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31. Isolation and Screening of Benzonitrile Degrading Actinomycetes A15

Ajane Vaishali H.
Department of Microbiology, Kohinoor Arts, Commerce & Science College Khultabad, Aurangabad (M.S.)
Kulkarni R. A.
Government Arts & Science College, Aurangabad. (M.S.)
S. M. Dharmadhikari
Government Arts & Science College, Aurangabad. (M.S.)

Abstract

Microorganism as biocatalyst have an important application in industry for transformation of waste nit rile to less toxic amides or carboxylic acids. Soil samples were collected from different areas like agriculture land and area surrounding pharmaceutical industries. By adaptation and acclimatization twenty actinomycetes cultures were isolated. All isolates were capable of growing on mineral medium containing benzonitrile as sole source of carbon and nitrogen. Out of these twenty isolates six actinomycetes 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 A11 at p^H-7 (24.62 µmole/min), strain A9at p^H – 4(26.80 µmole/min) & strain A15 at p^H at -9 (99.68 µmole/min). StrainA9, A11& A15 exhibited maximum benzonitrile degradation at temperature 30^0C (7.03 µmole, 9.33 µmole & 31.04µmole). 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 tostrainA9 & A11– 0.6 %(8.96 µmole &22.06 µmole),strain A15(18.68µmole). In these strain the enzyme activity was found to be maximum in cell lyzate (99.68µmole/ min as compair to cell supernatant (77.09µmole/min).

Keywords : Biodegradation.Benzonitrile,Benzonitrilase,Actinomycets

Introduction

Nitriles (ie.cyno group) arise in environment from two sources. Firstly, large quantities of nitriles originated as waste from industrial production and secondly, nitriles occur in the environment as a biological activity (Harris et al 1987).Some microorganism has the ability to utilize nitriles as carbon and nitrogen sources (Nagasawa &Yamada 1990). These nitrile wastes are highly toxic and conventional activated sludge system are highly susceptible to inactivation by them. The wastes are therefore extremely difficult to dispose off and thus create pollution Problems. The degradation of nitriles by microorganism constitutes an important aspect in industrial waste degradation.

Microorganisms utilizing benzonitrile as sole source of carbon and nitrogen source was isolated the enrichments culture technique and identified different microorganisms, Cell free extracts of benzonitrile grow cell contain an enzyme catalyzed conversion of benzonitrile directly into benzoic acid without intermediate formation of benzamide (Heper 1976). The ability to degrade nitriles is quite common among microorganism. The potential of nitriles - degrading enzymes for biotrasformation, waste treatment and production of herbicides-resistant has been assessed (kobayshi 1990).

Mustacchi et al (2005) reported that the nitrilase of *Rhodococcus rhodochrous* performance a one step biotransformation of nitrilase to their corresponding carboxylic acid. Knowles et al (1987) found that a one- step pathway nitrilase yielding the corresponding carboxylic acid and ammonia. Mahadevan and Thimann (1964) have examined a nitrilase the enzyme mediated the direct conversion of the nitrilase into the corresponding acid, with no detectable formation of amide.Yamada et al (1980) observed that biodegradation of glutaronitrile by *Pseudomonas* sp. K9 proceeds through cynobutyramide, 4-cynobutyaric acid. Perez et al (2005) reported that the nitrile hydratase activity of *Nocardia coralline* described. Only detected product during the biocatalysed hydrolyzed of selected nitrile corresponding amide. Tauber et al (2000) found that *Rhodococcus rhodochrous* NCIMB11216 produced the nitrile hydrates and amidase activity when grown on a medium containing the propinonitrile.The enzymes were able to hydrolyzed nitrile group of both granual polyacrylonitrile and acrylic fiberes.Nitrile groups of were converted into the corresponding carbonic acids.

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 pure cultures were maintained on basal salt agar for further studies.

Primary screening

Total 20 different actinomycetes cultures were isolated on basal salt media containing 0.2% benzonitrile. Actinomycetes 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 6 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 .

