Toward rapid and efficient utilization of non-conventional substrates by non-conventional yeast strains

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ABSTRACT

Economic and sustainable production of biofuels and chemicals necessitates utilizing abundant and inexpensive lignocellulosic biomass. Yet, *Saccharomyces cerevisiae*, a workhorse strain for industrial biotechnology based on starch and sugarcane-derived sugars is not suitable for lignocellulosic bioconversion due to a lack of pentose metabolic pathways and severe inhibition by toxic inhibitors in cellulosic hydrolysates. This review underscores the potential of non-conventional yeast strains, specifically *Yarrowia lipolytica* and *Rhodotorula toruloides*, for converting under-utilized carbon sources, such as xylose and acetate, into high value products. Multi-omics studies with non-conventional yeast have elucidated the structure and regulation of metabolic pathways for efficient and rapid utilization of xylose and acetate. The review delves into the advantages of using xylose and acetate for producing biofuels and chemicals. Collectively, value-added biotransformation of non-conventional substrates by non-conventional is a promising strategy to improve both economics and sustainability of bioproduction.

Key words: Xylose, Acetate, *Rhodosporidium*, *Rhodotorula*, *Yarrowia*

1. Introduction

To ensure sustainable and cost-effective production of biofuels and chemicals, it is crucial to use inexpensive and abundant feedstocks, as raw materials account for 50-70% of microbial fermentation operation costs [1, 2]. Lignocellulosic hydrolysates, containing diverse carbon sources like glucose, xylose, and acetate, offer a compelling advantage as they can be used directly for microbial growth and bioconversion, avoiding expensive purification steps to produce pure sugars of which utilization would compete with food industries (Fig 1a) [3].

However, conventional yeast species like *Saccharomyces cerevisiae* encounter challenges in efficiently growing on cellulosic hydrolysates. They lack the necessary genes to metabolize diverse carbon sources and are sensitive to fermentation inhibitors or extreme pH levels in lignocellulosic hydrolysates. Consequently, attention has shifted towards non-conventional yeast strains that can thrive under these conditions. *Yarrowia lipolytica* and *Rhodotorula* (*Rhodosporidium*) *toruloides*, known as oleaginous yeast strains, have been considered as a possible platform host for producing cellulosic biofuels and chemicals, as evidenced by significant increases of related research reports [4] (Fig. 1b, c). Both non-conventional yeast strains are under extensive investigation for their promising roles in bioproduction using cost-effective feedstocks.

Especially, *Y. lipolytica* is now considered a model non-conventional oleaginous yeast, with several established genetic engineering tools [5]. Although its toolkit is not as advanced as those available for conventional yeasts, *Y. lipolytica*'s genetic manipulation capabilities and ability to utilize acetate, coupled with its low baseline expression of enzymes for xylose utilization, suggest promising avenues for optimizing its use of non-conventional carbon sources. In contrast, while genetic engineering of *R. toruloides* is still in its nascent stages,

68 the strain naturally possesses high potential for utilizing diverse carbon sources, including
69 glucose, xylose, arabinose, acetate, glycerol, and more [6].
70 This review delves into recent advancements in utilizing unconventional carbon sources,
71 primarily xylose and acetate, by two leading non-conventional yeast strains: *Yarrowia*
72 *lipolytica* and *Rhodotorula toruloides*.
73
74 **2. Xylose utilization by non-conventional yeasts**
75 Xylose, the second most abundant sugar in nature, is primarily obtained from the
76 hydrolysis of lignocellulosic biomass. Additionally, compared to glucose, xylose has many
77 advantages for producing mitochondrial-targeted products [7] and acetyl-CoA-derived
78 products in yeasts [8]. While xylose production from the hydrolysis of hemicellulose offers a
79 good yield, purification procedures to produce pure xylose are complex and costly. As such,
80 direct utilization of hemicellulose hydrolysate without purification may significantly lower
81 the cost of biofuel and chemical production. Given that lignocellulosic hydrolysates contain
82 considerable amounts of glucose and xylose, efficient and rapid xylose utilization is crucial
83 for optimizing bioconversion using these hydrolysates as feedstocks.
84 This section summarizes how non-conventional yeasts, whether naturally evolved or
85 engineered, are capitalizing on xylose's potential, discussing associated metabolic pathways,
86 challenges, and biotechnological implications.
87
88 **2.1. *Y. lipolytica* engineering to enhance xylose utilization**
89 *Y. lipolytica* contains genes coding for xylose reductase (XR), xylitol dehydrogenase
90 (XDH), and xylulokinase (XK). Still, the inherent activities of these enzymes, particularly
91 XDH and XK, are not robust enough for effective xylose assimilation. Therefore, xylose is

not an appropriate carbon source of *Y. lipolytica* for producing biofuels and chemicals [9]. Since 2016, many efforts have been made to understand xylose metabolism and achieve efficient xylose assimilation in *Y. lipolytica*.

One approach was to identify putative xylose transporters through bioinformatics approaches. Ryu et al. [10] identified 22 native genes in *Y. lipolytica* associated with putative xylose transport using a blast analysis. Further mRNA analysis revealed that two genes, YALI0C04730 and YALI0B00396, exhibited a nearly 5 to 10-fold increase in expression when exposed to xylose. In a subsequent study, Ryu and Trinh [11] confirmed that YALI0C04730 and YALI0B00396 act as transporters for pentose sugars in *Y. lipolytica,* and among them, the efficacy of YALI0B00396g overexpression for improving xylose assimilation was revisited by another research [12].

The second approach to achieve efficient xylose assimilation was to explore xylose metabolic enzymes (oxidoreductase and isomerase pathway in *Y. lipolytica*. Li and Alper [13] introduced *XYL1* and *XYL2* genes, coding for XR and XDH from *Scheffersomyces stipitis*, into the *Y. lipolytica* PO1f strain to enhance its ability to metabolize xylose. This modification, combined with 14 days of adaptive laboratory evolution (ALE), resulted in an engineered and evolved strain capable of assimilating xylose efficiently. In a similar vein, Ledesma-Amaro R, Lazar Z, Rakicka M, Guo Z, Fouchard F, Coq A-MC-L and Nicaud J-M [14] boosted the expression of the endogenous XK and heterologous *S. stipitis* XYL1 and XYL2 to the PO1d strain, a parental strain of PO1f, which has additional AXP (encoding the acidic protease) gene deletion from PO1d, achieving growth rates on xylose comparable to those on glucose. Another study by Rodriguez GM, Hussain MS, Gambill L, Gao D, Yaguchi A and Blenner M [15] reported that the overexpression of endogenous XDH (YALI0E12463) and XK (YALI0F10923) was enough to achieve efficient xylose assimilation in *Y. lipolytica*,

even without additional overexpression of two possible functional xylose reductases (*ylXR1*:YALI0D07634 and *ylXR2*:YALI0F18590). A recent study by Yook et al. [16] introduced a heterologous XR, XDH, and XK from *S. stipitis* into *Y. lipolytica* PO1f and conducted ALE under xylose condition. Through the whole-genome sequencing followed by Cas9-based reverse engineering, the author identified that rapid xylose assimilation by the evolved strain was enabled by the amplification of heterologous xylose metabolic enzymes with a partial contribution of SNP in a cellular signaling protein.

