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Engineering Xylose Isomerase and Reductase Pathways in *Yarrowia lipolytica* for Efficient Lipid Production

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ABSTRACT

Xylose is a common monosaccharide in lignocellulosic residues that *Yarrowia lipolytica* cannot naturally metabolise for lipid production and therefore, heterologous xylose metabolic pathways must be engineered in this yeast to facilitate its consumption. We have compared the metabolic efficiency of two xylose metabolic pathways by developing three recombinant *Y. lipolytica* strains: one harbouring a xylose reductase pathway, one with a xylose isomerase pathway, and one combining both pathways, and the strains were tested for xylose consumption and lipid production at different scales. The recombinant strain with the reductase pathway that was directly isolated in selective xylose medium showed the highest lipid yield, producing up to 12.8 g/L of lipids, or 43% of the biomass dry weight, without requiring any other xylose consumption adaptive evolution process. This strain achieved a lipid yield of 0.13 g lipids/g xylose, one of the highest yields in yeast reported so far using xylose as the sole carbon and energy source. Although the strain harbouring the isomerase pathway performed better under oxygen-limiting conditions and led to higher lipid intracellular accumulation, it showed a lower xylose uptake and biomass production, rendering a lower yield under non-limiting oxygen conditions. Unexpectedly, the combination of both pathways in the same strain was less effective than the use of the reductase pathway alone.

1 | Introduction

Nowadays, the valorisation of waste to obtain biofuels and other value-added compounds is of great interest in order to mitigate environmental pollution. Lignocellulosic biomass, mainly composed of cellulose, hemicellulose and lignin, is the most abundant waste on the planet, which can result from different agroindustry activities. Hemicellulose, mainly composed of xylose, is the second most abundant carbohydrate in lignocellulosic biomass and has great potential for its saccharification, utilising enzymatic cocktails, and the valorisation of its monomers (Ye et al. 2019; Zhao et al. 2020). However, while the glucose from the cellulose is easily metabolizable by many microorganisms, xylose fermentation presents some limitations due to non-existent or inefficient native metabolism. Therefore, the metabolic engineering of glucose-fermenting microorganisms for efficient and rapid xylose utilisation has been undertaken to achieve economic bioconversion of lignocellulosic biomass into value-added products using a single microorganism (Lee et al. 2021).

Abbreviations: CDW, cell dry weight; FAMEs, fatty acid methyl esters; Kan, kanamycin; Leu, leucine; TAGs, triacylglycerols; Ura, uracil; XDH, xylitol dehydrogenase; XI, xylose isomerase; XK, xylulokinase; XR, xylose reductase; YNB, yeast nitrogen base; YPD, yeast extract peptone dextrose.

The oleaginous yeast *Yarrowia lipolytica* is a good candidate for the production of value-added compounds, since this species has been used to produce organic acids and heterologous proteins (Park and Ledesma-Amaro 2023). However, it stands out for its ability to accumulate high amounts of lipids intracellularly (Soong et al. 2023). These lipids consist mostly of triacylglycerols (TAGs) and are accumulated in specialised compartments known as lipid bodies (Beopoulos et al. 2009). Microbial oils have a great interest as an alternative source of renewable liquid fuels. Therefore, several technologies have already been developed for the production of lipids in *Y. lipolytica* using various agroindustry by-products and wastes as feedstock (Beopoulos et al. 2009; Zhao et al. 2020).

Y. lipolytica is generally classified as a non-xylose-utilising yeast despite it harbouring the genes from the xylose reductase pathway, coding for the enzymes: xylose reductase (XR), xylitol dehydrogenase (XDH), and xylulokinase (XK). However, the native XDH and XK are not active enough for efficient xylose utilisation (Lee et al. 2021). Nevertheless, *Y. lipolytica* is able to transform xylose into xylitol (Ledesma-Amaro and Nicaud 2016). Since *Y. lipolytica* practically cannot use xylose as a sole carbon and energy source (Ryu et al. 2016; Ryu and Trinh 2018), many efforts have been made to engineer metabolic pathways to facilitate its metabolism. In this sense, two pathways have already been expressed in *Y. lipolyitca* to facilitate xylose consumption (Lee et al. 2021; Sun et al. 2021), the reductase pathway that uses the enzymes XR, which transform xylose to xylitol consuming NADPH, and XDH, which transforms xylitol to xylulose producing NADH (Ledesma-Amaro et al. 2016; Li and Alper 2016; Rodriguez et al. 2016). The second pathway is the isomerase route that directly transforms xylose into xylulose using a xylose isomerase (XI) (Yook et al. 2020). The main difference between these two pathways is the production and consumption of reduced cofactors that, in the case of the reductase pathway, under certain conditions, may cause a cofactor imbalance (Lee et al. 2021). In this sense, although the yeast *Ogataea polymorpha* contains a reductase pathway to metabolise xylose, it does not efficiently consume xylose as the sole carbon source, since the pathway creates a cofactor imbalance (Gao et al. 2023). To circumvent this imbalance, the overexpression of a heterologous isomerase pathway in this yeast was studied in order to increase xylose consumption (Gao et al. 2023). The putative synergistic effect of both pathways on xylose consumption has been tested in *Sacharomyces cerevisiae*. Cunha et al. (2019) have compared the xylose fermentation efficiency of two industrial *S. cerevisiae* strains with separate or combined xylose reductase and isomerase pathways under anaerobic conditions to produce ethanol. In the presence of lignocellulosic-derived inhibitors, a positive synergistic effect resulted from the expression of both pathways (Cunha et al. 2019). Moreover, Wang et al. (2017) have cloned both xylose pathways together with an arabinose pathway in *S. cerevisiae*.

The natural coexistence of more than one xylose metabolic pathway in a single strain is a rare phenomenon, and, as far as we know, it has been only described in the bacterium *Sphingomonas sanxanigenens* NX02, where the xylose isomerase pathway coexists with the Weimberg and Dahms xylose catabolic pathways (Wu et al. 2021). As far as we know, nobody has reported the existence of a natural yeast strain that contains two alternative pathways to metabolise xylose. In this sense, the *O. polymorpha* recombinant strain constructed by Gao et al. (2023) and the *S. cerevisiae* recombinant strains constructed by Cunha et al. (2019) and Wang et al. (2017) were the only reported yeast strains used to investigate the effect of expressing two alternative xylose pathways in the same cell.

Y. lipolytica strains have been previously engineered with only one of the two optional reductase or isomerase pathways for the production of lipids using this monosaccharide as substrate. Table 1 shows the lipid titers and yields obtained with different recombinant strains on different genetic backgrounds. However, a direct comparison of the efficiency of these pathways on an isogenic background has not been performed yet. Moreover, the possibility to engineer and express both pathways together in the same *Y. lipolytica* cell to explore if the combination could increase the consumption of xylose and the production of lipids has not been investigated in this oleaginous strain.

