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A GREEN CHEMISTRY APPROACH TO BIOREMEDIATE ACETONITRILE.

***Pratiksha Alagh[1] and Gaganjyot Kaur[1], B. S. Ajit Kumar[2]**

[1]National Facility for Biopharmaceuticals, G N Khalsa College, Matunga, Mumbai, India.

[2]Department of Chemistry, G N Khalsa College, Matunga, Mumbai, India.

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***Correspondence for Author**
Pratiksha Alagh
National Facility for Biopharmaceuticals, G N Khalsa College, Matunga, Mumbai, India.

ABSTRACT

Nitriles are extremely toxic to the humans and cause environmental nuisance. Acetonitrile (ACN) is one such nitrile that is extensively used in many industries. There are chemical and physical methods of degradation of acetonitrile; however they have their own drawbacks. Green chemistry uses microbial source as an agent to clean the environment. Using this approach, we have identified six bacterial species using standard antibiotic sensitivity and 16s rRNA sequencing, which are able to tolerate and grow in a significant concentration of acetonitrile (5% w/v). The strains have been identified as *Stenotrophomonas maltophilia, Pseudomonas aeruginosa, Acinetobacter baumanii, Serratia plymuthica* and *Klebsiella pneumoniae.* Of this *Stenotrophomonas maltophilia, Acinetobacter baumanii, Serratia plymuthica* have never been reported before for such activity to the best of our knowledge. The strains were able to tolerate up to 10% v/v ACN. This has been the highest concentration reported so far. The bioremediation efficiency was studied and a putative enzymatic pathway for degradation was elucidated. These bacteria hold great potential as a tool to clean up the environment. Also the enzymes responsible for degradation can act as biocatalyst in various industrial processes.

KEYWORDS: Bioremediation, acetonitrile, enzyme assay, 16s rRNA, antibiotic sensitivity.

INTRODUCTION

Nitriles are cyanide substituted carboxylic acids (general formula R≡CN).[1, 2, 3, 4, 5, 6] Naturally they are found in plants, bone oil, insects, and microorganisms.[7, 8, 9] Synthetic nitriles have been used in the manufacture of feedstock, solvents, extractants, pharmaceuticals, drug intermediates, pesticides (dichlobenil, bromoxynil, ioxynil, buctril)

extensively used in rice, wheat, barley, corn and berry fields, used as intermediates in organic synthesis of amines, amides, amidines, carboxylic acid, esters, aldehydes, ketones, heterocyclic compounds, plastics and synthetic rubber and fibers.[4] Presence of nitriles in industrial, residual agricultural and chemical wastewater can pose severe health risks as they are highly toxic to the central nervous system where they alkylate the protein sulphdryl group.[10,11] Further they are mutagenic[4] and carcinogenic.[12] So monitoring and degrading of such compounds are important for the environmental safety[4, 8] as their removal from the waste is a difficult process.

Chemical methods like recycling, incineration, adsorption on ion exchange or charcoal columns[4] ozone and photocatalytic oxidation are available for the treatment of these compounds However, these ex-situ methods are cost intensive, involve harsh conditions[7] and generate secondary pollutants.[12] Hence biological methods, termed as bioremediation, have come to the forefront for treatment of wastes. Here, toxic substrates are converted to harmless products like water and carbondioxide by microbial enzymes.[8,10] Hydrolysis of nitriles are catalyzed by nitrile degrading enzymes which occur in a wide variety of plants and microorganisms.[7] Different strains have been reported to have nitrile degrading activity, for example *Pseudomonas*, *Acinetobacter*[13, 14], *Alcaligenes*[15], *Arthrobacter*, *Rhodococcus, Brevibacterium*[16], *Commamonas testosteroni*, *Acidovorax sp, Paracoccus thiophilus,* and *Klebsiella oxytoca.*[4 , 8] Industrial level application of these enzymes has limitation due to thermal instability.[7]

In the current research, we have isolated bacteria from soils and water samples which are previously not exposed to ACN. The aim was to identify the organisms which have an inbuilt mechanism of counter anthropogenic materials but have not been explored.

