

ADVANCED PRE-PROCESSING STRATEGIES FOR LIGNOCELLULOSIC BIOMASS APPLYING GENETIC ENGINEERING AND NANOTECHNOLOGY TO INCREASE BIOETHANOL YIELD

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Abstract

Production of Biofuel in higher yield by processing lignocellulosic biomass has been limited by the characteristics of conventional *saccharomyces cerevisiae* which can utilize lesser number of carbon sources and has very less thermotolerance. Further, complex structure of cellulose and hemicellulose bounded by fibrils of lignin makes it difficult to efficiently pre-treat these feedstocks by acidic and enzymatic hydrolysis. In current review, we discuss the plethora of methodologies which are a boon in production of biofuels from various lignocellulosic raw materials due to advancement in forte of Genome editing technologies, Plant Biotechnology, metabolic engineering and nanobiotechnology. Utilizing metabolically engineering strategies using non conventional microbial species, developing synthetic gene constructs, knocking out genes like COMT, mutating CESA genes using CRISPR Cas9 and RNA interference approaches has been shown to decrease the crystalline structure of Cellulose and enhance the availability of cellulose per unit area, expose lignin to efficient pre-treatment and accelerate conversion rate. Transferring gene encoding alpha amylase and glucoamylase(SWA2 and GAM1) from conventional yeast strains to uncommon strains like *H.polymorpha* has improvised the efficiency of glucose and xylose utilisation for industrial production of Biofuel. Application of metal oxides of nanomaterials and specifically gold nanoparticles have enhanced the yield of biofuels. Magnetic nanoparticles have been used to embed the enzymes involved in pre-treatment of Lignocellulosic material resulting in better encapsulation and sustainable use of these enzymes over multiple cycles.

Keywords: Biofuel, Microbes, Lignocellulose, Biomass, CRISPR-Cas9, RNA interference, Genome editing.

INTRODUCTION

With the constantly rising world population, there's a manifold increase in pollution with accelerating wear off in the energy sources. About 50% of fossil fuel reserves have been exhausted, raising an urge for an alternative environment-friendly approach that could lead to sustainability in the near future (Godbole et al., 2021; Fayyaz et al., 2020). Any hydrocarbon fuel that is produced from organic matter in a short period of time is considered as biofuel. It may be solid (wood, dried plant materials), liquid (bioethanol and biodiesel) or gaseous (biogas) in nature. Driving factors for inclining towards biofuel as a substitute for depleting some of the main reason for shifting to biofuels are rising prices of oil, emission of the greenhouse gases from fossil fuels and the interest for obtaining fuel from agricultural crops for benefit of farmers.

Recent investigations show that non conventional yeast species have been tremendously beneficial for bioethanol production Biofuels form a sound alternative renewable energy source for these fossil fuels, similarly, biomass becomes an alternative for these fossil resources (Kandel et al., 2018). Moreover, enhancement in genetic engineering and molecular biology tools is also keeping pace with the increasing population, exploring the biochemical pathways in organisms by facilitating an efficient approach of genome editing techniques like Zinc Finger nucleases (ZFNs), RNA interference (RNAi), (CRISPR-Cas9), and Transcription activator-like effector nucleases (TALENs) for the fulfilment of specific need (Jagadevan et al., 2018). RNAi genome editing is a reverse genetic approach that could knock off the expression of the target endogenous gene sequence (silencing) through antisense mechanism i.e. complementary binding of the injected RNA to the respective target mRNA

thereby suppressing the mRNA's expression, known as silencing of mRNA (Fayyaz et al., 2020; Fire et al., 1998). Because we have found evidence of RNAi in bacteria, fungi, algae, plants, and animals, we can conclude that this process occurs inside both cells (eukaryotic cells or prokaryotic cells), moreover, RNAi is a naturally occurring common cellular defense mechanism in eukaryotic cells which hinders the process of central dogma at the stage of mRNA so there will be no translation, no protein formation, no expression, and hence no enzyme formation (Rosa et al., 2018). This technique is under research exploitation for biofuel production primarily through standout bioenergy crop-sugarcane and switchgrass, and from the promising feedstock microalgae for a few decades (Fayyaz et al., 2020; Kandel et al., 2018; Baxter et al., 2014).

Among plants, sugarcane (*Saccharum* spp. hybrids) is a preeminent semi perennial bioenergy grass crop, researched as a major feedstock for global bioethanol production, having lignocellulosic biomass and high productive rate (Kandel et al., 2018; Jung et al., 2013). Aside from sugarcane, switchgrass (*Panicum virgatum* L.), a C4 perennial grass, forms another leading bioenergy crop with high production in biomass and minimum requirement of nutrients (Baxter et al., 2014). Despite the advantages of these bioenergy crops, the major hindrance to the cost-effective cellulosic biofuel production lies in the presence of recalcitrant lignin in the cell wall, which reduces the cellulolytic enzymes and microbial activity by limiting their accessibility to the fermentable walls i.e. cellulose and hemicellulose (Jung et al., 2013; de Souza et al., 2019; Baxter et al., 2014). The expensive thermochemical pretreatment researched for the degradation of the lignin-polysaccharide matrix has not been serving the purpose because it was generating the inhibitory molecules that further hindered the production of ethanol in downstream microbial fermentation (Alvira et al., 2010; Baxter et al., 2014). Thus, despite the advantages primarily, the switchgrass fails to become an economically competent fuel due to the high costs associated with the conversion of lignocellulose (Himmel et al., 2007). RNAi genome editing technique proved to be a boon to solve these issues with the least drawbacks. Conventional yeast species suffer from many limitations. *Saccharomyces cerevisiae* species utilized in biofuel production suffers from its limitation to work in high temperature and utilize only a limited number of glucose substrates.

1.GENOME EDITING APPROACHES TO IMPROVE PRETREATMENT EFFICIENCY OF LIGNOCELLULOSIC WASTE MATERIALS

Genome editing tools using TALENS and Crispr Cas 9 have been exploited to accelerate the biofuel production by increasing the pre treatment efficacy on lignocellulosic materials. Synthetic Gene constructs are made to increase saccharification in plant species. Genetic Engineering approaches have been implemented by employing the genome editing capability of CRISPR CAS 9 system for enhancing the thermo tolerance of biofuel producing microbes, making them more resistant towards presence of inhibitors, improving the pretreatment efficacy of cellulases and hemicellulases, altering the lignin content in the crops by mutating COMT gene and substantially increasing the tolerance capacity of the microbes towards the biofuel produced in the fermentative pathway.