In this work, we have analysed the efficiency of xylose consumption and lipid production in three recombinant strains of *Y. lipolytica* carrying the reductase pathway, the isomerase pathway or both pathways together. Using the reductase pathway alone, we have reached one of the highest lipid yields described in oleaginous yeast using xylose as a carbon source. We have determined that the isomerase pathway alone was less efficient for xylose composition and lipid production than the reductase route in the same genetic background. Surprisingly, the coexistence of both pathways produced an unexpected detrimental behaviour in the consumption of xylose and lipid production.

2 | Materials and Methods

2.1 | Strain and Culture Conditions

All microorganisms and plasmids used in this work are described in Table 2. YPD medium (DifcoTM BD) containing per litre: 10 g yeast extract, 20 g peptone and 20 g dextrose was used for routine culture of *Y. lipolytica* and generation of seed cultures. YNB medium (DifcoTM BD) containing per litre: 5 g ammonium sulphate, 1.0 g monopotassium phosphate, 0.5 g magnesium sulphate, 0.1 g sodium chloride, 0.1 g calcium chloride, 500.0 μg boric acid, 40.0 μg copper sulphate, 100.0 μg potassium iodide, 200.0 μg ferric chloride, 400.0 μg manganese sulphate, 200.0 μg sodium molybdate, 400.0 μg zinc sulphate, 2 μg biotin, 400 μg calcium pantothenate, 0.002 g, 2000 μg inositol, 0400 μg niacin, 200 μg, *p*-aminobenzoic acid, 400 μg pyridoxine hydrochloride, 200 μg riboflavin and 400 μg thiamine hydrochloride. This broth was used to select the transformants. YNB without ammonium sulphate (DifcoTM BD) was used for lipid production. The *Escherichia coli* cells harbouring the recombinant plasmids were cultured in LB (Sambrook and Russell 2001) medium with kanamycin (50 μg mL^{-1}) containing per litre: 10 g peptone, 5 g yeast extract and 5 g sodium chloride at 37°C.

Experiment in serum bottles was performed leaving a 90% or 10% headspace (i.e., air chamber). For this, bottles of 117 mL with 11.7 mL and 105.3 mL of culture medium were used, respectively. In these experiments, the medium used was YNB

TABLE 1 | Xylose consumption and lipid production using recombinant *Y. lipolytica* strains expressing the reductase or isomerase pathways. NA means not available.

Yarrowia lipolytica strain	Description	Initial xylose concentration (g L^{-1})	Culture conditions	Lipid titter (g L^{-1})	Lipid content (%)	Total yield (glip/gxyl)	Reference
YSX1	YSX Δpex10 XylA, XK	160	Batch, C/N:100	3.96	30.16	0.02	(Yook et al. 2020)
PO1f_XDH_XR	PO1f XDH XK	20	Batch, C/N: 8.8	0.3	10	0.02	(Rodriguez et al. 2016)
XYL+	PO1d ssXR ssXDH ylXK	150	Fed-batch until 250 g L^{-1} of xylose. C/N:45	5.9	12.7	0.02	(Ledesma-Amaro et al. 2016)
XYL + obese	PO1d Δpox1-6 Δtgl4 GDP1 DGA2 ssXR ssXDH ylXK	150	Fed-batch until 250 g L^{-1} of xylose. C/N:45	20.1	35	0.08	(Ledesma-Amaro and Nicaud 2016)
E26 XUS	E26 XR XDH (ALE strain)	160	Batch, C/N:35	15.06	NA	0.09	(Li and Alper 2016)
ALA-A x XUS-B	L36DGA1 2xRkD12-15 MATA1 MATA2 x E26XUS 4xXYL1 ylXKS MATB1 MATB2	110	Batch, C/N:NA	6	37.5	0.05	(Li and Alper 2020)
ADTP-XR-XDH evolved	ADTP-XR ΔintA::HisG⁻Ura⁻HisG, P_{TEFin}-SsXDH-xpr2t (ALE evolved)	130	Fed-batch until 440 g L^{-1}, C/N: NA	60	73.42	0.13	(Sun et al. 2023)
ADTP-XR-XDH evolved	ADTP-XR ΔintA::HisG⁻Ura⁻HisG, P_{TEFin}-SsXDH-xpr2t (ALE evolved)	200	Batch, C/N: NA	16.25	53.64	0.08	(Sun et al. 2023)

TABLE 2 | Strains and plasmids.

Strain	Description	Reference
Escherichia coli		
DH5α	F⁻ φ80l*acZΔM15* Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR17*(rK⁻, mK⁺) *phoA supE44* λ⁻ *thi*⁻¹ *gyrA96 relA*	Thermo Fisher Scientific
Yarrowia lipolytica		
Po1d	W29 *ura3-302 leu2-270 xpr2-322* (Leu⁻, Ura⁻, ΔAEP, Suc⁺)	(Tharaud et al. 1992) provided by R. Ledesma
DGA	Po1d with *dga1* gene from *Rhodosporidium toruloides* and *dga2* gene from *Claviceps purpurea* inserted in *mef1*gene. (Ura⁺, Leu⁻ strain)	CIB collection
YDGA	DGA transformed with plasmid pJMP62leu. (Ura⁺, Leu⁺ strain)	CIB collection
YXI	DGA transformed with plasmid pJMP62leu XI expressing xylulokinase (XK) from *Y. lipolyitca* W29 (YALI0F10923) and xylose isomerase (XI) from *Piromyces* sp. (XylA3). (Ura⁺, Leu⁺ strain)	This work
YXR	DGA transformed with plasmid pJMP62leu XR expressing NAD(P)H-dependent D-xylose reductase (XR) from *Scheffersomyces stipitis* (P31867), xylitol dehydrogenase (XDH) from *S. stipitis* (E5G6H4), and xylulokinase (XK) from *Y. lipolyitca* W29 (YALI0F10923). (Ura⁺, Leu⁺ strain)	This work
YXI-XR	DGA transformed with plasmid pJMP62leu XI-XR expressing NAD(P)H-dependent D-xylose reductase (XR) from *Scheffersomyces stipitis* (P31867), xylitol dehydrogenase (XDH) from *S. stipitis* (E5G6H4), xylulokinase (XK) from *Y. lipolyitca* W29 (YALI0F10923) and xylose isomerase (XI) from *Piromyces* sp. (XylA3). (Ura⁺, Leu⁺ strain)	This work

Plasmid	Description	Reference
pJMP62leu	Kan^R, Leu2 marker, TEF promoter and XPR2 terminator	(Luo et al. 2020)
pJMP62leu XI	pJMP62leu derivative expressing xylulokinase (XK) from *Y. lipolyitca* W29 (YALI0F10923) and xylose isomerase (XI) from *Piromyces* sp. (XylA3)	This work
pJMP62leu XR	pJMP62leu derivative expressing NAD(P)H-dependent D-xylose reductase (XR) from *Scheffersomyces stipitis* (P31867), xylitol dehydrogenase (XDH) from *S. stipitis* (E5G6H4), xylulokinase (XK) from *Y. lipolyitca* W29 (YALI0F10923)	This work
pJMP62leu XI-XR	pJMP62leu derivative expressing NAD(P)H-dependent D-xylose reductase (XR) from *Scheffersomyces stipitis* (P31867), xylitol dehydrogenase (XDH) from *S. stipitis* (E5G6H4), xylulokinase (XK) from *Y. lipolyitca* W29 (YALI0F10923) and xylose isomerase (XI) from *Piromyces* sp. (XylA3)	This work

supplemented with 30 g L^{-1} xylose or glucose and maintained for 72 h at 30°C and 200 rpm.

