

ISOLATION, IDENTIFICATION & PHYLOGENETIC ANALYSIS OF PHTHALIC ACID DEGRADING BACTERIA.

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ABSTRACT:

Phthalic acid is a precursor used in the manufacture of phthalate esters, which are used principally as plasticizers in plasticized polyvinyl chloride preparation. Phthalate esters make 40% of weight of some plastic materials. Phthalates are commonly found in child rearing products, blood bags, dialysis tubing, paints, lacquers, cosmetics, coating of capsules etc. Phthalates have been shown to be nervous system depressant and stimulators, teratogenic and estrogenic mimics. In the present study, five bacterial strains degrading phthalic acid were isolated using mineral salt media containing phthalic acid as a sole source of carbon. The two isolates showing effective degradation of phthalic acid were tested for the growth and tolerance limit on different concentrations (50- 3100 ppm) of phthalic acid in mineral salt medium with and without glucose & were used for further study. These were identified as *Pseudomonas aeruginosa* strain HNYM41 and *Bacillus cereus* strain BVC11.

Keywords: Phthalic acid, Degradation, *Pseudomonas*, *Bacillus*.

INTRODUCTION:

Bioremediation exploits the ability of microorganisms to degrade and detoxify organic contamination. It has been an efficient, economic, versatile and environmentally sound biological treatment method [5]. Phthalates belong to family of chemical compounds which are based on benzene ring, to which is attached a pair of carbonyl groups in consecutive positions on the benzene ring [2]. Phthalic acid is a precursor used in the manufacture of phthalate esters, which are used principally as plasticizers of vinyl products. Phthalate esters, which can make up 40% of the weight of some plastic materials, are produced in excess of 10^{10} lb (4.54×10^{12} g) per annum. Besides polyvinyl

chloride phthalates are often used in paints lacquers and cosmetics, nutritional supplements to viscosity control agents, gelling agents, film formers, stabilizers, dispersants, lubricants, binders, emulsifying agents and suspending agents. End applications include adhesives and glues, electronics, agricultural adjuvant, building materials, personal-care products, medical devices, detergents and surfactants, packaging, printing inks and coatings, pharmaceuticals, food products, and textiles. [6]. Phthalates are found in sediments, water and air [4] and have also been detected in foods as they can migrate out of food packaging materials [9] [10]. Low molecular weight phthalates such as Diethyl phthalate (DEP), Di-n-butyl phthalate (DBP),

may be dermally absorbed. Body-care products containing phthalates are a source of exposure for infants [7]. Phthalates have been shown to be both nervous system depressants and stimulator, teratogenic and estrogenic mimics [3].

MATERIALS AND METHODS:

Collection of soil samples:

The soil samples were collected from garbage area near ground and boys hostel of New Arts, Commerce and Science College, Ahmednagar, Maharashtra, India.

Isolation of phthalic acid degrading bacteria:

Isolation of Phthalic acid degrading microorganisms was done by inoculating the soil samples on mineral salt agar plates, containing Phthalic acid as a sole carbon and energy source. [8]. Five bacterial strains degrading phthalic acid were isolated using mineral salt media. The two isolates showing effective degradation of phthalic acid were tested for the growth and tolerance limit on different concentrations (50-3100 ppm) of phthalic acid in mineral salt medium(MSM) with and without glucose. Bacterial growth was measured by taking absorbance at 570nm [8] & were used for further study.

Identification of isolates:

For identification the morphological characterizations of both the isolates were done by observing colony characteristics and Gram character. The biochemical characterization included reactions like sugar fermentation, catalase and oxidase production was done according to the Bergey's Manual of Determinative Bacteriology[1].Molecular characterization included partial sequencing of 16s r- RNA genes of the isolates. For the same, the isolates were sent to NCCS (National Centre for Cell Sciences), university of Pune, Pune.

Sugar Fermentation test:

Fermentation of different sugars by the isolates was carried out by inoculating the organism in the Sterile peptone water base supplemented with, 1% of the required sugars (Glucose, Sucrose, Lactose, Maltose, Fructose) containing

phenol red as indicator and an inverted Durham's tube. After incubation for 24 hours at 37°C, the change in the colour of the medium from red to yellow was recorded as positive for acid production and formation gas bubble in Durham's tube indicated the formation of gas.

