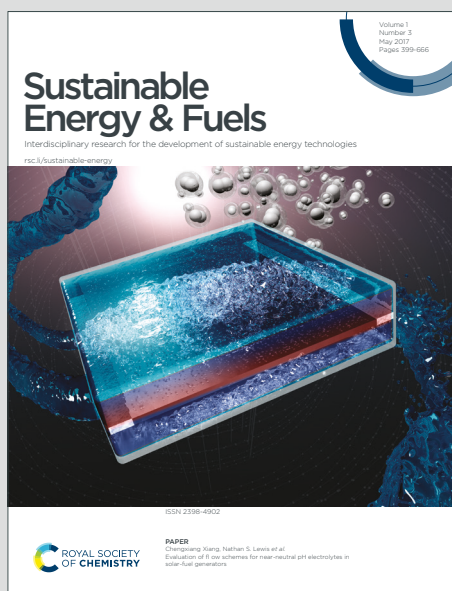


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# Valorisation of Xylose to Renewable Fuels and Chemicals, an Essential Step in Augmenting the Commercial Viability of Lignocellulosic Biorefineries

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## Abstract

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Biologists and engineers are making tremendous efforts in contributing to a sustainable and green society. To that end, there is a growing interest in waste management and valorisation. Lignocellulosic biomass (LCB) is the most abundant material on earth and an inevitable waste predominantly originating from agricultural residues, forest biomass and municipal solid waste streams. LCB serves as the renewable feedstock for clean and sustainable processes and products with low carbon emission. Cellulose and hemicellulose constitute the polymeric structure of LCB, which on depolymerisation liberates oligomeric glucose and xylose, respectively. The preferential utilization of glucose and/or absence of xylose metabolic pathway in microbial systems alienate and abandon xylose valorisation, a major bottleneck in the commercial viability of LCB-based biorefineries. Xylose is the second most abundant sugar in LCB, but a non-conventional industrial substrate unlike glucose. The current review sought to summarize the recent developments in biological conversion of xylose into a myriad of sustainable products and associated challenges. The review discusses the microbiology, genetics, and biochemistry of xylose metabolism with hurdles requiring debottlenecking for efficient xylose assimilation. It further describes the product formation by microbial cell factories which can assimilate xylose naturally and rewiring of metabolic networks to ameliorate xylose-based bioproduction in native as well as non-native strains. The review also includes a case study that provides an argument on suitable pathway for optimal cell growth and succinic acid (SA) production from xylose through elementary flux mode analysis. Finally, a product portfolio from xylose bioconversion has been evaluated along with significant development made through enzyme, metabolic and process engineering approaches, to maximize the product titers and yield, eventually empowering the LCB-based biorefineries. Towards the end, the review is wrapped up with current challenges, concluding remarks, and prospects with argument for an intense future research into xylose-based biorefineries.

**Keywords:** Xylose; Xylose reductase; Xylitol dehydrogenase; Redox balance; Carbon catabolite repression; Elementary flux mode.



**List of abbreviations**

|           |   |
|-----------|---|
| ATP       | Adenosine Triphosphate                      |
| BDO       | 2,3-Butanediol                              |
| cAMP      | 3',5'-Cyclic adenosine monophosphate        |
| CBP       | Consolidated bioprocessing                  |
| ccpA      | Catabolite control protein A                |
| CRE       | Catabolite repressive element               |
| CRP       | cAMP receptor protein                       |
| DA        | Dilute acid                                 |
| DHA       | Dihydroxyacetone                            |
| DHAP      | Dihydroxyacetone phosphate                  |
| DXD       | D-Xylose dehydrogenase                      |
| EMP       | Embden-Meyerhof-Parnas pathway              |
| E4P       | Erythrose-4-phosphate                       |
| F6P       | Fructose-6-phosphate                        |
| GlpF      | Glycerol facilitator protein                |
| GlfZ      | Glucose transporter                         |
| HT        | Hydrothermal                                |
| $K_{La}$  | Volumetric oxygen transfer coefficient      |
| $K_m$     | Michaelis Menten constant                   |
| LA        | Lactic acid                                 |
| LAB       | Lactic acid bacteria                        |
| LCB       | Lignocellulosic biomass                     |
| LHW       | Liquid hot water                            |
| NADH      | Nicotinamide adenine dinucleotide           |
| NADPH     | Nicotinamide adenine dinucleotide phosphate |
| SE        | Steam explosion                             |
| $P_A/P_E$ | Promoters                                   |
| PEP       | Phosphoenol pyruvate                        |
| PHB       | Polyhydroxybutyrate                         |
| PLA       | Polylactic acid                             |
| PPP       | Pentose phosphate pathway                   |
| SA        | Succinic acid                               |



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|                  |  |
|------------------|--|
| TCA              | Tricarboxylic acid cycle                     |
| $V_{\max}$       | Maximum velocity of enzyme mediated reaction |
| X1P              | Xylulose-1-phosphate                         |
| X5P              | Xylulose-5-phosphate                         |
| XDH              | Xylitol dehydrogenase                        |
| XI               | Xylose isomerase                             |
| XK               | Xylulose kinase                              |
| XR               | Xylose reductase                             |
| <i>Xyl1</i>      | Xylose reductase gene                        |
| <i>Xyl2</i>      | Xylitol dehydrogenase gene                   |
| <i>XylA</i>      | Xylose isomerase gene                        |
| <i>XylB/Xyl3</i> | Xylulose kinase gene                         |
| <i>XylC</i>      | Xylonolactonase                              |
| <i>XylD</i>      | Xylonate dehydratase                         |
| <i>XylX</i>      | 2-keto-3-deoxy-xylonate dehydratase          |

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## 1. Introduction

Biomass is potential alternative to non-renewable and non-sustainable fossil fuels causing massive harm to atmosphere through colossal carbon emission and generation of pollutants.<sup>1</sup> Analogous to petroleum refinery, a biorefinery process biomass into multiple products with a green and sustainable approach leading to low carbon biomanufacturing technologies.<sup>1,2</sup> The first generation biorefinery making use of edible feedstocks such as sugar, starch, and vegetable oils for generating biofuels is well-established, but pose a big concern and is a regular subject of food vs fuel debate.<sup>3</sup> On the other hand, second generation biorefinery based on non-edible feedstocks such as lignocellulosic biomass (LCB) does not interfere in any food chain and offers a clear value proposition for the production of bulk and speciality chemicals. LCB is the most abundant feedstock on the planet (~200 billion tonnes) with significant contribution stemming from post-harvest agricultural residues. It is composed of lignin (15-20%) the outermost protective layer, cellulose (40-50 %) inner amorphous and crystalline component of the secondary wall, and hemicellulose (25-30%) microfibrils connecting the outermost and inner cellulose layers (Figure 1A).<sup>4</sup> Cellulose is a linear homo-polymer of D-glucose units connected by  $\beta$  1,4-glycosidic bonds, and hemicellulose is a complex hetero-polymer containing D-xylose, L-arabinose, D-glucose, L-galactose, D-mannose, D-glucuronic acid and D-galacturonic acid (Figure 1B). Hemicelluloses constitute 26% dry weight in hard woods, 22% in soft woods, and up to 25% in agro-residues with various polymeric forms such as xylan, arabinoxylan, xyloglucan, and glucuronoxylan.<sup>5,6</sup> To utilize this three-dimensional polymeric structure as the feedstock for fermentative production of value-added chemicals, the polymer is converted into simple fermentable sugars. However, the major limitation is that most of the microorganisms are incapable of metabolizing all the fermentable sugars present in LCB, especially pentoses. The pentose sugars are present in hemicellulosic fraction with xylan as major polysaccharide which is composed of  $\beta$  1,4-linked xylose residues. The depolymerization of hemicellulosic fraction generates mixture of sugars containing ~ 90% xylose. In fact, xylose is the second most abundant sugar available after glucose in LCB (Figure 1A).<sup>7</sup> Despite this, the application of xylose as a potential feedstock is overlooked for biorefineries and discarded as waste or incinerated for energy purposes. This is due to a lack of efficient fermentation systems, as many of the microorganisms do not have native pathway for metabolizing xylose. In addition, uptake of xylose is suppressed in presence of glucose due to carbon catabolite repression.<sup>8</sup> That is why the number of the literature reports using glucose as substrate for bioproduction is much larger in comparison to xylose. However, while exploiting biochemical platform, the techno-commercial success of an LCB-based biorefinery largely thrives on the revival of carbohydrate economy, which in turn is dependent on efficient depolymerization of both the structural polysaccharides to simple sugars and their subsequent valorisation to various commercially important products either through chemical or biotechnological route.<sup>9,10</sup> Therefore, efficient conversion of xylose is necessary and it is imperative to find the robust microbial systems for metabolizing xylose for simultaneous assimilation of glucose and xylose for the pragmatic development of profitable LCB-based biorefineries.



Considering the challenges associated with xylose utilization, the current review, (i) covers the efficient pretreatment processes assisting in xylan extraction from different LCB residues, (ii) discusses on bottlenecks impeding xylose assimilation and strategies to overcome them, (iii) describes major native and engineered microbial cell factories available for efficient bioconversion of xylose to chemical building blocks, (iv) includes implementation of elementary flux mode analysis to understand the optimal pathway for xylose utilization to produce biomass and end metabolites with a case study of succinic acid, (v) briefly covers alternative chemical catalysis of xylose for manufacturing value-added products. Finally, the limitations and future perspectives for constructing microbial cell factories to effectively utilize xylose and produce a wide array of products is included.

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### 1.1 Pretreatment strategies for the extraction of fermentable sugars from LCB.

Recalcitrance is a natural and intrinsic feature of any LCB, originating from its three principal constituents, cellulose, hemicellulose, and lignin that chemically interact to form a complex network popularly known as lignin-carbohydrate complex (LCC).<sup>11,12</sup> During biorefining via biochemical route, pretreatment is an imperative module that disrupts the lignocellulosic matrix by breaking LCC linkages leading to delignification, partial or complete hydrolysis of xylan, thereby improvising the surface characteristics of biomass and enhancing accessibility of cellulose for enzymatic hydrolysis. Invariably, most of the traditional pretreatment strategies primarily result in the lignin removal releasing fermentable sugars from thermolabile hemicellulosic/xylan fraction or are focused on selective delignification enriching the biomass in glucan and xylan fraction<sup>13</sup>.

#### 1.1.1 Pretreatment method targeting xylan hydrolysis.

Conventional techniques like the steam explosion (SE), liquid hot water (LHW), dilute acid (DA), and hydrothermal (HT) pretreatment result in the solubilization of hemicellulose fraction and partial lignin removal<sup>14</sup>. However, the extent of xylan hydrolysis and release of inhibitors during pretreatment significantly depends on the process severity. Process variables such as solid loading during pretreatment, temperature, pressure, residence time and concentration of acid in case of DA pretreatment, biomass composition and pretreatment reactor configuration directly or indirectly govern the successful xylan extraction as monomers, oligomers or its degradation product like furfural, the release of lignin-derived inhibitory derivatives and loss of cellulose as glucose or its dehydrated product namely 5-hydroxymethylfurfural (HMF) in the hydrolysed fraction<sup>15,16,17</sup>. Generally, SE, LHW and HT pretreatment favours deacetylation of thermolabile acetyl groups attached to hemicellulose backbone and cause release of acetic acid in a temperature of 180–250°C.<sup>16</sup> Since the acetic acid is weak compared to inorganic acids, partial xylan hydrolysis occurs, and the resulting pre-hydrolysates are predominant in xylooligosaccharides (XOS) with fewer xylose monomers. Yao et al. have recently confirmed that the pH of the medium plays a decisive role in the breakage of LCC linkages<sup>15</sup>. Thus, HT pretreatment likely induces deacetylation and catalyses the cleavage of glycosidic linkages within the xylan





backbone, but the addition of strong acid even at low concentration reduces pH that preferentially breaks the esters linkages between lignin and xylan<sup>15</sup>. Therefore, during DA pretreatment, lower temperatures are recommended (120-180°C) as the addition of acid demands lower operating conditions favouring xylan hydrolysis. Further, combinatorial pretreatment involving a low concentration of inorganic acid and water facilitates the release of xylose monomers from hemicelluloses backbone. It enhances the efficiency of the process owing to milder operating conditions, less inhibitor generation while preserving the cellulosic fraction in the biomass. Table 1 exclusively showcases a few examples of previously published literature where SE, LHW and DA pretreatment and their combinations selectively hydrolysed xylan fraction hydrolysis (>85%) and < 25% delignification. Since HT and DA are among the most popular, efficient, and economically attractive pretreatment strategies that lead to selective xylan hydrolysis keeping the glucan fraction in the biomass intact, these technologies have been scaled up to semi-pilot and pilot plant level as well, as shown in Table 2. The forthcoming section describes conventional pretreatment methods, which lead to enrichment of xylan and glucan fraction in the biomass, targeting selective delignification.

### 1.1.2 Pretreatment strategies favouring glucan and xylan enrichment.

The use of sodium hydroxide (NaOH) during pretreatment is one of the most popular and industrially- scalable delignification technologies. It cleaves LCC linkages (phenolic  $\alpha$ -aryl, phenolic  $\alpha$ -alkyl, phenolic and non-phenolic  $\beta$ -aryl ethers linkages) between lignin and hemicellulosic fraction, improves the surface properties and digestibility of cellulose.<sup>19</sup> Unfortunately, hemicellulose being amorphous, acetylated and thermolabile, gets easily extracted when a high NaOH concentration is used above 70°C and account for significant losses ( $\geq 35\%$ ). Hence, there are isolated reports of alkali pretreatment wherein xylan enrichment in solid fraction has been successfully demonstrated. For example, Zhang and associates reported <20% xylan removal from wheat straw and sugarcane bagasse when they pretreated the biomasses with 0.5M NaOH for 80°C for 6 h. The resulted pretreated wheat straw and sugarcane bagasse contained 89.9% and 92.9% carbohydrate fraction, respectively.<sup>20</sup> Earlier, while evaluating various pretreatment methods for anaerobic digestion of *Miscanthus floridulus*, alkaline peroxide (2% H<sub>2</sub>O<sub>2</sub> at 35°C for 24h to pH 11.8) pretreatment removed >70% lignin, enriching pretreated biomass with 99.82 and 83.03% glucan and xylan fraction respectively.<sup>21</sup> In yet another variation, Gong et al. (2020) achieved >70% delignification of corn stover by treating it with 5% alkaline methanol at 80°C for an hour and retaining ~ 89.5 and 88.5% glucan and xylan fraction in solid biomass.<sup>22</sup>

### 1.1.3 Pretreatment strategies favouring biomass fractionation & holistic utilization of biomass components

The two-stage fractionation process has been another lucrative alternative for the xylan removal in the first stage, followed by delignification in the later stage. Recently, beechwood was subjected to a two-step





fractionation process in which pre-hydrolysis at 150°C for 90 min was performed with 20 mM H<sub>2</sub>SO<sub>4</sub>. As a result, ~85.8 wt% xylan was recovered in stage I. When in the second step, organosolv treatment was performed with a 1:1 ethanol-water mix and 80 mM H<sub>2</sub>SO<sub>4</sub> at 150°C for 70 min, ~82.7 wt% lignin yield were obtained in the liquid fraction leading to the generation of high-digestible cellulose-rich pulp.<sup>23</sup> Earlier, Smit and Huijen evaluated seven different feedstocks: wheat straw, corn stover, beechwood, poplar, birchwood, spruce, and pine for mild organosolv pretreatment with 50% acetone and <50 mM H<sub>2</sub>SO<sub>4</sub> at 140°C for 2h. Irrespective of biomass type, 87- 97% xylan hydrolysis was observed. Poor delignification yields were obtained only in spruce and pine, whereas glucan recoveries ranged between 68-94%. Later the group precipitated the dissolved lignin by diluting with water, leading to effective fractionation of all the three components of different LCB's.<sup>24</sup>

Recently, Xu et al. devised a mild technique for hemicellulose extraction from poplar wood with binary solvent system containing formic acid and water. Pretreatment at 90°C for 4 h resulted in 73.1% xylose yield while the solvent was recovered by fractional distillation and recycled back for second round of pretreatment<sup>25</sup>. The forthcoming section emphasizes the use of novel solvents for the complete LCB fractionation. Chen et al. used 1% H<sub>2</sub>SO<sub>4</sub> with 75% choline chloride to fractionate cellulose of switch grass from lignin and xylan fractions. Treatment with this acidified deep eutectic solvent (DES) at 120°C for 25 min removed 76% xylan fraction along with 51.1% delignification. Five cycles of recycling and reuse of this acidified liquor enriched the hydrolysed xylan and lignin fraction. Later, the group used xylose-rich liquor for furfural production at 160°C for 15 min with 2% w/v AlCl<sub>3</sub> and recovered lignin<sup>26</sup>. Very recently, a biphasic acidic water/phenol system was used for the fractionation of populus wood chips<sup>27</sup>. This unique biphasic system enriched the water-soluble phase with 77% xylose and negligible by-products when the chips were subjected to 120°C for an hour. In contrast, the phenolic phase contained 90% dissolved lignin (90%), leaving solids retaining 96% of the original cellulosic fraction<sup>27</sup>. Likewise, a novel biphasic system comprising 2-phenoxyethanol and acidified water (70:30) was used to fractionate rice straw<sup>28</sup>. Pretreatment at 130°C for 2 h led to cellulose-rich (86.48 % retained) biomass, facilitated by 92.1 and 63.16% removal of hemicellulose and lignin fraction, respectively. Later, 92.6% pure lignin was recovered by simple precipitation and 81.83% of xylan/xylose enriched in the aqueous phase<sup>28</sup>. Yang et al. evaluated the effect of p-toluenesulfonic acid (p-TsOH) on the fractionation of three feedstocks: corncobs, wheat straw, and miscanthus. Pretreatment at 80°C for 10 min resulted in significant removal of lignin and xylan, leaving cellulosic-rich pulp. Later, spent liquor was diluted to precipitate lignin, and the reusability of p-TsOH was shown ~5 times higher<sup>29</sup>. A similar attempt was made by yet another green hydrotrope, maleic acid (MA), for the effective fractionation of birchwood<sup>30</sup>. At 100°C and 50% wt MA, 94.5% of the cellulosic fraction was obtained as solids after 30 min. Lignin was precipitated by dilution, and the solubilised xylan was converted to furfural with ~70% yields. Furthermore, MA displayed ~3 times recyclability with comparable performance<sup>30</sup>. Earlier, the cosolvent enhanced lignocellulose fractionation (CELf) method was developed for pre-treating corn stover using 0.5% H<sub>2</sub>SO<sub>4</sub> and Tetrahydrofuran (THF) in the ratio of 1:1.



The dilute acid hydrolysed xylan to xylose which later dehydrated to furfural, while THF led to lignin dissolution enriching cellulosic biomass. Later, the group separated furfural from THF. The latter was recovered by vacuum distillation and recycled, leaving lignin as powder<sup>31</sup>.

## 2. Chemo-catalytic transformation of xylose to high-value chemicals

The chemo-catalytic routes are the conventional processes for the conversion of petroleum derivatives into bulk, fine and speciality chemicals. Like biological routes, several chemical routes exist via which xylose can be converted to a wide range of products such as furfural, furfuryl alcohol, xylitol, levulinic acid, levulinic ester, and other value-added chemicals, as shown in Figure 2.<sup>32,33</sup> In general, xylose conversion proceeds either via hydrogenation reaction in the presence of a metal catalyst to yield xylitol or isomerization reaction in the presence of a Lewis acid catalyst to produce xylulose. Xylulose further dehydrates to yield furfural in the presence of Brønsted acid catalyst. Notably, furfural estimated global market size was valued at \$1.2 billion in 2019 and expected to grow further to \$2 billion by 2027 which makes it the most attractive and widely produced product from xylose (<https://www.alliedmarketresearch.com/press-release/furfural-market.html>, accessed on 29-03-2021). Xylose can be converted to furfural via enol route,  $\beta$ -elimination, tautomerization and several other routes in the presence of a homogeneous or a heterogeneous catalyst, the necessity is the catalysts with acidic properties.<sup>34</sup> Therefore, a wide range of homogenous mineral acids such as sulfuric acid, hydrochloric acid, nitric acid, phosphoric acid, acetic acid, and formic acid have been used for xylose conversion to furfural.<sup>35</sup> It is noteworthy that 60-70% of total furfural produced globally is used for manufacturing furfuryl alcohol.

Interestingly, xylose can also be directly converted to furfuryl alcohol via the hydrogenation route by using a metal catalyst. The development of such processes may minimize the conventional multistep and tedious method of converting xylose to furfural and then hydrogenating it to produce furfuryl alcohol. In this regard, the Zhu group reported 87.2% furfuryl alcohol yield in the presence of Cu/ZnO/Al<sub>2</sub>O<sub>3</sub> catalyst in a continuous fixed-bed reactor at a temperature of 150°C.<sup>36</sup> Furthermore, the authors observed that increasing the reaction temperature to 190°C alters the xylose conversion pathway to yield 86.8% 2-methyl furan.<sup>36</sup> Therefore, the final product from xylose conversion via hydrogenation can be altered by tuning the operating parameters and catalytic materials. For example, Li and co-workers have carried out the xylose conversion to levulinic acid and levulinic esters in a high-pressure hydrogenation reactor using Pd/Al<sub>2</sub>O<sub>3</sub> catalyst. The conversion yields of levulinic acid and levulinic esters achieved were 40 and 10%, respectively.<sup>37</sup>

It is also worth mentioning that hydrogenation of xylose is being done since the 1970s at an industrial scale; to produce an essential chemical, xylitol.<sup>38</sup> In general, the xylitol production process takes place in the presence of a metal catalyst and hydrogen source at 353-413 K temperature and 1-8 MPa pressure for 15-360 minutes of reaction time.<sup>39</sup> The xylose to xylitol conversion is a surface controlling reaction; therefore, the interaction between adsorbed/unadsorbed xylose and chemisorbed hydrogen with the catalyst surface dictates the process's overall yield. In contrast, the product xylitol does not desorb easily from the catalyst surface,



thereby causing the catalysts' saturation.<sup>40</sup> Interestingly, some of these metal catalysts can also be used to convert xylose into xylaric acid via oxidation reaction. For example, Saha and co-workers have observed 60% xylaric acid yield in the presence of Pt/C catalyst via oxidation reaction. However, limited data is available for such reactions. The xylose-based conversion via chemical routes suffers from lower yields and furthermore, the use of acidic catalysts, and reactions operation at higher temperatures and pressures makes the process environmentally unfriendly. Although many chemical processes such as xylitol production are running at commercially scale, the long-term sustainability is doubtful due to high cost of production and environmental incompatibility.

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### 3. Xylose metabolism: Genetics, Biochemistry of enzymes and their regulation

Xylose valorisation through biotechnological intervention has the potential to become the most popular routes for producing various bio-based chemicals and fuels. A diverse group of microbes such as bacteria, yeast, and fungi, are known to assimilate xylose naturally through different metabolic pathways leading to formation of a range of products such as xylitol, 2,3-butanediol, ethanol- n-butanol, lactic acid, succinic acid etc. A considerate knowledge on these pathways can provide guidance in constructing efficient xylose assimilatory strains.

