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Complications remaining in the engineering of *Saccharomyces cerevisiae* towards the efficient hexose- and pentose-fermentation of lignocellulosic biomass

Pursuit for renewable energy sources

Bachelor thesis

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Abstract

Renewable energy sources are explored in order to replace the environmental harmful and depleting fossil fuel source. Sustainable second-generation based bioethanol is ought to be achieved by the biochemical use of microorganisms and enzymes. Nevertheless, no organisms have been discovered in nature that can convert both pentose and hexose sugars, present in the lignocellulosic biomass, at high rate and with high ethanol production yield. The current industrial relevance of *Saccharomyces cerevisiae* as hexose-fermenting agent makes it an appealing subject for bioengineering towards xylose fermentation and eventual co-consumption. Several strategies are engaged in this matter such as improvement of the endogenous enzymes and pathways, and the integration of the oxidoreductase or isomerase pathway. However, many issues exist in these endeavours. This report will discuss the complications remaining in the engineering of *S. cerevisiae* towards the hexose- and pentose-fermentation of lignocellulosic biomass.

Table of Contents

1. Introduction

After a period of using fossil fuels as the main energy source to power the engines of our transportation systems, the negative impact on the world's climate and environment is now measured and well known. Burning fossil fuels, resulting in the emission of greenhouse gasses (GHG), has contributed to the increase in average global temperature in the anthropogenic era (IPCC et al., 2011). The main effects of these anthropogenic GHG emissions are observed as receding of glaciers, a rise in sea level and loss of biodiversity. In addition, fossil fuel sources are finite and usage results in the rapid decrease of fossil fuel reserves (Shafiee & Topal, 2009).

In order to mitigate the impact of fossil fuels, technologies for the production of alternative transportation fuels is necessary. One of the leading alternatives is the production of first-generation bioethanol based on corn starch, and sugar cane. However, these raw materials also demand an environmental impact as the crop cultivation process causes ecological damages due to nitrogen and phosphorus fertilizers, pesticides, and erosion (Hill, 2009). In addition, the cultivation of first-generation bioethanol crops compete with food crops. This conflict results in an undesirable rise in the costs of both products (Nigam & Singh, 2011). It has been stated that the food vs. fuel competition has contributed to the 2007-2008 world food price crisis (Research Institute (IFPRI), 2012). Research has therefore been focussed on the production of second-generation biofuels, which are based on non-edible biomass and therefore do not require acreage.

Second-generation bioethanol is produced from lignocellulosic non-food biomass: residues, by-products and waste of the agricultural, industrial and forest industry. Examples of these materials are wheat straw, sugar cane bagasse or wood process wastes. Moreover, purpose-grown short rotation energy crops are also considered as second-generation feedstock. Although they will still require land, its energy yields are higher compared to purpose-grown first-generation biofuel crops with unnecessary by-products (Sims et al., 2009). Cellulose and hemicellulose are the main components of these biomasses. During the pre-treatment and hydrolysis of lignocellulosic biomass, polysaccharides, C6 (hexose) and C5 (pentose) sugars are released.

Up until now, no organisms have been discovered in nature that can convert both pentose and hexose sugars at high yield and high rate into ethanol (Ragauskas et al., 2006). Nevertheless, research has made great advances in bio-engineering microorganisms towards the co-consumption of both sugars (Kobayashi et al., 2018; Walfridsson et al., 1997; Watanabe et al., 2007). Although pentose fermentation has been achieved in laboratory conditions, progress still has to be made in order to produce a desired theoretical yield of 0.51 gram ethanol per 1 gram glucose or xylose from second-generation lignocellulosic feedstock.

Multiple species of bacteria, yeast and filamentous fungi are subjected to research or bioengineering to achieve efficient ethanolic pentose fermentation. For the production of bioethanol *Pichia stipitis, Escherichia coli* and *Candida shehatae* have been used as fermenting microbes. However, as *Saccharomyces cerevisiae* is already a relevant yeast in the large-scale industrial fermentation of first-generation biomass, it's potential for pentose fermentation is attentively explored. It tolerates high ethanol concentrations, withstands low pH conditions and is insensitive to bacteriophage infection, which is ideal for large industrial processes (Moysés et al., 2016). Moreover, as its convenient industrial use is already implemented, integrating large-scale lignocellulosic biomass into the process of existing bioethanol plants is approachable (Hahn-Hägerdal et al., 2007).

