COVALENT IMMOBILIZATION OF GLUCOSE ISOMERASE ON POLY(2-HYDROXYETHYL METHACRYLATE) PARTICLES

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ABSTRACT

Covalent Immobilization of Glucose Isomerase on Poly (2-hydroxyethyl methacrylate) Particles

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In this study, poly (2-hydroxyethyl methacrylate), P(HEMA), particles were prepared by suspension polymerization of the monomer 2-hydroxyethyl methacrylate with addition of ethylene glycol dimethyacrylate, EGDMA, as cross linker. Glucose isomerase, GI, enzyme was covalently immobilized on the prepared P(HEMA) particles after activation of the particles with cyanuric chloride. The activities of the free and immobilized enzymes were measured with Ethanol-Carbazole method. The immobilization of GI on P(HEMA) particles promoted enzyme stability and as a result, the enzyme became more stable to temperature, storage, and reuse. For maximum substrate conversion, optimum temperature was determined as 70 ^{o}C for free GI and this value shifted to 60 ^{o}C for immobilized enzyme. Optimum pH for maximum substrate conversion was found to be 7.0 for free GI and 8.0 for immobilized GI. The change of enzyme activity with substrate concentration were determined to calculate K_m and V_{max} values of the free and immobilized enzymes. K_m values were found to be $1.7x10^{-2}$ mol/L and $3.1x10^{-1}$ mol/L while V_{max} values were $1.01x10^{-4}$ mol/L.min, $1.65x10^{-3}$ mol/L.min for free and immobilized GI, respectively. Reuse capability of immobilized GI on

P(HEMA) particles was measured and compared with commercial GI. Both systems retained 80 % of their original activities after 40th use, within 6 days. The change of enzyme activities upon storage were detected at certain time intervals for the samples stored in buffer solution at 4 ^{o}C. Immobilized enzyme was retained 60% of its original activitiy in 60 days of storage at 4 ^{o}C. Immobilized GI and commercial GI both retained 90% of their activities under continuous flow after 180 mL of substrate solution passed through the column.

Key words: Glucose isomerase, poly(2-hydroxyethyl methacrylate), covalent immobilization, enzyme activity

ÖZ

Glukoz İzomeraz Enziminin Poli(2-hidroksietil metakrilat) Tanecikleri Üzerine Kovalent Tutuklanması

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Bu çalışmada poli(2-hidroksietil metakrilat); P(HEMA), tanecikleri 2-hidroksietil metakrilat monomerinden süspansiyon polimerleşmesi yöntemi ile etilen glikol dimetilakrilat, EGDMA, varlığında sentezlenmiştir. Glukoz izomeraz, GI, enzimi hazırlanan P(HEMA) tanecikleri üzerine siyanürik klorür ile aktifleştirildikten sonra kovalent olarak tutturulmuştur. Serbest ve immobilize enzim aktiviteleri Etanol-Karbazol metodu ile tayin edilmiştir. Glukoz izomerazın P(HEMA) tanecikleri üzerine tutuklanması ile enzim kararlılığında gelişme gözlenmiştir ve bunun sonucu olarak enzim sıcaklığa, depolanmaya ve tekrar kullanıma karşı daha kararlı hale gelmiştir. Maksimum substrat dönüşümü için optimum sıcaklık serbest enzim için 70 ^{o}C iken, bu değer tutuklanmış GI için 60 ^{o}C ye kaymıştır. Maksimum substrat dönüşümü için optimum pH serbest GI için 7.0 ve tutuklanmış enzim için 8.0 olarak bulunmuştur. Enzim aktifliğinin substrat konsantrasyonu ile değişimi tayin edilerek serbest ve immobilize enzim için K_m and V_{max} değerleri hesaplanmıştır. Serbest ve immobilize GI enzim için K_m değerleri sırasıyla $1.7x10^{-2}$ mol/L ve $3.1x10^{-1}$ mol/L olarak bulunmuş, V_{max} değerleri sırasıyla $1.01x10^{-4}$ mol/L.dk ve $1.65x10^{-3}$ mol/L.dk olarak bulunmuştur. Tutuklanmış GI için tekrar kullanma kapasitesi ölçülmüş ve ticari olarak bulunanlar ile

karşılaştırılmıştır. Her iki sistem, 6 gün içinde 40 kez tekrar kullanım halinde başlangıç aktifliklerinin %80'nini korumuştur. Enzim aktivitelerinin, depolanma ile değişimi, tampon çözelti içinde ve 4 ^{o}C de saklanan örnekler için belli zaman aralıklarında ölçülmüştür. Tutuklanmış GI, 60 gün 4 ^{o}C saklandığı koşulda başlangıç aktifliğinin %60'ını korumuştur. Tutuklanmış GI ve ticari olarak bulunan GI sürekli akış sisteminde denendiğinde, 180 mL substratın kolondan geçişinden sonra ilk aktifliklerinin %90'nını korumuştur.

Anahtar sözcükler: Glikoz izomeraz, poli(2-hidroksietil metakrilat), kovalent tutuklama, enzim aktivitesi

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CHAPTER 1

INTRODUCTION

1 General Informations

Ordinary sugar is a disaccharide called sucrose. Sucrose is the most widely occuring disaccharide found in all photosynthetic plants and is obtained commercially from sugarcane and sugar beets. Sucrose has a molecular formula $C_{12}H_{22}O_{11}$ with a structure shown in Figure1.

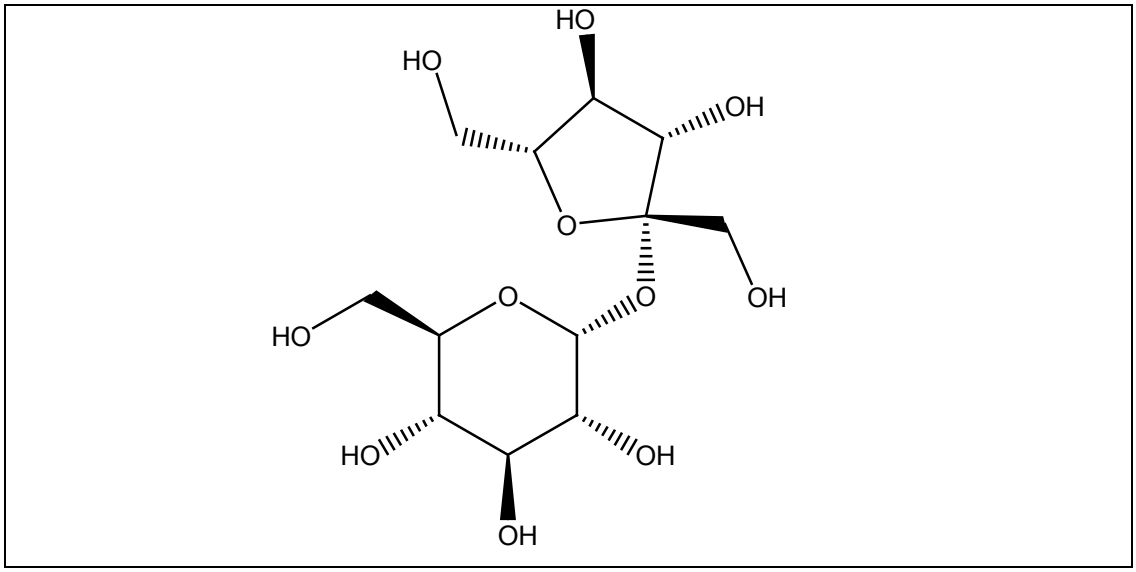

Figure 1 Structure of sucrose

Because of the increasing demand for sucrose and its continiously rising price a need for alternative sweeteners arose. Thus procedures for obtaining starch syrup became popular and the production of High Fructose Corn Syrup (HFCS) with very high sweetening degrees took the place of sucrose. In the last decades due to the limited supply of sucrose in the world a considerable amount of D-glucose is being manufactured by hydrolysis of

sweet patato starch as a substitute for sucrose. D-glucose has a structure shown in Figure 2. Enzymatic conversion of D-glucose to D-fructose was also well developed.However as the sweetness of D-glucose is usually about 60 to 70 % of sucrose, the marketing price of D-glucose (as the sweetener) is kept lower than that of sucrose.

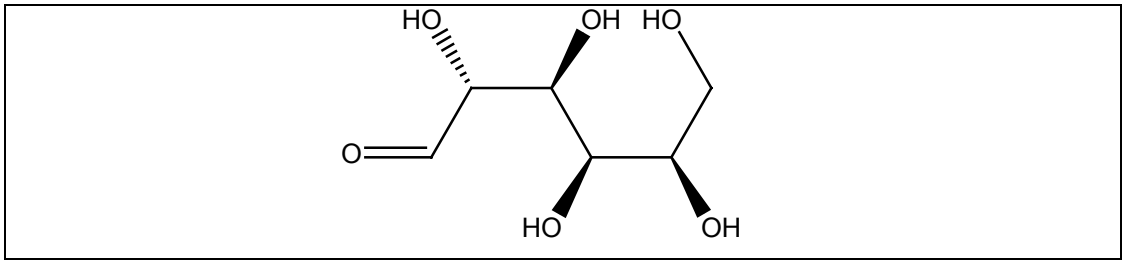

Figure 2 Structure of D-glucose

The purification, development and commercial application of Glucose isomerase (GI) is great success for enzyme technology. D-glucose isomerase is currently used in the industrial production of D-fructose from D-glucose in the form of HFCS when the initial syrup is obtained from corn starch and in the conversion process of hemicellulose to ethanol by coupling isomerisation and fermantation reactions (1).

Fructose is a six carbon monosaccharide found in relatively few forms in nature. The structure of D-fructose is shown in Figure 3. It occurs as the free sugar in many fruit, in combination with D-glucose in sucrose. Indeed isomerization is possible by chemical means but that is not economical, giving small amount of yields with many by-products (e.g. 0.1 M glucose 'isomerised' with 1.22 M KOH at 5°C under nitrogen for 3.5 months gives a 5% yield of fructose but only 7% of the glucose remains unchanged, the majority being converted to various hydroxy acids). Commercial interest in monomeric D-fructose has greatly increase due to the possibility of conversion by an enzymatic reaction using glucose isomerase.

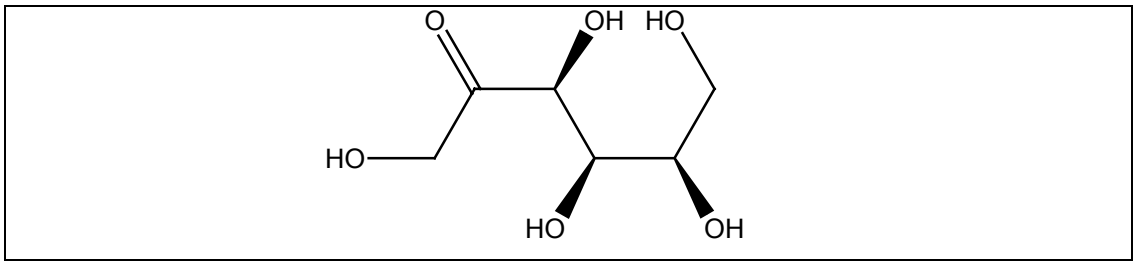

Figure 3 Structure of D-fructose

The main attraction of D-fructose is its sweetness which is 1.2 to 1.8 times higher than that of sucrose on a weight basis depending on the circumtances of measurement (Table1). High solubility, low crystallinity and easy dissolving, hygroscopicity are other important properties of D-fructose for its use in food industry. D-fructose is partly insulin independent metabolism and this makes it suitable and desired sweetener in diabetics diets. D-fructose for industrial use is more readily attainable due to the technological progress in the isolation of D-fructose as well as its consequently low price. Because of the benefical aspects of using glucose isomerase in the isomerization reaction of glucose to fructose, many scientists started to work about effective processes of production, purification and immobilization of glucose isomerase.

Table 1 Relative sweetness of fructose compared to other sugars and sweeteners (2)

D-Glucose	0.5-0.6
Maltose	0.6
Saccharose	0.8
D-Fructose	1.0
Lactose	0.27
Sucrose	0.8-0.9

Glucose Isomerase

According to the International Union of Biochemistry, Enzyme Commission classifies glucose isomerase (GI) as D-xylose ketol isomerase (the trivial name xylose isomerase, E.C.5.3.1.5) or D-glucose ketol isomerase (the trivial name, D-glucose isomerase, E.C.5.1.3.18). However it is better as most authors prefer so to call the enzyme as D-glucose isomerase as the object enzyme of the application is the conversion of D-glucose to D-fructose. When the object of application of the enzyme is conversion of xylose to xylulose, the name of xylose isomerase is readily appreciated. All known xylose ketol isomerases can also isomerase D-glucose, the presence of xylose isomerase, a true D-glucose isomerase which does not do so has been demonstrated by Slein (3).

Glucose isomerase is an inducible intracellular microbial enzyme and in general catalyses the reversible isomerisation of D-glucose to D-fructose, D-xylose to D-xylulose and D-ribose to D-ribulose.

GI catalyses the conversion of D-glucose to D-fructose and this reactions is one of the group reactions collectively known as the lobrey de Bruyn-Albreda van Ekenstein transformation. Such reactions are favored by alkali conditions and high temperatures. The isomerisation reactions of this type are thus called alkaline isomerisation (4).

