Iran. J. Biotechnol. January 2022;20(1): e2979 DOI: 10.30498/ijb.2021.272122.2979

Research Article

Molecular Cloning, Expression, and Enzyme Activity of Glucose Oxidase Gene from Soil Thermophilic *Streptomyces*

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Background: Glucose oxidase is an oxidoreductase that depletes oxygen in food processing and is used in biosensors, glucose diagnostic kits, food processing, cosmetics, and chemical industries. This enzyme is often isolated from fungi, such as Penicillium and *Aspergillus Niger*.

Objectives: The objective of this study was to clone and express a full-length GOX gene from soil thermophilic streptomyces for bioinformatic and enzyme activity evaluations.

Materials and Methods: After collecting samples from the Gandom Beryan area of Kerman province, Iran, Streptomyces strains were identified with specific biochemical and molecular tests. Streptomyces strains with glucose oxidase gene were detected by PCR, and the amplified gene fragment was cloned into *Escherichia coli* host bacterium using TA cloning technique. The expression of the cloned GOX gene in the host bacterium was measured using real-time PCR, and the recombinant plasmids were sequenced. The enzymatic activity was measured in the extracts of *E. coli* cells carrying the plasmids.

Results: After screening the samples, 12 strains of *Streptomyces* were identified, 4 of which carried the GOX gene. The GOX open reading frame, obtained by PCR, was cloned into a vector and transformed into *Escherichia coli origami* to generate GOX-producing bacteria. Enzyme activity was confirmed and a phylogenetic tree showed the degree of kinship between Streptomyces species and other species, including Streptomyces SP MI02-7b. The expression levels of GOX genes mRNA were found to be approximately 4-fold higher in transformed *E. coli* than in soil thermophilic Streptomyces ($P < 0.001$).

Conclusion: This study showed that natural thermostable streptomyces producing glucose oxidase enzyme could be found in Iran. The enzyme gene was successfully transformed into *Escherichia coli* generating a recombinant host with high yield capability that can be a major step towards the production of this enzyme from indigenous strains. It should be emphasized that the GOX enzyme produced by these strains is profitable due to high production levels correlated to the optimum condition in cheap culture media, short fermentation cycles, high expression capability, and ease of growth.

Keywords: Cloning, Expression, Glucose oxidase, Streptomyces

1. Background

Enzymes have long been used in the food preparation process. Today, with the advancement of science and technology, new enzymes are being produced with many applications in various fields, and this process is still in progress (1). Glucose oxidase (GOX) (EC number 1.1.3.4) is one of the most important commercial enzymes that was first isolated from *Aspergillus Niger* in 1928 by Detlev Muller and is currently widely used in various industries. This flavoprotein is an oxidoreductase that catalyzes the oxidation of glucose to hydrogen peroxide and gluconic

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acid (2)(3). This enzyme is utilized to measure the amount of free glucose in body fluids, the production of biosensors, food storage, and the chemical industry. Glucose oxidase is generated from many species of fungi and some insects and appears to be involved in the production of hydrogen peroxide to kill other bacteria. So far, this enzyme has been isolated from *Talaromyces flavus*, *Pleurotus ostreatus*, *Aspergillus Niger*, Penicillium spp., *Phanerochaete chrysosporium*, and *Botrytis cinerea*. Today, this enzyme is produced industrially from fungal sources, such as Aspergillus and Cladosporium (3) (4) (5).

GOX has a GRAS (Generally Recognized as Safe) status by the FDA (U.S. Food and Drug Administration) and is widely used as an additive in the food industry to improve the taste, aroma, and stability of food products by removing glucose and oxygen from diabetic drinks and egg albumen (5) (6). Its consumption in the cereal cooking industry is also increasing due to its oxidizing effect, which produces a stronger dough. In addition to its current applications, this enzyme is bound to find many more applications in the future.

