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OPTIMIZATION OF FACTORS AFFECTING BIODEGRADATION OF ACETONITRILE BY *ARTHROBACTER* ISOLATES OBTAINED FROM SOIL AND WASTEWATERS

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ABSTRACT

Wastes in the environment have always been broken down by microbes. Also, humans have used the microbes in domestic, industrial, and agricultural activities. Therefore, soil and wastewater niches are expected to harvest a variety of stress-resistant bacteria. *Arthrobacter*, a common occupant in these habitats can degrade organic pollutants, inorganic pollutants, and dyes. Nitriles are a group of toxic compounds that affect the central nervous system by the alkylation of protein sulfhydryl groups. They are also toxic to other microbes that degrade other wastes. The acetonitrile-grown cells of the isolates exhibited activities corresponding to nitrile hydratase and amidase, which mediate the two-step breakdown of acetonitrile into acetic acid and ammonia. The *Arthrobacter* isolates A11 and A40 were found to be potential biodegradors of acetonitrile. The optimization of factors affecting the biodegradation of acetonitrile by these isolates, identified as *Arthrobacters*, by 16SrRNA sequencing, was determined. Nutritional and physicochemical were the two categories of factors employed. Sucrose, glucose, lactose, sodium nitrite, peptone, and casein hydrolysate were employed to check their effect on degradation. The effect of other factors such as temperature, pH, incubation time, inoculum density and volume, aeration, and salinity, on degradation, was also studied. Both isolates showed maximum degradation of acetonitrile in presence of lactose and casein, room temperature, pH 7, 48 hours of incubation, static conditions, inoculum density of 1(optical density), inoculum volume of 3%, and salt concentration of 0.5% in the medium.

KEYWORDS: Pollutants, acetonitrile, physicochemical, inoculum, salinity.

INTRODUCTION

Nitriles have widespread use in the manufacture of plastics, pharmaceuticals, herbicides, synthetic rubber, and other chemicals. They are also used as industrial solvents and are synthesized on a large scale. Nitriles are notoriously poisonous compounds. They are toxic to (CNS) central nervous system by the alkylation of protein sulphydryl groups present in cells and have mutagenic activity due to conjugated bonds. Nitriles are also toxic to several microbes that degrade different types of waste. Therefore, it becomes necessary to treat waste containing nitrile, separately before mixing it with common waste.

The degradation of nitriles by the microbes is a two-step mechanism. The first step converts simple aliphatic nitriles to the corresponding amide via nitrile hydratase. The second step is the conversion of amide to carboxylate with the liberation of ammonia via amidase. Acetonitrile-grown cells of *Arthrobacter* exhibited activities corresponding to nitrile hydratase and amidase, which bring about the two-step breakdown of acetonitrile into acetic acid and ammonia. Acetonitrile can be degraded anaerobically and aerobically as a sole source of carbon and nitrogen by microbes.[1]

The successful execution of the selected bioremediation technology in polluted areas depends on the characteristics of the polluted site, the mechanism, and a complicated system of several factors that affect the processes of biodegradation. Thus, it becomes extremely important to take care of and understand those limiting factors.[2,3]

Nutritional and physicochemical factors were the two categories of factors studied in the current work. A11 and A40 were the isolates used for this experiment. They were identified as *Arthrobacters*, by 16SrRNA sequencing. The capability of the microorganism to grow in a given system depends on the organism's ability to consume any available nutrient. The carbon source in the medium serves as a source of energy. If the substrate in the surroundings is novel/different, it sometimes requires the microbe to start its degradation/utilization. However,

the presence of an easily degradable carbon source in the medium initiates metabolism and simultaneously co-metabolizes the novel substrate/pollutant. It could affect the degradation also.[2,4] Glucose, lactose, and sucrose were employed to check their effect on the degradation of acetonitrile, in this study. The growth of microbes is promoted by nitrogen sources. They aid in the synthesis of building blocks of cells, proteins, and enzymes. They are also reported to fasten the rate of biodegradation to a certain extent.[5]

The increased temperature expectedly increases the degradation capacity of microbes. The solubility of compounds increases with temperature. Also, the bioavailability of the molecules increases. Activity decreases as the temperature is lowered because of slow metabolism. The degradation activity is affected which may be due to a change in optimum temperature for the functioning of enzymes.[6] *Arthrobacter* is reported to survive extreme conditions of temperature. Acetonitrile degradation is better at neutral to alkaline pH. Many sites contaminated with pollutants are not at the optimal pH for biodegradation. This may be due to the absence of optimal pH for enzyme functioning and environmental changes.

