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PERSPECTIVE

Unraveling the potential of non-conventional yeasts in biotechnology

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One sentence summary: This article highlights the importance of NCY in research and industry and offers some perspectives to accelerate the application of NCY in biotechnology.

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ABSTRACT

Cost-effective microbial conversion processes of renewable feedstock into biofuels and biochemicals are of utmost importance for the establishment of a robust bioeconomy. Conventional baker's yeast *Saccharomyces cerevisiae*, widely employed in biotechnology for decades, lacks many of the desired traits for such bioprocesses like utilization of complex carbon sources or low tolerance towards challenging conditions. Many non-conventional yeasts (NCY) present these capabilities, and they are therefore forecasted to play key roles in future biotechnological production processes. For successful implementation of NCY in biotechnology, several challenges including generation of alternative carbon sources, development of tailored NCY and optimization of the fermentation conditions are crucial for maximizing bioproduct yields and titers. Addressing these challenges requires a multidisciplinary approach that is facilitated through the 'YEAST4BIO' COST action. YEAST4BIO fosters integrative investigations aimed at filling knowledge gaps and excelling research and innovation, which can improve biotechnological conversion processes from renewable resources to mitigate climate change and boost transition towards a circular bioeconomy. In this perspective, the main challenges and research efforts within YEAST4BIO are discussed, highlighting the importance of collaboration and knowledge exchange for progression in this research field.

Keywords: lignocellulosic sugars; volatile fatty acids; non-conventional yeasts; biochemicals; biofuels

INTEREST IN NON-CONVENTIONAL YEASTS

Yeast biotechnology has been applied for decades for production of foods and beverages, biopharmaceuticals, industrial biocatalysts and bioethanol. So far, the bioconversion processes have mainly relied on strains of conventional baker's yeast *Saccharomyces cerevisiae* as cell factories. In next generation production

processes, renewable and low-cost carbon sources including forestry, agricultural and industrial waste- and side-streams are converted to various bioproducts including biofuels, bulk and fine biochemicals and biomaterials (Mattanovich, Sauer and Gasser 2014; Kavšček et al. 2015). Such processes require a range of different cell factories tailored for the specific purposes, and

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S. cerevisiae lacks many of the traits needed for efficient conversion of these alternative substrates to products of interest. For example, wild type *S. cerevisiae* strains utilize primarily hexose sugars such as glucose and do not readily use multiple carbon sources simultaneously. This hampers the yeast's usefulness in fermentations of mixed-sugar substrates such as lignocellulosic hydrolysates, which apart from hexoses contain high concentrations of pentoses (Katahira et al. 2008). *Saccharomyces cerevisiae* is a good producer of alcohols, esters and organic acids but does not naturally accumulate high amounts of intracellular lipids and consume other residual carbon sources like short chain fatty acids, which limits its applications as cell factory (Zhang, Nielsen and Liu 2021). Thus, either extensive *S. cerevisiae* strain development is needed, or, alternatively, the biotech industry needs to turn to other microorganisms that are naturally more suited for intended tasks. In this respect, other yeast species, collectively referred to as non-conventional yeasts (NCY), are progressively gaining more attention as new potential workhorses for biotechnological applications (Navarrete and Martínez 2020).

To date, a few thousand different yeast species have been identified and described to various degrees. Only a small number of these species have been characterized in relatively large detail and are frequently used as cell factories, including but not limited to *Yarrowia lipolytica*, *Komagataella phaffii* (*Pichia pastoris*), *Hansenula/Ogataea polymorpha*, *Kluyveromyces lactis*, *Kluyveromyces marxianus*, *Zygosaccharomyces bailii*, *Rhodotorula toruloides* and *Phaffia rhodozyma* (Radecka et al. 2015; Rebello et al. 2018; Wen et al. 2020; Binati et al. 2021). Besides, a few hundred NCY species have now been genome sequenced and growth phenotyped (Shen et al. 2018), and the results show interesting traits in many of these emerging yeasts. It is projected that the vast majority of the existing yeast species have still not been isolated and identified (Lachance 2006), and thus yeast biodiversity can be considered as a largely untapped resource for biotechnological applications.

