DEPARTMENT OF AGRICULTURAL, FOOD AND ENVIRONMENTAL SCIENCES

DEGREE COURSE: Food And Beverage Innovation And Management

Structural investigations of *Candida antarctica* Lipase B in ethanol for catalysis in the food industry

TYPE OF DISSERTATION: research

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I dedicate this work to my dear parents, source of life, love, and affection, who have always guided and motivated me.
To my brothers, source of joy and happiness.
To all those I love and those who love me.

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Abstract

Candida antarctica lipase B (CALB) is one of the most widely studied enzymes, due to its high selectivity and catalytic activity in organic and polymer synthesis.

Given the great interest in knowing the behaviour of CALB in presence of specific organic solvents, the molecular mechanism of the deactivation of the enzyme by primary alcohols was already investigated by several researchers. In this contest, in order to understand the defective activity of the lipase, we addressed the inhibition pathways of CALB by ethanol.

The structural modifications of CALB in an ethanol-water mixture were evaluated in the present work considering that the catalytic activity decreases in such a mixture.

The present work reports two crystal structures of the lipase in presence of ethanol refined with specific crystallographic software tools, namely WinCoot and Phenix. WinCoot is the program used for any kind of correction at the atomic level to the crystal structure because capable to provide specific tools for intervening in the enzyme structure. Then, the obtained model was refined and validated the modifications with Phenix.

Having a molecular description of the interaction between solvent and enzyme is a prerequisite to a qualitative understanding of enzymatic activity in organic solvents.

Keywords: *Candida antarctica*, lipase B, esterification, ethanol inhibition

Work Aim

Study of the modifications in enzymatic structure by refining the crystallographic model of CALB in an ethanol-water mixture for understanding the catalytic activity.

I. Introduction

1. Lipases

Lipase enzymes are proteic-molecule soluble in water. Their normal function is to catalyze the hydrolysis of triglycerides into diglycerides, monoglycerides, fatty acids, and glycerol, without requiring any additional cofactor. Lipases are known as monomeric proteins that have a molecular weight in the range of 19–60 kDa.

Lipases differ from other enzymes by their high activity in the water-lipid interface which is significantly reduced if the enzyme is working only in water or oil (Tatsuo Maruyama, 2000), (Verger, 1998).

This interfacial activity is related to the lipases' lid (an α-helix above the active site that has an important role in the activity and the conformational stability of the enzyme) (F. Secundo, 2006).

Lipases were first discovered by Claude Bernard in 1856 in pancreatic juice as enzymes that solubilize hydrophobic oil droplets by hydrolysing them. The affinity of lipases is different depending on the type of lipase. In fact, there are those whose affinity is higher for short-chain fatty acids, those who prefer longer unsaturated fatty acids, and those that randomly cleave fatty acids from triacylglycerols. It is the triacylglycerol hydrolase that catalyzes the hydrolysis of triglycerides to free fatty acids, diacylglycerol, monoacylglycerol, and glycerol.
Therefore, we can say that the activity of lipases depends on the length, the cellular location, and the degree of unsaturation of the fatty acid (Nimkande, 2022).

Besides hydrolytic reaction, lipases can exhibit a variety of catalytic activities, such as interesterification, esterification, aminolysis, acidolysis, and alcoholysis, which contribute to its application in a wide range of industries (figure 1), (P. Chandra, 2020), (A. Houde, 2004).

Hydrolysis, esterification, and transesterification reactions are all reactions that happen in organic media (H. Deleuze, 1987).

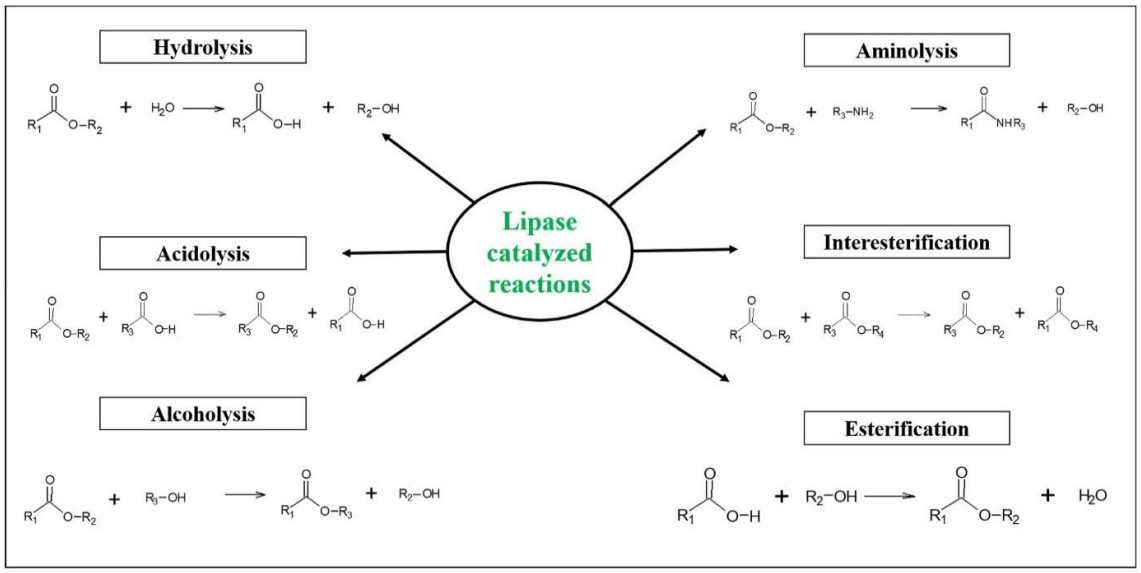

Figure 1: Catalytic activities of lipases

Microbial lipases are essential in the food industry since they are exploited in the formation of various products.

Lipases are widely used in industrial processes, such as biodiesel production (F.T.T. Cavalcante, 2021), production of enzyme-modified cheese (EMC) (P. Kendirci, 2020), enzyme-modified dairy ingredients (B. Ali, 2019), fatty acids (D.Sande, 2018) , etc (figure 2).

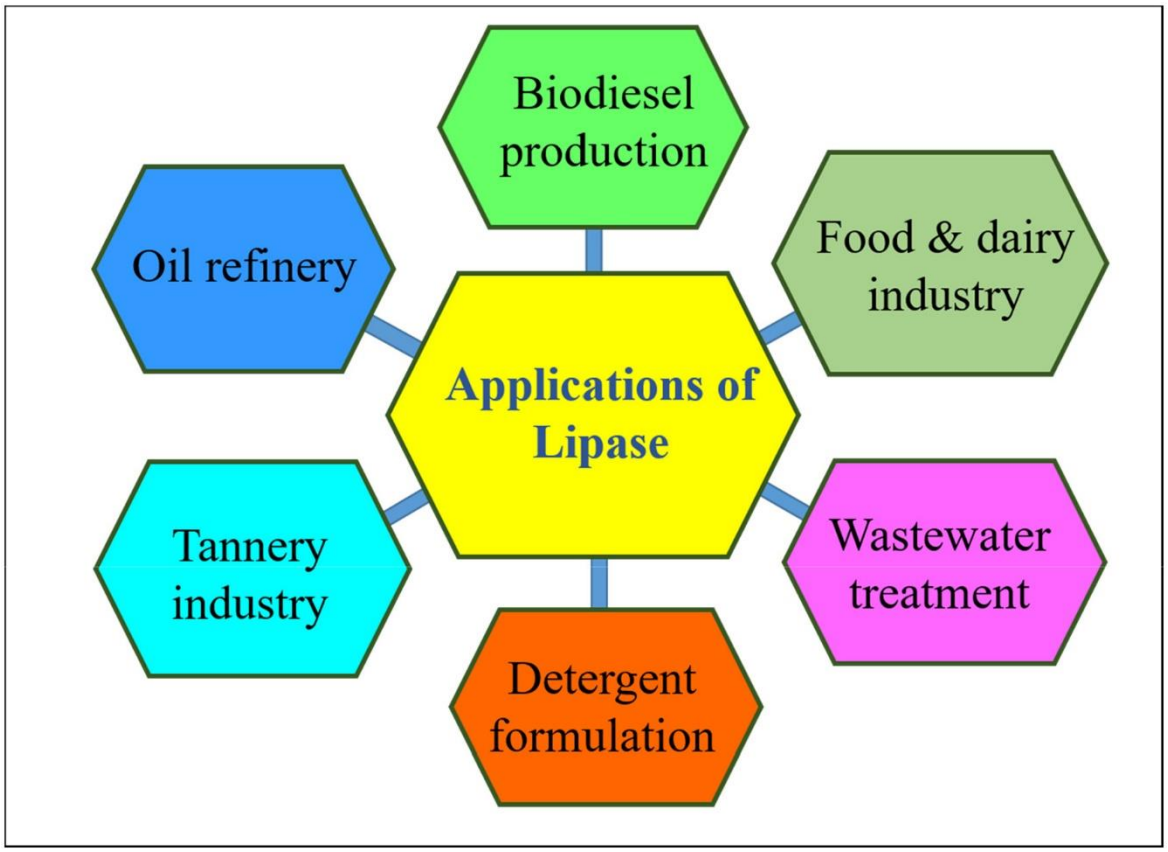

Figure 2: Industrial applications of lipases

Lipases are ubiquitous. They come from multiple and different natural sources. 45% of them come from bacteria, 21% from fungi, 18% from animals, 11% from plants, 3% from algae, and the rest from other sources (B. Bharathiraja, 2014). We can classify lipases based on their sources, from which the microbial ones are widely used due to their relatively high stability, selectivity, substrate specificity, and their easy production. The activity of the lipases depends on the microorganism from which they are screened. Therefore, a big range of microorganisms with different sources is being discovered to produce more enzymes with different properties (A. Houde, 2004), (B.S. Heater, 2018).

These enzymes can also be classified based on their substrate range (figure 3).

