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Mini review

Non-conventional yeasts as superior production platforms for sustainable fermentation based bio-manufacturing processes

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Abstract: Non-conventional yeasts are an excellent option for a number of different industrial bioprocesses. They possess beneficial natural phenotypes, which translates to several fermentation advantages when compared to traditional hosts, like *Saccharomyces cerevisiae*. The non-conventional yeasts *Yarrowia lipolytica*, *Trichosporon oleaginosus*, *Kluyveromyces marxianus*, *Dekkera bruxellensis*, *Pichia kudriavzevii*, *Debaryomyces hansenii* and *Hansenula polymorpha*, are considered desirable industrial hosts due to their natural characteristics, including tolerance to several by-products and inhibitors, thermotolerance, salt resistance or osmo- and xerotolerance. Therefore, they are a great alternative for the industrial production of bioethanol, fine chemicals, lipids and recombinant proteins, among others. In this review, we summarize the best natural characteristics of those seven non-conventional yeasts and their use in industrial biotechnology, as well as the molecular/synthetic biology tools available for their genetic modification. Moreover, possible limitations regarding their performance in industrial fermentations and a list of challenges to overcome in the future are also discussed.

Keywords: non-conventional yeast; production platforms, fermentation; *Yarrowia lipolytica*; *Trichosporon oleaginosus*; *Kluyveromyces marxianus*; *Dekkera bruxellensis*; *Pichia kudriavzevii*; *Debaryomyces hansenii*; *Hansenula polymorpha*

1. Introduction

Industrial biotechnology represents more than hundred billion market in the US, and is the fastest growing sector in the last decade [1]. Microbial chemical production has been covered by a

reduced number of model organisms, such as *Escherichia coli*, *Aspergillus* genus or *Pichia pastoris*, but *Saccharomyces cerevisiae* is one of the most widely utilized. This is partly because *S. cerevisiae* is classified as Generally Regarded as Safe (GRAS) organism, but also because it grows very well in environmental conditions normally associated to standard biotechnological processes, e.g. glucose fermentation during ethanol production [2].

General examples of industrially produced chemicals by *S. cerevisiae* are bioethanol or a number of recombinant proteins, like human insulin [2,3]. *Aspergillus* is well known for enzymes and citric acid production [4,5], and *E. coli* for vitamins and fine chemicals manufacture [6,7]. On the other hand, there are still important challenges regarding the production processes, sometimes due to limitations in the host organism used. Examples of this are *S. cerevisiae* and *E. coli*, which cannot tolerate certain environmental stresses. They normally have a better growth in moderate temperatures, and require a substantial microbial engineering effort in order to grow relatively well in such stressful conditions for them (e.g. to increase the ability to utilize carbon sources different to glucose or become thermotolerant) [8,9]. Similarly, many problems are associated to the filamentous growth of *Aspergillus* in bioreactors or industrial tanks, making the process more fastidious and expensive. Furthermore, expensive feedstocks, high energy and water use, loss of productivity due to contamination and downstream separation costs can be added to the list of challenges to be addressed [10].

The use of non-conventional microorganisms (the term "non-conventional" referring to any other microbe different from the classical aforementioned production workhorses) could easily overcome some of the challenges already mentioned. In fact, some of those microbes are already known for presenting a significant advantage compared to the model organisms. Some non-conventional microbes are able to withstand extreme environmental conditions, such as high temperature and osmotic pressure, while others show tolerance to inhibitors produced during different bioprocesses [10,11]. Moreover, an increased availability of molecular tools and synthetic biology methods is being lately offered for their use in non-model organisms (Figure 1).



Figure 1. A comparative overview of the strategies from lab-scale to industrial scale of production hosts, and the dedicated engineering efforts in each case, as well as the expected outcome, based on the initial microbial choice. ALE: adaptive laboratory evolution.

