**Optimization of Immobilized Aldose Reductase Isolated from Bovine Liver**

Sığır Karaciğerinden İzole Edilen İmmobilize Aldoz Redüktazın Optimizasyonu


## ABSTRACT

**Objectives:** Isolation of enzymes and experiments on enzymes require a great effort and cost and also they are time-consuming. Therefore, it is important to extend the usability of the enzymes by immobilizing. In this study our purpose was to immobilize the aldose reductase (AR) enzyme and to optimize the experimental conditions of the immobilized AR and compare them to the free AR's.

**Materials and Methods:** AR was isolated from bovine liver and immobilized the enzyme into the photographic gelatin by cross-linking with glutaraldehyde. Then the optimum conditions of free and immobilized AR for pH, temperature and storage were characterized by determining the enzyme activity.

**Results**: Following immobilization, the optimum pH and temperature levels of free AR which were pH 7.0 and 60 °C

slightly altered to pH 7.5 and 50 °C. The enzyme activity of the immobilized AR maintained for about 65% after reusing 15 times. Moreover, immobilized AR can maintain its original activity for 95% after 20 days storing at 4 °C, while the retained activity of the free AR was 85% of its original activity.

**Conclusion**: Our experiments indicated that the conditions which affect the enzyme activity might alter following immobilization. Once the optimum experimental conditions are fixed, the immobilized AR can be stored and reused with a higher efficiency than the free aldose reductase. Moreover, this study provides an insight into the advantages of using immobilized AR in the enzyme assays rather than the free one.

**KEYWORDS:** Aldose reductase, isolation, immobilization.

**ÖZ**

**Amaç:** Enzim izolasyonu ve enzimler üzerinde yapılan deneyler, büyük çaba, maliyet ve zaman gerektirir. Bu yüzden, enzimlerin kullanılabilirliğinin immobilizasyon ile uzatılması önemlidir. Bu çalışmadaki amacımız, aldoz redüktaz (AR) enzimini immobilize etmek ve serbest

AR'ninkiler ile karşılaştırarak immobilize AR'nin deney şartlarını optimize etmektir.

**Gereç ve Yöntemler:** AR, sığır karaciğer ve böbreğinden izole edildi ve gluteraldehid ile fotoğrafik jelatine çapraz bağlanarak immobilize edildi. Daha sonra, enzim aktivitesi belirlenerek serbest ve immobilize AR'nin optimum pH, sıcaklık ve depolama koşulları tespit edildi.

**Bulgular:** Serbest AR için pH 7,0 ve 60 °C olan optimum pH ve sıcaklık seviyeleri, immobilizasyondan sonra pH 7,5 ve 50 °C olarak belirlendi. İmmobilize AR'nin enzim aktivitesinin, 15 kez kullanımdan sonra %65 oranında korunduğu tespit edildi. Bununla birlikte, +4°C'de 20 gün boyunca saklanan serbest AR'nin aktivitesi %85 oranında korunurken immobilize AR'nin aktivitesinin %95 oranında korunduğu bulunmuştur.

**Sonuç:** Deneylerimiz, immobilizasyonu takiben enzim aktivitesini etkileyen koşulların değişebileceğini göstermektedir. Ayrıca, immobilize AR, serbest AR'ye göre daha yüksek aktiviteyle korunup tekrar tekrar kullanılabilmektedir.

**Anahtar kelimeler:** Aldoz redüktaz, izolasyon, immobilizasyon.

## 1. INTRODUCTION

Aldose reductase (AR) (EC 1.1.1.21) is expressed by AKR1B1 gene in human which locates on 7q35 chromosome, consists of 315 amino acids and its molecular weight is 36 kD. AR is a cytosolic protein which is monomeric.[1] It specifically catalyzes the polyol pathway which includes the conversion of glucose bound NADPH cofactor to sorbitol.[2,3] Moreover, it reduces the activity of aldehydes such as glutathione complexes.[4] Indeed, AR is crucial in detoxification of lipid aldehydes produced by oxidative stress.[5]

Under optimum conditions, enzymes are able to display their activities outside of their natural environment. Using this feature, they can be employed on a large scale of utilization in health science such as diagnosis and treatment of diseases and drug design. However, their structure tends to alter during the experimental process. Therefore, studies aim to improve conformational stability of enzymes.[6] Immobilization is a method reducing disruption of enzyme structure. Enzyme immobilization avoids majority of the instability and loss of the enzyme activity. This provides longer time and flexibility to work on

enzymes. Immobilization is not only useful to maintain the stability of biomolecule but also it provides lower expenses for the enzyme studies.

