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# Advances in genetic tools for metabolic engineering of non-conventional yeasts

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Non-conventional yeasts are emerging as powerful alternatives to *Saccharomyces cerevisiae* for metabolic engineering, owing to their innate stress tolerance, broad substrate utilization, and distinctive metabolic capabilities. These attributes position them as promising chassis for producing biofuels, pharmaceuticals, and specialty chemicals. This review synthesizes recent advances in genetic toolkits for four such species—*Pichia kudriavzevii* (*Issatchenkia orientalis*), *Starmerella bombicola*, *Debaryomyces hansenii*, and *Pachysolen tannophilus*—highlighting progress across plasmid architectures (episomal and integrative), identification of autonomously replicating sequences and centromeric elements, and the development of safe-harbor genomic loci. We summarize promoter and terminator libraries enabling tunable expression, the expansion of auxotrophic and antifungal selection markers with recycling strategies, and the rapid adaptation of CRISPR-based systems (Cas9 and Cas12a) with optimized guide RNA expression, multiplex editing, and approaches that enhance homologous recombination (e.g., KU70/80 disruption). We also review landing-pad platforms for modular, repeated integrations and transposon-based tools (e.g., piggyBac) that facilitate multigene pathway assembly. Collectively, these innovations are accelerating design-build-test-learn cycles and enabling precise, scalable engineering of non-conventional yeasts. Remaining challenges—including limited species-specific episomal systems, variable transformation efficiencies, genome-stability concerns, and alternative codon usage—define clear priorities for future toolkit development. Together, these advances and open needs chart a path toward robust, sustainable biomanufacturing using diverse non-conventional yeast chassis.

## KEYWORDS

*Debaryomyces hansenii*, genetic tools, genome engineering, metabolic engineering, non-conventional yeast, *Pachysolen tannophilus*, *Pichia kudriavzevii*, *Starmerella bombicola*

## 1 Introduction

Yeasts play a foundational role in biotechnology, supporting global food, beverage, and biomanufacturing industries. *Saccharomyces cerevisiae* has long been both a premier eukaryotic model organism and a dominant industrial chassis (Borodina and Nielsen, 2014). However, its usefulness is constrained by a relatively narrow substrate range, limited metabolic flexibility, and only moderate tolerance to industrial stresses such as high temperature, osmotic pressure, and inhibitory by-products. These limitations have fueled growing interest in non-conventional yeasts—phylogenetically diverse species that naturally exhibit robust metabolic capabilities and superior stress tolerance (Radecka et al., 2015; Wagner and Alper, 2016; Löbs et al., 2017; Wang et al., 2025).

Non-conventional yeasts span multiple genera, including *Pichia*, *Yarrowia*, *Kluyveromyces*, *Starmerella*, *Debaryomyces*, *Pachysolen*, and *Saturnispora* (Figure 1). Each genus is adapted to different ecological niches and displays unique physiological traits with biotechnological potential (de Souza Varize et al., 2019). Despite these advantages, the broader industrial adoption of non-conventional yeasts has historically been limited by insufficient genetic tools, low transformation efficiencies, and poorly characterized regulatory parts (Moon et al., 2025). Recent advances in genome sequencing, synthetic biology, and CRISPR-based genome engineering, however, are rapidly overcoming these obstacles. An expanding genetic toolbox—including episomal and integrative plasmids, diverse promoters and terminators, and increasingly efficient genome-editing systems—is now enabling the systematic domestication of these species (Table 1; Figure 1a). As a result, non-conventional yeasts are emerging as competitive, and in some cases superior, platforms relative to *S. cerevisiae* for industrial biotechnology (Moon et al., 2025).

