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Isolation, Characterization and Identification of Monochlorophenols Degrading Bacteria

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Abstract:

Soils from various agricultural fields having pesticides exposure since several years were used for the isolation of monochlorophenol degrading bacteria. Two strains designated as strain A2 and strain J3 were isolated. Strain A2 was able to mineralize 2-, 3- as well as 4-chlorophenol.The rates of degradation of the three isomers were in the following order: 4-chlorophenol (4-CP) >3-chlorophenol(3-CP)>2-chlorophenol (2-CP).The maximum degradable concentrations of 4-CP,3-CP and 2-CP were 900,800 and <300µM respectively. Strain J3 was able to mineralize 2-CP only and could not mineralize the 3-CP or 4-CP. The maximum degradable concentration of 2-CP was 2300µM. On the basis of various morphological, cultural, biochemical and 16 S rRNA gene sequence analysis the strain A2 was identified as a member of *Rhodococcus* genus (most probably *Rhodococcus coprophilus*) while J3 was identified as a member of *Achromo bacter* genus (most probably *Achromobacter xylosoxidans sub-sp. denitrificans*).

Keywords:
biodegradation, 2-,3-and4-chlorophenols.

Introduction:

Chlorophenols are toxic and carcinogenic in nature. These are very stable in nature.Due to uncontrolled industrial activities get discharged and pollute the environment. Monochlorophenols are used to prepare higher chlorinated phenols and certain dyes and pesticides. Ever since it was discovered that microbes have the ability to transform and/or mineralize xenobiotics, scientists have been exploring the microbial diversity, particularly of contaminated areas in search for organisms that can mineralize a wide range of pollutants. Microbes that transform specific compounds can be isolated, cultured, adapted, and enriched under laboratory conditions. Harnessing microorganisms to mineralize harmful compounds is an attractive option for coping up with the ever increasing pollution on this planet.

Traditionally, classification of bacteria has instead relied entirely on phenotypic characteristics, such as cell wall type, morphology, motility, nutritional requirements and fatty acid profile. Although these characteristics certainly give useful information for bacterial taxonomy, the problem is that individual microorganisms may exchange genes with one another via horizontal gene transfer and complicate the classification. An alternative approach to bacterial taxonomy is to study the genotype of a strain by analysis of its nucleic acids. A widely used method is cloning and

sequencing of the 16S rRNA genes. These genes have become a useful tool for phylogenetic analyses as they are present in all microorganisms, they are long enough to provide taxonomic information and they contain conserved regions as targets for PCR primers. The sequences of a large number of 16S rRNA genes are available in public databases and a novel strain can easily be compared to already studied microorganisms. Moreover, the 16S rRNA gene sequence can be used to trace the phylogenetic history (lineage) of an organism (Nordin, 2004).However, analysis of the 16S rRNA gene is in itself not enough to identify and assign a strain as a novel species, more data is necessary. The "gold standard" of bacterial phylogenetics is instead DNA:DNA hybridization between the total genomic DNA of two related microorganisms. Here, the entire genomes of two strains are compared, and this method is actually used to define a bacterial species; two strains with 70% or greater DNA similarity as determined by DNA:DNA hybridization are considered to belong to the same species (Wayne *et al.*, 1987). However, as cloning and sequencing of 16S rRNA genes is technically much simpler and cheaper than DNA:DNA hybridization, analysis of 16S rRNA gene has its place as an initial screening method to determine which the closest relatives of a novel/isolated strain are (Stackebrandt and Goebel, 1994).

Material and methods:

Chemicals: Chemicals 2-Chlorophenol (2-CP) and 4-Chlorophenol (4-CP) were obtained from Aldrich chemical co. Inc. 3-Chlorophenol (3-CP) from Fluka, Switzerland. Other chemicals were of analytical grade and purchased from Glaxo or S.D. Chem India.

Organisms: Out of various strains isolated from soil by enrichment technique, two strains were selected which showed ability to mineralize monochlorophenols and tentatively named as Strain A-2 and Strain J-3.