In this study for the first time the AR enzyme was isolated from bovine liver, immobilized and characterised in respect to several kinetical properties. In addition; AR derived from bovine kidney was used as a side control and simultanously applied to identical experiments. The AR enzymes from both sources were immobilised into the gelatin by gluteraldeyhde and washed to remove the free enzyme. We compared the attitudes of the free and the immobilised AR under different conditions of pH, temperature, reusing and storage. The difference in optimum conditions of the free and the immobilised enzyme may refer to alterations on the structure caused by several factors such as carrier material, immobilisation method and activation method.[7] Indeed it was previously indicated that the immobilisation significantly changes the conformation of enzymes and so their activity.[8]

## 2. MATERIAL & METHOD

*Chemicals materials*

In this research, ethylenediaminetetraacetic acid (EDTA), nicotineamide adenine dinucleotide phosphate (NADPH) and lithium sulphate ($Li_2SO_4$) was purchased from Gerbu, Germany. Ammonium sulfate was supplied from MERCK. Phenylmethylsulfonyl fluoride (PMSF), DL-glyceraldehyde and all other materials used were analytically graded and obtained from Sigma Aldrich, Germany.

*Isolation of Aldose Reductase Enzyme from Bovine Liver*

The bovine liver was provided from a slaughterhouse of Kazan, Ankara, Turkey. Small pieces of the liver samples were washed with 1.0 mM EDTA. Then it is measured and homogenized with threefold 1.0mM EDTA and 50 µM PMSF and spinned at +4 °C, 10,000 rpm for 30 min. To acquire 40% saturation, 22.6 gr ammonium sulfate was added to each 100mL supernatant and then rotated at 10,000 rpm at +4 °C for 25 min. To gain 50% and 75% saturations, the same method was carried out by adding 5.8 gr and 15.9 gr of ammonium sulfate to the 100 mL supernatant solution respectively. The pellets were dissolved with 50 mM sodium chloride and stored in deepfreeze at -80 °C.

*Determining the protein amount*

Following the isolation, amount of protein was detected by Bradford assay.[9] The bovine serum albumin (BSA) standards were used at the concentrations of 0.4, 0.6, 0.8, 1.0, 1.2, 1.4 mg/ml. BIO-RAD reagent was added into the BSA standards and the sample. Then, each solution was measured using spectrophotometer at the 595 nm wavelength. Finally, protein amount was calculated by using the standard graph which is generated with optical densities of the standards.

*Assay of AR enzyme activity*

AR activity was detected against DL-Glyceraldehyde, by monitoring the NADPH oxidation to $NADP^+$ at 340 nm.[10] The reaction consisted of AR enzyme (4.54 mg/mL), NADPH ($9\times10^{-5}$ M), $Li_2SO_4$ (320 mM-400mM), DL-GA ($6\times10^{-4}$ M), KP buffer (50mM, pH 6.2). $NADP^+$ oxidation was followed in 0.25mL reaction using multimode microplate reader at 340 nm for 4 min. Initial and final rates of enzymatic reactions were measured and recorded as nmol/ minute/ mg protein.

*Immobilization of AR enzyme*

The immobilization gel consisted of 0.025 g photographic gelatin and 0.125 M glutaraldehyde in 0.067 M phosphate tampon solution (pH 7.4). The photographic gelatin was prepared at 50 °C and then cooled to 30-35 °C. Different units of AR enzyme was added into the gelatin and was vortexed. Cross-linking glutaraldehyde was included at the concentration of 5 x $10^{-5}$ M. 100 µL of the mix was placed onto cellulose triacetate. Enzyme and gelatin complexes were dried for 24 hours at room temperature. The film strips carrying the immobilized AR were assayed for the enzyme activity. The reaction consisted of 2.7 mL potassium phosphate, 0.1 mL NADPH and 0.1 mL DL-glyceraldehyde. Absorbance of this reaction mixture was measured at 340 nm before and after 20 min incubation with the film strips to detect the enzyme activity.

