STUDIES IN THE BACTERIOLOGY OF SEWAGE

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Abstract

Over 400 bacterial cultures were isolated from raw sewage by employing the enrichment culture technique as well as by direct plating on nutrient agar. The enrichments attempted were nitrate-glycerol, nitrate-citrate, nitrogen-free-mannitol-sucrose, urea and pectin—ammonium sulphate. Quantitative data on the acrobic bacterial population of sewage and sludges were also obtained.

Introduction

Sewage by virtue of its nature and origin, acts as a repository for microorganisms of different types. Faeces brings with it the intestinal flora, the soil its autochtonous and zymogenous flora, the air its noncharacteristic flora and water its own typical flora. However, all the microorganisms that enter sewage do not survive and thus sewage soon acquires a characteristic flora of its own. The nature of this flora will be conditioned by the character of the sewage, *e.g.*, a specialised industrial waste will have a flora different from a purely domestic sewage. In the various biological methods of sewage purification this selection of types goes a step further with the result that a highly specialised flora, able to function properly only under the conditions that have favoured the selection, develops. Thus in sewage and more specially in the various methods of sewage treatment by biological agencies, we have a striking example of natural selection or the survival of the fittest.

In the above context it was considered desirable to examine the microbial flora of sewage by exploitation of the enrichment culture technique. The fact that sewage is an aqueous environ was an added impetus in selecting this methodology as this would obviate the objection often raised against liquid enrichments in that they usually favour the selection of the pseudomonads[1] irrespective of whether or not these bacteria constitute the dominant organism in the inocula. By isolating bacteria from sewage using aerobic enrichments it was considered possible to gain an insight into the flora responsible in the aerobic purification of sewage in general.

Another objective set forth in this study was to discover, if possible, bacteria exclusive to the human intestine. If successful the presence of such bacteria would prove useful as indices of faecal pollution of water, foods or crops grown in sewage farms.

Materials and Methods

All samples of sewage were obtained from the sewage works at this Institute; the sewage was domestic in origin. Almost all the samples were examined immediately on collection. Activated sludge was obtained from the laboratory units built up by the "fill-and-draw" technique; not more than one sample was collected within a period of one week. The aerobic enrichments were carried out either in 50 ml conical flasks or test tubes (1.9 × 15 cm) containing 10 ml of medium; test tubes were incubated in an inclined position. The anerobic enrichments were made in glass-stoppered bottles (30 ml) filled to the brim with enrichment medium. The inoculum in all cases was 1 ml of raw sewage or activated sludge. Irrespective of whether the enrichments were done aerobically or anaerobically, the plates on which the enrichments were seeded were always incubated aerobically. Except for the nitrate enrichments which were incubated both at room temperature (ca. 25°C) and 37°C, all other isolations were made at room temperature only. The following were the enrichment media employed:—(*a*) *Nitrate-glycerol enrichments*: 0.2% $NaNO_3$ and 1% glycerol were incorporated into a salt solution. The enrichments were done anaerobically. (*b*) *Nitrate-citrate enrichments*: 0.2% $NaNO_3$ and 0.5% $Na_3C_6H_5O_7$ were incorporated into the basal solution of Khambata *et al*[2]. and enriched anaerobically. (*c*) *Nitrogen-free-mannitol-sucrose enrichments*: A modification of Ashby's medium containing 1% each of mannitol and sucrose was employed. (*d*) *Urea enrichments*: 0.5% urea was incorporated into the basal solution of Khambata *et al*[2]. The medium was sterilsed by filtration. (*e*) *Pectin enrichments*: 0.5% pectin and 0.05% ammonium sulphate were incorporated into the basal solution of Khambata *et al*[2]. The medium was sterilised by filtration.

For the isolations on nutrient agar the sewage was stored for four hours at room temperature, serially diluted, and then streak plated on nutrient agar plates that had been dried at 37°C for 24 hours. From plates that contained about 200 colonies, 10 of the predominant types were picked off. The results, therefore, would represent only the dominant species occurring in sewage.

The population studies were made by the usual techniques. In examining the sludges effective dispersion was achieved by agitation in the presence of glass beads on a reciprocal shaker. Counts were made both on nutrient agar and on MacConkey's agar; the former counts referred to as the total count and the latter as the coliform count.

