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Degradation of Styrene by a New Isolate *Pseudomonas putida* SN1

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Abstract–Twelve styrene-utilizing bacteria were isolated from a biofilter used for treating gaseous styrene. A gram-negative strain had a high styrene-degrading activity and was identified as *Pseudomonas putida* SN1 by 16S rDNA analysis. The styrene degradation in SN1 was regarded to start with a monooxygenase enzyme which converted styrene to styrene oxide, a potentially important chiral building block in organic synthesis. SN1 could grow on styrene and styrene oxide, but not on benzene and toluene. The styrene degradation activity in SN1 was induced when incubated with styrene, and the induction was not inhibited by the presence of readily usable carbon sources such as glucose and citrate. The optimal activity was shown at pH 7.0 and 30 °C and estimated as 170 unit/g cell.

Key words: Styrene, Styrene Degrading Microorganisms, Styrene Monooxygenase (SMO), *Pseudomonas putida* SN1, Induction of SMO Activity

INTRODUCTION

Styrene is an important chemical compound used in a large quantity for synthesizing polymers such as polystyrene and styrene-butadiene rubber. It is also employed as a solvent in the polymer processing industry. Accordingly, styrene occurs in many industrial effluents, wastewater and waste gas as well. The emission of styrene lays a serious environmental concern even at low concentrations, due to the low odor threshold of 0.05-0.15 ppmv and its carcinogenic property. Many physicochemical and biological methods for treating industrial waste streams containing styrene have been studied and some of them are currently used [Panke et al., 1998].

Various microorganisms are known to be capable of degrading styrene under aerobic conditions. They include *Pseudomonas*, *Xanthobacter*, *Rhodococcus*, and several alkane-utilizing strains such as *Methylosinus*, *Methylococcus*, and *Brevibacterium* [Hartmans et al., 1990]. In these microorganisms, styrene degradation occurs by two different biochemical pathways. The first one is via a dioxygenase which attacks the aromatic nucleus of styrene and converts styrene to 3-vinylcatechol [Warhurst et al., 1994]. The other one, which is more popular than the first one among the bacterial species, is via a monooxygenase. Styrene monooxygenase (SMO) attacks the vinyl side chain of styrene and produces styrene oxide which is further oxidized to phenyl acetaldehyde and phenyl acetate. Some *Pseudomonas* strains having SMO have been investigated in detail for the genes and enzymes involved in styrene degradation. For example, Beltrametti et al. reported that *Pseudomonas fluorescens* ST had four open reading frames coding for the oxidation of styrene to phenylacetic acid, named styA, styB, styC, and styD. Although isolated by different research groups, *Pseudomonas* sp. Y2 and *Pseudomonas* sp. VLB120 also had very similar sets of genes and enzymes of styrene catabolism to *P. fluorescens* ST. In addition to styrene degradation pathway, a lower pathway which begins with phenylacetic acid, was also discovered [Beltrametti et al., 1997; O'conner et al., 1995; Panke et al., 1998].

The SMO is an important enzyme that often determines the degradation rate of styrene in many styrene-degraders. In addition, it has a significant potential as a biocatalyst in producing an enantiopure (*R*)- or (*S*)-styrene epoxide. Chiral epoxides are versatile building blocks for organic synthesis and can be used for synthesizing several drugs. In *Pseudomonas* strains, SMO was NADH-dependent, FAD-containing enzyme [Hollmann et al., 2003]. Since these cofactors should be regenerated for the continuous enzymatic reaction, whole-cell rather than purified enzymes have been developed as biocatalysts. Some recombinant strains possessing SMO and lacking other enzymes for further oxidation of styrene oxide have been reported [Panke et al., 1999].

The purpose of this study is to isolate and characterize an active styrene-degrading bacterium with a high SMO activity. Once a highly active strain is obtained, it can be applied for styrene degradation in biofiltration or for developing biocatalyst for the production of enantiopure styrene oxides. Composts and leachate samples from a biofilter operated for removal of gaseous styrene were screened for the strains growing on styrene as a sole carbon and energy source, and many new isolates were obtained. Among the isolates, a *Pseudomonas* strain had a high styrene-degrading activity and was studied in detail.

