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BIODEGRADATION OF POLY AROMATIC HYDROCARBONS USING NITRIFIFYING PSEUDOMONAS BACTERIA AND ITS MOLECULAR CHARACTERIZATION

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ABSTRACT

Biotechnology has applications in four major industrial areas, including health care (medical), crop production and agriculture, non-food (industrial) uses of crops and other products (e.g. biodegradable plastics, Vegetable oil, biofuels) and environmental uses.To attain the task microorganisms were isolated from the deliming water and air. The efficiency was assured by estimating the level of nitrogen content. It was found that all organisms were capable of performing the denitrifying reaction. The obtained isolates were inoculated in synthetic wastewater and their growth characteristics were studied by varying the pH between 4 to 10. The growth of organisms was maximum in between pH 7 to 9.The isolates were inoculated in deliming water (real life wastewater). Two efficient isolates were selected DN3 and DN5. The isolate DN5 was screened for the utilization of PAHs. The molecular identification of the isolate DN5 was identified based on 16S rDNA sequencing and the sequenced DNA was read in the Genbank databases (BLAST), compared with the other sequences bacterial class and confirmed as *Pseudomonas* sp.The strain DN5 showed considerable growth with on 1 mM concentration Naphthalene, Phenanthrene, Anthracene, Fluorene, as the sole carbon. *Pseudomonas* sp. DN5 showed greater degradation capability nearly around 100% against the PAHs tested.

KEYWORDS: Pseudomonas, Denitrification, PAHs, BLAST.

INTRODUCTION

Biodegradable matter is generally organic material such as plant and animal matter and other substances originating from living organisms, or artificial materials that are similar enough to

plant and animal matter to be put to use by microorganisms. Some microorganisms have a naturally occurring, microbial catabolic diversity to degrade, transform or accumulate a huge range of compounds including hydrocarbons (e.g. oil), polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs), pharmaceutical substances, radionuclides, pesticides and metals. Major methodological breakthroughs in microbial biodegradation have enabled detailed genomic, metagenomic, proteomic, bioinformatic and other high-throughput analyses of environmentally relevant microorganisms providing unprecedented insights into key biodegradative pathways and the ability of microorganisms to adapt to changing environmental conditions. Products that contain biodegradable matter and non-biodegradable matter are often marketed as biodegradable.

Biotechnology, particularly the use of enzymes, has greater role to play in Cleaner process development, pollution reduction and quality improvement in the leather manufacturing. Enzymes have an important role in the production of leather for centuries, but it is only in recent year that research and innovation have led to a greater understanding of how Biotechnology can be harnessed. As natural biological substances, Enzymes have much less impact on the environment, speed up processes and are very specific in their activity than chemicals.

Although nitrogen may appear in wastewater in four forms (as organic, ammonium, nitrite and nitrate nitrogen), the Predominant N fractions in tannery wastewaters are organic N, linked to proteins from hides and skins, and ammonia nitrogen (UNEP 1991). The presence of heavy metals, sulphides and ammonia is also a major problem encountered during the biological treatment of tannery wastewaters. At toxic levels these substances reduce the removal of biological oxygen demand (BOD) and chemical oxygen demand (COD) and they inhibit nitrification and dentrification processes in activated sludge (Cheremisinoff 1996; Bitton 1999).

A typical untreated tannery effluent contains

BOD 860 mg/l

COD 2380 mg/l

Total Nitrogen 110 mg/l

Ammonia nitrogen 75 mg/l

Chromium III 75mg/l

Sulphides 165 mg/l (UNEP 1991).

For environmental protection, tannery wastewaters should be treated and there are trends that effluent discharge permits for wastewater treatment plants are being revised to include restrictions on the discharge of various nitrogen compounds (Government of Ethiopia (GOE) 2002).

Biological Treatment of Nitrogenous Wastes: The processes for traditional nitrogen removal from wastewater consist principally of two sub-processes, nitrification and denitrification. In the nitrification process ammonium Is oxidized generally to nitrate by autotrophic bacteria; in the subsequent denitrification process, is reduced to dinitrogen gas (N2) by heterotrophic bacteria. For these reasons, biological Processes of nitrification and denitrification are commonly employed in many wastewater treatment systems.

