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ORIGINAL PAPER

Stereoselective epoxidation of *cis*-propenylphosphonic acid to fosfomycin by a newly isolated bacterium *Bacillus simplex* strain S101

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Abstract In industry, fosfomycin is mainly prepared via chemical epoxidation of *cis*-propenylphosphonic acid (cPPA). The conversion yield of fosfomycin is less than 50% in the whole process and a large quantity of waste is produced. Biotransformation by microorganisms is an alternative method of preparation. This kind of conversion is more delicate, environmentally friendly, and the conversion yield of fosfomycin would be higher. In this work, an aerobic bacterium capable of transforming cPPA to fosfomycin was isolated. The organism, designated as strain S101, was identified as *Bacillus simplex* by morphological and physiological characteristics as well as by analysis of the gene encoding the 16S rRNA. Fosfomycin was assayed by two means, bioassay and gas chromatography (GC). Glycerol was a good carbon source for growth and cPPA conversion of strain S101. When cPPA was used as the sole carbon source, neither growth nor conversion to fosfomycin occurred. The optimum cPPA concentration in the conversion medium was 2,000 μg ml^{-1}. After 6 days of incubation, the concentration of fosfomycin reached its maximum level (1,838.2 μg ml^{-1}), with a conversion ratio of 81.3%. Air was indispensable for the growth but not for the conversion to fosfomycin. Furthermore, vanadium ions were found to be essential for the conversion. High concentrations of cPPA had fewer inhibitory effects on the growth of strain S101.

Keywords *Bacillus simplex* strain S101 · *cis*-Propenylphosphonic acid · Fosfomycin · Transformation · Gas chromatography

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Introduction

Fosfomycin is a low molecular weight natural product as a secondary metabolite of many strains of *Streptomyces* [9] and *Pseudomonas* [25]. Structurally, fosfomycin is characterized by both an epoxide ring and a highly stable, covalent carbon-phosphorus bond which is resistant to acid and base hydrolysis, thermolysis, photolysis, and phosphotransferase-catalyzed cleavage [18].

As a phosphoenolpyruvate analogue antibiotic, fosfomycin irreversibly inhibits phosphoenolpyruvate UDP-N-acetylglucosamine-3-O-enolpyruvyltransferase (MurA), an enzyme that catalyzes the first step of peptidoglycan biosynthesis [14]. In addition, fosfomycin exhibits a broad spectrum of antimicrobial activity against gram-negative and gram-positive bacteria [20] and, as a result, has become the first choice for the treatment of certain types of infections [3, 4, 8, 26].

Presently, the worldwide increase in microbial resistance to antibiotics poses a genuine threat to human health. Therefore, the discovery of effective treatments against resistant pathogens is urgently required. Favorably, fosfomycin has been proven effective for the treatment of cephalosporin-resistant *Streptococcus pneumoniae* [21],

and is likewise effective against methicillin-resistant and vancomycin-resistant strains of *Staphylococcus aureus* [2, 11] and *Enterococcus faecium* [5].

For the large-scale production of fosfomycin, preparation is mainly by means of epoxidation of *cis*-propenylphosphonic acid (cPPA), which gives a racemic mixture of fosfomycin and its inactive isomer, (+)-(1R, 2S)-1, 2-epoxypropylphosphonic acid. This is then followed by an optical resolution of the racemic epoxide with phenethylamine. As a result, the yield of fosfomycin becomes less than 20% in the whole process [13]. In addition, a large quantity of waste is produced, which poses a threat to the environment. Consequently, alternative means of converting cPPA to fosfomycin are necessary.

Since several strains of *Penicillium* were initially found to be capable of transforming cPPA to fosfomycin (Fig. 1) [30], a number of microorganisms that can catalyze this kind of epoxidation have been reported [1, 13, 29].

In this article, we report the newly isolated *B. simplex* strain S101 that can transform cPPA to fosfomycin in the presence of a relatively high concentration of cPPA in the medium.