MATERIALS AND METHODS

1. Isolation of Styrene-degrading Microorganisms

Styrene degrading microorganisms were isolated from a biofilter that was used for treating gaseous styrene. Composts and leachate samples were taken, washed, diluted, and spread on agar plates with M9 mineral salts medium [O'Connor et al., 1997]. The agar plates were incubated at 30 °C in an air-tight container saturated with gas-phase styrene as a sole carbon and energy source. After one week, different colonies were selected and identified by API method (bioMerieux, France).

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Among 12 isolates, a gram-negative strain SN1 had a high activity on styrene degradation and identified by 16S rDNA analysis [Kim et al., 1995]. The partial segment of 16S rDNA was amplified with two universal primers 27f and 1392r [Kim et al., 1995] using a PCR, cloned and sequenced with ABI 3700 (Applied Biosystems). Molecular techniques were followed by standard protocols [Sambrook et al., 1989].

2. Growth and Styrene Degradation Activity of the Isolates

The isolates were cultivated in a 500 ml flask with 50 ml of M9 mineral salts medium supplemented with 0.5 g/L glucose and 1 g/L yeast extract (M9+ medium, hereafter). The cultivation was conducted on horizontal shakers at 30 °C and 250 rpm for 12 h. Styrene was added to the culture broth at 1 mM at 8 h to induce the styrene degradation activity.

To measure the styrene degradation activity, cells were centrifuged, washed twice with an ice-cold potassium phosphate buffer (50 mM, pH 7.0) and resuspended in the same buffer. A 165 ml serum bottle (working volume, 15 ml) fitted with a butyl rubber and aluminum cap was used. Cell concentration in dry weight was about of 0.06 g/L and gas-phase styrene was added to the headspace at 400-600 ppmv. The reaction was carried out in a reciprocally-shaking water bath at 30 °C where the shaking speed was 180 strokes/min. Gas samples from the headspace were analyzed periodically by gas chromatography, typically for 30 min. One unit was defined as the activity that degrades 1 μmol styrene in 1 min and the specific activity was expressed as unit $[\text{g cell}]^{-1}$.

3. Growth of *Pseudomonas putida* SN1 and Degradation of Aromatic Compounds

The isolate SN1, identified as *Pseudomonas putida*, was studied for growth on various aromatic compounds such as styrene, styrene oxide, benzene, and toluene as a sole carbon and energy source. The M9 mineral salts medium was used and each carbon substrate was supplied to the gas phase by placing an Effendorf microtube (2 ml) containing the substrate of 30 μl in the headspace of a 300 ml flask (working volume, 30 ml). In case of styrene oxide, an experiment with directly added substrate to the liquid medium (5 mM) was also carried out due to its low vapor pressure. The flasks were fitted with screw cap to prevent substrate evaporation and samplings were conducted through the screw-capped side-arm port sealed with a rubber septum.

The degradation of styrene, styrene oxide, benzene, and toluene by SN1 was studied in potassium phosphate buffer (50 mM, pH 7.0). Cells were cultivated in the M9+ medium and induced for 4 h before harvesting as described above. Reaction was carried out in a 165 ml serum bottle (working volume, 15 ml) at 0.3 g cell/L. Aromatic compounds except for styrene oxide were supplied as gas phase to the headspace at 600-660 ppmv. Degradation was monitored by measuring the gaseous concentration on a gas chromatograph. In case of styrene oxide, the reaction was conducted in a 30 ml vial with 10 ml reaction mixture. Racemic mixture of styrene oxide was added directly into the liquid mixture at 5 mM and its degradation was followed by a gas chromatograph after extracting styrene oxide from the reaction mixture with cyclohexane.

Styrene degradation was also measured with broken cell extracts. Fully induced cells were resuspended at 20 g/L in an ice-cold Tris-HCl buffer (20 mM, pH 7.5) containing 10% (v/v) glycerol and 1 mM DTT, and disintegrated by two passages through a French pressure cell (Thermo Electron Corporation, U.S.A) at 18,000 psi. The broken cells were centrifuged at 10,000 g to remove unbroken cell debris and were stored at 4 °C before use. The SMO activity was measured in the same way as described above.

