

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

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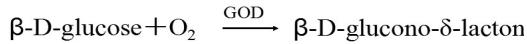
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12  
13 **Abstract** The gene encoding glucose oxidase from *Aspergillus niger* ZM-8 was cloned and  
14 transferred to *Pichia pastoris* GS115, a transgenic strain *Pichia-P. pastoris* GS115-His-GOD  
15 constructed. The growth rate of *Pichia-P. pastoris* GS115-His-GOD was similar to that of *Pichia*  
16 *pastoris* GS115 under non-induced culture conditions. While under Under the induction conditions,  
17 the growth rate of the GOD-transgenic strain was one-third of that of the wild-type *Pichia-P.*  
18 *pastoris*. The activity of glucose oxidase in the supernatant of the fermentation medium, the  
19 supernatant of the cell lysate, and the precipitation of cell lysate was 14.3 U/ml, 18.2 U/ml and  
20 0.48 U/ml, respectively. The specific activity of glucose oxidase was 8.3 U/mg, 6.52 U/mg and  
21 0.73 U/mg, respectively. The concentration of hydrogen peroxide formed by glucose oxidase from  
22 supernatant of the fermentation medium, the supernatant of the cell lysate, and the precipitation of  
23 cell lysate catalyzing 0.2 M glucose was 14.3 µg/ml, 18.2 µg/ml, 0.48 µg/ml, respectively. The  
24 combination of different concentrations of glucose oxidase and glucose could significantly inhibit  
25 the growth of *Agrobacterium* and *Escherichia coli* in logarithmic phase. The filter paper  
26 containing supernatant of the fermentation medium, supernatant of the cell lysate, and  
27 precipitation of cell lysate had no inhibitory effect on *Agrobacterium* and *Escherichia-E. coli*. The  
28 minimum inhibitory concentration of hydrogen peroxide on the plate culture of *Agrobacterium*  
29 and *Escherichia coli* was  $5.6 \times 10^3$  µg/ml and  $6.0 \times 10^3$  µg/ml, respectively.

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

32  
33 **Introduction**

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



Spontaneously

1  
2 GODs are produced by molds such as *Aspergillus niger* and *bacteria* such as *Penicillium* (Shaw *et*  
3 *al.*, 1986). Its antibiotic activity was considered as notatin (penicillin A *at first*) (Birkinshaw *et al.*,  
4 1943; Kocholaty 1942), penatin (Kocholaty 1942; Kocholaty 1943), and penicillin B (Harel and  
5 Kanner 1985) by early researchers who isolated it from extracts of *Penicillium*. Many documents  
6 reported that GOD could inhibit the growth of microbials-microbes in foods or food-prepared  
7 media due to production of hydrogen peroxide (Tiina *et al.*, 1989; Yoo *et al.*, 1995). The  
8 bacteriostatic effect of hydrogen peroxide is mainly attributed to the peroxidation of membrane  
9 lipids (Piard *et al.*, 1991; Roberts *et al.*, 1943). In  $\alpha$ -laboratory-scale testing, refrigerated shelf life  
10 of GOD-treated fish was improved by 67% over untreated fish (Field *et al.*, 1986). Moreover,  
11 GOD was able to inhibit growth of *Pseudomonas* spp. which are the main psychrotrophic spoilage  
12 microorganisms of chilled poultry (Barnes *et al.*, 1968; Cox *et al.*, 1975)

Commented [F1]: *Penicillium* like *Aspergillus* is a mold.

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13 GODs are also used in many medical applications. Sandholm and his co-workers suggested that  
14 all mastitismastitis pathogens were sensitive to the glucose oxidase-lactoperoxidase system  
15 (Sandholm *et al.*, 1988). GOD was also used as an antimicrobial agent in oral care (Szynol *et al.*,  
16 2004). The effect of honey on clearing infections in a wide range of wounds, which often did not  
17 respond to conventional therapy, was result of the antibacterial activity of hydrogen peroxide that is  
18 produced by GOD in honey (Molan *et al.*, 2001; Molan *et al.*, 1992; Bang *et al.*, 2003)

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

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## Materials and Methods

### Plasmid, Primers, and Strains

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

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1 methods (Manivasakam et al., 1993). The electroporation condition ~~were-was~~ 1.5 kV, 40.0  $\mu$ F, and  
2 150.0  $\Omega$  using a Gene ~~pulser-Pulser~~ (Eppendorf) according to manufacturer's instruction.

