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Introductory Chapter: Bioengineered Sugarcane - A Sustainable Biofactory of Renewable Energy

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1. Introduction

Engineered sugarcane provides superior genotypes with a regulated expression of endogenous and exogenous genes, improved enzymatic reactions and sugar production, resulting in increased bioconversion of lignocellulosic biomass, recovery of sugars, and lipids for ethanol and diesel production.

The genus *Saccharum* belongs to the family *Poaceae* with five major species; *officinarum*, *sinense*, *barberi*, *robustum*, and *spontanuem*, developed through complex hybridization. The *Saccharum officinarum* is mainly grown for sugar in tropical and subtropical regions of the world. Where this crop meets up to 80% of world sugar requirement [1] is facing several problems including biotic and abiotic that affect its production. Conventional approaches lagged in solving the problems due to the complex genome and narrow genetic pool of sugarcane, however, advanced tools are employed to address biotic and abiotic problems, and to increase agronomic traits including yield, juice, and sugar. Further, improved sugarcane is being grown for bioethanol production in countries like the USA, Brazil, European Union, Guatemala, and China. Recently, sugarcane is engineered not only for bioethanol also for oil, hence it opens a window to develop purpose-grown sugarcane in the world. The chapter specifically highlights the technologies exploited in improving the crop for sustainable production of biofuel, including ethanol and diesel.

Though fossil fuels meet approximately 80 percent of the energy demand of the world and are required for economic development, the system is not renewable and causing global warming due to heavy CO₂ emissions. Global warming may affect the agenda of sustainable development therefore it is imperative to address this issue. The development of modern approaches adds to our confidence in addressing these objectives in the energy system.

2. Sugarcane genetic improvement

Sugarcane has several unique characteristics compared to other cereals. Sugarcane is highly polyploid at its genome level, carries photosynthesis through C₄ mechanism, chloroplast distribution in mesophyll and bundle sheath cells are few unique traits of this monocotyledonous plant. These traits of sugarcane make this plant one of the valuable specimens to explore its genetic potential. Despite considerable varietal improvement using conventional interventions, a serious

effort to develop elite genotypes is required. Major hurdles in the genetic improvement of sugarcane are due to its genetic complexity, low fertility, long cycle, and climate-specific flowering nature of the crop. Under this scenario, biotechnology can play a leading role to improve and introduce commercially important traits into elite genotypes.

Starting from the somaclonal selection under *in vitro* conditions to genetically engineering the genome of the crop each of the approaches has made it possible to develop new traits in sugarcane; including insect resistance, herbicide- and abiotic stresses-tolerance. Interestingly, with the introduction of synthetic elements, the pathways have been engineered in the sugarcane, resulting in various industrial and non-industrial products. Briefly describing the technology, each cell carries three major organelles; namely, nucleus, chloroplast, and mitochondria. Each of these organelles has its genome where the nuclear genome is routinely manipulated to develop new traits by exploiting two major approaches of gene transfer; *Agrobacterium*-mediated and biolistic-mediated gene gun methods. Of these two methods, particle bombardment is more successful to develop transgenic plants in sugarcane [2]. Several steps are involved to complete the process of genetic modification of the genome, starting from the choice of a gene to develop a trait, selection of a promoter and a terminator to regulate the expression of the trait-conferring gene, selection of a selectable marker for selection and purification of transformed cells, selection of an explant and optimization of regeneration medium by fine-tuning the concentration of the growth regulators, supporting the cell to complete cycle of differentiation and regeneration into a shoot.

To transform the chloroplast genome, the *plastome*, requirements of the transformation process are different. In this process, the antibiotics that normally kill prokaryotic organisms are preferred, keeping in view the mode of actions of the drugs. For example, spectinomycin, an aminoglycoside antibiotic is used to select and purify the transgene-recipient cells on regeneration medium for several plants including members of families; Solanaceae, *Brassicaceae*, Apiaceae, and Asteraceae; including; tobacco, potato, tomato, cabbage, oil rapeseed, carrot, and lettuce. However, monocotyledonous crops of the Poaceae family, like, sugarcane and rice are resistant to spectinomycin, naturally; therefore, another antibiotic namely, streptomycin is added to the regeneration medium for selecting the transplastomic clones. Chloroplast transformation in sugarcane has been reported recently [3] using a vector carrying fluorescent antibiotic resistance marker gene, FALRE-S [4]. The transformed plants remained heteroplasmic after successive cycles of selection and regeneration owing to the complex anatomy and polyploidy of the plastome, though, homoplasmic clones have been successfully obtained in several plant species [5]. The sugarcane plastome is very attractive when talks about metabolic engineering where high-level expression of genes and accumulation of the product is required.

