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Improved FK506 production by the precursors and product-tolerant mutant of *Streptomyces tsukubaensis* based on genome shuffling and dynamic fed-batch strategies

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Abstract FK506, a secondary metabolite produced by *Streptomyces tsukubaensis*, is well known for its immunosuppressant properties to prevent rejection of transplanted organs and treat autoimmune diseases. However, the low titer of FK506 in the original producer strain limits the further industrialization efforts and restricts its clinical applications. To address this issue, a highly efficient method combined genome shuffling and dynamic fed-batch strategies was systematically performed in this work. Firstly, after five rounds of genome shuffling based on precursors and product resistances, a higher yielding strain TJ-P325 was successfully acquired, whose production reached 365.6 mg/L, 11-fold increase compared with the original strain. Then, the possible mechanism of different production capabilities between TJ-P325 and the wild type was explored through comparative gene expression analysis of key genes. Results showed that the transcription level of key genes was altered significantly in the mutant. Moreover, precursors addition enhanced the FK506 production by 28 %, as well as reduced the by-products biosynthesis. Finally, the disodium malonate and disodium methylmalonate dynamic fed-batch strategies dramatically led to the production of 514.5 mg/L in a 7.5-L bioreactor. These results demonstrated that genome shuffling and dynamic fed-batch strategies could be successfully applied to generate high-yield strains with value-added natural products during industrial microbial fermentation.

Keywords FK506 (tacrolimus) · Genome shuffling · Precursors resistances · Gene expression · Dynamic fed-batch strategies · *Streptomyces tsukubaensis* · Polyketide macrolide

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Introduction

FK506 (also named tacrolimus) is a 23-membered polyketide macrolide with immunosuppressant activity, which was originally isolated from the soil bacterium *Streptomyces tsukubaensis* in 1984 [16]. Since the Food and Drug Administration (FDA) approved the usage of FK506 for liver transplantation in 1994, it has become a clinically important drug used as an immunosuppressant after the transplantation of allogeneic kidney, liver and heart, as well as for the treatment of inflammatory skin diseases [27]. In addition, FK506 shows numerous promising therapeutic potentials, including neuroprotective and neuroregenerative activities [30].

Due to the pharmaceutical importance and broad applicability, FK506 and analogues (rapamycin, FK520) have raised researchers' great interest to improve the productivity during the fermentation process. Considerable efforts have been made to optimize fermentation conditions [35]. On one hand, the effects of cultivation conditions on FK506 biosynthesis were evaluated and the optimized medium was formulated to support the high production [21, 32]. On the other hand, the traditional breeding methods were undertaken for FK506 enhancement, such as mutagenesis [15]; however, the method is time-consuming and expensive. In addition, many attempts have been made to engineer strains with increased levels of FK506 [1, 11, 12, 22]. These rational strain improvement approaches and global techniques, nevertheless, are still limited and inefficient to gain the evolution of FK506-producing strains towards desirable phenotype. More importantly, the demand to engineer more complex phenotypes requires a more combinatorial approach. Genome shuffling has been demonstrated as an accelerated evolutionary tool for the rapid improvement of both antibiotic production and complex cellular phenotypes. As an efficient technology, it offers the advantage of simultaneous changes at different sites throughout the whole genome even without necessity of detailed genetic and network information. Particularly, two rounds of genome shuffling can enable the strain to achieve the results that had previously required 20 rounds of mutagenesis and screening [39]. Thus, genome shuffling represents a practical method to rapidly manipulate complex phenotypes from the whole cells and organisms. Currently, the method has been successfully used in some organisms to increase the metabolite productivities, such as tylosin [39], pristinamycin [34], rapamycin [2], spinosad [14], L-lactic acid [36], ε-poly-L-lysine [18], ethanol [33] and 1,3-propanediol [24]. In principle, this method can also be expected to increase the FK506 productivity in *S. tsukubaensis*. However, so far, there has never been a research to apply this method to improve FK506 biosynthesis, nor a possible mechanism for high yield. In addition, the key precursors can be successfully enhanced to improve the production via dynamic fed-batch strategies [12].

In this study, several strains with different productivities were first isolated by disodium malonate and disodium methylmalonate resistances screening. Then, the genome shuffling approach was performed to improve FK506 production based on enhanced FK506 resistance. Subsequently, the transcriptional level of key genes in metabolic pathways was determined to further understand the differential gene expression caused by precursors and product resistances. It might provide novel information on the metabolic mechanism of FK506 biosynthesis and further guide the strain development, fermentation technology improvement. Moreover, precursors addition and dynamic fed-batch strategies were also proposed towards the improvement of FK506 production. The results indicated that genome shuffling together with dynamic fed-batch method are efficient and powerful strategies for improving the important antibiotic biosynthesis.

Materials and methods

Microorganisms

S. tsukubaensis TJ-01 was used as the initial strain and was cryopreserved in 20 % (v/v) glycerol at −80 °C. *Candida albicans* ATCC 11651 was used as an indicator strain for the bioassay of FK506 and preserved in our laboratory.

Medium and culture conditions

Test medium for *C. albicans* ATCC 11651 growth contained 20.0 g/L glucose, 20.0 g/L peptone, 10.0 g/L yeast extract, and 8.0 g/L agar.

All the strains derived from *S. tsukubaensis* TJ-01 were maintained and subcultured on slant and plate media as described by Huang et al. [12]. Seed, fermentation medium and nutrient feeding solution were also prepared as reported by Huang et al. [12].

Liquid regeneration medium (LRM) was prepared as described by Hopwood et al. [10].

For FK506 fed-batch cultivation in a 7.5-L BIOFLO 110 bioreactor (New Brunswick Scientific Company, USA), a loop full of slant culture was inoculated into 100 mL of seed medium in a 500-mL Erlenmeyer flask. After 48 h, 100 mL of seed broth was transferred into fermentor with 3 L working volume and incubated at 28 °C, 200–1,000 rpm stirrer speed, and 1.0 vvm aeration rate. pH was controlled at 6.8 by 2 M HCl or 2 M NaOH. Foam was prevented by the automatic addition of 10 % (v/v) antifoam agent (Sigma 204). The nutrient feeding solution used for the fed-batch culture contained disodium malonate and disodium methylmalonate, which were supplemented into the bioreactor beginning at different time points (48, 60, 72, 84, 96 h) with the feeding rate of 0.05 and 0.03 g/L/h, respectively.

All the fermentative experiments were performed in triplicate.

