon sugars), and glucuronic acid. Lignin is present in the cellular wall to give structural support, mechanical resistance, impermeability and defence against microbial attack and oxidative stress. It is an amorphous heteropolymer formed by phenylpropane units joined together by non hydrolysable linkages [9].

Bioethanol production from lignocellulosic materials takes place in three phases: the first step consists in breaking the lignin barrier (pretreatment); further step involves the hydrolysis of cellulose and hemicellulose to generate fermentable sugars (saccharification) followed by the fermentation of mixed hexose and pentose sugars to produce ethanol. In this manuscript, the main routes to perform these steps and their components are described.

3. Pretreatment of lignocellulosic materials for ethanol production

The main goals and characteristics of the pretreatment of lignocellulosic materials are to:

- a) remove the barrier of lignin,
- b) expose plant cell wall polysaccharides,
- c) prevent a great degradation or loss of carbohydrate,
- d) minimize the concentration of by-products that are inhibitory to the following hydrolysis and fermentation processes, and (4) be economic and environmentally friendly. Numerous strategies of pretreatment have been developed belonging to different categories, i.e. physical (Pyrolysis, Microwave), physicochemical (Steam Explosion, Ammonia Fiber Explosion), chemical (Alkaline Hydrolysis, Acid Hydrolysis, Ozonolysis, Organosolv) and biological.

3.1. *Physical processes*

Pyrolysis

The pyrolysis pretreatment involves the decomposition of cellulose into H_2 , CO, and residual char by using temperatures higher than 300 °C; the process is enhanced when carried out in the presence of oxygen. The wide variety of pyrolysis products points to the need of separating and purifying them prior to use [10]. The recovered solution, after separation from the residual char is mainly composed of cellulose, which can be saccharified for the fermentation of ethanol. Different kind of food crops like corn, sugarcane and soybean were pretreated through pyrolysis to generate ethanol [11]. Ethanol can be produced on a large scale using bio-oil hydrolysate produced by fast pyrolysis of loblolly pine particles. The mainly issue is the presence of inhibitor compounds. These can be removed by economically friendly methods (activated carbon, air stripping and microbial), followed by microbial fermentation, reaching 0.4 g ethanol/g glucose produced with a 79% yield [12]. Pyrolytic sugars from poplar were separated from phenols by solvent extraction and were hydrolyzed into glucose using H_2SO_4 as catalyst. The fermentation of poplar hydrolyzed to produce ethanol was carried out by *Saccharomyces cerevisiae*. The yield obtained was 0.473 g ethanol/g glucose [13].

Microwave

The waves, with frequencies between 0.3 GHz and 300 GHz, are absorbed by water, fats and sugars and their energy is transferred to organic molecules generating heat. This leads to the degradation of lignin, making cellulose and hemicellulose more accessible to enzymatic hydrolysis [14]. Lignocellulose-rich sweet sorghum biogases were pretreated and hydrolysed at the same time using microwave irradiation. An ethanol yield based on total sugar of 480 g kg⁻¹ was obtained after 24 h of fermentation using a mixed culture of microorganisms [15]. Microwave pretreatment of oil palm empty fruit bunch beer, combined with alkaline conditions, was reported by Nomanbhay *et al.* [16]. A loss of 74% lignin, 24.5% holocellulose and a yield of total reducing sugars of 41% was obtained at the best operative conditions of 3% (w/v) NaOH at 180 W for 12 min [16].

3.2. Physicochemical processes

Steam Explosion

The steam explosion is one of the most currently used methods for the pretreatment of lignocellulosic biomasses. The high temperature (180 to 240 °C) combined to pressure from 1 to 3.5 MPa, followed by an explosive decompression allows one to obtain the breakdown of the rigid lignin structure fibers. The result is a better accessibility of cellulose for enzymatic hydrolysis and fermentation [17]. The optimal steam explosion conditions for sugarcane bagasse are 215 °C for 5 min at maximum operating pressure [18]. After pretreatment and enzymatic saccharification, an overall glucose yield of 86.8% of the content in raw material was achieved. The steam explosion of canola straw increases the glucose recovery in the saccharification process, reaching an increment of 153.22% compared to the control samples, as shown by Garmakhany *et al.* [19].

