Second Generation Bioethanol Production: The State of Art

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Abstract Second generation bioethanol from waste lignocellulosic biomass is a sustainable solution to the problems of diminishing petroleum reserves, issues over national security and environmental deterioration due to GHG emissions. The production of second generation bioethanol is a complex process and consist several steps including biomass pretreatment, saccharification of cellulosics followed by microbial fermentation and product recovery. In this chapter, an attempt has been made to review the process steps of bioethanol production from plant biomass and their respective scope of improvement. Afterwards, the global and national status of bioethanol production and various policies governing its commercialization have also been dealt with. The chapter also summarizes the energy balance, mass balance, life cycle analysis studies and techno-economic evaluation of lignocellulosic bioethanol production carried out by various researchers. Moreover, the technological barriers and alternatives investigated to overcome the challenges in second generation bioethanol production process are also discussed.

Keywords Second generation bioethanol · Lignocellulosics · GHG emission Pretreatment · Fermentation

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1 Introduction

Current scenario of declining fossil fuel reserves and soaring petroleum prices, concerns over the national energy security and in particular dependence on oil-import have led researchers all over the world to search for an alternative transportation fuel. Additionally, the global climate change and environmental impacts of fossil fuels have also heightened the awareness of replacing fossil fuels from our present way of life (Charles et al. 2007). According to reports from Environmental Protection Agency (EPA), since 1970, the CO_2 emission has almost doubled and GHG (Green House Gas) emissions have increased by 78% due to rapid industrialization and accelerated consumption of fossil fuels (US EPA 2016). As a consequence, governments and industries globally are putting various measures to provide suitable solutions to offset these problems; however, still petroleum is the chief source which is being used to meet the world's fuel demands.

For transportation sector, unconventional energy carriers like H_2 and electricity have been successfully developed but their large-scale application is marred by their lower energy density and storage-related issues (Agrawal et al. 2007). Therefore, it seems more convenient to use liquid transportation fuel through existing infrastructure. Biofuels in general and bioethanol, in particular, are the most promising clean fuel, which can be easily integrated in the prevailing transportation system. Although ethanol's energy content is roughly 2/3rd of gasoline, it has higher research octane number (107) than gasoline (91–99) (Lynd 1996). Moreover, researchers have shown that ethanol can be used up to 85% (v/v) in vehicles without major modifications (Balat et al. 2008) with associated benefits of being bio-renewable in nature, generation of less harmful emission and therefore, being environmentally sustainable and reduced dependence upon petroleum resources. Burning of petroleum-based fuels generate more harmful discharges when compared to that of ethanol (Wyman and Hinman 1990) and therefore, application of even E10 blend (10% ethanol in gasoline) results in up to 20% decreased GHGs. Further increase in ethanol blending has more prominent effect on reduced emissions of NO_x, SO_x, and particulate matter. Due to the associated benefits of using ethanol as an alternative or supplementary transportation fuel, there has been a global upsurge of interests in research and development of bio-based fuels from renewable biomass-based resources in a sustainable manner.

Currently, almost all of the commercially available bioethanol in United States, Brazil, and the European Union is produced from either starch- or sugar-rich crops, which is referred as 'first generation (1G) bioethanol'. For 1G bioethanol sugars derived from cane, molasses or corn starch are used as primary starting material. Production of bioethanol from such resources is expected to increase further in the coming few years (Goldemberg 2007). However, due to food nature of such resources, competition of the bioethanol fuel with the food is also expected to increase together with the expected deforestation to achieve higher production and further negative environmental impacts (Hahn-Hägerdal et al. 2006; Tenenbaum 2008). Therefore to combat the problems associated with the use of first generation bioethanol, interest

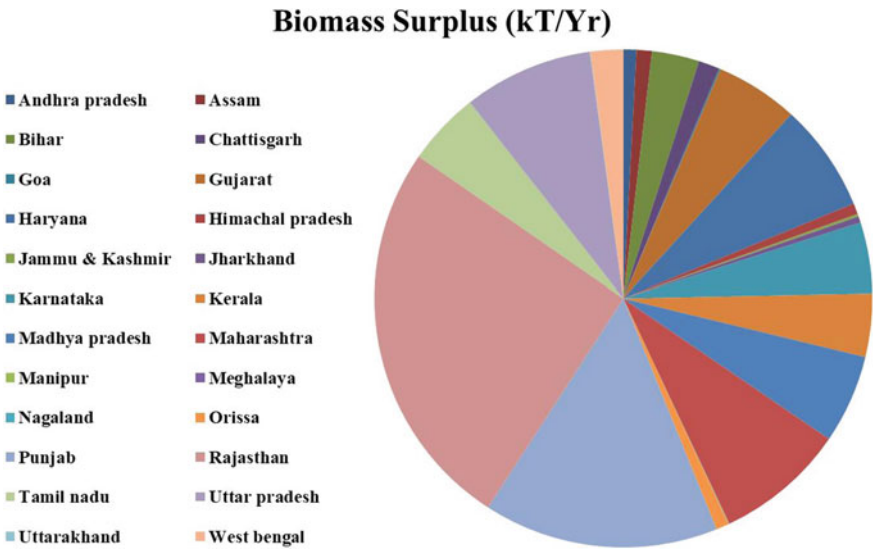

Fig. 1 Availability of lignocellulosic biomass in different states of India

was shifted to generate 'second generation or 2G bioethanol' from lignocellulosic non-food crops (e.g. *Prosopis, Miscanthus*) or waste plant biomass, such as crop wastes, rice and wheat straw, cotton stalk, etc.) or other waste resources like municipal solid wastes (MSWs) (Claassen et al. 1999).

Lignocellulosic biomass is the most promising feedstock considering its great availability, low cost and non-competence with the food demands. The availability of lignocellulosic biomass in Indian context is shown in Fig. 1. The conversion of lignocellulosic biomass to bioethanol is a multi-step process. The structural carbohydrate polymers in lignocellulose, i.e. cellulose and hemicellulose are first depolymerized through pretreatment and saccharification and the obtained monomeric sugars are subsequently fermented to ethanol. Lignocellulose conversion to bioethanol can be carried out in various manners, such as by employing biochemical/microbial/enzymatic route or by adopting thermochemical/chemical route, however, following are some common considerations that need to be taken care of (Kang et al. 2014):

- Complete or near complete conversion of holocellulose components to respective monomeric sugars
- Improved co-fermentation in presence of pretreatment derived toxins
- Integration of unit operations for minimal waste generation and maximum energy utilization
- Lignin valorization to increase the cost-competitiveness of bioethanol production process

Despite various reports on sustainable production of cellulosic bioethanol via enzymatic route, a common argument against biofuels production is their high production costs. In this regard, many countries are providing governmental subsidies and tax exemptions to biofuels in order to achieve economic competitiveness against oil-derived transportation fuels. Moreover, the whole process could be made more cost-effective by generating high-value products from side streams in an integrated biorefinery manner, especially finding better alternatives of utilizing lignin for value-addition in comparison to its conventional application in heat generation (Balat et al. 2008).

