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Original Research Article

Metabolic engineering of *Yarrowia lipolytica* to produce chemicals and fuels from xylose

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ABSTRACT

Yarrowia lipolytica is a biotechnological chassis for the production of a range of products, such as microbial oils and organic acids. However, it is unable to consume xylose, the major pentose in lignocellulosic hydrolysates, which are considered a preferred carbon source for bioprocesses due to their low cost, wide abundance and high sugar content.

Here, we engineered *Y. lipolytica* to metabolize xylose to produce lipids or citric acid. The overexpression of xylose reductase and xylitol dehydrogenase from *Scheffersomyces stipitis* were necessary but not sufficient to permit growth. The additional overexpression of the endogenous xylulokinase enabled identical growth as the wild type strain in glucose. This mutant was able to produce up to 80 g/L of citric acid from xylose. Transferring these modifications to a lipid-overproducing strain boosted the production of lipids from xylose. This is the first step towards a consolidated bioprocess to produce chemicals and fuels from lignocellulosic materials.

1. Introduction

Microbial oils (oils originating from microbial sources) are promising alternatives to fossil fuels in that they can be used for the production of biofuels and oleochemicals (Caspeta and Nielsen, 2013; Shi et al., 2011; Zhang et al., 2012). Additionally, these microbial oils present multiple advantages over plant oils or animal fats since they are not competitive with food, they are less susceptible to changes in seasonal availability, and they can be easily manipulated to be enriched in an oil of choice (Ledesma-Amaro, 2015). For this reason, many attempts have been made in recent years to maximize lipid production in different microbes (Georgianna and Mayfield, 2012; Janssen and Steinbuchel, 2014; Zhu et al., 2012).

Among the different microbial hosts tested for lipid production, the oleaginous yeast *Yarrowia lipolytica* has been by far the most studied and engineered (Ledesma-Amaro and Nicaud, 2015). In recent years, different approaches have led to a substantial increase in lipid production, mainly from glucose as a carbon source. For example, the availability of the precursors malonyl-CoA (Tai and Stephanopoulos, 2013) and G3P (Dulermo and Nicaud, 2011) for triglyceride (TAG) production has been enhanced. The synthesis of TAG has been increased through metabolic engineering of the Kennedy pathway (Beopoulos et al., 2012), the degradation of TAG by intracellular lipases has been avoided (Dulermo et al., 2013), and beta oxidation has been abolished, thus impairing the degradation of fatty acids (Beopoulos et al., 2008; Dulermo and Nicaud, 2011). The combination of some of these modifications has produced a strain able to accumulate more than 60% of its DCW as fatty acids (Qiao et al., 2015). Nonetheless, even with this high lipid production, the process is still not economically feasible and so far, *Y. lipolytica* has only been used industrially to produce high-value lipids, such as omega 3 fatty acids (Xie et al., 2015; Xue et al., 2013). Further attempts are therefore required to reduce the price of these microbial oils. Recently, *Y. lipolytica* has been engineered to secrete fatty acids to the culture media, reducing the cost required to break the cells and extract the oils

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from them (Ledesma-Amaro et al., 2016).

Another important way to lower the cost of a microbial oil is to use inexpensive carbon sources in place of glucose (Ledesma-Amaro and Nicaud, 2016). To this end, *Y. lipolytica* has been metabolically engineered to improve its performance in fructose (Lazar et al., 2014) and glycerol (Papanikolaou et al., 2013), and to permit growth in sucrose (Hong et al., 2012), cellobiose (Guo et al., 2015; Lane et al., 2015), starch (Ledesma-Amaro et al., 2015), and inulin (Zhao et al., 2010). Unfortunately, *Y. lipolytica* is unable to fully use the most abundant and inexpensive carbon source in nature, lignocellulose material. Economic conversion of lignocellulose requires use of both pentose, mainly xylose, and hexose sugars. (Sanchez Nogue and Karhumaa, 2015). Engineering the use of xylose has also been a main focus in the yeast *Saccharomyces cerevisiae* for the industrial production of bioethanol (Kim et al., 2013). In this organism, the expression of a xylitol dehydrogenase (*XDH*) and a xylose reductase (*XR*) from *Scheffersomyces stipitis* is enough to permit growth with xylose as the sole carbon source (Kotter et al., 1990).

In order to metabolize xylose, the host must be able to take up pentose inside the cell through sugar transporters. Then, a xylose reductase converts D-xylose into xylitol with the concomitant oxidation of NADPH into $NADP^+$. A xylitol dehydrogenase transforms this xylitol into D-xylulose, reducing NAD^+ to NADH. D-xylulose is further phosphorylated by a xylulokinase into D-xylulose-5-P, which can enter the pentose phosphate pathway (Fig. 1).

Generally speaking, *Y. lipolytica* is unable to grow with xylose as its sole carbon source; this has been reported for most of the biotechnologically important strains (Coelho et al., 2010; Kurtzman, 2011; Ruiz-Herrera and Sentandreu, 2002; Stephanopoulos and Tai, 2013; Zhao et al., 2015). Nonetheless, there are a few controversial reports claiming that strain Po1g can grow on xylose (Blazeck et al., 2014; Wang et al., 2014), in spite of others declaring that the same strain cannot use this pentose (Stephanopoulos and Tai, 2013).

Recently two attempts have been made to permit *Y. lipolytica* to grow on xylose. Firstly, Stephanopoulos and Tai (2013) overexpressed *XR* and *XDH* of *S. stipitis* in *Y. lipolytica* strain Po1g. Although the modified strain was unable to use xylose, the authors performed an adaptation that resulted in moderate growth on xylose (doubling time of around 25 h). The engineered strain was cultured with helper substrates such as glucose or glycerol, and the ability to produce lipids was tested. A biomass of 18 g/L (13 g/L generated by glycerol) and 7.64 g/L of lipids, as well as 9.13 g/L citrate, was achieved from 20 g/L glycerol and 80 g/L xylose. Secondly, Ryu et al. (2015) recently adapted a strain to enable some growth using xylose as the sole carbon source. The growth of the mutant strain on xylose had a final biomass that was 16 times lower than that of the wild type on glucose. Specifically, the authors identified *XYL2* (*XDH*) as the limiting factor and, after overexpressing this gene, obtained a strain improved for growth on xylose, but still grew 10 times slower than on glucose. These two attempts to modify *Y. lipolytica* to utilize xylose as sole carbon source both resulted in a very-slow-growth phenotype that was unsuitable for large-scale industrial production and therefore, further research is needed in this direction.