In parallel, the introduction of a xylose isomerase (*XylA*) from *Piromyces* sp. into *Y. lipolytica* has been explored. While Li and Alper [13] could not observe a drastic growth improvement under xylose conditions by introducing xylose isomerase into *Y. lipolytica*, another study by Yook et al. [17] using a modified XylA (E15D, E114G, E129D, T142S, A177T, and V433I), previously reported by Lee et al [18] followed by ALE showed potential in both xylose assimilation and lipid production. Although the xylose consumption rate after expression of the modified XylA was slower than those by engineered *Y. lipolytica* based on heterologous oxidoreductases, the study achieved the highest lipid production (12.01g/L and 0.16 g lipid/g glucose+xylose) from lignocellulosic biomass by preventing the consumption of NADPH by XR.

Additional strategies to improve the utilization of cellulosic sugars have been proposed for *Y. lipolytica*. The use of 2-deoxyglucose, an analog of D-glucose, combined with xylose in ALE experiments led to the generation of evolved strains exhibiting reduced carbon catabolite repression (CCR), enabling co-utilization of D-glucose and D-xylose [19]. Also, a mating approach between a haploid xylose-utilizing strain and engineering strains producing α-linolenic acid, riboflavin, and triacetic acid lactone (TAL), respectively, has

been reported as a feasible approach for combining advantageous phenotypes of multiple strains with independent phenotypes [20].

The above-mentioned efforts to improve xylose utilization resulted in enhanced production of lipids [21, 22] and diverse target molecules from xylose. For instance, overexpressing a native xylose metabolic pathway (*ylXR*, *ylXDH*, and *ylXK*) in the succinate-producing *Y. lipolytica* strain (PSA02004; PO1f Δ*sdh5*) led to the production of 22.3g/L of succinate with a yield of 0.15 (g succinate/g xylose) and a productivity of 0.14 ($gL^{-1}h^{-1}$) through fed-batch fermentation [23]. The overexpression of *ssXR*, *ssXDH*, *ylXK*, along with heterologous tNDPS1 (a truncated LS coding for orthogonal limonene synthetic enzyme) and mevalonate pathway enzymes (*HMG1* and *ERG1*) notably enhanced limonene production from xylose, reaching up to 9 mg/L within 72 hours from lignocellulosic hydrolysate [24]. Wu et al. [12] introduced ssXR, ssXDH, and ylXK combined with manipulated expression of native TKL and TAL genes to produce protopanaxadiol (PPD). The engineered *Y. lipolytica* strain exhibited significantly higher PPD production from xylose (300.63mg/L) than the engineered *S. cerevisiae* strain developed by the same research group [25], exhibiting only 152.37mg/L from a mixture of glucose and xylose. These results imply that engineered *Y. lipolytica* produced higher acetyl-CoA, a precursor for PPD biosynthesis, influencing higher PPD production than *S. cerevisiae* from xylose.

While extensive efforts have been to understand the xylose metabolism of *Y. lipolytica*, the endeavors to synthesize new target molecules have been relatively limited. There is a need for more reported cases introducing xylose metabolism to a broader range of engineered *Y. lipolytica* capable of producing various target molecules to better understand the correlation between *Y. lipolytica*'s xylose metabolism and product synthesis.

2.2. *R. toruloides* engineering to enhance xylose utilization

Rhodotorula toruloides naturally assimilates xylose at rates of 70-90% compared to that of glucose, even without genetic modifications [26, 27] (Fig. 2). However, secretion of xylitol and L-arabitol during xylose uptake results in its growth rate to 44-70% on xylose compared to that on glucose [26-28]. While improving xylose utilization by *R. toruloides* is crucial for utilizing cellulosic hydrolysates, limited genetic tools and an incomplete understanding of xylose metabolism in *R. toruloides* have hindered research progress. Recent years, however, have seen extensive efforts to elucidate xylose metabolism in *R. toruloides* through multi-omics analyses (Table 1) [26, 27, 29-33], bolstered by advancements in genetic engineering tools like CRISPR/Cas9 [34].

Tiukova et al. [26] identified thirteen sugar transporters in *R. toruloides* CBS14, eight of which (Rhto_01630, Rhto_03448, Rhto_07444, Rhto_06801, Rhto_06080, Rhto_01923, Rhto_00228, Rhto_07706) exhibited increased protein levels during xylose cultivations. Similarly, in *R. toruloides* IFO0880, two specific sugar transporters (RTO4_12976, RTO4_10452) showed 6-8 times higher protein levels under xylose than glucose. Yet, these transporters are repressed under glucose-rich conditions, hindering simultaneous uptake of xylose and glucose [31, 35]. Recently, deletion of genes (RTO4_11990, RTO4_9588, and RTO4_11924) homologous to glucose transceptors participating in carbon catabolite repression in *Neurospora crassa* (HGT-1/2) were investigated for understanding the roles of putative sugar transporters during xylose metabolism [33, 36]. Deletion of RTO4_11990 led to 6.5 times faster growth with xylose as a sole carbon source, with reduced or similar amount of sugar alcohol production. However, no significant difference was observed in other mutants or in the presence of glucose, leaving questions about the actual function of the RTO4_11990 protein encoded by HGT-1/2 homologues.

Once xylose enters the cells of *R. toruloides*, it is primarily metabolized by XR (RTO4_9774), XDH (RTO4_16452), and multiple reductase/dehydrogenase enzymes (RTO4_9990, RTO4_14368, RTO4_8988, RTO4_12977, RTO4_12974, RTO4_11882, RTO4_13562) with broad substrate specificities. Even with the deletion of XR or XDH, growth on xylose was not eliminated [37]. Additionally, by expressing heterologous XI from *Piromyces* sp. in the XR/XDH deleted strains, the cells were able to completely recover their growth on xylose [37]. Intriguingly, although XK is central to the standard fungal xylose pathway, its expression (*rt*XK, RTO4_16850), which had been annotated not based on experimental confirmation but based on bioinformatic homology analysis, was not detected during xylose fermentation in *R. toruloid*es [27, 31, 32]. This result suggests an alternate metabolic route for assimilating xylulose in *R. toruloides*. This is corroborated by the accumulation of substantial amounts of D-arabitol as a byproduct during xylose fermentation [29, 32, 38], and the observation that XK deletion did not impact growth in xylose media [37]. Subsequent studies identified two NAD+-dependent D-arabinitol dehydrogenase genes (RHTO_07702 and RHTO_07844), potentially catalyzing xylulose conversion to arabinitol in *R. toruloides* NP11 [39]. In *R. toruloides* IFO0880 growing on xylose, high expression levels of ortholog proteins D-arabinitol 4 dehydrogenase (DAD-4, RTO4_8905, RTO4_9837) and D-arabinitol 2-dehydrogenase (DAD-2, RTO4_9990), which sequentially convert xylulose into D-arabinitol and D-ribulose, were observed [31]. Adamczyk et al. found that DAD-2 deletion prevented the strain from using xylose as a sole carbon source [37]. Intriguingly, in *R. toruloides* CCT7815, DAD-2 was not identified, and it is suggested that the function is replaced by L-xylulose reductase (RHTO_00373), a NADPH-dependent enzyme capable of converting D-arabinitol to D-ribulose [32].

