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Thermodynamic and Kinetic Studies of α-Amylase Catalyzed Reaction in Free and Carrageenan/Chitosan Immobilized State

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The present study deals with the immobilization of α-amylase into carrageenan and chitosan medium and determination of kinetics as well as thermodynamic parameters of both free and immobilized enzyme catalyzed reaction to predict the extent of reaction and the position of equilibrium. At optimized condition, the K_m value derived from Lineweaver Burk plot for free enzyme (0.37 % w/v) was lower than the immobilized enzyme (0.48 % w/v for carrageenan and 0.59 % w/v for chitosan immobilized). The free enzyme had an E_a value of 1536 cal/mol compared to those of immobilized enzyme (5463.8 cal/mol for carrageenan and 4736 cal/mol for chitosan immobilized). Computed ΔS value for free enzyme is more negative than the immobilized enzyme. The increasing value ΔG° of in immobilized enzyme system indicates that the enzyme-substrate reaction is slower during immobilization. Immobilized enzyme could be reused up to 12 days of storage.

Keywords: α-Amylase, Kinetics, Enzymes, Immobilization, Carrageenan, Chitosan.

INTRODUCTION

Almost all processes in a biological cell need enzymes to occur at significant rates. Since enzymes are selective for their substrates and speed up only a few reactions from among many possibilities, the set of enzymes made in a cell determines which metabolic pathways occur in that cell[1,2]. Like all catalysts, enzymes work by lowering the activation energy (E_a) for a reaction, thus dramatically increasing the rate of the reaction.

Enzyme immobilization may be defined as the confinement of an enzyme in a distinct phase to a suitable inert support, where it can act upon its natural substrate repeatedly and continuously and can be renewed conveniently. Immobilization of enzyme molecules does not necessarily render them immobile, in some methods of immobilization *e.g.*, entrapment and membrane confinement, the enzyme molecules move freely within their phase, while in case of adsorption and covalent binding they are in fact immobile. The term immobilization was coined by Katchalskik and Kalzir in 1970. There are various methods of immobilization which include carrier binding (adsorption[3], ionic binding[4] and covalent binding[5]), cross linking[6] and entrapment[7] (gel, fibre and microencapsulation.). In the present study gel entrapment method was chosen to immobilize α-amylase in chitosan and carrageenan.

Amylase catalyses the breakdown of starch into sugars. Amylase is present in human saliva, where it begins the chemical process of digestion. The pancreas also makes amylase (α-amylase) to hydrolyze dietary starch into disaccharides and trisaccharides which are converted by other enzymes to glucose to supply the body with energy. α-Amylase is one of the largest selling enzymes for a wide variety of industrial applications[8].

The extent of reaction and the position of equilibrium for any process in which enzyme-catalyzed reactions occur is essentially played by thermodynamic data. Metabolic control analysis requires both thermodynamic and kinetic data[9,10]. It is therefore planned to study the kinetic and thermodynamic aspects of the enzyme substrate reactions using α-amylase as model enzyme to determine energy of activation (E_a), Arrhenius factor (A), standard enthalpy change (ΔH), Gibbs free energy (ΔG°), standard entropy change (ΔS), probability factor (P) and equilibrium constant (K) values of enzyme catalyzed reaction for both free and immobilized enzyme using following equations:

$$k = Ae^{E_a/RT} \quad (1)$$

$$A = \left(\frac{RT}{Nh}\right)e^{\Delta S/R} \quad (2)$$

$$P = e^{\Delta S/R} \quad (3)$$

$$\Delta G^\circ = \Delta H - T\Delta S \quad (4)$$

$$\Delta G^\circ = -RT \ln K \quad (5)$$

$$Z = \frac{RT}{Nh} \quad (6)$$

$$\log k = \log A - \frac{E_a}{2.303}\frac{1}{RT} \quad (7)$$

$$k = \left(\frac{RT}{Nh}\right) e^{\Delta S/R} e^{-\Delta H/RT} \quad (8)$$

$$k = (PZ)e^{-E_a/RT} \quad (9)$$

EXPERIMENTAL

α-Amylase and soluble starch were purchased from Sigma Chemical Co., USA and Central Drug House Pvt. Ltd., Mumbai, India, respectively. Chitosan and Carrageenan from Himedia, India. All other chemicals used were of Analytical Grade.

