Improvement of Amorpha-4,11-diene Production by a Yeast-Conform Variant of *Vitreoscilla* Hemoglobin

Junhao Shen[a,b], Haina Zheng[a], Xiaohui Zhi[a], Yanwei Shi[a], Yong Huang[a], Wei Wang[a], Yongqin Chen[b], Jianqiang Kong[a,*], and Ping Zhu[a,*]

[a] Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College (State Key Laboratory of Bioactive Substance and Function of Natural Medicines & Ministry of Health Key Laboratory of Biosynthesis of Natural Products), Beijing, 100050, China. E-mail: jianqiangk@imm.ac.cn and zhuping@imm.ac.cn

[b] Faculty of Life Sciences, Hubei University, Wuhan, 430062, China

* Authors for correspondence and reprint requests

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Amorpha-4,11-diene is the precursor of the antimalarial compound artemisinin. The effect of *Vitreoscilla* hemoglobin (VHb) and its yeast-conform variant (VHbm) on amorpha-4,11-diene production in engineered *Saccharomyces cerevisiae* was investigated. First, the *VHb* gene was mutated to the yeast-conform variant *VHbm* based on step-by-step extension of a short region of the gene through a series of polymerase chain reactions (PCR). The artificial *VHbm* gene contained codons preferred by the yeast translation machinery. Two yeast expression vectors containing *VHb* or *VHbm* gene were constructed and introduced into the amorpha-4,11-diene-producing strain *S. cerevisiae* WK1 to form WK1[VHb] and WK1[VHbm], respectively. Western blot and CO-difference spectrum absorbance assay showed that *VHb* and *VHbm* were successfully expressed. In shake flasks, VHbm expression conferred higher cell growth than VHb expression. GC-MS results indicated the amorpha-4,11-diene production in WK1[VHbm] and WK1[VHb] was 3- and 2-fold higher than that in WK1, respectively. This suggests that VHb might improve the amorpha-4,11-diene production in engineered *S. cerevisiae*.

Key words: Amorpha-4,11-diene, Engineered *Saccharomyces cerevisiae*, *Vitreoscilla* Hemoglobin

Introduction

Artemisinin, isolated from *Artemisia annua* L. (Asteraceae; commonly known as sweet wormwood), is a sesquiterpene lactone with an endoperoxide bridge efficient against both the chloroquine-resistant as well as the chloroquine-sensitive strains of *Plasmodium falciparum* and also against cerebral malaria. Currently, the primary method of obtaining artemisinin is by extraction of intact plants. However, yields of the endogenous bioproduction of artemisinin are relatively low (ranging from 0.1 to 0.5% of dry weight), thereby limiting its clinical utilization. It is of great importance to develop a new production method of artemisinin which can provide a stable supply and reduce the cost of artemisinin. Because of successively greater progress in the metabolic engineering of artemisinin production in microbes (Martin *et al.*, 2003; Ro *et al.*, 2006; Anthony *et al.*, 2009), microbial production of artemisinin may be the method of the future, which can dramatically reduce the cost of artemisinin and thus make artemisinin accessible to most of the people in the world.

The microbes used in the metabolic engineering of artemisinin include *Escherichia coli* (Martin *et al.*, 2003), *Saccharomyces cerevisiae* (Ro *et al.*, 2006), *Aspergillus nidulans* (Lubertozzi and Keasling, 2008) and others. *S. cerevisiae*, an eukaryotic microbe, is an attractive host for the production of artemisinin precursors because it can produce farnesyl pyrophosphate for its sterol biosynthesis. Moreover, *S. cerevisiae* offers promise for the heterologous expression of genes encoding membrane-bound protein, such as cytochrome P450-encoding genes that hardly have functions in bacterial systems. Engineered yeast can produce on a basis of biomass the 2- to 3-fold amount of artemisinic acid compared to *A. annua* L. and within a much shorter time (4–5 days for yeast versus several months for *A. annua* L.) (Ro *et al.*, 2006). As such, the productivity of the engineered yeast is nearly two orders of magnitude

higher than that of *A. annua* L., demonstrating an advantage for the large-scale production of artemisinic acid and other plant terpenes in yeast. In addition, the secretion of artemisinic acid to the medium by the yeast cells simplifies fractionation and purification of the product, representing another potential cost-saving advantage in industrial processing (Ro *et al*., 2006). Therefore, *S. cerevisiae* was chosen as the preferred host for microbial artemisinin production.

Large-scale production of the compounds of interest often requires the cultivation of genetically engineered microorganisms at high cell densities to maximize the volumetric productivity. Oxygen transfer is one of the key factors affecting growth and productivity in such a process. Oxygen transfer may be improved to a certain extent by the use of high-efficiency dispersion systems and modification of the growth medium to improve the solubility of oxygen. Alternatively, a metabolic engineering approach may be used, which is based on the observation that the expression of *Vitreoscilla* hemoglobin (VHb) enhances the growth and products of interest in microorganisms under oxygen-limiting conditions (Suthar and Chattoo, 2006; Chen *et al*., 2007).

VHb is an oxygen-binding protein produced by the aerobic bacterium *Vitreoscilla stercoraria*, which is proposed to enhance the respiration and energy metabolism by promoting oxygen delivery. Through a metabolic engineering strategy, it has been demonstrated that intracellular expression of VHb in a different host circumvented effects of reduced oxygen availability (Wilhelmson *et al*., 2007), promoted cell growth (Chen *et al*., 2007; So *et al*., 2004), improved protein expression (Kim *et al*., 2008; Suthar and Chattoo, 2006), and elevated chemical production (Chen *et al*., 2007).

In the present investigation, the *VHb* gene was transferred to a yeast strain engineered to produce amorpha-4,11-diene with the aim of enhancement of amorpha-4,11-diene productivity. Moreover, the effect of the yeast-conform variant of the *VHb* gene, VHb, on the production of amorpha-4,11-diene in a transformed yeast was also investigated.

Material and Methods

Strains and plasmids

Strains and plasmids used in this work are listed in Table I. *Escherichia coli* strain TG1 was used as a bacterial host for recombinant plasmid amplification. The strain was grown in Luria-Bertani medium (10 g L^{-1} Bacto-tryptone, 5 g L^{-1} Bacto-yeast extract, 10 g L^{-1} NaCl) supplemented with ampicillin (100 μg mL^{-1}) when required for selection.

