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Delrapport III:

FLOCCULATION OF BACTERIA FROM AN ACTIVATED SLUDGE BIOLOGICAL TREATMENT PLANT: SOME INITIATORY STUDIES

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Bacteria from an activated sludge treatment plant handling waste-water from a factory producing high-quality cardboard were isolated using a non-selective defined medium. A wide range of organisms was isolated and these were tested for their ability to produce flocculant growth in a defined medium. This was not produced by enteric bacteria but was observed with some pseudomonads and acinetobacters, a rose-coloured Gram negative organism and some filamentous Gram negative bacteria. Flocculation appeared to be substrate dependent and a few organisms produced flocculant growth even in rich complex media.

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1. INTRODUCTION

Sucessful operation of an activated sludge treatment system depends on effective phase separation following formation of flocs. A great deal of attention has therefore been directed towards understanding the mechanisms underlying floc formation and has resulted in an extensive literature: only a few of the most pertinent references will be cited here.

Although a large number of different bacteria have been isolated from activated sludge systems (e.g. Pike 1975: Tabor 1976), particularly in earlier studies, only a limited number have been held to be specifically responsible for floc formation e.g. _Zoogloea_ sp. (Crabtree et al. 1966), _Flavobacterium_ sp. (Tezuka 1969), _Pseudomonas_ sp. (Zevenhuizen and Ebbink 1974) and a variety of filamentous bacteria (Van Veen 1973: Eikelbloom 1975). Flocculation has also been studied in organisms not isolated from activated sludge e.g. _Micrococcus varians_ (Kamekura and Onishi 1978) and in yeasts (e.g. Patel and Ingledew 1975 a). It is also clear that, under certain circumstances, protozoans may make a significant contribution to flocculation (Curas 1975).

It therefore appears rational to seek a unified mechanism of flocculation for all of these different organisms rather than suggest the obligatory involvement of any one. One specially attractive hypothesis is that the organisms synthesize some form of polymer under suitable growth conditions, and that this is responsible for cell adhesion and flocculation. Considerable support for the validity of this may be cited (e.g. Friedman et al. 1969: Pavoni et al. 1972: Unz and Farrah 1976: Farrah and Unz 1976: Tago and Aida 1977). Most commonly, the polymers are exopolysaccharides although the importance of endogenous glycogen in yeast floccultion has been suggested (Patel

and Ingledew 1975 b). Particularly on the basis of earlier work, a role for poly-β-hydroxybutyrate in Zoogloea ramigera was proposed (Crabtree et al. 1965: Fukui et al. 1976) though this appears less attractive in spite of the fact that this is a widely distributed storage product in bacteria (Hayward et al. 1959: refs. in Fukui et al. 1976).

It may be noted parenthetically that synthesis of exopolysaccharide appears to play a cardinal role in a number of other quite unrelated situations e.g. infection of plants by pathogens, dental plaque formation and nitrogen fixation by strictly aerobic bacteria.

The hypothesis is valuable in focusing attention on the physiological conditions under which such polymers are formed, in providing an understanding of the role of various environmental parameters and in enabling a plausible physico-chemical model for flocculation to be proposed. Among environmental parameters of particular relevance are the concentration of divalent cations (Tezuka 1969: Anglebeck and Kirsch 1969) and nutrient limitation, especially nitrogen (e.g. Jarman et al. 1978: Souw and Demain 1979).

It would thus be predicted that specific organisms do not determine flocculation and that environmental factors play a fundamental role in determining flocculation.

Systematic control of these factors could therefore plausibly be expected to result in a predictable regulation of activated sludge treatment plants.

It was against this background that the present investigations were undertaken: their aim was, as far as possible randomly, to isolate bacteria from activated sludge, to determine which of them produced flocculant growth and to study the synthesis of biopolymers with a view to under-

standing its regulation and function. This report is concerned only with the first two of these and only parenthetically with a general overview of the micro-organisms present in the treatment system.

In view of the important influence of nitrogen limitation, these introductory studies were directed to activated sludge treatment plants handling industrial wastewater containing high concentrations of organic carbon. The present study was carried out on samples obtained from a factory producing high-quality cardboard: the incoming water contained 200-300 mg/l BOD_7.

The investigations were carried out during two widely separated periods; in period I, the system consisted of pre-sedimentation followed by a trickling filter and two activated sludge aerating units operated in series. Samples were taken daily over two weeks. During period II, the trickling filter was by-passed and the system operated with an abnormally high organic loading. Samples were taken twice weekly over two weeks.

