Exploring metal ion metabolisms to improve xylose fermentation in *Saccharomyces cerevisiae*

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Summary

The development of high-performance xylose-fermenting yeast is essential to achieve feasible conversion of biomass-derived sugars in lignocellulosic-based biorefineries. However, engineered C5-strains of *Saccharomyces cerevisiae* still present low xylose consumption rates under anaerobic conditions. Here, we explore alternative metabolisms involved in metal homeostasis, which positively affect C5 fermentation and analyse the non-obvious regulatory network connection of both metabolisms using time-course transcriptome analysis. Our results indicated the vacuolar Fe²⁺/Mn²⁺ transporter CCC1, and the protein involved in heavy metal ion homeostasis BSD2, as promising new targets for rational metabolic engineering strategies, enhancing xylose consumption in nine and 2.3-fold compared with control. Notably, intracellular metal concentration levels were affected differently by mutations and the results were compared with positive controls isu1Δ, a Fe-S cluster scaffold protein, and ssk2Δ, a component of HOG pathway. Temporal expression profiles indicate a metabolic remodelling in response to xylose, demonstrating changes in the main sugar sensing signalling pathways.

Introduction

The increasing environmental concerns regarding energy production from fossil sources have driven the development of cleaner alternatives to restructure the global energy matrix. A renewable and environment-friendly alternative to produce fuels and chemicals is the use of lignocellulosic biomass, a plant-derived feedstock essentially composed of cellulose (40–60%), hemicellulose (20–40%) and lignin (10–24%) (Sharma et al., 2019). The cellulose and hemicellulose fractions are mainly constituted of glucose (C6) and xylose (C5) monosaccharides and the maximum conversion by microbial platforms into higher added value bioproducts is essential for the feasibility of lignocellulose-based biorefineries.

The budding yeast *Saccharomyces cerevisiae* is the commonly eukaryotic organism used in industrial bio-based processes due to its robustness against several stresses (Attfield, 1997). However, although this yeast excels in fermenting glucose, wild type strains are unable to ferment pentose sugars as xylose, requiring synthetic biology strategies towards integration of heterologous metabolic pathways (Moysés et al., 2016). Two different xylose consumption pathways are commonly used to engineer a yeast cell: the oxidoreductive (OXR) pathway, which converts xylose to xylulose by an oxi-reduction reaction catalysed by two enzymes, xylose reductase (XR) and xylitol dehydrogenase (XDH); and the xylose isomerase (XI) pathway, which consists in a single-step reaction catalysed by the XI enzyme, encoded by xylA. Since XI does not require redox cofactors necessary for XR/XDH activity, the use of the isomerase pathway does not lead to a cellular redox imbalance present on the OXR alternative, being a more advantageous C5 metabolic route (Kwak and Jin, 2017). However, although *xylA* expressing strains achieve higher ethanol yields (Li et al., 2016), it still presents low anaerobic xylose consumption rates, demanding further genetic modifications to increase strain's performance. Other modifications, such as overexpression of genes from pentose phosphate pathway (PPP), deletion of the aldose reductase GRE3 or expression of heterologous transporters can be a relevant approach to improve strain fitness (Bueno et al., 2020).

Newly arising beneficial mutations fixed in cell population during evolution experiments are frequently off-pathway and can be a promising approach to explore molecular basis and alternative metabolisms that positively affect fitness in anaerobic C5 fermentation (Zhou et al., 2012; Qi et al., 2015). Recently, evolutionary engineering studies revealed a strong selective pressure.
on genes involved in metal homeostasis as deletion of the genes encoding a scaffold protein for mitochondrial iron-sulfur clusters (ISC) biogenesis, the ISU1, and a Ca\(^{2+}/\)Mn\(^{2+}\) Golgi ATPase, the PMRT gene, was reported to improve xylose-to-ethanol conversion on xyA strains (Santos et al., 2016; Sato et al., 2016; Verhoeven et al., 2017). As XI is a metalloenzyme that requires two divalent metal cations for the isomerization of xylose into xylulose (Kovalevsky et al., 2010; Lee et al., 2017); changes in intracellular metal homeostasis could enhance XI activity in vivo by increasing the availability of cofactors (Lee et al., 2020). Interestingly, deletion of ISU1 also had a positive effect on an XR/XDH strain (Osiro et al., 2019), suggesting a broader relation of metal homeostasis in improving xylose fermentation through optimization of other metal-dependent enzymes or different pathways of the cell metabolism. Additional gene mutations on the high osmolarity glycerol (HOG) pathway were also described by both studies that found the evolution-based mutations on ISU1 (Santos et al., 2016; Sato et al., 2016). The knock-out mutations in the mitogen-activated protein kinase (MAPK) gene, the HOG1, and in the SSK2 gene, encoding an upstream MAPKKK from HOG pathway (Saito and Posas, 2012), were described as capable to improve xylose metabolism on anaerobic conditions, however, few indications on how these mutations could interfere with xylose metabolism have been described (Wagner et al., 2019).

Recently, a mathematical genome-scale metabolic model was constructed to predict responses of metabolism and gene expression upon the availability of metal ions, demonstrating the dependence of enzymes on metal ions and the effect in yeast metabolism on a genome-scale (Chen et al., 2021). Heterologous pathways can be included in the model, amplifying the field for its application in the design and improvement of distinct bioproducts. The model was able to predict the influence of iron in an engineered yeast for biosynthesis of \(p\)-coumaric acid (\(p\)-HCA), demonstrating the important application of metal homeostasis in optimization of cell factories to the biotechnological industry and metabolic engineering studies.