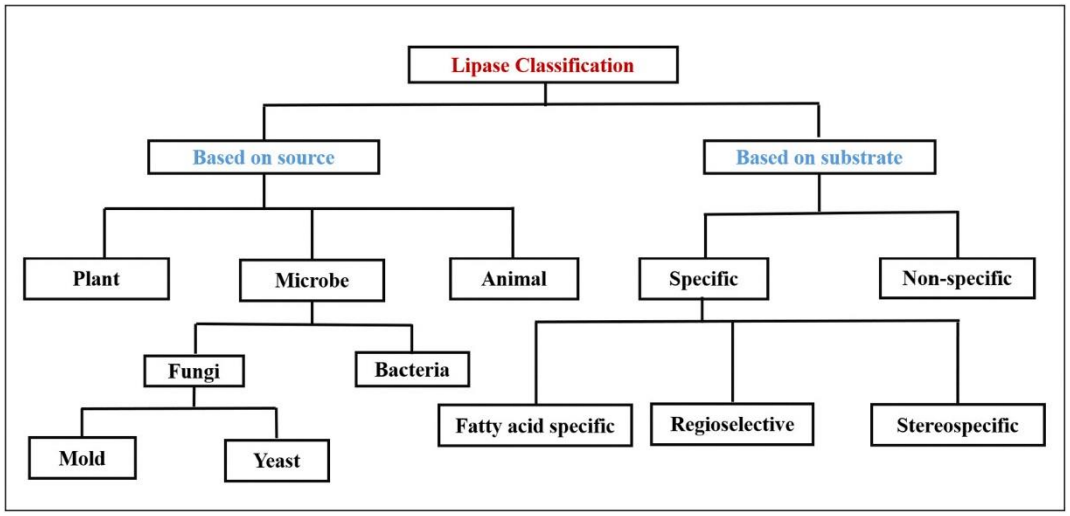

Figure 3: Lipases classification based on their substrates

2. *Candida antactica* lipase B (CALB)

The lipases A and B are enzymes produced from the basidiocete yeast *Candida antarctica*. Since the production of those isoenzymes from their original source is inconvenient, they have been recombinantly produced from *Aspergillus oryzae*. Despite its interesting characteristics, CALA is not yet well explored. CALA is a highly thermostable enzyme that exhibits an optimal activity at 70°C and its optimal pH is neutral even though it is more stable at acidic pH. By contrast, CALB is less stable, has a lower optimum temperature, optimal catalysis at neutral pH and has a lid that does not totally insulate the active site. Both CALA and CALB have an active site formed by a catalytic triad of serine hydrolases (K. Da S. Moreira, 2022).

Uppenberg et al. (1994) were the first to determine the 3-dimentional structure and sequence of CALB (J. Uppenberg, 1994). A year later (1995), CALB was first expressed in *Aspergillus oryzae* (D. Yu, 2021).

CALB (CalB, GenBank accession number LC215918) is considered an enzyme that is highly versatile due to the wide range of its possible applications (C. Ortiz, 2019).

Novozyme 435 (Melone Pharmaceutical Co., Ltd., Dalian, Liaoning China) is the immobilized CALB on microporous acrylic resin by physical adsorption. Due to the weakness of the immobilization method and the price, Novozyme 435 has seen limited applications in the industrial production (D. Yu, 2021).

An appropriate immobilization can improve the activity, stability, specificity, purity, and inhibition of an enzyme. In fact, the immobilization can change the molecular conformation of the enzyme, promote the combination of the enzyme to the substrate and increase or decrease the activity. Carrier may exhibit a protective effect on the proteins of an enzyme and reduce their denaturation which makes the enzyme able to show better activity in harsh environments such as high temperatures and extreme pH. Immobilization of CALB onto a carrier contributes to the reduction of processing cost by enhancing the stability of the enzyme, increasing the number of uses, and making the separation from the reaction medium easier.

Immobilization technology has been gradually improving. Now, even if the catalytic activity of the immobilized enzymes may decrease, the reuse and recycling of the enzymes are more important. The common methods of immobilization are physical adsorption, covalent bonding, cross-linking, and entrapment. Glutaraldehyde is often used as a reagent for enzyme immobilization because it is cost-effective and easy to obtain in large quantities (D. Yu, 2021).

3. Structure of CALB

CALB is an α/β hydrolase which active site consists of a conserved catalytic triad made of Ser, His and Asp/Glu (F. Secundo, 2006).

According to multiple molecular studies, whether CALB has a lid or not was a matter of debate. Since a closed conformation of CALB has only been recently reported (B. Stauch, 2015) and since this enzyme has never shown any significant interfacial activation leading to the conclusion that CALB has no lid that regulates the access to the active site (M. Martinelle, 1995).

α5 and α10 are two α-helices that surround the active site of CALB and are very flexible regions that could work as lipase lid by a relative motion between them. These helix motions are not large enough neither to produce variation in the hydrophobic surface and in surface-accessible area nor to prevent access to the active site. However, CALB sequence can stretch around the α-helix 5 and this significantly influences the catalytic properties of the enzyme. Therefore, the issue of the open and closed conformation of CALB is not just related to the interfacial activation, but it also influences the catalytic properties of the enzyme (B. Stauch, 2015).

4. Effect of the organic solvent on the catalysis

It is of great advantage to using organic solvents as reaction media since they allow a better solubility of the hydrophobic substrates, they increase the thermostability of the enzyme, and favor the synthesis over the hydrolysis. Though the enzymatic activity in an organic solvent is lower than in water solutions.

It is suggested that this effect is due to one of these phenomena:

1. Change in the enzyme conformation and/or flexibility

2. Change in the solubility and dissolvation of the substrates and the products
3. Competitive inhibition between the solvent molecules and the substrate (M. Graber, 2007).

In the last few years kinetic studies using lipases as catalysts in organic solvents have become important. Chulalaksananukul et al. (1990) proposed a kinetic model based on the Ping Pong Bi Bi mechanism for the reaction of esterification of oleic acid with ethanol using an immobilized lipase (W. Chulalaksananukul, 1990). These reactions (Bi Bi reaction) involve two substrates and two products and are reaction in which the enzyme, first, reacts with the first substrate (acid in this case) giving an acyl-enzyme intermediate and then releases the first product (water), then reacts with the alcohol, being the second substrate, and releases the second product which is in this case the ester. Many other studies experimentally confirmed that esterification, alcoholysis and ester exchange reactions catalysed by lipases in many different organic solvents fitted this model. In most cases, competitive inhibition by alcohol is reported in reaction catalysed by lipases in organic solvent (M.P. Bousquet-Dubouch, 2001). Competitive inhibition can be defined as a competition between the substrate and the inhibitor (that can be the organic solvent in this case) for the binding to one unique active site (figure 4).

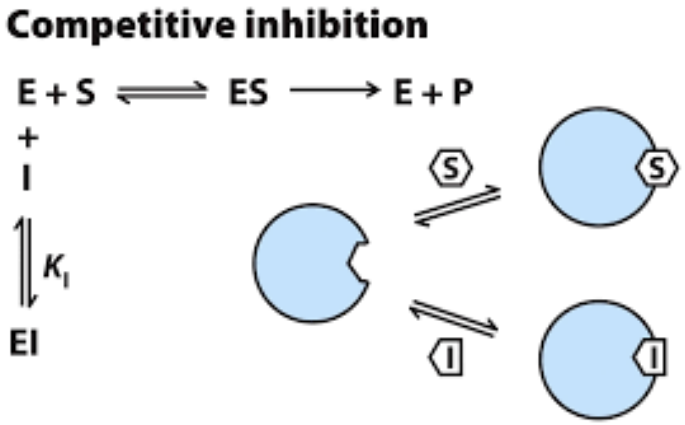

Figure 4: Competitive inhibition

Organic solvents used as reaction media in enzyme-catalyzed reactions are different and can affect the enzymatic activity differently. Polarity is one of the characteristics of the organic solvent that can affect the enzymatic activity and therefore, the reaction rate. In fact, enzymatic activity is higher in an environment surrounded by non-polar and mid-polar solvents that an environment surrounded by polar solvent (Z. Khan, 2021).

Polar solvents interact intensively with the active site of CALB leading to the breakdown of the hydrogen bond between Ser 105 and His 224. This hydrogen bond together with the one between Asp 187 and His 224 are very important for the stabilization of the transition state of the enzymatic reaction. Therefore, the corruption of such bonds leads to a lower activity of CALB (C. Li, 2010). Moreover, the activation energies of CALB and its active site are lower in the non-polar solvents that in polar solvent.

The effect of the solvent on the active site is the main cause of the differences in the activity of the active site. Therefore, the position of the solvent molecule in enzyme structure is important to determine its effect on the enzymatic activity (C. Li, 2010).

5. Odorant molecules for the food industry

Flavour esters are significant and versatile compounds widely used in food, beverage, pharmaceutical, cosmetic, and other industries due to their flavour and fragrance properties.

Aromatic esters are flavour esters that have an aromatic ring in their molecular structure. These esters can be obtained in two different ways. The first way is via extraction from plants or fruits which is disadvantageous due to its dependency upon the availability of the source, besides the low yield and high production costs needed for the extraction and purification. Therefore, another way to obtain these esters is employed, which is the chemical production (A.G.A. SÁ, 2017).

The reaction that produces esters is the esterification reaction that involves the heating of a mixture of carboxylic acid (acyl donor) and an excess amount of a corresponding alcohol (acyl acceptor) in the presence of a catalyst as it is shown in the figure 5. This reaction achieves the equilibrium after a certain period of time. To make the reaction proceed in the forward direction, one of the reactants must be added (usually it is the alcohol that is added) or water must be removed continuously from the reaction medium (Z. Khan, 2021).

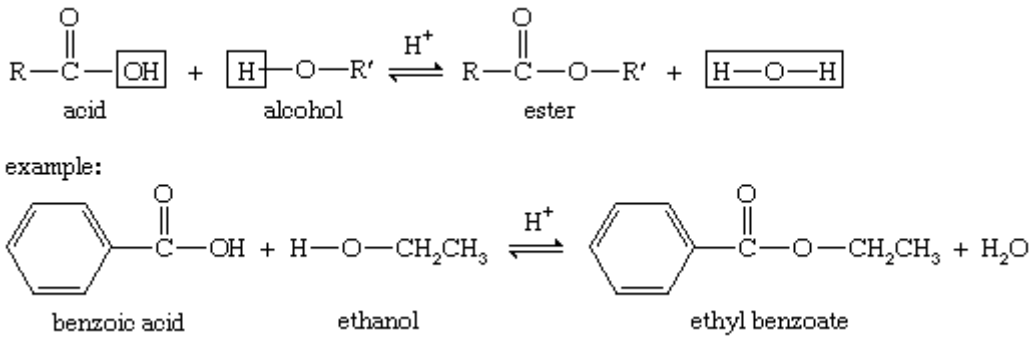

Figure 5: Esterification reaction

The type of ester depends on reactant used to produce it. Different combination of carboxylic acids and alcohols give different esters with different fragrances as shown in figure 6.

