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MINI-REVIEW

Yarrowia lipolytica: recent achievements in heterologous protein expression and pathway engineering

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Abstract The oleaginous yeast *Yarrowia lipolytica* has become a recognized system for expression/secretion of heterologous proteins. This non-conventional yeast is currently being developed as a workhorse for biotechnology by several research groups throughout the world, especially for single-cell oil production, whole cell bioconversion and upgrading of industrial wastes. This mini-review presents established tools for protein expression in *Y. lipolytica* and highlights novel developments in the areas of promoter design, surface display, and host strain or metabolic pathway engineering. An overview of the industrial and commercial biotechnological applications of *Y. lipolytica* is also presented.

Keywords *Yarrowia lipolytica* · Oleaginous yeast · Heterologous protein · Expression · Secretion · Surface display · Genetic engineering · Biotechnology · Lipid metabolism

Introduction

Yarrowia lipolytica is a dimorphic oleaginous yeast, belonging to the Ascomycota phylum (subphylum: Saccharomycotina, previously called Hemiascomycetes), that exhibits remarkable lipolytic and proteolytic activities. Its natural occurrence appears rather ubiquitous, including soils, marine waters, mycorrhizae, and a variety of foods (particularly dairy products, including numerous cheeses, and meat), as recently reviewed by Groenewald et al. (2014) and Zinjarde (2014). Since more than 50 years, this non-conventional yeast has promoted interest for industrial applications, firstly oriented toward the production of metabolites with commercial value and secondly aimed at producing heterologous proteins. The fact that *Y. lipolytica* has been frequently isolated from environments heavily polluted by hydrocarbons has also prompted interest for its use in bioremediation of oil-contaminated soils and waters or in upgrading oily wastes (Bankar et al. 2009). On account of its peculiar characteristics, *Y. lipolytica* has also become a model for basic research in the study of dimorphism, secretion, salt tolerance, mitochondrial complex, peroxisomes, or lipid accumulation (as reviewed by Nicaud 2012). More recently, the combination of increased knowledge of its metabolism, sequencing of its genome, and development of more efficient genetic tools offered new perspectives in metabolic pathway engineering, allowing using this yeast as a cell factory. In the rapidly developing domain of *Y. lipolytica* research and applications, publications abound but the most extensive reviews are published in books (such as the two recent volumes from Springer's Microbiology Monographs series—Barth 2013a, b), which limits their impact. This mini-review, besides providing a concise guide for the beginner, will attempt to be more synthetic than exhaustive and will focus on the most recent achievements in the domain of heterologous protein expression in *Y. lipolytica*, especially concerning the engineering of its metabolic pathways for various applications.

A brief history of *Y. lipolytica* use and safety

The industrial use of *Y. lipolytica* was pioneered in the 1950s by British Petroleum (UK), during the search for microorganisms

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growing on cheap substrates (especially on crude oil) and able to produce, on a large-scale, high-quality protein products for nutritional supply. Wild-type strains have been used to produce single-cell proteins (SCP) for livestock feeding until the 1970 oil crisis. Since then, *Y. lipolytica* has also been applied to the production of different organic acids, especially citric acid (Pfizer Inc. and ADM, USA), and of erythritol (Baolingbao Biology Co., China). More recently, the Polish company Skotan SA started to commercialize *Y. lipolytica* biomass, for use as fodder yeast in Europe, and to develop prebiotic and probiotic applications of this yeast for food industry. In the environmental domain, Artechno (Belgium) produces a starter for bioremediation of oily wastewaters, composed of freeze-dried wild-type *Y. lipolytica* cells and extracellular lipase. A lot of wild-type *Y. lipolytica* strains have been isolated from hydrocarbon-contaminated soils or waters, which makes them interesting candidates for bioremediation applications. Such studies are abundantly represented in the scientific literature, at the laboratory scale, but, to our knowledge, only one in situ field study has been conducted, for the bioremediation of a petroleum-contaminated soil (Bankar et al. 2009). Although the use of genetically modified microorganisms (GMM) could raise safety concerns, *Y. lipolytica* appears as one of the non-conventional yeasts that presents the best guarantees in this regard. With a longstanding history of safe use, *Y. lipolytica* is considered as a biosafety class 1 microorganism, namely a microorganism which is not known to cause disease in healthy adult humans (Groenewald et al. 2014). The industrial applications cited above have obtained a generally regarded as safe (GRAS) status from the Food and Drug Administration (FDA, USA). Consequently, *Y. lipolytica* is listed by many countries as a microorganism acceptable for constructing GMM as long as its large-scale production facilities comply with the good industrial large-scale practice (GILSP) level of physical containment (Groenewald et al. 2014). Since the 2000s, the use of genetically modified *Y. lipolytica* strains has developed and several products have been (or are on the edge to be) marketed. DuPont (USA) has extensively engineered *Y. lipolytica* metabolic pathways (Xue et al. 2013) for the production of eicosapentaenoic acid (EPA)-rich products: EPA-rich *Y. lipolytica* biomass is currently used as an ω-3 supplement in fish feed (harmoniously raised salmon Verlasso™), and an EPA-rich single-cell oil (SCO), for use as dietary supplement, has been marketed in 2010 under the name of New Harvest™. However, its commercialization has been recently discontinued (personal communication from DuPont) despite the obtention of a GRAS notice. DuPont and Microbia (USA—now a subsidiary from the Dutch-based multinational company DSM) have independently developed *Y. lipolytica* as a production host for carotenoids. Extensive data on the safety of β-carotene produced from GM *Y. lipolytica* has been recently disclosed by Microbia (Grenfell-Lee et al. 2014). Engineered *Y. lipolytica* strains are also used by two European companies for the production of recombinant enzymes, for enzyme replacement therapies (ERTs): human lysosomal enzymes, for treatment of lysosomal storage diseases (Oxyrane, Belgium—Tiels et al. 2012) and an overexpressed homologous lipase (YlLip2p) (Pignède et al. 2000) for treatment of exocrine pancreatic insufficiency (Mayoly Spindler, France). These ERTs are currently evaluated in clinical trials. In addition, engineered *Y. lipolytica* strains are employed in protein manufacturing platforms by Protéus (France) and Oxyrane (UK), both making use of technologies developed at Institut National de la Recherche Agronomique (INRA, France). More details on *Y. lipolytica* industrial use and safety assessment could be found in recent reviews by Groenewald et al. (2014) and Sibirny et al. (2014); the subject of bioremediation has been extensively treated by Bankar et al. (2009), and food-related applications have been recently reviewed by Zinjarde (2014).

Tools for heterologous protein expression, secretion, and surface display

The use of *Y. lipolytica* as a host for heterologous expression was initiated, 30 years ago, by the almost simultaneous development of transformation methods by Pfizer Inc. (USA) and a French INRA team (Davidow and DeZeeuw 1984; Gaillardin et al. 1984). Since then, more than 130 proteins, from more than 80 species, have been successfully produced in this yeast (Madzak and Beckerich 2013 and more recent survey of scientific literature). The use of synthetic genes, although relatively recent, represents 22 % of these cases. When the native genes were used, their phylogenic origins were diverse, with Ascomycota representing 27 % of the cases, mammals 20 % (human genes alone: 15 %), and Proteobacteria 14 %. The remaining 39 % of cases include viruses, Firmicutes, Actinobacteria, Basidiomycota, Zygomycota, Heterokonta, protists, plants (monocotyledons or dicotyledons), insects, and a mollusc. An overview of the possibilities offered by the *Y. lipolytica* expression system is schematized in the Fig. 1.

Strains

A few wild-type *Y. lipolytica* isolates have been selected, on the basis on noticeable properties, and engineered for peculiar applications. Thus, several marine *Y. lipolytica* strains from the Ocean University of China (OUC, Qindao) library have been recently engineered for use as citric acid, SCO, or SCP producers (respectively: Liu et al. 2010; Zhao et al. 2010; Cui et al. 2011). Similarly, the wild-type WSH-Z06 strain, a natural α-ketoglutaric acid (KGA) overproducer, has been engineered in order to optimize KGA production (Yin et al. 2012; Zhou et al. 2012; Guo et al. 2014) and the German wild-type isolate, H222 (*MatA*), or a KGA-overproducing derivative, were engineered for various organic acid production (Holz et al. 2009, 2011; Otto et al. 2012; Yovkova et al. 2014; Jost et al. 2015). Besides these examples, most heterologous expression works