Materials and methods

Chemicals

Fosfomycin was acquired from the National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China (NICPBP), and cPPA was acquired from the Northeast General Pharmaceutical Factory (China). Bis-trimethylsilylacetamide (BSA) and trimethylchlorosilane (TMCS) were acquired from Merck (Darmstadt, Germany). All other chemicals used in these experiments were of analytical grade.

Microorganisms

Bacillus simplex strain S101 was isolated by our laboratory from a mountain soil, using the screening medium described below. It was maintained at 4°C under lyophilized condition until use. *Proteus vulgaris* FI-7, which was used as an indicator strain in the microbiological assay, was acquired from the China General Microbiological Culture Collection Center (CGMCC).

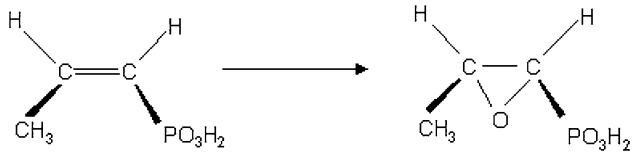

Fig. 1 Transformation of cPPA to fosfomycin

Medium

The medium for the growth of *P. vulgaris* FI-7 consisted of the following: tryptone, 5 g l^{-1}; yeast extract, 3 g l^{-1}; beef extract, 2 g l^{-1}; and agar, 15 g l^{-1}. Natural pH.

The screening and conversion medium consisted of the following: glycerol, 50 g l^{-1}; tryptone, 10 g l^{-1}; $Na_3VO_4 \cdot 12H_2O$, 0.1 g l^{-1}; $CoCl_2 \cdot 6H_2O$, 0.5 g l^{-1}; $MgSO_4 \cdot 7H_2O$, 1.5 g l^{-1}. The final concentration of 250 μg ml^{-1} cPPA was added for the screening medium and 2,000 μg ml^{-1} for the conversion medium. The pH was adjusted to 8.5 with 5 M NaOH.

Culture condition

A loop of *B. simplex* strain S101 was inoculated into 50 ml of Luria-Bertani (LB) medium (1% tryptone; 0.5% yeast extract; 1% NaCl, pH 7.0) in a 250-ml Erlenmeyer flask, which was subsequently aerobically cultured at 30°C for 24 h. Then, 4 ml of the cultured broth was inoculated to the conversion medium and incubated at 30°C on an orbital platform shaker at 200*g* for various periods of time.

Cell growth assay

Cell growth was monitored by measuring the increase in optical density at 600 nm (OD600) of the culture broth using a Hitachi 150-20 spectrophotometer.

Transforming with resting cells in anaerobic environment

B. simplex strain S101 was grown in the conversion medium minus cPPA. When the OD600 reached 4.0, cultures were aseptically centrifuged (10,000*g*, 10 min, 4°C). The supernatants were removed and the cell pellets were resuspended in the conversion medium minus cPPA to an OD600 nm of 9.0. 10 ml of the cell suspension was added to a screw-cap airproof tube, and cPPA was provided at a final concentration of 2,000 μg ml^{-1}. The tube was immediately degassed and filled with nitrogen to drive away the molecular oxygen, and incubated at 30°C for 24 h, then assayed for fosfomycin.

16S rDNA analysis

The 16S rDNA of strain S101 was amplified by PCR. Two universal 16S rDNA primers were used for the 30-cycle amplification PCR: 5′f (5′-AGAGTTTGATCCTGGCTC

AG-3′), which corresponds to positions 8–27 of *Escherichia coli* 16S rDNA and 3′r (5′-TACGGCTACCTTGT TACGAC-3′), which corresponds to positions 1479–1498 of *E. coli* 16S rDNA [31]. The 16S rDNA products were cloned into pGEMT vectors and sequenced on an Applied Biosystems (Foster, Calif., USA) Automatic Sequencer. The 16S rDNA sequences were aligned and compared with sequences deposited in the GenBank database using the BLAST program.

