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Isolation and Characterization of Bacterial Strains to be Used as Biosorbent for Removal of Atrazine from Wastewater

R. K. Pathak
Research Scholar: Centre for Environmental Science and Engineering , Indian Institute of Technology Bombay
Powai, Mumbai, India
e-mail: rkpathak2k@rediffmail.com

Anil Kumar Dikshit
Professor: Centre for Environmental Science and Engineering
Indian Institute of Technology Bombay
Powai, Mumbai, India
e-mail: dikshit@iitb.ac.in

***Abstract*—Due to the increasing population, less availability of land and significant loss of crops by weed, the use of atrazine is increasing significantly. Due to its low vapor pressure, longer half life (180 to 360 days) and apparently low biodegradability, atrazine has led to the contamination of terrestrial ecosystems and has been detected in ground and surface waters in many countries beyond permissible limits. Several fungi, bacteria and algae have been already reported as effective biosorbents for removal of dyes, metals and even pesticides due to its low cost, non-toxic approach, regeneration capability and high efficiency for pollutant uptake. This study aims to develop a bacterial biomass which can be used as adsorbent to remove the atrazine from wastewater.**

Keywords-Atrazine; Biosorption; Biosorbent; Herbicide

I. INTRODUCTION

Agriculture is the mainstay of the Indian economy. Agriculture and allied sectors contribute nearly 22 per cent of Gross Domestic Product (GDP of India), while about 65-70 per cent of the population is dependent on agriculture for their livelihood. The agricultural output, however, depends on monsoon as nearly 60 percent of area shown is dependent on rainfall [1-3].

Sinceland availability has not increased with the population, efforts have been made to increase the crop yield. This has forced the farmers to use more fertilizers and pesticides. Pesticides not only leaves residues in food items but also lead to wide spread pollution of surface and groundwaters.

Among various pesticides, atrazine is the most frequently found pesticide in the groundwater in the United States [4, 5]. According to Hallberg, the frequency of detection for atrazine is 10 to 20-fold greater than the next most frequently detected pesticides[4]. In a regional survey, Koterba reported that atrazine is the most commonly detected pesticide in the Delmarva Peninsula of Delaware, Maryland, and Virginia. Contamination was well correlated with the intensive use of atrazine in cornfields [6].Not only atrazine but its metabolites were also detectedin groundwater. DeLuca detected deethylatrazine in all 32 study wells where atrazine was found [7].

As can be seen from above, that due to its low vapor pressure, longer half life (180 to 360 days) and apparently low biodegradability, atrazine has led to the contamination of terrestrial ecosystems and has been detected in ground and surface waters in many countries beyond permissible limits. Several fungi, bacteria and algae have been already reported as effective biosorbents for removal of dyes, metals and even pesticides due to its low cost, non-toxic approach, regeneration capability and high efficiency for pollutant uptake [8]. This study aims to develop a bacterial biomass which can be used as a low cost bioadsorbent to remove the atrazine from wastewater.

II. MATERIALS AND METHODS

A. Glass wares and Chemicals

All the experimental work was carried out using Borosil glassware. All the glasswares were soaked in dilute chromic acid overnight, washed thoroughly using Lizol solution followed by washing with tap water. All chemicals and reagents used in this study were of analytical grade (AR). Technical grade atrazine (99.4%) was provided by M/s Divyalakshmi Banglore, India. Acetone and ethyl acetate were purchased from Merck India Ltd. Mumbai. Nutrient agar and nutrient were procured from Hi Media Laboratories Pvt. Ltd., Mumbai. Other chemicals like Concentrated Sulphuric Acid (98%), Crystal Violet, Ethanol, Gram's Iodine, Potassium dichromate, Saffranine, Sodium Sulphate, were analytical grade. The reagents were prepared in the lab using standard procedure.

B. Methods

All the initial experiments on biosorbent screening were done in triplicate. All remaining experiment were performed in duplicate.Wherever and whenever observations/results were found to be doubtful, one more duplicate set of those specific experiments were performed.

C. Isolation of *Bacteria* from Soil

Soil samples were collected in sterile containers from locations in Dahanu and from Meghmani Dyechem in Ahmedabad, India. These locations were chosen as they were rich in the residue of pesticide atrazine and so the probability of isolation of atrazine sorbing organisms was maximized.

Serial dilution was performed using the above soil sample. 10^{-7} and 10^{-8} tubes were plated on nutrient agar.

