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Synthetic biology and molecular genetics in non-conventional yeasts: Current tools and future advances

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ABSTRACT

Coupling the tools of synthetic biology with traditional molecular genetic techniques can enable the rapid prototyping and optimization of yeast strains. While the era of yeast synthetic biology began in the well-characterized model organism *Saccharomyces cerevisiae*, it is swiftly expanding to include non-conventional yeast production systems such as *Hansenula polymorpha*, *Kluyveromyces lactis*, *Pichia pastoris*, and *Yarrowia lipolytica*. These yeasts already have roles in the manufacture of vaccines, therapeutic proteins, food additives, and biorenewable chemicals, but recent synthetic biology advances have the potential to greatly expand and diversify their impact on biotechnology. In this review, we summarize the development of synthetic biological tools (including promoters and terminators) and enabling molecular genetics approaches that have been applied in these four promising alternative biomanufacturing platforms. An emphasis is placed on synthetic parts and genome editing tools. Finally, we discuss examples of synthetic tools developed in other organisms that can be adapted or optimized for these hosts in the near future.

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1. Introduction

The scope and prospect of synthetic biology is continuously evolving and expanding (Andrianantoandro et al., 2006; Cameron et al., 2014; Purnick and Weiss, 2009), yet fundamentally relies on the premise that biology can be built from a collection of well-characterized parts and subsystems. For more conventional, model organisms (such as *Escherichia coli* and *Saccharomyces cerevisiae*), this approach is aided by existing and ongoing efforts to catalogue synthetic parts and genome editing tools (Lee et al., 2015; Purnick and Weiss, 2009). The general tractable nature of genetic modifications and basic molecular biology and protein expression tools in these organisms aids such efforts (Ferrer-Miralles et al., 2009). In recent years, one of the major breakthrough areas for synthetic biology is expediting the often long and costly design-build-test cycle for industrial biotechnology. With the aid of modern genome editing tools, panels of production strains can now be created at unprecedented rates to support high throughput studies of genotype to phenotype relationships (Bao et al., 2015). The emergence of this capacity promises to spark a renaissance in the complex rewiring and engineering of organisms for production purposes. These synthetic biological capabilities have enabled impressive engineering efforts in model organisms, but there are many other non-conventional organisms of great interest to industrial biotechnology for which synthetic biology approaches are far more immature. The promise of synthetic biology in these organisms could significantly impact the metabolic engineering and recombinant protein production fields by establishing unique and robust expression and production platforms for biomanufacturing.

The potential of synthetic biology for rewiring organisms for production begets the question of which organism to choose, especially when not restricted to model organisms (Alper and Stephanopoulos, 2009). The ideal expression system would be a universal platform capable of efficiently upgrading a variety of low-cost feedstock into value-added, renewable products. At the

Abbreviations: ARS, autonomously replicating sequence; CEN, centromere; CRISPR, Clustered, Regularly Interspaced, Short Palindromic Repeats; DSB, double strand break; GFP, green fluorescent protein; GRAS, Generally Regarded As Safe; gRNA, guide RNA (for CRISPR); HR, homologous recombination; IRES, internal ribosomal entry site; FDA, Food and Drug Administration; NHEJ, non-homologous end-joining; RNAi, RNA interference; RNAP II/III, RNA polymerase II/III; TALEN, transcription activator-like effector nuclease; TF, transcription factor; UAS, upstream activation sequence; UTR, untranslated region; WT, wild type; ZeoR, Zeocin resistance cassette; ZFN, zinc finger nuclease.

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same time, the choice of host system imparts a substantial effect on industrial production (Westfall et al., 2012), as production-relevant phenotypes are usually multifactorial and difficult to unravel (Mateos et al., 2006; Stahmann et al., 2001). The innate advantages of a particular host or strain may include tolerance for high concentrations of product or precursor (Ling et al., 2014; Peralta-Yahya et al., 2012), moderate to high *de novo* synthesis of precursors or product (Blazeck et al., 2014), or robustness to processing conditions (Dijkstra et al., 2014; Hahn-Hägerdal et al., 2005). These complex traits are often difficult to characterize and reductively define, and thus they are not easily transferred into another preferred strain or organism.

Despite their higher relative complexity, yeasts tend to have many beneficial, industrially attractive traits relative to commonly used bacteria such as *E. coli* (Chen and Nielsen, 2013; Kim et al., 2014; Liu et al., 2013; Wildt and Gerngross, 2005). A few of these attributes include: growth to high cell densities on a wide variety of carbon sources, ability to perform a variety of post-translational modifications, potential to compartmentalize reactions in organelles, high secretion capacity, and a lack of susceptibility to infectious agents like bacteriophage (Blount et al., 2012). While filamentous fungi also share many of these advantageous characteristics, they are often more difficult to transform with exogenous DNA (Kawai et al., 2010) and less amenable to simple bioreactor cultivation. The process engineering complexity of large-scale growth of filamentous fungi is due in part to variations in hyphal morphology that can lead to rheological mixing challenges and mass transfer limitations (Gibbs et al., 2000).

The vast majority of yeast synthetic biology tools have been developed in *S. cerevisiae* due to its well-annotated genome, genetic tractability, and overall ease of use (Nielsen et al., 2013). Likewise, the model yeast *S. cerevisiae* has historically dominated the arena of modified yeasts for industrial processing. However, there are many other non-conventional yeast hosts with favorable traits (Buckholz and Gleeson, 1991; Gellissen et al., 2005), some of which are also used for industrial bioprocesses. In particular, with advances in synthetic biology, non-conventional yeasts such as *Hansenula polymorpha* (syn. *Ogataea polymorpha*), *Kluyveromyces lactis*, *Pichia pastoris* (syn. *Komagataella pastoris*), and *Yarrowia lipolytica* may take on expanded roles as industrial hosts.