Composition of medium: The basal medium consisted of following components per litre of distilled water : Dipotassium hydrogen phosphate,5g; Potassium dihydrogen phosphate,1g; Ammonium sulphate,1g; Magnesium sulphate, 100mg; Calcium nitrate, 50mg; Ferric ammonium citrate, 5mg.The stock solutions of various ingredients were prepared, sterilized and mixed in appropriate amounts to give required concentration of each ingredient. To this mineral medium filter sterilized required isomer of monochlorophenol solution was added, to obtain desired initial substrate concentration in the medium.

Monochlorophenol maintenance agar: Nutrient agar slants (pH 7.8) containing 500 µM each of 2-,3-.and 4-CP were used to maintain the stock cultures of strain A-2 while nutrient agar slants (pH 7.5) containing 1000 µM 2-CP were used to maintain the stock cultures of strain J-3. **Estimation of monochlorophenols/total phenols:** 4-aminoantipyrine (AAP) method

(APHA,1992) andFolin's method were used for estimation of phenols (Folin, 1927).

Chloride ions assay: The chloride ion concentration in the culture supernatant was determined by the method reported by Higson and Focht (1990).

PCR amplification of 16S rDNA: Amplification of 16S rDNA was performed by the method suggested by Arturo *et al.* (1995).16S rDNAwas amplified by Taq polymerase by using universal eubacteria primers; Bac 8f (20mer) forward primer 5'-AGAGTTTGATCCTGGCTCAG-3' and UNIV592r MPNU (15 mer) reverse primer 5'-ACCGCGGCKGCTGGC-3' (K= G/T). During the PCR the initial denaturation was done at 96°C for 5 min and then 40 cycles of 1 min at 94°C,1 min at 54°C and 1 min at 72°C; finally 10 min at 72°C. PCR was terminated after the programme and the amplified genes were subjected to 1 % agarose gel electrophoresis, sequencing and other studies.

Plasmid isolation: Plasmid isolation was done by alkali-lysis method (Sambrook 1989) as well as by rapid procedure for detection of large and small plasmids as suggested by Kado Liu (1981).

Soil samples: Soil samples were collected from several places, such as agricultural (cotton) fields in Tiosa (Dist: Amravati, India) and Bhandara (India) getting pesticide exposure since several years.

Soil suspensions: Soil suspensions were prepared by adding 10 g of soil in 50 ml of phosphate buffer pH 7.5 and mixed vigorously for few minutes. After that the soil particles were allowed to settle for around 15 minutes. 5 ml of supernatant was then transferred to conical flask containing 100 ml basal medium with 250 µM each of 2-CP, 3-CP and 4-CP and organisms were isolated by enrichment culture technique.

Isolation of 2-chlorophenol, 3-chlorophenol, 4-chlorophenol degrading bacteria: The bacterial strains were isolated from the mixed culture by repeated streaking on agar plates. The isolates were separated based on their morphological characteristics. Each colony was carefully separated and streaked on fresh agar plates, and this step was repeated three times. Thereafter, the cultures were streaked on agar slants and preserved at 4˚C for further experiments. Out of the various strains isolated two strains designated as Strain A2 and Strain J3 were selected for further study.

Result and discussion:

Biodegradation of monochlorophenols: Strain A-2 mineralized efficiently 4-CP as well as 3-CP. It also mineralized 2-CP but at a significantly slower rate. The maximum degradable concentrations of 4-CP, 3-CP and 2-CP were 900,800 and <300µM respectively. Strain J3 was able to mineralize 2-CP only and could not mineralize the 3-CP or 4-CP. The maximum degradable concentration of 2-CP was 2300µM.

Chloride release: Stoichiometric release of chloride was noticed in all the degradation experiments.

Identification of strain A2 and strain J3:(Table 1)

Gel electrophoresis and sequence analysis of amplified 16S rDNA : The amplified products were then loaded to agarose gel along with 1kb DNA ladder and observed after electrophoresis on UV transilluminator. In both the strains the amplified 16S rDNA was found to be around 2.0 kb in size (Figure3). The 16S rDNA of the both strains were subjected to sequencing(Figure4 and 7) and other studies.

