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Mineralization of aminobenzenesulfonates by a newly isolated bacterial co-culture (AS1 and AS2)

***Singh S[1], Awasthi SK[1], Iyengar L[3], Ashfaq M[2], Singh P[4]**

[1] Department of Life Sciences, C.S.J.M. University, Kanpur - 208024, India.
[2] Department of Microbiology, Jawaharlal Nehru Medical College, A.M.U., Aligarh, India.
[3] Department of Chemistry, Biotechnology Laboratory, Indian Institute of Technology (IIT), Kanpur - 208016, India.
[4] Division of Toxicology, Central Drug Research Institute, Lucknow - 226001, India.

***Corresponding Author:** surjeetunique@yahoo.co.in

Abstract

Aminobenzenesulfonates (ABS) are important building blocks of azo dyes and are used in the manufacture of few pharmaceuticals and pesticides. Thus, they are synthesized in large quantities and released into the environment through wastewaters emanating from these industries. Xenobiotic character of ABS renders these compounds resistant to degradation by unadapt activated sludge and bacterial species utilizing normal aromatics. Degradation of ABS isomers by a co-culture of *Agrobacterium* sp. strain PNS-1 and 2-ABS degrading bacterial consortium (BC) was studied. Among the ABS isomers, strain PNS-1 could only utilize 4-ABS as the growth substrate, whereas BC (AS1 & AS2) could degrade only 2-ABS. The co-culture, however, could completely mineralize both these isomers, whereas 3-ABS was not degraded. Studies on the effect of the presence of glucose, an easily assimilable and non-toxic substrate, on the degradation of 2- and 4-ABS by the co-culture showed that 4-ABS degradation rate was not significantly affected, whereas decrease was observed in 2-ABS removal rate. These observations show that a mixed ABS can only be mineralized by co-cultures of specialized bacterial strains, as individual strains have a very limited substrate range.

Keywords: Biodegradation; Mineralization; Aminobenzenesulfonates; Bacterial co-culture; Bacterial Consortium; ABS.

Introduction

Aminobenzenesulfonates (ABS) are important constituents of many azo dyes, pesticides and pharmaceuticals (Lidner, 1985). The presence of sulfonic acid group on the aromatic ring confers xenobiotic and polar character to these compounds, as these compounds are seldom synthesized in nature (Laskin and Lechevelier, 1984). Further, sulfonic acid group is completely dissociated at physiological pH making these compounds polar, thus limiting their entry into the cell in the absence of specific transport proteins. Nevertheless, few mixed cultures and pure bacterial strains, which could utilize ABS as the sole carbon and energy source, have been isolated (Thurnheer et al., 1986, 1988; Feigal et al., 1993; Perei et al., 2000; Coughlin et al., 2003; Tan et al., 2005; Singh et al., 2006). Many of these strains have narrow substrate specificity and often utilize only one benzenesulfonate isomer as the growth substrate. However, biodegradation of mixtures of ABS isomers is important, as industrial wastewaters often have a combination of these compounds. The studies on 2-ABS degradation, with *Alcaligenes* sp. strain O-1, have shown that this strain can utilize 2-ABS, 4-toluenesulphonate and benzenesulfonate as growth substrates (Kneimeyer et al., 1990). On the other hand, 4-ABS degrading pure cultures exhibit a very narrow specificity and can utilize this specific isomer. As per our knowledge, no detailed study on 3-ABS degrading culture has been reported so far. One approach for the treatment of wastewaters containing these sulfoaromatics is the genetic construction of strains with broader substrate specificity. However, their behavior with mixed substrates as well as their stability in treatment units cannot be predicted. Further, the regulation of their use in wastewater treatment units is still debated. Another approach is to develop separate enrichment or pure cultures for the degradation of each of these isomers and explore their suitability for the degradation of mixed substrates. Few ABS degrading cultures have been recently isolated in our laboratory. The aim of this study is on their specificity towards each ABS isomer as well as on the degradation of mixtures of these isomers using a combination of these cultures.

Materials and Methods

Bacterial cultures
4-ABS degrading bacterial strain PNS-1 was isolated from the enrichment culture developed using activated sludge from an aerobic biological unit treating Kanpur city domestic wastewater. Bacterial consortium AS1 & AS2, which can utilize 2-ABS as the sole carbon and energy source, was derived from the sludge taken from an effluent treatment plant of a large chemical manufacturing industry located in Rasayani, India.