2.2 | Plasmid Construction and Transformation

The genes used to construct the metabolic cassettes, that is, NAD(P)H-dependent D-xylose reductase (XR) from *Scheffersomyces stipitis* (P31867), xylitol dehydrogenase (XDH) from *S. stipitis* (E5G6H4), xylulokinase (XK) from *Y. lipolytica* W29 (YALI0F10923) and xylose isomerase (XI) from *Piromyces* sp. (XylA3*), were synthetically constructed using codon optimisation for *Y. lipolytica* by Genescript. The XR and XDH enzymes from the highly efficient xylose-utilising yeast *S. stipitis* were selected because these enzymes had been efficiently expressed in *S. cerevisiae* (Kötter et al. 1990; Jo et al. 2017) and in *Y. lipolytica* (Ledesma-Amaro et al. 2016; Li and Alper 2016). The enzymes ssXR, ssXDH and ylXK had been successfully tested in *Y. lipolytica* (Ledesma-Amaro et al. 2016), ensuring the correct expression and folding in our obese strain. XI from *Piromyces* sp. was selected because *Piromyces* is one of the few fungal strains that use the XI pathway to metabolise xylose and this enzyme has been expressed efficiently in *S. cerevisiae* (Kuyper et al. 2003) and in *Y. lipolytica* (Yook et al. 2020). We have used in this work the most efficient XI mutant (XylA3*) of *Piromyces* sp. containing the mutations E15D, E114G, E129D, T142S, A177T, and V433I (See *xylA3** gene sequence in Figure S1) previously described (Lee et al. 2012, 2014) and expressed in *Y. lipolytica* using codon optimisation for *Homo sapiens* (Yook et al. 2020). All genes have been constructed with a synthetic upstream constitutive P_{TEF} promoter and a synthetic XPR2 downstream terminator in the plasmid pJMP62 (Nicaud et al. 2002). Figures S1–S3 illustrate the plasmid constructions used for the transformation of *Y.*

lipolytica. The *E. coli* recombinants were obtained by using the method described by Sambrook and Russell (2001). This method is based on a thermal shock in the presence of rubidium chloride.

The constructed plasmids were transformed in *Y. lipolytica* DGA by the lithium acetate method, and the DNA cassettes integrated into the Z regions of the genome (Dall et al. 1994). Transformants were selected on YNB with xylose as a carbon source.

2.3 | Lipid Production Assays

Production experiments were carried out using YNB with a C/N ratio adjusted to 75. Xylose was used at 30 g L^{-1} in flasks and at 50 g L^{-1} and 150 g L^{-1} of xylose in bioreactor trials. Different concentrations of ammonium sulphate were added depending on carbon concentration to adjust the C/N ratio (0.77, 1.32 and 3.9 g/L, respectively).

Individual colonies were picked, inoculated into YPD medium, and grown at 30°C overnight (O/N). These cultures were further inoculated into 40 mL of YNB medium in a 250 mL flask with an initial cell density corresponding to $OD_{600} = 0.1$ and allowed to grow for 72 h at 200 rpm and 30°C. Samples were taken for analysis of biomass, sugar content and lipid production. Samples were maintained at -4°C until analysis.

The bioreactor (1 L, Multifors Infors, Switzerland) trials were carried out with 0.7 L working volume. The assays were inoculated with an initial cell density corresponding to $OD_{600} = 0.1$ using an O/N culture in YPD medium. Fermentation was carried out at 30°C, with a shaking speed of 800 rpm and an air flow rate of 0.3 vvm. The initial pH of the fermentation was established at 6.0. The pH decreases progressively when *Yarrowia* grows in xylose or glucose. Then, the pH was maintained at 3.5 by adding 2 M NaOH to facilitate the production of lipids and to reduce the production of citrate according to Friedlander et al. (2016). Antifoam (0.5 mL/L) was added to reduce foaming.

2.4 | Analytical Methods

The biomass time course evolution was measured by optical density of the cultures at 600 nm utilising a Shimadzu UV-260 spectrophotometer. Biomass was recovered by centrifugation at 3800 rpm (Eppendorf Centrifuge 5810R) for 5 min. The biomass concentration was then measured by dry weight after washing the pellet twice with Milli-Q water and subsequent lyophilisation.

The concentrations of sugars were determined through High-Performance Liquid Chromatography (HPLC) using an Agilent Technologies 1100 series instrument, equipped with a Rezex RHM-Monosaccharide-H+ 300 × 7.8 mm column (Phenomenex) and a refractive index detector (RID). A mobile phase consisting of 1 mM H_2SO_4 was employed at a flow rate of 0.5 mL min^{-1}. The column temperature was maintained at 80°C during analysis.

Lipids were quantified by two different methods, that is, by their transformation into fatty acid methyl esters (FAMEs) and by gravimetry. To produce the FAMEs, culture samples were centrifuged (Eppendorf Centrifuge 5810R) to separate the cells that were subsequently lyophilised. Afterwards, 2 mL of a solution of methanol/hydrochloric acid/chloroform (10:1:1) was used to break down 20–30 mg of lyophilised cells. The mixture was heated at 90°C for 60 min to transform lipids into FAMEs. FAMEs were extracted with hexane, and the samples were analysed by gas chromatography mass spectrometry (GC–MS). Heneicosanoic acid was added as an internal standard before adding the solution of methanol/hydrochloric acid/chloroform (10:1:1). Chromatographic analysis was performed using an Agilent 7890A GC–MS system equipped with an Agilent 122–5731 column (30 m × 250 μm × 0.1 μm), and H_2 as the gas carrier. An injection volume of 1 μL was employed with a split ratio of 10:1. The oven temperature started at 115°C, underwent a ramp-up to 210°C at a rate of 3°C min^{-1} (35 min), and was ultimately held at 280°C for an additional minute. Identification and quantification of fatty acids were conducted by comparing them to a standard mixture of FAMEs (Sigma). The fatty acid content was expressed as a percentage of fatty acids to cell dry weight (% CDW). Lipid content was also measured using the gravimetric method of Bligh and Dyer (1959). The lipid extraction was performed in triplicate in two steps. Firstly, we treated the lyophilised biomass (200 mg) with 2.5 mL of 2 M HCl at 80°C for 1 h. The sample was extracted with methanol-chloroform (2:1). The organic solvent was transferred into a previously tared tube, and chloroform was evaporated at 75°C. Finally, the tube containing the lipids was oven dried at 60°C to constant weight. Lipids were determined by weight on a precision balance. The yield of lipids has been calculated according to this equation:

Yield (g g^{-1}) = Total lipids produced (g L^{-1})/Total xylose used (g L^{-1}).