2.1 Genetic improvement for insect resistance

Insects are serious threats to the sugarcane industry, worldwide, though exact assessment of economic losses is difficult to record. However, fragmented information is available on individual pests. Major pests of the crop are chewing, sucking insects, and canegrubs, and termites. Amongst borers, the most common are top, stem, and root borers.

Engineering the genome for enhanced resistance to insects is one of the success stories of transgenic technology. Several molecules including proteinase inhibitors (PI), secondary metabolites, ribosome-inactivating proteins, lectins, and δ -endotoxins of *Bacillus thuringiensis* [6–8], have been identified for effectively

controlling the insects. Therefore, developing insect-resistant sugarcane through a transgenic approach is required. Several transgenic sugarcane plants have been developed in different regions of the world keeping in view the type of insects invading the crop and resulting in economic losses. Successful examples are the development of transgenic plants addressing the problems of top borers using δ -endotoxins of *Bacillus thuringiensis* in Indian subcontinent, American and African countries. The larvae invade the sugarcane crop two times, at early and at a later stage, during the growing season. Early infestation causes serious damage to the crop by developing a 'dead heart', young shoots died as larvae chew the base of the shoot while later benefits to increase population and feed, triggering yield loss from 15 to 30 percent. How the process of 'dead heart' starts, the young larvae tunnels into the nucleus of the spindle and damage the growing point, causing the shoot to wilt and die. This requires the development of resistance against several factors including biotic and abiotic in sugarcane [9], but traditional improvement strategies are hampered by high polyploidy, genetic complexity, environment-specificity, and low fertility [10]. Thus biotechnological approaches have the potential to address such stresses by engineering resistance. Since the first genetic manipulation of sugarcane [11, 12], the development of transgenic clones is a routine [13, 14]. Several traits including effective control of stem borers have been introduced into the sugarcane [15, 16]. In these studies, a synthetic *cry1Ab* gene was selected to introduce and express under a tissue-specific promoter, PEPC. After bombardment the transformed cells, subsequently shoots were recovered on phosphinothricin-containing regeneration medium. Expectedly, the primary clones accumulated varied amounts of expressed CRY protein, ranging from 20 to 40 ng/mg. The variation in expressed protein levels is perhaps due to the random insertion of the transgene, varied copy number of the transgene in transgenic clones. Once, homozygous clones were recovered, the varied expression was documented as developmental and photosynthetic control of expression of the transgene. However, the expression levels were regarded as the highest levels of toxin reported so far in the literature. These levels were 13- and 35-fold higher than the highest levels of modified *cry1Ac* and *cry1Ab* gene, respectively [16–18]. The maize ubiquitin promoter was used to drive the synthetically developed *cry1Ac* gene in sugarcane for stem borers [16, 17], which is five to six times higher compared to the levels obtained by a constitutive promoter, CaMV 35S, in sugarcane getting high expression under PEP-C promoter were perhaps of using a C4 plant-specific promoter.

Interestingly, the detectable endotoxins levels varied from base to tip and from first to the outermost leaf of the whorl, depending on the developmental stage of the leaf. Measurements of the toxin levels in leaves showed that the accumulation of transprotein was low in young emerging leaves and increased with the leaf development. The maximum amount was recorded in the fully developed leaf on the same plant [18]. In another investigation, the *cry1Ac* gene has been introduced using a vector-dependent method for borer-resistance in sugarcane. The transgene was successfully introduced and transformed plants showed a high level of toxicity to *Sesamia cretica* giving 100% mortality of the larvae. These two reports are success stories of developing borer-resistant transgenic sugarcane [19]. Other success stories of developing transgenic sugarcane with different genes used for effective control of diverse insects are not included intentionally due to the word limit of the article, hence, my apologies for not including such valuable published reports.