FK506 overproducing mutant screening using the bioassay method

Each well in aseptic 96-well microtiter plates was filled with 0.2 mL sterile production medium. During the inoculation, the 96-well plates were put into a 180 × 180 mm Petri dish with 100 mL sterile water added to keep humidity. The

dishes were incubated at 28 °C for 10 days in a biochemical oxygen demand incubator. Then, each medium column in one 96-well plate was picked up and transferred into a 1.5-mL Eppendorf tube containing 0.5 mL methanol. After 6 h shaking at 30 °C, methanol extracts were centrifuged for 15 min at 5,000×*g*, and the supernatants were then used for bioassay. If the strain in one well of plate showed high FK506 productivity by bioassay, the strain in the corresponding well of another plate will be further validated by liquid fermentation. Control plugs using *S. tsukubaensis* TJ-01 were also prepared with the same procedure.

FK506 was assayed by a paper disc-agar diffusion method using *C. albicans* ATCC 11651 as the indicator, which was activated at 28 °C for 24 h with YEPD medium. The cells were washed and suspended in sterile water, with a final concentration of 10^8 colony-forming units/mL. The agar medium was dispensed in a Petri dish composed of two separate layers. First, 15 mL of test medium formed the base layer in the Petri dish; then, after solidification, a 0.5 mL suspension of *C. albicans* ATCC 11651 mixed with 5 mL of sterile water containing 15.0 g/L agar at 40–50 °C was immediately poured onto the base layer to form the upper one. Subsequently, 6-mm-diameter paper disk was placed onto each dish, and 6 μL methanol extract from the agar plug of plate was transferred onto paper disks, respectively, with 6 μL pure methanol and 6 μL methanol extract from the parent strain used as negative and positive standard, respectively. In order to facilitate FK506 in the methanol extract diffusing into the medium of Petri dish, the plates were stored at 4 °C for 12 h, then incubated for 2 days at 30 °C. The diameter of the inhibitory zone was measured using a caliper to the nearest 0.1 mm. The strain with the largest inhibitory zone could be picked out and the corresponding strain in another plate would be further assayed by shaken flask submerged fermentation. The detailed procedure of this high-throughput screening technique is illustrated in Fig. 1a.

Preparation of starting strains for genome shuffling

A resistance two-layer plate was employed for screening mutants. The components of the lower layer were the same as the plate medium. The upper layer consisted of different concentrations of disodium methylmalonate or disodium malonate. Mutants screening was performed after ultraviolet (UV) and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) mutagenesis treatment. TJ-01 was transferred to an aseptic plate with cover removed and exposed to UV irradiation for 40 s at a distance of 30 cm from a UV lamp with the wavelength of 254 nm and the power of 15 W. After appropriate dilution, the suspension of survived spores was spread on the surface of a resistance two-layer plate containing 4 g/L disodium malonate, the concentration that inhibited growth of *S. tsukubaensis* TJ-01. Subsequently, the disodium malonate resistance strain with the highest FK506 yield was screened by the combination of agar block method and fermentation test. Using the same method, the selected strain was mutated by UV and incubated on the resistance plate containing higher disodium malonate concentration (8 and 12 g/L). After UV mutation, three populations showing different levels of resistance to disodium malonate were achieved and further treated by NTG at the concentration of 1 mg/L for 60 min. Similarly, the breeding of disodium methylmalonate-resistant population was performed and the unique difference was that 4, 8, and 12 g/L disodium methylmalonate were added to the resistance two-layer plates, respectively. Four mutants with higher FK506 production were selected from the above disodium malonate-resistant populations and disodium methylmalonate-resistant populations and then used as the starting strains for genome shuffling in the subsequent experiment.

Genome shuffling

Genome shuffling was performed through enhancing the product tolerance of strain by gradually increasing FK506 concentration in the medium. Specifically, spore suspensions of the starting strains were subsequently inoculated into 30 mL of seed medium in a 250-mL Erlenmeyer flask. After incubation for 48 h, mycelia were centrifuged at 4,000×*g* for 10 min at 4 °C, washed with 10 mL of P buffer twice [10] and incubated with 2 mg/mL lysozyme for 1 h at 35 °C. The protoplast formation was judged through observing the spherical cells by the phase-contrast microscopy. Subsequently, equal numbers of protoplasts from each parent strain were mixed, divided equally into two portions, and inactivated as follows: one portion was irradiated by UV for 120 s under a 15 W UV lamp with a distance of 30 cm, and another portion was incubated at 70 °C for 60 min. Then, the killed protoplasts were merged together and centrifuged for 10 min at 2,000×*g*. The collected pellets were resuspended gently in 5 mL of buffer P and mixed with the solution containing 50 % (w/v) polyethylene glycol (PEG) 4000, 0.05 mol/L $CaCl_2$, and 0.02 mol/L $MgCl_2$. After 10 min incubation at 25 °C, the fused protoplasts were collected, washed twice, resuspended in 5 mL of buffer P, serially diluted on LRM plate containing 150 mg/L FK506 for 7–10 days at 28 °C. Colonies were primarily screened by high-throughput cultivation and determination in a 96-well microplate, as illustrated above. Those strains with better FK506 yields were further validated by fermentation test in shaking flasks. The top four strains were used as the starting strains for the next rounds of genome shuffling. Five successive rounds of genome shuffling were performed by repeating the protoplast fusion by stepwise increase of the concentration