4. Induction of Whole-cell SMO Activity in *P. putida* SN1

Since the whole-cell SMO activity in *P. putida* SN1 appears only after induction by styrene, the induction conditions were further studied while supplying different nitrogen and carbon sources. Cells were cultivated for 8 h in the M9+ medium and washed twice with an ice-cold 50 mM potassium phosphate buffer. Induction was carried out with or without added glucose and/or yeast extract, or in fresh M9+ medium containing styrene. Specific activity of styrene degradation was measured in 50 mM potassium phosphate buffer as described above.

The change of specific activity during induction was also studied. Cells were cultivated and induced at 8 h by directly adding styrene to the culture broth at 1 mM. Cells were taken periodically for 6 h and measured for the whole-cell SMO activity.

5. Effect of Temperature, pH, and Styrene Concentration on Whole-cell SMO Activity of *P. putida* SN1

The effect of pH and temperature on the whole-cell SMO activity of SN1 was studied for the M9+ grown cells after induction. The pH effect was studied in the range of 5.8 and 8.0 at 30 °C, whereas temperature effect was between 20 °C and 45 °C at pH 7.0. Effect of styrene concentration was studied at 30 °C and pH 7.0 in varying liquid-phase styrene concentrations of 0.5-18 μM.

6. Analytical Methods

Styrene, benzene, and toluene in gas samples were analyzed by a gas chromatograph (HP6890, Hewlett Packard Inc., USA) equipped with a flame ionization detector. An HP-530 capillary column (Hewlett Packard Inc., USA, 15 m length, 0.53 mm ID, and 1.5 μm film thickness) coated with cross-linked 5% PH ME siloxane was used. Nitrogen gas was used as the carrier at a flow rate of 1 ml/min. The oven, injector, and detector temperatures were kept at 80 °C, 150 °C, and 300 °C, respectively.

Styrene oxide in cyclohexane phase was analyzed by a gas chromatograph (M600D, Young-Lin, Korea) equipped with a flame ionization detector and a Supelco β-DEX 120 column (fused silica cyclodextrine capillary column, 60 m, 0.25 mm ID, and 0.25 μm film thickness) with split injection (1 : 100). Helium was used as carrier gas at 0.5 ml/min, and the oven, injector, and detector were at 110 °C, 250 °C, and 250 °C, respectively.

Cell density was measured by a Lambda 20 spectrophotometer (Perkin-Elmer, USA) at 660 nm. One O.D. unit corresponded to 0.3 g cell/L.

RESULTS AND DISCUSSION

1. Isolation and Identification of Various Styrene Degrading Bacteria

Many different colonies were developed on minimal medium agar plates when the microbial sources from a biofilter were incubated with styrene as a sole carbon and energy source. Among the numerous colonies, twelve bacterial isolates that appeared to be morphologically different were selected. They were designated as SP1 to SP4 for gram-positive strains and as SN1 to SN8 for gram-negative strains. Table 1 shows identification of the isolates based on physi-

Table 1. The characterization and identification of 12 styrene-degrading strains

Isolated strains	Gram staining	Cell type	Oxidase test	Specific activity (U/g cell)	Identification[a] (%Id)
SP1	+	coccus	n[b]	21.4	*Micrococcus* sp. (98.6)
SP2	+	rod	n	71.26	*Corynebacterium* sp. (92.2)
SP3	+	coccus	n	35.59	*Micrococcus* sp. (98.8)
SP4	+	chain coccus	n	26.36	*Micrococcus* sp. (98.8)
SN1	-	short rod	+	170	*Pseudomonas putida* (99.7)
SN2	-	rod	-	30.65	*Flavimonas* sp. (<10)
SN3	-	rod	+	22.99	*Burkholderia* sp. (<10)
SN4	-	rod	+	125	*Brevundimonas* sp. (82.7)
SN5	-	chain rod	-	2.29	*Pasteurella* sp. (49.7)
SN6	-	coccus	-	28.74	*Neisseria* sp. (<10)
SN7	-	rod	-	5.79	*Flavimonas* sp.(<10)
SN8	-	coccus	-	1.1	*Neisseria* sp. (91.1)

