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## KEY WORDS

Enzymes; molecular modelling; solid/gas biocatalysis; structure activity relationship; thermodynamic activity of water

## ABSTRACT

The effect of water activity on enzyme catalyzed enantioselective transesterification was studied using a solid/gas reactor. The experimental results were compared with predictions from molecular modelling. The system studied was esterification of 2-pentanol with methyl propanoate as acyl donor and lipase B from *Candida antarctica* as catalyst. Experimental data showed a pronounced water activity effect on both reaction rate and enantioselectivity. The enantioselectivity increased from 100 at water activity close to 0 to reach a maximum of 320 at water activity 0.2. Molecular modelling revealed how a water molecule could bind in the active site and obstruct the binding of the slow reacting enantiomer. Measurements of enantioselectivity at different water activity and temperature showed that the water molecule had a high affinity to the stereospecificity pocket of the active site with a binding energy of 9 kJ $mol^{-1}$ and lost all its degrees of rotation, corresponding to an entropic energy of 37 J $mol^{-1}$ $K^{-1}$.

# INTRODUCTION

It is known that the enantioselectivity of enzymes depends on many different parameters such as temperature, substrate structure, reaction medium and thermodynamic water activity. Therefore, rational improvement of enzyme enantioselectivity such as solvent engineering is complicated and mostly a matter of trial and error. To improve the situation, a more detailed molecular level understanding of the solvent-substrate-enzyme interactions needs to be obtained. The influence of thermodynamic water activity, $a_w$, on the enantioselectivity of enzymes has been studied a lot during the last decades. There are reports of enantioselectivity increasing with thermodynamic water activity reaching a plateau value,[1,2] enantioselectivity decreasing with thermodynamic water activity,[2,3,4] and enantioselectivity being unaffected by thermodynamic water activity.[2,5,6,7] No general trends or rules have been found. [1,2,3]

In the present paper, a molecular explanation for the influence of thermodynamic water activity on *Candida antarctica* lipase B (CALB, EC 3.1.1.3) enantioselectivity is proposed. As a model reaction the resolution of racemic 2-pentanol was used (Figure 1). Two different and complementary methods were used to rationalise the effects of water. Firstly, an experimental study of the effect of thermodynamic water activity on enantioselectivity was performed in a continuous solid/gas bioreactor. This type of reactor has been used by several authors with immobilized lipases.[8-12] It allows a precise control of the thermodynamic parameters of the enzyme microenvironment and permits determination of the sole role of the addition of water.[8, 10, 13, 14] Secondly, molecular modelling was used to study the behaviour of water molecules in the active site of CALB, and their effect on enantioselectivity. Secondly, molecular modelling was used to study the behaviour of water molecules in the active site of CALB, and their effect on enantioselectivity.

## RESULTS

## The effect of thermodynamic water activity on CALB enantioselectivity in a continuous solid/gas bioreactor

The feasibility of studying lipase kinetics with substrates in gas phase was previously demonstrated.[8, 10, 13-16] In the same papers, the advantages of the solid/gas technology as a tool for studying the influence of the microenvironment on enzyme enantioselectivity have been explained. In particular this kind of reactor offers the possibility to independently control and adjust thermodynamic activities of all components present in the reaction medium, by varying the partial pressure of each compound in the carrier gas, which percolates the fixed catalytic bed.

### Influence of thermodynamic water activity on CALB activity

Results obtained are shown in Figure 2. Water can interact with enzymes and change their activity in several ways. Firstly, as lubricant, water should increase the total activity. Water would favour interaction between enzyme active site and ligands by acting as a molecular lubricant, increasing amino acid mobility[17], increasing active site polarity[18] and strengthening the hydrophobic interactions between apolar sites of the protein and apolar part of the substrates.[13] This effect might explain our observation of increased enzymatic activity for water activities up to 0.02. Secondly, water would be able to act as a competitive substrate resulting in a decreased transesterification and an increased hydrolysis rate.[10, 13] Both these effects were seen at water activities above 0.1. Finally, water can form a dead-end complex

with the free enzyme and decrease the total activity,[10, 13] again an effect that was seen at water activities above 0.1.

**Influence of thermodynamic water activity on CALB enantioselectivity**

The enantioselectivity of CALB towards 2-pentanol varied with thermodynamic water activity and had a maximum of 320 around 0.2 as seen in Figure 3. For a water activity close to zero we found an $E$-value of 101 ± 2 in this study, whereas we found it to be 176 ± 18 for the same reaction catalyzed by CALB from Roche Industry (Penzberg, Germany, CHIRAZYME® L-1, Lyo).[15] A study of the temperature dependence of the enantioselectivity allowed calculation of the entropic and enthalpic contributions to the enantioselectivity (Figure 4). From the data in Table 1 it can be seen that the preferred enantiomer (*R*)-2-pentanol, was favoured by enthalpy but disfavoured by entropy. The entropic term $T\Delta_{R\text{-}S}\Delta S^{\ddagger}$ was very significant and measured 33 to 67% of the enthalpic term $\Delta_{R\text{-}S}\Delta H^{\ddagger}$ of the differential free energy of activation $\Delta_{R\text{-}S}\Delta G^{\ddagger}$ (Table1). Enantioselectivity decreased with increasing temperature meaning that the experimental temperatures (ranging from 318 to 363 K) were below the racemic temperature ($T_R$), which is the general case for enzyme catalysis. Secondly, $\Delta_{R\text{-}S}\Delta H^{\ddagger}$- and $\Delta_{R\text{-}S}\Delta S^{\ddagger}$- values were both negative. This corresponds to the case in which enthalpic and entropic contributions counteract each other for defining enantioselectivity. A negative $\Delta_{R\text{-}S}\Delta H^{\ddagger}$ is consistent with a better conformity of the (*R*)-enantiomer than the (*S*)-enantiomer in the transition state comprising spatial and electrostatic interactions between the substrate and the enzyme active site. A negative $\Delta_{R\text{-}S}\Delta S^{\ddagger}$ corresponds to less conformational degrees of freedom for the (*R*)-enantiomer, protein and water molecules (when present in the reaction medium) in the transition state.