In this mini-review, we offer a fast and precise overview about the topic, which is already considered of great significance but will become more and more important in the coming years. We focus on yeasts as one of the most studied microbial group in nature and their consideration as a model for eukaryotic cell research. Therefore, in this manuscript, we present some of the most relevant non-conventional yeasts and their use as production platforms for sustainable fermentation based bio-manufacturing processes. *Yarrowia lipolytica*, *Trichosporon oleaginosus*, *Kluyveromyces marxianus*, *Dekkera bruxellensis*, *Pichia kudriavzevii*, *Debaryomyces hansenii* and *Hansenula polymorpha*, are reviewed for their use in biotechnology and/or their improved industrial potential for the near future.

2. Genetic engineering methods for non-conventional yeasts

Genetic and metabolic engineering is commonly more challenging in non-conventional yeasts than in other model organisms, especially due to a more limited knowledge about their metabolism and genomics. Even though non-conventional yeasts already present interesting native phenotypes for industrial production purposes, some metabolic engineering will be needed at some point to enhance those phenotypes and increase e.g. yields or production rates [12].

Gene expression methods are basically based on expression cassettes which can be expressed through episomal vectors or by integration in the host's genome. Some limitations are associated to gene expression through episomal vectors, e.g. lack of stable plasmids, low gene copy number or variable expression in the host's cells [13,14]. On the other hand, homogenous expression levels, increased stability of the expression cassette or lack of need for selection markers, are advantages while using genome integration methods. These methods can be divided into two different types, and are performed by the native DNA repair pathways in the host organism [15]:

- 1) Random integration via non-homologous end joining (NHEJ)
- 2) Targeted integration via homologous recombination (HR)

Although random integration via NHEJ is the preferred method to be used in non-conventional yeasts (HR is considered inefficient in this case), it can cause unwanted disruptions in genomic key elements or lead to variable expression levels across transformant cells [16,17]. For that reason, it was of especial importance the development of clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated 9 (CRISPR-Cas9) tools, for genome editing in non-conventional yeasts [18]. The use of these CRISPR tools allow to achieve a more efficient HR by the introduction of a genomic double strand break and a programmable endonuclease in the presence of a homologous repair template [12,19].

3. Non-conventional yeast platforms with important industrial applications

Yeast is one of the most studied microbial group in nature, as it has been associated e.g. with the production of fermented beverages and food for human consumption over thousands of years. They have provided an extensive scientific knowledge on basic and applied microbiology, and became model organisms for eukaryotic cell research [20]. A broad number of yeast genomes have been sequenced and uncountable molecular biology tools were described and are available for their genetic modification (some of which are reviewed along this manuscript). Besides, non-conventional yeasts are of great interest due to their unique characteristics and metabolism, which make them

suitable for different biotechnological processes at an industrial level (Table 1), in comparison with traditional model organisms.

Non-conventional yeast	Valuable phenotype	Product	Recombinant protein	Genetic tools	CRISPR system
Y. lipolytica	Oleaginous yeast, growth on organic acids, polyalcohols and paraffins [21,23]	Lipids, citric acid, erythritol, α-ketoglutaric acid, lycopene, Omega-3 eicosapentaenoic acid [116,117,118,119,120, 121,122]	Selenomethionyl lipase, erythritol dehydrogenase [24,25]	Highly developed	TEFintron promoter codon-optimized [31], UAS1B8-TEF promoter codon-optimized [32]
T. oleaginosus	Oleaginous yeast, metabolize recalcitrant feedstocks, tolerate by-products of lignocellulose pretreatments [33,36,37,38]	Lipids, modified fatty acids [36,37,39,40]	-	Need for improvement	_
K. marxianus	Thermotolerance, fast growth characteristics, utilization of sugar cane or molasses, growth on a range of sugars [44,45,46]	HTF-ethanol, 2-phenylethanol/2-phen yl ethyl acetate, hexanoic acid [46,51,123]	Inulinase, β-galactosidase, pectidases [47,48,124,125]	Highly developed	ScTEF1 promoter codon-optimized [61]
D. bruxellensis	Tolerance to ethanol and acetic acid, assimilation of nitrate, fermentation of cellobiose [63,64,66,70]	Ethanol [61]	-	Need for improvement	_

 Table 1. Overview of non-conventional yeast species, their industrially-relevant phenotypes/products and genetic tools availability.

