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Heterologous expression of VHb can improve the yield and quality of biocontrol fungus *Paecilomyces lilacinus*, during submerged fermentation

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ABSTRACT

Paecilomyces lilacinus is an egg-parasitic fungus which is effective against plant-parasitic nematodes and it has been successfully commercialized for the control of many plant-parasitic nematodes. However, during the large-scale industrial fermentation process of the filamentous fungus, the dissolved oxygen supply is a limiting factor, which influences yield, product quality and production cost. To solve this problem, we intended to heterologously express VHb in *P. lilacinus* ACSS. After optimizing the *vgb* gene, we fused it with a selection marker gene *npt*II, a promoter P*gpd*A and a terminator T*trp*C. The complete expression cassette P*gpd*A-*npt*II-*vgb*-T*trp*C was transferred into *P. lilacinus* ACSS by *Agrobacterium tumefaciens*-mediated transformation. Consequently, we successfully screened an applicable fungus strain PNVT8 which efficiently expressed VHb. The submerged fermentation experiments demonstrated that the expression of VHb not only increased the production traits of *P. lilacinus* such as biomass and spore production, but also improved the beneficial product quality and application value, due to the secretion of more protease and chitinase. It can be speculated that the recombinant strain harboring *vgb* gene will have a growth advantage over the original strain under anaerobic conditions in soil and therefore will possess higher biocontrol efficiency against plant-parasitic nematodes.

1. Introduction

Vitreoscilla hemoglobin (VHb) is a soluble homodimeric protein, which is synthesized by aerobic Gram-negative bacterium *Vitreoscilla* (Wakabayashi et al., 1986). Its oxygen dissociation rate constant is hundred times higher than that of other globins (Zhang et al., 2007). VHb can effectively enhance cellular capacity of oxygen utilization, strengthen cellular respiration intensity and reduce intracellular critical oxygen concentration. Moreover, VHb can maintain constant intracellular respiratory rate and dwindle the effects of variable oxygen environment on cell growth. Therefore, for industry-scale fermentation, heterologous expression of VHb has become a versatile tool to improve cellular growth, protein synthesis, metabolite productivity and biomass (Frey et al., 2011; Geckil et al., 2001; Liao et al., 2014; Zhang et al., 2007).

Plant-parasitic nematodes can cause a significant economic loss to almost all vegetables and field crops mainly in tropical and sub-tropical agricultural areas. Annual global yield loss caused by nematodes is about 80 billion (Nicol et al., 2011). Chemical control has been a widely used option for plant-parasitic nematode management. However, chemical nematicides are now being reappraised in respect of environmental hazard and their diminished effectiveness following repeated applications. For the biocontrol of the nematodes, the egg-parasitic fungus *Paecilomyces lilacinus* has been widely tested and shown promising application prospects (Anastasiadis et al., 2008; Atkins et al., 2005; Kiewnick and Sikora, 2006; Li et al., 2013; Siddiqui and Futai, 2009). Moreover, some excellent strains of *P. lilacinus*, such as strain 251 (Anastasiadis et al., 2008; Atkins et al., 2005; Kiewnick and Sikora, 2006) and strain PL9410 (Wang et al., 2010) have been successfully commercialized as these can deteriorate eggs of nematodes (Jatala, 1986) and survive for long period around rhizosphere (Hashem and Abo-Elyousr, 2011). However, the submerged fermentation of the filamentous fungus is a typically aerobic fermentation process with a long fermentation period (more than ten days). Therefore, it has a high demand for dissolved oxygen supply for large-scale and high-cell density industrial fermentation process, which is a crucial factor for product quality and production cost. These problems can be partially alleviated by improving bioreactor configuration (*e.g.* rational

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design of a stirrer and a diameter-to-height ratio) and optimization of fermentation parameters (Frey et al., 2011). However, significant improvements can only be achieved by metabolic engineering, especially by heterologous expression of VHb.

Therefore, the goal of this research was to transfer a functional and valid *vgb* gene into *P. lilacinus* genome and screen appropriate transformants to improve the utilization of oxygen, and thereby lessen ventilation requirement, reduce energy consumption, aggrandize output and ultimately bring down economic cost during large scale production of *P. lilacinus*. An optimized *vgb* gene was successfully transferred into *P. lilacinus* ACSS genome through *Agrobacterium tumefaciens*-mediated transformation (ATMT) and an applicable transformant PNVT8 was screened. Subsequently, submerged fermentation experiments were performed both at shake flask and bioreactor levels, and results demonstrated that PNVT8 grew well, produced more biomass, secreted more protease and chitinase, and had higher spore production as compared to original strain ACSS.

