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REVIEW PAPER

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Influence of reactors, microbial carbohydrate uptake, and metabolic pathways on ethanol production from grass biomass: A review

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Summary

Grasses are considered to be potential lignocellulosic feedstock for renewable and sustainable biofuels such as bioethanol. However, the process involved, ie, pretreatment, enzymatic saccharification, and fermentation in conversion of these lignocellulosic biomass to bioethanol, remains expensive and at present is not affordable for industrial production. Thus, the present review assesses the influence of the recent technologies that can be employed for the bio-refinery based pretreatment and enzymatic hydrolysis of grass biomass using advanced bioreactors. Since plant extracts have been seen to enhance the glucose uptake, an experiment was implemented to elucidate the role of plant extracts (bark extracts of *Xylocarpus granatum*) on glucose uptake capacity of microorganisms like *Saccharomyces cerevisiae*, *Pichia* sp., and *Zymomonas mobilis* and their subsequent ethanol production capability from glucose and xylose sugars. The results of these experiments indicated that supplementation of plant extracts promoted both glucose and xylose uptake in *S. cerevisiae* and *Pichia sp*. as compared with the control and *Z. mobilis* strain. Further, as *Pichia* sp. exhibited good uptake ability for both glucose and xylose, a model was proposed focusing on the gene silencing and operon concept in *Pichia* sp. for preferential pentose utilization during the fermentation of grass biomass to bioethanol.

KEYWORDS

bioreactor, glucose uptake, lignocellulose, metabolic pathway, pentose phosphate pathway

1 | INTRODUCTION

The fossil fuels which are the major source of energy supply are depleting at a faster rate and simultaneously generate the threat of environmental concerns such as global warming.[1] To address these challenges, bioethanol from lignocellulosic biomass, which is a form of quasi-renewable transport biofuel, is capable of reduction up to 80% greenhouse gas (GHG) emissions as compared with conventional fossil fuels have been considered.[2] The escalating interest for production of sustainable bioethanol can be exemplified by the production of 21 812 million gallons in 2012; 23 429 million gallons in 2013; 24 570 million gallons in 2014; 25 682 million gallons in 2015; 31 093 million gallons in 2016; and 30 961 million gallons in 2017.[3] Although a trivial

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decrease in bioethanol production is observed in 2017, its production is expected to accelerate to approximately 32 420 million gallons in 2018.[4]

According to an estimate by Kim and Dale[5] and Sarkar et al,[6] lignocellulosic biomass (agro-wastes, energy crops, etc) solely has the capacity to produce approximately 442 billion liters of bioethanol per year. If total crop residues and wasted crops are taken into consideration, the production can even mount up to 491 billion liters, which is about 16 times higher than the actual global production. A validation to this possibility was reported by Kim and Dale[5] who showed that 73.9 teragram of dry wasted crops (eg, agricultural residues) could possibly yield 11146.71 million gallons of bioethanol worldwide. Among the lignocellulosic biomasses, grasses have been reported to contain approximately 40% to 50% of fermentable sugar and are considered as a promising feedstock for bioethanol production.[7] They are cheap bio resources with high hexose and pentose contents, have higher yield rates, are suitable for low quality land, and offer non-hazardous environmental impacts. These properties mark them more convenient feed stock for bioethanol production in industrial sector as compared with other lignocellulosic substrates.[2]

Industrialization of bioethanol from lignocellulosic biomass at present is a challenging task because of the high cost factors that are involved in process parameters such as pretreatment, enzymatic hydrolysis, and fermentation. Among the different parameters, pretreatment is crucial in lignocellulosic bioethanol production since it facilitates the disruption of the recalcitrance and heterogeneous structure of the grass cell wall.[8] This disruption is essential for accessibility of hydrolytic enzymes for efficient saccharification of complex carbohydrates into simple sugars. Furthermore, currently available commercial enzymes with high hydrolytic activity enzyme activities have significantly high costs which also add to the production prices. It is interesting to note that despite a lot of research being focussed on increased enzyme activities, factors such as improper mixing of enzyme and substrates lead to lower ethanol yields. Apart from these, fermentation of the saccharified sugar (hexosans and pentosans) by fermentative microorganisms requires longer incubation periods. However, the utilization of hexosans is more at ease for the microorganisms as compared with pentosans. Although genetic modifications of microorganisms like *S. cerevisiae*, *Z. mobilis* and *Clostridium* sp.[9-11] have been attempted for utilization of both hexose and pentose sugars, but the process for modifications is expensive.

In the above context, this review attempts to highlight three important aspects of bioethanol production from grass biomass. In the first section, we emphasize the role of bioreactors in economical pretreatment and enzymatic saccharification of grass biomass, a promising lignocellulosic feedstock that belong to Poaceae family. Since the conventional methods of pretreatment and enzymatic hydrolysis have been extensively studied,[12] the focus of the present review has been limited to the recent adaptations in bioreactors that require less energy input with minimal loss of sugars along with higher delignification of grass biomass. Further, the mechanism of glucose uptake capacity, which has not been elucidated so far in detail for bioethanol production, has been highlighted. The role of plant extract in glucose uptake by fermenting microorganisms is experimented in the present study. A comparative glucose uptake study was carried out using three strains, ie, *S.cerevisae*, *Z. mobilis* and *Pichia* sp., with and without bark extracts of a mangrove plant ie, *Xylocarpus granatum*, at different concentrations. From the results of the glucose uptake experiments, a model has been proposed wherein we emphasize the utilization of *Pichia* sp. as a potential pentose fermenting microorganism even in the presence of glucose.

2 | COMPOSITION OF GRASS BIOMASS AND REACTORS FOR PRETREATMENT AND SACCHARIFICATION OF GRASS BIOMASS

The general composition of grasses varies with species, but it generally consists of ~26% to 47% of cellulose, ~25% to 43% of hemicellulose, and ~5% to 18% of lignin.[13] The lignin acts as a barricade for the enzymes that are required to saccharify the cellulose and hemicellulose in the grass cell wall. Therefore, pretreatment is generally required for disintegration of cell wall before enzymatic hydrolysis to enhance the digestibility of lignin for better accessibility of the saccharifying enzymes to the sugars. However, the pretreatment step should be economical and should not lead to significant loss of carbohydrates which are bounded to lignin.

Several conventional pretreatment techniques such as acid, alkaline, biological, and steam explosion have been studied intensely for lab-scale (shake-flask conditions) pretreatment of grass biomass.[12] However, these techniques add significant cost-factors to the process economics of bioethanol production when employed in industrial sectors. More recently, hydro-dynamic cavitation pretreatment technique has been proposed and implemented. It involves generation of cavitation in freely flowing liquid which is constricted with a venture tube or orifice plate.[14] The principle behind this technique is based on Bernoulli's equation, where the drop in pressure at the constriction (ie, venturi throat) below

the vapor pressure of the flowing liquid generates bubbles or hydrodynamic cavities. When the flowing liquid comes downstream of the constriction, an increase in the pressure leads to the collapse of cavities. Hydrodynamics cavitation reactors (HCR) are high-throughput systems which have been successfully used for delignification of reed grass (*Phragmites australis*)[15] and wheat straw for production of ethanol. The advantage offered by these reactors is that it requires much lower energy input compared with an ultrasonication reactor.[14] HCR has been also employed for delignification of sugarcane bagasse by many authors[16,17] and is observed to affect both delignification and crystallinity of cellulose for increase in bioethanol production. An extensive review on the use of HCR for delignification of lignocellulosic biomass has been reported by Hilares et al.[16] Apart from HCR, microwave reactors (MR) have also attracted the attention for lignocellulosic pretreatment in reactor scale.[18] MR have four sections, ie, a generator (magnetron is the most preferred generator having an anode and a cathode), a cavity section (which act electrical oscillator), a waveguide (an applicator which ensures the transfer of energy from magnetron till the sample), and an advanced control monitoring system (for power, temperature, and pressure).[18] The principle behind these reactors is based on Maxwell's equations, and these reactors are generally referred as monomode microwave or focused reactors. These reactors are easy to design and offer a uniform distribution of radiation throughout the sample.[19] Peng et al[20] developed the first reactor for lignocellulosic (corn strover) pretreatment in a pilot-scale continuous microwave irradiation, with a maximum work temperature of 300°C and biomass capacity of 5 kg/h. The ethanol production from the pretreated lignocellulosic biomass was 31.29 g/100 g of pretreated dry material, which offers a good productivity scale on a pilot scale production unit. To the best of our knowledge, there have been no reported data on pretreatment of grass biomass with these reactors, and hence the prospect of using MR for pretreatment of grass biomass can be explored for enhanced delignification.

Similar to the developments in designing of reactors for pretreatment, some research have also been focused for development of reactor configurations for efficient saccharification. The major reactor developments for saccharification from grasses have been focused on the improvement of configurations such as the agitation systems. For example, Palmqvist and Lidén[21] conducted a comparative study on the influence of water insoluble solids content on glucose yield during the hydrolysis of steam pretreated giant cane (*Arundo donax*) and spurce in a 3-L stirred tank bioreactor supplied with an anchor impeller. The authors adopted two different methods, wherein in the first instance the impeller speed was kept constant and in the second instance the power input for the impeller was constant. While the former decreased glucose yield of giant cane from ~ 40% to ~ 27%, the latter resulted in a decrease of ~45% to~ 35% of glucose in spruce. The results clearly indicate that differences in configurations yield different results for diverse variety of biomass. The relation between particle size distribution and the agitation rate has also been studied in a comparative analysis of the above-mentioned biomass.[22] However, a different impeller system, ie, a pitched blade impeller with three blades at an angle of 45° was used in the reactor. For this study also, two approaches were adopted. While in the first approach three different impeller speeds, ie, 100, 300, and 600 rpm (rotations per minute) were evaluated, different particle size of giant cane and spruce were assessed in the second approach. The authors concluded that while impeller speed adversely affected the saccharification efficiency of spruce, the lower particle size in giant cane improved its saccharification efficiency.

Innovative impeller concepts have also been experimented for enzymatic saccharification of wheat straw. Riedlberger and Weuster-Botz[23] suggested a precise high-throughput system for alkaline pretreated wheat straw to reduce the optimization cost for large scale enzymatic hydrolysis. The proposed system included 48 parallel stirred-tank bioreactors of 10-mL volume, equipped with novel S-shaped impeller. The authors analyzed the rate of hydrolysis of alkaline pretreated wheat straw in different solid concentration (4%–10% [w/w]). They observed glucose release up to ~111 mg/g of dry matter after 9 hours of hydrolysis, which was quite comparable to the 1-L scale revealing a scope towards an efficient and easy scale-up of this novel system for optimization of hydrolysis conditions. Vertical stirred tank with modified agitator systems have also been tested for bioethanol production from *Poaceae* family. Ludwig et al[24] designed a vertical stirred tank reactor supplied with segmented helical stirrer for the hydrolysis of alkaline-pretreated wheat straw and compared the rate of hydrolysis in both 250-mL Erlenmeyer flasks as well as stirred tank reactor at high solids loading. The authors found that, at a speed of 80 rpm, the glucose yield was increased from 70% to 76% from flask to bioreactor, corresponding to a hydrolysate with 110 g glucose kg^{-1} biomass in the reactor system. Modifications in agitator systems have also been observed for horizontal stirred tank reactors. Jørgensen et al[25] designed a reactor system containing horizontally placed drum divided into five independently sections equipped with three paddlers that were assembled around a horizontal rotating shaft for an efficient mixing during the liquefaction and

saccharification of the pretreated wheat straw. The authors analyzed the effects of the liquefaction and glucose releasing efficiency for 96 hours by varying the mixing speed and the initial dry matter content. They reported that while the mixing speed did not influence the cellulose conversion, the hemicellulose conversion was negatively influenced as it decreased with the increase in mixing speed. Furthermore, they obtained a maximum of 86 g/kg of dry biomass after 96 hours of treatment. Another interesting study on wheat straw was the investigation on solid recycling for the saccharification of the biomass by Pihlajaniemi et al.[26] The agitation system was used in the shape of a rotating paddle with the rotation speed of only 2 rpm and a solid loading of 16% (w/w). The differences were observed for hydrolysis rates and volumetric productivity. It was observed that while the hydrolysis rate was higher using rotating paddles, the volumetric productivity was significantly lower.

