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The lipases from *Yarrowia lipolytica*: genetics, production, regulation, biochemical characterization and biotechnological applications.

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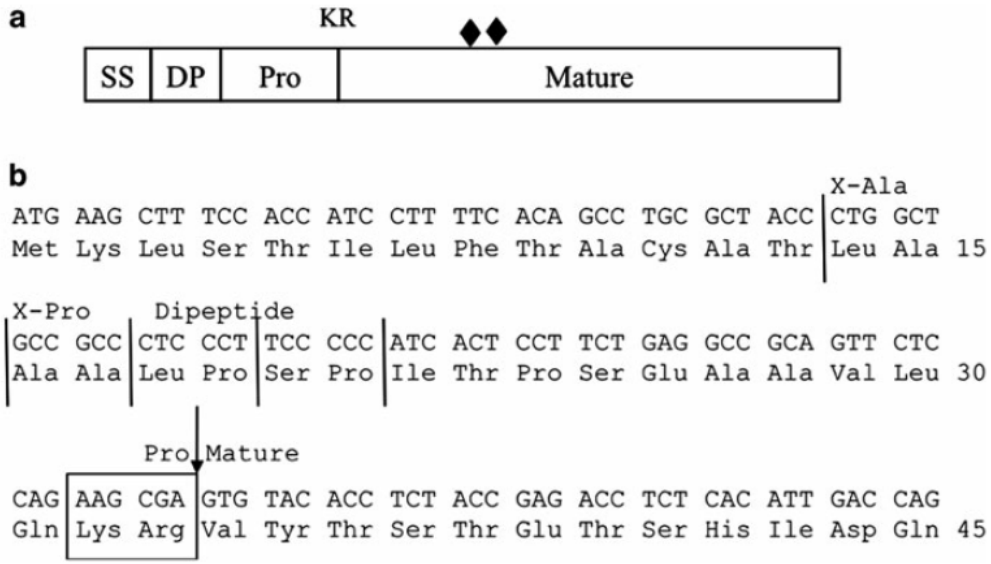
The lipases from *Y. lipolytica*

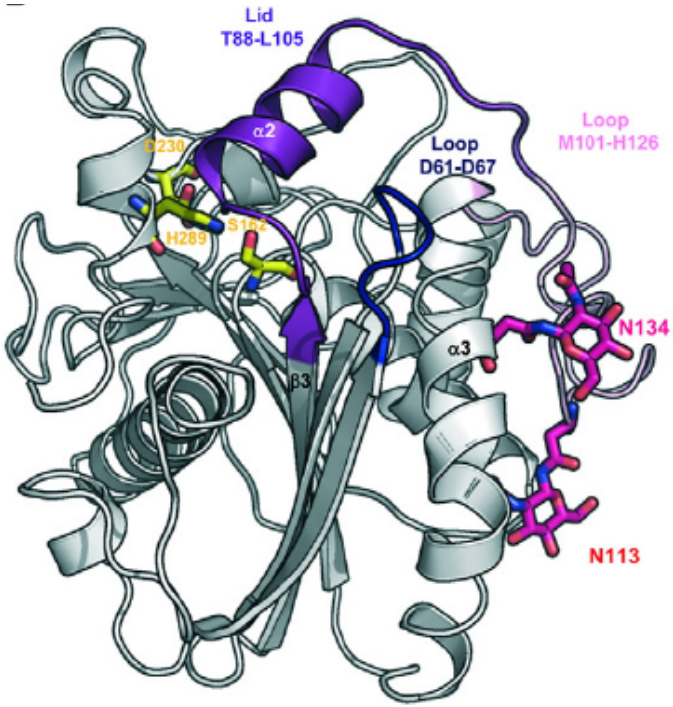
- From the genome sequence,16 lipases encoding gene have been highlighted.
- Lip2p is the main extracellular lipase.
- LIP2 code for a 334 aa precursor protein, processed by Kex2-like protease.
- Lip2p possess a pre-signal sequence (SS); a X-Ala, X-Pro motif (DP) and a pro-region with Lys-Arg cleavage site.



Pignede et al, 2000, Appli. Env. Microbiol.