The *Saccharomyces cerevisiae* strain WK1 (*MAT*a; *ade2*-1; *his3*-11,-15; *leu2*-3, -112; *ura3*-1; *trp1*-1), a derivative of W303-1B, was engineered to produce amorpha-4,11-diene and used as the parent strain for all engineered yeast strains (Kong *et al*., 2009). This strain grows in a non-selective YPD medium (10 g L^{-1} yeast extract, 20 g L^{-1} Bacto-peptone, 20 g L^{-1} glucose). Engineered yeast strains were generally grown at 30 °C in SD medium (0.67% Bacto-yeast nitrogen base with-

Table I. Strains and plasmids used in this study

Strain and plasmid	Description	Source or reference
WK1	The yeast strain W303–1B derivative containing *Artemisia annua* L. amorpha-4,11-diene gene	Kong *et al*. (2009)
TG1	K12 Δ(*lac-pro*) *supE thi hsd5*/F' *traD36 proA*$^+$*B*$^+$ *lacI*^{q} *lacZ*Δ*M15*	Laboratory stock
pYeDP60	2-μm plasmid with *GAL10-CYC1* promoter, *URA3* and *ADE2* marker	Pompon *et al*. (1996)
pYeDP60/GAPDH	pYeDP60 derivative, in which the *GAL10-CYC1* hybrid promoter was replaced with yeast GAPDH promoter	This study
pBSVHb	pBluescipt SK(+) derivative containing *VHb* gene	Laboratory stock
pMD18-T	pUC18 derivative used for T-A cloning	Takara Shuzo Co. Ltd
EZ-T	pBluescript SK(+) derivative used for T-A cloning	GenStar BioSolutions Co. Ltd
pMDVHb	pMD18-T derivative containing *VHb* gene	This study
pEZVHbm	EZ-T derivative containing *VHbm* gene	This study
pYeDP60/GAPDH/VHbm	pYeDP60/GAPDH derivative containing *VHbm* gene	This study
pYeDP60/GAPDH/VHb	pYeDP60/GAPDH derivative containing *VHb* gene	This study

out amino acid, 2% glucose) with omission of adenine and uracil where appropriate.

pBSVHb was a pBluescript SK(+) derivative containing the *VHb* gene. pYeDP60/GAPDH (Kong *et al.*, 2009) was a yeast vector derived from pYeDP60 (Pompon *et al.*, 1996), in which the *GAL10-CYC1* hybrid promoter was replaced with the yeast GAPDH (glyceraldehyde-3-phosphate dehydrogenase isozyme 3) promoter (constitutive).

Enzymes and chemicals

T4 DNA ligase, pMD18-T vector, and restriction enzymes were purchased from Takara Shuzo Co. Ltd. (Kyoto, Japan). EZ-T vector was obtained from GenStar BioSolutions Co. Ltd. (Beijing, China). KOD Plus Taq DNA polymerase was purchased from Toyobo Co. Ltd. (Osaka, Japan). The standard valencene was obtained from Fluka Co. Ltd. (Buchs, Switzerland). All other fine chemicals were analytical grade.

Design and synthesis of the VHbm gene

To obtain a high-level expression, a synthetic *VHb* gene (*VHbm*) optimized for the heterologous expression in *S. cerevisiae* was designed. Based on the original DNA sequence of the wild-type gene (GenBank accession no. AF292694), the *VHb* gene codons were modified to *S. cerevisiae*-preferred codons with the original protein structure. The table of codon usage in *S. cerevisiae* can be consulted at http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=4932.

De novo synthesis of the *VHbm* gene was achieved by successive and overlapping polymerase chain reaction (PCR) using 12 overlapping oligonucleotides (Table II). Ten of these primers (Fvm1–5 and Rvm1–5) were 59 bp in length and with 13 to 27 bp overlap. The detailed procedure was as follows (Fig. 1). First-round PCR was performed in a total volume of 50 μL containing 1 μL each of primer Fvm1 and Rvm1 (10 μM), 5 μL each of dNTP (2 mM) and 1 μL of KOD Plus DNA polymerase (Toyobo Co. Ltd.). The PCR product was diluted 100 times, and 2 μL of the diluted primary reaction mixture served as template in a secondary amplification round. After six rounds of PCR amplification, the resulting purified 400-bp PCR product was cloned into the EZ-T using *Nhe* I and *Bam*H I restriction sites to yield the vector pEZVHbm (Fig. 1).

Table II. Primers used in this study.

Primer	Sequences (5'-3') (restriction sites underlined)	Restriction enzyme
Fvm1	GTTTTGGCTGCTGCTCAAAATATTGAAAATTTGCCAGCTAT-TTTGCCAGCTGTTAAAAA	
Rvm1	AGCAGCAGCAACACCAGCTTGACAATGTTTAACAGCAATT-TTTTTAACAGCTGGCAAAA	
Fvm2	TAGACAAGAATCTTTGGAACAACCAAAAGCTTTGGCTATGAC-TGTTTTGGCTGCTGCTC	
Rvm2	TCTTTAATAGCACCCAACAATTCTTGACCAACAATTGGATAATGA-GCAGCAGCAACACC	
Fvm3	TGTTTGCTAAACATCCAGAAGTTAGACCATTGTTTGATATGGGT-AGACAAGAATCTTTG	
Rvm3	CCCAAGCATCCAAAATATCATCAGTAGCAGCATCACCCAA-AACTTCTTTAATAGCACCC	
Fvm4	AAAGAACATGGTGTTACTATTACTACTACTTTTTATAAA-AATTTGTTTGCTAAACATCC	
Rvm4	TCAACTTGAATAAAAACATCAGCAATAACACCATAAGCTT-TACCCCAAGCATCCAAAAT	
Fvm5	CAACAAACTATTAATATTATTAAAGCTACTGTTCCAGTTTTG-AAAGAACATGGTGTTAC	
Rvm5	ATGATGATGATGATGTTCAACAGCTTGAGCATACAAATC-AGCTTCAACTTGAATAAAAA	
Fvm6	GGCTAGCATGTTGGATCAACAAACTATTAAT	*Nhe* I
Rvm6	CGGATCCTTAATGATGATGATGATGATG	*Bam*H I
FVHb	GCTAGCATGTTAGATCAGCAAACCATT	*Nhe* I
RVHb2	GGATCCTTAATGATGATGATGATGTTCAACCGCTTGAGCGTACAA	*Bam*H I

