

# ***Yarrowia lipolytica* in Biotechnological Applications**

**Abstract** The nonconventional yeast *Yarrowia lipolytica* has been developed as a versatile and attractive tool for a large variety of biotechnological applications. This yeast has several physiological properties with industrial significance. *Y. lipolytica* uses hydrophobic substrates such as *n*-alkanes, oils, fats, and fatty acids as low-cost carbon sources. The yeast is able to produce a set of diverse added-value metabolites when grown on such low-value carbon sources. The useful physiological properties of the yeast have been used in various biotechnological processes ranging from enzymes, organic acids, single cell protein, single cell oil, or heterologous protein production to fatty acids bioconversions or bioremediation of environmental pollutants. This chapter presents a review of biotechnological applications of *Y. lipolytica* as high-throughput yeast in extracellular enzymes, organic acids and heterologous protein production, food and pharmaceutical industry, fine chemistry, and waste treatment as well as covers the recent developments in the application of the yeast in some fields.

**Keywords** *Yarrowia lipolytica* · Biotechnological applications · Extracellular enzymes · Organic acids · Biotransformations · Pharmaceutical · Bioremediation · Biodiesel · Biosensor · Heterologous protein expression

## **1 Extracellular Enzymes Production**

The *Yarrowia lipolytica* degrades very efficiently hydrophobic and unusual substrates. The yeast is well equipped to powerful enzymes in order to be able degrade these substrates (Bankar et al. 2009a).

This yeast has good potential to secrete a set of valuable proteins into the medium in interesting amounts for industrial applications. Extracellular enzymes are one of the most important proteins secreted by this microorganism. The extracellular enzymes included are lipases, alkaline or acid proteases,

phosphatases, RNase, and inulinase. The enzymes could be used in the detergent, food, pharmaceutical, and environmental industries (Beckerich et al. 1998).

## 1.1 Lipases and Esterases

Lipases (E.C. 3.1.1.3) are serine hydrolases defined as triacylglycerol acylhydrolases. They catalyze the hydrolysis of the ester bond of tri-, di-, and monoglycerides of long-chain fatty acids into fatty acids and glycerol. They differ from esterase (EC 3.1.1.1) due to their ability to hydrolyze triglyceride at the lipid-water interface (Fickers et al. 2011).

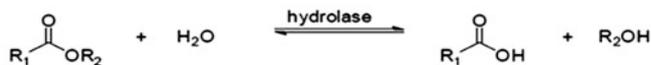
Lipases are primarily responsible for the hydrolysis of acylglycerides. However, a number of other low- and high-molecular weight esters, thiol esters, amides, and polyol/polyacid esters are accepted as substrates by this unique group of enzymes (Gandhi 1997). Some of the reactions catalyzed by lipase are summarized in Fig. 1.

The first step of triglycerides catabolism in *Y. lipolytica* involves their hydrolysis into free fatty acid and glycerol by lipases (Fickers et al. 2005a). This yeast produces several lipases, including intracellular, cell-bound, and extracellular enzymes (Pereira-Meirelles et al. 2000). Lipase production in this microorganism depends on media composition and environmental conditions (Sasarman et al. 2007; Lopes et al. 2008).

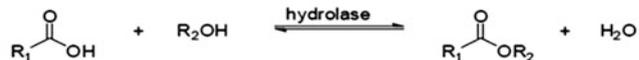
Lipase production in *Y. lipolytica* was first reported by Peters and Nelson in 1948, who described a single glucose-repressible activity with a pH optimum around pH 6.2–6.5 (Peters and Nelson 1948). Then, a cell-bound lipase was detected in this yeast (Sugiura et al. 1976). Ota et al. also described lipase I (39 kDa) and lipase II (44 kDa) as two cell-bound lipases, and an extracellular lipase (Ota et al. 1982). The extracellular lipase activity needs oleic acid as a stabilizer-activator (Ota et al. 1984). The *LIP1* and *LIP3* genes of *Y. lipolytica* were cloned that encode carboxylesterases containing 486 and 498 amino acids (aa), respectively (Choupina et al. 1999). Pignede et al. isolated and characterized the *LIP2* gene, which encodes the extracellular lipase Lip2p with 38.5 kDa molecular weight (Pignede et al. 2000a). Fickers et al. detected *LIP7* and *LIP8* genes belonging to two lipases cell-bound lipases that are easily released by washing the cells with phosphate buffer. A triple deleted strain  $\Delta lip2$ ,  $\Delta lip7$ ,  $\Delta lip8$  in successive gene disruption analysis unable to produce lipase suggested that all lipase genes had been identified (Fickers et al. 2005b).

However, the recent determination of the complete genome sequence of the haploid *Y. lipolytica* strain E150 (CLIB99) by the Génolevures consortium highlights the presence of 16 lipase encoding genes as well as four esterase encoding genes. The lipase family GL3R0084 contained genes *LIP2* (YALI0A20350g), *LIP4* (YALI0E08492g), *LIP5* (YALI0E02640g), *LIP7* (YALI0D19184g), *LIP8* (YALI0B09361g), *LIP9* (YALI0E34507g), *LIP10* (YALI0F11429g), *LIP11* (YALI0D09064g), *LIP12* (YALI0D15906g), *LIP13* (YALI0E00286g), *LIP14*

## 1. Hydrolysis

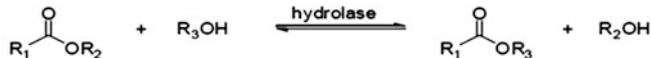


## 2. Esterification

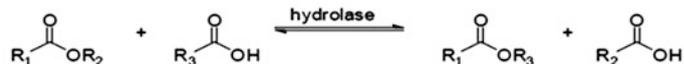


## 3. Transesterification

## alcoholysis



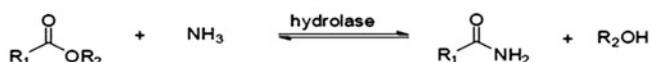
## acidolysis



## 4. Interesterification



## 5. Ammonolysis



**Fig. 1** Some reactions catalyzed by lipase

(YALIOB11858g), *LIP15* (YALIOE11561g), *LIP16* (YALIOD18480g), *LIP17* (YALIOF32131g), *LIP18* (YALIOB20350g), and *LIP19* (YALIOA10439g). Furthermore, the esterase family GL3C3695 presents four members: *LIP1* (YALIOE10659g), *LIP3* (YALIOB08030g), *LIP6* (YALIOC00231g), and *LIP20* (YALIOE05995g). Little information about all these paralogs is available yet (Fickers et al. 2011).

The *LIP2* gene encodes the major extracellular lipase, Lip2p. It is responsible for all of the extracellular lipase activity of *Y. lipolytica*, whose expression is repressed by glucose and induced by olive oil and oleic acid (Pignede et al. 2000a). The *Y. lipolytica* extracellular lipase as biocatalyst has various biotechnological processes in the pharmaceutical, food, and environmental industries. Hence, production of this enzyme on a large scale is of noticeable interest in the industrial microbiology and biotechnology sectors (Darvishi et al. 2009).

The classical and modern genetic methods have been used to reach a high level of lipase production (Thevenieau et al. 2007). In a classical genetic approach, Destain et al. isolated overproducing mutants by successive rounds of chemical mutagenesis using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) from the wild-type strain CBS6303. This led to the selection of the second-generation mutant LgX64.81, which produces about 1,200 U/mL in 500-L large-scale bioreactor. This amount is 25-fold more lipase than the wild-type (Destain et al. 1997). Darvishi et al. also obtained a mutant strain U6 from the wild-type strain *Y. lipolytica* DSM3286 using ultraviolet (UV) light. This mutant produced 356 U/mL of lipase in 20-L bioreactor

after 24 h, which is about 10.5-fold higher than the wild-type strain lipase production (Darvishi et al. 2011).

In a modern genetic approach, Pignede et al. cloned the *LIP2* gene under the control of the strong *POX2* promoter inducible by oleic acid in the vector JMP3 that a resulting multicopy strain JMY184 was able to produce 1,500 U/mL in a flask. The strain JMY184 represents a 30-fold increased lipase production over the wild-type strain level (Pignede et al. 2000a, b).

In a combined approach, Fickers et al. amplified the *LIP2* gene into the LgX64.81 mutant, whose resulting strain JMY1105 was able to produce 26,450 U/mL of lipase. Furthermore, lipase production was reached to 158,246 U/mL after 80 h of cultivation in a 20-L bioreactor by a feeding strategy using tryptone and olive oil at the end of the exponential growth phase (Fickers et al. 2005c).

Furthermore, the *LIP2* gene was cloned and expressed in other yeasts. Yu et al. cloned *LIP2* gene into the pPICZ $\alpha$ A and integrated into the genome of the methylotrophic yeast *Pichia pastoris* X-33. The lipase was successfully expressed and secreted with an apparent molecular weight of 39 kDa using *S. cerevisiae* secretion signal peptide ( $\alpha$ -factor) under the control of the methanol inducible promoter of the alcohol oxidase 1 gene (*AOX1*). The lipase activity of 12,500 U/mL was obtained in fed-batch cultivation when methanol feeding was linked to the dissolved oxygen content after initial glycerol culture (Yu et al. 2007). The *LIP2* gene was also cloned into a constitutive expression vector pGAPZ $\alpha$ A containing glycerol phosphate dehydrogenase (*GAP*) promoter and electrotransformed into the *P. pastoris* X-33 strain. The maximum lipase activity of the recombinant strain was 10,300 and 13,500 in 3- and 10-L bioreactors, respectively. The results show that the *GAP* promoter-derived expression system of *P. pastoris* is effective for the expression of *LIP2* by high cell density culture and is probably an alternative to the conventional *AOX1* promoter expression system in large-scale production of industrial lipases (Wang et al. 2012b). Wang et al. expressed the *LIP2* in *P. pastoris* using the formaldehyde dehydrogenase 1 promoter (*pFLD1*). The maximum lipase activity was 30,000 U/mL in a 10-L bioreactor after 143 h using methanol as the fed-batch feeding substrate, whereas the maximum lipase activity was further increased to 35,000 U/mL by adopting a co-induction strategy with methanol and methylamine as a mixed fed-batch substrates (Wang et al. 2012a). To further improve lipase production in *P. pastoris*, multicopy *LIP2* gene was constructed using high zeocin concentration screening. A recombinant strain obtained with three-copy integration of *LIP2* that produced 42,900 U/mL of lipase. Its lipase production was 2.5-fold higher than a single copy cloned strain (Yu et al. 2010).

The codon-optimized *LIP2* was expressed in *S. cerevisiae* under the control of the galactose-inducible *GALI* and fatty acid inducible *PEX11* promoters with some modifications, including replacement of native Lip2p prepro sequence with the *S. cerevisiae* carboxypeptidase Y (*CPY*) signal sequence, and adding a serine residue for optimal recognition and cleavage by the *S. cerevisiae* Kex2 protease (*LIP2* + Ser) with an inserted serine codon at the aa position 34 by site-directed mutagenesis in the *N*-terminal *LIP2* sequence. The recombinant strains produced

lipase on semisynthetic mineral media containing appropriate amino acid supplements, and either free linoleic acid or trilinolein as well as on laboratory and expensive media (Shockey et al. 2011). However, Darvishi cloned native and mutant *LIP2* genes in *S. cerevisiae* expression vector p426GPD containing strong constitutive *GPD* promoter without any modification from the wild-type strain *Y. lipolytica* DSM3286 and mutant strain U6, respectively. Surprisingly, recombinant *S. cerevisiae* strains expressed and secreted Lip2p on olive oil as cheap substrate. These results show that heterologous proteins of *Y. lipolytica* could be expressed in *S. cerevisiae* without any modifications. It is important because strong components of the *Y. lipolytica* expression/secretion system could be used for high-level production of recombinant proteins in *S. cerevisiae* (Darvishi 2012a).

Lipases from nongenetically modified organism (GMO) origin are required for many biotechnological applications. Hence, lipase production processes based on the nongenetically modified strain such as mutant LgX64.81 have been developed either in a batch or fed-batch bioreactor (Fickers et al. 2011). A stepwise strategy based on methylolate and tryptone feeding was employed for the production of lipase by mutant LgX64.81 in 2,000-L large-scale bioreactor. This strategy permitted a significant increase in lipase production up to 10,000 U/mL after 80 h of culture (Turki et al. 2010).

A common bottleneck for the production of commercial enzymes is their low-stability aqueous solutions. In the downstream processes, immobilization and dehydration methods are the possible ways for obtaining stable and storable enzymes. For immobilization of the extracellular lipase Lip2p, Alloue et al. used three methods including inclusion, adsorption, and covalent bond. Sodium alginate and chitosan polymers were selected to lipase immobilization in inclusion method. The beads of chitosan were more adapted to the inclusion of lipase. The immobilization by adsorption was carried out on both celite and silica gel. Maximum immobilization yield of 76 % was obtained with celite, followed by 43 % in silica gel. Immobilization by covalent bond formation on HiTrap NHS-activated matrix led to an immobilization yield of 70 %. The enzyme immobilized by covalent bond demonstrated greater activity (80 %) after 5 months of storage (Alloue et al. 2008a).

Furthermore, lipase activity and operational stability of *Y. lipolytica* lipase were improved by immobilization on ion-exchange resin D152H as carrier. Under the optimized conditions, the immobilization efficiency was 89.81 % and the specific activity was 2.1-fold higher than that of the free lipase. The immobilized lipase exhibited better thermal and pH stability and broader substrate specificity when used to enrich docosahexaenoic acid (DHA) from the algae *Chlorella protothecoides* oil (Yan et al. 2013).

Lipase liquid stabilization was achieved by monopropylene glycol (MPG), protease inhibitors (P2714 and P8215), and gamma irradiation. The enzyme solution containing 50 % (v/v) of monopropylene glycol and 0.1 % (v/v) of protease inhibitor PI 2714 lost its activity by 80 % at 20 °C after 2 months. To avoid microbial growth and contamination, liquid formulations were gamma-irradiated at 10 kGy. This process led to prevention of microbial growth at least for 24 weeks (Alloue et al. 2008b).

Both spray-drying and freeze-drying as dehydration methods are used to produce a stable lipase powder. Fickers et al. produced large-scale lipase with a lipolytic activity of approximately 1,100 U/mL in a 2,000-L bioreactor after 53 h of fermentation, and then added 12 % of milk powder and 3 % of gum arabic as additives to semi-purified and concentrated lipase solution before spray-drying, which led to production of a stable powder. The vacuum packed powders lose 2.7 % of lipase activity at 4 °C after 12 months. Casein and calcium ions from the milk powder were found to increase the enzymatic activity of the extracellular lipase (Fickers et al. 2006). Addition of 12 % maltodextrin, 6 % gum arabic, and 3 % calcium chloride had a positive effect on the enzyme and increase in lipase activity by 1.5 fold. The resulting powders in aluminum hermetic bags lose about 5 % of lipolytic activity after 30 weeks of shelf-storage at 20 °C (Alloue et al. 2007).

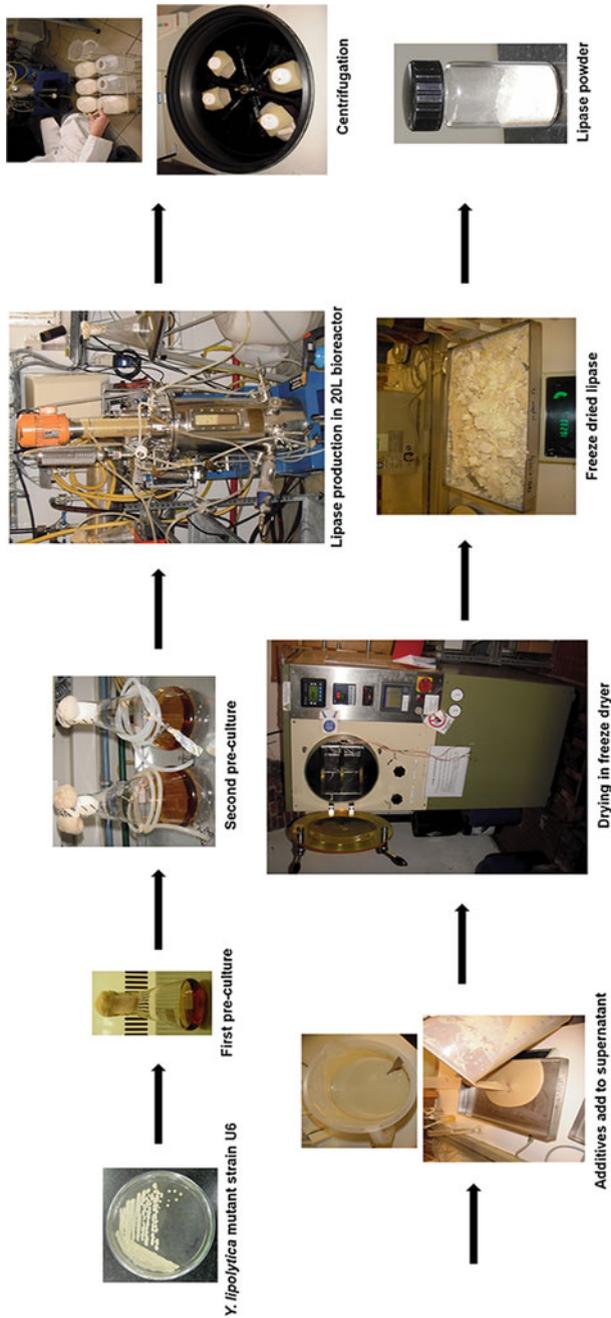
Darvishi et al. evaluated effects of additives on freeze-drying and storage of *Y. lipolytica* lipase (Darvishi et al. 2012). Nonconcentrated cell-free culture supernatant samples were supplemented with different concentrations (0.5–1 % v/v) of maltodextrin and glycerol as additives for freeze-drying process. The formulation with 0.5 % concentration of Maltodextrin gave the best protection of lipase during dehydration treatment, and its freeze-drying yield (77 %) is better than other formulations. Lipase powders were stored at 4 and 25 °C for 46 weeks. Periodic enzyme assays revealed a high stability of the powders, only a 4 % loss of activity was measured after 46 weeks. The fermentation and freeze-drying processes of the *Y. lipolytica* lipase are shown in Fig. 2. Therefore, immobilization and drying of the extracellular lipase from *Y. lipolytica* are valuable methods that lead to decreased cost of utilization, preservation, and transport.