Nitrification: Nitrification is the biological oxidation of ammonia with oxygen into nitrite Followed by the oxidation of these nitrites to nitrates. Degradation of ammonia to nitrite is usually the rate limiting step of nitrification. These bacteria known as “nitrifiers” are strict aerobes.

Polycyclic aromatic hydrocarbons: Polycyclic aromatic hydrocarbons (PAHs) are a group of chemicals that contain two or more fused aromatic rings in linear, angular, or cluster arrangements (Cheung and Kinkle, 2001).

Aim: To develop a methodology to reduce nitrogen from tannery effluent (deliming water) by bacteria and its effect on degradation of Polycyclic aromatic hydrocarbons.

Strategy: Removal of nitrogen from tannery effluent (deliming water) by bacteria and its effect on degradation of Polycyclic aromatic hydrocarbons.

OBJECTIVES

1. To isolate nitrifying bacteria from the tannery effluent.
2. To standardize the process conditions for nitrogen removal.
3. To validate the standardized process with real life wastewater.
4. To screen the denitrifying bacteria for utilization of PAHs.

Isolates DN3 & DN5 were inoculated in deliming water, incubated for 4 days at room temperature and the following tests were performed, shown in table 1.

Table. 1. Test with real life wastewater.

Isolates	DN3	DN5	CONTROL
Nessler reagent	-	-	+
Tromsdorff reagent	-	-	-
Diphenylamine reagent	-	-	-
Sulphanlic acid and alpha naphthylamine	+	+	-

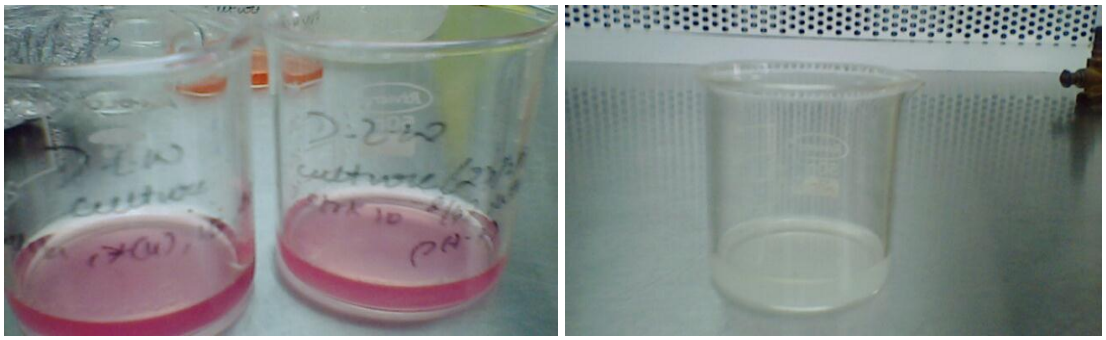


Fig. 1. Test with real life wastewater.

(a) DN3 & DN5 (b) CONTROL

Addition of sulphanilic acid and alpha naphthylamine results in cherry red color formation. It shows the presence of nitrite i.e. nitrate is converted to nitrite.Two efficient isolates were selected, which are capable of performing the denitrifying reaction.

- DN3
- DN5

The TKN for deliming water was noted before inoculating the isolates.

Table. 2. Initial TKN values.

S. No	Characteristics	
1	TDS	6730 mg/L
2	TKN	240 mg/L

Isolates DN3 & DN5 were incubated in the deliming water for 4 days, kept in room temperature. After incubation the level of nitrogen was checked by kjeldahl method.

