Transformation and expression of *Penicillium funicolusum* glucose oxidase gene in yeast

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ABSTRACT

Glucose oxidase is an important enzyme hydrolyzing for its hydrolyzing activity on glucos. It possesses and has a wide board of applications in different industries such as bakery, pharmaceutical, plant pathology and biosensors. In this study, yeast (*Saccharomyces cerevisiae*) was transformed successfully by the glucose oxidase gene (*GOX*) obtained from *Penicillium funicolusum*. The secreted glucose oxidase enzyme (GOX) by yeast transformants was characterized intra and extracellularly. The effect of different pH values, carbon sources and the duration of cultivation time on the gene expression were also studied in liquid and solid media. Results indicated that the produced enzyme had the maximum activity of 41and 38 U/ml for the intra and extracellular, respectively in pHs ranging between 4.5-5.5 at optimum temperature of 30°C after 3 days of yeast culture. Galactose was found to be the most efficient carbon source than the other sources and the maximum activity of target enzyme was observed at 1% (w/v) concentration of galactose. The presence of glucose in the culture media depressed the production of GOX. However, a high rate of growth of the yeast and the high yield of the target enzyme suggested its potential for application in the industry.

Keywords: Expression, Glucose oxidase, *Penicillium funiculosum*, *Saccharomyces cerevisiae*.

INTRODUCTION

Filamentous fungi are capable of producing large amounts of specific enzymes. One of such enzymes called glucose oxidase (GOX, β-D-glucose: oxygen 1-oxidoreductase, EC1.1.3.4) catalyzes the oxidation of β-D-glucose to glucono-δ-lactone and the concomitant reduction of molecular oxygen to hydrogen peroxide: β-D-glucose O_2 glucono-d-lactone H_2O_2 (Bhatti and Saleem, 2009; Sukhacheva *et al*., 2004). It has been first reported in the extract of *Aspergillus niger*. GOX is the most widely used enzyme in different industries and has attracted attention of many biological experts and industrialists. The mycelia fungi *Aspergillus* and *Penicillium* serve as industrial producers of glucose oxidase (Bankar *et al*., 2009). It is a commercially important enzyme which is applied in pharmaceutical industry as a biosensor for the enzymatic determination of glucose in the fermentation of liquor, beverages (Malherbe *et al*., 2003) and body fluids (Yang *et al*., 2011) and in the food industry for the removal of glucose and/or oxygen to improve color, flavor, texture and shelf life of various products (Parpinello *et al*., 2002) in baking industry for improving the texture of crumbs (Bonet *et al*., 2006). Recently, GOX has also been used in biofuel cells (Davis and Higson, 2007). GOX is also widely used to produce gluconic acid, which is used as a mild acidulant in the metal, leather and food industries (Znad *et al*., 2004). The enzyme was identified as a dimeric flavoprotein with a relative molecular mass of 161 KD containing two tightly bound FAD cofactors per monomer.

It has been purified from a range of different fungal sources, mainly from the genus *Aspergillus* (Tsuge *et al*., 1975; Frederick *et al*., 1990a; Fiedurek and Gromada, 2000) and *Penicillium* (Kusai *et al*., 1960; Sukhacheva *et al*., 2004; Simpson, 2005; Bhatti and Saleem, 2009), but *A. niger* is the most common fungus which utilized for the production of GOX (Pluschkell *et al*., 1996). However, the GOX from *Penicillium* species has been shown to exhibit more advantageous kinetics for glucose oxidation than that of *A. niger* (Kusai *et al*., 1960). During screening of *Penicillium* species for extracellular glucose oxidase production, a strain of *Penicillium* variable (P16) was selected which released high activities of enzyme into the culture medium. Maximum activity (5.49 U/ml) was observed 96 h after cultivation in shake-flask culture (Petruccioli *et al*., 1993).

