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(REVIEW ARTICLE)

Improvement of Large-Scale Production of Lignocellulosic Bioethanol through Synthetic Biology Approaches: A Comprehensive Review

Souvik Roy [1,*], Sampreet Manna [1], Subhayu Chowdhury [1] and Lopamudra Choudhury [2]

[1] *Post-Graduate & Research Department of Biotechnology, St. Xavier's College (Autonomous), 30, Mother Teresa Sarani, Kolkata-700 016, West Bengal, India.*
[2] *Department of Microbiology, Sarsuna College (under Calcutta University), 4/HB/A, Ho-Chi-Minh Sarani, Sarsuna Upanagari, Kolkata – 700 061, West Bengal, India.*

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Abstract

This review article explores the use of synthetic biology approaches like gene editing and metabolic engineering to create genetically-modified organisms that can produce commercially-valuable products on large-scale, clean environmental pollutants, and serve as efficient biosensors. The Biobrick system includes a registry of available genetic information and a set of standard protocols for assembling and testing them, which finds various significant uses in synthetic biology, such as in the production of "Fourth generation" biofuel. Genetic engineering techniques may be applied to manipulate crops or microbes for more efficient biofuel production. Such synthetic biology techniques also include modification of lignin structure and content, use of bacterial enzymes abundantly expressed in plants, and manipulation of hemicellulose biosynthesis. However, challenges such as high temperature, efficient conversion of all substrate molecules for maximum product yield, efficient pentose fermentation, and tolerance to acetic acid and bioethanol concentration can all impact the yield of the product. This article explores innovative genetic modification approaches designed to decrease lignin levels and enhance crop digestibility in plants, with a specific focus on the model plant *Arabidopsis thaliana* and herbaceous plants such as Alfalfa. The aim is to manipulate the Monolignol pathway and modify the CSE gene, known for its ability to decrease lignin content. These modifications have been shown to significantly improve saccharification efficiency and increase the yield of lignocellulosic bioethanol. Besides, the role of xylulose-5-phosphate as an intermediate in the non-oxidative pentose phosphate pathway and the oxidative pentose phosphate pathway, and the use of heterologous xylose transporters and modified sugar transporters as potential solutions to improve the biofuel production efficiency is also discussed in this review article.

Keywords: "Fourth Generation" Biofuel; Gene Editing; Genetically-Modified Organisms; Lignocellulosic Bioethanol; Metabolic Engineering; Synthetic Biology

1. Introduction

1.1. Fundamentals of Synthetic Biology

Synthetic biology is a rapidly expanding interdisciplinary field of science that brings together the knowledge and techniques from the multifarious fields of engineering, biology, and computer science to design and construct novel biological components, devices, and systems that are not present in nature [1]. The main goal of synthetic biology is to develop new tools for biotechnology, medicine, and environmental management and gain a deeper understanding of the fundamental principles of life [1]. Utilizing cutting-edge techniques such as gene editing and metabolic engineering, researchers in this field are working to create new genetically-engineered organisms that can produce commercially-

* Corresponding author: Souvik Roy

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valuable products on large-scale, clean environmental pollutants, and serve as efficient biosensors [2]. The potential applications of synthetic biology are thus vast and varied, and the field is poised to play a transformative role in addressing some of the most pressing challenges facing humanity today.[2]

1.2. Biobrick System

One of the most efficient tools of synthetic biology is the Biobrick system, based on the use of standardized genetic parts that can be easily assembled to create new biological systems [3]. The Biobrick standard, developed by the Registry of Standard Biological Parts at the Massachusetts Institute of Technology (MIT) in 2003, has since become a widely accepted standard in the field of synthetic biology [3]. Biobrick are DNA sequences that include promoters, coding sequences, and terminators, which are the basic building blocks of genetic engineering [3]. They are similar to electronic components in that they can be used to create complex systems by combining them in different ways. The Biobrick system also includes an archive of available genetic parts and a set of standard protocols for assembling and testing these parts [3]. One of the main benefits of using Biobrick is that they are exchangeable, meaning that they can be used in different biological systems without requiring significant modification [3]. This allows for the rapid and efficient construction of new biological systems, as well as the ability to test different combinations of parts to optimize performance [3]. Additionally, Biobrick are well-characterized, meaning that their function and behavior have been thoroughly studied and tested, which allows for greater predictability and reliability when building new systems [4]. Biobrick have been used in a wide range of applications in synthetic biology, including the production of biofuels, the detection of pollutants, and the creation of novel medical treatments. For example, Biobricks have been used to design bacteria that can convert plant material into biofuels, as well as to create biosensors that can detect pollutants in the environment [4].

1.3. Synthetic Biology and Biofuel Sector

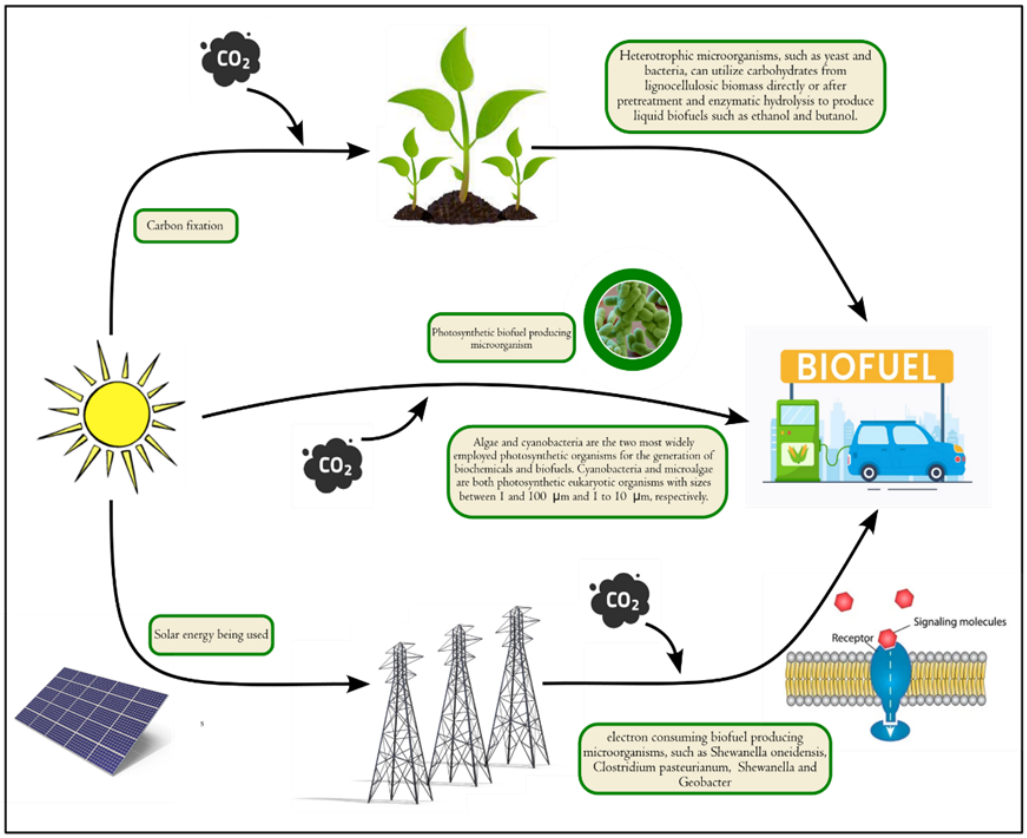

Figure 1 A visual representation of the processes involved in the production of biofuels using renewable sources