In this work, our goal was to provide deeper insights into how different metabolisms related to metal homeostasis could affect xylose fermentation by evaluating uncorrelated genes in a C5 engineered strain. Here, we explore how those mutations affected the yeast xylose fermentation, metal intracellular content, resistance to oxidative stress and gene expression, comparing with the previous phenotypes found on isu1.1 and ssk2.1 mutant cells. We also explore a global time-course transcriptional profile of ISU1 deletion to further enlightenment on the molecular mechanisms which allow an enhanced xylose consumption phenotype.

**Results and discussion**

**Rational selection of gene targets**

To further investigate the hypothesis whether modifications in metabolisms associated with metal homeostasis could impact xylose fermentation, six genes from different metabolic pathways related to metal homeostasis were rationally selected to be deleted on the xylose-consuming strain C5TY (C5-Trial Yeast), a yeast cell designed specifically to screen for mutations related to xylose metabolism. The yeast C5TY has an additional copy of each of the genes encoding a xylulokinase (XKS1) and of four genes from PPP (TAL1, RK11, TK1 and RPE1), deletion of GRE3 and multiple integrated copies of xyA.

The choice of the genes targets was based on distinct and distant functions from the genes previously described in the literature (Santos et al., 2016; Sato et al., 2016; Verhoeven et al., 2017) and their function alters the accumulation or intracellular flow of divalent metals as cobalt, manganese, magnesium and iron, which have been reported to be cofactors of XIs (Kovalevsky et al., 2010; Lee et al., 2017; Fig. S1). The genes selected includes BSD2, involved in heavy metal homeostasis (Liu et al., 1997); CCC1, a vacuolar Fe/Mn\(^{2+}\) membrane importer (Li et al., 2001); FRE6, a ferric reductase (Martin et al., 1998); HMX1, a heme oxygenase (Protzenko and Pilpott, 2003); MNR2, a vacuolar Mg\(^{2+}\) exporter gene (Pisat et al., 2009); and SMF3, a vacuolar Fe\(^{2+}\) exporter with the opposing function of Ccc1p (Portnoy et al., 2000).

**Genes involved in metal homeostasis impacts xylose fermentation**

To assess novel putative metabolisms associated with improved xylose fermentation, single deletions were performed in the background strain C5TY for the selected genes highlighted above and on ISU1 and SSK2 genes (as positive controls), which deletions have been previously described to positively affect xylose consumption (Santos et al., 2016; Sato et al., 2016). A double mutant ccc1.1ssk2.1 was also generated to verify the synergistic effect of these mutations in xylose consumption. Fermentation assays were performed with all mutants (bsd2.1, ccc1.1, fre6.1, hmx1.1, mnr2.1, smf3.1, isu1.1 and ssk2.1) and the control, C5TY, in YP-media supplemented with 50 g l\(^{-1}\) xylose as the sole carbon source. Maximum specific growth rate (h\(^{-1}\)) and the fermentative parameters ethanol productivity (g l\(^{-1}\) h\(^{-1}\)), ethanol yield (g-ethanol/g-xylose) and maximum specific xylose consumption rate (g g\(^{-1}\) CDW h) were evaluated for all strains (Table 1).
Table 1. Fermentation performance of mutants and parental strain C5TY.

| Strain | Max. specific growth rate (h⁻¹) | Ethanol productivity per hour (g l⁻¹ h⁻¹) | Ethanol yield per xylene (g g⁻¹) | Max. specific xylene consumption rate (g g CDW⁻¹ h⁻¹) | Xyitol yield per xylene (g g⁻¹) |
|--------|---------------------------------|------------------------------------------|---------------------------------|-----------------------------------------------|-------------------------------|
| C5TY   | 0.0535                          | 0.05 ± 0.00                              | 0.33 ± 0.04                     | 0.52 ± 0.07                                    | 0.05 ± 0.01                   |
| smf3Δ  | 0.0475                          | 0.03 ± 0.00                              | 0.32 ± 0.08 (–0.75%)            | 0.29 ± 0.01 (–43.20%)                          | 0.07 ± 0.02                   |
| mnr2Δ  | 0.0336                          | 0.04 ± 0.00                              | 0.22 ± 0.02 (–32.83%)           | 0.48 ± 0.02 (–6.06%)                           | ND                            |
| hmx1Δ  | 0.0319                          | 0.09 ± 0.02                              | 0.31 ± 0.04 (–6.29%)            | 0.54 ± 0.02 (4.50%)                            | ND                            |
| fre6Δ  | 0.0313                          | 0.10 ± 0.00                              | 0.36 ± 0.06 (10.85%)            | 0.52 ± 0.04 (0.87%)                            | ND                            |
| bsd2Δ  | 0.0642                          | 0.11 ± 0.02                              | 0.40 ± 0.02 (23.69%)            | 0.59 ± 0.16 (14.41%)                           | 0.02 ± 0.00                   |
| ssk2Δ  | 0.0619                          | 0.27 ± 0.02                              | 0.42 ± 0.00 (28.01%)            | 0.98 ± 0.13 (90.61%)                           | 0.01 ± 0.00                   |
| ccc1Δ  | 0.0687                          | 0.42 ± 0.01                              | 0.43 ± 0.00 (31.70%)            | 1.08 ± 0.05 (109.26%)                          | 0.01 ± 0.00                   |
| isu1Δ  | 0.0096                          | 0.53 ± 0.00                              | 0.43 ± 0.00 (31.58%)            | 1.25 ± 0.10 (142.08%)                          | 0.01 ± 0.00                   |

ND, not determined.