Ammonia Fiber Explosion (AFEX)

The process consists of treatment with liquid anhydrous ammonia at high temperature (60–100 °C) and pressure (250–300 psi) for 5 min, fol-

lowed by a rapid decrease of the pressure. This causes the disruption of the lignocellulosic structure and the de-crystallization of cellulose, but the lignin remains unchanged; the lignocellulosic biomasses characterized by a higher content of lignin are not efficiently pretreated with this method [20]. The AFEX pretreatment of lignocellulosic materials derived from forages and agricultural residues was performed by Belkacemi *et al.* [21]. The saccharification of the pretreated materials, without any detoxification, allows one to obtain a 60 to 80% of theoretical yield of sugars. Subsequent ethanol fermentation of the hydrolysate by *Pachysolen tannophilus* ATCC 32691 resulted in a theoretical yield of 40–60% after 24 h. AFEX followed by enzymatic hydrolysis has been applied to coastal Bermuda grass by Myoung *et al.* [22]. Pretreatment at 100 °C for 30 min produced 94.8% of theoretical sugar yield, corresponding to the maximum sugar yield achieved with the AFEX treatment.

3.3. Chemical processes

Alkaline Hydrolysis

Alkaline hydrolysis (mainly lime ($\text{CaO}/\text{Ca}(\text{OH})_2$), NaOH , Na_2CO_3) is effective in removing lignin and improving the subsequent enzymatic hydrolysis of the pretreated biomass. Its advantage with respect to other pretreatment technologies is the use of lower temperatures and pressures; however, it requires more time, hours or days rather than minutes or seconds. Compared with the other kinds of pretreatment, alkaline hydrolysis represents a slow process, requires neutralization, and the recovery of added alkali [23]. The optimization of alkaline pretreatment of coffee pulp for ethanol fermentation was performed by Menezes *et al.* [24]. The pretreatment using 4% (w/v) NaOH for 25 min gave the best results with a recovery of cellulose pulp of 69.18% and the production of 13.66 g/L of ethanol with a yield of 0.4 g ethanol/g glucose after the fermentation step. Alvarez *et al.* [25] used alkaline hydrolysis to pretreat different kinds of pine residues. They showed that the hydrolysis yield mainly depends on temperature and alkali concentration. Among the tested operative conditions, 2.5% NaOH for 90 min at 120 °C resulted in the best yield of glucose (41.33% w/w) after enzymatic hydrolysis.

The process could theoretically produce a maximum yield of 90.19% of ethanol/substrate (glucose) and about 80 L of bioethanol per dry ton of woody biomass.

Acid Hydrolysis

Acid hydrolysis represents a widely used pretreatment method because the use of concentrated or diluted acids (usually between 0.2% and 2.5% w/w) at temperatures between 130–210 °C allows one to obtain high yields of sugars [26]. The hemicellulose, easier to be hydrolyzed than cellulose, is attacked by acid solutions [20]. Depending on the reaction conditions, such as temperature, acid concentration and hydrolysis time, the amount of sugar decomposition products, e.g. furfural and 5-hydroxymethylfurfural (5-HMF), that inhibit the subsequent ethanol fermentation, changes [27]. The hydrolysate obtained by the sulphuric acid pretreatment of *Curcuma longa* waste was directly fermented without a detoxification step as shown by Nguyen *et al.* [28]. The acid hydrolysis was carried out at 122.68 °C with 4.91% sulphuric acid for 50 min, obtaining an ethanol yield of 30.57%. Some acid compounds can be used as pretreatment catalysts, but also as a nitrogen source in the fermentation process for bioethanol production. Rice straw was pretreated with 0.65% nitric acid at 158.8 °C for 5.86 min as reported by Kim *et al.* [29]. After neutralization, the pretreated rice straw was used in the fermentation run without adding any nitrogen sources, reaching an ethanol yield from 10.92 g/L to 14.50 g/L by using *Pichia stipitis*.

