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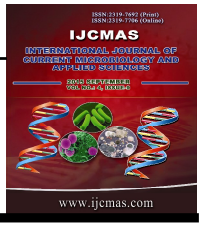

Original Research Article

Immobilization of *Bacillus pumilus*-8964 Xylanase Enzyme on C- PVA – g – poly (acrylamide) Hydrogel

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A B S T R A C T

Polyvinyl alcohol of high molecular weight was crosslinked using glutaraldehyde as a crosslinking agent (Resin I) and gamma radiation in nitrogen atmosphere (Resin II) was grafted with acrylamide monomer using ammonium persulphate (APS), ferrous ammonium sulphate (FAS) as chemical initiators, in aqueous medium. Effect of grafting was studied under various reaction parameters such as monomer concentration, ratio of the amount of APS and FAS, temperature and time of reaction. The resultant polymer with maximum grafting was successfully applied in immobilization of *Bacillus pumilus*-8964 xylanase enzyme by adsorption method. Effect of various parameters on immobilization of enzyme was studied. Results were compared with the immobilization of enzyme through silica gel using glutaraldehyde as crosslinking agent. Method of immobilization through hydrogel is simple and can be carried out by simple washing and filtration.

Keywords

Synthetic dyes, Textile industry, Decolourization, Bacteria

Introduction

Poly(vinyl alcohol), PVA, is a water soluble film forming polymer, with various applications in diverse fields (Kainer, 1949). PVA can be cross linked using cross linking agents such as Formaldehyde, glutaraldehyde, divinylsulfone, diisocyanates (Horkay and Nagi, 1982). Water soluble polymers derivatized with vinyl groups, can be crosslinked under the effect of high energy irradiation (Peppas and Merrill, 1977). Poly vinyl alcohol can be cross linked by heating with potassium persulfate and irradiation with Co-60 source. Enzyme immobilization is referred to as the restriction of enzyme mobility within a fixed space (Nasseri *et al.*, 2001).

Immobilization often stabilizes structure of the enzymes, thereby allowing their applications even under harsh environmental conditions of pH, temperature and organic solvents, and thus enables their uses at high temperatures in non aqueous enzymology.

Various improved techniques are available for immobilizing enzymes as well as the microbial cells. Hydrogels based upon PVA, silica particles, crosslinked poly(N-isopropylacrylamide-co-acrylamide) and polyacrylamide have been frequently used for the immobilization of the enzymes (Yildiz, 2002). Xylanase from *Bacillus pumilus* was also immobilized on different

matrices by various methods has showed different immobilization efficiency (Kapoor and Kuhad, 2007). Endo-1, 4-xylanase has been immobilized in hydrogel beads prepared by complexation between chitosan and xanthan (Dumitriu and Chornet, 1997). Among all techniques adsorption is the simplest of all the techniques and one which does not grossly alter the activity of the bound enzyme (Lim *et al.*, 2015; Kumar *et al.*, 2014). Enzymes like invertase (Bahuleker *et al.*, 1991) urease (Godbole *et al.*, 1990), and other enzymes have been bound through adsorption followed by cross-linking on polyethylenimine-coated supports. Silanization to activate supports and subsequent covalent binding of an enzyme to the carrier by using a coupling reagent, glutaraldehyde, is a common method used for immobilization (Cowan and Daniel, 1982).

Glutaraldehyde is toxic and causes the denaturation of immobilized enzymes. Keeping the above issues in consideration, the present article describe a novel method for immobilizing a bacterial enzyme by adsorption on the hydrogel prepared from poly vinyl alcohol (PVA) and assessed for its wide applicability in enzymology.