Determination of optimum pH and temperature: To determine the optimum pH for maximum enzyme activity, the reaction was carried out at different pH using 0.2 M acetate buffer of pH 3.6, 4.0, 4.4, 4.8, 5.0, 5.2 and 5.6. Triplicate sets of seven tests tubes were arranged and 0.5 mL enzyme solution was added to each test tube containing 1.75 mL of starch, 2.25 mL of buffer (pH range 3.6, 4.0, 4.4, 4.8, 5.0, 5.2 and 5.6) and 1 mL of distilled water. All the test tubes were incubated at 40 ºC in a thermostatic water bath. After 20 min, 0.5 mL of 1 M HCl was added to each test tube to stop the reaction. 0.2 mL of the reaction mixture from each test tube was diluted with 14.5 mL with distilled water. Then 0.2 mL of iodine reagent and 0.1 mL of 1 M HCl were added to each of them. The absorbance of the reaction mixture was recorded at 610 nm in a UV-visible spectrophotometer (Shimadzu UV-1800) against a blank[11-15].

Optimum temperature of the enzyme was determined by above procedure using buffer of optimum pH (5) and incubating at different temperatures *viz*. 30, 35, 40, 45, 50, 55 and 60 ºC using a thermostatic water bath.

Immobilization of α-amylase with carrageenan: Mixture of 0.5 mL 1 % α-amylase and 1.5 mL 3 % carrageenan was transferred in drop wise manner from a height of about 2 cm in a stirred solution of 3.5 % KCl using a 2 mL pipette. The beads were kept for 0.5 h in KCl solution for hardening and then filtered and washed with acetate buffer[16].

Immobilization of α-amylase with Chitosan: 0.5 mL of enzyme solution was mixed with 1.5 mL of 3 % Chitosan solution and mixed thoroughly. The chitosan-enzyme mixture was transferred in dropwise manner from a height of about 2 cm in a gently stirred solution of 3 M KOH using a 2 mL pipettes. The beads were kept for 0.5 h in KOH solution for hardening which were then filtered and washed with acetate buffer at pH 5[17].

Kinetic study: Triplicate sets of six test tubes were taken, each containing 2.25 mL of acetate buffer (pH 5.0), 1 mL of distilled water and 1.75 mL of different concentration *viz*. 2.4, 2.0, 1.6, 1.2, 0.8 and 0 % (blank) of soluble starch and maintained at 35 ºC in a thermostatic water bath. To each test tube 0.5 mL 1 % amylase solution was added. After 10 min, reaction in each test tube was stopped by addition of 0.5 mL of 1 M HCl. 0.2 mL of the reaction mixture was diluted by addition of 14.5 mL distilled water followed by addition of 0.2 mL of 2 % iodine reagent and 0.1 mL of 1 M HCl. The absorbance was measured at 610 nm in a UV-visible spectrophotometer. The above procedure was repeated at 40 and 45 ºC.

Above procedure was repeated for the kinetic study of immobilized enzyme but instead of using 0.5 mL free enzyme, carrageenan or chitosan immobilized enzyme beads prepared from 0.5 mL 1 % enzyme were used.

Self-life: Self-life of the entrapped α-amylase in carrageenan beads and Chitosan beads were evaluated by reusing the beads for digestion of starch (1.2 %) at pH 5 and temperature 40 ºC on 6th and 12th day. The beads were also inspected visually for any sign of physical change.

RESULTS AND DISCUSSION

Enzyme activities at different pH and temperature are plotted in Figs. 1 and 2 and optimum pH and temperature were found to be 5.0 and 40 ºC, respectively.