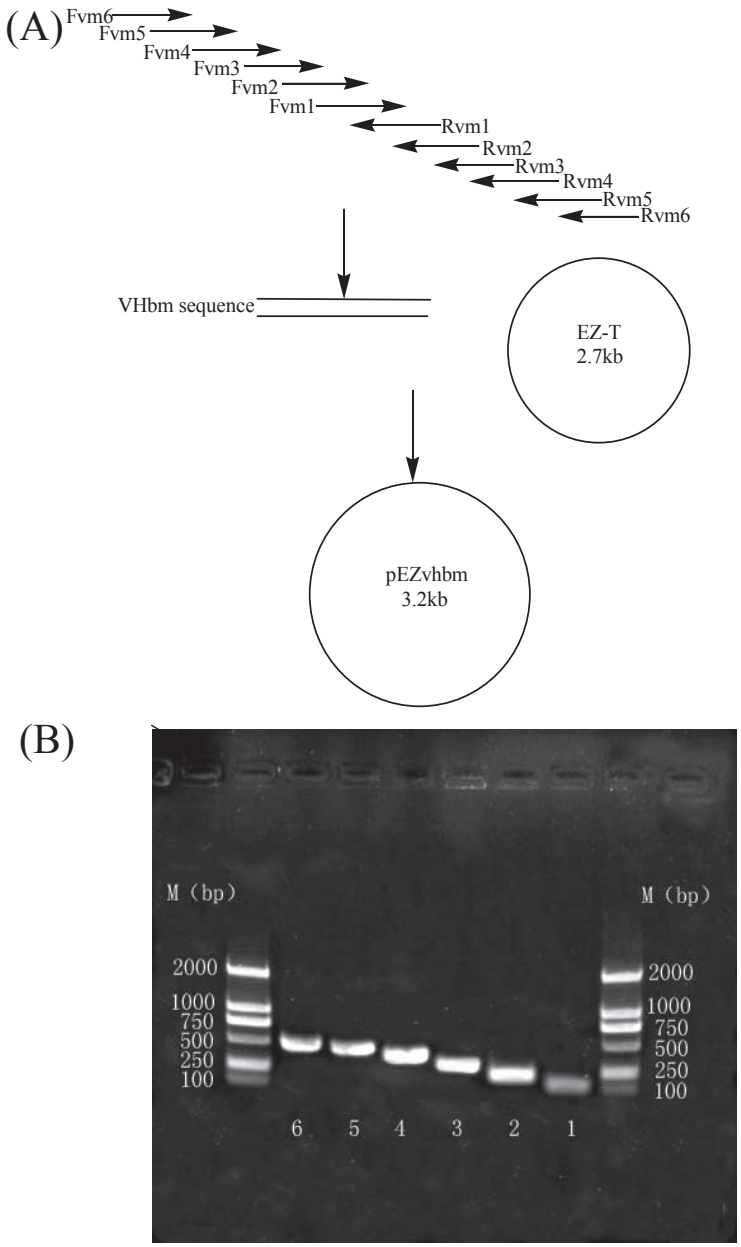

Fig. 1. Synthesis of the *VHbm* gene by successive PCR. (A) Scheme of the synthesis of the *VHbm* gene by successive PCR. (B) The PCR products obtained by amplification with the primer pairs Fvm1 – 6/Rvm1 – 6; lanes 1 – 6, the PCR products amplified by primers Fvm1/Rvm1, Fvm2/Rvm2, Fvm3/Rvm3, Fvm4/Rvm4, Fvm5/Rvm5, Fvm6/Rvm6, respectively; M, molecular marker DL2000.

Plasmid constructions

The *VHb* gene was amplified by PCR from pSKVHb using the forward primer FVHb harbouring a *Nhe* I restriction site and the reverse primer RVHb2 containing a *Bam*H I restriction site, and then was directly cloned into pMD18-T to obtain the plasmid pMDVHb (Table I). The *VHb* gene was cleaved from pMDVHb with *Nhe* I/*Bam*H I and cloned into pYeDP60/GAPDH digested by *Bam*H I/*Nhe* I. The resulting pYeDP60/

GAPDH/VHb was a plasmid containing *VHb* with the GAPDH promoter. *Nhe* I and *Bam*H I were used to double digest pEZVHbm, and the excised fragment (ca. 0.5 kb) was inserted into the *Nhe* I and *Bam*H I sites of pYeDP60/GAPDH to form the recombinant expression vector pYeDP60/GAPDH/VHbm. All clones were sequenced to confirm the expected sequences.

Transformation of yeast strains

The yeast expression vectors were transformed into *S. cerevisiae* WK1 by the LiAc/ss -DNA/PEG method according to Gietz *et al.* (1995). Two kinds of yeast strains, WK1[VHb] and WK1[VHbm], were constructed in this study. WK1[VHb] contained the plasmid pYeDP60/GAPDH/VHb, while the strain WK1[VHbm] was engineered by transformation with pYeDP60/GAPDH/VHbm. Transformants were selected using SD medium without adenine and uracil. Verification of positive clones was done by re-isolation of the plasmids and by colony PCR.

Yeast cultivation and determination of amorpha-4,11-diene

The engineered yeasts were grown in 10 mL liquid SD medium without adenine and uracil for 2 d at 30 °C until the optical density at 600 nm (OD_{600}) reached 1.0. Then 1 mL cell suspension was transferred into 50 mL fresh YPD medium and cultivated for two more days at 30 °C with continuous agitation (200 rpm) until OD_{600} reached 2.0. Two mL dodecane (4%, v/v) were added aseptically to the shake flasks to trap volatile amorpha-4,11-diene. This dodecane layer was sampled for determination of amorpha-4,11-diene production by GC-MS (gas chromatography-mass spectrometry).

