

Increasing Ascomycin Yield in *Streptomyces Hygroscopicus* var. *Ascomyceticus* by Using Polyhydroxybutyrate as an Intracellular Carbon Supply Station

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Research

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Abstract

Background: Ascomycin is a multifunctional antibiotic produced by *Streptomyces hygroscopicus* var. *ascomyceticus*. As a secondary metabolite, the production of ascomycin is often limited by the shortage of precursors during the late fermentation phase. Polyhydroxybutyrate is an intracellular polymer accumulated by various microorganisms. The development of polyhydroxybutyrate as a useful carbon reservoir for precursor synthesis is of great significance for improving the yield of ascomycin.

Results: The fermentation characteristics of the parent strain *S. hygroscopicus* var. *ascomyceticus* FS35 showed that the accumulation and decomposition of polyhydroxybutyrate were respectively correlated with the growth of strain and the production of ascomycin. The co-overexpression of exogenous polyhydroxybutyrate synthesis gene *phaC* and native polyhydroxybutyrate decomposition gene *fkbU* increased both strain biomass and ascomycin yield. Comparative transcription analysis showed that the storage of polyhydroxybutyrate during the exponential phase accelerated biosynthesis processes by stimulating the utilization of carbon sources, and the decomposition of polyhydroxybutyrate during the stationary phase increased the biosynthesis of ascomycin precursors by enhancing metabolic flux of primary pathways. The comparative analysis of cofactor concentration reflected that the biosynthesis of polyhydroxybutyrate depended on the supply of NADH pool. Under the condition of low sugar content in the later exponential phase, the optimization of carbon source addition further strengthened the polyhydroxybutyrate metabolism by increasing total concentration of cofactors. Finally, in the fermentation medium with 22 g/L starch and 52 g/L dextrin, the ascomycin yield of co-overexpression strain was increased to 626.30 mg/L, 2.11-fold higher than that of parent strain in the initial medium (296.29 mg/L).

Conclusions: This paper reported for the first time that the polyhydroxybutyrate was beneficial to the growth of strain and the production of ascomycin by working as an intracellular carbon reservoir, storing as polymers when carbon sources are abundant and depolymerizing to monomers for the biosynthesis of precursors when carbon sources are insufficient. The successful application of polyhydroxybutyrate in increasing the output of ascomycin provided a new strategy for the high yield of other secondary metabolites.

Background

Ascomycin (FK520) is a natural macrocyclic antibiotic produced by *Streptomyces hygroscopicus* var. *ascomyceticus* ATCC 14891 [1]. Because of its specific structure, FK520 exhibits diverse biological and pharmacological activities, for example antifungal [2], antimalarial [3], immunosuppressive [4] and anticonvulsive effects [5]. Therefore, FK520 has shown broad application prospect in the field of medicine and attracted extensive attention from researchers.

In recent years, to meet the growing market demand of FK520, many efforts have been done to improve the output of FK520, including random mutagenesis [6], genetic modification of key metabolic pathways

[7], and research on regulatory mechanism [8]. In our previous work, the strain *S. hygroscopicus* var. *ascomyceticus* FS35 with high FK520 production was selected via femtosecond laser irradiation [9]. The combined genetic manipulation of target genes *fkbO* and *pyc* predicted by ¹³C-metabolic flux analysis increased the FK520 production by 24.2% [10]. And the combined overexpression of the pathway-specific regulatory gene *fkbR1* and its target gene *fkbE* increased the FK520 production by 69.9% [11]. However, the carbon sources supply for the synthesis of FK520 precursors in the late phase of fermentation is still an urgent matter to be resolved. The excessive addition of carbon sources in fermentation medium would inhibit the growth of strain and decrease the yield of target products [12]. Therefore, the development and utilization of intracellular carbon sources reservoir is of great significance for the synthesis of antibiotics.

Polyhydroxybutyrate (PHB) is a multifunctional natural polymer accumulated in various microorganisms [13]. In vitro, it has been regarded as a green alternative to traditional petrochemical plastics because of its biocompatibility, thermoplasticity and biodegradability [14-16]. In vivo, it is accumulated as an intracellular carbon storage granules when carbon source is excess in the environment, and reused when there is no suitable carbon sources [17]. In previous studies, PHB had been demonstrated could improve the production of some primary metabolites [18]. For example, the introduction of the PHB biosynthesis pathway in *Escherichia coli* QZ1111 improved the output of succinate by increasing the flux of pyruvate into citrate cycle (TCA) [19]. And the introduction of the PHB synthesis pathway into *Corynebacterium crenatum* increased the L-arginine yield by regulating the corresponding carbon metabolic flows [20]. However, the effect of PHB on antibiotic production has not been reported to date.

In *S. hygroscopicus* var. *ascomyceticus*, FK520 is assembled from 12 molecules of precursors [21]. Among these precursors, nearly half of them (5 molecules of methylmalonyl-CoA and 1 molecules of ethylmalonyl-CoA) can be derived from the ethylmalonyl-CoA pathway (EMCP) (Figure 1a), which has been gradually revealed in recent years [22]. The EMCP is mainly responsible for the assimilation of C₂-dicarboxylic acids to C₄ or C₅-units [23]. In addition, in some *Streptomyces*, the assimilation products of EMCP supply CoA-ester precursors for the biosynthesis of antibiotics [24]. It is worth noting that the ethylmalonyl-CoA from the EMCP is the special precursor for FK520, which can be used to construct the specific structure for FK520 and be a reference to distinguish FK520 from other similar antibiotics [25, 26]. Therefore, maintaining the carbon source supply of EMCP is critical to the biosynthesis of FK520. The carbon flux of EMCP mainly depends on the supply of 3-hydroxybutyryl-CoA [27], which can not only be synthesized from the primary metabolite acetyl-CoA, but can also be obtained from the decomposition of PHB (Figure 1a). Thus, it is of great significance to explore the influence of PHB on the production of FK520 and develop PHB as an intracellular supply station of carbon source for the biosynthesis of FK520.

In this study, the effects of PHB metabolism on the strain growth and FK520 production were verified by the analysis of fermentation characteristics, and the genetic manipulations associated with the PHB biosynthesis and degradation. The underlying influence of PHB accumulation on carbon utilization and the deep mechanism of PHB decomposition on precursor synthesis was systematically analyzed to confirm the potential of PHB as an intracellular carbon reservoir. The full application of PHB in promoting

the FK520 production achieved satisfactory results, providing a new perspective for the high yield strategies of secondary metabolites.

Results

The correlation between the polyhydroxybutyrate content and ascomycin production

Various microorganisms are known to accumulated PHB [28], but its presence in *S. hygrosopicus* var. *ascomyceticus* was uncertain. Here, the parent strain FS35 was fermented for 8 days to investigate the change trends of PHB contents and residual sugars during the entire fermentation (Figure 1b). In strain FS35, the PHB content gradually increased along with the decrease of residual sugars during the first four days. This suggested that when carbon sources are abundant in the environment, PHB is accumulated as intracellular carbon storage granules. This was consistent with the study of PHB in a typical PHB-producing strain *Ralstonia eutropha* H16 [29]. However, in strain FS35, the PHB content was gradually reduced when residual sugar was already at low level during the last four days (Figure 1b). This implied that when residual sugar was insufficient at the stationary phase, PHB was depolymerized as an alternate carbon source. This change trend was different from that in *R. eutropha* H16, PHB content in *R. eutropha* H16 gradually increased, and then remained at a high level until the end of fermentation [30]. So it is speculated that in *S. hygrosopicus* var. *ascomyceticus*, the decrease of PHB content during the later fermentation stages might have other use.

To understand the temporal correlation between the PHB content, FK520 yield and strain biomass, fermentation characters of FS35 were recorded and analysis. As can be seen in Figure 1c, when the PHB content increased rapidly during the exponential phase, the biomass also increased correspondingly. Because both PHB accumulation and biomass increase require the consumption of carbon sources [31], so the synthesis of PHB might be associated with the growth of strain. Excitingly, FK520 yield rapidly increased when the PHB content gradually decreased during the stationary phase (Figure 1c). This indicated that the decomposition of PHB during the stationary phase might be conducive to the synthesis of FK520.

The promoting effect of polyhydroxybutyrate metabolism on strain growth and ascomycin production

In order to explore the influence of PHB metabolism on FK520 production, the PHB synthesis gene should theoretically be knocked out to observe the changes of fermentation characteristics. However, previous studies had shown that the deletion of PHB synthesis gene might cause a series of problems such as low growth rate, high rate of reverse mutation and deficient phenotype of sporulation [22, 32]. These indicate that PHB metabolism may be not a useless cycle, but plays an indispensable role in maintaining the strain growth. Moreover, the sequence of PHB synthesis gene is rarely revealed in *Streptomyces*, and it is unknown in *S. hygrosopicus* var. *ascomyceticus*. Therefore, this paper did not consider inactivating the PHB synthesis gene, but overexpressed the PHB degradation gene *fkbU* in the parent strain FS35 to construct the overexpressed strain OfkbU. However, the FK520 production in the strain OfkbU was not significantly improved (Additional file 1: Figure S2). This might because that in the strain OfkbU, the

amount of PHB synthesized in the exponential phase was not increased, so there were no more PHB available for degradation during stationary phase. Previous studies reported that the synthase and depolymerase of PHB are simultaneously expressed in most PHB-accumulating strains, but their activities are stringently regulated to avoid ineffective circulation [33, 34]. Therefore, the combined overexpression of the PHB synthesis gene and decomposition gene in strain FS35 was deemed a reasonable approach. The PHB synthesis gene (*phaC*) in *R. eutropha* H16 had been widely studied and used [35]. Therefore, the exogenous PHB synthesis gene (*phaC*) and the native PHB decomposition gene (*fkbU*) were co-overexpressed in the parent strain FS35 to construct the co-overexpression strain OphaCfkbU.

