Chapter 4

Immobilization of β-Galactosidase

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4.1. β-galactosidase :

β-Balactosidase (β-D- galactoside galactohydrolase) EC (3.2.1.23) typically catalyses the hydrolysis of lactose to its component monosaccharide , glucose and galactose and is also known as lactase. The numbers 3,2,1 indicate, respectively , that the enzymes are hydrolases , act on a glycosidic bond , and recognize the o-glycosyl bond. The use of β-galactosidase for the hydrolysis of lactose can become the basis for the production of new foodstuff such as lactose –hydrolyzed milk and whey, and products derived there from.

It is known that the glycosidase catalyzes both reactions , that is , the sugar residue forming the substrate may by transferred to either water or to some other hydrolytic acceptor, such as another sugar or an alcohol . This side reaction is called transgalactosidation when the substrate is lactose [1] . When the hydrolytic acceptor is another sugar the oligosaccharides are formed . If the sugar , which acts as acceptor is a monosaccharide , the product formed is a disaccharide, if the acceptor is a disaccharide the product is a trisaccharides and so on. β-galactosidase has two functional groups , sulfhydryl and imidazole in the active site . Wallenfels and Malhotra [2] proposed a mechanism for the transfer of a D-galactose residue from galactoside to an acceptor (water or another sugar or alcohol) .

Shukla [3] reviewed and summarized this proposed mechanism which is as follows. It includes three steps.

a. The hydrolysis reaction corresponds to a SN-2 like displacement mechanism , i.e a nucleophile reagent (donating an electron pair) , attacks C-1 to remove the galactosyl groups.
b. The sulfhydryl group at the active side as a general acid protonates the galactosidase oxygen atom , while the imidazole groups act as nucleophilic in the attack on the nucleophilic enter at C-1 in the glycone.

c. In the removal of the galactosyl group , the sulfhydryl anion acts as a general base to remove a proton from water which consists in the attack of OH- at the C-1 position.

β-galactosidase can be obtained from different sources such as yeast , mold , bacteria , plant and animal.

Source	***Name from which it is obtained***
Yeast	*Kefir Yeast* [4] *, Kluyvermyas fragilis* [5] *, Candida psudotropicalis* [6]*.*
Bacteria	*Bacillus anthracis* [7] *,B.megaterium* [8]*, E.Coli.*[9]
Molds	*Aspergillus sp* [10] *, Alternaria sp* [11] *, Neurospora Crassa* [12]
Plant	*Alfalfa seeds* [13] *, almond* [13]*.*
Animal	*Intestine* [13]

4.2. Literature Survey :

β-galactosidase is widely used enzyme in the industry for lactose hydrolysis. Much work has been carried out on β-galactosidase since ancient time. β-galactosidase has been immobilized on many natural and synthetic supports. The methods of immobilization may be entrapment , covalent coupling , ionic binding or crosslinking . A detail literature survey of the last two decades has been given.

Okos and Harper [14] covalently bound β-galactosidase to acrylamide derivatives of ZrO_2 porous glass They observed partial irreversible inhibition of immobilized

lactase activity during hydrolysis of whey. Further addition of 0.25 % of galactose in the feed solution showed 55 % inhibition of lactose hydrolysis at a conversion rate equivalent to 25 %.

Hustard et al. [15] have discussed immobilization of β-galactosidase from E.Coli onto polyisocynate based polymer. They observed that immobilized enzyme showed good stability and activity at pH 8.75 .Moreover the immobilized system showed 65 % retention of activity after 82 days.

Porous ceramics have been used for adsorption of β-galactosidase by Pye and Weetall [16] . The authors have optimized the operational conditions like pH, coupling efficiency , mass transfer effect and enzyme stability. Pastore et al.[17,18] used cellulose triacetate for entrapment of β-galactosidase . Skimmed milk was used as substrate for the activity and observed maximum percentage conversion was 71 – 81 % within 2 h reaction time.

Immobilization of β-galactosidase on amino silochrome S-80 using gossypol crosslinking agent was carried out by Ivanov et al.[19] The immobilized enzyme showed 3-4 % less activity compared to free enzyme. The immobilized enzyme system was used for 85 days in the continuos hydrolysis of cheese whey and 71 days in the hydrolysis of milk ultrafilterate .

β-galactosidase from A.niger and A.oryzae was used for covalent coupling to porous silica beads by Baret [20]. Lactose was used as a substrate and half life of the system was found to be 55 days. The immobilized process was scaled up from laboratory to an industrial application.

Narinesingh et al. [21] reported covalent binding of β-galactosidase from E.coli on fructogel support. They investigated the effect of pH, temperature and thermal stability using a plug flow β-galactosidase bioreactor .

PVA has been used as a support for immobilization of β-galactosidase by Ariga et al. [22] on hardening by freeze-thawing system. They observed leakage of the enzyme from PVA hydro gel beads and faster activity drop of the gel beads during prolonged incubation . They further observed that the stability of immobilized enzyme treated with boric acid at an adequate concentration restored to the extent of the free enzyme , although higher concentration of boric acid inhibited the free enzyme.

Sakurai et al. [23] used porous chitosan beads as supports for immobilization of β-galactosidase . They observed no reduction in enzyme activity even after continuous use for 8 cycles.

Immobilization of β-galactosidase on aminated silica particles using glutaraldehyde was carried out by Gonzalez et al. [24] The maximum activity on immobilization was observed when the particle had a specific surface area of 122 m^2/g and immobilization was carried out at pH 8.6 using 1.6 g protein /L.

Fungal β-galactosidase on silicon dioxide matrices with surface area of 70 – 100 m^2/g was carried out by Samoshina et al. [25] using glutaraldehyde .The method was found to be compatible with use in the food industry . When β-galactosidase 60 g with a specific activity of 360 units/g was immobilized on 100 g SiO_2 the specific activity of the preparation was 220 units/g . On further use in a column for the hydrolysis of deproteinized whey 95-100 % lactose hydrolysis was achieved maintaining stable activity at least for 36 h. The half-life of the immobilized system was calculated as 136 h. The immobilized enzyme showed greater efficiency for the production of glucose – galactose syrups from dairy products.

Porous glass has been used as a support for covalent attachment of β-galactosidase by Arora et al. [26] They observed that optimum temperature of

incubation , pH stability of the immobilized enzyme were similar to the native enzyme although K_m values of the immobilized enzyme were higher than those of the native enzyme with ONPG as a substrate . The immobilized enzyme was much more stable at 4°C and 37°C as compared to native enzyme.

β-Balactosidase has been immobilized on silanized silica gel by Kozhukharova et al. [27] by covalent binding using glutaraldehyde . The optimum pH values of 4.5 and optimum temperature of 55°C were established for the immobilized enzyme. Immobilization lead to stable enzyme preparation which was suitable for repeated application in lactose hydrolysis .They observed 80-85 % lactose hydrolysis.

β-galactosidase immobilized on a range of supports was reported to show suitable physicochemical characteristics for use in fluidized bed reactor by Bodalo et al.[28] Uncoated porous glass, alginate and K-carrageenan beads and chromosorb were used as carriers . The authors observed that highest percentage of immobilized protein and activity yielded when β-galactosidase was attached through its amine groups to aldehdye glass.

β-galactosidase was immobilized in a crosslinked poly (N-isopropyl acrylamide-co- acrylamide) hydrogel which exhibits an LCST behavior by Tac-Gwan [29]. The stability of immobilized enzyme was investigated at different temperatures which allowed different degrees of collapse in the hydrogels matrix. It was hypothesized that the immobilized enzyme was more stable in the collapsed matrix due to the physical restraint imposed on the enzyme entrapped.

β-galactosidase has been immobilized in triacetate fibers by Shulchishin et al. [30] The equilibrium and kinetic constants were calculated using computerized data of kinetic experiments performed according to a randomized block plan. It was observed that the kinetic parameters obtained were rather well reproduced in

lactose hydrolysis in milk whey both in a stirred tank reactor and in a column reactor with intensive substrate recycling.

β-galactosidase from Aspergillus oryzae was immobilized with PU foam (PVF) by Hu et al. [31] . The immobilization was carried out by adsorption , entrapment and covalent binding. Among several immobilization methods attempted here , the immobilized enzyme preparation by in-situ copolymerization between enzyme and pre-polymer HYPPL 3000 showed the highest activity.

Carrara et al. [32] used chitosan for immobilization of β-galactosidase . They observed that best results were obtained on chitosan beads of 2.2 mm diameter where the immobilization was carried out at 37°C using 1 % glutaraldehdye concentration . The higher activity values of the immobilized enzyme compared to those of free β-galactosidase were obtained over a large pH range and were at higher pH's and temperature but the highest activity was only 10.7 % of the free enzyme values.

Lopez et al. [33] carried out the immobilization of β-D-galactosidase from Kluyvermyces lactis on nylon – 6 microbeads and used it to hydrolyze lactose in skim milk using a novel spin basket reactor. The observed 75 % of lactose was hydrolyzed at 34°C within a short –space time without experiencing any pugging such as typically seen in packed columns.

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β-galactosidase (E.C.3.2.1.23) was immobilized onto a gelatin carrier system through crosslinking by Singur et al. [34] Glutaraldehdye and chromium (III) acetate were used as parameters and reusability of the immobilized enzyme were investigated . To obtain a better relative activity , a method was developed and further modified to increase the surface area of the support system by using polyacrylamide . Relative activity , enhancement of 68 % was achieved in the gelatin –glutaraldehyde system

β-galactosidase was immobilized on the surface of silica gel in the presence of polysaccharides derived from starch by Atyaksheva et al. [35] It was observed that immobilization not only increases the catatytic properties of the enzymes but also its stability . Moreover, the immobilized enzyme was active over greater pH range than free enzyme.

Copper , nickel , zinc, iron and calcium in the different chelating gels were used for immobilization of β-galactosidase by Brena et al.[36] The chelated ligand [Cu^{+2} –imino diacetate methyl (IDA)] , Cu^{+2} –Tris (carboxy methyl) ethylene diamine (TED) and Ni^{+2} derivative were more stable to high temperature and to storage than Cu^{+2} derivatives .Moreover , the temperature stability of the immobilized enzyme was improved on addition of strong metal-chelating gels such as carboxy methylated tetra ethylene pentamine-agarose .

Poly (vinyl alcohol)crosslinked with para-formaldehyde (PVA-F) and natural polysaccharides chitosan in bead form and salicylic acid-resorcinol-formaldehyde polymeric resin (SRF) in powder form were used for immobilization of β-galactosidase through covalent linkages by Rejikumar and Devi[37].The immobilized enzyme showed better storage stability at room temperature . No loss in enzyme activity was observed after 10 cycles when enzyme immobilized chitosan beads were taken for repeated use. Moreover the effect of Michaelis constant K_m and maximum reaction velocity V_{max} and energy of activation (Ea) was also examined for free and immobilized enzyme.