Key role of using genome editing tools is to increase the resistance of microorganisms towards the toxic compounds produced during the fermentation process like the specific product and growth inhibitors (Tkalec et al., 2014). Significant amount of cellulolytic enzymes and the microbial growth inhibitors get generated during the pre-treatment stages whereby Lignocellulosic biomass undergoes physical, chemical and enzymatic treatments. Growth inhibitors generated are classified in 3 categories- (a) Derivatives of Furan (b) Weak acids (c) Phenolic compounds (Pandey et al., 2011). By increasing the resistance of microbes towards these inhibitors, ethanol production is boosted. CRISPR CAS9 has been used as a genome editing tool to increase the furfural tolerance of *S. cerevisiae* by disrupting the SSK2 gene in it via integration of the transposon (Ulaganathan et al., 2017).

Barley is frequently used in production of bioethanol. It has been reported that by inducing mutation with the use of CRISPR CAS9 system in the caffeic acid O-methyltransferase 1 (HvCOMT1), the gene which is involved in the biosynthetic pathway for production of lignin by contributing in the formation of molecule syringyl in the lignin fibrils, there has been tremendous improvement in the recovery rate of the fermentable glucose and sugar production by the mutated plant as compared to the wild type Barley. Mutant plant had 14% reduction in the total content of lignin and 34% more bioethanol production was obtained.

In the desired environmental conditions, growth of mutant was very much similar to the wild type species but it served as a high yielding feedstock in terms of biofuel generation (Lee et al., 2021).

High temperature during ethanol production has been observed to decrease the growth of ethanol producing microbes. Making these microbes tolerant to high temperatures increases their capability to produce ethanol. Factors associated with thermotolerance are accumulation in the concentration of trehalose and higher number of heat shock proteins have been found to be associated with increased thermotolerance (Kandel et al., 2018). Elevated thermotolerance and accentuated production of ethanol in *Z. mobilis* and *S. cerevisiae* was attained by introduction of to single amino acid change in pyruvate kinase and NADH dehydrogenase (Benjaphokee et al., 2012; Benjaphokee et al., 2014). In continuation of the same approach, improvised thermostability was seen in *S. cerevisiae* on deletion of the gene of Dfg5 glycosyl phosphatidylinositol-anchored membrane protein (Nasution et al., 2015). CRISPR CAS9 has been tremendously exploited for these kind of genome editing approaches.

2. RNA INTERFERENCE APPROACHES

Recent approaches rely on using gene silencing approaches using RNA interference techniques. Lignin has been a recalcitrant in the availability of cellulose and hemicellulose for production of ethanol and hence reducing the amount of lignin exposes a greater of these two polymers. RNA interference mechanism has been successfully exploited for targeted silencing of specific genes. Lignin is a branched polymer of three alcoholic monomers-p-coumaryl, coniferyl and sinapyl alcohol. Once these subunits are incorporated in the lignin polymer, they are addressed respectively as p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units (Bout et al., 2003). It has been reported that the lignin content present in the grass family is enriched with sufficient amounts of the hydroxycinnamic acids like p-coumarate and ferulate. Predominant role of Ferulate has been observed in interconnecting the cell wall polysaccharides of hemicellulose and lignin (Barriere et al., 2004; Fu et al., 2011). In contrast to Ferulate, another moiety p-coumarate undergoes esterification in the gamma carbon of sinapyl alcohol (Grabber et al., 1996). Among monolignol biosynthetic genes, caffeic acid O-methyltransferase(COMT) encodes the enzyme that catalyses O-methylation at the C5 position of 5-hydroxyconiferaldehyde and 5-hydroxyconiferyl alcohol, yielding sinapaldehyde and sinapyl alcohol, respectively (Grabber et al., 1996; Ralph et al., 1994). Reduction in the expression of the COMT or knockout of COMT in various plants has shown significant decrease in lignin content and enhanced saccharification efficiency. RNA interference mechanism has been applied in sugarcane to generate transgenic sugarcane breeds with high production capacity of ethanol due to higher availability of cellulose and hemicellulose in these plants (Abramson et al., 2010).

In an experiment conducted through RNAi, Caffeic acid O-methyltransferase-suppressed transgenic sugarcane lines were created which marked the convincing reduction in the total lignin and s sub-content thereby increasing the fermentable glucose yield which ultimately contributed to an increase in the biofuel production from the lignocellulosic biomass of sugarcane and switchgrass (Grabber et al., 1996). During the enzymatic hydrolysis, COMT-suppressed sugarcane lines depicted the elevated saccharification efficiency of 19 to 32% in comparison to the wild-type or transgenic controls; 82-91% of suppressed COMT-transcript resulted in cut down of lignin by 6-12% and increase in conversion of lignocellulosic biomass to fermentable glucose (Ralph et al., 1994). The field study on the switchgrass, COMT down-regulation lines showed a remarkable reduction in S-lignin content thereby decreasing the S/G monomeric ratio, with 8-15% decrease in total lignin content in comparison to the non-transgenic lines (Louie et al., 2010). The boost in the production of sucrose from the lignocellulosic sugarcane biomass elevates the production of sucrose-derived biofuel from this most paramount biofuel crop.

The above approaches achieve efficiency in saccharification by silencing COMT-gene expression through RNA interference which was responsible for lignin synthesis; while in another study, the sugarcane saccharification efficiency was increased by silencing the BAHD01 gene which resulted in the decrease in biomass recalcitrance (Nasution et al., 2015). Sugarcane transgenic lines formed by the silencing of BAHD01 through RNAi showed no noticeable alteration in total lignin composition but showed a significant decrease in the level of FA(ferulate) and FA-Ara along with an increase in the biomass of fermentable glucose up to 24% in comparison to non-transformed lines (Rai et al., 2016; Harris et al., 2010). This was concluded as BAHD acetyltransferase gene silencing increased the saccharification level of biomass by decreasing the levels of FA in the cell wall resulting in a great contribution in increasing bioethanol production (Bhatia et al., 2017; Carpita and Mc Cann, 2010).

