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Expression, characterization and one step purification of heterologous glucose oxidase gene from *Aspergillus niger ATCC 9029* in *Pichia pastoris*

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ABSTRACT

Glucose Oxidase (GOD), is a common flavoprotein from *Aspergillus niger* ATCC 9029 with a broad application in biotechnology, food and medical industries. In this study, GOD gene was cloned into the expression vector, pPIC9 and screened by the alcohol oxidase promoter. The enzyme production increased at 28 °C. GOD activity induced by 1.0% methanol and the highest level of GOD production was the result of shaking rate at 225 rpm. The highest enzyme activity obtained at a pH value ranged from 5 to 7 at 50 °C. The enzyme was stable at a broad pH range and temperature.

1. Introduction

Glucose Oxidase (GOD; β-D-glucose: oxygen 1- oxidoreductase, EC 1.1.3.4) is a type of glycoprotein catalyzer used in oxidation process of D-β glucose molecules into D-glucono-δ-lactone and hydrogen peroxide [1,2]. GOD is a dimeric glycoprotein, with 150–180 kDa molecular weight, which is made up of two FAD molecules that are closely linked to the protein structure [3]. This enzyme is applicable in various industrial areas, such as food and medicine. Medically, this enzyme is commonly used in Glucose Assay Kits and employed in medical glucose sensors for identifying and quantifying blood glucose and urea. It is known as a source of hydrogen peroxide in food protection, as well [4,5]. Currently, the enzyme is primarily produced in industrial-scale cultivation using *Aspergillus niger* and *Penicillium* [6,7]. It should also be noted that, in comparison with other industrially significant enzymes (e.g. cellulase, catalase, and amylase), commercial GOD production, using these two species, is a hard task because of its low yield and production [7]. Furthermore, large-scale GOD application is also troublesome because there are problems concerning production cost, purification difficulty, and low productivity. In order to overcome these obstacles, heterologous expression system has been suggested by various researchers [8]. Until now, GOD production using different expression systems – such as *E-coli, H. polymorpha, Saccharomyces cerevisiae,* and *Pichia pastoris* − have been studied [9–14].

According to researches on *P. pastoris*, this yeast strain creates favorable condition for proper production of recombinant proteins with biological characteristics because of the presence of strictly structured methanol-inducible alcohol oxidase 1 promoter (AOX1) [15].

In the current study, we examined purification and description of rGOD by *P. pastoris* cultivation. Production of recombinant GOD (rGOD), which is explained in this study, has several benefits, including a one-step purification to maximize protein activity at an a favorable temperature (50 °C), which can be useful in sterilization procedures to prevent bacterial contaminants development in food industry

2. Material and method

2.1. Strains, plasmids and reagents

Aspergillums niger (ATCC 9029) is provided from Leibniz Institute **DSMZ-German** Collection of Microorganisms. *P. pastoris* GS115 (AOX1 mut+ his−) expression system, which secretes protein, available in vector pPIC9, is obtained from Invitrogen Corporation. Peroxidase (POD) is also purchased from Sigma.

2.1.1. Cloning of GOD gene

DNA fragment of gene encoding glucose oxidase, amplified by Polymerase Chain Reaction (PCR) using *A. niger* genomic DNA as a template. Primers employed in this study are:

Forward for pPIC9: GOP9-Sig F1:5′-CCC-TCG-AGA-AAA-GAG-

*Abbreviations:*ATCC, American type culture collection

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CTTTTACGACACTTGAGAAGATCAAAAAACAACTAATTATTCGAAGGATCCAAACGATGAGATTTCCTTCAATTT
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CCTACGGCGACATCTTTGGCAGCAGTGTAGACCACGCCTACGAGACCGTGGAGCTCGCTACCAACAATCAAACCG
CGCTGATCCGCTCCGGAAATGGTCTCGGTGGCTCTACTCTAGTGAATGGTGGCACCTGGACTCGCCCCCACAAGG
CACAGGTTGACTCTTGGGAGACTGTCTTTGGAAATGAGGGCTGGAACTGGGACAATGTGGCCGCCTACTCCCTCC
AGGCTGAGCGTGCTCGCGCACCAAATGCCAAACAGATCGCTGCTGGCCACTACTTCAACGCATCCTGCCATGGTG
TTAATGGTACTGTCCATGCCGGACCCCGCGACACCGGCGATGACTATTCTCCCATCGTCAAGGCTCTCATGAGCG
CTGTCGAAGACCGGGGCGTTCCCACCAAGAAAG