In this review, we highlight four *Saccharomycotina* yeasts, *Pichia kudriavzevii*, *Starmerella bombicola*, *Debaryomyces hansenii*, and *Pachysolen tannophilus*. We focused on these species because they collectively encompass a wide range of industrially relevant phenotypes that are not well represented in other, more extensively studied non-model yeasts (e.g., *Yarrowia lipolytica*, *Komagataella phaffii*, *Kluyveromyces marxianus*, *Schizosaccharomyces pombe*), and because recent efforts have substantially expanded the genetic tools available for each. For example, *P. kudriavzevii* thrives in low pH environments (~pH = 1.5) and is well suited for organic acid production, whereas *S. bombicola* is a natural producer of high-value sphorolipids (Tran et al., 2023; Wu et al., 2023; Frousnoon et al., 2025; Zhang et al., 2025). *D. hansenii* displays exceptional halotolerance and osmotolerance, allowing growth in high-salt environments (>1 M) (Breuer and Harms, 2006; Xelhuantzi et al., 2024; Estrada et al., 2023; Fukuda et al., 2004). Meanwhile, *P. tannophilus* efficiently ferments both glucose and xylose, making it attractive chassis for lignocellulosic biomass conversion (Maleszka et al., 1982; Slininger et al., 1982; Slininger et al., 1987; Mei et al., 2018). Collectively, these yeasts provide a representative view of the physiological and genetic diversity now accessible for systematic metabolic engineering.

## 2 Synthetic biology tools

### 2.1 Plasmids

Plasmids are naturally occurring extrachromosomal genetic elements capable of autonomous replication within host cells (Glass, 1982; Nora et al., 2019). In synthetic biology, engineered plasmids—commonly referred to as vectors—serve versatile molecular tools for applications such as cloning, heterologous gene expression, gene knockdown, reporter assays, and genome engineering. In yeast biology, vectors are generally categorized into episomal, centromeric, and integrative types, each offering distinct advantages and limitations (Nora et al., 2019).

Episomal and centromeric plasmids are circular DNA molecules that replicate independently of the host genome and are often maintained at high copy numbers (Gnügge and Rudolf, 2017; Nora et al., 2019). They are relatively easy to construct, introduce, and remove, making them ideal for rapid screening, transient expression, and high-level protein production. However, episomal and centromeric plasmids tend to be unstable without continuous selection pressure and can impose a metabolic burden on the host due to the energetic cost of maintaining multiple copies (Nevoigt, 2008). In contrast, integrative plasmids are typically linearized and stably integrated into the host genome via homologous recombination, often at defined chromosomal loci. Although they generally result in lower copy numbers and moderate expression levels compared to episomal systems, integrative plasmids offer superior stability across generations, even in the absence of selective pressure (Vještica et al., 2020). Despite being more time- and labor-intensive to construct, integrative plasmids are preferred for developing robust production strains suitable for industrial applications.

The functionality of a plasmid depends on several key genetic components that govern its replication and gene expression. These include promoters, terminators, and in the case of centromeric plasmids, autonomously replicating sequences (ARS) and centromeric (CEN) elements (Liachko and Dunham, 2014; Wagner and Alper, 2016; Vještica et al., 2020; Smith et al., 2024). Promoters, located upstream of the coding region, recruit RNA polymerase and transcription factors to initiate transcription. They can be constitutive, such as TEF1 promoter, or inducible, such as AOX1 promoter, which is activated only in response to specific stimuli. Terminators positioned downstream of the coding region ensure proper transcriptional termination and polyadenylation, contributing to mRNA stability and efficient expression.

The ARS enables autonomous plasmid replication within the host, typically resulting in high-copy-number maintenance. In contrast, the CEN element, derived from yeast centromeric DNA, allows plasmids to segregate similarly to chromosomes during cell division, resulting in stable inheritance at low copy number. CEN-based vectors are therefore useful for applications requiring controlled, low-level expression or stable maintenance without extensive selection pressure (Vještica et al., 2020).

TABLE 1 Genetic tools in non-conventional yeasts.