## 1.2 Proteases

The *Y. lipolytica* secretes acidic and alkaline proteases depending on the pH of the growth medium. Proteases production is also controlled by carbon, nitrogen, and sulfur starvation (Ogrydziak and Mortimer 1977). Both proteases are similarly induced at the end of the exponential phase on complex media containing proteins, whereas the type of protease synthesized is strictly dictated by ambient pH (Glover et al. 1997).

Extracellular alkaline protease (AEP) and acid extracellular protease (AXP) are encoded by *XPR2* and *AXP1* genes, respectively. Induction of the *AXP1* gene occurs at acidic pH which leads to secretion of an acid protease (Axp), whereas the *XPR2* gene induce at neutral pH and an alkaline protease (Aep) is produced (Glover et al. 1997).

The *XPR2* gene is one of at least 11 genes controlling extracellular alkaline protease synthesis, secretion, and/or activity (Simms and Ogrydziak 1981). Extracellular alkaline protease is a 32 kDa protease of the subtilisin family, which is intracellularly processed from a 55 kDa glycosylated precursor (Matoba and Ogrydziak 1989).



**Fig. 2** The fermentation and freeze-drying processes of the *Y. lipolytica* lipase (Photographs by Farshad Darvishi 2009)

Upstream activation sequences (UASs) are necessary for transcriptions which are targets for transcriptional activators. The *XPR2* promoter (p*XPR2*) has two major UASs (UAS1 and UAS2) essential for promoter activity. The UAS1 and UAS2 are located 700 and 40 bp upstream from the TATA box, respectively (Blanchinroland et al. 1994). UAS1 can increase activity in all media, but UAS2 seems to be involved in regulation by carbon and nitrogen sources and pH regulation. YIRim101p is a zinc-finger transcriptional activator in Rim pathway whose synthesis and activity are controlled by ambient pH. YIRim101p is activated by a C-terminal truncation at neutral or alkaline pH. This form can bind to PacC-like sites and activate p*XPR2* expression through UAS2 (Madzak et al. 1999).

Acid extracellular protease is a 397 amino acid pepsin-like protein that includes a 44 amino acid prepro-region. The 42 kDa precursor was secreted and at lower pHs (4.0 and 4.6) converted into mature acid extracellular protease; this conversion happened extremely slowly at pH 5.6 and 6.0 (Young et al. 1996; McEwen and Young 1998).

The Rim pathway is also involved in *AXP1* regulation and acid extracellular protease production. In little or no activation of Rim101p, alkaline genes are not induced and acidic genes are not repressed. The *AXP1* gene is not expressed at pH 7.0 when the Rim pathway is disrupted, because production of a specific *AXP1* inducer is dependent on alkaline protease activity. However, it is proposed that induction of *AXP1* is not dependent on Rim pathway for pH sensing. The non-Rim pathway genes *SSY5* and *OPT1* are involved in oligopeptide transport and extracellular amino acids sensing, respectively. At pH 4.0, both *ssy5* and *opt1* mutants make no *AXP1* mRNA suggesting that the signaling pathway(s) involving these genes has a role in *AXP1* expression (Gonzalez-Lopez et al. 2002).

In addition, the MEROPS Peptidase Database (Release 9.1) was used to predict potential extracellular proteases in *Y. lipolytica* CLIB122 (Rawlings et al. 2010). Three families had undergone lineage-specific expansion for *Y. lipolytica* extracellular proteases: Family A1 (pepsin family—endoproteinases) includes *AXP1* and has 30 potentially secreted members that are usually active at acidic pH with an aspartic active site. Family S8 (subtilisin family serine—endoproteinase) includes *XPR2* and has 16 potentially secreted members. The third expanded family is the serine carboxypeptidase family S10 (Ogrydziak 2013).

An integrated recovery method was developed for extracellular acid protease from *Y. lipolytica* cultures. Integrated fluidized bed adsorption was achieved by recirculating whole broth from 2-L bioreactor vessel (Hamilton et al. 2000).

Extracellular alkaline protease is secreted in large amounts (1–2 g/L). As a result, the extracellular alkaline protease promoter and prepro regions have been used to secrete heterologous protein in *Y. lipolytica* (Matoba et al. 1988; Ogrydziak 1988).

Heterologous expression using p*XPR2* in a  $\Delta axp1 \Delta xpr2$  host was commonly done using YPD medium at pH 6.8, while this host cannot degrade the proteins in YPD (Madzak et al. 2000).

*Y. lipolytica* proteases are of interest for biotechnological applications. However, unlike lipase, there are little or no reports on large-scale production and downstream processes of *Y. lipolytica* proteases till now.

### 1.3 Phosphatases

Several phosphatase activities have been found in the *Y. lipolytica*. A cell wall-bound acid phosphatase activity is induced when *Y. lipolytica* is grown on media depleted of inorganic phosphate sources (López and Domínguez 1988). The *PHO2* gene encoded an acid phosphatase with a narrow substrate spectrum. Its synthesis is induced in cells starved for inorganic phosphate (Tréton et al. 1992).

The *Y. lipolytica* produces four patterns of phosphatase activity during growth in the presence or absence of inorganic phosphate in the medium at different pH. The level of all four phosphatase activities depends on the presence of inorganic phosphate in the medium (Galabova et al. 1993).

PP2A as a major intracellular phosphatase has maximum activity in the early exponential growth phase and Pi-deficiency of the culture medium. Exogenous Pi level may control the synthesis and excretion of alkaline and acid phosphatases by *Y. lipolytica*. An extracellular phosphatase is characterized as a 33 kDa phosphotyrosine protein phosphatase with associated phosphoserine/threonine activity. Its optimum pH value is 6.1, apparent  $K_m$  for phosphotyrosine was calculated to be 760 mM, and Hill coefficient 3.2 indicating a rather high cooperativity (Jolivet et al. 1998). An extracellular phosphatase was significantly produced from *Y. lipolytica* W29 in the early stationary phase (Jolivet et al. 2001).

Ito et al. detected an acid phosphatase activity in *Y. lipolytica* with increasing  $\text{Cu}^{2+}$  concentrations in the medium. Furthermore, this enzyme activity was stimulated in vitro by  $\text{Co}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Mn}^{2+}$ , and  $\text{Mg}^{2+}$ . It was also inhibited by  $\text{Ag}^+$  and  $\text{Cd}^{2+}$ . The content of cellular phosphate involving polyphosphate was decreased by adding  $\text{Cu}^{2+}$  even if the medium was rich in inorganic phosphate (Ito et al. 2007b).

### 1.4 RNase

*Y. lipolytica* produces RNases during the exponential growth phase in the presence of high molecular weight nitrogen compounds. Highest levels of RNase production occur in media with pHs 5 and 7. The RNase was secreted rapidly, so that the time between synthesis and appearance in the extracellular medium was about 5 min (Cheng and Ogrydziak 1986).

Several RNases were detected in the supernatant medium. The RNases had estimated molecular weights of 45, 43, and 34 kDa. *Y. lipolytica* secretes only one 45 kDa RNase, where the 43 and 34 kDa RNases are degradation products of this

RNase by alkaline extracellular protease. The 45 kDa RNase could be purified from a mutant that does not produce the alkaline extracellular protease. The 43 kDa RNase is purified from a wild-type strain whose optimum pH is between 6.5 and 7.0 (Cheng and Ogrzydziak 1987).

### 1.5 Asparaginase

L-asparaginase (EC. 3.5.1.1; asparagine amidohydrolase) catalyzes the hydrolysis of L-Asparagine to L-aspartic acid and ammonia. This enzyme is used for the treatment of selected types of hemopoietic diseases such as acute lymphoblastic leukemia (ALL) and non-Hodgkin lymphomas (Pieters et al. 2011; Rytting 2012). It is also a model enzyme for the development of new drug delivery system and L-asparagine biosensor for leukemia. This enzyme was used in the food industry for the production of acrylamide free food (Kumar and Verma 2012; Dhanam Jayam and Kannan 2013).

*Y. lipolytica* is a potential producer of L-asparaginase. However, there are very few reports on L-asparaginase production by the yeast. Karanam and Medicherla optimized L-asparaginase production of *Y. lipolytica* NCIM 3472 in solid-state fermentation (SSF) using palm kernel cake as the substrate. The maximum L-asparaginase activity at optimum conditions was near 40 U/g of the initial dry substrate (U/gds) (Karanam and Medicherla 2010).

### 1.6 Laccase

Lee et al. isolated and identified an extracellular laccase-producing strain of *Y. lipolytica* from soil. Extracellular laccase (YILac) was purified by anion-exchange and gel filtration chromatography. YILac is a monomeric glycoprotein with 14 % carbohydrate content and a molecular weight of 67 kDa. It showed a higher catalytic efficiency toward 2,2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) ( $k_{\text{cat}}/K_m = 19.3 \text{ s}^{-1} \mu\text{M}^{-1}$ ) and 2,6-dimethoxyphenol ( $k_{\text{cat}}/K_m = 13 \text{ s}^{-1} \mu\text{M}^{-1}$ ) than any other reported laccases. This enzyme was able to oxidize phenolic compounds of pretreated rice straw. The use of YILac for the removal of cellulase inhibitory compounds from biomass slurries is a promising approach for improving the efficiency of biorefineries (Lee et al. 2012).

### 1.7 Mannosidase

Exo- $\alpha$ -mannosidases (EC. 3.2.1.24) hydrolyze terminal nonreducing residues of alpha-D-mannose residues in  $\alpha$ -D-mannosides. An exo- $\alpha$ -mannosidase was

characterized in *Y. lipolytica*. The enzyme located in a crude particulate fraction of the cell extract.

This enzyme has an optimum pH of 6.0, a  $K_m$  of 0.27 mM with *p*-nitrophenyl- $\alpha$ -D-mannopyranoside and is partially inhibited by D-mannose. It is not affected by ethylenediaminetetraacetic acid (EDTA), several cations, or sulfhydryl reagents. Its activity was increased by 25 % of  $Zn^{2+}$ . It can be partially solubilized by treatment with digitonine (Vega and Domínguez 1988).

## 1.8 Inulinase

Inulinase (EC. 3.2.1.7) hydrolyzes inulin that is in high demand for the production of high fructose syrups and fuel ethanol. A marine *Y. lipolytica* isolate OUC2 secreted 62.85 U/mL of inulinase. The enzyme has optimal pH and temperature at 5.0 and 60 °C, respectively.

However, no monosaccharides and disaccharides were detected after inulin hydrolysis by the crude inulinase produced by *Y. lipolytica* OUC2, suggesting that the crude inulinase had no exoinulinase activity (Gao et al. 2007).

## 2 Organic Acids Production

The global production of organic acids was estimated near 16 million tons in 2007, of which about 2 ton was produced by microbial process. The organic acids production is increasing at a rate of 5 % per year (Sauer et al. 2008). The organic acids represent the third largest category among the fermentation products (Soccol et al. 2008).

Organic acids constitute a key group among the building-block chemicals that can be produced by microbial processes from renewable carbon sources. Certain groups of bacteria (e.g., acetic acid and lactic acid bacteria) and mycelial fungi (e.g., *Aspergillus niger*) were conventional producers of organic acids. The yeast *Y. lipolytica* secretes high amounts of several organic acids such as citric acid, isocitric acid,  $\alpha$ -ketoglutaric acid, pyruvic acid, and succinic acid from different carbon sources under certain conditions. Furthermore, this yeast is able to produce 2-hydroxyglutaric and 2-ketoglutaric acids from glucose (Oogaki et al. 1983). A leucine auxotrophic strain was used for the production of isopropylmalic acid (Barth and Gaillardin 1996). Hence, *Y. lipolytica* is an attractive candidate for the development of microbial organic acids production processes (Finogenova et al. 2005; Otto et al. 2013).

## 2.1 Citric and Isocitric Acids

Global production of citric acid was over 1.6 million tons in 2007. There is an annual growth of 3.5–4.0 % in the demand/consumption of citric acid (Sauer et al. 2008; Soccol et al. 2006). Citric acid is used mainly in the food and beverage industry, primarily as an acidulant. Other applications of citric acid can be found in detergents and cleaning products, cosmetics and toiletries, and others (Soccol et al. 2006; Berovic and Legisa 2007).

Isocitric acid is formed by aconitase from citric acid via cis-aconitic acid, which can be used as ingredients of washing powder and detergents, as dietary supplement, or as active substance in pharmaceutical products. However, Isocitric acid is only available as expensive fine chemical on the market (Heretsch et al. 2008).

The filamentous fungus *A. niger* is used mainly for large-scale production of citric acid from beet or cane molasses or glucose syrup. *Y. lipolytica* would have several advantages compared to the *Aspergillus*, including a wider substrate range, a smaller sensitivity to low dissolved oxygen concentrations and heavy metals, higher product yield, simple process control, and waste and sewage minimization (Vandenbergh et al. 1999; Darvishi et al. 2009; Singh Dhillon et al. 2011).

Citric acid production and secretion of *Y. lipolytica* is triggered as a result of excess of carbon source and nitrogen source limitation (a high C/N ratio) (Anastassiadis et al. 2002; Il'chenko et al. 2003). Various substrates like alkanes, vegetable oils, fats, glycerol, ethanol, glucose, molasses, and starch hydrolysates were used for citric acid production by *Y. lipolytica* (Finogenova et al. 2005, 2008; Kamzolova et al. 2007, 2008; Levinson et al. 2007; Papanikolaou et al. 2008).

The synthesis of citric and isocitric acids started after complete consumption of the deficient component of the medium and the transition of the culture from the logarithmic phase to the retardation phase, which continued until the carbon source was consumed completely (Otto et al. 2013). Lipase and citric/isocitric acids could be produced by *Y. lipolytica* using renewable low-cost substrates such as plant oils. The maximum organic acids were produced after high level of lipase production, which is an interesting relationship between lipase and organic acids production (Darvishi et al. 2009).

The proportion of isocitric acid was increased by pH values of 5.5–6, oxygen concentration of 60–95 %, and increased ferrum ion concentration (Finogenova et al. 2002). Few studies were directed to improve the production of isocitric acid, because secretion of citric and isocitric acids in the same amount is a disadvantage for citric acid production by *Y. lipolytica*, which depends on the used carbon source. Citric acid was the predominant acid on media contain glucose, whereas citric and isocitric acids were found in equal amounts in media containing hexadecane or other *n*-alkanes. Wild-type strains secreted about 8–16 % isocitric acid on carbohydrates or glycerol, whereas approximately 35–50 % isocitric acid was secreted on alkanes, renewable triglycerides, ethanol, or acetate (Otto et al. 2013).

When the cells were supplied with monofluoroacetate, this compound was transformed into monofluorocitrate which competitively inhibited aconitase. This leads to improve the ratio of citric to isocitric acid to 85:15. In an effort to obtain a strain with low aconitase activity, a mutant fluoroacetate sensitive strain was obtained that produced citric and isocitric acids in a ratio of 97:3 (Barth and Gaillardin 1997). A mutant strain *Y. lipolytica* N1 was generated through random chemical mutagenesis that produced 217 g/L of citric acid on petrolatum as substrate in 5,000-L bioreactor. This mutant also produced 120 g/L of citric acid on ethanol (Finogenova et al. 2005).

