

Review

# The Xylose Metabolizing Yeast *Spathaspora passalidarum* is a Promising Genetic Treasure for Improving Bioethanol Production

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**Abstract:** Currently, the fermentation technology for recycling agriculture waste for generation of alternative renewable biofuels is getting more and more attention because of the environmental merits of biofuels for decreasing the rapid rise of greenhouse gas effects compared to petrochemical, keeping in mind the increase of petrol cost and the exhaustion of limited petroleum resources. One of widely used biofuels is bioethanol, and the use of yeasts for commercial fermentation of cellulosic and hemicellulosic agricultural biomasses is one of the growing biotechnological trends for bioethanol production. Effective fermentation and assimilation of xylose, the major pentose sugar element of plant cell walls and the second most abundant carbohydrate, is a bottleneck step towards a robust biofuel production from agricultural waste materials. Hence, several attempts were implemented to engineer the conventional *Saccharomyces cerevisiae* yeast to transport and ferment xylose because naturally it does not use xylose, using genetic materials of *Pichia stipitis*, the pioneer native xylose fermenting yeast. Recently, the nonconventional yeast *Spathaspora passalidarum* appeared as a founder member of a new small group of yeasts that, like *Pichia stipitis*, can utilize and ferment xylose. Therefore, the understanding of the molecular mechanisms regulating the xylose assimilation in such pentose fermenting yeasts will enable us to eliminate the obstacles in the biofuels pipeline, and to develop industrial strains by means of genetic engineering to increase the availability of renewable biofuel products from agricultural biomass. In this review, we will highlight the recent advances in the field of native xylose metabolizing yeasts, with special emphasis on *S. passalidarum* for improving bioethanol production.

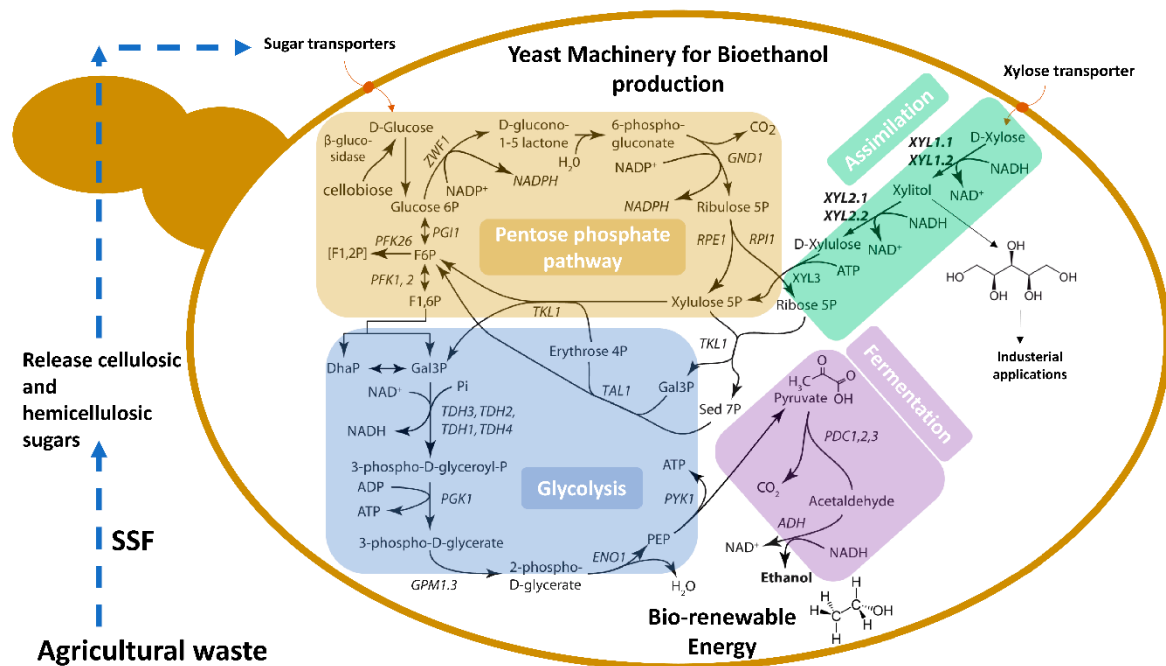
**Keywords:** fermentation; xylose metabolism; genetic engineering; biofuel; *Spathaspora passalidarum*; *Pichia stipitis*

## 1. Fermentation Technology and Challenges

The modern biotechnological applications for generation of alternative and renewable sources of biofuels are receiving more attention due to global worries over the climate change, rapid global warming, and the rising of fossil fuel costs. One of such growing biotechnological trends is the fermentation technology to convert the sugar-rich agriculture waste into bioethanol by conventional or non-conventional yeasts [1–4]. In general, yeasts have advantages over bacteria for commercial fermentation due to the thickness of their cell walls, less stringent nutritional requirements, large sizes, utmost resistance to contamination, and better growth at acidic pH of bioreactor fermenters.

