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Expression of *Aspergillus oryzae* Tannase in *Pichia pastoris* and Its Application in the Synthesis of Propyl Gallate in Organic Solvent

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Summary

Gallic acid esters could be synthesized biologically by tannase in organic media, among which is propyl gallate, an antioxidant widely used as a food additive. Efficient intracellular expression of *Aspergillus oryzae* tannase was achieved in *Pichia pastoris* under the control of the *AOX1* promoter, and the productivity of recombinant tannase was 960 U/L or 64 U/g dry mass. The recombinant *P. pastoris* was used to synthesize propyl gallate in organic solvent and the yield of propyl gallate was 53 %.

Key words: tannase, *Pichia pastoris*, recombinant protein production, propyl gallate, biosynthesis

Introduction

Tannase (tannin acyl hydrolase, EC 3.1.1.20) is an inducible enzyme in the presence of its substrate or a substrate analogue, such as tannic acid or its end product *e.g.* gallic acid (*1*). Tannase hydrolyses the ester and depside bonds of gallotannins and gallic acid esters in aqueous media and synthesizes gallic acid esters with a variety of alcohols and diols in organic media (*2*,*3*). Propyl gallate is a very important gallic acid ester, widely used as antioxidant in food, cosmetics, hair products, adhesives, and lubricant industries; and it is also used as a prodrug trimethoprim, a pharmaceutical antibacterial agent. At present, propyl gallate is mainly produced in large scale by a chemical method, which is a high-energy-consuming process under high temperature/pressure and strong acid conditions. Additionally, under such conditions the 3,4,5-trihydroxybenzene group would be oxidized easily, resulting in unusable products (*4*). However, producing propyl gallate by a biological method could be carried out under mild conditions and could produce fewer by-products. There have been several reports on the synthesis of propyl gallate by free or immobilized tannase (*3*,*5*–*7*). For the commercial realization, one promising approach would be to directly use whole cells as biocatalysts in organic solvents, thus avoiding the costly purification of enzymes (*8*). *Aspergillus niger, Aspergillus flavus* and *Aspergillus oryzae* were found to be the best tannase producers on tannic acid as a sole source of carbon. In our previous study *A. niger* was used to synthesize propyl gallate in organic solvents (*9*). However, the yield of propyl gallate was not high probably due to the low tannase productivity of *A. niger*. In order to improve the tannase productivity, researchers expressed the *Aspergillus* sp. tannase in heterologous expression systems. Albertse (*10*) expressed a tannase gene from *A. oryzae* in *Saccharomyces cerevisiae* at a very low level probably due to the phenomenon exhibited in *S. cerevisiae* to hyperglycosylate glycoproteins, therefore expressing catalytically inactive proteins. Zhong *et al.* (*11*) successfully expressed the *A. oryzae* tannase extracellularly in *Pichia pastoris* for generating large quantities of tannase.

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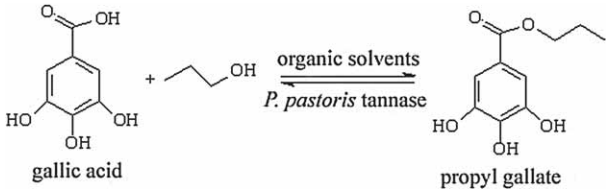
Over the last few decades, the *P. pastoris* expression system has been used successfully for the production of various recombinant heterologous proteins, such as *A. awamori* glucoamylase, *Candida rugosa* lipase, *Rhizopus oryzae* lipase, or *Escherichia coli* L-galactosidase (*12*). This process offers several advantages, such as alcohol oxidase 1 (*AOX1*) gene promoter tightly regulated by methanol, easy growth to high cell densities, high levels of protein expression at the intra- or extracellular level, and the ability to perform higher eukaryotic protein modifications (*12*). In this research, in order to achieve a microorganism with higher tannase productivity for the whole cell synthesis of propyl gallate in organic solvents, *A. oryzae* tannase gene was expressed intracellularly in *P. pastoris*, and the synthesis of propyl gallate by the recombinant *P. pastoris* in organic solvents was investigated. Scheme 1 depicts the esterification scheme:



Scheme 1. Esterification of propyl gallate by recombinant *P. pastoris* in organic solvent

Materials and Methods

Materials

Intracellular expression vector pPIC3.5K was from Invitrogen (Carlsbad, CA, USA) and vector pUCm-T was from Shenerg Biocolor (Shanghai, PR China). *E. coli* strain DH5α (*sup*E44Δ*lac*U169(φ80 *lac*ZΔM15)*hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1) was used for propagation of plasmids, and *P. pastoris* strain GS115 (*His*$^-$*Mut*$^+$, Invitrogen, USA) was used for protein expression. *A. oryzae* (Ahlb.) Cohn was purchased from China General Microbiological Culture Collection Center. Oligonucleotides were purchased from Sangon (Shanghai, PR China). Restriction enzymes were sourced from Toyobo (Japan).

Construction of plasmid

The tannase gene (*TAN*) was PCR-amplified using the forward and reverse primers (Forward *Eco*R I: 5′--GGGGAATTCACCATGGCTTCTTTTACCGATG-3′, Reverse *Not* I: 5′-TTATTGCGGCCGCCTAGTATACAGGGACCTTGAAGGCTG-3′) with the genomic DNA of *A. oryzae* as template. PCR products were purified on a PCR--prep column kit (BioDev-Tech, Beijing, PR China) and ligated into the linearized pUCm-T vectors. Cloned tannase gene was excised from pUCm-T by digestion with *Eco*R I/*Not* I and ligated into the same enzyme digested vector pPIC3.5K.

Pichia pastoris transformation and selection of His$^+$ multicopy recombinants

The plasmids (pPIC3.5K-*TAN* or pPIC3.5K) were linearized with *Bgl* II in order to be transformed into *P. pastoris* GS115 by electroporation, and selection of His$^+$ transformants was done on minimal dextrose (MD) medium plate. Multicopy recombinants were selected on yeast extract/peptone/dextrose medium plate containing 0.25–1.0 g/L of G418 (YPD-G418).

Single colonies of transformants from YPD-G418 plates were used to inoculate 5 mL of YPD overnight cultures, then the isolation of genomic DNA was performed with the Easy-DNA Kit from Invitrogen, and PCR amplifications were carried out according to Invitrogen's recommendations with 5 μL of genomic DNA and primers complementary to the 5′ and 3′ region of the *AOX1* gene (*13*).

Expression of tannase in Pichia pastoris

A plate assay was performed to visualize the expression of the recombinant tannase gene. *P. pastoris* transformants were cultivated on yeast extract/peptone/dextrose medium plate with 0.15 kg/m^3 of tannic acid (YPD-TA). After one day, 1 mL of methanol was added onto the lid of the plates daily to induce the expression of tannase.

P. pastoris transformants were cultured in 25 mL of buffered glycerol-complex medium (BMGY) shaken at 28 °C and 250 rpm in 250-mL glass flasks. When cultures reached an A_{600nm} of about 6, the cells were centrifuged and resuspended in 20 mL of buffered methanol--complex medium (BMMY) to an A_{600nm} of 1, shaken at 28 °C and 250 rpm in 100-mL glass flasks for 120 h. The cultures were supplemented daily with 0.5 % (volume fraction) methanol to induce the expression of tannase, and the pH was adjusted to 6.0 every 24 h. The cells were cleaved by glass beads, then the tannase in supernatants was analyzed by SDS-PAGE, and the enzyme activity was determined.

Assay of tannase activity

Tannase activity was assayed by a method based on the formation of a chromogen (2-thioxo-4-thiazolidinone) when gallic acid reacts with rhodanine (*14*). The absorbance value of the chromogen was recorded against water at 520 nm. All the assays were done in triplicate, and significant differences ($p<0.05$) were measured. One unit of tannase activity was defined as the amount of enzyme that releases 1 μmol of gallic acid per minute under the assay conditions.