Genetic Tool	Examples	Notes	References
<i>Pichia kudriavzevii</i>			
Promoters	FBA1, TEF1, PGK1, TDH3, PDC1, GAPDH, GPM1, SED1, indolepyruvate decarboxylase 6, inositol-3-phosphate synthase INO1, translation elongation factor EF-1 alpha, lipid-binding protein HSP12	Heterologous gene expression	Xiao et al. (2014), Sohn et al. (2019), Lee et al. (2022)
	tRNA promoter (Pol III), RPR1'-tRNA <sup>Leu</sup> promoter, RPR1, 5S RNA-tRNA <sup>Leu</sup>	Drives sgRNAs for CRISPR applications	Tran et al. (2019), Sun et al. (2020)
Terminators	CYC1, PGK1, PDC1, GAPDH, MDH1, PDC1, INO1	Constitutively expressed	Sohn et al. (2019), Cao et al. (2020), Lee et al. (2022)
Markers	URA3, LEU2, TRP1, HIS3	Leu2 from <i>S. cerevisiae</i>	Xiao et al. (2014), Tran et al. (2019), Cao et al. (2020), Sun et al. (2020)
	Nourseothricin N-acetyl transferase (NAT)	120 µg/mL	Lee et al. (2022)
	Zeoicin	200 µg/mL	Zhang et al. (2023)
	Aureobasidin A (AUR1-KP729614)	0.5 µg/mL	Yoo and Kim (2015), Sohn et al. (2019)
Integration Sites	21 loci were identified for high efficiency integration	Large pathways (18 kb) were integrated	Fatma et al. (2023)
<i>Starmerella bombicola</i>			
Promoters	GPD, eno, pGAPD, pUGTA1 pUGTB1, ura3	Constitutively expressed, native	Van Bogaert et al. (2007), 2013; Li et al. (2016a); Li et al. (2016b); Geys (2017); Jezierska et al. (2020) Lodens et al. (2020), Chatterjee et al. (2022)
	pCYP52M1	Promoter of a biosynthetic gene cluster	Geys, 2017; Lodens et al. (2020)
	TEF1, GAPD, PGK1	Native, constitutively expressed, used for SbCas9	Shi et al. (2022), Zhang et al. (2022)
	pGALK	Inducible galactokinase promoter	Shi et al. (2022), Zhang et al. (2022)
Terminators	TK (HSV thymidine kinase), Trpc	Non-native, TK is a viral terminator, Trpc is from <i>Aspergillus nidulans</i>	Van Bogaert et al. (2013), Li et al. (2016a); Li et al. (2016b); Chatterjee et al. (2022), Qazi et al. (2022)
	tGAL	Constitutively expressed	Lodens et al. (2018), 2020
	PGK1 (phosphoglycerate kinase), tCYP52M1, ura3, tUGTA1, tUGTB1	Native	Geys (2017), Chatterjee et al. (2022), Shi et al. (2022)
	Trpl41b	Strong terminator from the <i>S. cerevisiae</i> ribosome protein-encoding gene	Chatterjee et al. (2022)
	Tsyn7	Constitutively expressed, used for SbCas9	Shi et al. (2022), Zhang et al. (2022)
Markers	URA3	Native	Van Bogaert et al. (2007), Geys, 2017; Shi et al. (2022)
	Nourseothricin acetyl transferase (NAT), Hygromycin B	Hygromycin B- 500 µg/mL	Li et al. (2016a); Geys, 2017; Shi et al. (2022)
	GFP, SbGFP	SbGFP is codon-optimized yeGFP	Lodens et al. (2020), Shi et al. (2022)
	FBP	Can be used under oxygen-limitation	Lodens et al. (2020)
	Amylase system	Can be used as a reporter for extracellular protein expression	Lodens et al. (2020)
Integration Sites	AT locus, Ura3 locus, CYP52M1 locus, UGTA locus, UGTB locus	Large cassettes and biosynthetic clusters were integrated	Saerens et al. (2011); Van Bogaert et al. (2013), Geys, 2017; Lodens et al. (2018), 2020; Zhang et al. (2022)
	PXA1 locus, Sble locus	Used for CRISPR-Cas9 and to integrate Cas12a expression cassette respectively	Shi et al. (2022), Zhang et al. (2022)

(Continued on following page)

TABLE 1 (Continued) Genetic tools in non-conventional yeasts.

