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Exploring Scope for Breeding of Rice Varieties for Efficient Ethanol Production without Compromising Seed Yield

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ABSTRACT

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Rice straw being rich in cellulose and plentifully available, it can serve as the chief renewable biomass resource for biofuel production. Several protocols are now available for efficient recovery of ethanol from cellulosic substrates of rice straw. Rice varieties showing high lignocellulosic straw biomass inherently reveal low harvest index and consequently low grain yield potential. There is an urgent need to reorient the breeding strategies for efficient ethanol production without compromising seed yield. The authors reviewed the scope of rice straw for biofuel (ethanol) production and also the genetic basis for biosynthesis of lignocellulose components and their breeding perspective for development of bioenergy efficient smart varieties in rice.

Introduction

Petrochemicals are the chief inputs for transport and agricultural mechanization. India being an agrarian and second populous country next to China; petroleum becomes the lifeline for Indian economy. In recent years, climate change and energy security are the top agendas in almost all countries. Globally, rice covers about 160 million hectares with a quantum of 740 million tons grains and 730 million tons of straw (FAO, 2014). Asia alone produced 651.58 million tonnes, accounting for 90% of the world's rice output. To keep

pace with the world's growing population, rice production is to be increased by 40% by 2030 (Huang *et al.*, 2013). Rice straw is used as cattle feed, thatch of rural houses, mushroom production and rest as waste. With increasing scarcity of labour and difficulty in transport of huge bulk of rice stalks; large part of this undergoes field burning that increases the air pollution and consequently affects public health (Mussatto and Roberto, 2004). Alternatively, rice straw being an abundantly available lignocellulosic biomass (structural carbohydrates) and a non-food item, it can serve as potential substrate for biofuel

(ethanol) production. In the present pursuit, the authors reviewed the scope of rice straw for ethanol production and also the genetic basis for biosynthesis of lignocellulose components and their breeding perspective for development of bioenergy efficient smart varieties in rice.

Composition of lignocellulose components in rice straw

Rice straw contains higher amount of cellulose (32-47%) and hemicellulose (19–27%) as cell wall components (Saha, 2003). Cellulose part of cell wall contains parallel arranged long fibres while, hemicelluloses make cross-linking with cellulosic fibres. Structurally, cellulose is the major component of plant cell wall and it is composed of linear chain of D-glucose linked by β - (1, 4)-glycosidic bonds to each other. While, hemicelluloses is a heterogeneous sugar complex of pentoses (β -D-xylose, α -L arabinose), hexoses (β -D-mannose, β -D-glucose, α -D galactose) (Fig. 1) and/or organic acids (α -D glucuronic, α -D-4-O-methyl-galacturonic and α -D-galacturonic acids). Both of these are readily fermentable by microorganism mediated hydrolytic enzymes to form ethanol. The ethanol so produced may be blended with petrol and gasoline to reduce cost of petrol. But, higher amount of lignin (5-24%), ash (10-17%), silica (75% of the ash) and potassium in rice straw are the deterrent factors for conversion of cellulose and hemicellulose to fermentable sugars. However, different pre-treatment methods are now available to make the whole process economically viable. In fact, lignin is an aromatic, amorphous and hydrophobic polymer synthesized from phenylpropanoid precursors which forms inner and outer rigid cell wall matrix. High silica content wears out the cellulosic fibres and such structural aberration interferes sugar recovery in follow-up steps. Ash content in rice straw is much higher than that in wood. Ash and potassium

cause agglomeration of the components during processing; hence these seem to be barriers in conversion to sugar.

Estimation of cell wall components

Extraction of lignocellulose components is expensive and energy intensive. Rice straw treated with NaOH dissociates the hemicellulose component of the cell wall, while cellulose fraction is solubilised by treatment with concentrated H_2SO_4 . A number of methods are now available for isolation of cellulose, hemicelluloses and lignin component in rice straw depending upon kind of hydrolysing agent used (alkali, inorganic acids, organic acids) alone or in combination with thermal treatment, temperature, duration and pH condition during extraction process. However, a simple and cost effective direct method (Moubasher *et al.*, 1982) may be followed with minor modification. Air dried small pilled rice straw is boiled in ethanol for 15min, washed thoroughly with distilled water and kept in oven for dry weight at 40°C overnight, then divided into two parts in which one part is considered as A fraction. Second part of residue is refluxed at 80°C with 0.5M NaOH (100ml/g of biomass) for 4 h followed by filtering and washing the residue with plentiful of distilled water until pH 7.0, dried at 80°C over night and the dry weight taken as B fraction. A-B value is the hemicelluloses content in the rice straw. The same sample(B) is again soaked with concentrated H_2SO_4 (30ml/g of biomass) at room temperature for 24 h, and then refluxed at 100°C for 1h followed by filtering and washing the residue with distilled water until pH 7.0, dried at 80°C overnight and the dry weight taken as C fraction. B-C value gives cellulose content while, C value itself is the lignin content in rice straw. However, modern analytical methods e.g., Near Infrared Spectroscopy (NIRS) and Near-field Thermal Radiation (NFTR), Fourier-transform infrared

