Pak J Sci Ind Res 2000 43 (5) 289-293

The Influence of pH During Growth of Bacteria in Toluene

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(Received 15 September 1997; accepted 19 January 2000)

Five toluene tolerant species were isolated from the activated sludge of a wastewater treatment plant (Dublin). The isolate were investigated for influence of pH on the growth in toluene. Four of the bacteria have been identified as *Pseudomonas putida* and one as an *Aeromonas caviae*. When these bacteria were grown with toluene as the sole source of carbon and energy, the pH of the culture medium became acidic and dropped. 0.5 M sodium phosphate buffer was selected to investigate the optimum pH for growth in the presence of 500 µl of toluene. In general, the growth was optimum between pH 5.8 and 7.4.

Key words: Activated sludge, Toluene, pH, Wastewater, Biodegradation.

Introduction

Increased use of petrochemicals by modern society today has increased the amount of residual hydrocarbons found in air and soil samples. In 1989 the American Environmental Protection Agency found toluene in 54% of ground water samples near chemical waste sites (U.S. Public Health Service 1989). Significant contamination of ground water also arises from spillage of gasoline and/or other petroleum-based fuels and from leakage of gasoline from underground tanks. It is also used in plastic manufacture (Cagliati 1983) and in the chemical industry as a solvent. Therefore, it is not surprising that toluene is a common hydrocarbon pollutant found in wastewater, surface water and soil etc (Aracangeli and Arvin 1993).

The importance of microorganisms in decomposing natural organic residues in soil sediments and aquatic systems has long been recognised. The potential for harnessing such natural processes to deal effectively with treatment and pollution clean-up in an ecologically acceptable manner is attracting much attention (Bewly *et al* 1991).

Toluene waste has been successfully treated by activated sludge process for years. Bacteria play a significant role in treatment processes of industrial waste chemicals of activated sludge. Due to the volatile nature, toluene is a threat to the atmosphere and as such requires treatment both in the liquid and gaseous phases. Activated sludge processes are commonly employed for the treatment of wastewater of industrial effluents. Nowadays biofiltration is used to control air pollution from many industrial sources. Both of the systems perform best when inoculated with a suitable inoculum. For a metabolizing culture the pH of the growth medium is important that may reduce the degradation rate of toluene or other pollutants. The growth of bacteria on toluene is difficult because of the toxic effects of toluene when supplied directly to the growth medium. No detailed study has been published on the behavior and the role of pH during the biodegradation of toluene in uncontrolled pH condition. In biological growth experiment with uncontrolled pH, a variation of the initial value was observed. This phenomenon is described in relation to pure cultures (*Pseudomonas*), both free (Hill and Robinson 1975) and entrapped in polymer (Bettmann and Rehm 1984), as well as in relation to mixed cultures, both free (Kim and Armstrong 1981).

It was of interest to investigate the influence of decrease in pH on the growth of bacteria when toluene is used as the sole carbon source and energy. In the present study toluene was used as the growth limiting substrate due to the practical importance of its elimination from many industrial wastewaters, which are characterized by widely different pH values.

Materials and Methods

Strain. Two types of bacteria were isolated from the activated sludge sample of a pharmaceutical wastewater treatment plant in Dublin (Ireland Republic) and identified as *Pseudomonas* and *Aeromonas*. Strain numbers were given to the isolated bacteria according to the aromatic substrates from which they were isolated (To = toluene, Na = naphthalene). All the

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isolates were capable of growing with toluene as the sole source of carbon and energy.

Cultural conditions. The organisms were grown in *Pseudomonas* minimal medium (PMM) in the presence of different volumes of toluene. Toluene was supplied in a small tube hanging inside the flask and thus diffused as vapour phase without liquid toluene coming into direct contact with the culture. The pH experiment was carried out in 0.5M Na-phosphate-buffer with PMM instead of water. The pH values were 5.8, 6.6, 7.4, 7.8 and 8.0. All the cultures were incubated at 30°C and agitated at 200 rpm for the required time. The growth and pH of the culture medium was monitored for up to 7 days until O.D reached at constant value.

