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Ionic and covalent immobilization of glucose isomerase of thermophilic *Anoxybacillus gonensis* on DEAE-sepharose

Termofilik Anoxybacillus gonensis glukoz izomerazının DEAE-Sefaroz üzerine iyonik ve kovalent immobilizasyonu

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Abstract
High fructose corn syrup (HFCS), which is produced by the conversion of one sugar into another (glucose to fructose), has a marketing value. Hence, different glucose isomerases [(GI) (D-xylose ketol isomerase, EC 5.3.1.5)] isolated from different sources (macro-and microorganisms) were researched until today. In addition, the cost reduction of GI production for industrial applications has been investigated and applied with different techniques. Enzyme immobilization approaches have prominent features because they allow enzymes to be used repeatedly. In the current study, Anoxybacillus gonensis G2T glucose isomerase (AgoGI) (wild type) were immobilized with ionic and covalent binding on DEAE-sepharose matrix. Afterward, kinetic and biochemical parameters of the immobilized enzymes were evaluated. The pH and temperature parameters, in which the ionic and covalent immobilized enzymes showed the best activity, were determined as 6.50 and 85 °C, respectively. The kinetic data (Vmax and Km) of ionic bound AgoGI on DEAE-sepharose were 4.85±2.09 µmol/min/mg protein and 130,57±5,42 mM, as covalent immobilized AgoGI on the same matrix were 40.51± 0.81 µmol/min/mg protein µmol/min and 127,28±2,96 mM, respectively. Consequently, the usage of DEAE-sepharose for both covalent and ionic immobilization as immobilization matrix did not exhibit any negative effects on biochemical and kinetic parameters of glucose isomerase. Therefore, immobilized AgoGI on DEAE-sepharose was an excellent and promising tool for HFCS production.

Keywords: Anoxybacillus gonensis, DEAE-sepharose, Glucose isomerase, HFCS, Immobilization

Öz
Bir şekerin diğerine (glukozun fruktoza) dönüştürülmesiyle üretilen yüksek fruktozlu mısır şurubu (HFCS), pazarlama değerine sahiptir. Bu nedenle günümüze kadar farklı kaynaklardan (makro ve mikroorganizmalar) izole edilen farklı glukoz izomerazlar [(GI) (D-ksiloz ketol izomeraz, EC 5.3.1.5)] araştırılmıştır. Ayrıca endüstriyel uygulamalar için GI üretiminin maliyetinin düşürülmesi araştırılmış ve bunun için farklı teknikler uygulanmıştır. Enzim immobilizasyon yaklaşımları, enzimlerin tekrar tekrar kullanılmasına izin verdiği için öne çıkan özelliklere sahiptir. Bu çalışmada Anoxybacillus gönensis G2T (AgoGI) yabani tip enzimlerinin DEAE-sefaroz matriksi üzerinde immobilizasyonu (iyonik ve kovalent) gerçekleştirildi. Çalışmanın bir sonraki aşamasında elde edilen enzimlerin kinetik ve biyokimyasal özellikleri belirlendi. İmmobilize enzimler için optimum sıcaklık ve pH değerleri sırasıyla 85 °C ve 6.50 olarak belirlendi. DEAE-sefaroz üzerinde iyonik bağlı AgoGI'nin kinetik verileri (Vmax ve Km) 4.85±2.09 µmol/dk/mg protein ve 130,57±5,42 mM, aynı matris üzerinde kovalent immobilize AgoGI 40.51± 0.81 µmol/dk/mg protein ve 127,28±2,96 mM'dır. Sonuç olarak, DEAE-sefarozun hem kovalent hem iyonik immobilizasyon için immobilizasyon matriksi olarak kullanılması, glukoz izomerazın biyokimyasal ve kinetik parametreleri üzerinde herhangi bir olumsuz etki göstermedi. Bu nedenle, DEAE-sefaroz üzerinde immobilize edilmiş AgoGI HFCS üretimi için mükemmel ve umut verici bir araçtır.

