Yarrowia lipolytica

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After having drawn some industrialists' attention as early as the 1950s, the non-conventional oleaginous yeast *Yarrowia lipolytica* has been recognized since several decades, as a powerful host for heterologous protein expression, secretion and surface display. The development of sequencing and genetic engineering tools, combined with an increasing knowledge of its metabolism, have then facilitated the complex engineering of the metabolic pathways of this yeast for various applications. Since nearly two decades, numerous laboratories throughout the world have chosen *Y. lipolytica* as a chassis for designing microbial cell factories. White biotechnology applications of this yeast include notably single cell oil production, whole cell bioconversion and upgrading of industrial wastes.

Keywords: white biotechnology ; metabolic engineering ; non-conventional yeast ; oleaginous yeast ; cell factory ; heterologous expression ; biodiversity ; clade ; GMO

1. Introduction

A major challenge for our societies is to replace polluting technologies, based on fossil fuels, with clean ones, based on renewable resources. White biotechnology, using microorganisms and their enzymes to manufacture compounds of industrial interest (chemicals, biomaterials, biofuels, pharmaceuticals, feed, food), has an important role to play in this transition. This rapidly developing field aims to design industrial processes more environmentally friendly and making use of agricultural, forest and industrial waste or by-products. Among the microorganisms amenable for such industrial applications, yeasts cells present the cumulated advantages of high growth capacity, easy genetic manipulation and presence of a eukaryotic organisation allowing posttranslational processing, vesicular secretion and subcellular compartmentalization. Among non-conventional yeasts of industrial interest, the dimorphic oleaginous yeast *Yarrowia lipolytica* appears as one of the most attractive for a large range of white biotechnology applications, from heterologous proteins secretion to cell factories process development.

2. Main Characteristics

2.1. Natural Habitats

Y. lipolytica is a Crabtree-negative ascomycete yeast (class: Saccharomycetes, order: Saccharomycetales) that has been at first noticed for its remarkable lipolytic and proteolytic capacities. In accordance to these high levels of secreted enzymatic activities, wild-type isolates of this yeast generally originate from lipid-rich and/or protein-rich environments, notably from meat and dairy products (especially fermented ones, such as dry sausages and cheeses) and from sewage or oil-polluted waters [1][2]. In the last decades, the range of ecosystems from which *Y. lipolytica* strains has been isolated has broadened to encompass very diverse habitats, from marine waters, salt marshes and soils (especially oil-polluted ones) to a variety of consumable products (including fruits, vegetables or seafood) and even the excreta of insects or vertebrates that consume them [1][3][4][5][6][7]. This species thus appears to exhibit a rather ubiquitous distribution, in the natural world as well as in man-made extreme environments. The ecological significance of *Y. lipolytica* has been reviewed very recently, establishing this yeast as an eco-friendly organism able to develop symbiosis with some insects (beetles) and plants (microbial endophyte, mycorhizes) [7].

2.2. Safety

Since only a decade, *Y. lipolytica* is also considered as belonging to the normal human mycobiota, being found notably in the mouth and respiratory tract of adults, especially of diabetic people [8]. This yeast is also sometimes seen as a possible opportunistic emerging pathogen, since its biofilm formation capacity can be responsible of rare cases of catheter-related candidaemia [1][8]. Despite this, *Y. lipolytica* is classified as a Biosafety Level (BSL) 1 microorganism by the Public Health Service (Washington, DC, USA). It is also recognized as a "microorganism with a documented use in food" by the International Dairy Federation (IDF) and the European Food and Feed Cultures Association (EFFCA), and as a

“recommended biological agent for production purposes” by the European Food Safety Authority. This yeast has also gained a GRAS (generally recognized as safe) status, from the many GRAS notifications for its various applications that have been approved by the USA Food and Drug Administration (FDA) [1][8].

