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Research Article

Bioremediation Efficiency of P-Nitrophenol and Investigate the Macromolecular Profiles of Selected Two Bacterial Strains

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Abstract: Phenol and its compounds are one of the most important pollutants in the environment. P-Nitrophenol (PNP) is a toxic compound that enters the environment during manufacturing and processing of a variety of industrial products. In this context, the study was conducted on biodegradation of phenolic compound; PNP by *P. putida* MTCC 1194. The present study was also examined the efficiency of biodegradation of PNP on different pH. When the ph was increased up to 9, transformed organism possessed nullified effect towards the 72hrs of all the treated concentrations. Though, in wild type colonies had a remarkably significant ($p<0.0029$) biodegrading efficiency noted in each different concentrations with its stipulated period of time. Protein was isolated from *P. putida* and transformed *E. coli* they were run on SDS-PAGE and molecular weight was determined that *P. putida* have lower molecular weighed protein band such as 45.5 KDa, and transformed *E. coli* contains higher molecular weighed protein 62 KDa the GCMS spectral analysis for *P. putida*, It will showed that totally seven compounds has been identified. Amoung the seven compounds two peak compounds also analysed such as 5, 6-dibromo-N, N-dimethyltryptamine followed by 2, 3-dihydroxybiphenyl dioxygenase.
Keywords: *Pseudomonas putida*, Transformed *E. coli*, P-Nitrophenol, SDS-PAGE, AGE, GCMS

INTRODUCTION

Presence of phenolic compounds even at low concentration in the industrial waste water adversely affects aquatic as well as human life directly or indirectly when disposed off to public sewage, river or surface water [1]. The primary sources of phenolic compounds present in industrial effluents are petroleum refineries, plastic manufacturing plants, pharmaceutical industries, coal carbonization and tar distillation units, wood charcoal production units, coke ovens, phenol formaldehyde plants, bisphenol-A and other synthetic resin manufacturing units [2]. At the beginning of this century, around 50% of the world population consists of urban population, in which this issue seriously threatens water resources [3]. Phenolic compounds are serious pollutant for rivers and they have harmful effects such as growth inhibition, decrease of resistance against diseases, aquatic mortality and increase in growth of weedy plants. If phenolic pollution goes to underground water, it causes serious ecological problems [4]. Environmental pollution due to the release of natural phenolic compounds from agro- industrial operations has become widespread in the world [5]. The structure of the compounds present is similar in many industrial effluents and residues like those produced in wine-distillery, olive oil extraction, green olive debittering, cork preparation, wood debarking and coffee production described by the following researchers [5-7]. Earlier [8] reported that the Microbial degradation is the principal route of decomposition of many pesticides, ensuring a complete mineralization over a short period of time. In some cases the first products obtained in the degradation could be as toxic as or more toxic than the initial product [9]. However, phenol containing wastewater is difficult to treat because of substrate inhibition, whereby microbial growth and concomitant biodegradation of phenol are hindered by the toxicity exerted by high concentrations of the substrate itself [10]. One of the consequences of toxic compounds in biological wastewater treatment is process instability, which can lead to a washout of the effective organisms, sometimes providing little warning of the impending failure [11]. Still there is no other works has been done this kind of similar research, since the current research planned the following objectives such as to determine the p-nitro phenol biodegradation under different environmental factor such as different bacterial concentrations, different PNP concentrations and different pH condition using *pseudomonas Putida* and *Ecoli.* To identified the favorable optional pH for p-

nitrophenal degradation using *pseudomonas Putid*a and *transformed Ecoli* by ANOVA analysis. Demonstrated the Agarose and SDS protein profile of the both experimental strain followed by sequencing the from *pseudomonas Putida* and *Ecoli* finally elucidated the biodegradable bioactive compounds from the GCMS chromatogram

MATERIALS AND METHODS

Mass culture of *Pseudomonas putida* MTCC 1194

Peudomonas putida MTCC 1194 was cultured in LB broth and LB agar. The colonies were observed after 24 hours of incubation at 28°C. This broth was further used for biodegradation studies and Isolation studies.

