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# Expression of bacterial hemoglobin in the yeast, *Pichia pastoris*, with a low $O_2$-induced promoter

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**Abstract**

The *PsADH2*-promoter of *Pichia stipitis* alcohol dehydrogenase II (ADH II) gene was employed to control the expression of *Vitreoscilla* hemoglobin (VHb) gene in *Pichia pastoris*. As in *P. stipitis*, the promoter was also induced microaerobically in *P. pastoris*. The expression level of VHb in *P. pastoris* at low $O_2$ tension ($< 5\%$ air saturation) was 16 nmol/g dry cell wt, i.e. about 24-fold higher than that at 60% air saturation. The expressed VHb enhanced growth of *P. pastoris* under microaerobic conditions. The application of $O_2$-regulated promoter in *P. pastoris* revealed that induction of high-level expression of heterologous protein is feasible without addition of supplementary compounds.

## Introduction

The strong and tightly regulated, methanol-induced alcohol oxidase (*AOX1*) promoter (Cregg *et al.* 1993) is more often employed for protein expression in *P. pastoris*, compared with the constitutive glyceraldehyde-3-phosphate dehydrogenase (*GAP*) promoter (Waterham *et al.* 1997), because the growth and induction phases can be separated. In addition to the *AOX1*-promoter, other methanol-induced dihydroxyacetone synthase (*DHAS*) promoter (Tschopp *et al.* 1987) and formaldehyde dehydrogenase (*FLD1*) promoter (Shen *et al.* 1998) have also been studied to express recombinant proteins.

*Pichia stipitis* is one of the few yeasts that ferments xylose to ethanol (Jeffries & Kurtzman 1994). The regulation of its aerobic and fermentative metabolism is different from that of the model organism of yeast physiology, *Saccharomyces cerevisiae*. Two alcohol dehydrogenase (ADH) genes have been cloned from *P. stipitis*. One of them, *PsADH2*, is induced by $O_2$-limitation with its promoter being activated by $O_2$-limitation and employed to express heterologous green fluorescent protein (Passoth *et al.* 2003) and endo-1,4-xylanase (Passoth & Hahn-Hagerdal 2000, Gorgens *et al.* 2005) in *P. stipitis*. In this work, we are interested in applying this inducible *PsADH2*-promoter to control the expression of a heterologous protein in *P. pastoris*.

*Vitreoscilla*, a strict aerobic bacterium, synthesizes bacterial hemoglobin (VHb) to survive and grow in an $O_2$-poor environment. The VHb gene has been transformed into various host cells to increase their growth and metabolites productivity (Bulow *et al.* 1999, Frey & Kallio 2003). The effects of expressing VHb on cells growth of yeasts *S. cerevisiae* (Chen *et al.* 1994), *Yarrowia lipolytica* (Bhave & Chattoo 2003), and *P. pastoris* (Wu *et al.* 2003) are varied.

In this work, the low $O_2$-induced *PsADH2*-promoter was employed to express a heterologous bacterial hemoglobin VHb in *P. pastoris*.

The feasibilities of using *PsADH2*-promoter in *P. pastoris* to drive the expression of heterologous proteins as well as to enhance the cell growth of *P. pastoris* by expressing VHb at microaerobic condition were studied. To our knowledge this is the first report on the expression of a heterologous protein in *P. pastoris* using the low $O_2$-induced *PsADH2*-promoter.

## Materials and methods

### *Microorganisms and plasmids*

*Escherichia coli* TOP10 [$F^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC)Φ80*lac*ZΔM15Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L ($Str^R$) *end*A1 *nup*G] (Invitrogen) was used as a cloning host. *Pichia pastoris* strain X33 (wildtype, Invitrogen, San Diego, CA) was used as an expression host. *Pichia stipitis* CBS 6054 which contains the alcohol dehydrogenase gene, *ADH2*, was obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan). Plasmid pP-SVC containing the synthetic *Vitreoscilla* hemoglobin gene (*vgb*) (GeneBank Accession No. AY278220) was constructed in our laboratory and its construction has been described in previous report (Wu *et al.* 2003). Plasmid pGAPZαA (Invitrogen, San Diego, CA) which contains the strong constitutive promoter and zeocin resistance gene was used as a cloning vector. The bacterial strains were grown in Luria-Bertani medium, with 25 μg zeocin/ml for the selection of desired clones. *E. coli* transformations were performed by the $CaCl_2$ method according to a standard protocol. Transformation of *Pichia pastoris* was performed by the lithium acetate and dithiothreitol method (Wu & Letchworth 2004).