Genetic Tool	Examples	Notes	References
<i>Debaromyces hansenii</i>			
Promoters	GPD1 (glycerol-3-phosphate dehydrogenase)	Strong promoter from <i>S. cerevisiae</i> , constitutively expressed	Maggi and Govind (2004)
	GPD1d (Native GPD1), Dh_RNR2p and Dh_RHR2p	GPD1d is an endogenous constitutive strong promoter, Dh - <i>D. hansenii</i>	Maggi and Govind (2004), Strucko et al. (2021)
	HSP12, SME1	Heat Shock protein, protein kinase from <i>S. cerevisiae</i> used for heterologous expression, constitutively expressed, moderate levels of expression	Maggi and Govind (2004)
	CYC1, Cl_TDH3, Ag_TEF1p	Iso cytochrome C1 from <i>S. cerevisiae</i> , Cl - <i>Candida lusitanae</i> , Ag - <i>Ashbya gossypii</i>	Ricaurte and Govind (1999), Strucko et al. (2021)
	Cl_SNR52p, Dh_SRC1p, DhTEF1p	RNA pol III promoters for sgRNA expression cassette	Strucko et al. (2021)
Terminators	MF	Mating factor	Maggi and Govind (2004)
	CYC1, Ag_TEF1t, Ca_ENO1t, <i>A. adenivorans</i> -derived TEF1	CYC1 from <i>S. cerevisiae</i> , Ca - <i>Candida albicans</i>	Ricaurte and Govind (1999), Terentiev et al. (2004), Strucko et al. (2021)
	DhTEF1t	Native	Minhas et al. (2009); Strucko et al. (2021)
Markers	URA3	Auxotrophic marker from <i>S. cerevisiae</i>	Ricaurte and Govind (1999), Maggi and Govind (2004)
	Hygromycin B	100 µg/mL, expression of the cassette is weaker with respect to URA3	Ricaurte and Govind (1999)
	HIS4, ADE2, NAT	NAT <sup>CUG</sup> is used	Minhas et al. (2009); Spasskaya et al. (2021), Strucko et al. (2021)
	GFP, RFP	GFP is from the jellyfish <i>Aequorea victoria</i>	Maggi and Govind (2004), Terentiev et al. (2004), Minhas et al. (2009)
Integration Sites	25S rRNA gene locus	A conserved <i>A. adenivorans</i> -derived 25S rDNA sequence was used for targeting	Terentiev et al. (2004)
<i>Pachysolen tannophilus</i>			
Promoters	GAPDH	Native	Riley et al. (2016)
Terminators	GAPDH	Native	Riley et al. (2016)
Markers	Hygromycin, Leu2	CUG-leu codons in hygromycin were changed to other Leu codons	Riley et al. (2016), Mei et al. (2018)

