CHARACTERIZATION OF ACTIVATED SLUDGE IMMOBILIZED IN CALCIUM ALGINATE GEL WITH RESPECT TO BIODEGRADATION OF 2-CHLOROPHENOL AND BENZENE

by
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ABSTRACT

Title of Thesis: Characterization of Activated Sludge Immobilized in Calcium Alginate Gel with respect to Biodegradation of 2-Chlorophenol and Benzene.

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Activated sludges acclimated with phenol and 2-chlorophenol in one case, and a mixture of benzene, toluene, and o, m and p-xylene in another case, were characterized for biodegradation of 2-chlorophenol and benzene, respectively. Activated sludge was obtained from the Parsippany-Troy Hills Water Pollution Control Plant (NJ).

For both cases colonies were isolated before acclimation, after acclimation, and at the end of biodegradation studies. Since the unacclimated group was the

same in both cases, there were a total of five different groups for which characterization was carried out.

For all the five groups together, a total of 54 isolates were obtained and 35 of these could be assigned to a genus. The genera assigned included Bacillus, Lactobacillus, Microbacterium, Plesiomonas, Kurthia, Klebsiella, Corynebacterium and Pseudomonas.

Studies done with individual end run isolates and their mixture, in shaker flasks, established that the primary degrader of 2-chlorophenol was a Bacillus and that of benzene was a Pseudomonas.

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CHAPTER I

INTRODUCTION

Sawyer (1965) has given an excellent account of the developments in the activated sludge process in the first fifty years of its use. Activated sludge is the most widely used and effective method for wastewater treatment.

Activated sludge has been used by researchers to estimate the biodegradability of a host of organic compounds (Tabak et al., 1981; Kincannon et al., 1983; Petrasek et al., 1983; Kim and Maier, 1986; Lewandowski et al., 1986; Weber et al., 1987; Andrews et al., 1988 and Lakhwala et al., 1989). Starting with the pioneering work of Buswell and Long (1923) and Butterfield (1935), other researchers (Allen, 1944; Mckinney and Weichlein, 1953; Prakasam and Dondero, 1967a and 1967b; Adamse, 1968; Strom and Jenkins, 1984 and Lau et al., 1984a and 1984b) have attempted to characterize the complex microbial structure of activated sludge. These studies were aimed in part at understanding two important phenomena observed in activated sludge processes, namely, floc formation and bulking.

Biodegradation of chlorophenols and benzene related hydrocarbons has been studied using pure cultures and / or mixed cultures, with complete knowledge of the identity of the culture(s). In similar studies, where activated sludge

has been used, mixed cultures have not been characterized to find the microorganisms responsible for biodegradation of the substrate in question.

The present study was primarily a supporting work, using immobilized activated sludge for biodegradation of 2-chlorophenol, benzene, and a mixture of BTX. The mixed cultures were characterized in three groups, namely: unacclimated, acclimated and end run. These groups were formed primarily to study the changes in the microbial community of activated sludge that might occur as the mixed culture passes through the stages of acclimation, followed by biodegradation experiments. The unacclimated group is the same, while the acclimated and the end rum groups differ depending on whether the substrate is 2-chlorophenol or benzene.

CHAPTER II

LITERATURE SURVEY

Characterization studies of activated sludge date back to the beginning of the twentieth century. In a historic paper, Buswell and Long (1923) noted that the purification achieved in activated sludge processes is accomplished by ingestion and assimilation of the organic matter in the sewage and its resynthesis into the living material of the flocs. They established that activated sludge is heterotrophic in nature and is a flocculated mass composed mainly of bacteria and protozoa.

Butterfield (1935) was the first to isolate Zooglea ramigera. He showed that a pure culture of Zooglea ramigera produces floc simulated activated sludge and removed oxidizable material present in the polluted water.

Allen (1944) was also one of the early researchers to work on characterization of activated sludge. He found that homogenization of activated sludge facilitates the isolation of the predominant biota by liberating bacteria from the floc. He tested various media to investigate the bacteriology of activated sludge and found nutrient agar to be superior. The majority of the bacteria that he isolated were gram negative rods with no action on carbohydrates, and

were members of the genera Achromobacterium, Chromobacterium and Pseudomonas.

McKinney and Weichlein (1953) reported isolation of seventy two bacteria from sixteen different samples of activated sludge from four sources. Of these, two were municipal sewage treatment plants treating domestic sewage, one was treating industrial wastewater and one was synthetic activated sludge, produced in the laboratory. Of the 72 isolates, 14 were capable of floc formation in sterile synthetic sewage and 12 in sterile settled sewage.

Prakasam and Dondero (1967a and b) studied different agar media for enumeration and characterization of activated sludge. They found plating media containing solely activated sludge extracts to be the best in terms of obtaining higher viable counts as opposed to the nutrient agar of Allen (1944). The composition of activated sludge extract varies depending upon the variables of operation of the treatment plant.

Adamse (1968) compared the bacterial biota of a dairy waste activated sludge with that developed in the laboratory under comparable nutritional conditions and found them to be similar. He concluded that in dairy waste activated sludge, Arthrobacter-like bacteria were predominant.

Lighthart and Oglesby (1968) suggested that a large number of an organism's characteristics have to be

determined in order for one to understand its functional capabilities. They suggested that the wide array of data gathered must have a high degree of definitiveness so as to be processed in a binomial system for final classification.

Benedict and Carlson (1971) compared activated sludge samples from a laboratory unit and a domestic sewage treatment plant. They isolated 129 colonies from high dilution countable plates. They reported one type of yeast along with the different bacterial colonies.

In order to better understand the problem of flocculation, Lin (1984) studied the community structure of activated sludge from five different sources. He also studied samples from a trickling filter and a rotating biological contactor (RBC) type processes. The dominant species seemed fairly constant and belonged to genera of Acinetobacter, Flavobacterium or Pseudomonas.

Another prominent characteristic addressed regarding activated sludge process was that of bulking. Eikelboom (1975) did pioneering work in identifying filamentous microorganisms. He identified the microorganisms to type using microscopic examination coupled with two simple staining procedures.

Strom and Jenkins (1984) studied the correlation between the excessive growth of various types of filamentous organisms and the plant operating conditions associated with

sludge bulking. A total of 226 samples from 78 plants were examined. Using Eikelboom's key, filamentous types were identified. The same group developed a model for activated sludge bulking (Lau et al., 1984a), and studied kinetics of Sphaerotilus natans (a filamentous bacterium) and a floc former in pure and dual continuous culture (Lau et al., 1984b). They concluded that, S. natans predominated at low DO with low to moderate dilution rates, and the floc former at high DO or high dilution rates.

Activated sludge has been used to test the biodegradability of several organic compounds (Shamat and Maier 1980 and Tabak et al. 1981). Since activated sludge processes are used widely for treatment of both municipal and industrial wastes, it is an easily accessible source of biomass.

Degradation of chlorophenols using a mixed microbial community has been reported by Schmidt et al. (1983). The study was initiated to demonstrate that instabilities of the population due to the presence of chlorophenols can be counteracted by the addition of a chloroarene utilizing bacterium. Pseudomonas sp. strain B13 was added as a chlorocatechol dissimilating member of the community.

Rehm's group (1984, 1985 and 1987), studied degradation of 4-chlorophenol by free and immobilized Alcaligenes sp. A7-2. They used different methods of

immobilization: immobilization in polyacrylamide-hydrazide (PAAH), adsorptive immobilization and immobilization in calcium alginate gel. They showed that higher concentrations of 4-chlorophenol were better tolerated and more quickly degraded by immobilized cells than by free cells. Their study included batch as well as continuous operations; however, they did not consider the kinetics of biodegradation.

In a recent study, Crawford et al. (1987) investigated the ability of immobilized Flavobacterium cells to biodegrade pentachlorophenol. They reported substrate inhibition at higher concentrations and used a modified Haldane equation to model the kinetics.

Lee et al. (1976) and Thomas et al. (1986), studied biodegradation of benzene using microorganisms of marine origin. Although these studies point towards biodegradability of benzene and some of its derivatives, the studies were qualitative in nature.

Weber et al. (1987)studied the effect of addition of powdered activated charcoal to activated sludge to enhance the removal of organics. They concluded that benzene, toluene and o-xylene were effectively biodegraded in activated sludge systems under steady state conditions.

Andrews et al. (1988) used a consortium of microorganisms developed by municipal sewage enrichments

with a mixture of benzene, toluene and xylenes. The consortium so developed was immobilized on celite carrier (R-635). They reported a very high percentage of influent levels being removed biologically, but their influent concentrations were moderately low (40 ppm). They concluded that biological degradation of gasoline components contained in contaminated groundwaters using immobilized microorganisms is a viable remediation process.

CHAPTER III

OBJECTIVES

This study was primarily aimed as support for biodegradation studies of 2-chlorophenol (Yang, 1988) and benzene (Lodaya, 1989) using immobilized activated sludge. The objectives set forth included:

1. Characterization of mixed cultures in five groups namely, unacclimated, and acclimated and end run for both 2-chlorophenol and benzene.

2. To conduct biodegradation experiments with individual end run isolates and their mixtures, to identify the primary degraders of, 2-chlorophenol and benzene.

CHAPTER IV

MATERIALS AND EXPERIMENTAL METHODS

4.1 MICROORGANISMS

Activated sludge (a mixed microbial community) from the Parsippany-Troy Hills Water Pollution Control Plant (N.J.) was used in the study. Prior to the acclimation, a portion of the mixed liquor was centrifuged (3,000 rpm and 15^{o}C) soon after it was brought from the plant, and stored as unacclimated (UNA) pellets at 4^{o}C for one month.

For biodegradation studies of 2-chlorophenol, microorganisms were acclimated to phenol and 2-chlorophenol at room temperature. The microorganisms were spiked with 100 ppm phenol daily for five days followed by 10 ppm of 2-chlorophenol daily for another five days. Aeration was used to provide dissolved oxygen. During this period, the oxygen consumption of a fixed amount of sludge was measured daily in the microassay reactor (Figure 4.1), which is described later in the text. The oxygen consumption decreased initially, then remained constant, at which point the acclimation was considered to be complete. After the acclimation was complete, the mixed liquor was again centrifuged and the pellets obtained were stored as acclimated (AAC) pellets at 4^{o}C.

microorganisms were acclimated to a mixture of benzene, toluene and o, m and p xylene at room temperature. The microorganisms were spiked daily over a period of seven days with 10 ppm each of benzene, toluene and o, m and p xylene. A 3% solution of H_2O_2 was used to provide the dissolved oxygen. The measurments for oxygen consumption were done as described before. The pellets obtained after centrifuging were stored as acclimated (AAB) pellets at 4^oC.