2.3. Physico-Chemical Conditions for Growth

In contrast with most other hemiascomycetous yeasts, *Y. lipolytica* is an obligate aerobe, for which oxygen concentration constitute a limiting factor for growth. Its temperature limit is in the range of 32 to 34 °C for most strains, although a very few can grow as high as 37 °C. Most strains can be considered as psychrotrophic since they exhibit a residual growth when kept at 4–5 °C. The preferred growth temperature is however in the range of 25 to 30 °C [9]. *Y. lipolytica* is able to grow at a large range of pHs: most strains can be cultivated at pH 3.5 to 8.0 and a few can tolerate lower ones (2.0) or even very high pHs (9.7) [9]. In accordance with its presence in salty environments and foods, *Y. lipolytica* tolerates high salt concentrations, such as 7.5% NaCl for most strains and as high as 15% NaCl for a few of them [9]. This yeast is also known to be able to adsorb metallic atoms and has therefore been proposed for bioremediation of wastes containing heavy metals such as Cr, Fe, Ni, Cu, Zn and Cd [9].

2.4. Ploidy and Morphology

This yeast is heterothallic, with two mating types Mat A and Mat B, and natural isolates are in most cases haploid [10][11]. The mating frequency of two natural Mat-compatible strains is very low, but the resulting diploid state is stable under laboratory growth conditions [12]. Such hybrids exhibit a very low fertility, a problem that was alleviated through inbreeding programs to allow the establishment of the first genetic maps [10]. High sporulation rates can be obtained on peculiar media (yeast extract/malt extract or V-8 juice media, media with 1.5% sodium citrate as sole carbon source) and the shapes of asci and ascospores exhibit some strain-dependant variations [9]. Wild-type isolates of *Y. lipolytica* can present a large variety of colony aspects, ranging from smooth and glossy to strongly wrinkled and mat. This diversity reflects the fact that *Y. lipolytica* is a dimorphic yeast that can grow either as round multipolar budding cells, pseudohyphae (budding cells remaining attached) or mycelia with septate hyphae, depending on growth conditions [10][11][13]. This possibility of growth under different forms (dimorphic switch) is of practical importance regarding biotechnological applications of *Y. lipolytica*, since monitoring all environmental parameters will be crucial for the control of cellular morphology, from which the optimization of the bioprocess could depend on [14].

3. Physiology

3.1. Carbon Sources

Y. lipolytica is able to use as carbon source a large array of substrates of either hydrophilic or hydrophobic nature [10][11]. Water-soluble carbon sources include only a few sugars (glucose, fructose, mannose) but also glycerol and, to a lesser extent, organic acids and alcohols. The long-prevailing belief that *Y. lipolytica* could use only some hexoses, but no pentose, as sole carbon source has however been recently undermined in experiments on xylose assimilation by some strains, which revealed the presence of a dormant pentose pathway in *Y. lipolytica* [71, 72]. Similarly, the rather recent discovery of a wild-type strain able to metabolize lactose has undermined the previous belief that this sugar could not be a substrate for *Y. lipolytica* (lactose-positive B9 isolate [48]). Water-insoluble carbon sources comprise fatty acids, triglycerides and alkanes. Remarkably, the engineering of *Y. lipolytica* metabolism for the use of alternative substrates has been initiated very early in its history of genetic manipulation: heterologous expression of *Saccharomyces cerevisiae SUC2* gene was used more than three decades ago to confer the ability to grow on sucrose to some of the most used laboratory strains [15]. Wild-type *Y. lipolytica* isolates present a high potential for the valorization of liquid or solid wastes from various agricultural and industrial origins, notably crude or raw glycerol issued from biodiesel production processes, as reviewed previously [66,68]. In addition, genetic engineering of this yeast for use of other (agro)industrial wastes as alternative substrates for white biotechnology applications has become an important and rapidly developing research field, as exemplified in Section 5 below. This overall versatility that this yeast allows in the choice of possible substrates represents a valuable asset for the development of bioprocesses involving *Y. lipolytica*, especially those based on the valorisation of by-products or waste.