The parent strain FS35 and co-overexpressed strain OphaCfkbU were simultaneously fermented for 192 h to observe the changes of fermentation parameters caused by the co-overexpression (Figure 2a-d). Compared to the parent strain FS35, strain OphaCfkbU consumed more sugar and accumulated more PHB during the exponential phase (Figure 2a). This indicated that the increase of PHB biosynthesis promoted the consumption of carbon sources. Surprisingly, strain OphaCfkbU accumulated more biomass during the exponential phase than strain FS35 (Figure 2b). And the mycelia of strain OphaCfkbU in the fermentation broth at 96h and on plate culture at 20d were all stronger than that of strain FS35 (Figure 2d). This indicated that the PHB biosynthesis was beneficial to the growth of strain. A previous report had shown that in *Rhodospirillum rubrum* S1, PHB could promote the conversion of acetate into biomass [36]. Accordingly, PHB accumulation might promote the conversion of starch and dextrin into biomass in *S. hygroscopicus* var. *ascomyceticus*.

As expected, in strain OphaCfkbU, the more abundant supply of PHB during the exponential phase ensured the more decomposition of PHB during stationary phase (Figure 2c). This confirmed that the co-overexpression operation in strain OphaCfkbU did not cause an ineffective circulation, but instead promoted the accumulation of PHB during the exponential phase as well as its decomposition during the stationary phase. And this resulted in an increased of FK520 yield to 511.50 mg/L, 1.73-fold higher than that of strain FS35 (296.29 mg/L). This was the first time that PHB was found to promote the biosynthesis of antibiotics. And this means that the accumulation and depolymerization of PHB may be a very valuable cycle for the production of the secondary metabolites.

Transcriptomic evidences of the stimulating effect of polyhydroxybutyrate on strain growth

In order to fully verify the function of PHB as a carbon reservoir, the different patterns of PHB metabolism during the exponential phase and stationary phase were proved firstly through qRT-PCR. In *Streptomyces hygroscopicus* var. *ascomyceticus*, gene *hcd* is responsible for the synthesis of PHB monomer, and gene *fkbE* is responsible for the dehydration of PHB monomer (Figure 1a). Considering that the genes *phaC* and *fkbU* were co-overexpressed in this study, the different patterns of PHB metabolism during the exponential phase and stationary phase were testified by the comparative transcription analysis of genes *hcd* and *fkbE* (Figure 3a). During the exponential phase, the expression level of gene *hcd* in strain OphaCfkbU was higher than that in strain FS35, while the expression level of gene *fkbE* was lower. This

indicated that 3-hydroxybutyryl-CoA was used more to synthesize PHB than dehydrate to crotonyl-CoA during the exponential phase. On the contrary, during the stationary phase, the expression level of gene *hcd* in strain OphaCfkbU was lower than that in strain FS35, while the expression level of gene *fbkE* was higher. This reflected that during the stationary phase, the conversion of 3-hydroxybutyryl-CoA to crotonyl-CoA was mainly dependent on the depolymerization of PHB rather than the supply of acetoacetyl-CoA. These results demonstrated that PHB was indeed stored during the exponential phase and decomposed for reuse during the stationary phase. And the time difference between the accumulation and degradation ensured the double promotion of PHB on strain growth and FK520 production.

To provide further evidence of the stimulating effect of PHB metabolism on strain growth, comparative transcriptomic analysis between strain FS35 and OphaCfkbU was carried out using RNA samples drawn at 50h. Differential expression analysis revealed 285 up and 218 down-regulated genes in strain OphaCfkbU compared to strain FS35 (Figure 3b). These differentially expressed genes were clustered using GO enrichment analysis into three categories (biological process, cellular component and molecular function) (Figure 3c and 3d). The up-regulated genes were mostly related to the biosynthesis and metabolism of organic substances, which are necessary for cell growth (Figure 3c). The down-regulated genes were mainly involved in the biosynthesis of heterocyclic organics, which is unfavorable for biomass accumulation (Figure 3d). These results provided evidences for the stimulating effect of PHB on strain growth in both positive and negative aspects.

Furthermore, the significantly up-regulated genes in strain OphaCfkbU were mainly mapped to six metabolic pathways according to the Kyoto encyclopedia of genes and genomes (KEGG) enrichment analysis (Table 1). The up-regulation of genes in the starch and sucrose metabolism confirmed the promoting effect of PHB accumulation on the utilization of sugar. The up-regulation of genes in the ABC transport system reflected that PHB could not only accelerate the transport of sugar sources to provide more raw material for intracellular biosynthesis, but also provide more metal ions as cofactors for various enzymes. The up-regulation of genes in the ribosome biosynthesis pathway confirmed that PHB could increase the generation of ribosomes, which are key organelles responsible for translation. The up-regulation of genes in the pantothenate and coenzyme A biosynthesis pathway proved that PHB could promote the biosynthesis of coenzyme A and acyl-carrier protein (ACP), which participate in the metabolism of carbohydrates, fatty acids and energy [23]. The up-regulation of genes in the nicotinate and nicotinamide metabolism indicated that PHB could increase the biosynthesis of cofactors (NADH, NAD⁺, NADPH, NADP⁺), which are the important components of redox enzymes in the glycolysis pathway (EMP), citrate cycle (TCA) and electron transport chain [37]. The up-regulation of genes in sulfur metabolism could improve the generation of 3'-phosphoadenosine 5'-phosphosulfate (PAPS) and sulfide. PAPS is a sulfate donor for many sulfating reactions and sulfide is an important material for the biosynthesis of cysteine [38]. This indicated that the synthesis of PHB could promote the creation of sulfur-containing compounds and cysteine. All these data proved that PHB could stimulate the growth of strain by promoting the utilization of carbon sources for biosynthesis processes. As an intracellular

carbon reservoir, PHB not only stored carbon sources in the form of polymer, but also drove the flow of carbon sources to biomass.

The mechanism underlying the promoting effect of polyhydroxybutyrate on ascomycin production

In order to explore the influence mechanism of PHB depolymerization on FK520 biosynthesis, crucial genes in the primary metabolic pathways and all genes in the FK520 gene cluster were selected for the comparative analysis of transcription levels between FS35 and OphaCfkbU at 112 h (Figure 4). Compared to the parent strain FS35, the expression of *fbkU* in strain OphaCfkbU was up-regulated significantly as expected (Figure 4a). Moreover, the expression of all the selected genes in EMCP was also up-regulated significantly in this strain. This indicated that the degradation of PHB regulated the metabolic flow of EMCP to increase the biosynthesis of precursor ethylmalonyl-CoA and methylmalonyl-CoA. The *mut* and *aceB* genes encode the enzymes connecting EMCP with TCA. The up-regulation of their expression in strain OphaCfkbU promoted the conversion of metabolites between EMCP and TCA. The genes *gltA*, *idh* and *korB* encode key enzymes of TCA [39]. The up-regulation of their expression in strain OphaCfkbU indicated that the TCA was strengthened on account of the enhancement of PHB metabolism.

Oxaloacetate in the TCA is not only the source of the precursor pipicolate for FK520, but also the junction between the TCA and EMP. The up-regulated expression of *fbkL* and *pkcA* in strain OphaCfkbU indicated that the enhancement of the TCA promoted the conversion of oxaloacetate into precursor pipicolate and intermediate phosphoenolpyruvate. The latter increased the synthesis of precursor 4,5-dihydroxycyclohex-1-enecarboxylic acid (DHCHC), which was reflected by the up-regulated expression of the gene *fbkO* in strain OphaCfkbU. The up and down-regulated expression of selected genes in the EMP suggested that the enhancement of interconversion between the metabolites in TCA and EMP also regulated the metabolic flow of EMP. Furthermore, the up-regulated expression of the genes *fbkG*, *fbkH*, *fbkI*, *fbkJ* and *fbkK* in strain OphaCfkbU indicated that the synthesis of the precursor methoxymalonyl-ACP was also increased. All the data above illustrated that PHB could increase the biosynthesis of FK520 precursors by regulating the metabolic flows of the EMCP, TCA and EMP. On this basis, the expression of genes responsible for different functions in the FK520 gene cluster was also up-regulated accordingly to different degrees (Figure 4b). This confirmed that the enhancement of PHB metabolism increased the production of FK520 by promoting the biosynthesis of precursors.

FK523 is the main impurity in the production of FK520, resulting from the assembly of methylmalonyl-CoA onto the C21 position of macrolide skeleton instead of the specific precursor ethylmalonyl-CoA [6]. To rule out competitive use of these precursors by by-products, the yield of FK523 and the ratio of FK523/FK520 in the strain FS35 and OphaCfkbU was recorded as shown in Figure 4c. Although the yield of FK523 in the strain OphaCfkbU was higher than that in the parent strain FS35, the ratio of FK523/FK520 was decreased slightly. This indicated that the increase of precursors was mainly beneficial to the synthesis of FK520 but not FK523. This might be because that the degradation of PHB promoted the synthesis of ethylmalonyl-CoA more than methylmalonyl-CoA, which could be demonstrated by the change folds of genes *fbkE* (3.98), *fbkS* (4.12), *ecm* (3.35) and *bccA* (3.27) (Figure 4a). Previous study also showed that the overexpression of *fbkS* could promote the yield of FK520 but

decrease the production of FK523 by increasing the synthesis of ethylmalonyl-CoA [6]. Here, the priority supply of PHB to the specific precursor ethylmalonyl-CoA guaranteed the increase of FK520 production was not affected by the by-products.

The influence of cofactor concentration on polyhydroxybutyrate metabolism

To further develop the value of PHB as the intracellular carbon reservoir, the factors influencing the synthesis of PHB deserved to be explored. For the biosynthesis of PHB, the conversion of acetoacetyl-CoA to 3-hydroxybutyryl-CoA is an important step, which catalyzed by the NADH-dependent 3-hydroxybutyryl-CoA dehydrogenase (encoding by *hcd*) or the NAD(P)H-dependent acetoacetyl-CoA reductase (encoding by *phaB*) (Figure 5a). The NAD(P)H pool and the ratio of NAD(P)H/NAD(P)⁺ could significantly influence the content of PHB [40, 41]. The sequence alignment showed that there is a copy of *hcd* gene in the genome of *S. hygroscopicus* var. *ascomyceticus*. (Additional file 1: Figure S3), but no copy of *phaB* gene. Thus it was inferred that the synthesis of PHB in *S. hygroscopicus* var. *ascomyceticus* might be influenced by the pool of NADH and the ratio of NADH/NAD⁺ (Figure 5b). To test this inference, the concentrations of NADH, NAD⁺, NADPH, NADP⁺ and the ratio of NAD⁺/NADH, NADPH/NADP⁺ in strain FS35 and OphaCfkbU were measured at the end of the exponential phase.