## Molecular modelling

The enantioselectivity depends on energy differences in transition state for the competing enantiomers. For lipases and other serine hydrolases the transition state can be modelled as the tetrahedral intermediate in the active site of the enzyme.[19,20] Here we have modelled the tetrahedral intermediates when the alcohol attacks the acyl enzyme. The modelling has been done in vacuum, which should be a good model for gas phase experiments.

Models of the tetrahedral intermediates of (*R*)- and (*S*)-2-pentyl propanoate are shown in Figure 5. The intermediates were built in their fast reacting mode known from previous work.[19,20] According to that model the (*R*)-enantiomer reacts with its large substituent, a propyl group, of the alcohol sticking out towards the entrance of the active site, while the small substituent, methyl, is pointing towards Trp104. The (*S*)-enantiomer has to point its large substituent towards Trp104 to be able to form all hydrogen bonds needed for catalysis. Therefore, the main difference between the enantiomers was the substituent harboured in the restricted cavity of the stereospecificity pocket. Initial dynamics simulations confirmed earlier results that the propyl group of the (*S*)-enantiomer did not fit as well as the methyl group of the (*R*)-enantiomer. SURFNET was used to map residual volumes between the substrate in transition state and the enzyme in the energy minimized structures from the dynamics simulations. The structure with the (*R*)-tetrahedral intermediate had enough space to accommodate a water molecule in the stereospecificity pocket whereas the (*S*)-intermediate was totally lacking this possibility (data not shown). In conclusion a bound water molecule in the stereospecificity pocket would inhibit (*S*)-2-pentanol to react while it would not influence (*R*)-2-pentanol.

In another dynamics simulation a water molecule was inserted in the stereospecificity pocket before the simulation started. Figure 6 shows the energy-minimized structures of the (*R*)- and (*S*)-tetrahedral intermediates after dynamic simulations. For both enantiomers the water molecule remained in the active site pocket during the 200 ps dynamics simulation. The water molecule placed in the (*R*)-intermediate structure formed five hydrogen bonds with the enzyme. In the (*S*)-intermediate structure the water molecule formed only three hydrogen bonds with the enzyme. The enzyme could easily accommodate the (*R*)-enantiomer without any major structural changes from the crystal structure. On contrary, the (*S*)-enantiomers caused significant changes in the enzyme structure. For example the distance between $\alpha$-carbons of Thr40 and Thr42 increased by 1.4 Å. The conclusion was that the (*S*)-enantiomer should have problems to react with a water molecule present in the stereospecificity pocket, while the (*R*)-enantiomer would not be spatially hindered.

## Discussion

Water activity is an important parameter for the outcome of enzyme catalyzed reactions performed in organic solvents. The effect of water as a competitive substrate is obvious and well known for acyl transfer catalyzed by hydrolytic enzymes. In the present investigation we have investigated and quantified other functions of water in such reactions. Besides being a competitive substrate, water is also able to act as a competitive inhibitor. By means of molecular modelling, we were able to show the possibility for a water molecule to bind in the stereospecificity pocket of *Candida antarctica* lipase B. Being located in the specificity pocket, the water affects the two substrate enantiomers differently, causing a water activity-dependent enantioselective inhibition (fig. 6). This competitive inhibition caused by water could be described with a two-state system, in which the enzyme had or did not have a water molecule bound in the stereospecificity pocket. The distribution between these two states can

be analysed using the Hill equation for ligand binding, using the water activity as a measure of ligand concentration and enantioselectivity as a parameter of bound ligands, since enantioselectivity according to our model is directly affected by water occupancy of the stereospecificity pocket. The Hill equation analysis performed for $a_W$ values inferior to 0.1, had two outcomes. Firstly, the Hill coefficient was found to be 0.78, which is consistent with one water molecule bound as a ligand in the stereospecificity pocket. Of course, other sites on the enzyme might also bind water molecules, but without affecting the enantioselectivity. Secondly the water dissociation constant was calculated to 0.03, corresponding to a binding energy of 9 kJ $mol^{-1}$. As mentioned above the water molecule increased the enantioselectivity as the stereospecificity pocket became smaller. The binding energy of that water molecule is thus not directly related to the energy change in enantioselectivity, which only amounts to 3 kJ $mol^{-1}$. The enantioselectivity is instead related to the size of the newly formed pocket. It can also be seen that water binds tighter in the stereospecificity pocket than as a dead end inhibitor, as $K_i$ of water equals to 0.128 in a transacylation between methyl propionate and *n*-propanol catalyzed by the same enzyme in gas phase.[10]

A water molecule coming from gas phase and gets trapped in the stereospecificity pocket experiences a significant loss in entropy. In our experiment, using measurements of enantioselectivity to reflect the situation in transition state, the entropy changed from 19 to 56 $JK^{-1}mol^{-1}$ going from a state without water to a state with water saturation. This change of 37 $JK^{-1}mol^{-1}$ shows that the water molecule is very tightly bound in the stereospecificity pocket. The value is actually the same as for water in gas phase or bound in a salt hydrate.[21]

The influence of water on enzymatic reactions is complex, but essential to understand. In the present investigation we have seen water acting as a lubricant, increasing enzyme activity, as a competitive and enantioselective inhibitor, and as a competitive substrate. These effects, and maybe other, contribute to a complex and yet not understood scenery. The data shown in the

present investigation suggest that the impact of water activity on reaction systems, goes far beyond solubility issues.