2. Materials and methods

2.1. Strains, plasmids and growth conditions

P. lilacinus ACSS was obtained from the Agricultural Culture Collection of China (Beijing, China). *Agrobacterium tumefaciens* strains EHA105 and LB4404 were graciously provided by Professor Liang Chen (Xiamen University, China); plasmid pUR5750 was gifted from Dr. Rosa Elena Cardoza (University of Leo'n, Spain) (de Groot et al., 1998; Cardoza et al., 2006). The strains, plasmids and primers used in this study are listed in Table S1, Table S2 and Table S3, respectively. The *Escherichia coli* strains were grown in Luria-Bertani (LB) medium at 37 °C. *P. lilacinus* strains were grown in Potato Dextrose Agar (PDA) medium and Potato Dextrose Broth (PDB) medium at 28 °C as required. *A. tumefaciens* was grown on YEB medium (5 g/L tryptone, 1 g/L yeast extract, 5 g/L nutrient broth, 5 g/L sucrose, 0.5 g/L $MgSO_4 \cdot 7H_2O$, pH 7.2. To prepare YEB plates, 1.5% bacterial agar was added) at 28 °C. Liquid cultures were shaken at 200 rpm. Antibiotics were used at the following concentrations: 100 μg/mL of ampicillin, 50 μg/mL of kanamycin, 400 μg/mL of G418 and 200 μg/mL of cefotaxime.

2.2. Codon optimization and gene synthesis

The amino acid sequence of VHb (Genbank accession no. AAA75506) and corresponding nucleotide sequence of *vgb* gene are shown in Fig. S1. After codon optimization, the optimized *vgb* gene was synthesized by GENEWIZ, Inc in Soochow (Jiangsu, China). Alignment between original and optimized *vgb* gene sequences is shown in Fig. S1.

2.3. Susceptibility testing of ACSS to hygromycin B and G418

Before transformation, mycelia of ACSS were first inoculated onto PDA plates with 0, 50, 100, 200, 300, 400 and 500 μg/mL hygromycin B (HygB) or G418. The plates were then incubated at 28 °C for about 7 d. The available antibiotic and its appropriate concentration for the selection of transformants were determined according to the growing situation of ACSS.

2.4. Vector construction

E. coli DH5α was employed for the construction and amplification of plasmids. The neomycin phosphotransferase gene II (*npt*II) from bacterium was used as a selection marker for genetic transformation of ACSS. Glyceraldehyde-3-phosphate dehydrogenase gene promoter (P*gpd*A) and tryptophan synthetase C gene terminator (T*trp*C), both of which were from *Aspergillus nidulans*, were used as promoter and terminator for the construction of the integrating plasmid puPNVT. The construction strategy and process were described in Fig. S2.

2.5. ATMT of P. lilacinus

ATMT of *P. lilacinus* ACSS was carried out as described previously (de Groot et al., 1998; Wang et al., 2010) with some modifications.

The *Agrobacterium* strain containing plasmid puPNVT was grown overnight in YEB medium with 50 μg/mL kanamycin at 28 °C and 200 rpm. One milliliter of *Agrobacterium* culture was inoculated into 9 mL of induction medium (10 mM K_2HPO_4, 10 mM KH_2PO_4, 2.5 mM NaCl, 2 mM $MgSO_4$, 0.7 mM $CaCl_2$, 9 mM $FeSO_4$, 4 mM NH_4SO_4, 10 mM glucose, 40 mM 2-[N-morpholino] ethanesulfonic acid (MES), pH 5.3, 0.5% glycerol (w/v), 200 μM acetosyringone (AS)) and incubated under the same conditions until an OD_{660} of 0.8 was reached. After that, 100 μL of *Agrobacterium* culture (10^8 cfu/mL) was mixed with 100 μL of ACSS conidia. The mixtures were collected by centrifugation and resuspended in 200 μL of induction medium. Eppendorf tubes containing the resuspended mixture were statically incubated at 25 °C for 48 h. To determine whether the transformation of the fungal conidia was dependent upon the T-DNA transfer, the control experiments were performed in the absence of the virulence inducer AS. For screening the ATMT transformants of ACSS, the final co-cultures were spotted onto the PDA plates supplemented with 300 μg/mL G418 to select the transformants and 300 μg/mL cefotaxime to inhibit the growth of *Agrobacterium*. The plates were then incubated at 28 °C. When G418-resistance colonies became visible (about 3 to 5 d), they were transferred onto the fresh PDA plates containing 400 μg/mL G418. The *A. tumefaciens* strain EHA105 or LB4404 without plasmid puPNVT was used as the negative control. Each treatment was repeated five times. The results were subjected to single factor variance analysis using SPSS 10.0 statistical software.