3. REDUCING THE CRYSTALLINITY OF CELLOLUSE AND ENHANCING AVAILABILITY BY GENETIC ENGINEERING APPROACHES

Crystalline structure of Cellulose is due to inherent inter winding of fibres which in turn is attributed to the strong hydrogen binding between them. This strong crystallinity in cellulose causes resistance in breakdown of cellulose by enzymatic hydrolysis methods. So, in order to increase biomass conversion by increasing the susceptibility to pre treatment, crystalline forms of cellulose needs to be target for conversion to Amorphous forms (Abramson et al., 2010). Cellulose synthase (CESA) genes are involved in the synthesis of fibrils of cellulose and their quantity varies across various lignocellulosic residues (Rai et al., 2016). Harris and Trethewey (Harris et al., 2010) had performed an experimentation on Arabidopsis where they had mutated two of the cellulose synthase genes for a shorter timespan. This experimentation showed that the mutant plant demonstrated substantially decreased crystallinity in cellulose structure as compared to the wild type strain. Further, there was an enhancement of more than 40% in the saccharification yield of mutated plant.

Another technique to enhance yield of biofuel from lignocellulosic biomass is by increasing the amount of cellulose present per unit area of biomass (Bhatia et al., 2017). Research has shown the promising role of hexameric rosette complexes CESA in formation of cellulose by plants at the cell membrane (Carpita and Mc Cann, 2010). A targeted strategy for increasing the feedstock specific CESA complexes such as OsCESA 4, 7 and 9 can be targeted for multifold acceleration in the production of cellulose and hence a higher rate of glucose formation leading to enhanced production of biofuels (Chandel et al., 2012).

Discovery of the landmark enzymes involved in depolymerization of hemicellulose and lignin have resulted in production of higher yield of ethanol at economical rates and from novel resources (Banerjee et al., 2010). Several environmental samples such as compost, hot springs, forests wastes land have been investigated to isolate these pioneer enzymes which have high

depolymerization potential. Further insilico tools and big data analytics have been employed to explore these enzymes and fabricate designer enzymes with magnificent hemicellulase and cellulase activities (Voorend et al., 2016).

Another strategy that has revolutionized biofuel production from non conventional yeast includes fabrication and application of “tailored biomass”. Few paradigms of tailored biomass are, using the tailored maize crop which had increased expression of gene GA20-OXIDASE1 (Fan et al., 2016). This gene is involved in maintaining the endogenous concentrations of gibberellic acid. Higher the concentration of gibberellic acid, higher is the saccharification yield of fermentable glucose (Mohapatra et al., 2017). Another case is of when promoter of Arabidopsis cellulose synthase (AtCesA8) gene was applied as inducer in some varieties of rice, the resultant transgenic rice plants was observed to overexpress OsSUS3 gene and this lead to improvisation of the saccharification efficiency of the transgenic rice species (Bonawitz and Chapple, 2010; Grabber, 2005).

4. METABOLIC ENGINEERING STRATEGIES

Metabolic engineering is a promising technique to alter the metabolic or another pathway of any microorganism to increase the synthesized required product quantity of our interest and shorten the pathway or sometimes improve cellular properties of metabolic or another pathway. A compound that is already being produced by a metabolic pathway of a particular microorganism can be overproduced or force the organism to alter certain metabolic pathways so now synthesized a new compound which is normally not synthesized by that metabolic pathway (Stephanopoulos, 2012). This all can be done by the use of Metabolic Engineering. while the information and components of any metabolic or any other pathway are provided by synthetic biology which is utilized by metabolic engineering to optimize the pathway (Granados et al., 2019). Synthetic Biology and Metabolic Engineering in the present scenario where the world is looking for biofuel had very much important because these techniques have application in enhancing bio-fuel production (Rabinovitch-Deere et al., 2013). Application of metabolic engineering in various strains leading to substantial increase in production level of different types of biofuels on various substrates has been given in table 1.

Table 1: Application of metabolic engineering on various microbes to get higher yield of Biofuels as end products.

Metabolic engineered strains of microorganisms	Substrate /Carbon source	Biofuel (end product)	Reference
Synechocystis sp.	Glucose	Isobutanol	(Shabestary and Hudson, 2016; Varman et al., 2013)
Thermoanaerobacter mathranii	Glucose & glycerol	Ethanol	(Yao et al., 2010)
Clostridium thermocellum	cellulose	Ethanol & N-Butanol	(Tian et al., 2019)
Thermoanaerobacterium saccharolyticum	Cellobiose	Ethanol	(Hon et al., 2017; Herring et al., 2016)
Clostridium Tyrobutyricum	glucose, xylose, galactose & sucrose	N-Butanol & Butyrate	(Bao et al., 2020)
Geobacillus thermoglucosidasius	glucose & cellobiose	Ethanol	(Zhou et al., 2016)
Escherichia coli	Glucose	Isopropanol & N-butanol	(Liu and Khosla, 2010)
Escherichia coli	Glucose	Isobutanol,1-Butanol,Ethanol,N-Propanol and Isopropanol	(Koppolu and Vasigala, 2016)
Corynebacterium glutamicum	Glucose and Acetate	3-hydroxypropionic acid	(Chang et al., 2020)
Clostridium cellulovorans	cellulose	Butanol & Ethanol	(Yang et al., 2015)
Clostridium autoethanogenum	industrial waste gases	Ethanol	(Liew et al., 2017)
Clostridium carboxidivorans	syngas and glucose	Ethanol and Butanol	(Cheng et al., 2019)
Clostridium ljungdahlii	CO2	Butyrate	(Ueki et al., 2014)
Cupriavidus necator	CO2	Isobutanol and 3-methyl-1-butanol	(Lu et al., 2012)
Clostridium	cellulose	Isobutanol	(Gaida et al., 2016)