Compared with strain FS35, there were higher concentrations of NADH + NAD⁺ and NADPH + NADP⁺ in strain OphaCfkbU (Figure 5d). This reflected that the increase of PHB biosynthesis during the exponential phase promoted the biosynthesis of these cofactors. This was consistent with the transcriptome data above (Table. 1). The concentration of NAD⁺ in strain OphaCfkbU was higher than that in strain FS35 at the end of the exponential phase, and the concentration of NADH in strain OphaCfkbU was lower (Figure 5c). So, the ratio of NAD⁺/NADH in strain OphaCfkbU was significantly higher than that in strain FS35 (Figure 5d). This indicated that in OphaCfkbU, the increase of PHB biosynthesis during the exponential phase accelerated the consumption rate of NADH. On the contrary, the concentration of NADPH in strain OphaCfkbU was higher than that in strain FS35, but the concentration of NADP⁺ in strain OphaCfkbU was lower at the end of the exponential phase (Figure 5c). So, the ratio of NADPH/NADP⁺ in strain OphaCfkbU was significantly higher than that in strain FS35 (Figure 5d). This indicated that the production rate of NADPH in OphaCfkbU was increased during the exponential phase. This might because the increase of PHB synthesis in the exponential phase stimulated the carbon flux of pentose phosphate pathway (PPP) (Figure 5b). Since the NADPH is an essential reducing power in the assembly of FK520 precursors [42, 43], so the high production rate of NADPH also gave a reasonable explanation for the high yield of FK520 in the strain OphaCfkbU. On the whole, all the data above mean that the synthesis of PHB during the exponential phase depended on the conversion of NADH to NAD⁺. Therefore, increasing the intracellular concentration of NADH might be an effective strategy to further enhance the PHB metabolism.

Further increase of the ascocin yield by strengthening the polyhydroxybutyrate metabolism

Although PHB was proved could increase the synthesis of cofactors (NADH, NAD⁺, NADPH, NADP⁺) by stimulating the utilization of carbon sources (Table 1), the total concentrations of these cofactors in the

strain OphaCfkbU was only slightly higher than that in strain FS35 (Figure 5d). This might be caused by the insufficient of residual sugar at the later stage of exponential phase (Figure 2a). So the optimization of carbon source addition was carried out to explore its effects on NADH pool, the PHB accumulation and the FK520 production. As the main carbon sources in the fermentation medium, the addition of starch and dextrin was first optimized in single-factor experiments (Figure 6a and 6b). When the addition of starch increased to 24 g/L, the FK520 yield reached up to 576.14 mg/L, increased by 64.64 mg/L than that in the initial fermentation medium (511.50 mg/L) (Figure 6a). When the addition of dextrin increased to 56 g/L, the FK520 yield reached up to 602.73 mg/L, increased by 91.23 mg/L than that in the initial fermentation medium (511.50 mg/L) (Figure 6b). Then, the mixed addition of starch and dextrin were optimized in two-factor experiments. When the addition of starch and dextrin increased to 22 g/L and 52 g/L respectively, the FK520 production reached up to 626.30 mg/L, 1.22-fold higher than that in the initial medium (511.50 mg/L) and 2.11-fold higher than that of the parent strain FS35 in the initial medium (296.29 mg/L) (Figure 6c). Thus, 22 g/L starch and 52 g/L dextrin were determined as the optimal concentrations of carbon sources for the strain OphaCfkbU to produce FK520.

In the optimal fermentation medium, strain OphaCfkbU possessed a higher concentration of cofactor pool at the end of the exponential phase (Figure 6f). And the amount of NADH converted to NAD⁺ was increased during the exponential phase, even though a slightly decrease of conversion rate (Figure 6e and 6f). Furthermore, strain OphaCfkbU also possessed the more PHB content in the optimal fermentation medium (Figure 6d). These reflected that the addition of carbon sources enhanced the NADH-dependent PHB accumulation. Meanwhile, the amount of NADP⁺ converted to NADPH was also increased during the exponential phase (Figure 6e), providing more reducing power for the synthesis of FK520 during the stationary phase. On the whole, the addition of carbon sources led to more accumulation of PHB and more production of FK520, playing the role of carbon reservoir to a greater extent.

Discussion

For the biosynthesis of the secondary metabolites, the supply of precursors from the primary metabolism pathways is very important [44]. FK520 is a secondary metabolite produced by *Streptomyces hygroscopicus* var. *ascomyceticus*, so enhancing the supply of precursors is an efficient way to increase the production of this antibiotic [45]. It is well-known that the exogenous feeding of important metabolites is an effective method to increase the biosynthesis of precursors [46-48]. In our previous study, metabolic profiling analysis showed that lysine (Lys), used for the synthesis of precursors pipercolic, and valine (Val), used for the synthesis of precursors methylmalonyl-CoA, were the key primary metabolites in the production of FK520 under the condition of adding resin HP20. Adding 1.0 g/L Val to the fermentation broth at 48 h increased the yield of FK520 by 9.2 %, and adding 1.0 g/L Lys at 72 h increased the FK520 yield by 13.2 % [2]. Similarly, another previous research showed that for the shikimic acid-resistant strain SA68, adding 3 g/L shikimic acid, an important metabolite used for the synthesis of precursor DHCHC, to the fermentation broth at 48 h increased the yield of FK520 by 36 % [45]. Here, the decomposition of PHB was also proved to increase the production of FK520 by enhancing the supply of various precursors from

the main primary metabolic pathways. However, compared with strategy of exogenous feeding, the application of PHB metabolism seemed to be a more cost-effective strategy. Because the accumulation of PHB required only some cheap carbon sources, such as starch and dextrin, rather than some high-value amino acids or other primary metabolic intermediates. And these cheap carbon sources only need to be added in the preparation of fermentation medium, but not in the fermentation process, reducing the possibility of bacterial contamination. Furthermore, the decomposition of PHB during the stationary phase not only promoted the biosynthesis of all precursors through strengthening global carbon flux, but also ensured the priority supply to the specific precursor, eliminating the competitive use of these precursors by by-products. Therefore, the strategy of using PHB as an intracellular carbon reservoir increased the production of FK520 to a greater extent.

In fact, it had been reported that the introduction of PHB synthesis pathway could promote the production of some primary metabolites, such as L-threonine, succinate and L-arginine, by driving the carbon flux of related primary metabolic pathways [18-20]. Even so, PHB metabolism was also once thought to be an ineffective cycle in the biosynthesis of dicarboxylic acids in the EMCP. Previous study showed that in *Methylobacterium extorquens*, PHB metabolism decreased the yields of mesaconic acid and 2-methylsuccinic acid by competing the common source 3-hydroxybutyryl-CoA with methylsuccinyl-CoA and mesaconyl-CoA in the EMCP. However, although the knockout of PHB synthesis gene *phaC* temporarily increased the yield of these dicarboxylic acids, the growth rate of the mutant strain decreased significantly, and due to high genetic instability, the knockout strain frequently appeared suppressor cells, which grew at the same rate as the wild strain but produced less dicarboxylic acids than the wild strain [22]. Similarly, in *Bacillus thuringiensis*, the deletion of gene *phaC* resulted in low cell yield and even sporulation deficient phenotype. Proteomic analysis showed that in the knockout strain, the basic metabolism was disrupted and a variety of invalid products were synthesis [32]. All of these indicated that PHB metabolism is not a useless cycle, and it plays an indispensable role in maintaining strain growth and regulating intracellular carbon flux.

For the primary metabolites, the effect of PHB on their production may be uncertain. Because the primary metabolites are produced during the synthesis of PHB. This time coincidence makes it possible for PHB to increase the yield of some primary metabolites by driving the metabolic flow, or to reduce the production of some primary metabolites by competing for common substrates, as described above. In contrast, for the secondary metabolites such as FK520, this study showed that their biosynthesis was accompanied by the decomposition of PHB. When FK520 was synthesized during the later fermentation phase, PHB was decomposed into monomers, providing the available carbon source for the synthesis of precursors. At this point, the role of PHB metabolism is not as a competitor, but as a contributor to the production of the secondary metabolites. This is the first report that the decomposition of PHB can promote the production of the secondary metabolite by enhancing the carbon flux of the primary metabolic pathways. In reality, a previous study have speculated that PHB might serve as a carbon reserve material for the production of antibiotics in some *Streptomyces*, such as *Streptomyces venezuelae* and *Streptomyces coelicolor* A3 (2) [49], but there is no detailed evidence to support this speculation. This paper described the role of PHB as an intracellular carbon reservoir in enhancing the

output of FK520 in detail, so as to provide a reference for researching the effect of PHB on the production of other antibiotics.

In addition, instead of the single introduction or inactivation of PHB synthesis pathway, this work co-overexpressed the PHB synthesis gene *phaC* and the decomposition gene *fbu* in the parent strain. Different from the defective growth of knockout strains mentioned above, the co-overexpressed strain accumulated more biomass and produced more FK520 due to the time difference between the accumulation and degradation of PHB. Previous study showed that the introduction of PHB gene cluster in *Corynebacterium crenatum* could improve the growth level of strains by promoting the consumption of glucose [20]. Here, the comparative transcriptomic analysis reflected that when carbon sources were abundant in the environment, polyhydroxybutyrate stored in the form of polymer and stimulated strain growth by promoting the utilization of carbon sources. This is a systematic analysis of the influence mechanism of PHB accumulation on the growth of bacteria, providing a reference for the future research.

In the most PHB-producing bacteria, the synthesis of PHB monomer is catalyzed by the NADPH-dependent acetoacetyl-CoA reductase (encoding by *phaB*), and the accumulation of PHB mainly depended on the supply of NADPH [50]. However, NADPH is mainly responsible for the reduction in anabolism, so the biosynthesis of PHB is often limited due to the lack of NADPH. Unlike NADPH deficiency, NADH can be synthesized in large quantities via the EMP and TCA when the carbon sources are sufficient [41]. So previous studies have increased the production of PHB by replacing NADPH-dependent acetoacetyl-CoA reductase with the NADH-dependent acetoacetyl-CoA reductase [40, 41]. In this study, the synthesis of PHB monomer was proved to be catalyzed by the NADH-dependent 3-hydroxybutyryl-CoA dehydrogenase in *Streptomyces hygroscopicus* var. *ascomyceticus*. This is an example that the accumulation of PHB did not depend on NADPH but on NADH, providing a reference for the research of PHB synthesis in other bacteria.