Assay of fosfomycin

Two approaches were followed for the identification of fosfomycin:

1. Microbiological assay [13]. A 10-µl aliquot sample of the supernatant of the culture broth was applied to a sterile paper disc (6 mm). After drying under natural conditions, the disc was placed upon an agar plate seeded with *P. vulgaris* FI-7. This was cultured at 30°C for approximately 6 h. The size of the inhibition circle indicated the amount of fosfomycin present.
2. Gas chromatography (GC) according to Dios-Viéitez [6]. Briefly, a 100-µl sample of the supernatant of the culture broth was lyophilized or evaporated to dryness under a gentle stream of nitrogen. Then, 100 µl of silylating mixture (containing 47.5 µl BSA, 47.5 µl dichloromethane and 5 µl TMCS) was added to the residue for the silylation of fosfomycin, thoroughly mixed, and maintained for 15 min at 60°C. A 1-µl aliquot of the silylated solution was applied to the gas chromatograph.

Gas chromatographic analysis was performed using a Shimadzu GC-2010, which was coupled to a flame ionization detector (FID) equipped with a capillary DB-5 column (25 m length, 0.25 mm i.d., 0.25 µm film thickness; J&W Scientific, Folsom, CA).

The oven temperature program used in the analysis had an initial temperature of 105°C that was held for 3 min, and then ramped at 0.8°C min^{-1} to 115°C, and held for 5 min. Nitrogen was used as the carrier gas with a flow rate of 30 ml min^{-1}. The injection port and detector temperatures were both 250°C. This chromatography method was also used for monitoring the depletion of cPPA in the culture medium.

Fosfomycin in the culture broth was further confirmed by GC/MS. After silylation, a 1-µl aliquot of the solution was applied to a Shimadzu GCMS-QP2010 equipped with a DB-5 capillary column. The oven temperature was programmed similarly to that of the gas chromatography assay.

Results

Isolation and identification of *B. simplex* strain S101

During the course of the screening, the attempt to isolate cPPA-converting bacteria using the medium containing cPPA as the sole carbon source was unsuccessful under aerobic conditions due to the poor growth of the microorganisms in the culture mediums. Therefore, enrichment cultivation was performed at 30°C in the screening medium. Bacteria with the ability of converting cPPA to fosfomycin were isolated from the enrichment culture. Among the isolated bacteria, strain S101 was selected as having the most efficient ability to convert cPPA to fosfomycin. Strain S101 tested gram-positive and strictly aerobic. Its spores were oval and located subterminally to terminally. There was no accumulation of poly-β-hydroxybutyrate (PHB) in the cells. Its reaction to the Voges-Proskauer test was negative. The results of catalase, oxidase, and amylase tests were positive, and lipase and lecithinase tests were negative. It also had the ability to reduce nitrate. Due to the morphological and physiological characteristics of strain S101, we supposed that it belonged to the genus *Bacillus*. To ensure the genetic background of strain S101, the gene encoding the 16S rRNA was partially amplified by PCR and its nucleotide sequence was determined. The 16S rRNA gene sequence determined for strain S101 was 1452 nucleotides long, and was deposited in GenBank under the accession number FJ176468.

Furthermore, the 16S rRNA gene sequence of strain S101 was aligned automatically using the CLUSTAL X program (version 1.83) [28] to reference sequences of the genus *Bacillus* obtained from the GenBank, and a phylogenetic tree was constructed based on the neighbor-joining method using the software package MEGA (Molecular Evolutionary Genetics Analysis) version 4.1 [27]. The tree (Fig. 2) indicates that strain S101 was most closely related