Fig. 1. The partial nucleic acid sequence of GOD from *A. niger* (ATCC 9029). The deduced signal sequence is shown in bold and italic. The red letters are the initiation codon from *A. niger* ATCC 9029 and the underlined letters represent the EcoR1 site for cloning in pPIC9 vector.

AG*G-CTG-AAG-CTA-GCA-TTG-AAG −CCA-GCC-TC-3′ and Reverse: GOxP9S1R: 5′- CCG-GAT-CCG-CGG-CCG-CTC-ACT-GCA-TGG-AAG-CAT-AAT-CTT-CC- 3′,

The obtained product is embedded in the vector pMD18-T and yielded a cloned DNA with 1818 bp length.

PCR product is cloned in pPIC9 and the construct pPIC9-GOD is employed for ensuing large-scale production of glucose oxidase.

2.2. Culture media and growth conditions

Yeast Peptone Dextrose (YPD) medium contains the following elements ($g\,l^{-1}$): glucose, 20; yeast extract, 10 peptone, and 20 YPG (1% glycerol, 1% yeast extract, and 2% peptone) medium. Production medium (BMMY) contains ($g\,l^{-1}$): glucose, 20; yeast extract, 10; peptone, 20; potassium phosphate buffer (1 $mol\,l^{-1}$, pH 6.0) and 100 ml yeast nitrogen base, 13.4%. The culture medium is induced with various dose of methanol.

2.3. Optimization of culture medium

More production of glucose oxidase creates changes in parameters including temperature, methanol concentration, aeration rate, induction time, remove proteases. Recombinant strain GS115 is inoculated into 50 ml YPG (1% glycerol, 1% yeast extract, and 2% peptone) medium. Cells are incubated at 28 °C to level with logarithmic growth phase. Thereafter, the cells are centrifuged and put back into suspension of 100 ml BMMY medium in 1000 ml cultivation flask. Methanol is added with three concentrations; 1, 1/5 and 2%. Temperature is induced at 26, 28, and 30 °C. Aeration impact is examined using incubator with various circular speeds (180, 200, 220 rpm).

After adding amino acids or peptide supplements to the medium, proteases is removed. The medium pH is checked to understand where protein decomposition rate is reduced (e.g. pH 3). For about two days, GOD production is studied under optimal expression conditions.

2.4. Enzyme purification

In the first purification phase, floating cells are generated by centrifugation and moved into 50 $mmol\,l^{-1}$ Tris buffer (pH 6.5). In phase two, ion exchange chromatography is carried out, whereby enzyme solution is loaded onto a Q-Sepharose Fast Flow (F.F) column equilibrated with 50 $mmol^{-1}$ Tris buffer (pH 6.5). After washing it with the same buffer, a linear gradient of NaCl (250–500 mM) is applied to extract the column fractions of rGOD, which are pooled then.

2.5. Electrophoresis

Electrophoresis (SDS-PAGE) is performed in a gel composed of 10% polyacrylamide. After that, the resulting bands is stained with Coomassie Brilliant Blue R-250 for upcoming visualization.

2.6. Enzyme assay

After a few modification, activity of rGOD is evaluated based upon TOYOBO GLO201(Osaka,japan) method. 50 µl of enzyme, diluted with pH 6.0,50 $mmol\,l^{-1}$ K-PB buffer, is incubated with 1 ml substrate solution (131 $mmol\,l^{-1}$ D-Glucose in 79 $mmol\,l^{-1}$ MES-NaOH buffer, pH 6.0), at 40 °C for 5 min. The reaction is ended up with boiling water. In the next step, the mixture temperature is reduced to room temperature and 1 ml of chromogenic reagent (0.2 $mmol\,l^{-1}$ 4-aminoantipyrene, 0.3 $mmol\,l^{-1}$ *N*-Ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3-methylaniline, 4 U ml^{-1} peroxidase in 79 $mmol\,l^{-1}$ pH 6.0 MES-NaOH buffer) is added to each tube. By making use of a spectrophotometer, UV-2102, appearance of quinoneimine dye is measured at 555 nm. To explain enzyme unit, it is a formation of one mmol of hydrogen peroxide per minute under the above mentioned conditions.