#### 3.1 Xylose assimilation

The process of D-xylose assimilation is quite different from D-glucose, which is metabolized through Embden-Meyerhof-Parnas (EMP) pathway. D-xylose undergoes isomerization or reduction and subsequent oxidation to form D-xylulose. D-xylulose is the key intermediate for the pentose phosphate pathway, upon phosphorylation converted into xylulose-5-phosphate (X5P), which is funnelled to the central carbon metabolism to generate C3-C7 metabolites (Figure 3). These metabolites can be either precursors or intermediates for EMP, biosynthesis of amino acids, and nucleotides.<sup>41,42</sup>

##### 3.1.1 Xylose isomerase (XI) pathway

The XI pathway (Figure 3) is commonly found in prokaryotes. In this pathway, the initial isomerization of xylose to xylulose is mediated by xylose isomerase (XI), followed by phosphorylation of xylulose to xylulose-5-phosphate (X5P) by xylulose kinase (XK). The X5P enters the pentose phosphate pathway (PPP) and later central carbon metabolism through C3 metabolite, glyceraldehyde-3-phosphate.<sup>43</sup>

**Xylose isomerases** (EC. 5.3.1.5) (D-Xylose  $\rightarrow$  D-Xylulose) encoded by *XylA* gene are the metal dependent enzymes classified into two different classes, I and II. These two enzyme classes differ in length of the polypeptide chain, where class II enzymes have an additional 34 amino acid residues on N-terminus compared to class I. The catalytic activity of the XI is conserved at two sites of histidine residues H101 and H271 and induced in the presence of xylose. XI mediates the synthesis of xylulose via a hybrid shift



mechanism for ring opening to form open chair conformation.<sup>44</sup> The substrate binding at the active site was observed by fluorescence quenching at two conserved regions W29 and W188 with tryptophan residue at W29 being essential for catalytic activity. The genome mining and sequencing in thermophilic *Bacillus coagulans* strain identified *XylA* gene consisting of 1338 base pairs, encode for 50 KDa class II protein with 445 amino acids. The amino acid identity of *B. coagulans* XI gave homology of 65, 64, 58, 48 and 25% with *Lactobacillus brevis*, *L. pentosus*, *L. lactis*, *Piromyces* sp. E2, and *Streptomyces albus*, respectively.<sup>45</sup> Thermostable XI with maximum enzyme activity at 85°C and neutral pH were isolated from thermophilic strains like *Thermoanaerobacterium ethanolicus*.<sup>46</sup> Similarly, *Streptomyces* sp. F-1 strain, a new isolate, has two copies of *XylA* genes, and the biochemical characterization presented a significant difference in their optimal temperature. The protein coded from *XylA1* and *XylA2* displayed maximum activity at 60 and 75°C, respectively.<sup>47</sup> The structural characterization and enzyme kinetics of XI is well investigated. An interesting feature of XI is that the enzyme operates with high activity within a broad temperature range of 30 – 85°C. While it is sensitive to pH change and maximum specific activity was observed at physiological pH range of 6.0 – 8.0 which declined rapidly under strong acidic or alkaline conditions.<sup>46,48</sup> It was also observed that the divalent metal ions are pre-requisite for the activation and stabilization of the enzyme activity. The presence of Mg<sup>2+</sup>, Co<sup>2+</sup>, or Mn<sup>2+</sup> has profound positive effect than other divalent metal ions whereas Ni<sup>2+</sup> has been found to be inhibitory for the enzyme. These metallic cofactors also protect the enzyme from thermal denaturation.<sup>45</sup>

**Xylulokinase (XK)** (EC 2.7.1.17), is a substrate (D-xylulose) specific kinase enzyme catalysing the phosphorylation reaction: D-Xylulose + ATP → D-Xylulose-5-phosphate + ADP. XK in *B. coagulans* is 56 kDa protein consisting of 1536 bp with 511 amino acids. The amino acid identity of XK from *B. coagulans* revealed sequence homology of 56, 49, 38 and 25% with *L. pentosus*, *L. lactis*, *E. coli*, and *Scheffersomyces stipitis*, respectively. However, the homology between xylulokinase of *B. coagulans* and *L. brevis* was only 19 %. During the activity measurement at different pH, maximum activity of XK was observed at an optimal pH and temperature of 7 and 85°C, respectively, while the enzyme lost 20% and >50% of the activity when the pH was reduced to 6.0 or increased to 8.0, respectively. Similar to XI, the divalent ions Co<sup>2+</sup>, Mn<sup>2+</sup>, and Fe<sup>2+</sup> enhanced the activity of XK.<sup>45</sup>

### 3.1.2 Xylose reductase (XR) – Xylitol dehydrogenase (XDH) pathway

In yeast and fungi, xylose is assimilated through xylose reductase (XR) – xylitol dehydrogenase (XDH) pathway. In the first step, xylose is reduced to xylitol mediated by NAD(P)H dependent xylose reductase (XR; EC 1.1.1.21) followed by oxidation of xylitol to xylulose catalysed by NAD<sup>+</sup> dependent xylitol dehydrogenase (XDH; EC 1.1.1.9) (Figure 3). Identical to bacterial metabolism, xylulose is further phosphorylated to xylulose-5-phosphate and metabolised through pentose phosphate pathway.<sup>49-52</sup> The xylose fermenting yeasts (*Candida shehatae*, *Scheffersomyces (Pichia) stipitis*, *Pichia fermentans*, *Spathaspora* sp., etc) employ XR-XDH pathway for assimilation of xylose, and most of the yeasts utilize xylose under aerobic conditions.<sup>53-55</sup>



**Xylose reductase (XR; EC 1.1.1.21)** [D-Xylose + NAD(P)H  $\rightarrow$  Xylitol + NAD(P)<sup>+</sup>] is a 36 KDa protein containing 322 amino acid and a member of the aldoketoreductase family 2 (AKR2). AKRs are superfamily of enzymes that catalyse reversible reduction of aldehydes or ketones to their respective alcohols utilizing NADPH as a cofactor. XR is highly important enzyme when the desired product is xylitol, a molecule with nutritional and pharmaceutical value. Son et al., 2018 reported the crystal structure of XR from *S. stipitis*. XR is a dimer with two polypeptide chains made of 15  $\alpha$ -helices and 10  $\beta$ -strands each with conserved catalytic sites at Asp43, Tyr48, Lys77 and His110.<sup>51</sup> The literature describes the flexibility of XR in using NADPH as well as NADH as a cofactor. The  $K_m$  values of NADPH and NADH for XR in *S. stipitis* are 0.0277 and 0.136 mM respectively, indicating more affinity and specificity for NADPH than NADH. Although the physiological function of XR is to reduce D-xylose,  $K_m$  value of D-xylose for XR is very high (39.4 mM) indicating that a high level of xylose is needed to drive xylose metabolism efficiently. The structural conformation of the enzyme displays the presence of a hydrophobic binding pocket. It could be one of the reasons for the low affinity of XR towards xylose which has a high degree of hydrophilicity due to the presence of five hydroxyl groups. Similar to *S. stipitis*, a dimeric XR structure with  $K_m$  of 87 mM for D-xylose has been elucidated in *Candida tenuis*.<sup>56</sup> Recently new xylose utilizing yeasts of *Spathaspora sp.*, were characterized to have high XR activity mostly NADPH dependent, except three species, *Sp. arborariae*, *Sp. gorwiae*, and *Sp. passalidarum*.<sup>57,58</sup> The genome mining resulted in two putative XR genes, where *SpXYL1.2* has relatively higher XR activity with NADH, and strain could assimilate xylose effectively under anaerobic conditions. In *Sp. arborariae*, XR accepts NADH and NADPH as cofactors, with an affinity ( $K_m$ ) of 12.8 (NADH) and 26.1 (NADPH)  $\mu$ M, respectively. In the presence of xylose as substrate, the affinity was observed to strengthen with  $K_m$  of 29.5 (NADH) and 57.5 (NADPH) mM respectively.<sup>53,57</sup>

**Xylitol dehydrogenase (XDH; EC 1.1.1.9)** or xylulose reductase (Xylitol + NAD<sup>+</sup>  $\rightarrow$  D-Xylulose + NADH) mediates the conversion of xylitol to D-xylulose, is a well characterized enzyme encoded by nucleotide sequence of 1089 bp, and the operon reading frame codes for a protein containing 363 amino acids, with approximate mass of 38.5 KDa. The XDH mediates the oxidation of xylitol using NAD<sup>+</sup> as a cofactor.<sup>59,60</sup> The NAD<sup>+</sup>-dependent XDH is a homotetramer which forms heteronuclear multi-metal protein with 1 mol of Zn<sup>+2</sup> and 6 mol of Mg<sup>+2</sup> ions per mol of 37.4 kDa protomer (structural subunit of an oligomeric protein) with  $K_m$  of 39  $\mu$ M for xylitol. XDH enzyme displayed a half-life of 300 h in 50 mM Tris buffer at pH 7.5. The metal ions like Co<sup>+2</sup>, Mn<sup>+2</sup>, and Zn<sup>+2</sup> exerts inhibitory effect on the enzyme and the activity is completely ceased at 5.0 mM concentration of these metal ions. But complete dissociation of Zn<sup>+2</sup> from the enzyme was observed to inactivate XDH completely.<sup>61</sup>

### 3.1.3 Weimberg pathway

In 1946, Lockwood and Nelson identified a non-phosphorylative hexose and pentose sugar pathway in *Pseudomonas* and *Acetobacter sp.*, wherein the oxidation of sugars resulted in accumulation of respective





sugar (gluconic and pentonic) acids.<sup>62</sup> Later in 1961, Ralph Weimberg elucidated the pathway in *Pseudomonas fragi*, and the pathway was termed after Ralph Weimberg as Weimberg pathway. Analogous to glyoxylate cycle, it is a carbon conserving route for xylose metabolism to  $\alpha$ -ketoglutarate as there is no carbon loss like TCA cycle. The oxidative route consisted of five step enzymatic reactions converting pentose sugars to  $\alpha$ -ketoglutarate without any loss of carbon (Figure 3).<sup>63</sup> The pathway starts with oxidation of D-xylose to D-xylonolactone by D-xylose dehydrogenase (DXD encoded by *XylB*) which is further hydrolysed to D-xylonate by xylonolactone lactonase (XLA encoded by *XylC*) via a ring opening mechanism. The D-xylonate formed is dehydrated in subsequent reactions to form  $\alpha$ -ketoglutarate semialdehyde with 2-keto-3-deoxy xylonate as an intermediate. Both the dehydration reactions were predicted to be catalysed by xylonate dehydratase (XAD encoded by *XylD*) and 2-keto-3-deoxy-xylonate dehydratase (KDXD encoded by *XylX*). Finally, the  $\alpha$ -ketoglutarate semialdehyde is oxidized to form  $\alpha$ -ketoglutarate, by  $\alpha$ -ketoglutarate semialdehyde dehydrogenase enzyme (KGSADH encoded by *XylA*).<sup>64</sup>

The BLAST analysis of *P. fragi* genomic database revealed that *Caulobacter crescentus*, *Burkholderia xenovorans*, and *Chromohalobacter salexigens* have possible genes mediating the Weimberg pathway. In 2007, Craig Stephens and associates observed the expression of *XylXABCD* genes, when a freshwater bacterium *C. crescentus* was grown on D-xylose as the sole carbon and energy source. Recently, it has been found that *Pseudomonas taiwanensis* VLB120 can assimilate D-xylose through Weimberg pathway, but the initial oxidation and hydrolysis reactions are mediated by glucose dehydrogenase (EC 1.1.5.2) and gluconolactonase (EC 3.1.1.17) instead of DXD and XLA. Similar behaviour was also observed in *P. putida* strains wherein DXD and XLA are involved in gluconic acid production.<sup>65</sup> Further, determination of kinetic parameters,  $K_m$  and  $V_{max}$ , revealed two rate limiting reactions in the Weimberg pathway mediated by  $Mn^{+2}$  dependent XDH and  $NAD^+$  dependent KGSADH.<sup>66</sup> Therefore, D-xylose assimilation by Weimberg pathway requires external supplementation of metal ions ( $Mn^{+2}$ ) and availability of  $NAD^+$  for complete conversion of D-xylose into  $\alpha$ -ketoglutarate.

### 3.1.4 Dahms pathway

Until 1974, it was understood that xylose is metabolized via XI and XR-XDH pathway and rarely through Weimberg pathway. But, a novel aldolase (EC 4.1.2.18) was discovered by Stephen Dahms in *Pseudomonas* sp., cleaving 2-keto-3-deoxy xylonate, the intermediate of Weimberg pathway, to pyruvate and glycolaldehyde and the pathway was termed as Dahms pathway (Figure 3). The aldolase is specific to 2-keto-3-deoxy-D-xylonate, but not to L-isomers. Similarly, the pathway was also elucidated for L-arabinose assimilation where the enzyme mediating the conversion is an L-isomer specific aldolase.<sup>67-69</sup>

### 3.1.5 Non-natural or synthetic pathway



With the advancements in the genetic engineering approaches and availability of numerous genomic databases, it would be simplified to find the alternative routes for the natural pathways so that the end product can be achieved in few simple steps without imposing the metabolic burden and disturbing the microbial cell integrity. To this end, a non-natural synthetic pathway was constructed for xylose metabolism, where D-xylose is converted to D-xylose-1-phosphate (X1P) instead of X5P. This phosphorylation reaction leading to X1P is mediated by xylulose-1-kinase and the pathway is termed as X1P pathway. In further aldolytic cleavage, X1P is converted to glycolaldehyde and dihydroxyacetone phosphate (DHAP), an intermediate of EMP pathway (Figure 3). Through this non-natural or synthetic pathway, D-xylose is converted to DHAP in two sequential steps, whereas in PPP or through EMP pathways multiple steps are involved.<sup>70</sup>

### 3.2 Xylose operon

The genes responsible for the xylose transport, and assimilation are clustered into an open reading frame called as xylose (*xyI*) operon. In bacteria xylose metabolism is mediated through XI pathway.<sup>71</sup> The genes responsible for the metabolism of xylose was observed to be organized in two major transcriptional units *XylAB* and *XylFGHR* with promoters  $P_A$  and  $P_E$ , respectively. It was observed that the transcriptional activation is induced by xylose and repressed by glucose i.e. as long as glucose is available, the xylose assimilation will be suppressed.<sup>72</sup> The transcriptional units *XylAB* and *XylFGHR* were observed to be located at 80 min on the chromosome map.<sup>73</sup> The *XylAB* comprises of genes encoding for XI (*XylA*) and XK (*XylB*), respectively. The *XylFGHR*, a high affinity ABC type transporter for transport of xylose consist of four subunits where *XylH* acts as the transmembrane transporter, *XylF* binds to xylose and *XylG* is an ATP binding protein that mediates the phosphorylation of xylose. Subsequently, the transported xylose is acted up on by XI and XK, to form xylulose-5-phosphate. The *XylR* gene has been observed to be constitutively expressed under a weak promoter  $P_R$  regardless of xylose or glucose availability. The repressor tends to bind two DNA binding regions  $I_A$  and  $I_E$ , upstream to the transcriptional promoter's consensus sequences and adjacent to RNA polymerase binding site. In the presence of xylose, *XylR* forms a dimer with the xylose substrate, and causes activation of two promoters  $P_A$  and  $P_E$ , resulting in simultaneous transcription of *XylFGH* and *XylAB* genes. Any mutation in the repressor protein *XylR* was observed to abolish the expression of  $P_A$  and  $P_E$  promoters.<sup>74,75</sup> In *E. coli* the xylose transport into the microbial cell is mediated by low affinity transporter *xylE*, and the expression was observed to be 10-fold higher when the external medium was supplemented with xylose.<sup>76</sup> Though the xylose dissimilation follows the XI pathway in gram-positive bacteria, the regulation was observed to be different in few enteric bacteria like *B. subtilis* and *Lactobacillus* strains, where the *XylR* gene displays repressive behaviour rather than acting as a transcriptional activator. The *XylR* of *B. subtilis* and *Lactobacillus* strains is not homologous to its counterpart in *E. coli* and binds to a palindromic sequence upstream to transcriptional start codon, represses the transcription activation and the repression effect is relieved in the presence of xylose.<sup>77</sup> In *Staphylococcus xylosus*, three open reading frames containing 4520 nucleotide bases were





annotated as *XylR*, *XylA* and *XylB* genes. The BLAST studies of *XylA* gene presented 65% and 51% similarity with *B. subtilis* and *E. coli* respectively.<sup>78</sup>

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In the genera Clostridia, *C. acetobutylicum* is the representative strain for investigating different metabolic activities. The whole genome sequence is available along with the required genetic tools for strain engineering. Genome mining through subsystem-based approach revealed the presence of a novel XI (CAC2610), and further characterization observed that it is not homologous to known XI (*XylA*) genes. Along with XI, XK (*XylB*, CAC2612), xylose proton symporter (*XylT*, CAC1345), and transcriptional regulator (*XylR*, CAC3673) were also sequenced and characterized. The *C. acetobutylicum* strains are well known to utilize broad range of monosaccharides, disaccharides, starches, other polysaccharides like xylan and xyloglucan. Xylan and xyloglucan are the major components of the hemicellulosic fraction of plant. The depolymerization of xyloglucan and xylan, results in  $\alpha$ - and  $\beta$ -xylosides, respectively. These xylosides are transported into the cell and further degraded into xylose. The genetic make-up for utilizing these xylosides was mainly observed in firmicutes like *Bacillus*, *Lactobacillus* and *Clostridium* sp. In *B. subtilis* and *C. acetobutylicum*, xylose operon contains two clusters *XylAB* and *XynTB*. The *XynT* gene encodes ABC transporter that transport  $\beta$  and  $\alpha$ -xylosides into the microbial cell and further *XynB* gene converts xyloside into D-xylose.<sup>73,79</sup>

In yeast and fungi, xylose is sequentially metabolised through three cytosolic enzymes, XR, XDH and XK to convert it to xylulose-5-phosphate. *S. stipites* the most efficient xylose fermenting yeast was characterized to reveal the genes encoding XR (*Xyl1*), XDH (*Xyl2*), and XK (*Xyl3*) enzymes. The genes are either co-localized or distributed in the genome, for example in *S. stipitis*, *Xyl1* was observed on chromosome (Chr) V, *Xyl2* on Chr I, and *Xyl3* on Chr VIII. Although *S. cerevisiae* strains are not native xylose utilizing strains, a putative XDH (*Xyl2*) gene was identified on Chr XV, and XK on Chr VII.

### 3.3 Carbon catabolite repression or glucose effect: The natural phenomenon arresting the simultaneous conversion of mixed sugars

Microorganisms cultured on mixed sugar substrates displays a pattern of two successive exponential phases during the growth, called as diauxic growth. The occurrence of this growth pattern is due to utilization of preferred substrate which suppresses the uptake of other carbon sources present in the medium and this phenomenon is known as carbon catabolite repression (CCR) or glucose repression effect.<sup>80,81</sup> The diauxic growth significantly affects the utilization of mixed sugars and increase the length of fermentation (decreased productivity).

LCB or agricultural residues as the feedstock for the production of biofuels and bioproducts has received considerable interest.<sup>6</sup> It is not just the surplus agro-residual biomass, utilizing LCB as feedstock address various environmental concerns and food vs feed debate with first generation starchy feedstocks. As LCBs are polymers of celluloses and hemicelluloses, hydrolysates derived after pretreatment and saccharification contain mixture of hexoses (mostly glucose) and pentoses (mostly xylose).<sup>41,80</sup> The growth of



microorganism on hydrolysates containing mixed sugars results in suppression of pentose sugar utilisation. The mechanism and the strategies to overcome the limitation are discussed in this section. Aidelberg and co-workers observed a hierarchical fashion of utilization of hexoses and pentoses. When the microorganism is grown on mixed sugars (glucose, arabinose and xylose) as substrates, the most preferred carbon source has inhibitory effect on other sugars, for example glucose represses the uptake of arabinose and xylose, and upon glucose depletion, the next preferred substrate is arabinose, and xylose utilization mechanism is still inhibited.<sup>82</sup> From the literature, it was explained that mechanism occurs due to two instances; (i) inhibition of expression of genes involved in the non-glucose sugars by 3',5'-cyclic adenosine monophosphate (cAMP). The cAMP is a secondary messenger, derivative of ATP, synthesized by adenylate cyclase enzyme. In bacteria, cAMP levels depend on the type of growth medium. Intracellular transportation of glucose inhibits the adenylate cyclase enzyme and decreases the cAMP, and cAMP receptor proteins (CRP), which inhibits the transcription of the *xyl* operon,<sup>83</sup> (ii) inhibition of xylose transport mediated by dephosphorylated PTC component EIIA<sup>glc</sup> that binds to the cognate sugar transporter and prevents the transport, and the mechanism is *inducer exclusion* (Figure 4A).<sup>84</sup> Whereas in the phosphorylated form, the EIIA<sup>glc</sup> component activate the adenylate cyclase, which improves intracellular cAMP levels. Improved cAMP levels bind with CRP to form a complex, and the active cAMP-CRP complex that could bind to the ORF and express the permeases and other genes involved in metabolism of non-glucose sugars (Figure 4B).<sup>85</sup>

In *B. megaterium*, the glucose mediated xylose repression was 14-fold, *XylR* gene which regulates the transcription initiation by binding to promoters of *XylAB* and *XylFGH* was modified by incorporating a kanamycin resistance gene resulting in lowering the repression to 8-fold, and deletion of 184 bp at the 5'-end of *XylR* gene, further reduced repression by 2-fold.<sup>85,86</sup> Alternative CCR mechanism called feed-back inhibition was observed in few gram-positive bacteria, in which the catabolite control is exerted by catabolite control protein A (*ccpA*). The *ccpA* is a dimeric transcriptional regulator, expressed constitutively regardless of the carbon source. In the presence of glucose, and other glycolytic intermediates like fructose 1,6-bisphosphate, histone protein (HPr) component of the enzyme phosphorylation cascade (PTS enzyme I, HPr, and Enzyme II), the major facilitator of sugars, is phosphorylated at the serine residue (*HPr-Ser<sup>46</sup>-P*) instead of histidine residue. The phosphorylated *HPr*, binds to catabolite control protein (*ccpA*), and the complex binds to catabolite repressive element (*CRE*) within the transcriptional or coding sequence of upstream to the promoter region by blocking the transcription of pentose sugars.<sup>87</sup> In *C. acetobutylicum*, deletion of *ccpA* and enzyme II complex was attempted but it resulted in impaired growth rate and failure in metabolic flux.<sup>87</sup> But in *B. subtilis*, deletion of *CRE* protein could overcome the degree of repression from 13- to 2.5-fold.<sup>88</sup> The successful development of CCR negative strain would be of high value with a capacity to assimilate glucose and non-glucose sugars simultaneously causing increase in the yield and productivity.