Verification of VHb and VHbm expression by Western blot analysis

One mL of *S. cerevisiae* cells expressing the *VHb* and *VHbm* genes were harvested by centrifugation at 12,000 x *g* for 10 min. The resulting cell pellets were resuspended in 1 mL Y-PER (yeast protein extraction reagent; Thermo Fisher Scientific Inc., Waltham, MA, USA) and incubated for 1 h at room temperature to disrupt the cells. After incubation, the debris was removed by centrifugation at 4 °C and 12,000 x *g* for 10 min). The resulting supernatant was used directly for protein separation on a 15% polyacrylamide gel, and proteins were transferred onto a PVDF-membrane. A VHb-specific rabbit antibody and a horseradish peroxidase (HRP)/goat anti-rabbit IgG conjugate (Amersham-Pharmacia Biotech Europe GmbH, Dübendorf, Switzerland) were used to probe for VHb or VHbm protein bands. An ECL chemiluminescent reagent was used to visualize the antibody-decorated protein band (Amersham-Pharmacia Biotech Europe GmbH).

Analysis of biological activity of VHb and VHbm by CO-difference spectrum absorbance assay

The activity of the expressed VHb and VHbm was detected by CO-difference spectra. One mL cell suspension of WK1[VHb], WK1[VHbm], and WK1, respectively, was harvested from the culture medium by centrifugation at 12,000 x *g* for 10 min. The cells were resuspended in 1 mL Y-PER and incubated for 1 h at room temperature. The debris was removed by centrifugation at 4 °C (12,000 x *g*, 10 min), and the resulting supernatant was used directly for CO-difference spectral analysis using a Spectra Max190 Microplate Spectrophotometer (Molecular Devices, Inc., Sunnyvale, CA, USA).

Analysis of cell growth

Analysis of cell growth was performed in a 250-mL flask with a liquid volume of 50 mL. The yeast strains WK1[VHb], WK1[VHbm], and WK1 were grown in 10 mL liquid SD medium without adenine and uracil for 2 d at 30 °C. Then 1 mL cell suspension was transferred into 50 mL fresh YPD medium and cultivated at 30 °C until stationary phase. Samples of 1 mL were taken at 24-h intervals and OD_{600} was measured.

Analysis of amorpha-4,11-diene production in engineered cells

The GC-MS analysis of amorpha-4,11-diene production was performed as previously described (Kong *et al.*, 2009). GC-MS conditions were as follows: Shimadzu QP2010 system (Kyoto, Japan) equipped with a DB-5 ms column (30 m × 0.25 μm × 0.25 mm); injector temperature, 250 °C; flow rate, 2 mL min^{-1}; split ratio, 10:1; oven temperature, 100 °C for 2 min, 5 °C min^{-1} increase to 200 °C, 25 °C min^{-1} increase to 250 °C,

and 250 °C for 25 min. Total ion and selected ion chromatograms showed respective molecular ion and selected ions of *m/z* 204 and *m/z* 119, 121, 189.

Results and Discussion

Synthesis of VHbm gene

The accurate sequence of the synthetic *VHbm* gene is shown in Fig. 2. The *VHb* gene was 441 bp in length and encoded a polypeptide of 147 amino acids. The synonymous codons of the 20 proteinogenic of amino acids (AA) were changed into yeast-preferred codons in the *VHbm* gene, which can improve the abundance of cognate tRNAs during translation (Table III). The analysis of the relative adaptiveness (codon usage frequency) of the *VHbm* and *VHb* genes was performed with the gcua program (www.gcua.de). In the *VHb* gene, the relative adaptiveness of 81 codons was 100%, and 66 codons had a relative adaptiveness below 100%, even 26 codons had a relative adaptiveness below 50%. In the *VHbm* gene, the relative adaptiveness of all codons was 100%. The recombinant vector pEZVHbm was checked by *Bam*H I and *Nhe* I double digestion.

Construction of plasmids and yeast transformation

Two expression vectors, pYeDP60/GAPDH/VHb and pYeDP60/GAPDH/VHbm, were constructed for the functional expression of the *VHb* and the mutated *VHbm* gene (Fig. 3). The expression vector pYeDP60/GAPDH/VHbm harboured

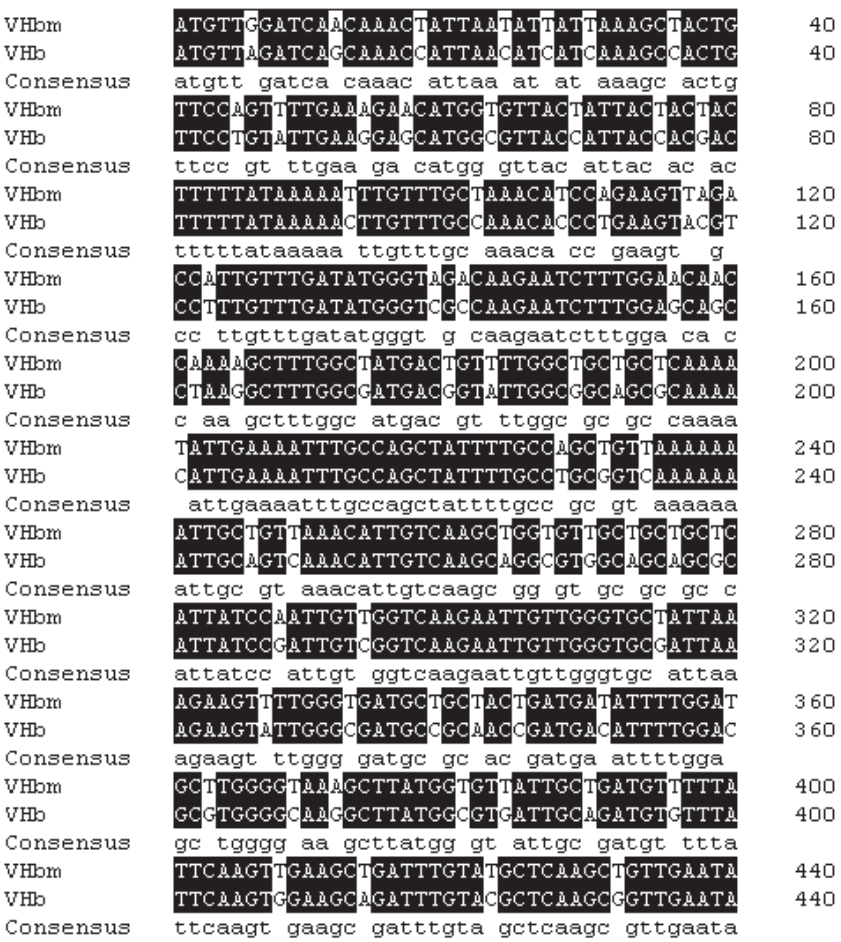

Fig. 2. Sequence comparison of the *VHb* and *VHbm* genes.

