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Research Paper

BIODEGRADATION OF DIMETHYLFORMAMIDE USING *Pseudomonas aeruginosa*

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Abstract

In the present study an attempt has been made to degrade DMF using a natural isolate *Pseudomonas aeruginosa* and the isolate was found to be efficient in degrading DMF based on the assessment of different parameters like pH, turbidity, biomass, carbon dioxide and ammonia released during the degradation process. Effective degradation and increased microbial growth occurred between the pH 7 and 9.2. The turbidity and biomass showed a gradual increase during the initial period of treatment and decreased at the end. Increase in carbon dioxide and ammonia production in a concentration dependent manner with increase in incubation time indicates utilization of DMF as carbon and nitrogen source by *P. aeruginosa.* HPLC analysis for 200µl of DMF degradation by the isolate showed peaks with different retention time. Thus the results indicated that the isolate was able to degrade DMF found in the textile industrial effluents.

Key words: Dimethylformamide, bioremediation*, P. aeruginosa,* HPLC.

INTRODUCTION

Industrial development has led to profound social and economic changes as well as significant impacts on the environment. Hence in many places the pollution load discharged into the environment has exceeded the assimilative capacity and caused severe degradation of ecosystems and ultimately affected the livelihood of the people who depend on the services provided by these ecosystems. The pollution impact is observed in the ground water, surface water bodies and soil and the damage cost is significant in agriculture, fisheries and domestic water supply and thereby degraded quality of life. Factory workers in areas with uncontrolled industrial pollution are especially vulnerable.

Being a versatile organic solvent, N, N-dimethylformamide (DMF) is widely used in several industrial applications. It is primarily used as a solvent in the production of polyurethane products and acrylic fibers. It is a colourless liquid with a faint amine odour and manufacturers use it as a solvent in a variety of applications, including textile coatings, production of electronic components and pharmaceutical products [8]. DMF is produced either with catalyzed reaction of dimethyl amine and carbon monoxide in methanol or the reaction of methyl formate with dimethyl amine. It may also be prepared on a laboratory scale by reacting dimethylamine with formic acid [13]. As most of the DMF is released into the effluents after recovering the solute, it

is regarded as one of the most common chemicals found in the industrial effluents [11]. In view of the established reports on its widespread occurrence and its adverse impacts on the environment and health, DMF is considered to be an increasing threat both for the environment and to humans [12].

DMF is a possible product of the photochemical degradation of dimethylamine and trimethylamine [3,7]. Both are commonly occurring natural substances and are also used in industrial applications. DMF does not occur naturally and there are few reports concerning environmental levels or the exposure of the general population to DMF [9]. Concentrations in the air in the range of 0.02-0.12 mg/m^3 have been found in residential areas, near industrial sites. DMF is released in to the environment through various ways. When emitted into air, most of the DMF released remains in that compartment, where it is degraded by chemical reactions with hydroxyl radicals. Indirect releases of DMF to air, such as transfers from other environmental media, play only a small role in maintaining levels of DMF in the atmosphere. When DMF is released into water, it degrades there and does not move into other media. Contamination of soil with DMF may occur through spillage or leakage during its production, transport, storage, or use. When releases are into soil, most of the DMF remains in the soil presumably in soil pore water until it is degraded by biological and chemical reactions. If DMF reaches groundwater, its anaerobic degradation will be slow [4].

DMF is readily absorbed following oral, dermal, or inhalation exposure. Following absorption, DMF is uniformly distributed, metabolized primarily in the liver, and rapidly excreted as metabolites in urine. Available data from case reports and studies in occupationally exposed populations indicate that the liver is the critical target organ for the toxicity of DMF in humans. Tests involving acute exposure of rats, mice, and rabbits have demonstrated DMF to have moderate acute toxicity from inhalation, oral, and dermal exposure [6].

Hazardous decomposition products of DMF include ammonia, carbon dioxide, amines, and nitrogen oxides when heated to decomposition. The overall rate of chemical degradation of DMF present in surface water is found to be extremely slow [1]. Bioremediation could be one of the viable alternatives for decontamination of DMF-polluted sites and it mainly depends on the isolation of microbes that use DMF as a source of carbon and energy [7]. Several microorganisms including algae degrade DMF either aerobically or anaerobically [2]. In the present study an attempt has been made to isolate a bacterial strain capable of degrading DMF and to identify and determine its efficiency of degradation by analyzing different parameters like pH, turbidity, biomass, CO_2 production and ammonia production.