3 | FERMENTATION OF GRASS BIOMASS FOR BIOETHANOL PRODUCTION

Enzymatic hydrolysis is followed by fermentation, which is a chemical process for converting simple monomeric sugars such as glucose and xylose to produce ethanol with the evolution of CO_2 via glucose and xylose utilizing pathways. Researchers have developed different modes of fermentation that includes batch, fed batch, and continuous operations depending on the quantitative ratio of the end products and direction of fermentation along with the characteristics of the fermenting microorganisms.[27] Bioethanol production from perennial crop could be a promising technology if an appropriate fermentation technique is applied. However, in order to achieve high ethanol titres from grass biomass, it is imperative to understand two vital points, ie, influence of fermentation parameters on microorganisms and the response of microorganisms to those parameters.

3.1 | Influential parameters on microorganisms

The most important influential parameters for fermentation include the carbon sources, pH, temperature, agitation system, and the type of fermentation strategy applied among the others. However, as the carbon sources and the fermentation strategy have the major roles with respect to fermentation yields, the discussion is focused on the same. The section also discusses about the brief role of inducers, such as plant extracts, in uptake of carbon sources into the microbial cells. Moreover, the mechanistic action on glucose uptake process by organisms such as bacteria and yeast is an interesting area which has not been discussed so far in literature studies. Therefore, a pioneering study has been undertaken to study the role of plant extracts on glucose and xylose uptake by the microorganisms. Consequently, their fermentation efficiency for bioethanol production from glucose and xylose as substrates, in the presence of plant extracts, was also evaluated.

3.1.1 | Carbon sources

The cell growth in microorganisms is greatly dependent on the given carbon sources as the cell regulates the flow of the substrate through metabolic network. It is interesting to note that the incoming sugar can either be fermented to a product or respired depending on the identity of the carbon source, the type of cell, and the growth conditions. An example of this was demonstrated by Velagapudi et al[28] where two structurally similar hexoses, ie, glucose and galactose, were used as the sole carbon sources. While glucose was efficiently fermented to ethanol with minimum oxygen requirement, galactose was used by the organism partly for respiration and partly for fermentation. Utilization of xylulose (an intermediate) as a sole carbon source, though is rare for naturally growing yeasts, but has been observed in some instances.[29] Briefly, the xylulose is transported by the hexose transporter family into the cells, which is then phosphorylated and through the pentose phosphate pathway (PPP) moves in to the central metabolism system.[30] Similar to glucose, xylulose may also be utilized for respiration or fermentation. This was exemplified by Mittelman and Barkai,[31] in which yeast cells (*S. cerevisiae*) that were grown on xylulose participated in activation of the transcription characteristic of respiring cells rather than fermenting cells. An important outcome of the research also highlighted that the fermentation capacity of the yeast cells appeared to correlate on the higher side with transcriptome characteristics related to the growth rate of the cells rather than respiration mechanism.

Glucose and xylose uptake in fermentative microbes: Mechanism and its applications in increased ethanol production

Fermentation efficiency can be enhanced by utilizing fermentative microorganisms to utilize more carbohydrate molecules present in the fermentation medium. In order to increase the cell absorption capacity, it is important to understand the underlying mechanism of intracellular carbohydrate uptake. Use of plant extracts (in the present study) has been observed to induce both glucose and

xylose uptake in yeast cells, and hence their mechanism is discussed below (Figure 1). A distinct glucose uptake pathway facilitates the acceptance of glucose molecule into the cell by expressing several genes encoding glucose transporters. Along with the glucose uptake, this pathway also aids the suppression of *Rgt1* (Restores Glucose Transport), a transcription factor that inhibits the expression of hexose transporters (*hxt*) by binding to them. Along with *Rgt1*, two proteins such as *Mth1* (MSN Three Homolog) and *Std1* (Suppressor of Tbp Deletion) also interact with *Rgt1* to suppress *hxt* gene expression and thereby inhibit the glucose uptake into the cell. In the cell membrane, glucose is sensed by two putative glucose sensor ie, *Rgt* 2 (Restores Glucose Transport) and *Snf* 3 (Sucrose Non-Fermenting). In the presence of extracellular glucose, these two sensors produce an intracellular signal that accelerates the expression of *hxt* (Hexose Transporter) gene for glucose uptake.[32] In case of high glucose concentration in the medium, the degradation of *Std 1* and *Mth 1* is accelerated which in turn aids the release of *Rgt1* from plasma membrane thereby preventing repression mediated by *Rgt1* and facilitating glucose uptake into the cell.

S. cerevisiae is considered as an important cell factory for industrial production of ethanol. Although yeast can utilize a wide range of carbon sources such as glucose, fructose, sucrose, lactose, and many pentose sugar molecules, but glucose is found to be the most preferred carbon source for fermentation and production of ethanol. In yeast cells, metabolism of glucose occurred mostly although alcoholic fermentation as fermentation process can utilize glucose aggressively and at a much faster rate. However, energy generation through fermentation of glucose is not so efficient; therefore, yeast cells consume the available glucose more efficiently by enhancing the glucose uptake. Hence, for optimal glucose utilization and growth rate, a fast and coordinated way to regulate gene expression is required.[33] In fact, *S. cerevisiae* has evolved a sophisticated system for sensing of glucose and its uptake. Two types of transport processes exist in yeast cells via. facilitated diffusion and active transport. The glucose transporter acts by facilitated diffusion mechanism in which intracellular transport of glucose molecules occurs by a concentration gradient. In *S. cerevisiae,* the glucose transport is a multi-factorial uptake system regulated by different *Hxt*. Out of the different hexose transporters, ie, *Hxt1-17*, *Gal 2 (GALactose metabolism)*, *Snf 3*, and *Rgt 2*;*Hxt 1* to *Hxt 7* function as prominent glucose transporters and metabolically more relevant.[34]

In case of *Z. mobilis*, a stereo-specific carrier-mediated, low-affinity, high-velocity, and non-concentrative D-glucose facilitated diffusion system has been proposed for transport of D-glucose.[35] The glucose facilitator protein (*Glf*) in *Z. mobilis* transports glucose

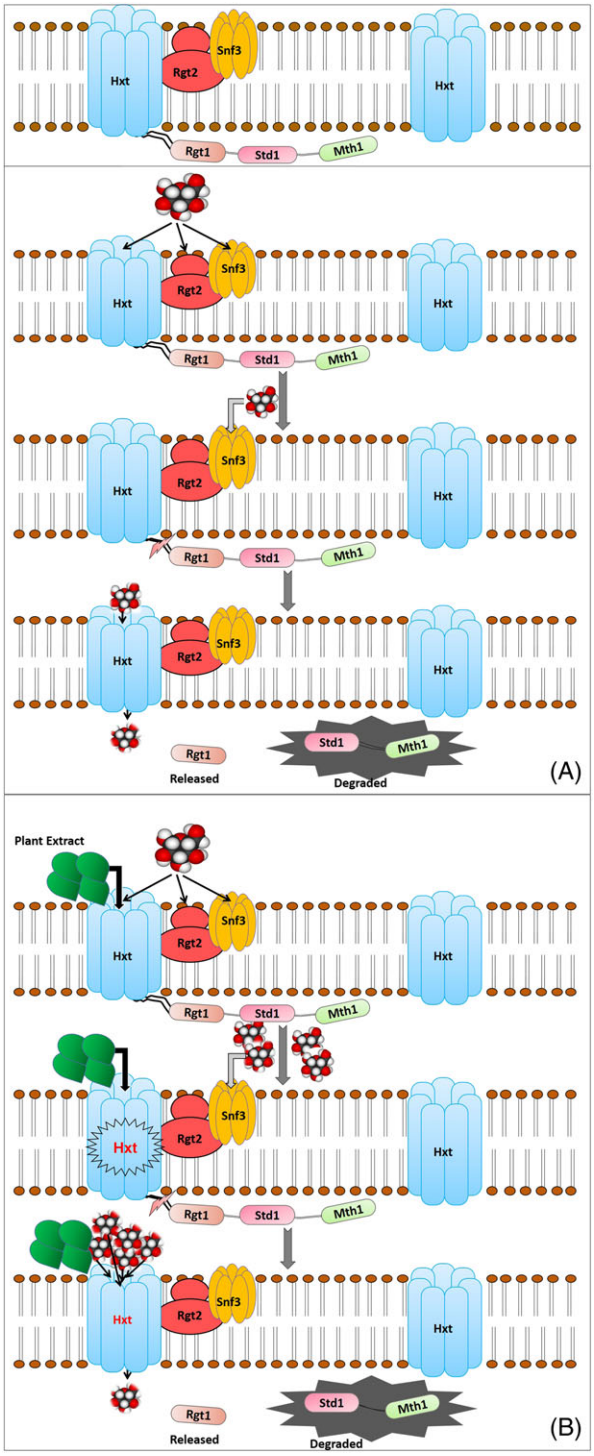


FIGURE 1 Mechanism of glucose uptake (A) in the absence of plant extract (bark extracts of *Xylocarpus granatum*) and (B) presence of plant extract. In the presence of plant extract, transportation of glucose across the yeast cell membrane was stimulated as comparison to the transportation of glucose in the absence of plant extract. The plant extract is supposed to have some effect on the glucose sensors (Rgt 2 and Snf 3) present on the plasma membrane of *S. cerevisiae*, responsible for the production of intracellular signal that causes the overexpression of hexose transporter gene (hxt) [Colour figure can be viewed at wileyonlinelibrary.com]

and is also involved in transport of fructose, xylose, and other non-metabolizable glucose analogs, such as 2-deoxyglucose.[36] However, glucose is more preferred over other sugars for transportation through the *Glf*. The glucose facilitator protein, *Glf* transporter encoded by the *Glf* gene, belongs to major facilitator super-family class of proteins. It functions without additional energy and relies on the cross-membrane concentration gradients of sugars. Several studies have reported to increase both glucose and xylose uptake by intervention of molecular techniques in the glf gene of *Z. mobilis*.[37]

Different studies have investigated the effect of inexpensive substances like soy flour, oils and fatty acids, fungal mycelia, and fruit pulp on yeast mediated improvement of ethanol production.[38] Lebaka et al[39] have shown that supplementation with fruit pulp of banana, mango, and chiku in small quantity during fermentation by *S. cerevisiae* increased the rate of alcohol production and alcohol yield in very high gravity fermentation. This may be due to the presence of high polyphenols content such as flavones, stilbenes, flavonones, isoflavones, catechins, chalcones, tannins, and antho cyanidins etc. that can stimulate high alcohol production. This may be attributed to antioxidant, metal ion-chelating, and/or free radical scavenging activity.[39] However, no studies have so far been attempted to evaluate the effect of plant extracts on glucose/xylose uptake by microorganisms and its subsequent effect in bioethanol production.

