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Biodegradation of Paranitro Phenol

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I. INTRODUCTION: BIODEGRADATION:

"Biodegradation" is the process by which organic substances are broken down by living organisms. "Degradation" means decay, and the "Bio"- prefix mans that the decay is carried out by a huge assortment of organisms including bacteria, fungi, insects, worms and other organisms that eat dead material and recycle it in to new forms. In nature, there is no waste material because everything gets recycled. The waste products from one organism become the food for others, providing nutrients and energy. Some organic materials will breakdown much faster than others, but all will eventually decay.By harnessing those natural forces of "Biodegradation", we can reduce wastes and clean up some types of environmental contaminants. Through composting, we accelerate natural "Biodegradation" and convert organic wastes to a valuable resource.

DEFINITION:

"Biodegradation" or biological degradation is the phenomenon of biological transformation of organic compounds by living organisms, particularity the microorganisms. "Biodegradation/Biotransformation" basically involves the conversion or Breakdown of complex organic molecules to simpler or smaller and mostly non-toxic or less toxic ones. The microbial organisms transform complex organic molecules through metabolic or enzymatic processes.A particular strain of microorganism may degrade one (or) more compounds. Sometimes, for the degradation of single compound, the synergetic action of few microorganisms i.e., a consortium or a Cocktail of Microbes may be more efficient. For instance, the insecticide parathion is more efficiently degraded by the combined action of pseudomonas aeruginosa and pseudomonas stulzer.

Co-Metabolism in Biodegradation:

In general, the metabolic breakdown of polluting chemicals is not associated with any advantage to the micro organism that is the pollutant chemical can not serve as a source of carbon (Or) energy for the organism. The term co-metabolism is often used to indicate the non beneficial (to the Micro organism) bio chemical pathways concerned with the Biodegradation of pollutants. How ever, co-metabolism depends on the presence of a suitable substrate for the micro organism and such compounds are referred to co-substrates.

Factors affecting biodegradation:

Several factors influence biodegradation. These include the chemical nature of compounds, the capability of individual microorganism, nutrient and oxygen supply, temperature, pH, and redox potential. Among these, the chemical nature of substrate that as to be degraded is very important. Some of the relevant features are given under here:

- In general, aliphatic compounds are more easily degraded than aromatic ones.
- Presence of cyclic ring structures and length chains or branches decreases the efficiency of Biodegradation.
- Water-soluble compounds are more easily degraded.
- Molecular orientation of aromatic compounds influences biodegradation i.e., Ortho > Para > Meta.
- The presence of halogens (in aromatic compounds) inhibits biodegradation.

Enzyme systems for biodegradation and their location:

Several enzymes system is in existence in the micro organisms for the degradation of chemical compounds.

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The genes coding for the enzymes involved in biodegradative pathways may be present in the chromosomal DNA or more frequently on the Plasmids. In certain microorganisms the genes of both chromosomes and plasmid contribute for the enzyme of Biodegradation.

Genetic Engineering of Biodegradation:

Despite the ability of many naturally occurring microorganisms to degrade a number of xenobiotic chemicals, there are limitations to the biological treatment of these waste materials. For example:

- No single microorganism can degrade all organic wastages.
- High concentration of some organic compounds can inhibit the activity or growth of degradation microorganisms.
- Most contaminated sites contain mixture of chemicals and organisms that can degrade one (or) more of the components of the mixture may be inhibited by other compounds.
- Many non-polar compounds adsorb on to particulate matter in soils or sediment and become less available to degradative microorganisms.
- Microbial biodegradation of organic compounds are often quite slow.

One way to address some of these problems is to transfer plasmids that carry genes for different degradative path ways in to recipient strain by conjugation If two resident plasmids contain homologous regions of DNA, recombination can occur and a single, larger, 'Fusion' plasmid with combined functions can be created. Alternately, if two plasmids do not contain homologous regions and, in addition, belong to different in compatibility groups, they can coexist within a single bacterium.Most of the degradative bacteria that have been genetically manipulated by plasmid transfer are mesophiles, organisms that grow well only at temperatures between 20 and 40º C.

