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ISOLATION OF PHTHALIC ACID DEGRADING *PSEUDOMONAS* SP. P1 FROM SOIL

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Abstract

Phthalates are common plasticizers used in *p*PVC (plasticized polyvinyl chloride) products. Phthalates are commonly found in the child rearing products (for teething, toys etc.), blood bags, dialysis tubing, paints, lacquers, cosmetics, coatings of capsules etc. The present study was conducted to explore the potential of new microbial strains for the biodegradation and biotransformation of phthalic acid. A bacterial isolate, *Pseudomonas* sp. P1, was found to degrade phthalic acid in agar plate assay as evident by the formation of clear zone around the colony. The strain was tested for the growth and tolerance limit on different concentrations (10- 3000 ppm) of phthalic acid in mineral salt medium with and without glucose. On mineral salt agar plates, containing phthalic acid as a sole source of carbon, rich growth along with the hydrolyzing zone was observed upto the maximum concentration of 2800 ppm without glucose and upto the maximum concentration of 2900 ppm of phthalic acid with glucose. Transformational studies were carried out in mineral salt liquid medium containing varying concentrations (100- 500 ppm) of phthalic acid. Microbial growth was checked at 570 nm at different time intervals and the cell free supernatant was analyzed for the disappearance of phthalic acid at 280 nm. The highest percentage of degradation of phthalic acid was found at 37°C and pH 8, i.e. 59% and 64% respectively. In mineral salt medium without glucose, phthalic acid degraded up to 72% at 500 ppm after 48 hours of incubation.

Introduction

Since 1930s plasticizers have been used to impart flexibility to an otherwise rigid polyvinylchloride (PVC). Besides PVC phthalates are used often in paints, lacquers and cosmetics (Harris *et al*., 1997; Niazi *et al*., 2001). Phthalates found in sediment, water and air (Fatoki *et al*., 1990) has also been detected in foods as they can migrate out of food packaging materials (Peterson *et al*., 1991; Sharman *et al*., 1994). Worldwide production of phthalate ester was estimated in 1993 to be 2.4 million metric tons per year. Effluents from the plastic and textile industries contain high concentrations of phthalates, which are listed as Priority Pollutants by the US Environmental Protection Agency (Kieth & Telliard, 1979).

Phthalates belong to a family of chemical compounds which are based on a benzene ring, to which is attached a pair of carbonyl groups in consecutive positions on the benzene ring. Representative examples of plasticizer in the phthalic acid group include dioctyl phthlate (DOP or DEHP), diisononyl phthalate (DINP) and diisodecyl phthalate (DIDP).

Phthalate (benzene-1, 2- dicarboxylate) is a central intermediate in the bacterial degradation of phthalate esters (Ribbons *et al.*, 1984) as well as of certain fused ring polycyclic aromatic hydrocarbons found in fossil fuels (Ribbons & Eaton, 1982) including phenanthrene (Kiyohara & Nagho, 1978), Fluorene (Grifoll *et al.*, 1994) and Fluoranthene (Sepic *et al.*, 1998).

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Due to wide spread use of phthalate there has been great concern about their release into the environment (Giam *et al.,* 1978; Mayer *et al.,* 1972) and their toxicity on human beings and other organisms (WoodWard, 1990). Phthalates have been shown to be both nervous system depressants and stimulators, teratogenic and estrogenic mimics (Ema *et al*., 1998). The present study was aimed to isolate the bacteria, having the ability to degrade phthalic acid and to study the effect of pH, temperature and presence or absence of glucose on biodegradation of Phthalic acid.

Material and Methods

Growth media, isolation and cultivation of bacterial strains: 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. The isolates, which showed clear zones of hydrolysis around their colonies were selected for further studies. The isolates were identified and characterized according to the Bergey's Manual of Determinative Bacteriology (Holt, 1993).