MATERIALS AND METHODS

Collection of sample

Soil samples were collected in sterile screw cap bottles from the sites contaminated with textile industry effluent containing dimethylformamide at Dindigul, fifty kilometers away from Madurai.

Isolation of DMF degrading bacteria

The collected soil samples were serially diluted and 0.1ml from the 10^{-6} dilution was taken and spread on minimal medium MM1 containing 50µl of DMF as a sole carbon source. The plates were incubated at 37°C for a week. The physico-chemical characteristics of DMF are given in Table 1. Its structure is depicted in Fig.1.

Identification of DMF degrading bacteria

From the different bacterial strains grown on agar plates one streak was selected. Gram staining was done and the isolate was grown in selective media like Pseudomonas agar. Biochemical tests like MR-VP, citrate utilization, oxidase, catalase, indole production, urease, Triple Sugar Iron agar and nitrate reduction tests were carried out for the tentative strain identification of the selected bacterial strain.

Degradation efficiency

The natural isolate *P. aeruginosa* was inoculated on minimal broth containing different concentrations of DMF (50, 100, 150 and 200µl). The flasks were incubated at room temperature for a period of ten days and the degradation was confirmed by analyzing the optical density, pH, biomass, CO_2 production and ammonia production. The above mentioned parameters were measured every 2days for 10 days.

pH

The pH of the sample was determined after 4, 6, 8 and 10 days of incubation using pH meter.

Biomass

Turbidometric method was followed for estimating the biomass by measuring the turbidity at 600nm. The biodegraded samples were taken and centrifuged. The pelleted biomass was taken and the wet biomass was calculated. After drying it in hot air oven, the dried biomass was determined.

CO_2 estimation

1ml of the sample was taken and titrated against 50ml of 0.05N NaOH solution prepared in CO_2 free distilled water. Phenolphthalein was used as an indicator and appearance of pink color was the end point. CO_2 liberated was calculated by using the following formula.

$$Free\ CO2\ \left(\frac{mg}{l}\right) = \frac{(Titre\ value\ \times Normality\ of\ NaOH) \times 1000 \times 44}{Volume\ of\ the\ sample}$$

Ammonia estimation

Two to three drops of Nessler's Reagent were added to one ml of the sample and the color change was noted. The concentration of ammonia was then estimated using colorimeter [10].

High Performance Liquid Chromatography (HPLC)

The samples from the minimal medium with 200µl DMF taken on 0^{th} day, 4^{th} day and 10^{th} day were subjected to HPLC analysis at CECRI, Karaikudi (Model: Shimadzu, Japan).

STATISTICAL ANALYSIS

Two way ANOVA was performed for the parameters like optical density, pH, biomass, CO_2 released and ammonia released using Microsoft Excel. Variability was considered significant only when the statistical value was greater than the tabulated value at P is less than or equal to 0.05.

RESULTS AND DISCUSSION

The bacterial strain isolated from the soil was identified as gram negative rod. Then the isolate was tentatively identified as *P. aeruginosa* on the basis of the results obtained in the biochemical tests (Table.2). The isolate, *P. aeruginosa* (Plate.1) was able to grow on minimal agae containing various concentrations of DMF.

Fig.2 illustrates the changes in the pH determined after the 4, 6, 8 and 10 days of treatment with *P. aeruginosa.* The pH of the medium was observed to increase from the 6^{th} day during the degradation of DMF by *P. aeruginosa.* The gradual increase in pH indicates the degradation of DMF and this increase in pH may be due to the constant release of dimethylamine and ammonia into the medium.

Veeranagouda *et al*. (2006) have isolated a novel bacterial strain *Ochrobactrum sp.* DGVK1 from the soil which is capable of producing dimethylformamidase to degrade DMF in which the DMF degradation witnessed concomitant increase in the growth of the culture and pH of the medium from 7 to 9.2.

