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Biosynthesis of terpene compounds using the non-model yeast *Yarrowia lipolytica*: grand challenges and a few perspectives

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Abstract

Yarrowia lipolytica has emerged as an important non-model host for terpene production. However, three main challenges remain in industrial production using this yeast. First, considerable knowledge gaps exist in metabolic flux across multiple compartments, cofactor generation, and catabolism of non-sugar carbon sources. Second, many enzymatic steps in the complex-terpene synthesis can pose rate-limitations, causing accumulation of toxic intermediates and increased metabolic burdens. Third, metabolic shifts, morphological changes, and genetic mutations are poorly characterized under industrial fermentation conditions. To overcome these challenges, systems metabolic analyses, protein engineering, novel pathway engineering, model-guided strain design, and fermentation optimization, have been attempted with some successes. Further developments that address these challenges are needed to advance the *Yarrowia* nonmodel yeast platform for industrial-scale production of high-value terpenes, including those with highly complex structures such as the anticancer withanolides and insecticidal limonoids.

Graphical Abstract

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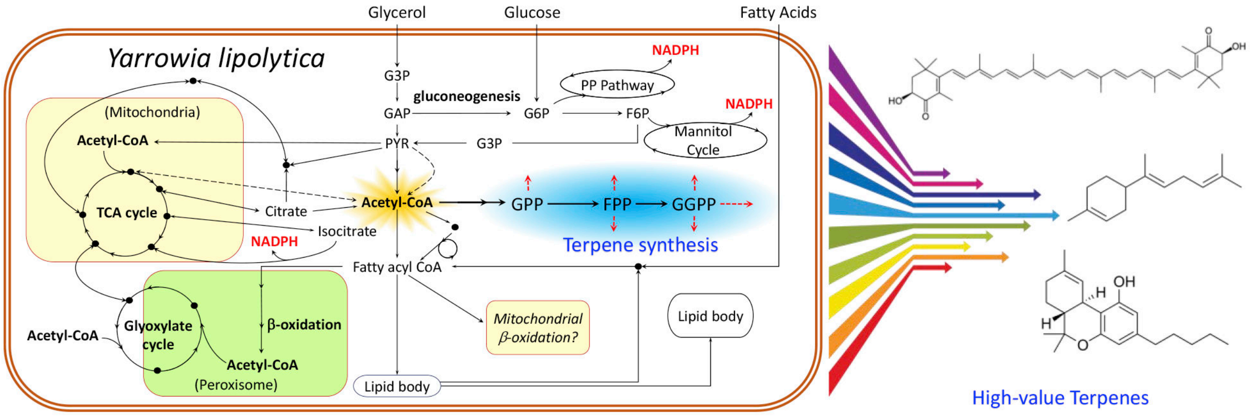
Credit Author Statement

Alyssa M. Worland: Writing – original draft; Writing – review & editing; Visualization. **Jeffrey J. Czajka:** Writing – review & editing. **Yanran Li:** Writing – review & editing. **Yechun Wang:** Writing – review & editing. **Yinjie J. Tang:** Writing – review & editing; Conceptualization; Supervision; Funding acquisition. **Wei Wen Su:** Writing – review & editing; Conceptualization; Supervision; Funding acquisition.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.



Keywords

acetyl-CoA; genetic stability; metabolic burden; metabolic shift; peroxisome

Introduction

Extensive research has been aimed at producing terpenes (a vast group of high value natural compounds) using microbial hosts. Model organisms like *Escherichia coli* and *Saccharomyces cerevisiae* have been widely used for the production of terpenes as nutraceuticals [1], fuels (e.g., bisabolene [2]), and pharmaceuticals such as cannabinoids [3], with encouraging results. The microbial hosts and production titers for terpenes of different molecular weights (monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), triterpenes (C_{30}), and tetraterpenes (C_{40})) are summarized in Figure 1. However, for commercial applications, *E. coli* has drawbacks such as bacterial toxin contamination, acetate inhibition, low activity of expressed plant-based enzymes, and an inability to post-translationally modify and compartmentalize complex proteins. *S. cerevisiae* also has drawbacks such as relatively slow growth rates, complex growth medium requirements, and the Crabtree effect. Recently, new microbial platforms, including non-model yeasts, microalgae, and consortia, have been developed via their unique traits for terpene biosynthesis [1,4,5]. For example, the red yeast *Rhodosporidium toruloides* is capable of using cheap feedstocks such as aromatics, glycerol, and methane, while cyanobacteria can utilize CO_2. Among these non-model hosts, oleaginous yeast *Yarrowia lipolytica* exhibits high chemical tolerance and robust secretion abilities, and has received strong research interests, facilitating the rapid development of relevant genetic and systems biology tools [6]. These tools include genome editing via CRISPR/Cas9, novel secretion pumps, promoter tuning, pathway assembly, and modular cloning strategies, as well as genome-scale modeling to guide rational metabolic engineering [7]. Thus, *Y. lipolytica* has emerged as an attractive non-conventional host, and has been demonstrated for its capability of high terpenoid production (e.g., 6 g/L β-carotene [8]).