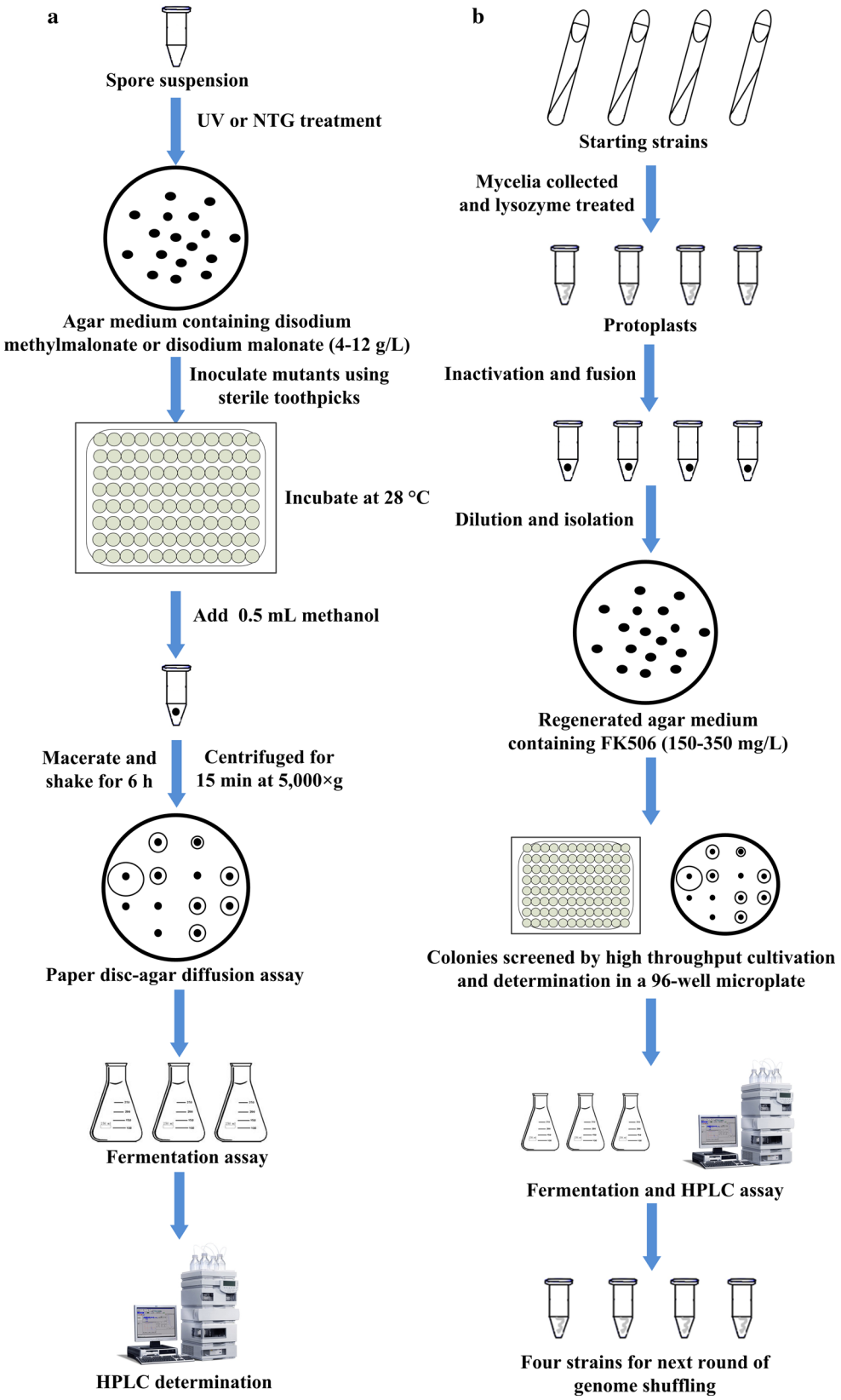


Fig. 1 The procedure of strain screening for enhancing FK506 production. **a** A procedure of the high-throughput screening technique; **b** a procedure of genome shuffling

of FK506 (200, 250, 300, and 350 mg/L in rounds 2–5, respectively) in LRM and agar media. The detailed procedure of genome shuffling is shown in Fig. 1b.

Analytical methods

The biomass, residual total sugar, FK506 as well as the by-products FK520 and 37,38-dihydro-FK506 (FK506D) were determined as described by Huang et al. [12].

Assay of gene expression by quantitative real-time RT-PCR (qRT-PCR)

Gene transcription profile was determined by qRT-PCR (BioRad) using the TransScriptTM II Green Two-Step qRT-PCR SuperMix (Trans). The samples were isolated from the wild-type and mutant strains of *S. tsukubaensis* at 24, 48, 72, 96 and 120 h, as described previously [12]. Total RNA was extracted by Trizol reagent (Qiagen) according to the manufacturer's instructions and then treated with DNase I (Fermentas). Primers were designed (Supplementary Table S1) based on published genome sequence. The RNA polymerase sigma factor *hrdB* was used as an internal control. Data analysis was performed according to the comparative C_T method. Fold change of the gene expression of the recombinant strain was calculated by normalizing the value of the control TJ-01. RT-PCR experiments were done in triplicate, using RNA samples from three independent cultures.

Extraction and analysis of CoA-esters

The preparation of the cell extracts and analysis of the CoA-esters were carried out as described by Park et al. [25]. The experiments were performed in three independent replicates. The concentrations of the intracellular CoA-esters of *S. tsukubaensis* were evaluated using a one-way analysis of variance. Differences among the sample groups were considered significant at $P < 0.05$.

Results

High-throughput screening of starting strains for genome shuffling

The FK506 production of initial strain *S. tsukubaensis* TJ-01 was only 30.5 ± 3.8 mg/L in shaken flask and still low efficient for using it in genome shuffling. Thus, an initial library of mutants with improved productivity was generated by the traditional mutation and precursors resistance screening. Moreover, in the fermentation process, without regard to the strain background, industrial processes for production and isolation of FK506 have been hampered by at least two FK506-related analogues: FK506D and FK520 [17]. These by-products compete with the target product FK506 for the carbon source and reducing power, thus decreasing the conversion of CoA-esters to FK506 and reducing FK506 fermentation titer. Therefore, it was necessary to comparatively analyze these by-products as evaluated factors. The wild strain was first treated by UV irradiation, and 1,500 colonies were examined by agar block method and fermentation test. As shown in Fig. 2a, the mutants exhibited a slight increase in FK506 production compared with *S. tsukubaensis* TJ-01. Specially, the mutant TJ-S316 with the higher FK506 yield and less by-products was selected and further treated with NTG. The precursors-resistant strains were generated by spreading the cell suspension onto the resistance two-layer plates containing disodium malonate and disodium methylmalonate, respectively. Four mutants (TJ-K168, TJ-K554, TJ-K627, and TJ-K945) with the best performance were selected as the starting strains for genome shuffling. These mutant strains

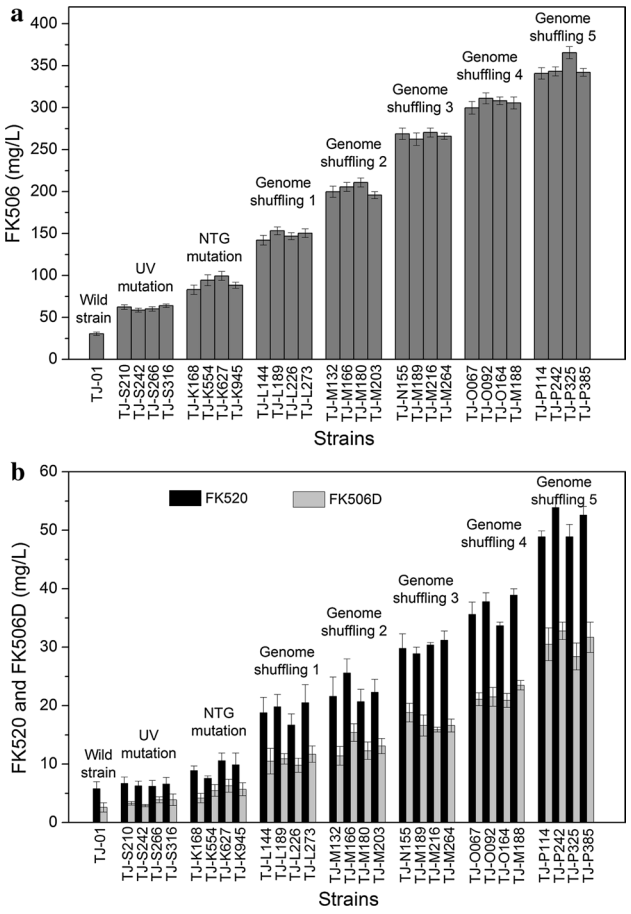


Fig. 2 The FK506 (**a**) and by-products (FK520, FK506D) (**b**) production profiles by different strain breeding methods. The values and *error bars* shown represent the means and standard deviations from three independent experiments