MATERIALS AND METHODS

1. **Media and culture conditions**. Synthetic media containing various concentrations of acetonitrile as a source of nitrogen was used[11]. The Minimal media composition used was: ($Na_2HPO_4.2H_2O$(50mM), KH_2PO_4 (100mM), $MgSO_4$(1mM), $CaCl_2$ (0.1 mM), Glucose (0.5%) maintained at a pH of 7.2[8]. The standard reaction conditions for study was 180 rpm and 30°C, and the standard centrifugation conditions were 10,000 rpm at 4°C for 10 minutes. All the analytical grade chemicals and bacteriological media were procured from HiMedia and Merck, Mumbai.

2. **Isolation, enrichment and characterization**. Bacterial samples were isolated from natural environment, previously not reported for ACN contamination. Eastern Express Highway water and soil sample (EEW, EHS), Tomato field soil (TO), Juhu beach water (JW), Grape field soil (GR), Garlic field soil (GA), Khopoli black soil (KH), Deonar Dumping ground water (DDW) were few locations of sampling. Bacteria from these samples were isolated in physiological saline and then transferred into Minimal media (MM) containing 0.1% (v/v) ACN at 10% initial inoculum. The samples were serially diluted and plated on MM containing 2% agar and 0.1% (v/v) ACN for isolating pure cultures. Subsequent enrichments were carried out in increasing concentration of ACN up to 10% v/v. At each stage of enrichment, the samples were plated to isolate pure cultures and growth was monitored by measuring change in absorbance. Culture characterization involved the identification based on the macroscopic and microscopic colony characteristics, metabolic activity on Mac Conkey's agar. Antibiotic sensitivity was also performed on pure cultures using automated BACTEC TM MGIT 960 analyzer. Molecular characterization was done by sequencing the conserved 16s rRNA homology region[17] using the 8F AGAGTTTGATCCTGGCTCAG[18] and 907R CCGTCAATTCMTTTRAGTTT primer.[19] A gene fragment of 900 bp was obtained on PCR which was sequenced on Beckman Coulter CEQ 8000 DNA Sequencer as per the protocol provided by the manufacturer.
3. **Growth curve.** The starter culture was inoculated at 2%(v/v) into St LB and optical density was measured at regular intervals.[7, 8, 20, 21, 22, 23] Growth curve was plotted and Generation time (g) and growth rate (μ) were calculated using the formula:
   $g = [2.303 (\log OD_2 - \log OD_1)]/ t_2 - t_1$ and $\mu = 0.693/g$.[7, 20]
4. **Enzyme assay**. The enzyme assay was performed on crude extract.[4] Cell free crude extracts were obtained by sonicating the sample at 169 μA, for 5 secs on for 15 cycles in 50 mM phosphate buffer pH 7.0. Post sonication, the crude extract was obtained by centrifugation under standard conditions. Enzyme assay was performed at 10mM ACN concentration.[2]
5. **Analytical methods**. Formation of ammonia was monitored and estimated through modified Fawcett and Scott method. Protein was estimated by Bradford method using BSA as standard.

RESULTS

Figure 1: 16s rRNA amplification using 8F and 907R primers on 2% agarose gel stained with 5 µg/µl ethidium. Lane 1 shows standard 100bp ladder; lane 2 shows a 900bp amplicon specific to the 16s rRNA region.

Table 1 shows the antibiotic sensitivity test for all the cultures. R: Resistant, S: Sensitive and NT: Not tested.