D-ribulose is then converted to D-ribulose-5 phosphate by D-ribulose kinase (RK, RTO4_14368) before entering the pentose phosphate pathway (Fig. 2) [37]. Yet, transcriptomic data reveals that RK expression is not highly induced under xylose conditions compared to other essential xylose metabolism genes [32], suggesting that RK may be a bottleneck for efficient xylose assimilation. Therefore, metabolic engineering strategies such as overexpressing RK or introducing heterologous XK coupled with cofactor regeneration, could be promising to enhance xylose utilization efficiency. Although these strategies have yet to be experimentally validated, a recent milestone in the enhancement of xylose assimilation was achieved by overexpressing the putative transcription factor, RTO4_12978 (Pnt1). This led to an 18% increase in the maximum specific growth rate and a significant reduction in the secretion of xylitol and D-arabinitol as byproducts [33].

While xylose enhances the flow towards acetyl-CoA in *Saccharomyces cerevisiae* [8], *R. toruloides* did not exhibit a significant improvement in the production of chemicals derived from acetyl-CoA from xylose as compared to glucose. Interestingly, a 66% increase in neutral lipid accumulation was observed when xylulose was used as the carbon source instead of glucose [40]. This implies that the comparable chemical productivity observed between xylose and glucose could be due to the inefficient metabolism of xylose in *R. toruloides*. This idea is further corroborated by recent progress, such as the 120% rise in fatty alcohol productivity observed in an engineered *R. toruloides* strain for improved xylose assimilation after Pnt1 overexpression [33].

Apart from pure xylose fermentation, numerous studies have successfully demonstrated the effective bioconversion of lignocellulosic hydrolysates into valuable chemicals such as carotenoids, lipids, TAL, and fatty alcohols using *R. toruloides* [41]. However, pinpointing the precise impact of xylose on their production is challenging due to

234 the complex compositions of cellulosic hydrolysates containing diverse carbon sources and
235 unidentified chemicals. Yet, as we deepen our understanding of the xylose metabolic pathway
236 in *R. toruloides*, and as genetic engineering techniques continue to advance, there is growing
237 anticipation that we can enhance the efficiency of lignocellulosic hydrolysate utilization by
238 strategically rewiring the xylose metabolism in *R. toruloides*.
239
240 **3. Acetate utilization by non-conventional yeasts**
241 Acetate, costing $300-450/ton, is a more economical carbon source compared to
242 glucose ($500/ton) [42]. Historically, its production primarily stemmed from yeast-driven
243 alcoholic fermentation, or the incomplete oxidation facilitated by acetic acid bacteria [2].
244 Additionally, the hydrolysis of lignocellulosic biomass leads to the co-production of acetate
245 alongside glucose and xylose, as a result of the degradation of acetylated hemicelluloses
246 under pretreatment conditions [3].
247 *Yarrowia* sp. and *Rhodotorula* sp. possess a natural capacity to ferment acetate and
248 resist against high levels of acetate. While acetate concentrations as low as 5 g/L can inhibit
249 many microorganisms [43], these oleaginous yeasts thrive even at higher concentrations of
250 acetate. Moreover, assimilation of acetate can be beneficial for cell growth and the
251 production of acetyl-CoA derived products as it can be directly converted into acetyl CoA, a
252 precursor for fatty acids and related chemical products [44]. Additionally, in these species,
253 acetate does not hinder the uptake of xylose, allowing for the co-consumption of these two
254 non-conventional carbon sources in lignocellulosic hydrolysates.
255
256 **3.1. *Y. lipolytica* engineering to enhance acetate utilization**

Y. lipolytica directly utilizes acetate as a carbon source, relying on the conversion of acetate into acetyl-CoA by acetyl-CoA synthase (ACS) [45, 46]. Another metabolic route, more common in prokaryotes, encompasses the combined action of acetate kinase (ACKA) and phosphotransacetylase (PTA). However, the eukaryotic organism *Y. lipolytica* lacks genes related to this indirect pathway and only possesses ACS (YALI0F05962p).

Under nitrogen-limited conditions, both WT (MTYL037, PO1g) and engineered strain (MTYL065) overexpressing *ACC1* and *DGA1* showed significant increases in the conversion of acetyl-CoA to lipids from glucose as compared to nitrogen-sufficient conditions [47]. Additionally, when acetate was used as a carbon source, both strains predominantly utilized the glyoxylate shunt and gluconeogenesis. However, the flux distributions through gluconeogenesis differed between the strains. The WT strain redirected a major flux of the glyoxylate shunt back to the TCA cycle via increasing metabolic fluxes catalyzed by pyruvate kinase. As a result, metabolic fluxes through gluconeogenic reduced accordingly. In contrast, the high-lipid accumulating strain (MTYL0645) via *ACC1* and *DGA1* overexpression shut off the metabolic flux by pyruvate kinase, increasing gluconeogenesis flux and maximizing the PPP fluxes. The increased metabolic fluxes through PPP can contribute to NADPH production essential for lipid (acetyl-CoA derived) synthesis. These results indicate that *Y. lipolytica* can produce diverse compounds from acetate, including organic acids from the TCA cycle, sugar alcohols from gluconeogenesis, and acetyl-CoA derived molecules, as reported by recent studies [48, 49]. In an endeavor to augment lipid biosynthesis from acetate, key enzymes, namely acetyl-CoA synthetase from *Saitozyma podzolica* (SpACS), in conjunction with endogenous acetyl-CoA carboxylase (YlACC1) and fatty acid synthase (YlFAS), were overexpressed in *Y. lipolytica* PO1f strain. These metabolic modifications led to a 4.71-fold greater lipid synthesis rate than that observed in

the wild-type when cultivated in a mixture of glucose and acetate. Concurrently, the intrinsic acetate consumption rate surged by 5.39-fold [50]. However, the engineered *Y. lipolytica* demonstrated suboptimal efficiency in utilizing acetate as its sole carbon source. Addressing this challenge, Narisetty et al. [51] employed an adaptive laboratory evolution (ALE) approach on a succinic acid-producing *Y. lipolytica* variant (PSA02004PP) with acetate concentrations ranging from 2-50g/L. This strategic adaptation not only improved the strain's tolerance against acetate but also amplified its succinic acid biosynthetic capacity. In batch fermentation trials, the evolved strain exhibited an acetate assimilation rate that was 2.86-fold higher than its parental strain, producing 5.1 g/L of succinic acid from an initial 20 g/L acetate with a yield of 0.23 g succinic acid/g acetate.

In light of these findings, acetate emerges as a promising substrate for the biosynthesis of both acetyl-CoA derivatives and TCA cycle-associated metabolites. Future endeavors should focus on devising metabolic engineering strategies to further improve acetate assimilation as rapid as glucose assimilation and diversify the portfolio of target molecules.