3.2. Secretion Pathway

The two more prominent characteristics of *Y. lipolytica* are its very efficient secretion pathway and its outstanding lipid storage capacity. Consequently, this yeast has become a research model in the domains of protein secretion and lipid metabolism [16][17]. The study of vesicular protein secretion in *Y. lipolytica* has demonstrated that the translocation of the nascent protein into the endoplasmic reticulum (ER) was mainly co-translational, as in the secretion pathway of mammals

[16]. This peculiarity, contrasting to the situation in *S. cerevisiae* and most yeasts for which secretion is mainly post-translational, allows *Y. lipolytica* to be very efficient in the folding and secretion of large and/or complex heterologous proteins and has contributed to its success as a heterologous production host [18][19][20]. In addition, *Y. lipolytica* is one of the few yeasts, with *Pichia pastoris*, which lacks an α-1,3-mannosyltransferase, a factor that limits the amount of excessive mannosylation of secreted heterologous glycoproteins and constitutes a valuable asset for the production of therapeutic proteins [21][22].

3.3. Lipid Storage

As an oleaginous yeast, *Y. lipolytica* can naturally accumulate lipids up to 30 to 50% of the cell dry weight (CDW), depending not only on each wild-type isolate genetic background but also on the carbon source used and the growth conditions. This lipid accumulation can reach up to 90% of CDW through genetic engineering, in obese *Y. lipolytica* cells [17] which have been derived from the wild-type W9 strain (isolated from Paris sewers) through extensive genetic engineering [178,179,180,181,182,183,184,185,186]. As regards growth conditions, the accumulation of lipids in this yeast is known for a long time to be favoured by nitrogen starvation [17]. Lipid storage in *Y. lipolytica* results from an effective *de novo* synthesis pathway for triacylglycerols (TAG) when sugars or similarly catabolized compounds such as polysaccharides or glycerol are used as carbon sources. It is however more remarkable when hydrophobic substrates are used, benefiting then from both an efficient uptake of lipids from the medium and an efficient *ex novo* synthesis pathway (biomodification) [23]. When grown on non-fatty substrates (such as crude glycerol) most wild-type *Y. lipolytica* strains are not able to accumulate high levels of lipids, even under nitrogen-limited conditions, since those produced during the early growth steps are submitted to degradation to the benefit of other compounds such as organic acids and polyols [24][25]. Thus, during growth of wild-type *Y. lipolytica* on glycerol in bioreactor, in repeated batch cultures, three succesive phases were identified: a biomass production phase, a lipogenic phase and a citric acid production phase [26]. There are only a few exceptions to this rule, such as notably the SKY7 isolate, able to convert efficiently crude glycerol into triacylglycerides [202,203]. Using double- or multiple-limitation media could however alleviate this problem, as was demonstrated for ACA-DC 50109 strain [130]. In this regard, wild-type *Y. lipolytica* strains appear somewhat atypical among oleaginous yeasts, for which lipid content is usually less substrate-dependent [27]. The physiological response of *Y. lipolytica* cells to the presence of hydrophobic substrates (such as alkanes, fatty acids or oils) consists in the production of biosurfactants (notably liposan), in a hydrophobization of the cell membrane and in the formation of protrusions on the cell surface [16]. These protrusions correspond to the hydrophobic binding structures of an interfacial transport system, composed of several dozens of multimeric protein complexes, which facilitate the uptake of hydrophobic compounds from the environment [17][28][29]. The very efficient secretion of the extracellular lipase *LIP2* also contributes to the effective uptake of lipids by this yeast, through a reduction in molecular weight of the hydrophobic substrates. The lipase family has known an expansion in *Y. lipolytica*, as in most oleaginous yeasts, with a total of 16 lipase genes. The storage lipids of *Y. lipolytica* consist mostly of TAG and sterol esters, more than free fatty acids (FFA) and accumulate in a specialized subcellular compartment, the lipid body (LB). These lipids can comprise as high as 80% of unsaturated fatty acids, which present some valuable health benefits. Notably, *Y. lipolytica* is the oleaginous yeast with the highest known percentage of linoleic acid (LA), namely more than 50% [17]. The lipid metabolism of this yeast is of particular relevance for some major white biotechnology applications, such as the production of single-cell oil (SCO) and of biofuel and has been the subject of numerous reviews [17][23][28][30]. Interestingly, the lipid profile of *Y. lipolytica* SCO can be modulated through the use of different mixtures of low-cost fatty substrates in order to provide tailor-made lipids, as was demonstrated notably by the obtention of cocoa-butter substitute from stearin, with chemically hydrolyzed rapeseed oil as co-substrate, using the wild-type strain ACA-DC 50109 [31][32].