Phthalic acid degrading microorganisms were isolated from soil by inoculating on mineral salt medium (MSM) (g/l): (K_2HPO_4 1, KH_2PO_4 0.2, NaCl 1, $CaCl_2$ 0.01, $MgSO_4$. $7H_2O$ 0.5, $FeSO_4$. $6H_2O$ 0.01, $CuSO_4$. $5H_2O$ 0.001, $ZnSO_4$. $7H_2O$, 0.001, $MnSO_4$. H_2O 0.001, Agar 15, pH 7.0) (modified from Nishida & Tokiwa, 1993), 5mM glucose supplemented with 10ppm of Phthalic acid. Bacterial growth was measured by taking absorbance at 570nm.

The bacterial strain, identified as *Pseudomonas* sp. P1, that showed best growth and also maximum hydrolyzing zone on different concentrations of the Phthalic acid (with and without glucose) were selected for further transformational studies.

Optimization of culture conditions for biodegradation of Phthalic acid: The *Pseudomonas* sp., P1 was grown in mineral salt liquid medium (MSM) (with and without glucose, 5mM) containing 100 ppm of Phthalic acid. The optimum conditions such as initial pH (4-10), incubation temperature (27, 37, 47˚C), required for the biodegradation of Phthalic acid by Pseudomonas sp. P1, were investigated. Samples were taken after every 24 hours (0-96 hours). Growth was monitored spectrophotometrically at 570 nm and cell free supernatant was analyzed for the disappearance of Phthalic acid at 280nm (Eaton & Ribbons 1982a,b, c). Percentage degradation was also calculated.

Results

Isolation of phthalic acid degrading bacteria: Soil samples were taken from different areas. Initially, 15 colonies grew and were isolated on MSM agar plates with phthalic acid as sole source of carbon and energy. Out of fifteen isolates, a bacterial strain, identified as *Pseudomonas* sp., P1, showed good growth up to 2900 ppm of Phthalic acid with glucose and up to 2800 ppm without glucose (Table 1).

Effect of pH on growth and degradation of phthalic acid by *Pseudomonas* sp. P1.: Degradation of phthalic acid (100ppm) increased by increase in initial pH of the medium. Best growth was observed at pH 8 after 48 hours, with maximum percentage degradation of phthalic acid (64%). whereas, at pH 7, 9 and 10, there was also good growth with percentage degradation as 63, 55, 54 and 44% after 48 hours of incubation (Figs. 1 & 2).

Table 1. Growth of *Pseudomonas* sp. PI on MSM agar plates containing Phthalic acid with and without glucose.

Growth	Concentration of Phthalic acid (ppm)										
	50	100	500	1000	1500	2500	2600	2700	2800	2900	3000
Without glucose	++	+++	+++	+++	+++	+++	+++	++	+	-	-
With glucose	++	+++	+++	+++	+++	+++	+++	+++	++	+	-