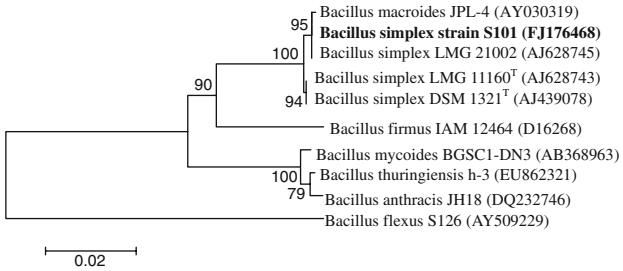

Fig. 2 Phylogenetic tree drawn from neighbour-joining analysis of 16S rRNA gene sequences, depicting the relationship of strain S101 with respect to closely related taxa. Bootstrap percentages above 50% (from 1,000 bootstrap replicates) are shown. Reference sequences were retrieved from GenBank under the accession numbers indicated. *Scale bar* 0.02 substitutions per nucleotide position

to *B. simplex* LMG21002. Therefore, strain S101 was tentatively referred to as *B. simplex* strain S101.

Identification of fosfomycin

After being cultured in the transformation medium for approximate 4 days, the supernatant from the culture broth was examined for biological activity by disc assay and subjected to GC and GC/MS as described in "Materials and methods". In the microbiological assay, an inhibition zone appeared around the filter paper disc indicating that there was a substance in the culture broth that could inhibit the growth of *P. vulgaris* FI-7. In the GC analysis, a peak with a retention time of 11.65 min had the same retention time as commercial fosfomycin (Fig. 3). The peak with retention time of 11.65 min had identical mass spectrum profiles to the standard, and the major fragment ions had m/e values of 267, 226, 211, and 73. There was no molecular ion for this compound (m/e 282), which was identical to the results done by Shafer [24].

The amount of fosfomycin determined by the bioassay and gas chromatography analysis coincided well with each other (data not shown). These data provided clear evidences that the *B. simplex* strain S101 can stereoselectively transform cPPA to fosfomycin.

In later experiments, we mainly assayed fosfomycin using GC methods.

Shaken flask studies

The ability of *B. simplex* strain S101 to use a range of carbon sources in a basic medium with a peptone concentration of 10 g l^{-1} was investigated. Eight carbon sources (glucose, sucrose, glycerol, maltose, xylose, starch, fructose, and lactose) were added separately to the medium to make a final concentration of 50 g l^{-1}. The growth and conversion to fosfomycin with the different saccharides are shown in Fig. 4. *B. simplex* strain S101 was able to use all the saccharides tested, yet no correlation was found between the biomass and conversion when S101 was cultured with the different carbon sources. Strain S101 grew well in the medium with the carbon sources indicated; however, the formation of fosfomycin varied greatly with different carbon sources. Glycerol was the best carbon source for the conversion, followed by glucose. Therefore, glycerol was used as the carbon source for the conversion of cPPA by strain S101.

The consumption of glycerol had positive effects on the production of fosfomycin (Fig. 5). When the glycerol concentration in the conversion medium was below 4%, conversion was moderate. However, the conversion began to increase when the glycerol concentration was above 4%.

cPPA concentration in the conversion medium was important for the growth and conversion by strain S101. However, when cPPA was used as the sole carbon source, neither growth or conversion to fosfomycin occurred. The conversion of cPPA to fosfomycin in various cPPA concentrations in the medium is shown in Table 1. When the cPPA concentration was low in the medium, the fosfomycin produced and the conversion ratio were moderate. When the cPPA concentration increased, the amount of fosfomycin produced also increased, as did the ratio of conversion. The maximum rate of conversion was reached when the concentration of cPPA was up to 2,000 μg ml^{-1}. However, the conversion ratio of fosfomycin decreased when the concentration of cPPA continued to rise. A high cPPA concentration (up to 10 mg ml^{-1}, data not shown) did not obviously restrain the growth of strain S101. Regardless of whatever the concentration of cPPA, strain S101 could not completely convert cPPA to fosfomycin during the whole process of cultivation.

