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trans-Stilbene degradation by *Arthrobacter* sp. SL3 cells

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Abstract *trans*-Stilbene degradation was examined by the reaction using resting cells of microorganisms isolated through the enrichment culture using *trans*-stilbene. The strain SL3, showing the highest *trans*-stilbene-degrading activity, was identified as *Arthrobacter* sp. One of the reaction products was identified to be *cis*,*cis*-muconic acid. *Arthrobacter* sp. SL3 cells also transformed benzaldehyde, benzoic acid and catechol into *cis*,*cis*-muconic acid, suggesting that one benzene ring of *trans*-stilbene was converted into *cis*,*cis*-muconic acid via benzaldehyde formed by its C_α=C_β bond cleavage.

Keywords *trans*-Stilbene · *Arthrobacter* sp. SL3 · *cis*,*cis*-Muconic acid · Double-bond cleavage

Introduction

Regarding the enzymatic cleavage of carbon double-bond, the cleavage of aromatic ring by dioxygenases such as catechol-1,2-dioxygenase (EC1.13.11.1) and indoleamine-2,3-dioxygenase (EC1.13.11.11) is known. With respect to the enzymatic cleavage of double-bond of alkenes, the following reactions are exemplified: (1) cleavage of ascorbate by ascorbate 2,3-dioxygenase (EC1.13.11.13); (2) C_α=C_β cleavage of lignin model compounds by lignostilbene-α,β-dioxygenase (Habe et al. 1989); (3) central cleavage of β-carotene by β-carotene15,15′-monooxygenase (Woggon 2002); (4) side-chain cleavage of *trans*-anethole (Shimoni et al. 2002). Recently, we have reported the oxidative cleavage of the side chain double-bond of isoeugenol by *Pseudomonas putida* cells (Yamada et al. 2007a), and the isoeugenol-degrading enzyme has been characterized (Yamada et al. 2007b). The degradation of isoeugenol is initiated by isoeugenol monooxygenase, which probably catalyze epoxidation of the side chain double-bond and subsequent hydrolysis to form isoeugenol-diol, followed by cleaving into vanillin and acetaldehyde. However, the detailed reaction mechanism of isoeugenol monooxygenase has not been clarified. In the present study, we used *trans*-stilbene as a model compound to elucidate the reaction mechanism for enzymatic cleavage of the side chain double-bond of aromatic compounds because *trans*-stilbene is a simple ethylene derivative substituted by two phenyl groups.

Stilbene is a raw material for the synthesis of azo dyes, and its derivatives are used as fluorescent whitening agents for textile and paper industries. In the river and ground waters, therefore, stilbene derivatives are detected as environmental pollutants. On the other hand, some of hydroxylated stilbene, such as 3,4′,5-trihydroxy-*trans*-stilbene (resveratrol) and 3,3′,4,5-tetrahydroxy-*trans*-stilbene (piceatannol),

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exhibit anticancer (Schneider et al. 2000), antioxidant (Martinez and Moreno 2000) and cardioprotective (Hung et al. 2000) effects. In an effort to demonstrate the existence of microbes able to detoxify stilbene-derivatives, several *trans*-stilbene-degrading *Pseudomonas* strains have been isolated from enrichment cultures (Yutani and Miyamono 1993; Leahy et al. 2003). In the studies on *trans*-stilbene degradation using *Pseudomonas* sp. strain L-1, Yutani et al. (1997) observed the formation of the metabolites: benzaldehyde, benzoic acid, catechol, 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, phenylacetic acid, *p*-hydroxyphenylacetic acid and mandelic acid. Leahy et al. (2003) also isolated *trans*-stilbene-degrading *Pseudomonas fluorescens* MN2, which formed metabolites with antioxidant activity. In the present study, we isolated a novel *trans*-stilbene-degrading bacterium, *Arthrobacter* sp. SL3, and investigated the microbial degradation of this compound by the resting cells.