A major drawback in lignocellulosic-based bioethanol production by *S. cerevisiae* is its incapability to transport, metabolize and ferment pentose sugars, such as D-xylose. By means of genetic and

metabolic engineering, *S. cerevisiae* strains capable of co-consuming hexose- and pentose-sugars were obtained. Extensive research has already tackled and thereby established improvements for certain components of the xylose to ethanol route. Examples are *e.g.* upregulation of pentose phosphate pathway genes and engineering of specific xylose transporters, which will be elaborated on in section 3.

Despite these findings, not all issues are resolved. Although *S. cerevisiae* has a metabolic pathway for the conversion of xylulose, it does not for xylose. Two pathways found in naturally xylose-fermenting microorganisms serve as the foundation commonly used for the integration of a xylose-converting system in *S. cerevisiae* (Figure 1). For one, as carried out in xylose-fermenting yeast, the oxidoreductase pathway occurs in two enzymatic reactions. Xylose reductase (XR) reduces xylose to xylitol, and xylitol dehydrogenase (XDH) oxidizes the latter to xylulose, both reactions demanding cofactors. On the other hand, as carried out in xylose-fermenting fungi, bacteria and yeast, the isomerase pathway occurs in which xylose isomerase (XI) is solely employed.

Nevertheless, integration of these pathways experience obstacles in which clarification is necessary. Therefore this report will deal with the following question: which complications remain in the engineering of *S. cerevisiae* towards the hexose- and pentose- co-consumption of lignocellulosic biomass for the production of bioethanol?

2. Engineering of endogenous enzymes and pathways

As mentioned, current research has improved *S. cerevisiae* strains towards more efficient ethanol production. Components of the natural metabolic system of the yeast involved in the conversion of xylulose to ethanol and their enhancement will be discussed in the following.

Upregulation of the pentose phosphate pathway

The non-oxidative branch of the pentose phosphate pathway (PPP) in *S. cerevisiae* converts xylulose-5-phosphate through a series of biochemical reactions into pyruvate, subsequently converting it to ethanol via the glycolysis (Figure 1).