***Anahtar kelimeler**: Anoxybacillus gönensis, DEAE-sefaroz, Glukoz izomeraz, HFCS, İmmobilizasyon*

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1. Introduction
1. Giriş

Xylose isomerase (D-xylose ketol-isomerase E.C 5.3.1.5) (XI) catalyzes the conversion of D-xylose to D-xylulose (Bhosale et al., 1996; Yanmis et al., 2014). XI is a key enzyme for the process of bioethanol production. The enzyme is also called glucose isomerase (GI) because of its ability to convert glucose to fructose (Jin et al., 2017). GI is an important enzyme for food industry because of its necessity and utilization in high-fructose corn syrup (HFCS) production processes (Bandlish et al., 2002; Mbagwu et al., 2018; Nguyen & Tran, 2018). HFCS is a mixture containing an equal amount of fructose and glucose, which is 1.3 fold sweeter and cheaper compound than saccharose (Jin et al., 2017; Tükel & Alagöz, 2008). D-fructose exhibits no effect on the glucose concentration of blood, as it is absorbed gradually. Therefore, D-fructose and HFCS including fructose and glucose is good sweetener choice for diabetics (Bhosale et al., 1996; Parker et al., 2010). According to the literature, it is thought that the isomerization by GI at high temperature and lower pH is better to fulfill the desired content of fructose in the syrup production as reaction equilibrium is a shift to fructose in the isomerization reaction (Hartley et al., 2000; Neifar et al., 2019). In addition, GI is preferred due to its ability to be used recursively in industrial applications. For this purpose, there have been many researches about immobilization of GI on the different matrix by different methods (Chen, 2010; Ge et al., 1998; Demirel et al., 2006; Rhimi et al., 2007; Tumturk et al., 2007; Illeova & Polakovic, 2018). The enzyme immobilization is a widely-used method to achieve enzyme usage in a cost-effective manner (Bashir et al., 2020). Enzyme immobilization is a method particularly designed to limit the enzyme movement by making the enzyme physically confined in a matrix or linked to a matrix. By this means, the enzymes are supported by the matrix used in the immobilization process, and therefore the enzymes are not released into the solution (Hassan et al., 2016). According to the previous studies, an ideal matrix should have some properties including physical strength, renewability, stability, inertness, non-specific adsorption, being affordable, etc. (Datta et al., 2013). Some of the frequently used matrices are alginate (Flores-Maltos et al., 2011), chitosan and chitin (Chang & Juang, 2007), cellulose (Klein et al. 2011), gelatin (Shen et al., 2011), starch (Raafat et al., 2011). Among them ion exchange polymers such as DEAE-cellulose and DEAE-sephadex have been preffered because of having renewable matrices with large surface area (Datta et al., 2013). A good deal of methods has been described for the immobilization of enzymes such as covalent bonding, adsorption, cross linking, entrapment, and encapsulation (Elnashar et al., 2014; Hassan et al., 2016; Marwa et al., 2020). In our previous study, the new GI gene of *Anoxybacillus gonensis* $G2^T$ (AgoG2GI) was revealed, cloned into the expression vector. Then, the gene product was expressed, purified, and biochemically characterized (Karaoglu et al., 2013). The data of that mentioned research revealed that AgoG2GI had promising advantages including high catalytic activity ($kcat/K_m$) high V_{max} and low K_m values for glucose, therefore, AgoG2GI seems to be a potential candidate enzyme compared to its counterpart used in the industry application. Taking into account these excellent properties of AgoG2GI, immobilization of this enzyme was investigated. In accordance with this purpose, we have performed ionic and covalent binding of *Anoxybacillus gonensis* $G2^T$ enzyme (wild type) on DEAE-sepharose matrix. Afterwards, parameters as kinetic and biochemical of immobilized enzymes were evaluated.

2. Material and method
2.Materyal ve metot

2.1. Chemicals
2.1. Kimyasallar

The chemicals used in the current study were reagent grade and supplied from a variety of commercial companies (Merck, Sigma, Fluka, Acumedia and Aldrich).

