Overexpression of *Shinorhizobium Meliloti* Flavohemoglobinefficiently Improved Cell Growth and Fatty Acid Biosynthesis in Oleaginous Fungus *Mucor Circinelloides*

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Abstract

Oxygen availability is a limiting factor for lipid biosynthesis in eukaryotic microorganisms. Two bacterial hemoglobins from *Vitreoscilla sp.* (VHb) and *Shinorhizobium meliloti* (SHb), which could deliver the oxygen to the respiratory chain to produce more ATP, were introduced into *Mucor circinelloides* to alleviate oxygen limitation, thereby improving cell growth and fatty acid production. VHb and SHb genes were integrated into the *M. circinelloides* MU402 genome through homologous recombination, and their protein expression was verified by carbon monoxide difference spectrum (CO-difference spectrum)analysis. SHb-expressing strain showed higher biomass than VHb-expressing strain. The biomass of the SHb-expressing strain was increased by about 50% and the total fatty acid (TFA) content was as high as 15.7% of the dry cell weight which was about 40% higher than that of the control strain in flask conditions. In the fermenter, the maximum biomass and TFA content was obtained in SHb-expressing strains, with the biomass being 12.1 g/L and the TFA being 21.1% of the dry cell weight. VHb and SHb expression also affected the fatty acid composition with the proportion of polyunsaturated fatty acids being increased. Over-expression of bacterial hemoglobins, especially SHb increased cell growth and TFA content in *M. circinelloides* at low and high aeration, suggesting that SHb is better than VHb in improving the fatty acid production in oleaginous microorganisms.

Introduction

Oleaginous microorganisms are able to accumulate lipid to >20% of their dry cell weight. Microbial lipid is believed to a promising raw material for biofuel production or an alternative source for polyunsaturated fatty acids (Adrio 2017; Ageitos et al. 2011; Hill et al. 2006). The typical oleaginous microorganisms include microalgae, fungi and yeasts. So far, the biochemical mechanisms of lipid accumulation in oleaginous microorganisms have been deeply explored to improve lipid production (Zhang et al. 2014; Wynn et al. 2001). The intracellular lipid content in oleaginous microorganisms can be increased to approach their theoretical level by genetical modification (Blazeck et al. 2014; Qiao et al. 2017). However, the fermentation technology can affect their productivity, particularly the level of dissolved oxygen (DO) in the medium (Yen and Zhang 2011; Zhang et al. 2019). Oxygen supply is an important parameter in aerobic fermentation, since it participates in numerous intracellular biochemical reactions. The Krebs cycle, glycolysis, and *de novo* fatty acid synthesis may require O_2 supply for the production of ATP via oxidative phosphorylation (Mannix et al. 1995; Martin et al. 2002). Low oxygen levels can reduce ATP production, and influence the intracellular biochemical reactions. Thus, sufficient oxygen availability during fermentation process could be a powerful process to enhance lipid production in oleaginous microorganisms.

The increased aeration and addition of surfactants are still the most common approaches to increase the concentrations of DO during the fermentation process. However, these approaches require high energy consumption or more specialized equipment. Previously, a bacterial hemoglobin, *Vitreoscilla* hemoglobin (VHb), has been introduced into a cell by genetic modification, for improving oxygen transfer in hypoxia conditions (Kallio et al. 1994). VHb is a well-characterized bacterial hemoglobin, owing to its effective

intracellular oxygen transfer to help aerobic organism adapt to the low oxygen conditions (Frey and Kallio 2003; Khosla et al. 1990). VHb has been used to alleviate oxygen-limiting conditions by improving oxygen supply, protein expression, cellular growth, and metabolic production during microbial fermentations. VHb-expressing *Pichia pastoris*, produced more lipase than the control cells under oxygen-limited conditions (Wang et al. 2012). Over-expression of VHb in *Schwanniomyces occidentalis* increased total protein secretion and alpha-amylase production (Suthar and Chattoo 2006). The mutant *Mortierella alpina* VHb-20, expressing a codon optimized VHb gene, has 4-fold and 8-fold increases in total lipid and arachidonic acid (ARA) yields, respectively, compared to control strains under microaerobic environments. In addition, under normal conditions, the level of ARA was 1.6-fold higher than that of wild-type (Zhang et al. 2017). A VHb-expressing *Aurantiochytrium* sp. mutant showed 44% increase in total fatty acid (TFA) and 9-fold elevated of astaxanthin concentrations at low DO conditions (Suen et al. 2014). The expression of VHb in *Yarrowia lipolytic*a remarkably improved the biomass, TFA content and citrate secretion under various DO conditions compared with control strains (Zhang et al. 2019). In addition to a common increase in DO levels of microbial fermentations for enhanced cell growth, the production of metabolites can also be improved by VHb during high cell-density fermentation (Pablos et al. 2011).

Flavohemoprotein shares high degrees of sequence and structural similarities in its globin domain with hemoglobin, and its expression have more positive influence on cell growth than VHb expression. One flavohemoprotein from *Alcaligenes eutrophus* was expressed in *Escherichia coli*, which led to 50% higher final cell density than VHb-expressing strain (Frey et al. 2000). Another flavohemoglobin (SHb), isolated from *Sinorhizobium meliloti* strain 1021, has a type globin domain fused with ferredoxin reductaselike FAD-binding and NAD(P)-binding domain (Lira-Ruan et al. 2003). *Streptomyces lividans* TK24 over-expressing SHb produced more secondary metabolites than VHb-transformant, because SHb expression could activate the pentose phosphate pathway by regulating oxidative stress and increasing NADPH levels (Kim et al. 2007).