In this study, the optimization of carbon addition increased the production of FK520 to a higher level by strengthening the PHB metabolism. In general, the excess addition of carbon source in the fermentation medium may inhibit the strain growth and the biosynthesis of target products. This problem is usually solved by fed-batch fermentation or adaptive evolution of tolerance [12, 51]. For example, high addition ration of glucose and maltose in the fermentation medium caused low cell density and less acarbose production in *Streptomyces* M37. Thus, a two-stage fermentation strategy was carried out to weaken the inhibition and increase the production of acarbose [52]. Similarly, the fed-batch fermentation of glucose achieved high cell density and high yield of valinomycin by reducing the glucose inhibition [51]. Besides, the glucose-tolerance strain was selected by the stepwise UV mutagenesis, achieving the high yield of rapamycin in *Streptomyces hygroscopicus* [12]. Here, PHB stored as polymers by accelerating the utilization of carbon sources when carbon sources are abundant, and depolymerized to monomers for the biosynthesis of FK520 precursors when carbon sources are insufficient. PHB acted as a buffer role to a certain extent, avoiding the inhibition phenomenon caused by the excessive carbon sources. Therefore, the enhancement of PHB metabolism could be used as a new strategy for high density cell culture and high yield of secondary metabolites. It is well known that nitrogen limitation at the present of sufficient

carbon sources would increase the NADH pool and repress the TCA cycle, resulting in more acetyl-CoA flowing into EMCP for the synthesis of PHB [41, 50]. So enhancing the PHB metabolism by triggering the nitrogen limitation will be our next goal.

Conclusions

Based on the systematic transcription analysis, this paper demonstrated for the first time that polyhydroxybutyrate worked as an intracellular carbon reservoir in *S. hygrosopicus* var. *ascomyceticus*. When carbon sources were abundant, polyhydroxybutyrate stored in the form of polymer and stimulated strain growth by promoting the utilization of carbon sources. And when carbon sources are insufficient, polyhydroxybutyrate depolymerized into monomers for the biosynthesis of ascomycin precursors. The gene manipulation associated with polyhydroxybutyrate metabolism and the addition optimization of carbon sources eventually increased FK520 yield by 2.11-fold (626.30 mg/L). This is the first report that polyhydroxybutyrate metabolism can promote antibiotic synthesis, providing a new strategy for the high yield of other secondary metabolites.

Materials And Methods

Strains, plasmids and growth conditions

S. hygrosopicus var. *ascomyceticus* FS35 selected from *S. hygrosopicus* var. *ascomyceticus* ATCC 14891 after femtosecond laser irradiation was used as the parent strain [2]. All the strains and plasmids used in this work are listed in Additional file 1: Table S1. Strain FS35 and its derivatives were cultured and passaged on the MS solid medium (a solid medium for the culture of *Streptomyces*, including 20 g/L soybean cake meal, 20 g/L mannitol and 20 g/L agar powder) at 28 °C. When there were black spores formed on the MS medium, then spores were inoculated into the liquid seed medium and shaken for 60 hours at 28 °C and 220 rpm. The composition of seed medium was same as described previously [45]. And then the seed cultures were transferred into fermentation medium with the inoculation of 10%. The fermentation broth was shaken for 192 hours at 28 °C and 220 rpm to detect various indexes. The fermentation medium used for the measurement of various fermentation parameters contained 20 g/L soluble starch, 40 g/L dextrin, 5 g/L yeast powder, 5 g/L peptone, 5 g/L corn steep liquor, 1 g/L K_2HPO_4 , 1.5 g/L $(NH_4)_2SO_4$, 0.5 g/L $MnSO_4$, 1 g/L $MgSO_4 \cdot 7H_2O$, 1 g/L $CaCO_3$, and 2.5 mL/L soybean oil. The optimum concentration of carbon sources suitable for mutant strain was presented in the results section. *Escherichia coli* DH5 α was used for plasmid construction. *Escherichia coli* ET12567 (pUZ8002) was used as the donor strain for intergeneric conjugation with *S. hygrosopicus* var. *ascomyceticus*. *E. coli* strains were cultured in Luria-Bertani (LB) medium at 37 °C. The plasmid pIB139, which contains the strong promoter *ermE***p*, was used to overexpress genes in strain FS35. The plasmid pBHR68, containing the PHB synthesis operon (*phaABC*) from *R. eutropha* H16, was kind gift from Professor Tao Chen (Tianjin University, China).

Construction of overexpression strain

To obtain the co-overexpression strain OphaCfkbU, the complete *phaC* gene was amplified from the plasmid pBHR68 using the primers OphaC-fkbU-F1/OphaC-fkbU-R1, and the complete *fbkU* gene was amplified from the genome of FS35 using the primers OphaC-fkbU-F2 /OphaC-fkbU-R2. All primers used for gene manipulations are listed in Additional file 1: Table S2. Then, the two fragments were fused by overlap-extension PCR using the primers OphaC-fkbU-F1/OphaC-fkbU-R2. The fusion fragment was inserted into the vector pIB139 between the NdeI and XbaI sites to construct the co-overexpression vector pIBOPF, in which the fusion fragment was under the strong constitutive promoter *ermE***p*. The recombinant plasmid pIBOPF was transformed into competent cell of *E. coli* ET12567 (pUZ8002) to obtain transformant. The transformant was cultured in liquid LB medium containing 50 µg/ml apramycin sulfate, 25 µg/ml kanamycin and 25 µg/ml chloramphenicol. When the OD value reached 0.4-0.6, the transformant was mixed with fresh spores of strain FS35 for conjugal transfer. Conjugal transfer was performed according to standard protocols [11]. Positive single colonies, which contained co-overexpression vector pIBOPF, were selected from MS solid medium containing 50 µg/ml nalidixic acid and 50 µg/ml apramycin sulfate. Finally, positive single colonies were relaxed cultured on MS solid medium without antibiotics to obtain co-overexpression strain OphaCfkbU. To obtain the overexpression strain OfkbU, the complete *fbkU* gene was amplified from the genome of FS35 using the primers OfkbU-F/OfkbU-R and inserted into the vector pIB139 in the way described above to obtain the overexpression plasmid pIBOF, which was introduced into FS35 by conjugal transfer to obtain the overexpression strain OfkbU.

Measurements of fermentation parameters

For the measurement of the yield of FK520 and FK523, 2 ml of fermentation broth was mixed with 3 ml of ethanol and was extracted by ultrasound for 30 minutes. After extraction, the mixture was centrifuged for 10 minutes at 8000 × g. The supernatant was filtered by a 0.22 µm of oily filter and quantified by liquid chromatography on a 1100 series instrument (Agilent, USA), equipped with a C-18 column (150 mm × 4.6 mm, 3.5 µm; Agilent). The column temperature was 60 °C and the detection wavelength was set to 205 nm. The mobile phase and the gradient elution method was same as described previously [6]. The flow rate was 2 mL/min, and the injection volume was 20 µL.

To obtain the biomass concentration, 5 ml of fermentation broth was washed once with 0.1 M-HCl solution and twice with Milli-Q water. After centrifugation for 10 minutes at 8000 g, the wet cell pellet was dried in oven at 80 °C until constant weight to measure the measurement of dry cell weight (DCW). The biomass concentration is the ratio of DCW to the volume of fermentation broth. The residual total sugars in fermentation broth were quantified in the same way as described previously [53].

To measure the intracellular content of PHB, the fermentation broth was centrifuged for 20 min at 8000 g and washed twice with deionized water. Wet hyphae was freeze-dried (Christ ALPHA 1-2 LD plus, Germany) and 60 mg of the lyophilized mycelium was put into an airtight tube with 2 ml of esterification reagent and 2 ml of chloroform. The esterification reagent was contained 0.1 g benzoic acid (internal standard), 3 ml concentrated sulfuric acid and 97 ml methyl alcohol. The esterification reaction was

carried out in a calorstat for 4 hours at 100 °C. The mixture were cooled to room temperature, and combined with 1 ml of deionized water. The lower chloroform phase was collected and quantified by gas chromatography (GC) on a 430-GC instrument (Bruker, Germany) equipped with a BR-5 capillary column (30 m, 0.32 mm, 0.25 µm; Bruker) according to a published method [54]. The detector for GC was flame ion detector (FID) and the temperature of detector was 250 °C. The heating program was 80 °C for 1.5 min, then heated up to 140 °C at a rate of 30 °C/ min, and then heated up to 250 °C at a rate of 40 °C/ min, finally held at 250 °C for 5min. Gradient poly-3-hydroxybutyric acid performed by above esterification reaction to build the standard curve.

Quantification of NADH, NAD⁺, NADPH and NADP⁺

For the measurement of the intracellular concentrations of NADH, NAD⁺, NADPH and NADP⁺, the samples was harvested at 96 h by centrifugation under 8000 g for 10 min. The wet hyphae were rapidly frozen in liquid nitrogen for later use. After washing twice with ice-cold PBS, the samples was mix with 100 µL extraction buffer and heat extracted at 60 °C for 5 min. The whole operation was carried out in strict accordance with the protocols of EnzyChrom™ NAD/NADH Assay Kit (Bioassay Systems, USA). The concentrations of NADH, NAD⁺, NADPH and NADP⁺ were measured at 565 nm.

RNA extraction and cDNA library construction

To record the changes in transcriptome data caused by the co-overexpression, the fermentation broth of FS35 and OphaCfkbU was collected at 50 h. After centrifugation at 8000 g for 10 min, the wet hyphae were rapidly frozen in liquid nitrogen. The total RNA was extracted from the frozen hyphae using the Trizol reagent (Invitrogen, USA). The rRNA was removed using the Ribo Zero rRNA Removal Kit (Epicentre, USA). RNA degradation and contamination was monitored on 1% agarose gels. RNA purity was checked using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). RNA concentration was measured using Qubit® RNA Assay Kit in Qubit® 2.0 Fluorometer (Life Technologies, CA, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). The cDNA libraries were generated using the NEBNext® Ultra™ Directional RNA Library Prep Kit for Illumina® (NEB, USA). The library fragments were purified using the AMPure XP system (Beckman Coulter, Beverly, USA). After terminal repair and PCR amplification, the quality of the cDNA libraries was assessed on the Agilent Bioanalyzer 2100 system.

