Appl Biochem Biotechnol (2011) 163:707–719
DOI 10.1007/s12010-010-9076-z

Enhanced Production of L-Arginine by Expression of *Vitreoscilla* Hemoglobin Using a Novel Expression System in *Corynebacterium crenatum*

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Received: 14 May 2010 / Accepted: 27 August 2010 /
Published online: 11 September 2010

Abstract *Corynebacterium crenatum* SYPA 5-5 is an aerobic and industrial L-arginine producer. It was proved that the *Corynebacterium glutamicum*/*Escherichia coli* shuttle vector pJC1 could be extended in *C. crenatum* efficiently when using the chloramphenicol acetyltransferase gene (*cat*) as a reporter under the control of promoter *tac*. The expression system was applied to over-express the gene *vgb* coding *Vitreoscilla* hemoglobin (VHb) to further increase the dissolved oxygen in *C. crenatum*. As a result, the recombinant *C. crenatum* containing the pJC-*tac*-*vgb* plasmid expressed VHb at a level of 3.4 nmol g^{-1}, and the oxygen uptake rates reached 0.25 mg $A_{562}{}^{-1}h^{-1}$ which enhanced 38.8% compared to the wild-type strain. Thus, the final L-arginine concentration of the batch fermentation reached a high level of 35.9 gL^{-1}, and the biomass was largely increased to 6.45 gL^{-1}, which were 17.3% and 10.5% higher than those obtained by the wild-type strain, respectively. To our knowledge, this is the first report that the efficient expression system was constructed to introduce *vgb* gene increasing the oxygen and energy supply for L-arginine production in *C. crenatum*, which supplies a good strategy for the improvement of amino acid products.

Keywords Amino acids · *Corynebacterium crenatum* · *vgb* · Fermentation · Dissolved oxygen

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Introduction

L-Arginine, as a conditionally essential amino acid in human nutrition, has numerous applications in food flavor and pharmaceutical. Therefore, it is regarded as an important "nutraceutical" [1, 2]. Microbial L-arginine production can be boosted to high levels by applying strategies based on the knowledge of enzyme properties and regulatory mechanisms or by repeated mutagenesis and screening [1, 3]. Since the 1960s, studies on the L-arginine production have been conducted using the mutants of *Corynebacterium*, *Bacillus*, and *Serratia*, but in industry, L-arginine initially produced by fermentation using the *Corynebacterium* (*Brevibacterium*) mutants [3]. More recently, the other elaborate strategies were devised to breed strains for efficient production of L-arginine on the basis of pathways, regulation, and metabolic reaction of amino acids [4–6]. In our previous work, *Corynebacterium crenatum* was successfully isolated from soil, and the final L-arginine production could reach 30 gL^{-1} under the optimal culture conditions by its mutated strain *C. crenatum* SYPA 5-5. *C. crenatum* SYPA 5-5 is an aerobic, Gram-positive, non-sporulating, and L-histidine auxotroph industrial bacterium.

Oxygen supply has an important influence on aerobic amino acids production by microorganisms, which generally requires a large amount of oxygen to re-oxidize NAD(P)H_2 or $FADH_2$ in order to generate ATP for metabolism effectively [7–10]. Maghnouj et al. reported that the dissolved oxygen (DO) levels largely determined the interactions between the metabolic reactions and genetic regulatory mechanism, as well as the formations of products and by-products in the production of L-arginine [11–14]. It has been reported that L-arginine production is seriously impaired under anaerobic conditions during the fermentation [3, 15]. Xu et al. [16] gained the metabolic flux distribution analysis of *C. crenatum* under various oxygen supply conditions. The metabolic flux analysis indicated that a relatively higher L-arginine production could be obtained under high oxygen supply condition overall. Oxygen as an indispensable raw material must be supplied in large amounts in industrial L-arginine biosynthesis. The constant DO level control by automatically changing either agitation or aeration is generally considered as the best and simplest way to control or optimize aerobic fermentations. However, high oxygen supply, obtained by increasing the agitation speed or injecting pure oxygen, demands power during the fermentation process and thus increases costs.

The *Vitreoscilla* hemoglobin (VHb) is one of the microbial hemoglobins, which was first discovered and probably best characterized [17, 18]. It has been demonstrated that engineered strains containing *vgb* gene often result in enhancement of cell density, oxidative metabolism, protein, and antibiotic production [19–22]. These beneficial effects result from the direct interaction of VHb with the terminal respiratory oxidase and delivering oxygen to enhance oxidative phosphorylation and thus the production of ATP [23–25]. The application of VHb provides a new approach for enhancing oxygen supply via increasing the oxygen uptake rates instead of more power consumption.