Plasmid Isolation: Alkali-lysis method (Sambrook et al. 1989) for plasmid detection was performed. A plasmid was detected in strain A2 having size more than 10,000 bp (Figure10). Rapid procedure for detection of large and small plasmids as suggested by Kado& Liu (1981) and Alkali-lysis method, both were tried but plasmid could not be isolated from strain J3.

Amplification of phenol hydroxylase gene complex: It was found that strain A2 mineralized phenol while strain J3 could not. By using Multicomponent phenol hydroxylase primer (Hiroyuki Futamata et al. 2001) the genome of strain A2 was amplified in the PCR, which resulted in to several, around 5, amplicons (Figure11).

Forward primer (24 mer) :5'-CCAGGCTGAGAAGGAGAGGAAGCT -3'

Reverse primer (20mer) : 5'- CGGAAGCCGCGCCAGAACCA -3'

From the results it appears that multicomponent phenol hydroxylase gene complex contains genes in the range of 600 to 1500 bp sizes (Figure11).

Identification of bacterial strains: On the basis of various morphological, physiological, cultural and biochemical characteristics and by referring Bergey's manual of determinative bacteriology 9thedi. 1994 and Bergey's manual of Systematic bacteriology vol Two Part C 2ndedi. 2004 ; strain A2 was identified as member of *Rhodococcus* genus (most probably *Rhodococcus coprophilus*) and strain J3 was identified as member of *Achromobacter* genus (most probably *Achromobacter xylosoxidans sub-sp. denitrificans*).

Although monochlorophenols are xenobiotic in nature, the bacterial strains degrading them without employing genetic manipulation methods were found in the soil, which suggests that several degrading strains have already evolved in the nature and can be exploited for our benefit.The unique feature of the Strain A-2 was that it could mineralize all the three monochlorophenols. The degradation of three isomers by strain A2 occurred in the following order: 4-CP > 3-CP > 2-CP. Contrary to the findings of the several studies (Beltrame *et al.*, 1988; Kirk-Othmer, 1979) the 3-chlorophenol which has been reported to be the most resistant amongst the three isomers; it was 2-CP which exhibited highest resistance in the biodegradation experiments conducted with

Strain A-2. Meta pathway has been reported to result in incomplete metabolism due to production of dead end or suicide metabolites (Schmidt *et al.*, 1983; Klecka and Gibson, 1981; Bartels *et al.* 1984).In our study as both the strains followed the ortho pathway for the monochlorophenol degradation along with stoichiometric release of chloride ions, suggests the complete degradation of the substrates. As plasmid was isolated from strain A2, it is possible that the chlorophenol degradation may be plasmid mediated however it could not be confirmed in our laboratory. Further study in this direction is needed. It was strange to note that strain J3 could not metabolize phenol but metabolized its chlorinated structural analogue 2-CP. Similar type of findings have been reported for 5-chlorosalicylate (Crawford *et al.*, 1979), 2-6 dichlorotoluene (Vandenbergh *et al.*, 1981).The reason for such type of behavior in the organisms is still not clear.When the genome isolated from the strain A2 wassubjected to amplification in the PCR in presence of gene specific primers for the largest subunit of the multicomponent phenol hydroxylase gene, several PCR amplicons were obtained, which could be sequenced and studied further for expression and other aspects.

Table 1: Morphological, biochemical and cultural characteristics of Strain A-2 and Strain J-3

S.No.	Characteristics	Strain A2	Strain J3
Morphological and cultural characteristics			
1.	Shape	Pleomorphic[a]	Rods
2.	Gram staining	+	-
3.	Acid fast staining#	+	-
4.	Pigmentation on Nutrient Agar	+[b]	-
5.	Motility	-	+
6.	Major pigment	+	-
7.	Growth		
	at 33°c	+	+
	at 37° c	+	+
	at 41° c	+	+
	at 45° c	-	-
8.	Growth rate	Slow[c]	Normal
9.	Colony characteristics	Moderately dry	Moist[d]
Acid production			
10.	D-Glucose in OF medium	-	-
11.	D-Xylose in OF medium	+	-
Other biochemical tests			
12.	Ring fission[e]	Ortho	Ortho
13.	Phenol (50) +	-	
14.	Starch hydrolysis	--	
15.	Gelatin hydrolysis	--	
16.	Tween 80 hydrolysis	--	
17.	Oxidase test	-	+
18.	Catalase	+	+

