

Construction-Expression of *Aspergillus niger* glucose oxidase-transgenic in *Pichia pastoris* and its antimicrobial activity against *Agrobacterium* and *Escherichia*

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Abstract The gene encoding glucose oxidase from *Aspergillus niger* ZM-8 was cloned and transferred to *Pichia pastoris* GS115, a transgenic strain *Pichia-P. pastoris* GS115-His-GOD constructed. The growth rate of *Pichia-P. pastoris* GS115-His-GOD was similar to that of *Pichia pastoris* GS115 under non-induced culture conditions. While under the induction conditions, the growth rate of the GOD-transgenic strain was one-third of that of the wild-type *Pichia-P. pastoris*. The activity of glucose oxidase in the supernatant of the fermentation medium, the supernatant of the cell lysate, and the precipitation of cell lysate was 14.3 U/ml, 18.2 U/ml and 0.48 U/ml, respectively. The specific activity of glucose oxidase was 8.3 U/mg, 6.52 U/mg and 0.73 U/mg, respectively. The concentration of hydrogen peroxide formed by glucose oxidase from supernatant of the fermentation medium, the supernatant of the cell lysate, and the precipitation of cell lysate catalyzing 0.2 M glucose was 14.3 µg/ml, 18.2 µg/ml, 0.48 µg/ml, respectively. The combination of different concentrations of glucose oxidase and glucose could significantly inhibit the growth of *Agrobacterium* and *Escherichia coli* in logarithmic phase. The filter paper containing supernatant of the fermentation medium, supernatant of the cell lysate, and precipitation of cell lysate had no inhibitory effect on *Agrobacterium* and *Escherichia-E. coli*. The minimum inhibitory concentration of hydrogen peroxide on the plate culture of *Agrobacterium* and *Escherichia coli* was 5.6×10^3 µg/ml and 6.0×10^3 µg/ml, respectively.

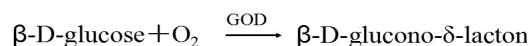
Keywords *Aspergillus niger*; *Pichia pastoris*; Glucose oxidase; Transgenic; Antimicrobial activity

Introduction

Glucose oxidase (β-D-glucose: oxygen oxidoreductase, GOD, EC 1.1.3.4) catalyzes the oxidation of glucose to gluconic acid and hydrogen peroxide in the presence of molecular oxygen according to the following reactions (Dobbenie *et al.*, 1995):

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Spontaneously

GODs are produced by molds such as *Aspergillus niger* and bacteria such as *Penicillium* (Shaw *et al.*, 1986). Its antibiotic activity was considered as notatin (penicillin A at first) (Birkinshaw *et al.*, 1943; Kocholaty 1942), penatin (Kocholaty 1942; Kocholaty 1943), and penicillin B (Harel and Kanner 1985) by early researchers who isolated it from extracts of *Penicillium*. Many documents reported that GOD could inhibit the growth of ~~microbials-microbes~~ in foods or food-prepared media due to production of hydrogen peroxide (Tiina *et al.*, 1989; Yoo *et al.*, 1995). The bacteriostatic effect of hydrogen peroxide is mainly attributed to the peroxidation of membrane lipids (Piard *et al.*, 1991; Roberts *et al.*, 1943). In a laboratory-scale testing, refrigerated shelf life of GOD-treated fish was improved by 67% over untreated fish (Field *et al.*, 1986). Moreover, GOD was able to inhibit growth of *Pseudomonas spp.* which are the main psychrotrophic spoilage microorganisms of chilled poultry (Barnes *et al.*, 1968; Cox *et al.*, 1975)

GODs are also used in many medical applications. Sandholm and his co-workers suggested that all ~~mastitismastitie~~ pathogens were sensitive to the glucose oxidase-lactoperoxidase system (Sandholm *et al.*, 1988). GOD was also used as an antimicrobial agent in oral care (Szynol *et al.*, 2004). The effect of honey on clearing infections in a wide range of wounds, which often did not respond to conventional therapy, was result of the antibacterial activity of hydrogen peroxide that is produced by GOD in honey (Molan *et al.*, 2001; Molan *et al.*, 1992; Bang *et al.*, 2003)

GODs currently used in industry are prepared mainly from the fermentation of *Aspergillus*, *Penicillium*, and transgenic *P. pastoris* ~~pastoris~~ (Fang *et al.*, 2015). Very little information is available whether a glucose oxidase-secreting microbe could inhibit growth of its surrounding living things. In this study, the GOD-encoding gene from *A. niger* ZM-8 was cloned and transferred into *P. pastoris* GS115 of which can excrete GOD to medium by the way of methanol induction. Its directly inhibitory effect on growth of bacteria was investigated and discussed.