The gene *vgb* was desired to express in *C. crenatum* with an aim to increase the oxygen and energy supply and, consequently, to improve the production of L-arginine. A suitable expression system was a key factor for the expression of heterologous gene, since there is no expression system reported in *C. crenatum* especially in the literature we had consulted. The value of expression system based on the efficient regulated promoter was well recognized in modern biotechnology. To test the transcription activity of promoters, in this work, we directly inserted the reporter gene chloramphenicol acetyltransferase gene (*cat*) as the selection marker [26, 27] based on the following considerations: *C. crenatum* is characterized by low-level resistant to chloramphenicol; thus, it is convenient and simple

Table 1 Strains, plasmids, and primers

Strains, plasmids, or primers	Characteristics	Source
Strains		
E. coli JM109	*recA1*, *endA1*, *gyrA96*, *thi*, *hsdR17*, *supE44*, *relA1*, Δ(*lac-proAB*), [*F'traD36*, *proAB*$^+$, *lac Iq*, *lacZ ΔM15*]	Invitrogen
E. coli JM109(DE3)	*recA1*, *endA1*, *gyrA96*, *thi*, *hsdR17*, *supE44*, *relA1*, λ–, Δ(*lac-proAB*), [*F'*, *traD36*, *proAB*, *lacIqZ ΔM15*], *lDE3*	Promega
Corynebacterium crenatum SYPA5-5	L-Arginine producer	Our labs
Plasmids		
pMD18-T	*E. coli* clone plasmid Amp^R, *Col* E origin	Takara
T-*vgb*	A derivative of pMD18-T, Amp^R, harboring *vgb* gene	This study
pACYCDuet-1	*E. coli* expression vector, Cm^R, P15A origin	Novagen
pET-28a	*E. coli* expression vector, Cm^R	Novagen
pET-28a-*cat*	A derivative of pET-28a, harboring *cat* gene, and *T7* promoter	This study
pGJ023	Host of *vgb* gene	[42]
pE*tac*	A derivative of pET28a harboring *tac* promoter, *E. coli* expression vector, Km^R	This lab
pE*tac-cat*	A derivative of pE*tac*, harboring *cat* gene, and *tac* promoter	This study
pJC1	*C. glutamicum–E. coli* shuttle vector without promoter	[43]
pJC-*tac-cat*	A derivative of pJC1, harboring *cat* gene and *tac* promoter	This study
pJC-T7-*cat*	A derivative of pJC1, harboring T7 promoter, and *cat* gene	This study
pC2	*C. glutamicum–E .coli* shuttle expression vector with *lac* promoter	[44]
pC2-*cat*	A derivative of pC2, harboring *lac* promoter, and *cat* gene	This study
pJC-*lac-cat*	A derivative of pJC1, harboring *lac* promoter, and *cat* gene	This study
pJC-*tac-vgb*	A derivative of pJC-*tac*, harboring *vgb* gene	This study
Primers 5′→3′		
P1	CGC*GAATTC*TTCGAATTTCTGCCATTCATC (*Eco*R I)	
P2	CGC*GGATCC*(*CTGCAG*)GCGGTGCTTTTGCCGTTACG (*Bam*H I/*Pst* I)	
P3	AAAA*CTGCAG*GGCTTTACACTTTATGCTTCCG (*Pst* I)	
P4	AAAA*CTGCAG*TATAGTGAGTCGTATTAATTTCG (*Pst* I)	
P5	AAAA*CTGCAG*GGAGCTTATCGACTGCACG (*Pst* I)	
P6	ACCCG*GAATTC*ATGTTGGATCAACAGAC (*Eco*R I)	
P7	ACCCG*GGATCC*(*CTGCAG*)TTATTCAACAGCTTGAG (*Bam*H I/*Pst* I)	

Amp^R ampicillin-resistant phenotype, *Cm^R* chloromycetin-resistant phenotype, *Km^R* kanamycin-resistant phenotype

for selection of transformed strains and evaluation of transcription activity of promoters in *C. crenatum*. Based on the expression system of *C. crenatum*, the coding gene of VHb was introduced into *C. crenatum* to enhance the production of L-arginine by increasing the oxygen and energy supply. To our knowledge, this is the first report that the efficient expression system was constructed and applied to express *vgb* gene for improvement of L-

Fig. 1 Map of plasmids constructed in this study. The construction of pJC-*lac-cat* (**a**), pJC-*T7-cat* (**b**), pJC-*tac-cat* (**c**), and pJC-*tac-vgb* (**d**) ▶

arginine production in *C. crenatum*, which supplies a good strategy for the improvement of amino acid products.

Materials and Methods

Strains and Plasmids

C. crenatum was isolated from soil and *C. crenatum* SYPA 5-5 was mutated by UV irradiation in our labs. The strains, plasmids, and primers (restriction sites were italicized) used in this study are listed in Table 1. Recombinant plasmids pC2-*cat*, pET-28a-*cat*, pE*tac-cat*, pJC-*lac-cat*, pJC-T7-*cat*, and pJC-*tac-cat* were constructed (Fig. 1). The gene *cat* was amplified from the plasmid pACYCDuet-1 using P1 and P2 and ligated into the plasmid pC2, pET-28a, and pE*tac* to construct pC2-*cat*, pET-28a-*cat*, and pE*tac-cat*, respectively. The gene fragment *Plac-cat* PCR from pC2-*cat* using P3 and P2 was ligated into the single *Pst* I site of pJC1 to get pJC-*lac-cat* (Fig. 1a). The gene fragment *PT7-cat* was subcloned from pET-28a-*cat* using P4 and P2 and inserted into the single *Pst* I site of pJC1 to construct pJC-T7-*cat* (Fig. 1b). The gene fragment *Ptac-cat* was amplified from pE*tac-cat* using P5 and P2 and constructed into the single *Pst* I site of pJC1 to create pJC-*tac-cat* (Fig. 1c). The recombinant plasmids pJC-*lac-cat*, pJC-T7-*cat*, and pJC-*tac-cat* carry *cat* cassette under the control of the *lac*, T7, and *tac* promoters, respectively.

