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**Denitrification, activity of bacterial flocs,
and growth of a filamentous bacterium in relation
with the bulking of activated sludge**

Proefschrift

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GENERAL INTRODUCTION

One of the prerequisites for an adequately functioning mechanical-biological water purification plant is the complete separation of sludge flocs and effluent. This depends on a ready sedimentation of the sludge flocs in the final settling tank. An inadequate settling of the sludge leads to a poor quality of the effluent because part of the flocs may flow off.

In addition to the settling characteristics of the flocs, suspended growth of the microorganisms in activated sludge plants is an adverse feature affecting the quality of the effluent in a negative way since the single cells and small cell aggregates do not settle in the final settling tank.

The various types of activated sludge with poor settling characteristics are mostly caught under the term "bulking sludge". The Sludge Volume Index, SVI, (sludge volume, ml, per gram dry weight) is often used to express the settling characteristics of the sludge. When the SVI is above 200, the sludge is characterized as bulking.

Some of the more important types of bulking sludge are:

- 1) Sludge containing an abundance of filamentous microorganisms.
- 2) Sludge characterized by excessive amounts of bacterial slime.
- 3) Flotating sludge caused by denitrification.

1) The most frequently encountered type of bulking depends on the presence of filamentous microorganisms. The filamentous bacteria protruding from the flocs can be easily observed microscopically. In some types of sludge only filamentous microorganisms are observed with some little flocs caught in the network of threads.

Different types of filamentous bacteria, either dominating or growing together in activated sludge, have been reported in the literature. Recently, van Veen (1973) and Eikelboom (1975) have made a survey of the filamentous bacteria occurring most frequently in different types of sludge. The characterization of these bacteria was based on morphological features owing to the lack of knowledge about the physiology of most of the organisms. One of the bacteria, which has been studied in more detail, is *Haliscomenobacter hydroxsis*, a sheath-forming organism, first isolated and described by van Veen *et al.* (1971).

The excessive growth of filamentous bacteria sometimes occurring in activated sludge plants is difficult to explain. One of the hypotheses is that the protruding filamentous bacteria are relatively more active as compared with unicellular

microorganisms occurring in flocs. The substrate and/or oxygen uptake by cells within flocs are supposed to be suboptimal owing to the existence of very low concentrations of substrate and/or oxygen within the flocs caused by the ready uptake of these compounds by the cells within the flocs and an incomplete replenishment by diffusion.

Another explanation as to the occurrence of excessive amounts of filamentous microorganisms in activated sludge may be the relatively strong resistance of these bacteria to low oxygen concentration presumably existing within the flocs.

The existence of oxygen and substrate gradients within flocs and the effect of these gradients on the growth of *Zoogloea ramigera*, a floc-forming bacterium, has been dealt with in chapter II of this thesis.

The substrate affinity of a microorganism is an important factor in the competition for nutrients. The relation between substrate uptake rate and substrate concentration in general can be reasonably described by two values: the maximum substrate uptake rate, V_m , and the substrate concentration, K_s , at which $V = 1/2 V_m$, assuming that one enzymic reaction limits the uptake rate. The strong competitive position of filamentous bacteria in some activated sludge plants might be explained by assuming that these bacteria have a relatively low K_s value i.e. high substrate affinity. As the substrate concentrations in most activated sludge plants are extremely low, especially within flocs, filamentous bacteria might compete favourably with unicellular microorganisms in the sludge.

Exact determinations of K_s and V_m values have not been made in this study. However, to obtain some information about the competition between *H. hydrossis* and *Z. ramigera*, continuous culture experiments have been carried out at different dilution rates (chapter III). In the same chapter, the behaviour of *H. hydrossis* in continuous culture (pure culture) using a complex medium has been reported.

2) Settling of activated sludge flocs may also be inhibited by excessive amounts of slime. A stimulated slime production may be expected at a high C/N ratio of the influent. The use of starch as C source in the influent may also cause deterioration of the settling characteristics of the flocs by extraordinary slime production (Janssen, J.M.A., personal communication). In the present study no attention has been paid to slime production by bacterial cells in activated sludge flocs.

3) The flotation of activated sludge flocs may result from denitrification occurring within the flocs. Denitrifying bacteria are able to respire nitrate to N_2 , N_2O or in some cases to NH_3 . This process takes mainly place under anaerobic conditions. Gas bubbles of mostly N_2 are generally responsible for the flotation of sludge flocs. Flotation due to denitrification may be observed in final settling tanks of activated sludge plants which have become anaerobic because of a long retention time of the sludge in the tank. Except in the final settling tank, denitrification may be

expected to occur to some extent in the aeration tank within anaerobic inner parts of the flocs.

Denitrification in flocs and suspensions at different oxygen concentrations has been dealt with in chapter IV. The experiments were carried out with a floc-forming, denitrifying bacterium of the *Alcaligenes* type (strain 15), isolated from denitrifying sludge. The flocs of this organism were dispersed by enzymic treatment with cellulase as floc formation depended on the presence of cellulose fibrils between the cells.

The study of strain 15 revealed that under distinct conditions, the dissimilatory nitrate reduction of both, cells in flocs and in suspension, proceeded aerobically at approximately the same rate as under anaerobic conditions. Aerobic dissimilatory nitrate reduction was studied in more detail in chapter V because of the possibly practical importance of this process in activated sludge plants.

Chapter VI contains the results of some studies about the influence of the oxygen concentration on the synthesis of the dissimilatory nitrate reductase in a number of denitrifying bacteria isolated from activated sludge.

SOME FACTORS AFFECTING FLOC FORMATION BY *ZOOGLOEA RAMIGERA*, STRAIN I-16-M

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Abstract—Floc formation by cells of *Zoogloea ramigera*, strain I-16-M, was observed after resuspending pre-cultivated cells in fresh medium. It was considerably stimulated by the additional supply of NaCl or $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$. Stimulated floc formation occurred also at an increased C/N ratio of the medium in which the pre-cultivated cells were resuspended. The growth phase of the cells had no effect on floc formation when measuring the process in suspensions of equal cell density. Metabolites, inhibiting floc formation, were found to be excreted during the pre-cultivation of strain I-16-M in a complex medium. Enzymatic treatment with cellulase completely dispersed "young flocs"; addition of toluol caused a partial dispersion. "Aged" flocs were more resistant towards the dispersing agents.

INTRODUCTION

The pseudomonad *Zoogloea ramigera* was considered for a long time as the principal floc-forming organism of activated sludge (Bloch, 1918; Butterfield, 1935; Heukelekian, 1939; McKinney & Weichlein, 1953; Dugan & Lundgren, 1960; Crabtree *et al.*, 1965, 1966; Unz & Dondero, 1967; Friedman & Dugan, 1968; Friedman *et al.*, 1968, 1969). Deinema & Zevenhuizen (1971) showed that flocs formed by *Zoogloea ramigera* were dispersible by treatment with the enzyme cellulase. The exopolymers, playing an important role in bacterial floc formation, were proved to be cellulose fibrils (Deinema & Zevenhuizen, 1971).

The growth of *Zoogloea ramigera* (strain I-16-M) in liquid media occurs sometimes without floc formation whereas in other experiments, carried out under the same conditions and by using the same media, good floc formation may be observed.

In the present paper some additional information is given about the factors affecting floc formation and floc dispersion of *Zoogloea* strain I-16-M.

METHODS

Bacteria

Zoogloea ramigera, strain I-16-M, was obtained from A.T.C.C. Other non-identified floc-forming bacteria were isolated from activated sludge.

Media

Medium A: 1 g Bacto-casitone (Difco), 2 g glycerol, 0.35 g Bacto-yeast extract (Difco), 1000 ml tap water.

Medium A₁: Similar to medium A, except that no glycerol is added.

Enzyme

Cellulase was obtained from Merck (ex Basidiomycetes, 90 mU). Flocs of strain I-16-M were dispersed with cellulase by shaking the suspended flocs with a cellulase solution of 10 mg/ml for 1 h at 30°.

Growth conditions and treatment of cells

Cells used in floc-forming experiments were pre-cultivated in 2 l. flasks filled with 1 l. medium A. The flasks were inoculated with a slant culture and slowly shaken at 30° for 2 days.

The suspended cells were centrifuged, washed twice and resuspended in demineralized water. This cell suspension was pipetted into tubes as indicated (Results).

Floc formation

Growth and floc formation during the pre-cultivation of *Zoogloea ramigera* were followed by taking samples after different incubation periods; the turbidity was measured after settling of the flocs (settling time: 15 min) and again after dispersion of the flocs with cellulase. The difference between the two turbidity values, measured at each sampling time, expressed in the corresponding nephelometric units, gives the amount of cells occurring in flocs.

Floc formation by pre-grown suspended cells was studied in tubes, into which the washed and resuspended cells were pipetted. The tubes were shaken vigorously at 30° for different periods and the turbidities of the suspended cells measured upon settling of the flocs (settling time: 15 min).

Nephelometer

Cell turbidities were measured with an E.E.L. nephelometer. A cell density of 100 nephelometric units corresponded with about 0.1 mg protein per ml when the cells of strain I-16-M were obtained from a culture at the end of the logarithmic growth phase.

Mechanical dispersion of flocs

Flocs were mechanically dispersed in a M.S.E. ultrasonic disintegrator during 2 min.

RESULTS

The effect of salts on floc formation

Pre-cultivated, centrifuged, washed and resuspended cells were pipetted into tubes containing 2 ml of medium A and solutions of the following salts: NaCl, CaCl_2 , Na_2SO_4 or $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ at final concentrations of 0.002, 0.01 and 0.02 M. Tubes

without additional salts were used as blanks. The total volume in the tubes was made up to 10 ml with demineralized water. The final cell density in each tube corresponded with 187 nephelometric units. The pH of the solutions was 6.5. The rate of cell aggregation was followed (Fig. 1). The pH of the solutions did not change during the first 5 h of incubation.

NaCl and $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ stimulated the aggregation of the resuspended cells. The highest tested salt concentrations were most effective. CaCl_2 and Na_2SO_4 at concentrations above 0.01 M had no favourable effect on floc formation. Some increased floc formation was observed at lower concentrations of these salts. It is clear that the stimulatory effect of NaCl and $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ on the cell aggregation of strain I-16-M can not be explained by the effect of ions on the ionic double layer since the multivalent ions were not more effective towards floc formation than the monovalent ions of NaCl, which is in contrast to the rule of Schulze and Hardy.

In a second series of experiments the concentration of medium A in the tubes was similar to that of the pre-cultivation medium. Addition of NaCl or $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ to these tubes also caused a strongly stimulated floc formation.

In pre-cultivation experiments, the addition of NaCl or $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ to medium A up to the final concentration of 0.01 M did not affect the growth rate of strain I-16-M. In these experiments floc formation varied widely and it was not reproducible.

The flocs of strain I-16-M, formed in the presence or absence of additional salts, can only be dispersed by the enzyme cellulase, showing that cellulose excretions are responsible for the cell aggregation of this strain. The stimulatory effect of NaCl and $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ on the floc formation of pre-cultivated, suspended cells of strain I-16-M might be explained by a stimulatory effect of these salts upon the production of cellulose fibrils.

The effect of different glycerol concentrations on floc formation

Pre-cultivated, resuspended cells were pipetted into tubes containing $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ (pH 6.5) and NaCl, final concentrations 0.01 M, and glycerol, final concentrations of 0.1 and 1.5 mM. Tubes without added glycerol were used as blanks. The total volume in the tubes was made up to 10 ml with demineralized water. The final cell density in each tube corres-

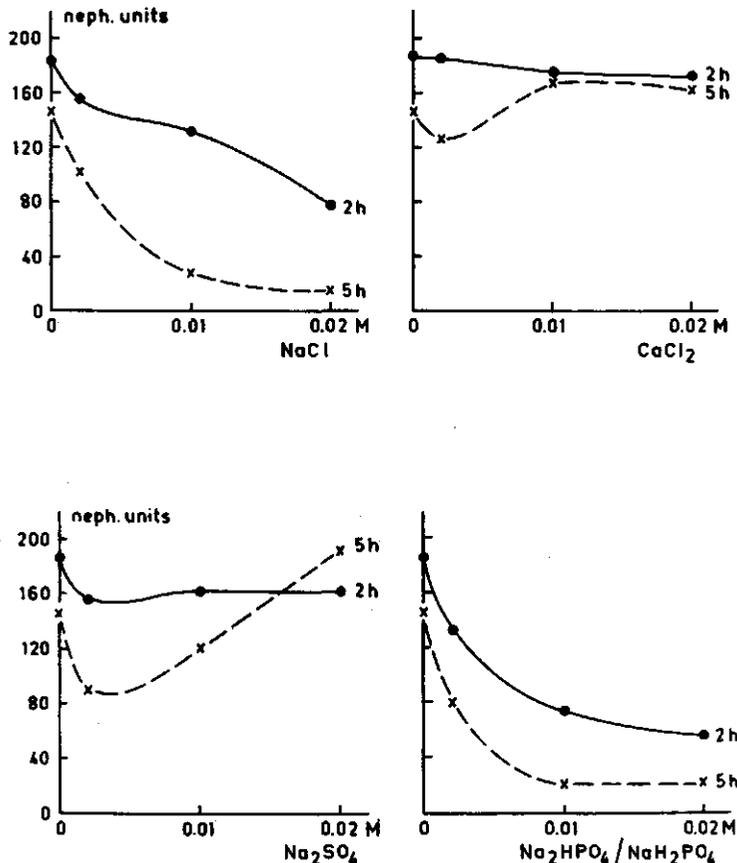


Fig. 1. The effect of NaCl, CaCl_2 , Na_2SO_4 and $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ on the floc formation by resuspended cells of strain I-16-M. Densities of suspended cells measured after 2, ●—● and 5 h, X—X.

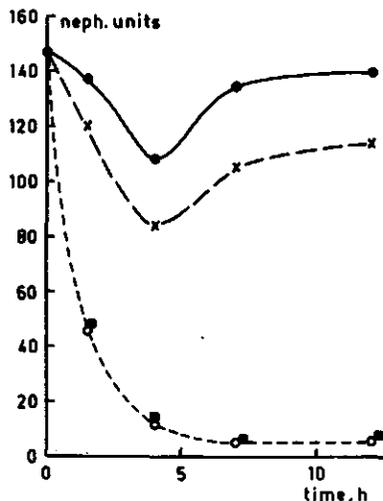


Fig. 2. The effect of glycerol on the floc formation by resuspended cells of strain I-16-M. Glycerol concentrations (mM): 0, ●—●; 0.1, X—X; 1.0, ○—○; 1.5, ■.

ponded with 147 nephelometric units. Glycerol stimulated the aggregation rate of the suspended cells (Fig. 2). In the absence of glycerol some initial floc formation was observed, followed by an increase of the turbidity of the cell suspension upon prolonged incubation. This effect of prolonged incubation was also measured at 0.1 mM glycerol, suggesting that dispersion of the flocs occurs in the absence of a C-source. The initial floc formation, observed in the tube without added glycerol, may have resulted from the production of cellulose fibrils derived from the conversion of internal reserve material as poly- β -hydroxybutyrate.

The effect of the C/N ratio of the medium on floc formation

Pre-cultivated, resuspended cells were pipetted into tubes containing 2 ml medium A₁, Na₂HPO₄/NaH₂PO₄ (pH 6.5) and NaCl, final concentrations: 0.1, 1.0 and 1.5 mM. Some tubes received no glycerol. The total volume in the tubes was made up to 10 ml with demineralized water. The final cell density in each tube corresponded with 147 nephelometric units.

The initial aggregation rates of cell suspensions with different amounts of glycerol were similar when measured within 2 h of incubation (Fig. 3). At prolonged incubation periods the turbidity of the cell suspensions increased, probably resulting from growth of the cells. In the medium with a high C/N ratio, the cells remained in the floc form upon prolonged incubation, probably due to a relatively high exopolymer production.

The effect of the density of the cell suspension on floc formation

Pre-cultivated, resuspended cells were pipetted into tubes containing Na₂HPO₄/NaH₂PO₄ (pH 7), final concentration 0.02 M, and 5 mM glycerol. The total

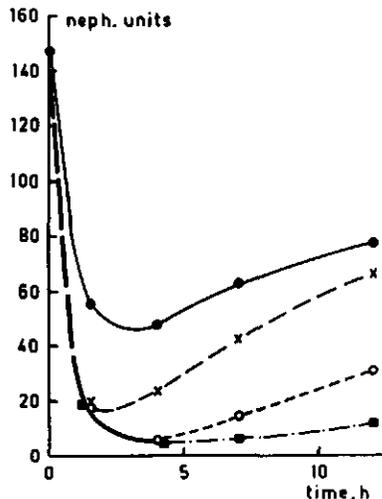


Fig. 3. The effect of the C/N ratio of the medium on floc formation by resuspended cells of strain I-16-M. The tubes contained per litre: 0.2 g casitone, 0.04 g yeast extract, additionally supplied with glycerol (mM): 0, ●—●; 0.1, X—X; 1.0, ○—○ and 1.5, ■—■.

volume in each tube was made up to 10 ml with demineralized water. The final turbidities of the suspensions equalled: 64, 320 and 640 nephelometric units.

As shown in Table 1, cell suspensions of a high cell density flocculated much better than suspensions of a low density. This may be explained by a greater probability of collision of the cells.

The effect of the growth phase of the pre-cultivated bacteria on floc formation

Growth and floc formation during pre-cultivation of *Zoogloea ramigera* were determined as described in Methods.

From the results of this experiment (Fig. 4), it will be seen that floc formation occurred during the second half of the logarithmic growth phase. Similar results were obtained by Deinema & Zevenhuizen (1971) who suggested that floc formation during this period was due to the increased number of cellulose fibrils per cell.

To eliminate the effect of density of the cell suspensions of different age upon floc formation (Table 1), suspended cells harvested after 12, 36, 50, 72 and 240 h (Fig. 4) were pipetted into tubes with a final turbidity of 187 nephelometric units. The tubes contained furthermore: Na₂HPO₄/NaH₂PO₄ (pH 6.5) at a final concentration of 0.02 M, glycerol, 5 mM, and demineralized water up to 10 ml. In a second series

Table 1. The effect of cell density on floc formation

$t = 0$		$t = 1.5 \text{ h}$		$t = 4 \text{ h}$	
Cell turb.	% of cells in flocs	Cell turb.	% of cells in flocs	Cell turb.	% of cells in flocs
64	0	63	1.5	40	37.5
320	0	157	50.9	133	58.4
640	0	206	67.8	175	72.6

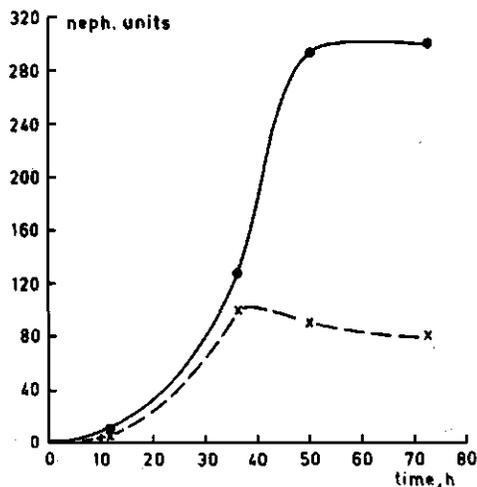


Fig. 4. Growth and floc formation of strain I-16-M in medium A: ●—●, cells in suspension and flocs (after dispersion by added cellulase); cells in suspension, X---X.

of tubes the glycerol was replaced by 2 ml of medium A.

The results of these experiments show (Figs. 5a and b) that suspended cells, harvested during the lag phase (12 h after inoculation), are aggregating at about the same rate as cells derived from the end of the logarithmic growth phase (50 h after inoculation). When the cells were too old, no floc formation occurred. From these results it was concluded that the low cell density may be responsible for the absence of floc formation in the early growth phase.

Substances inhibiting floc formation

In some batch experiments with medium A as culture medium, cells of strain I-16-M formed no flocs. However, the suspended cells could always easily be brought to floc formation when they were centrifuged, washed and resuspended in fresh medium A. This phenomenon might be explained by assuming that substances were excreted during the growth of strain I-16-M which inhibited floc formation. To confirm this hypothesis, strain I-16-M was pre-cultivated in

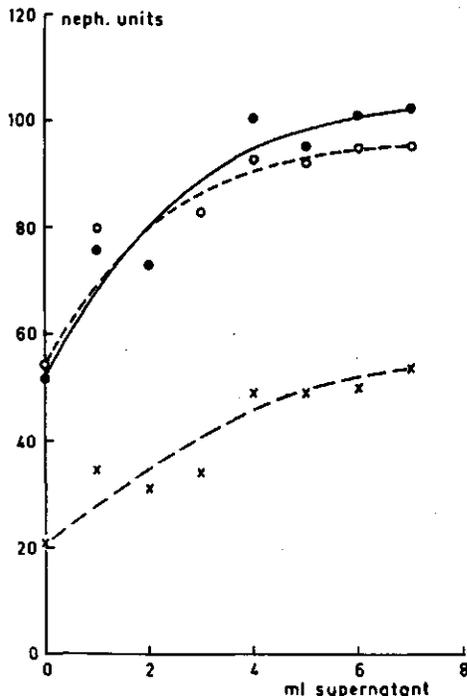


Fig. 6. The inhibitory effect of increasing amounts of the pre-cultivation medium on floc formation by resuspended cells of strain I-16-M. Turbidities measured after 1, ●—●; 2, X---X and 4 h, ○---○.

medium A for 2 days whereupon the suspended cells were harvested and transferred to tubes containing $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ (pH 7) at a final concentration of 0.01 M, 1 ml concentrated (10x) medium A, and 0, 2, 4 and 6 ml, respectively, of the supernatant derived from the pre-grown culture. The volume in the tubes was made up to 10 ml with demineralized water giving a final turbidity of the cell suspension that corresponded with 210 nephelometric units.

The results of the experiments show (Fig. 6) that metabolites, excreted in the growth medium during pre-cultivation of strain I-16-M, may inhibit floc formation. In some additional experiments it was shown that NH_4Cl , acetate and citrate had no effect

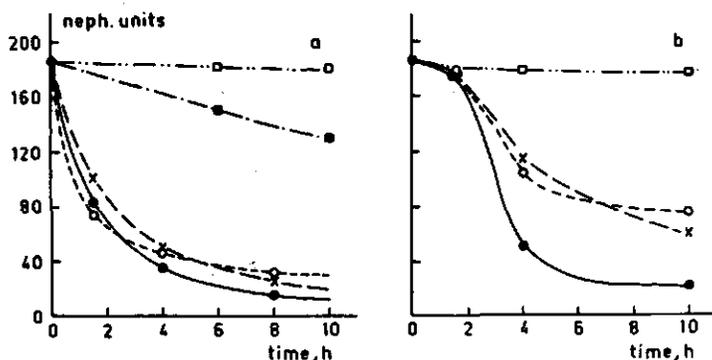


Fig. 5. Floc formation by suspended cells of strain I-16-M derived from different growth stages of the pre-cultivated culture of Fig. 4, measured with medium A (a) and with glycerol (b). Cells harvested after: 12, ●—●; 36, X---X; 50, ○---○; 72, ■—■ and 240 h, □---□.

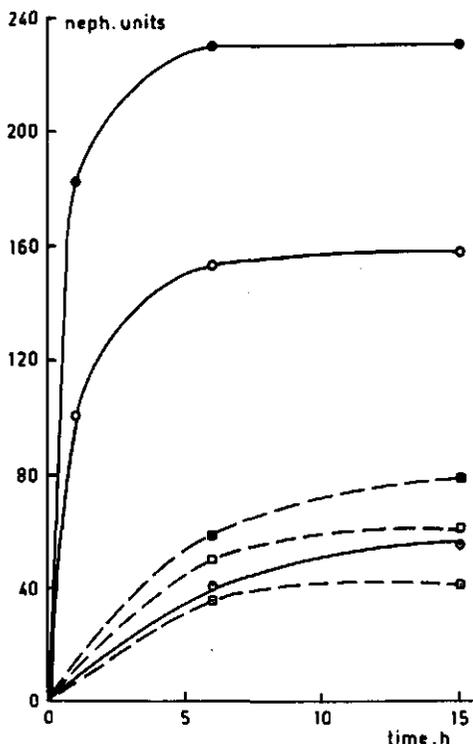


Fig. 7. Dispersion of "young" and "aged" flocs by cellulase and toluol. "Young" flocs: treated with cellulase, ●—●; with toluol, ○—○; untreated control, ○—○. "Aged" flocs: treated with cellulase, ■--■; with toluol, □--□; untreated control, □--□.

on floc formation. No further attempts were made to characterize the inhibiting substance(s) in the pre-cultivation medium. Upon incubation of the test tubes of this experiment, the turbidities increased, probably resulting from growth of suspended cells.