		S. plymuthica	*A. baumanii*	*K. pneumoniae*	*P. aeruginosa*	*S. maltophilia*
Sr No	**Antibiotics**					
1	Amikacin	S	R	S	S	NT
2	Ciprofloxacin	S	R	S	S	NT
3	Gentamycin	S	R	S	S	NT
4	Meropenem	S	R	NT	NT	NT
5	Tetracycin	S	NT	NT	NT	NT
6	Trimethoprin + sulfmethoxazole	S	S	NT	NT	S
7	Amoxicillin + clavulanic acid	R	NT	NT	NT	NT
8	Cefazolin	R	NT	NT	NT	NT
9	Cefapime	R	R	NT	S	NT
10	Cefotaxim	R	R	NT	NT	NT
11	Ceftazidime	R	S	R	R	R
12	Cefuroxime	R	NT	NT	NT	NT
13	Piperacillin + Tazobactum	R	R	NT	R	NT
14	Levofloxacine	NT	S	NT	S	S
15	Ceftriaxone	NT	R	R	NT	NT
16	Imipramine	NT	R	NT	NT	NT
17	Tobramycin	NT	NT	R	S	NT

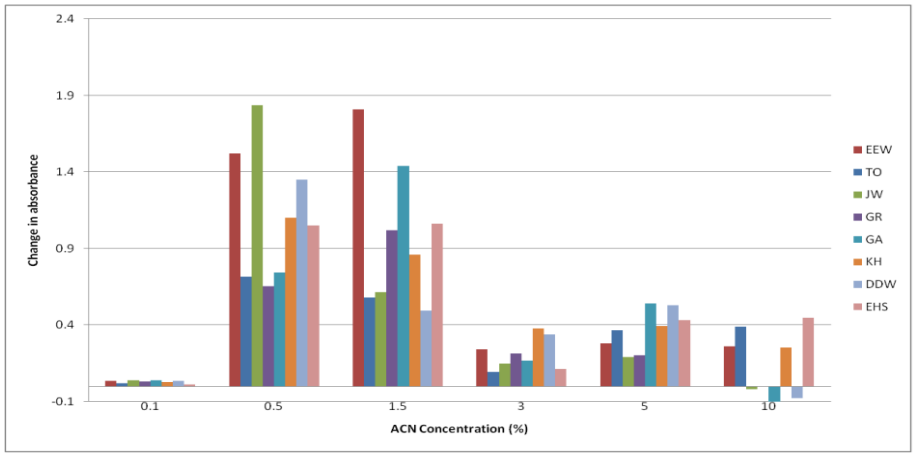


Figure 2: shows the change in absorbance of various cultures isolated from different regions namely Eastern Express Highway water and soil sample (EEW, EHS), Tomato field soil (TO), Juhu beach water (JW), Grape field soil (GR), Garlic field soil (GA), Khopoli black soil (KH), Deonar Dumping ground water (DDW)

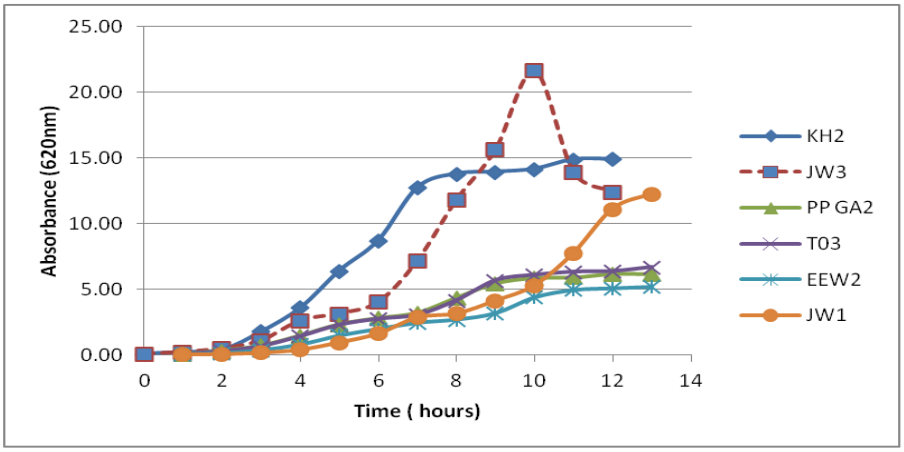


Figure 3: shows the growth curve of the 6 cultures in St. LB over time. The figure shows the various phases of growth curve viz. Lag, log or exponential, stationary and death phase. Cultures have been denoted by random names and code for the following: KH2 and TO3 - *Stenotrophomonas maltophilia*, JW3 - *Pseudomonas aeruginosa*, GA2- *Klebsiella pneumoniae*, EEW2- *Serratia plymuthica*, JW1- *Acinetobacter baumanii*.