All cultivations were performed in YPX50 and values represent the averages of three biologic replicates ± SD between 0 and 40 h. The percent difference of ethanol yield and maximum specific xylene consumption rate related to C5TY are represented between parentheses.

Consistent with a previous study (Santos et al., 2016), isu1Δ strain required only 40 h to consume all xylene present on the medium, exhibiting the highest ethanol productivity (0.53 ± 0.00 g l⁻¹ h⁻¹) and xylene consumption rate (1.25 ± 0.10 g g⁻¹ CDW h) among all strains (Fig. 1 and Table 1), showing an 11-fold enhancement of xylene fermentation when compared with the control. Deletion of CCC1, led to a ninefold increase in ethanol productivity (0.42 ± 0.01 g l⁻¹ h⁻¹), surpassing the performance of the previously described SSK2 loss-of-function mutation (Santos et al., 2016), which achieved an ethanol productivity of 0.27 ± 0.01 g l⁻¹ h⁻¹ in this study (Fig. 1 and Table 1). Interestingly, when deletion of CCC1 and SSK2 was combined to create the double mutant ccc1Δssk2Δ, the resulted strain showed a productivity sixfold higher than the single mutant ccc1Δ in 24 h, equalling the performance along with the fermentation (data not shown). Synergistic interaction between SSK2 and ISU1 have also been reported to enhance xylene metabolism (Santos et al., 2016).

The strain bsd2Δ also presented an increase of 2.3-fold in ethanol productivity compared with the control (Table 1). In contrast, strains harbouring the knockout of SMF3 and MNR2 encoding the vacuolar exporters of Fe²⁺ and Mg²⁺, respectively, demonstrated the worst performance in xylene among all deletants (Fig. 1 and Table 1), even though both deletants did not decrease glucose growth (data not shown).

Deletion of HMX1 and FRE6 showed more subtle changes on xylene fermentation (Table 1), suggesting that those deletions might have a more subtle impact on the cellular iron metabolism under iron-sufficient conditions than the other genes evaluated.

Intracellular metal levels were affected differently by mutations

The previously described improvement in xylene utilization due to PMR1 loss-of-function was associated with the
higher intracellular manganese content found in this mutant since the loss of function of PMR1 increases the cytosolic concentration of Ca^{2+}/Mn^{2+} by disabling their transport from cytosol into the Golgi lumen, increasing the available cofactor levels for xylA activity (Yu et al., 2012; Verhoeven et al., 2017.). Therefore, we speculated if the increase in intracellular metal ions could also trigger the enhancement of xylose consumption found in our strains.

To verify how mutations changed the intracellular metal content, we analysed the intracellular availability of calcium (Ca), cobalt (Co), iron (Fe), magnesium (Mg), manganese (Mn) and zinc (Zn) on the selected strains isu1Δ, ccc1Δ, ssk2Δ, bsd2Δ and smf3Δ compared with the control C5TY (Fig. 2A). Intracellular amounts of magnesium and zinc did not have expressive changes across the mutant strains when compared with C5TY, while cobalt content had a significant decrease in almost all strains (Student’s t-test, p < 0.05), except in ccc1Δ. Interestingly, ccc1Δ did not have expressive alterations in any metal concentration when compared with the control, even though its deletion is known to increase cytosolic amounts of Fe/Mn^{2+} by disabling vacuole storage of these ions (Li et al., 2001).

In contrast, deletion of BSD2 led to the highest intracellular accumulation of iron and manganese observed across the strains. Even though BSD2 deletion was reported to accumulate Co, Cd and Mn by increasing membrane stability of the heavy metal transporters Smf1p/Smf2p (Liu et al., 1997; Yu et al., 2012;), in our results even bsd2Δ strain showed a decrease in cobalt content compared with the control. Interestingly, it also shows an increase in intracellular iron that was not reported up to date.

Although it has been proposed that Smf3p acts as a Fe^{2+} vacuolar importer, smf3Δ also led to an increase in manganese content, agreeing with a previous report (Yu et al., 2012). This transporter is part of the family of the same Nramp family of transporters of Smf1p/Smf2p,
which are directly related to manganese homeostasis, suggesting that Smf3p could also be involved in manganese homeostasis. Since SMF3 deletion disables cells to access its vacuolar storages, it is suggested that besides cellular accumulation, metal ion compartmentalization is an important factor for xylose fermentation in XI-engineered strains.

As described in literature (Garland et al., 1999), isu1Δ exhibits an increased amount of iron and, curiously, it also had led to a significant increase in calcium content. On the other hand, ssk2Δ did not lead to an increase in intracellular metal concentration. SSK2 gene encodes an upstream MAPKKK of the HOG signalling pathway involved in the adaptation to high osmolarity stress (Saito and Posas, 2012) and its deletion was reported to lead to a decrease in active Hog1p (Lavinà et al., 2014), the kinase effector of the HOG pathway. Although ssk2Δ did not change intracellular metal levels, a link between the HOG pathway and iron homeostasis was reported as Hog1p was recently described to negatively regulate the iron uptake by favouring nuclear export of the transcription factor Aft1p (Martins et al., 2018).