[a]Identified by API except for SN1 that was identified by both API and 16S rDNA analysis
[b]Not determined

cochemical properties. The strains SN1, SN3 and SN4 identified as *Pseudomonas*, *Burkholderia* and *Brevundimonas*, respectively, might belong to a same species since they are very close phylogenetically to each other [Yabuuchi et al., 1992]. These microbes are known for having a degrading activity on styrene as well as other aromatic compounds including toluene [O'Connor et al., 1995]. The strains *Pasteurella* and *Neisseria* could grow on styrene but showed a very low or almost negligible activity in styrene degradation. They have not been reported as styrene degraders before. Among gram-positive bacteria, *Micrococcus* was dominant. In the literature, many microorganisms have already been reported to grow on styrene and the partial list includes *Pseudomonas* sp. [Velasco et al., 1998; Panke et al., 1998], *Pseudomonas putida* [Nothe et al., 1994; O'Connor et al., 1995], *Pseudomonas fluorescens* ST [Marconi et al., 1996], *Xanthobactor* sp. [Hartmans et al., 1989], *Corynebacterium* sp. [Itoh et al., 1996], and *Rhodococcus rhodochous* NCIMB 13259 [Warhurst et al., 1994]. The present study indicates that there might be more diverse styrene-degrading bacteria than discovered thus far.

Table 1 also shows the specific activity of styrene degradation of the new isolates. Among 12 strains, three strains, SP2, SN1 and SN4, had relatively high activities. The strains SP1 and SP2 exhibited large colonies in agar plate culture, but the activity was lower than SN1 and SN4. The strain SN1 showed the highest activity as 170 U/g DCW which is one of the highest values reported so far [O'Connor et al., 1995; Panke et al., 1998].

The strain SN1 was further identified by 16S rDNA analysis. When analyzed with NCBI BLAST program, the sequencing data of SN1 showed a high similarity (>99%) to those of *Pseudomonas putida* with bits score 825, E value 0. The strain was designated as *Pseudomonas putida* SN1 and chosen for further studies.

2. Growth of *P. putida* SN1 and Degradation of Aromatic Compounds

Fig. 1 shows the growth of *P. putida* SN1 on various aromatic compounds. Seed culture was conducted in M9+ medium and washed twice with M9 medium before inoculation to remove nutrients carryover from M9+ culture. *P. putida* SN1 could grow on styrene, but not on benzene and toluene. In case of styrene oxide, cells could

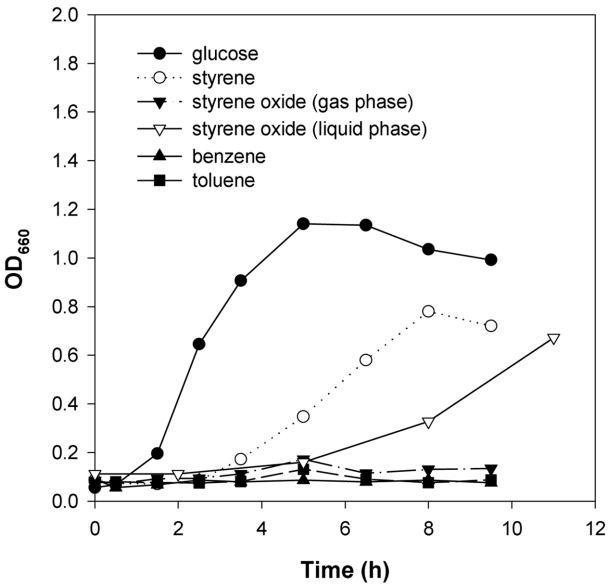

Fig. 1. Growth of *Pseudomonas putida* SN1 on different carbon sources.

grow when it was supplied to the liquid phase only (Fig. 1). This is attributed to the low volatility of styrene oxide, whose vapor pressure is 0.3 mmHg at 20 °C. In comparison, the vapor pressure of styrene is 4.5 mmHg at the same temperature [Chemical Safety Information from International Programme on Chemical Safety (www.inchem.org)]. Fig. 1 indicates that SN1 cannot utilize benzene or toluene as a carbon and energy source for its growth. The maximum specific growth rates in M9+, M9-styrene and M9-styrene oxide were estimated to be 1.13, 0.44, and 0.24 h^{-1}, respectively.

