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ENZYME CATALYZED SYNTHESIS OF DENDRIMER IN ORGANIC SOLVENTS: AN ENGINEERING APPROACH

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ABSTRACT

Enzyme catalyzed synthesis of zero generation (G0) polyamidoamine (PAMAM) dendrimer through Michael Addition has been studied in organic solvents. Lipase from *Candida antarctica* has been used for synthesis of G0 PAMAM dendrimer in ten different organic solvents. Initial rate of the reaction has been correlated with solvent properties such as hydrophobicity, water solubility, polarizability, dielectric constant, electron pair acceptance and donation index, etc. It is observed that there are good relationships of initial rate with solvent properties. Activity of the reaction has been correlated with initial rate and calculated the value of specific constant (K_{sp}) an intrinsic specific constant ($^{int}K_{sp}$). Solvent polarizability has been correlated with K_{sp} and $^{int}K_{sp}$ and observed a good relationship with 0.97 R^2 value. Effect of enzyme inhibition has also been observed for the reaction which is found to be uncompetitive in nature. The values of kinetic parameters are calculated for the reaction and reported. The enzyme remains its activity up to second cycle.

Keywords: *Candida antactica*, dendrimer, solvent properties, initial rate, activity.

INTRODUCTION

Synthesis of dendrimer and its derivatives has gained more attention in the past decades because of their wide applications in many fields (Gupta 2015; Smith 2006; Frank and Arno, 1997; Yoshitaka *et al*., 1988; Duan *et al*., 2008; Guobin *et al*., 2010). Generally dendrimer can be synthesized by Michael type addition reaction involving addition of primary amine to methyl acrylate (Torre *et al*., 2004). To increase the rate of the reaction with good yield, catalyst can be used in an effective way for dendrimer synthesis. In organic and bio-organic synthesis enzymes are more efficient catalyst due to their chemo-, regio- and sterio-selective properties (Klibanov and Zaks1986; Rubio *et al*., 1991; Stevenson and Storer, 1991). To our knowledge enzyme catalyzed synthesis of dendrimer is not reported elsewhere and hence we have planned this work using Lipase from *Candida antarctica* which has been selected from the reported data that it has the capability to form C-N bond (Kitazume *et al*., 1986). In enzymatic reaction solvents play an important role depending on the polarity of the solvent. The solvent selection is an important work for enzymatic reaction system. In this paper we describe a systematic study on the effect of solvents on synthesis of polyamidoamine (PAMAM) G0 dendrimer using lipase from *Candida antarctica*as enzyme catalyst as well as the activity of enzyme at different solvents. The mechanism of the reaction has also been described which gives an insight of the reaction. Different parameters have also been determined for establishing the solvent kinetics of enzymatic reaction.

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MATERIALS AND METHODS

Materials

Lipase from *Candida antarctica* was supplied by Sigma Aldrich Chemical Company, USA and stored at 4^0C. The lipase was lyophilized before use. Ethylene diamine and Methyl acrylate were purchased from Merck, Germany. All solvents used for this study were purchased from Sigma Aldrich and purified by steam distillation before use.

Methods

Kinetic experiments were carried out in a 100 mL round bottom flask by mixing the substrates and were agitated vigorously with a magnetic stirrer. The reaction was carried out using variables such as time, temperature, reactant ratio, enzyme concentration and agitation speed. After studying the detailed reaction kinetics using above variables the optimal conditions were establish as time=12 hours, temperature=45^0C, MA: EDA=1:4, [lipase]=1.5mg/mL (activity 0.5 U/mg), speed of agitation = 400 rpm. To study the solvent engineering, all reactions were carried out at optimum conditions. Experiments for

studying solvent effect, were carried out using a mixture of 2.12 mmol freshly prepared pure half generation PAMAM dendrimer (S_1) and 9 mmol of EDA in 40 mL of solvent in presence of 1.5 mg/mL lipase at room temperature. Aliquots of the samples were withdrawn at a regular interval of time and separated the catalyst by filtration with 0.2 µm filter paper fitted in HPLC filtration kit and the filtrate was analysed by UV-Vis spectrophotometer applying same conditions for storage of samples and analysis. Residual enzyme was added in the reaction mixture. The enzyme was recovered from the reaction mixture by filtration and washed with hexane and dried for further use. All samples were analysed three times repeatedly to avoid the analytical errors. Initial rate of the reaction was calculated from the percent conversion versus time profile from which initial rate of the reaction was calculated considering <10% conversion for which the plots were found to be linear. The initial rate was expressed as the concentration of substrate converted per unit time per unit weight of lipase (expressed as mmol $L^{-1}min^{-1}g^{-1}$) and calculated according to the following formula (Gogoi *et al*., 2010).

