Immobilization of Urease on k-carrageenan and Its use in urea hydrolysis

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Abstract: We have immobilized urease on k-carrageeenan beads using polyethylene imine as hardening agent. The method used for immobilization was entrapment. We have checked the effect of hardening agent and also the enzyme loading. We have also carried out the comparison study of free and entrapped enzyme such as pH, temperature, storage stability and reusability. We have also calculated the kinetic parameters such as Michealis Constant and Thermo deactivation constant. We have used the entrapped enzyme for hydrolysis of urea using a fixed bed reactor.

Keywords: k-carrageenan, urease, entrapment, urea hydrolysis.

Introduction: Biochemical reaction are accelerated by specific type of catalyst called enzymes. Enzymes are highly specific in action compared to conventional catalyst. Rate of reaction is increased and substrate is converted to product. Enzymes have varying industrial applications i.e. in chemical industry such as fine chemistry, food chemistry, and clinical analysis [1]. Use of free enzymes in biotechnological processes is limited owing to their high cost. Enzymes are dissolved in water as homogeneous catalysis system, which is why they contaminate the product and as a result cannot be recovered in the active form. Immobilized enzymes offer tremendous scope for analytical purposes as they can be easily separated from reaction medium. The alginate was used as a support material for urease immobilization. Generally, the immobilization of urease in alginate gel showed a marked increase in K_m and V_{max}. However, the thermal stability of the immobilized urease was much higher than that of the free enzyme. A stable immobilized system and long storage life are convenient for applications that would not be feasible with a soluble enzyme system. These results highlight the technical and biochemical benefits of immobilized urease over the free enzyme [2]. Urease has been immobilized earlier on many supports such as cellulose, alginate [3-7] ; including some natural seed coats [8]. Alginate as an immobilization medium has been extensively studied, mainly due to its ease of use, mild gelation conditions, biocompatibility and non toxicity. The mild conditions in which the immobilization procedure is carried out leads to its acceptability as food additive and use in oral drug delivery systems [9-10].

Chemicals:

Urease (200U/mg), Urea, Phenol, Hypochloride solution from Qualikems Fine Chem Pvt Ltd, k-carrageenan from TCI.

Experimental:

Preparation of Beads:

We have prepared the beads of k-carrageenan by modifying the method given by [11]. K-carrageenan solution (3%w/v) in water at 60^{o}C for 20-25 min and thencooled at ambient temperature. The resulting solution was dropped 1.5%polyethyleneiminesolution. The hardening time was kept for 2 hrs. The resulting beadswere taken for further studies.

Assay Method of Urease:

Take urease 0.1 ml and add urea solution 0.2 ml and incubate for 15min then add 5 ml of phenol solution and 5 ml of hypochlorite solution mix well and incubate for 30 minand then analysed by spectrophotometer.

Effect of PEI concentration:

Hardening and retention of enzyme activity was checked by using 0.5-3.5 % PEI concentration. The protein content of the bead and supernatant liquid was also calculated. The activity of the immobilized enzyme was checked by the method given in literature.

pH Study

The effect of pH on both free and entrapped urease was carried out by incubating both free and entrapped enzyme at 60^{o}C for 30 min in buffer solution of different pH. The amount of enzyme activity was checked by the assay method.

Thermal Stability:

The thermal stability of free and entrapped urease was checked by varying the temperature from 30-80^{o}C and the effect was observed. The thermos deactivation constant (*kd*) was calculated using the following equation.

$\ln A_t = \ln A_o - k_d(t)$ ----------(1)

Where A_o is initial activity of the enzyme, and A_t the activity after heat treatment for t min.

Storage Stability:

Storage Stability plays an important role in urease industrial applications.The free and immobilized enzyme were stored at 40^{o}C and the percentage residual activity was determined at different time interval.

Reusability of entrapped urease:

The reusability of immobilized urease was checked by fresh aliquots and using the same beads and the activity was measured after each turn. The process was continued with the beads showed 50% loss in enzyme activity.

Determination of Kinetic Parameters:

Substrate and enzyme concentration govern the kinetics of enzymatic reactions.Therefore, the kinetic parameters such as Maximum Reaction Velocity (V_{max}) and Michaelis Constant (K_m) were calculated by varying the substrate concentration and keeping enzyme concentration constant and vice-versa.

