Bull. Environ. Contam. Toxicol. (1995) 54:472–478

Environmental Contamination and Toxicology

Inactivation of Pathogens During Aerobic and Anaerobic Treatments at Low Temperatures

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Received: 6 October 1991/Accepted: 18 August 1994

Contamination of potable water with human and animal wastes lead to the outbreak of epidemics, such as diarrhoea, dysentery, cholera, typhoid, jaundice etc. There are several contrasting reports about the inactivation of intestinal pathogens during conventional biological waste treatment. Farrah and Bitton (1983) reported that the conventional aerobic and anaerobic waste treatment processes lead to inactivation of pathogens. Findlay (1973), Feachem *et al.* (1978) and Dudley *et al.* (1980) reported that pathogens like *Klebsiella, Shigella, Salmonella, Staphylococcus, Pseudomonads* survived anaerobic digestion. In contrast, Carrington *et al.* (1982) showed that *Salmonella duesseldorf* was inactivated in sewage-sludge digesters running at 10 to 20 days hydraulic retention time (HRT). Further, Gadre *et al.* (1986) and Olsen and Larsen (1987) reported that vegetative pathogenic bacteria were inactivated rapidly during mesophilic and thermophilic treatments. Most of these studies focussed on mesophilic and thermophilic temperatures and little attention was paid on the fate of the pathogens during aerobic and anaerobic treatments at psychrophilic temperatures. Since a large part of the earth is in the low temperature zone, the study on survival of pathogens during biological treatment will be useful for the proper disposal of wastes in these regions. Furthermore, the mechanisms of inactivation of pathogens during waste treatment is ill defined.

During the course of previous studies on aerobic treatment of organic wastes at low temperatures (5-20°C), *Arthrobacter* sp., an Antarctica isolate, was able to predominate over the other microorganisms (Aggarwal and Singh 1993). In the present study, we report the inactivation of pathogens during aerobic (by *Arthrobacter* sp.) and anaerobic treatments of rabbit

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waste at different temperatures. Further, an attempt was made to determine the factors responsible for destruction of pathogens during anaerobic treatment.

MATERIALS AND METHODS

Escherichia coli, salmonella typhi (antibiotic resistant strains) and *Staphylococcus aureus* were obtained from the All India Institute of Medical Sciences, New Delhi, India. *Arthrobacter* sp. was collected from the Centre for Cellular and Molecular Biology, Hyderabad,India.

The experiments were performed in serum bottles of 500 ml capacity containing 200 ml of diluted rabbit waste (6 % volatile solids). Fifty ml of a slurry, collected from a biogas digester running on rabbit waste at 15^{o}C for one year, was used as an inoculum. The head space of serum bottles was flushed with oxygen-free N_2 gas and the bottles were sealed with butyl rubber stoppers. Five ml of sterile saline suspension containing *E.coli, S.typhi and S.aureus* were added to the bottles aseptically after 10 days of fermentation when biogas production was in the exponential phase. The bottles were incubated at 10,20 and 37^{o}C. For aerobic treatment, 200 ml of a 1:10 diluted slurry was dispensed in 500 ml Erlenmeyer flasks and inoculated with *Arthrobacter* sp. at a concentration of 1x 10^3 colony forming units (cfu) ml^{-1}. After 4 days of incubation, the pathogens were added and incubation was continued on a rotary shaker.

The pathogens were enumerated at 4 day intervals (aerobic treatment) and 7 day intervals (anaerobic treatment) by plating 10-fold serial dilutions of samples on MacConkey agar containing 100 ug ampicillin ml^{-1} for *E.coli* and 25 ug tetracycline and 50 ug ampicillin ml^{-1} for S.typhi. The plates were incubated at 37^{o}C for 48hr. Ampicillin-resistant *S.typhi* growing on plates containing 100 ug ampicillin ml^{-1} were differentiated from *E.coli*, as *S.typhi* forms colorless colonies against the pink colonies of *E.coli*. Since *S. aureus* used in this study was not antibiotic resistant, its counts were monitored on nutrient agar containing 7% NaCl after incubation at 37^{o}C for 48 hr.