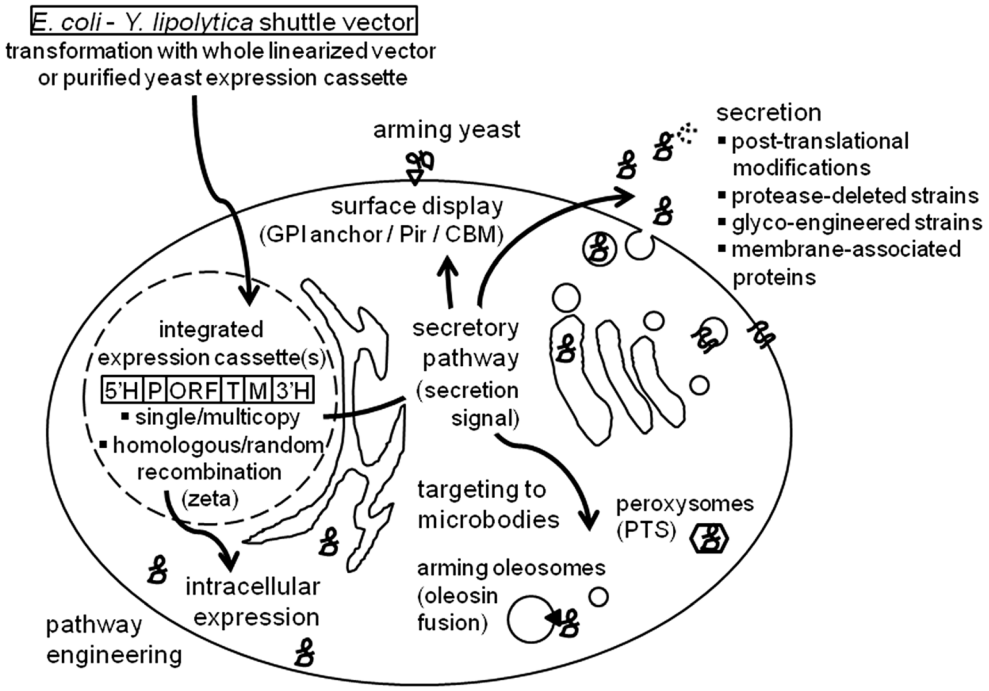

Fig. 1 Overview of possibilities for heterologous gene expression in *Y. lipolytica* expression vectors harboring one (or more) expression cassette(s) composed of a promoter (P), the open reading frame (ORF) of the gene of interest and a terminator (T), together with a selection marker (M), and bordered by sequences for integration, are linearized prior to transformation. Alternatively, a yeast expression cassette can be separated from the bacterial vector backbone and purified for transformation. Sequences for integration generally consist in 5′ and 3′ homologous regions (5′H and 3′H). Zeta sequences from Ylt1 retrotransposon can be used for that purpose in Ylt1-bearing strains or can promote random ectopic integration by non-homologous recombination in strains devoid of this retrotransposon. The additional use of proper signal sequence(s) will direct the recombinant protein to the secretory pathway (secretion signal) and, possibly, to surface display via fusion to anchoring domains (*open triangle*), notably homologous or heterologous glycosylphosphatidylinositol (GPI) anchor but also protein internal repeat (Pir) or chitin binding module (CBM). Other possibilities include targeting to cellular microbodies such as peroxysomes (peroxisomal targeting signal (PTS)) or oléosomes (fusion to heterologous oleosin domain: *black triangle*). Some glyco-engineered strains allow producing recombinant glycoproteins with human-compatible N-linked oligosaccharides for therapeutic applications. Others are able to produce heterologous lysosomal enzymes with high levels of mannose-6-phosphate which will target them to lysosomes in patient cells. Details and references are given in the text

have used only a limited number of inbred lines. An extensive list of these *Y. lipolytica* host strains can be found in Madzak et al. (2004), but the most used are E129 and the Po1 series of strains (Po1d, f, g and h), as more recently reviewed by Madzak and Beckerich (2013). Like the E150 strain (*MatB*, *his1*, *leu2-270*, *ura3-302*, *xpr2-322*), whose genome has been fully sequenced (Dujon et al. 2004), the E129 strain (*MatA*, *lys11-23*, *leu2-270*, *ura3-302*, *xpr2-322*) is issued from a mating followed by multiple back-crosses between derivatives of a French wild-type isolate, W29 (*MatA*), and an American one, YB423-12 (*MatB*), as reviewed by Barth and Gaillardin (1996). The Po1 series of strains are derived only from W29 and, consequently, do not bear the Ylt1 retrotransposon, which was absent from this French wild-type isolate. Po1d (*MatA*, *leu2-270*, *ura3-302*, *xpr2-322*) presents interesting features for heterologous protein production: high secretion levels, deletion of alkaline extracellular protease (AEP, encoded by *XPR2* gene), non-reverting Leu$^-$ and Ura$^-$ auxotrophies, and production of recombinant invertase (*ura3-302* allele corresponds to the disruption of *URA3* by the *SUC2* gene from *Saccharomyces cerevisiae* under the control of *XPR2* promoter) allowing to use sucrose or molasses as carbon source (Nicaud et al. 1989). A series of derivatives have been further adapted for heterologous protein production (Madzak et al. 2000 and 2004): Po1f, Po1g, and Po1h were further deleted for the acid extracellular protease (*axp1-2* allele). Po1g was also fitted with an integrated pBR322 docking platform (*ura3-302*::*URA3*:pBR322), for further integration of pBR322-based vectors, and retains only Leu$^-$ auxotrophy. Po1h was modified, using genic *LEU2* conversion, to retain only Ura$^-$ auxotrophy. These strains can be ordered from INRA's CIRM-Levures Yeasts Library (http://www7.inra.fr/cirmlevures/page.php?page=home&lang=en). A draft genome sequence of Po1f has recently been published (Liu and Alper 2014). Another derivative of Po1d strain has been engineered for high-throughput applications, especially in the domain of lipid metabolism: JMY1212 (*MatA*, *ura3-302*, *leu2-270*::*LEU2*:zeta, *xpr2-322*, Δ*lip2*, Δ*lip7*, Δ*lip8*), has the main lipases deleted, and was fitted with an integrated zeta (Ylt1 retrotransposon long terminal repeat (LTR) docking platform, for further

integration of zeta-based vectors; it retains only Ura$^-$ auxotrophy. JMY1212 is used as recipient strain in the high-throughput system developed by INSA, CNRS, and INRA (France), for screening new biocatalysts through expression cloning and for improving selected enzymes through directed enzyme evolution (Bordes et al. 2007). At last, the *ScSUC2* expression cassette has been recently modified in a new Po1d derivative, JMY2593, in order to provide a *Y. lipolytica* strain with improved growth on sucrose for industrial applications (Lazar et al. 2013).

Vectors

Two types of shuttle vectors can be used for transforming yeast cells: episomal (replicative) plasmids and vectors designed for integration into the yeast genome, for which linearization is required to direct targeting at a precise locus. No *Y. lipolytica* strain bears any natural episome, but replicative plasmids using chromosomal autonomously replicating sequence/centromer (ARS/CEN) replication origins have been designed (Fournier et al. 1993; Matsuoka et al. 1993). Such vectors are useful when transient expression is required, e.g., for the *Cre-lox* gene disruption method (Fickers et al. 2003), but their use for heterologous expression is limited by their low copy number (average of one to three plasmids per cell under selective conditions) and their high loss frequency (Vernis et al. 1997). Liu et al. (2014) have recently designed an improved *Y. lipolytica* ARS/CEN plasmid by fusing a promoter upstream of the centromeric region, thus increasing the relative copy number by 80 % and providing a starting point for more potent replicative plasmids in *Y. lipolytica*.

Nevertheless, the use of integrative vectors remains the preferred strategy for heterologous expression and/or genetic engineering in *Y. lipolytica*. As schematized in the Fig. 1, the integration of such vectors can be targeted to a genomic locus or docking platform, by linearization into a corresponding homologous region. However, in contrast to *S. cerevisiae* (but like other yeasts), *Y. lipolytica* uses mainly the non-homologous end-joining (NHEJ) recombination pathway and not the homologous recombination (HR) pathway for DNA double-strand break (DSB) repair (Richard et al. 2005). In consequence, homologous integration of exogenous DNA by a single crossing-over into *Y. lipolytica* genome can occur at acceptable rates (over 80 %) only in presence of large enough homologous 5′- and 3′-flanking regions, in the range of 0.5 to 1 kb (Barth and Gaillardin 1996; Fickers et al. 2003). In order to palliate this problem, a *Y. lipolytica* strain deleted for *KU70* gene, responsible for DSB repair in the NHEJ recombination pathway, was recently developed at INRA (France) (Verbeke et al. 2013). The *ku70* deletion significantly reduced transformation efficiency but promoted efficient HR with short length flanking regions. Targeted gene conversion, by double crossing-over, was obtained at a frequency of 43 % with 5′- and 3′-flanking homologous regions as short as 50 bp (Verbeke et al. 2013).

The requirement of large homologous flanking regions for efficient HR in *Y. lipolytica* has however not impaired the development of effective integrative expression vectors. The use of an integrated docking platform, usually a bacterial plasmid backbone (such as pBR322), allows targeting a unique copy at a precisely known genomic site (Madzak et al. 2000 and 2004; Bordes et al. 2007). This system is particularly adapted to the genetic engineering of proteins, since the effect of mutations on recombinant enzyme activity can be compared directly on the transformants. The transformation efficiency can be very high, in the range of 10^5 colonies per microgram of vector DNA for pBR322-based vectors transforming Po1g recipient strain (Madzak and Beckerich 2013). This efficient and easy-to-use expression/secretion system is commercialized under an INRA license: YLEX™ Expression Kit can be purchased from Yeastern Biotech Co. (Taiwan) or Gentaur (Belgium). In order to avoid the presence of bacterial DNA (especially antibiotic resistance genes) into recombinant yeast strains devoted to industrial applications, some autocloning vectors have been developed: The bacterial backbone can be excised, and only a yeast cassette (composed of the expression cassette and selection marker, bordered by sequences for integration) is used for transformation (Pignède et al. 2000; Nicaud et al. 2002).