Kravchenko et al.[38] checked the inclusion of β-galactosidase in poly-N-caprolactum . It was observed that 80 % of initial activity of enzyme remained in presence of resorcinol and stabilizer PVC and PEG 4000. Optimum pH, thermal stability and kinetic parameter were also investigated.

Trevisan et al. [39] used controlled pore silica for immobilization of β-galactosidase. Immobilization was performed on amino silica activated with glutaraldehdye and

the product was used for hydrolysis of lactose of whey. The behaviour of the soluble and immobilized enzyme was compared by using whey and a lactose solution as the substrate with the aim of optimizing the method. Parameters such as amount of glutaraldehdye and the size of the particles were evaluated by comparing activities and stability's in batch and continuously fluidized bed reactors.

β-galactosidase was immobilized by ionic adsorption on DEAE-Sepharose CL-6 by Zamich et al. [40].The immobilized enzyme retained 40 – 66 % of its activity and retained 100 percentage of its activity on storage at 4°C for 2 month and lost 50 % of its activity after 34 days at 25 °C. When subjected to lactose hydrolysis 80 % of conversion was achieved using column bio-reactor at 5 % lactose for 3 days at 2 °C .

β-galactosidase was immobilized on poly (hydroethylmethacrlate) membrane by Arica et al. [41] It was observed that immobilized enzyme activity increased from 1.5 to 10.8 U/g upon increase in enzyme loading . The K_m values for the free and the entrapped enzymes were found to be 0.26 and 0.81 mM respectively . The optimum temperature was found to be 50°C and optimum pH was found to be 7.5 for free and entrapped enzyme. The immobilization of β-galactosidase was characterized in a continuous system during lactose hydrolysis and the operational inactivation rate constant of the entrapped enzyme was found to be 3.1×10^{-5} min^{-1} .

Edin et al. [42] discussed the dependence of grafting and catalytic activity on the pore size of nylon membrane grafted with different DGMA concentration and loaded with β-galactosidase . It was observed that all experimental parameters decreased with increase in membrane pore size.

Sungur et al.[43] used gelatin, –CM-cellulose as carrier systems crosslinked with chromium acetate for β-galactosidase immobilization . It was observed that the

relative activity increased by 33 % on addition of CM-cellulose to gelatin. The effect of operational pH on relative activity and reusability of immobilized was investigated.

Szczodrak et al. [44] immobilized β-galactosidase from kluyveromyces fragilis on silanized porous glass modified by glutaraldehyde binding , with retention of more than 90 % of its activity . Marked shifts in optimum pH (7-6) , and temperature (35 -50°C) of the solid phase enzyme were observed together with high catalytic activity and reasonable stability at pH and temperature range from those of the free enzyme.

Lactose and milk whey hydrolysis using fixed bed reactor was carried out by Rejikumar and Devi [45]. β-galactosidase from Aspergillus oryzae was immobilized onto either cross-linked poly(vinyl alcohol) or natural polysaccharide chitosan . The optimum pH and temperature for the immobilized system in the reactor was observed to be 5 and 50-60 °C. The kinetic parameters were found to be K_m=7.1 x 10-3 and V_{max}=5.2 x 10-3M at 50°C and pH 5 in citrate-phosphate buffer. They observed almost (95 %) hydrolysis of lactose under optimized conditions . The immobilized systems were observed to retain their activity even after 12 – 30 cycles.

The behavior of five different hydrophobic β-galactosidase derivatives, obtained by grafting different amount of butyl-methaacrylate (BMA) on planar nylon membrane , under isothermal and non-isothermal conditions was studied by El Mastry et al.[46] Under isothermal conditions the effect of the grafting percentage on the enzyme activity was examined as a function of pH , temperature and substrate concentration . It was found that the apparent K_m values linearly increase with the increase in the grafting percentage , while the apparent V_{max} exhibits a maximum value.

β-galactosidase was immobilized on tosylated cotton cloth by Albayrak et al. [47]

Immobilization of Aspergillus oryzae β-galactosidase on cotton activated with p-tolyl sulphonyl chloride was by immobilization of enzyme on the tosylated cotton followed by a nucleophilic substitution . Mechanism is an evidence by uv spectra. NaOH mercerization before tosylation and adding pyridine at a tosyl (g/pyridine) (ml) ,ratio of 0.2 -0.4 increased the final activity of the immobilized enzyme by six-fold. The optimal pH for enzyme immobilization was found to be 4.5 when tested with different fibrous matrices tested. The knitted cotton cloth showed the highest immobilization enzyme activity. The enzyme immobilized on cotton cloth can be used in plug-flow reactor for continuous production of galacto-oligosaccharides from lactose . The reactor performances was stable , and there was immobilized enzyme also showed same pattern and level of (GOS) for lactose as those from free enzyme reaction, kinetic was got affected by immobilized enzyme reactors. The method to prepare tosylated cotton for enzyme immobilization is simple , inexpensive and scaleable for industrial applications.

β-galactosidase was immobilized effectively on a porous ceramic monolith by adsorption and intermolecular crosslinking by Papayannakos et al. [48] Maximum enzyme loading was found to be 80 % without any enzyme leaching even under vigorous agitation. The kinetics of lactose hydrolysis by both free and immobilized lactose was studied in a batch reactor system in absence of any mass transfer limitation.

β-galactosidase has been immobilized on graphite surface by Zhou et al. [49] The deactivation rate was found to follow the Arrhenius law with the activation energy of about 200 KJ/mol for both the free and immobilized enzyme. Moreover the mechanism of the hydrolysis process was discussed.

4.3. Experimental :

4.3.1. Chemicals :

β-galactosidase(E.C.3.2.1.23) from aspergillus oryzea of strength 15 U/mg.	Sigma Chemical , U.S.A. St. Louis MO 63103.
o-nitrophenyl β-D-galacto-pyranoside (ONPG)	
Glutaraldehyde	S.D. fine. Chem.Ltd .Boisar, India.
Lactose	
Acrylamide	Qualigens , Mumbai , India.
Follin reagent	
1,4 dioxane	
K-carrageenan	As a gift from Central Salt and Marine Chemical Research Institute ,Bhavnagar, India
Ascorbic Acid	Loba. Chemie, Mumbai, India.
p-benzoquinone	
2-hydroxyl ethyl methaacrylate (HEMA)	Fluka Chemie , Switzerland
Pyridine	Sulab , Baroda , India.
Sodium Carbonate	

4.3.2. Assay of β-galactosidase :

Assay of β-galactosidase was carried out by the method given by Cravens [50] using o-nitrophenyl -β-D-galactopyranoside as a substrate at 40 °C and using citrate phosphate buffer of strength 0.2 mM at pH 4.5. The reaction was stopped by using 2 ml of 1M Na_2CO_3 and the liberated o-nitrophenol was measured spectrophotometrically at 410 nm.

Lactose + H_2O $\xrightarrow{\text{β-galactosidase}}$ β-D-Galactose + D-glucose

4.3.3. Supports used for immobilization :

The natural support K-carrageenan used for entrapment of β-galactosidase was obtained as a gift from CSMCRI Bhavanagar, India and the preparation of beads was carried out as described in **sec.2.3.2.** and the covalent coupling to the copolymer of AAm-HEMA was carried out by using the method discussed in **sec.2.3.2.b.**

4.3.4. β-galactosidase Immobilization :

Entrapment of β-galactosidase in K-carrageenan beads was carried out and the effect of PEI concentration and enzyme loading was carried out as discussed in **sec.2.3.4.a**

The covalent coupling of β-galactosidase was carried out on copolymer of AAm-HEMA by activating only NH_2 or OH groups individually and also both simultaneously using different activating agents.

Activation of amino groups was carried out using 0.1 to 4 % of glutaraldehyde concentration , 1 – 8 h activation time , 1 – 8 h enzyme coupling time , enzyme

coupling pH 1 - 8 , and enzyme concentration from 1 to 10 mg . The activation of OH group was carried out by using PBQ and PTS respectively.

The effect of p-benzoquinone was examined by varying p-benzoquinone concentration from 0.15-0.1 g , activation time 1-8 h , enzyme coupling pH 1-6 , enzyme coupling time 1-14 h and enzyme concentration 0.5-10 mg.

The activation of hydroxyl group was also carried out by using p-tolyl sulphonyl chloride by varying its concentration from 0.15 –0.1 g , activation time from 0.5-8 h , enzyme coupling pH from 1-6 , enzyme coupling time of 2-10 h. The extent of enzyme loading was studied by varying the enzyme concentration from 0.5-10 mg of enzyme .

The simultaneous activation of OH and NH_2 groups was carried out using the optimized concentration of glu and pbq . The optimized glutaraldehyde concentration was taken and the PBQ activation time was varied from 1 - 10 h. The enzyme coupling time used was 1 - 14 h , and enzyme concentration was varied from 0.5-12 mg and the coupling pH was examined from 1-8.

4.3.5. Comparative study of free and immobilized β-galactosidase :

Every enzyme has a optimum pH for its best activity . Hence , stability of free and immobilized enzyme at various pH was examined by varying the pH from 1 - 7 at 40^0C to examine whether there is any change in optimum pH upon immobilization .

The storage stability of both forms of enzyme was carried out at 35^0C to examine the stability of enzyme upon storage and the residual activity was measured after different time intervals by using the assay method.

Kinetic parameters play an important role in immobilization of enzymes as conformational change may occur on immobilization and the affinity between enzyme and substrate may change. Hence the kinetic parameters K_m and V_{max} of free and immobilized enzymes were determined by using the Lineweaver Burk plots by first keeping the enzyme concentration constant and varying substrate concentration and also by varying the substrate concentration and keeping enzyme concentration constant .

The reusability of both entrapped and covalently bound enzyme was checked using 2mM ONPG as a substrate . The activity of immobilized system was measured till it lost 50 % of its activity.