Results and Discussion

Bacteria Isolated from Sewage

(*a*) *Nitrate Enrichments*: The results obtained at both room temperature and 37°C were essentially similar and are presented in Table I. It was observed that the nitrate-glycerol enrichments were conducive to the isolation

TABLE I

Bacteria encountered in nitrate enrichments

		Incubation temperature	
		R. T. (ca. 25°C)	37°C
A. *Nitrate-glycerol* :			
Total No. of samples analysed	.....	20	20
Aerobacter aerogenes	.....	14	18
Brevibacterium species	.....	5	1
Corynebacterium species	.....	1	0
Escherichia coli	.....	3	2
Escherichia freundii	.....	5	1
Paracolobactrum intermedium	.....	2	7
Pseudomonas aeruginosa	.....	0	2
Pseudomonas species	.....	1	0
B. *Nitrate-citrate* :			
Total No. of samples analysed	.....	20	20
Pseudomonas aeruginosa	.....	16	22
Pseudomonas stutzeri	.....	14	15
Pseudomonas species	.....	10	0

of the coli-aerogenes group while the nitrate-citrate enrichments resulted in the isolation of pseudomonads only. This would lead one to speculate on the possible role of these two groups of bacteria in the stabilisation of sewage—the enterobacters metabolising the carbohydrates and related compounds, and the pseudomonads in their turn, utilising the metabolic products derived therefrom. In fact preliminary investigations did reveal that sucrose enrichments (with ammonium sulphate) also lead to the isolation of the enterobacters. The observation of Shethna and Bhat[3] that four of the *Aerobacter aerogenes* cultures were able to bring about a 100% decomposition of 1% glycerol in the absence of either $CaCO_3$ or forced aeration, unlike any other bacterium tested, would lend support to this view. It may also be recorded that glycerol is an important constituent of sewage in that it is formed as a result of the hydrolysis of fats and oils which are normal constituents of sewage. Viswanathan and Pillai[4] found 25—85 ppm of fatty matter in the Institute sewage.

A point of interest revealed by these studies was that the nitrate-citrate enrichment was highly conducive to the isolation of *Pseudomonas aeruginosa*, an organism of both sanitary significance and medical importance. Only recently, nearly a year after these investigations were completed and presented in the form of a thesis[5] that another medium for the selective isolation of this bacterium was described.[6]

The isolation of *P. aeruginosa* from all the sewage samples analysed confirms the results of Ringen and Drake[7] who had reported its isolation from 97% of the samples analysed by them. The author would be thus inclined to agree with the view expressed by Reitler and Selligman[8] that the presence of *P. aeruginosa* in water should be considered as suggestive of faecal pollution. However, too much emphasis cannot yet be laid to its value as an indicator of faecal pollution as compared to the classical *Escherichia coli* inasmuch as *P. aeruginosa* has been isolated, though less frequently, from sources other than faeces and sewage.[7, 9, 10, 11, 12, 13, 14, 15.]

It is pertinent to record that six samples of activated sludge effluent were examined for the presence of *P. aeruginosa*. The bacterium was detected in as many as five samples. Since this bacterium has, specially in recent years, been associated with several infections[8] the possibility of its spread through effluents should be considered when discharging these into bodies of water used for drinking or recreational purposes.

(*b*) *Nitrogen-free Enrichments*: The isolates made from the nitrogen-free enrichments (mannitol-sucrose) could be classed as shown in Table II. *Azotobacter agilis* was found to be the dominant *Azotobacter* in sewage. Nonetheless, that only three cultures of *A. chroococcum* were isolated was surprising in that this species is so common in soil. Apparently, therefore, *A. chroococcum* is not able to survive in sewage. Some of the characteristics of the dominant sewage *Azotobacter* have been reported elsewhere.[16]

TABLE II

Bacteria encountered in the nitrogen-free enrichments

	Raw sewage	Activated sludge
Total No. of samples analysed	21	9
Azotobacter agilis	16	5
Azotobacter chroococum	3	0
Aerobacter aerogenes	17	9
Achromobacter species	9	3
Corynebacterium laevaniformans	10	9

