EFFECT OF NITROGEN SOURCE ON THE DEGRADATION OF NAPHTHALENE BY A *PSEDOMONAS SPS* NGK1 NCIM 5120.

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Abstract- Polycyclic aromatic hydrocarbons are ubiquitous environment pollutants.These compounds containing carbon and hydrogen with the carbon atoms arranged in a series of adjoining six-member as benzene rings. By its complex structure it cannot be degraded easily. Now a day most widely used co-products are naphthalene and it's by products. These are used in the textile areas as the co-functional compounds of the synthetic dyes, pharmaceuticals, agrochemicals, polymers etc. Accumulation of naphthalene is creating problems in nature which follows genotoxicity and potential hazards to environment, industries leave these PAHS without treatment, even some physicochemical techniques are available, but they lack the complete degradation of toxic compounds. Biological process of degradation through microorganisms is gaining importance. In this direction we used a potential strain *pseudomonas sp* strain NGK1 NCIM 5120, which is capable of degrading naphthalene.

Key Words - Genotoxicity, PAHS, polycyclic aromatic, hydrocarbons.

Introduction - PAHS are compounds which exhibit toxicological effects, mutagenic, carcinogenic properties (Pandey et al., 2010). Majorly three categories are available they are PAHs, heterocyclic and substituted aromatics. PAHS contain two or more aromatic rings in angular, linear, or in cluster arrangements, (Cheung, P.Y et al 2001). These are the lower molecular weight compounds, but significantly possess genotoxic effect (Juhasz etal 2000). Out of other PAHs naphthalene became a ubiquitous contaminant. It is widely used in the synthetic dye intermediate compound in pharmaceuticals, automobiles, production of pesticides etc. Through this compound is very helpful for the industrial purpose but it contains aromatic benzene ring which is highly nonbiodegradable in the environment. Several physicochemical methods are available but having disadvantages in the complete treatment of toxic compounds. Microbial degradation gave a promising result in the degradation of PAHs (Li et al., 2008). The PAHS Naphthalene is the simplest and widely used in the biodegradation purpose (Karl J Rockne etal., 2000). Bacteria such as *Pseudomonas putida, Rhodococcusopacus, Mycobacterium sp., Nocardiaotitidiscaviarum,* and *Bacillus pumilus* have been reported in biodegradionof naphthalene. (Soberon-Chavez G et al2005). *Pseudomonas* is the best strain in the degradation of naphthalene (Behrooz Kari et al., 2015:Wei Zhou, et al., 2013) and (HilorPathak et al., 2013).In this direction we used a potential pseudomonas strain which is able to degrade Naphthalene as the sole source of carbon. And to enhance degradation of strain

efficiency we optimized with different nitrogen source of energy and confirmed degradation of catechol and complete degradation by UV-VIS Spectroscopy.

Materials and Methods

Chemicals-All the inorganic chemical preparation used for the media are analytical grade and procured from Qualigens chemical co, India and Hi-media laboratories.Naphthalene was purchased from the S.D fine chemicals India.The constituents of nutrient broth and L-B medium and catechol were obtained by the Hi-media.

Microorganism – A naphthalene-degrading *pseudomona ssp* strain NGK1, NCIM 5120 was used for the investigation. The bacterium was sub cultured every 15 days on minimal medium supplemented with naphthalene. The organism was maintained on slants of mineral salt naphthalene agar. One set of bacterium maintained on LB slants.

COMPOSITION OF THE MINERAL SALT NAPHTHALENE MEDIUM:-

Microorganism detail :

A Collection of Samples

soil and effluent samples were collected from the textile industrial area and textile effluent
treatment unit, MIDC, Solapur, Maharashtra. Samples including untreated textile mill effluent, sludge soil, dye contaminated soil, greasy soil, colored charcoals from the textile industry, untreated synthetic dye contaminated water.Further some samples were collected from in and around of Kalaburagi, like dye contaminated soil from the M.S.K. Mill area, sewage samples, garbage soil, garden soil. Soil samples from Sharanabasaveshwara lake, Ganesh statue making place (colored soil), market area, dairy unit were also collected. All the samples were collected in a clean sterile bottle and other containers and brought to the laboratory. All the collected samples were processed immediately in the labs if delayed stored in the refrigerator (4^0C). out of samples one strain showing a good account of naphthalene degradation , further basic biochemical tests were done and tentatively identified as
pseudomonas sp strain and names as *pseudomonas sp* NGK1, NCIM 5120 was used for the investigation. The bacterium was subcultured every 15 days on mineral medium supplemented with naphthalene .