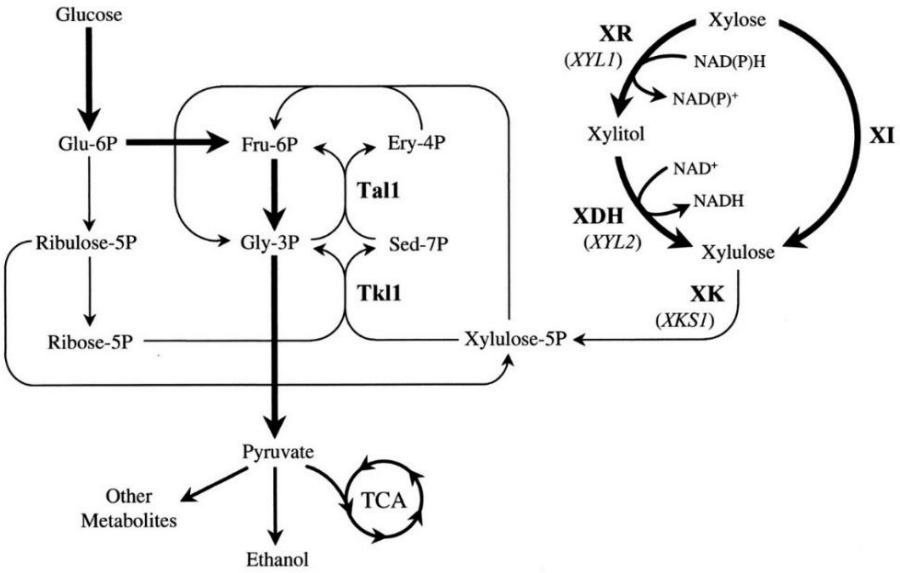

Figure 1
Overview of metabolic pathways for glucose and xylose metabolism. The glycolysis and the xylose utilization pathway are illustrated by thick lines. The PPP is indicated by thin lines. Abbreviations: TCA, tricarboxylic acid cycle; Tkl1, transketolase; Tal1, transaldolase; Glu-6P, glucose-6-phosphate; Fru-6P, fructose-6-phosphate; Gly-3P, glyceraldehyde-3-phosphate; Ery-4P, erythrose-4-phosphate; Sed-7P; sedoheptulose-7-phosphate. Adapted from "Metabolic Engineering of Saccharomyces cerevisiae" by S. Ostergaard et al., 2000, Microbiology and Molecular Biology Reviews.

The PPP limits the consumption of xylose and the conversion to ethanol due to insufficient capacity (Kötter & Ciriacy, 1993). Kötter and Ciriacy (1993) observed that sedoheptulose-7-phosphate (Sed-7P) accumulates in the cell and fructose-1,6-biphosphate (Fru-6P) is absent. PPP enzymes transketolase (TKL) and transaldolase (TAL) are responsible for the conversion of xylulose-5-phosphate (Xul-5P) to Sed-7P and subsequently to Ery-4P, respectively. Overexpression of both enzymes in *S. cerevisiae* leads to enhanced growth on xylose (Walfridsson et al., 1995). Moreover, upregulation of all non-oxidative PPP genes and thereby increasing their activity, such as ribulose-5-phosphate 3-epimerase and ribose-5-phosphate ketol-isomerase, results in improved xylose metabolism (Kobayashi et al., 2018).

Xylose uptake and transporter specificity

Non-specific sugar transporters facilitate the low affinity and inefficient transport of xylose in XR/XDH expressing strains. Glucose inhibits the uptake of xylose in *S. cerevisiae*, thereby hindering the simultaneous consumption. Therefore, improvement of sugar transporters, focussing on xylose affinity and efficiency, is necessary.

The endogenous hexose transporters Hxt1-7 and Gal2 are key components of glucose uptake of *S. cerevisiae*, with ten other, non-expressed Hxt transporters. Upon the deletion of the expressed genes, growth on glucose and xylose was prohibited (Reifenberger et al., 1995). After subsequently long-term chemostat cultivation, evolutionary adaption allowed the hexose transporter deletion strain to grow on xylose (Shin et al., 2015). Expression of *HXT11* was considered responsible for the redeemed cell growth. Using error-prone PCR the *HXT11* gene obtained a mutation at residue N366, which yielded improved xylose affinity and decreased glucose affinity. The mutated Hxt11 transporter was able to co-consume glucose and xylose (Shin et al., 2015). The mutated transporters is an efficient xylose specific transporter that can be used in *S. cerevisiae* strains for the production of bioethanol in glucose and pentose mixture cultivation.

Moreover, another study showed improved xylose transport allowing for co-consumption of glucose and xylose (Nijland et al., 2014). After evolutionary engineering, an endogenous hexose transporter (Hxt36) was obtained with the same asparagine mutation (N367I) as in Hxt11, providing the insight for the Hxt36-N367A variant. The engineered transporter possesses a tuneable specificity for glucose and xylose, and subsequent mutations in the ubiquitin binding sites decreased the turnover of the transporter in the absence of glucose (Nijland et al., 2016).

Heterologous expression of 26 sugar transporters from seven different xylose-fermenting organisms was examined (Young et al., 2011). Transporters from bacterial origin showed deficiencies, probably due to post-translational issues. The transporters isolated from *Pichia stipitis* and *Debaryomyces hansenii* allowed for growth on xylose. However, further genetic engineering is needed to achieve the desired functionality and sugar affinity for xylose.