Ozonolysis

Ozone attacks the aromatic rings of the lignin structure without damaging hemicellulose and cellulose. The process is affected by ozone concentration, biomass type and moisture content [14]. The ozonolysis of sugarcane bagasse in a fixed bed reactor at room temperature was performed by Travaini *et al.* [30] to evaluate the best samples moisture and ozone concentrations for sugar saccharification. The ozonolysis, under the best experimental conditions (40% sample moisture and 3.44% ozone), increased fermentable carbohydrate release considerably during enzymatic hydrolysis. Glucose and

xylose yields increased from 6.64% and 2.05%, for raw bagasse, to 41.79% and 52.44% for pretreated bagasse. The ozonolysis process is relatively expensive due to large requirements of ozone, but has some advantages: high dry matter concentrations (45–60%), very low production of inhibitory products, and reactions performed at atmospheric conditions [31]. The negative issues are the ineffective removal of lignin and the low yields of reducing sugars. However, combination of ozonolysis with other pretreatment processes has shown promising results. Ozonolysis was studied separately and in combination with wet disk milling (WDM) for the pretreatment of sugarcane bagasse and straw, with the aim of improving their enzymatic saccharification. The use of WDM followed by ozonolysis resulted in glucose yields of 81.1% for bagasse and 92.4% for straw, with shorter WDM times [32].

Organosolv

The use of organic solvent or mixtures of solvents in combination with water causes hydrolysis of the internal bonds in lignin and also of those between lignin and hemicellulose. Ethanol, methanol, acetone, and ethylene glycol are the solvents commonly used in the process. Besides the use of solvents, high temperatures (200 °C) are required, but depending on the type of biomass and the catalyst used, lower temperatures can be sufficient. It could be possible to use organic and inorganic acids as catalysts [14]. Geng *et al.* [33] showed that the addition of an acid catalyst in the organosolv pretreatment of horticultural waste, followed by H₂O₂ post pretreatment, was feasible. The enzymatic hydrolysis of the pretreated waste resulted in a hydrolysate containing 26.9 g/L reducing sugar, while the following fermentation by using *Saccharomyces cerevisiae* produced 11.69 g/L ethanol. Different organosolv operative conditions for the pretreatment of rice straw were tested by Amiri *et al.* [34], the best conditions resulting in 75% (v/v) aqueous ethanol containing 1% w/w sulphuric acid at 180 °C for 30 min. The enzymatic hydrolysis of the pretreated straw allowed one to obtain a glucose yield of 46.2%, which was then fermented to 22.5 g ethanol by *Clostridium acetobutylicum* NRRL B-591.

3.4. Biological processes

Biological pretreatment can represent the most environmentally friendly method for the delignification of lignocellulosic biomasses, since it does not require energy and chemical compounds. Although biological pretreatment is considered a promising technology because of its environmental advantages, the process has not been largely applied because it requires a long time (about 3–5 months), results in very high feedstock loss during pretreatment and because the rate of following hydrolysis is very low [35]. The best effective microorganisms that can be employed in the biological delignification are the brown and white rot fungi, which synthesize ligninolytic enzymes. Brown rot attacks cellulose while white and soft rots attack both cellulose and lignin [14]. The biological pretreatment of the rubber wood (*Hevea brasiliensis*) with the white rot fungus *Ceriporiopsis subvermispora* was investigated by Nazarpour *et al.* [36]. After 90 days of fermentation, the lignin and hemicelluloses loss was 45.06% and 42.08%, respectively, while the loss of cellulose was very low (9.50%). This treatment increased the sugar yield to about 27.67% during the subsequently hydrolysis. The hydrolysate obtained from wheat straw pretreated with the white-rot fungus *Irpex lacteus* was fermented by the yeast *Pachysolen tannophilus*. The ethanol yield obtained was between 23 and 35% greater than the yields typically obtained with a conventional process, in which wheat straw is pretreated using steam explosion and fermented with the yeast *Saccharomyces cerevisiae* [37].

4. Hydrolysis of polysaccharides fraction of lignocelluloses for ethanol production

After pretreatment, the released cellulose and hemicelluloses are hydrolyzed into fermentable monomeric sugars (hexoses and pentoses). The hydrolysis of (hemi)cellulose remains a major bottleneck for the efficient production of ethanol [38, 39].

