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Original Research Paper

Modification in Asphalt Texture by Heavy Metal Tolerant Bacteria Isolated From Industrial Effluent

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ABSTRACT

Asphalt is a sticky, black and highly viscous liquid or semi-solid form of petroleum. It is also known as bitumen which may be found in natural deposits or may be a refined product; classed as a pitch. Earlier reports show that most natural bitumen contains sulphur and several heavy metals, such as nickel, vanadium, lead, chromium, mercury, arsenic, selenium, and other toxic elements. The primary use of asphalt/bitumen is in road construction, where it is used as the glue or binder mixed with aggregate particles to create asphalt concrete. Many studies demonstrated microbial utilization of asphalt and related hydrocarbons, particularly the petroleum hydrocarbons. Investigation was carried out to isolate and characterize microorganisms and determine their heavy metal tolerance activity from such kind of ponds in which the pitch waste effluent was released. Later, it was checked whether they have any role in the modification of asphalt texture. Experiments were executed by first determining the physical and chemical characteristics (flame test) of the pitch effluent, then isolating the microorganisms from it and found Gram positive *Coccus*. Flame test revealed the presence of arsenic (As) and lead (Pb). Then, it was checked whether the isolated microorganisms are tolerant to these heavy metals. Later, the use of these heavy metals by the isolated microorganisms was detected by Atomic Absorption Spectrophotometric study. The pattern of growth indicated it to be *Staphylococcus* sp., and when incubated with asphalt, it not only utilizes the hydrocarbon, but also prevents it from hardening. Thus, a pathogenic bacteria can be utilized in recycling of complex hydrocarbon and it continue to sustain itself for a prolong period even in the absence of suitable substrate.

INTRODUCTION

Asphalt also known as bitumen, is a sticky, black and highly viscous liquid or semi-solid form of petroleum. It may be found in natural deposits or may be a refined product; classed as pitch. Most natural bitumen contain sulphur and several heavy metals, such as nickel, vanadium, lead, chromium, mercury, arsenic, selenium, and other toxic elements (John 1990). The primary use of asphalt/bitumen is in road construction, where it is used as the glue or binder mixed with aggregate particles to create asphalt concrete. Its other main uses are for bituminous water proofing products, including production of roofing felt and for sealing flat roofs.

Pit's oil and asphalt are extreme and hostile environments for life to survive. Microorganisms, which can be capable of growing on natural asphalt must contain previously undiscovered classes of enzymes that can naturally break down petroleum products (Harris et al. 1956).

In Rancho La Brea tar pits in Los Angeles (Ian & David 2010), hundreds of new species of bacteria with unusual properties were discovered, allowing them to survive and grow in heavy oil and natural asphalt. The living bacteria were most likely the progeny of soil microorganisms that were trapped in the asphalt, although some may also have been carried to the surface in the heavy oil that seeped upwards from deep underground oil reservoirs. While the bacteria remain to be grown in the laboratory, the researchers found that the closest relatives of many of the bacterial families are able to survive in high salt, toxic, and even radioactive environments. Continual production of bubbles of methane gas that come up through heavy oil overlying the asphalts that clued the researchers to the presence of bacteria in the asphalt. In the absence of oxygen, methane is produced by bacteria that use carbon dioxide for respiration instead of oxygen.

The bacteria and their enzymes have potential application for bioremediation (cleaning oil spills, heavy metals from water supplies), medical treatment (new medicines), alternative energy (biofuels), enhanced oil recovery, and industrial applications (biochemicals and biotechnology) (Lee et al. 2002).

Arsenic (As) and Lead (Pb) are the heavy metals causing physical, muscular, and neurological degenerative processes

that mimic Alzheimer's disease, Parkinson's disease, muscular dystrophy, and multiple sclerosis, allergies and repeated long-term contact with some metals (or their compounds) may cause cancer (CIS 1999). Long-term exposure to arsenic in drinking water can cause cancer in the skin, lungs, bladder and kidney, and skin changes such as thickening and pigmentation. Lead, which damages the nervous system, is a neurotoxin that accumulates both in soft tissues and the bones (Ferguson et al. 2000).

