

Improving Production of Tacrolimus In *Streptomyces Tacrolimicus* (ATCC 55098) Through Development of Novel Mutant by Dual Mutagenesis

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ABSTRACT

Tacrolimus is a polyketide macrolide produced by *Streptomyces* species which is widely used as anti-fibrotic agent and potent immunosuppressant. In this article dual mutagenesis approach using mutagens (NTG+EMS+UV) was used to develop a mutant strain of *Streptomyces tacrolimicus* (ATCC 55098) for higher tacrolimus production and this strain showed higher tacrolimus production at 82.5 mg/l. Interestingly; addition of L-Lysine (0.2 g/l) into the production medium further enhanced the tacrolimus production to ~102 mg/l at 7-L fed-batch bioreactor. To the best of our knowledge this is the first report mentioning efficient strain development for higher production of tacrolimus using dual mutagenesis. The obtained data presents an impressive model for higher production of tacrolimus and enhanced our understanding regarding improvement in production capacity of tacrolimus in *Streptomyces tacrolimicus*.

Key words: Immunosuppressant, Macrolide polyketide, Tacrolimus, Mutagenesis

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INTRODUCTION

Polyketides are a large and highly diverse group of natural products reported as antibiotic, antitumor and immunosuppressants. Tacrolimus (FK-506 or Fujimycin) is reported immunosuppressant and 822-Da polyketide extracted from the fermentation broth of *Streptomyces*¹. It is recommended to control rejection of organ transplants like kidney, heart and liver transplantation by reducing activity of the patient's immune system². It is reported act on T-cells and reduces the IL-2 production by T-cells¹. It is also used for inflammatory skin diseases². Tacrolimus is approximately 100-fold more potent than the structurally unrelated immunosuppressive compound CsA while having similar mechanism of action of inhibiting cell-mediated and humoral immune responses^{1,3,4}.

Macrocyclic polyketides are predominantly produced by *Streptomyces* and related filamentous bacteria, through the action of so-called type I modular PKS, multienzymes in which different sets (modules) of enzymatic activities catalyze each successive round of elongation⁵. A significant progress had been made in elucidation of tacrolimus biosynthesis. Researchers have findout gene cluster involved in tacrolimus biosynthesis, submitted draft genome of *Streptomyces tsukubaensis* and reported enhancement in production of tacrolimus by genetic manipulations^{5,6,7}. Including this insvestigators have studied the genetic mechinary involved in biosynthesis of tacrolimus and their regulation⁸.

Improvement in productivity of bacterial strain depends on isolation of high productivity strain, optimization of fermentation conditions, mutagenesis and metabolic engineering^{9,10,11}. Scientists have employed approaches such as development of novel strains by metabolic engineering and optimization of fermentation processes parameters for efficient and economical production of tacrolimus. Kim and Park, reported isolation of a novel tacrolimus-producing microorganism, *S. clavuligerus* CKD1119, from soil samples in Korea and this strain produced 58 mg/l tacrolimus in a 7-L jar fermenter¹². High tacrolimus yielding (500 mg/l) *Streptomyces* strain development has been reported by sequential adaptation and tacrolimus resistance method¹³. Supplementation of biosynthetic presursors^{14,15}, fermentation conditions optimization¹⁶, metabolic network guided engineering¹⁷, random mutagenesis and metabolic engineering^{18,19} are approaches used by researchers to enhance the production of tacrolimus in *Streptomyce tsukubaensis* and reported significant enhancement in tacrolimus yield.

Keeping aforesaid facts in view, author performed dual mutagenesis (random mutagenesis) in *Streptomyces tacrolimicus* ATCC 55098 to develop a high yielding strain for tacrolimus production and this is an innovative and productive scheme which could be immensely helpful for over production of tacrolimus at commercial level.

MATERIALS AND METHODS

Chemicals and Bacterial Culture Conditions

All the chemicals used were of analytical grade. EMS, NTG, lactose, glucose, yeast extract, peptone, NaCl, FeSO₄, KNO₃, acetone, acetonitrile, CaCl₂ and MgSO₄ and agar-type I were purchased from Hi-Media (Mumbai) Sigma, RFCL (India), SD Fine Chemicals (Mumbai), India. Tacrolimus standard was purchased from Sigma. *S. tacrolimicus* ATCC 55098 (Ref. no. 20) was obtained from American Type Culture Collection Centre. The bacterial culture was maintained on slants and petri plates containing M1 medium enriched with 4 g/l glucose, 10 g/l, malt extract, 2 g/l peptone and 17 g/l agar having pH 7.3, and finally incubated at 28°C.