Materials and Methods

Plasmid, Primers, and Strains

A 1749.0 bp GOD gene fragment was obtained by amplified from ~~nuclear-genomic~~ DNA of *Aspergillus niger* ZM-8 that was extracted by the method of CTAB (Porebski *et al.*, 1997). Primers for PCR were designed as sTable 1 based on conserved sequences of glucose oxidase gene (NO. JO5242) from GenBank Database, and then cloned into plasmid pUC19 which was linearized by *Sma* I to yield clone vector pUC19-His-GOD. And then inserted in frame with the *S. cerevisiae* α -factor secretion signal sequence under the control of the *AOX-I* promoter in pPIC9K (Invitrogen) resulting in an expression vector pPIC9K-His-GOD. The identified recombinant plasmid pPIC9K-His-GOD was linearized by *Bgl*-II and transformed into *P. pastoris* GS115 cells by electroporation. The electrocompetent *P. pastoris* GS115 cells were prepared using standard

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methods (Manivasakam et al., 1993). The electroporation condition ~~were-was~~ 1.5 kV, 40.0 μ F, and 150.0 Ω using a Gene ~~pulser-Pulser~~ (Eppendorf) according to manufacturer's instruction.

Screening of Clones and Determination of Biomass

The recombinant yeast clones were selected on yeast extract peptone dextrose (YPD) (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose, 2% (w/v) agar) plus 1 M sorbitol (YPDS) plates containing 100.0 μ g/ml G418 (Invitrogen) for 2.0 to 4.0 days. Potential high-level secretion transformants were obtained from the YPDS agar plates containing a higher G418 concentration (300.0 μ g/ml). All these potential high-level secretion clones were confirmed by PCR using genomic DNA as the templates. The colonies with the highest expression level were selected based upon on spectrophotometry, biomass in culture medium was determined by the cell density express as optical absorbance (OD₆₀₀). Pick one single-clone from high copies selected plate containing *P.pastoris* GS115-pPIC9k-His-GOD and *P.pastoris* GS115-pPIC9k that was negative control inoculated in Buffered Glycerol-complex Medium (BMGY) (1%, w/v) yeast extract, 2% (w/v) peptone, 100 mM Potassium Phosphate pH 6.0, 1.34% (w/v) YNB, 4×10^{-5} D-Biotin, 1% (w/v) glycerol) 30.0 °C cultured until OD₆₀₀=0.60, then 1% inoculated into Buffered Methanol-complex Medium (BMMY) (1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM Potassium Phosphate pH 6.0, 1.34% (w/v) YNB, 4×10^{-5} D-biotin, 0.5% (w/v) glycerol) 30.0 °C induced 51.0 hr and OD₆₀₀ were measured every 3.0 hours.

Expression of GOD in Transgenic *P. pastoris* GS115

100 ml inoculum cultures were prepared by cultivating producing *Pichia* strains in BMGY at 30.0 °C for ~24.0 h in 1 L shake flasks until the desired cell density was reached. After ~~an~~-initial glycerol as a carbon source phase, biomass was generated. Finally, to induce AOX I - depend protein expression, the methanol fed phase started with methanol feed rate of 0.5 ml/12.0 h. cell-free supernatant, the supernatant of cell lysate, and the precipitate of cell lysate from pellet which was disrupted by ultrasonic were collected and filter sterilized, the ultrasonic condition was 15.0 s, 25.0 s, 380.0 w, 99 times, stored at 4 °C (Cereghino & Cregg 2000).

Analysis of Glucose Oxidase Activity

Pichia pastoris GS115-His-GOD-01 and *Pichia pastoris*-pPIC9k ~~was-were~~ fed by 0.5 % methanol per 12 h, 30 °C introduction for 51 h. Activities of glucose oxidase from cell-free liquid, cell lysate supernatant and precipitation were determined by spectrophotometric that absorbance was measured at a wavelength of 615.0 nm of which in the condition of pH 5.2 (0.20 M Acetic acid-Sodium acetate) and heating for 13 min, hydrogen peroxide that ~~product~~ed by GOD catalyzed glucose (0.20 M) be able to discolor Indigo Carmine (1.0×10^{-3} M) and reaction rate in a certain range is proportional to the concentration of hydrogen peroxide (Gemba et al., 1971). Glucose oxidase activity was defined as follows: at 37.0 °C, glucose as substrate, within 1min catalytic reaction 1 μ g hydrogen peroxide (H₂O₂) as the amount of enzyme required 1U. ~~Formula-The formula~~ for enzyme activity as follows:

$$X_0 = [(A - A_0) \times K + C_0] \times 25 \times 10^{-3} \times 10^3 \times (4/1) \times (1/2) / 10$$