Tests for different enzymes:

The ability of organism to produce different enzymes like catalase, oxidase was checked.

Catalase: Hydrogen peroxide solution was taken in the tubes and single well isolated colony was immersed in to the tube and observed for effervescence.

Oxidase: Filter paper strip dipped in N,N,N,N tetra-methyl para-phenylene di-amine dihydrochloride reagent was smeared with single colony and observed for colour change.

Nitrate reduction:

In sterile nitrate reduction broth (5ml), one loop full of overnight grown suspension of the isolate was inoculated and kept for incubation for 5 days. After 5 days incubation test reagent 0.1ml was added and observed for formation of red colouration.

Test reagent:

Solution A:- Dissolve 8.0 gm of sulphanilic acid in 1 litre of acetic acid (5 mol/litre).

Solution B:- Dissolve 5.0 gm of alpha naphthalamine in 1 litre of acetic acid (5mol/ litre).

Immediately before use, mix equal volumes of Solution A and B to give the test reagent.

H₂S Production:

The test organism was streaked and stabbed on sterile TSI agar slant and then incubated for 24 hours at 37°C and observed for color change of slant and butt and formation of black color indicating H₂S gas production.

Indole Test-

The test organism was inoculated (0.1ml) in 1% tryptone broth. After incubation at 37°C for 24 hours, then Xylene was added followed by addition of Kovac's reagent. Development of Pink coloured ring on the surface indicates a positive test.

Methyl Red Test-

The test organism was inoculated (0.1ml) in Glucose phosphate peptone buffered broth. After incubation at 37°C for 24 hours, methyl red indicator was added. Development of red colour indicates a positive test.

Voges Proskauer Test-

The test organism was inoculated (0.1ml) in Glucose phosphate peptone buffered broth. After incubation at 37°C for 24 hours, 5% Alpha naphthol (as colour intensifier) and 40% KOH (as oxidizing agent) were added. Development of red colour indicates a positive test.

Citrate Utilization Test-

The test organism was streaked on Simon Citrate Agar slant and incubated at 37°C for 24 hours. Change in colour of slant from blue to green indicates a positive test.

The morphological characterization of the isolates was done by observing colony characteristics and Gram's staining character.

Study growth & tolerance limit on different concentrations of phthalic acid:

The growth & tolerance limit of phthalic acid on isolates were determined by observing the growth of the isolates in the MSM flask containing glucose at concentration of 5mM and Phthalic acid ranging from 50 to 3100 ppm.

Phylogenetic analysis of 16S r- RNA sequence of both the isolates-

Phylogenetic analysis of 16S r-RNA sequence of both the isolates was done using BLAST and Clustal-X which was as follows: Phylogenetic analysis is the analysis of groups of sequences that form gene families, requires the ability to make connections between more than two members of the group in order to reveal subtle conserved family characteristics.

Sequence analysis using BLAST:

Similarities between sequences can be studied using methods such as dot plot method & dynamic programming algorithms such as Needleman – Wunsch algorithm and the Smith – Waterman algorithm and word or k- tuple methods such as used by FASTA & BLAST programs.

The BLAST algorithm was developed as a new way to perform a sequence similarity search. A powerful computer system dedicated to running BLAST has been established as NCBI, National Library of Medicine. Access to this BLAST system is possible through the internet as a website and through a BLAST e-mail server.

Multiple sequence alignment & phylogenetic analysis using Clustal-X:

A multiple sequence alignment (MSA) is a sequence alignment of 3 or more biological sequence, generally protein, DNA or RNA. In many cases the input set of query sequences are assumed to have an evolutionary relationship by which they share a lineage and are descended from a common ancestor. From the resulting MSA, sequence homology can be inferred and phylogenetic analysis can be conducted to assess the sequence shared evolutionary origins.

RESULTS AND DISCUSSION:**Isolation of phthalic acid degrading bacteria:**

In present study the soil samples were taken from different areas. Initially in primary screening five isolates were obtained on MSM agar plates with phthalic acid as sole source of carbon and energy. Out of five isolates two isolates Phthalic acid degrading (PAD)-1 & (PAD)-2 showed a good growth up to 2000 & 3100 ppm of phthalic acid with glucose and up to 1500 & 3000 ppm without glucose respectively.

