CHAPTER III

MATERIAL AND METHODS

1. Microbiological techniques

1.1. Strains

The different strains and plasmids and their use in this study are presented in Table II-1.

Table II-1. Strains and plasmids used in this study

Strain	**Genotype**	**Use**	**Reference**
E. coli DH5α	F Φ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44-*thi*-1 *gyr*A96 *rel*A1 λ⁻	Plasmid amplification	Invitrogen
Y. lipolytica JMY1165	*MATA ura3-302 leu2-270 xpr2-322 lip2::LEU2 Δlip7 Δlip8*, Leu+, Ura–,	Production of Lipase, integration at random	Fickers *et al.*, 2005
Y. lipolytica JMY1212	*Mat*A *ura*3–302, *leu*2–270-LEU2-*zeta*, *xpr*2–322, *lip*2 ::*LEU*2, *Δlip2, Δlip7, Δlip8* Strain deleted for extracellular lipases Leu^+, Ura^-	Production of Lipase, integration at a targeted locus	Bordes *et al.*, 2009
Plasmid	**Characteristics**		**Reference**
JMP62 pPOX2	POX promoter inducible by oleic acid Selection marker in yeast: ura3d1 Selection marker in bacteria: kanR Used for expression of lipase		Nicaud *et al.*, 2002
JMP62 pTEF *Ura Ex*	TEF constitutive promoter Cre Lox recombinase system Selection marker in yeast: ura3d1 Selection marker in bacteria: kanR Used for expression of lipase		J.M. Nicaud, not published

1.2 Culture media

The culture media are sterilized for 20 minutes at 121°C. The described mediums are liquid medium. To obtain solid media, 15 g/L of agar is added.

1.2.1. Culture media for *E.coli*

Luria Bertani medium (LB) used for growth and glycerol stocks:

- 10 g/L Bacteriotryptone.
- 5 g/L Yeast Extract.
-10 g/L NaCl.

40 µg/mL kanamycine were used for selection of positive transformants.

SOC medium used for tranformation

- 0.2 g/L Bactotryptone
- 0.055 g/L Yeast Extract
- 1 % (v/v) 1 M NaCl
- 1 % (v/v) 1 M KCl
- distilled H_2O

After sterilization are added:

- 1 % (v/v) of 2M Mg^{++} (1M $MgCL_2$+1M $MgSO_4$)
- 1 % (v/v) of 2 M Glucose

Adjust pH 7.0

1.2.2. Culture media for *Y. lipolytica*

- YNBcasa medium used for the selection of *Ura+* transformants

- Yeast Nitrogen Bases without amino-acids or ammonium sulphate: 1.7 g/L (sterilized by filtration)
- Glucose: 10 g/L
- NH_4Cl: 5 g/L
- 50 mM Phosphates Buffer Na/K pH 6.8: 10 % (v/v)
- Casamino acids: 2 g/L (sterilized by filtration)

- Rich medium used for overnight pre-culture and glycerol stocks

YPD

- 10 g/L of Bactopeptone
- 10 g/L of Yeast Extracts
- 10 g/L glucose

- Rich medium used for growth and production in Erlenmeyer flasks

YTO medium:

- Yeast extract: 10 g/L
- Tryptone: 20 g/L

- 50 mM phosphates buffer Na/K pH 6.8
- Oleic acid: 30 g/L. The stock solution (200 g/L) was emulsified by sonication (three times one minute) with 0.5 % of Tween 40.

YTD medium:
- Yeast extract: 10 g/L
- Tryptone: 20 g/L
- 50 mM phosphates buffer Na/K pH 6.8
- Glucose: 50 g/L.

1.3. Transformation techniques

1.3.1. Transformation to *E. coli*

Plasmids or ligation products were transformed to *E. coli* DH5α competent cells by heat shock technique. 1-10 µL of DNA was added to 50-100 µL of bacterial suspension. The suspension was incubated at 4°C for 30 minutes, heat shocked for 45 second at 42 °C and returned to incubation at 4°C for 2 minutes. After that, 900 µL of SOC medium was added and the suspension was incubated at 37 °C with horizontal shaking for 1 h. The suspension was finally spread on selective media (LB supplemented with kanamycin).

1.3.2. Transformation to *Yarrowia lipolytica*

1.3.2.1. Preparation of *Y. lipolytica* competent cells

6 h pre-culture of *Y. lipolytica* strain in YPD supplemented with 10% citrate buffer (500 mM Citrate, pH 4.0) were used to inoculate 2 flasks ($1x10^5$ and $5x10^5$ cells/mL) containing 20 mL of the same medium. Cells were cultivated until a cell density ranging from $8x10^7$ to $1.5x10^8$ cells/mL (12-16 h). Cells were harvested 5 minutes at 5000 rpm, washed with 20 mL buffer (Tris 50 mM, EDTA 5 mM, pH 8) and further incubated in 20 mL of 0.1 M Litium acetate, pH 6.0 (LiAc) for 1 h at 28 °C without agitation. Cells were harvested for 2 minutes at 2000 rpm and finally gently re-suspended in 2 mL chilled LiAc ($5x10^8$ cells/mL).

1.3.2.2. Transformation to *Y. lipolytica*

5 µL of carrier DNA (5 mg/mL of Salmon DNA in TE buffer) were mixed with 1-10 µL of DNA to transform (1 µg) and 100 µL of competent cells. The suspension was incubated at 28 °C for 15 minutes without shaking. 700 µL of PEG 4000 (40% of PEG4000 in 0.1 M LiAC, pH 6.0) was added and the suspension was incubated at 28 °C with shaking for 1 h. Heat shock was done for 10 minutes at 39 °C. Finally, 1.2 mL of LiAc were added and the suspension was spread on selective media (YNBcasa).

2. Molecular biology techniques

2.1. Preparation of nucleic acids

2.1.1. Synthesis of *C. rugosa* synthetic genes

CRL genes were synthesized by Gene Art AG., Germany, by using Codon optimezer$^{(TM)}$ method. All non-universal codons encoding serine (CTG) were replaced by universal serine triplets TCT, and the nucleic sequence was optimized for expression in *Y. lipolytica*. The nucleic sequence of the synthetic genes encoding the mature proteins are shown in Figure II-1.

2.1.2. DNA extraction

a) Bacterial plasmidic DNA extraction

E. coli strain containing the gene of interest was grown in liquid selective media (LB kanamycine). Extraction and purification of the plasmids from overnight cultivated strains were carried out by using the QIAprep kit following the protocol provided by Qiagen. The method is based on the method of alkaline lysis (Sambrook *et al.*, 1989).

b) Yeast genomic DNA extraction

Yeast cells from 24 to 48 hours culture in 3 ml YPD were resuspended in 500 µL of sorbitol buffer (1 M Sorbitol, 0.1 M HCl pH 8, and 0.1 M EDTA). 50 µl of zymolyase 100 T (3 mg/mL) and 50 µl of β-mercaptoethanol 0.28 M were added. The mixture was incubated 1 h at 37°C. After 5 minutes centrifugation at 13 000 rpm, cells were resuspended in 500 µL of TE buffer (50 mM Tris-HCl pH 8,0, 20 mM EDTA) and 50 µL of SDS 10% was added. After 20 min incubation at 65°C, 200 µL of 5 M potassium acetate were added and the mixture was left 30 minute in ice.