The chapter aims to provide a concise overview of the basic concepts and newer developments as well as challenges and prospects of the state of the art related to the production of second generation biofuels. Various process steps in principle are discussed briefly and emphasis has been given on the advancement in each process step and to the challenges faced by the industries to make it commercially viable. Moreover, improvement of lignocellulose to bioethanol conversion process through genetic engineering approaches and development of biomass-based biorefinery has also been discussed.

2 Global Status of Second Generation Bioethanol Production

Advanced biofuels production over the world has been on rise since past few decades reaching more than 2×10^8 gallons annual production capacity and further developments and enhancement of production capacity in major biofuel producing nations is expected to nearly double current annual capabilities. Topmost nations on the list of global bioethanol producers are the United States, Brazil and China. The status of bioethanol production across the world is shown in Fig. 2. It is only recently that many advanced biofuel production plants, both demonstration as well as commercial scale, have been set up worldwide (US EPA 2016).

Different countries are using various substrates for bioethanol production depending on their regional availability, local climate and economic drivers. For example, in the US and Brazil, sugars derived from 1G resources such as maize and cane, respectively, are being used for ethanol production, whereas, China is using corn, wheat and sugarcane for production of bioethanol (Cardona and Sanchez 2007). The main drivers for biofuel development in India are secured energy supply by replacement or reduced usage of petroleum-based fuels. Indian biofuel policy targets to achieve 20% (volume) biofuels blending in fossil fuels by committing to establish various bioethanol and other advanced biofuel generation facilities in the whole country over a period of time and replacing the current sugar based substrates with lignocellulosic feedstock. A list of various first and second generation substrates currently used or proposed for bioethanol production is given in Table 1.

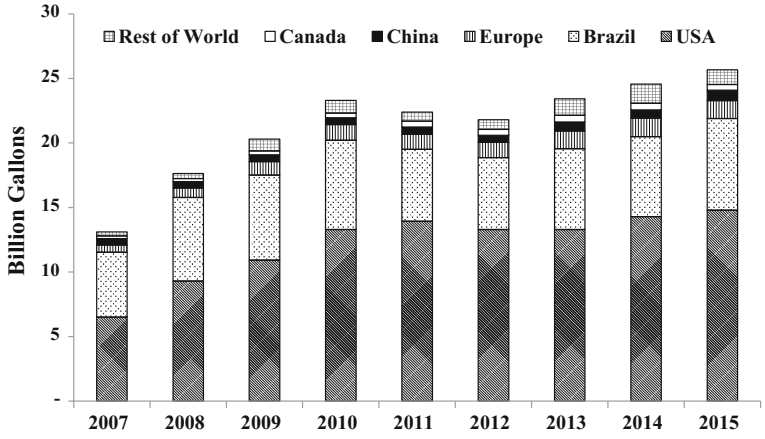

Fig. 2 Global trend for ethanol production (*Source* RFA 2015 www.ethanolrfa.org)

Table 1 List of various feedstock used for ethanol production in major biofuel producing countries

S. No.	Countries	Feedstock	
		First generation	Second generation
1.	US	Corn, Sugar beet	N.A.
2.	China	Corn, Wheat, Sugarcane	N.A.
3.	Germany	N.A.	Rye
4.	Brazil	Corn, Sugarcane	N.A.
5.	France	Sugar beet	N.A.
6.	Argentina	Soybean	N.A.
7.	Nigeria	Palm	Sorghum
8.	India	Wheat, Sugarcane	Sorghum
9.	Poland	N.A.	Rye
10.	Russia	Sugar beet	Rye
11.	Malaysia	N.A.	Palm waste
12.	Indonesia	Sugarcane molasses	N.A.
13.	Sudan	N.A.	Sorghum
14.	Columbia	Sugarcane	N.A.

Adapted and modified from (Araújo et al. 2017)
N.A. Not available

Since 2000, the global biofuels supply has increased by a factor of 8% and equalled 4% of the world's transport fuels in 2015. Global biofuels supply has improved enormously over past few years mainly due to adoption of biofuel policies by various countries with their own targets and mandates. The top two world-leading bioethanol

producing countries have alone produced more than 1/3rd of the global bioethanol in 2015. USA has committed to increase its biofuel production capabilities to a level of approximately nine times of the current scenario and the European Union target to increase biofuel/bioenergy share by more than 10% by the year 2020 (Yacobbi 2012; (http://ec.europa.eu/energy/renewables/biofuels/biofuels_en.htm), accessed on 20th Dec 2017).

The advanced biofuels commercialization is more expensive than original expected biofuels. The absence of any biofuel policy worldwide is the major concern regarding the decline in the cost of per barrel oil prices from June 2014 to 2015. Necessary time and funding are required to prevent the decline in the biofuel market. Globally, $3.1 billion were invested in biofuels in 2015, which is 35% decline relative to 2014. Later, billions of dollars were spent on various projects of advanced biofuels worldwide, but many of such projects have been closed after sometime mainly due to commercialization issues (www.worldenergy.org).

3 Second Generation Bioethanol Process

Second generation bioethanol can be considered more environmentally friendly. Lignocellulosic biomass can either be by-products of agro-based industries and comprises sugarcane bagasse, rice straw, rice husks, wheat straw, cotton stalks, corn cob, coconut shells and municipal solid waste (MSW), forestry waste counting bark and wood chips. Lignocellulose is mainly made up of cellulose (polymer of α-D-glucose), hemicellulose (heteropolymer of C5 and C6 sugars) and lignin (heteropolymer of phenylpropanoid units). Numerous lignocellulosic biomasses can be successfully utilized for producing bioethanol. Some of them with their compositions are listed in Table 2. The process of lignocellulosics to ethanol broadly comprises of four sequential steps; Deconstruction of biomass (pretreatment), saccharification, conversion of sugar to ethanol (fermentation) and purification of the product (Fig. 3).