As mentioned, the levels of enzyme production by fungi are relatively too low for commercial exploitation in the naturally occurring wild type strains, thus the strains may be mutated (Punt *et al*., 2002) or the genes coding for the enzymes of interest be cloned and over-expressed in suitable hosts. Mutagenesis of different fungi as a strategy for the improvement of glucose oxidase production has successfully improved glucose oxidase activities up to 77% (Ramzan and Mehmood, 2009). Cloning and over-expression of glucose oxidase gene in *Saccharomyces cerevisiae*, *Escherichia coli* and other fungal hosts from *Aspergillus* and *Penicillium* species, have been successfully carried out (Park *et al*., 2000; Kapat *et al*., 2001; Malherbe *et al*., 2003). Witt *et al.* (1998) cloned and expressed the gene encoding *P. amagasakiense* GOX in *E. coli*. The activity of the recombinant GOX, which was expressed in the form of insoluble inclusion bodies, was reconstituted, and then the active enzyme was shown to possess properties and secondary structure composition similar to those of native *P. amagasakiense* (Witt *et al*., 1998). Park *et al.* (2000) could express *A. niger* glucose oxidase gene successfully in *S. cerevisiae* containing different promoters and terminators and showed that the hybrid yeast ADH2-GPD promoter had high amounts of GOX production (Park *et al*., 2000). In a study, Malherb *et al.* (2003) expressed *A. niger GOX* gene in *S. cerevisiae* to evaluate the GOX producing yeast transformant under winemaking conditions for its ability to reduce the total amount of ethanol produced. Yamaguchi *et al.* (2007) presented a transformant cell by incorporating the *GOX* gene of *A. niger*, which is capable of constitutive as well as secretory expression, using the yeast strain *Pichia pastoris* for using in implantable biosensor in body. So far no studies have been reported regarding the use of *Penicillium* species *GOX* gene expression in yeast system to be used in industry with respect to its more advantageous kinetics for GOX compared to that of *A. niger*.

In this work, we expressed the *GOX* gene isolated from *Penicillium funiculosum* (native to Iran) in yeast (*Saccharomyces cerevisiae*, CEN.PK.113-5D) as a capable host for producing recombinant proteins and optimized the pH, temperature and time profile of enzyme activity.

MATERIALS AND METHODS

Bacterial and yeast strains, plasmids, media and gene manipulation

Escherichia coli strain DH5α was used for plasmid cloning and propagation. Yeast (*Saccharomyces cerevisiae*) strain CEN.PK 113-5D (MATα, MAL2-8c, SUC2, ura3-52), uracil mutated auxotroph strain was kindly gifted by Dr. Assadollahi (University of Isfehan, Iran).

The *E. coli* strain was grown in LB [1% bacto- tryptone (w/v), 0.5% yeast extract (w/v), 0.5% NaCl (w/v)] liquid or on solid (2% bacto agar) media at 37°C. Ampicillin (100 µg/ml) was added for the selection of transformed *E. coli*. Yeast cells were cultured in both liquid and solid yeast peptone dextrose (YPD) media [1% yeast extract (w/v), 2% peptone (w/v) and 2% glucose (w/v)] at 30°C. Yeast transformants were isolated on uracil selective agar plated SD-URA3 medium [0.67% yeast nitrogen base (w/v) without amino acids, 2% glucose (w/v), and yeast synthetic drop out supplements without uracil].

The *GOX* gene of *P. funiculosum* was amplified by polymerase chain reaction (PCR) from the recombinant plasmid PUC19-*gox* (Esmaiilpour *et al.*, 2014), using specific primers Es88GOX-1F (ATAGAGCTCACCATGGTGTCTGTATTTCTC), and Es88GOX-1R (ATAGAGCTCCT AGGCACTTTTGGCATAG). PCR was carried out in a final volume of 20 µl. The reaction was run for 30 cycles of denaturation at 94°C for 60s, annealing at 47°C for 60s, and extension at 72°C for 2 min. The resulted 1.8 kb PCR product (the *GOX* gene), was analyzed by standard agarose gel electrophoresis (Frederick *et al*., 2003).

DNA manipulation was carried out in the yeast-*E. coli* shuttle vector PYES2 containing the galactose de-

hydrogenase promoter (GAL1), the URA3 marker gene and cytochrome- c1 (CYC1) terminator. The recombinant episomal plasmid PYES2-*gox* was obtained by subcloning a 1.8 kb PCR *GOX* gene product, into the *Sac*I restriction site under the control of GAL1 promoter in PYES2 plasmid (Figure 1). Standard methods were used for plasmid DNA isolation, restriction enzyme digestion and ligation (Sambrook *et al*., 2001). Subsequently, a lithium acetate transformation method was used for the transformation of uracil mutated auxotroph yeast (Schiestl and Gietz, 1989).