However, rivers, lakes and oceans that are polluted generally have temperatures that range from 0 to 20 ºC.The term biodegradation is often used in relation to Environmental Pollution, Ecology, waste management, Environmental remediation (Bioremediation) and to degradation of plastic materials due to their long persistence in nature. Organic materials can be degraded aerobically, with oxygen or an anerobically, without oxygen.

II. REVIEW OF LITERATURE:

Since not much work has been reported on Biodegradation of Heterocyclic aromatic compound particularly on PNP, exhaustive literature on pollutant water has been reviewed and presented here under. Bruhn et al., (1987) reported that nitro aromatic compounds are raw materials for synthesis of pesticides, pharmaceutical, plastics, azo dyes, explosives and solvents and also found that Nitro groups reduce electron intensity of the nitroaromatic ring, there by impeding electrophilic attack by oxygenases and oxidative degradation of nitroaromatic compounds. Bhattacharya, and Uberoi (1997) reported that PNP and 2, 4 dinitrophenol as priority pollutants and recommended restricting their concentrations in natural waters to < 10 mg/lit. Lenke, H., and Knackmuss H.J. (1987) reported that various forms of PNP have become common environmental pollutants. Munnecke, D.M., (1974) reported that PNP is important as a basic material for medicines, dyes and explosives, and serves as a precursor in pesticides such as methyl parathion. PNP is a priority environmental pollutant occurring in industrial effluents and in the soil as a hydrolic product of parathion or methyl parathion. (Keith, L., H and W.A. Telliard 1970).Debomanda Ningthoujam., (2005) reported that Brevibacteria strain able to degrade PNP. This bacterium was isolated from garden soil in basal salts medium containing Para nitro phenol. This bacterium pure culture could degrade up to 300-mg/l. PNP presence of yeast extract.

Hanne et al., (1993) Pakel. (1957), and Spain, (1995) reported that Microbial degradation of PNP has been reported for several bacterial including Arthrobacter, Bacillus, Flavobacterium, Moraxella, Pseudomonas.Hanne, et al., Kirk L., L., (1993) reported degradation and induction specificity in Actinomycetes that degraded P-nitro phenol. Jain, R.K., J.H. Dreibanch, (1994) found that the Biodegradation of P-Nitro phenol via 1, 2, 4 – benzenetriol by Anthrobacter sp. This hydroxylates PNP to produce either 4-nitrocatechol (or) 4 –Nitro resorcinol.Marvin Sikkena, and de Bonkt, (1992) found that they are produced in the order of several thousands of tons annually.Ray, P., Ait onbelli, M., and Loeser, C.,(1985); found that activated sludge is used for the treatment of PNP contaminated wastewater. Kimura, N., Shinozyaki, Y., Suwa, Y., and Urushigawa, Y; (2000): found that Pseudomonas strain YTK 17 isolated using the medium supplemented with mineral salt medium with 10 mg PNP.

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The isolated bacteria were characterized by determining their partial 16S r DNA sequences (696 – 702 bp). Zaidi et al. Iman, S. H.,and Greene, R.V. (1996): isolated PNP degrading strains using a mineral salt medium containing 4.6mM K2HPO4, 1.5mM KHPO4 , 0.41mM MgSO4.7H20, 0.68mM CaCl2. H2O, 0.62mM NH4NO3 & 0.74mM FeCl3 6H20 supplemented with PNP as the sole carbon source. Different PNP degrading bacteria isolated from different places have been reported by Bhusan. Et.al (2000) to exhibit diverse kinetics with respect to PNP degradation. They proposed the possibility that the differences observed in PNP degradation activity are due to differences in the degradation enzyme which is responsible for the initial oxidation of PNP. Simpson, J.R., and W.C. Evans (1953);reported that the metabolism of nitro phenol by certain several aerobic pure culture of bacteria belonging to species Flavobacterium, Moraxella, Nocardia, and Arthrobacter metabolize PNP with removal of nitro group as nitrite. D.T. Gibson., (1991) proposed pathway for biodegradation of P-nitro phenol in Moraxella sp. This pathway is more common in Gram positive isolates and results in the formulation of hydroquinone from PNP, probably via 1, 4 – bezoquinone, with concomitant nitrite release.Ecker et al, (1989) suggested the involvement of a dioxygenase attack, in the displacement of nitro group from 2, 6 dinitrophenol.