# EXPERIMENTAL

### *Enzyme and chemicals*

*Candida antarctica* lipase B was produced and lyophilized in house.[22] All substrates were of the highest purity (99% minimum) and checked by gas chromatography before use. Chemicals were dried by distillation under argon prior to use. Substrates were purified and stored under argon atmosphere and over molecular sieves. (*R, S*)-2-Pentyl propanoate was synthesized from 2-pentanol and propanoic acid in the presence of *p*-toluene sulfonic acid.

### *Adsorption of lipase onto a solid support*

Enzyme adsorption was performed onto chromosorb P AW DMCS; (mesh 60-80, Acid Washed DiMethylChloroSilanized, Varian, France). In a typical adsorption procedure for solid-gas catalysis, wild-type enzyme (0.58 mg) was dissolved in sodium phosphate buffer (0.687 ml, *pH* 7.5, 10 mM), and dry chromosorb P AW DMCS (686 mg) was added to the solution. After vigorous shaking, the preparation was left one week under vacuum and over $P_2O_5$ at room temperature.

### *Experimental setup for solid-gas catalysis*

The bioreactor used in this study has already been described in a previous publication.[8] The packed bed bioreactor itself was composed of a glass tube (length 9 cm, 6 mm o.d., 4 mm i.d.) in which a known mass of the enzymatic preparation was packed between two glass wool layers. Substrate or solvent feeding was done by passing dried nitrogen, as carrier gas, through the substrate/solvent flasks. Substrates and solvent were continuously flown through the bioreactor, reacting with the lipase. Thermodynamic activities of the compounds in the

reactor were defined as the ratio of their partial pressure in gas entering the bioreactor to their saturation pressure at the working temperature. The desired thermodynamic activities were obtained by adjusting the volumetric flows of the carrier gas in the different lines at appropriate values, according to the calculations explained previously.[8] Calculations were performed considering that the gas was close to an ideal one. Then fugacity was not taken into account. The gas leaving the bioreactor was injected into a gas chromatograph for analysis. Acquisition and control of parameters (volumetric flows, molar flows, substrate and solvent thermodynamic activities, partial pressures, temperatures) were monitored on-line.

Experiments were run from 308 K to 363 K, with immobilized enzyme preparation (10 mg) diluted (8 times) with dried chromosorb P in order to obtain initial rates conditions. It was checked that the addition of dried support to the adsorbed enzyme had no influence on the enantioselectivity. The total flow passing through the reactor was set at 1.15 mmol $min^{-1}$.

***Chromatographic assays***

For the solid/gas system analyses, the vapour phase leaving the bioreactor was sampled using a loop (0.25 ml) on a six-way valve (Valco) maintained at 175°C. Samples were automatically injected in the split injector of a gas chromatograph (Agilent model 6890N Series) equipped with a flame ionization detector (FID) for detection of all products. The column used was a Chirasil-Dex CB composed of β-cyclodextrin (25 m x 0.25 mm i.d. x 0.25 μm film thickness, Chrompack, France). The injector and the detector were kept at 250°C. The column temperature was programmed to hold 30 min at 50°C. Nitrogen was used as carrier gas with a constant flow (1.8 mL $min^{-1}$). The split flow was 40 mL $min^{-1}$. Hydrogen and air were supplied to the FID (35 and 350 mL $min^{-1}$, respectively). Quantitative data were obtained after integration on an Agilent 3396 Series III integrator.

The external calibration of the two substrates (2-pentanol and methyl propanoate) was carried out by programming a range of their partial pressures in the bioreactor and by analyzing with the gas chromatograph. For the products (methanol and 2-pentyl propanoate), an internal calibration was carried out by using the 2-pentanol and the methyl propanoate as internal standards.

For accurate determination of E-values the vapour phase leaving the bioreactor was condensed for a period of time depending on the molar flows applied, and were then partially evaporated in order to enhance the detection and quantification of 2-pentyl esters. Then unreacted methyl propanoate (boiling point 78-79°C) was eliminated. This enrichment of reaction product enabled accurate measurements of the enantiomeric ratio.[15] Elution peaks of (*R*)-2-pentanol and (*S*)-2-pentanol were identified using pure commercial (*R*)-2-pentanol. The absolute configuration of the reaction products was established by esterification of commercial pure alcohol enantiomers.

***Determination of the enantiomeric ratio***

The enantiomeric ratio, $E$, Chen et al.[23], was calculated from $ee_s$ and $ee_p$.[24] The $E$-values were based on the average of three measurements for each condition; with a standard deviation was less than 10% in all cases. The equations $RT\ln E = -\Delta_{R\text{-}S}\Delta G^{\ddagger}$ and $\Delta_{R\text{-}S}\Delta G^{\ddagger} = \Delta_{R\text{-}S}\Delta H^{\ddagger} - T\Delta_{R\text{-}S}\Delta S^{\ddagger}$ were used to calculate enthalpic and entropic components of the enantiomeric ratio $E$.[25,26] Methyl propanoate and 2-pentanol thermodynamic activities were fixed at 0.1 and 0.05 in the solid/gas reactor respectively.

## Molecular modelling

All modelling was done on a SGI fuel station (Silicon Graphics Inc) using the Sybyl 7.0[27] package (Tripos Inc) and the SURFNET[28] program. For the dynamics simulations, the Kollman All Atom[29,30] force field was used. The study of cavities was done using

SURFNET, where gap spheres between 0.5 and 4 Å were used. A crystal structure of the empty enzyme, PDB code 1TCA,[31] was retrieved from RCSB data bank[32] and prepared for further modelling as previously described.[33]. The modelling was based on earlier work and the knowledge of binding modes of the enantiomeric substrates was taken from that.[19,20]

For each enantiomer, the tetrahedral intermediate form of the substrate was manually modelled into the active site. The manually found position was optimized by a dynamics simulation (1200 fs) on the alcohol and acyl part of the tetrahedral intermediate, whereafter the whole structure was minimized. Finally a dynamics simulation (100 ps) was run on the whole structure. The last structure from the dynamics simulation was minimized and used for SURFNET [28] calculations.