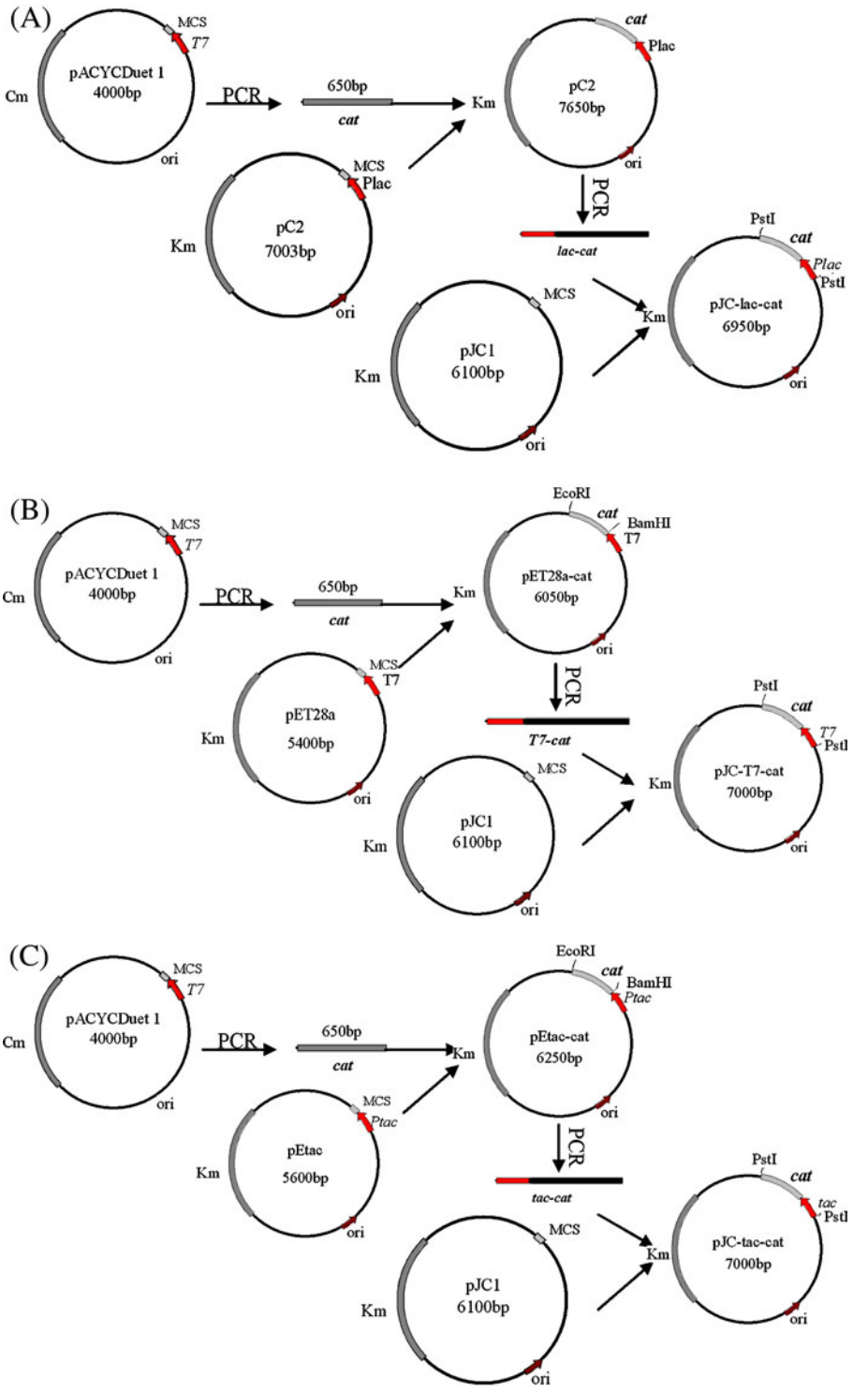
The *vgb* gene of 0.44 kb was amplified using P6 and P7 as primers and plasmid pYG013 as the template. Its *Eco*R I–*Bam*H I fragment was ligated into the corresponding site of pE*tac*, resulting in pE*tac-vgb*. The gene fragment *tac-vgb* PCR from pE*tac-vgb* using P5 and P7 and then ligated into the single *Pst* I site of pJC1 to construct pJC-*tac-vgb* (Fig. 1d) carried gene *vgb* under the control of the *tac* promoter.

Transformation and Screening

The ligation mixture was used to transform *Escherichia coli* by the calcium chloride method [28]. *C. crenatum* is cultivated at 31°C in Luria–Bertani media containing 0.5% glucose (LBG) and transformed using electroporation methods described by Tauch et al. [29]. When necessary, ampicillin or kanamycin was added at proper final concentration for transformant selections.