The 24 h old seed culture was used as inoculum, and 10% of medium volume was used as inoculum for inoculation of production medium having the composition of 35 g/l lactose, 11.25 g/l peptone, 0.477 g/l FeSO₄, 0.257 g/l MgSO₄, 10 g/l glucose, 10 g/l yeast extract, 1 g/l NaCl, 0.8 g/l CaCl₂ and 0.5 g/l KNO₃, and incubated at 28°C on a temperature controlled orbital shaker at 220 rpm for 120 h in a 500 ml Erlenmeyer baffled flask. Various amino acids used were filter-sterilized by membrane filter (0.22 µm, Millipore) and added after 28 h of inoculation.

Mutagenesis

Stock solutions (10 mM each) of EMS and NTG were prepared in phosphate buffer (pH 9.0). Spores were separated by passing the broth of a known cfu/ml through sterile cotton packed in a sterile column. Spores of wild-type were suspended in phosphate buffer (pH 7.0) and divided into aliquots of 0.1 ml containing 5x10⁶ spores. EMS and NTG were added to the spore suspension at concentrations of 50 µM, 100 µM, 300 µM, 500 µM, 800 µM, and 1000 µM, and incubated for 15 min in shake flasks at 28°C. The mutagenesis was terminated by diluting the spore suspensions with 0.16 M sodium thiosulfate^{21,22} and centrifuged at 3000xg for 5 min. The residue containing spores were washed twice with phosphate buffer having pH 7.0. The process was repeated and applied for other samples of diluted spore suspensions and the respective spore suspensions (20 µl) were spread into Petri dishes containing culture medium containing 4 g/l glucose, 10 g/l malt extract, 2 g/l peptone, 2% agar at pH-7.3.

The bacteria were cultivated in shake flask culture medium containing 4 g/l glucose, 10 g/l malt extract, 2 g/l peptone having pH 7.3 at 28°C for 72 h. The bacterial culture was diluted till OD₆₀₀ reached to 0.1, and 0.1 ml of diluted culture was streaked on GMP agar plates containing culture medium of 4 g/l glucose, 10 g/l malt extract, 2 g/l peptone, 2% agar with pH 7.3. The bacterial culture plates were then exposed to UV light (UV light source: TFP 20 M UV screen, 213 nm from Vilber Lourmat, France). The radiation (UV exposure time and intensity) led killing rate was found between 50% and 99%. The bacterial cells mutagenized by UV exposure were incubated at 28°C for 5 days, until colonies were visible. Well separated colonies were selected and transferred to the slants for incubation at 28°C for 5 days. Following the 5 days bacterial growth, a spore suspension was prepared as described earlier and an inoculum containing 10⁶ spores was added to each of 500 ml Erlenmeyer baffled flasks containing 100ml production medium. The flasks were incubated at 28°C on temperature controlled orbital shaker (Orbitek, Scigenics Biotech) for 5 days at 220 rpm. Afterwards, the culture broth was processed for tacrolimus quantification.

Transmission Electron Microscopy (TEM)

Control *S. tacrolimicus* ATCC55098 and newly developed mutant strain were grown and their cultures were harvested at stationary phase by centrifugation at 5000 rpm for 10 min. Bacterial samples were fixed in modified Karnovsky's fluid²³, buffered with 0.1 M sodium phosphate buffer at pH 7.4. The fixation step was performed for 10 - 18 h at 4°C temperature, afterwards the tissues were washed 2-3 times with fresh buffer, and after that it was post-fixed in 1% osmium tetroxide in the same buffer at 4°C for 2 h. After several washes in 0.1 M sodium phosphate buffer, the specimens were dehydrated in graded acetone solution and embedded in CY 212 araldite. Ultra thin sections of 60 - 80 nm thickness were cut by using an ultra cut E (Reichert Jung) Ultra microtome, and the sections were stained in alcoholic uranyl acetate and lead citrate for 10 min each, before proceeding for examining the grids in a transmission electron microscope (Philips, CM-100) operated at 60 - 80 kV. The

TEM studies were performed at Electron Microscopy facility of All India Institute of Medical Sciences (AIIMS), New Delhi, India.