Vectors carrying two or three expression cassettes, for co-expression of several heterologous genes, have been employed by different research groups (Chuang et al. 2010; Celińska and Grajek 2013). The possibility to increase and/or tune gene expression is crucial for both heterologous protein production and metabolic engineering. To this end, several research teams have developed various strategies, notably endogenous promoter characterization (Müller et al. 1998; Juretzek et al. 2000), hybrid promoter engineering (Madzak et al. 1995 and 2000; Blazeck et al. 2011 and 2013a), and multicopy integration (Le Dall et al. 1994; Juretzek et al. 2001).

Promoters

The characteristics of the major promoters used for heterologous expression in *Y. lipolytica* have been extensively described elsewhere (Madzak and Beckerich 2013) and are resumed in the Table 1. If the *XPR2* promoter (p*XPR2*) has been historically important during the development of *Y. lipolytica* expression system, its complex regulation and the cost of using peptones for its induction urged the search for new promoters, more fit for industrial constraints. Other major strong endogenous promoters are the constitutive p*TEF1* and some inducible promoters from the fatty acids pathway: p*POX2* and p*ICL1*. The functional dissection of p*XPR2* allowed to use one of its upstream activating sequences (UAS), poorly affected

Table 1 Major promoters used for heterologous expression in *Y. lipolytica*

Promoter	Corresponding gene	Regulation	Reference
p*LEU2*	β-Isopropylmalate dehydrogenase	Inducible by leucine precursor	Franke et al. 1988
p*XPR2*	Alkaline extracellular protease	Inducible by peptones, complex regulation	Franke et al. 1988
p*POX2*	Acyl-CoA oxidase 2	Inducible by fatty acids and alkanes	Juretzek et al. 2000
p*POT1*	3-Oxo-acyl-CoA thiolase	Inducible by fatty acids and alkanes	Juretzek et al. 2000
p*ICL1*	Isocitrate lyase	Same as above+inducible by ethanol and acetate	Juretzek et al. 2000
p*POX1*	Acyl-CoA oxidase 1	Weakly inducible by alkanes	Juretzek et al. 2000
p*POX5*	Acyl-CoA oxidase 5	Weakly inducible by alkanes	Juretzek et al. 2000
p*G3P*	Glycerol-3-phosphate dehydrogenase	Inducible by glycerol	Juretzek et al. 2000
p*MTP*	Metallothioneins 1 and 2	Bidirectional, inducible by metallic salts	Dominguez et al. 1998
p*FBA1*	Fructose 1,6-biphosphate aldolase	Inducible by glucose, very strong	Hong et al. 2012
p*FBA1*$_{IN}$ (p*FBA1*+*FBA1* intron included)		Same as above with 5-fold increased strength	Hong et al. 2012
hp4d recombinant promoter[a]		Growth phase dependent	Madzak et al. 2000
UAS1Bn-Leum series of recombinant promoters[b]		Same as above with increased tunable strength	Blazeck et al. 2011
p*RPS7*	Ribosomal protein S7	Constitutive	Müller et al. 1998
p*TEF1*	Translation elongation factor-1α	Constitutive	Müller et al. 1998
UAS1B$_{8/16}$-TEF recombinant promoters[c]		Same as p*TEF1* with increased tunable strength	Blazeck et al. 2011
p*TEF1*$_{IN}$ (p*TEF1*+*TEF1* intron included)		Same as p*TEF1* with 17-fold increased strength	Tai and Stephanopoulos 2013

[a] hp4d (standing for "hybrid promoter 4 direct") is composed of four direct copies of the p*XPR2* UAS1B enhancer sequence inserted upstream of a minimal p*LEU2* (reduced to its TATA box region)

[b] The UAS1Bn-Leum series are hybrid promoters constructed on the "hpnd" model, by inserting various (*n*) tandem copy numbers (from 1 to 32) of the p*XPR2* UAS1B element upstream of a minimal p*LEU2*

[c] The UAS1B$_{8/16}$-TEF are two hybrid promoters constructed by inserting 8 or 16 tandem copies of the p*XPR2* UAS1B element upstream of different size variants of p*TEF1*

by cultivation conditions (Madzak et al. 1999), to design the recombinant promoter hp4d (Madzak et al. 1995 and 2000). This recombinant promoter, able to drive a strong expression in various culture media, exhibits a growth phase-dependent expression pattern. Its expression increases at the beginning of the stationary phase, a characteristic particularly interesting for heterologous production since it allows a partial dissociation of the growth and expression phases. Due to these interesting characteristics, the use of hp4d has rapidly developed, especially since the YLEX kit for heterologous expression using this patented promoter has been commercialized by Yeastern Biotech Co. (Taiwan). This recombinant promoter has been employed up to now in more than half of the published examples of heterologous protein production in *Y. lipolytica* (Madzak and Beckerich 2013 and more recent survey of scientific literature). More recently, an important improvement in the design of strong and tunable *Y. lipolytica* promoters has been carried out at UT Austin (USA), based on an extension of the concept of the recombinant hp4d promoter. Blazeck et al. (2011) constructed a large set of hybrid promoters, carrying various copy numbers (from 1 to 32) of an UAS inserted upstream of a minimal or reduced endogenous promoter (Table 1). The strongest of these engineered promoters exhibited an unprecedented efficiency, 8-fold higher than that of any known endogenous *Y. lipolytica* promoter (Blazeck et al. 2011). In addition to providing a series of new hybrid promoters with tunable strength, this work proposed a general approach for improving selected endogenous promoters. Moreover, it also suggested that endogenous *Y. lipolytica* promoters were enhancer-limited and that this limitation could be alleviated through UAS addition, whereas transcription factor availability was not a limiting factor.

Selection markers

Either dominant or auxotrophic markers are available for selection in *Y. lipolytica*. Although naturally resistant to most antibiotics, *Y. lipolytica* is sensitive to hygromycin B and to antibiotics of the bleomycin/phleomycin group. Heterologous genes conferring resistance to these antibiotics can be used as dominant selection markers in *Y. lipolytica* (Gaillardin and Ribet 1987; Fickers et al. 2003). In contrast, heterologous expression of *S. cerevisiae SUC2* gene, which was developed at first as a dominant marker (Nicaud et al. 1989), is impaired by residual growth on sucrose plate impurities (Barth and Gaillardin 1996) and is used preferably for recipient strain improvement (in E129 and Po1d derivatives) than for

selection. A number of auxotrophic marker genes have been used for selection in *Y. lipolytica*, but the most widely employed are *LEU2* and *URA3*, for which non-leaky and non-reverting mutations are available: *leu2-270* and *ura3-302* alleles, present in E129 and in some Po1d derivatives, contain large deletions into their coding sequences (Nicaud et al. 1989; Barth and Gaillardin 1996). In order to increase heterologous protein production, some promoter-defective versions of *URA3* marker have been designed (Le Dall et al. 1994). For example, vectors carrying the *ura3d4* allele are unable to correct *ura3* auxotrophy when in single copy but can restore growth on selective medium after an amplification process leading to multiple integrations (Le Dall et al. 1994). Similar promoter-defective versions of *LEU2* marker are currently tested at INRA (Madzak, unpublished results).

Multiple integrations

Integrating multiple copies of a vector (or of an expression cassette) into the genome remains the preferred strategy for increasing heterologous gene copy number in *Y. lipolytica*. Selection of such events requires defective markers, especially the *ura3d4* allele which is employed in most multicopy vectors (Juretzek et al. 2001; Nicaud et al. 2002). This defective marker allows obtaining several tens of copies integrated at unique or multiple sites, especially when targeting the ribosomal DNA (rDNA) G unit or the zeta sequences (LTRs from Ylt1). However, such high copy number integrants were shown to be unstable under cultivation conditions inducing heterologous expression, their number of copies eventually stabilizing around ten (Juretzek et al. 2001). This low stability seemed to be linked to the fact that multiple integrations occurred almost always in tandem copies, at only one or two sites (Le Dall et al. 1994).

Due to *Y. lipolytica* efficient NHEJ recombination pathway, the zeta-based autocloning vectors are also able to transform effectively strains devoid of Ylt1 retrotransposon, such as W29 and its derivatives (like the Po1 series of strains), by random ectopic integration. When using a promoter-defective selection marker, this non-homologous integration process generates multiple copies which are more dispersed within the genome than those targeted by homology, ensuring a better stability of high copy number integrants (Nicaud et al. 1998). Such non-homologously transformed multicopy strains have allowed producing high yields of numerous heterologous proteins, as reviewed by Madzak and Beckerich (2013). However, they remain difficult to construct, due to a very low efficiency of the transformation step. Moreover, as the transformants can present alterations and/or large differences in production levels, both linked to random integration loci, a careful checking is required. High copy number strains were also reported to suffer from a productivity loss linked to metabolic burden (Roth et al. 2009), and their long-term stability appeared unable to comply with the high standard of GMP guidelines (Nicaud et al. 2008).

In order to overcome these limitations, Nicaud et al. (2008) have developed a more controlled amplification strategy: A genetically engineered strain, deleted for genes of major secreted homologous proteins (thus alleviating metabolic burden), presents several loci selected for the targeting of integrative vectors carrying different auxotrophic or dominant markers. Up to ten stable integrations can be performed sequentially, which can be checked easily since each step confers a new phenotype, detectable on plate, to the transformed strain. Although patented as a method for the obtention of stable multicopy transformants (Nicaud et al. 2008), this strategy appears however laborious compared to the recently developed alternative strategy of promoter tuning from Blazeck et al. (2011), if one considers only the purpose of increasing expression. In contrast, it seems much more interesting in the domain of genetic engineering of complex metabolic pathways, for which the expression of several different genes is required.