2.2 Genetic improvement for disease resistance

Sugarcane is vulnerable to several diseases caused by different organisms including; bacteria, fungi, viruses, and nematodes. The diseases damage the crop

throughout the growing season and multiple epidemics by 64 diseases are reported that have damaged the sugarcane crop, worldwide. Of these diseases, five are caused by bacterial and 40 by fungal pathogens. However, the most common are rust, wilt, red rot, and smut that have seriously affected the sugarcane, demanding the development of disease-resistant varieties. In this section, only two diseases; one of fungal and the other of bacterial origin, are discussed.

Amongst fungal diseases, red rot is the most common disease of sugarcane and is caused by *Colletotrichum falcatum* Went that attacks the sucrose accumulating parenchyma cells [20] of culmus stalk of sugarcane, causing severe losses; up to 29% in the cane yield and 25 to 75% in the sugar recovery [21, 22]. Since sugarcane is a vegetatively propagated plant hence the pathogens spread through diseased-culmus-setts, stressing the availability of healthy canes for propagation. Instead of chemical, biological control of the red rot in sugarcane using *Trichoderma* (*Trichoderma harzianum* and *Trichoderma viride*) is reported [23, 24]. Of these two species, *Trichoderma harzianum* is more effective in controlling the *Colletotrichum falcatum*. It is interesting to know how *Trichoderma* which is a fungus controls another fungus *Colletotrichum*? Such invading fungi control others through the mechanisms of mycoparasitism and antibiosis. Mycoparasitism is one of the most important mechanisms for biocontrol of pathogen fungi that work through three steps: chemotrophic growth and recognition; coiling and interaction of hyphae and secretion of specific lytic enzymes [25]. Whereas, antibiosis is a shift to the concept of mycoparasitism that is based on lethal principle. *Trichoderma* produces low molecular weight and diffusible substances that penetrate the host cell thereby inhibit the uptake of nutrients, sporulation, production of metabolites, and the synthesis of the cell wall of the target fungus [26, 27]. Nevertheless, antibiosis is a species-specific mechanism.

Transgenically, the red rot was controlled by expressing genes from *Trichoderma* into the genome of sugarcane. Encouraged from the inhibitory effects of β 1,3-glucanase, isolated from *Trichoderma*, against pathogenic fungi, Nayyar *et al.*, [28] expressed the gene in sugarcane and analyzed the regenerating plants for transgene integration and expression. The transgene was expressed up to 4.4-fold higher than the non-transformed wild-type plants, and resistance to two pathotypes of *Colletotrichum falcatum*. The expression levels of the transgene were up-regulated after infection compared to the levels recorded before infection in engineered resistant sugarcane plants. However, pathogenicity tests on transgenic sugarcane against the virulent strains of *Colletotrichum falcatum* will demonstrate the resistance level. To demonstrate the pathogenicity, the transgenic sugarcane plants were challenged with two virulent strains (CF08 and Cf09) of *Colletotrichum falcatum*, and plants exhibited moderate to high-level resistance against Cf09 and Cf08, respectively. The structural model of the β -1,3-glucanase encoded protein demonstrated that two active sites namely; Glutamate 628 and Aspartate 569 of the catalytic domain of the protein are the main sites that have catalyzed the cleavage of β -1,3-glycosidic bonds and lysis of pathogen hyphae [29].

Further, another disease caused by a bacterial pathogen in sugarcane, the leaf scald, is also a serious threat to the sugarcane industry as it severely affects the yield. The disease is mainly distributed in the Philippines, Thailand, Myanmar, Vietnam, Java, Laos, Australia, and USA [30, 31] but now it is the most important quarantine disease in Taiwan, Guangxi, Fujian, Jiangxi, Yunnan, Guangdong, and Hainan in China [32]. The leaf scald, also called leaf burning disease which is caused by *Xanthomonas albilineans* infects the xylem vessels of the sugarcane plant. Recently, it is reported that it also invades tissues of leaves and culmus stalks, predominantly sucrose accumulating cells. Unlike other phytopathogenic bacteria, *Xanthomonas albilineans* lacks a type 3 HRP secretion system, a system that enables most bacteria

to overcome the defense mechanism of their host plants [32]. Yet, it is capable of invading other tissues, and developing symptoms in three stages namely; latent, chronic, and acute phases. It is observed that the bacterium remains dormant for more than 12 months without any symptoms. The bacterium infection develops symptoms of chlorotic stripes and patches on leaves, side shooting starts at the base of culmus stalks and burning of the leaf tips, and well-defined white pencil-lines along the veins during the chronic phase. Sometimes, the sudden death of whole stools.