NCY is a hugely diverse group of organisms, where the phylogenetic span of just the hemiascomycetous yeasts is as big as the whole phylum of chordates (Dujon et al. 2004). This means that collectively, the NCY's are far more multifaceted than *S. cerevisiae* alone. Many NCY can grow and ferment at wide ranges of process conditions and can utilize, apart from glucose, a large variety of carbon sources present in renewable feedstock such as monosaccharides (xylose, galactose, fructose, arabinose), disaccharides (lactose, cellobiose, xylobiose) and even polysaccharides (xylan and cellulose) (Shen et al. 2018; Ravn et al. 2021). Some NCY species are highly tolerant to bioprocess-induced stresses including low pH, elevated temperatures and high osmolarities as well as inhibitory compounds formed during plant biomass pretreatment (Deparis et al. 2017; Mukherjee et al. 2017; Palma et al. 2017; Navarrete et al. 2021). On the product side, some NCY are superior to *S. cerevisiae* when it comes to accumulation of metabolites and synthesis and secretion of recombinant proteins (Corchero et al. 2013). Additionally, some oleaginous yeasts such as *Rhodotorula glutinis* and *Lipomyces starkeyi* can accumulate lipids up to 60%–70% of their biomass. Thus, the ability of non-conventional oleaginous yeasts to accumulate high quantities of lipids offers the commercial potential for production of lipids or 'single-cell oils', advanced biofuels generation and accumulation of lipid-soluble fine chemicals of high value, such as carotenoids and surfactants (Bharathiraja et al. 2017).

In the last decade, great progress has been made in NCY utilization for biotechnological applications, as well as in proposing solutions to improve their efficiency (Wagner and Alper 2016; Wang et al. 2020). Nonetheless, due to the many potential applications of these microorganisms that remain unexplored, there are numerous challenges that need to be addressed to fully exploit the advantages of NCY in biotechnology. First of all, to be able to use new strains and species in industrial production processes, extensive characterization and testing of the microorganism(s) of interest is needed. Second, when feeding NCY with alternative low-cost substrates, a detailed understanding of the substrate composition and its interaction with the microorganism of choice is required. Third, process development including optimization of the NCY cultivation parameters and modes is usually needed to boost production titers, yields and productivity. Fourth, strain development using adaptive laboratory evolution (ALE) is often essential to improve/enhance the interesting traits. Metabolic engineering was for long restricted to model organisms such as *S. cerevisiae*. Nowadays, it is an emerging technology in NCY research, much thanks to the recent technology revolution in terms of synthetic biology, genome sequencing and CRISPR/Cas9. However, for most NCY, metabolic engineering tools are still lacking or in the process of being developed, and great progress in this field is expected within the coming years (Raschmanová et al. 2018).

YEAST4BIO: DRIVING RESEARCH AND INNOVATION OF NCY-BASED BIOPROCESSES FORWARD THROUGH CROSS-DISCIPLINARY COLLABORATION

To address the many challenges with developing novel NCY cell factories and bioprocesses, collaboration, sharing of knowledge and cross-disciplinary research approaches are highly needed. Lately, this has been greatly facilitated through the 'YEAST4BIO' network funded by COST (European Cooperation in Science and Technology) (<https://yeast4bio.eu/>). The YEAST4BIO COST Action brings together yeast research communities from both industry and academia in Europe and beyond, to foster integrative investigations aimed at filling knowledge gaps and excelling research and innovation in the field. The relevance of YEAST4BIO is clear as there is an urgent need to improve biotechnological conversion processes from renewable resources to mitigate climate change and transition towards a stable and circular bioeconomy.

Meeting the challenges in NCY utilization for bioproduct generation requires a multidisciplinary approach. Therefore, YEAST4BIO has gathered researchers with a combination of skills and experience in biomass fractionation and hydrolysis, yeast cultivation and fermentation and NCY strain characterization and development through the means of molecular biology, genetics and physiology. Besides facilitation of networking, collaboration and knowledge exchange, YEAST4BIO aims to provide its members and in extension the global scientific community with: (i) a compilation of existing scientific knowledge of NCY and applications at the European level and beyond; (ii) standardized experimental techniques and procedures for production of carbon sources from low-cost alternative substrates to be used as platform molecules for NCY; (iii) access to genetic and systems/synthetic biology tools already available to be applied on NCY and (iv) a portfolio of bioproducts to be produced from both