2.2. Production of GI
2.2. GI'nın üretilmesi

In one of our previous studies, GI of *A. gonensis* $G2^T$ (NCIMB 13933^T) was cloned into $pET28a^+$ expression vector named pETG2GI (Karaoglu et al., 2013). In this study, the recombinant pETG2GI plasmid was transformed to *E. coli* BL21(DE) strains by standard calcium chloride transformation protocol (Bor et al., 1992; Karaoglu et al., 2013). To prepare pre-bacterial culture, the colonies containing pETG2GI were incubated at 37 °C in 10 mL of Luria-Bertani (LB) broth with kanamycin (0,05 mg/mL). After incubation, the new fresh bacterial culture was prepared as 0.1 optical density (OD_{600}) from pre-bacterial culture and was incubated under same conditions. As the OD of the bacterial culture came up to 0.8, 1 mM iso-propyl-d-thiogalactopyranoside (IPTG) was added to the medium to induce gene expression. The bacterial

culture was incubated for 4 h and then centrifuged at 11.000 rpm for 10 min. After removing supernatant, the harvested cells were solubilized in 50 mM of MOPS buffer and disrupted by a sonicator (0.6 cycle scale, 80% amplitude). The cell debris was removed by centrifugation (at 15.200 rpm for 15 min) of the extract and the supernatant was used as the crude extract (Karaoglu et al., 2013; Yanmis et al., 2014).

2.3. Purification of AgoGI
2.3 AgoGI'nın üretilmesi

2.3.1. Colon chromatography
2.3.1. *Kolon kromatografisi*

After the partial purification of crude extract by heat-shock application, the colon chromatographies (ion exchange and hydrophobic interaction) were applied for further purification (Karaoglu et. al., 2013). The purification assays were performed using a Biologic Lp System (Bio-Rad) instrument. All the chromatography studies were performed by using a chromatography column (1.5×30 cm) at flow rate of 1 mL/min. The fractions were collected in a volume of 3 mL and the fractions having GI activity were collected. The collected fractions from both of the chromatography steps were used to determine GI activity, total protein concentration, and the volume to prepare the purification table. In order to calculate GI activity, the obtained fraction was diluted 5 times. One Unit of GI activity was defined as the amount of enzyme that released 1 µmol of fructose/minute under the reaction conditions.

i) Ion exchange chromatography (IEC)
i) İon değişim kromatografisi

DEAE-sepharose fast flow was used as column material for IEC. The soluble fraction obtained from heat-shock application was loaded on a column of DEAE-sepharose pre-equilibrated with reaction buffer (100 mM MOPS, pH 6.5, containing 1 mM $CoCl_2$ and10 mM $MgSO_4$). The column was eluted with a linear gradient of (0–0.5 M) NaCl in the same buffer.

ii) Hydrophobic interaction colon chromatography (HIC)
ii) Hidrofobik etkileşim kolon kromatografisi

Phenyl-sepharose fast flow (Sigma) was used as the column material for HIC. At first, the column of phenyl-sepharose-6 (Sigma) was equilibrated with reaction buffer containing 1.3 M $(NH_4)SO_4$. The fractions obtained from IEC were saturated with ammonium sulfate solution to give its final concentration of 1.3 M $(NH_4)SO_4$ and loaded on the column. Then, the column was eluted with a 100 mL linear gradient of 1.3–0 M $(NH_4)SO_4$ (Yanmis et al., 2014). The fractions with GI activity were collected and used for immobilization applications.