Fig. 2 shows degradation of various aromatic compounds by *P. putida* SN1. The cells were cultured in M9+ medium and challenged to each substrate before and after induction with styrene. SN1, after induction, could degrade styrene rapidly from the beginning; whereas, the degradation by uninduced cells started after about 30 min passed. This indicates that the activity of styrene degradation

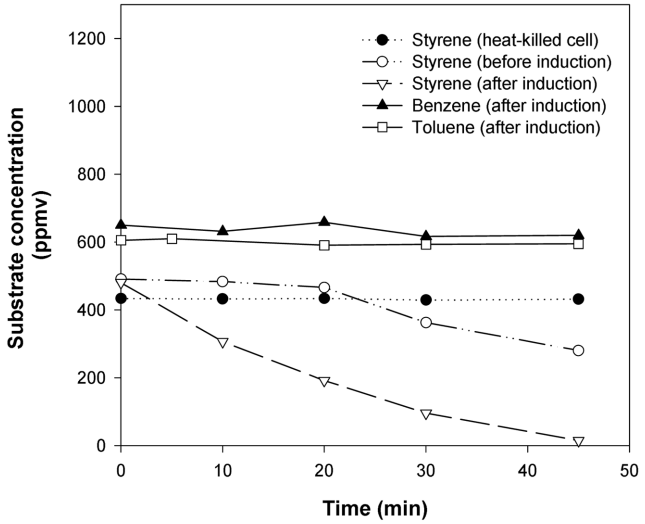

Fig. 2. Degradation of various aromatic compounds by *Pseudomonas putida* SN1.

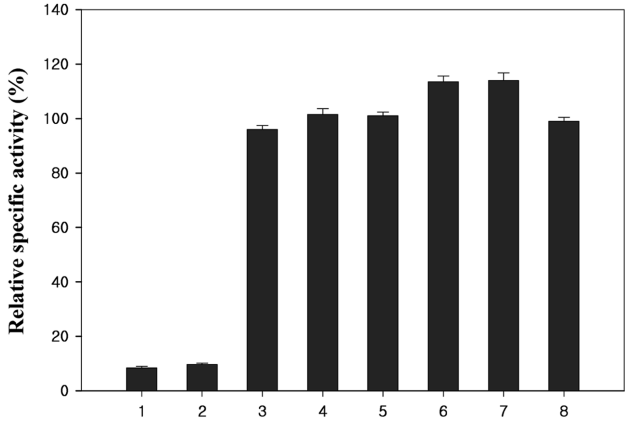

Fig. 3. Effect of medium composition on styrene-degrading activity of *P. putida* SN1. 1, culture broth without styrene (uninduced); 2, culture broth with rifampicin (20 μM); 3, culture broth; 4, phosphate buffer (Pi); 5, Pi plus 0.5 g/L yeast extract; 6, Pi plus 0.5 g/L glucose; 7, fresh M9 medium plus 0.5 g/L glucose; and, 8, M9+. The relative activity of 100% corresponds to 170±20 nmol/mg cell·min.

in SN1 appears only upon exposure to styrene. For several *Pseudomonas* strains [Beltrametti et al., 1997; Panke et al., 1998], the gene expression of SMO has been reported to be regulated by styrene at transcription level. Benzene and toluene were not degraded by SN1, suggesting that styrene-degrading enzyme did not have the activity on these compounds. These results, along with the growth experiments, propose that the styrene degradation in this strain involves an SMO rather than a dioxygenase, as in many bacterial strains such as previously reported *Pseudomonas* [Beltrametti et al., 1997; Panke et al., 1998]. Dioxygenase attacks the benzene ring of styrene and thus its activity coincides with that on benzene and toluene [Warhurst et al., 1994]. More direct evidence for the presence of SMO in SN1 can be obtained by detecting the formation of styrene oxide from styrene. However, it was not possible with SN1, probably due to the much faster rate of styrene oxide degradation than its formation from styrene.