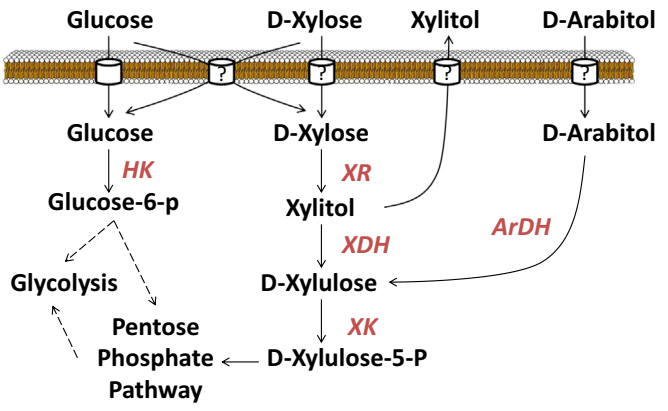

Fig. 1. Xylose utilization pathway, Schematic representation of the pathway leading to the consumption of glucose, xylose and arabitol. Unidentified transporters are represented by a question mark. XR: xylose reductase; XDH: xylitol dehydrogenase; XK: xylulose kinase; HK: hexokinase; ArDH: arabitol dehydrogenase. Dashed lines represent multiple metabolic steps.

Interestingly, *Y. lipolytica* is not only used for the production of lipids, but also for other biotechnological products such as organic acids, nutraceuticals, aromas, proteins, polyalcohols, emulsifiers, and surfactants (Ledesma-Amaro and Nicaud, 2015). Therefore, elucidating the key steps required for *Y. lipolytica* to grow well on xylose would have a tremendous impact on the production of these industrially relevant compounds, potentially reducing the cost of synthesis and making these green processes more competitive in the market.

In this work we aimed to generate a strain of *Y. lipolytica* able to use xylose efficiently. We found that the endogenous xylulokinase (*XK*) gene limits *Y. lipolytica*'s growth on xylose even with the expression of *S. stipitis XDH* and *XR*. We thus engineered a strain that was able to grow as well on xylose as glucose. Additionally, we combined these modifications into a strain that was able to accumulate high amounts of lipids and performed a fermentation strategy that led to the production of high titers of lipids. Finally, we also studied the ability of the engineered strain to produce another important biotechnological product, citric acid, using xylose as sole carbon source. Therefore, we describe for the first time the modifications that permit *Y. lipolytica* to efficiently use xylose to produce industrial products.

2. Results and discussion

2.1. The overexpression of heterologous xylose reductase, xylitol dehydrogenase, and endogenous xylulokinase are essential to permit Y. lipolytica to grow on xylose

Recently, Stephanopoulos and Tai (2013) patented an engineered strain of *Y. lipolytica* which overexpresses *ssXR* and *ssXDH* from *S. stipitis* under the control of the promoters *TEFin* and *hp4d*, respectively. In this work, we constructed a strain that overexpressed these genes under the control of the *TEF* promoter. The expression of both genes was checked by qPCR (Supplementary Fig 1). Like the previously patented strain, our modified strain was unable to grow in xylose (Fig. 2). However, the patented strain was able to slowly utilize xylose following an adaptation of the strain using serial dilutions, as previously reported for *S. cerevisiae* (Stephanopoulos and Tai, 2013). Unfortunately, the adapted strain had a doubling time of 25 h, which strongly limits its industrial application. In our hands, and probably due to the different strengths of the promoters, the strain that overexpressed both genes was unable to grow even after we attempted a similar adaptation. Likewise, strains Po1t (prototrophic strain of Po1g) and W29 (the parent of both Po1d and Po1g) were unable to grow with xylose as the sole carbon source (Fig. 2).

In order to study the transcription level of the first genes required for assimilating xylose, *ylXR* (YALI0D07634), *ylXDH* (YALI0E12463) and *ylXK* (YALIF10923), we performed qPCR analysis of RNA extracted from wild type *Y. lipolytica* grown either in glucose or in xylose containing media. The highest transcription level was found for *ylXR*, followed by *ylXDH* (Fig. 3), which seems to agree

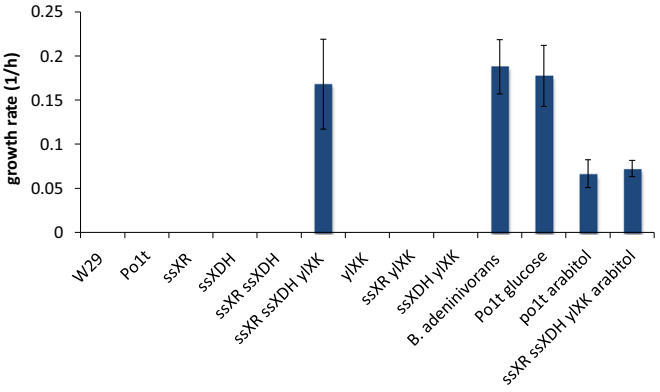

Fig. 2. Growth rate of different strains in microplate conditions. Bar plot representation of the growth rate of different *Y. lipolytica* strains and the LS3 strain of *B. adeninivorans*. The absence of a bar means that no growth was detected. The values represent the average growth rate (1/h) and the standard deviation of at least two replicates growing in microplates. Minimal media was used and the carbon source is xylose, unless specified in the figure as glucose or arabitol.

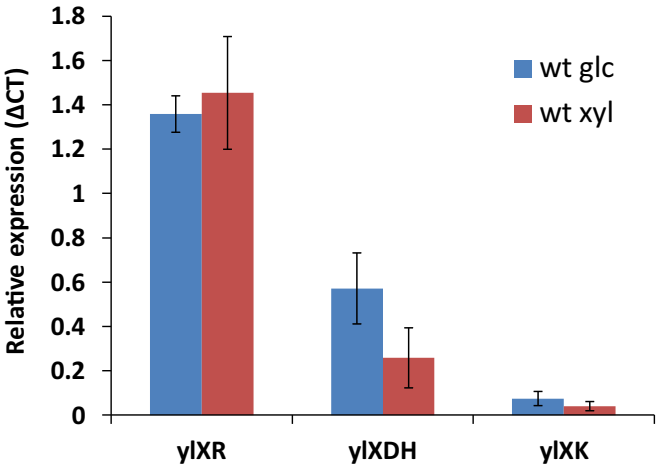

Fig. 3. Expression profile of the genes involved in xylose degradation. Cells of the wild type strain were first growing for 16 h in YNB medium with 6% glucose and then incubated for 4 h in YNB medium containing either 6% glucose (glc) or 6% xylose (xyl). Gene expression levels were normalized based on the expression levels of the actin gene (ΔC_T).

with the findings of Ryu et al. (2015) that associated *XDH* to the limiting step. *ylXK* transcription level was even lower (Fig. 3). The expression of none of the three genes was induced in xylose media (Fig. 3), as previously described (Ryu et al., 2015).