2.4. Covalent and ionic immobilization of GI
2.5. GI'nın kovalent ve iyonik immobilizasyonu

8000 µL DEAE-sepharose matrix was cleaned by washing 10 times with distilled water and equilibrated by washing 10 times with reaction buffer. After the excess reaction buffer was removed, 2.5 % glutaraldehyde [prepared in 100 mM MOPS buffer solution (pH 7.0)] was added to the mixture of matrix and the reaction buffer and incubated for 2 h at room temperature. Then, excess glutaraldehyde was removed by washing it 10 times with reaction buffer. After washing process, 4000 µL GI of *A. gonensis* (AgoGI) was incubated with glutaraldehyde-treated DEAE-sepharose matrix at room temperature for 16 h to perform covalent immobilization. Immobilized enzymes were stored +4 °C for further studies. Ionic immobilization of AgoGI on DEAE-sepharose matrix was performed according to the same procedure of covalent immobilization except adding glutaraldehyde (Singh et al., 2014).

2.5. Activity assay of immobilized GI
2.5 İmmobilize GI'nın aktivite deneyleri

Glucose isomerase activity was measured by incubating 250 µL of the reaction mixture (0.2 M glucose and the enzyme) at 85 °C for 30 min. The reactions were stopped by incubating on ice. The enzyme reaction was measured by the cysteine–carbazole–sulphuric acid method. 40 µL of 1.5 % cysteine hydrochloride, 40 µL of 0.12 % carbosol and 1.2 mL of 70 % sulphuric acid were added into 100 µL of the reaction mixture. The activity of enzyme was spectrophotometrically measured at 560 nm absorbance for fructose. One unit of activity was defined as the amount of enzyme that released 1 µmol of fructose per minute under the reaction conditions (Karaoglu et al., 2013).

2.6. Determination of protein concentration
2.6. Protein konsantrasyonunun belirlenmesi

At all the purification steps, protein concentrations of the examples were measured by Bradford's method (1976). Bovine serum albumin (BSA) was the standard for the procedure (Bradford, 1976).

2.7. Effects of temperature and pH on immobilized GI's

2.7. Sıcaklık ve PH'nın immobilize GI'lar üzerine etkileri

To calculate optimum temperature and pH, the reaction series were carried out between 30 °C and 100 °C and between pH 5.0 and pH 10.0 in suitable buffers [(sodium acetate (pH 5.0–6.0), potassium phosphate (pH 6.0–7.0), Tris–HCl (pH 7.0–9.0), and glycine–NaOH buffer (pH 9.0–10.0)] as described in the activity assay, respectively (Karaoglu et al., 2013; Yanmis et al., 2014).

2.8. Determination of kinetic parameters of immobilized GI's

2.8. İmmobilize GI'ların kinetik parametrelerinin belirlenmesi

The kinetic parameters of the enzymes were calculated, due to a serial reaction performed at 85 °C and pH 6.50 by increasing the glucose concentration from 0 mM to 700 mM. The kinetic parameters V_{max} (µmol/min/mg), K_m (Michaelis-Menten constant, mM) were calculated from Michaelis–Menten plots by the OriginPro8.1 program (OriginLab Data Analysis and Graphing Software) (Karaoglu et al., 2013).

2.9. Reuse capability and storage stability of GI's

2.9. GI'ların tekrar kullanım kapasitesi ve depolama stabilitesi

Immobilized GI was used repeatedly to evaluate the enzyme capacity for reuse according the method described by Yu et al (2011). Immobilized enzymes were used 10-15 times and GI activity was measured with the carbazole–sulphuric acid method with the procedure (Karaoglu et al., 2013). After each usage, the immobilized enzymes were cleaned with 50 mM MOPS buffer (pH 6.50). Both of ionic immobilized enzyme and covalent immobilized enzyme were stored at 4 °C in 50 mM MOPS buffer (pH 6.50). The enzyme activities were determined every other day. Storage stability experiments were performed for 10 weeks (Yu et al., 2011).

3. Results and discussion

3. Sonuçlar ve tartışma

3.1. Purification of AgoGI

3.1. AgoGI'nın üretilmesi

In the current study, glucose isomerase from *A. gonensis* was produced by pET28a$^+$/*E. coli* BL21(DE3) expression system and was successfully purified with heat shock application, ion-exchange, and hydrophobic column chromatography techniques. Biochemical parameters of purification steps are given in Table 1 and the SDS-PAGE image of the purifications steps is exhibited in Figure 1. The specific activity of the enzyme increased from 9.615 to 26.720 µmol/min/mg protein, while the enzyme was purified 2.78 fold with 60 % yield at the end of the purification steps (Table 1).