The filamentous fungus *Mucor circinelloides* has received growing attention from industrial field, as it can accumulate gamma linolenic acid (GLA) by more than 30% of the TFA content (Ratledge 2004). Now, *M. circinelloides* is considered as a model organism for studying lipid accumulation in oil producing filamentous fungi due to its clear genetic background and perfect molecular biology tools (Vellanki et al. 2018). Accordingly, to attenuate oxygen limitation, improve cell growth and promote the yields of lipid production in *M. circinelloides*, VHb and SHb genes were introduced into this fungus genome by homologous recombination, and then the influence of VHb and SHb overexpression on cellular growth and fatty acid production was investigated in this study.

Materials And Methods

Strains, plasmids and culture conditions

For plasmid amplification, *E. coli* TOP10 competent cells were cultured in Lysogeny Broth (LB) containing 100 μg of ampicillin/mL and maintained at 37°C with constant shaking. *Mucor circinelloides* MU502

(uridine auxotroph) was the recipient strain in transformation experiments for VHb and SHb genes overexpression. The VHb and SHb genes were cloned from plasmids pIB139-vhb and pIB139-sm (Mo et al. 2016). The MMC and YPG medium were supplemented with 200 μg uridine/mL if necessary, and used to culture strains at 26°C (Nicolas et al. 2007). The pH of media was fixed at 3.0 and 4.5 for colonial and mycelial growth, respectively.

Mutant strains and control strains were cultivated in 1-L baffled flasks supplemented with K&R medium (200 mL), modified to contain 80 g glucose/L and 2 g ammonium tartrate/L at 28°C with continuous shaking (150 rpm) for 96 h (Kendrick and Ratledge 1992). Fermentation was carried out a 2 L fermenter containing modified K&R medium (1.5 L) with inoculation at 10% (v/v). The fermentation process was maintained at 28°C with continuous stirring (700 rpm) and aeration (0.2 and 0.5 vvm). The pH was fixed at 6.0 by automatically adding 1 M HCl or 1 M KOH.

VHb- and SHb- expressing strains construction

The VHb and SHb genes were cloned into expression plasmid pMAT1552 for protein expression. The plasmid pMAT1552, containing the *M. circinelloides pyrG* gene surrounded down- and up-stream by 1 kb *CarRP* sequences, was employed for the construction of VHb and SHb genes overexpressing plasmids. The VHb and SHb genes were amplified using their primers with plasmids pIB139-vhb and pIB139-sm as the PCR template (Mo et al. 2016), which composes of 30-bp homologous sequences in pMAT1552 containing *Xho* I restriction sites. The PCR fragments were purified and cloned into the pMAT1552 at restriction endonucleases *Xho* I to yield pMAT1552-VHb and pMAT1552-SHb (In-Fusion HD Cloning kits, TaKaRa). The finished plasmids were linearized by restriction endonuclease *EcoR* I and *Srf* I, and then transformed into *M. circinelloides* cells through electroporation as described previously (Zhao et al. 2016), with the initial plasmid as the control. The transformants were screened by colony color and then subjected to PCR amplification. All the right transformants were named McVHb (VHb-expressing), McSHb (SHb-expressing) and Control strains.

Gene expression and CO-difference spectrum analysis

McVHb (VHb-expressing), McSHb (SHb-expressing) and the control mutant strains were cultured in modified K&R medium for 96 hours. Then, the mycelia were harvested by filtration, ground under liquid nitrogen, and dissolved in lysis buffer containing glycerol (5%), EDTA (1 mM), sodium phosphate (50 mM), pH 7.4. The cell lysate of expressed strains and control strains were centrifuged and supernatants were used for carbon monoxide (CO)-difference spectral analysis. The supernatants were divided into two aliquots and treated with sodium dithionite ($Na_2S_2O_4$, 2.5 mg/mL) to reduce the expressed VHb and SHb protein. Afterwards, CO gas was bubbled through an aliquot to coordinate SHb and VHb. The aliquot without CO exposure was used as a calibration control. CO-difference spectra were measured in the range of 400-470 nm using an UV-VIS spectrophotometer (SHIMADZU, Kyoto, Japan) according to previous methods (Liu and Webster 1974; Dikshit and Webster 1988).

Determination of dry cell weight, nitrogen and glucose in the medium

After fermentation, the cultivation was separated by filtering through a Buchner funnel at low pressure, and the filter liquor was used for determination of glucose and nitrogen concentration. The glucose oxidase Perid-test kit (Shanghai Rongsheng Biotech Co., Ltd) was used to measure the concentrations of glucose in the culture. Meanwhile, the indophenol method was employed to determine ammonium concentrations (Chaney and Marbach 1962). After washing with distilled water for 3 times, the mycelium was harvested on a weighed filter paper, followed by freeze-drying. The weight of the biomass was evaluated gravimetrically.

Determination of TFA content and fatty acid compositions

TFA was extracted from 20 mg mycelia with methanol/chloroform (1:2, v/v), then methylated with 10% (w/w) methanolic HCl for 3 h at 60°C and dissolved in n-hexane with pentadecanoic acid (15:0) as an internal standard. The content and composition of TFAs were assessed by GC with DB-Waxetr column (30 m × 0.32 mm, 0.25 μm film thickness). The following program was set: 3 min at 120°C, ramped to 200°C at 5°C/min, ramped to 220°C at 4°C/min, and held for 2 min (Zhang et al. 2018).

Statistical analysis

Statistical tests were conducted with SPSS v16.0 (SPSS Inc., IL, USA). All values were obtained from 3 independent experiments, and were presented as mean ± standard error of the mean. The difference between two groups was determined by Student's t test, and $P < 0.05$ was regarded as statistically significant.

Results

Construction of VHb- and SHb-expressing strains

The plasmid pMAT1552 with pyrG gene was chosen for VHb and SHb expression in *M. circinelloides.* This plasmid was chosen because of its designed homologous fragment, *CarRP*, which could be integrated into the chromosome via homologous recombination (Fig. 1). *CarRP* gene encodes lycopene cyclase/phytoene synthase, which is a key enzyme in β-carotene biosynthesis.[29] When VHb and SHb expression cassettes were integrated into the genomic DNA, the *CarRP* gene was broken and β-carotene biosynthesis was shutoff. Therefore, the correct mutants with homologous recombination were obtained by observing the color of single colonies. The transformants were screened until the whole colony turned white, because of multiple nucleuses in the fungus cell (Vellanki et al. 2018). All transformants were cultured in K&R medium and genomic DNA was extracted for PCR verification. The confirmed McVHb (VHb-expressing), McSHb (SHb-expressing) mutant strains, and the control strains were used for further analysis.