Industrial enzymes are generated by a wide range of microorganisms. *Aspergillus Oryza* and *Aspergillus Niger* are the most important fungi producing food processing enzymes, but the enzymes produced by microbial strains are superior to those generated by other microorganisms owing to their ease of reproduction and manipulation, safety, and cheapness (7). Streptomyces is aerobic, gram-positive bacteria with GC-rich genome; it is the largest genus of actinobacteria and one of the most valuable soil bacteria. Isolation and characterization of actinomycetes from diverse habitats not only helps understand the role of these microorganisms in ecosystems but is also beneficial to reaching aspects with industrial, pharmaceutical, and agricultural applications (8)(9). Actinomycetes cause complex physiological and morphological adaptations in the soil, hence they may be less affected by adverse environmental factors compared with other microbial groups. This group of microorganisms not only forms a dominant group of soil microflora but is also capable of forming spores and surviving under stress (10). Streptomyces is known by a process called secondary metabolism, and it produces approximately 70% of naturally occurring antibiotics, such as chloramphenicol and neomycin. various studies have identified and isolated genes producing industrial enzymes, including alditol oxidase, cholesterol oxidase, hyaluronidase, cellulase and xylanase from Streptomyces strains; it has also been proven that by isolating and identifying these genes, steps can be taken towards the industrial production of these enzymes (11) (12) (13). Even though many researchers were worked on the cloning of bacterial enzyme genes for a variety of applications, very few researchers were reported about Iran native species and their enzymatic capabilities. Furthermore, despite many advances in the development of bacterial enzymes in the food industry, heat tolerance of these enzymes is a huge problem.

Thermostability is one of the most important properties of industrial enzymes that could meaningfully increase the window for enzymatic bioprocess operations. This property is associated with higher resistance to chemical denaturants, easier purifying by heat treatment, higher substrate concentrations, lower viscosity, and fewer risks of microbial contaminations. Thermostable enzymes (also called thermozymes) could be produced by thermophilic microorganisms and genetic engineering. Intrinsically stable and active at high temperatures, thermophilic and hyperthermophilic enzymes offer major biotechnological advantages over mesophilic enzymes (8-12).

2. Objectives

For the first time, our study has tried to take a step towards isolating the enzyme glucose oxidase from bacteria in the Gandom Beryan-Iran, the warmest and driest region on Earth. This study aimed to clone the glucose oxidase gene from thermophilic Streptomyces, evaluate its expression in *Escherichia coli Origami*, and optimize the enzyme activity.

3. Materials and Methods

3.1. Bacteria Isolation

Sampling was performed from a depth of 6 to 10 cm of Gandom Beryan region soil, a 480 km^2 large plateau of Kerman province in southeastern Iran, which is the hottest place on earth (14). The coarse particles and pebbles of the samples were removed, transferred to the laboratory, and cultivated in nutrient agar (NA). The pure cultures of the bacterial colonies were grown overnight at 35 °C in nutrient broth (NB) and stored at 4 °C for further studies. For molecular methods, we selected Streptomyces colonies with Gram-positive

bacteria and a dry or gypsum appearance; they were also white or colored, attached to the medium, and with vegetative mycelium in the culture medium.

3.2. Polymerase Chain Reaction

To identify the Streptomyces strains containing the target gene, some isolates were analyzed for the presence of GOX gene cluster sequences in the genome. DNA extraction was carried out using QIAamp Minikit (QIAGEN, Germany) following the manufacturer's guidelines. The oligonucleotide concentration was determined spectrophotometrically by O.D. reading at wavelengths of 260 nm and 280 nm (Thermo Fisher Scientific, USA). The DNA purity was specified using an O.D. ratio; 260/280 nm (1.5-2), indicating that the DNA solutions were well purified. The conserved sequence of the GOX gene from streptomyces was identified from NCBI (National Center for Biotechnology Information, USA), and the primers were designed using the identified sequence. The target sequences were amplified using the PCR method as previously described (15). The PCR conditions were as follows: 94 °C for 2 min, followed by 37 cycles of 95 °C for 30 min, 56 °C for 1 min, and 72 °C for 1 min, and final synthesis at 72 °C for 5 min. The DNA fragments were separated by 2% agarose gel electrophoresis staining with erythrogel and then sequenced. Isolated strains were identified by 16SrRNA sequence analysis using universal primer pairs (Forward 5′ AGAGTTTGATCCTGGCTCAG3′ and reverse 5′AAGGAGGTGATCCAGCCGCA3′) and the phylogenic tree was built using the MEGA7 software and the neighbor-joining method.