Figure 6: Table of esters and their smells

The synthesis of these aromatic esters uses either liquid acids as catalysts which require post-treatments or enzymes as an alternative way to catalyse this reaction. In this contest, lipases are a good option of enzymes. Lipases from *Candida* sp. Are well-established enzymes for biocatalysis purposes especially *Candida antarctica* lipase B that has been studied for a potential application in flavour making due to its wide range of specificity for substrates, resistance to organic solvents, high thermal and pH stability, and stereospecificity. The use of the immobilized CALB is recommended to overcome some enzyme limitations such as: inhibition and poor stability.

Furthermore, immobilization presents the advantage of the reusability (M.C.M. de Souza, 2017).

6. Biodiesel production

Industry is developing year after year, and this means that also energy demand is increasing which makes it mandatory to search for new renewable energy sources to replace the sources of fossil fuels. One of these renewable energy sources is the biodiesel (figure 7). Biodiesel is produced by the transesterification of triglycerides from animal, vegetal or microbial sources with alcohols like ethanol, methanol, or other alcohols in the presence of a catalyst (B. Zieniuk, 2020). Glycerol is the second product of this reaction (B.D. Ribeiro, 2011). Catalysts used in the biodiesel production can be bases (such as sodium/potassium hydroxide or sodium/potassium methoxide), acids (such as hydrochloric or sulfuric acid) or enzymes. Alkaline catalysis has become dominant in the biodiesel production due to its lower costs and faster kinetics (B.D. Ribeiro, 2011). However, enzyme catalysis has more advantages namely avoiding the formation of soap and the occurrence of side reactions. Moreover, enzymes are biodegradable and environmentally friendly and thanks to their immobilization, they have become reusable, more stable, and easier to separate from the product of the reaction (B. Zieniuk, 2020). the most investigated enzymes in biodiesel production are *Candida* Antarctica lipase B (CALB/Novozym 435), Thermomyces lanuginosus (Lipolase 100 L or Lipozyme TL), *Candida* rugosa lipase, Pseudomonas fluorescens lipase, and porcine pancreatic lipase (PPL) (B. Zieniuk, 2020).

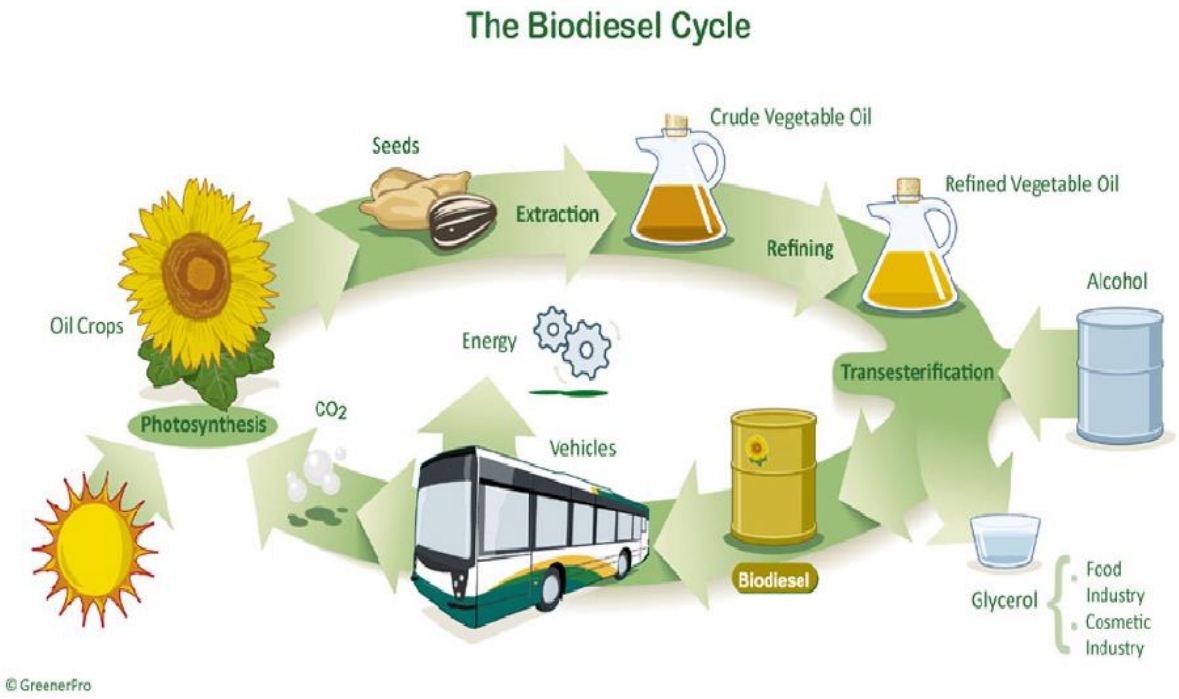

Figure 7: The biodiesel cycle

II. Materials and methods

1. *Candida antarctica* lipase B

Candida antarctica Lipase B (CALB) was purchased from Hampton Research and crystallized without further purification by Prof. M. Cianci at the European Molecular Biology Laboratory, c/o DESY, Hamburg (Germany).

2. Crystallisation conditions

Crystallization trials were performed at 293 K using the hanging-drop method using a Qiagen™ EasyXtal 15-well plate. 1.5 microliters of a 15 mg/ml CALB solution in 20 mM Na(CH 3COO) (pH 4.8) was diluted with 1.5 uL of the precipitant solution, made of 200 mM Na(CH 3COO) (pH 4.8), 20% (w/v) PEG4000, and 10–13% (v/v) ethanol. The drop was equilibrated by vapor diffusion against 500 ml of the precipitant solution.

3. X-ray diffraction data collection

Diffraction data were collected at 100 K using synchrotron radiation at the EMBL P13 beamline at the Petra III storage ring, Deutsches Elektronen-Synchrotron (DESY), Hamburg (Germany). The beam line was equipped with a Dectris Pilatus 6M detector and a MD2 goniometer (MAATEL-EMBL) with horizontal spindle axis. Crystals were cooled at 100 K with a cold nitrogen stream. The wavelength was set to 0.826 Å, using a Si(III) crystal monochromator (FMB-Oxford) (B. Stauch, 2015). Data were collected from four crystals oriented with different kappa angles while

performing helical scans. The data were integrated using the program XDS and scaled with XSCALE.

The structures of the ethanol-CALB complex were refined starting from the deposited structure of CALB as search model (Protein Data Bank (PDB): 5A71). The model was subjected to rigid-body minimization and subsequently to refinement steps with PHENIX. Manual rebuilding of the models was performed using the COOT graphic interface by inspecting the electron density map, calculated with $2F_{obs}$-F_{calc} or F_{obs}-F_{calc} coefficients and calculated phases from the model (B. Stauch, 2015).

4. Win COOT

COOT is a molecular-graphics program made for model building and validation of biological macromolecules (P. Emsley, 2010). The application displays electron-density maps and atomic models and allows model manipulation such as:

1- Idealization
2- Real space refinement
3- Manual rotation/translation
4- Rigid body fitting
5- Ligand search
6- Solvation
7- Mutation
8- Rotamers
9- Ramachandran idealization

The most important tool is the real space refinement engine that can optimise the feet of a section of atomic model to the electron density in real time with graphical feedback. The user is able also to intervene in this process dragging the atoms into the right places if the initial model is too far away from the corresponding electron density

Coot is a software that is freely available from the Coot website originally at the University of York and now at the MRC laboratory of molecular biology

Coot is a software that can read files containing 3-dimensional atomic coordinate models of macromolecular structures in different formats. the model can later be viewed from any viewpoint and rotated from all the direction The atomic model by default is represented using a stick model with factors representing chemical bonds each bond has two halves colour it with different colours according to the element of the atom at the end

of each bond allowing chemical structures to be visualised in a familiar manner to all chemists (figure 8).

