Review Paper

Bioethanol production from cereal crops and lignocelluloses rich agro-residues: prospects and challenges

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Abstract
The existing energy demand, fluctuating oil prices and repercussions due to usage of fossil fuels have enhanced the requirement for alternative energy sources. Bioethanol derived from cereal crops serve as a promising alternative to conventional gasoline owing to the advantages of feedstock availability, reduction in production costs coupled with significant low greenhouse gas emissions. Recent studies focused on agro-residues derived from cereal crops had illustrated potential technical advantages for bioethanol production. Conventional bioethanol process includes pretreatment, saccharification and fermentation. Unlike acid and base pretreatment methods, enzymatic and ionic liquid pretreatment methods showed promising results in delignification process. Besides, studies demonstrated that integrated processes like simultaneous saccharification and fermentation and consolidated bioprocessing showed significant reducing sugar release and higher bioethanol yield from cereal crops and their residues. Moreover, deploying advanced technologies such as genome editing and metabolic engineering techniques could not only enhance bioethanol content but also helps in development of biorefinery theme, which leads to development of inexpensive technology. These studies and know-how technologies imply that the cereal crops and their residues could be viable substrates for bioethanol production that ultimately bolster the energy security.

Keywords Bioethanol · Cereals · Paddy straw · Sugarcane

1 Introduction

Fossil fuel consumption and its repercussions on climate change is the driving force to search for renewable alternatives, which could be the ray of hope for sustainability and energy security [1, 2]. Conventionally, petroleum sources are finite, emit green house gas emmissions (GHG's) and cause air pollution, which triggered to look for an alternative biofuels [3]. In contrary, biofuels are eco-friendly and have tremendous potential to mitigate the emission of GHG. Besides, these fuels can be easily stored in the form of liquid fuels, unlike wind, water and photovoltaic energy [4, 5]. Among biofuels, bioethanol derived from agro-residues of cereal crops is having great potential owing to higher yield of hybrids, availability of substrate and know-how technology, circular economical approach for biorefinery development, cost-effective and eco-friendly [6, 7].

Moreover, advances in seed production, plant-breeding activities and agronomic practices have boosted the yield of cereals and has become a viable source for bioethanol production. Most of the cereal crops are C4 plants that have high yields due to higher photosynthetic capacity than C3 plants [8]. These C4 plants produce high yields of biomass with very few inputs and also survive in adverse climatic conditions. Among the major C4 cereal crops,

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maize, sugarcane, sorghum and switch grass are being in forefront as a promising feedstocks [9, 10]. In the process of bioethanol production, pretreatment, saccharification and fermentation are the predominant steps that need to be deployed in an effective manner. However, conventional operation of these steps individually have confronted feedback inhibition, lower tolerance of fermentive strains to ethanol concentrations, lower release of glucose from lignocellulose, inefficient utilization of pentoses etc.,

Recent studies demonstrated that adoption of newer methods such as biological and ionic liquid pretreatment, simultaneous saccharification and fermentation (SSF), consolidated bioprocessing (CBP) showed promising results [9, 10]. Besides, metabolic engineering and genome editing are gaining wide interest in addressing these gaps for development of cost-effective technology. Hence, in the present review, potential cereals and their residues for bioethanol production has been described. Moreover, technical know-how and advances in pre-treatment and saccharification for enhanced delignification and saccharification of cereals are illustrated. As the metabolic engineering and genome editing have potential to alter genomes for development of smart biofuel crops, a lucid explanation has been exemplified that would help for future research.

2 Cereal based substrates for bioethanol production

The cereal grains such as wheat, rice, maize, barley, sorghum, rye, oats etc. contain starch and protein as major constituents while the minor constituents include vitamins, phytic acid, lipids, non-starch carbohydrates and minerals. High starch content made cereals a viable substrate for ethanol production [11, 12]. As the initial step of ethanol production, the cereals are subjected either to dry grinding or wet grinding to release starch from the substrate. This is followed by gelatinization where, the starch is heated at high temperature (Table 1). The viscous slurry obtained through gelatinization is then acted upon by amylolytic enzymes during liquefaction and saccharification for the release of simple sugars which are further acted upon by yeast or any other microorganism for ethanol production through anaerobic fermentation. The ethanol produced along with CO_2 is separated and concentrated through distillation, rectification and dehydration processes. The ethanol produced depends upon the starch content of the substrate, process parameters and process implemented for ethanol production. Apart from separate hydrolysis and fermentation, simultaneous saccharification and fermentation, simultaneous saccharification and co-fermentation, an integrated process is being implemented to increase ethanol production and reduce the production cost and process time.