Molecular characterization:

Partial sequencing of 16s r- RNA genes of both the isolates PAD-1 & PAD-2 was done from NCCS (National Centre for Cell Sciences), university of Pune, Pune. The bacterial strains PAD-1 & PAD-2 were identified as *Pseudomonas aeruginosa* strain HNYM41 and *Bacillus cereus* strain BVC11 respectively, that showed best growth in MSM broth contains different concentrations of the Phthalic acid were selected for further transformational studies.

DISCUSSION:-

In the present study soil samples were taken from different locations and checked for the presence of phthalic acid degrading

microorganisms. The results suggest that soil contains both Gram positive and Gram negative bacteria, with the ability to utilize phthalic acid as a nutrient source. The isolated bacterial strains were adapted to increasing concentrations of phthalic acid. The tolerance limit in mineral salt medium broth (with and without glucose) containing phthalic acid was found to be (50-3100) ppm. The bacterial strain that showed that highest tolerance limit and degradation was identified as *Bacillus cereus* strain BVC11. The screening of isolates yielded five isolates which were good degraders of phthalic acid. Among the five isolates two of them showed promising degradation of phthalic acid in MSM liquid medium. The degradation was determined spectrophotometrically at 230 nm [8].

Degradation studies were carried out in minimal salt medium and it was found that the organism grew well in MSM with the addition of 100mM of Phthalic acid as sole carbon source reaching maximum growth at 48 hours. Glucose grown cells also oxidized phthalic acid suggesting the constitutive nature of enzymes, it was found that *Pseudomonas* strains could utilize phthalate as carbon source [8].

Pseudomonas and *Bacillus* are the mesophilic microbes that require certain range of temperature to obtain its maximum growth and to perform its metabolic activities. The isolates used to transform phthalic acid were able to degrade the test compound at 37°C as maximum degradation was observed at this temperature.

The ability of the bacteria to metabolize the target chemical whose degradation is in need, in the presence of these alternative compounds, gives promising hope for the pollution free environment. Our studies show that addition of glucose in the mineral salt medium have significant effect on the growth of PAD-1, but not on the PAD-2.

The 16S r-RNA sequences of both the isolates were analyzed using bioinformatics tools like BLAST and Clustal- X for their phylogenetic analysis. It was observed that PAD-1 was sharing distinct relationship with *Pseudomonas aeruginosa* strain F23 (JQ579643.1). While,

PAD-2 was closely related to *Bacillus cereus* (JQ 518346.1) and they are sharing common ancestor.

Conclusions:

The new bacterial strains with the capability of utilizing phthalic acid as the sole carbon source were isolated, identified and characterized. The adaptation of the microorganisms to grow on the increasing concentrations of phthalic acid shows the potential of the isolates to be used in bioremediation in the environment that might be polluted with a variety of compounds in variable concentrations. The 16S r-RNA sequences of both the isolates were analyzed using bioinformatics tools like BLAST and Clustal-X for their phylogenetic analysis. It was observed that PAD-1 was sharing distinct relationship with *Pseudomonas aeruginosa* strain F23 (JQ579643.1). While, PAD-2 was closely related to *Bacillus cereus* (JQ518346.1) and they are sharing common ancestor.

The growth & tolerance limit of bacterial isolates was observed in MSM media supplemented with different concentrations of Phthalic acid ranging from 50-31000 ppm.