Styrene degradation activity was also determined for crude cell extract. The activity was estimated as 144 nmol min^{-1} mg $protein^{-1}$, which was lower than that of unbroken cells. In many cases, broken cells give a higher activity than unbroken cells since the cell wall barrier for substrates transfer is removed. However, if a cofactor is required for the reaction or the multiple subunits of an enzyme dissociate upon cell breakage, the broken cells give a lower activity [Hollmann et al., 2003]. The SMO in many *Pseudomonas* strains consists of two components and its reaction is NADH-dependent [Beltrametti et al., 1997; Panke et al., 1998]. These seem to be related to the lowered activity in the broken cell extract of SN1.

3. Induction of Styrene Degrading Activity in *P. putida* SN1

Fig. 3 shows the effect of medium composition on the induction of the styrene degradation activity. When incubated with 1 mM styrene for 4 h, the cells exhibited almost the same specific activities regardless of the medium composition used. When rifampicin, a transcription inhibitor, is added during the induction, the activity did not appear. This indicates that the induction is associated with the synthesis of new protein(s) which did not exist during the cell growth in M9+ medium. The presence of carbon or nitrogen source or the kind of carbon source (glucose vs. citrate) did not affect the induction of styrene degrading activity. This suggests that the cells grown in M9+ medium do not need any supplemental carbon or nitrogen sources for the induction of styrene degrading activity, and the induction can be easily accomplished by simply exposing the cells to styrene. Especially, it should be pointed out that the presence of glucose or citrate did not inhibit the induction of the SMO enzyme in SN1. In some *Pseudomonas* strains, SMO has not been expressed when a readily usable carbon source is present due to catabolite repression [O'Leary et al., 2001]. Since glucose is often included in *Pseudomonas* culture medium for rapid cell growth or is required for cofactor regeneration during biocatalysis, it is an important merit for SN1 not to suffer from catabolite repression.

Fig. 4 shows induction profile of the styrene degrading activity. The cells were grown in M9+ medium for 8 h, and styrene was added

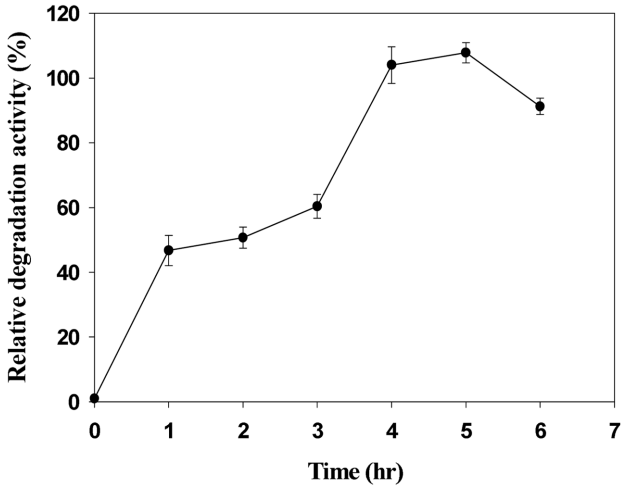

Fig. 4. Time course profile of styrene-degrading activity in *P. putida* SN1. The relative activity of 100% corresponds to 170 nmol/mg cell·min.