Supernatant was recovered and 700 µL iso-propanol was added; cells were then harvested, washed with 500 µL of ethanol 70%, and partially dried. 400 µL of TE-RNAse (100 µg/mL) was then added the mixture was incubated 1 h at 37°C. Finally, addition of 40 µL sodium acetate 2.5 M pH 5.2 and 1 ml of absolute ethanol allowed DNA to precipitate. DNA was washed with 700 µL of ethanol 70% and resuspended in 100 µL water.

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                10        20        30        40        50        60        70        80
      ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Lip1  GCCCCCACCGCTACCCTGGCTAACGGCGACACCATCACCGGCCTGAACGCCATCATCAACGAGGCCTTTCTGGGCATCCC
Lip2  GCCCCCACCGCCACCCTGGCCAACGGCGACACCATCACCGGCCTGAACGCCATCGTGAACGAGAAGTTTCTGGGCATCCC
Lip3  GCCCCCACCGCCAAGCTGGCCAACGGCGACACCATCACCGGCCTGAACGCCATCATCAACGAGGCCTTTCTGGGCATCCC
Lip4  GCCCCCACCGCCACCCTGGCCAACGGCGACACCATCACTGGCCTGAACGCCATCATCAACGAGGCCTTTCTGGGCATCCC
Lip5  GCCCCCACCGCCACCCTGGCCAACGGCGACACCATCACCGGCCTGAACGCTATCATCAACGAGGCCTTTCTGGGCATCCC

                90       100       110       120       130       140       150       160
      ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Lip1  CTTCGCCGAGCCCCCCGTGGGCAACCTGCGATTCAAGGACCCCGTGCCCTACTCTGGCTCTCTGGACGGCCAGAAGTTCA
Lip2  CTTCGCCGAGCCCCCCGTGGGCAACCTGCGATTCAAGCCCCCCGTGCCCTACTCTGCCTCTCTGAACGGCCAGCAGTTCA
Lip3  CTTCGCCGAGCCCCCCGTGGGCAACCTGCGATTCAAGGACCCCGTGCCCTACTCTGGCTCTCTGAACGGCCAGAAGTTCA
Lip4  CTTCGCCCAGCCTCCCGTGGGCAACCTGCGATTCAAGCCCCCCGTGCCCTACTCTGCCTCTCTGAACGGCCAGAAGTTCA
Lip5  CTTCGCCGAGCCCCCCGTGGGCAACCTGCGATTCAAGGACCCCGTGCCCTACCGAGGCTCTCTGAACGGCCAGTCTTTCA

                170       180       190       200       210       220       230       240
      ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Lip1  CCTCTTACGGCCCCTCTTGTATGCAGCAGAACCCCGAGGGCACCTACGAGGAGAACCTGCCCAAGGCCGCCCTGGACCTG
Lip2  CCTCTTACGGCCCCTCTTGTATGCAGATGAACCCCATGGGATCTTTCGAGGACACCCTGCCCAAGAACGCCCGACACCTG
Lip3  CCTCTTACGGCCCCTCTTGTATGCAGCAGAACCCCGAGGGCACCTTCGAGGAGAACCTGGGCAAGACCGCCCTGGACCTG
Lip4  CCTCTTACGGCCCCTCTTGTATGCAGATGAACCCCCTGGGCAACTGGGACTCTTCGCTGCCCAAGGCCGCCATCAACTCC
Lip5  CCGCCTACGGCCCCTCTTGTATGCAGCAGAACCCCGAGGGCACCTACGAGGAAAACCTGCCCAAGGTCGCCCTGGACCTG

                250       260       270       280       290       300       310       320
      ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Lip1  GTGATGCAGTCTAAGGTGTTCGAGGCCGTGTCTCCCTCGTCTGAGGACTGTCTGACCATCAACGTGGTGCGACCCCCCGG
Lip2  GTGCTGCAGTCTAAGATCTTCCAGGTGGTGCTGCCCAACGACGAGGACTGTCTGACCATCAACGTGATCCGACCCCCCGG
Lip3  GTGATGCAGTCTAAGGTGTTCCAGGCCGTGCTGCCCCAGTCTGAGGACTGTCTGACCATCAACGTGGTGCGACCCCCCGG
Lip4  CTGATGCAGTCTAAGCTGTTCCAGGCCGTGCTGCCCAACGGCGAGGACTGTCTGACCATCAACGTGGTGCGACCCTCTGG
Lip5  GTGATGCAGTCTAAGGTGTTCCAGGCCGTGCTGCCCAACTCTGAGGACTGTCTGACCATCAACGTGGTGCGACCCCCTGG

                330       340       350       360       370       380       390       400
      ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Lip1  AACCAAGGCCGGTGCCAACCTGCCCGTGATGCTGTGGATCTTCGGCGGAGGCTTCGAGGTGGGCGGCACCTCTACCTTCC
Lip2  AACCCGAGCCTCTGCCGGCCTGCCCGTGATGCTGTGGATCTTCGGCGGAGGCTTCGAGCTGGGCGGATCTTCTCTGTTCC
Lip3  AACCAAGGCCGGTGCCAACCTGCCCGTGATGCTGTGGATCTTCGGCGGAGGCTTCGAGATCGGCTCTCCCACCATCTTCC
Lip4  CACCAAGCCCGGTGCCAACCTGCCCGTGATGGTGTGGATCTTCGGCGGAGGCTTCGAGGTGGGCGGATCTTCTCTGTTCC
Lip5  CACCAAGGCCGGTGCCAACCTGCCCGTGATGCTGTGGATCTTCGGCGGAGGCTTCGAGATCGGCTCTCCCACCATCTTCC

                410       420       430       440       450       460       470       480
      ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Lip1  CACCTGCCCAGATGATCACCAAGTCTATCGCCATGGGCAAGCCCATCATCCACGTGTCTGTGAACTACCGAGTGTCCTCT
Lip2  CCGGCGACCAGATGGTGGCCAAGTCTGTGCTGATGGGCAAGCCCGTGATCCACGTGTCTATGAACTACCGAGTGGCCTCT
Lip3  CCCCTGCCCAAATGGTGACCAAGTCTGTGCTCATGGGAAAGCCTATTATCCACGTGGCCGTGAACTACCGAGTGGCCTCT
Lip4  CCCCTGCCCAAATGATCACCGCCTCTGTGCTGATGGGCAAGCCCATTATCCACGTGTCTATGAACTACCGAGTGGCCTCT
Lip5  CCCCTGCCCAAATGGTGTCTAAGTCTGTGCTGATGGGCAAGCCCATTATCCACGTGGCCGTGAACTACCGACTGGCCTCT