Knowledge gaps in *Y. lipolytica* metabolism

There are still grand challenges in using nonmodel yeasts for biomanufacturing of terpenes. Detailed metabolic understanding is necessary for performing rational strain engineering, but knowledge gaps still exist in *Y. lipolytica* metabolism [9,10]. First, cytosolic acetyl-CoA is the key terpene pathway precursor, but its generation is not completely elucidated, particularly when *Y. lipolytica* metabolizes non-sugar based feedstock. ATP-citrate lyase (ACL) is known to cleave cytosolic citrate to form acetyl-CoA and considered the major generation route. However, there are multiple putative acetyl-CoA generation and consumption routes, and acetyl-CoA translocation between compartments (mitochondria or peroxisome) is not yet fully understood. For example, *Y. lipolytica* can degrade fatty acids via β-oxidation and is able to grow on them as a sole carbon source. Acetyl-CoA is generated in the peroxisomes, but the transportation of peroxisomal acetyl-CoA to the cytosol is unclear. One likely route is shuttling via glyoxylate (Glyox) cycle intermediates, which can enter the mitochondria from the peroxisome. Another potential route is the carnitine shuttle that translocates acetyl-CoA between mitochondria and cytosol [11]. However, the activity of carnitine acetyltransferases in the oleaginous yeast is still not well-characterized compared to *S. cerevisiae* [12]. Finally, the cytosolic pyruvate bypass can produce acetyl-CoA via the intermediate acetate, but this pathway's contribution and activity during growth on non-acetate carbon sources are not quantified in *Y. lipolytica.* Different engineering efforts have been explored to enhance cytosolic acetyl-CoA and push the flux towards mevalonate. The pyruvate bypass and pyruvate dehydrogenase (PDH) overexpression have resulted in the highest increases of product titers; modification of the peroxisomal structural protein may also result in increased acetyl-CoA supplies for biosynthesis. However, enzyme engineering of ACL is less effective to rewire acetyl-CoA flux [11,13–15].

The second main knowledge gap involves NADPH generation, which is the reducing power for terpene synthesis. In *Y. lipolytica* grown using glucose as the carbon source, the oxidative pentose phosphate pathway was shown to be the primary source of NADPH for lipid overproduction [16]. However, it is unclear what is mainly responsible for NADPH generation when other carbon sources such as lipids are used. Most commonly, a cytosolic $NADP^+$-dependent malic enzyme can provide NADPH during oleaginous microbial growth [17]. However, *Y. lipolytica* lacks a cytosolic version of the malic enzyme, while the mitochondrial version is NAD^+-dependent [17]. Expression of a heterologous $NADP^+$-malic enzyme in the cytosol of *Y. lipolytica* did not lead to an increase in lipid productivity [18]. Therefore, *Y. lipolytica* may employ multiple cofactor generation routes, including a cytosolic $NADP^+$-dependent isocitrate dehydrogenase (ICDH) for NADPH synthesis. Another possible source of NADPH in many non-model yeasts is the mannitol cycle, which converts NADH and ATP to NADPH [19]. Transcriptional analysis of *Y. lipolytica* cultures indicated that both the mannitol cycle and ICDH were upregulated to support lipogenic activities [20]. Genome-scale modeling also showed that the ICDH and mannitol cycle could be two key NADPH sources in *Y. lipolytica* [21].