Tertiary Screening

Further for screening of single actinomycetes 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 standard reaction mixture consisted of 50 µmole of potassium phosphate buffer (pH 8.0), 3µmole of benzonitrile, and appropriate amount of enzyme in the total volume of 0.5 ml. the reaction was started by adding the substrate and was carried out at 30^0c for various time, depending on the experiment. The benzonitrilase assay was performed using both cell supernatant as well as cell lyzate. 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.

GC analysis method

The isolated strains were cultured aerobically at 28^0c for the 3 days on the isolation medium. The cells were centrifuged, washed with physiological saline and suspended in 0.1M potassium phosphate buffer, pH 7.0.The reaction mixture for the screening of benzonitrile producing strains contained 100 µmole of potassium phosphate buffer, 300 µmole of benzonitrile as substract, 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.

The mixture was determined with a Chemito Gas chromotograph, Model GC -7610 equipped with flame ionized detector. The column used was stainless steel silicon 30, packed with porapackQ (80 to 100 mesh) operational conditions were: column temperature,200^0c; injection and detector temperature 151^0c and 201^0c.The carrier gas was N_2 at 40 cm^3/min.

Optimization of growth parameters of A15 for benzonitrile degradation

For optimization of growth parameters amongst three isolates, strain A15 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 actinomycetes strain A15 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 10,000 rpm 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 no. 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.

Effect of various concentration of benzonitrile

Basal Salt broths with various concentrations of benzonitrile (0.2 to 1%) were prepared and inoculated with 0.5% intact cell suspension. These flasks were incubated on rotary shaker at 100 rpm for 72 hrs. The amount of ammonia produced was estimated by Standard Nesseler's method.

Growth and Activity Profile

Basal salt broth containing 0.2% benzonitrile was inoculated with 0.5% intact cells. This flask was incubated on rotary shaker at 100 rpm. The growth was monitored periodically every after 24 hours and the activity profile was prepared by estimating the amount of ammonia produced by Standard Nesseler's method.

Results and Discussion

By elective enrichment 20 different actinomycetes cultures were isolated from soil capable of utilizing benzonitrile as a sole source of carbon and nitrogen (Fig.1).Harper (1974) reported that number microorganisms isolated from soil were capable of utilizing benzonitrile as carbon and nitrogen source. The production of ammonia was estimated from culture filtrate of all twenty isolates. Harper et al (1977) has shown that a benzoate and ammonia. At primary level 6 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 degradation all 6 isolates were strain of the *Fusarium solani* is able to use benzonitrile as a source carbon and nitrogen and convert it into grown at three distinct pH. The results has shown that the strain A9, A11 and A15 were degrading benzonitrile at high rate in respective pH viz. 7, 9 and 4 (Table 1). Harper (1977) has

shown that in *Arthrobacter 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 A15 strain. Tentatively the A15 strain are showing the direct degradation of benzonitrile to benzoic acid and ammonia by enzyme nitrilase which was confirmed by Gas chromatography (A). Similar findings were observed by Thimann and Mahadevan (1964) during their studies on benzonitrile hydrolysis by *Fusarium sp., Aspergillums Niger* and *penicillium chrysogeum.*

In all three strains the enzyme was synthesized extract and highest nitrilase activity was observed in strain A15. By performing intact cell assay the strain A15 has shown maximum nitrilase activity (99.68 µmole/min) at pH 9 (Fig.3). Similarly the enzyme activity was maximum at mesophilic temperature 30^0C (31.04µmole). The enzyme is found to be stable up to 30^0C (Fig.4). The intact cells of A15 were showing maximum benzonitrile biodegradation at pH 9(Fig.5). High rate of degradation was observed at 72 hrs of incubation (Fig.6). The strain A15were found to tolerate benzonitrile up to 0.8% (Fig.7).The presence of casein as nitrogen and fructose as carbon source was found to accelerate the growth and benzonitrile hydrolysis (nitrilase activity for Casein-40.72 µmole, Fructose- 5.56 µmole) (Fig.8, 9). It seems likely that hydrolysis of the nitrile group is the limiting factor in the degradation of nitrilase herbicides. Hence adaptation, conservation and improvement in such actinomycetes are the essential tasks to carryout biodegradation of such recalcitrant compounds.