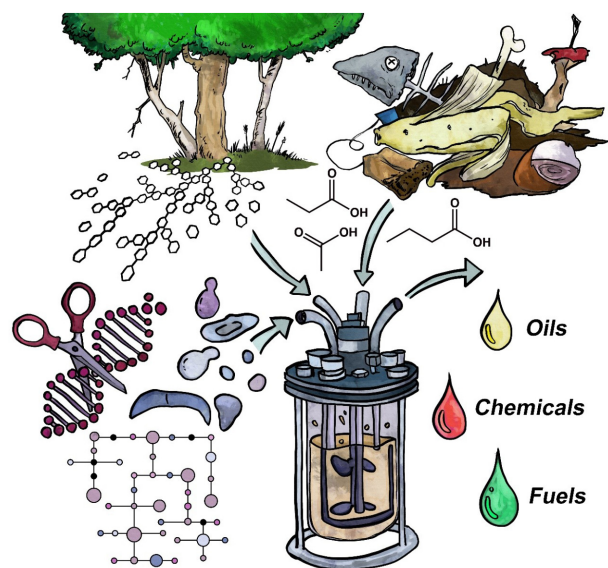


Figure 1. Overview of the main challenges addressed by YEAST4BIO, where renewable and low-cost carbon sources are generated and used as starting materials for generation of bioproducts using NCY, which can be characterized and developed using systems and synthetic biology techniques.

carboxylic and sugar platforms by means of NCYs. Altogether, these deliverables will help facilitating NCY-based conversion of renewable raw materials into biofuels and other important bioproducts in a zero-waste circular economy context.

YEAST4BIO FOCUS AREAS

YEAST4BIO has identified three main challenges of utmost importance for exploring and exploiting the full potential of NCY in biotechnology. These challenges, namely renewable and low-cost substrate generation, efficient NCY-based bioproduct formation and molecular strain characterization and development, are described in more detail below and depicted in Fig. 1.

Renewable and low-cost carbon source generation for NCY

Glucose and sucrose are classical carbon sources for industrial yeast processes, however, the price for these sugars can make up as much as 60%–80% of the overall production cost (Koutinas et al. 2014). Thus, the successful expansion of industrial biotechnology towards a sustainable economy drives research to the utilization of alternative low-cost carbon sources.

With the aim of converting renewable raw materials into fermentable carbon sources for NCY, two main routes can be considered: (i) enzymatic hydrolysis of biomass-contained carbohydrates, and (ii) production of carboxylic acids (i.e. volatile fatty acids, VFAs) from organic residues by anaerobic fermentation. Recent biotechnological advances in biomass deconstruction and anaerobic fermentation have facilitated utilization of both lignocellulosic sugars and carboxylates for the biotechnological production of biofuels and (fine) biochemicals. However, each carbon source generation route faces specific challenges that are assessed and studied within YEAST4BIO.

The Sugar Platform

The production of sugars as platform molecules is one of the most attractive biotechnological ways to valorize organic wastes

or lignocellulosic substrates. To date, there have been several initiatives to optimize the sugar release by means of pretreatment and enzymatic catalysts from biomass. Enzymatic hydrolysis is a relatively new concept and during the last two decades, significant advances have been made in the development of more efficient and cheaper enzymatic catalysts (Cannella and Jørgensen 2014). Notwithstanding, significant progress is still needed to increase performance and productivity of cellulosic enzymes to reduce the costs for production of sugars.

The organic residues are composed of different types of polysaccharides whose enzymatic hydrolysis ultimately releases a set of monosaccharides including glucose, galacturonic acid, arabinose, galactose, rhamnose, xylose and mannose, which can be transformed in bioproducts. For processes applying NCY that can utilize a wide range of sugars, optimization of the release of all kinds of sugars is of utmost importance. Hence, YEAST4BIO-associated researchers aim to boost the profitability of the bioconversion processes by maximizing the sugar production with a minimum requirement of enzyme loading. Furthermore, newly identified and characterized enzymes and recombinant enzymes are currently tested on lignocellulose (Agrawal, Tsang and Chadha 2021), and new enzymatic cocktails enriched in accessory enzymes are being formulated (Singhania et al. 2021).