Table III. Comparison of the codon usage in the *VHb* and *VHbm* gene targeted for *S. cerevisiae* expression.

AA	Codon	Frequency per thousand[a]	No.		AA	Codon	Frequency per thousand[a]	No.	
			VHb	VHbm				VHb	VHbm
Phe	ttt	26.1	4	4	Tyr	tat	18.8	3	4
	ttc	18.4	0	0		tac	14.8	1	0
Leu	tta	26.2	1	0	Stop	taa	1.1	1	1
	ttg	27.2	13	14		tag	0.5	0	0
	ctt	12.3	0	0	His	cat	13.6	3	4
	ctc	5.4	0	0		cac	7.8	1	0
	cta	13.4	0	0	Gln	caa	27.3	7	9
	ctg	10.5	0	0		cag	12.1	2	0
Ile	att	30.1	10	12	Asn	aat	35.7	1	4
	atc	17.2	2	0		aac	24.8	3	0
	ata	17.8	0	0	Lys	aaa	41.9	7	10
Met	atg	20.9	3	3		aag	30.8	3	0
Val	gtt	22.1	3	14	Asp	gat	37.6	6	8
	gtc	11.8	3	0		gac	20.2	2	0
	gta	11.8	4	0	Glu	gaa	45.6	7	9
	gtg	10.8	4	0		gag	19.2	2	0
Ser	tct	23.5	1	1	Cys	tgt	8.1	1	1
	tcc	14.2	0	0		tgc	4.8	0	0
	tca	18.7	0	0	Stop	tga	0.7	0	0
	tcg	8.6	0	0	Trp	tgg	10.4	1	1
Pro	cct	13.5	5	0	Arg	cgt	6.4	1	0
	ccc	6.8	0	0		cgc	2.6	1	0
	cca	18.3	1	7		cga	3	0	0
	ccg	5.3	1	0		cgg	1.7	0	0
Thr	act	20.3	2	8	Ser	agt	14.2	0	0
	acc	12.7	0	0		agc	9.8	0	0
	aca	17.8	0	0	Arg	aga	21.3	0	2
	acg	8	2	0		agg	9.2	0	0
Ala	gct	21.2	4	23	Gly	ggt	23.9	3	8
	gcc	12.6	2	0		ggc	9.8	5	0
	gca	16.2	8	0		gga	10.9	0	0
	gcg	6.2	9	0		ggg	6	0	0

[a] Refers to http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=4932.

the *VHbm* gene expression cassette, in which the *VHbm* gene is controlled by the GAPDH promoter. The plasmid had *URA3* and *ADE2* selectable markers. The constructed two plasmids were characterized by *Bam*H I/*Nhe* I double digestion.

After transformation, 12 clones of WK1[VHb] were picked out for further examination of pYeDP60/GAPDH/VHb. The positive clones containing pYeDP60/GAPDH/VHb were certified by colony PCR with the FVHb/RVHb2 pair. The results showed that all of the 12 clones were positive. With the same procedure, 3 clones out of 7 WK1[VHbm] were characterized to be positive clones harbouring the plasmid pYeDP60/GAPDH/VHbm.

Characterization of VHb and VHbm expression in engineered yeasts

The Western blot results showed that both the VHb and VHbm proteins reacted positively with the VHb-specific monoclonal antibody, while no band was observed in the negative control WK1 (Fig. 4A). Thus the VHb and VHbm proteins were expressed in the yeast cultures.

CO-difference spectrum absorbance analysis of VHb and VHbm activity

The functional expression of VHb and VHbm was confirmed by their CO-difference spectra. As shown in Fig. 4B, the CO-difference spectra of en-

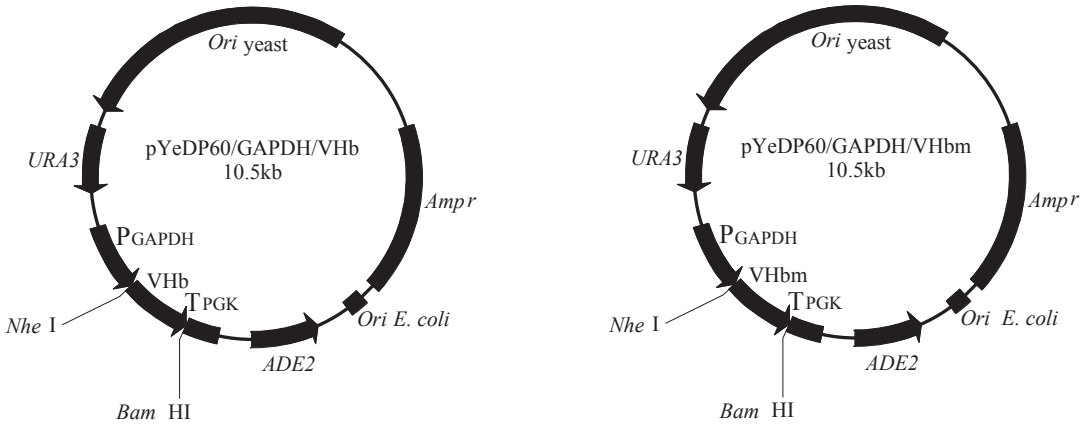

Fig. 3. Maps of pYeDP60/GAPDH/VHb and pYeDP60/GAPDH/VHbm.

gineered yeasts containing VHb and VHbm were typical for a VHb spectrum with a peak at around 420 nm, while the control strain did not show such VHb characteristic spetrum.