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Fig.1 Primary Screening for Selection of benzonitrile degraders

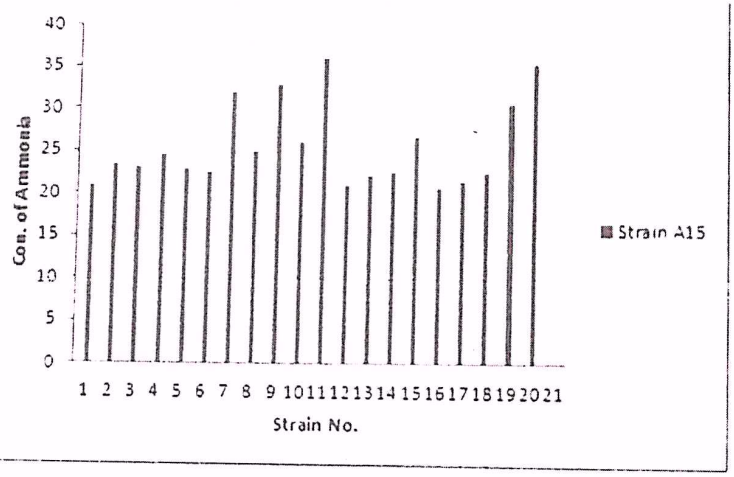


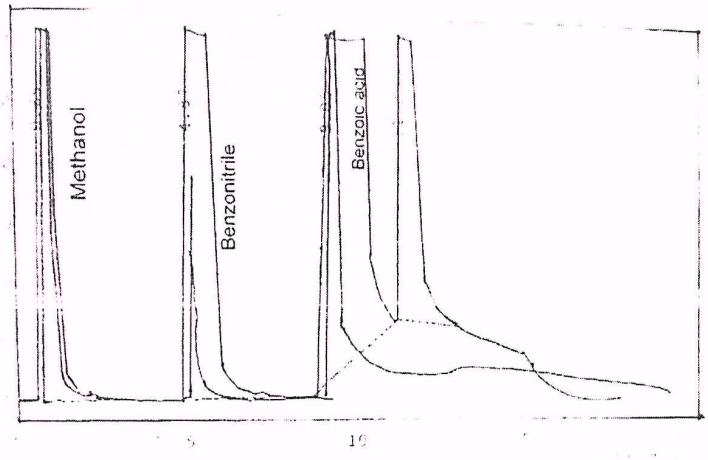
Table 1. Secondary Screening for Selection of benzonitrile degraders (Different pH)

Strain No.	pH 4	pH7	pH9
A7	8.28	8.48	16.04
A9	26.80	9.66	8.07
A11	13.53	24.62	20.42
A15	7.50	16.92	21.31
A19	5.90	14.86	13.92
A20	6.78	13.0	12.48

Table 2.Gas Chromatography analysis

Tue, 29th Jun 2010 20:18:34 /G44

Sample ID	B.Amide B.A Bn + met	Analyst	p
Sample	B.Amide B.A Bn + met		
Amount	2	Dilution	1
ISTD Amount	0	Inj Volume	3
Raw Data	Not Saved	From	Tue, 29th Jun, 2010 17:17:49
Primary	vg44	Calibration	(none)
Project	work4	Style	report



Result Table Calibration Table

Peak No.	Reten. time	Area [mV.s]	Height [mV]	W05 [min.]	Area [%]	Height [%]
1	[illegible]	6717.8921	625.3154	[illegible]	[illegible]	[illegible]
2	4.68	20809.8178	622.7547	[illegible]	[illegible]	[illegible]
3	[illegible]	[illegible]	595.1521	[illegible]	[illegible]	[illegible]
4	11.43	17037.5938	491.0359	[illegible]	[illegible]	[illegible]
-	Total	124140.3760	2319.3760			