Materials and methods

Isolation of *trans*-stilbene-degrading microorganisms

A basal medium containing 3 g $(NH_4)_2HPO_4$, 2 g KH_2PO_4, 1 g NaCl, 0.05 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.005 g $MnSO_4 \cdot xH_2O$, 5 ml metal solution (Uchida et al. 2003) and 5 ml vitamin mixture (Yoshida et al. 2004) in 1 l of tap water, pH 7.0, was used. Enrichment culture was aerobically carried out at 28°C in 500-ml shaking-flask containing 50 ml of the basal medium supplemented with 0.1–5 g/l *trans*-stilbene. Microorganisms isolated on agar plates were stored on slants of nutrient medium: 5 g peptone, 5 g meat extract, 0.5 g yeast extract and 2 g NaCl in 1 l of tap water, pH 7.0.

Culture conditions and degradation experiments with resting cells

The isolated microorganisms were cultivated at 28°C for 2 days in 300 ml of the basal medium, which was supplemented with 1 g/l *trans*-stilbene. Cells were harvested by centrifugation, followed by washing with 0.15 M NaCl and resuspended in 1.5 ml of the same sodium chloride solution. The cell growth of *Arthrobacter* sp. SL3 was estimated turbidimetrically at 610 nm, and 1.0 unit of optical density at 610 nm found to be equivalent to 0.363 mg dry cell weight ml^{-1}.

The *trans*-stilbene-degrading activity was determined in the mixture comprising 5 mM *trans*-stilbene, 100 mM potassium phosphate buffer (pH 7.0), 10% (v/v) ethanol and 0.5 ml cells suspension, which harvested from 100 ml of basal medium culture, in a total volume of 10 ml. Ethanol was added to enhance the solubility of *trans*-stilbene in the reaction mixture. The reaction was carried out for 30 min in a 50-ml Erlenmeyer flask with reciprocal shaking (160 strokes min^{-1}) at 30°C, started by adding *trans*-stilbene as ethanol solution, and stopped by the removal of cells by centrifugation. After centrifugation of the reaction mixture, the residual *trans*-stilbene was extracted twice with 10 ml of ethyl acetate. The ethyl acetate phase was directly analyzed by HPLC (Shimadzu LC-10AS and SPD-10A system) with a Wakosil-II 5C18 HG column (4.6 × 150 mm, Wako Pure Chemical Industries, Japan). Elution was done at 50°C with a 650:350:1 (v/v/v) mixture of acetonitrile, water and phosphoric acid. The flow rate was maintained at 1.0 ml min^{-1}, and the absorbance of eluates was monitored at 260 nm. *trans*-Stilbene-degrading activity was defined as the amount of *trans*-stilbene degraded per minute by 1 mg of resting cells as dry weight.

Identification of reaction products

One of the unknown products derived from *trans*-stilbene was isolated and identified as *cis*,*cis*-muconic acid. The produced *cis*,*cis*-muconic acid was extracted from the reaction mixture with ethyl acetate and purified by a silica gel column using a 2:1 (v/v) mixture of *n*-hexane and ethyl acetate as the eluent. The purified *cis*,*cis*-muconic acid was obtained as a white solid, with a purification yield of 36%. The product was identified by ^{1}H-NMR, ^{13}C-NMR and mass spectrometric analyses with authentic *cis*,*cis*-muconic acid as a reference; ^{1}H-NMR (500 MHz, CD_3OD): δ 5.87 (2H, dd, J = 1.8, 8.0 Hz) and 7.74 (2H, dd, J = 2.0, 8.3 Hz), ^{13}C-NMR (125 MHz, CD_3OD): δ 125.6, 138.7 and 168.8 and mass spectrometric analysis (EI+): 142, 124, 97, 96, 79

and 51. The NMR spectra were obtained by a Jeol model JNM-ECA500. Mass spectrum analysis was carried out using a Jeol model JMS-700/GI.

Chemicals

trans-Stilbene was purchased from Wako Pure Chemical Industries (Japan). 4-Hydroxy-*trans*-stilbene and *cis*,*cis*-muconic acid were obtained from Kanto Chemical Co. (Japan). Peptone was purchased from Nihon Pharmacy Co. (Japan). Meat extract was obtained from Kyokuto Seiyaku Co. (Japan). Yeast extract was purchased from Oriental Yeast (Japan). All other chemicals used were of analytical grade and commercially available.