Two major methods can be employed to carry out the hydrolysis process. The first method involves acids as catalysts, while the second uses enzymes known as cellulases [40, 41]. The acid hydrolysis degrades the cellulose and hemicelluloses polymers into monomeric sugars with-

out preliminary pretreatment of biomass. It can be carried out using concentrated or diluted acids. The concentrated acid hydrolysis, with sulphuric and hydrochloric acids, is the mainly used method for the hydrolysis of lignocellulosic biomass [27] producing high hydrolysis yields of cellulose [40]. However, this process requires large quantities of acids, expensive acid recycling and causes degradation of monosaccharides.

It follows that enzymatic hydrolysis is the generally preferred method due to the mild operation conditions, higher conversion yields, low energy cost, and less corrosive and toxic conditions compared to the acid hydrolysis [20, 42, and 43]. However, the enzymes involved in the enzymatic hydrolysis have higher costs and this represent the main bottleneck in lignocellulosic ethanol production [44].

4.1. *Enzymatic hydrolysis*

As reviewed in Kumar and Murthy [42], the enzymatic hydrolysis process can be divided into two phases. The first step is represented by enzymatic depolymerization in which long polysaccharide chains are hydrolyzed to soluble oligomers. In the second step, oligomers are hydrolyzed to sugar monomers. The rate-limiting step in the process is considered the first hydrolysis phase.

Due to the complexity of the lignocellulosic carbohydrates, the activities of multiple hydrolytic enzymes are required for complete deconstruction of the various components of the lignocellulosic biomass; these enzymes are produced as either *free cellulases* or *complexed cellulases*, called cellulosomes [43, 45]. The efficient enzyme mixture comprises ten different enzymes. Among these, at least 6 can be considered crucial, depending on the composition of the raw materials [46]. Enzymes involved in lignocellulosic biomass are following described.

Cellulase enzymes

Cellulases are glycosyl hydrolases (GH) involved in cellulose hydrolysis, whose classification is available on the CARbohydrate-Active ENzymes web site (CAZY: www.cazy.org). These enzymes are con-

ventionally divided in three major classes: endoglucanases, exoglucanases and β -glucosidases [44, 47, and 48].

Endoglucanases (EG) or 1,4-beta-glucanases (EC 3.2.1.4) randomly hydrolyze intramolecular β -1,4-glucosidic linkages, creating new chain-ends; exoglucanases (CBH) or cellobiohydrolases (EC 3.2.91) hydrolyze the 1,4-glycosidyl linkages from the reducing or non-reducing ends to form cellobiose; β -glucosidases (BG) (EC 3.2.1.21) hydrolyze cello-oligosaccharides and cellobiose into glucose eliminating cellobiose inhibition.

Fungi and bacteria have been reported to be able to produce cellulases. In particular, the fungus *Trichoderma reesei* is the preferred industrial source of cellulases and hemicellulases due to the high level of secreted enzymes. This fungus produces at least two CBHs, five EGs, and two BGs whose expression is finely regulated [49]. However, this fungus produces low levels of BGs if compared to *Aspergillus sp.* *Trichoderma* strains engineered with extra β -glucosidase have been studied. In particular Nakazawa *et al.*[50] reported a recombinant *T. reesei* strain expressing *A. Aculeatus* BGLI. The resulting strain appears more suitable for cellulose hydrolysis. Among bacteria, several *Bacillus* strains isolated from natural habitats were shown to be able to produce cellulase activities [51].

Cellulosome systems are multi-enzymatic complexes produced mainly by anaerobic bacteria but also by other bacteria and few anaerobic fungi [40, 48, and 52]. In these systems there are two subunit types, the scaffolding and the enzymatic subunits. The structural scaffolding subunits contain a carbohydrate-binding module that binds the cellulose surface and multiple copies of cohesins (named dockerins) that interact with the enzymatic subunits, CBD (cellulose binding domains) and CBM (carbohydrates binding modules).

Hemicellulase enzymes

In addition to the three major groups of cellulose enzymes, there is also a number of hemicellulases which hydrolyze hemicellulose. Due to the more varied composition of this polysaccharide, enzymes degrading hemicellulose are divided into two major groups: depolymerising enzymes, which cleave the backbone, and enzymes that remove substituents. These enzymes act synergistically to hydrolyze lignocellulosic polysaccharides [48].