Mutations did not impair strain tolerance to oxidative stress

High levels of metals can be toxic to the cells by the increase of oxidative stress (Morano et al., 2012), which is an undesirable trait to a scale up industrial strategy for engineered strains. Since it was observed changes in intracellular concentration of metal ions and/or its cellular distribution on mutant cells compared with control, a tolerance assay to oxidative stress with menadione or hydrogen peroxide (H2O2) was done (Fig. 2B) and the generation of cellular reactive oxygen species (ROS) was measured by fluorescent microscopy using the dye dihydorhodamine 123 (DHR 123; Fig. 2C).

Despite the increase of intracellular metal levels found in the mutants isu1Δ, ccc1Δ, ssk2Δ, bsd2Δ and smf3Δ strains, the oxidative stress response assay showed no change in ROS levels and growth in the presence of H2O2 and menadione when compared with the control.

Main differences in gene expression started at the beginning of fermentation

In order to understand the unique fermentation profile of each strain and to identify the main differences of gene expression shown on xylose, exploring individual cellular signatures, solutions and responses of each metabolism, we selected the four best mutants isu1Δ, ccc1Δ, ssk2Δ and bsd2Δ, based on their fitness, productivity and ethanol yield (Table 1), to analyse gene expression patterns and transcriptome profiles through fermentation.

We first compared the mRNA from parental strain, C5TY, with the mutant yeast cells isu1Δ, ccc1Δ, ssk2Δ and bsd2Δ at their first sample, corresponding to 8 h after the beginning of the experiment. Although xylose consumption profile at 8 h does not show significant differences between the selected strains (Fig. 1), isu1Δ and ssk2Δ surprisingly showed large changes in gene expression, presenting 1.199 and 997 differentially expressed genes (DEGs) relative to C5TY (Fig. 3A and B). However, ccc1Δ and bsd2Δ strains showed only 34 and 37 genes differentially expressed, respectively (Fig. 3C and D).

Among the genes differently expressed in the strains, deletion of CCC1 was the only to increase the expression of GRX4 and TYW1 genes, encoding a cytosolic monothiol glutaredoxin and an iron-sulfur protein, which protects cells from iron toxicity and is required for synthesis of Wybutosine-modified tRNA (Mühlenhoff et al., 2010; Li et al., 2011), respectively, remaining upregulated all long of fermentation. Both genes are under the control of the transcription factor Yap5p in iron-replete conditions (Martínez-Pastor et al., 2017), which suggests that ccc1Δ cells are sensing high levels of intracellular iron even though this strain did not present significant alterations on intracellular iron concentrations. Since the Ccc1p transporter works in the intracellular distribution of ions and its deletion increases cytosolic amounts of iron (Li et al., 2001), the iron sensed by the mutated cell might be differentially compartmentalized compared with the control.

To characterize the main transcriptional changes at the beginning of fermentation on isu1Δ and ssk2Δ strains, we performed the functional enrichment analysis of DEGs classified by biological process from gene ontology (GO) terms (http://www.geneontology.org/) on each mutant, considering only results for Benjamini-Hochberg corrected p-value < 0.05. Table S1 summarizes the top 10 GO terms classified by their biological process found on DEGs of each mutant strain for 8 h of fermentation.

The GO terms found in isu1Δ strain are similar to the transcriptional response to fermentable sugars, such as glucose, expressing upregulated genes enriched mainly in the glycolytic process (GO:0006096) and tRNA aminoacylation for protein translation (GO:0006418), and downregulated genes enriched for nitrogen utilization (GO:0019740), fatty acid beta-oxidation (GO:00066418), and mitochondrial gene expression (GO:0140053) and cellular respiration (GO:0045333), suggesting that the transcriptional responses necessary for xylose fermentation were present on isu1Δ strain since the beginning of the fermentation. Nevertheless, the term glucose import (GO:0046323), which includes hexose transporters responsible for xylose transport, was also overrepresented among the
downregulated genes, suggesting a non-efficient regulation of the transport system in the presence of this pentose.

In addition, as ISU1 is involved in mitochondrial iron-sulfur biogenesis, the expression of genes from this metabolism was explored more deeply (Fig. S2). As expected, isu1Δ was the only strain with a wide alteration in genes required for cellular iron-sulfur cluster assembly at this time point, exhibiting six genes of this metabolism downregulated (GRX5, ISA1, NFS1, ISD11, CIA2, NAR1) and two genes upregulated (MET18 and TAH18). Interestingly, the strain ssk2Δ, which presented the second most DEG profile at 8 h, also presented the upregulation of MET18 and TAH18, indicating the broad application of these genes in cellular metabolism.