3.1 Pretreatment: Deconstruction of Lignocellulosic Biomass

Production of bioethanol from second generation biomass requires efficient depolymerization of structural carbohydrate polymers to be fermented to ethanol. However, lignocellulosic biomass has evolved complex structural and chemical mechanisms, which provide recalcitrance to its structural sugars from the microbial and enzymatic attack. Therefore, a deconstruction of biomass is required to change the biomass size and structure as well as chemical composition so that hydrolysis of the carbohydrate portion to monomeric sugars can be attained rapidly with higher yields. The main aim of pretreatment is as follows:

Table 2 Composition of various substrates used for bioethanol production

Substrate	% Composition (dry wt.)			Substrate	% Composition (dry wt.)		
	Hexosans	Pentosans	Lignin		Hexosans	Pentosans	Lignin
Bamboo	49–50	18–20	23	Oat straw	41	16	11
Banana waste	13.2	14.8	14	Olive tree waste	25.2	15.8	19.1
Barley hull	34	36	19.3	Paper	85–99	0–5	0–15
Barley pulp	69.9	18.3	10.9	Pepper stalks	35.7	26.2	18.3
Bean stalks	31.1	26.0	16.7	Pine	41	10	27
Bermuda grass	25	35.7	6.4	Poplar	40	14	20
Birch wood	40	33	21	Reed	49.40	31.50	8.74
Chilli stalks	37.5	28.3	17.3	Rice husk	36	15	19
Coffee pulp	33.7–36.9	44.2–47.5	15.6–19.1	Rice straw	32	24	13
Corn cobs	42	39	14	Rye straw	31	25	7
Corn stover	38	26	19	Salix	41.5	22–25	25
Cottonseed hair	80–95	5–20	0–5	Sawdust	55	14	21
Cotton stalks	41.7	27.3	18.7	Softwood stem	45–50	25–35	25–35
Douglas fir	35–48	20–22	15–21	Sorghum straw	33	18	15
Eucalyptus	45–51	11–18	29	Soybean stalks	34	25	20
Flax sheaves	35	24	22	Spruce	45	26	28
Grapevine stems	43.1	19.4	26.6	Sugarcane bagasse	33	30	29
Grasses	25–40	35–50	10–30	Sweet sorghum	23	14	11
Groundnut shells	38	36	16	Switch grass	37	29	19
Hemp	53.86	10.60	8.76	Waste paper	60–70	10–20	5–10
Jute fibres	45–53	18–21	21–26	Water hyacinth	18.4	49.2	–
Miscanthus	43	24	19	Wheat straw	30	24	18
Municipal solids	8–15	NA	24–29	Willow	55.9	14	19

Sources Monsalve et al. 2006; Karp and Shield 2008; Kim et al. 2008; Alves et al. 2010; Singh et al. 2011; Garcia 2014; Ayeni et al. 2015; Raud et al. 2016; Bilal et al. 2017; Espinosa et al. 2017

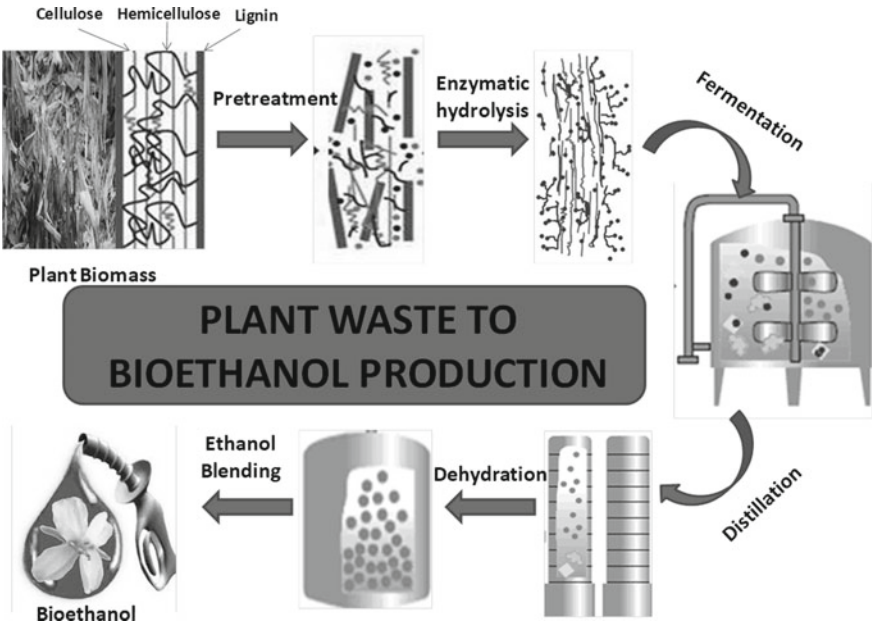

Fig. 3 Schematic illustration of second generation bioethanol production process

(1) To improve sugar yields during enzymatic hydrolysis by reduction of crystallinity of cellulose and enhanced porosity of the biomass;
(2) To minimize the emergence of fermentation inhibitors during deconstruction;
(3) To retrieve lignin from hydrolysate for converting it into valuable by-products and
(4) To make the process economic by making the operation easier (Aditiya et al. 2016).

Broadly pretreatment strategies are categorized into physical, physico-chemical, chemical and biological. With every different feedstock used for bioethanol production, the selection of pretreatment method varies due to distinct chemical composition and physical structure of feedstock. Factors like cellulose crystallinity, lignin content, cell wall porosity, hemicellulose side chain branching and crosslinking are critical in choosing the pretreatment method. Most chemical pretreatment modifies cellulose ultrastructure through certain physico-chemical modification, though it is possible to fractionate cellulose, hemicellulose and lignin by using pretreatment with some catalysts.

Pretreatment using acids or bases promote subsequent enzymatic hydrolysis by exposing cellulose and removing hemicellulose consequently enhancing the yield of glucose. The frequently used acid and base are H_2SO_4 and NaOH, respectively. Another additive, cellulose solvents have been used to liquefy cellulose in various cellulosic substrates which ultimately results in 90% conversion of cellulose to

glucose and substantiated raised enzymatic hydrolysis due to the deconstruction of biomass before the action of enzyme. Organosolvants like Lewis acids, $FeCl_3$ and $(Al)_2SO_4$, and alkaline-peroxide (H_2O_2) are known solvents to disintegrate lignocellulosic structure and facilitates hydrolysis (Coughlan 1992). Concentrated acids such as sulphuric acids (H_2SO_4) and hydrochloric acid (HCl), alkali solvents like NH_3 and hydrazine, aprotic solvents (DMSO), and some complexes of metal and wet oxidation enhance the porosity of biomass by interrupting the association of lignin with cellulose and also dissolving hemicellulose. Although the abovementioned methods are effective, the cost of these chemicals is high when compared with the value of the glucose and hence make their use impractical (Sun and Cheng 2002).