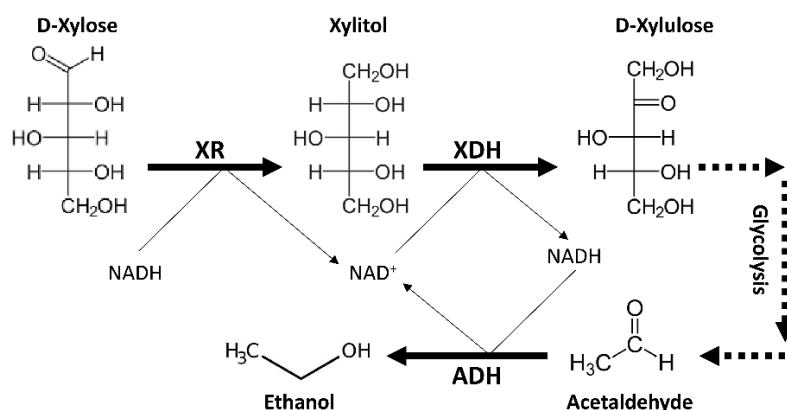
In nature, the second most abundant hemicellulosic sugar in fast-growing hardwoods and agricultural biomass is xylose. Xylose sugar forms up to 15–25% of all angiosperm biomass,

and it could supply an alternative fuel source for its ability to be commercially fermented into ethanol. Several approaches have been employed to engineer xylose assimilation metabolism into conventional fermenting yeasts, such as *Saccharomyces cerevisiae* [4–6]. Therefore, efficient hemicellulosic sugar fermentation is crucial for the economic conversion of lignocellulose biomass to renewable biofuels [4,6–8]. The discovery of xylose-fermenting yeasts in new niches and genetic engineering of yeasts to be capable of rapid fermentation of xylose and other sugars to recoverable concentrations of bioethanol could provide alternative biofuel sources for the future (Figure 1) [4,9].



**Figure 1.** Model of yeast fermentation machinery for bioethanol production using the agriculture waste to feed yeasts [4]. The metabolic pathways for xylose and glucose assimilation and fermentation are indicated including the pentose phosphate pathway and glycolysis. The agriculture waste is treated through the enzymatic and chemical simultaneous saccharification and fermentation (SSF) processes to release the cellulose and hemicellulosic sugars. The hexose and pentose sugars are transported by specific hexose and pentose sugar transporters into yeast for further metabolizing processes.

As a rule of thumb for metabolizing the xylose in most of xylose-fermenting yeasts [4], firstly the xylose is reduced by xylose reductase (XR) to xylitol. In the second step, the xylitol is oxidized by xylitol dehydrogenase (XDH) to xylulose. Afterward, the xylulose passes into the pentose phosphate pathway being metabolized into glyceraldehyde-3-P which is further reduced to pyruvate. Finally, the pyruvate is decarboxylated to acetaldehyde which is further reduced to ethanol by alcohol dehydrogenase (Figure 1). Notably, most xylose reductase enzymes have dual cofactor specificity, using both NADH and NADPH, but typically favor NADPH. However, xylitol dehydrogenase enzymes use NAD<sup>+</sup> specifically as a cofactor, which could cause imbalance between the cofactor’s source for the XR-XDH pathway and xylitol accumulation under uncontrolled oxygen conditions (Figure 2) [10].



**Figure 2.** Schematic model of the central metabolism for bioethanol production from xylose indicating the rate limiting steps (XR: xylose reductase; XDH: xylitol dehydrogenase, and ADH: alcohol dehydrogenase) and cofactors demand/balance in most of native xylose metabolizing yeasts. Xylose assimilation reactions starts with XR to produce xylitol. The xylitol is further metabolized by XDH to produce xylulose, which further metabolized to xylulose-phosphate to enter the glycolysis (indicate by black dotted arrow and summarized in Figure 1) to produce acetaldehyde. The acetaldehyde finally converted to ethanol by ADH.