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

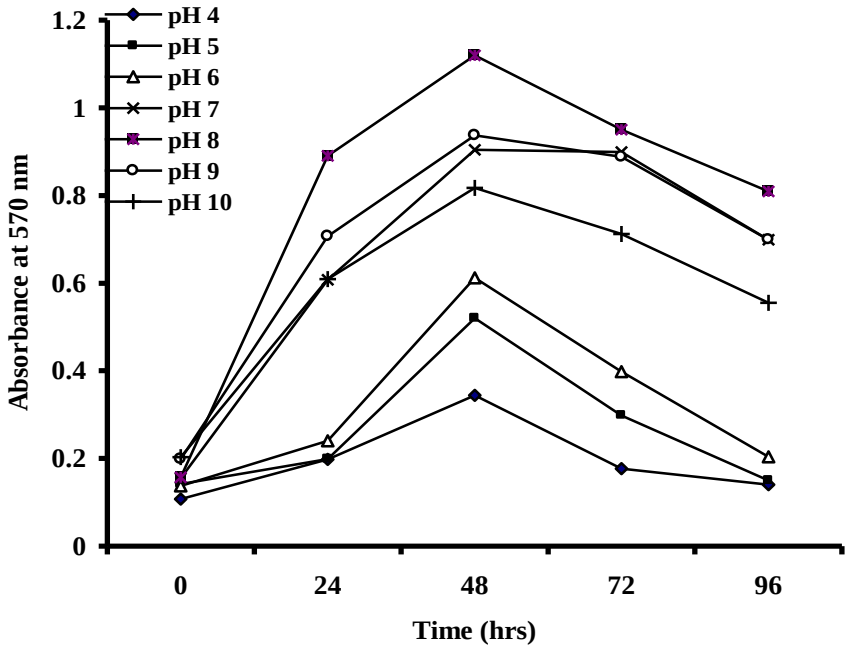

Fig. 1. Effect of pH on growth of *Pseudomonas* sp.,P1 in Phthalic acid at 100 ppm concentration.

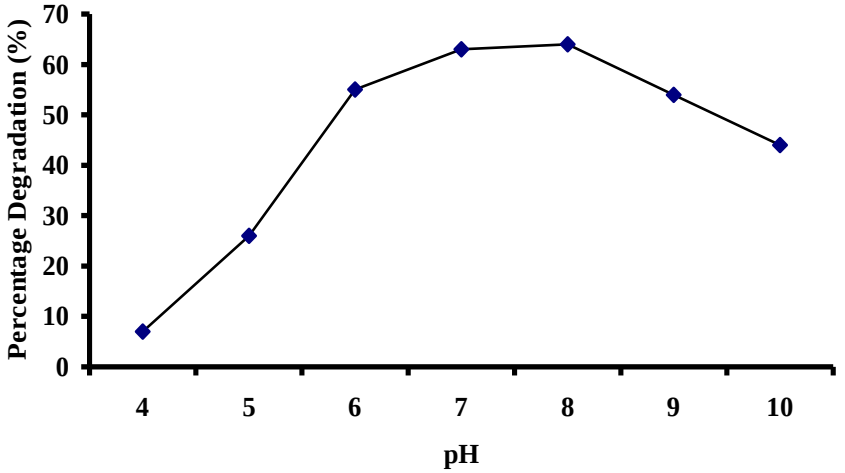

Fig. 2. Effect of pH on degradation of Phthalic acid by *Pseudomonas* sp. P1 at 100 ppm concentration after 48 hours.

Effect of temperature on growth and degradation of phthalic acid by Pseudomonas sp. P1. *Pseudomonas* sp., P1 showed best growth at 37ºC (Fig. 3) with maximum percentage degradation of phthalic acid (59%). Only 14.8% degradation was observed at 27ºC, while no degradation was seen at 47ºC (Fig. 4).

Effect of glucose on growth and degradation of phthalic acid by Pseudomonas sp. P1: Transformation of phthalic acid concentrations (100-500 ppm) was checked by *Pseudomonas* spf. P1 in MSM with and without glucose for 96 hours. In all the cases comparatively good growth was observed in the presence of glucose (5mM). Glucose is utilized as in co-oxidation process if compound is not utilized as sole source then can be degraded by co-oxidation mechanism using glucose as carbon source and donating reducing equivalents. There was no effect of glucose on the biodegradation of phthalic acid. At 100 ppm concentration of phthalic acid, the percentage degradation (76%) was observed both in the presence and absence of glucose whereas 72% degradation was observed at 500 ppm (Fig. 5 & 6).

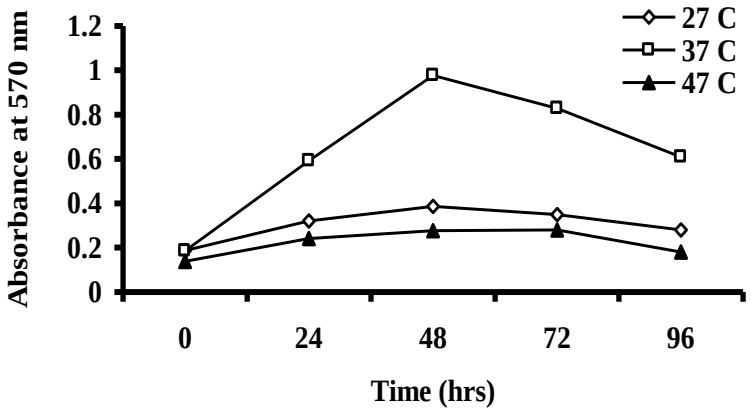

Fig. 3. Effect of temperature on growth of *Pseudomonas* sp., P1 in Phthalic acid at 100 ppm concentration.

