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Construction of a Genetic System for *Streptomyces albulus* PD-1 and Improving Poly(ε-L-lysine) Production Through Expression of *Vitreoscilla* Hemoglobin

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Poly(ε-L-lysine) (ε-PL) is a novel bioactive polymer secreted by filamentous bacteria. Owing to lack of a genetic system for most ε-PL-producing strains, very little research on enhancing ε-PL biosynthesis by genetic manipulation has been reported. In this study, an effective genetic system was established *via* intergeneric conjugal transfer for *Streptomyces albulus* PD-1, a famous ε-PL-producing strain. Using the established genetic system, the *Vitreoscilla* hemoglobin (VHb) gene was integrated into the chromosome of *S. albulus* PD-1 to alleviate oxygen limitation and to enhance the biosynthesis of ε-PL in submerged fermentation. Ultimately, the production of ε-PL increased from 22.7 g/l to 34.2 g/l after fed-batch culture in a 5 L bioreactor. Determination of the oxygen uptake rate, transcriptional level of ε-PL synthetase gene, and ATP level unveiled that the expression of VHb in *S. albulus* PD-1 enhanced ε-PL biosynthesis by improving respiration and ATP supply. To the best of our knowledge, this is the first report on enhancing ε-PL production by chromosomal integration of the VHb gene in an ε-PL-producing strain, and it will open a new avenue for ε-PL production.

Keywords: Poly(ε-L-lysine), submerged fermentation, *Vitreoscilla* hemoglobin, dissolved oxygen, *Streptomyces albulus*, genetic system

Introduction

Poly(ε-L-lysine) (ε-PL) is one of the four homopoly (amino acid)s discovered in nature until now. It is linked by the isopeptide bond between ε-amino and α-carboxyl groups of L-lysine residues [8]. Owing to its excellent properties, including antimicrobial activity, biodegradability, water solubility, edibility, and non-toxicity to humans and the environment, ε-PL has been used in many fields, such as food, medicine, cosmetics, and electronics [2, 26, 27]. As a novel biopolymer, ε-PL is mostly synthesized by filamentous bacteria through submerged fermentation, and various studies have reported on the achievement of efficient production of ε-PL, including pH regulation, dissolved oxygen (DO) regulation, *in situ* adsorption, and cheap raw materials application [1, 12, 16, 24, 25, 35]. However, because of the lack of a genetic system for most ε-PL-producing strains, very little research on improving ε-PL production by genetic manipulation has yet been conducted.

In the fermentation of *Streptomyces albulus* PD-1, a famous ε-PL-producing strain, it was shown that the DO concentration was a significant parameter for ε-PL production, and a higher DO level (about 30% saturation) was beneficial to ε-PL biosynthesis [35]. However, owing to the intertwined hyphae and high cell density, the culture broth became viscous during the fermentation process and oxygen transfer was limited, thus inhibiting cell growth and ε-PL biosynthesis. Although this issue can be partially settled by increasing agitation rates, the corresponding high shear stress will cause undesirable effects on the mycelium, and thus decrease product yields [7, 13]. Besides this, the

increasing agitation rate will also lead to additional energy cost. Thus, an effective approach is urgently needed to solve the oxygen limitation problem in ε-PL production. With the development of genetics and molecular biology, genetic manipulation provides new strategies that can complement the traditional methods to solve some traditional chemical engineering problems in bioprocesses. However, as mentioned above, owing to the lack of a genetic system for most ε-PL-producing strains, no research on solving the oxygen limitation by genetic manipulation has yet been reported in the ε-PL production process.

Vitreoscilla hemoglobin (VHb) was the first bacterial hemoglobin discovered in nature [31]. Its main function is to bind oxygen, especially under oxygen-limited conditions, and deliver the oxygen to the terminal respiratory oxidase, thus enhancing bacterial respiration and oxidative phosphorylation [4, 21]. Recent studies have demonstrated that heterogeneous expression of VHb can significantly enhance the production of many valuable compounds in microorganisms [11, 17, 28, 29, 39]. Thus, whether the heterogeneous expression of VHb could improve *S. albulus* PD-1 to produce more ε-PL will be of great significance.