Initial rate, $r = \frac{C_i - C_t}{t\,w}$ Eq. 1

Where C_i is the initial concentration of the limiting substrate (mole), C_t is the concentration after time t (min) during which the profile are linear and w is the weight of lipase.

Theoretical aspects

The reaction between EDA and MA is a two substrate reaction which is assumed to follow the Michaelis-Menten equation and the equation becomes:

$$v = \frac{[E]_0}{\frac{1}{k_0} + \frac{1}{k_A[A]} + \frac{1}{k_B[B]} + \frac{1}{k_{AB}[A][B]}}$$ Eq. 2

If one of the substrate is kept constant and one is varied then the equation 2 becomes

$$v = \frac{k_0^{app} k_A^{app} [E]_0 [A]}{k_0^{app} + k_A^{app}[A]}$$ Eq. 3

$$k_0^{app} = \frac{k_0 k_B [B]}{k_0 + k_B [B]}$$ Eq. 4

$$k_A^{app} = \frac{k_A k_{AB} [B]}{k_A + k_{AB}[B]}$$ Eq. 5

k_0^{app} is apparent catalytic constant

k_A^{app} is apparent specificity constant for A.

From equations 4 and 5, when [B] is extrapolated to an infinite value then,

$k_0^{app} = k_0$ and $k_A^{app} = k_A$

Equation 2 can be written as

$$v = \frac{V_{max}[A][B]}{k_A k_{mB} + k_{mB}[A] + k_{mA}[B] + [A][B]}$$ Eq. 6

Where V_{max} = maximum velocity

k_{mA} = Michaelis constant for A

k_{mB} = Michaelis constant for B

Apparent Michaelis constant for A may be written as

$$k_{mA}^{app} = k_0^{app} k_A^{app} = \frac{k_{iA} k_{mB} + k_{mA}[E]}{k_{mB} + [E]}$$ Eq. 7

When B is extrapolated to infinity. k_{iA} is inhibition constant for A. Here A refers to G-0.5 PAMAM, B refers to EDA and E refers to lipase from C*andida antarctica.*

RESULTS AND DISCUSSION

Effect of solvent hydrophobicity

Log P is the partition coefficient of a molecule between an aqueous and liophilic phases usually octanol and water. The log P value of the solvents is widely used parameter to describe solvent polarity (Vermue and Tramper, 1995) and their possible effects on the enzyme activity. We studied the enzymatic Michael addition in different organic solvents with log P values ranging from /-0.74 to 2.73. The relationship between initial rate and solvent hydrophobicity (log P) is shown in Figure 1 and its mathematical representation is

r = -0.0712 log P + 0.3539,(R^2= 0.98818) Eq. 8

From Figure 1, it is seen that the initial rate of the reaction decreases with increase in solvent hydrophobicity. The highest initial rate in case of less hydrophobic methanol is probably due to preferential partitioning behaviour of the substrate between the reaction medium and the active site of the enzyme used (Klibanov, 1990). This partitioning is diminishing as the solvent hydrophobicity increases and therefore a linear free energy relationship was existing between the catalytic efficiency and solvent hydrophobicity (Klibanov and Zaks, 1986). The effect of accessibility of hydrophobic solvent to the relatively polar phase of the enzyme for the contact with the catalytic surface is also found to play an important role in the phenomenon (Gogoi *et al*., 2009). Thus, from the above discussion we can conclude that, in our study the observed variation of initial rate with solvent hydrophobicity is considered to be reasonable.

Effect of water solubility

Water solubility of a solvent is an important polarity index for correlating it with enzyme activities [(Janseen *et al*., 1993; Valivety *et al*., 1994). The relation of water solubility with initial rate of dendrimer synthesis is shown in Figure 2. From this graph it was found that the initial rate of dendrimer synthesis increases with increasing the

value of water solubility. The empirical relationship between initial rate and water solubility is-

$$r = 0.04874 \log S_w + 0.24176 \quad \text{Eq. 9}$$
$$(R^2 = 0.9664)$$

On increasing polarity of solvent the interaction between substrate and enzyme increases. Methanol, Ethanol, acetone and isopropanol are highly polar solvent and completely miscible with water and hence the initial rate of the reaction in solvents (not shown in the figure) are significantly higher. It may, therefore, be inferred that solvent with high water solubility favour the synthesis of dendrimer which are opposite observation shown in esterification reactions reported elsewhere (Hazarika *et al*., 2002). Another study, Velivety *et al*. (1994) reported a relationship between log P and log Sw which also confirms our findings. The linear relationship of initial rate of reaction and log Sw inferring the favourable effect of polar solvent for the reaction.