4.3.6. Lactose Hydrolysis:

Lactose is a main component of the milk and whey and its concentration is about 6 % . Many people all over the world have deficiency of lactase enzyme in small intestine and when they take food containing high lactose concentration they are not able to hydrolyze it as the enzyme lactase which hydrolysis it is in less amount and hence it causes problems of lactose intolerance. Moreover lactose is also obtained as a byproduct of dairy industry and hence it also affects the environment [51,52,53]. The hydrolysis of lactose gives glucose and galactose . The hydrolysis of lactose is of considerable interest as the product of hydrolysis are in combination , sweeter , more soluble and directly absorbed from mammalian intestine. There are two methods for hydrolyzing lactose first by acid hydrolysis and second by enzymatic hydrolysis. The main advantage of using hydrolyzing enzyme is that it lowers the lactose content without adversely

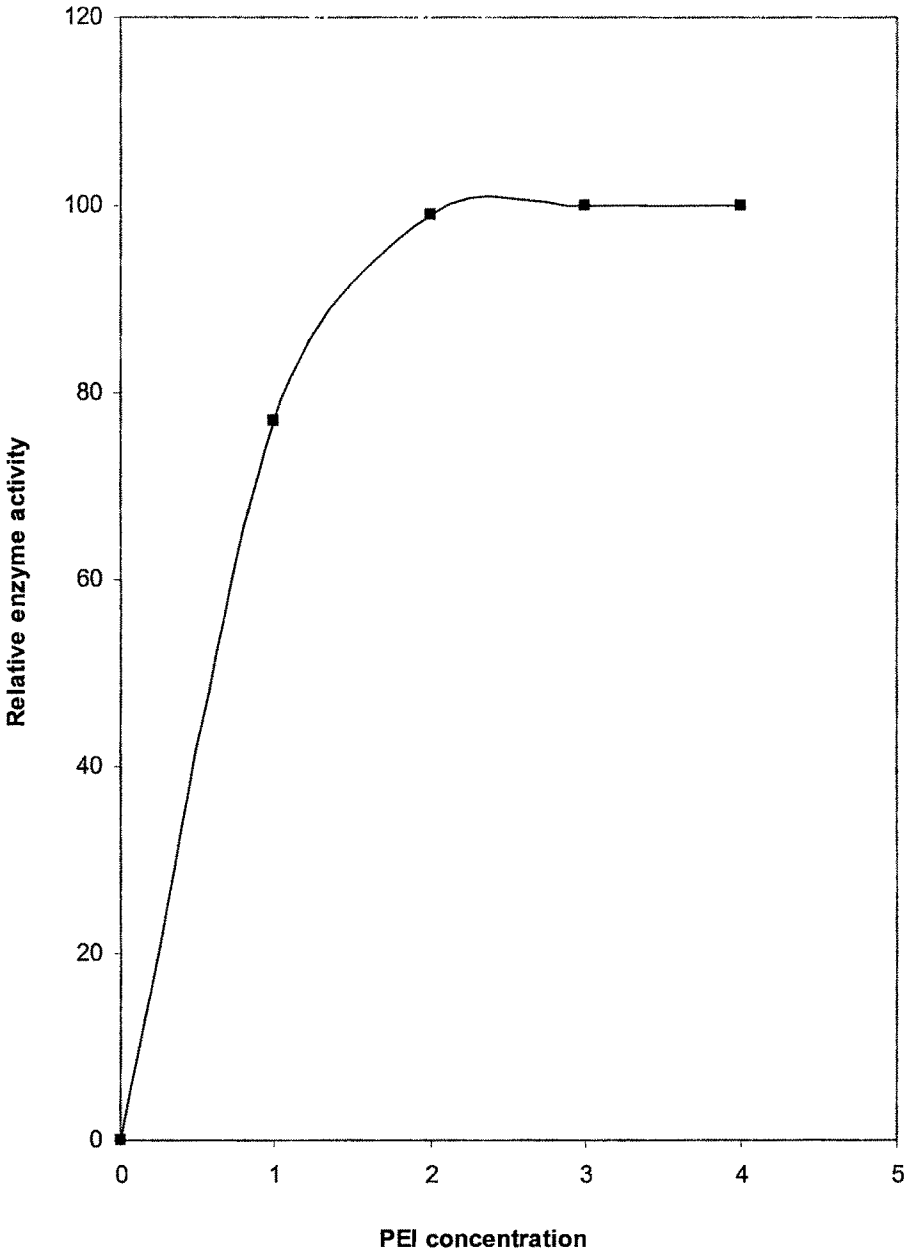

Figure 4.1. Dependence of enzyme activity on PEI concentration at 40 °C .

affecting the protein an other component in whey. The lactose hydrolysis was carried out by using fixed bed reactor as described in **sec 2.3.9.** Lactose hydrolysis was carried out by using 1 mM solution of lactose at different flow rate . The reactor parameters such as flow rate , lactose concentration , l/d ratio and temperature were also optimized.

4.4. Results and Discussion :

Immobilization of enzyme has been carried out by both physical and chemical methods . The entrapment of β-galactosidase was carried out in K-carrageenan by using PEI as hardening agent while the covalent coupling was carried out by using different activating agents such as glutaraldehdye , p-benzoquinone and p-tolyl sulphonyl chloride.

4.4.1. Effect of PEI concentration :

K-carrageenan beads were tested for hardening using 1-4 % PEI concentration and it was observed that beyond 2% PEI concentration and 2 h hardening time there was no further increase in hardening of the beads (**Figure 4.1**) with respect to PEI concentration.

4.4.2. Enzyme concentration :

The effect of enzyme loading on K-carrageenan beads was carried out by using 1 – 10 mg of β-galactosidase. It was observed that the enzyme loading increased with concentration and was found to be 771 μg/g of support at 7.5 mg . It was observed that the percentage immobilization increased with enzyme loading and reached upto 10 % of 7.5 mg enzyme concentration. **Table 4.1** shows the enzyme activity and percentage retention.

4.4.3.Covalent coupling conditions :

The copolymer of AAm-HEMA contains both hydroxyl and amino groups. The NH_2 group was activated using 10 ml of 2 % w/v solution of glutaraldehdye for 4 h followed by enzyme coupling for 14 h at pH 4 Maximum activity of enzyme retained on support was observed to be 118μg/g .

The OH groups were activated using 10 cm^3 of 5 mg 1 ml of p-benzoquinone solution for 6 h and coupling it with enzyme for 10 h at pH 4 to obtain maximum activity of 61 μg/g . The OH groups were also activated with 10 cm^3 of 5 mg / g p-tolyl sulphonyl for 6 h and then coupling with enzyme for 6 hr at pH 4 to get 41 μg/g enzyme coupling. The PBQ showed better activity compared to PTS and hence was used for further study. The simultaneous activation of both groups was also carried out and the results are given in **Table4.2. and Figure 4.2 – 4.6.**

4.4.4.Comparative study of Free and immobilized enzyme :

From the pH study we can come to know whether there is some change in the active site of enzyme during immobilization **Figure 4.7.** It was observed that the free, entrapped and covalently bound enzyme all showed optimum activity at pH 4. Thus indicating that there is no change in active sites on immobilization.

The storage stability of free and immobilized system was checked at 35°C and it was found that the free enzyme lost all its activity within 10 days , whereas entrapped enzyme showed 90 % of its activity after 15 days and the covalently bound enzyme showed 80 % of its activity after 15 days as shown in **figure 4.8.**

Table 4.1. Effect of enzyme loading on percentage retention of enzyme activity .

Enzyme taken for entrapment in mg (U)	*Total Protein Content in the Beads mg*	*Active Protein in beads mg*	*Percentage retention of enzyme activity in beads*
1 (200)	*0.287*	*0.076*	*26*
3 (600)	*0.436*	*0.153*	*35*
5 (1000)	*1.78*	*0.305*	*17*
7.5 (1500)	*2.648*	*0.771*	*29*

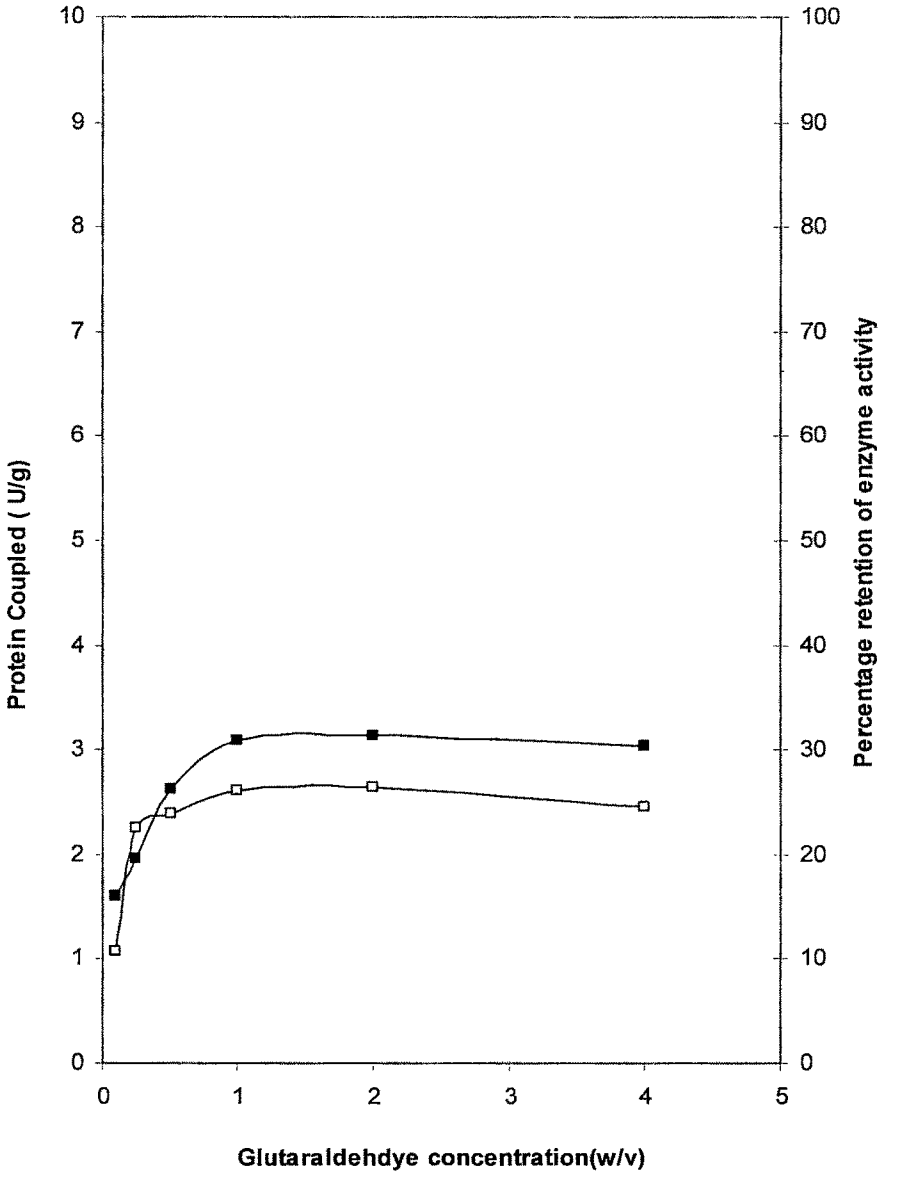

Figure 4.2. Effect of glutaraldehyde concentration on retention of enzyme activity.