Results

Screening of *trans*-stilbene-degrading microorganisms

Six *trans*-stilbene-degrading microorganisms were isolated from soils by an enrichment culture technique using *trans*-stilbene as the sole carbon source. Among them, the bacterial strain SL3 exhibited the highest activity, namely 19.2 nmol min^{-1} (mg dry cell weight)$^{-1}$. The 16S ribosomal DNA sequence (1,478 bp) of strain SL3 showed 99.9%, 99.9% and 99.6% identity to the corresponding sequences of *Arthrobacter* sp. Ellin158, *A. oxydans* DSM20119, and *A. polychromogenes* DSM20136, respectively, in analysis with FASTA WWW System. From the result, the strain SL3 was identified to be *Arthrobacter* sp.

To check if *trans*-stilbene-degrading activity was also present in other members of the *Arthrobacter* genus, we tested the corresponding strains contained in our laboratory collection. With 1 g/l *trans*-stilbene added as the sole source of carbon and energy to the basal medium, the degradation activity was observed with *A. atrocyaneus* IFO12956, *A. sulfurous* IFO12678 and *A. globiformis* IAM12438. The following strains did not assimilate *trans*-stilbene: *A. aurescens* IAM12340, *A. citreus* IAM12341, *A. luteus* IAM12312 and *A. pascens* IAM12343. However, in comparison to the laboratory collection strains, the soil isolate *Arthrobacter* sp. SL3 showed the highest *trans*-stilbene degradation under constant culture conditions.

The *Arthrobacter* sp. SL3 also grew on the basal culture medium containing benzoic acid, 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, glucose, glycerol and ethanol instead of *trans*-stilbene, but not 4-hydroxy-*trans*-stilbene, 3-hydroxybenzoic acid and methanol. The resting cells grown on the basal medium containing the above carbon sources or nutrient medium did not degrade *trans*-stilbene in the reaction mixture. Thus, *trans*-stilbene-degrading enzyme is specifically induced by *trans*-stilbene.

trans-Stilbene conversion by resting cells of *Arthrobacter* sp. SL3

In the experiments on *trans*-stilbene degradation using resting cells of *Arthrobacter* sp. SL3, without adding ethanol in the reaction mixture, no degradation of *trans*-stilbene was observed. Although the addition of methanol was also effective, ethanol was used in the following experiments because of higher solubility of *trans*-stilbene in ethanol. In the conversion of *trans*-stilbene by resting cells of *Arthrobacter* sp. SL3, benzoic acid and several unknown products were detected by HPLC-UV analysis, at 210 and 260 nm. One of the unknown products was isolated and identified as *cis*,*cis*-muconic acid as described in Materials and methods. The time course of *trans*-stilbene degradation by *Arthrobacter* sp. SL3 cells is shown in Fig. 1. After a 24-h incubation of 5 mM *trans*-stilbene with the bacterial cells, 3.02 mM *trans*-stilbene was consumed and 3.25 mM *cis*,*cis*-muconic acid stoichiometrically accumulated. During the reaction, benzoic acid was detected in trace amount (<0.1 mM) by HPLC-UV analysis.

Degradation of putative metabolites of *trans*-stilbene

The ability of *Arthrobacter* sp. SL3 to degrade putative metabolites of *trans*-stilbene was examined using the resting cells grown on *trans*-stilbene (Table 1). *trans*-Stilbene oxide and the different hydrobenzoin *stereo*-isomers, putative metabolites of *trans*-stilbene undergoing the cleavage of the C_{α}=C_{β} bond, were not degraded by *Arthrobacter* sp.