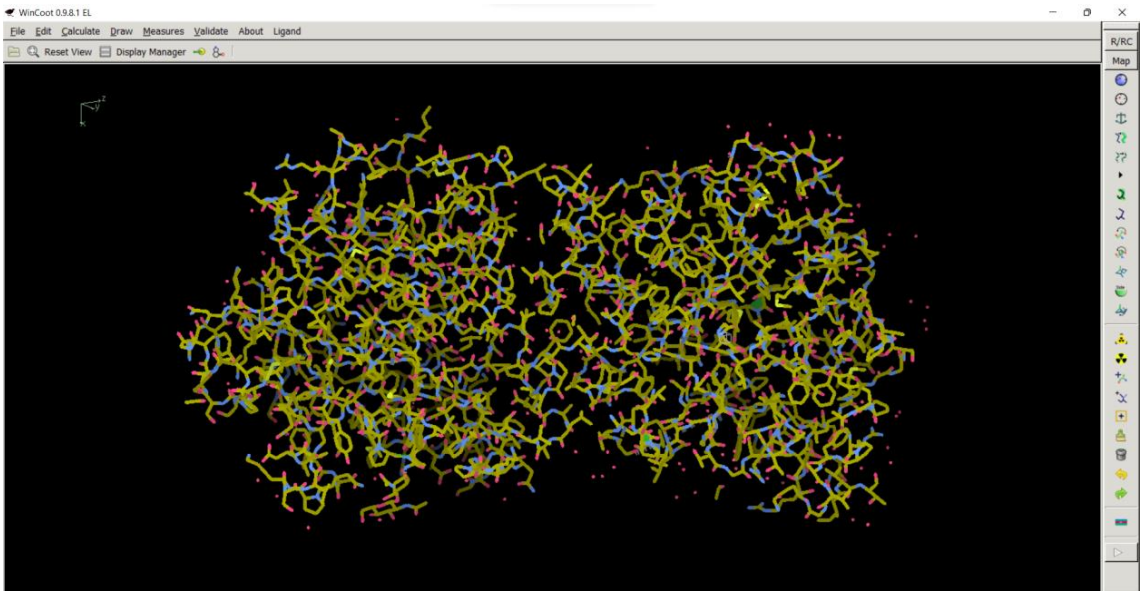

Figure 8: CALB view in WINCOOT software

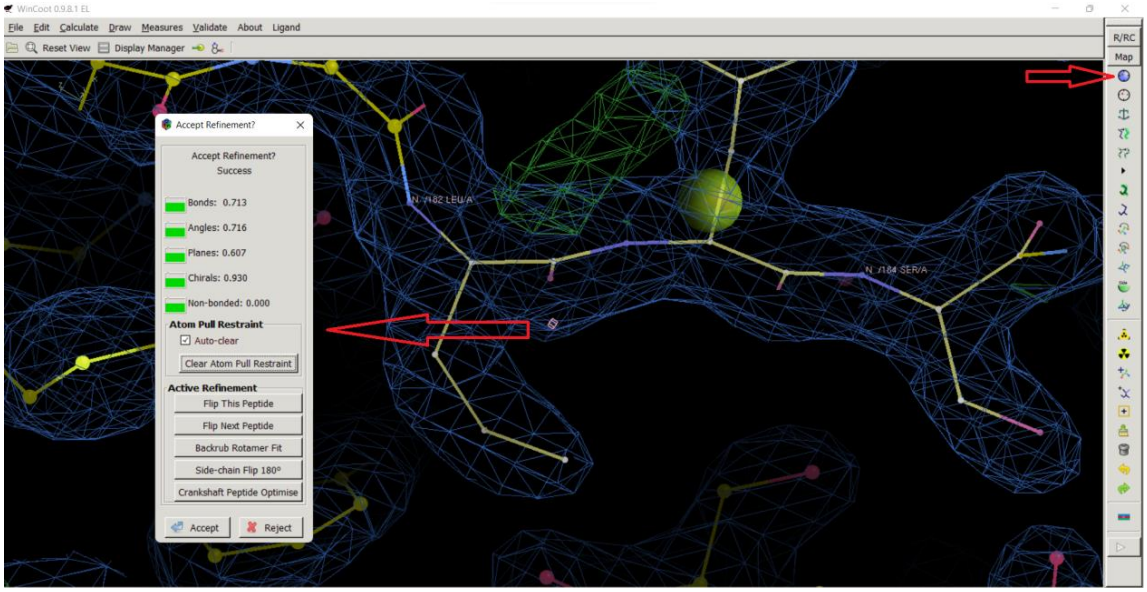

Figure 9: Real space refinement engine in WINCOOT

5. PHENIX

Phenix is a software suite that uses reduced data from X-ray diffraction, electron diffraction, neutron diffraction or cryo-EM to determine macromolecular structures (D. Liebschner, 2019). Each method has a different approach to derive structural information, with Phenix offering specific tools to address the unique properties of the experimental data (figure 10).

Macromolecules are essential for biological processes within organisms, engendering the need to understand their behaviour to explain the fundamentals of life. The function of macromolecules correlates with their three-dimensional structure, i.e. how the atoms of the molecule are arranged in space and how they move over time. Two major methods to obtain macromolecular structures are diffraction (usually using X-rays, but also neutrons or electrons) and electron cryo-microscopy, both of which are handled by Phenix.

Tasks performed with Phenix include data-quality assessment, map improvement, model building, the validation/rebuilding/refinement cycle and deposition. Each tool caters to the type of experimental data. The design of Phenix emphasizes the automation of procedures, where possible, to minimize repetitive and time-consuming manual tasks, while default parameters are chosen to encourage best practice. A graphical user interface provides access to many command-line features of Phenix and streamlines the transition between programs, project tracking, and re-running of previous tasks.

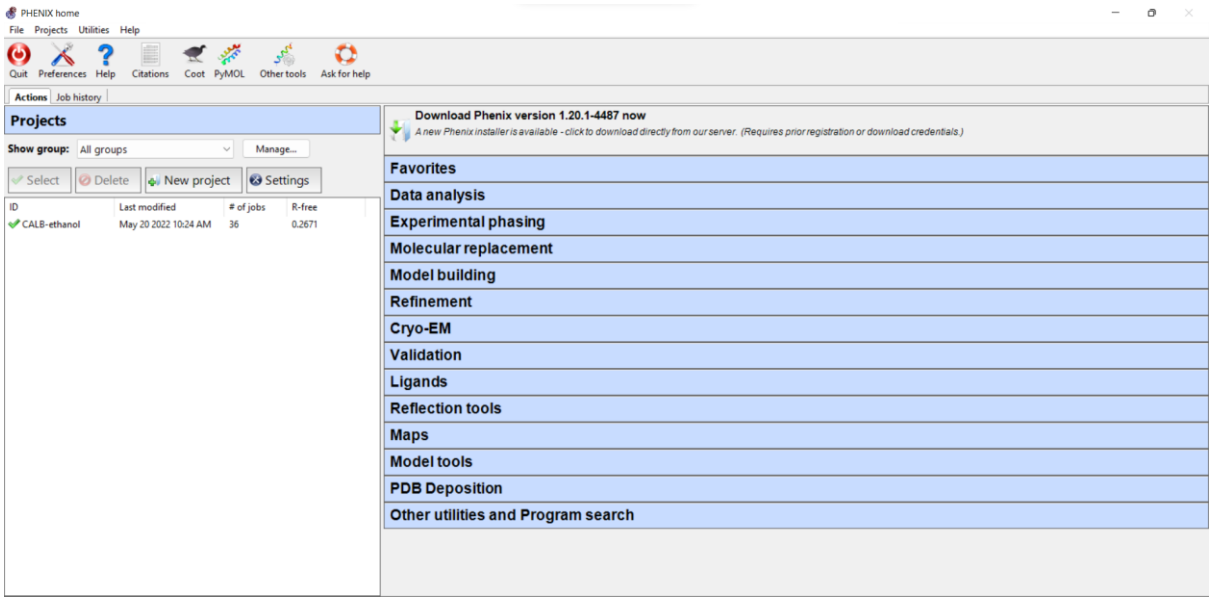

Figure 10: Tools offered by PHENIX

6. Structure analysis

The analysis of the structure was, directly, conducted on the density map of the CALB by looking for the ethanol molecules in CALB crystalised in presence of ethanol and for the methanol molecules in CALB crystallised in presence of methanol. The analysis involves checking if ethanol molecules were visible in the electron density maps and in the right place or they should be modified or eventually completely removed.

Ethanol molecules were identified thanks to the electron cloud that has a non-linear elongated shape as shown in the figure 11.

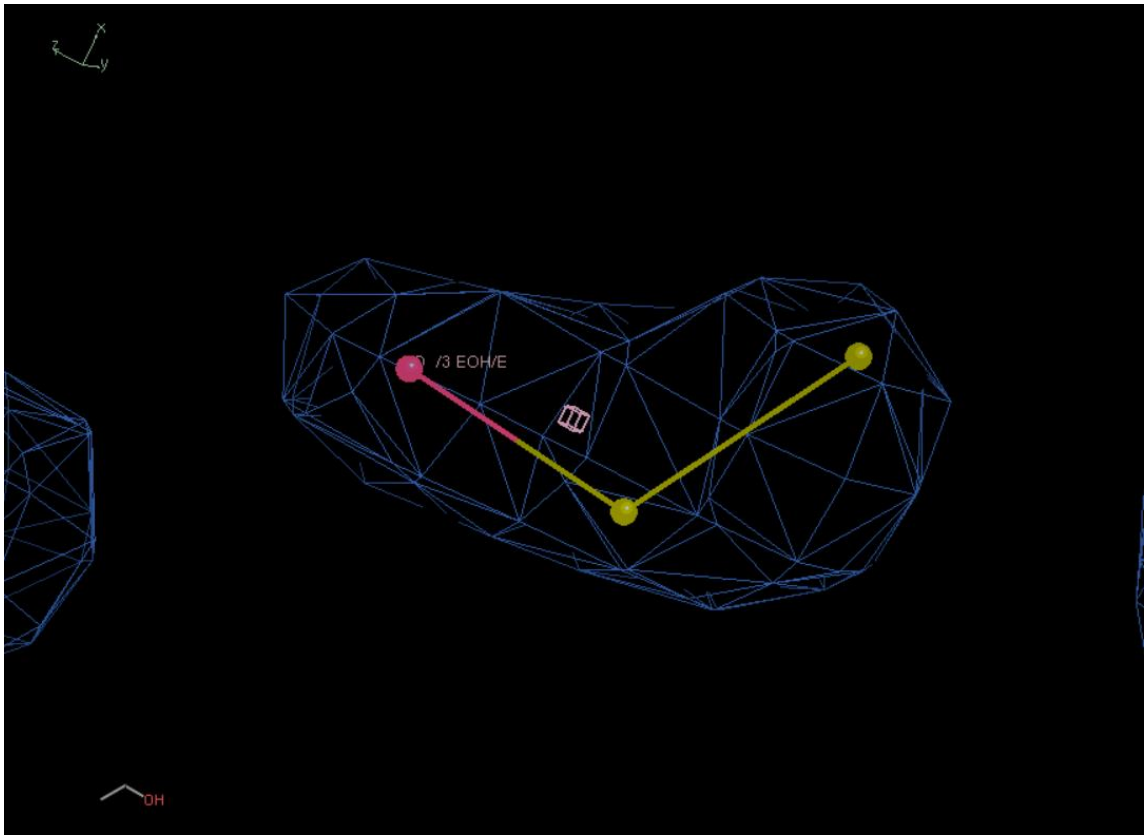

Figure 11: common shape of ethanol in CALB

This analysis involves checking if the enzyme's model fits well to the electron cloud and therefore do the modifications needed (figure12). WINCOOT provides all the required tools needed for the refinement. Analysis of the tertiary structure of the enzyme involves the control of the Ramachandran plot (figure 13). Ramachandran plot is a two-dimensional (2D) plot of the torsional angles of amino acids φ (phi) and ψ (psi) in a protein sequence. The φ represents the dihedral angle between N(i-1)-C(i)-CA(i)-N(i) and ψ is the backbone dihedral angle between C(i)-CA(i)-N(i)-C(i+1). The plot was developed by G.N. Ramachandran in 1963. The φ and ψ values are plotted to obtain the conformation of the peptide and the angular

spectrum lies between −180 and +180° on x-axis and y-axis. Ramachandran outlier is a representation of those amino acids which lie in the non-favorable regions of the plot (A. Pan, 2021)