Protein Coupled (■), Retention of enzyme activity (□)

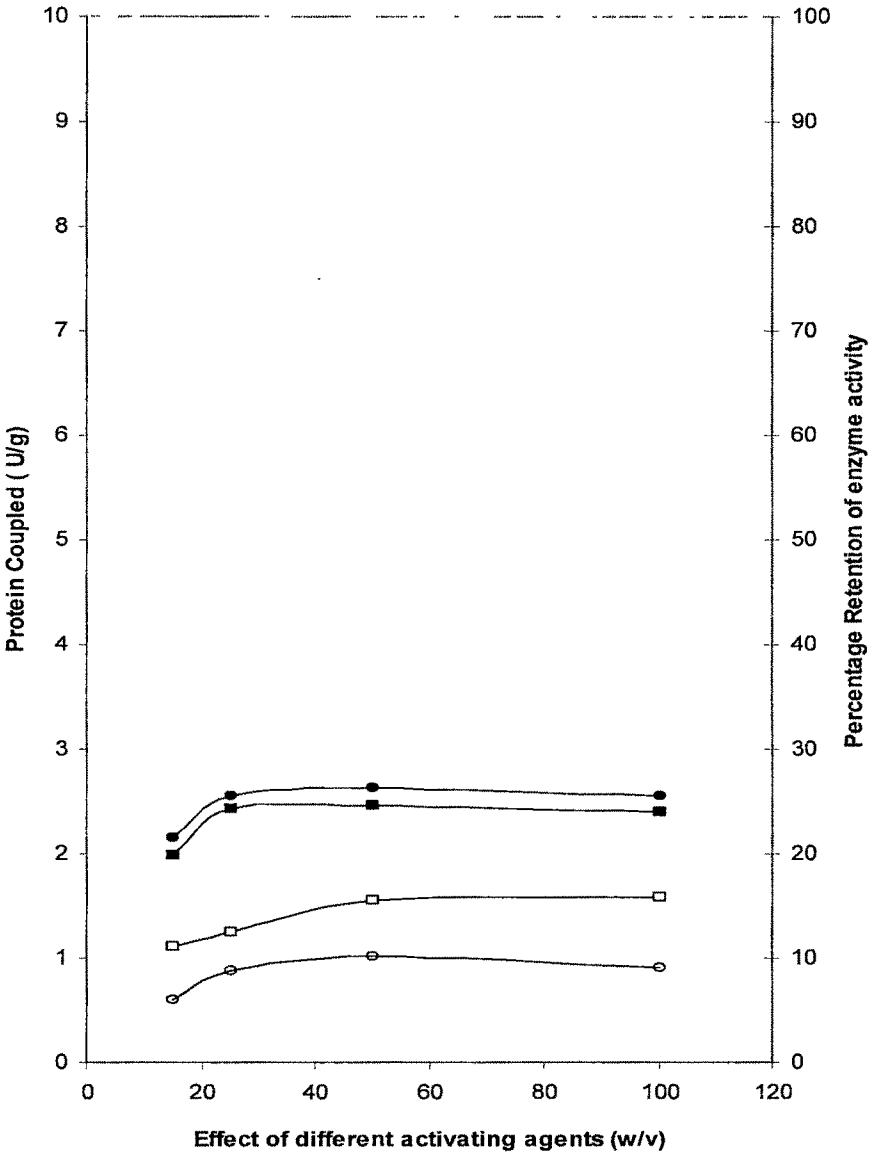

Figure 4.3.Effect of Different activating agents :

PBQ : Protein Coupled (■), retention of enzyme activity (□).

PTS : Protein Coupled (▲), retention of enzyme activity (Δ).

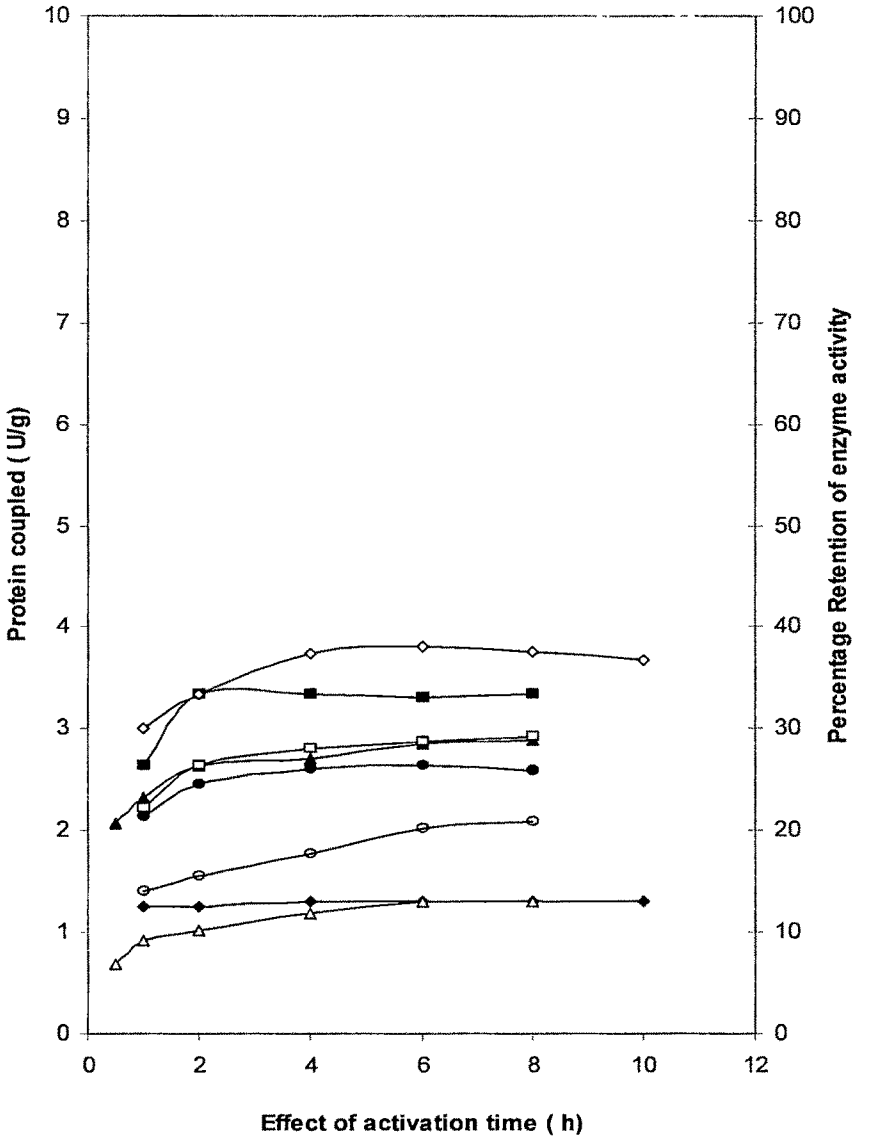

Figure 4.4. Effect of activation time on retention of enzyme activity.

GLU : Protein Coupled (■), retention of enzyme activity (□).

PBQ : Protein Coupled (●), retention of enzyme activity (○).

PTS : Protein Coupled (▲), retention of enzyme activity (Δ).

GLU+PBQ : Protein Coupled (◆), retention of enzyme activity (◇).

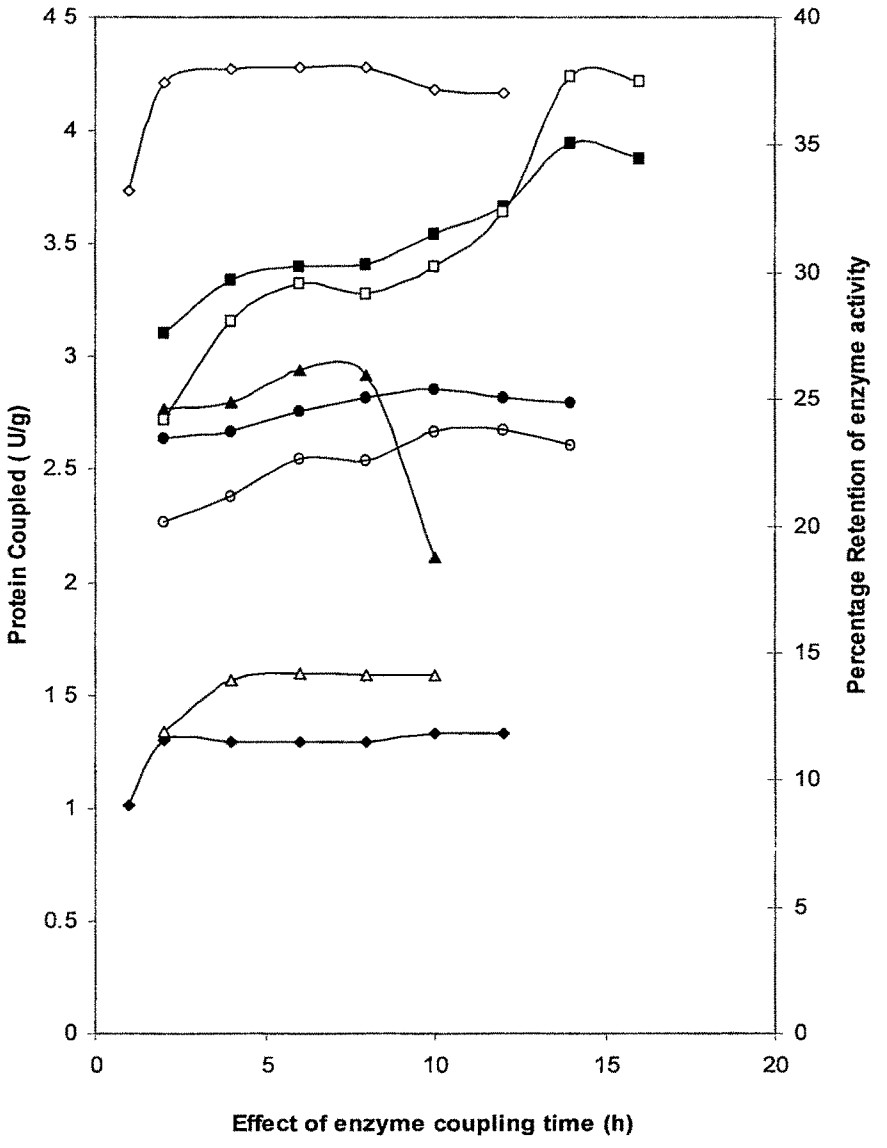

Figure 4.5. Effect of enzyme coupling time

GLU : Protein Coupled (■), Retention of enzyme activity (□).

PBQ : Protein Coupled (●), retention of enzyme activity (o).

PTS : Protein Coupled (▲), retention of enzyme activity (Δ).

GLU+PBQ : Protein Coupled (◆), retention of enzyme activity (◇).