The degradation percent of the compound increases with increased incubation time. The reason may be initial catabolite repression and the time taken by the microbe to synthesize enzymes necessary for degradation. However, with increasing time, percent degradation may decrease due to the accumulation of toxic products affecting the viability of microbes. It can also be due to the saturation of active sites of enzymes.[7]

The microbe's number and its catabolic activity decide the ability of the microbial community to degrade pollutants. For successful biodegradation, this number should not be lower than 10^3 microbes per gram of soil. On one hand, a smaller number of cells will slow the rate of degradation, but on other hand, denser culture may not be available because of cell packing.[8]

Inoculum volume is equally important as inoculum density. Less volume and more dense culture may not serve the purpose because the increase in the number of cells will again take a lag phase to multiply.[9]

Respiration of the microbe requires dissolved molecular oxygen and is used throughout the subsequent degradation pathway. Requirements for oxygen uptake are substantial. At sufficient depths and in deep water sediments, the degradation of pollutants can turn anaerobic when the oxygen supply is depleted. Oxygen is the rate-limiting for the activity of such organisms.[10] Microorganisms are generally well adapted to cope with the wide range of salinities common to the world's ocean.

However, increasing salinity in aquatic environments has had a negative impact on the biodegradation of various pollutants in soil and water ecosystems.[11,12]

Arthrobacter bacteria are also reported to survive in saline environments.

Two categories of limiting factors studied in the current work were nutritional and physicochemical factors and their effect on biodegradation by two identified *Arthrobacter* isolates. Glucose, lactose, sucrose, sodium nitrite, casein hydrolysate, and peptone were employed to check their effect on degradation. The effect of temperature, pH, incubation time, inoculum density and volume, aeration, and salinity, on degradation, was also studied. *Arthrobacter* can be used for bioremediation of acetonitrile contaminated niches. Thus, *Arthrobacter* could go a long way in the environmental clean-up of pollutants.

MATERIALS AND METHODS

The method used for Acetonitrile estimation i.e., Nesslerization spectrophotometric method remains the same throughout the study**.** All the experiments were carried out in triplicates. Positive and negative controls were kept wherever applicable. Appropriate dilutions were carried out as required.

Materials

Standard Ammonia estimation

Standard NH_3 solution-50 mcg/ml
0.5% K-Na tartrate (Rochelle salt) solution and Nessler's Reagent
D/W and ammonia free D/W
Sterile test tubes and pipettes
Centrifuge and Colorimeter
Culture suspensions of identified *Arthrobacter* isolate A11 and A40
Culture suspensions of Standard strains *Arthrobacter nicotinae mtcc no.*2* and *Arthrobacter chlorophenicolus*3706*

1. Nutritional Factors

Culture suspensions of identified *Arthrobacter* isolate A11 and A40

i. Effect of carbon sources

Sterile 100 ml Mineral salt broth media containing 100 mcg/ml of acetonitrile and100 mg. (1 gm/lit) of 3 selected carbon sources in 3 different flasks-2 sets
3 selected carbon sources in 3 different flasks -2 sets
Sterile 100 ml Mineral salt broth media containing 100 mcg/ml of acetonitrile and no carbon source - 3 flasks as controls
Carbon sources used - glucose, lactose, and sucrose

ii. Effect of nitrogen sources

Sterile 100 ml Mineral salt broth media containing 100 mcg/ml of acetonitrile and 100 mgs. of 3 selected nitrogen sources in 3 different flasks -2 sets
3 selected nitrogen sources in 3 different flasks -2 sets

Sterile 100 ml Mineral salt broth media containing 100 mcg/ml. of acetonitrile without nitrogen source -3 flasks as controls
Nitrogen sources used - sodium nitrite, casein hydrolysate, and peptone