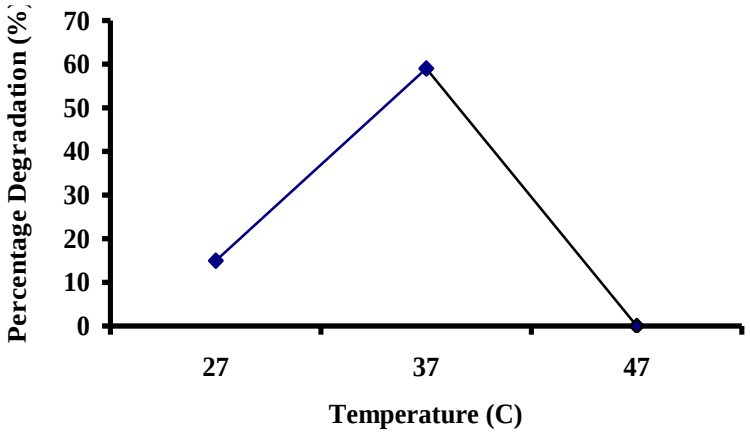

Fig. 4. Effect of temperature on degradation of Phthalic acid by *Pseudomonas* sp., P1 at 100 ppm concentration after 48 hours.

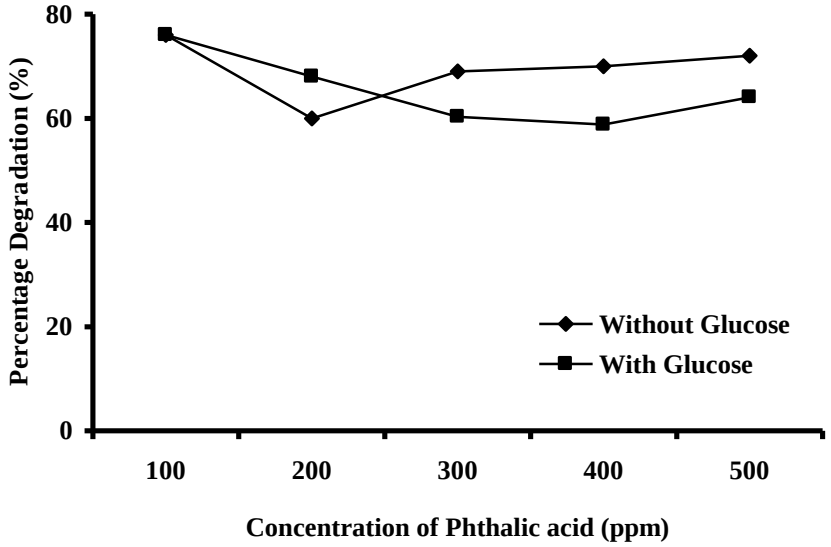

Fig. 5. Effect of Glucose on degradation of Phthalic acid by *Pseudomonas* sp., P1 at different concentrations.