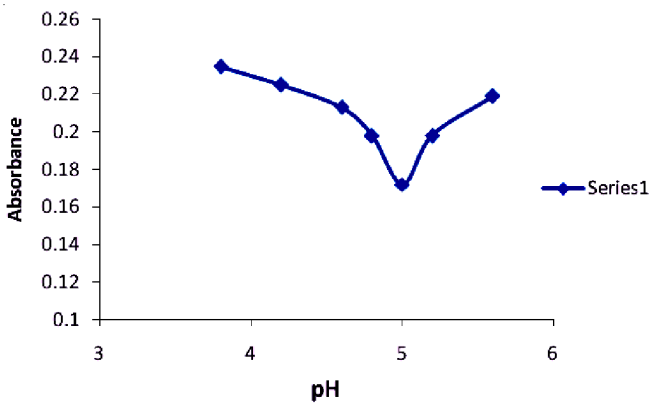

Fig. 1. Activity of α-amylase at different pH

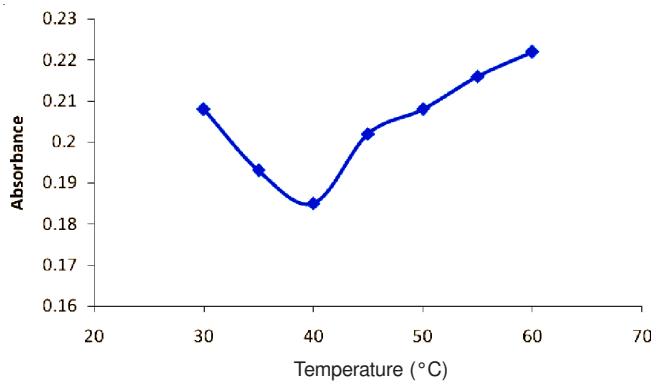

Fig. 2. Activity of α-amylase at different incubation temperature

The starch degradation activities of free α-amylase, carrageenan immobilized and chitosan immobilized enzyme at different temperature using different concentration of substrate are presented in Tables 1-3, respectively. The reciprocal velocity (1/V) at optimum temperature 40 ºC was plotted against reciprocal substrate concentration (1/[S]) in Figs. 3, 5 and 7, respectively. Extrapolation of the line gave the K_m values which are found to be 0.37, 0.48 and 0.59 %, respectively.

The K_m value of the free enzyme is lower than that of the values of carrageenan or chitosan immobilized enzyme. Low value of K_m indicate high affinity of the enzyme for the substrate[13]. The increase in K_m value might be either due to structural changes in the enzyme induced by the immobilization technique or due to lower accessibility of the substrate to the active site of the immobilized enzyme[14].

TABLE-1
STARCH DEGRADATION ACTIVITY OF FREE α-AMYLASE

Temp. (ºC)	Conc. of soluble starch (% w/v)			
	1.6	1.2	0.8	0.4
	Absorbance			
45	0.655 ± 0.002	0.439 ± 0.006	0.249 ± 0.022	0.125 ± 0.002
40	0.617 ± 0.002	0.386 ± 0.008	0.200 ± 0.013	0.113 ± 0.014
35	0.628 ± 0.002	0.400 ± 0.002	0.215 ± 0.012	0.120 ± 0.006

Values are mean ± SD (n = 3).

TABLE-2
STARCH DEGRADATION ACTIVITY OF IMMOBILIZED α-AMYLASE IN CARRAGEENAN

Temperature (ºC)	Conc. of soluble starch (%w/v)			
	1.6	1.2	0.8	0.4
	Absorbance			
45	0.766 ± 0.003	0.542 ± 0.005	0.327 ± 0.014	0.164 ± 0.002
40	0.724 ± 0.005	0.474 ± 0.006	0.259 ± 0.006	0.145 ± 0.014
35	0.742 ± 0.010	0.497 ± 0.022	0.284 ± 0.012	0.157 ± 0.008

Values are mean ± SD (n = 3).

TABLE-3
STARCH DEGRADATION ACTIVITY OF IMMOBILIZED α-AMYLASE IN CHITOSAN

Temperature (ºC)	Conc. of soluble starch (%w/v)			
	1.6	1.2	0.8	0.4
	Absorbance			
45	0.809 ± 0.004	0.576 ± 0.007	0.362 ± 0.012	0.176 ± 0.002
40	0.765 ± 0.006	0.510 ± 0.007	0.289 ± 0.003	0.159 ± 0.014
35	0.783 ± 0.004	0.528 ± 0.002	0.311 ± 0.014	0.169 ± 0.022

Values are mean ± SD (n = 3).