Arsenic and Pb compounds are frequently found to be present in the water bodies like ponds, rivers, lakes, underground water at much elevated concentration. Arsenic and its compounds are used in the production of pesticides, treated wood products, herbicides, insecticides, etc. Arsenic is notoriously poisonous to multicellular life, although a few species of bacteria are able to use arsenic compounds as respiratory metabolites (Mukhopadhyay et al. 2002). Lead is introduced into the different ore processing industries and their residual wastes are released into the water bodies. Drinking water also becomes polluted with these heavy metals.

Our aim is to isolate the environmentally important microorganisms helping in bioremediation and characterize them.

MATERIALS AND METHODS

Sample Collection

An amount of 70mL of sample (pitch effluent) was collected on 19/02/2013 from a pond in which the pitch manufacturing industry, Gladstone Lyall Employees Industrial Co-operative Society Ltd. near Kolkata releases the waste effluent.

Methods

Physical characterization of sample: Colour, aroma, texture, and turbidity of the industrial effluent (sample) were noted, and pH, dissolved oxygen and electrical conductance were measured. The results are given in Table 1.

Determination of metal ions in the effluent by flame test: In flame test, effluents are usually held on a platinum wire, cleaned repeatedly with hydrochloric acid to remove traces of previous analytes, to a hot, non-luminous flame, and observing the colour that results.

Isolation of microorganisms from the effluent and maintenance of pure culture: A small volume of effluent is transferred to the centre of a nutrient agar plate and is spread evenly over the surface with a sterile L-shaped glass rod and incubated at 37°C overnight. After a well-isolated colony has been identified, it can then be picked up and streaked onto a fresh agar plate to obtain a pure culture.

Table 1: Characteristics of pitch.

Colour	Yellowish pale green
Aroma	Fishy smell
Texture	Liquid
Turbidity	Non- turbid
pH	6.12
Electrical conductance	2 S/cm at room temperature

The pure culture was maintained in nutrient agar plate under sealed refrigerated condition.

Characterization of isolated microorganisms: Gram characterization of the isolated colonies was done. Then a single colony was picked up and inoculated in NB and kept at 37°C for overnight under shaking condition to carry out further experiment.

Size of the cell was measured by micrometry and the cell densities were determined by haemocytometer.

Determination of heavy metal tolerance by previously isolated microorganisms.

- Serial dilution ($NaAsO_2$) and [$Pb(CH_3COO)_2$] at ppm (parts per million) in NB:

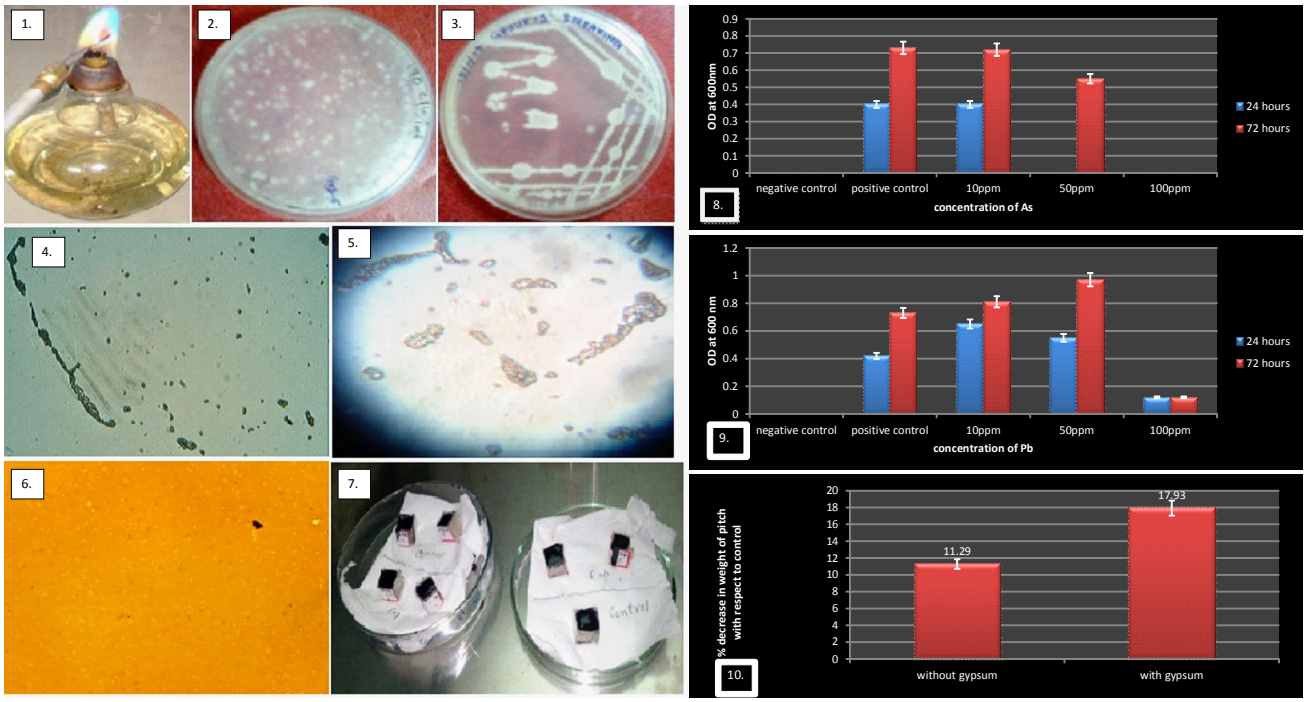
  1.73 mg of ($NaAsO_2$) was dissolved in 10mL of freshly prepared NB to prepare 100ppm arsenic stock solution. From this stock, the concentrations of 10ppm, 50ppm, 100ppm were made by serial dilution. In the same way 100ppm lead stock solution was prepared by dissolving 1.83mg of [$Pb(CH_3COO)_2$] in 10mL of NB and the concentration of 10ppm, 50ppm, 100ppm were made by serial dilution.

- Positive and negative controls were made which indicate no heavy metal salt (only NB) and no culture of tested microorganism respectively. The tubes were properly labelled and autoclaved and inoculated with 200µL fresh log phase culture (OD_{600} = 0.6) of previously isolated microorganism and incubated at 37°C on a shaker for 24 hours. A replica of all these tubes was made in the above manner and incubated for 72 hours at 37°C in shaker.

- OD measurement was taken for all the tubes incubated for 24 hours and 72 hours at 600nm.

Determination of appearance of treated microorganisms after heavy metal tolerance under inverted phase contrast microscope: After OD measurement, the metal tolerant microorganisms in the NB containing As and Pb at 10ppm concentration were taken to observe under phase contrast microscope.

Atomic absorption spectrophotometric study of heavy metals in bacterial cell free NB: The heavy metal tolerant organism's cultures (10ppm As and 50ppm lead containing



Figs. 1-10: 1. A blue white coloured flame was observed in flame test with test effluent; 2. Only one type of small round shaped whitish colonies were isolated in the Nutrient agar plate; 3. Pure culture was isolated from a single colony by streak plate method; 4, 5. Gram positive, small, round shaped bacterial (cocci) cells were observed under 40x magnification; 6. Under heavy metal stress condition, no such change in cell morphology and cell density was observed under inverted phase contrast microscope; 7. Experimental set up for determination of role of these microorganisms in the utilization of pitch; 8. Growth of the tested microorganism in As stress (OD_{600}); 9. Growth of the tested microorganism in Pb stress (OD_{600}); 10. Percent decrease in weight of pitch containing cardboard boxes with respect to control boxes.

tubes incubated for 72 hours) were centrifuged at 3500 rpm for 5min and supernatant was collected and tested for atomic absorption spectrophotometric study.