Since it has been claimed that nitrogen fixation occurs during the aerobic biological methods of sewage treatment[17, 18, 19] it was considered interesting to investigate quantitatively the azotobacterial population in activated sludge. The presence of a large population would provide indirect evidence for the fixation of atmospheric nitrogen specially when one considers the fact that nitrogen-rich enrichments in the laboratory never yield *Azotobacter*. However, it should be realised that a low azotobacterial count need not go against the concept of nitrogen fixation occurring during sewage treatment since several other bacteria, *e.g.*, *Aerobacter aerogenes*, are known to fix gaseous nitrogen. The *Azotobacter* counts were made by the "dilution to extinction" method using the enrichment medium described above as the growth substrate and by diluting the sludge in a ten fold series before inoculation. The results (Table III) revealed that activated sludge did contain a considerable azotobacterial population comparable to that found in soil. However, the numbers were not significant enough to justify any conclusion.

TABLE III

The numbers of *Azotobacter* and *Corynebacterium laevaniformans* in activated sludge

	Azotobacter/ml *wet sludge* *	Weight of ml of sludge mg	*C. laevaniformans* /g dry wt.
1	100	20	1.664×10^6
2	100	18.2	2.911×10^6
3	1000	16	0.91×10^6
4	10	15.8	3.16×10^5
5	1000	13	1.5×10^6
6	100	13.2	1.664×10^6

* The values represent the highest dilution showing growth and typical azotobacterial cells.

It may be mentioned that attempts were also made to see if nitrogen-free cellulose and pectin enrichments would lead to the isolation of *Azotobacter* specially in view of the fact that *A. agilis* is consistently present in strawboard waste water[24, 25]. However, no *Azotobacter* species could be isolated from either enrichment.

The other bacteria isolated from the nitrogen-free enrichments were *Aerobacter aerogenes* and *Achromobacter* species and nitrogen fixation is known to occur in both [20, 21, 22] and so no attempts were made to test these isolates for the property. On the other hand, attempts made to find if *Corynebacterium laevaniformans* could fix nitrogen showed its inability to do so. The turbidity formed by this species in nitrogen-deficient media was shown to be due to the formation of levan. A detailed report on its isolation and characterisation has appeared elsewhere.[23] Because of the interesting nature of this bacterium and

its possible role in the purification of sewage, studies were undertaken to count the *C. laevaniformans* population of activated sludge. Counts of this organism could be effectively made by plating out serially diluted sludge samples on the medium used for isolation. The typical colonies of *C. laevaniformans* could be easily distinguished and enumerated. The results are presented in Table III. The bacterium was present consistently and in fairly large numbers in activated sludge to warrant considering it a member of the normal flora of activated sludge. Another point worthy of mention in this connection is that for the isolation of this species from activated sludge no prior enrichment is necessary; to isolate it from raw sewage enrichment becomes imperative.

(*c*) *Urea Enrichments*: Most of the bacteria isolated from the enrichments were only weakly ureolytic and are apparently of no consequence in the transformation of urea in nature. The few strongly ureolytic bacteria that were encountered could be classed into two types—10 were coryneforms and 2 were *Pseudomonas* species. The weakly ureolytic bacteria were grouped as follows: 9 *Brevibacterium* species, 12 *Corynebacterium* species, 2 *Micrococcus* species and 1 *Bacillus subtilis*.

The ureolytic coryneforms, mentioned above, need special mention. On identification, they were believed to be strains of *Brevibacterium ammoniagenes* but a subsequent detailed comparison with typical strains of *B. ammoniagenes* showed this conclusion to be erroneous. The strains showed also a tendency towards a coryneform arrangement even as an authentic culture of *B. imperiale* did. However, it is possible that the latter species is not typical of the brevibacteria since it also displayed other attributes unlike those associated with the genus *Brevibacterium*,[26] namely, the ability to produce acid from lactose and polar flagellation. All this goes to show the subjective nature of the methods used in separating the brevibacteria from the coryneforms. From the characteristics presented in Table IV, it may be seen that the 10 cultures can be separated into two groups: one represented by culture UR 13 and comprising 9 cultures and the other represensed by a single isolate, UR 15.