Growth of VHb- and VHbm-expressing yeast cells

The VHb and VHbm transformants were grown in shake flasks to study the VHb effects on cell growth. In the first 24 h, growth of the three strains as monitored by OD_{600} was almost identical (Fig. 5). As expected, the expression of VHb increased the maximum cell density as compared to the control strain. In the two kinds of engineered yeasts, the VHbm strain maintained higher cell growth than the VHb cells during the entire culture period of 168 h.

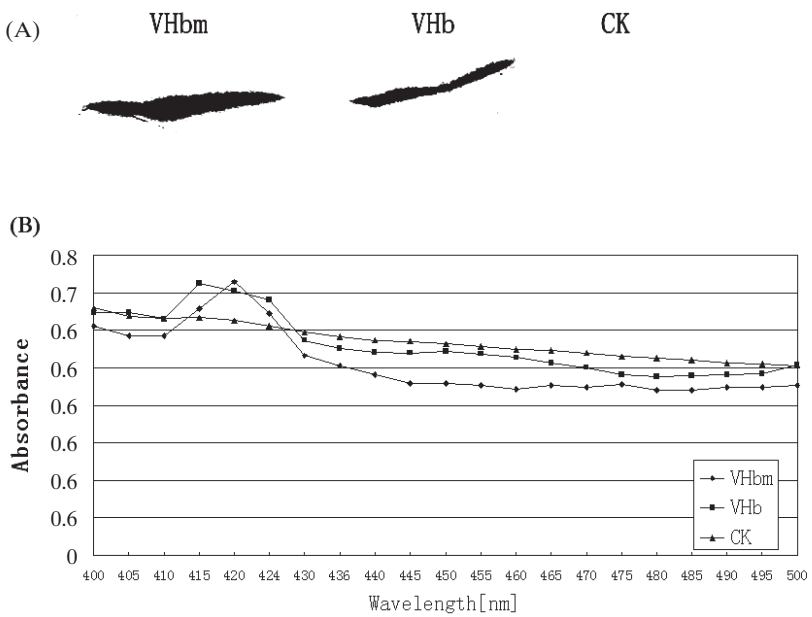

Fig. 4. Analysis of VHb and VHbm expression in the respective engineered *S. cerevisiae* strains. (A) Western blot analysis of VHb and VHbm proteins. (B) CO-difference spectra of the recombinant VHb and VHbm. CK stands for the yeast strain WK1.

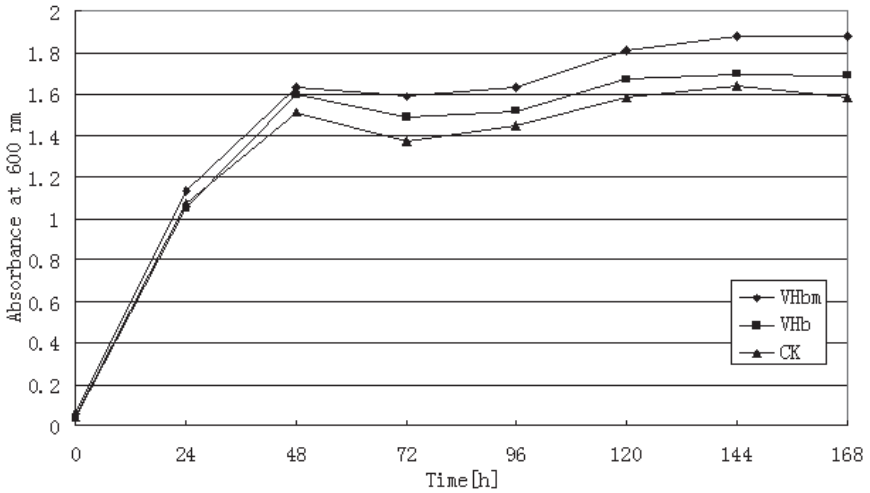

Fig. 5. Comparison of the growth of the yeast strains containing VHbm, VHb and CK. CK stands for the yeast strain WK1.

Determination of amorpha-4,11-diene in engineered yeasts

Previously, heterologous *VHb* gene expression had been reported to improve cell growth (Chen *et al*., 2007; So *et al*., 2004) and production of heterologous proteins and biomass (Kim *et al*., 2008; Suthar and Chattoo, 2006). With the aim of improving the amorpha-4,11-diene production, the *VHb* gene from *Vitreoscilla* was introduced into *S. cerevisiae* engineered to produce amorpha-4,11-diene. To determine the effect of VHb expression on amorpha-4,11-diene production, three positive clones of WK1[VHb] were cultured for product determination by GC-MS. As the control, three clones of WK1 containing the *ADS* gene only were also cultured and analysed by GC-MS. A standard curve for valencene was used for the quantification of amorpha-4,11-diene. The positive clones all produced amorpha-4,11-diene (Fig. 6), and the production of amorpha-4,11-diene produced by the strain containing the *VHb* gene was two times greater than that in the *VHb* non-expressing WK1 (Table IV).

It is well known that there is a strong correlation between the gene expression level and the degree of biased codon usage. Therefore, this work investigated whether codon usage indeed controls gene expression. The full *VHb* cDNA was rewritten according to the *S. cerevisiae*-preferred codon usage. The identity of amino acid sequences encoded by the two genes was 100%, while it was only 84.58% at the nucleic acid level. The GC-MS data indicated that the production of amorpha-4,11-diene in the engineered yeasts containing the *VHbm* gene is higher than that of the control not harbouring the *VHb* gene (Table IV). These results indicated the rewriting of the *VHb* gene can increase the amorpha-4,11-diene production.

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Table IV. Amorpha-4,11-diene production by engineered yeasts.