Besides, high temperature/pressure-based pretreatments and biological pretreatment with selected lignin degrading white rot fungi have been used successfully. Contrary to chemical based methods, input of energy in biological pretreatment is lesser as the reaction conditions are milder. White rot fungi can effectively degrade lignin by secreting hydrolases with lignin peroxidases which in the presence of H_2O_2 cleaves the backbone of lignin. A list of common pretreatment strategies used and their advantages and disadvantages are shown in Table 3.

This is interesting to note that while performing chemical-based pretreatments, generation of various fermentation inhibitors (furfural, hydroxymethyl furfural, phenolics, acetic acid, etc.) takes place. Therefore, prior to fermentation, removal of these inhibitors seems necessary. Several detoxification strategies such as liming, activated charcoal adsorption, ion-exchange resin treatment and enzymatic detoxification have been used to remove these fermentation inhibitors. An alternative and more sustainable way to tackle the problem of inhibitors is to use inhibitor resistant or tolerant enzymes and microbial strains.

3.2 Enzymatic Hydrolysis: Depolymerization of Structural Polymers

The hydrolysis of pretreated biomass is the most crucial step in the bioethanol production process. Although hydrolysis of biomass can be accomplished by using acid or enzymes, saccharification using enzymes is preferred due to milder processing conditions and environment-friendly nature. Nevertheless, depolymerization of biomass via enzymatic hydrolysis is a multi-enzymatic process with high complexities.

In nature, lignocellulosic biomass can be depolymerized by a number of hydrolytic enzymes that are produced by diverse fungi and bacteria. Cellulases are the representative class of enzymes involved in depolymerizing lignocellulosic substrate by synergistic action of all the three enzymes present in the complex. Cellulase complex consists of exoglucanases (cellobiohydrolases, CBH), endoglucanases (EG) and β-glucosidases (cellobiase, BG) (Behera and Ray 2016). EG acts upon cellulose chains and hence creates two types of reactive ends for CBHs. CBH I acts on reducing ends and CBH II on non-reducing ends of cellulose fragments thereby, catalysing step-

Table 3 Various pretreatment strategies with their specifications (Aditiya et al. 2016)

Pretreatment	Action	Advantages	Disadvantages
Dilute acid	Hydrolyses hemicelluloses, Concentrates cellulose enzymatic treatment, Alters lignin structure	Hemicellulosic removal	Low removal of lignin, low enzymatic hydrolysis (30–40%), Inhibitor generation
Dilute alkali	Eliminates lignin and hemicelluloses, Surface area exposed for enzyme access	High digestibility, high lignin removal	Hemicellulosic sugar loss, Low enzymatic hydrolysis (50–60%), Inhibitor generation
Ammonia fibre expansion (AFEX)	Surface area for access to enzyme upsurge after treatment Take out hemicellulose and lignin	Small amount of inhibitors formation	Not proficient for biomass with high level of lignin, High price of ammonia
Ionic-liquids	Increases proportion of amorphous cellulose, Lignin is separated	High dissolution, Environmentally safer	Scale-up is still a challenging
Alkaline peroxide	Removes lignin and solubilize most of the hemicellulose	Cellulose isolation	Loss of hemicellulosic sugars, Loss of lignin
Acid-chlorite	Reduces lignin content	Isolation of hemicellulose and cellulose	Loss of lignin, Costly method of pretreatment
Ammonia	Opens up cell wall and exposes celluloses and hemicelluloses	Lignin removal (partial)	Hemicellulosic sugar loss, Low enzymatic hydrolysis (50–60%), Inhibitor generation
Steam-explosion	Porosity of biomass increases, Hemi-cellulose solubilization	Deconstruction of structural polymer, Recovery of lignin, Lower loss of hemicellulose, Less amount of inhibitors generated, Higher yield of hemicellulose and, economic process	Generation of inhibitors, Partial degradation hemicellulosic components, Disrupted lignin-carbohydrate matrix is lacking
Biological	Ligninolytic and hemicellulolytic action	Partial deconstruction of lignocellulosics, Low energy consumption	Longer fermentation time
Lignin downregulation	Development of transgenic plants with downregulated lignin	Lower lignin content plants	Susceptible to disease

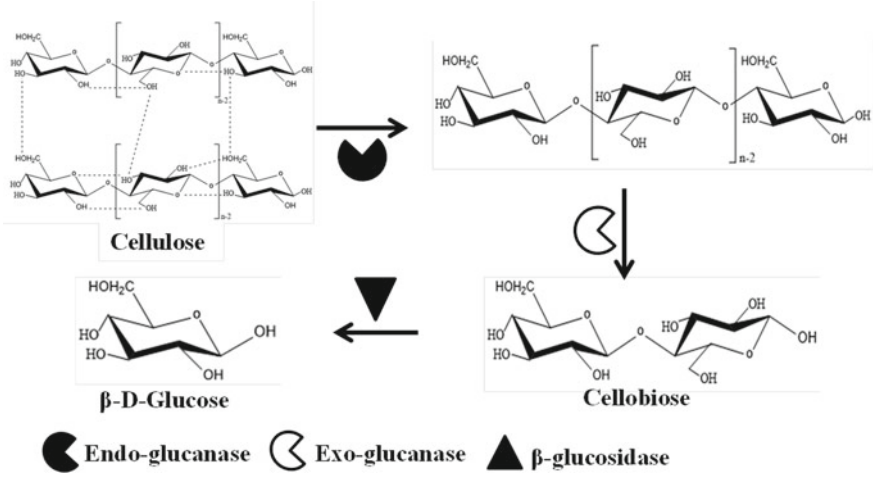

Fig. 4 Schematic diagram showing mechanism of enzymatic hydrolysis

wise degradation of cellulose to cellobiose. BG utilizes cellobiose and converts it into glucose (Kuhad et al. 2011b). CBH gets inhibited by cellobiose, therefore; BG plays a key role in reducing end-product inhibition and depolymerizing the cellulose completely. Modular structure with concluding catalytic and carbohydrate binding molecules (CBM) is the common feature of most of the cellulases. The carbohydrate binding molecules facilitate hydrolysis of biomass by fetching the catalytic domain in contiguity to the insoluble cellulose. Thus, the rate of enzymatic hydrolysis of the biomass is subjective to the substrate properties and catalytic performance both. The scheme of mechanistic action of cellulases over cellulose is shown in Fig. 4.