2.5 | Enzyme Assays

Crude extracts were prepared from overnight cultures of 50 mL YPD medium, using different strains (YXR, YXI-XR, and YDGA). After cultivation, cells were harvested by centrifugation and washed twice with 50 mM Tris/HCl buffer (pH 8.0). Pellets were resuspended in 3 mL of the same buffer. For cell disruption, 1 mL of the resuspended cells was transferred to 2 mL FastPrep plastic tubes. Each sample was mixed with 0.4 mL of glass beads and subjected to four 40-s pulses for grinding. The resulting lysate was centrifuged at 16,000 × *g* for 10 min at 4°C. The supernatant (crude protein extract) obtained after centrifugation containing about 50 mg protein per mL was used for enzymatic assays. Enzymes were assayed at 25°C using a Shimadzu UV-1900i spectrophotometer as described (Verduyn et al. 1985). Xylose reductase activity was measured in 50 mM Tris/HCl buffer (pH 8.0), adding 70 mM xylose as substrate and crude protein extract. The reaction was initiated by adding 0.2 mM NADPH. Xylitol dehydrogenase activity was measured in 50 mM Tris/HCl buffer (pH 8.0), adding 70 mM xylitol as substrate, 5 mM $MgCl_2$, and crude protein extract. The reaction was initiated by adding 0.2 mM NAD^+. Enzyme units are defined as μmol of nicotinamide nucleotide reduced or oxidised per min. Specific activities are expressed as U mg^{-1} protein.

3 | Results

3.1 | Engineering Xylose Metabolism in *Y. Lipolytica*

To test the efficiency of the isomerase and reductase pathways in the same isogenic background, we have constructed three recombinant strains using as host the obese *Y. lipolytica* DGA strain, which were named YXI, YXR, and YXI-XR, expressing the isomerase, the reductase or both synthetic pathways together, respectively. In all cases we have also overexpressed the homologous XK to improve the efficiency of the pathway, as proposed by Gao et al. (2023) in *O. polymorpha*.

The three recombinant strains were tested on xylose (30 g L^{-1}) under aerobic, low oxygen availability, and anaerobic conditions. These experiments were performed in open flasks or closed bottles with an air chamber of 90% or 10% of the total volume, respectively. Figure 1 shows that all recombinant strains can grow with xylose as the sole carbon source and that oxygen availability is crucial for biomass production. Under anaerobic conditions, cells reached an OD_{600} of 0.2 (Figure 1A). However, under low oxygen availability, cells reached an OD_{600} of 4–6 (Figure 1B) and under aerobic conditions, the OD_{600} was 15–20 (Figure 1C).

Under high oxygen availability, we observed that strain YXR grows better than the other two strains, YXI and YXI-XR (Figure 1C). In the 90% air chamber, the growth rate of YXR was higher than that of YXI-XR and YXI (Figure 1B). However, under anaerobic conditions, the strain YXI shows the best performance (Figure 1A).

3.2 | Consumption of Xylose in Flask Cultures by the Recombinant Strains

The consumption of xylose by the three recombinant strains was determined using xylose as the sole carbon source. Figure 2 shows that, as expected, the three constructed recombinant strains were able to consume xylose, whereas the parental strain was not. The YXR strain showed the highest xylose consumption rate, whereas the YXI strain showed the lowest (Figure 2B). This behaviour correlates with the biomass production (Figure 2A,C). It is worth mentioning that in the chromatograms used to quantify the consumption of xylose, we did not detect the accumulation of xylitol.

In addition, consumption of xylose by the three recombinant strains was determined using a mixture of sugars including glucose, xylose, and arabinose in order to mimic a lignocellulose hydrolysate as a carbon source and although very unlikely, to determine if the presence of arabinose might interfere in the consumption of xylose. Figure 3 shows that using the mixture of sugars a similar growth rate can be observed in the YXR and YXI-XR strains while the growth rate of YXI is lower (Figure 3A). Glucose is the preferred sugar since it is consumed faster than xylose by all recombinant strains. Although xylose can be consumed in the presence of glucose, its consumption is strongly inhibited. Interestingly, the glucose consumption was higher in the YXR and YXI-XR strains than in YXI. This behaviour correlates with the highest amount of biomass reached by the YXR and YXI-XR strains (about 7 g L^{-1}). Although *Y. lipolytica* cannot metabolise arabinose (Spagnuolo et al. 2018), we confirmed that the presence of arabinose in the culture medium did not impair the consumption of xylose by the recombinant strains.

Regarding the production of lipids in flask cultures, Figure 2D shows how the YXI strain is able to accumulate around 35% of lipids using xylose as a sole carbon source, whereas the other strains YXR and YXI-XR accumulate about 20%, suggesting that the type of xylose pathway used affects lipid accumulation. Nevertheless, the highest lipid yield (2 g L^{-1}) is obtained with strain YXR, because this strain reaches 9 g L^{-1} biomass while YXI only reaches 5 g L^{-1} biomass (Figure 2C,E).

In the assays carried out with a mixture of sugars, the lipid production responds to glucose metabolism, which is the main metabolised sugar and therefore, few differences in the lipid production are observed within the strains (Figure 3D). Using glucose, the strain YXI was able to accumulate 35% lipids.

3.3 | Consumption of Xylose and Lipid Production in Bioreactor by the Recombinant Strains

Xylose consumption and lipid production were evaluated in bioreactors with the recombinant strains as this allows control of pH and oxygen availability. Figure 4 shows the results of the batch experiments performed with 50 g L^{-1} of xylose

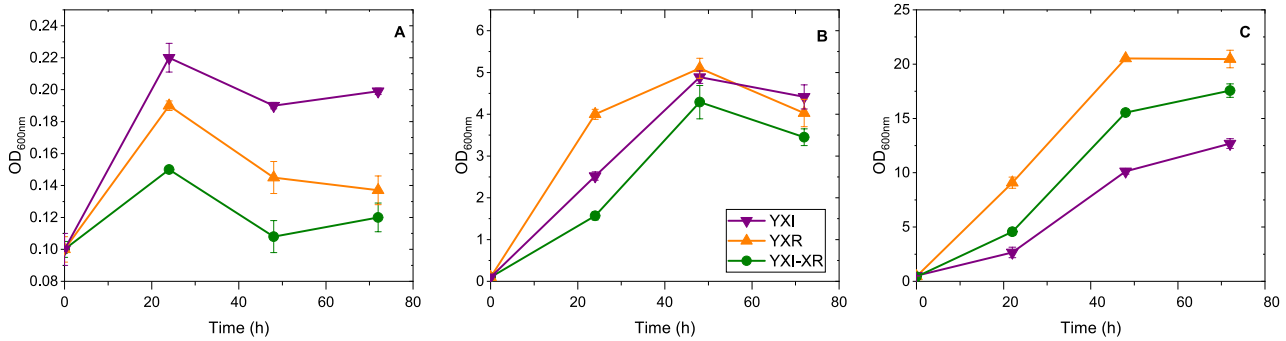

FIGURE 1 | Growth of recombinant and parental *Y. lipolytica* strains. Recombinant cells were cultured with xylose (30 g L^{-1}). (A) Cultures were performed in closed flasks with a 10% air chamber. (B) Cultures were performed in closed flasks with a 90% air chamber. (C) Cultures were performed in open flasks.