19.	Nitrite reduction	-	-
20.	Mannose	+	-
21.	Mannitol	?	-
22.	Fructose	+	-
23.	β- hydroxybutyrate	--	
24.	DL- Alanine+	+	
25.	DL- 2 Aminobutyric acid	--	
26.	L- Arginine HCl	+	+
27.	DL-Aspartic acid	+	+
28.	L-Cysteine HCl	+	+
29.	Cystine	+	+
30.	L-Glutamic acid	+	+
31.	Glycine	+	+
32.	L-HistidineHCl	-	+
33.	DL- Isoleucine	+	+
34.	DL- nor Leucine	+	+
35.	L-Leucine	+	+
36.	L-Lysine	-+	
37.	DL-Methionine	?	-
38.	DL- β Phenylalanine	-	+
39.	L-Proline	+	+
40.	DL-Serine +	+	
41.	DL-Threonine	+	+
42.	DL-Tryptophane	+	+
43.	L-Tyrosine -	+	
44.	DL-Valine	+	+
45. ONPG		-	-
46. Lysine decarboxylase		-	-
47. Ornithine		-	-
48. Urease		-	-
49. Phenylalanine deamination		-	-
50. Nitrate reduction		+	+
51. H_2S production		+	-
52. Citrate utilization		+/-	+
53. VogesProskauer test		-	-
54. Methyl red test		-	-
55. Indole test		-	-
56. Malonate		+	+
57.	Esculin	-	-
58.	Arabinose -		-
59.	Xylose	-	-
60.	Adonitol	-	-
61.	Rhamnose	-	-
62.	Cellobiose	-	-
63.	Melibiose	-	-
64.	Saccharose	-	-
65.	Raffinose	-	-
66.	Trehalose	-	-

67.	Glucose	-	-
68.	Lactose	-	-
69.	Succinic acid (50)	+	+
70.	Salicylic acid (50)	-	-

a – Small rods (pleomorphic in nature) as well as cocci are seen
b - Colonies show brick red colour pigmentation at Nutrient agar at 33° C. Intensity of the pigmentation goes on increasing and reaches its peak on 7th day.
c - Very small colonies form after 24 hrs and somewhat dry in nature.
d - Colonies moist and creamy. While picking up old (> 10 days) colonies with needle, string formation to some extent seen.
e - Ring fission test was performed as per the procedure given in Bergey's Manual of Systematic Bacteriology vol I (1984) p.159
- Modified acid fast staining was performed (i.e. 1% sulphuric acid was used as decolorizing agent) (Refer Bailey and Scott's Diagnostic microbiology 12thedi. Elsevier publication).
? - Results could not be confirmed.
Number in the parenthesis indicates initial concentration (mg/L) of substrate, while substrates with no parenthesis and number had initial concentration of 100 mg/L.

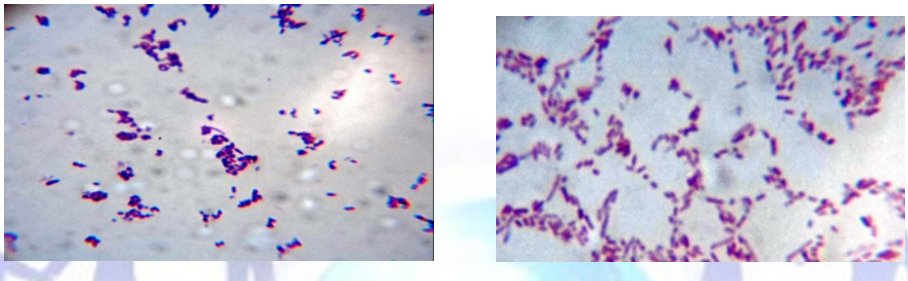

Figure 1: Slide of strain A2 (left) showing gm+ve pleomorphic rods and cocci. Slide of strain J3(right) showing gm-ve rods.