Dispersion of flocs

Deinema & Zevenhuizen (1971) showed that flocs of strain I-16-M, produced in medium A, can be dispersed by treatment with cellulase. This effect was confirmed in this study. Other conditions affecting the dispersion of the flocs such as ageing of the flocs and killing of the cells by toluol were also investigated.

"Aged" flocs of strain I-16-M were obtained from a 10-days old culture grown in medium A. "Young" flocs were derived from a 2-days old culture of this strain, after transferring the suspended cells to fresh medium A. The "aged" and "young" flocs were transferred to tubes with or without the additional supply of 0.05 ml toluol or 2 ml of a cellulase solution (50 mg/ml of demineralized water). The total volume in the tubes was made up to 10 ml with demineralized water. To determine the total amount of cells in the tubes, the "aged" and "young" flocs were dispersed with the M.S.E. ultrasonic disintegrator; the resulting turbidity corresponded with 600 and 240 nephelometric units, respectively.

As seen from Fig. 7, "young" flocs were completely dispersed by cellulase and partially by toluol. Without these treatments "young" flocs became also partially dispersed upon incubation. "Aged" flocs were resistant against the dispersing agents; however, upon prolonged incubation (more than 48 h) a substantial part of the "aged" flocs became suspended by cellulase. It is possible that in "aged" flocs, in addition to cellulose fibrils, a different aggregating agent is involved.

DISCUSSION

Stimulated floc formation of cells of *Zoogloea ramigera*, strain I-16-M, was obtained after resuspending pre-cultivated cells in fresh medium, additionally supplied with NaCl or $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$. Because floc formation took place within 1–2 h, it was possible to obtain flocs consisting of highly active cells. Such flocs may be used for studying substrate and oxygen gradients within cell aggregates.

Some details concerning the effect of cations on floc formation of *Zoogloea ramigera* were found in the literature. Tezuka (1967) reported that the presence of small quantities of magnesium ions in the growth medium inhibited the aggregation of the cells. This was confirmed by Angelbeck & Kirsch (1969) who found magnesium and calcium ions from a concentration of 1.2 mM reversed the aggregative growth of the organism. Manganese, iron and sodium ions up to a final concentration of 1.2 mM had no effect on either aggregative or non-aggregative growth in a peptone medium. The last-mentioned authors stated that floc formation by *Zoogloea ramigera* is specially related to the calcium or magnesium metabolism. The experiments presented in this paper showed that the addition of CaCl_2 or Na_2SO_4 to fresh medium in which pre-cultivated cells were resuspended, did not stimulate floc formation, but the addition of NaCl or $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ strongly stimulated this process. An explanation of this phenomenon could not be given.

The stimulated cell aggregation, shown after resuspending pre-grown cells of strain I-16-M in fresh medium, was not a general characteristic of floc-forming bacteria. Ten floc-forming strains, at random isolated from activated sludge, were tested for cell aggregation in the way described for strain I-16-M, but none of the isolates showed a strongly stimulated cell aggregation.

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ACTIVITY OF *ZOOGLOEA RAMIGERA* GROWING IN FLOCS AND IN SUSPENSION

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Abstract—A comparison was made between cells of *Zoogloea ramigera* occurring in flocs and in suspension. Suspended cells were obtained by adding cellulose to the growth medium, which prevented floc formation without damaging the cells. Differences in protein synthesis and poly- β -hydroxybutyrate production of cells in suspension and cells in flocs were only found between slowly shaken cultures. Increasing the oxygen content of such cultures did not raise the growth limitation of the cells in flocs. The oxygen uptake of the suspended cells was found to represent a zero order reaction at an oxygen concentration of the culture above 0.1 mg/l. and that of cells in flocs above 1.5 mg/l. Below 20 mg/ml glucose the glucose respiration rate of cells in flocs was lower than that of suspended cells. Above approx 20 mg/l. glucose, the glucose respiration of both types of cells was almost a zero order reaction.

INTRODUCTION

The availability of substrates and oxygen to bacterial cells occurring within flocs depends on the diffusion rates of these substances through the flocs. Wuhrmann (1963) applied the theoretical analysis of Gerard (1931) to estimate the extent of glucose diffusion through biological flocs. He estimated the glucose concentration in the surrounding medium at which glucose uptake might become a diffusion-controlled reaction to be approx six-times the limiting oxygen concentration of the floc particle. Baillo & Boyle (1970) measured the glucose uptake rates of *Zoogloea ramigera* cells in flocs and in suspension. The glucose uptake was found to be a zero-order reaction when the concentration was higher than approximately 2 mg/l. with suspended cells and higher than approx 8 mg/l. in case of flocs (at 30°).

Mueller *et al.* (1966) determined the limiting oxygen concentrations with suspended cells and cells in flocs of *Zoogloea ramigera*. The oxygen uptake of suspended cells was rate-limited when the oxygen concentration was below 0.1 mg/l. whilst the oxygen uptake by cells in flocs was reduced when the oxygen concentration in the surrounding medium was below approx 1.5 mg/l.

In the experiments with *Zoogloea ramigera*, carried out by Baillo & Boyle (1970) and Mueller *et al.* (1966), the cell suspensions were obtained by blending the flocs for 2 min. As was shown by Deinema & Zevenhuizen (1971), the flocs of *Zoogloea ramigera*, strain I-16-M, can easily be dispersed by enzymatic treatment with cellulose. By using this method, suspended cells can be obtained from flocs without mechanical damage to the cells.

In this paper the growth of suspended cells (obtained after enzymatic treatment) and of flocs have been compared. In addition, the relations: glucose concentration—glucose respiration and oxygen con-

centration—oxygen uptake rate were measured with suspended cells and with cells in flocs.

METHODS

Bacteria

Zoogloea ramigera, strain I-16-M, was obtained from A.T.C.C.

Media

Medium B: 1 g Bacto-casitone (Difco), 2.2 g glucose, 0.35 g Bacto-yeast extract (Difco), 0.02 M phosphate buffer (pH 6.5) and 1000 ml tap water.

Medium B₁: similar to medium B with extra addition of 10 g agar/l.

Enzyme

Cellulose was obtained from Merck (ex Basidiomycetes, 90 mU). The enzyme solution (10 mg/ml distilled water) was sterilized by filtration.

Growth conditions and cell treatment

Growth experiments were carried out in 300 ml flasks filled with 100 ml of medium B. The flasks were inoculated with a slant culture, kept on medium B₁. Suspended growth was obtained by adding 1 ml sterile cellulose solution per flask. Media additionally supplied with 1 ml of a boiled (i.e. inactivated) cellulose solution gave rise to floc growth. A number of flasks were shaken vigorously at 200 strokes/min (average floc dia approx 1 mm). Others were poorly aerated at 50 strokes/min (average floc dia approx 5 mm). In some experiments the cultures were placed in a desiccator which was flushed with oxygen containing 0.1% carbon dioxide. The desiccator was shaken slowly at 50 strokes/min. All growth experiments were carried out at 30°.

Cells used in respiration experiments were shaken at 100 strokes/min, giving rise to flocs with an average dia of approx 2 mm. These cells were harvested 48 h after inoculation, when the culture was in the stationary growth phase. After centrifuging and washing twice, the cells were resuspended in 0.02 M phosphate buffer (pH 7).

Oxygen uptake experiments

Oxygen uptake experiments were carried out in a respirometer as described by van Kessel (1975). The oxygen was

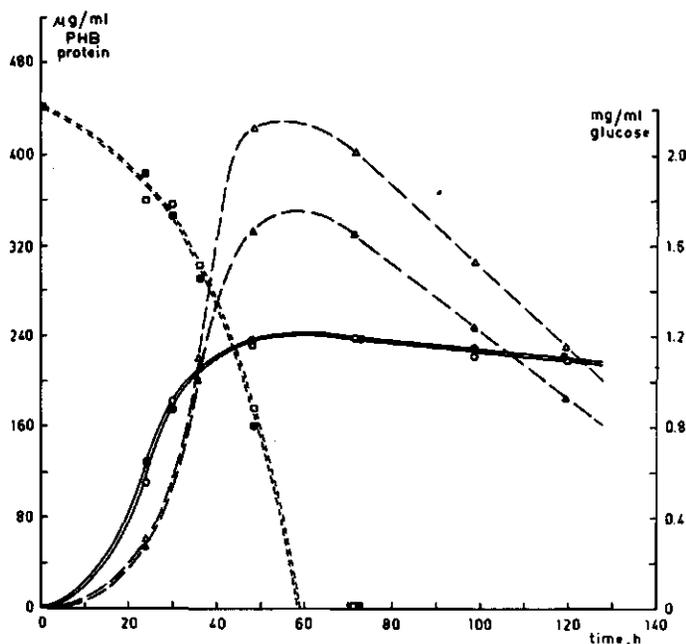


Fig. 1. Growth and PHB production of cells in suspension and cells in flocs in vigorously shaken cultures (200 strokes/min). Suspended cells: \circ — \circ , protein; \triangle --- \triangle , PHB; \square ---- \square , glucose. Cells in flocs: \bullet — \bullet , protein; \blacktriangle --- \blacktriangle , PHB; \blacksquare ---- \blacksquare , glucose.

measured with an oxygen macro-electrode connected with a physiological gas analyser. During the measurements, glucose was injected as desired. The relatively large volume of the respirometer (85 ml) proved to be of advantage in keeping the flocs intact.

Chemical analyses

Cell protein was assayed by the Folin-Ciocalteu method as described by Lowry *et al.* (1951) with modification of Herbert *et al.* (1971), using bovine serum albumin as the standard.

Poly- β -hydroxybutyrate (PHB) was determined by adding 1 ml of 2-N HCl to 1 ml of cell suspension. The mixtures were digested in loosely stoppered tubes at 100° for 2 h, cooled and extracted twice with 2.5 ml of chloroform by shaking. Samples containing 10–100 μ g of PHB were transferred to test tubes, the chloroform evaporated in a boiling-water-bath, 5 ml of 96% H_2SO_4 added and the mixture heated at 100° for 15 min. Ultraviolet absorption at 235 nm was recorded with a Gilford 2400 spectrophotometer ($E_{235} = 0.35$ per 10 μ g PHB).

Glucose was assayed by the method of Somogyi-Nelson (Somogyi, 1952).

RESULTS

Growth of cells in flocs and in suspension

The growth of cells in suspension and of cells in flocs proceeded similarly and at the same rate when the flasks were vigorously shaken at 200 strokes/min (Fig. 1). The average floc dia was approx 1 mm. The same results were obtained when the cultures were shaken at 100 strokes/min.

In the slowly shaken flasks (50 strokes/min), the protein synthesis and the production of PHB of cells in suspension and cells in flocs proceeded differently (Fig. 2). The average floc dia in these experiments was approx 5 mm. The cells occurring in flocs were

much retarded in their activity as compared with the suspended cells, which grew at the same rate as those of the strongly shaken cultures (Fig. 1). These differences in growth rate and in PHB production between both types of cells, may have been due to: (a) oxygen limitation within the relatively large flocs (dia approx 5 mm). (b) Substrate limitation within the flocs. (c) A possible accumulation within the flocs of growth-inhibiting metabolites, excreted by the bacteria in the flocs. As the substrate concentrations in the medium (per l.: casitone, 1 g; glucose, 2.2 g and yeast extract 0.35 g) were relatively high as compared with the dissolved oxygen concentration (at saturation approx 7.5 mg/l), the growth limitation was thought to be caused by oxygen limitation within the flocs. The potential oxygen demand of the cells in slowly shaken flasks (50 strokes/min) was determined by taking samples from the cultures at different stages of development and, after re-aeration, measuring the oxygen uptake rate in the respirometer (Fig. 3). It is obvious that the highest potential oxygen demand of the bacteria occurred in the early growth phase of the cultures. If shortage of oxygen would have limited the growth of the cells in the flocs of the slowly shaken cultures, the growth limitation must have been most pronounced when the culture was in the early growth phase. This was confirmed by the results presented in Fig. 2.

In a subsequent experiment the flasks were incubated in a slowly shaken desiccator filled with oxygen and 0.1% carbon dioxide. The dissolved oxygen concentration in the medium was at saturation about 40 mg/l. compared to 7.5 mg/l. under air saturation. Protein synthesis, production of PHB and glucose

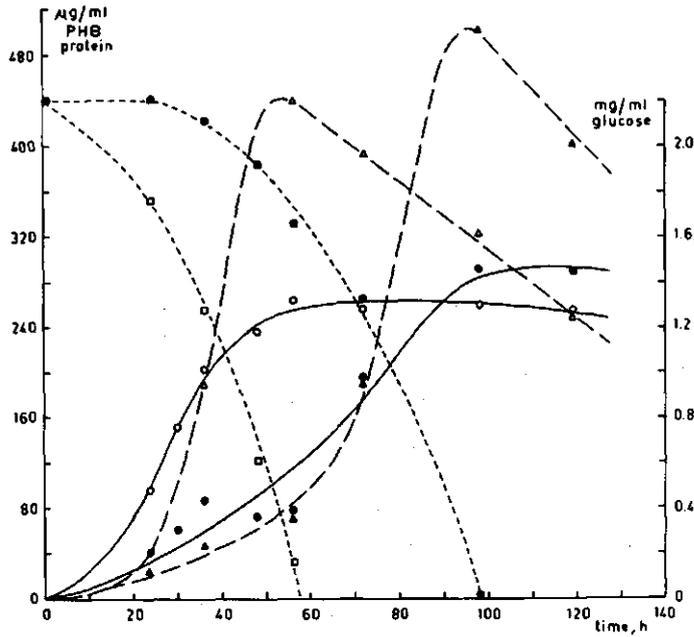


Fig. 2. Growth and PHB production of cells in suspension and cells in flocs in slowly shaken cultures (50 strokes/min). Suspended cells: ○—○, protein; △---△, PHB; □---□, glucose. Cells in flocs: ●—●, protein; ▲---▲, PHB; ■---■, glucose.

uptake of the suspended cells were measured whereas in the case of flocs only the glucose uptake was measured (Fig. 4). The growth and the production of reserve material of the suspended cells were similarly to those measured under air (Fig. 2). The glucose uptake of the cells in flocs under oxygen was comparable to that under air, indicating that oxygen deficiency has not been the growth-limiting factor in flocs of slowly shaken cultures.

Experiments with washed cells

The relation: oxygen concentration—oxygen uptake rate. To study the effect of oxygen concentration of

the medium on the oxygen uptake rate, cells were pre-cultivated in 300 ml flasks, which were additionally supplied with active and inactivated cellulase to obtain suspended and floc growth, respectively. The cultures were shaken at 100 strokes/min (the average floc dia was approx 2 mm). The cells were centrifuged, washed twice and resuspended in 0.02 M phosphate buffer (pH 7) with additional supply of 10 g glucose/l. The oxygen uptake of the suspended cells and of the cells in flocs was measured in the respirometer (Fig. 5a). The relation between the oxygen

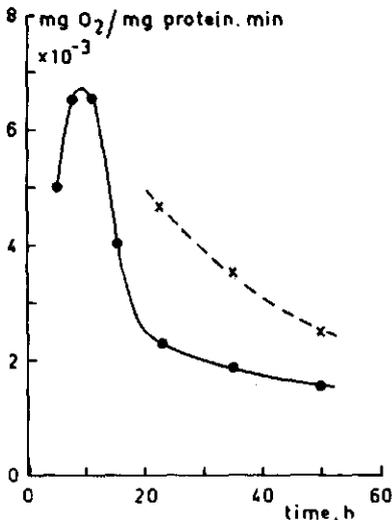


Fig. 3. The potential oxygen demand of cells in suspension and cells in flocs from slowly shaken cultures (50 strokes/min): —●—, cells in suspension; ×---×, cells in flocs.

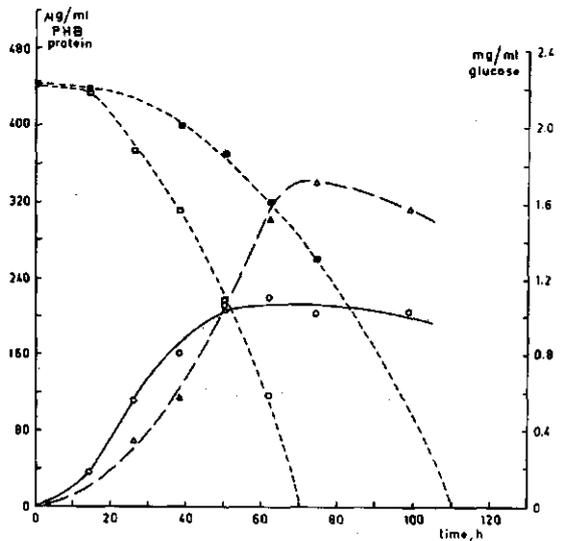


Fig. 4. Growth, and PHB production and glucose uptake of cells in suspension and glucose uptake of cells in flocs of slowly shaken cultures (50 strokes/min) under oxygen. Suspended cells: ○—○, protein; △---△, PHB; □---□, glucose. Cells in flocs: ■---■, glucose.

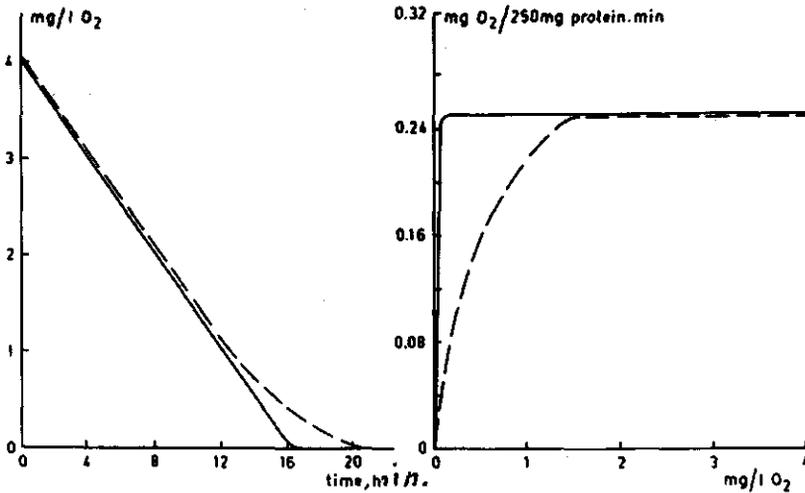


Fig. 5. Oxygen uptake rates of washed cells in suspension (—) and cells in flocs (---). The cell suspension corresponded with 250 mg protein/l.

uptake rate and oxygen concentration (Fig. 5b) can be derived from Fig. 5a by drawing tangents (i.e. oxygen uptake rates) at each oxygen concentration. The oxygen uptake of the suspended cells is shown to be a zero order reaction at oxygen concentrations higher than approx 0.1 mg/l., whereas the oxygen uptake of cells in flocs was a zero order reaction at oxygen concentrations higher than approx 1.5 mg/l. Comparable results were obtained by Mueller *et al.* (1966).

The relation: glucose concentration-glucose respiration rate. To study the effect of different amounts of glucose on the oxygen uptake rate of the cells, *Zoogloea ramigera* was pre-cultivated as described above. The cells were centrifuged, washed twice, re-suspended in 0.02 M phosphate buffer (pH 7) and transferred to the respirometer. After some minutes the oxygen uptake was linear and was considered to represent the endogenous respiration. Subsequently a small amount of a glucose solution was injected caus-

ing an almost immediate increase of respiration. After the glucose had disappeared and the oxygen uptake rate had fallen to the endogenous level, another amount of glucose was injected. The measurements were carried out at oxygen concentrations above 2 mg/l. to prevent oxygen limitation within the flocs. When the oxygen concentration had fallen to 2 mg/l. the suspension was re-aerated. The glucose respiration rate was determined by subtracting the endogenous respiration rate from the total oxygen uptake rate, measured directly after the glucose addition. In Fig. 6, the substrate respiration rate was plotted against the substrate concentration. The respiration rate of cells in flocs was found to be lower than that of cells in suspension below approx 20 mg/l. glucose in the surrounding medium. This suggests that below 20 mg/l. glucose in the medium, the glucose concentration within the flocs had decreased to such a level, that the substrate respiration was limited.

It was assumed that one enzyme step between the

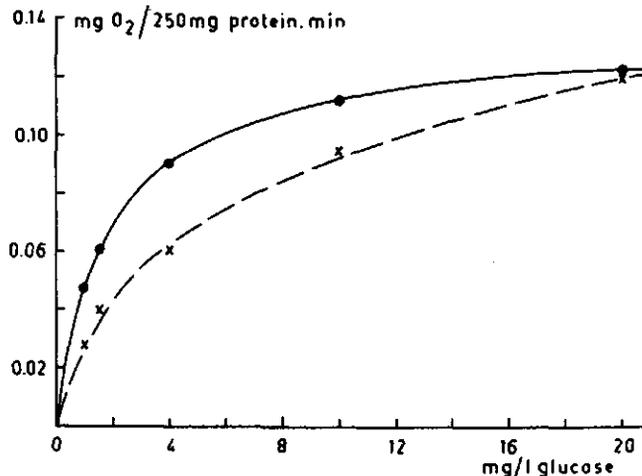


Fig. 6. Glucose respiration of washed cells in suspension (—●—) and cells in flocs (x---x). The cell suspension corresponded with 250 mg protein/l.

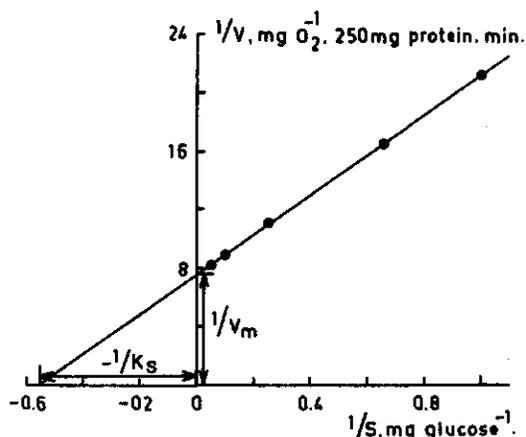


Fig. 7. Lineweaver & Burk plot of the glucose respiration by cells in suspension. The cell suspension corresponded with 250 mg protein/l.

addition of glucose and the substrate respiration was rate-limiting. The Michaelis-Menten equation was used to calculate the K_s value of the cells for the rate-limiting step in the substrate respiration.

$$V = V_m / (1 + K_s / S)$$

V = substrate respiration rate,

V_m = maximum substrate respiration rate,

K_s = substrate concentration at which $V = \frac{1}{2} V_m$,

S = substrate concentration.

K_s and V_m can be determined by plotting $1/V$ against $1/S$ according to the method of Lineweaver & Burk (Fig. 7). $K_s = 1.8$ mg/l. glucose and $V_m = 0.13$ mg O_2 /250 mg protein/min. The Michaelis-Menten equation cannot be used for flocs because of the interfering diffusion kinetics.

DISCUSSION

A study has been made of the effect of floc formation on growth, synthesis of PHB and respiration of *Zoogloea ramigera*. As the cells of this strain were kept together in flocs by cellulose fibrils (Deinema & Zevenhuizen, 1971), cultures of suspended cells can be easily obtained by the addition of small amounts of cellulose to the growth medium. In the absence of active cellulose in the growth medium more than 90% of the cells were found in flocs.

Differences in growth between cells in flocs and cells in suspension were shown to occur when the cultures were shaken very slowly (Fig. 2). Under these conditions both protein synthesis and PHB production of cells in flocs were retarded. After the "lag" period, PHB production of cells in flocs proceeded at almost the same rate as in suspended cells but protein synthesis proceeded at a considerably lower rate. The most significant growth limitation of cells in flocs occurred when the culture was in the early growth phase. This limitation might have been due to oxygen limitation within flocs resulting from the relatively high potential oxygen demand of the cells

during the early growth phase (Fig. 3). However, under pure oxygen, glucose uptake of cells in flocs (slowly shaken cultures) was still retarded as compared with the glucose uptake of suspended cells. The possibility that the substrate supply of the cells within flocs was insufficient for optimum growth might be considered. As the glucose concentration of the medium was relatively high, some other component of the complex medium rather than glucose may have been growth-limiting. To confirm this hypothesis, experiments were carried out with a medium, in which casitone yeast extract had been replaced by NH_4Cl . However, the growth in this medium proceeded only as suspended cells.

