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Comparative analysis of acetonitrile biodegradation in invitro environment by using *Pseudomonas* sps. isolated from industrial effluent

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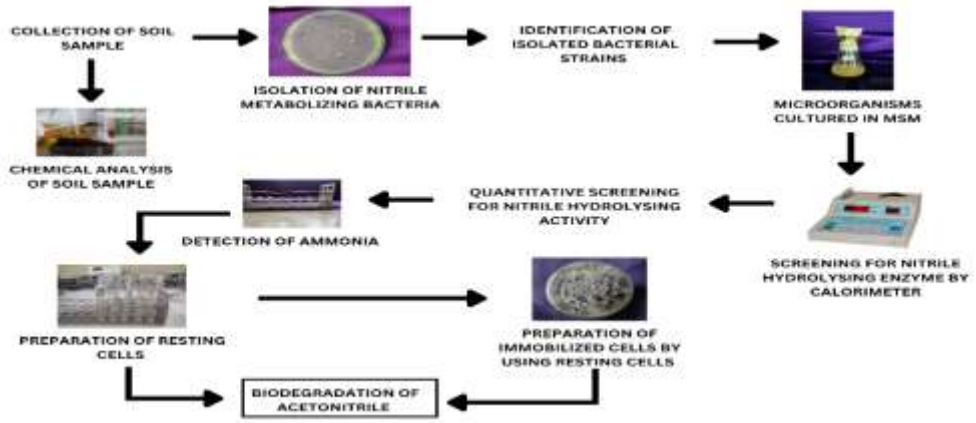
ABSTRACT:

Acetonitrile is a hazardous solvent that is used in different industrial processes. It is a pernicious to human and environmental health. In this study investigates biodegradation of acetonitrile by nitrile hydrolysing enzymes produced by *Pseudomonas* species isolated from soil from contaminated industrial site. Physical and chemical examination of soil samples revealed an array of trace elements, and a moderate alkalinity followed by morphological, biochemical, and 16S rRNA gene sequencing performed. Qualitative and quantitative analysis of nitrile metabolising activity using bromothymol blue based method was done. All the five isolated strains used nitriles exclusively as sole carbon and nitrogen source. Thus, all five Pseudomonas strains showed positive nitrilase enzyme activity, as conformed by qualitative screening. In quantitative screening Pseudomonas aeruginosa GPC1-C had the highest nitrilase activity by producing 1.6±0.02 U/mL of ammonia, furthermore the isolated strains are subjected to degradation of acetonitrile additionally the research included the optimization of degradation efficiency using immobilize the resting sells in agar-agar matrices. Pseudomonas aeruginosa GPC1-K demonstrated the highest degradation efficiency among the isolated strains at 72h, when both resting and immobilized cells were subjected to different concentrations of acetonitrile. Both resting and immobilised Pseudomonas species produced nitrilase enzymes, demonstrating increasing efficiency in degrading acetonitrile compounds. This enzyme activity offers a promising solution for environmental bioremediation and reduce pollution.

Keywords: Acetonitrile, Biodegradation, Nitrile hydratase, Pseudomonas species, Resting cells, Immobilized cells.



Graphical abstract

INTRODUCTION:

Acetonitrile is frequently used as a solvent in different sectors such as manufacturing of rubber, gloves, plastic and pharmaceutical industries (Babic et al., 2014; Nyalala et al., 2011). Because of its high solubility in nature, it is used in spectroscopy (Venables & Schmuttenmaer, 2000) and chromatography (Rudakov et al., 2018) for variety of compounds. Due to its wide range of importance acetonitrile molecule is used in both research and contexts and commercial for making sustainable contribution to disciplines of analytical science and chemistry (Goulding, 1995). Acetonitrile poisoning mostly affects the central nervous system in humans. Headache nausea and dizziness are a few symptoms that might results from inhalation, ingestion or skin contact. Acute poisoning may induce coma (Kurt et al., 1991; Pozzani et al., 1959). According to Gasparetto et al., 2012, acetonitrile exposure may negatively effects on the development and reproduction including possible injuries to the developing embryo during pregnancy. The acetonitrile exposure can cause acute poisoning in animals, particularly small mammals which may lead to seizures, breathing difficulties, and even death are possible symptoms. Apart from these, animals might suffer long term consequences from chronic acetonitrile exposure, including impaired reproductive and developmental process and organ damage to liver and kidney (Alonso et al., 2008).