PMM. The *Pseudomonas* minimal medium was prepared as outlined by Goulding *et al* (1988). 4.36g K_2HPO_4, 3.45g NaH_2PO_4, 1.0g NH_4Cl, 0.912g $MgSO_4$. $6H_2O$ and trace salt solution 1 ml in one lit 4.77g $CaCl_2.2H_2O$, 0.37g $FeSO_4.7H_2O$, 0.37g $CoCl_2.6H_2O$, 0.10g $MnCl_2$ and 0.02g $NaMoO_4$ in 100 ml H_2O.

Sodium phosphate buffer. 1M sodium phosphate buffer with different pH (5.8, 6.6, 7.4, 7.8 and 8.0) were prepared by adjusting pH with 1M NaOH.

Tris-HCl buffer. 0.033M Tris-HCl buffer was prepared by adjusting the pH to 7.6 with 2M HCl.

Inoculum. Isolated colonies from nutrient agar or aromatic plates were used to inoculate nutrient broth (10 ml). Cultures were incubated overnight at 30°C and agitated at 200 rpm. The cells were harvested at 5,000 rpm for 10 minutes in Labofuge 6000 bench-top centrifuge. The cultures were washed twice in sterile 0.033M tris-HCl buffer (pH 7.6). This cell suspension was then used to inoculate the growth medium at a concentration of 2% (v/v).

Specific growth rates. Specific growth rates were calculated using the computer software package Sigma plot (Version 4.0) Jandel Corporation. A mathematical transform was used to determine Ln (X/X_o) where X = absorbance at time t, X_o = initial absorbance.

A graph of Ln (X/X_o) vs t was plotted. Regresion analysis was performed on the exponential portion of the curve. The resulting slope being equal to the specific growth rate ($m=h^{-1}$).

Results and Discussion

Four isolates of the *Pseudomonas* spp and *Aeromonas* spp. were isolated from the activated sludge sample (one) of a wastewater treatment plant and capable of growing with toluence as the sole source of carbon and energy.

The pH of the isolates during growth on toluene was monitored (Fig 1). The pH dropped as the toluene was metabolized. This drop was greater at higher concentration of toluene, dropping from pH 7.0 to 5.24. However, good growth for all the isolates was observed in this pH range (Table 1). The nature of the activities of microorganisms is such that the pH of the growth medium of a metabolizing culture will not remain constant for long. Hydroxylated compounds might be expected in the early stages of toluene metabolism. The lowering of the pH of the growing cultures must be caused by the production of acidic substances. These were detected by Claus and Walker (1964) as acetic and pyruvic acids when *Pseudomonas* and *Achromobacter* were grown in media where toluene was the only carbon source. When naphthalene was metabolized by *Pseudomonas* spp. the pH of the culture medium dropped and the drop in pH increased with increasing concentration of naphthalene (Mulcahy 1993).

Lallai and Mura (1989) also reported the drop in pH in the culture during growth in phenol. The diminution of pH during the consumption of phenol can be attributed to the production of organic acids (Abson and Todhunter 1982) from the intermediates which are formed during the degradation of phenol by the mixed populations (Bayly and Dagley 1969; Neujahr and Varga 1970). The acids can reach such concentrations that they overcome the buffer capacity of the phosphate system resulting in a drop of pH. The subsequent degradation of the acids raises the pH.