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Non-conventional yeast	Valuable phenotype	Product	Recombinant protein	Genetic tools	CRISPR system
P. kudriavzevii	Thermotolerance, growth at low pH, growth on complex substrates, acetic acid, vanillin and furan derivative tolerance	Ethanol, succinic acid [67,74,75]	β-glucosidase [78]	Need for improvement	_
D. hansenii	[75,77,78,45,80,79] Halophilic and oleaginous yeast, osmotolerance, xerotolerance, resistance to inhibitory compounds, consumption of broad range of substrates [33,84,90,91]	Xylitol, trehalose, flavonoids, fatty acids, killer toxins, essential fat-soluble vitamins [84,90]	_	Need for improvement	_
H. polymorpha	Methylotrophic yeast, nitrate assimilation, thermotolerance [97,104,99]	Ethanol [101,102]	Hepatitis B surface antigen, insulin, hexose oxidase, phytase [108,109,107,106]	Highly developed	DH3 promoter human codon-optimized [115]

3.1. Yarrowia lipolytica

Y. lipolytica is an oleaginous non-conventional yeast and very attractive from the industrial point of view, due to its uncommon physiological characteristics [21]. It is classified as GRAS, obligate aerobe microbe and it can switch between hyphal and yeast morphology depending on the environmental conditions [22]. Moreover, it shows high secretory rates, low glycosylation rates and high cell densities during fermentation [21]. Especially interesting is its capacity to metabolize organic acids, polyalcohols and paraffins as sole carbon source [23]. Also biomass-derived sugars and industrial wastes, which makes the bioprocesses more cost effective and highly productive [11]. *Y. lipolytica* is actually a better candidate for recombinant protein expression compared to *S. cerevisiae* or *Kluyveromyces lactis*, for production of selenomethionyl lipase and erythritol dehydrogenase [24,25]. The best productivity level is obtained when *Y. lipolytica* is cultivated at 28–30 °C, pH 5.5–7.0 and dissolved oxygen levels at 20% of air saturation [22,26].

The genome of *Y. lipolytica* was sequenced and is available for researchers [27]. There are some examples of engineering methods already published for this yeast, being the ectopic integration of homology cassettes the preferred one [28–30]. Besides, CRISPR-Cas9 system has been successfully used [31,32]. On the other hand, homologous recombination is still a weakness that needs to be improved.

3.2. Trichosporon oleaginosus

T. oleaginosus is an oleaginous yeast with a lipid accumulation capacity between 20–60% of its biomass [33]. The optimal growth conditions for *T. oleaginosus* are within the range of 28–30 °C and pH 5.4–5.8, and it is able to utilize a broad range of carbon and nitrogen sources, being glucose the preferred substrate [34,35]. Moreover, it can metabolize a wide variety of recalcitrant feedstocks [33] and tolerate several by-products of lignocellulose pretreatments, like acetic acid, furfural and ammonia [36–38]. Due to its natural ability for lipid accumulation, *T. oleaginosus* has been mainly studied for its lipid production capability under different dissolved oxygen concentration or diverse substrates utilization [36,37,39,40]. Furthermore, its ability to tolerate and metabolize lignin-derived aromatic compounds, while remaining oleaginous, has been well described [41].

There are a couple of studies about genetic modification in *T. oleaginosus*, which was also successfully transformed by genomic integration for the production of modified fatty acids, although transformation efficiencies are not reported [42,43]. Nevertheless, an increased knowledge and development on molecular biology tools and genetic engineering are still needed for this yeast.

3.3. Kluyveromyces marxianus

K. marxianus is a hemiascomycetous non-conventional yeast, isolated mostly from cheese and other dairy products and with a respirofermentative metabolism (Crabtree negative yeast). It is considered an extreme thermotolerant microbe that can grow in temperatures up to 52 °C. Moreover, it shows acid tolerance (pH 3.8). *K. marxianus* is an interesting option for industrial bioprocesses, also because it presents high growth rates and it is able to utilize a number of industrially relevant substrates, such as sugar cane or molasses [44,45]. Those characteristics make *K. marxianus* a very good candidate for its use in biotechnology, presenting some advantages when compared to *S. cerevisiae*. For instance, it utilizes a broad range of different carbon sources in contrast to the model yeast: xylose, xylitol, cellobiose, lactose and arabinose. Additionally, it shows similar ethanol yield and glucose consumption as *S. cerevisiae* at 30 °C, although *K. marxianus* can also achieve glucose fermentation between 30–45 °C [46].