The influence of pH value of the conversion medium on the growth and conversion of strain S101 was significant, as shown in Fig. 6. The maximum conversion and the best growth were obtained at a pH of 9. Both the growth and the conversion were adequate when pH values ranged from 8.0 to 9.0. The growth was poor and the conversion was very low at pH values of less than 7.0. Both the growth and conversion began to decrease when the pH value increased to 10.0, the decrease in growth rate was much faster than the decrease in conversion. After 6 days of cultivation in the conversion medium described in "Materials and methods", the pH value of the culture broth decreased from 8.5 to 5.5–6.0. The reason for this decrease is not known.

Strain S101 was a strictly aerobic bacterial strain; it could not grow well without the supply of air. However, the air was not a critical factor for the conversion. When the resting cells were incubated for 24 h in an anaerobic environment, fosfomycin was produced at a concentration of 1,126.8 μg ml^{-1}.

The effect of various metal salts on the conversion of cPPA to fosfomycin was investigated. Vanadium salts were founded to be indispensable for conversion.

The courses of the growth and conversion by strain S101 are shown in Fig. 7. There were two steps in the course of growth. The first step of growth ended at the fourth day of cultivation, when the glycerol in the medium was exhausted, which was monitored by GC and GC/MS (data not shown). It was found that fosfomycin increased slowly in this step. In the second step, the fosfomycin increased rapidly until it reached its maximum level. At the end of the exponential phase (the second step of growth), the fosfomycin began to decrease.