## 3. RESULT & DISCUSSION

AR isolated from bovine liver was used to immobilize by cross-linking with glutaraldehyde into photographic gelatin. To detect the amount of AR protein we used Bradford assay and spectrophotometric measurements. The results indicated that the protein amount was 18.39 ± 0.09 mg/ml. In the standard group, the activity values for

only AR were 4.172 x $10^{-4}$ U/L in liver. These values are accepted as 100% enzyme activity.

Following immobilisation, the free enzyme was removed by washing the film strips three times with phosphate buffer (0.067 M, pH 6.2) at 25 °C for 5 min and spectrophotometry was used to detect the amount of free enzyme. Using the washed film strips, the effect of enzyme concentration on immobilized enzyme activity was tested to detect the immobilized enzyme leakage. Our results showed that AR leakage from the film strips increased with enzyme concentration while the enzyme activity was reduced after each wash. On the other hand, three times-wash was sufficient to remove the leakage completely (Table 1).

We demonstrated that enzyme leakage increased depending on the increasing enzyme unit. This is because of the constant concentrations of the gelatin and the gluteraldehyde. In other words, when there was not sufficient gelatin which is the immobilisation ground and/or gluteraldehyde which is the crosslinker in the immobilisation reaction, there was more unbound AR that released from the film strips at the high enzyme concentrations.

*Characterization of the Free and Immobilized AR*

Conditions of pH, temperature and storage were optimized to characterize the free and the immobilized AR. Moreover, effects of the storage stability and continual use on the activity of immobilized strips were tested.

*pH* To evaluate the optimum pH for the free (1.46 x $10^{-8}$ U) and the cross-linked (1.56 x $10^{-8}$ U) AR, we measured the levels of enzyme activity in a pH gradient between pH 4 and 9 at 25 °C for 5 min. It was demonstrated that the optimal pH values for the free and the immobilized AR from liver were respectively 7.0, 7.5 (Figure 1).

We observed that the optimum pH for AR shifted to 7.5 from 7.0 upon immobilisation. This alteration should be because of the $H^+$ and the $OH^-$ ions that are released to microenvironment during the reaction. Indeed, this result on the optimum pH alteration was expected because of the context of the environments of free and immobilised enzymes as it was previously indicated.[11] Moreover, previous studies showed that optimum pH of the enzyme was changed by the polar groups of gelatin during the immobilisation.[12] There are possible interactions such as hydrogen bond which are generated between enzyme and polymer carrier.[13] However, the immobilisation might not be significantly effective on the stability of optimum pH for

enzyme in some cases. For instance, NADH-cytochrome b5 reductase enzyme was isolated from the rabbit liver microsoms and it was immobilised into the photographic gelatin by chemical cross-linking using chrome (III) acetate. The experiments showed that the optimum pH for the free and the immobilised enzyme was at the range from 6.1 to 7.5.[14] Similarly, the free and the immobilised β-galactosidase on the gelatin carboxymethylcelulose carrier were assayed for the optimum pH. It was observed that immobilisation of β-galactosidase did not showed any significant effect on the pH stability.[15] Consequently, it can be foreseen that the values of optimum pH for free and immobilised enzyme can change, however, this might not stand for all the enzymes and the immobilisation methods.

*Temperature* We aimed to uncover the difference between optimum temperatures for the free and the immobilised AR that is important for the enzyme activity. To detect the optimum temperatures, activity of the enzyme was tested at different levels ranging from 5 °C to 70 °C. The highest enzyme activity was observed at 60 °C for the free AR from liver and this shifted to 50 °C after the cross-link immobilisation (Figure 2).