That the ureolytic *Pseudomonas* species as well as the coryneforms could grow on ordinary laboratory media at a neutral or even slighly acidic pH is in marked contrast to the much studied ureolytic *Bacillus* species and is of some ecological interest. For under normal conditions one should expect the less exacting species to play a dominant role in nature.

(*d*) *Pectin Enrichments*: The pectin enrichments yielded 52 isolates of which 24 were pectinolytic when tested by the qualitative technique described by Bilimoria and Bhat.[27] The pectinolytic cultures could be grouped broadly into (1) the gas producers resembling the enterobacters, and (2) the yellow chromogenic non-gas producers.

TABLE IV

Characteristics of the ureolytic coryneforms

Characteristic	Culture UR 13	Culture UR 15
Form	Straight rods with a tendency towards a coryneform arrangement	Straight rods with a tendency towards a coryneform arrangement
Gram stain	+	+
Motility	—	—
Flagella	—	—
Spores	—	—
Colonies on agar	Ivory white, opaque, round convex, moist, entire margin	Cream to orange, opaque, round, convex, moist, entire margin
NO_3 reduction	to NO_2	to NO_2
Indole	—	—
H_2S	—	—
Milk	Alkaline	Alkaline
Gelatin	+	+
Starch	—	—
Arginine	late +	late +
Urease	+	+
Glucose *	—	—
Sucrose	—	—
Lactose	—	—
Maltose	—	—
Glycerol	—	—
Growth at 37°C	+	+

* The—sign indicates that no acid was produced from these sugars incorporated in peptone water with bromocresol purple as the indicator.

The six gas producing cultures were classed as follows: 3 as *Escherichia freundii*, 1 *Aerobacter aerogenes*, 1 *Erwinia dissolvens* and 1 unidentified *Erwinia* species notwithstanding the objections that may be raised in classifying pectinolytic bacteria in the genus *Escherichia* and *Aerobacter* rather than among the *Erwinia*. The observation of Smith[28] that some coliforms isolated by conventional methods are pectinolytic is of significance in this connection.

The yellow chromogenic pectinolytic bacteria were of two types: 6 were coryneforms while the rest were members of the genus *Xanthomonas*. The coryneforms were described as strains of a new species and the name *Corynebacterium barkeri* was proposed for them.[29] The pectinolytic enzymes of this species were studied by Bilimoria[30] and he showed it to contain both a pectin polygalacturonase and a methylesterase. The need for calcium ions for pectinolysis[29] was also confirmed by him using an enzyme preparation. It is pertinent to mention here that one other coryneform has been reported to be pectinolytic since then.[30] The *Xanthomonas*, on the other hand, need no special mention as pectinolysis in this genus is a well established fact.

(*e*) *Nutrient agar isolates*: A total of 200 isolates were obtained from an examination of 20 samples of raw sewage.

TABLE V

Bacteria isolated at 25°C on nutrient agar inoculated with raw sewage

Bacteria	No. of Isolates
Aerobacter aerogenes	15
Aerobacter cloacae	10
Achromobacter species	17
Alcaligenes species	3
Brevibacterium species	17
Bacillus cereus	3
Bacillus megaterium	2
Bacillus subtilis	4
Bacillus species	3
Corynebacterium species	4
Escherichia coli	13
Escherichia freundii	7
Flavobacterium aquatile	6
Flavobacterium species	10
Micrococcus species	7
Proteus inconstans	3
Proteus morganii	7
Pseudomonas aeruginosa	8
Pseudomonas fluorescens	5
Pseudomonas ovalis	1
Pseudomonas species	12
Pseudomonas – *Alcaligenes* intermediates	20
Serratia marcescens	5
Xanthomonas species	18