Table 2: denotes the generation time and growth rate of the cultures.

Code	Culture	Generation time (hours)	Growth rate (hr^{-1})
GA2	*Klebsiella pneumoniae*	4.37	0.16
KH2	*Stenotrophomonas maltophilia*	1.57	0.44
TO3	*Stenotrophomonas maltophilia*	3.35	0.21
EEW2	*Serratia plymuthica*	2.88	0.24
JW1	*Acinetobacter baumanii*	2.71	0.26
JW3	*Pseudomonas aeruginosa*	1.79	0.39

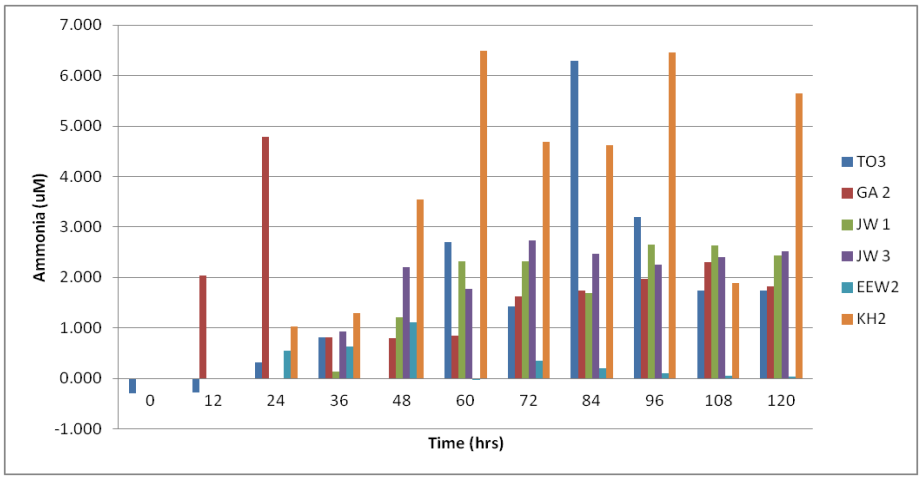


Figure 4 shows the trend of ammonia production over time for various cultures.

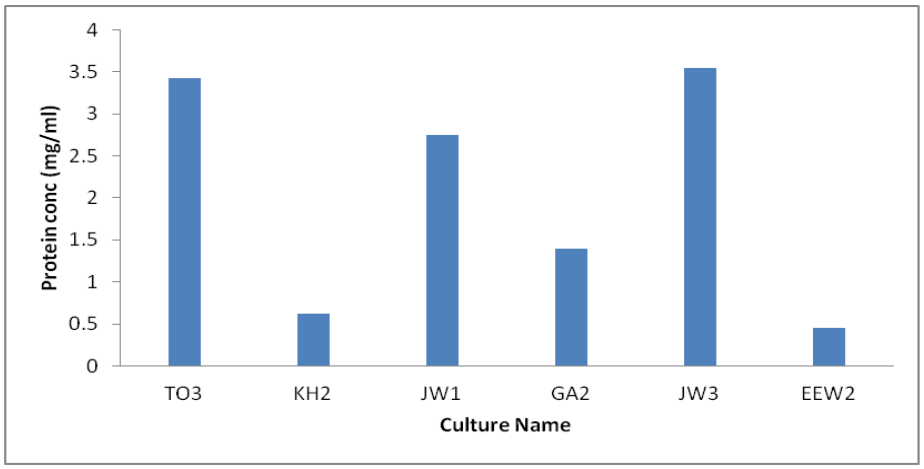


Figure 5: shows the protein concentrations of various cultures estimated using Bradford reagent and 1 mg/ml BSA as standard. Cultures have been denoted by random names and code for the following: KH2 and TO3 - *Stenotrophomonas maltophilia*, JW3 - *Pseudomonas aeruginosa*, GA2- *Klebsiella pneumoniae*, EEW2- *Serratia plymuthica*, JW1- *Acinetobacter baumanii.*

