First Generation Bioethanol

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Abstract At the beginning of 2016, first generation bioethanol still contributes to the majority of the 25 billion of gallons' bioethanol produced worldwide, with the United States and Brazil producing approximately 85 % of the global production predominantly based on corn and sugarcane, respectively. However, concerns over the long-term sustainability of first generation bioethanol, such as the impacts on land use, water resource, the potential contamination of soils with the distillation residues, and the competition for food and feed production is frequently highlighted. Current fuel ethanol research and development strives to minimize these negative externalities. The fundamental role that process design plays during the development of cost-effective technologies is evaluated through the modification of the major pathways in first generation ethanol synthesis. In this context, the central role that better performing enzymes and microorganisms play in the intensity and integration of the process, such as the typical example of simultaneous saccharification and fermentation from starchy material in first generation facilities is acknowledged. Compensating ethanol production costs by the integrated valorization of energy and by-products for feed and green chemistry in a typical

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biorefinery concept are striking outputs of the first generation ethanol real scale experiment. Finally, rather than a mistake, first generation bioethanol should be considered as the first step that made it possible to gain the necessary experience for the successful implementation of the future greener generations biofuels from the field to the tank, starting with second generation lignocellulosic that is now coming on the market. In this context, integrated biorefineries are a promising way to diversify the usable feedstocks, leading to reduced facilities size and optimized supply-chains, to valorize more efficiently bagasse's from sugarcane and corn stover or even to exploit the potential of microalgae to capture the carbon dioxide that is produced during the fermentation steps. Major stakeholders in bioenergy production are taking advantage of the large-scale successful development of first generation bioethanol, using the most promising processing schemes for next generation facilities, although the industry is still facing uncertainties with respect to its economic viability and longevity.

Keywords Sugarcane · Sugar beet · Corn · Cassava · Enzymatic treatments · Process engineering · High gravity fermentation · Integrated biorefineries

1 Introduction

Global population growth, projected to exceed 9 billion by 2050, will raise the average calorie intake thus pushing productivity from already scarce arable land to its limit. At the same time, the energy demand in developing nations is expected to increase by 84 % over the same period, with nearly one-third of this additional fuel probably needing to come from alternative renewable sources such as biofuels (Graham-Rowe 2011; Dutta et al. 2014). First generation ethanol (1G ethanol) processes utilize either soluble sugars or starch. In 2014 there were more than 200 starch-based bioethanol plants operating in the USA, with an average capacity of 260,000 m^3 ethanol produced per year from corn (maize) and sorghum (www.ethanolproducers.com). Figure 1 shows the global ethanol production by country or region, over the period 2007–2014. The United States is the world's largest producer of bioethanol, producing over 14 billion gallons in 2014 alone with more than 40 % of the US corn crop is being used to produce ethanol. Together, the U.S. and Brazil produce 83 % of the world's ethanol, which globally amounts to around 21 million m^3 ethanol produced from sugarcane and 60 million m^3 from corn and other grains (REN21 2012; Dutta et al. 2014; AFDC 2015). The fuels generated from these raw materials are readily used in today's petrol engines. However, there are country-specific mandates for blending biofuels, as there are concerns about possible food versus fuel conflicts of interest in land use.

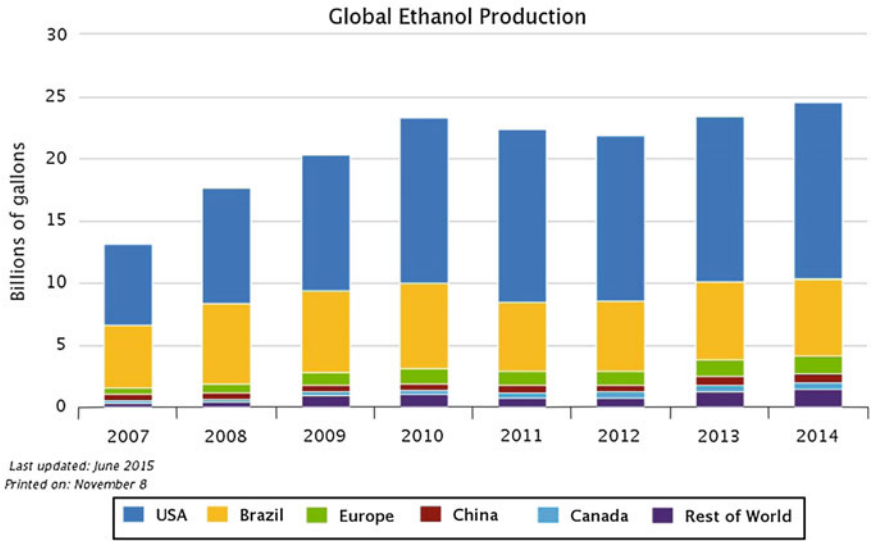

Fig. 1 Global bioethanol production from 2007 to 2014. *Source* Renewable Fuels Association, Ethanol Industry Outlook 2008–2015 reports (AFDC 2015)

2 Feedstocks for First Generation Bioethanol

Bioethanol feedstocks can be classified into three types: (i) sucrose-containing feedstocks (e.g., sugar beet, sweet sorghum, and sugarcane), (ii) starchy materials (e.g., wheat, corn, and barley), and (iii) lignocellulosic biomass (e.g., wood, straw, and grasses) (Balat et al. 2008). The availability of feedstocks for bioethanol can vary considerably from season to season and depending up on geographic locations. The changes in the price of feedstocks can highly affect the production costs of bioethanol (Yoosin and Sorapipatana 2007). Another point to consider is that, the major feedstocks for first generation biofuels are the sources of food, which may cause certain competition. Only 2 % of world's arable land is used to grow biomass feedstock for first generation biofuel production (OECD/IEA 2008), which may contribute to the increase of commodity prices for food and animal feeds. However, direct or indirect impact of biofuels on food price hike remains inconclusive.

1G bioethanol production in different producing countries and the main feedstock used is described in Table 1. With an increasing instability in petroleum prices, many countries have decided to direct their energy policy toward the use of biofuels. This imposes the production of crops such as maize, sugar beet, and others that can supply the demand for bioethanol, without conflict with food production. Cereal grains are the most abundant crops used at present for the generation of 1G ethanol. The infrastructure for growing, harvesting, and processing maize is well established, and the conversion of corn starch and corn syrups into ethanol is a relatively simple process. Corn grains contain approximately 65–76 % w/w starch,

Table 1 World's first generation ethanol production from different feedstocks

Country/continent	Major feedstock sugar and starchy crops	Ethanol production per year (billion liters)	Costs (US$/L)
Asia			–
China	Molasses, Sweet Sorghum	–	0.32, 0.29
Thailand	Cassava	1.0	0.18
Europe			
Belgium	Wheat	0.4	–
EU	Cereal and Sugar beet	4.5	–
France	Sugar beet	1.0	0.60–0.68
Spain	Barley, Wheat	0.4	–
Sweden	Wheat	–	0.40–0.45
Poland	Rye	0.2	0.55–0.65
North America			
US	Corn/Maize	50.3	0.25–0.40
Canada	Wheat/Cereal	1.8	–
South America			
Brazil	Sugarcane	25.5	0.16–0.22
Argentina	Sugarcane	0.5	–
Oceania			
Australia	Sugarcane	0.3	–

Source Modified from Gupta and Verma (2015), Haankuku et al. (2015)

while wheat (66–82 %), barley (55–74 %), sorghum (68–80 %), oat (45–69 %), and rice (74–85 %) are also rich sources of 1G raw materials.

Sugarcane is the second most used raw material for the production of bioethanol. The majority of the world's sugarcane is grown in Brazil. Unlike cereals, which produce starch as the source of fermentable sugars, sugarcane produces directly sugar, and so does not require an initial heating step prior to fermentation. As with maize, the infrastructure for the production, harvesting, and processing of sugarcane is well established.

Palm oil (SE Asia), sugarcane (Brazil), and sweet sorghum (China) appear to be the most sustainable crop for the generation of bioenergy as these crops make the most efficient use of land, water, nitrogen, and energy resources (de Vries et al. 2010). In comparison, maize (USA) and wheat (NE Europe) were poor performers for ethanol production, while rapeseed (NW Europe), cassava (Thailand), sugar beet (NW Europe), and soybean (USA, S. America) take a more intermediate position in feedstock sustainability factors. In temperate climates, first generation ethanol from maize and wheat appear not to be sustainable, as they are not fully meeting their primary goals of reducing fossil fuel consumption and GHG emissions.

Biofuels facilities can be broadly separated into two based on the feedstock, and thus the technologies required to produce the bioethanol. For instance, some of the

major producers include ADM Hamburg AG (Germany), a subsidiary of Archer Daniels Midland, producing bioethanol for transportation from food processing. LS9, Inc (USA), which uses synthetic biology to produce ethanol from sugarcane syrup, but is also investigating processes for sorghum and lignocellulosic 2G feedstock. Proalcool (Brazil) is the National State-sponsored company for supporting the production of bioethanol from sugarcane. Jilin Fuel Ethanol and the Henan Tianguan Alcohol Chemical Group Co (China) have plants with the capacity to produce 1.3 million tons of ethanol per year.

2.1 Sugarcane 1G Bioethanol

Sugarcane represents two-third of world sugar production and one-third is from sugar beet (Linoj et al. 2006). They are the most promising sources for bioethanol production (UNCTAD 2015). Both are produced in geographically distinct regions. Sugarcane is grown in tropical and subtropical countries, while sugar beet is only grown in temperate climate countries. (Balat et al. 2008). Unlike cereals, which produce starch as the source of fermentable sugars, sugarcane produces directly sugar, and so does not require an initial heating step prior to fermentation. As with maize, the infrastructure for the production, harvesting, and processing of sugarcane is well established. Brazil is the largest producer of sugarcane worldwide with 632 billion tons (Unica 2015). The center-south region of Brazil accounts for almost 80 % of its feedstock production (Zarrilli 2006). Sugarcane was chosen as the substrate for ethanol production due to its great adaptation to the Brazilian soil, the weather conditions and high sucrose juice composition. Due to this great interest, agricultural and technological studies were intensified. This fact, led Brazil to a very favorable position in terms of energy security. The sugarcane yields for 2015 and 2020 are estimated to be 79 and 84 t cane/ha, respectively, with an average increase rate of 1.3 % per year (Wang et al. 2014).