Biodegradation

The mineral salts basal medium used for the growth of the P-nitrophenol degrading microorganism contained the following (in grams per liter of distilled water):K_2HPO_4, 0.65; KH_2PO_4, 0.2; $MgSO_4.7H_2O$, 0.09 and $FeSO_4$, 0.01. Then it was autoclaved for 20 min at 121°C and cooled to room temperature before addition of a concentrated, filter-sterilized solution of mineral salts. The pH of the medium was adjusted to 5, 7 and 9 in different flasks. An inoculum of T*ransformed E.coli* strain was added to each flask at different concentration(100µl, 500µl, 1000 µl) and then same concentration of filter sterilized PNP (100µl) was also added to each flasks and incubated at 30°C in a rotary incubator-shaker at 200 rpm . Another set of experiment was also done by adding same inoculum of Transformed *E.coli* (100 µl) with different concentrations of PNP (50 µl, 100µl, 150 µl) to each flasks maintained at P^H 5, 7, and 9 [9].

Isolation of DNA from the experimental strain

Isolation of genomic DNA Total DNA was extracted by a modified [11].In brief, bacterial cells were collected by centrifugation at 13,000 rpm for 2 min followed by suspension in 564 µl Tris- HCl-EDTA buffer and incubation with 10 µg lysozyme (50mg/ml) at 37°C for 30 mins. 6 µl proteinase-K (20mg/ml) and 30 µl of 10% SDS were added, mixed and incubated for 1 hour at 37°C. To the lysis solution, 100 µl of 5M NaCl was added followed by incubation for 2 min at 65^0 C. This was followed by an addition of 80 µl CTAB/NaCl and a further incubation for 10 min at 65°C. The mixture was treated with phenol/chloroform/isoamyl alcohol (25:24:1). The supernatant was collected and precipitated with isopropanol by keeping at - 20°C overnight. Genomic DNA was washed in 70% ethanol and dissolved in 100 µl TE buffer. RNase treatment was carried out to remove traces of RNA from the sample.

Agarose Gel Electrophoresis

Agarose gel electrophoresis was carried out to confirm the presence of plasmid DNA.

Preparation of 1% Agarose: 1X TAE was prepared by diluting appropriate amount of 50X TAE buffer. Made up 4 ml of 50X TAE to 200 ml with distilled water.0.5 g of agarose was weighed and add to 50 ml of 1X TAE. This gives 1% agarose gel. Boiled till agarose dissolves completely and a clear solution was formed. Meanwhile the comb was placed approximately 2 cm away from the cathode. The agarose solution was poured in the central part of tank when the temperature reaches approximately 60°C. The gel thickness was should be around 0.5 to 0.9 cm. Keep the gel undisturbed at room temperature till they solidify. 1X TAE buffer was poured into the gel tank till the buffer level stands at 0.5 to 0.8 cm above the gel surface. Gently lifted the combs, ensuring that wells remain intact. 10. Add 2.5 µl of gel loading buffer to 25 µl of freshly extracted plasmid DNA samples.

SDS-Protein Profile

The power cord was connected to the electrophoretic power supply (red: anode, black: cathode.) the samples were loaded in the wells in the desired order. The voltage was maintained at 50 V and the power supply was switched on. When the tracking dye (bromophenol blue) reached ¾th of the gel, the power was switched off.

GC-MS Spectrogram

GC-MS (Agilent 6890 Series GC coupled to a GC Mate II mass spectrometer) analysis was done using a DB-5 capillary column (J&W Scientific, Inc.), 30 m length _ 0.25 mm i.d. _ 0.25 ím film, run under the following GC temperature program: initial, 70°C; held for 3.5 min; raised to 160 °C at 30 °C/min rate; raised to 270 °C at 70 °C/min rate; raised to 310 °C at 35 °C/min rate; and finally held at this temperature for 3 min. The injection port, GC interface, and ionization chamber were maintained at 260, 200, and 120 °C, respectively. The carrier gas was ultrahigh-purity helium at a 1 mL/min flow rate. The sample injection volume was 1 íL. The MS detector was a magnetic sector; spectra were acquired in the positive, lower solution, selected-ion monitoring mode. AMPA derivative was observed at 7:23 min (*m*/*z* 571, 502, 446, 372), and glyphosate derivative was observed at 7:59 min (*m*/*z* 611, 584, 460). Glyphosate and AMPA in the samples were quantified from a calibration curve of derivatized standards of glyphosate and AMPA. The Kovats index system has been widely used in the analysis of food flavors, pesticides and essential oil analysis. Kovats retention index, (1) is defined and calculated by following equation (Douglas, 2000).

$$1 = 100\,N + 100 \log'R\,(N+n) - \log'R(N).$$

Where,

t'R (N) = adjusted retention time of n paraffin hydrocarbon of carbon number eluting before solute-A.

T'R (N+n) = adjusted retention time of n paraffin hydrocarbon of carbon number (N+n) eluting after solute A.

T'R (A) = adjusted retention time of solute-A.