#### 4. Hurdles requiring debottlenecking for efficient xylose metabolism.

##### 4.1 Transport of xylose into the microbial cell



Xylose metabolism is well investigated and characterised in bacteria, fungi, yeast, and few archaea. In these prokaryotic and eukaryotic organisms, the xylose metabolism is either native or heterologously expressed to shape the cellular metabolism to rely on xylose as sole carbon and energy source. Before the start of xylose metabolism, the sugar must be transported into the microbial cell (Figure 5). The possible mechanisms for transport of sugars through the membrane are passive diffusion, facilitated diffusion or active transport.<sup>89</sup> The passive diffusion is the simplest process that occur based on the concentration gradient of the substrate between the intracellular environment and the extracellular medium, whereas in the facilitated diffusion a carrier protein mediates the transfer based on the concentration gradient. In active transport, the translocation of sugar through the transmembrane proteins happens with energy expenditure. Usually, transmembrane proteins that spans across the outer membrane mediates the translocation from extracellular space to intracellular environment. These transmembrane proteins belong to major facilitator superfamily (MFS), that are divided into three classes based on the functionality, (i) uniporter; transports single substrate across; (ii) symporter; transport of one substrate is coupled along with a charged molecule; and (iii) antiporter; two different substrates are translocated in the opposite directions.<sup>90</sup>

In bacteria there are three possible mechanisms known for the transport of xylose into the microbial cell, (i) H<sup>+</sup>/Na<sup>+</sup> - symporter, which is identified in *E. coli*,<sup>91</sup> *Salmonella typhimurium*, *B. megaterium*, *L. brevis*, and *B. subtilis*, (ii) PEP-Carbohydrate phosphotransferase system, identified in *E. coli* and use PEP as the source of energy, and (iii) ATP driven ABC transportation periplasmic binding protein, identified in *E. coli* and few other *Bacillus* sp. Facilitated diffusion is not well known in bacterial population, except glycerol facilitator protein (*GlpF*) in *E. coli* and glucose transporter (*GlfZ*) in *Zymomonas mobilis*. In lactic acid bacteria, phosphoenol pyruvate (PEP) D-mannose phosphotransferase system (PTS) with two integral membrane proteins EIICMan, EIIDMan and cytoplasmic phosphorylation proteins EIIMan and EIIBMan is observed to have significant role in xylose transport.<sup>89</sup> The endogenous transporters in the microbial cells, have an affinity for glucose from low ( $K_m$  50 – 100 mM) to high ( $K_m$  1 – 2 mM), whereas for xylose the numbers can be up to 10 times higher.<sup>92</sup> For example, *Glf*, a promiscuous glucose-facilitated diffusion protein from *Z. mobilis* expressed in *E. coli* has lower affinity towards xylose ( $K_m$  40 mM) than glucose ( $K_m$  4.1 mM), and  $V_{max}$  was observed to be two-fold higher for glucose, resulting in delayed xylose uptake and assimilation, when expressed in *E. coli* cells.<sup>91</sup>

There are two mechanisms identified in yeast for the xylose transport, (i) membrane potential due to proton symport, or (ii) facilitative diffusion through low affinity transporters. *Scheffersomyces stipitis*, a well know xylose metabolizing strain has low and high affinity carriers to mediate the xylose transport, whereas in *C. shehatae*, facilitated diffusion and low affinity symport mechanism was observed.<sup>93,94</sup> The sugar binding pockets in the transmembrane proteins have residues specific for sugar, any alteration or changes in the glucose specific residues could alter the D-glucose uptake rate and can increase the endogenous xylose transport. Farwick et al., 2014 implemented this method in altering the amino acids sequence which interact



with C6-hydroxymethyl group of D-glucose, but the deletion of those specific amino acids led to deleterious effects on the transport of glucose as well as xylose.<sup>92</sup> Wang and associates have studied the effect of 28 different site directed mutations on xylose uptake rate and metabolism in Mgt05196p transporter of *Meyerozyma guilliermondii*. The substitution mutations at Phe432Ala and Asn360Ser on Mgt05196p, improved the xylose uptake, but diminished the glucose uptake, whereas the mutation N360F specifically enhanced the xylose transport without any glucose inhibition.<sup>95</sup>

Evolution is the natural mechanism of adapting to new environmental or physiological conditions. To create an order of natural evolution, a new approach called adaptive laboratory evolution (ALE) was attempted to increase the efficiency of xylose transporters *XUT3* and *GXS1* from *S. stipitis* and *C. intermedia*, respectively.<sup>96</sup> The *XUT3* is one of the seven high affinity xylose (*XUT*) transporters annotated in *S. stipitis* with similar specificity towards both glucose and xylose. The *GXS1* in *C. intermedia* is a broad range sugar transporter with specificity to carbon sources like glucose, arabinose, ribose, and xylose.<sup>96</sup> In this directed evolutionary approach, the substitution mutations Phe40Val, Phe465Ser, and Phe500Ser in *GXS1*, and Leu122Val, Phe343Leu, Gln345Arg, Ala298Thr, Tyr304Phe, and Lys542Arg in *XUT3*, influenced the xylose uptake. The heterologous expression of these mutated transporters in *S. cerevisiae* resulted in 70% increase in the specific growth rate on xylose.<sup>96</sup> The mutant transporters also displayed phenomenal alteration in the diauxic growth and evolved strain could simultaneously utilize xylose and glucose. In the sugar transporters, motif G-G/F-XXX-G is a conserved sequence present on the transmembrane component. After site-directed mutagenesis or ALE, a modified motif GGFIMG, with larger side chains restricting the pore size for glucose transport and allowing smaller xylose molecules was identified. The alteration in the motif sequence because of point mutations increased the pentose specificity to the binding site by decreasing the pore size and transporting xylose efficiently than glucose.<sup>97</sup> However, bioprospecting for a novel xylose specific transporter or modification of an existing transporter to overcome the CCR induced by glucose will be beneficial and could significantly improve simultaneous glucose and xylose consumption.

#### 4.2 Availability of redox cofactors and homeostasis

Redox homeostasis is an important consideration in microbial cell factories as it affects a wide range of genes, cellular functions and metabolite profiles and. Redox balancing plays a critical role in coupling catabolism and anabolism. The co-factors involved in maintaining homeostasis are NADH and NADPH, usually acts as electron carriers, are involved in respiratory chain reactions (catabolism), and cell synthesis (anabolism) respectively. The NADH is the predominant redox product of catabolism while NADPH has a greater role in anabolism with major fraction coming from pentose phosphate pathway and a delicate balance in the intracellular level of these cofactors is required to ascertain an optimal metabolic output. The NADH/NAD<sup>+</sup> ratio which reflects the intracellular redox state of a living cell and is influenced by various factors such as the physiological state of the cell, oxidation state of the substrate, the nature and presence of electron acceptors,



as well as enzymes requiring redox factors.<sup>98-101</sup> The cells often start side reactions leading to byproducts formation which contributes towards the redox homeostasis. For example, during ethanol fermentation by *S. cerevisiae*, the yeast starts parallel formation of acetate and glycerol, NADH consuming reactions.

In the case of change in the substrate from glucose to xylose, there observed a myriad of changes in the metabolism, and the response observed were increased amino acid concentrations, increased TCA cycle intermediates, and reduction in sugar phosphates and reducing equivalents or redox cofactors.<sup>102</sup> One of the challenges with xylose metabolism is maintaining redox homeostasis. After initial 3-4 specific step of xylose metabolism, it is connected to central carbon metabolism. Majority of this problem stems from first two steps where xylose is isomerised to xylulose via XR and XDH. The higher preference of XR towards NADPH generates NADP<sup>+</sup> while second step require NAD<sup>+</sup>. The diminished synthesis of reducing equivalents and uneven demand during the xylose assimilation results in cofactor imbalance, which in turn affect the ATP yield, metabolic fluxes. The different cofactor preference of these two enzymes results in NAD<sup>+</sup> deficiency resulting in accumulation of the intermediate xylitol. The condition mostly prevails under anaerobic or oxygen limited conditions, where NADH cannot be oxidized to NAD<sup>+</sup>, due to absence of oxygen the final electron acceptor.<sup>103</sup> This problem could be overcome by continuous supply of NADPH and NAD<sup>+</sup>.

To prevent the xylitol accumulation and further direct the flux of xylose carbon to central carbon metabolism, NADH oxidase (NOX) can be used. NOX catalyses a water forming reaction using oxygen as the electron acceptor ( $\text{NADH} + \text{H}^+ + 0.5\text{O}_2 \rightarrow \text{NAD}^+ + \text{H}_2\text{O}$ ) and thereby regenerating the NAD<sup>+</sup> molecules. The xylose assimilation through XR-XDH pathway linked with NADH oxidase could render redox homeostasis. Zhang et al., 2012 constructed a cycle of regeneration using NOX, for regeneration of NAD<sup>+</sup>.<sup>104</sup> In a similar approach, NOX from *L. lactis* was heterologously overexpressed in *S. cerevisiae* harbouring XR-XDH from *S. stipitis* which resulted in 69.6% decrease in xylitol accumulation, and more carbon flux was directed towards ethanol leading to an improvement of 39.3% in molar yields.<sup>105</sup>

*Scheffersomyces stipitis* has the ability to ferment xylose under anaerobic conditions, thus under unfavourable conditions such as redox imbalance, the accumulated NADH can be utilized by NADH-dependent XR and circumvents the pathway.<sup>50</sup> In yeasts such as *S. stipitis*, XR has affinity for NADH as well as NADPH, hence using advanced genetic engineering techniques, the cofactor specificity of XR in required host strain can be altered, so that the cofactor requirement for first two steps can be compensated internally and the continuous availability of cofactors can lead to improved xylose uptake and fermentation efficiency. A mutant XR enzyme K270M, from *S. stipitis* with lower specificity to NADPH was expressed in *S. cerevisiae* and the cultivation of strain with mutated XR on xylose showed 16-fold reduction in NADPH and 4.3-fold increase in NADH specificity.<sup>93</sup> After a site directed mutagenesis approach on XR enzyme in *C. tenuis*, a 170-fold change in cofactor preference from NADPH to NADH was observed by in a Lys274Arg and Asn276Asp double mutant. When the mutant XR along with XDH from *Galactocandida mastotermitis* was expressed in *S. cerevisiae*, 42% increase in ethanol, 52 and 57% decrease in xylitol and glycerol yields was observed,

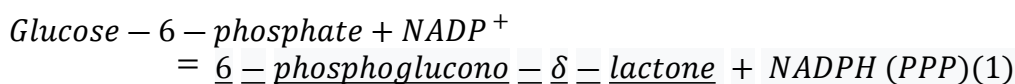




respectively, with xylose as the sole carbon source.<sup>106</sup> Similarly, a double mutant of *S. stipitis* XR (Arg276His, and Lys270Arg/Asn270Asp) showed decrease in catalytic efficiency and increase in  $K_m$  values towards NADPH, resulting in enhanced XR dependence on NADH. The strain expressing NADH-dependent XR, efficiently utilized xylose resulting in 20% increase in ethanol level and 52% decrease in xylitol accumulation.<sup>107</sup> A wild type NADH specific XR was identified from *C. parapsilosis*, in which the conserved motifs have arginine residues instead of lysine. Later, following the structural integrity of NADH specific XR of *S. stipitis* was altered (Lys270Arg), and the *S. cerevisiae* strain expressing this modified XR diverted the flux of carbon towards ethanol with reduced xylitol accumulation.<sup>108</sup>

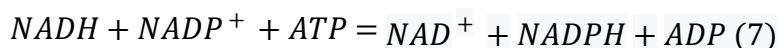
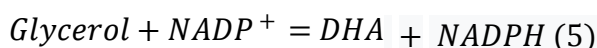
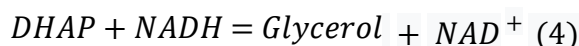
Besides overexpression of NOX and change in cofactor preference, a number of other approaches have been employed to alleviate problem of redox imbalance. Under anaerobic conditions, NADH molecules are oxidized through transhydrogenase shunt with malic enzyme (*MAE1*) (Malate + NADP<sup>+</sup> → Pyruvate + NADPH), malate dehydrogenase (*MDH2*) (Oxaloacetate + NADH → Malate + NAD<sup>+</sup>), and pyruvate carboxylase (*PYC2*) (Pyruvate + ATP → Oxaloacetate + ADP) that can regulate the redox balance in *S. cerevisiae*. In a combinatorial cassette along with xylose metabolizing (XR, XDH, and XK) genes, two different strains were constructed. Strain 1 expressing *MAE1* with *Xyl* genes was observed to improve the xylose uptake and caused increment in NADPH/NADP<sup>+</sup> ratio. The co-expression of *MAE1*, and *MDH2* along with *Xyl* genes resulted in 1.25-fold increase in ethanol titers due to regeneration of cofactors required for 1<sup>st</sup> and 2<sup>nd</sup> steps of XR-XDH pathway.<sup>109</sup> Alternative to multiple gene overexpression, a native NADH kinase (NADH + NADP<sup>+</sup> → NADPH + NAD<sup>+</sup>) enzyme was overexpressed replenishing the NADPH and NAD<sup>+</sup> cofactors.<sup>110</sup> In another study, NADPH dependent glutamate dehydrogenase (*GDH1*) (α-ketoglutarate + NH<sub>4</sub><sup>+</sup> + NADPH → Glutamate + NADP<sup>+</sup>) was deleted in *S. cerevisiae* and NAD<sup>+</sup> dependent glutamate dehydrogenase (Glutamate + NAD<sup>+</sup> + ATP + H<sub>2</sub>O → α-ketoglutarate + NH<sub>4</sub><sup>+</sup> + NADH + ADP) was overexpressed resulting in increased ethanol production and reduced xylitol accumulation.<sup>111</sup> Recently two NADH oxidation approaches were demonstrated in *L. lactis*, where external supplementation of hemin<sup>112</sup> and flavinium<sup>113</sup> catalyses the oxidation of NADH molecules in the presence of O<sub>2</sub>. Although the mechanism was demonstrated in *L. lactis*, this in-situ regeneration of reducing cofactors could be of wide significance on an industrial perspective.

Like NADH, NADPH is also a crucial electron donor in various metabolic pathways. Celton and associates reported that *S. cerevisiae* cells growing on pentose sugars respond to an increase in NADPH demand by directing the carbon flux through the pentose phosphate pathway (PPP), acetate synthesis pathway, as well as transforming NADH to NADPH in cytosol via transhydrogenase cycle. The enzymes involved in the regeneration of NADPH and NAD<sup>+</sup> cofactors are, glucose-6-phosphate dehydrogenase (Reaction 1), 6-phosphogluconate dehydrogenase (Reaction 2), and transhydrogenase (Reaction 3).



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In the case of higher xylose concentrations and increased uptake, the demand for NAD(P)H will be further increased, a predicted glycerol-DHA cycle has been reported to exchange NADH and NADP<sup>+</sup> for NAD<sup>+</sup> and NADPH, at the expense of ATP molecule. In the glycerol-DHA cycle, dihydroxyacetone phosphate (DHAP) is reduced to glycerol by NADH-dependent glycerol-3-phosphate dehydrogenase (Reaction 4), glycerol is then oxidised to dihydroxyacetone (DHA) by NADP<sup>+</sup> dependent glycerol dehydrogenase (Reaction 5) and finally DHA is phosphorylated to DHAP at the expense of one ATP molecule (Reaction 6).<sup>114</sup> Thus, glycerol-DHA cycle generates both the redox factors (NADPH and NAD<sup>+</sup>) required for the XR-XDH pathway.



## 5. Xylose as alternative carbon source for microbial growth and product development

The bioconversion of xylose into value-added chemicals has received a lot of attention in recent years. Many naturally occurring or engineering microbial strains have been discovered or designed to synthesize various industrially important chemicals and fuels using xylose as a sole carbon and energy source. Table 3 summarizes various chemicals that can be produced from xylose through biological route and their commercial applications.

### 5.1 Xylitol

Xylitol (C<sub>5</sub>H<sub>12</sub>O<sub>5</sub>), a platform chemical, is a five-carbon sugar alcohol with wide spectrum of applications in personal care, food, confectionary, and pharmaceutical industries.<sup>115</sup> Xylitol is equivalent to common table sugar with; lower calorific value (2.4 Vs 4 calories per gram), lower glycaemic index (7 vs 60 – 70 %) and insulin independent metabolism. In 2016, the global xylitol market was worth US\$ 725.9 million with production capacity of 190.9 thousand metric tons. It has been forecasted that with the increased global market demand and compound annual growth rate (CAGR) of 5.7%, the production capacity should be increased to 265.5 thousand metric tons (US\$ 1 billion) by 2022.<sup>116</sup> The commercial production of xylitol is performed via chemical route by catalytic dehydrogenation of pure xylose, involving expensive Ni based catalyst, sulfuric acid, calcium oxide, phosphoric acid and activated charcoal treatments at high pressure (5000 Kpa) and temperature (140 °C).<sup>117</sup> The process is uneconomical due to requirement of pure xylose as the substrate, the process generates heavy metal pollutants, and there is high risk associated with operating conditions and environmental pollution.<sup>117</sup> Alternative route for the production of xylitol is the biological process, where in whole/immobilized cells expressing XR or the cell free extracts with XR activity acts as biocatalysts (Figure 3), produce xylitol from pure and crude renewable sources rich in xylose. The process offers an advantage of mild operative conditions and non-requirement of purified xylose.<sup>118</sup>





Xylose is native substrate for xylitol which is accumulated due lack of synchronisation between steps catalysed by XR and XDH. Various microorganisms having natural xylitol producing ability include bacteria, yeast, and fungi. Among them yeasts are predominant such as *Candida athensensis*, *C. boidinii*, *C. guilliermondii*, *Debaryomyces hansenii*, *C. tropicalis*, *C. magnolia*, and *S. stipitis* that can accumulate xylitol with significant yields and productivities (Table 4). Later with introduction of heterologous pathway engineering *S. cerevisiae*, *Kluyveromyces* and other *Candida* sp., have been engineered to accumulate xylitol. Usually bacterial system presents XI pathway for xylose assimilation, but few bacterial strains like *Bacillus coagulans*, *Cellulomonas cellulans*, *Corynebacterium glutamicum*, *Corynebacterium ammoniagenes*, *Enterobacter liquefaciens*, *Mycobacterium smegmatis*, and *Serratia marcescens* have XR-XDH pathway for producing xylitol.<sup>119</sup> A new bacterial isolate *Pseudomonas putida* was screened for xylitol production and characterized to have XR activity of 48.7 IU/mg. The strain accumulated 35.2 g/L xylitol with productivity of 0.98 g/L/h when cultured on xylose under optimized growth conditions.<sup>118</sup>

One of the most critical environmental parameters to be considered during processing of yeast for xylitol production is dissolved oxygen concentration. Since oxygen limited conditions usually favour xylitol formation, as conversion of NADH to NAD<sup>+</sup> is hampered and the reduced availability of NAD<sup>+</sup> impedes xylitol to xylulose conversion and resulting accumulation of xylitol.<sup>59</sup> The XR catalysing reduction of xylose to xylitol is NAD(P)H dependent, hence to improve NAD(P)H levels, overexpression of glucose-6-phosphate dehydrogenase (Glucose-6-phosphate + NADP<sup>+</sup> → 6-Phosphogluconolactone + NADPH) from PP pathway in *S. cerevisiae* resulted in xylitol titer and productivity of 196.3 g/L and 4.27 g/L/h, respectively.<sup>120</sup> A new isolate *P. fermentans* subjected to chemical mutagenesis resulted in a strain with improved XR activity (34%) and reduced XDH (22.9%) activity. The fed-batch fermentation using mutant strain of *P. fermentans* produced 98.9 g/L xylitol with conversion yield of 0.67 g/g using pure xylose as substrate. Further, using non-detoxified xylose rich pre-hydrolysate from sugarcane bagasse, the strain amassed 79.0 g/L xylitol with an overall yield of 0.54 g/g respectively.<sup>55</sup> *S. stipitis* is well-known for its high xylose utilization rate, but as xylose assimilation leads to high ethanol production, the host is not suitable for xylitol production. *Yarrowia lipolytica*, an oleaginous yeast is well-known for production of lipids and TCA cycle intermediates. The yeast has cryptic xylose metabolic pathway or inactive xylose assimilatory enzymes. As a result of it, *Y. lipolytica* is unable to grow on xylose as sole carbon source but can biotransform xylose to xylitol when cultivated in xylose along with other carbon sources like glucose or glycerol. The accumulation of xylitol is due to. *Y. lipolytica* Polt strain accumulated 53.2 g/L xylitol with a yield of 0.97 g/g using pure glycerol and xylose as carbon sources where glycerol was used for biomass production. Similar results were obtained when pure glycerol was substituted with crude glycerol from the biodiesel industry (titer: 50.5 g/L; yield: 0.92 g/g).<sup>121</sup>

Other than the environmental characteristics, basic mechanisms like substrate and product mediated growth inhibitions limits the final product titers in biological processes. Xylitol, polyhydroxy compound can interfere with the membrane fluidity of the host cell's membrane, disrupting growth and increasing xylitol accumulation.



