



Development of FK506-hyperproducing strain and optimization of culture conditions in solid-state fermentation for the hyper-production of FK506

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Abstract FK506 hyper-yielding mutant, called the TCM8594 strain, was made from *Streptomyces tsukubaensis* NRRL 18488 by mutagenesis using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, ultraviolet irradiation, and FK506 sequential resistance selection. FK506 production by the TCM8594 strain improved 45.1-fold (505.4 µg/mL) compared to that of *S. tsukubaensis* NRRL 18488 (11.2 µg/mL). Among the five substrates, wheat bran was selected as the best solid substrate to produce optimum quantities of FK506 (382.7 µg/g substrate) under solid-state fermentation, and the process parameters affecting FK506 production were optimized. Maximum FK506 yield (897.4 µg/g substrate) was achieved by optimizing process parameters, such as wheat bran with 5 % (w/w) dextrin and yeast extract as additional nutrients, 70 % (v/w) initial solid substrate moisture content, initial medium pH of 7.2, 30 °C incubation temperature, inoculum level that was 10 % (v/w) of the cell mass equivalent, and a 10 day incubation. The results showed an overall 234 % increase in FK506 production after optimizing the process parameters.

Keywords FK506 · Immunosuppressant · Solid-state fermentation · *Streptomyces tsukubaensis* · Wheat bran

Introduction

Tacrolimus (FK506) has been used for patients undergoing organ and tissue transplants (Haddad et al. 2006). In particular, among the different commercially available immunosuppressant agents (cyclosporine, rapamycin, and mycophenolate), FK506 is one of the potent immunosuppressive agent to treat transplants rejection, marketed by Astellas under the name Prograf, which represented 27.6 % of annual immunosuppressant market sales in 2011 (Martínez-Castro et al. 2013). Recently, FK506 is also used for treatment atopic dermatitis and autoimmune disorder in recently (Barreiro and Martínez-Castro 2014). To meet the increasing demand for FK506, researchers have attempted to develop high producing strains and fermentation processes to produce FK506 more efficiently and economically (Yang et al. 2014). However, there are restraints on using high-yielding FK506 strains by companies that hold the patent for strain development. Hence, since FK506 was first isolated from the filamentous bacterium *S. tsukubaensis* in 1987, 18 FK506 producer strains have been reported to overcome this problem until now (Barreiro and Martínez-Castro 2014).

Random mutagenesis is the easiest and powerful tool to improve a strain. Ultraviolet (UV)-rays, X-rays, gamma-rays, lasers, neutrons, methyl methane sulfonate, hydroxyl amines, and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) have been extensively used as mutagenesis agents. NTG is able to lead to GC-AT transition mutations, which limits its usefulness in generalized mutagenesis (Baltz 1986a; b). In contrast, UV radiation is among the most effective, simple, and safe mutagenic agents. UV induces a broad range of single base mutations in *Escherichia coli* (Miller 1983) but knowns to be a weak mutagenicity in *Streptomyces* (Baltz 1986a; b). However, quite a lot of antibiotics have been procured from various high yielding mutants after treating *Streptomyces* spp. with UV irradiation. In particular, Xu et al. (2005) achieved three-fold increase in rapamycin yield by *Streptomyces hygroscopicus* HD-04-S after UV mutagenesis (Xu et al. 2005).

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Recently, a lot of solid-state fermentation (SSF) processes are used to produce microbial metabolites, including chemicals, enzymes, antibiotics, and immunosuppressants because of the many benefit over traditional submerged fermentation (SmF) (Kota and Sridhar 1998; Ellaiah et al. 2004; Rajoka et al. 2006). SSF is simpler, requires less working capital, results in superior productivity, reduces energy consumption, uses uncomplicated fermentation media, does not require exact parameters to control the fermentation, reduces water use, produces less wastewater, is easier to control microbial contamination, and requires lower cost for purification processing compared to those of SmF (Ghildyal et al. 1985). The significant constituents that affect the synthesis of bacterial secondary metabolites in a SSF system include selecting a proper substrate and microbe, pretreating the substrate, substrate particle size, substrate water content, relative humidity of culture environment, inoculum type and size, control of generation of heat in matter, fermentation duration, maintenance of O₂ consumption and CO₂ emission rates (Ghildyal et al. 1985; Rajoka et al. 2006; El-Naggar et al. 2009).

In this study, we studied the ability of a high yielding FK506 mutant obtained after NTG and UV mutagenesis of *S. tsukubaensis* NRRL 18488 to enhance FK506 production. We also defined various fermentation parameters necessary to maintain mutants that produced higher levels of FK506 under SSF. The production of FK506 by *S. tsukubaensis* under SSF conditions has not been reported before now.

Materials and Methods

Bacterial strain, culture conditions, and materials

S. tsukubaensis NRRL 18488 (wild-type) was obtained from the Agricultural Research Service Culture Collection (NRRL, Peoria, IL, USA) and its mutant (TCM8594) was developed after sequential FK506 resistance selection followed by NTG and UV mutagenesis and was used in this study. Wild-type and TCM8594 strains were grown on ISP4 plates at 28 °C for 6 days for complete sporulation and prepare the inoculum. The spore suspension of wild-type and TCM8594 was inoculated for seed culture into a baffled flask (250 mL) containing 50 mL of seed medium (0.5 % glucose, 1 % soluble starch, 0.5 % yeast extract, 0.25 % corn steep powder, 1 % glycerol, and 0.1 % CaCO₃, pH 7.2), and the baffled flask was then fermented at 28 °C for 3 days in an orbital shaking incubator at 180 rpm to develop the seed culture, respectively. Subsequently, 10 % (v/v) of the above seed culture was transferred to 250 mL baffled flasks containing 50 mL modified production medium (7.5 % dextrin, 2 % dried yeast powder, 0.5 % corn steep powder, 0.5 % K₂HPO₄, and 0.05 % CaCO₃, pH 7.2), and fermentation was carried out on an orbital shaking incubator at 180 rpm and 28 °C for 7 days according to the methods Martínez-Castro et al. (2013).