Growth Medium and Conditions for L-Arginine Production

C. crenatum SYPA 5-5 is auxotrophic for L-histidine. A stock culture was maintained on agar slants containing (in grams per liter) peptone 10, beef extract 10, yeast extract 5, NaCl 5, and agar 20. The seed culture medium (in grams per liter) was consisted of glucose 30, corn steep liquor 20, $(NH_4)_2SO_4$ 20, KH_2PO_4 1, $MgSO_4{\cdot}7H_2O$ 0.5, and urea 1.5. The seed was inoculated from agar slants and cultured at 31°C for 14–16 h in shake flask. The shake flask culture was then transferred into a 5-L bioreactor (BIOTECH-5BG, Baoxing Co., China) containing 3 L fermentation medium, which consisted of glucose 150, corn steep liquor 40, $(NH_4)_2SO_4$ 20, KH_2PO_4 1.5, $MgSO_4{\cdot}7H_2O$ 0.5, $FeSO_4{\cdot}7H_2O$ 0.02, $MnSO_4{\cdot}H_2O$ 0.02, biotin 8×10^{-5}, L-histidine 5×10^{-4} (pH 7.0). The batch fermentation was performed at


(A)
pACYCDuet 1
4000bp
MCS
T7
Cm
ori
PCR
650bp
cat
Km
pC2
7650bp
cat
Plac
ori
pC2
7003bp
MCS
Plac
Km
ori
PCR
lac-cat
pJC1
6100bp
MCS
Km
ori
pJC-lac-cat
6950bp
PstI
cat
Plac
PstI
Km
ori
(B)
pACYCDuet 1
4000bp
MCS
T7
Cm
ori
PCR
650bp
cat
Km
pET28a-cat
6050bp
EcoRI
cat
BamHI
T7
ori
pET28a
5400bp
MCS
T7
Km
ori
PCR
T7-cat
pJC1
6100bp
MCS
Km
ori
pJC-T7-cat
7000bp
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cat
T7
PstI
Km
ori
(C)
pACYCDuet 1
4000bp
MCS
T7
Cm
ori
PCR
650bp
cat
Km
pEtac-cat
6250bp
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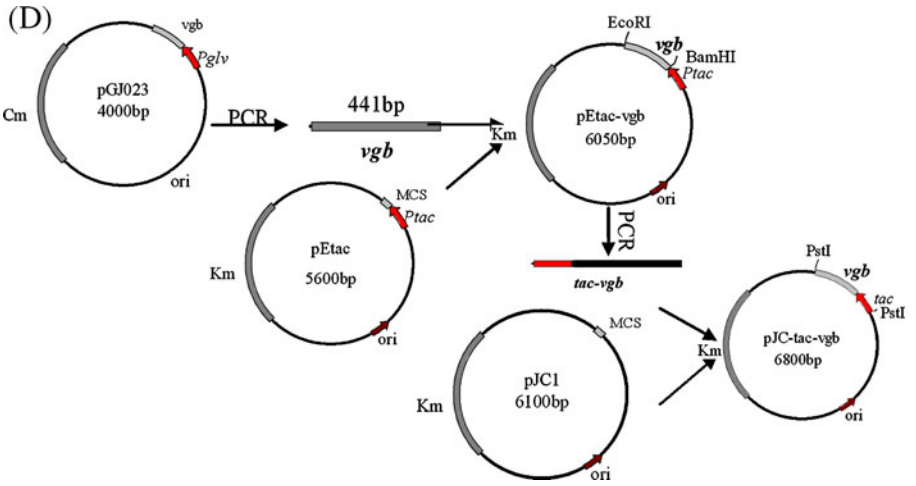

Fig. 1 (continued)

31°C for 108 h until the steady-strain growth. The aeration rate was controlled at 1 vvm for all the experiments. Studies in the bioreactor were carried out at the agitation rate of 600 rpm. The dissolved oxygen concentration (%) under the operation conditions was expressed in the term of DO saturation level, while 100% DO saturation level corresponded to an actual DO concentration of about 7.3 mgL^{-1} at 31°C, 1.0 atm. The DO was automatically recorded by the probe of bioreactor, and the slope of DO vs. time plots was used to determine oxygen uptake rates (OUR). The slope for OUR value was obtained from DO drop from 80% to 50% air saturation [30].

Assay of VHb Activity

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. The concentration of VHb produced by various recombinant strains was checked using CO-difference spectra (in which VHb has a characteristic absorption maximum at 419 nm and minimum at 436 nm) [19].

The Function of Different Promoters in *C. crenatum*

Several *C. crenatum*/*E. coli* promoter–probe shuttle vectors were constructed and used in trapping and analyzing the promoters (*lac*, T7, and *tac*). The chloramphenicol acetyltransferase (CAT) coding gene *cat* was used as a reporter. The CAT activity was measured according to the method described by Shaw [31].

Assays of Cell Concentration, Glucose, and L-Arginine

Cell concentration was firstly monitored at 562 nm, and the dry cell weight (DCW) was determined by a pre-calibrated relationship (1 OD=0.375 gL^{-1} DCW). Glucose concentration in the media was measured by using anthrone method [32]. Concentrations

of amino acids were measured by an Agilent 1100 HPLC, under the following conditions: Column Hypersil ODS-C18 4×125 mm, temperature 40°C, flow rate 1.0 mLmin^{-1}, detection fluorescence detector, Ex 340 nm Em 450 nm, eluent A 20 mmolL^{-1} Na acetate, eluent B 20 mmolL^{-1} of Na acetate/methanol/acetonitrile=1:2:2 (*v*/*v*). All of the measurements, particularly the most important state variables, such as the concentrations of cells, L-arginine, and glucose, were measured in three parallels.