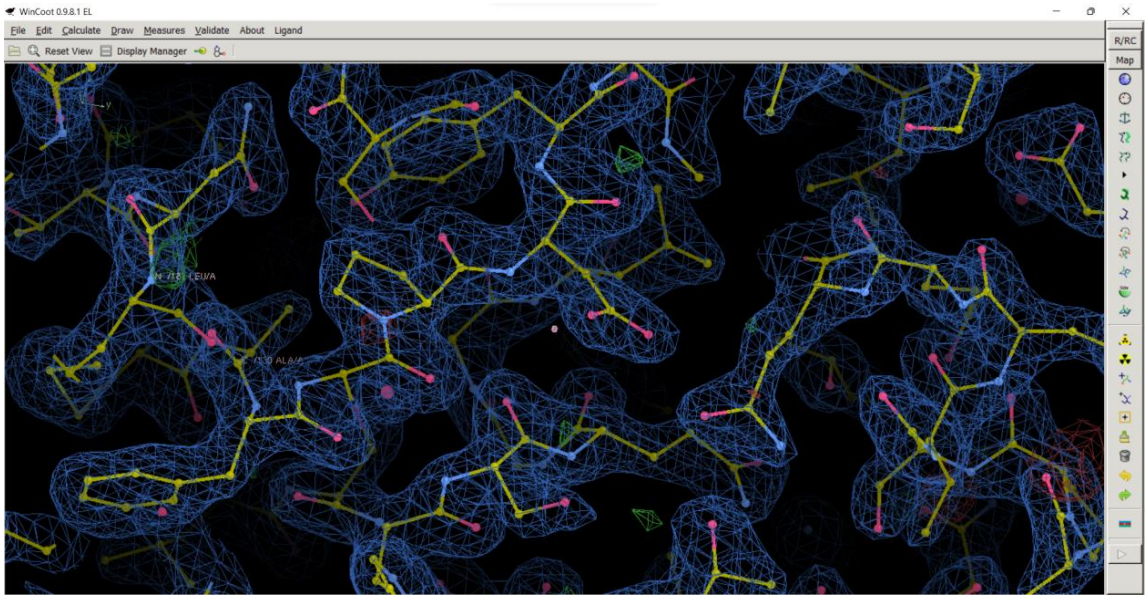

Figure 12: checking the fitness of the enzyme to the electron cloud

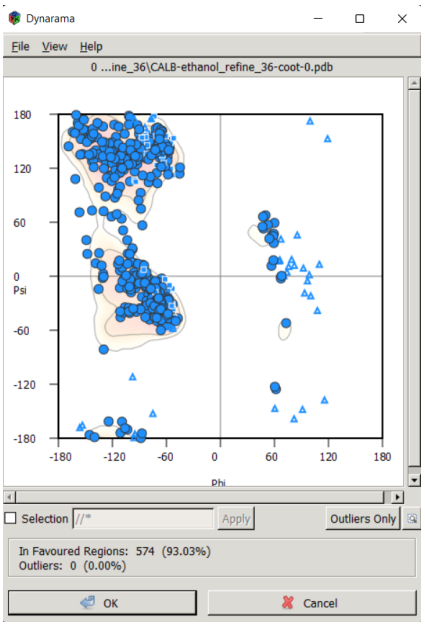

Figure 13: Ramachandran plot of CALB

III. RESULTS

The crystallization conditions produced two crystal forms of *Candida antarctica* lipase B in presence of ethanol. Two structures were resolved and analysed using the crystallographic software WINCOOT and Phenix.

One crystal (STRUCTURE 1) of the native enzyme in ethanol diffracted to 2.06 Å resolution with unit cell dimensions of a=39.13 Å, b = 44.79 Å, and c = 71.9 Å and space group P1. Refinement statistics are reported in Table 1.

Another crystal (STRUCTURE 2) of the native enzyme in ethanol diffracted to 1.94 Å resolution with unit cell dimensions of a=43.9 Å, b = 64.7 Å, and c = 89.0 Å and space group $P2_122_1$. Refinement statistics are reported in Table 2.

Table 1: Data collection and refinement statistics of structure 1.

Structure 1	
Resolution range	42.82 - 2.06 (2.134 - 2.06)
Space group	P 1
Unit cell	39.13 44.794 71.935 89.129 96.54 107.044
Unique reflections	25605 (2555)
Completeness (%)	89.33 (90.22)
Wilson B-factor	31.36

Reflections used in refinement	25603 (2555)
Reflections used for R-free	1230 (110)
R-work	0.2182 (0.3413)
R-free	0.2685 (0.4012)
Number of non-hydrogen atoms	4817
macromolecules	4575
ligands	74
solvent	168
Protein residues	623
RMS(bonds)	0.003
RMS(angles)	0.67
Ramachandran favoured (%)	95.62
Ramachandran allowed (%)	4.38
Ramachandran outliers (%)	0.00
Rotamer outliers (%)	2.58
Clash score	9.18
Average B-factor	33.48
macromolecules	33.32
ligands	42.31
solvent	33.76

Table 2: Data collection and refinement statistics of structure 2

Structure 2	
Resolution range	32.34 - 1.94 (2.009 - 1.94)
Space group	$P2_122_1$
Unit cell	43.964 64.683 89.009 90 90 90
Unique reflections	19257 (1904)
Completeness (%)	98.93 (99.06)
Wilson B-factor	31.30
Reflections used in refinement	19248 (1904)
Reflections used for R-free	915 (91)
R-work	0.1853 (0.2405)
R-free	0.2331 (0.3159)
Number of non-hydrogen atoms	2477
macromolecules	2316
ligands	145
solvent	94
Protein residues	316
RMS (bonds)	0.004
RMS (angles)	0.68

Ramachandran favoured (%)	97.45
Ramachandran allowed (%)	2.55
Ramachandran outliers (%)	0.00
Rotamer outliers (%)	1.18
Clash score	6.28
Average B-factor	31.77
macromolecules	30.82
ligands	52.62
solvent	40.47

Statistics for the highest-resolution shell are shown in parentheses.

Structure 1 and 2 were then compared with the structure of *Candida antarctica* lipase B in presence of methanol, previously refined in Prof. Cianci's laboratory. The analysis reveals differences in the alcohol molecule distribution and tertiary structure.

1. Overall structure of CALB structure 1 in ethanol

The first structure of *Candida antarctica* lipase B in ethanol shows two monomers A and B with different conformations. In fact, the residue range Leu140-Ala141-Gly142-Pro143-Leu144-Asp145-Ala146-Leu147, corresponding to alpha-helix 5, is different between the two molecules (figure 16). The molecule A shows a missing loop while the molecule B shows an alpha-helix that can be considered as an open conformation of the CALB.

There is no observed interaction between the two monomers of CALB.

The crystallographic model of CALB grown in presence of ethanol presents six ethanol molecules distributed all over the structure of CALB (figure 15).

One of the ethanol molecules was so close to the active site (figure 14) of the monomer A of CALB.