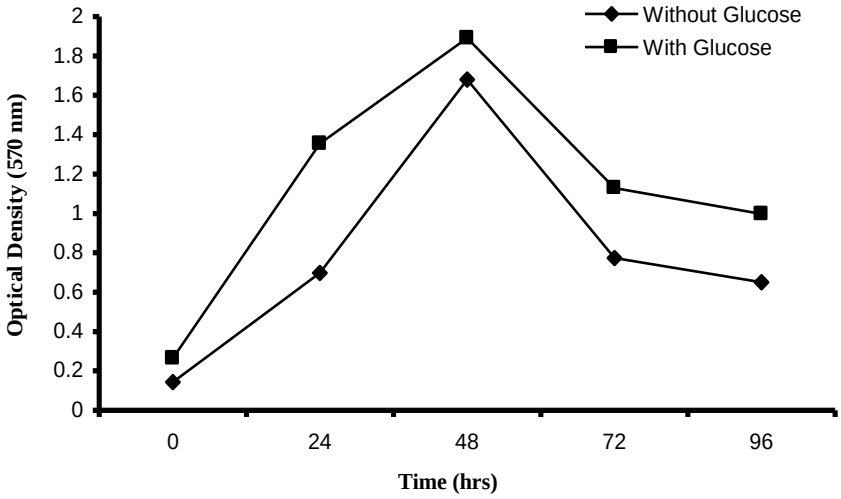

Fig. 6. Effect of Glucose on growth of *Pseudomonas* sp. P1 on 100 ppm of Phthalic acid.

Discussion

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 (Feidieker *et al.*, 1994). In the present study soil samples were taken from different locations and checked for the presence of phthalic acid degrading microorganisms. The results suggested that soil contains microorganisms,

mostly 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 on mineral salt medium (with and without glucose) agar plates containing phthalic acid was found to be 2900 ppm. The bacterial strain that showed that highest tolerance limit and degradation was identified as *Pseudomonas* sp. The first report about the degradation of ortho-phthalic acid was also by a soil *Pseudomonad*. One of several fluorescent *Pseudomonads* were isolated from enrichment cultures using o-phthalate as a sole carbon source (Ribbons & Evans, 1960). Another *Pseudomonad, P. ochraceae* was isolated with the ability to use o-phthalate as a sole carbon source (Maruyama *et al.,* 1978; Nomura *et al.,* 1990).

In the present study percentage degradation of phthalic acid by various microorganisms isolated from soil, was studied in mineral salt medium and was found to be about 72% at 500ppm initial concentrations. Karapagam & Lalithakumari (1999) isolated strain SKP3 capable of utilizing both ortho and para positioned phthalic acid from petroleum–polluted soil samples near Madras Refineries Limited, Madras, India. Degradation studies were carried out in minimal salt medium and it was found that the organism grew well in MSM with the addition of 6mM of Phthalic acid as sole carbon source reaching maximum growth at 30 hours. Glucose grown cells also oxidized phthalic acid suggesting the constitutive nature of enzymes. Stanier *et al.,* (1966) found that only one of 175 fluorescent *Pseudomonas* strains could utilize phthalate as carbon source. Phthalate degradation by *Burkholderia cepacia* DBO1 (ATCC 29424) has been well studied at both the genetic and biochemical levels (Battie *et al.,* 1987; Correl *et al.,* 1992; Gassner *et al.,* 1993). *Burkhoderia cepacia* DBO1 was originally isolated in Florida for its ability to utilize phthalate as the sole carbon and energy source. The ability of this particular strain to degrade phthalate has been well documented (Keyser *et al.,* 1976; Ribbons *et al.,* 1984).

Bogan *et al*., (2001), have identified 12 strains of polycyclic aromatic hydrocarbons (PAH)-degrading bacteria. Three of these isolates belong to the genus *Burkholderia*, with the remainder comprised of two *Pseudomonas* species and seven strains from the genus *Sphingomonas.* Only three of 12 strains were able to grow on phthalic acid, while three others (all *Sphingomonas*) could not grow. A *Morexella sp*., strain VG45 capable of utilizing o-phthalate as a sole source of carbon and energy was isolated (Maruyama *et al.,* 1978).

When effect of pH on degradation of phthalic acid was studied it was observed that alkaline pH favored degradation and very little removal was seen in acidic media. Wang & Barlaz (1997) demonstrated the decompositions of waste substances in landfills by the microbial population. Such populations were found to be able to degrade a number of aromatic compounds and their substituted compounds. They proposed that a relatively neutral pH was beneficial for phenol degradation. The production of carboxylic acid in the landfills as a result of phenol degradation resulted in drop of pH level. The later production of methane then balances the acidic pH, but in between the two phases, a long lag phase usually existed. Zeyer & Kearney (1984) have reported the high sensitivity of pH by *Pseudomonas putida* for the degradation of ortho- or meta-nitrophenol.