Hydrolysis of Urea:

Urease has been used for hydrolysis of urea using many supports. Urease was covalently bonded to polyacrlonitrile [12] . The effect of glutaraldehyde,pH, Temperature on the catalysis of urea were studied. Poly (vinyl alcohol) PVA membrane has been used for urea hydrolysis by [13]. Urea hydrolysis was also carried on ion-exchange resin using a fixed bed reactor by [14]. Poly (N-isopropyl amide-co-N-acrylosuccinimide-co-2-hydroxyethylmethacrylate) composite hydroxyl using immobilized urease [15]. We have made an attempt for hydrolysis of urea using a natural polysaccharide i.e. k-carrageenan. We have optimized various reaction parameters such as urease concentration, flow rate, L/D ratio, temperature on the hydrolysis of urea.

Result and Discussion:

PEI concentration:

KCl has been used by many researchers as hardening agent for k-carrageenan. But the retention of enzyme activity and hardening were not found to be desirable. Devi [11] used PEI as hardening agent and found it to be better compared to KCl. Hence we have used 0.5 - 3.5 % PEI solution for hardening of k-carrageenan beads. From **Figure 1** it is seen that maximum hardening is achieved at 1.5% PEI concentration and beyond that there was no further increase in hardening as well as enzyme activity.

pH Profile

pH determines the effectiveness of enzyme , every enzyme has an optimum pH at which it shows optimum activity. **Figure 2** shows thepH activity profile of free and entrapped urease. We have observed that both free and entrapped enzyme were showing maximum pH at 7.5 showing that there is no conformational change during immobilization.

Thermal Stability:

Enzyme are temperature dependent and with increase in temperature the enzyme activity increases and beyond certain limit the enzyme gets deactivated .**Figure 3** shows that entrapped enzyme showed better thermal stability compared to free enzyme. The thermos deactivation constant for free and entrapped enzyme are given in Table **1**. The entrapped enzyme showed better thermal stability as they are encapsulated with in the beads.

Effect of enzyme concentration:

The enzyme loading was carried out by varying enzyme concentration from 4-15 mg. It was observed that initially the entrapped enzyme showed good percentage immobilization but after 6-8 mg enzyme loading there was a decrease in enzyme loading as shown in **Table 2**.

Reusability:

Reusability has a great importance in industries. We have checked the reusability of entrapped enzyme as shown in figure 4 the entrapped enzyme retained 50 % of its enzyme activity after 8 cycles and 24 % activity after 10 cycles showing the advantage of immobilized enzyme and which increases its applicability.

Kinetic Parameters:

The kinetic parameters govern the reaction rate of the reaction. **Table 1** showed the kinetic parameter K_m and V_{max} . We have observed that there is not much change in the value of K_m and V_{max} for free and entrapped enzyme.

Storage stability:

The storage stability was carried out at 40^{o}C and it was observed that free enzyme lost all its activity within 15 days where as entrapped enzyme retained 50% of its activity even after 20 days as shown in **Figure 5** showing that entrapped enzyme can be used for long period of time with retention of activity showing its higher applicability.

Hydrolysis of Urea:

Figure 6 shows the effect of urea concentration from 1-5 mM and it was observed that at 3mM urea concentration maximum hydrolysis was achieved. **Figure 7** shows the effect of flow rate from 0.5-3.0 and we have observed that maximum of 90% hydrolysis was achieved at 1.0mM flow rate.We have also checked the effect of L/D ratio on the hydrolysis of urea as shown in **Figure 8**. We found that with the increase in l/d ratio the hydrolysis increased and maximum of 95% of hydrolysis was achieved at L/D ratio of 7, this can be attributed to the fact that with the increase in L/D ratio the contact time between the enzyme and the urea solution increases. The temperature dependence on urea hydrolysis was also checked and found that maximum 95% of hydrolysis was obtained at 60^{o}C temperature as shown in **Figure 9**.

Conclusion:

K-carrageenan was used as a support for immobilization of urease and it was seen that the immobilized system was better to free system under all tested conditions. The hydrolysis of urea was also carried out using free and entrapped enzyme and about 95% of hydrolysis was achieved at L/D ratio of 7 at 60^{o}C and urea concentration 1mM and flow rate of 3 mM.

Acknowledgement:

I am thankful to my guide **Prof. (Dr.) Dinesh S Patel** and Shree P. M Patel Institute of P.G. Studies and Research in Science, Anand. Recognized ResearchCenter for Ph.D. (Applied Chemistry), Ph.D. (Forensic Science) & M.Phil Chemistry) for their support and encouragement during my research work. I would also like to thank Mr. Bipin Vakil the owner of our Medicare group for providing me such a big opportunity in my life and making the research facilities available in our institute.

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Legend to Tables.

1. Kinetic parameters and Thermodeactivation constant of free and entrapped urease.
2. Effect of enzyme loading on k-carrageenan beads.