Changes in the turbidity of the medium during the ten days treatment of DMF by *P. aeruginosa* are illustrated in Fig.3. It seems to be fluctuating but the maximum growth of the

bacterium was observed after six and eight days of treatment. Fig.4 depicts the changes in biomass of *P. aeruginosa* during the treatment. There was a gradual increase in the biomass of the isolate which indicates an increase in bacterial growth utilizing DMF as a sole carbon and energy source. Increase in turbidity and biomass during the treatment period indicates the bacterial growth due to the utilization of DMF as a source of carbon and nitrogen. Growth of the bacterium is observed to increase till the eigth day of treatment and decreased afterwards. This indicates the degradation of DMF increasing as the incubation period increases. The decrease in the biomass may be due to the formation of degraded products like dimethylamine and ammonia.

The biodegradation of DMF resulted in the production of carbon dioxide which was found to increase linearly with the increasing concentrations of DMF. More amount of carbon dioxide was released during the degradation of 200µl of DMF by *P. aeruginosa*. The amount of carbon dioxide release during the treatment of DMF with *P. aeruginosa* is shown in Fig.5. The amount of carbon dioxide was the maximum for the 200µl DMF.

As proposed by Ghisalba *et al*. (1985), the DMF is mainly degraded either by the involvement of dimethylformamidase (DMFase) or by repeated oxidative demethylations. Depending on the pathway found in *Ochrobactrum* sp., it is expected to observe the release of nitrogen in the form of ammonia exclusively when more than one catabolic intermediate of a particular pathway is supplied to the resting cells. As expected, ammonia was released when dimethylamine and methylamine, considered to be the intermediates of DMF degradation pathway, were supplemented to the resting cells. Release of ammonia as a result of biodegradation of DMF by *P. aeruginosa* is shown in Fig.6. The concentration of liberated ammonia was found to increase till the eigth day and decreased in the tenth day of treatment for the various concentrations of DMF by *P. aeruginosa*. The maximum release of ammonia was observed after eight days of treatment of DMF.

Liberation of carbon dioxide and ammonia during degradation of DMF can be used as an indication for the activity of bacteria in the growth medium. The maximum release of carbon dioxide and ammonia was found during the degradation of 200 µl DMF by *P. aeuroginosa.* This clearly indicates that *P. aeuroginosa* can degrade DMF more effectively.

The model, phases (mobile and stationary), columns, detectors and flow rate used in the HPLC analysis are presented in the table 3. Table 4 shows the values of HPLC analysis for 200µl DMF before and after treatment with *P. aeruginosa.* Fig 7 divulges the HPLC analysis for 200µl of DMF before treatment (control). The peaks observed here were missing in the HPLC analysis of the same concentration of DMF on the 4th and 10th day of treatment with *P. aeruginosa* (Figures 8 and 9). There were few new peaks with different retention times indicating the formation of intermediates. HPLC analysis for 200µl of DMF degradation by the isolate showed peaks with different retention time. The report showed the difference in retention time between control and the biodegraded samples. The peaks obtained on the 10th day of treatment were different in their retention time from those on the 4th day of treatment indicating the mineralization of DMF into new intermediates.

Table 5 exhibits the two way analysis of variance for the factors such as pH, turbidity, biomass, carbon dioxide and ammonia with the variables, treatment period and DMF concentration. The variations due to treatment period and concentration were statistically significant for the isolate. Based on the assessment of different parameters, the isolate *P. aeruginosa* was found to be more effective in degrading DMF.

CONCLUSION

Extensive use of DMF as a versatile organic solvent in various processes makes its way into the environment. Though there are no reports on accidental release of DMF, the major route of DMF contamination is through the release of DMF into industrial effluents. From the assessment of various parameters, it can be concluded that *P. aeruginosa* efficiently degraded the DMF and certainly forms basis for generation of novel bioremediation strategies for effective removal of

DMF from the industrial effluents. Further studies on the enzymes involved in the conversion of DMF to carbondioxide and ammonia may strengthen the application of this bacterial strain in bioremediation programmes.