cellulolyticum			
<i>Saccharomyces cerevisiae</i>	cellulosic sugars	Ethanol	(Jin and Cate, 2017)
<i>Saccharomyces cerevisiae</i>	Glucose	Ethanol & fatty acids	(Hu et al., 2019)
<i>Saccharomyces cerevisiae</i>	Glucose and Xylose	Ethanol	(Ko et al., 2020)
<i>Saccharomyces cerevisiae</i>	Glucose & glycerol	Isoprenol (3-methyl-3-butene-1-ol)	(Kim et al., 2021)
<i>Synechococcus elongatus</i>	CO ₂	Photosynthetic 3-hydroxypropionic acid	(Kim et al., 2015)
<i>Synechococcus elongatus</i> PCC 7942	CO ₂	Isopropanol	(Hirokawa et al., 2017)
<i>Synechococcus elongatus</i> PCC 7942	CO ₂	1,3-propanediol	(Hirokawa et al., 2017)
<i>Saccharomyces cerevisiae</i> strain	Glucose & Galactose	2,3-Butanediol (BDO)	(Lian et al., 2014)
<i>Caldicellulosiruptor bescii</i>	Cellulose (lignocellulose)	Acetone and ethanol	(Straub et al., 2020)
<i>Caldicellulosiruptor bescii</i>	Glucose	Ethanol	(Straub et al., 2020)
<i>Chlamydomonas reinhardtii</i>	Geranylgeranyl diphosphate X 2	β-Carotene and Lutein	(Zhang et al., 2021)

5. USE OF NANOTECHNOLOGY STRATEGIES

Given recent breakthroughs in nanotechnology, it is expected that variety of different nanomaterials can be applied for enzyme immobilisation and increase the yield of biofuel after processing of lignocellulose rich materials. The use of enzymes on nanoscale materials is an unique and promising technology. The term 'nanobiocatalyst' refers to enzymes that have been immobilised using nanoparticles. The progression nanobiocatalyst is an example of a new and unique technology. Cross-linking molecules are used to encourage favourable chemical kinetics of enzymatic reactions as well as ensure substrate selectivity. Actually, the immobilisation of enzymes with the use of the cross-linking molecule acts as a spacer, reduces steric hindrance.

Magnetic micro ferrites doped with calcium have been found to have a substantial influence in biodiesel production, increasing yield by about 85 percent from soybean cooking oils (Dantas et al., 2017). Sugarcane leaves and MnO₂ were used to demonstrate that Bioethanol production was boosted using nanoparticles. It catalysed the process at several points. Sugarcane leaves are converted to bioethanol in this method, and due to their great size, they are an excellent source of bioethanol. MnO₂ nanoparticles' surface area is responsible for enzyme binding to their active sites, resulting in improved ethanol synthesis (Cherian et al., 2015). The immobilisation of yeast was discovered to be beneficial. The cells that were grown on magnetic nanoparticles produced more ethanol (Ueki, T et al, 2014; Ulaganathan, K. et al, 2017). Magnetic nanoparticles (MNPs) have previously been shown to have the potential to hydrolyze proteins. cell wall of microalgae by immobilising cellulase on MNPs with lipids.

By immobilising cellulase on MNPs and extracting lipids, previous research has proven the potential of using MNPs to hydrolyze the microalgae cell wall Mahmood et al. investigated the effects of adding iron nanoparticles over time in anaerobic digestion and hydrogen generation using water hyacinth, an aquatic weed as substrates (*Eichhornia crassipes*) (Duraiarasan et al., 2016). The findings of this research demonstrated that The hydrogen output was increased with the addition of iron nanoparticles, reaching 57 mL/g of hydrogen per gram of iron nanoparticles. Enhancing hydrogen production while utilising Glucose has also been used as a substrate in some research.

Metallic NPS of gold, platinum, and Pt_{0.75}-Tin_{0.25} were installed in acid-functionalized Multi-Walled NCTs to create hybrid nano-catalysts, whereas gold NPs were encased in a poly (amidoamine) PAMAM dendrimer structure in another technique. Dendrimer-encapsulated NPs are highly ordered and efficient, according to HR-TEM analysis. Gold, platinum, and Pt_{0.75}-Tin_{0.25} supported by MWCNTs have been used in biofuel cells, with gold NPs demonstrating greater electrical conductivity, biocompatibility, and catalytic activity than platinum NPs. For ethanol, a mixture of platinum and tin NPs demonstrated strong oxidation activity (Duraiarasan et al., 2016; Aquino Neto et al., 2014). Another study used laser ablation to create gold NPs in an aqueous solution, which showed good catalytic activity even on the 10th cycle, as well as high electrocatalytic efficiency. Even with a small sample size, the LA-Au NPs outperformed (Hebié et al., 2015; Zhang et al., 2007).

Various sorts of nanomaterials have application in the field of biohydrogen creation. Gold (Au) nanoparticles (5 nm) upgrade

the substrate use proficiency by 56% and increment the yield of biohydrogen creation up to 46% (Nagaragan et al., 2017). Gold nanoparticles upgrade the biohydrogen creation because of their little size and enormous surface region which are liable for the limiting of microbial cells to dynamic sites. Magnetic nanoparticles of metals additionally improve the movement of compounds of biohydrogen union hardware which is liable for the development of biohydrogen (Verma et al., 2013; Ramsurn and Gupta 2013).

CONCLUSION

There has been significant improvement in the production of Biofuel by using genome editing approaches by specifically utilizing Crispr Cas9, Talens and RNA interference methodologies. Knocking out of COMT gene and Cesa genes has shown to improve the lignin pretreatment efficacy. Usage of non conventional species and application of metabolic engineering has enhanced their thermotolerance and number of carbohydrate substrates can be effectively converted into bioethanol. Nanotechnology has given a promising hope to increase the enzyme retention rate by using magnetic nanoparticles for immobilization of enzymes like cellulases, xylanases which have crucial role in conversion of lignocellulosic biomass.