The effect of different volatile fatty acids (acetate, propionate, butyrate and valerate) and sulfide was determined by adding their respective sodium salts at 4 g l^{-1} concentration to 20 ml of sterile nutrient broth taken in 60- ml serum vials. The broth was later inoculated with exponentially growing cultures of *E.coli, S. typhi* and *S. aureus* (2 x 10^3 cfu ml^{-1}). The bottles were incubated at 20^{o}C for 72 hr and cfu of

pathogens were determined as described earlier.

RESULTS AND DISCUSSION

Arthrobacter sp. isolated from the Antarctica showed growth between 0-25^{o}C with an optimum of 20^{o}C. The inactivation of *E.coli, S.typhi and S. aureus* during aerobic degradation of rabbit waste by *Arthrobacter* sp. is shown in fig. 1. There was a linear fall in the viable counts of all the pathogens tested with time. In general, the degree of inactivation increased with increase in temperature. Table 1 shows the decimal reduction times (T_{90}) of the pathogens during aerobic treatment at different temperatures. *E.coli* displayed lower T_{90} values than *S. typhi* and *S. aureus* at all the temperatures. The T_{90} values varied from 5.7 to 4.6 days for *E. coli*, 10.2 to 7.8 days for *S.typhi* and 10 to 7 days for *S. aureus* at 5 to 20^{o}C (Table 1).

The survival of the pathogens when added at the exponential phase of the anaerobic digestion of rabbit waste is shown in fig.2. Like aerobic treatment,the increase in temperature from 10 to 37^{o}C resulted in an increase in percent destruction of the pathogens with time. *E.coli and S.typhi* were inactivated rapidly at 37^{o}C as compared to other temperatures (10 to 20oC). *S. aureus* was relatively resistant to anaerobic treatment even at 37^{o}C. The decimal reduction times of these pathogens varied from 5 to 1.4 for *E.coli*, 2.5 to 1.5 for *S.typhi* and 6.4 to 4.7 for *S.aureus* (Table 1).

In our study, unlike earlier observations (Gadre *et al.* 1987; Olsen & Larsen 1987), pathogen inactivation was influenced by the time of pathogen addition to the digester. The T_{90} values of pathogens were increased significantly at all the temperatures when the bacteria were added during the start up of the digester. The T_{90} values were 7.9 to 5.2, 8.0 to 5.4 and 9.6 to 5.5 for *E.coli, S.typhi and S.aureus*, respectively, at 10 to 37^{o}C.

Studies conducted in 20 litre digesters running at 20^{o}C in semicontinuous mode with a HRT of 25 days showed that all three pathogens tested were inactivated by > 99%.

To date, the biotic and abiotic factors involved in pathogen elimination during aerobic and anaerobic treatments are not well defined. The effect of biotic factors (competition from microflora) was determined by adding *E.coli* to sterile and unsterile rabbit waste slurry. After 3 days of incubation at 20^{o}C, the counts of *E.coli* decreased from 11 x 10^{8} ('0' time) to 4 x $10^{8}ml^{-1}$ in sterile and from 19 x 10^{8} to 12 x $10^{7}ml^{-1}$ in