On the other hand, the ssk2Δ strain showed different GO terms of downregulated and upregulated genes compared with the isu1Δ strain. As Ssk2p plays a role in the HOG stress pathway, its deletion has led to the downregulation of genes involved in different types of stress, such as response to H₂O₂ (GO:0042542), regulation of protein stability (GO:0031647), and cellular response to heat (GO:0034605; Table S1), even though it did not impair the tolerance to oxidative stress of this mutant (Fig. 2B and C). Although SSK2 deletion has been reported to lead to a decrease in active Hog1p (Laviña et al., 2014), HOG1 expression in ssk2Δ strain did not change compared with the control. Moreover, a HOG-independent function of Ssk2p has been described (Laviña et al., 2014) and thus we could not assume that the phenotype exhibited for ssk2Δ was only due to its connection to the HOG pathway.

Fig. 3. Graphical representation of DEGs at 8 h of fermentation. mRNA from parental strain, C5TY, was compared with the expression of the mutant yeast cells isu1Δ (A), ssk2Δ (B), ccc1Δ (C) and bsd2Δ (D) at 8 h of fermentation. Only transcripts with FDR < 0.01 and log2FC > 0.58 between the compared groups were considered as differentially expressed (red). Non-significant genes are shown in grey. Relative mRNA concentrations were calculated from three independent biological replicates.
Mutations favour the expression of genes required for xylose fermentation

The carbon flux through the PPP and glycolysis is a bottleneck in xylose fermentation. To investigate how the selected mutations altered the yeast primary metabolism, we analysed the temporal expression levels of genes from the central carbon metabolism and aerobic respiration (Fig. 4 and Fig. S3).

In strains in which xylose was completely or almost exhausted from media until 48h (isu1Δ and ccc1Δ), expression of genes related to the PPP and alcoholic fermentation is correlated with xylose concentration, decreasing its expression as the pentose is consumed from the medium (Fig. 4A and Fig. S3). In contrast, genes involved in the TCA, glyoxylate cycle and aerobic respiration were upregulated only when xylose starts being depleted (Fig. 4B and C and Fig. S3).

![Fig. 4. Temporal profiles of genes involved in the central carbon metabolism, aerobic respiration and TCA/glyoxylate cycle in each mutant relative to the control C5TY at specific time-points. Genes from alcoholic fermentation, xylose metabolism and PPP (A), aerobic respiration (B) and TCA and Glyoxylate cycles (C) are represented in this bubbleplot. Colours show up (red) and downregulation (blue) of genes and the bubble size is proportional to average Log2 fold change values of genes in each time-point compared with its respective time of C5TY.](image-url)
phosphoenolpyruvate carboxykinase (PCK1) and fructose-1,6-bisphosphatase (FBP1), gluconeogenesis specific enzymes, were downregulated at the initial fermentation points of almost all strains compared with the control, changing their expression only after the sugar exhaustion, corresponding to 40 h and 48 h for isu1.1 and ccc1.1, respectively (Fig. 4A).

Interestingly, although changes in expression of isu1.1 and ssk2.1 have started earlier in the fermentation when compared with the other mutants, ccc1.1 had a more similar temporal transcription profile to isu1.1 than the ssk2.1 mutant. Both deletions of CCC1 and ISU1 are known to increase the cytosolic amounts of Fe$^{2+}$ by different mechanisms: while CCC1 deletion disables vacuole storage of Fe/Mn$^{2+}$, ISU1 deletion has a wider effect on the iron homeostasis, leading to constitutive activation of the transcription factors Aft1/2p, which controls a set of genes related to iron homeostasis. However, comparing isu1.1 and ccc1.1 strains at their start of xylose consumption (when both strains had consumed around 10 g l$^{-1}$, corresponding to 16 and 24 h, respectively) to the control C5TY at its start of xylose consumption (48 h), deletion of ISU1 generated a high expression ($\log_{2}$FC > 3) in genes related to stressful conditions, as heat shock proteins, that was not observed in ccc1.1 (data available on YeastC5 platform), suggesting that its deletion is more stressful than CCC1 deletion, even though the strain resistance to oxidative stress was not affected (Fig. 2B and C).

In contrast, despite its faster xylose consumption and higher ethanol productivity when compared with C5TY, bsd2.1 did not show expressive differences in gene expression when compared with the corresponding time-point of the control.

**Temporal expression profile of isu1Δ indicates a metabolic remodelling in response to xylose**

Previous works have reported that yeasts engineered for xylose metabolism present under anaerobic conditions a respiratory response compared with glucose, which impairs xylose fermentation (Jin et al., 2004). However, as our time-course fermentation data does indicate a fermentative response in the presence of xylose when compared with the control and a respiratory response is present only after xylose is depleted, we selected the isu1.1 strain to perform a complete temporal analysis of mRNA expression to understand the regulation of metabolic changes that had led to an increase in xylose consumption rates. To capture the full xylose fermentation profile, we collected six successive 8 h intervals samples over 48 h after isu1.1 start of fermentation and all time-point were compared with its first sample after inoculation (8 h) to perform the time-course analysis.

As metabolic changes in the glycolytic pathway are linked to sugar sensing (Conrad et al., 2014), genes from the three main sugar signalling pathways (cAMP/PKA, Snf1/Mig1 and Snf3/Rgt2) were assessed (Fig. 5A). The SNF1 pathway, which is known to be expressed only in glucose-limited conditions, showed upregulation of the transcriptional activators involved in the metabolism of non-fermentable sugars, CAT8 and SIP4, as well as their targets FBP1, MLS1, ACS1 and PCK1, only after xylose depletion from the medium, suggesting that xylose was recognized as a fermentable sugar in this mutant.