Currently, *K. marxianus* is used in several industrial bioprocesses, e.g. to produce recombinant proteins and enzymes (inulinase and β -galactosidase) [47,48], to perform high-temperature fermentation of ethanol [49] or, due to its GRAS classification, to produce food-related compounds like aroma compounds and bioingredients [50,51].

As its genome was completely sequenced in 2012 [52], there are several tools already described for *K. marxianus*' genetic manipulation including transformation with linear DNA and simultaneous multiple integration [46,53]. Besides, genomics and transcriptomics analysis have been performed for this yeast [54–56], as well as genome-scale metabolic models [57–59]. The CRISPR system has been successfully applied in *K. marxianus* and used to characterize e.g. functional genes in biosynthesis pathway of ethyl acetate [60–62].

3.4. Dekkera bruxellensis

D. bruxellensis is a non-conventional yeast with an extremely complex genome, which cannot be defined as haploid or diploid, due to its genetic polymorphism. Similarly to S. cerevisiae, it has

been isolated from beer, wine (spoilage yeast) and cider, and classified as facultative anaerobic and Crabtree positive [45]. *D. bruxellensis* is able to produce and accumulate ethanol, being one of its main characteristic its tolerance to ethanol (10–16%) [63]. Similar ethanol yields to *S. cerevisiae* are obtained during glucose fermentation, although *D. bruxellensis* presents lower growth rates compared to the model yeast [64]. Positively, it can grow in acidic environments [65], produces low amount of glycerol [64], which leads to a more energy efficient process, and assimilates nitrate during industrial fermentation [66]. The yeast also shows a complex pattern of substrate consumption and metabolite production [67], and it is tolerant and able to accumulate acetic acid [64]. On the other hand, *D. bruxellensis* is thermosensitive already at 35 °C, being 30 °C its optimal growth temperature [68,69].

Its biotechnological potential mainly resides in its capability of fermenting cellobiose, which makes this yeast a good candidate for fermentation of lignocellulose to ethanol processes, and due to its utilization of nitrate as sole nitrogen source, for ethanol processes based on sugar cane [70].

As we mentioned before, D. bruxellensis has an important genome complexity that makes difficult its genetic modification even though its genome is fully sequenced [71,72]. Molecular tools are underdeveloped for this yeast and just one transformation method has been described so far, based non-homologous DNA integration, and with an efficiency on of only 0.6–20 transformants/µg [73]. Together with basic identification/manipulation molecular tools, transcriptomic analysis has been more recently carried out in D. bruxellensis in glucose and oxygen-limited cultures [74].

3.5. Pichia kudriavzevii

P. kudriavzevii has been isolated from several niches, including sourdough, cocoa bean fermentation, cereal-based beverages, sugar cane juice or rice straw. That gives an idea of its great ability to grow on complex substrates [45]. Carbon sources assimilated by *P. kudriavzevii* are glucose, sucrose, fructose, mannose and weakly galactose, but it is not able to utilize maltose, xylose, arabinose, cellobiose, raffinose and trehalose [75]. The yeast is classified as Crabtree negative [76], thermotolerant [75] and able to grow at low pH (lower than pH 2) [77,78].

P. kudriavzevii shows furan derivative tolerance, which makes this yeast very interesting in industrial bioprocesses. It tolerates up to 3 g/L of furfural, 5 g/L of 5-HMF, 8–10 g/L of acetic acid, 2 g/L of formic acid and 1.8–2 g/L of vanillin [75,79,80]. It is more efficient than *S. cerevisiae* in ethanol production at temperatures higher than 35 $^{\circ}$ C and can ferment at up to 45 $^{\circ}$ C. The same study also identified the salt and sugar tolerance of *P. kudriavzevii*, which can tolerate 5% (w/v) of NaCl (0.85 M) and 40% (w/v) of glucose [75].