The National Alcohol Program—ProAlcool, created by the government of Brazil in 1975 resulted in less dependency on fossil fuels (Rosillo-Calle and Cortez 1998; Soccol et al. 2005). In this way, the Brazilian government started its policy to substitute gasoline with sugarcane alcohol. The program saw the participation of several politicians and military, sugarcane producers, researchers, the alcohol industry, and the media. The use of a mixture of ethanol and gasoline (gasohol) to fuel common cars was then intensified. The addition of 25 % ethanol to gasoline reduced the import of 550 million barrels' oil and CO_2 emission by 110 million tons (Soccol et al. 2010). In March 2003, the introduction of flex fuel vehicles (FFVs) revitalized the Brazilian car industry. FFVs can use various mixtures of alcohol and gas, thus allowing the consumers to react to the different prices signals of the two markets (Hira and Oliveira 2009). Presently, Brazil has more than 90 % of flex fuel vehicles in its fleet (ANFAVEA 2015).

Nowadays, around 39.4 % of the Brazilian energy matrix is renewable and 157 % is derived from sugarcane (Unica 2015). Brazil has a land area of 851

million hectares, of which 54 % are preserved, such as the Amazon rainforest (350 million hectares). From the land available for agriculture (340 million hectares), only 2.59 % is used to produce sugarcane, representing 10 million hectares, showing a great expansion potential for this crop (Udop 2015; Unica 2015). Brazil ethanol production is entirely based on the fermentation of simple sugars extracted from harvested sugarcane stem either in autonomous distilleries or in annexed plants co-located with sugar mills that coproduce ethanol and crystalline sugar (Seabra et al. 2011; Wang et al. 2014). As one of the worlds' largest ethanol producers, Brazil has used sugarcane as feedstock to produce over 28 billion liters of ethanol in 2014/2015 (Unica 2015), which is destined for fuel. Currently, there are 403 bioethanol production units installed in the country (Udop 2015). Amongst these, 392 units are located in the south, southeast, and center-west and only five units in the Amazon region. However, an expansion of the ethanol production to 104 billion liters in 2025 will necessitate the reduction of production costs to sustain the transportation from more distant areas within Brazil to internal and external markets. In addition, advanced technology can provide better environmental performance and greater productivity per unit of land. However, this is also almost always bringing additional costs. A hectare of sugarcane can produce about 6000 liters of ethanol (Cerqueira Leite et al. 2009). Around 70 % of the ethanol production costs correspond to the raw materials (IBGE 2008).

2.2 Sugar Beet 1G Bioethanol

Based on the USA Energy Independence and Security Act (EISA) of 2007, sugar beets (*Beta vulgaris* L.) may be an eligible feedstock for advanced biofuel provided that production and conversion to biofuel meets the 50 % greenhouse gas reduction threshold required for advanced biofuel designation (Congress U.S. 2007; NREL 2014; Haankuku et al. 2015). The new energy strategy for Europe starting from 2011 to 2020 has been discussed in the European Union institutions (European Commission 2010; European Parliament 2010). This strategy has to be in line with the Lisbon Treaty to guide long-term emission- reduction goals, the so-called 20–20–20. To achieve energy and climate goals, the potential of bioenergy is a key issue. The main inputs in the production of bioethanol in the EU are sugar beet, wheat, corn, or barley (Salazar-Ordóñez et al. 2013). Wheat and sugar beet are frequently used in Northwestern Europe, while corn is employed in Central Europe and Spain, where barley is also often used. Thirty percent of bioethanol is produced from sugar beets (Agrosynergie 2011); around 24 % of the total production of this crop has been destined to bioethanol in the EU for the past three years (Eurostat 2011).

Sugar beets are tuber crops composed of about 75 % water, 18 % sugar (mainly sucrose), and 7 % insoluble and soluble materials (which are required to be at low levels). Unlike conventional sugar beets that are bred to produce sugar for table use, biofuel feedstock industrial beets are specialized nongrade varieties bred for total

sugar production (Haankuku et al. 2015). Some alternatives are being examined in order to reduce bioethanol production costs from sugar beet. New sugar beets varieties and multi-effect evaporation processes were proposed as the major factors in the future cost reduction. Although the costs of direct fermentation of sugar beet juice (adjust the sugar content by adding molasses) is lower than the process using sugar beet juice concentration, the multi-effect evaporation enables a high-sugar fermentation and saves distillation and equipment costs (Ruan et al. 2001). At the same time, it also reduces the microbial infection of the squeeze juice. Part of impregnated water and diluted water are the wastes from the distillation tower. Water can be recycled in the production process and therefore reduce emissions (Zhou et al. 2011). In addition, with this method, separating sugar beet pulps before fermentation improves the equipment utilization of fermentation and distillation, saves energy consumption and makes the comprehensive utilization of sugar beet pulps much easier. Enrichment process preserves the sugar, which will be able to extend the production period in ethanol plants.

2.3 Corn 1G Bioethanol

The U.S. Department of Agriculture (USDA) has a program devoted to the corn ethanol industry. Areas of scientific research address the establishment of new higher value ethanol coproducts, the development of microbes capable of converting various biomass materials into ethanol, improved processes for the enzymatic saccharification of corn fibers into sugars, and various methods of improving corn ethanol process efficiencies (McAloon et al. 2000). In the 2013/2014 USA's corn production reached nearly 13.8 billion bushels (351.3 million tons) of corn and roughly 11 % of the production was exported to more than 100 different countries. More than one-third of USA's corn crop is used to feed livestock, 13 % is exported and 40 % is used to produce ethanol. The remainder goes toward food and beverage production. Federal renewable-fuel standards require the blending of 13.2 billion gallons of corn ethanol with gasoline in 2012. This required 4.7 billion bushels of corn, which corresponds to forty percent of the annual crop (Carter and Miller 2012; EIA 2013).

Fuel ethanol production from corn can be described as a five-stage process: raw material pretreatment, hydrolysis, fermentation, separation and dehydration, and wastewater treatment. The production of bioethanol from starch includes the breakdown of this polysaccharide to obtain an appropriate concentration of fermentable sugars, which are transformed into ethanol by yeasts. After washing, crushing, and milling the corn grains (dry milling process), the starchy material is gelatinized in order to make the amylose and amylopectin susceptible for enzymatic attack in the following liquefaction step. This step is considered as a pretreatment process because of the partial hydrolysis of the starch chains using thermostable α-amylase. The hydrolysate obtained has reduced viscosity and contains starch oligomers called dextrins. Then, the fermentation process occurs where sugar is

immediately assimilated by the yeast *Saccharomyces cerevisiae* in the same reactor and converted into ethanol. The culture broth containing 8–11 % (w/w) ethanol is recovered in a separation step consisting of two distillation columns (Quintero et al. 2008).

2.4 Cassava 1G Bioethanol

Cassava is a shrub with tuberous roots. It is the third source of food calories in tropical countries after rice and corn. Cassava is used in both human and animal food, in many industrial sectors, particularly in the form of starch, and more recently to produce ethanol. Cassava is primarily grown for its roots but all of the plant can be used: the wood as a fuel, the leaves and peelings for animal feed, and even the stem as dietary salt (UNCTAD 2015). World production of cassava is around 281 million tons (Mt) a year. Africa contributes to more than half of the global supply. Asia encourages the development of cassava crops for industrial and energy purposes. This continent contributes to around one-third of the world production, with 26 Mt produced by Thailand and 28 Mt by Indonesia. In Latin America production is around 35 Mt where Brazil dominates with around 70 % of regional production and in third place in world production (Conab 2013). Cassava is still a small player on the biofuel scenario. For example, cassava roots, which have a starch content of 30 % (w/w), can generate 180 liters pure ethanol (96 %) per ton of raw material (500–4000 liters per hectare per year) (Larkin et al. 2004).

3 Enzymes for the First Generation Bioethanol

The starch hydrolysis by enzymes is a two-stage process involving liquefaction and saccharification. Liquefaction is a step where starch is degraded by α-amylase, which hydrolyzes only α-1,4 bonds between glucose units and causes a reduction in starch viscosity. Liquefying enzymes usually work at high temperatures (>85°C) so that the enzyme can help reduce starch paste viscosity during cooking. Dextrins, which are obtained after liquefaction, are further hydrolyzed by limit dextrins/pullulanases which can hydrolyze both α-1,4 and α-1,6 glycosidic linkage and then to glucose by glucoamylase. Glucose is then subsequentially converted to ethanol by yeast fermentation. After fermentation, approximately 10 % (v/v) ethanol is obtained and subjected to distillation and dehydration to remove water and other impurities, yielding anhydrous ethanol (Sriroth et al. 2012).

Biotech companies such as Dyadic, Amryis, and Gevo have focused on developing enzymatic solutions for the high-value steps. While enzyme production is considered to be an expensive step, accounting for nearly 50 % of the costs of 2G cellulosic ethanol production, the cost of enzyme production is being driven down by the manufacturers, such as Novozymes, Dupont, and Dyadic, from $2 per gallon

in 2010 to approx. $0.30 in 2015, together with much more efficient, feedstock specific pretreatment process development. This has also led to onsite enzyme production facilities provided by Dupont, Dyadic, and DSM. Dupont, who opened the world's largest bioethanol plant in Iowa (USA) in 2015 have developed enzyme technologies for the production of more than 68 billion liters of 1G ethanol per year from corn cobs, stems, and leaves. Enzyme companies are constantly trying to improve their products for 1G bioethanol production, where even the smallest improvement in hydrolysis can lead to an extra 1–2 % increase in ethanol production. Recent developments in the two main starch-degrading enzymes, α-amylase and glucoamylase, encouraged by the availability of various fungal and bacterial genome sequences, are moving in the direction of more robust products operating at pH 5 or below, and with limited supplementary calcium requirement (Harris et al. 2014). A number of enzymes, as described below, are required for the production of 1G ethanol.