### *Construction of plasmid pLJ02*

The construction scheme of pLJ02 plasmid is shown in Figure 1. *Pichia stipitis* genomic DNA was isolated by Wizard Genomic DNA Purification Kit (Promega, Madison, USA). The construction fragment of *PsADH2*-promoter was generated by PCR amplification from the *Pichia stipitis* genomic DNA using oligonucleotides 5′-CGC GGA TCC GCG GAA GCA CAG TCT AAT GC-3′ as a forward primer (ADH2-P1) containing an *Bam*HI site and 5′-TTG ATC CAA CAT GAT AAT TTG GAT GGA TCG CAG CAC-3′ as a reverse primer (ADH2-P2). The construction fragment of *PsADH2*-terminator sequence (TT) was also generated by PCR using oligonucleotides 5′-GCT GTT GAA CAT CAT CAT CAT CAT CAT TAA ACA AGC CGT GCT AGA TAG TGC-3′ as a forward primer (ADH2-T1) and 5′-CGC GAA TCC GCG GAG CTC AGG TAG AAG CCG AAA AGC-3′ as a reverse primer (ADH2-T2) containing an *Bam*HI site. The construction fragment of VHb gene was generated by PCR amplification from pP-SVC using oligonucleotides 5′-ATC CAA ATT ATC ATG TTG GAT CAA CAG ACT ATC AAC-3′ as a forward primer (VHb-M1) and 5′-ACG GCT TGT TTA ATG ATG ATG ATG ATG ATG TTC AAC AGC TTG AGC GTA CAA-3′ as a reverse primer (VHb-M2). After removing the excess primers and enzymes by the Qiagen PCR Purification Kit, an equal volume of PCR product of *PsADH2*-promoter gene and VHb gene were mixed for the second PCR using oligonucleotides ADH2-P1 and VHb-M2 as a forward and a reverse primer, respectively to generate PADH2-VHb gene fragment. An equal volume of PCR

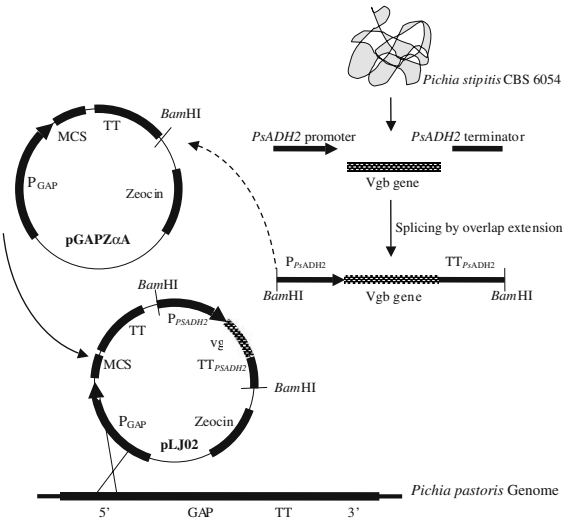


*Fig. 1.* Construction scheme of chromosome integrative plasmid pLJ02 for *Pichia pastoris*. *PsADH2*-promoter, terminator from *P. stipitis* and VHb gene were fused using splicing by overlap extension method. The fusion gene was inserted into the unique *Bam*HI sites of pGAPZαA and the strain X33 was transformed with pLJ02 to create $VHb^+$ strain for the expression of VHb under the control of $O_2$-regulated *PsADH2*-promoter.