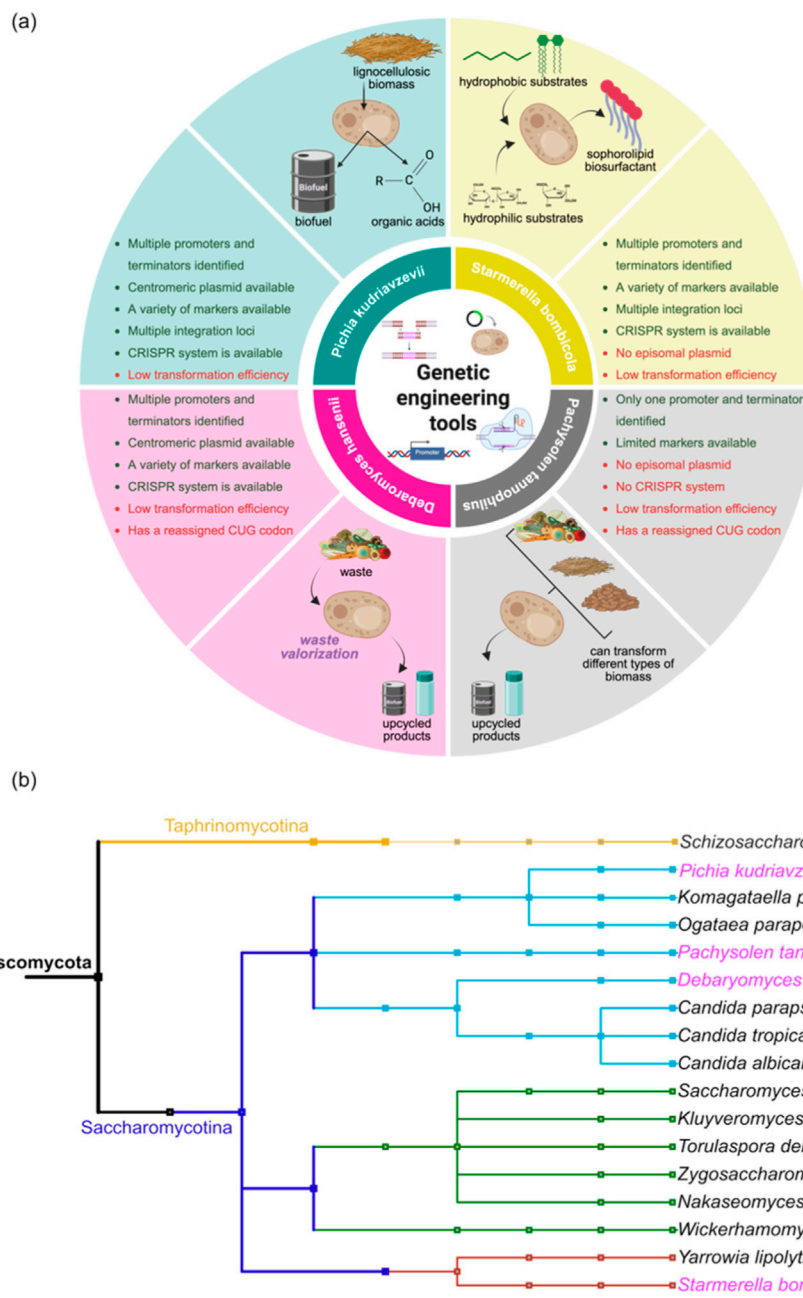
## 2.2 Markers

Markers are essential genetic elements used to verify successful transformation and to maintain plasmids or integrated constructs within host cells. They are broadly categorized into selection markers and reporter markers (Gnügge and Rudolf, 2017). Selection markers provide a growth advantage under specific conditions, allowing only transformed cells to proliferate. These are further divided into auxotrophic markers and antifungal-selection markers. Auxotrophic markers complement metabolic deficiencies in mutant host strains, enabling growth in selective media. For example, a *ura*-strain cannot grow in uracil-deficient media unless transformed with a plasmid carrying the *URA3* gene. Other common examples include *LEU2* and *HIS3*. Antifungal-selection markers, in contrast, confer resistance to antibiotics

such as hygromycin B or nourseothricin, and can be used in prototrophic strains without the need for auxotrophic backgrounds (Gnügge and Rudolf, 2017; Nora et al., 2019; Vještica et al., 2020). Meanwhile, reporter markers facilitate the visual or quantitative identification of transformed cells. They often encode easily detectable proteins or enzymes that reflect gene expression or cellular activity. The green fluorescent protein (*GFP*), for instance, enables real-time monitoring and quantification of expression levels in living cells (Soboleski et al., 2005).

## 2.3 Integration sites

Genomic integration sites are specific chromosomal loci within the host genome used for the stable insertion of



**FIGURE 1** (a) Summary of genetic tools in non-conventional yeasts. The tools currently available are denoted in green and the limitations/challenges are denoted in red. (b) Phylogenetic tree of selected non-conventional ascomycete yeasts belonging to the subphylum Saccharomycotina with *Schizosaccharomyces pombe* designated as the outgroup. The yeasts emphasized in this review, *Pichia kudriavzevii*, *Starmerella bombicola*, *Debaryomyces hansenii*, and *Pachysolen tannophilus*, are highlighted in pink.

foreign DNA. Integration at these sites ensures long-term maintenance and expression of the introduced construct without continuous selective pressure. Integration can occur either randomly or at defined loci that are known to tolerate insertions without disrupting essential host functions. Defined “safe harbor” sites are particularly valuable for achieving predictable expression and genetic stability in engineered yeast strains (Terentiev et al., 2004; Ronda et al., 2015; Babaei et al., 2021; Shi et al., 2022; Zhang et al., 2022; Fatma et al., 2023).