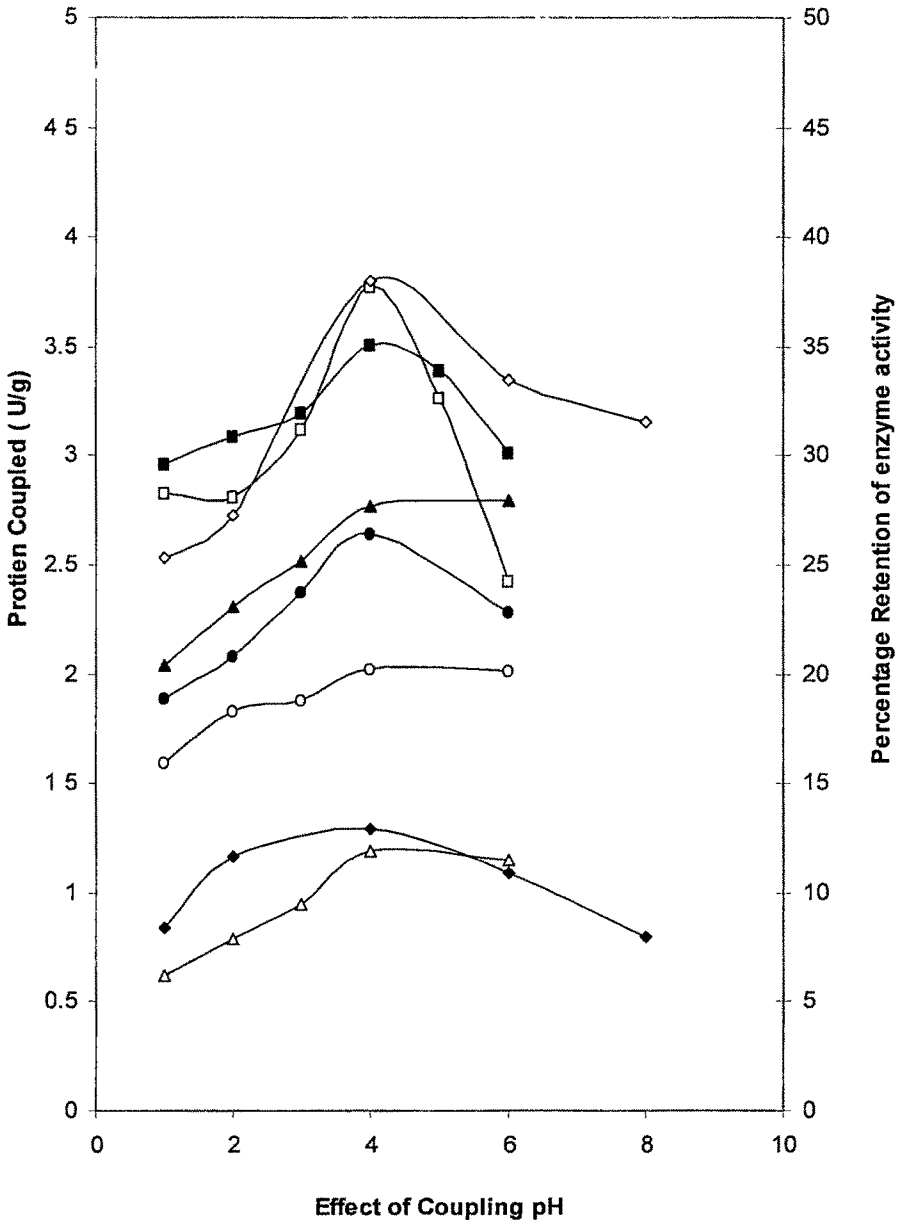

Figure 4.6. Effect of enzyme coupling pH .
GLU : Protein Coupled (■), Retention of enzyme activity (□).

PBQ : Protein Coupled (●), retention of enzyme activity (○).

PTS : Protein Coupled (▲), retention of enzyme activity (Δ).

GLU+PBQ : Protein Coupled (◆), retention of enzyme activity (◇).

Table 4.2. Effect of different activating agents on covalent coupling of enzyme.

Coupling Condition	Glutarald-aldehdye	Benzo-Quinine	Glutaraldehyde + p-Benzoquinone	p-Benzoquinone + Glutaraldehyde
Activating Agent Concentration	10ml of 2% (w/v)	50mg in 10 ml	10ml of 2% (w/v) + 50 mg in 10 ml	50mg in 10 ml + 10ml of 2% (w/v)
Activation Time (h)	4	6	4+4	4+4
Bound enzyme Concentration (μg/g)	118	61.2	364.74	122
Enzyme coupling time (h)	14	10	6	6
Coupling pH	4	4	4	4

The kinetic parameters K_m and V_{max} were obtained from Lineweaver Burk Plot from **Figure 4.9.a,b** . The values are given in **Table 4.3**. From the results we can see that there is no conformational change during immobilization.

The durability of entrapped and covalently bound enzyme was checked and it was found that entrapped enzyme lost 50 % of its activity after 5 cycles whereas covalently bound enzyme lost 50 % of its activity after 7 cycles as shown in **figure 4.10.**

4.4.5.Lactose hydrolysis :

Lactose hydrolysis was carried out by using both entrapped and covalently bound enzyme . Effect of different reactor parameters were studied by varying the flow rate from 1 – 8 cm^3 / min for both entrapped and covalently bound enzyme. The amount of lactose hydrolyzed was measured by determining the amount of glucose liberated during lactose hydrolysis by glucose- oxidase-peroxidase method and the product was measured at 510 nm [54,55]. It was observed that with the increase in flow rate from 1 - 8 cm^3 / min the percentage hydrolysis of lactose decreased. However , the covalently bound enzyme showed higher percentage hydrolysis of lactose compared to entrapped enzyme . This may be as covalent binding involves formation of bond between enzyme and support while entrapment is a physical process. Hence the bond formed in covalent binding are more strong than entrapment. The results are given in **figure 4.11.**

The effect of lactose concentration on hydrolysis was checked and it was found that with the increase in lactose concentration the percentage hydrolysis decreased **(Figure 4.12.)** Here also covalently bound enzyme showed better hydrolysis compared to free one. Maximum hydrolysis was observed at 1 mM

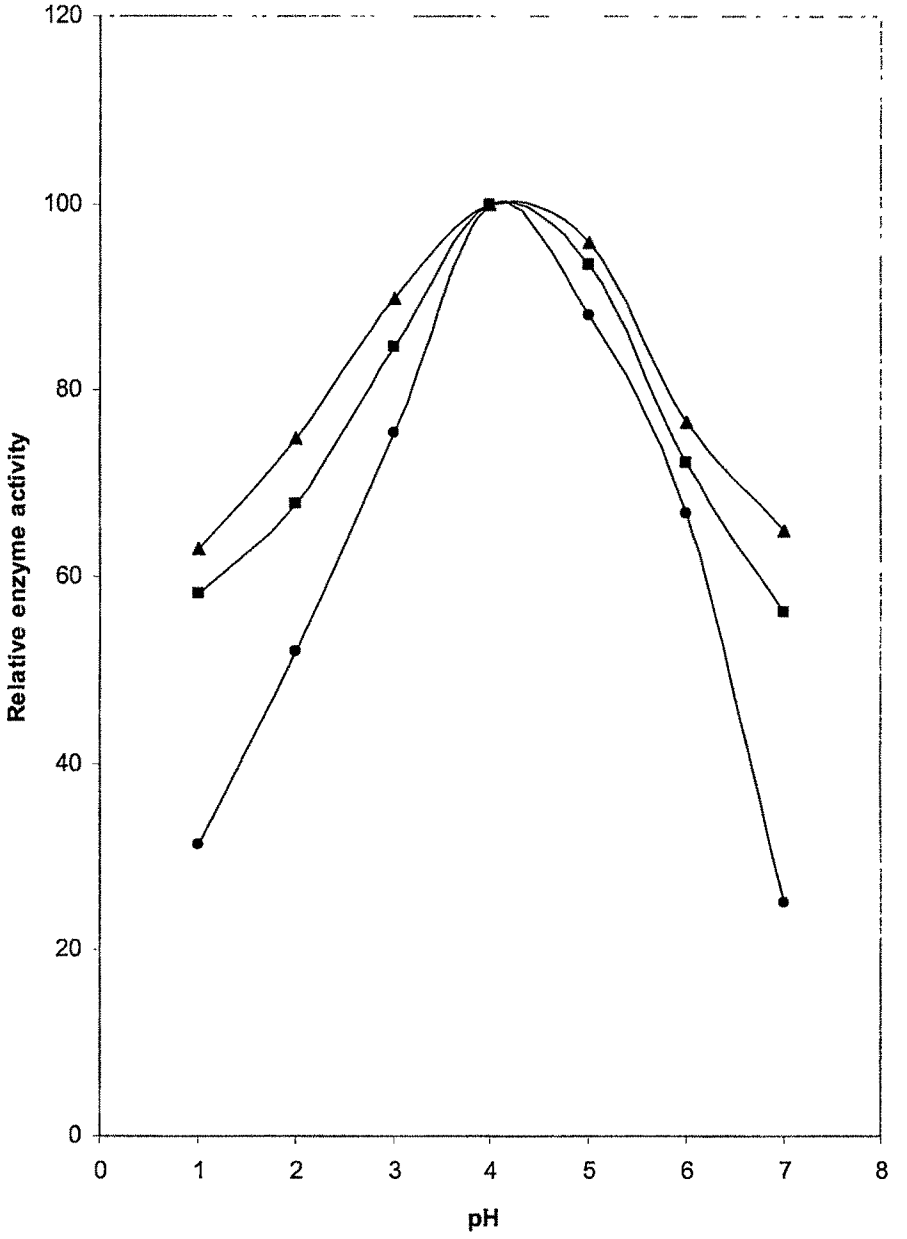

Figure 4.7. Effect of pH on free and immobilized enzyme.

Free (●), entrapped enzyme (■) and Covalently bound enzyme (▲).