PCR was done for confirming the existence of the gene of interest (*GOX*) and also to ensure the accuracy of the *GOX* gene cloning and inserted direction, the recombinant plasmid was digested by two restriction enzymes, *Sac*I and *Bam*HI.

Transformation of recombinant plasmid (PYES2-*gox*) into yeast cells

The recombinant plasmid, PYES2-*gox*, was transformed into yeast competent cells as described by Schiestl and Gietz (1989) using polyethylene glycol (PEG) and the heat shock method. A modified method of Hodgkins *et al*. (1993) was used for screening and the selection of GOX producing yeast colonies. Colonies that were previously grown on SD-URA3 medium (Uracil free medium) were considered as positive transformants and the untransformed yeast colonies were considered as negative controls.

Glucose oxidase plate assay

In order to assay GOX, both transformed and untransformed yeast colonies were spotted on YPD plates enriched with galactose (1%) and incubated for 2-3 days at 30°C. The plates were then overlaid with 100 mM molted potassium phosphate buffer, pH 7.0 [containing 1% (w/v) agarose, 10 g glucose/l, 100 mg o-dianisidine dihydrochloriede/l (Sigma) and 15 U/ml of horseradish peroxidase type II (Sigma)]. After setting the overlaid buffer, the cells were further incubated for 1h and observation of a brown halo was assumed as GOX production and activity (Hodgkins *et al*., 1993).

Glucose oxidase spectrophotometric assay

Based on Park *et al.* (2003) method, the transformed yeast cells containing the recombinant plasmids were inoculated in SD-URA3 liquid medium enriched with galactose (1%) for 3 days. After centrifugation, 1.5 ml of the cultured cells was centrifuged at 5000 rpm and the supernatant was used for the extracellular enzyme assay and the remaining cells were resuspended in the enzyme releasing buffer (containing 100 mM phosphate potassium) for intracellular enzyme assay. The intracellular enzyme extraction procedure was done as described by Kamangar and Haddad (2013). Glass beads were added, and total cell disruption was performed by vortexing five times for 1 min. Cell debris was separated by centrifugation and the supernatant containing the intracellular enzyme was removed and stored for further spectrophotometric assay. Briefly, 100 µl of the sample was incubated for 30 min in 0.1 M phosphate potassium buffer (pH 5.5) containing 0.3 ml of D-glucose, 0.1 ml of horseradish peroxidase (600 U/ml), 1.0 ml of 0.31 mM o-dianisidine dihydrochloride. The absorbance was measured at 500 nm. Three samples were used as replication (Malherbe *et al*., 2003).

Time profile of GOX production

The GOX intracellular production was studied by a spectrometric method as described above, during 5 days after culture in liquid medium to find out the best time for the maximum yield of enzyme production. This could be also useful for the determination of the length of lifetime of enzyme stability in industrial applications.

Temperature and pH characteristics of enzyme quantity

The stationary growth phase of transformed yeast solution was centrifuged at 14000 rpm for 2 min and the intracellular enzyme extraction procedure was carried out as described above. To investigate the pH dependence of GOX activity, 200 µl of phosphate potassium buffer with pH values between 4 and 8 was added to the 100 µl of intracellular enzyme extraction separately and the GOX activity was measured spectrophotometicaly at 30°C. Moreover, to investigate the effect of temperature on enzyme activity, 200 µl of phosphate potassium buffer with different temperatures (10, 20, 30, 40, 50, 60°C) was added to the 100 µl of the intracellular enzyme extraction and the GOX activity was measured at pH = 5.5 using a spectrophotometric method.

Effect of different carbon source on enzyme production

To evaluate the effect of different carbon source on the production of the target enzyme, transformed yeast cells were cultured on the YPD liquid medium supplemented with 1% galactose as control (Park *et al*., 2000), 1% glucose, 0.5% sucrose with 0.5% galactose and 1% galactose which then enriched by 1% glucose after