Claus and Walker (1964) reported that gradual acidification of a culture caused a decrease in the enzymatic activity of the organisms and also a slight loss of benzene or toluene from the aqueous solutions due to volatilization. The maintenance of a constant pH during growth of a culture is especially important for those organisms which produce acid but are not acid tolerant. Hence, investigation was carried out to see the

Table 1
The pH range of *Pseudomonas* and *Aeromonas* spp during growth in the presence of toluene

Isolate	Range of pH at different volumes of toluene			
	250 µl	500 µl	1000µl	2000µl
Pseudomonas putida (To-1)	6.35-7.0	6.06-7.0	5.63-7.0	5.32-7.0
Pseudomonas putida (To-3)	6.31-7.0	6.17-7.0	5.62-7.0	5.49-7.0
Aeromonas caviae (To-4)	6.38-7.0	6.28-7.0	5.83-7.0	5.24-7.0
Pseudomonas putida (To-5)	6.27-7.0	6.15-7.0	5.94-7.0	5.58-7.0
Pseudomonas putida (Na-13)	6.43-7.0	6.38-7.0	5.78-7.0	5.25-7.0

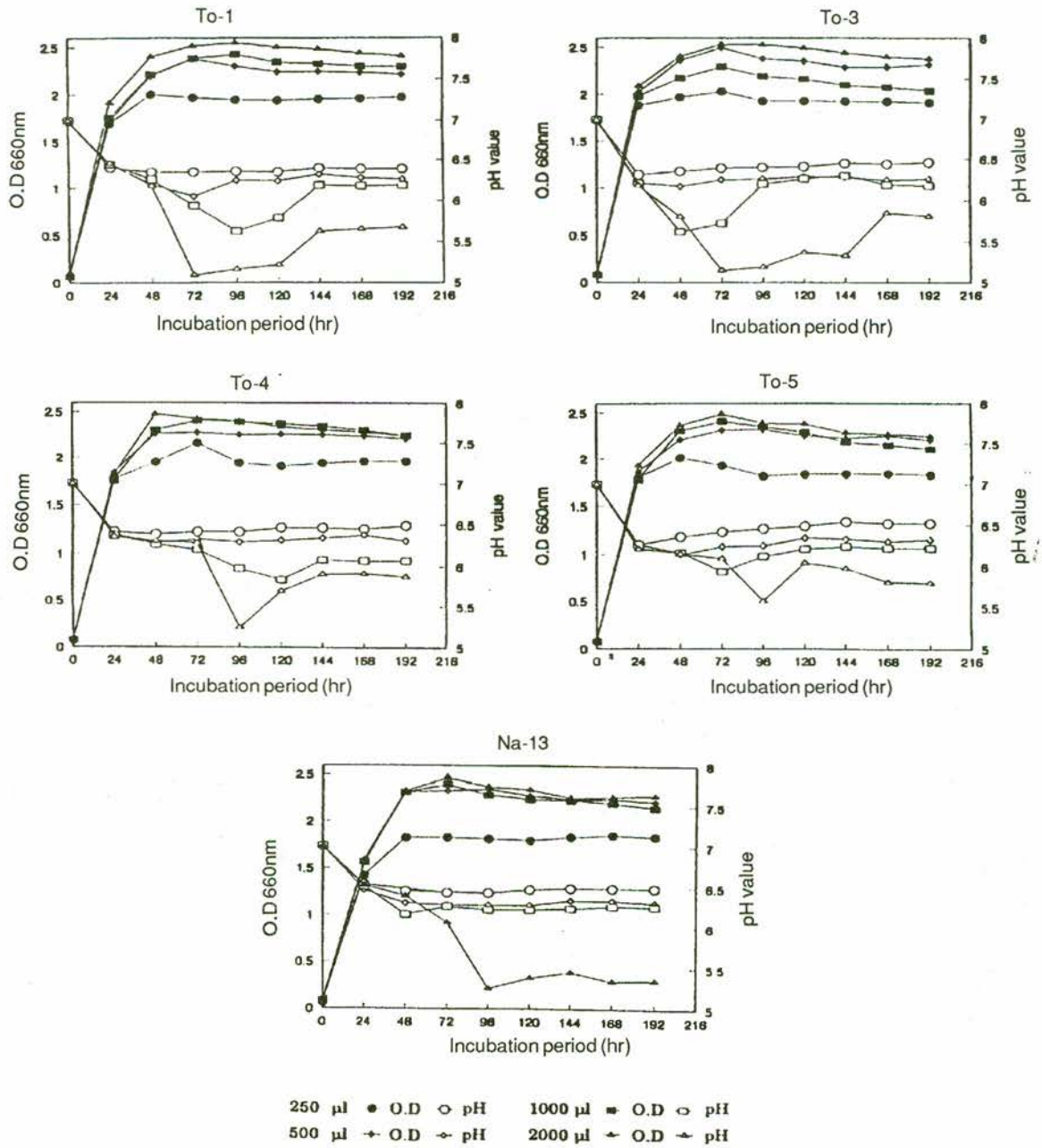

Fig 1. Growth of the *Pseudomonas and Aeromonas* spp in the presence of 250-2000 µl toluene (1 O D = 3.0 x 10^7 / ml)

influence of pH on the growth of the organisms when toluene was used as the sole source of carbon and energy.

Effect of pH was studied by growing bacteria in the presence of 500 µl of toluene at different pH values. The optimum pH for growth of five strains of bacteria was investigated using sodium phosphate buffer of 0.5M at pH values of 