Besides cellulases, several other auxiliary enzymes such as xylanases, mannases, feruloyl esterases, etc. also assist the enzymatic depolymerization of lignocelluloses. Recently, novel enzymes (non-hydrolytic) named lytic polysaccharide monooxygenases (LPMOs) have been reported to be capable in dropping cellulase dosages and finally the overall cost of the process (Vaaje-Kolstad et al. 2010; Horn et al. 2012). Although the mechanism is not clear yet, these LPMOs are believed to oxidize the highly recalcitrant crystalline regions of cellulose and create more reducing/non-reducing ends for cellulase components to attack (Horn et al. 2012). This may be due to the fact that LPMOs require an electron donor, e.g. oxygen, for their effective action (Hu et al. 2015).

Although saccharification using enzymes has more scope for improvements than those using chemicals, the high cost of cellulases is still a technical barrier (Hong et al. 2013; Culbertson et al. 2013). Fall in the cost of cellulase could be obtained by (a) intensive efforts which enquire more than a few aspects of enzymes with improved hydrolytic properties such as binding affinity, thermostability, etc. (b) by improvement of technologies for which are proficient for hydrolysis including of superior cocktails of enzyme and conditions for hydrolysis. In addition to enzyme character-

istics, substrate features such as the degree of polymerization, cellulose crystallinity and the existence of lignin and hemicellulose also affect the enzymatic hydrolysis. Therefore, to improve the overall process, upgrading in cellulase performance and enhancing the substrate-enzyme interaction are prerequisite.

Industrially, among all probable strategies, the optimization of the characteristics of cellulases like thermostability and end-product inhibition is crucial for large-scale application. Also, optimizing production medium by altering its components is an approach to enhance the enzymatic hydrolysis. Development of multi-enzyme cocktail secreted by various strains of fungi is also a good choice for improving the performance of cellulase as a complete system. Several studies have reported that synergistic action of cellulase is linked with the ratio of every enzyme in the system (Berlin et al. 2007; Hemansi et al. 2018).

3.3 Fermentation

As compared to simpler fermentation process of sugars derived from food-based feedstock, crop-waste based feedstock to ethanol conversion process is very tedious and involves many critical steps. Pentose-rich sugar syrup and hexose rich sugars coming from hydrolysis of hemicellulose and cellulose, respectively, are the major substrates after initial hydrolysis that can be further fermented to produce ethanol. There are many desirable characteristics of an ideal fermenting microorganism, such as high conversion efficiency both with respect to substrate utilized and time, robustness against inhibitory compounds and ability to withstand high ethanol concentrations.

Several laboratories have established the process of utilizing pentose sugars as well as hexose sugars by various yeasts, fungi and bacteria for the production of fermentation products including alcohols (Tables 4 and 5. Among these, the most common and efficient glucose fermenting microbes are brewer's yeast and *Zymomonas mobilis* (Hahn-Hägerdal et al. 2006), while for pentose fermentation are *Pichia stipitis* and *Candida shehatae*.

The process of ethanol production not always requires aerobic conditions. It is required only for the production of biomass (Agbogbo and Wenger 2007).

Further to enhance the ethanol production from pentose sugars, different detoxification strategies have been used by various researchers (Chandel et al. 2007). The elimination of inhibitors from fermentation broth considerably improved the yield and productivity of ethanol as compared to un-detoxified hydrolysate. Moreover, utilization of all the sugars including hexoses (C6; glucose, galactose, and mannose) and pentoses (C5 sugars; xylose and arabinose) in a single reactor can be another option to reduce the cost of producing cellulosic bioethanol.

Scientists around the world have employed different fermentation strategies for cost-effective processes for ethanol production from lignocellulosic biomass in a single reactor. These processes include separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), simultaneous saccharification and co-fermentation (SSCF), consolidated bioprocessing (CBP), and simultaneous

Table 4 Various pentose fermenting microorganisms

Bacteria	References	Fungi and yeasts	References
Klebsiella oxytocea	Ingram et al. (1999)	*Neurospora crassa*	Deshpande et al. (1986)
Lactobacillus pentosus	Chaillou et al. (1999)	*Pachysolen tannophilus*	Schneider et al. (1981)
Lactobacillus casei	Roukas and Kotzekidou (1997)	*Paecilomyces* sp NF1	Mountfort and Rhodes (1991)
Lactobacillus pentoaceticus	Chaillou et al. (1999)	*Pichia stipitis*	Gupta et al. (2009)
Lactobacillus plantanum	Sreenath et al. (1999)	*Rhizopus orizae*	Millati et al. (2005)
Lactobacillus xylosus	Sreenath et al. (1999)		

Table 5 Various hexose fermenting microorganisms

Hexose fermenting microorganisms			
Organisms	References	Organisms	References
Fusaruium sporium	Mamma et al. (1995)	*Rhizomucor pusillis*	Millati et al. (2005)
Kloeckera apiculata	Aguilera et al. (2006)	*Saccharomyces cerevisiae*	Kuhad et al. (2010)
Kluyeromyces marxianus	Ballesteros et al. (2004)	*S. bayarus*	Belloch et al. (2008)
Mucor indicus	Abdenifar et al. (2009)	*S. paradoxus*	Belloch et al. (2008)
Pachysolen tannophilus	Abbi et al. (1996)	*S. pastorianus*	Belloch et al. (2008)
Pichia stipitis	Gupta et al. (2009)	*Schizosaccharomyces pombe*	Hu et al. (2005)
Pichia membranifaciens	Aguilera et al. (2006)	*Terulospora delbruecki*	Aguilera et al. (2006)
Rhizopus oryzae	Abdenifar et al. (2009)	*Zymomonas mobilis*	

saccharification, filtration and fermentation (SSFF). All the processes have been shown in Fig. 5.