Legends to Figures :

1. Effect of Polyethyleneimine concentration of hardening of beads and enzyme activity.
2. pH activity profile of free and entrapped urease on k-carrageenan beads.

   Free Enzyme (- - -), Entrapped enzyme (----).
3. Effect of temperature on immobilized urease for free and entrapped enzyme.

   Free Enzyme (- - -), Entrapped enzyme (----).
4. Reusability of immobilized urease.
5. Storage stability of free and immobilized urease.

   Free Enzyme (- - -), Entrapped enzyme (----).
6. Effect of urea concentration on hydrolysis of urea.
7. Effect of Flow rate on urea hydrolysis by urease enzyme.
8. Effect of L/D ratio on hydrolysis of urea .
9. Effect of temperature on hydrolysis of urea.

Table 1:

Thermodeactivation Constant (K_d)(min^{-1})			
	50ºC	60ºC	70ºC
Free Urease	$2.10x10^{-2}$	$2.68x10^{-2}$	$4.1x10^{-2}$
Urease immobilized on k-carrageenan	$1.80x10^{-2}$	$2.36x10^{-2}$	$3.69x10^{-2}$
Kinetic Parameters			
	Michealis Constant $K_m(mM)$	Maximum Reaction velocity $V_{max}(mM/min)$	
Free Urease	2.53	$0.48x10^{-5}$	
Urease immobilized on k-carrageenan	1.12	$0.39x10^{-5}$	

Table 2

Effect of enzyme loading		
Units of enzyme taken(Units/mg)	***Unit of enzyme immobilized(Units/gm of support)***	***Percentage immobilization (%)***
800	739	92
1200	776	64
1600	1196	74
2200	1263	57
3000	1466	48

