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Original Article

Isolation and Screening of Benzonitrile Degrading Bacteria

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Abstract

Despite the widespread use of herbicides containing nitrile group comparatively little is known about the microbial metabolism of nitriles and in particular the mechanism of cleavage of the cyanide bond by microorganisms. Soil samples were collected from different areas like agricultural land and area surrounding pharmaceutical industries. By adaptation and acclimatization seven bacterial cultures were isolated. All isolates were capable of growing on mineral medium containing benzonitrile as sole source of carbon and nitrogen. Out of these 10 isolates 7 bacterial strains were screened based on maximum production of ammonia. Secondary screening was carried out based on benzonitrile biodegradation at various pH. Three strains were selected showing maximum biodegradation in terms of ammonia production at basic, acidic and alkaline pH strain K 8 at p^H-7 (8.78 micromole), strain K4 at p^H – 9 (6.45 micromole) & strain K7 at p^H at -4 (10.01micromole). The presence of casein as a nitrogen source & fructose as carbon source were found to enhance the benzonitrile hydrolysis. High rate of degradation was observed at 48 hrs of incubation. These isolates were found to tolerate benzonitrile up to 0.2%. In both strain the enzyme activity was found to be maximum in cell supernatant as compair to cell lyzate. The presence of benzonitrilase was confirmed qualitatively by detecting nonoccurrence of benzamide during hydrolysis.

Key words*: Benzonitrile, Benzamide, Biodegradation, Benzonitrilase, Bacteria*

Introduction

Nitriles are found in the environment due to the anthropogenic activities as well as natural synthesis. Nitrile compounds are widely manufactured and extensively used by chemical industries. Despite the widespread use of herbicides containing nitrile groups such as dichlorobenil C-2, 6- dichlorobenzonitrile, Bromoxynil C-3, 5- Dibromo-4- hydroxyl benzonitrile. Comparatively little is known about microbial metabolism of nitriles and in particular the mechanism of cleavage of C-N bond by microorganism. (Bandopadhayay 1985). Organic chemicals like nitrile compounds that are toxic to the human and to the environment and can be transformed and metabolized by the microorganisms. Some microorganisms have the ability to utilized nitriles as carbon and nitrogen source (Yamada 1979). Bandopadhyay (1985) reported two kinds of benzonitrilases, designated benzonitrilase A and B in a cell extract of *Arthobacter* sp. strain J-1 grown on benzonitrile as a sole source of carbon and nitrogen. Acrylonitrile- hydrating activity was found in various bacteria belonging to genera *Arthobacter, Cornybacterium, Pseudomonas* (Watanabe 1987). Duran (1991) reported *Brevibactrium* sp. is a nitrile degrading strain involved in several industrial processes. Harper (1974) states that an *Arthrobacter* utilize benzonitrile as sole source of carbon and nitrogen. Yamada (1990) reported some microorganisms having the ability to utilized nitriles as carbon and nitrogen source. .Benzonitrile and aromatic nitriles have been shown to be converted directly to corresponding acid and ammonia catalyzed by nitrilases. Aliphatic nitriles are breakdown to their respective acids and ammonia via the formation of the amides (Arnaud 1976; Digeronimo and Antonie 1976).

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Materials And Methods

Elective Enrichment and Isolation

1 gm of soil sample was suspended in Basal salt medium containing (KH_2PO_4 1.5 gm; K_2HPO_4 3.5 gm; $Mgso_4.7H_2O$ 0.19 gm; Yeast extract 50 mg; Trace element ; P^H 7.5; Distilled water 1000 ml.) Benzonitrile 0.05% was added aseptically to sterilized and cooled medium. The suspension (100ml) in 250 ml Erlenmeyer flask was incubated at 30^0 C on rotary shaker. After 7 days 2ml of this culture was transferred to 100ml of fresh medium with little rise in benzonitrile concentration. The process was repeated for a total four transfers by step by step raising the concentration of benzonitrile (0.05 to 0.2%). After one month of acclimatization, the last enrichment culture flask was used to isolate microorganisms on basal salt agar containing 0.2% benzonitrile. The colony characterization and gram staining of bacterial cultures were carried out. The pure cultures were maintained on basal salt agar for further studies.

Primary screening

Total 10 different bacterial cultures were isolated on basal salt media containing 0.2% benzonitrile. Bacterial cultures were found to be utilizing benzonitrile as a sole source of carbon and nitrogen. For primary screening all isolates were grown on basal salt broth containing benzonitrile (0.2%) for 48 hours and then potential cultures were screened out on the basis of ammonia production by standard Nessler's method.

Secondary Screening

Secondary screening was carried out on the basis of high rate of degradation as acidic, basic and alkaline pH. For this purpose all seven isolates were inoculated into basal salt broth containing benzonitrile(0.2%) at three distinct pH viz. 4.0, 7.0 and 9.0 After 48 hours of incubation the production of ammonia was measured .

