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ORIGINAL PAPER

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Biodegradation of 8-anilino-1-naphthalenesulfonic acid by *Pseudomonas aeruginosa*

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Abstract *Pseudomonas aeruginosa*, isolated from soil near tannery effluent was able to degrade 8-anilino-1-naphthalenesulfonic acid (ANSA), a sulfonated aromatic amine. The organism degraded this amine up to a concentration of 1,200 mg l^{-1} using glucose and ammonium nitrate as carbon and nitrogen sources respectively. The degradation started when the organism reached its late exponential growth phase. Salicylic acid and β-ketoadipic acid were identified as intermediate compounds using HPLC and GC–MS and provide evidence for ortho pathway reactions. Further proof for the pathway is obtained from the dioxygenase activity of the strain growing exponentially in medium with ANSA and glucose.

Keywords Biodegradation · 8-Anilino-1-naphthalenesulfonic acid · *Pseudomonas aeruginosa*

Abbreviation ANSA: 8-Anilino-1-naphthalenesulfonic acid

Introduction

Aromatic amines are widely used in the manufacture of dyes, inks, pesticides, rubber antioxidants, explosives, drugs, cosmetics, paints, varnish, lacquer, wood staining, curing agents for the synthesis of epoxy resins and polyurethanes [4] which makes them an indispensable group of chemicals. Aromatic amines can reach the environment from a variety of sources: Directly, as industrial waste from chemical manufacturing, energy technologies, coal conversion processing, resin and plastic manufacturing, textile and photographic industries and indirectly, from pesticide degradation or bacterial conversion of azo compounds into their original amines [3].

These aromatic amines are well known for their mutagenic and carcinogenic properties [4, 19, 23, 27]. Accumulation of these aromatic amines in the environment affects biogeochemical activities in the soil by inhibiting the growth of microorganisms and ultimately influencing the productivity of the soil [5, 6]. The sulfonic acid groups that are introduced to increase their water solubility make them recalcitrant to microbial degradation [7].

Peri acid and its derivatives are used extensively in dye industries for the synthesis of numerous dyes and dye intermediates. Reagent grade Phenyl peri acid is used as fluorescent probe for protein studies. ANSA (*N*-phenyl peri acid) was reported to be one of the intermediate compounds in the degradation of Navitan fast blue S5R [24, 25]. The objective of this investigation is to study the degradation of this intermediate compound and to identify the degradation products by various spectrophotometric and chromatographic methods.

Materials and methods

Microorganisms

The azo dye degrading strain of *Pseudomonas aeruginosa* used in this study was isolated from soil near the tannery site of the Central Leather Research Institute [24]. Stock cultures were stored at −20°C in 20% glycerol. The organisms from stock cultures were used after preculturing in nutrient broth.

Biodegradation studies

Biodegradation studies were carried out in duplicate in 250 ml conical flasks containing 50 ml of the culture medium. A stock solution of ANSA (Sigma chemicals, USA) was prepared by dissolving ANSA in 0.5 N NaOH

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Table 1 Biodegradation of ANSA at different concentrations by *Pseudomonas aeruginosa*

Concentration ($mg\ l^{-1}$)	24 h		48 h	
	Degradation rate ($mg\ min^{-1}$)	Growth rate ($mg\ min^{-1}$)	Degradation rate ($mg\ min^{-1}$)	Growth rate ($mg\ min^{-1}$)
100	0.055	0.647	0.03	0.317
200	0.085	0.574	0.055	0.265
250	0.094	0.551	0.062	0.239
500	0.163	0.48	0.092	0.191
1,000	0.227	0.398	0.133	0.167
1,200	0.178	0.346	0.122	0.153

and added separately to the medium. Care was taken so that the total volume in the flasks did not vary. The flasks were inoculated with 2% inoculum containing approximately 2×10^8 cells ml^{-1} of *P. aeruginosa*. The composition of the culture medium and culture conditions were as described previously [24]. Degradation of ANSA was followed spectrophotometrically by quantitatively estimating ANSA by the procedure described by Pielesz et al. [18], which utilizes the color reaction by diazotization of aromatic amines with α-naphthol (SD Fine chemicals, India).

Bacterial growth was monitored spectrophotometrically by reading the optical density at 600 nm. An absorbance of 1.0 at 600 nm corresponded to approximately 353 mg of cellular protein, estimated after boiling the cell pellets in 1 M NaOH for 20 min by Lowry method [13].