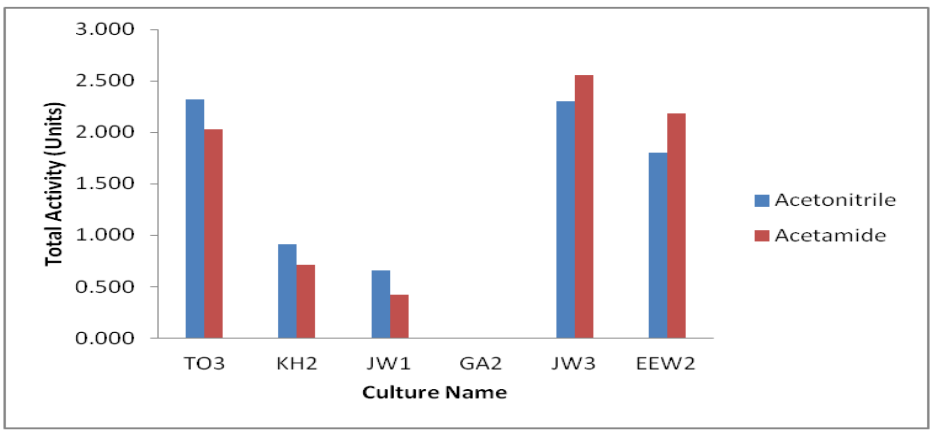


Figure 6 shows the enzyme activity of various cultures using acetonitrile and acetamide as substrates.

DISCUSSIONS

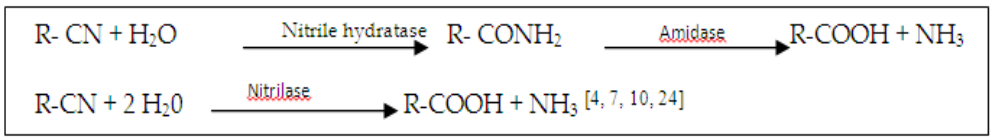
1. **Isolation, enrichment and characterization**: ACN degrading bacteria were isolated from six different virgin locations and enriched under standard reaction condition. Enrichment was done up to 10% v/v and the change in the absorbance as an indication of growth was measured as shown in figure 2. By far this is the highest concentration for enrichment not seen elsewhere for ACN to the best of our knowledge. Maximum growth was observed between 0.5% to 1.5% v/v ACN. A total of twenty four different isolates were obtained of which six cultures showed uniqueness in their morphology, growth as well as activity. All were microscopically identified as Gram negative with morphologies like short rods, cocci and bacilli. On further isolation on Mac Conkeys agar, two were identified as lactose fermentors while four were negative on Mac Conkey. Based on the 16s rRNA homology region (figure 1) and sequencing along with above results, these bacteria were identified as *Stenotrophomonas maltophilia, Pseudomonas aeruginosa, Acinetobacter baumanii, Serratia plymuthica* and *Klebsiella pneumoniae*. Of this, *S. maltophilia, S. plymuthica* and *A. baumanii* are reported for the first time for such ACN degrading activity.

   From the antibiotic profile of these bacteria tested against seventeen known broad spectrum antibiotics, it was evident that *S. plymuthica* was sensitive to six antibiotics and resistant to seven, *A. baumanii* sensitive to three and resistant to nine, *K. pneumoniae* sensitive and resistant to three, *P. aeruginosa* sensitive to six and resistant to one and *S. maltophilia* sensitive to two and resistant to one.