3 **Screening of Clones and Determination of Biomass**

4 The recombinant yeast clones were selected on yeast extract peptone dextrose (YPD) (1% (w/v)  
5 yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose, 2% (w/v) agar) plus 1 M sorbitol (YPDS)  
6 plates containing 100.0  $\mu$ g/ml G418 (Invitrogen) for 2.0 to 4.0 days. Potential high-level secretion  
7 transformants were obtained from the YPDS agar plates containing a higher G418 concentration  
8 (300.0  $\mu$ g/ml). All these potential high-level secretion clones were confirmed by PCR using  
9 genomic DNA as the templates. The colonies with the highest expression level were selected  
10 based upon on spectrophotometry, biomass in culture medium was determined by the cell density  
11 express as optical absorbance (OD<sub>600</sub>). Pick one single-clony from high copies selected plate  
12 containing *P.pastoris* GS115-pPIC9k-His-GOD and *P.pastoris* GS115-pPIC9k that was negative  
13 control inoculated in Buffered Glycerol-complex Medium (BMGY) (1%, w/v) yeast extract, 2%  
14 (w/v) peptone, 100 mM Potassium Phosphate pH 6.0, 1.34% (w/v) YNB, 4 $\times$ 10<sup>-5</sup> D-Biotin, 1% (w/v)  
15 glycerol 30.0 °C cultured until OD<sub>600</sub>=0.60, then 1% inoculated into Buffered Methanol-complex  
16 Medium (BMMY) (1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM Potassium Phosphate pH  
17 6.0, 1.34% (w/v) YNB, 4 $\times$ 10<sup>-5</sup> D-biotin, 0.5% (w/v) glycerol ) 30.0 °C induced 51.0 hr and OD<sub>600</sub>  
18 were measured every 3.0 hours.

19 **Expression of GOD in Transgenic *P.pastoris* GS115**

20 100 ml inoculum cultures were prepared by cultivating producing *Pichia* strains in BMGY at  
21 30.0 °C for ~24.0 h in 1 L shake flasks until the desired cell density was reached. After ~~an~~-initial  
22 glycerol as ~~a~~ carbon source phase, biomass was generated. Finally, to induce AOX I - depend  
23 protein expression, the methanol fed phase started with methanol feed rate of 0.5 ml/12.0 h.  
24 cell-free supernatant, the supernatant of cell lysate, and the precipitate of cell lysate from pellet  
25 which was disrupted by ultrasonic were collected and filter sterilized, the ultrasonic condition was  
26 15.0 s, 25.0 s, 380.0 w, 99 times, stored at 4 °C (Cereghino & Cregg 2000).

27 **Analysis of Glucose Oxidase Activity**

28 *Pichia pastoris* GS115-His-GOD-01 and *Pichia pastoris*-pPIC9k ~~was-were~~ fed by 0.5 %  
29 methanol per 12 h, 30 °C introduction for 51 h. Activities of glucose oxidase from cell-free liquid,  
30 cell lysate supernatant and precipitation were determined by spectrophotometric that absorbance  
31 was measured at a wavelength of 615.0 nm of which in the condition of pH 5.2 (0.20 M Acetic  
32 acid-Sodium acetate) and heating for 13 min, hydrogen peroxide that producted by GOD catalyzed  
33 glucose (0.20 M) be able to discolor Indigo Carmine (1.0 $\times$ 10<sup>-3</sup> M) and reaction rate in a certain  
34 range is proportional to the concentration of hydrogen peroxide (Gemba et al., 1971). Glucose  
35 oxidase activity was defined as follows: at 37.0 °C, glucose as substrate, within 1min catalytic  
36 reaction 1 $\mu$ g hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) as the amount of enzyme required 1U. ~~Formula-The~~  
37 ~~formula~~ for enzyme activity as follows:

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

2 A: Absorbance value of trichloroacetic instead of glucose as the control; A<sub>0</sub>: Absorbance value of  
3 the sample solution; K: Slope of the standard curve; C<sub>0</sub>: Intercept of the standard curve; 25: The  
4 reaction solution was diluted 25-fold; 10<sup>3</sup>: Milliliters converted to liters; 10<sup>3</sup>: Milligrams  
5 converted to micrograms; 4/1: Draw 1 ml for spectrophotometric from 4 ml reaction mixture; 1/2:  
6 2.0 ml of enzyme dilution used for the determination; 10: Reaction time, min.

#### 7 **The specific activity of GOD**

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

#### 12 **Antibacterial effects of Glucose and Glucose Oxidase System on Growth of Agrobacterium 13 and Escherichia coli in Liquid Medium**

14 Glucose oxidase and glucose were used in three dilution-set combinations. The concentrations  
15 for added glucose were: 1.0, 2.5, 5.0 mg/ml. The GOD was from fermentation supernatant of  
16 transgenic *P. pastoris* GS115-His-GOD-01 that was induced by methanol and fermentation  
17 supernatant from *P. pastoris* GS115-pPIC9K as control and concentrations for GOD were 1.0, 5.0,  
18 10.0 U/mL. The GOD and glucose solutions were added in the medium of YEP or LB and  
19 arranged in a Latin-square design to study the effects of substrates and enzyme on growth of  
20 *Agrobacterium* LBA4404 and *Escherichia coli* DH5 $\alpha$  by measuring optical density in 600 nm.

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

22 Antibacterial activity of Glucose oxidase that produced by *P. pastoris* GS115-His-GOD-01 to *A.*  
23 *tumefaciens* LBA4404 and *E. coli* DH5 $\alpha$  (Stored in Lamzhou University of Technology, Dr.  
24 Jianzhong Ma laboratory) were cultured until OD<sub>600</sub>=1.0, plated 200  $\mu$ L on YPE (1% (w/v) yeast  
25 extract, 1% (w/v) peptone, 0.5% (w/v) NaCl, 1.5% (w/v) agar) or LB (1% (w/v) yeast extract, 2%  
26 (w/v) peptone, 2% (w/v) NaCl, 1.5% (w/v) agar), after methanol introduced for 51 h, cell-free  
27 liquid, pellet ultrasonic disruption supernatant, and precipitation that resuspended in ice bath were  
28 collected and immersed on sterile filter paper and directly placed to surface of the 0.20 M glucose  
29 plates that were plated by *A. tumefaciens* LBA4404 or *E. coli* DH5 $\alpha$  cultured until OD<sub>600</sub>=1.0 and  
30 the antibacterial effect was observed.

#### 31 **Antibacterial Activity of Hydrogen Peroxide Solution to Agrobacterium and Escherichia coli 32 on Agar Plates**

33 To detect the inhibitory effect and the lowest hydrogen peroxide concentration be able to inhibit  
34 the growth of bacteria. *A. tumefaciens* LBA4404 shaking cultured at 28 °C until OD<sub>600</sub>=1.0,  
35 plated 200  $\mu$ L on YPE medium and then placed the filter paper ~~which~~ containing different  
36 concentrations of hydrogen peroxide solution, 28 °C stationary culture for 14 hr and the inhibitory  
37 effect were observed. *E. coli* DH5 $\alpha$  shaking cultured at 37°C until OD<sub>600</sub>=1.5, plated 200  $\mu$ L on  
38 LB medium and then placed the filter paper which containing 10  $\mu$ L different concentrations of