4.2 IMMOBILIZATION

For a given batch of pellets (50 g), a typical procedure for making beads is as follows. Distilled water and concentrated pellets (55 mg dry biomass/g of pellet) were taken in a ratio of 5:2 by weight along with sodium chloride (0.05% w/w) in a blender. Sodium alginate (1.5% w/w) was then added slowly over a period of 2 to 3 minutes to the mixture, with continuous stirring to obtain a homogeneous cell suspension. The homogeneous cell suspension was then extruded as discrete droplets using a syringe pump, into a slowly stirred 500 ml solution of 0.1 M calcium chloride. On contact with the calcium chloride solution, the droplets hardened to form beads about 3 to 3.5 mm in diameter. The beads were then cured in 0.1 M calcium chloride for 24 hours at 4^oC before use.

4.3 ISOLATION AND COUNTING OF VIABLE COLONIES

Alginate beads (25 g) of unacclimated, acclimated and end run (after running the biodegradation experiments) cultures for both 2-chlorophenol (ERC) and benzene (ERB) were seperately mixed with 25 ml of sterile distilled water each and blended for one minute at high speed. Then 0.5 ml of the resulting homogeneous mixture was added to 4.5 ml of 0.1% (w/v) tween solution to further break the floc. Following this, serial dilution to 10^{-7} times was done and 0.1 ml of each dilution was surface spread on agar plates (in triplicate). The selection of different agars used was based on Prakasam and Dondero (1967) which included, floc agar (FA) (Peptone, 2 g; Yeast Extract, 1 g; Agar, 20 g/l), plate count agar (PCA), MacConkey agar (MCA), trypticase soy agar (TSA) Nutrient agar (NA) and casitone glycerol yeast extract (CGY) (Casitone, 1 g; Glycerol, 2 g; Yeast Extract, 0.2 g; Agar, 15 g/l) agar. Whereas the other agars were obtained from BBL (Cockeysville, MD), the composition of CGY agar was based on Lin (1984), and the composition of floc agar was based on Benson (1985).

The highest number of characteristically different colonies grew on CGY agar plates. Following this, further isolation was done using CGY agar plates. Colonies showing the same characteristics on the agar plate were all considered as being of the same type.

Nutrient broth was used as the storage medium for the isolated colonies. Following the transfer of isolated colonies, a day's time was allowed for growth and subsequently the broth was stored at 4°C.

4.4 IDENTIFICATION OF ISOLATES

Colony characteristics (shape, size, margin elevation and color), morphological characteristics (cell size, shape and motility) and biochemical tests (Hugh, 1975) were used for identification of isolates. Incubations were performed at room temperature (25 to 27°C) and the inoculum was obtained from nutrient broth unless otherwise noted. The biochemical tests on different agar mediums were done using slants unless otherwise noted.

1. Colony characteristics.

The size, shape, margin, elevation and color of the colony were observed and noted after ten days of incubation on CGY agar plates. Appendix I illustrates the different combinations of shape, margin and elevation generally prevalent in bacterial colonies.

2. Morphological characteristics.

A young culture (18-24 hours) of each isolate grown in nutrient broth was examined in a wet mount to determine the

cell size, shape, and motility. Motility was also confirmed by observing the lateral movement of the colonies on SIM medium. For this purpose, SIM medium (BBL) was prepared in tubes and cultures were stabbed in the center of the agar. Standard gram stain procedures were adopted to identify gram positive and gram negative bacteria.

3. Carbohydrate reactions.

Oxidative fermentative (OF) basal medium containing 1% (w/v) glucose was used for detection of small quantities of oxidative acidity. For determining fermentation, a layer of mineral oil was aseptically added to the surface immediately after inoculation. A positive reaction was determined after 3 to 4 days of incubation in terms of a change in color of the medium from green to yellow.

4. Hydrogen sulfide production.

KIA agar (BBL) was used to detect the production of hydrogen sulfide. The positive reaction is observed in the form of a black butt at the base of the slant, after four days of incubation.

5. Indole production.

Indole production was detected by adding Kovac's reagent to cultures grown on slants of SIM medium (BBL).

Immediate formation of pinkish color indicated a positive reaction. The culture was grown for 4 days before testing.

6. Presence of β-D-galactosidase.

β-D-galactosidase was detected by adding 0.3 ml of o-Nitrophenyl-β-D-galactoside (ONPG) reagent, after incubating the one day old culture in CGY broth, at 37°C for ten minutes. A yellow color is a positive reaction.

7. Esculin hydrolysis.

Esculin hydrolysis was detected by observing for a black precipitate around the growth on a TSA agar (BBL) plate supplemented with 0.1% (w/v) esculin and 0.05% (w/v) ferric citrate. Culture was checked for positive reaction after 3 days of incubation.

8. Test for presence of catalase.

Catalase presence was evidenced by the formation of bubbles when a drop of hydrogen peroxide solution (30 g/l) was added to a smear on a slide. The inoculum was obtained from a CGY agar plate after seven days of incubation.

9. Nitrate reduction.

Nutrient broth supplemented with 0.1% (w/v) potassium nitrate was used to detect the reduction of nitrate to

nitrite. A pronounced red color upon addition of two drops of 0.8% (w/v) sulfanilic acid and two drops of 0.5% (w/v) N,N dimethyl-alpha-naphthyl amine to the culture broth after seven days of incubation indicates presence of nitrite. Reduction of nitrite to N_2 gas was detected by bubble formation in the tube prior to chemical addition. This can also be detected by adding a speck of zinc powder to the broth. Formation of red color only after addition of zinc indicates the presence of nitrate, whereas no color development will indicate that the reduction is complete.

10. Oxidase test.

The oxidase test was performed to determine the presence of cytochrome c. If present it oxidizes the oxidase reagent (N,N,N'tetramethyl-p-phenylenediamine). Formation of a blue color is a positive reaction. The inoculum was obtained from a CGY agar plate after seven days of incubation.

11. Urease production.

Urea agar (BBL) was used to detect the production of urease. A change in color of the indicator phenol red within 3 days of incubation, from yellow to red because of the production of ammonia, was considered as a positive test.

12. Growth on MacConkey agar.

Growth on MCA agar (BBL) was also tested. For this purpose, MCA agar plates were made. Inoculum was allowed to grow for 5 days.

13. Test for spore formation.

For identifying the sporeformers, soil extract agar was used (Gordon et al., 1973). Slants of the agar were streaked and incubated. Smears of culture were air dried and stained with safranin for 30 seconds. Unstained spores within and outside the sporangia were easily recognized. Isolates were checked for spore formation at the end of the 1st, 2nd, 4th, 6th and 8th day. Whereas most of the sporeformers showed positive results by the 2nd day, a few of them showed positive results as late as the 6th day.

14. Flagellar morphology.

The fuchsin-tannic acid method, described by Leifson (1960) was used. However, instead of transferring the growth on solid medium to distilled water for preparing the slide, young cultures (18-24 hours) grown in nutrient broth were used. Motility was checked by preparing a wet mount prior to slide making. A slide prepared from a culture in which a large proportion of the cells were motile, was generally a success. Among the tests done, this was the most difficult

test in terms of obtaining a definite outcome.

4.5 SHAKER FLASK EXPERIMENTS

The end run isolates were tested individually and collectively for biodegradation of 2-chlorophenol and benzene respectively.

Sterile Erlenmeyer flasks, each containing 150 ml nutrient medium (Table 4.1), were inoculated using 0.1 ml of nutrient broth stock cultures under sterile conditions. Whereas 2-chlorophenol was added at a concentration of 10 ppm, benzene was added at a concentration of around 150 ppm. Benzene or 2-chlorophenol were added as the only carbon source. Control flasks containing 150 ml of nutrient medium and 2-chlorophenol or benzene at the same respective levels, were also mounted on the shaker (Lab-Line, Model 3528, reciprocating type) along with the others.

Periodic samples were taken and analyzed by GC for removal of both 2-chlorophenol and benzene.

4.6 DISSOLVED OXYGEN MEASUREMENTS IN MICROASSAY REACTOR

The experimental set up of the microassay reactor is shown in Figure 4.1. The microassay reactor is a small jacketed vessel of 1.8 ml capacity, with provision for a Clark-type dissolved oxygen probe. This reactor has been a valuable tool in enzyme kinetic studies because the data

produced are accurate and reproducible. It is also economical as it requires very small amounts of reagents. In the present work, the reactor proved suitable for easy measurements of dissolved oxygen, the parameter to be monitored. Water at the required temperature was circulated through the jacket, and the reactor was mounted on a magnetic stirrer plate.

TABLE 4.1: COMPOSITION OF THE NUTRIENT MEDIUM

Magnesium chloride	100 mg
Manganese sulfate	10 mg
Ferric chloride ...	0.5 mg
Potassium phosphate	10 mg
Ammonium sulfate	10 mg
Distilled Water	100 ml

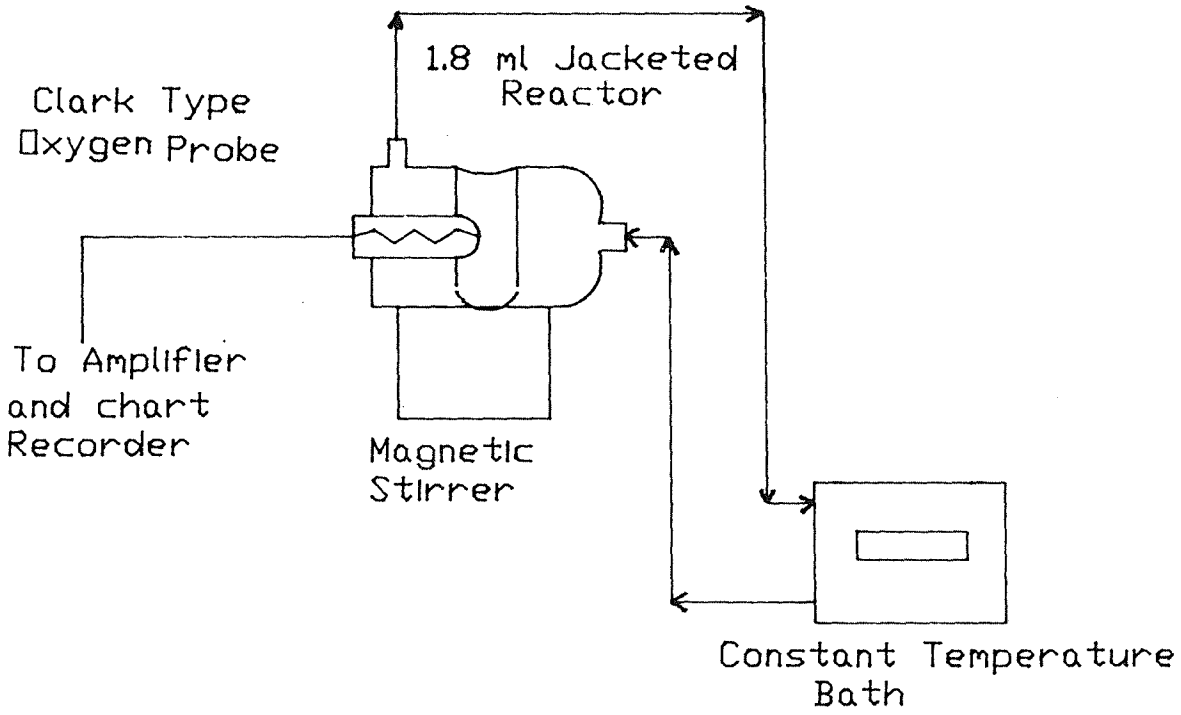

Fig. 4.1 Microassay Reactor

CHAPTER V

RESULTS AND DISCUSSION

5.1 COMPARISON OF THE MEDIA

Different agar media were used with a view to choosing the agar that offered the maximum number of characteristically different colonies. As can be seen from Table 5.1, the highest number of different colony types were seen on CGY agar. Thus, CGY agar was chosen as the isolation medium.