In the group of Prof. Barth, metabolic engineering was used for optimization of citric acid production. The *ScSUC2* gene encoding invertase was expressed in *Y. lipolytica* for the use of the cheap substrate sucrose, resulting in strain *Y. lipolytica* H222-S4 (p67ICL1) T5 that produces maximal citric acid amounts of 127–140 g/L with a citric acid yield of 0.75–0.82 g/g in a fed-batch cultivation process on sucrose (Forster et al. 2007a).

The formation of the citric and isocitric acids ratio in *Y. lipolytica* is obviously a complex process influenced by both the activity of tricarboxylic-acid and glyoxylate cycle enzymes (aconitase, citrate synthase, isocitrate dehydrogenases, isocitrate lyase, and malate synthase) and transport processes of citric and isocitric acids through the mitochondrial and plasma membranes (Holz et al. 2009).

For reduction of the proportion of isocitric acid to increase the proportion of citric acid, isocitrate lyase (ICL)—encoding gene *ICL1* copy numbers were increased in a strain. As a result, a strong shift of the citric and isocitric acids ratio in the direction of citric acid was observed. Furthermore, the *icl1* deletion strain showed a moderate 2–5 % increase in the isocitric acid proportion compared to isocitric acid wild-type strains on glucose or glycerol (Forster et al. 2007b).

A recombinant *Y. lipolytica* strain was constructed containing multiple copies of gene *ACO1* encoding aconitase (ACO), whose high-level expression of aconitase in the *ACO1* multicopy integrative transformant resulted in a shift of the citric and isocitric acids ratio product pattern in the direction of isocitric acid. This recombinant exhibits a 7–9 times higher aconitase activity than the original strain even during the production phase. The strain produces more than 70 % isocitric acid on sunflower oil, but the isocitric acid proportion increased only moderately from 10–12 to 13–17 % on glycerol, glucose, or sucrose (Holz et al. 2009).

## 2.2 $\alpha$ -Ketoglutaric Acid

$\alpha$ -Ketoglutaric acid is an intermediate of the tricarboxylic acid cycle and the main compound of amino acid and protein metabolism. This organic acid could be used as building-block chemical for the chemical synthesis of heterocycles, dietary supplement, component of infusion solutions, and wound healing compounds (Otto et al. 2013).

Tsugawa et al. discovered the ability of *Y. lipolytica* to synthesize  $\alpha$ -ketoglutaric acid in 1968, when they studied the production of microbial protein from oil paraffins. *Y. lipolytica* AJ5004 produced about 46 g/L  $\alpha$ -ketoglutaric acid from 8 % (w/v) *n*-paraffin after 72 h (Tsugawa et al. 1969; Tsugawa and Okumura 1969).

Thiamine deficiency is a critical factor in the formation of  $\alpha$ -ketoglutaric acid by *Y. lipolytica*. The yeast strain is unable to synthesize the pyrimidine structure of the thiamine molecule, or a medium with thiamine deficiency required for production of  $\alpha$ -ketoglutaric acid. The  $\alpha$ -ketoglutaric acid excretion was triggered after logarithmic growth phase when the yeast was grown on a medium containing hexadecane or other alkanes with low thiamine concentration (0.7  $\mu$ g/L). The biosynthesis of the acid continued in the stationary phase. It was possible to increase the concentration of  $\alpha$ -ketoglutaric acid to 108.7 g/L on petrolatum with a substrate-related yield of 120 % (Finogenova et al. 2005).

The hyper-producing strain H355 produced highest amounts of  $\alpha$ -ketoglutaric acid up to 195 g/L with a mixture of *n*-paraffins (C<sub>12</sub>–C<sub>18</sub>). Vegetable oils (olive, canola, sunflower, linseed, and rapeseed oil), ethanol, and glycerol were used as substrate for production of  $\alpha$ -ketoglutaric acid by *Y. lipolytica* (Chernyavskaya et al. 1997, 2000; Il'chenko et al. 2001, 2002, 2003; Finogenova et al. 2002; Otto et al. 2013; Kamzolova and Morgunov 2013).

The effects of different conditions on production  $\alpha$ -ketoglutaric acid, including initial substrate concentration, nitrogen source, exogenous vitamins, and calcium carbonate are investigated in some studies. The influence of thiamine limitation and excess of carbon and nitrogen source as well as a low pH on the intensive  $\alpha$ -ketoglutaric acid production are revealed in the previous work. Thiamine and calcium ion concentration had the greatest effect on this organic acid accumulation when glycerol was used as carbon source (Zhou et al. 2010). An increased amount of zinc and iron ions is required for the  $\alpha$ -ketoglutaric acid production from ethanol (Kamzolova et al. 2012a).

Holz et al. constructed strain containing multiple copies of all three  $\alpha$ -ketoglutarate dehydrogenase (KGDH) complex genes encoding the three subunits of the enzyme. The strain showed a reduced production of  $\alpha$ -ketoglutaric acid and an elevated production of pyruvic acid (Holz et al. 2011).

Secreted organic acids as by-products ( $\alpha$ -ketoglutarate and pyruvate as major by-product, fumarate, malate, and succinate as minor by-products) can be affected by enzymes (fumarase or pyruvate carboxylase) involved in  $\alpha$ -ketoglutaric acid production. Hence, the fumarase (FUM) or pyruvate carboxylase (PYC) genes (*FUM1*, *PYCI*) were overexpressed. The production of  $\alpha$ -ketoglutaric acid reached to the range of 137–147 g/L with the multicopy strains H355A (*FUM1*) and H355A (*FUM1*-*PYCI*) (Otto et al. 2012).

### 2.3 Pyruvic Acid

Pyruvic acid is a key position in cell metabolism and is involved in many catabolic and anabolic pathways, including glycolysis, gluconeogenesis, amino acid, and protein metabolism. Pyruvic acid is employed for the production of L-tryptophan, L-tyrosine, and 3,4-dihydroxyphenyl alanine in various industries. The diet supplementation with pyruvic acid increased fat loss and minimized the associated loss of body protein. Pyruvic acid is also used in biochemical researches and medicine as a substrate for assaying activities of such enzymes as pyruvate dehydrogenase, pyruvate carboxylase, and pyruvate decarboxylase (Nakazawa et al. 1972; Yamada et al. 1972; Stanko et al. 1992).

*Y. lipolytica* oxidize glucose and form pyruvic acid (75–80 %) and  $\alpha$ -ketoglutaric acid (20–25 %) under thiamine deficiency conditions. The synthesis of the acid was triggered by a decrease in intracellular thiamine concentration to 3.0  $\mu\text{g}$  per 1 g biomass. An approximately 3-fold increase in the amount of the biomass was associated with a subsequent decrease in thiamine content to the level of 1.0  $\mu\text{g}$  per 1 g biomass, whose maximum production of pyruvic acid was 50 g/L in this condition. In addition to glucose, thiamine-auxotrophic yeasts are capable of synthesizing pyruvic acid when grown on glycerol and propionic acid. Technical-grade glycerol is the most promising raw material for pyruvic acid production. Pyruvic acid was obtained at a concentration of 61 g/L with a yield of 71 % from glycerol (Morgunov et al. 2004; Finogenova et al. 2005).

### 2.4 Succinic Acid

Succinic acid and its derivatives are used as flavoring agents for food and beverages. This acid could be used as feedstock for dyes, insecticides, perfumes, lacquers, as well as in the manufacture of clothing, paint, links, and fibers (McKinlay et al. 2007). Succinic acid is widely used in medicine as an antistress, antihypoxic, and immunity-improving agent, in animal diets, and as a stimulator of plant growth. It is also a component of bio-based polymers such as nylons or polyesters (Kamzolova et al. 2012b). Succinate esters are precursors for the known petrochemical products such as 1,4-butanediol, tetrahydrofuran,  $\gamma$ -butyrolactone, and various pyrrolidinone derivatives (Bechthold et al. 2008).

Succinic acid production by *Y. lipolytica* was reported for the first time when it was grown on ethanol under aerobic conditions and nitrogen limitation. Succinic acid amount was 63.4 g/L as the major product of batch fermentation in this process. However, the disadvantage was low yield of succinic acid on ethanol (58 %), and a high cost of production (Kamzolova et al. 2009).

Kamzolova et al. developed a novel process for the production of succinic acid. It includes the synthesis of  $\alpha$ -ketoglutaric acid by a thiamine-auxotrophic strain *Y. lipolytica* VKMY-2412 from *n*-alkanes, and subsequent oxidation of the acid by

hydrogen peroxide to succinic acid. The concentration of succinic acid and its yield were found to be 38.8 g/L and 82.45 % of *n*-alkane consumed, respectively (Kamzolova et al. 2012b).

Succinic acid production was also studied by genetically modified strains using glucose and glycerol as substrates. Yuzbashev et al. constructed temperature-sensitive mutant strains with mutations in the succinate dehydrogenase encoding gene *SDH1* by in vitro mutagenesis-based approach. Then, the mutants were used to optimize the composition of the media for selection of transformants with the deletion in the *SDH2* gene. The defects of each succinate dehydrogenase subunit prevented the growth on glucose, but the mutant strains grew on glycerol and produced succinate in the presence of the buffering agent CaCO<sub>3</sub>. Subsequent selection of the strain with deleted *SDH2* gene for increased viability was allowed to obtain a strain that is capable to accumulate succinate at the level of more than 450 g/L with buffering and more than 17 g/L without buffering. Therefore, a reduced succinate dehydrogenase activity can lead to an increased succinate production (Yuzbashev et al. 2010). *Y. lipolytica* is able to produce succinic acid at low pH values. High amounts of succinate can be achieved by genetic engineering (Otto et al. 2013).

### 3 Fatty Acid and Alkane Bioconversions

The term bioconversion, also known as biotransformation, refers to the use of live organisms often microorganisms or their derivatives to carry out a chemical reaction that is more costly or not feasible nonbiologically. These chemical reactions are minor changes in molecules, such as the insertion of a hydroxyl, or keto function, or the saturation/desaturation of a complex structure. The growing or resting microorganisms or their enzymes act as biocatalysts in this process.

Bioconversions are normally performed at normal temperatures and pressures, so that no dangerous intermediates and wastes are generated. They have also become essential to the fine chemical industry because of the demand for single-isomer intermediates. The yeast *Y. lipolytica* is able to perform bioconversion of certain compounds including fatty acids, alkanes, steroids, and trinitrotoluene.

Fatty acids resulting from the hydrolysis of lipids by lipases can be entered to different oxidation pathways:  $\omega$ -oxidation of fatty acids to  $\alpha$ ,  $\omega$ -dicarboxylic acids, and the degradative  $\beta$ -oxidation of fatty acids to CO<sub>2</sub> and water. Alkanes are ultimately converted into fatty acids via  $\alpha$ -oxidation pathway (See “*Yarrowia lipolytica*: An Overview,” in Sect. “Physiology and Metabolism,” and also Fig. 5) (Fickers et al. 2005a).

The  $\omega$ -oxidation pathway can lead to production of musk fragrance precursors. These compounds can be obtained from alkanes or lipids through biocatalysis using the alkane-assimilating yeast species. The first step involves avoiding from the passage of the substrates through the  $\beta$ -oxidation pathway. This is done by disrupting the genes coding for the active acyl-CoA oxidases in the  $\beta$ -oxidation

pathway. This step is also enabled to redirect alkanes and fatty acids to  $\omega$ -oxidation. Then the flux of  $\omega$ -oxidation is increased by amplifying the cytochrome P450 monooxygenase and NADPH-cytochrome reductase genes. However, the  $\beta$ -oxidation pathway generates volatile fatty acids, esters, and lactones in fermented food and can be involved in the production of vanillin. The *Y. lipolytica* is an excellent biocatalyst in the field of alkane, lipid, and fatty acid transformation into dicarboxylic acids and lactones (Wache et al. 2006).

There is a current interest in the production of  $\gamma$ - and  $\delta$ -lactones as flavors through microbial procedures, because the procedures are simple, less expensive, and more environment friendly routes than chemical pathways. Furthermore, transforming natural substrates into lactones with a natural label has made them the subject of much research (Waché 2013). The production of lactones from hydroxy fatty acids is needed for the  $\beta$ -oxidation cycle and intramolecular esterification. Lactonization can give  $\gamma$ -,  $\delta$ - or  $\epsilon$ -lactones depending on the position of the hydroxyl group of the carboxylic acid.

$\gamma$ -decalactone ( $\gamma$ -C<sub>10</sub>) is a peach-like and creamy aroma compound of industrial interest that can be produced biotechnologically by some microorganisms. This compound results from the lactonization of 4-hydroxydecanoic acid at low pH. Farbood and Willis patented the  $\gamma$ -decalactone production by *Y. lipolytica* from castor oil (Farbood and Willis 1985). Castor oil is natural and nontoxic oil, biodegradable, and a renewable resource obtained from the seeds of the castor plant *Ricinus communis*. Ricinoleic acid (a hydroxylated C<sub>18</sub> fatty acid; 12-hydroxyoctadec-9-enoic acid) is a major component (about 86 %) of castor oil (Puthli et al. 2006). Castor oil was also used as substrate for production of  $\gamma$ -decalactone by *Y. lipolytica* in other studies (Gomes et al. 2013; Moradi et al. 2013; Braga and Belo 2013). The maximum  $\gamma$ -decalactone concentration of 11 g/L was obtained by *Y. lipolytica* HR145 (DSM12397) on castor oil in >70 h (Wache et al. 2003). *Y. lipolytica* is able to transform ricinoleic acid and methyl ricinoleic acid into  $\gamma$ -decalactone (Ercoli et al. 1992; Pagot et al. 1997; Gomes et al. 2012). *Y. lipolytica* PO1d (*ura3* auxotrophic strain derived from ATCC 24060) produced 9.5 g/l of  $\gamma$ -decalactone from methyl ricinoleic acid after 75 h (Wache et al. 2003).

Peroxisomal  $\beta$ -oxidation was shown to be responsible for the bioconversion ricinoleic acid and methyl ricinoleic acid into  $\gamma$ -decalactone (Endrizzi et al. 1993). *Y. lipolytica* possesses a six-member family of acyl-CoA oxidases (Aox1 to 6 encoded by *POX1* to 6), with the enzymes catalyzing the first step of  $\beta$ -oxidation (Wang et al. 1999).

The Aox3 is involved in the short-chain-specific oxidation after the C<sub>10</sub> level and the disruption of the *POX3* gene decreases lactone degradation (Wache et al. 2000, 2001). Aox4 and Aox5 are non-chain-length-specific acyl-CoA oxidases and their activity is weak. However, they are directed toward the wide range of substrates whereas Aox1 is inactive (Wache et al. 2002). The long-chain-specific Aox2 was significant for conversion of ricinoleic acid into the  $\gamma$ -decalactone. Deleting all the *POX3–5* genes resulted in an increased accumulation and an inhibition of  $\gamma$ -decalactone degradation. Aox4 exhibits a slight activity on a broad spectrum of substrates and is involved in lactone degradation. Hence, a strain was

constructed lacking this activity which produced 10 times more lactone than the wild-type strain in 48 h (Groguenin et al. 2004).

A recombinant of the diploid strain *Y. lipolytica* was constructed with expression of *POX2* gene and disruption of *POX3* genes on two chromosomes, but without disruption of *POX4* and *POX5* genes. This mutant could be grown in the continuous fermentation of methyl ricinoleate and produced 4-fold  $\gamma$ -decalactone compared with the wild-type strain. It could be concluded that the positive effect of Aox2 had a greater influence than negative action of Aox3 on the  $\gamma$ -decalactone production (Guo et al. 2012).

The  $\beta$ -oxidation flux is an important determining factor in the production of  $\gamma$ -decalactone and 3-hydroxy- $\gamma$ -decalactone. The 3-hydroxy-acyl-CoA dehydrogenase activity is reduced in low aeration conditions, because its cofactor regeneration ( $\text{NAD}^+$ ) is not sufficient. Lactone accumulates when the regeneration of NAD is not sufficient, because a decrease in the  $\beta$ -oxidation flux decreases demand for NAD and thus the cofactor is not anymore a limiting compound for the pathway. This phenomenon occurs also when the aeration of cells is modified and this accumulation can be a symptom of upscaling problems in industry. The 3-hydroxy lactone accumulates when aeration is decreased as well as the flux in the oxidation pathway decreases, and thus NAD is again sufficient and 3-hydroxy lactone does not accumulate (Romero-Guido et al. 2011; Świzdor et al. 2012).