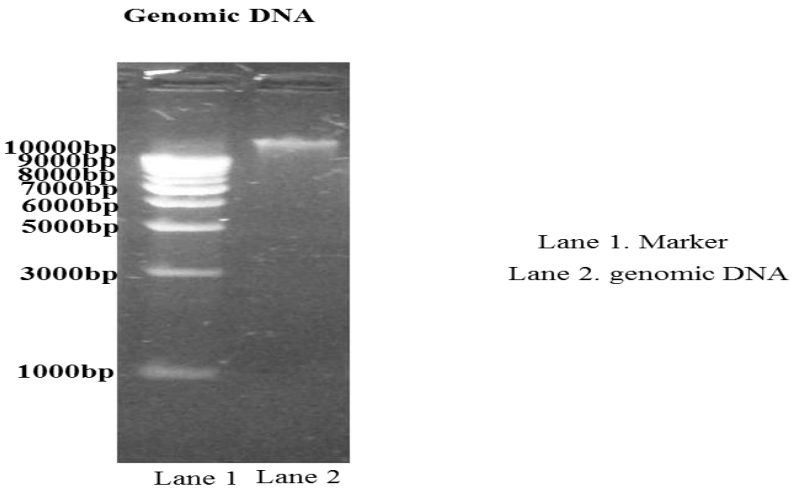
Table. 3. Reduced TKN values.

S. No	Characteristics	DN3	DN5

1	Reduced TKN	48 mg/L	88 mg/L
2	Reduced TDS	1240 mg/L	1840 mg/L

Molecular identification of DN5

Genomic DNA was isolated from *DN5* by following the phenol: choloroform.



Sequencing of DNA

Polymerase Chain Reaction was performed and the sequenced DNA was read in the Genbank databases (BLAST), compared with the other sequences bacterial class and its phylogeny was analyzed.

Pseudomonas sp. DN5 and further confirmation was done by sequencing the 16S rDNA gene and compared with the GenBank databases using the BLASTN program. The 16S rDNA sequence of the isolate revealed a close relatedness to *Pseudomonas* sp. with 98% similarity. Hence the strain was confirmed as *Pseudomonas* sp. DN5.

The Pseudomonas sp strain DN5 16S ribosomal RNA gene, partial sequence is given below

AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAG
TCGAGCGGATGAAGAGAGCTTGCTCTCTGATGCGGCGGACGGGTGAGTAATGCC
TAGGAATCTGCCTGGTAGTGGGGGACAACGTTTCGAAAGGAACGCTAATACCGC
ATACCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCT
AGGTCGGATAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAAC
TGGTCTGAGAGGATGATCAGTCACACTGGAGAGACACGGTCCAGACTCCTACGG
GAGGCAGCAGTGGGGAATATTGGACAATGGCGAAAGCCTGATCCAGCCATGCCG

CGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGC
GTAAAATACTTTGCTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTG
TGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGC
GTAAAGCGCGCGTAGGTGGTTTGTTAAGTTGGATGTGAAAGCCCCGGGCTCAAC
CTGGGAACTGCATTCAAAACTGACAAGCTAGAGTATGGTAGAGGGTGGTGGTTC
CTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGA
CCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGAT
TAGATACCCTGGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGGAGCCTT
GAGCTCTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCA
AGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGG
TTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTTGACATCCAATGAACTTT
CCAGAGATGGATTGGTGCCTTCGGGAACATGAGACAGGTGCTGCATGGCTGTCG
TCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTC
CTTAGTTACCAGCACGTAATGGTGGGCACCTAAGGAGACTGCCGGTGACAAACC
GGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACA
CACGTGCTCAATGGTCGGTACAGAGGGTTGCCAAGCCGCGAGGTGGAGCTAATC
TCATAAAACCGATCGTGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGG
AATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTCCCGGGCCTTGT
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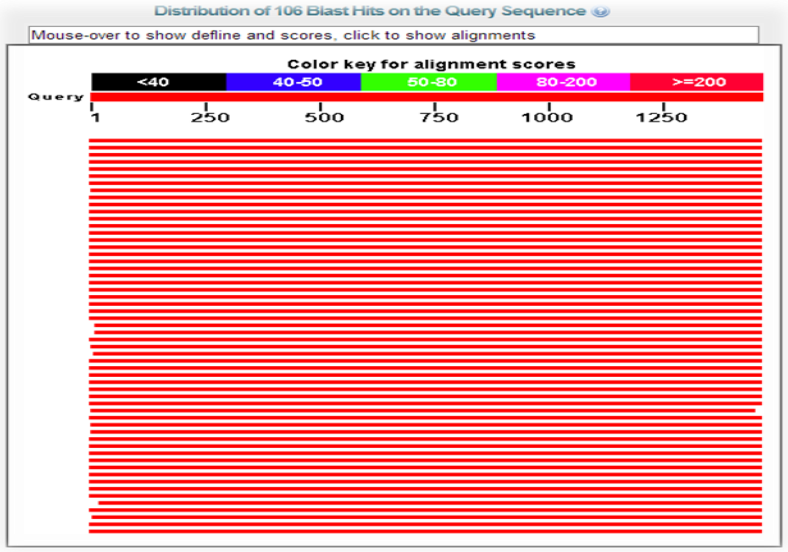
AAGGT