5.2 PLATE COUNT OF VIABLE COLONIES

Plate counts were carried out for the unacclimated (UNA) group of isolates, both of the acclimated groups (AAC and AAB), and both the end run groups (ERC and ERB). When expressed as 10^6 per gram of wet beads, the counts were 210 for unacclimated, 0.063 and 9.8 for AAC and AAB respectively, and 1.3 and 12 for ERC and ERB respectively.

The reduction in viable count by more than an order of magnitude as a result of acclimation could be due to competition between different microorganisms, or may be that toxicity of 2-chlorophenol or benzene to some microorganisms, or the inability of some of the microorganisms to use 2-chlorophenol or benzene as a carbon

source.

Plate counts were primarily done to observe the relative change in the viable count among unacclimated, acclimated and end run cultures. The actual numbers may have been different since the blending time was not optimized.

5.3 IDENTIFICATION OF THE ISOLATES

In order to facilitate analysis, the tests upon which assignments to genus usually would be made using Skerman's (1974) key were expressed as a 4 digit number (Table 5.2). This method of expression has been used by Lin (1984), and is based on API (Analytab Products Co., Plainview, NY) data handling procedure for identification of Enterobacteriaceae. The individual tests used for this purpose were different. The results of other biochemical tests performed are recorded (Tables 5.3, 5.4, 5.5, 5.6 and 5.7). These results were used for additional confirmation of the genera and possibly for species identification. An exception to the use of Skerman's key was identification of Bacillus. Any isolate showing endospore formation was identified as Bacillus, irrespective of whether it was gram positive or gram negative (Gordon et al., 1973). Details of endospores of some isolates are shown in Appendix II. Appendix III contains, pictures showing flagellar morphology of some isolates.

5.3 (i) Identification of bacteria in the unacclimated group

Seven of the 13 different isolates obtained under this heading were assigned to five different genera, namely Bacillus (3), Lactobacillus (1), Microbacterium (1), Plesiomonas (1) and Pseudomonas (1). The remaining six isolates could not be assigned a genus name.

An unusual feature noted here when compared to earlier studies done with activated sludge identification was that over half (7/13) of the isolates were gram positive. A majority of isolates were rods and produced acid from glucose. Motility was also observed to be prevalent. Spore formation was observed in 3 isolates. Table 5.3 shows the results of other biochemical tests and the coding factor and the genus assignment obtained for each isolate.

5.3 (ii) Identification of bacteria in the acclimated group for 2-chlorophenol (AAC)

In this group 8 different isolates were obtained. Of these, 4 could be assigned to a genus. The genera identified were, Bacillus (1), Microbacterium (1), Plesiomonas (1), and Kurthia (1).

In this case also the majority of the isolates were rods producing acid from glucose. A predominance of gram negative isolates was observed. Six isolates were motile and

spore formation was observed in one. Table 5.4 lists the results of other biochemical tests, the coding factor and genus assignment obtained for each isolate.

5.3 (iii) Identification of bacteria in the end run group for 2-chlorophenol (ERC)

Of the 7 different isolates obtained, 5 were assigned a genus name. These were, Bacillus (4) and Microbacterium (1).

All but one of the isolates were rods, with about even distribution of gram positive and gram negative bacteria. Spore formation was observed in 4 of them and 4 were motile. Table 5.5 lists the results of other biochemical tests, the coding factor and the genus assignments obtained for each isolate.

5.3 (iv) Identification of bacteria in the acclimated group for benzene (AAB)

In this group 11 different isolates were obtained. Of these, 9 could be assigned to a genus. The genera identified were, Bacillus (4), Microbacterium (1), Plesiomonas (1), Kurthia (1), Klebsiella (1) and Pseudomonas (1).

More than 50% of the isolates were gram positive, all of them being rods. Spore formation was observed in 4 and a high percentage were found to be motile. Table 5.6 lists the

results of other biochemical tests the coding factor and the genus assignment obtained for each isolate.

5.3 (v) Identification of bacteria in the end run group for benzene (ERB)

Of the 15 different isolates obtined, 10 were assigned a genus name. These were, Bacillus (3), Microbacterium (1), corynebacterium (1), Plesiomonas (1), and Pseudomonas (4).

Here also, all the isolates were rods, with about even distribution of gram positive and gram negative. Spore formation was observed in 3 of them and 10 were motile. Table 5.7 lists the results of the other biochemical tests, the coding factor and the genus assignments obtained for each isolate.

5.3 (vi) Difficulties in identification of cultures

Identification of bacteria in activated sludge flocs is difficult and extremely complex. This has been observed by earlier workers also. Lack of a systematic approach and use of contradictory names for the same bacteria makes the task of identification doubly difficult. This is reflected in the number of unidentified isolates (19) from the total of 54 isolates from the five different groups.

Because of its poor success, use of Skerman's (1974) key to the genera may not be an ideal method to identify

isolates from samples such as activated sludge. Furthermore, if the bacteria in activated sludge were not found before in other environments, they would not be included in Bergey's Manual.

5.4 SHAKER FLASK EXPERIMENTS

Isolates from the end run group of both 2-chlorophenol and benzene were each tested individually and collectively for 2-chlorophenol or benzene consumption respectively, in the shaker flasks.

As seen from Table 5.8, ERC_3 which was identified as a Bacillus, shows distinct removal of 2-chlorophenol. This establishes a Bacillus as the primary degrader of 2-chlorophenol among the isolates present.

As seen from Table 5.9, ERB_1 which was identified as a Pseudomonas, shows distinct removal of benzene. This establishes a Pseudomonas as the primary degrader of benzene among the isolates present.

TABLE 5.1 COMPARISON OF THE ISOLATION MEDIA

Inoculum: Unacclimated group

Dilutions plated: 4^{th}, 5^{th} and 6^{th}

TYPE OF AGAR	NUMBER OF DIFFERENT COLONY TYPES
TSA	1
FA	2
PCA	3
MCA	3
NA	7
CGY	13

TABLE 5.2: CODING OF TEST RESULTS

Test	Coding Factor	Example Response	Octonary Code
Gram Stain	1	+	1
OF-O	2	–	
OF-F	4	–	
Motility	1	+	3
Polar Flagella	2	+	
Peritrichous Flagella	4	–	
Rod Shape	1	+	1
Size (≥1μm)	2	–	
Size (≤0.5μm)	4	–	
Spore Formation	1	+	5
Oxidase	2	–	
Catalase	4	+	
Coded Result 1315			

Each positive response is considered a one, and each negative zero. Each binary coded response is multiplied by its coding factor, and the results for each triplet are summed. This procedure is repeated for all isolates.

TABLE 5.3: TEST RESULTS FOR THE UNACCLIMATED GROUP OF ISOLATES

Unacclimated (UNA)		1	2	3	4	5	6	7	8	9	10	11	12	13
Coded Result from the 12 primary tests		1331	6315	5330	5024	6350	4354	5310	5314	6310	7016	1512	0316	0015
Identified Genus		G1	G1	G5	G6	G7	–	–	–	–	–	–	G8	G1
Colony Color		–	–	–	Y	–	–	–	–	–	R/V	–	–	Y
Urease		–	+	+	–	–	–	+	–	+	+	+	–	+
Esculin		+	+	–	+	–	–	–	–	–	+	–	+	–
Nitrate Reduction		+	+	–	+	–	+	–	–	–	+	+	+	–
Growth on MacConkey		–	+	–	–	+	–	+	+	+	–	+	+	–
Indole Production		+	+	+	–	+	+	–	+	+	–	+	–	–
H_2S Production		+	+	+	+	–	–	–	–	+	–	+	+	–
β–D–Galactosidase		–	+	+	+	–	–	–	–	–	–	–	–	–
Colony Characteristics	Configuration	CC	LF	CM	CM	FM	IS	RS	CM	LF	CC	W	IS	RA
	Margin	WA	WA	WA	S	BR	LO	S	LO	S	S	WA	IR	WA
	Elevation	HI	CN	HI	CN	CN	UM	CN	HI	UM	CN	HI	F	UM

BR = Branching
CI = Ciliate
CC = Concentric
CM = Complex
CN = Convex
CR = Crateriform
DL = Drop-Like
F = Flat
FF = Filiform
FM = Filamentous
+ = Positive Response
HI = Hilly
HL = Hair-Lock Like
IR = Irregular
IS = Irregular and Spreading
– = Negative Response
LF = L-Form
LO = Lobate
R = Round
RH = Rhizoid
RI = Raised
RR = Round with Raised Margin
RA = Round with Radiating Margin
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WA = Wavy
WO = Wooly
UM = Umbonate
Y = Yellow
R/V = Red/Violet
G1 = Bacillus
G2 = Corynebacterium
G3 = Klebsiella
G4 = Kurthia
G5 = Lactobacillus
G6 = Microbacterium
G7 = Plesiomonas
G8 = Pseudomonas

TABLE 5.4: TEST RESULTS FOR ACCLIMATED GROUP OF ISOLATES (2-CHLOROPHENOL)

Acclimated 2-Chlorophenol (AAC)		1	2	3	4	5	6	7	8
Coded Result from the 12 primary tests		6315	7006	6314	1314	7014	1314	1514	6356
Identified Genus		G1	–	G7	–	G6	–	G4	–
Colony Color		–	Y	–	–	–	–	–	–
Urease		+	–	+	+	+	+	–	+
Esculin		+	–	–	+	–	+	–	+
Nitrate Reduction		+	+	–	–	–	–	+	–
Growth on MacConkey		–	–	+	–	+	–	–	+
Indole Production		–	–	+	+	–	–	–	–
H_2S Production		–	+	–	+	–	+	+	+
β–D–Galactosidase		+	–	–	–	–	–	–	–
Colony Characteristics	Configuration	LF	R	RS	W	IS	CC	RA	FM
	Margin	HL	S	WA	IR	LO	S	BR	BR
	Elevation	UM	CN	CN	CN	UM	DL	CN	F