Therefore, in order to further engineer our strain to grow in xylose, we focused our efforts on the third enzyme of the pathway, the xylulokinase (*ylXK*). This enzyme is presumably encoded in *Y. lipolytica* gene *YALI0F10923* and it presents 40% and 52% identity with the XKs of *S. cerevisiae* and *S. stipitis*, respectively. We therefore cloned this gene into *Y. lipolytica* under the control of the *TEF* promoter. The overexpression of *ylXK* was checked by qPCR (Supplementary Fig 1). The generated strain was able to grow in a medium containing xylose as the sole carbon source (Fig. 2). Thus, the strain overexpressing *ssXR*, *ssXDH*, and *ylXK* was designated XYL+. These experiments were carried out in microplates, and *Blastobotrys (Arxula) adeninivorans* was used as a positive control for growth on xylose since this organism is well-known for its ability to utilize xylose (Boer et al., 2005). Instead, Po1t growing on glucose was a control that represented *Y. lipolytica*'s regular growth (Fig. 2). Interestingly, the XYL+ strain did not demonstrate improved growth in D-arabitol over the wild-type strain (Fig. 2), despite the fact that the metabolism of this substrate may also have a step involving D-xylulose (Fig. 1). This may suggest either the existence of a different catabolic pathway for arabitol, or that the same pathway is limited by inefficient transport into the cell, insufficient arabitol dehydrogenase activity, or putative toxicity of the alcohol. In microplate conditions, the XYL+ strain had a growth rate in xylose ($0.17 \pm 0.05\ h^{-1}$) similar to that of the parental strain in glucose ($0.18 \pm 0.04\ h^{-1}$). We therefore decided to study its behavior in flasks, where better aeration conditions and higher biomass formation are often achieved.

2.2. *Y. lipolytica strain XYL+ is able to grow efficiently on xylose but still prefers glucose*

The engineered strain of *Y. lipolytica* that overexpressed *ssXR*, *ssXDH*, and *ylXK* (XYL+) was able to use xylose as the sole carbon source for growth, and, interestingly, it was able to do so efficiently, reaching a dry cell weight (DCW) of 13 g/L in flask (Fig. 4A). In addition, its growth in xylose was also comparable with the growth of the wild type in glucose (Fig. 4B). This also suggests that the mutant strain can cope with the cofactor imbalance provoked by the heterologous enzymes, since XR consumes NADPH and XDH generates NADH. It has been previously suggested (Stephanopoulos and Tai, 2013) that, in the presence of oxygen, mitochondrial function actively controls and maintains NADPH/NADH equilibrium (Singh and Mishra, 1995). We could observe slight xylitol accumulation when growth had ceased, reaching 1.24 g/L (Fig. 4B). Additionally, in these conditions and similarly to the wild type in glucose medium, the excess carbon flux was also redirected towards citric acid, a preferred metabolic sink in this yeast (Fig. 4B). However, in *S. cerevisiae* the XR/XDH pathway impairs both growth and product formation in anaerobic conditions, and the xylose isomerase (XI) pathway has been required to permit efficient ethanol production in anaerobiosis from xylose (Kuyper et al., 2004). Recent publication in *Y. lipolytica* suggests a positive effect in lipid production when 0_2 is limited in the productive phase (Kavscek et al., 2015; Qiao et al., 2015) due to a favored metabolic flux towards fatty acid synthesis rather than citric acid. More studies are required to evaluate the efficiency of the XR/XDH pathway in limited aeration in *Y. lipolytica*, and in case of deleterious effect due to cofactor imbalance, XI pathway might be considered. Furthermore, we investigated xylose consumption in media that also contained glucose. Similarly to what had been found with engineered *S. cerevisiae* (Subtil and Boles, 2012), our mutant strain only consumed xylose when glucose in the media had dropped (Fig. 4C). This may suggest specific regulation at the transporter level; more experiments are required to characterize and optimize co-consumption. Protein engineering of the common transporter to selectively transport xylose without inhibition by glucose has solved the same issue in baker's yeast (Farwick et al., 2014). In the co-substrate scenario, xylitol and citric acid increased slightly, to 2.7 g/L and 2.2 g/L, respectively (Fig. 4C). Interestingly, the wild-type strain was also able to consume xylose after depletion of glucose, but in this case the pentose was almost completely transformed into xylitol, with a yield of $Y_{XOH/S}=0.92$ g/g (Fig. 4D). This result deserves further investigation due to industrial interest in xylitol as a sweetener and anti-cariogenic, antiketonic, and anti-infection agent, and since such a high yield is more than that generated from many of the microorganisms proposed for this biotransformation (Cheng et al., 2011; Guo et al., 2006). The production of xylitol in the wild type strain may indicate a endogenous XR activity and therefore, in order to determine if the heterologous expression of *ssXR* is essential or not to permit growth in xylose, a strain overexpressing *ssXDH* and *ylXK* was constructed. Additionally the strain overexpressing *ssXR* and *ylXK* was also generated. None of them was able to grow in xylose media (Fig. 2), although a residual growth was appreciated after a

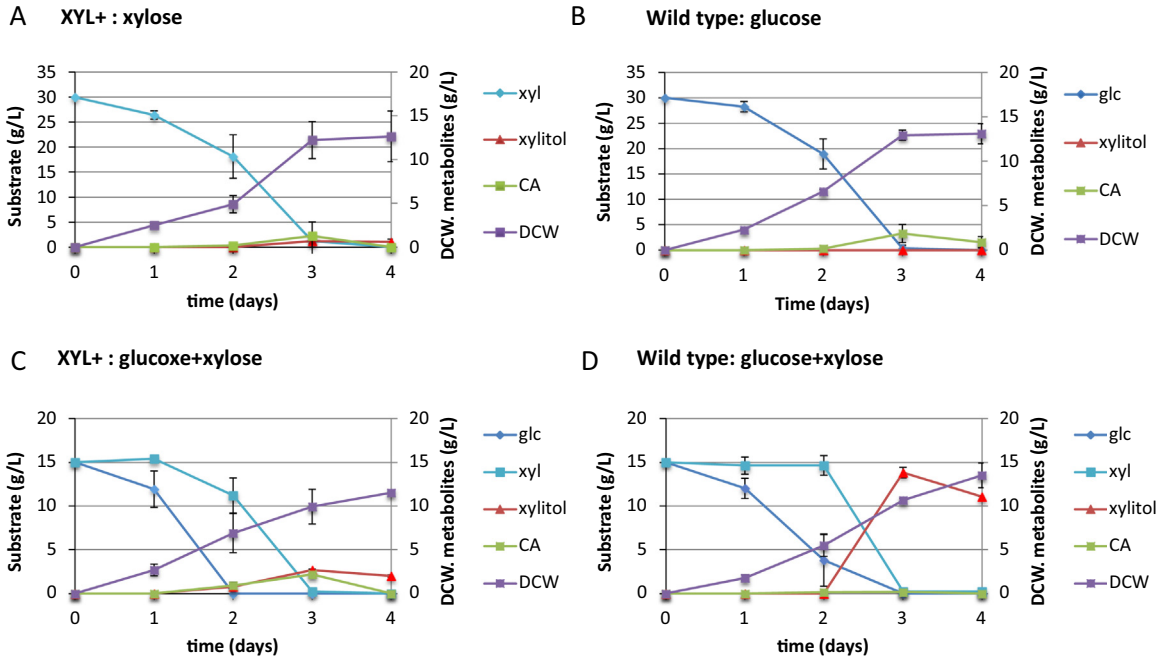

Fig. 4. Growth of the wild-type and XYL+ strains in different substrates. Behavior of the strain XYL+ (A and C) and the wild-type (B and D) growing in xylose (A), glucose (B), or a mixture of both (C and D). The graphics show the consumption of substrates (xylose and glucose, g/L), the DCW (g/L), and the production of metabolites (xylitol and citric acid, g/L).

long incubation of the plate (3 weeks), only in the strain overexpressing *ssXDH* and *ylXK*, which seems to support the presence of endogenous xylose reductase activity (Supplementary Fig 2).