The characteristics of VHb and SHb protein and their expression level in M. circinelloides

VHb and SHb proteins, which belong to the hemoglobin family but originate from different species, can transfer oxygen for physiological activity. However, the number of amino acid residues of SHb (403 aa) is about three times larger than that of VHb (146 aa). Alignment with VHb sequence showed that SHb contains two additional, highly conserved motifs: oxido-reductase FAD-binding and NAD(P)-binding domain (the sequences), except the heme-binding domain (Fig. 2A).

The expression of VHb and SHb in *M. circinelloides* was verified by CO-difference spectral analysis (Fig. 2B). On treatment with CO, there was a typical peak at about 419 nm in mutant strains with VHb or SHb expression, whereas the control strain did not exhibit this peak, demonstrating that active SHb or VHb was successfully expressed in *M. circinelloides* mutant strains.

Effects of VHb and SHb expression on cellular growth and TFA content in M. circinelloides in flask

The transformants and the control strains were cultivated in flasks with glucose as a carbon source, to assess the effects of VHb and SHb expression on the biomass and TFA content in *M. circinelloides* (Fig. 3). Compared to the control strains, the biomass were improved in VHb- and SHb- expressing strains, and SHb-expressing strains had larger amount of biomass than VHb-expressing strains. The biomass of SHb-expressing strain No 1 reached a maximum of 9.8 g/l, which was 50% higher than that of control strains (Fig. 3A). The biomass showed a significant difference among the transformants with the same gene integration, however, there was only a slight difference in TFA content (Fig. 3B). Like the results of biomass, both VHb and SHb expression in *M. circinelloides* led to increased TFA content. TFA content in SHb-expressing strain number 1 was up to 15.7% of dry cell weight, which was about 40% higher than in control strains. There was also a better impact on the TFA content in SHb-expressing strains than VHb-expressing strains.

Verification of the effect of VHb and SHb expression on cellular growth of M. circienlloides grown in fermenter

VHb and SHb expression had positive influence on cell proliferation and lipid biosynthesis in *M. circinelloides* cultivated in flask. Since the fermentation condition of the flask was unstable and the ventilation was limited, the fermenter was used to verify the results obtained in flask conditions. The mutant strains were cultivated in a fermenter at 0.2 and 0.5 vvm, and the samples were collected for residual glucose concentration, ammonia concentration, biomass, and TFA content determination.

The aeration rate is an extremely important parameter for cell growth of *M. circinelloides*. At the aeration rate of 0.2 vvm, the biomass of the control and the VHb-expressing transformants was up to ~ 60% lesser (from ~8.0 g/L to ~5.0 g/L) than the biomass at the relatively high aeration rate (0.5 vvm). However, the cell growth in SHb-expressing transformants was not virtually inhibited at low oxygen condition (0.2 vvm). There was significant difference among the mutant strains, the maximum biomass of McSHb was 12.1 g/L (Fig. 4), which was higher than the control and the VHb-expressing transformants, especially at low oxygen conditions (0.2 vvm). Therefore, the expression of SHb clearly promoted cell growth in *M. circinelloides*.

During fermentation, residual glucose and ammonia concentration was measured to monitor the nutrition condition in the medium. The results showed that the rate of glucose consumption in strain McSHb with high aeration (0.5 vvm) slowed down, however the rate of ammonia consumption became faster compared to the control strain and McVHb. When these mutant strains were cultivated at low aeration (0.2 vvm), there was a same trend in the rate of ammonia consumption among the transformants, while the rate of glucose consumption showed no difference between the control strain and McVHb, and the residual glucose of strain McSHb was lower than these two strains (Fig. 5).

Effects of VHb and SHb expression on TFA content and composition of M. circienlloides grown in fermenter

When these transformants were cultivated in fermenter at high aeration condition (0.5 vvm), hemoglobin expression led to improvement of the TFA content. The TFA content of SHb-expressing strain was up to 21.1% of dry cell weight, increased by 35.2% compared to that of control strains. However, the TFA content of VHb-expressing strain was only increased by 12.8%, further. The TFA content of these mutant strains, at low aeration rate (0.2 vvm), was significantly lower than at high aeration rate. The TFA content of VHb-expressing strain markedly reduced (from 17.6–14.3%) at low aeration rate, whereas TFA content of McSHb was only weakly affected. Irrespective of the aeration rate, the TFA content of McSHb still remained at a relatively high level, and the maximum lipid content of McSHb was more than 20% of the dry cell weight (Fig. 6).

The fatty acid profile of VHb- and SHb-expressing transformants was also analyzed (Table 1). The profile revealed the main contributors to the fatty acid content of triacylglyceride were saturated fatty acid (C16:0), oleic acid (C18:1) and polyunsaturated fatty acids (C18:2 and C18:3). Stearic acid (C18:0) occured in a lower amount and measurable tetradecanoic acid (C14:0) was detected. The fatty acids of VHb- and SHb-expressing transformants presented similar values with the control strain when cultured in fermenter, and SHb expression resulted in more change in fatty acid profile than VHb expression in *M. circinelloides*. The SHb expression led to an increase in polyunsaturated fatty acids constituents and a decrease in the monounsaturated fatty acid relative to the control strain, while no obvious saturated fatty acid change was observed in two fermenter conditions. The proportion of polyunsaturated fatty acids in McSHb was clearly higher than McVHb and the proportion of C18:3 in SHb-expressing strain was up to 22.3% of TFA.