BR = Branching
CI = Ciliate
CC = Concentric
CM = Complex
CN = Convex
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G1 = Bacillus
G2 = Corynebacterium
G3 = Klebsiella
G4 = Kurthia
G5 = Lactobacillus
G6 = Microbacterium
G7 = Plesiomonas
G8 = Pseudomonas

TABLE 5.5: TEST RESULTS FOR END-RUN GROUP OF ISOLATES (2-CHLOROPHENOL)

End Run 2-Chlorophenol (ERC)		1	2	3	4	5	6	7
Coded Result from the 12 primary tests		1517	6356	1017	6316	6317	0025	7014
Identified Genus		G1	–	G1	–	G1	G1	G6
Colony Color		–	Y	–	–	–	–	–
Urease		+	+	+	+	+	+	+
Esculin		+	–	–	–	+	+	+
Nitrate Reduction		+	+	–	–	–	–	+
Growth on MacConkey		–	–	–	–	–	+	+
Indole Production		–	–	–	–	–	–	–
H_2S Production		–	+	–	+	–	–	+
β–D–Galactosidase		+	–	+	–	+	+	–
Colony Characteristics	Configuration	FM	RR	LF	R	R	RA	FM
	Margin	BR	S	IR	S	S	BR	BR
	Elevation	CN	CR	UM	DL	CN	UM	UM

BR = Branching
CI = Ciliate
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CM = Complex
CN = Convex
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HI = Hilly
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R/V = Red/Violet
G1 = Bacillus
G2 = Corynebacterium
G3 = Klebsiella
G4 = Kurthia
G5 = Lactobacillus
G6 = Microbacterium
G7 = Plesiomonas
G8 = Pseudomonas

TABLE 5.6: TEST RESULTS FOR THE ACCLIMATED GROUP OF ISOLATES (BENZENE)

Acclimated Benzene (AAB)		1	2	3	4	5	6	7	8	9	10	11
Coded Result from the 12 primary tests		1315	6314	0314	5054	0315	1017	6316	1514	1515	6014	1554
Identified Genus		G1	G7	G8	G6	G1	G1	–	G4	G1	G3	–
Colony Color		Y	R/V	–	Y	Y	–	–	Y	R/V	–	–
Urease		+	+	–	+	+	–	+	–	–	–	–
Esculin		+	–	–	+	+	–	–	+	–	+	–
Nitrate Reduction		+	–	–	–	+	–	+	–	+	+	+
Growth on MacConkey		–	+	+	–	–	+	+	+	+	+	–
Indole Production		–	–	–	–	–	–	–	–	–	–	–
H_2S Production		–	–	–	–	–	–	+	–	–	–	–
β–D–Galactosidase		–	–	–	–	–	–	–	–	–	+	–
Colony Characteristics	Configuration	RA	R	R	R	R	FF	RS	CC	RA	LF	R
	Margin	BR	WA	S	S	S	HL	WA	S	BR	WA	S
	Elevation	UM	CN	UM	CN	CN	HI	F	UM	CN	F	DL

BR = Branching
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CM = Complex
CN = Convex
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WO = Wooly
UM = Umbonate
Y = Yellow
R/V = Red/Violet
G1 = Bacillus
G2 = Corynebacterium
G3 = Klebsiella
G4 = Kurthia
G5 = Lactobacillus
G6 = Microbacterium
G7 = Plesiomonas
G8 = Pseudomonas

TABLE 5.7: TEST RESULTS FOR THE END-RUN GROUP OF ISOLATES (BENZENE)

End Run Benzene (ERB)		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Coded Result from the 12 primary tests		6316	1315	1314	1117	1054	7316	6314	5316	0356	0354	1014	6517	1017	0316	1014
Identified Genus		G8	–	G2	G1	–	–	G7	G6	G8	G8	–	G1	G1	G8	–
Colony Color		Y	–	Y	–	–	–	–	–	Y	–	–	–	–	Y	–
Urease		–	–	–	–	–	+	+	+	–	–	+	+	+	+	+
Esculin		+	–	+	–	+	–	+	–	+	+	+	+	+	+	+
Nitrate Reduction		+	+	+	+	+	+	+	+	–	–	+	+	+	+	+
Growth on MacConkey		+	–	–	+	–	+	+	+	–	–	–	+	+	–	–
Indole Production		–	–	–	–	–	–	–	–	+	–	–	+	–	–	–
H_2S Production		–	–	–	–	–	+	–	–	+	–	–	–	–	–	–
β–D–Galactosidase		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Colony Characteristics	Configuration	R	R	R	RR	RA	RA	RR	R	R	R	CN	R	CN	R	RA
	Margin	RS	S	S	S	BR	S	S	S	S	S	WA	S	WA	S	IR
	Elevation	UM	CN	CN	CR	RI	DL	CR	CN	DL	UM	CN	RI	F	CN	RI

BR = Branching
CI = Ciliate
CC = Concentric
CM = Complex
CN = Convex
CR = Crateriform
DL = Drop-Like
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FF = Filiform
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G2 = Corynebacterium
G3 = Klebsiella
G4 = Kurthia
G5 = Lactobacillus
G6 = Microbacterium
G7 = Plesiomonas
G8 = Pseudomonas

TABLE 5.8: SHAKER FLASK EXPERIMENTS FOR REMOVAL OF 2-CHLOROPHENOL BY INDIVIDUAL END RUN (ERC) ISOLATES

Temperature : 25°C
Shaker speed : 130 rpm
Reaction volume: 150 ml
Sampling period: 24 h

SPIKE #	1	2	3	4
SAMPLE	(mg of 2-chlorophenol removed)			
CONTROL	0.50	0.52	0.51	0.53
ERC_1	0.54	0.51	0.52	0.57
ERC_2	0.61	0.56	0.58	0.60
ERC_3	0.70	0.75	0.95	1.05
ERC_4	0.57	0.55	0.53	0.52
ERC_5	0.51	0.56	0.58	0.65
ERC_6	0.52	0.54	0.55	0.61
ERC_7	0.50	0.55	0.57	0.52
MIXTURE	0.59	0.66	0.84	0.99

TABLE 5.9: SHAKER FLASK EXPERIMENTS FOR REMOVAL OF BENZENE BY INDIVIDUAL END RUN (ERB) ISOLATES

Temperature : 25°C
Shaker speed : 60 rpm
Reaction volume: 150 ml
Sampling period: 6 h

SPIKE #	1	2	3	4
SAMPLE	(mg of benzene removed)			
CONTROL	17.78	15.31	16.51	15.35
ERB_1	20.18	20.41	24.31	28.57
ERB_2	17.02	14.70	15.12	15.60
ERB_3	16.05	15.52	15.15	16.05
ERB_4	17.27	13.57	16.34	16.35
ERB_5	16.05	15.90	15.45	15.45
ERB_6	18.00	14.12	16.02	15.75
ERB_7	15.83	15.45	15.95	14.70
ERB_8	14.59	16.20	16.10	16.50
ERB_9	17.37	16.35	15.00	13.45
ERB_{10}	16.75	13.85	15.00	16.65
ERB_{11}	14.76	14.32	15.15	13.35
ERB_{12}	18.13	13.56	14.50	14.48
ERB_{13}	14.96	14.85	16.25	15.30
ERB_{14}	16.88	15.90	14.85	14.40
ERB_{15}	15.97	14.25	14.35	14.40
MIXTURE	18.59	19.56	23.34	25.89

CHAPTER VI

CONCLUSIONS

The community of bacteria present in activated sludge was studied with respect to biodegradation of 2-chlorophenol and benzene. Based on the results obtained, the following conclusions can be derived.

CGY agar was found to be the best as an isolation medium.

Gram positive and gram negative rods were equally prominent. A good percentage of isolates found were motile. Glucose fermentors and non-fermentors were both common.

For the unacclimated group (which was common to both 2-chlorophenol and benzene), 7 of the 13 isolates were assigned to a genus. These were Bacillus, Microbacterium, Plesiomonas, Lactobacillus, and Kurthia.

In the case of 2-chlorophenol, 9 of the 15 isolates (combining acclimated and end run groups) could be assigned to a genus. These were, Bacillus, Microbacterium, Plesiomonas and Kurthia.

In the case of benzene, 19 of the 36 isolates (combining acclimated and end run groups) could be assigned to a genus. These were, Bacillus, Microbacterium, Plesiomonas, Kurthia, Klebsiella, Lactobacillus, and Pseudomonas.

An inability to identify all of the isolates is common in the literature.

When the results are compared on the basis of the 4 digit code representing the 12 primary test factors, similarity is observed among the groups, although that is not always the case within a group. For the end run group, 4 isolates belonged to the genus Bacillus in the case of 2-chlorophenol, and the same number of isolates belonged to Pseudomonas in the case of benzene. On the other hand if the results of all the tests (not just the 12 primary ones) are considered for comparison, there is hardly any similarity that can be observed. This is expected, as the colonies were picked selectively.

For 2-chlorophenol, the primary degrader was identified to be a Bacillus, while for benzene it was found to be a Pseudomonas.

CHAPTER VII

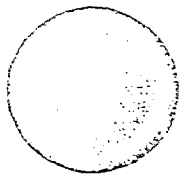
SUGGESTIONS FOR FURTHER WORK

In the present study the community structure of the activated sludge used in biodegradation studies of 2-chlorophenol (Yang, 1988) and benzene (Lodaya, 1989), respectively was identified. The most eminent suggestions that fall out from the study are discussed below.

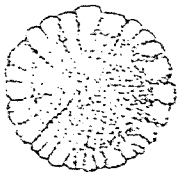
Activated sludge as such was used in the present work. It will be worthwhile to compare the removal rates obtained, in each case when the primary degrader is used instead of the mixed culture.

Further identification of the primary degrader to the species level if possible, for both 2-chlorophenol and benzene will be appropriate.