Oxygen affects aroma production by *Y. lipolytica*. Cellular growth was stimulated under increased air pressure up to 5 bar and the influence of oxygen has been highlighted on the activities of enzymes of  $\beta$ -oxidation pathway in *Y. lipolytica* (Aguedo et al. 2005). The addition of small amounts of Tween 80 in the medium strongly increases the oxygen transfer and thus improves  $\gamma$ -decalactone production by *Y. lipolytica* (Gomes et al. 2007).

Another factor influencing the yield is the toxicity of the  $\gamma$ -decalactone and its  $\text{C}_{10}$ -precursor against the producing strains. A concentration of 3 g/L of  $\gamma$ -decalactone leads to a 50 % cell death when cultured in glucose while this concentration exhibits no effect on the viability of cells grown in methyl oleate. Lactone caused a sterol depletion which altered cell physiology and an incorporation of sterol into the cellular membrane could make the cells more resistant. Cellular resistance is observed to be linked with the presence of intracellular lipid bodies as the cells possessing more lipid bodies are more resistant toward lactone (Ta et al. 2010, 2012).

The  $\gamma$ -decalactone production was compared using *Y. lipolytica* strain HR 145 (DSM 12397) in 10- and 300-L bioreactors. In the 10-L lab scale bioreactor, the optimum conditions of agitation and aeration for  $\gamma$ -decalactone production were 400 rpm and 3 L/min leading to a production of 12.5 g/L after 52 h. For the 300-L large-scale bioreactor, the best agitation and aeration were 180 rpm and 35 L/min, which yielded similar production after 69 h (Romero-Guido et al. 2011).

In an interesting project entitled LipoYeasts, the *Y. lipolytica* was developed into a versatile and high-throughput microbial factory using specific enzymatic pathways from hydrocarbonoclastic bacteria to efficiently mobilize lipids by directing its versatile lipid metabolism toward the production of industrially

valuable lipid-derived compounds like wax esters (WE), isoprenoid-derived compounds (carotenoids, polyenic carotenoid ester), polyhydroxyalkanoates (PHAs), and free hydroxylated fatty acids (HFAs). Different mutant and recombinant strains of *Y. lipolytica* are being assessed to bioconversion of various lipid stocks (petroleum, alkane, vegetable oil, fatty acid) and combinations thereof as substrates to the added-value products. The main metabolic routes to be optimized are  $\beta$ - and  $\omega$ -oxidations and lipid accumulation. It will be concentrated on the construction of strains with reduced capacity in the storage of fatty acid into lipid bodies and with reduced capacity to degrade fatty acids by either  $\beta$ - or  $\omega$ -oxidation, as shown in Fig. 3 (Sabirova et al. 2011).

## 4 Importance in Food and Feed Industries

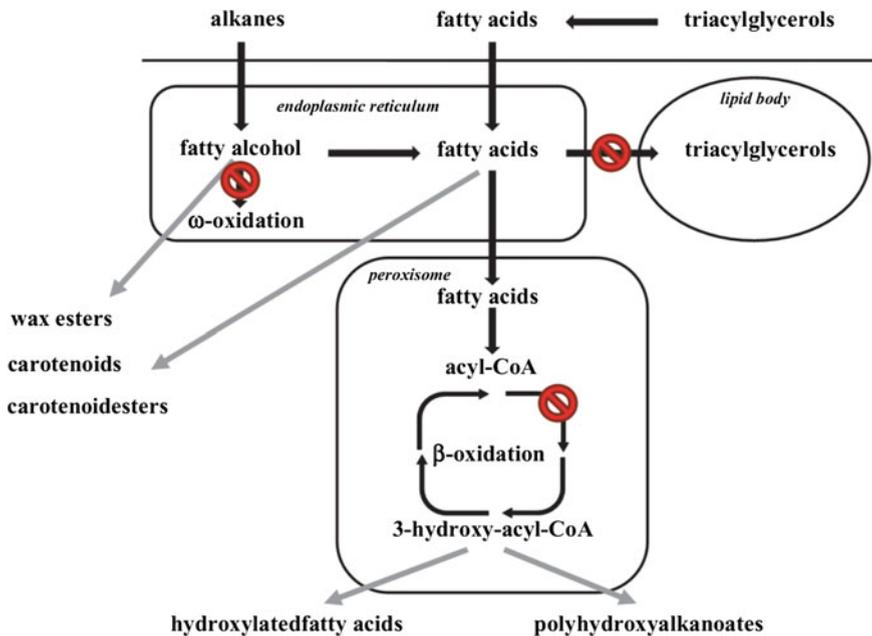
### 4.1 Traditional Food Making

*Y. lipolytica* is lipolytic and proteolytic yeast and its strains are readily isolated from food products containing lipids and proteins. This yeast was frequently observed in milk, meat, fish, and their derived products.

*Y. lipolytica* has been identified in a number of fermented milk products at lower frequencies such as yogurt, kefir, *nunu* and *amasi*, as well as in butter, cream, and margarine (Prillinger et al. 1999; Lourens-Hattingh and Viljoen 2002; Viljoen et al. 2003; Lopandic et al. 2006). The yeast has been found in a variety of cheeses such as fresh, blue-veined, Danish farmhouse, Slovakian bryndza, Cantalet, Pecorino Crotonese, smear-ripened, and mold-ripened cheeses. A higher prevalence of *Y. lipolytica* was found in ewe, goat, and buffalo cheese compared to cow cheese, possibly due to the differences in fat and crude protein content in the milk (Mounier et al. 2005; Gardini et al. 2006; Gente et al. 2007; De Freitas et al. 2009; Larpin-Laborde et al. 2011; Chebeňová-Turcovská et al. 2011; Gkatzionis et al. 2013; Golić et al. 2013; Gori et al. 2013; Groenewald et al. 2014).

*Y. lipolytica* strains have been detected in salami, Spanish-fermented sausages such as chorizo, longaniza, and salchichon. This yeast has also been reported to occur in ham, salted bacon, beef, poultry, biltong, other meat samples, and fish (Fung and Liang 1990; Abunyewa et al. 2000; Encinas et al. 2000; Wolter et al. 2000; Gardini et al. 2001; Mirbagheri et al. 2012).

*Y. lipolytica* is generally regarded as a biosafety class 1 microorganism. This biosafety class encompasses microorganisms which are not known to cause disease in healthy adult humans. The safety issues of *Y. lipolytica* were thoroughly evaluated and this yeast was labeled as a “safe-to-use” organism (Groenewald et al. 2014). The aspects regarding the safety of the yeast are evident because (i) it is inherently associated with dairy, poultry, and meat products, (ii) yeast biomass is a safe nutritional supplement, (iii) it is consumed as food and feed, and (iv) food-grade additives have been obtained from this yeast (Zinjarde 2014).



**Fig. 3** Metabolic engineering of *Yarrowia lipolytica* for production of value-added products. Lipids such as triacylglycerols (TAGs), fatty acids, and *n*-alkanes are used as carbon sources. They get hydrolyzed (TAGs) extracellularly and get transported into the cell, where fatty acids get either stored as TAGs in lipids bodies or get further oxidized in  $\omega$ - or  $\beta$ -oxidation cycles. Storage of fatty acids as TAGs will be blocked. The  $\beta$ -oxidation cycle provides precursors for hydroxylated fatty acids (HFAs) and polyhydroxyalkanoates (PHAs). Modifications of *POX1-6* genes catalyzing the first step of  $\beta$ -oxidation will be explored for modulating monomer composition of the produced HFAs and PHAs. Finally, the  $\omega$ -oxidation will be also blocked to avoid the production of undesired dicarboxylic fatty acids. The resulting pool of fatty acids and fatty acid alcohols will be used for the production of wax esters (WEs), carotenoids, or carotenoid esters by expressing the corresponding bacterial biosynthetic genes (Sabirova et al. 2011). Reprinted with permission from John Wiley and Sons (license number: 3326191231944)

*Y. lipolytica* has positive effect as ripening/maturation agent and/or negative effect as spoilage agent of food products. There is eventually a narrow border in between the two effects.

*Y. lipolytica* so far has not been included deliberately in ripening cultures in commercial cheese production. Therefore, its occurrence in cheese must be due to either its presence in milk or through contamination of equipment, bodily surfaces of operators, or aprons in the cheese-making environment (Welthagen and Viljoen 1998; Larpin-Laborde et al. 2011).

Nevertheless, *Y. lipolytica* has been considered for probiotic starter or ripening cultures in cheese manufacturing (Freitas et al. 1999; Wyder and Puhán 1999a; van den Tempel and Jakobsen 2000; Ferreira and Viljoen 2003; Kumura et al. 2004; De Wit et al. 2005; Lanciotti et al. 2005b; Foschino et al. 2006).

*Y. lipolytica* contributes to create body and/or texture of the cheese and its organoleptic characteristics like taste and aroma (Lanciotti et al. 2005b). Cheese aroma is generated due to the production of volatile sulfur compounds, including methanethiol, dimethylsulfide, or dimethyldisulfide (Arfi et al. 2002; López del Castillo-Lozano et al. 2007). Additional benefits include a reduction in ripening times and extended shelf life of the cheese (Ferreira and Viljoen 2003). Furthermore, *Y. lipolytica* has anti-listerial activity, and to inhibit the growth of *Bacillus cereus* and green mold (Addis et al. 2001; Goerges et al. 2006; Lanciotti et al. 2005b). In cottage cheese, *Y. lipolytica* increased toward the end of the shelf life. This yeast was not identified in the visible spoilage area of the cheese (Brocklehurst and Lund 1985). *Y. lipolytica* supports the growth of probiotic bacteria in yogurt starter cultures (Lourens-Hattingh and Viljoen 2002).

There are some reports on the undesirable effects of *Y. lipolytica* on cheese ripening process: off-flavors production, negative effects on cheese texture, stimulates the formation of biogenic amines and surface browning defects of cheese, and inhibits the growth of *Penicillium roqueforti* (Bintsis and Robinson 2004; Westall and Filtenborg 1998; Gardini et al. 2006; Wyder and Puhán 1999b; Ross et al. 2000).

*Y. lipolytica* produces brown extracellular pigments that correlate with tyrosine catabolism. During tyrosine depletion, the yeast accumulated homogentisic acid, p-hydroxyphenylethanol, and p-hydroxyphenylacetic acid in the medium. Brown pigments are produced when homogentisic acid accumulates in the medium. This acid can spontaneously oxidize and polymerize, leading to the formation of pyomelanins (Carreira et al. 2001). *Y. lipolytica* either had no effect or even reduced browning when used in combination with *Penicillium candidum* (Carreira et al. 2002).

## 4.2 Single Cell Protein

Single cell protein (SCP) refers to biomass or total protein extracted from pure or mixed cultures of microorganisms used as a substitute for proteins in human foods and animal feeds. In the 1950s–1970s, British Petroleum (BP) used *Y. lipolytica* for the production of SCP using *n*-alkanes derived from crude oil as substrates. BP built the large-scale production plant in Grangemouth, UK which produced 4,000 ton per annum of SCP by this yeast (Toprina G). In addition, Italproteine built a 100 kton per annum production plant for Toprina G in Sarroch, Sardinia.

Toprina products were used for livestock nutrition as well as in the diet of broilers, pigs, lambs, calves, and rainbow trout. Furthermore, the Polish company Skotan SA employed *Y. lipolytica* for prebiotic and probiotic production to use in feed and food (Groenewald et al. 2014).

Juszczuk et al. used *Y. lipolytica* S6 for biomass production using glycerol wastes. When 25 g/L pure and raw glycerol were used, this strain produced 11.7

and 12.3 g/L of the biomass with 1.30 and 1.37 g/L h productivity, respectively (Juszczak et al. 2013).

### 4.3 Carotenoids

Carotenoids are used commercially as food colorants, feed supplements, nutraceuticals, and for cosmetic and pharmaceutical purposes. The main dietary sources of carotenoids are fruits and vegetables. Microbial processes are developed for the production of carotenoids using bacterial, fungal, and algal strains (Schmidt-Dannert et al. 2009). The genetically modified *Y. lipolytica* has been developed and patented as an alternative source of bio-based carotenoids (Groenewald et al. 2014).

The genotoxic and subchronic toxicity potential of  $\beta$ -carotene from *Y. lipolytica* was determined to support the use of this compound as a food ingredient. The  $\beta$ -carotene administered orally to Sprague Dawley rats for 90 days was considered to be at least 500 mg/kg body weight. Adverse effects were not observed following clinical, clinical pathology, and histopathological evaluations of dosed rats. All results show no significant difference in the safety of the product derived from *Y. lipolytica* (Grenfell-Lee et al. 2014).

Matthäus et al. produced lycopene in a recombinant *Y. lipolytica* strain. The codon optimized genes *crtB* and *crtI* of *Pantoea ananatis* were expressed in *Y. lipolytica* under the control of the *TEF1* promoter. The rate limiting genes for isoprenoid biosynthesis in *Y. lipolytica*, *GGSI* and *HMGI*, were overexpressed to increase the lycopene production. All genes were also expressed in a *Y. lipolytica* strain with *POX1*, *POX2*, *POX3*, *POX4*, *POX5*, *POX6*, and *GUT2* deletions, which led to an increased size of lipid bodies and a further increased lycopene production. Lycopene is mainly located within lipid bodies and increased lipid body formation leads to increased lycopene storage capacity in *Y. lipolytica*. This yeast produced 16 mg/g lycopene in fed-batch culture (Matthäus et al. 2014).

## 5 Fine Chemistry and Pharmaceutical Applications

The need for enantiomerically pure molecules has grown since the legislation required investigations on the pharmacological effects of both enantiomers, especially in the pharmaceutical industry. The market for drugs sold as single-enantiomer was \$160 billion worldwide in 2002 and it has a 10 % growth per year.

Chemical asymmetric synthesis and enzymatic procedure are two classical ways to obtain pure enantiomers. Stereoselective crystallization and chiral chromatography were used in chemical asymmetric synthesis, but this process is often expensive.

The enzymatic procedure of resolving a racemic mixture is very attractive. Lipases are the most frequently used catalysts in this method. The reasons for this interest lie in their high stability, their nonrequirement of co-factors, their synthetic activity in organic solvents, and mainly in the wide range of substrate specificities. They are also capable of catalyzing reactions on non-natural substrates. Whole cells or enzymes of *Y. lipolytica* have been applied in enantioselective resolution (hydrolysis, oxidation, or reduction) and re-esterification reactions (Guieysse et al. 2004; Fickers et al. 2005a).

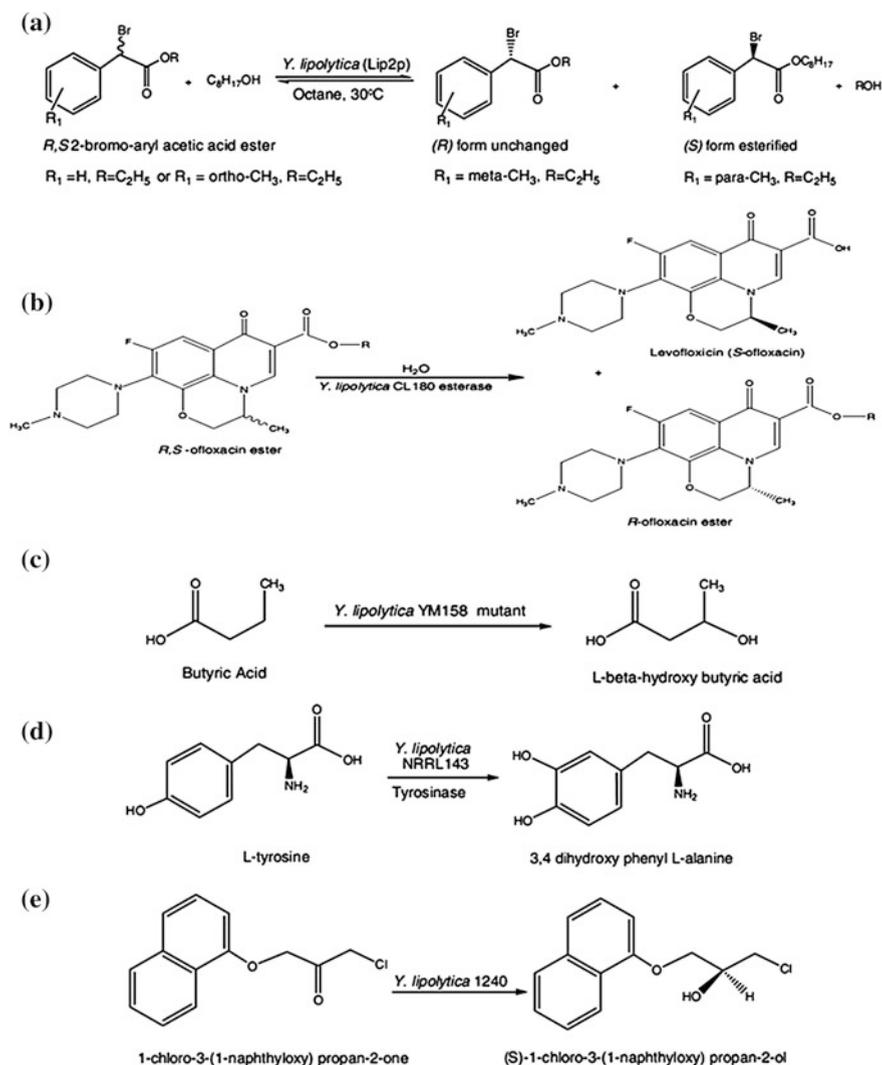
### 5.1 Resolution of 2-Bromo-arylacetic Acid Esters

Enantiopure carboxylic acids are important building blocks for the synthesis of many pharmaceuticals, pesticides, and natural compounds such as pheromones. These compounds are important intermediates found in the synthetic pathways of a number of drugs, such as prostaglandin, prostacyclin, semisynthetic penicillin, and thiazolium salts.