Production of Glucose Isomerase

Majority of microorganisms producing GI require the presence of xylose or xylan in the fermentetion medium to induce the enzymatic synthesis (5). For instance Streptomyces phaeochromogenus (Table 2) produced GI when grown on straw hemicellulose and sulphiric acid hyrolysate of ryegrass straw which is xylose containing material (6,7). Carbon sources; lactose, mannose, lactate, glycerol were inducers for enzyme production (8). Both organic and inorganic nitrogen sources are vital for production of GI. Organic nitrogen sources such as corn peptone, polypeptone yeast extract, casein and soy flour have been used for GI production. NH_4Cl, $(NH_4)_2SO_4$,

$(NH_4)_2HPO_4$ are some inorganic nitrogen sources. As mineral salts; $MgSO_4$. $7H_2O$, $MnSO_4$. $7H_2O$ and $CoCl_2$. $6H_2O$ are the common mineral salts that have been used in the production of GI. During the production of GI, pH of the enviroment changes between 6.8-9.0 while temperature varies between 35^oC – 70^oC (9,10).

Table 2 Selected list of Glucose Isomerase producing organisms

Microorganism	Form of Enzyme preparation	Optimal pH for reaction	Optimal T(^{o}C) for reaction	Inducer used	Metal ion requirement	Conversion (%)
Pseodomanos hydrophila	Dried whole cell enzyme (DWCE)	8.5	42	xylose	Mg, Mn, As	33
Aerobacter cloacea	Ammonium sulfate Cr treatment	7.6	50	xylose	Mn, Co, Mg	45-48
Bacillus Megaterium	Aqueous Suspension of cells	7.0	35	glucose	Mg	55
Aerobacter aerogenes	DWCE	6.8	39	xylose	As	30
Streptomyces phaeochromogenus	DWCE	8 to 9	60	xylose	Mg, Co	52
Streptomyces albus	DWCE	7.0	68	xylose	Co	50

Assay of Glucose Isomerase

The isomerisation reaction of GI is usually monitored by quantification of the ketose reaction products D-xylulose or D-fructose (forward reaction), or by quantification of D-glucose or D- xylose (reverse reaction). Until now the most widely used method for ketose determination has been the colorimetric cysteine-carbazole method based on the reaction of keto sugars with carbazole in sulphuric acid (11). Some of the other colorimetric methods that

have been used are skatole (12), resorcinol (13), anthrone (14) and phenol acetone boric acid (15) methods. Glucose isomerase in the reverse reaction can be quantified by measuring D-glucose amount by the glucose oxidase method (16). The isomerisation reaction has also been analyzed polarimetrically (17,18) or high performance liquid chromatography (HPLC) (9,19, 20).

Isomerisation Conditions of Glucose isomerase

Isomerisation reaction of GI is usually carried out in the presence of an appropriate buffer with desired pH, containing activators and stabilizators (Mg^{2+} and Co^{2+}) and the substrate solution. The mixture is then incubated for some time at desired temperature and the formed D-fructose is quantifed by application of one of the methods (see assay of GI in section 2.5). One unit activity (U) of GI is usually defined as the amount of enzyme which produces 1μ mole of D-fructose in one minute under the specified assay conditions (21) or alternatively the amount of enzyme which produces 1mg of D-fructose in one hour under the specified assay conditions (6).

Many factors influence the activity, stability, and thus productivity of this enzyme. The activity of the enzyme indicates the rates of D-glucose conversion to the D-fructose at any given temperature. This depends on the pH, substrate specificity, substrate concentration, contact time, presence of activating metal ions and its concentration and presence of inhibitors. The stability reflects the ability of the enzyme to retain its activity on various different conditions. The productivity is the combined effect of activity and stability of the enzyme in HFCS production process, and is defined as the weight amount in kg of 42% D-fructose syrup (dry substance) produced per kg of enzyme throught its life time. Related with these terms there is also D-glucose to D-fructose equilibrium constant (K_{eq}) meaning % of D-fructose produced over % of D-glucose remained after an isomerisation reaction (Figure 4). One more term related with immobilized enzyme and isomerisation potency, is the half life which is defined useful life time of the

enzyme from the start time of the experiment until the enzyme activity has dropped to 50% of the initial activity and enzyme becomes unusefull if this value drops below 25%.

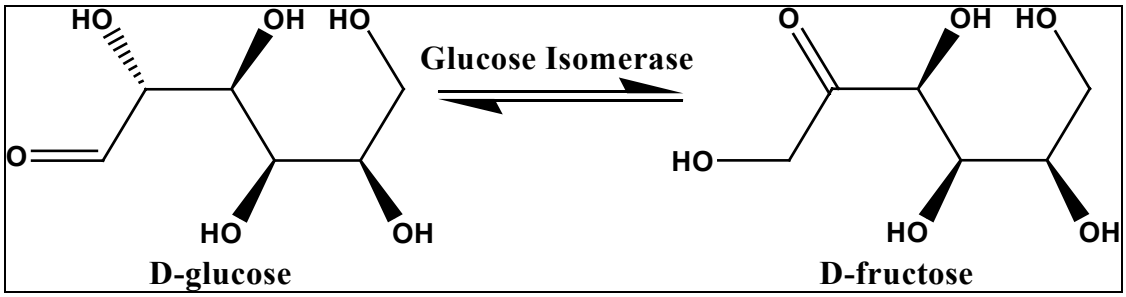

Figure 4 Isomerization reaction of D-glucose to D-fructose

Importance of Glucose Isomerase

Glucose (xylose) isomerases are a paradigm for enzyme studies because of their commercial importance in production of high-fructose syrups from starch. The thermodynamic equilibrium fructose/glucose ratio increases with temperature, so commercial processes are carried out with immobilized enzymes at around 60^{o}C, which is the effective upper limit of their thermostability. These yield 45% fructose 55% glucose syrups, but 55% fructose syrups are desirable for food and soft drinks use, so the former are fortified with chromatographically purified fructose. This expensive step would been eliminated if the isomerization could be performed at 90-95^{o}C where 55% fructose syrups could in theory be produced directly, so there has been a widespread search to get for glucose isomerases stable at these temperatures (22, 23, 24).

Recent researches are concentrated on genetic modification of glucose isomerase in order to increase the stability of the enzyme. By changing the sequence of enzyme protein, denaturation was tried to be avoided. The protein denaturation is in practice an irreversible kinetic process rather than a reversible thermodynamic phenomenon. It was claimed that, the rate limiting step of denaturation takes place at some certain local structure whichi could be relatively immune to sequence changes arising from natural selection or protein engineering in more distant parts of the structure. A chain will

break at its weakest point when put under stress, so one can call this region the weak point. Attempts to engineer and increase thermo stability of glucose isomerases will therefore benefit from elucidation of the thermal weak point. Thermal stability of glucose isomerase is increased with modification of protein sequences of enzyme and it was shown that with this genetic modification glucose isomerase could be stable even at 95^{o}C (25, 26).

Immobilization of Enzymes

Enzymes are macro molecules which serve to accelerate the chemical reactions of living cells. Without enzymes, most biochemical reactions would be too slow to even carry out life processes. Enzymes show great specificity and are not permanently modified by their participation in biological reactions. As catalysts enzymes are not consumed during the reactions, but when the reactions are carried out as batch type (enzymes are in solution with the substrate and / or products) it becomes difficult to separate the enzyme from the whole solution and reuse it again. For a sufficient separation process, enzymes can be attached and immobilized on to the reactor in some ways, and can be used repeatedly and continiously after the products have been removed. In immobilization process; an enzyme is physically attached to a solid support, the substrate is passed over this immobilized enzyme and converted to product. Some changes in physical and chemical properties of the enzyme would be expected to upon immobilization and this changes may affect the rate of catalytic activities of the enzymes. For immobilized enzymes, changes in activities, stabilities and in kinetic properties may also be observed due to the changes in micro environment of the immobilized site of the enzyme. Immobilization of enzymes often cause an additional expense and is only undertaken if there is economic or process advantages in the use of the immobilized rather than free (soluble) enzymes. The most important benefit derived from immobilization is the easy separation of the enzyme from the products of the catalysed reaction. This prevents the enzyme contaminating the product,

minimising downstream processing costs and possible effluent handling problems, particularly if the enzyme is noticeably toxic or antigenic. It also allows continuous processes to be practicable, with a considerable saving in enzyme, labour and overhead costs. Immobilization often affects the stability and activity of the enzyme, but conditions are usually available where these properties are little changed or even enhanced. The productivity of an immobilized enzyme, is greatly increased as it may be more fully used at higher substrate concentrations for longer periods than the free enzyme.

1.6.1 Methods of Immobilization

In enzyme immobilization it is very important to select a method of binding that will not cause loss of enzyme activity by not changing the chemical nature or reactive groups in the binding site of the enzyme. Considerable knowledge of the active site of the enzyme will prove this task. It is desired to avoid binding of the essential sites of the enzyme which give reactions with the substrates. Alternatively, the active site can be protected during attachment as long as it is needed and the protective groups can be removed later on without any loss in enzyme activity.

The surface on which the enzyme is immobilized, is effective for retaining the structure of the enzyme through hydrogen bonding or the by formation of electron transition complexes. These links will prevent vibration of the enzyme and thus increase thermal stability (27). The micro environment of the surface and the enzyme immobilized on this surface has a charged nature that can cause a shift in the optimum pH of the enzyme up to 2 pH units compare to free one (28). This may be accompanied by a general broadening of the pH region in which the enzyme can work effectively, allowing enzymes that normally do not have similar pH regions to work together. The immobilization methods can be classified as:

- **Carrier Binding** : binding of enzymes to water-insoluble carriers.
- **Cross-Linking** : intermolecular cross-linking of enzymes by bi-functional or multi-functional reagents.

- **Entrapping** : incorporating enzymes into the lattices of semi-permeable gels or enclosing the enzymes in semi-permeable polymer matrices.

1.6.1.1 Carrier-Binding

The oldest immobilization technique for enzymes is the carrier-binding method. In this method, the nature of the carrier influence the amount of the enzyme bound to the carrier and the activity after immobilization. Figure 5 schematically shows how an enzyme is bound to a carrier.

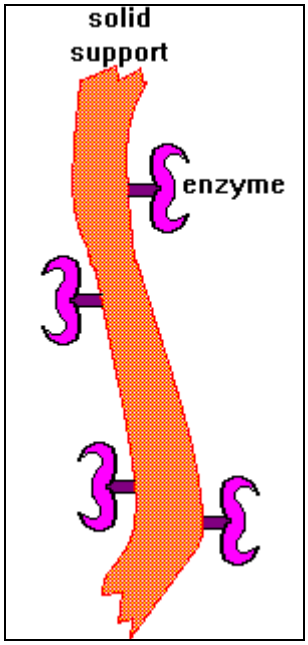

Figure 5 Binding of enzyme on solid support material

The selection of the carrier depends on the nature of the enzyme. Following properties of the solid support material effect the binding capacity of an enzyme;

- Physical shape (particle, membrane, pellet, ect)
- Surface / volume ratio
- Molar ratio of hydrophilic to hydrophobic groups
- Chemical composition

Generally, increase in surface area and in the ratio of hydrophilic groups

leads to an increase in the concentration of bound enzymes, which result higher activity for the immobilized enzymes. Polysaccharide derivatives such as cellulose, dextran, agarose, and polyacrylamide gel are some of the most commonly used carriers for enzyme immobilization. According to the binding mode of the enzyme, the carrier-binding method can be further sub-classified as given below

- Physical Adsorption
- Ionic Binding
- Covalent Binding

1.6.1.1.1 Physical Adsorption Mode

This method for the immobilization of an enzyme is based on the physical adsorption of enzyme protein on the surface of water-insoluble carriers (29). The method causes little or no conformational change of the enzyme or destruction of its active center. If a suitable carrier is found, this method is simple and cheap. However, it has the disadvantage that the adsorbed enzyme may leak from the carrier during use due to the weak binding forces between the enzyme and the carrier. The earliest example of enzyme immobilization using this method is the adsorption of beta-D-fructo-furanosidase onto aluminum hydroxide. The processes available for physical adsorption of enzymes are: static procedure, electro-deposition, reactor loading process, mixing or shaking bath loading

A major advantage of adsorption as a general method of immobilizing enzymes is that usually no reagents and only a minimum of activation steps are required. Adsorption tends to be less disruptive to the enzymatic protein than chemical means of attachment because the binding is mainly by hydrogen bonds, multiple salt linkages, and Van der Waal's forces (30). In this respect, the method bears the greatest similarity to the situation found in natural biological membranes and has been used to model such systems. Because of the weak bonds involved, desorption of the protein resulting from

changes in temperature, pH, ionic strength or even the mere presence of substrate, is often observed. Another disadvantage is non-specific, further adsorption of other proteins or other substances as the immobilized enzyme is used. This may alter the properties of the immobilized enzyme or, if the substance adsorbed is a substrate for the enzyme, the rate will probably decrease depending on the surface mobility of enzyme and substrate. Stabilization of enzymes temporarily adsorbed onto a matrix can be achieved by cross-linking or by binding the enzyme by a chemical reaction subsequent to its physical adsorption.