Table.1. Details of dimethylformamide

Parameters	**Values**
CAS number	68-12-2
Chemical formula	C_3H_7NO
Molecular weight	73.09
Appearance	Colorless to slight yellow liquid
Odour	Faint amine
Boiling point	153.0 ºC
Flash point	58 °C
Density	0.944 g/cm^3, liquid
Solubility	Water: Freely soluble Alcohols: Soluble
Vapour density	2.51
Vapour pressure	490 Pa at 25ºC

Table.2. Tests for the identification of the isolate *P. aeruginosa*

S.No.	**Test**	**Response of the organism**
1.	Gram staining	-
2.	Methyl Red	-
3.	Vogus proskauer	-
4.	Simmons citrate agar	+
5.	Oxidase	+
6.	Catalase	+
7.	Indole	-
8.	Urease	-
9.	Sucrose	-
10.	Lactose	-
11.	Triple Sugar iron Agar	+
12.	Nitrate reduction	-
13.	Glucose fermentation	-

(+ve = Positive reaction, - ve = Negative reaction)

Table 3: High Performance Liquid Chromatography

Model	: SHIMADZU, JAPAN
Stationary Phase	: SILICA GEL [reversed phase]
Mobile Phase	: 100% METHANOL
Main Column	:Analytical– Shim–Pack CLC - OCTA DECYL SILANE (ODS-C18) [4.6 mm ID * 25cm]
Guard Column	: Shim – Pack G-ODS [4mm ID * 1cm]
Detector	: UV-Spectrophotometric
Flow Rate	: 1ml per minute
Column head Pressure	: 125 kgf/cm2
Injection per sample	: 20 micro liter
Wave Length	: 254 nana meter

Table.4. HPLC analysis report for 200µl DMF before and after treatment with *P. aeruginosa*

Sample	Retention Time [min]	Height [µV]
Before treatment	2.849	87
	3.292	143.6
	3.473	198.7
	3.695	99.9
	Total	529.2
After 4 days of treatment	2.88	172.5
	3.34	265.1
	3.519	241.9
	3.726	192.1
	Total	871.6
After 10 days of treatment	2.89	150.2
	3.403	195.7
	3.579	160.2
	3.828	113.2
	Total	619.3

Table .5. Two way analysis of variance for the factors with the variables, treatment period and DMF concentration for *P. aeruginosa*

Factor	Source of variation	df	MS	Calculated F value	Table F value	Level of Significance at 5% level
pH	Treatment period	3	0.057292	1.941176	3.862548	Not significant
	Concentration	12	0.036458	13.23529	3.862548	Significant
Turbidity	Treatment period	3	0.000769	3.322581	3.862548	Not significant
	Concentration	12	0.006095	0.936131	3.862548	Significant
Biomass	Treatment period	3	0.07204	106.1791	3.862548	Significant
	Concentration	12	0.029566	43.56295	3.862548	Significant
Carbon dioxide	Treatment period	3	52433.33	2.294118	3.862548	Not significant
	Concentration	12	262166.7	11.47059	3.862548	Significant
Ammonia	Treatment period	3	0.08279	17.54998	3.862548	Significant
	Concentration	12	0.041906	8.883409	3.862548	Significant

Fig.1 Structure of dimethylformamide

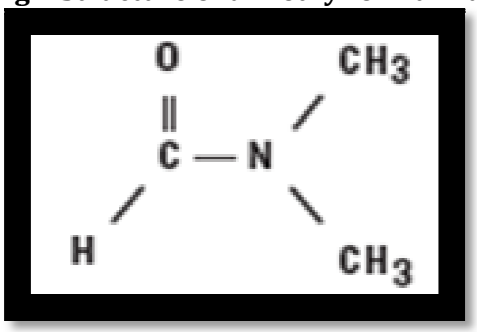


Plate1.Growth of *P.aeruginosa* in Pseudomonas agar

Plate 2. Growth of *P. aeruginosa* in minimal medium containing 200µl of DMF

Fig. 2 Changes in the pH of the medium during the degradation of DMF by *P. aeuroginsa*