spectroscopy (FT-IR), Scanning electron microscope (SEM), Energy-dispersive X-ray analyzer (EDX), Thermo-gravimetry Differential Thermal Analyzer (TG-DTA) and (Gas Chromatography-Mass Spectrometry (GC-MS) can be used for rapid assessment of polysaccharide components in rice straw.

Ethanol production from rice straw

Rice straw is the cheap source of abundant lignocellulosic waste materials for ethanol production. Rice straw is grounded by several methods (ball milling, roll milling, wet disk milling etc.) prior to its pretreatment to permit enzymatic saccharification. Cellulose and hemicellulose components are required for ethanol recovery while lignin component of rice straw needs to be separated as waste material. Cellulose is first converted to fermentable sugars (glucose) by cellulase, while, hemicelluloses are hydrolysed to produce pentose sugar (xylose) and small fraction of other sugars e.g., arabinose, galactose, and mannose) by hemicellulases produced by specific microorganisms (biological saccharification). These simple sugars are fermented to produce ethanol using yeasts. Ethanol so produced is then fractionated through a series of purification steps.

Pre-treatment

Pretreatment of rice straw plays an important role to increase the efficiency of enzymatic saccharification. This allows structural loosening of cellulose and hemicelluloses (decrease the crystallinity and degrees of polymerization of cellulose) from the lignin component (Fig. 2) and thereby enhances the accessibility of the cellulosic substrate (carbohydrate polymers) for conversion to fermentable sugars in the follow-up step. A number of physical, chemical, biological and thermal pre-treatment methods and their

combinations are now available. Acid digestion in dilute HCl, sulphuric acid(0.6 % v/v), acetic acid or formic acid, fumaric acid, maleic acid, lactic acid, oxalic acid etc. at 140°C is needed to solubilise hemicelluloses. Alternatively, alkali (NaOH and KOH) (Gaspar *et al.*, 2007), hydrogen peroxide (oxidizing agent) (Hon and Shiraishi, 2001) or ammonia (Kim and Lee, 2007) may be used for the purpose. 2% NaOH pretreatment for 3 hours results high total carbohydrate content while high concentration of NaOH (12%w/v) for 1 hour is best for delignification of rice straw. A two-step pretreatment of rice straw with dilute sulfuric acid and aqueous ammonia is reported to offer more efficient conversion to fermentable sugar (Kim *et al.*, 2013). Besides, thermal treatment e.g., microwave (Xiong *et al.*, 2000), electron beam irradiation (Jin *et al.*, 2009), ultrasonic (Wongjewboot *et al.*, 2010) and popping method (Wi *et al.*, 2013) proved beneficial prior to enzymatic hydrolysis. Lu and Minoru (1993) reported radiation pretreatment of rice straw in the presence of NaOH solutions using an electron beam accelerator. A 0.16 kW/L ultrasonic treatment helps in breaking of lignin and exposition of cellulose surface. Popping method offers two fold increase in surface area of rice straw than control for more accessibility to hydrolytic enzymes in the follow-up step. However, biological pretreatment using white-rot fungi (*Pleurotus ostreatus*) belonging to Basidiomycetes seems to be a safe, cost effective and environment-friendly method (Taniguchi *et al.*, 2005) for lignin removal from lignocellulose.