1.6.1.1.2 Ionic Binding Mode

The ionic binding method relies on the ionic binding of the enzyme protein to water-insoluble carriers containing ion-exchange residues. *Polysaccharides* and *synthetic polymers* having ion-exchange centers are usually used as carriers (29). The binding of an enzyme to the carrier is easily carried out, and the conditions are much milder than those needed for the covalent binding method. The ionic binding method causes little changes in the conformation and the active site of the enzyme and therefore, this method yields immobilized enzymes with high activity in most cases. Leakage of enzymes from the carrier may occur in substrate solutions of high ionic strength or upon variation of pH. This is because the binding forces between the enzyme proteins and the carriers are weaker than those in covalent binding. The main difference between ionic binding and physical adsorption is that the enzyme to carrier linkages are much stronger for ionic binding although weaker than that of in covalent binding.

1.6.1.1.3 Covalent Binding Mode

The most intensely studied immobilization technique is the formation of covalent bonds between the enzyme and the support matrix. When trying to select the type of immobilization reaction for a given protein, the choice is

limited by two characteristics: first one is the conditions of binding reaction should not cause loss of enzymatic activity, and second is the active site of the enzyme should not be affected by the linker reagents used (31, 32). The covalent binding method is based on the binding of enzymes and water-insoluble carriers by covalent bonds. The most general functional groups that may take part in this binding are : amino, carboxyl, sulfhydryl, hydroxyl, imidazole, phenolic, thiol, threonine and indole groups.

This method can be further classified into diazo, peptide and alkylation methods according to the mode of linkage. The conditions for immobilization by covalent binding are much more complicated and less mild than in the cases of physical adsorption and ionic binding. Therefore, covalent binding may alter the conformational structure and active center of the enzyme, resulting in major loss of activity and/or changes of the substrate. However, the binding force between enzyme and carrier is so strong that no leakage of the enzymes occurs, even in the presence of substrate or solution of high ionic strength.

Covalent attachment to a support matrix must involve some functional groups of the enzyme those are not essential for catalytic action. These functional groups can be α amino groups of poly peptide chains, α amino groups of argenine and lycine (33), α carboxyl groups of glutamate and aspartanine imidazol ring of histidine and indole ring of tryptofan (34). Higher activities result from prevention of inactivation reactions with amino acid residues of the active sites. A number of protective methods have been improved such as covalent attachment of the enzyme in the presence of a competitive inhibitor or substrate, a reversible, covalently linked enzyme-inhibitor complex, a chemically modified soluble enzyme whose covalent linkage to the matrix is achieved by newly incorporated residues. Covalent binding can be achived by the groups tabulated in Table 3.

Table 3 Selected list of functional groups which are used in covalent binding of enzyme on to solid support

Diazotization	**SUPPORT--N=N--ENZYME**
Amide bond formation	**SUPPORT--CO-NH--ENZYME**
Alkylation and Arylation	**SUPPORT--CH_2-NH--ENZYME** **SUPPORT--CH_2-S--ENZYME**
Schiff's base formation	**SUPPORT--CH=N--ENZYME**
Amidation reaction	**SUPPORT—CH_2NH-NH--ENZYME**
Thiol-Disulfide interchange	**SUPPORT--S-S--ENZYME**

For covalently bound enzymes the active site of the enzyme must be free or must not be hindered. It is essential that enough space for the substrate to diffuse between the enzyme and the support should exist. In some cases it is possible to increase the number of reactive residues of the support in order to increase the amount of the immobilized enzyme. This provides alternative reaction sites to those essential for enzymatic activity. Covalent bonding should provide stable immobilized enzymes that do not leach into the surrounding solution. The wide variety of binding reactions and insoluble carriers (with functional groups capable of covalent coupling or being activated to give such groups) makes covalent bonding a popular and generally applied method of immobilization. This is true even if very little is known about the protein structures or active sites of the enzymes to be coupled.

1.6.1.2 Cross-Linking

Immobilization of enzymes has been achieved by intermolecular cross-linking of the protein, either to other protein molecules or to functional groups on an insoluble support matrix, Figure 6 represent immobilization of enzyme by

crosslinking. Cross-linking of an enzyme to itself is both expensive and insufficient, as some of the protein material will inevitably be acting mainly as a support. This will result in relatively low enzymatic activity.

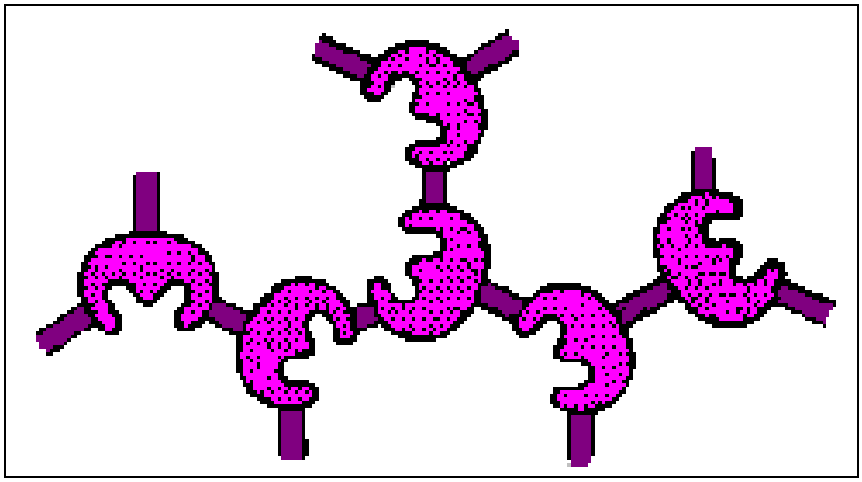

Figure 6 Illustration of croslinked enzymes

Generally, cross-linking is best used in conjunction with one of the other methods. It is used mostly as means of stabilizing adsorbed enzymes and also for preventing leakage from polymeric gels. Since the enzyme is covalently linked to the support matrix, very little desorption is expected. For example for carbamy phosphokinase cross-linked to alkyl amine glass with glutaraldehyde it is reported that only 16% of its activity was lost after continuous use in at room temperature for fourteen days (19).

Glutaraldehyde is the most common reagent used for cross-linking. In general cross-linking reactions are carried out under relatively severe conditions and these harsh conditions can change the conformation of active center of the enzyme; and so may lead to significant loss of activity.

1.6.1.3 Entrapping Enzymes

The entrapment method of immobilization is based on the localization of an enzyme within the lattice of a polymer matrix (34). Enzyme is dissolved in aqueous monomer solution or in polymer solution. Synthesis of polymer and

crosslinking are initiated with application of gamma ray, UV radiation or thermally by heat (35, 36). Polymerization and formation of matrix is done in such a way that protein gets entrapped in the matrix and demonstrates its activity while substrate penetrates in to the matrix. Figure 7 shows schematically entrapping process.

Figure 7 Shematic representation of entrapping of enzymes

This method differs from the covalent binding and cross linking since the enzyme does not bind to itself or to the support matrix. In general the conditions, used in the chemical polymerization reaction and in the formation of matrix, are relatively severe and this result a decrease in the immobilized enzyme activity. Therefore, careful selection of the most suitable conditions for the immobilization of various enzymes is required. The most generally used entrapping techniques are lattice-type and microencapsule formations.

1.6.1.3.1 Lattice-Type

Lattice type entrapment involves immobilization of enzymes within the interstitial spaces of a cross-linked water-insoluble polymer. In this method, enzyme containing monomer or polymer solutions are crosslinked by UV radiation or gamma ray applications and entrapped enzyme molecules can not penetrate out from the polymer matrix while substrate and product molecules freely move in to the matrix. Some synthetic polymers such as

polyarcylamide, polyvinylalcohol, etc... and natural polymers such as starch, cellulose, etc. have been used to immobilize enzymes using this technique.

1.6.1.3.2 Microcapsule-Type

Microcapsule type entrapping involves enclosing the enzymes within semi permeable polymer membranes. In the preparation of enzyme containing micro capsules extremely well-controlled conditions and the procedures are required. Micro capsulation of enzymes can be classified as; interfacial polymerization, liquid drying and phase separation.

In interfacial polymerization method enzymes are enclosed in semi permeable membranes of polymers. An aqueous mixture of the enzyme and hydrophilic monomer are emulsified in a water-immiscible organic solvent. Then the same type of hydrophilic monomer is added to the organic solvent by stirring while polymerization of the monomers occurs at the interface between the aqueous and organic solvent phases in the emulsion. The result is that the enzyme in the aqueous phase is enclosed in a membrane of polymer (37).

In liquid drying process, a polymer is dissolved in a water-immiscible organic solvent which has a boiling point lower than that of water. An aqueous solution of enzyme is dispersed in the organic phase to form a first emulsion of water-in-oil type. The first emulsion containing aqueous micro droplets is then dispersed in an aqueous phase containing protective colloidal substances such as gelatin, and surfactants, and a secondary emulsion is prepared. The organic solvent is then removed by warming in vacuum. A polymer membrane is thus produced to give enzyme micro capsules.

Phase separation process, one purification method for polymers involves dissolving the polymer in an organic solvent and re-precipitating it. This is accomplished by adding another organic solvent which is miscible with the first, but which does not dissolve the polymer.

1.6.2 Selection of Immobilization Method

For a successful immobilization method the following conditions are essential.

- Enzyme should be stable under reaction conditions
- Cross-linkers should not react with the active site of the enzyme
- Active site of enzyme should be protected during immobilization process (for instance active site of sulfydryl enzymes can be protected reacting with cystein after completion of immobilization enzyme can be reactivated by removing cystein)
- Washing step to remove unbound enzyme from the immobilized system should not effect immobilized enzyme
- Mechanical stability and physical form of support material should be suitable for immobilization (The solid supports used for enzyme immobilization can be *inorganic* or *organic.* Some organic supports include: polysaccharides, proteins, polystyrenes, polyacrylates, maleic anhydride based copolymers, polypeptides, vinyl and allyl polymers, and polyamides)

1.6.3 Forms of Immobilized Enzyme Systems

The forms of an immobilized enzyme system can be classified into four types: particles, membranes, tubes, and filters. Most immobilized enzymes are in particle form for ease of handling and ease of application.

Particles - The particles can be prepared as granules, microcapsules, microspheres or as nanoparticles. They can be inorganic solids (ceramics, glass ect) or organic materials (natural or synthetic polymers)

Membranes - Enzyme membranes can be prepared by attaching enzymes to membrane-type carriers, or by molding into membrane form. The molding is done after the enzymes have been enclosed within semi-permeable

membranes of polymer by entrapment.

Tubes - Enzyme tubes are produced using Nylon or polyacrylamide tubes as carriers. The polymer tube is first treated in a series of chemical reactions to activate the surface and the enzyme is bound by covalent binding to give final form of the tube.

Fibers - Enzymes can be immobilized by entrapment or binding to surface of the fibers to form enzyme fibers.

1.6.4 Aim of the Work

The aim of this study is to prepare an effective glucose isomerase immobilized system that can be used for the conversion of glucose to fructose which is highly used in food industry as sweetener. For this purpose the following milestones were followed :

- synthesis of poly(2-hydroxy ethyl methacrylate), P(HEMA), as enzyme carrier support material
- activation of P(HEMA) particles by treatment with cyanuric chloride
- achievement of covalent immobilization of glucose isomerase (enzyme) on activated P(HEMA) particles
- determination of the kinetic constants, optimum parameters, reuse and storage capabilities of free and immobilized glucose isomerase
- comparision of activities of free and immobilized glucose isomerase in batch and in continuous systems
- comparasion of activities of immobilized enzyme with commercially available enzyme.

CHAPTER 2

EXPERIMENTAL

2.1 Materials

In this study, the followimg chemicals and solutions are used.

- Glucose isomerase [D-xylose ketolisomerase, E.C. 5.3 1.5], Activity : 3000 GIU/g, Sp. Gravity : 1.11-1.30 g/mL Gensweet (USA)
- Commercial immobilized enzyme on cellulose based rigid carrier resin IGI-SA Gensweet (USA)
- D(+)-Glucose [$C_6H_{12}O_8$, M_W:180.16 g/mol], Acros (Germany)
- D(+)-Fructose [$C_6H_{12}O_8$, M_W:180.16 g/mol], Acros (Germany)
- Sodium dihydrogen phosphate [$NaH_2PO_4.2H_2O$, M_W:156.01 g/mol], Merck AG (Germany)
- Disodium monohydrogen phosphate [$Na_2HPO_4.12H_2O$, M_W:358.15 g/mol], Merck AG (Germany)
- Magnesium Sulfate [$MgSO_4.7H_2O$, M_W:246.48 g/mol], Merck AG (Germany)
- L-Cystein [$C_3H_8ClNO_2S.H_2O$, M_W:175.64 g/mol], Merck AG (Germany)
- Carbasole [$C_{12}H_9N$, M_W:167.2 g/mol], Acros (Germany)
- Benzoyl peroxide [$C_{14}H_{10}O_4$, M_W:242.23 g/mol] Merck AG (Germany).
- 2-Hyroxyethyl methacrylate, P(HEMA) [$H_2C=C(CH_3)CO_2CH_2CH_2OH$, M_W:130.14 g/mol], Acros (Germany)
- Ethylene glycol dimethacrylate (EGDMA) [$(H_2C=C(CH_3)CO_2CH_{2-})_2$, M_W:198.22 g/mol], Acros (Germany)

- Cyanuric chloride [$C_3N_3Cl_3$, M_W:183.14 g/mol], Acros (Germany)
- Phosphate buffer (PB pH:7.0, 0.5 M) (contains 11.9738 g $NaH_2PO_4.2H_2O$, 17.2807 g $Na_2HPO_4.12H_2O$ in 1 L distilled water pH was adjusted to pH 7.0)
- L-cystein (%2) solution, 2g L-cystein was dissolved and diluted to 100 mL with distilled water.
- Ethanol-Carbazol (%0.12), 60 mg carbazol dissolved in 50 mL ethanol.
- Buffer solutions used in this study and their compositions are given in Table 4. Buffers were prepared by using Sodium dihydrogen phosphate [$NaH_2PO_4.2H_2O$] and disodium monohydrogen phosphate [$Na_2HPO_4.12H_2O$] in 250 mL water.