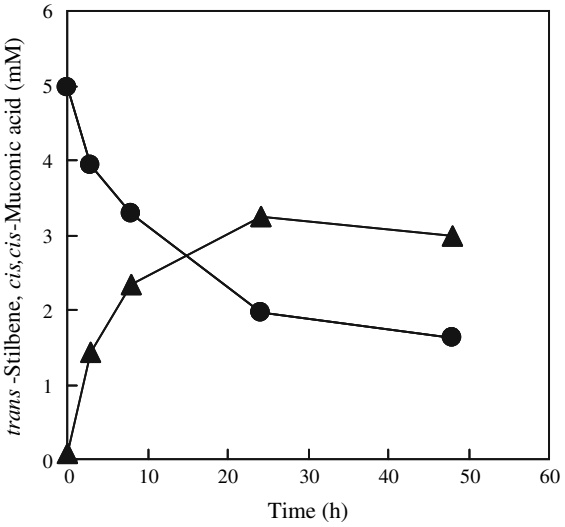

Fig. 1 Time course of *trans*-stilbene degradation by *Arthrobacter* sp. SL3 cells. The reaction mixture contained 5 mM *trans*-stilbene, 11.8 mg cells as dry weight, 100 mM potassium phosphate buffer (pH 7.0) and 10% (v/v) ethanol in a total volume of 10 ml. Symbols: circles, *trans*-stilbene; triangles, *cis*,*cis*-muconic acid

SL3 cells. Instead the cells showed high activity for benzaldehyde, benzoic acid and catechol. In line with the observed degradation of benzaldehyde, benzoic acid and catechol, nearly stoichiometric amounts of *cis*,*cis*-muconic acid were formed (Fig. 2). When 4-hydroxy-*trans*-stilbene was used as the substrate, 4-hydroxybenzoic acid, benzoic acid and *cis*,*cis*-muconic acid were formed (Fig. 3).

To elucidate the degradation pathway of *trans*-stilbene, various hydroxybenzaldehydes and hydroxybenzoic acids were tested as the substrate for resting cells reaction. *Arthrobacter* sp. SL3 cells rapidly degraded hydroxybenzaldehydes, and formed the corresponding hydroxybenzoic acids (Fig. 4). Whereas 2-, 3-, 4- and 2,3-hydroxylated benzaldehydes were stoichiometrically converted to the corresponding hydroxybenzoic acids by 1-h incubation, 3,4-dihydroxybenzaldehyde was degraded without stoichiometric accumulation of 3,4-dihydroxybenzoic acid. With regard to the benzoic acid derivatives, 3,4-dihydroxybenzoic acid and 3-hydroxybenzoic acid were quickly degraded by the bacterial cells (Fig. 5). The degradation of 2- and 4-hydroxybenzoic acids was observed after a lag time, suggesting that the degradation activity was induced during 1-h incubation. After the reactions

Table 1 Degrading activity of *Arthrobacter* sp. SL3 cells for *trans*-stilbene and its putative metabolites

Compound	Activity (nmol min^{-1} (mg dry cell weight)$^{-1}$)	
	Cells grown on *trans*-stilbene	Cells grown on nutrient medium
trans-Stilbene	19.2	0
trans-Stilbene oxide	0	n.t.
meso-Hydrobenzoin	0	n.t.
(1*R*,2*R*)-hydrobenzoin	0	n.t.
(1*S*,2*S*)-hydrobenzoin	0	n.t.
4-Hydroxy-*trans*-stilbene	23.6	0
Benzaldehyde	27.3	9.4
Benzoic acid	17.4	0
Catechol	230	0.3
cis,*cis*-Muconic acid	0.1	0
2-Hydroxybenzaldehyde	83.3	7.1
3-Hydroxybenzaldehyde	23.5	2.0
4-Hydroxybenzaldehyde	45.7	0.6
2,3-Dihydroxybenzaldehyde	66.8	1.3
3,4-Dihydroxybenzaldehyde	31.0	0.6
2-Hydroxybenzoic acid	0.3	0
3-Hydroxybenzoic acid	2.4	0.3
4-Hydroxybenzoic acid	0.3	0
2,3-Dihydroxybenzoic acid	0	0
3,4-Dihydroxybenzoic acid	2.5	0.3

The reaction mixture contained 0.5 mM each compound described above, 0.927, 9.27 or 65.6 mg cells as dry weight, 100 mM potassium phosphate buffer (pH 7.0) and 5% (v/v) ethanol in a total volume of 10 ml. The composition of nutrient medium is described in Materials and methods. n.t., not tested

using hydroxybenzaldehydes and hydroxybenzoic acids, *cis*,*cis*-muconic acid and other compounds were not detected by HPLC-UV analysis at 210 and 260 nm.