COMPOSITION OF THE MINERALS SALT NAPHTHALENE MEDIUM

Mineral salts solution (MS medium) (Karim ME et al.,2018 ; kehra et al.,2005) was employed. The pH was adjusted to 6.8 with HCl and/or NaOH. For the preparation of a solid medium, 1.5% agar was added. For cultivation in liquid medium, organic arsenic compounds were dissolved in water and then sterilized by filtration (Puradisc 25 AS, Whatman, Middlesex, UK), Bushnell Haas (BH) medium composed of KH2PO4 0.1%, K2HPO4 0.1%, MgSO4·7H2O 0.02%, CaCl2·2H2O 0.002%, NH4Cl 0.1%, NH4NO3 0.1%, NaCl 0.01%, and FeCl3–6H2O 0.005% at pH 7 may be used with 1gm/L napthlene added.

Experimental methods:

The various methods used in the study are described in the following lines.

Medium Conditions:

The sterilized medium (5ml) such as the medium containing ammonia (NH_4) and nitrate (NO_3) nitrogen source, supplemented with naphthalene and was inoculated by the freshly grown bacterium and incubated at room temperature (30ºC) for3 to 4 days. This freshly grown pure culture was then inoculated (1ml culture) into 250ml

conical flasks containing 25ml mineral salt medium, NH_4Cl orKNO_3 as nitrogen source and supplemented with naphthalene. The flasks were incubated till the appearance of bacterial growth. Several subcultures in respective media were done in the growth limiting naphtelene as the sole carbon source.

Analytical methods:

Analysis of naphthalene :

The growth of the bacterium on naphthalene at concentrations ranging from 0.1% to 0.5% /L was monitored spectrophotometrically at 660nm. A 3ml culture was withdrawn aseptically from the culture flask at different incubation periods, and centrifuged at 10000rpm for the duration of 10 minutes and supernatant was collected finally O.D was checked at 660nm. control one and calculate the percent decolorisation of initially added dye using the following
formula (Dave and Dave, 2009).
Decolorization=initial optical density-final optical density×100/initial optical density

Utilization of naphthalene :

The utilization naphthalene by the bacterium during growth was determined at different incubation periods. The spent medium at different incubation period was drawn and residual naphthalene was estimated in the culture medium. A plot of residual naphthalene at different incubation period was made.

Analysis of catechol:

Catechol was analyzed in the spent medium by UV/Visible spectrophotometer at wavelength 275nm, the wavelength of catechol. A dark bluecolor developed was an indicative of catechol

Rapid screening of bacterial colonies for catechol-2-3 oxygenase(c230):

Isolated bacterial sp ,those that can utilize naphthalene. This strain one was belonging to *pseudomonas sps* . A bacteria was inoculated on mineral salt agar containing salicylic acid and plates were incubated. The bacterial colonies appeared after two to three day of incubation, were sprayed with catechol (1mm in 0.5M tris buffer, p^H 8.3). The colonies belonging to *pseudomonas sp* rapidly turned florescent yellow which absorbed the light of 373nm, meta cleavage product of catechol, the α-hydroxymuconicsemialdhyde.

Analysis of α-hydroxymuconic semialdehyde:

The α-hydroxymuconic semialdehyde was analyzed and detected at 373nm in UV/Visible spectrophotometer. This was done by inoculating the bacterium with naphthalene in a mineral salt agar. The bacterial colonies those grew on the mineral salts agar medium were added with catechol, the development of florescent yellow color that absorbs maximally at 373nm is the meta cleavage product of catechol , a- hydroxymuconic semialdehyde.