These were classified as shown in Table V. It was not possible to classify all the isolates upto the species level though most of them could be classified upto the generic level. It should be mentioned that the designation "species" in Table 5 after the generic name, (*e.g.*, *Pseudomonas* species) does not mean that all the isolates were similar but refers only to the fact of their generic status. The differences in characteristics of the various isolates have been detailed elsewhere.[5] A group of 20 bacteria, however, were difficult to classify even at the generic level. They were motile with polar flagella but did not metabolise glucose either oxidatively or fermentatively. The former attribute is a characteristic of the pseudomonads, the latter of the genus *Alcaligenes*. The pseudomonads attack glucose oxidatively[31] while the *Alcaligenes* are either peritrichate or nonmotile. The only species which the isolates resembled was *Vibrio alcaligenes* in that this species is polar flagellated and does not attack glucose.[35] However, unlike this species the present isolates were straight rods and so cannot be reasonably included in the genus *Vibrio*. They have therefore been referred to as *Pseudomonas-Alcaligenes* intermediates.

Similar bacteria, having the flagellar pattern of the pseudomonads and the metabolic attributes of the *Alcaligenes*, have been previously described and appear to occur in a variety of environs. The first detailed report on such organisms was by Galarneault and Leifson.[32] All their isolates were lophotrichous and they proposed the name *Lophomonas alcaligenes*, nov. gen., nov. spec., for them. (Whether the creation of a new genus on the basis of flagellar pattern is justifiable, specially in the context of the recent reports by Leifson and Hugh[33] and Sneath[34] on the variability of flagellar patterns is a debatable point). More recently, Moore and Pickett[35] have described similar organisms isolated from a variety of sources including pathological material.

Apparently, these organisms represent a phylogenetic link between the existing genera of bacteria, the *Pseudomonas* and the *Alcaligenes*. Their existence lends support to the view that the concept of well defined species does not hold strictly in classifying bacteria. The observations on the genus *Bacillus*[36] are in accordance with this view, and it has been possible to identify several microbes as intermediates between the well known species. Similar questionable taxonomic situations have been recognised in recent times.[37]

Observations of large number of nutrient agar plates seemed to suggest that in sewage chromogenic and fluorescent bacteria were predominant. A large number of sludges and effluents on examination also showed that whereas activated sludge and the effluent therefrom contained a preponderance of chromogenic and fluorescent bacteria (even more than in raw sewage, thus indicating their role in the activated sludge process) in septic tank sludge and effluent opaque white colonies were the dominant aerobic flora. The samples of septic sludge and effluents examined however revealed a large number of moulds.

Quantitative Studies on the Bacterial population of Sewage and Sludges

A large number of sewage samples were examined for their total bacterial load as well as coliform population. A great variation in the bacterial population of sewage was observed from day to day, and this variation could not be correlated with the chemical quality of the sewage (S. S. Rao, unpublished data). However a proportionality—though not in absolute numbers—existed between the total bacterial load and the coliform population (Figure 1.) This ratio between the total count and the coliform count, in

