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KEY WORDS

Lipase B from *Candida antarctica* – alcoholysis – water activity– activation thermodynamic parameters – enzyme solvation

Summary

The effect of water on the alcoholysis of methyl propionate and n-propanol catalyzed by immobilized *Candida antarctica* lipase B (CALB) has been compared in a continuous solid/gas reactor and in an organic liquid medium. The enthalpic and entropic contributions of water to the Gibbs free energy of activation in the gas phase were different from the ones in the organic phase, the inverse trends being observed for the variation of both ΔH^* and ΔS^* with water activity.

Different phenomena were identified for their influence on the thermodynamic parameters. When increasing a_W, the enhanced flexibility of the enzyme was predominant in the gas phase whereas enzyme-solvent interactions due to an increased polarity of the solvent affected mainly the thermodynamic parameters in the organic phase. The observed variations of ΔG^* with water activity were in accordance with kinetics results previously obtained in both reaction media.

Introduction

Biocatalysis in continuous gas phase reactor is emerging as a new technology, offering numerous advantages for organic gas bioremediation [1] and modification or conversion of compounds generated as vapor [2]. The use of this system in which a solid packed enzymatic sample is percolated by a carrier gas, which simultaneously carries gaseous substrate to the enzyme and removes reaction products, is also a powerful tool for studying the influence of enzyme microenvironment on catalysis [3]. Indeed, by varying the partial pressure of each compound in the carrier gas, it is possible to control its thermodynamic activity around the enzyme. This parameter which measures the availability of the species for the biocatalyst, has been recognized as the key parameter for studying enzymes in non-conventional media [4]. It enables an easier comparison of enzyme activity in organic solvent and gaseous environment to be achieved, as substrate-solvent interactions are taken into account [5,6]. Indeed, depending on their polarity, solvents show different ability to solvate the substrates, and this influences the thermodynamic activity of the substrates and thereby the measured enzyme activity [7].

In this paper, immobilised lipase B from *Candida antarctica* (CALB) was used as biocatalyst for the alcoholysis of methyl propionate and 1-propanol. Its crystal structure has been determined and its biochemical and catalytic properties have been well described [8,9]. Previous paper provided a detailed kinetic investigation of the alcoholysis of methyl propionate and propanol catalyzed by CALB both in the solid/gas system and in organic media [3]. It was shown that an increase of water activity in the medium is associated with a decrease of enzyme activity, but it is still necessary to accomplish an investigation on how the solvent of the reaction medium affects catalyst performance. To elucidate this point, a determination of thermodynamic parameters of the reaction was carried out both in an organic liquid medium and in a solid/gas reactor, where no solvent is added. Indeed in

this last case, the microenvironment of the enzyme is solely composed of substrates and products molecules plus water when added. No attemps have been previously made to generate such comparisons.

The goal of this study was also to provide precious information about the role played by water, in particular its enthalpic and entropic contribution to the Gibbs free energy of activation, as well as a thermodynamic explanation of the relationship between water activity and catalytic efficiency of CALB.

Materials and Methods

Enzyme and chemicals

Chirazyme L2 (Type B lipase from *Candida antarctica* lyophilised) was a gift from Roche Industrie (Penzberg, Germany). It was adsorbed onto Chromosorb P AW DMCS (Acid Washed DiMethylChloroSilanized), mesh 60-80 (Prolabo, France) as explained in a previous paper [3]. It was checked that no desorption of the enzyme occured, when the enzyme was placed in 2-methyl-2-butanol over three days. The preparation was dried over P_2O_5. The dry Chromosorb loaded with enzyme was then stored at 4°C over silica gel.

All the substrates were of the highest purity (99% minimum) and checked by gas chromatography before use. Chemicals were dried over molecular sieves and filtered just before use. Pure water was obtained via a Milli Q system (Millipore, France).