3.1 α-Amylases

α-Amylases (EC 3.2.1.1) are 1,4-α-D-glucan glucanohydrolases that catalyze the cleavage of internal α-1,4-glycosidic bonds in starch in a random manner, releasing dextrins and gluco-oligosaccharides with the reducing groups liberated in the α-configuration. The α-1,4 bonds close to the α-1,6 branch points in amylopectin are resistant to hydrolysis by α-amylases. Prolonged hydrolysis of the amylopectin with α-amylases yield limit dextrins. Most a-amylases belong to the CAZy family GH13 (Lombard et al. 2014), together with pullulanases, cyclomaltodextrinases, and trehalose-6-phosphate hydrolase. This classification is based on the direct relationship between sequence and folding similarities. Some α-amylases belong to GH Family 57. The sequences of α-amylases from different origins have very few discernable similarities but the catalytic mechanism requires three catalytic residues (two Asp and one Glu), as well as residues involved in substrate binding. These residues are all found in four highly conserved regions. Despite the low sequence similarity, α-amylases from different sources display remarkably similar tertiary folding, with a $(\alpha/\beta)_8$ barrel central core. The enzyme contains a calcium-binding site, similar to that of other amylolytic enzymes, and the removal of the calcium leads to irreversible inhibition. Cereal α-amylases contain starch granule binding sites, which interact with a host of different substrates, from granular starch to cyclodextrin, through two consecutive Trp residues (Lundgard and Svensson 1987). This feature is not present in animal of microbial α-amylases. The SPEZYME® line of α-amylases from Dupont claim to offer robust liquefaction of starch and viscosity reduction over a range of temperatures and pH (http://biosciences.dupont.com/industries/biofuels/bioethanol-from-starch/). Proteinaceous α-Amylase inhibitors have been identified in cereal grains, which are implemented in plant defense and endogenous enzyme regulation (Sancho et al. 2003; Nielsen et al. 2004).

The presence of such inhibitors in the raw materials utilized for 1G production may have a serious impact on the efficiency of the process.

3.2 β-Amylases

β-Amylases (EC 3.2.1.2) are exo-acting hydrolases removing successive β-anomeric maltose units from α-1,4-glucans. such as starch and glycogen. They belong to the GH 14 family of CAZy. Sweet potato contains a high level of this enzyme as soluble protein in its tubers, with only trace amounts of α-amylase. As with α-amylase, β-amylases adopt a large $(\alpha/\beta)_8$ barrel central core, with a catalytic pocket containing the two catalytic Glu residues, compared to α-amylases which have their catalytic mechanism in a long cleft open at both ends. Substrate binding causes a structural shift where a flexible loop moves 11 Å upon maltose binding, effectively closing over the substrate like a hinged lid (Rockey et al. 2000; Kang et al. 2005). A single enzyme can release several maltose molecules in a phenomenon where the β-amylase "slides" on the substrate (Ishikawa et al. 2007). Such enzymes can be used to make maltose syrups for further processing into fermentable sugars for 1G ethanol production. More resistant starch can be formed through the action of β-amylases (Luckett and Wang 2012).

3.3 Glucoamylase

Glucoamylase (GA; EC 3.2.1.3), also known as amyloglucanases, is an inverting exo-acting multi-domain enzyme which attacks starch from the nonreducing end to produce glucose. The catalytic domain of GA belongs to CAZy family GH15 and has a $(\alpha/\alpha)_6$ barrel structure. The active site is described as a well of $\sim$10 Å deep by $\sim$15 Å wide so the substrate must penetrate deep into the well before cleavage can occur. This means that the cleaved glucose and the remaining chain must leave the well before the next reaction can proceed. To overcome this, filamentous fungi, such as *Aspergillus* sp., produce very large amounts of GA. Hydrolysis occurs via multichain attacks, but at high glucose concentrations, GA reforms all the glycosidic bonds that it hydrolyses, condensing glucose to form isomaltose, isomaltotriose, and other derivatives (Nikolov et al. 1989). Purified GA has been used to make glucose syrups from maltodextrins produced by the action of α-amylases. A standard industrial saccharification process using GA starts with dextrins of DP 10–15. The most studied GAs are those from *Aspergillus awamori* and *A. niger*, and are composed of three separate structures: a catalytic and a starch-binding domain separated by a rigid, highly glycosylated linker (Kramer et al. 1993). The binding domain can be proteolytically cleaved to leave only the catalytic domain, which is then incapable of acting on granular starch. The intact multi-domain molecule can degrade the whole starch granule. The starch-binding domain belongs

to CBM20 family of the CAZy database (Lombard et al. 2014) and is ~108 residues long, extending from the C-terminus of the linker. This binding domain has two binding sites for starch and related substrates, where a Trp in each side has been implicated in the interaction between the parallel strands of the amylosic double helix (Morris et al. 2005). Saccharification of liquefied starch-containing substrates requires addition of glucoamylase (e.g., Spirizyme® from Novozyme) to ensure maximum conversion of dextrins to glucose. New generation glucoamylase preparations have been developed to work directly on the corn-fiber matrix to degrade trapped starches down to glucose. More than 100 glucoamylases with a huge diversity (40–50 % identity) were recently cloned and characterized by Novozymes with respect to ethanol stability, activity in high-density solids and preference for branched dextro-oligosaccharides (Harris et al. 2014).

3.4 Pullulanases or Limit Dextranases

Pullulanases (EC 3.2.1.41) or limit dextranases (EC 3.2.1.142) catalyze the hydrolysis of the α-(1,6)-D-glucosidic linkage in amylopectin. They are usually more active on dextro-oligosaccharides than on polymeric starch. This enzyme, together with the amylases and GA help bring about the complete degradation of starch to glucose and maltose. They belong to the GH13 family and are distributed widely amongst microorganisms (mainly thermophilic bacteria and archaea) and plants. A conformational difference around the active site cleft together with domain organization determines the different substrate specificities between pullulanases and the other α-1,6-glucan debranching enzyme, isoamylase (Mikami et al. 2006).

3.5 Lytic Polysaccharide Monooxygenase (LPMO)

Very recently, a lytic polysaccharide monooxygenase (LPMO) specifically acting on starch has been reported (Harris and Wogulis. 2010; Horn et al. 2012; Lo Leggio et al. 2015). This enzyme belongs to the AA13 family of the CAZy database, are metalloproteins with a histidine-ligated mononuclear copper and in one case have been associated with a starch-binding CBM20 module (Horn et al. 2012; Lo Leggio et al. 2015). A highly starch-specific AA13 showed moderate activity on retrograded starch, degrading it through oxidation at the C1 position to aldonic acids dependent on the presence of copper and the reducing cofactor cysteine, and acted in synergy with β-amylase to release maltose (Lo Leggio et al. 2015). The starch-acting LPMO has a conserved central β-sandwich core with the active site common to other fungal LPMOs, such as the AA9s (see Couturier et al. Chapter "Fungal Enzymatic Degradation of Cellulose" of this book), with the active site presented to the solution in a shallow groove along the protein surface which leads

to the bound copper. Lo Leggio and coworkers postulate that this difference in structure to AA9s is likely to accommodate the more contoured surface of retrograded starch.

3.6 Phytases

Phytases can be included in the list of enzymes involved in 1G ethanol production through its ability to stabilizing the α-amylase by degrading phytic acid that could detach vital calcium ions from the α-amylase. The phosphate moieties of phytic acid are able to bind di- and trivalent metal ions such as calcium, magnesium, zinc, and iron. Phytases (EC 3.1.3.8 and 3.1.3.26) de-esterify the phosphate groups from phytate, and are most commonly used in animal feed to date. Two main groups of phytases exist, hence the two EC numbers. Plant-derived enzymes belong to the 3-Phytase (EC 3.1.3.8) group, while most microbial ones belong to the 6-phytase group (EC 3.1.3.26). The number indicates the position of the ester bond on the substrate. Fungal phytases are active in the acid-neutral region, while bacterial ones are more active in the neutral-alkaline range. They hydrolyze phytate and other phosphoesters in a two-step mechanism (Ping-Pong) involving a covalent phosphorylated histidine adduct enzyme intermediate (Ostanin et al. 1992).

3.7 Proteases

Proteases (also called peptidases) have been typically used in the manufacturing of bioethanol as a way of degrading protein present in the raw material providing free amino nitrogen for yeast growth, thus replacing the need to add an exogenous source such as urea during fermentation (Lei et al. 2013; Vidal et al. 2009) but can also be used to breakdown starch-gluten complexes through weakening the endosperm-associated protein matrix encapsulating the starch granule (Wang et al. 2009), thereby providing further accessibility of the starch to the α-amylases and glucoamylases (Alvarez et al. 2010). This increased the fermentation rate and ethanol yield in a dry-grind ethanol production process (Johnston and McAloon 2014). This in turn increases the specific gravity of the mash and improves germ recovery. Proteases are of two kinds: exoproteases and endoproteases. The exoproteases remove one amino acid from the protein chain at a time and can be further subclassified into aminopeptidases and carboxypeptidases. Aminopeptidases cleave the amino acids from their amino (N-) terminus while the carboxypeptidases cleave from the carboxy (C-) terminus. The carboxypeptidases are further subdivided based on their active site mechanism, e.g., metallo-carbopeptidases, serine carboxypeptidases, and cysteine carboxypeptidases. Exoproteases are generally not commercially available but are present in enzymatic cocktails or culture supernatants. Endoproteases act randomly along the polypeptide chain and are subdivided into

four classes differing in their catalytic mechanism: serine proteases (e.g., chymotrypsin, trypsin, subtilisins), cysteine proteases (e.g., papain, ficin, bromelain), aspartic proteases (e.g., pepsin) and metalloproteases (e.g., thermolysin, neutral proteases). Acidic and metalloproteases activate a water molecule which then performs a nucleophilic attack on the peptide bond resulting in hydrolysis, while serine, threonine, and cysteine proteases uses a nucleophilic residue and a catalytic triad to form intermediate complexes between the enzyme and the substrate, releasing one part of the product, then activated water performs the second catalytic step to release the second half of the product, regenerating the free enzyme. The addition of and endoprotease from *A. niger* (Genencor International/DuPont GC 100) and an exoprotease from *A. oryzae* (Novozyme 50045) was shown to result in a higher ethanol concentration (mean 0.3−1.8 % v/v) and lower DDGS yield compared to a no protease control (Wang et al. 2009). The addition of an acid protease with or without urea during the fermentation step was calculated to decrease overall process costs by $0.01/L (Johnston and McAloon 2014).

Most of these enzymes are present in commercially available blends or as Trade names from the major enzyme producers. Novozymes attributes 18 % of its US turnover to selling enzymes for 1G starch-to-ethanol. A recent product, Avantec® is designed to make more corn starch available for hydrolysis and thus fermentation through reducing viscosity and so allowing refining plants to run at higher solid loading or higher run rates. The Liquozyme® ranges are enzymatic preparations with low pH tolerance and high thermostability, which reduces the starch down to an optimal dextrin profile. Viscozyme® was designed specifically for cereal crop utilization to degrade the β-glucan and arabinoxylan and other cereal-specific components, which can lead to high viscosity in the process. High viscosity limits the dry feedstock you can add to the process, increasing water consumption, and affecting downstream processing, such as the efficiency of separation, evaporation, and heat exchange, and thus lowering ethanol yield. This allows a smoother flow of the liquefaction process.