In this context, supplementation with plant extracts having high poly phenol content may help in high ethanol production by protecting the yeast cells from osmotic stress and overcoming the nutritional deficiencies of yeast. The supplementation would allow them to stay longer in the growth phase thereby resulting in a higher ethanol yield. For an elucidation of this hypothesis, an experiment was designed to investigate the relationship between cell growth and bioethanol fermentation. The experiment consisted of four parts viz. screening of three organisms for their ability to grow on different sugar sources. The organisms, ie, *S. cerevisiae*—a kind gift from Dr R. C Roy, Central Tubercrop Research Institute, Odisha, India; *Pichia sp*.—isolated in microbiology laboratory, CET, Odisha, India, and *Z. mobilis*—a commercial strain[40] were evaluated for their growth (540 nm) on four different sugar sources. Following it, the optimized sugar sources (glucose and xylose) along with bark extracts of *X granatum* with different concentrations (1, 3, and 5 mg/10 mL of sugar solution) were utilized for the growth of the three organisms over an incubation period from 0 to 72 hours. The ethanol bark extracts of *X. granatum* have been reported earlier for its potential to increase glucose uptake in yeast cell during the assessment of antidiabetic activity.[41] The reason for such activity may be attributed to the fact that *X. granatum* plant being rich in polyphenols has prominent free radical scavenging potential[41] that might play an important role in protecting yeast cells from osmotic stress allowing them to have prolonged growth period. In the third part, the optimized concentration of plant extract was used against a constant weight of glucose (3 g) and xylose (3 g), and subsequently the growth was observed for the same time period. The final part of the experiment included the fermentation of the sugar media (both glucose and xylose separately) in the optimized conditions, to understand the relationship between growth pattern and fermentation ability. The observations of the above experiments (from second to final part) were conducted under both control (without plant extract) and addition of plant extract in glucose/xylose medium with the fermentative microorganisms. The data obtained were analyzed by statistical analysis of variance (ANOVA). Statistical significance was assessed at $P < 0.05$, and Turkey's HSD multiple range test was conducted to separate the means.

The observations for the above experiments are as follows. In the screening part from the four sugars that were considered, ie, glucose, xylose, raffinose, and arabinose. Glucose and xylose showed the highest growth rates for *S. cerevisiae*, *P. besseyi*, and *Z. mobilis* at 24, 12, and 24 (h) respectively (Figure 2A,B,C). Following it when the organisms were evaluated for their growth on glucose and xylose sugars (separately) along with different concentrations of plant extract, *Z. mobilis* and *S. cerevisiae* were observed to have enhanced growth at 5-mg supplementation of plant extract, while for *Pichia* sp. it was at 3 mg of plant extract. Although the highest growth rates were observed at significantly greater incubation periods as compared with the controls (without plant extract), an interesting observation was the viability of cells (for all the three organisms) which persisted beyond 48 hours of incubation (Figure 3A). While supplementation of carbon sources with plant extract showed visibly higher growth rates in *Z. mobilis* and *S. cerevisiae* (Figure 3B, C), *Pichia* sp. exhibited higher growth rates for glucose without plant extract (Figure 3D). However, supplementation of plant extract increased its growth rate in xylose as compared with the control. The remaining glucose and xylose in the above conditions were evaluated by di-nitrosalicylic acid method and bail's reagent method, respectively.[42,43] Since the reducing sugar concentrations were lower in glucose and xylose sugars supplemented with plant extract, this indicated a higher uptake of the sugars into the cells, and hence ethanol yield was only observed for the same (Figure 4). Surprisingly, when glucose was taken as the sole carbon source with supplementation of plant extract, higher ethanol yield was observed

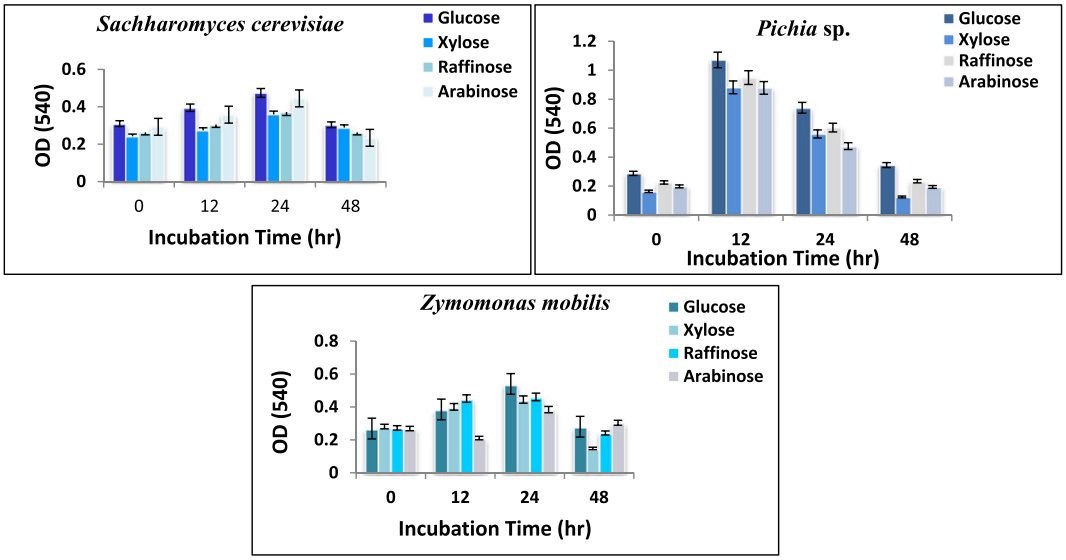


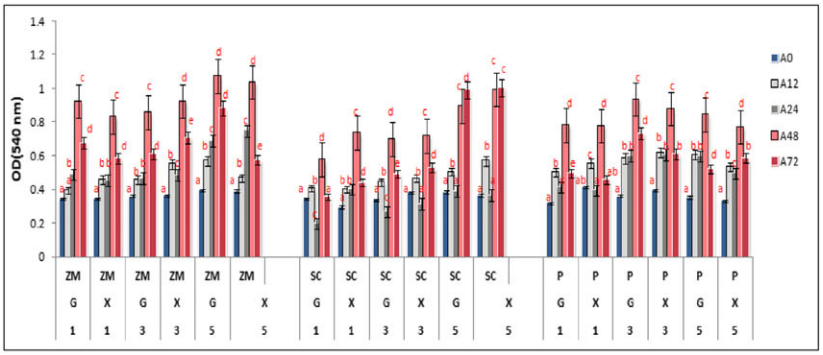
FIGURE 2 Growth kinetics of *S. cerevisiae* (A), *Z. mobilis* (B), and *Pichia sp.* (C) on different hexose and pentose sugar. Four sugars, ie, glucose xylose, raffinose, and arabinose, have been taken into consideration, and the growth rate was observed at OD 540. *S. cerevisiae* exhibited the highest glucose uptake capability up to 24 h, and then glucose consumption decreased. *Pichia sp.* showed simultaneous consumption of glucose and xylose up to 24 h after which the consumption is significantly decreased. The optimum glucose and xylose uptake was observed after 12-h incubation. The rate of glucose uptake is more than that of the xylose uptake rate for *Pichia sp.* [Colour figure can be viewed at wileyonlinelibrary.com]

for *Pichia sp* followed by *S. cerevisiae* and *Z. mobilis*. Similarly, when xylose was supplemented with plant extract, highest ethanol production was observed for *Pichia* sp.as compared with *Z. mobilis* and *S. cerevisiae*.

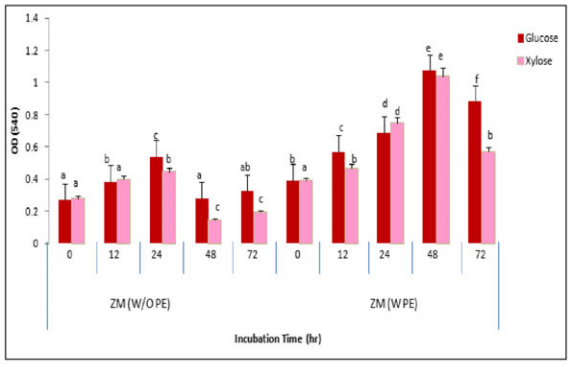
So, it may be hypothesized that the extracts of *X. granatum* triggered an increase in ethanol production in yeast cells by enhancing the growth of the microbial cells. It is anticipated that the bark extracts of *X. granatum* may have stimulated the glucose transport across the yeast cell membrane. The increased ethanol production with supplementation of lower concentrations of plant extracts can be beneficial in economical bioethanol production in biorefinery-based concepts. Nevertheless, these preliminary studies elucidating the effect of plant extracts on the carbohydrate uptake in microorganisms can be explored and the mechanisms can be more intensely studied. Further, using these concepts consolidated bio-processing (CBP) approaches can be targeted for commercial bioethanol from grass biomass.

3.2 | Metabolic pathways for bioethanol by bacteria and yeast using dominant pentose sugars of grass biomass as a potential substrate

Fermentation of grass biomass by several microorganisms such as *Bacillus macerans*, *Bacteroidespolypragmatus*, *Z. mobilis*, *Clostridium saccharolyticum*, *Saccharomyces cerevisiae*, *Pichia stipitis*, *Candida shehatae*, and *Pichia tannophilus* etc. have been exploited for efficient production of ethanol from grass biomass.[44] A list of microorganisms observed to ferment different types of sugars for ethanol production is listed in Table 1. Among all the microorganisms studied till date, *Z. mobilis*, *S. cerevisiae*, and *Pichia* sp. are the most commonly studied microorganisms for both laboratory and industrial scale bioethanol production from different biomass. The reason for selection of these organisms mostly is due to the endogenous metabolic pathways posed by these organisms for production of other metabolic byproducts, such as lactate, levan, acetate, acetoin, isoprenoids, gluconic acid, sorbitol, bionic acid, and succinic acid.[40] However, knowledge corresponding to the response of the fermenting organisms using grass as a substrate is an interesting area to be explored. Therefore, in this section, we attempted to provide an overview of discrete pathways followed by three different microorganisms, ie, *Z. mobilis*, *S. cerevisiae*, and *P. stipitis*, for production of bioethanol. This overview will help us in understanding the metabolic engineering of the genes in the above-mentioned organisms that are responsible for uptake and transport of xylose monomers within their cell. Table 2 depicts the genes responsible for substrate uptake and its transportation within the cell through different pathways in the above-mentioned microorganisms. Since the glucose uptake and transport mechanisms are explained in the preceding section, we emphasize on the uptake and



(A)



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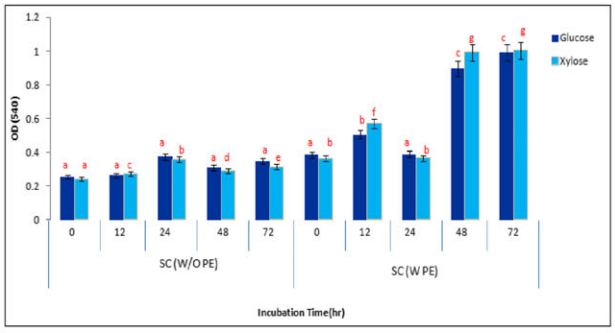

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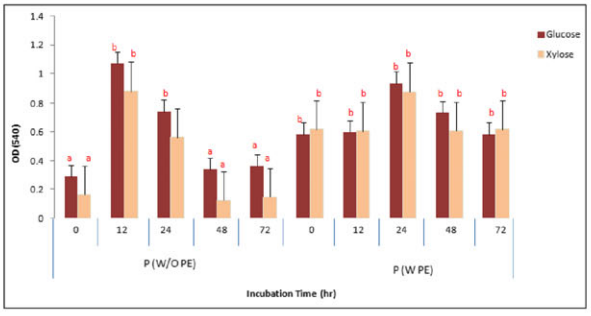

(D)

FIGURE 3 (A) Growth pattern of, *Z. mobilis* (ZM), *S. cerevisiae* (SC), and *P. beseyi* (PB) on glucose (G) and xylose (X) medium with different concentrations (1, 3, and 5 mg) of plant extract; (B) growth kinetics of *Z. mobilis* without plant extract (w/o PE) and with plant extract (w PE—3 mg/mL); (C) growth kinetics of *S. cerevisiae* without plant extracts (w/o PE) and with plant extracts (w PE—3 mg/mL); (D) growth kinetics of *Pischia* sp. without plant extracts (w/o PE) and with plant extracts (w PE- 3 mg/mL). The results were expressed as the mean ± standard deviation. Bars represent the standard deviations of the mean. Data were subjected to one-way analysis of variance (ANOVA) with Tukey test for multiple comparisons. Values with different letters indicate significant differences ($P < 0.05$) [Colour figure can be viewed at wileyonlinelibrary.com]

fermentation of predominant pentose sugar (ie, xylan and arabinoxylan) in grasses in this segment. Furthermore, although the pentose sugars, xylan, and arabinoxylans share a common pathway that involves xylitol and genes, ie, *Xyl1* and *Xyl2* (Xylitol dehydrogenases), L-arabinose induces *Xyl2* much more whereas *Xyl2* is not specifically induced by xylitol.[85] Therefore, arabinose-specific induction pathways are implicated, and most of the recombinant xylose fermenting microorganisms with their respective accountable genesis is shown in Table 3.