One of pioneer xylose fermenting yeasts is *Pichia stipitis*. *P. stipitis* is heterothallic ascomycetous yeast, predominantly haploid and related to pentose fermenting yeasts, such as *Candida shehatae* [1,4,11–15]. *P. stipitis* was recently renamed to be *Scheffersomyces stipitis* [15] and it is natively one of the highest xylose-utilizing and fermenting yeasts. In type culture collections, the *P. stipitis* strains are among the best xylose-metabolizing microbes [16]. Under controlled low O<sub>2</sub> conditions, *P. stipitis* is able to consume xylose and produce up to 57 g/L of bioethanol at 30°C [4,13,14,17]. *Pichia* uses an alternative nuclear genetic code (ANGC) in which CUG encodes for Ser rather than Leu [17], which makes the genetic manipulation of *Pichia* with the commercial drug resistance markers unusually problematic because essentially all of these markers are derived from bacteria that use the universal codon system. Moreover, one of classical challenges in fermentation technology is that some of key enzymes of bioethanol production pathway are expressed relatively in low levels [1,4,13,14]. Therefore, the metabolic engineering of the bioethanol pathway in yeasts, which can ferment the sugars of the agriculture biomass with considerable and recoverable bioethanol concentrations, could enhance the productivity and sustainability of renewable biofuel sources [1,13,14]. To improve bioethanol production and xylose metabolism, a stable and manipulatable genetic system that enables overexpression or deletion of one or more of key enzymes and sugar transporters in xylose-fermenting yeast *P. stipitis*, was developed [1,3,18]. This approach comprises modelling, metabolic and flux analysis, quantitative metabolomics and transcriptomics followed by the targeted overexpression or deletion of genes of the rate-limiting steps [1,3,4,13,14]. Since there is reasonable information about the metabolic capacity of *P. stipitis* to ferment xylose on various omics levels, this makes it an attractive model system for metabolic engineering.

Recently, a new xylose-fermenting yeast *Spathaspora passalidarum* was discovered, which naturally co-ferments xylose, glucose, and cellobiose and demonstrates potentials in the effective conversion of mixed sugars from hemicellulosic hydrolysates into ethanol [9]. *S. passalidarum* was initially isolated from extremely O<sub>2</sub>-limited and hemicellulosic sugar rich environments from the gut of a wood-boring beetle (as will be discussed below). Although the anaerobic fermentation of glucose is broadly known, the xylose fermentation typically needs a controlled oxygen condition. Uncontrolled oxygen conditions in another xylose-fermenting yeast, such as *P. stipitis*, leads to accumulation of xylitol due to insufficient amounts of NAD<sup>+</sup>, and as consequence the xylose metabolism will be blocked (Figure 2) [9,13,14]. To solve this problem, precise controlled O<sub>2</sub> (very low O<sub>2</sub> concentrations) during the xylose fermentation is required in *P. stipitis* to generate NAD<sup>+</sup> from NADH.

The bioethanol production from the bioconversion of lignocellulose biomass must be achieved at high rates and yields for economically recoverable concentrations. The achieving of such targets for efficient bioethanol production are more difficult with cellulose and hemicellulose. The major barrier for cellulose utilization is enzymatic saccharification, while for hemicellulose it is the utilization of mixed sugars (hexose sugars: glucose, galactose, mannose, and rhamnose; and pentose sugars: xylose and arabinose) in the presence of ferulic and acetic acids along with other byproducts of the thermochemical pretreatment of the hydrolysates [13,14]. However, *S. passalidarum* and *P. stipitis* yeasts possess a set of unique physiological merits that make them very useful biodegradable organisms for bioconversion of lignocellulosic biomass [4,9,13,14]. *Pichia* can utilize and ferment effectively cellobiose, glucose, galactose, and mannose along with xylan high oligomeric sugars xylan and mannan, in addition to its extensively studied ability to metabolize and ferment the xylose [4,13,14]. The primary sugar released in enzymatic hydrolysis is cellobiose and, remarkably, *P. stipitis* and *S. passalidarum* have the capability to utilize the cellobiose, which make such yeasts potent organisms for simultaneous saccharification and fermentation (SSF) or hydrolysate, because most commercially available cellulase products are often deficient in  $\beta$ -glucosidase enzyme so the accumulation of cellobiose inhibits cellulase activities. Since *P. stipitis* and *S. passalidarum* can directly metabolize the cellobiose, they have the potential to improve SSF processes [4,9,10,13,14,19,20].

Collectively, the native ability of *P. stipitis* and *S. passalidarum* to metabolize the oligomeric sugars is of high importance as the mild acidic pretreatments of agriculture waste biomass can prevent the formation of the sugar degradation byproducts, which could inhibit significantly the fermentation process, but can release about 15–55% of soluble oligomeric sugars. Therefore, with low cost and high yield, the hemicellulosic sugars can be more readily recovered and underutilized from cellulose biomass than glucose. Although such easy recoverable sugars can be utilized for formation of a number of useful products such as xylitol, butanol, lactic acid, and other chemicals, bioethanol is still the major product with the largest potential market. Hence, bioethanol production from the lignocellulosic biomasses is receiving a lot of attention as a consequence of agriculture policies and energy demands to improve the production of alternative renewable biofuels and to reduce CO<sub>2</sub> emissions [2,4,13,14].