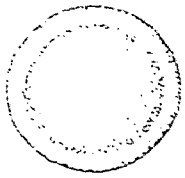
APPENDIX I



1. Round

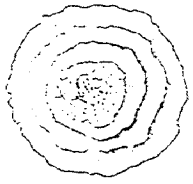


2. Round with Scalloped Margin



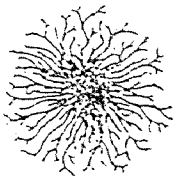
3. Round with Raised Margin

4. Wrinkled

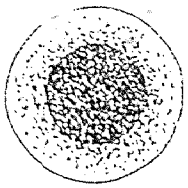


5. Concentric

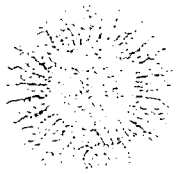
6. Irregular and Spreading



7. Filamentous



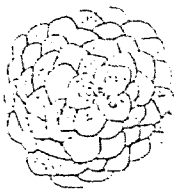
8. L-Form



9. Round with Radiating Margin

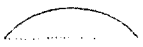
10. Filiform

11. Rhizoid

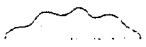


12. Complex

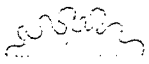
CONFIGURATIONS



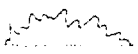
1. Smooth (Entire)



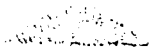
2. Wavy (Undulate)



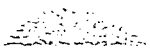
3. Lobate



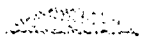
4. Irregular (Erose)



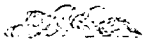
5. Ciliate



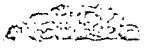
6. Branching



7. Wooly



8. Thread-Like

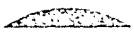


9. "Hair-Lock"-Like

MARGINS

1. Flat

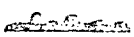
2. Raised



3. Convex

4. Drop-Like

5. Umbonate



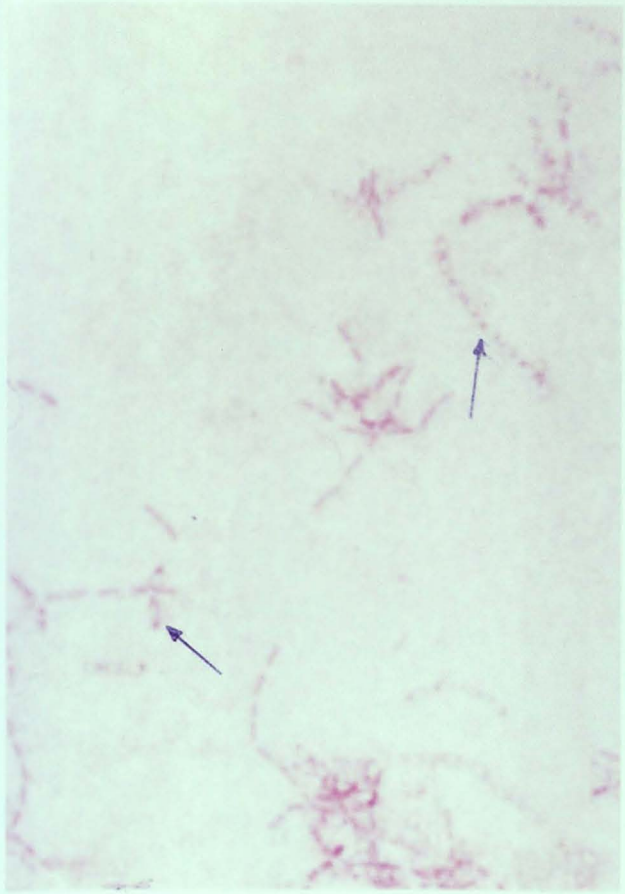
6. Hilly

7. Ingrowing Into Medium

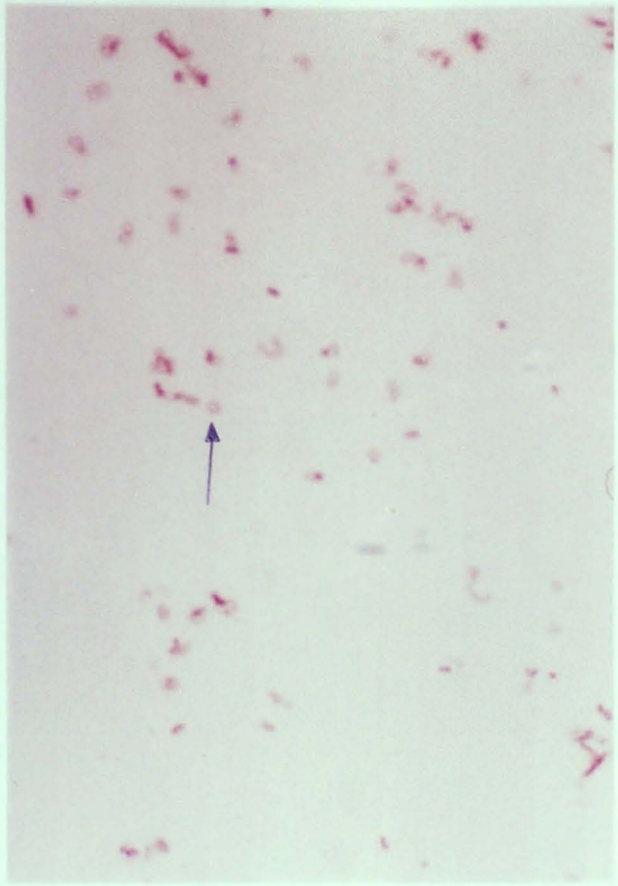
8. Craterform

ELEVATIONS

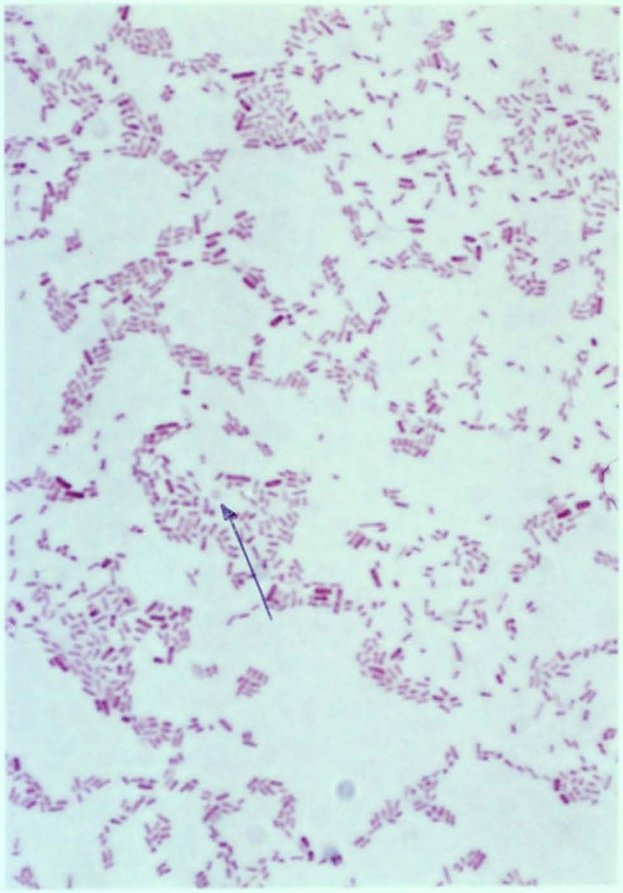
Colony characteristics.