REFERENCES

1. Abramson M, Shoseyov O, Shani Z (2010) Plant cell wall reconstruction toward improved lignocellulosic production and processability. *Plant Sci* 178:61–72
2. Ali A, Mahar R, Soomro R, Sherazi S (2017) Fe₃O₄ nanoparticles facilitated anaerobic digestion of organic fraction of municipal solid waste for enhancement of methane production. *Energy Sources, Part A* 37:1815–1815
3. Alvira, P., Tomás-Pejó, E., Ballesteros, M., & Negro, M. J. (2010). Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: a review. *Bioresource technology*, 101(13), 4851–4861.
4. Aquino Neto, S.; Almeida, T.S.; Palma, L.M.; Minter, S.D.; De Andrade, A.R. Hybrid Nanocatalysts Containing Enzymes and Metallic Nanoparticles for Ethanol/O₂ Biofuel Cell. *J. Power Sources* 2014, 259, 25–32. [CrossRef]
5. Banerjee G, Car S, Scott-Craig J, Borrusch M, Walton J (2010) Rapid optimization of enzyme mixtures for deconstruction of diverse pretreatment/biomass feedstock combinations. *Biotechnol Biofuels* 3:22
6. Bao, T., Feng, J., Jiang, W., Fu, H., Wang, J., & Yang, S. T. (2020). Recent advances in n-butanol and butyrate production using engineered *Clostridium tyrobutyricum*. *World Journal of Microbiology and Biotechnology*, 36(9), 1–14.
7. Barri_ere, Y., Ralph, J., M_echin, V., Guillaumie, S., Grabber, J.H., Argillier, O., Chabbert, B. and Lapierre, C. (2004) Genetic and molecular basis of grass cell wall biosynthesis and degradability. II. Lessons from brown-midrib mutants. *C. R. Biol.* 327, 847–860.
8. Baxter, H. L., Mazarei, M., Labbe, N., Kline, L. M., Cheng, Q., Windham, M. T., ... & Stewart Jr, C. N. (2014). Two-year field analysis of reduced recalcitrance transgenic switchgrass. *Plant Biotechnology Journal*, 12(7), 914–924.
9. Benjaphokee, S., Koedrih, P., Auesukaree, C., Asvarak, T., Sugiyama, M., Kaneko, Y., Boonchird, C., Harashima, S., 2012. CDC19 encoding pyruvate kinase is important for high-temperature tolerance in *Saccharomyces cerevisiae*. *New. Biotech.* 29(2), 166–176.
10. Bhatia R, Gallagher JA, Leonardo D, Bosch GM (2017) Genetic engineering of grass cell wall polysaccharides for biorefining. *Plant Biotechnol J* 15:1071–1092
11. Bonawitz, N.D. and Chapple, C. (2010) The genetics of lignin biosynthesis: connecting genotype to phenotype. *Annu. Rev. Genet.* 44, 337–363.
12. Bout, S. and Vermeris, W. (2003) A candidate-gene approach to clone the sorghum brown midrib gene encoding caffeic acid O-methyltransferase. *Mol. Genet. Genomics* 269, 205–214.
13. Carpita NC, McCann MC (2010) The maize mixed-linkage (1/3), (1/4)-b-d-glucan polysaccharide is synthesized at the Golgi membrane. *Plant Physiol* 153:1362–1371
14. Caspeta, L., Chen, Y., Ghiaci, P., Feizi, A., Buskov, S., Hallström, B.M., Petranovic, D., Nielsen, J., 2014. Altered sterol composition renders yeast thermotolerant. *Science.* 346(6205), 75–78.
15. Chandel AK, Chandrasekhar G, Silva MB, da Silva SS (2012) The realm of cellulases in biorefinery development. *Crit Rev Biotechnol* 32:187–202
16. Chang, Z., Dai, W., Mao, Y., Cui, Z., Wang, Z., & Chen, T. (2020). Engineering *Corynebacterium glutamicum* for the efficient production of 3-hydroxypropionic acid from a mixture of glucose and acetate via the malonyl-CoA pathway. *Catalysts*, 10(2), 203.
17. Cheng, C., Li, W., Lin, M., & Yang, S. T. (2019). Metabolic engineering of *Clostridium carboxidivorans* for enhanced ethanol and butanol production from syngas and glucose. *Bioresource technology*, 284, 415–423.
18. Cherian, E.; Dharmendirakumar, M.; Baskar, G. Immobilization of Cellulase onto MnO₂ Nanoparticles for Bioethanol Production by Enhanced Hydrolysis of Agricultural Waste. *Chin. J. Catal.* 2015, 36, 1223–1229. [CrossRef]
19. Clara Navarrete and Jos eL.Mart nez(2020),Non-conventional yeasts as superior production platforms for sustainable fermentation based bio-manufacturing processes.*Aims biotechnology*7(4):289-305.
20. Cletus P. Kurtzman, Pichia E.C.Hansen (1904)Chapter :-57
21. Dantas, J.; Leal, E.; Mapossa, A.B.; Cornejo, D.R.; Costa, A.C.F.M. Magnetic nanocatalysts of Ni_{0.5}Zn_{0.5}Fe₂O₄ doped with Cu and performance evaluation in transesterification reaction for biodiesel production. *Fuel* 2017, 191, 463–471. [CrossRef]
22. Das D, Khanna N, Veziroglu NT (2008) Recent development in biological hydrogen production processes. *Chem Ind Chem Eng Q* 14:57–67
23. de Souza, W. R., Pacheco, T. F., Duarte, K. E., Sampaio, B. L., de Oliveira Molinari, P. A., Martins, P. K., ... & Molinari, H. B. C. (2019). Silencing of a BAHD acyltransferase in sugarcane increases biomass digestibility. *Biotechnology for biofuels*, 12(1), 1–14.
24. Durairasan, S.; Razack, S.A.; Manickam, A.; Munusamy, A.; Syed, M.B.; Ali, M.Y.; Ahmed, G.M.; Mohiuddin, M.S. Direct Conversion of Lipids from Marine Microalga C. Salina to Biodiesel with Immobilised Enzymes Using Magnetic Nanoparticle. *J. Environ. Chem. Eng.* 2016, 4, 1393–1398. [CrossRef]
25. Fan X, Tang Z, Tan Y, Zhang Y, Luo B, Yang M, Lian X, Shen Q, Miller AJ, Xu G (2016) Overexpression of a pH-sensitive nitrate transporter in rice increases crop yields. *PNAS* 113:7118–7123