Strain development and screening study for the FK506 high-yielding strain

Wild-type spore suspensions (10⁸ spores/mL) were processed with NTG (0.5–5 mg/L) in Tris/malate buffer (50 mM, pH 9.0) for 60 min, collected by filtration, washed three times with sterile saline, and plated on Bennett's (BN) agar plates for 5 days at 28 °C. The resulting colonies were overlaid with soft (7 %, w/v) malt extract agar seeded with 10⁵ cells/mL of *Aspergillus niger* ATCC 6275. After 48 h cultivation at 28 °C, colonies with large zone of fungal inhibition were selected. These colonies were stabilized on ISP4 plates, and the spores were collected again using the method described above. The spores were diluted and plated on BN agar plates, and the open plates were UV irradiated for 30–120 s 30 cm from UV (253.7 nm/30 W). The BN agar plates were incubated under the same conditions described above for overlay-selection. After 48 h incubation at 28 °C, colonies showing wide inhibition zones were selected (Lee et al. 2003). These colonies with wide inhibition zones were cultivated on ISP4 medium for spore formations. The spores were smeared on agar medium plates with 50 µg/mL FK506 for sequential adaptation and incubated at 28 °C for 5 days. Among the strains with resistant to FK506, an FK506 high-yielding strain was screened using the *A. niger* bioassay and high performance liquid chromatography (HPLC) analysis according to the methods of Mo et al. (2013). The calibration curve of FK506 was created using an FK506 standard (Sigma-Aldrich, USA). The calibration curve for quantitative analysis of FK506 is shown in Supplementary Fig. 1 in the supplement materials.

The concentration of FK506 eventually increased to a final concentration of 500 µg/mL on FK506 resistance selection agar plates. The detailed procedure for the development of FK506 high-yielding strain by random mutagenesis and sequential FK506 resistance selection is illustrated in Fig. 1.

Gene expression analysis by semi-quantitative RT-PCR

For RNA isolation, wild-type and TCM8594 strains were grown on R2YE medium for 72 h. Mycelia of wild-type and TCM8594 were harvested and immediately stabilized in RNAProtect Bacteria Reagent (Qiagen, Valencia, CA, USA), and suspensions were kept at -80 °C. Total RNA was extracted according to the manufacturer's protocol by using a RNeasy mini kit (Qiagen). The RNA quantity and quality were measured via a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis. RT-PCR was carried out by OneStep RT-PCR kit (Qiagen) using 1 µg of total RNA as a template. The conditions were as follows: for first stand cDNA synthesis, 50 °C for 30 min, followed by 95 °C for 15 min; for PCR amplification, 35 or 38 cycles of 97 °C for 1 min, 60–70 °C (depending on the set of primers used) for 1 min, 74 °C for 1 min, and a final elongation step of 5 min at 75 °C. All primers used for RT-PCR amplification are shown in Supplementary Table 1 in the

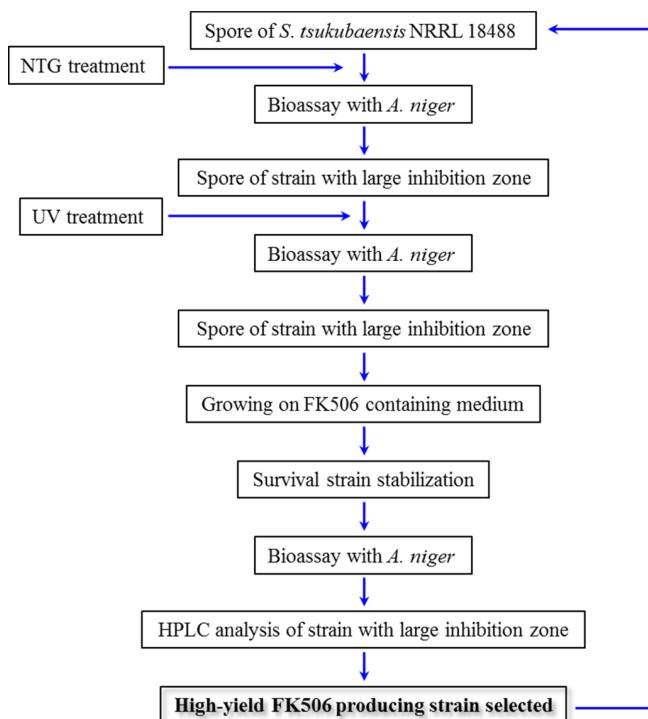


Fig. 1 Development strategies of high-yield FK506 producing strain via repeated and sequential mutagenesis and HPLC analysis of developed strain. Flow charts for development of FK506 high-yielding strain by random mutagenesis and sequential FK506 resistance selection

supplement materials. Negative control (the absence of reverse transcriptase) experiments were performed to check for DNA contamination in the RNA preparations. The 16S rRNA were used as positive markers for RNA quality (Mo et al. 2016). All experiments were performed in duplicate RNA sample, using two independent cultures.