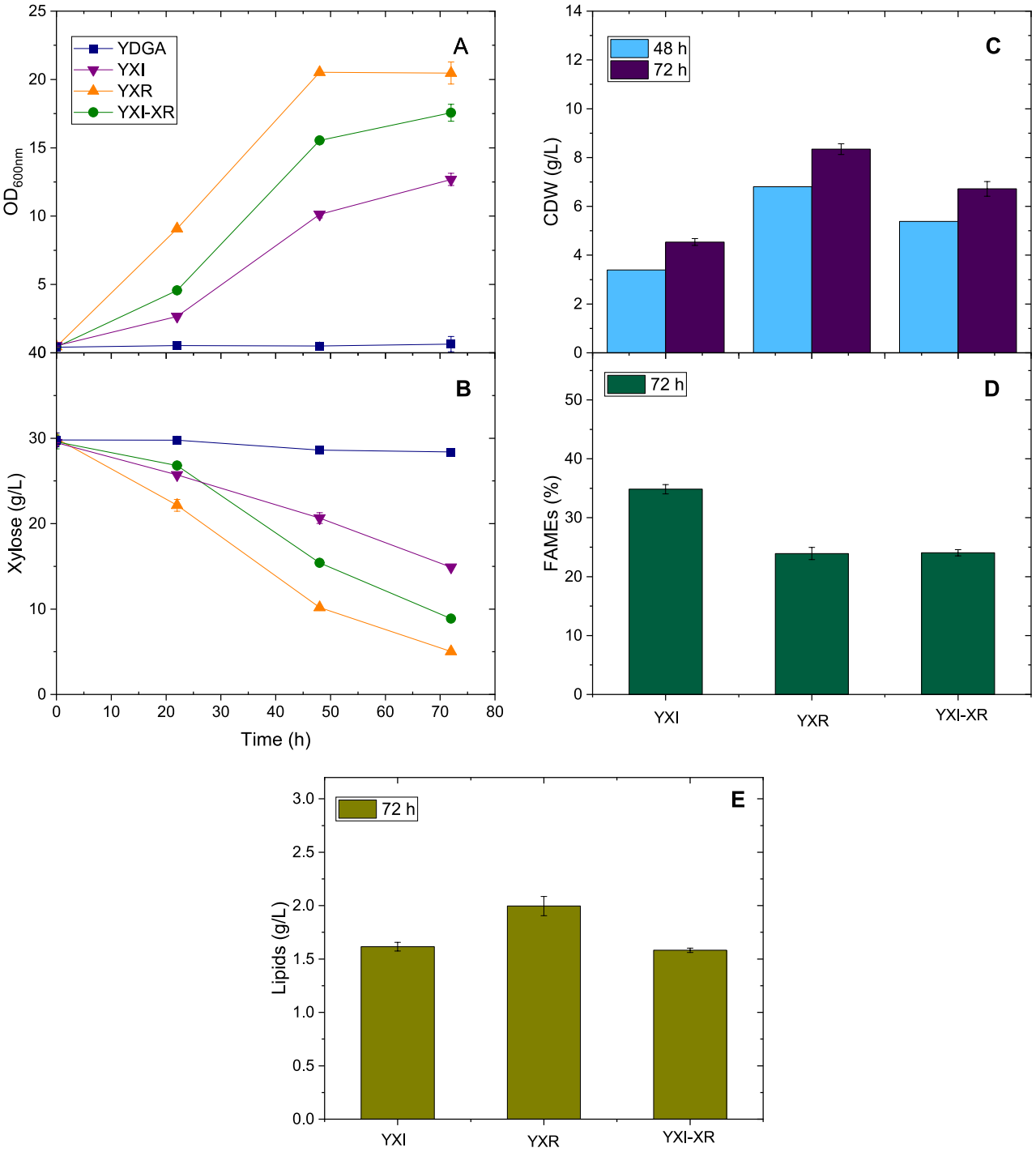

FIGURE 2 | Growth and lipid production of the recombinant *Y. lipolytica* strains. Cells were cultured in flasks with 30 g L^{-1} of xylose. (A) Optical density time course. (B) Xylose consumption. (C) Cell dry weight (g L^{-1}); (D) FAMEs percentage. (E) Lipids concentration (g L^{-1}).

and a C/N ratio of 75. In these bioreactor assays, the pH was maintained at 3.5 since this is the optimal pH to accumulate lipids (Friedlander et al. 2016). Figure 4A shows that strain YXR is able to reach the highest OD_{600} values of 50, whereas strains YXI and YXR-XI reach only OD_{600} values of 40. These results correlate with cell dry weight (Figure 4C) where YXR reaches about 13 g L^{-1} of biomass, whereas the other two strains reach only 10 g L^{-1}. Xylose is consumed almost completely by the YXR strain, whereas in the other strains, the xylose consumption rate decreased after 52 h (Figure 4B). Strain YXR is the best lipid producer, being able to accumulate under these culture conditions 40% of lipids, rendering a total yield of 4.5 g L^{-1} (Figure 4E).

Considering that the YXR strain was the best producer, we decided to test the lipid production by culturing the cells at a higher xylose concentration (150 g L^{-1}) maintaining the same operational conditions (i.e., pH 3.5 and C/N: 75). Figure 5 and Table 3 show that strain YXR is able to accumulate 43% of lipids with a final yield of 12.8 g L^{-1}.