Above mentioned methods (SSF, SSCF, CBP) are preferred over separate enzymatic deconstruction and fermentation (SHF) strategy. Despite it, in current scenario, SHF is the mostly used method for bioethanol production. During the first step of SHF, cocktail of lignocellulolytic enzymes is produced so that lignocellulosic biomass can be converted into a syrup of monomeric sugars (hexoses/pentoses). This solution is further used to produce bioethanol with the help of pentose/hexose fermenting microbes in a separate step. For the first step, i.e. hydrolysis, optimum temperature ranges from 45 to 50 °C, whereas for fermentation, the optimal range is near 30 °C, so both steps are performed sequentially. In SSF, enzymatic hydrolysis of pretreated lignocellulosic biomass to release monomeric sugars for subsequent

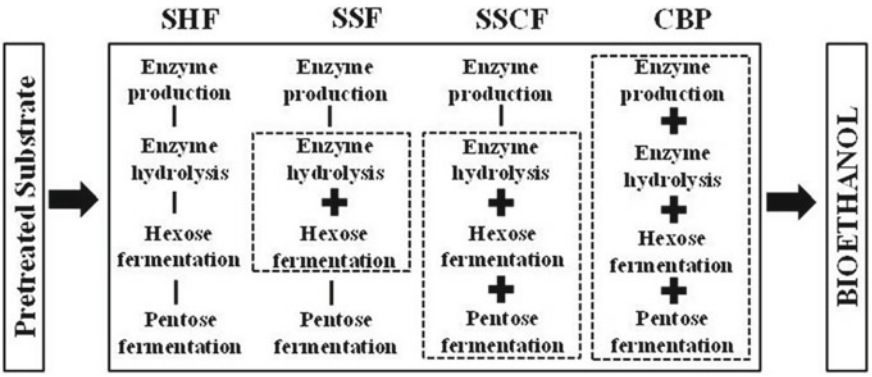

Fig. 5 Overview of various fermentation strategies

microbial conversion to ethanol is performed in the same vessel. Hallmark of this type of process is the compromise between optimum temperatures of both hydrolysis and fermentation (Choudhary et al. 2016). SSF is important over SHF as it delimits repression of cellulases (by glucose) via feedback inhibition, so improves the efficiency of saccharification as well as ethanol yield.

Further improvements in the ethanol titres and yields can be achieved if saccharification and simultaneous conversion of both five- and six-carbon sugars can be carried out (SSCF method) depending upon the fermentation capacity of the microorganisms. During the process, cellulases feedback inhibition also gets inhibited in a similar way to that of SSF, enhancing the efficiency of co-fermentation. Consolidated bioprocessing (CBP) is a relatively newer process configuration in which various biomass conversion steps such as synthesis of lignocellulolytic enzymes, feedstock deconstruction and final conversion to ethanol are performed in an integrated manner by a single microorganism. It is a comparatively promising, long-lasting and cost-effective approach for ethanol production, because of lesser requirements than other process configurations. However, current research shows that CBP-based configuration is still in its infancy and there is a lot of scope for the development of better and robust CBP organisms through molecular biology and recombinant DNA-based approaches.

SSFF is another integrated process where saccharification and fermentation chambers are separated by a membrane filtration chamber. Most of the genetically engineered or natural yeasts do not efficiently convert hexoses as compared to pentose conversion and thus, fermentation of pentose begins after that of hexose sugars. SSFF is more efficient in comparison to separate or simultaneous fermentation approaches as it provides conditions for hydrolytic enzymes and the fermenting microbes that can be maintained separately. In brief, hydrolytic enzymes carry out hydrolysis in a separate chamber and are filtered and recycled back using a tangential flow membrane filtration system. The filtrate rich in sugar is further put back into the compartment where final fermentation can take place chamber and hence, both the chambers are

maintained at similar working capacity. Furthermore, applications of flocculating yeasts can help in cell harvesting and recycling of the settled microbial biomass in the fermenter (Ghose and Bandyopadhyay 1980).

4 Genetic Engineering Approach for Bioethanol Process Improvement

Yeasts belonging to genera like *Saccharomyces, Candida, Kluyveromyces, Pachysolen, Pichia, Brettanomyces* and *Schizosaccharomycesetc* are used for bioethanol production. Out of these, *Saccharomyces cerevisiae* is commonly employed in bioethanol production due to higher productivity, high ethanol tolerance and the ability of fermenting hexoses rapidly. However, it cannot utilize pentose sugar (mainly xylose) due to the absence of key enzymatic machinery required for pentose sugar metabolism. There are two pathways present naturally among fungi and bacteria. The pathway present in fungi utilizes xylulo-reductase (XR) and xylose dehydrogenase (XDH) enzymes for the conversion of D-xylose into its isomer D-xylulose while another pathway present in bacterial utilizes xylulo-isomerase (XI) that converts the same in single step. Xylulose then enters the pentose phosphate pathway in the form of xylulose-5-phosphate by the activity of the enzyme xylulo-kinase (XKS) common to every pathway for sugar metabolism (Fig. 6).

Though *S. cerevisiae* harbours XKS gene but does not have XR/XDH (XYL1/XYL2) or XI (Xyl a) gene. Besides, various transporters are also needed for the entry of pentose sugar. Various combinations of these key genes in vector based transformation and genomic integration have been widely attempted for higher production of ethanol (Table 6) but these combinatorial approaches pose two major limitations: (a) Xylitol Accumulation: the main problem of XR–XDH pathway is incomplete recycling of redox co-substrates ($NADPH/NAD^+$) during catalysis of NADPH dependent XR and the NAD^+ preferring XDH which forms xylitol (a valuable by-product) and hence it lowers overall yield of ethanol from xylose. (b) Lower catalytic efficiency of XI. One practical solution can be the replacement of XI by XR-XDH pathway to overcome cofactor preference, but its catalytic efficiency is much lower and slower.

These limitations have been addressed by (i) XR mutation for preference of cofactor via genetic engineering for higher ethanol yield, and (ii) improvement in its genetic makeup or codon optimization. Moreover, it was also observed that overexpression of XKS1 and TAL1 (transaldolase), TKL1 (transketolase), RPE1 (ribulose5-phosphate epimerase) and RKI1 (ribose 5-phosphate keto-isomerase) (Genes of Non-oxidative pathway of *S. cerevisiae*) may lead to enhanced production of ethanol and reduction in xylitol production. In addition, enhancement in ethanol production could be accomplished by decreasing glycerol, the main side-product during glucose fermentation. In *S. cerevisiae*, GPD1 and GPD2, two ample NAD-dependent glycerol-3-phosphate dehydrogenases, are main enzymes in the synthesis of glycerol with NADH produc-