Table 1 Starch content, gelatinization temperature and ethanol yield of various cereal substrates

Substrate	Starch content (%)	Gelatinization temperature (°C)	Ethanol yield (L/100 kg)
Wheat	58–62	58–65	36–39
Rice	55–70	62–80	48–57
Barley	54–65	53–63	34–41
Maize	60–63	68–74	38–40
Sorghum	55–65	70–78	36–42
Rye	56–70	57–70	35–42
Oats	54–64	75–80	36–42

3 Bioethanol production from agro-residues derived from cereal based substrates

The cereals are harvested and the cereal waste that remains in the field is used to certain extent as animal feed and remaining is disposed by burning. The smoke released during burning is causing severe health hazards. The cereal waste is lignocellulosic in nature and predominantly comprises of lignin (10–20%), cellulose (40–50%) and hemicellulose (20–30%) [13]. Cellulose is a glucose polymer responsible for mechanical strength of the plant, while hemicellulose is a heteropolysaccharide of hexoses and pentoses. Cellulose and hemicellulose are bound to one another by non-covalent attractions. Similarly, lignin comprising of various alcohols such as coniferyl, sinapyl and coumaryl alcohols, acts as a protective seal around holocelluloses. The composition of various cereal wastes has been tabulated (Table 2) [14, 15].

Value added products such as biofuels and other chemicals are produced from the lignocellulosics through thermo-chemical methods such as gasification and pyrolysis. Thermolysis of biomass produce syngas and bio-crude that serve as precursors for drop-in fuel. During pyrolysis, the biomass is exposed to 500–600 °C in absence of oxygen to produce bio-oil which upon hydroprocessing gets converted to precursor for drop-in fuel. At higher temperature, above 700 °C, under controlled oxygen, biomass can be converted to liquid fuel via gasification. The syngas produced during this process can be converted to bioethanol either by microorganism such as *Butyribacterium methylotrophicum*, *Clostridium ljungadahlii*, *C. autoethanogenum*, *C. carboxydivorans*, *Methanosarcina barkeri* and *Rhodospirillum rubrum* [16] or by metal catalysts [Fischer–Tropsch (FT) synthesis] such as aluminium, cobalt etc. [17]. The major drawbacks of the FT synthesis are high cost, fixed H_2:CO

Table 2 Lignocellulosic composition of varied agro-residues derived from cereals

Substrate	Cellulose (% dry wt)	Hemicellulose (% dry wt)	Lignin (% dry wt)
Cornstalk	39–47	26–31	3–5
Corn cobs	45	35	15
Corn stover	38–40	28	7–21
Rice straw	28–36	23–28	12–14
Wheat straw	33–41	26–32	13–19
Wheat husk	36	18	16
Barley straw	31–45	27–38	14–19
Sorghum stalks	27	25	11
Sorghum straw	32	24	13
Rye husk	26	16	13
Sweet sorghum bagasse	34–45	18–28	14–22

(2:1) ratio, catalyst poisoning and high operating conditions. The advantages of the biocatalysts are specificity, independence of H_2:CO ratio, no requirement of metal catalysts and operation of bioreactor under ambient conditions.

Among the various ethanol production processes, 50% of ethanol yield was obtained through gasification. In some processes, methanol was produced first which upon catalytic shift produces bioethanol whose yield is approximately 80%. Gas to liquid mass transfer, solubility of syngas and meager yield are considered as constrains for commercialization of syngas fermentation technology [6]. Despite of improvement in reactor design, process optimization and appropriate catalyst, the ethanol produced from syngas is only 30 g/L due to which the cost of ethanol recovery is too high. The ethanol recovery will become cost effective only when the ethanol concentration is around 15% (v/v).

In contrary to thermochemical method, the biochemical route includes transformation of polysaccharides of the biomass into monosaccharides and its conversion into ethanol. The multi-step process of biochemical method for ethanol production from lignocellulosics includes (1) pre-treatment/delignification (2) enzymatic hydrolysis/saccharification (3) fermentation process [9, 10] (Fig. 1).

4 Technical know-how of bioethanol production

The competence of the biomass to biofuel conversion process primarily depends upon pretreatment, which is required to break the mechanical barrier i.e. lignin, to utilize its holocellulose constituents. Lignin removal increases the biomass digestibility, porosity and surface area that enhance the accessibility of hydrolytic enzymes towards holocelluloses for improved reducing sugar yield.