Technological difficulties in constructing multi-step pathways for terpene synthesis

Engineering terpene synthesis requires multiple heterologous enzymes that may pose rate limitations, flux imbalances, accumulation of toxic intermediates, and metabolic burdens. Some enzymes, such as the cytochrome P450 oxygenase [26,27], are difficult to functionally express in heterologous hosts [28]. Further, many secondary metabolite pathways involve metabolite channeling via membrane or covalent binding of multiple pathway enzymes [29]. The multi-enzyme complexes help to channel the flux of substrates into different branches of the pathways [30]. A better understanding of the native channeling mechanisms will improve the efficacy of the heterologously expressed pathways while also reducing metabolic burdens and metabolite transport challenges [31–33]. Some successes in synthetic multi-enzyme complexes have been met by utilizing protein scaffolds [34,35] and by natural organelle engineering to cluster cascade enzymes. In addition, innate channels may prevent the release of intermediate metabolites to engineered pathways. For example, heterologous enzymes could not effectively hijack the geranyl diphosphate (GPP) precursor for monoterpene synthesis in *Yarrowia* [36,37]. Thus, a rich area for future research will be compartmentalization of native biosynthesis pathways with downstream heterologous enzymes, as well as the development of membraneless synthetic channels with minimal metabolic disruptions.

Metabolic shifts, morphological changes, and genetic instability under bioreactor conditions

Y. lipolytica can ferment diverse substrates to various organic acids and its overflow metabolism is highly sensitive to growth conditions and fermentation stages. For example, *Y. lipolytica* ionone fermentation has been observed to secrete mevalonate and other organic acids in the early fermentation stage and reuse these acids during the late production phase [38]. Moreover, *Y. lipolytica* is dimorphic, i.e., capable of transition between ovoid and filamentous morphologies, which is influenced by pH, dissolved oxygen, temperature, and nutrient conditions, and can alter fermentation broth rheological property and mixing efficiency [39,40]. The mechanism of such morphological changes is not entirely clear, but it is believed to result from the interplays among complex genetic and environmental factors [41]. In general, industrial-scale bioreactor operations tend to intensify cell stress responses due to constant exposure of the cells to highly varying hydrostatic pressures and ununiformed O_2 and substrate concentrations [24]. Decoupling the growth and production phase or the use of consortia systems could potentially alleviate stresses on the engineered yeast cells [42].

Furthermore, *Y. lipolytica* strains isolated from different sources are known to contain high genetic and metabolic variabilities [43], and these issues have not been fully studied [44]. The inherently stochastic nature of cellular machinery often leads to DNA mismatch during replication, and the accumulation of these defects can lower productivity [45,46]. In general, the combined effect of growth stresses and long replication generations leads to genetic instability [47]. Particularly, highly transcribed genes tend to accumulate mutations at higher rates [48], and synthetic biological components (such as efflux pumps) may increase the propensity for strain mutations [49]. Overall, overcoming *Y. lipolytica* genetic instability associated with bioreactor conditions and fermentation scale-up is an emerging area deserves more investigations to succeed in industrial applications.

Future perspective

There has been considerable progress in developing non-model yeast platforms. However, challenges are still present, as shown in *Y. lipolytica* studies (Figure 3). Traditional strain development relies on the design-build-test-learn (DBTL) cycle, and significant time is spent on strain development and testing of desired phenotypes. Computational designs can reduce the duration of DBTL cycles and facilitate strain development. For example, genome-scale modeling can predict flux organization for optimal biosynthesis in oleaginous yeast *Y. lipolytica* [50]. However, such techniques rely on the completeness of metabolic knowledge. Thus, further characterization of *Y. lipolytica* using ^{13}C-metabolic flux analysis is necessary to quantify cofactor balances, cellular maintenance costs, acetyl-CoA fluxes, and metabolic burdens. Moreover, machine learning approaches can analyze omics data to decipher dynamic metabolic regulations and to estimate the hidden constraints/limitations on strain engineering outcomes [51]. The data-driven approaches may also assist genome-scale modeling to capture cellular features and predict effective strain engineering targets [52].

Recently, the biosynthesis of terpenoids with highly complex structures has been elucidated, and engineered into *S. cerevisiae*, including steroid-derived metabolites such as the anticancer withanolides [53] and insecticidal limonoids [54]. Intertwining long-step terpenoid pathway with the endogenous yeast pathway requires intensive engineering efforts on regulations of enzyme assemblies and temporal control of metabolic reactions. These successful engineering strategies in *S. cerevisiae* can be potentially applied to the non-model yeasts to promote production yields [55–57]. Finally, with the success already demonstrated for producing the more conventional terpenoid products such as β-carotene that accumulated to a titer over 6 g/L, meeting the challenges including those highlighted in Figure 3 will likely advance the *Yarrowia* non-model yeast platform to produce even more diverse and complex terpenoid compounds.