Fouling of the bioreactors costs a 1G biorefinery plant time and money. Fouling reduces the efficiency of the heat exchangers and normally will involve the use of additional sulphuric acid and/or hydroblasting to remove the material accumulated in the reactors. Enzymatic treatments have been designed to be added during the fermentation stage to avoid metal chelation and fouling due to the unfermented material present after liquefaction and saccharification.

4 Overview of Processes for the First Generation Bioethanol

Currently, industrial production of first generation bioethanol is made from agricultural products rich in starch or sucrose which are readily fermentable. These sugars serve as an energy reserve for plants and as such are stored in specific tissues

for each plant. Two general processes could be highlighted: one using sugar crops and another based on starchy plants.

4.1 Sugar Beet and Sugarcane Processing

Sugarcane, with its widespread use in Brazil, is the sugar producing plant most used for the production of bioethanol. In 2010, sugarcane ethanol represented a third of the ethanol production worldwide (Linoj et al. 2006). But it grows only in tropical areas. Sugar beet is much better suited to Europe and temperate climate. There is also a third plant used to a lesser extent, sweet sorghum. It comes from Africa, but is adaptable to temperate climate and has the advantage of having reduced water needs to grow. The sugar-containing plants have the drawback of being rich in water and therefore cannot be stored over time for later use. Indeed, their sugar content decreases quickly after uprooting or cutting.

Sugarcane contains 12–17 % total sugars on a wet-weight basis with 68–72 % moisture (90 % sucrose and 10 % glucose or fructose). The average extraction efficiency to produce cane juice by crushing is approximately 95 % and the remaining solid residue is cane fiber (bagasse). In factories that only produce ethanol, the cane juice is heated up to 110 °C to reduce microbial contamination, decanted, sometimes concentrated by evaporation and then fermented. In combined sugar–ethanol plants (annexed distilleries), sucrose crystals that are formed after cane juice concentration are removed by centrifugation, leaving a syrup (molasses) that contains up to 65 % w/w sugars. Both sugarcane juice and molasses (after adjusting the sugar concentration) normally contain sufficient minerals and organic nutrients to be immediately suitable for ethanol production by fermentation with Saccharomyces cerevisiae (Wheals et al. 1999). Sugar beets can be used directly in dedicated plants for ethanol production or processed for sugar production. In such a case, clear juices, syrups, and molasses obtained from the clarification steps, evaporation, and crystallization can be used for ethanol production. Table 2 shows the average compositions of these juices.

The maximum theoretical yield of glucose to ethanol conversion is 0.511 g of ethanol per gram of glucose corresponding to the so-called Gay–Lussac equation giving 2 mol of ethanol by mole of glucose. For sucrose, this yield is 0.538 g/g, one mole of sucrose giving 4 mol of ethanol. The actual yield in industrial units is around of 90 % of these maxima. Clear juices have the required sugar concentration to obtain fermented juice with ethanol concentration of 10–15 % (v/v). A high alcohol content is necessary to limit energy consumption and to optimize ethanol recovery during the distillation step (Bai et al. 2008). The upper limit depends on the sugar concentration before fermentation and the greater or lesser adaptation of the strains to high levels of ethanol. In France, the average production of sugar beet is around 70 T/ha, with a sugar content of 17 % (on wet basis) representing an average production of 12 T of sugar/ha (Data from SNSF). Figure 2 illustrates the process of ethanol production from sugar beet described hereafter.

Table 2 Composition of different juices produced during sugar beet processing (adapted from Reiss 2012)

	Diffusion juice	Clear juice	Syrup	Molasses
Dry matter (% m/m)	14.70	14.50	58.80	80.80
Sucrose (% m/m)	12.85	13.13	53.00	49.20
Total nitrogen (% m/m)	0.13	0.13	0.14	1.82
Reducing compounds (% m/m)	0.07	0.01	0.47	0.86
Ashes (% m/m)	0.28	0.34	1.85	9.86
pH	6.3	9.25	7.27	6.98

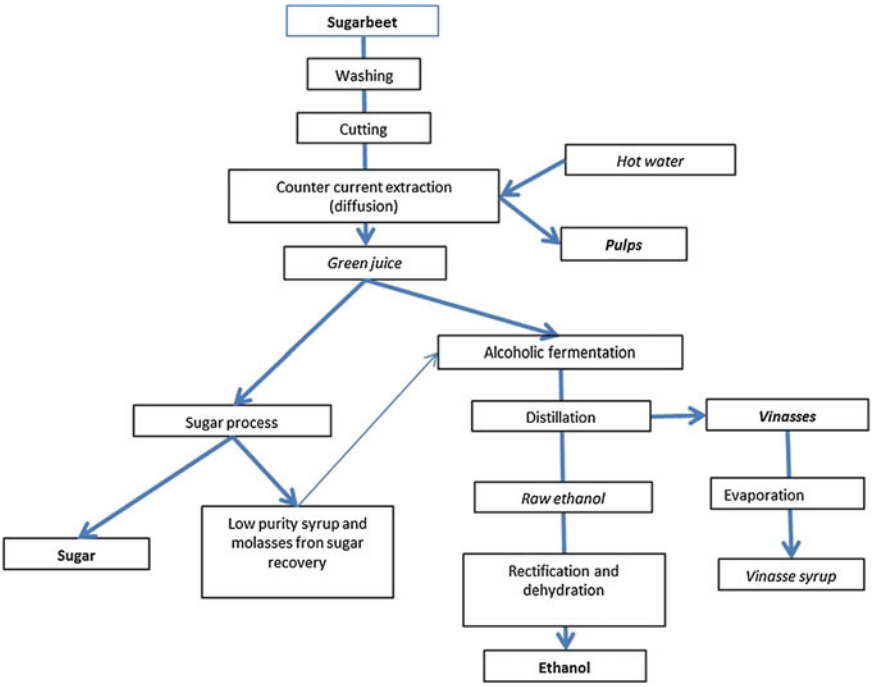

Fig. 2 Diagram of ethanol production from sugar beets. Alcohol should be produced directly from green juice produced by counter-current extraction or from syrups and molasses coming from crystal sugar production (adapted from Reiss 2012)

Ethanol production from sugar beet begins, as for sugar production, with the washing of roots and cutting them into chips (thin strips of 5–6 cm long) in a root cutter. The shape of chips is optimized for good extraction of sugar in water. The chips are then transported to the diffusion step, a counter-current extraction by hot water. The enriched water, the "diffusion juice" is recovered at diffuser head and chips "exhausted" out tail diffuser in the form of pulps. After extraction, pulps contain about 92 % water. Much of this water is separated from the pulp by

pressing or dewatering and recycled. Dehydrated pulps are used in animal feed. The extraction juice called also green juice can be used directly for the fermentation by *Saccharomyces cerevisiae* during the sugar campaign (September to January in Europe). Fermented juice containing 10–14 % v/v of ethanol is called beer or wine. The next step is the distillation of beer to recover ethanol.

4.1.1 Purification

The purification of diffusion juice aims to remove some of the insoluble impurities they contain. A lime treatment (liming) results in the precipitation of impurities. Under the action of the lime, mineral acids and number of organic materials, such as pectins and proteins are converted into insoluble salts. This is followed by a double carbonation (adding CO_2) which serves to precipitate the lime remaining in the juice. These insoluble salts and calcium carbonate form a precipitate entraining the removal of some soluble impurities. Liming leads the virtual elimination of iron. Iron can catalyze oxidation reactions leading to the color formation and conducing to the formation of grayish-white sugars (Borges et al. 2012), which is undesirable for sugar production. The liquid fraction, called clear juice is then recovered. Carbonated lime and precipitated impurities are filtered off.

4.1.2 Evaporation

The purified juice still contains 85 % water. Evaporation allows the thin juice concentrate to obtain a syrup with a concentration close to saturation. Evaporation takes place in a multiple "effects" evaporator (generally four successive evaporators). Every evaporator uses the vapor produced by the precedent and the pressure in each evaporator is reduced to compensate for the reduction in temperature of vapors and to decrease correlatively the boiling point. Moreover, the low temperature avoids the cooking of sugar at a stage dedicated to evaporate the juice.

4.1.3 Crystallization

Crystallization involves the separation of sucrose (as crystals) from impurities that remain in the concentrated juice. The crystallization is carried out in 2 or 3 stages called "jets." Each jet is made of a proper crystallization stage, mixing, and centrifugation. The concentrated juice is heated and stirred in large boilers operating under partial vacuum. Its concentration continues and very fine sugar crystals are introduced therein to initiate crystallization (coarsening of crystals). The mixture syrup-crystals obtained then passes into a stirring tank to cool while continuing the crystallization. Finally, it is centrifuged in turbines or centrifuges to recover crystals. Drains still contain sugar as well as impurities that were not removed during the purification. Drains from the third jet are molasses, still rich in sugar but hardly

extractable due to the high glucose and fructose content. Molasses are used in fermentation industries and in animal feed. Sugar plants with an annex distillery usually operate only 2 jets and route the drains of the second jet directly at the distillery. Pulps and vinasses (bottom residues of distillation) produced during the process can be valued in animal feeding or by anaerobic digestion. They are mainly recycled at different stages of the process (Reiss 2012).

4.2 Sweet Sorghum Processing

Sweet sorghum is a C4 crop in the grass family belonging to the genus *Sorghum bicolor* L. Moench which also includes grain and fiber sorghum and is characterized by a high photosynthetic efficiency. The average productivity is around 49.7 t/ha of fresh stem producing roughly 63 % of juice (Gnansounou et al. 2005). The total dissolvable content of juice is around 17.9 % (°Brix) with 69.5 % of sucrose (Woods 2001). In addition to sugar, the juice contains other compounds and impurities, which have to be eliminated before crystallization to obtain white sugar. Furthermore, sweet sorghum sugars consist of 85 % sucrose, 9 % glucose, and 6 % fructose—on average—and only sucrose may readily be converted to white sugar (Woods 2000). Juice purification is operated by liming (addition of lime milk and precipitation by carbon dioxide). The lime milk precipitates and captures the impurities in the raw juice. The settled solids (mainly calcium carbonate and nonsugars) from the clarifier are filtered and sent to the spent lime storage area, while the filtrate is again saturated in a second carbonation station. The purified juice obtained after the subsequent filtration is called thin juice and is thickened in a multi-effect evaporator into thick juice. High-pressure steam produced in the boiler provides the energy for evaporation, and the condensed steam is returned to the boiler or used as technical water. The thin juice that has been diluted with water during extraction and purification enters the evaporating station with an average sugar content of 15 % while the thick juice leaving the evaporator contains approximately 70 % sugar.