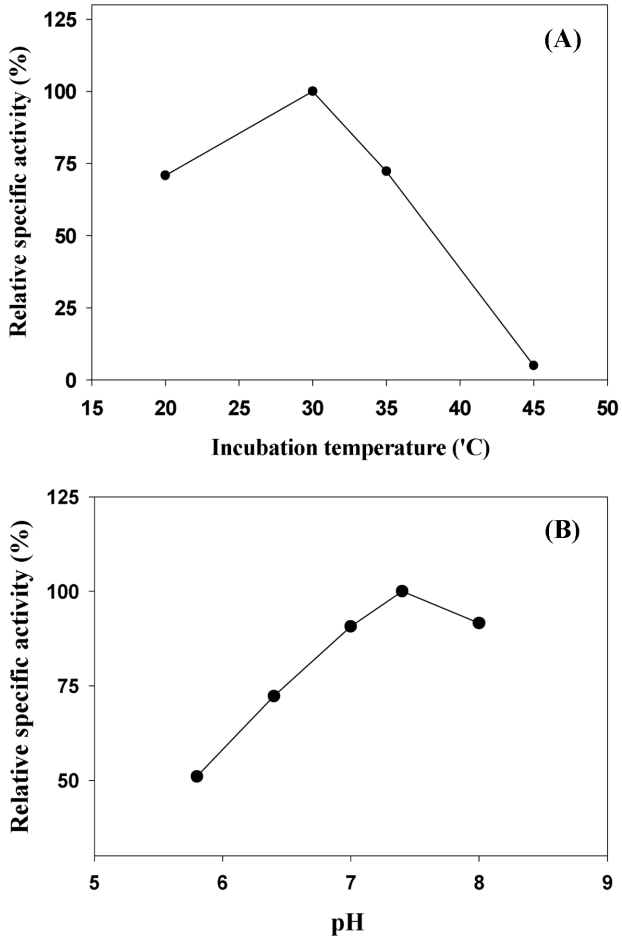

Fig. 5. Effect of temperature (A) and pH (B) on styrene-degrading activity. The relative activity of 100% corresponds to 170 ±20 nmol/mg cell·min.

to be 1 mM to the culture without any nutrient supplementation (Fig. 3). Two separate experiments were conducted and the results were averaged. The activity increased rapidly during the first 1 h and between 3 and 4 h, and rather slowly in other periods. The rapid increase between 3 and 4 h is unexpected, but no further efforts to reveal the reason were attempted. The maximal activity was observed at 4-5 h, which decreased slightly at 6 h.

4. Effect of Temperature and pH on Styrene Degrading Activity in *P. putida* SN1

Fig. 5 shows the effect of temperature and pH on styrene degrading activity in *P. putida* SN1. Cells were cultivated in M9+ medium and induced with styrene for 4 h. Initial styrene was about 450-500 ppmv and cell was 0.06 mg/ml. When measured at a constant pH of 7.0, the whole cell activity was maximal at 30 °C. Effect of pH was studied at 30 °C and the maximum activity was observed at pH 7.5.

5. Effect of Substrate Concentration: Michaelis-Menten Kinetics

Styrene biodegradation in this study is a two-phase reaction. Most styrene is present in the gaseous phase and should be transported to the liquid phase to be degraded. The degradation rate is dependent on the liquid-phase concentration rather than the gas-phase concentration. However, the liquid-phase concentration is hard to measure, since the solubility of styrene in water is low as 0.029% (w/v) at 20 °C and the liquid-phase styrene is continuously consumed by cells. For sparingly soluble gases, Klasson et al [1993] has suggested a simple method to estimate liquid-phase concentration from gas-phase concentration and gas-liquid mass transfer coefficient. The same method was employed to estimate liquid-phase styrene concentration.

Styrene balance in the gas-phase is expressed as:

$$-\frac{K_La}{H}(C_G-C_L)V_L=\frac{dN_G}{dt} \quad (1)$$

While, styrene balance in the liquid-phase is:

$$\frac{K_La}{H}(C_G-C_L)V_L-qXV_L=\frac{V_L}{H}\frac{dC_L}{dt} \quad (2)$$

Eq. (1) can be rearranged as:

$$C_L=C_G+\frac{H}{K_LaV_L}\frac{dN_G}{dt} \quad (3)$$

Symbols are: C, styrene concentration (ppmv); H, Henry's law constant for styrene (L ppmv/mol); K_La, overall mass-transfer coefficient (h^{-1}); N_G, moles of gaseous styrene (mol); V_L, Volume of liquid-phase (L); q, specific styrene uptake rate (mol/g·h); X, cell concentration (g/L).

Eq. (1) indicates that the mass-transfer coefficient can be determined from the plot $-1/V_L$ [dN_G/dt] vs. C_G when C_L is zero. When C_G is very low, overall reaction rate is controlled by gas-liquid mass transfer rate and the liquid-phase concentration is assumed to be practically zero. In Fig. 6, several batch experiments at varying cell concentrations were given. There was a linear relationship between $-1/V_L$ [dN_G/dt] and C_G at low C_G regardless of cell concentrations (r^2=0.99 when the data were taken up to C_G=100 ppmv). From the slope (K_La/H), the volumetric mass-transfer coefficient (K_La) was estimated to be 0.28 nmol/ml/min/ppmv. Once K_La is determined, C_L is calculated from from C_G and dN_G/dt from Eq. (3). Fig. 7 shows