Biotechnological interventions to address diseases are primarily focused on engineering the sugarcane genome and molecular breeding. The complete genome has been sequenced despite the complexities of ploidy levels, individual genes are transferred to engineer resistance traits. The coding region of an albicidin detoxifying gene (*albD*) from *Pantoea dispersa* has been expressed under *ubi* promoter from the maize and the *nos* terminator using *Agrobacterium* into the nuclear genome of the sugarcane plants found susceptible to leaf scald [33]. However, the transgenic plants were resistant to the disease since no chlorotic disease symptoms appeared in inoculated leaves, whereas non-transgenic plants developed severe symptoms. Further, a high concentration of accumulated transprotein has protected young stems against the multiplication of the pathogen. Thus, transgenic sugarcane clones conferred resistance to the disease as the plants showed no symptoms and multiplication of the bacterium [34, 35]. Introducing transgenes into sugarcane represents an important step forward, however, the growing investment in genomics and identification of resistance gene analogs (RGA), present in a majority of indigenous genotypes, might become an attractive alternative in disease resistance management through transgenic technology, and the development of screening tools of resistant genotypes.

2.3 Genetic improvement for sugar production and recovery

Sugarcane leaves contain both the mesophyll and bundle sheath cells. Photosynthesis occurs in the chloroplasts of both cells. Sugarcane unlike other organisms accumulates photosynthetic assimilates in the form of sucrose in the culmus stalk, thereby directly providing sugar for human consumption. Sucrose produced in the leaves is translocated from the source (leaves) to sink (parenchyma cells of the culmus stalk) through the phloem. The sucrose-loving parenchyma cells of the stalk accumulate the sucrose to an exceptionally high concentration i.e. up to 25–27% of the fresh weight [36, 37]. However, there is a correlation between photosynthetic activity and the sucrose contents in sugarcane. Toward maturity of the cane, the sucrose contents in the stem are increased whereas photosynthesis in leaves is reduced [38], indicating the regulatory mechanism of sucrose accumulation in the stalk cells [39]. Sucrose synthesis, transport, and accumulation in sugarcane are continuous processes.

Sucrose is accumulated in the stalk for metabolism and storage, and this accumulation is believably dependent on source supply, the storage capacity of the sink, and the metabolism in the parenchyma cells of the cane stem. Conceptually, the sucrose supply and demand situation determines whether the plant is source-limited or sink-limited. Accordingly, when source supply is limited in a plant to meeting the demand of the sink, then it will be a source-limited plant and when the demand is less than the source supply then it will be the sink-limited plant [40]. Generally, the photosynthetic activity falls with the maturation of the culmus stalk, and the rate of photosynthesis in high sucrose-accumulating elite lines is reduced to two-third of low sucrose accumulating lines, suggesting the role of the source-sink communication in the sucrose accumulation [38, 41]. For example,

Saccharum spontaneum accumulates sucrose at a lower level with high photosynthesis compared to the noble canes [42].

Sugarcane has developed a special mechanism of carbon fixation, and fix CO₂ in the form of four carbon molecules. This carbon fixation is facilitated by the coordination of cell types; mesophyll and bundle sheath cells, and hence photosynthetic functions are divided between these two cells. Carbon dioxide is initially fixed in the mesophyll cells, where it is converted into bicarbonate by phosphoenolpyruvate carboxylase. The product formed is oxaloacetate, which is reduced to malate by the NADP-dependent malate dehydrogenase. The malate is transported to the bundle sheath cells, located near the vascular system of the leaf, where it is converted to pyruvate, releasing CO₂ in the vicinity of the ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO). In bundle sheath cells the carbon dioxide is fixed by RuBisCO to energy-rich molecules such as glucose. After this step, the Calvin cycle proceeds as in C₃ plants. Knowledge of C₄ photosynthesis has made it possible to understand sugarcane productivity and highlights its importance to become one of the principal sources of carbohydrates for human consumption and bioethanol production.