                490       500       510       520       530       540       550       560
      ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Lip1  TGGGGCTTCCTGGCCGGCGACGAGATCAAGGCCGAGGGCTCTGCCAACGCCGGCCTGAAGGACCAGCGACTGGGAATGCA
Lip2  TGGGGCTTCCTGGCCGGACCCGACATCCAGAACGAGGGCTCTGGCAACGCCGGCCTGCACGACCAGAGACTGGCCATGCA
Lip3  TGGGGCTTCCTGGCCGGCGACGACATCAAGGCCGAGGGCTCTGGCAACGCCGGCCTGAAGGACCAGCGACTGGGAATGCA
Lip4  TGGGGCTTCCTGGCCGGACCCGACATCAAGGCCGAGGGCTCTGGCAACGCCGGCCTCCACGACCAGCGACTGGGCCTGCA
Lip5  TTCGGCTTCCTGGCCGGACCCGACATCAAGGCCGAGGGATCTTCTAACGCCGGCCTGAAGGACCAGCGACTGGGAATGCA
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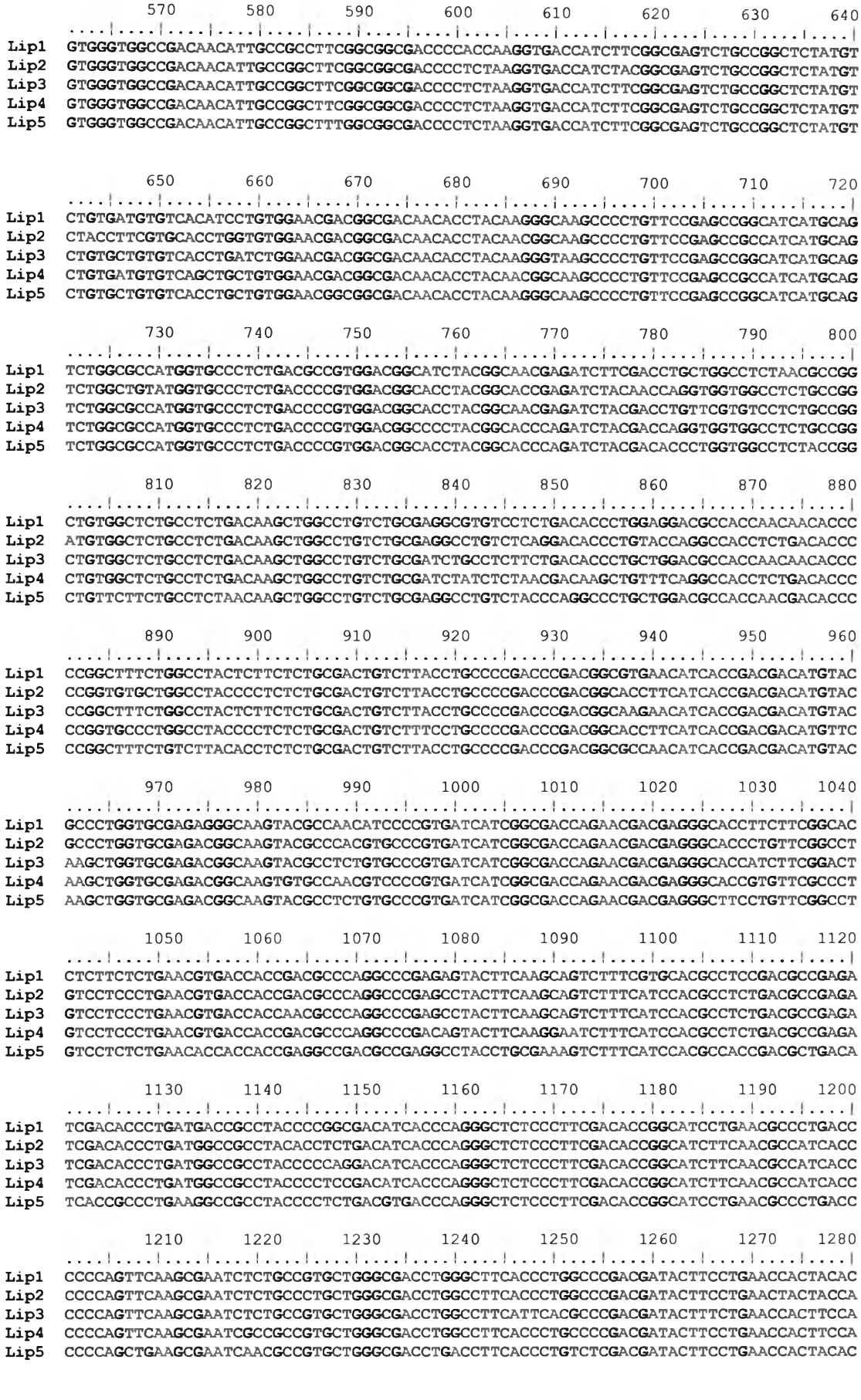
570 580 590 600 610 620 630 640
Lip1 GTGGGTGGCCGACAACATTGCCGCCTTCGGCGGCGACCCCACCAAGGTGACCATCTTCGGCGAGTCTGCCGGCTCTATGT
Lip2 GTGGGTGGCCGACAACATTGCCGGCTTCGGCGGCGACCCCTCTAAGGTGACCATCTACGGCGAGTCTGCCGGCTCTATGT
Lip3 GTGGGTGGCCGACAACATTGCCGGCTTCGGCGGCGACCCCTCTAAGGTGACCATCTTCGGCGAGTCTGCCGGCTCTATGT
Lip4 GTGGGTGGCCGACAACATTGCCGGCTTCGGCGGCGACCCCTCTAAGGTGACCATCTTCGGCGAGTCTGCCGGCTCTATGT
Lip5 GTGGGTGGCCGACAACATTGCCGGCTTTGGCGGCGACCCCTCTAAGGTGACCATCTTCGGCGAGTCTGCCGGCTCTATGT
650 660 670 680 690 700 710 720
Lip1 CTGTGATGTGTCACATCCTGTGGAACGACGGCGACAACACCTACAAGGGCAAGCCCCTGTTCCGAGCCGGCATCATGCAG
Lip2 CTACCTTCGTGCACCTGGTGTGGAACGACGGCGACAACACCTACAACGGCAAGCCCCTGTTCCGAGCCGCCATCATGCAG
Lip3 CTGTGCTGTGTCACCTGATCTGGAACGACGGCGACAACACCTACAAGGGTAAGCCCCTGTTCCGAGCCGGCATCATGCAG
Lip4 CTGTGATGTGTCAGCTGCTGTGGAACGACGGCGACAACACCTACAACGGCAAGCCCCTGTTCCGAGCCGCCATCATGCAG
Lip5 CTGTGCTGTGTCACCTGCTGTGGAACGGCGGCGACAACACCTACAAGGGCAAGCCCCTGTTCCGAGCCGGCATCATGCAG
730 740 750 760 770 780 790 800
Lip1 TCTGGCGCCATGGTGCCCTCTGACGCCGTGGACGGCATCTACGGCAACGAGATCTTCGACCTGCTGGCCTCTAACGCCGG
Lip2 TCTGGCTGTATGGTGCCCTCTGACCCCGTGGACGGCACCTACGGCACCGAGATCTACAACCAGGTGGTGGCCTCTGCCGG
Lip3 TCTGGCGCCATGGTGCCCTCTGACCCCGTGGACGGCACCTACGGCAACGAGATCTACGACCTGTTCGTGTCCTCTGCCGG
Lip4 TCTGGCGCCATGGTGCCCTCTGACCCCGTGGACGGCCCCTACGGCACCCAGATCTACGACCAGGTGGTGGCCTCTGCCGG
Lip5 TCTGGCGCCATGGTGCCCTCTGACCCCGTGGACGGCACCTACGGCACCCAGATCTACGACACCCTGGTGGCCTCTACCGG
810 820 830 840 850 860 870 880
Lip1 CTGTGGCTCTGCCTCTGACAAGCTGGCCTGTCTGCGAGGCGTGTCCTCTGACACCCTGGAGGACGCCACCAACAACACCC
Lip2 ATGTGGCTCTGCCTCTGACAAGCTGGCCTGTCTGCGAGGCCTGTCTCAGGACACCCTGTACCAGGCCACCTCTGACACCC
Lip3 CTGTGGCTCTGCCTCTGACAAGCTGGCCTGTCTGCGATCTGCCTCTTCTGACACCCTGCTGGACGCCACCAACAACACCC
Lip4 CTGTGGCTCTGCCTCTGACAAGCTGGCCTGTCTGCGATCTATCTCTAACGACAAGCTGTTTCAGGCCACCTCTGACACCC