2. Physicochemical Factors
i. Effect of temperature
Sterile 100 ml Mineral salt broth media containing 100 mcg/ml. of acetonitrile - 5 flasks for 5 different temperatures (4, RT, 37, 45, and 55^{0}C) – 2 sets and 1 set of flasks as a control for each temperature.
RT—Room temperature
ii. Effect of pH
Sterile 100 ml Mineral salt broth media containing 100 mcg/ml of acetonitrile 5 flasks with different pH (5, 6, 7, 8, and 9) – 2 sets and 1 set of flasks as a control for each pH
iii. Effect of incubation time
Sterile 100 ml Mineral salt broth media containing 100 mcg/ml of acetonitrile – 2 flasks plus 1 flask as control
iv. Effect of inoculum density
Culture suspensions of identified *Arthrobacter* isolates A11 and A40 with 5 different inoculum densities 0·6, 0·7, 0·8, 0·9 and 1.0 (at 660 nm)
Sterile 100 ml Mineral salt broth media containing 100 mcg/ml of acetonitrile - 5 flasks for 5 different inoculum densities –2 sets and 1 flask as control
v. Effect of inoculum volume
Sterile 100 ml Mineral salt broth media containing 100 mcg/ml of acetonitrile - 5 flasks for 5 different inoculum volumes of cultures – 2 sets and 1 flask as control
vi. Effect of aeration
Sterile 100 ml Mineral salt broth media containing 100 mcg/ml of acetonitrile- 2 flasks-2 sets
vii. Effect of salinity
Sterile 100 ml Mineral salt broth medium containing 100 mcg/ml of acetonitrile 5 flasks with different salt concentrations of 0.5,1, 1.5, 2, 3, and 4 % – 2 sets and 1 set of flasks as controls. Sodium chloride

METHOD
Degradation assay of acetonitrile. Sterile Mineral salt liquid medium (100 ml) containing acetonitrile and different carbon/nitrogen sources was taken in different flasks and inoculated with 2 ml of *Arthrobacter* isolate A11 (0.5 @ 660 nm). A similar setup was done for isolate A40. A control flask without any carbon/nitrogen source was also kept. A control flask without any culture was also included. **A similar setup was employed for all nutritional and physicochemical conditions mentioned above.** They were all incubated at RT for 48 hrs. Aliquots were removed from the medium at end of 24 and 48 hrs of incubation. Growth, ammonia release, and change in pH were checked in the supernatant of aliquots.

As mentioned earlier growth of the isolate was checked colorimetrically at 660 nm. The pH of aliquots removed was read with a potentiometric pH meter. For ammonia concentration, Nesslerization spectrophotometric method was used. A set of standards was run by using NH_4Cl as standards ranging from 1-5 mcg/ml. The absorbance was measured at 425 nm using D/W as blank.[13,14,15] The standard graph was plotted and the unknown values were determined from the graph. The degradation Percent was calculated by the standard formula.

RESULTS AND DISCUSSION

Standard Ammonia estimation was carried out by Nesslerization spectrophotometric method and the values were obtained from the standard graph depicted in figure 1. These values were correlated with the concentration of acetonitrile and percentage degradation was calculated. The regression statistics of the standard graph of ammonia are shown in tables number 1 and 2.

Table number 3 depicts the growth of *Arthrobacter* isolates and standards whereas table number 4 shows the changes in pH, in the mineral salts medium containing acetonitrile.