Inappropriate disposal of acetonitrile by the industries may disturb the natural ecosystem of the soil, which may have detrimental effect on plant development and disturb soil microbiota leads to affect the soil structure. Contamination of water can result from acetonitrile can negatively affects aquatic life and disturbs the aquatic ecosystem. Crop development and growth may be impacted by exposure to acetonitrile. The toxin may seep into plants altering their metabolism and perhaps accumulating in the food chain (Bhalla and Kumar, 2005).

The nitrile hydrolysing enzymes primarily composed of nitrilase, (Thimann & Mahadevan, 1958) amidase and nitrile hydratase (Kobayashi et al., 1995). Microorganisms degrade nitrile compounds through two main pathways. Nitrilase enzymes hydrolyse these compounds into carboxylic acids and ammonia (Howden & Preston, 2009). Subsequently, nitriles hydratase converts nitriles into their corresponding amides, which are then broken down by amidase into

carboxylic acids and ammonia (Maestracci et al., 1984). Nitrilases, which hydrolyse nitriles, are crucial for the industrial synthesis of carboxylic acids and the biodegradation of cyanides and nitriles, with extensive applications in industry and biotechnology (Howden and Preston, 2009). Microbial cells can convert nitriles into valuable organic compounds such as thiophenamide, nicotinic acid, acrylamide, pyrazinoic acid, benzamide, and P-aminobenzoic acid (Brady et al., 2003).

The *Pseudomonas* genus includes a diverse array of gram-negative bacteria noted for their extensive metabolic capabilities and skills in using different organic compounds as carbon and energy sources. These bacteria can break down a variety of xenobiotics, such as nitriles like acetonitrile through specialized enzymatic process. To less harmful products is a testament to the microbial world's remarkable capacity to facilitate the removal and detoxification of hazardous chemicals from the environment. Acetonitrile is readily biodegradable by bacteria like *Arthrobacter* (Sandhya & R, 2022), *Corynebacterium*, *Azobacter*, *Pseudomonas* etc. It is well known that some *Pseudomonas* spices can produce enzymes required to breakdown acetonitrile. Pseudomonas pieces can produce nitrilase and nitrile hydratase and can also break the acetonitrile in a sequence. The production of these enzymes is tightly regulated by the bacteria in response to the presence of acetonitrile as a carbon and nitrogen source (Kobayashi *et al*., 1995b).

The synthesis of pharmaceutical compounds, specialty chemicals, and commodity chemicals is facilitated by immobilised enzymes. For example, the synthesis of acrylamide has been facilitated by effective immobilization of NHase, an enzyme, on a variety of matrices (Nagasawa and Yamada, 1990). Various materials such as agar-agar, sodium alginate, polyacrylamide, di-ethyl aminoethyl cellulose, and soldier matrix, have been used in previous studies to immobilise biocatalyst containing cells. This has facilitated the development of enzymatic processes for the bioconversion of acetonitrile into acrylamide. An enzymatic process of bioconversion of acetonitrile to acrylamide has been developed by immobilised biocatalysts containing cells. The potential for enhanced stability, economic efficiency and versatile applications across various disciplines is offered by enzyme immobilization, which offers a promising solution to the challenges associated with their industrial useful. The development of a standardised, robust immobilisation procedure is necessary to the diversity of substrates and enzymes. The synthesis of valuable compounds and bio catalytic processes remains a promising area of research.

Hence, this study was conducted to investigate acetonitrile biodegradation by nitrile hydrolysing enzyme produced by Pseudomonas species isolated from soil in contaminated industrial site for sustainable environmental remediation Protection.

MATERIALS AND METHODS:

Chemicals and reagents:

All the chemicals used in this study were analytical or HPLC quality. Avra Synthesis Pvt LTD supplied the acetonitrile, and MERCK, the bromothymol blue. We used Himedia to buy all the other media components.