Sucrose synthesized in the leaves of sugarcane has to be accumulated into the parenchyma cells of a stalk, experimentally it has been documented that sucrose is transported mainly through the symplastic system [43–45]. However, it is imperative to understand the distribution and accumulation of sucrose in three compartments namely; apoplast, cytoplasm, and vacuole to comprehensively overview the accumulation of sucrose in the parenchyma cells [46]. The transported sugar is unloaded into the apoplast and then transported to vacuoles. This transport of sugar to vacuoles is carried out by the sucrose transporters under low turgor conditions. Another way to translocate the unloaded sucrose is that the sucrose is hydrolyzed into glucose and fructose in the apoplast by the cell wall acid invertase and the end products (hexoses) are transported by hexose carriers, allowing the continued efflux of sucrose from the phloem. In this context, the cooperation of both the hexose transporters and the cell wall invertase is important and has been extensively reviewed elsewhere [47]. In plants sucrose is hydrolyzed by two enzymes; the sucrose synthase and cell wall invertase. Of these two enzymes, the sucrose synthase reversibly converts sucrose into glucose and fructose for utilization in respiration and cellulose biosynthesis [48], invertase irreversibly converts sucrose into glucose and fructose, exerting a pivotal role in carbon utilization and distribution.

Sucrose starts accumulating in the internodes at the early growth stage but the accumulation sharply increases when internode elongation stops [49], coinciding with the development of the stem and its elongating internodes [50]. Further, elongating internodes are characterized by a high ratio of hexoses to sucrose [51]. Higher concentrations of hexose in the developing internodes could be due to the predominant apoplasmic phloem unloading pathway as a result of sucrose cleavage by cell wall invertase and cellular uptake by hexose transporters [52]. Experimentally it has been demonstrated in radiolabeled sucrose transport studies that the majority of the translocated sucrose is taken into parenchyma cells of both developing and mature internodes, extensively reviewed [53], despite cell wall invertase activity was present in the apoplasm. Initially, vacuoles in the parenchyma cell of sugarcane stalk function as a sink to store sucrose through growth and development-dependent reversible process, but at a later stage when elongation of internodes stops then mature culm stores sucrose in the apoplast, intercellular spaces outside the plasma membranes.

Through the interventions of genetic engineering approaches, several attempts have been made to improve the sucrose contents of the sugarcane genotypes.

Sucrose synthase and cell wall invertase are the main regulatory enzymes of sucrose metabolism. When sucrose synthase was overexpressed in sugarcane, the contents were not improved [54]. Similarly, cell wall invertase was regulated to improve the sucrose contents of the sugarcane but no significant increase in sucrose accumulation was observed [55]. Though sucrose contents were increased in sugarcane cell suspension cultures by suppressing the invertase [56] yet it was not reproducible in stable transgenic sugarcane plants [57], indicating the role of the regulatory feedback mechanism between source and sink during accumulation [58]. Recently, two homologs of invertase inhibitors are identified in sugarcane, believed to be members of the superfamily of pectin methylesterase inhibitor, moderately conserved in plants [59]. To demonstrate where both enzymes are precisely located in the plant, both genes were fused with the green fluorescent protein and experimentally observed that ShINH1 is targeted to the apoplast. Further, expressed tissue specifically and is developmentally regulated, suggesting its role in metabolic regulation of sucrose between source and sink during sucrose accumulation in sugarcane. The gene expresses at relatively high levels in leaves and stalk but decreases significantly in stalk toward the maturity of the internodes. Experimentally, ShINH1 potently inhibited acid invertase, making it a candidate for controlling the deterioration of sucrose in sugarcane. However, experimental and *in silico* studies have revealed that both ShINH1 and ShINH2 have a role in sucrose accumulation and may contribute to the improvement of sugar yield and recovery in sugarcane.

2.4 Genetic improvement for bioconversion of biomass

Commercially, the juice is extracted from sugarcane and processed through multistep procedures to develop sugar, ethanol, or biodiesel, leaving fibrous material, bagasse, in a large volume of approximately 540 million metric tons on yearly basis, worldwide, which is used in sugar mills as a furnace fuel and apparel industry for paper or board manufacturing, however, its use as a feedstock for cellulosic ethanol is gaining importance and several independent reports have been published on its chemical composition, constituents preparation, and utilization. Sugarcane bagasse consists of 41 to 55 percent cellulose, 20 to 27.5 percent of hemicellulose, 18 to 26.3 percent of lignin, and around 7 percent of others by weight. Of these three major constituents, cellulose and lignin have gained much attention to develop byproducts, and/or for value addition [60].