Calibration curve of naphthalene:

The salicylic acid was determined spectrophotometrically using Fecl3 reagent. 0 to 1 ml of the standard solution of salicylic acid (100ug/ml) was pipeted into series of test tubes and the volume was made to 1ml by adding distilled water. A 100ml of 5% aqueous Fecl3 solution was added to all tubes. The purple color obtained was read spectrophotometrically at 550 nm against reagent blank. A graph of O.D v/s naphthalene concentration was then plotted.

Results- A naphthalene degrading *pseudomonas sp,* which is also capable of utilizing naphthalene as the sole source of carbon and energy was used to study the effect of nitrogen source on the metabolism of salicylic acid.

Studies on growth behavior:

The naphthalene at initial concentrations (10-100mm) was supplemented as sole carbon source. It is evident from figure 1 that growth of bacterium after an initial 12 hour lag phase entered into the exponential phase. The maximum growth was observed at 22 to 24hours incubation of bacterium in the medium containing NH_4Cl as nitrogen source, supplemented by naphthalene .During the utilization of naphthalene by the bacterium, the pH of the medium remained constant up to initial 12 hours and there after a slight increase of the pH of the culture medium to 7.3 has occurred after 24hours. The initial cell population of $5x10^6$ colony forming units (CFU) per ml has increased to about $2x10^{10}$ cfu per ml after 24hours incubation in the mineral salts medium containing NH_4Clas nitrogen source. The results of the growth of the bacterium in the mineral salts medium containing KNO_3 as nitrogen source, supplemented by naphthalene as the sole carbon source is shown fig 2.Itwas observed that the growth of the bacterium occurred with the increase in the incubation time and the maximum growth observed with catechol observation. The color of the medium which received NH_4Cl as nitrogen source turned florescent yellow whereas the culture medium that received KNO_3 and naphthalene turned brown.

Studies on the utilization:

The utilization of naphthalene was observed at different incubation period by estimating the residual salicylic acid by UV spectrophotometer at 234nm and also by measuring the purple color produced by FeCl3 reaction at 550nm in a spectrophotometer. It was observed that naphthalene was completely degraded within 24 hours of incubation of the bacterium in the mineral salt medium containing NH_4CL as ammonical nitrogen source.

The mineral salts medium that contained KNO_3 as nitrogen source showed a maximum naphthalene degradation after 24 to 48 hours incubation of bacterium with naphthalene (20mm).The naphthalene utilization by the bacterium was accompanied with the concomitant production of detectable levels of catechol and α-hydroxymuconic semialdehyde in the culture medium which received NH_4CLas nitrogen source and high concentration of catechol and the brown colored product in the medium containing KNO3 nitrogen source.

Metabolite Characterization:

In order to have an insight into the degradative sequence of naphthalene by metabolite characterization, the bacterium was grown in mineral salts medium containing $NH_4CL$and KNO_3 as nitrogen source and supplemented by 20 to 160mM naphthalene. The spent medium was extracted and the metabolite characterized and quantified by paper chromatography, and reacting the culture medium by UV spectrophotometer. The NH_4Cl containing mineral salt medium showed the accumulation of catechol and the yellow product,α-hydroxymuconicsemialdehyde. The wavelength of yellow color compound was 373nm that correspond wellwiththat of α- hydroxymuconic semialdehyde. The culture medium, which received the nitrate (NO3) nitrogen source, supplemented by salicylate, turned brown after two days and thecolor intensitydecreased by passing time. At around 10-12 hour incubation the culture medium showed detectable levels of catechol. The medium was extracted at different time incubation and the metabolite characterized by a spectrophotometer and also by the paper chromatography. The metabolite identified was catechol, was found to be accumulated even after 24-48 h in the medium supplemented by 20 and 160mM naphthalene

Rapid detection of C230:

The bacterial colonies after incubating with salicylate readily converted the catechol into its meta –cleavage product theα-hydroxymuconic semialdehyde which indicates that the bacterium possess very high activities of catechol-2, 3-hydroxylase.