Collection of soil samples and soil analysis:

Soil samples were collected in sterilised screw capped glass tubes from soil in contaminated industrial sites of Gajulamandyam, (13.59703211°N, 79.51194742°E) situated at Tirupati district of Andhra Pradesh, India. Upon the arrival of the soil sample at the laboratory, the initial step involves the air drying of the soil at ambient room temperature, typically ranging between 25ºC to 35ºC. The dried samples were sieved to 2mm particle size, and the primary physical and chemical properties were analysed (Dandwate, 2020).

Isolation of nitrile metabolising bacterial strain from collected soil sample:

One gram of soil was dissolved in 100 ml minimal salt medium and incubated at 160 rpm for 24 h (Sahu *et al*., 2019; B et al., 2013). One ml of the suspended culture was serial diluted and plated on enriched minimal salt agar media by pour plate technique supplemented with acetonitrile as substrate and incubator at 37ºC (Kobayashi *et al*., 1989). The pure culture of the isolated colonies was maintained in nutrient agar slants.

Identification of isolated bacterial strains:

Bergey's manual systematic bacteriology is employed to identify the isolated strains, and morphological and biochemical tests are conducted (Williamson et al., 2010). Followed by genomic DNA was isolated from the isolated bacterial species and subsequently amplified to obtain the 16th RNA gene sequence. The amplified sample was submitted to CSIR national chemical Laboratory, Pune, India for 16S rRNA gene sequencing. The sequencing files were the analysed using then analysed using blast compared them to the most similar culture sequence obtained from the. NCBI database.

Screening for nitrile hydrolysing enzyme:

The isolated bacteria were subjected to screen for the synthesis of nitrile hydrolysing enzyme following the procedure (Banerjee et al., 2003; Xue *et al*., 2016). This screening involved monitoring changes in pH of the acetonitrile substrate as it was hydrolysed into amides or carboxylic acid and ammonia (Xue *et al*., 2016). The experiments basic idea is that the blue to green pH indicates amid formation by nitryl hydrates activity (Banerjee *et al*., 2003). Conversely, a transition of the pH indicator dye from blue to yellow signals the accumulation of carboxylic acid and ammonia, which is indicative of nitrilase enzyme activity (Howden & Preston, 2009).

Detection of ammonia:

The bacterial isolates that produced nitriles war identified by measuring ammonia according to Nessler's method (Nxumalo et al., 2020). The experiment's reaction mixture consists of 2 ml of cell suspension, 0.5 ml of 500 mM acetonitrile and 2 ml of 10 mM phosphate buffer with 0.001% bromothymol blue. The controverse maintained, and the tubes were incubated 30 ºC for 3 to 12 h. To quantify the quantity of ammonia that was liberated during the process, 100 µl of Nessler reagent was introduced to the reaction after incubation (Jeong et al., 2013).

Quantification of nitriles Production:

Spectrometric assay was employed to quantify the production of nitriles by the bacterial isolates (Xue et al., 2016b). Each isolate was inoculated into 50ml of minimal medium containing yeast extract (5g/L), peptone (5g/L) glucose (10g/L), at pH 7 and incubated at 30 ºC for 24 h to prepare pre cultures, after the incubation 2ml of pre culture was transferred to

50ml freshly prepared medium supplemented with 1% acetonitrile and incubated at 30 ºC for 36h. following incubation cells were collected by centrifugation at 6000 RPM for 5mins. The resulting pellets were washed twice with 100mM K_2PO_4 buffer at pH 7.2 and then resuspended in the same buffer for subsequent enzyme assay (Hjort et al., 1990).

Preparation of resting cells:

The isolates were cultured in minimal basal broth media under optimised conditions. After 42h of incubation, the cells were collected by centrifugation at 600o RPM for 10 min. the resulting pellets were washed twice with 0.1M K_2PO_4 buffer and then resuspended in the same buffer. (Raj et al., 2007).

Immobilization of resting cells in agar-agar:

To immobilise the resting cells, agar matrices were utilised. According to Shinde and Karegoudar (1998), a particular quantity of agar- agar was dissolved in 0.1M potassium phosphate buffer pH 7.0 and then autoclaved. The molten Agar was aseptically mixed with 2 ml of resting cell suspension (30mg dwc/ml) and then transferred to Petri plates with flat bottoms. The ingredients were left to cool to room temperature until they solidify. Down to 50 ºC, the molten Agar was lowered. A sterile corkborer was used to separate the agar gel into small 5 mm diameter and 5 mm thick discs. The agar beads were rinsed 3 times with buffer and once with distilled water before being transferred to a fresh beaker and kept at a low temperature of 4 ºC (Raj et al., 2007).