The possibility of growth limitation of cells in flocs by produced metabolites has not to be excluded. In this respect the accumulation of carbon dioxide in the flocs in the early growth phase may be considered.

The production of PHB by *Zoogloea ramigera* was found to be independent of the aeration rate when the cells were in suspension (Figs. 1, 2 and 4), indicating that the PHB production was independent of the oxygen concentration. For this reason no increased PHB production was expected to take place in cells within flocs in slowly shaken cultures. This was confirmed by the results plotted in Fig. 2. The independence of PHB production of the oxygen concentration is in contrast with the results obtained by Senior *et al.* (1972), who found an increased PHB production of *Azotobacter beyerinckii* cultures at limiting oxygen concentrations.

The oxygen uptake rates of *Zoogloea ramigera* cells in suspension and in flocs, measured at different oxygen and glucose concentrations, give an orientating rather than a precise description of the affinities of the cells towards oxygen and glucose. Some critical remarks concerning the method and the interpretation of the measurements are: (a) The substrate respiration may not be equal to the glucose uptake rate; (b) When calculating the substrate respiration rate, the endogenous respiration was extrapolated during the substrate respiration. However, it has been shown by Dietrich & Burris (1967) that the endogenous respiration may be considerably stimulated or reduced during substrate respiration, dependent on the organism tested; (c) When oxygen and substrate affinity values of the cells are obtained from batch experiments with non-growing cells, the use of these parameters may be erroneous when applied in continuous processes as for example the activated sludge process. In this respect the adaptation of cells to low oxygen and/or substrate concentrations may be mentioned. Harrison & Pirt (1967) for instance found an increase of the oxygen uptake rate at very low dissolved oxygen concentrations.

It has been clearly shown, however, that the oxygen uptake rate and the glucose respiration rate of cells of *Zoogloea ramigera*, when occurring in flocs, were considerably reduced at a very low concentration of oxygen and glucose, respectively.

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EXPERIMENTS WITH *HALISCOMENOBACTER HYDROSSIS* IN CONTINUOUS CULTURE WITHOUT AND WITH *ZOOGLOEA RAMIGERA*

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Abstract—A study has been made of the sheath-forming bacterium *Haliscomenobacter hydrossis*, by growing the organism in continuous culture. Glucose together with components from trypticase soy broth/yeast extract were used as the energy source. At low dilution rates glucose was a more important energy source than components from trypticase soy broth/yeast extract. *H. hydrossis* was unable to produce large amounts of reserve material even in the presence of an excess of glucose. The minimum doubling time was approximately 13 h in the complex medium. The uptake sequence of amino acids derived from the trypticase soy broth yeast extract was found to be as follows: glutamic acid, glycine, methionine, tryptophan, lysine and arginine were easily consumed by the micro-organism, whereas the other amino acids were taken up mainly at low dilution rates.

Competition experiments were carried out with *H. hydrossis* and *Z. ramigera* at two different dilution rates (0.016 and 0.005 h⁻¹). At both dilution rates *Z. ramigera* was dominantly present in the cultures.

Finally the glucose respiration and glucose uptake of a washed cell suspension of *H. hydrossis* was studied. The oxygen uptake rate increased only very slightly upon addition of glucose. The difference between endogenous respiration and oxygen uptake rate in the presence of glucose was increased when the washed cells had been aerated a period of time.

INTRODUCTION

Haliscomenobacter hydrossis (syn. *Streptothrix hyalina*, *Bergey's Manual*, 1975), is a sheath-forming bacterium, isolated and described by Van Veen *et al.* (1971, 1973). The organism is often observed in activated sludge (Eikelboom, 1975) and when present abundantly it may cause 'bulking' by deteriorating the settling characteristics of the sludge flocs. The competitive position of this thread-forming micro-organism in bulking sludge is not completely understood. Heukelekian (1941) suggested that filamentous micro-organisms, occurring in activated sludge flocs, are less sensitive to the very low oxygen concentrations within the flocs as compared to the unicellular floc-forming bacteria. Chudoba *et al.* (1973) discussed the possibility of relatively high substrate affinities of filamentous micro-organisms being responsible for excessive growth of these bacteria in activated sludge plants; however, without presenting experimental results.

In the present study, continuous culture experiments were carried out with *H. hydrossis* to obtain more information about growth conditions and parameters. In a further series of experiments, the competition between *H. hydrossis* and *Zoogloea ramigera* was studied. Washed cells of *H. hydrossis* were used for measuring the glucose respiration and glucose uptake rates.

MATERIALS AND METHODS

Bacteria

Zoogloea ramigera, strain I-16-M, was obtained from American Type Culture Collection (19623). *Haliscomeno-*

bacter hydrossis, American Type Culture Collection 27775, was provided by Van Veen, Laboratory of Microbiology, Wageningen.

Media

Medium B, g l⁻¹: glucose, 1; casitone, 1; yeast extract, 0.35; vitamin B₁₂, 5 × 10⁻⁶. Medium C, g l⁻¹: glucose 1.5; trypticase soy broth BBL, 1; yeast extract, 0.25; thiamine, 2 × 10⁻⁴; vitamin B₁₂, 5 × 10⁻⁶. Medium C₁: similar to medium C with additional supply of 10 g l⁻¹ agar. Medium D, g l⁻¹: glucose, 1; KNO₃, 1.5; and the basal salts solution, mg l⁻¹: KH₂PO₄, 77; K₂HPO₄, 40; Na₂HPO₄·2H₂O, 40; CaCl₂·2H₂O, 50; MgSO₄·7H₂O, 100; FeCl₃·6H₂O, 5; MnSO₄·H₂O, 3; ZnSO₄·7H₂O, 0.1; CuSO₄·5H₂O, 0.1; H₃BO₃, 0.1; Na₂MoO₄·2H₂O, 0.05; and CoCl₂·6H₂O, 0.05.

The vitamins were sterilized separately by filtration through a Jena G5F filter. Glucose, dissolved in demineralized water, was added separately after heat sterilization.

Enzymes and co-enzymes

Cellulase (ex. Basidiomycetes, 90 mU) was obtained from Merck, Darmstadt, West Germany. Hexokinase, glucose-6-P-dehydrogenase, NADP and ATP were purchased at Boehringer, Mannheim, West Germany.

Chemical analyses

Total carbohydrate was assayed by using the anthrone method as described by Trevelyan and Harrison (1952). Glucose was used as the standard. Total organic carbon (TOC) was determined with a Beckman TOC apparatus connected with an i.r. spectrophotometer, after elimination of carbonates and CO₂ by the addition of HCl to the solution and aeration. Cell protein was assayed with the biuret method as described by Robinson and Hogden (1940). Bovine-serum albumin was used as the standard. Ninhydrin-positive components were determined according to the method described by Rosen (1957). Leucine was used as the reference standard. Separation of a mixture of ninhydrin-positive components was performed with the

aid of a Biocal-200 amino acid analyser following the procedure of Moore and Stein (1954). To determine the amino acid composition in a mixture of proteins and free amino acids, the samples were diluted 20-fold with 6 N HCl after the addition of a known amount of a standard solution of norvaline. Thereafter, the hydrolysis was carried out in sealed glass tubes for 16 h at 110°C. HCl was evaporated under vacuum at 40°C until dry and the residue was dissolved in a buffer solution of pH 2.2. The loss of norvaline was considered to be similar to the loss of the other amino acids during the process of hydrolysis. Free amino acids were directly determined without hydrolysis of the samples.

Enzymatic determinations

Glucose in small amounts from approximately 5 μ m was determined enzymatically with hexokinase, glucose-6-P-dehydrogenase, ATP and NADP according to the method described by Ruchti and Kunkler (1966). The increase of NADPH extinction at 340 nm was measured.

Oxygen uptake experiments

Oxygen uptake experiments were carried out in a Yellow Springs Instruments respirometer (Yellow Springs, Ohio, U.S.A.). During the measurements glucose was injected as required.

Growth conditions

Batch culture; two flasks of 2-l. capacity were filled with 1 l. medium B. One of the flasks was inoculated with *H. hydrossis* and slowly shaken during 3 days at 20°C. After this incubation period the flask, which contained small flocs of *H. hydrossis*, was inoculated with *Zoogloea ramigera*. Simultaneously, another sterile flask was inoculated with the last-mentioned organism. The two flasks were shaken slowly for 5 days at 20°C, whereafter the flocs were harvested.

Continuous culture

Two-litre fermentors (Eschweiler, Kiel, West Germany) were used in continuous-culture experiments. The oxygen content was measured with an oxygen electrode connected to an oxygen detector (Eschweiler). The dissolved oxygen in all experiments was kept above 75% of air saturation by controlling the air flow rate and the stirring rate. The temperature was 20 or 30°C. The pH was controlled automatically and kept at 7.8 or 7.5. The interval between successive samplings was normally three turnovers of the working volume.

Glucose uptake by *H. hydrossis*

The bacterial cells, used in batch glucose uptake experiments, were taken from a continuous culture (medium D, 30°C and pH 7.5). The cells were centrifuged, washed twice, resuspended in 0.0025 M HEPES buffer (sodium salt of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.5 with the addition of 1.5 g l⁻¹ NaCl, and brought into a 300-ml flask, which was stirred continuously at 30°C. After the addition of a known amount of glucose, samples were taken after different incubation periods and the cells quickly separated by vacuum filtration. The glucose in the filtrate was determined enzymatically.

RESULTS

The effect of *H. hydrossis* on the settling characteristics of *Z. ramigera* flocs

Flocs composed of *Z. ramigera* and those composed of *Z. ramigera* and *H. hydrossis* were grown in batch culture. Flocs and suspended cells were separated by settling of the former. After washing free from suspended cells, the settling of both types of

flocs was followed in 100-ml cylinders by measuring the movement of the division line between floc mass and clear water (Fig. 1). It was shown that the flocs, composed of both *Z. ramigera* and *H. hydrossis* cells, settled at a much slower rate than those consisting of *Z. ramigera* only. The sludge volume indices (S.V.I.), expressed as ml volume of the flocs per g dry weight, calculated after 30 min settling time, were found to be 58 for the pure culture and 114 for the mixed culture.

The composition of the mixed culture flocs was estimated by incubating 100 ml of flocs at 30°C for 30 min with 2 ml of a solution containing 10 mg cellulase (ml distilled water)⁻¹. This treatment releases the cells of *Z. ramigera* which are kept together by cellulose fibrils (Deinema and Zevenhuizen, 1971). The filamentous flocs of *H. hydrossis* were not affected by cellulase. Microscopic examination revealed that more than 90% of the suspended cells belonged to *Z. ramigera*, whereas the remaining flocs consisted largely of *H. hydrossis* cells. The *H. hydrossis* flocs were separated from the suspended *Z. ramigera* cells by settling. By determining the dry weights of flocs and suspended cells, it was calculated that approximately 20% of the dry weight of the original mixed flocs was derived from *H. hydrossis*.

Continuous cultures of *H. hydrossis*

H. hydrossis was cultivated in medium C in continuous culture at pH 7.8 and at \pm 20°C.

Good growth of *H. hydrossis* in this medium was observed by van Veen *et al.* (1973), and no indications were obtained that one or more essential compounds were lacking.

Samples of the culture were taken at different residence times and the composition of the cells and of

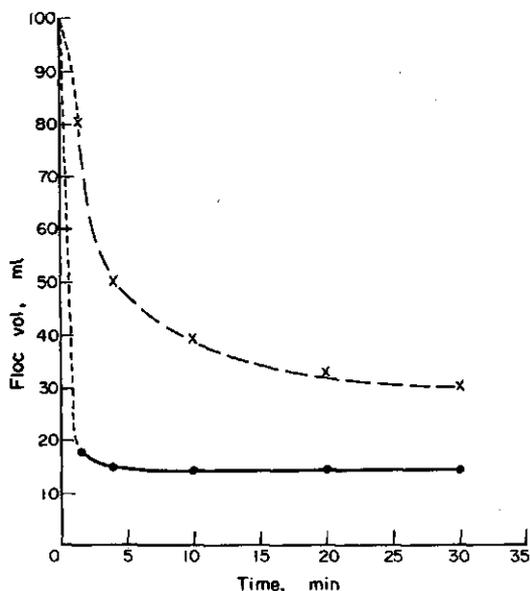


Fig. 1. Settling characteristics of flocs consisting of *Z. ramigera* (80%) and *H. hydrossis* (20%), X--X; and of *Z. ramigera* only, ●—●. The mixed culture contained 2.5 g cell dry weight l⁻¹, the pure culture 2.6 g l⁻¹.

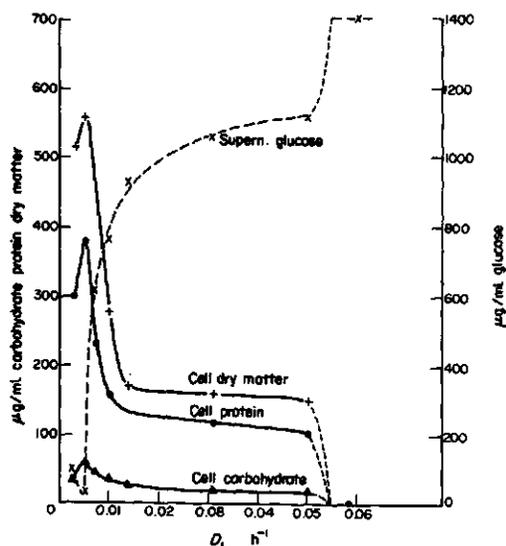


Fig. 2. The amount of cell dry matter, cell protein, cell carbohydrate, and glucose in the supernatant, determined at different dilution rates of a continuous culture of *H. hydrossis*.

the medium analysed. From the results obtained (Fig. 2) it was concluded that the culture was not glucose-limited at a dilution rate above approximately 0.005 h^{-1} . The growth limitation above $D = 0.005 \text{ h}^{-1}$ might have been due to a limited availability of amino acids, caused by a suppressive effect of glucose upon the uptake of (some) amino acids as has been reported by Stokes and Powers (1967) with a *Sphaerotilus* sp.

To decide whether glucose or components derived from trypticase soy broth and/or yeast extract were used as carbon and energy source by *H. hydrossis*, cell carbon and total organic carbon (TOC) of the medium were estimated at different dilution rates. The

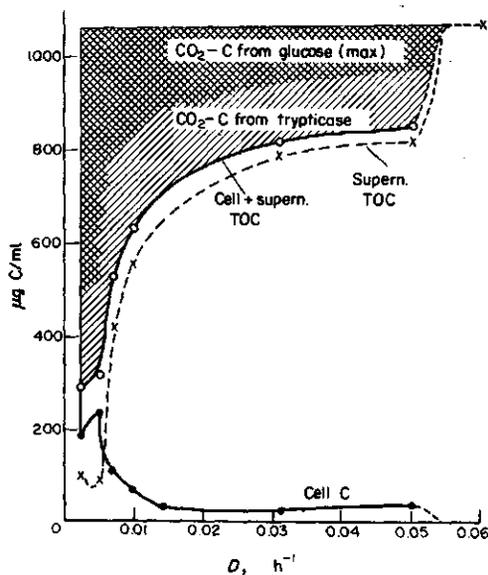


Fig. 3. The amount of cell carbon, TOC of the supernatant and cell carbon + TOC of the supernatant, determined at different dilution rates of a continuous culture of *H. hydrossis*.

Table 1. The cell composition of *H. hydrossis* at various dilution rates

%	$D \text{ (h}^{-1}\text{)}$			
	0.005	0.010	0.031	0.050
Dry cell matter	100	100	100	100
Cell protein	69	(56)	70	70
Cell carbohydrate	11	12	13	11

difference between cell carbon + supernatant TOC, and TOC of the influent was assumed to correspond with the produced $\text{C}-\text{CO}_2$ at a certain dilution rate. The theoretical maximum amount of CO_2 derived from glucose was calculated from the amount of glucose which had disappeared at a certain residence time assuming that all of this glucose had been respired. The results of these calculations are shown in Fig. 3. Especially at higher dilution rates, at least some components from trypticase soy broth and yeast extract were relatively more important for respiration, whereas at lower dilution rates relatively more glucose was respired.

It is obvious that *H. hydrossis* did not accumulate substantial quantities of reserve materials under the experimental conditions (Table 1).

The minimum doubling time of *H. hydrossis* was estimated by using the growth equation $\mu = \ln 2/t_d$, where μ is the specific growth rate and t_d the doubling time. When the culture is in steady state $\mu = D$. The minimum doubling time in medium C appeared to be approximately 13 h.

The dilution rate $D = 0.05$ used in the formula was the estimated value at which the cells were washed out of the fermentor.

The formation of ninhydrin-positive compounds from trypticase soy broth and yeast extract was studied more in detail. The concentration of these compounds increased at dilution rates between approximately $D = 0.0075$ and $D = 0.05 \text{ h}^{-1}$, in contrast to the ammonium content of the medium which increased only slightly during cultivation (Fig. 4). This

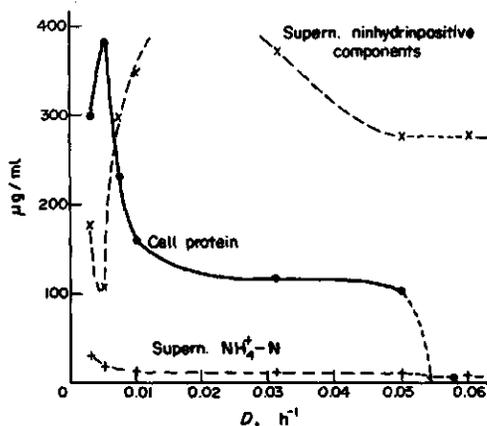


Fig. 4. The amount of ninhydrin-positive components in supernatant, $\text{NH}_4^+\text{-N}$ in the supernatant, and cell protein determined at different dilution rates of a continuous culture of *H. hydrossis*.

Table 2. The occurrence of amino acids in the cell-free filtrate so a culture of *H. hydrossis* growing continuously on a medium of trypticase/soy broth/yeast extract at different dilution rates (*D*)

Amino acid (mM)	<i>D</i> = 0.005 h ⁻¹			<i>D</i> = 0.010 h ⁻¹			<i>D</i> = 0.014 h ⁻¹			<i>D</i> = 0.031 h ⁻¹			Influent		
	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
Asp	0.107	0.096	0.011	0.115	0.087	0.028	0.231	0.177	0.054	0.403	0.334	0.069	0.465	0.420	0.045
Thr	0.020	0.015	0.005	0.019	0.010	0.009	0.055	0.025	0.030	0.110	0.070	0.040	0.131	0.106	0.025
Ser	0.104	0.098	0.006	0.128	0.065	0.063	0.238	0.095	0.143	0.363	0.166	0.197	0.371	0.328	0.043
Glu	0.338	0.332	0.006	0.335	0.313	0.022	0.612	0.563	0.049	0.983	0.916	0.067	1.218	1.146	0.072
Prol	0.093	0.089	0.004	0.100	0.077	0.023	0.281	0.119	0.162	0.406	0.288	0.118	0.452	0.405	0.047
Glyc	0.071	0.068	0.003	0.078	0.050	0.028	0.136	0.091	0.045	0.219	0.153	0.066	0.343	0.284	0.059
Ala	0.092	0.089	0.003	0.253	0.100	0.153	0.374	0.165	0.209	0.459	0.197	0.262	0.476	0.377	0.099
Val	0.048	0.045	0.003	0.171	0.086	0.085	0.451	0.196	0.255	0.529	0.288	0.241	0.535	0.459	0.076
Meth			0.004			0.013			0.030			0.048			0.078
Isoleu	0.058	0.053	0.005	0.095	0.070	0.025	0.140	0.090	0.050	0.234	0.157	0.077	0.248	0.209	0.039
Leu	0.070	0.059	0.011	0.118	0.077	0.041	0.539	0.310	0.229	0.788	0.394	0.394	0.826	0.477	0.349
Tyr	0.003	0.004	0.001	0.022	0.008	0.014	0.043	0.014	0.029	0.061	0.025	0.036	0.098	0.080	0.018
Pheala	0.030	0.024	0.006	0.070	0.031	0.039	0.137	0.049	0.088	0.231	0.103	0.128	0.254	0.162	0.092
Tryp			0.0001			0.006			0.007			0.021			0.040
Lys	0.015	0.014	0.001	0.060	0.054	0.006	0.268	0.177	0.091	0.435	0.242	0.193	0.687	0.501	0.186
His	0.010	0.010	0.0005	0.022	0.018	0.004	0.083	0.056	0.027	0.112	0.064	0.048	0.130	0.113	0.017
Arg	0.010	0.010	0.0001	0.013	0.013	0.0004	0.045	0.034	0.011	0.084	0.043	0.041	0.163	0.083	0.080
Sum without meth and tryp	1.071	1.096	0.065	1.599	1.059	0.540	3.633	2.161	1.472	5.417	3.440	1.977	6.397	5.150	1.247

I = total, II = amino acids contained in peptides, III = I - II = free amino acids.

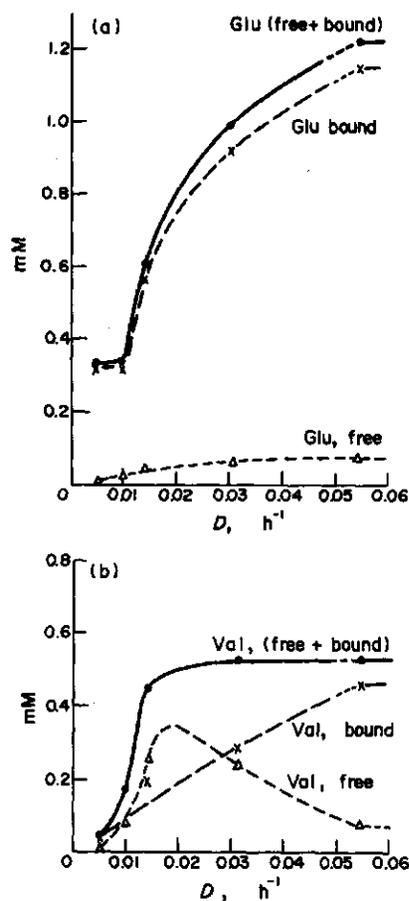


Fig. 5. (a) The concentrations of total glutamic acid, bound in peptides and of free glutamic acid. (b) the same for valine, determined at different dilution rates of a continuous culture of *H. hydrossis*.

implies that the peptides were broken down, but a considerable part of the amino acids and other ninhydrin-positive compounds were not taken up by the bacteria unless the dilution rate was below approximately 0.0075 h⁻¹.

To collect more information about the uptake sequence of amino acids by *H. hydrossis*, the samples taken at different dilution rates were centrifuged and their supernatant analysed for amino acids, as described in 'Methods'. Total amino acids, derived from peptides and free amino acids, were determined after hydrolysis of the samples, free amino acids by analysing the samples without preceding hydrolysis and amino acids in peptides by subtracting the latter from the former (Table 2). At *D* = 0.031 h⁻¹ a considerable part of the peptides had been hydrolysed, which resulted in a pronounced rise in concentration of most of the free amino acids. In the case of glutamic acid, glycine, methionine, tryptophan, lysine and arginine, this increase was small or absent because of uptake by the bacteria. At lower dilution rates practically all of the free amino acids were finally taken up. However, a considerable amount of most of the bound amino acids remained in the medium. In Figs. 5a and b the fate has been shown of glutamic acid, an example of an amino acid which was readily consumed, and valine, an example of an amino acid which was taken up poorly.

Competition experiments with *Haliscomenobacter hydrossis* and *Zoogloea ramigera*

To investigate the competitive ability of *H. hydrossis* in activated sludge plants, model experiments were carried out with *H. hydrossis* and *Z. ramigera*

Table 3. Cell protein, cell carbohydrate and supernatant carbohydrate in continuous cultures of pure cultures of *H. hydrossis* and *Z. ramigera* and mixed cultures of both organisms

	Protein	Cells $\mu\text{g ml}^{-1}$ Carbohydr.	Supernatant $\mu\text{g ml}^{-1}$ Carbohydrate
$D = 0.005 \text{ h}^{-1}$			
<i>H. hydrossis</i> (A)	380	59	32
<i>Z. ramigera</i> (C)	207	37	50
<i>H. hydrossis</i> + <i>Z. ramigera</i> * (B)	592	38	20
<i>Z. ramigera</i> in mixed culture† (B)	444	n.c.	
<i>H. hydrossis</i> in mixed culture† (B)	148	n.c.	
$D = 0.016 \text{ h}^{-1}$			
<i>H. hydrossis</i> (A)	225	45	650
<i>Z. ramigera</i> (C)	217	47	250
<i>H. hydrossis</i> + <i>Z. ramigera</i> * (B)	557	45	21
<i>Z. ramigera</i> in mixed culture† (B)	533	n.c.	
<i>H. hydrossis</i> in mixed culture† (B)	24	n.c.	