For each enantiomer a water molecule was manually fitted into the stereoselectivity pocket of the CALB structure containing the bound tetrahedral intermediate. The position of the water molecule was optimized by repeated short dynamics simulations and minimizations with the rest of the structure kept rigid. The structure generated after the last minimizations was used as a starting point for a dynamics simulation (200 ps) started with a warm-up phase (30 ps). In the long simulation the whole structure was free to move. The last structure from each dynamics simulation was then minimized.

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## REFERENCES


[1] A. Jönsson, E. Wehtje, P. Adlercreutz, B. Mattiasson, *Biochim. Biophys. Acta* **1999,** *1430*, 313-322.

[2] M. Persson, D. Costes, E. Wehtje, P. Adlercreutz, *Enzyme Microb. Technol.* **2002**, *30*, 916-923.

[3] A. Ducret, M. Trani, R. Lortie, *Enzyme Microb. Technol.* **1998**, *22*, 212-216.

[4] C. Orrenius, T. Norin, K. Hult, G. Carrea, *Tetrahedron-Asymmetry* **1995**, *6*, 3023-3030.

[5] P. Pepin, R. Lortie, *Biotechnol. Bioeng.* **1999,** *63*, 502-505.

[6] R. Bovara, G. Carrea, G. Ottolina, S. Riva, *Biotechnol. Lett.* **1993**, *15*, 937-942.

[7] E. Wehtje, D. Costes, P. Adlercreutz, *J. Mol. Catal. B: Enzym.* **1997**, *3*, 221-230.

[8] S. Lamare, M. D. Legoy, *Biotechnol. Bioeng.* **1995**, *45*, 387-397.

[9] J.W. Barton, E. K. Reed, B. H. Davidson, *Biotechnol. Tech.* **1997,** *11,* 747-750 .

[10] M. P. Bousquet-Dubouch, M. Graber, N. Sousa, S. Lamare, M. D. Legoy, *Biochim. Biophys. Acta*, **2001**, *1550*, 90-99.

[11] P.A. Cameron, B. H. Davison, P. D. Frymer, J. W. Barton *Biotechnol. Bioeng.* **2002**, *78,* 251-256.

[12] T. Debeche, C. Marmet, L. Kiwi-Minsker, A. Renken, M.-A. Juillerat, *Enzyme Microb. Technol.* **2005**, *36*, 911-916.

[13] M. Graber, M. P. Bousquet-Dubouch, S. Lamare, M. D. Legoy, *Biochim. Biophys. Acta, Proteins Proteomics* **2003**, *1648*, 24-32.

[14] M. Graber, M. P. Bousquet-Dubouch, N. Sousa, S. Lamare, M. D. Legoy, *Biochim. Biophys. Acta, Proteins Proteomics* **2003**, *1645*, 56-62.

[15] V. Leonard, S. Lamare, M. D. Legoy, M. Graber, *J. Mol. Catal. B: Enzym.* **2004**, *32*, 53-59.

[16] F. Letisse, S. Lamare, M.-D. Legoy, M. Graber, *Biochim. Biophys. Acta, Proteins Proteomics* **2003**, *1652*, 27-34.

[17] J. L. Schmitke, C. R. Wescott, A. M. Klibanov, *J. Am. Chem. Soc.* **1996**, *118*, 3360-3365.

[18] P. P. Wangikar, T. P. Graycar, D. A. Estell, D. S. Clark, J. S. Dordick, *J. Am. Chem. Soc.* **1993**, *115*, 12231-12237.

[19] F. Haeffner, T. Norin, K. Hult, *Biophys. J.* **1998**, *74*, 1251-1262.

[20] D. Rotticci, F. Haeffner, C. Orrenius, T. Norin, K. Hult, *J. Mol. Catal. B: Enzym.* **1998**, *5*, 267-272.

[21] J. D. Dunitz, *Science* **1994**, *264*, 670.

[22] J.C. Rotticci-Mulder, M. Gustavsson, M. Holmquist, K. Hult, M. Martinelle, *Protein Expression Purif.* **2001**, *21*, 386-392.

[23] C.-S. Chen, Y. Fujimoto, G. Girdaukas, J. C. Sih, *J. Am. Chem. Soc.* **1982**, *104*, 7294-7299.

[24] J. L. L. Rakels, A. J. J. Straathof, J. J. Heijnen, *Enzyme Microb. Technol.* **1993**, *15*, 1051-1056.

[25] R. S. Phillips, *Enzyme Microb. Technol.* **1992**, *14*, 417-419.

[26] R. S. Phillips, *Trends Biotechnol.* **1996**, *14*, 13-16.

[27] Tripos Inc., 1699 South Hanley Rd., St. Louis, Missouri, 63144, USA.

[28] R. A. Laskowski, (Ed.: S. R.R), *J. Mol. Graphics* **1995,** *13*: 323-330.

[29] S. J. Weiner, P. A. Kollman, D. A. Case, U. C. Singh, C. Ghio, G. Alagona, S. J. Profeta, P. K. Weiner, *J. Am. Chem. Soc.* **1984**, *106*, 765-784.

[30] S. J. Weiner, P. A. Kollman, D. T. Nguyen, D. A. Case, *J. Comput. Chem.* **1986**, *7*, 230-252.

[31] J. Uppenberg, M. T. Hansen, S. Patkar, T. A. Jones, *Structure* **1994**, *2*, 293-308.

[32] H. M. Berman, J. Westbrook, Z. Feng, G. Gilliland, T. N. Bhat, H. Weissig, I. N. Shindyalov, P. E. Bourne, *Nucleic Acids Res.* **2000**, *28*, 235-242.

[33] S. Raza, L. Fransson, K. Hult, *Protein Sci.* **2001**, *10*, 329-338.