26. Farshad Darvishi, Zahra Fathi, Mehdi Ariana, Hamideh Moradi (2017), *Yarrowia lipolytica* as a workhorse for biofuel production. *Biochemical engineering journal* 127(2017)87-96.
27. Fayyaz, M., Chew, K. W., Show, P. L., Ling, T. C., Ng, I. S., & Chang, J. S. (2020). Genetic engineering of microalgae for enhanced biorefinery capabilities. *Biotechnology advances*, 43, 107554.
28. Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., & Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *nature*, 391(6669), 806-811.
29. Fu, C., Mielenz, J.R., Xiao, X., Ge, Y., Hamilton, C.Y., Rodriguez, M. Jr, Chen, F., Foston, M., Ragauskas, A., Bouton, J., Dixon, R.A. and Wang, Z.Y. (2011) Genetic manipulation of lignin reduces recalcitrance and improves ethanol production from switchgrass. *Proc. Natl Acad. Sci. USA*, 108, 3803–3808.
30. Gaida, S. M., Liedtke, A., Jentges, A. H. W., Engels, B., & Jennewein, S. (2016). Metabolic engineering of *Clostridium cellulolyticum* for the production of n-butanol from crystalline cellulose. *Microbial cell factories*, 15(1), 1-11.
31. Godbole, V., Pal, M. K., & Gautam, P. (2021). A critical perspective on the scope of interdisciplinary approaches used in fourth-generation biofuel production. *Algal Research*, 58, 102436.
32. Grabber, J.H. (2005) How do lignin composition, structure, and cross-linking affect degradability? A review of cell wall model studies. *Crop Sci.* 45, 820–831.
33. Grabber, J.H., Quideau, S. and Ralph, J. (1996) p-Coumaroylated syringyl units in maize lignin: Implications for β-ether cleavage by thioacidolysis. *Phytochemistry*, 43, 1189–1194.
34. Granados, R., Lerma-Escalera, J. A., & Morones-Ramírez, J. R. (2019). Metabolic engineering and synthetic biology: synergies, future, and challenges. *Frontiers in bioengineering and biotechnology*, 7, 36.
35. Gustavo Graciano Fonseca, Elmar Heinzle, Christoph Wittman, Andreas K.Gombert (2008) The yeast *Kluyveromyces marxianus* and its biotechnological potential. *Appl microbial biotechnol.* 79:339:354.
36. Harris P, Trethewey JK (2010) The distribution of ester-linked ferulic acid in the cell walls of angiosperms. *Phytochem Rev* 9:19–33
37. Hebié, S.; Holade, Y.; Maximova, K.; Senti, M.; Delaporte, P.; Kokoh, K.B.; Napporn, T.W.; Kabashin, A.V. Advanced Electrocatalysts on the Basis of Bare Au Nanomaterials for Biofuel Cell Applications. *ACS Catal.* 2015, 5, 6489–6496. [CrossRef]
38. Herring, C. D., Kenealy, W. R., Shaw, A. J., Covalla, S. F., Olson, D. G., Zhang, J., ... & Hogsett, D. A. (2016). Strain and bioprocess improvement of a thermophilic anaerobe for the production of ethanol from wood. *Biotechnology for biofuels*, 9(1), 1-16.
39. Higashide, W., Li, Y., Yang, Y., & Liao, J. C. (2011). Metabolic engineering of *Clostridium cellulolyticum* for production of isobutanol from cellulose. *Applied and environmental microbiology*, 77(8), 2727-2733.
40. Himmel, M. E., Ding, S. Y., Johnson, D. K., Adney, W. S., Nimlos, M. R., Brady, J. W., & Foust, T. D. (2007). Biomass recalcitrance: engineering plants and enzymes for biofuels production. *science*, 315(5813), 804-807.
41. Hirokawa, Y., Dempo, Y., Fukusaki, E., & Hanai, T. (2017). Metabolic engineering for isopropanol production by an engineered cyanobacterium, *Synechococcus elongatus* PCC 7942, under photosynthetic conditions. *Journal of bioscience and bioengineering*, 123(1), 39-45.
42. Hirokawa, Y., Matsuo, S., Hamada, H., Matsuda, F., & Hanai, T. (2017). Metabolic engineering of *Synechococcus elongatus* PCC 7942 for improvement of 1, 3-propanediol and glycerol production based on in silico simulation of metabolic flux distribution. *Microbial cell factories*, 16(1), 1-12.
43. Hon, S., Olson, D. G., Holwerda, E. K., Lanahan, A. A., Murphy, S. J., Maloney, M. I., ... & Lynd, L. R. (2017). The ethanol pathway from *Thermoanaerobacterium saccharolyticum* improves ethanol production in *Clostridium thermocellum*. *Metabolic engineering*, 42, 175-184.
44. Hu, Y., Zhu, Z., Nielsen, J., & Siewers, V. (2019). Engineering *Saccharomyces cerevisiae* cells for production of fatty acid-derived biofuels and chemicals. *Open biology*, 9(5), 190049.