Table 1. Purification Table for AgoGI
Tablo 1*. AgoGI'nın saflaştırma tablosu*

Purification Steps	Total Volume (ml)	Protein (mg/ml)	Total Protein (mg)	Activity (µmol/dk/µl)	Total Activity (µmol/dk)	Specific Activity (µmol/dk/mg protein)	Yield	Fold
CE	16.3	9.36	152.57	0.090	1467.00	9.615	100	1
HSA	15.1	4.87	73.58	0.090	1359.00	18.471	92.6	1.92
IEC	16.5	2.60	42.91	0.066	1089.00	25.376	74.2	2.64
HIC	20	1.65	32.93	0.044	880.00	26.720	60.0	2.78

CE: Cell Extract, HSA: Heat-shock application, IEC: ion exchange colon chromatography, HIC: Hydrophobic interaction colon chromatography

3.2. Covalent and ionic immobilization of GI

3.2. GI'nın kovalent ve iyonik olarak immobilizasyonu

Ion-exchange material has been widely used for protein purification since twentieth century. Recently, Ion-exchange material such as DEAE-sepharose has been studied for enzyme immobilization. Since, the usage of DEAE-sepharose is simpler exceptionally simpler when compared to other matrices. It basically involves electrostatic/ionic interactions of weak nature between protein and resin. These weak interactions generate minimal conformational changes, improving enzyme chemical and physical stabilities, and increases specificity, enzyme selectivity, and catalytic activities (Vaz & Filho, 2019). Therefore, in this study, DEAE-sepharose was prefferred for immobilization process. The purified enzyme was separately immobilized

covalent and ionic on DEAE-sepharose matrix. After immobilization processes, the enzyme activities and the protein amounts were calculated for both of the liquid remained on the matrix (nonbinding enzyme) and immobilized enzymes. It was concluded that all enzymes were immobilized on the matrix because of the absence of GI activity and protein in the liquid. The total protein and unit amount of the immobilized enzymes are given in Table 2. The total amount of enzyme added for covalent immobilization was 37.33 U, as the amount of enzyme added for ionic immobilization was 36.83 U. The enzymes were immobilized in the rate of 99.5 % and 99.2 % by covalent and ionic immobilization, respectively (Table 2).

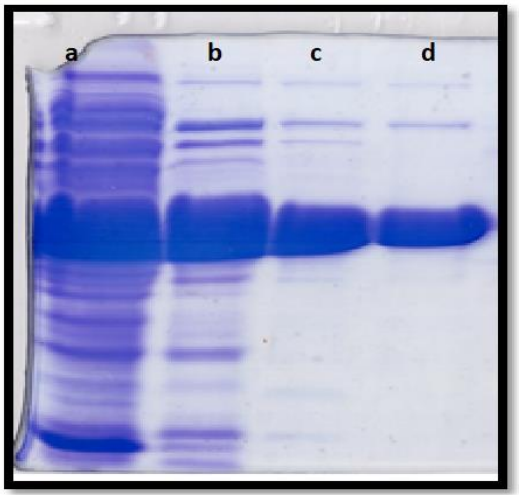

Figure 1. The SDS-PAGE imagination of purified wild-type GI for every purification steps. (a: Cell extract, b: Heat-shock application c: Ion-exchange chromatography, d: Hydrophpbic-interaction chromatography)
Şekil 1. *Saflaştırılan yaban tip GI için saflaştırma bas….amaklarına ait SDS-PAGE analizi (a: Kaba ekstrakt, b: Sıcak şoku uygulaması, c: İyon değişim kromatografisi, d: Hidrofobik etkileşim kromatografisi)*

Table 2. Total unit of immobilized enzymes
Tablo 2. *İmmobilize enzimlerin total protein ve ünite miktarları*