Discussion

Oleaginous microorganisms can synthesize intracellular fatty acids to >20% of their dry cell weight by using series of carbon source. The pathways for *de novo* fatty acid biosynthesis in oleaginous microorganisms have been fully mapped out (Ageitos et al. 2011). Two substrates, NADPH and acetyl-CoA, are required for fatty acid synthesis in all organisms. At present, many metabolic engineering processes are carried out to improve the supply of substrates for producing more fatty acids in oleaginous microorganisms. Along with these substrates, the biochemical pathways of fatty acid synthesis also need energy supply. ATP, the main energy donor in the cells, is mainly provided by

oxidative phosphorylation through electron respiratory chain (Mannix et al. 1995; Martin et al. 2002), and O_2 availability is one of the main factors for ATP production. VHb, an oxygen-binding protein with extremely high oxygen dissociation rate, can enhance DO diffusion especially in microaerophilic conditions. More O_2 delivery to cytochrome *d* and *o* complex, can improve the activity of the respiratory chain. This was demonstrated in VHb-expressing *Saccharomyces cerevisiae* and *E. coli* (Khosla et al. 1990; Chen et al. 1994). Consequently, the relative shift in respiratory chain activities could improve the proton-pumping efficiency, extruding protons for ATPase complexes to generate ATP. Hence, VHb over-expressing has been confirmed to be an efficient way for improving the fatty acid production in bacteria or fungi microbial fermentations. VHb-expressing *E. coli* mutant accumulated 30% and 70% higher amounts of biomass and free fatty acids, respectively, with comparable amount of glucose consumption compared with wild-type strain (Liu et al. 2017). The heterologous expression of VHb in *Aurantiochytrium sp.* mutant exhibited 44% and 9-fold higher levels of TFA and astaxanthin than that of wild-type strain in an aerobic environment (Suen et al. 2014). Moreover, the codon-optimized expression of VHb gene showed 8- and 4-fold increases in ARA and total lipid contents compared with *Mortierella alpina* wild-type strain under microaerobic conditions, and a 1.6-fold increase in ARA yield under standard growth conditions (Zhang et al. 2017). In the present work, a similar result was obtained. The TFA content and biomass of *M. circinelloides* increased because of VHb expression, irrespective of the aeration rate.

The hemoglobin domain of flavohemoprotein has high degrees of sequence and structural similarities with that of VHb. Thus, flavohemoprotein, like VHb, also had been applied to reduce the detrimental effect of oxygen limitation on microorganisms. The upregulated expression of VHb and flavohemoglobin genes in *Aureobasidium melanogenum* P16 could render to overproduce the pullulan concentrations, microbial production and glucose consumption compared with the wild-type strain P16 (Xue et al. 2019). However, SHb exhibited two extra domains, FAD-binding and NAD(P)-binding domain, which had more positive influence on cell growth and metabolite synthesis than VHb. The hemoglobin domains of flavohemoprotein and VHb shares of 51% sequence homology in the gram-negative, hydrogen-oxidizing bacteria *Alcaligenes eutrophus*. Furthermore, the expression cassettes encoding amino-terminal hemoglobin (FHPg), FHP, VHb or VHb-FAD-NAD activities were constructed. Compared with VHb, the VHb-FAD-NAD and FHP could increase the final cell densities of wild-type *E. coli* about 75% and 50, respectively (Frey et al. 2000). Another flavohemoprotein from *Sinorhizobium meliloti*, SHb, also had been proved that its expression in *Streptomyces lividans* TK24 by genetic engineering was superior to VHb for biomass production and metabolic regulation (Kim et al. 2007). Our results showed that the biomass of SHb-expressing strain was markedly increased by approximately 35% at high aeration rate and by approximately 50% at low aeration rate compared to the VHb-expressing strain.

A similar result was also found that SHb-expressing *Streptomyces actuosus* in our group led to higher nosiheptide production than a VHb-expressing strain (Mo et al. 2016). SHb expression could enhance the NADPH supply, one substrate of fatty acid biosynthesis, and it was rightly suited for the production of fatty acids in oleaginous microorganisms. Previous studies have found that NADPH supply was a limiting factor for fatty acid accumulation in oleaginous microorganisms, and many molecular biological

evidences had proved it. The overexpression of $NADP^+$-dependent malic enzymes in *Rhodococcus jostii* RHA1 and *Rhodococcus opacus* PD630 strains grown on glucose could elevate total $NADP^+$-malic enzyme level and showed 1.9-fold increase in TFA production, without affecting the cell biomass (Hernandez and Alvarez 2019). Glucose-6-phosphate dehydrogenase enzyme level towards NADPH generation was increased by 2.19-fold in the engineered microalgae *Chlorella pyrenoidosa.* Lipidomic analysis revealed a 3.09-fold increase in total lipid content in the engineered strains, and its yields gradually elevated during the growth phase and remained constant at the stationary phase (Xue et al. 2019). It had been proved that the improvement of NADPH supply could increase TFA production in *M. circinelloides*. In this study, heterologous SHb expression in *M. circinelloides* enhanced the pentose phosphate pathway and increased the production of NADPH for biosynthesis, in turn positively inspired the cellular growth and the fatty acid production.

In a word, to improve the fermentative DO condition of oleaginous fungus *M. circinelloides*, VHb and SHb genes were successfully expressed by homologous recombination and it was found both VHb and SHb expression could increase the cell growth and TFA content at either high aeration or low aeration. Furthermore, SHb expression exhibited more positive influence on cell growth and TFA content than VHb expression in *M. circinelloides*, especially at low aeration.

Declarations

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Conflict of interest: All authors declare that they have no competing interests.