4.1 Pretreatment

Various pretreatment approaches have been employed to breakdown the complex holocellulosic polymers into simple fermentable sugars. The pretreatment process should avoid the degradation of pentose sugars, minimize the inhibitor formation, recover lignin for the formation of value added products, minimize heat and power requirement for making the process cost effective [1, 9]. Various pretreatment methods viz, physical, chemical, physico-chemical and biological are being used for lignin removal [10]. Physical pretreatment process such as grinding, milling etc. reduces the crystalinity and size of biomass [12]. The energy requirement for the process depends upon the final particle size and crystalinity of the biomass. This process is quite expensive and not advisable at large

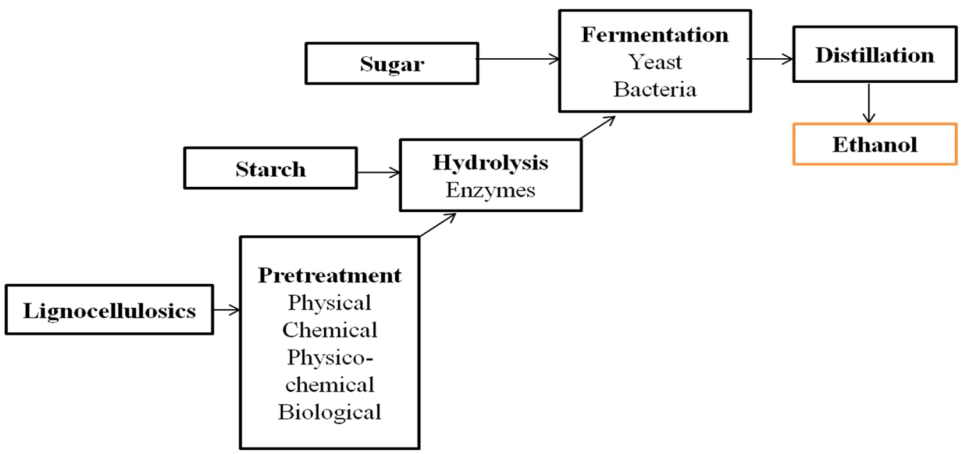

Fig. 1 Schematic representation of ethanol production from various feedstocks

scale hence, other pretreatment processes have gained importance.

4.1.1 Acid pretreatment

The concentrated and diluted sulphuric acid, nitric acid, phosphoric acid and hydrochloric acid [18, 19] are used to break the lignocellulosic structure. Acid hydrolyze hemicellulose into simple sugars such as xylose and convert into furfurals [6]. To accomplish pretreatment, acid (0.2–2.5%, w/w) is added to biomass, mixed constantly and temperature is maintained between 130 and 210 °C. Depending upon the conditions, the acid pretreatment completes in short time [14, 20]. Sometimes there is no requirement of enzymatic hydrolysis due to acid pretreatment but this process needs a detoxification step to remove acid from the biomass for smooth operation of fermentation process. Moreover, it causes corrosion to the reactor and requires an adequate reactor material to withstand the acid pretreatment process.

4.1.2 Alkaline pretreatment

The structural alterations in the lignocellulosics occur in the presence of bases viz., sodium, calcium, ammonium hydroxide and potassium. These chemicals degrade the glycosidic and ester bonds in lignin, cause cellulose swelling and decrystallization, partial digestion of hemicellulose and increases the enzyme accessibility towards holocelluloses [21]. This technique is mostly implemented for pretreatment of corn stover, wheat and rice straw [22]. Kumar and Sharma [23] employed alkaline pretreatment on wheat straw and observed 60% delignification with 1.5% NaOH at approximately 20 °C and 144 h of incubation period. Similarly, sodium hydroxide pretreatment on wheat straw resulted in 26% reduction in lignin content [24]. Alkaline pretreatment occurs under mild conditions but takes longer incubation period. This process involves the soaking of biomass in alkaline solution for certain period with constant mixing. The alkaline pretreatment is generally succeeded by a neutralization step for removal of inhibitors and lignin. For instance, neutralization of lime with carbon dioxide enhanced the glucose recovery by 89% in rice straw [25]. Though the lime pretreatment is energy intensive its recovery requires precipitation with CO_2.