Table 4 Preparation of different phosphate buffer solutions

pH	$Na_2HPO_4.12H_2O$ (g)	$NaH_2PO_4.2H_2O$ (g)
6.0	2.66	18.34
6.5	7.45	16.25
7.0	17.28	11.97
7.5	29.83	6.50
8.0	38.65	2.66
8.5	42.63	0.92

2.2 Preparation of P(HEMA) Particles

Synthesis of P(HEMA) was carried out by suspension polymerization. For this purpose, $MgSO_4.7H_2O$ (12g), distilled water (100 mL) and benzoyl peroxide (0.1 g) were added in to a three-necked flask (250 mL) fitted with a condenser and thermometer. HEMA (15 mL) and EGDMA crosslinker (1mL) were added and suspension was formed by stirring vigorously for 5 h at 80 ^oC in oil bath. N_2 was flushed through the system all the time during the polymerization process. The mixture was kept at room temperature overnight and the precipitated P(HEMA) particles were separated by filtration, washed three times with acetone, three times with distilled water and dried in oven

at 35 ^{o}C. Dried polymer was sieved and the polymer particles that have sizes bigger than 0.5 mm were used in further studies. Average particle size and particle size distributions were obtained by using a particle size analyser (Master Sizer E Malvern Instruments Ltd., UK)

2.3 Activation of Support Material P(HEMA) with Cyanuric Chloride

P(HEMA) particles (0.1g) were added to a cyanuric chloride solution (0.1g $C_3N_3Cl_3$ in 5 mL dioxane), kept in a shaking waterbath for 4h at 60 ^{o}C and left at room temperature overnight (38). The activated particles were separated and washed three times with acetone and three times with phosphate buffer (pH 7.0, 0.5 M). Figure 8 shows the activation reaction of P(HEMA).

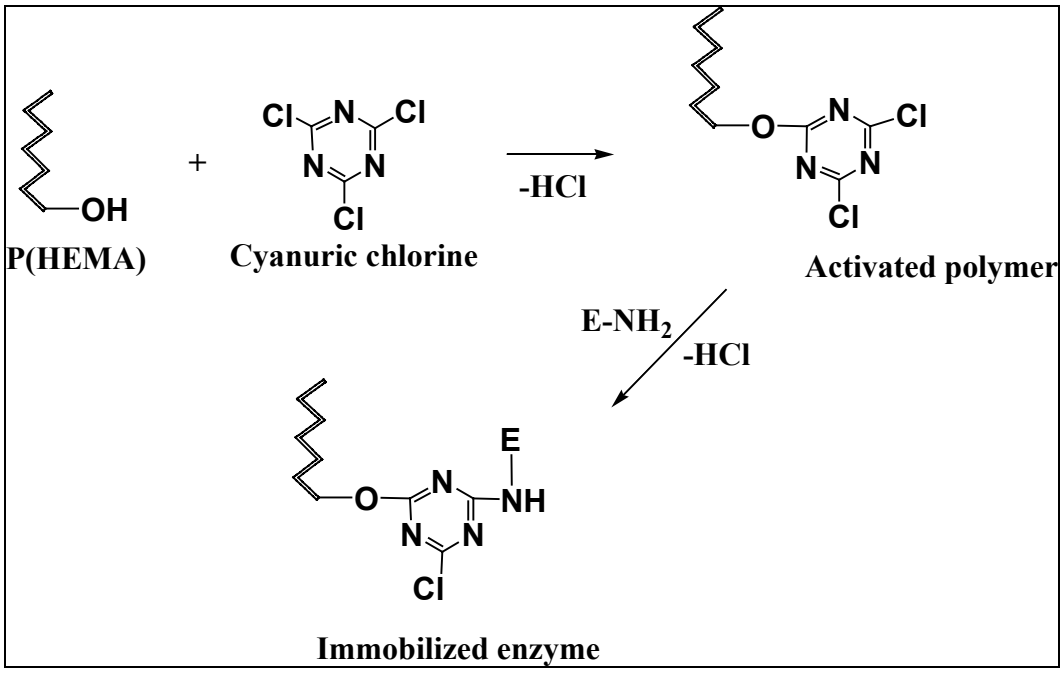

Figure 8 Activation of support material P(HEMA) with cyanuric chloride

2.4 Immobilization of Glucose Isomerase (GI) on P(HEMA) Particles

The activated particles were added in to enzyme solution (2.25 mL GI in 50 mL distilled water) and immobilization reaction was carried out for 4 h at 60 ^{o}C in a shaking waterbath. Particles were separated and unbound enzyme was removed by washing three times with phosphate buffer (PB).

2.5 Fructose Calibration Curve

Ethanol-carbazole method (34) was based on the determination of fructose in reaction medium. In order to get calibration curve, fructose solutions were prepared in different concentrations (0.5 mg, 1.0 mg, 2.0 mg, 4.0 mg, 8.0 mg, 10.0 mg in 100 mL). L-Cystein (0.1mL, 2%) was added in to each of 1 mL of fructose solutions and then 5 mL H_2SO_4 (75 %, 5 mL) was added. Ethanol carbazole (0.15 mL, 0.12 %) was added in to these mixtures in ice bath. Solutions were kept in ice bath for 5 minutes and then solutions were stored in water bath at 40 ^{o}C for 30 minutes for the completion of the complex formation reaction. The solutions were again dipped in to ice bath for 1.5 minutes followed by keeping at room temperature for 4 minutes. Absorbance measurements of solutions were carried out at 566 nm.

2.6 Assay of GI Activity

Activities of free enzyme were determined with ethanol-carbazole method (38). Stock enzyme solution was prepared by dissolving 2.25 mL of GI enzyme in 50 mL of distilled water. 0.5 mL of this solution was mixed with PB (1 mL, pH 7.0, 0.5 M), and $MgSO_4$ solution (1 mL, 0.05 M) and added in to aqueous glucose solutions ($2x10^{-4}$ M , 3 mL). The solutions were stored in waterbath for 15 minutes at 60 ^{o}C. $HClO_4$ solution (0.5 M, 5 mL) was added in to each assay solution to stop the reaction. 1 mL of these solutions were drawn in to tubes and put in ice bath. In to these solutions, L-cystein (0.1 mL, 2 % w/w) , H_2SO_4 (75 %, 5 mL) and ethanol - carbazole

(0.15 mL, 0.12 %) were added and kept in ice bath for 5 minutes, and then put in water bath at 40 ^{o}C for 30 minutes for the completion of the complex formation reaction. The solutions were again dipped in to ice bath for 1.5 minutes followed by keeping at room temperature for 4 minutes. Absorbance measurements of the formed complex were carried out at 566 nm. The same activity measurement assay procedure was followed for immobilized enzyme by using 0.1 g of P(HEMA) particles instead of free enzyme.

2.7 Effect of pH on Activity of GI

In order to explore effect of pH on activity of free enzyme, the similar enzyme assays which were described in section 2.6 were achieved at various pH buffer solutions where pH values were in the range of 6.0 to 8.5. All experiment were carried out at constant temperature (60^{o} C) and constant substrate concentration ($2x10^{-4}$ M , 3 mL). Immobilized enzyme activity were measured at various pH buffers (pH = 6.0 to 8.5) in the same way as described but instead of free enzyme by using 0.1g of P(HEMA) particles which contains immobilized GI.

2.8 Effect of Temperature on Activity of GI

Temperature effect was determined by measuring activities of the enzyme at different temperatures at 50^{o}C, 55^{o}C, 60^{o}C, 70^{o}C and 80^{o}C. Activities of free glucose isomerase enzyme were determined with the described ethanol-carbazole method (section 2.6). During the reaction, pH was 7.0 (0.5 M) and initially added glucose solution concentration was $2x10^{-4}$ M (3mL). Effect of temperature on the activity of immobilized enzyme was determined with ethanol carbazole method in the same way as described but by using 0,1 g of P(HEMA) particles that contain immobilized enzyme instead of free GI.

2.9 Effect of Substrate Concentration on Activity of GI

In order to search effect of substrate concentration on the activity of GI, aqueous glucose solutions were prepared in different concentrations (0.02M, 0.01M, 0.005M, 0.002M, 0.001M). Glucose solutions were (3 mL) put in to the shaking water bath at 60^{o} C and enzyme was added in to these solutions. For free enzyme assay, 5mL of enzyme solution (stock solution: 2.25 mL GI / 50 mL distilled water), for immobilized enzyme assay, 0.1 g P(HEMA) particles on which enzyme was immobilized were added. In certain time intervals, solution samples were drawn out from the reaction medium. Enzyme activities and fructose concentrations in each sample were determined by applying the method described previously.

2.10 Reuse Capacity of GI

Immobilized GI was used repeatedly to determine the reuse capacity of the enzyme. In order to designate repeated use capacity, immobilized GI (0.1 g) was used 40 times in six days and activity of GI was measured by using the ethanol-carbazole method with the procedure as described in section 2.6. After each use, the immobilized enzyme was washed with PB (pH 7.0, 0.5 M) and put in refrigerator. During reuse experiments, temperature (60^{o} C), substrate concentration (2x10^{-4} M , 3 mL) and pH (7.0) were kept constant. The reuse experiments were repeated for commercially available immobilized GI (GenSweet USA) and the obtained results were compared.

2.11 Storage Stability of GI

Immobilized GI (0.1 g) was stored at 4 ^{o}C in 5 mL buffer solution (pH 7.0) and activity of enzyme was determined periodically at certain time intervals. Storage stability experiments were carried out for 8 weeks. Activity determination assays for the enzyme were the same as the method

described in section 2.6. After each assay, immobilized enzyme was washed with PB (pH 7.0, 0.5M) and replaced in refrigerator. During storage stability experiments, temperature (60^{o} C), substrate concentration ($2x10^{-4}$ M, 3mL) and pH (7.0) of the medium were kept constant. These storage stability experiments were repeated for commercially available immobilized GI (GenSweet USA) by using the similar amount of material and the obtained results were compared.

2.12 Continuous Flow Activity Measurements

GI was used for continuous substrate conversion system. Immobilized GI packed in to a small (5cm x 1 cm) column and activity of GI was detected. Figure 9 shows the continuous system. For this purpose, 200 mL substrate ($2x10^{-4}$M glucose) solution was prepared. 66 mL PB (pH 7.0), and 66 mL $MgSO_4$ solution (0.05 M) were added in it was heated to 60^{o}C and passed through the column which is also at 60^{o}C. The flow rate was kept constant as 3ml/min. In every minute 3 mL samples were drawn out and put in to glass tubes. 5 mL $HClO_4$ solution (0.5M) was added in to every collected samples to stop reaction. These samples were treated according to procedure given in section 2.6 to determine activity of enzyme. The same procedure applied for commercially available immobilized GI (GenSweet USA) and results were compared.