Figure 1

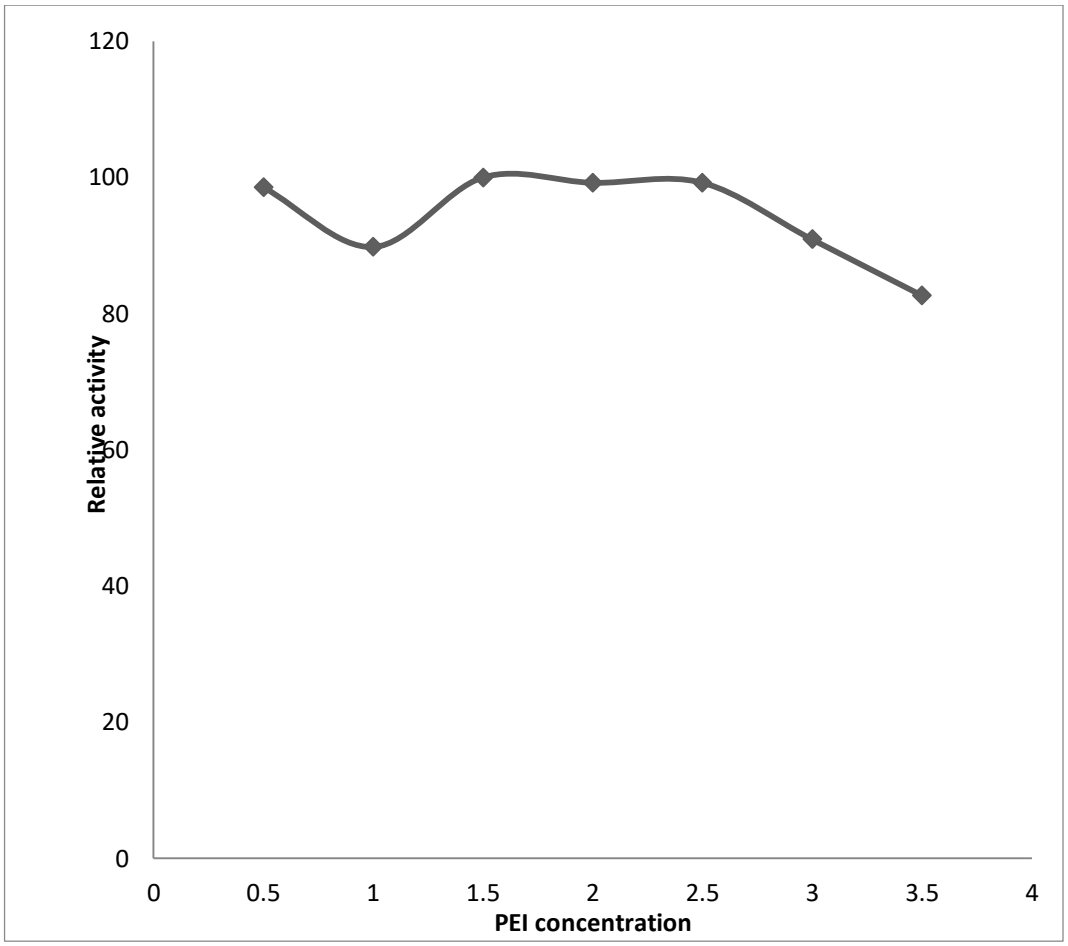
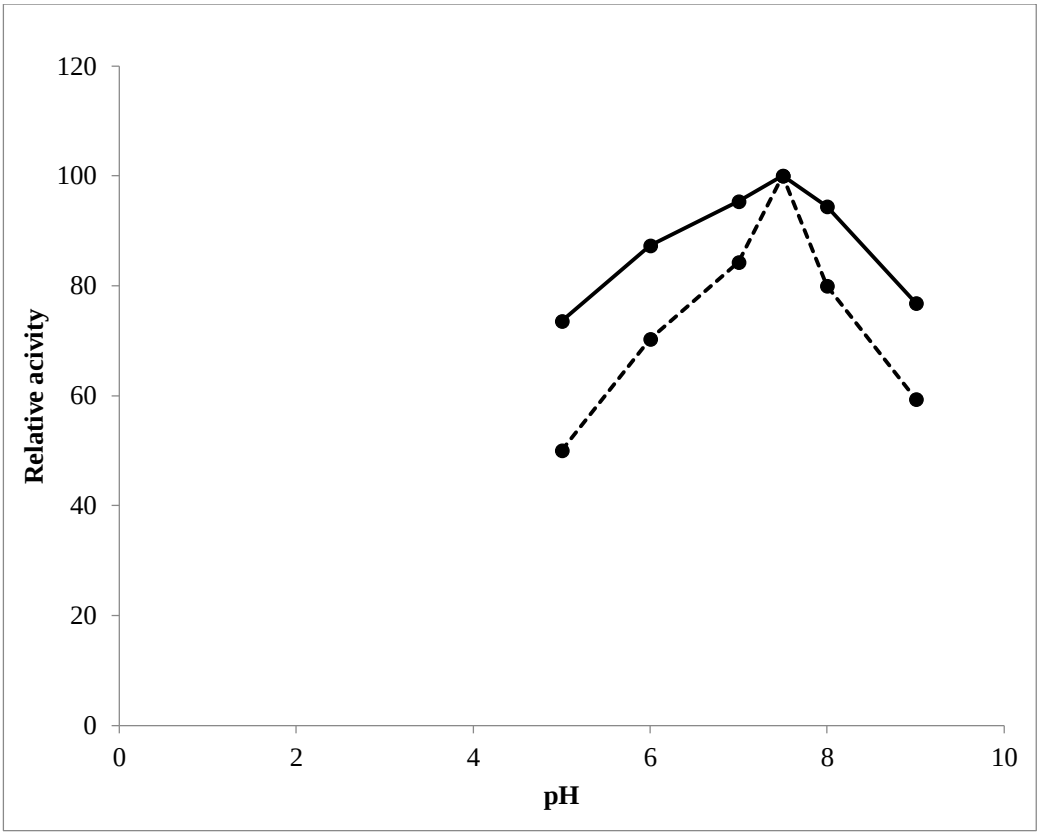


Figure 2 .