The genome of *P. kudriavzevii* is also sequenced [81], but the molecular tools available for its genetic manipulation are still very limited. A first attempt to engineering the yeast was performed in 2010, in which study the authors successfully developed a β -glucosidase expression system for conversion of cellobiose to ethanol [78]. Besides, a metabolic engineered strain was generated for industrial scale production of succinic acid. While *P. kudriavzevii* is not a natural producer, its engineered version was able to produce and tolerate succinic acid [82,83].

3.6. Debaryomyces hansenii

D. hansenii is a halophilic, xerotolerant and oleaginous non-conventional yeast that was originally isolated from seawater and commonly found in high osmotic and saline environments, such as cured meats and cheeses. This yeast is able to grow in a broad range of temperatures (20–35 °C) and pHs (3–10), and up to 25% of NaCl (4M). Besides, it respires a broad number of carbon sources [33,84]. Several studies have demonstrated that sodium protects *D. hansenii* against oxidative stress and other abiotic stresses [85–87]. Moreover, glycerol is produced and accumulated as a compatible solute under osmotic pressure [88,89].

D. hansenii is used in biotechnology for production of fine chemicals, such as xylitol and flavonoids, killer toxins and others [84,90]. Furthermore, this yeast shows resistance to a variety of inhibitory compounds including chlorine dioxide, penconazole, benomyl, and cycloheximide [90,91]. Apart from its great biotechnological potential, which mostly resides in its improved performance under very harsh conditions (high salinity, osmotic pressure, media acidification or nutrient scarcity), the use of pure water sources will not be necessary when industrially growing *D. hansenii*. That could lead to a decrease in production costs whilst the production yields are increased. Furthermore, when growing in high salt concentration media, the risk of contamination is also highly reduced.

The only limitation when working with *D. hansenii*, whose genome was sequenced in 2004 [27], is the lack of molecular tools for engineering this yeast. Although some methods for transformation and heterologous gene expression have been published, there is still low transformation efficiency and low reproducibility between labs [92–96].

3.7. Hansenula polymorpha

H. polymorpha is a thermotolerant methylotrophic yeast used for production of traditional fermented wine in Asia [97]. It optimally grows at 37 °C, although it has been described as able to grow up to 48–50 °C [98,99]. *H. polymorpha* is capable to metabolize a range of different carbon sources such as glycerol, C_5 and C_6 sugar monomers and C_{12} disaccharides [100]. Moreover, it ferments glucose, xylose, cellobiose and other lignocellulose sugars to ethanol, at high temperature [101]. The yeast also shows resistance to several growth inhibitors and can utilize lignocellulosic as crude substrate streams [102].

H. polymorpha was first studied as model organism for peroxisome function and biosynthesis, as well as nitrate metabolism and assimilation [103,104]. Its ability to grow on methanol promotes peroxisome proliferation, which was used for penicillin production, partially located in this organelle [105]. From an industrial point of view, *H. polymorpha* is considered a powerful production platform for heterologous protein biosynthesis, based on a strong inducible expression system coupled with effective protein secretion and glycosylation. Actually, it is less prone to toxic hyperglycosylation (compared to *S. cerevisiae*), as it does not produce alpha-1,3-linked residues which triggers immunogenicity in humans [106]. Biopharmaceutical examples, industrially produced by *H. polymorpha*, are insulin [106,107] and proteins for hepatitis B vaccine [108,109].

The *H. polymorpha* genome was sequenced in 2003 [110]. Several engineering tools have been described for this yeast, being random integration and homologous recombination into telomeric regions the most successful ones. On the other hand, non-homologous end-joining mechanism makes it hard to disrupt genes in this yeast [12]. Expression cassettes used in *H. polymorpha* are commonly

constructed using inducible methanol oxidase promoter and terminator (pMOX, MOXt) [111,112], although other endogenous promoters has also been used [113]. *H. polymorpha* plasmids containing autonomously replicating sequence (ARS) elements have been isolated and demonstrated to have highly transformation efficiency and episomal replication [114]. CRISPR-Cas9 system has also been successfully used in *H. polymorpha*, obtaining gene disruption rates up to 71% [115].