## 2. *Spathaspora passalidarum* a Promising Genetic Source

The *Spathaspora* clade contains many bioethanol producer yeasts, including *Spathaspora arborariae*, *Spathaspora brasiliensis*, *Spathaspora gorwiae*, *Spathaspora hagerdaliae*, *Spathaspora passalidarum*, *Spathaspora roraimanensis*, *Spathaspora suhii*, *Spathaspora xylofermentans*. They are usually endosymbiotically associated with wood-boring-beetles that occupy rotting wood. *Spathaspora passalidarum* (Figure 3), the first identified species of genus *Spathaspora*, was isolated from the gut of passalid beetle *Odontotaenius disjunctus* [9,21–24]. Notably, *S. arborariae*, *S. gorwiae*, *S. hagerdaliae*, and *S. passalidarum* ferment xylose to produce bioethanol, whereas the rest within the *Spathaspora* clade are thought to be xylitol producers [9].



**Figure 3.** *Spathaspora passalidarum* budding cells with characteristic curved and elongated ascospore.

*S. passalidarum* was firstly described in 2006 by Nguyen et al. [21]. The authors speculated that *Spathaspora* mainly exists in the beetle's biosphere rather than the beetle's gut microbiota, and it may be only by coincidence that *O. disjunctus* beetles ingested decaying wood contaminated by yeasts. Later in 2012 and 2017, another 12 strains were described in two independent studies from wood-boring beetles and wood samples of Amazonian forest in Brazil [23,25]. Among these strains, only one isolate was obtained from the gut of *Popilus marginatus* beetle, while the rest of the strains were obtained from the woody samples inhabited by the beetles [23,25]. In 2014, two more strains were isolated from rotted wood in China [26]. Additionally, Rodrussamee and colleges in 2018 reported a new thermotolerant strain, named *S. passalidarum* CMUWF1–2, which was isolated from Thailand soil [27]. The frequency of finding *S. passalidarum* mainly among the woody samples supports the notion that those yeasts are probably associated with decaying wood niches rather than with the gut microbiota of wood-boring beetles. However, the fact of the low frequency of finding *S. passalidarum* among other yeast species keeps an open possibility that they inhabit mainly the wood-related beetles [9].

### 3. Fermentation Capability of *Spathaspora passalidarum*

It is believed that the beetle's gut is truly anaerobic or microaerobic, therefore it was speculated that *S. passalidarum* possess a unique adaptation capability to survive under oxygen-depleted conditions on mixtures of hemicellulosic sugars in the midgut of wood-boring beetles [10,19]. Currently, *S. passalidarum* is among the best xylose-utilizing and fermenting yeasts. Under anaerobic or microaerobic conditions, *S. passalidarum* possess rapid utilization and consumption rates for xylose and produces up to 0.48 g/g bioethanol (near to the maximum theoretical bioethanol production of 0.51 g/g), in contrast to *P. stipitis* which can hardly metabolize xylose anaerobically, accumulating xylitol and a very low yield of bioethanol [10,19,20,28,29]. Under anaerobic conditions, Hou in 2012 showed that *S. passalidarum* has a high growth rate with rapid consumption rate of sugars and can ferment xylose into a high yield of bioethanol with higher production efficiency than *P. stipitis* [10]. Similarly, Veras and colleges in 2017 showed that under anaerobic conditions, *S. passalidarum* accumulates 1.5 times more bioethanol than *S. stipitis*, while both stains accumulate around 0.44 g/g under O<sub>2</sub> limiting conditions [30]. The previous work by Hou (2012) defined strictly that *S. passalidarum* can metabolize and ferment xylose in tightly capped flasks [10]. In contrast to the previous report by Hou (2012) [10], under stringent O<sub>2</sub> limiting conditions, the *S. passalidarum* was not able virtually to utilize the sugars, indicating that native wild-type *S. passalidarum* does not ferment sugars under truly anaerobic conditions [19,20]. Therefore, it is still under debate whether *S. passalidarum* can ferment xylose truly anaerobically or whether it requires a controlled microoxygenic condition similar to *P. stipitis*.

One of the major challenges in fermentation technology is the inability of the majority of known microbes to co-ferment xylose and glucose, since glucose usually inhibits the metabolization of the other sugars in lignocellulose hydrolysate, as in the case of *P. stipitis* [13,14]. Astonishingly, in a recent study to address the metabolic profiling and fermentation capacity of *S. passalidarum*, *S. passalidarum* was found to co-ferment xylose, cellobiose, and glucose simultaneously with high bioethanol yields ranging from 0.31 to 0.42 g/g [19,20]. Moreover, an adapted *S. passalidarum* strain was found to accumulate up to 39 g/L bioethanol with a 0.37 g/g yield from a lignocellulosic hydrolysate. The specific production rate of bioethanol on xylose as a carbon source was superior with three times more than the corresponding rate on glucose, where the flux of glycolytic intermediates was meaningfully lower on glucose than on xylose and its xylose reductase enzyme had a higher affinity for NADH than NADPH [19,20]. Thus, the allosteric activation of glycolytic routes associated with the xylose utilization and the NADH-dependent xylose reductase are most likely the causes for such unique ability of *S. passalidarum* to co-ferment mixed sugars [19,23]. Later, such results were confirmed in a metabolic flux study, where *S. passalidarum* showed about 1.5–2 times high flux rate in the NADH-dependent xylose reductase reaction [31], which caused continuous recycling and reduction of xylitol levels. Such directed high flux rates to glycolytic routes and pentose phosphate pathway was the cause for high levels of bioethanol production in *S. passalidarum* [31]. In large scale fed-batch fermentation study, *S.*

