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The Role of Cellulose Binding Heptapeptide in Immobilizing Glucose Oxidase to Cellulosic Surfaces

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Abstract

This study aimed to produce a recombinant version of glucose oxidase enzyme and introduce an economical method for enzyme immobilization. The pGAPZαA expression vector was utilized, which has a continuous expression and does not need an inducer and medium replacement. Enzyme immobilization was accomplished by adding WHWTYYW heptapeptide, which tends to bind to cellulosic and nitrocellulose surfaces, to bind the enzyme to the above-mentioned surfaces. In this study, the glucose oxidase (GOX)-encoding gene from Aspergillus niger ATCC 9029 was amplified by the polymerase chain reaction (PCR) and cloned into the pGAPZαA vector, and pGAPZαA/GOX was obtained as a control construct. On the other hand, a primer containing the heptapeptide sequence was designed, followed by adding the heptapeptide by the PCR technique to the end of the carboxyl in the GOX-encoding gene in the pGAPZαA/GOX construct and obtaining the pGAPZαA/GOX–heptapeptide construct. Both constructs were integrated into the Pichia pastoris genome by electroporation, and enzymatic activity and protein expression of the recombinant enzyme from both constructs underwent investigation. The amount of GOX production in the pGAPZαA/GOX wild-type construct and pGAPZαA/GOX with heptapeptide was estimated at 0.234 and 0.135 g/l, respectively. The stability of both constructs was evaluated on different surfaces and then compared as well. It was revealed that the enzyme with WHWTYYW heptapeptide has an increased affinity to bind in cellulose and nitrocellulose surfaces compared to the wild-type enzyme, while demonstrating no affinity for binding to the polyvinylidene fluoride paper.

Keywords: Glucose Oxidase; Heptapeptide; pGAPZA/GOX; Pichia pastoris; Nitrucellulose.

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Introduction

Enzymes are attracting extensive attention because of their high activity, selectivity, and specificity, allowing researchers to catalyze numerous complex chemical processes in an adverse experimental environment. However, enzymes' low operating stability and insufficient reusability severely limit their industrial applicability [1].

Immobilizing biomolecules on insoluble carriers is essential for the fabrication of a wide range of functional materials. Some benefits of immobilizing enzymes include improved stability, simple separation from reaction mixtures, and the capacity to modulate catalytic characteristics. The enzyme immobilization process is one of the most important fields in biotechnological studies because of its relevance to medical, pharmaceutical, food, environmental, and biotechnological applications, and biological research [2].

GO_X (EC number 1.1.3.4) β-D-oxygen glucose-1-oxidoreductase is a glycoprotein that catalyzes the oxidation of β-D-glucose to gluconic acid by using molecular oxygen as an electron acceptor to produce hydrogen peroxide [3]. It is used to preserve the color and taste of food and beverages [4], as well as a commercial source of gluconic acid production [5]. Gluconic acid is employed as a food preservative, acidity regulator, sterilization solution in food production, and as salt in the chemical industry [6].

To improve its recyclability, controllability, recovery, and shelf-life, GO_X has been immobilized in different studies using various immobilization techniques [1, 2, 7, 8].

The immobilization of enzymes frequently leads to decreased stability and activity of surface-attached enzymes. The key factors to such activity decreases are orientation control and molecule-level contacts at solid support surfaces. For complicated processes requiring the exact operation of multiple components functioning in unison, biological systems generally use multicomponent self-assembly. There is an increasing interest in using self-assembly to influence molecular-level interactions between enzymes and surfaces. However, the effective assembly of multicomponent systems at the nanoscale continues to be a considerable difficulty [9-13].

Recently, affinity adsorption-based immobilization has attracted extensive attention and has been widely employed to enhance the efficiency of enzyme-based biosensors [14]. The contact between the enzyme and carrier is highly selective and specific. In addition, the orientation of the immobilized enzyme can be regulated, and minimum structural changes and good retention of the immobilized molecular activity have been accomplished by this type of binding [15]. Enzymes with genetic tag modifications are often utilized in affinity adsorption immobilization. Affinity tags linked to the enzymes' amino or carboxyl terminals, away from the enzyme's active site, can keep the fusion enzyme operational and prevent steric barriers from interfering with substrate binding and electron transfer following immobilization [16-18].

Surface-recognition peptides are now used as surface-specific bio-linkers as an alternative to chemical coupling methods. Peptides are chosen from combinatorial peptide phage or cell surface display libraries [19, 20]. These peptides' characteristics have been thoroughly investigated in the study of surface functionalization, self-assembly, and the production of different nanostructures. They have also been confirmed to be effective surface linkers for merging with other biomolecules [21-23].

Methylotrophic yeast is a yeast species that can use methanol as its primary carbon and energy source for cell development. The most common example is *Pichia pastoris* [24], which has emerged as an essential host organism for high-level protein synthesis. The key benefits of *P. pastoris* are high-level expression, high cell density, and simple scaling up, as well as powerful and tightly controlled promoters [24-26]. Furthermore, this system shares most of the structure and function of the eukaryotic secretory system and can fold to process proteolytically and glycosylate and exude huge amounts of disulfide-bonded proteins [27-29]. In the 1980s, *P. pastoris* was used as an effective heterologous protein expression method. Numerous recombinant proteins and enzymes, including nitrate reductase, insulin, and GO_X, have so far been successfully produced in *P. pastoris* [30-32]. Two simple expression methods for *P. pastoris* are AOX (methanol-inducible) and GAP (constitutively) promoter-based systems [33]. The technology of using a combination of both AOX1 and GAP promoters for recombinant proteins has been employed by many research teams [31, 32, 34].