Extraction and Quantification of Tacrolimus

The pH of the bacterial culture broth was adjusted to 10 and mixed with equal volume of acetone, and incubated at 50°C for 2 h at 180 rpm. The flask containing the culture broth was allowed to settle for 1 h for proper separation. The supernatant collected from above was passed through a filter having Whatmann No. 1 filter paper. The solvent layer was collected and used for quantification of tacrolimus. Stock solution of tacrolimus standard (1 mg/ml) was prepared in acetone and diluted with acetone to 5, 10, 20, 40 and 50 µg/ml for calibration studies. The sample for HPLC analysis was prepared by diluting the tacrolimus acetone extract with acetone. HPLC analysis for tacrolimus quantification was performed with Shimadzu LC20 HPLC system. The HPLC system was equipped with UV detector, column oven and C18 column. During sample analysis 2.0 ml/min flow rate was maintained having column temperature of 50°C. Mixture of acetonitrile-water (60:40, v/v) was used as mobile phase and UV detector was monitored at 210 nm. An injection volume of 20 µl was used for sample analysis.

RESULTS AND DISCUSSION

Isolation and Screening of Mutants

There is sufficient scientific data supporting the enhanced production of metabolites using mutagenesis. UV mutagenesis has been reported to enhance production of clavulanic acid in *Streptomyces clavuligerus*²⁴. Chemical mutagens particularly NTG and EMS have been used successfully for various strain improvement of bacteria^{16,19,24,25} and fungi^{26,27} for variety of purposes. Among these two chemical mutagens NTG alone has been proved to be the most potent mutagen for *Streptomyces* spp. for enhanced production of macrolides^{17,28}.

Similarly, in this study, *S. tacrolimicus* ATCC55098 went through treatments of various mutagens (NTG, EMS and UV) with varying time and quantity. To optimize the quantity of mutagens, survival rate and LD₅₀ values were calculated, and it was found that at ~100 µM of NTG and ~150 µM EMS concentration survival rate was 50% and time of UV treatment was ~3 min, for 50% survival (data not shown). Screening of various mutants was performed on the basis of tacrolimus yield, and it was noticed that control *S. tacrolimicus* ATCC55098 was able to produce 10.5 mg/l of tacrolimus, whereas different mutants like, 3 min UV treated mutant produced 33±1.96 mg/l, 100 µM NTG treated mutant produced 45±2.34 mg/l, 100 µM EMS treated mutant produced 41±2.10 mg/l. Newly developed NTG mutant was further subjected to strain improvement by combined treatment of EMS and UV for dual mutation. The combined treatment of chemical and physical mutagens (dual mutation) caused significant increase in tacrolimus production as compared to the previously isolated single mutant of NTG+EMS+UV (physical and chemical mutagen treated dual mutant) treated mutant produced maximum amount of tacrolimus as 82.5±3.53 mg/l, however NTG+UV treated mutant produced 65.8±2.68 mg/l and NTG+EMS treated mutant produced 50.4±2.78 mg/l (Table 1).

Table 1. Specific growth rate and tacrolimus production of control and various mutant strains of *S. tacrolimicus* ATCC 55098 at shake flask level

Mutagen treatment	μ_{\max} (/h)	$Y_{p/x}$ mg/g	Tacrolimus production (mg/l)	Productivity (g/l/h)
Control	0.047	0.47±0.02	10.5±1.63	0.09
A	0.042	1.53±0.05	33±1.96	0.28
B	0.038	2.11±0.08	45±2.34	0.38
C	0.041	1.74±0.05	41±2.10	0.34
D	0.039	3.34±0.07	65.8±2.68	0.55
E	0.031	2.57±0.11	50.4±2.78	0.42
F	0.043	4.46±0.14	82.5±3.53	0.69

S. tacrolimicus ATCC55098 used as Control; and A= 15 min UV treated; B= 100 μ M NTG treated; C= 100 μ M EMS treated; D= NTG+UV treated; E= NTG +EMS treated; F= NTG+EMS+UV treated *S. tacrolimicus* ATCC55098. Experiments were carried in triplicate and each data point is mean value±SD.

Note 1. Culture conditions were same as mentioned in materials and method section.