4.3 Corn and Starchy Grains

Today, most fuel ethanol is produced from corn by either the dry-grind (67 %) or the wet mill (33 %) process. The two processes differ with respect to complexity, associated capital costs, the numbers and types of coproducts produced, and the flexibility to produce different kinds of primary products. The principal differences between the ethanol dry-grind process and the wet mill process are the feedstock preparation steps and the numbers and types of coproducts recovered. Once the starch has been recovered the process of converting it to fuel ethanol and recovering the ethanol is similar in both wet mill and dry-grind facilities (Bothast and Schlicher

2004). Production of ethanol from starch needs its depolymerisation to obtain a glucose syrup suitable for fermentation. The hydrolysis of starch may be considered as a first and key step in corn and other starchy plants processing for bioethanol production. The main role of this step is to effectively provide the conversion of two major starch polymers: amylose, a mostly linear α-D-(1–4)-glucan and branched amylopectin, α-D-(1–4)-glucan, which has α-D-(1–6) linkages at the branch points, into fermentable sugars that could subsequently be converted to ethanol by yeasts or bacteria. Recent advances in the developing of thermostable α-amylases, the starch liquefying enzymes which catalyze the hydrolysis of internal α-D-(1–4)-glucosidic linkages in starch in a random manner and effective glucoamylases, the starch saccharifying enzymes which catalyze the hydrolysis of α-D-(1–4) and α-D-(1–6)-glucosidic bonds of starch from the nonreducing ends giving glucose as the final product, have led to commercial establishment of the so called 'two enzyme cold process' (Baras et al. 2002). The traditional thinning agent used in starch technology was acid (hydrochloric or oxalic acids, pH 2 and 140–150 °C for 5 min). The introduction of thermostable α-amylases has meant milder processing conditions (Aiyer 2005). The formation of by-products is reduced and the main advantages of this process are lower energy consumption and a lower content of non-glycosidic impurities and thus much better suitability for ethanol production.

4.3.1 Wet Milling Process

In the wet milling process, illustrated in Fig. 3, grains are dipped into an aqueous solution containing sulphuric acid, which facilitates the separation of the different components including starch, fiber, gluten, and germ. A grinding is then realized to separate these components. The germ is removed from the kernel and corn oil is extracted from the germ. The remaining germ meal is added to fibers and the hull to form corn gluten feed. Gluten is also separated to become corn gluten meal, a high-protein animal feed. A starch solution is separated from the solids and the starch so obtained is then liquefied and saccharified by enzymatic way to yield corn syrup that can be processed for ethanol production (Bothast and Schlicher 2004). However, secondary reactions occur due to the acid used during the stage of soaking (Sanchez and Cardona 2008). Furthermore, during this process several compounds of the plant are extracted. The composition of juices is thus very variable.

4.3.2 Dry Milling Process

In the case of "dry milling," illustrated Fig. 4, grains are cleaned and crushed in ball milling apparatus or hammer mill. The flour obtained is hydrolysed in two stages by enzymatic way. The first stage, realized by means of one α-amylase is called liquefaction. The second stage is called saccharification and uses a glucoamylase (GA). This ends in the formation of a syrup of glucose. This syrup will then be used

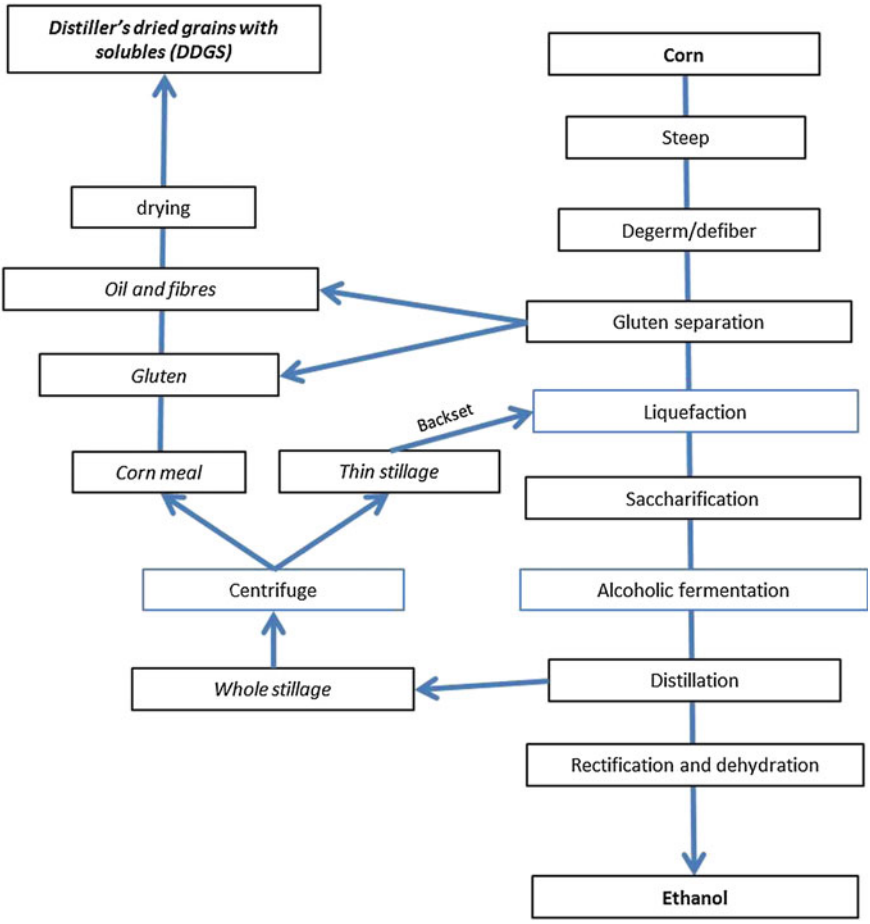

Fig. 3 Schematic representation of the wet mill process for starchy materials (adapted from Reiss 2012 and Bothast and Schlicher 2004). After steeping, crushing of hydrated grains gives fiber and oil from germs, proteins (gluten) and starch. Associated to corn meal recovered after distillation, these products are known as Distiller's dry grain with solubles (DDGS)

to produce ethanol by fermentation. Spent grains and cheap wines will be valued in animal feed. The stages of saccharification and of fermentation can be associated to decrease the duration of the process. This allows the glucose release at the desired rate in the medium by the regulation of the amylolytic activity.

4.3.3 Liquefaction, Saccharification, and Fermentation

The ground corn is first sent to a slurry tank along with process water, thermostable alpha-amylase, ammonia, and lime. After the slurry is prepared, the mixture

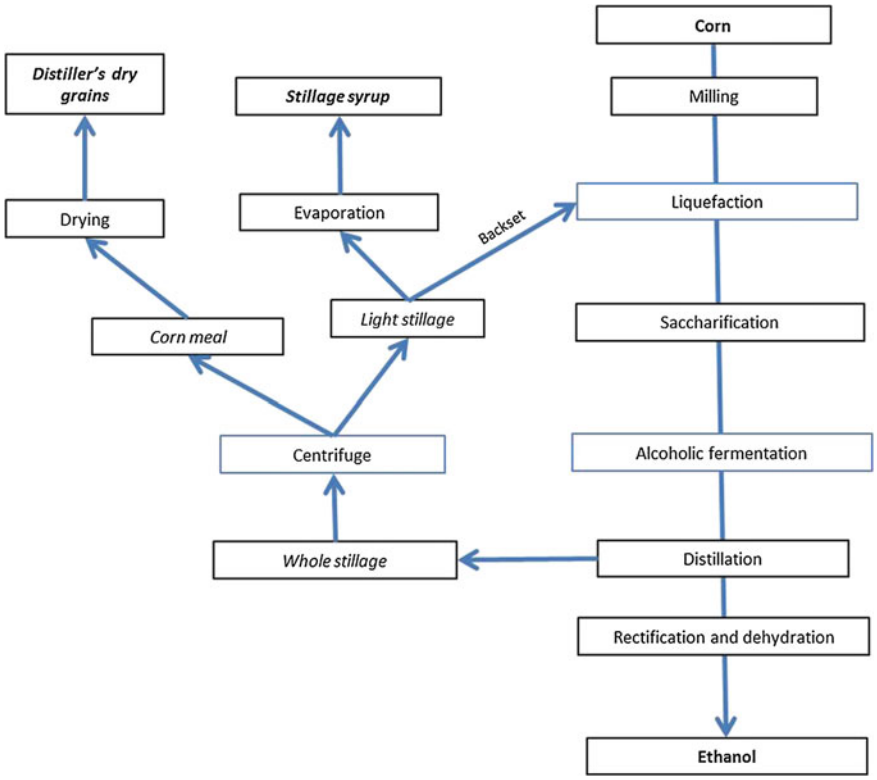

Fig. 4 Schematic representation of the dry grind process (adapted from Reiss 2012 and Bothast and Schlicher 2004). All grains components follow the entire process yielding only one coproduct: Distiller's dried grains (DDG)

undergoes liquefaction, where starch is gelatinized using a "jet-cooker" (steam injection heater) and hydrolysed with thermostable alpha-amylase into oligosaccharides also known as dextrins. During the gelatinization step, there is a sharp rise in the slurry viscosity that is rapidly decreased as the alpha-amylase hydrolyses the starch. Liquefaction is done at pH 6.5 and is initially held for 60 min at 88 °C with agitation. The output from the initial liquefaction step is combined with "backset," a recycled stream taken from the liquid portion of the "stillage" separated by centrifugation after the distillation step. The backset provides critical nutrients for the yeast later in fermentation.

These combined streams are "cooked" (i.e., held at 110 °C for 15 min), cooled, and then transferred to the saccharification tank. The resulting solution contains mainly dextrins, shorts oligosaccharides. Addition of glucoamylase converts dextrins into glucose. During this incubation at a temperature of 60 °C, almost all of the dextrins are converted to glucose. Glucoamylase continues to be active and can further hydrolyse during fermentation if there are any remaining dextrins.