The lipase lip2 : structure

- Lip2 is a lipase sensus stricto: GH**S**LGG/AA motif (Pignede et al 2000)
- Lip2 is a N-glycosylated protein: $Man_8GlcNac_2$ at $N^{113}IS$ and $Man_9GlcNAc_2$ at $N^{134}NT$ (Jolivet et al 2007)
- Cristalogarphic structure has been solved at 1,7 A (Bordes et al 2010)



The lipase lip2 is a secreted protein

- Lip2 is a secreted protein. However, with an uncomon secretion kinetic as evidenced by western blot experiments

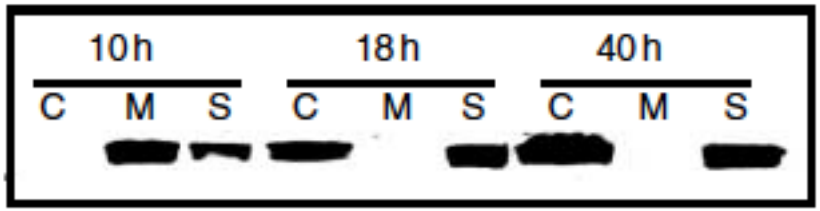


Fig. 5 Western blot analysis of the *Yarrowia lipolytica* JMY1098 culture in YNBO after 10, 18 and 40 h of growth. S, 200 µl of culture supernatant; C, cytosolic fraction from 0·5 mg of cell extract; M, membrane fraction from 0·5 mg of cell extract

- C : cytosolic location
- M : membrane location
- S : supernatant location

During cell growth, lip2 remains cell-associated before being released in the medium during the stationery phase

Fickers et al, 2004

The lipase lip2 : regulation

- LIP2 regulation was investigated using a **pLIP2-LacZ** reporter gene and β-galactosidase measurments
- LIP2 expression is induced by :
  - **Oleic acid**

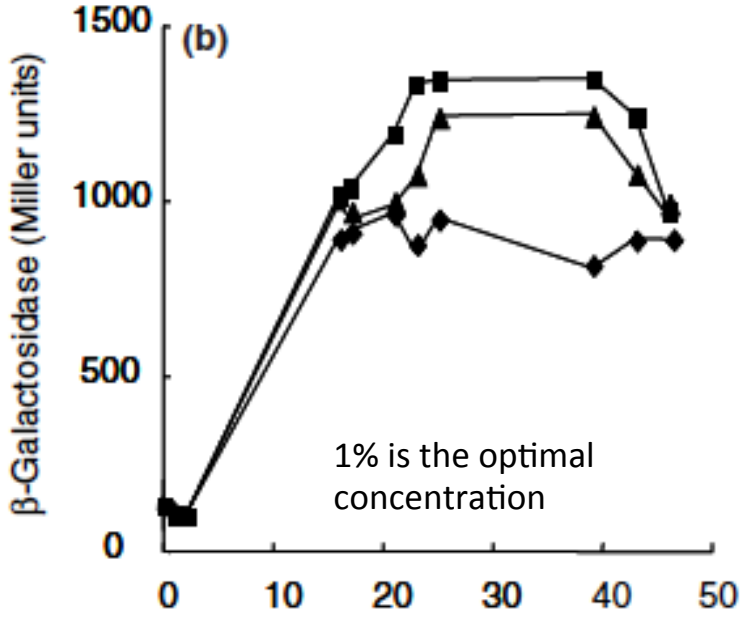


Fig. 4 Time course of lipolytic productivity (a), β-galactosidase activity (b) and oleic acid concentration (c) obtained for JMY775 growing in YNBO containing 0·5% of tryptone N1 and 0·5% (♦), 1% (■) and 3% (▲) of oleic acid. Results are mean values of three independent experiments. Standard deviation were <10% of average value