In the present study, to open up the possibility of genetic manipulation for *S. albulus* PD-1, we directed our efforts toward the development of a genetic system for it. By using the established system, the VHb expression cassette was integrated into the chromosome of the ε-PL-producing strain for the first time. The fermentation results indicated that the ε-PL titer was significantly improved by the expression of VHb. Furthermore, to investigate the effects of VHb on *S. albulus* PD-1 in submerged fermentation, the oxygen uptake rate, transcriptional levels of ε-PL synthetase gene, and ATP level were also detected. This study would open a new avenue for enhancing ε-PL biosynthesis by genetic manipulation and become a good example for solving oxygen-limited problems in submerged fermentation processes.

Materials and Methods

Microorganisms, Plasmids, and Media

S. albulus PD-1 (Accession No. M2011043), a well-known ε-PL-producing strain, was employed as the wild-type strain in this study. *Escherichia coli* ET12567 (pUZ8002) was employed as the donor in intergeneric conjugal transfer. The site-specific integration vector, pIB139, which is a pSET152 derivative with a strong constitutive *ermE** promoter, was used for intergeneric conjugal transfer and VHb expression.

Luria-Bertani (LB) medium (yeast extract, 5 g/l; tryptone, 10 g/l; NaCl, 10 g/l) was used for *E. coli* cultivation. Mannitol-soy flour (MS) agar medium (mannitol, 20 g/l; soy flour, 20 g/l; agar, 20 g/l), AS-1 (soluble starch, 5 g/l; yeast extract, 10 g/l; L-arginine, 0.5 g/1; L-alanine, 0.2 g/l; Na_2SO_4, 10 g/l; NaCl, 2.5 g/l; agar, 20 g/l; pH 7.5), ISP-2 (yeast extract, 4 g/l; malt extract, 10 g/l; glucose, 4 g/l; agar, 20 g/l), and ISP-4 (soluble starch, 10 g/l; $(NH_4)_2SO_4$, 2 g/l; $MgSO_4{\cdot}7H_2O$, 2 g/l; $CaCO_3$, 2 g/l; NaCl, 1 g/l; K_2HPO_4, 1 g/l; $ZnSO_4$, 1 mg/l; $FeSO_4{\cdot}7H_2O$, 1 mg/l; $MnCl_2$, 1 mg/l; agar, 20 g/l; pH 7.2) media were used for conjugal transfer. Medium 3G (M3G) (glucose, 50 g/l; $(NH_4)_2SO_4$, 10 g/l; yeast extract, 5 g/l; KH_2PO_4, 1.36 g/l; K_2HPO_4, 0.8 g/l; $MgSO_4{\cdot}7H_2O$, 0.5 g/l; $ZnSO_4{\cdot}7H_2O$, 0.04 g/l; $FeSO_4{\cdot}7H_2O$, 0.03 g/l; pH 6.8) was used for seed culture, as well as for fed-batch fermentation of *S. albulus* PD-1.

Conjugal Transfer Method

Conjugal transfer was conducted as described previously with some modifications [15]. *E. coli* ET12567 (pUZ8002) harboring plasmid pIB139 was grown in 10 ml of LB medium to an OD_{600} of 0.45 in the presence of kanamycin (50 μg/ml), chloramphenicol (50 μg/ml), and apramycin (50 μg/ml). Cells were then washed twice to remove the antibiotics and were subsequently resuspended in 500 μl of fresh LB medium. While washing the *E. coli* cells, *S. albulus* PD-1 spores (about 10^8) were suspended in 500 μl of LB medium. Subsequently, *E. coli* donor cells and *S. albulus* PD-1 spores were mixed thoroughly and spread on 20 ml of agar medium (MS, ISP-2, ISP-4, or AS-1) containing 10 mM $MgCl_2$. The plates were incubated at 30°C for about 18 h and then overlaid with 1 ml of sterile water containing 0.05 mg of nalidixic acid and 0.1 mg of apramycin. The plates were further incubated at 30°C for about 3 days, and the ex-conjugants were counted.