Effect of solvent polarizability

Solvent polarizability is an important factor to correlate between rate of enzyme catalysis reaction and substrate (Gogoi *et al*., 2009). It represents the ability of solvent to stabilize the change of a dipole in solution by virtue of its dielectric constant. The correlation between initial rate and polarizability is shown in Figure 3. The relation between polarizability and initial rate can be represented as,

$$r = -0.03156\,\delta + 0.54083 \quad \text{Eq. 10}$$
$$(R^2 = 0.96784)$$

Where, δ is polarizability. From this figure it indicates that the initial rate of reaction decreases with increasing the value of polarizability. Polarizability indicates the percentage covalent character of a compound and with increasing polarity, covalent character of a compound increases. Therefore, polar nature of solvent decreases with increasing polarizability and thus, the interaction of solvent with reactants decrease with increasing polarizabilty, as a result of which initial rate of the reaction decreases with increasing polarizability. In some cases this type of correlation is not observed due to shortage of data of polarizability however considering the available data (Hazarika *et al*., 2002; Hazarika *et al*., 2003; Valivety *et al*., 1994).

Effect of dielectric constant

Dielectric constant is a function of polarizibility; therefore an attempt was made to correlate the initial rate with dielectric constant. It is an independent parameter and the correlation with initial rate is shown in Figure 4. From the figure it is observed that the initial rate increases with increase in the values of dielectric constant of the solvent. This increasing reaction rate with dielectric constant is due to increasing interaction between solvent and reactants. The observed relationship between reaction rate and dielectric constant is-

$$r = -0.032\,\mu + 0.549 \quad \text{Eq. 11}$$
$$(R^2 = 0.9754)$$

The increase in reaction rate with increase in value of dielectric constant is due to the conformational rigidity of enzyme in anhydrous media which is due to the result of non covalent interactions, essentially for the electrostatic origin (Klibanov, 1990). The strength of these interactions is inversely proportional to the dielectric constant and is higher for water than organic solvent (Reichardt, 1988). The conformational rigidity increases the lipase activity in the reaction media and thus observed correlation between dielectric constant and initial reaction rate is reasonable.

Effect of electron donor and acceptor index

For establishing the performance of the reaction media using polarity as one of the criteria, there are also some other solvent properties like electron pair donor and acceptor number which gives information on donor acceptor interactions of the solvent including hydrogen bonding capacity. Solvation of water requires both donation and acceptance of hydrogen bond or other dipole-dipole interactions. To measure the effect of electron pair acceptance index and Gutmann's donor number we have calculated the value of summation of normalized electron pair index and Gutmann's donor number and plotted the value against the value of initial rate of reaction and the correlation is shown in Figure 5. We established a good correlation with the available data of $E_T^N + D_N^N$ and initial rate. From the figure it is seen that the curve is polynomial in nature and the initial rate of the reaction increases with increasing the value of $E_T^N + D_N^N$. It is also deduced that the hydrogen bond donation and accepting capacity of the solvent determines both water solubility and equilibrium of the reaction in that solvent (Kadish *et al*., 1989). The observed increase of initial rate with $E_T^N + D_N^N$ seems to be reasonable and may be explained from the solvation effect of G-0.5. The differential solvation which would affect the equilibrium position involves additional acceptor and donor interactions (Hazarika *et al*., 2003).

Reusability of enzyme

The enzyme was reused in three successive batches of experiments in methanol (Table 1). The activity of the enzyme was dropped almost 100% after second batch of experiments as shown in Table 2. Usually, free enzyme lost their activity after the first batch. However, in case of dendrimer synthesis the enzyme showed 50% activity after first batch. This may be due to the stability of lipase in methanol. We tasted the reusability of lipase in other solvents and confirmed the inactiveness of the lipase in other solvents. However, ethanol like methanol is quite better than other solvents reported here. Immobilized lipases show better stability than free lipases and works on some other enzymes are reported in earlier works (Gogoi

et al., 2006; Hazarika and Dutta, 2004; Ursoiu *et al*., 2012; Sheldon and Pelt, 2013; Adlercreutz, 2013). Thus our next plane is to established kinetics of immobilized lipase for some other organic synthesis.