Interestingly, SUC2, another glucose repressed gene encoding an invertase, showed its expression downregulated when compared with the control C5TY (data available on YeastC5 platform) and decreases its expression overall fermentation time (Fig. 5A). A recent study using a fluorescent biosensor coupled to the promoter region of SUC2 showed that even high xylose concentrations (50 g l$^{-1}$) fully induce its expression in the same manner that it does in low glucose conditions, whereas low C5 concentration (5 g l$^{-1}$) slightly affect this biosensor (Osiro et al., 2018). Curiously, our results showed that SUC2 is downregulated even in the presence of xylose concentration (50 g l$^{-1}$) when compared with the control C5TY (data available on YeastC5 platform), unlike reported with SUC2-biosensor. These results suggest that the presence of xylose in our mutant might mimics signals of glucose availability as it shows the genetic patterns of carbon catabolite repression in the presence of xylose. Additionally, the HXK2 gene, which product is also known to be involved in SNF1 inactivation and cooperates in the regulation of glucose-repressed genes (Conrad et al., 2014), was upregulated at the beginning of fermentation, when the sugar was present, becoming downregulated only after 40 h, reinforcing an inactive SNF1 pathway in the presence of xylose in our strain isu1.1. Curiously, the downregulation of HXK2 at the end of fermentation was observed for all mutants, except for bsd2.1 (data available on YeastC5 platform).

Expression of HXK2 is also related to an active PKA pathway (Conrad et al., 2014). The genes required for intracellular PKA activation, RAS1 and CYR1, as well as two catalytic subunits of PKA, TPK1/2 genes, showed a decrease in expression throughout fermentation time, while the RAS/PKA inhibitor IRA2 increased its expression only after xylose depletion. Decreased expression on IRA2 was related to improve xylose consumption by constitutive activation of PKA (Myers et al., 2019). This result suggests an active PKA in the isu1.1 strains at the beginning of fermentation, agreeing with the GO enrichments related to fermentation previously described.

Although these results suggest that xylose triggered in isu1.1 mutant similar metabolic changes as glucose, most of the hexose transporters maintained its expression
downregulated all long of fermentation, except for HXT1 and HXT4 (Fig. 5B). HXT1 is a low-affinity glucose transporter which expression is induced under presence of glucose and, interestingly, its expression was also found in strains genetically modified to improve xylose consumption, harbouring the disruption of IRA2 and ISU1 genes (Osiro et al., 2019).

Expression of hexose transporters in yeast is controlled by the sugar sensors Snf3p and Rgt2p in the presence of low and high glucose concentrations, respectively (Bisson et al., 2016). These sugar sensors did not show high expression at the beginning of fermentation, suggesting that these sensors did not sense xylose as a fermentable sugar (Fig. 5A). This hypothesis agrees with the finding that, although cells cannot sense extracellular xylose, it responds towards intracellular xylose (Osiro et al., 2019).

As ISU1 is related to the control of metal homeostasis, we also examine genes involved in iron metabolism. Isu1p is a scaffold necessary for assembly of the mitochondrial iron-sulfur clusters (ISCs), which generates an intermediated compound, called (Fe-S)int, necessary for production of the cytosolic ISCs by the cytosolic iron-sulfur cluster assembly (CIA) machinery (Pandey et al., 2019). In conditions of iron sufficiency, cytosolic ISCs regulates the expression of the transcription factors Aft1/2p, which induce the expression of a set of genes in response to iron deprivation called the iron regulon (Fig. 6). Deletion of ISU1, as disruption of other genes of mitochondrial ISC assembly, has been reported to increase the mitochondrial iron content and constitutively activate the transcription factors Aft1/2p (Hausmann et al., 2008; Lill et al., 2014). Interestingly, although isu1Δ does have an increase in intracellular iron content (Fig. 2A), almost all Aft1/2p targets were downregulated at the beginning of fermentation (Fig. 5C). An alternative activation of the iron regulon relies on the connection between iron homeostasis and aerobic respiration (Casas et al., 1997), and a regulatory connection between the main sugar signalling pathways and the iron regulon has been described. While an active cAMP/PKA pathway was described to inhibit Aft1 expression (Robertson et al., 2000), the low-glucose sensing pathway Snf1/Mig1 is known to positively regulate Aft1/2p,
thus helping the switch from a fermentative to respiratory growth on glucose-limited conditions (Haurie et al., 2003; Fig. 6). This result agrees with the sensing profile presented by isu1p and reinforces the evidence of PKA activation in the presence of xylose and activation of SNF1/MIG1 pathway after xylose exhaustion.

Conclusion

In this study, we explore different metabolisms associated with metal homeostasis and the non-obvious connection with improved xylose conversion rates into bioproducts. Our results point to BSD2 and CCC1 as promising novel targets to engineer S. cerevisiae towards xylose fermentation. Specific transcriptional responses point unique solutions for each mutant while a temporal transcriptional profile demonstrates a global metabolic reprogramming favouring the expression of essential genes for xylose metabolism and downregulation of a respiratory response. Although S. cerevisiae is not able to sense extracellular xylose, the engineered yeast demonstrated changes in gene expression patterns in the main sugar sensing signalling pathways.