Experimental setup for solid-gas catalysis

The bioreactor used in this study has already been described in a previous publication [2]. The packed bed bioreactor itself was composed of a 9 cm long glass tube (6.5 mm O.D., 3.5 mm I.D.) in which the enzymatic preparation was packed between two glass wool layers. Substrate feeding was done by passing dried nitrogen, as carrier gas, through the

substrate flasks. Substrates were continuously flowed through the bioreactor, reacting with the lipase. Thermodynamic activities of these compounds in the reactor are 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 [2]. Calculations were performed considering that the gas was close to an ideal one. The 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 water thermodynamic activities, partial pressures, temperatures) were monitored on-line using an IBM personal computer.

A typical experiment was run, with 5 to 15 mg of adsorbed enzyme, 1 mg of adsorbed enzyme preparation corresponding to 3.32 µg of lyophilised lipase. The total flow passing through the reactor was set at 1 $mmol.min^{-1}$ giving a volumetric flow of 28.5 $mL.min^{-1}$. In these conditions, less than 5% of the substrates were converted allowing thus to assume that initial rates (expressed in µmoles of product formed per minute and per mg of immobilized enzyme preparation) were measured and that inhibition by the products did not occur. Mass balances were checked at the end of each experiment.

Experimental setup for organic phase synthesis

Alcoholysis reactions run in organic media were carried out using 15 mg of adsorbed enzyme and 4 ml of a solution containing water, methyl propionate, propanol and 2-methyl-2-butanol at specified thermodynamic activities. All components were previously dried over molecular sieves overnight. The concentrations for each component were calculated from molar fractions obtained using the UNIFAC group method [10] and they

corresponded to specified thermodynamic activities. Such calculations were performed for each experimental point of the curves displayed. The mixture was incubated at 70°C for 20 min. under magnetic stirring. Samples were taken at intervals. After a 9-fold dilution in a mixture of 2-methyl-2-butanol and methyl butyrate, the biocatalyst was removed by centrifugation and the samples were analyzed by gas chromatography.

Chromatographic assays

For the solid/gas system analyses, the vapor phase leaving the bioreactor was sampled using a 0.25 mL loop on a six-way valve (Valco) maintained at 150°C. Samples were automatically injected in the split injector of a gas chromatograph (Hewlett Packard model 5890 A) equipped with a Flame Ionization Detector (FID) for detection of all products. The column used was a CP sil 19-CB fused silica capillary column (25 m x 0.32 mm i.d. x 1.2 µm film thickness, Chrompack, France). The split ratio was 150:1. The injector and the detector were kept at 220°C and 250°C respectively. The column temperature was held at 60°C for 1 min., then programmed to increase at 5°C.min^{-1} to 100°C. Carrier gas was nitrogen and the flow rate in the column was 2 mL.min^{-1}. Hydrogen and air were supplied to the FID at 39 and 375 mL.min^{-1} respectively. Quantitative data were obtained after integration on a HP 3396A integrator. An external standard method was used for the calculations.

For the analyses after reactions run in 2-methyl-2-butanol, a gas chromatograph with an automatic sampler (Hewlett Packard model 5890 A) equipped with a FID was used. The column was a OV 1701 (30 m x 0.32 mm i.d. x 1 µm film thickness, Chrompack, France). The split ratio was 220:1. The injector and the detector were kept at 220°C and 250°C respectively. The initial temperature was 35°C and it was increased to 80°C using two different rates : 0.5 °C.min^{-1} from 35°C to 39°C and 5°C.min^{-1} from 39°C to 80°C.

Carrier gas was hydrogen and the flow rate in the column was 0.9 mL.min^{-1}. Hydrogen and air were supplied to the FID at 24 and 364 mL.min^{-1} respectively. Quantitative data were obtained after integration on a HP 3396A integrator. Methyl butyrate was used as internal standard.

Determination of thermodynamic activation parameters

Thermodynamic activation parameters based on the transition state theory as described by Eyring [11] were determined. Measurements of initial rates of reaction at various temperatures were used to calculate the energy of activation (E_a) of the reaction from the slope ($-E_a/R$) of an Arrhenius plot. This graph is normally obtained by plotting ln Vmax versus 1/T [12]. In the present study sufficiently high methyl propionate activity to reach Vmax could not be used. For this reason it was checked that the experimental values of E_a obtained in the gas/solid reactor were identical for two different methyl propionate activities (0.15 and 0.3). This point was also checked in the organic phase, with methyl propionate activities of 0.5 and 0.8. The highest values of methyl propionate activities were finally chosen for the experiments.