Discussion:

Naphthalene is a key phenolic compound biosynthesized by many plants and some microorganisms. It is a member of an important anti-inflammatory drug class used widely in human medicine. In microorganisms naphthalene is a biosynthetic precursor of secondary metabolites [Nair et al.,2005]. The isolated *pseudomonas sp* which was isolated by successive enrichment technique on salicylate was acclaimed the limiting carbon source. The bacterium tolerated the salicylate concentration as high as 160mM which indicates that the salicylate is non-toxic to the bacterium. It is evident from the studies on growth behavior that the growth of the bacterium increased with the incubation time and perhaps the degradation of naphthalene is associated with the growth of the bacterium. The results indicate that the complete utilization of naphthalene occurred by the bacterium with in of incubation of the bacterium in the mineral salts medium containing ammonical (NH_4)nitrogen source. Where the salicylate was completely degraded by the bacterium after 22hour and 48hour of incubation in the same medium and similar fermentation conditions. The bacterium degraded naphthalene after 48hours incubation of the bacterium in the mineral-salt medium containing nitrate (NO3) as nitrogen source and no further degradation occurred. In the mineral salt medium containing NH_4Cl as nitrogen source and salicylateas the limiting carbon source the initial cell population of $5x10^6$ increased about 10^{10} cfu per ml whereas the medium that received KNO_3 as nitrogen source the initial cell population of 10^6 increased only to 10^7 cfu perml. The culture medium that received NH_4Cl as nitrogen source showed, a slight increase in the pH of the culture medium from 7to 7.4 whereas the rapid increase of the pH, from 7 to 9.3 occurred in the culture medium which received KNO_3 as the nitrogen source and salicylate as the limiting carbon source.

The results on the metabolite characterization revealed, the transient accumulation of catechol α-hydroxymuconic semialdehyde occurred in the mineral saltmedium, containing NH_4Cl as nitrogen source. It was also observed that the *pseudomonas sp*that grew in the mineral salts medium containing NH4Cl and salicylate, upon spraying with catechol, readily transformed catechol into a yellow coloured product, theα-hydroxymuconic semialdehyde, which absorbs strongly at 373nm. Theα-hydroxymuconic semialdehydethe metacleavage product of catechol also is readily disappeared suggesting that the bacterium grown in the medium containing NH_4Cl contains high levels of salicylate hydroxylase activity which transform salicylate into catechol), the catechol 2,3 dioxygenase activity (which transform the catechol into α-hydroxymuconic semialdehyde into and a α-hydroxymuconic semialdehyde)degrade enzyme activities. The degradation of naphthalene, is degraded via catechol, a common intermediate in the degradation of many aromatic compounds, including benzoate, benzene and phenol. Catechol can be subject to either intradiol cleavage by a catechol 1,2-dioxygenase (the *ortho* cleavage pathway) or extradiol cleavage by a catechol 2,3-dioxygenase (the *meta* cleavage pathway). This pathway has been demonstrated in both bacteria by [Yamamoto et al.,1965, Bosch et al., 1999a, Camara et al.,20 07].The naphthalene degradation by the bacterium in the medium containing KNO3 has resulted in the concomitant increase of the pH in the culture medium to 9.3 and the accumulation of catechol and a brown colored metabolite. The naphthalene degradation was accompanied by the increase of pH 9.3 which lowered or completely utilized the catechol-2, 3 oxygenase enzyme activities that lead to the accumulation of catechol in the medium.The combined effect of pH, catechol and other phenolic acidthose were accumulated in the culture medium may have lysed bacterium and inducesthe killing effect. It is evident from the results of our

investigations that the nitrogen source plays a highly significant role in the regulation of naphthalene degradation by the bacterium .It is observed that the rapid and more complete degradation of naphthalene occurred in the medium containing NH4Cl as nitrogen source as it is compared with that degradation of naphthalene by the culture medium which received KNO3 as the nitrogen source. To our knowledge this is the first report suggesting the regulation of naphthalene metabolism by changing the nitrogen source and the complete degradation of naphthalene by a *pseudomonas sp* at the concentration as high as 160mM.

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