45. Ivanova, V.; Petrova, P.; Hristov, J. Application in the Ethanol Fermentation of Immobilized Yeast Cells in Matrix of Alginate/ Magnetic Nanoparticles, on Chitosan-Magnetite Microparticles and Cellulose-Coated Magnetic Nanoparticles. *arXiv preprint 2011, arXiv:1105.0619.*
46. Jagadevan, S., Banerjee, A., Banerjee, C., Guria, C., Tiwari, R., Baweja, M., & Shukla, P. (2018). Recent developments in synthetic biology and metabolic engineering in microalgae towards biofuel production. *Biotechnology for biofuels*, 11(1), 1-21.
47. Jiaoqi Gao, Wenjie Yuan, Yimin Li, Ruijuan Xiang, Shengbo Hou, Shijun Zhong, Fengwu Bai (2015), Transcriptional analysis of *Kluyveromyces marxianus* for ethanol production from inulin using consolidated bioprocessing technology. *Biotechnology for biofuels*, Vol 8:115
48. Jin, Y. S., & Cate, J. H. (2017). Metabolic engineering of yeast for lignocellulosic biofuel production. *Current opinion in chemical biology*, 41, 99-106.
49. Jung, J. H., Vermeris, W., Gallo, M., Fedenko, J. R., Erickson, J. E., & Altpeter, F. (2013). RNA interference suppression of lignin biosynthesis increases fermentable sugar yields for biofuel production from field-grown sugarcane. *Plant biotechnology journal*, 11(6), 709-716.
50. Kandel, R., Yang, X., Song, J., & Wang, J. (2018). Potentials, challenges, and genetic and genomic resources for sugarcane biomass improvement. *Frontiers in plant science*, 9, 151.
51. Kim, J., Baidoo, E. E., Amer, B., Mukhopadhyay, A., Adams, P. D., Simmons, B. A., & Lee, T. S. (2021). Engineering *Saccharomyces cerevisiae* for isoprenol production. *Metabolic Engineering*, 64, 154-166.
52. Ko, J. K., Enkh-Amgalan, T., Gong, G., Um, Y., & Lee, S. M. (2020). Improved bioconversion of lignocellulosic biomass by *Saccharomyces cerevisiae* engineered for tolerance to acetic acid. *GCB Bioenergy*, 12(1), 90-100.
53. Koppolu, V., & Vasigala, V. K. (2016). Role of *Escherichia coli* in biofuel production. *Microbiology insights*, 9, MBI-S10878.
54. Kwon, C.H.; Ko, Y.; Shin, D.; Kwon, M.; Park, J.; Bae, W.K.; Lee, S.W.; Cho, J. High-Power Hybrid Biofuel Cells Using Layer-by-Layer Assembled Glucose Oxidase-Coated Metallic Cotton Fibers. *Nat. Commun.* 2018, 9, 1–11. [CrossRef] [PubMed]
55. Lan, E. I., Chuang, D. S., Shen, C. R., Lee, A. M., Ro, S. Y., & Liao, J. C. (2015). Metabolic engineering of cyanobacteria for photosynthetic 3-hydroxypropionic acid production from CO₂ using *Synechococcus elongatus* PCC 7942. *Metabolic engineering*, 31, 163-170.
56. Lee JH, Won HJ, Hoang Nguyen Tran P, Lee S, Kim H, Jung JH (2021). Improving lignocellulosic biofuel production by CRISPR/Cas9-mediated lignin modification in barley. *GCB Bioenergy*;13:742–752. <https://doi.org/10.1111/gcbb.12808>
57. Lee, K.H.; Choi, I.S.; Kim, Y.-G.; Yang, D.-J.; Bae, H.-J. Enhanced Production of Bioethanol and Ultrastructural Characteristics of Reused *Saccharomyces Cerevisiae* Immobilized Calcium Alginate Beads. *Bioresour. Technol.* 2011, 102, 8191–8198. [CrossRef]
58. Lian, J., Chao, R., & Zhao, H. (2014). Metabolic engineering of a *Saccharomyces cerevisiae* strain capable of simultaneously utilizing glucose and galactose to produce enantiopure (2R, 3R)-butanediol. *Metabolic engineering*, 23, 92-99.
59. Liew, F., Henstra, A. M., Köpke, M., Winzer, K., Simpson, S. D., & Minton, N. P. (2017). Metabolic engineering of *Clostridium autoethanogenum* for selective alcohol production. *Metabolic engineering*, 40, 104-114.
60. Liu, T., & Khosla, C. (2010). Genetic engineering of *Escherichia coli* for biofuel production. *Annual review of genetics*, 44, 53-69.
61. Louie, G.V., Bowman, M.E., Tu, Y., Mouradov, A., Spangenberg, G. and Noel, J.P. (2010) Structure-function analyses of a caffeic acid O-methyltransferase from perennial ryegrass reveal the molecular basis for substrate preference. *Plant Cell*, 22, 4114–4127.
62. Lu, J., Brigham, C. J., Gai, C. S., & Sinskey, A. J. (2012). Studies on the production of branched-chain alcohols in engineered *Ralstonia eutropha*.