This project mainly focused on studying the production of recombinant GO_X protein bound to heptapeptide in the yeast of *P. pastoris* to improve the binding property of this enzyme to the substrate and enzymatic stabilization.

Materials and Methods

Strains

Escherichia coli DH5α was purchased from Invitrogen, USA, and P. pastoris GS11 was provided by

the National Institute of Genetic Engineering and Biotechnology.

Primers

In this study, the intended primers (Table 1), which were designed by Gene Runner software, were used for the polymerase chain reaction (PCR) of the native GOX gene and GOX gene with heptapeptide. Initial denaturation is performed by placing the samples at 97-92 °C for 3-5 minutes; in this study, the samples were placed at 95 °C for 5 minutes. For this purpose, the PCR was performed at 45 °C for 45 seconds in each cycle. In addition, the annealing of the template DNA was performed for 1 minute at 59 °C for reciprocating primers.

Plasmid extraction

Plasmid DNA extraction was performed using a Roche kit as follows:

First, 1-1.5 ml of the overnight culture (usually 16 hours) of bacteria was poured into a microtube and precipitated by a centrifuge at 4000 rpm for 5 minutes. The supernatant was discarded, and then 250 µl of the suspension buffer was added to the sediment and vortexed to form a uniform suspension. Next, 250 µl of the lysing buffer was added to the above-mentioned bacterial suspension, and its contents were mixed by inverting the microtube 5-6 times and placed at room temperature for 5-6 minutes. Moreover, 350 µl of the binding buffer was added to the above mixture, and its content was mixed by inverting the microtube several times and then placed on ice for 5 minutes. The binding buffer must be cold. The above mixture was centrifuged at 13000-14000 rpm for 10 minutes. Next, the purification column was placed in the vial. Additionally, the supernatant from the previous step was transferred to the column and then centrifuged for 30-60 seconds at 13000 rpm. The contents of the vial under the purification column were thrown away, and the column was put back in the same vial. Then, 500 µl of Washing Buffer I was added to the column and centrifuged at 13000 rpm for 30-60 seconds. The contents of the vial under the column were discarded, and the column was placed in the same vial, followed by adding 700 µl of Washing Buffer II to the column and centrifuging at 13000 rpm for 30-60 seconds. Further, the contents of the vial under the purification column were discarded, and the column was placed in a new sterile 1.5 ml vial. Finally, 50-100 microliters (Elution Buffer) were added to the column and centrifuged at 13000 rpm for 30-60 seconds. It is noteworthy that sterile distilled water can also be used instead of Elution Buffer.

Gene cloning and construction of the expression vector

The primers related to the native GOX gene and GOX gene with heptapeptide, along with the cutting site of specific restriction enzymes (XhoI and XbaI, Fermentase Company) and wild GOX gene fragment with heptapeptide were obtained by the PCR technique from the Aspergillus niger (A. niger) genome. Then, both fragments were separately cloned in the pGAPZαA vector with the cutting sites of the predetermined restriction enzyme. The pGAPZαA vector has a resistance sequence to the zeocin antibiotic, which is used in the screening stage.

Connecting foreign DNA to the vector is called ligation, which is performed by the T4 DNA ligase enzyme. This step was conducted with the ligation kit provided by Sina Clone.

To obtain and observe recombinant plasmids obtained from cloning, the content of the microtube was sent into the host susceptible to accepting the foreign gene (bacteria). Next, 5 micrograms of plasmid DNA containing the desired fragment was added to 1200 µg of susceptible cells and mixed, and the sample was placed on ice for 30 minutes. In this case, plasmid DNA was attached to the host cell wall. By creating a heat shock at 42 ºC for 1.5 minutes, the pores in the host cell wall were opened, and the plasmid was transferred into the cell. Then, 180 µ of the LB medium without antibiotics was added to the sample and placed in a 37 ºC incubator shaker for 1 hour. In this case, the cells get a chance to repair and rearrange. Eventually, 100 µ of this sample was spread on the LB-Zeocin plate and placed at 42 ºC overnight.

Table 1. Materials and steps required to perform the polymerase chain reaction

Length	Tm (°C)	Type of primer	Gene name of primer sequence ´3 → ´5	Gene
22 Nt	53.3	Forward	TCTCTCGAGAAGAGACAGACC	Glucose Oxidase
35 Nt	53.3	Reverse	TGTTCTAGAGCGGCCGCTTACTGCATGGAGGCGT	
35 Nt	53.3	Forward	TCTCTCGAGAAGAGACAGACC	Glucose oxidase
51 Nt	62.6	Reverse	TTCTAGATTACCAATAGTAAGTCCAGTGCCACTGCA TGGAGGCGTAGTCC	with heptapeptide

Transformation into the yeast genome

To integrate plasmids containing recombinant protein into the genome of the host yeast (P. pastoris), the recombinant plasmid was cut with the AvrII enzyme and linearized so that it could enter the host genome by homologous recombination. The single enzyme digestion method was performed according to the instructions mentioned in the enzyme digestion kit of Takara Company. Further, electroporation was conducted to integrate a plasmid containing the gene encoding the recombinant protein into the genus P. pastoris using the method mentioned by Invitrogen.