However, sequential gene integration methods remain laborious and time-consuming. The DNA assembler method, previously used only on a few other yeast species, was very recently proposed by Gao et al. (2014) as an effective alternative strategy for *Y. lipolytica* genetic engineering. This method, which allows assembling multiple fragments in a single step via *in vivo* homologous recombination, was successfully used to integrate an entire β-carotene biosynthesis pathway (a selection marker and three expression cassettes, with a total size near 11 kb) at the rDNA locus of *Y. lipolytica*. Despite the fact that efficient homologous recombination in *Y. lipolytica* requires large 5′- and 3′-flanking regions (0.5 to 1 kb), the total efficiency of in vivo one-step assembly of the four DNA fragments was around 20 % with overlaps between two successive cassettes of only 65 bp (Gao et al. 2014). The choice of the β-carotene biosynthesis pathway for making a proof of concept of the DNA assembler method in *Y. lipolytica* allowed an easy visual screening of the transformants, since the colonies bearing a functional set of genes turned orange/red. Gao et al. (2014) suggested that the overall efficiency of this method for one-step multiple gene transformation could be higher in strains deleted for *KU70* gene, impaired in NHEJ mechanism (Verbeke et al. 2013). This time-saving DNA assembler method is expected to greatly facilitate metabolic engineering in *Y. lipolytica*.

Signals for secretion, targeting, or display

In order to direct a heterologous protein to the secretory pathway of *Y. lipolytica*, with the purpose to obtain its release into the cultivation medium, its targeting to cellular microbodies, or its display on the cell surface, a translational fusion with a proper signal is required. The characteristics of the major

signals used are resumed in the Table 2. Efficient secretion of heterologous proteins, involving mainly the co-translational pathway (Boisramé et al. 1998), is a key strength of the *Y. lipolytica* expression system: A signal sequence is fused upstream from the mature sequence of the protein of interest, in order to target the nascent polypeptide to the secretion pathway. Most secretion signals used are derived from *Y. lipolytica XPR2* or *LIP2* genes, but a few heterologous secreted proteins from fungi or plants have also been produced successfully using their own secretion signal (reviewed by Madzak and Beckerich 2013).

Although of more restricted use, the possibility to target heterologous proteins to cellular microbodies, such as peroxisomes or oleosomes, constitutes an interesting tool for peculiar high value applications. Targeting of heterologous proteins to *Y. lipolytica* peroxisomes can be performed via fusion of a peroxisomal targeting signal (PTS) domain to their C terminus. The tripeptide AKI, considered as a consensus PTS for the related *Yarrowia* and *Candida* genera, has been used for directing a bacterial polyhydroxyalkanoate synthase to *Y. lipolytica* peroxisomes (Haddouche et al. 2010), and the tripeptide SKL, closer to the *S. cerevisiae* consensus PTS, has been used for targeting green fluorescent protein (GFP) (Xue et al. 2013). Recently, *Y. lipolytica* has been developed as a platform for producing tunable arming oleosomes (Han et al. 2013) that could serve multiple purposes, like cell targeting and reporting activities (targeted drug delivery, pathogen detection) or self-assembly of functionalized nanostructures (nanofactories). Several heterologous proteins have been targeted to *Y. lipolytica* oleosomes and anchored on their surface via their fusion to the C-terminal domain of a plant oleosin (Han et al. 2013), a structural protein embedded in

Table 2 Major signals used for heterologous expression in *Y. lipolytica*

Secretion signals	Gene function	Description	Reference
XPR2 prepro	Protease	157 aa, KR cleavage site for Xpr6p endoproteinase	Franke et al. 1988
XPR2 pre	Protease	15 aa, LA cleavage site for signal peptidase complex	Franke et al. 1988
XPR2 pre+dipeptides[a]	Protease	17-19 aa, XA or XP cleavage site for diamino-peptidase	Tharaud et al. 1992
LIP2 prepro	Lipase	32 aa, KR cleavage site for Xpr6p endoproteinase	Pignède et al. 2000
LIP2 pre+dipeptides[b]	Lipase	21 aa, XA or XP cleavage site for diamino-peptidase	Gasmi et al. 2011a
Anchor signals for surface display	Type of linkage	Description	Reference
C terminus of YlCWP1p	Covalent: GPI anchor of CWP[c]	110 aa, in Ct	Yue et al. 2008
C terminus of YlCWP3p	Covalent: GPI anchor of CWP	121 aa, in Ct	Yuzbasheva et al. 2011
C terminus of YlCWP6p	Covalent: GPI anchor of CWP	139 aa, in Ct	Yuzbasheva et al. 2011
C terminus of YlYWP1p	Covalent: GPI anchor of CWP	242 aa, in Ct	Moon et al. 2013
C terminus of *S. cerevisiae* FLO1p	Non-covalent: flocculation domain	1097 aa, in Nt	Yang et al. 2009
N or C terminus of YALI0C09031p[d]	Non-covalent: flocculation domain	662 or 668 aa	Yuzbasheva et al. 2010
C terminus of YALI0B20306p[e]	Covalent: Pir domain of Pir-CWP[e]	100 aa, in Nt	Duquesne et al. 2014
C terminus of YALI0D22396p[f]	Non-covalent: chitin binding module	87 aa, in Nt	Duquesne et al. 2014
Termination signals	Gene function	Description	Reference
*XPR2*t	Protease	430 bp of non-coding 3′ sequence	Franke et al. 1988
Short *XPR2*t	Protease	130 bp of non-coding 3′ sequence	Tharaud et al. 1992
Minimal *XPR2*t	Protease	100 bp of non-coding 3′ seq.+*Asc*I restriction site[g]	Swennen et al. 2002
*LIP2*t	Lipase	150 bp of non-coding 3′ sequence	Pignède et al. 2000

aa amino acids, *K* lysine, *R* arginine, *L* leucine, *A* alanine, *X* any aa, *P* proline, *in Ct* added in C-terminal of the target protein, *in Nt* added in N-terminal of the target protein

[a] In the *XPR2* prepro, the pre region (15 aa) is separated from the pro region (124 aa) by a stretch of nine XA or XP dipeptides

[b] In the *LIP2* prepro, the pre region (13 aa) is separated from the pro region (12 aa) by a stretch of four XA or XP dipeptides

[c] Glycosyl phosphatidyl inositol anchor domain of a *Y. lipolytica* cell wall protein

[d] YALI0C09031p is the closest homologue of *S. cerevisiae* Flo1p detected in *Y. lipolytica*

[e] YALI0B20306p is homologous to *S. cerevisiae* Pir4p, a protein internal repeat-cell wall protein (Pir-CWP)

[f] YALI0D22396p is homologous to *S. cerevisiae* endochitinase CTS1p, which carries a chitin binding module (CBM)

[g] The minimal *XPR2*t was engineered to contain an *Asc*I site that allows targeting integration into the genomic *XPR2*t region

the monolayer phospholipidic membrane of plant oleosomes. The engineered oleosomes were purified, using floating centrifugation, as highly stable nanoparticles with a mean diameter of 200 to 300 nm (Han et al. 2013). The feasibility of co-displaying multiple proteins on *Y. lipolytica* oleosomes was demonstrated by using the high-affinity binding properties of the cellulosome scaffolding system from *Clostridium cellulolyticum*: Oleosomes displaying a cohesin domain were able to interact with different fusion proteins bearing a dockerin domain, thus forming multi-functional nanoparticles able to target specific cells and to provide new catalytic functions at their surface (Han et al. 2013).

The relatively recent development of several surface display systems now allows using *Y. lipolytica* as arming yeast, which increases its possibilities for various applications such as immobilized biocatalysis or bioconversion, biosensor and live vaccine development, or high-throughput screening of new enzymatic activities. The first example of surface displaying a heterologous protein in *Y. lipolytica* used its fusion to the glycosylphosphatidylinositol (GPI) anchor domain of a cell wall protein (Yue et al. 2008). In this GPI-anchoring system, the C-terminal domain of YlCWP1p served as a signal for creating a covalent bond between the fusion protein and cell wall β-1,6 glucans, via a GPI anchor (post-translational modification including a phosphoethanolamine linker, a glycan core, and a phospholipid tail). Several heterologous proteins have been displayed successfully using this system (reviewed by Madzak and Beckerich 2013), and GPI anchor domains from other *Y. lipolytica* cell wall proteins have also been used for the same purpose (Table 2) (Yuzbasheva et al. 2010; Moon et al. 2013). Besides GPI anchor domains, three alternative systems have been used for surface displaying heterologous proteins in *Y. lipolytica* (Table 2): (i) fusion with homologous (Yuzbasheva et al. 2010) or heterologous (Yang et al. 2009) flocculation domains which bind non-covalently to cell surface mannan chains, (ii) fusion with a protein internal repeat (Pir) domain which binds covalently to cell wall β-1,3 glucans and, via disulfide bonds, to structural proteins, and (iii) fusion with a chitin binding module (CBM) which binds non-covalently to cell surface via interactions with chitin from cell wall (Duquesne et al. 2014).