The application of synthetic biology in the biofuel sector primarily concentrates on the implementation of metabolic engineering and gene editing to enhance the efficiency and output of biofuel production, thus making it more cost-efficient and eco-friendly [1]. Metabolic engineering is the manipulation of metabolic pathways to improve the production of biofuels [5]. This can be achieved through the introduction of new genes, the alteration of existing genes,

or the deletion of genes that are not essential for biofuel production, for example, to increase the number of sugars converted into biofuels, or to improve the efficiency of enzymes involved in biofuel production (Fig.1) [5].

One application of synthetic biology in large-scale biofuel production is based on the use of genetically-engineered algae [1]. Due to their rapid growth and capacity to produce enormous quantities of lipids that may be converted into biodiesel, algae are a good commercial source of biofuels [1]. More so, by using synthetic biology techniques, scientists have been able to modify the metabolic pathways of algae to increase their lipid production to maximize bioethanol production, and also improve their tolerance to environmental stresses [1]. Another efficient approach is the use of engineered yeasts or bacteria to produce biofuels through fermentation [5]. By manipulating the genetic makeup of these microorganisms, scientists can improve their ability to ferment sugars or other carbon sources into bioethanol or other biofuels. This can increase the yield and efficiency of biofuel production while reducing costs [5].

1.4. Four Generations of Biofuels

The biofuels are categorized into four generations based on the feedstock used and the production processes involved. (Fig.2) [2].

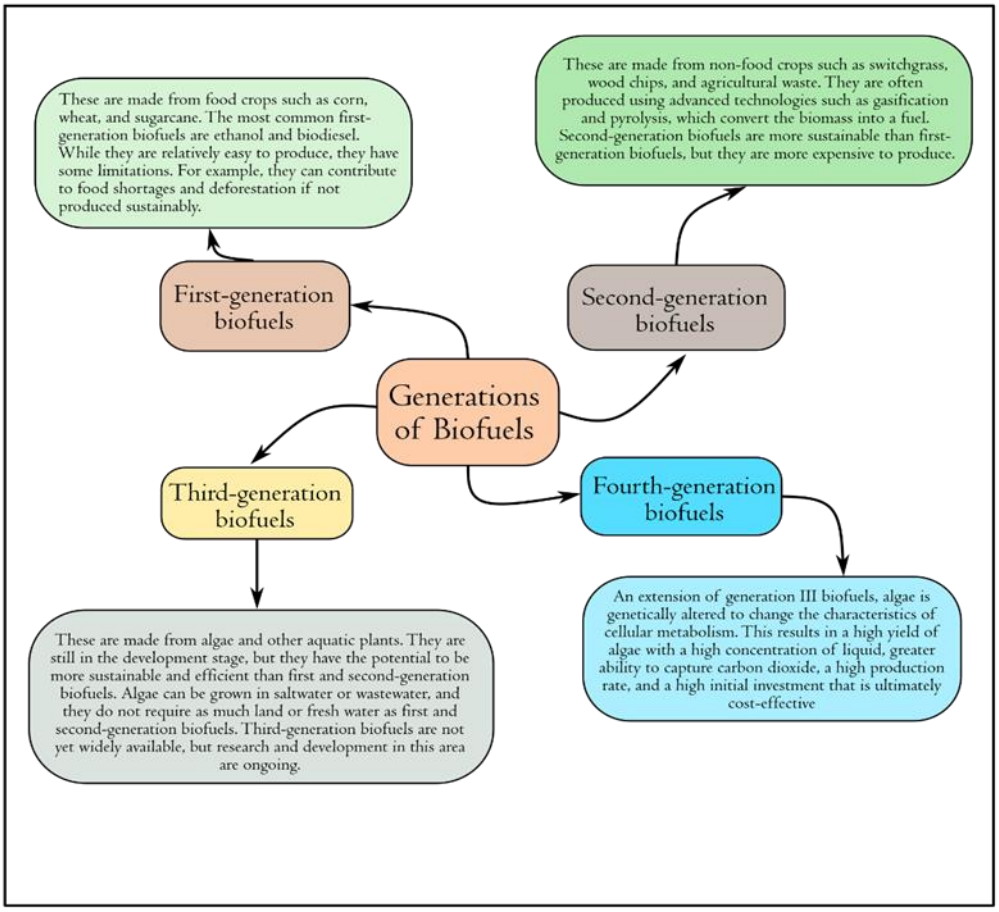

Figure 2 Generations of Biofuels

The First-generation biofuels are made from food crops such as corn, wheat, and sugarcane [6]. The most common first-generation biofuels are bioethanol and biodiesel [6]. While they are relatively easy to produce, they can contribute to food shortages and deforestation if not produced sustainably [6]. The Second-generation biofuels are made from non-food crops such as switchgrass, wood chips, and agricultural wastes [6]. They are often produced using advanced technologies such as gasification and pyrolysis, which convert biomass into fuel [6]. Second-generation biofuels are more sustainable than first-generation biofuels, but they are more expensive to produce [6]. Third-generation biofuels are made from algae and other aquatic plants [6]. Although they are still in their nascent stages of production, they have the potential to be more sustainable and efficient than first and Second-generation biofuels [6]. Algae can be grown in saltwater or wastewater, and they do not require as much land or fresh water as first and Second-generation biofuels [6]. Third-generation biofuels are not yet widely available, but research and development in this area are ongoing. The

term "Fourth-generation biofuel" is a less commonly used and less defined term compared to the first three generations of biofuels [7]. It generally refers to biofuels that are produced using advanced technologies and innovative approaches that are still in the research and development stage [7]. Like Second-generation biofuels, Fourth-generation biofuels are also made from non-food biomass, which would include agricultural wastes, wood wastes, or algae [7]. However, cutting-edge technologies like genetic engineering and synthetic biology would be used to design, engineer, and develop crops or algae which could be used in more efficient and effective biofuel production processes [7].

1.5. Lignocellulosic Biofuel

The use of plant, animal, or microbial biomass as an energy source is considered to be a form of renewable energy, being replenishable through natural processes [2]. Lignocellulosic biomass is one of the most commonly used types of biomass for large-scale energy production [2]. It includes materials such as wood, grasses, and agricultural residues, which are particularly abundant and relatively inexpensive [2]. The conversion of lignocellulosic biomass into biofuels typically involves several steps, including pre-treatment, hydrolysis, fermentation, and purification [2].