Sequencing and data analysis

For the analysis of the transcriptome changes caused by genetic manipulation, the cDNA libraries were clustered on a cBot Cluster Generation System with TruSeq PE Cluster Kit v3-cBot-HS (Illumina) according to the manufacturer's instructions, and sequenced on a Illumina HiSeq platform to generate the paired-end reads. The clean reads were obtained by removing reads containing adapter, reads containing poly-N and low quality reads from raw reads of fastq format. Then they were aligned to the sequenced genome of *S. hygroscopicus* var. *ascomyceticus* FS35 using Bowtie 2-2.2.3 [55]. The numbers of reads mapped to

each gene were counted by HTSeq v0.6.1. In order to estimate gene expression level of each gene, Fragments Per Kilobase of transcript sequence per Millions base pairs sequenced (FPKM) were calculated based on the length of the gene and reads count of mapped genes. After reads counts adjusted using edgeR program package through one scaling normalized factor, differential expression analysis was performed using the DESeq R package (1.20.0) [56]. A corrected P-value of 0.005 and \log_2 (fold change) of 1 were set as the thresholds for significantly differential expression analysis. Gene ontology (GO) enrichment analysis of the differentially expressed genes was implemented using the GOseq R package [57]. The statistical enrichment of differentially expressed genes into Kyoto encyclopedia of genes and genomes (KEGG) pathways was tested on the KEGG Orthology Based Annotation System (KOBAS) [58]. The raw transcriptomic data and genome sequences had been uploaded to the Gene Expression Omnibus (GEO) database in National Center for Biotechnology Information (NCBI) (accession number: GSE 143832).

Quantitative real-time PCR (qRT-PCR) analysis

To verify the different pattern of PHB metabolism during the exponential phase and stationary phase, the transcriptional levels of gene *hcd* and *fkfE* at 50 h and 112 h were measured by qRT-PCR. And to explore the influence mechanism of PHB degradation on FK520 production, the transcriptional levels of the selected genes at 112 h were measured by qRT-PCR. All primers were listed in Additional file 1: Table S3 and S4. The sample collection was the same as described above. The total RNA was extracted from frozen hyphae using the RNeasy Pure Cell/Bacteria Kit (Qiagen, Beijing, China). The RNA sample was reversely transcribed into cDNA using PrimeScript™ RT reagent Kit (Takara, Japan). The integrity and concentration of RNA sample was detected by 1% agarose gel electrophoresis. Then the RNA sample was reversely transcribed into cDNA for qRT-PCR using PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Japan). With the cDNA as template, qRT-PCR was carried out on a LightCycler® 480 using SYBR Green Master Mix (Roche, Switzerland). To exclude DNA contamination, the RNA sample which treated by gDNA Eraser but not reverse transcription was used as a template for negative control. The 16S rRNA was used as the internal reference gene, and the change folds of the selected genes were quantified relatively using comparative C_T method [44].

To verify the accuracy of transcriptome data, several genes were selected for qRT-PCR analysis. All primers used for accuracy verification of transcriptome data were listed in Additional file 1: Table S5. The procedure was the same as description above. As shown in Additional file 1: Figure S1, the high correlation ($r^2=0.9637$) between the qRT-PCR validation data and transcriptome data proved the reliability of the transcriptomic results.

Statistical analysis

In this study, the samples for the measurement of fermentation parameters were all taken in five independent biological replicates. The samples for quantitative real-time PCR were all taken in three

independent biological replicates. All data were presented as the mean values of respective independent biological replicates and the error bars indicate the standard deviations (SD).

Abbreviations

NADH: Nicotinamide adenine dinucleotide (Reduction State)

NAD⁺: Nicotinamide adenine dinucleotide (Oxidation State)

NADPH: Nicotinamide adenine dinucleotide phosphate (Reduction State)

NADP⁺: Nicotinamide adenine dinucleotide phosphate (Oxidation State)

FK520: Ascomycin

FK523: A structural analogue of ascomycin

PHB: Polyhydroxybutyrate

EMCP: Ethylmalonyl-CoA pathway

EMP: Glycolysis pathway

PPP: Pentose phosphate pathway

TCA: Citrate cycle

DCW: Dry cell weight

LB: Luria-Bertani

GC: gas chromatography

GO: Gene ontology

KEGG: Kyoto encyclopedia of genes and genomes

KOBAS: KEGG Orthology Based Annotation System

NCBI: National Center for Biotechnology Information

GEO: Gene Expression Omnibus

qRT-PCR: Quantitative real-time PCR

ACP: Acyl-carrier protein

PAPS: 3'-phosphoadenosine 5'-phosphosulfate

DHCHC: 4,5-dihydroxycyclohex-1-enecarboxylic acid

SD: standard deviations

FPKM: Fragments Per Kilobase of transcript sequence per Millions base pairs sequenced

FID: Flame ion detector

Lys: lysine

Val: valine

MS: a solid medium for the culture of *Streptomyces*

Declarations

Authors' contributions

PW carried out the experimental work, analyzed the data and wrote the manuscript. YY performed the experimental analysis and helped in editing and revising the manuscript. XW Helped to analyze the data and write the manuscript. JPW designed the experiments and supervised the work. All authors read and approved the final manuscript.

Consent for publication

All authors consent to publish this manuscript.

Competing interests

The authors declare that there are no conflict of interests.

Availability of data and supporting materials

The dataset related to transcriptomic and genome sequences is available in the GEO database, [accession number: GSE 143832, <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE143832>]. And the data generated and analyzed during this study are included in the article and its additional file 1.

Ethics approval and consent to participate

This manuscript does not involve the data collected from animals or humans.

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Tables

Table 1 Main enriched metabolic pathways of up-regulated genes

Gene no.	KEGG ID	Enzyme name	Log ₂ (change fold)	P value
Starch and sucrose metabolism				
1_2422	SHJG_3718	alpha-glucosidase	3.0752	6.06×10 ⁻¹⁴⁸
1_2417	SHJG_3717	alpha-amylase	2.5774	1.81×10 ⁻¹⁶
1_3621	SHJG_4827	trehalose-6-phosphate synthase	1.266	2.10×10 ⁻⁵⁹
1_6067	SHJG_7159	alpha-amylase	1.2268	4.07×10 ⁻⁰⁷
1_7923	SHJG_1896	beta-glucosidase	1.1619	6.11×10 ⁻⁰⁶
ABC transporters				
1_1441	SHJG_3980	zinc transport system substrate-binding protein	4.3311	7.75×10 ⁻⁵⁸
1_2420	SHJG_3720	maltooligosaccharide transport system permease protein	4.2287	8.35×10 ⁻⁴¹
1_1444	SHJG_3235	iron complex transport system ATP-binding protein	4.0095	1.55×10 ⁻²⁹
1_2421	SHJG_3721	maltooligosaccharide transport system substrate-binding protein	3.9837	0
1_2419	SHJG_3719	maltooligosaccharide transport system permease protein	3.6265	5.76×10 ⁻³²
1_2743	SHJG_3235	iron complex transport system ATP-binding protein	2.9746	4.61×10 ⁻⁵⁷
1_2741	SHJG_3982	zinc transport system permease protein	2.9356	2.58×10 ⁻²⁵⁷
1_2742	SHJG_8417	iron complex transport system ATP-binding protein	2.9089	1.10×10 ⁻⁸³
1_1279	SHJG_8416	iron complex transport system permease protein	1.8523	5.37×10 ⁻⁰⁸
1_1280	SHJG_8416	iron complex transport system permease protein	1.7734	8.39×10 ⁻⁰⁷
1_3705	SHJG_4851	multiple sugar transport system ATP-binding protein	1.518	6.19×10 ⁻²⁹
1_1281	SHJG_8418	iron complex transport system substrate-binding protein	1.4464	1.39×10 ⁻²³
1_4985	SHJG_3981	zinc transport system ATP-binding protein	1.0094	8.97×10 ⁻¹¹
Ribosome				

1_1485	SHJG_6688	50S ribosomal protein L32	5.1594	7.62×10 ⁻¹⁸¹
1_7134	SHJG_5164	30S ribosomal protein S18	3.8835	3.44×10 ⁻⁵²
1_7129	SHJG_7368	30S ribosomal protein S14	3.8384	1.82×10 ⁻¹⁴
1_7130	SHJG_7369	50S ribosomal protein L28	3.7143	1.33×10 ⁻⁰⁵
1_2812	SHJG_4055	30S ribosomal protein S20	1.1893	9.08×10 ⁻¹²
1_5742	SHJG_6711	30S ribosomal protein S16	1.0786	4.86×10 ⁻²⁵
1_6570	SHJG_3036	large subunit ribosomal protein L20	1.0423	1.61×10 ⁻¹⁰
1_4696	SHJG_5834	30S ribosomal protein S9	1.0196	8.92×10 ⁻²⁵
Pantothenate and CoA biosynthesis				
1_2437	SHJG_3745	3-methyl-2-oxobutanoate hydroxymethyltransferase	1.9693	5.22×10 ⁻⁵⁶
1_8015	SHJG_7832	L-aspartate-alpha-decarboxylase	1.7699	4.69×10 ⁻⁰⁷
1_3771	SHJG_5555	pantoate-beta-alanine ligase	1.6344	1.24×10 ⁻⁰⁵
1_3768	SHJG_5558	type III pantothenate kinase	1.4657	5.69×10 ⁻⁰⁷
1_5666	SHJG_6609	acetolactate synthase	1.1284	1.07×10 ⁻¹⁵
nicotinate and nicotinamide metabolism				
1_7327	SHJG_8783	nicotinate phosphoribosyltransferase	2.8566	3.75×10 ⁻⁰⁹
1_3770	SHJG_5556	L-aspartate oxidase	1.7152	6.70×10 ⁻¹³
1_7638	SHJG_2643	succinate-semialdehyde dehydrogenase	1.7112	3.27×10 ⁻²³³
1_3769	SHJG_5557	nicotinate-nucleotide pyrophosphorylase	1.5887	9.02×10 ⁻⁰⁶
1_3164	SHJG_4395	nicotinamidase	1.1981	5.28×10 ⁻¹⁸
Sulfur metabolism				
1_1485	SHJG_6688	50S ribosomal protein L32	5.1594	7.62×10 ⁻¹⁸¹
1_7134	SHJG_5164	30S ribosomal protein S18	3.8835	3.44×10 ⁻⁵²
1_7129	SHJG_7368	30S ribosomal protein S14	3.8384	1.82×10 ⁻¹⁴
1_7130	SHJG_7369	50S ribosomal protein L28	3.7143	1.33×10 ⁻⁰⁵

1_2812	SHJG_4055	30S ribosomal protein S20	1.1893	9.08×10^{-12}
1_5742	SHJG_6711	30S ribosomal protein S16	1.0786	4.86×10^{-25}
1_6570	SHJG_3036	large subunit ribosomal protein L20	1.0423	1.61×10^{-10}
1_4696	SHJG_5834	30S ribosomal protein S9	1.0196	8.92×10^{-25}

The data was the comparative values between the strain FS35 and strain OphaCfkbU, with the strain FS35 as the control.