These results suggested that the immobilisation of AR from liver reduces the optimum temperature. However, in our experiments we also showed that the immobilisation increased the thermal stability of the enzyme between 60 °C and 70 °C (Figure 2). These results indicated that immoblization generated an optimum temperature range from 50 °C to 70 °C, while free enzyme showed the highest activity only at 60 °C. In other words, immobilization maintained the enzyme stability against to increasing temperature. It was previously demonstrated that the optimum temperature of NADH-cytochrome b5 reductase enzyme from rabbit was 30 °C, while it reduced to 25 °C upon cross-link immobilisation of the enzyme.[14] Conversely, free β-galactosidase showed the highest enzyme activity at 47 °C while it was at 57 °C after the enzyme was cross-linked to the gelatin carboxymethlyselulose carrier.[15] This might suggest that the carrier system preserves the immobilised enzyme from the thermal denaturating.

*Reusing* To test the activity of the reused AR, it was immobilised to the photographic gelatin by gluteraldehyde at 5 x $10^{-8}$ M concentration. Upon reusing the immobilized AR from liver (1.46 x $10^{-8}$ U) for 15 times a day at 25 °C, 65% of its original activity was recovered (Figure 3a).

Similarly, we applied the same approach to the different concentrations of the immobilized AR at different conditions. For instance, the immobilized AR from liver at the concentration of 2.08 x $10^{-8}$ U conserved the 35% of the beginning enzyme activity after using 10 times at 25 °C (Data not shown). We also tested the immobilised AR from liver for the concentrations at 1.04 x $10^{-8}$ U, 1.46 x $10^{-8}$ U and 2.08 x $10^{-8}$ U after reusing 4 times and reported that its activity was conserved for about 74%, 90% and 72%, respectively (Figure 3b).

*Storage* To evaluate the storage period effect on the enzyme activity, we stored the free and the immobilised AR at 4 °C for 20 days and at 25 °C for 10 days. It was demonstrated that after 20 days of storage at 4 °C, activity of the immobilized AR was 95% of its original activity from liver (Figure 5). Moreover, the free and the immobilized liver AR preserved 54% and 78% of the enzyme activity capacities following the storage at 25 °C for 10 days (Figure 6).

Enzyme stability during storage is an important parameter for enzyme studies. Although it is expected that the enzyme activity is lower than the beginning, the decrease should be minimized. This impairment on the activity is most probably because of the denaturation of the

enzyme structure.[16] In Yıldırım's[14] research the free and the immobilized NADH-cytochrome b5 reductase enzymes were compared for their activities after 60 days-storage at -7 °C, +4 °C and +25 °C. They recorded that immobilization protects activity of the stored enzyme with a higher efficiency in high temperature (+25 °C) than lower temperatures (-7 °C and +4 °C).[14] Similarly, Kim et al.[6] showed that immobilized lipase enzyme maintained 82% of the activity after 30 days at room temperature, while the free lipase lost the activity completely.[6] These results suggested that immobilization is a good tool to protect enzyme activity against to temperature increase during storage. Efficiency of immobilization can be increased by carrying out the immobilization in a specific manner. For instance, it was demonstrated that the aldo/keto reductase AKR1A1 was immobilized as specific-oriented and so it was surface-bound. On the other hand, the unspecific immobilization was adsorptive, therefore, it showed less activity relative to the specifically-immobilized aldo/keto reductase AKR1A1.[17]

This study showed that the immobilized AR enzyme can be more preferable to work with than the free one. Our results showed that the enzyme activity is retainable following immobilization as long as the experimental

conditions are specifically fixed according to the immobilized AR.

## CONCLUSION

Consequently, we indicated that immobilization is a convenient method to utilize the AR enzyme in multiple experiments. While free enzymes can be used only once in the experiments, immobilized enzymes can be used several times due to the protective features of glutaraldehyde crosslinking. In this case, optimal conditions for the immobilized AR should be indicated and applied to the experiments since these conditions might be different than that of the free AR.