However, this conversion of lignocellulose to biofuel is currently hindered by the high costs associated with chemical pre-treatment and enzymatic hydrolysis required for cell wall degradation [2]. Also, despite ongoing efforts to utilize yeast for conversion, the selection of an appropriate host organism remains a complex issue, requiring a careful balance between ease of genetic manipulation and the potential for robust industrial phenotypes [2]. Hence, biotechnological interventions are nowadays much sought after for reducing the recalcitrance of plant cell walls to deconstruction, such as modifying the lignin content and structure therein, employing bacterial and fungal polysaccharide hydrolase enzymes abundantly expressed in plants, and hemicellulose biosynthesis manipulations to break down lignin-carbohydrate complex interconnections [2]. These modifications often result in improved saccharification yields and higher bioethanol production.

The ability to genetically modify plant materials to improve their saccharification efficiency has been made possible by recent developments in our understanding of the biosynthesis of the plant cell wall constituents, such as cellulose, hemicellulose, and lignin. Cell wall polymers must first undergo pre-treatment to be hydrolyzed into simple sugars for fermentation to bioethanol to occur [8]. However, lignocellulosic materials are highly resistant to microbial and enzymatic degradation, as cellulases cannot easily access cellulose because of its highly crystalline structure, majorly cross-linked with lignin and hemicelluloses [8]. So, pre-treatment strategies are required to either remove lignin and hemicelluloses or to interrupt their interactions with cellulose and lower its crystallinity to hydrolyze cellulose efficiently [8]. Currently, chemical and thermo-chemical pre-treatments are most effective for industrial applications [8]. Upon such pre-treatment, polysaccharide hydrolases can hydrolyze additional cellulose into glucose [8]. The biomass feedstock undergoes a series of harvesting and processing steps aimed at converting complex carbohydrates into glucose, which serves as a precursor for bioethanol production by fermentation [9]. Subsequently, the bioethanol is recovered using distillation techniques [9]. However, the conversion of lignocellulose to fermentable sugars is a costly process, primarily attributed to the expenses associated with the cultivation, collection, transportation, storage, and chemical pre-treatment of biomass, as well as the cost of exogenous enzymes [9]. Pre-treatment alone accounts for a significant proportion (approximately 20%) of the total cost of this conversion [10]. *In planta* expression of cell wall polysaccharide-degrading enzymes from bacteria and fungi can increase the efficiency of saccharification [10]. Transgenic plants are engineered to reduce recalcitrance without compromising yield or plant development hence offering a promising avenue for cost-effective biofuel production [10].

Also, to date, *Saccharomyces cerevisiae* has emerged as the premier host for metabolic engineering of biofuel pathways, owing to the availability of a wide range of genetic-systems, and synthetic biology tools [2]. However, alternative yeasts, which may possess superior phenotypes, such as thermotolerance, resistance to toxic compounds generated during plant biomass deconstruction, and enhanced carbon consumption capabilities, are yet to be widely exploited [2]. In contrast, the methods for manipulating the metabolism of oleaginous yeasts like *Yarrowia lipolytica*, which may create high titers of lipids, are fast improving [2].

1.6. Utilizing Engineered *Saccharomyces cerevisiae* for Enhanced Biofuel Production

The term “Engineered consortia” is applied to the synergistic use of a defined set of organisms towards biofuel production. These consortia may or may not include genetically modified organisms (GMOs), and this terminology is adopted for convenience rather than consensus. Yeast, a widely-employed microorganism in bioethanol production, plays a crucial role in converting sugars into bioethanol [11]. This study highlights the impact of yeast strains, fermentation processes, and yeast immobilization on bioethanol yield [11]. A diversity of yeast strains with the ability to produce bioethanol from a range of feedstock have been identified globally [11].

Continuous solid-state fermentation (SSF), which gives a high bioethanol concentration and productivity, shows that the fermentation process has a substantial impact on the production of bioethanol [12]. Pre-treatment, hydrolysis and fermentation are the three basic steps in bioethanol production, where the amount of bioethanol produced is influenced by several variables, including temperature, sugar content, pH, fermentation time, agitation rate, and inoculum size [12]. Hybrid, recombinant and wild-type yeasts are used in the manufacturing of bioethanol [12]. These yeasts can directly ferment simple sugars into bioethanol, unlike conventional feedstock which must first be transformed into fermentable sugars [12]. Yeast cell immobilization also offers a means of enhancing the efficiency and productivity of bioethanol [11]. The use of yeast cell immobilization was assessed with calcium alginate serving as the optimal yeast carrier and the adsorption technique serving as the most preferable method of immobilization [11]. Immobilized yeast cells offer several benefits for the production of bioethanol, including high cell density, simple medium separation, effective substrate conversion, less inhibition, quicker reaction times, and reusable cells [13]. Therefore, yeast cell immobilization represents an economically viable approach for the commercialization of bioethanol production. However, challenges in yeast fermentation such as high temperature, high bioethanol concentration, and pentose sugar fermentation limitations can majorly impact bioethanol yield [13].

1.7. Challenges for lignocellulosic biofuels

The large-scale production of lignocellulosic biofuels suffers from difficult challenges strewn in its path (Table 1). The utilization of agricultural and forestry wastes, as well as energy crops as feedstocks for the production of bioethanol, is nowadays being extensively investigated [14]. Existing lignocellulosic feedstocks with a variety of chemical compositions include switch grass, wheat straw, sugarcane bagasse and maize stover [14]. These raw materials are used both in full-scale and pilot facilities [14]. However, seasonal fluctuations, weather patterns, climatic conditions, crop maturity and storage conditions are some of the variables that might affect the composition and quality of lignocellulosic feedstocks [14].

Table 1 Challenges for Lignocellulosic Biofuels

Challenges	**Description**	**Trouble-shooting by Synthetic Biology approaches**	**Reference**
Feedstock variability	Variables such as seasonal fluctuation, weather patterns, climatic conditions, crop maturity, and storage conditions can affect the composition and quality of lignocellulosic feedstocks.	By leveraging genetic engineering and synthetic biology techniques, researchers can develop new strains of yeast that are better able to tolerate and utilize lignocellulosic feedstocks with a variety of chemical compositions.	[18]
Heterogeneity of feedstock composition	The heterogeneous composition of lignocellulosic feedstocks makes it challenging to develop yeast strains that can efficiently ferment all types of feedstocks.	By using genetic engineering and metabolic engineering approaches, synthetic biologists can develop yeast strains with enhanced tolerance to the inhibitors generated during biomass deconstruction, such as 5-Hydroxymethyl-2-furaldehyde, 2-furaldehyde, Formic acid, Levulinic acid, phenolic inhibitors, and inorganic salts.	[18]
Pentose utilization	Lignocellulosic biomass contains pentoses, such as D-xylose and L-arabinose, which cannot be fermented by wild-type *S. cerevisiae* strains.	Synthetic biology can be used to engineer yeast strains that can efficiently ferment pentose sugars such as D-xylose and L-arabinose. This involves introducing heterologous genes encoding enzymes for pentose metabolism.	[18]
Acetic acid inhibition	Hemicellulose hydrolysis releases acetic acid, which can inhibit anaerobic growth and sugar fermentation by yeast.	Synthetic biology can be used to develop yeast strains with improved tolerance to acetic acid. This can involve engineering yeast to express acetic acid resistance genes, or modifying metabolic pathways to reduce the impact of acetic acid on yeast growth and metabolism.	[7]

Inhibitors from a biomass deconstruction	Several inhibitors of yeast performance are generated during the process of biomass deconstruction, such as 5-hydroxymethyl-2-furaldehyde, 2-furaldehyde, Formic acid, Levulinic acid, phenolic inhibitors, and inorganic salts.	By engineering yeast strains with improved tolerance to these inhibitors, synthetic biology can increase the efficiency of bioethanol production from lignocellulosic biomass.	[16]

The development of yeast strains suited for Second-generation bioethanol production has several generic obstacles due to the heterogeneity of these feedstock compositions and the techniques used for decomposing biomass [14].