The resting cells grown on *trans*-stilbene showed high degrading activities for benzaldehyde, benzoic acid, hydroxybenzaldehydes and hydroxybenzoic acids than those grown on nutrient medium (Table 1), suggesting that some of these compounds are intermediates in the degradation pathway of *trans*-stilbene.

Discussion

The previously described *trans*-stilbene assimilating microorganisms all belong to the genera *Alcaligenes*

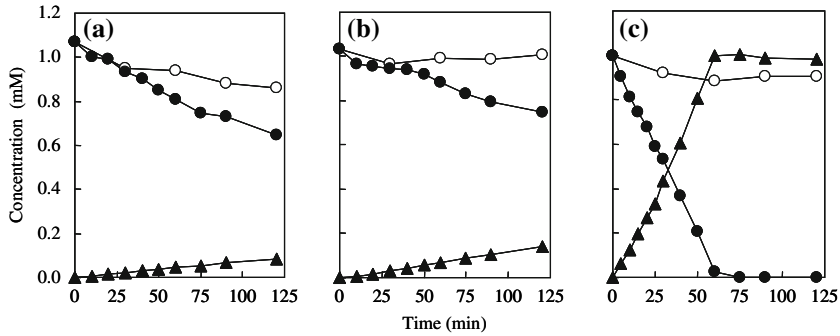

Fig. 2 Degradation of benzaldehyde (**a**), benzoic acid (**b**) and catechol (**c**) by *Arthrobacter* sp. SL3 cells. The reaction mixture contained 1.0 mM substrate, 2.58 mg cells as dry weight, 100 mM potassium phosphate buffer (pH 7.0) and 10% (v/v) ethanol in a total volume of 10 ml. Symbols: closed circles, benzaldehyde (**a**), benzoic acid (**b**) and catechol (**c**); triangles, *cis*,*cis*-muconic acid. Open circles indicate benzaldehyde (**a**), benzoic acid (**b**) and catechol (**c**) concentrations in the reaction mixture without adding *Arthrobacter* sp. CL3 cells

and *Pseudomonas* (Tsuchii et al. 1977; Takase et al. 1986; Yutani and Miyamono 1993; Yutani et al. 1997; Leahy et al. 2003). Yutani et al. (1997) proposed a pathway of degradation of *trans*-stilbene by *Pseudomonas* sp. L-1, in which the C_{α}=C_{β} bond of *trans*-stilbene was initially cleaved to form benzaldehyde. In the present study, using soil isolates and our laboratory collections, we found *trans*-stilbene-degrading microorganisms in the genus *Arthrobacter*. A soil isolate, *Arthrobacter* sp. SL3, showed the highest *trans*-stilbene-degrading activity, and this activity was induced by *trans*-stilbene. The observed

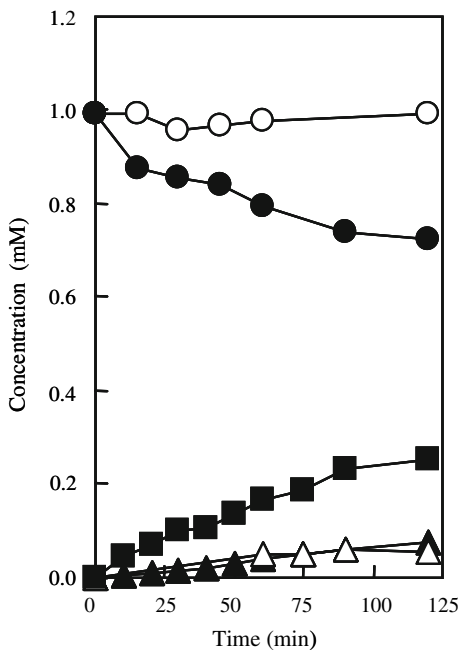

Fig. 3 Degradation of 4-hydroxy-*trans*-stilbene by *Arthrobacter* sp. SL3 cells. The reaction mixture contained 1.0 mM 4-hydroxy-*trans*-stilbene, 2.58 mg cells as dry weight, 100 mM potassium phosphate buffer (pH 7.0) and 10% (v/v) ethanol in a total volume of 10 ml. Symbols: closed circles, 4-hydrpxy-*trans*-stilbene; closed triangles, *cis*,*cis*-muconic acid; Open triangles, benzoic acid; squares, 4-hydroxybenzoic acid. Open circles indicate 4-hydrpxy-*trans*-stilbene concentrations in the reaction mixture without adding *Arthrobacter* sp. CL3 cells