Experimental

Poly vinyl alcohol (PVA), Glutaraldehyde (crosslinking agent) and Dinitrosalicylic acid (DNS) were received from E. Merck Schuchardt. Acrylamide, ammonium persulfate and ferrous ammonium sulfate were of AR grade. Samples of poly vinyl alcohol was irradiated under nitrogen atmosphere from a 2100 ci cobalt-60 γ-radiation source installed in a gamma chamber 900 supplied by BARC (Mumbai) India. Xylanase enzyme was Isolated from the degraded wood, sugarcane bagasse, cow feed and soil. The reagents used to prepare the buffers were of AR grade and prepared in distilled water.

Isolation and screening of microorganism

Samples of degraded wood, sugarcane bagasse, cow feed and soil sample were collected from various sites of Solan and Shimla (H.P.), India, in sample containers and stored at 40°C for further processing. Samples were enriched in nutrient medium supplemented with xylan (0.5%) at 40°C for 24h at pH 5.5 in an incubator shaker at 135 rpm and plated on PDA containing oat-spelt xylan (0.5% w/v). All the isolates were screened for xylanase release ability in production medium containing potato 5.0%, dextrose 2.0%, yeast extract 0.5%, oat spelt xylan 0.5% (pH 5.5) and incubated at 40°C for 48h. Only the hyper xylanase producer isolate (X1) was selected for further optimization and characterized according to the given method (Harrigan, 1998).

Xylanase assay

The supernatant obtained after centrifugation of fermentation broth at 5000 rpm for 15 min at 40°C was used as a crude enzyme. Xylanase activity was determined by measuring the reducing sugar by the dinitrosalicylic acid (DNS) method (Miller, 1959) using D-xylose as the standard.

The enzyme assay was carried out at 40^0C using 0.5% (w/v) oat spelt xylan (50 milli molar acetate buffer, pH 6.0) as substrate.

Preparation of crosslinked polyvinyl alcohol(c-PVA) using glutaraldehyde (Resin I)

Poly (vinyl alcohol) PVA (5.0 g), of molecular weight 72,000 was dissolved in 100 mL of distilled water by stirring and heating at 100°C. The aqueous solution was cooled to room temperature and then glutaraldehyde (25%) was added with varying concentration in order to obtain optimum conditions for suitable gel (high

yield and good swell ability). The wet gel thus formed was crushed and refluxed in distilled water, DMF and dioxane then thoroughly washed with water, finally with methanol and dried at 50°C. Results are presented in table 1.

Preparation of cross linked Poly (vinyl alcohol) c-PVA using γ-rays irradiation method (Resin II)

Poly vinyl alcohol (PVA) (5.0 g), of molecular weight 72,000 was dissolved in 100 mL of distilled water by stirring and heating at 50°C. The aqueous solution was cooled to room temperature and nitrogen was passed. The solution of PVA was irradiated with γ-rays from Co-60 source at the dose rate of 2.0 kGy/h to a total dose of 47 kGy. The wet gel was thoroughly washed and dried as discussed above. Resulting c-PVA was refluxed for 3h with 1N HCl to break the sessile ketal linkages and release the soluble uncrosslinked PVA by filtration. Yield of c-PVA was 88% based on the PVA initially taken.

General Procedure of Grafting of Acrylamide onto Resin I and Resin II

Cross linked poly (vinyl alcohol), (c-PVA) (100 mg) was taken in a predetermined amount of water. To the reaction mixture, predetermined amounts of acrylamide and initiators ammonium persulfate and ferrous ammonium sulfate were added. The reaction mixture was stirred at 70ºC temperatures for 3hrs. The homopolymer, poly(AAm) formed during the reaction was completely removed by thoroughly washing with warm distilled water. The grafted polymer was refluxed with methanol. The gel was dried at 50°C to a constant weight. The percentage of grafting was calculated from the increase in the initial weight of the c-PVA and was expressed as follows:

$$\text{Percentage of grafting} = \frac{W_2 - W_1}{W_1} \times 100$$

Where

W_1 = Wt. of ungrafted c—PA

W_2 = Wt. of grafted c—PA after complete removal of homopolymer

The percentage of grafting of acrylamide onto c-PVA (resin I & resin II) was studied as function of monomer concentration, ratio of concentration of APS and FAS, time of reaction and temperature of reaction medium.