product of VHb gene and ADH2 terminator gene were mixed for the second PCR using oligonucleotides VHb-M1 and ADH2-T2 as a forward and a reverse primer, respectively to generate a VHb-TADH2 gene fragment. After removing the excess primers and enzymes by the Qiagen PCR Purification Kit, an equal volume of PADH2-VHb and VHb-TADH2 PCR product were mixed for the third PCR using oligonucleotides ADH2-P1 and ADH2-T2 as a forward and a reverse primer, respectively. After purification, the obtained PADH2-VHb-TADH2 fusion gene fragment was singly digested with *Bam*HI and ligated into the vector pGAPZ$\alpha$A. The ligation products were transformed into *E. coli* TOP10 and selected on LB plates containing 25 $\mu$g zeocin/ml. The plasmids isolated were screened by colony PCR and restriction digestion. The resultant plasmid containing VHb fused with a $6\times$His tag at C-terminus flanked by *PsADH2*-promoter and terminator was denoted as pLJ02.

*Transformation and screening*

The integration vector pLJ02 was linearized by digesting with *Avr*II. *Pichia pastoris* strain X33 was made competent and transformed with linearized pLJ02 by the electroporation according to the methods reported by Wu & Letchworth (2004). Electro-competent cells were mixed with 10 $\mu$g linearized pLJ02 plasmid in a 0.2 cm electroporation cuvette and pulsed for $\sim$10 ms with a field strength of $\sim$7500 V/cm using a Bio-rad Gene Pulser (Bio-Rad Lab, Hercules, CA). The transformants X33/vgb ($VHb^+$) was selected on zeocin (0.1 mg/ml) containing YPD plate. The putative clones harboring multiple copies of the ADH2-VHb expression cassette were selected on YPD plate containing 0.5, 1, 1.5, 2 mg zeocin/ml, respectively. After 2–4 days of incubation at 30 °C, clones that survived the highest antibiotics concentrations were cultured in test tubes using YPD as medium to screen their VHb expression level by Western blotting.

*Assay of VHb activity*

The cell pellet, collected by centrifugation, was suspended in assay buffer (pH 7.5, 100 mM Tris/HCl and 50 mM NaCl). After disruption by sonication, VHb activities in the cell lysate were assayed using CO-difference spectra as described by Liu & Webster (1974). The total [ferric (oxidized) plus ferrous (reduced)] VHb prepared by sonication was determined after reduction with sodium dithionite. The difference in absorbance was taken between the VHb in the sample cuvette, which had been bubbled with CO, and an otherwise identical sample of VHb which had not been bubbled with CO. VHb levels were calculated using the extinction coefficient $E_{419-436nm} = 274\ mM^{-1}\ cm^{-1}$ (Dikshit & Webster 1988).

*SDS-PAGE and Western blot*

The recombinant proteins were analyzed by SDS-PAGE (15%) and stained with Coomassie Blue. The proteins separated by SDS-PAGE were transferred onto PVDF membrane (Hybond-P, Amersham Pharmacia Biotech, Uppsala, Sweden) by electroblotting (semi-dry Trans-Blot system, Bio-Rad, Hercules, CA). After blotting, the VHb on the membrane was detected by monoclonal anti-polyhistidine clone His-1 antibody (Sigma) and followed by anti-mouse antibody conjugated with alkaline phosphatase (Sigma).

*Cultivation of microorganisms*

For microaerobic cultivation, both the $VHb^+$ and $VHb^-$ (host) clone were grown in 250 ml flasks containing 50 ml YPD medium (1% yeast extract, 2% peptone, and 2% glucose) at 30 °C and 250 rpm. After 24 h, the flasks were sealed with Parafilm to prevent the air from flowing into the flasks. Every 12 h the sealed flask was opened and an aliquot of culture was taken for estimating the cells growth by measuring the optical density at 600 nm ($OD_{600}$) which was converted into the cell dry wt by using the relation of 1 OD = 0.3 g dry cell wt/l. This method allowed parallel estimation the growth of $VHb^+$ and $VHb^-$ strains. In order to monitor the dissolved oxygen concentration during the cultivations, $VHb^+$ and host strain were grown, respectively at 30 °C and 500 rpm in a 2.5 l computer-controlled, DO probe equipped fermentor (Biostat B, B. Braun Biotech International, Germany) containing 1 l YPD medium with aeration rate of 2 vvm

(1 vvm air plus 1 vvm pure $O_2$). In other words, the gas sparging into the medium contains about 60% $O_2$.