Fickers et al, 2004

The lipase lip2 : regulation

- LIP2 expression is **induced** by :
  - **Organic nitrogen**

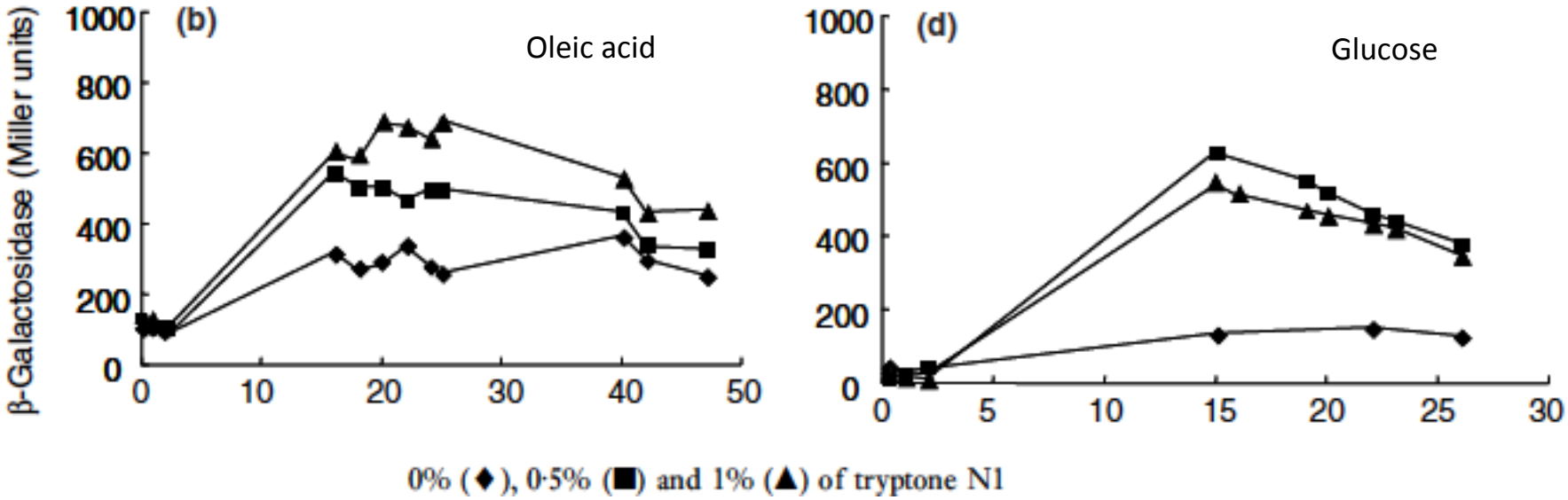
Nitrogen source	Biomass (mg DW ml^{-1})	pU (U mg^{-1})
Casamino acids	3·2 ± 0·3	6·6 ± 2·3
Peptone ET1	4·5 ± 0·2	109·0 ± 5·3
Peptone N1	3·2 ± 0·5	77·1 ± 3·3
Peptone PLUS	2·4 ± 0·6	96·8 ± 2·7
Tryptone N1	2·3 ± 0·5	484·7 ± 59·1
Urea	2·7 ± 0·2	59·1 ± 2·7
Yeast extract	2·5 ± 0·3	38·1 ± 10·1
NH_4Cl	2·0 ± 0·1	1·6 ± 0·1
$(NH_4)_2SO_4$	1·9 ± 0·1	1·7 ± 0·5
None	1·8 ± 0·1	2·9 ± 0·1

DW, dry weight; pU, lipolytic productivity.
Mean and standard deviations were calculated on four replicates.

Fickers et al, 2004

The lipase lip2 : regulation

- LIP2 expression is **induced** by :
  - **Organic nitrogen (casein tryptone)**



Fickers et al, 2004

The lipase lip2 : regulation

- LIP2 expression is **repressed** by :
  - **Glycerol**

	Lipase productivity	
Strain	YNBG (glycerol) pU ($U\ ml^{-1}\ h^{-1}\ A_{600}^{-1}$)	
	Phase I	Phase II
CBS6303	0.01	0.02[b]

Phase I : glycerol

Phase II : glycerol starvation

A very low level of lipase production could be observed even after glycerol depletion in the medium

Fickers et al, 2003

The lipase lip2 : regulation

- LIP2 expression is **repressed** by :
  - **Glucose**

	Lipase productivity	
Strain	YNBD (glucose) pU ($U\ ml^{-1}\ h^{-1}\ A_{600}^{-1}$)	
	Phase I	Phase II
CBS6303	0.1	0.5[b]

Phase I : glucose

Phase II : glucose starvation

Lipase production increases after glucose depletion

Fickers et al, 2003

The lipase lip2 : regulation by glucose

- LIP2 repression by glucose : further investigations with regulatory mutants

Lipase productivity

Strain	YNBD (glucose) pU ($U\ ml^{-1}\ h^{-1}\ A_{600}^{-1}$)	
	Phase I	Phase II
CBS6303	0.1	0.5[b]
LgX64.81	5.6	6.2[a]

Phase I : glucose

Phase II : glucose starvation

LgX64.81 : NTG mutagenesis
Regulatory mutant

High level of lipase production in the presence of glucose

Fickers et al, 2003

The lipase lip2 : regulation by glucose

- Mutant LgX64.81 presents a **growth defect** on glucose medium Vs WT
- LgX64.81 presents **a lower glucose uptake capacity**