Effect of the concentration of monomer on grafting

Samples of resin I (100 mg) were taken in tubes. Initiators Ammonium persulfate 0.028mmol and ferrous ammonium sulfate 0.020 mmol were added. After adding varying amounts of AAm, the volume of reaction mixture in each tube was made up to 5.0 mL. The mixture was stirred for 3h at 70°C. Similar experiments were run with c-PVA crosslinked with γ- radiation (resin II). The results are presented in figure 1.

Effect of the ratio of initiator concentration on grafting

The percentage of grafting was determined as a function of the concentration of

initiator. The concentration of APS was varied while keeping the concentration of FAS (0.020 mmol) and acrylamide (16.90 mmol) constant. Then keeping the concentration of APS (0.028mmol) and acrylamide as constant and the concentration of FAS was varied. The results are represented in table 2.

C-PVA = 100 mg, Temperature = 70^{0}C, AAm = 16.90 mmol, Time of reaction = 3h

Effect of the time of reaction on grafting

Samples of Resin I and Resin II (100 mg) each were taken in tubes. APS (0.028 mmol), FAS (0.020 mmol), acrylamide (16.90 mmol) and H_2O (4.25 mL) were added. The mixture was magnetically stirred for different periods of time at 70°C. The results are presented in figure 2.

Effect of temperature on grafting

The percentage of grafting was studied as a function of temperature of the reaction. Resin I and Resin II (100mg) were taken. APS (0.028 mmol), FAS (0.020 mmol), acrylamide (16.90mmol) and H_2O (4.25 mL) was added and the mixtures were stirred for 3h at different temperatures. The results are presented in figure 3.

Immobilization of *Bacillus pumilus*-8964 xylanase enzyme on c-PVA-g- (AAm) (Resin I and resin II) and silica gel

Immobilization of xylanase on silica gel

Silica (100-200 mesh size; 6 g) was pretreated with glutaraldehyde (1%, v/v; 15 mL of 0.05 M Tris HCl buffer of (pH 8.5 for) for 1 h at 37^{0}C. The supernatant was retained by centrifugation for assay of unbound xylanase. The sedimented matrix-bound biocatalyst was washed once with 0.05 M Tris HCl buffer (pH 8.5) to get rid of unbound activating agent. The immobilized enzyme in matrix was determined by subtracting unbound xylanase in the supernatant from the total enzyme used for immobilization. All the experimental studies were done using silica-bound xylanase (100 mg) under shaking (120 rpm).

Immobilization of xylanase enzyme on c-PVA-g- poly(AAm) hydrogel

100 mg of c-PVA-g- poly(AAm) hydrogel (resin I and resin II) with maximum grafting was washed with phosphate buffer pH 7 and immersed in 20 mL solutuion of xylanase and then stirred for 3hrs at room temperature. After stirring gel was separated by filtration and again washed with phosphate buffer. The resulting polymer was tested for immobilization according to following procedure.

To the two test tubes containing substrate (xylan) 500 µL and sodium phosphate buffer 300 µL, resin I and resin II was added (100 mg each). After incubation for 15 minutes at room temperature, DNS reagent 1.5 mL to each test tube was added. Then the mixture was again incubated for 15 minutes at room temperature. The activity of enzyme was determined at 575 nm in UV Spectrophotometer. Similar experiments were run without Resins (blank) and by adding both the resins in each mixture after incubation (control experiments). The activity of enzyme was calculated according to following relationship:

Enzyme activity

$$DF = V_f / V_i$$

Where, DF = Dilution factor

V_f = Final volume

V_i =Initial volume

Incubation time = 15 minutes

Effect of different physico- chemical parameters on the activity of immobilized enzyme

The matrix bound-immobilized enzyme was evaluated to study the effect of various physico-chemical parameters such as pH, temperature and incubation time. The activity of immobilized enzyme was calculated with variation of pH and temperature. The effect on the activity of enzyme was expressed in terms of % Relative activity (RA). The activity of immobilized enzyme by grafted gel was compared with immobilization of enzyme through silica gel.