Nucleotide Sequencing and Alignment

16S rDNA sequencing of the isolated strain was carried out by Inqaba Biotec (South Africa). Sequences obtained were compared to the non-redundant nucleotide database at the National Center for Biotechnology Information by using their World Wide Website, and the BLAST (Basic Local Alignment Search Tool) algorithm.

RESULT

Biodegradation of pnp with same inoculum concentration but different concentration

Biodegradation of PNP by *P. putida* and Transformed *E.coli* in mineral salt medium maintained at three different pH ANOVA has shown that there is significant difference is found between the rows in inoculum concentration and incubation duration in the biodegradation of PNP by Transformed *E.coli* and non transformed *P. putida* (Figure 1, 2, 3).

The fig. 1 shows that Amoung the three treated days PNP biodegradation was maximum possibility occurred at 24 hrs on both strains. Though, in the wild type organism clearly denoted when ever the concentration increased the PNP degradation capability also been increased. But in the wild type treatment explained the biodegration ability is very poor at 48 and 72 hrs on 100 as well as 150µl concentration noted in pH-5.

Similarly, when the ph range was seven the PNP biodegrading ability also notably changed, the result showed when the duration of the treated days has been prolonged degradation performance also been neglected on both experimental organisms Fig.2. In the figure 3 showed that when the ph was increased up to 9, transformed organism possessed nullified effect towards the 72hrs of all the treated concentrations. Though, in wild type colonies had a remarkably significant ($p<0.002974$) biodegrading efficiency noted in each different concentration with its stipulated period of time.

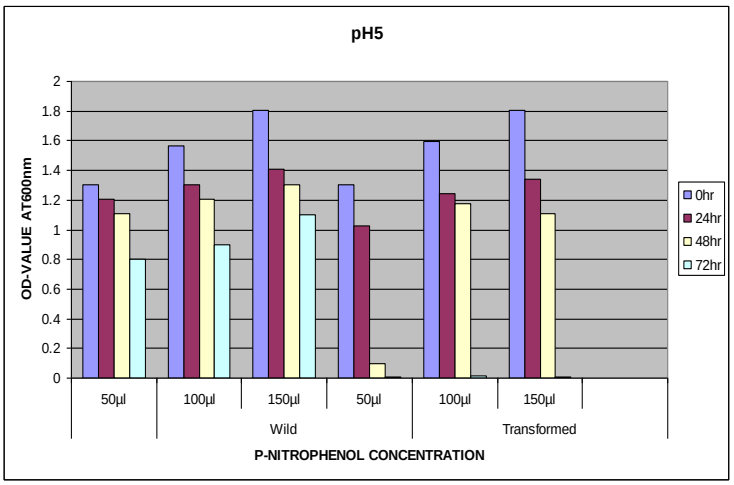

Fig. 1(A)

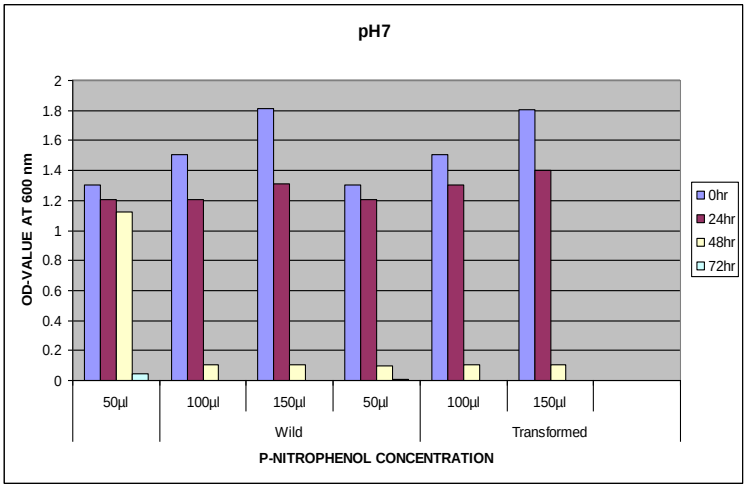

Fig. 1(B)