Zeng *et al*., (2000) extracted a phthalic acid ester (PAE) degrading enzyme located in the soluble part of the cytoplasm in *Pseudomonas fluorescens* FS1, isolated from the activated sludge at a petrochemical factory. The results showed that the optimum temperature and pH for the PAE degrading enzyme activity to di (2-ethylhexyl) phthalate were 20 and 6.5, respectively. The enzymatic metabolism of di-n-butyl phthalate under aerobic conditions starts by PAEs-degrading enzyme to monoester, further enzymatic degradation of monoester proceeds *via*., phthalic acid by benzoate, 4-hydroxy benzoic acid and phenol (Zeng *et al.,* 2000).

The microorganisms bear considerable sensitivity towards the environmental factors like pH, temperature and oxygen availability. Thus temperature optimization is an important parameter to enhance the degradative capability of microbes. Pitter & Chudoba (1990) emphasized on the significance of the environmental conditions on the growth of the strains and the rate of degradation they conduct. *Pseudomonas* is a mesophilic microbe that requires 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. Nishino & Spain (1993) while studying the degradation of nitrobenzene by *Pseudomonas pseudoalcaligenes* used 30°C temperature and reported it to be optimum for this strain. Takenaka *et al*., (1998) used *Pseudomonas* sp. Ap-3 and during its growth and metabolic activity study the optimum temperature was found to be 30°C. This temperature thus has been found suitable for *Pseudomonas* by most of the scientists.

The biodegradation of synthetic chemicals in the presence of a mixture of alternative carbon sources is a matter of great practical importance. This is because in the environment several organic substrates in addition to the synthetic aromatic compounds exist. 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 showed that addition of glucose in the mineral salt medium have effect on the growth of microorganisms as better growth was seen in the presence of glucose. But very little effect was observed on degradation, rather there was better degradation at higher concentrations without glucose as compared to medium with glucose. The efficacy of naphthalene degradation in the presence of glucose by a recombinant *Pseudomonas* mutated in glucose metabolism was determined and compared to the non-mutated strain. Results obtained indicated that impairment of glucose metabolism leads to better degradation of naphthalene in the presence of glucose (Samarta *et al.,* 2001). The effect of alternate carbon source (glucose) over the metabolic activity of aromatic amines by the bacteria has been studied widely. Zeyer *et al*., (1985) and Nakanishi *et al.,* (1991) suggested the repression of aniline metabolism in the presence of glucose. However, some microbes have been reported to conduct the degradation mechanism effectively in the presence of high levels of glucose (Aoki *et al.,* 1983: Konopca *et al.,* 1989). Zissi & Lyberators (1999) observed that the utilization of one type of organic substrate effects the utilization of other. The presence of glucose thus makes available extra carbon and energy for the microbes. This enhances the activity of the degrading bacteria to utilize the resistant aromatic amines.

Conclusions and future prospects: The newly isolated bacterial strain with the capability of utilizing phthalic acid as the sole carbon source was isolated, identified and characterized. The adaptation of the microorganism to grow on the increasing concentrations of phthalic acid showed the potential of the isolate to be used in bioremediation in the environment that might be polluted with a variety of compounds in variable concentrations. The bacterial strain P1 showed best degradation at pH 8. Enrichment of the medium with glucose had no significant effect on the degradation capability of the selected isolates to degrade the aromatic compound. Effects of other inducers and vitamins on the degradation capacity can also be studied further. HPLC, Mass spectra, and GC can be used to further study the degradation products. The same strain can be used to check the degradation of other toxic polyaromatic hydrocarbons like phenanthrene and naphthalene whose degradation products include phthalic acid. For further study the plasmid involved in the degradation of the phthalic acid can be isolated

and it can be checked if the organisms degradation abilities can be enhanced by the genetic engineering. Hydrolytic enzymes secreted by these organisms could be studied. The microorganisms could also be studied as potential candidate for the degradation of plastics.

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