Bioconversion of lignocellulosic biomass from bagasse has gained much attention of the plant biotechnologists and microbiologists for bioethanol production and value-added products as bagasse has long been considered in bioethanol industries for ethanol production, referred to as 'second-generation ethanol'. Biotechnological interventions have enabled the efficient bioconversion of bagasse through the introduction of improved microbial strains, media formulations, and product recovery processes. As the bagasse has a complex structure, therefore it is pretreated to dissociate the lignin-cellulose and to increase the surface area for better enzymatic activities to convert the biomass to fermentable sugars. Different approaches including; biological treatment, dilute acid hydrolysis, alkali hydrolysis, and solvent-based pretreatment have been reported for *saccharification* of the bagasse. However, all such processes significantly add to the cost of bioethanol production from lignocellulosic biomass [61].

The saccharification process is affected by the high contents of lignin and lignin syringyl to guaiacyl (S/G) ratio in the bagasse hence the composition of the lignin is needed to be changed as they prevent cellulase from accessing the cellulose molecules in the process of ethanol production [61]. Biotechnological approaches may help in altering the composition of this fibrous material by regulating the genes

involved in its biosynthesis pathway. Three key genes; namely, *COMT* (caffeic acid *O*-methyltransferase), *CCoAOMT* (caffeoyl-CoA *O*-methyltransferase), and *F5H* (ferulate 5-hydroxylase) have been targeted employing techniques; RNAi (RNA interference), TALEN (transcription activator-like effector nuclease), and CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/case9. The gene, *COMT* was downregulated from the biosynthesis pathway of lignin using the RNAi approach, and the expression of the gene was lowered by 67–97%, and the lignin contents were reduced by 3.9% to 13.7%, respectively. Further, the S/G ratio in the lignin was reduced from 1.47 in the wild type to values ranging between 1.27 and 0.79. Consequently, levels of fermentable sugar were increased up to 29% without pretreatment however, these levels were further increased by 34% when biomass was treated with dilute acid [62]. Further, *COMT* mutant lines developed by targeting the conserved region of the gene through the TALEN approach showed lignin reduction up to 19.7% with 43.8% improved saccharification efficiency. The lignin contents were further reduced from 29 to 32% in *COMT* mutants [63]. Owing to the highly complex and polyploid genome, targeted mutagenesis using CRISPR/Cas9 could be a valuable tool to reduce the lignin contents.

As far as the bioconversion of the lignocellulosic biomass to fermentable sugars is concerned, significant quantities of cellulolytic and hemicellulolytic enzymes are required, and currently, microbes are used to produce these enzymes that have substantially increased the production cost of bioethanol. Therefore, *in planta* production of enzymes could have a significant impact on the economics of bagasse-based ethanol production. Several independent reports have been published describing the hydrolysis of pretreated biomass by mixtures of purified cellulases from wheat and barley straw, corn stover, switchgrass, and poplar. Afterward, genes encoding the enzymes like; cellobiohydrolase I (CBH I), cellobiohydrolase II (CBH II), and endoglucanase (EG) were stably introduced into the genome of the sugarcane, and the accumulation of enzymes was confirmed in the leaves of plants [64]. However, the enzymes CBHI and CBHII of fungal origin remained resistant to proteolysis during sugarcane leaf senescence, while bacterial origin (EG) was degraded, demonstrating the stability of recombinant cellulase in transgenic sugarcane. When tissue-specific regulatory elements, with or without targeting sequences, were used to control the expression of transgenes the accumulation of enzymes was variably enhanced. All three enzymes accumulated to higher levels when targeted to vacuoles however, the highest levels of accumulation of endoglucanase were recorded when the enzyme was targeted to the chloroplasts. This practically demonstrates a significant step forward for the economic production of lignocellulosic ethanol. Hence, such initiatives could advance cellulosic ethanol technology.

2.5 Genetic improvement for ethanol production

Renewable fuels are an attractive alternative to petroleum-derived gasoline since it potentially lowers the production of greenhouse gases, and add to the country's economy. Amongst biofuels, bioethanol is being produced from non-food as well as food parts of the plants including sugarcane. It is prepared from can juice, molasses, and bagasse. The renewable bioethanol industry has been successfully developed in countries like the USA, Brazil, Mexico, the EU, and China, and bioethanol is being used to unravel the energy crisis. Developing bioethanol from non-food sources has emerged as a trend due to several advantages like availability of abundant raw materials, low price, and renewability. Such bioethanol industry is strategically being developed in China.