It is well known that transition state theory is very simplistic to describe a complex phenomenon such as an enzymatic reaction and contains several simplifications. A way to attenuate the errors inherent to this theory is to take into account the variation of the value of the thermodynamic parameters, using the same substrate in identical conditions. Therefore in the present study, $\Delta\Delta G^*$, $\Delta\Delta H^*$ and $\Delta\Delta S^*$ (equal to the differences of the thermodynamic parameters obtained between a_w equal to 0 and a_w values of 0.1, 0.3 or 0.5, $\Delta\Delta X^* = \Delta X^* (a_w=0) - \Delta X^* (a_w>0)$ with X = H, S or G) were determined by plotting the activation factor defined as the logarithm of the ratio of initial rate of reaction at a_w =0 to the initial rate of reaction at a_w =0.1, 0.3 or 0.5 versus 1/T. The slope of this plot is equal to

$-\frac{\Delta\Delta H^*}{R}$ and the intercept with Y-axis gives $\frac{\Delta\Delta S^*}{R}$. The temperature range was 45°C to 65°C. It was experimentally impossible to broaden this interval; indeed above 65°C enzyme stability is not sufficient at high a_w [3] and below 45°C, the control of temperature was not accurate enough.

Results and Discussion

Figure 1 shows the Arrhenius plots obtained for different water activities in the gas/solid reactor. The calculated activation energies are displayed in Table 1.

The temperature dependence of the activation factor is shown in figure 2. As explained in the experimental part, this plot enables ΔΔH*, ΔΔS* and ΔΔG* (differences of activation thermodynamic parameters in absence and presence of water) to be calculated (Table 1).

Experimental results obtained in the organic medium were processed by the same way (figures not shown) and the data obtained are summarized in Table 1.

Concerning the enthalpic part of the Gibbs energy of activation, it was found that ΔH* decreases sharply when a_w increases (ΔΔH*>0) in the solid/gas reactor. On the contrary, ΔH* slightly increases with a_w in the organic liquid medium (ΔΔH*<0). To explain these trends, it is important to dissociate the energetic level of the ground state ES complex and the one of the transition state ES*, which both can be influenced when changing water activity. As far as the reaction in the organic liquid medium is concerned, the polarity of the reaction medium increases with the water activity which induces a better solvation of the substrates and which modifies interactions between the enzyme and the solvent (2-methyl 2-butanol plus water). This moderately polar tertiary alcohol (log*P*=0.89, [13]) is likely to interact with the protein and to solvate the reactants. Indeed it was shown that organic solvents compete with water to adsorb on proteins : the most polar solvents (like n-propanol, log*P*=0.3) are able to replace water at the ionizable groups on the surface of the

protein, less polar organic solvents (like di(n-butyl)ether, log*P*=3.08) can not displace water at these sites, but can compete for moderately polar and non polar sites that are hydrated at higher water activities [14]. In the present case 2-methyl 2-butanol is thus probably able to interact with the protein, creating a kind of solvation shell. Therefore in liquid system, in order for substrate and enzyme to react, both have to get rid of their solvation shells, and new interactions have to form around the transition state.

However in the present work, the use of thermodynamic activities (instead of concentrations) is a method that has been proposed for correcting for solvation of substrates and products. Therefore for a similar value of thermodynamic activity, substrates disponibilities are similar in gaseous and liquid media. Nevertheless differences between enzyme-solvent interactions in gaseous and liquid phase still linger since it was shown that solvents interfere with the binding process between substrate and enzyme (immobilized or not) and/or with the catalytic mechanism [15]. In other studies solvent effects on the enzyme have been ascribed to an effect of the solvent on the conformational mobility of the enzyme [16], or to specific effects of the solvent on the enzyme, e.g. by binding of solvent molecules to the enzyme active site [17,18], or binding of the solvent to the hydrophobic part of the enzyme surface [19]. Van Tol *et al.* [20] proposed a competitive inhibition of the solvent by non-specific interactions of the solvent and the active site. According to Ryu and Dordick [21], organic solvents have an effect on the local polarity in the active site, accounting for varying oxidation rate of phenols catalyzed by horseradish peroxydase.