Expression and Purification of recombinant proteins

Electrophoresis SDS-poly acrylamide gel was used to separate and determine the molecular weight of proteins [31]. Ion exchange chromatography is one of the most powerful and common methods for separating proteins, polypeptides, nucleic acids, polynucleotides, and other charged biological molecules. One of the advantages of protein separation by this method is that washing is usually performed in mild conditions, thus the protein retains its original conformity in ion exchangers more than the other methods used in protein purification. Other reasons for the success of ion exchange chromatography are the principles of easy separation and ease of method control. In addition, ion exchange resins are highly stable and easily synthesized and can be applied hundreds of times. The produced recombinant protein was purified by the following method:

Stability test of the native and the recombinant enzymes

As the enzyme is extracellularly produced in the host, the culture medium contains large amounts of the enzyme. The enzymatic activity of the recombinant protein produced by the host yeast during 4 days of culture was measured under optimal conditions for GOX activity (pH of 5.5 and temperature of 30 °C) according to previous research (Belyad et al., 2018). The highest activity was determined in both enzyme-producing clones (enzyme without peptide tag and enzyme with heptapeptide tag). The produced enzyme was incubated with cellulose, nitrocellulose, and polyvinylidene fluoride (PVDF) substrates on a shaker for 3 hours, and then the enzyme activity was measured again. The difference in enzyme activity at the beginning and end of the experiment indicates the tendency of the enzyme together with the heptapeptide to bind to cellulose derivatives. This experiment was repeated at least 2 times.

Statistical analysis to evaluate the binding of the enzyme to the substrate

The degree of the binding of the native enzyme with heptapeptide to the substrate was expressed as a percentage. The binding percentage was obtained by dividing the mean enzyme uptake in OD600 after binding by the initial OD uptake of the enzyme multiplied by 100.

Results

The PCR of the native and recombinant GOX genes

The accuracy PCR fragment of the pGAPZαA/GOX and recombinant pGAPZαA/GOX-heptapeptide was confirmed by gel electrophoresis (Figure 1A).

Electrophoresis of pGAPZαA, GOX gene, and GOX with heptapeptide

PCR products containing native GOX gene and GOX gene with heptapeptide at the carboxyl end of the gene were digested by XhoI and XbaI enzymes and purified to clone the fragments into the pGAPZαA vector, which was simultaneously digested with XhoI and XbaI enzymes (Figure 1B).

Cloning of PCR product of GOX gene in PGAPZαA vector

The PGAPZαA vector and the PCR products (from both native and heptapeptide genes) digested by two enzymes were combined in the mentioned ratios in the presence of T4-Ligase and incubated at 16 °C for 24 hours. The recombinant construct was transferred into the bacterial host, and the fragment was extracted as the confirmation method. Plasmid extraction and digestion of two enzymes to evaluate cloning accuracy. To this end, the transformed clones were re-cultured overnight, and the extracted double digested the recombinant genes from both clones (native and with heptapeptide). The fragments obtaining double digestions confirmed the entry of the gene into the vector (Figure 1C).

Sequencing of cloned genes

The sequencing of the cloned gene was performed, and the result confirmed the correct frame of the clone. The confirmation of sequencing is presented in the appendix (Supplementary Data Figure 1).

Transfer of structures containing the recombinant gene into the yeast of P. pastoris

Plasmids containing the GOX gene and GOX gene with heptapeptide were linearized and then cleaved by

the AvrII restriction enzyme to be separately and linearly integrated into the genus P. pastoris, and were finally sent into the yeast genome by electroporation (Figure 1D).