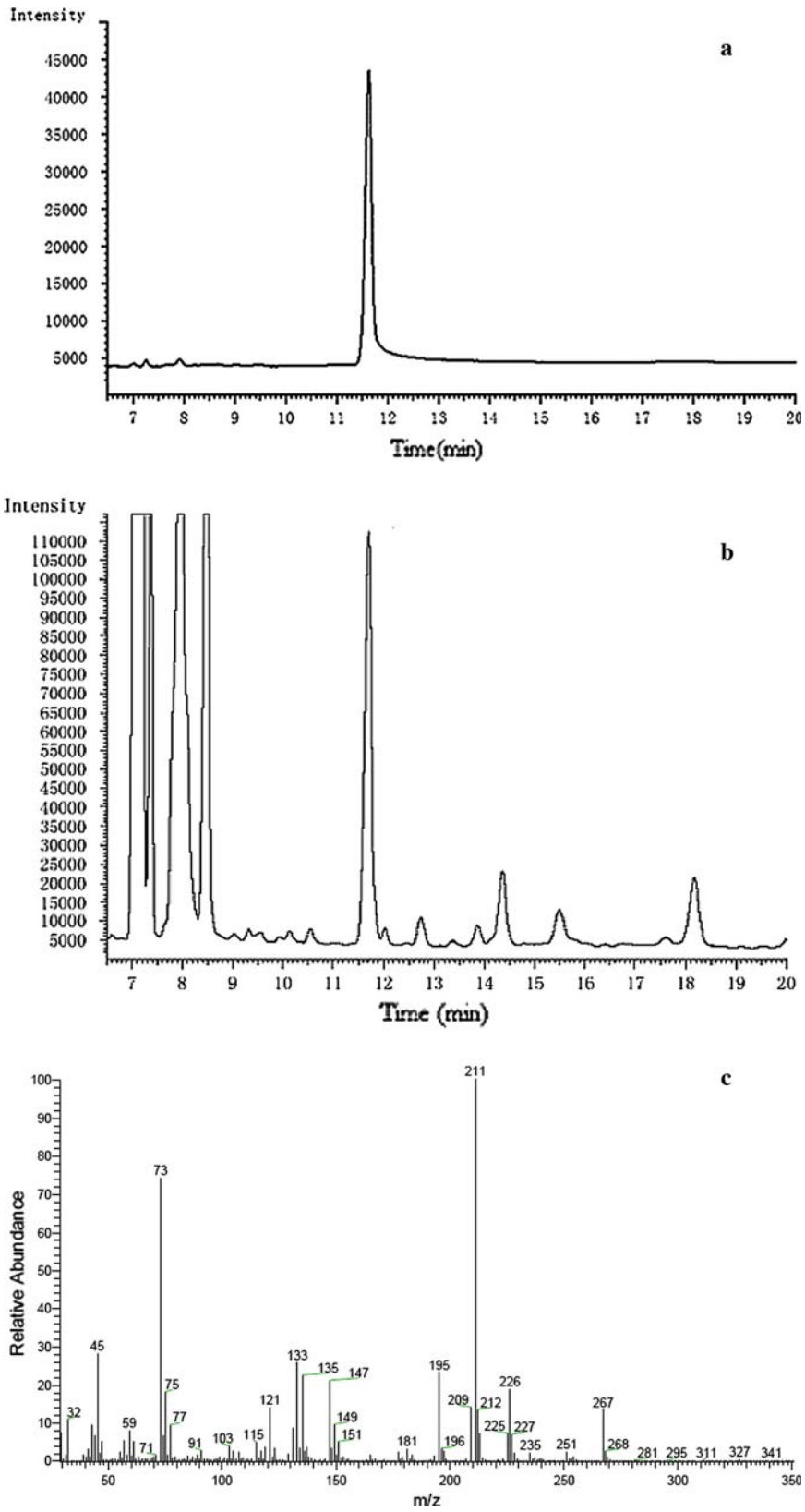

Fig. 3 Gas chromatography of the di-trimethylsilyl derivatives of **a** standard fosfomycin, **b** the fermentation supernatant, **c** the mass spectrum profile of the di-trimethylsilyl derivative of standard fosfomycin, showing the peak with retention time of 11.65 min of the transformation supernatant obtained via GC/MS

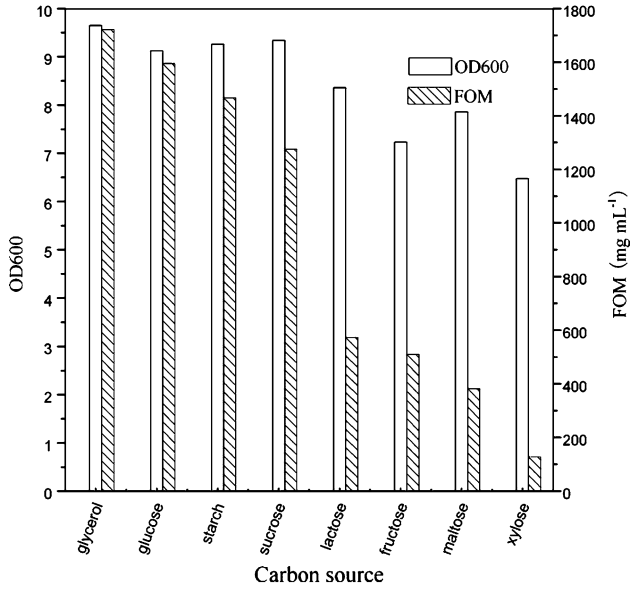

Fig. 4 Effect of different carbon sources on the biomass and transformation of cPPA to FOM by *B. simplex* strain S101

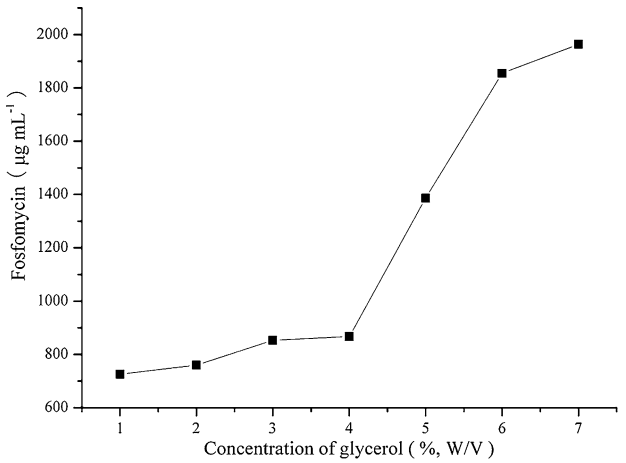

Fig. 5 Effects different concentration of glycerol on the production of fosfomycin