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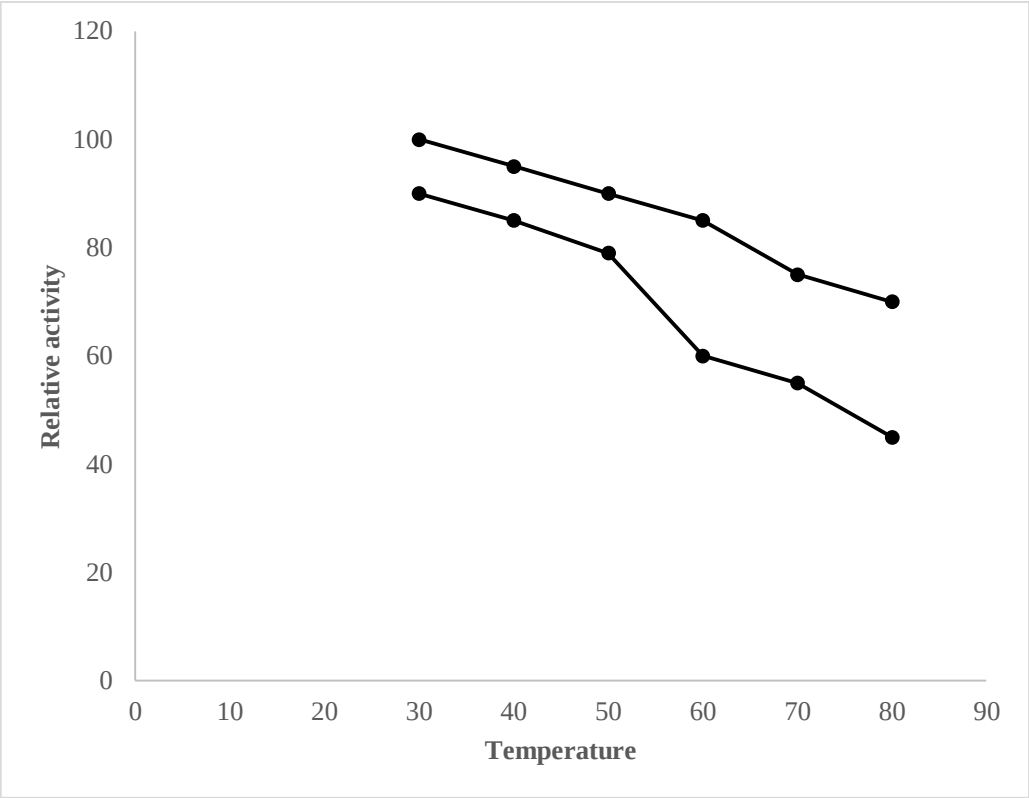
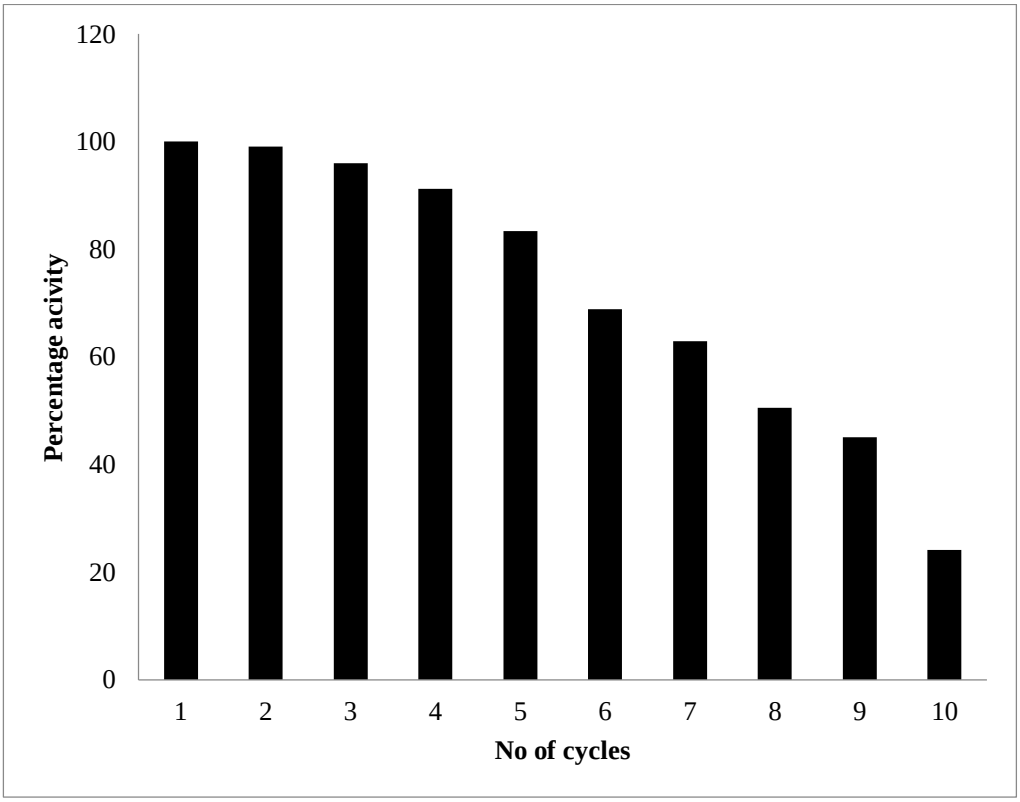