The Carboxylic Acids Platform

Besides being interesting bioproducts, carboxylic acids can also be used as renewable carbon sources for NCY-based biosynthesis (Llamas et al. 2020b). Given the great heterogeneity of food wastes, agricultural residues or even lignocellulosic feedstock, the anaerobic fermentation is considered a very technologically favorable process for obtaining an assimilable carbon source for the yeasts. Furthermore, the anaerobic fermentation process is almost completely independent of the composition of the substrate. When limiting the anaerobic digestion to the first three stages to avoid the production of biogas, the organic matter contained in the waste results in a mixture of VFAs (Magdalena and González-Fernández 2019). The VFAs can have different chain lengths (between C2 and C6), and their prevalence depends on the composition of the substrate and on the conditions of the anaerobic reactor (pH, temperature, organic load, hydraulic time of residence etc.) (Magdalena, Greses and González-Fernández 2019; Llamas et al. 2021). VFA production is nowadays considered as a novel technology for the development of future biotechnology industries based on these alternative carbon sources.

To date, research efforts addressed towards the implementation of a stable carboxylate platform are scarce compared to the numerous studies on the sugar platform. Thus, YEAST4BIO research activities aim to advance the understanding on how feedstock composition may affect carbon source output and VFA profiles for robust anaerobic fermentation technologies.

Bioproduct Formation using NCY

Numerous NCY are more efficient than *S. cerevisiae* in converting carbon sources from alternative feedstock such as lignocellulosic wastes and organic residues into bioproducts. These microorganisms will surely play a key role in production of bio-based chemicals from both sugar and carboxylate platforms in the near future.

Bioproducts using NCY and the sugar platform

The most typical bioproduct associated with yeast is ethanol. In contrast to wild type *S. cerevisiae* that most readily ferments hexose sugars to ethanol, multiple NCY including *Scheffersomyces*

stipitis and *Spathaspora passalidarum* can also produce ethanol from xylose, the second to glucose most abundant carbon source in plant biomass (Veras, Parachin and Almeida 2017). Other NCY bioproducts from the sugar platform include lipids, 2-phenylethanol, flavin coenzyme, weak acids, industrially relevant enzymes, bioplastics, etc. Some relatively well characterized yeasts that are used as production hosts include *K. marxianus* for production bioethanol, aroma compounds and biosurfactants (Karim, Gerliani and Aider 2020), *K. phaffii* (*Pichia pastoris*) for recombinant protein and enzyme production (Heisting, Gasser and Mattanovich 2020) and *Y. lipolytica* for production of biosurfactants, carotenoids and lipids (Ledesma-Amaro and Nicaud 2016). A number of less well-characterized yeasts are also emerging as potential cell factories, including, *Z. bailii* for organic acids and both *Candida intermedia* and *Candida boidinii* for sugar alcohols (Bedř et al. 2021; Kuanyshv et al. 2021). However, as most NCY still await identification and/or detailed characterization, the scientific community and biotechnological industries are most likely only beginning to realize the vast range of other bioproducts that NCY can generate through the use of the sugar platform.

With the goal of developing economically-viable production processes, large research efforts within the YEAST4BIO community are directed to push product titers, yield and productivity toward theoretic limits. To reach this goal, more knowledge on NCY metabolism, genetics and physiology is essential. There is also a need to carefully tailor the cultivation parameters, growth medium composition and yeast species and strain of choice for each specific raw material and targeted bioproduct. The unique nature of each biotechnology process adds to the complexity and difficulty of scale-up, which is also being addressed within YEAST4BIO.

Bioproducts using NCY and the carboxylic acids platform

Some NCY such as *Y. lipolytica*, *Cutaneotrichosporon oleaginosus* (previously known as *Trichosporon oleaginosus*, *Cryptococcus curvatus*, *Apiotrichum curvatum* or *Candida curvata*) *R. toruloides*, *Lipomyces lipofer* and *Williopsis saturnus* have demonstrated their ability to utilize VFAs as platform molecules for production of biochemicals (Llamas et al. 2020a). However, high concentrations of VFAs can exert inhibitory effects on yeast affecting final product titers and yields (Ma et al. 2019; Llamas, Tomás-Pejoj and González-Fernández 2020). The maximum concentrations of the different VFAs that can be used without compromising process yields remain to be elucidated. Thus, exploiting the intrinsic tolerance to acid stress present in some NCY species is of utmost importance when using these carbon sources. Remarkably, the metabolic pathways involved in the conversion of VFAs to targeted products by NCY are still unknown or only partially elucidated, and many research efforts are directed to unravel the metabolism behind VFAs utilization in NCY (Park et al. 2021).