*Northern blot analysis*

Total RNA was extracted from *P. pastoris* $VHb^+$ using $TRI_{ZOL}$ reagent (Invitrogen) and following the manufacturer's protocol. Digoxigenin (DIG)-labeled DNA probe and tPA control probe were prepared by PCR DIG probe synthesis kit (Roche). Total RNA of *P. pastoris* $VHb^+$ and tPA control probe were subjected to electrophoresis on 1.2% agarose/2% (v/v) formaldehyde denaturing gels, transferred to Nylon$^+$ membrane and UV-crosslinked. The membrane was hybridized overnight with DIG-labeled DNA probe at 50 °C, washed once at room temperature with 2× SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) and 0.1% SDS. DIG nucleic acid detection kit (Roche) was employed to detect the DIG-labeled probes on the membrane. The membrane was rinsed with washing buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5, 0.3% Tween 20) for 5 min and transferred to blocking buffer (Roche) for 30 min. Anti-Digoxigenin-AP conjugate antibody was added and incubated for another 30 min. The membrane was washed twice with washing buffer and equilibrated 2–5 min in detection buffer. The membrane was then incubated in 10 ml freshly prepared color substrate solution in an appropriate container and stored in the dark. The color precipitate started to form within a few minutes. The reaction was stopped by using water or TE-buffer.

## Results and discussion

The DNA fragments of *PsADH2*-promoter, *vgb* gene and *PsADH2*-terminator were fused together by PCR using the method of splicing by overlap extension (SOEing) as described by Horton *et al.* (1989). The size of this trifusion product was determined to be about 1.4 kbp, which corresponds to the expected size. It was cloned into the unique *Bam*HI site of pGAPZαA vector, generating vector pLJ02. The vector was amplified in *E. coli* TOP10. After linearized by *Avr*II cutting at *GAP*-promoter region, it was transformed into *P. pastoris* X33 and expected to be integrated into the chromosome at the *GAP*-promoter region. Several of the zeocin selected transformants were tested for the production of *Vitreoscilla* hemoglobin in the test tube cultivations which were carried out under microaerobic condition. All the tested transformants did show different VHb activities in the cell crude extract. The one showed the highest VHb level was employed for further investigation.

In order to confirm that the expression of VHb results from the low $O_2$ induction, *P. pastoris*, transformed with pLJ02 ($VHb^+$), was grown in a fermentor supplied with 60% $O_2$ enriched air with an aeration rate of 2 vvm. Since the DO probe was calibrated by using air (ca. 20% $O_2$) to saturate the fermentation medium before the start of fermentation, dissolved oxygen (DO) concentration of 200% air saturation was observed at the beginning of the fermentation (Figure 2). DO value dropped to about 60% air saturation when cells were cultivated to 2.4 g DCW/l. After 25 h cultivation, cells concentration increased to 9.6 g DCW/l and DO decreased to a level of < 5% air saturation. Sample A at 12 h (DO of 60% air saturation) and sample B at 24 h (DO < 5% air saturation) were collected for analyzing the expressed VHb activity. After these two samples were adjusted to the same cell concentration and disrupted, the supernatant of the cell extracts were subjected to CO-difference spectrophotometry analysis. Sample B showed a typical VHb CO-binding absorbance spectrum (inset of Figure 3), a peak at 419 nm and a