2.7. The effect of pH and temperature on rGOD activity and stability

Favorable pH for rGOD is specified through monitoring pH range from 2 to 12 which is prepared using a combination of citrate buffer containing phosphate buffer (pH = 2–6), Tris–HCl buffer (pH = 7.5–9), and glycine-NaOH buffer (pH = 10–12). Optimum temperature for rGOD, ranging from 10 to 100 °C, is analyzed by incubating the sample for 5 min.

In order to examine pH stability at different temperature, at first, enzyme is incubated 90 min at 4 °C. Then, enzyme activity and stability is analyzed at temperatures ranging from 10 to 90 °C and 10 to100 °C, respectively.

3. Results

3.1. GOD cloning

The results of sequencing analysis indicated that partial sequence of GOD gene is composed of a signal peptide and an *Eco*R1 site for cloning in pPIC9 vector (Fig. 1).

GOD gene is inserted into the vector, pPIC9, via EcoR1 restriction site and the construct is then presented in *P. pastoris* GS115 genome (Fig. 2).

Fed-batch cultivation is used to obtain the most cell growth; yeast cells are bred in 50 ml of BMGH medium (buffered glycerol-complex

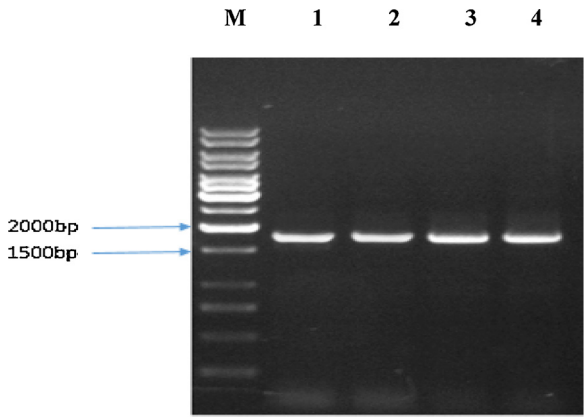

Fig. 2. Polymerase chain reaction (PCR) of genomic DNA from *Aspergillus niger* (ATCC 9029) transformants. Lane M DNA marker (1 kb, Thermo Fisher Scientific), lanes 1 to 4, 1818 bp DNA fragment of glucose oxidase gene from *Aspergillus niger* (ATCC 9029).

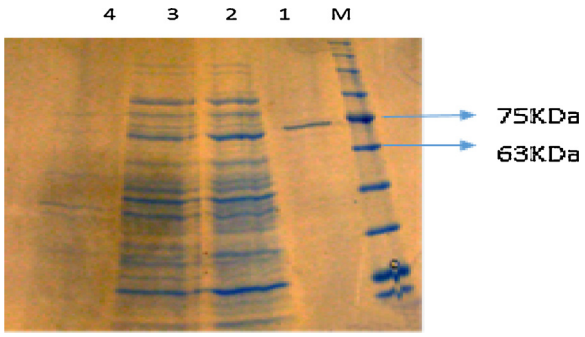

Fig. 3. Polyacrylamide gel electrophoresis (SDS- page) to investigate the expression of recombinant glucose oxidase at temperatures of 26,28,30 °C. Lane M molecular weight markers, lane 1 Ppic9/GOX + sig at 28 °C, lane 2 Ppic9/GOX + sig at 30 °C, lane 3 Ppic9/GOX + sig at 26 °C, lane 4 *Pichia pastoris* yeast lacking the glucose oxidase gene as a negative control.