Nystatin a membrane porin agent that can increase the permeability of the lipid membranes to ions, water and non-electrolytes was used to increase the xylitol transport from the cell. The *C. tropicalis* ATCC 13803 strain cultured along with nystatin could accumulate 197 g/L xylitol with 0.75 g/g and 3.9 g/L/h yield and productivity respectively.<sup>122</sup>

## 5.2 Lactic acid

Lactic acid (LA) or 2-hydroxypropanoic acid is an optically active compound and exist in L and D forms. Being a platform chemical, LA has diverse industrial applications in food, cosmetic, polymer and packaging. The most valued application of LA as monomer in the production of poly lactic acid (PLA), an alternative for commercial petrochemical polymers. The market price for food grade LA is approximately \$1400- \$1600/metric ton It has been estimated that the global market size of LA would reach ~\$8.7 billion by 2025, with a CAGR of 18.7 %.<sup>123</sup>

Various microorganisms like bacteria, fungi and yeast have been employed for the production of LA using xylose as the sole carbon and energy source (Table 4). Lactic acid bacteria (LAB) are considered predominant for industrial scale LA production. Based on the end products, LAB strains can be divided into two categories: homofermentative (*Lactobacillus delbrukii*, *L. acidophilus*, *L. plantarum*, *L. helveticus*) and heterofermentative (*L. brevis*, *L. diolivorans*, *L. fermentum*, *L. reuteri*) lactic acid fermentation. In homofermentative bacteria, the carbon flux is directed only to lactic acid (LA) with no by-product formation, whereas in case of heterofermentative, mixture of LA, acetic acid and ethanol are obtained with LA as main product. One of the most common routes for LA production is the simple reduction reaction of pyruvate obtained from EMP and/or PP pathway catalysed by NADH-dependent lactate dehydrogenase enzyme (Figure 6A). *Lactobacillus pentosus* and *L. brevis* can naturally ferment xylose through pentose phosphate and phosphoketolase pathway, producing LA and mixture of acetic and LA, respectively.<sup>124</sup> Wischral et al., 2019, investigated different *Lactobacillus* sp., for fermentation of xylose-rich hemicellulosic hydrolysates and identified *L. pentosus* strain efficiently utilizing the xylose-rich SCB hydrolysate obtained from combined alkali and acid pretreatment and accumulated 65 g/L LA with yield and productivity of 0.93 g/g and 1.01 g/L/h, respectively.<sup>125</sup> Similarly an engineered *E. coli* JU15 strain was supplemented with 32 g/L xylose and 42 g/L glucose, a simulated corn stover hydrolysate, resulting in 40 g/L LA with a conversion yield of 0.60 g<sub>LA</sub>/g<sub>sugar</sub>.<sup>126</sup> *Pediococcus acidilactici* is a facultative anaerobic lactic acid producing strain with specificity to wide range of substrates including xylose, but the strain *P. acidilactici* TY112 was not able to utilise xylose. Qui and associates, blocked the PK pathway by deleting the *pkt* gene, and overexpressed heterologous xylose assimilation pathway genes *xylA* and *xylB* along with transketolase (*tkt*) ( $X5P + E4P \rightarrow F6P + G3P$ ) and transaldolase (*tal*) ( $G3P + S7P \rightarrow F6P + E4P$ ) genes, directing the carbon flux from phosphoketolase to pentose phosphate pathway. The recombinant strain accumulated 130.8 g/L LA with 0.68 g/g yield in simultaneous saccharification and fermentation mode using dilute acid pretreated wheat straw as feedstock.<sup>124</sup>



The group have conducted similar overexpression and deletion strategy in D-LA producing *P. acidilactici* ZP26 strain, and the modified strain was adapted in laboratory conditions with xylose as the sole carbon source. The adapted strain *P. acidilactici* ZY15 accumulated 97.3 g/L D-LA with 0.92 g/g conversion yield through simultaneous saccharification and co-fermentation strategy using dry dilute acid pretreated and detoxified corn stover feedstock.<sup>127</sup> However the CCR limits the performance of various strains on LCB hydrolysates as they consists of both hexose and pentose sugars, *E. coli* JH15 strain was engineered to overcome CCR by deletion of the *ptsG* gene which encodes for *IIBCglc* (a PTS enzyme for glucose transport). The engineered strain produced 83 g/L D-LA with 0.83 g/g yield and 0.86 g/L/h productivity from co-fermentation with a mixture containing glucose and xylose 1:1 ratio.<sup>128</sup>

The commercially viable yeast *S. cerevisiae* produces LA in very minute quantities, hence requires the either homologous or heterologous expression to increase the titers. Ethanol is the natural and dominant product by *S. cerevisiae* and would be the major competitor for LA production. The competition is for the precursor/substrate, pyruvate, and cofactor NADH between pyruvate decarboxylase and alcohol dehydrogenase. In order to prevent this, a *pdh* deficient strain was constructed which exhibited poor growth and productivities.<sup>129</sup> In *S. cerevisiae*, heterologous overexpression of *Xyl1*, *Xyl2*, and *Xyl3* from *S. stipitis*, cellodextrins transporter (*cdt-1*) and a  $\beta$ -glucosidase (*gh1-1*) from the cellulolytic fungi *Neurospora crassa*, and additional laboratory evolution on medium containing cellobiose, resulted in a strain that could produce 83 g/L LA, with 0.66 g/g yield when cultivated on LCB hydrolysate containing 10 g/l glucose, 40 g/L xylose and 80 g/L cellobiose.<sup>130</sup>

### 5.3 Succinic acid

Succinic acid (SA) is an aliphatic dicarboxylic acid containing four carbon atoms with potent application as a precursor in pharmaceutical, polymers, and chemical industries. Like LA, SA is a platform chemical and due to the presence of two carboxyl acid groups, SA can be converted into a variety of products such as succinic anhydride, succinic esters, 2-pyrrolidine, and polyesters for synthesizing biodegradable plastics.<sup>131</sup> According to global market research, the market size was expected to reach \$237.8 million by 2022 with a CAGR of 9.2% (Succinic Acid Market Size & Share | Industry Analysis Report, 2022 (grandviewresearch.com)). SA production from pure sugars and LCB hydrolysates has been reported using natural producers *Anaerobiospirillum succiniciproducens*, *Actinobacillus succinogenes*, *Mannheimia succiniciproducens*, *Basfia succiniciproducens* and genetically engineered strains *E. coli* and *Y. lipolytica* (Table 4).<sup>132,133</sup> The three different biochemical pathways for SA production are: oxidative tricarboxylic acid (TCA) cycle, reductive branch of TCA cycle, and the non-frequent pathway is glyoxylate pathway (Figure 6B).<sup>134</sup>

*Actinobacillus succinogenes* and *Basfia succiniciproducens* are the most evaluated and predominant native SA producing strains, with ability to utilise either pure sugars or LCB hydrolysates.<sup>135</sup> *Actinobacillus succinogenes* 130Z, a natural SA producer, was immobilized and continuously fed with xylose-rich hydrolysate



from corn stover and generated 39.6 g/L SA, with yield and productivity of 0.78 g/g, and 1.77 g/L/h, respectively.<sup>136</sup> In another study, when the same strain was cultivated using dilute acid pretreated corn stover hydrolysate, SA titer, yield and productivity of 42.8 g/L, 0.74 g/g and 1.27 g/L/h was obtained, respectively.<sup>137</sup> Pateraki et al. (2016) cultivated *A. succinogenes* and *B. succiniciproducens* on mixed sugar feedstock (synthetic solution) containing 72% xylose, 12.2% galactose, 10.9% glucose, 4.2% mannose, and 0.1% arabinose. The SA titer, yield and productivity achieved with *A. succinogenes* were 26 g/L, 0.76 g/g and 0.66 g/L/h, respectively. Similar results were obtained with *B. succiniciproducens*: 27.4 g/L, 0.69 g/g and 0.60 g/L/h.<sup>138</sup> In addition to single strains, microbial consortiums have been used for SA production. A microbial consortium containing *Thermoanaerobacterium thermosaccharolyticum* M5 and *A. succinogenes* 130Z were employed to utilize hemicellulosic derived sugars to produce SA. *T. thermosaccharolyticum* M5 strain has an ability to saccharify the LCB components, by secretion of extracellular enzymes like xylanase where xylose obtained was converted into SA by *A. succinogenes*. The consortium with consolidated bioprocessing approach was able to generate 32.5 g/L SA with yield of 0.39 g/g.<sup>139</sup>

*E. coli* KJ122 strain was previously modified to reduce the by-products by deleting the respective genes, and increased the SA yield, titers and productivity when cultivated on glucose and sucrose in mineral medium under anaerobic conditions.<sup>140</sup> The strain was observed to be defective in growth and SA production during cultivation on xylose medium. It was speculated that the major reason behind this could be energy limitations as transport and phosphorylation of 1 mole of xylose requires 2 moles of ATP, but only 1.67 moles of ATP are generated when xylose is biosynthesis of SA.<sup>141</sup> Thus, xylose as the sole carbon source, cannot provide efficient energy currency to the cell growth and development. Hence Khunnonkwao and associates deleted the *xyIFGH* (ATP dependent ABC Transporter), which is energy expensive xylose transporting transmembrane protein and the resultant mutant strain was subjected to adaptive evolution on xylose media. When the recombinant *E. coli* KJ12201-14T strain was cultured on a glucose and xylose mixtures, it utilized both the sugars and accumulated 84.6 g/L SA with yield and productivity of 0.86 g/g and 1.01 g/L/h, respectively.<sup>142</sup> An engineered *E. coli* strain YL104H, with deleted pathways for LA, ethanol and other byproducts was evaluated for SA production using corn-based liquor containing glucose and xylose in a ratio of 2:1. Alternative to strategy followed where ABC transporter was deleted, Zhang and associates attempted a process modification approach where intracellular ATP concentration was maintained by co-substrate fermentation with supplementing glucose and xylose in 2:1 ratio. The process resulted in accumulation of 61.66 g/L SA, with 0.95 g/L.h productivity.<sup>143</sup>

Bacterial strains are more sensitive to changes in the physiological pH and require continuous addition of neutralizing agents. The addition of neutralizing agents not only dilute the concentration of SA in the fermented broth but also convert organic acids into salt form which complicate the downstream processing and increase the production cost. On the other hand, yeast strains are more promising for SA production, as they have better tolerance and can withstand lower pH. In a study Prabhu and associates (2020) engineered *Y.*



*lipolytica* PSA02004 strain to utilize xylose as the sole source of carbon and energy by overexpressing the pentose phosphate pathway comprising XR, XDH and XK under a strong constitutive promoter. The recombinant strain accumulated 22.3 g/L SA using xylose-rich hydrolysate from SCB hydrolysate.<sup>144</sup>

#### 5.4 2,3-Butanediol

2,3-butanediol (BDO) is a 4-carbon diol, with applications in food, cosmetics, fuel-additive, agrochemicals, and pharmaceuticals. One of the major applications of BDO is production of methyl ethyl ketone (MEK), an organic solvent used in production of resins and lacquers.<sup>145,146</sup> Microbial physiology adaptation for BDO pathway is hypothesized to prevent intracellular acidification and balancing the reducing equivalents. Two moles of pyruvate undergo sequential oxidoreductive reactions to form BDO with  $\alpha$ -acetolactate, acetoin/diacetyl as intermediates as shown in Figure 6C.

In biological BDO synthesis, the main factor influencing the economy of the process is the substrate cost which accounts to 50% of the total production cost.<sup>147</sup> Microorganisms of different genera *Klebsiella*, *Lactobacillus*, *Enterobacter*, and *Bacillus* (Table 4) have been reported to accumulate large amount of BDO (50 – 120 g/L) from variety of renewable feedstocks like cane molasses, cane sugar, SCB, fruit and vegetable waste. In our recent study, we evaluated the performance of a mutant *Enterobacter ludwigii* strain on pure xylose, non-detoxified and detoxified xylose-rich hydrolysates obtained from the thermochemical pretreatment of SCB. During the fed-batch cultivation, the strain produced 71.1 g/L BDO using pure xylose with a conversion yield and productivity of 0.40 g/g, and 0.94 g/L/h, respectively. In case of non-detoxified and detoxified hydrolysates, BDO titers of 32.7 and 63.5 g/L with yield of 0.33 and 0.36 g/g, and productivity of 0.43 and 0.84 g/L/h, was achieved, respectively.<sup>148</sup> A study conducted by Wang and associates implemented a process engineering approach by optimizing the media components to improve the BDO titers and productivity using *Klebsiella pneumoniae*. The *K. pneumoniae* strain with optimal media components and physiological conditions could produce 42.7 g/L BDO with 95% theoretical maximum yields and 99% xylose sugar uptake efficiency.<sup>147</sup> Although *Klebsiella* is a known work horse in production of value-added chemicals, its resistance to xylose is not satisfactory, xylose concentration >70 g/L was observed to be inhibiting the growth and metabolic performance of the strain. A global transcription regulating sigma ( $\sigma$ ) factor encoded by *rpoD* gene was observed to improve the substrate consumption rate and metabolic behaviour in *E. coli* strains. Hence, to overcome the xylose mediated inhibition, *rpoD* gene was overexpressed in *K. pneumoniae* which caused increment in substrate tolerance up to 125 g/L xylose, and product tolerance by 200%. Xylose transport, glycerol-3-phosphate acyl transferase, and phosphate kinase genes were observed to be upregulated by 5.7, 2.2 and 3-fold respectively.<sup>149</sup>

To modulate the commercially viable *S. cerevisiae* for BDO production, the biochemical pathway for BDO should be overexpressed and biosynthetic pathways leading to byproducts (ethanol, acetic acid and glycerol) formation must be eliminated. Kim et al., 2015 constructed the BDO producing *S. cerevisiae* strain by





introducing BDO pathway and to this end,  $\alpha$ -acetolactate synthase (*AlsS*),  $\alpha$ -acetolactate decarboxylase (*AlsD*), from *B. subtilis*, endogenous BDO dehydrogenase (*BDH1*) and *NoxE* from *L. lactis* was overexpressed. Further, production of ethanol (*adh 1-5*) and glycerol (*gpd 1* and *gpd 2*) was blocked by deleting the relevant genes. The resulting engineered strain produced 72.9 g/L BDO with 0.41 g/g and 1.43 g/L.h yield and productivity, respectively, using glucose as the carbon source.<sup>150</sup> Extending the work, Later Kim and associates constructed a xylose assimilatory *S. cerevisiae* strain by overexpressing the *S. stipitis* transaldolase ( $S7P + G3P \rightarrow E4P + F6P$ ) and endogenous NADH preferring XR. The recombinant strain showed 2.1-fold increase in xylose consumption rate and 1.8-fold in BDO productivity. Further NOX and PDC1 genes from *L. lactis* and *C. tropicalis* were heterologously overexpressed resulting in BD5X-TXmNP strain. The fed-batch cultivation of resultant strain on xylose produced 96.8 g/L BDO with 0.58 g/L/h productivity.<sup>151</sup>

## 5.5 Ethanol

Ethanol/ethyl-alcohol/bioethanol is most widely used biofuel in transportation sector and offer several advantages such as higher-octane number, high combustion efficiency and increasing heat of vaporization. Bioethanol is less toxic, readily biodegradable and produces lesser air-borne pollutants in comparison to petroleum fuel and most promising alternative to gasoline. However, due to its hygroscopic nature, complete replacement of gasoline with ethanol is not possible as water vapours can corrode the engine.<sup>152,153</sup> Currently, ethanol is blended with gasoline at different levels (5-20%) across the globe. It has been found that the blended fuel cause substantial reduction in emission of hydrocarbons and greenhouse gases.<sup>154</sup> The commercial production of ethanol from various renewable feedstocks has gained significant interest due to its increased application as a fuel component in gasoline. In 2019, the global ethanol production was 115 billion litres (\$38.83 billion), and with CAGR of 1.77% the demand has been expected to increase to \$43.14 billion by 2025. (Global Ethanol Market - Forecasts from 2020 to 2025 (researchandmarkets.com)). Ethanol fermentation by *S. cerevisiae* is one of the oldest practices in Biotechnology. *S. cerevisiae* is the most promising cell factory for ethanol production and employed at industrial level. In current times, the yeast is used for generating ethanol from a variety of feedstocks.<sup>155</sup>

The ethanol production from xylose follows production of X5P through pentose phosphate pathway and further proceeds through EMP pathway. Pyruvate, final product of EMP pathway, is converted to ethanol through acetaldehyde as intermediate as shown in Figure 6D. However, *S. cerevisiae* lacks pentose assimilatory pathways and can generate ethanol from xylose only after introducing the enzymes connecting xylose to central carbon metabolism.<sup>156</sup> Even though few strains like *S. stipitis*, *P. fermentans*, *P. kudriavzevii*, and *Spathaspora* (*S. passalidarum*), are well-known xylose assimilating yeast, the processes are limited due to substrate and product mediated inhibition. For example, the strains *S. stipitis* and *S. passalidarum* on xylose fermentation resulted in maximum ethanol titers of 29.9 g/L and 25 g/L, with a conversion yield of 0.47 and 0.41 g/g, and productivity of 1.5 and 1.04 g/L/h, respectively.<sup>157-161</sup>



The possibility of xylose as the feedstock to produce ethanol was explored in *S. cerevisiae* and to this end, XR and XDH genes from *S. stipitis* were overexpressed in *S. cerevisiae*. The heterologous expression resulted in lower ethanol titer of 10.7 g/L with a conversion yield of 0.19 g/g, and xylitol titers and yield of 14.3 g/L and 0.26 g/g, respectively. In *Pichia* sp., there is a competition between ethanol and xylitol formation for carbon flux. The carbon flux towards xylitol synthesis can be reduced by altering the cofactor specificity. Xiong et al., 2013 expressed a mutant form of XR (K270R) in *S. cerevisiae* with higher specificity of XR for NADH than NADPH which resulted in a higher ethanol (0.38 g/g) and reduced xylitol yield (0.08 g/g).<sup>162</sup> Along with the ALE, the polyploidy was also considered as an accelerative solution for adaptation of yeast. In the process, either the native or mutant haploid strains are subjected to mating to produce diploid or triploid strains. These strains were observed to have improved phenotypic and genotypic characteristics compared to the parent strains. Using this approach, *S. cerevisiae* XR-K270R mutant strain diploids and triploids were produced by Liu and associates. Furthermore, the comparative analysis between the haploid, diploid and triploid strains, displayed better performance of triploid on dilute acid and alkali pretreated corn cob and corn stover hydrolysates resulting in maximum ethanol production yield of 87.3%, whereas diploid strain yielded 76.2% ethanol.<sup>163</sup> Recent discovery of non-conventional yeast *S. passalidarum*, with xylose fermenting ability and possessing NADH dependent XR, could provide alternative research focusing on heterologous expressions rather than protein or cofactor engineering of known XR from *S. stipitis*.<sup>157</sup> Further research towards expression of XR and XDH from *Spathaspora* sp., could result in increased ethanol titers and yields in commercial yeasts.

### 5.6 n-Butanol

n-Butanol is a four-carbon straight chain alcohol and is considered better biofuel than ethanol due to high octane number, higher heating value, lower volatility, ignition problems, low miscibility with water and higher viscosity.<sup>80</sup> In a chemical approach, aldol condensation (oxo process) can produce n-butanol by hydroformylation and hydrogenation of propylene. In the biological route, n-butanol is the part of acetone-butanol-and ethanol (ABE) fermentation and *Clostridium* sp. are well known cell factories with ABE fermentation (Figure 6E).<sup>164,165</sup> However, the bio-butanol production suffers from low titers, yield, and product mediated inhibition. Clostridiums strains can naturally ferment xylose into n-butanol via ABE fermentations. *C. beijerinckii* could accumulate 26.3 g/L ABE with a yield of 0.44 g/g using Ca(OH)<sub>2</sub> detoxified xylose-rich corn stover hydrolysate.<sup>166</sup> Although the n-butanol yield is 20% lower than the ethanol, the energy generated from n-butanol is 32% higher than ethanol.<sup>167</sup> Currently with the available titers and yield, the cost of biobutanol production is around \$1.8/L, but further optimization of the biocatalysts, process conditions could reduce the production cost to \$0.6/L which is comparable to gasoline and other fossil fuels.<sup>167</sup> Jiang and associates implemented the process of consolidated bioprocessing (CBP), where a xylan degrading and n-butanol producing strain *Thermoanaerobacterium* sp. M5 was evaluated. The strain was able to grow at 55 °C, with efficient expression of xylanase, β-xylosidase and alcohol dehydrogenase for the conversion of xylose to n-





butanol through ABE fermentation.<sup>168</sup> As *Thermoanaerobacterium* sp. M5 strain has efficient xylan degradation efficiency, a co-cultivation strategy was investigated along with solventogenic strain *C. acetobutylicum* N4. The co-cultivation of these strains resulted in 13.3 g/L n-butanol with yield of 0.19 g/g.<sup>169</sup> Supplementing a crude hemicellulosic hydrolysate may be toxic to the microbial cells in the initial lag phases, hence in a study, *C. saccharoperbutylacetonicum* DSM 14923 after growing on sugarcane molasses for 24 hours, hemicellulosic hydrolysate was added into the media resulting in 10 g/L butanol, with yield and productivity of 0.31 g/g and 0.14 g/L/h, respectively.<sup>164</sup>

### 5.7 Polyhydroxybutyrate (Polyhydroxyalkanoates)

Polyhydroxyalkanoates (PHA) are the hydroxy alkanolic polyesters which are stored as intracellular granules in various prokaryotic microorganisms and are accumulated when carbon source is in surplus along with limitation of a key nutrient.<sup>170,171</sup> Although the primary function of these polyesters are the storage of carbon and energy, they also play a role in preventing the microbial cell from stress. Poly-3-hydroxybutyrate (PHB) and its derivatives like PHB-co-3-hydroxyvalerate (PHB-co-HV),<sup>171</sup> polylactate-co-3-hydroxybutyrate (PL-co-HB),<sup>172</sup> and 4-hydroxyhexanoate are type of PHA produced by prokaryotes (Figure 6F). PHA's have ample applications in the field of nanotechnology, drug delivery, medical prosthetics etc. Although PHB is the well-known and characterized, its brittle and crystalline structure limits its industrial relevance, but its derivatives like PHB-co-HV have impressive biomedical applications. A halophilic *Bacillus* sp., isolated from mangrove soil was observed to utilize wide range of carbon sources. The strains could accumulate PHB-co-HV up to 73% of biomass weight on xylose rich acid hydrolysates of sugarcane trash, under optimal conditions.<sup>171</sup>

*Burkholderia sacchari*, an industrially viable strain for the production of xylitol, xylonic acid and PHB, was engineered by overexpressing the xylose transporters (*XylE*, and *XylFGH*), metabolic genes (*XylA*, and *XylB*), and the regulatory gene (*XylR*). The engineered *B. sacchari* strain showed 55, 77.3 and 71% improvement in the growth rate, polymer yield and cell dry weight, respectively.<sup>170</sup> As explained in section 5.3., *E. coli* strain was engineered to hydrolyse xylan fraction of hemicellulose by heterologous overexpression of  $\beta$ -xylosidase and an endoxylanase, further the saccharified xylose was converted to PLA-co-HB. The resulting strain on xylan based production medium with additional pentose sugar as co-substrate increased the polymer yield up to 37% in comparison to the strain cultivated on pure xylose as sole carbon source.<sup>173</sup> In a lignocellulosic biorefinery, thermophiles are of utmost importance due to the benefit of simultaneous saccharification and fermentation as most of enzymes utilized for hydrolysis of hemicellulosic and cellulosic residues are active around 50 °C. The thermophilic bacterium *Schelegella thermodepolymerans* DSM 15344 is a natural polymer degrading microorganism with optimal growth at 55 °C. The genome mapping revealed a conserved PHA biosynthesis pathway, with 70 -76% similarity to the model PHA accumulating microorganism *Cupriavidus necator* N-1. The Interesting feature identified in *S. thermodepolymerans* strain is accumulation of more PHB on xylose (54%) in comparison to glucose (37%) as carbon source.<sup>174</sup> The interesting and highly



investigated strain for PHA production is *Ralstonia eutropha* which lacks the ability to metabolize xylose. The recombinant *R. eutropha* strain expressing the *E. coli XylAB* genes was able to accumulate 33.7 g/L PHB which is 79% weight of biomass, and the same strain when cultured on the hydrolysate solution of sunflower stalk consisting of 16.8 g/L glucose and 5.9 g/L xylose, resulted in production of 7.86 g/L PHB corresponding to 72.5 % CDW.<sup>175</sup> As PHB accumulation in the microorganism is growth dependent, the optimal conditions for the cell growth would be favouring PHB accumulation. A new isolate *B. megaterium* J-65 was able to accumulate 35 % CDW under optimal conditions with 2% xylose as the sole carbon source.<sup>176</sup> Supplementing pretreated corn husk hydrolysate along with nitrogen deficient production media to *B. megaterium* could accumulate 57.8% PHB which is almost 3-fold higher than on glucose as sole carbon source.<sup>177</sup>

In the earlier years, the researchers were more focussed towards valorisation of both the carbohydrate fractions of lignocellulosic biomass to ethanol. But lately, the trend has changed, and diverse product portfolio is preferred as it has been found more profitable as compared to targeting single product. Particularly, in the last five years the researchers have attempted to integrate the developed process modules with the techno-economics to understand the benefits associated with holistic utilization of all biomass components. For instance, Ou et al. (2021) showed that if 1500 tonnes of miscanthus was processed for sugar production per day, the minimum sugar selling price (MSSP) was \$446/tonnes.<sup>178</sup> However, when the xylose stream obtained after auto-hydrolysis was diverted for xylitol production, the MSSP was reduced to \$347/ tonnes. Similar observations were made by Giuliano et al., (2018) who found that if only cellulosic ethanol was targeted from steam exploded corn stover, the payback ethanol price was €1.62/kg.<sup>179</sup> But when the hydrolysed xylose stream was bio-transformed to xylitol, it reduced the overall cost of ethanol by 50.9%. In yet another study, xylitol co-production could raise the profitability of cellulosic ethanol by 2.3-folds during sugarcane biorefining, when fed-batch strategy fermentation was adopted.<sup>180</sup> Lately Ranganathan (2020) showed that when glucose derived from rice straw was used for ethanol production, but xylose was kept intact, the cost of ethanol was \$0.627/L. But when xylose was converted to furfural and lignin was upgraded to biochemical its cost reduced to merely \$0.25/L.<sup>181</sup> Thus, all these recent studies give a fair indication on how xylan/xylose valorisation can augment carbohydrate economy and increase the profitability of LCB-based biorefinery. The researchers are relentlessly working towards accelerating the biotechnological production of some bio-based and commercially important chemicals through genetic and protein engineering approaches as shown in Table 5.