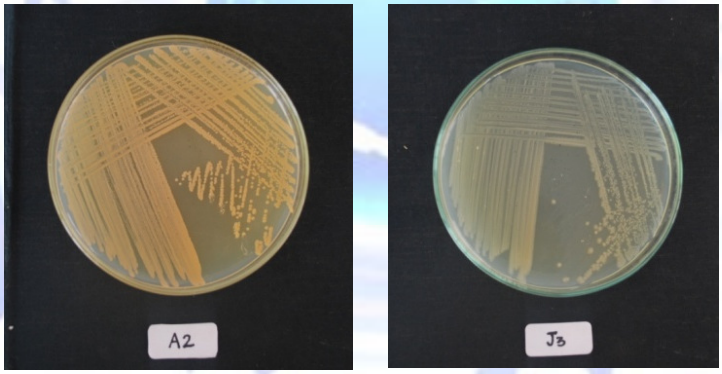

Figure 2: Photographs showing colony characteristics of Strain A2 and strain J3 on Nutrient agar plates after 4 days incubation

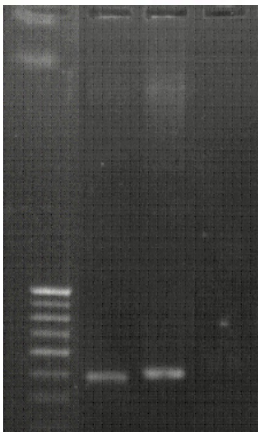

Figure 3: Gel dock picture of amplified products after gel electrophoresis: Left to right lane: control genome ladder of 1kb bp, amplified 16S rDNA of strain A2, amplified 16S rDNA of strain J3 and the control sample with no genomic material.

GCTGTCCTGCTTCGTGTTTGATCATGGCTCAGGTTCGGACACCTCCTTAGTAGTTGGGTGGATTAGTGGCGAACGGGTGAG
TAACACGTGGGTGATCTGCCCTGCACTCTGGGATAAGCCTGGGAAACTGGGTCTAATACCGGATATGACCTCGGGATGCAT
GTCCTGGGGTGGAAAGTTTTTCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCG
ACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTG
GGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTC
AGCAGGGACGAAGCGCAAGTGACGGTACCTGCAGAAGAAGCACCGGCCAACTACGTGCCAGCAGCCGCGGTAATACGTAGG
GTGCGAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCGTCTGTGAAATCCCGCAGCTCAA
CTGCGGGCTTGCAGGCGATACGGGCAGACTCAAGTACTGCAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAG
ATATCAGGAGGAACACCGGTGGCGAAGGCGGGTCTCTGGGCAGTAACTGACGCTGAGGAGCGAAAGCGTGGGTAGCGAACA
GGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCGCTAGGTGTGGGTTTCCTTCCACGGGATCCGTGCCGTAGCC
AACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCG
GAGCATGTGGATTAATTCGATGCAACGCGAAGAACCTTACCTGGGTTTGACATGTACCGGACGACCGCAGAGATGTGGTTT
CCCTTGTGGCCGGTAGACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCG
CAACCCTTGTCCTGTGTTGCCAGCACGTGATGGTGGGGACTCGCAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGA
CGACGTCAAGTCATCATGCCCCTTATGTCCAGGGCTTCACACATGCTACAATGGTCGGTACAGAGGGCTGCGATACCGTGA
GGTGGAGCGAATCCCTTAAAGCCGGTCTCAGTTCGGATCGGGGTTTGCAACTCGACCCCGTGAAGTCGGAGTCGCTAGTAA
TCGCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCATGAAAGTCGGTAACACC
CGAAGCCGGTGGCCTAACCCCTCGTGGGAGGGAGCCCTCCAAGGAGGGATAGGCGGATGGGAGGAGGAACACCTCCATAGA
GTTGACGCAG