Construction of Recombinant Plasmid pIB139-*vgb*

The VHb gene (*vgb*) (Accession No. AF292694, gifted by the Institute of Biochemistry and Cell Biology, SIBS, CAS, China) was amplified by PCR using the following primers: P1 (5'-GGA ATTCCATATGGTGCTGGACCAGCAAAC-3', *Nde*I site underlined) and P2 (5'-GCTCTAGATTATTCAACCGCTTGAG-3', *Xba*I site underlined). Subsequently, the PCR product was digested with *Nde*I and *Xba*I, gel purified, and ligated into the corresponding sites of pIB139 to generate pIB139-*vgb* (Fig. 1A). The plasmid pIB139-*vgb* was identified by restriction digestion and DNA sequencing. Then, the identified recombinant plasmid was introduced into *S. albulus* PD-1 *via* the conjugal transfer method mentioned above.

Cultivation Conditions

Submerged fermentation experiments were performed in flasks and a bioreactor. For seed cultures, a loop of 1-week-old fully grown spores was inoculated into 100 ml of M3G medium contained in a 500 ml flask and then incubated at 30°C and 200 rpm for 24 h as stock culture. To explore the VHb effects on ε-PL biosynthesis in flask fermentation, especially under oxygen-limited conditions, 10% seeds were inoculated into 45, 90, 135, and 180 ml of M3G medium in 500 ml flasks, and the yield of ε-PL was

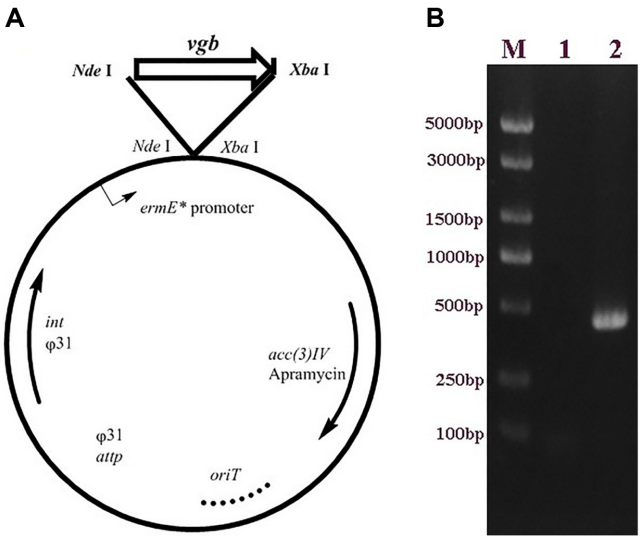

Fig. 1. (**A**) Construction of recombinant plasmid pIB 139-*vgb*. and (**B**) PCR amplification for identification of the ex-conjugant. Lane M, DL 5000 marker; lane 1, the PCR fragment using the chromosome of *S. albulus* PD-1 as template; lane 2, the PCR fragment using the chromosome of ex-conjugant as template.

determined after the cultivation was incubated at 30°C and 200 rpm for 72 h. Fed-batch fermentations were conducted in a 5 L bioreactor (KoBio Tech Co., Ltd., Korea) by using a two-stage pH control strategy [12, 34]. In the first stage, the pH was controlled at 6.0 for cell growth, and in the second stage, the pH was controlled at 4.0 for ε-PL biosynthesis. When the glucose concentration in the culture broth decreased to about 10 g/l, the feeding solution (glucose, 500 g/l; $(NH_4)_2SO_4$, 50 g/l) was pumped into the broth to keep the glucose concentration at approximately 10 g/l.

Determination of Cell Growth, Glucose Concentration, pH, DO, and ATP Level

Cell growth was measured in terms of dry cell weight (DCW). The harvested culture sample was filtered; the mycelia were washed and dried at 65°C until constant weight was achieved. Glucose concentration in the culture broth was determined by using a biosensor (SBA-40C; Shandong Science Academy, China). The yield of ε-PL was determined by high-performance liquid chromatography, following the method reported previously [34]. Intracellular ATP concentration was quantified by a chemiluminescence response method as reported before [35]. The pH and DO were measured by probes of the bioreactor. The oxygen uptake rate (OUR) of bacteria was analyzed with a process mass spectrometer (SHP8400PMS-162R). All assays were performed in triplicate, and experimental errors were <5%.