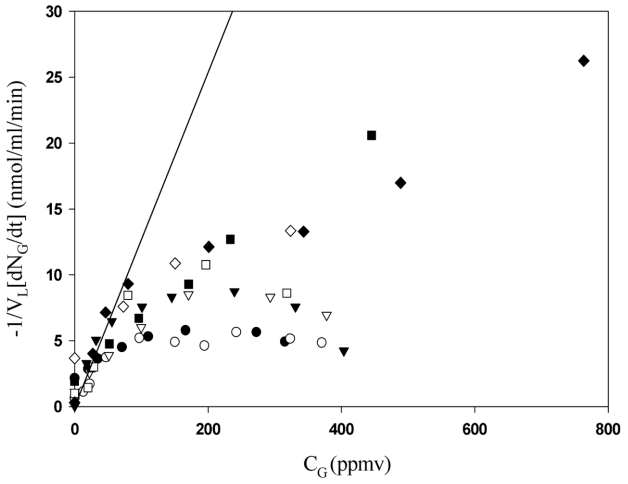

Fig. 6. Determination of the volumetric mass-transfer coefficient. Duplicate experiments at each cell concentration were performed and the results were averaged. Cell concentrations were 0.15 (◆), 0.12 (■), 0.09 (▼) and 0.06 g/L (●).

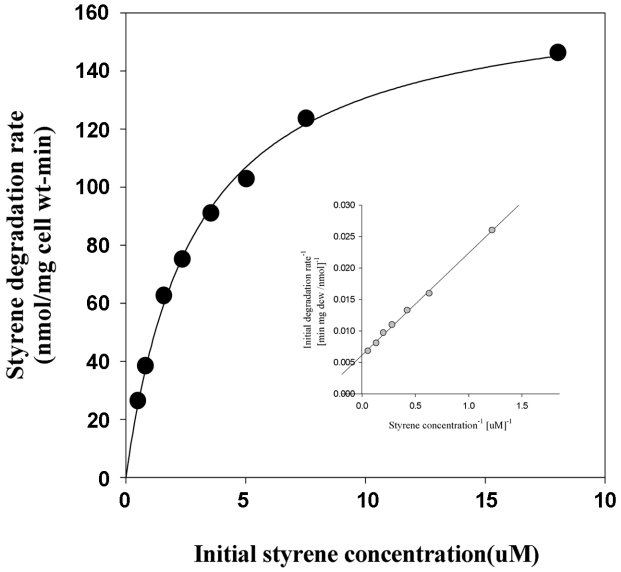

Fig. 7. Effect of styrene concentration on its degradation rate. Symbols represent experiments and solid line indicates theoretical calculation based on Michaelis-Menten model equation. Insert is a double-reciprocal plot to calculate kinetic constants.

the relation between specific styrene degradation rate and liquid-phase styrene concentration, which was obtained from initial rate experiments. The experiments were conducted at varying initial gas-phase concentrations (20-600 ppmv) and varying cell concentrations (0.02-0.06 g/L) for 10 min. Then, the rate of styrene degradation was measured from gas-phase concentration between 5 and 10 min under the assumption that liquid-phase concentration was constant during that period. The liquid-phase concentration was estimated from Eq. (3) with the K_La value obtained from Fig. 6. As shown in Fig. 7, the styrene degradation kinetics followed well the Michaelis-Menten kinetics. The maximum specific degradation rate (V_{max}) and saturation constant (K_m) were determined from the Lineweaver-Burke plot (Fig. 7) as 180 nmol/mg cell/min and 2.5 μM respectively. Substrate inhibition was not observed up to 1,200 ppmv styrene.

CONCLUSIONS

Twelve styrene-degrading bacteria have been isolated and one strain *Pseudomonas putida* SN1 that had a high styrene degradation activity was obtained. SN1 was speculated to have a styrene monooxygenase as the first enzyme that initiates styrene catabolism. The styrene degradation activity in SN1 was induced when incubated with styrene and the induction was not inhibited by the presence of readily usable carbon sources such as glucose and citrate. The fully induced SN1 had a high SMO activity of 180 nmol/mg cell/min. SN1 is regarded as a potentially useful strain to be developed into biocatalysts for producing styrene oxide or treating styrene-containing wastewater or waste gas.

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