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Effect of pH on the activity of immobilized enzyme on Resin I, Resin II, Silica Gel

The Relative activity was determined at different pH of the medium using different buffer (acetate buffer pH 3.0-5.0), (Phosphate buffer pH 6.0-7.0) Glycine NaOH buffer (pH 8.0-11.0) ranging from 3.0 to 11.0.

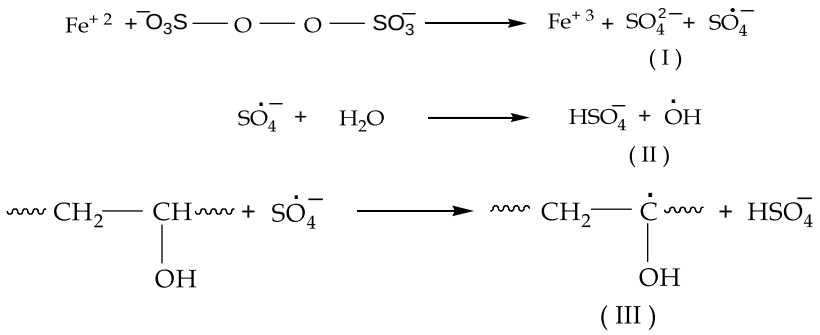
Effect of temperature on immobilization of enzyme

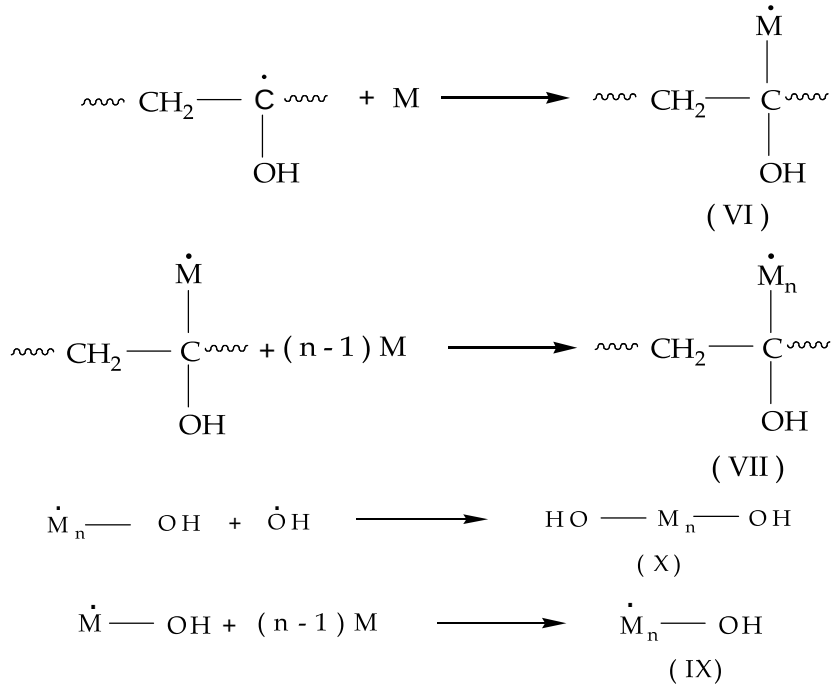
The effect of temperature on the immobilization of enzyme was carried out at a particular pH of the medium with maximum percentage of relative activity. In each case the temperature of the incubation was varied from 30°C to 60°C after 2 hours. The results are presented in figure 5.

Results and Discussion

In the course of redox initiation free radicals are generated as transient intermediates. These create reactive sites both on the polymer and the monomer. Transient radical formed by a redox initiator pair of $S_2O_8^{2-}$ and Fe^{2+} abstracts a hydrogen atom from the polymer backbone forming a macroradicals while Fe^{+2} ions are oxidized to Fe^{+3}. The free radical sites of backbone react with the olefinic bond of a monomer present, leading to the formation of a graft copolymer.