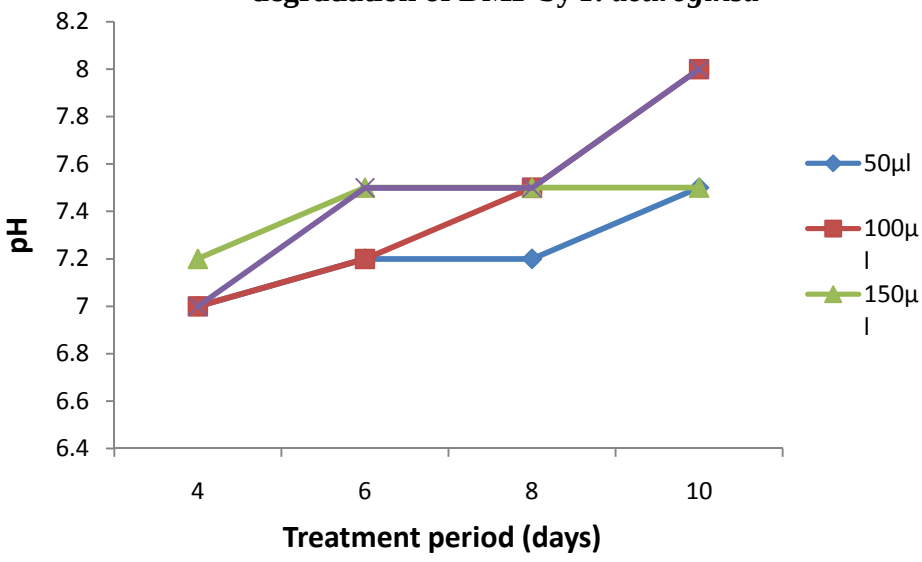


Fig.3 Changes in turbidity during the degardation of DMF by *P. aeuroginosa*

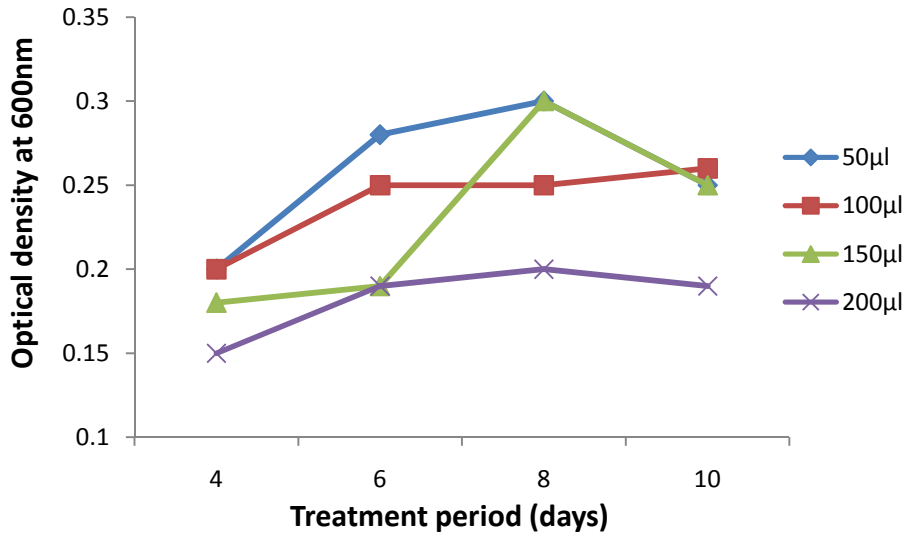


Fig.4 Changes in the biomass (g dry weight /L) during the degradation of DMF by *P. aeuroginosa*

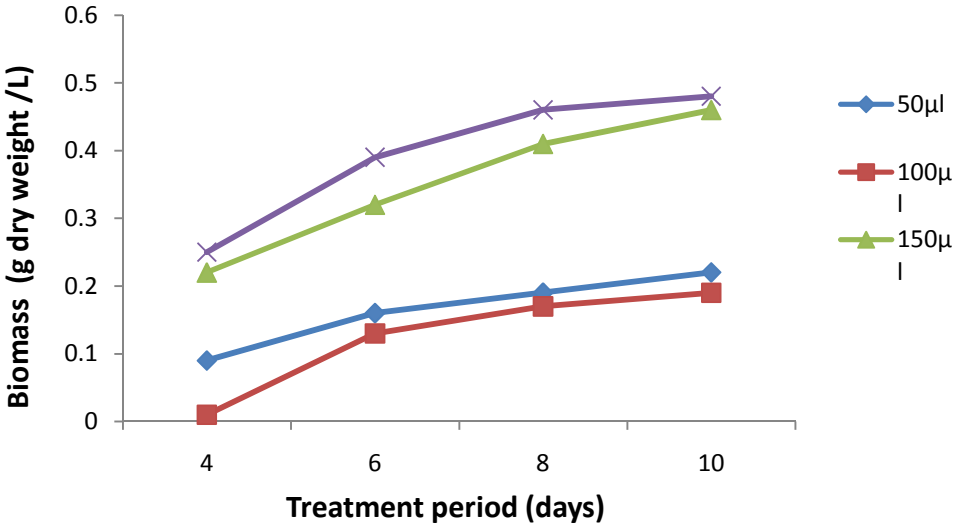


Fig.5 Carbon dioxide released (mg/L)during the degradation of DMF by *P. aeuroginosa*