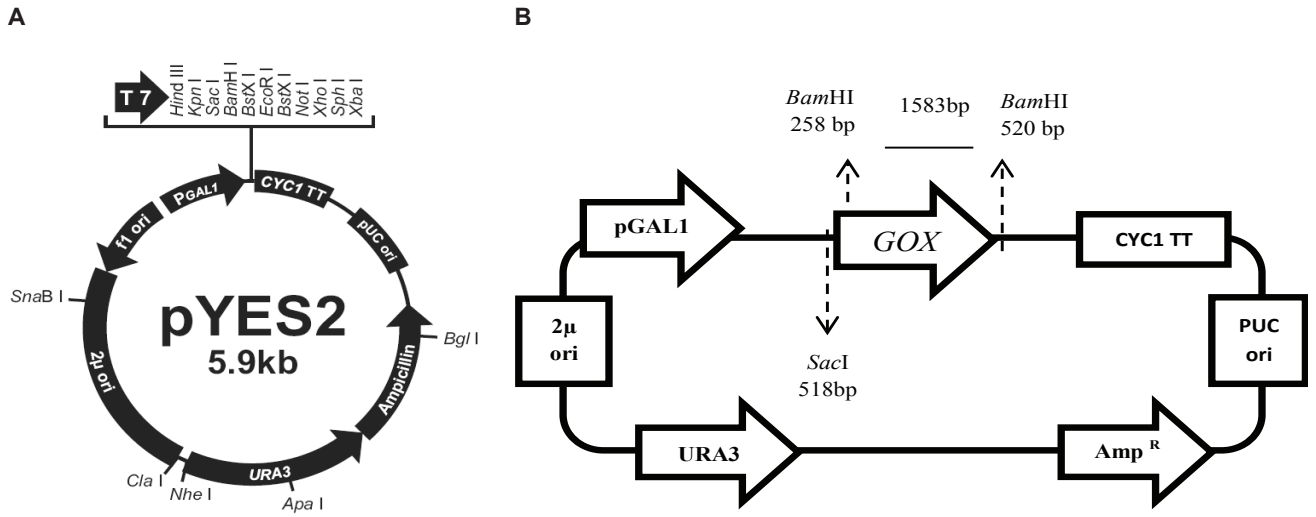

Figure 1. Construction of PYES2–*gox* vector. **A:** The schemic presentation of PYES2 vector (www.lifetechnolgy.com); **B:** Manipulated PYES2-*gox* expression construct. pGAL1: galactose dehydrogenase gene promoter; CYC1 TT: cytochrome-c1 terminator, a transcriptional terminator for efficient termination of mRNA; pUC ori: sequence for maintenance and high copy replication in *E. coli*; URA3: URA3 gene for selection of yeast transformants in uracil deficient medium; 2µ ori: sequence for maintenance and high copy replication in yeast; f1 ori: rescue of single-stranded DNA.

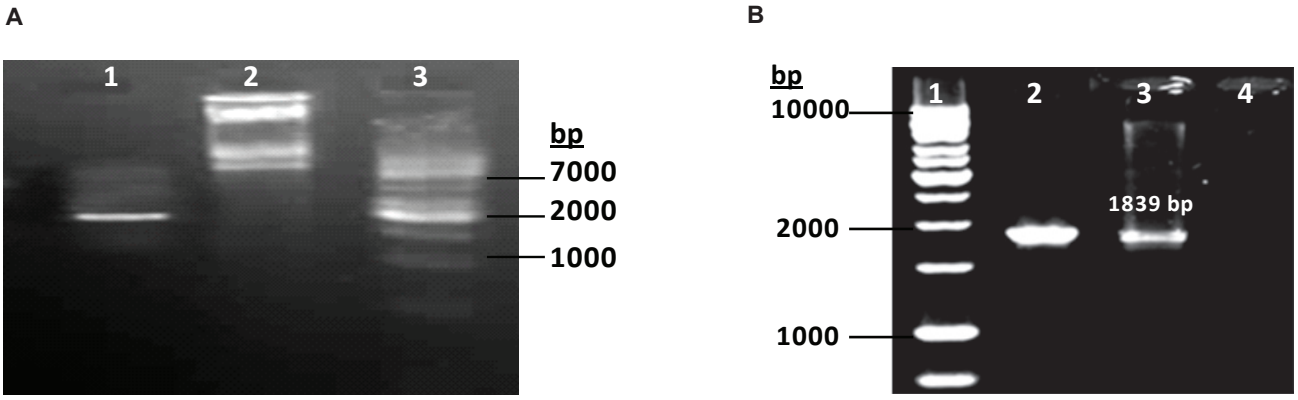

Figure 2. Agarose gel electrophoresis of PCR products of *GOX* **A:** Lane 1) PCR product of *GOX* gene, Lane 2) PCR product of recombinant PYES2-gox using DNA extracted from *E. coli*. Lane 3) 1Kb ladder; **B:** Lane 1) 1Kb ladder DNA, Lane 2, 3) *GOX* PCR products of genomic DNA isolated from two transformed yeast colonies, Lane 4) PCR product of non-transformed yeast colonies.

reaching the stationary phase. Subsequently, following intracellular enzyme extraction, enzyme activity and quantity of the produced enzyme were measured by a spectrometric method (Malherbe *et al*., 2003).