Lip5 CTGTTCTTCTGCCTCTAACAAGCTGGCCTGTCTGCGAGGCCTGTCTACCCAGGCCCTGCTGGACGCCACCAACGACACCC
890 900 910 920 930 940 950 960
Lip1 CCGGCTTTCTGGCCTACTCTTCTCTGCGACTGTCTTACCTGCCCCGACCCGACGGCGTGAACATCACCGACGACATGTAC
Lip2 CCGGTGTGCTGGCCTACCCCTCTCTGCGACTGTCTTACCTGCCCCGACCCGACGGCACCTTCATCACCGACGACATGTAC
Lip3 CCGGCTTTCTGGCCTACTCTTCTCTGCGACTGTCTTACCTGCCCCGACCCGACGGCAAGAACATCACCGACGACATGTAC
Lip4 CCGGTGCCCTGGCCTACCCCTCTCTGCGACTGTCTTTCCTGCCCCGACCCGACGGCACCTTCATCACCGACGACATGTTC
Lip5 CCGGCTTTCTGTCTTACACCTCTCTGCGACTGTCTTACCTGCCCCGACCCGACGGCGCCAACATCACCGACGACATGTAC
970 980 990 1000 1010 1020 1030 1040
Lip1 GCCCTGGTGCGAGAGGGCAAGTACGCCAACATCCCCGTGATCATCGGCGACCAGAACGACGAGGGCACCTTCTTCGGCAC
Lip2 GCCCTGGTGCGAGACGGCAAGTACGCCCACGTGCCCGTGATCATCGGCGACCAGAACGACGAGGGCACCCTGTTCGGCCT
Lip3 AAGCTGGTGCGAGACGGCAAGTACGCCTCTGTGCCCGTGATCATCGGCGACCAGAACGACGAGGGCACCATCTTCGGACT
Lip4 AAGCTGGTGCGAGACGGCAAGTGTGCCAACGTCCCCGTGATCATCGGCGACCAGAACGACGAGGGCACCGTGTTCGCCCT
Lip5 AAGCTGGTGCGAGACGGCAAGTACGCCTCTGTGCCCGTGATCATCGGCGACCAGAACGACGAGGGCTTCCTGTTCGGCCT
1050 1060 1070 1080 1090 1100 1110 1120
Lip1 CTCTTCTCTGAACGTGACCACCGACGCCCAGGCCCGAGAGTACTTCAAGCAGTCTTTCGTGCACGCCTCCGACGCCGAGA
Lip2 GTCCTCCCTGAACGTGACCACCGACGCCCAGGCCCGAGCCTACTTCAAGCAGTCTTTCATCCACGCCTCTGACGCCGAGA
Lip3 GTCCTCCCTGAACGTGACCACCAACGCCCAGGCCCGAGCCTACTTCAAGCAGTCTTTCATCCACGCCTCTGACGCCGAGA
Lip4 GTCCTCCCTGAACGTGACCACCGACGCCCAGGCCCGACAGTACTTCAAGGAATCTTTCATCCACGCCTCTGACGCCGAGA
Lip5 GTCCTCTCTGAACACCACCACCGAGGCCGACGCCGAGGCCTACCTGCGAAAGTCTTTCATCCACGCCACCGACGCTGACA
1130 1140 1150 1160 1170 1180 1190 1200
Lip1 TCGACACCCTGATGACCGCCTACCCCGGCGACATCACCCAGGGCTCTCCCTTCGACACCGGCATCCTGAACGCCCTGACC
Lip2 TCGACACCCTGATGGCCGCCTACACCTCTGACATCACCCAGGGCTCTCCCTTCGACACCGGCATCTTCAACGCCATCACC
Lip3 TCGACACCCTGATGGCCGCCTACCCCCAGGACATCACCCAGGGCTCTCCCTTCGACACCGGCATCTTCAACGCCATCACC
Lip4 TCGACACCCTGATGGCCGCCTACCCCTCCGACATCACCCAGGGCTCTCCCTTCGACACCGGCATCTTCAACGCCATCACC
Lip5 TCACCGCCCTGAAGGCCGCCTACCCCTCTGACGTGACCCAGGGCTCTCCCTTCGACACCGGCATCCTGAACGCCCTGACC
1210 1220 1230 1240 1250 1260 1270 1280
Lip1 CCCCAGTTCAAGCGAATCTCTGCCGTGCTGGGCGACCTGGGCTTCACCCTGGCCCGACGATACTTCCTGAACCACTACAC
Lip2 CCCCAGTTCAAGCGAATCTCTGCCCTGCTGGGCGACCTGGCCTTCACCCTGGCCCGACGATACTTCCTGAACTACTACCA
Lip3 CCCCAGTTCAAGCGAATCTCTGCCGTGCTGGGCGACCTGGCCTTCATTCACGCCCGACGATACTTTCTGAACCACTTCCA
Lip4 CCCCAGTTCAAGCGAATCGCCGCCGTGCTGGGCGACCTGGCCTTCACCCTGCCCCGACGATACTTCCTGAACCACTTCCA
Lip5 CCCCAGCTGAAGCGAATCAACGCCGTGCTGGGCGACCTGACCTTCACCCTGTCTCGACGATACTTCCTGAACCACTACAC