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Highlights

- *Yarrowia* has shown the potential for producing complex terpenoids.
- *Yarrowia* may hold multiple cytosolic acetyl-CoA and NADPH generation pathways.
- Metabolite channeling is a key consideration in terpene pathway engineering.
- Knowledge gaps exist in compartment-specific fluxes for utilizing alternative carbon sources (e.g., lipids).
- Metabolic shift, genetic stability, and dimorphism in large-scale *Yarrowia* fermentation need further studies.

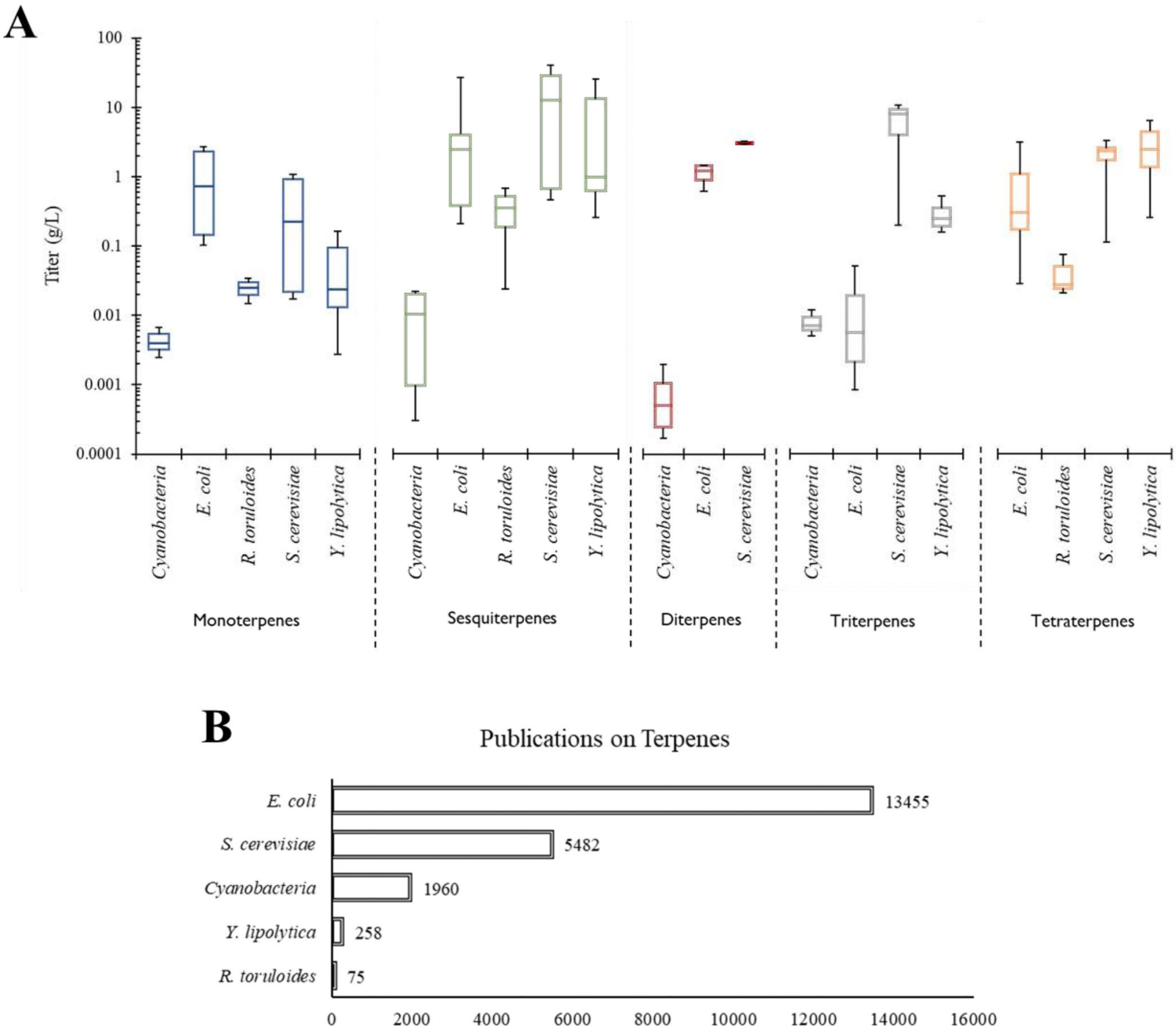

Figure 1: A survey of microbial terpene production.

A. Terpene production titers relative to hosts and terpenes, with the data mostly derived from a recent review [4]. **B**. The number of terpene-related publications for each host, according to a PubMed (PMC) search conducted on January 15, 2020, based on the keyword combination “(host) AND (isoprenoid or terpene or terpenoid)”.