Medium and culture conditions
The growth medium (MM) used, in the present study, consisted of the following constituents: 12.0 g Na_2HPO_4, 2.0 g K_2HPO_4, 0.5 g NH_4Cl, 0.1 g $MgCl_2.6H_2O$ and 0.05 g $CaCl_2.2H_2O$ per litre of distilled water. 1 ml filter sterilized trace element solution (Kneimeyer et al., 1990) and required volumes of ABS from a stock solution (5 gL^{-1} neutralized to pH 7.0 using 1N NaOH) were added to the growth medium after sterilization. Final pH of the medium was 7.0 ±0.2. Cultures were grown in 100 ml liquid medium taken in 250 ml Erlenmeyer flasks, which were kept in a rotary shaker incubator (120 rev min^{-1}) at 35±2^oC.

ABS degradation and bacterial growth
ABS degradation was monitored at an initial concentration of 400 mgL^{-1}. Strain PNS-1 or BC (AS1 & AS2), grown up to a late exponential phase, was used as the inoculum (10% v/v) for studies on the degradation of individual isomers. Initial biomass optical density at 555 nm was generally below 0.1. Aliquots were withdrawn periodically. Bacterial growth was determined by measuring the turbidity at 555nm. Samples were then centrifuged at 1100×g (3400 rpm) and ABS was estimated in the supernatant. Uninoculated controls with the organic carbon source were always included in experiments.

Degradation of ABS mixtures was studied at an individual ABS isomer concentration of 400 mgL^{-1} and cultures of strain PNS-1 and BC were used as the inocula. Kinetic studies on ABS removal were carried out, after growing the mixed culture for three cycles on mixed ABS substrates. Chemical oxygen demand (COD) was determined with 0.45 µ membrane filtered culture samples taken out just after inoculation and at the end of the exponential growth phase.

Effect of glucose on ABS degradation
Removal of mixed ABS substrates was studied in the presence of alternate organic carbon source. For adapting the culture to these growth substrates i.e. glucose, 2-ABS and 4-ABS, cultures were grown for three growth cycles prior to the determination of growth and substrate removal kinetics.

Analytical procedures
Biomass growth was monitored at 555 nm against distilled water in a UV-Visible Spectrophotometer (Shimadzu, Japan, model 160A). An optical density of 1.0 represented 340 mg cell dry weight per litre. ABS isomers were estimated by measuring the absorbance at their λ_{max} in the UV-Visible Spectrophotometer. When used in combination, 2 and 4-ABS were quantified by measuring the absorbance at 244 nm for total ABS removal and at 290 nm for 2-ABS removal, as 4-ABS did not exhibit the absorbance at 290 nm. HPLC method was also used in few experiments for the quantification of ABS. 20µl of membrane (0.45 µm) filtered sample was injected to 4.0x250 mm ODS C_8 column (Hypersil MOS2 SU). Acetic acid solution (0.5 %) was used as the solvent and flow rate was maintained at 0.3 ml/min. Emerging peaks were detected at 237 nm (2- & 3- ABS) and 248 nm (4-ABS) using a UV-Visible detector (Amersham Pharmacia Biotech-900, UV-900). Under these experimental conditions, 2-, 3- and 4-ABS had retention times of 14.2 min, 11.8 and 10.1 min respectively. Chemical oxygen demand (COD) was determined by close reflux method as per the procedure given in the standard methods (Greenberg et al., 1992). Glucose in culture filtrates was determined using glucose oxidase-peroxidase method (Erba Mannheim test kit).

Results

ABS degrading bacterial cultures
Enrichment cultures for 2-ABS and 4-ABS degradation were developed in batch cultures under aerobic conditions with the specific isomer as the growth substrate. Strain PNS-1 was isolated from 4-ABS degrading enrichment culture. Cultivation of an enrichment culture for more than a year on 2-ABS, as the sole carbon and energy source, yielded a stable bacterial consortium (BC), which consisted of two bacterial strains. Present investigations were carried out with 4-ABS degrading *Agrobacterium* sp. strain PNS-1 and 2-ABS degrading BC (AS1 & AS2).