3.3. Gene Cloning

To clone the GOX gene from the isolated strains, its conserve sequence was identified from the NCBI conserved domain site. Cloning was performed by the TA cloning vector kit (Invitrogen, USA) according to the manufacturer's instructions. The total DNA was extracted using a DNA extraction kit (QIAGEN, USA) and PCR was carried out as follows: initial denaturation at 94 °C for 7 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 62 °C for 40 sec, extension at 72 °C for 120 sec, and final extension at 72 °C for 10 min in a thermal cycler. The PCR products were analyzed by electrophoresis in 1% agarose, purified using a QIAEXII Gel extraction kit (QIAGEN, USA), and ligated into the pTG19 vector. The ligated plasmid (pTG-GOX) was transformed to the competent bacteria, *E. coli origami* blue strain in line with Mirzaei *et al*. (16), and incubated in Luria-Bertani (LB) broth medium free antibiotic for 55 min at 35 °C. The transformed bacteria were cultivated on the LB agar medium (peptone 1% (w/v), yeast extract 0.5% (w/v), agar 1.2% (w/v), NaCl 1% (w/v)) containing IPTG 200 $mg.mL^{-1}$, ampicillin 100 $mg.mL^{-1}$, and X-Gal 20 $mg.mL^{-1}$ and incubated at 37 °C for 24h. Gene cloning was confirmed by a screening of blue (non-recombinant) and white (recombinant and effective ligation) colonies. The recombinant plasmid DNA was extracted from positive colonies incubated in the liquid LB medium containing ampicillin (100$\mu g.mL^{-1}$) for 24 h using a plasmid extraction kit (QIAprep Spin Miniprep Kit, QIAGEN, Germany). The products were analyzed by electrophoresis on 0.6% agarose gel, and their size was compared with a 1kb DNA ladder (Fermentase, USA), and the recombination was verified by sequencing.

3.4. Quantitative Analysis of GOX mRNA Expression

Non-recombinant and successful ligated *E. coli origami* colonies were separately washed twice with PBS to assess the GOX gene expression. To induce expression, methanol was added every 24 hours to gain a final concentration of 0.75%. Afterward, the samples were cumulated every 12 hours and the supernatant was collected as crude enzyme fluid following centrifugation at 3500 g for 5 min. Total RNA was extracted using an RNA isolation kit (QIAGEN, Germany) and stored at -80 °C; cDNA was synthesized in an RNase-free environment using Quantitech Reverse Transcriptase RT kit (QIAGEN, Germany), and the real-time PCR was performed using a Real-time PCR kit (QIAGEN, Germany) according to the manufacturer's protocols. Real-time PCR was done on a StepOneplus System (Applied Biosystems, USA) using SYBR Green PCR Master MIX (Applied Biosystems). Relative quantification of gene expression was performed using the 16SrRNA gene as a reference in REST2018 software. The specificity of the PCR reaction was checked with a melting curve analysis following the final step of the PCR.

3.5. Enzyme Activity Detection

Induction was stopped once the enzyme activity of the GOX was tested in the crude enzyme fluid (96 h).

The crude enzyme fluid was freeze-dried into powder, and the powder was redissolved in double-distilled water. As previously described, a turbidimetric assay was performed in 570 nm to determine the enzymatic characteristics of the recombinant protein, including the effect of substrate concentration, pH value, and temperature on enzyme activity (8).