2. MATERIALS AND METHODS

A <u>General microbiological examination</u>. Enumeration of the frequencies of occurrence of heterotrophic bacteria, enteric bacteria, Gram negative bacteria, fungi and protozoa were carried out by standard methods used in this laboratory (Neilson and Wikström 1977) and will be only briefly summarized here. The total number of heterotrophic bacteria was determined using plates of tryptose-glucose-beef extract agar and incubation at 22°C for 5 d: of enteric bacteria using aniline blue agar and triple sugar iron agar and incubation at 35°C for 24 h and 48 h: of Gram negative bacteria using aniline blue agar and incubation at 35°C for 48 h: of fungi using Sabouraud glucose agar with penicillin and streptomycin and incubation at 22°C for 5 d. Protozoa were determined by direct counting after fixing the sample in Lugol's iodine solution.

B Isolation of organisms was carried out using a medium (VV 2) modified form that of Van Veen (1973): this is a non-selective medium incorporating only low concentrations of carbon substrate and contained (g/l distilled water): $(NH_4)_2SO_4$, 0.1 : $MgSO_4 \cdot 7H_2O$, 0.05 : $Ca(NO_3)_2$, 0.01 : K_2HPO_4, 0.05 : $Fe(NH_4)_2(SO_4)_2$, 0.02: trace element solution (see below), 1.0 ml: vitamin mixture (see below), 2 ml. This basal medium was prepared at twice the finally desired concentration and sterilized by autoclaving.

After cooling to 50°C, it was mixed with an equal volume of sterile agar (2 % v/v, Oxoid No. 1) at the same temperature and a solution of glucose sterilized by autoclaving added to give a final concentration of 0.15 g/l.

The trace element mixture contained (mg/l): $ZnSO_4 \cdot 7H_2O$, 100: $MnCl_2 \cdot 4H_2O$, 30: H_3BO_3, 300: $CoCl_2 \cdot 6H_2O$, 200: $CuCl_2 \cdot 2H_2O$, 10: $NiCl_2 \cdot 6H_2O$, 20: $Na_2MoO_4 \cdot 2H_2O$, 300. The vitamin mixture contained (per 1 distilled water): thiamin hydrochloride 1.0 g: biotin, 10 mg, pantothenic acid, 0.8 mg and vitamin B_{12}, 0.8 mg.

Serial dilutions of the samples were spread onto the surface of the medium and the plates incubated at 22°C for up to 7 d. Isolated colonies (often extremely small due to the low substrate concentration) or portions of filamentous growth were removed and restreaked onto the same medium. Further tests of purity were carried out wherever possible using nutrient agar or soy tryptone media: not all of the isolates were able to grow on these redia however.

C Tests for utilization of a restricted range of carbon substrates (glucose, sucrose, xylose, mannitol, glycerol, glucuronate, gluconate, starch, inulin and polygalacturonic acid) were carried out using the above basal medium replacing glucose with the desired substrate at a concentration of 2 g/l. The carbohydrates and polyols were

sterilized by filtering 10 % w/v solutions, the starch by autoclaving a 1 % w/v solution and all of these added to the mixture of basal medium and agar after autoclaving and cooling to 50°C. Polygalacturonic acid and inulin were sterilized by autoclaving with the agar.

D <u>Tests for flocculation</u> were carried out in the above medium lacking agar and supplemented with an amino acid mixture (15 ml/l) containing 60 mg/100 ml of each of the following L-amino acids: proline, tryptophane, histidine, methionine, cystein, threonine and leucine. Cultures were shaken at 25°C for up to 6 d and flocculation ability estimated visually after allowing the cultures to stand for 30 min using a standardized procedure.

3. RESULTS

A <u>General microbiological examination of the system</u>. The frequencies of occurrence of the several broad classes of micro-organisms are given in Tables 1 and 2: for period I, averages over the whole two-week period are given since no significant variations in the micro-flora were observed during the whole period. During period II, however, there was an abrupt change in the microflora towards the end of the first week and averages for the two single weeks have been given separately.

B <u>Studies with pure cultures isolated from the system</u>.

1. During period I, a total of 72 strains of bacteria were isolated on VV 2 medium and 19 of these were selected for further study. On the basis of the oxidase reaction (Kovacs), Gram staining and capacity for anaerobic growth with glucose, four broad groups of organisms could be readily distinguished:

Group 1. enteric bacteria all of which were shown by standard biochemical reactions to belong to the species Klebsiella pneumoniae.