*passalidarum* was able to metabolize around 90% of xylose sugar and all of glucose of sugarcane bagasse hydrolysate, even so glucose had approximately three-fold higher xylose content; and produced a high ethanol yield of 0.46 g/g with volumetric productivity of 0.81 g/L/h in contrast to *P. stipitis* which produced 0.32 g/g ethanol with productivity of 0.36 g/L/h [32]. In follow up study, *S. passalidarum* UFMG-CM-Y473 strain was able to simultaneously utilize and co-ferment about 78% of the released sugars (xylose, glucose, and cellobiose) of pretreated sugarcane bagasse hydrolysate (delignified and enzymatically hydrolyzed) to yield up to 0.32 g/g bioethanol with productivity of 0.34 g/L/h without any nutritional supplementation [33]. Moreover, the new thermotolerant strain, *S. passalidarum* CMUWF1–2, was able to co-ferment various sugars (mannose, galactose, xylose, and arabinose) of lignocellulosic biomass, even in presence of glucose, to accumulate considerable amounts of bioethanol and low amounts of xylitol at higher temperatures. For example, it was able to accumulate 0.43, 0.40, and 0.20 g/g ethanol per xylose at 30, 37, and 40 °C, respectively [27]. Constant with absence of the glucose repression effect on the utilization of other sugars, *S. passalidarum* CMUWF1–2 exhibited a resistance to 2-deoxy glucose, the nonmetabolizable glucose analog, and tolerance to elevated levels of glucose (35.0% of *w/v*) and ethanol (8.0% of *v/v*) [27]. In contrast, the first discovered *S. passalidarum* NRRL Y-27907 strain was sensitive to 2-deoxy glucose, as 2-deoxy glucose suppressed the xylose consumption under anaerobic conditions. While under aerobic conditions, the 2-deoxy glucose inhibited, only partially, *S. passalidarum* NRRL Y-27907 [10]. Therefore, the author speculated that xylose uptake in *S. passalidarum* NRRL Y-27907 may take place by different xylose transport systems under aerobic and anaerobic conditions. Under aerobic conditions, xylose is taken up by means of ATP-dependent/high affinity xylose-proton symporter and low affinity transporter via facilitated diffusion driven only by the sugar gradient. While, under anaerobic condition, the yeasts are most likely to use only the low affinity xylose pump, as the active transport via xylose-proton symporter will deplete the ATP levels. The inhibitory effect of 2-deoxy glucose on *S. passalidarum* NRRL Y-27907 can be therefore explained by (1) blocking of the low-affinity-facilitated diffusion transporters which are occupied with transporting 2-deoxy glucose, and (2) the inhibition xylose active transport due to the depletion of the intracellular ATP levels to actively phosphorylate the 2-deoxy glucose into the non-metabolizable phospho-2-deoxy glucose [10].

#### 4. Genetic and Physiological Features of *Spathaspora passalidarum* Emphasis Special Roles for Xylose Reductase and Xylitol Dehydrogenase

These unusual unique traits of *S. passalidarum* are very attractive for studying on a molecular level. The complete genome sequence of xylose-fermenting yeast *S. passalidarum* was therefore necessary and it was accomplished and published for first time in 2011 [34]. The comparative genomic, transcriptomic, and metabolomic analysis between two of the native xylose-fermenting yeasts, the relatively newly discovered *S. passalidarum*, and the deeply studied *P. stipitis*, allowed a better understanding of the regulatory mechanisms of lignocellulose utilization, and identified the target key genes involved in xylose metabolism [9,10,19,31,34]. The comparative genomic and phylogenetic analysis clearly revealed that *S. passalidarum* is one of the CUG yeast clades, similar to *P. stipites* [34]. In addition, the transcriptome analysis indicated upregulation of the genes implicated in transporting carbohydrate and xylose- and carbohydrate-metabolisms under xylose growth. Several of genes, which are involved in regulation of redox balance and recycling of NAD(P)H<sup>+</sup>, were upregulated to probably keep the redox balance during xylose utilization. Additionally, the genes encoding for cellulases and  $\beta$ -glucosidases were also upregulated, which suggests a positive feedback of xylose on the upstream genes to activate its own liberation from the higher oligomeric sugars of hemicelluloses by means of the catalytic activities of cellulases and  $\beta$ -glucosidases [34].