Xylanase

Most studies on hemicellulases were focused on enzymes that hydrolyze xylan. According to the CAZY database, these enzymes include GH 3, 30, 39, 43, 52, 54, 116 and 120.

The complete degradation of xylan requires the cooperative action of endoxylanase and xylosidase.

Endo-1,4- β -xylanase (1,4- β -D-xylan xylanohydrolases, EC 3.2.1.8) cleaves the glycosidic bonds in the xylan backbone releasing shorter xylo-oligosaccharides; β -xylosidase (1,4- β -D-xylan xylohydrolase, EC 3.2.1.37) cleaves the small xylo-oligosaccharides and cellobiose into xylose. These enzymes are produced by several microorganisms such as fungi, bacteria, yeast, and marine algae. Filamentous fungi are interesting producers because the enzymes are secreted at levels higher than those of yeasts and bacteria.

As reviewed by Van Dyk and Pletschke, [48] endoxylanases have different specificities. For example, family 11 xylanases prefer cleaving the xylan backbone in unsubstituted regions, while family 10 xylanases are able to cleave the xylan backbone closer to the substituents.

The synergic activity of several accessory enzymes which remove the various substituents linked to the backbone is also essential.

α -arabinofuranosidase

α -L-arabinofuranosidase (EC 3.2.1.55) removes the L-arabinose substituents from the xylan backbone. According to the CAZY database, arabinofuranosidases are present in GH 3, 43, 51, 54 and 62. All these families, except for GH 43 and 62, perform hydrolysis with retention of the anomeric configuration.

As reviewed by Van Dyk and Pletschke [48], α -L-arabinofuranosidases have different specificities cleaving 1,2 linkages or 1,3 linkages. Moreover, these enzymes are able to cleave doubly substituted arabinose residues from arabinoxylan. As reported by Lagaert et al [53], complete degradation of arabinoxylan needs the synergistic action of arabinofuranosidases and also of β -xylosidases. Rasmussen *et al.* [54] demonstrated that optimal hydrolysis of soluble wheat arabinoxylan requires the combined action of an endoxylanase, a β -xylosidase and the two types of arabinofuranosidases.

α -glucuronidase

α -glucuronidase (EC 3.2.1.139) removes 4-O-methyl glucuronic acid substituents from the glucuronoxylan. This enzyme hydrolyses the α -1,2 bonds between the glucuronic acid residues and β -D-xylopyranosyl backbone units [44].

Esterases

Esterases hydrolyse the ester linkages between xylose units of the xylan and acetic acid (Acetylxylan esterase, EC 3.1.1.72) or between arabinose side chain residues and phenolic acids such as *p*-coumaric acid (*p*-coumaric acid esterase EC 3.2.1.73) or ferulic acid (ferulic acid esterase, EC 3.2.1.73).

Acetylxylan esterase removes the O-acetyl groups from positions 2 and/or 3 on the β -D-xylopyranosyl residues of acetyl xylan.

Ferulic acid and p-coumaric acid esterases (EC 3.1.1.73) hydrolyze ester linkages on xylan, liberating the respective phenolic acids linked to the arabinofuranoside residues [48].

Mannanases

Mannose residues have α -1,6-galactose as side groups and these are acetylated at the O-2 and O-3 positions. The core enzymes for complete degradation of mannan into simple sugars are the endomannanase and β -mannosidase.

Endo- β -1,4-mannanases (EC 3.2.1.78) catalyze hydrolysis of the β -1,4-linked backbone within different mannans. In the CAZy database enzymes are classified into three different glycoside hydrolase families: GH5, GH26 and GH113 [55].

β -mannosidase (EC 3.2.1.25) is the key enzyme responsible for catalyzing random hydrolysis of manno-glycosidic bonds in the main chain. All plant mannanases belong to family GH5 subfamily 7 (GH5_7) [56].

Additional enzymes, such as α -galactosidases (EC 3.2.1.22), β -glucosidase (EC 3.2.1.21) and acetyl mannan esterases are required to remove side chain sugars on mannans.

5. Sugars fermentation for bioethanol production

Once lignin has been removed and the saccharification of the free accessible (hemi)cellulose portions of the biomass has been carried out, the final step to be performed is the fermentation of sugars into bioethanol.