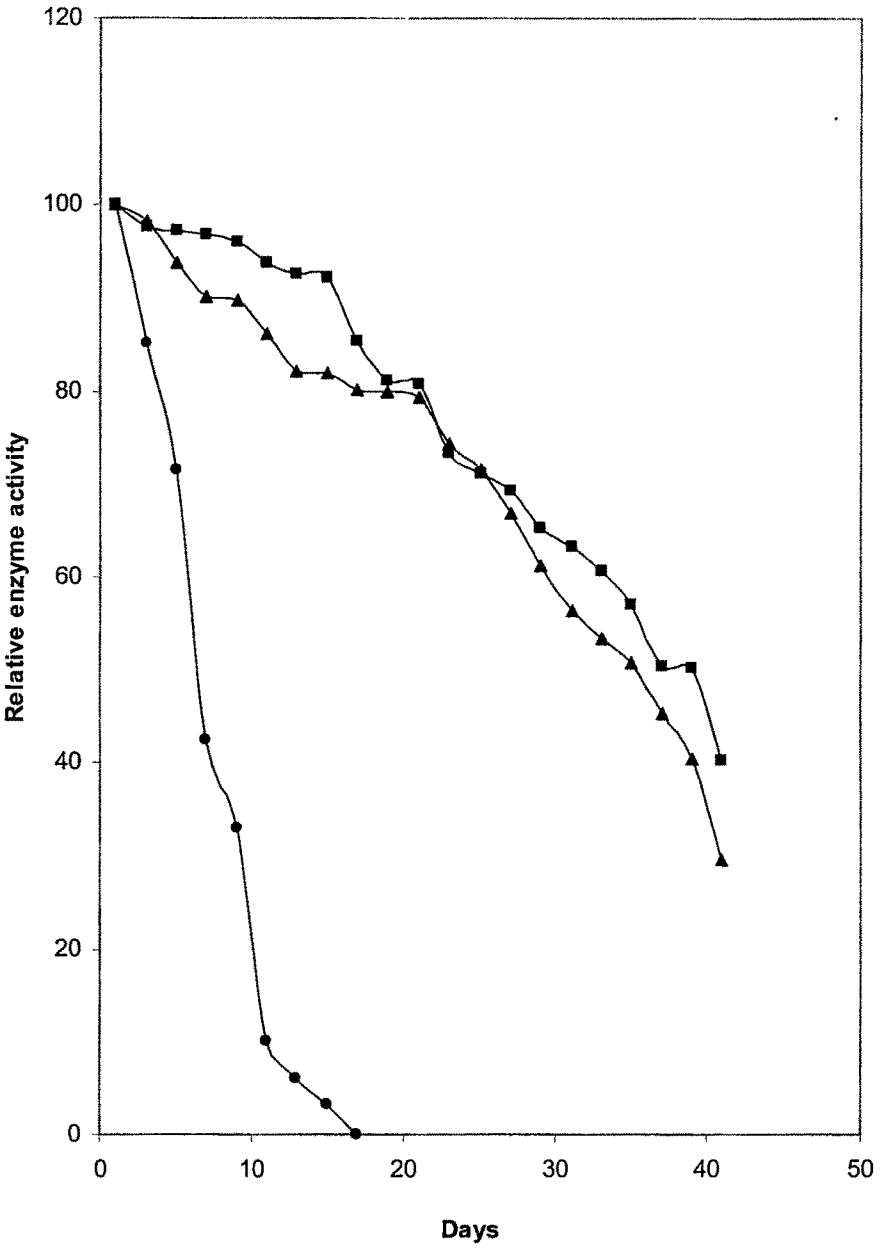

Figure 4.8. Storage stability of free and immobilized enzyme.

Free (●), entrapped enzyme (■) and Covalently bound enzyme (▲).

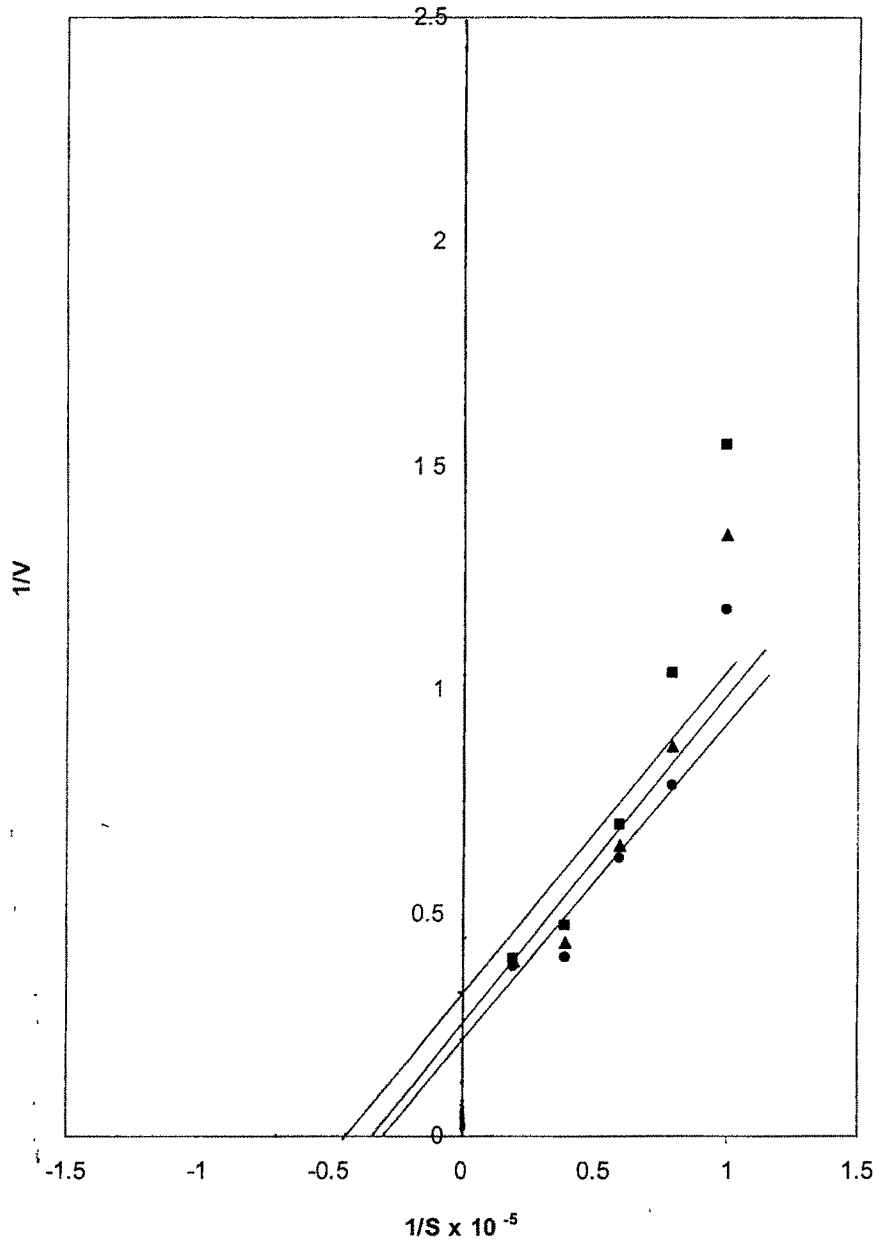

Figure 4.9. (a) Lineweaver Burk plot at 40°C , pH 4 for 1 min .When enzyme concentration is constant and substrate concentration is varied .

Free (●) , entrapped enzyme (■) and Covalently bound enzyme (▲).

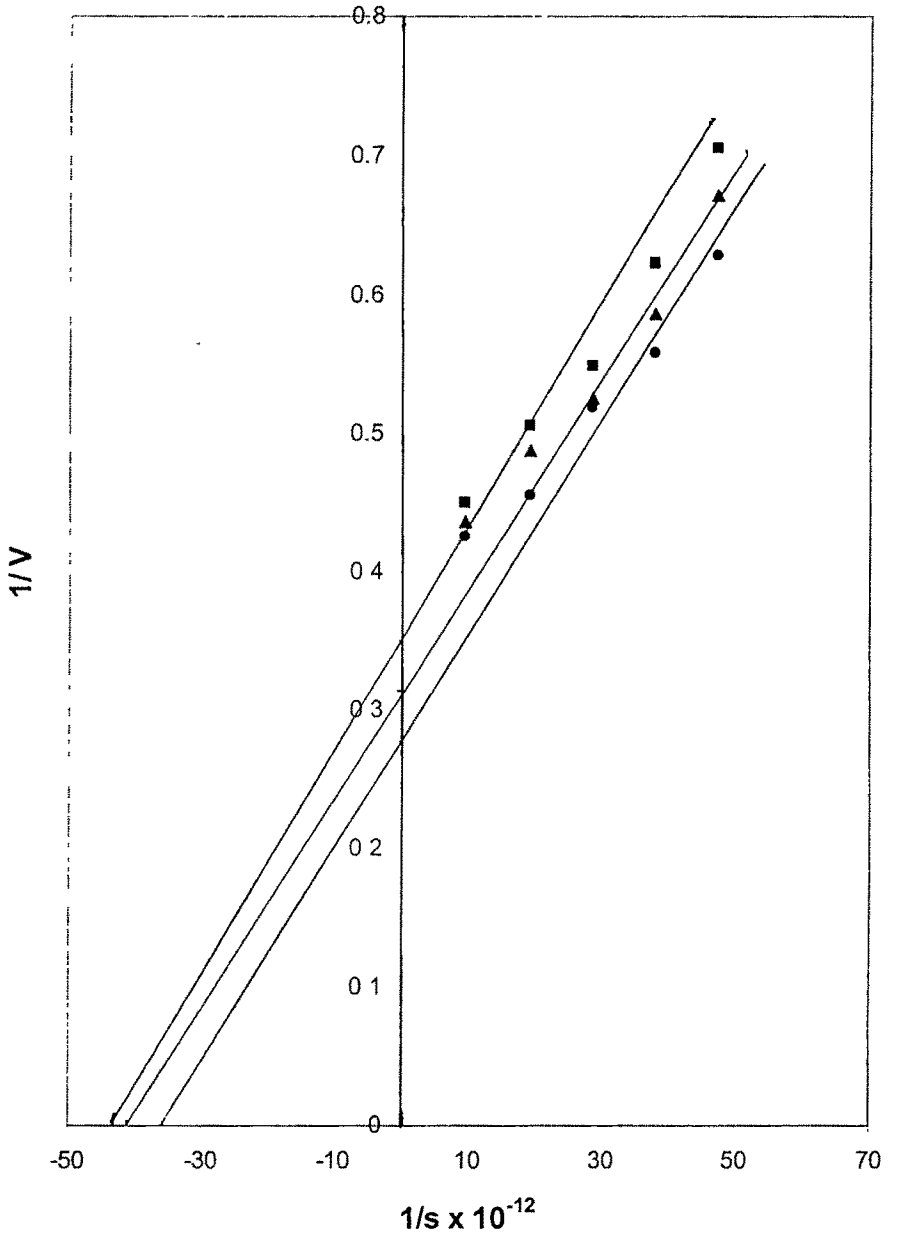

Figure 4.9.(b) Lineweaver Burk plot at 40°C , pH 4 for 1 min .When substrate concentration is constant and enzyme concentration is varied .

Free (●) , entrapped enzyme (■) and Covalently bound enzyme (▲).