So solvent molecules that may have been lying around the active site have to be displaced before the substrate gets in.

These enzyme-solvent interactions increase with a_w .Indeed when a_w increases in the organic liquid medium, interactions between the catalyst and the solvent (2-methyl 2-

butanol plus water) are modified, as these two different molecules compete to adsorb on the surface of the protein. Polar interactions with water are increased and hydrophobic interactions between apolar sites of the protein and apolar part of 2-methyl 2-butanol are strengthened. These interactions have to be broken for the transition state to be reached. Therefore they weigh unfavorably in the activity of the enzyme, and could induce a lower energy level of the ground state and thereby an increase of the enthalpy of activation with a_w. This effect will be more important in the organic liquid medium than in the gas phase where no solvent is added.

Previous study of activity of dry Alcohol dehydrogenase from baker's yeast (YADH) demonstrated that the reaction of conversion of gaseous 3-methyl-2-buten-1-ol to 3-methyl-2-buten-1-al (with simultaneous reduction of NAD+ to NADH) had an activation energy of 7.47 kcal/mol, which was independent on the hydration level of the enzyme [22]. On the contrary, Barzana and co-workers [23] noted that Ea for alcohol-oxidase-catalyzed ethanol oxidation in gaseous phase was a function of water activity. As in the present study, these authors showed that Ea decreased when water activity increased, approaching the value found for Ea in aqueous solution (5.5 kcal/mol). The variations in Ea were attributed to the rigidity of the enzyme at low hydration levels.

A more recent study [24] about reduction of ketones catalyzed by alcohol dehydrogenase from *Thermoanaerobium brockii* in different organic liquid media revealed that an increase of a_w led to an increase of ΔH^*. According to these authors, the higher solvation of the substrate in the liquid medium as a_w increases (which tends to increase ΔH^*) is predominant over a favorable effect coming from an enhanced flexibility of the enzyme as a_w increases. This last effect involves a larger number of possible interactions between the enzyme and the substrate, when the hydration level of the enzyme increases, leading to a stabilization of the transition state and thereby to a lowering of the activation energy

(which means that ΔH* decreases). They finally conclude that the overall effect of increasing a_w is that the ΔH* increases.

However, in the solid/gas system, the unfavorable solvation of the substrate by the solvent can be neglected whereas the increased flexibility of the enzyme at high a_w can be considered as a predominant effect. Indeed, no solvent is present to solvate them. As a consequence, the overall effect of increasing a_w is that ΔH* decreases.

When comparing energies of activation in the gas phase and in liquid organic medium, it appears that in the range of a_w studied (0 to 0.5), the energies of activation in the organic liquid phase are in the range of 29-37 kJ/mole which is similar to energies of activation in the gas phase for a_w in the range of 0.1 to 0.3. Indeed, in the gas phase, the Ea at a_w equal to 0 is higher (41 kJ/mol) while it is far lower at a_w equal to 0.5 (17.5 kJ/mol).

The high energy of activation at very low a_w in the gas phase is likely to be related to a lack of enzyme flexibility as explained before, whereas in the organic phase, methyl-2 butanol-2 can replace water in the interaction with water binding sites [25] and this would account for the lower Ea in the organic medium compared to the one in the gas phase at a_w =0.

For a_W between 0.1 and 0.3, the enzyme has probably reached its optimal conformation in the solid/gas reactor therefore the variations of energies of activation are quite smoother and stay in the range of Ea values observed in the liquid organic medium.