2.3. *Y. lipolytica XYL+ can produce lipids from xylose as the sole carbon source*

Once we had proved that *Y. lipolytica* strain XYL+ was able to efficiently consume xylose, we wanted to investigate its ability to produce lipids from this substrate. Because differences in the amounts of carbon and nitrogen sources are important in triggering lipid accumulation in this oleaginous yeast, we tested lipid production in four different media: YNB (2% xylose), YNB 30 (3% xylose), YNB 60 (6% xylose), and YNB 90 (9% xylose). As expected, lipid accumulation from xylose increased with higher amounts of xylose, reaching a fatty-acid content (with respect to the DCW) of 6% in YNB, 10.2% in YNB30, 13.3% in YNB60, and 16.9% in YNB90 (Fig. 5A). Accordingly, the production yields increased with increased xylose content in the medium, from 0.03 to 0.04, 0.07, and 0.10 g fatty acids produced per gram of xylose consumed, respectively (Table 1). These fatty acids were accumulated in lipid bodies, as observed by fluorescence microscopy (Fig. 5A and Supplementary Fig 4A). The lipids synthetized from xylose were rich in 18:1, 16:0, 18:2, 18:0, and 16:1 fatty acids (Table 2); this profile was similar to one previously obtained from growing an engineered *Y. lipolytica* strain on starch, which was predicted to fit different biodiesel quality standards (ASTM D 6751 in USA and EU14214 in Europe; (Ledesma-Amaro et al., 2015). The final biomass produced was also correlated to the amount of xylose present in the media and ranged from 7.5 to 17.2 g/L (Supplementary Fig 3 and Table 3). It was observed that, when its initial concentration was above 3%, the xylose was not completely consumed after 6 days (Supplementary Fig 3). At the same time, the excess xylose also boosted byproduct formation during fermentation: when the initial substrate contained at least 6% xylose, xylitol and citric acid were produced in substantial amounts, ranging from 1.8 to 3.0 g/L and 6.3–7.5 g/L, respectively (Fig. 5, Supplementary Fig 3, and Table 1). We therefore prove here that metabolically engineered *Y. lipolytica* can produce lipids while using xylose as a carbon source. However, the relatively low lipid yield and the flux of the substrate towards fermentation products such as xylitol or citric acid prompted us to transfer the xylose utilization pathway into another modified strain that was able to overproduce lipids, in order to further increase bio-oil production.

2.4. *Y. lipolytica strain XYL+ Obese is able to accumulate high amounts of lipids from xylose*

We have previously engineered a strain of *Y. lipolytica*, JMY3501, that is able to accumulate high amounts of lipids (Lazar et al., 2014). In this strain, lipid synthesis has been enhanced through overexpression of GPD1 and DGA2. GPD1 is involved in glycerol-3-phosphate formation, a precursor of TAG (Dulermo and Nicaud, 2011), while the *DGA2* gene encodes the acyltransferase involved in the last step of TAG formation (Beopoulos et al., 2012). In addition, lipid degradation and remobilization have been eliminated through deletion of the *POX* genes and the gene *TGL4*. The deletion of *POX1–6* completely blocks beta-oxidation in the peroxisome (Beopoulos et al., 2008), whereas deletion of *TGL4* prevents the release of fatty acids from the lipid body (Dulermo et al., 2013). We therefore overexpressed *ssXR*, *ssXDH*, and *ylXK* in the obese strain JMY3501, named this new strain XYL+ Obese, and observed its growth in xylose as sole carbon source (Supplementary Fig 5). Following the same rationale as for our descriptions of XYL+, we studied lipid production in xylose media at different sugar concentration and keeping constant and low the nitrogen source: YNB (2% xylose), YNB 30 (3% xylose), YNB 60 (6% xylose), and YNB 90 (9% xylose). As expected, lipid accumulation was higher in XYL+ Obese than in XYL+ for all the tested media, and reached a fatty-acid content with respect to DCW of 16.0% in YNB, 23.7% in YNB30, 36.5% in YNB60, and 21.8% in YNB90 (Fig. 5B); this represented up to 2.75 times more lipids than those produced by the XYL+ strain. Interestingly, the highest lipid content was found in YNB60, instead of 90 as in the XYL+ strain. This was consistent with a previous report that YNB60 (60 g/L of fructose) was best for lipid accumulation in fructose (Lazar et al., 2014). Because xylose