PNP monoxygenase activity was determined by measuring the nitrite released from the substrate (PNP or 4-nitrocatechol) at 30 C. The standard reaction mixture contained in 1 ml of TE buffer (50mM Tris-HCl, 0.25% ethanol, 0.2mM NAD, 0.02mM FAD, 1mM MgSo4, and various amounts of protein. The reaction was initiated by addition of either PNP or 4-nitrocatechol (0.08 mM). The substrates were omitted from the control reaction mixtures. After 30 min, nitrite was determined by the method adapted by Daniels et al. Barth F., Smelis., Jim C Spain: reported a preliminary characterization of a novel monoxygenase from Bacillus sphaericus JS 905 that catalyzes the first two steps in the degradation of PNP Via 4-nitrocatechol and then in second step removes the nitro group. The reactions are very specific, and the enzyme does not release nitrite directly from PNP. Zeyer and Kocher (1984); purified a soluble nitro phenol oxygenase from Pseudomonas putida B2 that Converts ortho nitrophenol to catechol and nitrite. The relative molecular mass of the native proteins were determined by gel filtration on a Sephacryl S-300 column (1.5 by 107 cm) at a flow rate of 1.0 ml./min with 100mM NaCl in TE buffer the calibration standards were ferritin, catalase, aldolase, and ovalbumin.

Partially purified proteins were separated by sodium dodecyl sulfate poly acryl amide gel electrophoresis, and the gels were stained with Comassie brilliant blue to visualize proteins. This method was proposed by Spain, J.C., & D.T. Gibson (1997). PNP induced cells were suspended to an A 600 of 1.5 in 50 ml. of MSM containing 0.1% yeast extract, 0.2mM PNP or 4-nitrocatechol, and 1mM either 2, 2 -dipyridyl or o-phenanthroline for inhibition of THB oxidation. Nitrite release was determined, and the THB formed was detected by HPLC. In a separate experiment, cell extracts prepared in phosphate buffer (20mM, pH 7.2) containing no added FAD were incubated with an inhibitor at 30°C. For 15 min. prior to the addition of NADH, FAD, MgSO4, and substrate (PNP or 4-nitrocatechol) to the reaction mixture. This method was proposed by Chapman P., J. Hooper D.J., (1968) Mithra D, and Viadyanathan C S., reported the purification and characterization of P-nitrophenol -2-hydroxylase enzyme for conversion of PNP to 4-nitrocatechol from a Nocardia sp. In another study, hydroquinone and 1, 2, 4 -benzenetriol were detected as intermediates during PNP degradation by a Pseudomonad. Raymond and Alexander :(1971) suggested that Flavobacterium Sp. converted PNP to 4 - Nitro catechol as the first step in complete degradation.

Grown on PNP cells released synoichiometric amounts of nitrite and produced a purple compound rules incubated in media containing PNP (or) 4-nitrocatechol and iron cheaters such as O-phenanthroline. The color was identical to that produced when 2-hydroxy – 1, 4 – benzoquinone was added to reaction mixtures (Join, R.K., J.H. Dreisbach J.C. Spain (1994). Identification of the products of PNP oxidation, cell extracts incubated in potassium phosphate buffer with NADPH converted PNP to a yellow metabolite. Addition of 2.5N NaOH to reaction mixture after incubation at 30°C gave a deep red solution, which is a characteristic of 4 - nitro catechol (Mitra, D., & C.S. Vaidynatham). NADPH was also an electron donor for the reductase activity. A flavoportein and a second protein of the ferredoxin type are required for cytochrome C reductase activity L.M. siegel (1980). Marvin.sikkema,. FD., (1994); reported that a Moraxella species was isolated from activated sludge by selective enrichment with PNP cultures were grown in a minimal salts medium at pH 7.0 with forced aeration at 30 C. The basal medium was supplemented with PNP (150 mg/l) and yeast extract (0.1%). Simpson. And Evans. (1953) suggested that the initial step in the bacterial degradation of PNP involved a similar oxidative removal of the nitro group. Mynnecke and Hsien.