4.1.3 Ionic liquid pretreatment

Ionic liquids (ILs) are referred as salts composing of large cations and small anions that act as nucleophile and play a crucial role in the delignification process. These exist in liquid state at room temperature and have low vapour pressure. Due to this property, the ionic liquids such as 1-Ethyl-3-methylimidazolium hydroxide ([EMIM]OH), 1-Butyl-3-methylimidazolium chloride ([BMIM]Cl) and -ethyl3-methylimidazolium acetate ([EMIM][OAc]) exhibits maximum recovery which leads to cost reduction [26]. Ionic liquids find its application in pretreatment of cereal waste such as wheat and rice straw, corn stover etc. The extent of lignin removal due to application of 1-ethyl-3-methylimidazolium acetate ([EMIM][OAc]) was reported to be 29–64% at 150 °C for 1.5 h in triticale straw; 44% at 125 °C for 1 h in corn stover; and 37% at 120 °C for 5 h in rice straw [27]. Though ILs carryout pretreatment of lignocellulosic effectively it cannot be employed at large scale due to its high cost. Moreover, the effect of ILs on the fermenting microbes needs to be studied extensively.

4.1.4 Biological pretreatment

Biological pretreatment is conducted either by using microorganisms or enzymes. It requires mild operation conditions and meagre energy unlike the other methods of pretreatment. Lignin removal is conducted by brown, white and soft rot fungi and bacteria. White and soft rot fungi predominantly act on lignin and cellulose; while the brown rot fungi attack only on cellulose. Degradation of lignin by white rot fungus *Phanerochaete chrysosporium* has been extensively studied making white rot fungi an effective microorganism for delignification [9, 28]. These microbes secrete enzymes such as laccase, manganese peroxidase (MnP), lignin peroxidase (LiP) and versatile peroxidase (VP) that specifically degrade lignin. Along with these ligninolytic enzymes, accessory enzymes such as aryl alcohol oxidase and glyoxal oxidase are also reported to produce hydrogen peroxide that acts as an oxidant during the oxidation of lignin [10, 29]. LiP and MnP belong to the class of peroxidases. LiP, due to its high redox potential oxidises both non-phenolic and phenolic substrates in the presence of H_2O_2 whereas, MnP oxidises phenolic substrates in the presence of manganese. Versatile peroxidase oxidises both non-phenolic and phenolic compounds in the absence of manganese [30]. The biological pretreatment of cereal waste has been depicted in Table 3.

4.2 Saccharification of substrates for ethanol production

Lignocellulosic biomass constitutes 70–75% of cellulose and hemicelluloses, which upon enzymatic hydrolysis releases soluble sugars. The cellulolytic enzymes are synthesized by several bacteria such as *Cellulomonas fimi*, *Clostridium thermocellum* and *Bacillus subtilis*; and fungi such as *Aspergillus niger*, *Penicillium funiculosum, Rhizopus oligosporus* and *Trichoderma viride*. Among these

Table 3 Biological pretreatment of various agro-residues derived from cereals

Lignocellulosics	Microbes/enzymes	Reference
Rice straw	*Fungal consortium*	[31]
Rice straw	LiP, MnP, laccase, cellulase	[32, 33]
Rice husk	*Phanerochaete.chrysosporium*	[34]
Rice bran	Protease	[35]
Corn stalks	*Irpex lacteus*	[36]
Corn stover	Fungal consortium	[37]
Corn stover	*Ceriporiopsis subvermispora*	[38]
Corn cobs	LiP, MnP, protease, laccase, xylanase	[33]
Wheat straw	*Ceriporiopsis subvermispora*	[39]
Wheat bran	MnP, protease, endoglucanase, β-glucosidase, laccase	[35, 40]
Wheat straw	LiP, MnP, laccase, cellulases xylanase	[32, 33, 41]

microorganisms, *Trichoderma reesei*, a filamentous ascomycete isolated on the Solomon Islands is well known for its simultaneous production of cellulase and xylanase. *T. reesei* is capable of producing saccharifying enzyme with endoglucanase, β-glucosidase, cellobiohydralase and xylanase activities that hydrolyses holocellulose of lignocellulosic biomass [42]. Production of cellulolytic enzymes by *T. reesei* RUT-C30 using various lignocellulosics such as wheat bran, millet husk and rice husk as substrates was investigated by Olsson et al. [43]. Jeya et al. [44] reported the production of 685 mg/g of reducing sugar from rice straw after 48 h of incubation time with cellulase and β-glucosidase produced from *Trametes hirsute*. Similarly, Wood et al. [45] reported the production of 208.40 mg/g reducing sugar from wheat straw using *T. reesei* cellulase.