* Average values.

† Average values, indirectly calculated.

in continuous culture. Two different dilution rates were used to imitate differently loaded activated sludge plants.

The continuous culture experiments were carried out at 20°C and at pH 7.8. Medium C was used as the growth medium. After pumping 1 l. of the medium into three fermentors A, B and C, two of the fermentors, A and B, were inoculated with *H. hydrossis* and the medium was continuously stirred. Good growth was obtained after 7 days incubation. During this period no fresh medium was pumped into the vessel. Fermentors B and C were now inoculated with *Z. ramigera*. The growth of this bacterium proceeded only as suspended growth. After another incubation period of 7 days, the pumps were switched on and the dilution rates fixed at $D = 0.005 \text{ h}^{-1}$. Samples were taken after 1.6 turnovers (320 h) and the numbers of viable *Zoogloea* cells in the fermentors B and C determined by making a series of dilutions in sterile tap water and plating 0.1 ml of suitable dilutions on medium C₁. The plates were incubated for 5 days at 30°C, whereupon the colonies were counted. Only colonies of *Z. ramigera* developed on the counting plates as the filamentous, floc-forming cells of *H. hydrossis* were lost at high dilutions. After taking the samples, the pumping rates were changed and fixed at $D = 0.016 \text{ h}^{-1}$. Samples were taken after 1.6 turnovers (100 h) and the pumps changed again to return to the dilution rate of 0.005 h^{-1} . The procedure of changing the pumping rates was repeated several times. The protein (biuret) and carbohydrate (anthrone) contents of the cells and the carbohydrate content (anthrone) of the supernatant were determined (Table 3 and Figs. 6 and 7).

Z. ramigera (fermentor C). At $D = 0.016 \text{ h}^{-1}$, a considerable amount of carbohydrate was still present in the supernatant, indicating that glucose was not growth-limiting (Table 3). The relatively low numbers of cells counted at both $D = 0.005 \text{ h}^{-1}$ and $D = 0.016$

h^{-1} (Fig. 6) confirm the observation of Friedman and Dungan (1968) that *Z. ramigera*, strain I-16-M, is unable to hydrolyse proteins.

From the results obtained, the relationships between number of cells and quantity of cell protein were derived: $71 \mu\text{g protein} (10^9 \text{ cells})^{-1}$ at $D = 0.005 \text{ h}^{-1}$ and $80 \mu\text{g protein} (10^9 \text{ cells})^{-1}$ at $D = 0.016 \text{ h}^{-1}$ (Figs. 6 and 7).

H. hydrossis (fermentor A). The cell concentration at $D = 0.005 \text{ h}^{-1}$ was higher than that at $D = 0.016 \text{ h}^{-1}$ (Table 3). A substantial portion of the carbohydrate (glucose in the medium was not used at $D = 0.016 \text{ h}^{-1}$).

Mixed culture of Z. ramigera and H. hydrossis (fermentor B). The total amount of cell protein at both

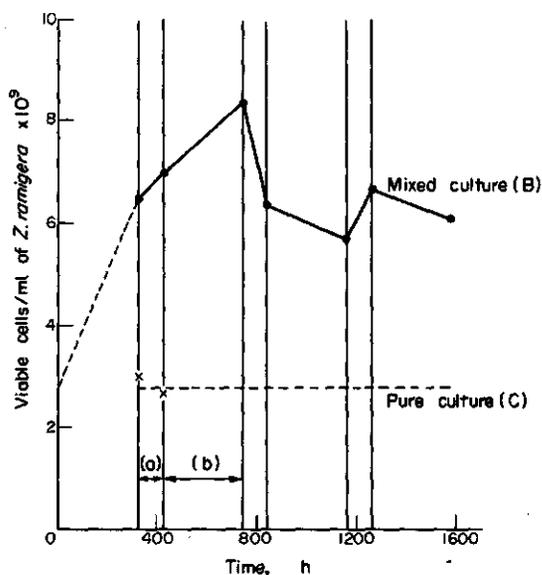


Fig. 6. Numbers of viable *Z. ramigera* cells in pure culture (C), and in mixed culture with *H. hydrossis* (B), at different dilution rates.

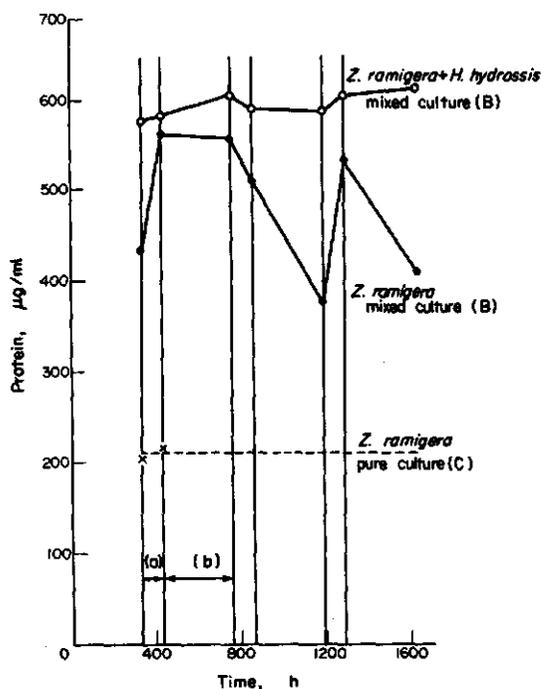


Fig. 7. The amount of cell protein of *Z. ramigera* in pure culture (C), in mixed culture (B), and of *Z. ramigera* + *H. hydrossis* (B).

$D = 0.005 \text{ h}^{-1}$ and $D = 0.016 \text{ h}^{-1}$ was higher than with each of the pure cultures of *Z. ramigera* (C) and *H. hydrossis* (A). The carbohydrate contents of the supernatants at the two dilution rates were low (Table 3).

To estimate which part of the total cell protein of the mixed culture was derived from *Z. ramigera*, the

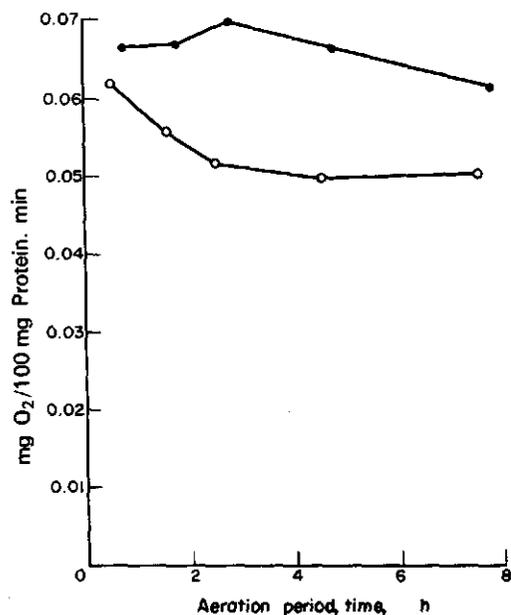


Fig. 8. Endogenous respiration, \circ — \circ ; and substrate respiration (after adding glucose), \bullet — \bullet of *H. hydrossis*, measured after different aeration periods of the washed cells.

quantity of protein of the counted *Z. ramigera* cells (fermentor B) was calculated by using the data obtained with the pure culture of this organism in fermentor C. It was assumed that the quantity of protein per *Z. ramigera* cell was constant in fermentors B and C as it was, for example, in the experiments at different dilution rates (Table 1).

The results obtained clearly show that *Z. ramigera* dominated in the mixed cultures at the two different dilution rates. The number of viable *Z. ramigera* cells in the mixed culture was considerably higher than in the pure culture. The stimulated growth of *Z. ramigera* in mixed culture with *H. hydrossis* can be explained by an improved availability of amino acids and/or small units of peptides resulting from the proteolytic activity of *H. hydrossis*. The differences in the composition of the bacterial populations at $D = 0.005 \text{ h}^{-1}$ and $D = 0.016 \text{ h}^{-1}$ (Table 3) were assumed to be non-significant considering the relatively inaccurate counting method used.

Glucose respiration and glucose uptake by *H. hydrossis*

Cells of *H. hydrossis* were taken from a continuous culture fed with medium D at the dilution rate of 0.01 h^{-1} , at 30°C . After centrifuging and washing twice, the cells were resuspended in 0.0025 M Hepes buffer, pH 7.5, with additional supply of 1.5 g l^{-1} NaCl (to obtain roughly the same osmotic value as in the pre-cultivation medium). The washed cell suspension was aerated continuously at 30°C . After different incubation periods, samples were taken and the endogenous respiration and the oxygen uptake after addition of 10 mg l^{-1} glucose were measured in the respirometer at 30°C (Fig. 8). The oxygen uptake rate in the absence of glucose, decreased slightly during the aeration period of the washed cells, but addition of glucose to the samples restored the respiration almost to the initial level. Increasing the glucose concentration from 10 to 1000 mg l^{-1} did not affect the respiration rate. It was evident that K_s values for glucose respiration could not be measured in this way (Krul, 1976).

The fact that washed cells of *H. hydrossis* measured for O₂ uptake without a preceding aeration period hardly respond to added glucose (Fig. 8), suggests the presence of sufficient amounts of easily respirable substrates within the cells or adsorbed to the cells.

To determine the glucose uptake rate directly, the following procedure was carried out. Centrifuged and washed cells of *H. hydrossis* were supplied with a small amount of glucose. The cell suspension was aerated continuously. Samples were taken after different incubation periods and the cells immediately separated from the medium by filtration. The glucose concentration in the filtrate was determined enzymatically (Fig. 9).

The glucose uptake appeared to be non-linear below a glucose concentration of 10 mg l^{-1} . Adding a second amount of glucose to the washed cells which had just completed the respiration of the first amount

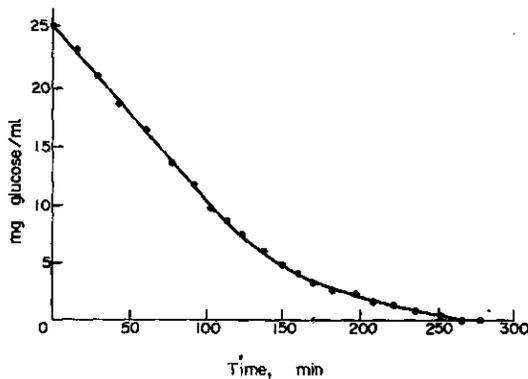


Fig. 9. The disappearance of glucose from the medium by washed cells of *H. hydrossis*.

gave a similar uptake rate. The K_s value, defined as the substrate concentration at which $V = \frac{1}{2} V_{max}$, was approximately 5 mg l^{-1} , which is relatively high compared to the K_s values of glucose uptake, measured with a number of other bacteria: *Zoogloea ramigera*, 1.6 mg l^{-1} (Kruel, 1976); *Pseudomonas aeruginosa*, 2 mg l^{-1} (Eagon and Phibbs, 1971); *Archromobacter aquamarinus*, 0.007 mg l^{-1} (Vaccaro and Jannasch, 1966).

Subsequently, the following rough calculation was made. The oxygen uptake rate in the presence of glucose was approximately $0.066 \text{ mg O}_2 (100 \text{ mg protein})^{-1} \text{ min}^{-1}$ (Fig. 8), corresponding with the complete respiration of $0.062 \text{ mg glucose } (100 \text{ mg protein})^{-1} \text{ min}^{-1}$. During respiration, the rate of glucose disappearance from the medium was about $0.120 \text{ mg } (100 \text{ mg protein})^{-1} \text{ min}^{-1}$, which is about twice as much as the amount respired.

SUMMARY AND DISCUSSION

The presence of relatively small amounts of filamentous bacteria may cause a serious deterioration of the settling characteristics of bacterial flocs.

To understand the appearance of filamentous bacteria in activated sludge, a thorough study has to be made of the physiology of these bacteria and their growth conditions. In the present study, *H. hydrossis* was chosen as the filamentous micro-organism which was investigated more extensively. The bacterium was grown in continuous culture at different dilution rates using a complex medium (trypticase soy, yeast extract and glucose). The following results were obtained:

(a) significantly higher cell contents were obtained at dilution rates lower than 0.015 h^{-1} (Fig. 2);

(b) glucose was not growth-limiting at dilution rates higher than 0.005 h^{-1} (Fig. 2);

(c) *H. hydrossis* was unable to store large amounts of reserve materials, even in the presence of an excess of glucose (Table 1);

(d) amino acids derived from trypticase soy/yeast extract were used both as nitrogen source and energy source in the presence of glucose. The degree of remo-

val by respiration of at least some compounds derived from trypticase soy broth/yeast extract was relatively high at low retention times (Fig. 3);

(e) glutamic acid, glycine, methionine, tryptophan, lysine and arginine derived from trypticase soy/yeast extract as a result of the proteolytic activity of *H. hydrossis*, were relatively easily taken up by *H. hydrossis*. The uptake of the remaining amino acids was apparently suppressed (by glucose), resulting in an accumulation of these amino acids in the free state at relatively high dilution rates. At dilution rates below 0.015 h^{-1} all the amino acids were taken up more or less completely by *H. hydrossis* (Table 2).

The high cell concentrations at dilution rates below 0.015 h^{-1} as recorded under (a) were due to the fact that a number of less easily usable amino acids were taken up in considerable amounts only at prolonged retention times.

The growth limitation above $D = 0.005 \text{ h}^{-1}$ might have been due to a limited uptake of amino acids, caused by a suppressive effect of glucose. Such an effect has been reported by Stokes and Powers (1967) with a *Sphaerotilus* sp.

The uptake of amino acids by *H. hydrossis* is in accordance with the common uptake pattern of amino acids of micro-organisms. Only glycine is rarely found to be easily usable.

The mixed-culture experiments, carried out with *H. hydrossis* and *Z. ramigera* revealed that the growth of the latter organism was stimulated considerably as compared to its growth in pure culture (Table 3). This was probably due to the proteolytic activity of *H. hydrossis*, which favoured the supply of amino acids of the nonproteolytic *Z. ramigera*. The mixed-culture experiments were carried out at two different dilution rates, $D = 0.005 \text{ h}^{-1}$ and $D = 0.016 \text{ h}^{-1}$, which succeeded each other after 1.6 turnovers. A steady-state situation had certainly not been reached. The counting of the numbers of viable *Z. ramigera* cells, used to estimate the composition of the mixed cultures, proved to be too inaccurate to clarify differences between the composition of the bacterial populations at the two dilution rates. Moreover, the number of the non-viable cells of *Z. ramigera* was not taken into account.

The observation that the growth of *Z. ramigera* in mixed culture with *H. hydrossis* was stimulated at the dilution rate $D = 0.005 \text{ h}^{-1}$ does not support the hypothesis that unicellular bacteria are unable to compete with filamentous bacteria at low substrate concentrations because of assumed relatively low substrate affinity of the unicellular micro-organisms as compared with that of filamentous bacteria.

One of the typical characteristics of *H. hydrossis* is the poor response of the O_2 uptake of washed cells to added glucose. The response became more significant upon prolonged aeration of the washed cells, owing to the reduced endogenous respiration, whereas the glucose respiration rate was unaltered (Fig. 8). In spite of the poor response of the O_2 uptake

of washed cells to the addition of glucose, a clear uptake of this compound was observed (Fig. 9).

The glucose uptake was non-linear below approximately 10 mg l^{-1} . The K_s value of the glucose uptake was found to be approximately 5 mg l^{-1} , which is relatively high when compared to K_s values of the glucose uptake, measured at many other bacteria.

From the results obtained in this study it was concluded that the filamentous bacterium *H. hydrossis* has a poor competition ability in heavily loaded activated sludge plants because of a low growth rate (low maximum substrate uptake rate). In under-loaded plants, this micro-organism may have a better competition position because of a possibly high affinity for some substrates; however, no evidence has so far been given for this assumption.

Acknowledgement—The research was supported by the Deutsche Forschungsgemeinschaft.

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THE RELATIONSHIP BETWEEN DISSIMILATORY NITRATE REDUCTION AND OXYGEN UPTAKE BY CELLS OF AN *ALCALIGENES* STRAIN IN FLOCS AND IN SUSPENSION AND BY ACTIVATED SLUDGE FLOCS

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Abstract—The oxygen uptake and the dissimilatory nitrate reduction by anaerobically grown cells of a denitrifying *Alcaligenes* strain, occurring in floc form or in suspension, were studied at different oxygen concentrations in the surrounding medium. When the oxygen concentration in the medium fell below 1.5 mg l^{-1} , the nitrate reduction by the cells within floes increased considerably. The cells in suspension showed an increased nitrate reduction when the oxygen concentration was below 0.1 mg l^{-1} . When anaerobically grown cells had been aerated for 24 h in a nitrogen-free medium, the cells became sensitive to respiration inhibition by nitric oxide, resulting from nitrate reduction. This gave rise to an increased nitrate reduction below $2.5 \text{ mg oxygen l}^{-1}$ when the cells were aggregated in floes and below $0.1 \text{ mg oxygen per litre}$ when the cells were in suspension. The nitrate reduction by the denitrifying, floc-forming pure culture was compared with that of activated sludge floes.

INTRODUCTION

The diffusion of oxygen into bacterial floes of *Zoogloea ramigera* was studied by Mueller, Boyle and Lightfoot (1966). The maximum oxygen uptake of cells in suspension was measured when the dissolved oxygen content was above 0.1 mg l^{-1} . With floes the maximum oxygen-uptake rate was reached when the dissolved oxygen in the surrounding medium was maintained between 0.6 and 2.5 mg l^{-1} or above, depending on floc size and form. Oxygen transfer into mycelial pellets of *Aspergillus niger* was studied by Kobayashi, van Dedem and Moo-Young (1973). The oxygen-uptake rate became nonlinear below $1.6 \text{ mg dissolved oxygen l}^{-1}$. Wuhrmann (1964) calculated the diffusion coefficient for oxygen into spherical floes. He concluded that the oxygen-uptake rate of the innermost cells in a spherical floc was not limited above a critical oxygen tension of 0.1 mg l^{-1} . This oxygen tension within the floc was attained at an oxygen tension of 2 mg l^{-1} in the mixed liquor of activated sludge plants. Ruffer (1964) considered the anaerobic innerpart of sludge floes responsible for the loss of nitrogen by denitrification. This loss ranged from 28.8 to 36.1% of the influent nitrogen at an oxygen tension of 0.5 mg l^{-1} . When the oxygen concentration was 10 mg l^{-1} , this loss ranged from 3.9 to 9%. In this paper we report the effect of oxygen gradients in floes of a pure culture and in sludge floes upon the dissimilatory nitrate reduction.

MATERIALS AND METHODS

Activated sludge

Denitrifying and aerobically grown activated sludge were produced in two laboratory installations described by Klapwijk (1975). The aerobically grown activated sludge

was strongly aerated. The denitrifying sludge was stirred slowly thus favouring anaerobic conditions. The sludges were fed intermittently with artificial sewage for 1 yr. The load of both installations was about 0.1 g COD g^{-1} sludge day $^{-1}$.

Media

Artificial sewage (g l^{-1}). Skim-milk powder, 9; urea, 0.03; gelatine, 0.06; starch, 0.12; $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.03; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.003; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.004; KCl, 0.004; with additional supply of 1.2 g l^{-1} KNO_3 in the influent of the denitrifying installation.

Medium P (g l^{-1}). Peptone, 4; KNO_3 , 2 and glycerol, 5.

Medium Y (g l^{-1}). Casitone, 1; yeast extract, 0.2 and glycerol, 2.

Medium Y-1. The same as medium Y with in addition 10 g agar l^{-1} .

Medium D (g l^{-1}). Peptone, 4; KNO_3 , 10; glycerol, 5 and agar, 10.

Medium G (g l^{-1}). Glycerol, 10; KH_2PO_4 , 0.027; K_2HPO_4 , 0.04; $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.04; CaCl_2 , 0.05; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.075; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.005; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.005; $\text{ZnSO}_4 \cdot \text{H}_2\text{O}$, 0.0001; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.0001; H_3BO_3 , 0.0001; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.00005; $\text{CoCl}_2 \cdot \text{H}_2\text{O}$, 0.00005.

All of the media received a concentrated phosphate solution, sterilized separately, to a final concentration of 0.02 M , pH 7.

Isolation of bacteria

Sludge samples of 1 ml were diluted 10^{-1} in sterile 0.02 M phosphate buffer of pH 7. The tubes were placed for one minute in an ultrasonic cleaning bath in order to disperse the floes (Report, W.P.R., 1970). The dispersed floes were diluted 10^{-4} in sterilized water and spread on previously dried agar plates containing medium Y-1. After 5 days incubation at 25°C , about 100 colonies from both aerobically grown activated sludge and denitrifying sludge were isolated at random.

Floc-forming ability

The isolates were tested for floc-forming ability by inoculating the bacteria in tubes containing 7 ml medium Y. The tubes were vigorously aerated for 3 days at 25°C .

When more than approximately 50% of the cells were found to occur in flocs (visually estimated) the isolates were classified as floc-forming bacteria.

Dispersion of flocs by cellulase

Deinema and Zevenhuizen (1971) reported the formation of extracellular cellulose fibrils by floc-forming bacteria; the flocs could be dispersed by the addition of cellulase. To estimate which percentage of the floc-forming bacteria formed cellulose fibrils, the following procedure was carried out. The settled flocs were separated from the suspended cells and resuspended in 5 ml 0.02 M phosphate buffer, pH 7, supplied with 1 ml cellulase solution (5 mg cellulase per ml distilled water). Control tubes with resuspended flocs received 1 ml of boiled (inactivated) cellulase solution. The tubes of both series were shaken for 1 h at 30°C and thereafter visually judged: when more than approximately 50% of the flocs were dispersed by cellulase the flocs were considered to be cellulase-sensitive.

Denitrification

Denitrifying ability of the isolated bacteria was tested by inoculating test tubes containing 10 ml melted medium *D* at 40°C. The tubes were incubated for 5 days at 25°C. The formation of large amounts of gas was considered as a positive indication of denitrification.

Cell material used for respiration and nitrate-reduction experiments was cultivated in 2-l. flasks containing 1 l. medium *P*. After inoculation, the flasks were shaken slowly (to stimulate floc formation) at 30°C for 3 days. The cells produced large amounts of nitrogen gas during the cultivation period. Most of the growth took place under anaerobic conditions.

Treatment of cell material used for respiration and nitrate-reduction experiments

After anaerobic growth in medium *P*, the flocs were harvested, washed and resuspended in the nitrogen-free medium *G*. The resuspended flocs were divided into two portions, *A* and *B*. Portion *A* was again divided into two portions, *A*₁ and *A*₂, of which portion *A*₁ was supplied with 0.1 mg active cellulase per mg cell dry matter and portion *A*₂ with the same amount of boiled (inactivated) cellulase. Both portions *A*₁ and *A*₂ were vigorously aerated for 1 h at 30°C. After this aeration period more than 90% of the flocs of portion *A*₁ were dispersed. Portion *B* was aerated for 24 h at 30°C and thereafter the flocs were harvested, washed and resuspended in medium *G*. These resuspended flocs were divided into two portions *B*₁ and *B*₂ of which the flocs in portion *B*₁ were dispersed as described for the flocs of *A*₁. Portion *B*₂ was treated similarly to *A*₂. Respiration and nitrate-reduction experiments were performed with the cell suspensions of *A*₁ and *B*₁ and the flocs of *A*₂ and *B*₂.

Oxygen and nitrate measurements in the respirometer

Oxygen uptake and nitrate-reduction experiments were carried out in a respirometer with an oxygen and a nitrate electrode as described by van Kessel (1975).

Chemical analyses

Nitrite was determined chemically by the method of Griess-Romijn-van Eck (NEN 1056 IV.2, 1966). Protein was assayed by the Folin-Ciocalteu method as described by Lowry *et al.* (1951), using bovine serum albumin as the standard.

Gas analyses

Oxygen and nitrogen in the gas phase were separated on a 900 × 0.2 cm steel column containing Poropak 80-100 mesh at 50°C and measured by thermal conducti-

city in a Packard-Becker, multigraph 409, gas chromatograph using hydrogen as a carrier gas (25 ml min⁻¹).