Bioethanol production in the USA is from corn with a maximum production of 56.7 billion liters from cereal grains, though the focus has been shifted recently

from corn to cellulosic ethanol, facing many challenges like feedstock availability and high conversion costs. Brazil, the largest sugarcane-based ethanol producer in the world is pioneering in using bioethanol as a motor fuel. In 2019–2020, Brazilian ethanol production reached 34 billion liters to meet the domestic market demand. To increase the target of 50 billion liters of ethanol the Brazilian industry is increasing its fermentation efficiency from 83–90%. This will enable the ethanol industry to meet the environmental targets established by Brazil at COP21, The *United Nations Climate Change Conference*, which was held in Paris, France, from 30 November to 12 December 2015. In the market, it is being sold in the form of either pure ethanol fuel (E100) or blended with gasoline (E27). Presently, ethanol production from sugarcane produces nine times more energy than the energy consumed during its production process [65]. Hence, bioethanol is produced directly from the sugarcane juice converting this into ethanol through the fermentation process using microorganisms as sugarcane juice is rich in sucrose, glucose, and fructose. During the fermentation process, the sucrose is readily broken down into glucose and fructose by the yeast invertase. During the process, the sugarcane stalk is crushed and milled with water. The extracted juice is heated at a temperature of 115°C. After heating, the juice is treated with either sulfuric acid or lime, and the excessive inorganic compounds are precipitated. Afterward, the heated juice is cooled down and yeast is added along with nutrients for yeast growth. Fermentation can be carried out in either batch or continuous reactors, however, continuous reactors are used in Brazil.

Biotechnological attempts have been made to improve the sugarcane for agronomic traits and recently, several interventions have been made to improve the genetic makeup of the plant as well as of microorganisms used to develop ethanol from sugarcane. The *Saccharomyces cerevisiae* is commonly used in the fermentation process of sugarcane to develop ethanol. One of the potential candidate genes associated with ethanol fermentation is PHO4, a phosphate regulon [66]. The replacement of the gene from fast-growing strain MC15 to a high ethanol-producing strain MF01, exploiting the homologous recombination approaches, has improved the ethanol yield to 5.30% as the maximum yield harvested was 114.71 g/L and has decreased the fermentation time to 12.5%, compared to the non-recombinant MF01 strain. So, the engineered strain will not only be improving the yield of the ethanol also reducing the fermentation time. In another experiment, the F-514 strain of the *S. cerevisiae* was used to ferment sugars from sugarcane molasses in hot and dry weather, and it was reported that by improving the fermentation parameters ethanol yield was improved. In addition to yeast strains, the pretreatment of molasses or can juice with lime or sulfuric acid affect the ethanol yield in the fermentation process. Raharja et al. [67] used commercial instant dry yeast to simplify the production process and reduce the bacterial contamination risk. Using the molasses as starting material, containing a very high concentration of sugar (30%), and pretreated with sulfuric acid produced ethanol at a very low level, lower than the levels attained using bagasse as feedstock. Hence, producing ethanol from feedstock like sugars and molasses and its application as an energy source may not be desirable on a long-term basis as the demand for fuel, food, and feed is rising, demanding alternative means of cost-competitive and sustainable supply of feedstock. Further, the bioethanol producing industries in the USA as well as in Brazil have decided to develop second-generation ethanol (2G) from lignocellulose materials, requiring new production models [68]. Biomass, which is burnt to produce energy in sugar industries could be used as feedstock to convert polysaccharides into ethanol [68]. The pretreatment of biomass to extract hemicellulose is carried out either by biological or chemical (diluted acids) treatment or through steam. Bioethanol is produced from hemicellulosic hydrolysate using yeast, immobilized on magnetic

particles, in the fermenters under a magnetic field. It has been reported that ethanol production was 34% higher in bioreactors assisted with axial rather than by transversal magnetic field.