Xylulokinase activity

Besides the xylose transporter limitations, the endogenous *S. cerevisiae* xylulokinase XK is also considered as rate limiting step in the fermentation of xylulose since it has a rather low activity rate (Richard et al., 2000). The enzyme catalyses the ATP-depended phosphorylation of xylulose to xylulose-5-phosphate, which is then channelled into the pentose phosphate pathway (PPP) for further metabolism. Deletion and overexpression of the *XKS1* gene led to defective cell growth when grown on xylulose as the sole carbon source, whereas only the wild-type and minor upregulation of XKS1 yielded growth on xylulose (Rodriguez-Pena et al., 1998). This explains the contradicting findings on the effect of overexpression of XK in recombinant XR/XDH *S. cerevisiae* strains. Toivari et al. (2001) observed that overexpression of XK leads to increased production rates and ethanol yields in strains grown on xylose. Similarly, Johansson et al. (2001) found improved ethanol yields, however they

observed reduced xylose consumption. They hypothesized that the extensive overexpression of XK has a negative effect on the metabolic pathway by accumulation of xylulose-5-phosphate and ATP depletion. The high activity of an enzyme involved in an early stage of the metabolic pathway apparently takes its toll on the system. The XK activities of Toivari et al. (2001) and Johansson et al. (2001) were 0.42 U/mg protein and 28 to 36 U/mg protein respectively.
Jin et al. (2003) investigated the effect of *S. cerevisiae* (*XKS1*) and *Pichia stipites* (*XYL3*) xylulokinase activity on xylose metabolism in *S. cerevisiae* by varying the copy number and promoter strength for both genes. They observed that a moderate (*ps*)XK activity yields the most efficient ethanol production. However, the definition of 'moderate' XK activity seems debatable as the optimal activity depends on multiple factors. Jin et al. suggests that intrinsic strain-specific properties such as XR and XDH activity, and (oxidative) PPP capacity matter in the equation as they are all related to X5P and ATP. Extrinsic factors such as aeration, which controls the generation of ATP, also influence the XK activity which make it difficult to determine the actual in-vivo value. For further improvements Jin et al. propose a reverse engineering strategy. By means of a yeast integration vector, Parekh et al. (1996) constructed *S. cerevisiae* strains with 1 to 30 copy numbers after a single transformation. This enables different levels of copy number amplification tuned with antibiotic resistance, which is stable for over 50 generations. When combined with *XKS1* or *XYL3*, the optimal XK activity can be fine-tuned according to the intrinsic and extrinsic factors.

GRE3 and ScXYL2

Although *S.* cerevisiae has been considered incapable of xylose to xylulose conversion, an endogenous aldose reductase (Gre3), capable of converting xylose to xylitol, has been discovered (Garay-Arroyo & Covarrubias, 1999). Strains in which *GRE3* is deleted show no xylitol formation (Träff et al., 2002), indicating that the *GRE3* is the major xylose reductase of *S. cerevisiae*. Therefore, the effect of *GRE3* activity on xylose-fermenting recombinant *S. cerevisiae* was investigated. It was observed that in a recombinant strain expressing XDH and an extra copy of XK, GRE3 was responsible for the xylose to xylitol conversion (Träff et al., 2002). Overexpression of *GRE3* resulted in increased xylose consumption and ethanol formation. This opens the possibility for a semi-endogenous metabolic xylose pathway in *S. cerevisiae*.
Furthermore, xylitol accumulation is considered as a rate-limiting factor in the integration of the oxidoreductase pathway in *S. cerevisiae*. As *GRE3* contributes to the xylitol formation, deletion of the gene in a recombinant strain with XR, XDH and an extra copy of XK was also examined. Träff et al. (2002) showed decreased xylitol formation and an increased ethanol yield in this strain. Despite this improvement, biomass was reduced by 31%. The detoxification of methylglyoxal via Gre3 is given as a possible explanation.
Xylitol has also been known to inhibit the xylose isomerase pathway in *S. cerevisiae* (Yamanaka, 1969). *GRE3* deletion in an XI expressing strain resulted in two- to three-fold decreased xylitol formation compared to the XI expressing reference strain. However, a low xylose consumption rate was measured in both strains (Träff et al., 2001). As the integration of the isomerase pathway in *S. cerevisiae* has been challenging, it is hard to ascribe the culprit of the results in this study. Nevertheless, *GRE3* expression levels should be taken into consideration for finetuning the xylose isomerase pathway.