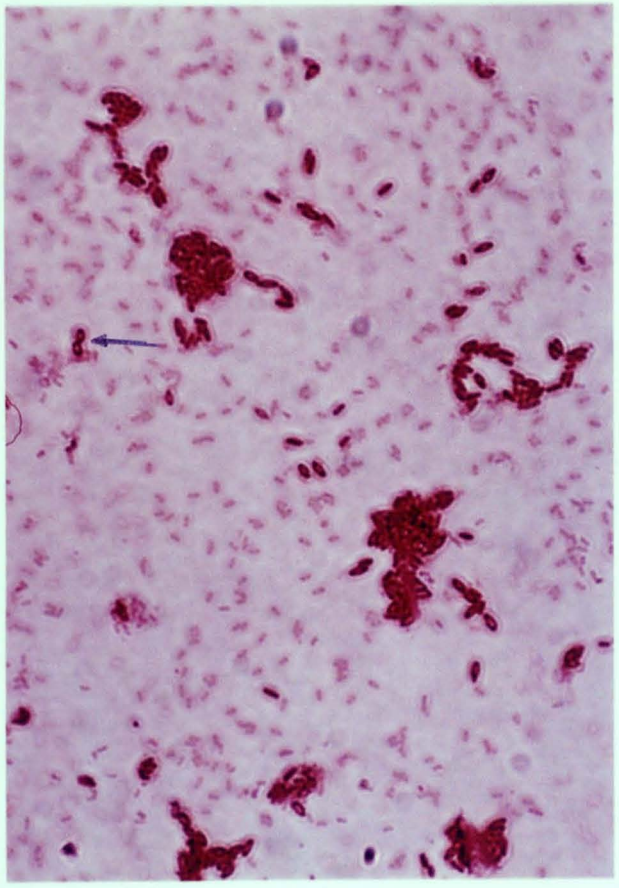
AAC_1



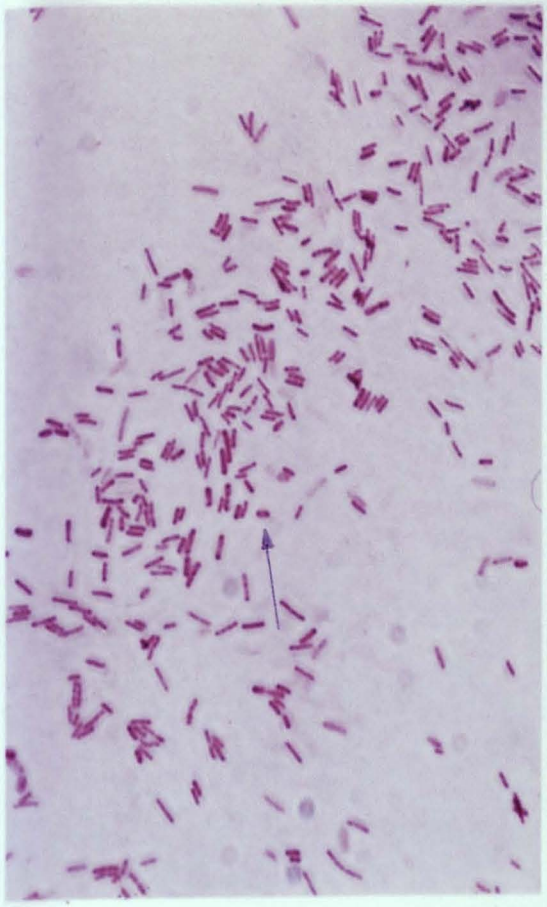
BAA_{11}



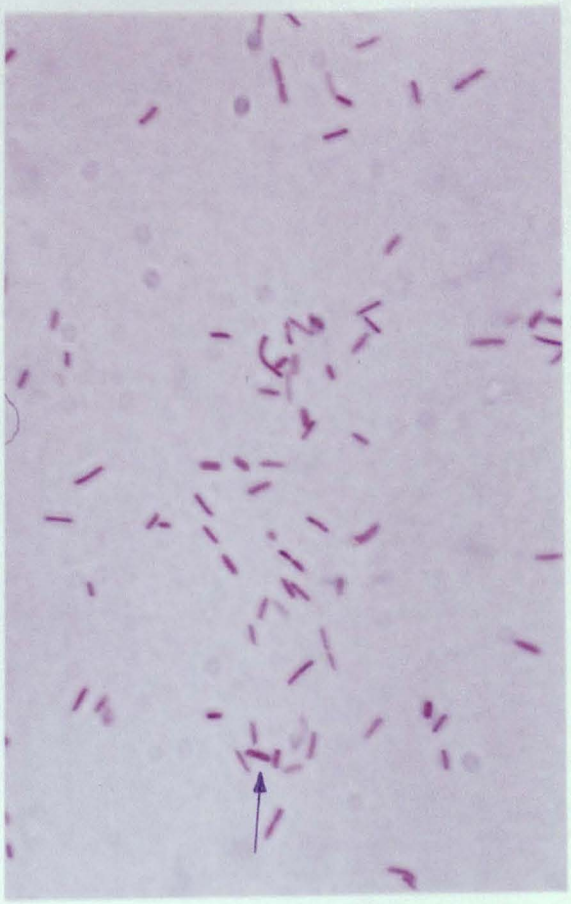
UNA_2



UNA_{14}



ERC_1



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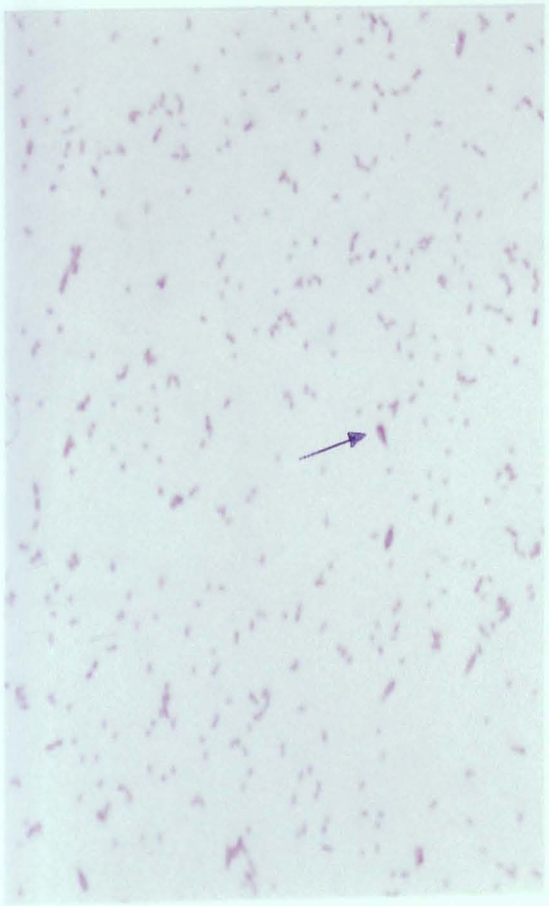

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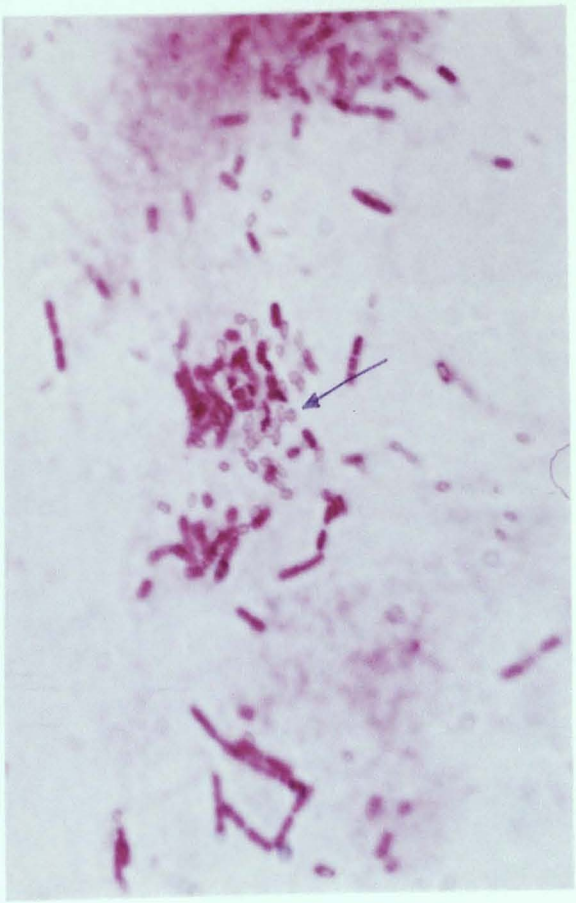

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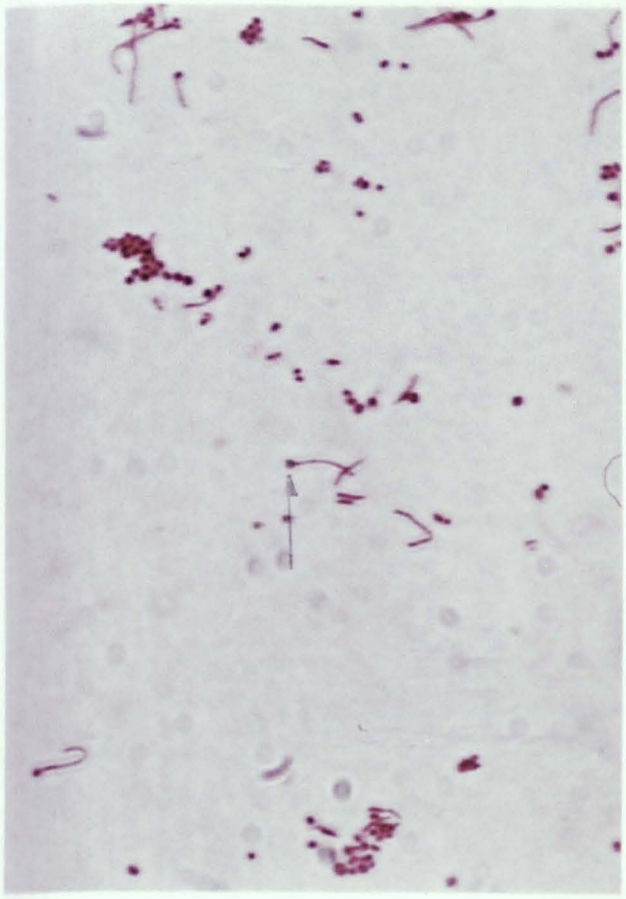

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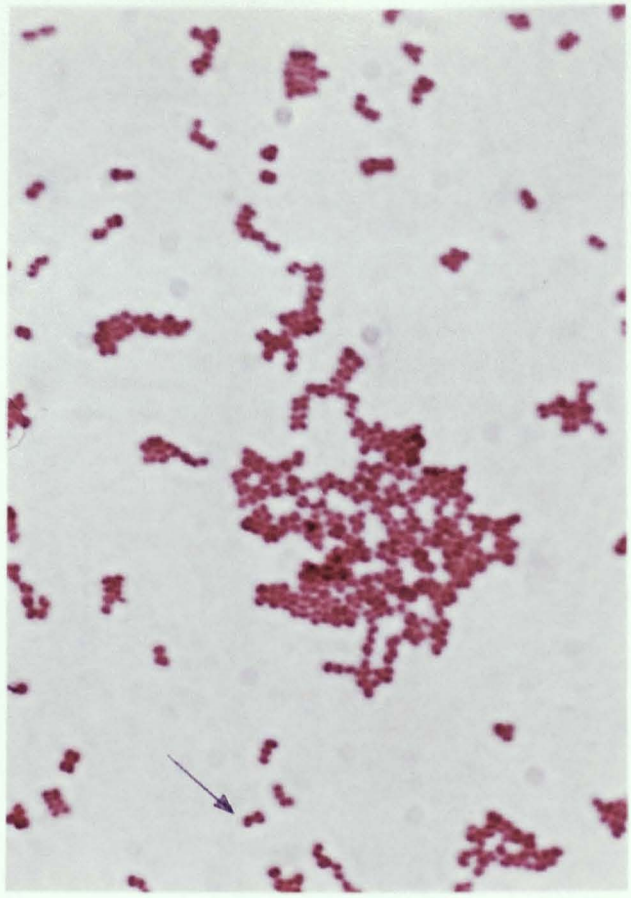

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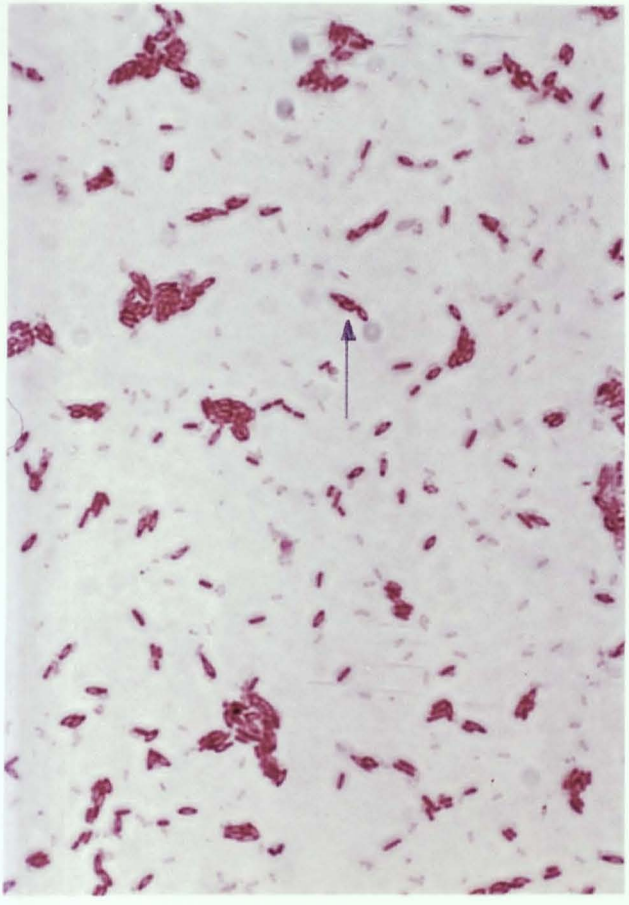