For an a_w increment from 0.3 to 0.5, a drop of 10 kJ/mol is observed for the energy of activation in the solid/gas system. This might not be attributed to a higher flexibility of the enzyme since this state probably corresponds to a maximal structured water state and precedes the apparition of free water [2]. A reason for the sharp decrease of Ea at a_w superior to 0.3 in the gas phase might be that gaseous substrate condensation provides a part of the energy required to reach the transition state. Barzana *et al* [23] noticed that

water facilitates the adsorption of ethanol vapors by the enzyme support (DEAE Cellulose) when conducting enzymatic oxidation of gaseous ethanol. Experiments of competitive isothermal adsorption in the presence of methyl propionate and water shall provide evidence of such phenomenon. However, the condensation heat of methyl propionate, which is the first substrate of the alcoholysis described in the present work [3], is equal to 32.2 kJ/mol which is twice higher than the difference in Ea between the liquid phase and the gaseous phase (~15.5 kJ/mol).

Concerning the entropic part of the Gibbs energy of activation, it was shown that ΔS^* decreases when a_w increases ($\Delta\Delta S^*>0$), in the solid/gas reactor.

The activation entropy ΔS^* of an enzyme-catalyzed reaction may be found to be either positive or negative [26]. A negative ΔS^* is the most common case, as the reaction between E and S to form the ES* complex frequently involves a considerable loss of entropy, due to the loss of the reactant and catalytic groups translational and rotational degrees of freedom. Nevertheless a positive ΔS^* may also occur, especially when molecules of water are displaced by the substrate during activation, leading to a more disordered system.

Whatever the sign of ΔS^* may be, the increased flexibility of enzyme at water activities equal to 0.3 or 0.5 is probably the factor explaining the observed positive $\Delta\Delta S^*$ in the solid/gas system. Indeed as a consequence of the presence of more water, the ground state enzyme-substrate complex ES occupies a broader distribution of conformational states which involves an increased entropy of this state, compared to that of the enzyme with less water.

A graphical representation for the possible origin of positive $\Delta\Delta S^*$ is shown in figure 3. The same explanation was proposed by Lonhienne *et al.* [26] for reactions involving psychrophilic and mesophilic enzymes.

On the contrary, for the reaction in organic medium, the inverse effect is observed: ΔS^* increases when a_w increases ($\Delta\Delta S^*<0$). When water is added, the precedent effect of increased flexibility of the ground state ES complex is likely to be much less pronounced, as the 2-methyl 2-butanol partially plays this role, as explained before. The trend observed in the organic medium may be due to a decreased entropy level of the ground state ES complex when the water activity is enhanced. Indeed polarity of the solvent increases with water activity, leading to a strengthening of hydrophobic interactions between the first substrate (methyl propionate) and the solvent (2-methyl 2-butanol). This hypothesis seems to be in accordance with the results for variations of ΔS^* in the liquid medium which tend to be smaller than in the gas phase reactor (Table 1).

As far as the overall free energy, ΔG^*, is concerned, it was shown for the gas/solid reaction that $T\Delta\Delta S^*$ is superior to $\Delta\Delta H^*$ whatever the temperature in the range studied (45 to 65°C) (Table 1). Consequently, $\Delta\Delta G^*$ is inferior to 0 and the reaction is thus favored when a_w decreases. In the range of a_w studied, the advantageous entropic part of the Gibbs energy is predominant over the unfavorable enthalpic part. This is in accordance with previous results, which show that the initial velocity decreases when a_w increases [3].

In organic medium, $\Delta\Delta H^*$ is negative and superior to positive ($- T\Delta\Delta S^*$), between 45 and 65°C and when a_w decreases. As explained before, the Gibbs free energy of activation therefore decreases when decreasing the water content, which can be related to a decrease of enzyme activity as a_w increases. In this case, the enthalpy of activation is the dominating factor.

Conclusions

This study demonstrates the real interest to compare the variations with water of thermodynamic activation parameters in the gas/solid reactor and in more frequently used

liquid organic phase, so that understanding of enzyme mechanism in the gas phase can be improved.