The production of large-volume products such as bioethanol requires efficient conversion of all substrate molecules in the feedstock for maximum product yield [15]. Lignocellulosic biomass contains pentoses like D-xylose and L-arabinose [15]. These pentoses respectively makeup 10-25% and 2-3% of the carbohydrate content in lignocellulosic feedstocks, but can even be up to 10-fold higher in some feedstocks [15]. However, these cannot be fermented by wild-type, *Saccharomyces. cerevisiae* strains [15].

Hemicellulose, when hydrolyzed, releases acetic acid, whose volume increases by bacterial contamination during the bioethanol production process [7]. The low pH used in the First-generation bioethanol production processes causes undissociated acetic acid to easily diffuse into yeast cells and dissociate, causing the yeast to expend ATP to prevent cytosolic acidification [7]. High levels of acetic acid in lignocellulosic hydrolysates can thus inhibit anaerobic growth and sugar fermentation by yeasts [7]. Therefore, tolerance to acetic acid at low pH is an important target in developing yeast strains for Second-generation bioethanol production [7].

Biomass deconstruction is a process to convert biomass into fermentable sugars while minimizing the generation of compounds that affect yeast performance. It involves three steps, namely size reduction, thermal pre-treatment, and hydrolysis [16]. During the process, several inhibitors of yeast performance are generated, including 5-hydroxymethyl-2-furaldehyde, 2-furaldehyde, Formic acid, Levulinic acid, phenolic inhibitors and inorganic salts [16]. These inhibitors can interact with each other and bioethanol in complex ways, and their impact can change over time [16]. As the detoxification of hydrolysates has been extensively researched, its cost and complexity have generally been deemed prohibitive. Therefore, the yeast strain's ability to tolerate the chemical environment resulting from current biomass deconstruction is increasingly recognized as a pivotal factor in their advancement [17].

2. Modifications to Overcome the Challenges

2.1. Modified Cell Wall biosynthesis

Cell wall engineering is a field of research aimed at improving the sustainability of biofuels by overcoming the natural resistance of Lignocellulosic Biomass (LCB) [19]. To achieve this, researchers have employed various strategies such as pre-treatments and bioprocessing consolidations, which can solubilize lignocellulose polymers into fermentable sugars [17]. However, these methods also generate complex and expensive downstream processes due to the destruction of valuable sugars and the production of inhibitor compounds [17].

Current developments in metabolic engineering have made it possible to modify the production of cell wall polymers to improve hydrolase access to polysaccharides and decrease processing inhibitors [20]. Researchers have used metabolic engineering to regulate genes involved in cellulose biosynthesis and remodeling in lignocellulosic feedstocks to increase cellulose content and biomass and reduce crystallinity [20]. Additionally, researchers have targeted mixed-linked glucan, xylan backbone and lignin to alter recalcitrance and improve saccharification efficiency [20]. The expression of enzymes involved in xylan biosynthesis has been a target to increase biomass saccharification effectiveness, and overexpression of genes similar to OsAT10 in switch grass has shown enhanced saccharification [20]. Lignin, the end product of the phenylpropanoid pathway, is the main cause of LCB recalcitrance, and recent progress has been made to manipulate the genes encoding enzymes of the phenylpropanoid pathway [21].

2.1.1. Modification of Monolignol pathway in the model plant Arabidopsis thaliana

Genetic modification is utilized in the model plant *Arabidopsis* (*A. thaliana*) with the aim of altering the Monolignol pathway, leading to a reduction in lignin levels and an improvement in crop digestibility (Fig.3) [22]. This approach is motivated by the detrimental impact of lignin on the activity of enzymes responsible for breaking down polysaccharides [22, 23]. The C3H (4-coumaroylshikimate 3-hydroxylase) gene mutation in the *Arabidopsis ref8* mutant has been shown to enhance the degradation of cell walls by polysaccharide hydrolases as compared to the wild type [24]. The bulk of the mutants showed higher cellulose conversion efficiency, with the *ccr* (cinnamoyl-CoA reductase) mutant showing the greatest conversion rate at 88%, according to a study on 20 *Arabidopsis* mutants from 8 gene families involved in Monolignol production [23]. This highlights the potential for manipulating the Monolignol pathway to optimize cellulose saccharification in other species. However, it is important to note that different species may have different pathways for producing Monolignol, and therefore, the downregulation of specific genes involved in the Monolignol process may not produce consistent results across species [23].

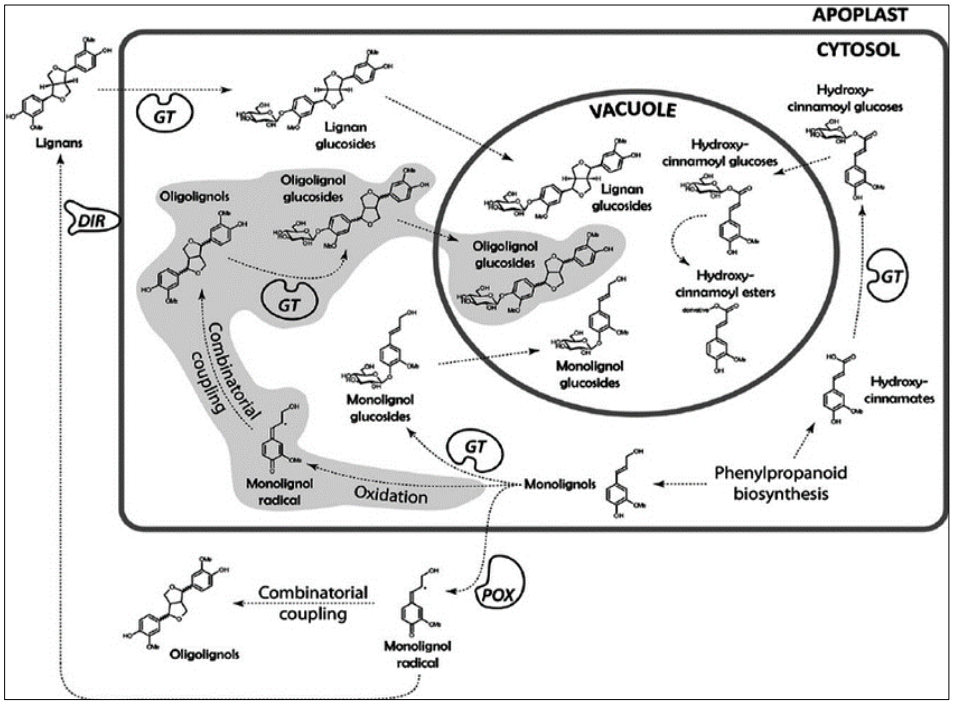

Figure 3 Monolignol-Biosynthesis-Dependent Pathways in *Arabidopsis thaliana* [25]