Experimental procedures

Growth conditions

Yeast cells were cultured at 30°C in YP medium (10 g l\(^{-1}\) yeast extract, 20 g l\(^{-1}\) peptone) supplemented with 20 g l\(^{-1}\) p-glucose as carbon source (YPD). For plates, 2% agar was added to the medium. Transformants were selected in YPD plates supplemented with 200 mg l\(^{-1}\) of Hygromycin B or 200 mg l\(^{-1}\) of geneticin.

Batch fermentations were performed in YP medium supplemented with 50 g l\(^{-1}\) of xylose as the sole carbon source (YPX). The cultures started at an initial OD\(_{600}\) of 1.0 and were incubated at 30°C and 200 rpm for 72 h. Fermentations were performed in biological triplicates using 100 ml sealed bottles containing a working volume of 70 ml. Samples for OD\(_{600}\) measurements, extracellular metabolites and RNA sequencing were collected and stored at −20°C.

Strains construction

The C5TY (C5-trial yeast) background strain used in this study was designed to express the complete xylose
pathway, including multi-copy integration of xyIA and without any beneficial mutation fixed by adaptive evolution experiments. A MATz haploid spore from PE-2 diploid strain was transformed with an additional copy of the genes xylulokinase (XKSI), transaldolase (TAL1), ribose-5-phosphate ketol-isomerase (RKI1), transketolase (TKL1), ribulose-5-phosphate 3-epimerase (RPE1) and the xyIA gene from Orpinomyces sp. (Madhavan et al., 2009) flanked by δ LTR sequences, stably integrated at the GRE3 locus under control of different strong constitutive promoters (Table S2). After adaptive evolution in xylose, the xyIA gene was tandem amplified in 30 copies and a mutation Ser98Phe in ISU1 gene was identified as responsible to improve xylose fermentation rates (personal communication). The mutation in ISU1 was reverted to its original wild-type allele, resulting in the C5TY strain.

Strains with specific deletions (bsd2Δ, ccc1Δ, isu1Δ, smf3Δ, ssk2Δ1, mnr2Δ, fre6Δ, hmx1Δ) were created by PCR amplifying the hphMX6 cassette from plasmid pAG32 (Goldstein and McCusker, 1999), or the KanMX4 cassette (Wach et al., 1994) from plasmid pFA6-kanMX4 for the double mutant ccc1Δ ssk2Δ4, with primers containing 42 bp homology to the upstream and downstream regions of target genes and then using the amplicon to delete genes on CSTY by homologous recombination in vivo. The transformation procedure was performed by lithium acetate using standard methods (Gietz and Schiestl, 2007) and all strains were confirmed for gene deletion by PCR with external flanking primers. Primers used for amplification and gene deletion confirmation are listed in Table S3.

**Intracellular metal content analysis**

Intracellular metal concentrations were analysed with an inductively coupled plasma optical emission spectrometer (ICP-OES, Optima™ 8000) using an adapted protocol from Eide et al. (2005) The yeast strains C5TY, smf3Δ, bsd2Δ1, ccc1Δ1, isu1Δ1 and ssk2Δ1 were grown in 100 ml of YPD medium at an initial OD600 of 0.5 and incubated at 30°C, 200 rpm, overnight. The culture was collected by vacuum filtration using 0.2 μm pore size membrane filters and cells were washed three times each with 30 ml of 1 μM EDTA disodium salt solution, pH 8.0, followed by three additional washes with 30 ml of deionized water. Filtered cells were incubated at 65°C for 2 h to obtain the dry mass weight. Cells were then digested by microwave digestion (ETHOS™ Easy) through the addition of 14 ml of HNO3 2.0 M at each sample followed by 1.0 ml of H2O2 30% v/v, with a heating profile of 200°C over 20 min and then held in 200°C for 15 min, 1800 W. The final sample solution was then subjected to the measurements. A standard curve of 0.1–10.0 mg l⁻¹ was prepared using a 1000 mg ml⁻¹ multi-element standard solution from PerkinElmer. All experiments were performed in triplicate.

**Oxidative stress resistance on spot-assay growth**

Cells grown overnight on YPD medium at 30°C and 200 rpm were harvested by centrifugation, washed three times, and suspended in sterile water at an initial OD600 of 1.0 and serial-diluted to give concentrations of 10⁻¹, 10⁻², 10⁻³ or 10⁻⁴. Samples (5 μl) of each suspension were inoculated on solid YPD medium supplemented with 80 and 100 μM of Menadione, 3 mM of H2O2 or without supplementation. All experiments were made in triplicate and plates were incubated at 30°C for 48 h.

**ROS assay by microscopy**

ROS were measured using DHR (Sigma-Aldrich) with a protocol adapted from previously described reports (Madeo et al., 1999; Peeters et al., 2017). The yeast strains C5TY, smf3Δ, bsd2Δ1, ccc1Δ1, isu1Δ1 and ssk2Δ1 were grown overnight on YPD medium and menadione was added to a final concentration of 100 μM in samples of each strain and incubated for 2 h at 30°C, 200 rpm. DHR was added to a final concentration of 10 μg ml⁻¹ on each yeast sample and incubated for additional 2 h in the dark. Cells were pelleted and resuspended on 1× PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4) for microscopy visualization. Stained S. cerevisiae cells were visualized on fluorescence microscopy (Carl Zeiss, Jena, Germany) equipped with Compact Light Source HXP 120C (Jena, Germany) using the 100× oil immersion objective lens. The captured images were further processed using AxiosVision software version 3.1.