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Detected hydroquinone as an early metabolite in PNP degradation by a pseudomonad and proposed that it was hydroxylated to form 1, 2, and 4 – benzenetriol prior to ortho ring fusion. Described an enzyme in a Moraxella strain that replaced the nitro group with a hydroxyl group as the initial reaction in PNP degradation. Degradation of o – Nitrophenol by Pseudomonas putida involves replacement of nitro group with hydroxyl group and subsequent conversion of the catechol to Ketoadipate Via, Cis, Cis – muconate. (Nishino, S, F.and Spain 1993); Aerobic granules to treat waste water containing PNP were successfully developed in a sequencing batch reactor (SBR) using activated sludge as inoculums. The aerobic granules were cultivated at a PNP loading rate of 0.6 kg/m 3 day, with glucose to boost the growth of PNP – degrading biomass (Carlton, B.C., and B.J. Brown 1981).Aerobic granules had specific PNP degradation rates that increased with PNP concentration from 0 to 40.1 mg. of PNP / lit., (Chapman, 1976).

Microbial analysis of activated sludge reactor after repeated exposure to 100 mg. of PNP per liter resulted in the isolation of three Pseudomonas species able to utilize PNP as a sole source of carbon and energy (J.C. Spain., S.F Nishino, 1991). Bacillus sphaericus Js 905, isolated from an agricultural soil by selective enrichments, transforms PNP releasing only nitrite in stoichrometric amounts (Barth F Smetes., 1993) M.Cordero, Ran do,, M.Barea., Zamora proposed the electrochemical technique of differential pulse voltammentry has been used to measure PNP in seawater and can potentially be used for online monitoring. J.I. Lee, I.Karube, (1983);reported that Microbial biosensors are cheap and easy to operate and sense are suitable for online process control and environmental monitoring several microbial biosensors, such biological O2 demand, cyanide, etc., have been developed for these applications. A.Oubina, B.Ballesteros, Regale(1987); found that the detection of PNP with the new microbial biosensor is simple, direct, single step and rapid the analysis time for each sample was less than 5 min., which is significantly shorter than the hours required for immuno assays. Jain .et. Al (1986); described the isolation of Arthobacter sp. That degrades PNP through 4-nitrocatechol, 1, 2, 4 – benzenetriol, and B –Ketoadipate to tricarboxylic acid intermediates while releasing nitrite and consuming 02 .Suzuki, K., T. Giomi, and P.C..(1991); suggested that bacteria can oxidatively remove nitro groups from ortho, Meta, Para micro phenol.Mitra, D., and C.S. Vaidyanathan proposed that A new 4- nitrophenol

III. MATERIALS & METHODS:

Materials used for the investigation and various methods standardized for the biodegradation of Heterocyclic aromatic compound, Para nitrophenol (PNP) are described below:

SITE DESCRIPTION AND SAMPLE COLLECTION:

Hussain Sagar Lake is one of the biggest lakes in the located between the twin cities of Hyderabad and Secunderabad in Andhra Pradesh. It is being polluted by many anthropogenic ways. Daily waste water from the industries and residential areas are being discharged into the lake. Many of the wastes are composed of many organic and toxic chemicals. The soil in this lake is loaded with many toxic chemicals and metals and is rich sources of pollutants. Hence Soil samples are collected from different places from the lake and are immediately transferred to the laboratory and stored at –20 ^{0}C freezer.

ISOLATION OF THE PNP DEGRADED BACTERIA:

Serial dilution of 1 gram of soil sediments up to 10–9 concentration was carried out under laminar airflow. Glassware used for the serial dilution was autoclaved at 15 lbs for 15 minutes. Aliquots of 10^{-1}, 10^{-3}, 10^{-5}, 10^{-7}, 10^{-9} dilutions are inoculated into the minimal salt media containing 0.1 mg. of PNP.

Composition of minimal salt medium:

Glucose	:	1 gram
Peptone	:	1 gram
Nacl	:	0.5 gram
PNP	:	0.1 mg.
D.W.	:	100 ml.
P^{H}	:	7.0

Note: In the above medium glucose concentration is gradually replaced by increasing concentration of PNP. Minimal salt medium was sterilized at 15 lbs. Pressure for 15 minutes in an autoclave. From the 5th serial diluted of Hussain Sager Soil sample was inoculated into 100 ml. Of minimal salt medium and incubated at 37 0C. with aeration speed 200 rpm for 48 hours.