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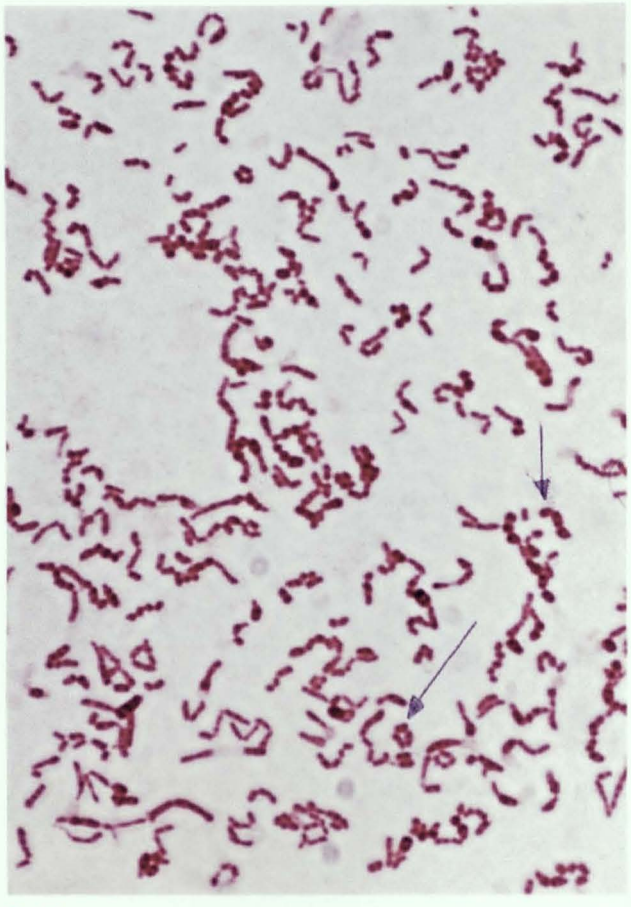

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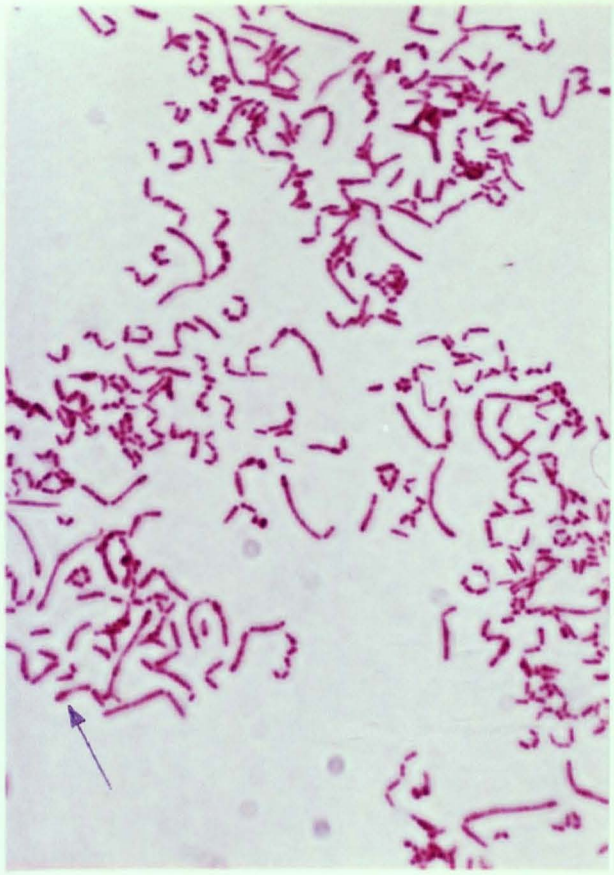

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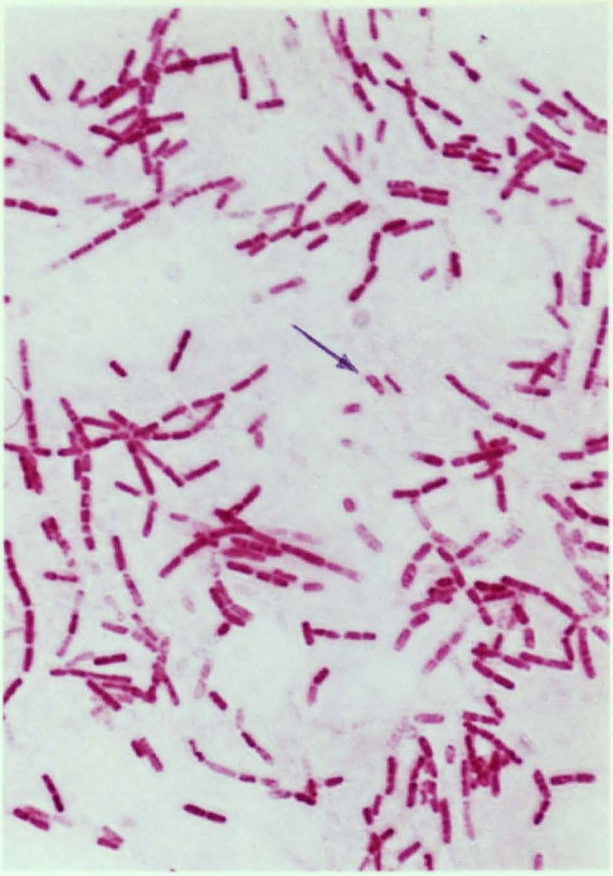

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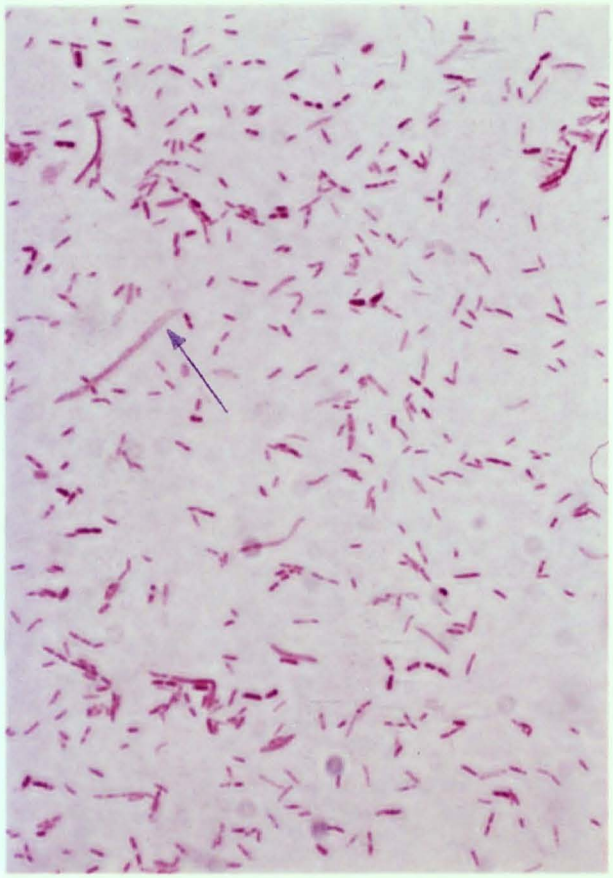

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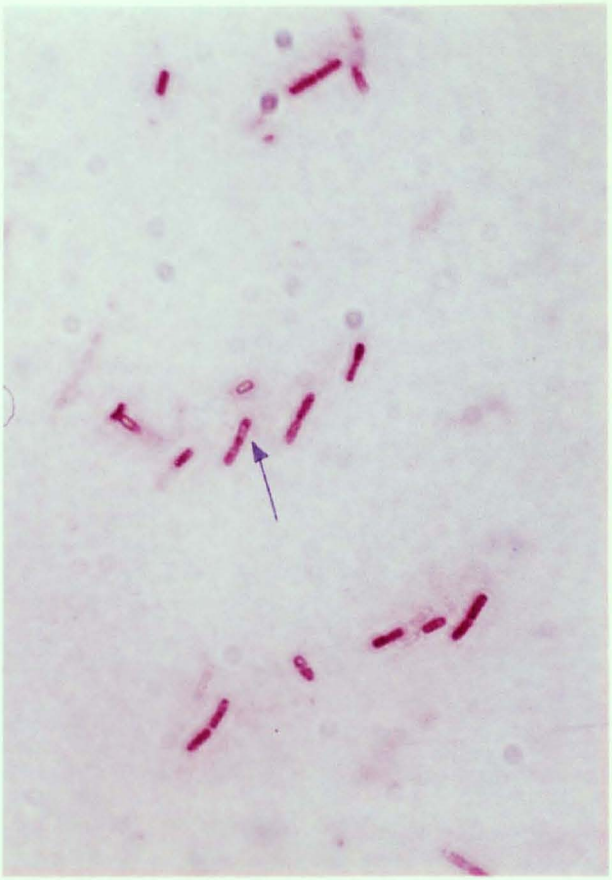

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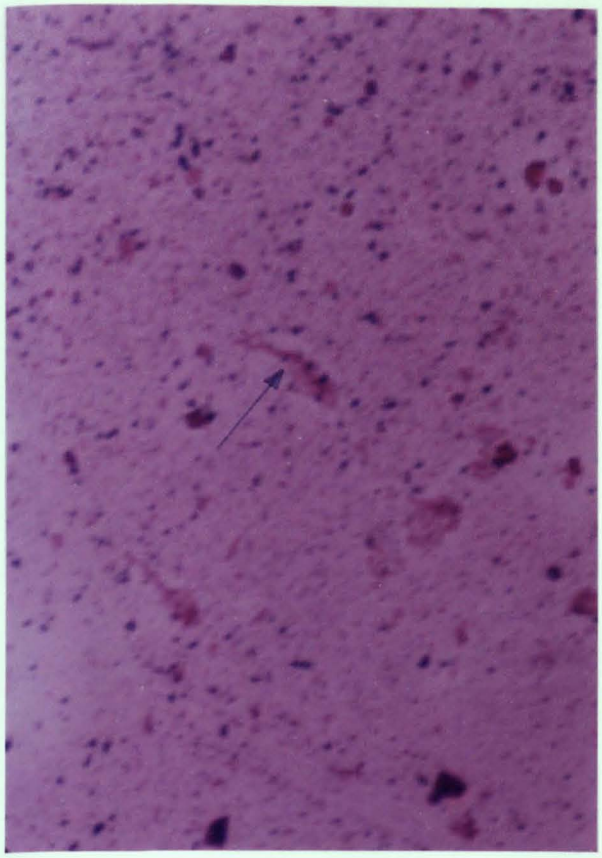
APPENDIX III