4. Results

4.1. Bacterial Isolation

In this study, a total of 23 bacterial strains were isolated from 30 soil samples. Based on the biochemical tests, morphological examinations, and molecular analysis data, 12 bacterial strains isolated from soil were *Streptomyces spp*. The extracted DNA oligonucleotide concentration was in the standard range, and gel electrophoresis did not show either smear or abnormal fragments. Molecular analysis for the identification of isolates with the GOX gene showed that four isolates (30%) had this enzymatic region (**Fig. 1**). **Figure 2** shows the analysis of 16SrRNA gene sequences of isolated strains to determine the phylogenetic relationships. The results of the phylogenetic tree by neighbor-joining (NJ) method showed that the species of Streptomyces sp. BPSEAC7 with 81% bootstrap with Streptomyces sp. MI02-7b (EU080955.1) were placed in a cloud, which indicates the close relationship between them.

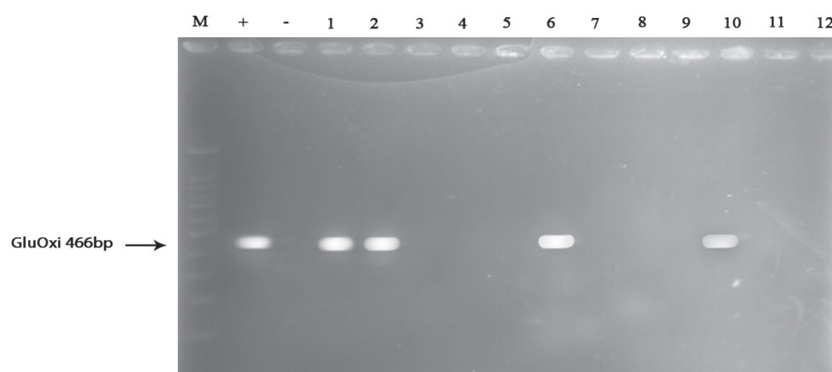

Figure 1: Gel electrophoresis analysis of GOX gene fragments amplification by PCR method. Lane +: positive control, Lane -: blank, molecular weight marker (100bp ladder), Lane 1 to 12: samples. Positive control is a 466 bp GOX fragment.

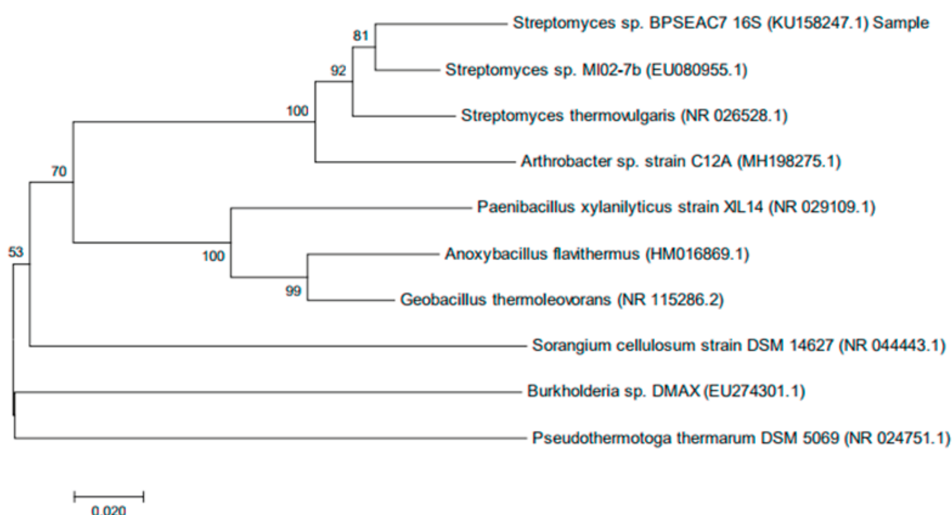

Figure 2. Molecular phylogenetic trees of 16SrRNA based on the neighbor-joining method. Numbers at nodes indicate the levels of bootstrap support based on 1000 replicated datasets.