Optimization of SSF

Factors affecting FK506 production by wild-type and TCM8594 strain were optimized in wide mouth Erlenmeyer flasks (500 mL) each containing 20 g of substrate (wheat bran, rice husk, rice bran, sesame oil cake, and soy flour) and 2 mL of element solution in triplicate. The composition of the element solution was (w/v): 0.05 % KH₂PO₄, 1.0 % MgCl₂·6H₂O, 1 % CaCl₂·2H₂O, 0.05 % FeCl₃·6H₂O, and 0.05 % ZnCl₂. Experiments were carried out in wide mouth Erlenmeyer flasks (500 mL) containing 20 g substrate moistened with distilled water to produce 50 % (v/w) moisture content. Initial pH was adjusted to 7.2, the Erlenmeyer flasks were plugged with non-absorbent cotton, and autoclaved for 20 min at 121 °C (Khaliq et al. 2009). After cooling the substrate at room temperature, 10 % (v/w) of a 3-day old seeding culture was inoculated, and the flasks were fermented at 28 °C for 12 days under appropriate experimental conditions. After fermentation, samples (three whole flasks) were analyzed the FK506 production by HPLC.

Table 1 Improved FK506 production by random mutations of *S. tsukubaensis* NRRL 18488

Mutagenic treatment round ^a	Mutant	FK506 production (μg/mL) ^b	Relative productivity (fold)
None	Control	11.2±0.6	1
1 st NTG+UV	TCM324	25.1±0.9	2.2
2 nd NTG+UV	TCM935	75.4±1.1	6.7
3 rd NTG+UV	TCM2104	141.9±4.7	12.7
4 th NTG+UV	TCM3174	219.3±5.1	19.6
5 th NTG+UV	TCM4817	296.5±9.2	26.5
6 th NTG+UV	TCM6048	353.8±10.9	31.6
7 th NTG+UV	TCM7129	431.7±12.4	38.5
8 th NTG+UV	TCM8594	505.4±11.7	45.1

^aThe strain was developed by NTG treatment and UV irradiation to obtain a superior strain combined with cultivation on medium containing stepwise increasing concentrations of FK506

^bThe mycelia were collected on filter paper by vacuum filtration after a 6 day cultivation. Cell-free culture broth was extracted twice with equal volumes of ethyl acetate. The organic extract was evaporated to dryness under reduced pressure and dissolved in 0.2 mL methanol for the HPLC analysis. The level of FK506 production reported is the mean of two series of duplicate separate cultivations and extractions

Optimization of process parameters for FK506 production in SSF

The influence of various physicochemical and nutritional parameters on FK506 production was investigated. Wheat bran was used as the best substrate for FK506 production in all studies. Moisture contents of 20, 30, 40, 50, 60, 70, and 80 % (v/w) and incubation times of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12 days as well as inoculum levels of 2, 5, 10, 15, and 20 % (v/w) of a 3-day old seeding culture were investigated. Incubation pH of the medium was studied between 4 and 9. The pH was adjusted with 0.5 N NaOH or HCl. The effects of temperatures on FK506 production were investigated at 25, 30, 35, 40, and 45 °C. Studies were also conducted to examine the effects of various carbon and nitrogen sources supplemented into the wheat bran solid culture on FK506 production. The effect of adding 5 % (w/w) carbon sources (glucose, lactose, maltose, mannose, sorbitol, sucrose, starch, and dextrin) was evaluated using appropriate culture conditions (Asagbra et al. 2005; El-Naggar et al. 2009; Khaliq et al. 2009). Also more studies were conducted to determine the significance of various organic nitrogen sources (yeast extract, tryptone, peptone, casamino acids, soytone, malt extract, and corn steep powder at 1 g/20 g substrate) in the existence of wheat bran and dextrin on FK506 production (Khaliq et al. 2009). SSF was carried out at 30 °C for 12 days under optimal fermentation conditions (initial pH of 7.2, initial moisture content of 70 % (v/w), inoculum level of 10 % (v/w, 3-day old seeding culture). Each experiment was conducted in triplicate.

FK506 extraction and HPLC analysis

The cell biomass was homogenized with three volumes of

methanol by blender after SSF. The sample was kept on rotary shaker (180 rpm) for 3 h. After centrifugation, the extract was then filtered with filter paper (Advantech 5A) and 0.45 µm GD/XP nylon syringe filter (GE Healthcare, Clifton, NJ, USA) to remove additional fine particles and obtain a clear solution. The extract (2 mL) was evaporated to dryness in a speed vacuum concentrator. The dried extract was dissolved in 0.2 mL methanol, and FK506 was estimated by HPLC as mentioned above.

Antifungal assay of SmF and SSF

Samples of SmF and SSF were extracted twice with ethyl acetate and methanol, respectively. The crude extracts were dried using an evaporator. The dried SmF and SSF extracts were redissolved in methanol to make a concentration of 100 mg/mL. Methanol was served as a negative control and 1 mg/mL of Amphotericin B (Sigma-Aldrich, USA) was served as a positive control for this assay. Sterile paper discs (8 mm in diameter, Advantec Co., Tokyo, Japan) were impregnated with 4 mg (40 µL) of each extract. Cell suspension of *A. niger* (at a concentration of 10^5 cells/mL) was spread onto the surface of potato dextrose agar (BD Difco, Sparks, MD, USA) media before addition of the impregnated papers discs. The inhibition zone diameter of around the discs was measured after incubating fungal plates at 28 °C in the dark for 24 h. This assay was done in three replicates.