Results

Comparison of Expression System of *C. crenatum*

In order to determine whether the three different promoters based on *C. glutamicum*/*E. coli* shuttle vector pJC1 could be extended to express heterologous gene in *C. crenatum* efficiently, we used *cat* gene as a selectable marker and constructed its different kinds of recombinant vectors: pJC-*lac-cat*, pJC-*T7-cat*, and pJC-*tac-cat* using standard techniques in "Materials and Methods." These plasmids were transformed into *E. coli* JM109/JM109 (DE3) and *C. crenatum*. The recombinant colonies were obtained by selection of 30 mgL^{-1} kanamycin on LB or LBG medium (Fig. 2). The results testified that the T7 promoter is silent in *C. crenatum*, although it is a strong promoter in *E. coli* (DE3). To compare the expression level of *cat* gene under the control of two different active *lac* and *tac* promoters, the chloromycetin-resistant phenotype of *E. coli* JM109 and *C. crenatum* SYPA were selected by the addition of chloromycetin with different concentrations into their culture media (Fig. 3). Meanwhile, their CAT-specific activities were determined. It is demonstrated that JM109 (pJC-*tac-cat*) and SYPA (pJC-*tac-cat*) exhibited much higher specific activities than JM109 (pJC-*lac-cat*) and SYPA (pJC-*lac-cat*). In addition, the specific activities of JM109 (pJC-*tac-cat*) and SYPA (pJC-*tac-cat*) presented 4.6- and 5.8-fold increase than those of JM109 (pJC-*lac-cat*) and SYPA (pJC-*lac-cat*) with the induction of isopropyl-β-D-thiogalactopyranoside (IPTG; Table 2). The above results suggested that the promoter *tac* was the most suitable for the CAT protein expression in both *E. coli* JM109 and

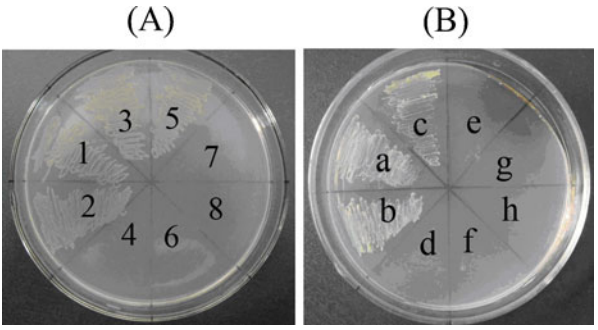

Fig. 2 Growth of transformants on LB and LBG medium containing 30 mgL^{-1} kanamycin. **a** *E. coli* JM109 transformants: *1*, *3*, *5*, *7*, and *8*, JM109 (pJC-*tac-cat*), JM109 (pJC-*lac-cat*), JM109 (DE3) (pJC-*T7-cat*), JM109, and JM109 (pJC1) induced with 0.2 M IPTG, respectively; *2*, *4*, and *6*, JM109 (pJC-*tac-cat*), JM109 (pJC-*lac-cat*), and JM109 (pJC-*T7-cat*) (DE3) without induction by IPTG. **b** *C. crenatum* SYPA transformants: *a*, *c*, *e*, and *h* SYPA (pJC-*tac-cat*), SYPA (pJC-*lac-cat*), SYPA (pJC-*T7-cat*), and SYPA (pJC1) induced with 0.2 M IPTG; *b*, *d*, *f*, and *g*, SYPA (pJC-*tac-cat*), SYPA (pJC-*lac-cat*), SYPA (pJC-*T7-cat*), and SYPA (pJC1) without induction by IPTG

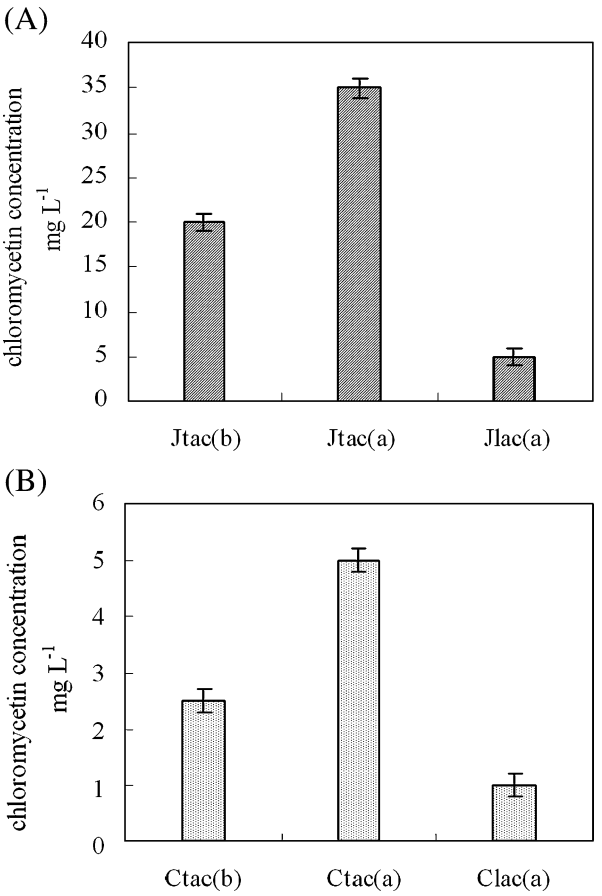

Fig. 3 The initial determination of the promoter intensity. **a** *E. coli* JM109 transformants: *Jtac*(*b*) JM109 (pJC-*tac-cat*) without IPTG, *Jtac*(*a*) JM109 (pJC-*tac-cat*) induced with 0.2 mM IPTG, *Jlac*(*a*) JM109(pJC-*lac-cat*) induced with 0.2 mM IPTG; **b** *C. crenatum* SYPA transformants: *Ctac*(*b*) SYPA (pJC-*tac-cat*) without IPTG, *Ctac*(*a*) SYPA (pJC-*tac-cat*) induced with 0.2 mM IPTG, *Clac*(*a*) SYPA (pJC-*lac-cat*) induced with 0.2 mM IPTG

C. crenatum SYPA strains among the T7, *lac*, and *tac* promoters. The vector pJC-*tac* was more efficient for heterologous gene expression in *C. crenatum*. The effectiveness of this novel system could be applied to improve L-arginine production in *C. crenatum*.