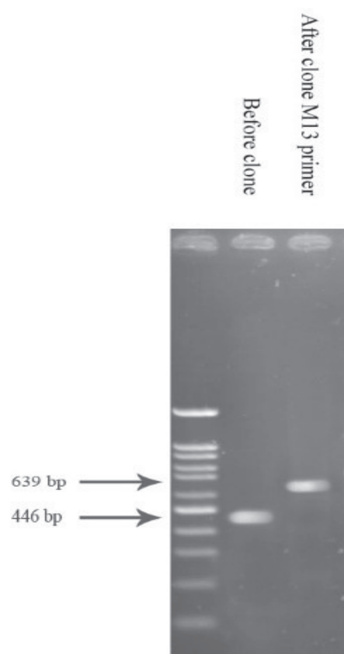

Figure 3. Gel electrophoresis analysis of PCR product. Lane 1, ladder; lane 2, pTG19T vector without insert; lane 3, pTG19T vector containing GOX gene.

4.2. Cloning and Gene Expression Data

The cloning GOX gene in pTG19 vector was confirmed by evaluating the white and blue colonies. The pTG-crt was successfully constructed and sequenced using the TA cloning method (**Fig. 3**). The sequenced GOX gene showed 99% homology of the original gene in NCBI GenBank. **Figure 4** compared the expression levels of GOX gene in cloned and control *E. coli* bacteria. These amplification plots represent the accumulation of product throughout the real-time PCR experiment. The samples used to create the plots are a dilution series of the target genomic sequence. The expression levels of GOX genes mRNA were found to be approximately 4-fold higher in transformed *E. coli* in comparison with soil thermophilic Streptomyces (P <0.001) (**Fig. 4**).

4.3. Glucose Oxidase Activity

4.3.1. Effects of Substrate Concentration on GOX Activity

According to the standard glucose curve shown in **Figure 5**, the activity of the GOX enzyme changes at different concentrations of the substrate (glucose) (**Fig. 6**). In these examinations, optical density values decreased with the increase in the substrate viscosity. The data showed that glucose oxidase had the highest activity at 0.4 g.mL^{-1} concentration, and the lowest activity was shown at 0.1 g.mL^{-1} concentration.

4.3.2. Effects of pH On GOX Activity

Optimal pH was determined at the optimum reaction times, buffer concentrations, temperatures, and enzyme concentrations (**Fig. 7**).