Analysis for the Biological Activity of VHb

The CO-difference spectral analysis was performed to determine the biological activity of VHb according to the previously described method [39].

Analysis of the Transcriptional Level of ε-PL Synthetase Gene

As the fermentation process reached 110 h, the maximum specific ε-PL production rate was reached. At this moment, the transcriptional level of the ε-PL synthetase gene (*pls*) was determinated by quantitative real-time PCR (qRT-PCR) according to the method mentioned before [35]. The relative gene transcriptional level was calculated by the $2^{-\Delta\Delta Ct}$ method, with *hrdB* (a housekeeping sigma factor gene) as the endogenous control gene [32, 38]. For *pls* and *hrdB*, the following primers were used: *pls*-F, 5'-CGGATTCGTCCAACTCCT-3' and *pls*-R, 5'-GACGATGATCAGCCACCA-3'; *hrdB*-F, 5'-CGACTACACCAAGGGCTACA-3' and *hrdB*-R, 5'-TTGTTGATGACCTCGACCAT-3'.

Results and Discussion

Construction of a Genetic System for *S. albulus* PD-1

With the development of molecular biology, intensive genetic engineering applications have been applied in biotechnology [19, 20]. In such applications, the target DNA fragments should be transferred into candidate cells first. Streptomycetes are well known for their ability to produce many valuable secondary metabolites. In order to obtain the genetically modified streptomycetes, one of the key challenges is delivering the target DNA fragments into living streptomycetes cells efficiently. However, owing to the slow growth and thick cell wall of most streptomycetes, the genetic system of these organisms has obstacles compared with manipulations in *E. coli*, yeast, and other commonly used industrial strains [18]. With the continuous efforts of researchers, some kinds of genetic systems were developed for streptomycetes, including polyethylene glycol-mediated method, electrotransformation, and intergeneric conjugal transfer [5, 10, 18, 22, 23]. Among the three methods, intergeneric conjugal transfer has been performed in many streptomycetes because of its high transformation efficiency, and reproducibility. Thus, we attempted to construct an intergeneric conjugal transfer system for *S. albulus* PD-1.

It has been reported that the type of medium used has a significant effect on the efficiency of conjugation. Thus, four representative media (AS-1, ISP-2, ISP-4, and MS) were used for conjugal transfer for *S. albulus* PD-1 [3, 22]. As shown in Table 1, the highest transformation frequency ($4.0 \pm 0.5 \times 10^{-7}$ per recipient) was observed when MS medium was used for conjugal transfer. Thus, MS medium was selected as the most appropriate medium and was

Table 1. Effect of medium type on the intergeneric conjugation efficiency.

Medium	Transformation frequency
AS-1	$(6.0 \pm 0.3) \times 10^{-8}$
ISP-2	$(2.0 \pm 0.5) \times 10^{-8}$
ISP-4	0
MS	$(4.0 \pm 0.5) \times 10^{-7}$

employed in the subsequent experiments. To confirm whether pIB139 was integrated into the chromosome of *S. albulus* PD-1, the apramycin resistance gene was amplified from the chromosome of transformants, but not from wild-type strain *S. albulus* PD-1 (data not shown). In particular, the integration of plasmid pIB139 had no effect on cell growth and ε-PL production of *S. albulus* PD-1, and it was stable after passing several generations. In previous studies, Hamano *et al.* [9] made a lot of effort to construct the genetic system for *S. albulus* IFO 14147, which is the only ε-PL-producing strain owning the genetic systems. In their studies, they spent much time constructing free-replicating plasmids for *S. albulus* IFO 14147. Even though the free-replicating plasmids have many uses for genetic operation, stable maintenance of a free-replicating plasmid requires selection *via* expensive antibiotics. Compared with free-replicating plasmids, the plasmid pIB139 (capable of integration into φ31 attB site in *S. albulus* PD-1) could be more stable in streptomycetes, which would not only reduce the cost of additional antibiotics but also prevent the contamination caused by antibiotics. Thus, pIB139 was used for VHb expression in the following work.