The previously mentioned capabilities of *S. passalidarum* to co-ferment different sugars and accumulate high levels of bioethanol with very low concentrations of xylitol, can be explained by the presence of a set of physiological characters encoded by unique set of genes [10,19,34]. Thus, the high capacity of xylose fermentation and low levels of xylitol accumulation by *S. passalidarum* was speculated

to be due to the cofactor's equilibrium between the intracellular demand and supply of the cofactors via NADH-favored xylose reductase enzyme and NAD<sup>+</sup>-specific xylitol dehydrogenase enzyme, the key enzymes of the xylose utilization pathway [10,28]. Normally, the xylose metabolization occurs through the reduction of xylose to xylitol with xylose reductase, which requires NADPH or NADH as a cofactor with preference for NADPH. Only few NADH-favored xylose reductase enzymes have been described so far [10,35–37]. Then the xylitol is metabolized further by xylitol dehydrogenase which is strictly NAD<sup>+</sup> dependent (Figure 2). The unbalance between NAD<sup>+</sup> supplement and requirement can block the xylose metabolization and leads to accumulation of xylitol. Later, *S. passalidarum* was found to harbor two genes encoding for xylose reductase (*SpXYL1.1* and *SpXYL1.2*) [28]. The *SpXYL1.1* gene product is more equivalent to *XYL1* found in other yeasts. The expression levels of *SpXYL1.2* were found to be higher than *SpXYL1.1* and bioethanol production in *S. passalidarum* was attributed to higher xylose reductase activity with NADH than with NADPH [28]. The *SpXYL1.2* was found to use both NADH and NADPH with preference for NADH, while *SpXYL1.1* was stringently NADPH-dependent. Furthermore, the transformation of *S. cerevisiae* with *SpXYL1.2* of *S. passalidarum* enabled the overexpressing *S. cerevisiae::SpXYL1.2* strain to grow anaerobically on xylose and to ferment it to higher ethanol yield than the isogenic *S. cerevisiae* TMB 3422 strain, which overexpresses *P. stipitis* *XYL1*. While, the *S. cerevisiae::SpXYL1.1* overexpressing strain was not able to grow on xylose [28]. Similarly, in the yeast-like fungus *Aureobasidium pullulans*, the overexpression of *SpXYL1.2* xylose reductase along with *S. passalidarum* xylitol dehydrogenase encoded by *SpXYL2.2* enhanced the xylose metabolization by 17.76% and improved the fermentation capability and the pullulan production by 97.72% of the overexpressing mutants compared with the parental strain [38].

Finally, a metabolic analysis of *S. passalidarum* speculated that NADH-preferred xylose reductase and NAD<sup>+</sup>-dependent xylitol dehydrogenase would tend to drive both of xylose assimilation via the oxidoreductase pathway and the acetaldehyde reduction to ethanol by the alcohol dehydrogenase enzyme [19]. Recently, a metabolic flux analysis of different xylose-fermenting yeasts confirmed a better cofactors balance within *S. passalidarum* cells during xylose catabolism to bioethanol production than within *P. stipitis* cells [31], which further supports the growth characteristics of *S. passalidarum*.

Collectively, those unique and unusual traits of *S. passalidarum* encourage using it as a source for genes to improve xylose utilization and bioethanol production from lignocellulosic biomass in the current xylose fermenting yeasts, such as *P. stipites*, or to introduce xylose metabolism genes to develop industrial strains of *Saccharomyces cerevisiae* capable of co-fermentation of pentose and hexose sugars. Or alternatively, to domesticate it given the excellent results already accomplished by wild-type representatives of that species for co-fermentation of mixed sugars. To facilitate that purpose, Li et al. (2017) developed a stable genetic expression system compatible with the CUG yeasts clade for genomic integration of Gene Of Interest (GOI) into several yeasts [39]. The developed multi-host integrative system was functional in several of the xylose-fermenting yeasts including *S. passalidarum*, *P. stipitis*, and *Candida jeffriesii* and *Candida amazonensis*, as well as in a hexose metabolizing yeast *Saccharomyces cerevisiae*, for heterologous expression of green fluorescent protein (GFP) or lactate dehydrogenase. For lactate dehydrogenase overexpressing strains, all the engineered yeast strains were able to metabolize either glucose (in case of *S. cerevisiae*) or xylose (in case of xylose-fermenting yeasts) to produce lactate [39].

## 5. New Adaptive Strains of *Spathaspora passalidarum* for Potential Industrial Applications

One of the unique features of those xylose metabolizing yeasts, is the ability to use not only the monomeric hexose and pentose sugars but also the high oligomeric disaccharide sugar in mixed co-fermentation [9,19], which can be an advantage for large scale industrial applications. The mild acid pretreatment of agriculture waste biomasses is relatively cheap and prevents the accumulation of harmful compounds, which inhibits the fermentation processes, but releases the sugars in higher oligomeric stats. Therefore, the ability of such native xylose fermenting strains, *P.*

*stipitis*, and *S. passalidarum*, to use the high oligomeric sugar can be a great advantage for various biotechnological applications.