Figures

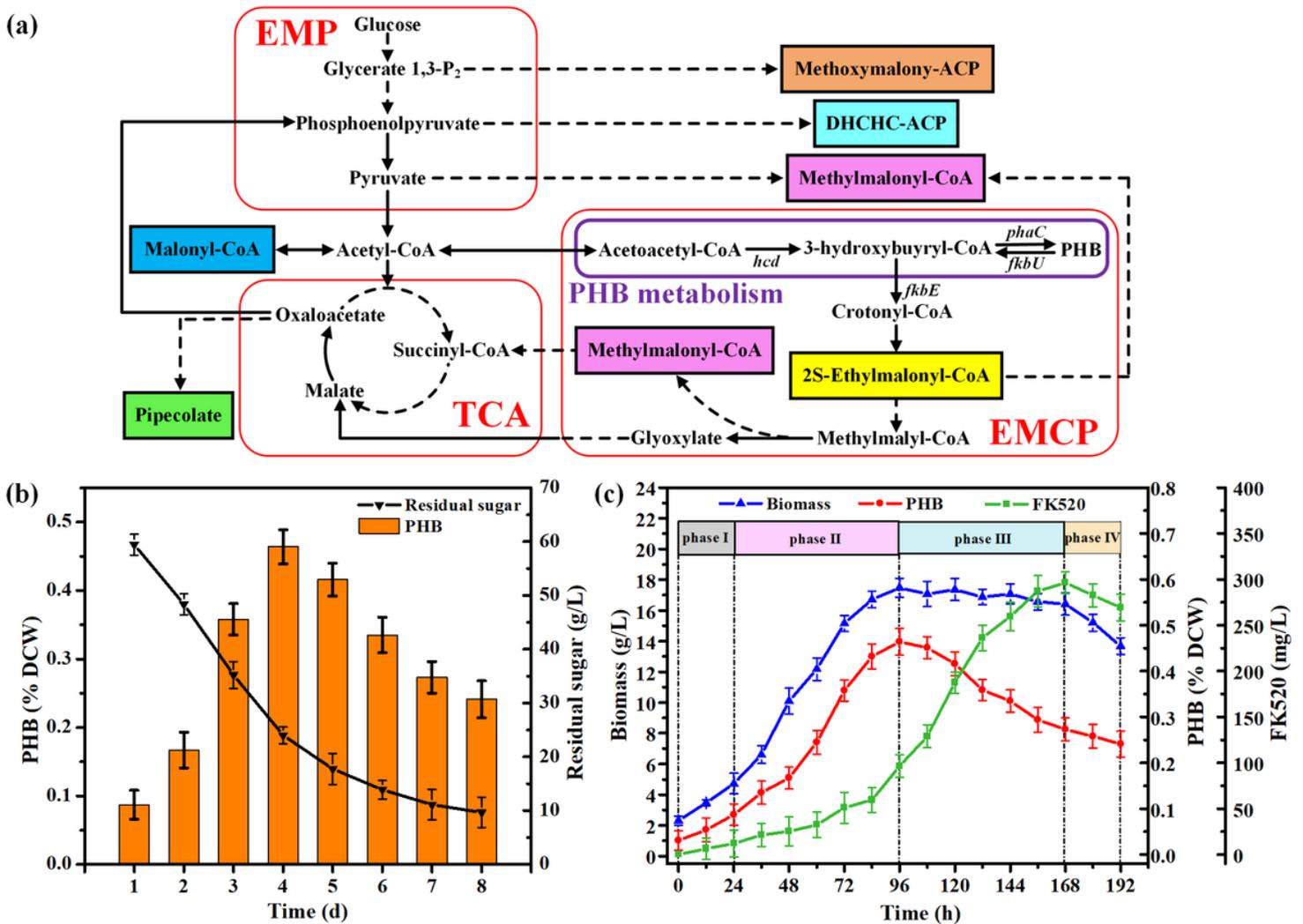


Figure 1

Correlation between polyhydroxybutyrate (PHB) metabolism and ascomycin (FK520) biosynthesis. (a) An general view of polyhydroxybutyrate metabolism and ascomycin biosynthesis pathway. The solid arrows represent one-step reactions, and the dotted arrows represent multi-step reactions. The words in the

colored shaded boxes represent the precursors of ascomycin. The italics represent genes associated with polyhydroxybutyrate metabolism. (b) The change trends of polyhydroxybutyrate content and residual sugar in the parent strain FS35. (c) The correlation between the polyhydroxybutyrate content, biomass and ascomycin production in the parent strain FS35. Phase I, lag phase; Phase II, exponential phase; Phase III, stationary phase; Phase IV, decline phase. The data represent the mean values of five independent biological replicates, and the error bars represent the standard deviations.

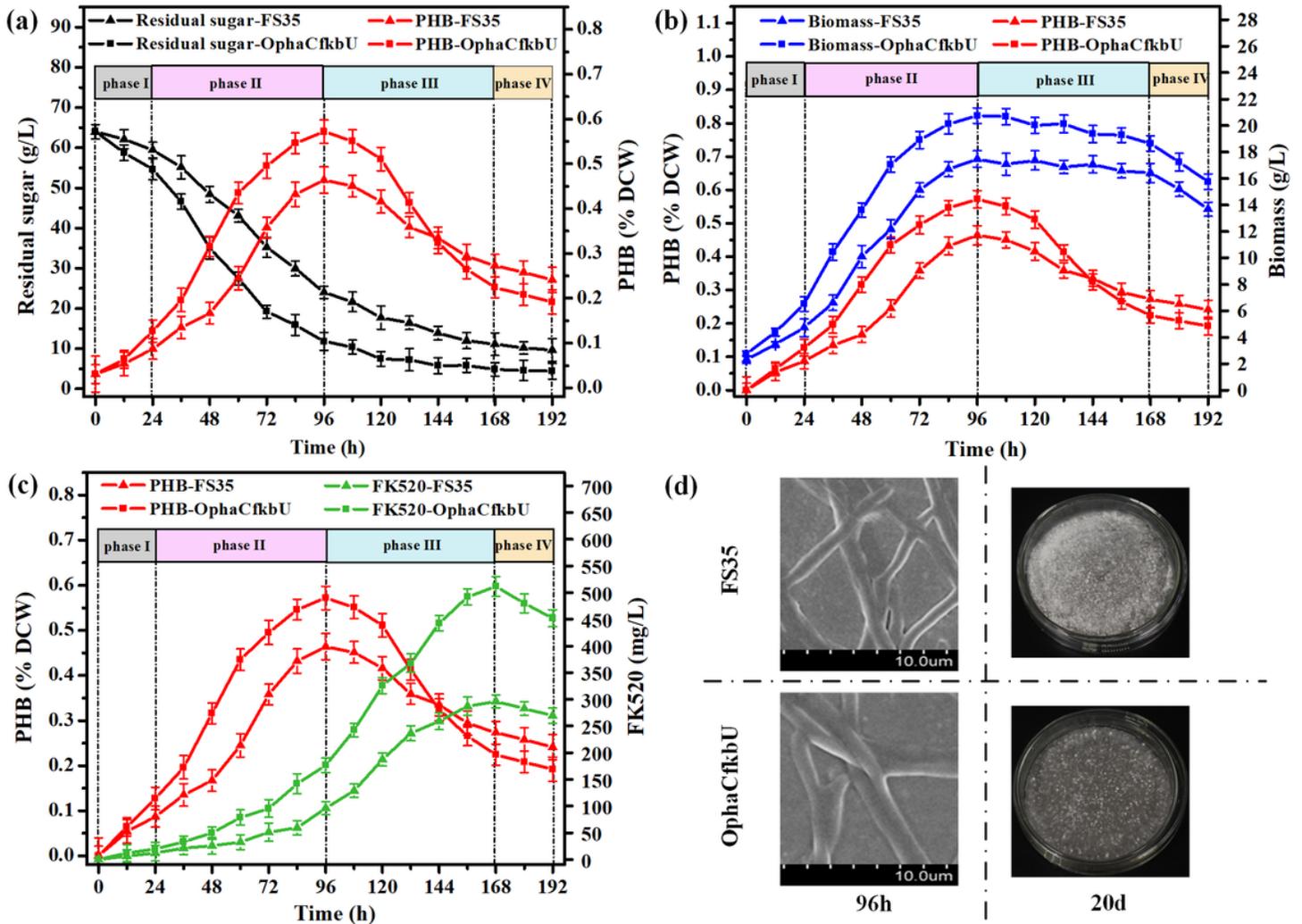


Figure 2

Comparison of fermentation characteristics and growth states between parent strain FS35 and co-overexpression strain OphaCfkbU. (a) The comparison between the strain FS35 and OphaCfkbU in the changes and correlation of polyhydroxybutyrate (PHB) content and residual sugar during the different fermentation phases. (b) The comparison between the strain FS35 and OphaCfkbU in the changes and correlation of polyhydroxybutyrate content and biomass during the different fermentation phases. (c) The comparison between the strain FS35 and OphaCfkbU in the changes and correlation of polyhydroxybutyrate content and ascomycin (FK520) production during the different fermentation phases. Phase I, lag phase; Phase II, exponential phase; Phase III, stationary phase; Phase IV, decline phase. The data represent the mean values of five independent biological replicates, and the error bars

represent the standard deviations. (d) The different growth states of the strain FS35 and OphaCfkbU in the fermentation broth at 96h and in the flat plate at 20d. The hyphae in the fermentation broth was photographed under the scanning electron microscopy, and the scale shows the magnification.