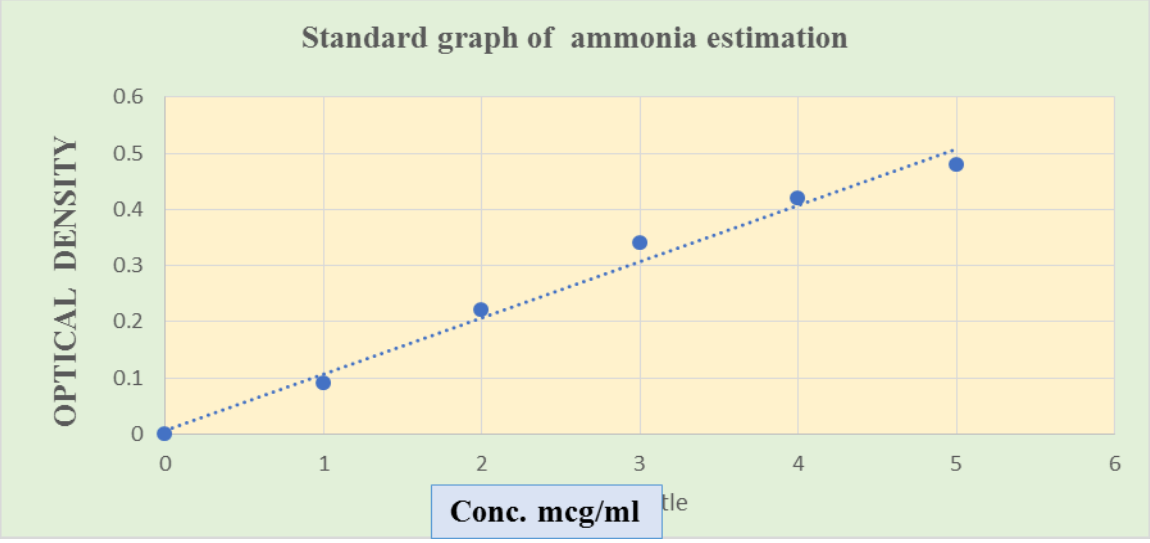

Figure 1: Standard graph for ammonia estimation by Nesslerization method.

Table 1: Regression statistics of the standard plot of Ammonia estimation.

Regression Statistics					
Multiple R	0.993023				
R Square	0.986094				
Adjusted R Square	0.982618				
Standard Error	0.024909				
Observations	6				
ANOVA					
	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	0.176001	0.176001	283.6554	7.28501E-05
Residual	4	0.002482	0.00062		
Total	5	0.178483			

Table 2: Regression statistics of the standard plot of Ammonia estimation.

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	0.007619	0.018028	0.422621	0.69430	-0.0424	0.05767	-0.042434	0.0576
X Variable 1	0.100285	0.005954	16.84207	7.2905	0.0837	0.11681	0.083753	0.1168

Table 3: Growth of the identified *Arthrobacter* isolates and *Arthrobacter* standard strains measured as optical density /absorbance at 660nm in acetonitrile medium.

	Growth measured at 660 nm	
Isolate no	**24 hrs**	**48 hrs**
A11	**0.06**	**0.12**
A40	**0.12**	**0.13**
AS1	0.06	0.08
AS2	0.03	0.09

Table 4: pH of the acetonitrile medium inoculated with probable *Arthrobacter* isolates and *Arthrobacter* standard strains.

	pH at		
Isolate number	**0 hr**	**24 hrs**	**48 hrs**
A11	7.84	7.69	7.49
A40	7.84	7.85	7.36
AS1	7.75	7.55	7.63
AS2	7.84	7.44	7.58
Control	7.1	7.4	7.4

Nutritional Factors

The results of the effect of Carbon and Nitrogen sources on biodegradation by *Arthrobacter* isolates A11 and A40 are tabulated in tables 5 and 6.

I. Effect of carbon sources

Table 5: Degradation of Acetonitrile in presence of different carbon sources at 24 and 48 hrs, by *Arthrobacter*, isolates A11 and A40.

Isolate/Carbon source	Glucose		Lactose		Sucrose	
	24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs
A11	73.17%	78.24%	**75 %**	**87.2 %**	15.7 %	20 %
A40	41.65%	62.92%	**50.8 %**	**64.7%**	14.1 %	21.2 %

ii. Effect of Nitrogen sources

Table 6: Degradation of Acetonitrile in presence of different nitrogen sources at 24 and 48 hrs, by *Arthrobacter*, isolates A11 and A40.

Isolate/Carbon source	Peptone		Casein hydrolysate		Sodium nitrite	
	24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs
A11	**71.18%**	**75.42%**	66%	66.2%	14.5%	18.1%
A40	39.65%	49.92%	**47.8%**	**58.7%**	9%	11.2%

2. Physicochemical Factors

The results of the effect of temperature, pH, incubation time, inoculum density and volume, aeration, and salinity, on degradation by *Arthrobacter*, isolates A11 and A40 are tabulated in **tables 7 to 13.**

i. Effect of temperature

Table 7: Degradation % of Acetonitrile at different temperatures by *Arthrobacter* isolates A11 and A40 at 24 and 48 hrs.

Isolate/Temp	4^0C		RT		37^0C		45^0C		55^0C	
	24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs
A11	14.3%	17.1%	**70.19%**	**74.2%**	64%	64.2%	60%	61%	51%	51.7%
A40	9%	10.2%	**38.55%**	**49.2%**	48.8%	58.7%	42%	45.5%	35.7%	36.7%

ii. Effect of pH

Table 8: Degradation % of Acetonitrile at different pH by *Arthrobacter* isolates A11 and A40 at 24 and 48 hrs.