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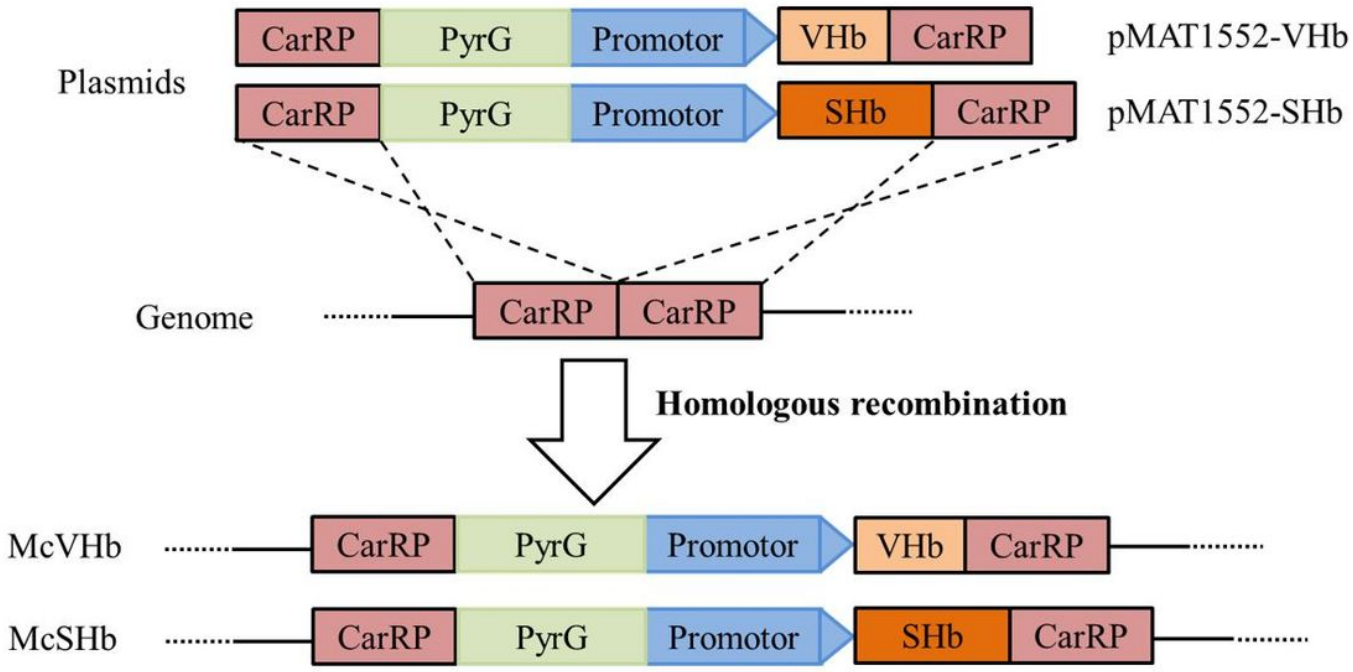
Tables

Table 1
Fatty acid compositions of McVHb, McSHb and the control strains cultivated in fermenter with aeration at 0.5 vvm and 0.2 vvm

Strains	Aeration (vvm)	Fatty acid profile (%, w/w)					
		C14:0	**C16:0**	**C18:0**	**C18:1**	**C18:2**	**C18:3**
Control	0.2	2.3 ± 0.3	21.4 ± 1.2	5.9 ± 0.7	33.2 ± 2.1	20.3 ±1.5	16.9 ± 1.6
	0.5	2.1± 0.4	20.3 ± 1.5	5.7 ± 0.5	31.3 ± 1.6	21.7 ±1.6	18.9 ± 1.1
Mc-VHb	0.2	1.9 ± 0.2	22.3 ± 1.7	6.3 ± 0.7	30.7 ± 2.5	21.5 ± 1.4	17.3 ± 0.5
	0.5	2.0 ± 0.1	21.8 ± 1.1	6.1 ± 0.3	28.2 ± 2.7	21.8 ± 2.3	20.1 ± 0.7
Mc-SHb	0.2	2.2 ± 0.4	21.7 ± 0.8	6.0 ± 0.6	26.9 ± 1.9	22.9 ± 0.4	20.3 ± 0.4
	0.5	2.4 ± 0.2	20.5 ± 1.3	5.6 ± 0.3	25.1 ± 1.2	24.3 ± 0.9	22.3 ± 2.1

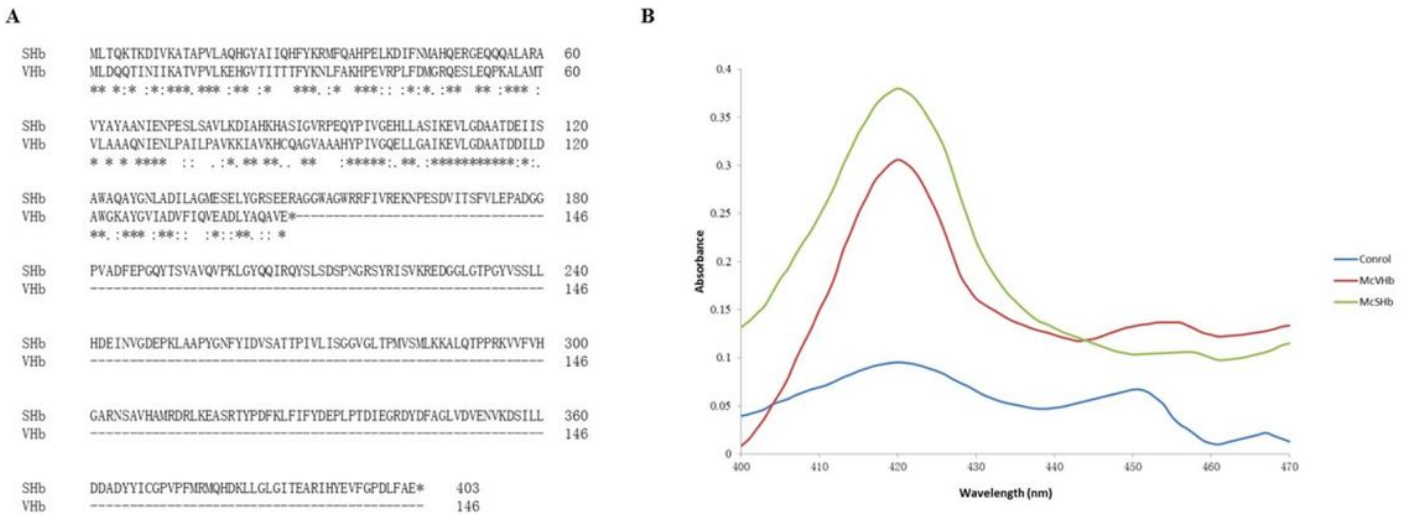
Figures



(Fig. 1)