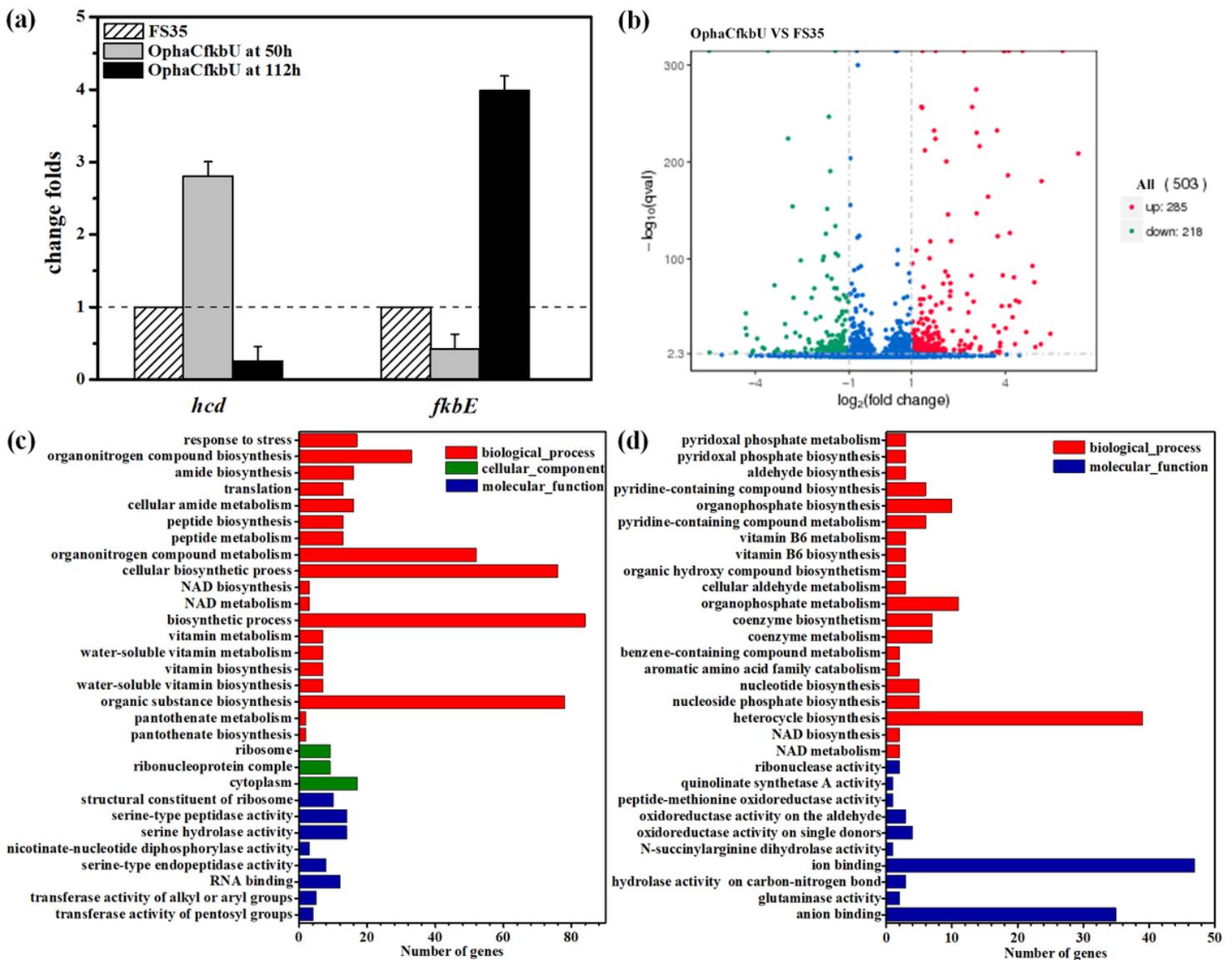


Figure 3

Patterns of polyhydroxybutyrate (PHB) metabolism at different phases and its effect on strain growth. (a) The transcriptional change folds of genes *hcd* and *fkbE* in the strain FS35 and OphaCfkbU at different fermentation phases. The transcriptional levels of the genes in strain FS35 were defined as 1 and used as the control. The data represent the mean values of three independent biological replicates, and the error bars represent the standard deviations. (b) The quantity of differential genes and degree of differential expression analyzed by the comparative transcriptomic between strain FS35 and OphaCfkbU at the exponential phase. The X-axis represents the logarithmic change folds of differential expression. The Y-axis represents the statistical significance of differential expression. Red dots represent the genes whose expression is significantly up-regulated. Green dots represent the genes whose expression is significantly down-regulated. Blue dots represent the genes with no significant difference in expression.

(c) The enrichment categories and degrees of the up-regulated genes in strain OphaCfkbU reflected by the comparative transcriptomic analysis. (d) The enrichment categories and degrees of the down-regulated genes in strain OphaCfkbU reflected by the comparative transcriptomic analysis. The Y-axes represent different enrichment categories. The X-axes represent the quantity of genes enriched in the corresponding categories, which reflect the enrichment degrees.

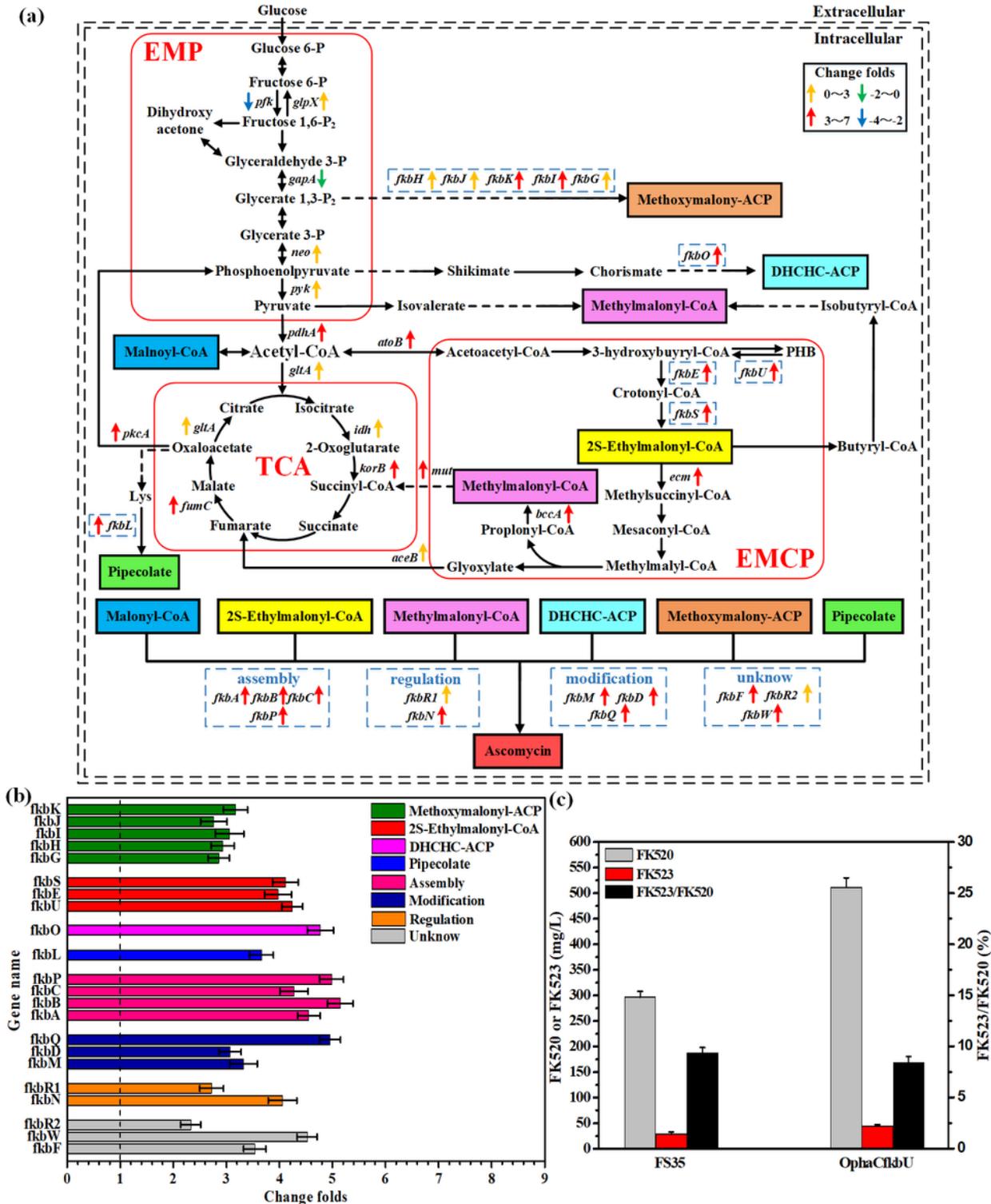


Figure 4

Influence of polyhydroxybutyrate (PHB) on ascomycin (FK520) synthesis and by-product (FK523) yield.

(a) An schematic view of the major primary metabolic pathways for the synthesis of ascomycin precursor. And the transcriptional changes of key genes responsible for precursor synthesis, assembly and modification at the stationary phase. The solid lines represent one-step reactions. And the dotted lines represent multi-step reactions. Different colored shaded boxes represent different precursors of ascomycin. The italics represent genes. Blue dotted boxes represent the genes in the gene cluster of ascomycin. The up- and downward facing arrows in different colors represent the different change folds of transcriptional levels as shown in the legend.