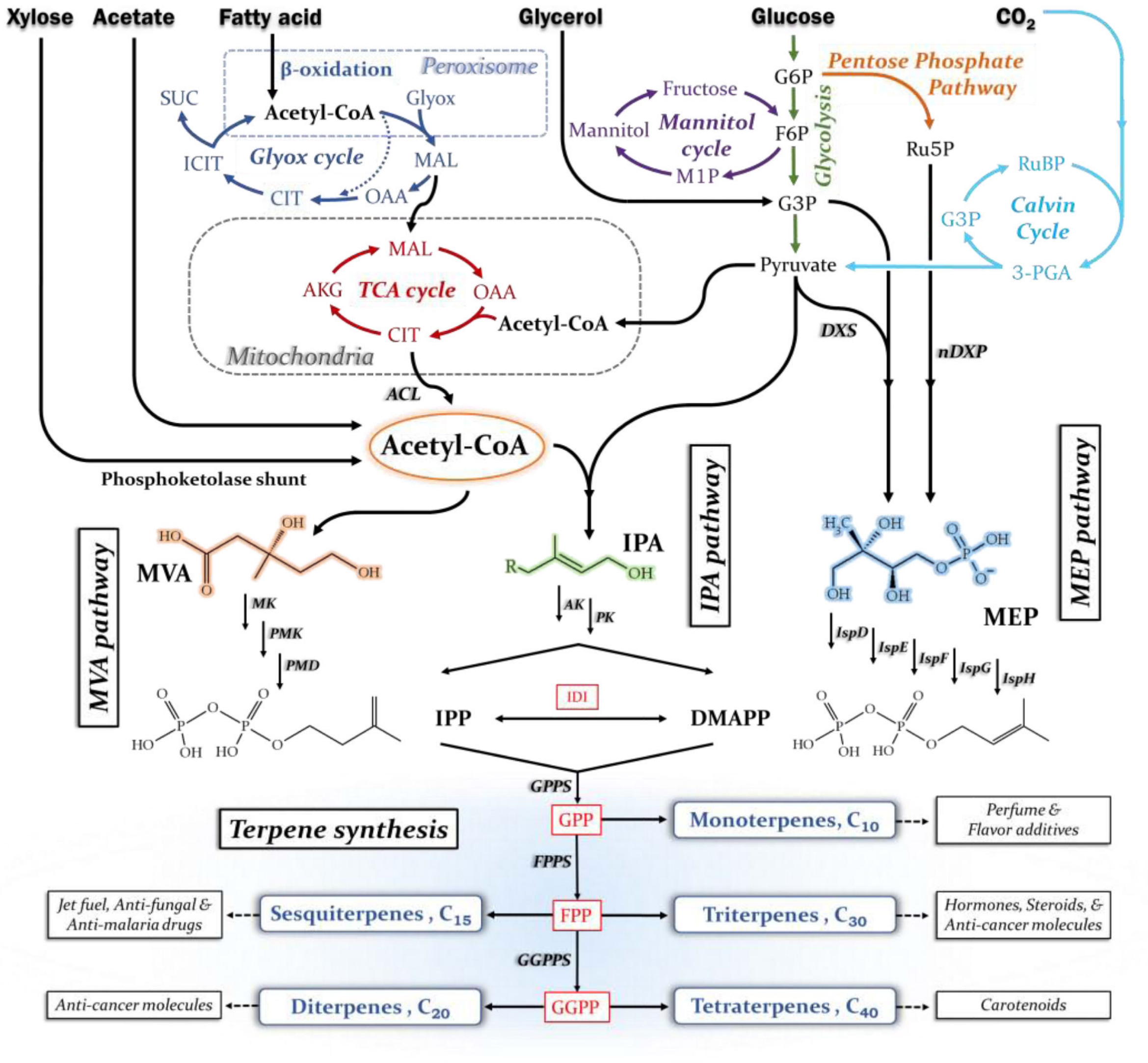

Figure 2: Pathway map for the production of terpenoid compounds.