Activity of enzyme

Lipase activity was determined considering the values of initial rate against substrate concentration of one substrate at a fixed concentration of other substrate. Figure 7 shows the reciprocal of [EDA] versus reciprocal of initial rate which gives straight line. With decreasing concentration of EDA the value of initial rate also decreases. The values of inhibition constant calculated from Eq. 6 are shown in Table 3.

The initial rate of the reaction was found to be highest in methanol and lowest in chloroform. However the nature of variation of initial rate versus activity for all the solvent systems appears to be identical as shown in the Figure 6. From the above discussion, methanol is considered as the best solvent for dendrimer synthesis as the activity of the lipase is maximum in this solvent. The reaction mechanism has been determined by a plot of reciprocal of both initial rate and EDA concentration shown in Figure 7. From the figure it is seen that an increase of EDA concentration at a constant G-0.5 concentration increases the initial rate. The observation of decrease in initial rate with increase of G-0.5 concentration indicates the G-0.5 inhibition effect (Fig. 7) which is uncompetitive in nature due to multi-substrate enzyme reaction.

The effect of solvent was assessed by evaluating $V_{max}$and K_m values and plotting against log P as shown in the Figure 8a, 8b. V_{max} and K_m values were calculated from Line weaver-Burke Plot using the equation

$1/V_0 = (Km/Vmax) (1/[S]+1/Vmax$ Eq. 12

The apparent kinetic parameters for one substrate depend on the thermodynamic activity of the other substrate. In support of this, specificity constant K_{sp} of lipase is calculated by the equation $K_{sp} = V_{max}/ K_m$ and $^{int}K_{sp}$is calculated bythe equation $^{int}K_{sp}= K_{sp}/\gamma$ where γ is the activity coefficient and determined by ASOG method. The values of specific constant (K_{sp}) and intrinsic specific constant ($^{int}K_{sp}$) for different solvents are given in Table IV.

From the Figure 8a and 8b the significant correlations are obtained as represented by the following empirical equations:

$V_{max}= -0.68258 (\log P) + 0.88642$ Eq. 13

$K_m = -0.73131 (\log P) + 0.59629$ Eq. 14

with correlation coefficient 0.78 and 0.58 for V_{max} and K_m respectively. We also correlated K_{sp} and $^{int}K_{sp}$ values with solvent polarizability values and from the plot (Fig. 9) it is found that the values increases with increasing polarizability and follows the equation shown below with R^2 value 0.97.

$K_{sp}= -0.15215 \delta + 0.37737$ Eq. 15

$R^2= 0.97516$

$^{int}K_{sp}= -0.14318 \delta + 0.35468$ Eq. 16

$R^2= 0.97521$

CONCLUSION

The effect of solvent on synthesis of G0 PAMAM was studied in presence of lipase from *Candida Antarctica*. The reaction rate was found to correlate well with solvent properties such as hydrophobicity, water solubility and dielectric constant. From these solvent properties it was found that the rate of reaction was maximum when methanol was used as a solvent. From our study we found that the initial rate decreases with increase in solvent hydrophobicity. Again it was found that the initial rate of dendrimer synthesis increases with increasing water solubility. Similarly, on increasing polarizability of the solvent the initial rate of dendrimer synthesis decreases. The dielectric constant and electron donor-acceptor index gives similar observation which increases the initial rate of the reaction. From the reaction mechanistic studies it was also observed that G-0.5 has uncompetitive inhibition effect due to multi substrate enzyme.

Table 1. Characteristics of the solvents used for this study.

Solvent	Log P	Log S_W	E^T_N	D^N_N	$E^T_N+D^N_N$	Dielectric constant	Polarizibility	Initial rate
Methanol	-0.74	m	41.5	19	60.5	32.50	3.26	0.43
Ethanol	-0.32	m	37.9	19.2	57.1	24.6	5.13	0.392
Acetone	-0.24	m	12. 5	17.0	29.5	20.70	6.47	0.354
Isopropanol	0.14	m	33.8	21.1	54.9	18	6.98	0.336
Isobutanol	0.65	1.176	-	-	-	-	-	0.307
Diethyl ether	0.77	0.8754	3.9	19.2	23.1	4.3	-	0.294
1-butanol	0.89	0.799	-	-	-	-	-	0.286
Chloroform	1.97	-0.0996	0.26	0.10	0.36	4.81	9.50	0.222
Benzene	2.13	-0.7447	8.2	0.10	8.3	2.28	10.44	0.198
Toluene	2.73	-1.8	E	e	E	2.38	12.4	0.16

e- These values have not been reported in the literature. However, calculations indicate they are close to benzene.