Group 2. comprised a variety of pseudomonads and acinetobacters.

Group 3. rose-coloured Gram negative organisms.

Group 4. filamentous Gram negative bacteria unable to grow on complex organic media.

These four groups were first tested for their ability to use a variety of compounds as sole source of carbon and then for flocculating ability in liquid media containing those substrates able to support growth. Sucrose which was generally utilized for growth, did not, however, result in flocculant growth of any strain examined. Flocculation was not observed for any of the enteric isolates: the ability of strains belong to the other groups to flocculate during growth with various substrates is given in Table 3. In addition, flocculant growth was observed for strains of groups 2 and 3 during growth in soy tryptone broth containing 2.5 g/l glucose. Group 4 strains were unable to grow in this or any other complex medium.

Organisms belonging to groups 3 and 4 could be isolated from all sections of the treatment plant, from the trickling filter as well as from both aeration units.

2. A more restricted investigation was carried out during period II. Based on the results from period I, attention was focused primarily on organisms belonging to groups 3 and 4 above. Flocculation was not observed with any of the enteric bacteria or pseudomonads isolated. The results have been collected in Table 3: it should be noted that no isolate was able to grow at the expense of acetate, sucrose or polygalacturonate. Group 3 strains grew luxuriantly in soy tryptone broth producing flocculant while group 4 strains were unable to grow in this

medium. The frequency of occurrence of group 3 strains increased dramatically after the first week, so that by the end of the second the rose-coloured organism was the single diminant bacterium.

Representatives of all of the floc-forming organisms have been retained for further study and taxonomic determination which was not included in this preliminary study.

4. CONCLUSIONS

The results of this study may be summarized as follows:

1. use of a non-selective defined medium (VV 2) allowed the isolation of a wide range of bacteria including some unable to grow on complex organic media. Such 'non-selective' complex media may therefore be highly restrictive.

2. flocculating ability was not restricted to any one type of bacterium but was found in widely differing groups. During period I of the investigation, flocculating organisms were successfully isolated from all stages of the treatment system. Detailed taxonomic determination has not been attempted but representative strains have been retained; the 'rose bacterium' belongs to a group of organisms not hitherto encountered in this laboratory.

3. flocculating capacity was substrate dependent but was not simply correlated with growth.

4. in limited experiments, no effect could be demonstrated by increasing the C/N ratio from 20 to 75. It is, however, possible that the range of ratios chosen was too close. A few strains produced flocculant growth even in complex organic media containing glucose (2.5 g/l

The investigation has resulted in the isolation of some bacterial strains valuable for further studies of flocculation and has simultaneously provided basic data on their nutritional capacity and ability to produce flocculant growth. The next stage of the investigation should clearly be directed to the following issues:

1. is there a correlation between flocculation and synthesis of bio-polymer?

2. if so, under what physiological and environmental conditions is synthesis of polymer and therefore flocculation optimal?

It is suggested that, when answers to these questions become available, some significant progress will have been made towards an understanding of the mechanism of flocculation and towards means for its regulation on a scientific basis.

5. ACKNOWLEDGEMENT

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Table 1. Frequencies of occurrence of various groups of microorganisms during Period I ($\log_{10}$/ml).

	Filter	Unit 1	Unit 2
Total heterotrophic bacteria	7	8.5	7.8
Enteric bacteria	5.95	5.8	5
Gram negative bacteria	6.85	6.5	5.8
Fungi	5.5	5.6	4.85
Protozoa	N.D.	4	3.85

N.D. = not determined

Table 2. Frequencies of occurrence of various groups of microorganisms during Period II ($\log_{10}$/ml).

	Unit 1		Unit 2	
	Week 1	Week 2	Week 1	Week 2
Total heterotrophic bacteria	7.5	7.4	7.4	7.7
Enteric bacteria	5.3	5.55	5	5.3
Gram negative bacteria	7	6.7	6.8	6.8
'Rose bacterium'	0	6.65	0	6.3
Fungi	2	2	7	2
Protozoa	<3	<3	3	<3

Table 3. Distribution of the ability of organisms in Groups 2, 3 and 4 to produce flocculant growth at the expense of selected substrates.

Substrate	Group	
	Period I	Period II
Succinate	2	3,4
Glucuronate	3	3,4
Gluconate	N.T.	4
Acetate	3	N.G.
Polygalacturonate	3.4	N.G.
Glucose	4	3,4
Mannitol	4	4
Glycerol	4	3
Starch	4	3,4

N.T. = not tested : N.G. = no growth