Using isoprene, a C_5 terpene compound, as a building block, larger terpene molecules are created. Bacterial hosts employ the methylerythritol 4-phosphate (MEP) pathway (the non-mevalonate pathway, also called the DXP pathway) to synthesize isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). The Entner-Doudoroff Pathway [22] or nDXP route (synthesis of 1-deoxy-d-xylulose 5-phosphate from pentose) [23] can increase the flux of the MEP pathway. In contrast, eukaryotes employ the mevalonate (MVA) pathway to synthesize IPP. In *S. cerevisiae*, cytosolic acetyl-CoA generation can be promoted via the pyruvate dehydrogenase bypass or novel phosphoketolase shunt [24]. The newly discovered isoprenoid alcohol (IPA) pathway can convert pyruvate and acetyl-CoA to IPP and DMAPP [25].

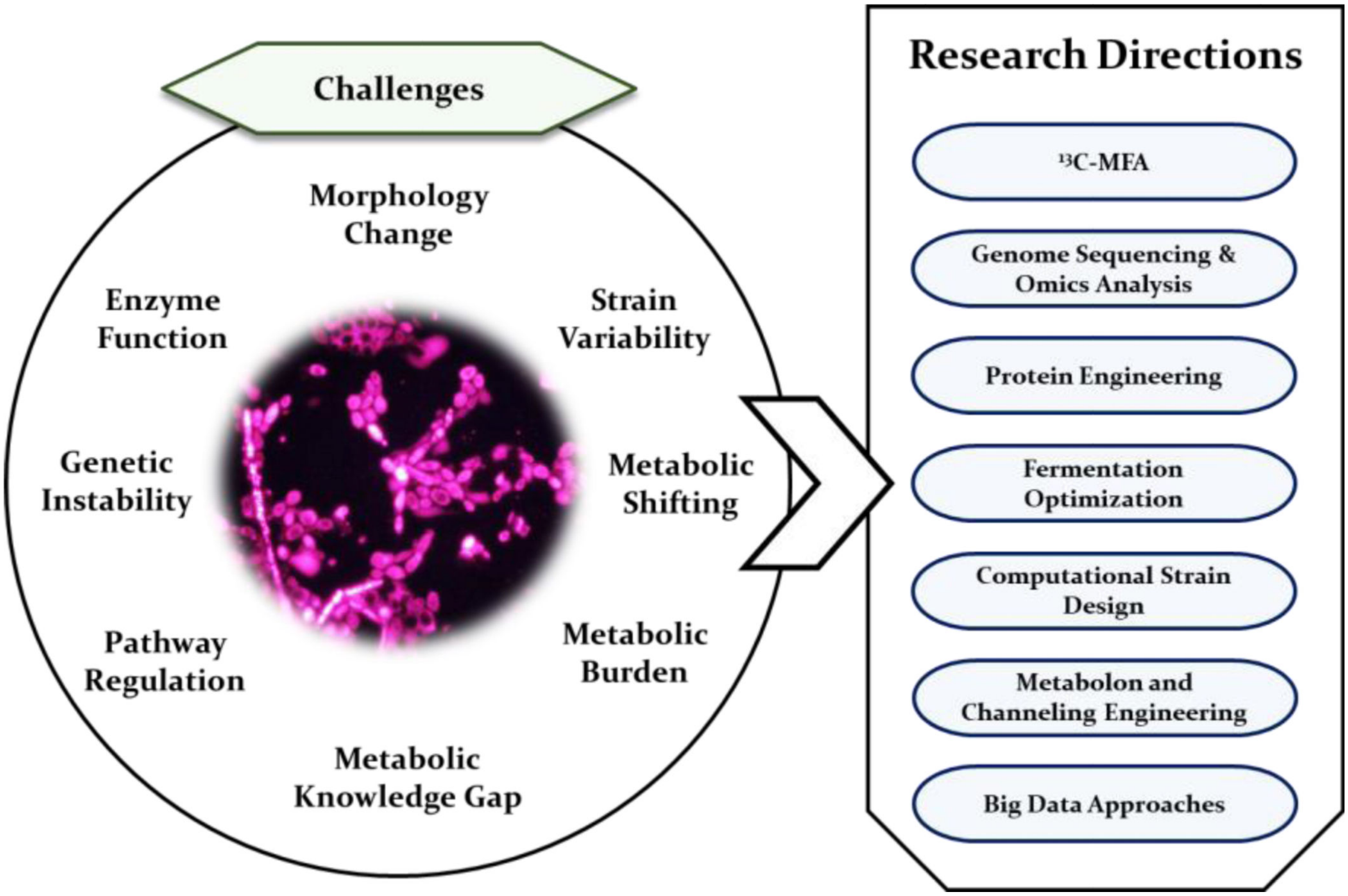

Figure 3:

Challenges and future perspective of terpene production from non-model yeasts.