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

3 **Results**

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

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

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

13 Since H<sub>2</sub>O<sub>2</sub>, one of the products by GOD, injures living cells, growths of the GOD-transgenic  
14 strain, *P. pastoris* GS115-His-GOD 01, were firstly determined if they were inhibited by the  
15 transgenic GOD. Compared to *P. pastoris* GS115-pPIC9K, the growth of *P. pastoris*  
16 GS115-His-GOD 01 was slightly decreased during most time of the 51-hour incubation if the  
17 GOD was not induced (Fig. 1a). Its optical density at 600 nm was 0.95-fold of that of *P. pastoris*  
18 GS115-pPIC9K at the time point of 51.0 h. However, the growth of *P. pastoris* GS115-His-GOD  
19 01 was significantly lowered if the GOD was induced by methanol (Fig. 1b). During the growth of  
20 51.0 h, the optical densities of *P. pastoris* GS115-His-GOD 01 were 0.54-fold of that of *P. pastoris*  
21 GS115-pPIC9K at 18.0h, 0.43-fold at 36 h, and 0.37-fold at 51.0 h, respectively. The inhibited  
22 growth of the GOD-transgenic *P. pastoris* could be attributed to the expression of the foreign  
23 GOD and, hereafter, accumulation of H<sub>2</sub>O<sub>2</sub>.

24 **Activities of the Glucose Oxidase**

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

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

### 3 **Concentration** **The concentration** of Hydrogen Peroxide from GOD Catalyzed Glucose

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

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

#### 8 **Liquid Medium**

9 The combination of 1.0, 2.5 or 5 mg/ml glucose and 1.0, 5.0 or 10 U/ml glucose oxidase were  
10 added to the medium of *A. tumefaciens* LBA4404 and *E. coli* DH5a. The GOD was contained in  
11 fermentation supernatant of *P. pastoris* GS115-His-GOD 01 and in Fig. 4a showed a marked  
12 inhibition to the growth of *A. tumefaciens* LBA4404 12 hours later compared with the control that  
13 was added with equal volume of *P. pastoris* GS115-pPIC9K fermentation supernatant, and the  
14 impact increased with substrate concentration. Conclusions were drawn from Fig. 4b, it showed  
15 these combinations did not completely inhibit growth of *E. coli* DH5a but influenced the time at  
16 which growth was initiated. Delay of growth initiation was greatest with the enzyme concentration,  
17 5.0 U/mL, and the impact increased also with substrate concentration.

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### 18 **Antibacterial effects of Glucose and Glucose Oxidase on Growth of *A. tumefaciens* and *E.coli* 19 on Agar Plates**

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

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### 27 **Antibacterial Activity of Hydrogen Peroxide Solution to *A. tumefaciens* LBA4404 and *E.coli* 28 DH5a**

29 To detect the minimum concentration of hydrogen peroxide solution inhibit the growth of *A.*  
30 *tumefaciens* LBA4404 and *E.coli* DH5a, the sterile filter papers were soaked with a volume of 10  
31  $\mu$ l hydrogen peroxide that were-was diluted to different concentrations. Different concentrations of  
32 hydrogen peroxide solution effect on *A. tumefaciens* LBA4404 were showed-shown in Fig. 6a and  
33 the diameters of inhibition zone were showed-shown in Fig. 6c, it showed that the minimum  
34 concentration of hydrogen peroxide solution inhibits the growth of *A. tumefaciens* LBA4404 was  
35  $5.6 \times 10^3$   $\mu$ g/ml; Different concentrations of hydrogen peroxide solution effect on *E.coli* DH5a  
36 were showed-shown in Fig. 6b and the diameters of inhibition zone were showed-shown in Fig. 6d,  
37 it showed that the minimum concentration of hydrogen peroxide solution inhibits the growth of *A.*  
38 *tumefaciens* LBA4404 was  $6.0 \times 10^3$   $\mu$ g/ml.

1    **Discussion**

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

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

28   **Compliance with ethical standards**

29   **Conflict of Interest**

30   No conflict of interest declared.

31   **Acknowledgement**

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34   Screening and Deep Processing for Traditional Chinese and Tibetan Medicine of Gansu Province  
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