(b) The transcriptional change folds of the genes with different functions in the gene cluster of ascomycin at the stationary phase. The transcriptional levels of the genes in the parent strain FS35 were defined as 1 and used as the control. Different color bars represent different groups of genes with specific functions as shown in the legend. The data represent the mean values of three independent biological replicates, and the error bars represent the standard deviations.

(c) The yield and ratio of ascomycin and by-product in the strain FS35 and OphaCfkbU. The data represent the mean values of five independent biological replicates, and the error bars represent the standard deviations.

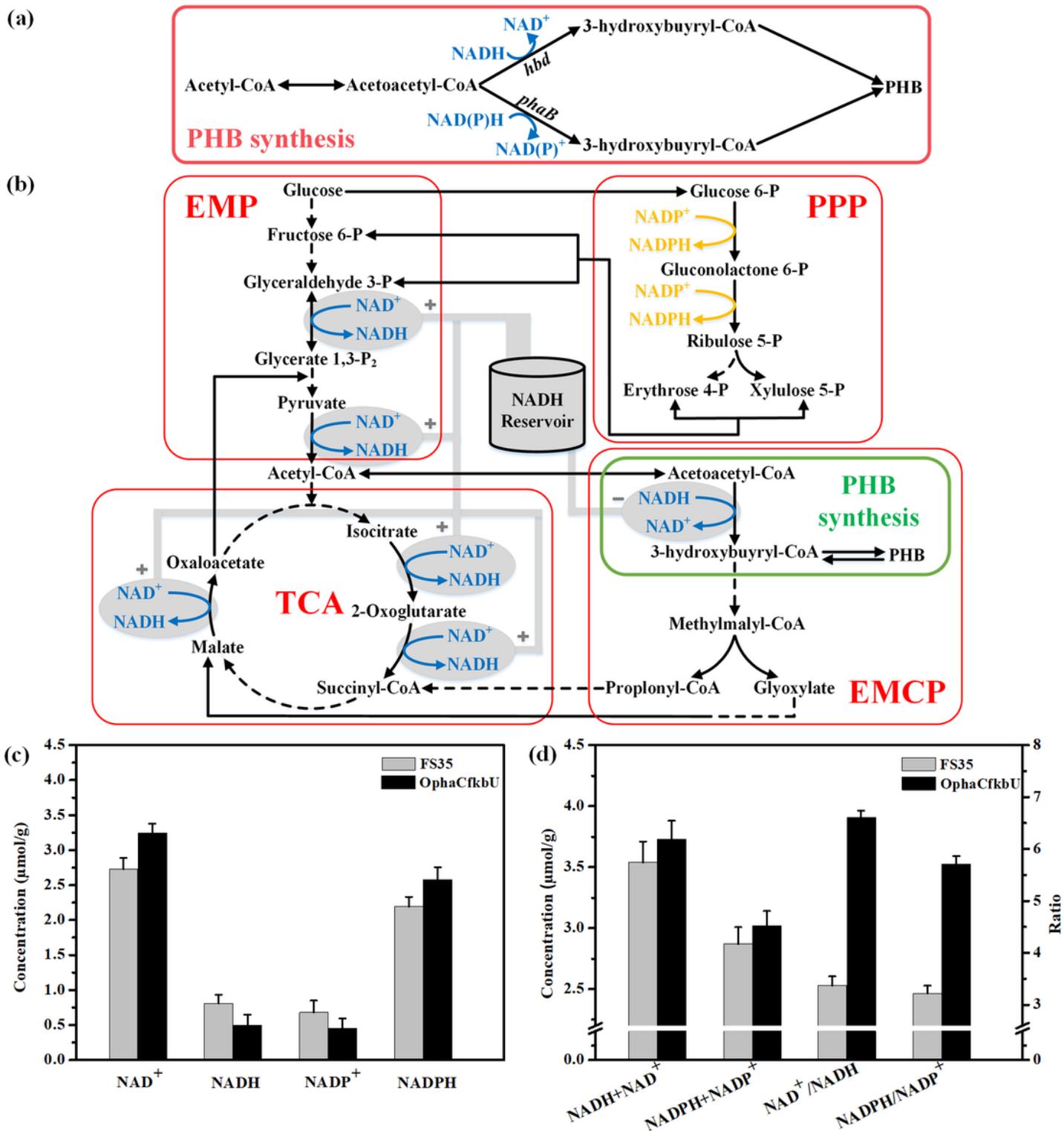


Figure 5

Influence of cofactors (NADH , NAD^+ , NADPH , NADP^+) on the biosynthesis of polyhydroxybutyrate (PHB). (a) The general view of polyhydroxybutyrate biosynthesis pathway and the role of these cofactor in this pathway. The italics represent the genes encoding the corresponding enzymes. (b) The main generation pathways of NADH and NADPH , and the supply of NADH pool to the cofactor used in polyhydroxybutyrate biosynthesis. (c) The comparison of intracellular concentration of NADH , NAD^+ ,

NADPH, NADP⁺ between the strain FS35 and OphaCfkbU at the end of exponential phase. (d) The comparison of intracellular concentration of NADH+NAD⁺ and NADPH+NAD⁺, and the ratio of NAD⁺/NADH and NADPH/NADP⁺ between the strain FS35 and OphaCfkbU at the end of exponential phase. The data represent the mean values of five independent biological replicates, and the error bars represent the standard deviations.

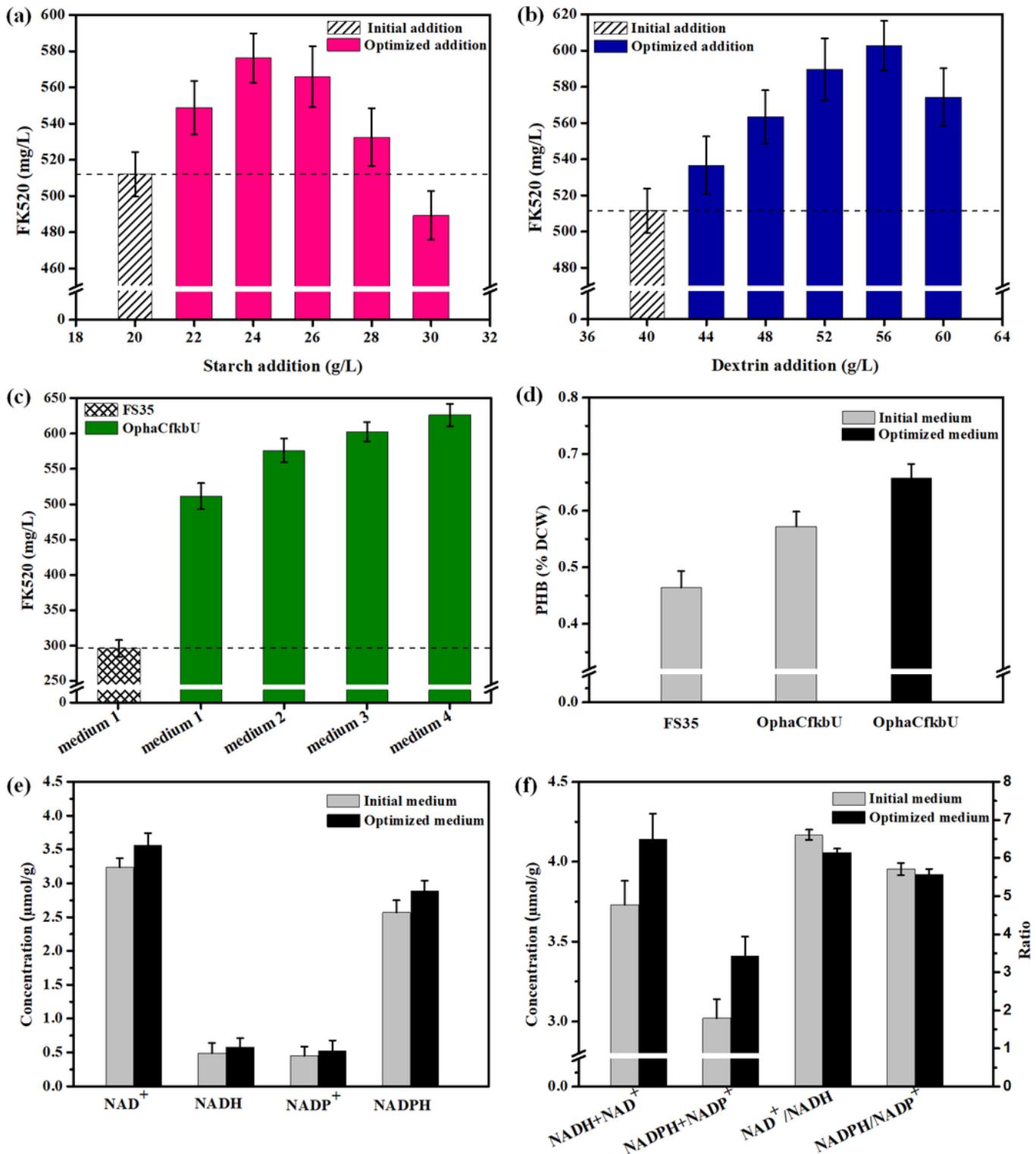


Figure 6

Effect of carbon source addition on cofactor concentration, polyhydroxybutyrate (PHB) content and ascomycin (FK520) yield. (a) The effect of starch addition on the ascomycin yield in the strain OphaCfkbU. (b) The effect of dextrin addition on the ascomycin yield in the strain OphaCfkbU. (c) The comparison of ascomycin yield in different strains or different fermentation mediums. Medium 1 represents the initial fermentation medium. Medium 2 represents the optimized fermentation medium of starch addition. Medium 3 represents the optimized fermentation medium of dextrin addition. Medium 4 represents the final optimized fermentation medium. (d) The comparison of polyhydroxybutyrate content in different strains or different fermentation mediums. (e) The comparison of intracellular concentration of NADH, NAD⁺, NADPH, NADP⁺ in the strain OphaCfkbU between the initial medium and the final optimized fermentation medium at the end of exponential phase. (f) The comparison of intracellular concentration of NADH+NAD⁺ and NADPH+NAD⁺, and the ratio of NAD⁺/NADH and NADPH/NADP⁺ in the strain OphaCfkbU between the initial medium and the final optimized fermentation medium at the end of exponential phase. The data represent the mean values of five independent biological replicates, and the error bars represent the standard deviations.

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