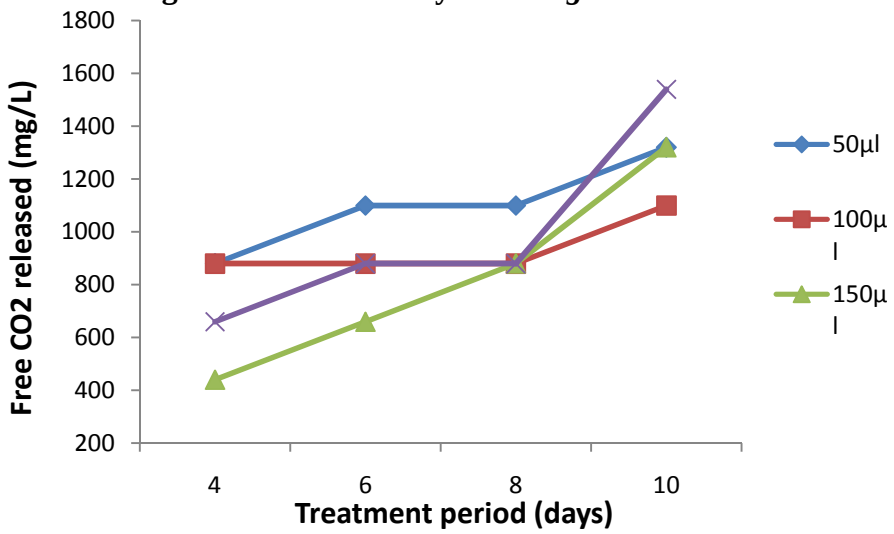


Fig. 6 Ammonia released during the degradation of DMF by *P. aeuroginosa*