Discussion

S101 is a new strain of *B. simplex*, which is capable of converting cPPA to fosfomycin without the concomitant production of the unwanted (+) isomer. To the best of our knowledge, this work is the first to report on the biotransformation of *B. simplex*. Compared to chemical methods, one of the possible advantages of the biotransformation is avoidance of loss of the majority of the substrate, making it more environmentally friendly and possibly more cost-effective in the long term. However, the current situation for the biotransformation is not cost-competitive to the chemical process. What is most needed

Table 1 Effects of cPPA concentration in the transformation medium on the transformation of cPPA to fosfomycin

Concentration of cPPA (μg ml^{-1})	Fosfomycin (μg ml^{-1})	Ratio of transformation (%)
250	138.2	48.9
500	392.7	69.4
1,000	813.9	72.0
1,500	1,364.1	80.4
2,000	1,838.2	81.3
2,500	1,789.2	63.3
3,000	2,021.0	59.6
3,500	2,154.6	38.1
4,000	1,020.8	22.6
4,500	1,125.3	22.1
5,000	831.7	21.0

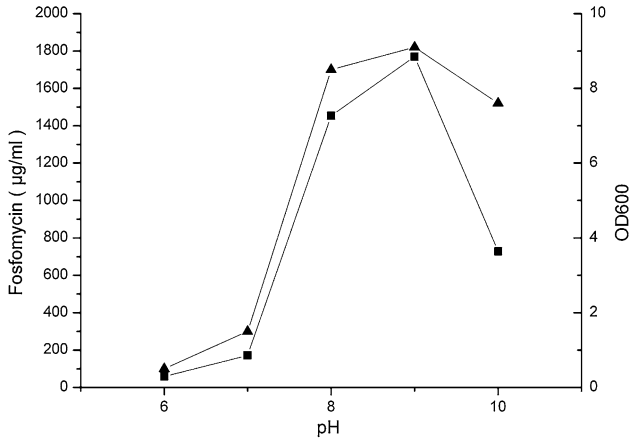

Fig. 6 Effects of pH on the growth of *B. simplex* strain S101 (*filled square*) and the epoxidation of cPPA to fosfomycin (*filled triangles*)

is to substantially increase the conversion ratio, resulting in a significant decrease in cost in the end.

Compared to other cPPA conversion microorganisms, such as *Penicillium spinulosum* MF2843 [30], *Cellvibrio gilvus* [1], and *Flavobacterium esteroaromaticum* IFO3751 [13], when cPPA concentration in the conversion medium was high (higher than 1,000 μg ml^{-1}), *B. simplex* strain S101 had the highest conversion ratio of 81.3%. High cPPA concentration in the medium has less inhibition effect on the growth of strain S101. Therefore, *B. simplex* strain S101 could be a potential converter of cPPA to fosfomycin in industry.

There have been only a few reports on the microbial enzyme system that converts cPPA to fosfomycin. In *F. esteroaromaticum* IFO3751 and *Pseudomonas putida* IK-8, a two-enzyme system was responsible for the conversion [13]. cPPA was first converted to bromohydrin, which was then epoxidized to fosfomycin, catalyzed by

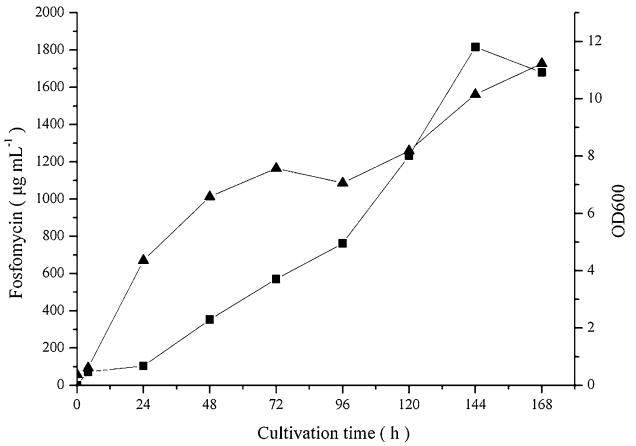

Fig. 7 Time course of the growth of *B. simplex* strain S101 (*filled triangles*) and the transformation of cPPA to fosfomycin (*filled squares*) during cultivation