Construction of *S. albulus* PD-2

For heterologous expression of VHb in *S. albulus* PD-1, the *vgb* was cloned and placed under the control of *ermE** promoter in plasmid pIB139 to create pIB139-*vgb* (Fig. 1A). Subsequently, the plasmid pIB139-*vgb* was introduced into *S. albulus* PD-1 *via* the established genetic system. The ex-conjugant was designated as *S. albulus* PD-2, which was verified by PCR amplification by using the primer pair P1/P2.

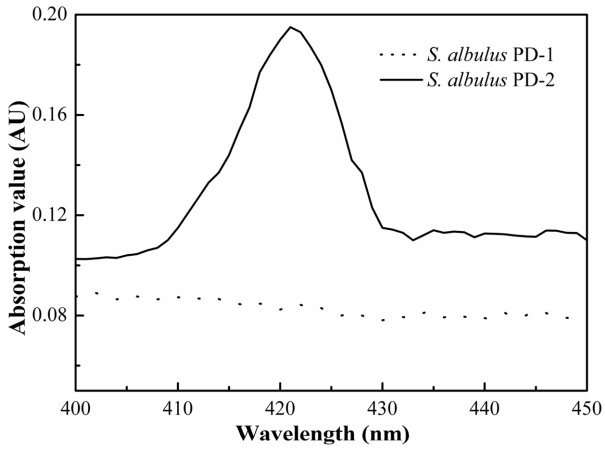

Fig. 2. CO-difference spectral analysis of crude extracts of *S. albulus* PD-1 and *S. abulus* PD-2.

A PCR product of 450 bp was obtained during electrophoresis when chromosomal DNA from *S. albulus* PD-2 was used as the PCR template, whereas no band was observed when chromosomal DNA from *S. albulus* PD-1 was used (Fig. 1B). This observation confirmed that the *vgb* had been successfully integrated into the chromosome of *S. albulus* PD-2.

The biological activity of VHb was measured using the CO-difference spectra. The CO-difference spectra showed a significant VHb CO-binding absorbance peak at 420 nm from the crude cell extract of *S. albulus* PD-2 compared with that of the wild-type strain (Fig. 2). This typical peak of VHb protein demonstrated that the expressed VHb in *S. albulus* PD-2 was biologically active [17, 39]. Taken together, by using the established genetic system, the biologically active VHb was successfully expressed in *S. albulus* PD-2.

Comparison of ε-PL Production for Different Loading Volumes in Flask Culture

Heterologous expression of VHb has been demonstrated in many strains to improve cell growth and secondary metabolite productivity, especially under oxygen-limited conditions [33, 39]. To reveal the effect of VHb expression on ε-PL production, *S. albulus* PD-1 and *S. albulus* PD-2

Table 2. Summary of ε-PL yield in different volumes of culture broth.

Liquid volume (ml in 500 ml flask)	DCW (g/l)		Yield of ε-PL (g/l)		ε-PL increase rate
	S. albulus PD-1	*S. albulus* PD-2	*S. albulus* PD-1	*S. albulus* PD-2	
50	10.36 ± 0.31	11.27 ± 0.26	1.21 ± 0.07	1.41 ± 0.08	0.16
100	9.92 ± 0.24	10.58 ± 0.27	0.91 ± 0.05	1.27 ± 0.06	0.39
150	8.95 ± 0.17	9.91 ± 0.21	0.76 ± 0.04	1.17 ± 0.06	0.54
200	8.52 ± 0.21	9.43 ± 0.17	0.43 ± 0.02	0.98 ± 0.05	1.28