2.1.2. Monolignol pathway engineering in herbaceous plants

Because of their high biomass yields and ability to thrive in a variety of soil conditions, perennial herbaceous plants like Alfalfa are thought to be promising sources of biofuel [26]. Previous investigations have provided a complete characterization of the roles of four different essential enzymes [Cinnamate 4-hydroxylase (C4H), Caffeic acid *O*-methyltransferase (COMT), Cinnamyl alcohol dehydrogenase (CAD), and CCR] in the monolignol pathway in Alfalfa [26, 27].

Such herbaceous plants can have their lignin biosynthesis pathway altered through the use of synthetic biology to enhance the output of biofuel [26]. In order to better understand the connection between lignin content and biofuel generation, a series of transgenic Alfalfa lines were used, each of which was separately down-regulated in six different lignin biosynthesis enzymes [C4H, Hydroxycinnamoyl CoA:shikimate hydroxycinnamoyl transferase (HCT), C3H, Caffeoyl CoA 3-O-methyl transferase (CCoAOMT), Ferulic acid 5-hydroxylase (F5H), and COMT) [26]. The results revealed a clear inverse relationship between lignin content and sugar release during enzymatic hydrolysis, with the HCT line with the lowest lignin concentration having the maximum saccharification efficiency [26]. This study also discovered that transgenic lignin modification boosted the accessibility of the residual hemicelluloses to degradative enzymes, resulting in more sugar release from xylan than the wild type [26]. The Alfalfa COMT transgenic with reduced S-lignin was further studied to evaluate bioethanol production, whose results showed that the bioethanol yield from the transgenic plants could reach 277 L/ton, 19.7% more than the wild type [28]. These findings show the potential of Alfalfa as a biofuel crop, and the significance of lignin modification for improving the efficiency of biofuel production from it and other similar herbs.

2.1.3. CSE gene modification in Arabidopsis thaliana

The *caffeoyl shikimate esterase (CSE)* gene has recently been identified in *Arabidopsis* species and is found to convert caffeoyl shikimic acid to caffeic acid [29]. Metabolite profiling demonstrated an increase in the accumulation of the lignin-intermediate caffeoyl shikimic acid in the CSE mutants of *Arabidopsis thaliana* as compared to the wild-type plants, suggesting that caffeoyl shikimic acid serves as a substrate for CSE enzyme [29]. The investigation of *CSE* mutants revealed a decrease in the cell wall lignin content, with the remaining lignin exhibiting enrichment in p-hydroxyphenyl units, thereby exhibiting a saccharification efficiency (i.e. conversion of cellulose to glucose) four times higher than the wild-type, without any pretreatment [30].

2.2. Genetic approaches to reduce plant Lignin content for an improved Bioethanol production

Lignin is a key component of plant growth and development and is crucial for the proper physiological functioning of plant cells [31]. Hence, the reduction of lignin content can negatively impact plant growth and ultimately, the efficiency of bioethanol production [31]. The decreased biomass yield can be attributed to the perturbation of auxin signal or alteration of Salicylic acid levels [31].

The reduction of the *hct* gene in *Arabidopsis* was found to have a significant impact on lignin content, independent of Salicylic acid levels [32]. A methylene-tetrahydrofolate reductase that is involved in the production of S-adenosyl methionine (SAM) was found to be encoded by the *bm2 (maize brown-midrib 2)* gene [32]. It was found that the low-lignin *bm2* mutant of maize has a longer flowering time than the wild type, thus demonstrating the potential to generate transgenic plants with low lignin, but without negatively impacting their agronomic performance [32]. Also, the manipulation of specific genes and transcriptional regulatory machinery may provide a means of reducing lignin content without affecting plant growth and performance [33]. Like, the disruption of the transcriptional coregulatory complex Mediator *(MED5a* and *MED5b*) in the *Arabidopsis C3H* missense mutant resulted in the biosynthesis of novel lignin consisting primarily of p-hydroxyphenyl lignin subunits, which led to a substantial increase in the conversion of cellulose to glucose [33]. The changed lignin content and structure may be responsible for the rise in saccharification efficiency [33].

2.3. Modified Xylose metabolism

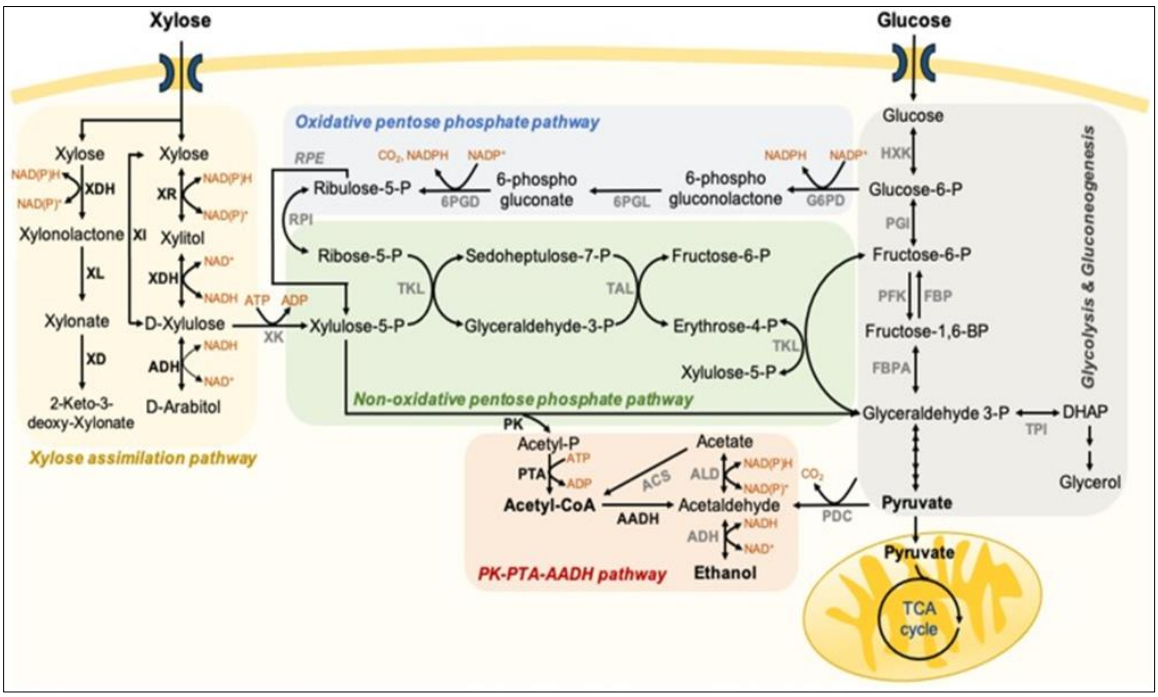

Figure 4 Engineering xylose metabolism in yeasts to produce Biofuel: Xylose assimilation pathway followed by Oxidative and Non-oxidative Pentose Phosphate Pathway [35]