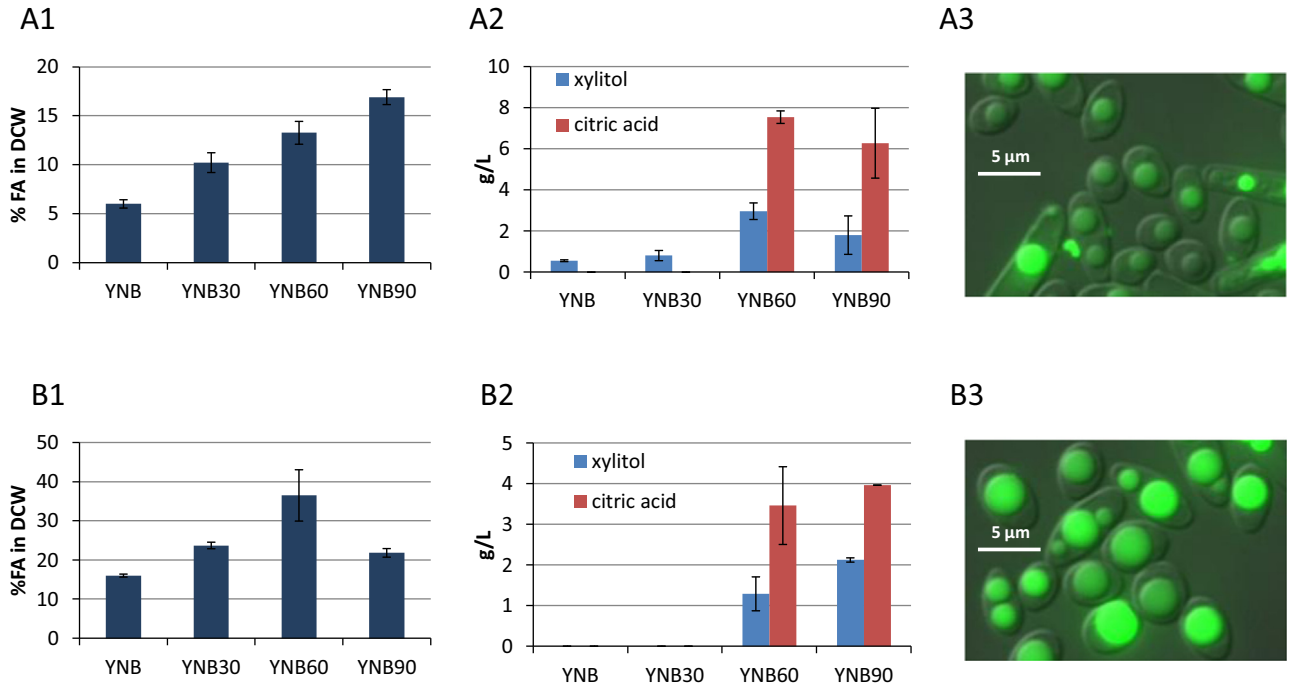

Fig. 5. Lipid production by XYL+ and XYL+ Obese strains. A) Experiments carried out using the XYL+ strain in media with different concentrations of xylose (YNB, YNB30, YNB60, and YNB90; see main text). A1) The percentage of fatty acids in the DCW after 6 days of culture. A2) The amounts of the secreted metabolites citric acid and xylitol (g/L) in the culture media after 6 days of culture. A3) Fluorescence microscopy image showing the cells and their lipids stained with Bodipy in the YNB90 medium. B) The same experiments carried out using the XYL+ Obese strain. B3) Cells and lipids after growth in YNB60 medium.

Table 1
Titers and yields in flasks with xylose as sole carbon source. This table represents the titers in g/L and the yields in g/g of xylose after 6 days of flask culture of the engineered strains in YNB, YNB30, YNB60, and YNB90 media. X: biomass as DCW, FA: fatty acids, CA: citric acid, and XOH: xylitol. The values represent the averages of two independent experiments. On the top are the values for the experiments that used XYL+, and on the bottom, those that used XYL+ Obese.

Strain	Media	Xylose consumed (g/L)	X (g/L)	Y X/S (g/g)	FA (g/L)	Y FA/S (g/g)	CA (g/L)	Y CA/S (g/g)	XOH (g/L)	Y XOH/S (g/g)
XYL+	YNB	20	7.58	0.38	0.61	0.03	0.00	0.00	0.55	0.03
	YNB30	30	8.38	0.28	1.15	0.04	0.00	0.00	0.80	0.03
	YNB60	31	12.83	0.41	2.26	0.07	7.53	0.24	2.96	0.07
	YNB90	38	17.23	0.45	3.88	0.10	6.27	0.16	1.80	0.05
XYL+ Obese	YNB	20	8.68	0.43	1.85	0.09	0.00	0.00	0.00	0.00
	YNB30	30	10.88	0.36	3.44	0.11	0.00	0.00	0.00	0.00
	YNB60	50	12.98	0.26	6.16	0.12	3.46	0.07	1.29	0.03
	YNB90	31	18.33	0.59	5.32	0.17	3.96	0.13	2.12	0.07

Table 2
Lipid profile of the modified strains growing on xylose. Percentage of each fatty acid produced after growth of the engineered strains in different xylose media (YNB, YNB30, YNB60, and YNB90). The values represent the averages and the standard deviations of two independent experiments.

Strain	Media	C16:0	C16:1	C18:0	C18:1	C18:2	Others
XYL+	YNB	8.8 ± 1.4	5.4 ± 0.2	5.5 ± 1.4	64.8 ± 3.1	12.9 ± 0.4	2.7 ± 0.0
	YNB30	12.1 ± 1.1	6.2 ± 0.1	11.4 ± 2.2	53.5 ± 5.8	13.9 ± 2.1	2.9 ± 0.4
	YNB60	15.2 ± 0.4	4.5 ± 0.2	14.3 ± 0.6	50.6 ± 0.5	11.6 ± 0.1	3.8 ± 0.4
	YNB90	13.8 ± 0.3	6.1 ± 1.3	10.9 ± 1.5	57.6 ± 0.6	8.4 ± 0.1	3.2 ± 0.6
XYL+ Obese	YNB	24.3 ± 0.7	5.8 ± 0.5	10.0 ± 1.4	49.1 ± 3.1	8.0 ± 0.5	2.9 ± 0.3
	YNB30	27.6 ± 0.6	7.2 ± 2.4	8.7 ± 3.1	47.0 ± 1.7	7.3 ± 0.1	2.2 ± 0.5
	YNB60	25.0 ± 2.5	5.1 ± 1.3	11.0 ± 3.3	50.6 ± 4.6	5.4 ± 0.1	2.8 ± 0.2
	YNB90	25.9 ± 0.4	5.5 ± 0.2	11.2 ± 0.1	49.8 ± 0.3	4.7 ± 0.1	2.8 ± 0.0

was utilized less effectively at a high xylose concentration, the highest titer was achieved in YNB60; however, the highest yield was still obtained in YNB90 ($Y_{FA/S}$=0.17 g/g) (Table 1). Huge lipid bodies could be observed inside the cells, likely correlating to increased TAG accumulation (Fig. 5B and Supplementary Fig 4B). The lipid profiles in the XYL+ Obese (Table 2) and XYL+ backgrounds (Table 2) were slightly different, with an increase in 16:0 and a decrease in 18:1 and 18:2 in the former compared to the latter. Similar variation in fatty-acid composition has been found in strains with the obese background that were modified for growing on starch (Ledesma-Amaro et al., 2015) and fructose (Lazar et al., 2014), suggesting that these variations are independent of the carbon source and are more likely related to the strain background. Xylose was completely consumed only at lower

concentrations (2% and 3%) and some substrate remained after 6 days when higher concentrations (6% and 9%) were used. As expected, final biomass correlated with the amount of xylose in the media, ranging from 8.7 to 18.3 g/L (Supplementary Fig 5). Again, at high xylose concentrations the byproducts xylitol and citric acid were found in the media (Fig. 5B and Supplementary Fig 5), with the former present at 1.3 and 2.1 g/L in YNB60 and YNB90, respectively, and the latter at 3.4 and 4.0 g/L, respectively. Interestingly, in the case of YNB60, more xylose was consumed by XYL+ Obese (9.9 g/L remained) than by XYL+ (28.9 g/L remained), suggesting that the extra xylose consumed was redirected to lipid accumulation. Therefore we can conclude that the modifications in XYL+ Obese efficiently enhanced the flux towards lipid metabolites, thus reducing the production of citric acid. This can be also noted in the reduction of the citric acid yield in XYL+ Obese ($Y_{CA/S}$=0.07 g/g at YNB60) compared to XYL+($Y_{CA/S}$=0.24 g/g at YNB60). Consequently, these results led us to study two different processes: first, the use of strain XYL+ for the production of citric acid from xylose, and second, the use of strain XYL+ Obese for the production of lipid-derived compounds from xylose.