showed approximately twofold increase in the yield over the wild-type strain, with titers of 83.1 ± 5.5, 94.3 ± 6.5, 99.5 ± 5.4, and 88.4 ± 3.6 mg/L, respectively. Besides, these strains generated only minor amount of FK520 and FK506D, with 7.6–10.8 and 3.8–6.1 mg/L, respectively (Fig. 2b). These results were different from the case of classically improved industrial polyketide-producing actinomycetes in which the overproduction of target compounds was coupled with the overproduction of by-products. One possible explanation for this is that the key genes responsible for the precursor biosynthesis of by-products (*tcsABC* for propylmalonyl-CoA biosynthesis, *tcsC* for ethylmalonyl-CoA biosynthesis) were downregulated after mutation. The FK506 yield and positive mutation were increased consistently with the enhancement of disodium malonate and disodium methylmalonate resistances. These disodium malonate-resistant and disodium methylmalonate-resistant mutants produced higher FK506, which was mainly due to disodium malonate and disodium methylmalonate being the precursors of FK506 biosynthesis. Therefore, the disodium malonate and disodium methylmalonate resistances screening were much more efficient for phenotypic evolution.

High-yield FK506 producing strain by genome shuffling

Five successive rounds of genome shuffling were carried out with the above four mutants which possessed enhanced resistance to disodium malonate and disodium methylmalonate as the starting strains. The evolution pressure during genome shuffling was FK506-resistant. From the first to the fifth rounds of genome shuffling, 200, 188, 168, 137, and 115 colonies appeared on the agar plates, which contained 100, 150, 200, 250, and 300 mg/L of FK506, respectively. The colonies were picked out for agar block bioassay and fermentation test. As shown in Fig. 2a, the FK506 production and product tolerance in *S. tsukubaensis* were increased gradually after each round of genome shuffling. Moreover, the increase of FK506 resistance was consistent with FK506 production improvement. Finally, after the fifth round of protoplast fusion, a recombinant resistant to 350 mg/L FK506, *S. tsukubaensis* TJ-P325, was isolated. Its yield reached 365.6 ± 7.8 mg/L, which was 2.67-fold higher than that of the parent strain TJ-K945 (Fig. 2a). In addition, it should be noted that the by-products FK520 and FK506D yields of TJ-P325 reached up to 48.9 ± 3.5 and 28.4 ± 3.3 mg/L, respectively, compared with the values of starting strains (TJ-K168, TJ-K554, TJ-K627, and TJ-K945) (Fig. 2b). This might be ascribed to the reason that although the bottleneck of the pivotal biosynthetic pathways for FK506 were relieved by increasing the product resistance of strain, the by-products shared the common polyketide synthase genes (*fkbABC*), nonribosomal peptide synthetase gene (*fkbP*) and post-modification genes (*fkbD* and *fkbM*) with FK506 [11, 17]. The application of genome shuffling raised benefit to complex strain improvement, particularly for the case that was hampered primarily by the inhibition from fermentation product or substrate [18, 34, 36]. It was reported that both mycelium growth and secondary metabolite biosynthesis were inhibited by product itself [20]. Therefore, there may exist a certain relationship between FK506 resistance and yield. So it was speculated that the high product tolerance strains would be helpful to resist the product inhibition and survive in the culture broth containing higher concentrations of product.

Genetic stability of recombinant TJ-P325

The genetic instability is a very important issue for the high-producing strains originated from various treatments of mutation or recombination. In this work, the genetic stability of *S. tsukubaensis* TJ-P325 with the highest FK506 production was evaluated by ten successive subcultivation tests. The ranges of cell-specific growth rate and FK506 production among ten generations remained from 0.0504 ± 0.0003 to 0.0511 ± 0.0006/h and from 342.5 ± 10.4 to 368.5 ± 8.8 mg/L, respectively, indicating that the hereditary character of the strain was stable. Genome shuffling in the mutants led to a dramatic increase in the volumetric productivity and specific production rate, demonstrating the application potential of these methods. These results were consistent with those previous studies in which notable improvements were shown [2, 14, 18, 36]. An indication of the possible mechanism underlying the improved productivity was that the mutants produced more biomass than the wild type and that higher biomass may confer the capability of tolerance. The enhanced growth rate may reflect the mutations that allow the cells to use nutrients in the medium more effectively or to export inhibitory molecules more rapidly. The stable and high-efficient production performance made TJ-P325 a promising microbial cell factory that can be used in the industrial process.

Transcription comparison of key genes related to FK506 biosynthesis between TJ-P325 and TJ-01

The genome shuffling method seems to play an important role in the FK506 biosynthesis of microorganism. However, it is difficult to determine the potential bottlenecks limiting the production based on such limited information. Therefore, it is necessary to get further insight into the underpinning metabolism. For this purpose, expression profiles of key genes involved in FK506 metabolic pathways as described previously were conducted to unravel the possible mechanism of FK506 overproduction in this study. The transcription level of FK506 biosynthesis structural genes (*tcsABCD*, *fkbGHIK*, *fkbL*, *fkbO*, *fkbABC*, *fkbP*,

fkbDM) and putative regulatory genes (*afsR*, *glnR*, *fkbR*, *fkbN*) were monitored during fermentation.