4. Genomic Organization

The first *Y. lipolytica* strain to be completely sequenced and fully assembled and annotated, E150, a genetically engineered strain derived from the sporulation of a diploid issued from the mating of W29 and ATCC wild-type isolates, constitutes the reference strain for genome structure studies. Its genome of 20.5 Mb comprises six chromosomes which sizes range from 2.6 to 4.9 Mb [33][34]. This genome size is almost twice those of most other yeasts, including *S. cerevisiae* (12 Mb) [34]. Several other genomic characteristics make *Y. lipolytica* clearly stand out from the crowd of other hemiascomycetous yeasts. Notably, the G/C content, of 49% in average and near 53% in the genes, and the proportion of intron-containing genes, of 15%, are strikingly higher than for other yeasts (respectively, 38%, 40% and 5% in *S. cerevisiae*) [34][35]. In contrast, the number of genes, although on the strong side of the range for hemiascomycetous yeasts, is not as high as may be inferred from the large genome size. Namely, *Y. lipolytica* totalizes 6703 genes, more than the 5807 ones from *S. cerevisiae* but less than the 6906 ones from *Debaryomyces hansenii*, which both have genomes of around 12 Mb [34].

Numerous *Y. lipolytica* strains have been sequenced, which correspond to a total of ten different completely independent genetic backgrounds and constitute the start of a pan-genome representing the genetic diversity of this species [33,34,36,37,48,125,156,168,171]. The already assembled genomes show some chromosomal rearrangements compared to the reference strain, despite a nearly constant genome size [36][37]. This is consistent with the previous observation, in karyotypic analyses, of an important polymorphism in the length of various chromosomes between different *Y. lipolytica* strains. Such a high level of chromosomal rearrangements between strains could explain the poor fertility that was observed for the hybrids [38].

Among yeasts, *Y. lipolytica* presents atypical ribosomal DNA units, with several rRNA gene clusters scattered on different chromosomes (six clusters on four chromosomes in E150). In addition, the 5S RNA gene is not included in those rDNA unit but present as separated copies scattered throughout the genome [38]. These characteristics, such as the co-translational secretion pathway mentioned above, are closer to those of mammals than to those of other yeasts, confirming the eccentric phylogenetic position of *Y. lipolytica* based on the comparison of 18S and 26S rDNA sequences [38]. Some expression vectors for *Y. lipolytica* genetic engineering make use of rDNA sequences as targeting elements for integration into the genome [158,218].

The first *Y. lipolytica* retrotransposon identified, Ylt1, was detected in the E150 genome [33][34]; this element can only be found in a few wild-type isolates and in their derivatives, such as the genetically modified (GM) E150 strain. Ylt1 belongs to the Ty3/gypsy group and is bordered by unusually large (more than 700 bp) long terminal repeats (LTRs) termed zeta sequences, which can also be found as solo elements in the genome [39]. The numbers of Ylt1 and of solo zeta sequences present in a genome vary for each Ylt1-bearing strain but is of at least 35 copies for the retrotransposon and more than 30 copies for the solo LTRs [39]. A number of other retrotransposons have since been identified in other *Y. lipolytica* strains [36][40][41], but the presence of Ylt1 in a genome is relevant for some metabolic engineering strategies, since zeta sequences have been used as targeting elements in some expression vectors or cassettes [42][43]. In contrast to *S. cerevisiae*, *Y. lipolytica* does not bear any retrotransposon of the Ty1/Copia group, which are usually abundantly found in eukaryotic genomes. Interestingly, the presence in some strains of several retrotransposons and LTR-like sequences near to RNA polymerase III-transcribed genes, which number is almost twice that in most other yeasts, seems to indicate that these retro-elements may have played an active role in the evolution of *Y. lipolytica* [36].