Determination of role of these microorganisms in modification of asphalt texture (Traxler et al. 1965): Eight equally shaped 1cm^3 card board boxes were prepared for this experiment. The weight of all the empty boxes was taken. At first, 20g of pitch and 2g of gypsum (solidifying agent) were weighed separately. Then by heating, the pitch was melted, with the help of laquor by stirring with the glass rod. Now, the boxes were properly labelled and equal amount of melted pitch was given to each box by using spatula. Then, two sets were prepared and named as "without gypsum" and "with gypsum" in which 20µL of the experimental microorganisms were added in each box along with the control boxes in which no organism was added. Boxes containing pitch were allowed to solidify at room temperature in laminar air flow. After 2 hours, weight of the pitch-filled boxes was measured. All the boxes were incubated at 25°C for 72 hours. After 3 days, observations were made.

RESULTS

The results of the study are shown in Figs. 1-10.

Determination of metal ions in the sample by flame test: A blue white coloured flame was observed as shown in the photograph taken. From the result we may say that the liquid sample (pitch effluent) may contain Arsenic (As) and Lead (Pb) on the basis of the emission spectra of metal cations.

Isolation of microorganisms from the sample and maintenance of pure culture: Only one type of small round shaped whitish single colonies were observed in the nutrient agar plate having 170 colonies and pure culture was made.

Characterization of isolated microorganisms: By Gram characterization, Gram positive, some single and some clustered, small, round shaped bacterial (cocci) cells were observed under 40x magnification.

The actual size of the each microorganism (coccus) isolated from pitch effluent is 1.887 µm in diameter.

Determination of heavy metal tolerance by previously isolated microorganisms: Microorganisms isolated from pitch effluent, were able to tolerate and grow at high concentrations of As and Pb as observed by OD_{600} measurement of bacterial suspension.

Determination of appearance of microorganisms after

heavy metal tolerance under inverted phase contrast microscope: Single, small, round shaped healthy cells (cocci) were observed under 40x magnification. Under heavy metal stress condition, no such change in cell morphology and cell density was observed.

Atomic absorption spectrophotometric study of heavy metals in bacterial cell free NB: From the result of atomic absorption spectrophotometry, it was found that isolated microorganisms were utilizing these heavy metals as evident in decrease of As concentration from 10 ppm to 1.0017 ppm, and Pb concentration from 50 ppm to 10.0179 ppm.

Determination of role of these microorganisms isolated from pitch effluent in the utilization of pitch: Performing the above experiment, it was observed that the weight of the cardboard boxes containing the asphalt (in both "without gypsum" and "with gypsum" set) was decreased upon incubation with the microorganisms with respect to control boxes.

DISCUSSION

Colony characteristics and the appearance of the microorganisms isolated from asphalt effluent observed under microscope (by Gram staining) have suggested that it might be species of *Staphylococcus* (most likely *Staphylococcus aureus*) (King et al. 1984). It was also observed that the isolated organisms were more resistant to lead than arsenic.

Atomic absorption spectrophotometric study also reveals the utilization of heavy metals (As, Pb) by the experimental microorganisms.

From the experiments, it can be concluded that microorganisms have the ability to oxidize hydrocarbons present in the pitch which was evident from the decrease in the weight of the pitch inoculated with isolated bacteria after 3 days of incubation. It may happen due to the release of green house gases like-carbon dioxide, methane, etc. Hence, we can say that if those microbes as contaminants were present in the pitch during road construction then the pitch will take a longer time to solidify or asphalt setting.

It was also observed that the boxes containing gypsum showed more decrease in weight than those which did not contain the solidifying agent. As the asphalt contains different types of metals, the asphalt utilizing bacteria may utilize the calcium sulphate (gypsum) for their metabolic needs as a result of which subsequent change in weight and also in density were occurring.

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