Fig 2 : Screening of Isolation of Benzonitrile degraderes

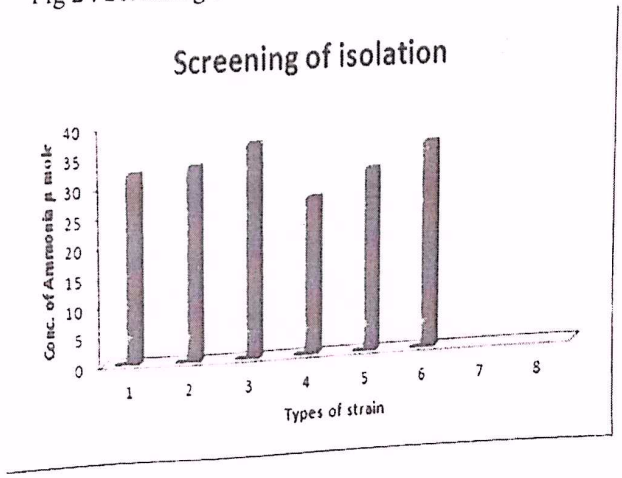


Fig 3: Effect of Temperature

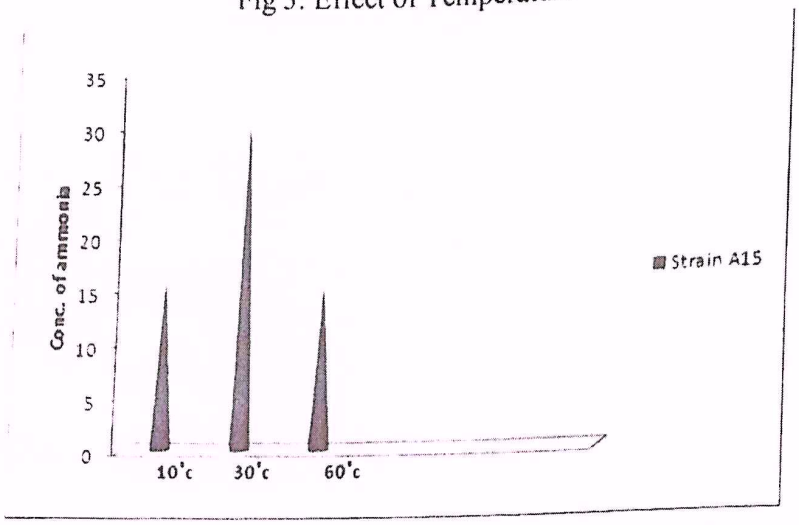


Fig 4: Effect of pH

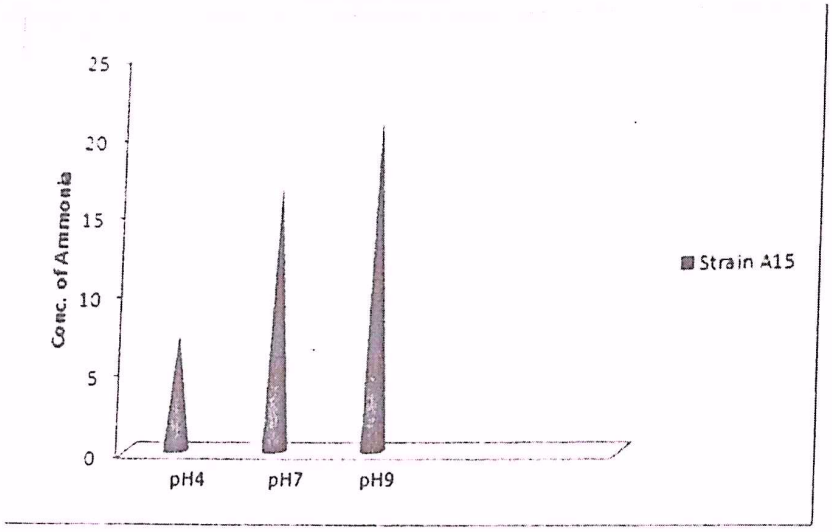


Fig 5 : Effect of growth activity and profile