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          1290       1300       1310       1320       1330       1340       1350     1360
     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Lip1 CGGCGGCACCAAGTACTCGTTTCTGTCTAAGCAGCTGTCTGGCCTGCCCGTGCTGGGAACCTTCCACTCTAACGACATCG
Lip2 GGGCGGCACCAAGTACTCGTTTCTGTCTAAGCAGCTGTCTGGACTGCCCGTGCTGGGAACCTTCCACGGCAACGACATCA
Lip3 GGGCGGCACCAAGTACTCGTTTCTGTCTAAGCAGCTGTCTGGCCTCCCTATCATGGGAACCTTCCACGCCAACGACATCG
Lip4 GGGCGGCACCAAGTACTCGTTTCTGTCTAAGCAGCTGTCTGGACTGCCCGTGATCGGCACCCACCACGCCAACGACATCG
Lip5 CGGCGGACCCAAGTACTCGTTCCTGTCTAAGCAGCTGTCTGGCCTGCCCATCCTGGGCACCTTTCACGCCAACGACATCG

          1370       1380       1390       1400       1410       1420       1430     1440
     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Lip1 TGTTCCAGGACTACCTGCTGGGCTCTGGATCTCTGATCTACAACAACGCCTTCATTGCCTTCGCCACCGACCTGGACCCC
Lip2 TCTGGCAGGACTACCTGGTGGGCTCTGGCTCTGTGATCTACAACAACGCCTTCATTGCCTTCGCCAACGACCTGGACCCC
Lip3 TGTGGCAGGACTACCTGCTGGGCTCTGGCTCTGTGATCTACAACAACGCCTTCATTGCCTTCGCCACCGACCTGGACCCC
Lip4 TGTGGCAGGACTTTCTGGTGTCTCACTCTTCTGCCGTGTACAACAACGCCTTCATTGCCTTCGCCAACGACCTGGACCCC
Lip5 TGTGGCAGCACTTTCTGCTGGGCTCTGGCTCTGTGATCTACAACAACGCCTTCATTGCCTTCGCCACCGACCTGGACCCC

          1450       1460       1470       1480       1490       1500       1510     1520
     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Lip1 AACACCGCCGGACTGCTGGTGAAGTGGCCCGAGTACACCTCTTCGTCCCAGTCTGGCAACAACCTGATGATGATCAACGC
Lip2 AACAAGGCCGGACTGTGGACCAACTGGCCCACCTACACCTCTTCGTCCCAGTCTGGCAACAACCTGATGCAGATCAACGG
Lip3 AACACCGCCGGACTGCTGGTGAACTGGCCCAAGTACACCTCTTCGTCCCAGTCTGGCAACAACCTGATGATGATCAACGC
Lip4 AACAAGGCCGGACTGCTGGTGAACTGGCCCAAGTACACCTCTTCGTCCCAGTCTGGCAACAACCTGCTGCAGATCAACGC
Lip5 AACACCGCCGGACTGTCTGTGCAGTGGCCCAAGTCTACCTCTTCGTCCCAGGCCGGCGACAACCTGATGCAGATCTCTGC

          1530       1540       1550       1560       1570       1580       1590     1600
     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Lip1 CCTGGGCCTGTACACCGGCAAGGACAACTTCCGAACCGCCGGCTACGACGCCCTGTTCTCTAACCCCCCCTCGTTCTTCG
Lip2 CCTGGGCCTGTACACCGGCAAGGACAACTTCCGACCCGACGCCTACTCTGCCCTGTTCTCTAACCCCCCCAGCTTCTTCG
Lip3 CCTGGGCCTGTACACCGGCAAGGACAACTTCCGAACCGCCGGCTACGACGCCCTGATGACCAACCCCTCTAGCTTCTTCG
Lip4 CCTGGGACTGTACACCGGCAAGGACAACTTCCGAACCGCCGGCTACGACGCCCTGTTCACCAACCCCTCTTCGTTCTTCG
Lip5 CCTGGGCCTGTACACCGGCAAGGACAACTTCCGAACCGCCGGCTACAACGCCCTGTTCGCCGACCCCTCTCACTTCTTCG

     ....|
Lip1 TGTAA
Lip2 TGTAA
Lip3 TGTAA
Lip4 TGTAA
Lip5 TGTAA
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Figure II-1. Alignment of the genes synthesized by GeneArt and encoding mature proteins Lip1, Lip2, Lip3, Lip4 and Lip5 from *C. Rugosa*

2.1.3. Polymerase Chain Reaction (PCR) for the construction of specific mutants by site directed mutagenesis

PCR consists in the synthesis of a given DNA fragment from a matrix DNA, by means of two oligonucleotidic primers surrounding the DNA fragment and a DNA polymerase. PCR leads to an exponential increase of the desired DNA fragment quantity. PCR is held in several successive cycles (approximately 25 cycles), each cycle will enable to double the quantity of the desired DNA fragment. Each cycle includes:

- Denaturation step at 98°C: formation of single strand DNA

- Hybridization step: oligonucleotidic primers will hybridize to matrix DNA, the temperature of this step is chosen depending on the intrinsic hybridization temperatures of the two primers used

- Elongation step, whose duration depends on the polymerase used and the size of the fragment to be amplified.

In this study, PCR was used to generate single (the expression vector carrying the wild type gene was used as DNA matrix, i.e. JMP62 pTEF *Ura Ex*) and double (the expression vector carrying single mutated gene was used as DNA matrix) mutants of the desired lipase gene. The reaction requires the use of two reverse complementary oligonucleotidic primers of approximately 20-30 bp, carrying the mutation to be introduced. These oligonucleotidic primers were synthesized by Eurogentec Company and are summarized in Table II-2. The protocol is schematized in Figure II-2.

The final volume of reaction medium was set to 50 µl and included:

- 5 to 50 ng of parental DNA
- 1U Phusion DNA polymerase (New England Biolab, 2-4 kb/min)
- 100 pmol of each oligonucleotide (list provided in Table II-2.)
- deoxyribonucleotides (dNTP): 250 µM each
- Phusion Buffer HF 1X

The conditions used were:

	Temperature	**Duration**
Denaturation step	98 °C	2 min
Amplification cycles: *25	98 °C	15 sec
	65 to 55 °C	30 sec
	72 °C	4 min
Final elongation step	72 °C	7 min
Storage	4 °C	∞

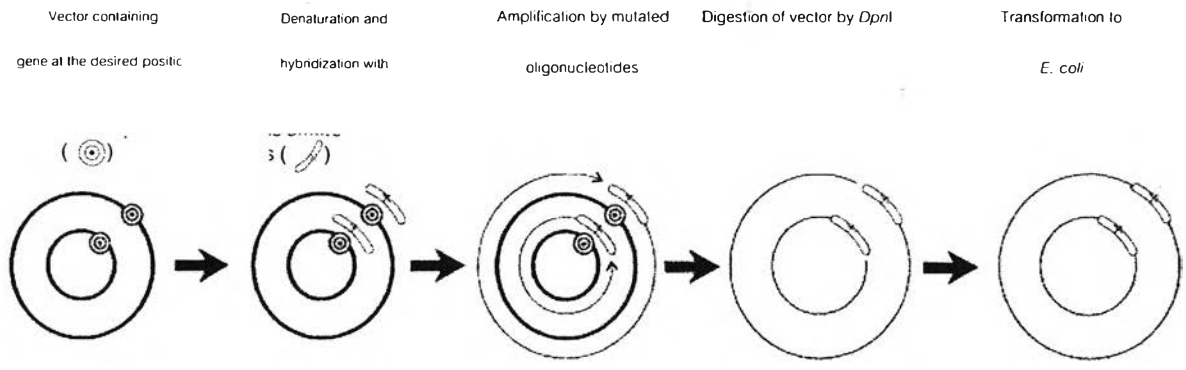

Figure II-2. Schematic method for site directed mutagenesis by PCR (adapted from Bauer *et al.*, 2007, Patent No. US7176004).

Table II-2. List of oligonucleotides used for amplifications of DNA by PCR; Sequence and temperature of hybridization are showed. The oligonucleotidic primers are named as follow: CR1, CR3 and CR4 primers were used to generate mutants of Lip1, Lip3 and Lip4 respectively; suffixes _d and _r stands for direct and reverse oligonucleotidic primers, respectively. The number corresponds to the position mutated in the mature protein sequence and the following letter is the one letter code for the amino acid with which the wild type amino acid was replaced.