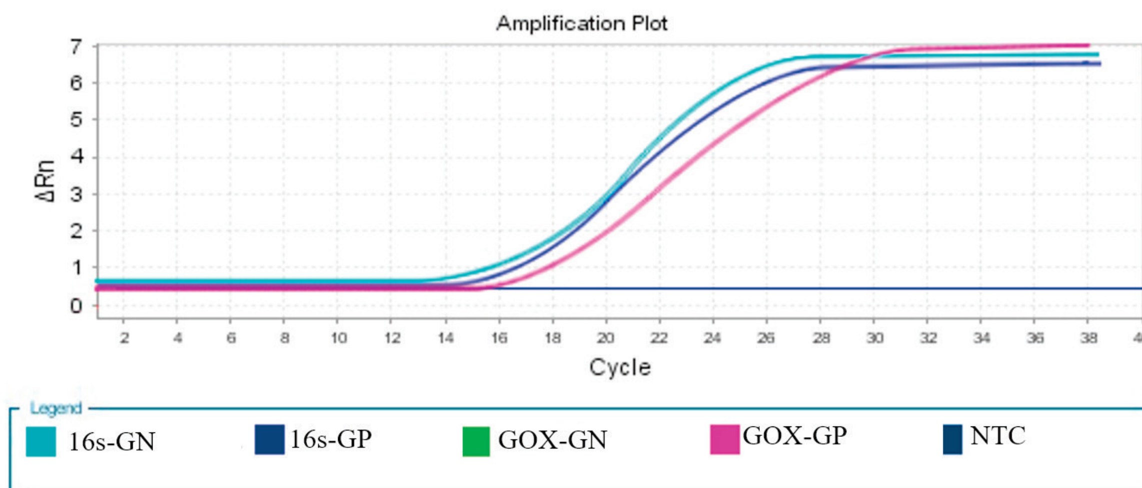

Figure 4. Real time PCR amplification plot of 16SrRNA (housekeeping) and glucose oxidase genes in cloned and control (GOX negative) *E. coli* bacteria. NTC= No template control, GN = GOX negative *E. coli*, GP = GOX positive *E. coli*.

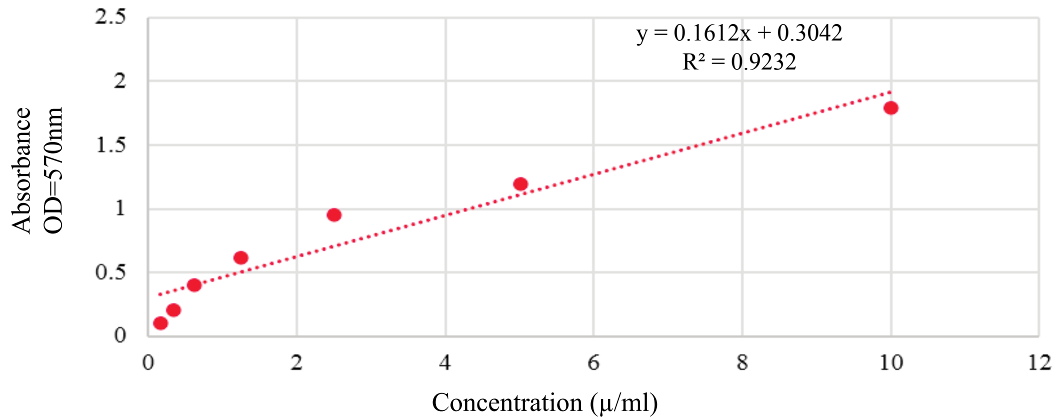

Figure 5. Example of calibration curve obtained for D-glucose standards with concentrations ranging from 2 to 12 μ/ml (absorbance recorded at 570 nm).

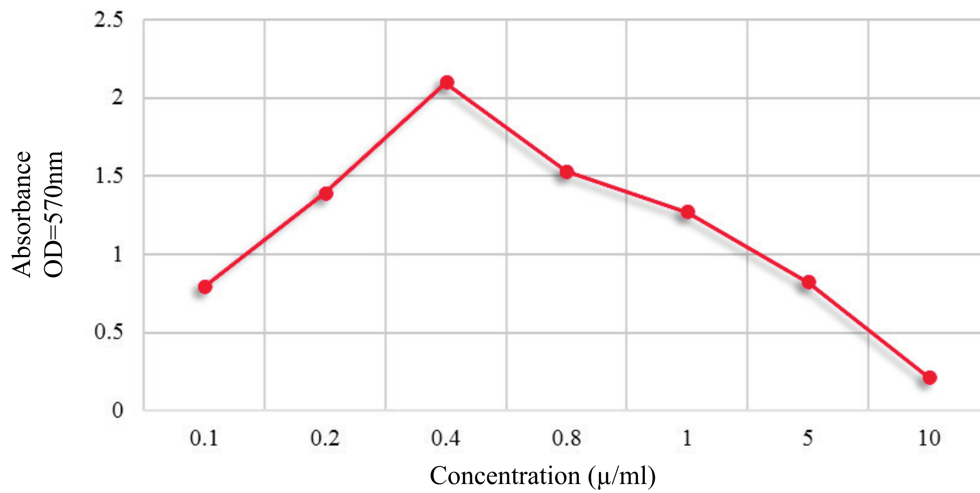

Figure 6. The optical density (OD) (at 570 nm) as a measure of the enzyme activity in isolates cultures supplemented with seven concentrations of glucose (0.1 g/ml, 0.2 g/ml, etc.) as substrate. Error bars represent the standard deviation between 3 replicates.