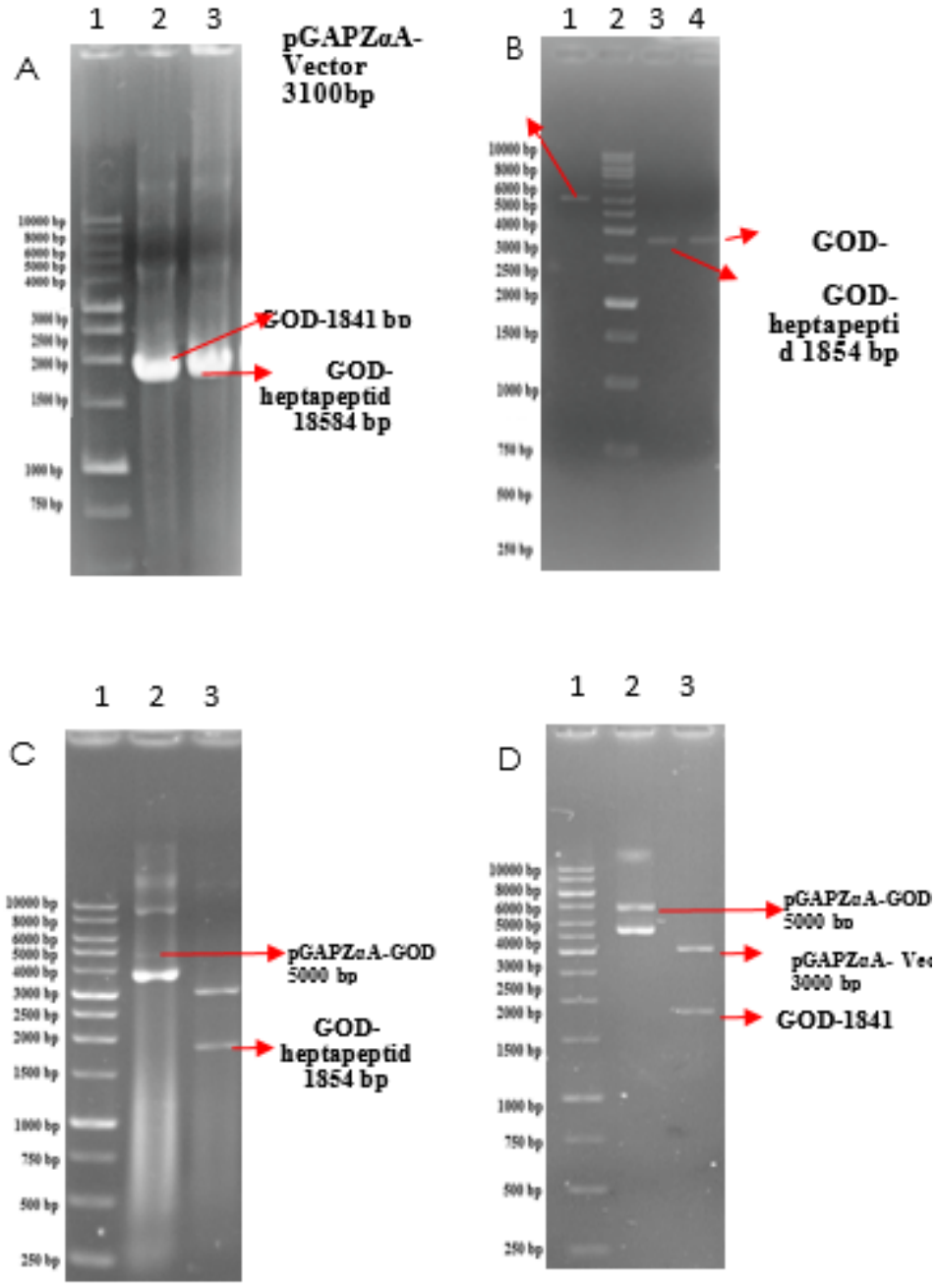

Figure 1. (A) PCR product image of agarose gel electrophoresis from GOx gene: **1.** Molecular marker (1 kb), **2.** PCR product of native GOx gene from *A. niger*, and **3.** PCR product of recombinant GOx gene with heptapeptide; **(B)** The electrophoresis of the purified pGAPZαA vector and GOx gene: **1.** Purified vector, **2.** Molecular marker (1 kb), **3.** Agarose gel image of purified GOx gene native, and **4.** Agarose gel image of purified GOx gene with heptapeptide; **(C)** Extraction of recombinant plasmids and evaluation of cloning accuracy by double digestion: **1.** Molecular marker (1 kb), **2.** An extracted construct containing native GOx gene, and **3.** Double digestion of construct by Xho*I* and Xba*I*; **(D) 1.** Molecular marker (1 kb), **2.** An extracted construct containing GOx gene with heptapeptide, and **3.** Digestion of recombinant construct by Xho*I* and Xba*I*

Electroporation into the genus P. pastoris

The linearized constructs were transformed into the hosts by electroporation (Figure 2A). Colonies were formed 72 hours after electroporation. Then, to identify colonies containing more copies of the gene, the colonies were passed into new media and incubated again for 72 hours to form colonies. This step was repeated twice, increasing the concentration of antibiotic each time to remove colonies that had fewer plasmids inserted into their genomes.

Confirmation of the transfer of structures containing recombinant genes into the yeast genome

Five colonies of transformed yeast cells containing two controlled and tested constructs were randomly selected after performing several passages of transformed yeast cells and obtaining pure cells with a high number of copies of the desired genes. Next, the genome of the host yeast was isolated, and the PCR was performed to confirm the entry of the desired genes into the genome using primers specific to each gene. The