## 6. Exploring the efficiency of multiple xylose assimilatory pathways for carbon flux towards SA and biomass production using the established genome scale models.

Small scale metabolic networks were constructed by retrieving information from genome scale metabolic models. Xylose assimilation pathways were incorporated into the metabolic network of *C. glutamicum*, *E. coli*, *A. succinogenes* and *Y. lipolytica*. Elementary flux mode analysis was implemented to elucidate optimal



pathways for producing biomass or succinic acid (SA) through different xylose assimilation pathways.

Theoretical maximum yields are summarised in the Table 6.

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### 6.1 *Corynebacterium glutamicum*

*C. glutamicum* is a well-known industrially relevant bacteria that is widely engineered to produce value-added products from wide range of carbon sources.<sup>182</sup> Xylose isomerase pathway (XI) was previously implemented into *C. glutamicum*.<sup>183</sup> which showed 30% of theoretical maximum yields of succinic acid from xylose. As seen from Table 6, XI and XR-XDH pathways has the potential to produce yields equivalent to that on glucose on carbon basis. Theoretical maximum yields of up to 80% can be achieved when Weimberg (WB) pathway is used while lowest possible maximum yields of 40% is observed with Dahms pathway. Optimal routes using the different xylose assimilating pathways are shown in Figure 7. As seen from the Figure 7, XR-XDH and XI show similar optimal routes for SA production and about 2 mol/mol of O<sub>2</sub> demand is observed for both the pathways. WB pathway seems to be the most efficient route that can reach maximum yields of 1mol SA/ 1mol xylose. This is attributed to alpha-ketoglutarate that is generated in the upper xylose assimilation pathway which directly enters the TCA cycle. As seen, biomass yields are significantly lower compared to XI and XR-XDH pathways (~20%) in both the WB and Dahms pathways. When Dahms pathway is used only 40% of carbon can be theoretically converted to SA under non-biomass production conditions. Under non-biomass conditions which would be normally implemented for succinic acid production (i.e. dual fermentation mode), surplus ATP must be replenished which is seen as output of ATP for maintenance purposes. Futile cycle could be generated which can replenish this surplus ATP under non-biomass production conditions. NADPH required for xylose assimilation via the XR-XDH pathways is mainly supplied by the isocitrate dehydrogenase. Optimal succinate production modes were also observed (data not shown) where glyoxylate cycle can be active and malic enzyme could be providing the required NADPH. XW pathway was implemented previously in *C. glutamicum*,<sup>184</sup> which showed growth inhibition due to accumulation of xylose 5-phosphate. As per the advantage of using the XW pathway is to prevent loss of carbon via CO<sub>2</sub> production, there is about 40% less CO<sub>2</sub> being produced and 25% less O<sub>2</sub> demands in comparison to XR-XDH and XI pathways. The XD pathway loses about 80% more carbon in the form of CO<sub>2</sub> and demands 30% more O<sub>2</sub> compared to the XR-XDH and XI pathways.

### 6.2 *Escherichia coli*

*E. coli* is more suitable for achieving higher biomass yields from xylose as seen in Table 6 due to its wider flexibility. The two transhydrogenase demonstrate their advantage in the *E. coli*'s added metabolic flexibility. About 8% more biomass can be achieved on all the different pathways in *E. coli* compared to *C. glutamicum*. Similar theoretical maximum yields of succinate were observed in *E. coli* when compared to *C. glutamicum*. *E. coli* naturally harbours the XI pathway which enables it to assimilate xylose naturally.<sup>185</sup> Several anaerobic strategies and metabolic engineering routes have been reported for enhanced succinic acid production in *E.*



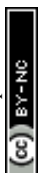
*coli* on different carbon sources.<sup>186</sup> Only aerobic related succinic acid production strategies are depicted in Figure 8. Under anaerobic conditions, pyruvate carboxylase or PEP carboxykinase overexpression together with succinate dehydrogenase deletion were proven to be efficient targets for enhanced succinic acid production implementing mainly the reductive TCA cycle. The shown optimal strategies using xylose are under aerobic conditions. As seen from the optimal strategies, owing to the flexibility of metabolism i.e. balancing reducing equivalents in *E. coli*, strategies were observed for both reductive as well as oxidative TCA cycle routes. As seen in Figure 8, both the XR-XDH and XI pathways rely on the KDPG pathway and NADPH is mainly generated via malic enzyme for the XR-XDH pathway. Excess NADPH is converted back to NADH in the XI pathway via the soluble transhydrogenase. Similar to that observed in *C. glutamicum*, the XW pathway demands less O<sub>2</sub> per mol xylose assimilated and produces less CO<sub>2</sub> with a theoretical maximum yield of 1 mol succinate/mol xylose. XW and XD pathways were not implemented in *E. coli* to produce succinic acid till date. When XD pathway is implemented a potential overexpression target would be the glyoxylate cycle, but as shown in Figure 8, higher O<sub>2</sub> demands and carbon loss in the form of CO<sub>2</sub> will make this pathway inefficient to produce succinate from xylose.

### 6.3 *Actinobacillus succinogenes*

The acid tolerant strain *A. succinogenes* is known for its high production capacity of succinic acid. This facultative anaerobic bacterium can assimilate both C6 and C5 sugars. Certain studies also showed that higher biomass and succinic acid production was observed when CO<sub>2</sub> and/or H<sub>2</sub> are supplied additionally.<sup>187</sup> *A. succinogenes* does not harbour a complete TCA cycle and it is auxotroph of glutamine, methionine and cysteine. A detailed flux analysis has been performed on *A. succinogenes* giving insights into its metabolism.<sup>188</sup> It is also identified that it does not comprise the glyoxylate cycle. As shown in Figure 9, both the XR-XDH and the XI pathway yields are 1 mol succinate per mol xylose. Interestingly most of the optimal succinic acid production pathways showed to produce ethanol as a by-product indicating excess NADH to be replenished. NADPH is mainly supplied by the PP pathway for the XR-XDH based xylose consumption. On xylose, the transhydrogenase present in *A. succinogenes* would be a very essential reaction in balancing NADH and NADPH. To incorporate XW pathway into *A. succinogenes*, alpha-ketoglutarate dehydrogenase, and a succinyl-CoA synthetase have to be expressed. This could also eliminate the glutamine auxotroph. For the XD pathway, the glyoxylate cycle genes, isocitrate lyase and malate synthase must be expressed which would enable the uptake of glyoxylate produced from the XD pathway.

### 6.4 *Yarrowia lipolytica*

Owing to its well-known potential in producing lipids, citric acid and other value-added products, *Y. lipolytica* has been demonstrated for its effectiveness in producing succinic acid from xylose.<sup>144</sup> Availability of genetic tools and engineering for wider substrate spectrum, tolerance at reduced pH and flexible metabolism makes



this yeast a potential cell factory for succinic acid production. As depicted in Figure 10, optimal production of succinic acid can reach maximum yields of 1 c-mol succinic acid per c-mol xylose in XR-XDH and XI pathways. The PP pathway is shown to be the main NADPH supplying route for xylose uptake in the XR-XDH pathway which is also observed during lipid production.<sup>189</sup> O<sub>2</sub> demand is significantly lower per mol xylose when compared to the bacterial O<sub>2</sub> demands for succinic acid production. Interestingly, the XW pathway is not the optimal pathway for producing succinic acid in *Y. lipolytica* compared to what has been observed in *E. coli* and *C. glutamicum*. Carbon loss in the form of CO<sub>2</sub> is observed in XW pathway in *Y. lipolytica*. As also observed in bacterial XW and XD pathway demand higher O<sub>2</sub> compared to XR-XDH and the XI pathways. Addition of external CO<sub>2</sub> to *A. succinogenes* and this CO<sub>2</sub> being consumed via the PEPC or PYC will reduce the overall succinic acid yield (0.56 c-mol/c-mol). Reductive TCA cycle is probably the best option, but optimal strategies can also be envisaged as observed in *E. coli* or *C. glutamicum* optimal pathways. As observed previously, enhanced flux through the PP pathway especially for the XR-XDH pathway would be beneficial for enhanced xylose uptake and succinic acid production.

## 7. Challenges for xylose based bioproduction.

Xylose, a renewable sugar with greater potential, but overlooked due to in-efficient metabolic capabilities and process limitations. Although various bacteria, yeast and fungi do assimilate xylose, they utilize in a hierarchical fashion, and these are the bottlenecks that are limiting the commercial perspective.

### 7.1 Inefficient transport of pentose or absence of xylose specific transporters in the microbial cell

Xylose transport into the bacteria, yeast or fungal cells through native or heterologous transporters as explained in Section 4.1. In all these microorganisms, the major limiting factor, and a prerequisite objective to be addressed is xylose uptake rate or transport efficiency of the individual cell. Most of the attempts to improve the xylose utilization efficiency of native transporter or heterologous expression were made in *E. coli* and *S. cerevisiae*, but still the results are incomparable to glucose uptake rates. For example, the glucose uptake rate in *S. cerevisiae* cell is 0.085 C-mol/g<sub>CDW</sub>/h, whereas for xylose it is approximately three-times slower (0.027 C-mol/g<sub>CDW</sub>/h). In recent years, with the availability of advanced systems/synthetic biology tools and metabolic engineering techniques, there lies a scope of either engineering native promoters or investigating novel high efficiency xylose transporters through genome mining.

Relative modifications in genotype and phenotype can be achieved by subjecting the microbial consortia under selective pressure.<sup>190</sup> Radek et al., 2017 developed automated and miniaturized ALE approach based on repetitive batch cultivations in microtiter plates. They subjected *Corynebacterium glutamicum pEKEx3-xyIXABCDCc* bearing Weimberg (WMB) pathway to ALE for improving xylose consumption. The evolved strain showed 260% increase in xylose consumption efficiency.<sup>191</sup> Overexpression of *S. stipitis Sut1* xylose transporter gene in *S. cerevisiae* improved xylose uptake rate and ethanol yield by 25





and 17%, respectively, whereas xylose assimilation was enhanced by 25 and 40% with introduction of *Arabidopsis thaliana* xylose transporter genes, *At5g17010* and *At5g59250*, respectively.<sup>192</sup> A large improvement (75%) in xylose transport was achieved with *Gxf1*, a MFS transporter identified from *Candida intermedia*.<sup>193</sup> This traditional xylose transporter displayed improved xylose uptake efficiency at lower xylose concentrations (~10 g/L) while at higher levels, the efficiency was reduced drastically. Later, comparative genome analysis of *C. sojae* revealed the presence of two xylose specific transporters encoded by *Cs3894* and *Cs4130* genes, exhibiting a substantial xylose uptake rate at concentrations up to 50 g/L.<sup>193</sup> The quest for novel and efficient xylose specific transporters and expression of those xylose transporters could allow rapid transport of xylose, bypass the glucose mediated repression mechanisms and enable simultaneous fermentation of mixed sugars.<sup>194</sup>

## 7.2 Glucose imposed carbon catabolite repression

After xylose transport, the next challenge with xylose-based cell factories is the phenomenon of carbon catabolite repression or glucose mediated inhibition which impedes the simultaneous consumption of xylose and glucose. So, first question comes into mind, why we need to supplement both glucose and xylose together? LCB is the most abundant material, and the abundance is so high that it can replace all the carbon coming from fossil sources. The production and process economics of LCB-biorefineries can be improved if the host strain can simultaneously utilize both glucose and xylose saccharified from the LCB. The three basic mechanisms mediating CCR has been explained in section 3.3 (Figure 4). As per EIIA<sup>Glc</sup> mediated catabolite repression, high levels of cAMP activate the expression of genes responsible for the metabolism of non-glucose sugars. In a study by Ammar et al., 2018 when the culture medium was supplemented with 5–10 mM cAMP the CCR was not observed in glucose – galactose co-fermentations, but 10 mM cAMP concentration was not enough to overcome CCR for simultaneous utilization of glucose and xylose.<sup>72</sup>

## 7.3 Regulation of intracellular xylose metabolism

After passing the gateway and co-substrate mediated repression, the next obstacle to address would be the slow rate of biochemical reactions using xylose and its derivatives as substrates making the overall process sluggish. That is why cell growth and metabolites production rates on xylose are slower in comparison to glucose. *S. cerevisiae* has been in commercial use and known to be best hexose utilizer, the strain lacks an active xylose utilization pathway and most of the studies were concentrated on heterologous expression of *S. stipitis* xylose assimilatory pathway in *S. cerevisiae*, but the results observed were not satisfactory.<sup>195</sup> *S. cerevisiae*, for example, has a specific growth rate of 0.25 h<sup>-1</sup> on glucose vs 0.05 h<sup>-1</sup> on xylose, a nearly 5-fold difference, that not only limits the biomass, but also influences cell physiology and metabolism. This indicates the insufficient understanding of metabolic network and to decode this problem, it is very important to have deep understanding of kinetics of xylose related reactions and underlying complex regulation as *in vivo* activity



is burdened with several types of regulations. Hence exploring the innate regulatory mechanisms of native and non-native xylose assimilatory microorganisms, and rational design and metabolic engineering leading to optimal metabolic flux and energy metabolism during the xylose assimilation is necessary.

#### 7.4 Maintaining the redox homeostasis

It is challenging for most of the biological processes to maintain redox homeostasis. Any deficiency of redox cofactors leads to expression of alternative metabolic pathways leading to by-product synthesis. In xylose oxidoreductive (XR-XDH) pathway, regeneration of NAD<sup>+</sup> is very important to direct the carbon flux into central carbon metabolism to allow smooth xylose assimilation. The imbalance between the enzymatic activities of XR and XDH results in NAD<sup>+</sup> limitation which leads to xylitol accumulation. Under aerobic conditions, microbial cell has ability to regenerate NAD<sup>+</sup>, whereas oxygen limited, or anaerobic conditions renders shortage of NAD<sup>+</sup> supply. Thus, maintaining optimal oxygen levels or alternative routes to generate NAD<sup>+</sup> without interfering the fermentation capability of the microbial strain is very much important to facilitate xylose metabolism. Carlos Roseiro and associates observed the relationship between the volumetric oxygen transfer coefficient ( $K_La$ ), a parameter which is reflection of ease of oxygen supply and the xylitol production in a yeast *Debaryomyces hansenii*. They found that increasing the  $K_La$  ( $h^{-1}$ ), from 0.2 to 1.8 caused improvement in the xylitol accumulation and an increase beyond 1.8, boosted the ethanol production.<sup>196</sup> Bonan et al., 2020 attained highest ethanol titers of 28.6 g/L with 0.31 g/g yield and 1.12 g/L/h productivity by *Spathaspora passalidarum* at  $K_La$  ( $h^{-1}$ ) of 45.<sup>54</sup> Hence, an ideal process engineering aspect would be to determine optimal  $K_La$  value which maximize xylose flux towards desired product with minimal or no secretion of xylitol and eventually benefiting the cell growth and product formation. Alternative approaches such as overexpression of NOX, modification of the cofactor specificity of XR, or expression of NADH specific XR over NADPH-dependent XR can alleviate problem of redox imbalance and replenish the flux towards central carbon metabolism.<sup>197</sup> Most of them have been attempted and only limited success has met so far.

#### 8. Conclusion and future perspective

Xylose is a readily available sugar with potential to serve as feedstock for biorefineries. For the economic viability of lignocellulose biorefineries, the efficient conversion of hemicellulosic sugars into value-added products is mandatory. Glucose-based commercially developed bioprocesses are prevalent while xylose-based are evolving at industrial scale. Recent developments in biomass pretreatment technologies have led the way to extract xylose from hemicellulosic fraction of plant cell wall with desired yields with low amount of plant cell wall inhibitors. In nature, the xylose metabolising microorganisms are scanty compared to glucose. Therefore, bioprospecting of novel microorganisms that could assimilate xylose separately or in combination with glucose with faster conversion rates will significantly promote efficiency of LCB-based biorefineries. However, the xylose uptake rates of the well-known xylose assimilating microorganisms is significantly lower



than glucose. Despite the exemplary developments made in xylose bioconversion, there are still several challenges which need to be fixed for the developing efficient microbial cell factories for high level manufacturing of biochemicals and biofuels. These challenges include the efficient xylose transportation into microbial cell, faster uptake & metabolism of xylose similar to glucose, continuous availability of redox cofactors for maintaining homeostasis, glucose repression during co-fermentations, feedback, substrate, and product mediated inhibition. Recent advancements in enzyme/metabolic/pathway engineering along with system/synthetic biology approaches have been employed to overcome these challenges but has met with limited success. Though, xylose-based bioproduction has shown significant progress in last few decades, many obstacles still need to be addressed to realize xylose as a feedstock at industrial level.

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## Legends to figures

**Figure 1:** (A) Structural components of Lignocellulosic biomass (LCB), (B) Illustration of composition of individual subunits of LCB and compositions of sugar and sugar acids in hemicellulosic fraction.

**Figure 2:** Catalytic routes for xylose conversion to value-added chemicals

**Figure 3:** Illustration of xylose transport inside the microbial cell and further dissimilatory pathways directing the carbon flux into central carbon metabolism. Abbreviations: DXD: Xylose dehydrogenase; XLA: Xylonate synthase; XAD: Xylonate dehydratase; KDXD: 2-Keto-3-deoxyxylonate dehydratase; KGSADH: Ketoglutarate semialdehyde dehydrogenase; XI: Xylose isomerase; XR: Xylose reductase; XDH: Xylitol dehydrogenase; X1K: Xylulose-1-kinase; X1PA: Xylulose-1-phosphate aldolase; DAL: 2-Keo-3-deoxyxylonate aldolase; GLX: Glyoxylate shunt; XLK: Xylulose-5-kinase; XPK: Phosphoketolase; PPP: Pentose phosphate pathway; EMP: Emden Meyerhof Parnas pathway; Weimberg pathway (blue); Isomerase pathway (Green); Dahms pathway (brown); XR-XDH pathway (yellow); Phosphoketolase pathway (Violet); Synthetic pathway (red).

**Figure 4:** Illustration of a possible mechanism for carbon catabolite repression A) Inducer exclusion, and B) cAMP mediated inhibition.

**Figure 5:** Transport of xylose into the microbial cell and further flux into central carbon metabolism

**Figure 6:** Biochemical pathways (A) Lactic acid, enzymes and genes involved: 1, lactate dehydrogenase (*ldh*). (B) Succinic acid, enzymes and genes involved: 1, acetyl-CoA synthetase (*acs*); 2, citrate synthase (*gltA*); 3, aconitase (*acnAB*); 4, isocitrate lyase (*aceA*); 5, pyruvate carboxylase (*pyc*); 6, malate dehydrogenase (*mdh*); 7, fumarase (*fh*); 8, fumarate reductase (*frd*) or succinate dehydrogenase (*sdh*). (C) 2,3-Butanediol, enzymes and genes involved: 1,  $\alpha$ -acetolactate synthase (*alsS*); 2, spontaneous reaction; 3, diacetyl reductase (*butA*); 4,  $\alpha$ -acetolactate decarboxylase (*aldc*); 5, butanediol dehydrogenase (*bdh*). (D) Ethanol, enzymes, and genes involved: 1, pyruvate decarboxylase (*pdc*); 2, alcohol dehydrogenase (*Adh*). (E) Butanol, enzymes and genes involved: 1, acetyl-CoA synthase (*acs*); 2, thiolase (*thl*); 3, 3-hydroxybutyryl-CoA dehydrogenase (*Hbd*); 4, crotonase (*Crt*); 5, butyryl-CoA dehydrogenase (*EtfAB*); 6, butyraldehyde dehydrogenase (*AdhE2*). (F) Polyhydroxybutyrate (PHB), enzymes and genes involved: 1, acetyl-CoA synthase (*acs*); 2, 3-ketothiolase (*PhaA*); 3, acetoacetyl-CoA reductase (*PhaB*); 4, PHB synthase (*PhaC*).

**Figure 7:** Theoretical maximum (optimal) production of SA via A) XR-XDH pathway B) XI pathway C) XW pathway and D) XD pathway in *C. glutamicum*. Values normalised to 100% xylose uptake rate (mmol/g\*h).

**Figure 8:** Theoretical maximum (optimal) production of SA via A) XR-XDH pathway B) XI pathway C) XW pathway and D) XD pathway in *E. coli*. Values normalised to 100% xylose uptake rate (mmol/g\*h).

**Figure 9:** Theoretical maximum (optimal) production pathways of SA via A) XR-XDH pathway B) XI pathway in *A. succinogenes*. Values normalised to 100% xylose uptake rate (mmol/g\*h).