## Figure legends

**Figure 1**. Reaction catalyzed by immobilized *Candida antarctica* lipase B with substrates and products in gas phase.

**Figure 2.** Influence of thermodynamic water activity, $a_w$, on the alcoholytic (♦), hydrolytic (□) and total (Δ) activity for the *Candida antarctica* lipase B catalyzed acylation of 2-pentanol with methyl propanoate as acyl donor. At low water activities, up to 0.02, the alcoholytic activity increased due to water acting as a lubricant, facilitating the reaction. As the thermodynamic water activity increased, water started acting as a competitive substrate, contributing to a hydrolytic activity. In parallel to water acting as a competitive substrate, it was also acting as a competitive inhibitor causing a decrease in the total activity.

**Figure 3**. Influence of thermodynamic water activity, $a_w$, on enantioselectivity for the acylation of 2-pentanol with methyl propanoate as acyl donor. In the thermodynamic water activity interval 0 to 0.2, the enantioselectivity increased from 101 to 320. Around the thermodynamic water activity 0.2, the enantioselectivity has reached a plateau value. At higher water activities, the enantioselectivity decreased, possibly due to an increased flexibility of the enzyme.

**Figure 4.** Linear regression of ln$E$ versus $T^{-1}$ at different water activities used for obtaining the thermodynamic components $\Delta\Delta H^{\ddagger}$ and $\Delta\Delta S^{\ddagger}$ of the enantioselective *Candida antarctica* lipase B catalyzed acylation of 2-pentanol using methyl propanoate as acyl donor. The data obtained are shown in Table 1.

**Figure 5A-B.** Stick models of the tetrahedral intermediates of (*R*)- (fig 4A) and (*S*)-2-pentyl propanoate (fig 4B surrounded by space fill amino acids important for catalysis and selectivity. The surrounding amino acids consist of the catalytic triad, (Ser105, Asp187 and His224, shown in red), the oxyanion hole (Thr40 and Gln106, shown in brown), the specificity pocket (Thr42 and Ser47, shown in blue), and the active site space limiter 'the floor' (Trp104 shown in green). Catalytically important hydrogen bonds are marked with yellow dotted lines. In the stereospecificity pocket, the available space was much larger for

the alcohol of the (*R*)-enantiomer than for the (*S*)-enantiomer, for which a propyl group is pointing directly at the surface Trp104 (in green). This difference is the origin of enantioselectivity.

**Figure 6A-B.** A stick model of the (*R*)- (fig. 5A)- and (*S*)- tetrahedral intermediates (fig. 5B) of 2-pentyl propanoate together with a water molecule introduced into the stereospecificity pocket. The surrounding amino acids shown consist of the catalytic triad, (Ser105, Asp187 and His224, shown in red), the oxyanion hole (Thr40 and Gln106, shown in brown), the specificity pocket (Thr42 and Ser47, shown in blue), and the active site space limiter 'the floor' (Trp104 shown in green). A schematic drawing of the tetrahedral intermediate complex is shown as an inset. The water molecule in the (*R*)-intermediate structure had five hydrogen bonds to the enzyme, whereas the water molecule in the (*S*)-intermediate structure only had three. This indicated a better fit for the water molecule in the (*R*)-intermediate structure. The orange arrows indicate the distance between Thr 40 and Thr 42. This distance was significantly longer for the (*S*)- enantiomer than for the (*R*)- enantiomer, which indicated that an unfavourable enzyme conformational change would be necessary for the (*S*)- enantiomer to react.

**Table 1:** Thermodynamic components of the enantiomeric ratio, $E$, for *Candida antarctica* lipase B transesterification of 2-pentanol with methyl propanoate at different thermodynamic water activities, $a_w$.

| $a_w$ | $E$ at 318 K[a] | $\Delta_{R\text{-}S}\Delta G^{\ddagger}$ at 318 K (kJ mol$^{-1}$)[b] | $\Delta_{R\text{-}S}\Delta H^{\ddagger}$ (kJ mol$^{-1}$)[c] | $-T\,\Delta_{R\text{-}S}\Delta S^{\ddagger}$ at 318 K (kJ mol$^{-1}$)[d] | $\Delta_{R\text{-}S}\Delta S^{\ddagger}$ (J mol$^{-1}$ K$^{-1}$)[c] | $T_R$ (K) | $N$[e] |
|---|---|---|---|---|---|---|---|
| 0 | 101±5 | -12.2±0.1 | -18±1 | 6±1 | -19±4 | 953 | 18 |
| 0.1 | 318±75 | -15.2±0.7 | -30±1 | 15±1 | -48±3 | 627 | 18 |
| 0.3 | 309±60 | -15.2±0.6 | -33±1 | 18±1 | -56±4 | 595 | 17 |
| 0.5 | 194±35 | -13.9±0.5 | -41±1 | 27±1 | -86±3 | 481 | 11 |

[a] Standard deviations were calculated with 3 values of $E$ and correspond to 95% confidence intervals.

[b] Values calculated with: $-RT\ln E$. Standard deviations were obtained from standard deviations of $E$.

[c] $\Delta_{R\text{-}S}\Delta H^{\ddagger}$ and $\Delta_{R\text{-}S}\Delta S^{\ddagger}$ were calculated using the least square method, using between 11 and 18 measurements of $E$ as a function of $T$. Standard deviations correspond to 99% confidence intervals.

[d] $T\Delta_{R\text{-}S}\Delta S^{\ddagger}$ values were calculated at 318 K. Standard deviations were obtained from standard deviations of $\Delta_{R\text{-}S}\Delta S^{\ddagger}$.

[e] Total number of $E$ measurements.