Table 2. Determination of the doubling time (g) and sugar consumption rate (R_s) for *Y. lipolytica* CBS6303 wild-type and LgX6481 mutant growing in glucose (YNBD) and fructose (YNBF) media. Values are the mean and standard deviation of three separate experiments

Strain	Glucose		Fructose	
	g (min)	R_s (g/(LhmgDW)	G (min)	R_s (g/(LhmgDW))
CBS6303	171 ± 7	0.18 ± 0.04	182 ± 6	0.28 ± 0.02
LgX64.81	322 ± 12	0.09 ± 0.02	215 ± 8	0,18 ± 0.07

Fickers et al, 2003

The lipase lip2 : regulation by glucose

- This lower uptake capacity is due to a **lower hexokinase activity** of LgX64.81
- Is glucose transport involved in LIP2 regulation ?

Table 3. Determination of hexokinase activity during growth of *Y. lipolytica* strains CBS6303 and LgX64.81 in YNBD medium. Hexokinase activity was assayed with 20 mM fructose. Values are the mean and standard deviation of three separate experiments. Hexokinase activity was expressed as mU per milligram cells (DW)

Time (h)	CBS6303	LgX64.81
16	1.10 ± 0.01	0.82 ± 0.01
24	1.11 ± 0.03	0.87 ± 0.04
44	1.07 ± 0.02	0.86 ± 0.03

Fickers et al, 2003

The lipase lip2 : regulation by glucose

- Over-expression of hexokinase *HXK1* in LgX64.81 restore glucose repression

	Hexokinase (mU /mg)	Lipase productivity (U /mL mg DW)	β-galactosidase activity (Miller units)
WT	1.10 ± 0.10	1.0 ± 0.1	36 ± 4
LgX64.81	0.80 ± 0.05	7.4 ± 0.1	612 ± 14
LgX64.81 / HXK1	2.40 ± 0.40	1.3 ± 0.2	168 ± 23

Fickers et al, 2003

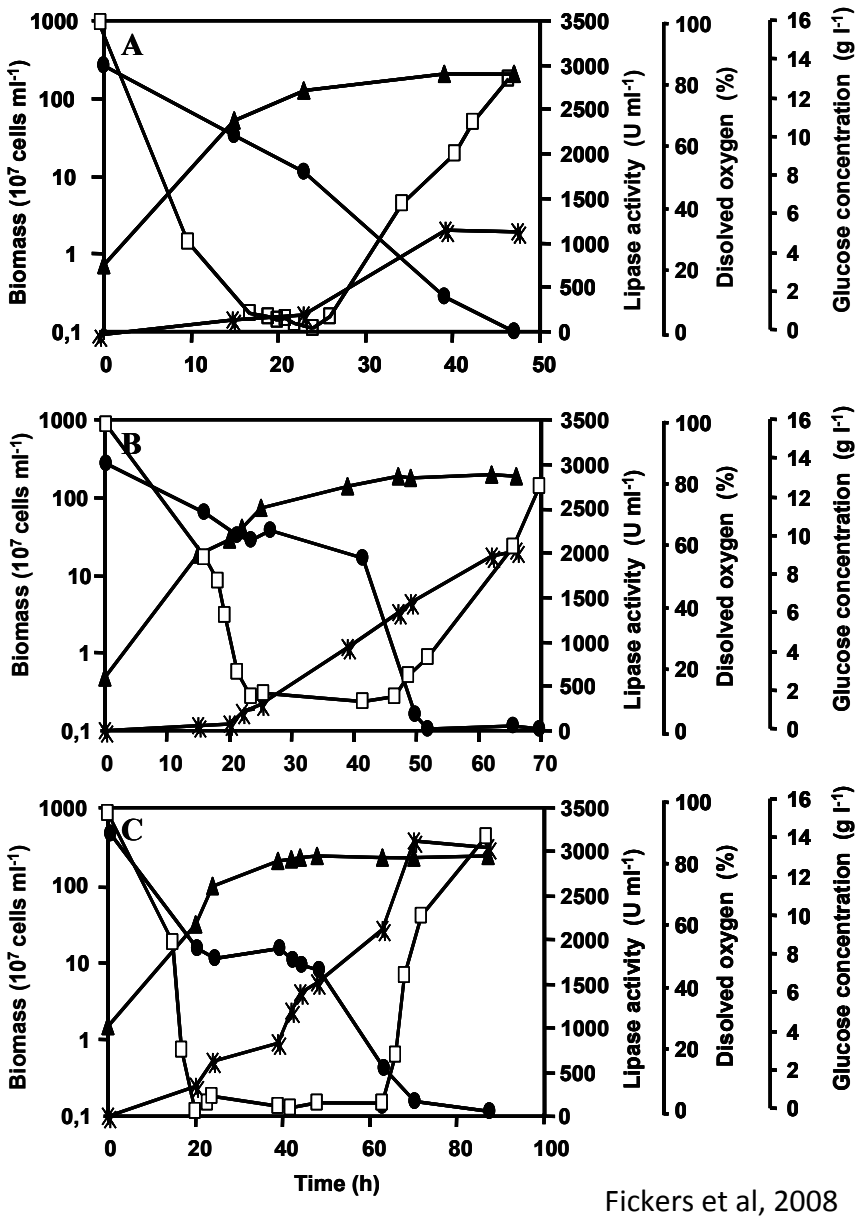
Production in bioreactor with non-GMO strain

