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# Expression of *Penicillium variabile* P16 glucose oxidase gene in *Pichia pastoris* and characterization of the recombinant enzyme

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**Abstract**

Glucose oxidase (GOX) is a glycoprotein that finds wide application in food industry and clinical analysis. The gene encoding the GOX from *Penicillium variabile* P16 was expressed in *Pichia pastoris* X 33 using the methanol inducible AOX1 promoter. Among 11 transformants resistant toward high zeocin concentrations, six Mut$^+$ strains were screened in shaken flasks and the strain X33 c9, producing 0.33 U ml$^{-1}$ of heterologous GOX after 11 days of fermentation, was selected. Recombinant GOX (ca. 50 U ml$^{-1}$) was produced in a 3-l fermenter under not optimized conditions, recovered and purified in order to characterize and to compare it with the native one. The GOX from *P. pastoris* had a molecular weight of 82 kDa. Comparison of carbohydrate moieties showed a slight over-glycosylation of the GOX from *Pichia* over the native enzyme (17 and 14%, respectively). pH behavior of the recombinant enzyme, in terms of both activity and stability, was similar to that of the native one; on the other hand, a certain difference was observed in optimal temperature for activity and in thermal stability. *P. pastoris* appears to be a good expression system for GOX production.


*Keywords:* Glucose oxidase; Heterologous expression; *Penicillium variabile*; *Pichia pastoris*

## 1. Introduction

Glucose oxidase (GOX) is a glycoprotein that catalyzes the oxidation of β-D-glucose to gluconic acid and hydrogen peroxide.

At present time, the enzyme is commercially produced using selected fungal strains of *Aspergillus niger* and *Penicillium amagasakiense*; its considerable importance depends on the fact that it is already used as analytical reagent in test kits and in biosensors for the determination of D-glucose in clinical chemistry and industrial processes monitoring [1]. Potentially, GOX might be used on a large scale in food technology as oxygen and/or glucose scavenger from foods and beverages and to improve their shelf life [2,3].

The fungal glucose oxidases are homodimers of approximately 150–170 kDa containing two tightly, but non-covalently bound FAD cofactors, and about 11–13% carbohydrate moiety of the high-mannose type [4,5]. Typical problems that are usually encountered during their production are, mainly, either low productivity or concomitant production of other enzymes such as catalase [6]. To overcome these problems, use of genetically modified microorganisms rather than natural sources for the expression of this enzyme has been strongly suggested [7].

In the last 10–15 years, yeasts such as *Hansenula polymorpha* and *Saccharomyces cerevisiae* have been investigated (as promising high-yield production systems) and suggested for heterologous GOX production; in the case of the latter yeast, however, hyperglycosylation may occur leading to serious limitations of usage [8].

The metylotrophic yeast *Pichia pastoris* has proved to be an efficient host for expression of both secreted and intracellular heterologous proteins [8]; success of the *Pichia* system is strictly related to presence of the strong and tightly regulated promoter of alcohol oxidase, enzyme that catalyzes the first step of methanol metabolism [9]. Major advantage of *P. pastoris* expression over bacterial systems is that the yeast has potential to perform many post-translational modifications, typically associated with higher eukaryotes, such as processing of signal sequences, folding, formation of disulfide bridges, certain types of lipid addition, and O- and N-linked glycosylation [2].

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Other key features are high efficiency of secretion, capacity to grow at very high cell densities and, above all, in minimal salt media, feature particularly interesting since it would positively contribute to cost effectiveness of eventual industrial production processes.

In previous research works, a strain (P16) of *Penicillium variabile* was selected for its high levels of GOX activity [10] and for the ability to grow in submerged culture in stirred tank reactors [11] releasing the enzyme extracellularly. Recently and in view of possible genetic improvement, its GOX-encoding gene was cloned, characterized and compared with that of another strain of the same species (NRRL 1048) [12]. In the present paper, the gene was used to transform *P. pastoris* X33, a strain largely used for selection on zeocin and large scale growth studies; the recombinant enzyme was purified, characterized and compared with the native fungal GOX.

## 2. Materials and methods

### 2.1. Bacterial and yeast strains

*Escherichia coli* DH5α and *P. pastoris* X-33 were used for plasmid construction and propagation and for expression of GOX, respectively. Yeast growth media were prepared according to the *Pichia* expression system manual from Invitrogen (Groningen, ND).

### 2.2. Construction of the expression plasmid

Recombinant GOX protein was produced using the *P. pastoris* expression system from Invitrogen (Groningen, ND). The GOX DNA sequence was amplified by PCR as described previously [12] using the oligonucleotides: 5′-CGGAATTCATGGTGTCTGTATTTCTCAG-3′ and 5′-GCTCTAGACTAGGCACTTTTGGCATAGT-3′(MWG Biotech, Eberseberg, Germany).

The primers used for PCR introduced *Eco*R I and *Xba* I restriction sites permitting directional cloning of the amplified DNA in frame with the α-factor leader sequence in the pPICZαA expression vector.

The amplified fragment was first subcloned into pGEM-Teasy cloning vector (Promega, Madison, WI, USA). The recombinant vector was digested with *Eco*R I and *Xba* I to generate a 1800 bp fragment which was subsequently introduced into the pPICZαA expression vector using *Eco*R I and *Xba* I sites. Construct correctness was confirmed by DNA sequencing.