Enzymatic saccharification

The cellulose usually contains only glucans, whereas hemicellulose contains polymers of several sugars such as, xylan, glucan, galactan, mannan and arabinan. Readily decomposable rice straw is all time needed for higher recovery of ethanol. The saccharification step

involves conversion of cellulose and hemicelluloses to fermentable sugars (Fig. 3). By enzymatic hydrolysis with a commercial cellulase and β -glucosidase preparation for 48 h, about 52% holocellulose (cellulose + hemicellulose) and 44% cellulose in the pre-treated rice straw were solubilised. *Trichoderma koningii* BTS120, *Aspergillus niger* BTS149, *Aspergillus tubingensis* BTS83 and *Penicillium roquofrti* BTS12 culture filtrates are the best producer of extracellular cellulases and hemicellulases and crude enzymes in liquid culture. The concentration and rate of glucose production depends on pretreatment of rice straw, substrate concentration and cell loading with microbe filtrates. Glucose yield from rice straw by *Aspergillus niger* is reported to increase from 43 to 87% (Aderemi *et al.*, 2008). Besides, the supplementation of non-cellulolytic enzymes such as xylanase, pectinase, feruloyl esterase is reported to enhance hydrolysis of lignocellulosic biomass (Tabka *et al.*, 2006).

Fermentation

Glucose and xylose are two dominating sugars in the lignocellulosic hydrolysates produced at the saccharification step. For follow-up fermentation, two specific microorganisms are necessary for co-fermentation of these sugars at suitable temperature and duration (Fu and Peiris, 2008) for production of ethanol. At this step, yeast converts the carbohydrates into alcohol and CO₂. Belal (2013) reported highest ethanol concentration (about 10 and 11 g/l) after 7 days fermentation with yeast (*Saccharomyces cerevisiae*). A thermophilic yeast *Kluyveromyces* sp. IPE453 can be suitably used for fermentation process. Kundu *et al.*, (2015) reported that theoretical ethanol yield can be as high as 90.93% by simultaneous saccharification and fermentation (SSF) at the optimum conditions. Wang *et al.*, (2015) has shown 77.3% increase in bioethanol production by SSF using a

specific strain of yeast *S. cerevisiae* KF-7 as compared with the 74.4% glucose recovery efficiency of enzymatic saccharification (Wang *et al.*, 2015). However, microorganism e.g., *Clostridium phytofermentans* which can efficiently ferment cellulose (in the straw) directly to ethanol, may be considered as the most suitable candidate for ethanol production. Besides, the advent of genetically modified yeast and suitable synthetic hydrolysing enzymes may also improve bioethanol yield from rice straw.

Fractional distillation and purification

The alcoholic mixture referred to as 'mash' produced in the previous step is fed into a fractional distillation apparatus to separate the alcohol from other components of 'mash'. Further, the raw alcohol so produced is cleaned again following dehydration/drying process to remove water contents. The final product after a series of cleaning and drying process is the bioethanol with an extremely high purity of 99.7% (v/v).

Rice- a candidate crop for biomass production

A kilogram of milled rice is produced against roughly 0.7–1.4 kg of rice straw depending on cultivars. On an average, 60% of a rice plant at maturity is straw, 14% constitutes bran and husk; while rest 26% is milled rice. Elevated (CO₂) significantly increased straw biomass (Zhu *et al.*, 2016). Usually, landraces have lower rates of CO₂ assimilation, increased transpiration via greater stomatal conductance, and a higher ratio of internal to atmospheric CO₂ than advanced varieties. Stems constituted the largest component (34–55%) of total dry biomass compared to leaf (23–37%) and leaf sheath (23–33%) (Jahn *et al.*, 2011). Therefore, selection of thick tillers is necessary for high cellulosic biomass. Leaf traits, such as leaf thickness, size, and shape,

leaf number, and orientation, plant height, tiller diameter, tiller length, tiller number, and straw girth are key factors influencing biomass formation (Yang and Hwa, 2008). Physiological traits related to growth and carbon capture, including leaf-area based photosynthesis, transpiration, stomatal conductance, and water use efficiency (WUE) can elucidate the process of biomass accumulation. In rice, erect leaves have a higher leaf area index that increases photosynthetic carbon assimilation rates through increased light capture and nitrogen use efficiency (Sakamoto *et al.*, 2006).

Selection for high harvest index is expected to favour alleles that reduce vegetative biomass and favour grain yield. Hence, there is need to assess genetic variation and heritability of key traits that significantly impact biomass and the genomic regions associated with this variation. Biomass genetic variation in the OryzaSNP set can be a good option for biomass gene discovery. The dry biomass yield of Pokkali was about 11-fold higher than other low biomass varieties (Jahn *et al.*, 2011).