	Unite(µmol/dk)
Total amount of AgoGI added for covalent immobilization	37.33
The amount of AgoGI added for ionic immobilization	36.83
Unit amount of AgoGI covalent attached to the matrix	37.14
Unit amount of AgoGI that ionic binds to the matrix	36.54

AgoGI: glucose isomerase of *Anoxybacillus gonensis* $G2^T$

3.3. Temperature and pH effects on immobilized GI's
3.3. Sıcaklı ve pH'nın immobilize GI'lar üzerine etkileri

In addition, in this study, biochemical parameters of the immobilized enzymes were determined. The best temperature for the immobilized enzymes were determined as 85 °C (Fig 2). According to the report of Karaoglu et al. (2013), the optimum temperature of recombinantly produced *A. gonensis* GI was found to be 85 °C (Karaoglu et al., 2013). In this case, the optimum temperatures of immobilized enzymes and free enzyme are same. It is clear that immobilization applications cause no differences on the optimum temperatures. In this study, the maximum pH values of the immobilized enzymes were found to be 6.50 (Fig 3). Similarly, according to the study performed by Karaoglu et al. (2013), the optimum pH value of free enzyme was 6.50. After immobilization of the enzymes on any matrix, different changes can occur on the surface of enzyme because of the different charge distribution of the matrix surface and the interaction of the enzyme with the matrix. These changes can cause differences between the optimum pH values of the immobilized enzymes and the free enzymes. In this study, immobilizations of the enzyme did not cause any changes in the optimum pH value. The obtained results indicated that the interaction between enzyme and DEAE-sepharose in both immobilization techniques did not generate significant conformational changes on AgoGI. According to the literature, the matrices with ion-exchange material such as DEAE produce a

minimal conformational changes (Vaz & Filho, 2019). Besides, there are many immobilization researches for GI, that the free and immobilized enzymes exhibited the same optimum pH and temperature (Han & Juan 2000; Tumturk et al., 2007; Demirel et al., 2006). The results of optimum parameters were supported with previous studies.

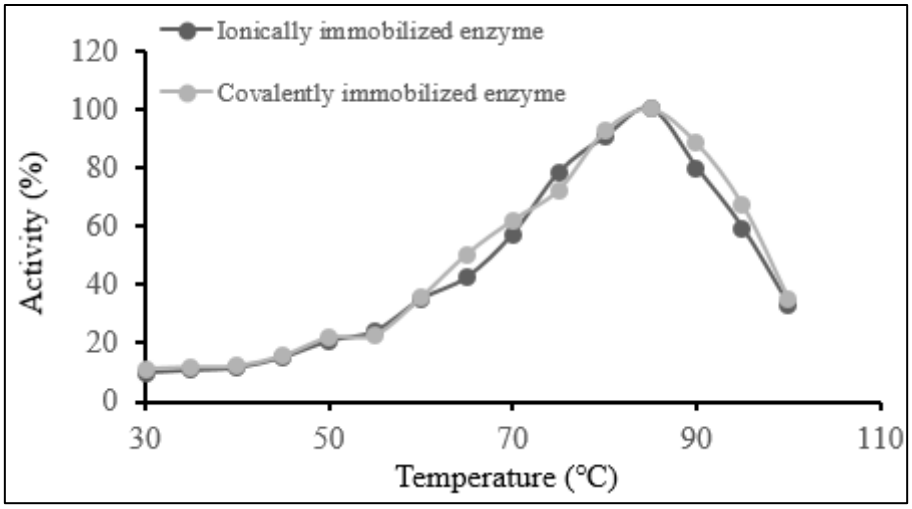

Figure 2. Temperature effect on immobilized AgoGI activity
Şekil 2. *İmmobilize AgoGI'nın aktivitesine sıcaklığın etkisi*