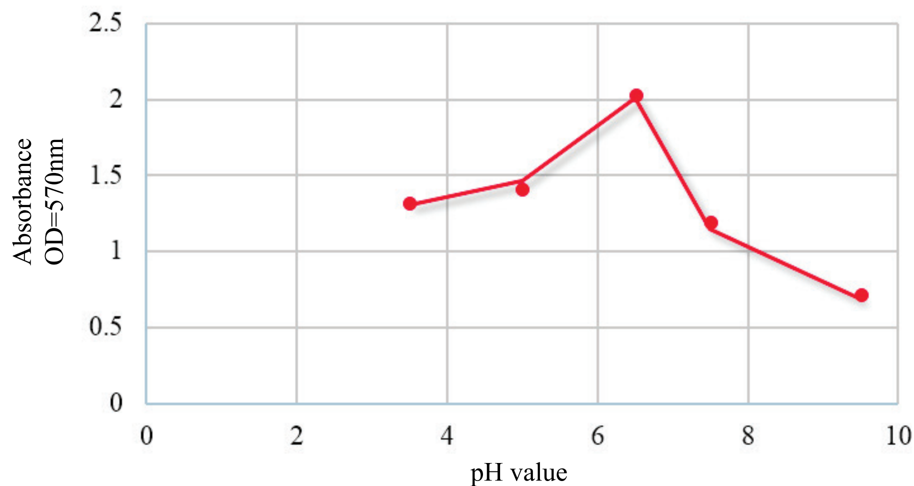

Figure 7. Glucose oxidase enzyme activity in five different pH values.

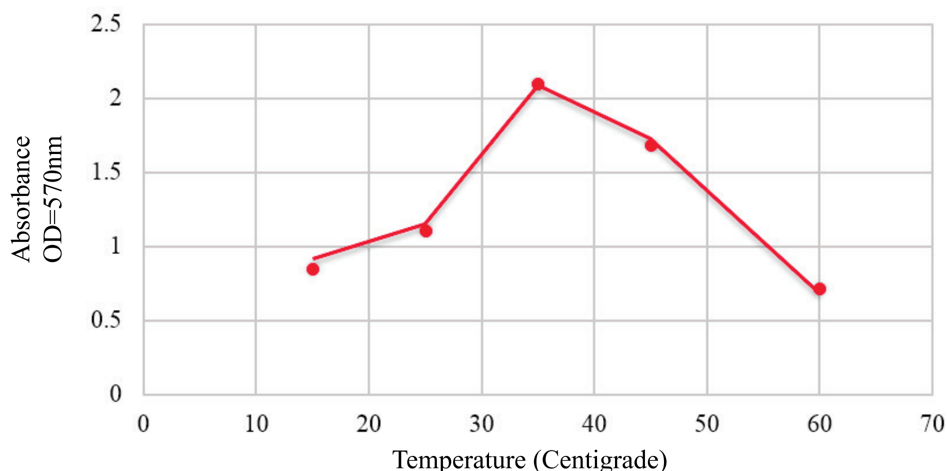

Figure 8. Glucose oxidase enzyme activity at different temperatures.

The maximum enzyme activity was observed at pH 6.5 (actually observed near-neutral pH), and the lowest activity of this enzyme was detected at alkaline pH.

4.3.3. Effects of Temperature on GOX Activity

The optimal temperature for GOX activity was specified at the optimum reaction times, buffer concentrations, pH, and enzyme concentrations (**Fig. 8**). Maximum enzyme activity was determined at 35 ºC, and the lowest activity was seen at 60 ºC.