Figure 1

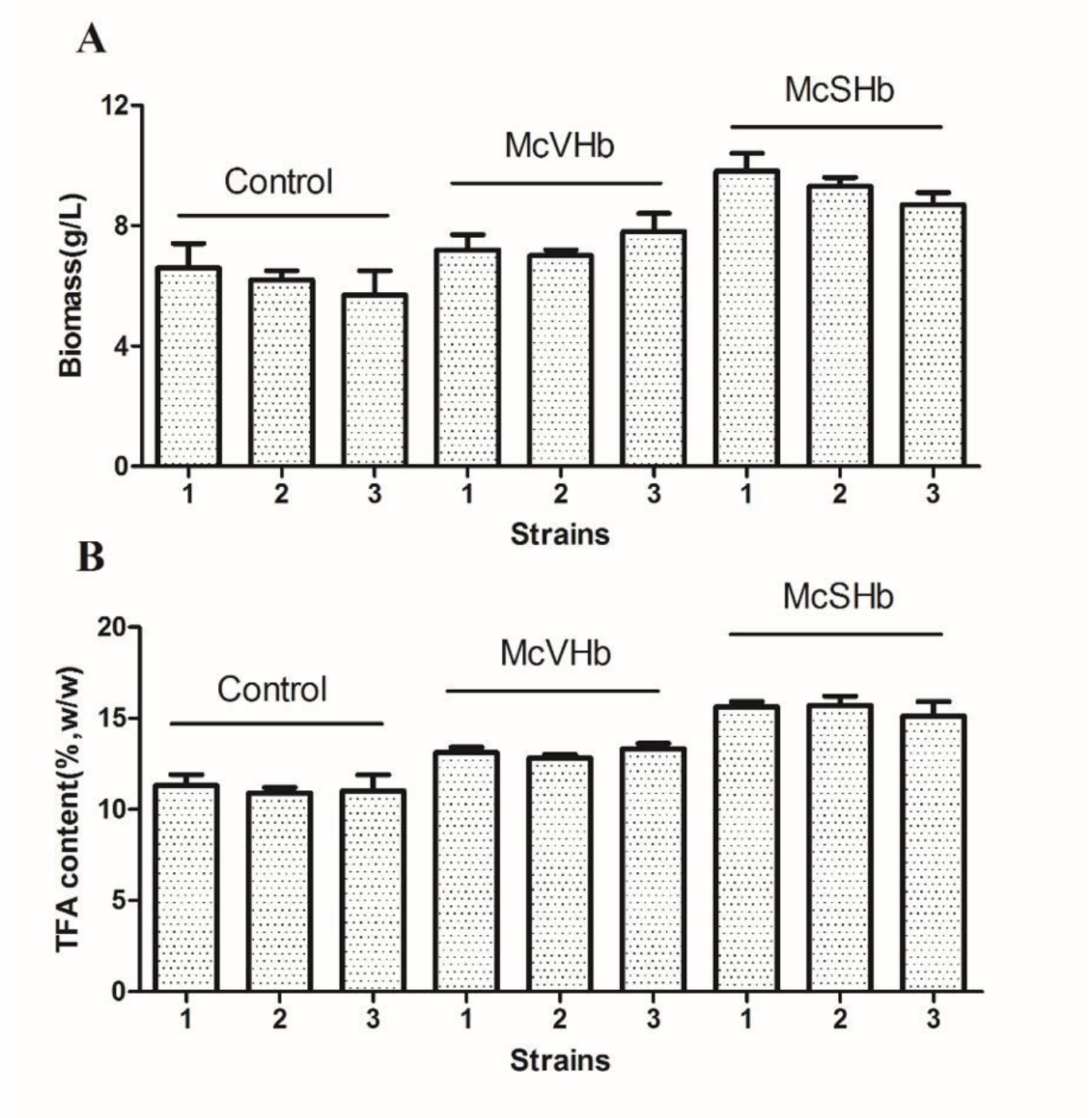
The construction strategy of VHb- and SHb-expressing strains. The plasmid pMAT1552 for the VHb and SHb genes expression containing the pyrG gene surrounded down- and up-stream by 1 kb CarRP sequences, which could be integrated into the chromosome via homologous recombination.



(Fig. 2)