## 2.4 Genome-editing tools

Genome editing tools enable precise and efficient manipulation of yeast genomes to engineer metabolic pathways and optimize strain performance. In *S. cerevisiae*, genome modification has traditionally relied on homologous recombination (HR), which occurs with high efficiency. However, non-conventional yeasts exhibit relatively low HR efficiency, promoting the development of alternative tools for targeted genome modification (Ji et al., 2020; Strucko et al., 2021). The advent of nuclease-based editing

technologies, particularly CRISPR-Cas systems, has revolutionized genome engineering (Doudna and Charpentier, 2014). Enzymes such as Cas9 and Cas12a (Cpf1) introduce targeted double-strand breaks, enabling site-specific base editing, gene knockout, marker-free integration, and multiplex genome modification (Tran et al., 2019; Spasskaya et al., 2021; Strucko et al., 2021; Shi et al., 2022; Zhang et al., 2022). Beyond nuclease-based systems, landing pad approaches have been developed to facilitate repeated or modular genome engineering (Fatma et al., 2023). These systems introduce defined genomic docking sites (e.g., *loxP* or *attP*) that allow site-specific integration of genetic constructs via recombination (Santos et al., 2013). Additionally, transposon-based systems such as piggyBac allow random integration of genetic cassettes at TTAA sites without requiring homologous recombination or site-specific nucleases (Li et al., 2013). Such tools expand the flexibility and scalability of genome manipulation, accelerating the development of non-conventional yeast platforms for biotechnological applications.

### 3 Non-conventional yeasts

#### 3.1 *Pichia kudriavzevii*

*Pichia kudriavzevii* (also known as *Candida Krusei* or *Issatchenkia orientalis*) is a ubiquitous non-conventional yeast known for its exceptional tolerance to high temperature, low pH, elevated salinity, and high concentrations of lignocellulosic inhibitors (Douglass et al., 2018; Lee et al., 2022; Chu et al., 2023; Wang et al., 2025). These robust traits make it an attractive platform for the bioproduction of organic acids and other value-added chemicals from renewable feedstocks (Sun et al., 2020; Tran et al., 2023; Wu et al., 2023; Tan et al., 2025). The species is predominantly diploid, although triploid and aneuploid variants have also been identified (Douglass et al., 2018; Hsieh et al., 2025).

Genetic modification of *P. kudriavzevii* has traditionally relied on linear integrative plasmids employing homologous recombination, commonly with the *URA3* selection marker (Xiao et al., 2014; Wu et al., 2023). The introduction of episomal plasmids became feasible with the incorporation of an *S. cerevisiae* ARS element and a native centromere-like sequence (CEN-L), which together improved plasmid replication and segregation stability (Tran et al., 2019; Cao et al., 2020). Recent progress includes multicopy and multigene integrations using piggyBac transposon and landing pad systems targeting defined intergenic loci (Fatma et al., 2023; Wu et al., 2023). The development of CRISPR-Cas-based tools now allows efficient, marker-free genome editing without reliance on auxotrophic or antifungal selection markers (Tran et al., 2019). Transformation is most commonly achieved through the lithium acetate (LiAc) method, which remains the standard protocol for *P. kudriavzevii*.