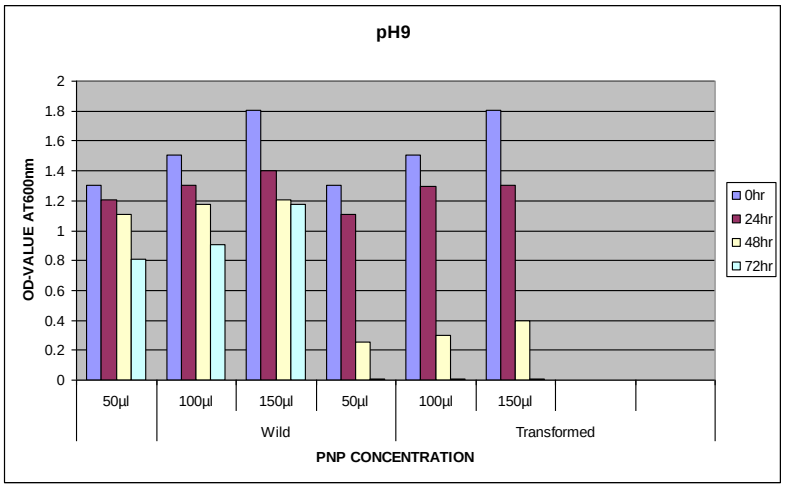

Fig. 1(C)

Fig. 1: Effect of PH and its biodegration of PNP by transformed and wild type clonies (A) PH-5 (B) PH-7 and (C) PH-9

The agarose gel electrophoresis well defined the quantitatively when compared with marker the plasmid DNA of *Pseudomonas putida* showed the highest (2300bp) as well as lowest (820bp) basepairs appeared within the level of thickened and faded bands.

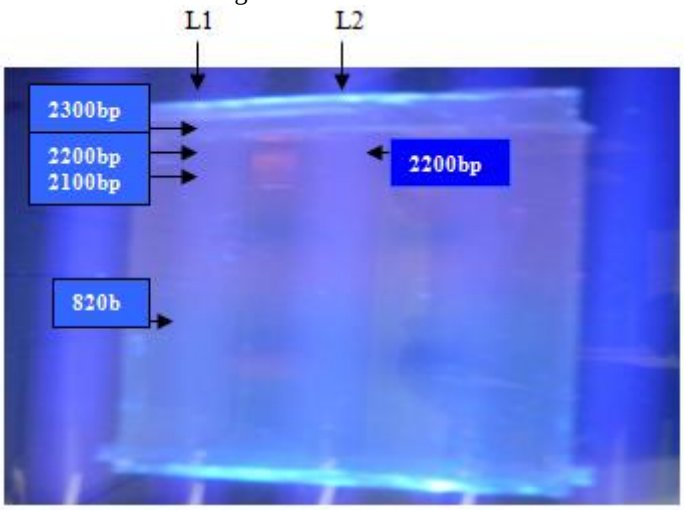

Fig. 2: Agarose Profile of Plasmid DNA of *Pseudomonas putida* (Lane 1 – Plasmid DNA of *Pseudomonas putida* (15 kb), Lane 2 – Marker DNA (20 kb)

DNA Sequencing

The base sequence of both plasmid DNA of Pseudomonas *putida* and *Transformed E. coli* are given below.

Pseudomonas putida

ATGCAAGTCGAACGATGAACCGGCTTCGGCTG
GGGATTAGTGGCGAACGGGTGAGTAACACGTG
GGCAATCTGCCCTGCACTCTGGGACAAGCCCTG
GAAACGGGGTCTAATACCGGATATGACCTGGT
GAGGCATCTCATTGGGTGGAAAGCTCCGGCGG
TGCAGGATGAGCCCGCGGCCTATCAGCTTGTTG
GTGGGGTGATGGCCTACCAAGGCGACGACGGG
TAGCCGGCCTGAGAGGGCGACCGGCCACACTG
GGACTGAGACACGGCCCAGACTCCTACGGGAG
GCAGCAGTGGGGAATATTGCACAATGGGCGAA
AGCCTGATGCAGCGACGCCGCGTGAGGGATGA
CGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGG
AAGAAGCGCAAGTGACGGTACCTGCAGAAGAA
GCGCCGGCTAACTACGTGCCAGCAGCCGCGGT
AATACGTAGGGCGCAAGCGTTGTCCGGAATTA
TTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCGC
GTCGGATGTGAAAGCCCGGGGCTTAACCCCGG
GTCTGCATTCGATACGGGCAGGCTAGAGTTCGG
TAGGGGAGATCGGAATTCCTGGTGTAGCGGTG
AAATGCGCAGATATCAGGAGGAACACCGGTGG
CGAAGGCGGATCTCTGGGCCGATACTGACGCT
GAGGAGCGAAAGCGTGGGGAGCGAACAGGATT
AGATACCCTGGTAGTCCACGCCGTAAACGTTGG
GAACTAGGTGTGGGCGACATTCCACGTTGTCCG
TGCCGCAGCTAACGCATTAAGTTCCCCGCCTGG
GGAGTACGGCCGCAAGGCTAAAACTCAAAGGA
ATTGACGGGGGCCCGCACAAGCGGCGGAGCAT
GTGGCTTAATTCGACGCAACGCGAAGAACCTT
ACCAAGGCTTGACATACATCGGAAACATCCAG