Degradation of acetonitrile:

Log phase culture of bacterial strain was evaluated for the rate of acetonitrile degradation percentage which slight modification following procedure of (Nagasawa and Yamada, 1990b). Bacterial cells were grown in rotary shaker with different concentration of acetonitrile (1%, 2%, 3%) in minimum salt broth at 30 ºC for 24, 48, 72 h and 96 h of incubation. The experiment was performed in triplicates. The following equation is used to determine the percentage of degradation.

$$\text{Degradation \% } (\eta) = \frac{C0-C}{C0} \times 100 \text{ (Sen } \textit{et al.}\text{, 2022)}$$

Where C_0 indicates initial concentration of the acetonitrile (%) before biodegradation and C: final concentration of acetonitrile (in %) after degradation.

Evaluation of ammonia release:

Acetonitrile degradation results in the production of ammonia as a byproduct, which can be quantified quantitatively measured calorimetrically by the Berthelot method (Nawaz and Chapatwala, 1991b), thereby allowing indirectly estimation of the amount of acetonitrile degraded (Nxumalo *et al*., 2020).

RESULTS AND DISCUSSION:

The primary physical and chemical properties of the soil are given in Table 1. The findings offer significant information on the properties of the soil and the possible influence of the effluent on the quality of agricultural soil. pH measures the relative concentration of hydrogen ions in the solution. As per Jackson's method, the soil pH was measured using a

potentiometer pH meter with the soil water suspension ratio of 1: 2:5. The pH of both control and the GPC-1 soil samples ranged between pH 8.2 and pH 8.3, respectively, which indicates the Slight alkalinity of the soil.

Table – 1: Physical and chemical analysis of soil samples

S.NO	PARAMETERS	UNITS	CONTROL	GPC 1
1	pH	pH	8.2	8.3
2	Electrical Conductivity (EC)	m.mhos/cm	2.02	2.95
3	Organic Carbon	%	0.34	1.1
4	Boron	Mg/L	0.081	0.534
5	Calcium	Mg/L	70.98	63.06
6	Copper	Mg/L	0.739	0.33
7	Iron	Mg/L	361.5	142.4
8	Potassium	Mg/L	135.4	19.17
9	Magnesium	Mg/L	180.2	12.88

An essential characteristic of the soil is its electrical conductivity. It shows the soils overall soluble salt content, the number of ions in the soil sample is indicated by conductivity value, the cations of the clay or colloidal matter and the cations of soil and soil solution are exchanged in proportions during the process. The anions such as Co_3^-, HCo_3^- and Po_4^- while the cations such as Ca^+, K^+, Na^+, and Mg^+. The conductivity readings can change depending on the soil's chemical composition. The soil type is acceptable if the E.C. is less than 4 m.mhos/cm. The electrical conductivity (E.C.) values of the control and GPC-1 soil samples range between 2.02-2.95 m.mhos/cm (millimhos per centimetre).

Soil organic carbon is an essential component of soil fertility as it releases nutrients that are essential for plant growth, soil fertility and health, or enhanced by increases in organic carbon (Johnston *et al*., 2009). The organic carbon percentage of control and GPC-1. Soil samples ease about 0.34% and 1.1%, respectively. Potassium is an essential nutrient for plant physiological function and superior crop production. The potassium content in the control and GPC-1 is about 135.4 mg/L and. 19.17 mg/L respectively. Phosphorus plays a crucial role in energy storage and transfer root development, and fruit and flower formation in the plant (Dandwate, 2020). In the study area, the phosphorus range in control and GPC-1 is 0.042 mg/L and 3.251 mg/L respectively. The trace elemental content in the control soil is boron 0.081 mg/L, copper 0.739 mg/L. Iron 361.59 mg/L. Magnesium 180.2 mg/L, manganese 5.695 mg/L, molybdenum 8.596 mg/L, zinc 0.968 mg/L. Whereas in GPC-1 soil sample Boron 0.534 mg/L. Copper 0.33 mg/L. Iron 142.4 mg/L. Magnesium 12.88 mg/L. Manganese 1.509 mg/L. Molybdenum 3.141 mg/L. and zinc, 0.439 mg/L.