5. Engineering *Y. lipolytica* strains into cell factories

The process of transforming a selected *Y. lipolytica* host strain into a successful cell factory represents a long journey, through multiple technical steps requiring complementary expertises, that have already been extensively reviewed previously [19,20,54,55,204,205,206,207,208,209]. Briefly, remodelling the metabolic pathways of *Y. lipolytica* for the production of a compound of interest can be obtained via deletion/repression/activation/overexpression of endogenous genes combined with (over)expression of a few heterologous genes as well as introduction of complete new metabolic pathways, all steps achieved through classical or more recently developed engineering/editing methods such as CRISPR-derived strategies. In addition, new strategies for metabolic engineering take also into account the availability of cofactors, the reduction of oxidative compounds and the compartmentalization of the modifications in different cell organelles, in a holistic view of the metabolic fluxes. The obtained GM strain can also benefit of multi-omics technologies which, by allowing in silico modelling of genome-scale metabolic pathways, could contribute to identify limiting factors and bottlenecks, suggesting future genetic engineering targets in a virtuous circle. Adaptative evolution strategies could also be applied for further improvement and, at last, bioprocess engineering will permit the valorisation of the laboratory achievements into an industrial-scale economically viable bioprocess.

5.1. Brief history of industrial use

The high potential of *Y. lipolytica* for industrial applications has been exploited since more than 70 years, at first in the fields of biomass and valuable metabolites production, using proprietary wild-type isolates or traditionally improved strains (mutants, strains issued from hybridizations and crossings), as reviewed previously [1,44,45,50]. Notable applications of wild-type strains include the production of single-cell protein (SCP) from crude oil until the oil crisis of the 1970s (Toprina G, for livestock feeding) and, presently, industrial citric acid production (ADM, Chicago, IL, USA), erythritol production (Baolingbao Biology Co., Yucheng, Shandong, China), use of *Y. lipolytica* biomass as fodder yeast for farm and pet animals (Skotan SA, Chorzów, Poland). The outstanding capacity of *Y. lipolytica* for degrading hydrocarbons, and especially alkanes, explains that wild-types isolates were frequently found in oil-polluted environments and justifies the use of this yeast in bioremediation projects [44,51,52]. A starter for depolluting wastewaters is commercialized by Artechno (Isnes, Belgium), based on traditionally-obtained highly lipolytic mutants of ATCC 48436 strain.

In the 1980s, the newly developed technics of molecular biology rejuvenated the interest in *Y. lipolytica*, this time as an expression host for producing heterologous proteins [18]. Metabolic engineering of this yeast ensued rapidly, following the development of transformation methods, shuttle vectors and non-leaky non-reverting auxotrophic strains [15]. As *Y. lipolytica* started, in the 2000s, to be recognized as a valuable host for recombinant protein production [19,20], the YLEX kit for expression/secretion of heterologous proteins in this yeast was commercialized in 2006 (Yeastern Biotech Co., Taipei, Taiwan), based on a GM derivative of W29 wild-type isolate. Other W29 derivatives have been established as commercial protein production platforms by Protéus (Sequens Group, Ecully, France) and Oxyrane UK (Manchester, UK). With the continuous progress of genetic engineering technics, increasingly complex modifications of *Y. lipolytica* metabolism, such as the introduction of complete heterologous metabolic pathways, could be performed. Proofs of concept of the use of this yeast as cell factory for the production of valuable compounds or as arming yeast for bioconversion processes are abundantly reported in the scientific literature since a few decades [54,55,56]. However, most of the proposed applications for these GM *Y. lipolytica* strains remain, until now, only at an exploratory stage and are not developed further to the industrial stage. This matter of fact could be attributed at least in part to social acceptance issues concerning GM microorganisms, especially in the domain of food applications. Until now, only a few commercial or industrial applications of GM *Y. lipolytica* strains can be reported [1,45]. GM *Y. lipolytica* cell factories are presently used for industrial production of two kinds of food/feed additives: carotenoids [45] (DSM, Heerlen, The Netherlands) and polyunsaturated fatty acids (PUFAs)-rich SCOs (DuPont, Wilmington, DE, USA). The technology of PUFAs-rich SCOs production by a heavily engineered *Y. lipolytica* strain derived from the ATCC 20362 wild-type isolate was more particularly applied to industrial production of ω-3 eicosapentaenoic acid (EPA)-rich products [57,58], such as notably EPA-rich *Y. lipolytica* biomass marketed (in joint venture with AquaChile, Puerto Montt, Chile) as an ω-3 feed supplement for “harmoniously raised” salmon Verlasso^{TM}.