Polycyclic aromatic hydrocarbons accumulate in high concentrations in terrestrial environments near coal gasification sites and tar oil distillation plants (Capotori *et al*., 2004). Major sources of PAHs are incomplete combustion of organic materials, gas production, wood treatment facilities, and waste incineration (Kim *et al*., 2003). PAHs are formed naturally during thermal geologic reactions associated with fossil-fuel and mineral production, and during burning of vegetation in forest and bush fires (Juhasz and Naidu, 2000). The strain DN5 showed growth over important PAHs such as Naphthalene, Phenanthrene, Anthracene, Fluorene, as the sole carbon source with 1 mM concentration in MSM agar plates after 24 h (Table 4).

Table. 4: Qualitative growth of the strain DN5 on PAHs.

PAHs	Qualitative growth in MSM agar plate
Naphthalene	++
Phenanthrene	+++
Fluorene	++
'+' indicates visible colony growth	

Low molecular weight PAHs are compounds with less than three benzene rings and having a molecular weight in the range of 128–178 g/mol (eg : naphthalene, fluorene, phenanthrene and anthracene) (Arulazhagan *et al*., 2010). The test isolate *Pseudomonas* sp. DN5 utilized naphthalene (3 mg/L) as sole carbon source. The growth of the test isolate was steady till 96h. Naphthalene was readily degraded by the consortium and nearly 95% of the compound was degraded in 96 h (Fig. 1).

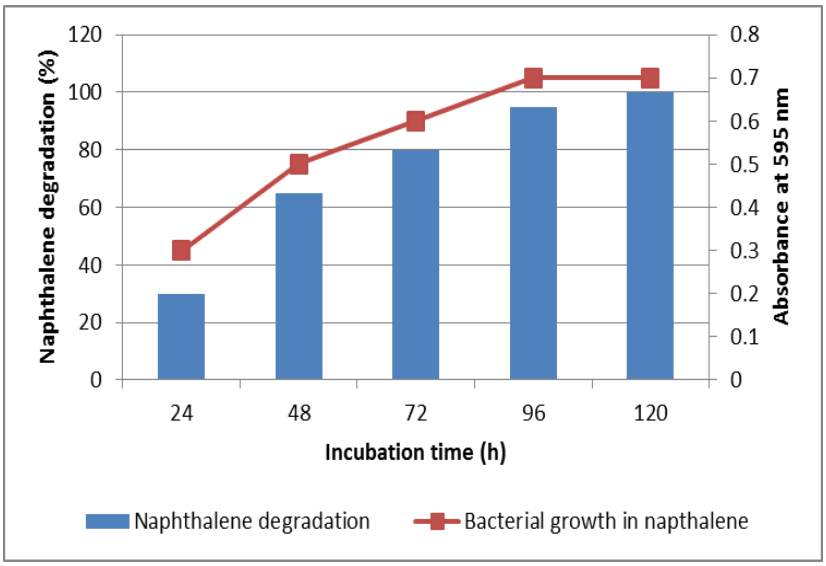


Figure. 1: Growth of Pseudomonas sp. DN5 and naphthalene degradation.