Purification of recombinant tannase

Cell lysis was aided with acid-washed glass beads (0.5 mm). Supernatants were concentrated and interchanged with 10 mM citric acid (pH=5.5) by ultrafiltration using 30-kDa membranes (Millipore). The solution was loaded onto DEAE-Sepharose fast flow column equilibrated with 10 mM citric acid (pH=5.5). Protein was eluted with a gradient of 0.0–0.5 M NaCl. Then the active fraction was mixed with an equal volume of 0.02 M sodium acetate buffer (pH=5.0) containing 0.1 M NaCl. The diluted solution was placed on a Sepharose 6B column equilibrated with the same buffer containing 0.1 M NaCl and eluted with the same buffer containing NaCl.

Synthesis of propyl gallate

Enzymatic reactions were carried out by adding the pellet of 2 mL of transformant cultures to the reaction mixture, which consisted of 5.56 mmol/L gallic acid, 7.3 % (volume fraction) of 1-propanol and 92.7 % (volume fraction) of benzene with a total volume of 10.8 mL in 25-mL glass flasks shaken at 40 °C and 200 rpm. The molar conversion was defined as (mol of propyl gallate/mol of the initial fed gallic acid)×100 %.

HPLC analysis

Analyses were performed on a Waters 2690 HPLC with a LiChrospherC18 column (LiChroCART 125×4, Merck KGaA, 64271 Darmstadt, Germany). Samples (200 μL) removed from the systems were vacuum evaporated, diluted with 0.15 mmol/L of ethyl *p*-hydroxybenzoate in methanol and a volume of 20 μL was injected into the HPLC. The solvent system/mobile phase was comprised of methanol/water in the ratio of 55:45 adjusted to pH=3 with phosphoric acid at a flow rate of 1 mL/min for 8 min. Ethyl *p*-hydroxybenzoate was the internal standard. The absorbance analysis was carried out at 275 nm. The reaction product was calculated from a calibration curve plotted (Y=0.03144X–0.00194, R=0.9999) as propyl gallate concentration (X, μg/mL) *vs.* the ratio (Y) between propyl gallate peak area and internal standard peak area.

All the assays were done in triplicate and significant differences ($p<0.05$) were measured.

Results and Discussion

Cloning and sequence analyses of the tannase gene

Hatamoto *et al.* (*15*) had cloned and sequenced the tannase gene from *A. oryzae* TH (GenBank accession no. D63338) and found no intron in *A. oryzae* tannase gene. According to the reported sequence, the tannase gene from *A. oryzae* (Ahlb.) Cohn coding for mature tannase was PCR-amplified directly from the genome of *A. oryzae* and submitted to GenBank with the accession no. DQ988081. The gene sequence and the amino acid sequence of the tannase was 97.26 % (1666/1713) and 98.42 % (561/570) identical, respectively, to that from *A. oryzae* TH (*15*).

Screening of multicopy transformants and PCR analysis of Pichia pastoris transformants

The experimental results showed that all of the His^+ transformants could grow on YPD-G418 plate of 0.25––0.5 g/L G418 and 30 % of them could grow at the highest resistance concentration of 0.75 g/L G418. Multiple insertions of the kanamycin resistance gene into the *P. pastoris* chromosome increase the resistance to G418. Since the kanamycin resistance gene is linked to the tannase gene, isolation of hyper-resistant G418 transformants might indicate that the tannase gene is present in multiple copies.