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Growth was observed by the turbidity in the conical flask. 1 ml. of growth was transfer to the minimal salt medium with glucose concentration at 0.8 Gms. And PNP concentration at 0.2 mg. and for the incubated for 48 hours. The Process was repeated with successive transfer of 1 ml. growth to fresh minimal salt medium with decreased glucose concentration and increased.

PNP concentration as follows:Glucose concentration was 0.8 Gms. To 0.1 gm. and PNP concentration was 0.2 mg. to 0.9 mg. after 7 such transfers, 0.1 gm. concentration of glucose and 0.9 mg. concentration of PNP were transferred to medium containing only PNP at 1 mg. without glucose and with other ingredients.

This composition step will select bacteria that can utilize PNP as the only source of carbon and energy.

Isolation of pure culture of PNP degrading bacteria:

Minimum salt agar medium with following composition was used to isolate the pure culture of PNP degrading bacteria.

Agar	:	2 gms.
Peptone	:	1 gm.
Nacl	;	0.5 gms.
PNP	:	0.8 mg.
D.W.	:	100 ml.
Ph	:	7.0

100 ml of the of minimal salt agar medium was prepared and sterilized in the autoclave at 15 lbs. Pressure and 121 0C. temperature.0.5 Ml. of growth from final medium (minimal salt medium) was transferred to solidified Minimal Salt Agar medium by streak plate method.

Characterization of PNP degrading bacteria:

Preliminary characterization of PNP degrading bacterial isolates obtained by enrichment technique was done by morphological identification that includes:

- Gram's staining,
- Endospore staining,
- Capsule staining,
- Motility test by hanging drop preparation

GRAM STAINING METHOD:

- Smear was prepared from the instructions given for sample stain.
- The reagent crystal violet (Primary Stain) was added to that covers the whole smear. Allowed to react for 1 minute.
- Rinsed with tap water.
- Few drops of Grams Iodine (Mordant) were added to cover the smear. Allowed to react for 30 sec to 1 minute.
- Rinsed with tap water.
- 95% Ethanol (decolorizing agent) added to covers the whole smear allowed reacted for 1 minute.
- Rinsed with tap water.
- Covered the smear with safranine (counter strain) and allowed the stain react for 1 minute.
- Rinsed with tap water.
- Bolt dried, & examined under micro scope.
- Result was recorded.

BACTERIAL ENDOSPORE STAINING METHOD:

- Prepared the smear and air dried and fixed with heat.
- Covered the smear with Malachite green and allowed it to act for 10 to 20 minutes.
- Rinsed with Tap Water.
- Bolt dried it and allowed the slide to dry.
- A drop of mountain was added covered it with a cover glasses and examine under micro scope.
- Result was recorded.

MOTILITY TEST (HANGING DROP METHOD):

- A square sized clean cover slip is placed on the white sheet.
- A drop of cell suspension is recovered with the help of an inoculated loop and placed at the centre of the cover slip.
- Vaseline is placed on the four corners of the cover slip.
- A clean cavity slide is held, cavity side down, over the drop on the cover slip. The cavity slide and the cover slip are brought into contact.
- The slide is inverted quickly so as to bring the cover slip on top and to avoid running off the drop to one side.
- The hanging drop preparation is then placed on the microscope stage.
- Result was recorded.

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ANALYSIS OF PNP RESISTANCE:

To determine the tolerance of the isolated bacteria obtained by enrichment technique. Isolated organism was incubated at various PNP concentrations ranging from 0.8 mg. to 10 mg. Viability of resistant bacterial isolates is checked by screening on mineral salt medium supplemented with PNP.

Composition of Mineral Salt Medium:

$Fecl_3\ 6H_2O$	:	1 mg.
$Cacl_2\ 2H_2O$	:	27.5 mg.
$Cacl_2\ 2H_2O$	:	27.5 mg.
$MgSo_4\ 7H_2O$	:	22.5 mg.
$Kh_2\ Po_4$	:	108.75 mg.
$Na_2\ HPO_4\ 7H_2O$	:	166 mg.
$NH_4\ Cl$	:	1.7 mg.
D.W.	:	1000 ml.
PH	:	7.0

PNP was added into each 100 ml salt medium in conical flask respectively, 0.8 mg, 2 mg, 5 mg, 8 mg, 10 mg, The above mineral salt media supplemented with PNP was sterilized in an autoclave at 15 labs pressure and 121o C. PNP degrading microorganism culture was inoculated into sterilized mineral salt medium under aseptic conditions and incubated at 37 o C. With aeration speed 200 rpm. For 48 hours. Growth was observed by the development of turbidity in the conical flask from low concentration to high concentration of PNP Results were recorded.