Moreover, the endogenous xylitol dehydrogenase (*ScXYL2*, *Sc*XDH) also contributes to xylose metabolism of *S. cerevisiae* (Richard et al., 1999). However, it is of low expression in wild-type yeasts. Both *Sc*XDH and Gre3 enzymes are repressed by glucose (Batt et al., 1986). Nevertheless, it was found that *S. cerevisiae* can grow on xylose when the endogenous enzymes are overexpressed. Compared to recombinant strains expressing the heterologous XR/XDH pathway, it was observed that the non-

recombinant strains grow slower and accumulate more xylitol. The imbalance of redox cofactors NADPH/NAD^+ is a limiting factor also seen in the integration of heterologous XR. However Gre3 solely accepts NADPH as a cofactor, heterologous XR also accepts NADH. This creates an even more severe redox imbalance compared to XR (Toivari et al., 2004). It seems that the strong evolutionary directed preference for glucose of *S. cerevisiae* cannot easily be surpassed. Reversely, Attfield et al. (2006) obtained a non-recombinant strain to grow on xylose by natural selection and breeding, recurrently very slow. Combination of strategies of both Toivari et al. and Attfield et al. might enhance the industrial potential of non-recombinant *S. cerevisiae.* Note that this type of strategy does not involve genetically modified organisms (GMOs), which to some consumers is a benefit in regard to growing concern about GMOs (National Science Foundation USA, 2018).

3. Integration of the xylose reductase and xylitol dehydrogenase pathway

As mentioned, several yeasts (e.g. *Pichia stipitis, Pachysolen tannophilus* and *Candida shehatae*) can convert xylose to xylulose. In previous studies, recombinant *S. cerevisiae* strains have been shown to grow on xylose as sole carbon source by introducing genes *XYL1* and *XYL2* obtained from *Pichia stipitis*, encoding the NAD(P)H dependent XR and NAD^+ dependent XDH respectively (Tantirungkij et al., 1993)(Kötter & Ciriacy, 1993)(Walfridsson et al., 1997)(Meinander et al., 1999). However, the desired ethanol yield and production rate were not obtained and high amounts of xylitol were observed. This is supposedly due to the formation of xylitol and the imbalance of redox cofactors involved in the conversion, in which a shortage of NAD^+ occurs. Multiple strategies have been employed in order to rectify this.

Expression levels and ratios of xylose reductase and xylitol dehydrogenase

To reduce xylitol formation, Walfridsson et al. (1997) studied the influence of different XR and XDH ratios on ethanol production during xylose utilisation of recombinant *S. cerevisiae* strains, harbouring overexpressed *TAL1* and *TAL2,* encoding transaldolases. This study showed that an optimal XR:XDH ratio of 0.06 prevented xylitol formation and produced the highest ethanol yield compared to ratios of 5.0 and 17.5.

Similarly, Eliasson et al. (2001) study is in unison with the substantial effect of the enzymatic ratio effects on product formation. Xylulokinase (XK) was added to the XR/XDH ratio optimization for the desired decrease in xylitol formation and a kinetic model predicted that with a XR/XDH ratio of less than 0.1 minimized the xylitol accumulation. Experiments with recombinant *S.* cerevisiae were done in high-performance bioreactors and initial XR/XDH/XK ratio of 1:15:16 lead to minimal xylitol and enhanced ethanol formation.

According to Walfridsson et al., the composition of the formed products is probably due to both a changed equilibrium of XR and XDH, and a favourable redox balance. Glycerol and acetic acid, by-products of the metabolic pathway, are both relevant to the redox balance in the cell. On the contrary, ethanol formation is redox-neutral. Overexpression of XDH activity compared to XR activity can therefore possibly mitigate the redox imbalance.

However, cultivation of the strains in shake-flasks (batch) are not suitable to obtain significant quantitative flux data. Controlled chemostatic cultivations should therefore be performed in order to determine the metabolic changes in the XR/XDH ratio. Furthermore, the amount of aeration influences the reaction (Jin et al., 2003) and therefore must be studied as well.