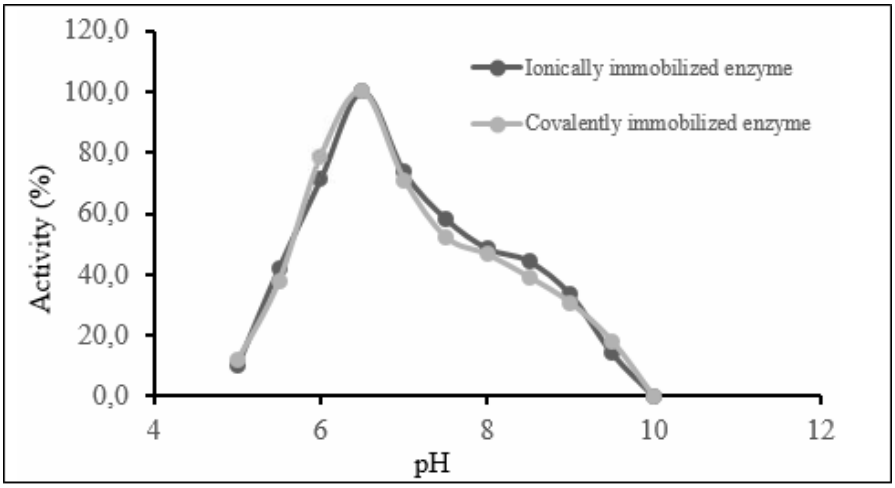

Figure 3. pH effect on immobilized AgoGI activity
Şekil 3. *İmmobilize AgoGI'nın aktivitesine pH'nın etkisi*

3.4. Kinetic parameters of immobilized GI's
3.4. İmmobilize GI'ların kinetik parametreleri

The K_m values and V_{max} values of the ionic immobilized and covalent immobilized enzyme were determined as 130.57±5.42 mM, 44.85±2.09 µmol/min/mg protein and 127.28±2.96 mM, 40.51± 0.81 µmol/min/mg protein, respectively. The Michaelis-Menten curves drawn with the origin program are shown in Fig 4. K_m and V_{max} values of the free enzyme (wild type) were found as 138.37 mM and 40.51 µmol/min/mg protein in our previous study (Karaoglu et al., 2013). Immobilization of enzymes often causes changes in the K_m and V_{max} values of the enzymes. However, a significant change in the kinetic parameters of the immobilized enzymes did not occur. In other words, ionic and covalent immobilization of AgoGI has been achieved on DEAE-sepharose matrix without any significant change in kinetic parameters compared to its free counterpart (Table 3). According to the literature, K_m and V_{max} values generally exhibited differences for immobilized enzyme and free enzyme (Han & Juan 2000; Tumturk et al., 2007; Demirel et al., 2006). The results of kinetic parameters were not compatible with the literature. It could be explained with being use DEAE-sepharose for immobilization which generated insignificant changes in the structure of GI.

Table 3. Comparison of enzymes in terms of kinetic parameters
Tablo 3. *Enzimlerin kinetic paramatrelerinin karşılaştırılması*

Glucose Isomerase	K_m (mM)	V_{max} (µmol/dk/mg protein)	Reference
AgoG2GI-wt	146.08 ± 9.50	43.72 ± 1.01	Karaoğlu (2013)
AgoG2GI-rec	138.37 ± 7.63	40.51 ± 0.81	Karaoğlu (2013)
IIAgoGI	130.57 ± 5.42	44.85 ± 2.09	In this study
CIAgoGI	127.28 ± 2.96	41.26 ± 1.97	In this study

AgoG2GI-wt: *Anoxybacillus gonensis* $G2^T$ glucose isomerase-wild type, AgoG2GI-rec: *Anoxybacillus gonensis* $G2^T$ glucose isomerase recombinant, IIAgoGI: ionic immobilized *Anoxybacillus gonensis* $G2^T$ glucose isomerase, CIAgoGI: covalent immobilized *Anoxybacillus gonensis* $G2^T$ glucose isomerase