Program PCR:
Required materials:

• DNA template	:	5 µl
• d NTPS	:	4 µl
• Taq polymerase buffer	:	2.5 µl
• Forward primer	:	2.5 µl
• Reverse Primer	:	2.5 µl
• Tag DNA polymerase	:	0.33 µl
• $Mg\ Cl_2$	:	1.5 µl
• Sterile double distilled water	:	7.5 µl

Amplification reaction was performed in a final volume of 25 □1 mixer and the process as follows:

- Label 0.2 ml PCR tubes for each genomic DNA and arrange the tubes in eyes containing rack.
- 5 □1 of template DNA was taken to the PCR tubes and 0.5 □1 of priers was added.
- 10 □1 of cocktail was added.
- 7.5 □1 of sterile doubled distilled water was added.
- Sterile double distilled water was added to adjust the final reaction volume to 25 □1.

- All the above components are mixed in a microfuge tube and the reaction is carried out in a thermal cycler, which is an automated instrument that's takes reaction through a series of different temperatures for ranging amount of time.

Each PCR cycle theoretically doubles the amount of target template sequence in the reaction.

The amplification profile was as follows:
The amplification was carried for 35 cycles.

Step	Name	Temperature	Time
1	Initial Denaturation (Hot start)	95° C.	3 Minutes
2	Denaturation	94° C.	1 Minute
3	Primer annealing	55° C.	37 Seconds
4	Primer Extension	72° C.	1 Minute
5	Steps 2 – 4 where done 35 cycles of	94° C. 55° C. 72° C.	5 Seconds 15 Seconds 1 Minute
6	Final extension	72° C.	7 Minutes

IV. RESULTS:

This PNP depredating bacterium was grown on Nutrient Agar medium by Streak plate technique.

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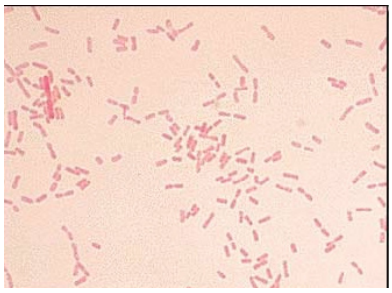
GRAM STAINING
Observation

On Gram staining pink colored Rods were observed. Hence it is a Gram negative Bacterium.

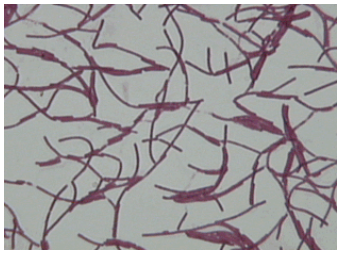
Result:
From above observation it is said that is a Gram-negative bacterium.



NEGATIVE STAINING:
Observation:

On negative staining spherical cells occurring in clusters not appears transparent (colorless) against a blue-black ground.

Result:
From above observation it is a capsulated.



Spore staining:
Observation: green color spores are observed.
RESULTS: from above observation it is spore containing bacteria.

VII. Conclusions and Summary:

Screening of Hussain Sagar soil samples resulted in isolation of and efficient PNP degrading bacterium that was characterized as Gram positive, endosporforming, and motile bacillus.The efficient isolate was identified as sulfido bacillus sp.

The isolate was efficient in metabolizing 10 mg of PNP added to 100ml of degradation mineral salt medium used in this study.Hydroxylated derivatives were found to be intermediates of PNP degradation pathway.PNP degradation genes were found to be located on main chromosome of sulfido bacillus sps.

Isolated during the study. Para Nitrophenol Degrading Bacteria were successfully isolated from Hussain Sagar Soil Sample in Minimal Salt Medium containing a Low and High concentration of PNP. PNP acclimated (0.1 to 1 mg. / 100 ml. culture were plated on minimal salt media & minimal salt agar media by streaking after serial dilution.

Subsequently subcultures in minimal salt agar plates and agar slant by streaking led to isolation of pure culture colonies. The pure culture could degraded up to 10 mg./l. PNP. PNP as their sole source of carbon and energy.

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