Identification of isolated bacterial strains.

All five strains are identified as gram negative motile, rod shaped and non-sporulating bacteria. All five strains show positive for catalase tests followed by molecular identification

done by 16s rRNA sequencing at CSIR labs, Pune, India, and nucleotide sequence data were deposited to gene bank and obtained the assession number listed in table 2.

Table 2: **Identification of isolated bacterial strains**

Isolated Strains	Assession number	Bases	Name of the strains
GPC1- C	PP967784	726 bp	*Pseudomonas aeruginosa* GPC1- C
GPC1- E	PP967928	726 bp	*Pseudomonas aeruginosa* GPC1- E
GPC1- G	PP967860	726 bp	*Pseudomonas aeruginosa* GPC1- G
GPC1- J	PP967931	726 bp	*Pseudomonas aeruginosa* GPC1- J
GPC1- K	PP967932	726 bp	*Pseudomonas aeruginosa* GPC1- K

The Nitrile. metabolising bacteria where isolated using serial dilution and enrichment culture techniques (Sahu *et al*., 2019). The study found five bacterial strains using nitrile as their sole source of carbon and nitrogen where isolated from industrial area Gajulamandyam, Andhra Pradesh.

All the five isolates were subjected to screening for nitrile hydrolysing enzyme by dye-based method using acetonitrile as substrate. The basic principle of the method is changing in pH which results changing the colour pH. Indicator by the optimum pH of the nitrile hydrolysing enzyme is in between 7.0 to 7.5. Bromothymol blue is used as pH indicator dye which is blue at 7.4 pH

Nitrile hydrolysing enzymes have potential applications in industries as preferred biocatalyst for the synthesis of carboxylic acid amides and ammonia. Nitriles is the first nitrile metabolising enzyme discovered four decades ago in plants (Thimann & Mahadevan, 1958) and in bacteria (Hook & Robinson, 1964). The isolated *Pseudomonas* species was tested for nitrile hydrolysing enzyme activity, sceptically nitrilase enzyme. The bromothymol blue technique was used for qualitative screening and all five *Pseudomonas* strains tested positive for nitrilase activity. This method is based on the colour changing of bromothymol blue indicator from yellow to greenish blue measured in Colorimetry, which indicates the production of ammonia from the hydrolysis of acetonitrile.

Following qualitative screening, a quantitative analysis of a nitrilase activity was conducted. The quantitative screening of all five nitrilase producing bacteria strains was carried out using spectrometric essay, at 660nm O.D., (Xue *et al*., 2016b). Shown in fig 1. Among all bacterial strains *Pseudomonas aeruginosa* GPC1- C exhibited maximum nitrilase activity releasing 1.6± 0.02 U ML^{-1} ammonia Shown in Table 3. *Pseudomonas aeruginosa* GPC 1-E, GPC1-C and GPC1- J were the next in line of ranking. *Pseudomonas aeruginosa* GPC1-G demonstrated minimum activity of nitrilase releasing. 0.6 ± 0.01 U ML^{-1} of ammonia. According to the results, *Pseudomonas aeruginosa* GPC 1-C, *Pseudomonas aeruginosa* GPC1-J and *Pseudomonas aeruginosa* GPC1-K are the most effective in production of nitrilase among all isolated *Pseudomonas* species, although their enzyme activity levels differ (Zhang et al., 2022).

Table 3: Qualitative and quantitative nitrilase activity of different *Pseudomonas Sps*

Sl No	Name of the Isolates	Inference	Enzyme	Ammonia Released (U ML^{-1}) 660nm O.D.
1.	ACETONITRILE (C1)	Blue	Control-1	0

2.	BUFFER (C2)	Blue	Control-2	0
3.	MEDIA (C3)	Blue	Control-3	0
5.	*Pseudomonas aeruginosa* GPC1- C	Yellow	Nitrilase	1.6 ±0.02
6.	*Pseudomonas aeruginosa* GPC1- E	Yellow	Nitrilase	0.8 ±0.04
7.	*Pseudomonas aeruginosa* GPC1- G	Yellow	Nitrilase	0.6 ±0.01
8.	*Pseudomonas aeruginosa* GPC1- J	Yellow	Nitrilase	1.3 ±0.03
9.	*Pseudomonas otitidis* GPC1- K	Yellow	Nitrilase	1.04 ±0.02