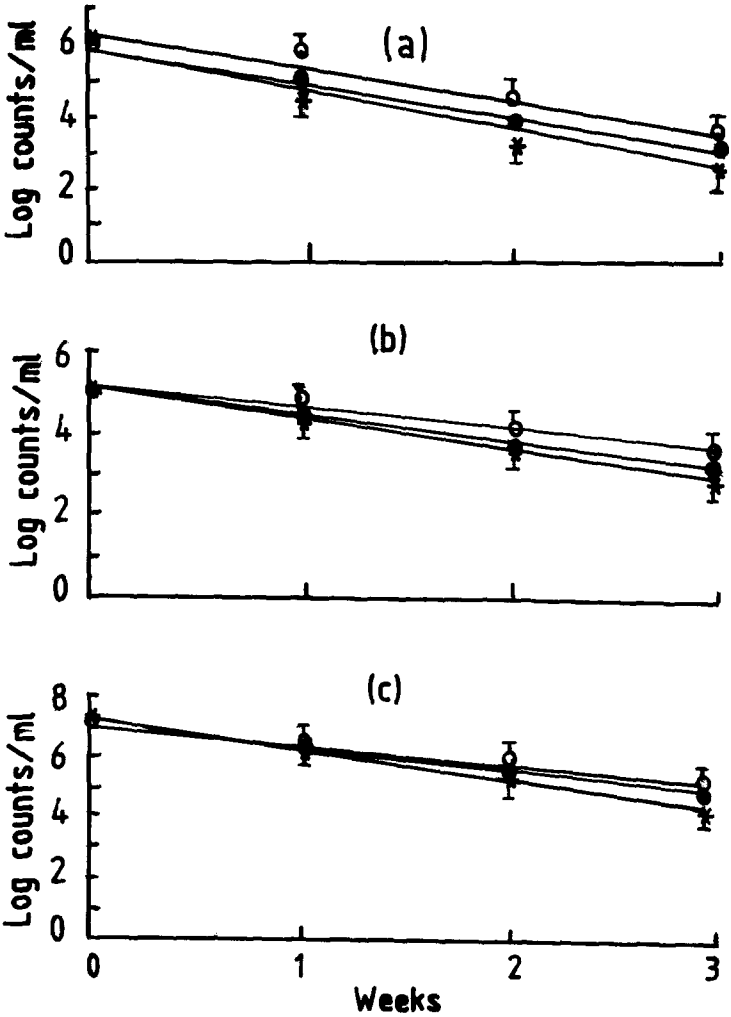

Figure 1.Effect of aerobic treatment of rabbit waste on pathogen inactivation by *Arthrobacter* sp. a) *E.coli*; b) *S.typhi*; c) *S.aureus*. ,5°C; ,10°C; , 20°C.

Table 1. Influence of temperature on decimal reduction times in days (T_{90}) of pathogens (Mean ± SE, n=4)

	Aerobic treatment			Anaerobic treatment		
	5°C	10°C	20°C	10°C	20°C	37°C
E.coli	5.7 ±0.4	5.4 ±0.2	4.6 ±0.3	5.0 ±0.6	3.3 ±0.3	1.4 ±0.2
S.typhi	10.2 ±0.3	9.6 ±0.7	7.8 ±1.0	2.5 ±0.1	2.1 ±0.4	1.5 ±0.2
S.aureus	10.0 ±0.3	9.1 ±0.6	7.0 ±0.2	6.4 ±0.1	5.3 ±0.3	4.7 ±0.1

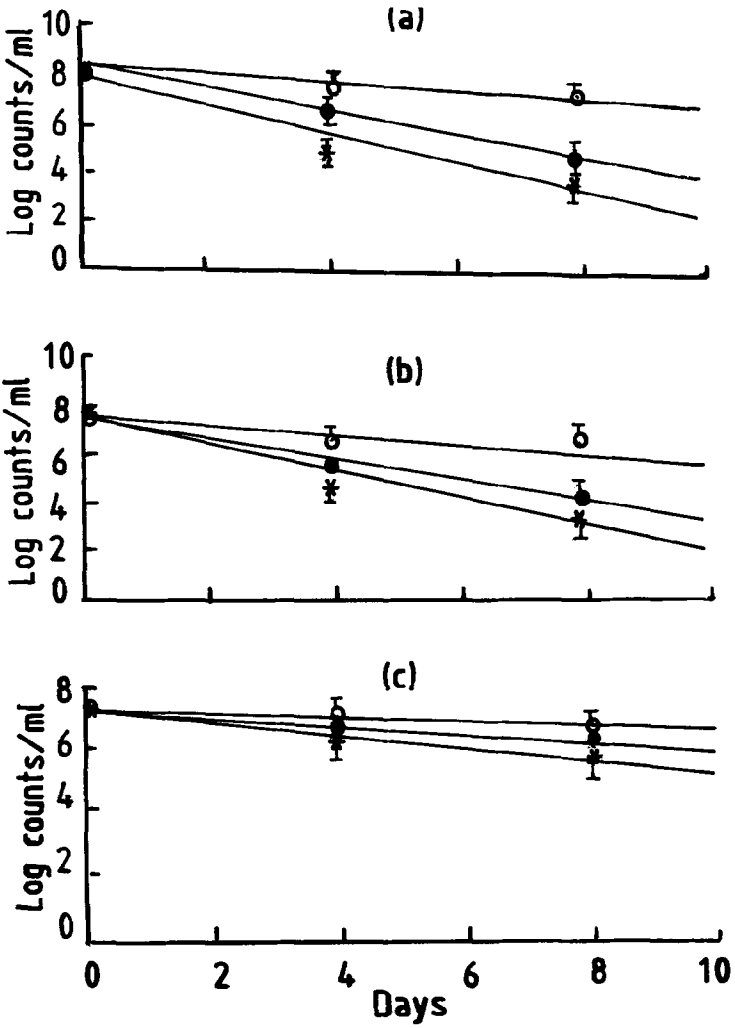

Figure 2. Effect of anaerobic treatment of rabbit waste on pathogen inactivation. a) *E.coli*; b) *S.typhi*; c) *S. aureus*. , 10°C; , 20°C; ,37°C.