Results and Discussion

Selection of FK506 high-yielding strain by random mutagenesis and FK506 resistance selection

S. tsukubaensis NRRL 18488 spores were subjected to NTG, UV, and sequential FK506 resistance selection to improve an FK506 yield of mutants. A number of colonies obtained after NTG treatment of the wild-type strain were preliminarily screened for a wide inhibition zone against *A. niger*, and the spores of selected colonies were UV irradiated. The rates of survival of the parent strain after the 3 mg/mL NTG treatment for 1 h and UV irradiation for 120 s were 10 and 1 %, respectively. Among them, colonies with the maximum diameters of growth inhibition zone against *A. niger* were smeared on agar medium plates containing FK506. An FK506 resistance colonies were isolated in a growth medium containing FK506, and the mutant strains with the highest FK506 yields were selected using the agar plug method and HPLC analysis. One colony with the largest inhibitory zone diameter against *A. niger* was selected from 418 FK506 resistance colonies and designated TCM324. The TCM324 mutant strain increased FK506 yield by approximately 2.2-fold (25.1 µg/mL) compared with that by the wild-type strain (11.2 µg/mL; Table 1). The TCM8594 mutant strain, which was selected after eight selection rounds among 10,798 mutagenized colonies after NTG treatment, UV irradiation, and sequential FK506 resistance selection, showed the highest FK506 production, and increased

FK506 yield by approximately 45.1-fold (505.4 µg/mL) compared with that from wild-type strain (11.2 µg/mL; Table 1) without any improvement in biomass yield. This result shows that NTG and UV were the most powerful and efficient methods to generate the optimal FK506 yield from the mutants. Furthermore, the FK506 yield by the TCM8594 strain increased to 505.4 µg/mL, which was close to that observed by *S. tsukubaensis* CZ-19 previously (Yang et al. 2014).

Transcription level analysis of *tcsB*, *fkbB*, and *fkbN* genes in wild-type and TCM8594 strains

To investigate the difference in expression profiles of *tcsB*, *fkbB*, and *fkbN* transcripts level, transcription analysis of these genes was conducted by semi-quantitative RT-PCR in the wild-type and TCM8594 strains. This was because these genes are an essential gene for FK506 biosynthesis (Mo et al. 2012). Total RNA was isolated from the wild-type and TCM8594 strains after 24, 48, and 72 h of cultivation and was prepared as the template for analysis of gene expression level by RT-PCR. All primers were specific to the sequences within the *tcsB*, *fkbB*, and *fkbN* genes and were designed to amplify approximately 500 bp of each cDNAs (Supplementary Table 1). The levels of the unrelated transcript, constitutively expressed 16S rRNA gene, assayed as a control for RNA purity, integrity and loading levels, did not change culture times. The Fig. 2 presents the results of these RT-PCR experiments. The transcripts of these genes were not observed a change at any point during a 3-day time course using semi-quantitative RT-PCR in the wild-type strain, suggesting that these genes might constant the onset of FK506 biosynthesis. While the transcripts of these genes increased slowly during 24 h of fermentation and relatively quickly between hours 48 and 72 h in the TCM8594 strain (Fig. 2). This result is corresponding with the FK506 production time course of the wild-type and TCM8594 strains in the production medium because the maximal levels of FK506 were revealed at approximately 6 and 4 days, respectively (data not shown). Indeed, RT-PCR results at the different time points in cultivation accurately showed that the transcript levels of the *tcsB*, *fkbB*, and *fkbN* genes had apparently increased in TCM8594 strain. This strongly demonstrated the idea that an increase in an essential gene transcription gives rise to the improvement in FK506 production.

FK506 production in SSF using various substrates

Various low-cost agricultural wastes were used as substrates to produce FK506 by the TCM8594 strain during SSF. Among the five inexpensive and easily available substrates screened, the maximum FK506 yield (382.7 µg/g substrate) was achieved with wheat bran (Supplementary Table 2). Comparatively less FK506 was produced with all other substrates, such as rice husk, rice bran, soy flour, and sesame oil cake. This result indicates that wheat bran is the most suitable substrate for producing FK506 in the TCM8594 strain, which may have been due to the fact that

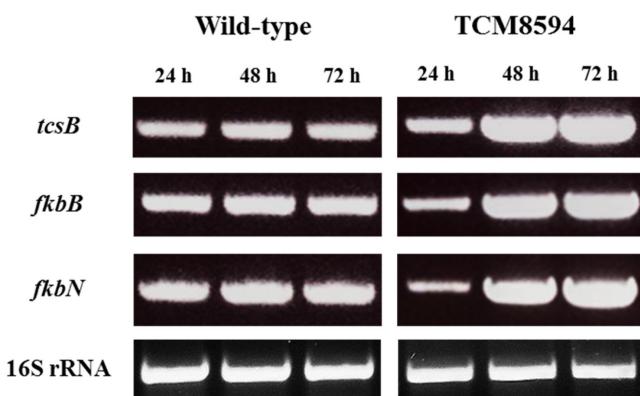


Fig. 2 Gene expression analysis of *tcsB*, *fkbB*, and *fkbN* genes by RT-PCR. Analysis was carried out on *S. tsukubaensis* NRRL 18488 and the TCM8594 strain as indicated in Materials and Methods. For transcript analysis of each strain, the experiments were carried out on three independent isolates after 24, 48, and 72 h of fermentation. The amplified PCR product of 16S rRNA gene was served as a control for RNA integrity and loading levels

wheat bran contains sufficient nutrients and remained loose under moist conditions, thereby creating a large surface area (Babu and Satyanarayana 1995). It was previously reported that wheat bran helped to produce the highest titers of tylosin, meroparamycin, and oxytetracycline (El-Naggar et al. 2009; Khaliq et al. 2009; Lazim et al. 2010). However, the wild-type strain produced a very small amount of FK506 during SSF, using five substrates (Supplementary Table 2).