2.6. Verification of the transformants

2.6.1. PCR verification

To verify the transformants, the mycelia from the margin of the G418-resistance colonies were inoculated into PDB supplemented with G418. After growth for 3–4 d, the mycelia were collected and frozen in liquid nitrogen and then ground with a mortar in ice-bath. Genomic DNA was isolated from ACSS and its transformants as described by Reader and Broda (1985). Putative transformants were verified by the PCR detection with the primers NPTF and NPTR, and *vgb*F and *vgb*R (Table S3). Plasmid puPNVT and ACSS genomic DNA were used as the positive and negative controls, respectively.

2.6.2. Southern blotting

Southern blotting was performed with 15–25 μg DNA for each sample. The genomic DNA samples of putative transformants were separately digested by *Spe*I. The *npt*II fragment amplified from puPNVT with the primer pair *npt*F and *npt*R were used as DNA probes. The *npt*II fragment excised from puPNVT by *Xho*I and *Eco*RI was used as a positive control, while the genomic DNA of ACSS served as a negative control. DNA probe labeling, prehybridization and hybridization were performed according to the manufacturer's protocols (Roche, Basel, Switzerland).

2.6.3. RT-PCR analysis of the transcription of nptII and vgb genes

The primer pairs NPTF and NPTR and *vgb*F and *vgb*R were used to amplify the *npt*II gene (839 bp) and *vgb* gene (463 bp), respectively. Isolation of total RNA and synthesis of cDNA were performed according to the reference (Wang et al., 2010).

2.6.4. CO-difference spectrum analysis

CO-difference spectrum analysis was carried out as described previously (Liao et al., 2014).

2.7. Testing of the transformant stability

Single spore isolate of transformant PNVT8 was continuously grown on PDA plates with 400 μg/mL G418 for three generations and then transferred onto PDA plates without G418 for five generations. After that, the transformants were tested on PDA plates containing 400 μg/mL G418.

2.8. Shake flask culture

Submerged fermentation experiments were carried out in shake flasks and bioreactor, respectively. The mycelia in pure cultures of ACSS and PNVT8 at the late exponential growth phase were inoculated into three 250-mL shake flasks, each containing 100 mL PDB, and incubated at 28 °C and 100 rpm in an orbital shaker for 6 d. Spores were obtained by filtration of the cultures with 4–6 layers of cotton gauze. For the determination of spore count, samples were spread on plate after serial dilution. After inoculation with a culture of 9×10^6 spores/mL, a 250 mL Erlenmeyer flask (with a silicone plug) containing 100 mL PDB was incubated at 28 °C and 100 rpm for 4 d, then the mycelia and mycelial pellets were observed.

In order to study the influence of expression of VHb on the growth of mycelial pellets, especially under the oxygen restricted conditions, we added 20, 40, 60 and 80 mL of PDB to each of 100-mL glass infusion bottles (narrow-neck bottle), inoculated with spores at 2.4×10^5 spores/mL, The culture bottles were tightly closed with rubber plugs and sealed with aluminum foil. The cultures were incubated at 28 °C for 7 d, and then a 5 mL of 1% Tween 80 was added to a 5 mL suspension in graduated plates for counting mycelial pellets (Xu et al., 2000).

Supernatants from glass infusion bottles with 60 mL PDB were precipitated by saturated ammonium sulfate, the precipitates were resuspended in phosphate buffer and filtered to achieve crude protease solutions. After the determination of protein concentrations, 200 μL crude protease solutions (ten-fold concentrate) of PNVT8 and ACSS were added into oxford cups on skim milk agar plates and incubated at 25 °C, the diameters of clearing zone were measured (Wang et al., 2010).

All tests were repeated three times.

2.9. Batch fermentation at bioreactor scale

2.9.1. Fermentation condition

Bioreactor cultivations were performed in a 15-L in situ sterile bioreactor (Wuhan, Hubei, China) with an effective working volume of 10 L. For inoculation purposes, a 500 mL flask containing 200 mL PDB was inoculated with spores (2.4×10^5 spores/mL) and incubated at 28 °C for 48 h. The 48-h-grown culture in the flask was transferred to the bioreactor containing PDB supplemented with 2% chitin and 50 μg/mL kanamycin. The cultivation conditions were 28 °C temperature, 200-rpm agitation, and 0.037 L/min ventilation. In these experiments, 20 mL samples were taken at 24-h intervals. The samples were centrifuged (10,000 × *g*) for 10 min at 4 °C, and supernatants and precipitates were stored at −80 °C for the analyses of chitinase activity and spore production, respectively. Fermentation was terminated at the end of the eleventh day.