Table 2. Repeated use of Lipase.

Enzyme	No of use	Concentration of EDA after 12 hours ($mmolL^{-1}$)	Activity
Lipase	First	3.226	100%
	Second	19.078	50%
	Third	31.39	2%

*Reaction conditions: lipase 1.5mg/ml, EDA 9 mmol, G-0.5 PAMAM 2.12 mmol, temperature 45^0C, reaction time 12 hours.

Table 3. Inhibition constants of lipase from *Candida antarctica* in various solvents for G0 PAMAM dendrimer synthesis reaction.

Solvent	k_A ($g^{-1}min^{-1}U^{-1}$)	k_B ($g^{-1}min^{-1}U^{-1}$)	k_0 ($mmolL^{-1}g^{-1}min^{-1}U^{-1}$)	k_{mA} (k_0/k_A)	k_{mB} (k_0/k_B)	k_{mA}^{app}	k_{mB}^{app}	k_{iA}
Methanol	0.775	0.182	0.065	0.0838	0.3559	0.0503	0.0118	1.4395
Ethanol	0.706	0.166	0.059	0.0835	0.3554	0.0416	0.0097	1.4538
Acetone	0.638	0.150	0.054	0.0846	0.3600	0.0344	0.0081	1.4687
Isopropanol	0.605	0.142	0.051	0.0842	0.3591	0.0308	0.0072	1.4732
Isobutanol	0.553	0.130	0.046	0.0831	0.3538	0.0254	0.0059	1.4861
Diethyl ether	0.530	0.124	0.044	0.0830	0.3548	0.0233	0.0054	1.4831
1-butanol	0.515	0.121	0.043	0.0834	0.3553	0.0221	0.0052	1.4920
Chloroform	0.400	0.094	0.033	0.0825	0.3510	0.0132	0.0031	1.5101
Benzene	0.357	0.084	0.030	0.0840	0.3571	0.0107	0.0025	1.5173
Toluene	0.288	0.067	0.024	0.0833	0.3582	0.0069	0.0016	1.5076

Table 4. Specific constants ($K_{sp} = V_{max}/K_m$) of lipase from *Candida antarctica* in various solvents for G0 PAMAM dendrimer synthesis reaction.

Solvent	K_{sp}(mM)	$^{int}K_{sp}$ (mM)
Methanol	0.9149×10^{-2}	0.8599×10^{-2}
Ethanol	1.7548×10^{-2}	1.649×10^{-2}
Acetone	2.634×10^{-2}	2.475×10^{-2}
Isopropanol	2.4864×10^{-2}	2.337×10^{-2}
Isobutanol	2.3402×10^{-2}	2.199×10^{-2}
Diethyl ether	1.2737×10^{-2}	1.197×10^{-2}
1-butanol	4.9061×10^{-2}	4.611×10^{-2}
Chloroform	3.4228×10^{-2}	3.217×10^{-2}
Benzene	3.6029×10^{-2}	3.386×10^{-2}
Toluene	4.5614×10^{-2}	4.287×10^{-2}

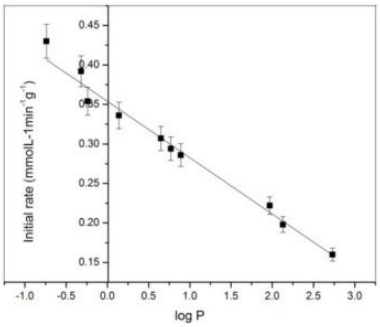


Fig. 1. Initial rate as a function of solvent hydrophobicity

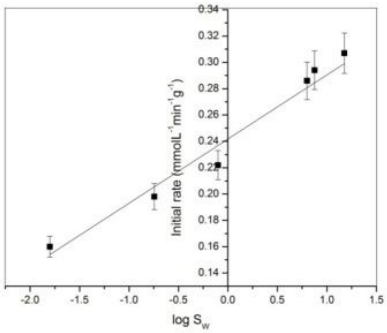


Fig. 2. Initial rate as a water solubility.

