Journal of Biotechnology 187 (2014) 147–153

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

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

Shumeng Zhang [a], Jieping Wang [a,b], Yale Wei [a], Qing Tang [a], Maria Kanwal Ali [a], Jin He [a,*]

[a] State Key Laboratory of Agricultural Microbiology, College of Life Science and Technology, Huazhong Agricultural University, Wuhan, Hubei 430070, PR China
[b] Agricultural Bio-resource Institute, Fujian Academy of Agricultural Sciences, Fuzhou, Fujian 350003, PR China

ARTICLE INFO

Article history:
Received 6 May 2014
Received in revised form 18 July 2014
Accepted 23 July 2014
Available online 1 August 2014

Keywords:
Paecilomyces lilacinus
Vitreoscilla hemoglobin (VHb)
Agrobacterium tumefaciens-mediated transformation (ATMT)
Fermentation, Biomass
Spore production

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

* Corresponding author.
E-mail addresses: hejin@mail.hzau.edu.cn, hejinhz@yahoo.com (J. He).

http://dx.doi.org/10.1016/j.jbiotec.2014.07.438
0168-1656/

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).