Figure 4: Aligned sequence data (1468bp) of strain A2

ID	Alignment results	Sequence Description
A2	1.00	Studied Sample
AF420423	0.94	*Rhodococcus sp. ARG-BN062*
EF990683	0.96	*Rhodococcus sp. A2*
AY771328	0.93	*Gordonia rubripertincta st. ATCC 21930*
GU191923	0.94	*Rhodococcus pyridinivorans st. SB 3094*
FJ468342	0.94	*Rhodococcus rhodochrous st. DSM 43241*
AF459741	0.93	*Rhodococcus pyridinivorans st. R04*
NR_025730	0.95	*Rhodococcus gordoniae st.W4937*
NR_029206	0.92	*Rhodococcus coprophilus st. CUB 687*
AM933579	0.92	*Rhodococcus phenolicus DSM 44812T*
AF191343	0.92	*Rhodococcus zopfii*

Figure 5: Alignment results of strain A2 using combination of NCBI genebank and RDP database

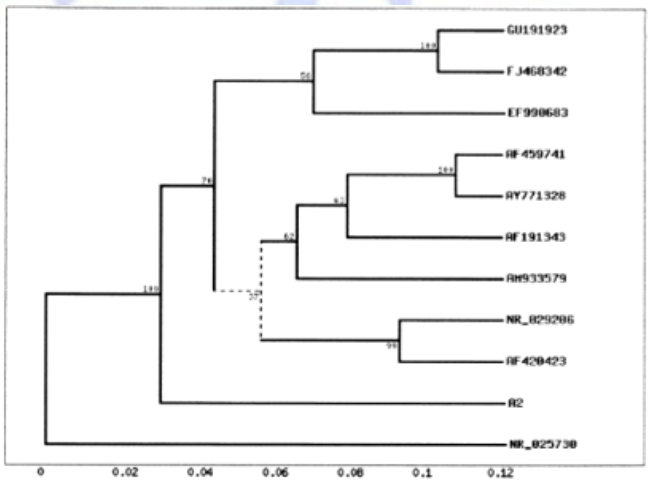

Figure 6: Phylogenetic tree in relation to strain A2 made in MEGA 3.1 software using neighbor joining method

GCTGACGCTCGCGGGATGCCTTGCACATGCAAGTCGAACGGCAGCACGGACTTCGGTCTGGTGGCGAGTGGCGAACGGGTG
AGTAATGTATCGGAACGTGCCCAGTAGCGGGGGATAACTACGCGAAAGCGTAGCTAATACCGCATACGCCCTACGGGGGAA
AGCAGGGGATCGCAAGACCTTGCACTATTGGAGCGGCCGATATCGGATTAGCTAGTTGGTGGGGTAACGGCTCACCAAGGC
GACGATCCGTAGCTGGTTTGAGAGGACGACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGT
GGGGAATTTTGGACAATGGGGGAAACCCTGATCCAGCCATCCCGCGTGTGCGATGAAGGCCTTCGGGTTGTAAAGCACTTT
TGGCAGGAAAGAAACGTCGCGGGTTAATACCTCGCGGAACTGACGGTACCTGCAGAATAAGCACCGGCTAACTACGTGCCA
GCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTCGGAAAGAAAG
ATGTGAAATCCCAGAGCTTAACTTTGGAACTGCATTTTTAACTACCGGGCTAGAGTGTGTCAGAGGGAGGTGGAATTCCGC
GTGTAGCAGTGAAATGCGTAGATATGCGGAGGAACACCGATGGCGAAGGCAGCCTCCTGGGATAACACTGACGCTCATGCA
CGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCAACTAGCTGTTGGGGCCTTCG
GGCCTTGGTAGCGCAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGG
GGACCCGCACAAGCGGTGGATGATGTGGATTAATTCGATGCAACGCGAAAAACCTTACCTACCCTTGACATGTCTGGAATC
CCGAAGAGATTTGGGAGTGCTCGCAAGAGAACCGGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTT
GGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCATTAGTTGCTACGAAAGGGCACTCTAATGAGACTGCCGGTGACAAACC
GGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTATGGGTAGGGCTTCACACGTCATACAATGGTCGGGACAGAGG
GTCGCCAACCCGCGAGGGGGAGCCAATCCCAGAAACCCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGT
CGGAATCGCTAGTAATCGCGGATCAGCATGTCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGG
GAGTGGGTTTTACCAGAAGTAGTTAGCCTAACCGTAAGGAGGGCGATTACCACGGTAGGATTCATGACTGGGGGAAGTCGT
AACAAGTGGGCTTATCGCAG