Construction of *vgb*-Bearing *C. crenatum*

The significance of a novel expression construction is to pave the way to improve the *C. crenatum*. The expression vector pJC-*tac* was employed to construct the recombinant *C.*

Table 2 Specific activities of CAT in the different strains (mean ± SD, n=3)

Strains	CAT specific activity (Umg^{-1})
JM109 (pJC-*tac-cat*)[a]	10.3±0.8
JM109 (pJC-*tac-cat*)[b]	18.5±1.2
JM109 (pJC-*lac-cat*)[b]	3.3±0.3
SYPA (pJC-*tac-cat*)[a]	2.6±0.2
SYPA (pJC-*tac-cat*)[b]	5.4±0.2
SYPA (pJC-*lac-cat*)[b]	0.8±0.1

[a] Without IPTG

[b] Induced with 0.2 mM IPTG

crenatum carrying the gene *vgb* to express VHb protein. The recombinant plasmid was transformed into *C. crenatum* SYPA with kanamycin as the selectable marker using the standard protocols in "Materials and Methods." The presence of gene *vgb* in the recombinant *C. crenatum* strain (SYPA (pJC-*tac*-*vgb*)) was confirmed by digestion of the plasmid pJC-*tac*-*vgb* extracted from the recombinant with *Pst* I and PCR method using the wild-type *C. crenatum* SYPA 5-5 as a negative control. The results showed that the plasmid pJC-*tac*-*vgb* was obtained from SYPA (pJC-*tac*-*vgb*), and only the recombinant plasmid from the recombinant *C. crenatum* released a 0.44-kb fragment identical in size to that amplified using plasmid pJC-*tac*-*vgb* from *E. coli* as template.

The expression of VHb was checked using CO-difference spectra (in which VHb has a characteristic absorption maximum at 419 nm and minimum at 436 nm) [19, 33]. Cell-free extracts from *C. crenatum* showed neither peak nor trough, while the recombinant *C. crenatum* cell-free extracts had a peak at approximately 419 nm (Fig. 4). The VHb concentrations expressed as nanomole active protein per gram wet cells were assayed at 24 h after the cell growth. The expression of level VHb induced by IPTG was about 3.4 nmol/gram wet cells, resulting in an increase of 28.5 % than that without IPTG; however, the recombinant *C. crenatum* has significantly inferior cell growth and L-arginine production when induced by IPTG. Therefore, it was reasonable to observe the fermentation by recombinant *C. crenatum* without IPTG.

Improved Production of L-Arginine by Recombinant *C. crenatum* Carrying *vgb*

The gene *vgb* from *Vitreoscilla* was introduced to express VHb in *C. crenatum* under the control of *tac* promoter to enhance the DO. The time-changing profiles of DOs of the two strains are depicted in Fig. 5a. Starting from the 100% air saturation point, during the growth phase of the recombinant *C. crenatum*, DO dropped rapidly and reached the lowest levels of 35.3% at 18 h under the fermentation conditions, while in the wild-strain, DO dropped to 42.0% at 21 h. Then, the DOs of the two strains began to rebound gradually, and the DO of the recombinant *C. crenatum* stayed at a lower level until 100 h at the late of the fermentation. This result explained an increasing utilization of oxygen in the *vgb*-bearing *C. crenatum* SYPA. At the same time, OUR value of the wild-type *C. crenatum* and the VHb-expressing strain were measured (Table 3). For the VHb-expressing strain, the OUR was 0.25 mg $A_{562}{}^{-1}h^{-1}$, much higher than that only 0.18 mg $A_{562}{}^{-1}h^{-1}$ in the wild-type strain. The recombinant strains with higher OUR indicated that VHb could promote cell respiration rate. Then the effect of VHb on the cell growth, glucose consumption, and L-arginine accumulation of the recombinant *C. crenatum* were determined. Starting from the 100% air saturation point, the fermentation curves including concentrations of cells, glucose, and L-arginine, as well as the L-arginine production rate of wild-type strain and *vgb*-bearing *C. crenatum* compared in the same media for the proper aeration fermentation are described in Fig. 5. The results illustrated that the presence of VHb could enhance cell growth and L-arginine production in the recombinant strain. The final biomass was increased to 6.45 gL^{-1}, which were 10.5% higher than 6.16 gL^{-1} obtained by the wild-type strain (Fig. 5b). The glucose consumption rate in the *vgb*-bearing strain remained faster than the control (Fig. 5c). The recombinant and wild-type *C. crenatum* produced L-arginine with final concentrations of 35.9 and 30.6 gL^{-1} under the same fermentation conditions, respectively, suggesting that L-arginine production of *vgb*-bearing *C. crenatum* was 17.3% higher than that of wild-type strain (Fig. 5d). The L-arginine production rate was almost 11.4% higher in the recombinant strain than that in the wild-type strain at these peaks (Fig. 5e). It could