2.9.2. Determination of chitinase activity

Colloidal chitin was prepared by partial hydrolysis of chitin (Sigma–Aldrich, St. Louis, MO, U.S.A.) using 10 M HCl and left at 4 °C overnight (Roberts and Selitrennikoff, 1988). Subsequently, the mixture was added to 95% ethanol and kept at −20 °C overnight. The precipitate was collected by centrifugation at 4000 × *g* for 20 min at 4 °C. The colloidal chitin was washed several times with sterile distilled water till pH 7.0. A 1% colloidal chitin was prepared and stored at 4 °C for the chitinase activity experiments.

Chitinase assay mixture consisted of 0.6 mL of sample and 0.4 mL of 1% colloidal chitin in 50 mM sodium acetate buffer (pH 5.0) (Monreal and Reese, 1969). The reaction was maintained at 40 °C for 60 min. Subsequently, the mixture was heated in boiling water bath for 10 min and centrifuged at 10,000 × *g* for 5 min, to remove the insoluble chitin. The resultant reducing sugar was calculated using the dinitrosalicylic acid method (Miller, 1959). The standard curve was generated from known concentrations of GlcNAc (0–100 μg/mL). One unit of chitinase activity was defined as the amount of enzyme that released 1 μmol of GlcNAc per hour. Protein concentrations were measured using the Pierce BCA assay kit (Pierce Biotechnology, Rockford, IL, USA).

2.9.3. Measurement of biomass dry weight

To measure biomass, fermentation broth was mixed well after the end of fermentation and three 250 mL mixtures were taken randomly. The sample was filtered through a preweighed Xinhua No. 1 quantitative filter paper (Hangzhou Xinhua Paper Industry Co., Ltd., Hangzhou, Zhejiang, China), and then washed with sodium chloride solution (1%). The washed biomass was oven dried at 80 °C for 48 h and weighed.

2.9.4. Quantification of fungal spores

Spore production was determined after macerating the fungal precipitates in a Waring blender for 2 min at high speed in PDB (10 mL) and the mixture filtered through glass wool (Pitt and Poole, 1981). Flask containing spore suspension was shaken on a vortex mixer for 1 min. Three 1-mL aliquots were diluted to 5 mL separately. They were then counted by dilution spread plate method. The spore concentration was determined as millions of spores per mL of sample. Culture samples were taken after 1–11 d of incubation for spore quantification.

3. Results

3.1. Susceptibility of ACSS to HygB and G418

The HygB resistance gene (*hph*) has been widely used as a selection marker for the transformation of many filamentous fungi (de Groot et al., 1998; Kemppainen et al., 2005; Michielse et al., 2005, 2008; Rolland et al., 2003; Sugui et al., 2005). Accordingly, we first tested the susceptibility of ACSS to HygB. However, the results showed that ACSS could normally grow on all selective plates with 50–500 μg/mL HygB, suggesting that ACSS was highly resistant to HygB. Therefore, HygB is not a suitable selection antibiotic for the transformation of ACSS.

Subsequently, we tested the susceptibility of ACSS to other antibiotics that had been used in the transformation of other fungi. Fortunately, ACSS was found susceptible to G418, an aminoglycoside antibiotic that specifically inhibits protein synthesis. The growth of ACSS was completely suppressed when the concentration of G418 reached 200 μg/mL. Thus, it was decided to use the G418-resistant gene *npt*II as the selection marker for the genetic transformation of ACSS. In fact, the *npt*II gene has been used for the genetic transformation of filamentous fungi, such as *Fusarium oxysporum* (Inoue et al., 2002) and *Trichophyton mentagrophytes* (Yamada et al., 2008). Consequently, we used 200 μg/mL and 400 μg/mL G418 to screen and preliminarily identify the transformants of ACSS, respectively.