### 2.3. Transformation of P. pastoris and screening of recombinant colonies

Plasmid pPICZαA-GOX was digested with *Pme* I and integrated into pretreated *P. pastoris* X33 by electroporation using a Gene pulser apparatus (Biorad, Hercules, CA) according to Invitrogen instructions.

Transformants of *P. pastoris* containing GOX DNA were selected on the basis of zeocin resistance using YPDS (1% yeast extract, 2% peptone, 2% dextrose and 1 M sorbitol) agar plates with zeocin from 100 to 2000 μg/ml. Zeocin-resistant colonies were replicaplated onto minimal methanol (MM) plates containing 1.43% yeast nitrogen base, 0.5% methanol, 0.4 μg ml$^{-1}$ biotin, and minimal dextrose (MD) plates (same composition as MM but with 2% dextrose instead of methanol) to determine methanol-utilizing phenotypes. After 3–4 days of incubation, the Mut$^+$ phenotypes grew normally on both MM and MD whereas the Mut$^-$ phenotypes grew very slowly on MM plates. Screening of the most efficient GOX-producing transformants under the methanol-inducible AOX1 promotor was carried out by growing the Mut$^+$ transformants in 50 ml of buffered glycerol complex medium (BMGY) in 500 ml flasks at 28 °C and 250 rpm till the cell density reached an $OD_{600}$ value between 2 and 6. Yeast cells from the culture suspension were harvested by centrifugation and resuspended in 50 ml of buffered methanol complex medium (BMMY) in 500 ml flasks to a final $OD_{600}$ value of 1 and cultured as above.

### 2.4. Production of recombinant GOX

Submerged fermentation of transformed *P. pastoris* X33 was performed in a 3-l fermenter (Applikon Dependable Instruments, Schiedam, ND) under the following conditions: temperature, 28 °C, stirrer speed, 750 rpm, air flow 1.0 vol vol$^{-1}$ min$^{-1}$. The inoculum, obtained as above, was added to reach an initial $OD_{600}$ value of 1.0. Cultivation of *P. pastoris* transformant included two phases: a growth phase on glycerol and, then, an induction phase on methanol. Fermentation began with a batch growth phase (24 h) in a basal salt medium containing glycerol (1.0%, w/v), followed by a glycerol fed-batch phase (24 h) in order to maintain the glycerol concentration at 0.5%. During the induction phase, methanol was added continuously to keep its concentration at 0.5%.

Agitation was set to be controlled automatically by the reactor's microprocessor in the range 500–1000 rpm to keep the dissolved oxygen (DO) concentration well over 20% of the saturation level.

### 2.5. Purification of the recombinant GOX

Culture broth was centrifuged at 5000 × *g* for 10 min and filtered. The filtrate was concentrated by ultrafiltration using 10 kDa cut-off membranes (Amicon) and equilibrated in 10 mM potassium phosphate (KP) buffer, pH 6.0. The raw extract was chromatographed on a DEAE-Sepharose Fast Flow (Pharmacia, Uppsala, Sweden) column (1.0 cm × 18 cm) equilibrated with the same KP buffer as above at a flow rate of 1 ml min$^{-1}$. The gradient program was as follows: washing with 30 ml of the equilibrating buffer; linear gradient (25 ml) of 0.05–0.075 M of NaCl in the same buffer; isocratic 0.075 M of NaCl (25 ml); linear gradient (75 ml) of 0.075–0.3 M of NaCl; linear gradient (15 ml) of 0.3–1 M NaCl; washing with 15 ml of NaCl 1 M in KP buffer. Protein was measured via absorbance (*A*) at 280 nm and the collected fractions (1 ml each) were tested for GOX activity.

Fractions containing GOX activity were pooled, concentrated by ultrafiltration using a 10 kDa cut-off membrane and loaded on Superdex 200 column (1.0 cm × 100 cm) equilibrated with 10 mM KP buffer pH 6.0, at flow rate of 1 ml min$^{-1}$.

After each step, protein concentration was determined according to Bradford [13] and the enzyme purity evaluated by SDS/PAGE.

### 2.6. Molecular weight of the recombinant GOX

The molecular weight of the recombinant enzyme was determined by both gel filtration and gel electrophoresis.

Gel filtration was performed using the same Superdex 200 column calibrated with a protein standard mixture containing bovine thyroglobulin (670 kDa), bovine gamma globuline (158 kDa), chicken ovalbumin (44 kDa), horse myglobin (17 kDa) and Vitamin B-12 (1.35 kDa), under the same conditions used for the last step of purification.

The electrophoresis gel was calibrated using a protein standard mixture (Biorad low range) containing phosphorilase b (103 kDa), bovine serum albumin (77 kDa), ovalbumin (50 kDa), carbonic anhydrase (34 kDa), soybean trypsin inhibitor (28.8 kDa) and lysozyme (20.7 kDa). SDS/PAGE was performed as specified below.

### 2.7. Enzyme assay

GOX activity was measured by the spectrophotometric method of Ciucu and Patroescu [14] as modified by Markwell et al. [15], following the enzymatic reduction of benzoquinone to hydroquinone at 290 nm ($\varepsilon = 2.31$ mM$^{-1}$ cm$^{-1}$).

### 2.8. Enzyme characterization

The apparent values of GOX Km for glucose were determined by measuring the initial velocities at pH 5.0 over a range of substrate concentrations (2–500 mM). Apparent values were calculated by non-linear regression analysis utilizing the Mac Curve Fit program.

Effect of temperature on enzyme activity was determined by the standard assay in the temperature range 30–60 °C. Optimal pH was determined by the