In this review, we highlight the advances of synthetic biology in light of these four promising non-conventional yeast strains. Specifically, we describe the importance and status of basic synthetic parts, enabling molecular biology techniques, and genome-editing technologies in these organisms. Initially, we establish the importance and current uses of these four non-conventional yeasts. Next, we evaluate the state of synthetic parts including promoters, terminators, and episomal replication elements. Subsequently, we review techniques for site-specific cassette integrations, controlled and scarless excisions, and related genome editing methods including CRISPR–Cas. Ultimately, we provide a prospective for the future with respect to the application of synthetic biology in these promising non-conventional yeasts.

2. Non-conventional yeast systems

Non-conventional yeasts can provide many potential advantages over *S. cerevisiae* with respect to pathway requirements, desired product profile, and gross physiology (Gellissen and Hollenberg, 1997; Wolf, 2012). While some non-conventional yeasts have served as long-standing industrial protein production platforms, their utility in the small molecule chemical space is sometimes limited to native metabolite overproduction phenotypes. However, the increasing availability of high-quality yeast genome sequences (De Schutter et al., 2009; Dujon et al., 2004; Ramezani-Rad et al., 2003; Sherman et al., 2009), effective vectors and transformation methods (Bianchi et al., 1987; Cregg et al., 1985; Das and Hollenberg, 1982; Davidow and Dezeeuw, 1984; Faber et al., 1994; Gaillardin et al., 1985; Roggenkamp et al., 1986), and emerging synthetic tools are beginning to change this picture. This review focuses on four non-conventional yeasts of significant biotechnological interest: *H. polymorpha*, *K. lactis*, *P. pastoris*, and *Y. lipolytica*. Each of these organisms presents with different advantages, commonalities, and differences when compared with *S. cerevisiae*. A short survey of basic characteristics for these yeasts is illustrated in Fig. 1, and a selection of significant products currently produced from these hosts is provided in Table 1. A brief summary of several unique and promising traits is also presented in the text that follows. For more detailed descriptions of these individual yeast systems, the authors refer the reader to organism-specific reviews for *H. polymorpha* (Saraya et al., 2012), *K. lactis* (Rodicio and Heinisch, 2013), *P. pastoris* (Vogl et al., 2013), and *Y. lipolytica* (Madzak, 2015), and to several previous non-conventional yeast reviews (Dominguez et al., 1998; Gellissen et al., 2005; Kim et al., 2014; Radecka et al., 2015; Wolf, 2012).

Products derived from all four of these systems have been assigned Generally Regarded As Safe (GRAS) status by the United States FDA (Dominguez et al., 1998; Saraya et al., 2014; Vogl et al., 2013), which eases potential regulatory concerns when selecting an expression system for a new product. Whereas *S. cerevisiae* is a poor host for some human therapeutic proteins due to its tendency to hyperglycosylate (Bretthauer and Castellino, 1999) and produce potentially allergenic glycan structures, *H. polymorpha* and *P. pastoris* have both been extensively glycoengineered to produce strains with humanized N-glycosylation (Cheon et al., 2012; Wildt and Gerngross, 2005). *K. lactis* has also been engineered to reduce the hypermannosylation of expressed proteins (Liu et al., 2009). *Y. lipolytica* is an oleaginous, alkane assimilating yeast with high potential for use as a platform for the production of lipid and fatty acid-derived products (Blazeck et al., 2014; Tai and Stephanopoulos, 2013) and has been used for recombinant carotenoid and polyunsaturated fatty acid production industrially (Xue et al., 2013). *H. polymorpha* is a highly thermotolerant yeast capable of converting glucose and xylose to ethanol at temperatures up to 45–50 °C (Suwannarangsee et al., 2010).

All of these hosts possess a broader or diversified catabolic repertoire compared to *S. cerevisiae*, thus enabling lower cost bioprocesses (Gellissen et al., 2005; Rymowicz et al., 2010). High level recombinant protein expression has also been achieved using all four of these systems, and production titers similar to or greater than *S. cerevisiae* are possible (Dominguez et al., 1998). Additionally, each of these organisms has substantial protein secretion capacity that can exceed that of *S. cerevisiae* by several orders of magnitude (Muller et al., 1998). Finally, all of these yeast species are 'Crabtree-negative' and as such, do not undergo aerobic alcoholic fermentation (Kiers et al., 1998). This can be an advantageous phenotype for increasing biomass accumulation in aerated cultures without losing overflow carbon to undesired alcohol production.

Despite the advantages described above, each of these hosts is considered non-conventional and suffers from some molecular genetic intractability compared with *S. cerevisiae*. In particular, one significant characteristic that has historically hampered modification of non-conventional yeast systems relative to *S. cerevisiae* is DNA repair pathway preference. *S. cerevisiae* strongly favors homologous recombination (HR), whereas all four non-conventional yeasts covered by this review display a stronger preference for non-homologous end-joining (NHEJ) when exogenous DNA is introduced (Vogl et al., 2013). NHEJ results in random, spontaneous integrations of all or part of a cassette or vector instead of the precise integration that is possible with highly active

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