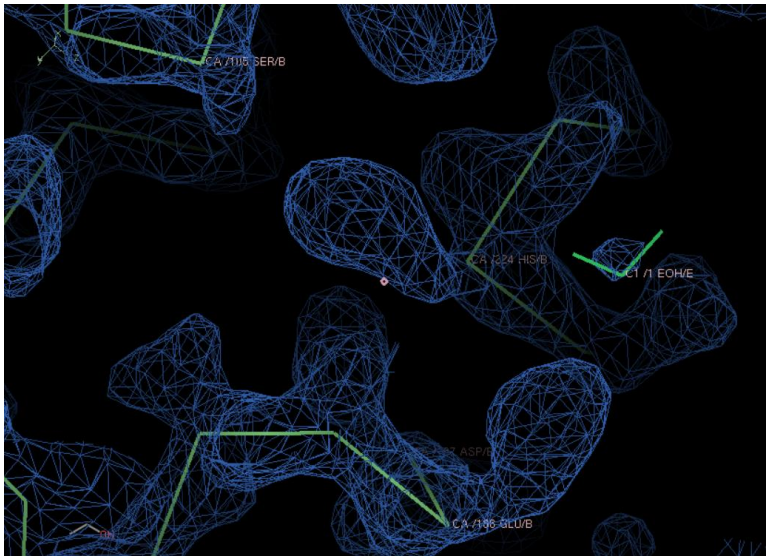

Figure 14: Ethanol (EtOH) molecule near the active site of CALB structure 1

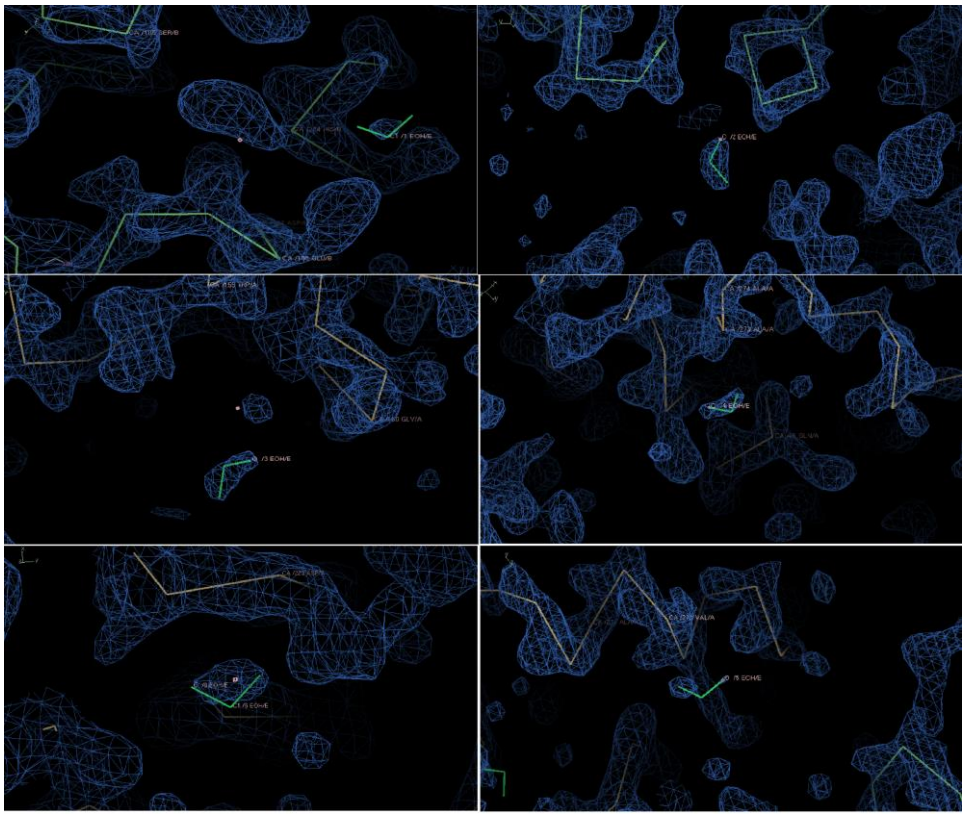

Figure 15: Ethanol molecules in CALB structure 1

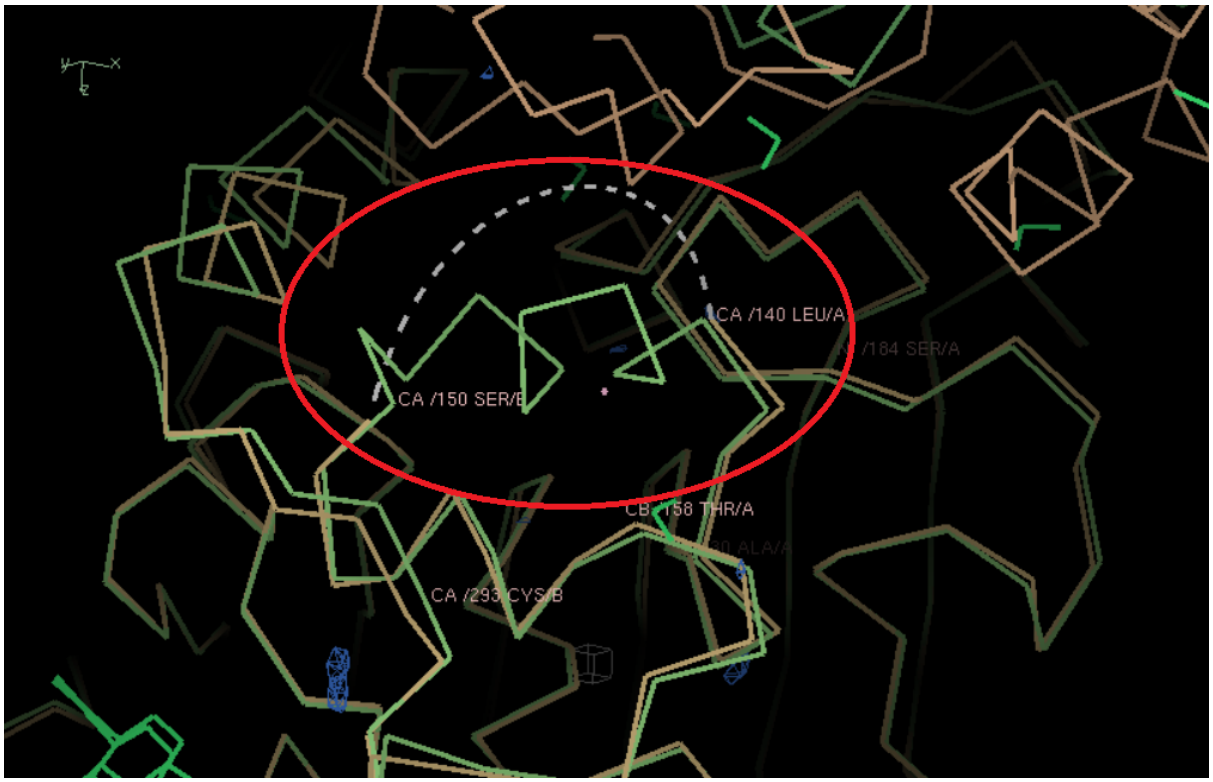

Figure 16: residue range Leu140-Ala141-Gly142-Pro143-Leu144-Asp145-Ala146-Leu147 in monomer A in orange and in monomer B in green of CALB structure 1

2. Overall structure of CALB structure 2 in ethanol

The structure of the second *Candida antarctica* lipase B in ethanol (chain C) shows only one monomer that can be considered as an open structure of CALB (figure 17). In fact, the residue range Leu140-Ala141-Gly142-Pro143- Leu144-Asp145-Ala146-Leu147, corresponding to alpha-helix 5, is identical to the one of the monomer B of the first structure of CALB crystalised in presence of ethanol in folded open conformation.

The residue range Gln 23-Gly 24-Ala 25-Ser 26-Pro 27-Ser 28-Ser 29-Val 30-Ser 31-Lys 32 of the chain of the second structure of CALB is different from the other structures' one. In fact, the chain follows a different path leaving the place for an ethanol molecule (figure 18).

Crystallography showed 13 ethanol molecules dispersed all over the structure of the enzyme. One of the ethanol molecules was close to the alpha-helix 5 of the CALB, one other ethanol molecule was found next to the ligands and one molecule was in the active site

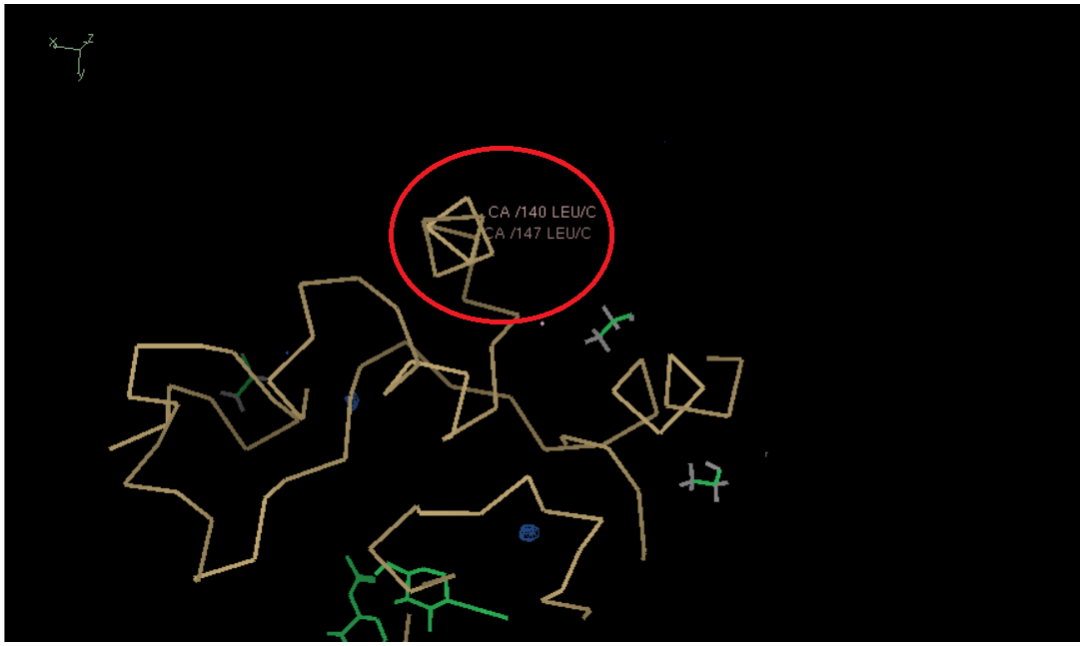

Figure 17: Alpha-helix 5 of CALB 2

Figure 18: residue range Gln 23-Gly 24-Ala 25-Ser 26-Pro 27-Ser 28-Ser 29-Val 30-Ser 31-Lys 32 in CALB structure 1 (purple) and in CALB structure 2 (in yellow)

3. Distribution of ethanol molecules in the active site of CALB 1 and 2

The active site of CALB is a catalytic triad made of a nucleophilic serine 105 activated by a hydrogen bond in relay with histidine 224 and aspartate 187 or glutamate 188.

In both the lipases, molecules of ethanol were found dispersed all around the structure of the enzyme but not always found in the active site. In fact, CALB structure 1 had one monomer (monomer B) that had one ethanol molecule in its active site (figure 20), as well as CALB structure 2 (figure 19) while the monomer A of CALB structure 1 didn't have any (Table 1).