**Figure 10:** Theoretical maximum (optimal) production of SA via A) XR-XDH pathway B) XI pathway C) XW pathway and D) XD pathway in *Y.lipolytica*. Values normalised to 100% xylose uptake rate (mmol/g\*h)

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Figure 1

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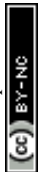
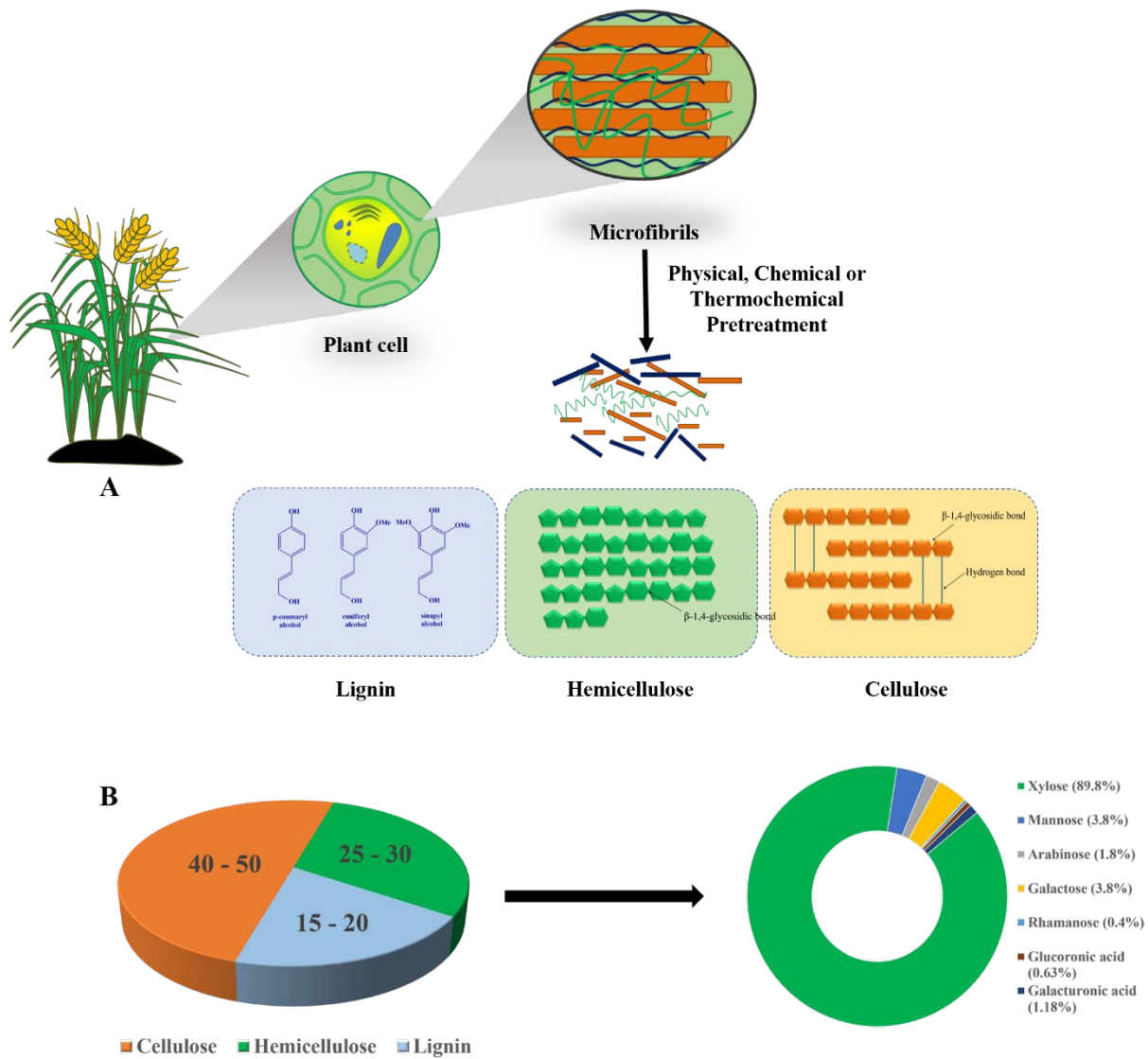


Figure 2

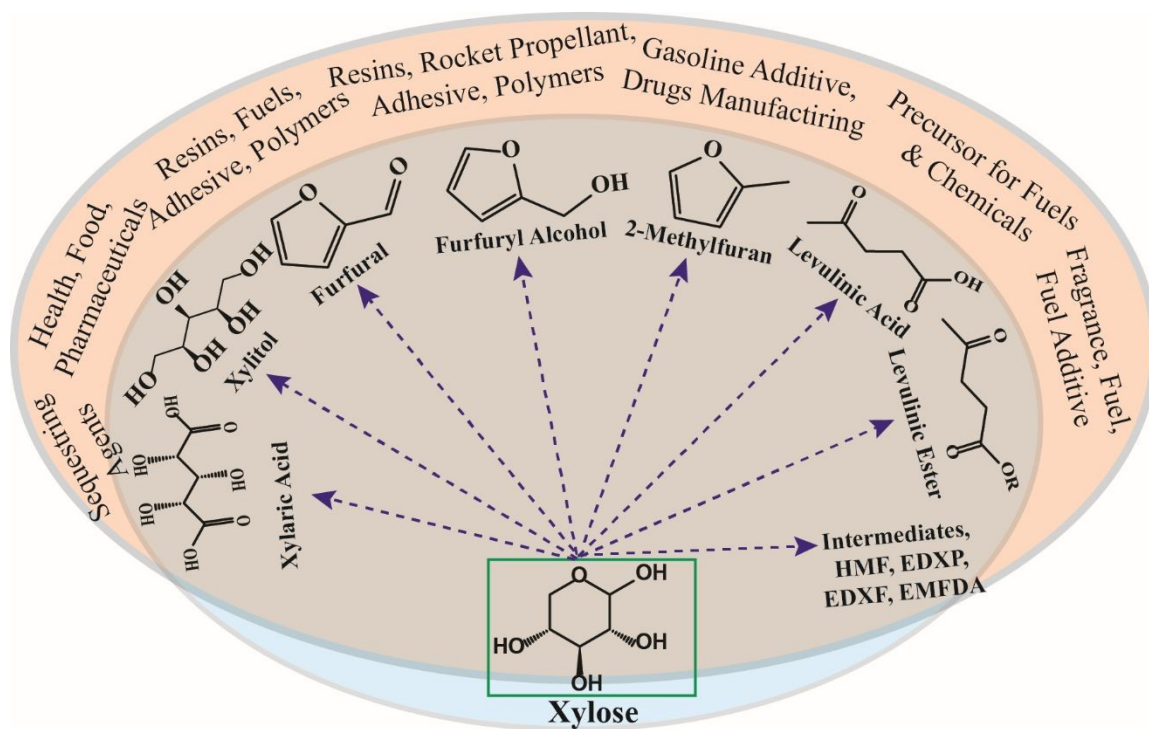
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Figure 3

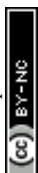
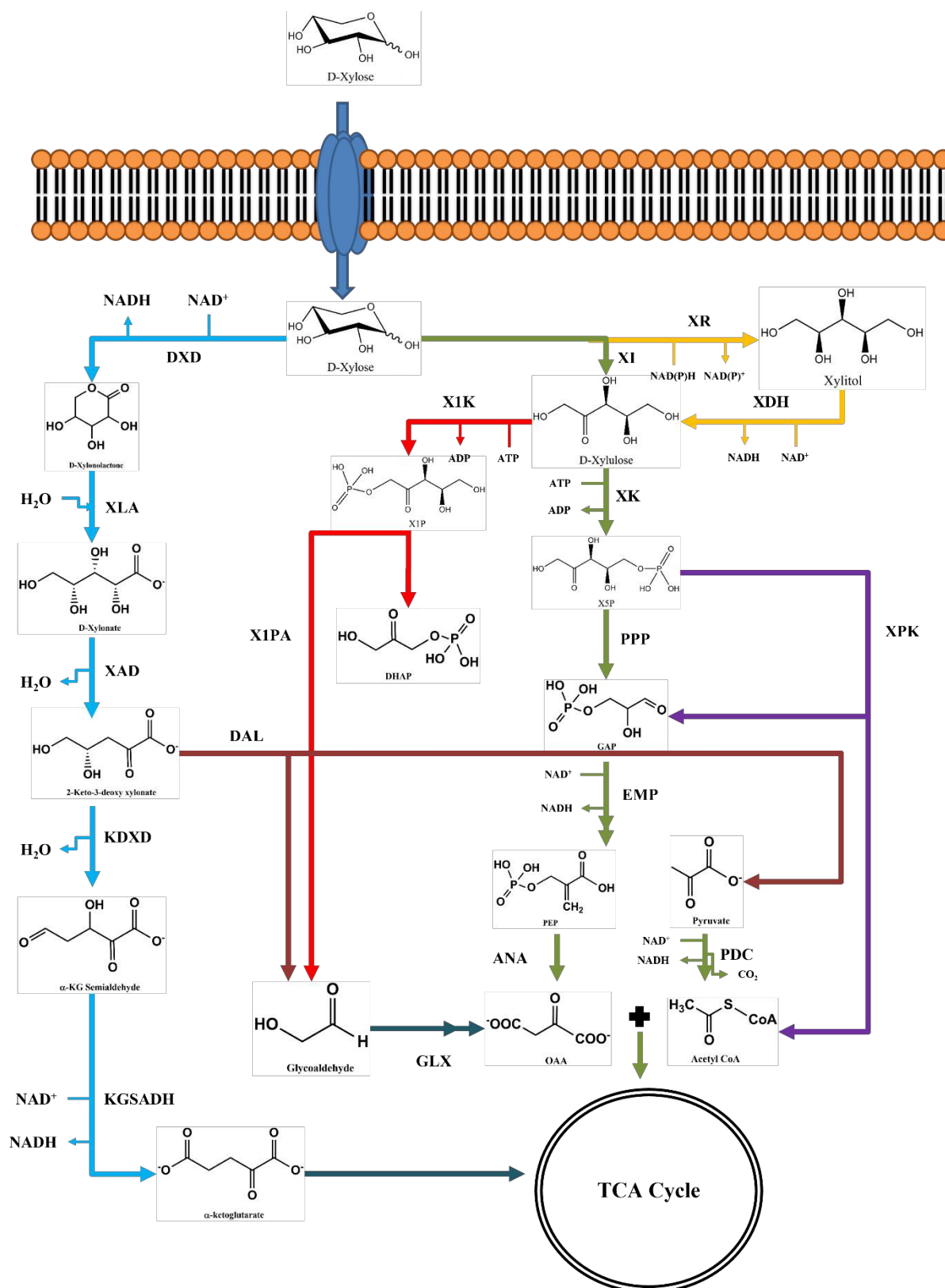
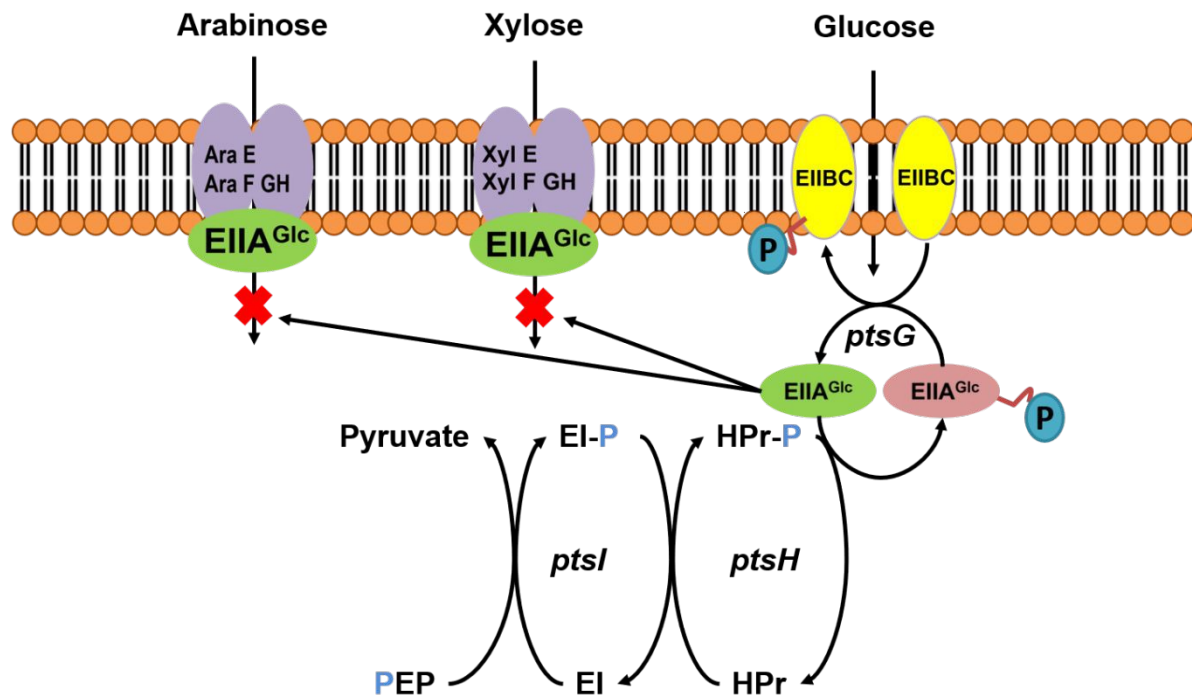
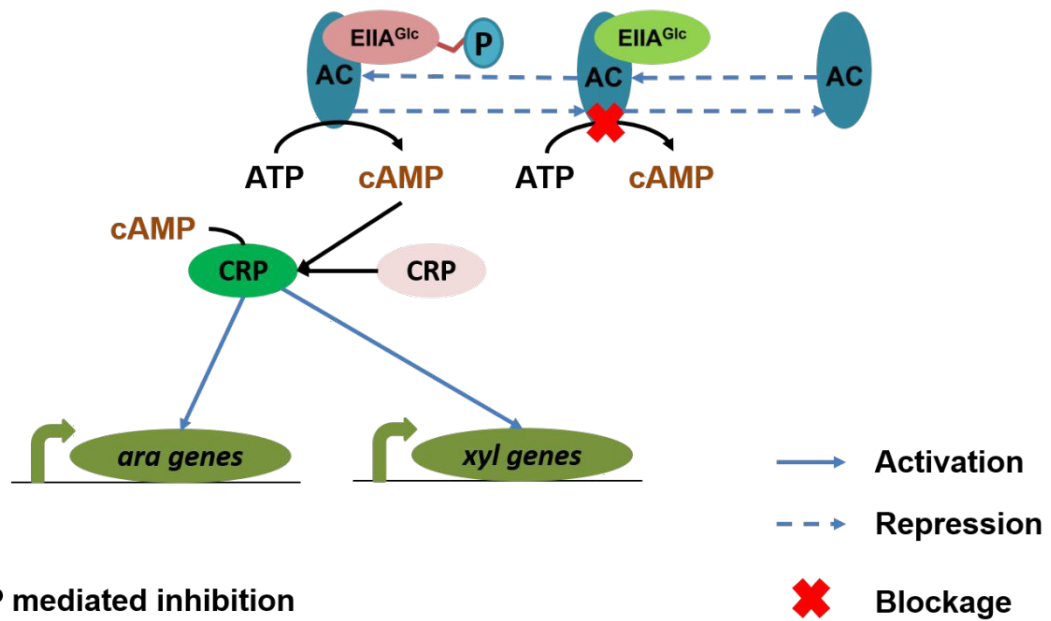
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Figure 4

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A) Inducer exclusion



B) cAMP mediated inhibition



Figure 5

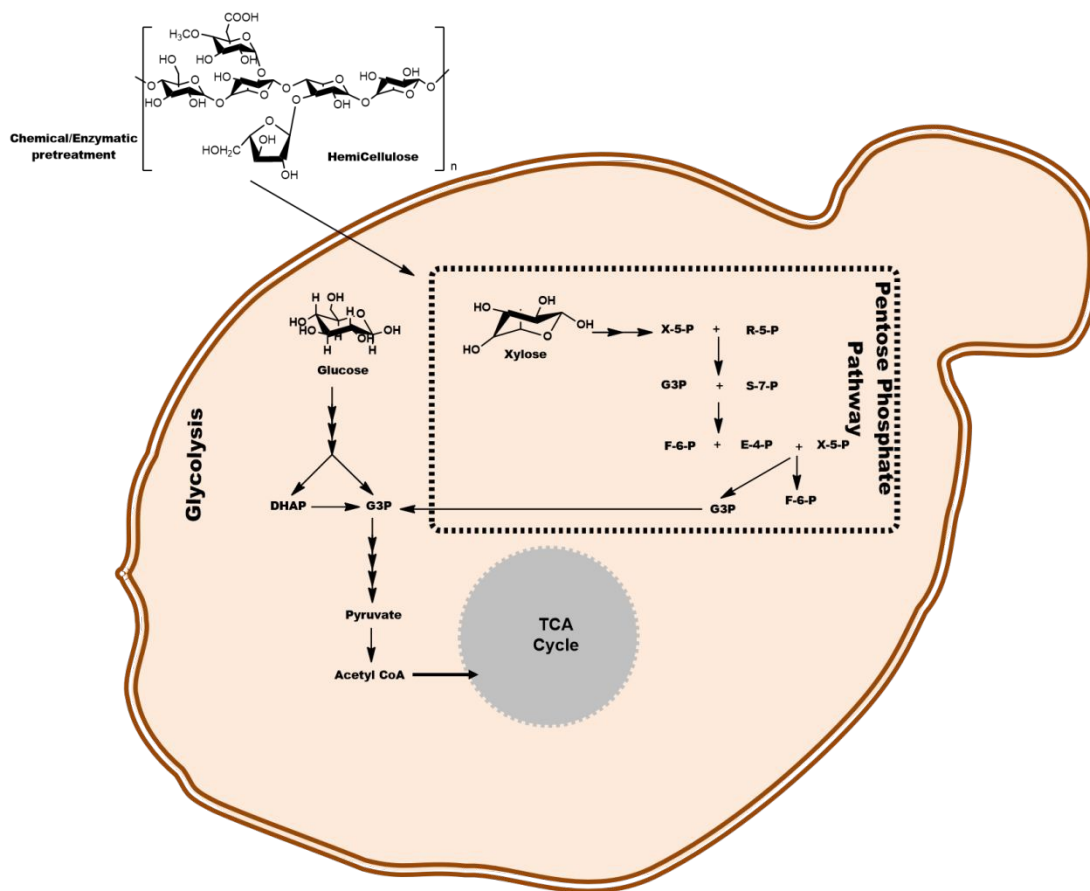


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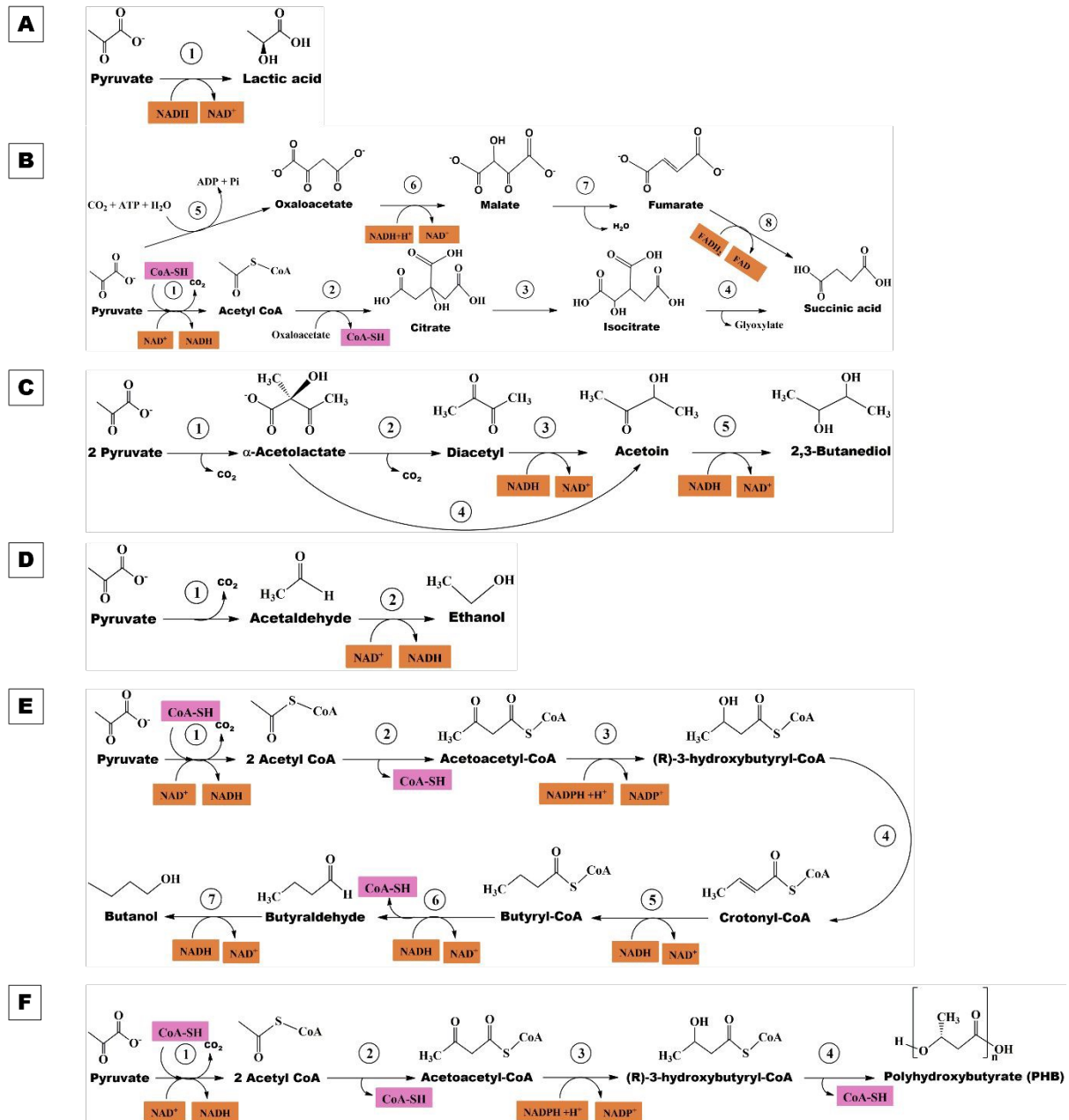
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Figure 7