Downregulation of the oxidative pentose phosphate pathway

Another way of influencing the redox balance is by reducing the oxidative pentose phosphate pathway flux (Jeppsson et al., 2002). By means of lowering phosphoglucose isomerase (PGI) activity, deleting the *GND1* or *ZWF1* gene encoding 6-phosphogluconate dehydrogenase (6-PGDH) and glucose-6phosphate dehydrogenase (G6PDH), the oxidative PPP activity was lowered or inactivated. Although reduced PGI and deletion of *GND1* have negative effects on glucose utilization (Genet, 1986)(Sinha & Maitra, 1992), strains lacking G6PDH grow normally and although this is suggested as the main source of NADPH in yeast (Nogae & Johnston, 1990). Jeppsson et al. constructed *S. cerevisiae* strains which harbours one of these modifications, together with PsXR and PsXDH. They found that the G6PDH deficient strain produced an ethanol yield of 0.41 g/g consumed sugar and a xylitol yield of 0.05 g/g consumed sugar. As the reaction catalysed by G6PDH was absent the NADPH level in the cell was depleted. According to Jeppsson et al. this resulted in the low xylitol amount. They proposed that with the decreased NADPH cofactor source of XR, its cofactor use shifted to NADH. Thereby producing NAD^+ and creating the cofactor source for XDH, which in turn converts this to NADH for its reaction. In this way, XI and XDH cofactors have an improved balance.

On the downside, a low xylose consumption was observed due to the strictly NADH-based xylose conversion. For efficient and sustainable biomass usage, a high xylose consumption rate is necessary. In order to pursue this strategy, a greater NADH source should be considered. The upregulation of other biochemical pathways generating NADH can be endeavoured in order to create a viable NADH source for the XR/XDH driven xylose conversion. For example, the high specificity of G6PDH for $NADP^+$ could be altered to NAD^+ (Fuentealba et al., 2016). Instead of downregulation, an upregulated NAD^+ coenzyme-specific G6PDH can contribute to an increased NADH concentration in the cell.

Altered coenzyme preference for xylose reductase and xylitol dehydrogenase

As mentioned, the NAD^+ dependent XDH is restricted due to the NADPH preference of XR. By means of genetic engineering, the coenzyme preference of the xylose reductase should, preferably, be altered towards a preference for NADH (Jeppsson et al., 2005)(Petschacher & Nidetzky, 2008)(Bengtsson et al., 2009). Multiple site-specific mutagenesis was performed on *P. stipitis* xylose reductase, targeting the Lys270. This residue was considered as it is important in the binding site for the phosphate group of NADPH in human aldose reductase and it is located in the conserved coenzyme binding Ile-Pro-Lys-Ser motif found in all yeast xylose reductases. Petschacher et al. (2008) and Jeppsson et al. (2005) confirmed the critical role of Lys270 in the IPKS motif in NADPH binding. It was shown that the Lys270Met mutant significantly decreases the affinity for NADPH. However, the NADH affinity remained unaltered, indicating that further research of the enzyme-cofactor binding is needed.

Bengtsson et al. (2009) constructed an XR carrying a Lys270Arg mutation. This was based on an NADH-preferring XR of *Candida parapsilosis*, which carries this arginine instead of a lysine in the IPKS motif. The acquired recombinant *S. cerevisiae* strain showed a high ethanol yield (0.39 g/g consumed sugar) and low xylitol formation (0.05 g/g consumed xylose).

Likewise, the NAD^+ coenzyme preference of xylitol dehydrogenase is also pursued towards an altered preference for $NADP^+$. It was shown that a complete reversal of coenzyme specificity from NAD^+ to $NADP^+$ (Watanabe et al., 2004) was possible. A triple and quadruple mutant, D207A/I208R/F209S and D207A/I208R/F209S/N211R respectively, of *P.* stipitis XDH showed an 4500-fold higher values in Kcat/Km with $NADP^+$ coenzyme specific XDH than the wildtype XDH with $NADP^+$. Similar to the XR mutations, the D207/I208/F209(/N211) residues were targeted as they are considered to be part of the coenzyme binding site. In-vitro addition of a zinc atom, to guarantee thermostability of the enzyme, yielded a significant catalytic activity with $NADP^+$. When expressed in *S. cerevisiae*, the