4. Current challenges and future perspectives

One of the biggest challenges in industrial biotechnology is coping with the host limitations normally found for a specific production process. Dealing with high osmotic stress, high salt or sugar concentrations, high temperature processes or product inhibition are the most common problems found among the sector [45]. As we mentioned before, model organisms such as *S. cerevisiae* or *E. coli* are very well-studied, both physiologically and genetically, and have been highly genetically modified for an improved performance under certain stress conditions. Still, they have their limitations regarding metabolic engineering to mitigate some of those detrimental effects. In that sense, non-conventional organisms (yeast) represent a better natural choice to overcome the problems related to stress tolerance during industrial bioprocesses.

In this review, we have presented some of the best examples of non-conventional yeasts with a strong potential in biotechnology, and able to naturally deal with the biggest problems mentioned above. E.g. *T. oleaginosus* tolerates several by-products like acetic acid, furfural and ammonia, *K. marxianus* is thermotolerant, *D. bruxellensis* tolerates up to 16% of ethanol, *P. kudriavzevii* shows furan derivative and vanillin tolerance, *D. hansenii* is a halophilic yeast, and *H. polymorpha* is a methylotrophic yeast. Besides, their genomes are sequenced and several genetic tools are already available to the scientific community. Nevertheless, there is still room for improvement when industrially using these microbes or any other non-conventional yeast in general, especially related to the development of new molecular biology methods and the investigation of the molecular mechanisms underlying their tolerance, which can lead to their maximum fermentative capacity and/or best optimal behavior.

In this respect, some of the biggest challenges that need to be addressed in the future are the following:

1) Development, and improvement, of new genetic tools for non-conventional microorganisms.

2) Investigation of the molecular mechanisms underlying their natural tolerance.

3) Implementation of CRISPR-Cas systems for highly efficient genome editing, which allows getting the most desired phenotypes for industrial bioprocesses.

4) Improvement of random integration engineering of non-conventional yeast for stable genetic modifications by disrupting/repressing competing DNA repair through non-homologous end-joining (NHEJ).

5) Design of Genome Scale Models (GSMs) based on experimental data-sets relevant to metabolic engineering and synthetic biology, and specifically developed during industrial performances.

6) Development of metabolic models based on genomic and transcriptomic information, which allows designing genomes for improved productivity.

5. Concluding remarks

Industrial biotechnology is a field in exponential expansion, and fermentation-based manufacturing of bio-based chemicals has attracted a considerable attention within the last decade. Given the current global situation, there is an increasing need of novel green technologies to mitigate CO_2 emissions, exploring sustainable ways of production that favor a competitive shift from oil-based technologies towards biosustainable alternatives. The concept of circular economy and the waste revalorization into high-value products strategy, demand better large-scale production strategies. A new generation of microbial production hosts may tackle the challenge and replace the classical production workhorses, as they lack of the capacity of withstanding large-scale production setups and require substantial engineering efforts.

In this review we suggest a series of alternative yeast hosts, that could serve as a better starting point for bio-based production processes of chemicals or other molecules of interest (industrial enzymes, peptide based biotherapeutics, food ingredients, etc). Because of i) their inherent and evolutionary acquired higher tolerance in hostile environments, ii) their higher tolerance to fermentation inhibitors normally produced as by-products from biomass hydrolysates, or iii) their ability to consume a wide range of different carbon sources, they certainly show in all aspects greater advantages than the current available options.

With the recent advances in microbial engineering technologies, such as the implementation of CRISPR-based technologies, and the fast (and cheaper than ever) capacity of genome sequencing and annotation of novel microbial isolates, it has become easier to engineer these microbes for increasing production yields instead of modifying the classical hosts to increase their tolerance. Overall, using one of these non-conventional microbes from the start will significantly decrease the initial engineering efforts, therefore speeding up the initial design and test process and the transition from lab to industrial scale of novel production hosts.

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Conflict of interest

The authors declare no conflict of interest.

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