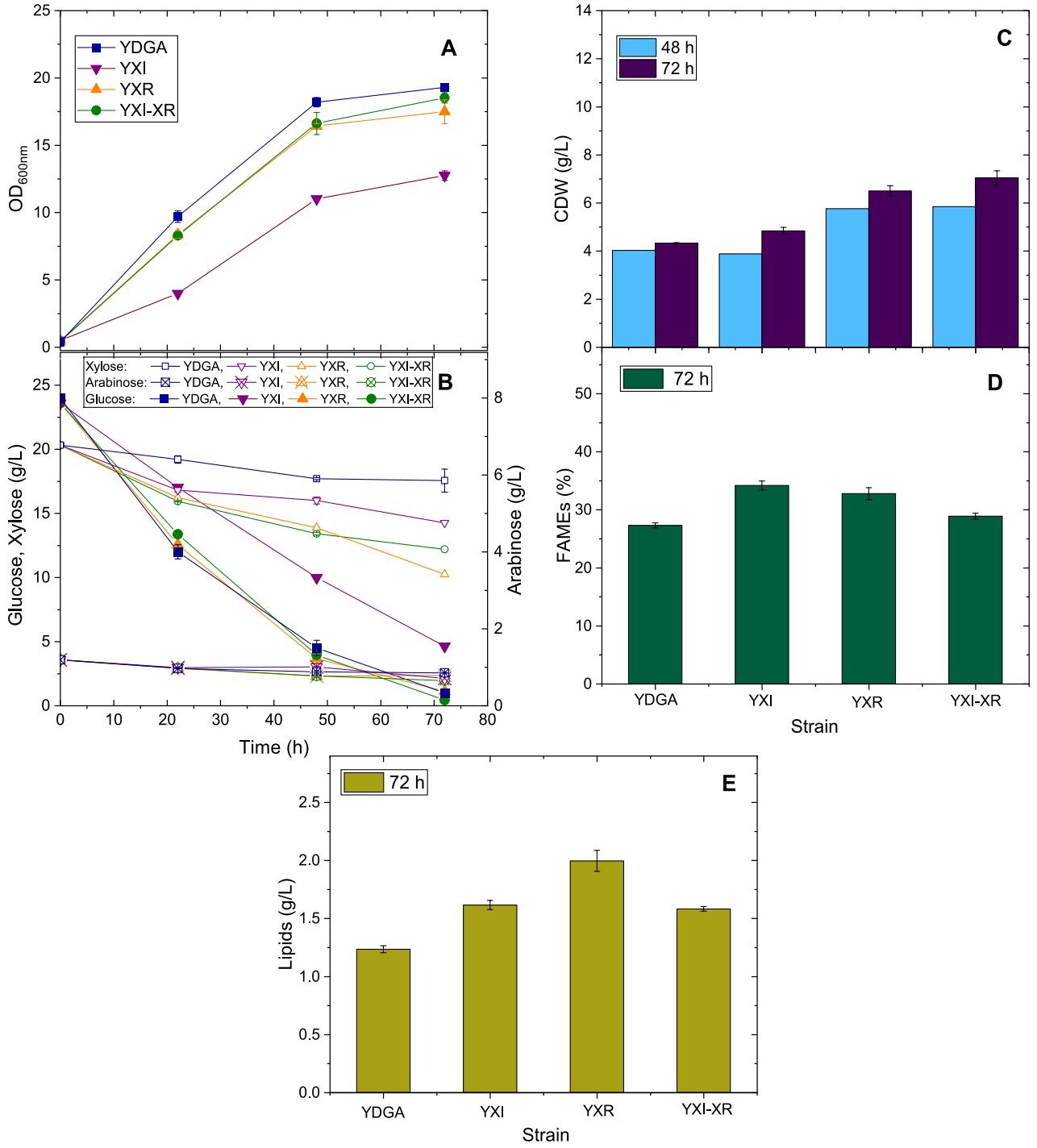

FIGURE 3 | Growth and lipid production of the recombinant *Y. lipolytica* strains cultured with a sugar mix (25 g L^{-1} glucose, 20 g L^{-1} xylose and 4 g L^{-1} arabinose). (A) optical density time course. (B) Sugar compositions. Glucose (closed symbol), xylose (open symbol) and arabinose (crossed-out symbol). (C) Cell dry weight (g L^{-1}). (D) FAMEs percentage. (E) Lipids concentration (g L^{-1}). Times for sampling is indicated by colours.

4 | Discussion

Our results show that the behaviour of the three recombinant strains developed in this work, capable of utilising xylose through two synthetic pathways, is different when cultured in xylose as a sole carbon source. The YXR strain uses the oxide-reductive pathway present in fungi, which requires two enzymes: NAD(P)H-dependent XR and NAD-dependent XDH. In this case, xylose metabolism is highly oxygen-dependent because the pathway consumes NAD(P)H and NAD+ and under low oxygen availability, it might create a cofactor imbalance resulting in xylitol accumulation (Jefries 2006; Liang et al. 2014). This is the reason why the YXR strain grows best in xylose under high oxygen concentration conditions. In contrast, the isomerase pathway transforms xylose into xylulose without the need for cofactors (Bergdahl et al. 2012) and this pathway should be advantageous regardless of oxygen concentration. This is the reason why YXI grows better under oxygen-limited