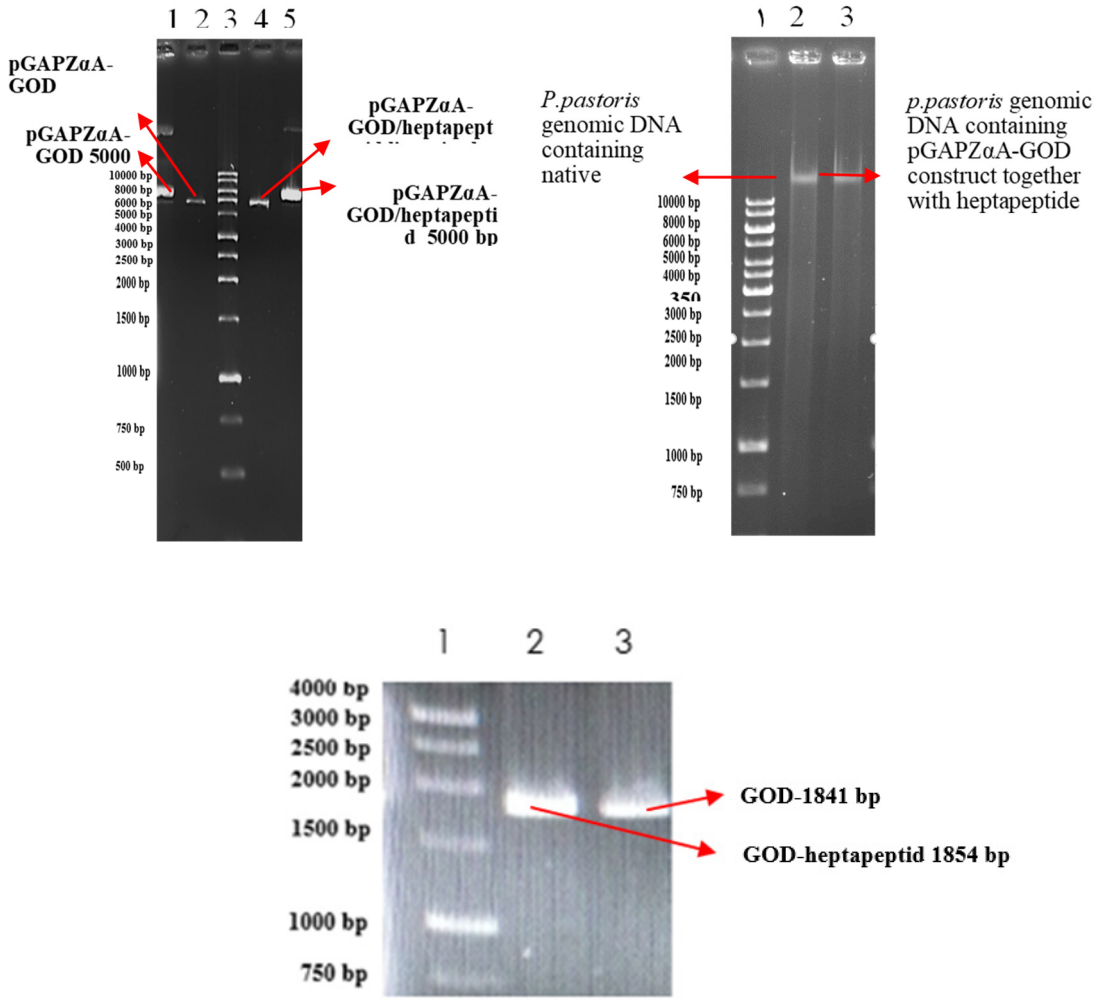

Figure 2. (A) Linearization results of recombinant GOx gene: **1.** Native recombinant plasmid pGAPZαA-GOD, **2.** A recombinant plasmid containing linear localized GOx gene, **3.** Molecular marker (1 kb), **4.** A plasmid containing GOx gene with linear heptapeptide, and **5.** Recombinant plasmid with pGAPZα; **(B)** Genomic DNA of the *P. pastoris*: Molecular marker (1 kb), **2.** Pichia genomic DNA containing native pGAPZαA-GOD construct, and **3.** Pichia genomic DNA containing pGAPZαA-GOD construct with heptapeptide; **(C)** Confirmation PCR of a yeast genome-containing structure with GOx gene: **1.** Molecular marker (1 kb), **2.** GOx gene together with heptapeptide, and **3.** Native GOx gene

extracted genomic DNA image of P. pastoris is shown in Figure 2B. Figure 2C confirms the validity of GOX genes (native and associated with heptapeptide) entering the host yeast genome (P. pastoris) by gene-specific primers via PCR. Both constructs containing the native GOX gene and GOX with the heptapeptide were inserted into the host yeast genome with a suitable orientation (´3 →5).

Expression and purification of the recombinant protein produced by SDS-PAGE

The analysis of the SDS-PAGE results of protein and enzyme activity in the yeast culture medium containing the recombinant construct demonstrated that the cloned gene was translated into protein, and a band of 65.9 kDa, which is similar to the size of GOX protein, was found in both clones with and without heptapeptide. Additionally, the examination of the extracellular proteins of the host yeast culture medium (lacking the pGAPZαA/GOD construct) is illustrated as a negative control in Figures 3A and 3B, which can be a confirmation of this claim. The recombinant GOX enzyme was purified in one-step based on ion exchange chromatography (Figure 3C).

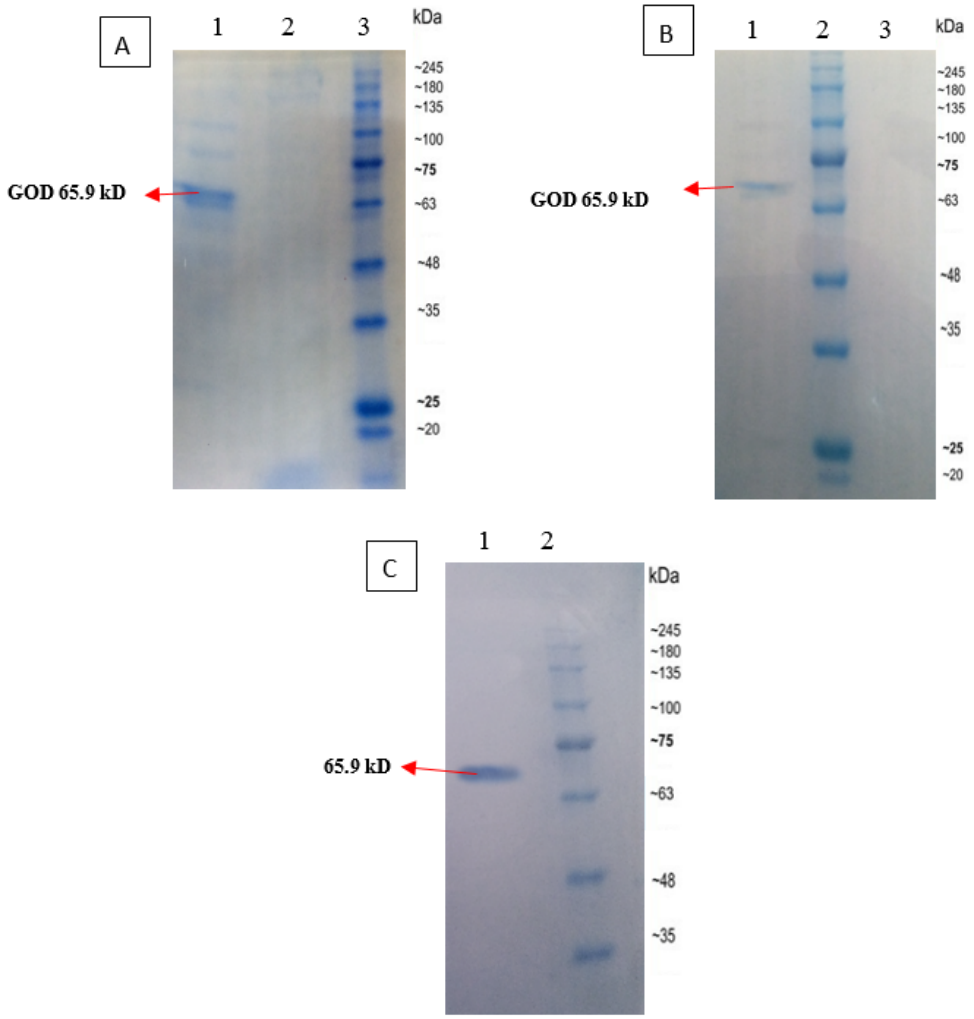

Figure 3. (A) Polyacrylamide gel electrophoresis to evaluate the expression of native GO_X gene in yeast with a pGAPZαA/GO_X structure: **1.** GOD protein of a recombinant ppGAPZαA/GO_X construct, **2.** *P. pastoris* without GO_X gene as a negative control, and **3.** Molecular marker; **(B)** Polyacrylamide gel electrophoresis of the expression of GO_X gene with heptapeptide in *P. pastoris* with a pGAPZαA/GOX construct: **1.** *P. pastoris* with the pGAPZαA/GO_X construct with heptapeptide, **2.** Molecular marker, and **3.** *P. pastoris* without GO_X gene, and **(C)** Gel electrophoresis (SDS-PAGE) results of the purified GO_X enzyme: **1.** Purified recombinant GOD and **2.** Molecular marker