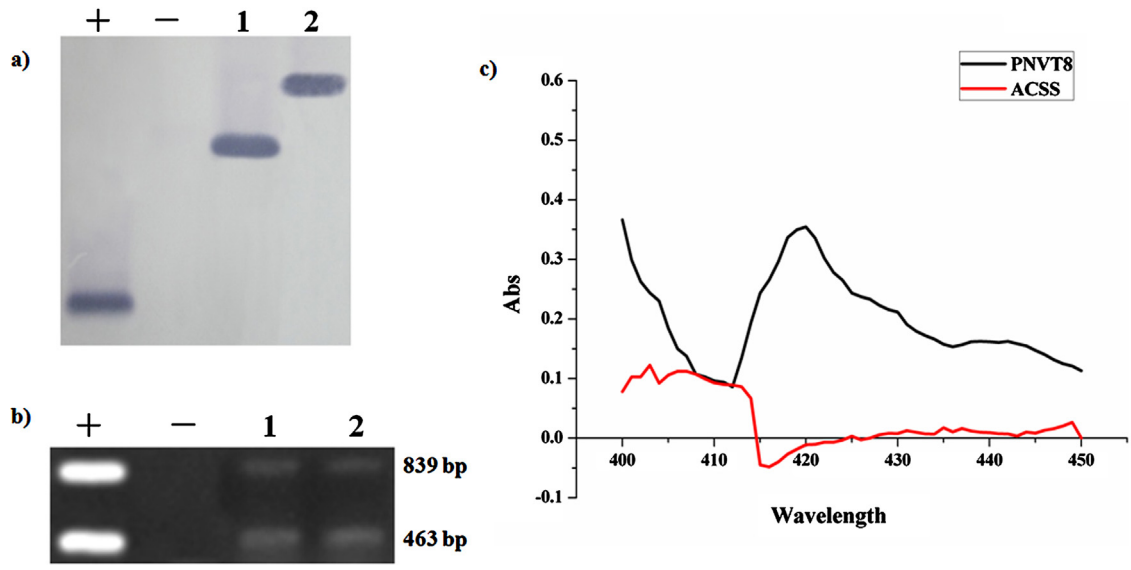

Fig. 1. Transformant verification. (a) Southern-blot analysis of *npt*II gene in transformants of *P. lilacinus* (+, positive control, plasmid puPNVT; −, negative control, original strain ACSS; 1, transformant PNVT8; 2, transformant PNVT18); (b) RT-PCR analysis of transformants of *P. lilacinus* (+, positive control, plasmid puPNVT; −, negative control, original strain ACSS; 1, transformant PNVT8; 2, transformant PNVT18); (c) CO binding spectra of cell extracts from original strain ACSS and transformant PNVT8.

3.2. Characterization of transformants

We randomly selected ten putative transformants to perform the conidial germination assay, and observed that the conidial germination of each transformant was not inhibited by 400 μg/mL G418, while that of the original strain ACSS was completely inhibited by 200 μg/mL G418. These results further verified that the *npt*II gene was a suitable and reliable selection marker for the genetic transformation of ACSS.

To confirm the integration of the T-DNA into the genomes, genomic DNA of ten representative G418-resistant colonies were independently isolated and detected by PCR. As shown in Figs. S3 and S4, each tested transformant contained both the *npt*II and *vgb* genes as expected. However, we failed to amplify the two genes from the original strain ACSS, indicating that the T-DNA had been integrated into the genomes of the tested transformants. The genomic DNA isolated from two representative transformants, PNVT8 and PNVT18, were selected for Southern blotting to determine the integration pattern of T-DNA in the genomes. The single bands in various places (Fig. 1A) revealed that the tested transformants contained a single copy of T-DNA which integrated randomly in the genomes.

RT-PCR result indicated that both *npt*II and *vgb* genes were co-transcripted and transcription levels of the two genes were approximate equal in each transformant (PNVT8 and PNVT18) (Fig. 1 B).

In order to confirm the functional expression of VHb, the supernatant of the cell extracts was subjected to CO-difference spectrum analysis (Fig. 1C). PNVT8 cells showed a typical VHb CO-binding absorbance spectrum with a peak at 420 nm, whereas there was no such peak apparent in the spectrum of the original strain ACSS.

The resistance stability of the transformants was also tested to determine the stability of the integrated T-DNA. It was observed that PNVT8 and other nine representative transformants were still able to grow normally on the PDA plates with 400 μg/mL G418 after continuously growing for five generations without the G418 stress. Thus, it was inferred that the *npt*II-*vgb* gene could stably inherit in the tested transformants.

3.3. Shake flask assay

In the 250 mL shake flask fermentation, PNVT8 grew faster, had more uniform hypha, formed more mycelial pellets, and existed in more turbid culture than the original strain ACSS did under low dissolved oxygen (at a low rotational speed 100 rpm) for 4 d.

The effect of loading volume on the fungus fermentation was also investigated. Fig. 2 shows that the mycelial pellet number decreased with increasing loading volume from 20 mL (20%) to 80 mL (80%), indicating that more dissolved oxygen resulted in more mycelial pellets. This tendency is consistent with most researches. Importantly, PNVT8 can form at least 1.2-fold mycelial pellets compared to the original strain under the same conditions.