Following the saccharification reaction, the slurry is transferred to the fermentation vessel and cooled at the yeast's optimal temperature (around 30 °C) prior to yeast addition. Often, ammonium sulfate or urea is added as a nitrogen source for the growth of yeast. Proteases can also be added. They break down the corn proteins to free amino acids, which serve as an additional source of nitrogen for the yeast. The fermentation requires 48–72 h to reach a final ethanol concentration of 10–12 %. The pH of the beer declines during the fermentation below pH 4, because of the carbon dioxide formed during the ethanol fermentation. This decrease in pH is important both for increasing the activity of glucoamylase and inhibiting the growth of contaminating bacteria. Either batch, fed-batch or continuous fermentation systems may be used, although batch processing is more common (Bothast and Schlicher 2004).

4.4 Cassava

After harvesting, the roots are chopped into chips for drying. Chips are usually sun dried. Dry chips are packed in bags and can be stored for months. Their starch content is more than 65 % (Sriroth et al. 2012). However, during storage, the starch yields decreases somewhat, depending on storage temperature: typically, 5 % reduction of starch yield is observed after 8 months of storage (Abera and Rakshit 2004). Another advantage of chips is the easy transportation. A big advantage of cassava over many other traditional crops is that it can be grown and harvested throughout the year. This results in a constant supply of cassava to the ethanol production facility in contrast to more seasonally crops. As for other starchy materials, the process described in Fig. 5 is carried out with two distinguishable technologies: wet milling process and dry-grinding process. Currently, most new facilities use the dry grinding process. The wet milling process starts with soaking the cassava chips in an acid to soften the material which results in the separation of starch from other components. The fibers are recovered in several separation steps. Next, the starch and protein are separated. In this process the streams are fractionated and several coproducts can be recovered. Most streams are recovered before the fermentation step. The dry grinding process starts with grinding the chips. This is done by hammer mills or roller mills. Next the ground material is mixed with water, cooked and mixed with enzymes. Cassava starch has a lower gelatinization temperature and offers a higher solubility for amylases in comparison to corn starch (Sanchez and Cardona 2008). This process produces only one coproduct that is separated at the end of the whole process, after fermentation, distillation, and drying: distiller dried grains with solubles. This is mostly used as animal feed. The use as animal feed is, however, limited due to the high fiber content.

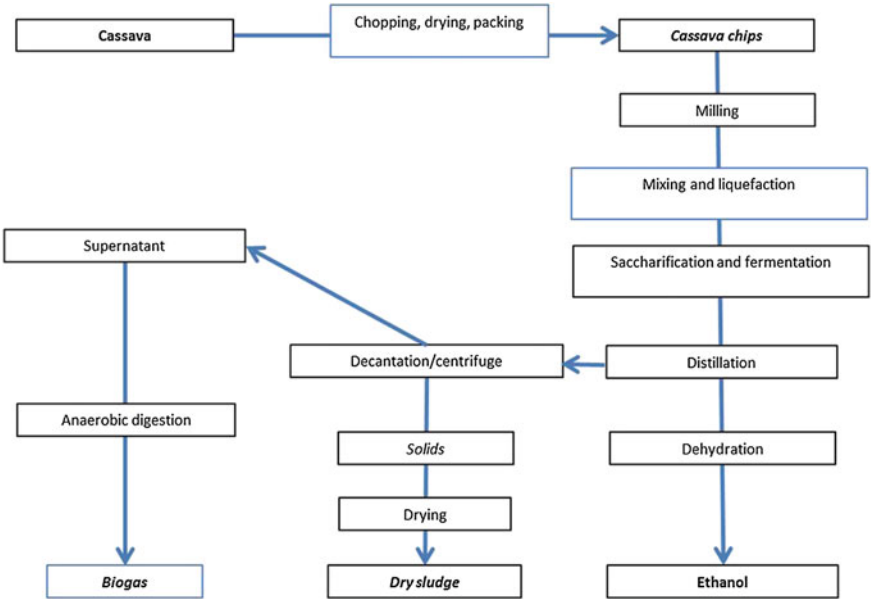

Fig. 5 Cassava processing for ethanol production. In a first step, starch-containing roots are chopped and dried. The resulting chips are further processed in ethanol facilities either by wet or dry milling

4.4.1 Fermentation

Ethanol fermentation can be realized classically in batch bioreactors of high volumes, typically around 500 m^3. These types of cultures have no constant yields throughout the process because of the change of medium composition during the fermentation. The cultures in batch impose time-outs during the filling and during the draining of tanks, their cleaning, and sterilization. The semi-continuous techniques called fed-batch are often used. These processes are said semi-continuous and allow limiting the sugar concentration in the medium as it is added in a progressive manner, limiting inhibition by the substrate or by the fermentation products which can be removed in the same way. Some precursors could also be added when it is necessary allowing a very precise regulation of the strain metabolism (Echegaray et al. 2000). The continuous systems are opened systems in which the cellular population is constantly maintained in a stable environment and a state of balanced growth, by removing continuously a part of the culture and by replacing it by fresh medium. Classically, they can be operated in chemostat or turbidostat mode.

To increase the productivity and decrease the cost of the production of ethanol, many researches are performed on high-density fermentation (Das Neves et al. 2006). These fermentations are said at high density because the fermentation medium contains more than 250 g/L of sugar, which in theory allows to obtain

more than 15 % (v/v) of ethanol instead of 10–12 % generally obtained in most of the distilleries. The high-density fermentations possess numerous advantages. Indeed, these fermentations allow increasing production capacities without any modification of the structure of production (Bvochora et al. 2000; Puligundla et al. 2011). However, high-density cultures are very sensitive to temperature, concentration and cellular viability, medium composition, oxygen concentration. Furthermore, strains selected for strong concentrations in ethanol must be used (D'Amore 1992).

Nowadays, the production process of bioethanol from starch feedstock is developed to significantly reduce processing time and energy consumption by conducting saccharification and fermentation in a same step. This process is called "Simultaneous Saccharification Fermentation," or SSF process (Sriroth et al. 2012). In this SSF process, the liquefied slurry is cooled down to 32 °C, afterward glucoamylase and yeast are added together. While glucoamylase produces glucose, yeast can use glucose to produce ethanol immediately. No glucose is accumulated throughout the fermentation period (Rojanaridpiched et al. 2003).

4.4.2 Distillation and Ethanol Recovery

The fermentations presented above allow the obtaining of wines containing between 10 and 12 % (v/v) of ethanol in the case of sugar plants and until 18 % for the starchy plants, it is thus necessary to separate the ethanol of the water contained in beers. The mixture obtained from fermentation is not a water-ethanol binary system, even if it represents the main part, but a complex mixture containing volatile secondary products of the fermentation as aldehydes, esters, methanol, or higher alcohols possessing more than two carbons. The presence of these secondary products is regulated, for fuel alcohol, by the US or European standards.

The first step in ethanol recovery is the beer column, which recovers nearly all of the ethanol produced during fermentation in the distillate. An almost equal amount of water is also distilled that must be separated from the ethanol in the next stage of rectification/stripping. To obtain anhydrous ethanol, two stages are necessary after the distillation. The first part of this process is intended to extract the head products (aldehydes, ethyl acetate). The second part, is intended to concentrate the alcohol and to eliminate the tails (superior alcohols). Finally, the last part eliminates the methanol contained in the alcohol. The second stage for the obtaining of the pure ethanol consists in eliminating the residual water. Indeed, by distillation, one can obtain only a composition near the azeotrope composition, which is around 96 % (v/v) of ethanol for 4 % (v/v) of water. The alcohol so produced can serve then directly as biofuel. But if this one must be mixed with gasoline it is necessary to add a stage of dehydration to obtain anhydrous alcohol. The solution used in industry is the use of molecular sieve allowing the separation of ethanol from water according to the existing size difference between these two molecules.

5 Microorganisms for the First Generation Bioethanol

Technological development can help to diminish the environmental impact and the prices of the ethanol fuels. Numerous research has been conducted in order to obtain better fermentation conditions including organisms, low cost substrates, and process optimization to achieve optimal environmental conditions (Siqueira et al. 2008). Many microbial species are able to metabolize sugars and convert it into ethanol. However, only a few have proven sufficient efficiencies to be deployed at industrial scale. The yeast *Saccharomyces cerevisiae* and the bacteria *Zymomonas mobilis,* are the two main microorganisms traditionally found in first generation facilities. Although *Zymomonas mobilis* gave better yields, lower biomass production, and does not require any addition of oxygen contrary to *Saccharomyces cerevisiae*, it is also more sensitive to the environmental contaminations, it only uses a limited range of substrates and the produced biomass is not reusable for feeding purposes. Consequently, *Saccharomyces cerevisiae i*s most of the time the microorganism of choice for the large-scale bioethanol production.

5.1 Metabolic Pathways Towards Ethanol Production

Three metabolic behaviors can be considered in yeasts. They are depending on the way the carbon source is used to produce the energy necessary for the cellular machinery. This type of metabolic behavior is strongly dependent on the environmental conditions such as what are the sugars available as a substrate, what is the local oxygen level available for the yeast and on the yeast itself. Purely oxidative yeasts will never produce ethanol, yeasts sensitive to the oxygen concentration will produce ethanol only in the case of an oxygen limitation and yeasts sensitive to the glucose content will also produce ethanol in the case of an excess of glucose even if oxygen is present. This last effect is known as the Crabtree effect. *Saccharomyces cerevisiae* is the typical example of a Crabtree positive yeast having an oxidative metabolism in the presence of oxygen at very low glucose concentrations (0.1–0.5 $g{\cdot}L^{-1}$ depending on the strain) and a mixed metabolism when the glucose concentration increases above that threshold. Soluble glucose penetrates into the yeast cell and is converted by a series of enzymatic reactions into pyruvate according to two major pathways. Pyruvate is further converted to carbon dioxide, energy, and eventually into ethanol. Some of the released energy is used by the yeast cells to support their growth and maintenance reactions during the fermentation. The rest of the energy is converted into heat and must be taken out of the fermenter or it will cause a temperature increase. Ethanol and carbon dioxide are taken out of the yeast cells.

5.2 Conversion of Glucose to Pyruvate

The conversion of glucose (or fructose) into pyruvate inside the cytosol of the yeast is known as the Embden-Meyerhof-Parnas pathway (EMP). This path is common for both aerobic and anaerobic conditions and includes 11 individual enzymatic steps. For instance, Cheng (2009) proposed a detailed description of the EMP pathway. Besides the EMP pathway, a second carbohydrate breakdown pathway is widely used among bacteria. It was discovered by Entner and Doudourof in *Pseudomonas saccharophilia.* Glucose-6-Phosphate is first dehydrogenated into 6-phosphogluconate by the glucose-6-phosphate dehydrogenase and this is further converted by the 6-gluconate dehydratase and the 2-keto-3-deoxy-6-phosphate-gluconate aldolase into one molecule of pyruvate and one molecule of 3-phosphoglyceraldehyde. The 3-phosphoglyceraldehyde can be further oxidized to pyruvate by the enzymes of the EMP pathway. *Zymomonas mobilis* degrades sugars into pyruvate with this pathway.