- Culture in 20 L bioreactor of LgX64.81

Batch
1000 U/ml

Fed batch with full medium
2000 U/ml

Fed batch with glucose/oleic acid
3000 U/ml



Fickers et al, 2008

Production in bioreactor with non-GMO strain

- Culture in 2.000 L bioreactor in an olive oil based medium of LgX64.81

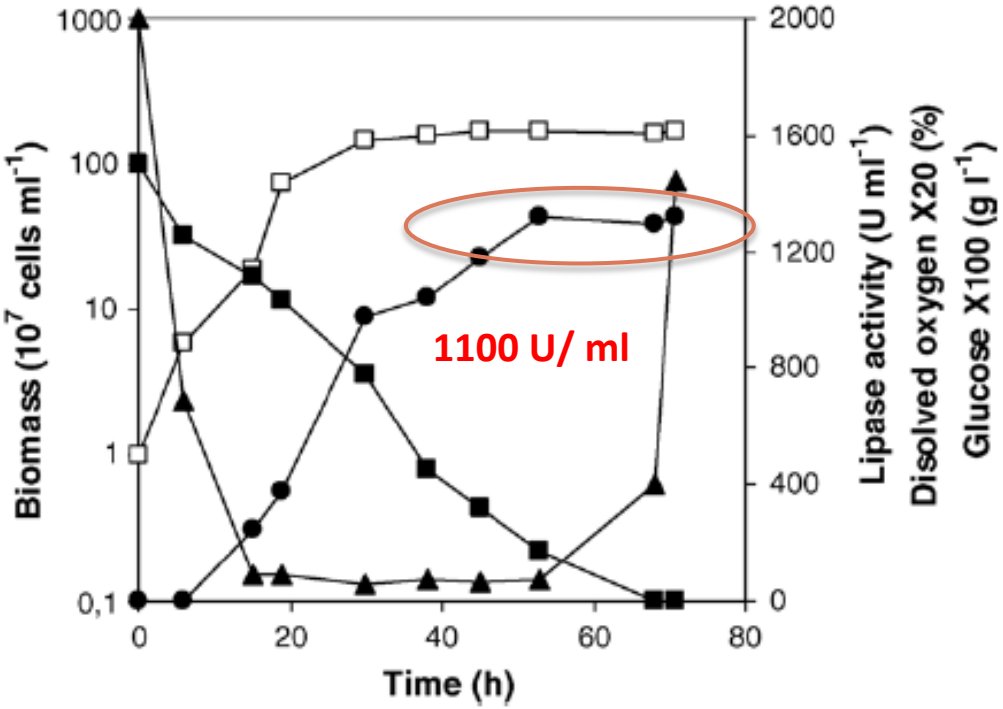


Fig. 1. Cell growth and lipase production by *Y. lipolytica* LgX64.81 during culture in 2000 L bioreactor. Cell growth (□) is expressed in 10^7 cells mL^{-1} and lipolytic activity (●) in U mL^{-1}. Dissolved oxygen (▲) and glucose concentration (■) were multiplied by a factor of 20 and 100, respectively.

Fickers et al, 2006

DSP with non-GMO strain

- Lip2 downstream process is a three steps procedure based on filtration and ultrafiltration

Table 1
Evolution of the lipase activity during the down-stream process

Down-stream step	Volume (l)	D.W. (%)	Lipase activity U g D.W.$^{-1}$
Culture broth	1100	–	ND
Centrifugation	950	7.6	12828
Plate filtration	950	5.6	13160
Ultra-filtration	75	9.0	108166

Yields in lipase activity were calculated based on the activity expressed in U g D.W.$^{-1}$. D.W., dry weight.