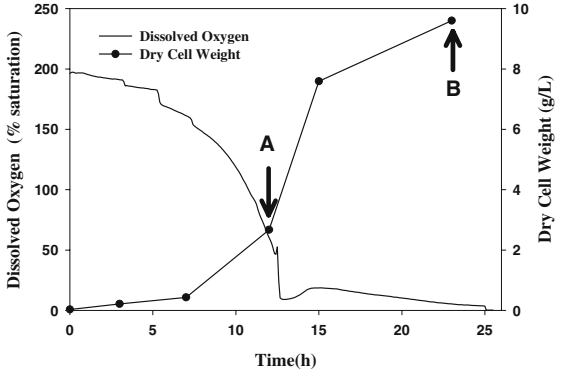


*Fig. 2.* The growth curve and dissolved $O_2$ concentration profile of $VHb^+$ strain cultivated in YPD medium at 30 °C and 500 rpm in a 2.5 l computer-controlled fermentor with 2 vvm aeration rate of 60% oxygen enriched air. Sample A and B are collected for VHb expression level analysis.

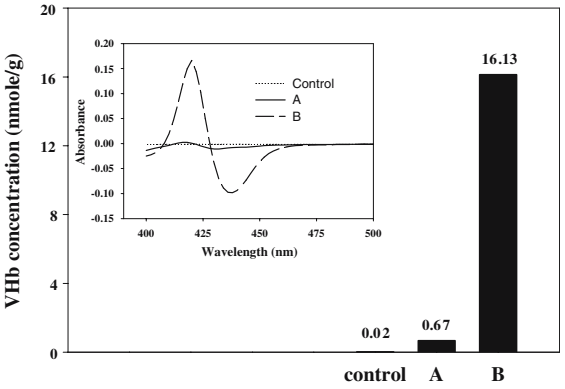


*Fig. 3.* The VHb expression levels of control and VHb$^+$ cells collected at high oxygen tension (sample A at 60% air saturation) and low oxygen tension (sample B at $<5\%$ air saturation). The inset is the CO-difference spectrophotometry analysis of VHb activity in each sample.

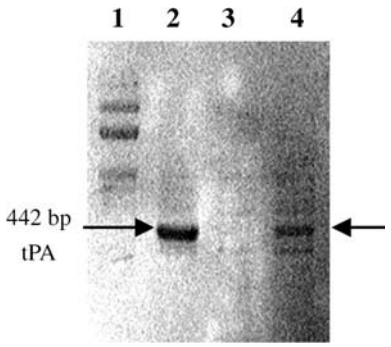


*Fig. 4.* Northern blot analysis of mRNA for VHb of VHb$^+$ cells collected at high and low $O_2$ culture conditions. Total RNA of cells samples was separated by electrophoresis and hybridized with a VHb-specific DNA probe. Lane 1, DNA marker; lane 2, tPA control probe; lane 3, high $O_2$ tension; lane 4, low $O_2$ tension.

minimum at 436 nm. The absorbance peak and minimum of sample A were insignificant. No typical VHb absorbance peak and minimum was observed for the control host strain.

The VHb expression level of VHb$^+$ strain collected at high $O_2$ (sample A) and low $O_2$ (sample B) were 0.67 and 16.13 nmol/g DCW, respectively. The specific VHb expression level of VHb$^+$ strain at low $O_2$ condition is about 24-fold higher than that at high $O_2$ condition. This result demonstrates that the VHb gene of VHb$^+$ strain was induced to express under microaerobic condition. In order to further confirm that the expression of VHb is resulted from the regulation of *PsADH2*-promoter, Northern blot analysis was carried out to check whether the size of the mRNA for VHb is the expected size for transcription initiating from the *PsADH2*-promoter. Figure 4 shows Northern blot analysis of the mRNA for VHb of VHb$^+$ strain cultivated under high and low $O_2$ condition. No single band was observed at high $O_2$ condition (lane 3). On the other hand, at low $O_2$ condition (lane 4) a prominent band was observed at the location close to the tPA control probe (lane 2) which has a size of 442 bp. Therefore, the observed prominent mRNA band for VHb should have a size around 442 bp which is very close to the expected size (456 bp) of mRNA for VHb initiating from the *PsADH2*-promoter. This result indicates that the expression of VHb gene was driven by *PsADH2*-promoter under microaerobic condition.