Effect of initial moisture level

The effect of moisture content of substrate (wheat bran) on FK506 production is shown in Fig. 3A. The maximum FK506 production (523.8 µg/g substrate) by the TCM8594 strain was accomplished at 70 % (v/w) moisture content on day 12 of the incubation. When moisture content was lower or higher than 70 % (v/w), FK506 production was low because the substrate was too dry or wet for cell growth and antibiotic production. Improvement of FK506 production was not observed in wild-type strain.

Effect of inoculum level

To study the influence of inoculum level on FK506 production, various cell concentrations (equivalent to 2, 5, 7, 10, 15, and 20 % (v/w) cell culture after 72 h) were added to each of six flasks. Fermentations were then performed for 12 days, and the results are presented in Fig. 3B. Higher FK506 titers were obtained at an inoculum level of 10 % (v/w), and FK506 production by the TCM8594 strain increased up to 654.5 µg/g substrate on day 12 of incubation. Wild-type strain showed a very small in FK506 yield (28.8 µg/g substrate).

Effect of initial pH

Among the physical parameters occurring during SSF processes, initial pH of the SSF plays a key role by inducing secondary metabolite production by *Streptomyces* spp. (Khaliq et al. 2009). The effect of initial substrate pH on FK506 production is

displayed in Fig. 3C. The optimal pH for maximum FK506 production was 7.2 (721.6 µg/g substrate) when grown in SSF. Less FK506 was produced when initial pH was lower or higher than the optimal. In particular, FK506 yield decreased significantly when the initial pH was adjusted to 8.2 and 9.2. The same FK506 production result was observed for wild-type strain.

Effect of incubation temperature

Maintaining an optimal process temperature is a major factor and strongly affects the economy of the SSF process. Fermentation temperature affects microbial cell growth, sporulation and germination of spore, and microbial cell physiology; thus, affecting product formation. The optimal temperature for maximum FK506 production by the TCM8594 strain was 30 °C (754.6 µg/g substrate) (Fig. 3D). The highest FK506 production by wild-type strain was achieved at 30 °C. A similar observation was observed by *S. marinensis* NUV 5 in SSF by Ellaiah et al. (2004). However, Yang and Ling found that the optimal temperature for *Streptomyces viridifaciens* for tetracycline production was 26 °C using sweet potato residue (Yang and Ling 1989). This result indicates that the optimal temperature for antibiotic production is dependent on the test strain.

Effect of incubation times

Fig. 3E exhibits the influence of incubation time on FK506 production in SSF. Different incubation times were evaluated under optimized conditions (70 % (v/w) substrate moisture level, 10 % (v/w) inoculum, initial pH 7.2, and incubation temperature of 30 °C) to study their effect on FK506 production over 12 days. FK506 was produced in the beginning of the exponential growth phase at day 6 of incubation, and maximum FK506 production (806.7 and 39.4 µg/g substrate) occurred on day 10 in the TCM8594 strain and wild-type strain cultures, respectively, after which there were slight decreases in FK506 production. The most remarkable increase in antibiotic yield was observed during