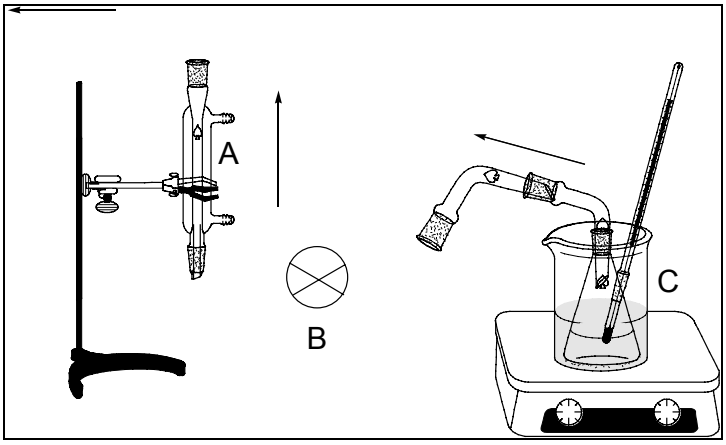

Figure 9 Continuous flow system A: Column, B: Peristaltic pump, C: Substrate solution D: Collected sample

CHAPTER 3

RESULT and DISCUSSION

3.1 Properties of P(HEMA) Particles

P(HEMA) particle preparation was carried out by suspension polymerization of HEMA monomers in aqueous media by using a crosslinker, EGDMA. The particles were sieved and the ones size larger than 500 μm were used in the experiments. The average particle size distribution curve of these particles were obtained by using a particle size analyser and the obtained result are given in Figure 10 and Table 5. Average particle size was found as 603.43 μm. The distribution curve shows that about 2% of the particles were smaller than 100 μm, about 23% were in the range of 100-500μm and about 75% were larger than 500μm

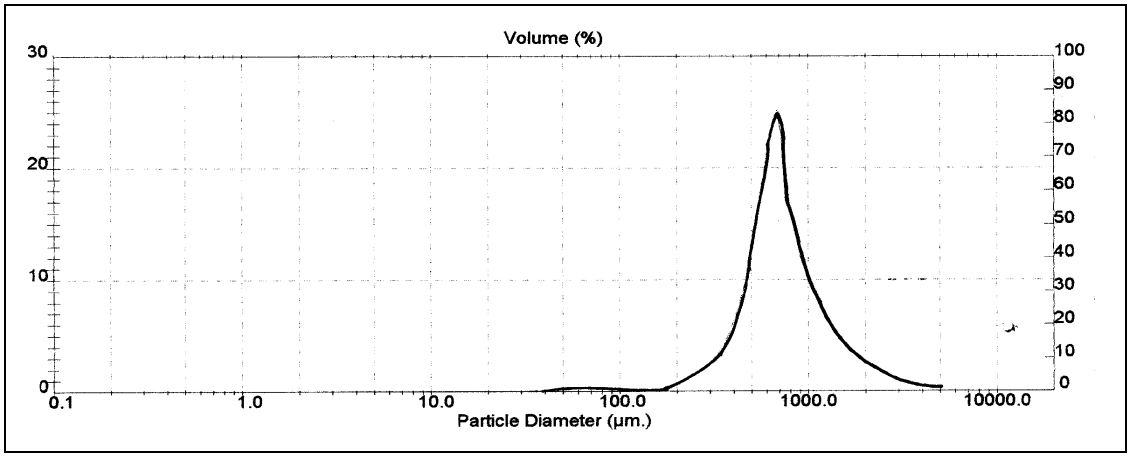

Figure 10 Particle size distribution of P(HEMA)

Table 5 P(HEMA) particle size distribution and ratios

Range: 300 mm Beam: 14.30 mm Sampler: MS7 Obs': 21.5 %
Presentation: 3OHD Analysis: Polydisperse Residual: 2.664 %
Modifications: None

Conc. = 0.2227 %Vol Density = 1.000 g/cm^3 S.S.A.= 0.0151 m^2/g
Distribution: Volume D[4, 3] = 577.11 um D[3, 2] = 397.29 um
D(v, 0.1) = 339.55 um D(v, 0.5) = 603.43 um D(v, 0.9) = 784.65 um
Span = 7.376E-01 Uniformity = 2.247E-01

Size (um)	Volume In %	Size (um)	Volume In %	Size (um)	Volume In %	Size (um)	Volume In %
0.49	0.00	3.60	0.00	26.20	0.09	190.80	0.74
0.58	0.00	4.19	0.00	30.53	0.11	222.28	1.33
0.67	0.00	4.88	0.01	35.56	0.15	258.95	2.15
0.78	0.00	5.69	0.01	41.43	0.20	301.68	3.34
0.91	0.00	6.63	0.01	48.27	0.25	351.46	5.28
1.06	0.00	7.72	0.02	56.23	0.28	409.45	8.65
1.24	0.00	9.00	0.03	65.51	0.31	477.01	14.68
1.44	0.00	10.48	0.04	76.32	0.31	555.71	21.03
1.68	0.00	12.21	0.05	88.91	0.28	647.41	24.76
1.95	0.00	14.22	0.06	103.58	0.22	754.23	14.73
2.28	0.00	16.57	0.07	120.67	0.15	878.67	
2.65	0.00	19.31	0.08	140.58	0.17		
3.09	0.00	22.49	0.08	163.77	0.35		
3.60		26.20		190.80			

3.2 Parameters Affecting Enzyme Activity

The activities of free and immobilized GI were calculated by measuring the absorbances of the fructose-carbazole complexes at 566 nm. The reactions were carried out at various pH values, temperatures and substrates concentrations and the effects of these parameters on activities as well as storage stabilities and repeated use capabilities were examined.

3.2.1 Effect of pH on Activity of Enzyme

In order to examine the effect of pH on activity of free and immobilized enzyme, assay procedure given in section 2.6 was followed. Glucose solutions ($2x10^{-4}$ M) prepared in different buffer media in different pH values ranging between 6.0-8.5 were used in these assays. Temperature was kept constant at 60^{o} C.

3.2.1.1 Effect of pH on Activity of Free Enzyme

Free GI activity values in varied pH media were tabulated in Table 6

Table 6 Effect of pH on activity of free GI

pH	absorbance	relative activity
6.0	0.040	0.274
6.5	0.078	0.530
7.0	0.147	1.000
7.5	0.099	0.673
8.0	0.070	0.476

Table 6 shows that pH 7.0 is the optimum pH for free GI. The activity at pH 7.0, was accepted as maximum activity and all the activities at different pH values were divided to this value to find relative activity values. In both sides acidic and basic regions certain decrease on relative activity of free GI was observed. Enzymes are proteins and the groups which locate in active site or the globular body of the enzyme have electrolytic character which can change by altering pH (39). The presence of acidic and basic groups in the enzyme, substrate and coenzyme molecules effects formation and the stability of enzyme-substrate complex (ES) at different pH media. Reaction rate and the formation of product has a maximum value for a stable ES complex. Therefore optimum pH values should be determined for every enzyme in order to find the maximum reaction rate to get the maximum amount of product. In general, optimum pH value is in between 3 – 8 for enzymes. In very acidic or basic media, enzyme denatures and reaction rate decreases if the amount of denatured enzyme increases. In this study it was found that the optimum activity of free GI is at pH 7.0. Figure 11 shows the activity of free enzyme in different pH values. Activity of free glucose isomerase decreased in acidic and basic region. This can be explained by instability of ES complex in very acidic and basic regions by denaturation of enzyme.

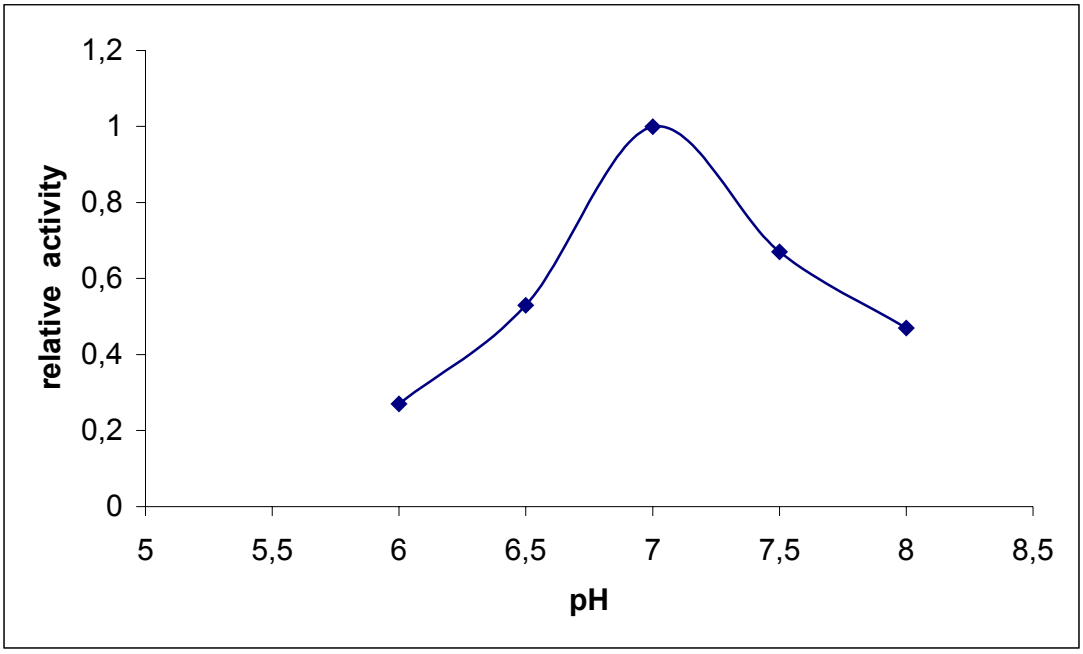

Figure 11 Effect of pH on activity of free GI

3.2.1.2 Effect of pH on Activity of Immobilized GI

In order to search effect of pH on activity of immobilized GI on P(HEMA) particles, glucose solutions ($2x10^{-4}$ M) prepared in different pH media (6.0-8.5) were used. Activities were determined by following ethanol carbazole method given in section 2.6. Obtained activity values are given in Table 7.

Table 7 Effect of pH on activity of immobilized GI

pH	absorbance	relative activity
6.0	0.093	0.462
6.5	0.097	0.482
7.0	0.114	0.567
7.5	0.170	0.845
8.0	0.201	1.000
8.5	0.113	0.562

Optimum pH value for immobilized GI is found as 8.0. The changes in the activies of immobilized GI with respect to pH is schematically represented in Figure 12.

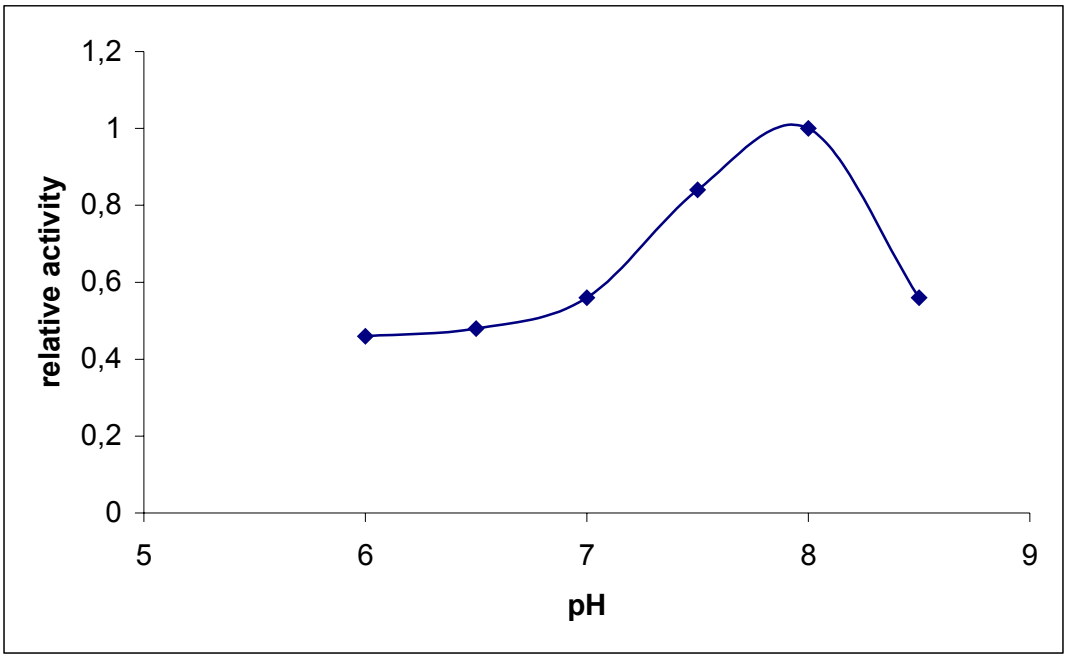

Figure 12 Effect of pH on activity of immobilized GI

While optimum pH was 7.0 for free GI this value is shifted to 8.0 in case of immobilized GI. This shift can be explained by presence of support material in the immobilized system. P(HEMA) is polyionic matrix and it causes different proton concentrations between enzyme microenviroment and reaction medium. Polyanions want to attract protons around the enzyme however polycations repels these protons. Because of these interaction pH is different around support material from bulk of the solution. Other factors of pH shift can be; enzymatic reaction type, structure of matrix, and diffusional limitations which causes a pH gradient between raction media and matrix. pH can also be influenced by the interactions between the enzyme and the support material such as hydrogen bonding and dipol-dipol interactions. In literature optimum pH value was found as 7.5 for free enzyme and 8.0 for immobilized GI on porous glass and cellulose (40-44).

3.2.2 Effect of Temperature on Activity of Enzyme

The temperature effect on the activities of free and immobilized enzyme were determined by ethanol-carbazole method in different temperatures ranging between 50^{o} C-80^{o} C. pH was constant at 7.0 and the concentration of glucose solution was $2x10^{-4}$ M.

3.2.2.1 Effect of Temperature on Activity of Free GI

Optimum temperature was found as 70^{o} C for free enzyme. The activity at 70^{o} C was accepted as 1, and the other activity values were divided to this maximum activity. Obtained relative activities were tabulated in Table 8.

Table 8 Effect of temperature on activity of free GI

T(^{o}C)	absorbance	relative activity
50	0.020	0.125
55	0.074	0.462
60	0.108	0.675
65	0.154	0.962
70	0.160	1.000
80	0.030	0.187

Alterations in the activities of free enzyme with respect to temperature is schematically represented in Figure 13.