RESULTS AND DISCUSSION

Confirmation of recombinant plasmid construction and yeast transformation

The *P. funiculosum* structural glucose oxidase (*GOX*) gene was successfully placed under the control of the galactose dehydrogenase gene promoter (GAL1) and cytochrome-c1 (CYC1) terminator of an episomal yeast-*E. coli* shuttle expression vector (PYES2) (Figure 1A). Successful integration of the *GOX* gene segment in the expression vector was confirmed by PCR. Electrophoresis results elucidated a 1.8 kb band of *GOX* gene using vector and genomic DNA extracted from both transformed *E. coli* and yeast, respectively (Figure 2), which confirmed the accuracy of the gene cloning

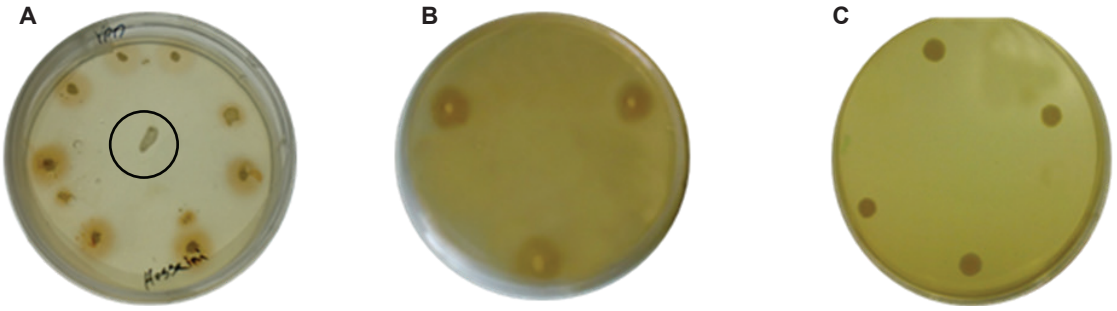

Figure 3. Glucose oxidase plate assay on the transformed yeasts grown on the YPD medium supplemented with **A:** 1% galactose. The transformed yeast colonies secreting active recombinant GOX are surrounded by a brown halo and are considered as positives (colonies around of the plate) and the untransformed yeast host strain (the colony in circle in the middle of the plate) was used as the negative control; **B:** 0.5% sucrose and 0.5% galactose; **C:** 1% glucose.

and precise size of the target sequence. Yeast genomic DNA was extracted from a 2-3 days grown transformed yeast colonies on SD-URA3 medium.

Glucose oxidase plate assay

GOX enzyme metabolizes glucose into gluconic acid and H_2O_2 is formed as a byproduct. The H_2O_2 is then used by the horseradish peroxidase to oxidize o-dianisidine and a color change could be visible in the agar plates. Based on this phenomenon, transformed yeast colonies grown on selective SD-URA3 plates, could be screened for the secreting yeast transformants and also for screening biologically active GOX by selecting the colonies surrounded by a brown halo in the GOX agar plate assay. The result of GOX plate enzyme assay indicated the true activity of GOX from predicted yeast tranformants (Figure 3). Different sizes of browning zone and also darkness of halo among the transformants (Figure 3A), suggested a possible different copy number of episomal plasmids in the transformants (Park *et al*., 2000).

Production of glucose oxidase

The *GOX* gene was placed under the control of GAL1 inducible promoter. Based on result from Park *et al*. (2000), 1% galactose was added to the medium for further comparisons of GOX production. Park *et al.* (2000) could achieve the maximum amount of 32 U/ml using the GAL1 promoter. The *GOX* gene used in this study had a leader peptide that causes secreting of the enzyme into the medium (Esmaiilpour *et al.*, 2014). The GOX activity was measured both intra and extracellularly. The maximum yield of GOX production was achieved after 3 days of cultivation in SD-URA containing 1% galactose, with 45 and 32 U/ml for the intra and extracellular enzyme activity, respectively (Figure 6). A correlation between gene dosage and expression level was observed, although the yield per expression unit is usually reduced at very high copy numbers (Park *et al.*, 2000).