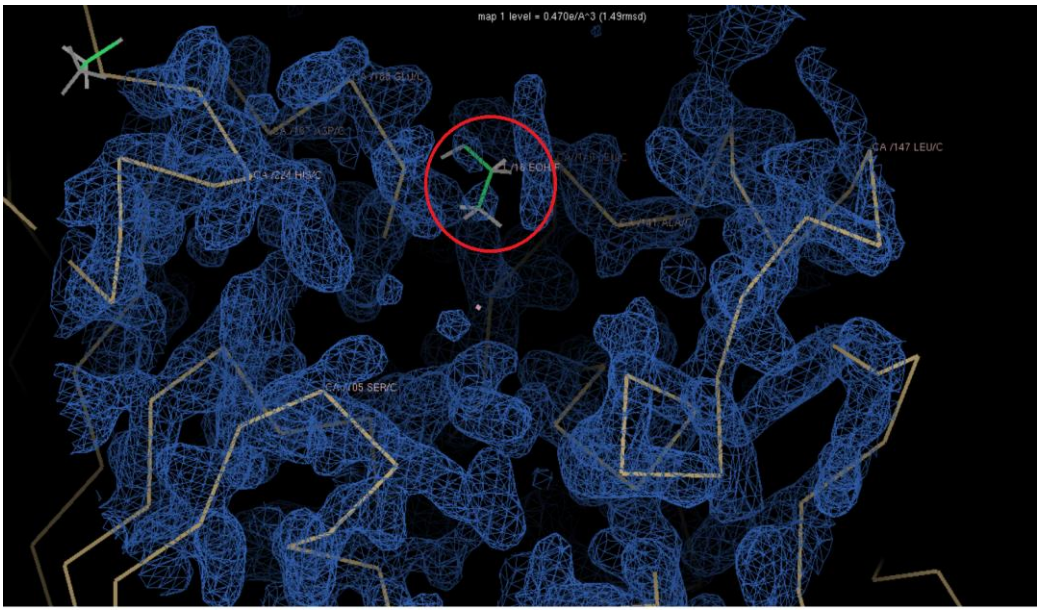

Figure 19: Active site of CALB structure 2

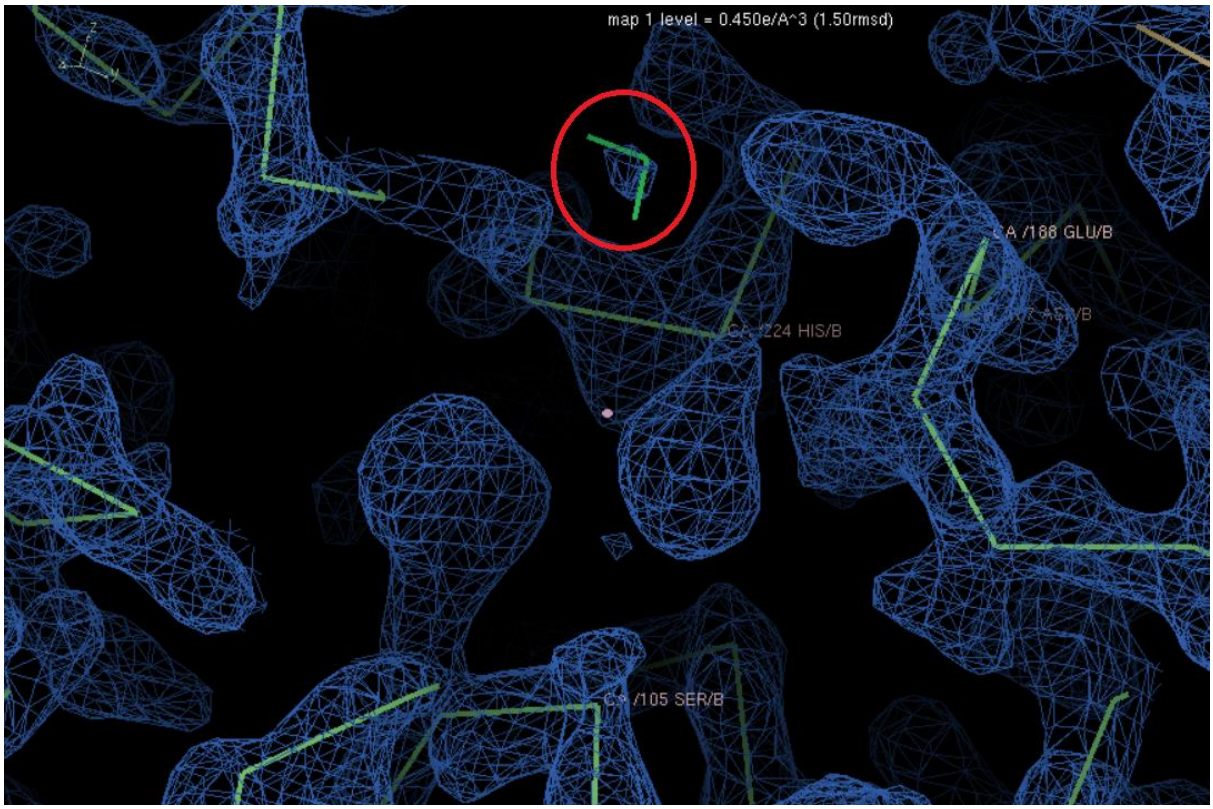

Figure 20: Active site of monomer B of CALB structure 1

4. Differences between ethanol structure and methanol structure (Structure 3)

Crystallography in presence of methanol showed two different monomers (A and B) that can be defined as a monomer with an open conformation (figure 21, monomer A) and with a closed one (figure 22, monomer B). In fact, the monomer A, like the structure of CALB crystallised in the presence of ethanol, has an alpha-helix which can be considered in an open conformation while the monomer B, by contrast, in the residue range Leu140-Ala141-Gly142-Pro143- Leu144-Asp145-Ala146-Leu147, clearly shows an unfolded loop that is associated to the closed conformation of CALB.

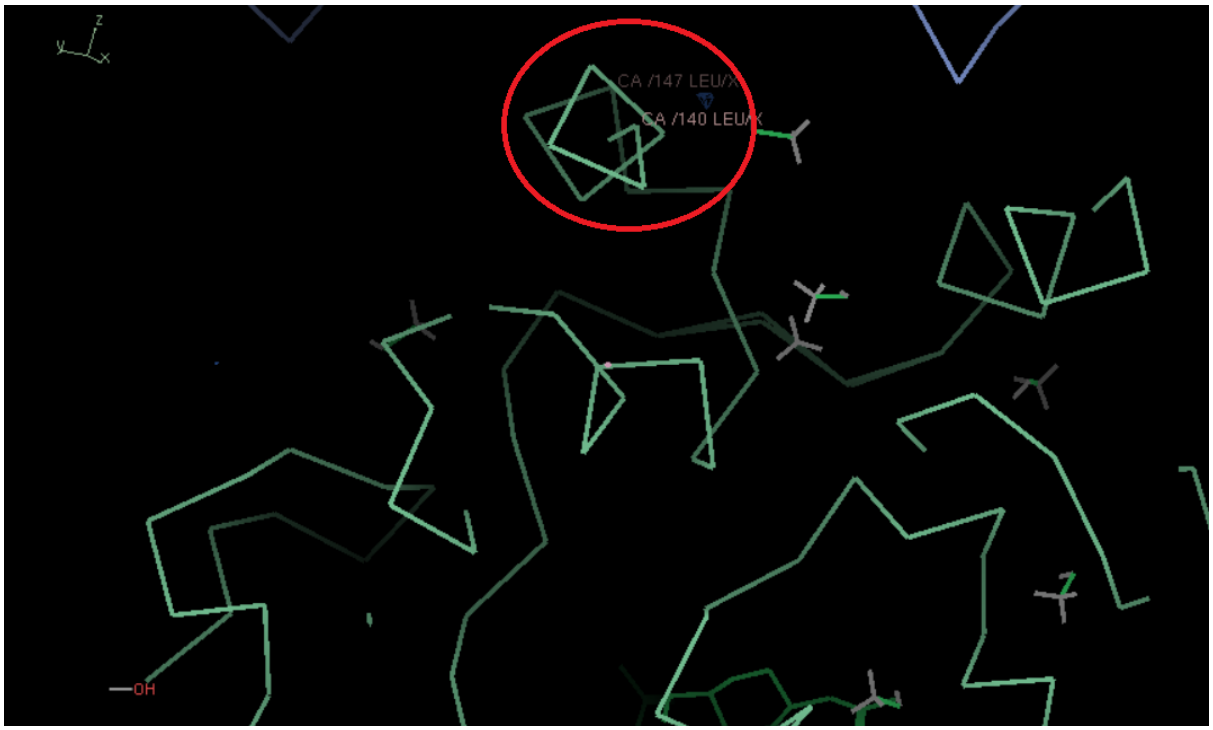

Figure 21: Open conformation of CALB crystallized in presence of methanol

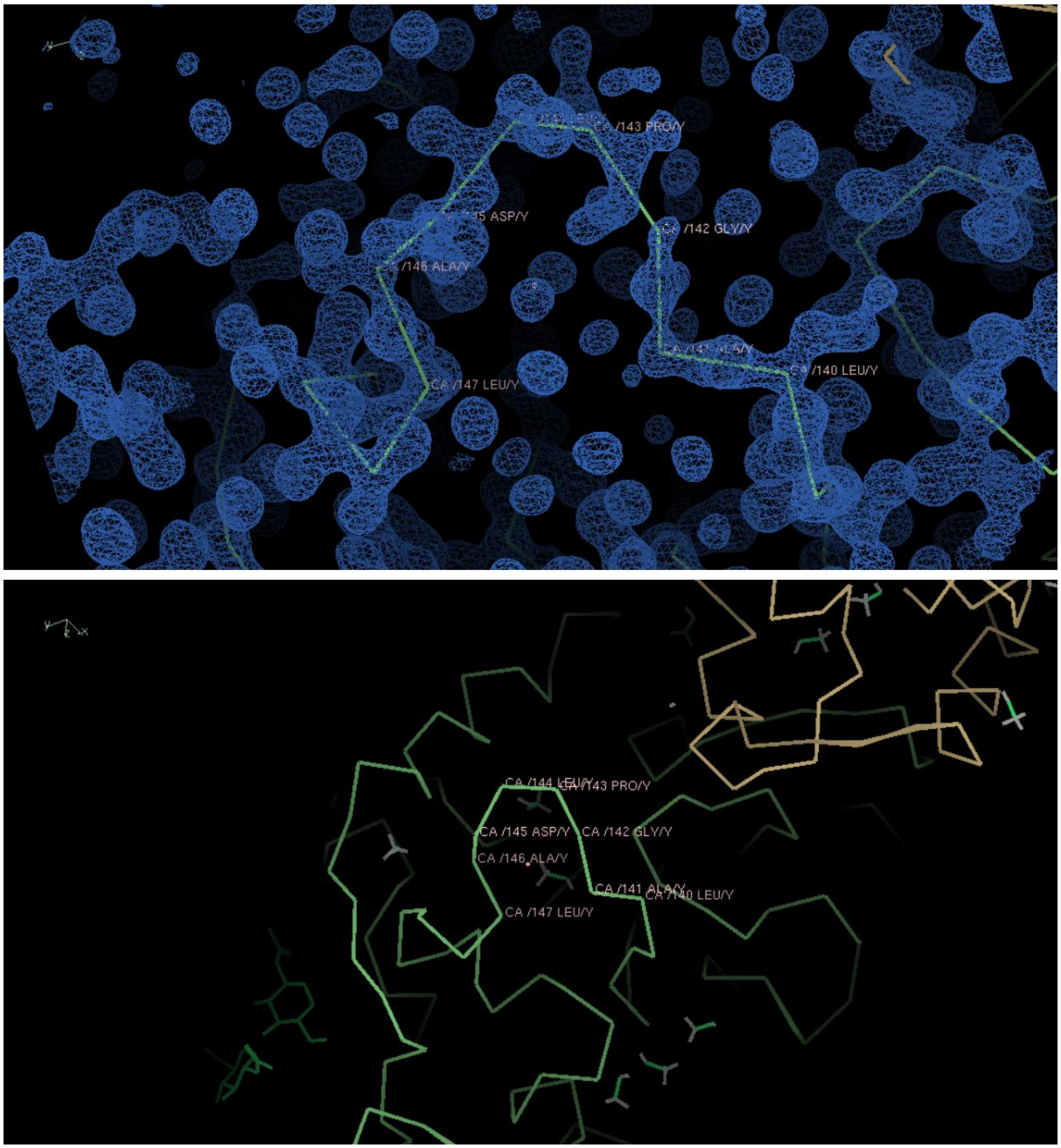

Figure 22: Open conformation of CALB crystallized in presence of methanol

The crystallographic model of CALB grown in presence of methanol presents 36 methanol molecules, a number which is higher than the ethanol molecules found in the two CALB structures. Three methanol molecules were found in the active site of the monomer A (figure 23) and two of them in the active site of the monomer B of CALB crystallised in presence of methanol (figure 24). Whereas, only one ethanol molecule was found in the

active site of the monomer B (the one with the open conformation) of CALB structure 1 crystallised in presence of ethanol (figure 20) and one other ethanol molecule in the active site of CALB structure 2 crystallised in presence of ethanol which, same as the other enzyme, has an open conformation (figure19) (Table 1). The methanol structure 3 is very similar to the 2-propanol structure (structure 4) already reported by (B. Stauch, 2015) (Table 3).