The ethyl ester derivatives of 2-bromo-*o*-tolylacetic acid are used as precursors for the synthesis of analgesics and nonpeptide angiotensin II-receptor antagonists. The lipase (Lip2p) from *Y. lipolytica* is an active and selective catalyst in the transesterification of 2-bromo-phenyl and 2-bromo-tolyl acetic acid ethyl esters. The enzyme efficiently brought about a preferential transesterification of the (*R*) form with 1-octanol in *n*-octane and the (*S*)-enantiomer was thus enriched (Fig. 4a). *Y. lipolytica* lipase presents a higher catalytic activity and an (*S*)-enantiopreference, while *Burkholderia cepacia* lipase is (*R*)-enantiomer selective. The most interesting result is that *Y. lipolytica* lipase has until now been the only enzyme able to catalyze the resolution of 2-bromo-*o*-tolylacetic acid ethyl ester (Guieysse et al. 2004).

### 5.2 Ofloxacin Synthesis

Ofloxacin is a fluoroquinolone antibiotic present as a racemic mixture. Levofloxacin, *S*-isomer of ofloxacin, shows a broad spectrum of antibacterial activity against both gram-positive and gram-negative bacteria. The antibacterial activity of levofloxacin is 8–128 times greater than that of the corresponding *R*-isomer. A novel esterase of type B1 carboxylesterase/lipase family from a marine isolate *Y. lipolytica* CL180 was used to resolve a racemic mixture of ofloxacin ester (Fig. 4b). This esterase showed an enantioselectivity toward *R*, *S*-ofloxacin ester, and levofloxacin was produced with an enantiomeric excess of 52 % (Kim et al. 2007).



**Fig. 4** Some valuable compounds with pharmaceutical applications produced by *Y. lipolytica*. **a** Resolution of 2-bromo-arylacetic acid esters by Lip2p. **b** Ofloxacin synthesis by strain CL180 esterase. **c** Conversion of butyric acid into L-hydroxybutyric acid. **d** L-dopa production from L-tyrosine. **e** Production of halohydrin precursor of (*S*)-propranolol

### 5.3 L-Hydroxybutyric Acid Production

A mutant strain *Y. lipolytica* YM158 converts butyric acid into L-hydroxybutyric acid of 8.0 g/L in a batch culture (Fig. 4c). The optimum culture conditions in the bioreactor for maintenance of high cell activity were a glucose concentration of

2.0 g/L and a butyric acid concentration of 8.1 g/L. A fed-batch fermentation was performed under these conditions resulting in an L1-hydroxybutyric acid yield of 31 g/L. The wild-type strain used butyric acid as a carbon source and was unable to accumulate  $\beta$ -hydroxy butyric acid (Kyong and Shin 2000; Kim et al. 1999).

### 5.4 Production of L-Dopa

The L-dopa (3,4-dihydroxy phenyl L-alanine) is a drug of choice for Parkinson's disease, controlling changes in energy metabolism enzymes of the myocardium following neurogenic injury. *Y. lipolytica* NRRL-143 was used for the transformation of L-tyrosine to L-dopa (Fig. 4d). The addition of 2.0 mg/mL diatomite (2:1 clay mineral) markedly improved the biotransformation of L-tyrosine to L-dopa. The yeast produced 2.96 mg/mL of L-dopa from 2.68 mg/mL of L-tyrosine when 2.0 mg/mL diatomite was added 15 min after the start of the reaction. Diatomite addition after 15 min led to a 35 % higher substrate conversion rate compared to the control. Furthermore, L-dopa production rate reached to 12.5-fold when biomass concentration of 2.5 mg/mL and reaction time of 30 min were optimized (Ali et al. 2007).

### 5.5 Production of Halohydrin Precursor of (S)-Propranolol

Propranolol is a sympatholytic nonselective beta blocker. Sympatholytics are used to treat hypertension, anxiety, and panic. Chiral alcohols with an additional functional group are promising building blocks for the synthesis of enantiomeric pure pharmaceuticals and other chemicals. The halohydrine 2 is an intermediate model in the preparation of homochiral  $\beta$ -blockers with aryloxy-2-propanolamine structure and very useful in the treatment of many diseases. Only the (S) enantiomers of these kinds of drugs are active, being the (R) counterpart inactive or toxic.

*Y. lipolytica* CECT 1240 was tested for the synthesis of (R) or (S)1-chloro-3-(1-naphthyl)propan-2-ol, the precursor of 2-propranolol. The yeast produced an enantiomeric excess (ee) of the (S) isomer, as shown in Fig. 4e.

*Y. lipolytica* CECT 1240 resting cells gave 87 % yield in (S) 1 (ee = 99 %). This strain was able to resolve racemic halohydrins to the (S) forms with 99 % ee at pH 7.0 (Lagos et al. 2002, 2004).

*Y. lipolytica* has been immobilized in calcium alginate for reduction of ketones. The maximum productivity of alcohol dehydrogenases is achieved at the beginning of the stationary phase (48 h). The immobilization experimental conditions have been optimized by factorial experimental design. The influence of the volume of sodium alginate, alginate percentage, cell loading, and amount of ketone has

been studied. *Y. lipolytica* carries to *S*-enantiomer of the halohydrin with 80 % yield and ee 95–97 % (Martinez-Lagos and Sinisterra 2005).

## 5.6 Terpenes

Terpenes are derived biosynthetically from units of isoprene, which are the largest class of plant secondary metabolites and also produced by some animals. They are used widely as natural flavor additives for food, as fragrances in perfumery, and as healing agents in aromatherapy and traditional and alternative medicine. Terpenes may be used as substitutes for chlorinated solvents in applications such as cleaning of electronic components and cables, degreasing of metal, and cleaning of aircraft parts. Terpenes biotransformation is an attractive alternative to production of aromas, because it occurs under mild conditions, does not generate toxic wastes, and allows producing “natural” aromas that can be used as fragrances and flavors in industry (de Carvalho and da Fonseca 2006; Trytek et al. 2009).

*Y. lipolytica* is able convert *R* (+)-limonene into perillic acid. Perillic acid and its derivatives as terpenoid compounds are increasingly important due to their flavoring and antimicrobial properties as well as their potential as anticancer agents (Ferrara et al. 2014). Maximum concentration of perillic acid was 1 g/L with 50 % yield on the substrate.

The yeast also transformed (–) piperitone into 7-hydroxy-piperitone so that this monoterpene concentration reached to 0.4 g/L with 20 % yield on the substrate (van Rensburg et al. 1997; van Dyk et al. 1998).

A wide range of microorganisms and higher eukaryotes cyclize 2,3-oxidosqualene to polycyclic triterpenes such as lanosterol by a well-known single-enzyme-catalyzed reaction. Aniol and Huszcza tried to find microorganisms that are capable to cyclize the 6,7-epoxides of geraniol and nerol. A high yield (85–99 %) cyclization of 6,7-epoxygeraniol to 2-methyl-2-(2-hydroxyethyl)-5-(2-hydroxyprop-2-yl)tetrahydrofuran was achieved using *Y. lipolytica* and *Botrytis cinerea*.

*Y. lipolytica* gave the highest cyclization yield of 99 % within 5 days. The mechanism of cyclization of 6,7-epoxygeraniol to the furanoid oxide occurred via an intermediate formed by hydration of the 2,3-double bond. Closure of the furan ring resulted from the oxirane cleavage, which is a consequence of the C3-hydroxyl group attack on C6 (Aniol and Huszcza 2005).

## 6 Environmental Applications

*Y. lipolytica* could be adapted to polluted environments with oily and organic pollutants, heavy metals, and salts. *Y. lipolytica* strains had been isolated and employed in the bioremediation and detoxification of such environments (Bankar et al. 2009a).

## 6.1 Waste Treatment

*Y. lipolytica* has been used for the treatment or upgradation of a variety of wastes. Current agricultural and industrial practices have led to the generation of large amounts of various low-value or negative cost crude wastes, which are difficult to treat and valorize. Production of agro-industrial waste pollutants has become a major problem for many industries.

The olive oil industry generates large amounts of olive mill waste (OMW) OMW as by-product has significant polluting properties due to high levels of chemical oxygen demand (COD) and biochemical oxygen demand (BOD) as well as phosphate, polyphenols, polyalcohols, and metals.

Generally, OMW has BOD values ranging from 12,000 to 63,000 mg/L, and COD values from 80,000 to 200,000 mg/L. These concentrations are around 200–400 times higher than a typical municipal sewage. The average amount of OMW produced during the milling process is approximately 1,000 kg/ton of olives. It should be noted that 10 million m<sup>3</sup> per year of liquid effluent produced from olive mill systems corresponds to an equivalent load of the wastewater generated from about 20 million people (Azbar et al. 2004; Lanciotti et al. 2005a; Darvishi 2012b).

Different strains of *Y. lipolytica* have been effective in the treatment of OMW. *Y. lipolytica* ATCC 20255 was used in the treatment of OMW. The yeast was capable of reducing the COD level by 80 % despite the presence of large amount of phenols (200 mg/L) after 24 h when grown in a 3.5-L bioreactor. It also produced a useful biomass of 22.45 g/L as SCP and 770 U/L of extracellular lipase. During this process, most of the organic and inorganic substances were consumed (Scioli and Vollaro 1997).

Lanciotti et al. tested the ability of 62 strains of *Y. lipolytica* to grow in undiluted OMW and reduce its COD level. Strain PO1 was most effective and reduced the COD by 41.22 % in the presence of 578 mg/L of polyphenols. Furthermore, this strain produced 925 U/L of lipase and 4.2 g/L of citric acid. Strains Y9, Y17, B16, C11, RO18, PO1, PO18, PO20, B7, and Y17 showed a high lipase activity, and in addition induced a reduction of COD value ranging between 20 and 40 % with respect to the uninoculated OMW. Some strains Y17, RO18, B16, PO20, and C21 induced a reduction of polyphenol content. The strains Y9, Y2, B16, C11, and Y17 were able to produce high concentration (up to 5.2 g/L) of citric acid (Lanciotti et al. 2005a).

*Y. lipolytica* strain W29 produced 3,500 U/L lipase on OMW supplemented with yeast extract and ammonium chloride. This particular strain could be used for the scaleup of lipase production from OMW. The strain W29 was a reduction in COD by 80 % and total phenol by 70 % (Gonçalves et al. 2009).

Wu et al. immobilized *Y. lipolytica* W29 cells in calcium alginate for the degradation of oil wastewater. Immobilized cells degraded 2,000 mg/L of oil and reduced 2,000 mg/L of COD within 50 h at 30 °C. The beads could be stored at 4 °C for 30 days and reused 12 times. The COD degradation rate of immobilized

cells was maintained at 82 % even at the sixth cycle. The factors affecting oil degradation and COD reduction by immobilized cells were investigated. The results showed that immobilized cells had high thermostability compared to that of free cells, and substrate concentration significantly affected degrading ability of immobilized cells (Wu et al. 2009).

*Y. lipolytica* ACA-DC 50109 was cultivated on OMW enriched with commercial–industrial glucose. A notable quantity of total citric acid (28.9 g/L) was produced on OMW medium with initial sugar concentration of 65 g/L. OMW had a noteworthy stimulating effect on the production of citric acid, since both final citric acid concentration and conversion yield of citric acid produced per unit of sugar consumed were higher compared to the respective parameters obtained from trials without added OMW. This strain also synthesized cellular unsaturated fatty acids, principally of oleic and palmitoleic acids. Additionally, a non-negligible decrease of the phenolic compounds in the growth medium [up to 15 % (wt/wt)], a slight decrease of the phytotoxicity, and a remarkable decolorization of the OMW were observed (Papanikolaou et al. 2008).

Lopes et al. investigated the ability of two different wild-type strains, W29 and IMUFRJ 50682, to grow on OMW and their potential to produce high-value products such as lipases. Both strains were able to grow in OMW with 19 g/L of COD and approximately 800 mg/L of total phenols concentration. The strain IMUFRJ 50682 has been reported to be an efficient lipase producer, but the strain W29 showed a higher potential for lipase production in OMW-based medium. Lipase productivity was improved by the medium supplementation with ammonium sulfate up to 6 g/L, leading to 80 % of COD and 70 % of total phenols reduction. The surfactant Tween 80 enhanced cell growth and COD reduction, but it had a negative effect on lipase activity (Lopes et al. 2009).

Therefore, OMW as fatty low-value renewable carbon sources could be used for production of various added-value metabolites such as lipases, organic acids, microbial biopolymers and lipids, single cell oil, single cell proteins, and biosurfactants.

Palm oil mill effluent (POME) composition is somewhat different from OMW. It contains mainly lignocellulosic wastes with a mixture of carbohydrates and oil. POME also presents very high BOD and COD values of 246,000 and 11,000 mg/L, respectively.

The *Y. lipolytica* NCIM 3589 was used for treatment of POME without any addition of nutrient or dilution. The strain reduced the COD of the effluent by 95 % within 48 h. Treatment with a chemical coagulant further reduced the COD and a consortium developed from garden soil clarified the effluent and adjusted the pH to between 6 and 7. The complete treatment reduced the COD content to 1,500 mg/L which is a 99 % reduction from the original (Oswal et al. 2002).

*Y. lipolytica* MBRC-10073 has been isolated and employed in the upgradation of fishmeal. This strain showed the highest efficiency for reducing the lipids by 29 % under solid-state fermentation. In the fermentation with intermittent mixing during 96 h incubation, reduction efficiency for crude lipids reached to 46 %.

The results suggest that the fermentation can improve the quality of fishmeal from fish waste, which is rich in lipids (Yano et al. 2008).

*Y. lipolytica* was successfully used in the treatment of sewage sludge from the food industries containing up to 90 % of grease. The best extracellular-lipase-producing strains were selected and tested in a 6,000-L grease tank with a feeding rate of 6,000 L every 24 h. This led to a reduction in the lipid content and maintaining the COD at a value of 3,000 mg/L during 33 weeks of treatment (Fickers et al. 2005a).

Domínguez et al. investigated the degradation waste cooking oil and its application as lipase inducer by *Y. lipolytica* CECT 1240. The ability of this strain to degrade the spent oil was evaluated by monitoring COD throughout the cultures. The addition of waste cooking oil to the medium led to a significant augmentation in extracellular lipase production by the yeast. The COD was diminished up to nearly 90 % after 3 days in the presence of high levels of lipolytic activity (Domínguez et al. 2010).

## 6.2 Bioremediation and Biodegradation of Environmental Pollutants

Oil pollution occurs in terrestrial, marine, and freshwater environments, which is a major cause for ecological and environmental damage. The potential of hydrocarbon-degrading microorganisms has led to the development of bioremediation techniques for contaminated soil and water. Different strains of *Y. lipolytica* were isolated and used in the bioremediation of oil-contaminated soils (Bankar et al. 2009a).

A psychrotrophic strain of *Y. lipolytica* RM7/11 was tested on diesel oil biodegradation in a mineral medium and in soil. This strain was able to grow in a temperature ranging from 10 to 30 °C and degrade 68 % of diesel oil after 10 days (Margesin and Schinner 1997). This strain also degraded 39.9 % of *n*-hexadecane and 35.4 % of *n*-dodecane during 8 days at 10 °C. After 5 days at 15 °C, 50 and 73 % of hexadecane and dodecane were degraded (Margesin et al. 2003).

Zogała et al. used *Y. lipolytica* A-101 for bioremediation of petrol-contaminated soil in situ study. A suspension of the strain was introduced into 12 one-meter deep boreholes. The process of bioremediation was controlled using electromagnetic and resistivity methods. The study showed the ability of *Y. lipolytica* to remedy petrol contaminated soils (Zogała et al. 2005).