For an efficient implementation of the carboxylate platform in NCY-based industrial processes, it is crucial to determine the capacity of NCY to convert these acids into products of interest. In this sense, YEAST4BIO aims to shed light both on the NCY metabolic pathways involved in VFAs utilization and the process conditions favoring VFAs conversion into targeted products.

NCY potential and application of systems/synthetic biology techniques

The implementation of novel system and synthetic biology tools for NCY is highly required to facilitate the utilization of

these microorganisms in biotechnological applications. However, there are still some challenges that should be addressed to make the application of these new techniques for NCY a reality. The limited -omics (genomics, proteomics, transcriptomics and metabolomics) studies so far on NCY and the lack of knowledge on their wide variety of metabolic pathways and production capacity are among the main challenges to progress in the application of NCY. Systems biology approaches, using proteomic, transcriptomic, metabolomic, and fluxomic analyses can be potentially applied to NCY for the identification and characterization of DNA parts as well as for understanding the metabolic networks and regulation. Furthermore, -omic information such as genomics and transcriptomics can be used to create reliable genome-scale metabolic models, which can guide strain design strategies. These genome scale metabolic models can also integrate further -omic results such as proteomics, metabolomics and fluxomics to more reliably simulate metabolic fluxes in silico. Some NCY have curated genome-scale metabolic models that are available to be used. Examples of these are *Y. lipolytica* (Kerkhoven et al. 2016), *K. phaffii* (Cankorur-Cetinkaya, Dikicioglu and Oliver 2017), *O. polymorpha* (Liebal et al. 2021), *K. lactis* (Dias et al. 2014), *K. marxianus* (Marcisauskas, Ji and Nielsen 2019), *R. toruloides* (Dinh et al. 2019; Tiukova et al. 2019).

In addition, identified and characterized DNA parts such as promoters and terminators can be used to generate novel synthetic biology tools tailored for each NCY. Some of these tools being developed include Golden Gate libraries of parts and CRISPR technologies for genome engineering and transcriptional regulation. Developing novel NCY strains requires a multidisciplinary approach involving metabolic engineering for new pathways and enzymes to expand the substrate usage by the microorganism; engineering microorganisms for better stress response to industrial conditions and for higher productivity, as well as biochemical engineering to apply these organisms under optimal process configurations. Different NCY have different degrees of development regarding systems and synthetic biology tools, being some of the most advanced *Y. lipolytica* (Larroude et al. 2018), *K. marxianus* (Rajkumar and Morrissey 2020), *K. phaffii* (Gao, Jiang and Lian 2021) and *D. hansenii* (Strucko et al. 2021). In any case, the developments of these tools and their efficiency are still behind those of *S. cerevisiae*, and there is still much that need to be done to domesticate these organisms. YEAST4BIO will explore these limitations and will bring together researchers working with different NCY to address current challenges by sharing learnings and brainstorming.

YEAST4BIO THEMATIC ISSUE COVERING DIFFERENT ASPECTS OF NCY IN BIOTECHNOLOGY

Some of the challenges mentioned in the previous sections are covered in the different review articles included on this Thematic Issue. These review articles show both the breadth and the depth of research within the Action and gather different expertise.

In this Thematic Issue, different systems and synthetic biology tools and their application for NCY are reviewed. Godinho and co-authors (2021) present N.C.Yeasttract and CommunityYeasttract databases to study gene and genomic transcription regulation in non-conventional yeasts. Binati and colleagues (2021) discuss the applications of NCY in food and food additive production. Ergün and collaborators (2021) describe the interplay between metabolic model and transcriptional control of *K.*

phaffii and their use in the production of proteins. In addition, Ata and colleagues (2021) review the features of *K. phaffii* that makes it non-conventional and discuss its potential in industrial applications. In another work, Solieri and collaborators (2021) digest and discuss the current knowledge about life cycle and cell identity regulatory circuits with special focus on the genetic improvement of NCY. Park and co-workers (2021) offer their perspective on the bioproducts generated by NCY using carboxylic acids as substrates with *Y. lipolytica* while Karaalioglu and Yuceer (2021) review how NCY can produce volatile compounds. Altogether, these collaborative works exemplify the importance of NCY in research and industry, identify current challenges and future perspectives and clearly imply one step forward towards the application of NCY in biotechnology.

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