Lohani *et al.* (1958) reported that the kinetics of the persulfate initiated reaction has effects similar to that of Fenton's reagent. The decomposition of persulfate and activation by reducing agents has been summarized by Bacon in his view (Mishra and Yagci, 2008). The activation of persulfates by various reductants has been reported (Sorenson *et al.*, 1961). The APS-FAS catalyzed graft copolymerization of a vinyl monomer on crosslinked PVA occurs through the following possible rout:





The species (III) and (IV) give rise to grafting on c-PVA and (VI) leads to homopolymerization.

Reaction parameters like concentration of monomers and initiator, reaction time and temperature affect the grafted contents and hence the graft–copolymer. Effect of these parameters has been evaluated in terms of the yield of network formed and discussed below:

The percentage of grafting was studied as a function of monomer concentration (Fig. 1). On variation of concentration of monomer acrylamide between 2.84 mmol and 21.09 mmol respectively, it was observed that percentage of grafting increases with maximum at 16.90 mmol of acrylamide at a fixed concentration of APS/FAS.

The percentage of grafting increases with increase in conc. of acrylamide (16.90 mmol) and acrylic acid (29.17 mmol) after that it decreases. This trend is understandable because at higher amount of acrylamide, higher amount of their polymeric radicals are available. Beyond the optimum concentration homopolymer formation becomes the preferred process. The soluble polymer forms a viscous solution in water which lowers the mobility of the growing polymeric chain. In addition, it becomes more difficult for large radicals to diffuse into c-PVA gel. Therefore, a high concentration of large radicals with low mobility will favour dimerization leading to the formation of homopolymer at the cost of grafting.

Table 2 presents the percentage of grafting as a function of the conc. of APS at a fixed concentration of FAS and acrylamide. The percentage of grafting increases as the conc. of APS was raised. Higher yield was achieved at 0.028 mmol of APS. Further increase in the concentration of APS lowers the percentage of grafting. At 0.028 mmol of APS and keeping other reaction parameters constant, concentration of FAS was varied. The percentage of grafting continues to increase up to 0.020 mmol of FAS after it slides to lower values.

Thus, this initiator system operates best at APS: FAS ratio of 0.028 mmol: 0.020 mmol for grafting of acrylic acid onto crosslinked PVA. It is known that FAS is good homopolymer suppressor by removing. OH radicals, which are responsible for the formation of homopolymer at a critical concentration, above which the macro-radical concentration drops to a very low value which lowers the grafting yield.

Figure 2 shows the effect of time of reaction on the percentage of grafting of acrylamide onto crosslinked poly (vinyl alcohol) when other parameters were constant. There was increase in the percentage of grafting as the time of reaction was increased from 1h to 3h beyond which there was a decrease in the percentage of grafting. It is evident that the percentage of grafting decreases possibly as a result of the scission of the grafted chains brought about through "backbiting mechanism". Backbiting may be interchain, or between a grafted chain and a homopolymer radical. Backbiting should increase the formation of homopolymer at the cost of grafting (Pande *et al.,* 1995).

Keeping other reaction parameters unchanged the effect of temperature on the percentage of grafting of acrylamide onto c-PVA is presented in figure 3. As the temperature of reaction varied from 40^0C to 80°C, the percentage of grafting continues to increase up to 70°C. After that increase in temperature decrease the percentage of grafting. This implies that there exists an optimum temperature to afford maximum grafting. At higher temperature the rapid decomposition of redox system generates free radicals at a higher rate. In addition the solubility and hence the mobility of monomer molecules and macro radicals in the reaction medium is increased, this accelerates the grafting process. In a heterogeneous system, where diffusion to the interior of crosslinked PVA is a key process for grafting, higher temperatures should be a facilitating factor. However, further increase in reaction temperature favour more homopolymerization due to enhanced mutual termination of growing polymeric chains results in the wastage of monomer.