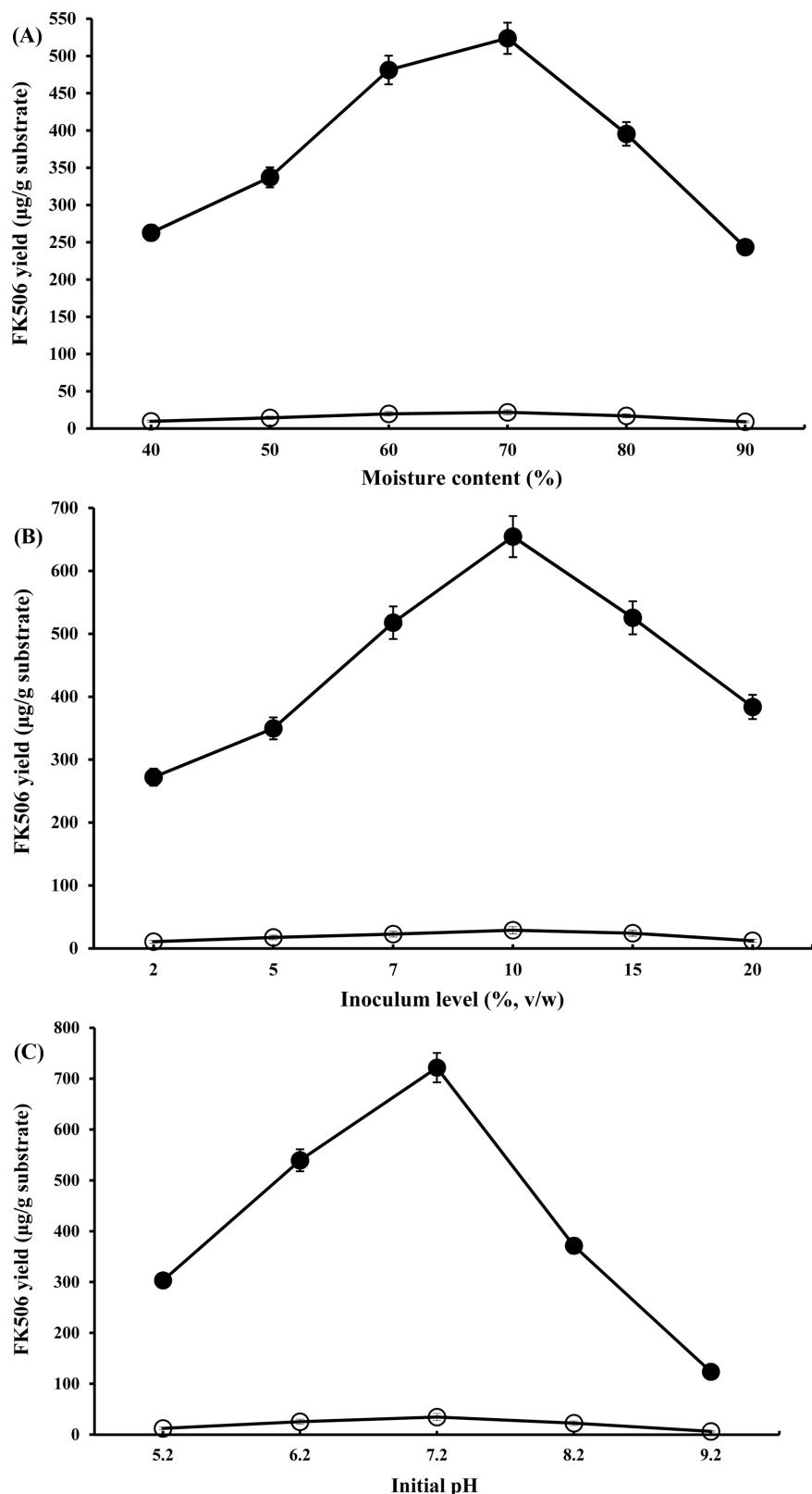
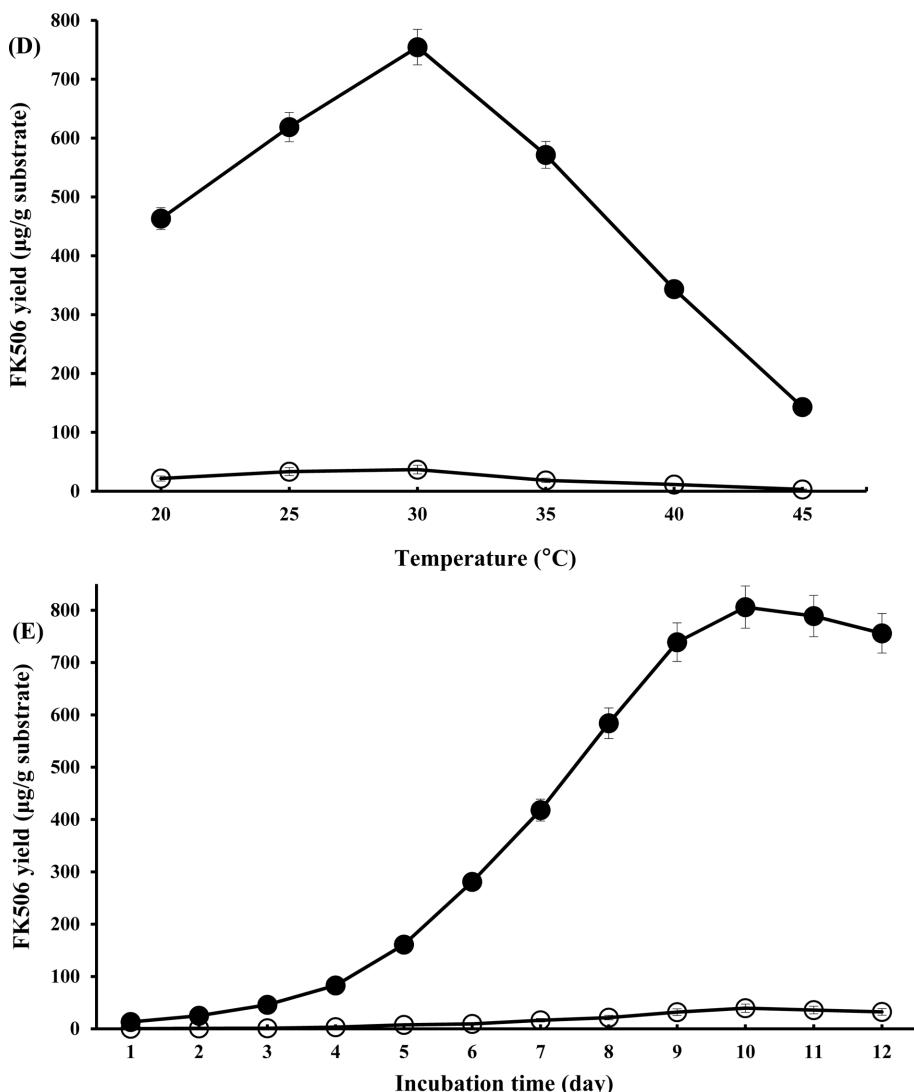


Fig. 3 Effects of various process parameters and additives on FK506 production by *S. tsukubaensis* NRRL 18488 and the TCM8594 strain under SSF with wheat bran as the substrate. The process parameters were: (A) Initial moisture content, (B) Inoculum level, (C) Inoculum level, (D) Incubation temperature, and (E) Incubation time. Solid circle, FK506 yield by TCM8594 strain; open circle, FK506 yield by *S. tsukubaensis* NRRL 18488

**Fig. 3** Continued

SSF between days 8–10 (Ellaiah et al. 2004; Khaliq et al. 2009). However, Kota and Sridhar (1998) reported that production of cephamycin C reached a maximum on day 15 in SSF. Lazim et al. (2010) also reported that oxytetracycline production reaches its maximal value on day 5. This result indicates that optimal production of secondary metabolites depend on the growth kinetics of the microbes.