Primers	**Sequence 5' – 3'**	**Temperature of hybridization (°C)**	**Use for**
Lip aval	CGGATGACTAACTCTCCAGAGCG	55.0	Sequencing
CR_296_AM	CCGACAACATTGCCGGCTTCGG	72.0	
CR3_296A_d	CCAACAACACCCCCGGCGCTCTGGCCTACTC	65.9	Directed mutagenesis
CR3_296A_r	GAGTAGGCCAGAGCGCCGGGGGTGTTGTTGG	65.9	
CR3_296G_d	CCAACAACACCCCCGGCGGTCTGGCCTACTC	65.9	
CR3_296G_r	GAGTAGGCCAGACCGCCGGGGGTGTTGTTGG	65.9	
CR3_296L_d	CCAACAACACCCCCGGCCTGCTGGCCTACTC	65.9	
CR3_296L_r	GAGTAGGCCAGCAGGCCGGGGGTGTTGTTGG	65.9	
CR4_296F_d	CCTCTGACACCCCCGGTTTCCTGGCCTACC	64.7	
CR4_296F_r	GGTAGGCCAGGAAACCGGGGGTGTCAGAGG	64.7	
CR4_296V_d	CCTCTGACACCCCCGGTGTCCTGGCCTACC	66.1	
CR4_296V_r	GGTAGGCCAGGACACCGGGGGTGTCAGAGG	66.1	
CR4_296G_d	CCTCTGACACCCCCGGTGGCCTGGCCTACC	67.4	
CR4_296G_r	GGTAGGCCAGGCCACCGGGGGTGTCAGAGG	67.4	

2.2. Analysis of nucleic acids

2.2.1. Purification of DNA from agarose gels

After migration on agarose gel, fragments resulting from either PCR or enzymatic digestion were purified using the Qiaquick kit gel extraction kit (Qiagen) following the protocol provided by Qiagen. The visualization of DNA fragments was done on a UV-transilluminator light (Safe image by Invitrogen) after direct insertion of Syber safe (Invitrogen) in the agarose gel.

2.2.2. DNA analysis

a) DNA electrophoresis

The separation of DNA fragments was carried out by electrophoresis (25 minutes migration at 135V) on agarose gel (0.8 % w/v for a separation of fragments > 1 KB) in TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.0). The loading buffer (5 % glycerol, 0.04 % bromophenol blue, 0.04 % xylene cyanol) was added to samples before their deposit. The standard size marker used was smart ladder (New England Biolabs). DNA fragments were visualized with UV (λ=254 nm) after a 15 minutes incubation in ethidium bromide (0.5 μg/mL).

b) DNA sequencing

Plasmidic DNA obtained after transformation to *E. coli* of PCR reaction (site directed mutagenesis) was sequenced by Beckman Coulter Genomics with oligonucleotides CR_296_AM and lip aval (sequences given above).

c) DNA quantification

The quality and quantity of purified DNA was calculated by measuring the absorbance at 260 and 280 nm on a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, USA). The $A_{260\ nm}$/ $A_{280\ nm}$ ratio allows the estimation of DNA quality: when this ration is inferior to 1.65 or superior to 2, DNA is considered as contaminated by proteins or RNA, respectively.

2.3. Construction of Expression vectors

a) DNA digestion

For cloning, plasmidic DNA, corresponding to subcloning vector and insert was double digested by restriction enzyme (BamHI and AvrII) according to the recommendations of the suppliers (New England Biolabs).

For transformation to *E. coli*, PCR products were digested by DpnI according to the recommendations of the suppliers (New England Biolabs). This enzyme only acts on the methylated DNA and enables to get rid of the matrix DNA.

For transformation to *Y. lipolytica*, plasmids extracted from *E. coli* were linearised by NotI according to the recommendations of the suppliers (New England Biolabs) to get rid of the bacterial part of the expression vector.

b) DNA Ligation

The ligation of DNA fragments with cohesive ends was carried out over night at 16°C in the presence of 400 units of T4 DNA ligase (Biolabs) in the buffer recommended by supplier (New England Biolabs). The insert was added in molar concentrations 3 to 5 times higher than the vector.

2.4. Construction of expression plasmid and production of *Candida rugosa* lipases

Strains and plasmids

Escherichia coli strain DH5α [(F⁻Φ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44-*thi*-1 *gyr*A96 *rel*A1 λ^-); Invitrogen] was used for plasmid amplification.

Y. lipolytica strain JMY1212, [*MAT*A *ura*3–302, *leu*2–270-LEU2-*zeta*, *xpr*2–322, *lip*2 ::*LEU*2, *Δlip2*, *Δlip7*, *Δlip8*] (Bordes et al. 2007) was used as host for recombinant expression.

Plasmids JMP62-TEF-*Ura-Ex* was used for expression of recombinant CRL. It contains the U*ra3d1* marker for selection of Ura^+ transformants in *Y. lipolytica* and the kanamycin gene resistance (KanR) for selection in *E. coli*. In JMP62-TEF-*Ura-Ex*, the lipase gene is under the control of the constitutive TEF promoter.

Construction of expression plasmids

The LIP1-LIP5 optimized *C. rugosa* lipase-encoding genes were obtained by Gene optimizer® method from GeneArt, AG, Germany, from the available sequenced sequences (SwissProt and GenBank accession number of proteic and nucleotic sequences of CRL: *LIP1*: P20261, X64703.1; *Lip2*: P32946, X64704.1; *LIP3*: P32947, X66006.1; *LIP4*: P3294, X66007.1; *LIP5*: P32949, X6608.1). They were synthesised with BamHI and AvrII surrounding restriction sites to facilitate subcloning.

The GeneArt plasmids containing synthesised LIP1-LIP5 genes were digested by AvrII and BamHI, according to the suppliers recommandations (New England Biolabs), and the gene fragments were purified from agarose gels by Qiaquick extraction kit (Qiagen) before subcloning into the BamHI/AvrII double digested expression plasmids JMP62-TEF-*Ura-Ex*, leading to JMP62-TEF-LipX (where LipX refers to Lip1 to Lip5 of CRL). The plasmids constructed were finally digested by NotI to liberate the expression cassette, and directly used for transformation to *Y. lipolytica* stain JMY1212 by the lithium acetate method as previously described (Le Dall et al. 1994). Selection of Ura+ transformants was performed on YNBcasaD plates (YNB with 2 g of casamino acids/L and 1 g/L glucose).

Construction of expression vectors

The derivative plasmids JMP62Tef-CR3F296A, JMP62Tef-CR3F296G, JMP62Tef-CR3F296L, JMP62Tef-CR4A296F, JMP62Tef-CR4F296V, JMP62Tef-CR4F296G, carrying single amino acid changes in the active site gene were constructed by site-directed mutagenesis using the QuikChange™ site-directed mutagenesis kit (Stratagene). The technique used the JMP62-Tef-*Ura-Ex* double-stranded DNA vector and two synthetic oligonucleotide primers containing the desired mutation (Eurogentec); each primer is complementary to opposite strands of the vector. The following forward primers and their complementary reverse counterparts were used to construct the variants.