Heterologous protein production and pathway engineering

The use of *Y. lipolytica* for obtaining industrially valuable compounds has developed in two interconnected directions: as a host for production of heterologous proteins and as an engineered whole cell biocatalyst. Using *Y. lipolytica* as a host sometimes requires modifying its metabolism, notably developing humanized glyco-engineered strains. Using *Y. lipolytica* as a microbial factory often requires introducing new enzymatic activities by expressing foreign proteins. A genome-scale metabolic model for *Y. lipolytica* has been recently produced by a consortium of French research teams (Loira et al. 2012), using a scaffold derived from the well-studied but phylogenetically distant yeast *S. cerevisiae*. This metabolic model constitutes a powerful tool that will be helpful for metabolic engineering and high-throughput data analysis. As production of heterologous proteins in *Y. lipolytica* has been extensively reviewed recently (Madzak and Beckerich 2013), this mini-review will focus on the production of enzymes of economic significance (notably lipases, proteases, amylases, mannanases, and laccases) and on recent advances in genetic engineering of metabolic pathways. Among paramount data, a few selected examples of recombinant proteins or metabolic products production in *Y. lipolytica* are shown in Table 3, especially those highlighting successful effects of expression amplification, gene design, optimized cultivation, or metabolic engineering.

Heterologous production of industrially relevant enzymes

Lipases are a subclass of esterases that catalyzes the hydrolysis of lipids. They have various applications in food and detergent industries, in bioremediation (wastewater treatment), or as biocatalysts for organic chemistry and biodiesel production. They can also be used in exocrine pancreatic insufficiency treatments, like the ERT developed by Mayoly Spindler (France) with a recombinant strain overexpressing homologous YlLip2p (Pignède et al. 2000). An arming *Y. lipolytica* strain displaying YlLip2p has also been designed for use as whole-cell biocatalyst (Yuzbasheva et al. 2011). Extracellular lipases from *Rhizopus* genus are noticeable industrial enzymes, in which properties vary depending on post-translational modifications. One of these fungal lipases has been displayed on *Y. lipolytica* cells, for bioremediation (Song et al. 2011). Another was secreted by *Y. lipolytica* as a post-translationally modified form exhibiting increased thermostability (Yuzbashev et al. 2012). The *Y. lipolytica* high-throughput expression platform (INRA-CNRS-INSA, France—Bordes et al. 2007) has been applied to the engineering of *Candida antarctica* lipase B, one of the main enzymes for industrial biocatalysis, and allowed identifying new mutants with higher catalytic efficiencies (Emond et al. 2010).

Proteases include different classes of enzymes able to hydrolyze peptide bonds by various catalytic mechanisms. They have numerous applications, mainly in food and detergent industries, but also in wastewater treatment and in the medical domain. Two fungal alkaline proteases, with potential applications in the production of bioactive peptides, have been either secreted by *Y. lipolytica* (Ni et al. 2008) or displayed on its cell surface (Ni et al. 2009). A fungal acid protease has also been either secreted by *Y. lipolytica* (Yu et al. 2010a) or

Table 3 Selected examples of recombinant proteins or metabolic products production in *Y. lipolytica*

Protein (size) from species (phylogenic origin) or engineered metabolic pathway		
Construction (A/, B/) or product (C/)	Yield/activity (cultivation conditions)	Reference
A/ Production of industrially relevant or therapeutic enzymes		
Overexpressed extracellular lipase *YlLIP2* (39 kDa)		
Secretion from multi-integrative vector (16 copies) using p*POX2* and *LIP2* prepro	1,500,000 U/l (×30 compared to 50,000 U/l for untransformed strain) and 560 mg/l (shake flask) 10,000,000 U/l (×6.7) (batch)	Pignède et al. 2000
Secretion from multi-integrative vector using hp4d and *LIP2* prepro	11,500,000 U/l (batch) 90,500,000 U/l (×7.9) (fed-batch)	Nicaud et al. 2002
Lipase B (34 kDa) from *Candida antarctica* (Ascomycota)		
Secretion from mono-integrative vector using p*POX2* and *LIP2* prepro	510 U/l (shake flask) 5090 U/l (×10) and 190 mg/l (batch)	Emond et al. 2010
Lipase (30 kDa) from *Rhizopus oryzae* (Zygomycota)		
Secretion from mono-integrative vector using p*XPR2* and either of native prepro signal, *XPR2* prepro, or *XPR2* pre+native pro signal	Respectively: 3230, 1450, and 7610 U/l (shake flask)	Yuzbashev et al. 2012
α-Amylase (45 kDa) from *Oryza sativa* (Angiosperms, Monocotyledons)		
Secretion from mono-integrative vector using p*XPR2* and native secretion signal	31,200 U/l (fed-batch)	Chang et al. 1998
	88,000 U/l (one-step feeding fed-batch)	Kim et al. 2000
Endo-β-1,4-mannanase (42 kDa) from *Aspergillus aculeatus* (Ascomycota)		
Secretion from mono-integrative vector using hp4d and *LIP2* prepro	123 nkat/ml (shake flask) 685 nkat/ml (×5.6) (batch)	Roth et al. 2009
Same construction with multi-integrative vector (two copies)	496 nkat/ml (×4) (shake flask)	
Secretion from multi-integrative vector (nine copies) using hp4d and native secretion signal	13,073 nkat/ml (shake flask) 26,139 nkat/ml (×2) (fed-batch)	
	40,835 nkat/ml (×3) (improved fed-batch)	van Zyl 2010
Laccase I (54 kDa) from *Pycnoporus cinnabarinus* (Basidiomycota)		
Secretion from mono-integrative vector using hp4d and prepro	8.5 mg/l (shake flask) 20 mg/l (×2.4) (batch)	Madzak et al. 2005
Prochymosin (40 kDa) from *Bos taurus taurus* (Mammals)		
Secretion from mono-integrative vector using hp4d and *XPR2* prepro	20 mg/l (shake flask) 160 mg/l (×8) (batch)	Madzak et al. 2000
α2b-interferon (19 kDa) from *Homo sapiens sapiens* (Mammals)		
Secretion from mono-integrative vector using p*POX2* and *LIP2* prepro	5 mg/l (shake flask)	Gasmi et al. 2011a
Same construction with synthetic gene adapted to *Y. lipolytica* codon bias	56 mg/l (×11) (shake flask)	
Same construction with synthetic gene using *LIP2* pre+dipeptides	95 mg/l (×19) (shake flask)	
	425 mg/l (×85) (fed-batch)	Gasmi et al. 2011b
Leucine aminopeptidase II (90 kDa) from *Aspergillus oryzae* (Ascomycota)		
Secretion from mono-integrative vector using hp4d and hybrid *LIP2*/*XPR2* prepro	320 U/l (batch)	Nicaud et al. 2002
Same construction with multi-integrative vector	2500 U/l (×7.8) (batch) 28,000 U/l (×88) (fed-batch)	
B/ Recombinant *Y. lipolytica* strains for use as whole-cell biocatalysts		
Strain expressing epoxide hydrolase (46 kDa) from *Rhodotorula araucariae* (Basidiomycota)		
From mono-integrative vector using hp4d	2400 U/l (shake flask)	Maharajh et al. 2008a
Same construction with multi-integrative vector	5400 U/l (×2.2) (shake flask) 22,750 U/l (×9.5) (batch) 194,700 U/l (×81) (fed-batch)	
	206,000 U/l (×86) (+exponential feed rate)	Maharajh et al. 2008b

Table 3 (continued)

Protein (size) from species (phylogenic origin) or engineered metabolic pathway		
Strain expressing cytochrome P450 1A1 (16 kDa) from *Homo sapiens sapiens* (Mammals)		
From mono-integrative vector using p*POX2*	32 u (pM/min/dw) (shake flask)	Nthangeni et al. 2004
Same construction with surexpression of *YlCPR* using pPOX2	65 u (×2) (shake flask)	
From multi-integrative vector using p*POX2*	129 u (×4) (shake flask)	
Same construction with surexpression of *YlCPR* using pPOX2	1645 u (×51) (shake flask)	
C/ Engineering *Y. lipolytica* strains for products production (see details on genetic engineering in the text)		
Metabolic engineering for production of organic acids		
Citric acid (CA) produced from sucrose	140 g/l (fed-batch)	Förster et al. 2007
Citric acid (CA) produced from inulin	84 g/l (batch)	Liu et al. 2013
Isocitric acid (ICA) produced from sunflower oil	105 g/l, with ICA/CA ratio of 70 % (shake flask)	Holz et al. 2009
α-Ketoglutaric acid (KGA) produced from glycerol	62.5 g/l (batch)	Yin et al. 2012
α-Ketoglutaric acid (KGA) prod. from raw glycerol	186 g/l (batch)	Yovkova et al. 2014
Succinic acid (SA) produced from glycerol	25 g/l (fed-batch)	Jost et al. 2015
Metabolic engineering for production of polyunsaturated fatty acids (PUFAs)		
α-Linolenic acid (ALA)	28 % of DCW (shake flask)	Damude et al. 2006
Eicosapentaenoic acid (EPA)	15 % of DCW and 57 % of lipids (shake flask)	Xue et al. 2013
γ-Linolenic acid (GLA)	20 % of lipids and 44 % of TAG fraction (shake flask)	Chuang et al. 2010
Metabolic engineering for production of carotenoids		
Lycopene	16 mg/g DCW (fed-batch)	Matthäus et al. 2014
β-carotene	2 mg/g DCW	Gao et al. 2014

displayed on its cell surface (Yu et al. 2010b), for potential use as rennet substitute in cheese industry.