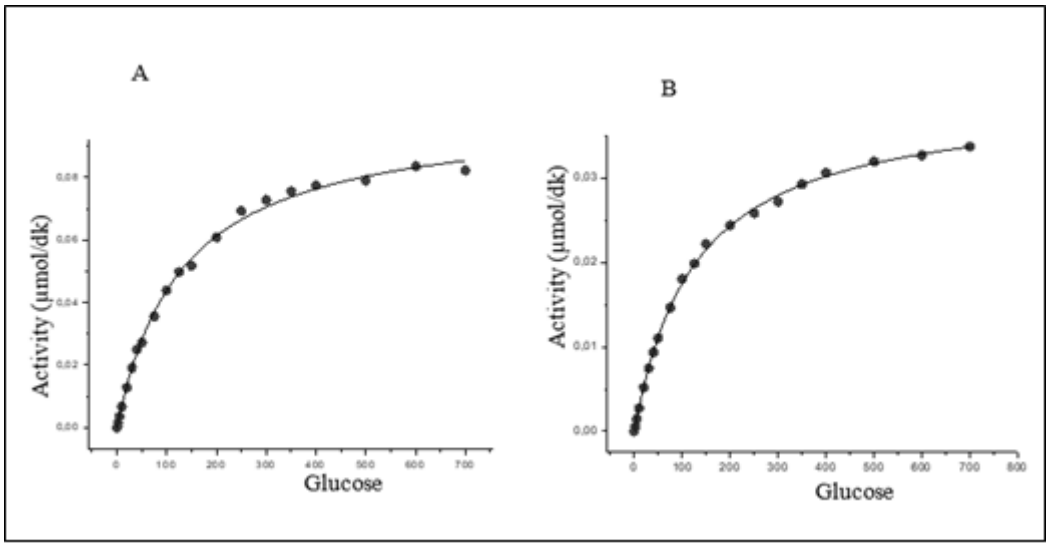

Figure 4. The Michaelis-Menten curves for immobilized enzymes (A: Ionic immobilized enzyme, B: Covalent immobilized enzyme)
Şekil 4. *İmmomilize enzimlere ait Michaelis-Menten eğrisi enzymes (A: İyonik immobilize enzim, B: Kovalent immobilize enzim)*

3.5. Reuse Capability and Storage Stability of GI
3.5. GI'nın tekrar kullanım kapasitesi ve depolama stabilitesi

Immobilized enzymes were used repeatedly to determine reuse capability. Ionic immobilized enzyme lost 50 % activity in its 5^{th} utilization, while covalent immobilized enzyme lost 50 % activity in its 10^{th} utilization. To determine of storage stability of immobilized GIs were incubated at + 4 °C. Both free enzyme and immobilized enzymes did not lose activity even after 75 days at + 4 °C.

4. Conclusion
4. Sonuç

Recombinant glucose isomerase of *Anoxybacillus gonensis* $G2^T$ was effectively immobilized on DEAE-sepharose matrix by using covalent and ionic immobilization methods. The covalent and ionic immobilization did not exhibit a significant change in the values of biochemical and kinetic parameters because of electrostatic/ionic interactions of weak nature between protein and matrix. Therefore, DEAE-sepharose is a good candidate to be used for GI immobilization in industrial applications. However, covalent immobilization of glucose isomerase on DEAE-sepharose was more efficient than ionic immobilization and had a great potential to be used for immobilization of glucose isomerase.

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Autor contribution
Yazar katkısı

Experiment performation (cloning of gene, expression and purification of protein, enzyme immobilization, and enzyme characterization) were performed by Zuleyha AKPINAR. Merve

KIZAKLI YILDIRIM was helped during the characterization of immobilized enzymes. Hakan KARAOGLU organized all the steps of the study, wrote the manuscript and performed revision of manuscript.

Declaration of ethical code
Etik beyanı

The authors declare that all of the rules stated to be followed within the scope of the "Higher Education Institutions Scientific Research and Publication Ethics Directive" were followed, and none of the actions specified under the title of "Actions Contrary to Scientific Research and Publication Ethics" have been taken.

Conflict of interest
Çıkar çatışması beyanı

All authors declared that there is no conflict of interest.

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