One of the major problems that hinders the use of *S. passalidarum* for industrial bioethanol production, even with its remarkable ability for bioethanol production, is the high sensitivity of *S. passalidarum* to the chemical inhibitors, such as ferulic and acetic acids, which are released in preparation of hemicellulosic hydrolysates [9]. Several elaborative studies have focused mainly on improving the tolerability of *S. passalidarum* to the hydrolysates inhibitors with keeping in mind the bioethanol productivity of the strains [19,40–43]. Hou and Yao in (2012) reported a strong strain [40], which is able to grow on furfurals and many other inhibitors of wheat straw hydrolysate (75%) and able to accumulate up to 0.40 g/g ethanol. Such strain was generated through hybridization of a *S. cerevisiae* and a UV-mutagenized *S. passalidarum* [40]. In 2012 also, another resistant strain was developed under O<sub>2</sub> limiting conditions through several passage of the wild-type *S. passalidarum* NRRL Y-27907 on wood hydrolysate, followed by adaptive growth of the strain on corn stover AFEX (ammonia fiber expansion) hydrolysate [19]. Even with such efforts, the strain was not able to accumulate significant amounts of ethanol during the fermentation of the AFEX hydrolysate, despite its ability to grow in AFEX hydrolysate media. When the acetic acid was depleted from AFEX hydrolysate media, ethanol production was surprisingly observed with a yield of 0.45 g/g and most of the xylose content was consumed [19]. Later in 2017, Morales and colleagues developed an evolutionary adapted strain [41] with high tolerance toward the classical inhibitor of the fermentation processes, acetic acid, and that produces ethanol with a yield of 0.48 g/g. In a non-detoxified hydrolysate of *Eucalyptus globulus*, the authors reported also the ability of this strain to co-utilize mixed sugars of xylose, glucose, and cellobiose under microaerobic conditions [41]. This strain was generated by UV irradiation followed by successive growing of the strain under elevated acetic acid concentrations [41]. In similar way, another group also obtained a mutated *S. passalidarum* strain but via plasma mutagenesis and continuous cultivation in alkaline liquor pretreated corncob [42]. Under a simultaneous saccharification and co-fermentation, the obtained strain produced bioethanol with efficiency of 75% [42]. Finally, Su et al. in 2018 developed an adaptive *S. passalidarum* strain (named YK208-E11) [43], which is designated for resistance to AFEX hydrolysate inhibitors, from the wild-type NRRL Y-27907 through high-throughput screen via combining several approaches of batch adaptation, cell recycling, and cell mating [43]. The *S. passalidarum* YK208-E11 strain produced less biomass (about 40% compared to the wild-type), co-metabolized mixed sugars of xylose, glucose, and cellobiose, and exhibited a three-fold improvement in the ethanol production rate with a yield of 0.45 g/g. The whole genome sequence of *S. passalidarum* YK208-E11 strain revealed a deletion of about 11 kb in this strain. The ORF, which was deleted in *S. passalidarum* YK208-E11, is encoding for proteins predicted to be involved in cell division and respiration. Therefore, the authors speculated that this deletion may account for those unique adaptive/physiological features of this AFEX-acclimatized *S. passalidarum* YK208-E11 strain [43].

## 6. Future Perspective for Engineering New Strains for Better Bioethanol Production

The metabolic engineering approaches involve targeted overexpression and/or deletion of fermentative key genes that facilitates quick and efficient conversion of sugars into bioethanol with high recoverable yields [1,3]. As we discussed above, *S. passalidarum* xylose reductase and xylitol dehydrogenase are among the promising candidates for targeted overexpression. The cumulative knowledge of the transcriptomics, metabolomics, and comparative genomics studies for *P. stipitis* and *S. passalidarum*, identified other key enzymes controlling the xylose assimilation, rather than XDH and XR (Figure 2). One of such promising key genes is *adh* that encodes for fermentative isozyme alcohol dehydrogenase (ADH), which is vital for production and/or assimilation of ethanol (Figure 2). Generally, ADH catalyzes the final (rate limiting) step in the yeast glycolytic pathway, the reduction of acetaldehyde to ethanol and NAD<sup>+</sup>, and therefore it accepts NADH as a co-factor [44,45]. However, ADH enzymes are also able to perform the reverse reaction from ethanol to acetaldehyde, enabling the yeasts to oxidize and grow on ethanol as a carbon source. In *P. stipitis*, the ADH fermentative activities



is crucial not only for ethanol production and/or consumption but also for maintenance redox balance within the yeast cell, so it is considered to be a part of the cofactor balance system in *P. stipitis* [44].