Applied microbiology and biotechnology, 96(1), 283-297.

63. Lukajits R, Holowacz I, Kucharska K, Glinka M, Rybarczyk A (2018) Hydrogen production from biomass using dark fermentation. *Renew Sust Energy Rev* 91:665–694
64. Melanie M.Lane , Niall Burke , Rob Karreman , Kenneth H. Wolfe , Conor P.O'Byrne , John P. Morrissey(2011)Physiological and metabolic diversity in the yeast *Kluyveromyces marxianus*.*Antonie Van Leeuwenhoek*100:507-519.
65. Mochamad Nurcholis , Noppon Lertwattanasakul , Nadchanok Rodussamee , Tomoyuki Kosaka , Masayuki Murata , Mamoru Yamada (2019), integration of comprehensive data and biotechnological tools for industrial application of *Kluyveromyces marxianus*.*Applied microbiology and biotechnology*.
66. Mohapatra S, Mishra C, Behera SS, Thatoi H (2017) Application of pretreatment, fermentation and molecular techniques for enhancing bioethanol production from grass biomass—a review. *Renew Sustain Energy Rev* 78:1007–1032
67. Nagaragan D, Lee D, Kondo A, Chang J (2017) Recent insights into biohydrogen production by microalgae – from biophotolysis to dark fermentation. *Bioresour Technol* 227:373–387
68. Nasution, O., Lee, J., Srinivasa, K., Choi, I.G., Lee, Y.M., Kim, E., Choi, W., Kim, W., 2015.Loss of Dfg5 glycosylphosphatidylinositol- anchored membrane protein confers enhanced heat tolerance in *Saccharomyces cerevisiae*. *Environ. Microbiol.* 17(8), 2721-2734
69. Nuttaporn Chamnipa, Sudarat Thanonkeo, Preekamol Klanrit, Pornthap Thanonkeo (2018) ,The potential of the newly isolated thermotolerant yeast *Pichia kudriavzevii* RZ8-1 for high -temperature ethanol production. *Brazilian journal of microbiology*(2018)378-391.
70. Palaniappan K (2017) An overview of applications of nanotechnology in biofuel production. *World Appl Sci J* 35:1305–1311
71. Pandey, A., 2011. Biofuels: alternative feedstocks and conversion processes. Academic Press. plants. *Plos One.* 9(1), e87582.
72. Rabinovitch-Deere, C. A., Oliver, J. W., Rodriguez, G. M., & Atsumi, S. (2013). Synthetic biology and metabolic engineering approaches to produce biofuels. *Chemical reviews*, 113(7), 4611-4632.
73. Rai KM, Thu SW, Balasubramanian VK, Cobos CJ, Disasa T, Mendu V,(2016) Identification, characterization, and expression analysis of cell wall related genes in *Sorghum bicolor* (L.) Moench, a food, fodder, and biofuel crop. *Front Plant Sci* 7:1287
74. Ralph, J., Hatfield, R.D., Quideau, S., Helm, R.F., Grabber, J.H. and Jung, H.-J.G.(1994) Pathway of p-coumaric acid incorporation into maize lignin as revealed by NMR. *J. Am. Chem. Soc.* 116, 9448–9456.
75. Ramsurn H, Gupta R (2013) Nanotechnology in solar and biofuels. *ACS Sustain Chem Eng* 1:779–797
76. Rosa, C., Kuo, Y. W., Wuriyangan, H., & Falk, B. W. (2018). RNA interference mechanisms and applications in plant pathology. *Annual review of phytopathology*, 56, 581-610.
77. Salman Zafar, Mohammad Owais (2006),Ethanol production from crude whey by *Kluyveromyces marxianus*. *Biochemical engineering journal* 27,295-298.
78. Sandeep Singh Dhaliwal , Hrinder Singh Oberoi , Simranjeet Kaur Sandhu , Dhiraj Nanda , Dinesh Kumar , Satinder Kaur Uppal (2011), Enhanced ethanol production from sugarcane juice by galactose adaptation of a newly isolated thermotolerant strain of *Pichia kudriavzevii*.*Bioresource technology* 102, pg 5968-5975.
79. Serrano, E., G. Rus and J. Garcia-Martinez, 2009. Nanotechnology for sustainable energy. *Renewable and Sustainable Energy Reviews*, 13(9): 2373-2384
80. Shabestary, K., & Hudson, E. P. (2016). Computational metabolic engineering strategies for growth-coupled biofuel production by *Synechocystis*. *Metabolic engineering communications*, 3, 216-226.
81. Stephanopoulos, G. (2012). Synthetic biology and metabolic engineering. *ACS synthetic biology*, 1(11), 514-525.
82. Straub, C. T., Bing, R. G., Otten, J. K., Keller, L. M., Zeldes, B. M., Adams, M. W., & Kelly, R. M. (2020). Metabolically engineered *Caldicellulosiruptor bescii* as a platform for producing acetone and hydrogen from lignocellulose. *Biotechnology and Bioengineering*, 117(12), 3799-3808.
83. Tian, L., Conway, P. M., Cervenka, N. D., Cui, J., Maloney, M., Olson, D. G., & Lynd, L. R. (2019). Metabolic engineering of *Clostridium thermocellum* for n-butanol production from cellulose. *Biotechnology for biofuels*, 12(1), 1-13.
84. Tkalec, M., Štefanić, P.P., Cvjetko, P., Šikić, S., Pavlica, M., Balen, B., 2014. The effects of cadmium-zinc interactions on biochemical responses in tobacco seedlings and adult
85. Tomohisa Hasunuma , Akihiko Kondo (2012) , Consolidated bioprocessing and simultaneous saccharification and fermentation of lignocellulose tp ethanol with the thermotolerant yeast strains. *Process biochemistry* 47(2012) 1287-1294.
86. Ueki, T., Nevin, K. P., Woodard, T. L., & Lovley, D. R. (2014). Converting carbon dioxide to butyrate with an engineered strain of *Clostridium ljungdahlii*. *MBio*, 5(5), e01636-14.
87. Ulaganathan, K., Goud, S., Reddy, M., Kayalvili, U., 2017. Genome engineering for breaking barriers in lignocellulosic bioethanol production. *Renew. Sustainable. Energy. Rev.* 74, 1080-1107.
88. Varman, A. M., Xiao, Y., Pakrasi, H. B., & Tang, Y. J. (2013). Metabolic engineering of *Synechocystis* sp. strain PCC 6803 for isobutanol production. *Applied and environmental microbiology*, 79(3), 908-914.
89. Verma, M.L., CJ. Barrow and M. Puri, 2013. Nanobiotechnology as a novel paradigm for enzyme immobilisation and stabilisation with potential applications in biodiesel production. *Applied Microbiology and Biotechnology*, 97(1): 23-39.
90. Voorend W, Nelissen H, Vanholme R, De Vliegheer A, Van BF, Boerjan W, Roldán-Ruiz I, Muylle H, Inzé D (2016) Overexpression of GA20-OXIDASE1 impacts plant height, biomass allocation and saccharification efficiency in maize. *Plant Biotechnol J* 14:997–1007
91. Yang, X., Xu, M., & Yang, S. T. (2015). Metabolic and process engineering of *Clostridium cellulovorans* for biofuel production from cellulose. *Metabolic Engineering*, 32, 39-48.
92. Yao, S., & Mikkelsen, M. J. (2010). Metabolic engineering to improve ethanol production in *Thermoanaerobacter mathranii*. *Applied microbiology and biotechnology*, 88(1), 199-208.
93. Zhang Y, Shen J (2007) Enhancement effect of gold nanoparticles on biohydrogen production from artificial wastewater. *Int J Hydrog Energy* 32:17–23
94. Zhang, K., Zhao, W., Rodionov, D. A., Rubinstein, G. M., Nguyen, D. N., Tanwee, T. N., ... & Zhang, Y. (2021). Genome-Scale Metabolic Model of *Caldicellulosiruptor bescii* Reveals Optimal Metabolic Engineering Strategies for Bio-based Chemical Production. *Msystems*, 6(3), e01351-20.
95. Zhang, Y.; Shen, J. Enhancement Effect of Gold Nanoparticles on Biohydrogen Production from Artificial Wastewater. *Int. J. Hydrog. Energy* 2007, 32, 17–23. [CrossRef]
96. Zhou, J., Wu, K., & Rao, C. V. (2016). Evolutionary engineering of *Geobacillus thermoglucosidasius* for improved ethanol production. *Biotechnology and bioengineering*, 113(10), 2156-2167.