Analytical methods

Lyophilized culture supernatants containing the degradation products of ANSA (at a concentration of $100\ mg\ l^{-1}$) were subjected to Thin Layer Chromatographic analyses on fluorescent silica plates (Polygram Sil G/UV, 40 × 80 mm, Germany) using chloroform and ethanol in the ratio of 9:1. The samples were visualized by exposing the plates to UV light. The same samples as well as the chloroform extract of the spot obtained in the TLC analysis were subjected to FTIR analysis in a Perkin–Elmer RX1-FTIR spectrometer.

The culture medium containing the degradation products of ANSA was centrifuged and was filtered through 0.22 μm filters. Twenty-five microliter of this filtrate was subjected to HPLC analysis using a C-18 reverse phase column equipped with UV-visible spectrophotometer detector set at 235 nm and a solvent system consisting of methanol and water [24]. The ethyl acetate extract of the centrifuged culture supernatant was subjected to GC–MS analysis in a Perkin–Elmer Autosystem XL GC with a Turbomass MS Spectrometer [25].

The CO_2 [1] and NH_3 [12] released during degradation were studied by the procedure described earlier.

Oxygenase assay

Protocatechuic acid (3,4-dihydroxybenzoic acid) dioxygenase assays were carried out according to the method described by Stanier and Ingraham [21]. The bacterial cells grown in medium containing ANSA at a concentration of $50\ mg\ l^{-1}$ were harvested by centrifugation after 24 h of incubation and sonicated at 4°C (15 s, 70% output, 5 ×). Sonicated cells were centrifuged and the supernatant was assayed for protocatechuate 3,4-dioxygenase activity. The assay mixture contained 100 mM phosphate buffer (pH 7.0), 50 μl crude extract containing 56 μg of protein and 0.25 mM protocatechuic acid and readings were taken at regular intervals at 288 nm.

Results and discussion

Pseudomonas aeruginosa which had been isolated in an attempt to identify azo dye (Navitan Fast Blue S5R) degrading bacteria was able to degrade ANSA, one of the products of the bacterial degradation of this dye [24]. The efficiency of *P. aeruginosa* to degrade ANSA was checked by studying the degradation of ANSA at different concentration in the range of $100–1,500\ mg\ l^{-1}$ and the results are presented in Table 1. It was found that this organism was able to grow and degrade ANSA at concentrations up to $1,200\ mg\ l^{-1}$. No growth was observed above this concentration. The rate of degradation increased as the concentration of the amine was increased up to $1,000\ mg\ l^{-1}$. In contrast, the rate of bacterial growth decreased as the concentration of the amine was increased. The lower growth yield at higher ANSA concentration even in the presence of glucose in the medium suggested that ANSA is intrinsically toxic for this strain. When the period of incubation was increased to 48 h, the degradation rate for this amine decreased in all the concentrations studied. Changes in the uninoculated control were negligible.

Growth and ANSA degradation (at $100\ mg\ l^{-1}$) by *P. aeruginosa* were followed simultaneously at regular intervals. It was found that the degradation of ANSA started when the organism reached the late exponential growth phase and nearly 80% of the ANSA was degraded by 24 h (Fig. 1). Interestingly, the process is not under catabolic repression, in contrast with the situation for degradation of alkanes and toluene [9, 15].

TLC analysis of the lyophilized culture medium containing the degradation products of ANSA (at a

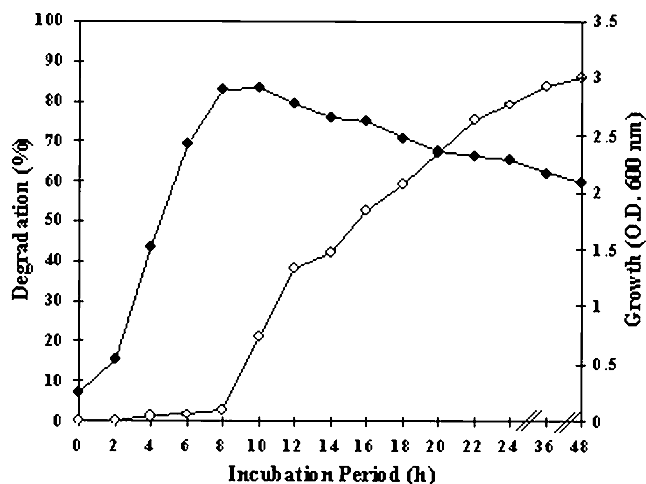

Fig. 1 Time course of ANSA degradation (100 mg l^{-1}) by *Pseudomonas aeruginosa*. ANSA degradation (*open circle*), bacterial growth (*filled circle*)