AGATGGGTGCCCCCTTGTGGTCGGTGTACAGGT
GGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGA
TGTTGGGTTAAGTCCCGCAACGAGCGCAACCCT
TGTTCTGTGTTGCCAGCATGCCCTTCGGGGTGA
TGGGGACTCACAGGAGACTGCCGGGGTCAACT
CGGAGGAAGGTGGGGACGACGTCAAGTCATCA
TGCCCCTTATGTCTTGGGCTGCACACGTGCTAC
AATGGCCGGTACAATGAGCTGCGATACCGTGA
GGTGGAGCGAATCTCAAAAAGCCGGTCTCAGT
TCGGATTGGGGTCTGCAACTCGACCCCATGAAG
TCGGAGTCGCTAGTAATCGCAGATCAGCATTGC
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CGCCCGTCACGTCACGAAAGTCGGTAACACCC
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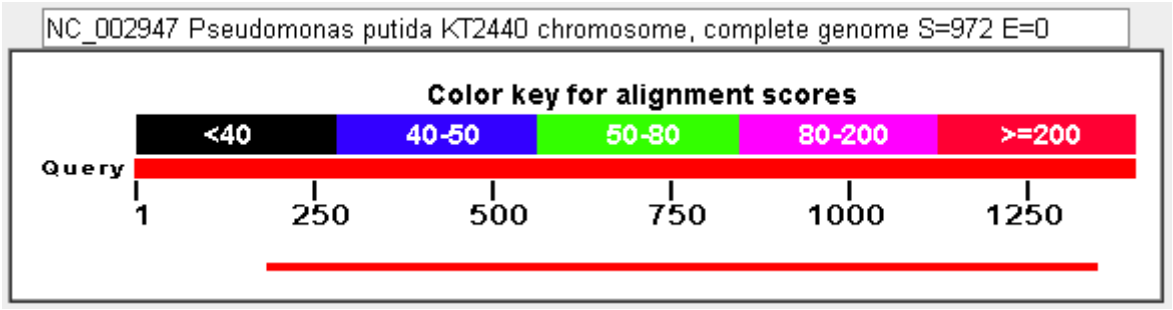

Fig. 3: Distribution of 7 Blast Hits on the Query Sequence (*P. putida*)

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Pseudomonas putida KT2440 chromosome, complete genome	972	6790	83%	0.0	82%	NC_002947.3

Fig. 4: Sequences producing significant alignments (*P. putida*)

Apart from the result of the sequence Blast analysis the above said alignment score highly signified similarity with the quary sequence data. In order to that E value denoted the zero also stands dof beeter matching accompanied with its original sequence.

Transformted *E. coli*

CGGGGGGGCCGCCTATACATGCAAGTCGTACG
CTTCTTTTTCCACCGGAGCTTGCTCCACCGGAA
AAAGAGGAGTGGCGAACGGGTGAGTAACACGT
GGGTAACCTGCCCATCAGAAGGGGATAACACT
TGGAAACAGGTGCTAATACCGTATAACAATCG
AAACCGCATGGTTTTGATTTGAAAGGCGCTTTC
GGGTGTCGCTGATGGATGGACCCGCGGTGCATT
AGCTAGTTGGTGAGGTAACGGCTCACCAAGGC
CACGATGCATAGCCGACCTGAGAGGGTGATCG
GCCACATTGGGACTGAGACACGGCCCAAACTC
CTACGGGAGGCAGCAGTAGGGAATCTTCGGCA
ATGGACGAAAGTCTGACCGAGCAACGCCGCGT
GAGTGAAGAAGGTTTTCGGATCGTAAAACTCT
GTTGTTAGAGAAGAACAAGGATGAGAGTAACT
GTTCATCCCTTGACGGTATCTAACCAGAAAGCC
ACGGCTAACTACGTGCCAGCAGCCGCGGTAAT
ACGTAGGTGGCAAGCGTTGTCCGGATTTATTGG
GCGTAAAGCGAGCGCAGGCGGTTTCTTAAGTCT
GATGTGAAAGCCCCCGGCTCAACCGGGGAGGG
TCATTGGAAACTGGGAGACTTGAGTGCAGAAG
AGGAGAGTGGAATTCCATGTGTAGCGGTGAAA
TGCGTAGATATATGGAGGAACACCAGTGGCGA
AGGCGGCTCTCTGGTCTGTAACTGACGCTGAGG
CTCGAAAGCGTGGGGAGCAAACAGGATTAGAT
ACCCTGGTAGTCCACGCCGTAAACGATGAGTG
CTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCT
GCAGCTAACGCATTAAGCACTCCGCCTGGGGA
GTACGACCGCAAGGTTGAAACTCAAAGGAATT
GACGGGGGCCCGCACAAGCGGTGGAGCATGTG
GTTTAATTCGAAGCAACGCGAAGAACCTTACC
AGGTCTTGACATCCTTTGACCACTCTAGAGATA
GAGCTTCCCCTTCGGGGGCAAAGTGACAGGTG
GTGCATGGTTGTCGTCAGCTCGTGTCGTGAGAT
GTTGGGTTAAGTCCCGCAACGAGCGCAACCCTT
ATTGTTAGTTGCCATCATTCAGTTGGGCACTCT
AGCAAGACTGCCGGTGACAAACCGGAGGAAGG
TGGGGATGACGTCAAATCATCATGCCCCTTATG
ACCTGGGCTACACACGTGCTACAATGGGAAGT
ACAACGAGTTGCGAAGTCGCGAGGCTAAGCTA
ATCTCTTAAAGCTTCTCTCAGTTCGGATTGCAG
GCTGCAACTCGCCTGCATGAAGCCGGAATCGCT
AGTAATCGCGGATCAGCACGCCGCGGTGAATA
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TATAGCACC