Z. mobilis is a promising ethanol producing microorganism as it possesses higher glucose fermenting capacity

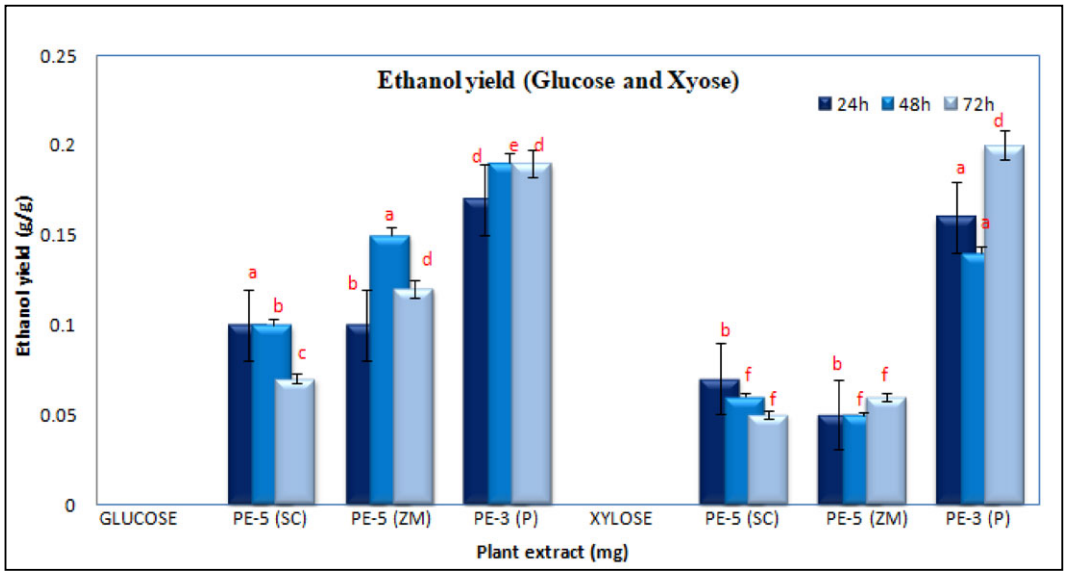

FIGURE 4 Ethanol yield from glucose and xylose sugars with PE—5 mg/mL for SC and ZM and 3 mg/mL for P. The results were expressed as the mean ± standard deviation. Bars represent the standard deviations of the mean. Data were subjected to one-way analysis of variance (ANOVA) with Tukey test for multiple comparisons. Values with different letters indicate significant differences ($P < 0.05$) [Colour figure can be viewed at wileyonlinelibrary.com]

as compared with other bacterial sp.[106,107] Its unique capacity to tolerate high titres of ethanol as those of yeasts and robustness to phase contaminations makes the organism a preferred bacterium than others. However, limited utilization of xylose and arabinose is a primary drawback with the organism, and hence many engineered sp. of *Z. mobilis* have been designed to overcome the limitation. Since hydrolysis of grass biomass releases a rich quantity of arabiono-xylans, the genetically modified bacterium should be targeted for utilization of both xylose and arabinose sugars along with glucose. Deanda et al[108] isolated genes related to PPP from *E. coli* and incorporated it in *Z. mobilis*. An extension to this work was conducted by the same authors wherein the metabolism of D-xylose and L-arabinose in *Z. mobilis* was studied.[109] In summary, the study led to the conclusion that seven genes (xylose isomerase, xylulokinase, L-arabinose isomerase, L-ribulokinase, L-ribulose-5-phosphate 4-epimerase, transketolase, and transaldolase) which are responsible for encoding the enzymes necessary for conversion of xylose and arabinose to common intermediates of *Z. mobilis* in the central glycolytic pathway were simultaneously introduced into *Z. mobilis*. Further, the introduction of these genes was done under the control of strong promoters that possess the ability to direct their expression, even if glucose is present as a substrate. The resultant genetically engineered strain was capable of utilizing xylose, arabinose, or glucose either individually or in mixed forms to bioethanol. In a similar approach, Chou et al[110] conducted an experiment where glucose, xylose, and arabinose utilizing *Z. mobilis* strain was incorporated with catabolic pathway genes, namely araBAD encoding L-ribulokinase, L-arabinose isomerase, and L-ribulose-5-phosphate- 4-epimerase. The research group used a transposition integration approach for genetic modification of the strain and further improved it by application of a second transposition to create a genomic knockout mutant library. The resultant organism indicated remarkable improvement in breakdown of arabinose in acid pretreated and enzymatically saccharified corn stover slurry for efficient bioethanol production. While these experiments have been targeted for fermenting of arabinoxylans, Dunn and Rao[37] engineered a *Z. mobilis* strain directed towards increasing its arabinoxylan uptake capacity. The authors suggested that the limiting factor in transportation of xylose and related polysaccharides is not the specificity, but the sugar uptake capacity of the organism.

Among hexose fermenting yeasts, *S. cerevisiae* is preferred as the most potential candidate for industrial bioethanol production. It is because of its high ethanol productivity and its tolerance towards wide range of pH and inhibitors. Although many studies have been aimed for engineering the organism for xylose uptake and transport,[78,111] very few studies have been aimed for arabinoxylan uptake and fermentation. In one such study, Sun et al[112] developed recombinant *S. cerevisiae* strains which were capable of combining hemicellulase production, xylan hydrolysis, and hydrolysate fermentation in a consolidated bio-processing step. The strains exhibited a series of unifunctional, bifunctional, and trifunctional mini-hemicellulosomes that comprised a

TABLE 1 Microorganisms that can ferment different types of pentose sugars to produce ethanol

Sl No.	Microbe	Substrate	Product	Reference
1	*Fusarium lini*	Xylose	Ethanol	32
2	*Moniliamurmanica* and *Oidium laminarium*	Xylose	Ethanol	45
3	Homogenates of *S. cerevisiae*	D-ribose-5-phosphate, D-xylose-5-phosphate, and L-arabinose-5-phosphate	Ethanol	46
4	*Candida tropicalis*	D-xylose and L-arabinose	Ethanol	47,48
5	*Hansenula*, *Debaromyces*, and*Schwanniomyces*	Xylose	Ethanol	48
6	*S. cerevisiae*	Xylulose	Ethanol	49,50
7	*Kluyveromyces lactis*	Xylulose	Ethanol	51
8	*Schizosaccharomycespombe*	Xylulose	Ethanol	52
19	*Pachysolen tannophilus*	Xylose	Ethanol	53,54
10	*Candida tropicalis*	Xylose	Ethanol	52
11	*Candida* sp.	Xylose	Ethanol	55
12	*Candida shehatae*	Xylose	Ethanol	56
13	*Pichia stipitis*	Xylose	Ethanol	57
14	*Fusarium oxysporum*	Xylose	Ethanol	58,59
15	*Lenzites betulinus* IUM 5468	Xylose	Ethanol	60
16	*Lenzites betulinus* IUM 5468	Arabinose	Ethanol	60
17	*Mucor*	Xylose	Ethanol	29,61
18	*Rhizopus javanicus,*	Xylose	Ethanol	62
19	*Rhizopus oryzae*	Xylose	Ethanol	62
20	Several species of *Aspergillus*	Xylose	Ethanol	62
21	*Penicillium chrysogenum*	L-arabinose	Ethanol	62
22	Mutant of *Aspergillus niger*	L-ribose, L-xylulose	Ethanol	63
23	*Neurospora crassa*	D-xylose	Ethanol	53
24	*Neurospora crassa*	D-mannose	Ethanol	53
25	*Neurospora crassa*	D-galactose	Ethanol	57
26	*Neurospora crassa*	D-arabinose	Ethanol	52
27	*Fusarium oxysporum* VTT-D-80134	D-xylose	Ethanol	64

mini-scaffoldin (CipA3/CipA1) and up to three chimeric enzymes. High-affinity cohesin-dockerin interactions were utilized for assembling three types of hemicellulases (endoxylanase [XynII], an arabinofuranosidase [AbfB], and a β-xylosidase [XlnD]) onto the mini-scaffoldin. The resultant organisms with unifunctional, bifunctional, and quaternary trifunctional mini-hemicellulosomes showcased an increased arabinoxylan hydrolysis, with the quaternary complex showing the highest hydrolysis rate. However, no reports have been cited for monitoring the uptake of arabinoxylans in *S. cerevisiae* so far and the available literatures are for comparative uptake rates of xylose and glucose. Jeffries et al[113] and Du et al[111] characterized three xylose-specific transporters such as *An 25*, *Xyp 29*, and *Xut 1* gene with both glucose and xylose uptake activity, but the relative uptake rate of xylose was comparatively lower as compared with glucose. Although these transporters have a high affinity for xylose compared with other transporters, the uptake activity was observed to be very slow, and the affinity for glucose was higher as compared with xylose.

Similarly among different pentose fermenting yeasts, *P. stipitis* possess the highest native capacity for xylose fermentation along with utilization of glucose, mannose, galactose, and cellobiose to some extent.[114] Furthermore, *P. stipitis* also modifies low-molecular-weight lignin moieties,[115] reduces acyclic enones to the corresponding alcohols,[116] and possesses resistance to inhibitors like furfural and hydroxy-methyl furfural.[117] These remarkable abilities have led to the modification of genes of different

TABLE 2 List of enzymes that catalyzes the glycolytic and pentose utilizing pathway for *Z. mobilis*, *S. cerevisiae*, and *Pichia* sp.