5.3 Fermentative Pathway

In the absence of oxygen, since there is no other electron acceptor *Saccharomyces cerevisiae* has a fermentative metabolism. Under these conditions, the energy is only produced via the EMP pathway. Pyruvate is then catalyzed by a pyruvate decarboxylase into acetaldehyde (with the release of one carbon dioxide) and finally acetaldehyde is handled by the alcohol dehydrogenase and reduced into ethanol. This further allows the recovery of the reduced cofactors NADH, H^+ into its oxidized form NAD^+. In addition to ethanol, other by-products are formed. The most important is glycerol. The purpose of the glycerol production pathway is to balance the redox balance in response to the biomass production associated with the fermentation reaction. The theoretical ethanol yield is 0.51 g ethanol per gram of glucose consumed while the biomass yield is approximately 0.10–0.12 g biomass per gram of glucose consumed. However, maintenance reactions, synthesis of the cellular infrastructure and the formation of secondary compounds (glycerol, acetic acid, reserve substances) limit this efficiency to approximately 90 % of its theoretical value.

5.4 Oxidative Pathway

The oxidative metabolism of glucose result in the complete degradation of the molecule into water and carbon dioxide with the simultaneous production of energy through the successive involvement of the EMP pathway, the conversion of pyruvate into acetyl-CoA through the pyruvate dehydrogenase reaction, the

production of reduced cofactors ($NADH,H^+$ and $FADH_2$) into the tricarboxylic acid cycle (TCA cycle), the conversion of these reduced cofactors into a proton gradient and finally into adenosine triphosphate in the oxidative phosphorylation pathway. In this metabolic configuration, the biomass yields for *Saccharomyces cerevisiae* are approximately of 0.45–0.50 g biomass per gram of glucose without any production of ethanol.

5.5 Crabtree Effect

In order to explain the origin of the Crabtree effect, two main hypotheses have been considered. However, the exact origin of this saturation mechanism is not clearly established yet. The respiratory capacity might be limited due to the repression of some specific enzymes responsible for the transportation of the reducing power from the cytosol into the mitochondria by glucose. Thus, the decrease in the cell capacity to reoxidize NADH into NAD^+ will favor the ethanol production. As the conversion of pyruvate into ethanol produces little energy in comparison to the oxidative phosphorylation, the glycolytic flow will increase to meet the cellular needs. The metabolic saturation might be located at the pyruvate node. A too high glycolytic flux might progressively induce an over-accumulation of pyruvate and cause its redirection toward the production of ethanol and the others by-products. The Crabtree effect is not energetically interesting for the yeast as it has for first consequence a much lower biomass to glucose yield. However, the secondary consequence is a drastic increase of the growth rate from 0.08–0.15 h^{-1} to 0.4–0.45 h^{-1}. This energetic waste tendency turns out to be advantageous in the case of a competition for the substrate between microorganisms.

5.6 High Gravity Fermentation

The minimal ethanol concentration step that is necessary to achieve an economically viable process has been estimated at approximately 60 $g{\cdot}L^{-1}$ at the end of the fermentation step. This threshold is due to the high energetic costs for the evaporation and rectification unit operations (Fig. 2) of the ethanol process. In the current industrial processes, yields were estimated to be around 90–92 % of the maximal Gay–Lussac theoretical yield (being 0.511 g ethanol produced per g of glucose consumed). The productivity is approximatively of 2 kg ethanol produced per cubic meter of fermentation medium per hour. At the laboratory scale, the optimization of the fermentation conditions such as medium composition, vitamins and nitrogen feeding strategies, aeration, thermal settings, and strain selection makes it possible to enhance these performances significantly. For instance, it has been possible to obtained final concentrations with ethanol content higher than 180 $g.L^{-1}$ (Thomas and Ingledew 1992) or productivity records equivalent to the production of 3.5 kg

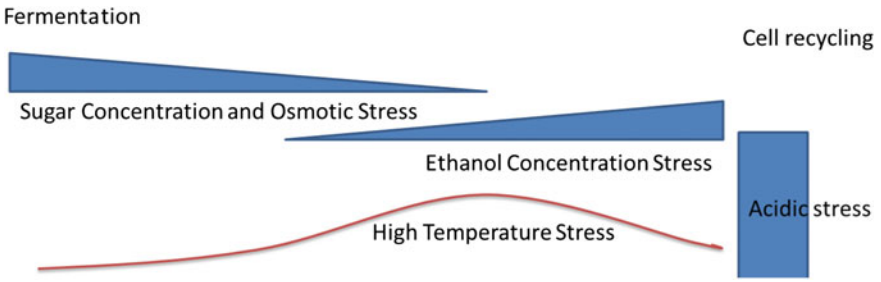

Fig. 6 Various environmental stresses that are traditionally occurring during the alcoholic fermentation (adapted from Della-Bianca et al. 2013; Koppram and Olsson 2014)

ethanol per cubic meter of fermentation medium per hour in a 45 h long fed-batch process (Alfenore et al. 2004). These high substrate concentration processes (>300 $g{\cdot}L^{-1}$) are not only essential for the first generation bioethanol but also for the successful implementation of a cost competitive second generation. Several technological advantages have to be taken into consideration such as a drastic decrease in the water and energy needs (Mussatto and Roberto 2004). However, the implementation of these conditions causes high loading of raw materials (up to 20 % mass concentration) and implies mixing conditions and increased amount of fermentation inhibitors. In fact, the environmental conditions found in the industrial production processes of first and second generation are far from the optimal physiological condition for yeast. It is therefore necessary that high gravity and very high gravity fermentations uses robust industrial strains to deal with these unfavorable environments (Koppram and Olsson 2014).

Figure 6 represents the various stresses that a yeast cell can encounter during single batch fermentation.

According to Della-Bianca et al. (2013), high-sugar concentration at the beginning of the fermentation and high ethanol concentration during the last stage, pH variations, high temperature, and the presence of toxic compounds are the most significant stresses. Furthermore, in the case, of a SSF configuration, the yeast strains have to be active near to the optimal activity of the amylase (pH 7) or cellulases (pH 6, 40–50 °C). It is also important to take into consideration the risk of contamination by bacteria during the fermentation process. In fact, in order to achieve low costs ethanol yields the fermentation is carried out nonaseptically in most of the facilities. It can deviate carbon away from ethanol production due to the bacterial cells metabolites and have detrimental effects on yeast performances (Basso et al. 2014). In this context, yeasts cells are usually recycled between two fermentations runs with the addition of sulfuric acid in order to reduce the bacterial contamination (Della-Bianca et al. 2013). Because they are commonly facing simultaneously or sequentially, a wide variety of stresses conditions, it is generally admitted that industrial strains are better and faster to adapt at these stresses (Pizarro et al. 2008). It is also usually believed that under selective industrial conditions, fermentation requires the use of industrial wild strains (Albers and Larsson 2009).

For instance, ethanol plants in Brazil are traditionally using selected baker's yeast as a starter. However, these yeasts were unable to survive the many recycling processes and were progressively replaced by indigenous yeasts strains during the season (Basso et al. 2014). This has adverse effects on the antifoam consumption, on the ethanol yield and consequently on the global performance of the whole process.

5.7 Strain Selection and Improvement

Given the large volumes of ethanol being produced at the facility scale, an improvement of the fermentation yields of only 1 % will have huge consequences on the overall profitability and environmental impact of the installation. Consequently, research efforts are now focusing on the improvement of the fermentation steps, including more resistant strains to the various stresses encountered during the alcoholic fermentation. Further efforts are also made toward the diversification of the usable carbon sources and on the improvement of the yields. One last focus is the consolidation of processes making it possible to carry out two or many unit operations in a single vessel in order to reduce investments and operating costs. Several strategies such are genome shuffling (Wang et al. 2014; Snoek et al. 2015), transcription machinery engineering, random mutagenesis have been developed with the aim to modify specific metabolic pathways and substrate transport systems in laboratory strains (Pereira 2014; Steensels et al. 2014). Results obtained by Pagliardini (2010) clearly demonstrate that the metabolic engineering of microorganism for the production of interesting molecules including ethanol is very difficult to implement as soon as it changes the central carbon metabolism. In fact, the major interconnections between the different metabolic pathways through energetics and redox couplings make it almost impossible to change one or the other pathway without affecting the system as a whole. In addition, certain metabolic pathways are leading to the production of molecules possessing fundamentals physiological roles. Changing these pathways can consequently affect the behavior of the mutant strains. The author was able to produce mutants that exhibited a reduction of up to 80 % in the glycerol production in comparison with the wild strain. The ethanol to glucose consumptions ratios increased from two to five percent depending on the mutant strain. For some of the mutants a decrease in the production of other organic acids and biomass was also observed. However, growth rates as well as ethanol tolerance and the ability to handle osmotic stresses were drastically reduced in the mutant strains in particular in anaerobic conditions making these new strains hardly usable for industrial purposes.

A second strategy aims at gaining and integrating physiological knowledge for strain improvements. The microflora of traditional and industrial fermentation

processes is the potential source for discovering natural microbial strains with the desired physiological properties in order to resist better to the environmental stresses they are facing. Wild yeasts could be found for example in industrial alcoholic fermentation processes for beverage or bioethanol production plants. Once the right host strain is selected, metabolic engineering approaches might be used in a second step (Pereira 2014).