Strain	Amorpha-4,11-diene production [mg L^{-1}]
WK1-1	0.191
WK1-2	0.172
WK1-3	0.156
VHb-1	0.378
VHb-2	0.376
VHb-3	0.359
VHbm-1	0.491
VHbm-2	0.501
VHbm-3	0.513

(A)

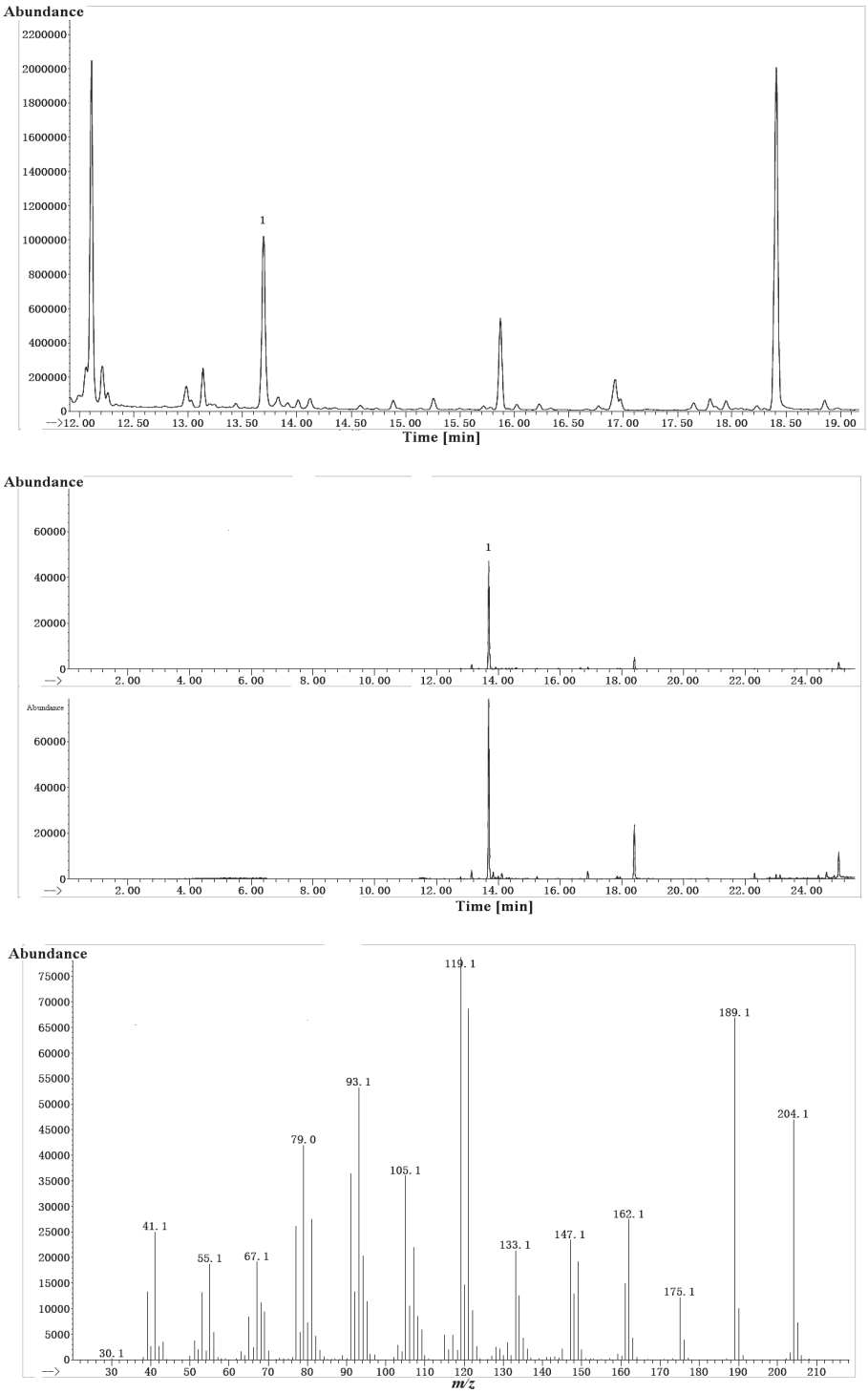
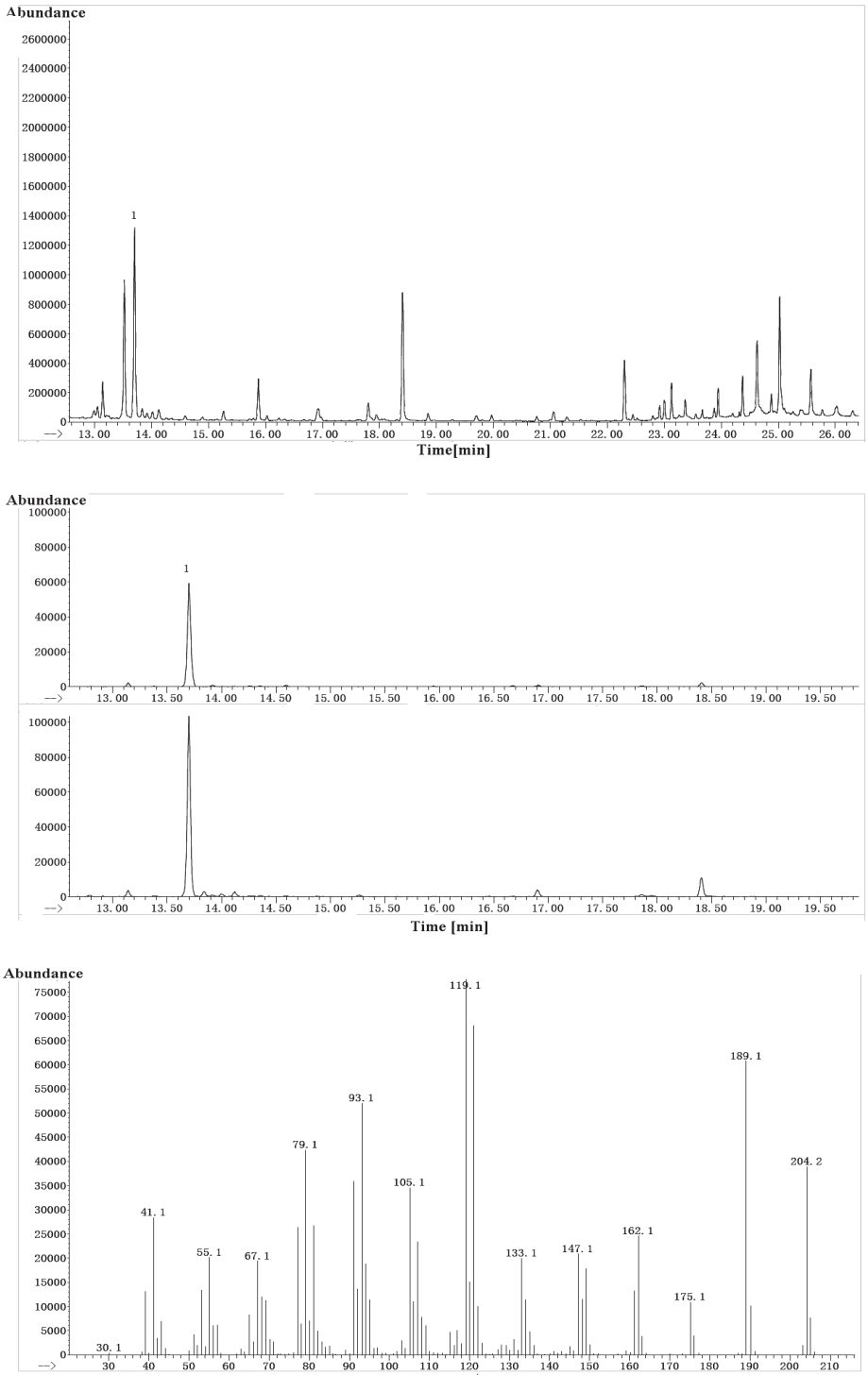