Microorganism	Gene	Enzyme	Function	References
Z. mobilis	*glf*	Glucose transporter	Facilitates intracellular glucose transport via diffusion in sugar-rich medium.	65
Z. mobilis	*glk*	Glucokinase	Facilitates the entry of glucose to ED pathway via ATP dependent phosphorylation of gluconate to 6-Phosphogluconate which is the substrate for EDD enzyme.	65
Z. mobilis	*frk*	Fructokinase	Aids entry of fructose into ED pathway by ATP dependent phosphorylation	66
Z. mobilis	*pgi*	Phosphoglucoisomerase	Facilitates fructose to enter the central pathway by conversion of fructose to glucose-6-phospate	67
Z. mobilis	*zwf*	Glucose-6-phosphate dehydrogenase	Partially facilitates the conversion of glucose-6-phospate to 6-phosphogluconate. This enzyme uses NAD^+ or $NADP^+$ to produce reducing equivalents in the form of NADPH as well auto balance oxido-reduction of acetaldehyde by alcohol dehydrogenase.	65
Z. mobilis	*edd*	6-Phosphogluconnate dehydratase	Catalyzes dehydration of 6-phosphogluconate to form KDPG via an enol-KDPG intermediate that undergoes spontaneous rearrangement to keto form by an irreversible reaction sequence.	65
Z. mobilis	*eda*	2-KDPG aldolase	Catalyzes an aldol cleavage for inter-conversion of 2-keto-3-deoxy-6-phospho-gluconate with glyceraldehydes-3-phosphate and pyruvate.	68
Z. mobilis	*gap*	Glyceraldehyde-3-P-dehydrogenase	Catalyzes the oxidation of glyceraldehyde 3-phosphate at C^1via conversion of an aldehyde to carboxylic acid with simultaneous reduction of NAD^+ to NADH. The energy released by this reaction impels the transfer of inorganic phosphate to the GAP intermediate to form 1,3-bisphosphoglycerate.	69
Z. mobilis	*pgk*	Phosphoglycerate kinase	Catalyzes the reversible transfer of a phosphate group from 1,3-bisphosphoglycerate to ADP producing 3-phosphoglycerate and ATP.	70
Z. mobilis	*pgm*	Phosphoglycerate mutase	Catalyzes the internal transfer of a phosphate group from C_3 to C_2 which results in the conversion of 3-phosphoglycerate to 2-phosphoglycerate through a 2, 3-bisphosphoglycerate intermediate.	71
Z. mobilis	*ano*	Enolase	Catalyzes the conversion of 2-phospho-glycerate to phosphoenolpyruvate	72
Z. mobilis	*pdc*	Pyruvate decarboxylase	Catalyzes the decarboxylation of pyruvic acid to acetaldehyde and CO_2	69
Z. mobilis	*adhA*	Alcohol dehydrogenase I	Catalyze the reduction of acetaldehyde to ethanol.	73
Z. mobilis	*adhB*	Alcohol dehydrogenase II	During fermentation, *adhII* is activated by ethanol accumulation while *adhI* is inhibited.	73
Z. mobilis	*gfor*	Glucose-fructose oxidoreductase	Leads to the formation of gluconic acid which after phosphorylation enters the ED pathway after the NAD(P) H formation	74
Z. mobilis	*gi*	Gluconolactonase	These enzymes specifically act on carboxylic ester bonds and produces D-gluconate via pentose phosphate pathway,	75

(Continues)

TABLE 2 (Continued)

Microorganism	Gene	Enzyme	Function	References
Z. mobilis	*PdhAα, pdhAβ pdhB lpd*	Pyruvate dehydrogenase complex	Catalyzes non-oxidative decarboxylation to form acetaldehyde and CO_2 from pyruvate.	[75]
S. cerevisiae	*pgi1*	Glucose-6-phosphate isomerase	Catalyzes the inter-conversion of glucose 6-phosphate and fructose 6-phosphate via opening glucose ring, isomerizing glucose into fructose through an enediol intermediate and closing the fructose ring.	[76]
S. cerevisiae	*pfk1, pfk2*	Phosphofructokinase	Catalyzes the conversion of fructose 6-phosphate and ATP to fructose 1,6-bisphosphate and ADP.	[76]
S. cerevisiae	*fba1*	Aldolase	Catalyzes the aldol condensation of dihydroxyacetone phosphate with glyceraldehyde 3-phosphate to form fructose 1,6-bisphosphate in gluconeogenesis and the reverse reaction in glycolysis.	[76]
S. cerevisiae	*tpi*	Triosephosphate isomerase	Catalyzes the synthesis of D-glyceraldehyde 3-phosphate from glycerine-phosphate	[76]
S. cerevisiae	*tdh1*	Glyceraldehydes-3-phosphate dehydrogenase	Catalyzes oxidation of glyceraldehyde 3-phosphate (G3P) at the C_1, and reduction of NAD^+ to NADH.	[75]
S. cerevisiae	*tdh2*	Glyceraldehyde 3-phosphate dehydrogenase	Synthesizes pyruvate from D-glyceraldehyde 3-phosphate	[76]
S. cerevisiae	*tdh3*	Glyceraldehydes-3-phosphate dehydrogenase	Synthesizes pyruvate from D-glyceraldehyde 3-phosphate	[76]
S. cerevisiae	*pgk1*	3-phosphoglycerate kinase	Catalyzes the transfer of a high-energy phosphoryl group from the acyl phosphate of 1,3-diphosphoglycerate to ADP to produce ATP.	[75]
S. cerevisiae	*gpm1*	Phosphoglycerate mutase	Catalyzes the internal transfer of a phosphate group from C_3 to C_2that results in the conversion of 3-phosphoglycerate to 2-phosphoglycerate through a 2,3-bisphosphoglycerate intermediate.	[76]
S. cerevisiae	*gpm3*	Glyceratephospho mutase	Initiates final capture of energy by shifting the phosphate from the end of the molecule to a strategic place in the centre.	[76]
S. cerevisiae	*err3*	Phosphopyruvate hydratase	Catalyzes the conversion of 2-phospho-glcerate with similar kinetic to phosphoenolpyruvate	[76]
S. cerevisiae	*err2*	Enolase		[77]

(Continues)

TABLE 2 (Continued)

Microorganism	Gene	Enzyme	Function	References
			Catalyzes the conversion of 2-phospho-glycerate to phosphoenolpyruvate.	
S. cerevisiae	*err1*	Enolase	Catalysis of the conversion of 2-phospho-glycerate to phosphoenolpyruvate.	[77]
S. cerevisiae	*eno2*	Enolase	Catalyzes the conversion of 2-phospho-glycerate to phosphoenolpyruvate.	[76]
S. cerevisiae	*eno1*	Enolase I	Removes a water molecule, forming a new double bond in an awkward place in the carbon skeleton of the molecule, making it easy to remove to form ATP	[76]
S. cerevisiae	*pvk2*	Pyruvate kinase	Removes the remaining phosphates and places them on ADP, to create new ATP molecules that allow the unstable little sugar fragments to rearrange into stable pyruvate molecules that subsequently burned up completely into carbon dioxide and water.	[76]
S. cerevisiae	*adh1*	Alcohol dehydrogenase I	Catalyzes reduction of acetaldehyde to ethanol	[76]
P. stipitis	*zwf1*	Glucose 6-phosphate dehydrogenase	Catalyzes oxidation of aldehyde at C_1 of glucose-6-phosphate, to a carboxylic acid in ester linkage. $NADP^+$ serves as electron acceptor.	[78]
P. stipitis	*gnd1*	6-Phosphogluconate dehydrogenase	Catalyzes oxidative decarboxylation of 6-phosphogluconate at C_3 position to yield ribulose-5-phosphate that promotes loss of carboxyl at C_1 as CO_2 whereas $NADP^+$ serves as oxidant.	[78]
P. stipitis	*rpe1*	D-Ribulose-5-phosphate 3-epimerase	Catalyzes inter-conversion of ribulose-5-phosphate and xylulose-5-phosphate	[79]
P. stipitis	*rki1*	Ribose-5-phosphate ketol-isomerase	Converts the ketose ribulose-5-phosphate to the aldose ribose-5-phosphate.	[80]
P. stipitis	*tkl1*	Transketolase	Catalyzes transfer of a C_2 fragment (glycoaldehyde) from xylulose-5-phosphate to either ribose-5-phosphate or erythrose-4-phosphate.	[81]
P. stipitis	*tal1*	Transaldolase	Catalyzes transfer of a C_3 dihydroxyacetone moiety, from sedoheptulose-7-phosphate to glyceraldehyde-3-phosphate.	[82]

(Continues)

TABLE 2 (Continued)

Microorganism	Gene	Enzyme	Function	References
P. stipitis	*pgi1*	Phosphoglycerate isomerase	Interconversion of glucose to fructose	82
P. stipitis	*xyl1, xyl1p*	D-xylose reductase	Catalyzes the initial reaction in the xylose utilization pathway, the NAD(P) H dependent reduction of xylose to xylitol	81
P. stipitis	*xyl2, xyl2p*	Xylitol dehydrogenase	Produce xylutol	82
P. stipitis	*xyl3, xks1*	D-xylulokinase	Catalyzes the phosphorylation of D-xylulose	83,84
P. stipitis	*xyla*	Xylose isomerase	Catalyzes the interconversion of D-xylose and D-xylulose.	78
P. stipitis	*araA*	L-Arabinose isomerase	Catalyzes the conversion of L-arabinose to L-ribulose	78
P. stipitis	*araB*	L-Ribulokinase	Facilitates the conversion of L (or D)-ribulose to L (or D)-ribulose 5-phosphate (Rhodopirellulabaltica)	78
P. stipitis	*araD*	L-Ribulose-5-phosphate 4-epimerase	Synthesizes D-xylulose 5-phosphate from L-arabinose	78
P. stipitis	*xfp*	Phosphoketolase	Splits xylulose-5-phosphate (or fructose-6-phosphate) to acetate precursor acetyl phosphate	78
P. stipitis	*ackA*	Acetate kinase	Facilitates the production of acetyl-CoA by phosphorylating acetate in the presence of ATP.	78
P. stipitis	*pta*	Phosphotransacetylase	Catalyzes the transfer of an acetyl group between acetylphosphate and acetylcoenzyme A	78
P. stipitis	*adhE*	Acetaldehyde dehydrogenase	Catalyzes the conversion of acetaldehyde into acetic acid with the formation of NADH.	78
P. stipitis	*adh1, adh2*	Alcohol dehydrogenase	Alcohol dehydrogenase oxidizes ethanol into acetaldehyde.	78
P. stipitis	*pdc1*	Pyruvate decarboxylase	Catalyzes the decarboxylation of pyruvic acid to acetaldehyde and carbon dioxide.	78
P. stipitis	*ald6*	Aldehyde dehydrogenase	Utilizes $NADP^+$ as the preferred coenzyme; required for conversion of acetaldehyde to acetate	78

Pichia sp. for either glucose or xylose fermentation.[118] However, alike *S. cerevisiae* fermentation of arabinoxylans by *P. stipitis* is not much studied. Shi et al[119] explored a mutant strain of *P. stipitis* which was deficient in *Xylose isomerase dehycrogenase* (*Xid*), D-arabinitol dehydrogenase (*Dad*), L-arabinitol dehydrogenase (*Lad*), and D-ribitol dehydrogenase which initially did not exhibit any fermentation in either xylose or arabinose medium. Nevertheless, complementation with *Psxyl2* (Xylitol dehydrogenase) restored growth on L-arabinose followed by its efficient fermentation. Different recombinant microorganisms along with their engineered genes for enhanced glucose and xylose uptake and their subsequent effect on their fermentation ability are listed in Table 4. The results of these investigations are expected to improve our knowledge on the functioning of genes

TABLE 3 List of recombinant xylose fermenting microorganisms and their respective accountable genes

Microbe	Enzyme	Gene	Fermenting Sugar	Reference
S. cerevisiae	Xylose isomerase	*xylA*	Xylose	75,76,79-84
S. cerevisiae	Xylose isomerase	*xylA*	Xylose	86
Rhodosporidiumtoruloides	Xylose isomerase	*xylA*	Xylose	87
E. coli	Xylose isomerase	*xylA*	Xylose	88
Klebsiellaoxytoca	Xylose isomerase	*xylA*	Xylose	87,89-92
Zymomonasmobilis	Xylose isomerase	*xylA*	Xylose	77,93
S. cerevisiae	Xylose isomerase	*xylA*	Xylose	94,95
C. shehatae	Xylose isomerase	*xylA*	Xylose	96,97
P. stipitis	Xylose isomerase	*xylA*	Xylose	98
P. stipitis	Xylose reductases	xyl1	Xylose	99-101
P. stipitis	Xylose transporter	stu1	Xylose	102
S. cerevisiae hxt1-7	Xylose transporter	psstu1	Xylose	92
S. cerevisiae	Xylose transporter	*stu*1	Xylose	93
P. stipitis	Xylose reductase	*xyl1*	Xylose	81,103
C. tropicalis	Xylose reductase	*xyl1*	Xylose	99
K. *lactis*	Xylose reductase	*xyl1*	Xylose	100
P. tannophilus	Xylose reductase	*xyl1*	Xylose	101
C. guilliemondii	Xylose reductase	*xyl1*	Xylose	103
S. cerevisiae	NADPH specific aldose reductase	*xyl1*	Xylose	104
S. cerevisiae	Xyulokinase	*xks1*	Xylose	102,105

that are responsible for the glucose and xylose uptake in different fermenting microorganisms and thus assist us in structuring more robust microorganisms.