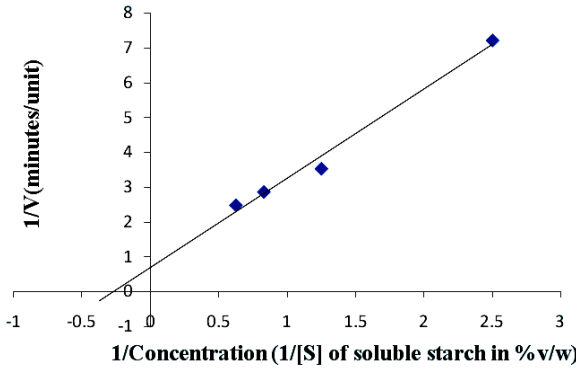

Fig. 3. Lineweaver burk plot of α-amylase (free)

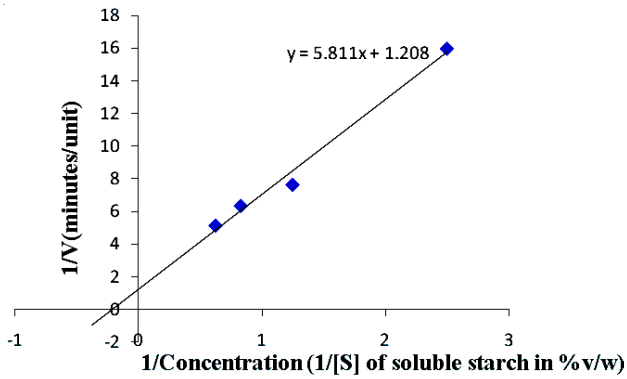

Fig. 5. Lineweaver burk plot of α-amylase immobilized in carrageenan

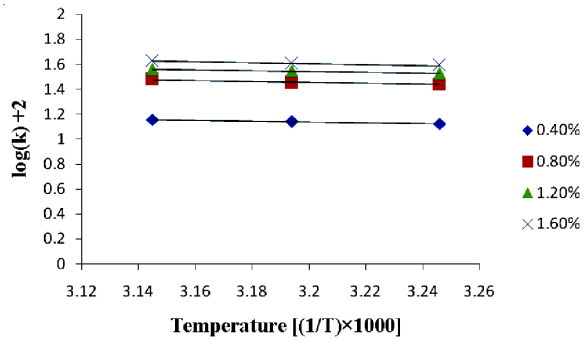

Fig. 4. Arrhenius plot of free enzyme (α-amylase)

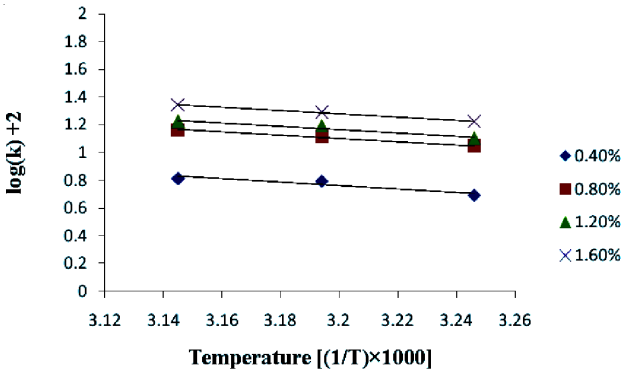

Fig. 6. Arrhenius plot of α-amylase immobilized in carrageenan

Arrhenius plot of the free and immobilized enzyme are presented in Figs. 4, 6 and 8, respectively and from the slope, energy of activation (E_a) was calculated for each case. Subsequently, Gibbs free energy (ΔGº) entropy of activation (ΔS) equilibrium constant (k), probability factor (P) for free and immobilized enzyme were computed using above mentioned equations and are presented in Table-4.