Figure 4



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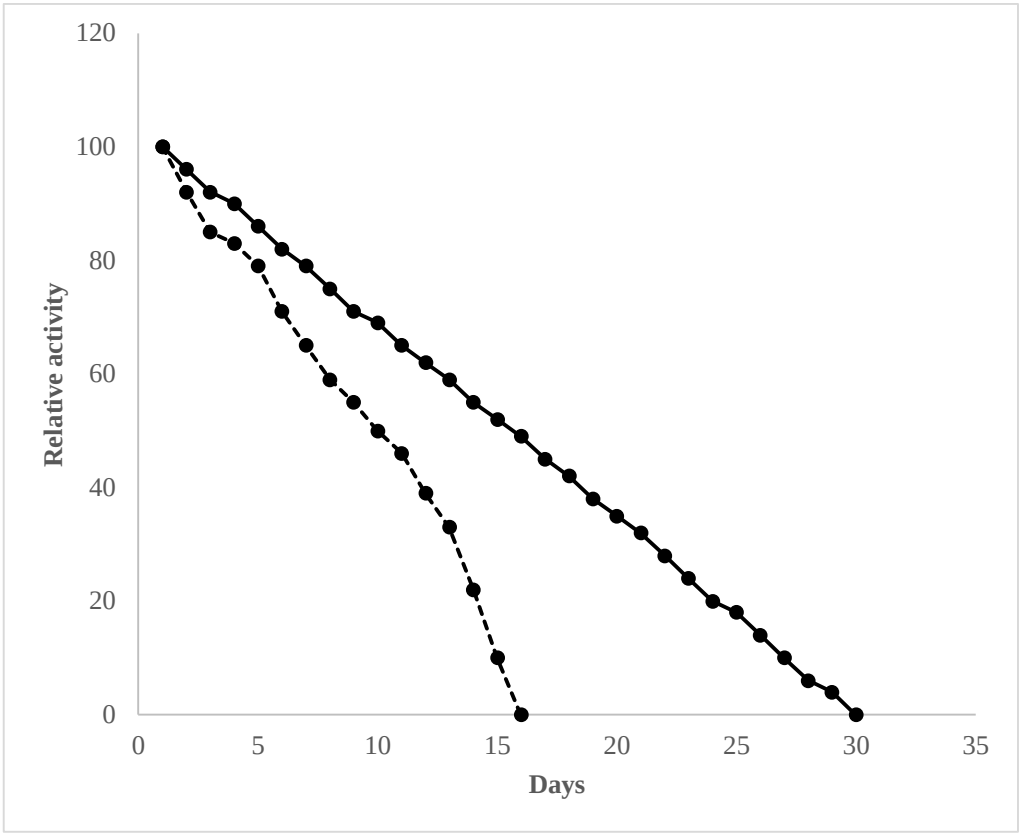