Standard GOX curve and calculation of the enzyme production

The slope equation of the line obtained from the standard diagram of native GOX was inserted, and the amount of the produced enzyme was obtained accordingly.

Y = 9899/0 x – 062/0

The highest OD_{500} was obtained in yeast culture media containing a native pGAPZαA/GOX structure (0.3) and a pGAPZαA/GOX structure with heptapeptide (0.2), which was satisfied after placement in the line equation.

Y = 9899.0 × - (3/0) 062/0 = 234.0 g/l

Y = 9899.0 × - (2/0) 062/0 = 135.0 g/l

The production of the GOX enzyme in the pGAPZαA/GOX construct and pGAPZαA/GOX construct with heptapeptide was estimated to be 0.87614 and 0.63776 g/l, respectively. The reason for the production reduction in the structure with heptapeptide is the optimization conditions for the production of previously used recombinant enzymes.

Assay of the enzyme activity of GOX

The highest OD_{500} was observed in the p. pastoris culture medium containing a native pGAPZαA/GOX structure (0.3) and the yeast culture medium containing a pGAPZαA/GOX structure with heptapeptide (0.2), which was satisfied after placement in the line equation.

The activity of the control and constructs was tested, and constructs with the highest enzymatic activity in the clones containing native GOX and GOX with heptapeptide were determined and selected for stability testing.

Evaluation of the stability of constructs with native GOX and GOX with heptapeptide

To evaluate the tendency to bind to cellulose, nitrocellulose, and PVDF paper surfaces, the enzymatic activity of both structures was measured and incubated on the mentioned substrates for 3 hours in a shaker. The enzyme activity was then measured again. This experiment was performed at least 2 times at three different levels. The difference in enzyme activity at the beginning and end of the experiment represents the extent that each enzyme binds to the substrate (Table 2).

The binding percentage of the native enzyme with heptapeptide was obtained by dividing the average of OD_{500} after binding by the initial OD_{500} multiplied by 100.

Immobilized wtGOX and Heptapeptide-GOX chimera adduct on different materials based on the retained GOX activity were measured at 500 nm. Each value is the mean of three replicates (Figures 4A and 4B).

Discussion

GOX is an enzyme with highly important applications in various fields, including a food preservative in the food industry and a biomedical sensor to detect blood sugar in the medical industry, as well as in the process of producing organic acids and in the bread and cosmetic industries. This enzyme was first purified from the extract of *Aspergillus* species. Numerous microorganisms were then screened on an industrial scale for the production of this enzyme, and *A. niger* was the best producer of this enzyme [35]. The main problem with using GOX isolated from a native source is that it produces an enzyme that may be contaminated, which is unsuitable for specific uses of the desired protein. Because GOX is used to preserve food, and its main source of production (i.e., *A. niger*) is highly allergenic and thus is not approved for use in the food industry. In addition, the precise purification methods of this enzyme are extremely expensive. GOX is also an intracellular enzyme, making its extraction and purification difficult. These problems can be solved by producing this enzyme in recombinant systems [35]. The commercial production of this enzyme is in the possession of large commercial companies, and the production method has a patent. To this end, numerous efforts have been made by other countries to produce this enzyme economically on a large scale by genetic engineering methods. In addition to cost-effective production, methods to stabilize this enzyme for use as a biosensor are a challenge. The recombinant production

Table 2. The comparison results of the binding affinity of native GO_X and GO_X with heptapeptide on different surfaces

Surface-GO type	Primary OD(500nm)	After Binding 1	After Binding 2	After Binding 3	Average	Bound/ Total Ratio	Standard Deviation	Bound (%)
Cell-WT	0.355	0.11	0.083	0.082	0.092	0.258	0.000505	25.82
Cell-Hept	0.263	0.127	0.133	0.151	0.137	0.520913	0.000312	52.09
Nitro-WT	0.263	0.142	0.137	0.141	0.140	0.532319	0.000014	53.23
Nitro-Hept	0.355	0.291	0.287	0.29	0.289	0.815023	0.000009	81.50
PVDF-WT	0.263	0.185	0.179	0.183	0.182	0.693283	0.000019	69.33
PVDF-Hept	0.355	0.093	0.109	0.101	0.101	0.284507	0.000128	28.45