4.3 Fermentation process of saccharified broth for ethanol production

The hydrolysate obtained from lignocellulosic biomass after enzymatic saccharification contains reducing sugars rich in C-6 (glucose, mannose and galactose) and C-5 sugars (xylose and arabinose). The efficiency of the fermentation process for ethanol production depends mostly on the strain employed for fermentation along with the process parameters such as temperature, pH, mixing, media composition etc. The utilization of microorganisms with high ethanol tolerance; and hexose and pentose sugar fermenting ability are mostly economical. Besides *Saccharomyces cerevisiae,* ethanol producing bacteria (EPB) like *Zymomonas mobilis* is grabbing the attention due to its fast growth, high sugar uptake, high ethanol tolerance (up to 16%, v/v) and low oxygen requirement [46]. Another strategy to enhance the ethanol production is to co-ferment the pentose utilizing microorganisms (*Candida shehatae*, *Kluyveromyces marxianus Pichia stipitis and Pachysolen tannophilius*) along with C6 utilizing yeast (*S. cerevisiae*) [46]. Various strategies adopted to obtain ethanol from lignocellulosics are shown in Fig. 2.

4.3.1 Separate hydrolysis and fermentation (SHF)

The process in which saccharification and fermentation are conducted in two separate fermenters under various reaction conditions is known as SHF. The saccharifying enzymes (cellulases and xylanases) efficiently hydrolyze at 45–50 °C, while the fermenting strains produce ethanol at 30–37 °C. SHF provided flexibility to carry out both the processes at their optimum conditions. The ethanol yield during SHF could be improved by fermenting with co-culture or with the strain capable of fermenting both hexose and pentose sugars and the process is referred as separate hydrolysis and co-fermentation (SHCF). The main disadvantage in SHF is inhibition of cellulolytic

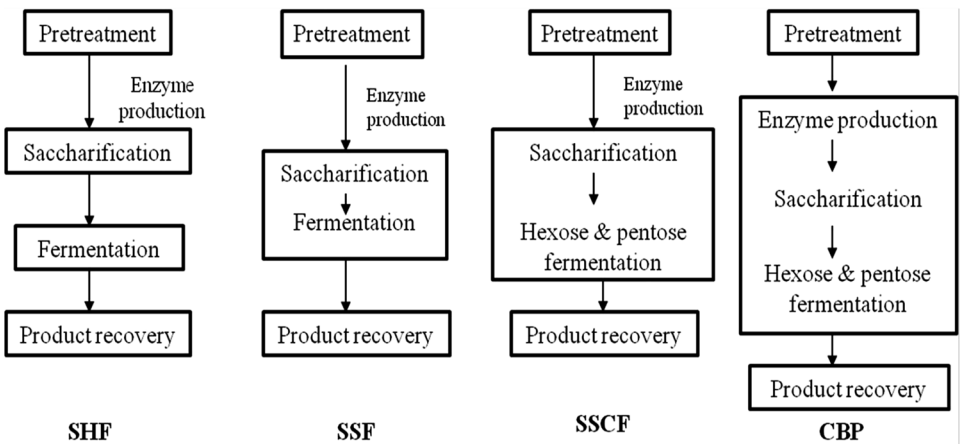

Fig. 2 Various strategies for bioethanol production from cereals and their residues. (SHF, Separate hydrolysis and fermentation; SSF, simultaneous saccharification and fermentation; SSCF, simultaneous saccharification and co-fermentation; CBP, consolidated bioprocess)

enzyme activity due to accumulation of reducing sugars [47]. Moreover, as the process takes place in two reactors that incur additional cost besides longer processing time. These drawbacks of the SHF may be avoided by simultaneous saccharification and fermentation process.

4.3.2 Simultaneous saccharification and fermentation (SSF)/co-fermentation (SSCF)

SSF is the process where saccharification of the pretreated biomass and fermentation of reducing sugars occurs simultaneously within a single reactor. This process is feasible, only when the optimum conditions of saccharifying enzyme are in close proximity with that of the fermenting microbial strain. Fermentation with *Saccharamyces cerevisiae* should cope with temperature, as the yeast may not sustain the optimum temperature of saccharifying enzymes. Thermophilic microorganisms such as *C. acidothermophilum* and *K. marxianus* are being used for fermentation without compromising the optimal temperature of saccharification. Enzymatic saccharification and fermentation of cassava waste resulted in the ethanol productivity of 9.3 g/L in 36 h of incubation period [48].