concentration of 100 mg l^{-1}) indicated the disappearance of ANSA (R_f value of 0.2) from the culture broth incubated up to 48 h and revealed a spot with the R_f value of 0.12 in the sample incubated for 48 h. Analysis of this spot by FTIR spectroscopy after extraction with chloroform showed peaks corresponding to C–H stretch at 2,917 and 2,841 cm^{-1} and C=O stretch at 1,727 cm^{-1} characteristic of aliphatic carboxylic acid. The IR spectra of the lyophilized culture broth drawn at different incubation period showed the disappearance of peak at 1,385 cm^{-1} assigned for sulfonic acid (SO_3H) group in the samples incubated for 24 h, which gave a clear indication for the desulfonation. The first step in the degradation of aromatic amines in most cases has been found to be desulfonation, which facilitates the transport of the compound across the membrane [8].

HPLC analysis of the culture supernatant containing the degradation products of ANSA at a concentration of 100 mg l^{-1} indicated the presence of salicylic acid having R_T value of 3.4 and 34.3 mg l^{-1} of salicylic acid could be found after 24 h of incubation which decreased to 19.2 mg l^{-1} after 48 h of incubation. Salicylic acid has been reported to be the first intermediate compound isolated from microbial degradation of naphthalene [2]. This salicylic acid might have been converted further into catechol through a decarboxylation reaction [22], which through the ortho cleavage reactions forms β-ketoadipic acid that ultimately enters the TCA cycle [14].

The GC chromatogram and the mass spectra obtained when ethyl acetate extract of the culture supernatant containing the degradation products of ANSA (at a concentration of 100 mg l^{-1}) was subjected to GC–MS analysis are given in Fig. 2. Fragments with *m*/*z* 101 and 115 and the strong *m*/*z* 43 signal (Fig. 2b) characteristic of keto group corresponding to R_T value of 4.585 strongly suggested the presence of β-ketoadipic acid [20].

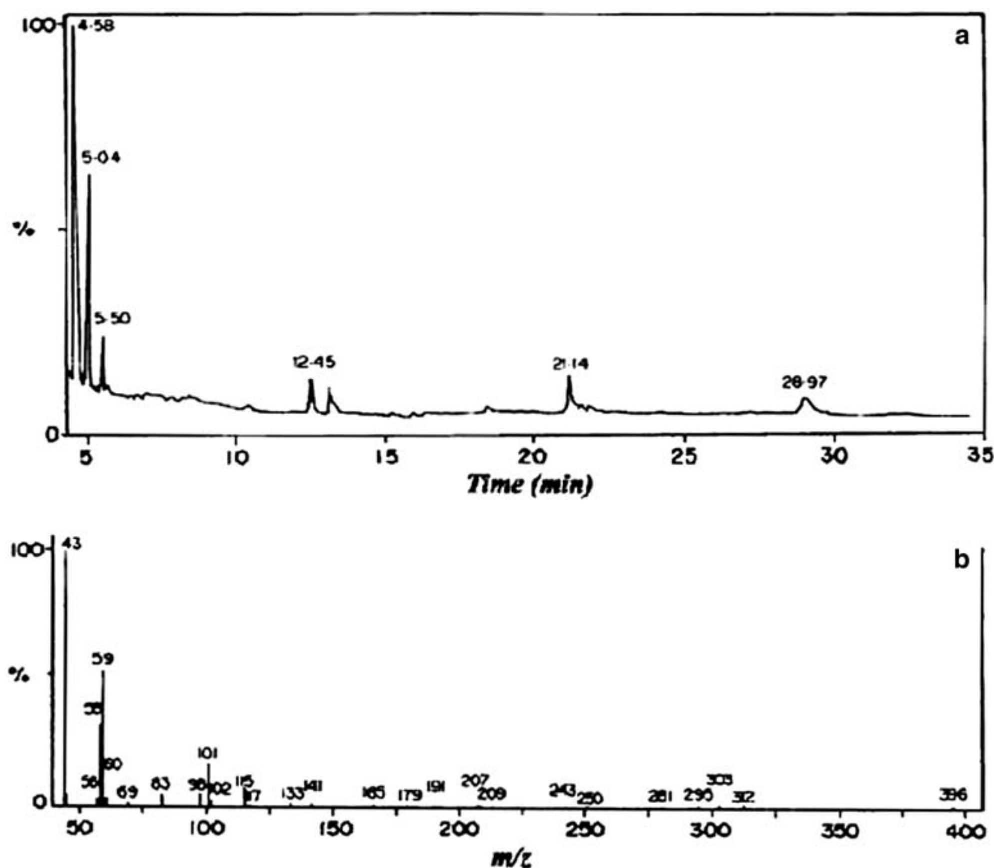

Fig. 2 GC–MS analysis of ethylacetate extract of the culture filtrate containing the degradation products of ANSA (at 100 mg l^{-1}) degraded by *Pseudomonas aeruginosa* after 48 h incubation. **a** Gas chromatogram **b** mass spectra for the peak with R_T value of 4.59 min