Figure 2

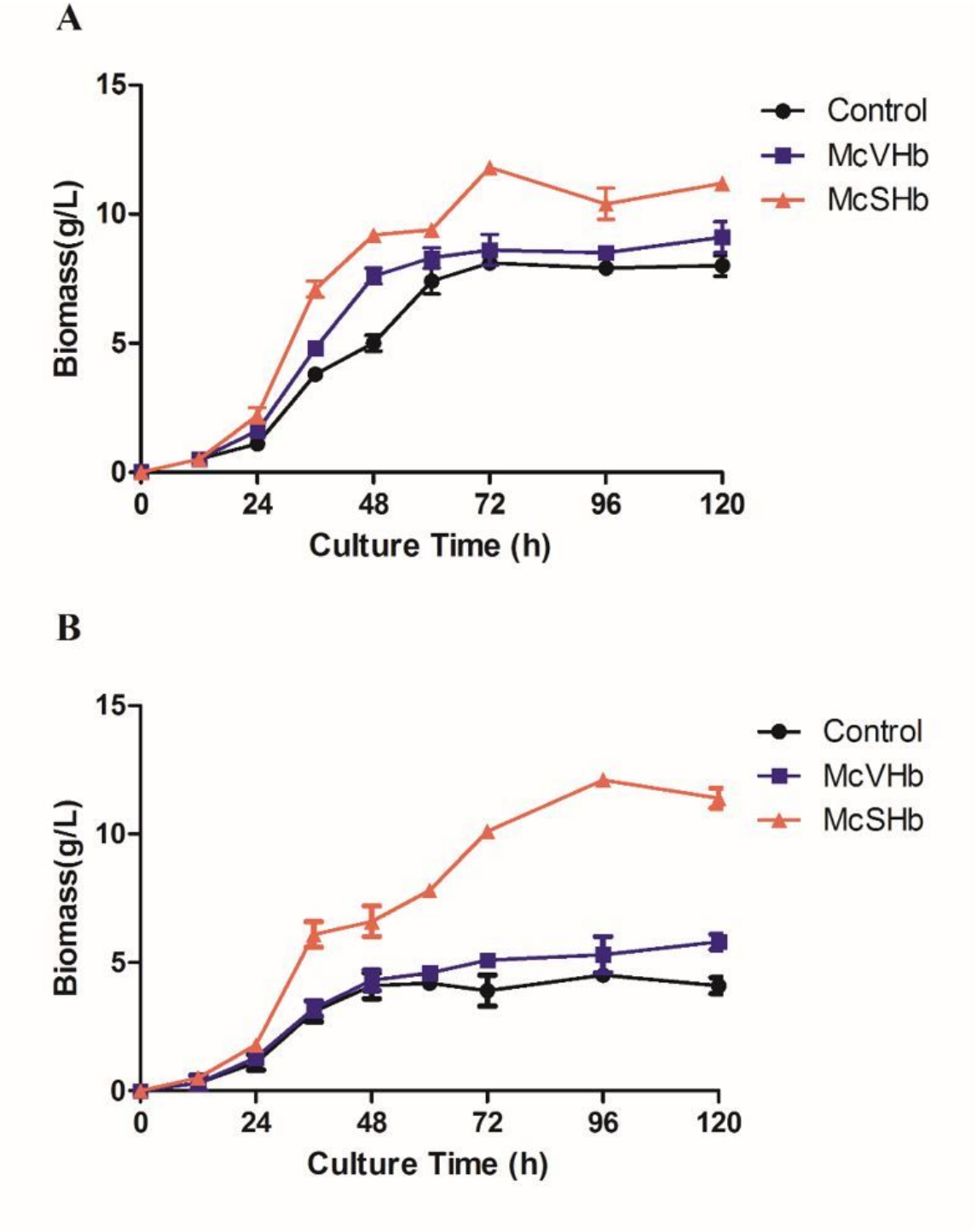
The amino acid sequence alignment (A) of VHb and SHb and their protein expression (B) in M. circinelloides. The amino acid sequence of VHb and SHb were aligned by Clustal Omega, and the protein expression of SHb and VHb was analyzed by CO-difference spectrum described in Methods.



(Fig. 3)

Figure 3

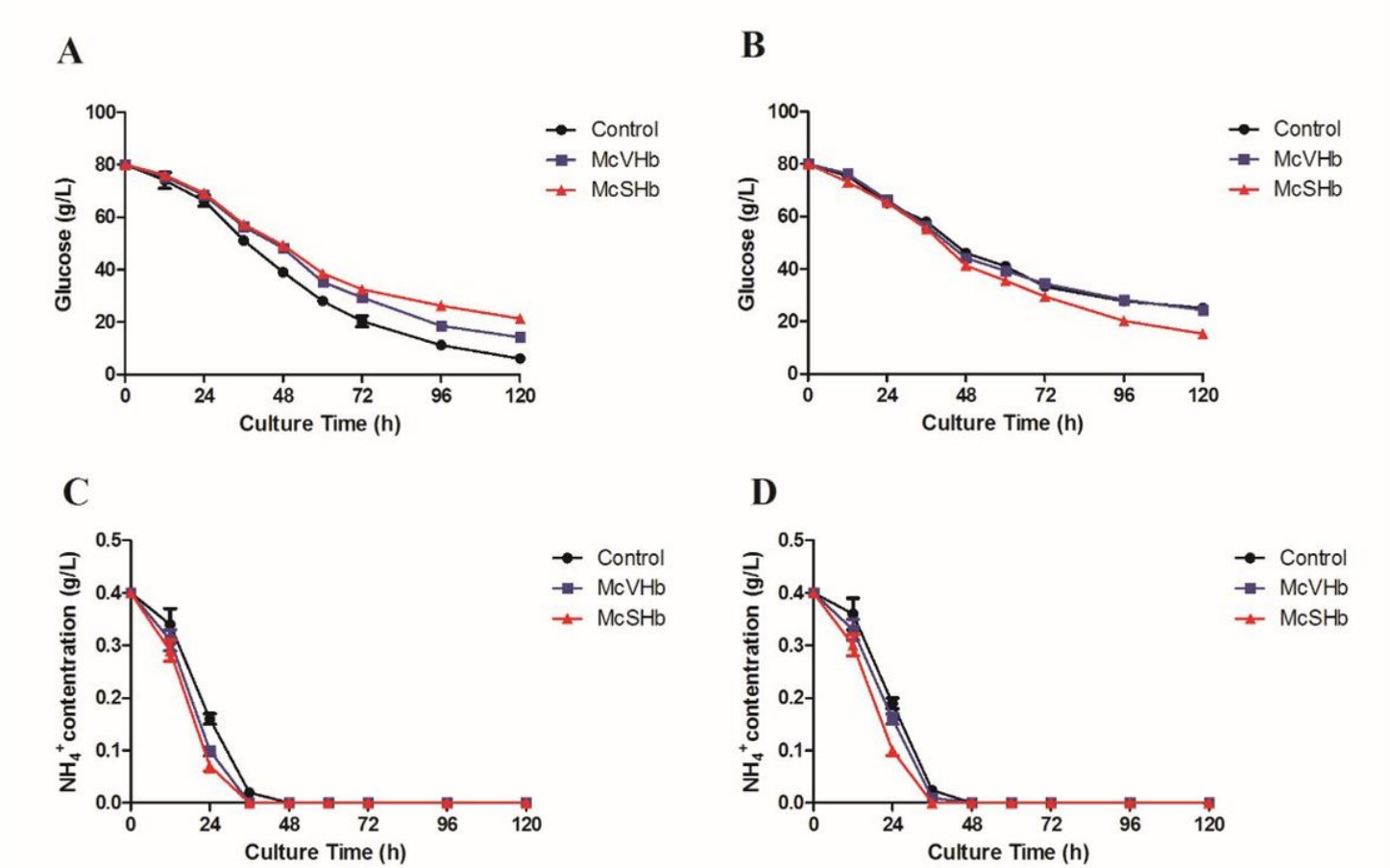
The biomass and TFA content of various transformants cultivated in flask. The transformants were cultivated in 1-L flask with modified K&R medium (200 mL) for 96 h, and then the mycelium was harvested to determine the biomass and TFA