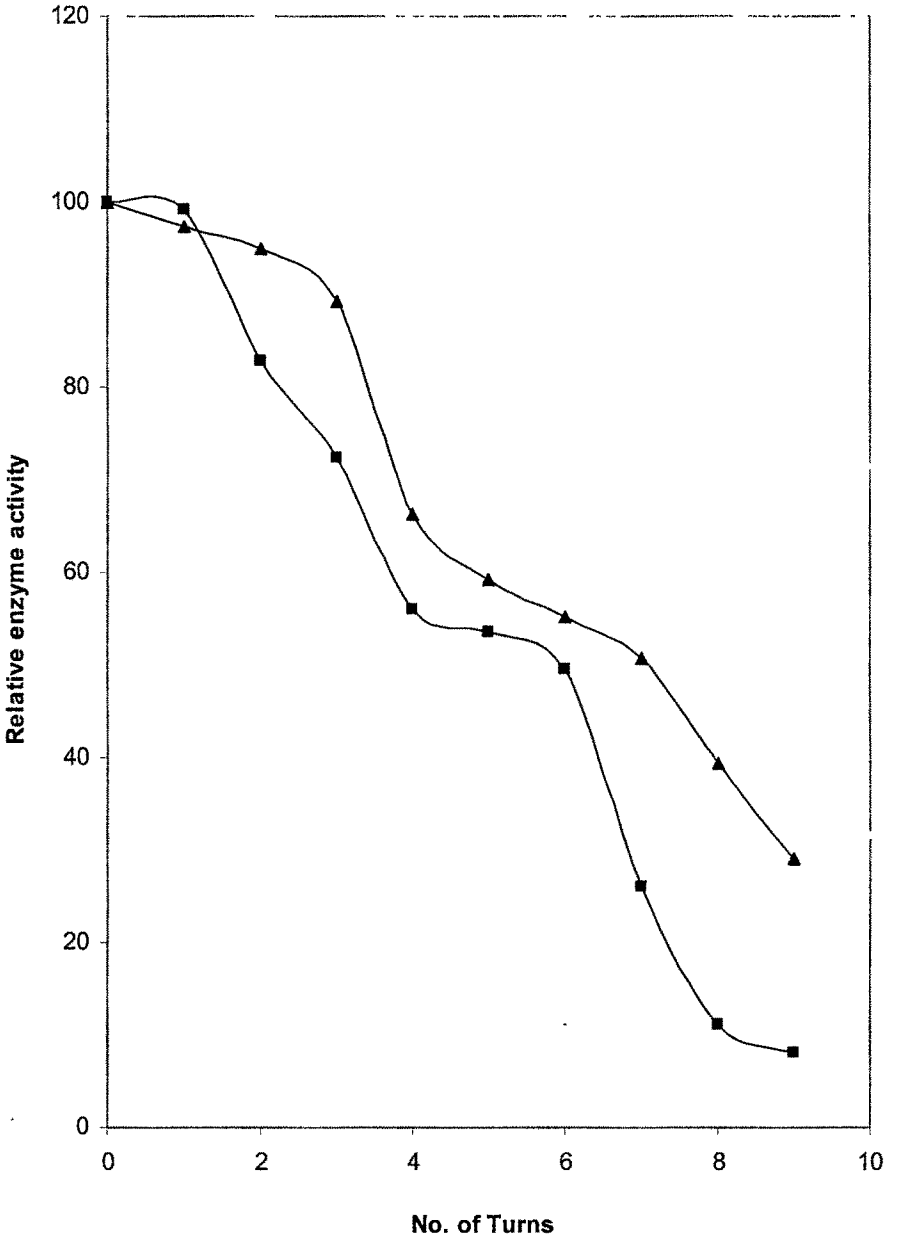

Figure 4.10. Stability of entrapped (■)and covalently bound enzyme(▲).

Table 4.3. Kinetic parameters of free and covalently bound enzyme.

*Conditions	Free enzyme		Entrapped enzyme		Covalently bound enzyme	
	K_m (mM)	V_{max} (mM/min)	K_m (mM)	V_{max} (mM/min)	K_m (mM)	V_{max} (mM/min)
When substrate con Is varied and enzyme concentratio n is varied.	0.22×10^{-5}	0.16	0.38×10^{-5}	0.27	0.27×10^{-5}	0.20
When enzyme Concentratio n Is varied and substrate concentratio n is varied .	3.44×10^{-3}	0.26	4.3×10^{-13}	0.33	4.0×10^{-13}	0.30

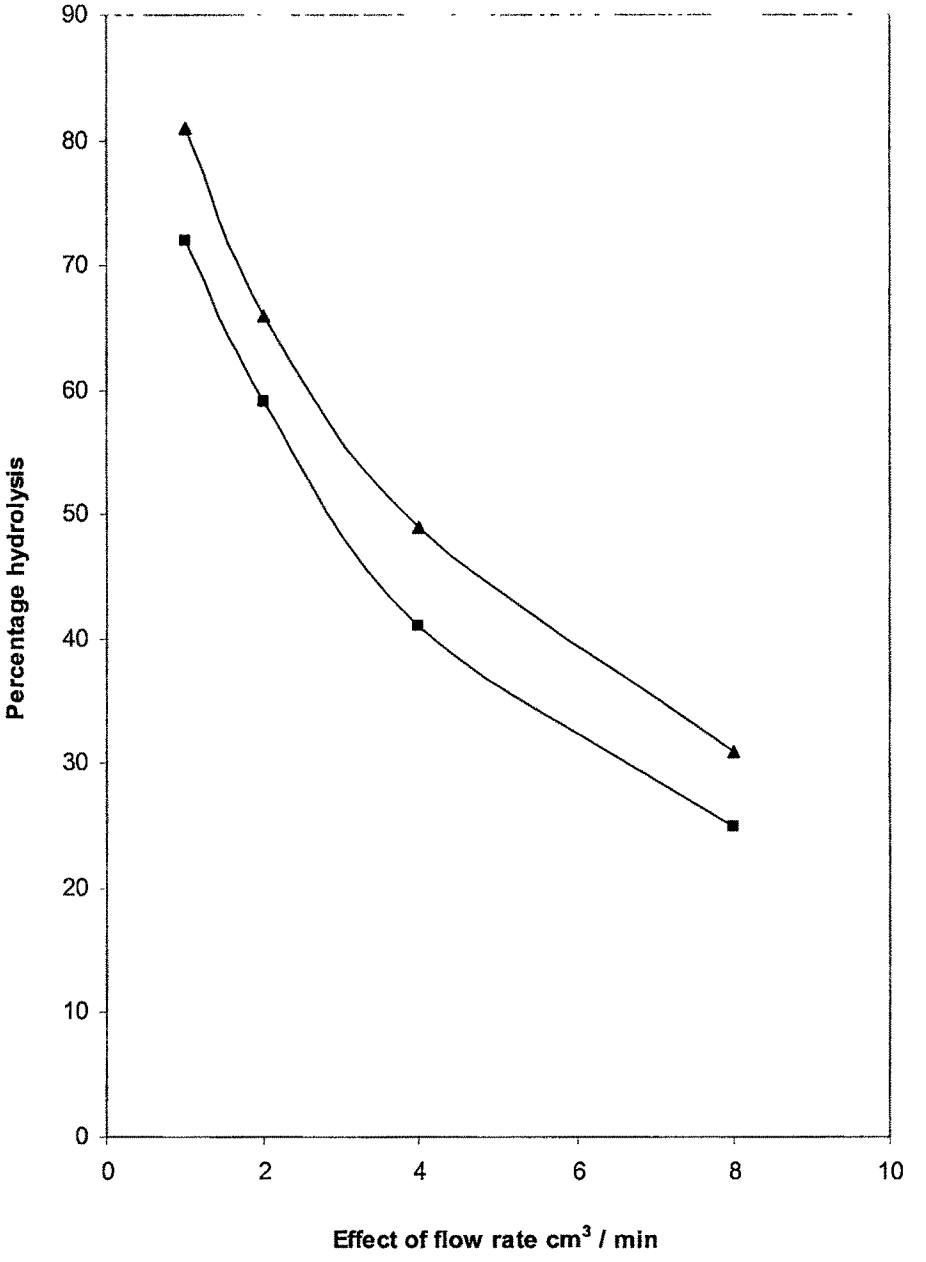

Figure 4.11. Effect of flow rate on hydrolysis of lactose .

Entrapped (■)and covalently bound enzyme(▲).

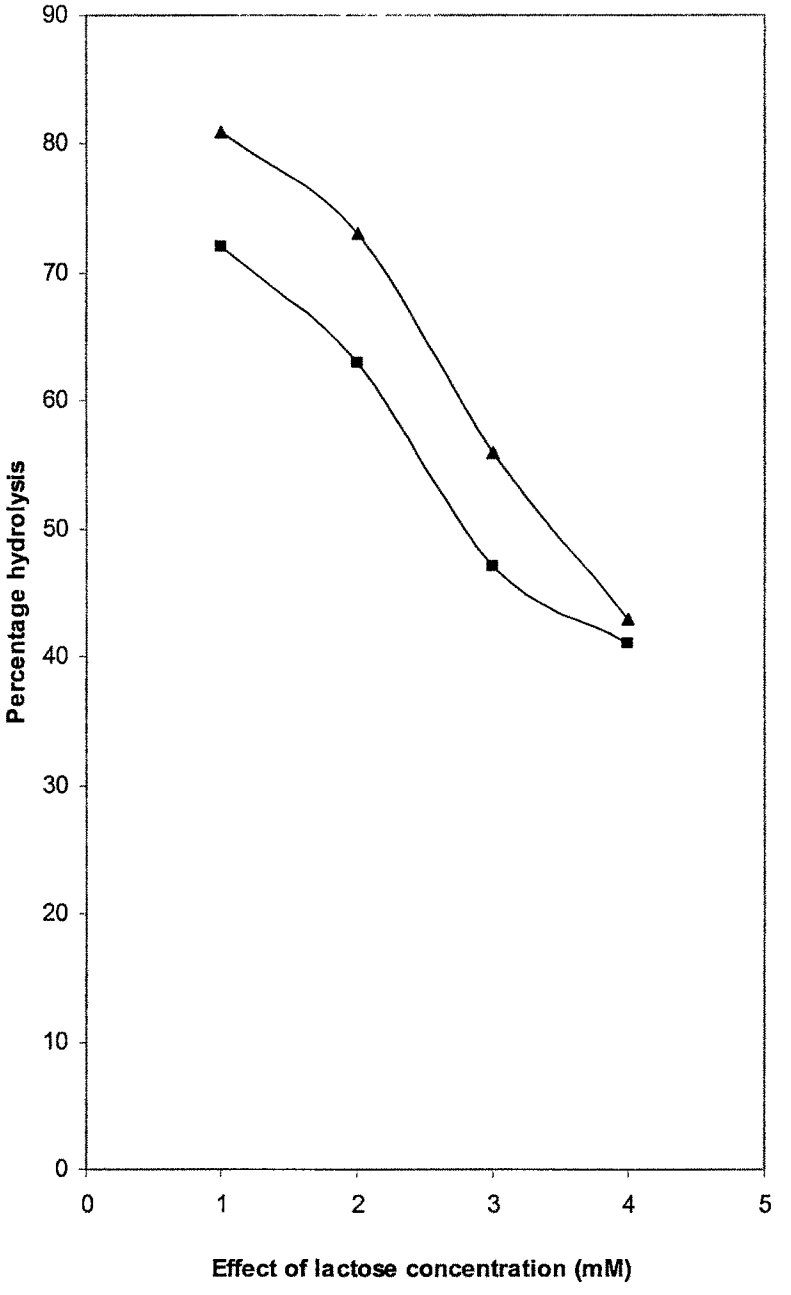

Figure 4.12. Effect of lactose concentration .