Figure 6

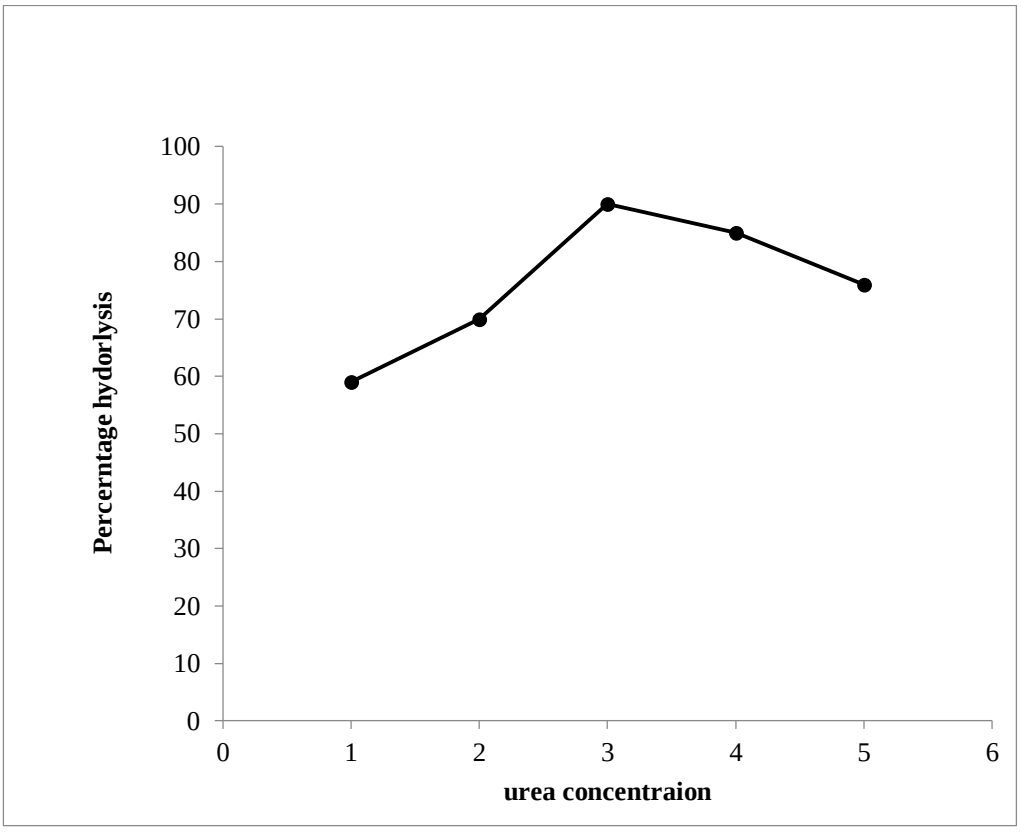


Figure 7

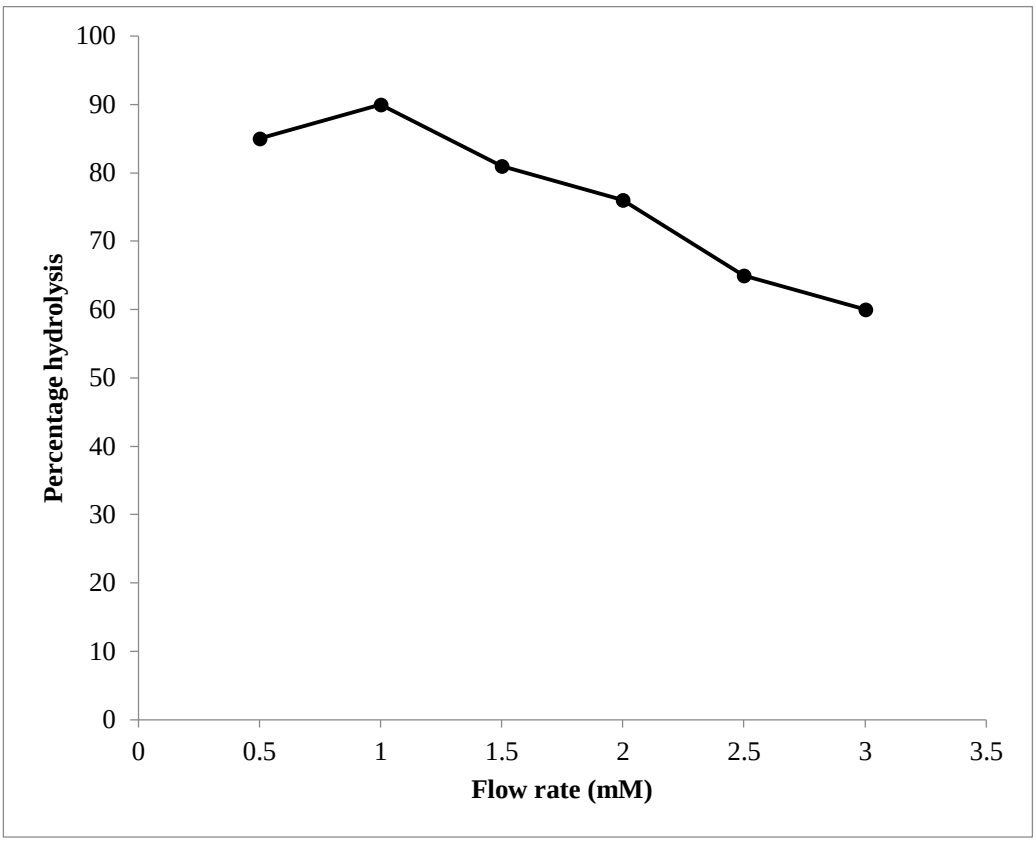


Figure 8:

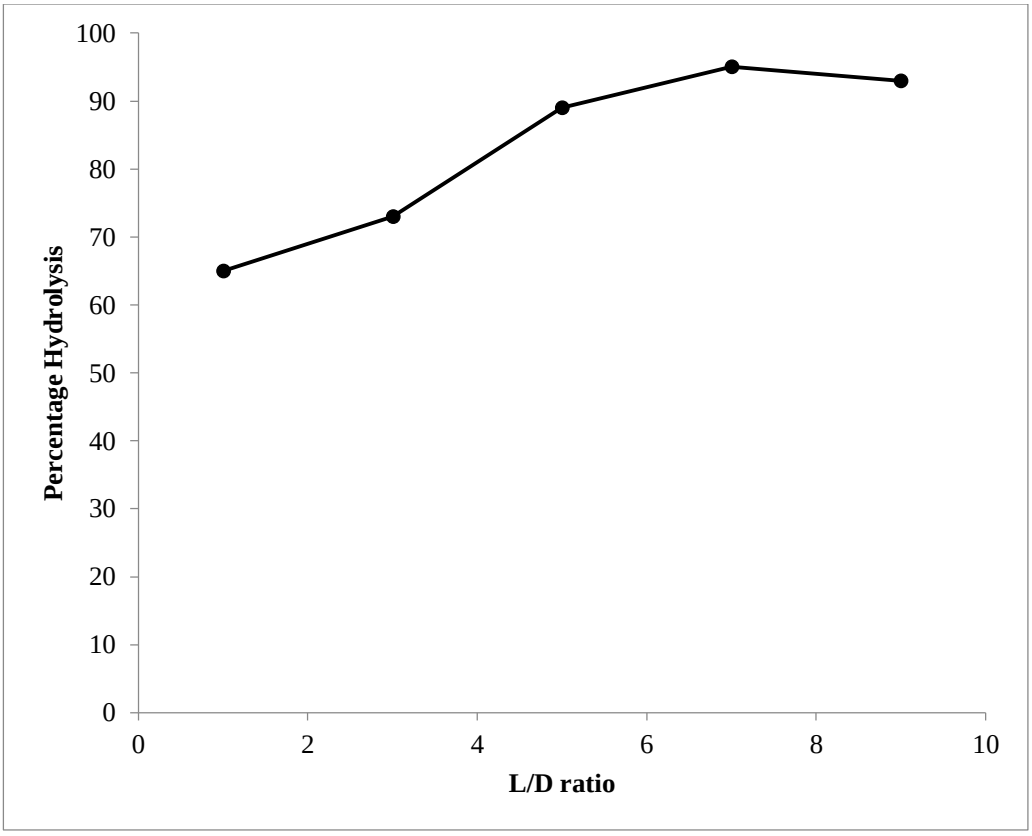
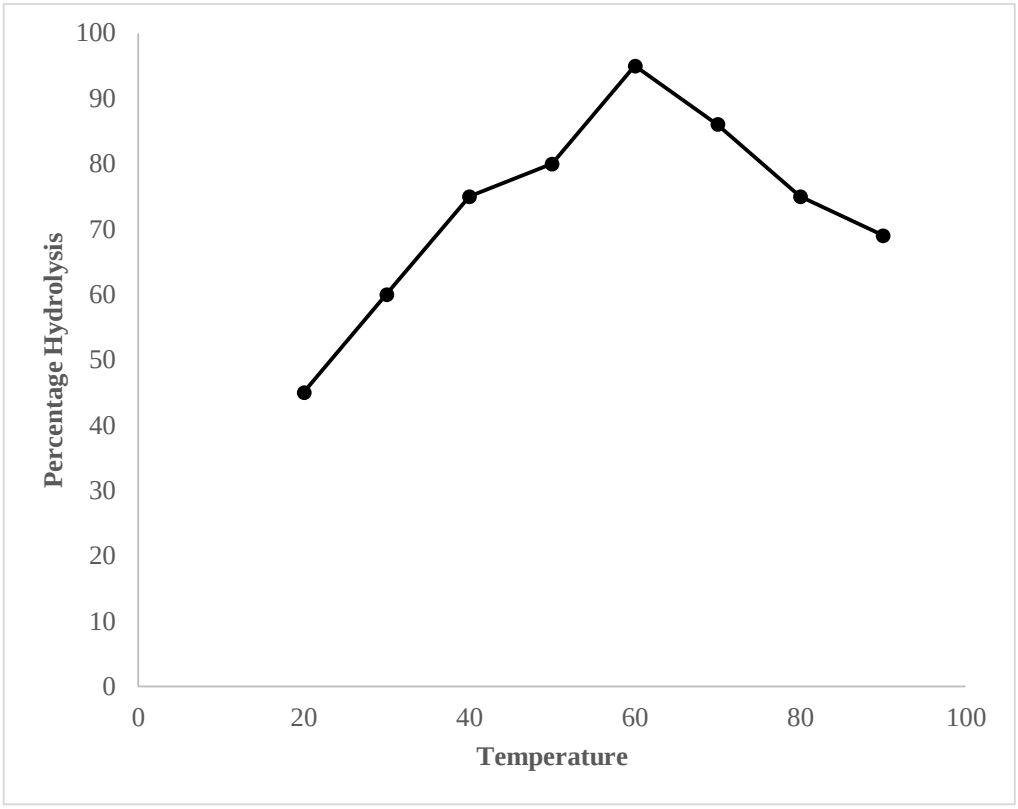


Figure 9: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