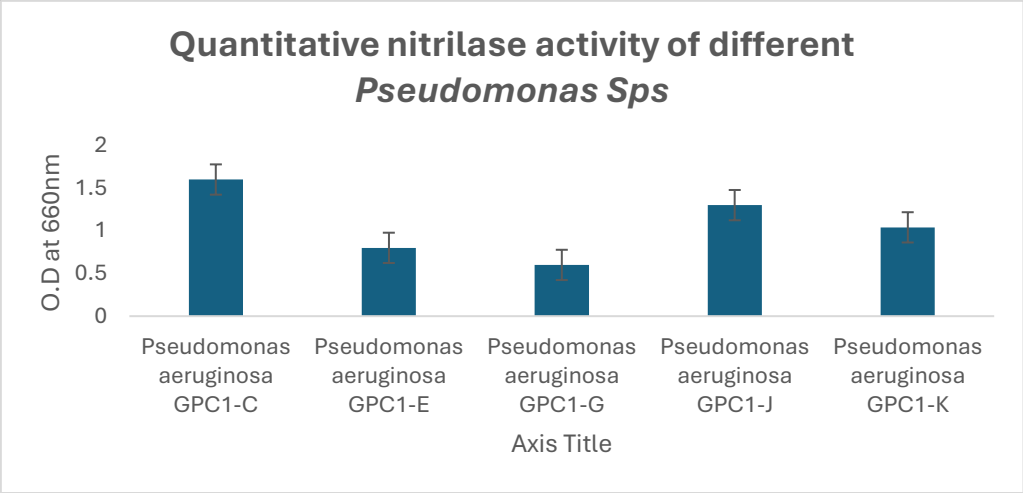


Fig 1: Quantitative nitrilase activity of different *Pseudomonas Sps*

Ammonia releases during the degradation of acetonitrile was quantified using Nessler's method (Jeong *et al*., 2013). Examining the amount of ammonia release provides the further validation the effectiveness of the screening method in differentiating between nitrilase and nitrile hydratase activity. Nitrilase and nitrile hydratase often catalyse the degradation of nitriles, leading in the release of carboxylic acid and ammonia into the surrounding medium (Nxumalo *et al*., 2020). The quantification of ammonia produced during the reaction was carried out by adding Nessler's reagent. The extent of the color change from colourless to orange directly indicates the level of nitrilase enzyme activity. All the five isolated bacterial strains showed positive for nitrilase activity.

The immobilization of the microorganisms has been suggested as a method to improve degradation efficiency in wastewater treatment system (Kumar *et al*., 2018). Various methods were employed for immobilising bacteria such as entrapment, adsorption, encapsulation, biofilm to enhance to degrade pollutants has been developed resulting in increased effectiveness in wastewater treatment (Li *et al*., 2013). The immobilized microorganisms were achieved using agar-agar as the support metrics after solidification. The agar gel was then cut into small discs of 5mm in diameter and 5mm in thickness using sterile corkborer (Sahu *et al*., 2019). Immobilised cell having demonstrate remarkable degradation capacity at high acetonitrile concentrations under long incubation times, demonstrated their unique efficiency in harsh environmental conditions (Kumar *et al*., 2022).

In this study, the degradation of acetonitrile was measured in the form of ammonia which is liberated by as byproduct during the breakdown of acetonitrile (Sandhya & Mulchandan, 2022), catalysed by enzyme nitrilase produced by *Pseudomonas* species. The concentration of the acetonitrile decreases by increasing ammonia, although the accumulation of ammonia in the media was detected acetonitrile degradation. Individual analysis of conducted for each

Pseudomonas species regarding acetonitrile biodegradation. Various *Pseudomonas* species perform degradation in different concentration, which is mentioned in the Table 4. Among the five isolated *Pseudomonas* strains evaluated for acetonitrile degradation, *Pseudomonas aeruginosa* GPC 1-K at 96 h, 3% concentration of acetonitrile for resting cells and 2% acetonitrile concentration at 24 h for immobilised cells shown in Figure – 2 exhibited maximum acetonitrile degradation activity.