2.5. Bioreactor-controlled conditions boost lipid production in Y. lipolytica from xylose and glycerol

Because the metabolically engineered *Y. lipolytica* XYL+ Obese strain was able to produce lipids in flask, we further investigated its ability to produce lipids in a 5 L bioreactor under more controlled conditions. We therefore carried out fed-batch fermentation using both strains (XYL+ and XYL+ Obese) using xylose as sole carbon source (Fig. 6 and Table 3). 150 g/L of xylose were provided initially and when pentose level dropped below 50 g/L, extra 100 g/L of xylose were added. The fermentation ended at 117 h (when all xylose was consumed). Final biomass was higher for the XYL+ Obese (56.6 g/L) than for the XYL+(46.4 g/L). As expected lipid accumulation was more efficient in XYL+ Obese (35% vs 12% of the DCW). In accordance with the flask experiments, the XYL+ strain produced more citric acid (44.2 g/L, 3 times more than XYL+ Obese) and xylitol (8.6 g/L, 4 times more than XYL+ Obese). In these conditions, XYL+ Obese accumulated

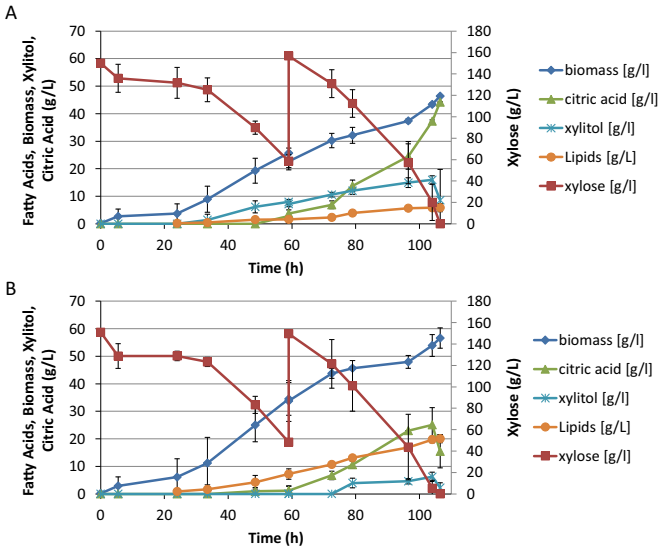

Fig. 6. Lipid production in bioreactor conditions. Kinetics of fed-batch bioreactor experiments for strains XYL+(A) or XYL+ Obese (B). Average concentrations with standard deviations for the following compounds are shown (g/L): Lipids, Biomass, xylitol, citric acid and xylose. The bioreactor was fed initially with 150 g/L of xylose and after with additional 100 g/L of xylose as described in Materials and Methods.

a total of 20.1 g/L with a yield of 0.08 g/g and a productivity of 0.19 g/L/h, proving the feasibility of producing a high amount of lipids from xylose.

In a previous work, additional substrates (glucose, glycerol or arabitol) have been tested as co-feeding together with xylose using *Y. lipolytica* in flask, which resulted beneficial, specially with glycerol (Stephanopoulos and Tai, 2013). We therefore decided to investigate the effect of co-feeding substrates in XYL+ and XYL+ Obese in bioreactor conditions. We declined to use arabitol since it is an expensive substrate and our strains are not able to assimilate it properly (Fig. 2). For this purpose, fed-batch fermentations were carried out using two different co-substrates in addition to xylose: glucose or glycerol. The preferable lignocellulosic hydrolysates are mainly composed not only of xylose but also of large amounts of glucose whereas glycerol is the major byproduct of the biodiesel industry and might be advantageously recycled into the process. Both lignocellulosic and glycerol wastes are cheap and widely available substrates which are highly desirable for the biotechnology industry (Sanchez Nogue and Karhumaa, 2015; Yang et al., 2012). When comparing XYL+ and XYL+ Obese strains in both co-feeding experiments, XYL+ Obese showed higher biomass and lipid production as well as lower citric acid and xylitol production than XYL+, in both titer and yields, in accordance with our previous results (Supplementary Fig 6 and Table 3). However the co-feeding with glucose boosted citric acid production (up to 67.2 g/L and 0.18 g/g in XYL+ Obese), which might limits lipid production (22.5 g/L and 0.06 g/g in XYL+ Obese) while the co-feeding with glycerol produced limited amount of citric acid (1 g/L and 0.02 g/g in XYL+ Obese) and improved lipid production (50.5 g/L and 0.12 g/g in XYL+ Obese) (Supplementary Fig 6 and Table 3). The better performance of the co-feeding with glycerol could be in part due to the additional supply of reducing power which may favor the metabolic flux towards lipid production (specially in XYL+ Obese strain, which overexpresses *GDP1* (G3P dehydrogenase) and to a lesser extent due to the direct conversion of glycerol by *GUT1* (glycerol kinase), into G3P, an important precursor metabolite for TAG synthesis (Beopoulos et al., 2008). Interestingly, in *C. guilliermondii*, the addition of glycerol as co-substrate enhanced the bioconversion of xylose into xylitol (Arruda and Felipe, 2009). However, further experiments are required to fully explain these results in Y. lipolytica.

2.6. Citric acid production from xylose in Y. lipolytica

As the XYL+ strain was able to produce significant amounts of citric acid in flask experiments ($Y_{CA/S}$=0.24 g/g), we decided to analyze this process in a 5 L bioreactor using a culture medium designed specifically for citric acid secretion (Rywinska et al., 2010). The process was carried out in a batch culture using 150 g/L of initial xylose. The final biomass obtained in this process reached 23.6 g/L (Fig. 7), likely due to the nitrogen limitation. Citric acid secretion began after 24 h of the process and occurred at a constant production rate (0.91 g/L/h) until the end of the culture. The final titer of this metabolite reached 80 g/L with a yield $Y_{CA/S}$=0.53 g/g (Table 4). It is important to note that this production level is similar to ones previously found with citric-acid-overproducing strains growing in glucose and glycerol (Lazar et al., 2011; Rywinska et al., 2010). Because of this, production from xylose seems very promising. Additionally, during the process, a small quantity of xylitol was also secreted to the culture medium; however, this metabolite was completely re-consumed at the end of the process. These results serve as solid evidence that the engineered *Y. lipolytica* strain can efficiently produce citric acid using xylose as the sole carbon source.

Table 3
Titers and yields in bioreactor conditions to promote lipid production.