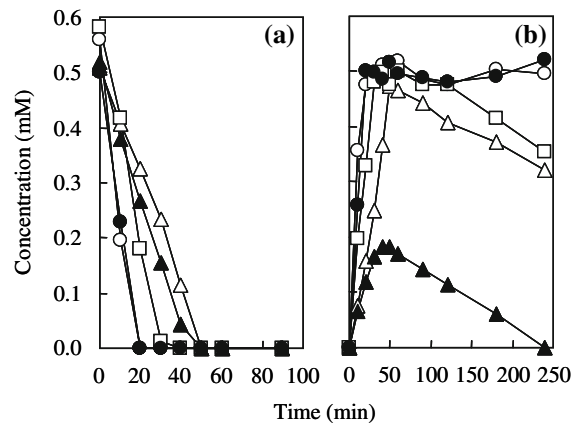

Fig. 4 Time course of hydroxybenzaldehydes degradation (**a**) and hydroxybenzoic acids formation (**b**) by *Arthrobacter* sp. SL3 cells. The reaction mixture contained 0.5 mM hydroxybenzaldehyde, 4.07 mg cells as dry weight, 100 mM potassium phosphate buffer (pH 7.0) and 5% (v/v) ethanol in a total volume of 10 ml. Symbols: open circles, 2-hydroxybenzaldehyde (**a**) and 2-hydroxybenzoic acid (**b**); open triangles, 3-hydroxybenzaldehyde (**a**) and 3-hydroxybenzoic acid (**b**); squares, 4-hydroxybenzaldehyde (**a**) and 4-hydroxybenzoic acid (**b**); closed circles, 2,3-dihydroxybenzaldehyde (**a**) and 2,3-dihydroxybenzoic acid (**b**); closed triangles, 3,4-dihydroxybenzaldehyde (**a**) and 3,4-dihydroxybenzoic acid (**b**)

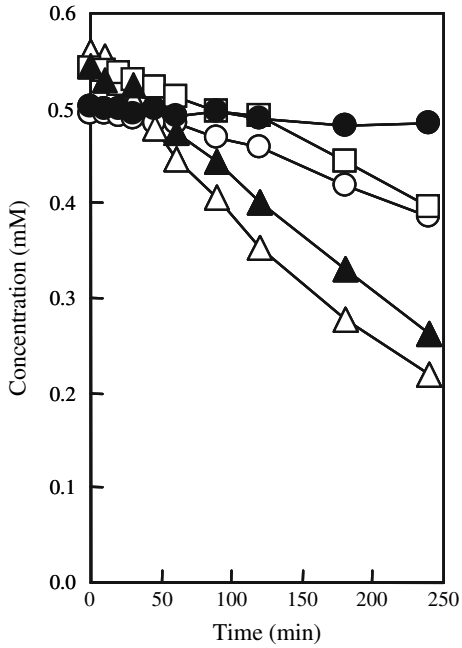

Fig. 5 Time course of hydroxybenzoic acids degradation by *Arthrobacter* sp. SL3 cells. The reaction mixture contained 0.5 mM hydroxybenzoic acid, 5.59 mg cells as dry weight, 100 mM potassium phosphate buffer (pH 7.0) and 5% (v/v) ethanol in a total volume of 10 ml. Symbols: open circles, 2-hydroxybenzoic acid; open triangles, 3-hydroxybenzoic acid; squares, 4-hydroxybenzoic acid; closed circles, 2,3-dihydroxybenzoic acid; closed triangles, 3,4-dihydroxybenzoic acid