Table 4: Quantitative estimation of ammonia liberation

Time	Resting cells			Immobilized cells		
	1%	2%	3%	1%	2%	3%
Pseudomonas (*aeruginosa*) GPC 1 K isolates						
24 hours	0.2 mg/L	0.4 mg/L	0.6 mg/L	0.25 mg/L	0.6 mg/L	0.7 mg/L
48 hours	0.4 mg/L	0.54 mg/L	1.2 mg/L	0.56 mg/L	0.56 mg/L	1.4 mg/L
72 hours	0.6 mg/L	0.75 mg/L	2.4 mg/L	0.64 mg/L	0.8 mg/L	2.6 mg/L
96 hours	0.75 mg/L	1.2 mg/L	3.0 mg/L	0.78 mg/L	1.4 mg/L	3.5 mg/L

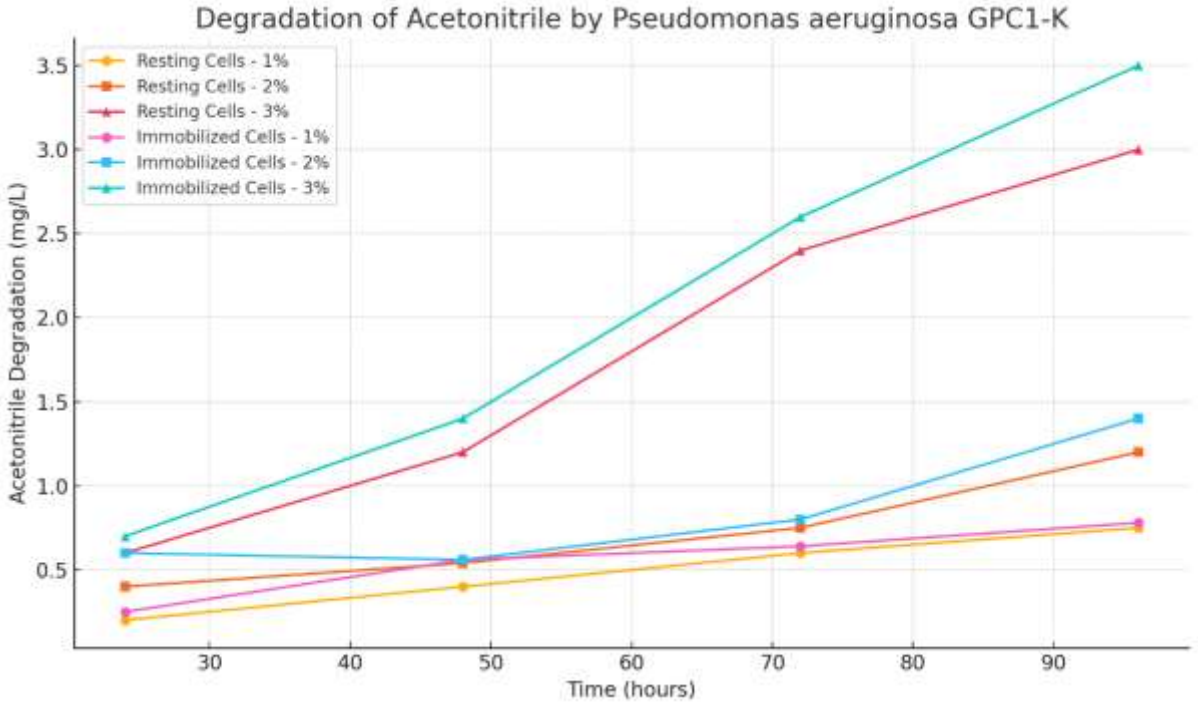


Figure 2: Degradation of Acetonitrile by Pseudomonas aeruginosa GPC1-K

Statistical Analysis:

The statistical analysis performed using IBM SPSS. 25.0. software all the experiments were conducted in triplicates and the results was presented as mean value ±P is equal to 0.032031 as shown in Table 5 and in figure 3 Regression analysis of nitrilase activity over time provided.