5.8, 6.6, 7.4, 7.8 and 8.0. Phosphate buffers are widely used in the preparation of media because they are the only inorganic buffers in the physiologically important range around neutrality and are relatively non-toxic to microorganisms. In addition they provide a source of phosphorus, which is essential for growth (Stainer *et al* 1988).

The growth of five isolates was different at different pH values (Fig 2). Strain To-1, To-4 and Na-13 could grow at all pH values. Strain To-3 and To-5 grew at pH 6.6 and 7.4 but did not grow at 7.8 and 8.0. A lag was experienced in most cases except in the case of To-4 which grew readily at pH 6.6 and 5.8 and To-5 which grew readily at pH 5.8, 6.6 and 7.4. In all the other cases the duration of lag increased with increasing pH values. In general, growth was optimum between pH 5.8 and

Table 2

Influence of pH on the growth of the isolates in the presence of 500 ml toluene

	Duration of lag (h) at different pH values					Specific growth rates (μ=h-1) at different pH values				
	5.8	6.6	7.4	7.8	8.0	5.8	6.6	7.4	7.8	8.0
Pseudomonas putida (To-1)	24	24	48	72	72	0.052	0.060	0.100	0.070	0.042
Pseudomonas putida(To-3)	24	24	48	72	72	0.070	0.070	0.062	0.030	0.020
Aeromonas caviae (To-4)	12	12	48	48	48	0.040	0.040	0.070	0.063	0.030
Pseudomonas putida (To-5)	12	12	12	24	24	0.080	0.071	0.061	0.013	0.021
Pseudomonas putida (Na-13)	24	24	48	48	72	0.060	0.071	0.063	0.043	0.060