Fickers et al, 2006

DSP with non-GMO strain

- Lip2 dehydration by spray-drying to obtain lipase powder
- Formulation : milk powder and gum arabic

Table 3
Effect of additives on the lipase activity before and after spray-drying

Additives	Before	After	Yield (%)
6% MP	12898	9862	76
12% MP	8687	7522	86
12% MP + 3% GA	7857	6667	84
12% MP + 6% GA	6751	5572	82

Lipase activities, expressed in U mL^{-1}, are means of two experiments. MP, milk powder; GA, gum arabic.

Fickers et al, 2006

DSP with non-GMO strain

- Lip2 powder is very stable

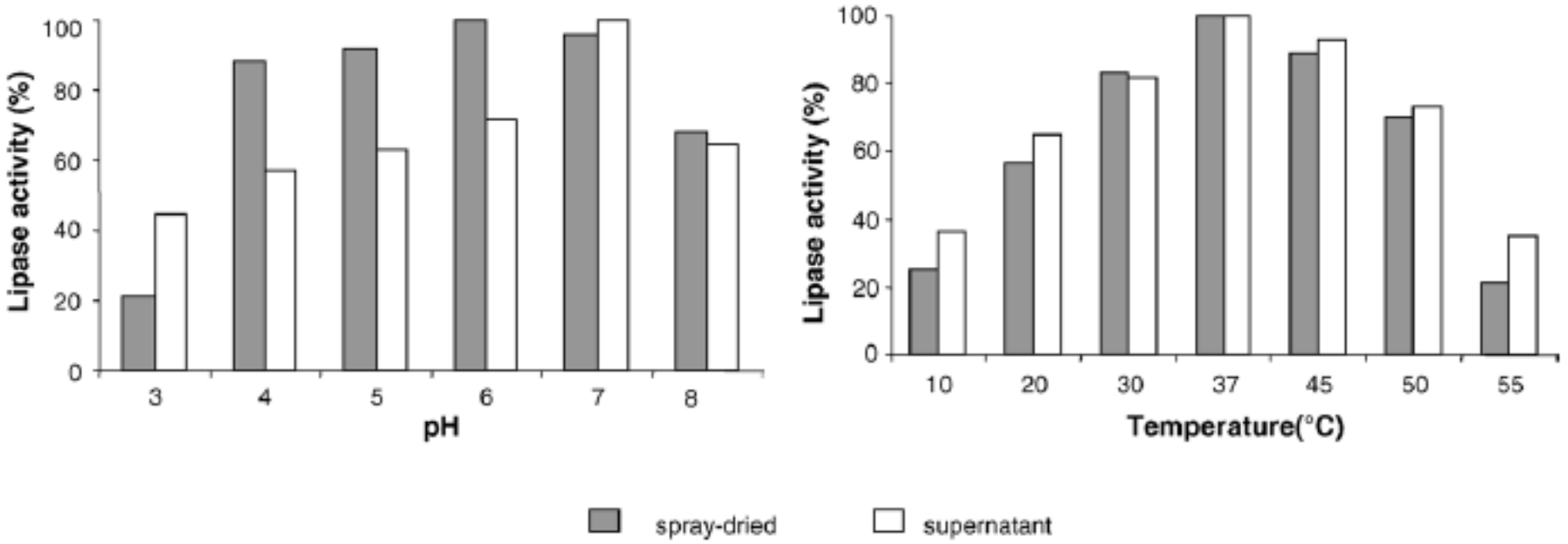


Fig. 2. Temperature and pH stability of *Y. lipolytica* extracellular lipase after dehydration by spray drying (grey) and in the culture supernatant (white). Relative enzyme activities are means of two experiments. Fickers et al, 2006

Production in bioreactor with GMO strain

- Lgx64.81 derivative with pLIP2-LIP2
- Tryptone and oleic acid fed-batch culture in 20L bioreactor