PCR amplification results (Fig. 1) show that the parent plasmid pPIC3.5K produced a 220-bp product, recombination plasmid pPIC3.5K-*TAN* produced a 1.95-kb product containing the tannase gene (1.73 kb) and 220-bp sequence from plasmid pPIC3.5K, positive recombinant *P. pastoris* produced a 1.95-kb product, negative transformants only produced the *AOX1* gene (2.2 kb) from GS115 genomic DNA, control strains transformed by plasmid pPIC3.5K produced a 220-bp product. This suggests that linearized plasmids pPIC3.5K-*TAN* or pPIC3.5K by *Bgl* II were integrated into the *P. pastoris* genome *via* a double crossover between the *AOX1* promoter and 3′ *AOX1* regions of the vector, which disrupted the wild-type *AOX1* gene and created the His^+Mut^s transformants, here named GS115/pPIC3.5K-*TAN* Mut^s transformant. Since the pPIC3.5K plasmid has two *Bgl* II sites, linearized by *Bgl* II, there are more chances to create His^+ Mut^s transformants than to make His^+Mut^+ transformants *via* gene insertion at the *AOX1* locus. In our study, no His^+Mut^+ transformants were detected.

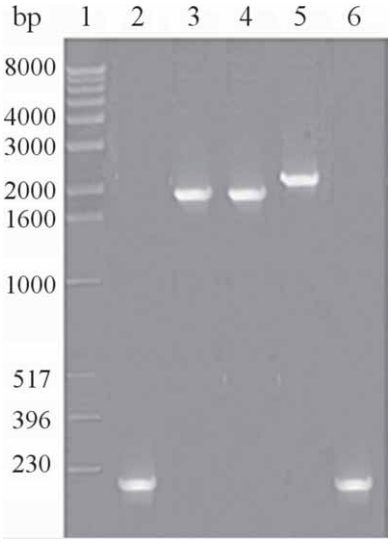


Fig. 1. PCR analysis of *P. pastoris* colonies. From left to right, lane 1: DNA ladder marker, lane 2: PCR product of pPIC3.5K, lane 3: PCR product of pPIC3.5K-*TAN*, lane 4: PCR product of GS115/pPIC3.5K-*TAN* Mut^s transformant, lane 5: PCR product of control strain GS115, lane 6: PCR product of GS115/pPIC3.5K Mut^s transformant

Induced expression of tannase by recombinant P. pastoris, tannase activity assay and SDS-PAGE

Biochemical studies showed that methanol utilization by *P. pastoris* requires a specific metabolic pathway involving several unique enzymes (*16*). The enzyme alcohol oxidase (AOX) catalyzes the first step in the methanol utilization pathway, the oxidation of methanol to formaldehyde and hydrogen peroxide. There are two genes that encode alcohol oxidase in *P. pastoris*: *AOX1* and *AOX2*; *AOX1* is responsible for a vast majority of alcohol oxidase activities in the cell. Expression of the *AOX1* gene is controlled at the level of transcription. The presence of methanol is essential to induce high levels of transcription (*12*,*17*). The pPIC3.5K expression vector has an expression cassette composed of a 0.9-kb fragment from *AOX1* composed of the 5′ promoter sequences and a second short *AOX1*-derived fragment with sequences required for transcription termination (*18*). Between the promoter and terminator sequences is a site or multiple cloning site (MCS) for insertion of the

foreign coding sequence, here the tannase gene. Through electroporation, the tannase gene was integrated into the *P. pastoris* genome under the control of *AOX1* promoter induced by methanol.

A total of fifty *P. pastoris* colonies resisting 0.75 g/L of G418 were randomly picked and grown on YPD-TA. After induction by methanol for 72 h, 10 colonies that produced the largest clear zone were chosen to express tannase in glass flasks. The clear zone is shown in Fig. 2. Upon expression of the recombinant tannase gene, the tannic acid is hydrolyzed into gallic acid and glucose, which leads to the formation of a clear zone around the tannase-expressing yeasts.