**Figure 1**

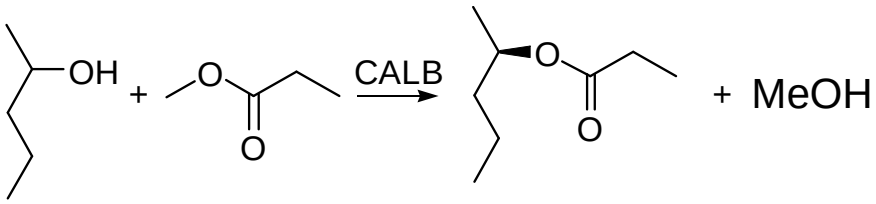


**Figure 2**

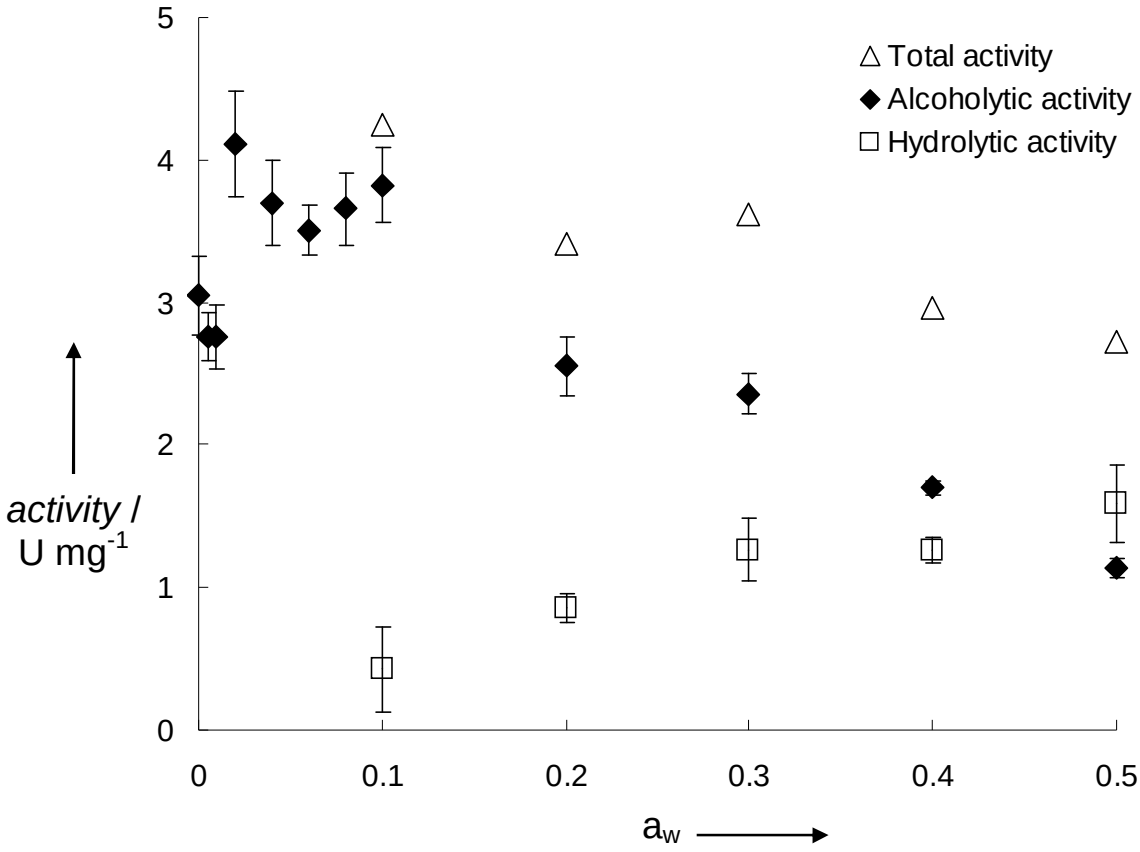



**Figure 3**

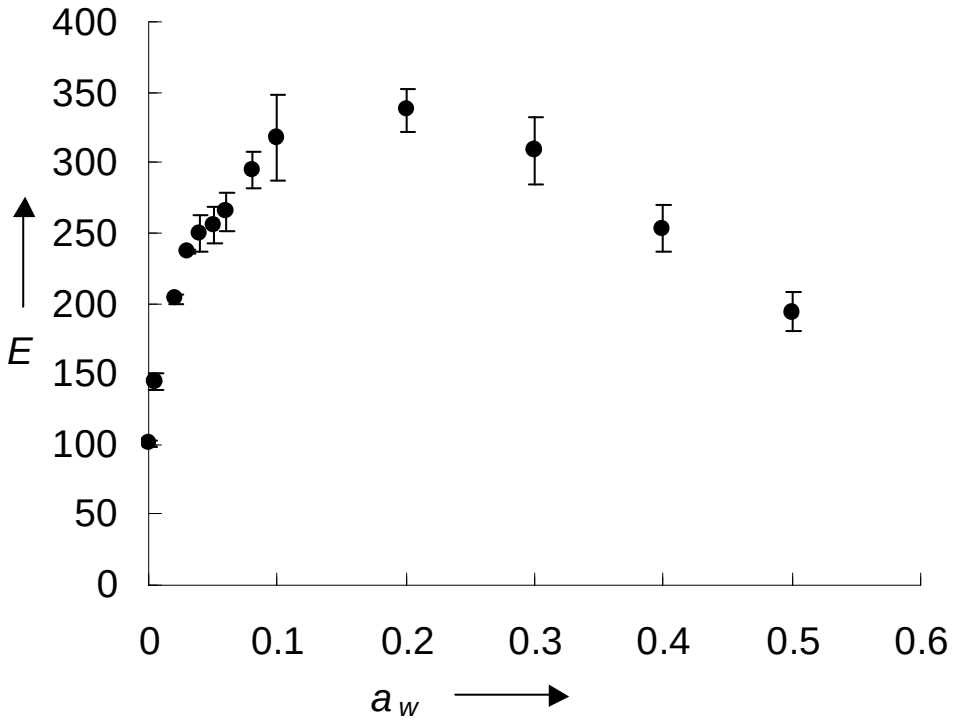


**Figure 4**

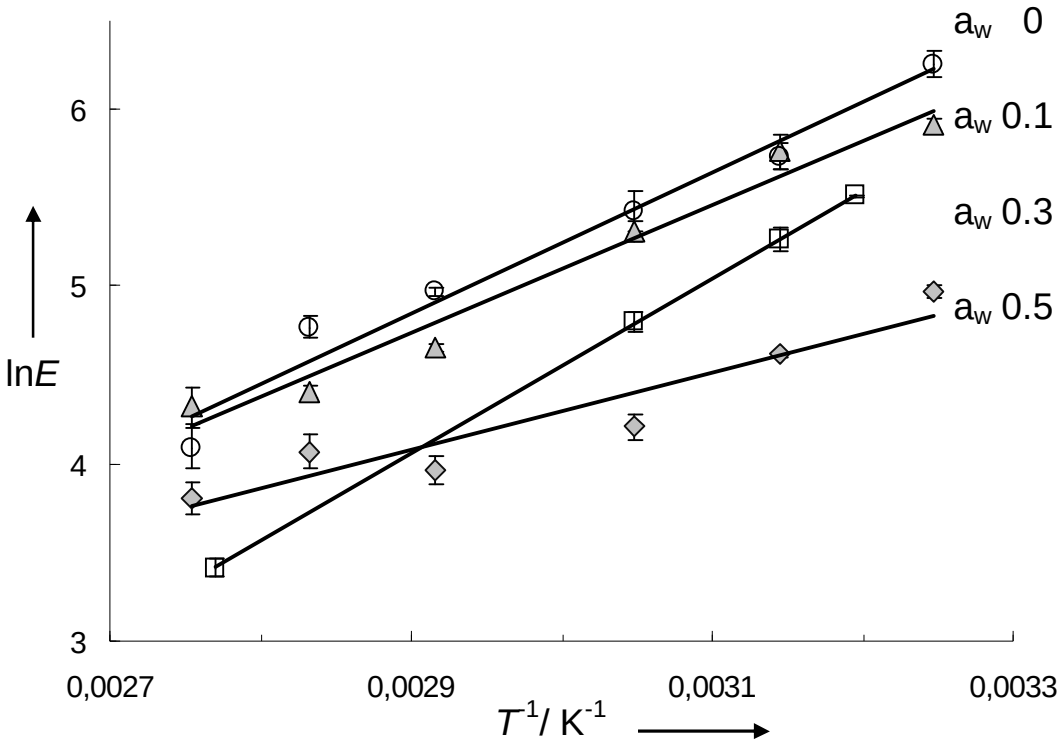


**Figure 5**

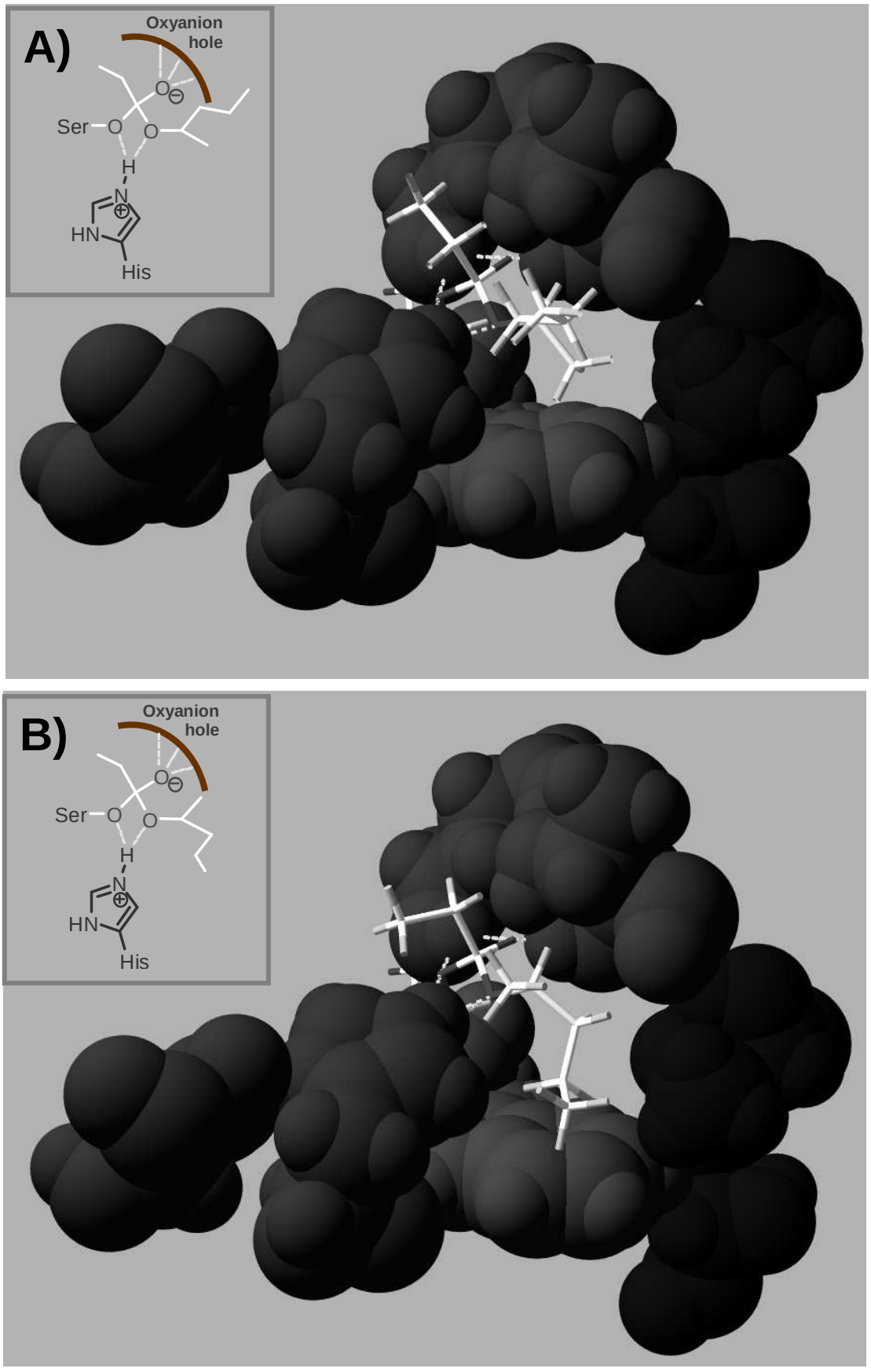