As shown in Fig. 3a, an increased transcription for all structural genes in FK506 biosynthetic pathway was noticed at 48 h for TJ-P325 strain, comparing with the wild type. It was found that enhancement of gene expression of polyketide synthase and nonribosomal peptide synthetase genes (*fkbABC*, *fkbP*) occurred prior to secondary metabolite stimulation, which corresponded positively to the rapid accumulation of FK506 in the stationary phase. Particularly, it was observed that *fkbC* transcription was much higher than other structural genes at 120 h, indicating that it played a pivotal role in FK506 biosynthesis. On the other hand, as the precursor biosynthetic pathways, the *fkbO* and *fkbL* transcription were more pronounced than other structural genes, since *fkbO* and *fkbL* encoded chorismatase and lysine cyclodeaminase, respectively, which were the rate-limiting enzyme in the first step and cyclization step of the FK506 biosynthesis. It is suggested that the transcription of the key genes *fkbO* and *fkbL* could be considered as a signal of FK506 overproduction. Indeed, expression of *fkbO* and *fkbL* was tightly associated with FK506 production and overexpression of these genes could enhance titer of FK506 [12]. Therefore, their up-regulation in TJ-P325 may result in the improved FK506 production. Additionally, as shown in Fig. 3a, the expression level of post-modification genes *fkbM* and *fkbD* were also strengthened, suggesting a stimulated effect on FK506 production based on initial macrolactone intermediate (unmodified FK506).

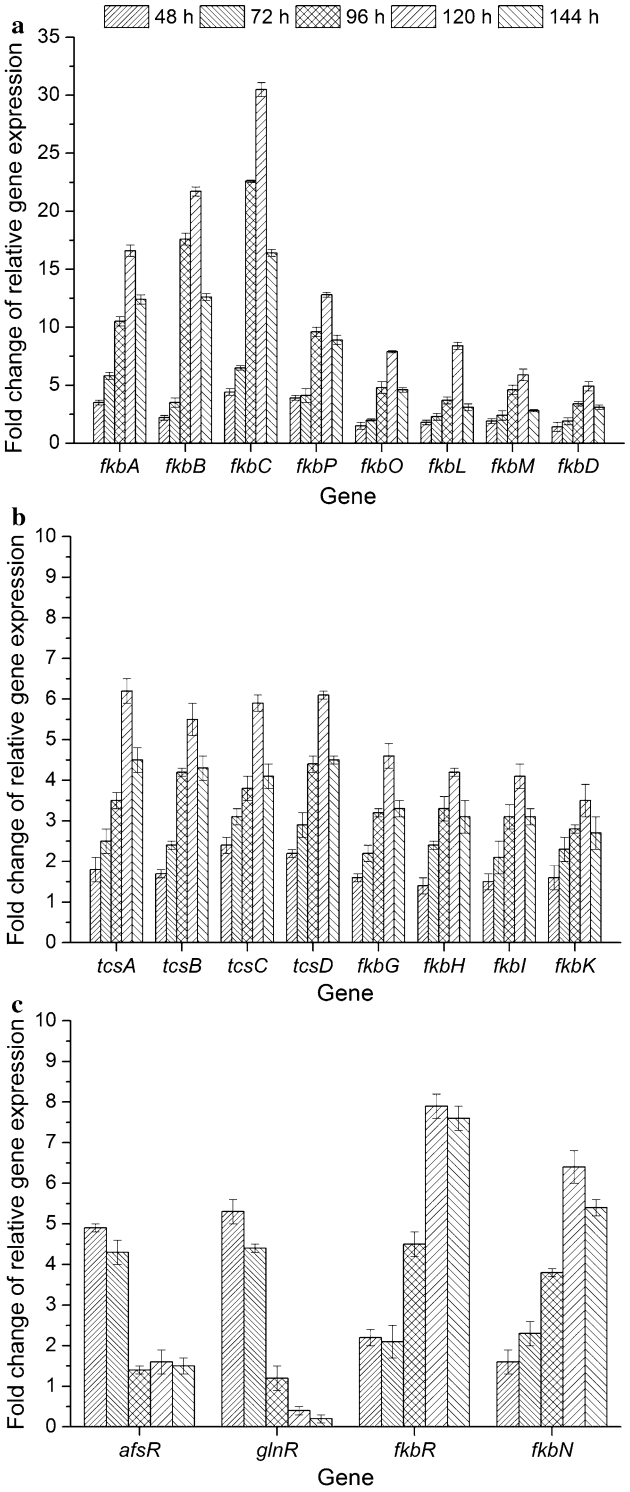
To confirm the improved performance of FK506 resulted from the changed transcriptional levels of whole gene cluster, the genes expression of another two intermediates (allylmalonyl-CoA and methoxymalonyl-ACP), including *tcsABCD* and *fkbGHIK* (*fkbJ* cannot be carried out by qRT-PCR because of short nucleotide sequence) were assayed. Actually, it was reported that the formation of allylmalonyl-CoA and methoxymalonyl-ACP were the rate-limiting step during the FK506 biosynthesis process [11]. As shown in Fig. 3b, these genes were all enhanced in TJ-P325, as expected, indicating the shuffling strain efficiently synthesizing these two precursors.

Furthermore, when fermentation proceeded to the stationary phase, the transcript level of structural genes in TJ-P325 were still maintained at higher levels or decreased slightly, compared with the transcript level in TJ-01 (Fig. 3a, b). This result was consistent with previous studies that the overproduction of target compounds in classically improved industrial polyketide-producing actinomycetes strains was mainly attributed to the lengthened expression of biosynthetic genes [19].

In addition to the significant structural genes described above, there are numerous pleiotropic regulatory genes present in genomes of secondary metabolite-producing actinomycete strains. Among them, *glnR* is a global regulator with a dual functional impact upon nitrogen metabolism and related antibiotics production [31]. The global regulatory gene *afsR* has been reported to stimulate antibiotics and other secondary metabolites [5, 26]. Besides, pathway-specific regulators *fkbR* and *fkbN* involved in FK506 biosynthesis have been studied recently [8, 23]. As shown in Fig. 3c, the transcription level of *afsR* in TJ-P325 was approximately 4.9- and 4.3-fold over TJ-01 at 48 and 72 h, respectively. Later, no remarkable transcriptional change of *afsR* was observed. In the case of *glnR*, the expression level increased 5.3-fold in TJ-P325 at 48 h but up-regulation lasted for no more than 48 h. Subsequently, *glnR* showed opposite changes, with a 2.5-fold and 3.6-fold decrease over TJ-01 at 120 and 144 h, respectively. For the gene *fkbR* and *fkbN*, both the transcript levels showed evident increase in TJ-P325 throughout the whole fermentation process compared with TJ-01 (Fig. 3c). Contrary to the pleiotropic regulatory genes *asfR* and *glnR*, the levels of pathway-specific regulators *fkbR* and *fkbN* during stationary phase were significantly higher, which could directly enhance the expression of other genes in the secondary pathway. Therefore, it seemed that pathway-specific regulators (*fkbR* and *fkbN*) were involved in the regulation of FK506 biosynthesis by stimulating the structural genes in *S. tsukubaensis*. Together, these findings provided new insights into the link between high-yielding *S. tsukubaensis* and specific cellular metabolic regulation.