2. **Growth curve, generation time and growth rate**: When bacteria are placed under stressful environment like solvents, it spends a long time and energy to acclimatize to the micro-niche. Once acclimatized, it begins utilizing its resources in order to produce energy and grow, as evident from these bacteria. The generation time is the indication of how these cultures adjust to the environment. Cultures grown under metabolic pressure have a longer generation time in comparison to those grown under conducive environments. Those having relatively shorter generation time are more comfortable in the environment and readily adapt to the toxic condition. To understand the adaptation and mechanism of growth, all the cultures were inoculated at the same OD. Growth curve was plotted and various phases of growth were observed as shown in figure 3. Highest

OD was achieved by *P. aeruginosa* in the shortest span of time i.e. 10 hours whereas *S. maltophilia* has the shortest doubling time which is 1.75 hours.

3. **Ammonia estimation**: The physiological phenomenon of adaptation is evident from ammonia production. Cultures have taken 48 hours to adjust to ACN condition, post which, they begin the enzymatic degradation of ACN. Degradation of ACN is reported to take place by production of ammonia. In the current research, ammonia is the sole indicator of ACN degradation as no external organic or inorganic nitrogen source is supplied to the bacteria. ACN gets degraded to produce acetic acid and ammonia through two enzymatic pathways namely.



Cultures were grown under standard reaction condition and ammonia was estimated using modified Fawcett and Scott method. Ammonia was estimated every 12 hours and its profile against time was plotted as shown in figure 4. It is evident that ammonia production begins after 48 hours and increases up to 120 hours. *S. maltophilia* shows maximum ammonia production with time and *P. aeruginosa* is consistent in terms of ammonia production. However *K. pneumoniae* reverses the pattern and shows maximum production in 24 hours. The probable source of early nitrogen may be through cellular respiration or breakdown of nitrogenous compounds during cell death. This is in correlation to its slow growth rate and longer generation time.

4. **Enzyme assay**: Besides ammonia production, the total protein content in bacteria is also an indirect measure of its metabolic activity. Cultures showing increased protein content are metabolically active. Under stressful conditions, these proteins serve as energy source to the bacteria. Crude enzyme extract was obtained post sonication. Protein content was estimated using Bradford's reagent and BSA as standard. The profile is shown in figure 5. *S. maltophilia* and *P. aeruginosa* have shown good protein content in comparison to other cultures. This could probably be due to increased metabolic activity. . Conversely, *S. plymuthica* shows decreased protein content. Equal amount of protein was incubated with ACN and acetamide under standard reaction condition. On analysis, it was observed that *S. maltophilia, P. aeruginosa* and *S. plymuthica* besides having low protein content,

showed significant activity. This may be attributed to the increase in turn over number of the enzyme. *S. maltophilia* isolated from Khopoli soil showed lesser activity. This could be because of loss in enzyme activity post sonication or incomplete protein extraction due to rigidity of the cell wall. *S. maltophilia* and *A. baumanii* showed increased activity in acetonitrile thereby indicating a single step enzymatic system using nitrilase, in comparison to *P. aeruginosa* and *S. plymuthica* which displayed better activity with acetamide as substrate which indicate that these cultures possibly could be using the two step enzymatic degradation pathway ie nitrile hydratase and amidase, where the reaction catalyzed by nitrile hydratase is the rate limiting step. However, further proof needs to be developed for this concept.

CONCLUSION

Six different bacteria housing the potential to degrade ACN have been isolated from virgin soil and water samples. Of these *S. maltophilia*, *S. plymuthica* and *A. baumanii* have been reported for the first time for such an activity. Also these bacteria have been enriched up to 10% where previously reported is 3%. This has been the highest reported concentration for tolerance of ACN. These bacteria have a specific enzymatic pathway for degradation which has been elucidated by the study. Further work can be carried on for increasing the threshold for degradation and characterization at the genomic and proteomic level. It is also evident from the antibiotic sensitivity profile that these bacteria are resistance to many antibiotics. This resistance can be attributed to many environmental factors.

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