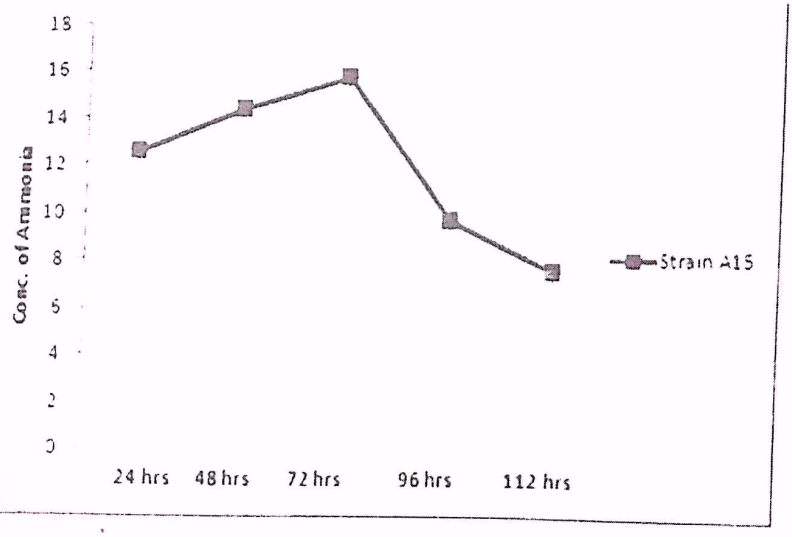


Fig 6: Effect of differnant Concentration of benzonitrile

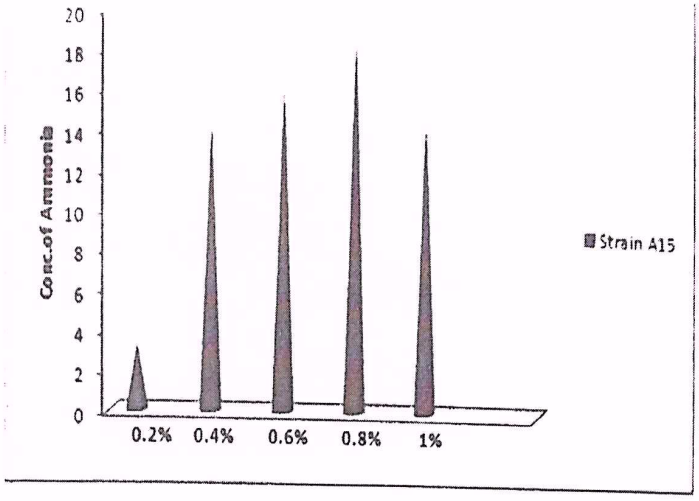


Fig 7: Effect of carbon sources

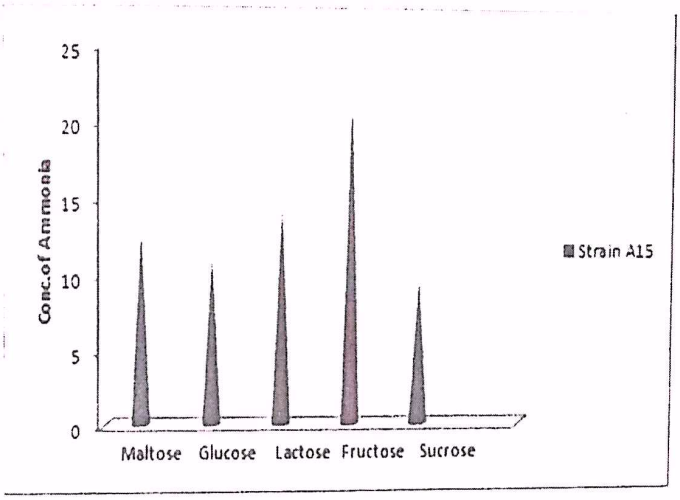


Fig 8: Effect of nitrogen source