A: Absorbance value of trichloroacetic instead of glucose as the control; A₀: Absorbance value of the sample solution; K: Slope of the standard curve; C₀: Intercept of the standard curve; 25: The reaction solution was diluted 25-fold; 10⁻³: Milliliters converted to liters; 10³: Milligrams converted to micrograms; 4/1: Draw 1 ml for spectrophotometric from 4 ml reaction mixture; 1/2: 2.0 ml of enzyme dilution used for the determination; 10: Reaction time, min.

The specific activity of GOD

Protein concentrations of cell-free liquid, cell lysate supernatant and precipitation from *P. pastoris* GS115-His-GOD-01 and *P. pastoris* GS115-pPIC9K were determined by the method of Bradford (Hammond *et al.*, 1988), measured absorbances at 615nm wavelength and specific activity of GOD was the value of activity divided by the value of protein concentrations.

Antibacterial effects of Glucose and Glucose Oxidase System on Growth of *Agrobacterium* and *Escherichia coli* in Liquid Medium

Glucose oxidase and glucose were used in three dilution-set combinations. The concentrations for added glucose were: 1.0, 2.5, 5.0 mg/ml. The GOD was from fermentation supernatant of transgenic *P. pastoris* GS115-His-GOD-01 that was induced by methanol and fermentation supernatant from *P. pastoris* GS115-pPIC9K as control and concentrations for GOD were 1.0, 5.0, 10.0 U/mL. The GOD and glucose solutions were added in the medium of YEP or LB and arranged in a Latin-square design to study the effects of substrates and enzyme on growth of *Agrobacterium* LBA4404 and *Escherichia coli* DH5α by measuring optical density in 600 nm.

GOD Antibacterial Activity to *Agrobacterium* and *Escherichia coli* on Agar Plates

Antibacterial activity of Glucose oxidase that produced by *P. pastoris* GS115-His-GOD-01 to *A. tumefaciens* LBA4404 and *E. coli* DH5α (Stored in Lamzhou University of Technology, Dr. Jianzhong Ma laboratory) were cultured until OD₆₀₀=1.0, plated 200 μL on YPE (1% (w/v) yeast extract, 1% (w/v) peptone, 0.5% (w/v) NaCl, 1.5% (w/v) agar) or LB (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) NaCl, 1.5% (w/v) agar), after methanol introduced for 51 h, cell-free liquid, pellet ultrasonic disruption supernatant, and precipitation that resuspended in ice bath were collected and immersed on sterile filter paper and directly placed to surface of the 0.20 M glucose plates that were plated by *A. tumefaciens* LBA4404 or *E. coli* DH5α cultured until OD₆₀₀=1.0 and the antibacterial effect was observed.

Antibacterial Activity of Hydrogen Peroxide Solution to *Agrobacterium* and *Escherichia coli* on Agar Plates

To detect the inhibitory effect and the lowest hydrogen peroxide concentration be able to inhibit the growth of bacteria. *A. tumefaciens* LBA4404 shaking cultured at 28 °C until OD₆₀₀=1.0, plated 200 μL on YPE medium and then placed the filter paper which-containing different concentrations of hydrogen peroxide solution, 28 °C stationary culture for 14 hr and the inhibitory effect were observed. *E. coli* DH5α shaking cultured at 37°C until OD₆₀₀=1.5, plated 200 μL on LB medium and then placed the filter paper which containing 10 μl different concentrations of

hydrogen peroxide solution, 37 °C stationary culture for 14 hr and the inhibitory effect were observed. *Agrobacterium* and *Escherichia coli*.

Results

Vector construction and Screening of transgenic *P. pastoris* Clones

Pichia pastoris strain GS115 was transformed using linearized pPIC9K-His-GOD as described in materials and methods to yield *P. pastoris* GS115-His-GOD (sFig. 1). Twelve clones were obtained and confirmed by PCR-testing for the gene integration. These clones were then screened on YPDS plates with different concentrations of Geneticin (G418), i.e. 100 mM, 200 mM, and 300 mM, respectively. A positive transgenic clone, designated as *P. pastoris* GS115-His-GOD 01, can be grown on the YPDS plate with a high Geneticin concentration and was chosen for subsequent experiments.