quadruple mutant strain achieved an ethanol yield of 0.46 g/g consumed sugar and a xylitol formation of 0.2g/g consumed sugar (Watanabe et al., 2007). However, in the experiment all glucose was consumed in 15h, whereas only 68% of the xylose was consumed after 72h. Evidently, higher consumption rates and absolute consumption of xylose are needed and therefore further research is necessary. The yeast strain used in this research did not possess the upregulated pentose phosphate pathway or engineered high-affinity sugar transporters. Therefore, a combination of these modifications could generate an improved and efficient metabolic flux in order to obtain efficient ethanol production. In conclusion, Watanabe et al. strategy resulted in promising findings which significantly improve the xylose reductase and xylitol dehydrogenase pathway

4. Integration of the xylose isomerase pathway

As mentioned in the introduction, the enzyme xylose isomerase is responsible for the conversion of xylose to xylulose in bacteria and some fungi. This reaction does not require co-factors and will therefore not affect the redox balance in the cell, in contrast to the oxidoreductase pathway. Research focussed on the expression of the bacterial XI gene (*xylA*) in *S. cerevisiae* inevitably encounters complications. By some means, a low enzymatic activity in the yeast is observed, even though the theoretically required amount of enzyme was produced (Sarthiy et al., 1987). Further research used the expression of *Thermus thermophiles xylA* and high enzymatic activity at 85°C, contrasting a low activity at the optimal growth temperature of *S. cerevisiae* of 30°C (Walfridsson et al., 1996). After random PCR mutagenesis of the gene encoding the XI enzyme, efficient xylose conversion was still not achieved (Lo et al., 2002). The reason for this deficiency remains unclear.

However, a xylose isomerase pathway was discovered in the fungus *Piromyces* sp. strain E2 (Harhangi et al., 2003) and unlike other xylose-fermenting yeasts employing the oxidoreductase pathway, this unicellular eukaryote converts xylose to xylulose using a xylose isomerase. Functional expression of the xylose isomerase was observed in recombinant *S. cerevisiae* containing the *Piromyces* XylA gene (Kuyper et al., 2003). High anaerobic xylose consumption was achieved when the engineered strain sequentially was subjected to directed evolution, anaerobic selection and extensive metabolic engineering (Kuyper et al., 2005). This metabolic engineering includes, amongst others, the upregulation of the pentose phosphate pathway. An ethanol yield of 0.43 g/g consumed sugars in anaerobic batch cultivation was measured. Van Maris et al. (2007) found similar results to this in terms of ethanol yield of 0.40 g/g consumed sugar by integrating *Pichia stipitis* xylose isomerase. Kuyper et al. argues that the theoretical yield of 0.51 g/g consumed sugar cannot be reached due to suppression of biomass formation by maintenance energy requirements. Moreover, in glucose-xylose co-cultivation a commonly lower xylose consumption rate is observed. As the endogenous sugar transporters remained untouched, the low affinity for xylose is hence stated as bottleneck. With further development, this research reports a promising prospect.

Metal homeostasis: *PMR1*

Although the reaction catalysed by XI does not involve redox cofactors, divalent cations are required in order to yield an active enzyme. Common sources of these metal ions are Co^{2+}, Mn^{2+} and Mg^{2+}. (Bhosale et al., 1996). Verhoeven et al. (2017) reported, after evolutionary engineering, mutations in the *PMR1* gene of xylose-adapted *Piromyces* XI expressing *S. cerevisiae* strains. This gene encodes a Ca^{2+}/Mn^{2+} ATPase located on the Golgi membrane, facilitating Ca^{2+} and Mn^{2+} transport into the apparatus. XI-expressing strains with deleted *PMR1* allowed for instantaneous growth anaerobic growth on xylose, in contrary to a 12-day adaption period required by the parental XI-expressing strain. Relatively high intracellular Mn^{2+} concentrations were measured in the *PMR1* strains. Although this improved the anaerobic growth on xylose, it negatively affected aerobic growth. In addition, high

intracellular Mn^{2+} concentrations can lead to several complications in the cell: extreme sensitivity for manganese ion toxicity, impaired mitochondrial function and Mn^{2+}-induced apoptosis (Verhoeven et al., 2017). Despite these factors, Verhoeven et al. (2017) study emphasises the influence of metal homeostasis in assimilation of xylose isomerase in recombinant *S. cerevisiae*.