medium) for two days at 28 °C and shaking at 200 rpm. After that, the culture sample is centrifuged. The obtained cell pellet is collected after the removal of culture supernatant. The cell pellet is then suspended in 50 ml of BMMY medium (Buffered complex methanol medium) in order to induce AOX_1 promoter. In a 96-day induction period, methanol is added every 24 h with the final concentration of 1%. During the expression experiments, yeast containing the expression vector without the glucose oxidase gene is used as a negative control. In the first place, expression of recombinant glucose oxidase enzyme is examined using a qualitative Omodeo method. Then, protein expression and secretion are analyzed using Sodium Dodecyl Sulfate (SDS) Poly-Acrylamide Gel Electrophoresis (PAGE) and dot blot procedures. To do so, the cells' pellets are gathered together after 96 h of induction and treated with 12% trichloro acetic (TCA). The obtained precipitate is dissolved in electrophoresis buffer and boiled for 5 min to be used in SDS-PAGE electrophoresis. Findings regarding SDS-PAGE, with temperature range and methanol concentration of induction element (Figs. 3 and 4) indicated the existence of a protein band of about 69 kDa in yeast which is consisted of recombinant plasmid Ppic9/GOX + sig equivalent to the molecular weight of glucose oxidase protein.

In the previous experiment, the major antibody is rabbit serum immunized with pure recombinant glucose oxidase produced in *Escherichia coli* and the minor one is HRP (horse radish peroxidase- goat anti-rabbit IgG). Also, Dot Blot experiment is carried out and the result is shown in Fig. 5.

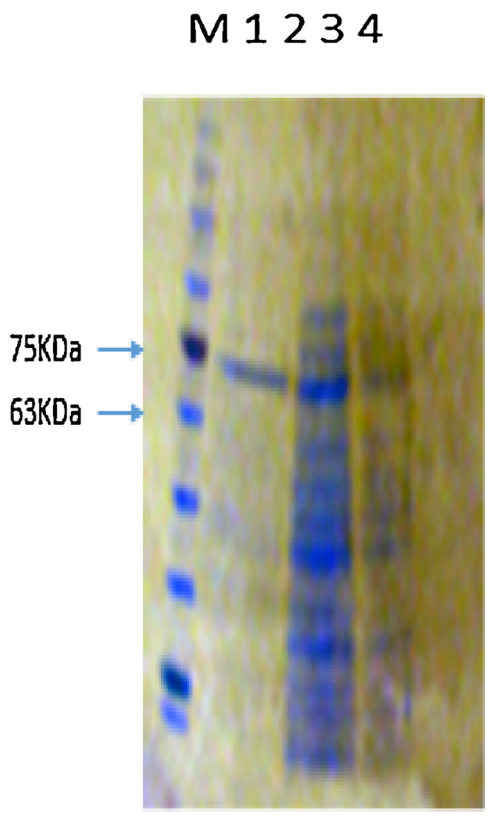

Fig. 4. polyacrylamide gel electrophoresis (SDS-page) to investigate the expression of recombinant glucose oxidase enzyme in Ppic9/GOX + sig at different concentrations of methanol. Lane M molecular weight markers, lane 1Ppic9/GOX + sig With 1% of methanol, lane 2 Ppic9/GOX + sig With 1.5% of methanol, lane 3 Ppic9/GOX + sig With 2% of methanol, lane 4 *Pichia pastoris* yeast lacking the glucose oxidase gene as a negative control.

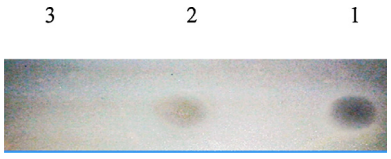

Fig. 5. Dot blot procedure to detect specific glucose oxidase enzyme in yeast containing the recombinant plasmid pPIC9/Gox + sig. Lane 1 the enzyme glucose oxidase as a positive control, lane 2 pPIC9/Gox + sig, lane 3 yeast cells lacking the glucose oxidase gene as a negative control.

3.2. Optimization of expression conditions

Glucose oxidase generation increases after 48 h of cultivation and induction with 1.0% methanol and an agitation rate of 225 rpm, at 28 °C. It is worthwhile noting that methanol accumulation is associated with adverse impacts on cell growth and reduces protein expression level. Thus, access to optimized methanol levels to conduct protein expression is necessary [16]. Lower induction temperature (28 °C) is also preferred since intermolecular disulfide bonds commonly form at higher temperatures and result in increased collection of proteins and misfolded structures that are more sensitive to intracellular proteolytic decomposition [16].