Qualitative detection for nonoccurrence of benzamide

The culture flasks contents of all three isolates were detected qualitatively for presence or absence of benzamide by performing thin layer chromatography. Silica gel containing plates were spot inoculated and then they were placed on a chromatography chamber containing the solvent systems .Chloroform: Butonol: Water (10: 25:65).They was allowed to run for 20 minutes. Then the plates are removed from the chamber and were dried in an oven. Locating reagent Ninhydrin (0.2% in ethanol) was sprayed over the plate. The results were record based on the observations that the development of violet spots indicates the presence of benzoic acid in the samples but nonoccurrence of brown colour spots indicates absence of benzamide .

Tertiary Screening

Further for screening of single bacterial culture , all three strains were subjected for detection of extracellular as well as intracellular activity of nitrilase.

Preparation of cell free extract: Benzonitrilase activity was measured by preparing cell free extract. The cells were centrifuged, washed with physiological saline and suspended in 0.1M potassium phosphate buffer, pH7.0. The assay mixture contained 100 micromole of potassium phosphate buffer pH 7.0, 300 micromole of benzonitrile as substracte, washed cells from 3 ml of culture broth in a total volume of 1.0 ml. The reaction was carried out at 30^0c for 1 hr. with moderate shaking and terminated by addition of 0.2 ml of 1 N HCL. After 20 minutes the activity was estimated in terms of ammonia production. Protein was determined by the method of Lowry.

Enzyme Unit: One unit of Benzonitrilase was defined as the amount of enzyme which catalyzed the formation of 1 micromole of ammonia per min.

1) Optimization of growth parameters of K5 for benzonitrile degradation

For optimization of growth parameters amongst three isolates, strain S 15 was selected expressing higher benzonitrilase activity in cell lyzate.

Intact cell preparation

Basal salt broth containing 0.2% benzonitrile was prepared and it is inoculated with selected bacterial strain S 15... This flask was incubated on rotary shaker at 100 rpm for 72 hrs. After 72 hrs cells were harvested by centrifuging the culture flask at 1000rpm for 10 min. washing of cell pallet was carried out using saline. These intact cells were suspended in saline and used further to study growth parameters.

Effect of pH:

Basal salt broth containing 0.2% benzonitrile adjusted to various pH viz. 4 to 9 was prepared and inoculated with 0.5% intact cells. All flasks were incubated on rotary shaker at 100 rpm for 72 hrs and the amount of ammonia produced was estimated by Standard Nessler's method.

Effect of temperature

Basal salt broths containing 0.2% benzonitrile were prepared and inoculated with 0.5% intact cell. All these flasks were incubated at various temperature viz. 10^0c to 60^0c. for 72 hours. The amount of ammonia produced was estimated by Standard Nessler's method.

Effect of carbon and nitrogen sources

Basal salt broths containing 0.2% benzonitrile were prepared. The flasks were supplemented with various carbon and nitrogen sources as mentioned in Fig. 7 and 8. After inoculation with 0.5% intact cells the flasks were incubated on rotary shaker at 100 rpm for 72 hrs. The amount of ammonia produced was estimated by Standard Nessler's method.

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Results and Discussion

By elective enrichment 10 different bacterial cultures were isolated from soil capable of utilizing benzonitrile as a sole source of carbon and nitrogen.(Heper 1977) show that cultures actively growing on benzonitrile as carbon and nitrogen source were tested for their for their ability to oxidized possible intermediate in the degradation of benzonitrile. Out of 10 bacterial strains, 4 belongs to gram positive and 6 belongs to gram negative (Fig.1). The production of ammonia was estimated from culture filtrate of all ten isolates. At primary level 7 isolates were screened out on the basis of maximum production of ammonia as one of the metabolite of degradation (Fig.2). To study the impact of pH on benzonitrile degredation all 7 isolates were grown at three distinct pH. The results has shown that the strain A8 , A4 and A7 were degrading benzonitrile at high rate in respective pH viz. 7, 9 and 4 (Table 1). Harper (1977) has shown that in Arthobacter sp.J-1 benzonitrile was directly hydrolyzed to benzoic acid and ammonia by nitrilase. In course of time benzonitrile was degreaded by accumulating benzoic acid and ammonia but benzamide was not detected throughout the cultivation of all three selected strains. Tentatively all three strains are showing the direct degredation of benzonitrile to benzoic acid and ammonia by enzyme nitrilase which was confirmed by thin layer chromatography. The expression of nitrilase was studied by observing enzyme activity in cell supernatant as well as in cell lysates. all these three strains the enzyme activity was more in cell supernatant as compare to cell lysates. Further the strain k5 was showing highest activity which was selected for optimization of physiological parameters (Fig.3). By performing intact cell assay the strain A5 has shown maximum nitrilase activity (10.31µmole) at pH 4 (Fig.4). Similarly the enzyme activity was maximum at mesophilic temperature 30 ^{o}c (5.72 µmole). The enzyme is found to be stable up to 60^{o}C (Fig.5). To accelerate the biodegradation the cells were grown in basal medium supplemented with various carbon as well as nitrogen sources. Even if the strain k7 was capable of utilizing benzonitrile as sole source of carbon as well as nitrogen, the presence of casein as a nitrogen source and fructose as carbon source enhances the nitrilase activity(Casein-40.72 µmole, Fructose- 5.56 µmole) (Fig.6,7). It seems likely that hydrolysis of the nitrile group is the limiting factor in the degradation of nitrile herbicides. Hence adaptation, conservation and improvement in such bacteria is an essential task to carryout biodegradation of such recalcitrant compounds.