#### 3.2 *Starmerella bombicola*

*Starmerella bombicola* (formerly *Candida bombicola*) is a non-pathogenic yeast renowned for its natural production of sphorolipids—biodegradable glycolipid surfactants with broad applications in pharmaceuticals, cosmetics, and environmental

remediation (Kurtzman et al., 2010; De Graeve et al., 2018; Zhang et al., 2025). The species can metabolize both hydrophilic carbon sources (e.g., sucrose and fructose) and hydrophobic substrates (e.g., fatty acids, fatty alcohols, and long-chain alkanes), enabling valorization of inexpensive waste feedstocks for sphorolipid and long-chain dicarboxylic acid production (Li et al., 2016b; Chatterjee et al., 2022; Lee et al., 2025; Wang et al., 2019).

Genetic modification of *S. bombicola* has been challenging due to low HR efficiency (Shi et al., 2022; Zhang et al., 2022). Although extending homology arms to 1 kb can improve HR frequency, colony recovery on selective media remains slow (Saerens et al., 2011; Shi et al., 2022; Zhang et al., 2022). The species appears to rely minimally on non-homologous end joining (NHEJ) for DNA double strand break repair. Transformation is typically achieved via LiAc-mediated chemical transformation or electroporation, whereas *Agrobacterium Tumefaciens*-mediated transformation (ATMT) has proven ineffective (Lodens et al., 2018).

Plasmids have been derived from the pGEM-T and pJET backbones (Saerens et al., 2011; Geys, 2017; Lodens et al., 2018). The activities of 14 native promoters were characterized and shown to vary with carbon sources; among them, *TEF1*, *GAPD*, *ENO*, *PGK1*, *TPI*, and *TDH1* promoters showed moderate to strong expression based on GFP assays (Shi et al., 2022). A codon-optimized *Streptococcus pyogenes* Cas9 (SbCas9) was developed by removing inhibitory secondary structures to enhance expression. Although RNA polymerase III promoters for sgRNA expression have not yet been identified in this species, a CRISPR-Cas12a (Cpf1) system employing a codon-optimized *Acidaminococcus sp.* Cas12a driven by the *TEF1* promoter enabled efficient single and multiplex genome editing (Zhang et al., 2025). Using donor DNA with ~300 bp homology arms further enhanced editing efficiency, outperforming both HR- and NHEJ-based approaches (Zhang et al., 2025).

Despite these editing strategies, further advances are limited by the lack of a functional episomal plasmid in *S. bombicola*. Attempts to identify ARS elements from *Kluyveromyces lactis*, *S. cerevisiae* (ARS/CEN4 and 2-micron plasmid), or its own genome have not yielded self-replicating constructs (Geys, 2017).

#### 3.3 *Debaryomyces hansenii*

*D. hansenii* is a non-pathogenic, halotolerant, osmotolerant, xerotolerant, and oleaginous, haploid yeast (Gunge et al., 1993; Maggi and Govind, 2004; Breuer and Harms, 2006). It secretes a NaCl-enhanced killer toxin that is lethal to other yeasts (Gunge et al., 1993; Breuer and Harms, 2006). Sodium ions appear to protect its growth even under extreme pH conditions (Prista et al., 2005; Navarrete et al., 2022). *D. hansenii* utilizes diverse carbon sources, including raffinose, xylose, and n-alkanes, and can thrive in highly saline (>1 M) or nutrient-limited environments, making it well suited for waste revalorization processes (Estrada et al., 2023; Navarrete et al., 2022; Breuer and Harms, 2006; Fukuda et al., 2004).

Stable plasmid replication in *D. hansenii* typically requires high salt conditions (~2.1 g/L) (Gunge et al., 1993; Fukuda et al., 2004). Several episomal plasmids—pRGM, pMR95, pMR96, pDH4, and pDH11—derived from the pUC19 backbone contain *D. hansenii* ARS elements, while plasmids such as pDhARS2, pDhARS3, and

pDhARS9 derived from pGEM7Z harbor *C. famata* ARS sequences for heterologous expression (Ricaurte and Govind, 1999; Maggi and Govind, 2004; Minhas et al., 2009). CRISPR<sup>CUG</sup>-tRNA plasmids (pDIV series) have also been developed, incorporating replication origins from *Candida famata*, *K. lactis* and *S. cerevisiae* (Strucko et al., 2021).