5.8 Resistance to Ethanol Stresses

Cot (2006) studied the physiological adaptation of yeasts strains to very high ethanoic content. According to the author, the key toward high ethanol content lies in the yeast's ability to maintain a high metabolic activity as long as possible. The selected yeast should demonstrate the best ability to withstand stressful conditions induced by ethanol accumulation. Ethanol is known to induce disturbances in the membranes (Walker-Caprioglio et al. 1990). However, in the case of very high gravity fermentations, this can be the cause not only of modifications of the activities of the membrane transporters but also of an irreversible partial or total loss of the membrane integrity. Lipid analysis clearly shows a correlation between the phospholipids content and the cellular viability (Cot 2006). The adaptation of yeast must depend on its ability to maintain its phospholipids content (Alexandre et al. 1994). Monitoring the gene expression reveals the establishment of a general stress response that has already started during the growth phase and reached its paroxysms at approximately 100 grams ethanol per litter when ethanol started to be produced without any production of biomass. Transcription and translation-related genes are downregulated and some genes necessary to enter the stationary phase are on the contrary being induced (SSA3, HSP12…). Carbohydrates and lipids reserves are accumulating, cells are less budding and close to a quiescent physiological state (Cot 2006). According to Herman (2002), quiescent cells are more resistant to ethanoic stresses. However, the mechanisms leading to this quiescent state remain hypothetical. As quiescent states are often found after an essential nutrient deficiency (Gray et al. 2004) and as ethanol is reported to inhibit many active transportation systems (Walker-Caprioglio et al. 1990), a possible mechanism would be that high ethanoic concentrations are responsible for active transportation systems damages and therefore causing indirectly nutritional starvation. In the same temporal windows, the overexpression of genes coding for active transportation systems was also reported. Under high ethanoic pressure, the cell population divides into two or more subpopulations that have different properties: quiescent and nonquiescent cells. Quiescent cells are usual daughter cells without any budding scar; they are accumulating glycogen and have different transcriptomic profiles that those of the nonquiescent cells (Cot 2006).

5.9 Resistance to Temperature Stresses

The temperature is known to affect the membrane fluidity (Kim et al. 2006). It may even cause an increase in permeability and ion leakage (Pipper 1995). It has been reported that the membrane composition might change in order to preserve its fluidity (Suutari et al. 1990). The conformation of proteins is also affected and may cause denaturation and aggregations. A large number of heat-shock genes might be induced in order to express chaperones proteins activities and prevent the denaturation of proteins (Morano et al. 1998). Postmus (2011) demonstrated that glycolytic flux is increased in C- and N-limited chemostats at higher temperatures and that the energetic cost for maintaining a proper protein folding is higher at 38 C than at 30 C. He also observed that transient increases of glycolytic flux are occurring immediately after an increase of the cultivation temperature in order to restore the balance between growth and maintenance and to produce ATP at a level that matches with the new energetic needs. The author reported that it might even cause a metabolic switch from respiratory to fermentative metabolism at high temperature. According to the author, the morphology of the mitochondria was severely perturbed. The author hypothesizes that in the 30–37 °C range, the energetic needs for the maintenance increases and at higher temperature, the energetic yield of the respiratory metabolism was not sufficient to support the investments in the mitochondria and causes the shift toward fermentative metabolism.

6 Environmental Assessment of First Generation Bioethanol

Environmental studies on large-scale production of first generation biofuels have shown that the net energy output is generally favorable to bioethanol. Similarly, greenhouse gas emissions are reduced in comparison to fossil fuels. This general assessment must, however, be strongly nuanced when the good agricultural practices are not respected. The gains expected by the technological process improvements are significant. However, they do not constitute the most promising source for further environmental savings (Benoist 2009). In the case of ethanol from wheat, the potential benefit achievable with technical savings is estimated at +0.24 MJ produced per MJ of fossil fuel consumed (−38 %) and at −11.6 g CO_2 equivalent for greenhouse gases emissions (−21 %). A more efficient valuation of the agricultural coproducts is the key to improve the life cycle analyses balance sheets whatever the bioethanol production pathway being considered. When these coproducts are recovered energetically as heat and electricity cogeneration, this allows an important reduction of greenhouse gas emissions potential. However, this gain is hampered by the significant decrease in the apparent productivity of the cultures. It is therefore appropriate to consider the apparent productivity of crops that can include the effects on land use of the by-products generated by these crops,

especially their use in the animal feed sector. Consequently, the environmental evaluations must be considered not only in terms of MJ produced at the ethanol plant but rather in terms of kilometers traveled per MJ of fuel consumed and ideally per hectare of crops mobilized.

First generation bioethanol has been pointed out since 2008 because of its possible competition with food use and because of its impact on the local biodiversity (Koh and Ghazoul 2008). It this context, the use of alternative crops that are able to use on marginal lands (such as salted soils) that are not usable for the cultivation of conventional edible crops) is a topic of interest toward better environmental balances of first generation biofuels. More recently, concerns are also raising about the efficiency of the biofuels policies with respect to the climate change mitigation. The major assumption is that biofuels replace the consumption of fossil fuels. However, recent evidences suggested that because an increased use of biofuels will lower oil prices and consequently result in an increase of the crude oil consumption. This so-called rebound effect of biofuels can significantly lower the effectiveness of the biofuel policies in reducing the greenhouse gases emissions (Smeets et al. 2014). Financial mechanisms need to be set on the international trade system in order to offset this rebound effect. This is a major point in the international negotiations on climate change (De Perthuis and Trotignon 2015). Would it be possible to set a unique price for the carbon emissions or are we heading towards a global economy with variable ecological balances depending on the differentiated national development needs (Gollier and Tirole 2015)? Nevertheless, a sustainable and economically viable development of the green energy including bioethanol will probably never be possible without any satisfactory international agreement.

7 Toward Integrated First-, Second-, and Third Generation Biorefineries

Due to the high investment, costs, and uncertainties about the second generation bioethanol process (see Sigoillot and Faulds, Chapter "Second Generation Bioethanol" of this book), one solution is to consider an integration of first and second generation plants (Lennartsson et al. 2014) and even integrated third generation (Maranduba et al. 2015). This strategy is schematically represented in Fig. 7. In order to be functional, it requires the adaptation of the fermentation step to new substrates. Again, yeasts are at the center of this feedstock diversification. In the case of the second generation processes, the crucial point is the ability to convert xylose into ethanol without being negatively affected by inhibitory compounds such as furfural or acetic acids that are produced during the various pretreatments of the biomass. In the case of the third generation many new substrates such as Mannose, Rhamnose, Uronic acid, Glucuronic acid, or even N-acetylglucosamine may be used as a carbon sources. *Pichia stipites, Pichia angoraphorae, Candida shehatae, or Zimomonas palmae* have already proven their potential for fermenting part of these

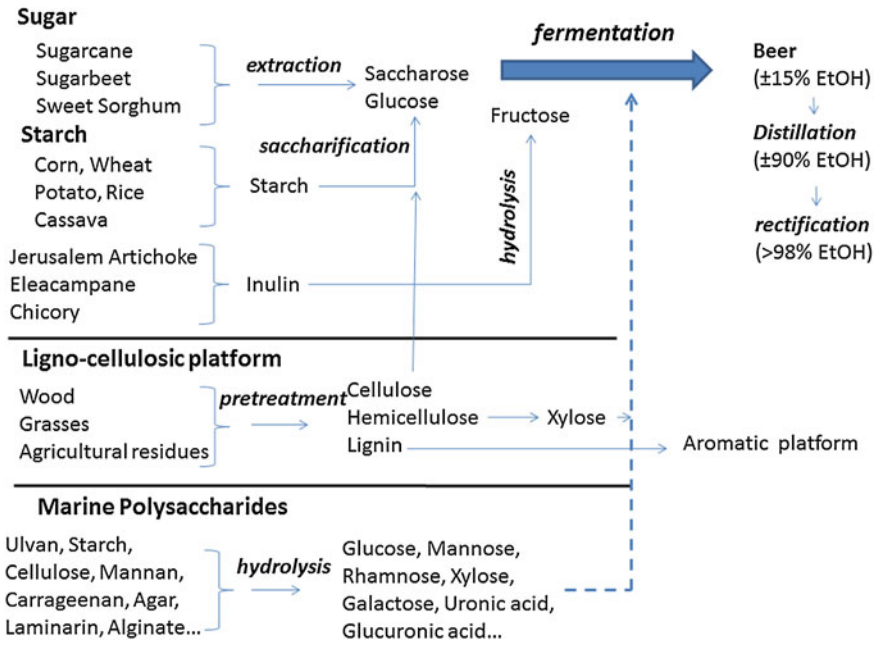

Fig. 7 Substrate diversification strategies for the integration of first, second, and third generation feedstocks into a single integrated biorefinery concept (adapted from Souza et al. 2015)

new substrates and to be more tolerant to inhibitory compounds (Gonçalves et al. 2015; Trivedi et al. 2015).

First generation grain mills currently dominate the world market through the production of Distiller's Dried Grains and Solubles (DDGS), predominantly a high-protein product, which is sold as low cost animal feed. The 1G refineries are usually located close to the production fields and so would evoke low transportation costs for the incorporation of straw, corncobs or bran as 2G raw materials. The combination of the high yielding 1G ethanol process would require less conversion of the 2G feedstock in order to make the biorefinery profitable. Sugarcane provides an attractive raw material feedstock for such a process, with a combined plant being projected to potentially outperform a 1G plant if all the plant is used, e.g., leaves and pentoses (Macrelli et al. 2014). Recent life cycle analysis assessment on the integrated first, second, and even third generation bioethanol facilities prove its potential in terms of reduction of the environmental weaknesses of the first generation facilities (Souza et al. 2015; Maity et al. 2014; Maity 2015a, b). Fungi have probably received the least attention in the biofuel arena. However, there are many studies, which show that fungi may make it easier to process biofuels from plant feedstock. Food grade fungi, such as *Rhizopus* sp., *Aspergillus oryzae, Fusarium vebebatum, Neurospora intermedia,* or *Monoacus purpureus*, already used in the manufacturing and processing of human food, can be used to utilize the remaining

pentose sugars and so improve the overall economy of the first generation process (Lennartsson et al. 2014). In addition, those fungi are less prone to inhibition by the inhibitors generated by the process, which can interfere with the yeast metabolic processes. The fungus is easily removed from the liquid broth and can then be dried as a feed source, thus contributing to the improved economics of an integrated processing plant.

8 Conclusion and Perspectives

First generation bioethanol is produced at large industrial scale and has proven its economic viability. The United States and Brazil are the two leaders for production with the predominant use of corn and sugarcane respectively. Despites of the many concerns on the long-term sustainability of the first generation bioethanol, such as the impacts on land use change, the water use, the potential contamination of soils with the distillation residues, and the competition for food and feed production, many potentials routes are arising in order to make this production greener. In this context, integrated biorefineries are a promising way (i) to diversify the feedstocks usable, leading to reduced facilities size and optimized supply chains, (ii) to valorize more efficiently bagasse's from sugarcane and corn stover or even (iii) to exploit the potential of microalgae to capture the carbon dioxide that is produced during the fermentation steps and use it for example after transformation as the biodiesel that is needed for the agricultural machineries. This is the opportunity to take advantage of this large-scale successful deployment to get experience for the development of the most promising processing schemes for the next generation facilities that are still facing uncertainties with respect to their economic viability.

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