*Y. lipolytica* NCIM 3589 degraded 78 % of the aliphatic fraction of Bombay High crude oil in the free form under optimal conditions. Immobilized cells in porous agar beads degraded up to 92 % of the aliphatic fraction of supplied oil at 30 °C. The immobilized cells were effective up to five cycles each of 5 days with 28 % loss of activity in batch culture. In a continuous flow reactor, immobilized cells were active for 30 days with no loss of activity.

Strain NCIM 3589 in the yeast form degraded the aliphatic fraction of crude oil as well as the pure alkanes, *n*-hexadecane (60 %), *n*-tetradecane (50 %), *n*-octadecane (45 %), *n*-decane (40 %), and *n*-dodecane (40 %) within 24 h under aerobic conditions. Hence, transition of mycelium to yeast form may be the prerequisite for effective alkane degradation (Zinjarde et al. 1998; Zinjarde and Pant 2000, 2002b).

Two *Y. lipolytica* strains PG-20 and PG-32 were used for degradation of crude oil. The strains PG-20 and PG-32 degraded 68 and 58 % of crude oil, respectively. The optimal growth condition and biodegradation of hydrocarbons was in ONR medium with an acidic pH after 1 week at 30 °C. These two strains may degrade aliphatic hydrocarbons more efficiently than aromatic hydrocarbons, although strain PG-20 had better degradation than strain PG-32. The strains also reduced surface tension when cultured on hydrocarbon substrates (1 % v/v). These strains showed a cell surface hydrophobicity higher than 70 %. The *Y. lipolytica* strains have high crude oil degrading activity due to their high emulsifying activity and cell hydrophobicity. They could be used for the bioremediation process in the Persian Gulf and decreasing oil pollution in this marine ecosystem (Hassanshahian et al. 2012).

Phenol and phenolic compounds are ubiquitous pollutants due to effluents of a variety of chemical industrial such as cool refineries, phenol manufacturing, pharmaceuticals, and industries of resin paint, dyeing, textile wood, petrochemical, pulp mill, etc. They induce genotoxic, carcinogenic, immunotoxic, hematological, and physiological effects.

*Y. lipolytica* Y103 degraded phenol and 4-chlorophenol to catechol. The catechol then will be further degraded to produce 2-hydroxymuconic semialdehyde via meta-cleavage. The most active degradation of phenol by this strain occurred with a 0.5 mM phenol concentration at 30 °C and pH 7.0 (Lee et al. 2001).

*Y. lipolytica* LPS 605 degraded biphenyl to 4-hydroxy biphenyl and an additional hydroxylated product (3,4-dihydroxy biphenyl) within 24 h. The cleavage product 4-phenyl-2-pyrone-6-carboxylic acid was observed after 4 days (Romero et al. 2001).

Romero et al. isolated an *Y. lipolytica* strain that was able to hydroxylate dibenzofuran and formed 3-hydroxydibenzofuran as major metabolite, and 4-hydroxydibenzofuran and 2-hydroxydibenzofuran as minor metabolites (Romero et al. 2002).

*Y. lipolytica* NBRC 1658 was used to decolorize Reactive Black 5 dye through biodegradation. This strain decolorized 97 % of 50 mg/L Reactive Black 5 within 24 h and tolerated up to 300 mg/L of dye. Decolorization process occurred during the exponential growth phase. Aerobic batch culture with 5 g/L glucose and 1 g/L ammonium sulfate at pH 7 was optimum decolorizing conditions (Aracagok and Cihangir 2013).

### 6.3 Bioaccumulation of Heavy Metals

Heavy metals as important pollutants are routinely released into the aquatic and terrestrial environments as a result of industrial processes and anthropogenic activities. Many of these are toxic to human beings and also pose a serious threat to the environment. *Y. lipolytica* used different mechanisms to tolerate and detoxify heavy metals which are summarized in Fig. 5.

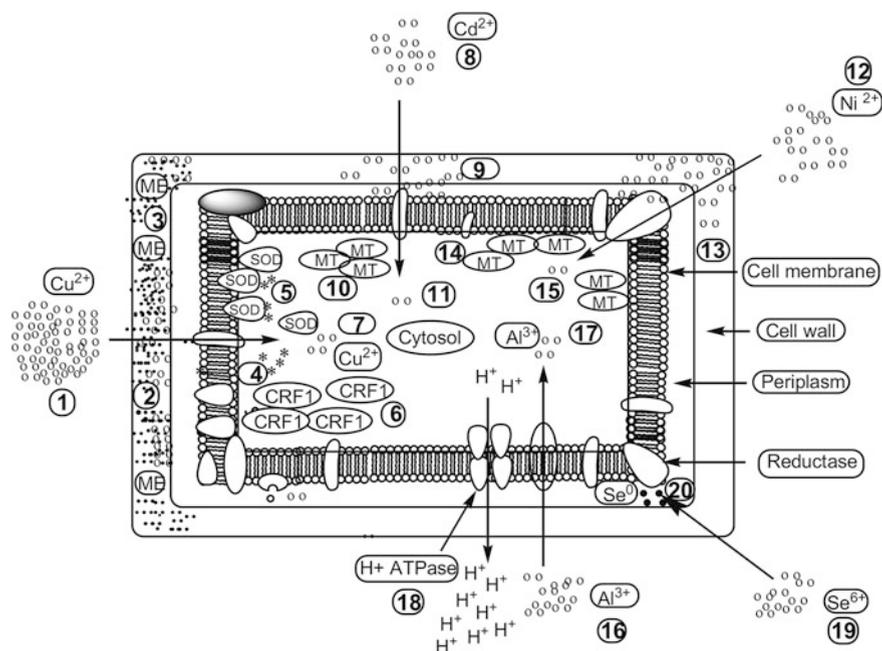
High concentration (2–4 mM) of copper ( $\text{Cu}^{2+}$  ion) did not significantly affect the growth rate of *Y. lipolytica* cells at the logarithmic phase, but increased the lag period. The intracellular copper content of *Y. lipolytica* progressively increased in direct correlation with increasing copper content in the medium. Copper precipitates at the cell wall or the formation of copper complexes by cell wall components. Furthermore, melanin contributes to metal binding and accumulation at the cell wall.  $\text{Cu}^{2+}$  ions could be reacted with oxygen and produce toxic free radicals. Level of Zn-superoxide dismutase (SOD) is increased due to the accumulation of  $\text{Cu}^{2+}$  ions. This matter avoids toxic reactive oxygen damage generated by transition metal ions. A copper responsive factor (CRF1) synthesis plays an unidentified novel role in metal detoxification (Ito et al. 2007a; Garcia et al. 2002).

In high concentrations of  $\text{Cd}^{2+}$  (30  $\mu\text{M}$ ) and  $\text{Ni}^{2+}$  (600  $\mu\text{M}$ ) ions, large amounts of these ions deposit at cell wall and cell membrane. Then, high levels of metallothioneins (MT) synthase in the cytosol of cell in response to these ions. Aluminum accumulates during growth of the yeast cell in presence of high  $\text{Al}^{3+}$  ion concentrations (0.5–1 M). A stronger efflux of  $\text{H}^+$  by  $\text{H}^+$ ATPase induces in response to accumulation of  $\text{Al}^{3+}$  ions.  $\text{Se}^{6+}$  ions reduce to  $\text{Se}^0$  by reductases in high  $\text{Se}^{6+}$  ion concentrations (1–10 M) in the outside of cells (Bankar et al. 2009a).

Chromium is an important toxic heavy metal that is encountered in groundwater. The removal of chromium (VI) ions from aqueous solutions by the biomass of two marine strains of *Y. lipolytica* NCIM 3589 and 3590 was studied with respect to environmental conditions. Maximum biosorption was observed at pH 1.0 and at a temperature of 35 °C. Increase in biomass and sea salts resulted in a decreased metal uptake. Under optimum conditions, biosorption was enhanced with increasing concentrations of Cr (VI) ions. Strains NCIM 3589 and 3590 displayed a specific uptake of Cr (VI) ions of 63.73 mg/g at a concentration of 950 ppm and 46.09 mg/g at 955 ppm, respectively (Bankar et al. 2009b).

In another study,  $\text{Fe}^0/\text{Fe}_3\text{O}_4$  nanocomposites were used for surface modification of these strains for increase in efficiency of Cr (VI) biosorption. Absorption capacity in the magnetically modified cells of NCIM 3589 and 3590 reached to 186.32 and 137.31 mg/g, respectively.

The enhanced detoxification of Cr (VI) ions by this composite material could be attributed to the reductive power of the  $\text{Fe}^0/\text{Fe}_3\text{O}_4$  nanocomposites as well as the yeast cell surface functional groups. Magnetic modification of cells thus resulted in the development of a “smart biosorbent” that could be recovered by applying



**Fig. 5** Proposed mechanisms of metal detoxification and tolerance in *Y. lipolytica*: 1 high  $\text{Cu}^{2+}$  ion concentration (2–4 mM) present outside the cell, 2 deposition of Cu at the cell wall, 3 production of melanin (ME) that has a role in the binding and accumulation of  $\text{Cu}^{2+}$  ions, 4 production of toxic free radicals after reaction of  $\text{Cu}^{2+}$  ions with oxygen, 5 enhanced levels of Zn-superoxide dismutase (SOD) due to the accumulation of  $\text{Cu}^{2+}$  ions, 6 synthesis of a copper responsive factor (CRF1) possibly playing an unidentified novel role in metal detoxification, 7 low concentrations of  $\text{Cu}^{2+}$  ions in the cytosol, 8 high concentrations of  $\text{Cd}^{2+}$  ions (30  $\mu\text{M}$ ) outside the cell, 9 deposition of large concentrations of Cd at cell wall and cell membrane, 10 synthesis of high level of metallothioneins (MT) in the cytosol of cell in response to  $\text{Cd}^{2+}$  ions, 11 low concentrations of  $\text{Cd}^{2+}$  in the cytosol, 12 high concentrations of  $\text{Ni}^{2+}$  (600  $\mu\text{M}$ ) outside the cell, 13 deposition of large concentrations of Ni at cell wall and cell membrane, 14 synthesis of high level of metallothioneins (MT) in the cytosol of cell in response to  $\text{Ni}^{2+}$  ions, 15 low concentrations of  $\text{Ni}^{2+}$  in the cytosol, 16 high  $\text{Al}^{3+}$  ion concentrations (0.5–1 M) present outside the cell, 17 accumulation of  $\text{Al}^{3+}$  ions during growth of the yeast cell in presence of 0.5–1 M  $\text{Al}^{3+}$  ion concentrations, 18 induction of a stronger efflux of  $\text{H}^+$  by  $\text{H}^+$ ATPase in response to accumulation of  $\text{Al}^{3+}$  ions, 19 high  $\text{Se}^{6+}$  ion concentrations (10–1 M) outside the cell, and 20 reduction of  $\text{Se}^{6+}$  ions to  $\text{Se}^0$  by reductases (Bankar et al. 2009a). Reprinted with permission from Springer (license number: 3326701356942)

external magnetic fields thereby avoiding the risk of direct contact with toxic metal ions (Rao et al. 2013).

*Y. lipolytica* NCIM 3589 was used for the synthesis of gold nanoparticles from chloroauric acid ( $\text{HAuCl}_4$ ). The reduction of gold occurred in a pH-dependent manner. Acidic pH favored nucleation on the cell surfaces and the subsequent formation of gold crystals. At pH 7.0 and 9.0, there was nanoparticle synthesis with a size of 15 nm. SEM and transmission electron microscopy (TEM) showed

that nanoparticles were associated with the cell wall. This strain was able to synthesize gold nanoparticles in seawater and in freshwater. The inherent presence of reductases or proteases may be playing a role in the reduction of the gold salt into nanoparticles (Agnihotri et al. 2009).

#### 6.4 Trinitrotoluene Biotransformation

TNT (2,4,6-Trinitrotoluene) is one of the most common explosive components. It is produced by the military industry since 1902 and continues today. TNT is an extensive pollutant of soils, surface water, and groundwater due to its large-scale production. It has a high toxic potential for the ecosystem, including humans and aquatic and terrestrial organisms, and is also listed as class C potential human carcinogen (Smets et al. 2007).

The *Y. lipolytica* is efficiently transformed and detoxified TNT via two approaches: (a) direct aromatic ring reduction and (b) nitro group reduction.

Jain et al. used a tropical marine strain *Y. lipolytica* NCIM 3589 that cannot utilize TNT as the sole carbon or nitrogen source, but reduces the nitro groups in TNT to aminodinitrotoluene (ADNT). This strain has two types of TNT reduction reactions depending on presence or absence of glucose. In the presence of glucose, the yeast preferentially modulates the choice of the reaction to the ring reduction mode and forms the hydride-Meisenheimer complex ( $H^-$  TNT) as a transiently metabolite that subsequently denitrates to 2,4-dinitrotoluene (2,4-DNT) and 2-nitrotoluene (2-NT). In the absence of glucose, the nitro groups are preferentially reduced to amino derivatives such as 4-aminodinitrotoluene (4-ADNT) and 2-aminodinitrotoluene (2-ADNT). The reduction of the nitro groups to amino groups was the major functional pathway. The resulting products metabolize by other microorganisms, therefore the detoxification process could be complete. This yeast is able to completely transform 1 mM (227 ppm) of TNT in a complete medium (Jain et al. 2004).

Ziganshin et al. used an acid-tolerant strain *Y. lipolytica* AN-L15 for TNT biotransformation. In this strain unlike NCIM 3589 direct aromatic ring reduction was the predominant pathway through hydride ion-mediated reduction of the aromatic ring. Eight distinct mono- and dihydride complexes were produced during TNT ring reduction. The nitro group reduction was observed to be a minor pathway which produces hydroxylamino or amino derivatives (Ziganshin et al. 2007).

The effects of pH and aeration were investigated on TNT transformation during *Y. lipolytica* AN-L15 growth. Aerobic conditions stimulated strain to reduce TNT nitro groups to mostly HADNTs including 2-hydroxylamino-4,6-dinitrotoluene (2-HADNT) and 4-hydroxylamino-2,6-dinitrotoluene (4-HADNT), while static conditions increased the yield of ADNTs including 2-ADNT and 4-ADNT. The pH value has a significant influence on the transformation of the TNT-hydride complexes. While transformation of TNT into TNT-monohydride complex  $3-H^-$ -TNT (as one of the key intermediates in the TNT transformation process) occurs at

initial pH values of 6 and 7, this reaction is inhibited at pH values below 4. In contrast, the formation of other hydride complexes from 3-H<sup>-</sup>-TNT is promoted at pH values of 4 and below. Furthermore, nitrite eliminated from the protonated TNT–dihydride complex is quickly converted into nitrate at a pH lower than approximately 4. The *Y. lipolytica* is a strictly aerobe and unable to grow fermentatively, hence the production of organic acids and TNT transformation were dependent on the aeration of the medium. The optimal conditions for TNT biotransformation by *Y. lipolytica* AN-L15 are intensive aeration of neutral or slightly acidic medium with an initial pH of 6.0, which is being acidified over time by the yeasts to a pH below 4.2 (Ziganshin et al. 2010).

*Y. lipolytica* formed colored metabolites (H<sup>-</sup>-TNT, wine red, or ADNT, orange) during TNT biotransformation, hence they could be used in the development of biosensors for the detection of TNT (Bankar et al. 2009a).

## 7 Heterologous Proteins Expression System

Yeast expression systems combine the ease of manipulation and growth of unicellular organisms with eukaryotic posttranslational processing and modifications. *Y. lipolytica* provides an attractive expression platform. Low overglycosylation, high secretion efficiency, good product yield, and performance reproducibility are the advantages of this yeast compared to other yeasts (Madzak et al. 2004).

An efficient integrative transformation system became available in the mid-1980s. The powerful genetic and molecular tools such as well-characterized strains and expression/secretion vectors are available now for transformation, expression, and secretion of foreign genes in this yeast. Episomal (replicative) vectors and vectors designed for integration into the yeast genome as two main types of shuttle vectors can be used in *Y. lipolytica*. No natural episomal DNA was ever found in this yeast. Replicative vectors have been engineered based on autonomously replicating sequences (ARS68 and ARS18). The copy number of ARS-based vectors is limited to 1–3 copies/cell and the correlated gene expression is also limited. In addition, ARS-based vectors require the maintenance of a selective pressure, which may not be always compatible with efficient industrial management (Madzak et al. 2000).