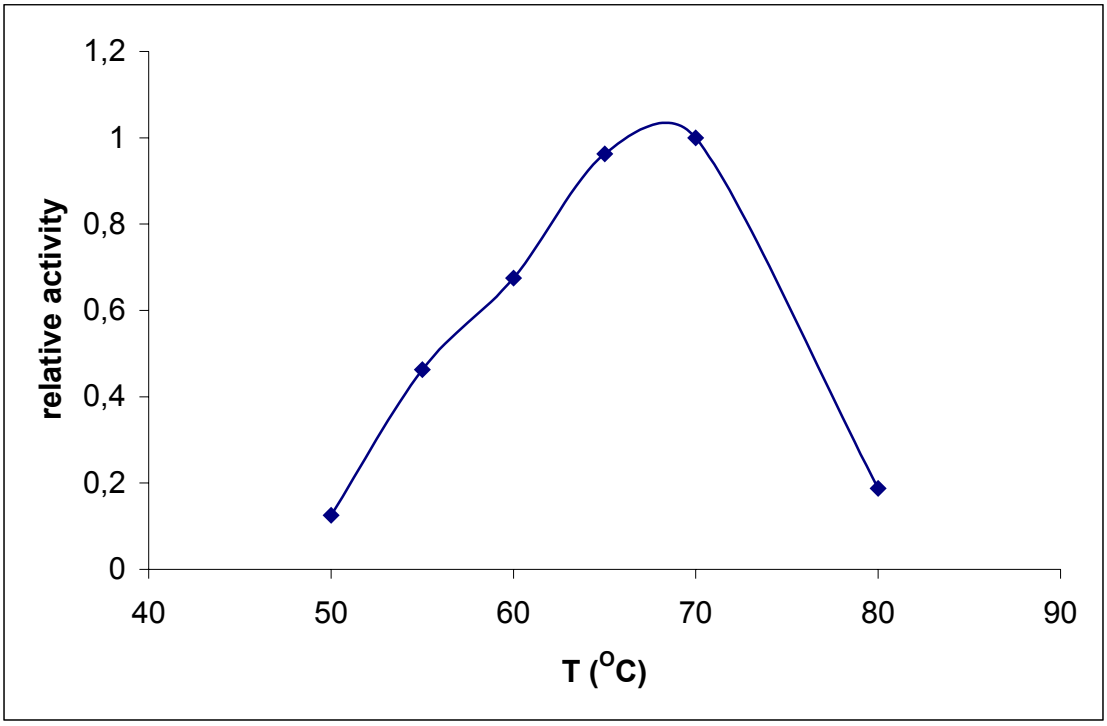

Figure 13 Effect of temperature on activity of free GI

Activity of free enzyme decreased above and below 70° C. Kinetic energy of reactions increase with increasing temperature. In biochemical reaction the same trend is also observed. However above certain temperatures enzymes start to denature because of their balanced protein structure. Tertiary and secondary structure of enzyme collapse with denaturation and the active site of the enzyme loss its activity. In this study it was observed that activity of GI suddenly decreased above 70° C. This shows that after 70° C, GI start to denature and loss its activity.

3.2.2.2 Effect of Temperature on Activity of Immobilized GI

In order to search effect of temperature on the activity of GI which is immobilized on P(HEMA) particles the assay described in section 2.6 was followed in different temperatures in the range of 50° C -70° C. During the reaction pH (7.0) and substrate concentration ($2x10^{-4}$ M) were kept constant. Table 9 tabulates the relative activities of immobilized GI in different temperatures.

Table 9 Effect of temperature on activity of immobilized GI

T(°C)	absorbance	relative activity
50	0.039	0.354
55	0.090	0.818
60	0.110	1.000
65	0.024	0.218
70	0.010	0.091

While 70° C is optimum temperature for free enzyme, immobilized GI demonstrated its maximum activity at 60° C. The main reason of this decrease in activity is conformational limitations of enzyme because of covalent bond formation between enzyme and polymer support material (P(HEMA)). In other words immobilization process prevent free motion of enzyme residues in some extent compared with free enzyme and enzyme exhibits its catalytic activity in lower activation energy than normal state. Lowering in enzyme catalytic activity cause shift in optimum temperature to lower values as observed for immobilized GI. Figure 14 shows the trend in temperature optimization of immobilized GI.

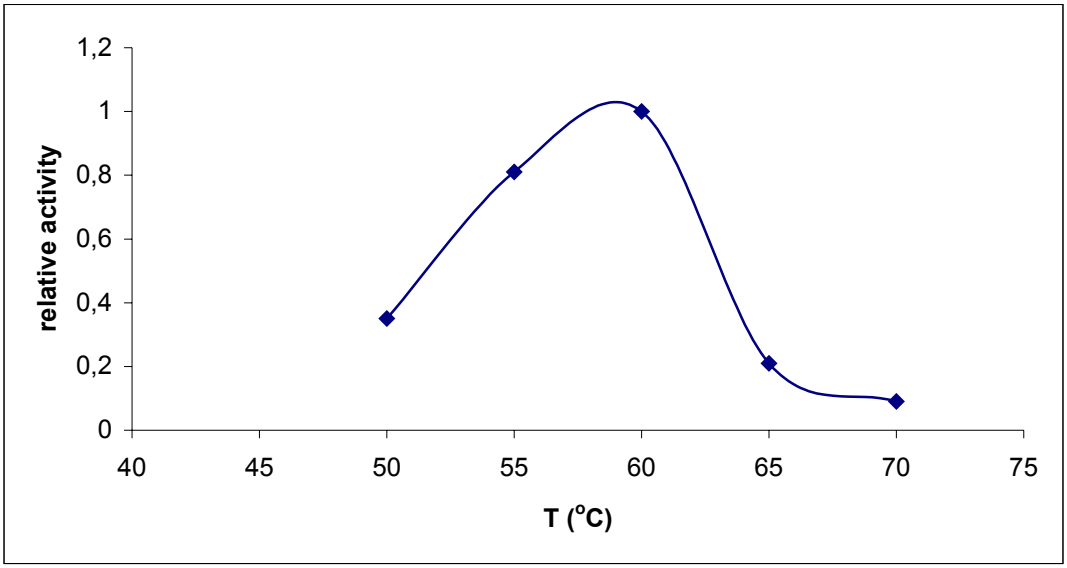

Figure 14 Effect of the temperature on activity of immobilized GI

In literature it is given that immobilized glucose isomerase on silica and polyurethane exhibits optimum activity at 75^{o} C, this value shift to 65^{o} C for free enzyme (45, 46). Depending on the support, the activation process, and the binding process of the enzyme; shifts towards to higher or lower temperatures are given in the literature for various enzymes.

3.2.3 Effect of Substrate Concentration on Activity of GI

Kinetic parameters K_m and V_{max} for free and immobilized enzymes were determined by using soluble glucose as substrate. The measurements were achieved in the way described previously in section 2.6 in order to determine the initial rates for conversion of glucose to fructose. Calibration curve was obtained by plotting absorbance values to fructose concentrations as shown in Figure 15.

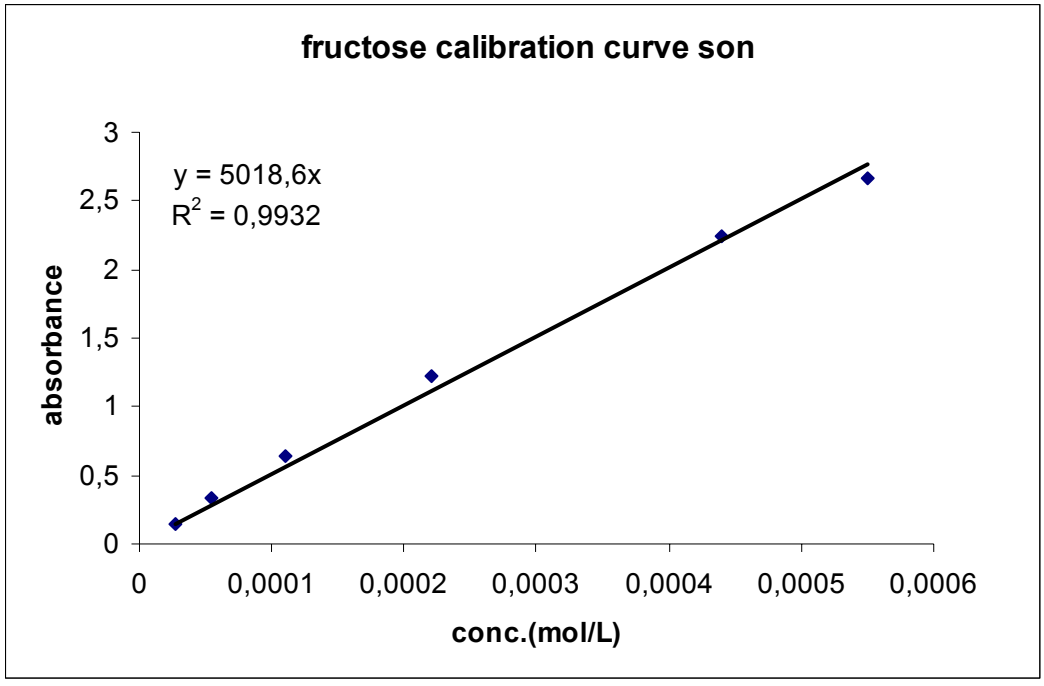

Figure 15 Calibration curve of fructose

In order to determine effect of substrate concentration on the activity of GI, glucose solutions in various concentrations (0.02M, 0.01M, 0.005M, 0.002M, 0.001M) were prepared. Free and immobilized GI activities were measured by using these glucose solutions.

Enzyme and substrate reactions can be shown as;

$$E+S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E+P$$

In this equation E represents enzyme S is substrate, ES is enzyme-substrate complex, and P is product.

Initial rate of reaction is given by Michaelis-Menten Equation as;

$$V_0 = \frac{V_{max} * S}{K_m + S}$$

Lineweaver-Burk Equation is obtained from Michaelis Menten Equation by arrenging it. K_m and V_{max} values of free and immobilized enzyme for various substrate concentrations were calculated from Lineweaver-Burk Equation given below.

$$\frac{1}{V_0} = \frac{K_m}{V_{max}} \times \frac{1}{S} + \frac{1}{V_{max}}$$

In the equation V_0 is the initial rate, V_{max} is the maximum rate and K_m is Michaelis constant. S represents the substrate concentration. V_{max} and K_m values are determined from this equation by using known amount of substrate and measuring the initial rate of reaction. The rates of free and immobilized enzyme for various substrate concentrations are plotted in the form of Lineweaver-Burk plots to obtain K_m and V_{max} values in both systems.

3.2.3.1 Effect of Substrate Concentration on Activity of Free GI

In order to search effect of substrate concentration on the activity of free GI,

glucose solutions (40 mL) were prepared in various concentrations (0.02M, 0.01M, 0.005M, 0.002M, 0.001M). 13.3 mL $MgSO_4$ and 13.3 mL PB were added each glucose solution was stored at 60^{o} C in water bath after adding free enzyme (5mL) at to these solutions. Samples (3 mL) were taken from each glucose solution at certain time intervals (at 1, 3, 5, 7, 13, 15, 20, 25, 30, 40, 50, 60, min) and enzyme assays were carried out. Activities versus time plots were used for determination of the initial of reaction rates which are the initial linear parts on the graph. Absorbance values of free GI are tabulated in Table 10 and schematically represented in Figure 16.

Table 10 Effect of substrate concentration on absorbance of free GI

minute	0.02M	0.01M	0.005M	0.002M	0.001M
1	0.860	0.495	0.260	0.130	0.051
3	0.975	0.523	0.270	0.104	0.056
5	1.060	0.532	0.266	0.100	0.054
7	1.400	0.715	0.335	0.140	0.075
13	2.040	1.010	0.510	0.199	0.110
15	2.428	1.218	0.612	0.239	0.133
20	3.034	1.517	0.753	0.300	0.156
25	3.011	1.990	0.920	0.388	0.201
30	3.150	2.430	1.202	0.478	0.235
40	3.100	2.480	1.237	0.490	0.246
50	3.090	2.430	1.244	0.496	0.254
60	3.100	2.510	1.260	0.495	0.269

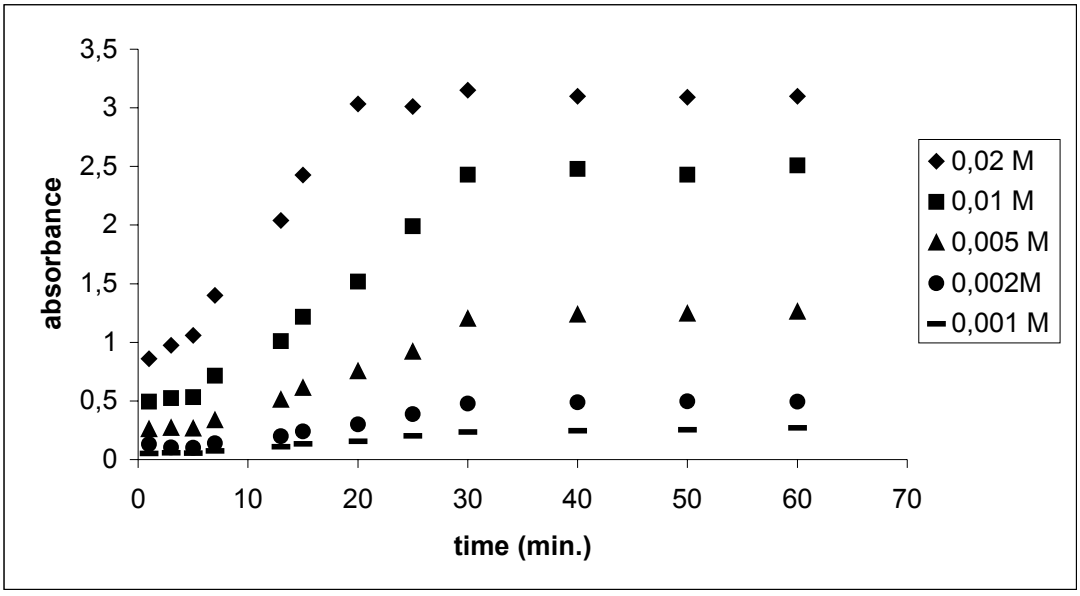

Figure 16 Effect of substrate concentration on absorbance of free GI

By using these activity values obtained from Table 10, Lineweaver-Burk graphs were plotted for free GI. Actvities were calculated from the slopes of the linear parts of the graphs using the absorbance values at 15min. Activities and substrate concentrations (at 15 min.) were tabulated in Table 11. Reaction rate V is calculated with the formula given below.

$$\text{Rate(V)} = \frac{\triangle c}{\triangle t} = \frac{\triangle A_{566}}{\triangle t} \text{ x } \frac{\triangle c}{\triangle A_{566}}$$

Table 11 Activities of free GI at 15 min in different concentrations of glucose

absorbance	V (mol/L.min.)	1/V (L.min/mol)	S (mol/L)	1/S (L/mol)
2.428	3.22×10^{-5}	3.10×10^{4}	0.020	1.66×10^{2}
1.218	1.61×10^{-5}	6.17×10^{4}	0.010	3.33×10^{2}
0.612	$8.13x \times 10^{-6}$	1.23×10^{5}	0.005	6.66×10^{2}
0.239	3.12×10^{-6}	3.15×10^{5}	0.002	1.66×10^{3}
0.133	$1.75x \times 10^{-6}$	5.66×10^{5}	0.001	3.33×10^{3}

Reciprocal values of substrate concentrations and initial rates were used to obtain Lineweaver-Burk plot given in Figure 17.