Genetic basis of cell wall components

Paddy straw with high cellulose content results better saccharification yields. Genetic variation in saccharification yields do exists among rice cultivars (Abe *et al.*, 2016) and thus, it will be a breeding target for biofuel production. Saccharification yield is high at tillering and before heading, but reduced after heading.

Stems show highest response at all growth stages while; leaf blades usually show lowest saccharification yields. Teramura *et al.*, (2015) reported that the glucose content in the liquid hydrolysate is highly correlated with the resulting ethanol yield. So, glucose content can be considered as factor in selection of rice cultivars most suitable for bioethanol

production. Goda *et al.*, (2016) evaluated the natural variation of glucose content in dilute sulfuric acid pre-treated rice straws of 208 diverse cultivars. The glucose content varied across cultivars although largely affected by temperature. Hence, there is ample scope for development of novel rice cultivars for bioethanol production.

Recently, a novel mutant allele Osfc16 of cellulose synthase gene CESA9 showed normal plant growth with 25–41% more biomass yield as compared to wild type (Nipponbare, a japonica variety) and the mutant enhanced enzymatic saccharification of biomass up to 2.3fold and ethanol production by 34.42% (Li, 2017).

Reddy and Yang, (2006) reported a novel long natural cellulose fibre variant in rice with tensile strength similar to linen fibre. In contrast, Sharma (1986) isolated a brittle stem mutant of rice in which the so called parallel arranged cellulosic fibres are frequently broken resulting lower cellulose content. But, surprisingly, such mutants revealed higher hemicelluloses and lignin content than normal rice genotypes.

Cellulose fibrils are synthesized at the plasma membrane, whereas matrix polysaccharides (pectin and hemicellulose) are assembled in the Golgi cisternae and exported to the cell surface by Golgi-derived vesicles to form cell wall. Hemicellulose biosynthesis in the Golgi apparatus is mediated by seven GT (glycosyl transferases) gene families. On the other hand, Xie *et al.*, (2011) identified sixteen QTLs for cellulosic fiber and lignin contents from an interspecific cross *Oryza sativa* × *Oryza rufipogon*. Most of the QTLs revealed small genetic effects and none of these were common in two environments suggesting significant environmental influence in biosynthesis of fibres.

Fig.1 Chemical structure and composition of cellulose and hemicellulose of rice straw