Improvement of FK506 production based on precursor addition by TJ-P325

According to the enhancement measures described before, precursor addition strategies were proposed as described before [12]. The addition strategies were as follows: soybean oil and lactate were added at 24 and 36 h at a final concentration of 5 and 15 g/L, respectively; shikimate, chorismate, lysine and pipecolate were added at 48 h at 0.5, 0.25, 1.0 and 0.25 g/L, respectively; succinate, isoleucine and valine were added at 96 h at 1.5, 1.0 and 1.5 g/L, respectively. The performance of the high-producing recombinant TJ-P325 was carried out in shaken flask, and the products characterizations in batch fermentation were monitored. As shown in Fig. 4a, FK506 production of recombinant TJ-P325 with precursors addition was remarkably enhanced and reached its peak of 443.8 $\pm$ 6.3 mg/L at 144 h, compared to the value without addition. Besides, TJ-P325 attained 3.22 mg/L/h of FK506 productivity, leading to about a 28 % improvement over the control (data not shown). Moreover, the by-products were comparatively analyzed with and without precursors addition by TJ-P325, so as to get a profound metabolism insight. Surprisingly, both by-products displayed a remarkable decrease at the



◀**Fig. 3** Relative transcriptional levels determined by qRT-PCR for key genes involved in the biosynthesis and regulation of FK506 during the fermentation process by recombinant TJ-P325 and the initial strain TJ-01. **a** Polyketide synthase genes, nonribosomal peptide synthetase gene, initiative and cyclized genes as well as post-modification genes; **b** genes involved in two intermediates (allylmalonyl-CoA and methoxymalonyl-ACP) biosynthesis; **c** global and pathway-specific regulatory genes. The values and *error bars* shown represent the means and standard deviations from three independent experiments. All the values were statistical significance ($P < 0.05$ or $P < 0.01$) compared to TJ-01

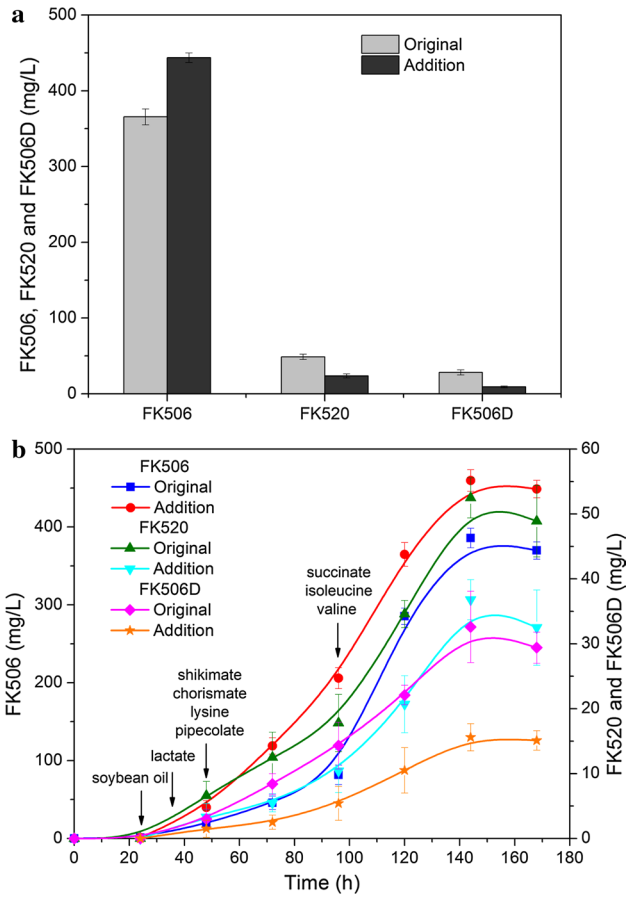


Fig. 4 The effect of precursor addition on the FK506 and by-products (FK520, FK506D) production profiles of the recombinant TJ-P325 in shaken flask (**a**) and in 7.5-L bioreactor (**b**). The values and *error bars* shown represent the means and standard deviations from three independent experiments

end of synchronous fermentation (Fig. 4a). The precursors addition resulted in a 67.9 % reduction of FK506D and a 51.6 % decrease of FK520, compared with those values obtained without addition. Furthermore, in order to verify the conclusion that TJ-P325 screened by multi-round breeding could display the significant increase in product as well as decrease in by-products, the cultivation was carried out in a 7.5-L bioreactor. As shown in Fig. 4b, the FK506 production was greatly improved by feeding various nutrients at late-exponential stage, as expected. In addition, the fermentation profile of by-products revealed that less FK520 and FK506D were observed after precursors addition (Fig. 4b). It was consistent with the result of shaken flask, confirming that the exogenous precursors supplemented into medium directed TJ-P325 to produce less by-products. To a certain extent, the decreased

by-products might be due to the fact that the strain made a shift to carbon metabolism, where the expression fold change of FK506 biosynthetic gene *tcsD* was significantly higher than *tcsAB* (also encoding FK506D CoA ester) and *tcsC* (also encoding FK520 and FK506D CoA-esters) under the added condition (data not shown). Moreover, it was noted that the by-products were even lower than our previous engineering strain HT-FKBOPLMD [12], demonstrating that different strains possessed different production capacities of by-products, though they were cultured under the same condition with exogenous precursor addition. It is very important in industry application, since FK520 and FK506D represent the major hurdle and complicate the subsequent extraction and purification of FK506 from the fermentation broth during the industrial production processes [17]. Therefore, these exciting results further indicated that the precursors addition was a powerful tool to improve the FK506 production via lessening carbon fluxes of the branched pathway.

Enhancing the FK506 production by dynamic fed-batch of disodium malonate and disodium methylmalonate

The above results (Fig. 2) have shown that, TJ-P325 was screened by disodium malonate and disodium methylmalonate resistances. Hence, fed-batch strategies with dynamic disodium malonate or disodium methylmalonate supplement were carried out to enhance the FK506 production, as described in Materials and methods. Here, the FK506 production for control TJ-P325 cultivation without adding the precursors was 367.5 ± 5.8 mg/L (Fig. 5a). It was noted that 0.05 g/L/h disodium malonate supplemented at 60 h displayed the most obvious role, up to 485.8 ± 5.7 mg/L (Fig. 5a). Likewise, 0.05 g/L/h disodium methylmalonate supplemented in the bioreactor at 84 h resulted in the highest titer (499.1 mg/L). It was also observed that FK506 production reached 495.4 mg/L with 0.03 g/L/h disodium methylmalonate added at 84 h, similar with the value of 0.05 g/L/h disodium methylmalonate, indicating that the precursor has saturated the FK506 production and excess disodium methylmalonate could not further improve the yield. In light of the production cost, 0.03 g/L/h disodium methylmalonate at 84 h was chosen as the optimized condition. In addition, when disodium malonate or disodium methylmalonate was fed too early (48 h) or too late (96 h), it exhibited no positive effect on FK506 enhancement compared with the control, which might be due to its negative effects on the strain growth. On the basis of the above single factor optimization, combination of feeding strategies in the fermentation process was proposed; that was, 0.05 g/L/h disodium malonate supplemented at 60 h and 0.03 g/L/h disodium methylmalonate added at 84 h. The result showed that the recombinant