**RNA extraction and library preparation**

Cells for transcriptional analysis were harvested in biological triplicates at specific times from fermentation culture: all strains were collected at 8, 32, 40 and 48 h (bsd2Δ1, ccc1Δ1, isu1Δ1, ssk2Δ1 and C5TY), and additional samples were collected at 12 h for isu1Δ1 and 24 h for the strains ccc1Δ1 and isu1Δ1. Total RNA was extracted using MasterPure™ Yeast RNA Purification Kit (Lucigen) according to the manufacturer’s directions. The RNA integrity and purity were checked by Agilent RNA 6000 Nano kit on Agilent 2100 Bioanalyzer (Agilent) and the RNA quantity was determined by Qubit™ RNA BR Assay Kit (Thermo Fisher Scientific, MA, EUA), both following the manufacturer’s protocols.

The RNA extracted was used to perform the Illumina® TruSeq Stranded mRNA Sample Prep (Low-Throughput
Protocol, according to the manufacturer’s protocol. Library was validated by quality control using Agilent DNA 1000 kit on the 2100 Bioanalyzer (Agilent) and the DNA concentration was determined by qPCR using KAPA qPCR MasterMix (Roche) on the Viia7 Real-Time PCR System (Thermo Fisher Scientific). DNA template for cluster generation was prepared with the indexed DNA library normalized to 10 nM and then pooled. All libraries were made manually.

RNA analysis and data processing
The quality of the reads was assessed with FastQC (v.0.11.6) (bioinformatics.babraham.ac.uk/projects/fastqc/). Low-quality reads and adapters were removed by Trimmomatic (v. 0.38) with default parameters (Bolger et al., 2014). SortMeRNA (v. 2.0) was used for rRNA filtering (Kopylova et al., 2012). The reads were then mapped against the genome of S. cerevisiae strain LVY34.4 using the mapping tool STAR (v 2.14; Dobin et al., 2013); allowing the detection of splicing events. The BAM files generated by STAR were provided to BRAKER annotation tool (v. 1.8; Hoff et al., 2019), which combines the tools GeneMark-ET (v. 4.29) and AUGUSTUS (v. 3.1.0) and the aligned reads as evidence to generate full gene structure annotations. The functional annotation of the identified genes was carried with the webtool eggNOG-Mapper (standalone version; Huerta-Cepas et al., 2017).

In order to quantify the transcript abundance the RSEM tool (v1.3.1) was employed with STAR as the mapping tool, and the genome of S. cerevisiae strain LVY34.4 and the transcriptome annotation generated by BRAKER as references. Differential expression analysis was made with the R package DESeq2 (v. 1.28.1; Love et al., 2014), transcripts with FDR < 0.01 and log2FC > | 0.58| between the compared groups were considered as differentially expressed. The RNA-Seq Illumina reads were deposited in the National Center for Biotechnology Information Short Read Archive (NCBI-SRA) and are publicly available under the accession number PRJNA708278 (BioProject ID).

YeastC5: A user-friendly application for transcriptome analysis
To facilitate transcriptome data visualization, an RNA-Seq bioinformatics tool was developed for a comprehensive automated analysis of the next-generation sequencing data produced in the time-course fermentations. The YeastC5 platform allows the user to explore the expression patterns ofisu1 Δ, ccc1 Δ, ssk2 Δ and bsd2 Δ mutants intra and inter-strains compared with the C5TY control. Additionally, pathway plots can be explored for each mutant for different primary metabolisms: aerobic respiration, glycolysis and gluconeogenesis, oxidative stress, PPP, TCA and glyoxylate cycles. The gene_ID is unique and guides the individual analysis in a user-friendly interface. The application is available on https://phybio.shinya pps.io/YeastC5/

Analytical procedures
The cellular growth was monitored by optical density (OD) measurements at a wavelength of 600 nm (OD₆₀₀) using an Ultrospec 2100 Pro spectrophotometer (Amersham Biosciences). Quantification of xylose, xylitol, acetate acid, glycerol and ethanol in the cultures was determined via high-performance liquid chromatography using chromatograph Alliance (Waters) with refractive index detector (Waters 2414) using ion exclusion HPX-87H column (300 x 7.8 mm², BioRad®), heated in an oven at 35°C, a 2 mM H₂SO₄ solution as the mobile phase at a flow 0.6 ml min⁻¹. A standard curve with known concentrations of compounds of interest was also analysed using the same procedure.

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Conflict of Interest
All authors declare that they have no conflict of interests.
Author Contribution

Gisele Cristina de Lima Palermo: Investigation, Methodology, Visualization, Writing - Original Draft, Writing - Review & Editing. Natalia Coutone: Data Curation, Writing - Original Draft. Joao Gabriel Ribeiro Bueno: Investigation, Writing - Original Draft. Lucas Ferreira Maciel: Software, Data Curation, Visualization. Leandro Vieira dos Santos: Conceptualization, Methodology, Supervision, Visualization, Project administration, Writing - Original Draft, Writing - Review & Editing, Funding acquisition.

Data Availability Statement

The sequence data have been submitted to the NCBI-SRA and are publicly available under the accession number PRJNA708278 (BioProject ID). Datasets related to this article can be found at https://phybio.shinyapps.io/

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