Table 1. Effect of the enzyme concentration on the immobilized AR from liver.

| Enzyme volume ($x10^{-8}$ U) | Activity (Enzyme Unite/μ mol/min/L) | | |
|---|---|---|---|
| | Number of washes | | |
| | 1 | 2 | 3 |
| 1.04 | 0.017 | 0.007 | 0.000 |
| 1.46 | 0.130 | 0.015 | 0.000 |
| 2.08 | 0.347 | 0.024 | 0.000 |

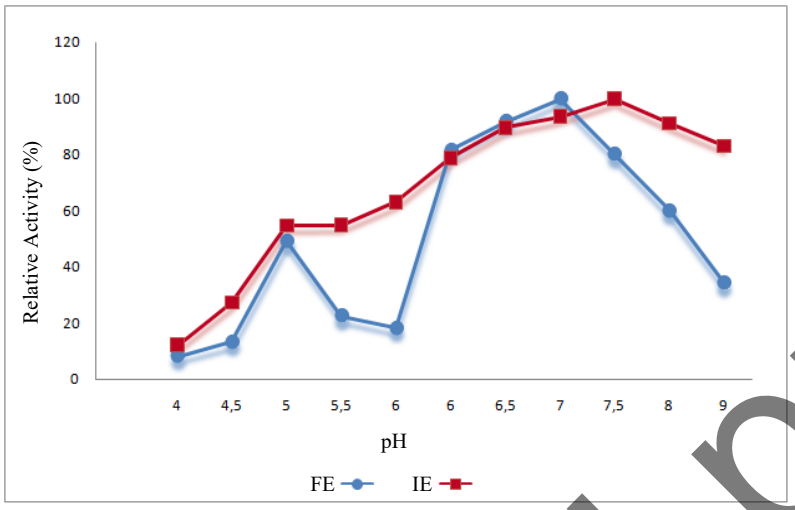



Figure 1. The enzyme activity levels of the free (FE) and the immobilized (IE) AR enzyme from liver according to pH.

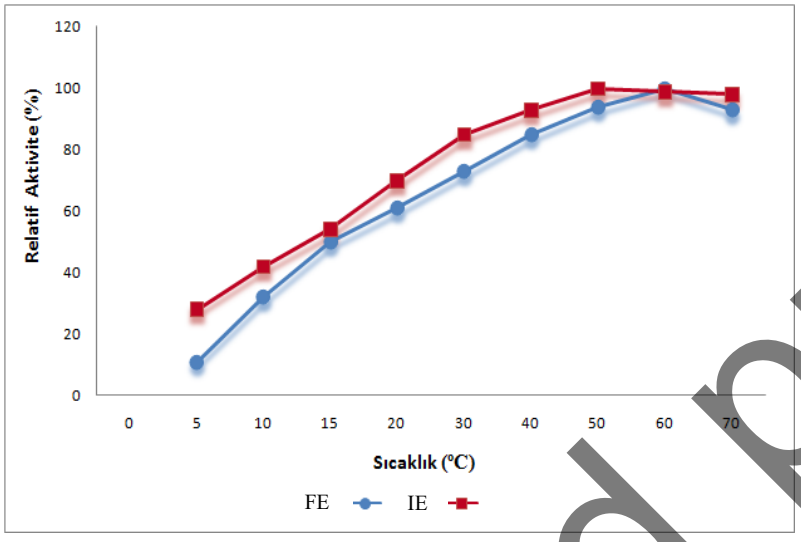
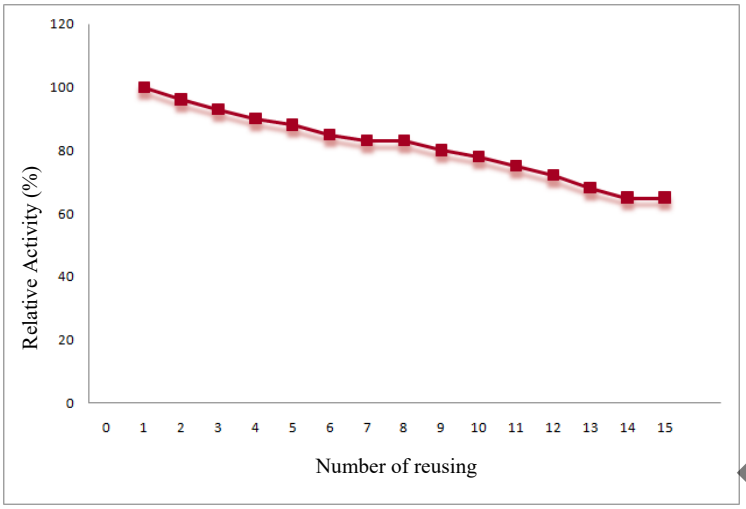