unsterile slurry. Since the reduction in *E.coli* counts were higher in unsterile rabbit waste than the sterile one, this indicated that the competetion from saprophytic microorgamisms also play an important role in destruction of the pathogens. Findlay (1973) found that *Salmonella* can multiply in sterile sewage sludge in the absence of competetion from other microbes.

The ten-fold decrease in number of *E.coli* when added to sterile rabbit waste slurry indicated that some chemical components present in the rabbit waste slurry were also contributory to the observed inhibition. The effects of different metabolites of anaerobic digestion, such as volatile fatty acids and sulfide, on the growth of *E.coli, S.typhi* and *S.aureus* are shown in

Table 2. Acetate had no effect, whereas propionate, butyrate and valerate inhibited the growth of all the pathogens tested. The increase in chain length of fatty acids didnot appear to influence the inhibitory effect. By and large, the dissolved sulfide (Na_2S) inhibited the growth of pathogens more than the other compounds tested.

Table 2. Effect of different chemical parameters on the growth of pathogens at 20°C. (Mean±SE, n=4)

Chemical parameters	% of control		
	E.coli	*S.typhi*	*S. aureus*
Acetate	92±0.4	93±0.1	97±0.5
Propionate	60±0.2	73±0.3	46±0.1
Butyrate	60±0.5	75±0.1	52±0.4
valerate	54±0.1	71±0.9	48±0.8
Mixed acids	40±1.0	67±0.3	49±0.3
Na_2S	20±0.5	10±0.5	7±0.8

100% counts - *E.coli*, 97 x 10^4 cfu ml^{-1}; *S.typhi*, 3 x 10^5 cfu ml^{-1}; *S.aureus*, 4 x 10^5 cfu ml^{-1}.

Both aerobic treatment by *Arthrobacter sp* and anaerobic treatment were effective in eliminating the pathogens at all the temperatures. In general, an increase in temperature resulted in a decrease in T_{90} values. Earlier Olsen and Larsen (1987) observed that the decimation times decreased with increase in temperature in the ranges of 30 to 53°C. Although the T_{90} values observed for *E.coli and S.typhi* during anaerobic treatment at 37°C was in agreement, the T_{90} value of *S.aureus* (4.7 days) was found to be much higher. This may have been due to the differences in the strains used.Of the three pathogens, *E.coli* was found to be more sensitive than the other two to both the treatments. *S.typhi and S.aureus* showed relatively higher resistance to aerobic and anaerobic treatments, respectively, at all temperatures. Competition by saprophytic microflora, production of slime and sedimentation during aerobic degradation appeared to play an important role in eliminating the pathogens.

Further experiments with different chemicals revealed that the volatile fatty acids (VFA) such as propionate, butyrate, valerate and sulfide had a significant inhibitory effect on the growth of pathogens. Thus, a combination of several factors such as temperature, competing microflora,products of anaerobic fermentation such as VFA and sulfide play an important role in the elimination of pathogens during anaerobic treatment.

In low temperature areas, usually the anaerobic digesters are operated at 50 to 60 days HRT. Though the T_{90} values of the pathogens observed in the present study were higher (< 10 days) at low temperatures, the digester with 50 days HRT can eliminate 99.999 % of pathogen population. Aerobic treatment with *Arthrobacter* sp. also resulted in T_{90} values of about 10 days at $10^{o}C$; this will be of great help to control the organic pollution and spread of diseases in Antarctica.

Acknowledgments. The authors thank Dr. R.V. Swamy, Director and Shri. K.M. Rao, Jt. Director, DRDE, Gwalior, India for giving guidance and encouragement. The help rendered by Pancham Singh in the preparation of the illustrations is acknowledged.

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