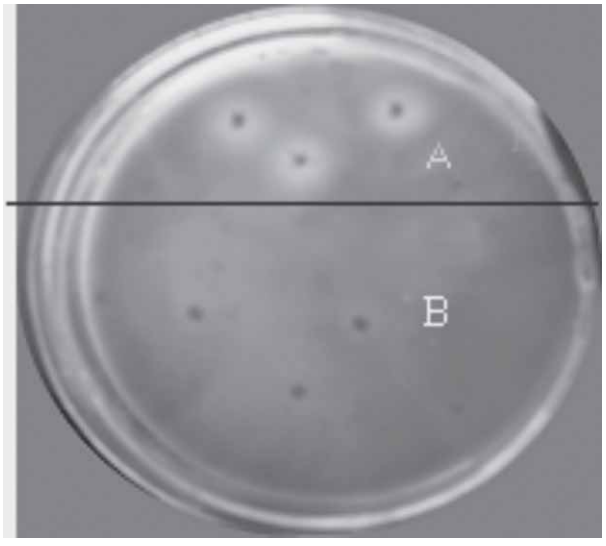


Fig. 2. Tannase activity examined by clear zone method. Label A: around the GS115/pPIC3.5K-*TAN Mut*^{s} transformants showed the clear zones. Label B: the GS115/pPIC3.5K *Mut*^{s} transformant, GS115 served as negative control

As shown in Fig. 3, crude or purified tannase loaded onto SDS-PAGE without 2-mercaptoethanol migrated as one broad band at about 100 kDa (lanes 3–6); in lane 7, purified tannase with 2-mercaptoethanol appears as a blurred band at about 45 kDa. As shown in lane 8, the

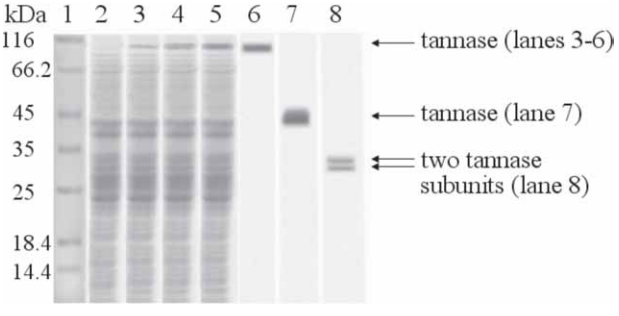


Fig. 3. SDS-PAGE analysis. From left to right, lane 1: protein molecular mass marker; lanes 2–5: extract of GS115/pPIC3.5K--TAN *Mut*^{s} transformant loaded without 2-mercaptoethanol (induced time: 0, 24, 48, 96 h, respectively), lane 6: purified tannase without 2-mercaptoethanol, lane 7: purified tannase with 2-mercaptoethanol, lane 8: purified tannase digested with *N*-glycosidase F and loaded with 2-mercaptoethanol

two clear separated bands suggest that the tannase was composed of two subunits (about 30 and 33 kDa) linked by disulphide bond(s) and high glycosylation since the blurred tannase band in lane 7 became clearer after the tannase was treated with *N*-glycosidase F.

The relationship between fermentation time and tannase productivity or biomass is indicated in Fig. 4. After 24 h of fermentation, the dry mass of biomass reached about 1.6 g/L, and methanol was added to the medium to induce the expression of tannase. Experimental results showed that the biomass increased quickly with the increase of tannase productivity, which reached the maximum of 960 U/L or 64 U/g dry mass after 72 h of induction, which was approximately 2.5-fold of tannase productivity compared to that of *A. niger* under the same enzyme assay conditions (*9*,*19*). Other researchers reported higher activity of tannase (*20*,*21*), while comparison of tannase activities is very difficult because of the use of different assay methods and activity units. After 96 h of induction tannase productivity began to decrease, probably due to the sharply decreased biomass. The cell lysis contributes to increased proteolytic activity and eventual degradation of recombinant protein, which has been a perpetual problem when yeasts are employed to express recombinant proteins (*22*,*23*). The transformants were quite stable since their activity showed hardly any decrease after five passages (experimental data not shown).