In the process of yeast xylose metabolism, xylose is transformed into xylulose through the process of isomerization, which occurs under aerobic conditions [34]. Two distinct routes – the oxidoreductase pathway and the isomerase

pathway, are used by xylose-fermenting bacteria to transform xylose into xylulose [34]. However, xylose-fermenting yeasts utilize the oxidoreductase pathway, which consists of two enzymatic reactions [34].

The first reaction in the oxidoreductase pathway is performed by the enzyme xylose reductase (XR), which uses either NADH or NADPH, contributed by the oxidative pentose phosphate pathway, as a cofactor [35]. The second reaction is performed by the enzyme xylitol dehydrogenase (XDH), which uses NAD^+ as a cofactor (Fig.4) [35]. This pathway converts xylose into xylulose via xylitol [35].

The isomerase pathway, prevalent mostly in bacteria, consists of a single enzymatic reaction catalyzed by xylose isomerase (XI) [34]. XI catalyzes various sugar conversions, including the conversion of xylose into xylulose, without the need for a cofactor. Most XIs have been identified in bacterial strains, although some anaerobic fungi, such as *Piromyces* and *Orpinomyces* that utilize XI for xylose assimilation have also been discovered [2].

After this conversion, xylulose is phosphorylated by xylulokinase to produce xylulose-5-phosphate (Fig.4) [35]. Both the phosphoketolase process and the non-oxidative phase of the pentose phosphate pathway use xylulose-5-phosphate as an intermediary [35]. In the non-oxidative pentose phosphate pathway, xylulose-5-phosphate is converted in yeast cells into a variety of phosphorylated sugars with three to seven carbon atoms [35]. These sugars serve as glycolytic intermediates or as cellular building blocks like nucleotides and amino acids [35].

2.3.1. Designing a competent xylose-fermenting S. cerevisiae for bioethanol production

The construction of a highly efficient xylose-fermenting yeast strain of *S. cerevisiae* for bioethanol production has been an area of interest due to the abundance of xylose in nature [36]. Although many yeasts possess the genes for the xylose metabolic pathway, only a few are naturally capable of metabolizing xylose [36]. Native xylose-fermenting yeasts such as *Scheffersomyces stipitis* and *Spathasporapas salidarum* have been studied, but they have limitations in terms of bioethanol tolerance and resistance to inhibitors in lignocellulosic hydrolysates [36]. Also, the efficiency of xylose fermentation by these native yeasts depends on the culture conditions [36].

To overcome these limitations, metabolic engineering approaches have been employed to introduce novel xylose utilization pathways into *S. cerevisiae* [36]. *S. cerevisiae* is an important microbe in the Food and Biotechnology industries due to its high fermentation rate and robustness, including tolerance to bioethanol [36]. The goal of the metabolic engineering approaches is to develop an efficient xylose-fermenting *S. cerevisiae* strain by optimizing its internal metabolism and introducing heterologous xylose utilization pathways [36].

2.3.2. Engineering Strategies for Xylose Fermentation in S. cerevisiae

Genetic engineering strategies for xylose fermentation in *S. cerevisiae* are nowadays been well harnessed for improved bioethanol production (Table 2).

The use of the xylose isomerase pathway for fermentation of xylose by engineered yeasts for bioethanol production has been a challenge due to the difficulties in expressing bacterial XI genes in yeast [37]. Nevertheless, the discovery and use of eukaryotic XI coding genes from anaerobic fungi and prokaryotic XI genes from bacteria like *Thermus thermophiles*, *Clostridium phytofermentans* and *Bacteroides stercoris* that can functionally be expressed in *S. cerevisiae*, have enabled effective xylose fermentation [37]. Besides, codon optimization and increased gene dosages of XI have been shown to enhance XI activity in *S. cerevisiae* [37]. A successful approach for fine-tuning the kinetic characteristics of XI expressed in *S. cerevisiae* is through the application of directed evolution, a powerful method that harnesses the principles of genetic variation and selection to drive targeted improvements [39]. Additionally, new engineering targets to enhance xylose usage have been described in recent investigations [37]. Loss-of-function mutations in *isu1* (*Iron sulfur cluster assembly protein 1*) gene, which encodes a conserved mitochondrial matrix protein involved in iron-sulfur (Fe-S) cluster assembly, have been found to improve xylose fermentation in *S. cerevisiae* strains that produce XI [37]. Additionally, it has been noted that loss-of-function mutations in *ssk2 (Suppressor of Sensor Kinase)* and *hog1 (High-Osmolarity Glycerol)*, which are genes involved in stress response and osmotic regulation enhance xylose assimilation in the *isu1* mutant of *S. cerevisiae* [37]. Due to these mutations, iron is made more readily available that affect the production of the Fe-S cluster, which may activate metalloenzyme XI, or encourage aerobic sugar catabolism [37]. The up-regulation of GRE3, which codes for a NADPH-dependent aldose reductase involved in xylitol formation, is controlled by *hog1p* [37]. Because less xylitol is produced when *hog1p* function is lost, XI-expressing *S. cerevisiae* may be better able to assimilate xylose [37]. The lower *gre3* expression levels due to the *hog1p* mutation, meanwhile, might not be enough to support anaerobic xylose fermentation [37]. Besides, it has been studied that, a loss-of-function mutation in *ira2*, which encodes an inhibitor that lowers cAMP levels, and the direct loss of gre3function as a result of laboratory evolution under anaerobic conditions [38]. The *ira2* mutation necessitates the *isu1* mutation for beneficial effects during

anaerobic xylose absorption; however, it may boost xylose intake and bioethanol generation of engineered *S. cerevisiae* by increasing glycolytic flux via the cAMP-protein kinase A pathway [38]. These findings offer new insights into the engineering of yeast for xylose fermentation, and suggest potential targets for further optimization of fermentation parameters.