The integration of exogenous DNA into *Y. lipolytica* genome using integrative vectors occurs almost exclusively by homologous integration (in *LEU2*, *URA3*, *XPR2* terminator, rDNA, or when present, in zeta or pBR322 docking platform) and nonhomologous integration in Ylt1-devoid strains. The integration of a shuttle vector by single crossover can thus be efficiently directed by linearization. In more than 80 % of the cases, a single complete copy of the vector will be integrated at the chosen site. Multicopy shuttle vectors have been developed so that they can integrate foreign gene into the genome either by homology with multiple target sites or nonhomologously (Madzak et al. 2004; Böer et al. 2007; Barth and Gaillardin 1996).

The presence of bacterial DNA (especially of the antibiotic resistance gene) into the producing yeast strain using classical shuttle vectors could be a drawback for acceptance by regulatory authorities for commercial applications. Several auto-cloning expression vectors were developed in order to alleviate this problem. Auto-cloning vectors are composed of an auxotrophy marker, the expression cassette, and elements for integration into the recipient strain (Madzak et al. 2004).

Different types of promoters have been employed to drive transcription, some of the most common used promoters are constitutive (pTEF), inducible (pXPR2, pPOX2, and pICLI), synthetic (hp4d), and bi-directional (pMTP). New promoters containing UAS from hp4d and TEF have been combined resulting in promoters with a large range of efficiency (Blazeck et al. 2011).

Auxotrophy or dominant (acquisition of antibiotic resistance or new metabolic property) markers can be used for selection in *Yarrowia*. The most commonly used auxotrophy marker genes are *LEU2* and *URA3*. The yeast is unable to use sucrose as sole carbon source, hence sucrose utilization gene (*SUC2*) from *S. cerevisiae* was used as a dominant marker (Nicaud et al. 1989). This yeast is sensitive to the bleomycin/phleomycin group of antibiotics and to hygromycin B. Resistance genes to these antibiotics have also been employed as dominant markers (Otero and Gaillardin 1996).

Protein secretion is directed by the targeting sequence of the alkaline extracellular protease (XPR2p) or the extracellular lipase (LIP2p) signal sequence. The shuttle vectors can be introduced into host strains using either the lithium acetate method in the case of integrative vectors or electroporation in the case of replicative ones. Host strains (especially Po1 series) were constructed with nonreverting auxotrophic markers, deletions of protease-encoding genes, and carrying a docking platform (Nicaud et al. 2002).

More than 100 heterologous proteins from viruses, bacteria, fungi, protists, plants, insects, mammals (nonhuman), and humans have been successfully produced in this yeast (Table 2.1). A detailed description of the available strains, vectors, and cloned genes as well as recent developments of the *Yarrowia* expression system, such as surface display vectors, engineered strains, and high-throughput screening processes is given in the review by Madzak and Beckerich (Madzak and Beckerich 2013).

## 8 Miscellaneous Applications

### 8.1 Biosensor

Biosensor is a device for the detection of an analyte that combines a biological component with a physicochemical detector component. Microbial biosensors are analytical devices that integrate microorganism(s) or their derived enzyme(s) with a physical transducer to generate a measurable signal proportional to the concentration of analytes (Su et al. 2011).

**Table 2.1** Heterologous protein expression/secretion in *Y. lipolytica*<sup>a</sup>

Protein	Promoter:secretion signal	Vector <sup>b</sup> /production <sup>c</sup>
<i>Viruses</i>		
Hepatitis B virus pre-HBs antigen (30 kDa)	pXPR2:XPR2 prepro	Int., Mono/batch: (85 mg/L)
Bacteriophage P1 Cre recombinase (41 kDa)	hp4d:none	Rep./flask: [intra: +]
<i>Bacteria</i>		
<i>E. coli</i> Tn5 phleomycin resistance gene (15 kDa)	pLEU2:none	Int., Mono/flask: [intra: +]
<i>E. coli</i> $\beta$ -galactosidase (116 kDa)	pLEU2:none	Int., Mono/flask: [intra: +]
	pXPR2:none	Int., Mono/flask: [intra: 400 U/L]
	hp4d:none	Rep. and Int., Mono/flask: [intra: 420 U/L]
	pG3P, pPOX1 or pPOX5:none	Int., Mono/flask: [intra: 200–1000 U/L]
	pICL1, pPOX2 or pPOT1:none	Int., Mono/flask: [intra: 800–4000 U/L]
	pICL1:none	Rep./flask: [intra: 300 U/L] Int., Multi/flask: [intra: 1,300–3,400 U/L]
<i>E. coli</i> $\beta$ -glucuronidase (68 kDa)	pALK1, pRPS7, pICL1 or pTEF1:none	Rep./flask: [intra: 265–575 U/mg protein]
	pTEF1, pEXP1, hp4d, hp8d, hp12d, hp16d, hp20d, hp28d or hp32d:none	Rep./flask: [intra: 26–1200 U/L]
	pLEU2:none	Int., Mono/flask: [intra: +] Rep./flask: [intra: +]
<i>E. coli</i> hygromycin B resistance gene	pTEF1, pGPM1, pGPD, pFBA1, pGPAT, pYAT1 or pEXP1:none	Rep./flask: [intra: +]
	pTEF1, pGPM1, pTDH1 or pFBA1:none	Int., Mono/flask: [intra: 650–18,000 nmol/min/mg]
<i>E. coli</i> XylE catechol dioxygenase	pXPR2 or hp4d:none	Int., Mono and Rep./flask: [intra: +]
<i>E. coli</i> XylE catechol dioxygenase	pXPR2:none	Int., Mono/flask: [intra: +]
<i>E. coli</i> amylolytic enzyme (85 kDa)	hp4d:modified XPR2 prepro	Int., Mono/flask: 1 g/L
<i>Vitreoscilla stercoraria</i> single-chain hemoglobin VHb	pXPR2 or pICL1:none	Int., Mono and Multi/batch: [intra (+)]
<i>Agrobacterium radiobacter</i> soluble epoxide hydrolase (33 kDa)	pTEF1:none	Int., Mono/flask: [intra: +]
<i>Vibrio harveyi</i> haemolysin (46 kDa)	hp4d:XPR2 pre [Y1CWPI]	Int., Mono/flask: Surface display 100 % cells
<i>Vibrio</i> sp. QY101/alginate lyase (38 kDa)	hp4d:XPR2 pre [Y1CWPI]	Int., Mono/flask: 208 U/g (dw) Surface display

(continued)

**Table 2.1** (continued)

Protein	Promoter:secretion signal	Vector <sup>b</sup> /production <sup>c</sup>
<i>Erwinia chrysanthemi</i> L-asparaginase (39 kDa)	pPOX2:LIP2 prepro	Int., Mono and Multi/flask: [intra (+)]
<i>Pseudomonas aeruginosa</i> polyhydroxyalkanoate synthase (62 kDa)	pPOX2:none	Int., Mono/flask: [intra: Peroxisomal targeting]
<i>Bacillus subtilis</i> endo- $\beta$ -1,4-annanase (41 kDa)	hp4d:XPR2 pre [ScFLO1]	Int., Mono/flask: 62 I U/g (dw) Surface display
<i>Thermobifida fusca</i> thermostable $\alpha$ -amylase (65 kDa)	hp4d: XPR2 pre	Int., Mono/flask: 730 U/L
<i>T. fusca</i> AXE thermostable esterase (28 kDa)	hp4d: XPR2 pre	Int., Mono/flask: 71 U/L
<i>Arthrobacter</i> sp. S37 endo-inulinase (79 kDa)	hp4d: XPR2 pre	Int., Mono/flask: 17 U/mL
<i>Fungi</i>		
<i>Saccharomyces cerevisiae</i> invertase (85 kDa)	pXPR2: XPR2 pre	Int., Mono/flask: 1,400 U/L
	pFBA1: Native + FBA1 pFBA1:Intron XPR2 prepro + 13 aa +FBA1 intron	Int., Mono/flask: 45 U/mL Int., Mono/flask: 39 U/mL
<i>Aspergillus aculeatus</i> cellulase I (29 kDa)	pXPR2:native	Rep./flask: (+)
<i>A. aculeatus</i> galactanase I (44 kDa)	pXPR2:native	Rep./flask: (+)
<i>A. aculeatus</i> polygalacturonase I (45 kDa)	hp4d:XPR2 pre	Int., Mono/flask: 3 mg/L
	pXPR2:native	Rep./flask: (+)
<i>A. aculeatus</i> endo- $\beta$ -1,4-mannanase (42 kDa)	hp4d:LIP2 pro, LIP2 prepro or Native	Int., Mono and Multi/flask and batch: 123–40,835 nkat/mL
<i>A. oryzae</i> leucine aminopeptidase II (90 kDa)	hp4d:hybrid LIP2/XPR2 prepro	Int., Mono/batch: 320 U/L Int., Multi/fed-batch: 28,000 U/L
	hp4d:none	Int., Mono/flask: 11 U/mg
<i>A. niger</i> soluble epoxide hydrolase (CAB59813–44 kDa)	pTEF1:none	Int., Mono/flask: (+)
	pTEF1:none	Int., Mono/flask: (+)
<i>A. niger</i> SQ-6 soluble epoxide hydrolase (AAX78198–44 kDa)	hp4d: XPR2 pre	Int., Mono/flask: 5 mg/L
	pXPR2, pTEF or pRPS7:native	Rep./flask: 5–8 mg/L
<i>H. insolens</i> xylanase I (27 kDa)	pXPR2, pTEF or pRPS7:native	Rep./flask: 250 $\mu$ g–2 mg/L
<i>Thermomyces lanuginosus</i> lipase I (35 kDa)	pXPR2:native	Rep./flask: (+)

(continued)

**Table 2.1** (continued)

Protein	Promoter:secretion signal	Vector <sup>b</sup> /production <sup>c</sup>
<i>Trichoderma reesei</i> endoglucanase I (45 kDa)	p <i>XPR2</i> : <i>XPR2</i> prepro	Int., Mono/flask: (±)
	p <i>XPR2</i> :native	Int., Mono/flask and fed-batch: 5–100 mg/L
<i>Arxula adenivorans</i> glucoamylase (90 kDa)	hp4d: <i>XPR2</i> pre	Int., Mono/flask: (+)
	hp4d: <i>XPR2</i> prepro	Int., Mono/flask: (+)
<i>Alternaria alternata</i> recombinant Alta I p allergen	p <i>MTP</i> :native	Rep. and Int., Mono/flask: (+)
<i>Fusarium moniliforme</i> Δ15- desaturase (46 kDa)	p <i>GPD</i> :none	Int., Mono/flask: (+)
<i>F. moniliforme</i> Δ12-desaturase (53 kDa)	pFBA1, p <i>GPD</i> or p <i>YATI</i> : none	Int., Mono/flask: (+)
<i>Candida albicans</i> epoxide hydrolase (34 kDa)	p <i>TEF1</i> :none	Int., Mono/flask: (+)
<i>Aureobasidium pullulans</i> 10 alkaline protease I (43 kDa)	hp4d: <i>XPR2</i> pre	Int., Mono/flask: 49 U/L
<i>A. pullulans</i> HN2-3 alkaline protease II (43 kDa)	hp4d: <i>XPR2</i> pre [ <i>Y1CWPI</i> ]	Int., Mono/flask: 691 U/g (dw) Surface display
<i>Candida antarctica</i> lipase B (34 kDa)	p <i>POX2</i> : <i>LIP2</i> prepro	Int., Mono/flask and batch: 510–5,090 U/L
<i>Saccharomycopsis fibuligera</i> A11 acid protease (50 kDa)	hp4d: <i>XPR2</i> pre [ <i>Y1CWPI</i> ]	Int., Mono/flask: 1,140 U/ mL Surface display
<i>Kluyveromyces marxianus</i> exo- inulinase (59 kDa)	hp4d: <i>XPR2</i> pre	Int., Mono/flask, batch: 42–43 U/mL
	hp4d:idem + [ <i>Y1CWPI</i> ]	Int., Mono/flask: 23 U/mg cell dry weight
<i>Williopsis saturnus</i> exo-β-1,3- glucanase (46 kDa)	hp4d: <i>XPR2</i> pre	Int., Mono/flask: 16 U/L
<i>Pycnoporus cinnabarinus</i> laccase I (54 kDa)	hp4d:native, <i>XPR2</i> pre or <i>XPR2</i> prepro	Int., Mono/flask, batch: up to 20 mg/L
<i>Trametes versicolor</i> laccase IIIb (58 kDa)	hp4d:native or <i>XPR2</i> pre	Int., Mono/flask: 2.5 mg/L
<i>Rhodosporidium paludigenum</i> epoxide hydrolase (46 kDa)	p <i>TEF1</i> :none	Int., Mono/flask: (+)
<i>Rh. toruloides</i> epoxide hydrolase (45 kDa)	p <i>TEF1</i> :none	Int., Mono/flask: (+)
<i>Cryptococcus neoformans</i> epoxide hydrolase (45 kDa)	p <i>TEF1</i> :none	Int., Mono/flask: (+)
<i>Rhodotorula araucariae</i> epoxide hydrolase (46 kDa)	p <i>TEF1</i> :none	Int., Mono/flask: (+)
	hp4d:none	Int., Mono and Multi/flask, batch and fed-batch: 2,400–206,000 U/L
<i>Rh. minuta</i> cytochrome P450 53B1 (58 kDa)	p <i>POX2</i> :none	Int., Multi/flask: (+)

(continued)

**Table 2.1** (continued)

Protein	Promoter:secretion signal	Vector <sup>b</sup> /production <sup>c</sup>
<i>Rh. mucilaginosa</i> epoxide hydrolase (44 kDa)	pTEF1:none	Int., Mono/flask: (+)
<i>Mortierella alpina</i> Δ5-desaturase (50 kDa)	pTEF1:none	Int., Mono/flask: (+)
<i>M. alpina</i> Δ6-desaturase (51 kDa)	pFAB1:none + FBA1 intron hp4d:none	Int., Mono/flask: (+) Int., Mono/flask: (+)
<i>M. alpina</i> Δ12-desaturase (44 kDa)	hp4d:none	Int., Mono/flask: (+)
<i>Rhizopus stolonifer</i> lipase (43 kDa)	hp4d:XPR2 pre [ScFLO1]	Int., Mono/batch: (+) Surface display
<i>Rh. oryzae</i> lipase (30 kDa)	pXPR2:native, XPR2 pre or XPR2 prepro	Int., Mono/flask: 1.45–7.61 U/L
<i>Pythium aphanidermatum</i> Δ17-desaturase (40 kDa)	pFAB1:none + FBA1 intron pEXPI:none	Int., Mono/flask: (+) Int., Mono/flask: (+)
<i>Protists</i>		
<i>Euglena gracilis</i> Δ9-elongase (30 kDa)	pGPD:none	Int., Mono/flask: (+)
<i>E. gracilis</i> Δ5-desaturase (50 kDa)	pFAB1:none + FBA1 intron	Int., Mono/flask: (+)
<i>Plants</i>		
<i>Oryza sativa</i> α-amylase (45 kDa)	pXPR2:native, XPR2 pre or XPR2 prepro	Int., Mono/flask: (+)
<i>Zea mays</i> cytokinin oxidase I (55 kDa)	hp4d:XPR2 prepro	Int., Mono/flask: 12 mg/L
<i>Z. mays</i> cytokinin oxidase III (55 kDa)	hp4d:native or XPR2 prepro	Int., Mono/flask: (+)
<i>Theobroma cacao</i> aspartic proteinase II (62 kDa)	hp4d:hybrid LIP2/XPR2 prepro	Int., Multi/flask: (+)
<i>Capsicum annuum</i> fatty acid hydroperoxide lyase (cytochrome P450) (55 kDa)	pPOX2:none	Int., Multi/flask: 1,200 U/L
<i>Arabidopsis thaliana</i> soluble epoxide hydrolase (36 kDa)	pTEF1:none	Int., Mono/flask: (+)
<i>Insects</i>		
<i>Trichoplusia ni</i> (Lepidoptere) gut epoxide hydrolase (51 kDa)	pTEF1:none	Int., Mono/flask: (+)
<i>T. ni</i> microsomal epoxide hydrolase (51 kDa)	pTEF1:none	Int., Mono/flask: (+)
<i>Mammals (nonhuman)</i>		
Bovine prochymosin (40 kDa)	pLEU2:XPR2 prepro pXPR2:XPR2 pre + dipept. pXPR2:XPR2 prepro	Int., Mono/flask: (+) Int., Mono/flask: (+) Int., Mono/flask: (+) Rep./ flask: (+)
	hp4d:XPR2 prepro	Int., Mono/flask: 20 mg/L Int., Mono/batch: 160 mg/L

(continued)