The sequencing projects of *S. passalidarum* NRRL Y-27907 and *P. stipitis* CBS6054 (JGI-MycCosm) revealed the presence of several/different alcohol dehydrogenase (ADH) encoding genes. For example, in *P. stipitis*, seven genes were predicted to encode for alcohol dehydrogenase (*PsADH1* to *PsADH7*) enzymes [13,17]. Among them *PsADH1* and *PsADH2* were found to be essential for xylose assimilation and ethanol production [44,46]. Each of the ADH proteins in *S. passalidarum* and *P. stipitis* are supposed to have different kinetic properties. Some of the enzymes could be mainly responsible for producing ethanol while others might be responsible for oxidizing it. In *S. passalidarum*, the gene encoding for *SpADH1* was found to be expressed at a very high level during xylose metabolization [34]. In addition, metabolic analysis and metabolic flux analysis revealed that alcohol dehydrogenase is one of key enzymes driving ethanol production in *S. passalidarum* [19,31]. Notably, owing to relative *SpADH1* abundance, the *SpADH1* promoter was used to develop a multi-host integrative system for xylose-fermenting yeast [39].

While in *P. stipitis*, the function of some ADH enzymes are better understood, in particular *PsADH1* and *PsADH2* [13,17,44,46–49]. Transcriptomic studies of the *P. stipites adh* system indicated that the *PsADH* activities are correlated with and induced under O<sub>2</sub> limited/microaerobic conditions [46,48]. Under xylose fermentation, the *PsADH1* was found to be the primary key enzyme among the *PsADH* system. The deletion of *PsADH1* caused a reduction in *P. stipites* growth rate and a notable increase in xylitol accumulation accompanied with a dramatic decrease in ethanol production, due to intracellular cofactors imbalance [44]. The *PsADH2* is not expressed under microaerobic or aerobic conditions unless *PsADH1* is deleted [44,46], which further confirms that the significant role of *PsADH1* is in sugar assimilation and ethanol production. The levels of *PsADH1* and *PsADH2* transcripts were observed, however, to be low through xylose metabolism relative to the transcript levels of other fermentative and glycolytic enzymes [13,17]. In addition, *PsADH1* and *PsADH2* were able to complement the growth of the *S. cerevisiae*  $\Delta adh$  mutant on ethanol as a sole carbon source [47]. Moreover, *PsADH3* to *PsADH7* were speculated to keep the balance between the cofactors NADPH and NADH [17]. However, the expression patterns of the other *PsADHs* on xylose and glucose under microaerobic conditions, in particular, for *PsADH7* and *PsADH4* are not fully understood [13]. *PsADH5* was found in proximity to NADPH dehydrogenase, implying a function in maintenance the intracellular cofactors balance, however, it is not proven yet. Notably, *PsADH7* was found to be upregulated under aerobic growth on xylose [50]. *PsADH7* was described as a strictly NADP(H) dependant enzyme with broad spectrum for substrates-specificity, including variety of aromatic and linear aldehydes (e.g., acetaldehyde, butanal, propanal, and furfural) and alcohols (e.g., ethanol, butanol, pentanol, hexanol, and octanol) for forward and reverse reactions, respectively [50]. Surprisingly, *PsADH7* was able to utilize xylitol as a substrate too with moderate activity. In the same context, the overexpression of *PsADH7* into a *P. stipites* xylitol dehydrogenase mutant ( $\Delta PsXDH$ ) [18], which cannot metabolize xylitol and therefore cannot grow on xylose as a sole carbon source, was able exclusively to complement the growth of  $\Delta PsXDH$  on xylose, in contrast to *PsADH1*, 2, 4, and 5 [50]. Hence, there is a need to understand the kinetic characteristics of each of *PsADH* and *SpADH* enzymes in order to target the correct genes for overexpression and/or deletion. Finally, we would like to state that genes encoding for *adh* isozymes are worth studying, especially of *S. passalidarum*, owing to their significant functions in bioethanol production/consumption and/or intracellular cofactor balance.

## 7. Conclusions

Taken together, the advances in fermentation performance by *S. passalidarum* pave the way for engineering the conventional and the nonconventional fermenting yeasts, such as *S. cerevisiae* and *P. stipites*, for economical fermentation of hexose and pentose sugars in hemicellulosic hydrolysates on industrial scales. Keeping in mind that the efficient metabolization and fermentation of xylose is essential for the bioconversion of lignocellulosic biomasses into biofuels and chemicals, but the

conventional wildtype strains like *S. cerevisiae* cannot use the xylose. Therefore, researchers keep trying to engineer the xylose utilization pathway into the conventional yeast. The genomes of the natural xylose-fermenting yeasts, in particular of *P. stipitis* and *S. passalidarum*, are of huge importance, as their genomics features and regulatory patterns can serve as guides and genomic resources for further genetic engineering development in those native xylose-metabolizing yeasts or to engineer non-xylose fermenting yeasts. Therefore, *S. passalidarum* and *P. stipitis* can be considered as genomic treasure sources for various genes to engineer the xylose metabolism and to improve the bioethanol production [1,24,34].

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