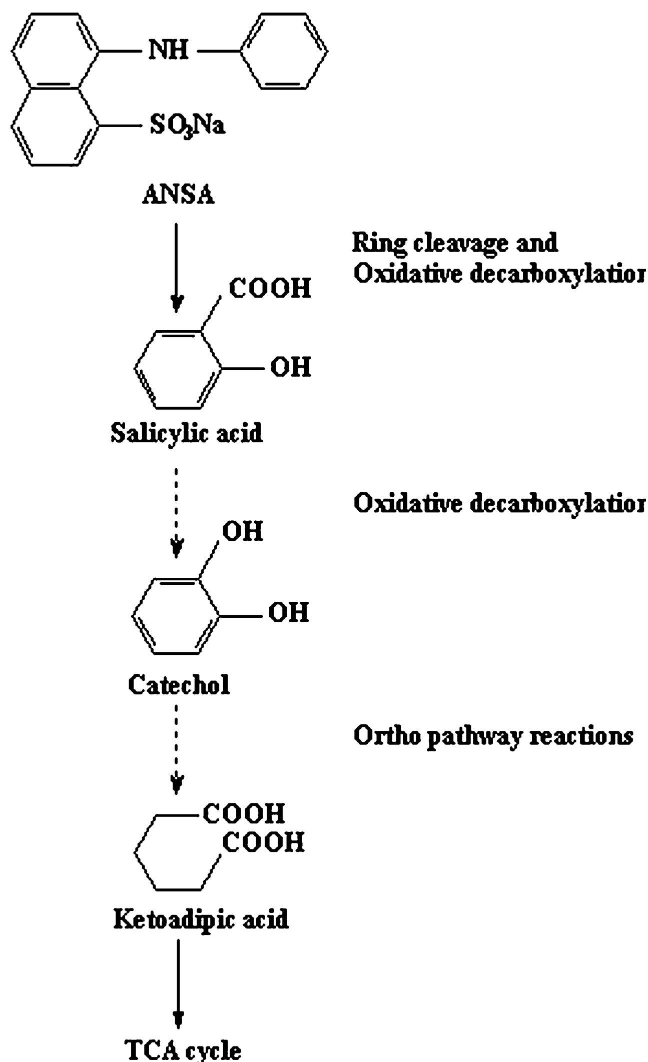

Fig. 3 Proposed pathway for the degradation of ANSA by *Pseudomonas aeruginosa*

β-ketoadipic acid has been found to be one of the intermediates in the degradation of a number of aromatic compounds [7, 15]. The very low R_T value for β-ketoadipic acid may be explained in two ways. It may be acetylated during extraction with ethyl acetate or it may be acetylated by the enzymes of bacteria [16].

Further evidence of the degradation pathway is provided by the presence of protocatechuate 3,4-dioxygenase, a key enzyme in the β-ketoadipate pathway, also known as the ortho pathway [10, 11, 17]. The enzyme activity from the *P. aeruginosa* cells grown in the presence of ANSA and glucose was found to be 26.2 μmol min^{-1} mg^{-1} of protein whereas an insignificant level of protocatechuic acid degrading activity was detectable in the crude extract of *P. aeruginosa* cells cultivated in medium without ANSA.

Nearly 57% of the carbon and 64% of the nitrogen present in ANSA at a concentration of 100 mg l^{-1} were converted into CO_2 and NH_3 respectively indicating the complete mineralization of this aromatic amine.

Based on the above findings a probable mechanism for the degradation of ANSA by *P. aeruginosa* has been proposed (Fig. 3).

Conclusion

ANSA has been selected for this biodegradation study as it is one of the substrates for the synthesis of most of the azo dyes. Several reports indicate the formation and accumulation of aromatic amines when these azo dyes are degraded by bacteria under anaerobic conditions. Reports regarding the degradation of aromatic amines are rather scanty and most of them are related to the degradation of substituted anilines. Complete pathways have been proposed for only a few aromatic amines. An attempt has been made to find the possible mechanism by which ANSA is degraded. The azo dye degrading strain which was used may be of potential use for the complete degradation of azo dyes as well as their intermediate compounds.

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