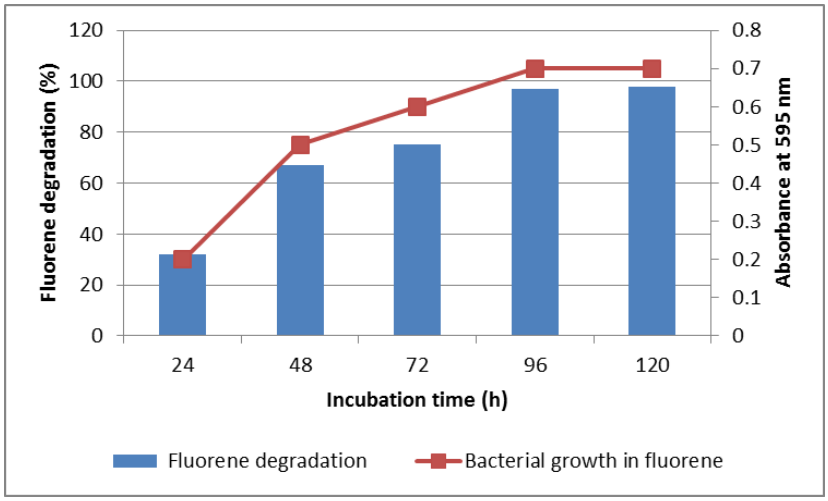


Figure. 2: Growth of Pseudomonas sp. DN5 and fluorene degradation.

Fluorene consists of two benzene rings coupled with a pentagonal ring (cyclopentane ring) and listed as a priority pollutant by Environmental Protection Agency (EPA) (Gomes *et al.*, 2006). In this study, the test isolate utilized fluorene (3 mg/L) as the sole carbon source and showed a maximum degradation of 97% (Fig 2).

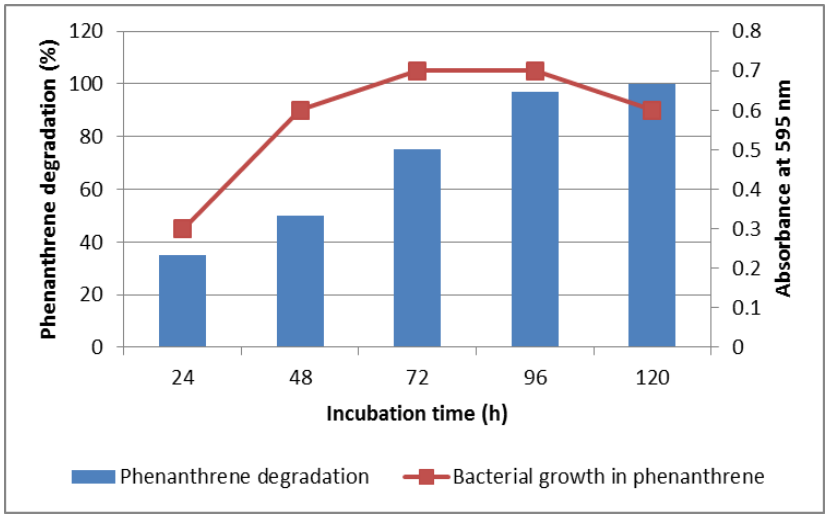


Figure. 3: Growth of Pseudomonas sp. DN5 and phenanthrene degradation.

The bacterial growth was initially acclimatized on phenanthrene (3 mg/L). The growth of the test isolate was steady till 96 h. Nearly 50% of phenanthrene was degraded in 48 h and 100% degradation was recorded in 96 h. The degradation of phenanthrene was 75% on the 3rd day (Fig. 4). The test isolate utilized anthracene (3 mg/L) and the growth increased steadily in 96 h. The results were analysed for reproducibility of the PAHs degradation by the bacterial consortium using phenanthrene and fluorine showed no major change in the percent degradation (97-98%) was observed.

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