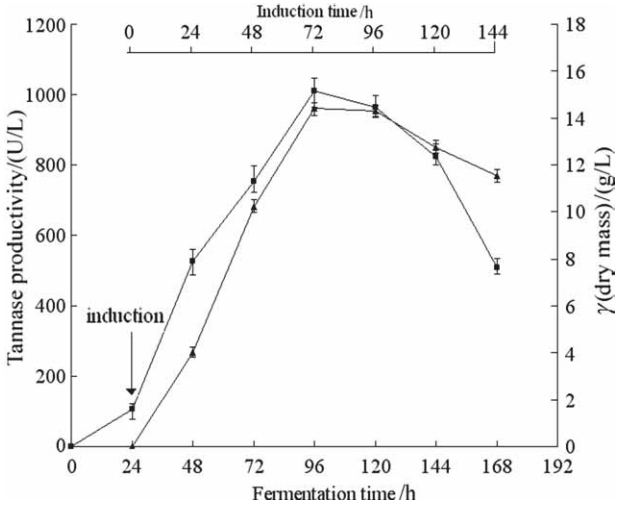


Fig. 4. Expression of tannase by GS115/pPIC3.5K-*TAN Mut*^{s} transformant induced by methanol; ■ dry mass; ▲ tannase productivity

Synthesis of propyl gallate

It is suggested that the mechanism of synthesis of propyl gallate by tannase involves an attack by the acyl donor (gallic acid) to form an acyl enzyme, which then reacts with an alcohol (1-propanol) to yield the ester, which could be confirmed by the kinetic model of esterification by tannase based on the ping-pong bi-bi kinetic mechanism (*24*), while there is still no evidence of the mechanism on a molecular level.

The time course of the synthesis of propyl gallate in benzene by GS115/pPIC3.5K-*TAN Mut*^{s} transformant is shown as an equilibrium-controlled reaction (Fig. 5). The catalytic rate rose quickly in the first hours of incubation, and the reaction equilibrium was reached at about 24 h with the yield of 53 %, which was higher than that catalyzed by *A. niger* no. 3.315 (36 % after 72-hour reaction) (*9*). Higher yields of propyl gallate by *P. pastoris* tannase were probably due to higher tannase productivity of *P. pastoris* (64 U/g dry mass) than that of *A. niger* (25 U/g dry mass) (*9*). In the control test, no product could be detected with the GS115/pPIC3.5K *Mut*^{s} transformant or without any transformants.

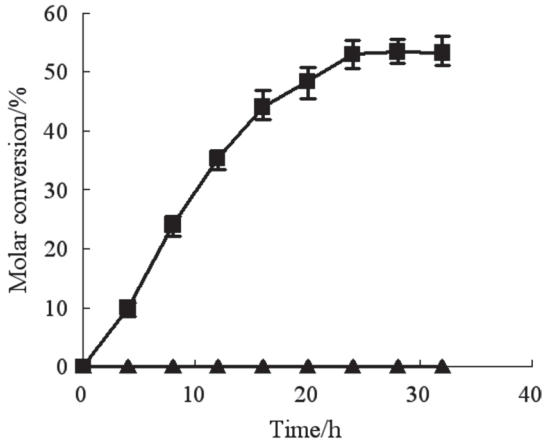


Fig. 5. Time course of propyl gallate catalyzed by recombinant *P. pastoris;* ■ GS115/pPIC3.5K-*TAN Mut*^{s} transformant; ▲ negative control strain GS115/pPIC3.5K *Mut*^{s} transformant

Conclusions

Depending on the type of fermentation the tannase from most fungi can be completely extracellular or intracellular (*2,25*). In order to directly employ whole cells as biocatalysts in organic solvents, which offers several advantages, such as avoiding costly purification of enzymes and improving enzyme stability, in this research efficient intracellular expression of *A. oryzae* tannase was achieved in *P. pastoris* under the control of *AOX1* promoter, and the productivity of recombinant tannase was 960 U/L or 64 U/g dry mass. The recombinant *P. pastoris* was used to synthesize propyl gallate in organic solvent and the yield of propyl gallate was 53 %, which suggests that this expression system of tannase is expected to be a powerful tool in industrial production of gallic acid esters.

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