Note 2. The data had been taken after 5 days of culture when the bacteria attended the steady state of growth.

Morphological Comparison of Control and Mutant Cells

The developed mutant was compared for probable changes in their morphological characters. Fig. 1 shows the morphological changes occur in control *S. tacrolimicus* ATCC55098 cells after exposing to UV, NTG and combination of physico-chemical mutagens (UV+EMS+NTG).

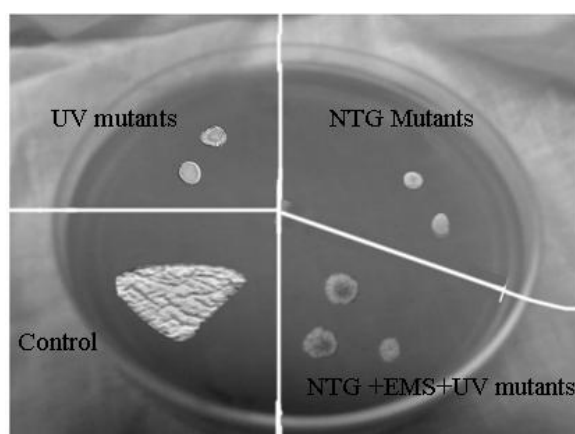


Fig. 1. Morphological changes observed in control and in mutagen treated *S. tacrolimicus* ATCC. **Control:** colony size $975\pm 4.65\mu\text{M}$; margin of colony is smooth; color of colony is white. **UV mutants:** colony size $3850\pm 9.82\mu\text{M}$; margin of colony is thick; color of colony is Off-gray. **NTG mutants:** colony size $2730\pm 5.75\mu\text{M}$; margin of colony is irregular; color of colony is Off-gray with fuzz at center. **NTG+EMS+UV mutants:** mutants colony size $2730\pm 5.75\mu\text{M}$; margin of colony is irregular wavy; color of colony is Off-blackish with fuzz at center.

At Macroscopic Level

Fig. 1 showed the bacterial colonies were rounded, smooth and milky white in control cells. Significant morphological differences were noticed in the bacterial colonies exposed to various mutagens. Control *S. tacrolimicus* ATCC55098 cells showed white colonies having diameter of $975\pm 4.65\mu\text{M}$ with smooth margins whereas colonies of UV treated *Streptomyces* mutants were off-white in colour with thick margins showing diameter of $3850\pm 9.82\mu\text{M}$. Similarly, the colonies of NTG treated mutants were off-gray in colour and fuzz at the center with irregular margin having colony diameter of $2730\pm 5.75\mu\text{M}$. Colonies of EMS treated mutant were grey in colour with irregular margins having diameter of $1575\pm 5.43\mu\text{M}$, colonies of dual mutants showed irregular and wavy margins with off blackish colour with fuzz

at center having diameter of $5510 \pm 11.57 \mu\text{M}$. The NTG and EMS exposed colonies were off white having blackish fuzz at the centre of the colony and the size of these colonies were noticed to be bigger as compared to control *S. tacrolimicus* ATCC55098 (Fig. 1). The UV exposed colonies were noticed as blackish with wavy margins. The colonies of control *S. tacrolimicus* ATCC55098 cells exposed to combination of mutagens (NTG+EMS+UV radiation) were observed to be significantly different into their morphological structure from the colonies of other individual mutants. Prominent dark fuzz was noticed at the centre of each colony of combined mutagen treated (NTG+EMS+UV) *S. tacrolimicus* ATCC55098 (Fig. 1) and the colony size was significantly bigger ($5510 \pm 11.57 \mu\text{M}$) than that of control ($975 \pm 4.65 \mu\text{M}$) *S. tacrolimicus* ATCC55098.

At Microscopic Level

Fig. 2 showed the characters of the fine structure of the cell wall of the control *S. tacrolimicus* ATCC55098 and mutant strain. Under controlled growth conditions, the shape of the cell wall appear to be long filamentous having $736.18 \pm 0.274 \text{ nm}$ and $794.28 \pm 0.208 \text{ nm}$ breadth. A significant increase in cell wall thickness was noticed in mutant cells which results into greater black color pigmentation in the bacterial cell wall and ultimate reflection into appearance of black color bacterial colonies.