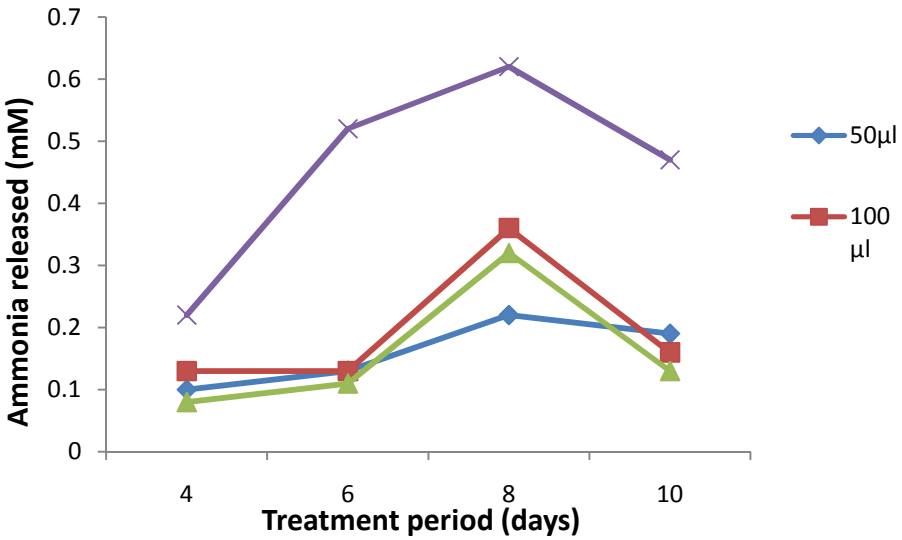
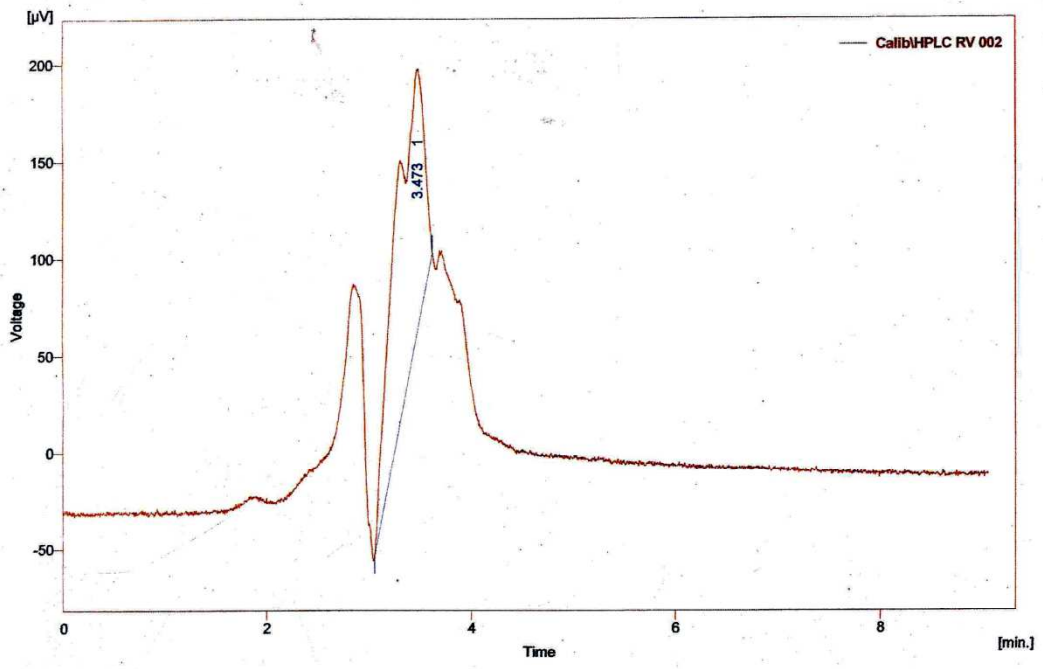
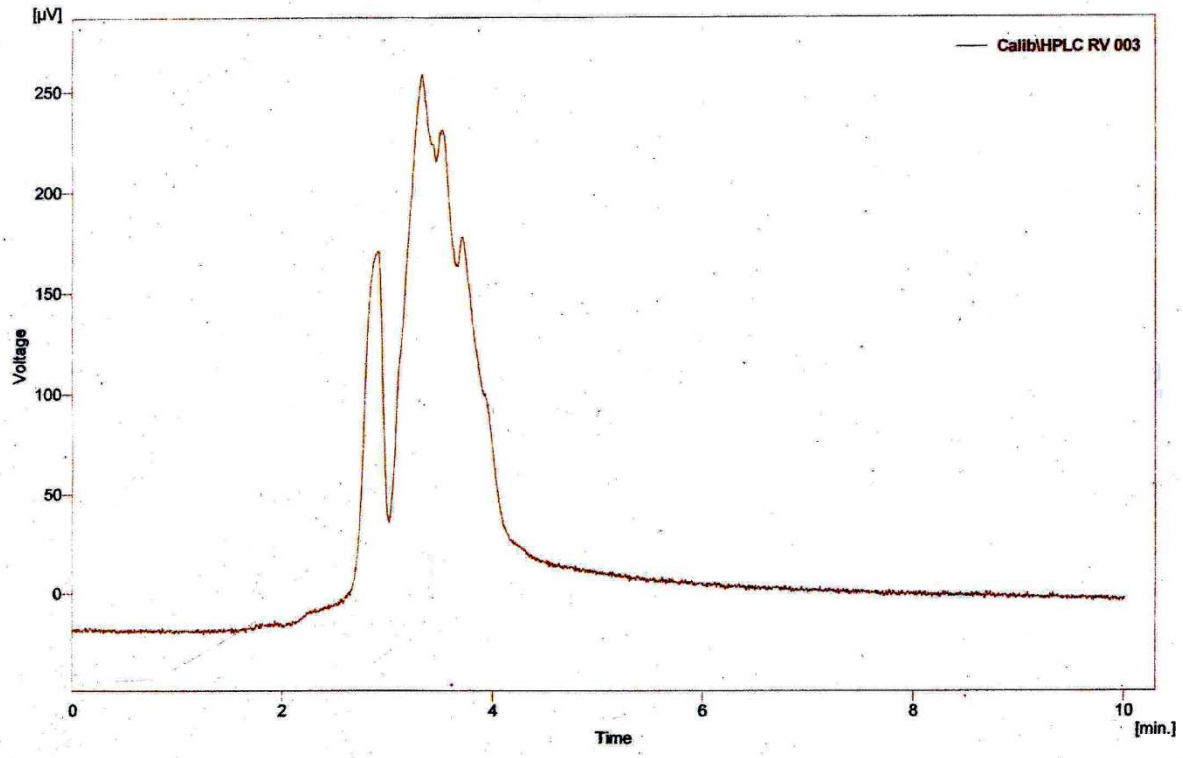