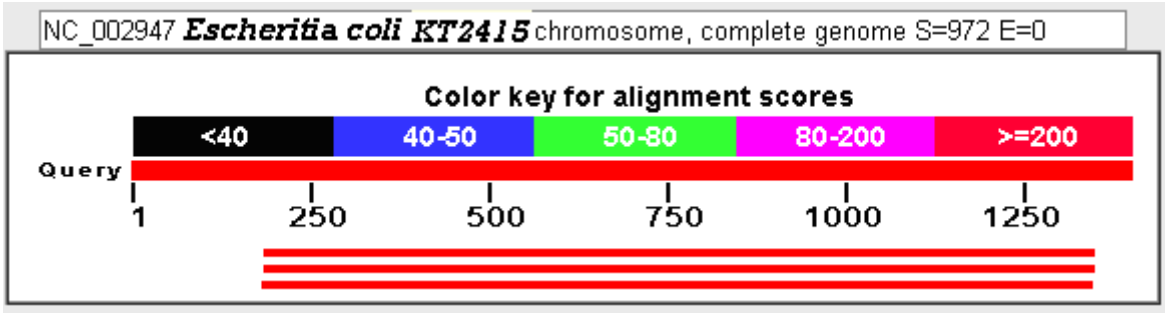

Fig. 5: Distribution of 7 Blast Hits on the Query Sequence (*E. coli*)

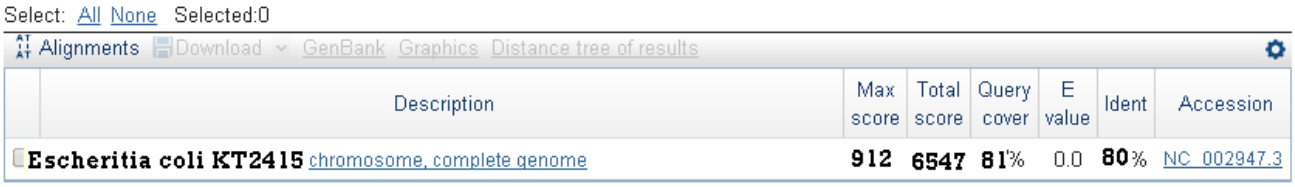

Fig. 6: Sequences producing significant alignments (*E. coli*)

Separation of proteins by SDS-PAGE

Protein was isolated from *Pseudomonas putida* and transformed *E. coli.* The protein bands were identified by SDS-PAGE and molecular weight was determined as KDa. Both of the experimental strain Highest protein band appeared 62KDa in an organism of Transformed *E.* coli followed by *P. putida* also possessed only one protein band lowest kilo Dalton protein band such as 45.5KDa.