The major advantage with SSF is abatement of feedback inhibition by glucose and cellobiose, as they are simultaneously converted to ethanol. Thus, SSF not only enhances the ethanol yield in short incubation time but also reduces the operation cost; since, one reactor suffices *in lieu* of two [49]. Besides, microbial contamination of sugars is checked due to the presence of ethanol in the same vessel [50, 51]. The ethanol yield can be further enhanced by employing the strains that could ferment both C6 and C5 sugars known as simultaneous saccharification and co-fermentation (SSCF).

4.3.3 Consolidated bioprocess

Another advanced technology associated with bioethanol production is consolidated process, where enzyme production, saccharification and fermentation takes place in a single step in a reactor [52]. During consolidated bioprocess (CBP), the microorganisms produces their own saccharifying enzymes for decomposition of lignocellulosics, compensating the need for exogenous enzymes into the system; thereby, resulting in cost reduction [53]. This process improves the cellulose conversion efficiency and decreases processing cost of bioethanol and other value-added products. Ethanol yield of 0.35 g/g and 0.45 g/g has been reported from wheat and rice straw through CBP using *Pichia stipitis* NRRL Y-7124 and *Candida shehatae* NCL-3501 respectively [54]. The microbial conversion of the biomass into bioethanol and other useful products can be enhanced by using genetically engineered organism with cellulolytic and ethanologenic activities. Therefore, an efficient ethanol producing strain could be genetically modified to express genes for cellulases and xylanases that could be engineered metabolically to form a superbug capable of fermentation of both hexoses and pentoses [55].

During the process, when a thermotolerant strain is not used, the temperature and other operation conditions have to ensure that they are optimal for all the steps during the process. Ethanol yield can be improved by bringing slight modification in CBP. The temperature of the system could be maintained optimum for the saccharifying enzymes for a short span of time and thereafter the fermenting stain is added to the system. Optimum temperature for all the process steps needs to be controlled in such an effort. This modified process is referred to as partially consolidated bioprocess (PCBP). Partially consolidated bioprocess is a combination of simultaneous pretreatment and saccharification (SPS) and fermentation. The temperature and pH optimum for both ligninase and cellulolytic enzymes are maintained for short span, followed by fermentation. It is worth mentioning that the bio-processing technologies for biofuel production are focusing more and more on consolidation. Though, the research on the CBP configuration is in its infancy, it has huge scope to be adopted in the near future.

5 Metabolic engineering and genome editing techniques for enhanced bioethanol production

Bioethanol derived from cereal crops confronts several problems such as exorbitant cost of cellulase production in microbial bioreactors at commercial level. Besides, another important issue is pretreatment of lignocellulosic substrates that need to break down into individual constituents for efficient removal of lignin to facilitate the accessibility of cellulases to biomass cellulose. The cost incurred on cellulosic ethanol production is several folds higher than the corn grain ethanol price. Recent advances in genetic engineering technology particularly metabolic engineering and genome editing offer huge potential to circumvent the cellulosic ethanol production. So as to make the process viable, sustainable production of cellulases and hemicellulases in the plants could alleviate the production need in bioreactors. Further, alteration of lignin content or configuration using metabolic engineering/genome editing techniques could significantly make the pretreatment process inexpensive. And the last approach for cost effective ethanol production, upregulation of hemicellulose and cellulose enzymes for higher

polysaccharides in the future prospects could enhance the potential for increased cellulosic bioethanol production.

5.1 Genetic manipulation mode in cereals crops

Genetic transformation in most of the food crops is reported either using *Agrobacterium tumefaciens* or biolistics based gene transfer. Some efficiently transformant crops at commercial scale are rice, sorghum, poplar maize, and switchgrass [56]. Generally, *Agrobacterium* mediated transformation is successful in dicotyledonous crops; however, fewer strains have showed promising results in transformation process of corn, wheat, rice, sorghum and switchgrass. The prominent feature for efficient transformation is establishing a genotype-nonspecific genetic engineering process. Besides, understanding the biological basis for incompetence should also be ensured before choosing of cultivars/varieties for genetic transformation. For instance, in switchgrass genotypes, very few (02) cultivars can be efficiently genetically engineered. On the other hand, well established genetic transformation process has been deployed in cereal crops such as barley, maize and oat using biolistics bombardment with multiple meristem primordial explants [57]. Apart from genetic transformation, development of suitable feedstock with resistance to biotic and abiotic factors is essential. Breeding strategies play vital role in the improvement of feedstocks from their wild ancestors through the years. Amalgamation of traditional breeding, marker assisted selection breeding, genetic markers and genome sequencing could further help in improvement of efficient feedstock from cereal crops.