CR3F296A for: 5'- CC AAC AAC ACC CCC GGC GCT CTG GCC TAC TC-3';
CR3F296G for; 5'- CC AAC AAC ACC CCC GGC GGT CTG GCC TAC TC-3'

CR3F296L for; 5'- CC AAC AAC ACC CCC GGC CTG CTG GCC TAC TC-3'
CR4A296F for; 5'- CC TCT GAC ACC CCC GGT TTC CTG GCC TAC C-3'
CR4A296V for; 5'- CC TCT GAC ACC CCC GGT GTC CTG GCC TAC C-3'
CR4A296G for; 5'- CC TCT GAC ACC CCC GGT GGC CTG GCC TAC C-3'

Expression of variants CRL in *Y. Lipolytica*

Erlenmeyer flasks (500 mL) containing $Y_1T_2D_5$ (50 mL total) made of yeast extract (10 gL^{-1}), bactotryptone (20gL^{-1}), and glucose (50 gL^{-1}), buffered with phosphate buffer (100 mM, pH 6.8) were inoculated with cells pregrown in YPD containing of yeast extract (10 gL^{-1}), bactopeptone (10 gL^{-1}), and glucose (10 gL^{-1}) at an initial cell density of OD_{600}=0.2. Stock solutions containing glucose (500 gL^{-1}). Cells were incubated at 28 °C for 24 h until complete glucose consumption. The cells were removed through centrifugation (10 000 rpm for 10 min). Supernatants were directly used in reactions.

3. Enzyme production and characterisation

3.1. Production of the enzyme in Erlenmeyer flasks

The cultures were inoculated with 1/10 volume of overnight pre-culture in YPD medium. They were carried out in erlenmeyer flasks with baffles filled to maximum 1/5 of their total volume with production medium (YTO or YTD). They were placed at 28°C under agitation until total consumption of the carbon source (see below). The supernatant containing the produced enzyme was recovered by centrifugation (5 minutes 5000 rpm). For long time conservation, the supernatant was filtered on 0.2 µm and store at 4°C. Proteins were concentrated on Amicon membranes (Millipore) with cut off 10 kDa.

3.2. Yeast growth monitoring: consumption of the carbon source

3.2.1. Residual glucose concentration

When the culture was carried out in YTD, 3,5-dinitrosalycilic acid (DNS) was used to assay residual glucose (Sumner and Howell, 1935). One volume of sample

was mixed with en equal volume of reagent (see composition below). After 5 minutes at 95°C, 10 volumes of water were added and the absorbance was read at 540 nm. A standard curve for glucose (0 to 2 g/L) was realised.

The reagent containing DNS:

- 3, 5-dinitrosalycilic acid: 10 g/L
- Sodium Potassium tartrate: 300 g/L
- NaOH: 16 g/L

3.2.2. Oleic acid consumption

When the culture was realised in YTO medium, residual oleic acid in the supernatant was checked by centrifugation (3 minutes at 13000 rpm). The culture was stopped and the enzyme recovered once oleic acid was completely consumed (clear supernatant).

3.2.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Produced proteins were analysed by SDS-PAGE. Proteins were denatured (10 minutes at 70°C in the presence of reducing agent) and loaded on 10% Bis-Tris NuPAGE®. The migration was done in MOPS buffer (50 minutes at 200 V). After migration of proteins, gel was submitted to colloidal blue staining according to furnisher (Invitrogen).

4. Procedure for enzymatic reactions

4.1. Lipases production

Erlenmeyer flasks (500 mL) containing 50 mL medium $Y_1T_2O_3$/ $Y_1T_2D_5$ made of yeast extract (10 g/L), bactotryptone (20 g/L), and either oleic acid (30 g/L) or glucose (50 g/L), buffered with phosphate buffer (100 mM, pH 6.8) were inoculated with overnight preculture grown in YPD (yeast extract 10 g/L, bactopeptone 10 g/L, and glucose 10 g/L) at an initial cell density of OD_{600} = 0.5. Cells were incubated at 28 °C until complete oleic acid/glucose consumption. Cells were removed by centrifugation (10 000 rpm for 10 min) and supernatants were directly used in reactions.

4.2. Enzyme characterization

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Produced proteins were analysed by SDS-PAGE. Proteins were denatured (10 minutes at 95°C in the presence of reducing agent) and loaded on 10% Bis-Tris NuPAGE®gels. The migration was done in MOPS buffer (50 minutes at 200 V). After migration of proteins, gel was submitted to colloidal blue staining according to furnisher (Invitrogen).

4.3. Lipase activity assay

Lipase activity in the culture supernatant was determined by monitoring the hydrolysis of *p*-nitrophenyl butyrate (*p*-NPB) into butyric acid and *p*-nitrophenol. The method was optimized using 2-methyl-butan-2-ol (2M2B) as solvent to solubilise *p*-nitrophenyl butyrate. Lipase activity was measured in 96-well microplates filled with 20 µL of the supernatant containing Lipase, 175 µL of a 100 mM phosphate buffer pH 7.2 containing 100 mM NaCl. The reaction was started with addition of 5 µL *p*-NPB (40 mM in 2M2B) and activity was measured by following absorbance at 405 nm at 25 °C for 10 min using the VersaMax tunable microplate reader apparatus (Molecular Devices, Rennes, France). One unit of lipase activity was defined as the amount of enzyme releasing 1 µmol of fatty acid per min at 25 °C and pH 7.2.

4.4 Procedure for enzymatic reactions

Hydrolysis of methyl ester of different carbon chain lengths

Hydrolysis reactions were carried out at 25 °C. in 1.5 mL eppendorf tubes containing a biphasic medium composed of 1 mL decane containing the methyl esters of different chain lengths (15 mM of methyl hexanoate (C6), octanoate (C8), decanoate (C10), laurate (C12), myristate (C14), palmitate (C16), stearate (C18) and oleate (C18:1)) and 0.5 mL of aqueous enzymatic solution. The mixture was shaken in a Vortex Genie 2 (D. Dutscher, Brumat, France). At regular time intervals, the progress of the reaction was followed by taking samples from the organic phase after phase separation by centrifugation. Samples were directly analysed by GC.

Analyses were performed with the GC device (6890N, Algilent technologie) equipped with a capillary HP-5 column (30 m length x 0.32 mm internal diameter and 0.25 µm thickness, Variant Inc., USA) connected to a FID detector. The following conditions were used: carrier gas He (25 ml/min), air and hydrogen flow of 300 mL/min and 30 mL/min, respectively, split ratio of 1/100, injector and detector temperature of 250 °C and 270 °C, respectively, temperature programme: 70 °C to 75 °C at 1.79 °C/min, 75 °C to 140 °C at 20 °C/min, 140 °C to 143 °C at 2.5 °C/min and 143 °C to 240 °C at 10 °C/min.

4.5. Purification of DHA from fish oil

The reaction was carried out at room temperature in 1.5ml eppendorf tubes containing 0.5 ml of 100 mM Ethyl Ester of Fish Oil (EEFO) containing 25% DHA and 5% EPA and 0.5 ml of aqueous enzymatic solution. The mixture was shaken in a Vortex Genie 2 (D. Dutscher, Brumat, France). At regular time intervals, the progress of the reaction was followed by taking samples from the organic phase after phase separation by centrifugation. 50 µL of organic phase were taken and dissolved in 450 µL hexane and the free fatty acids (FFA) were removed by saponification with 500 µL NaOH 1N. The resulting organic phase was analyzed by GC. GC analysing equipment is described above. The temperature program used for the ethyl esters analysis is the following: 180°C for 15 minutes, 180°C to 250°C at 7 °C/ min, and hold for 10 minutes, 250°C to 280°C at 10 °C/min, and hold for 10 minutes.