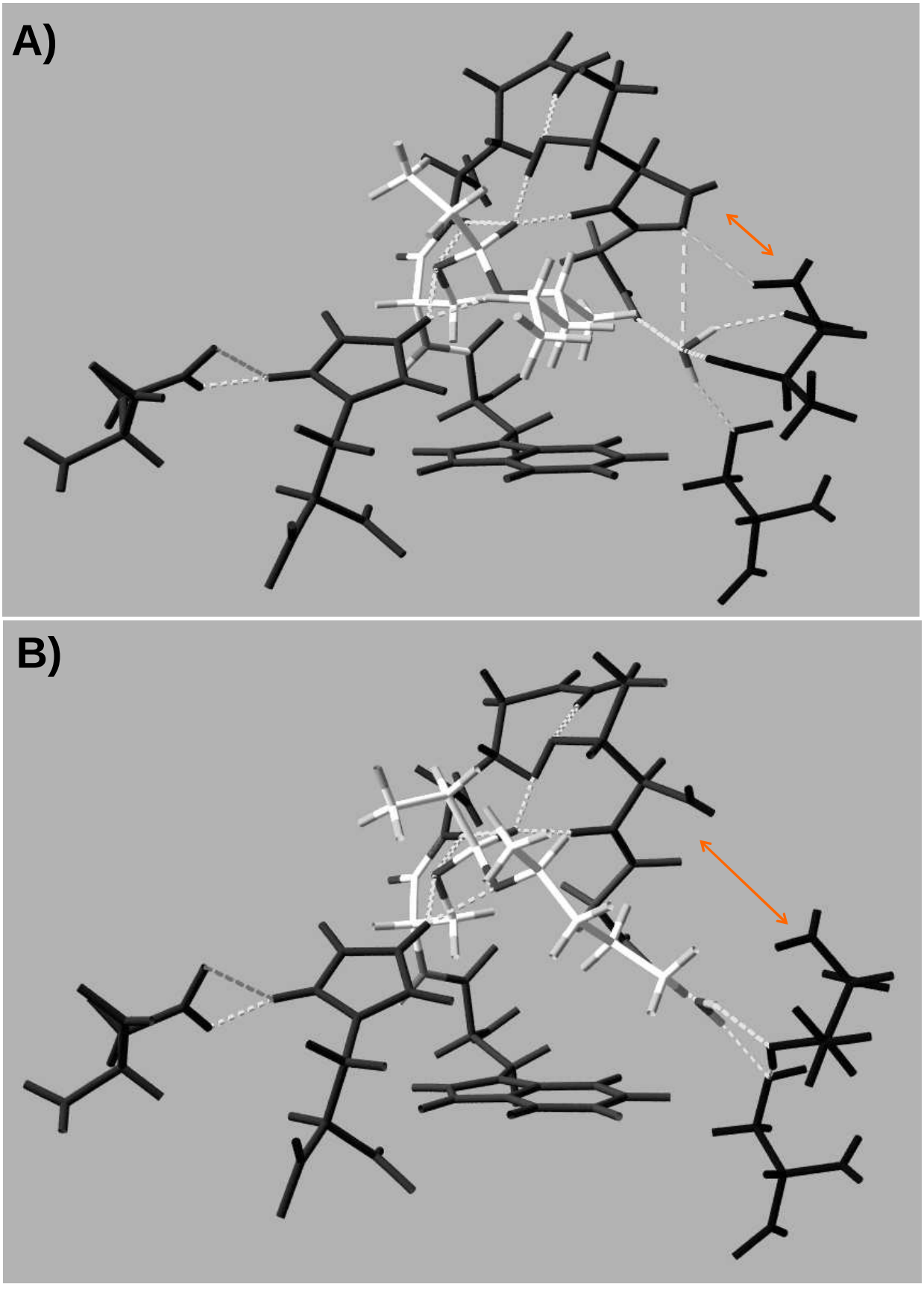
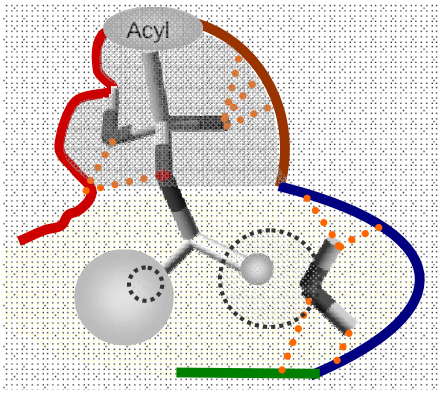


**Figure 6**



# Table of contents

A water molecule bound in the stereospecificity pocket of lipase B from *Candida antarctica* causes an enantioselective inhibition in the acylation of 2-pentanol, increasing the enantioselectivity three fold. The bound water molecule loses all its rotational freedom equivalent to 37 J $mol^{-1}$ $K^{-1}$.





## Keywords

Enantioselectivity, enzyme catalysis, solid/gas biocatalysis, hydrolase, thermodynamic water activity