The existence of the leader peptide with 17 amino acids in the *GOX* gene sequence, directed GOX out of *Penicillium* cells. Researches have indicated that signal peptides in eukaryotic organisms show the divergence in amino acid residues with a conserved hydrophobic core region of 6-8 amino acids long. It works well in other organisms as long as they can maintain the conserved core (Walter and Johnson, 1994). The 17 amino acid leader peptide of GOX was shown to direct the secretion of GOX from yeast (Frederick *et al*., 1990b). Thus, it was not necessary to alter the GOX leader peptide in recombinant yeast. The results of this study were consistent with this idea and our recombinant yeast cells were able to produce GOX extracellularly. In a study done by Malherb *et al.* (2005), they used yeast mating pheromone α-factor secretion signal as leader peptide. Most of the GOX activity was measured in the extracellular fraction and only a small fraction remained intracellular (Malherbe *et al*., 2003).

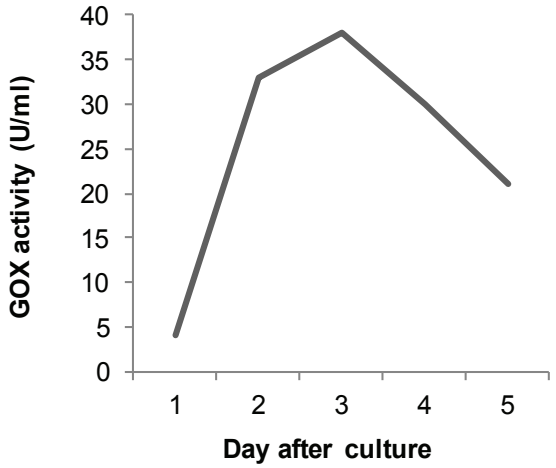

Figure 4. Production of GOX during 5 days of culturing transformed yeasts in SD-URA3 containing 1% galactose.

Time profile of GOX production

Enzymes like other living things have actualy a lifetime. Lifetime or half-life of an enzyme is very important in reactions and industrial applications. Its importance is for understanding the time for the maximal production of enzyme and also the length of enzyme stability. Some enzymes survive only for twenty minutes or so, while others can survive for many weeks before some other enzymes come along and seal their fate. In this study, the highest GOX activity (38 U/ml) was observed after 3 days of culture (Figure 4). Malherbe *et al*. (2003) found the highest enzyme activity 4 hrs after culture hich is disagreeing with our findings. Howevere, the-conditions in which they carried out the experiments are not mentioned. After 3 days, the enzyme production decreased severely. This could indicate that 3 days is the enzyme lifetime. However, due to its importance, many efforts have be made to enhance the stability of glucose oxidase (Nursel *et al*., 2005; Chaiyasit *et al*., 2010).

pH and temperature characteristics

The results indicating the pH and temperature characteristics of the GOX produced by transformed yeast are shown in (Figure 5). The optimal pH was found to be 5.5 (Figure 5A). This result agrees with the information of the enzyme extracted using Protparam and expasy software (Esmaiilpour *et al.,* 2014). The activity was not less than 50% of the maximum in the pH ranging between 5 and 7. However, the optimal temperature was found to be 30°C (Figure 5B).

Effect of different carbon sources

The major effecting factor on the expression of glucose oxidase from the yeast transformant by PYES2-GOX expression vector is the carbon source. Galactose is the most efficient carbon source for the expression of glucose oxidase carrying this vector. In this project, the effect of other carbon sources like glucose and sucrose was examined. Among the different carbon sources used, galactose was found to be the most efficient one. The maximum activity for galactose was observed at 1% concentration (w/v). The presence of