Table 2 Engineering Strategies for Xylose Fermentation in *S. cerevisiae*

Engineering Strategy	Target	Effect	Reference
Eukaryotic and bacterial xylose isomerase (XI)	Anaerobic fungal and bacterial genes	Functionally expressed in *S. cerevisiae*	[32], [33], [34], [36]
Codon optimization and increased gene dosage	XI encoding genes	Enhance XI activity	[33], [36]
Directed evolution	XI encoding genes	Optimize kinetic characteristics	[36]
Loss-of-function mutations in *isu1*	Conserved mitochondrial matrix protein involved in iron-sulfur cluster assembly	Improve xylose fermentation	[37]
Loss-of-function mutations in *ssk2* and *hog1*	Genes involved in stress response and osmotic regulation	Enhance xylose assimilation in *isu1* mutant *S. cerevisiae*	[37]
Up-regulation of *gre3*	Aldose reductase involved in xylitol formation	Controlled by *hog1p*	[38]
Loss-of-function mutations in *ira2*	Encodes an inhibitor that lowers cAMP levels	Increase glycolytic flux via cAMP-protein kinase A pathway	[38]

2.3.3. Utilization of Engineered Xylose Reductase-Xylitol Dehydrogenase (XR-XDH) pathway

Metabolic engineering studies in *S. cerevisiae* mostly concentrate on the expression of the heterologous Xylose Reductase-Xylitol Dehydrogenase (XR-XDH) pathway [40]. This has mostly been accomplished by using the genes *S. stipitis xyl1* and *xyl2*, which, respectively, code for XR and XDH [40]. In *S. cerevisiae*, these genes have been used to close the metabolic gap between xylose and xylulose [40]. According to earlier studies, compared to *S. cerevisiae* modified via the XI route, the XR-XDH pathway exhibits a higher rate of xylose assimilation and a higher bioethanol titer [40]. However, the XR-XDH pathway does have a limitation in terms of the cofactor imbalance between the XR and XDH enzymes [38]. In particular, the XR enzyme relies primarily on NADPH, whereas the XDH enzyme relies primarily on NAD^+ [40]. When oxygen cannot be used as an electron acceptor to convert NADH to NAD^+ under anaerobic conditions, this imbalance is especially hazardous [40]. Therefore, NAD^+ deficit (or excess NADH) may emerge from the differing cofactor preferences of XR and XDH, which may result in the build-up of xylitol under anaerobic conditions [40]. Therefore, compared to yeasts that were developed to use the XI pathway, yeasts that were developed to use the XR-XDH pathway may produce less bioethanol [40].

Dynamic flux balance analysis predicted that a cofactor-balanced oxidoreductase pathway would result in faster xylose assimilation and higher bioethanol titers [41]. By combining either wild-type XR and NADH-specific mutant XDH or wild-type XR and $NADP^+$-specific mutant XDH, protein engineering techniques have been used to change the cofactor preferences of XR and XDH, reducing xylitol accumulation and improving bioethanol production from xylose [40, 41]. Increasing the activity of XDH compared to XR has also been shown to reduce xylitol accumulation and increase bioethanol yield (Fig.5) [42].

The NADH/NADPH ratio in *S. cerevisiae* has been altered by modifying endogenous oxidoreductase pathways, such as by knocking out genes in the oxidative phase of the pentose phosphate pathway, which generates NADPH [44]. By the introduction of heterologous electron sink reactions in engineered yeasts with the oxidoreductase pathway, it has been demonstrated that cofactor imbalance can be resolved and xylitol accumulation reduced by methods such the overexpression of *noxE*, which encodes a water-forming NADH oxidase [44]. The use of the acetate reduction pathway, which consist of acetyl-CoA synthetase (ACS) and acetylating acetaldehyde dehydrogenase (AADH), has also been

explored in a similar fashion [45]. It is possible to use the Ribulose-1, 5-bisphosphate carboxylase/oxygenase (RUBISCO) and Phosphoribulokinase (PRK) enzymes in the reductive pentose phosphate pathway as an electron sink reaction to re-assimilate carbon dioxide [45]. To form two molecules of 3-phosphoglycerate (3-PG) from ribulose-5-phosphate (Ru-5-P) and carbon dioxide (CO_2), the PRK and RuBisCO reactions are carried out sequentially [45]. While bioethanol is eventually produced from 3-PG, CO_2 can be used as an electron acceptor to reoxidize it [45]. By including chaperonins, PRK, and RuBisCO into the bioethanol fermentation process to reassimilate CO_2 generated by pyruvate decarboxylase, a viable reductive pentose phosphate pathway has been developed in an effective xylose-fermenting *S. cerevisiae*, improving the bioethanol output [45]. The yields of glycerol and xylose, which were generated to offset the excess NADH, were noticeably reduced when the reductive pentose phosphate pathway was functionally expressed in engineered S*. cerevisiae* strains, and the yield of bioethanol during xylose fermentation was dramatically enhanced [45]. Nevertheless, during the fermentation of glucose, the expression had no discernible impact on the yields of glycerol and bioethanol [45]. Due to a greater supply of Ru-5-P and an abundance of NADH during xylose fermentation compared to glucose fermentation, there was a synergistic impact between the utilization of xylose and the reassimilation of CO_2 [41, 45].