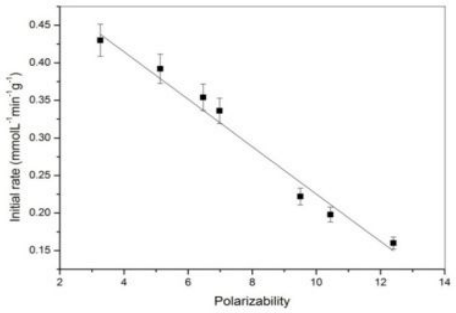


Fig. 3. Initial rate as a function of Polarizability.

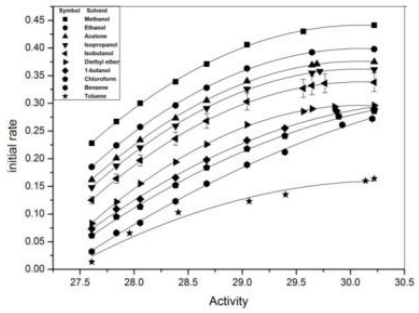


Fig. 6. Initial rate versus activity of lipase.

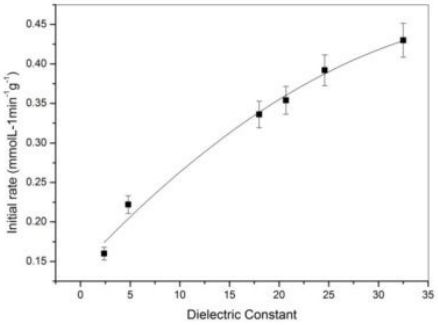


Fig. 4. Initial rate as a function dielectric constant.

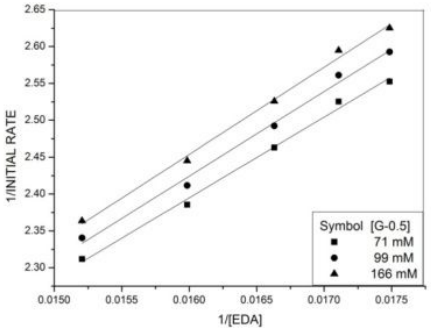


Fig. 7. Reciprocal coordinate representation of lipase activity on G-0.5 and EDA Concentration.

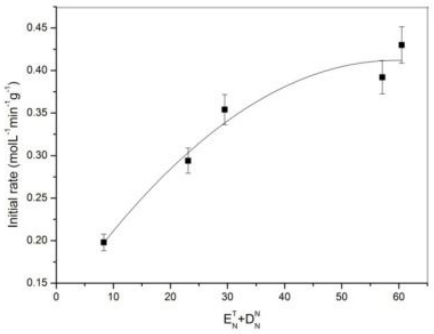


Fig. 5. Initial reaction rate as a function of (E_T^N+ D_N^N).

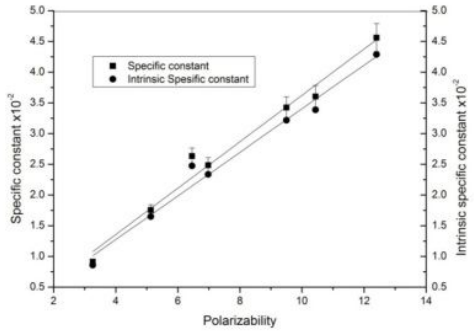
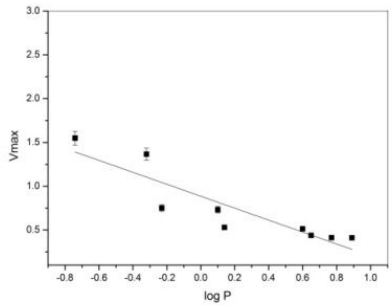
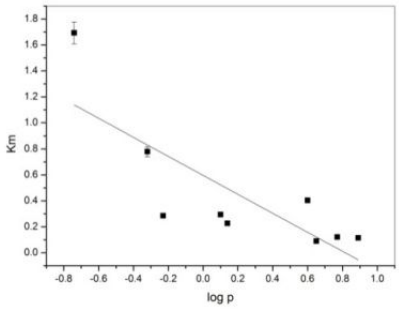


Fig. 8. Polarizability as function of Specific constant and intrinsic specific constant.



a



b

Fig. 9. V_{max} and K_m as a function of log P.

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