Table 5: Statistical Analysis of Nitrilase Activity

Regression Statistics analysis

Statistic	**Value**

Multiple R	0.967969
R Square	0.936964
Adjusted R Square	0.905446
Standard Error	0.073601
Observations	4

ANOVA

Source	df	SS	MS	F	Significance F
Regression	1	0.161041	0.161041	29.728	0.032031
Residual	2	0.010834	0.005417		
Total	3	0.171875			

Coefficients

Coefficient	Standard Error	t Stat	P-value	Lower 95%	Upper 95%
Intercept	-0.088476	0.111865	-0.79091	0.511888	-0.569791
X Variable 1	1.03314	0.189486	5.45233	0.032031	0.217848

Regression Analysis Graph

Statistical Analysis of Nitrilase Activity (Corrected)

Time (hours)	Nitrilase Activity (U/mL)
24	0.2
48	0.4
72	0.6
96	0.75
24	0.25
48	0.56
72	0.64
96	0.78

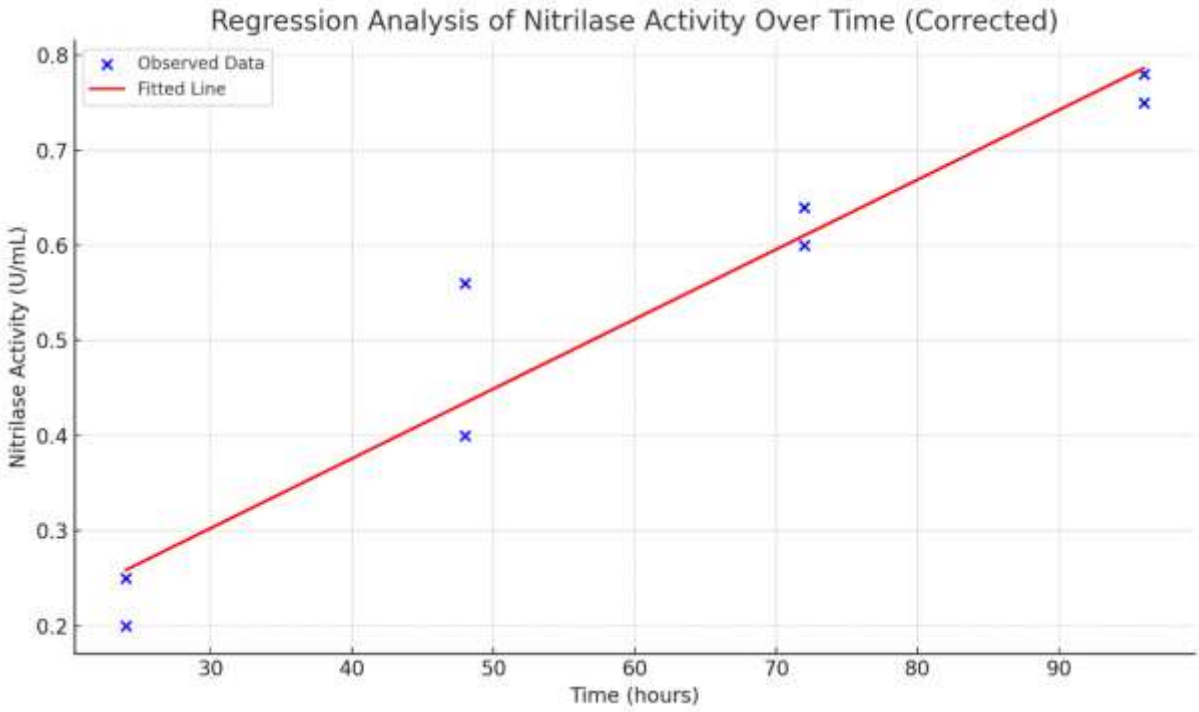


Figure 3: Regression analysis of nitrilase activity over time (corrected)

Conclusion.

The result of the study shows that GPC 1-K strain of *Pseudomonas aeruginosa* can biodegrade the toxic industrial solvent acetonitrile. The strain showed high level of nitrilase activity in both qualitative and quantitative screening. The efficiency of degradation was enhanced by the presence of immobilised cells. The bioremediation. Potential of GPC1-K was highlighted by its maximum degraded activity under different settings. The strain shows promise for reducing acetonitrile contamination in industrial effluent, according to the results. Immobilization technique improvement and scaling up for. Industrial applications should be the focus for future study.

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