3.2. *R. toruloides* engineering to enhance acetate utilization

Wild-type *R. toruloides* can grow on acetate as a sole carbon source, even at high concentrations of 20 ~ 26 g/L [52, 53]. However, the consumption of acetate increases the pH of a culture medium up to pH 8-9, requiring pH control between pH 6 and 7.5 for optimal growth on acetate. Although *R. toruloides* can tolerate a wide range of pH (2.2 – 7.5) [54], at pH lower than 4.75 (pKa of acetic acid), the protonated form of acetate can freely diffuse across the cellular membrane and can generate protons at a neutral cytosolic pH via dissociation. This intracellular pH reduction can cause growth inhibition [32]. In *R.*

305 *toruloides*, two permeases (ID: 11,570, 10,804) exhibited increased expression in an acetate-
306 rich medium compared to a glucose-rich medium [27]. Given that multiple studies observed
307 co-consumption of acetate with glucose [55], these potential acetate transporters are not
308 likely to be repressed by glucose, but their overexpression has not been tested for the
309 enhanced utilization of acetate. More comprehensive studies focusing on these transporters,
310 encompassing substrate identification and targeted overexpression, are crucial to enhance
311 acetate utilization efficiency in *R. toruloides*.

312 Like *Y. lipolytica*, *R. toruloides* also directly converts acetate into acetyl-CoA, but it
313 utilizes both ACS and acetyl-CoA hydrolase (ACH1) [27]. Rekena et al. [32] conducted a
314 metabolic flux analysis using acetate as a sole carbon source in a synthetic medium and
315 predicted three major routes for acetyl-CoA metabolism. The glyoxylate shunt, involving
316 isocitrate lyase (ICL1-2) and malate synthase (MLS), accounts for 51% of the flux, while 18%
317 is used for lipid biosynthesis by acetyl-CoA carboxylase (ACC). Additionally, a significant
318 portion of the flux (29 %) is predicted to enter the mitochondrial TCA cycle via the carnitine
319 carrier (CRC) pathway. Multiple studies observed high expression levels of enzymes in the
320 glycolysis, gluconeogenesis, glyoxylate cycle, pentose phosphate pathway, and lipid
321 synthesis when acetate is utilized as compared to glucose [27, 32]. While no studies have yet
322 been published on genetic engineering to improve acetate assimilation of *R. toruloides*,
323 various strategies have been explored to leverage acetate utilization for enhancing the
324 production of target molecules. For instance, a 2-pyrone synthase-expressing *R. toruloides*
325 strain, designed to produce TAL from malonyl-CoA, exhibited a 78% increase in TAL titer
326 when 1% sodium acetate was added to the YPD media [55]. Moreover, implementing a two-
327 stage pH regulation strategy with acetate as a substrate yielded a high lipid productivity of
328 0.18 $gL^{-1}h^{-1}$ [53]. A recent experiment involving the co-cultivation of *Rhodotorula glutinis*

and the microalgal species *Chlorella vulgaris*, using acetate as a carbon source, resulted in a lipid titer increase of over 2.6 times compared to single-strain cultivation, thanks to the synergistic exchanges of CO_2 and O_2 between the microalgae and yeast strains [56].

4. Conclusion and prospect

This review is centered on the exploration of non-conventional carbon sources, specifically xylose and acetate, by non-conventional yeast strains *Y. lipolytica* and *R. toruloides*. Extensive genetic engineering has been pursued in the relatively well-researched *Y. lipolytica*, while in *R. toruloides*, a comprehensive multi-omics analysis was necessary to decipher its unique fermentation characteristics under xylose and acetate conditions. Notably, recent endeavors have begun to unravel the intricate web of xylose and acetate metabolism in *R. toruloides*, spearheading efforts to bolster xylose consumption efficiency.

While significant strides have been made in improving xylose and acetate utilization, through genetic perturbation and adaptive laboratory evolution (ALE), the translation of these strategies into actual production of chemicals remains limited. One primary challenge is that engineering these non-conventional strains proves less straightforward than with established model organisms. Moreover, the concurrent application of numerous genetic alterations—for both chemical production and efficient xylose/acetate use—often presented complexities that warranted individual study considerations. Currently, numerous successful endeavors have been made in producing a variety of chemicals from these non-conventional species, as illustrated in (Fig. 3). With the potential of enhanced xylose and acetate consumption, especially in the context of utilizing lignocellulosic hydrolysates, there emerges a promising juncture. This paves the way to integrate strategies for optimized utilization of non-conventional carbon sources alongside diversifying chemical production.

In summary, the latest advancements in metabolic engineering for acetate and xylose utilization include regulating redox balance via cofactor regeneration, overexpressing metabolic pathway genes, discovering and regulating related transcription factors, modifying sugar transporters, and deleting repressor genes. Nonetheless, the functionalities of several genes linked to xylose and acetate metabolism remain elusive. This presents ripe opportunities for magnifying xylose and acetate utilization efficiency. Additionally, ongoing efforts in optimizing culture conditions, such as pH control, and creating symbiotic systems through co-cultivation with microalgae, indicate promising avenues for further progress.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT in order to improve language and readability. After using ChatGPT, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

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Utilizing multi-omics analysis, the intricacies of lignocellulosic carbon processing in *Rhodosporidium toruloides* were unraveled, revealing its metabolic intricacies. Drawing on model organisms and an expansive omics dataset, this study has sculpted one of the most comprehensive metabolic model for *R. toruloides* to date.

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This research offers a profound insight into the metabolic shifts in *Rhodotorula toruloides* by assimilating intricate physiological datasets across glucose, xylose, and acetate consumption. A standout discovery was the alternative xylose assimilation mechanism that harnesses D-arabinitol and D-ribulose. By blending enzyme-constrained modeling with quantitative proteomics in a comprehensive multi-condition study, this work illuminates previously uncharted territories in yeast metabolism and establishes a robust platform for future explorations. The unveiled models emerge as a precious asset for the broader scientific community.

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Harnessing high-throughput fitness data, this study identifies key regulators to amplify pentose catabolism in *R. toruloides*. Emphasis was placed on two genes: a projected transcription factor (RTO4_12978, Pnt1) and a carbon catabolite repression homolog (RTO4_11990). Overexpressing Pnt1 yielded promising results with elevated xylose growth rates, minimized by-products, and a 120% boost in xylose-to-fatty alcohols conversion in batch cultures. Although deleting RTO4_11990 enhanced xylose growth, it couldn't negate glucose's carbon catabolite repression. With *R. toruloides*' repression mechanisms remaining somewhat elusive, this groundbreaking paper stands as the first to notably amplify xylose utilization in *R. toruloides* through targeted genetic engineering.

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This study delves into the unique metabolic pathways of *R. toruloides*, an oleaginous yeast with versatile metabolic capabilities and high resilience to inhibitory compounds found in plant biomass hydrolysates. The research sheds light on the non-traditional L-arabinose and D-xylose metabolism in *R. toruloides*, suggesting its progression via D-arabitol and D-ribulose. By employing genetic and metabolite analyses, the study pinpoints specific enzymes, such as D-arabitol-2-dehydrogenase (RTO4_9990) and D-ribulose kinase (RTO4_14368), that play crucial roles in pentose metabolism. This work not only refines the understanding of pentose metabolism in *R. toruloides* but also underscores the intricate microbial mechanisms behind pentose utilization, paving the way for enhanced biomass-derived sugar conversions to value-added bioproducts.