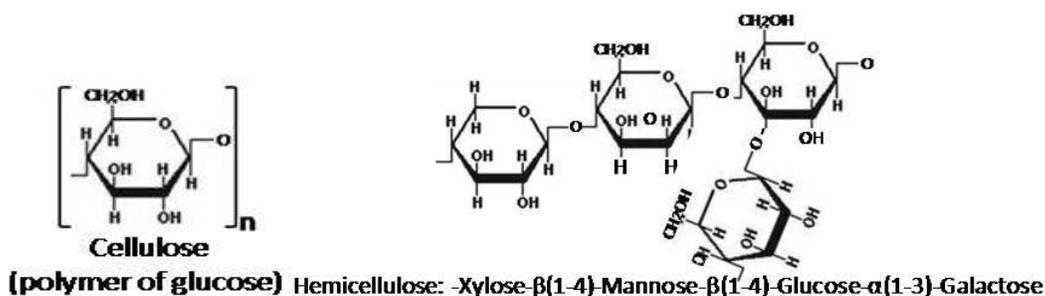


Fig.2 Effect of pre-treatment on lignocellulose complex

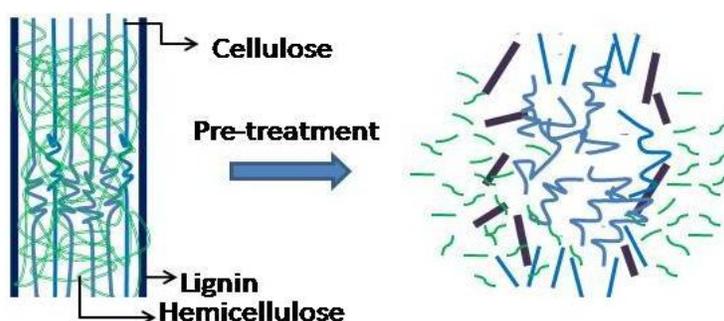
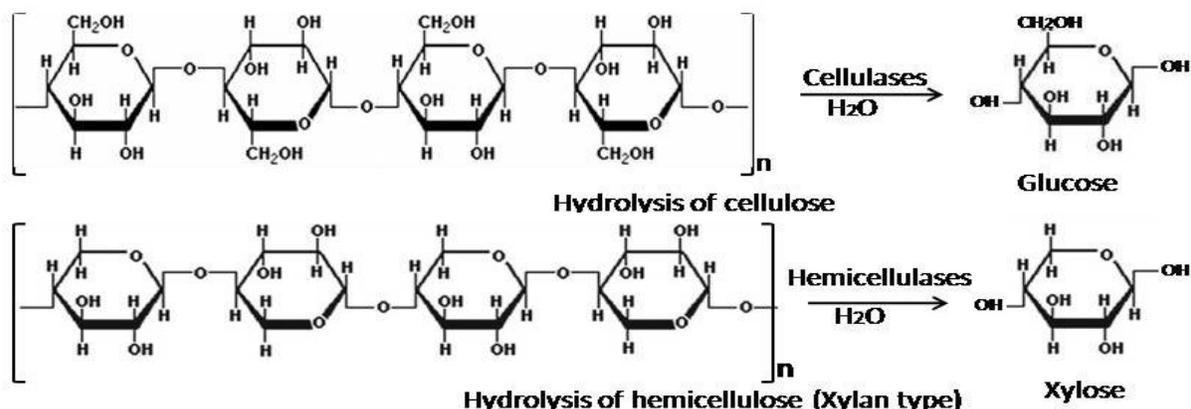


Fig.3 Enzymatic saccharification of cellulose (top) and hemicelluloses (below)



Further, they identified co-location of yield and fertility QTLs with those for fibre content within same marker interval in most of the cases which paves the way for development of dual-purpose rice varieties that are high in grain yield and are also high in straw quality.

Liu *et al.*, (2016) developed recombinant inbred lines (RILs) from a cross between IR1552 (lowland rice) x Azucena (upland

rice) and identified several cell-wall associated candidate genes using quantitative trait SNP (QTS) analysis using 271 molecular markers. One QTS locus and 4 pairs of epistatic loci had shown positive contribution to the enzymatic digestibility phenotype.

These loci revealed inverse relationship between reducing sugar and lignin content for aminability to enzymatic digestibility. The

above QTS may be monitored using marker-assisted breeding to develop high saccharification rice varieties.

Genotypic variation in lignin content in rice straw is reported to be ranged from 1 to almost 12% (Arai-Sanoh *et al.*, 2011) although the estimated values largely differs between laboratories and method used. Ookawa *et al.*, (2014) identified a natural long-culm rice variant with increased lodging resistance and low lignin (gh2) content amenable for bioenergy production. The gh2 locus is located in chromosome 2 and reduced synthesis of cinnamyl-alcohol dehydrogenase (CAD). Reduction of lignin in rice has been also achieved by RNA interference of the lignin biosynthesis gene Os4CL3 (Vanolme *et al.*, 2010).

Genome wide association (GWAS) survey using high throughput SNPs can help to identify candidate genes (Wood *et al.*, 2017) without complete elucidation. Mining the wide diversity of candidate genes associated with lignin and cellulosic fibre biosynthesis is now possible (Naz *et al.*, 2017) using GWAS.

The analysis allowed us to identify regions of the genome containing significant associations between molecular markers and phenotypic data of the trait, but it is beyond reach for cell wall genes owing to difficulty in unbiased phenotyping of large number of genotypes using conventional analytical approaches. Recently, relative quantification of cell wall components has been made possible using carbohydrate microarrays and the technique could be suitably used for phenotypic characterization of genotypes. This paves the way for cell wall candidate gene identification through GWAS (Wood *et al.*, 2017). Houston *et al.*, (2015) detected a few putative cell wall candidate genes in barley that co-expressed with members of the cellulose synthase gene family.