Flasks of 35 ml capacity, containing 10 ml cell suspension (medium *G*) were flushed with helium after which oxygen was added as indicated. Gas samples of 0.1 ml were analysed.

RESULTS

Isolation and some characteristics of the isolated strain 15

In order to compare the oxygen uptake and the dissimilatory nitrate reduction by cells in suspension and cells in flocs, a number of bacteria were isolated from aerobically grown and denitrifying activated sludge. The isolated bacteria were tested for the following characteristics: (1) the capacity to form flocs, (2) the sensitivity of the flocs towards dispersion by cellulase and (3) the ability to denitrify.

The percentage of floc-forming bacteria, 31 and 36% (Table 1), respectively, was higher than that reported by Deinema and Zevenhuizen (1971). These authors found that the percentage of floc-forming bacteria was between 10 and 20% of the total number of bacteria isolated from aerobically grown activated sludge. More than 50% of the newly isolated floc-forming bacteria had flocs which were dispersible after enzymatic treatment with cellulase.

All of the following experiments with pure cultures were carried out with one isolate from group IV, obtained from denitrifying sludge. The isolate, strain 15, belonging to the genus *Alcaligenes*, was obligatory aerobic, Gram-negative, peritrichously flagellated and was able to use lactate, glycerol, propanol, mannitol, sorbitol, mannose, lactose, glucose, galactose, saccharose, and cellobiose as C-source. No growth was obtained with citrate and butyrate. Suitable N-sources were ammonium chloride and nitrate. No vitamins had to be given. Flocs of strain 15, grown anaerobically in medium *P*, had a diameter range from 500 to 5000 μm. The flocs settled within 10 min after harvesting and resuspending in 0.02 M phosphate buffer, pH 7. Addition of 1 g KNO₃ l⁻¹ resulted in flotation of the flocs within 10 min (Fig. 1). The phenomenon of flotation can also be observed in final settling tanks of activated sludge plants at a prolonged retention-time of the sludge and in the

Table 1. Floc-forming ability, sensitivity of the flocs towards dispersion by cellulase and capacity to denitrify of 93 strains isolated from aerobically grown activated sludge and of 94 strains, isolated from denitrifying sludge

Property	Strains isolated from	
	aerobically grown activated sludge	denitrifying activated sludge
I Floc-forming capacity	31%	36%
II Floc-forming capacity and cellulase sensitivity of the flocs	16%	26%
III Denitrifying capacity	31%	60%
IV Floc-forming capacity, cellulase sensitivity of the flocs and denitrifying capacity	10%	20%

* Percentage related to the total number of isolates from each sludge

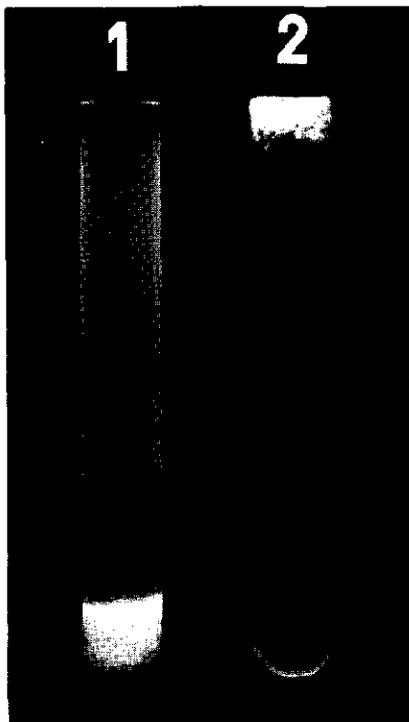


Fig. 1. Settled flocs in medium without nitrate (tube 1) and flotated flocs in medium with $1 \text{ g KNO}_3 \text{ l}^{-1}$ (tube 2).

presence of adequate amounts of nitrate. Some additional characteristics of strain 15 have been reported more extensively by Krul (1976).

Oxygen uptake and nitrogen-gas production by cells in suspension and in flocs

After the anaerobic cultivation of strain 15 in medium *P*, cells in suspension and in flocs were obtained as described in "Materials and Methods" (suspended cells A_1 and cells in flocs A_2). Ten millilitre of both, A_1 and A_2 , containing 2.5 mg ml^{-1} dry cell matter, were put into 35 ml flasks. The flasks were flushed with helium followed by the injection of oxygen to obtain 5% oxygen in the gas phase. Hereafter nitrate was added to a final concentration of $1.5 \text{ g KNO}_3 \text{ l}^{-1}$. Oxygen and nitrogen were measured in the gas phase (gaschromatographically; Fig. 2). The oxygen concentration in the gas phase above the suspended cells decreased faster than that above the cells in flocs. This was explained by a relatively faster oxygen-uptake rate of the suspended cells resulting in a lower dissolved oxygen concentration in the liquid phase. Owing to this difference in dissolved oxygen content, the cells in suspension showed a faster nitrogen gas production, measured at a certain oxygen concentration in the gas phase, as compared to the gas production by cells in flocs, measured at the same oxygen concentration in the gas phase.

From these results it was concluded that dissimilatory nitrate reduction in relation to the oxygen concentration should be studied by measuring the oxygen and nitrate contents of the medium.

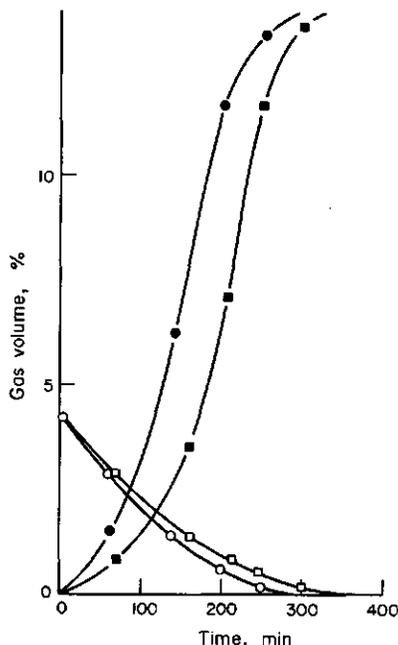


Fig. 2. O₂-uptake and N₂-production of cells in suspension A_1 and in flocs A_2 : ○—○ O₂, suspended cells; □—□ O₂, flocs; ●—● N₂, suspended cells; ■—■ N₂, flocs.

Oxygen uptake and nitrate reduction by cells in suspension and in flocs

The oxygen uptake and nitrate disappearance from suspension A_1 , and flocs A_2 were followed by using an oxygen and a nitrate electrode in a respirometer at 30°C. Nitrate was injected as indicated (arrow). Fig. 3 shows a linear oxygen uptake by suspended cells. The nitrate reduction sharply increased when the oxygen concentration was about zero.

The oxygen uptake and nitrate reduction by flocs (A_2) are graphically shown in Fig. 4. The oxygen uptake was linear up to approximately $1.5 \text{ mg oxygen l}^{-1}$. Below this oxygen concentration the nitrate reduction was considerably enhanced. From both experiments it is concluded that the oxygen concentration within the flocs must have been, at least partially, close to zero at $1.5 \text{ mg oxygen l}^{-1}$ in the surrounding medium, because nitrate reduction increases only when the oxygen concentration around the cells is practically zero.

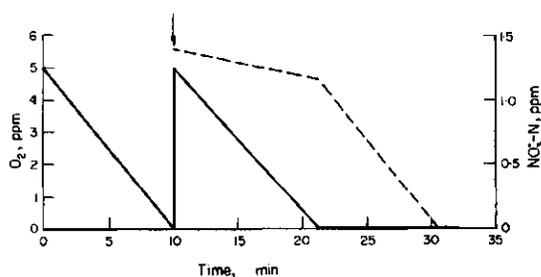


Fig. 3. O₂-uptake and NO₃⁻-reduction by suspended, anaerobically grown cells A_1 of strain 15: — O₂; - - - NO₃⁻. The cell suspension contained $0.1 \text{ mg protein ml}^{-1}$.

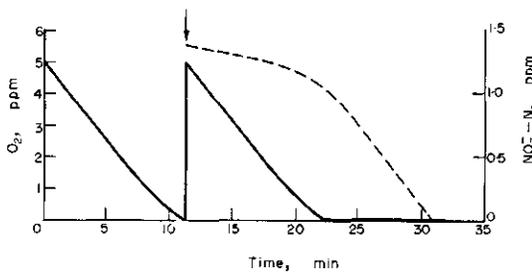


Fig. 4. O_2 -uptake and NO_3^- -reduction by flocs of anaerobically grown cells A_2 of strain 15: — O_2 ; - - - NO_3^- . The cell suspension contained $0.1 \text{ mg protein ml}^{-1}$.

Similar experiments were performed with cell suspension B_1 (Fig. 5), and floc suspension B_2 (Fig. 6). These cells had additionally been aerated for 24 h after anaerobic growth. As demonstrated elsewhere (Krul, 1976), the aeration period causes a partial inactivation of the nitric-oxide reductase resulting in the accumulation of nitric oxide after addition of nitrate or nitrite at low dissolved oxygen concentrations. Nitric oxide strongly inhibits the oxygen uptake.

The oxygen uptake and nitrate-reduction rates of the aerated suspension B_1 (Fig. 5) were lower than when measuring the cells immediately after the anaerobic growth, A_1 (Fig. 3). In both suspensions A_1 and B_1 , the nitrate reduction showed a pronounced rise when the dissolved oxygen was nearly zero. The oxygen uptake of the cells in suspension B_1 was also linear down to a very low oxygen concentration in the surrounding medium.

The oxygen uptake of flocs B_2 was linear down to approximately $1.5 \text{ mg oxygen l}^{-1}$ in the absence of nitrate (Fig. 6). Linearity of the oxygen uptake in the presence of nitrate was observed above $2.5 \text{ mg oxygen l}^{-1}$ in the surrounding medium. Nitrate reduction increased below $2.5 \text{ mg dissolved oxygen per litre}$, where the oxygen uptake was inhibited.

The inhibition of the oxygen uptake below $2.5 \text{ mg oxygen l}^{-1}$ in the presence of nitrate, observed in the aerated flocs B_2 (Fig. 6) is thought to have been caused by the accumulation within the flocs of small amounts of nitric oxide, resulting from nitrate reduction followed by nitrite reduction (Krul, 1976). The production of toxic amounts of nitric oxide, was initiated at a very low oxygen tension. This is concluded from the inhibition of the oxygen uptake in the aer-

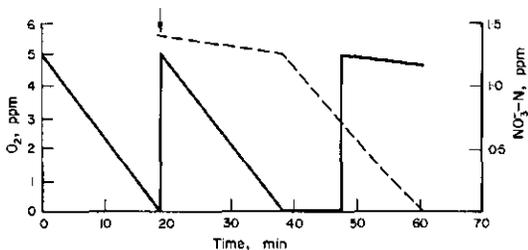


Fig. 5. O_2 -uptake and NO_3^- -reduction by pre-aerated, suspended, anaerobically grown cells B_1 of strain 15: — O_2 ; - - - NO_3^- . The cell suspension contained $0.1 \text{ mg protein ml}^{-1}$.

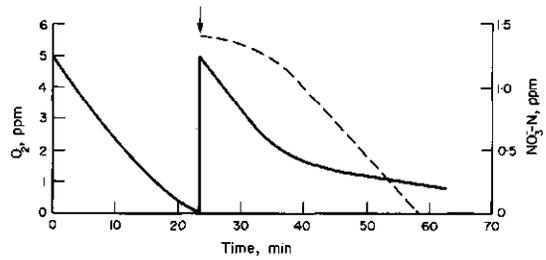


Fig. 6. O_2 -uptake and NO_3^- -reduction by pre-aerated flocs of anaerobically grown cells B_2 of strain 15: — O_2 ; - - - NO_3^- . The cell suspension contained $0.1 \text{ mg protein ml}^{-1}$.

ated suspension (Fig. 5) which was only observed when the cells had been supplied with nitrate and the suspension had been re-aerated after the dissolved oxygen had been entirely consumed. During the inhibition of the oxygen uptake, nitrate reduction proceeds rapidly despite the presence of $5 \text{ ppm } O_2$.

From the experiments with aerated cells it is concluded that the oxygen concentration within the flocs was at least partially below 0.1 ppm when the surrounding medium still contained 2.5 ppm oxygen.

Oxygen uptake and nitrate reduction by activated sludge flocs

To investigate the influence of an oxygen gradient in activated sludge flocs upon the dissimilatory nitrate reduction, experiments were carried out with denitrifying activated sludge flocs. Aerobically grown activated sludge was not investigated because the presence of both nitrifying and denitrifying bacteria rendered the experiments unnecessarily complicated.

Samples of denitrifying activated sludge were centrifuged, washed and resuspended in the nitrogen-free medium *G*. The oxygen uptake and the nitrate reduction were measured in the respirometer with oxygen and nitrate electrodes. The oxygen uptake was linear above approximately $0.5 \text{ mg oxygen l}^{-1}$. Below this oxygen concentration, the nitrate reduction readily increased (Fig. 7).

Since it is very difficult to disperse activated sludge flocs without damaging the cells, no information was obtained with dispersed flocs. It is assumed that the nitrate reduction of dispersed flocs increases when the oxygen concentration is below 0.1 ppm , as in the case of suspended cells of pure cultures.

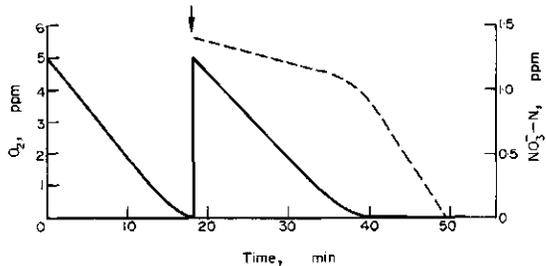


Fig. 7. O_2 -uptake and NO_3^- -reduction by flocs of denitrifying activated sludge: — O_2 ; - - - NO_3^- . The sludge suspension contained $0.1 \text{ mg dry matter ml}^{-1}$.

From the results with denitrifying activated sludge flocs it is concluded that the dissolved oxygen concentration within the flocs was at least partially below 0.1 ppm at 0.5 ppm oxygen in the surrounding medium. As a consequence, dissimilatory nitrate reduction in this sludge suspension occurred at 0.5 ppm O₂ outside the flocs. This is in contrast to the results with the floc-forming denitrifying strain 15 which was able to reduce nitrate at an oxygen concentration below 1.5 ppm outside the flocs.

DISCUSSION

In most denitrifying bacteria the dissimilatory nitrate reductase, once synthesized, is mainly active in the absence of oxygen or at a very low oxygen tension. Inactivation of this enzyme by oxygen in coliform bacteria was reported by Pichinoty (1965), Van't Riet, Stouthamer and Planta (1968) and de Groot and Stouthamer (1970). This inactivating effect of oxygen upon the dissimilatory nitrate reductase was also observed in the isolated strain 15 when tested immediately after anaerobic growth.

Oxygen consumption by bacterial cells in flocs like activated sludge causes an oxygen gradient. Because of the relatively low oxygen concentrations within these cell aggregates, dissimilatory nitrate reduction within the flocs was expected to occur at relatively high oxygen concentrations in the surrounding medium. Flocs of the anaerobically grown cells of strain 15 showed strongly increased nitrate reduction below approximately 1.5 mg oxygen l⁻¹ in the surrounding medium (Fig. 4) in contrast to cells in suspension which did not show this rise in nitrate reduction until the pO₂ had approached zero (Fig. 3). This difference between denitrifying bacteria in flocs and as suspended cells was much more pronounced when both, flocculated and suspended cells, had been pre-aerated in a nitrogen-free medium for 24 h before being tested for oxygen uptake and nitrate reduction (Figs. 5 and 6). Pre-aerated flocs of strain 15 showed an increased nitrate reduction below approximately 2.5 mg oxygen l⁻¹ in the surrounding medium. NO was probably produced below this oxygen concentration resulting in respiration inhibition (Krul, 1976). Suspensions of pre-aerated cells of strain 15 had to be practically free from dissolved oxygen before nitrate reduction and NO accumulation started. Upon re-aeration, the oxygen uptake was almost completely inhibited. Once the oxygen uptake was inhibited the nitrate reduction rate under aerobic conditions was nearly the same as under anaerobic conditions.

The respiration of bacterial cells in sludge flocs was non-linear below approximately 0.5 mg oxygen l⁻¹, measured in the medium. Below this concentration an increased nitrate reduction was observed. Exact calculations of oxygen gradients in the flocs have not

been made because floc-size distributions were not determined.

The anaerobic, denitrifying innerpart of flocs occurring under aerobic conditions may be responsible for the loss of nitrogen in activated sludge plants as reported by Wuhrmann (1954); Johnson (1958); Dept. Scientific and Industrial Research (1962); Ruffer (1964).

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Dissimilatory Nitrate and Nitrite Reduction under Aerobic Conditions by an Aerobically and Anaerobically Grown *Alcaligenes* sp. and by Activated Sludge

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The oxygen uptake of an *Alcaligenes* sp., isolated from activated sludge, was inhibited by small amounts of nitric oxide. The occurrence of this inhibition was dependent on the growth conditions and the pretreatment of the cells. Anaerobically grown cells, which had subsequently been aerated in a nitrogen-free medium, accumulated nitric oxide, after the addition of nitrate or nitrite. When the oxygen uptake was inhibited by nitric oxide, dissimilatory reduction of nitrate and nitrite proceeded under aerobic conditions at the same rate as in the absence of oxygen. Activated sludge removed nitric oxide actively under aerobic conditions and as a consequence the oxygen uptake of the sludge was not inhibited in the presence of nitrite. The rate of nitrate reduction under aerobic conditions was about 20% of that in the absence of oxygen.

IN A NUMBER of bacteria, nitrate may function instead of oxygen as an electron acceptor. Some of these bacteria e.g. *Pseudomonas aeruginosa* are able to grow under aerobic and anaerobic conditions with nitrate as the only nitrogen source. Denitrifying bacteria like *Bacillus licheniformis* can utilize nitrate as a nitrogen source under aerobic conditions but they require amino acids for anaerobic growth (Woldendorp, 1963).

Pichinoty (1965) demonstrated the existence of two types of nitrate-reducing enzymes: nitrate reductase B, a soluble enzyme which has a nutritive rather than a respiratory function, and nitrate reductase A, a particle-bound enzyme which functions mainly in nitrate respiration and which also has the capacity to reduce chlorate.

The assimilatory nitrate reductase can be formed both in the presence and absence of oxygen (Pichinoty, 1970). In some facultative bacteria, such as *Aeromonas hydrophyla*, formation of the assimilatory nitrate reductase is greatly increased in the presence of nitrate by anaerobiosis.

Dissimilatory nitrate reductase is usually induced in the presence of nitrate under anaerobic conditions (Pollock, 1946; Delwiche, 1956; Wainwright & Nevill, 1956). The rate of dissimilatory nitrate reduction is quite different in the presence of oxygen. Sacks & Barker (1949) demonstrated that reduction of nitrate to nitrite and of nitrite to nitrogen gas under aerobic conditions by anaerobically grown *Ps. denitrificans* proceeded at rates which were 29% of those occurring under anaerobic conditions. However, the oxygen concentration of the medium was not measured. Mechsner & Wuhrmann (1963) isolated denitrifying bacteria from activated sludge and noted a significant variation among these organisms in their response to oxygen. Some strains required anaerobic conditions for denitrification, whereas others were more tolerant

of limited oxygen conditions. One isolate continued denitrifying at an oxygen tension in the medium as high as 153 mmHg. Skerman & MacRae (1957*a,b*) suggested that the occurrence of nitrate dissimilation in the presence of a positive, though low, dissolved oxygen concentration would be the result of an oxygen gradient in the cell suspension.

The present paper reports experiments on oxygen uptake and nitrate reduction in relation to the concentration of dissolved oxygen in the medium. The experiments were carried out with a bacterium isolated from activated sludge. The relevance of this phenomenon to the activated sludge process has been studied.

Materials and Methods

Activated sludge

Denitrifying and aerobically grown activated sludges were produced in two laboratory installations. The denitrifying sludge was stirred slowly thus favouring anaerobic conditions. The aerobically grown activated sludge was strongly aerated. Both types of sludge were fed discontinuously (once per 48 h) with an artificial sewage during a period of 1 year with a load of about 0.1 g COD g⁻¹ MLSS day⁻¹.

Media

Artificial sewage: (g l⁻¹) skim-milk powder, 9; urea, 0.03; gelatine, 0.06; starch, 0.12; Na₂HPO₄·2H₂O, 0.03; MgSO₄·7H₂O, 0.003; KCl, 0.004; FeCl₃·6H₂O, 0.004; and in the influent of the denitrifying installation additionally: KNO₃, 1.2.

Medium A: (g l⁻¹) NH₄Cl, 2; glycerol, 5; and mineral salts: KH₂PO₄, 0.027; K₂HPO₄, 0.04; Na₂HPO₄·2H₂O, 0.04; CaCl₂, 0.05; MgSO₄·7H₂O, 0.075; FeCl₃·6H₂O, 0.005; MnSO₄·H₂O, 0.005; ZnSO₄·H₂O, 0.0001; CuSO₄·5H₂O, 0.0001; H₃BO₃, 0.0001; Na₂MoO₄·2H₂O, 0.00005 and CoCl₂·H₂O, 0.0005.

Medium B: (g l⁻¹) KNO₃, 2; glycerol, 5; and the mineral salts mixture.

Medium G: (g l⁻¹) Glycerol, 10; and the mineral salts mixture.

Medium P: (g l⁻¹) Peptone, 4; KNO₃, 2 and glycerol, 5.

A concentrated phosphate solution, sterilized separately, was added to all media to a final concentration of 0.02 M, pH 7.

Isolation of denitrifying bacteria

The isolation of denitrifying bacteria from denitrifying and aerobically grown activated sludge was carried out as described by Krul (1976).

Cultural conditions

Anaerobic growth was obtained in 2-l flasks filled with 1 l of Medium P. After inoculation, the flasks were incubated at 30° for 3 days, without shaking.

Aerobic growth was obtained (1) in 300-ml flasks filled with 100 ml of medium. The cultures were strongly aerated for 36 h at 30° on a shaker. At the end of the logarithmic

growth phase the concentration of dissolved oxygen was measured with an electrode. In all cases the oxygen concentration was above 3 mg/l. (2) Controlled aerobic growth was obtained in a 2-l fermentor. The oxygen concentration was measured with an Eschweiler oxygen probe. The dissolved oxygen concentration was kept above 5 mg/l.

Treatment of cell material

Cells grown anaerobically were centrifuged, washed and resuspended in Medium G. Each cell suspension was divided into two equal portions, one of which was directly used in oxygen uptake and nitrate-reduction experiments, and the other strongly aerated for 24 h at 30° after which the suspended cells were harvested, washed and resuspended in Medium G. Sludge samples were treated in the same way as the anaerobically grown cells.

Cells grown aerobically in Media A and B were used for respiration and nitrate-reduction experiments immediately after centrifuging, washing and resuspending in Medium G.

Respirometer

Oxygen uptake was measured with an electrode connected to a YSI respirometer (Yellow Springs, Ohio, U.S.A.). Simultaneous measurements of O₂ and nitrate concentrations were carried out in a respirometer as described by van Kessel (1975). Oxygen was measured with a Beckman oxygen macro-electrode connected to a physiological gas analyser. Nitrate was measured with an electrode (Orion, model 92-07) connected to an ion analyser (Orion, model 407). The uptake of nitrate was corrected for the nitrite produced. In some experiments, nitrite was measured with the electrode, in the absence of nitrate. Calibration curves were made before performing the experiments. All of the experiments were carried out at 30°.

Chemical analyses

Nitrite was determined chemically following the method of Griess-Romijn-van Eck (1966). Cell protein was assayed by the Folin-Ciocalteu method as described by Lowry *et al.* (1951), using bovine serum albumin as the standard.

Gas analyses

Nitrogen, nitric oxide and nitrous oxide in the gas phase were separated on a 900 × 0.2 cm steel column, containing Poropak 80-100 mesh, at 50° and measured by thermal conductivity in a Packard-Becker, Multigraph 409, gas chromatograph using hydrogen as the carrier gas (25 ml min⁻¹). Flasks of 35 ml capacity, containing 10 ml cell suspension (Medium G), were flushed with helium. Nitric oxide (1 ml) or nitrite (1 mg NO₂⁻ N/10 ml) was injected as required. Gas samples of 0.1 ml were analysed.