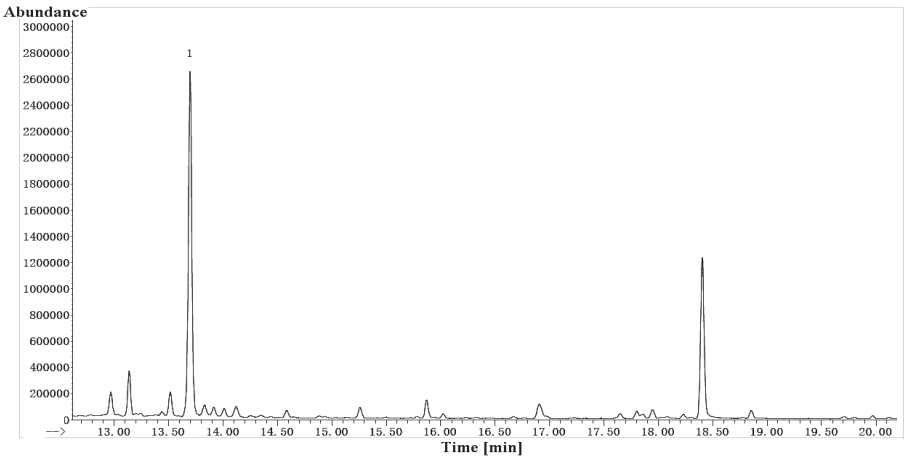
Fig. 6. GC-MS identification of amorpha-4,11-diene produced by the strains (A) WK1, (B) WK1[VHb], and (C) WK1[VHbm], respectively. Upper, the total ion chromatogram of the strains WK1, WK1[VHb], and WK1[VHbm], respectively; middle, selected ion monitoring of *m/z* 204 and *m/z* 119 of the strains WK1, WK1[VHb] and

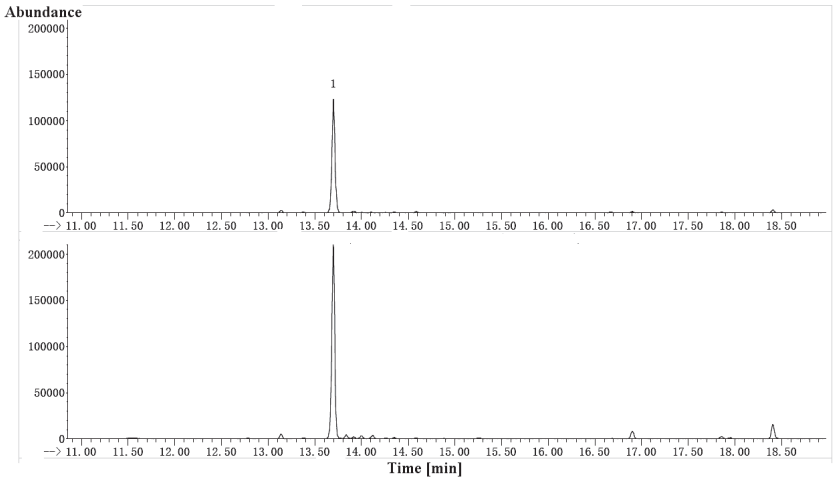
(B)



WK1[VHbm], respectively; bottom, MS spectra of the peak of retention time 13.695 (WK1), 13.69 (WK1[VHb]), and 13.7 (WK1[VHbm]) min, respectively.

(C)





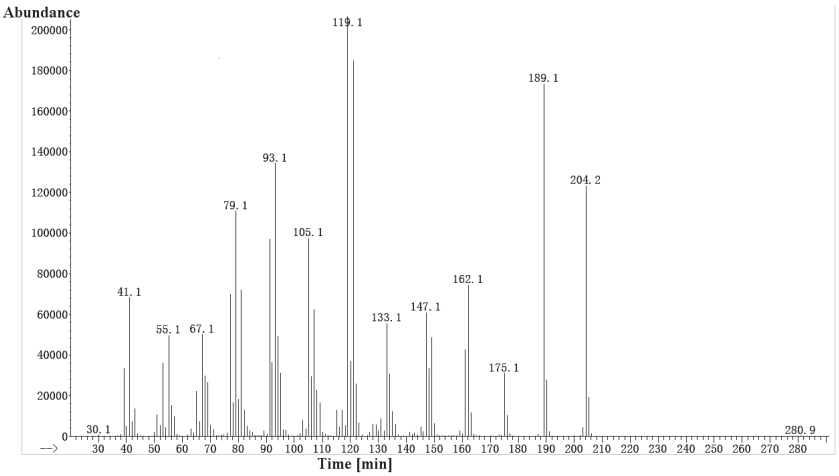


Fig. 6 continued.

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