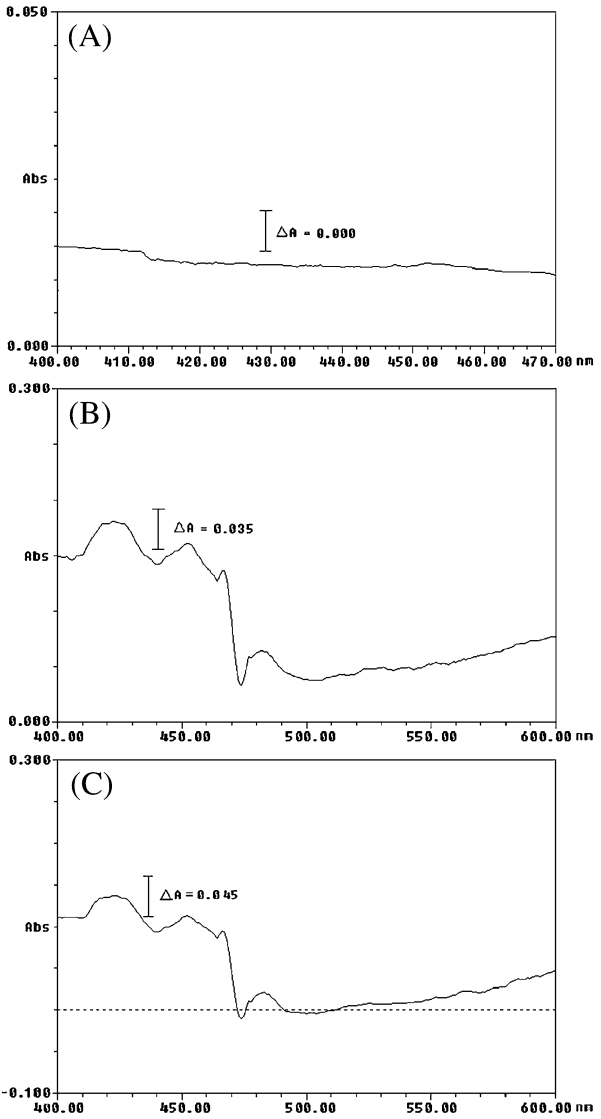

Fig. 4 CO-difference spectra of wild-type strain *C. crenatum* SYPA 5-5 and the recombinant *C. crenatum* strain cell-free extracts. **a** *C. crenatum* SYPA 5-5, **b** SYPA (pJC-*tac*-*vgb*) recombinant without IPTG, **c** SYPA (pJC-*tac*-*vgb*) recombinant induced with 0.2 mM IPTG

be clearly observed that the expression of *vgb* gene led to more oxygen utilization and thus fast growth, enhancement of L-arginine production, and increase glucose consumption for recombinant *C. crenatum*.

Discussion

The *C. glutamicum*/*E. coli* shuttle vector pJC1 could be extended to *C. crenatum* efficiently. Using the *cat* gene as a reporter, the *tac* promoter fragment was found to exhibit high expression strength both in *E. coli* and *C. crenatum*, and the recombinant *C. crenatum* had a