Effect of additional nutrients

Although wheat bran can support FK506 production, it may not provide sufficient carbon source for maximum FK506 production by the TCM8594 strain. Hence, adding different carbon sources to the solid medium may enhance FK506 production. We tested the effects of adding glucose, lactose, maltose, mannose, sorbitol, sucrose, starch, and dextrin (5 %, w/w), and the results are shown in Fig. 4A. Maximum FK506 production (854.7 µg/g substrate) was achieved when 5 % (w/w) dextrin was added to the SSF

medium. Starch also increased FK506 yield slightly, whereas glucose, sucrose, lactose, maltose, mannose, and sorbitol decreased FK506 production. These results agree with those reported previously showing that starch and dextrin stimulate FK506 production (Martínez-Castro et al. 2013).

The influence of different organic nitrogen sources on FK506 production was also studied using wheat bran medium supplemented with dextrin, and the results are shown in Fig. 4B. Maximum FK506 yield (897.4 µg/g substrate) was obtained after adding yeast extract whereas a reduction of FK506 yield was observed when other nitrogen sources were added to the TCM8594 strain culture. This result agrees with that reported previously showing that yeast extract improves the yield of FK506 in *S. tacrolimicus* B3178 (Singh and Behera 2009). Under the optimum culture conditions, the TCM8594 finally yielded 897.4 µg per g of substrate. This yield is 2.3-fold higher than that obtained in before optimum culture condition (Fig 5A).

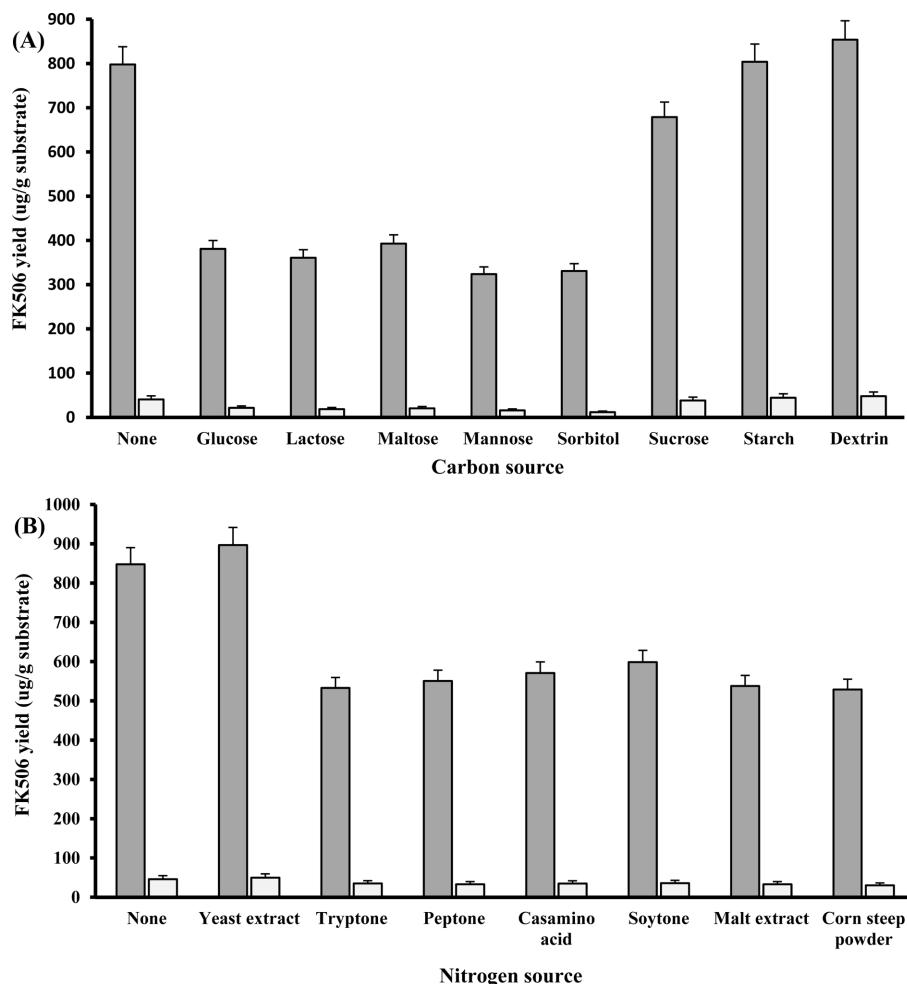


Fig. 4 Effect of various additives on FK506 production. (A) Effect of additional carbon sources in the presence of wheat bran on FK506 production by *S. tsukubaensis* NRRL 18488 and the TCM8594 strain during SSF. All carbon sources were added at 5 % (w/w). (B) Effect of additional organic nitrogen sources in the presence of wheat bran and dextrin on FK506 production by *S. tsukubaensis* NRRL 18488 and the TCM8594 strain during SSF. Dark grey, FK506 yield by TCM8594 strain; Light grey, FK506 yield by *S. tsukubaensis* NRRL 18488. Data are means \pm standard deviations of three independent experiments

Comparisons of antifungal activity of SmF and SSF

The antifungal activities of extracts against microorganisms were investigated in this study and their potency was evaluated by the diameter of presence inhibition zones. The effect of two different culture systems on FK506 titer against *A. niger* in TCM8594 strain is shown Fig. 5B. For comparison of the antifungal activity of FK506 by SSF and SmF, the dried SmF and SSF extracts were dissolved in methanol to give a final concentration of 100 mg/mL. The clear inhibitory zone of 15 and 20 mm against *A. niger* was observed for crude FK506 extracted from SmF and SSF. According to the results obtained, the final concentration of SmF and SSF extracts are adjusted similarly, however, extracts of SSF proved to have more antifungal activity against *A. niger* than extracts of SmF. This result showed that effect of the growth inhibition of *A. niger* by the crude extracts depended on fermentation process. Thus, it can be concluded that SSF was more efficient way to increase in the FK506 productivity and titer.