Fig .7 HPLC analysis report for 200µl of dimethylformamide



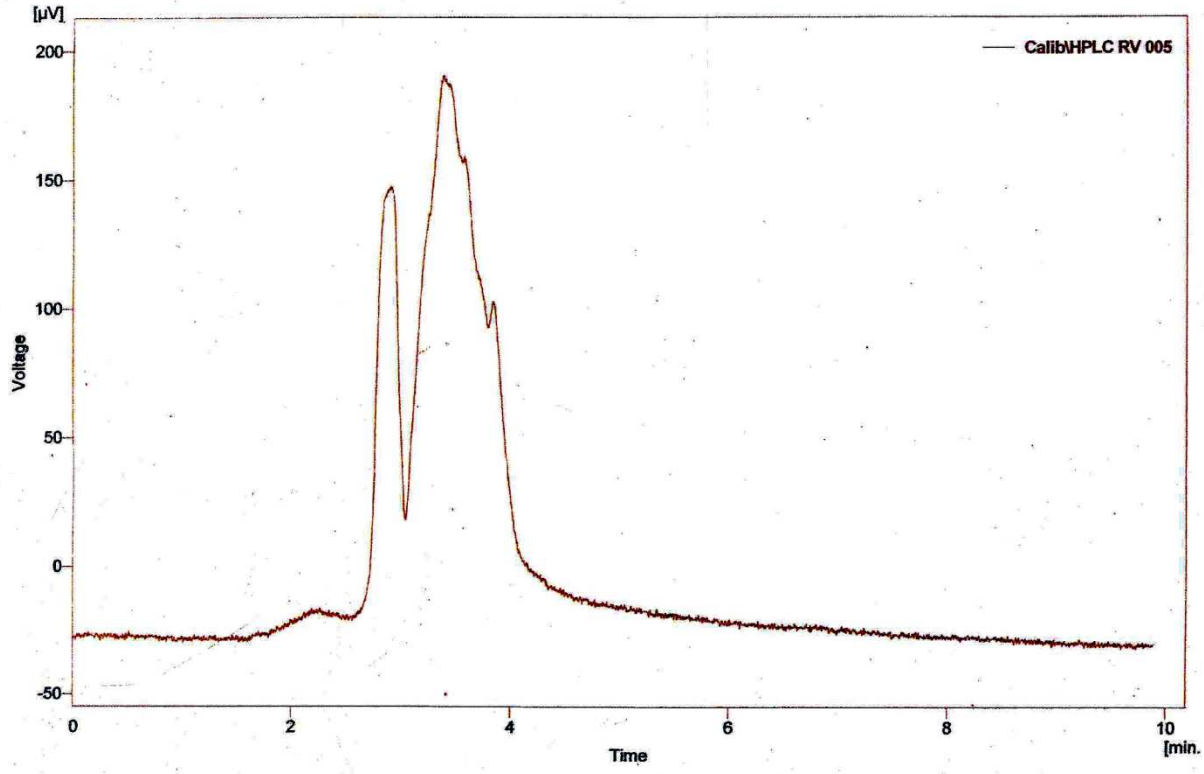
Retention Time [min]	Height [µV]
2.849	87
3.292	143.6
3.473	198.7
3.695	99.9
Total	529.2

Fig .8 HPLC analysis report for200µl of dimethylformamide treated with *P. aeruginosa* (4th day)



Retention Time [min]	Height [µV]
2.88	172.5
3.34	265.1
3.519	241.9
3.726	192.1
Total	871.6

Fig .9 HPLC analysis report for200µl of dimethylformamide treated with *P. aeruginosa* (10th day)



Retention Time [min]	Height [µV]
2.89	150.2
3.403	195.7
3.579	160.2
3.828	113.2
Total	619.3

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