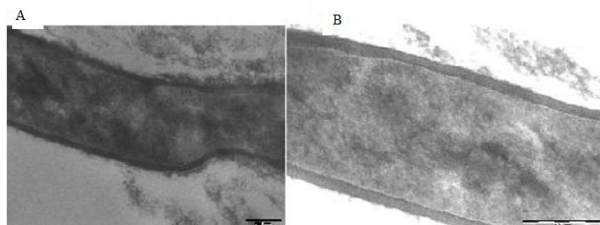


Fig. 2: Electron micrograph of *S. tacrolimicus* ATCC 55098. Cell size $736.18 \pm 0.274 \text{ nm}$; Cell wall $33.33 \pm 0.019 \mu\text{m}$; Pigmentation in cell wall: No pigmentation (A) and *Streptomyces* spp. ATCC 55098 Cell size $794.28 \pm 0.208 \text{ nm}$; Cell wall $48.44 \pm 0.094 \mu\text{m}$; Pigmentation in cell wall Black pigmented (B) vegetative cells).

Optimization of Growth and Yield in Batch Culture

Maximum production of tacrolimus ($\sim 102 \text{ mg/l}$) was obtained after 120 h of culture with dual mutant strain having lesser biomass than the control strain. Synthesis of tacrolimus was initiated after 40 h of bacterial growth in broth, attained just before completion of log phase in control strain, whereas, in case of dual mutant strain, tacrolimus production was noticed after 32 h.

Fed-Batch Culture in 7-L Bioreactor

Effect of Amino Acid (L-Lysine) Addition on Tacrolimus Yield

In our earlier report²⁶, we have tested the effect of various amino acids on tacrolimus yield; among the various amino acids tested to the culture broth of all developed mutant strains of *S. tacrolimicus* ATCC 55098, 0.2 g/l L-Lysine was proved to be helpful in tacrolimus over-production. Exogenous addition of L-Lysine (0.2 g/l) into the bacterial production medium surprisingly resulted in significant increase in tacrolimus yield to (102 mg/l) in comparison to control (12.5 mg/l) strain (Table 2).

Table 2. Testing of various developed mutants when they were grown into fed batch conditions and studying the tacrolimus yield at shake flask level

Mutagen treatment	Tacrolimus yield in fed Batch culture (mg/l)		
	L-Lysine ^a	Shikimic acid ^b	L-Lysine and shikimic acid combined ^c
Control	12.5±1.36	25±0.91	27±1.21
A	41±1.50	56±1.24	58±1.52
B	45±1.32	42±1.19	43±2.06
C	81±1.12	74±1.56	69±1.24
D	51±1.42	67±2.12	64±0.98
E	64±2.23	72±1.65	70±1.67
F	102±1.31	98.5±1.60	101±1.61

Experiments were carried in triplicate and each data point is mean value±SD.

^aAfter 28 h of incubation filter sterilized 0.2 g/l L-lysine was added

^b2 g/l shikimic acid was added during medium preparation

^c0.2 g/l L-lysine + 2 g/l shikimic acid were added in same flask as per time mentioned in above conditions

Effect of Shikimic Acid Addition on Tacrolimus Yield

Shikimic acid (2 g/l) was added into the culture broth of control, NTG treated and dual mutated strains, after 36 h of inoculation. Effect of shikimic acid addition on tacrolimus production has been shown in Table 2. Addition of shikimic acid in the production medium of control culture resulted into 2.5 folds increase in tacrolimus production in comparison with control growing in production medium without addition of shikimic acid. However, mutant strains i.e., NTG treated, EMS treated, UV treated and combined all three mutagen treated did not showed significant response in tacrolimus production with shikimic acid addition into culture medium. These results were confirmed by shikimic acid quantification, and it showed that the amount of shikimic acid present in control strain was nearly one fourth of the amounts present in *S. tacrolimicus* ATCC55098 and *S. tacrolimicus* ATCC55098 mutants. Hence, obtained data depicts an impressive model for the regulation of tacrolimus production (Fig. 3).