Degradation of ABS isomers by Agrobacterium sp. *strain PNS-1*

Kinetics of 4-ABS degradation by strain PNS-1 is shown in Fig. 1. 4-ABS was rapidly utilized and >95% degradation was observed in 12 h. Specific growth rate (μ) and mean generation time were calculated to be 0.19 h^{-1} and 3.6 h respectively. Biomass yield was 0.34 mg/mg 4-ABS degraded. Mineralization of the substrate was ascertained by COD analysis of the culture filtrate prior to and after the growth of strain PNS-1 on 4-ABS up to a late exponential phase (Table 1). Theoretical COD for ABS is 1.4 mg/mg ABS. Thus, expected initial COD at 400 mgL^{-1} ABS is 560mgL^{-1}. Observed COD of the culture medium, just after inoculation, was 640mgL^{-1}. At the end of the exponential phase, COD of the culture filtrate was 60mgL^{-1}. This clearly indicated the extensive mineralization of 4-ABS by the strain PNS-1.

2 and 3-ABS (400 mgL^{-1}) were tested as the sole carbon/energy source for the growth of *Agrobacterium* sp. strain PNS-1. Results showed that neither growth nor decreases in the substrate concentration/COD were observed with 2-ABS, while a marginal decrease (<10%) was observed with 3-ABS in 24 h, which remained constant even after 48 h of incubation.

Degradation of ABS by BC (AS1 & AS2)

Fig. 2 presents the kinetics of 2-ABS removal and the growth of BC. Degradation of 400 mgL^{-1} 2-ABS required around 21 h. Specific growth rate and the mean generation time for BC were calculated to be 0.104 h^{-1} and 6.65 h respectively. Biomass yields of 0.38 mg/mg 2-ABS degraded was marginally lower to that observed with 4-ABS. Thus, the growth rate of BC with 2-ABS as the growth substrate was approximately half as compared to the strain PNS-1 on 4-ABS at equimolar concentrations.

UV-Visible spectra of culture filtrates drawn at different time intervals, during the growth phase, did not show any changes except for the absorbance decrease at 237 nm (spectra not shown). This showed that there was no accumulation of any intermediate during 2-ABS degradation. No change in 2-ABS concentration was observed in the absence of the culture or in the presence of heat killed BC. These observations showed that the decrease in absorbance was due to biodegradation of 2-ABS. COD analysis indicated extensive mineralization of 2-ABS (Table 1). BC could not utilize 4-ABS as the growth substrate. However, marginal decrease (<15%) in 3-ABS concentration was observed in 48 h, which remained constant even up to 120 h.

Degradation of a combination of ABS isomers by the co-culture consisting of Agrobacterium sp. *strains PNS-1 and BC*

Batch degradation studies were conducted, using either 2- and 4-ABS or 2-, 3- and 4-ABS at an initial substrate concentration of 400 mgL^{-1} of each isomer, using the co-culture of strain PNS-1 and BC. When 2- and 4-ABS were used together as growth substrates, 4-ABS was undetected beyond 12 h and >90% 2-ABS degradation was observed in 21 h (Fig. 3). Further, neither 2- nor 4-ABS was preferentially utilized. UV-Visible spectrum as well as percent COD removal (Table 1) after the growth of the co-culture indicated mineralization of both these isomers.

Effect of the addition of 3-ABS (400 mgL^{-1}) along with 2- and 4-ABS in the growth medium of co-culture was also studied. Initial (total) ABS concentration was 1200 mgL^{-1} and COD was in the range of 1580-1600 mgL^{-1}. COD removal was only around 72%, as compared to 91% with 2- and 4-ABS at the end of the growth phase (data not shown).

Degradation of ABS by co-culture in the presence of glucose

Co-culture, consisting of strains PNS-1 and BC (AS1 & AS2), was acclimatized to the presence of glucose, 2- and 4-ABS as growth substrates. MM medium, supplemented with 400 mgL^{-1} of each of these substrates was used for three growth cycles prior to the use of co-culture as an inoculum for kinetic studies. Substrate removal as well as biomass growth is presented in Fig. 4. More than 85% biomass growth was observed during initial 9 h. Simultaneous removal of glucose and 4-ABS was observed, although glucose removal rate was higher. Substrate degradation rate of 2- and 4-ABS by BC and PNS-1, under different experimental conditions, is presented in Fig. 5. 4-ABS degradation was not significantly changed in the presence of either 2-ABS or 2-ABS and glucose. On the other hand, 2-ABS removal rate was affected in the presence of 4-ABS and glucose.

Discussion

There are few reports on mixed and pure bacterial cultures, which can utilize specific ABS isomers as the sole carbon and energy source (Thurnheer et al., 1986, 1988; Feigal et al., 1993; Perei et al., 2000; Coughlin et al., 2003;

Tan et al., 2005; Singh et al., 2006). Studies on the mineralization of a combination of these isomers by a co-culture are reported in this communication.