Figure 7: Aligned sequence data (1478bp) of strain J3

ID	Alignment results	Sequence Description
J3	1.00	Studied Sample
FJ919599	0.99	*Alcaligenes sp. DG-5*
DQ450530	0.98	*Alcaligenaceae bacterium LBM*
EU727196	0.98	*Achromobacter sp. MT-E3*
AF411020	0.98	*Achromobacter xylosoxidans st.AU1011*
EU214611	0.98	*Achromobacter xylosoxidans isolate ybb5*
EU221379	0.98	*Achromobacter insolitus st. Y2P1*
Y14907	0.98	*Alcaligenes denitrificans*
AJ002802	0.98	*Alcaligenes sp. isolate 151*
FJ810080	0.98	*Achromobacter denitrificans st.22426*
AB161691	0.98	*Achromobacter xylosoxidans st. NFRI-A1*

Figure 8: Alignment results of strain J3 using combination of NCBI gene bankand RDP database

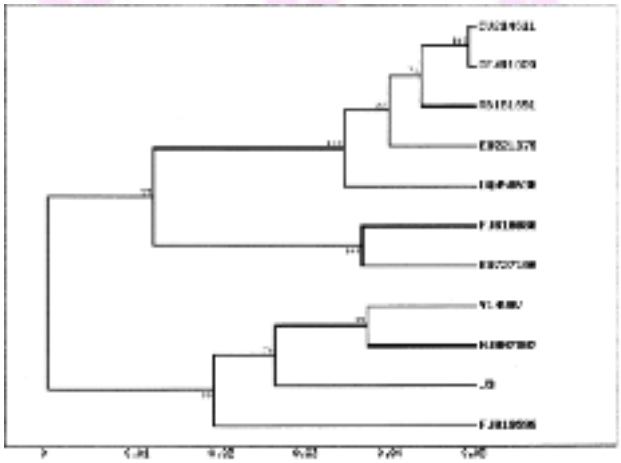

Figure 9: Phylogenetic tree in relation to strain J3 made in MEGA 3.1 software using neighbor joining method

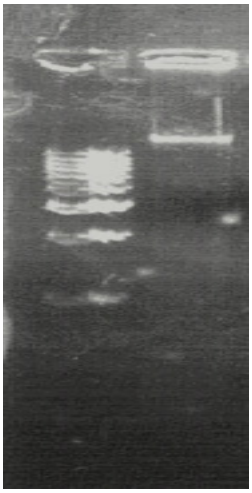

Figure 10: Lane 1 (on left)- 1 kb DNA Ladder (1000-10000 bp);Lane 2 (on

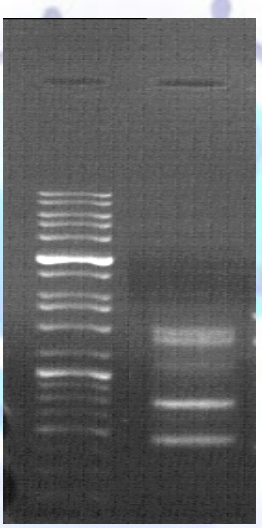

Figure 11: Lane 1(on left)- High range DNA Ladder (100 to 10,000 bp ladder) Lane 2(on right)- Amplified phenol hydroxylase gene complex of strain A2 (600 to 1500 bp).

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