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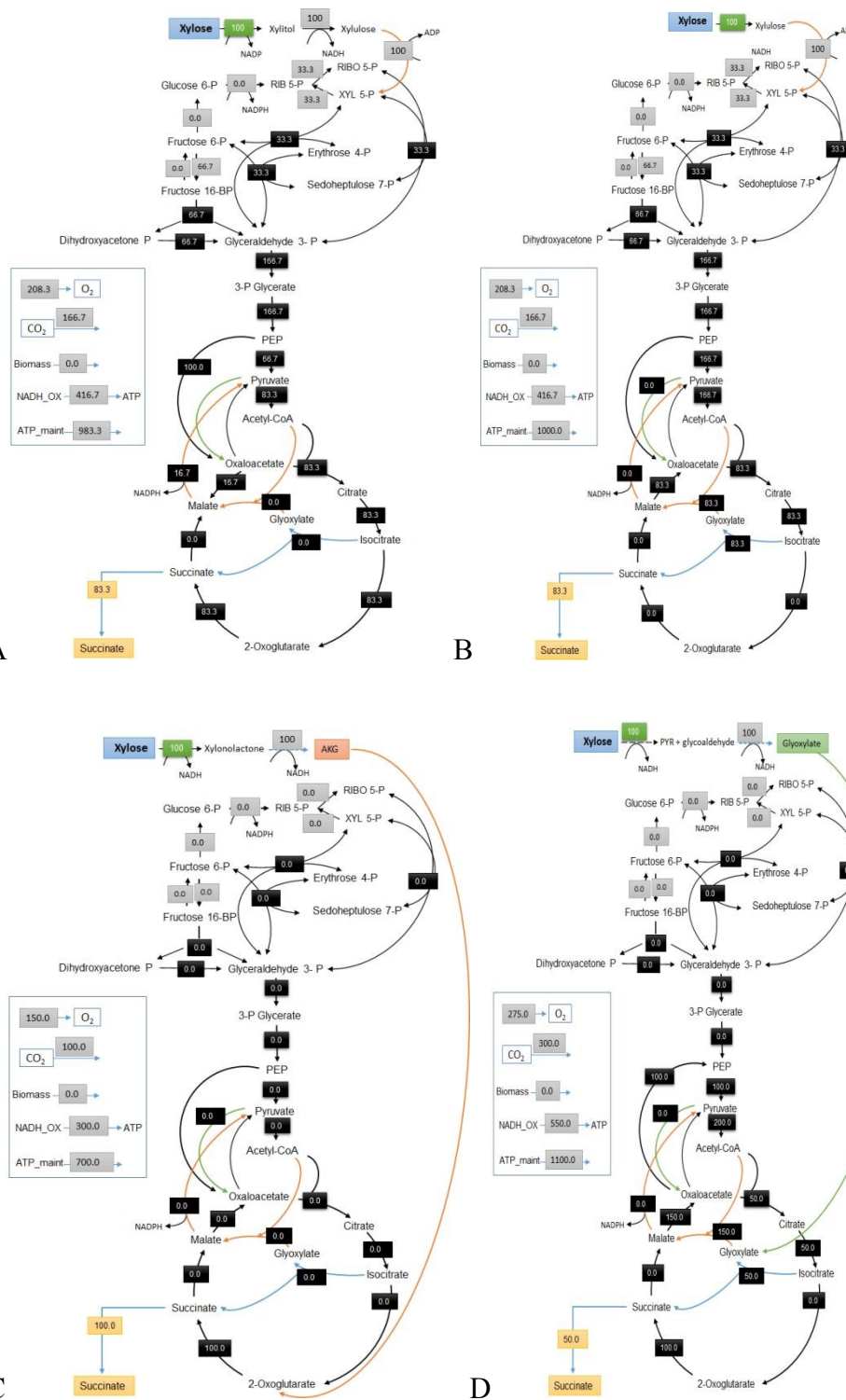


Figure 8

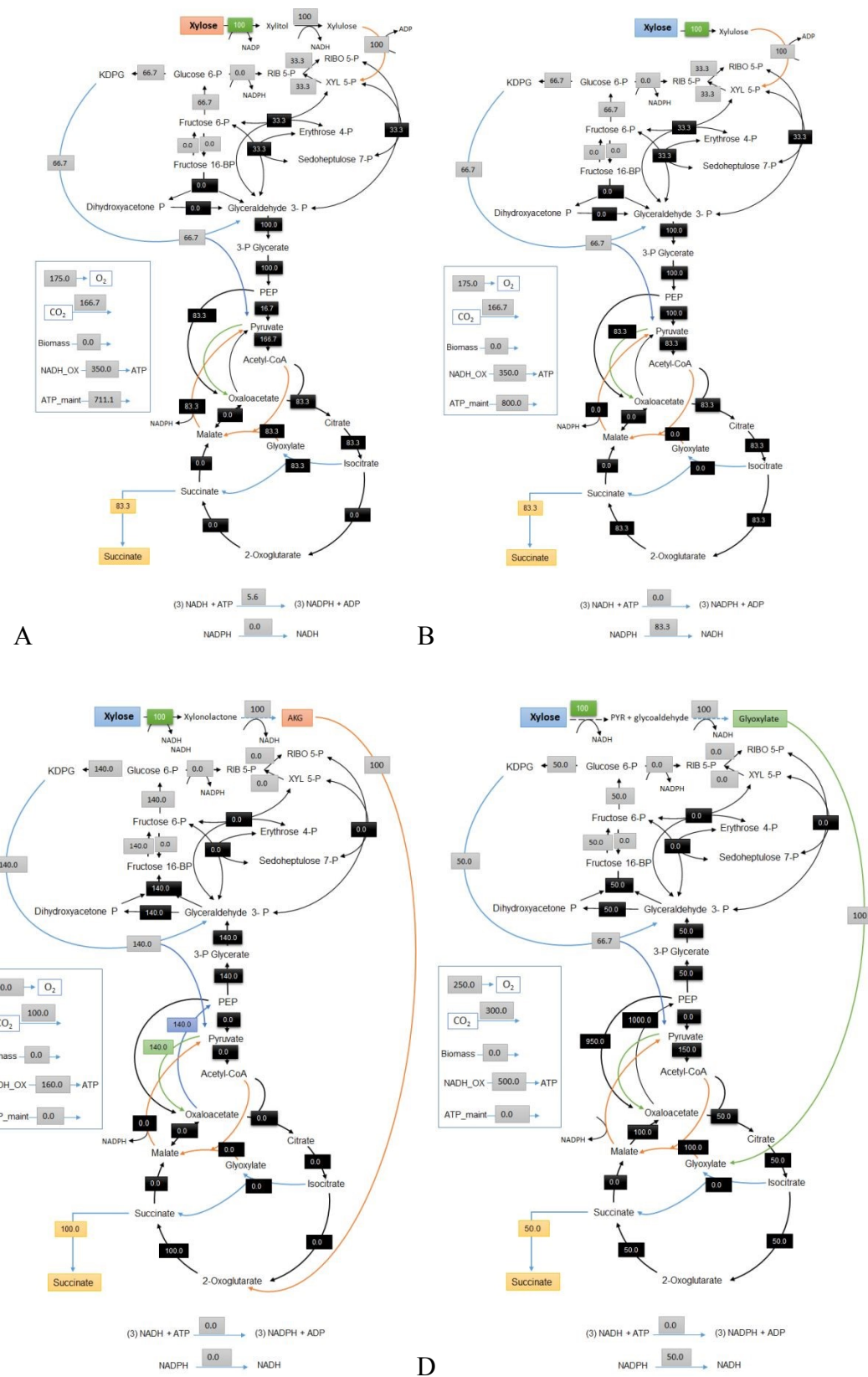
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Figure 9

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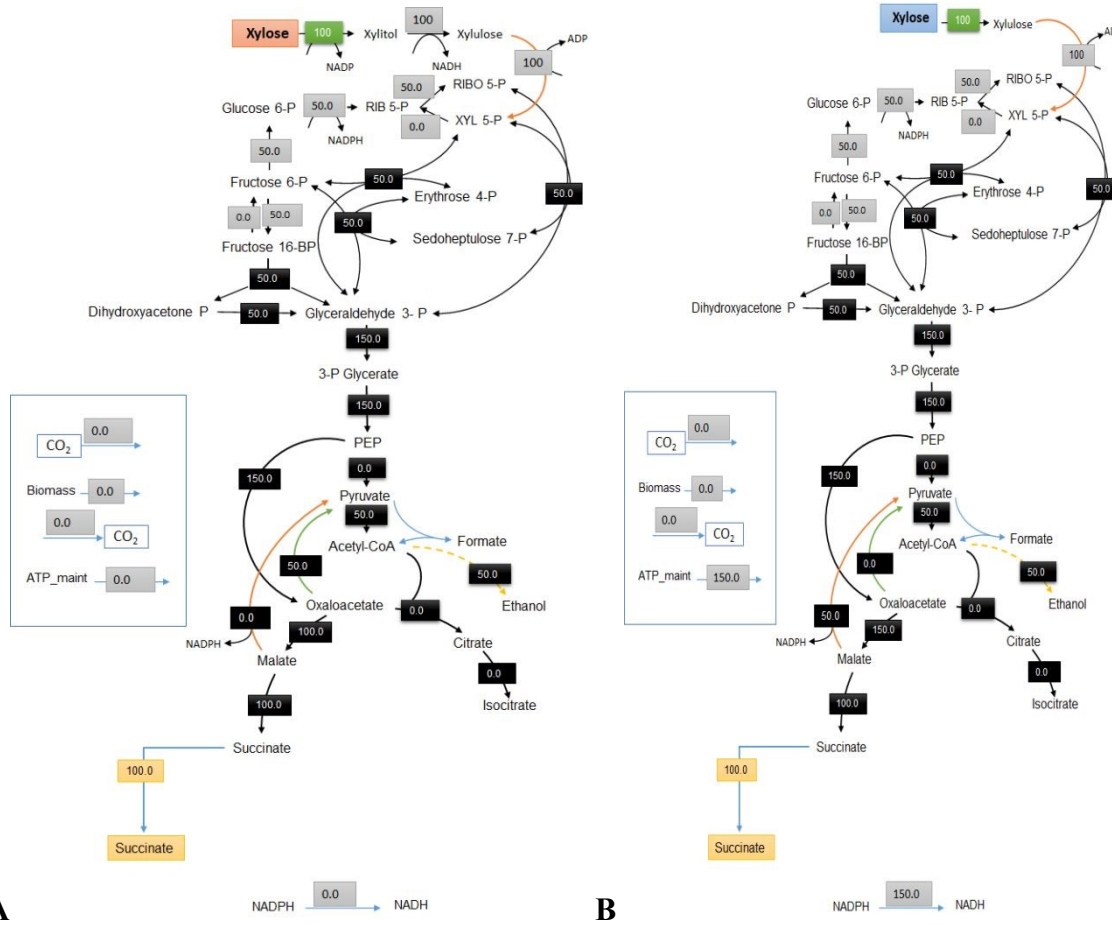
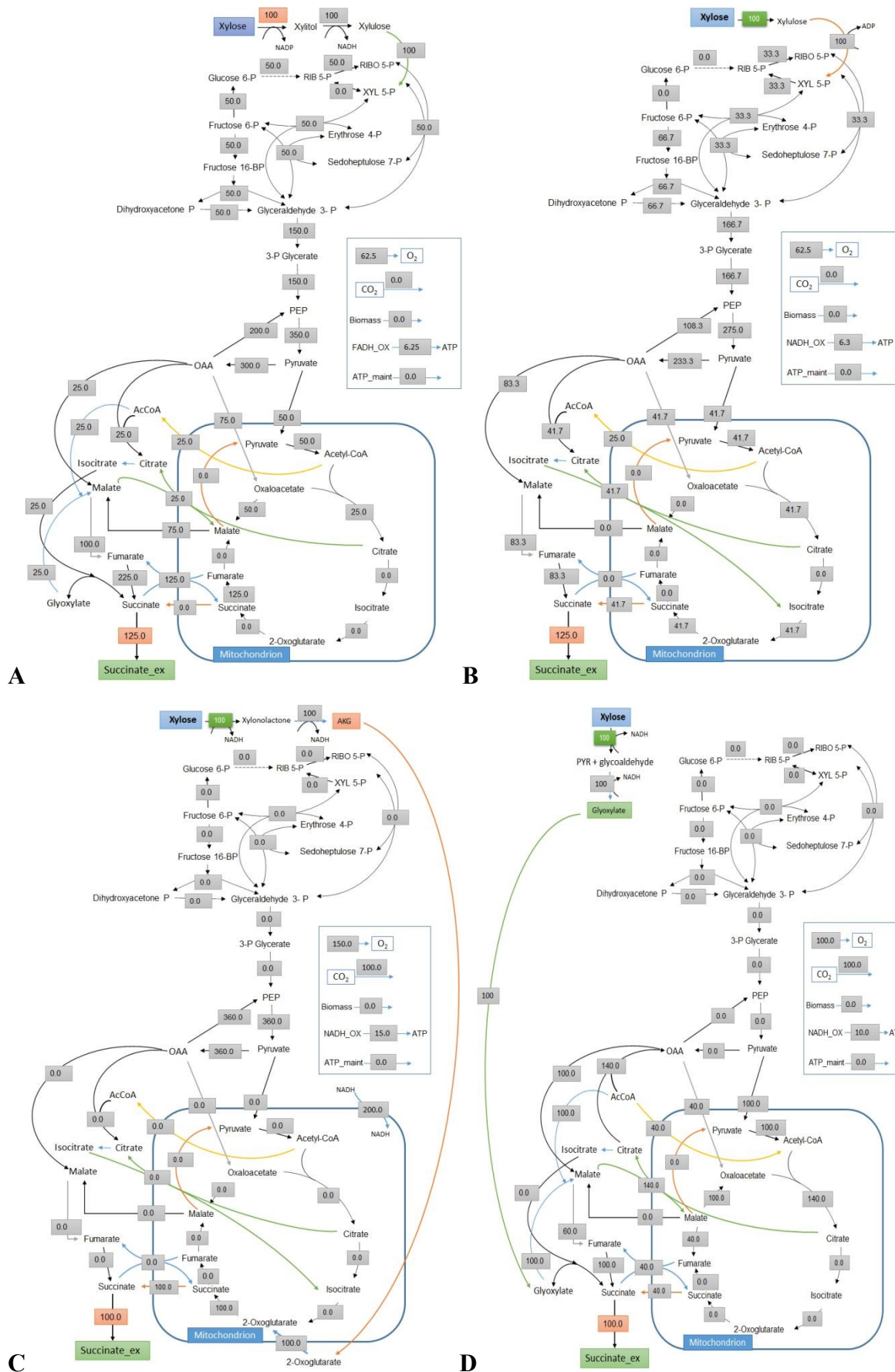


Figure 10

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**Table 1: State of the art showcasing pretreatment strategies leading to selective xylan hydrolysis**

| Type of LCB | Type of pretreatment                        | Pretreatment conditions  | Biomass Composition (%)           |                                  | Removal (%) |        | References |
|-------------|---|--|-----------------------------------|----------------------------------|-------------|--------|------------|
|             |   |  | Before pretreatment               | After pretreatment               | Xylan       | Lignin |            |
| Poplar      | DA  | Temp:170°C; Time: 8.5 min<br>H <sub>2</sub> SO <sub>4</sub> : 0.5% (w/w)     | Gln-57.9; HC-17.5;<br>KL-24.6     | Gln-74.2; HC-<2.0;<br>KL-25      | 99          | -      | <u>198</u> |
| MW          | DA  | Temp: 160°C; 27.5 min Oxalic acid: 0.5% (w/w)                                | Gln-41.8; HC-21;<br>TL:27.1       | -                                | 87.5        | -      | <u>199</u> |
| CS          | DA  | Temp:160°C; Time: 40 min<br>H <sub>2</sub> SO <sub>4</sub> : 0.5% (w/w)      | Gln-36.1; Xln-21.4;<br>TL-13.6    | Gln-31.1; Xln-1.6;<br>TL-13.1    | 92.5        | -      | <u>200</u> |
| SCB         | DA  | Temp: 140°C; Time: 8 min<br>H <sub>3</sub> PO <sub>4</sub> : 0.2% (w/v)      | Gln- 40.1; HC -27.5;<br>TL- 18.5  | Gln- 58.5; HC -1.8<br>TL- 29.05  | 96.5        | 14.8   | <u>201</u> |
| SG          | DA  | Temp: 160°C; Time: 30 min;<br>H <sub>2</sub> SO <sub>4</sub> : 1% (w/w)      | Gln- 33.5; Xln -22.7;<br>KL- 16.3 | Gln- 53.2; Xln -0.8<br>KL- 33.3  | 98.6        | 18.3   | <u>202</u> |
| WS          | DA  | Temp: 140°C; Time:90 min;<br>H <sub>2</sub> SO <sub>4</sub> : 0.5% (w/w)     | Gln- 43.2; Xln -24.4<br>KL- 20.8  | Gln- 59.1; Xln -2.4<br>KL- 30.7  | 91.5        | -      | <u>203</u> |
| CS          | HT  | Temp: 180°C  | Gln-36.1; Xln-21.4;<br>TL-13.6    | Gln-33.0; Xln: 5.4;<br>TL-13.5   | 74.9        | -      | <u>200</u> |
| CC          | HT  | Temp: 207°C  | Gln- 28.8; Xln -29.6<br>KL- 18.6  | Gln- 54.5; Xln -10.2<br>KL- 21.8 | 80.4        | 33.1   | <u>204</u> |
| SS          | LHW   | Temp: 220°C;Time: 5 min  | Gln- 33.13; HC- 26.2<br>KL- 18.2  | Gln- 56.7; Xln -2.0<br>KL- 37.0  | 96.5        | 6.6    | <u>205</u> |
| SCB         | H <sub>3</sub> PO <sub>4</sub> catalysed SE | Temp: 195°C; Time: 7.5 min;<br>H <sub>3</sub> PO <sub>4</sub> : 0.95 % (w/w) | Gln- 31.8; Xln -12.2<br>KL- 24.3  | Gln- 49.7; Xln -2.3<br>KL- 31.9  | 90.6        | 14.4   | <u>206</u> |
|             | H <sub>2</sub> SO <sub>4</sub> catalysed SE | Temp:195°C; Time :7.5 min;<br>H <sub>2</sub> SO <sub>4</sub> : 0.2% (w/w)    |                                   | Gln- 49.4; Xln -3.3<br>KL- 31.5  |             |        |            |

MW: Maple wood;SCB: Sugarcane bagasse; SG: Switchgrass; WS: Wheat straw; CC: Corn Cob; SS: Sugarcane straw; DA: Dilute Acid; SE: Steam Explosion; LHW: Liquid hot water; HT: Hydrothermal; Gln: Glucan; Xln: Xylan; HC: Hemicellulose; KL: Klason lignin; TL: Acid soluble and insoluble lignin

**Table 2: Acid catalysed SE and DA pretreatment carried out at semi-pilot and pilot-scale with different types of lignocellulosic feedstocks.**

| LCB type | Reactor type  | Reaction conditions  | Biomass composition (%)              |                                   | Composition of pre- hydrolysate  |   | Reference  |
|----------|---|--|--------------------------------------|-----------------------------------|--|---|------------|
|          |   |  | Untreated                            | Pretreated                        | Sugars   | Non-sugar component   |            |
| CC       | Screw steam explosive extruder                      | Pressure: 15.5 bar;<br>Time: 5.5 min<br>H <sub>2</sub> SO <sub>4</sub> : 2.4% (w/w) + Steam explosion                          | Gln- 42.23<br>HC -39.01<br>KL- 14.42 |                                   | Xylose: 27.5 wt %<br>XOS: 2.4 wt %<br>Glucose: 3.9 wt %<br>Arabinose: 3.7 wt % | Acetic acid: 1.1 wt%<br>TP: 1.7 wt%<br>Furfural: 0.5 wt%<br>5 HMF: 0.2wt% | <u>207</u> |
| SCB      | 350-L SS reactor with stirrer & thermal oil heating | Temp: 120°C; Time: 10 min;<br>H <sub>2</sub> SO <sub>4</sub> : 1% (w/v)  | Gln- 45.1<br>HC -26.9<br>KL- 22.2    | Gln- 54.6<br>HC -10<br>KL- 32     | C5: 17.4 g/L<br>C6: 1.6 g/L  | Acetic acid: 2.3 g/L<br>TA: 7.5g/L<br>Furfural: 0.8 g/L<br>5 HMF: 0.2 g/L | <u>208</u> |
| WS       | Continuous pretreatment reactor (250 kg/day)        | Temp: 160°C; Pressure: 5.2 bar<br>; Time: 10 min<br>H <sub>2</sub> SO <sub>4</sub> : 0.5% (v/v)                                | Gln- 47.1<br>HC -24.3<br>KL- 28.5    | Gln- 63.1<br>HC -1.0<br>KL- 35.8  | Xylose: 29.2 g/L<br>Glucose: 8.4 g/L<br>Arabinose: 2.6 g/L                     | Acetic acid: 1.9 g/L<br>Furfural: 0.9 g/L<br>5 HMF: 0.6 g/L               | <u>209</u> |
| EG       | 150 L horizontal Andritz reactor                    | Temp: 180°C; Time: 15 min;<br>H <sub>2</sub> SO <sub>4</sub> : 2.4% (w/w) + Steam explosion                                    | Gln- 38.5<br>Xln -11.0<br>KL- 25.2   | Gln- 55.5<br>Xln: 0.8<br>KL- 37.1 | 92% Xylan recoverable and 74% as xylose  | Acetic acid: 2.9 wt %<br>Furfural: 0.9 wt%<br>5 HMF: 0.2 wt%              | <u>210</u> |
| RS       | Continuous pretreatment reactor (250 kg/day)        | Temp: 162°C; Time: 10 min<br>Final H <sub>2</sub> SO <sub>4</sub> : 0.35% (w/w);<br>(pre-soaking in acid: 0.5h acid)           | Gln- 37<br>Xln -20<br>TL- 13.4       | Gln- 51.8<br>Xln -3.6<br>TL- 28.8 | 100g xylose in hydrolysate/kg initial dry substrate                            | Acetic acid: 2 g/L<br>Furfural: 1.2 g/L<br>5 HMF: 1.1 g/L                 | <u>211</u> |
| WS       | Steam Explosion in a 30L rig                        | Pressure : 12 bar; Time: 12 min;<br>Final H <sub>3</sub> PO <sub>4</sub> : 1.2% (w/v);<br>(Acid pre-soaked biomass introduced) | Gln- 41.6<br>Xln -30.3<br>TL- 19.3   |                                   | Xylose: 17.7 wt%   | -   | <u>212</u> |
| CS       |   |  | Gln- 38.5<br>Xln -24.3<br>TL- 18.3   |                                   | Xylose: 13.9 wt%   | -   |            |
| MS       |   |  | Gln- 47.0<br>Xln -25.1<br>TL- 26.15  |                                   | Xylose: 14.7 wt%   | -   |            |

CC: Corn cobs; SCB: Sugarcane bagasse; RS: Rice Straw; WS: Wheat Straw; EG: Eucalyptus grandis; CS: Corn Stover; MA: Miscanthus; Gln: Glucan; Xln: Xylan; HC: Hemicellulose; KL: Klason lignin; TL: Acid soluble and insoluble lignin; C5: Pentose sugars; C6: Hexose sugars; XOS: Xylooligosaccharides; GOS: Glucooligosaccharides; DA: Dilute Acid; TPL: Total phenolics; TA- Total aromatics; wt% : Wt in g/100 g biomass.