Another domain of successful applications for GM *Y. lipolytica* strains is the therapeutic use of recombinant enzymes: several enzyme replacement therapies (ERTs) based on this yeast are now marketed or on the edge to marketing stage [54,55]. The first of these ERTs, developed by Mayoly Spindler (Chatou, France), uses a recombinant extracellular LIP2 lipase [60] for the treatment of exocrine pancreatic insufficiency (also under Phase 2 clinical trial for two other fat malabsorption diseases, cystic fibrosis and chronic pancreatitis). More recently, Oxyrane (Ghent, Belgium) established a proprietary *Y. lipolytica* engineering platform able to produce recombinant glycoproteins, with the possibility of added mannose-6-phosphate (M6P) glycan residues [61], for treatment of different lysosomal storage diseases. The presence of M6P on therapeutic glycoproteins improves their internalization into the patient’s cells and addresses them to lysosomes, their targeted subcellular site of action. A recombinant human acid α-glucosidase produced in *Y. lipolytica*, OXY2810, is currently marketed for use as ERT in Pompe disease (in which glycogen accumulates in the patient’s tissues) and recombinant glucocerebrosidases are in preclinical testing for treatment of Parkinson’s disease or neuronopathic Gaucher disease [54,55], while other new ERTs are in project.

5.2. Rewiring the metabolism for a bio-based economy

In an environmentally friendly concept of circular bioeconomy, it is of major importance to base industrial processes on the use of substrates issued from agricultural, forest and industrial waste or by-products. This aim towards a bio-based economy often imply rewiring the metabolism of *Y. lipolytica* strain for allowing them to use alternative renewable substrates. Such innovations have been abundantly described in many recent reviews [45,50,54,55,56,62,63,64,65] and are schematically depicted in the Cover Figure that represents a state of the art for substrates availability and biotechnological applications for GM *Y. lipolytica* strains. Natural substrates and traditional applications of wild-type *Y. lipolytica* strains are indicated in green. Alternative substrates and new applications, requiring metabolic remodelling of *Y. lipolytica*, are indicated in blue (including pentoses and lactose, despite the recent reports of strains being able to metabolize these sugars [48,71,72]). Substrates issued from waste or by-products are underlined. Abbreviations used, per order of occurrence in the figure: SCP, single cell protein; SCO, single cell oil; PUFA, poly-unsaturated fatty acids; EPA, eicosapentaenoic acid; ARA, arachidonic acid; ERTs, enzyme replacement therapies; α-KG, α-ketoglutarate; FFA, free fatty acids; FAEE, fatty acid ethyl esters; FAME, fatty acid methyl esters; PHA, polyhydroxyalkanoates.

6. Conclusion

The wide range of engineering tools and strategies now available will contribute to establish *Y. lipolytica* as a workhorse for a wide range of applications in the very competitive world of white biotechnology. However, for an optimal development of *Y. lipolytica* cell factories, it is to hope that a future easing of the regulation policy for the new GMOs (especially for gene edited/CRISPR-generated organisms) could allow the relieving of the regulatory constraints that presently limit their use in some of their numerous domains of application. Even though it would be difficult to determine what influence GMO regulations and societal acceptance could have had on the strategic choices of laboratories and companies, we can note

that the major food-oriented applications of *Y. lipolytica* strains (citric acid, erythritol, KGA) have majorly favoured traditionally improved strains. If this tendency was to increase in the future, a more systematic exploration of the natural *Y. lipolytica* biodiversity for potential applications, leveraged by new mutagenesis technics (ARTP: atmospheric and room temperature plasma), adaptative evolution strategies and high-throughput screening technologies, would constitute a valuable asset. Therefore, *Y. lipolytica* is in good position to become a biotechnological workhorse, through both traditional and genetic engineering pathways.

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