Saccharomyces cerevisiae is the most widely used organism for ethanol production from hexoses, whilst *Pichia stipitis* and *Candida shehatae* are the main yeasts able to ferment both hexose and pentose sugars to ethanol. Bacteria belonging to the species *Clostridia* and *Zymomonas*, and fungi such as *Fusarium* spp. have been also demonstrated to be useful for ethanol production.

The current process is optimized for 6-carbon atoms sugars fermentation since most yeasts cannot ferment 5-carbon atoms sugars. Given that the economically competitive ethanol production from lignocellulosic materials requires the efficient use of both hexose and pentose monosaccharides, research is now strongly exploring new engineered yeasts able to ferment 5-carbon sugars with high yields. The main routes for ethanol fermentation are described below.

5.1. Ethanol production by separate hydrolysis and fermentation (SHF)

Two main routes can be followed for ethanol production, such as separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF).

In SHF, the bioconversion of lignocellulose takes place in two separate reactors, thus separating the saccharification and the fermentation processes, resulting in the quick and easy possibility to optimize each production step, with conduction of each process at the optimal conditions of pH and temperature.

An example of SHF process application for ethanol production is described by Erdei *et al.* [57], who reported an experiment of separate hydrolysis and co-fermentation (combining xylose and glucose fermentation) of steam-pretreated wheat straw (SPWS) combined with wheat starch hydrolysate feed, achieving an average yield of ethanol up to 86%.

5.2. Ethanol production by simultaneous saccharification and fermentation, SSF

In SSF, enzymatic hydrolysis and fermentation are carried out together, using a unique reactor. By taking place in the same reactor, this kind of process has a number of advantages such as the reduction of costs and the increase of hydrolysis rate, besides the possibility to make the fermentable sugars immediately available to yeasts.

However, the ideal pH or temperature conditions for the saccharification step may differ from those of the fermentation step. Thus, it is very difficult to find favorable conditions for both processes.

As generally stated, SSF is a much more competitive process in comparison to SHF from the economical point of view, since the use of a unique bioreactor results in a total reduction of investment and operational costs.

Recent works regarding SSF are those by Nahar and Pryor [58] and Rezić *et al.* [59], who used as feedstock for bioethanol production crushed whole sugar beets (ethanol yield up to 92%) and sugar beet pulp (ethanol yield up to 49%), respectively.

Hydrolysis and fermentation conditions for production of ethanol from very high-gravity cassava mash during a simultaneous saccharification and fermentation (SSF) process were optimized using a statistical methodology [60], reaching a final ethanol yield of 15.03% (wt.%) in 72 h.

5.3. Ethanol production by consolidated bio processing, CBP

Consolidated bio processing (CBP) has been so far recognized as the best system to reduce the cost of biomass processing, by combining the hydrolysis of the polysaccharides and the subsequent fermentation of the hexoses/pentose sugars.

Differently from SSF, besides the use of a unique reactor, in CBP a single engineered microorganism is used, which is able to directly convert (hemi)cellulose into ethanol using its own enzymatic machinery.

So far, there are no microorganisms able to perform both the enzymatic hydrolysis and the fermentative steps with high yield of both processes, thus genetic engineering must be applied to create suitable microorganisms for the CBP process.

It is worth noting that there are two main routes to perform CBP: CBP I route, characterized by the engineering of a cellulase producing microorganism to make it able to ferment sugars; CBP II route aimed at the engineering of an ethanologenic microorganism to make it able to produce cellulases or hemicellulases.

As recently reviewed by Olson *et al.* [39], progress in the development of genetic tools for fungal systems has been obtained, focusing mostly on the increase of cellulase production, especially for fungi like *Fusarium oxysporum* and *Trichoderma reesei*. There has also been a substantial progress in the development of genetic tools for free-enzyme bacterial systems, including *Clostridium phytofermentans*, *Clostridium japonicas*, *Thermoanaerobacter* and *Thermanaero-bacterium* sp. The latter, a thermophilic anaerobe that utilizes a broad range of substrates including xylan, is a prominent example of engineered organisms with recently developed genetic tools to produce a biofuel at high yield [61, 62].