Nutrient agar was prepared by standard protocol. Visibly distinct colonies were, again, inoculated on different nutrient agar plate. This procedure was followed till pure bacterial colonies were isolated

D. Plating *Methods*

1) Spread plate method

0.1 mL of 10^{-3} dilution was added on the nutrient agar plate. It was spread evenly with a glass spreader in laminar cabinet. This was repeated for 10^{-4} to 10^{-7} dilutions. The Petri plate was kept in an inverted position. The plates were incubated at 37 ± 2^0C for 24 hours.

2) Streak plate method

A loop-full culture was added from the 10^{-3} dilution test tube. It was evenly streaked on the nutrient agar plate. This was repeated for 10^{-4} to 10^{-7} dilutions. The petriplate was kept in inverted position. The plates were incubated at 37 ± 2^0C for 24 hours. The plates were incubated till substantial growth of colonies was observed.

E. Preservation of Cultures

Preservation was done by inoculating the nutrient agar slants with pure bacterial inoculums. These slants were incubated overnight in the incubator at 37 ± 2^0C. The stock cultures were sealed with paraffin tape and the working subcultures were preserved at 4^0C.

F. Growth *Studies* of Cultures

Growth studies of four bacterial cultures were done to check the growth rate of each bacterial culture. A loopful of culture was inoculated in 50 mL nutrient broth in 00 ml conical flasks. They were, then, incubated in shaking conditions for 24 hours at 37 ± 2^0C. 5 mL of these cultures were inoculated in 25 mL nutrient broth in side-arm conical flasks and optical density was measured for each colony at 540 nm with Thermo Spectronic spectrophotometer (model Helιose, USA). After optical density measurement, flasks were kept in continuous rotary shaker. After interval of fifteen minutes, optical density of the culture was measured again and then, kept in shaker. This process was continued till the optical density of the culture reached to maximum and started decreasing. Optical density versus time graphs were plotted to identify the optimum time required to give maximum growth by bacterial culture. Nutrient broth was used as a blank.

G. Characterization of Bacterial Isolates

The isolated bacterial colonies were characterized for various characters such as shape, colour, opacity, elevation, consistency, surface and margin. Gram Characterization was checked for fast growing strains.

H. Gram *Staining* for Characterization of Isolated Strains of Bacteria

The culture was taken on the loop and placed on the clean glass slide. A drop of standard saline solution was put on the culture and a smear was made by mixing properly. Heat fixing was done by moving the slide over the flame for 2-3 times keeping the lower side of slide towards the flame. A drop of Crystal Violet stain (primary stain) was added and kept for 30 seconds. The slide was washed with distilled water. A drop of Gram's Iodine (mordant) was added and kept for 60 seconds. The slide was washed with 95% of alcohol (decolorizing agent). The slide was then washed with distilled water. A drop of Saffranine stain (counter stain) was added and kept for 30 seconds. The slide was washed with distilled water and air dried. The slide was observed under oil immersion lens at 100X magnification.

I. Mass Cultivation in Nutrient Broth

A loopful of culture was inoculated in six 250 mL flasks containing 50 mL Nutrient Broth.The flasks were maintained in shaking conditions for 48 hours. The cell mass obtained by centrifuging the sample at 3000 rpm for 30 minutes was separated and collected in sterile petri plates. The cell mass was kept for drying in the hot air oven at 70^0C till completely dry. The dry mass was weighed.

J. Gas Chromatography (GC) Analysis

The sorption analysis was done using GC (model 2014, Shimadzu, Japan) with the injection port SPL1 of split type with injector temperature of 280^oC and carrier gas as N_2. The column (Restek RXI-17) had a temperature range of 120-220^oC (atrazine boiling point at 178^oC) with an inner diameter of 0.25 mm, thickness 0.25 µm and length of 15 m. The ECD detector was used with detector temperature 300^oC.

K. Biosorption *of Atrazine using the Selected Strains*

Atrazine solution of 5 ppm atrazine was prepared by dissolving 0.005g of atrazine powder in 1L distilled water.20 mg of dried culture sample was added in 20 ml of 5 ppm stock solution. The samples were incubated for 0 hr, 24 hrs, 48 hrs, 72 hrs, 96 hrs. 0 hr sample was shaken in shaker at 250 rpm and 31^oC temperature. The samples were kept for 1 hr for settling and then it was filtered. Using a Whattman's filter paper of pore size 2.5 µm. 10 ml of the filtrate was put in the separating funnel. The serial extraction was done by 3 step extraction method by taking 2 ml of ethyl acetate and two ml of sample each time. The extract was collected and volume was made to 6 ml by adding ethyl acetate. The volumes of both aqueous phase and solvent phase were measured. The solvent phase was passed 2-3 times through a sodium sulphate bed to remove any water or dust molecule if present. The solvent phase was collected in a clean vial for GC analysis.