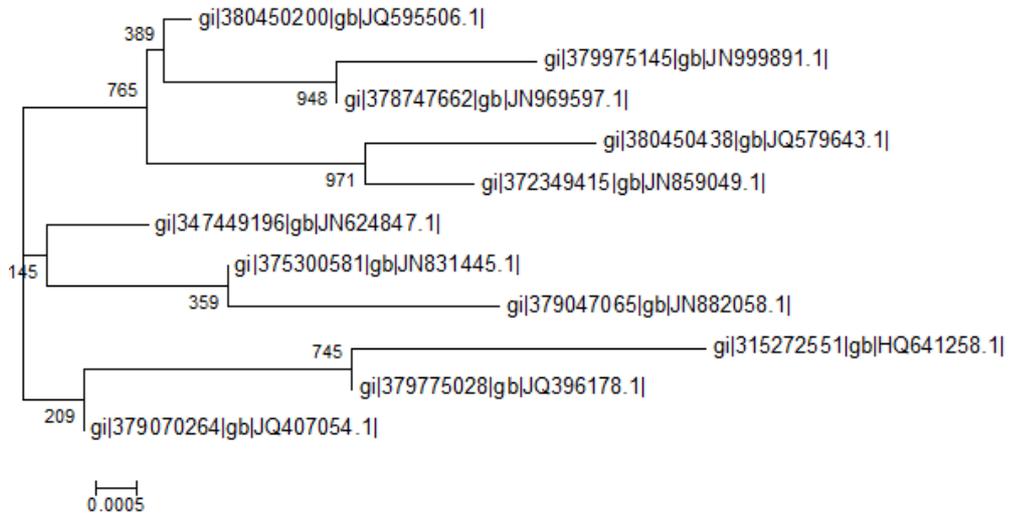
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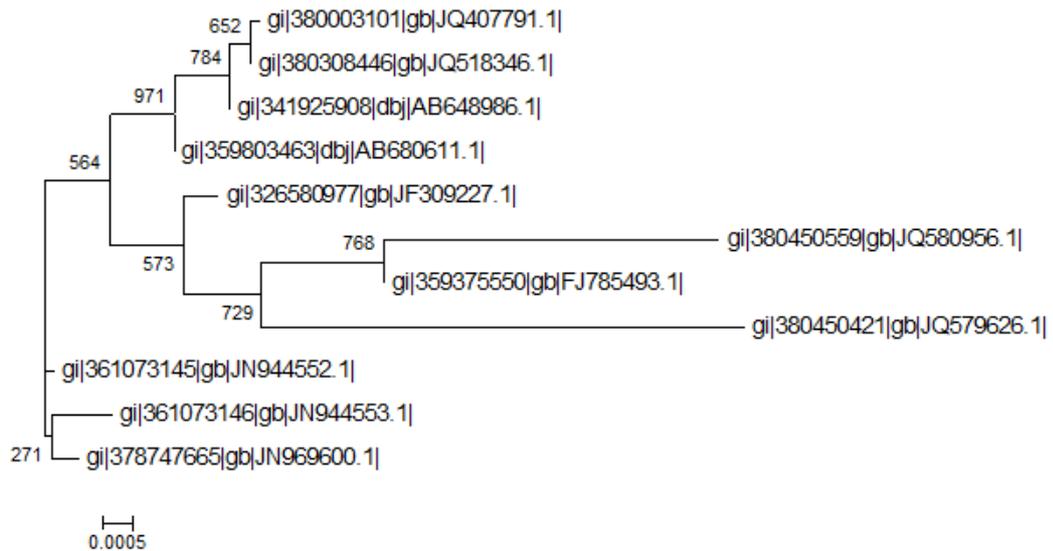
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Phylogenetic tree obtained by Clustal -X:



Phylogenetic tree of PAD-1



Phylogenetic tree of PAD-2

Table: 1 Biochemical characterization of PAD-1 & PAD-2

Biochemical test	PAD-1			PAD-2				
	Acid	Gas		Acid	Gas			
Sugar fermentation								
Glucose	+	+		-	-			
Maltose	+	+		+	+			
Fructose	+	+		+	-			
Lactose	-	-		-	-			
Sucrose	-	-		+	+			
Indole	-			-				
Methyl Red	-			-				
Voges-Proskaur Test	-			-				
Citrate Utilization	+			+				
Nitrate reduction test	+			-				
Catalase	+			+				
Oxidase	+			-				
TSI test	Slant	Butt	Gas	H ₂ S	Slant	Butt	Gas	H ₂ S
	Alkaline	Acidic	+	-	Alkaline	Acidic	-	-

Table 2: Growth of PAD-1 in MSM broth

	Concentration of Phthalic acid (ppm)							
	50	100	200	500	1000	1500	2000	2500
Without glucose	++	+++	+++	++	++	++	-	-
With glucose	++	+++	+++	+++	++	++	+	-

+++ = Rich growth, ++ = Good growth, + = Slight growth, - = No growth.

Table 3: Growth of PAD-2 in MSM broth

	Concentration of Phthalic acid (ppm)							
	50	100	200	500	1000	1500	2000	2500
Without glucose	++	+++	+++	++	++	++	-	-
With glucose	++	+++	+++	+++	++	++	+	-

+++ = Rich growth, ++ = Good growth, + = Slight growth, - = No growth.