From figure 4 it follows that resin I shows maximum relative activity at pH 7.0 (81.40 U/mL) while resin II shows maximum relative activity at pH 5.0 (48.09). It follows that the activity of enzyme decreases with increase in pH of the medium when silica gel was used. c-PVA-g-AAm hydrogel shows similar trends. But the enzyme immobilized on Resin I shows increase in % relative activity (RA) with increase in upto pH 7. Hence immobilization of enzyme on Resin I is better than silica gel in neutral medium. The % relative activity (RA) of immobilized xylanase on Resins I and II is near about equal to the silica gel. From figure 5 it follows that Immobilized xylanase on c-PVA-g- poly(AAm) (Resin I) shows maximum activity 81.40 U/ mL, while Resin II shows maximum activity 43.80 U/ mL at 50°C. In comparision to resin II, resin I shows high % relative activity. The increase in % relative activity of enzyme is due to the fact that at temperature 50°C (pH 7), functional groups in Resin I came in contact with medium of outside environment to maximum extent in comparision to the resin II and form stable bonds with carboxylate ion of the enzyme with acetal linkage formed by glutardehyde during crosslinking of polyvinyl alcohol.

Xylanase was immobilized on a porous polymer matrix which was formed by chemical and radiation polymerization of bifunctional monomers i.e. PVA. The porous structure of the polymer matrix was found to change markedly with monomer concentration and nature of crosslinking. The enzyme activity is strongly related to

pore size, degree of hydration of polymer matrix and particle size of the composite. In case of enzymes immobilized through ionic interactions, adsorption and desorption of the enzyme depends on the basicity of the ion exchanger. Moreover, a dynamic equilibrium is normally observed between the adsorbed enzyme and the support which is often affected by pH as well as the ionic strength of the surrounding medium. This property of reversibility of binding has often been used for the economic recovery of the support. This has been successfully adapted in industry for the resolution of racemic mixtures of amino acids, using amino acid acylase1,2. A variety of commercially available ion exchangers have been investigated for this purpose.

Traditional enzyme immobilization procedures involve isolation of the enzyme, followed by use of several steps for the immobilization. The immobilization technique through adsorption can have future potentials especially in the downstream processing and immobilization of enzyme/proteins obtained by recombinant DNA technology. Immobilization of enzyme by various methods involves use of glutaraldehyde in activation process which is toxic in nature. Hence large processes of washing and cleaning involved. The use of Resins like present one the possibility of toxicity is negligible. The use of polymer matrix involves simple and rapid process. The polymer is cross linked hence reuse of Resin is possible by simple cleaning and filtration.

Table.1 Preparation of c-PVA using different concentration of Glutaraldehyde (GA)

S. No.	Amount of PVA (g)	Amount of GA (mL)	H_2O (mL)	% Crosslinking
1.	5	2	98	76%
2.	5	3	97	90%
3.	5	5	95	70%

Table.2 Effect of the ratio of initiator concentration on grafting (Resin I and Resin II)

S. No.	Amount of FAS mmol	Amount of APS mmol	H_2O (mL)	% grafting (Resin I)	% grafting (Resin II)
1.	0.018	0.028	4.27	155	180
2.	0.020	0.028	4.25	180	240
3.	0.022	0.028	4.22	170	156
4.	0.027	0.028	4.17	162	135
5.	0.036	0.028	4.07	156	140
6.	0.041	0.028	4.02	130	126
7.	0.020	0.016	4.48	144	150
8.	0.020	0.018	4.43	152	154
9.	0.020	0.021	4.38	173	160
10.	0.020	0.024	4.33	175	170
11.	0.020	0.027	4.28	178	192
12.	0.020	0.028	4.25	180	240