3.3. Purification of the rGOD enzyme

Purification of rGOD protein is a one-step process. SDS-PAGE of the purified enzyme manifested a single band with 69 kDa molecular weight (Fig. 10). So far, researchers applied similar strategies – such as lyophilization and size exclusion chromatography [14] – yet, the one

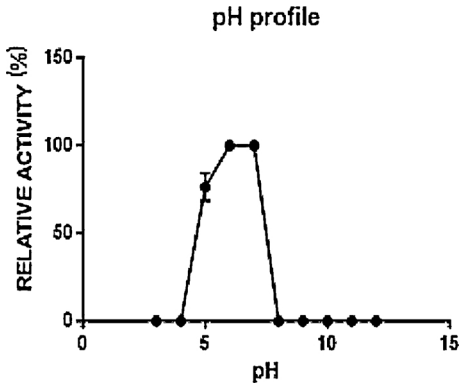

Fig. 6. The activity of the glucose oxidase enzyme determined at a pH range of 2 to 12.

that is used here only has one step; thus, it provides a more plain purification process for protein products which require less protein degradation as well as less production costs.

3.4. Effect of pH and temperature on activity and stability of rGOD

Using D-glucose as substrate, the effect of pH on rGOD activity is measured for different pH levels. For pH levels < 5 and > 7, rGOD becomes inactivated rapidly. For pH levels between 5 and 7, it becomes more active and showes optimum activity at pH = 7. Research findings suggest that this enzyme prefers acidic conditions because more than 50% of its activity is maintained after 90 min incubation under acidic conditions (Fig. 6).

The result of pH impact on stability of glucose oxidase is depicted in Fig. 7.

What is more, temperature impact on enzyme activity is examined between 10 and 100 °C. It seems that activity slowly increases with temperature from 20 to50 °C and then decreases at higher temperatures. As regards, preferred temperature for enzyme activity seems to be 50 °C (Fig. 8).

After that, thermal stability is measured at 10–100 °C. It is observed that 50% of enzyme activity is maintained after 90 min incubation at 20–40 °C (Fig. 9).

Finally, result of this one-step purification process of GOD protein is depicted in Fig. 10.

4. Discussion

Due to the potential of growing at high growth rates, in the presence of methanol and leading to increased production of recombinant products [16], *Pichia pastoris* strain (GS115) is a widely known expression host in biotechnology [17]. However, besides its benefit, it has a major problem, i.e. presence of vacuolar proteases which are regarded as

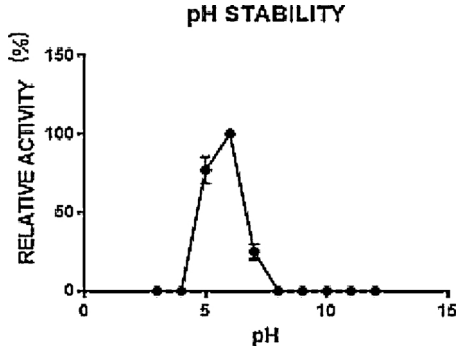

Fig. 7. Effect of pH on stability of the glucose oxidase enzyme.

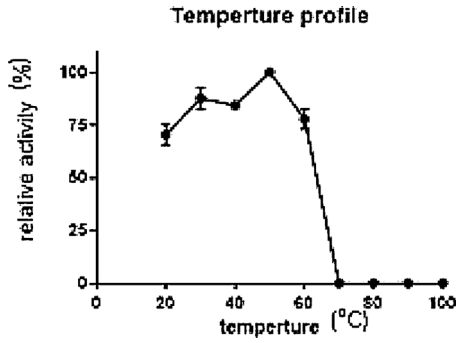

Fig. 8. The activity of the glucose oxidase enzyme determined at a temperature range of 10 to 100°.