5.2 Production of cell-wall degrading enzymes in plants

Production of cell-wall hydrolyzing enzymes in microbial bioreactors is expensive and needs to produce in other alternative medium such as plants for cost effective process. Several reports substantiate that the plants have been explored for carbohydrates, lipids, proteins, enzymes, pharmaceuticals and industrial polymers at industrial scale [58, 59]. The main advantage of enzymes production in plants requires significantly low energy input than microbial production of hydrolysis enzymes. Besides, technical know-how of genetic transformation, biopharming, harvesting and logistics have been available and further to make the process cheaper, heterologous expression of hydrolysis enzymes has been targeted [60]. However, a major setback for enzyme production in plants is proper misfolding in the desired transformant environment.

5.3 Expression of enzymes in cytosol versus compartmentalization

Expression of hydrolysis enzymes in subcellular compartments is most favored than expression in the cytosol. Enzymes expression in subcellular compartments can facilitate proper folding and activity, post translational modifications and increased stability over their accumulation in the cytosol [58]. Sub-cellular targeting of enzymes could be advantageous due to several reasons. They are:

- It helps the foreign enzymes from potential damage by alleviating from cytoplasmic metabolic activities.
- It enhances enzyme stability avoiding exposure to proteases and accumulation.
- It enables better protein folding because of molecular chaperones available in sub-cellular compartments.
- Cell organelles like chloroplasts, apoplast, vacuoles and mitochondria are favourable for targeting the enzymes due to retention signal peptides.
- In addition to these organelles, endoplasmic reticulum (ER) organelle has been one of the important organelles for efficient targeting of proteins owing to abundance of molecular chaperones (few proteases) coupled with oxidizing atmosphere [59]. Recent study showed that the proteins targeted in ER lumen had increased stability and greater activity (two–tenfold) than the cytosol [61]. In another study, targeting of antibodies in sub-cellular organelles showed increased protein accumulation than the cytosol.

Studies that have been done on targeting of hydrolysis enzymes in plants particularly in alfalfa and tobacco have been shown in Table 4.

Major drawback suffer with sub-cellular targeting of cell wall degrading enzymes is with optimal pH. It is well known that the pH is one of the important factors for efficient function, which should match with the organelle pH; otherwise, enzyme biological activity could be hampered. For instance, in chloroplasts at night the pH is 7.5 and during day time the pH is 8.0, which implies that the enzymes targeted for expression in chloroplasts could not maintain the same biological activity [58]. Another factor that needs to be pondered with bioconfinement of genetically engineered biomass crops [62, 63]. To apply in bioethanol production process, cell degrading enzymes can be extracted either from dry or fresh transgenic crop biomass as total soluble protein (TSP). This can be added to pretreated biomass to convert into fermentable sugars [71]. TSP extraction from dry or fresh biomass is quick easy and hence, it could be included in ethanol extraction process. In addition to sub-cellular targeting of enzymes for cost effective enzyme production, other options such

Table 4 Heterologous expression of cell-wall-deconstructing enzymes in plants

Plant/crop	Heterologous enzyme	Subcellular storage compartment	References
Arabidopsis thaliana	*A. celluluolyticus* $E1_{CAT}$	Apoplast	[62]
Tobacco	*A. celluluolyticus* E1	Endoplasmic reticulum	[63]
Rice	*A. celluluolyticus* $E1_{CAT}$	Apoplast	[64]
Potato	*A. celluluolyticus* E1	Apoplast	[63]
Tobacco	*A. cellulolyticus* E1	Cytosol	[65]
Alfalfa	*T. fusca* E2 and E3	Cytosol	[66]
Potato	*S. olivaceoviridis Xyn*B	Apoplast	[67]
Barley	*N. patriciarum XynA*	Cytosol	[68]
Maize	*A. celluluolyticus* $E1_{CAT}$	Apoplast	[69]
Tobacco	*A. celluluolyticus* E1 and $E1_{CAT}$	Apoplast	[70]
Potato	*A. celluluolyticus* E1	Vacuole	[63]
Maize	*A. celluluolyticus* $E1_{CAT}$	Apoplast	[71]
Rice	*C. thermocellum* $XynA_{CAT}$	Cytosol	[72]
Potato	*A. celluluolyticus* E1	Chloroplast	[63]

E1, E2 and E3, endoglucanases (endocellulases); CAT, catalytic domain; *XynA*, *XynB* and *XynZ*, xylanases (hemicellulases); CBH1, celluobiohydrolase 1

as increase of plant cell biomass [66, 67], lignin modification [73] and modification of cellulose [74] have been studied. However, efficient method to deploy for viable technology is still a challenge and need to include innovative techniques such as CRISPR/Cas-9 (genome editing) for further improvement. Although, studies in this aspect is very scanty only basics have been dealt for better understanding and implementation of the technique in desired modification of lignin and cellulose.