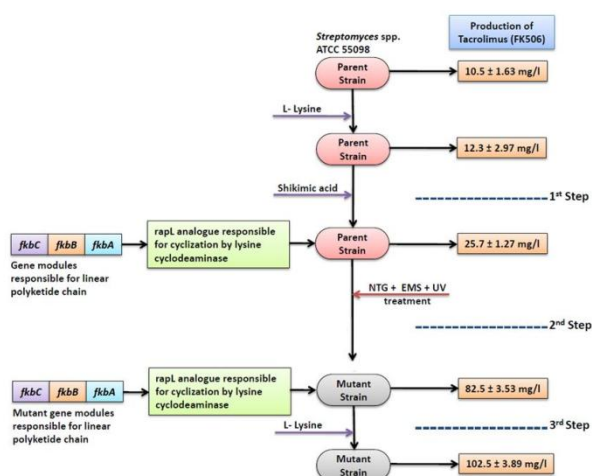


Fig. 3: Showing the metabolic regulation of tacrolimus production in mutant strain of *S. tacrolimicus* (ATCC 55098) B3178 possibly via mutation in *fkBC*, *fkBB* and *fkBA* genes which are responsible for linear polyketide chain formation.

Feeding of L-Lysine and shikimic acid at 28 h showed increase in tacrolimus yield and the probable reason for this might be because of inability of cell membrane of mutant strains to permit shikimic acid into the bacterial cell. The electron

microscopic studies showed that the cell wall thickness of mutant strains was significantly greater than the control strain (Fig. 2). The possible increase in compactness and thickness of mutant bacterial cell wall may work as a strong barrier for shikimic acid intake; hence its addition in the mutant strain's culture medium doesn't affect the tacrolimus production.

On the basis of aforesaid facts it can be hypothesized that rapL gene which encodes for lysine cyclodeaminase putative enzyme is suggested responsible for cyclization of linear chain of hydrocarbons^{29,30} is not affected by physical and chemical mutagens used in this strain improvement study. Hence, it can be predicted that simultaneous treatment of *Streptomyces* cells with chemical and physical mutagens caused mutation among the cluster of genes i.e., *fkfB*, *fkfC* and *fkfA* (Fig. 3) involved in the formation of linear polyketide chain^{7,8}. On the basis of results obtained in the above discussed study, it can be proposed that the over production of tacrolimus can be regulated by mediating three steps (Fig. 3):

- i) In the first step, addition of shikimic acid in the culture broth of mutant strain at the beginning of the scale-up process is not required, as practiced earlier³¹. As already stated, the shikimic acid is not permeable through the cell wall of the mutant strain; hence, depriving the bacterial culture broth from shikimic acid could be helpful in reducing the cost of tacrolimus production.
- ii) In the second step, novel and potent mutant strains having high potential for tacrolimus can be produced by combined treatment of both, physical and chemical mutagens.
- iii) In the third step, the novel mutant obtained after second regulatory step can be commercialized through metabolic regulation by exogenous supply of L-Lysine before the culture broth attend the idiophase.

CONCLUSIONS

Researchers have reported the enhanced yield of metabolites in *Streptomyces* spp.^{7,9,16,19,24,25,31} and other bacterial species³² using optimization of process conditions and strain improvement methods. In this study results showed that NTG treated *S. tacrolimicus* ATCC55098 mutants produced higher tacrolimus in comparison to EMS or UV treated mutants. Additionally, it was also observed that combined treatment of NTG, EMS and UV for strain improvement cause significant increase in tacrolimus production. By following the proposed metabolic regulation process (Fig. 3), further enhancement in production of tacrolimus may be obtained. This study also proposes an impressive model (Fig. 3, Table 3) of metabolic regulation for enhanced tacrolimus production through newly developed dual mutant of *Streptomyces tacrolimicus* ATCC55098 and this model might be helpful to obtain higher production of tacrolimus.

Table 3. Combined (dual) mutant of *S. tacrolimicus* ATCC 55098 tested in different culture systems at 7-L bioreactor level

Parameters	^a Batch culture	^b Fed-Batch culture
μ_{max} (/h)	0.068	0.058
Dry cell weight g/l	16.4±0.51	15.8±0.62
Tacrolimus yield (mg/l)	90±1.32	110±1.57
Productivity (g /l/h)	0.75	0.91
Fermentation time (h)	120±1	120±1.2

Experiments were carried in triplicate and each data point is mean value±SD.

^aBatch conditions: dissolved oxygen 40%, agitation 450 rpm.

^bFed-batch conditions: after 28 h of incubation 0.2 g/l L-lysine and 2 g/l shikimic acid was fed and other conditions were similar as batch culture experiment.

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