Expression of the GOD Affecting the Growth of the GOD-transgenic Strain

Since H₂O₂, one of the products by GOD, injures living cells, growths of the GOD-transgenic strain, *P. pastoris* GS115-His-GOD 01, were firstly determined if they were inhibited by the transgenic GOD. Compared to *P. pastoris* GS115-pPIC9K, the growth of *P. pastoris* GS115-His-GOD 01 was slightly decreased during most time of the 51-hour incubation if the GOD was not induced (Fig. 1a). Its optical density at 600 nm was 0.95-fold of that of *P. pastoris* GS115-pPIC9K at the time point of 51.0 h. However, the growth of *P. pastoris* GS115-His-GOD 01 was significantly lowered if the GOD was induced by methanol (Fig. 1b). During the growth of 51.0 h, the optical densities of *P. pastoris* GS115-His-GOD 01 were 0.54-fold of that of *P. pastoris* GS115-pPIC9K at 18.0h, 0.43-fold at 36 h, and 0.37-fold at 51.0 h, respectively. The inhibited growth of the GOD-transgenic *P. pastoris* could be attributed to the expression of the foreign GOD and, hereafter, accumulation of H₂O₂.

Activities of the Glucose Oxidase

After 51 hour-induced incubation, the cultures were processed into three parts of which were the cell-free supernatant, the supernatant and the precipitation of the cell lysates. The activities of the GOD preparations from *P. pastoris* GS115-His-GOD 01 were 14.27 U/ml in the cell-free supernatant, 18.2 U/ml in the supernatant of the cell lysate, and 0.48 U/ml in the precipitation (Fig.2a). As a control, the activities of the three GOD preparations from *P. pastoris* GS115-pPIC9K were 3.22 U/ml, 1.76 U/ml and 0.41 U/ml, respectively (Fig. 2a). The specific activities of the three GOD preparations from *P. pastoris* GS115-His-GOD 01 were 8.30 U/mg in the cell-free supernatant, 6.52 U/mg in the supernatant of the cell lysate, and 0.73 U/mg in the precipitation, respectively (Fig. 2b). The specific activities of the three preparations from *P. pastoris* GS115-pPIC9K were 0.859 U/mg, 1.483 U/mg, and 0.529 U/mg, respectively (Fig. 2b). According to the specific activities, the cell-free supernatant of *P. pastoris* GS115-His-GOD 01 had the highest value, but the supernatant of the cell lysate of *P. pastoris* GS115-pPIC9K gave the highest specific activity. These results suggested that the native GOD of *P. pastoris* GS115 was mainly an intracellular enzyme. In the GOD-transgenic *P. pastoris* GS115, the enzyme was mainly

secreted. This is in accordance with that the recombinant GOD was directed to an extra-cellular fraction by a signal peptide, α -mating factor.

~~Concentration~~The concentration of Hydrogen Peroxide from GOD Catalyzed Glucose

The concentration of hydrogen peroxide produced by GOD from *P. pastoris* GS115-His-GOD-01 catalytic glucose in cell-free supernatant, the supernatant of cell lysate, and the precipitate of cell lysate was 14.3 $\mu\text{g/ml}$, 18.2 $\mu\text{g/ml}$ and 0.48 $\mu\text{g/ml}$, respectively as shown in Fig. 3(a, b).

Inhibition of the GOD preparations on the Growth of *A. tumefaciens* LBA4404 and *E. coli* in Liquid Medium

The combination of 1.0, 2.5 or 5 mg/ml glucose and 1.0, 5.0 or 10 U/ml glucose oxidase were added to the medium of *A. tumefaciens* LBA4404 and *E. coli* DH5a. The GOD was contained in fermentation supernatant of *P. pastoris* GS115-His-GOD 01 and in Fig. 4a showed a marked inhibition to ~~the~~ growth of *A. tumefaciens* LBA4404 12 hours later compared with the control that was added with equal volume of *P. pastoris* GS115-pPIC9K fermentation supernatant, and the impact increased with substrate concentration. Conclusions were drawn from Fig. 4b, it showed these combinations did not completely inhibit growth of *E. coli* DH5a but influenced the time at which growth was initiated. Delay of growth initiation was greatest with the enzyme concentration, 5.0 U/mL, and the impact increased also with substrate concentration.