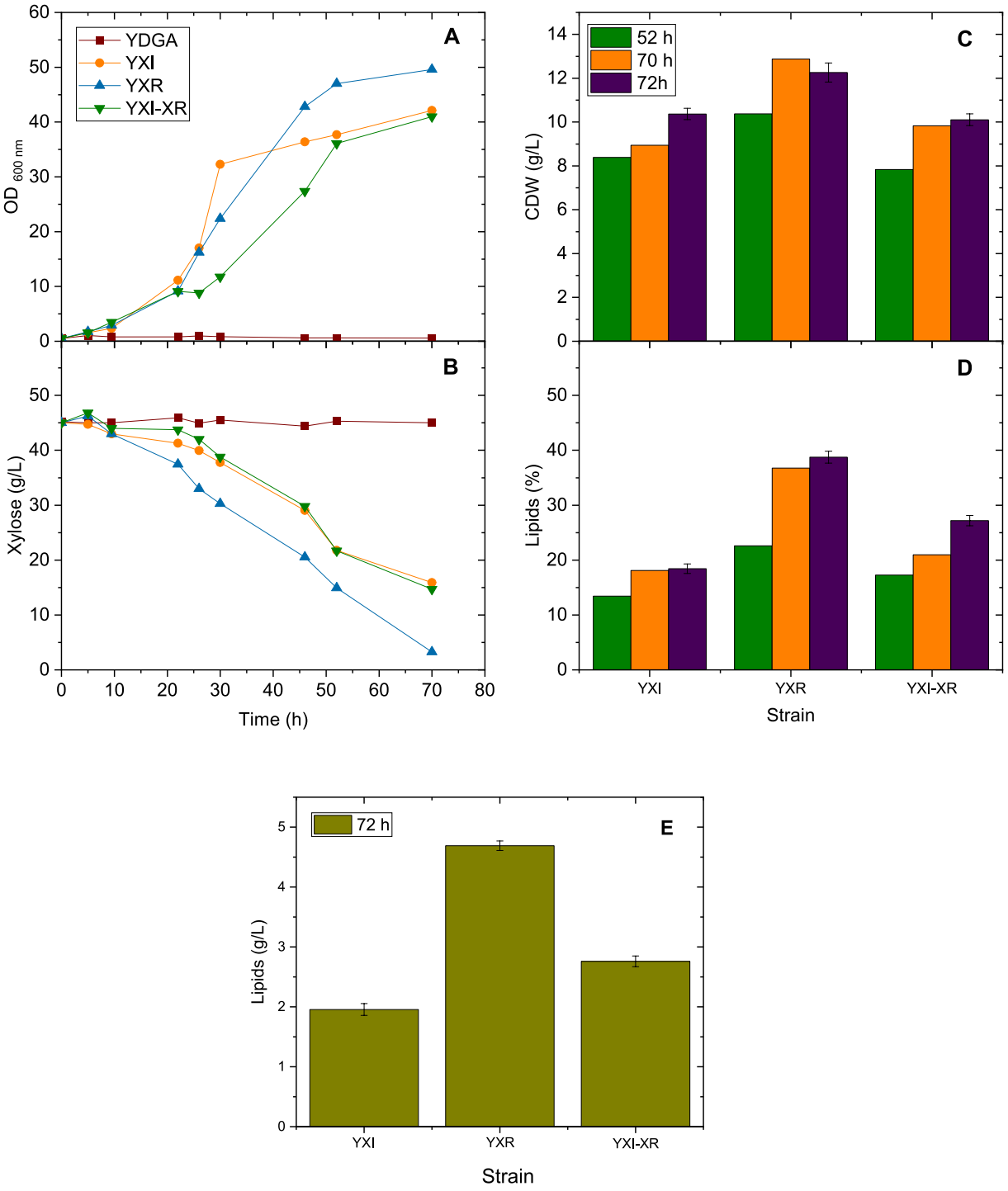

FIGURE 4 | Bioreactor tests performed with 50 g L^{-1} of xylose. (A) Optical density time course. (B) Xylose consumption. (C) Cell dry weight (g L^{-1}). (D) FAMEs percentage. (E) Lipids concentration (g L^{-1}).

atmospheres. Finally, in principle, the YXI-XR strain that harbours both pathways (reductase and isomerase) should be able to adapt the xylose metabolism depending on the availability of oxygen and cofactors. Nevertheless, this assumption is not so simple, since it has been described that xylitol might inhibit the xylose isomerase activity (Brat et al. 2009; Yamanaka 1969) and that the isomerization of xylose to xylulose is a reversible reaction where the xylose formation is more favourable than xylulose formation at equilibrium by 80:20 (Chiang et al. 1981; Young et al. 2010). These factors should be considered since the reductase and the isomerase pathways converge in the same xylulose intermediate.

Figure 1 shows how the YXI strain performs the best under anaerobic conditions; however, the strain YXI-XR grows poorly in xylose in all cases compared with the YXR strain. This suggests that the presence of the isomerase pathway impairs the consumption of xylose in contrast to the hypothesis that the presence of both pathways together could improve xylose consumption. This result correlates with the observation that the

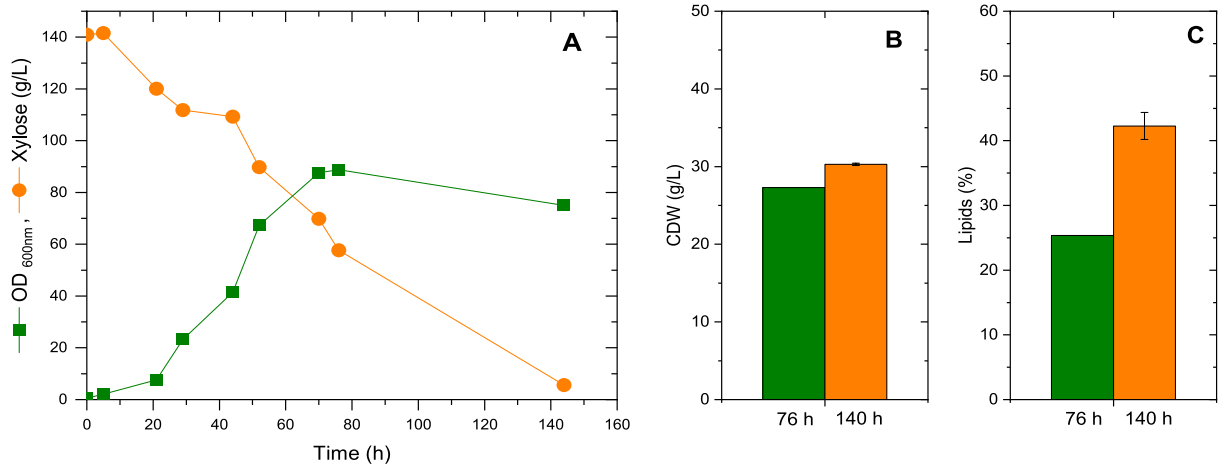

FIGURE 5 | Bioreactor tests performed with 150 g L^{-1} of xylose with YXR strain. (A) Optical density and xylose concentration time course. (B) Cell dry weight (g L^{-1}). (C) FAMEs percentage. Times for sampling is indicated by colours.

TABLE 3 | Xylose consumption and lipid production using recombinant *Y. lipolytica* strains produced in this work expressing the reductase, the isomerase, or both pathways.

Yarrowia lipolytica strain	Description	Initial xylose concentration (g L^{-1})	Culture conditions	Lipid titter (g L^{-1})	Lipid content (%)	Total yield (glip/gxyl)	Reference
YXR	DGA ssXR ssXDH ylXK	45	Batch, C/N:75	4.8	39	0.110	This study
YXI-XR	DGA ssXR ssXDH XylA ylXK	45	Batch, C/N:75	2.9	25	0.060	This study
YXI	DGA XylA ylXK	45	Batch, C/N:75	2.0	20	0.040	This study
YXR	DGA ssXR ssXDH ylXK	150	Batch, C/N:75	12.8	43	0.090	This study

xylose consumption rate of the YXI strain is lower than that of the YXR strain. Our results suggest that not only the isomerase pathway is less efficient than the reductase pathway when oxygen is not limited, but also the combination of both pathways causes a metabolic burden that reduces the xylose consumption rate under aerobic conditions. This happens in spite of the fact that we have overexpressed the homologous XK of *Y. lipolytica* in all the recombinant strains to favour the phosphorylation of the xylulose intermediate produced by both pathways.

When we have analysed the XDH and XR activities of the YXI-XR and YXR clones, we have observed that these activities are highly expressed in both clones (Figure S4). The slightly lower activities observed in the YXI-XR clone should not explain the different behaviour of both clones. The presence of XI in the YXI-XR appears to create a metabolic cycle that reduces the efficiency of the reductase pathway. This artificial cycle might explain why the XDH activity cannot completely transform all NAD^+ into NADH in the presence of XI. After a while, an equilibrium is reached between NAD^+ and NADH because XI transforms xylulose into xylose and XR transforms xylose into xylitol using the NADH produced by XDH. However, the XR clone completely transforms NAD^+ into NADH since xylulose cannot be transformed into xylose and the NADH cannot be recycled by XR. It is worth mentioning that we were able to see this artificial cycle when we used xylitol and NAD^+ in the reaction because, although XR uses preferably NADPH as cofactor, it can also use NADH. This is not the case for XDH, which only uses NAD^+.