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●50. Chen L, Yan W, Qian XJ, Chen MJ, Zhang XY, Xin FX, Zhang WM, Jiang M, Ochsenreither K: **Increased Lipid Production in Yarrowia lipolytica from Acetate through Metabolic Engineering and Cosubstrate Fermentation.** *Acs Synth Biol* 2021, **10:**3129-3138.
This study focuses on the bioconversion of acetate into microbial lipids using engineered *Y. lipolytica*. By overexpressing key enzymes such as acetyl-CoA synthetase (ACS), acetyl-CoA carboxylase (ACC1), and fatty acid synthase (FAS) in *Y. lipolytica*, lipid content increased significantly. The most effective strategy involved cosubstrate fermentation using glycerol and acetate, leading to a lipid content of 41.7%. This approach presents a promising method for efficient microbial lipid production from waste acetate.
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The study focuses on developing a hypertolerant strain of *Yarrowia lipolytica* capable of accumulating succinic acid using high levels of acetate. The research introduced acetyl-CoA synthase (ACS) in *Yarrowia lipolytica* PSA02004PP, followed by adaptive laboratory evolution (ALE) to enhance tolerance toward acetate. The adapted strain demonstrated rapid growth and accumulation of lipids and succinic acid at high acetate concentrations. This work provides a pathway for valorizing biomass hydrolysates with high acetate levels into value-added products.
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610 **Figure captions**

611 **Fig. 1.** The rising significance of non-conventional carbons and yeast organisms. (a)
612 Composition of hydrolysate from high-solid lignocellulosic biomass (20-30% high solids
613 loading) [3]. (b) Number of published research articles in the field of fermentation with
614 'hydrolysate,' 'xylose,' or 'acetate.' (c) Trends in the number of publications for each yeast
615 strain: *S. cerevisiae*, *Y. lipolytica*, and *R. toruloides*. Data on published works were retrieved
616 from the Web of Science (https://www.webofscience.com/).

617

618 **Fig. 2.** Important pathways for xylose and acetate metabolism in *R. toruloides* and *Y.*
619 *lipolytica*. Red and yellow lines represent unique pathways found in *R. toruloides* and *Y.*
620 *lipolytica*, respectively. Enzymes: XR, xylose reductase; XDH, xylitol dehydrogenase; XK,
621 xylulose kinase; DAD, D-arabinitol dehydrogenase; RK, ribulose kinase; RPE, ribulose-
622 phosphate 3-epimerase; PK, phosphoketolase; PTA, phosphotransacetylase; ACC, acetyl-coa
623 carboxylase; FAS, fatty acid synthase; RPI, ribose 5-phosphate isomerase; GND, 6-
624 phosphogluconate dehydrogenase; PGLS, 6-phosphogluconolactonase; ZWF, glucose-6-
625 phosphate 1-dehydrogenase; PGI, phosphoglucoisomerase; TKT, transketolase; TAL,
626 transaldolase; FBP, fructose-1,6-bisphosphatase; PFK, phosphofructokinase; TPI,
627 triosephosphate isomerase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FBA,
628 fructose-bisphosphate aldolase; GPD, glycerol-3-phosphate dehydrogenase; GK, glycerol
629 kinase; GPM, phosphoglycerate mutase; ENO, enolase; PYK, pyruvate kinase; HXK, hexose
630 kinase; MPDH mannitol-P dehydrogenase; PDC, pyruvate decarboxylase; ALDH, aldehyde
631 dehydrogenase; ACS, acetyl-coenzyme a synthetase; ACH, acetyl-CoA thioesterase; ACL,
632 atp citrate synthase; ACO, aconitate hydratase; ICL, isocitrate lyase; ME, malic enzyme;
633 PYC, pyruvate carboxylase; MDH, malate dehydrogenase; MLS, malate synthase; PEPCK,

phosphoenolpyruvate carboxykinase; PDH, pyruvate dehydrogenase; CIT, citrate synthase; IDP, isocitrate dehydrogenase; IDH, isocitrate dehydrogenase; FUM, fumarate hydratase; KGD, α-ketoglutarate dehydrogenase; LPD, oxoglutarate dehydrogenase; LSC, succinate--coa ligase; SDH, succinate dehydrogenase; FUM, fumarate hydratase

Fig. 3. Diverse chemicals that can be produced from *R. toruloides* and *Y. lipolytica* from xylose and acetate. Enzymes: XR, xylose reductase; XDH, xylitol dehydrogenase; XK, xylulose kinase; ADH, aldehyde dehydrogenase; ER, erythrose reductase; M1P, mannitol-1-P dehydrogenase; SCS, succinyl coenzyme A synthetase; LDH, lactate dehydrogenase; ACS, acetyl-CoA synthetase; 2PS, 2-pyrone-synthase; MCR, malonyl-CoA reductase; FAS, fatty acid synthase; FAR, fatty acyl-coenzyme A reductase; DGAT, diacylglycerol acyltransferase; CrtE, geranylgeranyl diphosphate synthase; CrtB, phytoene synthase ; CrtI, phytoene desaturase; PPDS, protopanaxadiol synthase; FPP*, mutated farnesyl diphosphate synthase; KS, kaurene synthase; LS, limonene synthase; BIS, bisabolene synthase; ADS, amorphadiene synthase

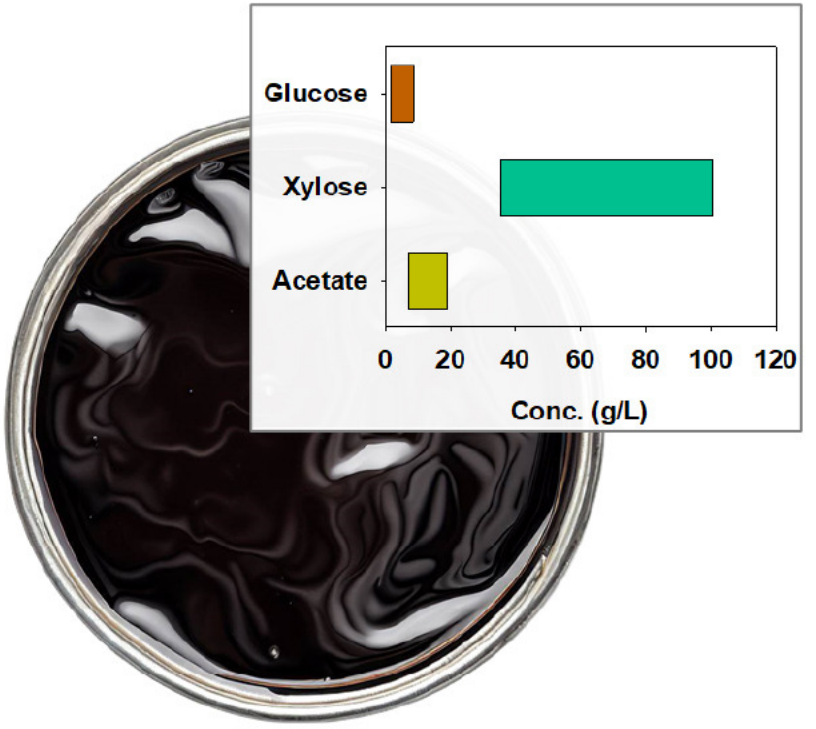
Table 1. Summary of multi-omics analysis conducted in *Rhodotorula* spp. and *Yarrowia spp*. using xylose or acetate.