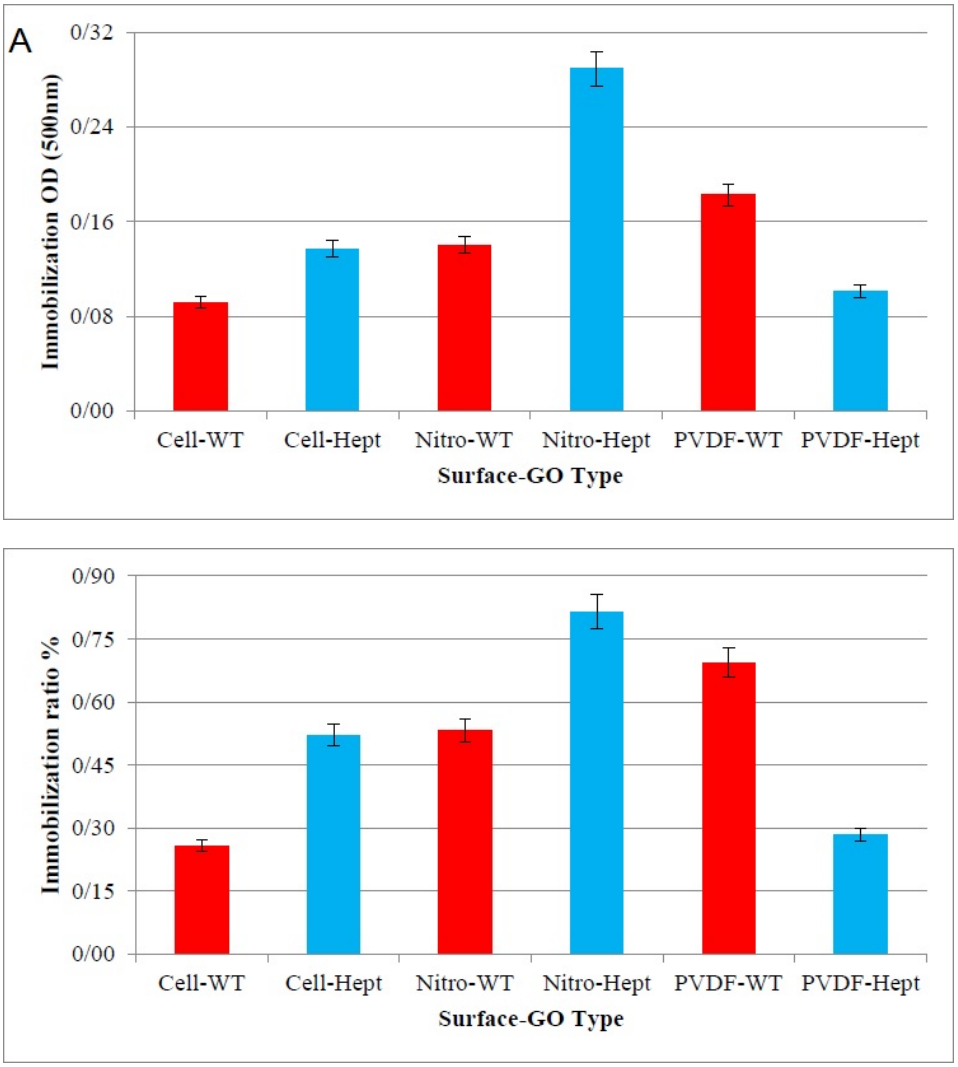

Figure 4. Comparison of the effects of different substrates on the stability of GO enzyme activity: **(A)** Immobilized wt-GO and Heptapeptide-GO chimera adduct on different materials based on retained GO activity measured at 500 nm and **(B)** Immobilization rate

of this enzyme has been performed in multiple hosts and under several promoters.

This study evaluated the partial production and purification of this enzyme under the GAP promoter, which is a permanent promoter and is permanently induced by the GAPDH gene, as well as the effect of a short sequence of aromatic amino acids on binding to the substrate that were attached to the end of the gene by the PCR. Previously, the production of this enzyme under the promoter was used in a similar study from a group of engineered hosts producing recombinant proteins, which are protease-free and lack protein-degrading enzymes. Furthermore, the production process was performed on an industrial and optimized scale. In this study, enzyme production in the GS115 strain from P. pastoris was used, and the important point in the GS115 strain P. pastoris was the presence of vacuole proteases, which was one of the problems of this project. Vacuole proteases are highly essential factors in protein breakdown and cause the instability of the recombinant protein produced in the culture medium. The production of the recombinant glucosidase enzyme was estimated at 0.234 and 0.135 g/l in the native pGAPZαA/GOX construct and pGAPZαA/GOX construct with heptapeptide, respectively. The reduction in production in the structure with heptapeptide is related to optimization conditions for the production of recombinant enzymes [31]. Moreover, partial purification was performed from the native pGAPZαA/GOX construct, and the SDS-PAGE results

confirmed the presence of a purified enzyme band with a size of 66 kD. As mentioned earlier, the present project mainly sought to investigate the effect of a heptapeptide with a tendency to bind to cellulose crystals on the stabilization of the GOX enzyme to cellulose and nitrocellulose levels. This heptapeptide is found in cellulose-degrading enzymes in nature, and NMR studies have confirmed the tendency of this heptapeptide to bind to cellulose [36].

Some cellulose hydrolyzing enzymes contain cellulose-binding sequences. Additionally, some cellulose-binding sequences are attached to the surface of the ligand by a physical hydrophobic bond that includes aromatic amino acid residues. These aromatic amino acids are W, Y, and sometimes H and F, which often interact with cellulose binding [37-39]. Other cellulose-binding sequences have been identified by creating gaps to replace polysaccharide chains for enzymatic digestion [40]. In a typical protein-carbohydrate bond, the hydrogen bond and the Wonder Waltz force, especially in the two-way interaction, are usually involved between the aromatic amino acid side chains and glucose rings [40]. Although the structures of some sequences binding to cellulose have been exploited, little is known about the structural determinants required to bind these sequences to the crystals of cellulose substrates due to the complexity of the cellulose structure and the complex formed with the cellulose. Moreover, although several studies have been performed on the detection of important universal residues (aromatic amino acids) for binding, it has not yet been determined whether specific amino acids are responsible for identifying cellulose crystals or a peptide arrangement of amino acids. They make this connection possible.