It was interesting to notice that we have not observed the secretion of xylitol to the culture medium in the YXR and YXI-XR clones when cultured with 30 g L^{-1} of xylose, indicating that the reductase pathway does not cause a critical cofactor imbalance promoting the accumulation of xylitol. In this sense, Ledesma-Amaro et al. (2016) have observed that the secretion of xylitol to the culture medium by a *Y. lipoltica* recombinant carrying a heterologous reductase pathway depends on the concentration of xylose used to grow the cells, since they were only able to detect xylitol using more than 30 g L^{-1} of xylose. Moreover, the overexpression of XK in our clones might also explain that we could not find xylitol in the extracellular medium, since most probably the internal concentration of xylitol is highly reduced by the overexpression of XK, as observed in *O. polymorpha* (Gao et al. 2023). These authors have demonstrated that the overexpression of XK in *O. polymorpha*, but not the expression of XI, is the essential factor that facilitates growth on xylose, and apparently reduces the cofactor imbalance and consequently the accumulation of xylitol. The additional expression of XI only

slightly increases the consumption of xylose, but, at the same time slightly reduces the lipid accumulation under aerobic conditions (Gao et al. 2023).

The behaviour of both xylose pathways has also been studied in *S. cerevisiae*, showing a very low rate of xylose uptake by the strain harbouring the isomerase pathway (Bettiga et al. 2008; Bergdahl et al. 2012). This lower uptake rate with the isomerase pathway has also been observed in an XI engineered strain of *Y. lipolytica*, which required a further adaptative evolution to efficiently consume xylose (Yook et al. 2020), in accordance with our results (Figure 3). The precise accumulated mutations responsible for such adaptative improvement were not identified (Yook et al. 2020).

On the other hand, we observed that glucose competes with xylose uptake in all recombinant strains (Figure 3). This effect cannot be attributed to a catabolic repression, since glucose does not cause this effect in *Y. lipolytica* (Mori et al. 2013; Worland et al. 2020), and the heterologous xylose pathways that we have introduced are constitutively overexpressed because the genes are expressed under the control of the strong constitutive P_{TEF} promoter, as demonstrated by Ledesma-Amaro et al. (2016). Considering that *Y. lipolytica* is not able to grow on xylose, it has not developed an inducible specific xylose transporter; therefore, xylose must compete with glucose for the high-affinity transport systems induced in the yeast to uptake glucose or other sugars. The competition between xylose and glucose for the sugar-binding proteins of the uptake systems has already been observed in other yeasts (Subtil and Boles 2012). Therefore, xylose transport can only be improved by introducing modifications in the glucose transport proteins to reduce the affinity for glucose without affecting their low xylose affinity (Zhou et al. 2020; Zhang et al. 2023).

Concerning lipid metabolism, cells use two NADPH molecules for each fatty acid elongation step, and one NADPH is consumed for each fatty acid desaturation reaction (Wasylenko et al. 2015). Then, the xylose isomerase pathway, which does not consume this cofactor, does not compete with lipid synthesis and should be in principle more efficient for lipid production when compared with the xylose reductase pathway that consumes NADPH. This reason can explain why strain YXI is able to accumulate up to 35% lipids, while the other recombinant strains accumulate only 25% of lipids (Figures 2 and 3). However, the final lipid yield is lower in the YXI strain because the biomass production is lower since the isomerase pathway is less efficient in xylose uptake.

According to Table 1 that compiles the results from other authors on the lipid production by *Y. lipolytica* using xylose as a carbon source, the best results have been obtained with a recombinant strain also evolved by ALE. This strain produces 60 g L^{-1} of lipids and is able to accumulate up to 73% cell dry weight of lipids with a yield of 0.13 g/g (Sun et al. 2023). Our results (Table 3) show how the YXR strain was able to produce 5 g L^{-1} of lipids, achieving a yield of 0.11 g/g, that is one of the highest yields obtained using xylose as a substrate in a non-evolved recombinant strain. These results were obtained under batch conditions using 45 g L^{-1} of xylose and were further improved using 150 g L^{-1} of xylose, where YXR accumulates 43% lipids and produces 12.8 g $L^{-1}$of lipids (Table 3). The reason for these high yields with xylose without any further evolution might be due to the fact that the transformants of *Y. lipolytica* were screened directly on minimal medium plates containing xylose as the sole carbon source, allowing us to select the clones that grow more efficiently in xylose in a single selective step.

Summarising, in this work we have demonstrated that it is possible to select a recombinant strain of *Y. lipolytica* with a highly efficient consumption of xylose by a direct screening of the recombinant clones in a selective xylose medium. On the other hand, our results suggest that the reductase route is more efficient than the isomerase pathway without a further evolution when compared in the same isogenic background. Moreover, our results demonstrate that the joint expression of the two routes within the same cell, far from producing a metabolic advantage, generates a decrease in xylose consumption in *Y. lipolytica* in contrast with the results obtained in other yeasts.

5 | Conclusions

In this study, a comprehensive comparison of the efficiency of two main xylose metabolic pathways—reductase and isomerase—has been conducted in *Y. lipolytica*. Our findings reveal that *Y. lipolytica* exhibits a higher efficiency in xylose consumption when the reductase pathway is expressed. Conversely, the efficiency diminishes when either the isomerase pathway alone or both pathways (isomerase and reductase) are expressed. A significant outcome of this research is the development of a *Y. lipolytica* strain capable of producing up to 12.8 g/L of lipids, which constitutes 43% of the dry biomass weight from 150 g/L of xylose. Moreover, this strain provided one of the highest lipid yields reported to date using xylose as the sole carbon source, with a conversion rate of approximately 0.13 g of lipids per gram of xylose. Consequently, this new strain emerges as a promising candidate for the production of lipids from lignocellulosic residues rich in xylose. This capability highlights its potential for contributing to sustainable bioconversion processes, transforming xylose-rich lignocellulosic residues into valuable lipid-based products. Furthermore, we cannot discard the possibility of further optimising our strain through additional Adaptive Laboratory Evolution (ALE) in xylose, which could potentially lead to even higher lipid yields as demonstrated in other strains.

Author Contributions

Isabel De La Torre: conceptualization, methodology, investigation, formal analysis, writing – original draft, writing – review and editing. **Miguel G. Acedos:** conceptualization, investigation, writing – original draft, writing – review and editing, methodology, formal analysis. **Juan J. Cestero:** conceptualization, formal analysis, writing – review and editing. **Jorge Barriuso:** project administration, supervision, writing – review and editing. **José L. García:** conceptualization, project administration, writing – original draft, writing – review and editing.

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Ethics Statement

The authors have nothing to report.

Consent

The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.