CHAPTER V
CONCLUSION

In the past two decades, the major novelty in the field of enzymology was the development of concepts and tools to enable the improvement of enzyme performances, with the objective to adapt them to extreme industrial conditions. Thanks to these genetic techniques, it is now possible to improve the activity of an enzyme, its selectivity, optimal temperature or thermostability, and even its resistance to extreme pH or tolerance to organic solvent. Two strategies can be adopted to improve an enzyme. The first approach is a rational one, through the study of the relation between the structure and the function of the biocatalyst, leading to the fine comprehension of mechanisms involved at the molecular level. This strategy starts with the choice of amino-acids, for instance in the active site of the enzyme, to modify by site-directed or saturation mutagenesis. The pre-requisite of such a strategy is to know the three-dimensional structure of the enzyme of interest. On the contrary, the second strategy, an evolutionary approach, does not imply any knowledge of structural or functional data, because variants are randomly generated by genetic engineering. Therefore, the primordial necessity for this second approach is the development of an adapted high throughput screening method to be able to efficiently and quickly assay tens, or even hundreds of thousands of variants.
My doctorate work deals with such concerns.

1. Evaluation of a new expression system for enzyme evolution

An essential need for both strategies is an efficient and reproducible expression host to be able to rapidly compare enzyme variants. Therefore, we first tested a new expression system, a specific strain of *Y. lipolytica*, for the expression of variants obtained by site-directed mutagenesis. This *Y. lipolytica* strain, namely JMY1212, enables the integration of the expression cassette to be targeted to a given locus of the *Y. lipolytica* genome.

We demonstrated that it was possible to statistically compare the activities of different enzymatic variants directly from the supernatant of the culture. A statistical

difference in activity between two enzymes can then be attributed to a difference in catalysis potentialities at the molecular level and not to a difference in protein expression levels. This strategy was applied in the following experiments.

2. Expression of *Candida rugosa* lipases in strain JMY1212 of *Y. lipolytica*.

C. Rugosa expresses 5 main lipases. Despite their high homology (superior to 70%), they display different selectivities. Although they were greatly studied in the past, the difficulty to study them separately and to express them as heterologous proteins, because this yeast has an original non-universal codon usage, impeded their development in the last years. These difficulties were circumvented here by the use of synthetic genes. Three lipases of *C. rugosa,* Lip1, Lip3 and Lip4, were cloned and successfully produced in *Y. lipolytica* strain JMY1212. An individual characterization, from the point of view of thermostability and specificity, as well as evolution of the performances of these isoenzymes for industrial applications was thus rendered possible.

Lip2 and Lip5 could not be produced, neither in the extracellular medium, nor inside the cells. An extensive analysis of the synthetic genes, together with the study of transcriptional products, should enable to understand, and hopefully solve this expression problem.

3. Purification of DHA using *Candida rugosa* lipases

Lip1 and Lip3 present specificity for fatty-acids of medium chain length (C8-C12), whereas Lip4 prefers C18: 1. This difference is specificity was exploited for the purification of polyunsaturated fatty acids (PUFAs). *cis*-5, 8, 11, 14, 17-eicosapentaenoic acid (EPA) and *cis*-4, 7, 10, 13, 16, 19-docosahexaenoic acid (DHA) are health beneficial PUFAs with a great potential in prevention and/or treatment of cardiovascular diseases and as non steroidal anti-inflammatory drugs. Although they are major components of fish oils, it is still difficult to separate them from such a complex lipidic mixture.

For the first time, purification of DHA and EPA from a mixture of ethyl esters issued from tuna oil was realised with the three *C. rugosa* lipases used separately. Whatever the enzyme, the recovery of DHA was superior to 90% (97, 100 and 93%

for Lip1, Lip3 and Lip4 respectively). The maximal DHA purity ~60% was obtained with Lip3 and Lip4, with an initial ethyl ester mixture containing 25% DHA. A remarkable difference between these enzymes lies in the fact that Lip4 is able to better hydrolyse EPA esters (60% against 15% for Lip1 and Lip3). This work opens very interesting perspectives of enzyme evolution with the goal to improve CR lipases to obtain DHA of very high purity. Molecular modelling studies will be realised in order to better understand the difference in selectivity between the 3 enzymes. Especially, it appears as crucial to understand why Lip4 is able to catalyse the hydrolysis of EPA. One can imagine modulate the length of the tunnel composing the active site, in order to favour EPA hydrolysis.

4. Resolution of a racemic mixture using *C. rugosa* lipases.

The reaction of interest was the resolution of a racemic mixture of pharmaceutical interest, pertaining to the family of 2-halogeno aryl acetic acids. These compounds are intermediates in the synthetic pathways of many drugs, such as prostaglandin, prostacyclin, semi-synthetic penicillin or thiazolium salts, and analgesics.

This study focussed on the racemic mixture of 2-bromo -phenyl acetic acid octyl esters. Lipases Lip1, Lip3 and Lip4 from *C. rugosa* were assessed for their aptitude to resolve such mixture.

Lip1 and Lip3 lipases from *C. rugosa* were proven to be remarkable from the point of view of their enantioselectivity. Indeed, both of them are totally selective with a preference for the *S*-enantiomer (E-value >200). In spite of its high homology, Lip4 presents *R* specificity (E-value=15). The analysis of amino acids located in the active site of the 3 enzymes suggested that the amino acid at position 296 could be crucial for enantiomer recognition. In lip1 and Lip3, this amino acid is a phenylalanine, whereas it is an alanine in Lip4. Site-directed mutagenesis at this position was thus realised, and we could demonstrate that the nature of the amino acid in position 296 is a key factor for the enantiomers discrimination by these enzymes. The presence of a small amino acid, such as a glycine or an alanine, leads to *R*-selectivity, whereas a leucine or a phenylalanine transforms the enzyme in an *S*-selective enzyme. Molecular docking of the *S* and *R* enantiomers in the active site of

Lip1 and Lip4 enables the observed differences in specificity to be better understood. However it will be necessary to make important efforts in molecular modelling to fully understand the discrimination phenomenon.

5. Biodiesel production catalyzed by *C. rugosa* lipase

Lipase is and interesting enzyme that can hydrolysis triglyceride to free fatty acids and synthesis of alkyl ester for application in biodiesel production. Results from screening of yeast producing lipase showed that *Candida rugosa* has potential for hydrolysis of high conversion for free fatty acids from palm oil in the present water. For biodiesel production, 75 % conversion to methyl ester from palm oil and methanol catalyzed by immobilized lipase.

The macroporous adsorption resins are well desirable to immobilize the *Candida rugosa* lipase. The remarkable immobilization effect was found when the coupling procedure was performed in the presence of heptane. Additionally, lipase adsorbed onto non-polar resin H103 could effectively catalyze the free fatty acid production via hydrolysis of palm oil. NKA-9 exhibits as support for lipase immobilization for transesterification of methyl ester. Optimization conditions for biodiesel production were investigated as 1 to 4, molar ratio of oil and methanol substrate. At 30 ^{o}C was suitable for transesterification of immobilized lipase.

The results obtained for the different lipases used in this thesis project demonstrate the high potential of rationally engineered mutations to further enhance enzyme activity, to modulate selectivity and broaden applications for industrial.