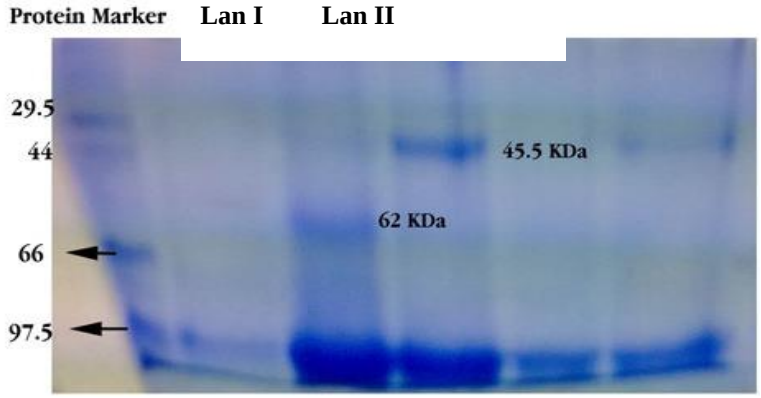

Fig. 7: SDS-PAGE proteins profile of Transformed *E.* coli and *P. putida*(Lan 1- Transformed *E. coli*, Lan 2- *Pseudomonas putida*)

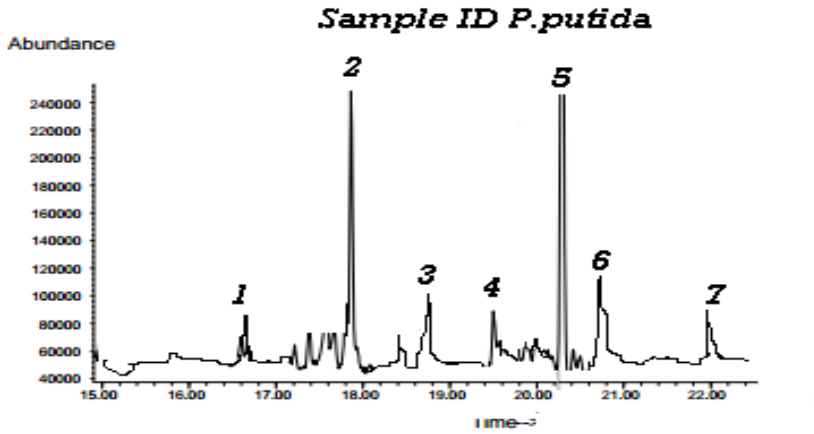

Fig. 5: Targeted detection of Biodegradation of PNP by Transformed *Escherichia coli* extracts, by reconstructing GC-MS chromatogram

Table 1: GCMS spectrogram analytes (separated bioactive compounds) of the experimental organism of *P. putida*

Sl. No.	Retention Time (RT)	Compounds separated (Name of the Analytes)	Abundance (%)
1.	15.6	Cyclo-3-hydroxy-2',4' diphromophenoxy-phenol	**85000**
2.	18.1	5,6-dibromo-N,N-dimethyltryptamine	**248000**
3.	18.94	1, 2,4-benzenetriol	**88000**
4.	19.65	Cryptophycin	**84000**
5.	20.3	2,3-dihydroxybiphenyl dioxygenase	**240000**
6.	20.8	4-methyldeconyl dibromobarretin	**10000**
7.	**22.0**	**α' β-Benzene Triol**	**70000**

Fig. 6: Targeted detection of Biodegradation of PNP by Transformed *Escherichia coli* E. coli extracts, by reconstructing GC-MS chromatogram

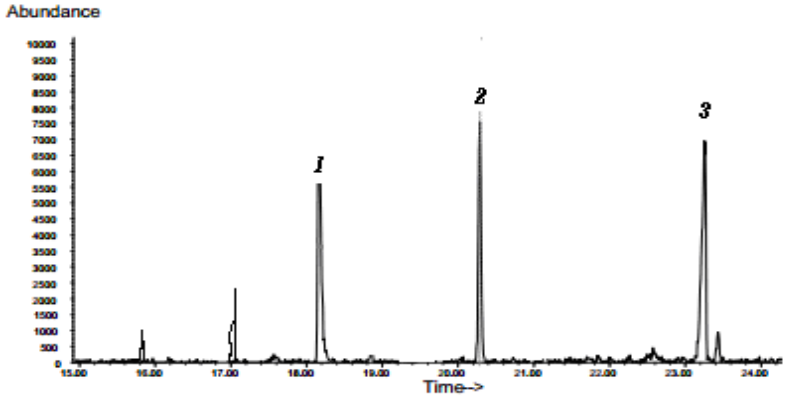

Table 2: GCMS spectrogram analytes (separated bioactive compounds) of the experimental organism of *E.coli*

Sl. No.	Retention Time (RT)	Compounds separated (Name of the Analytes)	Abundance (%)
1.	18	3-hydroxy- dicyclohexylphthalate	55000
2.	22	5,6-N,N-diphenyl Fucosterol	85000
3.	23.5	5-7-octa salicylihalamide	82100