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Fig.1 Distribution of microorganisms degrading benzonitrile

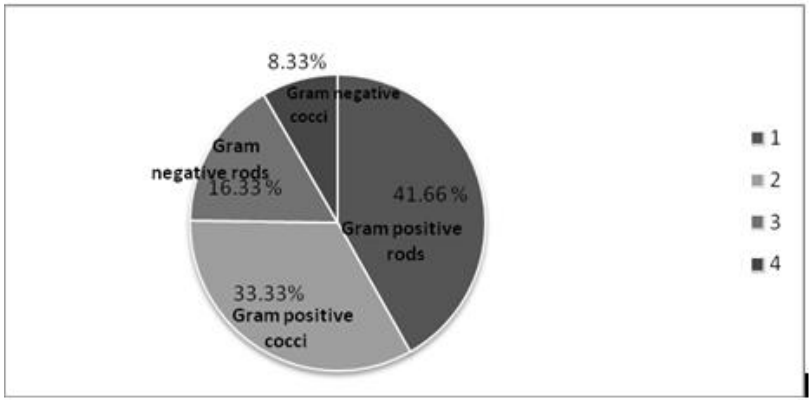

Fig.2 Primary screening for selection of benzonitrile degraders

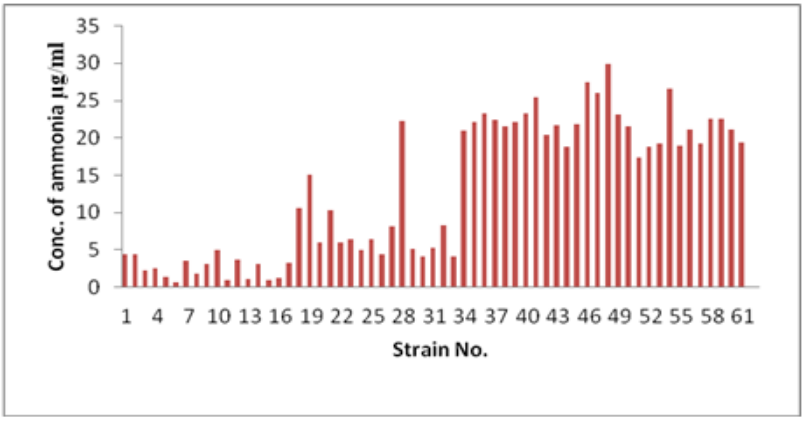

Fig.3: Location of Enzyme

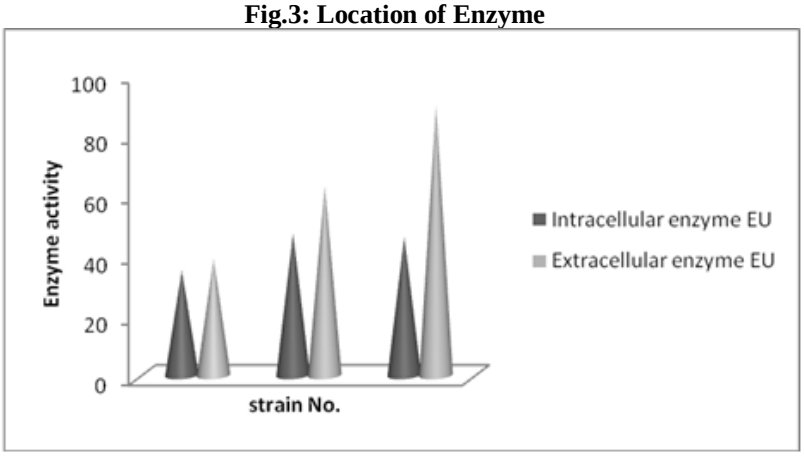

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Table 1: Secondary screening for selection of benzonitrile degraders (Different pH)

Sr.No	Strain No	Acidic pH 4	Alkaline pH7	Basic pH 9
1	K1	4.27	4.45	5.92
2	K2	4.72	6.84	4.86
3	K3	9.54	5.68	5.12
4	K4	3.30	5.45	10.77
5	K5	10.31	6.51	5.53
6	K6	4.19	7.04	9.54
7	K7	5.13	6.10	4.04
8	K8	3.56	9.78	3.45
9	K9	6.15	6.72	3.80
10	K10	5.54	3.62	6.65

Table 2 : Effect of pH, temperature, carbon and nitrogen source (strain k5)

Sr. No	**Carbon source**	**Enzyme activity µmol**	**Nitrogen source**	**Enzyme activity µmol**	**pH**	**Enzyme activity µmol**	**Temperature**	**Enzyme activity µmol**
1	Fructose	5.56	Peptone	14.16	4	10.31	10^0c	5.59
2	Maltose	3.22	Tryptone	8.24	7	6.51	30^0c	5.72
3	Lactose	2.84	Casein	40.72	9	5.530	60^0c	5.40