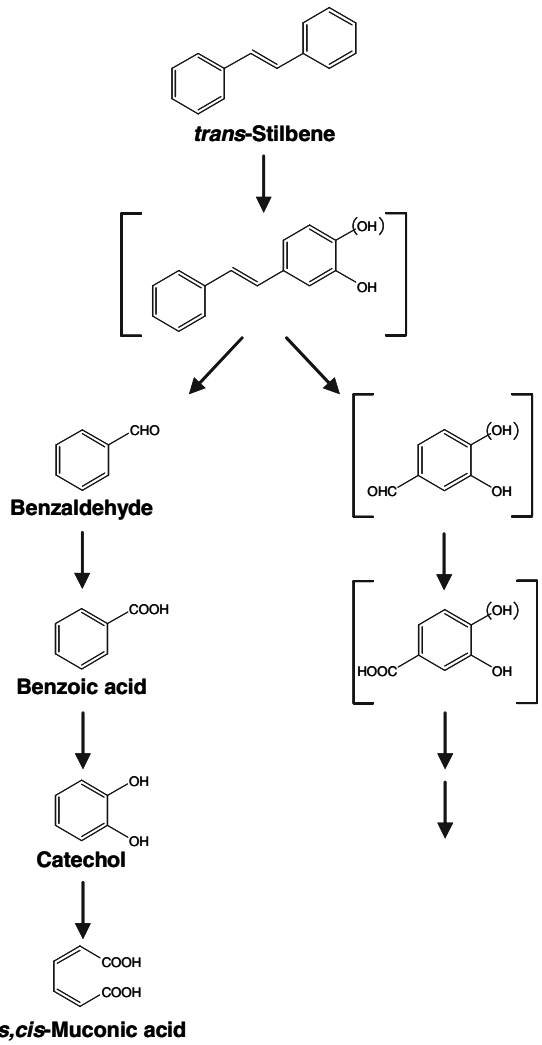

Fig. 6 Proposed pathway for *trans*-stilbene degradation by *Arthrobacter* sp. SL3 cells

stoichiometric accumulation of 1 mol of *cis*,*cis*-muconic acid per mol of *trans*-stilbene consumed (Fig. 1) suggests that the two benzene rings in the *trans*-stilbene molecule might be metabolized by two different routes (Fig. 6). Resting cells of *Arthrobacter* sp. SL3 exhibited high degrading activity toward 4-hydroxy-*trans*-stilbene as well as *trans*-stilbene (Table 1), and the C_{α}=C_{β} bond of 4-hydroxy-*trans*-stilbene cleaved by the resting cells, resulting the formation of 4-hydroxybenzoic acid, benzoic acid and *cis*,*cis*-muconic acid (Fig. 3). Interestingly, the lignostilbene-α,β-dioxygenase of *Sphingomonas paucimobilis* TMY1009 is able to cleave the C_{α}=C_{β} bond of 4-hydroxy-*trans*-stilbene, but not that of *trans*-stilbene (Kamoda et al. 2003). Taking into account this observation and based on our results, we propose that the initial step in the degradation of *trans*-stilbene by *Arthrobacter* sp. SL3 consists in the hydroxylation of one of the two benzene rings and is followed by the cleavage of the C_{α}=C_{β} bond, resulting in the formation of benzaldehyde on the one hand and of hydroxylated benzaldehyde on the other hand. *Arthrobacter* sp. SL3 cells exhibited high degradation activity for benzaldehyde, benzoic acid and catechol, and these substrates were transformed into *cis*,*cis*-muconic acid. In contrast, the activity towards *cis*,*cis*-muconic acid was considerably low. The degradation of *trans*-stilbene resulted therefore in the accumulation of *cis*,*cis*-muconic acid in the reaction mixture.

Arthrobacter sp. SL3 cells oxidized various hydroxybenzaldehydes to the corresponding hydroxybenzoic acids and degraded 3,4-dihydroxybenzoic acid and 3-hydroxybenzoic acid quickly. In line with these observations, the initial ring hydroxylation of

trans-stilbene by an oxygenase might occur at the positions C3 and C4, or alternatively, at the position C3 alone. To elucidate the mechanism of the double bond cleavage in *trans*-stilbene, further degradation experiments with *trans*-stilbene derivatives such as 3-hydroxy-*trans*-stilbene and 3,4-dihydroxy-*trans*-stilbene will be required.

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