Strain	Carbon source	Features	Transcript omics	Prote omics	Metabol omics	Lipid omics	Flux analysis	Ref
R. toruloides CCT 7815	Xylose (70g/L)	μ_{max}: 0.06 h^{-1}		●			●	[29]
R. toruloides CCT 0783	Xylose Acetic acid Glycerol	μ_{max}: 0.09 h^{-1} μ_{max}: 0.11 h^{-1} μ_{max}: 0.24 h^{-1}					●	[30]
R. toruloides CBS 14	Glucose Xylose	μ_{max}: 0.14 h^{-1} μ_{max}: 0.07 h^{-1}		●				[26]
R. toruloides IFO0880	Glucose Xylose Arabinose p-Coumaric acid	n.a n.a n.a n.a	●	●	●	●	●	[31]
R. toruloides IFO0880	Glucose Xylose Acetate Soybean oil	$OD_{600, max}$: 50 $OD_{600, max}$: 39 $OD_{600, max}$: 22 $OD_{600, max}$: 20	●		●			[27]
R. toruloides CCT7815	Glucose Xylose Acetate	$\mu_{max: 0.19}$ h^{-1} $\mu_{max: \sim 0.08}$ h^{-1} $\mu_{max: \sim 0.08}$ h^{-1}		●		●	●	[32]
R. toruloides IFO0880, WT-OE-Pnt1 WT ΔPnt1	Xylose (Xylose + Glycerol)	Doubling time: 6 ~ 10 h Doubling time: 4 h Doubling time: 7 ~ h		●				[33]
Y. lipolytica XYL+ (PO1d derived)	Xylose (Xylose + 2-deoxyglucose)	$OD_{600, max}$: 60	●					[19]
Y. lipolytica YB420 *Y. lipolytica* CBS7504	Xylose (Xylose + Glucose)	$OD_{600, max}$: 45 (YB420) $OD_{600, max}$: 40 (CBS7504)		●				[57]
Y. lipolytica MTYL037 *Y. lipolytica* MTYL065	Sodium acetate	μ_{max}: 0.055 μ_{max}: 0.055					●	[47]

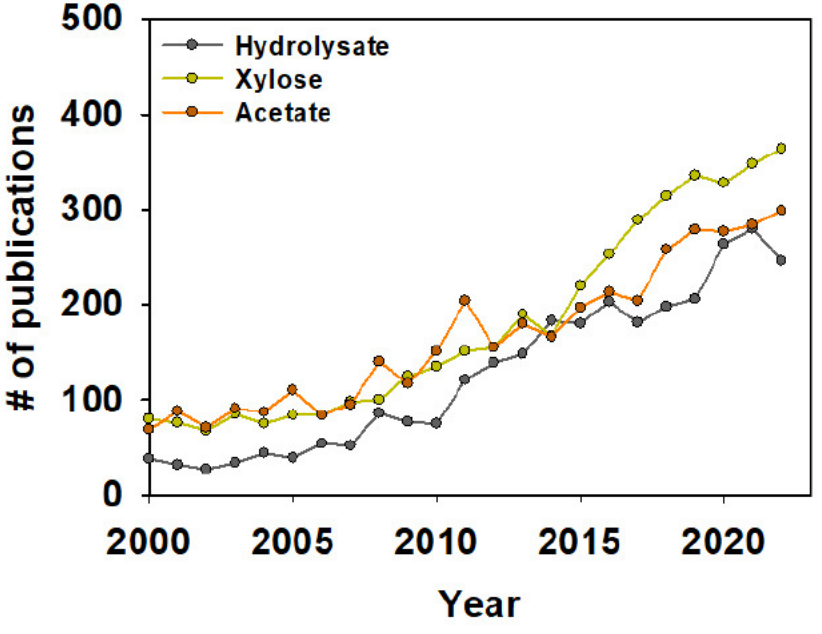
(a)

High-solid lignocellulosic biomass
(>15% solids)