Figure 2. The enzyme activity levels of the free and the immobilized AR from liver according to temperature.

a)




b)

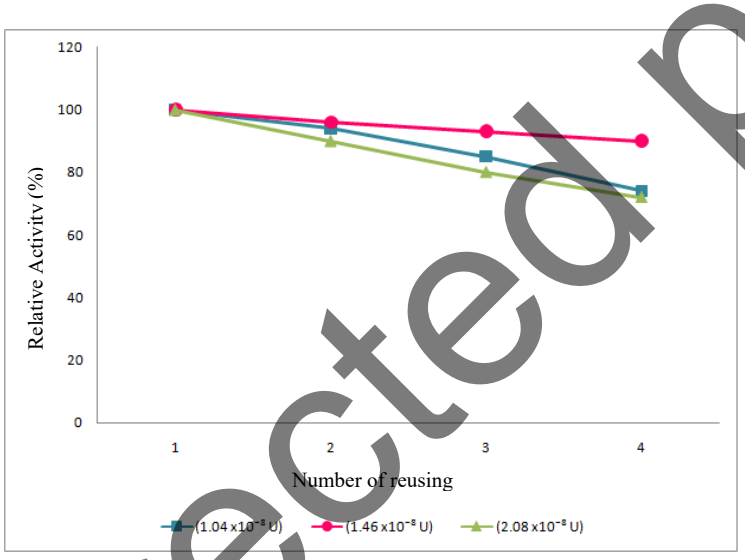
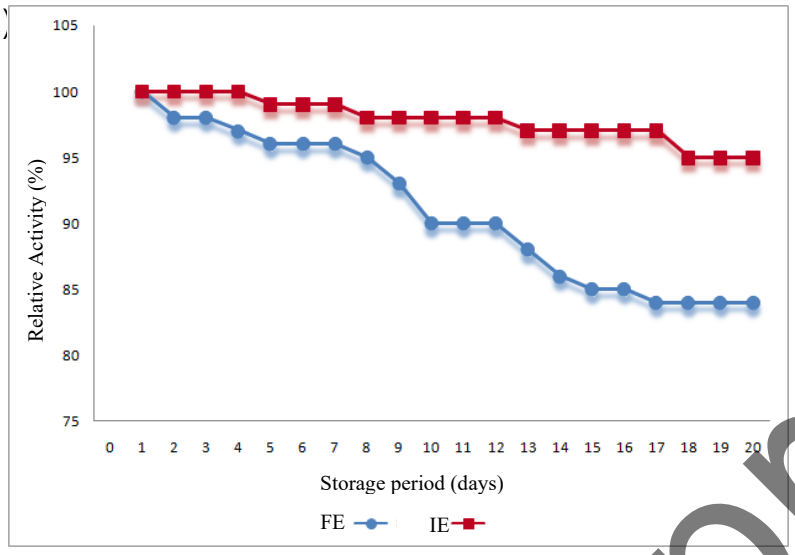



Figure 3. a) The enzyme activity levels of the immobilized AR from liver ($1.46 \times 10^{-8}$ U) according to reusing stability. b) The enzyme activity levels of the immobilized AR from liver ($1.04 \times 10^{-8}$ U) and ($1.46 \times 10^{-8}$ U) ($2.08 \times 10^{-8}$ U) according to reusing stability.

a)




b)