4.6. Hydrolysis resolution of 2-Bromo-phenyl acetic acid octyl ester

Hydrolysis was carried out in 1.5 mL eppendorf tubes containing a biphasic medium composed of 0.5 mL decane containing the ester (50 mM) and 0.5 mL of the aqueous enzymatic solution. The mixture was shaken in a Vortex Genie 2 (D. Dutscher, Brumat, France). Reactions were realized at 25 °C. At regular time intervals, the progress of the reaction was followed by taking samples after phase separation by centrifugation (100 µL diluted in 1 mL hexane).

4.7. HPLC analysis

The HPLC device was equipped with a chiral column: Chiralpack OJ (25 cm x 4.6 mm) (Daicel Chemical Industries Ltd., Japan) connected to a UV detector (at 254 nm). A flow rate of 1.0 mL/min and a 40 °C column temperature were used. The mobile phase was composed of a mixture of n-hexane/isopropanol (80:20 v/v).

4.8. Determination of the enantiomeric excess (*ee*), conversion and enantioselectivity (E)

From HPLC results, enantiomeric excesses of the substrate (ee_s) and the product (ee_p), the total conversion (C) of both enantiomers and *E*-values (*E*) were calculated as defined below:

ee_s (%) = $(S\text{-}R)_t/(R+S)_t * 100$

ee_p (%) = $(S\text{-}R)_t/[(R+S)_{t0}\text{-}(R+S)_t] * 100$

$C=1\text{-}[(R+S)_t/(R+S)_{t0}] * 100$

with *R* and *S* corresponding to the concentrations of *R* and *S* enantiomeric substrates

E=(v*i*S/v*i*R) for a *S* enantioselectivity

E=(v*i*R/v*i*S) for a *R* enantioselectivity

With v*i*S and v*i*R corresponding to the initial rates of *S* and *R* enantiomer consumption respectively. The initial rates were determined by linear regression.

5. Model building

A three dimensional model of Lip4 Lipase from *C. rugosa* was constructed using the program MODELLER implemented in the HOMOLOGY module of the InsightII suite of programs (Accelrys, San Diego, CA, USA) and the sequence alignment with LIP1 Lipase from *C. rugosa*.

The lowest energy structure predicted using MODELLER was further refined using the CFF91 forcefield implemented within the DISCOVER module of InsightII software suite (Accelrys, San Diego, CA, USA). For the minimization, the CFF91cross terms, a harmonic bond potential, and a dielectric of 1.0 were used. An initial minimization with a restraint on the protein backbone was performed using a steepest descent algorithm followed by conjugated gradient minimization steps until

the maximum RMS was less than 0.5. In a subsequent step, the system was fully relaxed. Calculations were performed on a Silicon Graphics O2 workstation.

Tetrahedral intermediate models were built for both (*R*, *S*)-2-bromo-phenylacetic acid octyl ester enantiomers. The substrates were placed in the active site of Lip1 and Lip4 from *C. rugosa* so that they were covalently bound to catalytic Ser206. The covalent intermediates were then minimized using the CFF91 forcefield implemented within the Discover module of InsightII in order to generate a low-energy starting conformation with suitable bond distances and angles. The covalent docking of the enantiomers was carried out using the automated flexible docking program FlexX (Biosolveit) (Kramer et al. 1999; Rarey et al. 1996). All parameters were set to the standard values as implemented in Version 3.1.1. The docking region was defined to encompass all protein amino acids for which at least one heavy atom was located within a 6.5 Å radius sphere, whose origin was located at the centre of mass of catalytic Ser 162. For each docking, the top 30 solutions corresponding to the best FlexX scores were retained. Visualization and graphics were done using PyMol software, (DeLano, 2002).

6. Immobilization and Biodiesel production from C. rugosa lipase

6.1. Support for immobilization

Macroporous adsorption resin NKA-9, AB-8, H103, D4020 and NKA were purchased from Chemical plant of Nankai University (Tianjin, China). Palm oil was purchased from the local market.

6.2. Lipases production

Erlenmeyer flasks (500 mL) containing lipase production medium (50 mL total) made of yeast extract (5 g/L), Potassium hydrogen phosphate (KH_2PO_4; 10 g/L), Magnesium sulphate ($MgSO_4 \cdot 7H_2O$; 1g/L) and palm oil (1% w/v), were inoculated with overnight preculture grown in YM (yeast extract 3 g/L, malt extract 5 g/L, peptone 5 g/L, and glucose 10 g/L) at an initial cell density of OD_{600} = 0.2. Cells were incubated at 30 °C for 120 h Cells were removed through centrifugation (10 000 rpm

for 10 min) and supernatants were partially purify by filtration and dialysis at molecular weight cut off 10 kDa. Later, lipase was lyophilized and keeps at 4 °C until used in reaction.

6.3. Lipolytic activity assay

Lypolytic activity was measured by spectrophotometry method using *p*-nitrophenyl palmitate (*p*NPP) as substrate and the absorbance was measured spectrophotometrically at 410 nm. One unit (U) was defined as the amount of enzyme that liberated 1 µmol of *p*-nitrophenol per min. (Maia et al., 2001)

6.4. Immobilization of lipase

Lipase immobilization in non-aqueous media (Gao et al., 2006); 0.6 g of lyophilized lipase, 1 g of resin and 5 ml of heptane were mixed under low stirring for 12 h at temperature ambient. The particles were filtered and washed with heptane under reduced pressure, and then dried at room temperature.

6.5. Biodiesel production: Enzymatic reactions

Hydrolysis of palm oil

The enzymatic hydrolysis reactions were carried out in vial under magnetic stirring for 48 hr, using 3 g of palm oil, 50% distilled water (v/oil w) and 1 g of immobilized lipase. Sampling were analysed by HPLC.

Transesterification of palm oil and methanol

Transesterification reaction were carried out in vial containing 3 g of palm oil and 1 g of 1 g of immobilized lipases under magnetic stirring for 48 h. at 40 °C and 600 rpm stirring. Samples were centrifuged 13 000 rpm for 15 min and upper phase were analyzed by HPLC.

Determination of fatty acids content

The fatty acid contents in the reaction mixture were quantified using high performance liquid chromatography (HPLC) and thin layer chromatography (TLC) (Samukawa et al., 2000)

The HPLC device was equipped with an Apollo Silica column (25 cm x 4.6 mm) (Alltech, USA) connected to an ELSD detector with Nitrogen gas equipped. A flow rate of 1.5 mL/min and a 40 °C column temperature were used. The mobile phase was composed of a mixture of Solution A, n-hexane:isopropanol: Ethyl acetate: formic acid (85:10:10:0.1 v/v) and Solution B, h-hexane:formic acid (100: 0.2 v/v).

Protein assay

Protein concentration was determined according to the method of micro lowry. Bovine serum albumin was used as a standard. (Held and Hurly, 2001)