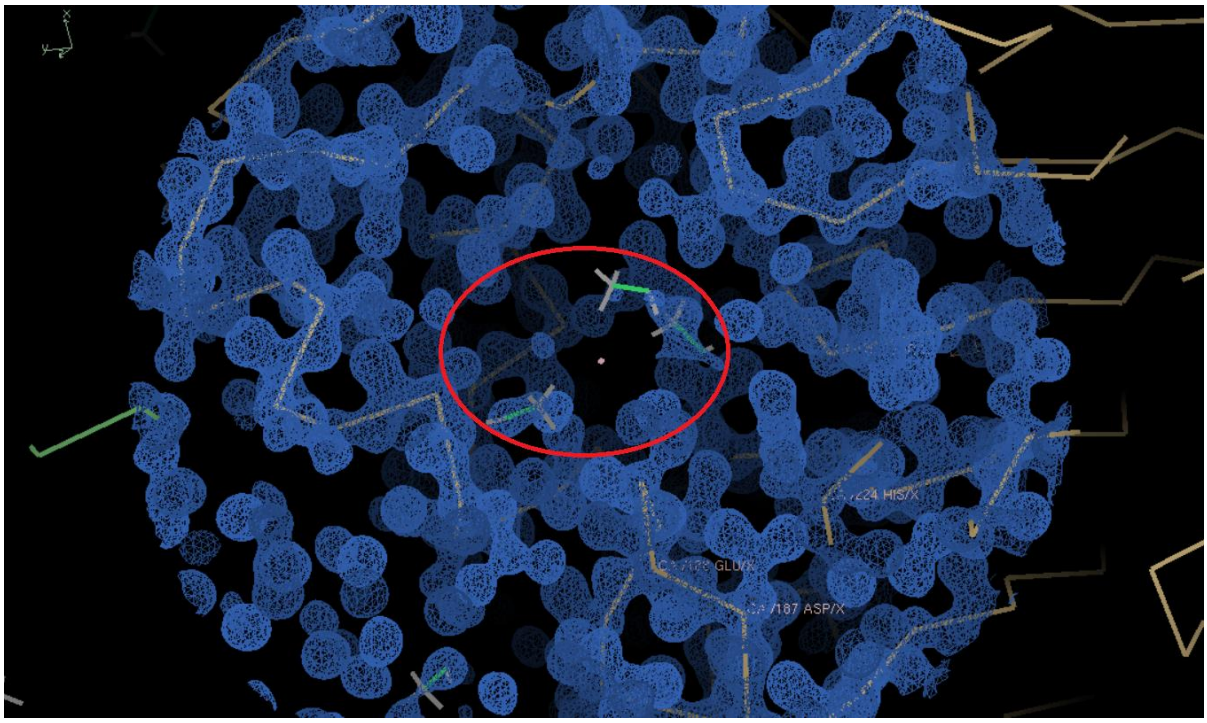

Figure 23: Active site of monomer A of CALB crystallized in presence of methanol

Table 3: Comparison between CALB structures.

	Solvent	Monomer	Secondary structure	conformation	Alcohol molecules
Structure 1	Ethanol	A	Not visible	Disordered	0
	Ethanol	B	Alpha-helix	Open	1
Structure 2	Ethanol	B	Alpha-helix	Open	1
Structure 3	Methanol	A	Alpha-helix	Open	3
		B	loop	Closed	2
Structure 4	Propanol	A	Alpha-helix	Open	1
	Propanol	B	loop	Closed	1

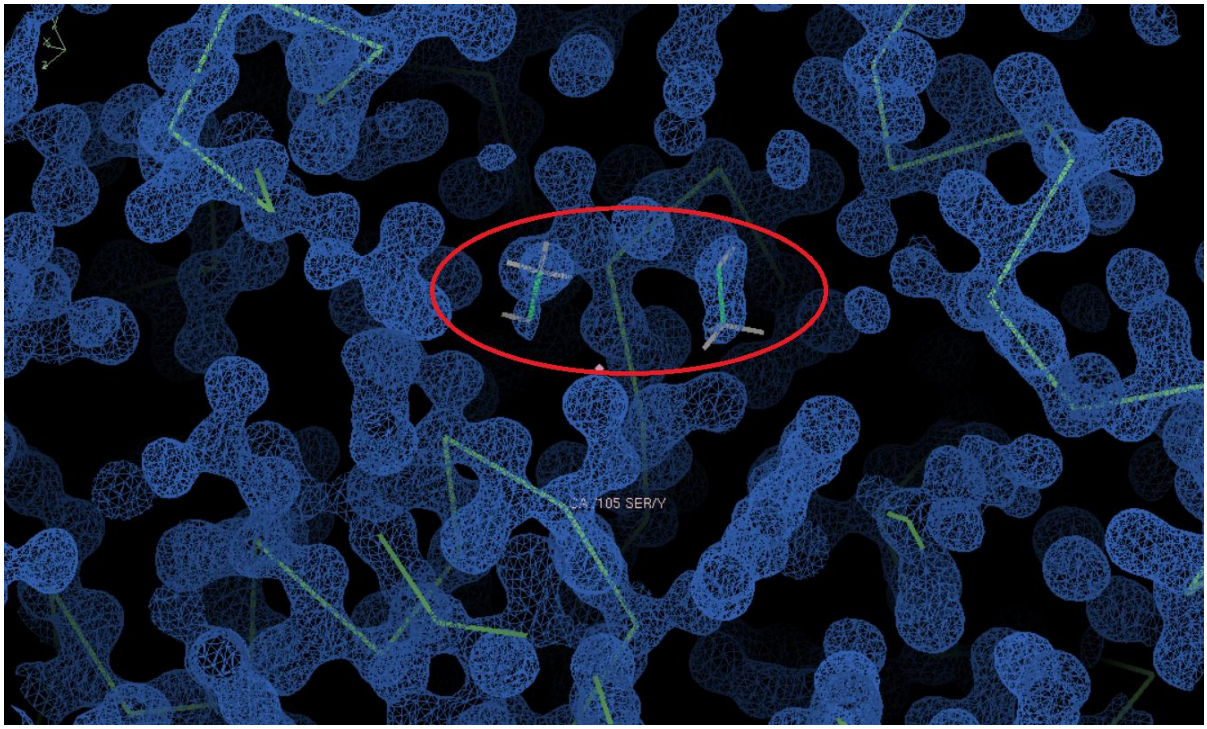

Figure 24: Active site of monomer B of CALB crystallized in presence of methanol

IV. DISCUSSION

1. Influence of the solvent type on the tertiary structure

The structure 1 and 2 in ethanol reported in this study present all monomers in open conformations, while the monomers of the structure in methanol and 2-propanol have one open and one closed conformation.

The polarity of the solvent used in the crystallization is:

Table 4 : relation between the polarity of the solvents and the conformation of CALB

Solvent	Polarity	Formula	Conformation
2-propanol	0.546	C_3H_8O	Open/Closed
Ethanol	0.654	C_2H_6O	All open
Methanol	0.762	CH_3OH	Open/Closed
Water	1.000	H_2O	All open

(https://sites.google.com/site/miller00828/in/solvent-polarity-table)

So, while a direct link between conformation of alpha helix 5 and polarity of the solvent cannot be made, a more evident link could be with the size and number of the solvent molecule and the conformation of the loop.

The superposition of the different structures of CALB crystallized both in the presence of ethanol and methanol gave the possibility to notice six methanol molecules that share the same position with other six ethanol molecules.

In only one case the difference of the solvent did not affect the structure while in the 5 other cases differences in the structure were noticed. In fact, in one case, it was able to observe an open conformation in presence of ethanol and a closed conformation in the presence of methanol. In 3 other cases, the chain of the CALB crystallized in the presence of methanol and of

the one crystallized in presence of ethanol follow slightly different paths. In the last case, the deviation of the chains is higher. Knowing that the size of the two studied solvents is different with ethanol being bigger, we can link the size and number of the solvent molecules to the effect on the tertiary structure.

2. Principles of CALB inhibition by ETHANOL

If in one hand the utility of the organic solvents could give many advantages to the activity of the enzyme because of their capability to increase the solubility of the substrates; in the other hand it should be taken into consideration the concentration of it in the enzymatic environment.

Since the organic solvents can modify the thermodynamics of the complex enzyme-substrate it could be seen some side effects potentially negative concerning the catalytic activity. These effects are surely affecting the reaction velocity of the enzyme and so are considered inhibitor effects because reduce the enzymatic activity.

As introduced at the beginning of this paragraph the organic solvent concentration is a potential factor affecting the reaction velocity. To this regard Lotti, Pleiss, Kulschewski, Sasso and Secundoc, in the Journal of Biotechnology 2013, have thoroughly described the behaviour of *C. antarctica* lipase B in toluene–methanol–water mixtures. They concluded that the "two major factors contribute to the observed concentration dependency of the catalytic activity of CALB are the thermodynamic properties of the non-ideal substrate-solvent mixture and the molecular interactions between substrate and enzyme" (T. Kulschewski, 2013)

Considering all the above-mentioned properties of the methanol-substrate-enzyme complex and by comparing the structures of the same enzyme in

ethanol and methanol is possible to talk about a reversible competitive inhibition pathway thanks to the distribution of ethanol molecules in the overall structure.

Since ethanol is present in the active site and that the site itself is not large enough for accommodate two different substrates, it can be assumed that it is reversible because of the non-covalent nature of the bonds and competitive because of the sharing of the same site with the substrate.

V. Conclusions

The structural analysis of *Candida antarctica* lipase B in presence of ethanol lead to the following conclusions:

- The structure of the active site was not affected but the overall structure of the enzyme was affected making methanol a better solvent since it did not affect any part of the enzyme structure
- Ethanol molecules were found all over the structure of CALB and in its active site
- It is possible to talk about a reversible inhibition of CALB in ethanol
- There is no direct link between the conformation of alpha helix 5 and polarity of the solvent but there is a possibility to link the size of the solvent and its effect on the tertiary structure of the enzyme
- This implies that smaller solvents and solvents that do not access the active site might be better.

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