Alpha-amylases are glycoside hydrolases of major importance for starch processing applications. *Y. lipolytica* has been used as production host for two industrially relevant α-amylases, from a mesothermophilic bacteria (Yang et al. 2010) and from an insect pest of rice (Celińska et al. 2014).

Beta-mannanases are hemicellulases able to hydrolyze 1, 4-β-D-mannosidic linkages from the main chains of mannan and derivatives. As such, they play a significant role in the bioconversion of lignocellulosic biomass and can be applied to pulp bleaching. They are also employed in food and feed industries, in detergent industry and in oil drilling (Yang et al. 2009). A fungal β-mannanase has been secreted in *Y. lipolytica* and its production optimized from a multicopy strain (Roth et al. 2009). An arming *Y. lipolytica* strain displaying a bacterial β-mannanase has been constructed and proposed for use as whole-cell biocatalyst in industrial processes (Yang et al. 2009).

Laccases are multi-copper oxidoreductases able to oxidize a large range of phenols and arylamines. Fungal laccases are involved in lignin catabolism and can be applied to the degradation of pesticides, dyes, and polycyclic aromatic hydrocarbons (PAHs), which makes them environmentally relevant enzymes (Mougin et al. 2003). Three laccases from Basidiomycota have been secreted in *Y. lipolytica* (Madzak et al. 2005; Jolivalt et al. 2005; Theerachat et al. 2012) and, for two of them, improved by genetic engineering (Madzak et al. 2006; Theerachat et al. 2012). These enzymes could find applications in the biodegradation of xenobiotics (Galli et al. 2011) and/or in the development of oxygen cathodes in microbial biofuel cells (Sané et al. 2014).

Design of glyco-engineered strains for therapeutic applications

Using yeasts as host for producing recombinant therapeutic proteins has been at first impaired by the differences existing between their N-glycosylation pathway and the mammalian one. Namely, yeasts modify glycoproteins with high mannose-type N-glycans, which often reduces in vivo protein half-life and can also be immunogenic in humans and other mammals. This prompted the development of glyco-engineered "humanized" strains in the few yeast species commonly used for heterologous production, as recently reviewed by Kim et al. (2014). Exploration of the genetics of N-glycosylation in *Y. lipolytica* has already allowed some research groups from South Korea and Belgium to design and

patent strains producing more human-compatible glycoproteins, which will serve as starting hosts for further glycoengineering (Kang et al. 2007; Callewaert et al. 2007). A *Y. lipolytica* strain deleted for α-1,6-mannosyltransferase YlOCH1p was shown to synthesize only the core oligosaccharide $Man_8GlcNAc_2$ (Song et al. 2007), when wild-type strains synthesize oligosaccharides with heterogeneous sizes (up to $Man_{12}GlcNAc_2$). After demonstrating that YlMPO1p was necessary for the mannosylphosphorylation of N-linked oligosaccharides, the same consortium of South Korean research groups constructed a Δ*och1* Δ*mpo1* double mutant strain lacking yeast-specific hypermannosylation and mannosyl phosphorylation (Park et al. 2011). In order to develop a glyco-engineered *Y. lipolytica* strain with mannose trimming activity, this Δ*och1* Δ*mpo1* strain was recently engineered further by surface display of a fungal α-1,2-mannosidase. This arming strain was able to convert $Man_8GlcNAc_2$ to $Man_5GlcNAc_2$, not only in activity tests, but also on a large portion of its own cell wall mannoproteins (Moon et al. 2013), demonstrating its efficiency not only for in vitro trimming but also for in vivo trimming of N-glycans during the secretion process.

A consortium of Belgian research groups, including Oxyrane (Belgium), also designed *Y. lipolytica* engineered strains producing glycoproteins homogenously carrying either $Man_8GlcNAc_2$ or $Man_5GlcNAc_2$. They constructed a strain lacking both yeast-specific Golgi α-1,6-mannosyltransferases YlOch1p and YlMnn9p, which produced homogeneous $Man_8GlcNAc_2$ N-glycans. This strain was modified further by heterologous expression of an ER-targeted fungal α-1,2-mannosidase, in order to be able to produce homogeneous $Man_5GlcNAc_2$ residues (De Pourcq et al. 2012a).

The same Belgian consortium has also engineered N-glycan biosynthesis in *Y. lipolytica* for production of N-glycoproteins homogenously carrying a $Man_3GlcNAc_2$ core. The involved steps were the following (De Pourcq et al. 2012b): (i) disruption of *YlAlg3* α-1,3-mannosyltransferase, (ii) overexpression of *YlAlg6* glucosyltransferase, (iii) heterologous overexpression of a fungal glucosidase, and (iv) overexpression of the same ER-targeted fungal α-1,2-mannosidase. Interestingly, the resulting $Man_3GlcNAc_2$ core produced is common to all mammalian N-glycan structures and can be modified further in vitro to yield any of the complex type N-glycans, providing that adequate glycosyltranferases and sugar-nucleotide donors were used (De Pourcq et al. 2012b). These new expression platforms will promote *Y. lipolytica* as a competitive heterologous host able to produce humanized N-linked oligosaccharides compatible with therapeutic applications.

The same consortium of Belgian research groups, including Oxyrane (Belgium), has also developed a glyco-engineered *Y. lipolytica* strain expressing a bacterial glycosidase, in which the level of mannose-6-phosphate modification of glycoproteins was increased. This strain was designed for producing recombinant human lysosomal enzymes for ERTs of lysosomal storage diseases. It was applied to the production of a recombinant Pompe disease enzyme, an α-glucosidase enriched in mannose-6-phosphate, which enabled its efficient targeting to the lysosomes of diseased cells via interaction with specific receptors (Tiels et al. 2012). This technology has high potential for the production of cost effective and more efficient enzymes for replacement therapy of lysosomal diseases.

Use of recombinant *Y. lipolytica* strains as whole-cell biocatalysts

Epoxide hydrolase-expressing Y. lipolytica strains

Epoxide hydrolases (EHs) are able to catalyze the hydrolysis of epoxides into their corresponding diols. Fungal EHs are particularly enantioselective in this process, which makes them interesting as catalysts in the kinetic resolution of various epoxides for applications in fine chemicals and pharmaceutical industries (Labuschagné 2005). Several heterologous EH genes of various phylogenetic origins (from a bacterium, fungi, a plant, an insect, or a mammal) have been expressed intracellularly in *Y. lipolytica* by a consortium of South African research groups (Labuschagné 2005; Labuschagné and Albertyn 2007; Maharajh et al. 2008a, b). For recombinant strains producing fungal EHs from *Rhodotorula* genus, both activities and enantiomeric selectivities in the whole-cell biotransformation of a racemic substrate were higher than those observed with the native fungi (Labuschagné and Albertyn 2007; Maharajh et al. 2008a). The process was optimized using a multicopy strain and an exponential feed model of fermentation (Maharajh et al. 2008a, b). The spin-off company Oxyrane (UK) has constructed a portfolio of EH-engineered *Y. lipolytica* strains that constitutes a biotransformation platform for whole-cell enantiomeric bioconversion of racemic epoxides (Pienaar et al. 2008).

Cytochrome P450-expressing Y. lipolytica strains

Eukaryotic cytochromes P450 (CYP450s) are a superfamily of membrane-bound, haem-containing mono-oxygenases involved in the metabolism of drugs, steroid hormones, and fatty acids. Their heterologous expression is both of fundamental (investigation of their role in xenobiotics and drug degradation pathways) and of economical interest (use in bioconversion for synthesis of hydroxylated biomolecules in biotechnological or therapeutic applications), as reviewed by Novikova et al. (2009). Several heterologous CYP450 genes of various phylogenetic origins (from a fungus, a plant, or mammals) have been expressed intracellularly in *Y. lipolytica*, from fatty acid inducible promoters (p*POX2*, p*ICL1*), for use in whole-cell biocatalysis (Shiningavamwe

et al. 2006; Bourel et al. 2004; Nthangeni et al. 2004; Braun et al. 2012). As, in contrast to plant CYP450, fungal and mammalian CYP450s require a cofactor, the concomitant expression of the corresponding heterologous cytochrome P450 reductase (CPR) (Braun et al. 2012) or overexpression of the homologous *YlCPR* (Shiningavamwe et al. 2006; Nthangeni et al. 2004; Braun et al. 2012) was necessary. These studies demonstrated the potential of *Y. lipolytica* for expression of fungal or mammalian CYP450s and the in vivo efficiency of *YlCPR* in shuttling electrons to these CYP450s, although human CPR was found to be a more efficient redox partner for human CYP450 (Braun et al. 2012). The *Y. lipolytica* strain expressing a plant CYP450 (a fatty acid hydroperoxide lyase from *Capsicum annuum*) was proposed as a whole-cell biocatalyst for the synthesis of volatile aldehydes, for use in the aroma industry (Bourel et al. 2004). A research group from the Graz University of Technology (Austria) demonstrated the potential of two recombinant *Y. lipolytica* strains expressing human liver CYP450s (CYP2D6 or CYP3A4 genes) and human CPR as whole-cell biocatalysts for the oxidation of hydrophobic steroids in a two-liquid biphasic culture (Braun et al. 2012). In this biphasic system, cells and water-soluble nutrients were maintained in an aqueous phase, when substrates and most of the products were in a second water-immiscible organic solvent phase, which enabled more efficient bioconversion and simplified continuous process (Braun et al. 2012). This work took advantage of the lipophilic properties of *Y. lipolytica* to propose it as a model for the hydroxylation of other poorly soluble substrates to molecules of biotechnological or therapeutic interest.