Antibacterial effects of Glucose and Glucose Oxidase on Growth of *A. tumefaciens* and *E. coli* on Agar Plates

Analysis of ~~the~~ antibacterial activity of hydrogen peroxide (H_2O_2) produced by GOD catalyzed substrates glucose. *A. tumefaciens* LBA4404 (Fig. 5a) and *E. coli* DH5a (Fig. 5b) were plated on YEP or LB which were contained 0.2 M glucose. Filter papers were soaked by cell-free supernatant, the supernatant of cell lysate, and the precipitate of cell lysate from *P. pastoris* GS115-His-GOD 01, cell-free supernatant of *P. pastoris* GS115-pPIC9K as the negative control. ~~It showed that the H_2O_2 from GOD catalyzed substrates glucose were completely effected the growth of neither *A. tumefaciens* LBA4404 nor *E. coli* DH5a.~~

Antibacterial Activity of Hydrogen Peroxide Solution to *A. tumefaciens* LBA4404 and *E. coli* DH5a

To detect the minimum concentration of hydrogen peroxide solution inhibit the growth of *A. tumefaciens* LBA4404 and *E. coli* DH5a, the sterile filter papers were soaked with ~~a~~ volume of 10 μl hydrogen peroxide that ~~were-was~~ diluted to different concentrations. Different concentrations of hydrogen peroxide solution effect on *A. tumefaciens* LBA4404 were ~~showed-shown~~ in Fig. 6a and the diameters of inhibition zone were ~~showed-shown~~ in Fig. 6c, it showed that the minimum concentration of hydrogen peroxide solution inhibits the growth of *A. tumefaciens* LBA4404 was $5.6 \times 10^3 \mu\text{g/ml}$; Different concentrations of hydrogen peroxide solution effect on *E. coli* DH5a were ~~showed-shown~~ in Fig. 6b and the diameters of inhibition zone were ~~showed-shown~~ in Fig. 6d, it showed that the minimum concentration of hydrogen peroxide solution inhibits the growth of *A. tumefaciens* LBA4404 was $6.0 \times 10^3 \mu\text{g/ml}$.

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Discussion

Glucose oxidase acts as a bacteriostatic agent by catalyzing hydrogen peroxide production via glucose oxidation (Wong *et al.*, 2008). Compared with glucose oxidase as an antibacterial agent applied in food preservation, direct uses of the GOD-transgenic strains or their fermented supernatants are easily and widely available, and inexpensive. However, little information is available whether a glucose oxidase-secreting microbe could inhibit the growth of its surrounding living things. In this paper, the GOD-encoding gene from *A. niger* ZM-8 was cloned and transferred into *P. pastoris* GS115 to yield a transgenic strain, ~~ef~~ which can excrete GOD to medium by the way of methanol induction. Although the growth of *P. pastoris* GS115-His-GOD was found to be seriously inhibited during the period of methanol induction, its fermented supernatants containing the GOD activity can really reduce the growth of *E. coli* and *A. tumefaciens* in liquid culture (Fig. 5). But, in contrast, the GOD-soaked filter papers didn't exhibit any inhibition to the growth of *A. tumefaciens* and *E. coli* on the solid medium (Fig. 6). At present, it was not sure that it ~~was~~ resulted from no enough oxygen or no enough GOD. As shown in Fig. 7, hydrogen peroxide can inhibit growth of *A. tumefaciens* and *E. coli* on solid medium, but, with the concentrations of at least 5.6×10^3 $\mu\text{g/ml}$ and 6.0×10^3 $\mu\text{g/ml}$, respectively. To reach the concentration of hydrogen peroxide, the activity of the GOD produced from the transgenic strain should be increased at least 300-fold.

According to our results, the GOD-transgenic *P. pastoris* has to produce more enzyme molecules or higher active enzymes in order to inhibit microbes. Recently, Gu et al. reported that a yield of GOD reached 21.81 g/L, with an activity of 1972.9 U/mL, in *P. pastoris* S17 of which is a genetically modified strain by manipulating genes involved in protein folding machinery and abnormal folding stress responses (Gu *et al.*, 2015). Kovačević *et al.* (2014) cloned several mutated glucose oxidase genes from *A. niger* M12 and expressed them in *P. pastoris* KM71H. The highest activity of the GOD came up to 17.5 U/mL of fermentation media. To achieve directly antibacterial applications by GOD-transgenic *P. pastoris*, there will be more studies to be done in enzyme activity improvement and oxygen-offering system.

Compliance with ethical standards

Conflict of Interest

No conflict of interest declared.

Acknowledgement

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