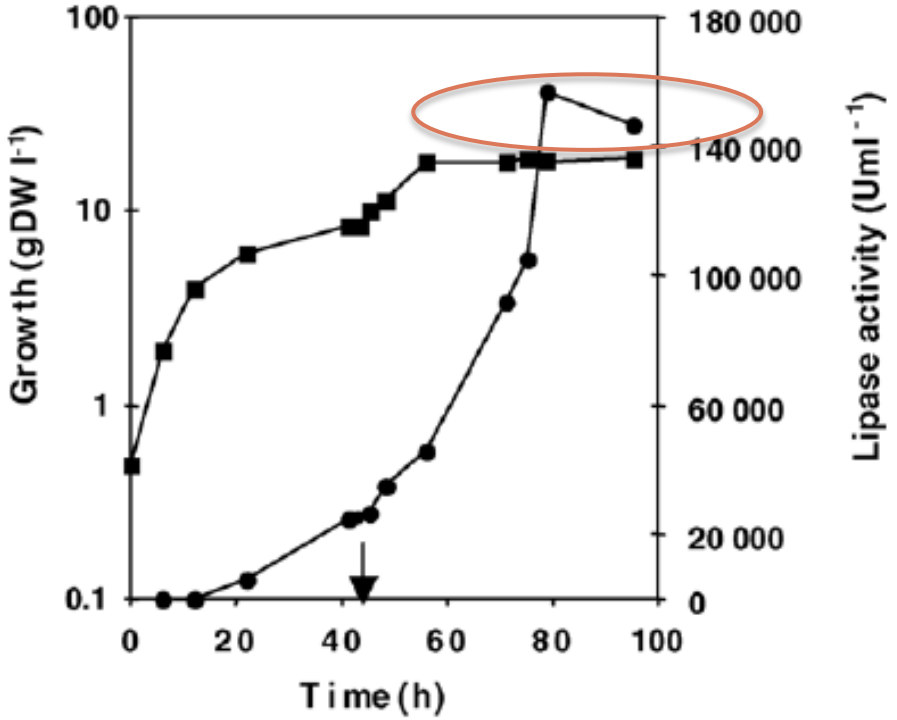


Fig. 4. Lipase production by JMY1105 during fed-batch fermentation in a 20 l bioreactor. Cell growth (solid square) is expressed in mg DW l^{-1} and lipolytic activity (solid circle) in U^{-1}. Tryptone (1%) and olive oil (2%) were added after ~45 h (indicated by the arrow) when cells entered the stationary phase.

Fickers et al, 2006

Biotechnological applications

- Waste treatment : oil mill waste water

Table 1
Application of *Yarrowia lipolytica* in the treatment of oil mill effluent.

Strain	Type of waste	Reduction	Lipase (U/L)
ATCC 20255	OMW + $(NH_4)_2$ SO_4 + YE	80% COD	770 cell free 980 cell bound
62 different strains	OMW (diluted or not)	1.5 to 41% COD 0 to 18% polyphenol	35 to 2315
ACA-DC 50109	OMW + glucose	15% polyphenol	
W29	OMW + $(NH_4)_2$ SO_4 + YE	61 to 79% COD 57 to 72% polyphenol	49 to 78
IMUFRT 50682	OMW + $(NH_4)_2$ SO_4 + YE	75 to 80% COD 39 to 68% polyphenol	16 to 27
W29	Different crude OMW	21 to 36% COD 30% polyphenol	320 to 451
CBS 2073	Different crude OMW	23 to 51% COD 25% polyphenol	828 to 1041
IMUFRJ 50682	Different crude OMW	23 to 50% COD 20% polyphenol	317 to 533
W29 immobilized	Oil waste water	82% COD	n.d.
W29	Oil waste water	67% COD	n.d.
NCIM 3589	POME	97% COD 80% BOD	n.d.

n.d.: not determined; YE: yeast extract; CA: citric acid; OMW: olive mill waste water; POME: palm oil mill effluent.

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Biotechnological applications