bromoperoxidase and bromohydrin epoxidase, respectively. Using 2-D electrophoresis, Watanabe [29] found a cPPA-induced protein, epoA, in *Penicillium decumbens*. This is a 31 kDa protein, and has some similarity to oxygenases. When the gene epoA was subcloned back into *Penicillium decumbens*, a fourfold increase in epoxidation activity was achieved.

Like the majority of such epoxidation reactions [1, 29], the cell-free extract of *B. simplex* strain S101 could not epoxidize cPPA to fosfomycin. The reason for this is presumably that there are multiple-protein complexes that participate in this reaction. Upon the course of cell disruption, there is at least one component of the complex that had ceased to comply with its functions.

We tried to illustrate the mechanism of the transformation by means of proteomic methods. Unfortunately, we failed to identify any proteins related to the transformation (data not shown). The major drawback of protein identification based on MALDI-TOF MS and MS/MS is that they are highly dependent on the availability of genome data or protein sequence identity. In the case of *B. simplex*, its genome is not yet available. Up to now, there are only four protein sequences of *B. simplex* that can be found in the annotated protein database Swiss-Prot. Therefore, it is normal that we could not find any protein candidates from the databases. However, we believe that an oxidation system indeed exists in the cells of strain S101. In fact, aside from converting cPPA to fosfomycin, it can also convert glycerol to 2, 3-dihydroxy-propanoic acid at the same time (data not shown).

Several species of *Streptomyces* [9, 22] and *Pseudomonas* [25] were found to produce fosfomycin naturally, and the fermentation details for *Streptomyces fradiae* [22] have been studied. Compared to biotransformation of cPPA to fosfomycin, the level of fosfomycin produced was low. On the other hand, the biosynthetic pathway for production of fosfomycin by *Streptomyces wedmorensis* and other bacteria have been well studied [10, 15, 16, 23]. Fosfomycin was synthesized from phosphoenolpyruvate via four steps including the formation of a C–P bond and an epoxide. In the last step, fosfomycin was produced by dehydrogenation of (S)-2-hydroxypropylphosphonic acid (HPP) catalyzed by HPP epoxidase (HppE) [11, 17, 32]. HppE is a mononuclear nonheme iron-containing enzyme and requires reducing equivalents and molecular oxygen for activity. However, it was found that HPP epoxidase could not convert cPPA to fosfomycin [23]. The air was an indispensable factor for the growth of *B. simplex* strain S101 but not for the conversion, suggesting that the molecular oxygen probably metabolizes other reactions aside from the conversion of cPPA to fosfomycin, and the atom incorporated into cPPA to form fosfomycin does not originate from molecular oxygen. This demonstrates that cPPA is probably epoxidized to fosfomycin in the same manner as the epoxidation of HPP to fosfomycin.

Traditionally, fosfomycin is measured by means of bioassay. This has some disadvantages, such as being imprecise and not able to monitor the depletion of the substrate cPPA. In our experiments, we found that cPPA had some repressive effects on the growth of *P. vulgaris* FI-7. As a consequence, it can interfere with the precision of the results. Compared to the bioassay, GC analysis is more accurate and can evaluate the depletion of cPPA.

Vanadium ions are essential for strain S101 to convert cPPA to fosfomycin. They exhibit a rich redox chemistry and can act as cofactors to some enzymes, such as nitrogenase [7] and bromoperoxidase [19]. In fact, vanadium ions can even act as a catalyst for asymmetric epoxidation in organic synthesis [12]. However, for the epoxidation of cPPA to fosfomycin in strain S101, the exact function of vanadium ions remains unknown.

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