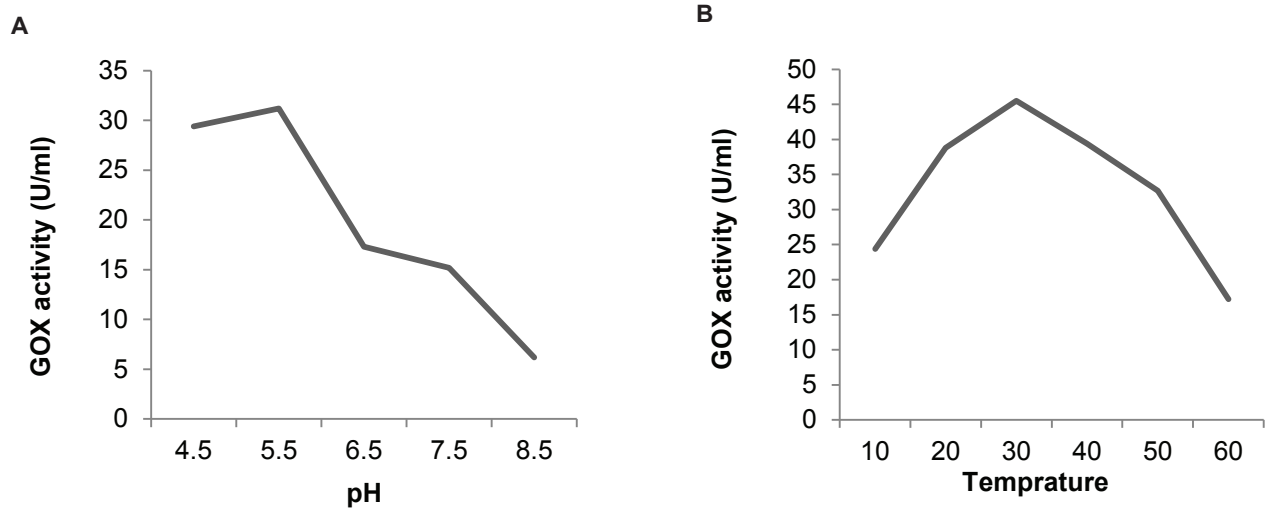

Figure 5. Effect of **(A)** pH and **(B)** temperature on GOX activity.

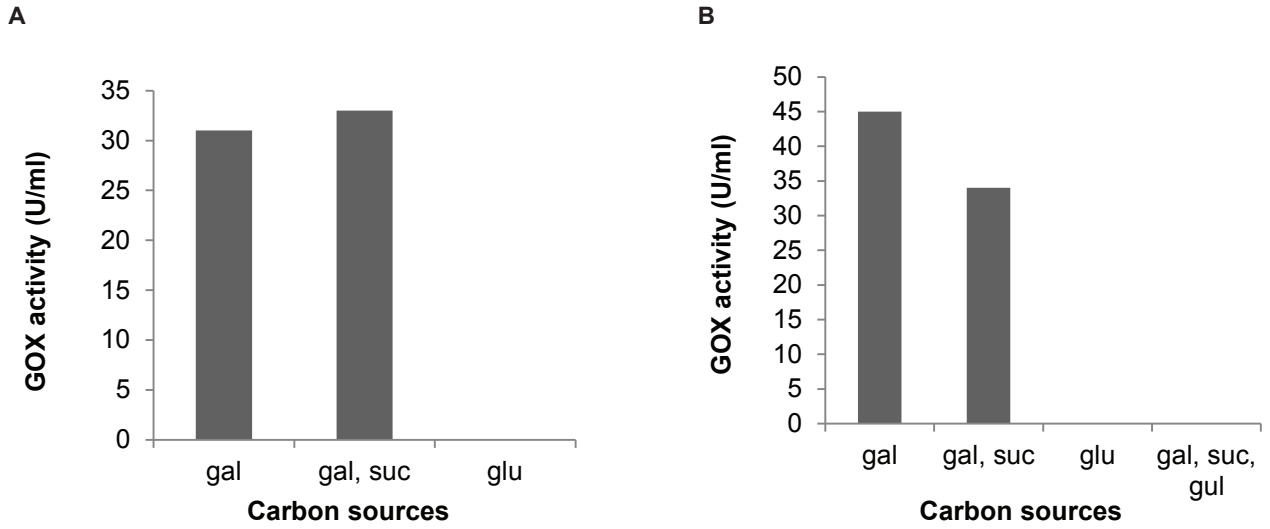

Figure 6. Effect of different carbon sources on the production of GOX in, **A:** extracellular; **B:** intracellular GOX activity the of transformed yeasts (gal: galactose, glu: glucose, suc: sucrose).