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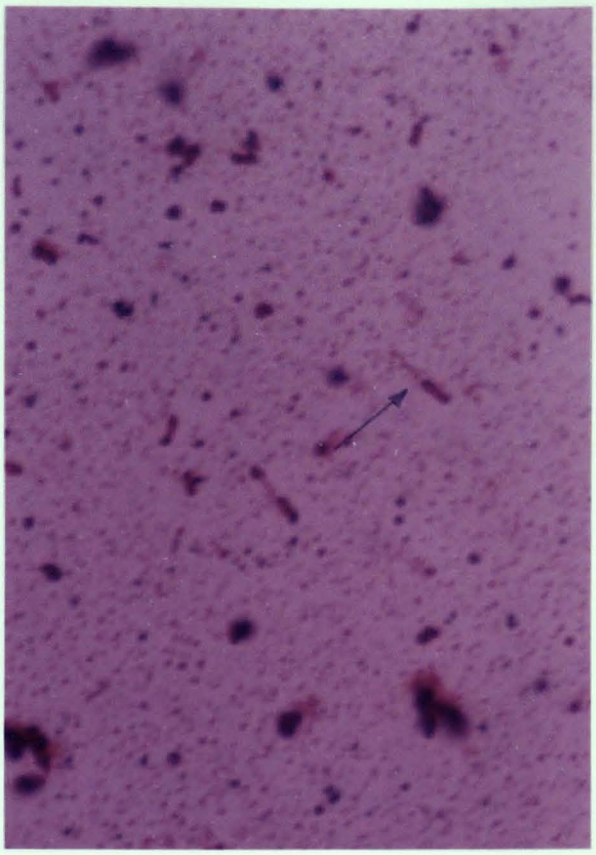

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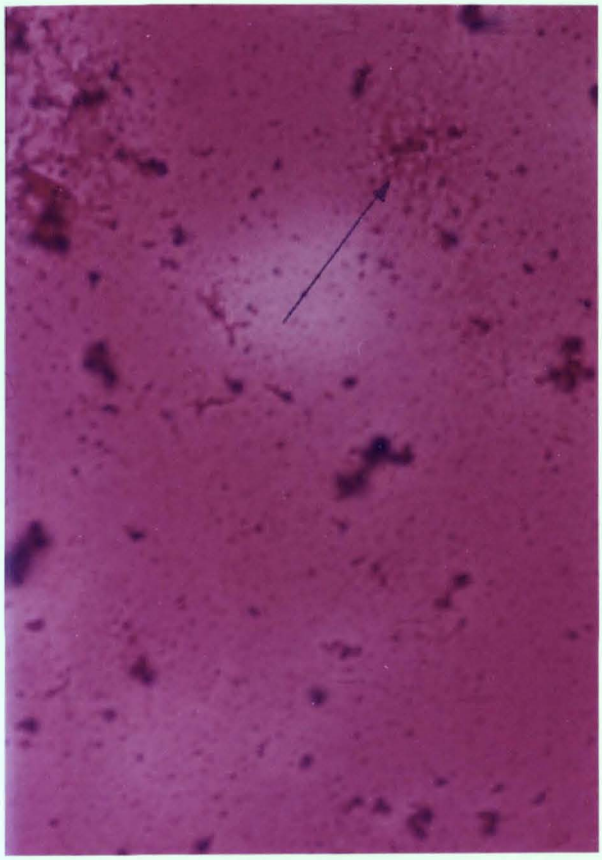

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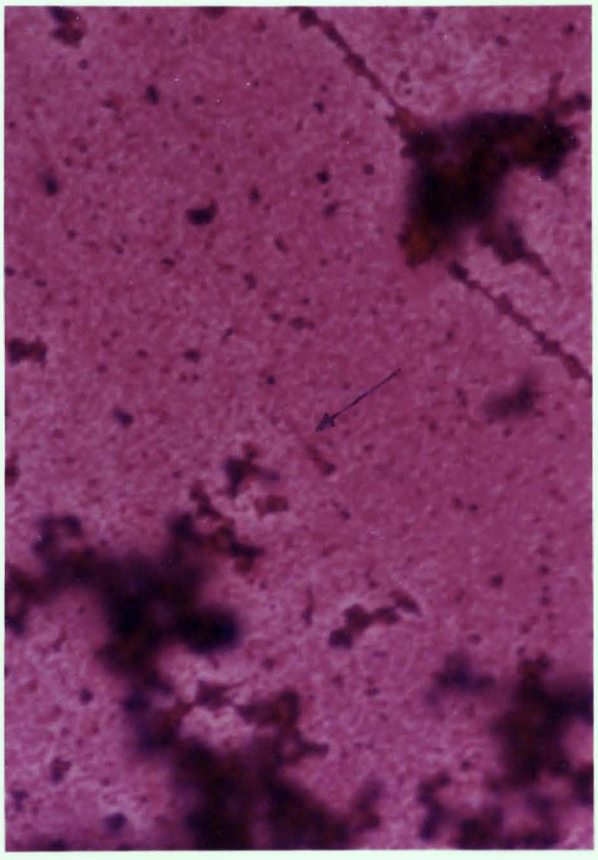

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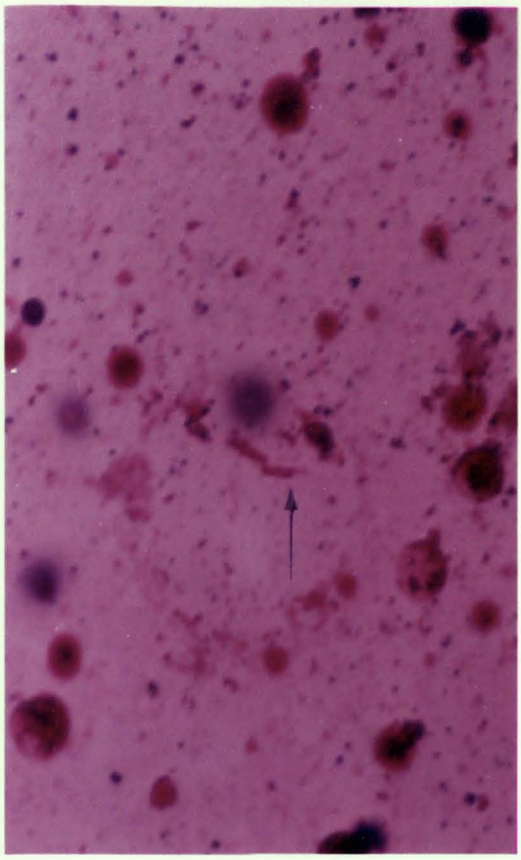

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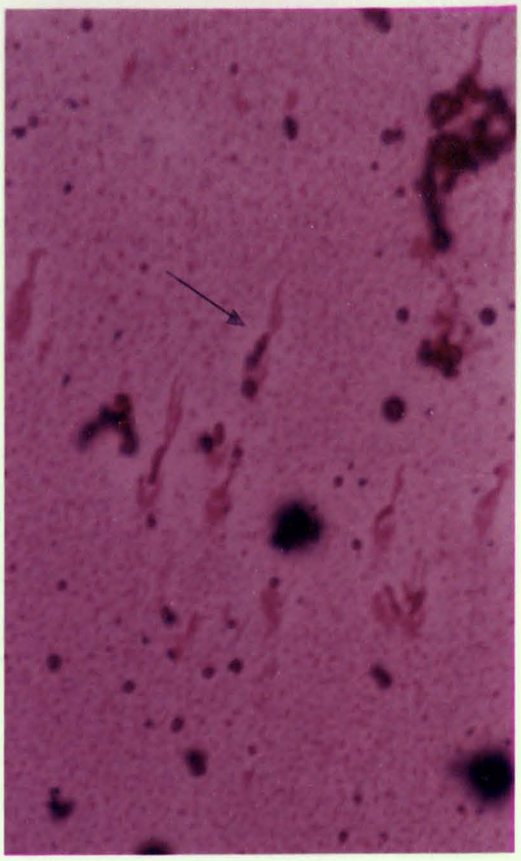

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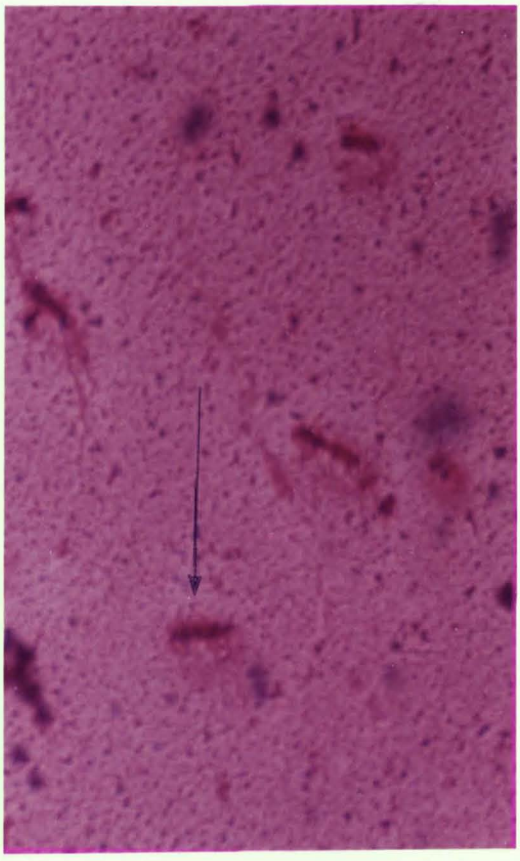

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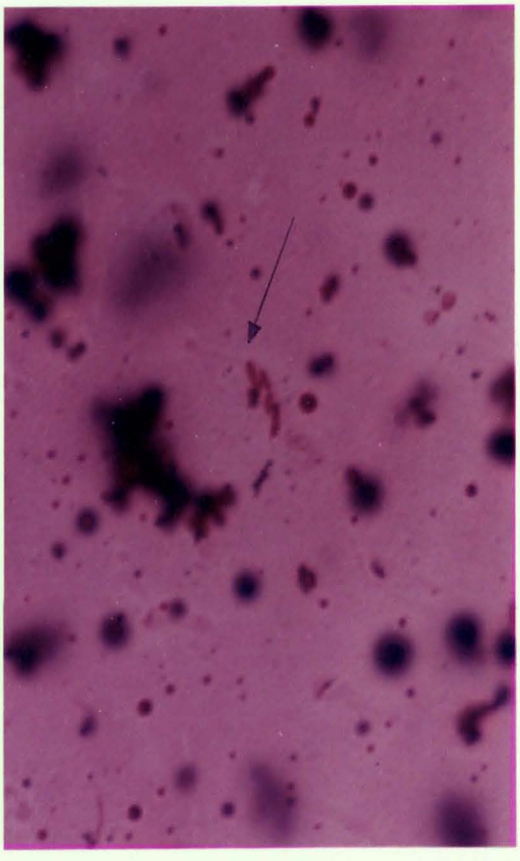

BER_6

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