To allow parallel estimation of the growth of VHb$^+$ and VHb$^-$ strains so that the effect of VHb expression on cell growth could be studied, both strains were grown in shake-flasks under aerobic and microaerobic conditions. The microaerobic cultivation condition was simulated by sealing the cap of shake-flask with Parafilm. As shown in the growth curves (Figure 5), both VHb$^+$ and VHb$^-$ strains had the same growth rate during the first 24 h aerobic cultivation. When the cultivation condition was shifted to microaerobic, VHb$^+$ still kept the same growth rate. The VHb$^-$ strain, on

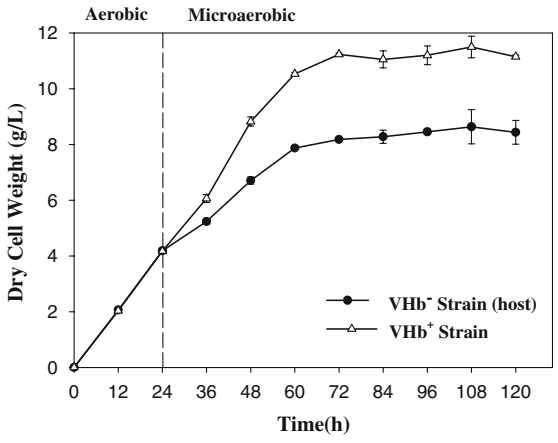


*Fig. 5.* Growth curves of the VHb$^+$ (Δ) and VHb$^-$ (●) strain cultivated in 250 ml culture flask containing 50 ml YPD medium at 30 °C and 250 rpm. The microaerobic cultivation condition was simulated by sealing the cap of the flask by parafilm during the cultivation.

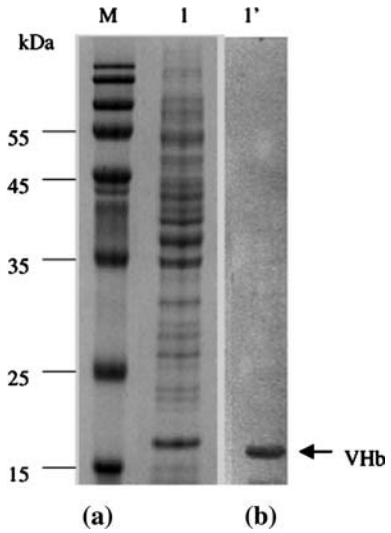


*Fig. 6*. SDS-PAGE (a) and Western blot (b) analysis of the expression of VHb in recombinant *P. pastoris* $VHb^+$ strain. The $VHb^+$ cell was cultured in YPD medium at microaerobic condition.

the other hand, decreased its growth rate. Both strains achieved their stationary phase at about 60 h. The $VHb^+$ resulted in a cell density of about 3 g DCW/l higher than that of $VHb^-$ at microaerobic condition. The $VHb^+$ cells collected at the end of cultivation (120 h) were disrupted to analyze their VHb expression level by Western blotting. As expected, a prominent band with molecular weight corresponding to that of VHb (~16 kDa) was observed (Figure 6). Probably, the higher growth rate and cell density achieved by $VHb^+$ strain under microaerobic condition is resulted from the expression of VHb which can enhance the $O_2$ uptake during the $O_2$-limiting condition. Our results show, for the first time, that a low $O_2$-induced *PsADH*-promoter can be used for the expression of heterologous protein in *P. pastoris* and VHb expression at low $O_2$ can improve the cells growth under microaerobic condition.

## Conclusion

The VHb gene under the control of *PsADH*-promoter was integrated into the chromosome of *P. pastoris* X33 via the plasmid pGAPZαA and expressed under microaerobic condition. The resultant strain $VHb^+$ had a higher specific growth rate and achieved a higher cell density under microaerobic condition. The *PsADH*-promoter is not only activated in *P. stipitis* as reported by Passoth *et al*. (1998, 2003) and Passoth & Hahn-Hagerdal (2000) but also in *P. pastoris* under low $O_2$ condition. The application of this $VHb^+$ strain for the high-level expression of other interested heterologous proteins in high-cell-density cultivation is currently carried out in our laboratory.

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