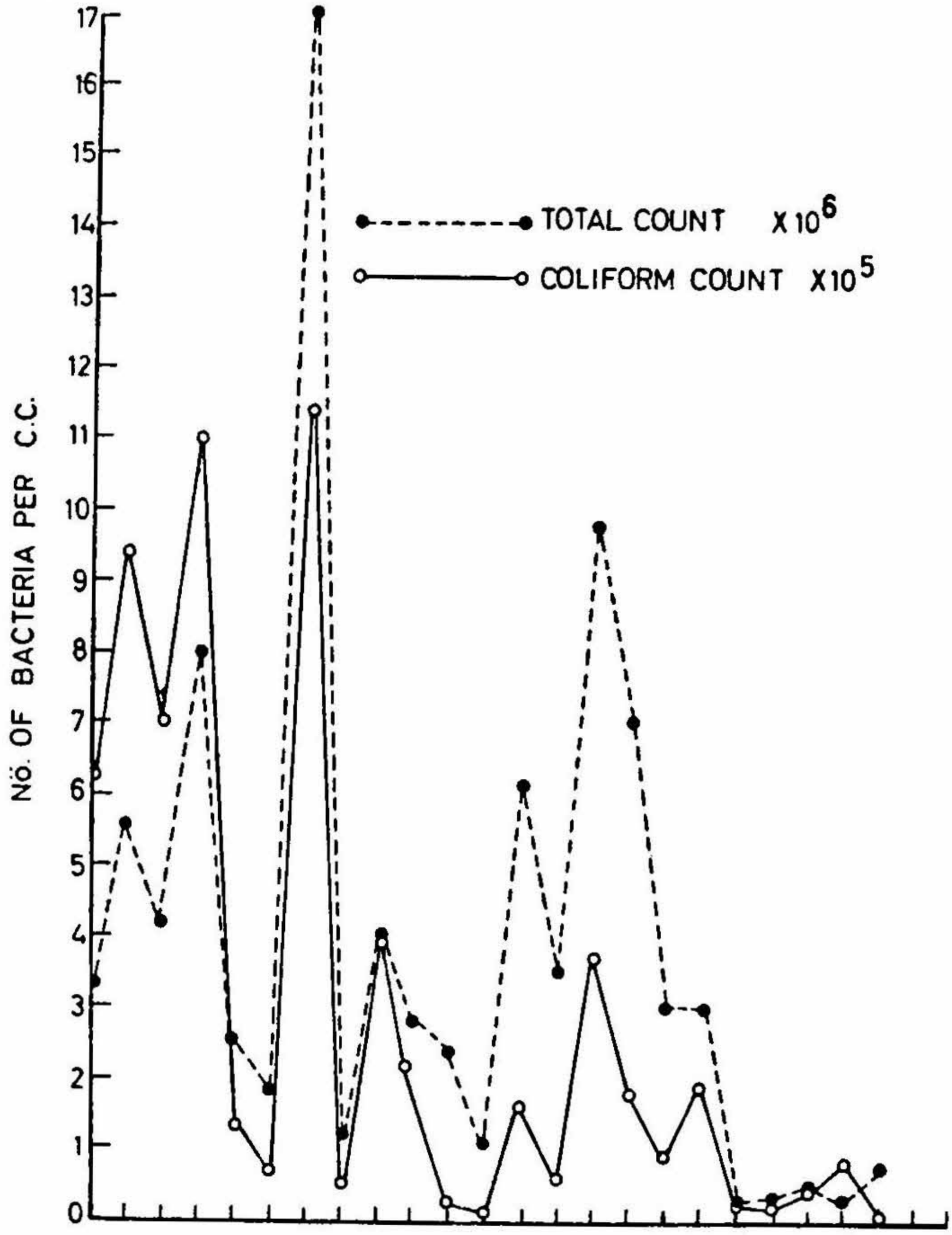

Fig. 1

Comparative Coliform to total Bacterial population of Raw Sewage

contrast to that of faeces, was always greater than one and usually greater than five. With faeces, however, this ratio was always less than one when the same techniques of enumeration were employed. The results for a dilute faecal suspension are recorded in Table VI. Similar results have been previously

TABLE VI

Bacterial population of a dilute faecal suspension (bacteria/ml suspension)

		Total count $\times 10^6$	Coliform count $\times 10^6$
Sample 1	.....	1.06	1.70
Sample 2	.....	3.21	4.01
Sample 3	.....	1.89	2.89

reported by Lobo[38]. That the recorded value of the coliform count was greater than the total count is not as paradoxical as it appears when one considers that MacConkey's agar is a richer medium than nutrient agar. Therefore, in actual fact the difference between the coliform count and total count will be much greater than the observed value. One possible explanation for this observation is that in sewage bacteria other tnan the coliforms are better suited to survive.

TABLE VII

Bacterial population of activated and septic sludges (bacteria/g dry weight)

		Total count $\times 10^6$	Coliform count $\times 10^6$
Activated sludge			
Sample 1	.....	1870	59
Sample 2	.....	2000	58
Sample 3	.....	1000	12.7
Septic sludge			
Sample 1	.....	840	0.15
Sample 2	.....	1000	1.2
Sample 3	.....	55	0.08

The aerobic bacterial population of activated and septic sludges were also enumerated (Table VII.) That the septic sludge contained a smaller number of coliforms than activated sludge was surprising, as these being facultatively anaerobic, a higher population might be expected to persist under the anerobic conditions. However, that the coliforms from only a small portion of the total population lends support to the view that in sewage stabilisation bacteria other than the coliforms play an important part.

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