TABLE-4
COMPUTED DATA

Enzyme	E_a or ΔH (cal/mol)	A (s^{-1})	ΔS (cal/mol)	ΔG°	K	K_m (% w/v)	P
α-Amylase (free)	1536	1.62×10^{2}	-48.52	16722	2.119×10^{-12}	0.37	2.490×10^{-11}
Immobilized in carrageenan	5463.8	3.84×10^{4}	-37.6	17232	9.240×10^{-13}	0.48	5.900×10^{-9}
Immobilized in chitosan	4736	9.93×10^{3}	-40.34	17363	7.51×10^{-13}	0.59	1.524×10^{-9}

TABLE-5
STARCH DEGRADATION ACTIVITIES OF IMMOBILIZED α-AMYLASE AT OPTIMUM pH AND TEMPERATURE AFTER DIFFERENT TIME OF STORAGE

Enzyme	Absorbance on 1st day	Absorbance on 6th day	Absorbance on 12th day
Carrageenan immobilized α-amylase	0.474 ± 0.006	0.496 ± 0.002	0.510 ± 0.006
Chitosan immobilized α-amylase	0.483 ± 0.007	0.510 ± 0.003	0.514 ± 0.006

Values are mean ± SD (n = 3).

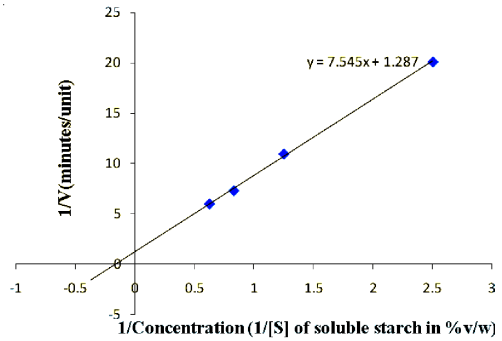

Fig. 7. Lineweaver burk plot of α-amylase immobilized in chitosan

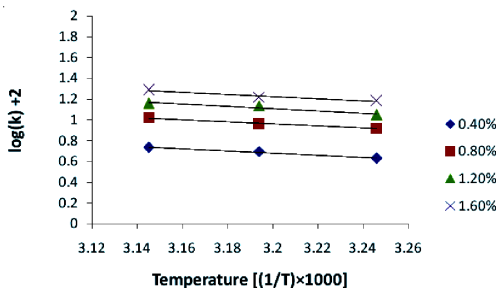

Fig. 8. Arrhenius plot of α-amylase immobilized in chitosan

Lower the entropy values of the enzyme, more efficient are the formation of transition state or activated complex between enzyme-substrate[18]. The present study showed the lower entropy value for the free enzyme but higher entropy value for the immobilized enzyme indicating the good efficiency in the formation of transition state for free enzyme in comparison to immobilized enzyme. Computed ΔS value for free enzyme (-48.52 cal/mol-deg) is more negative than the carrageenan immobilized beads (-37.6 cal/mol-deg) or chitosan immobilized beads (-40.34 cal/mol-deg).

The reaction rate is proportional to formation of transition state complex by lowering the Gibb's Free Energy of activation (ΔG°). The increasing value of ΔG° in immobilized enzyme (17232 cal/mol for carrageenan immobilized and 17363 cal/mol for chitosan immobilized) than in free form (16722 cal/mol) indicates that the enzyme substrate reaction is slower during immobilization.

K value for free enzyme (2.119×10^{-12}) is more than that of carrageenan immobilized or chitosan immobilized enzyme (9.240×10^{-13} and 7.51×10^{-13}, respectively) which indicated that free enzyme was more specific than immobilized enzyme. Starch degradation activities of immobilized enzyme after different time of storage are presented in Table-5. It is evident from the table that the immobilized enzyme retained is activity even after 12 days. However the beads developed small fissure or softened or partially broken in the reaction mixture after more than 12 days storage.

Immobilized enzymes are generally more stable compared to free enzymes, due to the curtailment of their degrees of freedom of rotation. Entrapped enzymes are unable to rotate freely. Prevention of the unfolding of its structure could be preserving its function[19]. The present work demonstrated a promising application potential of the carrageenan and chitosan beads for enzyme immobilization.

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