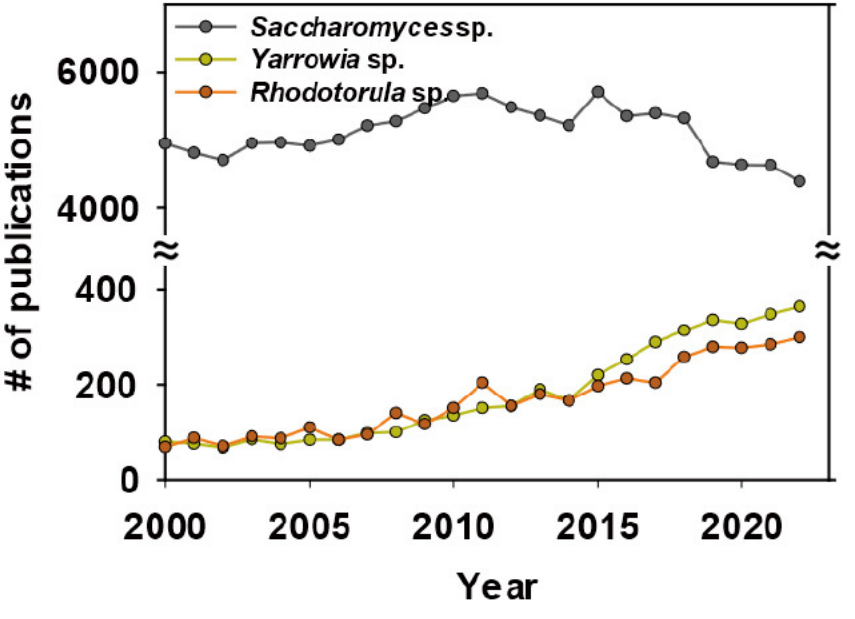
Hydrolysis

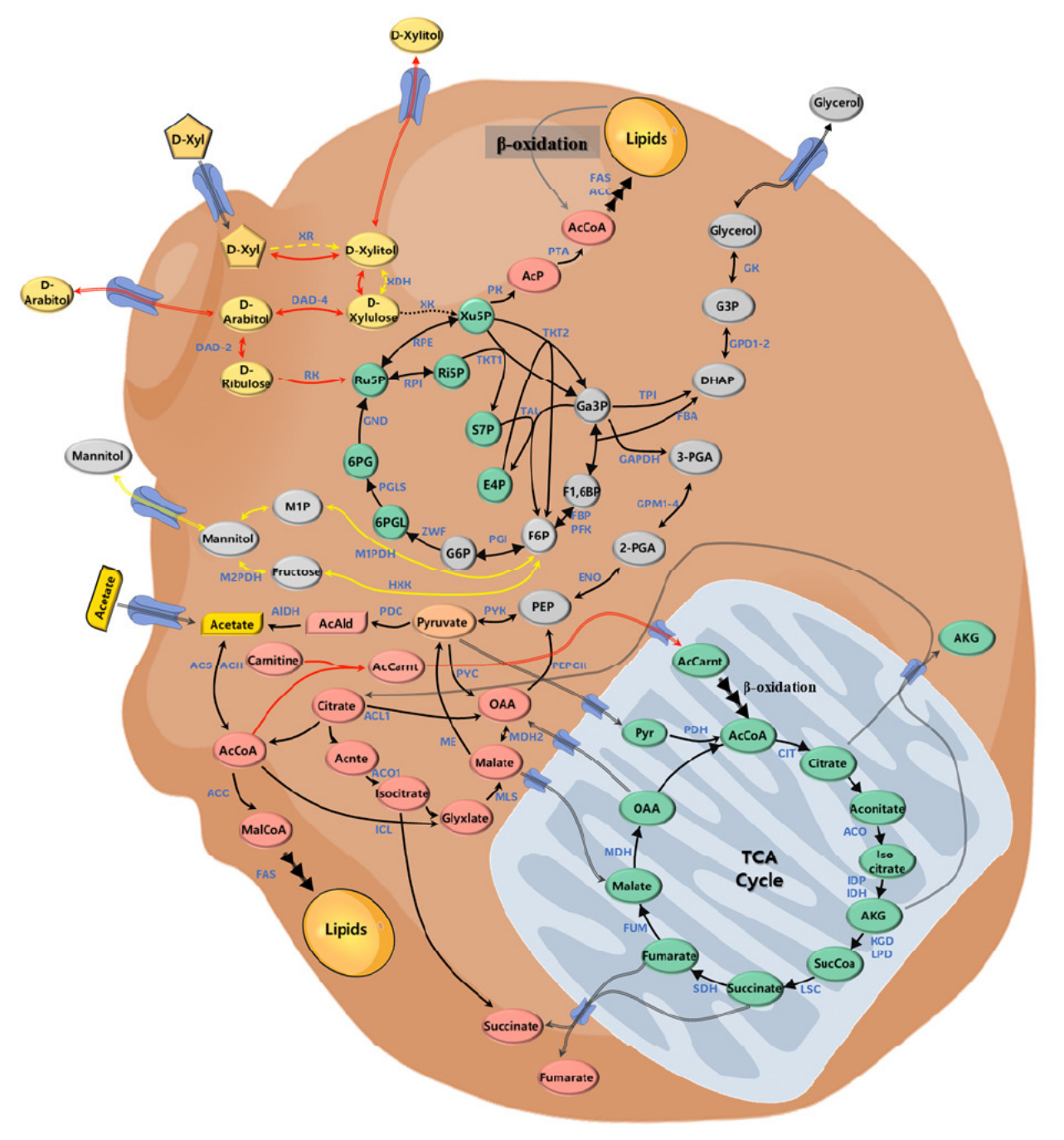


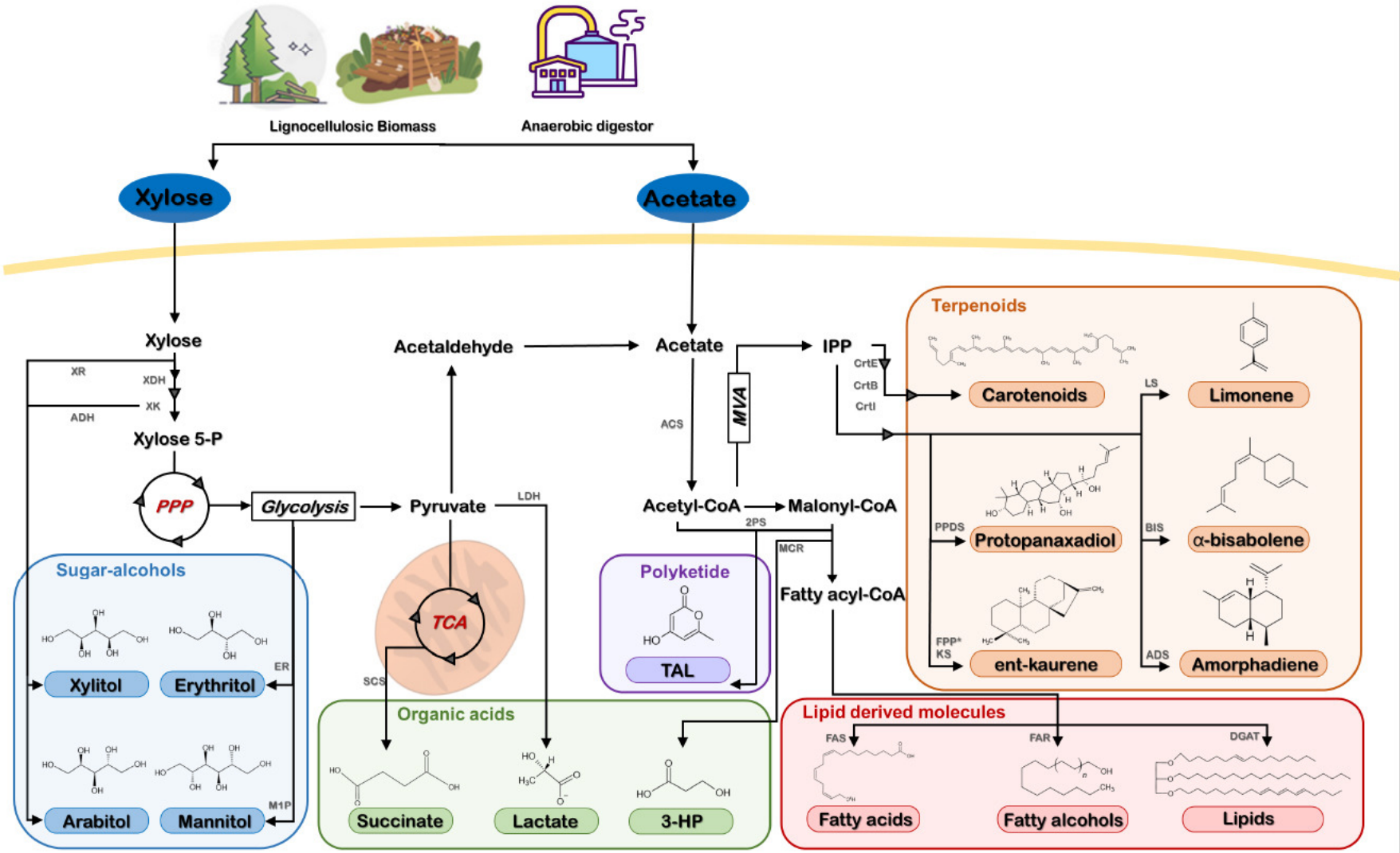
(b)



(c)




D-Xylitol
D-Xyl
XR
D-Xylitol
XDH
D-Arabitol
DAD-4
D-Xylulose
XK
Xu5P
DAD-2
D-Ribulose
RK
Ru5P
RPE
RPI
Ri5P
TKT2
TKT1
Ga3P
TAL
S7P
E4P
TPI
DHAP
FBA
GAPDH
3-PGA
GPM1-4
2-PGA
ENO
PEP
PYK
Pyruvate
PDC
AcAld
AIDH
Acetate
ACS
ACH
Carnitine
AcCarnt
PEPCK
PYC
OAA
Citrate
ACL1
AcCoA
Acnte
ACO1
Isocitrate
ICL
Glyxlate
MLS
Malate
ME
MDH2
ACC
MalCoA
FAS
Lipids
PTA
AcP
AcCoA
FAS
β-oxidation
Lipids
Glycerol
GK
G3P
GPD1-2
DHAP
PK
PFK
FBP
F1,6BP
PGI
F6P
G6P
ZWF
6PGL
PGLS
6PG
GND
Ru5P
M1P
M1PDH
Mannitol
M2PDH
Fructose
HXK
Mannitol
Acetate
AKG
AcCarnt
β-oxidation
Pyr
PDH
AcCoA
CIT
Citrate
Aconitate
ACO
Iso citrate
IDP IDH
AKG
KGD LPD
SucCoa
LSC
Succinate
SDH
Fumarate
FUM
Malate
MDH
OAA
TCA Cycle
Succinate
Fumarate