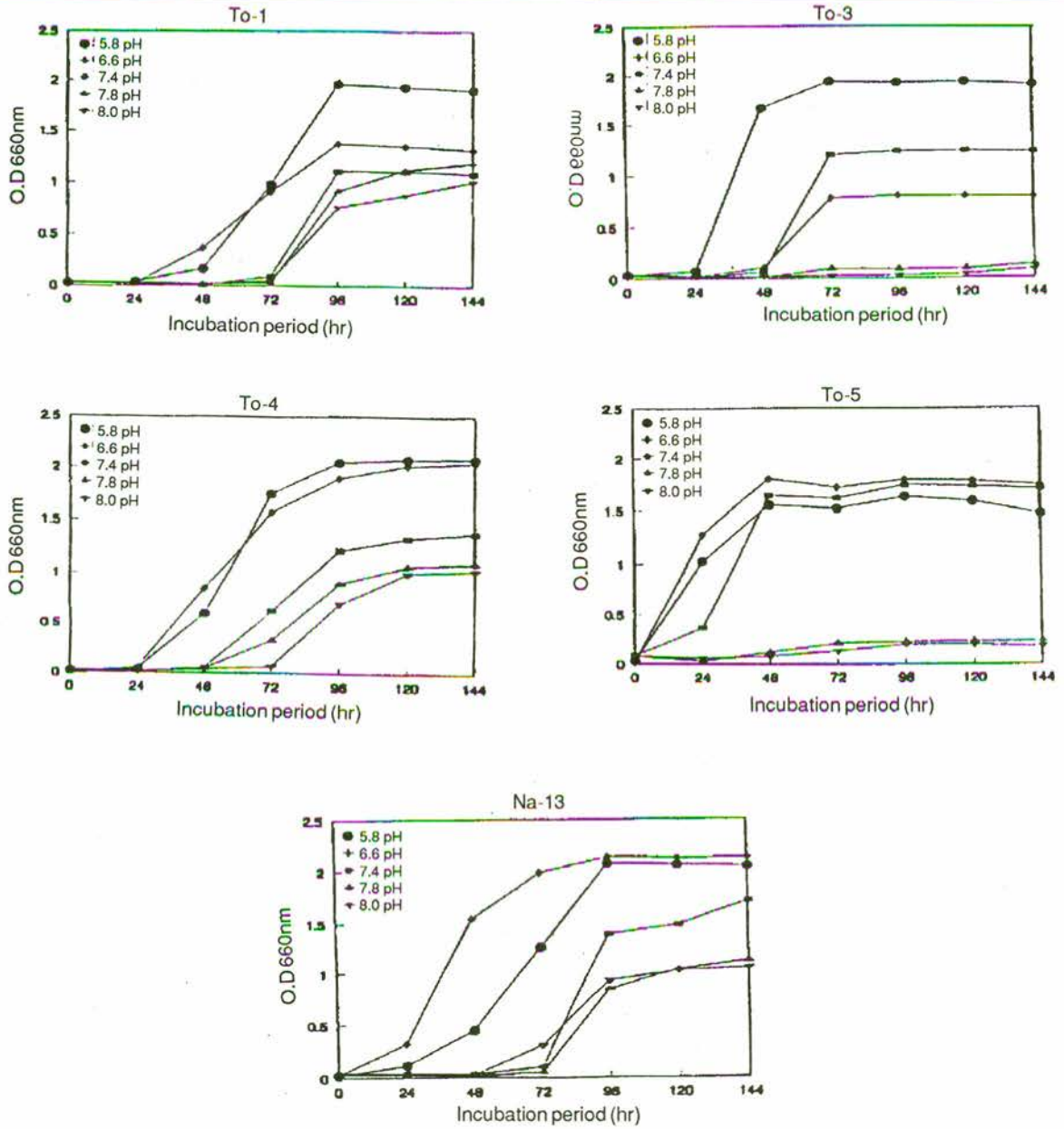

Fig 2. Growth pattern of *Pseudomonas* and *Aeromonas* spp at different pH values in 0.5 M sodium phosphate buffer in the presence of 500 μl toluene.

7.4 (Table 2) which suggested that though the duration of lag increased at higher pH values but the growth was faster.

Duration of lag increased with increasing pH of the culture medium which is probably due to the time taken to adapt to a higher pH environment. The duration of lag also increased with increasing pH of the culture medium during phenol degradation by a *Pseudomonas* sp. (Bettman and Rehman 1984). The growth rate was maximum for most of isolates in buffered media between pH 6.6 and pH 7.4 suggesting that the organisms favoured neutral pH during growth in the presence of toluene. The maximum growth rate observed for phenol degradation by a mixed culture *(Achromobacter, Flavobacterium, Pseudomonas)* (Lallai *et al* 1987) were between pH 6.0 and pH 7.0. Therefore, it can be concluded that the specific growth rates of the organisms depend on pH values of the growth culture during biodegradation of aromatic hydrocarbons.

The findings in the present study can help in the control of growth culture used for the treatment of toluene containing effluents. By measuring the pH of the growth culture only, useful information can be obtained about the process of purification in terms of disappearance of toluene (minimum of pH) and of the end of fermentation (stabilized pH).

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