In conclusion, we optimized the SSF conditions to produce FK506 from a TCM8594 strain developed by random mutagenesis and FK506 sequential adaptation. The results clearly indicate that *S. tsukubaensis* TCM8594 can be successfully cultivated under SSF conditions to produce FK506 using wheat bran as the substrate. The maximum FK506 production (897.4 $\mu\text{g/g substrate}$) was accomplished by employing wheat bran with dextrin and yeast extract as additional nutrients and with optimized process parameters, such as 70 % (v/w) initial moisture content, initial pH 7.2, incubation temperature 30 °C, inoculum 10 % (v/w) cell culture, and incubation time of 10 days. Overall, FK506 production finally increased 234 % after optimizing the process parameters. In addition, antifungal activity shows that comparative advantage of FK506 production under SSF and SmF indicated better production with the SSF technique. Our results show for the first time that high yields of FK506 can be produced using SSF with an improved strain and optimized parameters, and that this is a

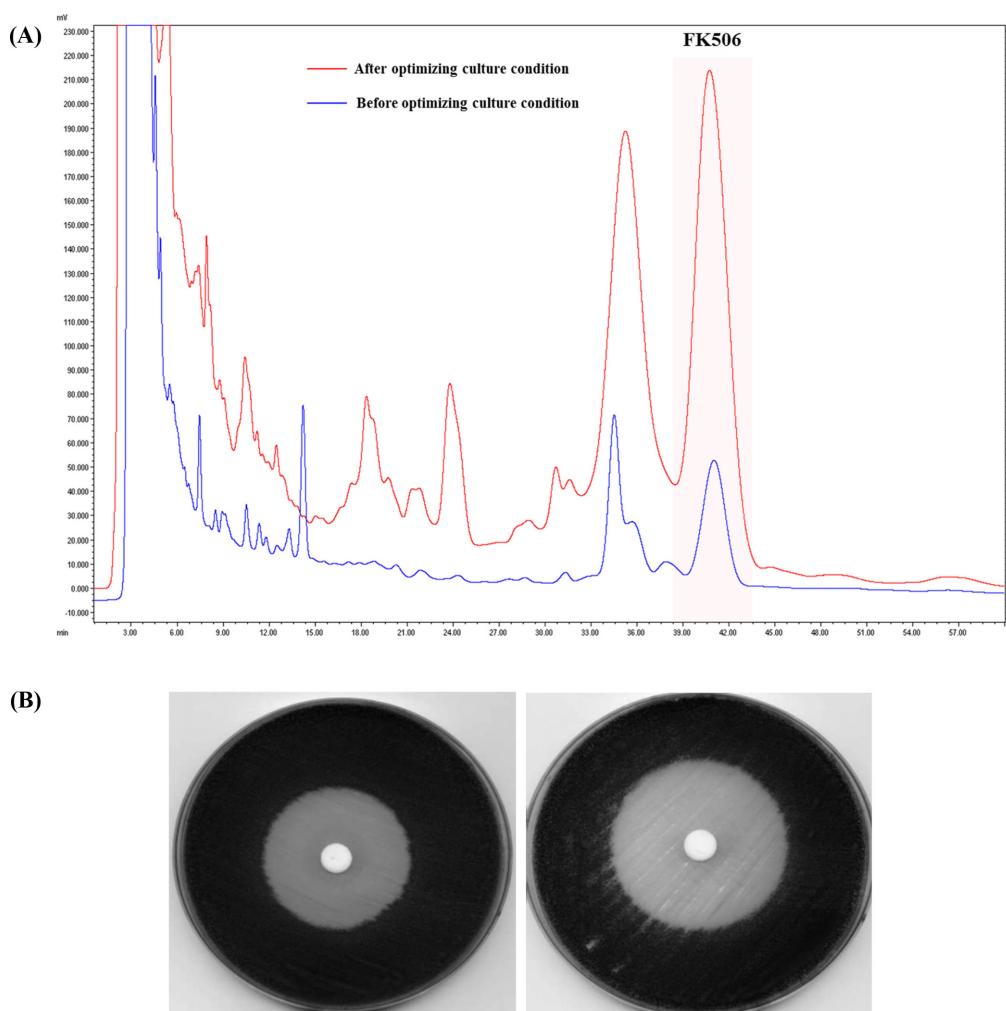


Fig. 5 HPLC trace of FK506 titer after optimizing culture condition and antifungal activity comparision of SmF and SSF. (A) HPLC analyses from methanol extracted solid medium from before optimizing culture condition (blue line) and after optimizing culture condition (red line). The shade indicates the identity of FK506 (in red). Extracted samples were loaded onto a Discovery HS C18 column (Supelco Analytical, USA), which was maintained at 50 °C. The linear elution gradient from 20 to 100 % acetonitrile, the flow rate was 1 mL/min, and detection was carried out with a UV detector at 205 nm. (B) Antifungal activities of submerged culture (left) and solid culture (right) extracts of TCM8594 were examined against *A. niger* by paper disc diffusion assay. Equal amounts of crude extracts were impregnated and observed for zone of inhibition after 2

good alternative technology to SmF.

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