Chemicals

Nitric oxide was obtained from Matheson, Gas products, East Rutherford, New Jersey, U.S.A. For the respiration experiments, nitric oxide solutions were made by dissolving the gas in acidified, oxygen-free, demineralized water.

Results

Denitrifying bacteria were isolated from both denitrifying activated sludge and aerobically grown sludge (Krul, in press). One isolate, strain 15, obtained from denitrifying sludge was studied more extensively. Strain 15 was a rod-shaped, Gram-negative, bacterium with peritrichous flagella of the genus *Alcaligenes*.

The effect of nitrate, nitrite and nitric oxide upon the oxygen uptake of pre-cultivated cells

Anaerobically grown cells. Strain 15 was grown anaerobically in the nitrate-containing Medium P. The cells were treated as described in 'Methods'. The oxygen uptake of the non-aerated cells was measured directly in the YSI respirometer. Nitrate, nitrite and nitric oxide were added as indicated [Fig. 1(a)]. The respiration rate of these cells was

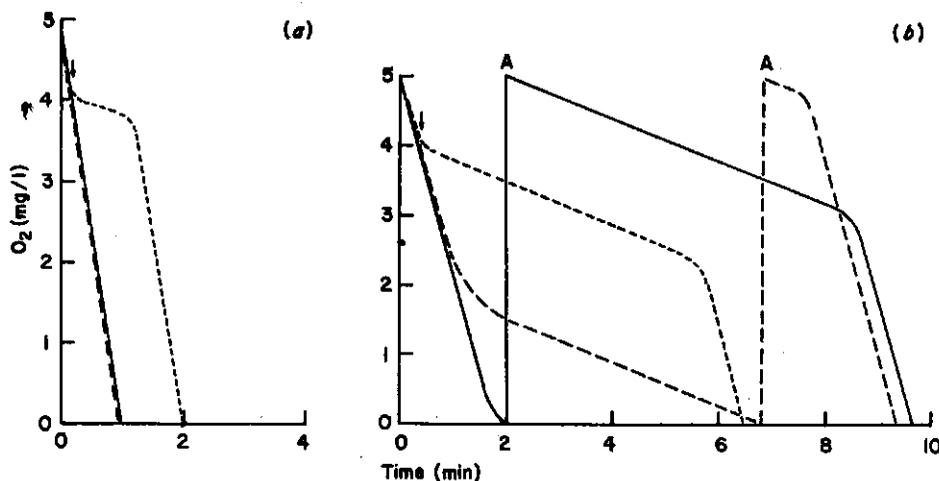


Fig. 1. Effect of nitrate (—), nitrite (---) and nitric oxide (· · ·) on the oxygen uptake of non-aerated (a), and aerated (b), anaerobically grown cells of strain 15 at pH 7: 1 mg/l $\text{NO}_3^- \text{N}$; $\text{NO}_2^- \text{N}$ and NO N , respectively, added at 4 mg/l O_2 (arrow). Each cell suspension contained 1 mg cell protein/ml. A; re-aeration of cell suspensions.

influenced by neither nitrate nor nitrite, but nitric oxide caused a brief inhibition of oxygen uptake. Below 0.5 mg/l of added NO N the uptake of O_2 was not affected. Lowering the pH value to 6 by adding HCl did not change the rate of respiration.

Analogous experiments were carried out with anaerobically grown cells which had been pre-aerated for 24 h in the nitrogen-free Medium G at pH 7 [Fig. 1(b)]. When the concentration of dissolved oxygen was 4 mg/l, addition of nitrate caused no inhibition of respiration before the oxygen level had fallen to 0.5 mg/l. Nitrite inhibited respiration at about 2 mg/l oxygen, whereas nitric oxide caused an almost immediate inhibition which lasted much longer than with non-aerated cells. Re-aeration of the cells did not reverse the inhibition of oxygen uptake.

At pH 6 a different inhibition pattern was obtained (Fig. 2). Both nitrate and nitrite caused inhibition of oxygen uptake at higher oxygen tensions in the medium, respectively, at 3 and 4 mg/l oxygen. At pH 8 the inhibition of respiration caused by nitrate, nitrite and nitric oxide was reversed. Further experiments showed that the lowest nitric oxide concentration which caused inhibition of the oxygen uptake of pre-aerated cells decreased from 0.1 mg/l NO N at pH 7 to 0.005 mg/l NO N at pH 5.5. At the lower pH-values the inhibition lasted much longer (about 5–10 times longer than at pH 7).

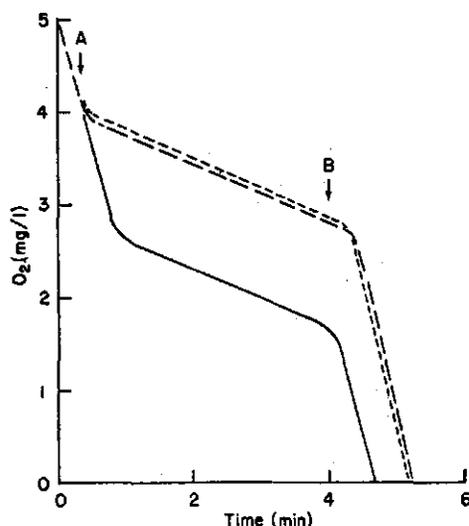


Fig. 2. Effect of nitrate (—), nitrite (----) and nitric oxide (-·-) on the oxygen uptake of aerated, anaerobically grown cells of strain 15 at pH 6 and pH 8 (after addition of NaOH, arrow B): 1 mg/l $\text{NO}_3^- \text{N}$; $\text{NO}_2^- \text{N}$ and NO N , respectively, added at 4 mg/l O_2 (arrow A). Each cell suspension contained 1 mg cell protein/ml.

The results of these experiments show that both anaerobically grown cells examined immediately after harvesting, and cells which in addition had been aerated in a nitrogen-free medium for 24 h, are sensitive to inhibition of oxygen uptake by nitric oxide. The aeration period caused an increased sensitivity of the cells to inhibition by nitric oxide. The inhibition of oxygen uptake by pre-aerated cells, observed after the addition of nitrate or nitrite, was also thought to be caused by the production of nitric oxide.

These results suggest that nitric oxide is removed more readily by non-aerated cells than by pre-aerated cells. This assumption is supported by the following experiment. When the pre-aerated cells were mixed with non-aerated cells, the period of respiration inhibition by nitric oxide was equal to the period of inhibited oxygen uptake obtained with non-aerated cells (Fig. 3).

Cells grown aerobically with ammonium N as nitrogen source. Strain 15 was grown in the ammonium-containing Medium A in continuous culture. The dilution rate was 0.08 and the dissolved oxygen content was kept above 5 mg/l. The oxygen uptake of the cells was measured in the YSI respirometer (Fig. 4). Small amounts of nitrate

(0.05 mg/l $\text{NO}_3^- \text{N}$) or nitrite (0.05 mg/l $\text{NO}_2^- \text{N}$) at pH 7 did not affect the rate of oxygen uptake, but small amounts of nitric oxide (0.05 mg/l NO N) caused a moderate degree of inhibition which was reversible. At pH 6 the oxygen uptake was inhibited shortly after the addition of nitrite (0.01 mg/l $\text{NO}_2^- \text{N}$). When nitrate (0.01 mg/l $\text{NO}_3^- \text{N}$) was added at this pH value the respiration was inhibited as the dissolved

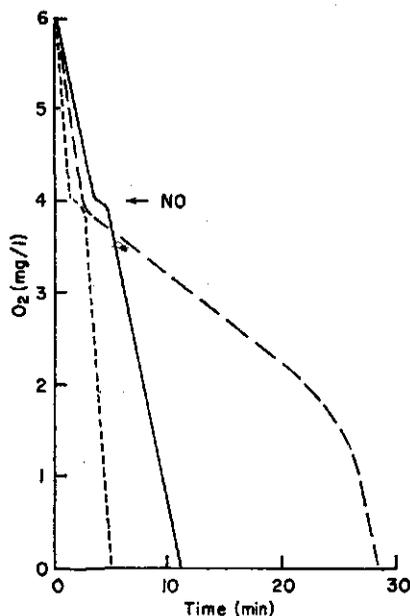


Fig. 3. Effect of nitric oxide on the oxygen uptake of non-aerated (—), aerated (---) and a mixture of non-aerated and aerated (— · —), anaerobically grown cells of strain 15 at pH 7: 0.32 mg/l NO N added at 4 mg/l O_2 (arrow). Non-aerated, aerated and the mixture of non-aerated and aerated cells contained 0.116, 0.300 and 0.416 mg protein/l, respectively.

oxygen fell below 1 mg/l. The inhibition by nitric oxide (0.002 mg/l NO N) at pH 6 was maintained for a much longer period than at pH 7 in spite of the much lower concentration used.

The inhibition of oxygen uptake (at pH 6) after the addition of nitrate or nitrite indicates the presence of nitrate and nitrite reductases in these cells assuming that nitric oxide is the inhibiting agent. The prolonged inhibition of respiration caused by the addition of small amounts of nitric oxide suggests that the NO -reducing enzyme is not present in these cells.

Cells grown aerobically with nitrate as nitrogen source. Strain 15 was grown aerobically in the nitrate-containing Medium B in 300 ml flasks containing 100 ml of medium. The flasks were aerated vigorously so that the dissolved oxygen content was kept above 3 mg/l. The oxygen uptake was measured in the YSI respirometer (Fig. 5). The inhibition pattern of oxygen uptake by these cells was similar to that obtained with cells grown in Medium A with ammonium chloride as the nitrogen source except that

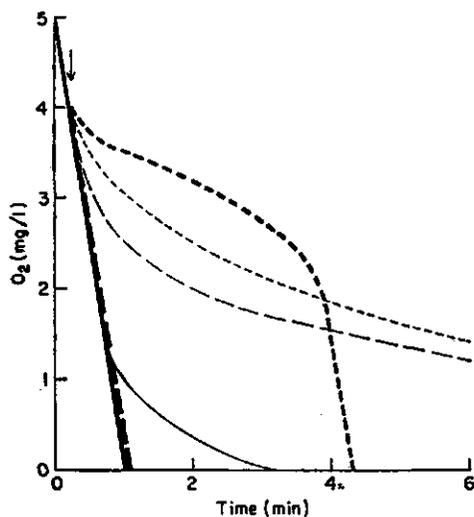


Fig. 4. Effect of nitrate, nitrite and nitric oxide on the oxygen uptake of cells of strain 15 grown aerobically with an ammonium salt as nitrogen source: 0.05 mg/l $\text{NO}_3^- \text{N}$, pH 7 (—); 0.01 mg/l $\text{NO}_3^- \text{N}$, pH 6 (---); 0.05 mg/l $\text{NO}_2^- \text{N}$, pH 7 (· · · · ·); 0.01 mg/l $\text{NO}_2^- \text{N}$, pH 6 (- · - · -); 0.05 mg/l NO N , pH 7 (— — — —) and 0.003 mg/l NO N , pH 6 (- - - -). Nitrate, nitrite or nitric oxide was added at 4 mg O_2/l (arrow). Each cell suspension contained 1 mg cell protein/ml.

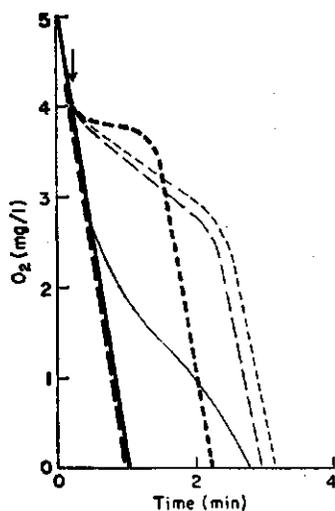


Fig. 5. Effect of nitrate, nitrite and nitric oxide on the oxygen uptake of cells of strain 15 grown aerobically with nitrate as nitrogen source: 0.05 mg/l $\text{NO}_3^- \text{N}$, pH 7 (—); 0.04 mg/l $\text{NO}_3^- \text{N}$, pH 6 (---); 0.05 mg/l $\text{NO}_2^- \text{N}$, pH 7 (· · · · ·); 0.04 mg/l $\text{NO}_2^- \text{N}$, pH 6 (- · - · -); 0.05 mg/l NO N , pH 7 (— — — —) and 0.02 mg/l NO N , pH 6 (- - - -). Nitrate, nitrite or nitric oxide was added at 4 mg O_2/l (arrow). Each cell suspension contained 1 mg cell protein/ml.

reduced to nitrous oxide and nitrogen gas. This resulted in the accumulation of nitric oxide [Fig. 7(b)]. Similar reduction products were obtained when nitrate was added instead of nitrite under anaerobic conditions to pre-aerated and non-aerated cells, respectively, of strain 15. If nitrate and nitrite reduction by pre-aerated cells proceed at a comparable rate under anaerobic conditions and also under aerobic conditions when oxygen uptake is inhibited, an accumulation of nitric oxide from nitrate or nitrite can be expected under aerobic conditions.

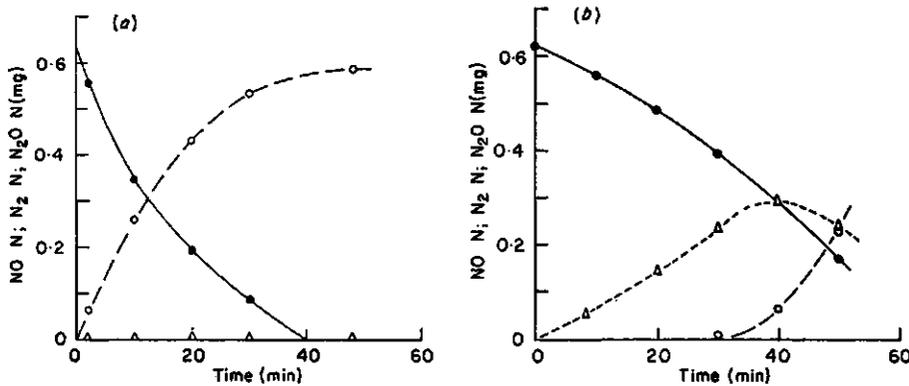


Fig. 6. Reduction of nitric oxide under anaerobic conditions by non-aerated (a), and aerated (b), anaerobically grown cells of strain 15. NO (●—●); N₂ (○—○) and N₂O (Δ—Δ). The cell suspension contained 50 mg cell protein/10 ml (pH 7, 30°). 1 ml NO added.

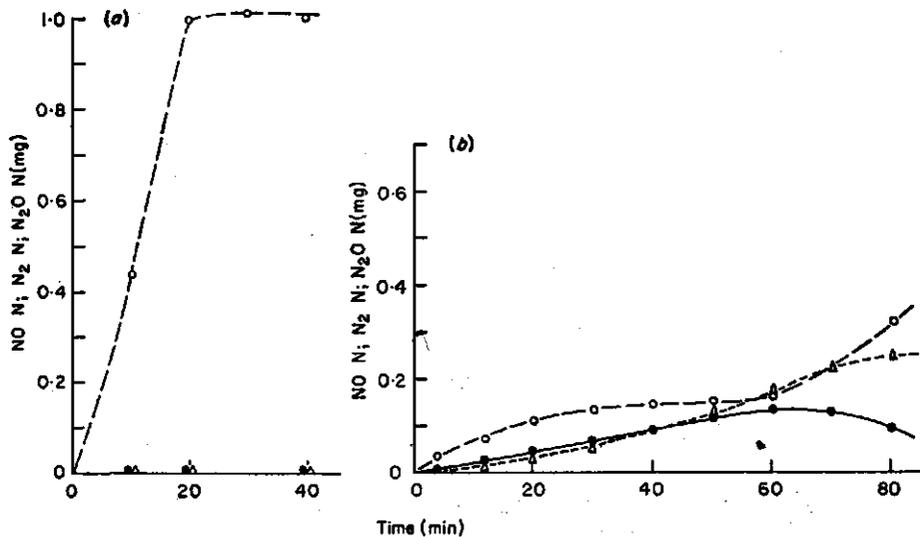


Fig. 7. Production of nitric oxide, nitrogen gas and nitrous oxide from nitrite under anaerobic conditions by non-aerated (a), and aerated (b), anaerobically grown cells of strain 15. NO (●—●); N₂ (○—○) and N₂O (Δ—Δ). The cell suspension contained 50 mg cell protein/10 ml (pH 7, 30°). 1 mg NO₂ N added.

Interrelationships between oxygen uptake and the reduction of nitrate and nitrite in suspensions of strain 15

Anaerobically grown cells. Cells grown anaerobically in Medium P containing nitrate were tested for oxygen uptake and nitrate reduction in the respirometer containing an oxygen and a nitrate electrode. The cells were measured immediately after harvesting and after an aeration period of 24 h in the nitrogen-free Medium G. The non-aerated cells showed a sharp increase in reduction of nitrate after the dissolved oxygen had disappeared. Following re-aeration of the cell suspension, the oxygen-uptake rate remained unchanged, but the rate of nitrate reduction decreased sharply [Fig. 8(a)].

Cells which had been pre-aerated for 24 h in the nitrogen-free Medium G showed the same sharp increase in rate of nitrate reduction after the dissolved oxygen had been consumed as was found with non-aerated cells. However, upon re-aeration of the cell suspension, the rate of oxygen uptake was strongly diminished and the cells continued to reduce nitrate to nitrite at nearly the same rate as under anaerobic conditions. The nitrite derived from nitrate was subsequently reduced further. When the nitrite had nearly disappeared, the inhibition of oxygen uptake was removed [Fig. 8(b)]. The compounds obtained from the reduction of nitrite by pre-aerated cells under aerobic conditions at an inhibited oxygen uptake, are probably similar to those formed under anaerobic conditions [Fig. 7(b)]. This implies that nitric oxide is also produced under aerobic conditions and is responsible for inhibition of oxygen uptake.

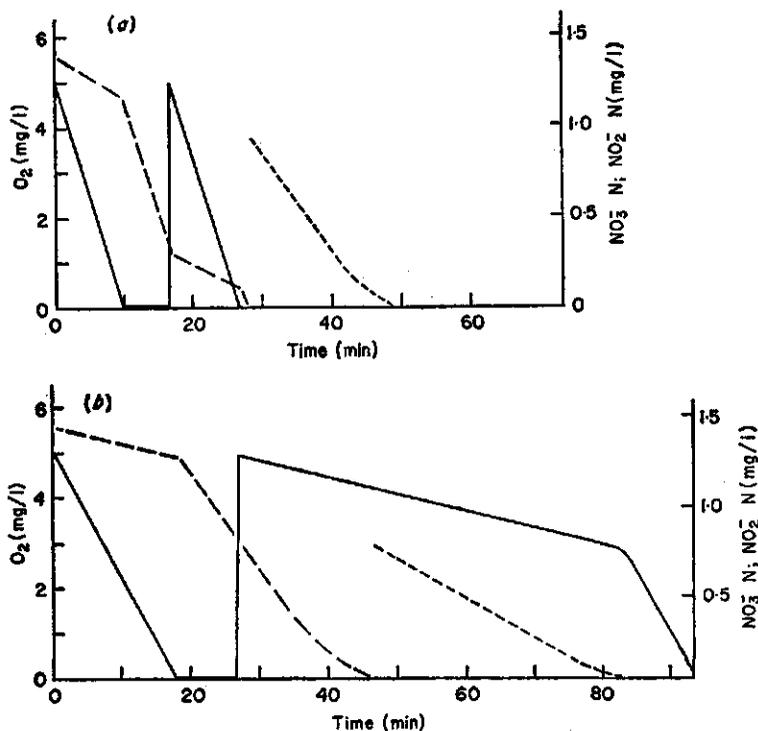


Fig. 8. Oxygen uptake and reduction of nitrate and nitrite by non-aerated (a), and aerated (b), anaerobically grown cells of strain 15. O_2 (—); NO_3^- (---) and NO_2^- (· · ·). The cell suspension contained 0.1 mg cell protein/ml (pH 7, 30°).

Nitrite reduction under aerobic conditions in respiration-inhibited cells was studied more extensively by resuspending anaerobically grown cells in the nitrogen-free Medium G at two different pH values: 5.8 and 7.0, after aeration of these cells for 24 h in Medium G at pH 7. Nitrite was added and the flasks were aerated vigorously. Nitrite was determined chemically after different time intervals (Fig. 9). Nitrite

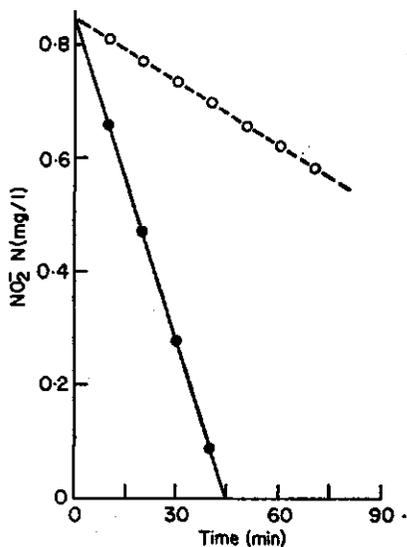


Fig. 9. Nitrite reduction at pH 5.8 (●—●) and at pH 7 (○—○) under aerobic conditions by aerated, anaerobically grown cells of strain 15. The cell suspension contained 0.1 mg cell protein/ml.

reduction proceeded much faster at pH 5.8 than at pH 7.0. The high rate of nitrite reduction at pH 5.8 is explained by assuming that the dissimilatory nitrite reductase had regained its activity owing to the inhibited oxygen uptake, but this did not occur at pH 7 (Fig. 9) at which there was no inhibition of respiration because of the lower sensitivity of the cells to NO at this pH_a value. Inhibition of oxygen uptake at pH 7 in the previous experiment [Fig. 8(b)] was associated with the less vigorous aeration which failed to remove nitric oxide.

Cells grown aerobically with an ammonium salt as nitrogen source. Strain 15 was grown in the ammonium-containing Medium A in continuous culture. The dilution rate was 0.08 and the dissolved oxygen content was kept above 5 mg/l. After centrifuging, washing and resuspending the cells in Medium G, oxygen uptake and nitrate reduction were measured in the respirometer with an oxygen and a nitrate electrode (Fig. 10). The nitrate reduction increased considerably after the oxygen had been consumed but the reduction of nitrite under these conditions was extremely low. From this experiment it was concluded that in strain 15 the dissimilatory nitrate reductase is a constitutive enzyme and the dissimilatory nitrite reductase an adaptive enzyme. The presence of nitrate reductase provoked the inhibition of oxygen uptake by these cells upon the addition of nitrate at pH 6 (Fig. 4), assuming that nitrite was reduced to nitric oxide.

Nitrite reduction under aerobic conditions was determined by following the nitrite concentrations in strongly aerated cell suspensions at different pH values. Under such conditions only the induction of assimilatory nitrite reductase can be expected. Nitrite was determined chemically. From the results of this experiment, it will be seen (Fig. 11) that the assimilatory nitrite reductase of these cells is an adaptive enzyme (addition of 10 $\mu\text{g/ml}$ of chloramphenicol at pH 7 prevented the synthesis of the enzyme). Before the cells had become adapted to nitrite, the nitrite-reduction rate was

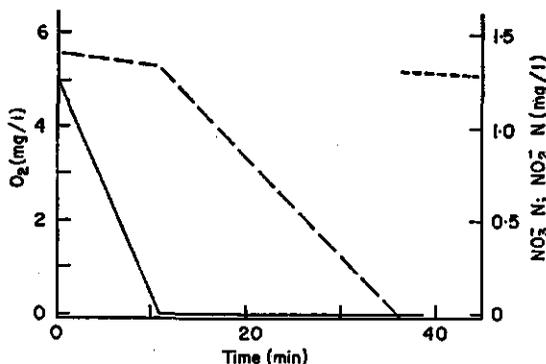


Fig. 10. Oxygen uptake and reduction of nitrate by cells of strain 15 grown aerobically with ammonium-N as nitrogen source. O₂ (—); NO₃⁻ (---) and NO₂⁻ (-·-·-). The cell suspension contained 0.1 mg cell protein/ml (pH 7, 30°).

very low and independent of the pH values tested. However, the very low nitrite-reducing activity of the bacteria under aerobic conditions may have been responsible for the formation of sufficient nitric oxide to explain the observed inhibition of oxygen uptake upon the addition of nitrate and nitrite (Fig. 4). The chemical decomposition of nitrite according to the reaction:



which mainly takes place at a low pH value, may also have been responsible for the formation of toxic concentrations of nitric oxide.