L. *Sorption* Calculations

Calculation of percent sorption by various strains using following formula:

$$\% \, Sorption = 100 - \{ (GC \, reading / 5) \, x100\}$$

III. RESULT

A. Isolation

A total of four bacterial strains were isolated from the soil samples collected.

B. Characterization

The four colonies grown on nutrient agar were studied for various characteristics such as size, shape, colour, opacity, elevation, consistency, surface and margin the result is shown in Table I.

TABLE I. CHARACTERIZATION OF BACTERIAL STRAINS

Colony	A	A	A	A_4
Size (mm)	2-3	Thread Like	2-3	Pinpoint
Shape	Circular	Threads	Circular	Circular
Color	Off-white	Off-white	Orange	Yellow
Opacity	Opaque	Opaque	Opaque	Opaque
Elevation	Fla	Fla	Flat	Flat
Consistency	Sticky	Sticky	Sticky	Sticky
Surface	Smooth	Rough	Smooth	Smooth
Margin	Irregular	Regular	Regular	Regular

C. Gram Staining

Gram Staining of various bacterial strains is shown in Table II.

TABLE II. GRAM CHARACTER OF BACTERIAL STRAINS

Colony	**Gram Character**	**Type**
A_1	Positive	Bacillus
A_2	Negative	Cocci
A_3	Positive	Bacillus
A_4	Positive	Bacillus

D. Growth curve

The growth curves for four selected strains were done and result is shown in Figure1. The strain A_2 showed the fast growth. Therefore, this strain was selected for further study.

E. Biosorption

The percentage biosorption was measured using GC analysis. The percentage biosorption by obtained by various strains after 24 hrs is given in Table III. Effect of temperature on the biosorption of atrazine is shown in Figure 2.

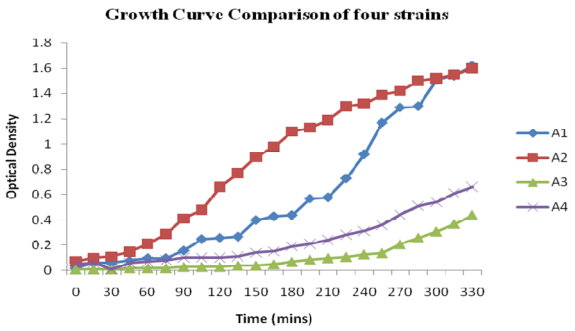

Figure 1. Growth Curves for Strains of Bacteria

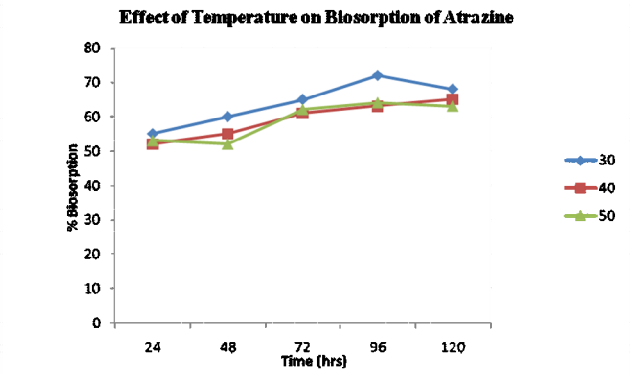

Figure 2. Effect of Temperature on Biosorption of Atrazine by Strain A2

TABLE III. PERCENTAGE BIOSORPTION BY VARIOUS STRAINS AFTER 24 HRS

Stra	% Biosorption
A	43.2
A	55.3
A	39.0
A	37.0

The strain A_2 showed maximum biosorption while the strain A_4 showed the minimum biosorption.

IV. CONCLUSION

Bacteria were isolated from soil samples that were known to contain the herbicide atrazine as a contaminant. This was an attempt to increase the probability of the isolation of strains with potential biosorbent capacity. Initial isolation from the soil suspensions was carried out on Nutrient Agar. Four test strains were selected on the basis of their fast growth, for biosorption analysis.

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