Exploring breeding strategy in rice

Continued use of semidwarf varieties in conjunction with higher rates of nitrogen application has resulted in doubled grain yields. Seed yield usually negatively correlated with biomass and cell wall components. Modern high yielding cultivars are dwarf with moderately short erect leaves which reduce biomass yield. Cytokinin oxidase (OsCKX2) degrades Cytokinin resulting suppressed canopy growth and improved panicle characteristics and consequently increasing grain production in dwarf cultivars without lodging. Conversely, the tall indica rice varieties inherently results high biomass production with less partitioning of the same to the sink (low yield). A QTL-based strategy can improve lodging resistance even in tall plant types. In this context, it becomes a challenging task to breed a novel genotype with high grain yield alongside high biomass production. This can be achievable if the molecular mechanisms (genes) that regulate grain productivity, biomass production and aminability to its decomposition are critically elucidated. From productivity point of view, an ideal ideotype of rice should be intermediate in plant height with deep root system, moderate tillering, erect leaves, long heavy panicle, high grain number and resistance to biotic and abiotic stresses to improve yield potential. Rice varieties differ in composition and configuration of lignocellulose components. Rice straw with higher amount of cellulose in relaxed form (preferably less complexed with hemicelluloses) and low lignin content are most preferred for its easy decomposition and efficient ethanol production. The thumb rule is that the green plant stalks are more decomposable than the same after harvest. Hence, cultivars with stay green (SGR) trait will be rewarding. In wheat, functional stay green mutants maintained longer photosynthetic activity along with more seed

weight and seed yield than the parent genotype. Post-flowering drought adaptation is reported to be associated with the stay-green phenotype in sorghum (Harris *et al.*, 2007). The wild SGR allele (conditioning chlorophyll degradation by chlorophyllase) is expressed in green tissues and it is down regulated in leaves at post flowering stage causing senescence (Rong *et al.*, 2013). Currently, a functional SGR mutant trait has been elucidated in japonica rice (Fu *et al.*, 2009). Stay green rice cultivars retain green flag leaves and their stalks still maintain more water content even at physiological maturity. Such elite cultivars are expected to be high yielding with ease in decomposition of plant stalks.

Chlorophyll degradation by SGR gene (wild allele) is stimulated/triggered by ABA but inhibited by cytokinins (6-benzyladenine) (Park *et al.*, 2007). Specific chlorophyllase gene(s) can be induced by methyl jasmonate and ethylene to stimulate chlorophyll breakdown similar to chlorophyll degradation under dark (dark induced senescence). Antisense suppression of a senescence related chlorophyllase gene in transgenic broccoli resulted reduced rate of post-harvest chlorophyll loss (Barry, 2009). Two separate stay green QTLs, one in flag leaf and other on penultimate leaf are co-located between flanking markers RM 455 and RM 505 on ch.7 and another two stay green QTLs specific to above respective leaves are co-located between flanking markers RM 410 and RM 566 on ch. 9. Besides, two other QTLs one on Chr. 5 and another on Chr.3 have been identified by molecular marker RM 440 and RM 228 respectively using single marker analysis (Yoo *et al.*, 2007).

Rice genome being small in size (390mbp) and the genome has synteny with those of other cereals; it becomes a good candidate for molecular dissection and genetic

transformation. Targeting of promoter sequence of cellulose synthase gene underlying cellulose biosynthesis is the direct approach to improve cellulose content using genetic transformation. Alternatively, the sucrose synthase gene *Susy* has been reported to improve the cellulose content in cell wall without any adverse effects on plant growth (Colman *et al.*, 2009). Besides, enhanced photosynthesis under elevated CO₂ is reported to increase total biomass and cellulose fibre production. Lignin in rice straw limits decomposition of plant stalks and follow-up fermentation. Down-regulation of genes involved in lignin biosynthesis seems to be a direct approach to reduce lignin content and alter lignin structure (Li *et al.*, 2008). Xia (2013) reported silencing of C4H (cinnamate-4- hydrolase) gene using RNAi technique which reduced lignin content significantly with minimum effect on morpho-economic traits. Besides, identification of cell wall degrading genes and/or QTLs for reduced lignin content can be explored and suitably applied in marker-assisted selection (Frei 2013) for high ethanol yield.

Paddy straw is a better choice of substrate for ethanol production as it is structurally composed of higher amount of cellulose. This results better glucose yields by saccharification and follow-up efficient ethanol recovery by fermentation and purification steps. Development of dual purpose rice (high grain yield and high ethanol recovery) is a challenging task as straw biomass production and grain yield is often negatively correlated. Nonetheless, opportunities do exist to make it a reality by breaking yield barrier. Selection of high yielding rice varieties with moderately high biomass and low lignin, silica, ash and potassium content in straw are most preferred for accessibility of enzymes for saccharification. Besides, mining candidate genes for cellulose synthase, stay green trait

and low lignin content in combination with exploring QTLs for high yield potential would certainly help breeders to develop bioenergy efficient smart varieties in rice using marker assisted selection.

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