Entrapped (■)and covalently bound enzyme(▲).

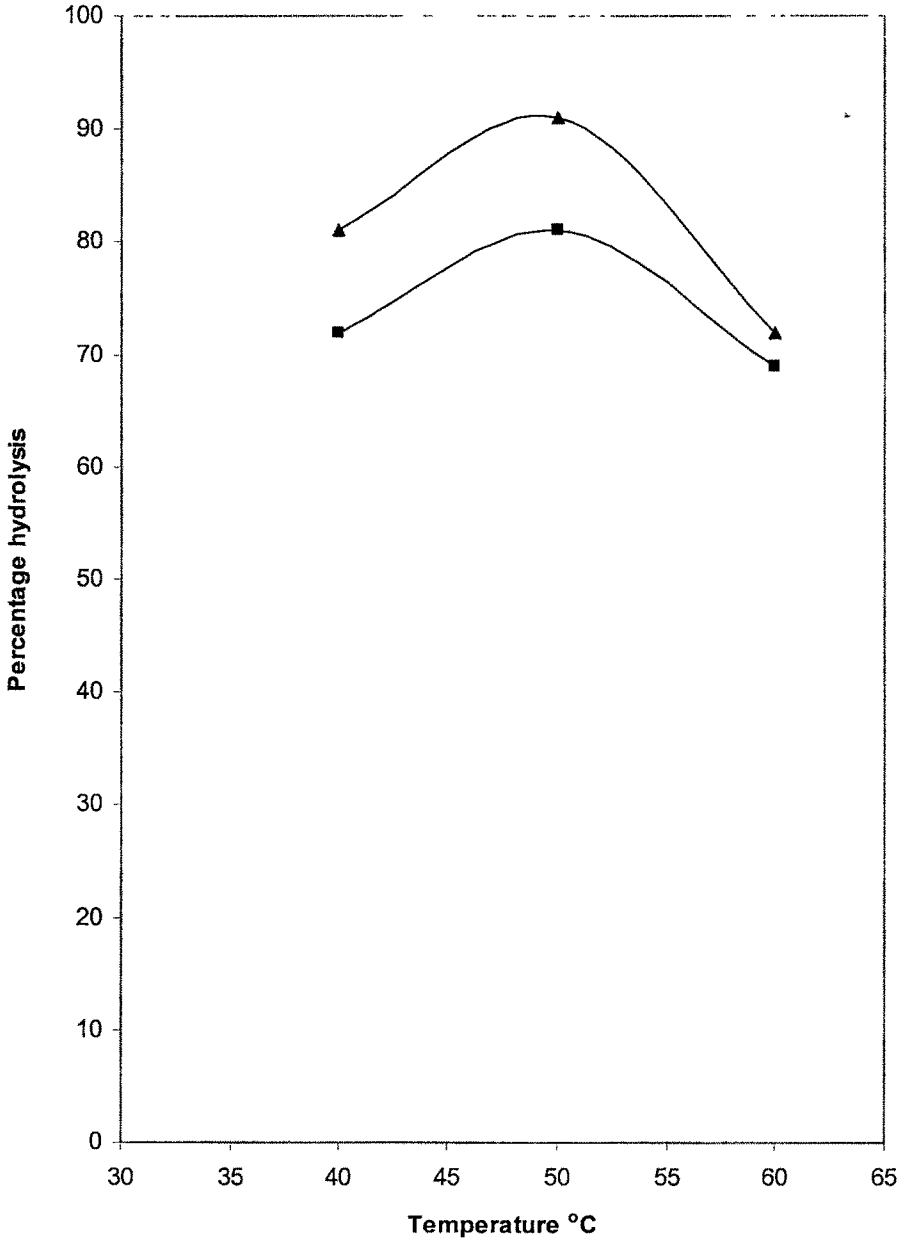

Figure 4.13. Effect of temperature on lactose .

Entrapped (■)and covalently bound enzyme(▲).

lactose concentration.

Temperature being an important parameter in enzymatic reaction and also in industries the effect of temperature was checked by varying the temperature from 40 – 60 °C and it was found that with the increase in temperature the hydrolysis increased but it decreased after certain temperature as each enzyme has an optimum temperature . **(Figure 4.13.)**

The length to diameter ratio is also an important parameter and hence it was also checked and found that with the increase in l/d ratio the percentage hydrolysis of lactose increased upto a l/d ratio of 8 but after that it did not show any more increase. **(Figure 4.14)**

From all the study we found that maximum of 94 % hydrolysis for covalently bound enzyme and 91 % hydrolysis for entrapped enzyme was found at 1 cm^3 / min flow rate at 50 °C and a l/d ratio of 8 at 1mM lactose concentration.

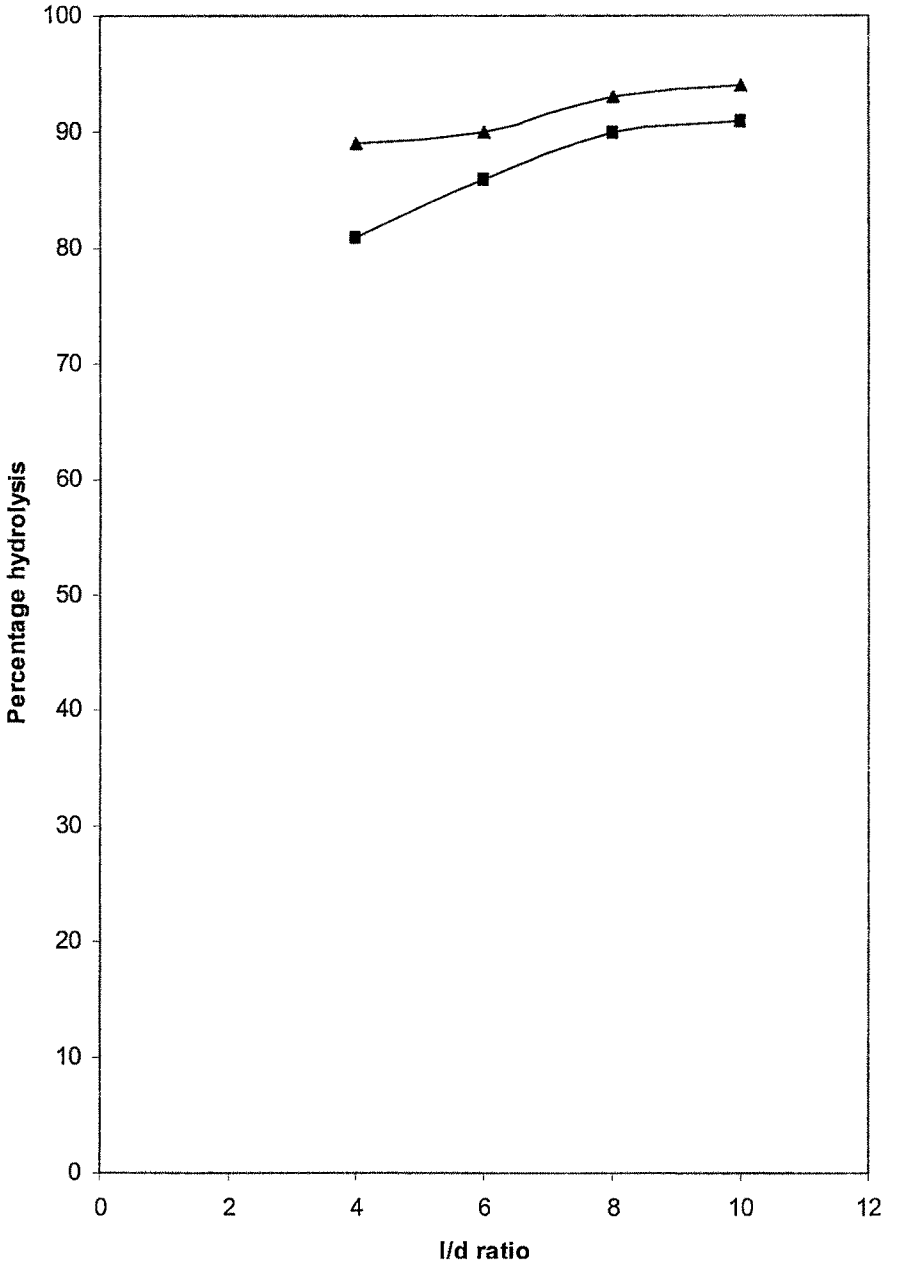

Figure 4.14. Effect of l/d ratio.

Entrapped (■)and covalently bound enzyme(▲).

4.5. Summary and Conclusion :

We have successfully immobilized β-galactosidase on natural and synthetic support. Conditions for entrapment and covalent coupling of enzymes were optimized. Maximum loading of enzyme was 11.56 U/g of support for entrappment and 1.77 U/g of support for glutaraldehyde activation and 0.91 U/g of support for p-benzoquinone activation and 0.68 U/g of support for p-tolyl sulphonyl chloride activation . Simultaneous activation by glutaraldehyde and p-benzoquinone gave 5.48 U/g enzyme coupling.

The comparative study of both forms of enzymes was carried out and it was observed that immobilized system shows better performance .

The lactose hydrolysis using both entrapped and covalently bound system was carried out and it was observed that entrapped enzyme gave 91 % hydrolysis while covalently bound enzyme showed 94 % hydrolysis at optimum conditions such as 1 cm^3 / min flow rate at 50 ^oC and l/d ratio 8 at 1 mM lactose concentration.

Table 4.4. Comparison of properties of free and immobilized enzyme.

Properties	Free enzyme	Entrapped enzyme	Covalently bound enzyme
Optimum pH	4	4	4
Optimum Temperature (° C)	40	40	40
Storage stability at room temperature till 50 % loss in enzyme activity (days).	7	37	35
Durability (Cycles)	-	5	7
Michaelis constant (K_m) (mM)	0.22×10^{-5}	$0\ 38 \times 10^{-5}$	$0\ 27 \times 10^{-5}$
Maximum velocity (V_{max}) (mM/min)	0.16	0.27	0.20
Turnover number	-	1.7×10^{4}	1.14×10^{4}

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