It appears that ΔH^* and ΔS^* variations with a_w are opposite in the two media. Upon increasing a_w, the effects involved in the gas phase was a decrease of the reaction energetic barrier and an increase of enzyme flexibility (which means that the ground state ES complex occupies a broader distribution of conformational states). In the organic medium, the predominant phenomenon affecting thermodynamic parameters was an increased polarity of the solvent at high a_w, leading to an increase of the reaction energy of activation and to a decrease entropy level of the ground state ES complex.

Whatever the medium, $\Delta\Delta G^*$ is negative which is in accordance with previous results showing that the initial velocity decreases when a_w increases [3]. Nevertheless in the gas/solid reactor, the predominant contribution to $\Delta\Delta G^*$ is the entropic part whereas it is the enthalpic part in the liquid organic medium.

Experimental studies are currently in progress to precise the role played by the solvent, depending on its polarity, on the enzyme flexibility and activity in the solid-gas reactor. This study will be focused on the kinetic and thermodynamic parameters of the reaction and should give further information about the mechanism of the reaction in the gas phase.

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FIGURE LEGENDS

Figure 1: Determination of the energies of activation at different a_w values in the gas-solid reactor.

Synthesis was carried out in the solid/gas bioreactor at temperatures between 45° and 65°C, at a molar flow of 1 $mmol.min^{-1}$ in the presence of adsorbed CALB onto Chromosorb AW DMCS (5 to 15 mg depending on temperature). Methyl propionate and propanol thermodynamic activities were set at 0.3 and 0.1 respectively. Water activity was varied from 0 to 0.5.

Figure 2: Determination of $\Delta\Delta H^*$ and $\Delta\Delta S^*$ at different a_w values in the gas-solid reactor.

The experimental procedure is the same as the one described in figure 1.

The activation factor is the ratio between the initial rate at a_w equal to 0 and the initial rate at a_w equal to 0.1, 0.3 or 0.5.

Figure 3: Graphical representation for the origin of the positive sign of $\Delta\Delta S^*$ in the gas/solid reactor, with $a_{w1} < a_{w2}$

The transition state intermediate ES* can be reached through a decrease ($\Delta S^* < 0$) or an increase ($\Delta S^* > 0$) of the activation entropy S*. At a_{w2}, the distribution of conformational states for ES is broader than at a_{w1} and is translated into a higher level of S*. It follows that $\Delta\Delta S^*$ is always positive.

Table 1: Thermodynamic activation parameters

Alcoholysis reactions were carried out at temperatures between 45° and 65°C.

In the solid/gas bioreactor, experimental conditions were as follows: molar flow of 1 $mmol.min^{-1}$ in the presence of adsorbed CALB onto Chromosorb AW DMCS (5 to 15 mg depending on temperature). Methyl propionate and propanol thermodynamic activities were set at 0.3 and 0.1 respectively.

Alcoholysis reactions run in organic media were carried out using 15 mg of adsorbed enzyme and 4 ml of a solution containing water, methyl propionate, propanol and 2-methyl-2-butanol. Thermodynamic activities of methyl propionate and propanol were set at constants values of 0.8 and 0.1 respectively.

In both media, water activity was varied from 0 to 0.5.

	a_w	**Ea** **(kJ/mol)** (1)	**ΔΔH*** **(kJ/mol)** (2)	**ΔΔS*** **(J/mol.K)** (2)	**T.ΔΔS*** **(kJ/mol)** (2)		**ΔΔG*** **(kJ/mol)** (2)	
					45°C	**65°C**	**45°C**	**65°C**
Solid-gas reactor	0	40.7						
	0.1	31.3	9.48	30	9.54	10.14	- 0.07	- 0.66
	0.3	28.0	12.60	42	13.36	14.20	- 0.76	- 1.60
	0.5	17.5	23.08	76	24.17	25.69	- 1.09	- 2.61
Organic medium	0	28.9						
	0.1	29.1	- 0.21	-0.3	- 0.09	- 0.10	- 0.12	- 0.12
	0.3	32.6	-3.56	-10	- 3.18	- 3.38	- 0.38	- 0.17
	0.5	36.7	-7.83	-22	- 7.00	- 7.44	- 0.82	- 0.39