2- and 4-ABS degrading cultures were developed in the laboratory using batch enrichment technique. It was observed that 4-ABS degrading enrichments could be developed with several inocula. *Agrobacterium* sp. strain PNS-1 was isolated from one such enrichment. On the other hand, 2-ABS degrading bacterial consortium could be derived only from one source inoculum. Both strains, PNS-1 and BC, were highly specific and could utilize only 4-ABS and 2-ABS respectively. However, it should be mentioned that the strain PNS-1 and BC (AS1 & AS2) could degrade nonsulfonated aromatic compounds (data not shown).

Earlier studies have also shown that 4-ABS degrading bacterial strains, *Hydrogenophaga intermedia* strain S-1 and *Pseudomonas paucimobilis*, could not utilize 2- and 3-ABS. Detailed studies on 2-ABS degradation has been carried out only with *Alcaligenes* sp. strain O-1 (Thurnheer et al., 1986). This strain could also utilize benzenesulfonate and toluene-4-sulphonate as growth substrates (Thurnheer et al., 1986). Further studies with strain O-1 showed that cell free extracts could desulfonate these as well as 3-aminobenzenesulphonate, 4-aminobenzenesulfonate and 4-hydroxybenzenesulphonate on which the strain was unable to grow. Based on these observations, Thurnheer et al. (1990) proposed that strain O-1 was unable to utilize later three aromatic sulfonates due to the lack of specific transport proteins. Tan et al. (2005) have recently reported that their enrichment culture could utilize 2-ABS and 4-ABS, but not 3-ABS. In the present study, BC could utilize only 2-ABS.

HPLC analysis as well as UV-spectral analysis showed that there was no accumulation of any detectable intermediate, during the degradation of 4-ABS or 2-ABS under the experimental conditions used in this study. Feigal and Knackmuss (1993) have showed that catechol-4-sulphonate accumulated in the culture filtrate, when *Hydrogenophaga intermedia* strain S-1 was grown in a medium containing 4-ABS and other complex organics. Catechol-3-sulphonate was detected during 2-ABS degradation by *Alcaligenes* sp. O-1 only in the presence of 3-chlorocatechol (Junker et al., 1994).

Conclusion

A co-culture of strain PNS-1 and BC (AS1 & AS2) could utilize 2- and 4-ABS. Complete mineralization of 2- and 4-ABS by the co-culture was shown by COD analysis. 3-ABS could not be utilized by the co-culture. Preliminary studies with the immobilized co-culture in an aerated sequential batch reactor have shown that the degradation of both these isomers can be maintained for extended periods (results not shown). Thurnheer et al. (1988) have reported that a co-culture consisting of five defined bacteria was able to degrade at least five substituted benzenesulfonates and even after operating for a long time, there was no competition was detected. Further, they were able to isolate few strains after continuous culturing for 30 months, which could utilize all five sulfonates. However, as per our knowledge, no further studies have been reported on these isolates. Recent studies by Tan et al. (2005) showed that both 2- and 4-ABS were degraded in a bioreactor bioaugmented with a 4-ABS degrading culture derived from Rhine sediment, whereas 3-ABS could not be degraded. It is generally observed that sulfonated aromatic amines are difficult to degrade and requires enrichment of specialized microbes. This is mainly due to their polar nature, which obstructs membrane transfer. Further, many of these isolated strains exhibit narrow substrate specificity for a specific isomer. Thus, biodegradation of mixed aminobenzenesulfonates may only be possible with mixed bacterial consortia.

Bacterial genes encoding enzymes required for the biodegradation of aromatic pollutants are often regulated in response to the availability of the respective substrate. However, if a rapidly metabolizing carbon source, such as glucose, is additionally present (which is often the case in wastewaters), then the synthesis of peripheral enzymes required for the pollutant degradation, can be affected. Hence, the effect of glucose on 2- and 4-ABS removal by the co-culture was studied. Results showed that glucose did not significantly affect 4-ABS removal. A longer lag period and degradation time was observed with 2-ABS. to the availability of the respective substrate. Present observation shows that their degradation is feasible even in the presence of glucose, if the inducers are present. However, the rate of degradation may be affected.