Media	Strain	Carbon consumed (g/L)	X (g/L)	Y X/S (g/g)	FA (g/L)	Y FA/S (g/g)	CA (g/L)	Y CA/S (g/g)	XOH (g/L)	Y XOH/S (g/g)	P FA (g/L/h)
Xylose	XYL+	250	46.4	0.19	5.9	0.02	44.2	0.18	8.6	0.03	0.06
	XYL+ Obese	250	56.6	0.23	20.1	0.08	15.5	0.06	2.3	0.01	0.19
Xylose + Glucose	XYL+	375	57.25	0.15	7.8	0.02	102.8	0.27	14.2	0.04	0.05
	XYL+ Obese	375	73.6	0.20	22.5	0.06	67.2	0.18	0.0	0.00	0.15
Xylose + Glycerol	XYL+	429	58.2	0.14	7.3	0.02	21.5	0.05	18.6	0.04	0.03
	XYL+ Obese	429	120.7	0.28	50.5	0.12	1.0	0.00	11.1	0.03	0.23

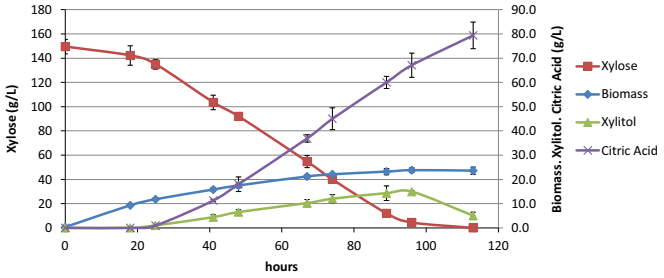

Fig. 7. Citric acid production from xylose in bioreactor conditions. Kinetics of batch bioreactor experiments using strain XYL+. Average concentrations with the standard deviations for the following compounds are shown: Biomass (g/L); xylitol, citric acid, and xylose (g/L of DCW).

Table 4
Titers and yields in bioreactor conditions to promote citric acid production using XYL+.

Carbon consumed (g/L)	X (g/L)	Y X/S (g/g)	CA (g/L)	Y CA/S (g/g)	XOH (g/L)	Y XOH/S (g/g)
150	23.6	0.16	79.4	0.53	5.0	0.03

3. Conclusions

One of the first steps in engineering *Y. lipolytica* to produce microbial oils from lignocellulosic materials is to give it the ability to utilize xylose efficiently. In this work we not only demonstrated that the expression of the first two enzymes of this pathway are important, but also that overexpression of the endogenous xylulokinase represents a key step for growing *Y. lipolytica* using xylose as the sole carbon source. Interestingly, this is not the first time that a sugar kinase has been found to be the limiting factor in an assimilation pathway; a similar case was reported for hexokinase in *Y. lipolytica* growing in sucrose or fructose (Lazar et al., 2014).

Importantly, we showed that the strategy of overexpressing *ssXDH*, *ssXR*, and *ylXK* can be transferred to other strains, in this case to one that had been previously engineered with the ability to overaccumulate lipids. The resulting mutant strain was able to accumulate up to 42% of its DCW as fatty acids on xylose as the sole carbon source, which represented 3.4 times more lipids than generated by the wild type in the same media. These results indicate that the described modifications can be combined in other published strains that are enhanced for lipid production on xylose.

Additionally, this work demonstrates how tuning the bioreactor conditions can result in different biotransformations. For example, a high xylose/nitrogen ratio, promoted high lipid production, up to a titer of 20 g/L on xylose and 50 g/L in xylose co-fed with glycerol. Instead, a lower C/N ratio and a higher pH promoted citric acid production up to 80 g/L. This sets up the basis and serves as a proof of concept for the transferability of the presented strategy among different strains and different products.

Y. lipolytica has been recently engineered to grow in other components present in lignocellulosic materials, such as celluloses and cellobiose (Guo et al., 2015; Wei et al., 2014). The future combination of such approaches could lead to the first strain with the consolidated bioprocess ability to produce industrial compounds from lignocelluloses, not only microbial oils for fuels and oleochemicals, but also other fermentative products such as organic acids, polyalcohols, and proteins (Ledesma-Amaro and Nicaud, 2015).

4. Materials and methods

4.1. Strains and media

The *Y. lipolytica* strains used in this study were the wild-type strain W29 (ATCC20460) and its derived strains Po1d (Ura- Leu-) and Po1t (Ura+ Leu+) (Le Dall et al., 1994; Madzak et al., 2004). The prototrophic strains generated in this study are listed in Supplementary Table 1. Media and growth conditions for *Escherichia coli* and *Y. lipolytica* have been described elsewhere (Dulermo et al., 2015). Minimal medium contained 0.17% (wt/vol) yeast nitrogen base (YNBww); 2, 3, 6, or 9% xylose (wt/vol; Merck, Fontenay-sous-Bois Cedex, France) for YNB, YNB30, YNB60, and YNB90, respectively; 0.15% (wt/vol) NH_4Cl; and 50 mM phosphate buffer (pH 6.8). Growth tests were carried out in similar minimal medium containing 0.5% (wt/vol) NH_4Cl and the indicated carbon source (0.5%).*Blastobotrys* (*Arxula*) *adeninivorans* strain LS3 was used as a positive control for growth in xylose (Kunze et al., 2014).

4.2. Cloning and expression of heterologous and endogenous genes

The NAD(P)H-dependent D-xylose reductase (*XR*) and D-xylulose reductase (xylitol dehydrogenase) (*XDH*) genes were amplified by PCR from genomic DNA of *Scheffersomyces stipitis* (ATCC® 58,785™) using SXRA/SXRB and SDHA/SDHB as primers (Supplementary Table 2), respectively. The PCR fragments were digested using *Bam*HI/*Avr*II and inserted into the plasmid JMP62 UraTEF (Nicaud et al., 2002; Pignede et al., 2000) at the corresponding sites, creating plasmids JMP62 UraTEF *SsXR* and JMP62 UraTEF *SsXDH*, respectively. Then the URA marker was exchanged with the LEU marker in order to generate the plamids JMP62 LeuTEF *SsXR* and JMP62 LeuTEF *SsXDH*. Additionally, xylulokinase (*XK*) was amplified from genomic DNA of *Y. lipolytica* Po1d and cloned into the JMP62 HygTEF plasmid to yield JMP62 HygTEF *YlXK*.

Expression vectors were digested with *Not*I, purified on gel, and used for transformation. Expression cassettes were integrated into the *Y. lipolytica* genome as described previously (Bordes et al., 2007; Pignede et al., 2000). The overexpression cassettes were used for transformation by the lithium acetate method (Le Dall et al., 1994). Transformants were selected on YNBUra, YNBLeu, YPDHyg, or YNB media depending on their genotype. Then genomic DNA from yeast transformants was prepared as described in (Querol et al., 1992). Positive transformants were checked by PCR. The removal of the selection marker was carried out via the LoxP-Cre system widely used in *Y. lipolytica* (Fickers et al., 2003). The wild-type strain Po1d (JMY195) transformed with *ssXR*, *ssXDH* and

ylXK was named XYL+, while the modified strain JMY3501 transformed with the same three genes was named XYL+ Obese.