$\Delta\Delta X^* = \Delta X^* (a_w=0) - \Delta X^* (a_w>0)$ with X = H, S or G

(1) determined from Fig. 1

(2) determined from Fig. 2

FIGURE 1

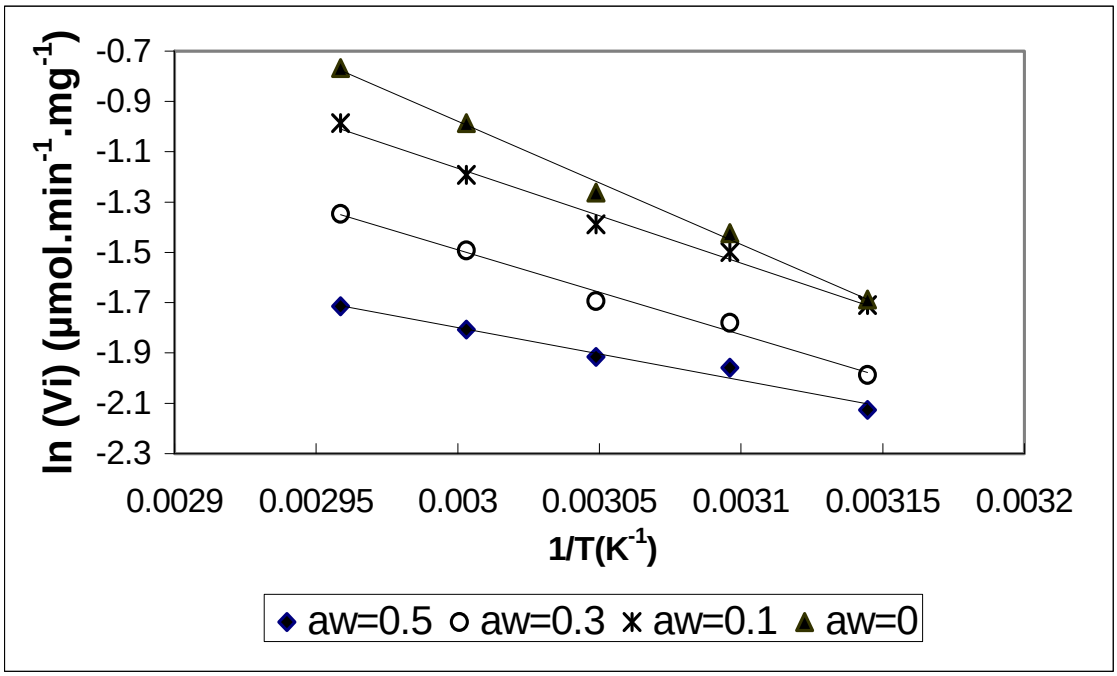

ln (Vi) (µmol.min-1.mg-1)
-0.7
-0.9
-1.1
-1.3
-1.5
-1.7
-1.9
-2.1
-2.3
0.0029
0.00295
0.003
0.00305
0.0031
0.00315
0.0032
1/T(K-1)
aw=0.5
aw=0.3
aw=0.1
aw=0