**Table 2.1** (continued)

Protein	Promoter:secretion signal	Vector <sup>b</sup> /production <sup>c</sup>
Bovine cytochrome P450 17 $\alpha$ (56 kDa)	p <i>ICL1</i> :none	Rep./flask: [intra (+)] Int., Multi/flask: [intra (+)]
Porcine $\alpha$ 1-interferon (21 kDa)	p <i>XPR2</i> : <i>XPR2</i> prepro	Int., Mono/flask: 40 U/L Rep./flask: 120 U/L
Murine interleukin 6 (20 kDa)	hp4d: <i>XPR2</i> prepro	Int., Mono/flask: 15 mg/L
Llama anti-ACE VHH antibody (30 kDa)	hp4d: <i>XPR2</i> pre	Int., Mono/flask: (+)
<i>Human</i>		
Anaphylatoxin C5a (74 kDa)	p <i>XPR2</i> : <i>XPR2</i> prepro	Int., Mono/flask: (+)
Blood coagulation factor XIIIa (80 kDa)	p <i>XPR2</i> : <i>XPR2</i> prepro	Int., Mono/flask: (-)
	p <i>XPR2</i> : <i>XPR2</i> pre + dipept.	Int., Mono/flask: 1 mg/L
	p <i>XPR2</i> : <i>XPR2</i> pre	Int., Mono/flask: (-)
Proinsulin analog (10 kDa)	p <i>XPR2</i> : <i>XPR2</i> prepro	Int., Multi/flask: (+)
Insulinotropin (4 kDa)	p <i>XPR2</i> : <i>XPR2</i> prepro	Int., Multi/flask: (+)
Epidermal growth factor (6 kDa)	p <i>XPR2</i> : <i>XPR2</i> prepro	Int., Mono/flask: 100 $\mu$ g–2 mg/L
Tissue plasminogen activator (59 kDa)	p <i>XPR2</i> : <i>XPR2</i> prepro	Int., Mono/flask: (+)
$\alpha$ -Foetoprotein (74 kDa)	hp4d: <i>XPR2</i> prepro	Int., Mono/flask: 250 $\mu$ g/L
$\beta$ 2-Microglobulin (12 kDa)	hp4d: <i>XPR2</i> prepro	Int., Mono/flask: 5 $\mu$ g/L
Soluble CD14 variants (48 kDa)	hp4d:hybrid <i>LIP2/XPR2</i> prepro	Int., Multi/fed-batch: 500 mg/L
Cytochrome P450 1A1 (16 kDa)	p <i>POX2</i> :none, or surexpression of <i>Y1CPR</i> under p <i>ICL1</i> or p <i>POX2</i>	Int., Mono and Multi/flask: 32–1,645 U (pM/min/dw)
Anti-Ras single-chain antibody scFv (30 kDa)	hp4d: <i>XPR2</i> pre or <i>XPR2</i> prepro	Int., Mono/flask: 20 mg/L
Anti-estradiol scFv (30 kDa)	hp4d: <i>XPR2</i> pre	Int., Mono/flask: (+)
Microsomal epoxide hydrolase (51 kDa)	p <i>TEF1</i> :none	Int., Mono/flask: (+)
estrogen receptor $\alpha$ (67 kDa)	p <i>XPR2</i> , p <i>TEF</i> , p <i>ICL1</i> or p <i>RP57</i> :native	Rep./flask: (+)
$\alpha$ 2 $\beta$ -interferon (19 kDa)	p <i>POX2</i> : <i>LIP2</i> prepro	Int., Mono/flask: 5 mg/L

<sup>a</sup> Modified from (Madzak et al. 2004; Madzak and Beckerich 2013). Furthermore, more than 28 synthetic constructs and variants (their genes adapted to *Yarrowia* codon bias) of heterologous protein were expressed in *Yarrowia lipolytica*

<sup>b</sup> *Vector-type* integrative vectors (Int.) can be either monocopy (Mono), or multicopy (Multi) when using promoter-defective marker genes; centromeric replicative vectors (Rep.) can maintain 1–3 copies per cell

<sup>c</sup> Cultures were performed in shake flasks or in bioreactor (batch or fed-batch cultivation). When unspecified, the production was measured as heterologous activity in the culture supernatant, and thus corresponds to secreted active heterologous protein. When no secretion signal is present, the production is specified to be intracellular ([intra])

A microbial biosensor was developed based on immobilized psychrotrophic yeast *Y. lipolytica* integrated to flow injection analysis (FIA) for the determination of middle chain alkanes. The system responded very well to middle chain alkanes even at low operational temperatures down to 5 °C. The maximum sensitivity was obtained at 15 °C. A linear relationship was observed between the sensor response and dodecane concentration up to 100 μM. This system is suitable for the continuous monitoring of soil bioremediation processes at cold climates and also for the in situ analysis of groundwater samples (Alkasrawi et al. 1999).

Cho et al. constructed a strongly sensitive detection system with *Y. lipolytica* for detection of environmental estrogens. The detection system was constructed with different promoters (*ALK1*, *ICL1*, *RPS7*, and *TEF1*) linked to the upstream of the expression vector for the human estrogen receptor  $\alpha$  (hER $\alpha$ ) gene transformed into the *Y. lipolytica* with a chromosome-integrated *lacZ* reporter gene under the control of estrogen response elements (EREs). A combination of pTEF1p-hER $\alpha$  and CXAU1-2XERE was the most effective system for the 17 $\beta$ -Estradiol (E<sub>2</sub>)-dependent induction of the  $\beta$ -galactosidase activity. This system showed the highest  $\beta$ -galactosidase activity at 10<sup>-6</sup>–10<sup>-10</sup> M E<sub>2</sub>. The system could be used for characterizing endocrine disruptors, such as natural/synthetic hormones, pesticides, and commercial chemicals (Cho et al. 2010).

Surface display system allows the use of *Yarrowia* as a whole-cell biocatalyst (Yue et al. 2008; Yang et al. 2009). This yeast also produces environmentally significant enzymes. Therefore, *Y. lipolytica* has good potential for design and creates powerful biosensors.

## 8.2 Surface-Active Compounds Production

Surface-active compounds are amphiphilic molecules that display surface activity and emulsifying properties. Microorganisms produce biosurfactants and bio-emulsifiers as two main types of surface-active compounds. Biosurfactants reduce surface tension at the air–water interface. Bioemulsifiers reduce the interfacial tension between immiscible liquids, or at the solid–liquid interface. Biosurfactants usually exhibit emulsifying capacity but bioemulsifiers do not necessarily reduce the surface tension (Batista et al. 2006).

*Yarrowia* produce a variety of different emulsifiers, which are critical factors in biodegradation and bioremediation of hydrophobic substrates. They may also be applied to food processing, cosmetic formulations, textile manufacture, leather processing, enhance oil recovery, and crude oil drilling (Trindade et al. 2008).

*Y. lipolytica* ATCC 8662 produced liposan as an inducible extracellular bio-emulsifier when this yeast was grown on a number of water-immiscible carbon substrates. In hexadecane-supplemented cultures, emulsification activity was first detected after 36 h of growth and its maximum production was after 130 h.

Maximum emulsification activity was obtained from pH 2–5. Liposan is a heat stable bioemulsifier and loses a 60 % activity after heating for 1 h at 100 °C (Cirigliano and Carman 1984, 1985).

A tropical marine strain of *Y. lipolytica* NCIM 3589 produced emulsifier in a medium containing alkanes or crude oil with initial pH of 8.0 and the presence of sodium chloride at a concentration of 2–3 % (342–513 mM). In the stationary phase, the yeast produced the emulsifier extracellularly under conditions of carbon excess and nitrogen limitation (Zinjarde and Pant 2002a). Sarubbo et al. used babassu oil as substrate to produce bioemulsifiers by *Y. lipolytica* UCP 0988 (Sarubbo et al. 1999).

Amaral et al. used *Y. lipolytica* IMUFRJ50682 to produce a bioemulsifier in YPD medium containing glucose as carbon source. This bioemulsifier was named Yansan, which presents high emulsification activity and stability in the pH range of 3.0–9.0 and is capable of stabilizing oil-in-water emulsions with several aliphatic and aromatic hydrocarbons (Amaral et al. 2006).

*Y. lipolytica* IA 1055 produced extracellular biosurfactants with emulsification activity by fermentation using babassu oil and D-glucose as carbon sources. Natural seawater diluted at 50 % supplemented with urea, ammonium sulfate, and phosphate was used as economic basal medium. The best results were achieved under fed-batch fermentation for 60 h with 5 % of babassu oil (Vance-Harrop et al. 2003).

Sarubbo et al. have produced a biosurfactant using a combination of canola oil and glucose by *Y. lipolytica* UCP 0988. The yield was 8.0 g/L and the biosurfactant decreased the surface tension to 30 mN/m (Sarubbo et al. 2007). This strain is able to produce the biosurfactant when grown on a vegetable oil refinery residue. The isolated biosurfactant corresponds to a yield of 4.5 g/l, and the surface tension of water was reduced from 71 to 32 mN/m (Rufino et al. 2007). A soybean oil refinery residue was used for optimizing levels of biosurfactant production by *Y. lipolytica* UCP 0988. The concentration of the soybean residue (6 %) and glutamate (1 %) gave the best yields and the surface tension reached to 25.29 mN/m (Rufino et al. 2008).

Fontes et al. used a factorial design to optimize biosurfactant production. They also studied the effects of carbon sources (glycerol, hexadecane, olive oil, and glucose) and nitrogen sources (urea, ammonium sulfate, yeast extract, and peptone) on maximum variation of surface tension ( $\Delta$ ST) and emulsification index (EI). Using the response surface method (RSM) analyses, optimal concentrations for glucose (4 % w/v), glycerol (2 % w/v), ammonium sulfate (10 g/L), and yeast extract (0.5 g/L) were identified for the production of an EI of 81.3 % and a  $\Delta$ ST of 19.5 mN/m. The experimental design optimization enhanced EI and  $\Delta$ ST of the standard biosurfactant process by 110.7 and 108.1 %, respectively (Fontes et al. 2010).

### 8.3 Single Cell Oil

Oleaginous microorganisms (bacteria, fungi, and microalgae) accumulate lipids to more than 20 % of their dry weight. These lipids form the storage lipid fraction, which consists mostly of triacylglycerols (TAG) and steryl esters (SE) (Beopoulos et al. 2009). The production of microbial lipid or single cell oil (SCO) attracts much attention because of their bi-function as a supplier of functional oils and feedstock for the production of biodiesel (Huang et al. 2013).

SCO as edible oils obtained from microorganisms is now accepted as biotechnological products fulfilling key roles in the supply of major polyunsaturated fatty acids (PUFA), which are known to be essential for human nutrition and development. The commercial niche targeted by SCO is that of dietary supplements enriched in docosahexaenoic acid (DHA), arachidonic acid (AA), and  $\gamma$ -linolenic acid (GLA) (Beopoulos et al. 2009).

Lipid content of *Y. lipolytica* can reach to 40 % (w/w) of the dry cell weight. Fatty acid composition of lipid produced by this yeast is palmitic acid (C16:0, 11 %), palmitoleic acid (C16:1, 6 %), stearic acid (C18:0, 1 %), oleic acid (C18:1, 28 %), linoleic acid (C18:2; 51 %), and linolenic acid (C18:3, 1 %) (Li et al. 2008).

However, *Y. lipolytica* possesses only the  $\Delta 9$  and  $\Delta 12$  desaturases and thus, wild-type strains can only produce linoleic acid as PUFA. A engineered strain produces  $\omega$ -3 and  $\omega$ -6 fatty acids (e.g., 18:3, 18:4, 20:3, 20:4, 20:5, 22:6) by introducing and expressing heterologous genes encoding the  $\omega$ -3/ $\omega$ -6 biosynthetic pathway from fungi *Schizochytrium aggregatum* and *Mortierella alpina* known to synthesize PUFAs. Dupont company has patented several strains in order to commercialize PUFA as nutrition complements against cardiovascular disease (Beopoulos et al. 2010).

Conjugated linoleic acid (CLA) has been extensively studied because of its health benefits including cancer prevention, anti-diabetic, anti-atherogenic, and anti-obesity effects, and immune system modulation. Zhang et al. have successfully constructed a de novo conjugated linoleic acid (CLA) biosynthesis system by transforming *Y. lipolytica* Polh with the recombinant linoleate isomerase gene from *Propionibacterium acnes*. The yeast strain could produce up to 5.9 % of CLA of the total fatty acid yield using glucose as the sole carbon source (Zhang et al. 2012).

The  $\Delta 12$ -desaturase gene (*FADS12*, *d12*) from *Mortierella alpina* together with the codon-optimized linoleic acid isomerase (*opai*) gene were co-expressed in *Y. lipolytica*. The recombinant strain accumulated CLA at a level of up to 44 % of total fatty acids when grown on soybean oil, which represented 30 % of DCW after 38.5 h of cultivation. In addition, CLA was also detected in the growth medium up to 0.9 g/L (Zhang et al. 2013).

The consumption of large amounts of vegetable oils for biodiesel production could result in a shortage of edible oils and would increase the price of food. SCO like fossil hydrocarbons is a highly concentrated store of saturated hydrocarbons

that can be oxidized to generate energy. The SCO could be used as alternative lipid feedstock for biodiesel production, but its high cost is the main problem. To reduce the cost of microbial oil production, many efforts focused on using low-cost materials as media for SCO production (Huang et al. 2013).

Yarrowia was used to produce SCO using low-cost substrates such as wheat straw, sugarcane bagasse and rice bran hydrolysates, glycerol and industrial fats (Papanikolaou et al. 2002; Papanikolaou and Aggelis 2002, 2003, 2009; Papanikolaou et al. 2007; Yu et al. 2011; Tsigie et al. 2011, 2012).

Some metabolic pathways in this yeast can be improved by genetic engineering for SCO production using cheap raw materials. Enhancement of lipid level and modification of lipid profiles were performed by targeting the four following approaches: (1) Increasing the level on the two main precursors G3P and acyl-CoA; (2) boosting the TAG synthesis pathway; (3) preventing TAG remobilization and acyl-CoA degradation; (4) modification of the fatty acid profiles (Thevenieau and Nicaud 2013).

SCO production from renewable low-cost substrates offers a new direction for biorefinery, and it will have a great impact on the industrialization of SCO production process.

## 8.4 Polyols Production

Sugar alcohols are a class of polyol biological sweeteners with applications in food and pharmaceuticals. Yarrowia has noticeable potential for the production of polyols such as erythritol, mannitol, and arabitol (Tomaszewska et al. 2012).

Erythritol (four-carbon sugar alcohol) has been produced commercially using a mutant of *Aureobasidium*. However, *Y. lipolytica* is able to produce erythritol from renewable low-cost carbon substrates which is important for an economically competitive fermentation process. Glycerol is a by-product generated in large amounts during the production of biofuels. An acetate-negative mutant of *Y. lipolytica* Wratislavia K1 produced 170 g/L erythritol after 7 days when grown with 300 g/L raw glycerol at pH 3 (Rymowicz et al. 2009).

Repeated batch cultures were performed to improve the productivity of erythritol production from pure and crude glycerol by the strain Wratislavia K1. The amount of erythritol reached to 220 g/L, which corresponded to a 0.43 g/g yield and a productivity of 0.54 g/L h (Mirończuk et al. 2014).

Three strains A UV'1, A-15, and Wratislavia K1 were used for the production of erythritol or/and mannitol in bioreactor using batch cultures and fed-batch modes. The best results for erythritol biosynthesis were achieved in medium with crude glycerol supplemented with 2.5 % NaCl. Wratislavia K1 strain produced up to 80.0 g/L erythritol with 0.49 g/g yield and productivity of 1.0 g/L h. Erythritol biosynthesis by A UV'1 strain was accompanied with the simultaneous production of mannitol and arabitol up to 27.6 and 3.4 g/L, respectively. The highest production rate of arabitol (9.2 g/L) was obtained by A-15 strain. The addition of

NaCl to the medium improves erythritol biosynthesis, and simultaneously inhibits mannitol formation (Tomaszewska et al. 2012).

Since 2003, Baolingbao Biology Co., Ltd., from Shandong, China, produces erythritol with *Y. lipolytica*, for addition to foods as a nutritive sweetener, flavor enhancer, formulation aid, humectant, stabilizer and thickener, sequestrant, and texturizer. This product is distributed in China, Japan, Korea, and Norway (Groenewald et al. 2014).

## 9 Conclusion

The yeast *Y. lipolytica* degrades efficiently low-cost hydrophobic substrates for the production of various added-value products. Therefore, the yeast has interesting characteristics for biotechnological applications. On one hand, this strain degrades hydrophobic compounds and reduces their environmental pollutant effects. On the other hand, it secretes a set of valuable metabolites and proteins in noticeable amounts for industrial applications.

Since the *Y. lipolytica* genome has been sequenced, it is possible to use new recombinant technology and metabolic engineering in order to improve metabolic pathways involved in the production of desirable metabolites and products.

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