Lignocellulosic Biomass
Anaerobic digestor
Xylose
Acetate
Xylose
XR
XDH
XK
ADH
Xylose 5-P
PPP
Glycolysis
Pyruvate
LDH
Acetaldehyde
Acetate
ACS
MVA
Acetyl-CoA
Malonyl-CoA
2PS
MCR
Fatty acyl-CoA
IPP
CrtE
CrtB
CrtI
Terpenoids
Carotenoids
LS
Limonene
PPDS
Protopanaxadiol
BIS
α-bisabolene
FPP*
KS
ent-kaurene
ADS
Amorphadiene
Sugar-alcohols
Xylitol
Erythritol
ER
Arabitol
Mannitol
M1P
TCA
SCS
Polyketide
TAL
Organic acids
Succinate
Lactate
3-HP
Lipid derived molecules
FAS
FAR
DGAT
Fatty acids
Fatty alcohols
Lipids

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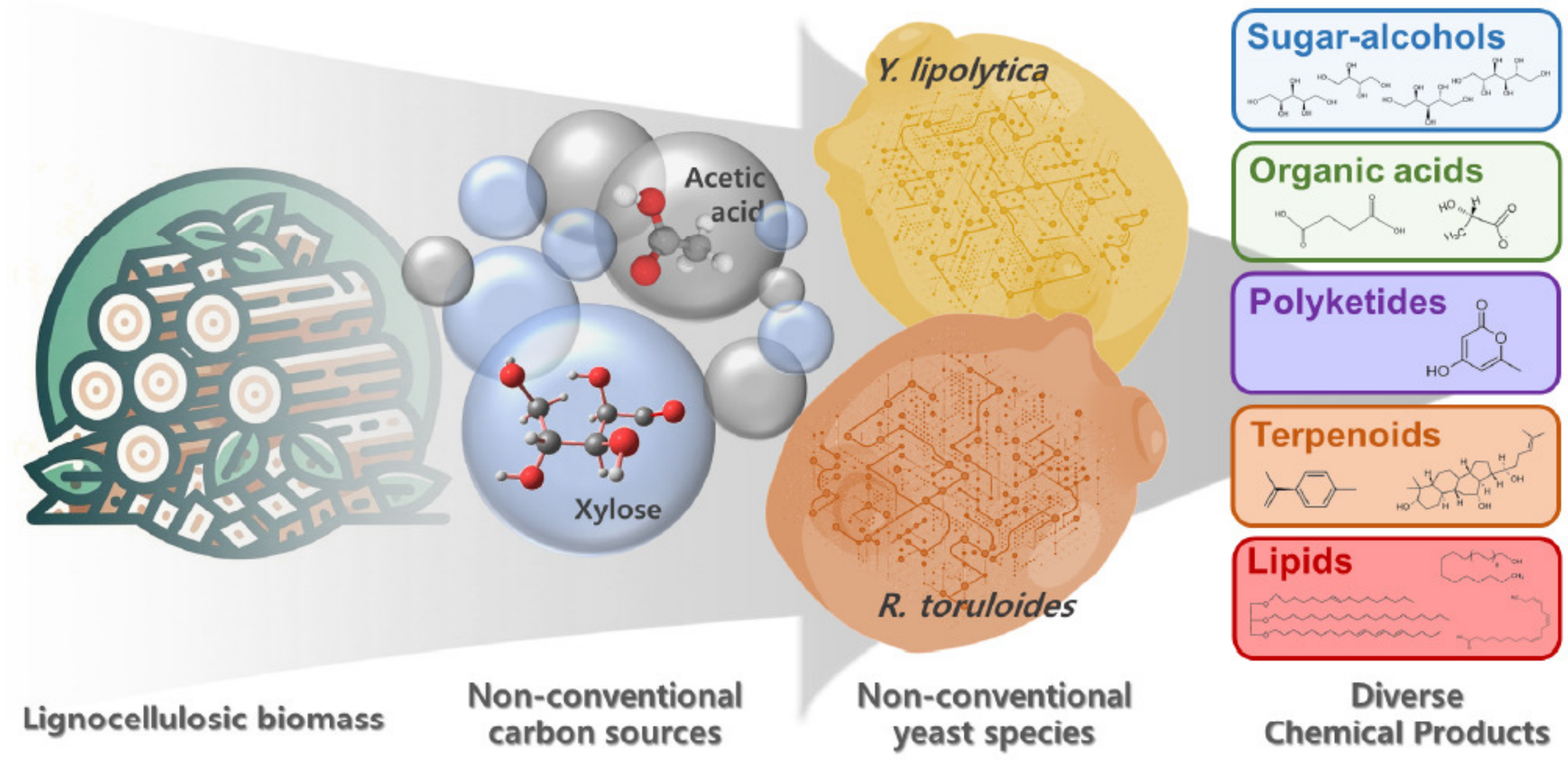
R. toruloides CCT 0783	Xylose Acetic acid Glycerol	μ_{max}: 0.09 h^{-1} μ_{max}: 0.11 h^{-1} μ_{max}: 0.24 h^{-1}					●	[29]
R. toruloides CBS 14	Glucose Xylose	μ_{max}: 0.14 h^{-1} μ_{max}: 0.07 h^{-1}		●				[25]
R. toruloides IFO0880	Glucose Xylose Arabinose p-Coumaric acid	n.a n.a n.a n.a	●	●	●	●	●	[30]
R. toruloides IFO0880	Glucose Xylose Acetate Soybean oil	$OD_{600, max}$: 50 $OD_{600, max}$: 39 $OD_{600, max}$: 22 $OD_{600, max}$: 20	●		●			[26]
R. toruloides CCT7815	Glucose Xylose Acetate	$\mu_{max: 0.19}$ h^{-1} $\mu_{max: \sim 0.08}$ h^{-1} $\mu_{max: \sim 0.08}$ h^{-1}		●		●	●	[31]
R. toruloides IFO0880, WT-OE-Pnt1 WT ΔPnt1	Xylose (Xylose + Glycerol)	Doubling time: 6 ~ 10 h Doubling time: 4 h Doubling time: 7 ~ h		●				[32]
Y. lipolytica XYL+ (PO1d derived)	Xylose (Xylose + 2-deoxyglucose)	$OD_{600, max}$: 60	●					[18]
Y. lipolytica YB420 *Y. lipolytica* CBS7504	Xylose (Xylose + Glucose)	$OD_{600, max}$: 45 (YB420) $OD_{600, max}$: 40 (CBS7504)		●				[55]
Y. lipolytica MTYL037 *Y. lipolytica* MTYL065	Sodium acetate	μ_{max}: 0.055 μ_{max}: 0.055					●	[45]


Acetic acid
Xylose
Y. lipolytica
R. toruloides
Sugar-alcohols
Organic acids
Polyketides
Terpenoids
Lipids
Lignocellulosic biomass
Non-conventional carbon sources
Non-conventional yeast species
Diverse Chemical Products