glucose in the culture media depressed the production of GOX. In fact, glucose inhibits the GAL1 promoter to function correctly and expresses the gene under its control (Demir and Kurnaz, 2006). Using 0.5% (w/v) of galactose and sucrose in the culture media did not cause a decrease in the GOX production (Figure 6B). Different carbon sources affected cell density and GOX production. As shown in Figure 7, cells cultured in the medium containing glucose had the maximum OD_{600} compared to the other two carbon sources. In the absence of galactose or the presence of glucose with its inhibitory influence, the GAL1 promoter was not active and therefore no GOX was produced (Figure 6). Also according to Figures 6 and 7 it seems that there was an inverse relationship between OD_{600} and GOX production among three carbon sources, such that the medium containing galactose had a lower OD_{600} but higher GOX production. It is due to the fact that glucose is the main carbon source for yeast and in its absence, other carbon sources like galactose, fructose and sucrose are consumed which results in a slow growth rate of yeast cells. In the case of medium supplemented by both galactose and sucrose, it seems that yeast cells used sucrose as the carbon source for their growth and used galactose for launching GAL1 promoter; consequently they showed a better growth rate compared to when they were grown in the medium containing galactose and they produced more GOX.

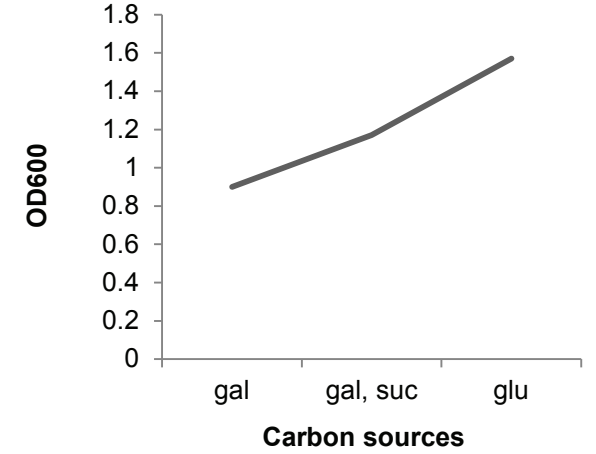

Figure 7. Effect of different carbon sources on the growth of transformed yeasts. gal: galactose, glu: glucose, suc: sucrose.

In this project, we also examined the effect of addition glucose to the medium containing galactose and sucrose. For this purpose, after yeast cells reached the stationary phase (OD600> 1.5), glucose was added and the cultures were further incubated for about 7 hours. Then, GOX enzyme activity was measured. As shown in Figure 6B, no enzyme was produced, the following reasons can be considered 1: the glucose was decomposed by the produced enzyme in the medium and so the amount of GOX decreased, 2: the inhibitory effect

of glucose prevented yeast to produce enzyme, 3: the low stability of enzyme (about 30 min). However, results revealed that the amount of GOX production in intracellular was more than extracellular matrix for both galactose and combination of galactose and sucrose as carbon source. Whittington *et al*. (1990) could obtain 21.1 U/ml of GOX activity on YEP medium (1% w/v yeast extract, 2% w/v peptone) supplemented with 2% w/v sucrose by *Aspergillus niger* glucose oxidase gene inserted in *S. cerevisiae* strain DB1using λ base expression vectors, pSHK1 and pSHKI01 In another study using *A. niger gox* gene but different yeast strain, vector,media and conditions to the previous study, about 110 U/ml GOX was produced (Malherbe *et al.*, 2003). In both studies results differ from those achieved in this study. It seems that apart from the origin of gox gene, host yeast strain, expression construct, medium content, other environmental conditions should be important in this context.

CONCLUSION

Glucose oxidase is an important enzyme that has board industrial applications. Up to now, extracted *gox* gene from *Aspergillu niger* has been used in many basic and applied studies. In this study, the extracted *gox* gene from *Penicillium funiculosum* (native to Iran) was cloned in the PYES2 expression vector and introduced successfully into yeast (*Saccharomyces cerevisiae*) strain CEN.PK 113-5D, an uracil mutated auxotroph strain. Some concepts of the target gene expression were studied. The GOS plate assay revealed a clear color change around the transformed colonies. The results indicated a considerable intracellular and extracellular enzyme activity. The highest enzyme activity was observed between pH 5 and 7 and 30°C. It was revealed that the transformed yeast cells prefered galactose or a combination of galactose and sucrose. However, glucose alone depressed the GOX production. Time profile study showed the maximum enzyme activity after 3 days of transformed yeasts culture. Nevertheless, this study demonstrated a progress towards the development of new microbial strains for producing glucose oxidase as a useful enzyme in many industries. There are still many ambiguities in this context which should be clarified in the future researches.

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