The effect of nitrate, nitrite and nitric oxide upon the oxygen uptake of activated sludge

Oxygen-uptake experiments with activated sludge were carried out with a denitrifying sludge. Aerobically grown activated sludge was not investigated because the presence of both nitrifying and denitrifying bacteria rendered the experiments unnecessarily complicated. In denitrifying sludge no nitrification occurred. This was concluded from the fact that no nitrite or nitrate formation was detected after the addition of ammonium and nitrite, respectively.

Washed denitrifying sludge was resuspended in the nitrogen-free Medium G, and aerated for 24 h. After this aeration period the sludge was washed and resuspended in Medium G, pH 7. This suspension was transferred to the respirometer containing

oxygen and nitrate electrodes and after a short anaerobic period, it was aerated continuously. Nitrate, nitric oxide and HCl to give a pH value of 5.8 were injected separately as indicated (Fig. 12).

From the data presented it can be seen that the nitrate-reduction rate under aerobic conditions was about 20% of the reduction rate measured in the absence of oxygen.

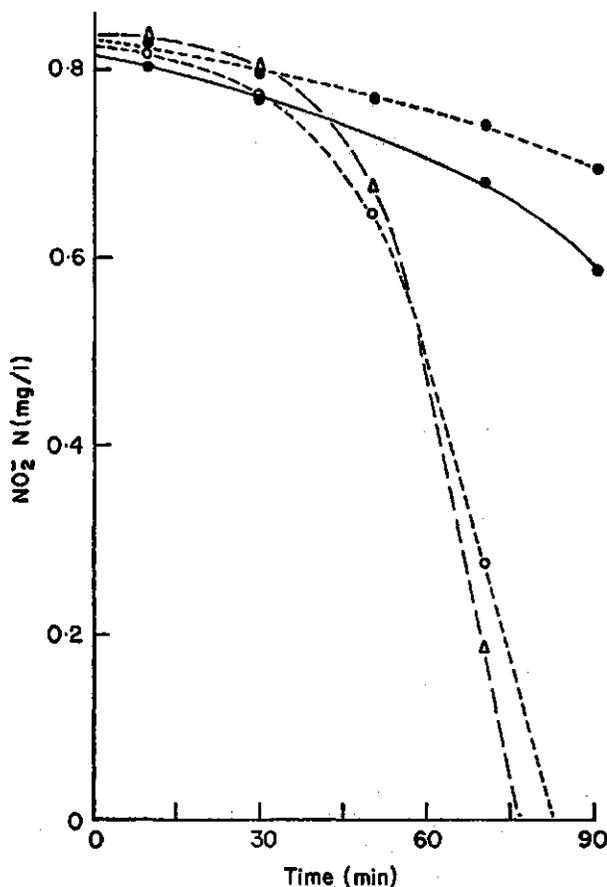


Fig. 11. Nitrite reduction at pH 5.8 (●—●), at pH 7 in the absence (○—○) and presence (●—●) of 10 µg chloramphenicol per ml and at pH 8 (△—△) under aerobic conditions by cells of strain 15 grown aerobically with ammonium-N as nitrogen source. The cell suspension contained 1 mg cell protein/ml.

As long as nitrate was present, the dissolved oxygen concentration was higher, indicating a decreased rate of oxygen uptake during nitrate reduction. Under aerobic conditions the nitrate was reduced to nitrite, which was very slowly reduced further. Addition of HCl to give a pH value of 5.8 did not affect the oxygen-uptake rate of the sludge. Nitric oxide caused only a brief inhibition of this process. The absence of such an inhibition in the presence of nitrite may indicate the presence of bacteria which were able to reduce the inhibitory nitric oxide as soon as it was produced. The fact that the

dissimilatory nitrate reduction still proceeded under aerobic conditions at a rate which was 20% of the reduction rate under anaerobic conditions could not be explained.

Discussion

The oxygen uptake of strain 15, a denitrifying bacterium of the genus *Alcaligenes*, isolated from activated sludge, was frequently found to be inhibited shortly after the addition of nitrate, nitrite or nitric oxide. The extent of the inhibition depended on the growth conditions, the pre-treatment of the cells and on the pH of the medium during the measurements. The inhibition of the oxygen uptake occurred with anaerobically grown cells, which had been aerated in a nitrogen-free medium for 24 h, and with aerobically grown cells. The inhibition was thought in all cases to be caused by nitric oxide.

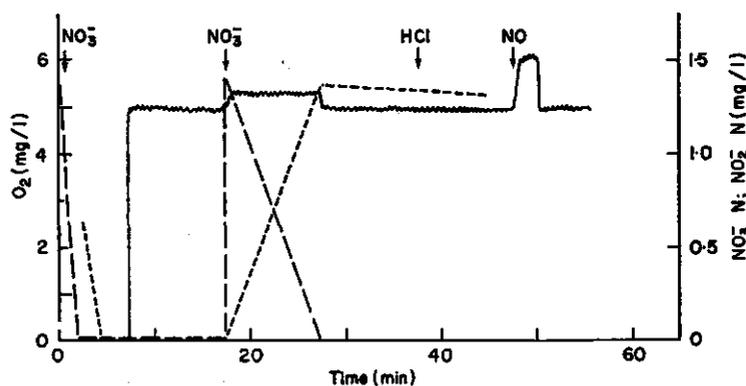


Fig. 12. The effect of nitrate, nitrite and nitric oxide on the oxygen uptake of aerated activated sludge. O_2 (—); NO_3^- (---) and NO_2^- (-·-·-). Nitrate (1.4 mg NO_3^- N/l), nitric oxide (1 mg NO N/l) and HCl (to final pH 6) additions are indicated by arrows. The sludge contained 1 mg dry matter/ml.

It was shown that, after addition of nitrite, under anaerobic conditions nitric oxide accumulated in anaerobically grown cells, which had been aerated for 24 h in the nitrogen-free medium. Formation of nitric oxide from nitrite was also reported by Verhoeven (1956). He detected this compound in cultures of *Thiobacillus denitrificans* when these bacteria were physiologically old and were exposed to cyanide at pH 7.2. *Pseudomonas aeruginosa* formed NO from nitrite under these conditions at pH 6.4.

The pronounced sensitivity of aerobically-grown cells, particularly those grown with NH_4^+ , to the inhibition of oxygen uptake by nitric oxide may be explained by the absence of the dissimilatory nitric oxide reductase in these cells. Inhibition of oxygen uptake measured after the addition of nitrate and nitrite (at pH 6) suggests the presence of nitrate and nitrite reductases in these cells. The dissimilatory nitrate reductase was found to be present and was shown to be a constitutive enzyme (Fig. 10). However, the dissimilatory nitrite reductase was largely absent in aerobically grown cells of strain 15 (Fig. 10). The synthesis of this enzyme is induced only under anaerobic conditions. The assimilatory nitrite reductase is also an adaptive enzyme which was found to be

synthesized under aerobic conditions in the presence of nitrite in cells grown aerobically with an ammonium salt as nitrogen source (Fig. 11). Before adaptation the nitrite-reductase activity was very low. The reduction of nitrate or nitrite to nitric oxide in cells grown aerobically with an ammonium salt as nitrogen source (necessary to explain the observed inhibition of oxygen uptake), is difficult to understand. In addition to the low nitrite-reductase activity in the non-adapted cells, the chemical decomposition of nitrite according to the reaction:



which mainly takes place at a low pH, may be involved. The presence of the assimilatory nitrite-reductase complex, in cells grown aerobically with nitrate as nitrogen source, accounts for the relatively short periods of inhibition of oxygen uptake after the addition of nitrate, nitrite and nitric oxide to such cells, Fig. 5 (the enzyme complex is also able to reduce nitric oxide).

Nitrate and nitrite reduction generally proceed at a low rate under aerobic conditions and have mainly assimilatory functions. Dissimilatory nitrate and nitrite reduction generally take place in the absence of oxygen. The differences between the nitrate-reduction rates of anaerobically grown cells of strain 15 under aerobic and anaerobic conditions are shown in Fig. 8(a). In pre-aerated, anaerobically grown cells, in which the oxygen uptake was inhibited by nitric oxide, nitrate and nitrite reduction proceeded at the same rate in the presence of oxygen as under anaerobic conditions [Figs 8(b) and 9]. Hartingsveldt & Stouthamer (1974) observed a non-inhibited nitrate and nitrite reduction under aerobic conditions by a mutant of *Pseudomonas aeruginosa* which was affected during aerobic growth because of blocked haem synthesis.

Dissimilatory nitrate and nitrite reduction might be responsible for the shortage of the nitrogen balance which is often observed in the aerobic activated sludge process. Apart from any denitrification under the anaerobic conditions existing in the final sedimentation tanks, especially when retention times are unduly long, the dissimilatory nitrate and nitrite reduction under aerobic conditions might be due to inhibited oxygen uptake by nitric oxide as demonstrated for strain 15. Nitrate-reduction experiments were carried out with denitrifying activated sludge, which had been aerated in a nitrogen-free medium for 24 h. The nitrate-reduction rate under aerobic conditions was shown to be 20% of the reduction rate measured in the absence of oxygen. The aerobic reduction of nitrate was probably dissimilatory since the rate of oxygen uptake decreased during the process. In contrast to the results obtained with strain 15, no inhibition of oxygen uptake was observed in the presence of nitrite (Fig. 12).

In many experiments with discontinuously aerated activated sludge, oxygen uptake was inhibited by relatively high concentrations of nitric oxide but for a short time only. This indicates the presence in the sludge of bacteria which were able to reduce nitric oxide below the toxic level. Owing to this activity oxygen uptake by the denitrifying bacteria was not inhibited and as a consequence no nitrite reduction occurred under aerobic conditions. It is uncertain if the same results would have been obtained if the activated sludge had contained a different microbial population.

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THE SYNTHESIS OF THE DISSIMILATORY NITRATE REDUCTASE UNDER AEROBIC CONDITIONS IN A NUMBER OF DENITRIFYING BACTERIA, ISOLATED FROM ACTIVATED SLUDGE AND DRINKING WATER

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Abstract—A number of denitrifying bacteria were isolated from activated sludge and drinking water. These bacteria were tested for the synthesis of the dissimilatory nitrate reductase under aerobic conditions (dissolved oxygen concentration above $4 \text{ mg} \cdot \text{l}^{-1}$). The synthesis of this enzyme varied from total repression by oxygen in some bacteria, especially those isolated from drinking water, until a nearly non oxygen-repressed synthesis in other bacteria (strains 15 and N4). The effect of the dissolved oxygen concentration during growth of the bacteria on the synthesis of the dissimilatory nitrate reductase in cells of strain 15 was studied more extensively. A considerable repression of the enzyme synthesis was obtained when the dissolved oxygen concentration was relatively high (approx $15 \text{ mg} \cdot \text{l}^{-1}$). Addition of chlorate to the growth medium of strain 15 (using $\text{NH}_4^+ \cdot \text{N}$ as nitrogen source) also resulted in a serious repression of the nitrate reductase synthesis during aerobic growth (dissolved oxygen above $4 \text{ mg} \cdot \text{l}^{-1}$). The dissimilatory nitrate reductase of aerobically grown cells of strains 15 and N4 was found to be mainly localized in the membrane fraction.

INTRODUCTION

In a number of bacteria, nitrate may function as electron acceptor instead of oxygen. Pichinoty (1965) demonstrated the existence of two types of nitrate-reducing enzymes: nitrate reductase B, a soluble enzyme which has a nutritive rather than a respiratory function, and nitrate reductase A, a particle-bound enzyme which functions mainly in nitrate respiration and which also has the capacity to reduce chlorate. Most nitrate-reducing bacteria contain nitrate reductase A or nitrate reductase B (Pichinoty *et al.*, 1966; Pichinoty, 1966). Some contain both types of reductases (Chippaux & Pichinoty, 1968; de Groot & Stouthamer, 1970). It was shown that the differentiation between the two nitrate reductases with different functions, as made by Pichinoty, cannot be applied to all nitrate-reducing bacteria.

The dissimilatory nitrate reductase of most microorganisms can be induced by nitrate under anaerobic conditions. However, sometimes nitrate needs not to be present to induce this enzyme (Schulp & Stouthamer, 1970; Payne *et al.*, 1971). The mechanism by which oxygen and nitrate control the enzyme activity is not entirely clear. It has been found that oxygen represses the synthesis of the dissimilatory nitrate reductase (Pichinoty, 1965; Pichinoty & d'Ornano, 1961; de Groot & Stouthamer, 1970; Downey *et al.*, 1969; Showe & DeMoss, 1968). Wimpenny & Cole (1967) suggested that the redox potential of the medium is the regulating factor rather than the concentration of oxygen.

In the present study, some more information has been given about the synthesis of the dissimilatory nitrate reductase under aerobic conditions by a number of denitrifying bacteria isolated from activated sludge and drinking water.

MATERIALS AND METHODS

Activated sludge

Denitrifying and aerobically grown activated sludge were produced in two laboratory installations as described by Klapwijk (1976). The latter type of sludge was strongly aerated. The denitrifying sludge was stirred slowly thus favouring anaerobic conditions. The sludges were fed discontinuously with artificial sewage during 1 yr. The load of both installations was about $0.1 \text{ g COD} \cdot \text{g}^{-1} \text{ MLSS} \cdot \text{day}^{-1}$.

Media

Artificial sewage ($\text{g} \cdot \text{l}^{-1}$). Skim-milk powder, 9; urea, 0.03; gelatine, 0.06; starch, 0.12; $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.03; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.003; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.004; KCl, 0.004; with the additional supply of $1.2 \text{ g} \cdot \text{l}^{-1} \text{ KNO}_3$ in the influent of the denitrifying installation.

Medium A ($\text{g} \cdot \text{l}^{-1}$). NH_4Cl , 2; glycerol, 1; mineral salts: KH_2PO_4 , 0.027; K_2HPO_4 , 0.04; $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.04; CaCl_2 , 0.05; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.075; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.005; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.005; $\text{ZnSO}_4 \cdot \text{H}_2\text{O}$, 0.0001; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.0001; H_3BO_3 , 0.0001; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.00005; $\text{CoCl}_2 \cdot \text{H}_2\text{O}$, 0.00005, and vitamins: B12, 10^{-5} ; thiamine, $8 \cdot 10^{-4}$; biotine, $2 \cdot 10^{-5}$. Medium P ($\text{g} \cdot \text{l}^{-1}$). KNO_3 , 2; peptone, 4 and glycerol, 5. Medium S ($\text{g} \cdot \text{l}^{-1}$). Casitone, 1; yeast extract, 0.2; and glycerol, 2. A concentrated phosphate solution, sterilized separately, was added to all media to a final concentration of 0.02 M, pH 7.

Isolation of denitrifying bacteria

The isolation of denitrifying bacteria from denitrifying and aerobically grown activated sludge was carried out as described by Krul (1976). Six denitrifying organisms, isolated from drinking water, were kindly supplied by van der Kooy (KIWA, Rijswijk, The Netherlands).

Cultural conditions

Aerobic growth was obtained in 300 ml flasks, filled with 100 ml of medium. The cultures were vigorously aerated at 30° in a Gallenkamp orbital incubator, at 250 rev/min. Under these conditions the oxygen transfer rate (OTR) was found to be 10–12 mmol O₂·l⁻¹·hr⁻¹, as measured by the sulphite method described by Cooper, Fernstrom & Miller (1944). The dissolved oxygen concentration during cultivation was ≥4 mg·l⁻¹ for all bacteria isolated from activated sludge and ≥mg·l⁻¹ for the strains isolated from drinking water.

Aerobic growth of strain 15 with the dissolved oxygen concentration above air saturation was obtained in 300 ml flasks, filled with 100 ml medium, in which the air was replaced by pure oxygen. The flasks were closed with rubber stoppers and vigorously shaken at 250 rev./min at 30°. When the cell-density of the cultures was approx half of the possible maximum, the flasks were flushed again with pure oxygen. During growth, the oxygen content of the medium in all cases was maintained above approximately 15 mg·l⁻¹.

Cultivation of strain 15 under poor aeration conditions took place in 300 ml flasks, filled with 100 ml medium. The flasks were aerated very slowly (20 strokes/min) at 30°. When the culture was in the logarithmic growth phase, the dissolved oxygen concentration was below 0.1 mg·l⁻¹.

Anaerobic growth was obtained in 2 l flasks, filled with 1 l medium P. The flasks were incubated without shaking at 30°. Nitrogen gas production was observed from the early logarithmic growth phase and the measured dissolved oxygen concentration was lower than 0.05 mg·l⁻¹, when the nitrogen gas production was observed.

Treatment of cell material used in respiration and nitrate reduction experiments

The cells were harvested when the culture had reached the end of the logarithmic growth phase unless otherwise stated. Before centrifuging, 20 µg·ml⁻¹ chloramphenicol was added to the culture to prevent protein synthesis. The cells were washed twice with 0.02 M phosphate buffer, pH 7, additionally supplied with chloramphenicol, whereafter they were resuspended in the same buffer (containing chloramphenicol) supplied with 10 g·l⁻¹ glycerol.

Oxygen and nitrate measurements in the respirometer

Oxygen and nitrate measurements were carried out in a respirometer containing an oxygen and a nitrate electrode as described by van Kessel (1975). The oxygen uptake rate of the resuspended cells was determined. When the dissolved oxygen content of the suspension was zero NaNO₃ 5 mg·l⁻¹ was added. The nitrate electrode was also able to detect nitrite but with much less sensitivity than nitrate was detected. Nitrate reduction rate in cell suspensions which were unable to reduce nitrite, was calculated by measuring the time necessary to reduce the added nitrate to nitrite, assuming that the nitrate reduction proceeded linearly with time. This assumption was proved to be correct by following the nitrate concentration chemically.

When measuring the nitrate reduction in cell suspensions which were also able to reduce nitrite under anaerobic conditions, the nitrate measurements were corrected for the amount of nitrite which was produced. Nitrite was determined chemically.

Chemical analysis

Nitrite was determined chemically with the method of Gries-Romijn-van Eck (NEN 1056. IV. 2, 1966). Protein was assayed by the Folin-Ciocalteu method as described by Lowry *et al.* (1951), using bovine serum albumin as the standard.

Preparation of cell-free extracts

Cell-free extracts were prepared from cells of two isolates, strains 15 and N4. After centrifuging, washing and resuspending the cells in 0.025 M Tris buffer, pH 7, additionally supplied with 20 µg·ml⁻¹ chloramphenicol, the bacteria were extracted by using a French press at 20,000 psi. Thereafter the bray of strain 15 was treated for 2 min with an ultrasonic (MSE). The cells of strain N4 were more resistant against the destruction methods, for this reason the bray was treated for 30 min with the ultrasonic.

The bray was centrifuged for 20 min at 20,000 rpm. The supernatant (R) was centrifuged for 90 min in an ultracentrifuge at 40,000 rpm (pellet P and supernatant S). Samples of R, S and P were stored below 0° (the pellet P was resuspended in 0.025 M Tris-buffer, pH 7).

Nitrate reduction measured in the cell-free extracts

Nitrate reduction in cell-free extracts was measured according to the modified procedure described by van 't Riet *et al.* (1968). Five ml of a mixture consisting of 2.5 ml 0.15 M Tris-buffer, pH 7; 1 ml 0.1 M KNO₃; 1.25 ml benzylviologen (0.2 mg·l⁻¹) and 0.25 ml demineralized water were pipetted into 100 ml flasks which were flushed with argon and closed with rubber stoppers. Thereafter 0.5 ml of a mixture consisting of 10 mg·l⁻¹ Na₂S₂O₄ and 10 mg·l⁻¹ NaHCO₃ was injected. After 15 min shaking in a waterbath at 30°, 1 ml of one of the (diluted) fractions R, S, or P was injected. Samples were withdrawn during a period of approx 1 hr. The reaction was stopped by aerating the samples and the nitrite formed determined chemically.

RESULTS

The effect of aerobic cultivation on the synthesis of the dissimilatory nitrate reductase

Twenty-four denitrifying bacteria, isolated from both aerobically grown activated sludge and denitrifying sludge, and 6 denitrifying strains, isolated from drinking water, were cultivated in 300 ml flasks filled with 100 ml of medium S. The flasks were shaken vigorously (250 rev·min⁻¹) at 30°. The strains, isolated from activated sludge, were relatively slow-growing bacteria. The dissolved oxygen concentration during cultivation must have been certainly above 4 mg·l⁻¹. The strains, isolated from drinking water were very fast-growing *Pseudomonas* spp resulting in dissolved oxygen concentrations below 1 mg·l⁻¹. The cells of the fast-growing strains were harvested after 16 h incubation, whereas the other strains were incubated for more than 2 days. After centrifuging and washing the cells, oxygen uptake rates and nitrate reduction rates under anaerobic conditions of the 30 strains of denitrifying bacteria, were determined. The results obtained are calculated as ratios: nitrate reduction rates (mg NO₃⁻·g⁻¹·min⁻¹)/oxygen uptake rate (mg O₂·g⁻¹·min⁻¹).

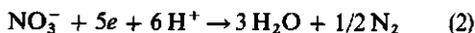
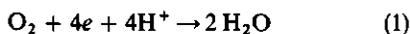
The data of Table 1 show that a number of bacteria isolated from activated sludge possessed a consider-

Table 1. The ratio of dissimilatory nitrate reduction rate (measured under anaerobic conditions) and oxygen uptake rate, of 30 aerobically grown denitrifying bacteria, isolated from drinking water and activated sludge

Number of strains	Group	Isolated from	Diss. nitrate reduction rate/oxygen uptake rate (mg NO ₃ ⁻ ·g ⁻¹ ·min ⁻¹) (mg O ₂ ·g ⁻¹ ·min ⁻¹)
6	I	drinking water	0.00
7	I	activated sludge	0.00
7	II	activated sludge	0.02-0.20
5	III	activated sludge	0.20-0.40
3	IV	activated sludge	0.40-0.90
2	V	activated sludge	0.90-1.50

able dissimilatory nitrate reductase activity when grown aerobically in a medium without nitrate.

The values of the two strains of group V (Table 1) approximated the theoretical values obtained from the reactions (1) and (2) assuming that the respiration rates of glycerol with O₂ and NO₃⁻ were equal.



(1 mg O₂ corresponds with 1.55 mg NO₃⁻).

All the aerobically grown cultures were unable to reduce nitrite when incubated under anaerobic conditions.

Some additional experiments were carried out with strains N4 and 15, both belonging to group V. Strain 15 has been described more in detail by Krul (1976a, b). Strain N4 was a short rod of 1-1.5 μm.

The effect of the oxygen concentration during the growth of strain 15 on the synthesis of the dissimilatory nitrate reductase

Strain 15 was cultivated at different dissolved oxygen concentrations: (a) above 15 mg·l⁻¹, (b) above 4 mg·l⁻¹, (c) above 0.1 mg·l⁻¹, all in medium A (with NH₄⁺-N as nitrogen source) and (d) anaerobically in medium P. The growth of the cells at (a), (b) and (c) proceeded at similar rates. The bacteria were harvested as soon as the cultures had reached the stationary growth phase. After washing the cells, the oxygen uptake rate and dissimilatory nitrate reduction rate under anaerobic conditions were measured in the respirometer. From the results obtained (Table 2) it can be seen that the oxygen uptake rates of cells derived from cultures grown at a high dissolved oxygen concentration (≥15 mg·l⁻¹) and grown at poor aeration (≥0.1 mg·l⁻¹), were lower than those of cells above 4 mg·l⁻¹ dissolved O₂. The synthesis of the dissimilatory nitrate reductase decreased with increasing dissolved oxygen concentration during growth of the cells. However, with strain 15 this decrease was much less pronounced than with most other denitrifying bacteria tested.

Table 2. The effect of the dissolved oxygen concentration during cultivation on the synthesis of the dissimilatory nitrate reductase in strain 15

Dissolved oxygen conc. mg O ₂ ·l ⁻¹	Oxygen uptake rate mg O ₂ (100 mg protein) ⁻¹ ·min ⁻¹	Diss. nitrate reduction mg NO ₃ ⁻ (100 mg protein) ⁻¹ ·min ⁻¹
0	0.300	0.510
≥0.1	0.308	0.531
≥4.0	0.422	0.380
≥15.0	0.335	0.110

The localisation of the dissimilatory nitrate reductase in cells of strains 15 and N4

The assumption of Pichinoty (1965), that the assimilatory nitrate reductase is a soluble enzyme, whereas the dissimilatory nitrate reductase is a membrane-bound enzyme generally has been accepted.