Isolate/ pH	5		6		7		8		9	
	24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs
A11	3%	4%	16.3%	18.1%	**67.9%**	**77.2%**	56%	59%	51%	51%
A40	4%	4%	7%	11.2%	**41.5%**	**49.9%**	44%	46.5%	36.7%	37%

iii. Effect of Incubation time

Table 9: Degradation % of Acetonitrile by *Arthrobacter* isolates A11 and A40, at 24 and 48 hrs.

Isolate / Incubation time	24 hrs	48 hrs
A11	73.17%	**78.24%**
A40	41.65%	**62.92%**

iv. Effect of inoculum density

Table 10: Degradation % of Acetonitrile using different inoculum densities by *Arthrobacter* isolates A11 and A40, at 24 and 48 hrsv.

Isolate/ Inoculum density	0.6		0.7		0.8		0.9		1	
	24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs
A11	28%	30%	47.9%	48%	50%	52%	67.5%	72.9%	**69%**	**74.2%**
A40	21%	24.7%	25.6%	26%	28%	30%	38%	43%	**39.5%**	**45.9%**

v. Effect of inoculum volume

Table 11: Degradation % of Acetonitrile using different inoculum volumes by *Arthrobacter* isolates A11 and A40, at 24 and 48 hrs.

Isolate/Inoculum volume-%	1		2		3		4		5	
	24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs
A11	23%	38%	50%	65%	**69%**	**75.2%**	59%	57%	45%	34%
A40	21.5%	20.4%	34%	35%	**43.2%**	**47.9%**	35%	34%	22%	22.6%

vi. Effect of aeration

Table 12: Degradation % of Acetonitrile under Static and Shaker conditions by *Arthrobacter* isolates A11 and A40, at 24 and 48 hrs.

Isolate / Aeration condition	Shaker		Static	
	24 hrs	48 hrs	24 hrs	48 hrs
A11	73.17%	**78.24%**	73.17%	**78.24%**
A40	41.65%	**62.92%**	41.65%	**62.92%**

vii. Effect of salinity

Table 13: Degradation % of Acetonitrile using different salt concentrations in medium, by *Arthrobacter* isolates A11 and A40, at 24 and 48 hrs.

Isolate/Salt concentration %	0.5		1		2		4		6	
	24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs
A11	**69.9%**	**87.8%**	55.1%	55.7%	35.3%	35.9%	10.5%	10.2%	0%	0%
A40	**85%**	**88%**	60.2%	60.8%	25.4%	25.1%	5.6%	5.3%	0%	0%

CONCLUSIONS

Arthrobacter is indigenous to soil and wastewater ecosystems. It can biodegrade a wide array of pollutants including Acetonitrile. The contemporary times require the development of microbial inocula for bioremediation of such polluted habitats. The knowledge about the degrading abilities of native microbial populations is also important.[16] Bioremediation requires the optimization of parameters used in cultivating the organism. Biodegradation can be achieved efficiently if the optimum conditions for degradation are provided to the microbe. Biostimulation is a widely used approach to bioremediation. It involves additions of electron donors, electron acceptors or nutrients, or changes in pH, moisture, and aeration. Bioremediation can also be achieved through bioaugmentation.[17] Bioaugmentation is a type of bioremediation that requires studying the indigenous forms present in the location to assess if biostimulation is possible.[18] Hence optimum nutritional and physicochemical conditions were determined that can be employed for acetonitrile degradation by *Arthrobacter* isolates A11 and A40. Both these isolates showed maximum degradation of Acetonitrile in presence of lactose and casein as carbon and nitrogen sources respectively. Other optimum conditions for degradation were room temperature, pH 7, 48 hrs of incubation, static conditions and inoculum density of 1, inoculum volume of 3%, and salt concentration of 0.5% in the medium. *Arthrobacter* species are non-fastidious and can easily grow in simple media. *Arthrobacter is* one such sole organism that can degrade/bioremediate a multitude of pollutants from almost all the categories of pollutants. Due to their ubiquitous presence in soil, it can be explored to bioremediate subsurface pollution and thus the environmental clean-up. Any bioremediation strategy needs to be applied practically. It should be economically and technically feasible. This remains an important challenge to overcome.[16]

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