3.2.1 | Model proposed for strain improvement in *Pichia stipitis* for targeting improved xylose utilization

In the previous section, we have presented a concise depiction about the glucose and xylose utilization pathways as well as molecular alteration of glycolysis for engineered *S. cerevisiae* strain for effective uptake and consumption of pentose sugar mainly xylose (major constituent of grass cell wall) via PPP. However, efficient pentose utilization requires PPP optimization and may interfere with its role in NADPH and pentose production. Likewise, *P. stipitis* can be exploited as a pentose fermenting yeast, with the capacity of utmost utilization of xylose present in the medium along with the consumption of glucose.[140]

Recent study about the regulation of fermentation revealed that, in a mixed sugar medium, *P. stipitis* cannot utilize glucose and xylose simultaneously[141] as glucose represses the genes required for xylose assimilation[78] and has the tendency to ferment glucose prior to xylose (as glucose metabolism requires fewer steps and less energy to break down than xylose) resulting in underutilization of xylose.[142] Moreover, unlike *S. cerevisiae, P. stipitis* is "Crabtree-negative," ie, they generate energy through fermentation reaction in anaerobic or low oxygen conditions.[143] However, this crabtree-negative effect is beneficial for *P. stipitis* as the low oxygen concentration helps in the activation of enzymes that are necessary for fermentation of xylose sugars. Another field of study for the glucose and xylose consumption in *P. stipitis* is an integral membrane protein, ie, the proton symporter. There are two types of proton symporter, ie, low and high affinity proton symporter. Among these two, high-affinity transporter can transfer xylose whereas low affinity system can mediate the uptake of glucose along with xylose.[144] Transportation of both glucose and xylose by same transporter leads to complete inhibition of xylose transporters in the presence of slightest amount of glucose.[145] This may direct towards the incomplete consumption of D-glucose and D-xylose, present in lignocellulosic biomass.[146] Apart from these, the uptake of the substrate also depends on the number of carrier proteins present in the plasma membrane of the cell.[146] Therefore, by manipulating the carrier proteins, the

TABLE 4 List of recombinant microorganisms utilizing different sugars for glycolytic and pentose utilizing pathway

Microbe	Host Cell	Substrate	Product	Gene	Enzyme	Findings	Reference
C. guilliermondii	*P. pastoris*	Xylose	Ethanol	*xyl1*	Xylose reductase	*xyl1* stimulates the heterologous expression of aldose reductases enzyme in P. Pastoris, as thesecreted enzyme utilizes NADPH as a cofactor whereas the intracellularenzyme uses both NADH and NADPH that enhance the intracellular redox potential that drives the reaction rates.	103
P. stipitis		Xylose	Ethanol	*xyl1*	Xylose reductase	Overexpression of *Psxyl1* in *P. stipitis* exhibited up to 1.8-fold higher Xor activity on xylose medium and up to 10 timeshigher on glucose under than that of the control strain (without *XYL1) under* aerobic condition. The increased Xor activity did not affect the fermentation rate significantly which explained that *xyl1* has no significant role in fermentation in *P. stipitis*.	104
S. cerevisiae	*P. stipitis*	Xylose, glucose, mannose, fructose	Ethanol	*Psstu1*	Xylose transporter	Xylose uptake was effectively inhibited by glucose, mannose, and fructose present in the medium which reflected a competitive inhibition of xylose transport.	120
C. tropicalis		Xylose	Ethanol	*xyl1*	Xylose reductase	*xyl1* was cloned in *C. tropicalis* successfully.	99
Candida tropicalis		D-xylose and L-arabinose	Ethanol	*xyl1*	Xylose reductase	Directly convert D-xylose and L-arabinose to ethanolunder low aeration conditions.	47
P. stipitis		Xylose	Ethanol	*xyl1, xyl2, xyl3*			96,97

(Continues)

TABLE 4 (Continued)

Microbe	Host Cell	Substrate	Product	Gene	Enzyme	Findings	Reference
	S. cerevisiae				Xylose reductase, xylose dehydrogenase	Absence of *XYL1* and *XYL2* influenced the growth of *S. cerevisiae* on xylose medium, whereas lack of all the three genes, ie, XYL1, XYL2. andXYL3 simultaneously, inhibit the fermentation activity.	
P. stipitis	*P. stipitis*	Xylose	Ethanol	*xyl1, xyl2, xyl3,*	Xylose reductase, xylose dehydrogenase	Over expression of *XYL1* in the presence of abundant *XYL2* and *XYL3*, did not increase the fermentation activity significantly, that validate the *XYL1* expression as non-limiting step for fermentation in *P. stipitis*.	121
	S. cerevisiae	Xylose	Ethanol	*xks*	Xylulokinase	The *xks1* mutant complimented with *XKS1* gene grew better than the wild type did on xylulose.	122
	S. cerevisiae	Xylulose	Ethanol	*xks1*	Xylulokinase	Overexpression of xks1 gene in wild *Saccharomyces* strain deterred the growth on xylulose medium.	123
Aspergillus nidulans		L-xylulose	Ethanol	*lad*	L-arabitol dehydrogenase	Deletion of *Lad* gene led to the accumulation of ambitol in the mutant	124
A. niger		L-xylulose	Xylitol	*lxd*	NADPH dependent L-xylulose reductase	NADPH dependent L-xylulose reductase converted L-xylulose to xylitol	63

(Continues)

TABLE 4 (Continued)

Microbe	Host Cell	Substrate	Product	Gene	Enzyme	Findings	Reference
A. niger		Xylitol	D-xylulose	*dxd*	NAD + − dependent xylitol dehydrogenase	NAD$^+$ dependent xylitol dehydrogenase (Dxd) converted xylitol to D-xylulose.	[63]
P. Stipitis		Xylose	Xylitol	*xor*	Xylose reductase	Three cysteine residues in Xor were mutagenised to serine and showed reduced catalytic activities while retaining the original function.	[125]
P. stipitis		Xylose	*Ethanol*	*xid*	Xylitol dehydrogenase	Activity of Xid decreased under anaerobic or oxygen-limited conditions.	[114]
C. shehatae		Xylose	*Ethanol*	*Xor, xid*	Xylose reductase, xylitol dehydrogenase	Oxygen limitation had a slight positive effect on Xor and Xid activities.	[126]
S. cerevisiae		Xylose	*Ethanol*	*Psxyl2*	Xylose reductase	Mutant strain could not ferment xylose effectively and produced xylitol in mixed sugar medium.	[97,120]
*P. stipitis*CBS 6054		Xylose	*Ethanol*	*xyl2*	Xylose reductase	While the mutant strain grew well on D-xylose and L-arabinose, the wild strain showed very less L-arabinitol dehydrogenase (NAD$^+$) activity on L-arabinose due to lack of L-arabinitol dehydrogenase.	[127]
P. stipitis CBS 6054		D-arabinitol	D-ribulose	*a short chain dehydrogenase gene*	Arabitol dehydrogenase	Cloned gene encoded enzyme similar to NAD+-dependent D-arabitol dehydrogenase cloned from *Candida*	[128]

(Continues)

TABLE 4 (Continued)

Microbe	Host Cell	Substrate	Product	Gene	Enzyme	Findings	Reference
						albicans and had highest D-arabinitol activity along with the production of D-ribulose as product.	
Candida auringiensis, Candida succiphila, Ambrosiozymam onosporaand Candida sp. (YB-2248)		L-arabinose	Ethanol			Microbes were screened for their ability to ferment L-arabinose and showcased a positive result in low aeration along with D-xylose utilization with low ethanol production.	129
P. tannophilus		Xylose, Galctose	Ethanol	*GAL10*	UDP-galactose-4-epimerase.	Enzyme encoded by this gene was equally induced by D-galactose and D-xylose.	130
S. cerevisiae		Xylose	Ethanol	*UDP-galactose-4-epimerase*	DHOdase	Enzyme was inactivated by D-xylose or L-arabinose owing to the reduction of NAD^+ which was essential for epimerase activity.	131
	S. cerevisiae	Xylose	Ethanol	*Scpdc1*	Pyruvate decarboxylase	Over-expression of *Scpdc1* led to the overproduction of *pdc* that influenced the competition for pyruvate between Pdc and the mitochondrial Pdh complex.	132
P. stipitis	*S. cerevisiae*	Xylose	Ethanol	*xyl1*	Xylulokinase	Heterologous production of *xyl1* in *S. cereviaise* did not stimulate the xylitol production from	120,124

(Continues)

TABLE 4 (Continued)

Microbe	Host Cell	Substrate	Product	Gene	Enzyme	Findings	Reference
						xylose but the concurrent presence of *xyl1* and *xyl2* genes facilitated the growth on xylose. However, overexpression of *xks1* significantly influenced the ethanol production onxylose.	
	S. cerevisiae	Glucose, xylose	Ethanol	*Pxyl1, Psxyl2,xks1*		Expression of *Pxyl1, Psxyl2, xks1 in S. cerevisiae* strain showed higher fermentative capability for both glucose and xylose.	101
P. stipitis	*S. cerevisiae*	Xylose	Ethanol	*tal1,tkl1, Xyl, xyl2*	Transketolase, transaldolase	Introduction of both genes into *S. cerevisiae* did not show substantial results unless *xyl1* and *xyl2 genes* are overexpressed at the same time.	133
S. cerevisiae		Xylulose	Ethanol	*tkl1, tkl2*	Transketolase	Mutant ion in TKL2 gene did not affect the growth on xylulose medium, while deletion of TKL1 gene led to very slow growth on xylulose medium. Simultenious mutation in TKL1 and TKL2 led to the non-utilization of xylulose, signifying the limitation of transketolase activity.	