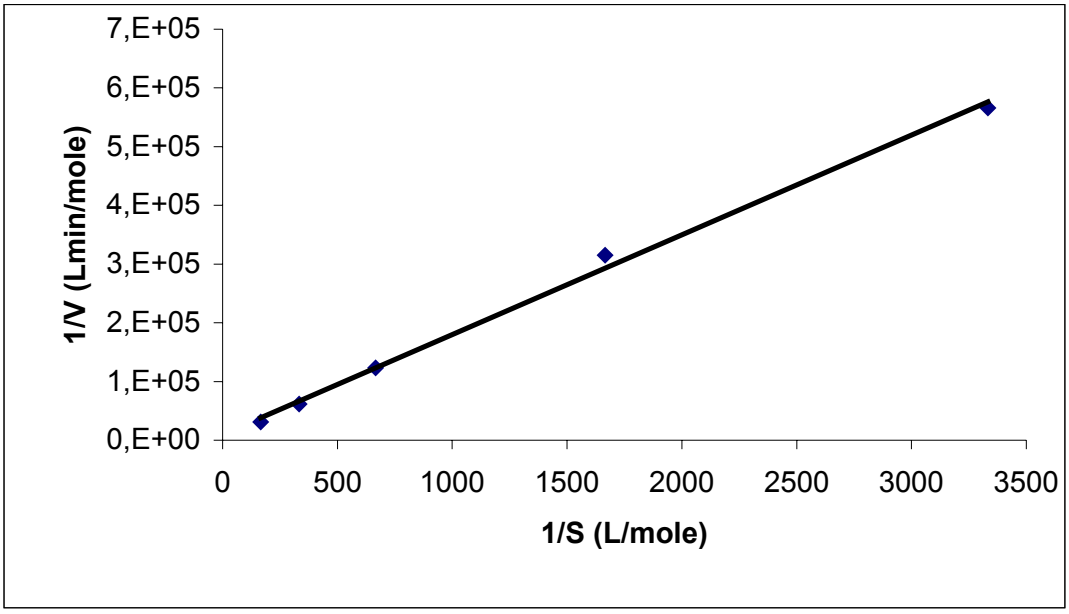

Figure 17 Lineweaver-Burk plot of free GI

V_{max} and K_m values of free GI were determined from Lineweaver-Burk equation. K_m value found as $1.7x10^{-2}$ mol/L and V_{max} value is found as $1.01x10^{-4}$ mol/L.min. When K_m increases V_{max} increases as well. In Kenneth's work for glucose isomerase K_m is given as 0.411 mol/L and V_{max} value is given as $2.2x10^{-4}$ mol/L.min (46). These variations in the values mat come from the varitions in the origin of enzymes.

3.2.3.2 Effect of Substrate Concentration on Activity of Immobilized GI

Glucose solutions (40 mL) were prepared in various concentrations (0.02M, 0.01M, 0.005M, 0.002M, 0.001M). 13.3 mL $MgSO_4$ and 13.3 PB were added. Each glucose solution was stored at 60^o C in water bath after adding immobilized enzyme (1.33g) in to these solutions. Samples (3 mL) were taken from each medium and amount of fructose were determined at certain time intervals (at 1, 3, 5, 7, 13, 15, 20, 25, 30, 40, 50, 60 min). This time scan was used for the determination of initial rate which is the linear part of the absorbance time graphs. Absorbance values of immobilized GI are tabulated in Table 12 and schematically represented in Figure 18.

Table 12 Effect of substrate concentration on absorbance of immobilized GI

minutes	0.02M	0.01M	0.005M	0.002M	0.001M
1	1.300	0.610	0.294	0.117	0.069
3	1.470	0.640	0.310	0.125	0.062
5	1.640	0.660	0.324	0.129	0.064
7	1.730	0.823	0.397	0.167	0.081
13	2.480	1.190	0.575	0.235	0.119
15	2.740	1.218	0.591	0.237	0.121
20	3.510	1.817	0.880	0.360	0.177
25	3.600	2.540	1.190	0.497	0.248
30	3.620	2.900	1.400	0.556	0.287
40	3.590	3.250	1.610	0.584	0.317
50	3.590	3.330	1.617	0.650	0.325
60	3.570	3.800	1.615	0.741	0.381

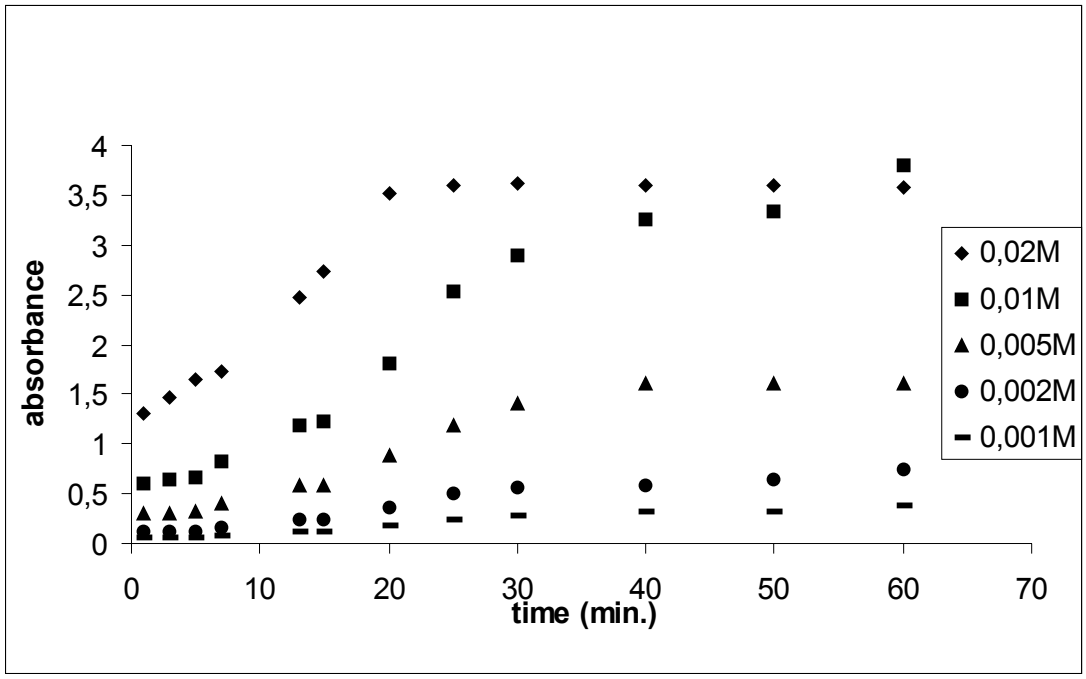

Figure 18 Effect of substrate concentration on absorbance of immobilized GI

Activities of immobilized GI at 15 minute were tabulated in Table 13. Reciprocal values of substrate concentrations and rates were used to obtain Lineweaver-Burk plot given in Figure 19.

Table 13 Activities of immobilized GI at 15 min in different concentrations of glucose

absorbance	V (mol/L.min.)	1/V (L.min/mol)	S (mol/L)	1/S (L/mol)
2.740	3.63×10^{-5}	2.75×10^{4}	0.020	1.66×10^{2}
1.218	1.60×10^{-5}	6.25×10^{4}	0.010	3.33×10^{2}
0.591	7.29×10^{-6}	1.37×10^{5}	0.005	6.66×10^{2}
0.237	3.14×10^{-6}	3.18×10^{5}	0.002	1.66×10^{3}
0.121	1.60×10^{-6}	6.22×10^{5}	0.001	3.33×10^{3}

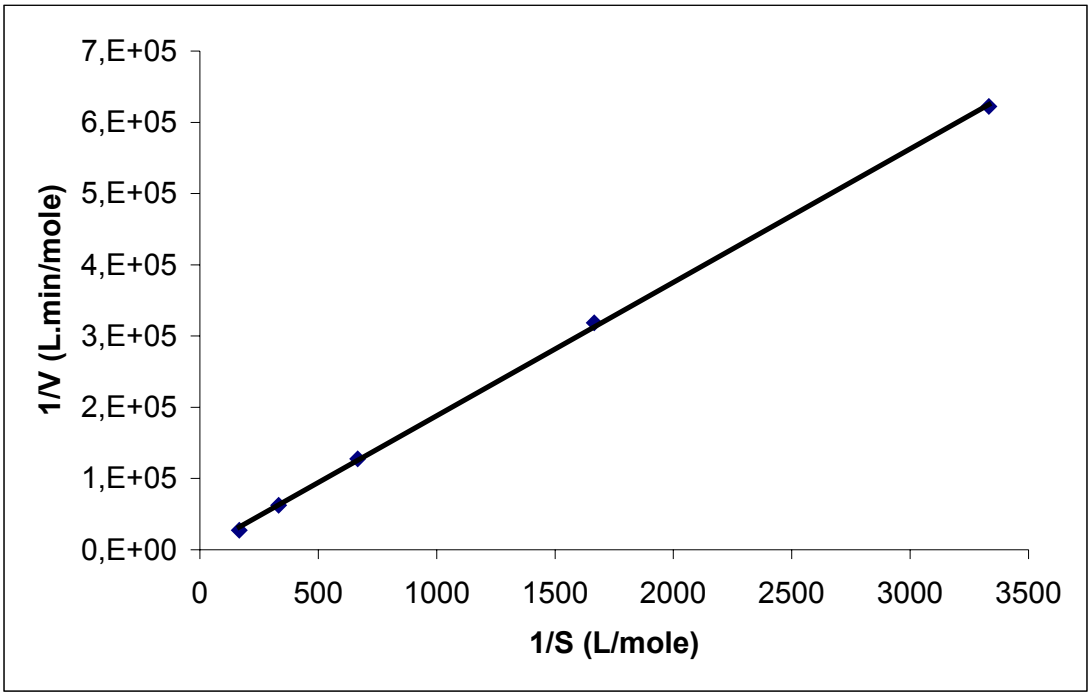

Figure 19 Lineweaver-Burk plot of immobilized GI

V_{max} and K_m values of immobilized GI were determined by Lineweaver-Burk equation. K_m value is found as 3.1×10^{-1} mol/L and V_{max} value is found as 1.65×10^{-3} mol/L.min.

V_{max} of GI which is immobilized by using cyanuric chloride onto P(HEMA) particles were higher than V_{max} of free GI. This situation can be explained with covalent bonding. Because of covalent bonding GI changes its conformation and active groups of the enzyme becomes more susceptible to react with substrate. K_m value of immobilized GI increased comparing with

K_m value of free GI. Increasing of K_m value with immobilization is the result of steric effects and difusional limitations of substrate to enzyme upon immobilization. Conformational changes of globular structure of enzyme alters the interactions between substrate and polymer matrix and decrease the possibility of formation of enzyme-substrate complex. This effect cause an increase in K_m value of immobilized enzyme compare to free enzyme. Chemical structure of support materials effects K_m and V_{max} values of enzymes. In Lee's work K_m is given as 0.21 mol/L and V_{max} is given as 0.087 mol/L.min. for GI immobilized on porous glass (47).