Here, diameters of hydrolysis zones formed on the skim milk agar were used to indirectly estimate protease activities. We found

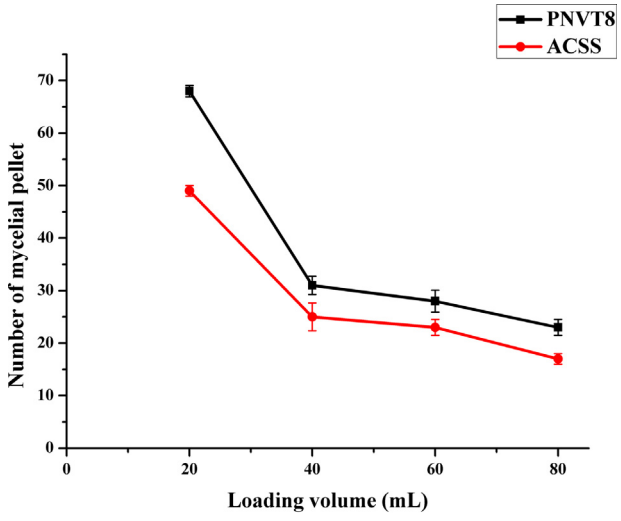

Fig. 2. Relationship between the fluid loading volumes and the numbers of mycelial pellets. Different volumes of PDB were added to each of 100-mL glass infusion bottles (narrow-neck bottle), they were then inoculated with spores at 2.4×10^5 spores/mL. The cultures were incubated at 28 °C for 7 d.

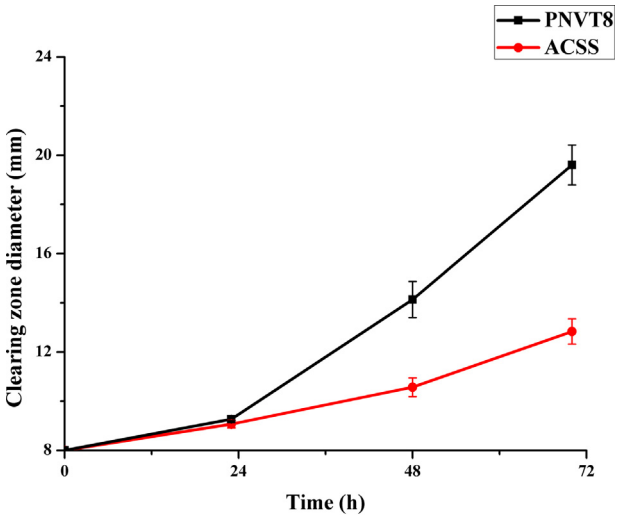

Fig. 3. Comparison of the protease activities of transformant PNVT8 with that of the original strain ACSS. 200 μL crude protease solutions (ten-fold concentrates) of PNVT8 and ACSS from glass infusion bottles with 60 mL PDB were added into oxford cups on skim milk agar plates and incubated at 25 °C, the diameters of clearing zone were measured at 24-h intervals.

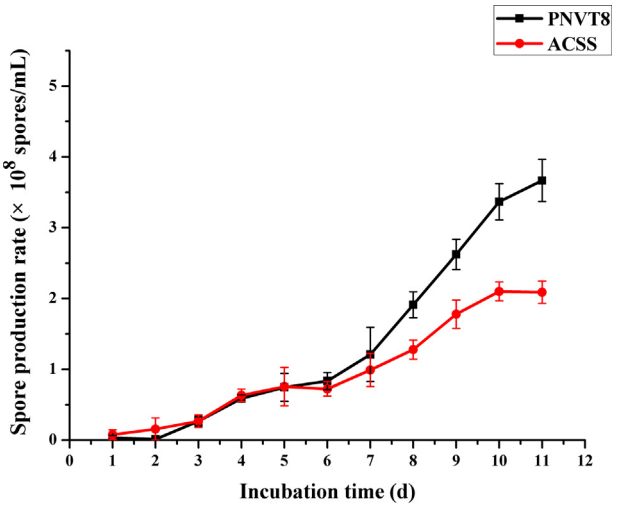

Fig. 5. Comparison of the spore production rates of transformant PNVT8 with that of the original strain ACSS. Bioreactor cultivations were performed in a 15-L in situ sterile bioreactor with an effective working volume of 10 L. The cultivation conditions were 28 °C temperature, 200-rpm agitation, and 0.037 L/min ventilation. 20 mL samples were taken at 24-h intervals. The spore production was determined by dilution spread plate method.

that the hydrolysis zones became visible after incubation with crude protease solutions at 25 °C for about 12 h. The diameter of hydrolysis zone increased with longer incubation time. As shown in Fig. 3, the diameters of the hydrolysis zones formed by the ten-fold concentrates of PNVT8 and ACSS after fermentation for 4 d display significant differences ($P < 0.05$ according to the Dunnet two-sided t test): the hydrolysis zone forming ability of PNVT8 increases by 86% compared to ACSS at 72 h (23.9 ± 0.5 mm *vs* 12.9 ± 0.6 mm). It is likely connected with the increase in secretary protein concentration caused by the VHb expression.