2.6 Genetic improvement for oil production

Biofuel's largest markets in the world are in the USA, Brazil, the European Union, and China, collectively producing 85% of global biofuel with a 48% share of the USA alone. Of the total biofuel production, 82% share is of bioethanol, which is majorly produced by the USA and Brazil. However, biodiesel is the second majorly produced biofuel in the world, with a 49% contribution from the European Union. The biodiesel contribution of the EU, USA, and Brazil is 34.1%, 19.5 and 12%, respectively. The feedstock used to extract oil is rapeseed and or cooking oil in the EU whereas in the USA and Brazil is soybean. The soybean has been grown on 33 million hectares in the US with per hectare oil production from 0.36 to 0.61 MT/ha [USDA, 2015]. However, total biodiesel production would have been only about one-tenth of the US distillate fuel oil consumption (about 155 million MT) by crushing the total soybean produced [69]. This situation demands the introduction of other crops, and interventions of recombinant technology to engineer metabolic pathways in plants to accumulate oil, and could be grown on marginal land avoiding any possible competition with major food and feed crops. Metabolic pathways have been engineered in tobacco and *Arabidopsis*, highly regarded model plants used in genetic engineering experiments, to accumulate triacylglycerol [70]. In another independent study three genes namely; WRI1 (WRINKLED1), DAGA T1–2 (diacylglycerol acyltransferase1–2), and OLE1 (Oleosin1) were expressed, and the triacylglycerol was accumulated in leaves and culmus stalk of sugarcane [71]. The accumulation of triacylglycerol added to the total fatty acid contents of up to 4.7% and 1.7% of dry weight in mature leaves and stems, respectively. Interestingly, confocal micrographs have shown the presence of lipid droplets within the transgenic mesophyll cells, indicating a step forward in the accumulation of high levels of triacylglycerol in sugarcane. In another independent study, multiple genes were either expressed or suppressed in sugarcane to increase the lipid contents in the vegetative biomass. The genes CYSOLE1, DAGAT1–2, OLE1, WRI1 were co-expressed, whereas, *tgdl* and *sdp1* were simultaneously suppressed in the sugarcane and an elevated amount of the TAG was recorded. The transgenic plants with constitutively co-expressed CYSOLE1, DAGAT1–2, OLE1, WRI1, and simultaneously suppressed *tgdl* in different plasmids elevated the TAG accumulation by 277-fold and 109-fold in leaf and stem tissues, respectively [71]. Constitutive co-expression of CYSOLE1, DAGAT1–2, WRI1, and co-suppression of *tgdl* and *sdp1* in a single construct elevated the TAG content by 404-fold in leaves. These findings need further confirmation of TAG accumulation with large-scale field testing. Developing dual-purpose feedstock by either overexpressing or suppressing the genes to produce both ethanol and biodiesel is becoming a routine. Transgenic sugarcane plants were developed by co-expressing genes, namely; WRINKLED1, DAGAT1–2, Oleosin1, and suppressing the AGPase and PXA1 genes using the RNAi approach. The AGPase encodes ADP-glucose pyrophosphorylase while PXA1 is a subunit of the peroxisomal ABC transporter1. The engineered plants accumulated triacylglycerol to 5% higher than the non-transformed plants [69]. The TGA levels were 31–33% of the total lipid contents in the transgenic plants. From these canes (**Figure 1**), both sugars and lipids were extracted where the sugar extraction efficiency was as high as 90% with repeated hot water while of lipids was 60%, demanding an improved process to increase the lipid extraction efficiency and for commercialization.

Figure 1.
Schematic representation of improvement of sugarcane genotype for renewable energy and byproducts.

3. Conclusion

The sugarcane genome is highly polyploid with specialized leaf anatomy, the Kranz anatomy where mesophyll cells are arranged around the bundle sheath cells. Mesophyll cells are better connected to the environment and perform functions of phosphoenolpyruvate carboxylase (PEPC). Whereas, bundle sheath cells are rich in chloroplasts to carry out functions of Ribulose-1,5-bisphosphate carboxylase-oxygenase (rubisco) to fix CO₂ to synthesize sugars in the Kelvin cycle. The CO₂ concentration mechanism in sugarcane allows the development of the highest annual yield of biomass. Plant biotechnologists have engineered sugarcane for agronomic as well as value addition traits. Hence, the yield of fermentable sugars could be enhanced by improving the translocation and by bioconversion of lignocellulosic biomass for bioethanol and biodiesel production, sustainably. Further, Cellulose and lignin could be separated and used to develop high-quality fiber and lignosurfactants, respectively.

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