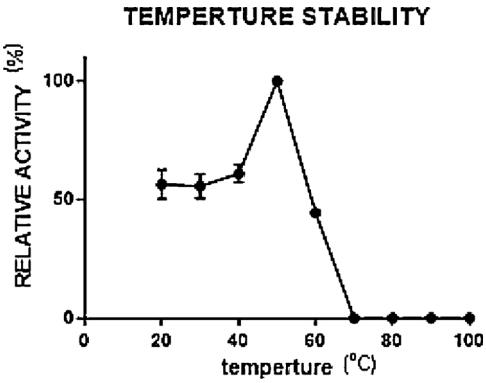

Fig. 9. Effect of temperature on stability of the glucose oxidase enzyme.

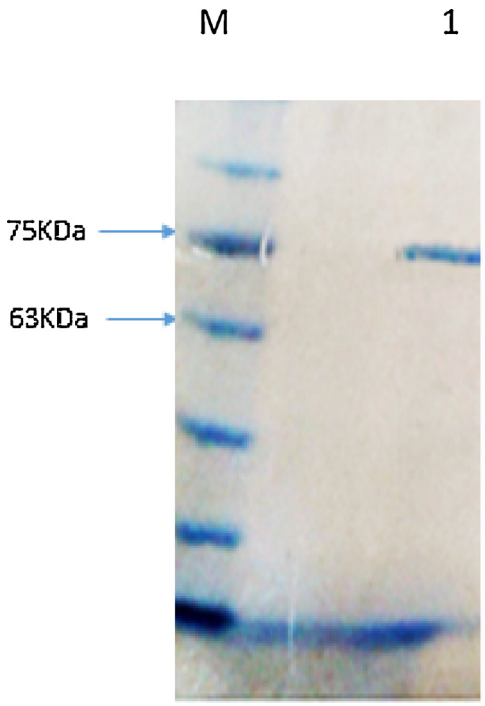

Fig. 10. Polyacrylamide gel electrophoresis(SDS-PAGE) of the purified enzyme. .Lane M moecular weight markers, lane 1 proteins purified by column.

significant factors in protein degradation. As a result, some proteins are unstable in *Pichia pastoris* culture because they are sensitive to the presence of proteases so they rapidly go through degradation. Specifically, these proteases are available in large-scale cultures because of having high cell densities [18]. Yet, when using shake flask cultivation,

increased yield of recombinant enzymes is observed in the current research. Many strategies are proposed to increase GOD production in *P. pastoris*, all of which contained proteolysis reduction through decreased induction time and temperature. According to experimental observations, low temperatures can increase cell membrane stability and reduce cell protease secretion [19]. Employment of skim milk and ammonium phosphate in induction medium of this study preserves the recombinant enzyme from protease degradation [20,19]. During the induction, methanol is used as a source of energy and carbon for cell growth and protein biosynthesis [15]. Therefore, methanol concentration of culture medium is a significant element which should be considered during the production of rGOD enzyme. Concentrations of methanol that are generally used in *P. pastoris* cultures range from 0.5 to 1% [19]. In this study, 1.0% is a highly favorable methanol concentration to maximize the induction of GOD expression.

What is more, high methanol concentrations are associated with adverse impact on cell growth and lower protein expression [20,19].

Added to this, above normal temperatures may result in the formation of intramolecular disulfide bonds and then increased aggregation of proteins which are usually more sensitive to intracellular proteolysis [21].

As for the induction period, more induction time may result in decreased number of living cells as well as increased amount of cellular degradations [22].

Augmented cell concentrations give rise to more production of secreted proteins, but this will also increase accumulation of other cellular material, protein-degrading enzymes, in particular [23].

The current research examined purification, description, and biochemical properties of glucose oxidase from *Aspergilus niger*.

Ethics approval and consent to participate

Not applicable.

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

Consent for publication

Not applicable.

Availability of data and materials

Authors agreed to publish this article.

Competing interests

Authors declares that she has no conflict of interest.

All authors have seen a copy of the paper AND have approved its submission.

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Authors' contribution

JR Supplies and equipment testing coordinator and guidance of Genetics section in the Project, conceived of the study, and participated in its coordination.

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