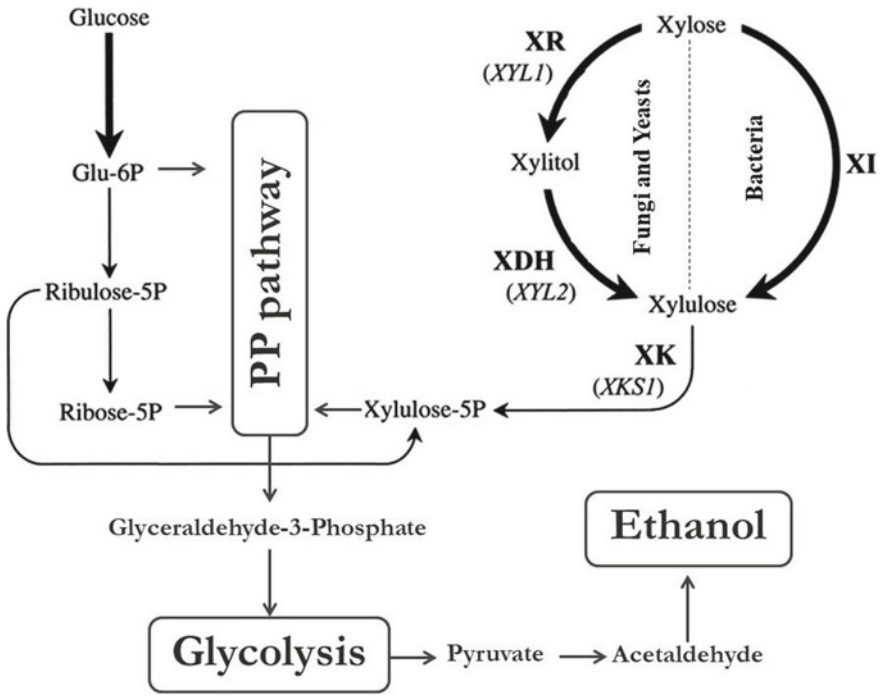

Fig. 6 Schematic diagram for xylose fermentation pathway for ethanol production

tion. This problem can be addressed by deletion of glycerol metabolism genes and overexpression of genes of glutamate pathway (Glutamate synthase, GLN1 or GLT1), which can increase ethanol production and reduce glycerol production significantly.

5 Energy and Mass Balance for Cellulosic Ethanol Production

The application of energy and mass balance regularities appear to be useful for the estimation of the efficiency of bioethanol production. The use of lignocellulosic biomass feedstock will markedly decrease energy input/output ratio. During the process of bioethanol production, the feedstock runs through a series of process steps and to make the process sustainable and economic, maximum output of energy should be attained. For this, energy inputs and outputs at every step of typical ethanol production process are analysed. Moreover, a detailed analysis of mass balance should be prepared. A schematic diagram of typical bioethanol production process comprising acid pretreatment, detoxification, delignification, pentose fermentation and hexose fermentation under SHF fermentation strategy is shown in Fig. 7.

Table 6 List of few pentose fermenting recombinant *S. cerevisiae* and their ethanol production potential

Strain	Sugar used (g/L)	Ethanol yield (g/g)	Ethanol productivity (g/L/h)	References
S. cerevisiae TMB 3001	G:X 5:15	25	0.15	Eliasson et al. (2000)
S. cerevisiae TMB 3001	10 X	48	NA	Sonderegger and Sauer (2003)
S. cerevisiae F12	G:X 50:50	52	NA	Sonderegger et al. (2004)
S. cerevisiae TJ1	50 X	10.6	0.02	Tantirungkij et al. (1993)
S. cerevisiae TMB 3001	10 X	88	0.061	Träff-Bjerre et al. (2004)
S. cerevisiae H 2673	50 X	46	NA	Verho et al. (2003)
S. cerevisiae ZU-10	80 X	75.6	0.50	Zhao and Xia (2009)

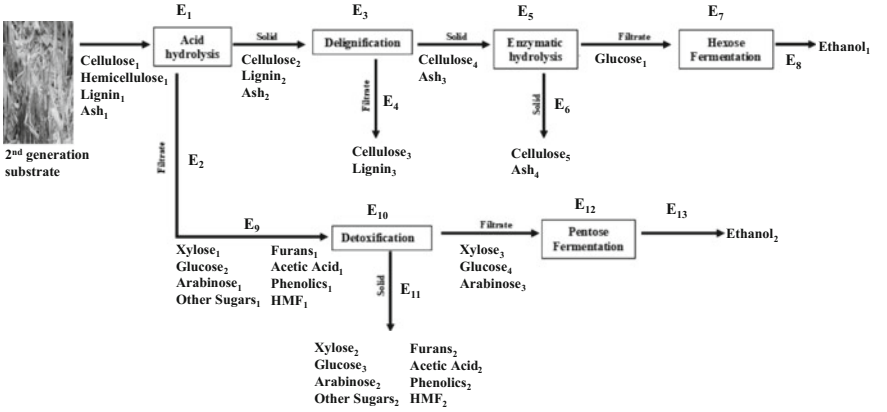

Fig. 7 Schematic overview of various steps for energy and carbon evaluation in a process for production of bioethanol from second generation feedstock using SHF strategy

6 Life Cycle Analysis or Assessment of Cellulosic Ethanol Production Processes

Besides manufacturing expenses and method, which determine the overall economic sustainability, various environmental and social criteria must also be considered for designing the biofuel production process (IEA technology 2011). Few regulatory as well as volunteer bodies (GBP 2011; ISO 2009; RSB 2012) have been instrumental in formulating set of standards and benchmarks for sustainable biofuel manufac-

turing. Many reports in the literature are available as far as the comparison of the socio-econo-environmental sustainability aspects of second generation bioethanol production processes is concerned.

The LCA is an assessment of contributions and productions to determine the effect of products formed on environment throughout the life cycle. LCA is created to compare the impacts of a product, process and/or service to generate environmental awareness in customers, governments and companies (ISO 2006). LCA could also be functional to evaluate improvement in product, its designing and comparison. It considers four phases: (a) Defining limits and objectives of system, (b) to access the inventory of life cycle, (c) quantification of life cycle impacts, and (d) results interpretation (Morales et al. 2015).

Environmental effects target to enumerate the effect of global warming, ozone depletion, photochemical oxidation and others (Roy et al. 2012). A number of software such as SimaPro, LCAmanager, Umberto, etc. have been designed to help assessing the LCA, which also involves database from various economic sectors, which may differ in their quality. These software quantify the effects of emissions on different objectives and are in favour for the different effects like depleting ozone layer, eutrophication, global warming, etc. LCA analysis of few commonly used substrates in different countries is listed in Table 7.