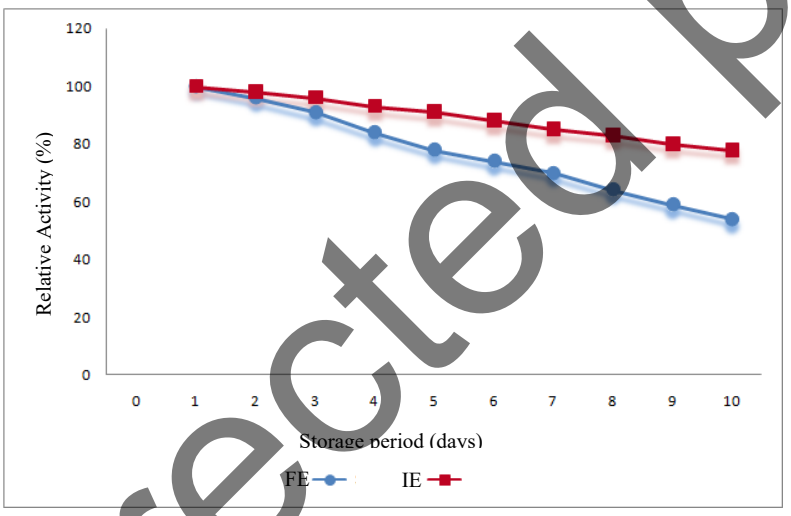



Figure 4. a) The enzyme activity levels of the immobilized AR from liver according to storage stability at 4 ºC. b) The enzyme activity levels of the immobilized AR from liver according to storage stability at 25 ºC.

## REFERENCES


1. Ramana KV, and Srivastava SK. Aldose reductase: A novel therapeutic target for inflammatory pathologies. Int. J. Biochem. Cell Biol. 2009;42:17–20.
2. Petrash JM. All in the family: Aldose reductase and closely related aldo-keto reductases. Cell Mol. Life Sci. 2009;61(7–8):737–749.
3. Reddy GB, Satyanarayana A, Balakrishna N, Ayyagari R, Padma M, Viswanath K and Petrash JM. Erythrocyte aldose reductase activity and sorbitol levels in diabetic retinopathy. Mol. Vis. 2008;14:593-601.
4. Srivastava S, Chandra A, Bhatnagar A, Srivastava SK and Ansari NH. Lipid peroxidation product, 4-hydroxynonenal and its conjugate with GSH are excellent substrates of bovine lens aldose reductase. Biochem. Biophys. Res. Commun. 1995;217:741–746.
5. Srivastava SK, Ramana KV and Bhatnagar A. Role of aldose reductase and oxidative damage in diabetes and the consequent potential for therapeutic options. Endocr. Rev. 2005;26:380–392.
6. Kim M, Park JM, Um HJ, Lee DH, Lee KH, Kobayashi F, Iwasaka Y, Hong CS, Min J and Kim YH.

Immobilization of cross-linked lipase aggregates onto magnetic beads for enzymatic degradation of polycaprolactone. J. Basic Microbiol. 2010;50:218–226.

7. Yemul O, and Imae T. Covalent-bonded immobilization of lipase on poly (phenylene sulfide) dendrimers and their hydrolysis ability. Biomacromolecules. 2005;6(5):2809–2814.
8. Atia KS, Ismail SA and Dessouki AM. Immobilization of β-amylase using polyacrylamide polymer derivatives. J. of Chem. Technol. and Biotechnol. 2003;78:891–898.
9. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry. 1976;72:248-252.
10. Hayman S, Kinoshita JH. Isolation and properties of lens aldose reductase. J. Biol. Chem. 1965;2:240.
11. Hasırcı N, Aksoy S and Tümtürk H. Activation of poly(dimer acid-co alkylpolyamine) particles for covalent immobilization of a-amylase. React. Funct. Polym. 2006; 66:1546–1551.

12. Emregül E, Sungur S, and Akbulut U. Immobilization of glucose oxidase onto gelatin for biosensor construction. J. Biomater. Sci. Polym Ed. 2009;16(4):505-519.
13. Chen KC and Wu JK. Substrate protection of immobilized glucose isomerase. Biotechnol. Bioeng. 1987;30:817-824.
14. Yıldırım Ö, Akbulut U, Arınç E, and Sungur S. Immobilization of NADH-cytochrome b5 reductase into gelatin by crosslinking. Macromolecular Reports. 1994;A31:19–28.
15. Sungur S and Yıldırım Ö. Batch and continuous hydrolysis of lactose using β-galactosidase immobilized on gelatin-CMC. Polym. Plast. Technol. Eng. 1999;38:821–829.
16. Chellapadian M. Preparation and characterization of alkaline protease immobilized on vermiculite. Process Biochem. 1997;33:169-173.
17. Holland-Nell K and Beck-Sickinger AG. Specifically immobilized aldo/keto reductase AKR1A1 shows a dramatic increase in activity relative to the randomly immobilized enzyme. Chem Bio Chem. 2007;8:1071-1076.