CBP category I

Trichoderma, *Aspergillus*, *Rhizopus* and *Fusarium* are the potential filamentous fungi suitable for development of CBP type I. All of them produce high amounts of both cellulases and hemicellulases, leading to high yields of fermentable sugars. Particularly, *T. reesei* is the best producer of such activities, so far described.

These fungi have been reported as ethanol producers, even if with very low yield and high concentration of by-products, such as lactic acid.

As reviewed by Amore and Faraco [63], many efforts are focused on the elucidation of regulatory mechanisms, in order to advance the knowledge on the metabolic pathways involved in ethanol production by filamentous fungi recognized as potential candidates for CBP type I.

CBP category II

S. cerevisiae is the most used yeast for ethanol production from C6 sugars and it represents the best candidate for CBP type II. Thus, a big challenge is to confer to this microorganism the capability to produce cellulase and/or hemicellulase activities.

S. cerevisiae has been so far described as a suitable host for recombinant production of different enzymes, among which are also enzymes involved in the conversion of polysaccharides.

As previously reported, for the complete conversion of cellulose there is the need of three different enzymes namely endoglucanases, cellobiohydrolases and β -glucosidases, whose most common sources are fungi, especially *Trichoderma reesei*, and bacteria.

Cellobiohydrolases (CBH) and endoglucanases have been successfully expressed in *S. cerevisiae*, whilst very low yields of fungal β -glucosidase have been achieved.

Kluyveromyces marxianus is another interesting candidate for CBP II. It has been used with good results as host for heterologous proteins, including enzymes involved in cellulose hydrolysis.

Among bacteria, *E. coli* represents the main candidate for the CBP II category. It is able to metabolize a wide spectrum of sugars and a well-known microorganism to be genetically engineered.

Despite the extensive research performed to optimize *E. coli* recombinant cellulase production and reduce the addition of external cellulases, the potential of the obtained recombinant strains to directly grow on plant biomass cannot be proved, mainly due to the complex cocktail of enzyme needed for the complete lignocelluloses conversion into fermentable sugar, as reviewed by Amore *et al.* [64].

Zymomonas mobilis, *Klebsiella oxytoca* and *Bacillus subtilis* are other candidates for the CBP II process, being known for ethanol production and tolerance to the toxicity of the final products.

6. Conclusions

To overcome bottlenecks associated to first generation bioethanol, produced from traditional agricultural crops, second generation bioethanol production from non edible renewable lignocellulosic biomass is attracting strong interest, as it minimizes the conflict between land use for food (and feed) and energy production. Lignocelluloses are the most abundant renewable resources on Earth and include residual biomass such as agro-industrial wastes, agricultural and forest crop residues and the organic and paper fractions of municipal solid waste (MSW). Moreover, second generation ethanol

production and use show lower green-house gas emissions than the first generation fuels, reducing environmental impact. However, the process currently adopted for second generation bioethanol production is not competitive as yet. Lignocellulose consists of three types of polymers — cellulose, hemicellulose and lignin — bonded by both non-covalent and covalent cross linkages. Bioethanol production from lignocellulosic materials takes place in three phases, namely a pretreatment step to remove the lignin barrier, hydrolysis of cellulose and hemicellulose to generate fermentable sugars (saccharification) and fermentation of mixed hexose and pentose sugars to produce ethanol.

As regards the pretreatment step, all the techniques so far used (physical, physico-chemical and chemical processes), are not eco-friendly and generate inhibitors which negatively affect the next step of lignocellulose saccharification into fermentable sugars. Biological pretreatment could represent a valid alternative, but the long treatment time and the low final yield do not make it a feasible process, as yet.

Despite the high costs of enzyme production, enzymatic hydrolysis is preferred to the acid one. A wide spectrum of enzymes is required to completely convert pretreated lignocellulose into fermentable sugars, both cellulases, acting on the cellulose polymer for C₆ sugars release, and hemicellulases, for C₅ sugars production.

Simultaneous saccharification and fermentation (SSF) and separate hydrolysis and fermentation (SHF) are the main routes used to produce bioethanol by fermenting the hydrolysate from cellulose, even if consolidated bioprocessing (CBP) has been recognized as the best system to reduce the cost of biomass processing, by combining the hydrolysis of the polisaccharides and the subsequent fermentation of the hexose/pentose sugars.

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