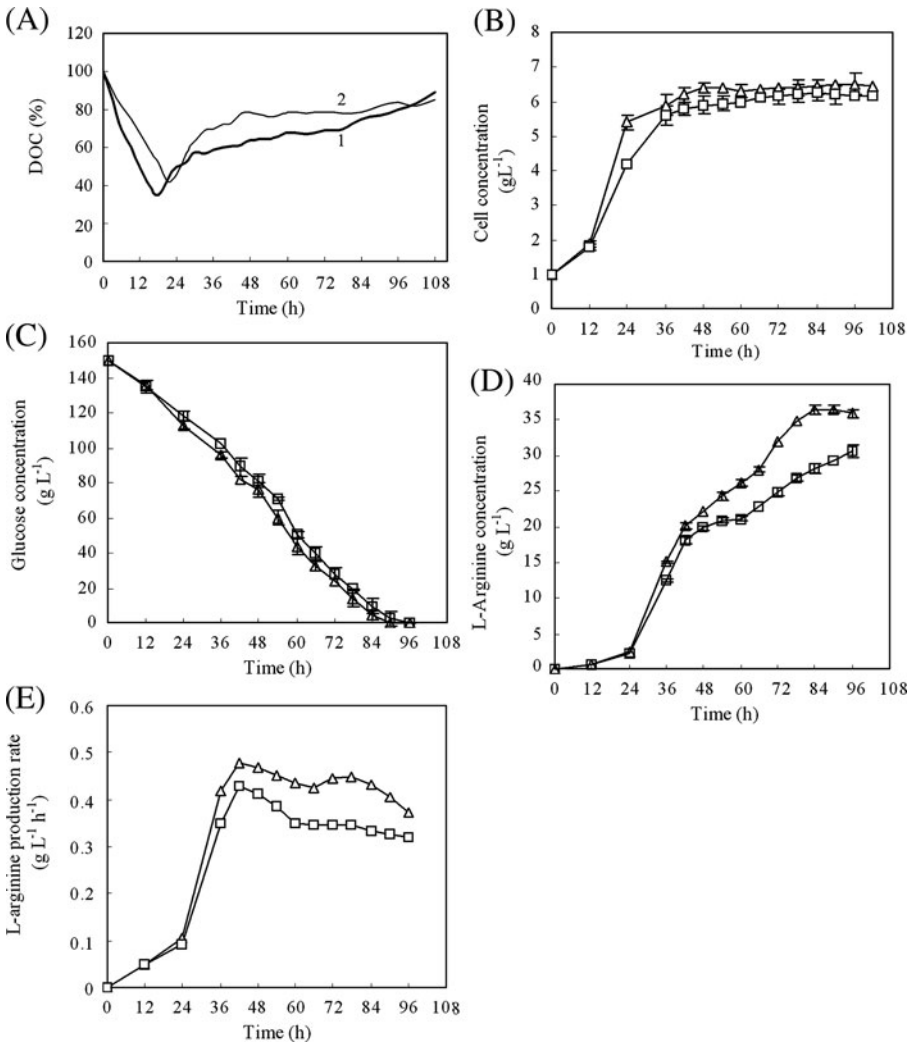

Fig. 5 Comparison of L-arginine production between the *vgb*-bearing *C. crenatum* SYPA and *C. crenatum* SYPA 5-5. **a** The DO changing patterns under the proper oxygen supply condition. *1* SYPA (pJC-*tac*-*vgb*) recombinant, *2 C. crenatum* SYPA 5-5, **b** cell concentration, **c** glucose concentration, **d** L-arginine concentration, **e** L-arginine production rate. *Empty triangles* SYPA (pJC-*tac*-*vgb*) recombinant, *empty squares C. crenatum* SYPA 5-5

high-level resistance to chloramphenicol, which revealed that the pJC-*tac* expression vector was efficient in *C. crenatum*. In fact, T7 promoter must be under control of strong bacteriophage T7 transcription and translation signals. In other words, T7, promoter only works in host cells (*E. coli* (DE3)) expressing the T7 polymerase gene whereas no

Table 3 Comparison of the OUR between wild-type *C. crenatum* SYPA 5-5 and its recombinant harboring *vgb* in LBG medium

Strains	Oxygen uptake rates (mg $A_{562}{}^{-1}h^{-1}$)
C. crenatum SYPA 5-5	0.18±0.03
SYPA (pJC-*tac*-*vgb*)	0.25±0.04

expression in *C. crenatum*. The *tac* promoter is a functional hybrid promoter derived from the *trp* and *lac* UV5 promoters. The *tac* promoters are about eleven and seven times stronger, respectively, than the *lac* UV5 promoter in *E. coli* [34]. The results indicated the *tac* promoter also provides a stronger efficiency than the *lac* promoter in *C. crenatum*. Consequently, the pJC-*tac* system was applied to the overexpression of gene *vgb* to improve the DO levels during the accumulation of L-arginine in *C. crenatum*.

The presence of VHb is usually, although not always, most effective regarding its positive effects when cells are grown in limited aeration conditions. It has been reported that the introduction of VHb could enhance flux towards ethanol production in *Saccharomyces cereviseae* [35], increase the recombinant β-galactosidase production in *Pichia pastoris* [36], and elevate levels of extracellular α-amylase in *Schwanniomyces occidentalis* [37]. The different oxygen supply strategy and thus the DO changing patterns have significant impacts on the cell growth, glucose consumption, and L-arginine production during the fermentation by *C. crenatum*. Our previous study had demonstrated that improved L-arginine production in a way of enhancing the flux distribution ratio directed to glutamate synthesis in TCA cycle during late fermentation phases and maintaining the ATP generation rate throughout the fermentation at the high oxygen supply condition. TCA cycle has to be activated in order to satisfy the increased ATP requirements for both L-arginine synthesis and metabolic maintenance of the cells [16]. Although, in the present study, we did not analyze the effect of VHb on energy metabolism, there is evidence that the beneficial effects in bacteria are the result of direct interaction of VHb with the terminal respiratory oxidase, delivering oxygen to enhance oxidative phosphorylation and thus the production of ATP [30, 38–40]. The recombinant strain containing the pJC-*tac*-*vgb* plasmid expressed VHb at a level of 3.4 $\mathrm{nmol\,g^{-1}}$ hold reasonably higher OUR which enhanced 38.8% indicating that VHb enhanced cell respiration rate compared to the wild-type strain. The higher OUR in the *vgb*-carrying recombinant strain suggested that VHb could enhance the cell respiration rate. Instead, such improvements might be due to the effect of VHb on ATP synthesis rate [39, 40]. The most significant characteristics of the recombinant *C. crenatum* were the enhanced cell growth rate and L-arginine production with the increased consumption rate of glucose compare to the wild-type strain. The introduction of gene *vgb* encoding VHb can be considered as an important tool for microbial metabolic engineering including energy regulation and oxygen consumption [41]. So, the genetic engineered *C. crenatum* with gene *vgb* supplies an effective method to increase L-arginine production by improving DO levels of the fermentation. In conclusion, this work provides a good perspective for the application of VHb in the biotransformation, especially in the amino acid aerobic processes in which oxygen was required as a substrate.

Acknowledgments This work was supported by the High-tech Research and Development Programs of China (2006AA020301, 2007AA02Z207), the National Basic Research Program of China (2007CB707800), the National Natural Science Foundation of China (20676053, 30970056) and the Program for New Century Excellent Talents in University (NCET-07-0380), Fok Ying Tung Education Foundation (121020), the Fundamental Research Funds for the Central Universities (JUSRP31001).

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