Engineering *Y. lipolytica* metabolic pathways for organic acid production

Although wild-type *Y. lipolytica* strains have for decades attracted interest as producers of several organic acids, such as pyruvic (PA), citric (CA), isocitric (ICA), α-ketoglutaric (KGA), and succinic acids (SA), engineering strains for such applications is much more recent. Selected examples and yields are given in Table 3 (part C/). A German research group from Dresden University reported that overexpressing aconitase gene (*YlACO1*) in a multicopy system, in the wild-type H222 strain, resulted in a shift toward ICA of the CA/ICA ratio in organic acids produced from sunflower oil: ICA proportion increased from approximately 40 % in the parent strain to around 70 % in the engineered strain (Holz et al. 2009). The same research group used an engineered H222 strain, harboring an invertase cassette (p*XPR2*:*ScSUC2*) and multiple (15) *YlICL1* copies to enhance CA production (Förster et al. 2007). A Chinese research group from OUC has displayed a fungal exo-inulinase on the surface of marine SWJ-1b *Y. lipolytica* strain in order to produce CA and ICA from inulin-containing low-cost raw materials (Liu et al. 2010). This recombinant strain was engineered further, by deleting ATP-citrate lyase gene (*YlACL1*) and overexpressing *YlICL1*, in order to increase CA production from inulin (Liu et al. 2013). A consortium of research groups from Jiangnan University (China) explored several engineering strategies on WSH-Z06 strain, a natural KGA overproducer, in order to enhance KGA production while reducing by-product (mainly PA) accumulation (Yin et al. 2012; Zhou et al. 2012; Guo et al. 2014). The most successful approaches were the expression of a fungal pyruvate carboxylase gene (Yin et al. 2012), the heterologous expression of a mammalian ATP-citrate lyase gene (Zhou et al. 2012), or the overexpression of the α subunit of the E1 component from the pyruvate dehydrogenase complex (*YlPDA1*) (Guo et al. 2014), with increases of KGA yields in the range of one third compared to the wild-type strain. Similarly, a consortium of German research groups (centered on Dresden University and UBZ from Leipzig) engineered H355 strain (a derivative of the wild-type H222 strain with high KGA productivity from alkanes) for improved KGA production using raw glycerol as a renewable carbon source. The effects of overexpressing the following genes in a multicopy system were investigated: three genes encoding the KGA dehydrogenase complex (*YlKGD1*, *YlKGD2*, *YlLPD1*), the fumarase (*YlFUM1*), and the pyruvate carboxylase (*YlPYC1*) genes. These studies showed that modifying the expression level of these genes affected both organic acid yields and product spectrum, in a production condition-dependent manner (Holz et al. 2011; Otto et al. 2012). The overexpression of *YlFUM1* was shown to drive a substantial increase in KGA yield under adapted production conditions (Otto et al. 2012). More recently, the same Dresden and Leipzig research groups established that overexpression of *YlIDP1* gene (encoding $NADP^+$-dependent isocitrate dehydrogenase) or preferably simultaneous overexpression of *YlIDP1* and *YlPYC1* had the strongest positive effects on KGA production from raw glycerol (Yovkova et al. 2014). Under adapted production conditions, the engineered strain co-overexpressing *YlIDP1* and *YlPYC1* produced nearly 20 % more KGA than the parent H355 strain (Yovkova et al. 2014). Very recently, the same German research groups showed that reducing the expression of *Y. lipolytica* succinate dehydrogenase gene by a promoter exchange, in wild-type H222 strain, increased SA production by a more than 12-fold factor (Jost et al. 2015).

Engineering *Y. lipolytica* lipid metabolism pathways

As attested by the presence in its genome of several multigene families involved in lipid metabolism pathways, *Y. lipolytica* is an oleaginous yeast able to use a wide range of hydrophobic substrates (notably n-alkanes, fatty acids, and triacylglycerols (TAGs)). It can accumulate lipids up to more than 40 % of its dry cell weight (DCW) and is the only yeast known in which

the proportion of linoleic acid is so high (more than 50 % of fatty acids). It is also the only one for which efficient genetic tools are available. *Y. lipolytica* constitutes a model organism for hydrophobic substrate degradation and lipid accumulation, which have been studied notably at INRA (France) (Beopoulos et al. 2008, 2009a, b) and at Tokyo University (Japan) (Fukuda 2013; Iwama et al. 2014). Depending on C/N ratio, different metabolisms predominate in *Y. lipolytica*: cell growth, organic acid production, or lipid accumulation (mainly TAGs and steryl esters), which occurs notably under nitrogen limitation via de novo synthesis or ex novo accumulation (Beopoulos et al. 2009a). Lipid accumulation has been shown to depend on the peroxisomal β-oxidation flux, regulated by six acyl-CoA oxidases with different substrate specificities (genes *POX1* to *POX6*). The size and fatty acid composition of intracellular lipid bodies is determined by the *POX* genotype. For example, the overexpression of *POX2* in a quadruple mutant Δ*pox2-5* resulted in an "obese yeast" phenotype (Beopoulos et al. 2009a). The same INRA research group obtained, by combining mutations in the glycerol-3-phosphate shuttle pathway (Δ*gut2* and overexpression of *YlGPD1*) with inactivation of the β-oxidation pathway (*POX1-6* or *MFE1* genes), strains that accumulated lipids up to 75 % of DCW (Dulermo and Nicaud 2011). Several research groups from Jiangnan University (China) enhanced lipid accumulation in *Y. lipolytica* by increasing conversion of citrate to acetyl-CoA via expression of a mammalian ATP-citrate lyase gene (Zhang et al. 2014). In consequence of the oleaginous status of *Y. lipolytica*, most proposed applications involve engineering its lipid metabolism pathways. They took advantage of the very effective uptake of lipids, characteristic of this yeast, which can be attributed to several factors: production of bio-surfactants (e.g., liposan), action of extracellular lipases, modification of cell surface facilitating adhesion of hydrophobic droplets (formation of protrusions), and complex transport mechanisms (Beopoulos et al. 2009a). Some major examples of the engineering of *Y. lipolytica* lipid metabolism pathways for bioconversion will be examined further.

Single-cell oil production for health applications

Microorganisms can be a valuable source of edible SCOs enriched in essential fatty acids, namely polyunsaturated fatty acids (PUFAs) not synthesized by mammals but essential for health. PUFAs must be supplied by diet, and SCOs can play a role in their supply, as a cheaper and environment-friendly alternative to plant or fish oils. There is a strong economic interest in engineering *Y. lipolytica* ω-3/ω-6 biosynthetic pathway for producing SCO enriched in ω-3 or ω-6 fatty acids for use as dietary supplements in feed or food. The main targets are the ω-3 eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and α-linolenic acid (ALA), and the ω-6 arachidonic acid (AA) and γ-linolenic acid (GLA). Selected examples and yields are given in Table 3 (part C/). Research groups from DuPont Company (USA) have engineered a *Y. lipolytica* strain for ALA production by expressing a bifunctional fungal Δ12/ω3 desaturase (Damude et al. 2006). DuPont Company has also patented a process for optimizing EPA production in *Y. lipolytica* (Picataggio et al. 2003) and has marketed EPA-rich *Y. lipolytica* biomass for use in fish feed (Verlasso™) and EPA-rich SCO for use as dietary supplement (New Harvest™). The corresponding *Y. lipolytica* production strain constitutes the first recombinant microorganisms able to achieve the productivity necessary for commercial EPA production (Xue et al. 2013). It was obtained through extensive remodeling of its metabolic pathways: In order to produce EPA from endogenous LA, while avoiding GLA buildup, the Δ-9 pathway was selected by overexpressing several (more than 20) heterologous enzymes (Δ-9 elongases, Δ-8 desaturases, Δ-5 desaturases, Δ-17 desaturases, Δ12 desaturases, and C16/18 elongases) and by knocking out three homologous genes encoding a lipase, a sterol carrier, and a protein involved in peroxisomal import (Xue et al. 2013). More recently, a consortium of Taiwanese (including Yeastern Biotech Co.) and French (INRA) research groups obtained efficient GLA production in *Y. lipolytica* by co-expressing two Δ6 and Δ12 fungal desaturases (Chuang et al. 2010).