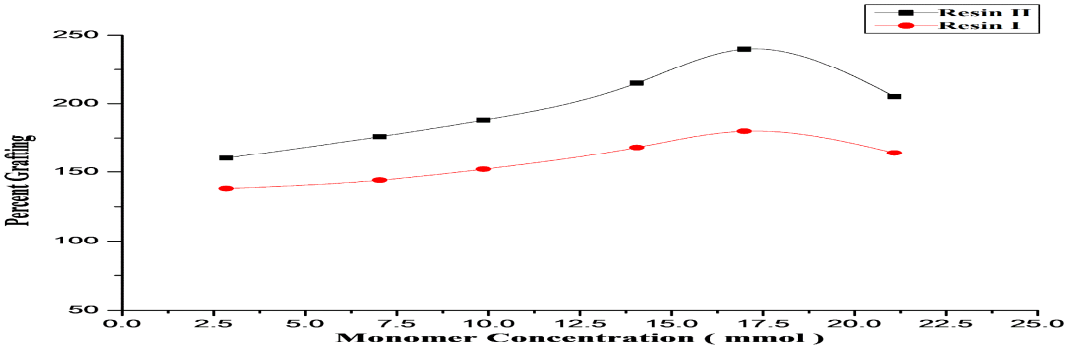


Figure-1 : Effect of Acrylamide Concentration on Grafting
c-PVA = 100mg, APS = 0.028 mmol, FAS = 0.020 mmol, H_2O = 4.25 mL
Temperature = 70°C, Time of Reaction = 3h

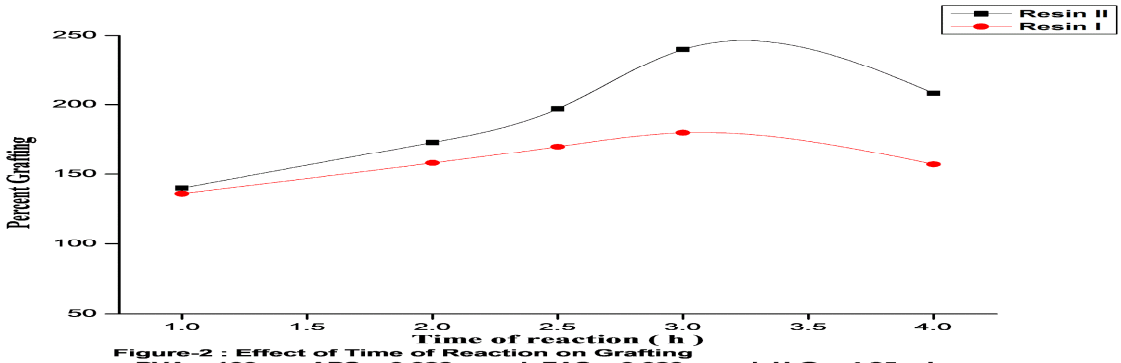


Figure-2 : Effect of Time of Reaction on Grafting
c-PVA = 100mg, APS = 0.028 mmol, FAS = 0.020 mmol, H_2O = 4.25 mL
Temperature = 70°C, Acrylamide = 16.90 mmol