3.2.4 Repeated Use Capability of GI

The main benefit of immobilization of enzyme is using the enzyme repeatedly. Immobilization protects enzyme to loss its activity and therefore increase its stability. This kind of immobilization applications decrease the cost of the enzyme systems in industrial applications. Reuse capacity of immobilized GI were designated by using the same immobilized enzyme system many times successively. In this study immobilized enzymes (0.1 g) were stored in (5 mL) buffer solution (pH 7.0) at 4^o C and activities were detected at certain time intervals. Assay reactions were achieved by using $2x10^{-4}$ M glucose at 60^o C by following the described procedure given in section 2.6. Same conditions were applied for commercially available immobilized GI and reuse capacity of this enzyme was also found. Measured activities of the immobilized samples that were used repeatedly 40 times in 6 days, are presented in Figure 20. It was observed that after 40th use, immobilized GI and Commercial GI retained almost the same amount, ~80%, of their original activities.

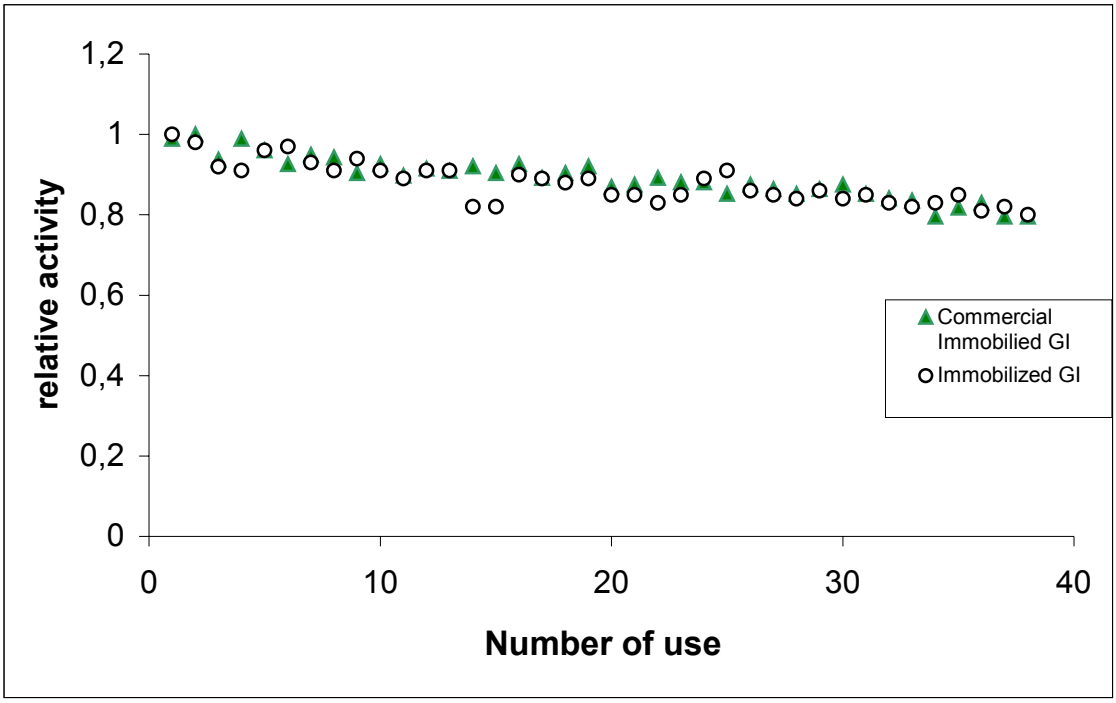

Figure 20 Effect of reuse on activity of immobilized GI

The reason of retaining their high amount of activity is the increase of stability of enzyme upon immobilization on a solid support or polymer matrix. Polymer matrix can protect enzyme against to denaturation by fixing its conformation with covalent bonds. In literature for α amylase immobilized on poly(methyl methacrylate-acrylic acid) microspheres, it is given that enzyme retained 90.0% of its original after using repeatedly 20 times within 3 days (48). Immobilization of polyphenoloxidase in conducting copolymers (menthyl monomer with pyrrole) makes enzyme stable for repetitive uses, polyphenoloxidase exhibits very high repeated use capability and retained 85% of its activity up to 40 th use (49).

3.2.5 Storage Stability of GI

Enzymes in solution are not stable and during storage their activities decrease gradually with time. They, either free or immobilized, have certain half lives and can be effective for limited times. Therefore the storage stabilities of enzymes are very important. In this study 0.1g of P(HEMA)

particles with immobilized GI were stored in 5mL PB (pH 7.0) at 4 ^{O}C and stabiltiy of immobilized GI was investigated by measuring the enzyme activities at certain time intervals by following the procedure given in section 2.6. The results are given in Figure 20. Immobilized enzyme was retained more than 90% of its orginal activity for 3 weeks. Retained activity decreased to 80% in 4 weeks and to 60% in 8 weeks, when stored in buffer at 4^{o}C. Figure 21 shows the effect of storage on the activity of immobilized GI.

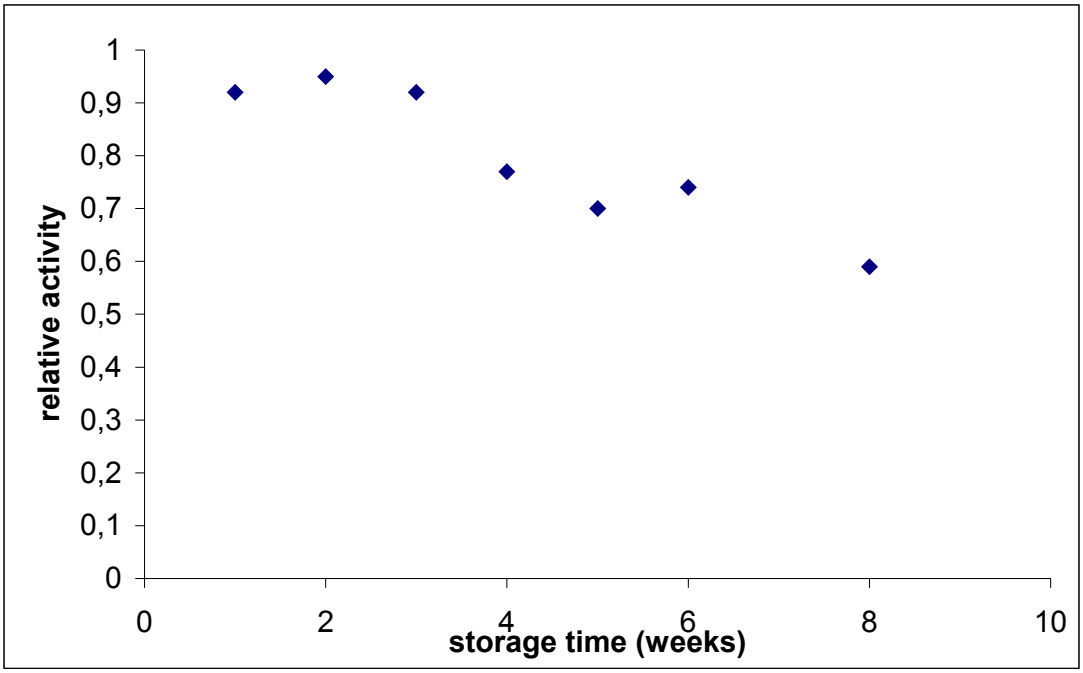

Figure 21 Effect of storage on activity of Immobilized GI

Immobilized enzymes were stored in buffer solution at pH 7.0 and at 4^{o} C when they were not used in the experiments. The decrease in activity with time is the result of denaturation of enzyme. In literature it is given that α amylase immobilized on poly(methyl methacrylate-2-hydroxyethyl methacrylate) microspheres retained 90% of its activity after 20 days of storage at 4^{o} C (50). Immobilization of glucose oxidase in poly pyrrole-poly 2-methylbutyl-2-(3-thienyl) acetate matrix improves its storage stabilty, glucose oxidase retained 40% of its activity after 35 days of storage (51).

3.2.6 Continuous Flow Application

Immobilized enzyme was packed in to small glass column (with sizes of 5 cm lenght, 1 cm diameter) heated by water circulation to 60°C. 200 mL of substrate solution ($2x10^{-4}$M glucose) in water, 66 mL of PB (pH 7.0), and 66 mL of $MgSO_4$ solution (0.05 M) were mixed and this solution (332 mL) was heated to 60°C and passed through the column with a rate of 3 mL/min. In every minute, 3 mL samples were collected into glass tubes, up to 180 mL. 5 mL $HClO_4$ solution (0.5M) was added into every collected sample. These samples were treated according to the procedure given in section 2.6 to determine the content of fructose and to find activity of enzyme. Figure 22 shows the trend of immobilized enzyme in continuous flow system. Absorbance values of two immobilized systems were given in Table 14. Same experiment were repeated with the commercial enzyme. It was observed that both immobilized enzyme systems retain 90% their activity under continuous flow .

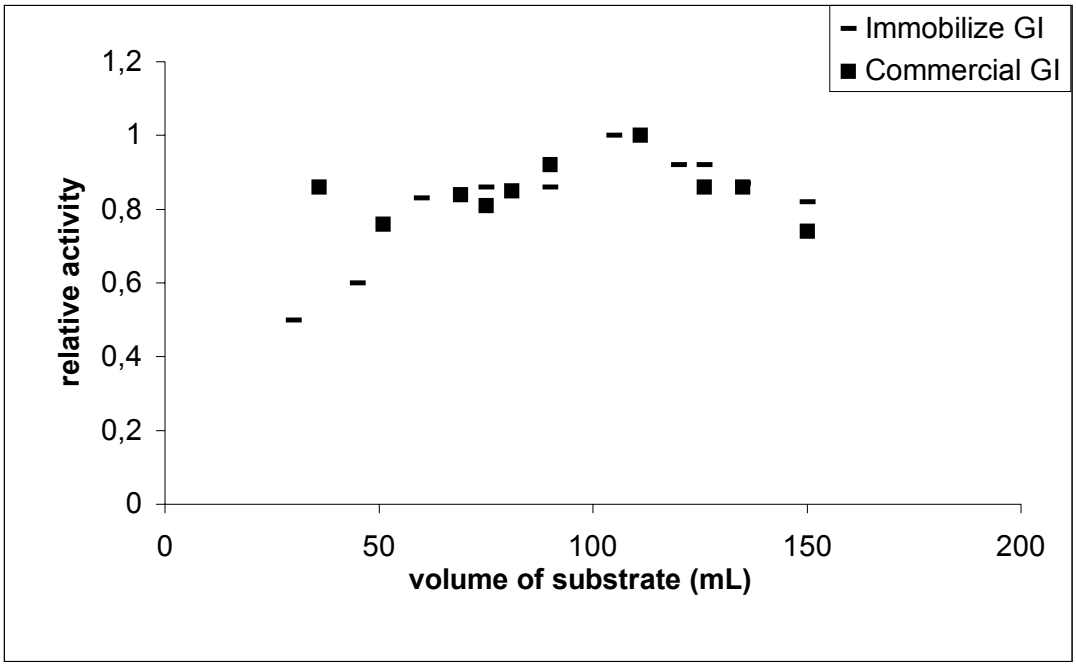

Figure 22 Activity of immobilized GI in continuous system

Table 14 Absorbance values of Immobilized GI and Commercial GI under continuous flow

Time (min.)	Absorbance	
	Immobilized GI	Commercial GI
1	-	-
5	-	-
7	-	-
10	0,11	-
12	-	0,183
15	0,132	-
17	-	0,161
20	0,18	-
23	-	0,179
25	0,187	0,172
27	-	0,185
30	0,188	0,196
35	0,216	-
37	-	0,211
40	0,199	-
42	-	0,176
45	0,188	0,177
50	0,178	0,157
55	0,173	-
60	0,133	-

CHAPTER 4

Conclusions

The present study shows that the P(HEMA) polymer particles are promising carriers for the immobilization of GI enzyme. The immobilization of GI on P(HEMA) particles promoted enzyme stability and as a result, the enzyme became more resistant to temperature and storage and became available for repeated or continuous use. For immobilized GI system, the activities were measured at different conditions and the observed optimum values were compared with the values of free enzyme. The obtained results are summarized as follows;

- Optimum temperature was determined as 70 ^{o}C for free GI and 60 ^{o}C for immobilized GI.
- The pH for maximum substrate conversion was found to be 7.0 for free GI and 8.0 for immobilized GI.
- K_m value was found as $1.7x10^{-2}$ mol/L and V_{max} $1.01x10^{-4}$ mol/L.min. for free enzyme. K_m value was found as $3.1x10^{-1}$ mol/L and V_{max} value was found as $1.65x10^{-3}$ mol/L.min for immobilized system.
- Immobilized GI on P(HEMA) particles and commercial GI retained 80% of their original activities after 40th use, within 6 days.
- Storage stability of GI was improved via immobilization. Immobilized enzyme was retained more than 90% of its orginal activity in 3 weeks and 60% of its original activitiy in 60 days of storage in buffer(pH 7.0) at 4 ^{o}C.
- Immobilized GI and commercial GI retained 90 % of their activity under continuous flow.
- Immobilized GI and commercial GI convert 36% of the glucose to fructose under continuous flow .

Immobilized GI allows continuous processes to be practicable, with a considerable saving in enzyme, labour and overhead costs. Immobilization of GI often affects the stability and activity of the enzyme. The productivity of an immobilized enzyme greatly increases as it may be more fully used at higher substrate concentrations for longer periods than the free enzyme. The prepared immobilization system exhibited the similar behaviours and almost the same stability values as the ones commercially available. Therefore, the prepared system can be a good candidate to be used in food industry.

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