FIGURE 2

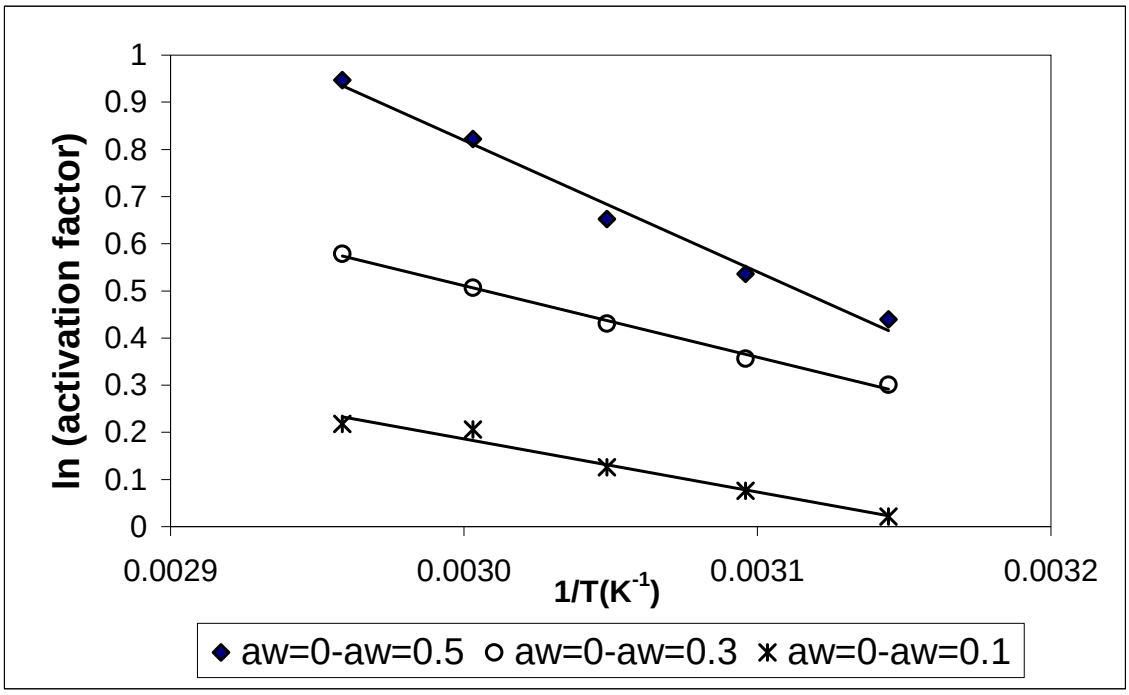
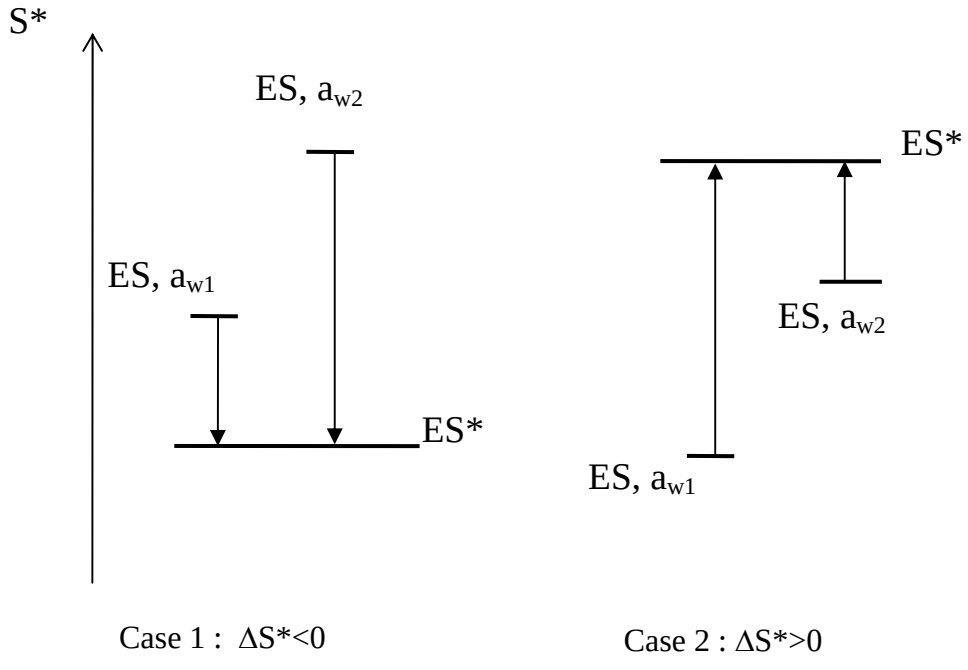

ln (activation factor)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.0029
0.0030
0.0031
0.0032
1/T(K-1)
aw=0-aw=0.5
aw=0-aw=0.3
aw=0-aw=0.1

FIGURE 3



Case 1 : $\Delta S^* < 0$

Case 2 : $\Delta S^* > 0$