3.4. Bioreactor cultivation

The secreted chitinase and serine protease are the main active substances for attacking nematodes (Gintis, 1983; Khan et al., 2004). The protease activity of PNVT8 was nearly two-folds of that of the original strain as mentioned above. The chitinase activity of fermentation broth at different period is shown on Fig. 4, which demonstrates that PNVT8 yields more chitinase than ACSS at the mycelium growth period. Especially on the first two days of fermentation, the chitinase activity of PNVT8 is about two-folds of that of ACSS (44.2 ± 1.5 U/mL *vs* 23 ± 1.3 U/mL on the first day, and 10.0 ± 1.1 U/mL *vs* 33.0 ± 1.4 U/mL on the second day).

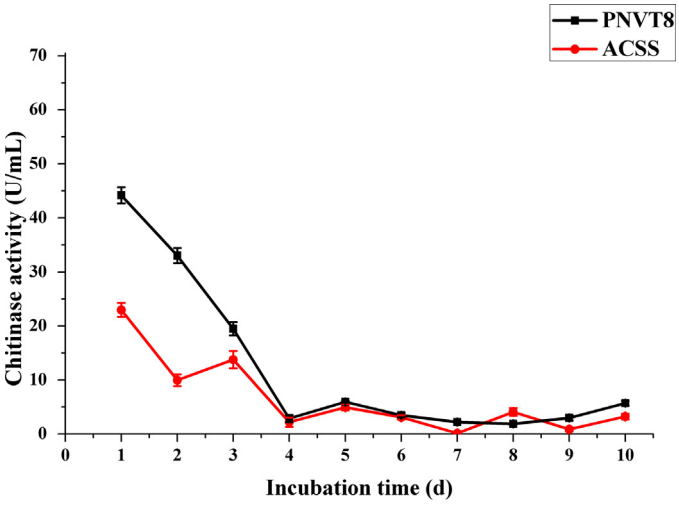

Fig. 4. Comparison of the chitinase activities of transformant PNVT8 with that of the original strain ACSS. Bioreactor cultivations were performed in a 15-L in situ sterile bioreactor with an effective working volume of 10 L. The cultivation conditions were 28 °C temperature, 200-rpm agitation, and 0.037 L/min ventilation. 20 mL samples were taken at 24-h intervals. The method for chitinase activity determination was described in the experimental Section 2.8.

Biomass and the spore production reflected the same tendency after transferring *vgb*; at the end of fermentation, PNVT8 acquired 11.0% larger biomass than ACSS (90.02 ± 0.67 g *vs* 81.27 ± 0.14 g). Fig. 5 shows that from the 6th day of fermentation, the spore production of PNVT8 increased gradually and exceeded that of ACSS. At the end of fermentation, spore production of PNVT8 was at least 1.5 times higher than that of ACSS ($(3.7 \pm 0.3) \times 10^8$ spores/mL *vs* $(2.1 \pm 0.2) \times 10^8$ spores/mL).

4. Discussion

In order to efficiently express VHb in *P. lilacinus*, we designed and optimized *vgb* gene. The amino acid sequence (Genbank accession no. AAA75506) was translated back into a homologous DNA sequence. Because of the codon degeneracy, a practical consequence error in the third position of the triplet codon would not affect the encoded amino acid. Consequently, the DNA sequence was changed according to the codon preference in the filamentous fungus. Simultaneously, we optimized the sequence to keep both the lengths of repeated sequences and the stem lengths of stem-loop structures to be less than seven. Thus, the transcripted mRNA is more stable and has high translation efficiency.

In this study, we fused the *npt*II and *vgb* genes together using a connective peptide (amino acids sequence, GPGGGSGGGGS) and used the same promoter P*gpd*A and the same terminator T*trp*C and inserted the expression cassette P*gpd*A-*npt*II-*vgb*-T*trp*C behind the original cassette P*gpd*A-*hph*-T*trp*C (Fig. S2). The fusion expression was succesful and efficient. There are many advantages for this: P*gpd*A and T*trp*C are highly active (Wang et al., 2010); *hph* gene is also expressed and useful if required; repeated vector construction and transformation are avoided; the detection of the gene *vgb* or *vgb* expression is more convenient because closely-linked gene *npt*II can be indirectly detected (Fig. 1A).