Single-cell oils for biofuel production

The search for renewable fuels as petroleum alternatives has prompted study of SCOs as a potentially viable source of fatty acids for biodiesel synthesis. As an oleaginous yeast able to grow on various agro-industrial by-products or wastes, *Y. lipolytica* constitutes an interesting target for biofuel production. The yeast TAGs can be transesterified into fatty acid methyl esters (FAMEs), which constitute the main component of biodiesel. Transesterification can occur as a distinct in vitro process or can be integrated as a direct in vivo step, in an engineered cell factory. In order to make the use of SCOs for biofuel production economically feasible, low-cost feedstock needs to be employed as the carbon source for both biomass production and neutral lipid synthesis. Various waste feedstocks have been proposed for that purpose, such as biodiesel-derived waste glycerol, lignocellulosic hydrolysates, used oils, sewage, and agricultural or food-processing wastes (Cheirsilp and Louhasakul 2013; Sestric et al. 2014 and references therein). A Chinese research group from OUC has expressed a fungal exo-inulinase into a marine *Y. lipolytica* strain in order to obtain high SCO yields (more than 50 % of DCW) for biofuel production from inulin-containing low-cost raw materials (Zhao et al. 2010). Like other oleaginous yeasts, *Y. lipolytica* lacks the array of

cellulolytic enzymes required to break down cellulose and cannot therefore use vegetal biomass directly as a carbon source. In order to develop the production of SCOs from lignocellulosic biomass, composed of carbohydrate polymers (cellulose, hemicellulose) and lignin (aromatic polymer), research groups from the National Renewable Energy Laboratory (USA) have engineered *Y. lipolytica* strains for expression of fungal cellulases and xylanases (Wei et al. 2014; Wang et al. 2014). A chimeric cellobiohydrolase I, secreted by *Y. lipolytica*, exhibited a synergistic effect on the degradation of cellulosic substrates when associated with an endoglucanase II and a cellobiohydrolase II in a consortia system approach (Wei et al. 2014). Similarly, a synergistic effect on the conversion of xylan to xylose was observed when two fungal xylanases (an endo-xylanase and an exo-xylosidase) secreted by *Y. lipolytica* strains were used (Wang et al. 2014).

In order to establish *Y. lipolytica* as a platform for biofuel production, its metabolic flux needs to be redirected toward TAG synthesis. Besides the obtaining of obese yeast phenotypes at INRA (France), as cited above (Beopoulos et al. 2009a; Dulermo and Nicaud 2011), a research group from Massachusetts Institute of Technology (MIT, USA) co-overexpressed diacylglycerol acyltransferase (final step of the TAG synthesis) and acetyl-CoA carboxylase (first step of fatty acid synthesis), thus obtaining a synergistic effect on lipid production which reached 62 % of DCW (Tai and Stephanopoulos 2013).

Very recently, the department of Chemical engineering from the University of Texas at Austin (USA) brought unprecedented improvement in harnessing *Y. lipolytica* for lipid and biofuel production. They rewired *Y. lipolytica*'s metabolism for maximum de novo lipogenesis by using a combinatorial multiplexing approach (Blazeck et al. 2014). The overexpression of five lipogenesis enzymes was multiplexed with deletions that reduced fatty-acid catabolism by impairing β-oxidation (*mfe1* deletion), peroxisome biogenesis (*pex10* deletion) or both, and coupled with phenotypic induction. This combinatorial multiplexing produced 57 genotypes that were analyzed for their lipogenesis capacity, in comparison with the wild-type strain. The Δ*pex10*, Δ*mfe1*, leucine$^+$, uracil$^+$, and *YlDGA1* (acyl-CoA:diacylglycerol acyltransferase isozyme I) overexpression genotype was selected as the strain exhibiting the highest lipogenesis potential: It allowed increasing the lipid content up to 90 % of DCW, which represents an enhancement of de novo lipid accumulation of 60-fold compared to the parental Po1f strain (Blazeck et al. 2014). This study also advanced the fundamental understanding of lipogenesis by presenting evidence that two of its central tenets, the necessity for nitrogen starvation and citric acid cycling, are not universal. It reported the highest lipid titer obtained for *Y. lipolytica* (25 g/l) and constitutes a stepping stone in creating a robust and efficient platform for industrial production of value-added oleochemicals and biofuels. Previously, the same research groups had also engineered *Y. lipolytica* for pentane production from linoleic acid, by overexpressing a soybean lipoxygenase, revealing the potential of this yeast for short chain n-alkane synthesis (Blazeck et al. 2013b).

Engineering Y. lipolytica for carotenoid production

Carotenoids are lipophilic organic pigments widely used as natural coloring and antioxidant agents for food and feed and also as dietary supplements. DuPont (USA) and Microbia (DSM, USA) (safety data of recombinant β-carotene disclosed in Grenfell-Lee et al. 2014) are independently using GM *Y. lipolytica* for carotenoid production (Groenewald et al. 2014). Selected examples and yields are given in Table 3 (part C/). DuPont Company has engineered *Y. lipolytica* for the production of lycopene, a carotene that is also an intermediate in the biosynthesis of other carotenoids, by expressing three bacterial genes from the CRT cluster (Ye et al. 2012). More recently, the production of unprecedented lycopene levels for a eukaryotic host was reported by a research group from Dresden University (Germany) by using an "obese" strain, developed at INRA (France), in which two bacterial genes from the CRT cluster were expressed and two *Y. lipolytica* rate-limiting genes for isoprenoid biosynthesis were overexpressed (Matthäus et al. 2014). At last, Gao et al. (2014) recently applied a DNA assembler method to engineer *Y. lipolytica* for carotenoid production: Two distinct β-carotene biosynthesis pathways were targeted at the rDNA locus of a *Y. lipolytica* strain, using an in vivo multiple fragment assembly method. The best results, in terms of both pathway construction efficiency and β-carotene production level, were obtained by integrating a selection marker and three expression cassettes, composed of different promoters and terminators, driving expression of two fungal genes from *Mucor circinelloides* (*carB* encoding phytoene dehydrogenase and *carRP* encoding a bifunctional enzyme with lycopene cyclase and phytoene synthase activities) and overexpression of *YlGGS1* (rate-limiting gene for isoprenoid biosynthesis). As high as 20 % of the transformants were found to produce β-carotene, and the best production levels were in the range of 2 mg per g DCW (Gao et al. 2014).

Y. lipolytica as a source of valuable lipid-derived compounds for the future?

Besides its potential as provider for PUFA-enriched SCOs, biofuels, and carotenoids, *Y. lipolytica* is also expected to become a source for other high-value lipid-derived products like polyhydroxyalkanoates (PHAs), wax esters (industrial lubricants), and free hydroxylated fatty acids (HFAs, used as chiral building blocks in fine chemical synthesis). A consortium of research groups, including notably laboratories from Ghent

University (Belgium) and INRA (France), is currently using specific enzymatic pathways from hydrocarbonoclastic bacteria and from other hydrocarbon or lipid-degrading bacteria isolated from contaminated environments, to redirect *Y. lipolytica* lipid metabolism toward the production of valuable lipid-derived compounds (Sabirova et al. 2011). Up to now, the most advanced projects concern PHAs, biodegradable linear polyesters naturally produced by some bacteria from excess supply of sugar or lipids, which can be used for producing bioplastics. Recombinant *Y. lipolytica* strains expressing a peroxisome-targeted bacterial PHA synthase gene were shown to produce various PHA yields, depending on their *POX* genotype (Haddouche et al. 2010). The same research groups were then able to increase PHA production up to more than 7 % of DCW by deleting several genes from *Y. lipolytica* neutral lipid synthesis pathway (*LRO1*, *DGA1*, *DGA2*, and *ARE1*), thus redirecting the fatty acid flux toward β-oxidation (Haddouche et al. 2011). The composition of the PHAs produced was also shown to be dependent on the *Y. lipolytica* β-oxidation multifunctional enzyme, since the use of different variants (deletions or inactivations of the R-3-hydroxyacyl-CoA dehydrogenase domain) influenced the PHA structure (hetero- or homo-polymers) (Haddouche et al. 2011). These studies constitute a stepping stone in establishing *Y. lipolytica* as a potential platform for converting cheap and vastly available lipid substrates into high-value biotechnological, nutritional, or pharmacological products.

Future perspectives

Since several decades, *Y. lipolytica* has promoted interest for multiple uses such as organic acids, SCP, and SCO production or bioremediation and upgrading of industrial wastes. *Y. lipolytica* has also become a model for basic research, and the development of advanced genetic and molecular tools has established it as an efficient platform for heterologous protein expression/secretion. The relatively recent development of surface display and oleosome targeting methods has opened new possibilities of using arming *Y. lipolytica* cells or derived nanoparticles for applications in molecular recognition or bioconversion. Further improvements of the *Y. lipolytica* expression system will probably be realized in the near future, through strain engineering. Complete genome, transcriptome, and proteome data will allow to identify bottlenecks in protein secretion and quality control pathways and to study the response of cells challenged with the stress of overproducing heterologous proteins. The development of high-throughput screening platforms will favor the use of *Y. lipolytica* for protein evolution, production, and structure analysis. The development of -omic approaches and of metabolic modeling will offer new perspectives in metabolic pathway engineering, allowing a better use of *Y. lipolytica* as cell factory. The recent proof of concept of the possible use of DNA assembler methods in *Y. lipolytica* will also facilitate the engineering of this yeast. Some new tools would however be desirable, such as ORFome, knockout or overexpression libraries, a two-hybrid system or fully repressible strong inducible promoters. It remains also critical to know if, despite its GRAS status, *Y. lipolytica* could be socially acceptable for use as a GMM, especially for whole-cell applications in the environmental domain or in food industry.

Conflict of interest The author declares no conflict of interest.

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