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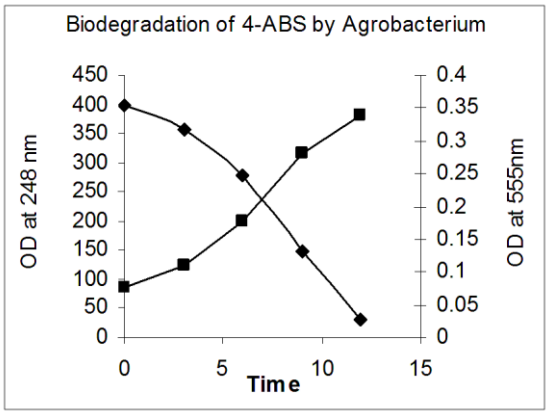

Figure 1. Degradation of ABS by *Agrobacterium* sp. strain PNS-1 (■) 4-ABS and (●) Growth.

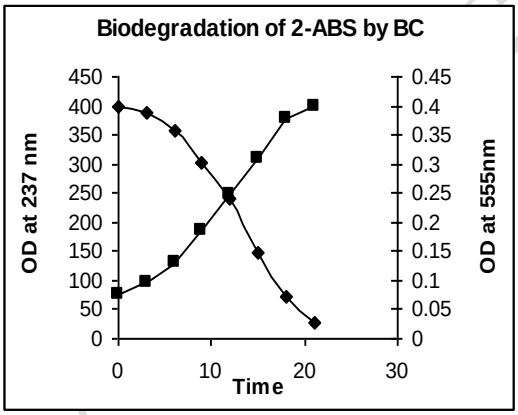

Figure 2. Degradation of 2-ABS by Bacterial Consortium (■) 2-ABS and (●) Growth.

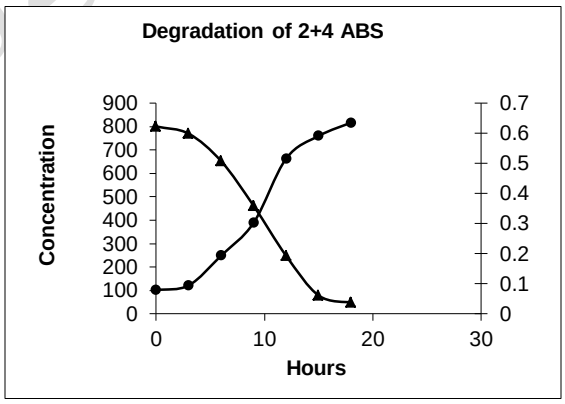

Figure 3. Degradation of 2+4 -ABS by Bacterial Co-culture (■) 2-ABS and (●) Growth.

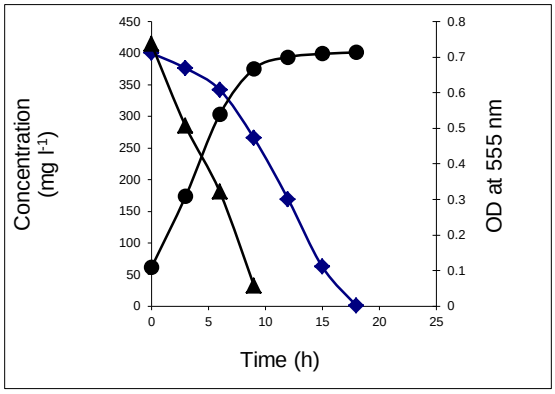

Figure 4. Kinetics substrate removal and growth of 2-ABS and glucose acclimated culture. (▲) Glucose removal, (■) 2-ABS degradation and (●) 2-ABS growth.

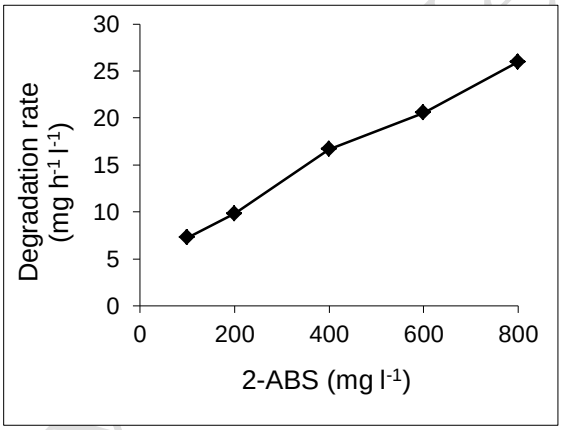

Figure 5. Relation between substrate utilization rate and initial 2-ABS concentration.

Table 1. Analysis of Chemical Oxygen Demand.

Influent COD (mg/l)	Effluent COD (mg/l)	% of COD Removal
1180	180	83.63
1100	80	92.72
1120	100	91.07

COD removal for 2-ABS (400 mg/l) + 4-ABS (400 mg/l)