(Continues)

TABLE 4 (Continued)

Microbe	Host Cell	Substrate	Product	Gene	Enzyme	Findings	Reference
						On the other hand, over expressivity by about 100 fold compared with the wild strain	
S. cerevisiae		Xylulose	Ethanol	*tal1*	Transaldolase	Mutant strain exhibited very slow growth in xylulose as sole carbon source.	134
P. stipitis	*S. cerevisiae*	Xylose	Ethanol		*xyl1,xyl2,* xks1	Ethanol production from xylose deceptively increased with the expression of *xyl1, xyl2* along with *xks1*thatenabledenhancedxylose metabolism in this organism.	135
P. stipitis		Glucose, xylose	Ethanol	*Pyruvate decarboxylase*	*Pspdc1,Pspdc2*	While *Pspdc1* gene was properly expressed on glucose and xylose medium with maximum growth on glucose, *Pspdc2* expression was restricted to glucose medium only. Moreover, the expression level was considerably enhanced in anaerobic conditions.	136
S. cerevisiae	*P. stipitis*	Glucose, xylose	Ethanol	*ScURA1*	DHOdehase	Expression of scura1 in *P. stipitis* enabled rapid anaerobic growth in glucose medium and produced ethanol higher and faster than control. Moreover, mutant strain were also viable in anaerobic xylose media for a long period while growth of the control strain was limited on xylose medium	137

(Continues)

TABLE 4 (Continued)

Microbe	Host Cell	Substrate	Product	Gene	Enzyme	Findings	Reference
CYC1 P. stipitis				*xyl1, xyl2*	Xylose reductase and xylitol dehydrogenase	Xylose and glucose were utilized simultaneously in mutant strain as compared with control, signifying incomplete de-repression of xylose assimilation. By reduced ATP generation.	[138]
P. stipitis		Xylose	Ethanol	*xyl1*	Xylose reductase	Enhanced *xyl1* expression led to improve the enzymatic hydrolysis about two fold though no positive outcome has observed the production of ethanol.	[81]
P. stipitis				*adh1, adh2*	Alcohol dehydrogenase	Deletion of *adh1* and *adh2* genes led to decreased ethanol production and increased xylitol production.	[138]
S. cerevisiae				*PsXR, PsXDH*	Xylose reductase, xylose dehydrogenase	Mutant strain ($PsXR^+$, $PsXDH^+$) utilized xylose to ethanol with the production of xylitol.	[139]

enhancement of xylose uptake could be achieved. Hence, considering the above facts, we have proposed a brief idea about an alternative model for *P. stipitis* in the present study which is expected to address the challenge concerning the xylose transport (Figure 5). This model includes an operon that operates, on over-expression of two xylose transporters which are insensitive to the presence of glucose and by-passes the conventional glycolysis to enter the PPP by the production of 6-phosphogluconolactone for efficient conversion of xylose to ethanol.

Studies regarding the sugar transporter present in *P. stipitis* convey a greater facilitation mechanism for glucose transportation into the cytoplasm as compared with xylose. Hence, the first fundamental of proposed pathway is based on the uptake of glucose to form glucose-6-phosphate followed by its entrance onto PPP by silencing *pgi1* gene that encodes phosphoglucoisomerase (responsible for the conversion of glucose-6-phosphate to fructose-6-phosphate). This particular gene has been considered as it is the key factor to decide the fate of sugar metabolism in *P. stipitis*. Hence, silencing this gene may lead to the oxidation of glucose-6-phosphae at C_1 position to form 6-phosphogluconolactone by glucose-6-phosphate dehydrogenase (encoded by *zwf1* gene) which eventually enters the PPP with the reduction of $NADP^+$ to NADPH. The 6-phosphogluconolactone formed during shunting the glycolysis pathway earlier will be converted to erythrose-4-phosphate and xylose-5-phosphate by a series of reaction.

The second part of the proposed pathway model involves the construction of an operon to facilitate efficient xylose uptake and utilization for PPP. The operon model is so designed that it would turn on in the presence of high concentration of xylose and would stimulate *Gal2* and *Hxt7* gene to uptake xylose and turn off when the xylose is fully utilized from the fermentation medium. When xylose is transported into the cell by xylose transporters encoded by gene *ScURA1* and *xyp29* in *P. stipitis*, a chain of reaction occurs in the presence of a series of enzymes to form xylose-5-phosphate without being affected by the presence of glucose in the medium. This may occur due to the mutation at two conserved amino acid positions, asparagine 376/370 and threonine 219/213 in *ScURA1* and *xyp29*, respectively, might result in abating the inhibition by glucose.[147] As a result, xylose will be transported to the cell even in presence of glucose and converted to xylulose which is the key element to form xylulose-5-phopshate, the major component of PPP. Subsequently, erythrose-4-phosphate and xylose-5-

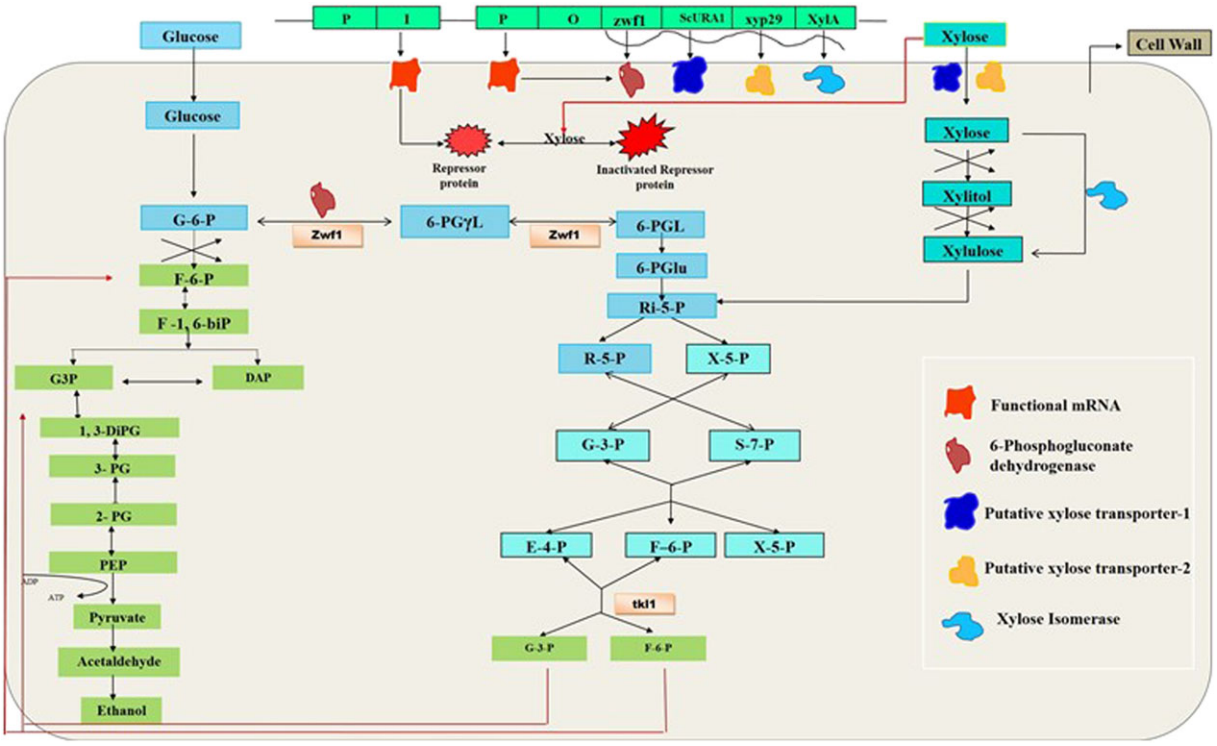

FIGURE 5 This pathway includes two improvements. Firstly, "***pgi***" gene encoding phosphoglucoisomerase will be silenced which will lead the pathway to enter the pentose phosphate pathway (PPP) by the formation of 6-phosphogluconolactone by glucose-6-phosphate dehydrogenase (encoded by *zwf1* gene). Secondly, an operon is constructed to facilitate efficient xylose uptake even in the presence of glucose in the medium. The operon consists of functional genes like ***ScURA1*** and ***xyp29*** which can transport xylose even in the presence of glucose in the medium [Colour figure can be viewed at wileyonlinelibrary.com]

phosphate formed by merging ribose-5-phosphate and xylyulose-5-phospahte will eventually be converted into fructose-6- phosphate by a series of intermediary reactions involving three different enzymes.

Proposed structure of the operon model

Proposed operon model is constructed to mediate the xylose uptake in *P. stipitis* as well as combining PPP with glycolytic pathway for the production of ethanol. It consists of four genes, ie, *Zwf1*, *ScURA1*, *xyp29*, and *XylA* as depicted in Figure 6, that encode the proteins for glucose-6-phosphate dehydrogenase, xylose transporters, and xylose isomerase that stimulate the oxidation of glucose-6-phosphate to 6-phosphogluconolactone, mediate the intracellular xylose transport, and accelerate the formation of xylulose respectively. While the former step is necessary for the formation ribulose-5-phosphate, the first molecule of the non-oxidative phase followed by the transportation of xylose into the cell membrane, the latter step is necessary to avoid the difference in the co factor requirement of XR (NADPH) (xylose reductase) and XDH (NAD^+) (xylitol dehydrogenase) that may cause a redox imbalance under anaerobic conditions.

The proposed operon model consists of three regions such as promoter region (P), regulatory gene (I), and operator region (O). RNA polymerase attaches to promoter region "P" that assists the transcription of mRNA which is carried out by the regulator gene "I." The transcribed RNA is eventually translated to a repressor protein followed by a termination signal at the end of the gene. Operator region "O" is composed of a short sequence of triplet bases that acts like a switch which is recognized by the repressor protein. A long mRNA is constructed for these four genes, ie, *Zwf1* (6-phosphogluconate dehydrogenase), *ScURA1* (Xylose transporter), *xyp29* (Xylose transporter), and *XylA* (xylose isomerase) that code for enzymes and responsible for their expressions on that particular substrate and forms the basis of the operon model system. Furthermore, the operon requires xylose present in the medium as a sensor/repressor to be turned on/off by regulating transcription of those four genes.

The operon model should work on two conditions elaborated in Figure 7A,B. I the first condition, the operon will be inactive in the absence of adequate amount of xylose along with glucose in the medium. Consequently, the second condition involves an opposite phenomena leading the operon to be active/switched-on. When xylose is present in the medium adequately along with glucose, the promoter for "I" gene is bound by an RNA polymerase to initiate transcription. The "I" mRNA is translated into the repressor protein to which xylose binds very efficiently and converts the repressor into an inactive state, so that it cannot bind to the Operator. When a promoter for transcription of the mRNA of those three functional genes is not blocked, many copies of

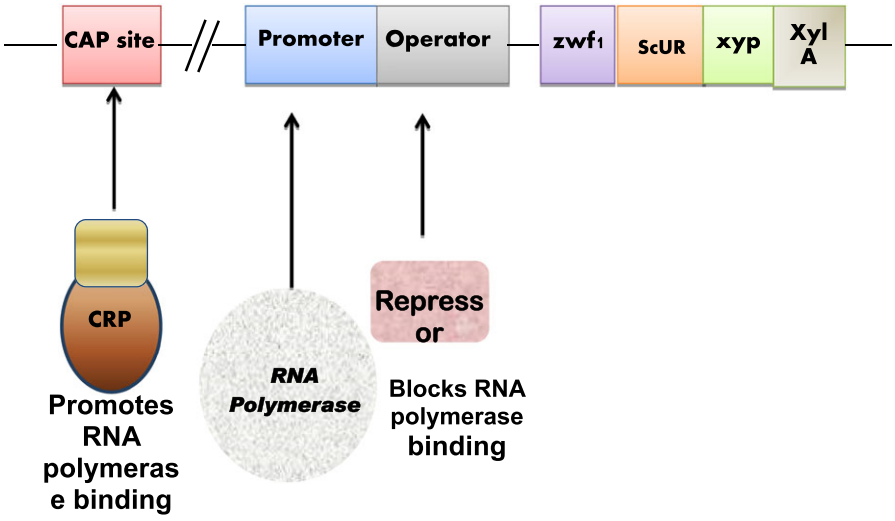

FIGURE 6 Structure of the *operon model proposed to facilitate the xylose uptake in Pichia sp. This operon is proposed to consist of four genes* such as *Zwf1, ScURA1, xyp29,* and *XylA* that encode the proteins for glucose-6-phosphate dehydrogenase, xylose transporters, and xylose isomerase. In addition to the four functional genes, this operon also comprises a number of regulatory DNA sequences to which regulatory proteins bind and control the transcription of the functional genes. A catabolite activator protein (CAP) is bound to the CAP region in the operon. Binding of CAP to CAP region promotes the binding of RNA polymerase to the promoter. The operator region is a negative regulatory site which is responsible for terminating the transcription. This region usually overlaps with the promoter region and facilitates termination by being bound to repressor protein. Binding to repressor protein ceases the binding of RNA polymerase to the promoter. Transcription of functional gene can only begin if the repressor protein does not bind to the operator region [Colour figure can be viewed at wileyonlinelibrary.com]