7 Techno-economic Evaluation

Stone & Webster Engineering Corporation (1987) evaluated the feasibility of wood-based cellulosic ethanol plant, which includes feedstock handling, acid catalysed steam explosion pre-treatment, enzyme production and hydrolysis, concentration of glucose, fermentation, distillation and anaerobic digestion and the ethanol selling price was estimated to be $0.93/l or $3.5/gal. Similarly, another report released by Chem Systems, Inc. (1987) which consisted of separate saccharification and ethanol fermentation of hardwood, enzyme production, CO_2 recovery and furans production, estimated an ethanol selling price of 0.54/l or $2.06/gal. Later on, NREL reported the lignocellulose conversion to ethanol following acid hydrolysis at a cost of ~$0.05/l or $ 0.20/gal ethanol. They also reported that though enzymatic hydrolysis has great potential for improvement, the saccharifying enzymes are very expensive (~US$0.08–0.13/l ethanol or 0.3–0.5/gal ethanol) (Aden et al. 2002). In the past decades, maximum efforts were focussed to reduce the enzyme production cost. Aden et al. (2002) estimated that if the enzyme cost comes less than 2.67 cents/l or 10 cents/gal ethanol, the cost of ethanol production could drop as low as $0.28/l or $1.07/gal and in another report NREL has aimed to achieve this goal by 2012 (Aden 2008). Concerning the R&D in cellulosic ethanol, a multi-year program was planned, which has to be updated every 2 years, including 2005 (US DOE, 2005), 2007 (US DOE, 2007) and 2009 (US DOE, 2009). The detailed updates of the technology model are provided by Aden and Foust (2009). In the European Commission, seven EU institutes evaluated the biofuels potential and costs (Hamelinck et al. 2005;

Table 7 LCA of 1G and 2G bioethanol processes

S. No.	Substrate	Type of feedstock	Growth time (Months)	Growth temperature	Water requirement	g CO_2/MJ	Energy balance	References
1.	Corn	1st	4–5	18–20 °C	50–80 cm	90	1.25	Thompson (2012)
2.	Sugar beet	1st	4–6	20–25 °C	55–75 cm	30–106	1.4–2.1	Araújo et al. (2017)
3.	Sugarcane	1st	15–16	32–38 °C	150–250 cm	5.06–59.3	4.4	Araújo et al. (2017)
4.	Wheat	1st	3–4	15–20 °C	45–65 cm	56–77	1–1.2	Araújo et al. (2017)
5.	Lignocellulose	2nd	3–4	ND	Very little	4–40	1.23–2.2	Araújo et al. (2017)
6.	Agriculture waste (Sorghum, Rye straw)	2nd	3.5–5	20–35 °C	45–65 cm	23–72	0.78–1.79	Araújo et al. (2017)

Table 8 Major technological bottlenecks in bioethanol development process

Pretreatment	Enzymatic hydrolysis	Fermentation
• Single or universal pretreatment • Lignin recovery • No inhibitor generation • Efficient Conditioning Strategy • Recovery or reuse of input energy • Recovery or reuse of used water Fully integrated process	• Availability of low-cost enzyme • Development of substrate-specific enzyme formulation • Specially designed reactor for high substrate consistency • Capability of converting unreacted xylan/xylo-oligomers • Operation in whole slurry mode (inhibition tolerance)	• Inhibitor tolerant microbes • The approach of SSF or CBP should be used • Bioprospecting for efficient pentose fermenting strain • Efficient conversion of hemicellulose sugars to other value-added product such as xylitol • Genetically modified strain for mixed sugar fermentation

Gnansounou and Dauriat 2010). The economic evaluation took into account the manufacturing cost of $0.90/l or €0.62/l in 2010, $0.85/l or €0.59/l in 2020 and $0.72/l or €0.50/l in 2030. In another case study, Sassner et al. (2008) compared the economic performances for the conversion of different lignocellulosics (Spruce, corn-stover and salix) to ethanol, which required estimation of annual production cost including annualized capital cost and annual operation costs. According to them, the annual production costs (US$) vary significantly, i.e. $0.66–0.69/l ethanol (spruce), 0.67–0.86 (corn stover) and 0.72–0.87 (salix). Reports on LCA of cellulosic bioethanol from Indian researchers are very few in comparison to other countries.

8 Future Prospects

Development of cellulosic ethanol as a biofuel is very much needed at present, as it will have the potential to make countries self-sufficient in the energy sector and make the environment more safer and greener. Globally, the focus has already shifted from food-based resources towards non-food crop wastes (Saha et al. 2005; Himmel et al. 2007; Kuhad et al. 2011a; Saini et al. 2015). However, to reduce the final production costs, major cost-contributing steps have to be optimized from a technical as well as economical point of view (Table 8).

Priority should be on development of highly efficient and cheaper cellulolytic enzymes that can be produced economically and can act very fast even at a minimal dose. Additionally, an environmentally greener as well as cheaper and highly efficient pretreatment technology has to be used that will further reduce the efforts and costs in subsequent steps. As far as improvement of fermentation technology is concerned, there is still a very large scope for development of very robust and efficient pentose fermenting microorganisms. Priority should be developments in research and technological advancements in co-fermentation of hexose and pentose sugars

simultaneously at a greater ease. It will definitely require more robust applications of molecular biology and metabolic engineering approaches (Galazka et al. 2010) as well as adjustments of metabolic flux (Matsushika et al. 2008). Another robust technology could be the development of consolidated bioprocessing microorganisms that have better catalytic abilities (Zhang et al. 2009). And finally, the successful transition of the lab or demonstration scale technologies to a large industrial scale will finally help in establishing commercial level cellulosic ethanol plants based upon currently available processes. In short, concerted efforts by experts from various science and technological disciplines will be required to tackle the hurdles that the current cellulosic ethanol industry is facing.

9 Conclusion

The potential to use lignocellulosic biomass from various sectors to produce second generation bioethanol underscores the need of technological advancement in each and every process step. The impediments of lower sugar recovery, hemicellulose fermentation, enzyme recycling, etc. need extensive inputs to be taken care of. The technological interventions for better biomass deconstruction strategies in conjunction with better process integration and optimization are required. One of the better strategies may be development and application of most efficient organisms in association with smart integration of various processes in an integrated biorefinery approach, where a multitude of products can be obtained in addition to bioethanol only and this may also include applications of consolidated bioprocessing microorganisms.

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