**Table 3:** Commercial products from xyloseView Article Online  
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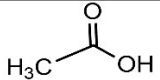
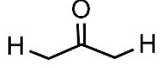
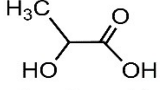
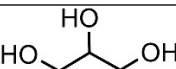
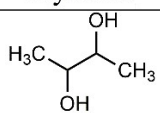
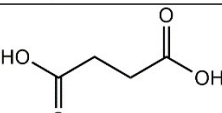
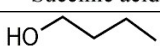
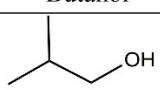
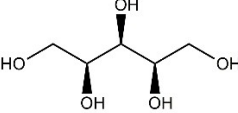
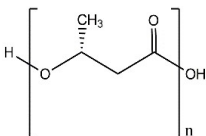
| Chemical  | Commercial applications   |
|---|---|
| <br>Ethanol        | Solvent, automotive gasoline, alcohol beverages, distilled spirits, hand sanitizers and medical antiseptics |
| <br>Acetic acid    | Polymeric monomers, paints, adhesives, inks, coatings, and food additives.                                  |
| <br>Acetone        | Plastics, cosmetics, and solvents   |
| <br>Lactic acid    | Food, beverages, polyesters, textiles, and pharmaceuticals  |
| <br>Glycerol       | Pharmaceutical, food, polymer, humectant, solvent, lubricant, personal care, and household products         |
| <br>2,3-Butanediol | Polymers, solvents, fine chemicals, lactones, fuel additives.   |
| <br>Succinic acid | Pharmaceutical products, surfactants, detergents, plastics and food grade ingredients.                      |
| <br>Butanol      | Lubricants, brake fluids, synthetic rubber, polymers, and automotive fuels                                  |
| <br>Isobutanol   | Coatings, chemical derivatives, paints, fuel additive, and solvents   |
| <br>Xylitol      | Confectioneries, chewing gums, syrups, odontological, and pharmaceutical products                           |
| <br>PHB          | Biodegradable plastics  |





Table 4: Summary of products obtained from conversion of pure and crude xylose by native microorganisms.

| Product     | Pretreatment                                  | Form of substrate                       | Additional steps prior to fermentation | Microorganism                              | Mode of Fermentation | Titer (g/L)       | Yield (g/g)        | Productivity (g/L/h) | Reference  |
|-------------|---|---|--|--|----------------------|-------------------|--------------------|----------------------|------------|
| Xylitol     | Hydrothermally pretreated SCB                 | Xylose- rich hydrolysate                | None                                   | <i>Pichia fermentans</i>                   | Batch                | 79                | 0.67               | 0.47                 | <u>55</u>  |
|             | HNO <sub>3</sub> pretreated CC                | Xylose-rich hydrolysate                 | Detoxification & decolorization        | <i>Candida tropicalis</i> MTCC 6192        | Batch                | 34.35             | 0.62               | 0.26                 | <u>213</u> |
|             | Pure Xylose                                   | Pure Xylose + Pure glycerol             | None                                   | <i>Yarrowia lipolytica</i> Polt            | Batch                | 53.2              | 0.97               | 0.36                 | <u>121</u> |
|             | Pure Xylose                                   | Pure Xylose + Crude glycerol            | None                                   | <i>Y. lipolytica</i> Polt                  | Batch                | 50.5              | 0.92               | 0.35                 | <u>121</u> |
|             | Steam exploded poplar                         | Xylose-rich hydrolysate                 | Detoxification                         | <i>C. guilliermondii</i>                   | Batch                | 28.78             | 0.59               | 0.81                 | <u>214</u> |
|             | Steam exploded Eucalyptus globulus chips      | Xylose- rich hydrolysate                | Detoxification                         | <i>Kluyveromyces marxianus</i> NRRL Y-6373 | Batch                | 28.05             | 0.68               | 0.16                 | <u>215</u> |
|             | H <sub>2</sub> SO <sub>4</sub> pretreated SS  | Xylose-rich hydrolysate                 | None                                   | <i>Corynebacterium glutamicum</i> Cg-ax3   | Fed-batch            | 27.0              |                    | 2.25                 | <u>216</u> |
|             | Hydrothermally pretreated CC                  | Whole slurry of corn cobs               | None                                   | <i>Saccharomyces cerevisiae</i> PE-2 (GM)  | Fed-batch            | 29.61             | 0.93               | 0.54                 | <u>115</u> |
|             | CC  | Corn cob hydrolysate                    | Detoxification                         | <i>S. cerevisiae</i> IS5-d                 | Fed-batch            | 143               | 0.93               | 1.83                 | <u>217</u> |
|             |   |   | <i>S. cerevisiae</i> 2bpgi             | Fed-batch                                  | 162                  |                   | 2.13               |                      |            |
| Lactic acid | HCl pretreatedSCB                             | Xylose- rich hydrolysate                | None                                   | <i>Bacillus coagulans</i> DSM ID 14-300    | Batch                | 55.99             | 0.87               | 1.70                 | <u>218</u> |
|             | H <sub>2</sub> SO <sub>4</sub> pretreated SCB | Xylose- rich hydrolysate                | None                                   | <i>Lactobacillus pentosus</i> ATCC 8041    | Batch<br>Batch SScF* | 42.4<br>64.8      | 0.71<br>0.93       | 1.02<br>1.01         | <u>125</u> |
|             | DryPB pretreated CS                           | Carbohydrate rich biomass               | None                                   | <i>Pediococcus acidilactici</i> ZY1        | Fed-batch<br>SScF    | 97.3 <sup>§</sup> | 0.926 <sup>#</sup> | 1.01                 | <u>219</u> |
|             | NaOH pretreated CS                            | Carbohydrate rich & delignified biomass | Washed                                 | <i>P. acidilactici</i> PA204               | Fed-batch<br>SScF    | 104               | 0.69               | 1.24                 | <u>220</u> |
|             | NaOH pretreated CC                            | Carbohydrate rich & delignified biomass | None                                   | <i>K. marxianus</i> YKX071 (GM)            | Fed-batch<br>SScF    | 101.7             | 0.56               | 1.10                 | <u>221</u> |
|             | Organosolv pretreated                         | Cellic CTec2 & HTec2 hydrolysed         | None                                   | <i>B. coagulans</i> DSM ID 14-298          | Batch                | 88.09             | 0.674              | 2.93                 | <u>222</u> |



|   |  |  |  |  |  |       |       |            |            |
|---|--|--|--|--|--|-------|-------|------------|------------|
|   | beechwood  | mixed sugars   |  |  |  |       |       |            |            |
| Succinic acid                             | NaOH pretreated SCB  | Delignified carbohydrate rich biomass hydrolysed by Cellulclast 1.5L | None   | <i>Y. lipolytica</i> PSA02004                | Batch  | 33.2  | 0.58  | 0.33       | <u>223</u> |
|   | Sulfuric acid pretreated CS                                      | Mixed hydrolysate (81% xylose; 14% glucose)                          | None   | <i>Escherichia coli</i> BA408                | Batch  | 23.1  | 0.85  | 0.24       | <u>224</u> |
|   | Sulfuric acid pretreated EG                                      | Mixed hydrolysate (69% xylose; 29.4% glucose)                        | None   | <i>E. coli</i> M6PM                          | Dual phase (Aerobic growth and anaerobic production phase) | 30.03 | 0.64  | 0.41       | <u>225</u> |
|   | Acid sulphite pulping of Eucalyptus globulus                     | Xylose-rich spent sulphite liquor                                    | Nanofiltration using vibratory shear-enhanced processing filtration unit | <i>Actinobacillus succinogenes</i> DSM 22257 | Continuous (Dilution Rate 0.2 h <sup>-1</sup> )            | 19    | 0.48  | -          | <u>226</u> |
|   |  |  |  |  |  | 22    | 0.55  | -          |            |
|   | CS hydrolysate   | Glucose: Xylose (70:30)  |  | <i>C. glutamicum</i> CGS5 (GM)               | Two stage (Initial growth and production stage)            | 98.6  | 0.87  | 4.29       | <u>227</u> |
|   | Dilute acid pretreated CC  | Xylose rich hydrolysate  | Detoxification   | <i>C. glutamicum</i> NC-2 (GM)               | Two stage (Initial growth and production stage)            | 40.8  | 0.69  | 0.85       | <u>228</u> |
| SCB                                       | Hydrolysate (Glucose: Xylose; 2:1)                               | None   | <i>E. coli</i> BA305   | Fed-batch                                    | 39.3   | 0.97  | 0.327 | <u>229</u> |            |
| 2,3 Butanedio I                           | Hydrothermally pretreated SCB                                    | Xylose-rich hydrolysate  | None   | Mutant of <i>E. ludwigii</i>                 | Fed-batch  | 32.3  | 0.36  | 0.84       | <u>148</u> |
|   |  |  | Detoxification   |  |  | 63.5  | 0.33  | 0.43       |            |
|   | KOH pretreated corn cobs followed by Hemicellulose precipitation | Enzymatically derived Xylose-rich hydrolysate                        | None   | <i>K. oxytoca</i> XF7                        | Batch  | 12.18 | 0.97  | 0.50       | <u>230</u> |
|   | H <sub>2</sub> SO <sub>4</sub> pretreated soybean hulls          | Xylose-rich hydrolysate  | None   | <i>Pantoea agglomerans</i> BL1               | Batch  | 20.5  | 0.50  | 0.55       | <u>231</u> |
|   |  |  | None   | <i>K.pneumoniae</i> BLh-1                    |  | 21.9  | 0.40  | 0.30       |            |
| H <sub>2</sub> SO <sub>4</sub> pretreated | Xylose-rich  | None   | <i>P. agglomerans</i> BL1  | Batch  | 14.02  | 0.53  | 1.17  | <u>232</u> |            |

|           |  |   |                                    |  |   |       |      |      |                     |
|-----------|--|---|------------------------------------|--|---|-------|------|------|---------------------|
|           | soybean hulls  | hydrolysate                                   |                                    |  |   |       |      |      |                     |
|           | H <sub>2</sub> SO <sub>4</sub> pretreated wheat straw                  | Xylose-rich hydrolysate + exogenous glucose   | None                               | <i>Paenibacillus polymyxa</i> DSM 365          | Batch   | 23.4  | 0.27 | 0.28 | <a href="#">233</a> |
|           | Sulphite pretreated Oil palm empty fruit bunches                       | Hydrolysed slurry + Xylose-rich spent liquor  | None                               | <i>K. pneumoniae</i> PM2,                      | SHF   | 75.03 | 0.43 | 0.78 | <a href="#">234</a> |
|           | Corn Cobs pretreated with mixed acids                                  | Xylose-rich hydrolysate                       | None                               | <i>Enterobacter cloacae</i> M22                | Batch   | 23.2  | 0.44 |      | <a href="#">235</a> |
|           | Hot water extracted Birchwood  | Xylose-rich hydrolysate                       | Detoxification by ultra-filtration | <i>B. vallismortis</i> B-14891                 | Batch   | 22.7  | 0.43 | 0.63 | <a href="#">236</a> |
| Ethanol   | NaOH pretreated SCB  | Xylose-rich hydrolysate                       | None                               | <i>Scheffersomyces stipitis</i> Y-7124         | Sequential fed-batch with cell-recycle  | 18.52 | 0.32 | 0.36 | <a href="#">158</a> |
|           | NaOH pretreated SCB  | Xylose-rich hydrolysate                       | None                               | <i>Spathaspora passalidarum</i> Y-27907        | Sequential fed-batch with cell-recycle  | 23.3  | 0.46 | 0.81 | <a href="#">158</a> |
|           | Steam exploded WS  | Whole biomass slurry                          | None                               | <i>S. cerevisiae</i> F12 (GM)                  | Batch   | 23.7  | 0.43 | 0.7  | <a href="#">237</a> |
|           | 1.5% v/v H <sub>2</sub> SO <sub>4</sub> pretreated SCB                 | Xylose-rich hydrolysate                       | Detoxified by Ca(OH) <sub>2</sub>  | <i>Spathaspora passalidarum</i> Y-27907        | Batch   | 19.4  | 0.4  | 0.8  | <a href="#">238</a> |
| n-Butanol | Hydrothermally pretreated SCB  | Xylose-rich hydrolysate fortified with xylose | None                               | <i>C. saccharoperbutylacetonicum</i> DSM 14923 | Batch   | 5.8   | 0.22 | 0.08 | <a href="#">239</a> |
|           | Hot water extracted debarked hybrid poplar                             | Xylose-rich hydrolysate                       | Detoxification                     | <i>C. acetobutylicum</i> ATCC 824              | SScF  | 6.8   | 0.16 | 0.07 | <a href="#">240</a> |
|           | Xylan  | Pure Xylan                                    | None                               | <i>C. acetobutylicum</i> NJ4                   | Batch-CBP involving co-culturing with hemicellulase secreting <i>Thermoanaerobacterium thermosaccharolyticum</i> M5 | 13.28 | 0.19 | 0.08 | <a href="#">241</a> |
|           | Hot water extraction and steam treatment with chips (60% aspen and 40% | Xylose-rich hydrolysate                       | Laccase mediated detoxification    | <i>C. acetobutylicum</i>                       | Batch   | 4.17  | -    | -    | <a href="#">242</a> |



|  |       |  |  |  |  |  |  |  |
|--|-------|--|--|--|--|--|--|--|
|  | maple |  |  |  |  |  |  |  |
|--|-------|--|--|--|--|--|--|--|

\*SCB: Sugarcane bagasse; CC: Corn cobs; CS: Corn stalk; EG: Elephant grass; SHF: Separate hydrolysis and Fermentation; SScF: Simultaneous Saccharification and Fermentation; GM: Genetically modified.

**Table 5:** Performance of engineered strains for product formation from xylose via microbial route.

| Product     | Microorganism                    | Modification   | Improvement  | Reference  |
|-------------|----------------------------------|--|--|------------|
| Xylitol     | <i>Meyerozyma guilliermondii</i> | Cloning and overexpression of XR, and knockout of XDH.   | Engineered strain was able to improve 3-fold xylitol yield   | <u>243</u> |
|             | <i>E. coli</i>                   | Expression of XR and glucose dehydrogenase   | Immobilized recombinant cells could maintain the enzyme activity up to 80% after repeated 10 batches.                      | <u>244</u> |
|             | <i>K. marxianus</i>              | Expression of XR and transporter genes   | Engineered strain efficiently utilized glucose and xylose from xylose rich hydrolysate for production of xylitol > 100 g/L | <u>245</u> |
|             | <i>S. cerevisiae</i>             | Expression of constitutive GPD promoter for ZWF1(cytoplasmic G6P dehydrogenase) gene to increase NADPH pool, a cofactor for XR   | The heterologous expression of a constitute promoter resulted in 12% increase in xylitol yield(0.78 Vs 0.88).              | <u>246</u> |
| Lactic acid | <i>S. cerevisiae</i>             | Heterologous overexpression of lactate dehydrogenase ( <i>ldhA</i> ) gene from <i>Rhizopus oryzae</i> under the control of PGK1 promoter through chromosome integration. | High lactate dehydrogenase activity and produced LA titers of 28.9 g/L with 0.69g/g yield.                                 | <u>247</u> |
|             | <i>Scheffersomyces stipitis</i>  | Heterologous overexpression of <i>ldh</i> from <i>L. helveticus</i> under the control of native ADH1 promoter.   | Ethanol production decreased 15 to 30% and carbon flux is shifted towards LA from xylose resulting in 58                   | <u>248</u> |

|               |                                     |   |   |            |
|---------------|-------------------------------------|---|---|------------|
|               | <i>Pediococcus acidilactici</i>     | Heterologous expression of xylose assimilating genes <i>XylA</i> and <i>XylB</i> , substitution of endogenous phosphoketolase, with heterologous transketolase and transaldolase. | g/L LA with 0.58 g/g yield.<br>The metabolic carbon flux is concentrated towards LA biosynthesis resulting in 97.3 g/L LA with 0.93 g/g conversion yield. | <u>219</u> |
|               | <i>Lactococcus lactis</i>           | Disruption of phosphoketolase gene, introduction of transketolase gene.   | High LA titers of 50.1 g/L with 1.58 mol/mol yield and 99.6 % purity after the downstream processing was quantified.                                      | <u>249</u> |
|               | <i>Candida sonorensis</i>           | Integrating lactate dehydrogenase gene from <i>L. helveticus</i> .  | Engineered strains was able to accumulate 31 g/L LA with 0.62 g/g yield under microaerobic conditions.  | <u>250</u> |
| Succinic acid | <i>Yarrowia lipolytica</i> PSA02004 | Overexpressing pentose pathway cassette (XR, XDH and XK genes)  | Engineered strain could growth on xylose as sole energy and carbon source producing 22.3 g/L SA.  | <u>144</u> |
|               | <i>Corynebacterium glutamicum</i>   | Heterologous expression of XI, overexpression of XK, transaldolase, 6-phosphogluconate dehydrogenase and phosphoketolase.   | Recombinant strain enhanced the growth and xylose consumption rate resulting in 7.22 g/l SA with 0.18 g/g yield.  | <u>251</u> |
|               | <i>Escherichia coli</i> KJ122       | Deletion of <i>XylFGH</i> and <i>XylE</i> genes and ALE   | Improved succinate titers of 85 g/L with, 0.85 g/g yield, and 0.7 g/L/h productivity.   | <u>142</u> |
|               | <i>E. coli</i> K12                  | Inactivation of pyruvate formate lyase ( <i>pflB</i> ) and lactate dehydrogenase ( <i>ldhA</i> )  | Significant increase in cell mass (2.5 g/L) and succinate (11.6 g/L) production.  | <u>252</u> |
|               | <i>Aspergillus niger</i>            | Overexpression of fumarate reductase, disruption of gluconic acid and oxalic acid production  | Engineered strain was able to utilize xylose rich hydrolysates derived from sugar beet and wheat straw to produce 23 and 9 g/L SA.                        | <u>253</u> |







|                |                             |  |   |            |
|----------------|-----------------------------|--|---|------------|
| 2,3-Butanediol | <i>S. cerevisiae</i>        | Heterologous expression of BDO biosynthetic pathway, deletion of ethanol and glycerol assimilatory genes. Restoration of redox balance by overexpression of NADH oxidase | Highest yield (0.41 g/g) and productivity (1.43 g/L/h) using <i>S. cerevisiae</i> as the host   | <u>85</u>  |
|                | <i>Enterobacter cloacae</i> | Expression of BDO dehydrogenase, inactivation of glucose transporter and overexpression of galactose permease.   | Engineered strain could overcome CCR and able to utilize glucose and xylose simultaneously producing 119.4 g/L BDO with 2.3 g/L/h productivity. | <u>254</u> |
|                | <i>Zymomonas mobilis</i>    | Heterologous expression of BDO biosynthetic pathway consisting of acetolactate synthase, acetolactate decarboxylase, and butanediol dehydrogenase                        | Engineered strain was able to produce BDO (13.3 g/L) utilizing both C5 and C6 sugars.   | <u>255</u> |
|                | <i>S. cerevisiae</i>        | Expression of <i>S. stipitis</i> transaldolase, xylose reductase, <i>L. lactis</i> NADH oxidase and overexpression of <i>pdh</i> from <i>C. tropicalis</i> .             | Increase in 2.1-fold xylose consumption and 1.8-fold in BDO productivity  | <u>256</u> |
|                | <i>K. pneumoniae</i>        | Overexpression of transketolase, NADP transhydrogenase subunit alpha, and NADH dehydrogenase subunit F   | Engineered strain increased the xylose consumption and BDO production resulting in 38.6 g/L BDO with 0.62 g/L/h productivity.                   | <u>149</u> |
| Ethanol        | <i>S. cerevisiae</i>        | Overexpressing pentose pathway genes (XR and XDH)  | Assimilation of xylose towards ethanol biosynthesis with a conversion yield of 0.19 g/g.  | <u>162</u> |
|                | <i>S. cerevisiae</i>        | Overexpression of mutant XR K270R  | Increased specificity towards NADH rather than NADPH, resulting in increased ethanol yield (0.38 g/g) and reduced xylitol yield (0.08 g/g).     | <u>162</u> |
| n-Butanol      | <i>E. coli</i>              | Expression of a synthetic butanol pathway  | In a defined medium 4.32 g/L n-butanol was produced.  | <u>257</u> |

|     |                                  |   |   |            |
|-----|----------------------------------|---|---|------------|
|     | <i>Clostridium tyrobutyricum</i> | Heterologous expression of <i>XylT</i> , <i>XylA</i> , and <i>XylB</i> from <i>C. acetobutylicum</i> and overexpression of native alcohol dehydrogenase           | Engineered strain could accumulate 12 g/L n-butanol with 0.12 g/g yield.          | <u>258</u> |
| PHB | <i>E. coli</i>                   | Heterologous expression of XI, XK and pentose transport protein from <i>B. subtilis</i> in <i>E. coli</i> harbouring PHB pathway from <i>Ralstonia eutropha</i> . | Simultaneous utilization of glucose-xylose increased 2-fold PHB titers            | <u>259</u> |
|     | <i>S. cerevisiae</i>             | Heterologous overexpression of PHB biosynthesis pathway from <i>Cupriavidus necator</i>   | Engineered strain could produce 1.99 mg PHB/g xylose.                             | <u>260</u> |
|     | <i>S. cerevisiae</i>             | Heterologous overexpression of NADH dependent acetoacetyl-CoA from <i>Allochroamtiumvinosum</i> replacing the gene from <i>C. necator</i>                         | PHB titers increased 5-fold in aerobic and 8.4-fold in oxygen limited conditions. | <u>261</u> |



**Table 6. Theoretical maximum yields of biomass and succinic acids from xylose assimilation via different pathways**

| <i>C. glutamicum</i>                                     |         |                                   |                                  |                                 |                              |  |
|--|---------|-----------------------------------|----------------------------------|---------------------------------|------------------------------|--|
| Yield  | Glucose | Xylose-XR-XDH pathway<br>(XR-XDH) | Xylose-isomerase pathway<br>(XI) | Xylose-Weimburg pathway<br>(XW) | Xylose-Dahms pathway<br>(XD) |  |
| Biomass yield<br>(c-mol biomass/c-mol substrate)         | 0.84    | 0.81                              | 0.84                             | 0.59                            | 0.54                         |  |
| Succinic acid yield<br>(c-mol succinate/c-mol substrate) | 0.67    | 0.67                              | 0.67                             | 0.80                            | 0.40                         |  |
| <i>E. coli</i>   |         |                                   |                                  |                                 |                              |  |
| Biomass yield<br>(c-mol biomass/c-mol substrate)         | 0.90    | 0.90                              | 0.90                             | 0.63                            | 0.58                         |  |
| Succinic acid yield (c-mol<br>succinate/c-mol substrate) | 0.67    | 0.67                              | 0.67                             | 0.80                            | 0.40                         |  |
| <i>A. succinogenes</i>                                   |         |                                   |                                  |                                 |                              |  |
| Biomass yield<br>(c-mol biomass/c-mol substrate)         | 0.34    | 0.34                              | 0.34                             | NA                              | NA                           |  |
| Succinic acid yield<br>(c-mol succinate/c-mol substrate) | 0.67    | 0.80                              | 0.80                             | NA                              | NA                           |  |
| <i>Y. lipolytica</i>                                     |         |                                   |                                  |                                 |                              |  |
| Biomass yield<br>(c-mol biomass/c-mol substrate)         | 0.73    | 0.70                              | 0.72                             | 0.49                            | 0.46                         |  |
| Succinic acid yield<br>(c-mol succinate/c-mol substrate) | 0.90    | 1.0                               | 1.0                              | 0.80                            | 0.80                         |  |



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