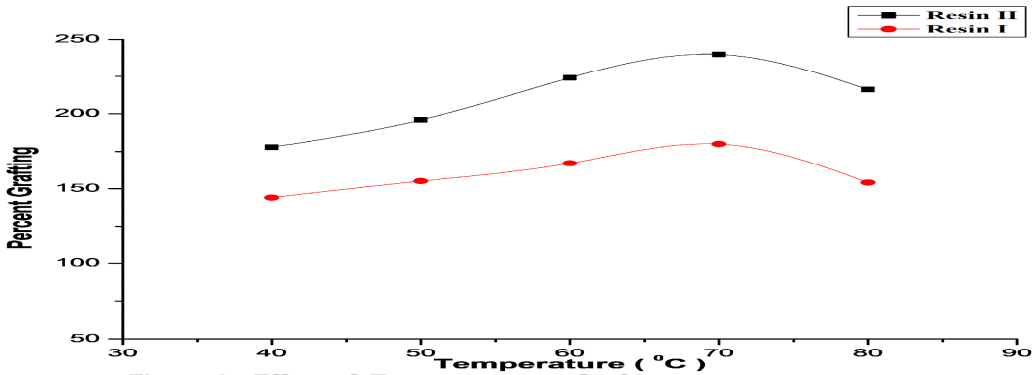


Figure-3 : Effect of Temperature on Grafting
c-PVA = 100mg, APS = 0.028 mmol, FAS = 0.020mol, H_2O = 4.25 mL
Time of Reaction = 3h, Acrylamide = 16.90 mmol

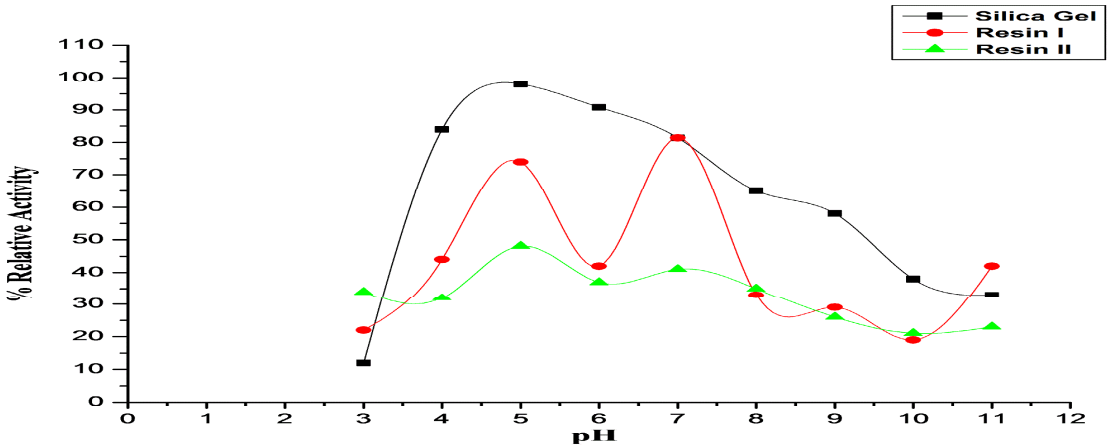


Figure-4 : Effect of pH of the Medium on % Relative Activity of Immobilized Enzyme on Resin I, Resin II and Silica Gel

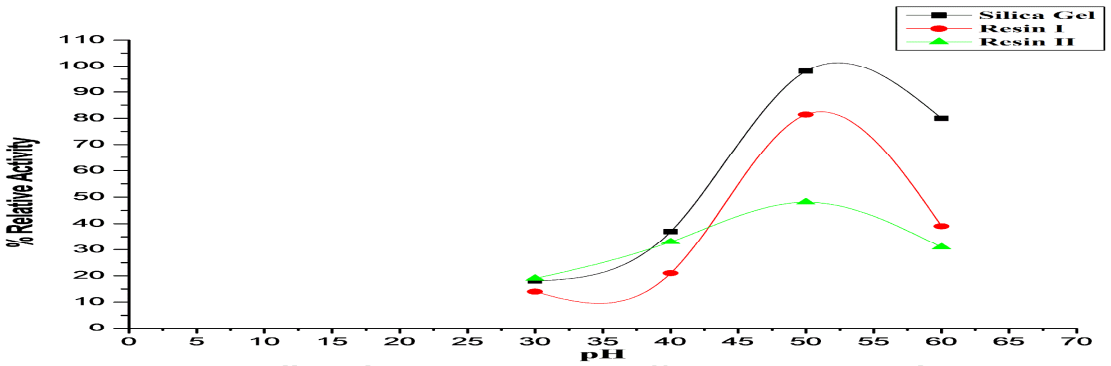


Figure-5 : Effect of Temperature on the % Relative Activity of Immobilized Enzyme on Resin I, Resin II and Silica Gel

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