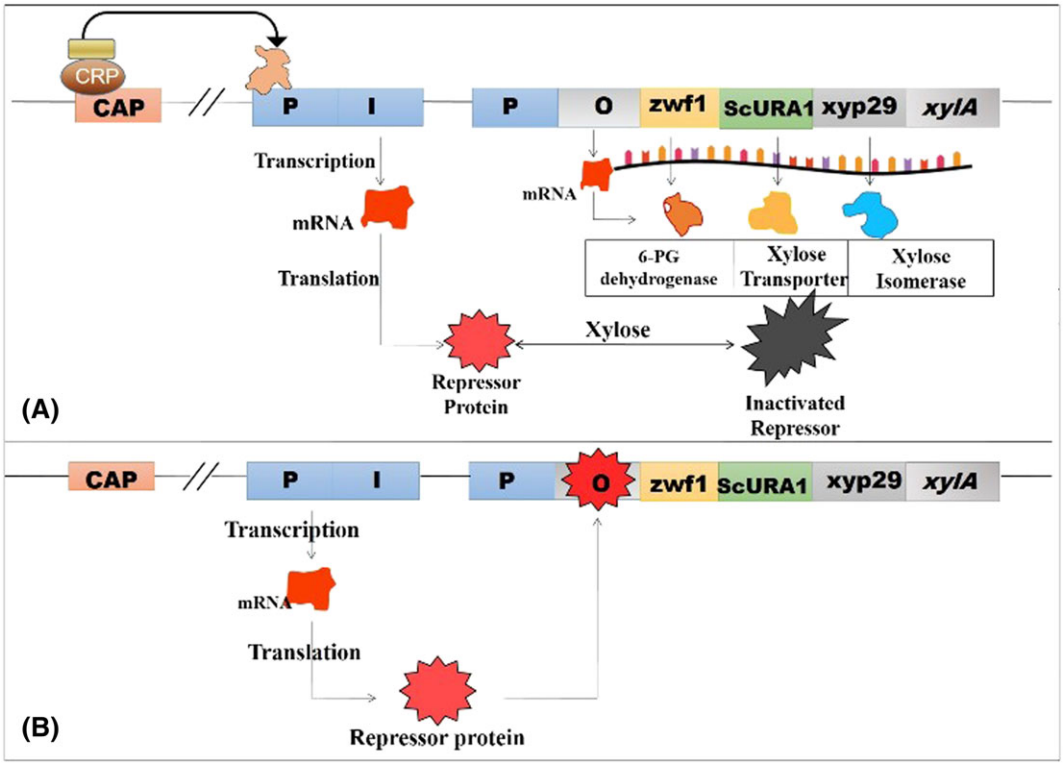

FIGURE 7 Function of proposed operon model in the presence of xylose (A) and in absence of xylose (B). In the absence of xylose, almost no mRNA will be transcribed as the repressor protein would bind to the operator region. In contrast, in the presence of xylose, alloxylose will bind to the repressor protein and inactivate it. Inactivation of repressor protein will lead to the transcription of four functional genes in the operon which will further be translated to produce proteins/enzymes that will facilitate the proposed pathway [Colour figure can be viewed at wileyonlinelibrary.com]

mRNAs for those three genes are transcribed. Xylose will act as sensor to initiate the induction of transcription of the mRNA for *Zwf1*, *ScURA1*, *xyp29*, and X*ylA*. After the construction of mRNA, translation begins at the 5′ end by constructing glucose 6-phosphate dehydrogenase from the *Zwf1* gene followed by the formation of putative xylose transporters from *ScURA1 andxyp29*and then xylose isomerase from *XylA*.

Glucose 6-phosphate dehydrogenase stimulates the oxidation of glucose-6-phosphate and NADPH as a by-product that would enter the PPP and produce ribulose-5-phosphate the first component of oxidative stage. Hence, after the formation of the first component of PPP, xylose transporters (coded by gene *ScURA1 and xyp29*) will mediate the putative xylose transportation without being affected by the presence of glucose in the medium. Afterwards, xylose would be isomerised to form xylulose by xylose isomerase (XI). The xylulose is subsequently phosphorylated, channeled into the pentose phosphate shunt, and converted to C_6 and C_3 compounds.

When xylose is utilized completely in the medium, the Promoter for "I" gene remain "on," but due to less strength, it is transcribed very rarely. The mRNA for I gene is translated into a repressor protein that would bind to the operator in the absence of fructose-6-phosphate thereby preventing the transcription from second promoter. Almost no mRNA is further created for the three genes in operator region. When the repressor binds to the operation region, it "folds" the DNA so that the promoter will not be accessible to transcribe and the function of the operon would stop there. For complete utilization of both glucose and xylose present in the medium, it is anticipated that the yeast must express the operon genes, which encode key enzymes to continue the glucose metabolism after the complete utilization of xylose.

4 | VALIDATION OF THE PROPOSED MODEL

Various genome scale metabolic network-based model organisms have been constructed to gain fundamental insights into their biochemistry, genomics, and genetic makeup. This systems biology approach is an inventive platform to gain a systems level understanding of the cellular/molecular phenomena occurring inside a cell, to identify and predict the important metabolic targets and pathways. Constraint-based reconstruction and analysis

TABLE 5 List of reactions and their descriptions for the proposed model by flux variability analysis (FVA)

Sl. No.	Reaction Affected in the Proposed Model:	Reaction description
1.	r328: Beta-D-fructose 6-phosphate: D-glyceraldehyde-3-phosphate	Beta-D-fructose 6-phosphate + D-glyceraldehyde 3-phosphate <= > D-Xylulose 5-phosphate + D-Erythrose 4-phosphate
2.	r329: Sedoheptulose-7-phosphate: D-glyceraldehyde-3-phosphate	D-glyceraldehyde 3-phosphate + Sedoheptulose 7-phosphate <= > beta-D-fructose 6-phosphate +D-Erythrose 4-phosphate
3.	r563: ATP:D-xylulose 5-phosphotransferase	ATP + D-Xylulose - > ADP + D-Xylulose 5-phosphate
4.	r550: ATP:D-fructose 6-phosphotransferase	ATP + beta-D-fructose - > ADP + beta-D-fructose 6-phosphate
5.	r981:D-mannose-6-phosphate aldose-ketose-isomerase	D-mannose 6-phosphate <= > beta-D-fructose 6-phosphate

(COBRA) method remains the most preferred systems biology method to interrogate phenotypic properties of the target organism (https://opencobra.github.io/cobratoolbox/latest/index.html). Constraint-based metabolic model of the target organism comprises metabolic reactions, metabolites participating in these reactions, and genes that encode the enzymes catalyzing these reactions.

Our model organism, *P. stipitis*, was obtained from http://www.sysbio.se/BioMet. Metabolic flux analysis was then performed to understand and support the feasibility of the proposed model of *P. stipitis* using COBRA Tool Box. The uptake and release rates of metabolite in a reaction were modulated by changing the lower and upper bounds of the reaction. To model the first part of the proposed pathway, the gene encoding phosphoglucoisomerase which is responsible for the conversion of glucose-6-phosphate to fructose-6-phosphate was targeted. For this specific enzyme catalyzed reaction, the lower and upper bounds were set to zero (0), to silence/knock out the gene. r982 was the reaction corresponding to the activity of *pgi1* gene in the in silico downloaded model. To understand the effects of the silenced gene on the sugar metabolism in the organism, flux variability analysis (FVA) was performed as given in Table 5. FVA is another tool to maximize or minimize the flux values for each reaction in the metabolic network, while simultaneously satisfying all imposed constraints on the optimized objective function value. It provides a span or range of allowable fluxes that can exist within the optimized solution space as defined by the linear program. Consequently, FVA provides useful insight into the network variability. The focus was on checking the flux variability for reactions involving sugar metabolism. The fluxes through the reactions involving PPP changed, highlighting the effects of gene silencing on glucose metabolism. An increase in the uptake rates (reaction r328 and r329) of D-fructose 6- phosphate (M_m64) and D-glyceraldehyde 3-phosphate (M_m67) was observed, which resulted in increased generation of D-Xylulose 5-phosphate (M_m128) and D-Erythrose 4-phosphate (M_m129).

The model was then further expanded by incorporating the second part of the proposed pathway, involving constructing an operon for efficient xylose uptake and utilization in PPP. The xylose uptake rate (r1091) was changed to 2.855 g/100 mL, which corresponds to the maximum uptake rate, whereas, the glucose uptake rates were not changed and set to default. To maximize xylose uptake rates and entry inside cells, the xylose transporter (xylTrans) bounds were changed to permit higher xylose transfer. On making these changes to the metabolic model, the reaction r563 (catalyzed by ATP: D-xylulose 5-phosphotransferase) was metabolically affected, showing a change in flux value, when compared with the original in silico model. This enzyme catalyzed reaction generates xylulose 5-phosphate, which is a major component in PPP, thereby utilizing xylose as the carbon source despite the presence of glucose (an alternative, yet major carbon source). Consequently, an influence on fructose 6-phosphate metabolism was also observed. The reactions generating this metabolite (r550 and r981) showed changes in the positive or maximum flux values for the proposed model, thereby highlighting the involvement of activating xylose transporters and other key intermediary metabolic enzymes (such as ATP:D-fructose 6-phosphotransferase and D-mannose-6-phosphate aldose-ketose-isomerase).

5 | RESEARCH OUTLOOKS AND CONCLUDING REMARKS

Pretreatment followed by enzymatic hydrolysis are the vital steps that lead to efficient bioethanol production from lignocellulosic biomass. The recent development of modern fermenters for efficient pretreatment and enzymatic saccharification of lignocellulosic biomass will play

a significant role in biorefinery-based concepts for bioethanol production from grass biomass. Further, efficiency of microorganisms plays a major role in fermentation and ethanol production which depends upon uptake of carbohydrates. Our experiments reveal strong correlation between supplementation of plant extracts with enhanced cell growth followed by increased bioethanol production from glucose and xylose by yeast and bacteria. However, further research elucidating the molecular mechanisms which lead to enhanced glucose and xylose uptake with the supplementation of plant extracts need to be explored. Likewise, the uptake of pentose sugars like arabinoxylans (that are present in substantial quantities in grass biomass) by both bacteria and yeast for enhanced bioethanol production requires significant research. Moreover, the model proposed for preferential use of xylose/arabinoxylan followed by uptake of glucose in *P. stipitis* can be explored for complete lignocellulosic substrate utilization. This can be achieved by targeted manipulation technologies which can result in activation of genes that are responsible for activating the operon and thereby producing robust microorganisms for higher titers of bioethanol production from grass biomass.

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