It should be noted that Watanabe et al. (2007) improved the thermostability and catalytic efficiency of an engineered $NADP^+$-dependent XDH by providing specific cysteine ligands creating an additional zinc-binding site for the enzyme. Thereby also confirming the importance of the role of metal ions in enzyme fine-tuning.

5. Conclusion

Several strategies are engaged in the engineering of *S. cerevisiae* towards the co-fermentation of glucose and xylose, with some studies yielding more prosperous results than others.

S. cerevisiae strains harbouring the oxidoreductase pathway show several complications in the metabolic pathway. High amounts of xylitol are produced and the imbalance of cofactors is a bottleneck. By fine-tuning the expression levels of xylose reductase and xylitol dehydrogenase, the amount of xylitol formation can be reduced (Walfridsson et al, 1997)(Eliasson et al., 2001). However, an efficient ethanol yield is still not achieved. Although Jeppsson et al. (2002) observed a promising ethanol yield of 0.41 g/g consumed sugar by means of downregulating G6PDH, this strategy had a major downside. A low xylose consumption prevented efficient use of the provided biomass. A strategy of altering the coenzyme preference of XDH resulted in an ethanol yield of 0.46 g/g consumed sugar. Once again, the xylose uptake, and consumption rate, were very low (Watanabe et al., 2007).

The most promising strategy is the integration of the xylose isomerase pathway (Table 1). Both integration of *Piromyces* sp. and *Pichia stipitis* XI observed a high ethanol yield of 0.43 g/g consumed sugar and 0.40 g/g consumed sugar, respectively (Kuyper et al., 2005)(Van Maris et al., 2007). Only minor complications occur in these applications. In combination with enhanced xylose transporters and the upregulation of PPP, an industrial strains seems to come in sight. Deletion of *PMR1* increased the intracellular Mn^{2+} concentration which improves the XI activity. However this did not yield the desired results. Verhoeven et al. (2017) emphasized the influence of metal homeostasis in assimilation of xylose isomerase in recombinant *S. cerevisiae*.

It is clear that upregulation of the non-oxidative branch of the pentose phosphate pathway is an effective method in order to improve ethanol yield (Table 1). Kobayashi et al. (2018) showed that overexpression of *RK1, TKL1* and *TAL1* in xylose isomerase strains showed an high ethanol yield of 0.45 g/g consumed sugar. In addition, mutations in Hxt7, Hxt11 and Hxt36 show to yield efficient transporters for the co-uptake of glucose and xylose (Farwick et al., 2014)(Nijland et al. 2014)(Shin et al. 2015). The combination of the isomerase pathway and the upregulation of PPP genes makes for an appealing industrial strain.

Table 1: xylitol formation (g l^{-1}) and ethanol yield (g l^{-1}) in different strategies of engineering

Target	Strategy	Xylitol formation	Ethanol yield	Cultivation	Source
Integration of XR/XDH	XR/XDH ratio of 1:15	None	0.35	Oxygen limited, 20 g l^{-1} glucose and 20 g l^{-1} xylose	Walfridsson et al., 1997
Oxidative PPP in XR/XDH expressing *S. cerevisiae*	Downregulation of oxidative PPP G6PDH	0.05	0.41	Batch cultivation, 50 g l^{-1} xylose (sole carbon source)	Jeppsson et al., 2002
Coenzyme specificity of XDH	$NADP^+$ preference	0.2	0.46	Oxygen limited, 5 g l^{-1} glucose and 15 g l^{-1} xylose	Watanabe et al., 2007
Integration of XI	*Pichia stipitis* XI	0.07	0.40	Anaerobic, 20 g l^{-1} glucose and 20 g l^{-1} xylose	Van Maris et al., 2007
PPP in XI expressing *S. cerevisiae*	Overexpression of *RKI1, TKL1, TAL1*	5.03	0.45	Micro-aerobic, 85 g l^{-1} glucose and 35 g l^{-1} xylose	Kobayashi et al., 2018

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