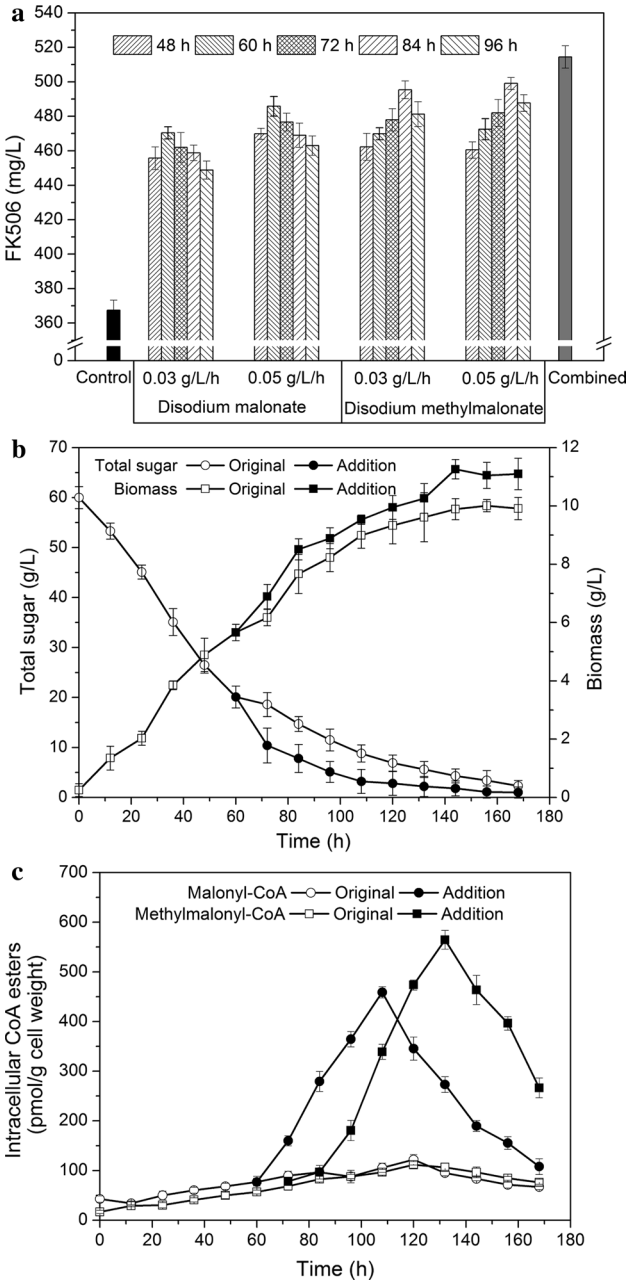


Fig. 5 The effects of disodium malonate and disodium methylmalonate dynamic fed-batch strategies on the FK506 production and intracellular CoA-esters of TJ-P325 at different feeding times and different feeding concentrations. **a** FK506 production by different feeding strategies; **b** dry cell weight and total sugar by combinatorial feeding strategies; **c** intracellular CoA-esters by combinatorial feeding strategies. The values and *error bars* shown represent the means and standard deviations from three independent experiments

strain was able to produce 514.5 ± 9.5 mg/L of FK506. Meanwhile, the profile of cell growth during fermentations is presented in Fig. 5b. After adding precursors in combination, the strain TJ-P325 had a shorter lag phase, compared with the control. Dry cell weight of TJ-P325 without

addition increased slightly in the first 36 h of cultivation while TJ-P325 with addition exhibited better growth. The growth characterization was consistent with FK506 biosynthesis. The biomass of TJ-P325 reached a maximum of 11.26 ± 0.33 g/L at 144 h, whereas the control was 10.01 ± 0.21 g/L at 156 h, suggesting that the supplementation of precursors significantly enhanced the strain growth. As shown in Fig. 5b, total sugar was consumed rapidly in the exponential growth phase. In the stationary phase, the mycelium growth almost stopped, so the consumed total sugar was mainly used for FK506 biosynthesis and mycelium maintenance. Figure 5b also showed that the differences between the precursors addition and control group with regard to consumption of total sugar in the first 24 h of fermentation were not obvious. With the extension of fermentation, the consumption rate of total sugar of the recombinant TJ-P325 with precursors addition accelerated and evidently outstripped that of the control. Moreover, intracellular CoA-esters were comparatively analyzed to examine the effects of the additives. As expected, both malonyl-CoA and methylmalonyl-CoA, the major precursors of FK506, were greatly improved after the exogenous addition (Fig. 5c). It was indicated that the mutant may strengthen the efficiency of the transporters involved in these two precursors. These results demonstrated that exogenous precursor feeding strategies played a significant role in the availability of malonyl-CoA and methylmalonyl-CoA towards improved FK506 biosynthesis.

Discussion

Genome shuffling has become a promising technique to enhance secondary metabolite production in many microorganisms since first discovered [20, 28, 38–40]. As a laboratory evolution method, it addresses the limitations of classical strain improvement, which is often based on high-throughput screening of mutants generated by mutagenesis. The method takes the advantage of multiparental crossing facilitated by recombination of an entire genome associated with conventional breeding applications and, thus, acts as a combined method to improve phenotype. Our study demonstrated that genome shuffling was very useful in the improvement of immunosuppressant FK506 based on the product tolerance (Fig. 2a). In fact, it is significant to enhance the screening efficiency in the acquisition of important phenotype during the course of genome shuffling, based on the resistance to condition stresses [18, 28, 38]. Particularly, increased resistance to the target product in the evolved process often results in an improvement of production [24, 34]. So it is not surprising to speculate that through increasing the product concentration in the medium, the strain resistant to higher concentration can also improve production titers. In addition to the disodium malonate and disodium methylmalonate resistances, pipecolate, chorismate (shikimate) were also used to screen the tolerant strains. But the FK506 yield did not increase (data not shown), which might be due to the fact that the strain has saturated these precursors level and additional evolution under these conditions cannot support the FK506 improvement. It should be noted that the FK506 yield of another original strain D852 is 143.5 ± 8.7 mg/L [11, 12], higher than that of strain TJ-01 (30.5 ± 3.8 mg/L) in this work. Actually, D852 were firstly used as the parent strain to carry out the genome shuffling and amount of recursive work was carried out in order to screen the positive results. Unfortunately, there was no overproduction strain isolated (data not shown). From this point, it can be concluded that although initial production yield by D852 was higher, the potential improvement by TJ-01 based on genome shuffling could be exploited. On the other hand, a multi-scale analysis of metabolic network is necessary to fully understand the intracellular behavior differences between TJ-01 and D852. Therefore, further work will be carried out to provide insight into the potential mechanism. Besides, based on our engineered strains (HT-FKBOPLMD with *fkbOPLMD* gene overexpression, HT-ΔGDH–DAZ with *gdhA*-deletion and *dahp*-, *accA2*-, *zwf2*-overexpression) [11, 12], genome shuffling can be applied to improve the carbon fluxes of both secondary and primary pathways in the evolved recombinant strain. It will provide the possibility of evolving a rationally engineered phenotype, which is difficult to manipulate by metabolic engineered approach, especially for multi-genes construction of *Streptomyces*.