To better understand the structural determinants of important peptides for binding to cellulose crystals, the phage display technique has been used to screen for short peptides that have the property of binding to cellulose crystals [36]. Phage display is a selective method. There is a biology to identify new peptides/proteins with desirable binding traits. The phage display method has been widely employed to produce peptides and proteins with the property of binding to surfaces. Different peptide-protein libraries can be constructed and displayed on the surface of bacteriophages, then as sources for selecting types of peptides and/or proteins with binding properties to be applied at the desired level [41]. Short peptides (heptapeptides) that can tightly bind to nano-viscous cellulose crystals have been screened, and it was revealed that some of the screened heptapeptides are found in many naturally occurring cellulose-binding sequences [42]. As a result, these peptides bind to cellulose. They can also be potentially used as binders or receptors for many applications, including protein peptides specifically bound to cellulose to improve the mechanical or chemical properties of cellulose compounds.

One of these identified peptides is WHWTYYW, which is a sequence identified by the bio-screening process. The repetition of aromatic amino acids W and Y is highly common in these peptides, and W residue, among others, is more abundant. The WHWTYYW heptapeptide appears to have an extremely high affinity for cellulose binding. In particular, the five aromatic amino acids in this coordinated sequence indicate the preferential involvement of aromatic amino acids in cellulose binding [43]. Observations suggest that the binding affinity of the screened heptapeptides may be due to the known interaction of van der Waals interaction and hydrogen bonding in cellulosic crystals. Similar to cellulose, they are significantly higher than those without aromatic amino acid peptides [36]. Another peptide called WHWRAWY has many similarities to the peptide sequence consisting of amino acids with a higher affinity for cellulose, preferably binding specifically to cellulose crystals. In this sequence, the aromatic amino acids W and Y predominate and make up five of the seven highly conserved amino acids among the screened heptapeptide sequence. In addition, aromatic amino acids are examined among all the peptide sequences. Additionally, these amino acids interact with the glucose ring of cellulose crystals [44].

In this study, a genetically engineered structure of the GOX gene was synthetically synthesized with the WHWTYYW heptapeptide sequence. The purpose of this structure was to compare the binding of the produced enzyme to the cellulose substrate surface and cellulose derivatives including nitrocellulose and PVDF paper and then compare it with the enzyme without heptapeptide (native). According to experiments performed with WHWTYYW heptapeptide and repeated tests and statistical analysis of the results, WHWTYYW heptapeptide is effective in binding enzymes to cellulose and nitrocellulose substrates in comparison with the wild enzyme. The role of this heptapeptide in binding to the PVDF paper is estimated without any effects. As previously mentioned, this tendency to bind to cellulose and nitrocellulose substrates seems to be due to the interactions of amino acid charges and the substrate structure. Given that the designed heptapeptide tends to bind to cellulose crystals, it justifies its binding to cellulose and nitrocellulose. The reason for the reluctance to bind the

heptapeptide to the PVDF paper could be the polymeric structure of the membrane and the charge compounds present on the surface of the membrane, creating a repulsive state and preventing the binding of the enzyme with the heptapeptide to the membrane surface.

The stabilization of covalent enzymes on the secondary substrate reduces their activity to such an extent that due to the chemical bonding of parts of the enzyme with the substrate, this bonding may occur in the active part of the enzyme so that up to 70% of the total space of the catalyst is occasionally occupied by the gel or polymer. A substance called glutaraldehyde is used in cross-linking stabilization. Zhou et al. (2010) first used graphene plates to stabilize the enzyme GOX [44]. They employed graphene to disperse graphene in the structure of L. They further used functionalized polyethyleneimine with an ionic liquid that has good dispersion and high solubility in water. A sensor was introduced that combines chitosan and graphene, a biocompatible compound. The bonds between chitosan and graphene are esterified. As mentioned earlier, glutaraldehyde is used as a linker between the enzyme and chitosan to stabilize GOX on chitosan [44). Glutaraldehyde with its two aldehyde terminal groups can bind to the amine group of enzymes, which reduces the activity of the enzyme and changes its physical and chemical properties. Nonetheless, the resulting compound is economically viable due to its solubility and reuse [45]. The substrate is bonded and does not require intermediate composition. Furthermore, the binding site of the enzyme to the substrate surface is known and does not prevent the activity of the active site of the enzyme. Test substrates are also widely available and are highly economical for enzyme stabilization. They can also bind the enzyme with the heptapeptide to the substrate from the end of the heptapeptide by chemical engineering methods.

An important feature of most P. pastoris GS115 strains is the presence of vacuole proteases, which was one of the problems of this project. Vacuole proteases are extremely important factors in protein breakdown. For example, some proteins are produced unstable in the culture medium of Picapasturis because proteins are sensitive to the presence of proteases and are rapidly degraded by them. Particularly, in large-scale culture conditions, due to high cell density, these enzymes are found in abundance in the culture medium. Several enzymes have been reported as successful strains in reducing the breakdown of some foreign proteins. Strains with this property can grow at high cell concentrations and with a small percentage of cell degradation but have a slow growth rate. These strains have low viability and are difficult to transform [39]. Regarding the tendency of GOX to bind to heptapeptide compared to GOX without heptapeptide, the following suggestions are made:

It has been shown that WHWRAWY peptide has a higher affinity for cellulose crystals than WHWTYYW peptide, this affinity can be more due to the positive charge of R amino acid charge and negative charge of cellulose charge and electrostatic interaction between these charges [36]. In addition, no agreed sequence fully confirms the 100% probability of cellulose binding due to substrate variability and binding mechanisms between peptide and cellulose [46]. Therefore, membrane structures such as cellulose and nitrocellulose, as well as computer simulations, were further investigated to select the best substrate-binding peptide.

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