Restriction enzymes were obtained from OZYME (Saint-Quentin-en-Yvelines, France). PCR amplifications were performed in an Eppendorf 2720 thermal cycler with GoTaq DNA polymerases (Promega). PCR fragments were purified with a QIAgen Purification Kit (Qiagen, Hilden, Germany). All the reactions were performed using the manufacturers' instructions.

4.3. Growth rate calculation and DCW

To determine DCW in flask experiments, 2 mL of the culture were washed and lyophilized in a pre-weighed tube. The differences in weight corresponded to the mg of cells found in 2 mL of culture.

Growth tests were performed in 100-μL cultures in 96-well plates, with constant shaking, in the presence of 0.5% glucose, xylose, or arabitol as carbon source at 28 °C. Growth was monitored by measuring the optical density ($OD_{600\ nm}$) at different intervals with a microtiter plate reader (Biotek, Colmar, France). For each strain and set of conditions, we used at least two biological replicates. The growth rate was calculated in the exponential phase for each strain and condition.

4.4. Determination of sugars, acids, and alcohols

Glucose, xylose, xylitol, and citric acid were identified and quantified by HPLC (UltiMate 3000, Dionex-Thermo Fisher Scientific, UK) using an Aminex HPX87H column coupled to UV (210 nm) and RI detectors. The column was eluted with 0.01 N H_2SO_4 at room temperature and a flow rate of 0.6 mL/min. Identification and quantification were achieved via comparisons to standards. Before being subjected to HPLC analysis, samples were filtered on membranes of 0.45-μm pore-size.

4.5. Lipid quantification

Lipids from aliquots of 10–20 mg of cells were converted into their methyl esters with freeze-dried cells according to Browse et al. (1986) and used for gas chromatography (GC) analysis. GC analysis of fatty acid (FA) methyl esters was performed with a Varian 3900 instrument equipped with a flame ionization detector and a Varian FactorFour vf-23 ms column, where the bleed specification at 260 °C is 3 pA (30 m, 0.25 mm, 0.25 μm). FAs were identified by comparison to commercial FA methyl ester standards (FAME32; Supelco) and quantified by the internal standard method, involving the addition of 50 μg of commercial C17:0 (Sigma).

4.6. Microscopic analysis

Images were acquired using a Zeiss Axio Imager M2 microscope (Zeiss, Le Pecq, France) with a 100 × objective and Zeiss filters 45 and 46 for fluorescence microscopy. Axiovision 4.8 software (Zeiss, Le Pecq, France) was used for image acquisition. Lipid body visualization was performed by the addition of Bodipy® Lipid Probe (2.5 mg/mL in ethanol; Invitrogen) to the cell suspension (A_{600} of 5) and after incubation for 10 min at room temperature.

4.7. Bioreactor culture and conditions

Lipid biosynthesis was evaluated using fed-batch cultures that were kept in 5-L stirred-tank BIO-STAT B-PLUS bioreactors (Sartorius, Frankfurt, Germany) under the following conditions: 2-L working volume, 28 °C, 800 rpm of agitation, and 1.0 L/min aeration rate. The initial production medium contained (in 1 L of tap water): 150 g xylose, 1.6 g NH_4Cl, 1.0 g $MgCl_2 \times 7H_2O$, 0.5 g H_2PO_4, 1.0 g YE, and 1.0 g YNB. The addition of substrate varied for the three conditions, xylose only, xylose and glucose and xylose and glycerol. Additional xylose was added once when its concentration dropped below 50 g/L, 100 g/L in the case of xylose only and xylose and glucose, and 40 g/L in the case of xylose and glycerol, due to the slower consumption of xylose in this case.

After 2 days of the process, the medium was supplemented either with 400 mL of 95% glycerol at a rate of 3.5 mL/h (included in total working volume of 2 L) or with 500 mL of 50% glucose at a rate of 4 mL/h. Glucose feeding was maintained low in order to avoid repression of xylose uptake. During the cultures, 1 mL/L of mineral salts solution (20 g/L $Ca(NO_3)_2 \times 4H_2O$, 2 g/L $FeCl_3 \times 6H_2O$, 0.5 g/L H_3BO_3, 0.4 g/L $MnSO_4 \times 4H_2O$, 0.4 g/L $ZnSO_4 \times 7H_2O$, 0.2 g/L Na_2MoO_4, 0.1 g/L $CuSO_4 \times 5H_2O$, 0.1 g/L $CoCl_2$, and 0.1 g/L KI), 1.6 g/L NH_4Cl, and 300 μg/L thiamine were added to the medium after 2, 4, and 6 days of culture. The pH was kept at 3.5 using a 30% (w/v) NaOH solution. The pre-cultures were grown in 0.1 L of YPD medium in 0.5-L flasks at 170 rpm, at 28 °C for 48 h. The volume of the inoculum added to the bioreactor cultures was equal to 5% of the total working volume. The fermentation ended when all the xylose was consumed. Citric acid biosynthesis was evaluated using batch cultures that were carried out for 113 h in the same bioreactors under the conditions described above. The difference was that the pH of the process was maintained at 5.0 using 30% (w/v) NaOH solution. The production medium contained (in 1 L of tap water): 150 g xylose, 3.0 g NH_4Cl, 0.2 g KH_2PO_4, 1.0 g $MgSO_4 \times 7H_2O$, and 1.0 g yeast extract. An inoculum of 0.2 L was introduced into a bioreactor containing 1.8 L of the production medium.

Samples for analysis were taken from the bioreactor at the time points indicated in the figures. The samples were centrifuged (5000 rpm, 5 min), then the cells were washed twice with distilled water, harvested by filtration on 0.45-μm pore-size membranes, and dried to a constant weight at 105 °C. The remaining supernatant was analyzed for citric acid, xylose, glycerol, and xylitol concentration.

4.8. Analysis of gene expression

For the expression experiments, the wild-type strain and the XYL+ strain were grown at 28 °C in YNB60 g (60 g/L glucose) medium. After 16 h, the cell suspensions were washed twice with distilled water and transferred into fresh either YNB60 g (60 g/L of glucose) or YNB60 (60 g/L xylose) medium. The samples were harvested at 4 h post inoculation. The experiments were conducted in triplicates. All the samples were frozen in liquid nitrogen and stored at −80 °C.

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany), and 1.5 μg of each sample was treated with DNase (Ambion, Life Technologies). cDNA was synthesized using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific). PCR reactions were performed using specific primers that targeted the 3′ end of the genes (Supplementary table 3). For the quantitative RT-PCR analyses, gDNA dilutions were first tested for primer efficiency and then retained if their efficiency rating was higher than 90%. Amplifications were carried out using the Sso Advanced Universal SYBR Green Supermix Kit (BIO-RAD). The following program was used: 98 °C for 3 min, followed by 40 cycles of 98 °C for 15 s, 58 °C for 30 s, and 72 °C for 30 s. Finally, melting curves were generated to confirm amplification specificity. ΔC T method was used to calculate relative expression levels; a constitutive gene, actin, was utilized as the reference control (Schmittgen and Livak, 2008).

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ymben.2016.07.001.

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