Because *D. hansenii* favors NHEJ over HR, a  $\Delta$ KU70 mutant strain was engineered to enhance targeted gene integration (Minhas et al., 2009; Strucko et al., 2021; Navarrete et al., 2022). CRISPR-Cas9 transformation achieves approximately 3000 CFU/ $\mu$ g of DNA via electroporation (Ricaurte and Govind, 1999; Minhas et al., 2009). Improving transformation efficiency and toolkits for this species will expand functional genomics and pathway engineering. Furthermore, *D. hansenii* reassigns the CUG codon to serine instead of leucine, necessitating codon optimization for all heterologous genes (Strucko et al., 2021).

### 3.4 *Pachysolen tannophilus*

*Pachysolen tannophilus* is a haploid, non-conventional yeast with a broad substrate range, capable of metabolizing glucose, glycerol, galactose, and xylose, making it a promising host for industrial conversion of diverse hydrolysates (Maleszka et al., 1982; Slininger et al., 1982; Slininger et al., 1987; Yang and Jeffries, 1997; Wedlock et al., 1989). However, *P. tannophilus* is relatively sensitive to lignocellulosic inhibitors (Harner et al., 2014). Genome sequencing in 2012 revealed that its CUG codon is reassigned to encode alanine rather than leucine, placing it among the alternative-codon-usage yeasts (Liu et al., 2012; Riley et al., 2016; Muhlhausen et al., 2016).

Early transformation studies employed *S. cerevisiae*-derived plasmids such as YRp7, YEp13 and pACT containing 2- $\mu$ m ARS sequences, supported limited replication in *P. tannophilus* (Mei et al., 2018; Wedlock et al., 1989). However, plasmids bearing *S. cerevisiae* ARS/CEN elements were not stably maintained, likely due to sequence divergence (Mei et al., 2018). Furthermore, previous work reported that successful transformation of *P. tannophilus* occurred only with plasmids bearing a codon-optimized hygromycin resistance gene, while plasmids containing the native version failed to produce transformants (Riley et al., 2016). Optimization of the LiAc-mediated transformation protocol improved transformation efficiency, yet the lack of native replication and centromeric elements remains a bottleneck for stable episomal maintenance.

The reassignment of the CUG codon further complicates heterologous expression, as genes encoding leucine via CUG may produce mistranslated proteins. Identifying native ARS/CEN sequences, together with species-specific selection markers and strong regulatory elements, will be critical for developing a reliable genetic toolkit. Establishing high-efficiency transformation and genome-editing systems will be key to advancing *P. tannophilus* as a robust microbial cell factory.

## 4 Discussion

Despite significant progress in developing genetic tools for non-conventional yeasts, several challenges remain before these

species can be fully established as robust industrial platforms. The lack of species-specific plasmid systems, low transformation efficiencies, and issues of genome stability continue to constrain efficient genetic manipulation and metabolic engineering. Although many non-conventional yeasts display exceptional stress tolerance and metabolic versatility, the underlying molecular mechanisms and regulatory networks governing these traits remain underexplored. Additional challenges are presented by species such as *D. hansenii* and *P. tannophilus*, which exhibit CUG codon reassignment. This deviation from the universal genetic code prevents the direct use of conventional plasmids and heterologous genes without codon optimization. Similarly, *S. bombicola* currently lacks a functional episomal plasmid, limiting its genetic tractability despite its natural capacity to produce biodegradable and non-toxic surfactants. Identifying native ARS/CEN elements and establishing species-specific expression systems will therefore be essential to expand their biotechnological potential. To unlock the full capabilities of these emerging yeast platforms, future research should prioritize the development of stable genomic integration loci, high-efficiency transformation methods, and tailored genome-editing strategies that accommodate species-specific characteristics. Such advancements will be critical to broaden the genetic toolbox and realize the full potential of non-conventional yeasts as sustainable microbial chassis for next-generation biomanufacturing.

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## Conflict of interest

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