5. Discussion

GOX enzyme is an effective industrial oxidant with important applications in food preservations, bread, gluconic acid, and alcohol production; it can be produced through biochemical, bio electrochemical, and fermentative processes. Fermentative methods are expensive, and chemical methods of producing enzymes, even under optimum reaction conditions, have shown low and limited specificity, resulting in undesirable byproducts and unsatisfactory yield. Nowadays, biotechnology has improved the production of microorganism-derived enzymes, and Streptomyces strains are considered as powerful sources in the production of GOX. The objective of this study was to prepare recombinant plasmid with GOX gene and elements necessary for gene expression(pTG-Gox) that were constructed using TA cloning method. This recombinant plasmid makes it possible to easily clone target genes in this expression vector and with different promoters in prokaryotic or eukaryotic systems; in addition to evaluating these promoters, gene expression can be further assessed in the expression vector. Esmaeilpour *et al*. (17), Lee *et al*. (18), and Raheb *et al*. (19) cloned and expressed GOX enzyme using pUC19, pCGI38, and pTA57RGO vectors, respectively; the present work is another successful cloning and expression of this enzyme.

Recent studies have reported the presence of several bacterial and yeast species producing glucose oxidase enzyme (2). According to these studies, Streptomyces spp. is among the most promising bacteria for the commercial production of enzymes. There are studies on the production of enzymes, especially GOX, in numerous isolates of genera Streptomyces spp. such as *Streptomyces aegyptia* (20), *Streptomyces rochei* (21), *Streptomyces aegyptia* (22), and *Streptomyces anulatus* (23); these studies have shown that the catalytic efficacy of this enzyme is highly dependent on its stability and thermostability in particular.

Several studies have been conducted to optimize GOX activity through biotechnological engineering methods. Qingxuan *et al* (8) purified the thermostable GOX, secreted by *Aspergillus Niger*, using *in silico* design method, and similar studies on other microorganisms were performed by Ning *et al*. (24), TU (25), Derakshan (9), and Jiang (26). Our study optimized naturally thermostable wild-type GOX enzyme, and the optimum pH, temperature, and substrate concentration for enzyme activity were 6.5, 35 ºC, and 0.4 g.moL^{-1}, respectively. In the study carried out by Qingxuan, wild-type GOX enzyme lost its activity when pH reached 7.0; it lost 70% of its activity when incubated at 50 ºC whereas mutants showed improved thermostability retaining 60% of the initial activity at 50 ºC. Our enzyme is

another type of natural thermostable enzyme showing 50% activity at 60 ºC. Belyad *et al*. (27) examined the purification and description of recombinant GOX by *P. pastoris* cultivation. The highest enzyme activity was obtained at a pH value ranging from 5 to 7 at 50 °C. The enzyme was stable at a broad pH range and temperature, which is in line with our data. Mobayen *et al*. optimized and immobilized GOX on Gum Tragacanth carrier (28). GOX enzyme was stabilized in polymer gel, and the effects of pH, temperature, substrate, and enzyme concentration on its activity were evaluated. Optimum reaction condition for enzyme activity reached pH=6, 36 ºC, 100 $mg.mL^{-1}$ glucose and 1 $lit.min^{-1}$ oxygen pressure, which is in accordance with our findings.

6. Conclusion

Enzymes such as GOX are gaining importance in the food and other industries as ecologically friendly alternatives to the outdated chemical methods. Our findings indicated that this study succeeded in finding native thermostable streptomyces spp. that produce GOX. The thermostable enzyme gene was extracted from Streptomyces in Kerman province and successfully cloned and expressed in *E. coli origami*. On a large scale, the GOX enzyme produced by these strains in the optimized condition is cost-effective due to high production levels related to standard expression, cheap culture media, ease of growth, and short fermentation cycles.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Ethical statement

This article does not contain any studies with human participants or animals performed by any of the authors.

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