were cultured in 500 ml flasks first, with increasing loading volumes from 50 ml to 200 ml. As shown in Table 2, the loading volume had a strong effect on cell growth and ε-PL production. In all these cases, the concentration of ε-PL and DCW decreased with the increasing loading volume. According to a report, the increasing liquid volume decreases the volumetric oxygen mass transfer rate and leads to oxygen limitation [30]. This phenomenon could be the main reason for the decrease in ε-PL production and cell growth. However, the expression of VHb in *S. albulus* PD-2 was functional for reducing the unsatisfactory effect caused by the high liquid volume. In addition, when the liquid volume was increased, the effect of VHb was more significant. With 50 ml of broth in a 500 ml flask, the expression of VHb could only contribute to 0.16-fold increase of ε-PL titer. However, with 200 ml of broth in a 500 ml flask, the expression of VHb could lead to as much as 1.28-fold increase of ε-PL titer. Thus, our results supported that VHb expression in *S. albulus* PD-2 can solve the problems caused by limited oxygen and eventually increase ε-PL production.

Performance of Fed-Batch Operation Using Recombinant Strain

To verify the feasibility of VHb expression in actual production, *S. albulus* PD-1 and *S. albulus* PD-2 were further incubated in a 5 L bioreactor. As illustrated in Fig. 3C, for both strains, the DO concentrations in the culture broth decreased continuously at the early stage of the fermentation period. Finally, the DO concentration maintained at about 23.5% after 55 h, compared with 18.9% after 52 h in *S. albulus* PD-2 fermentation process. In another words, the DO level in the culture broth of *S. albulus* PD-2 was lower compared with that in *S. albulus* PD-1. A similar phenomenon has been reported for *S. diastatochromogenes* [17]. In previous studies, researchers found that VHb can increase the intracellular effective DO concentrations by allowing a more effective intracellular delivery of oxygen [6, 28]. The lower DO in *S. albulus* PD-2 culture broth may be because more oxygen was transported to cells and metabolized rapidly. Therefore, the oxygen uptake rate (OUR) values of the wild-type strain and of *S. albulus* PD-2 were determined. As depicted in Fig. 3D, the OUR values of *S. albulus* PD-2 were higher than those of the wild-type strain regardless of the limited DO concentration during the 55th–168th h or not (0–55 h). The high OUR supported the increased respiration of the recombinant strain with VHb expression. The enhancement of respiration will provide more energy for both cell growth and ε-PL biosynthesis. These results explained the growth advantage (Fig. 3B) and high ε-PL production (Fig. 3A) exhibited by *S. albulus* PD-2.

Ultimately, *S. albulus* PD-2 obtained a final biomass concentration of 33.4 g/l and ε-PL of 34.2 g/l, which corresponded to 27.5% and 50.7% increase, respectively, compared with the wild strain. Moreover, the specific ε-PL production rate of *S. albulus* PD-2 was higher than that of the wild-type strain (Fig. 3E), indicating that the improvement of ε-PL production was not only due to the higher biomass volume of *S. albulus* PD-2 (Figs. 3B and 3F) but that the expression of VHb also strengthened the ε-PL synthesis ability of the signal cell. Recently, oxygen-vectors were added in culture broth to alleviate the oxygen-limited problem in the fermentation process of *S. albulus* PD-1 [35]. As a result, 30.8 g/l of ε-PL could be produced with 0.5% *n*-dodecane in the culture broth. Compared with oxygen-vector applications, the expression of VHb not only resulted in a higher ε-PL production but also saved the cost brought by oxygen-vectors addition. Thus, the expression of VHb provides a new strategy that can complement the existing methods for alleviating low DO concentrations in the ε-PL production process.

Changes of ATP Level in *S. albulus* PD-2

Until now, several hypotheses have been raised to elucidate the function of VHb. The dominant view at present is that VHb could bind oxygen and deliver the oxygen to the terminal respiratory oxidase and/or oxygenases, thus enhancing bacterial respiration and oxidative phosphorylation [4, 14, 21]. In short, VHb can take part in one or more steps of the respiratory chain and thus promote respiration of bacteria. The increase in OUR of *S. albulus* PD-2 agreed with those perspectives, and the enhancement of respiratory action may probably lead to a higher ATP, which is an essential cofactor in ε-PL biosynthesis. To verify this speculation, the ATP level was also determined during the fermentation process.