- Fine chemistry : drug synthesis

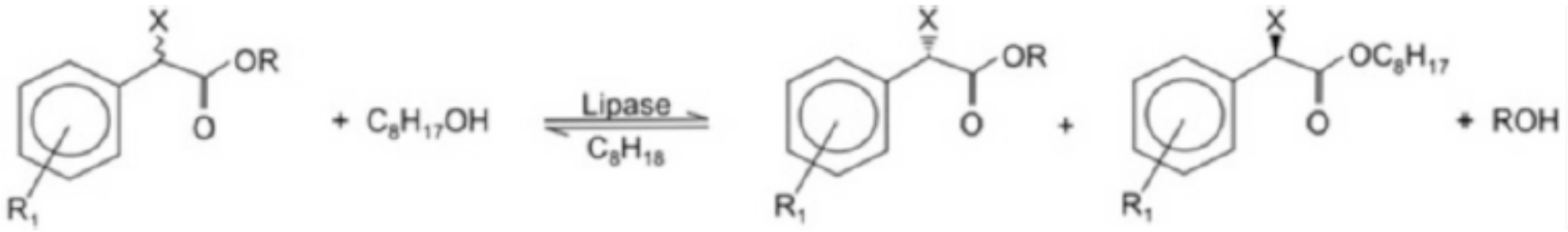

Fig. 1 Transesterification reaction between 2-halogeno-carboxylic acids and 1-octanol catalyzed by the extracellular lipase Lip2p from *Y. lipolytica* in *n*-octane

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Biotechnological applications

- Fine chemistry : synthesis of polyether

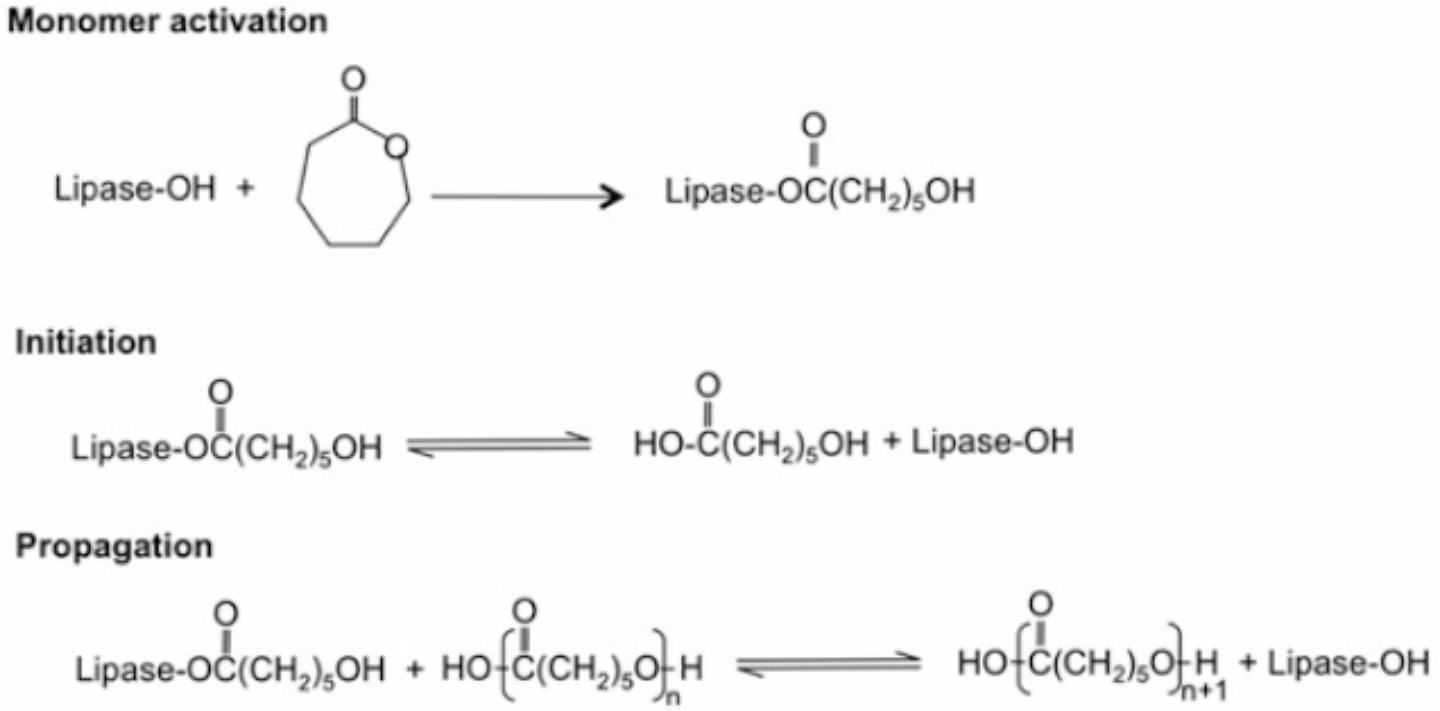

Fig. 2 Scheme for the polyether synthesis by the so-called ring-opening polymerization (ROP)

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Traditional applications

- Food applications :
  - Cheese ripening and maturation
  - Flavor development in dry fermented sausage

Thank you for your attention