Fig. 5 revealed that the GCMS spectral analysis for *P. putida*, It will show that totally seven compounds has been identified. Amoung the seven compounds two peak compounds also analysed such as 5, 6-dibromo-N, N-dimethyltryptamine followed by 2, 3-dihydroxybiphenyl dioxygenase and its retention time was 18.94 , 20.3 also abundance 248000, 240000 respectively. Subsequently other five compounds were more or less similarly present in the experimental strain of *P. putida* since the reason this organism possessed the abilityoot degrade the PNP. The present result was interestingly observed little amount PNP degradable compound also been present this organism (Table 2). Similarly, another experimental strain *E.coli* also separated by GCMS chromatogram. It denoted mainly three compounds were analyzed. Initial compound named as 3-hydroxy- dicyclohexylphthalate followed by 5, 6-N, N-diphenyl Fucosterol and 5-7-octa salicylihalamide also its retention time and abundance such as 18, 22 and 23.5. While, the GCMS result explained analytes having the abundance range was 55000, 82100 for 3-hydroxy- dicyclohexylphthalate followed by 5,6-N,N-diphenyl Fucosterol and 5-7-octa salicylihalamide respectively (Fig. 6); (Table-2).

DISCUSSION

Previously [12] viewed the similar kind of observation through other ability bacterial organism, they involves an initial monooxygenase-catalyzed removal of the nitro group. The resultant hydroquinone is subject to ring fission catalyzed by a dioxygenase enzyme. Studies have been done on the degradation of PNP by various scientists [6, 10, 13]. In the present study degradation of p- nitrophenol was done under various pH conditions. *Pseudomonas putida* was capable of metabolizing p-nitrophenol (PNP) as a sole source of carbon, nitrogen and energy [13-16]. To explore the applicability of this strain for bioremediation for controlling environmental PNP pollution, its degradation potential at 300 and 500ppm was examined in a medium devoid of carbon and nitrogen source (minimal medium) also degradation was accompanied by release of stoichiometric amount of nitrite depicted by Czekalowski and Skarzynski [10].

In the current study biodegradation at different pH (5, 7, and 9) with different inoculum concentration and same PNP concentration with different inoculum concentration and same PNP concentration was also done in MSG medium using non transformed *Pseudomonas putida* (MTCC 1194). Effect of glucose and nitrogen on PNP degradation under similar conditions revealed that (i) glucose (0.4 g/l) at 20 and 50 ppm PNP did not accelerate the rate of PNP degradation, while glucose (0.4 g/l) at 300 ppm PNP inhibited its degradation, nitrogen supplement viz. sodium nitrate and ammonium sulphate (0.04 and 0.4 g/l) in minimal medium with PNP (300 ppm) showed no effect on PNP degradation published by Choi and Gu, (2001) , while glutamate alone (0.04 and 0.4 g/l) showed mere rise in biomass (from 0.5 to 1.6 OD units), and (iii) acidic pH (4.0-6.5) did not support PNP degradation, while alkaline pH (7.5-9.5) significantly enhanced the rate of PNP degradation previously reported by [15]. The complete degradation of PNP at high concentration (300 ppm) was confirmed previously by HPTLC analysis [16-17]. In order to probe root cause of higher PNP degradation, preliminary studies on genetic analysis of *P. putida* were undertaken, which revealed the prevalence of a degradative plasmid of approximately 15 kb, while cured derivatives of *P. putida* (PNP-) Further conjugal transfer of PNP+ phenotype from *P. putida* to standard strain of *E. coli* Nova blue (PNP) confirmed the degradative type of plasmid [6]. This study also involves the isolation of the plasmid from the non transformed *E. coli*. Then plasmid DNA from *Pseudomonas putida* was isolated by modification method of alkaline lysis method and run in agarose gel and bands were viewed under UV transilluminator. Then isolated plasmid transformed and the transformed *E. co*li appears as blue colonies.

CONCLUSION

The increase in degrading activity of the transformed *E. coli* suggests that the transformed plasmid DNA changed their base pair composition compared to wild *Pseudomonas putida*, So it has lost its originality and the transformed *E. coli* has increased its protein concentration or vigor sufficient enzyme production. From the current study it could be reveal that p-nitrophenol degrading activity was higher in the transformed *E.coli* strain than the *P. putida* (MTCC 1194). The nuteral pH (7) has support PNP degradation, while alkaline pH (7.5-9.5) and acidic pH did not significantly enhance the rate of PNP degradation.

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