Previous studies showed that the transformation efficiencies mediated by different *A. tumefaciens* strains in different fungi were various. For example, the transformation efficiency of the strain AGL-1 was higher than that of the strain LBA1100 in *Laccaria bicolor* (Kemppainen et al., 2005) and the efficiency of strain AGL-1 was higher than the strain C58C1 in *Colletotrichum graminicola* (Flowers and Vaillancourt, 2005). In this study, we compared the transformation efficiencies of *A. tumefaciens* strains EHA105 and LB4404 in ACSS. The results showed no significant differences ($P > 0.05$). Consequently, we only used *A. tumefaciens* EHA105 to transfer T-DNA from its tumor-inducing plasmid to a recipient's genome. The tumor-inducing plasmid contains a set of genes termed *vir* that are essential for the T-DNA transfer (de Groot et al., 1998). Induction of the *vir* genes by certain chemical signals, such as AS, initiates the process of transfer and integration of the T-DNA into the recipient's genome (de Groot et al., 1998). Consequently, the effect of AS on the ATMT of ACSS was analyzed. Once AS was added, the high numbers of transformants could be obtained, while no transformant could be observed in the absence of AS during co-cultivation. So, AS was necessary for the ATMT of ACSS.

The submerged fermentation experiments verified that transformant PNVT8 grew well, produced more than 11.0% biomass, secreted protease and chitinase to nearly two-folds, and had 1.5 times higher spore production compared to the original strain ACSS. The results showed that the expression of VHb not only increased production traits of *P. lilacinus*, but also improved the product quality and application value as a result of the secretion of more protease and chitinase.

Although killing mechanism of *P. lilacinus* against plant-parasitic nematodes has not been well demonstrated, electron microscopy observation found that *P. lilacinus* could secret chitinase and serine protease while attacking nematodes (Yang et al., 2011). Specifically, chitinase could hydrolyze the innermost layer of the egg to destroy its integrity and then brought big vacuoles to chitinous layer, exposing lipid layer on the inner layer. Simultaneously, serine protease could substantially alter the structure of egg chorion, completely vanished lipid layer, adequately hydrolyzed chitinous layer and finally resulted in egg death (Gintis, 1983; Khan et al., 2004). Our results showed that the protease and chitinase activities of PNVT8 were obviously higher than those of the original strain, which indicates that PNVT8 possesses a higher nematode-killing activity. Our preliminary results were in good accordance with the inference, and to further confirm it, we are now carrying out on a large number of nematode-killing experiments.

The mechanism of VHb on enhancing oxygen utilization has not been fully known, and several hypotheses were proposed. Wittenberg (1966) claimed that hemoglobin enhanced oxygen utilization by facilitating the diffusion of oxygen. In 1990, it was claimed that VHb improved the efficiency of ATP production in oxygen-limited *E. coli* and two possible models for the mechanism of action of VHb: the facilitated diffusion hypothesis and the intracellular redox effector hypothesis, were proposed (Khosla et al., 1990). Afterwards, Chinese scientists proposed terminal electron acceptor hypothesis that VHb works as terminal electron acceptor and thus increases the number of whole terminal electron acceptors in terminal respiratory chain in *E. coli* (Wu and Yang, 1997). Recently, some scholars found that VHb not only possesses the capacity of delivery oxygen but also has peroxidase feature *in vitro* (Kvist et al., 2007; Isarankura-Na-Ayudhya et al., 2010; Liao et al., 2014), which promotes intimacy between VHb and oxygen utilization. In short, VHb can take part in one or more steps of respiratory chain. By regulating relevant breathing oxidase, VHb is able to improve the efficiency of oxidative phosphorylation and then refresh intracellular metabolic processes, which facilitates cell growth and promotes protein expression under low oxygen condition. Even so, in-depth mechanisms of VHb on enhancing oxygen utilization should be constantly excavated in the future.

One of the important characteristics of *P. lilacinus* is that it has strong colonization ability in soil and strong parasitic ability to plant-parasitic nematodes. The transformant PNVT8 can efficiently expresse VHb, so we can speculate that once PNVT8 colonizes in soil, it will have a growth advantage over the original strain and therefore will possess higher control efficiency against plant-parasitic nematodes under anaerobic conditions, it had been verified by us in a short-term survival survey and the long-term survival experiments are being undertaken.

5. Conclusion

In this study, we optimized *vgb* gene, fused it with a selection marker gene *npt*II, and transferred the expression cassette P*gpd*A-*npt*II-*vgb*-T*trp*C into *P. lilacinus* ACSS by ATMT. Consequently, we successfully selected an applicable transformant PNVT8 which can efficiently express VHb. Importantly, the submerged fermentation experiments demonstrated that the expression of VHb not only increased the production traits of *P. lilacinus*, such as biomass and spore production, but also improved the beneficial product quality and application value due to the secretion of more protease and chitinase.

Conflict of interest

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2014.07.438.

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