5.4 CRISPR/Cas system: a promising technique for alteration of genomes

CRISPRs are DNA loci with diminutive base sequence reiteration which are available in 40% of sequenced bacteria and 90% of archaea genomes. CRISPRs are associated with Cas genes that specifically code for CRISPR-proteins and forms a CRISPR/Cas system that provides immunity against foreign genetic elements like phages and plasmids [75]. Moreover, each repetition is linked with short spacer DNA segments of virus which recognize foreign genetic elements and cut them in a manner analogous to RNAi in eukaryotes [76].

Approximately eleven CRISPR/Cas systems were identified which were mainly categorized into type I, type II and type III. Among these, type II CRISPR/Cas systems are unique due to the occurrence of protospacer adjacent motif (PAM) and trans-acting CRISPR RNA (tracrRNA, a second RNA) with crRNA. This facilitate in maturation and recruiting the Cas9 nuclease to DNA [77, 78]. Moreover chimeric 'guide' RNA (sgRNA/gRNA) was created by a simplified three-component system by fusing crRNA and tracrRNA which is widely used for genome engineering [78, 79].

CRISPR/Cas9 system uses RNA to channels the nuclease towards specific nucleic acid present in the genome. CRISPR necessitate a single construct for synthesizing RNA which is easier than synthesizing protein domains of ZFN and TALEN [80, 81]. Through multiple gRNAs, CRISPR/Cas system can introduce mutations simultaneously in multiple genes. Even though, CRISPR/Cas system is more efficient than ZFN and TALEN, the complication associated with this system is that it introduces mutations at non-specific loci called as off-site effect. This effect results in cell toxicity and creates hurdles in transgenic plant production through micro propagation.

A mutated version of Cas9 avoids off-target effect and enhances the specificity by inducing nicks (SSBs) in genome and by using gRNAs with target sequence of 20 nucleotides [81, 82]. Thus, design and optimization of gRNA, besides Cas9 expression play crucial role in avoiding off-target effects [82]. The Cas9–guide RNA complex showed constant and extensive interaction with target site containing PAM whereas binding at non-target sequences without PAM is transient thus inferring that PAM is important for stimulating Cas9 activity [80].

6 Challenges and opportunities for bioethanol production from cereals

Plant genetic engineering for biofuel production is in its infancy despite of advances that have laid for way forward [82]. However, a great challenge would be to make the process viable by developing an efficient, genotype-nonspecific transformation system in feedstock crops. In addition, to make the process cheaper,

enzymes production should be done in plants rather than in bioreactors. Although, it offer several drawbacks, which needs to address the bottlenecks of succinct supply, matching of pH ad maintaining its biological activity. Apart from genetic engineering, storage, transport issues and utilization of agricultural land for fuel rather than food purpose, has been a major concern.

Finally, questions are looming large over the utilization of ethanol as ideal biofuel than butanol. Because the former is difficult to be transported by normal pipelines owing to its hydrophilic nature that cause corrosion of pipeline and would be expensive to transport by trains or tankers. The viable options to use ethanol can be exploited using plant genetic engineering on the following themes such as deconstruction of plant cell-wall polysaccharides, suppression of lignin biosynthesis enzymes, increase of polysaccharides level or the overall plant biomass.

7 Conclusion

Cereal based crops and their residues are potential substrates for bioethanol production. However, lack of commercially viable technologies for efficient conversion of these substrates into bioethanol production is scarce. Besides, added downstream steps for bioethanol production could makes the process exorbitant. Integration of SSF and CBP processes could be accomplished in a single step, which reduce the number of downstream steps and ultimately reduce the cost of the process. Besides, biological pretreatment and ionic liquids are greener, eco-friendly and remain robust techniques that have potential to replace convention acid and alkaline pretreatments. Embracing advanced breeding strategies such as association mapping, marker assisted selection for crop improvement, speed breeding, TILLING and EcoTILLING could help in development of energy dedicated crops for important traits such as higher yield, resource use efficiency, low recalcitrance and stress tolerance. In addition, genome editing and metabolic engineering techniques not only help in development of specifically designed energy crops but also bolster the biorefinery theme that ultimately aid in development of commercially viable techniques.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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