(Fig. 4)

Figure 4

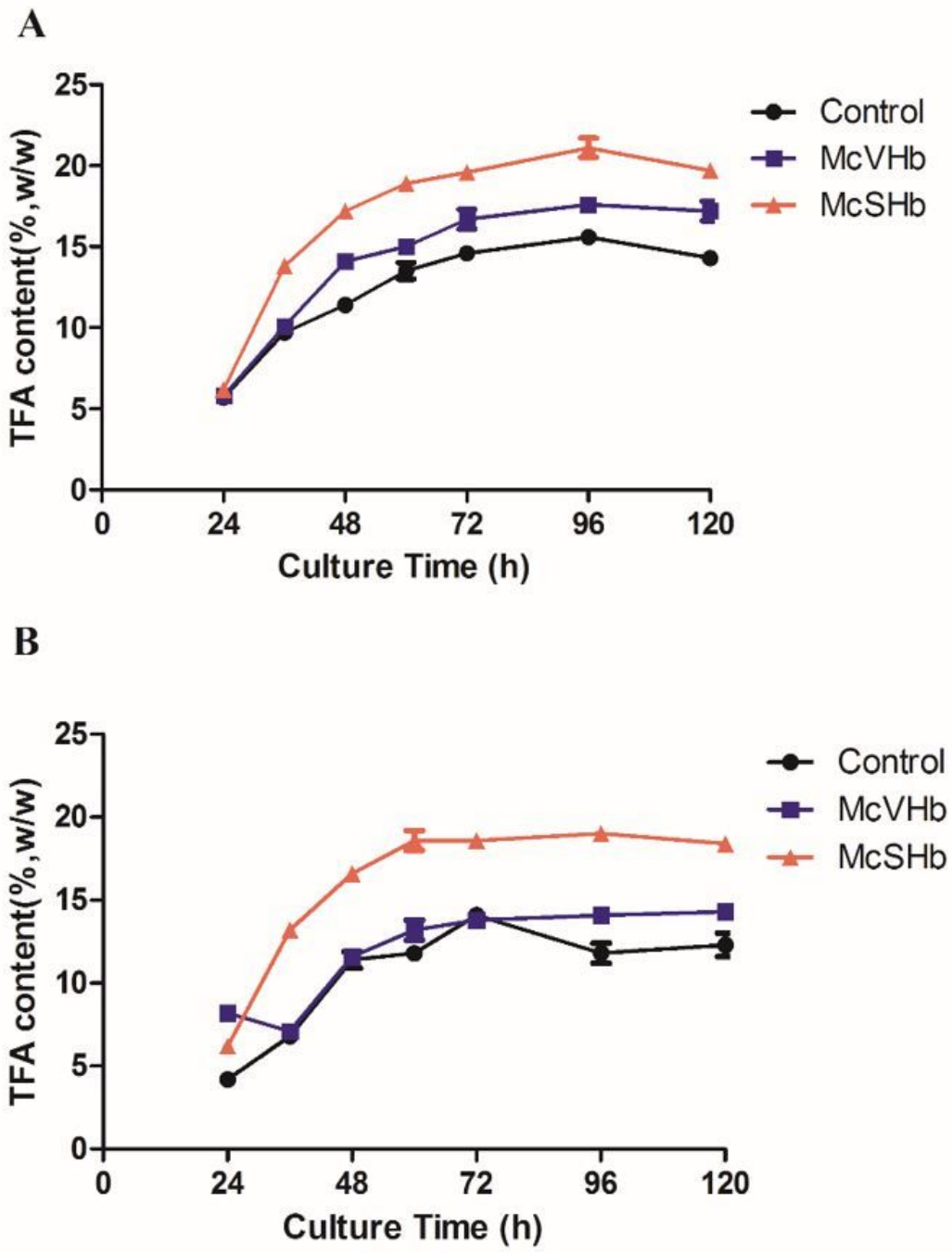
The biomass of McVHb, McSHb and the control strains cultivated in fermenter with aeration at 0.5 (A) and 0.2 (B) vvm.



(Fig. 5)

Figure 5

The residual glucose and NH4+ concentration in the fermenter with aeration at 0.5 vvm (A, C) and 0.2 vvm (B, D). The mutant strains were grown in 2 L fermenter, and supernatants of medium were collected to detect the residual glucose and NH4+ concentrations in the medium according to the operational approach described in Methods.



(Fig. 6)

Figure 6

The TFA content of McVHb, McSHb and the control strains cultivated in fermenter with aeration at 0.5 (A) and 0.2 (B) vvm.