Fig. 4 shows the ATP level of *S. albulus* PD-2 and the wild-type strain. Amongst the variation trends, the ATP level in *S. albulus* PD-2 was higher than that of the wild-type strain at all times. The fact that high ATP level is a benefit for ε-PL synthetase (Pls) has been demonstrated in some ε-PL-producing strains [35, 36]. As reported, ATP regulates ε-PL in two ways: first, ATP is directly involved in the assembly of ε-PL through activing lysine to lysyl-*O*-AMP [37]; second, a high ATP level is essential for the expression of *pls* [36]. The increase of transcriptional level of *pls* in *S. albulus* PD-2 also validated the improvement of ATP level indirectly (Fig. 5). Thus, we hold the opinion that

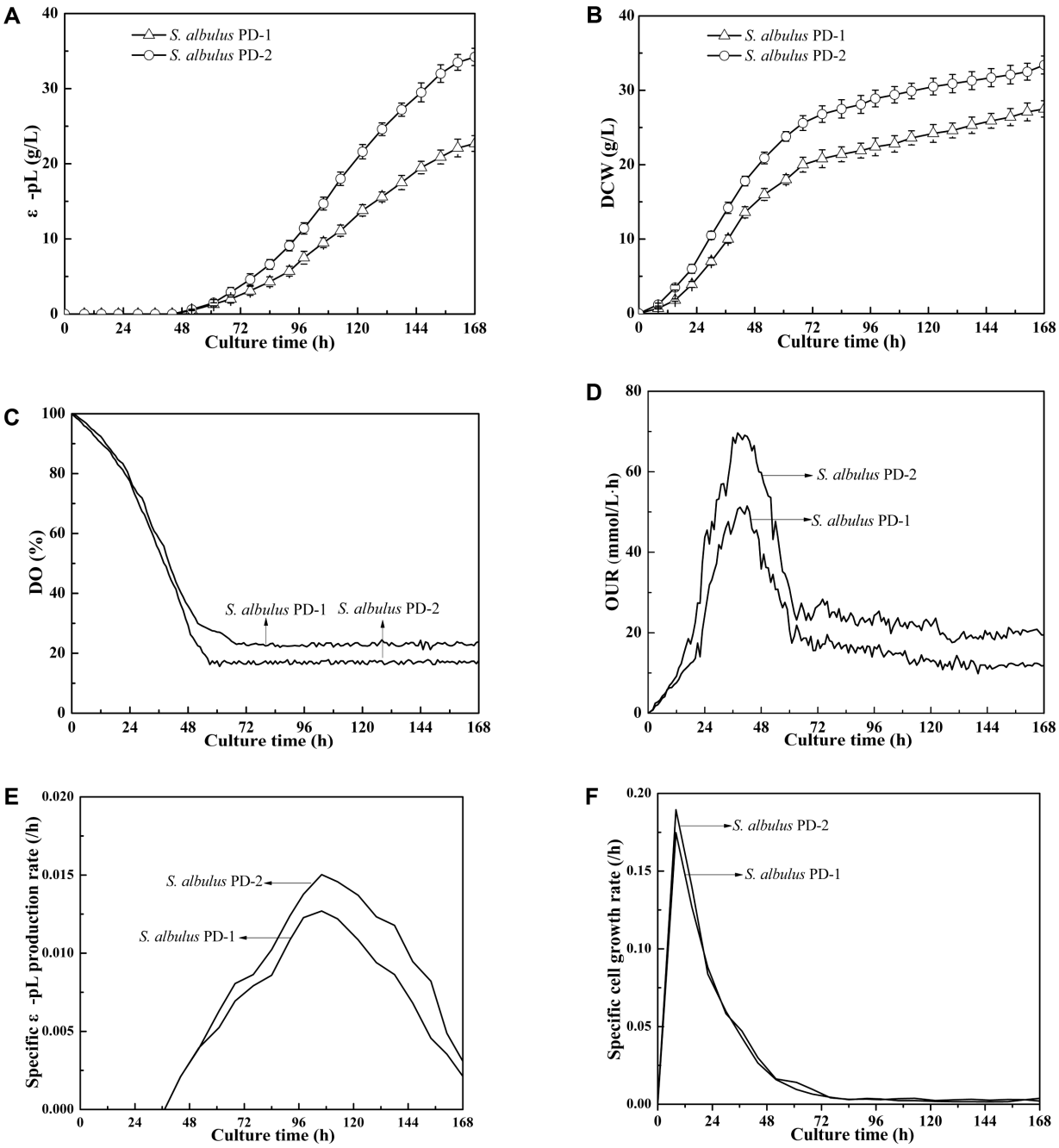

Fig. 3. Time curve analysis of ε-PL production (**A**), cell growth (**B**), DO concentration (**C**), OUR (**D**), specific ε-PL production rate (**E**), and specific cell growth rate (**F**) of *S. albulus* PD-1 and *S. albulus* PD-2 in a 5 L bioreactor culture experiment.

with the expression of VHb in *S. albulus* PD-2, the respiratory action was enhanced and more ATP was generated. The high ATP level in *S. albulus* PD-2 stimulated ε-PL biosynthesis by improving the transcriptional level of *pls* and activing more lysine to participate in ε-PL assembly.