The transcriptional changes of several key genes were analyzed to uncover the possible mechanism of high-yield FK506 strain TJ-P325 isolated by multi-round screening of mutagenesis and genome shuffling. In fact, the shuffling phenotypes may redistribute carbon fluxes, regulate metabolic network, change transport ability, and improve substrate availability. Many studies have suggested that it is the gene expression changes of the key pathways through the whole genome that contributed to the products enhancement. Using qRT-PCR approach, Jin et al. [13] showed that the expression of two genes *snbA* and *snaB*, which were involved in the last step of the pristinamycin IIA biosynthesis, displayed higher in the shuffling recombinant than that of the parent strain during the prolonged fermentation, indicating that their expression changes might be key factors during antibiotic biosynthesis. Furthermore, a third gene *ptr* involved in resistance to the antibiotic started high-level expression ahead of the onset of the antibiotic production in the recombinant strain, compared with the parent [13]. Similar conclusions also pointed out that surfactin synthetase gene expression in evolved mutant was 15.7-fold higher than in the wild type [40]. These results indicated

that the discrepancy in expression changes might play a key role in antibiotic biosynthesis. In our study, a possible mechanism for FK506 overproduction was described by analyzing the gene expressions of structural genes in FK506 biosynthetic pathway and regulatory genes in shuffling and parent strains. The relative expression analysis revealed that all the structural genes of mutant increased transcription compared with that of the parent strain (Fig. 3a, b), which was consistent with the other antibiotic. Based on the comparative analyses of the regulatory genes, the transcriptional change of *afsR* was higher in mutant at 48 and 72 h (Fig. 3c). Previous reports validated that *afsR* controlled expression of the biosynthesis of actinorhodin and undecylprodigiosin by activating the pathway-specific regulatory genes *actII-ORF4* and *redD* [5]. For another regulator *glnR*, it displayed a different trend that the fold change of the expression level by shuffling mutant decreased significantly from 48 to 144 h, whereas the fold change of pathway-specific regulators (*fkbR* and *fkbN*) involved in the regulation of FK506 biosynthesis were both enhanced during late fermentation (Fig. 3c). This might be due to the fact that *glnR* was a global regulator with a dual functional impact upon secondary metabolism and antibiotics production in *Streptomyces*, which was consistent with previous reports [4]. Taken together, these transcriptional level changes can partly explain the mechanism of high-yield shuffled strain. Further work such as knocking out the putative *afsR* and *glnR* genes in *S. tsukubaensis* is necessary to clarify the role of global regulators in FK506 biosynthesis regulation. It should be noted that, however, it is the whole-genome sequencing that can enable the detailed investigation of genetic changes (single-nucleotide polymorphism) and transcriptional changes in genome-shuffled recombinants. In addition, the intracellular metabolite concentrations will be assayed to unravel the precursors and product-tolerant mechanisms of *S. tsukubaensis* at micro-environmental levels [9]. Even so, our results may provide important physiological information that can guide the metabolic engineering of *S. tsukubaensis* for FK506 overproduction which is underway in our groups.

Precursors addition and dynamic fed-batch strategies play an important role in secondary metabolite production. In this work, the supplementation of soybean oil, lactate, shikimate, chorismate, lysine, pipecolate, succinate, isoleucine together with valine markedly enhanced the FK506 production of the shuffling strain, as well as reduced by-products production dramatically (Fig. 4a, b). These findings indicated that precursors addition approach can be applied to the production improvement of other immunosuppressant such as rapamycin and FK520. Besides, malonyl-CoA and methylmalonyl-CoA characterized in this work were revealed to be two key limiting factors in FK506 production under a dynamic fed-batch fermentation culture condition. On the basis of combinatorial dynamic addition of disodium malonate and disodium methylmalonate, the shuffling strain TJ-P325 could accumulate 514.5 ± 9.5 mg/L of FK506 (Fig. 5a), showing the advantage of dynamic fed-batch strategies in elevating production at a significant level. To further increase the FK506 production, the combination of precursors supplementation and dynamic fed-batch of disodium malonate and disodium methylmalonate were performed in the bioreactor. However, compared with the control (precursors or dynamic fed-batch supplementation), no higher amounts of FK506 were observed (data not shown). A possible explanation for this low amount of product accumulation is that the FK506 production machinery may already be abundant within the cell, and the existing level can manage increased metabolic flux. Although the production titer in this work is relatively competitive, further enhancement of FK506 titer is still necessary for commercial applications in future.

Nowadays, it is necessary to apply raw materials to effectively reduce the costs of industry production [3, 6, 29]. Therefore, further research will be investigated to characterize a global profile of the intracellular metabolites [37], further to provide a more rational guidance in control of FK506 production and get optimal cultivation conditions by using the cheaper resources such as glycerol or molasses [7]. Besides, the further efforts to reduce the usage of relatively expensive ingredients including disodium malonate and disodium methylmalonate would be appreciated at a commercially meaningful level.

In conclusion, the current study showed that FK506 production enhancement was achieved by the rational breeding of strains based on genome shuffling. The gene expression analysis of key genes in metabolic pathways provided new insights into the potential factors of FK506 overproduction. Moreover, the production of FK506 in *S. tsukubaensis* TJ-P325 based on precursors supplementation was ultimately enhanced up to 514.5 mg/L. This study demonstrated that more effective genome shuffling and more appropriate dynamic fed-batch strategies can provide a promising platform for immunosuppressant FK506 production improvement. The simple and effective strategies developed here can be extended to titer improvement of other important natural products and process optimization on a commercial scale. To determine the exact mechanisms of FK506 yield improvement by genome shuffling, further genomic, transcriptomic, proteomic and metabolomic investigations are needed.

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