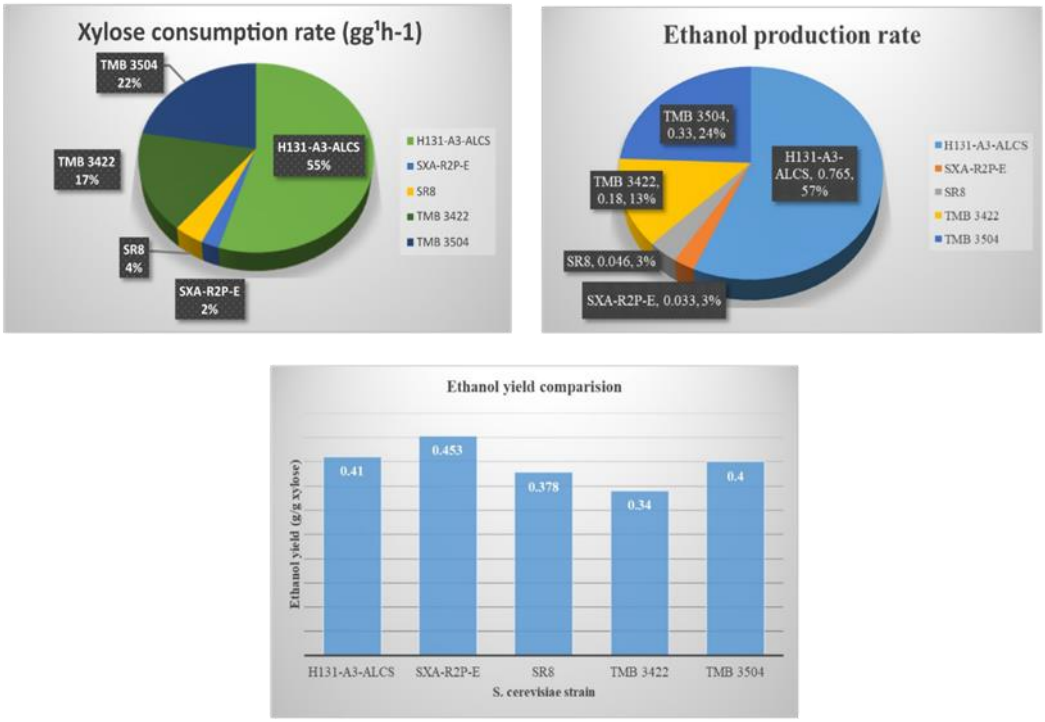

Figure 5 Comparative analysis of xylose consumption and bioethanol production, as represented by pie chart. SXA-R2P-E is an evolved strain of SXA-R2P (deleted the *pho13* gene in the rationally engineered strains SXA-R2) [42]; the strain TMB is constructed by chromosomal integration of the genes encoding D-xylose reductase (XR), xylitol dehydrogenase (XDH), and xylulokinase (XK), strains TMB 3402 and 3504 are the results of chemical mutagenesis [42]; SR8 is an engineered *Saccharomyces cerevisiae* strain that has been developed using a combination of rational and inverse metabolic engineering strategies, thereby improving its xylose fermentation and bioethanol production abilities [42]; and H131-A3-ALCS is a genetically modified yeast strain with multiple copies of *Piromyces xylA*, overexpression of *S. stipitis* XYL3 and PPP genes, and the introduction of ARG4 and LEU2 for auxotrophic marker recovery [43].

However, the availability of NADPH and ATP for XR and RuBisCO, respectively, during xylose fermentation of modified S*. cerevisiae* may be constrained [40]. Through the co-fermentation of maltose and xylose, the problem of NADPH and ATP limitation during CO_2 reassimilation under xylose culture conditions has recently been proven and solved [40].

2.4. Engineered Xylose-specific Transporters

Xylose-specific transporters are absent in the yeast *S. cerevisiae* [46]. Therefore, non-specific hexose transporters (Hxt) such Hxt1p, Hxt2p, Hxt4p, Hxt5p, Hxt7p, and Gal2p can be used by *S. cerevisiae* to utilize xylose [46]. These transporters are ineffective at lower xylose concentrations, and have a significantly lower affinity for xylose than glucose [46]. As a

result, developing effective xylose-fermenting yeast strains for industrial purposes is significantly hampered by the restricted capacity of native sugar transporters [46]. Two main approaches have been used by researchers to get over this bottleneck - investigating novel heterologous xylose transporters and modifying already-existing sugar transporters [46].

The ability of heterologous transporters from naturally occurring xylose-fermenting yeasts like *Candida intermedia* and *S. stipitis* in *S. cerevisiae* to transport xylose has been studied [46]. In contrast to the expression of endogenous transporters like Hxt7p, the expression of *C. intermedia GXF1* (glucose/xylose facilitator 1) and *GXS1* (glucose/xylose symporter 1), and *S. stipitis* XUT1 and XUT2 xylose transporters had little effect on growth in hexose transporter null mutants of *S. cerevisiae* (*hxt1-17, gal2, stl1, agt1, mph2* and *mph3*) [46]. However, the extra expression of GXF1 considerably increases the xylose absorption rate and cell proliferation at low xylose concentrations in transporter-positive *S. cerevisiae* [46]. The xylose absorption rates and specific growth rates of industrial *S. cerevisiae* harboring XI are also increased by the overexpression of *S. stipitis* transporters such as XUT4, XUT5, XUT6, XUT7, RGT2 (Restores Glucose Transport 2) and SUT4 (Sucrose Transporter 4) [47].

2.5. Inhibitor Tolerance

The detoxification of particular inhibitors by yeast enzymes offers potential targets for metabolic engineering [42]. It has been demonstrated, for instance, that overexpression of native $NAD(P)^+$-dependent alcohol dehydrogenases (ADH) promotes the conversion of furfural and hydroxymethyl furfural (HMF) into less toxic alcohols, such as Furanmethanol and furan-2,5-methanol, respectively [42]. Additionally, the combined over-expression of the yeast Alcohol acetyltransferases (Atf) 1 and 2, the decarboxylase Pad1 (Phenylacrylic acid decarboxylase-1) and Ald5 (Aldehyde dehydrogenase-5), can result in increased resistance to a number of phenolic inhibitors [42].

Complex, strain- and context-dependent stress-response networks have been identified as the main drivers of inhibitor tolerance in genome-wide expression investigations [42]. SFP1 and ACE2, which encode transcriptional regulators involved in ribosome biogenesis and septum degradation during cytokinesis, respectively, have been discovered by in-depth transcriptome analysis as key players in the phenotype of acetic acid and furfural-tolerant strains [42]. Surprisingly, in the presence of these inhibitors, overexpression of these transcriptional regulators greatly increases bioethanol productivity [42].

By performing whole-genome re-sequencing of these strains, researchers have been able to identify the mutations responsible for their increased tolerance [43]. To further understand the mechanisms underlying tolerance, physiological and evolutionary engineering experiments have been conducted [43]. These have demonstrated that high rates of sugar fermentation are crucial for acetic acid tolerance [43].

It is interesting to note that the evolved cultures required pre-exposure to lower acetic acid concentrations in order to fully show their improved tolerance, indicating that their tolerance was inducible, rather than constitutive [42]. On the contrary, constitutive tolerance refers to the proportion of yeast populations that may start growing after being exposed to acetic acid stress [42].

To select constitutive acetic acid tolerant strains, scientists have utilized an evolutionary engineering strategy that alternates batch cultivation cycles in the presence and absence of acetic acid [42]. This approach has been successful in identifying genes and alleles that contribute to tolerance by exploring the natural diversity of inhibitor tolerance among *S. cerevisiae* strains [42]. To this end, the combination of whole genome sequencing and classical genetics has proven to be a powerful approach for identifying genomic loci, genes, and even individual nucleotides responsible for tolerance [43].

3. Conclusion

Plant biotechnology studies have made significant progress in modifying plant cell walls to enhance the production of lignocellulosic biofuels. A key focus has been on understanding lignin production and finding effective interventions to target the monolignol pathway and reduce cross-linking between lignin and carbohydrates. However, most of the technological advancements for bioethanol production have been observed in controlled facilities. One crucial aspect in lignocellulosic materials is the presence of xylose, the second most abundant monosaccharide. To facilitate cost-effective and sustainable conversion of lignocellulosic biomass into chemicals and fuels, researchers have explored various methods to develop efficient xylose-utilizing microorganisms. One such candidate is *S. cerevisiae*, which possesses durability under industrial fermentation conditions and can be engineered to consume xylose, eliminating the need for naturally occurring xylose-fermenting yeast strains. To achieve effective xylose fermentation in *S.*

cerevisiae, two pathways have been utilized: the XR-XDH pathway and the XI pathway. Through manipulating the efficiency ratio of the XR/XDH pathway, introducing recombinant electron sink processes, and designing optimized proteins, researchers have minimized a major drawback of *S. cerevisiae*, which is its dual cofactor preference. These advancements pave the way for the development of robust xylose-fermenting *S. cerevisiae* strains, which hold promise for a more efficient and sustainable bioethanol production on a large-scale.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare that there is no potential conflict of interest between them.

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