In conclusion, in the present study, we constructed a genetic system for *S. albulus* PD-1. By using the established genetic system, *vgb* was integrated into the chromosome of *S. albulus* PD-1 for the first time. The expression of VHb in *S. albulus* PD-1 relieved the unsatisfactory effect caused by limited oxygen in the culture broth, and significantly enhanced ε-PL production compared with the wild-type strain. By measuring the oxygen uptake rate, the transcriptional

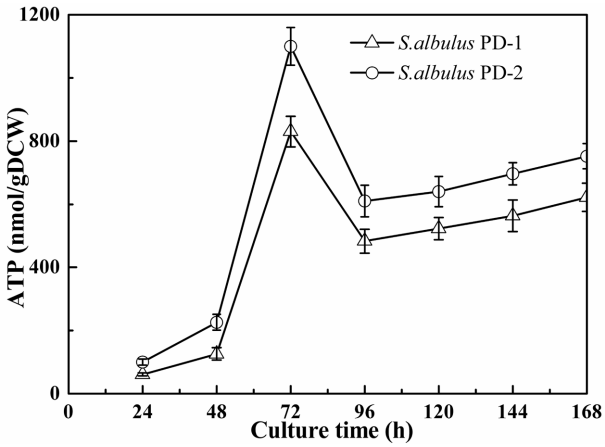

Fig. 4. Comparison of ATP level changing patterns between *S. albulus* PD-1 and *S. albulus* PD-2 in a 5 L bioreactor culture experiment.

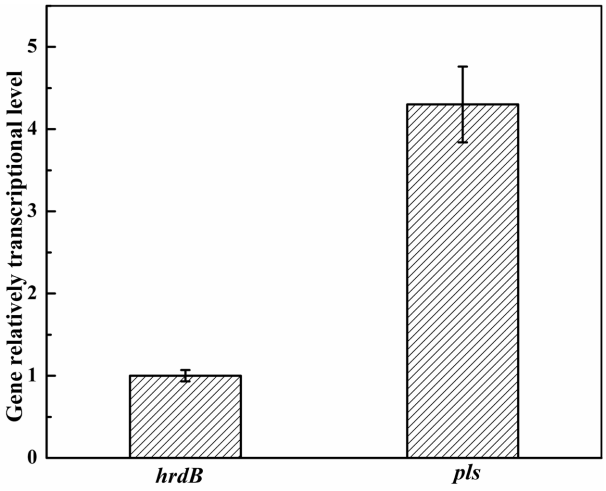

Fig. 5. qRT-PCR analysis of the change of *pls* transcriptional level with VHb expression.

level of *pls*, and ATP level, it can be concluded that the expression of VHb in *S. albulus* PD-1 enhanced ε-PL biosynthesis through improving respiration and the ATP supply. We believe that this study will open a new avenue for ε-PL production by genetic engineering.

Acknowledgments

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