To investigate the localization of the nitrate reductase in cells of strains 15 and N4, these bacteria were cultivated aerobically in medium A (NH₄⁺-N as nitrogen source). The dissolved oxygen concentration during cultivation was maintained above 4 mg·l⁻¹. The ratios nitrate reduction rate/oxygen uptake rate of these cells were similar to those obtained with the cells grown in medium S (Table 1). Cell-free extracts were made as described in "Methods". The nitrate reductase activities of fractions R, P and S were measured under anaerobic conditions (Table 3). It was clearly shown that most of the nitrate reductase activity was localized in the membranes of the aerobically grown cells of strains 15 and N4. This confirms the hypothesis that the nitrate reductase found in these bacteria, is a dissimilatory enzyme.

The effect of chlorate and nitrate on the synthesis of the dissimilatory nitrate reductase of strains 15 and N4

Pichinoty (1965) reported that chlorate (ClO₃⁻) can be used as substrate by the dissimilatory nitrate reductase. However, the reduction product, chlorite (ClO₂⁻) is very toxic to bacteria. (Because of this feature chlorate is often used to obtain mutants which have lost the ability to synthesize the dissimilatory nitrate reductase.) In a subsequent series of experiments a study was made of the effect of chlorate or nitrate added to medium A (with NH₄⁺-N as nitrogen source), on the synthesis of the dissimilatory nitrate reductase during aerobic growth of strains 15

Table 3. Anaerobic nitrate reduction by cell-free extracts of aerobically grown strains 15 and N4 (with NH₄⁺-N as nitrogen source)

	Nitrate reduction, measured under anaerobic conditions	
	Strain 15	Strain N4
Supernatant of 20 min, 20,000 r.p.m.	100%	100%
Pellet of 90 min, 40,000 r.p.m.	75%	66%
Supernatant of 90 min, 40,000 r.p.m.	5%	12%

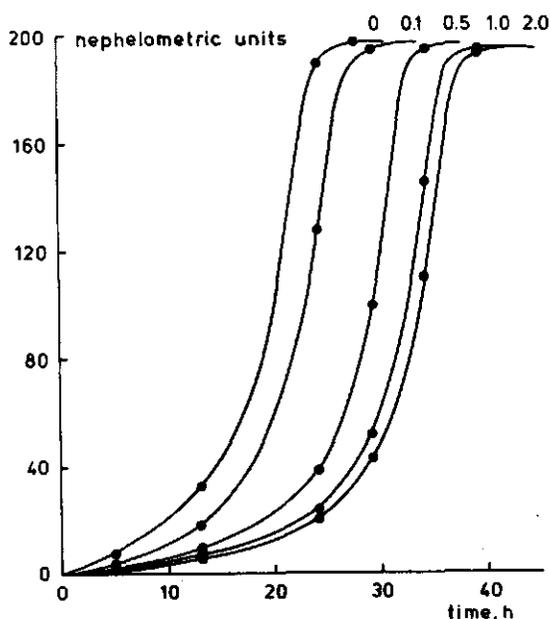


Fig. 1. The growth of strain 15 in medium A additionally supplied with 0.01, 0.5, 1.0 and 2.0 $\text{g}\cdot\text{l}^{-1}$ KClO_3 . The dissolved oxygen concentrations during cultivation were maintained above $4\text{ mg}\cdot\text{l}^{-1}$.

and N4. The dissolved oxygen concentration during the cultivation of strain N4 remained above $4\text{ mg}\cdot\text{l}^{-1}$, whereas strain 15 was cultivated at 3 different dissolved oxygen levels viz. (a) $\geq 0.1\text{ mg}\cdot\text{l}^{-1}$, (b) $\geq 4\text{ mg}\cdot\text{l}^{-1}$, and (c) $\geq 15\text{ mg}\cdot\text{l}^{-1}$. The growth of strain 15 proceeded similarly at the different dissolved oxygen concentrations. The growth of the culture, incubated above $4\text{ mg}\cdot\text{l}^{-1}$ dissolved O_2 was followed by measuring the cell densities (Fig. 1). It was observed that under these conditions chlorate only retarded the growth of strain 15. Nitrate did not affect growth rate and cell yield. The same results were observed with strain N4.

The cells were harvested as soon as the cultures had reached the stationary growth phase. After washing, the bacteria were resuspended and the oxygen uptake rate and the nitrate reduction rate (under anaerobic conditions) measured in the respirometer.

The results obtained (Table 4) show that the addition of nitrate to the growth medium did not affect the synthesis of the dissimilatory nitrate reductase in

both strains 15 and N4. (Approx 1% of the added nitrate was reduced to nitrite during cultivation.) Chlorate added to the growth medium of the bacteria caused a serious repression of the dissimilatory nitrate reductase synthesis in strain 15, especially when the cells were grown at oxygen concentrations above $0.1\text{ mg}\cdot\text{l}^{-1}$ and above $4\text{ mg}\cdot\text{l}^{-1}$. This repressing effect of chlorate on the synthesis of nitrate reductase was much less significant in strain N4. Addition of chlorate (and/or NH_4^+) during the nitrate reduction measurements did not affect the nitrate reduction rate in both strains.

The reduced dissimilatory nitrate reductase synthesis during cultivation which chlorate cannot have been due to a possible selection of nitrate reductase less mutants because it was found that upon re-inoculation of the cells pre-grown with chlorate into a medium without chlorate resulted in a culture with the same dissimilatory nitrate reductase activity as measured in cultures derived from cells which had never been in contact with chlorate.

Wimpenny & Cole (1967) suggested that the redox potential of the medium is the regulating factor for the synthesis of the dissimilatory nitrate reductase rather than oxygen. The repressing effect of chlorate (or chlorite), especially with strain 15, might be explained by the redox potential-increasing effect of these compounds.

DISCUSSION

It has been clearly shown that in a number of denitrifying bacteria the synthesis of the dissimilatory nitrate reductase was only partially repressed by oxygen (Table 1). Especially cells of strains 15 and N4 were found to synthesize a considerable amount of respiratory nitrate reductase, when grown at a dissolved oxygen concentration above $4\text{ mg}\cdot\text{l}^{-1}$. However, increasing the oxygen concentration during the growth of the bacteria above air saturation, a significant repression of the nitrate reductase synthesis was observed in cells of strain 15 (Table 2).

The dissimilatory nitrate reductase of strains 15 and N4 was mainly localized in the membrane-fractions (Table 3). The activity was not significantly influenced by the preparation of the cell-free extract nor by stor-

Table 4. The effect of nitrate and chlorate added to the growth medium on the synthesis of the dissimilatory nitrate reductase of strain 15 and N4

Pre-cultivation medium ($\text{g}\cdot\text{l}^{-1}$)			Oxygen uptake rate $\text{mg O}_2\cdot(100\text{ mg protein})^{-1}\cdot\text{min}^{-1}$			Diss. nitrate reduction $\text{mg NO}_3^-\cdot(100\text{ mg protein})^{-1}\cdot\text{min}^{-1}$				
			Dissolved oxygen concn during growth $\text{mg}\cdot\text{l}^{-1}$			Dissolved oxygen concn during growth $\text{mg}\cdot\text{l}^{-1}$				
NH_4Cl	KNO_3	KClO_3	≥ 0.1	≥ 4	≥ 15	≥ 0.1	≥ 4	≥ 15	≥ 4	
				strain 15		str. N4	strain 15		str. N4	
1	0	0	0.308	0.422	0.335	0.170	0.531	0.380	0.110	0.430
1	2.0	0	n.d.	0.422	0.335	0.170	n.d.	0.380	0.110	0.430
1	0	0.1	0.295	0.369	0.388	0.140	0.251	0.164	0.050	0.350
1	0	0.5	0.281	0.438	0.348	0.110	0.106	0.128	0.070	0.340
1	0	2.0	0.308	0.442	0.370	0.100	0.066	0.105	0.067	0.230

n.d.: not determined.

age below 0°. As nitrate reductase with an assimilatory function is very labile in cell-free extracts (Lowe & Evans, 1964; van 't Riet *et al.*, 1968) it was concluded that cells of strains 15 and N4 when grown aerobically, synthesize a nitrate reductase with a respiratory function. This was confirmed by the fact that growing these bacteria in a medium with only NH_4^+ -N as nitrogen source (which seriously represses the synthesis of the assimilatory nitrate reductase) did not reduce the synthesis of the nitrate reductase in strains 15 and N4.

Chlorate was shown to repress the synthesis of the dissimilatory nitrate reductase, especially in cells of strain 15 (Table 4). This was explained by the redox potential-increasing effect of chlorate assuming that the redox potential is the regulating factor for the synthesis of the dissimilatory nitrate reductase (Wimpenny & Cole, 1967).

The nitrate reductase synthesis in cells of strain N4 was not seriously repressed by chlorate suggesting a different regulation mechanism. It is also possible that the dissimilatory nitrate reductase in this organism is a constitutive enzyme.

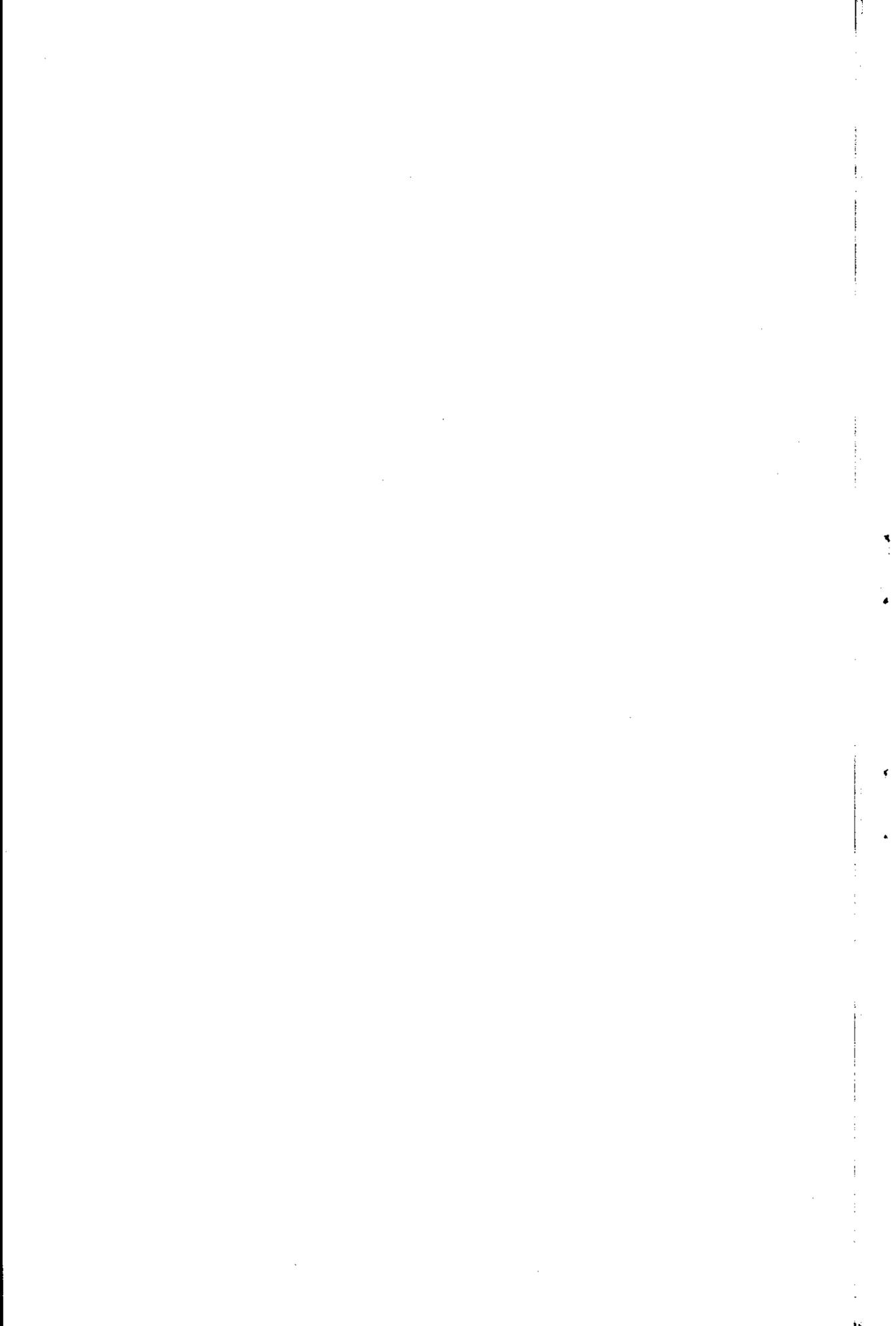
In this respect it may be of importance that the nitrate reductase activity, measured with cells pre-cultivated in the presence of high amounts of chlorate, is comparable with the activity measured in cells, grown at a dissolved oxygen concentration above $15 \text{ mg} \cdot \text{l}^{-1}$ (without chlorate) (Table 4). This suggests that a redox potential sensitive repressor was activated to the same extent in the presence of high amounts of oxygen and of chlorate.

From the results obtained in this investigation it was clearly shown that a number of bacteria in activated sludge are able to synthesize dissimilatory nitrate reductase under aerobic conditions, independent of the N-source present. This implies that as soon as the aeration of the sludge ceases, nitrate reduction may be expected to occur, resulting in a temporary accumulation of nitrite as the synthesis of dissimilatory nitrite reductase is strongly repressed by oxygen.

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DISCUSSION AND SUMMARY

Activated sludge with poor settling characteristics is caught under the term "bulking" sludge. Various types of bulking activated sludge can be distinguished.

- 1) Sludge containing an abundance of filamentous microorganisms.
- 2) Sludge, characterized by excessive amount of bacterial slime.
- 3) Flotating sludge caused by denitrification.

The excessive growth of filamentous bacteria, sometimes occurring in activated sludge plants, is difficult to understand. One of the hypotheses is that the filamentous bacteria protruding from flocs have a better competitive ability as compared with unicellular microorganisms which occur mostly in flocs, in which the growth conditions are assumed to affect adversely the development of these bacteria.

As activated sludge flocs are difficultly to disperse without damaging the cells, it was decided to study the effect of the aggregative condition on the bacterial activity by using floc-forming strains of which the flocs were easily dispersible by adding the enzyme cellulase. Most experiments were carried out with *Zoogloea ramigera*, strain I-16-M, and an *Alcaligenes* sp., strain 15.

As floc formation turned out to be a rather unreproducible and unpredictable phenomenon, it was often difficult to obtain sufficient amounts of flocs. For this reason it was necessary to study floc formation by some pure cultures. In chapter I it was shown that floc formation of *Zoogloea ramigera* can be strongly stimulated by adding small amounts of NaCl or $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ to suspended cells. Unless the cells were very old, the growth phase of the culture was unimportant in respect to floc formation. The stimulatory effect of the salts could not satisfactorily be explained.

By studying the activities of cells in flocs and in suspension, it was shown (chapter II) that the oxygen uptake rate of cells in flocs and of cells in suspension, in the presence of a high level of substrate, represents a zero order reaction above oxygen concentrations in the medium of approx. 1.5 ppm. and approx. 0.1 ppm., respectively. In activated sludge, where only part of the flocs consists of living bacteria, the rate-limiting oxygen concentration is approx. 0.5 ppm. Glucose respiration becomes rate-limiting when the glucose concentration is below approx. 20 ppm. with cells in flocs and below approx. 8 ppm. with cells in suspension. In the same chapter it was shown that the protein and poly- β -hydroxybutyrate syntheses of cells in flocs of slowly shaken cultures were strongly retarded as compared with these activities in cells occurring in suspension.

The rate-limiting concentrations of oxygen and substrate (glucose) for the uptake of these compounds by cells in flocs are in the same range as the values found in many reactors of activated sludge plants.

To compare the competitive ability of *Haliscomenobacter hydrossis*, a filamentous bacterium, with that of the unicellular *Zoogloea ramigera*, continuous culture experiments were carried out. *Zoogloea ramigera* was the most abundant organism present in the mixed cultures at all tested dilution rates (using a complex medium). The filamentous microorganism was shown to have a low affinity towards the uptake of glucose. Both, amino acids and glucose were used as energy and C sources by this organism (chapter III).

Floc flotation, caused by N_2 gas bubbles resulting from the dissimilatory reduction of nitrate, is another aspect of the phenomenon of bulking sludge. Denitrification occurs only under anaerobic conditions. These conditions were shown to occur within flocs at relatively low oxygen concentrations of the surrounding medium. In chapter IV dissimilatory nitrate reduction in the flocs of the *Alcaligenes* sp. strain 15, was shown to take place as soon as the supply of oxygen within parts of the flocs was inadequate. By studying the denitrifying ability of this strain, the dissimilatory nitrate reduction turned out to be even independent of the presence of oxygen when the cells had been subjected to a special treatment. Upon aerobic precultivation of strain 15 with NH_4^+-N or $NO_3^- -N$ as nitrogen source, aerobic nitrate respiration of the washed cells was measured after nitrate addition as soon as the oxygen uptake was inhibited. This inhibition was caused by NO, the reduction product of nitrite (chapter V). The same aerobic dissimilatory nitrate reduction was observed with anaerobically precultivated cells which afterwards had been aerated for some hours in a nitrogen-free medium. It was shown that in these cells during the aeration period the NO reductase and N_2O reductase were inactivated/broken down giving rise to the accumulation of NO and N_2O during subsequent anaerobic nitrate respiration. When the cells were re-aerated, even very small amounts of the produced NO were sufficient to inhibit the oxygen uptake, resulting in a continued aerobic nitrate respiration.

This type of aerobic dissimilatory nitrate reduction as found with strain 15 was also found with a number of different denitrifying strains, isolated from activated sludge. The oxygen uptake of activated sludge itself could not be inhibited for a long time upon addition of NO as the presence of many NO-reducing bacteria (denitrifying bacteria) readily removed the inhibiting agent (chapter V).

In the aeration tank of an activated sludge plant, the oxygen concentration sometimes drops temporarily to such a level that the interior parts of the flocs become anaerobic. Denitrification in these anaerobic innerparts starts only when the denitrifying bacteria present possess the enzymes for the reduction reactions. In chapter VI it was proved that a number of denitrifying bacteria have the ability to synthesize the dissimilatory nitrate reductase in the presence of oxygen and

ammonium ions so that these bacteria are able to start dissimilatory nitrate reduction as soon as all the oxygen has been consumed.

A more detailed study of the "constitutive" character of the synthesis of the dissimilatory nitrate reductase in strain 15 and another denitrifying strain, N4, showed that the synthesis of the enzyme by strain 15 was strongly repressed when the growth medium was over-satisfied with oxygen or upon the addition of chlorate. The synthesis of the dissimilatory nitrate reductase in strain N4 was hardly affected by the mentioned repressing agents. The repressing effect of chlorate on the synthesis of the dissimilatory nitrate reductase in cells of strain 15 was explained by the redox potential-increasing effect of this agent assuming that the redox potential is the regulating factor in the synthesis of this enzyme, rather than oxygen.

DISKUSSIE EN SAMENVATTING

Aktief slib met slechte bezinkings-eigenschappen wordt meestal "licht" slib genoemd. Er bestaan verschillende soorten licht actief slib:

- 1) slib, waarin grote hoeveelheden draadvormige bacteriën aanwezig zijn
- 2) slib, gekarakteriseerd door de aanwezigheid van grote hoeveelheden exopolymeren
- 3) opdrijvend slib, veroorzaakt door denitrifikatie

Het voorkomen van grote hoeveelheden draadvormige mikroorganismen in sommige actief-slib-installaties is moeilijk te verklaren. Een hypotese is dat de uit de vlokken stekende draden beter kunnen concurreren met de zich in de vlokken bevindende eencellige bacteriën. Hierbij wordt aangenomen dat de groei-omstandigheden binnen de vlok de ontwikkeling van laatstgenoemde bacteriën nadelig beïnvloeden.

Omdat het moeilijk is actief-slib-vlokken te dispergeren zonder de cellen te beschadigen, werd besloten het effect van de vlok-toestand op de activiteit van de bacteriën te bestuderen door gebruik te maken van vlokvormende reinkultures waarvan de afzonderlijke cellen door cellulose-fibrillen aan elkaar hechten. Door toevoeging van het enzym cellulase lossen de cellulose-draadjes op en worden de vlokken snel gedispergeerd. De meeste proeven werden uitgevoerd met *Zoogloea ramigera*, stam I-16-M, en een *Alcaligenes* sp., stam 15. Allereerst moest de vlok-vorming enigermate bestudeerd worden omdat de vlokopbrengst van de gebruikte kultures nogal varieerde. In hoofdstuk I wordt aangetoond dat de vlok-vorming bij *Zoogloea ramigera* sterk gestimuleerd wordt na toevoeging van kleine hoeveelheden NaCl of $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ aan gesuspendeerde cellen. De groeifase van de kultuur was niet van belang bij de stimulatie van vlok-vorming door de zouten, tenzij de cellen al te oud waren. Het genoemde "zout-effekt" kon niet bevredigend worden verklaard.

In hoofdstuk II worden de activiteiten van cellen in vlokken en in suspensie beschreven. De zuurstofopnamesnelheid van cellen in vlokken, gemeten in aanwezigheid van een substraatovermaat, is onafhankelijk van het zuurstofgehalte d.w.z. is een nulde orde reactie bij een zuurstofconcentratie in het medium hoger dan ongeveer 1,5 mg/l, en van cellen in suspensie hoger dan ongeveer 0,1 mg/l. In actief-slib-vlokken die slechts voor een gedeelte bestaan uit levende bacteriën, is de snelheidsbeperkende zuurstofconcentratie ongeveer 0,5 mg/l. De glukose-respiratie is snelheidsbeperkend bij een glukoseconcentratie lager dan ongeveer

20 mg/l voor cellen in vlokken en lager dan ongeveer 8 mg/l voor cellen in suspensie.

In hetzelfde hoofdstuk wordt beschreven dat de eiwit- en poly- β -hydroxybutyrate-syntese bij cellen in vlokken van een langzaam schuddende cultuur sterk wordt vertraagd in vergelijking met dezelfde activiteiten in gesuspendeerde cellen. De snelheidsbeperkende concentraties van zuurstof en substraat (glukose) voor de opname van deze componenten door cellen in vlokken liggen in dezelfde orde van grootte als de gemeten concentraties in de reaktors van actief-slib-installaties.

In hoofdstuk III wordt een aantal kompetitie-experimenten met de draadvormende bacterie *Haliscomenobacter hydrossis* en de ééncellige *Zoogloea ramigera* beschreven. Uit de in continue cultuur uitgevoerde proeven bleek bij alle uitgeteste verblijftijden dat *Zoogloea ramigera* de meest dominante bacterie was in de mengcultuur (bij gebruikmaking van een complex medium). *Haliscomenobacter* blijkt een lage affiniteit te hebben wat betreft de glukose-opname. Zowel aminozuren als glukose kunnen worden gebruikt als C- en energie-bron door deze bakteriestam.

Een ander aspekt van het verschijnsel "licht slib" is het opdrijven van vlokken veroorzaakt door gasbelletjes afkomstig van de opeenvolgende reducties van nitraat tot gasvormige stikstof. Denitrifikatie vindt alleen plaats onder anaërobe omstandigheden (in aanwezigheid van nitraat en een H-donor). Deze anaërobe omstandigheden ontstaan in vlokken als de zuurstofconcentratie van het omringende medium relatief laag is. In hoofdstuk IV wordt aangetoond dat dissimilatorische nitraatreductie in de vlokken van stam 15 plaats vindt zodra de zuurstoftoevoer naar het inwendige van de vlokken onvoldoende is om de vlokken aëroob te houden. Bij het bestuderen van deze bakteriestam bleek dat dissimilatorische nitraatreductie zelfs kon plaats vinden met een snelheid onafhankelijk van de zuurstofconcentratie, indien de cellen op een bepaalde wijze voorgegroeid en/of voorbehandeld waren. Indien cellen van stam 15 aëroob worden voorgekweekt met NH_4^+ -N of NO_3^- -N als stikstofbron blijkt dat aërobe nitraatrespiratie mogelijk is na toevoeging van NO_3^- -N zodra de zuurstofopname geremd is. De remming wordt veroorzaakt door NO, een reductieproduct van nitriet (hoofdstuk V). Dezelfde aërobe dissimilatorische nitraatreductie vindt plaats bij anaëroob voorgekweekte cellen die daarna enkele uren waren geaëreerd in een stikstof-vrij medium. Aangetoond werd dat in de laatstgenoemde cellen gedurende de aëratie-periode een inaktivering/afbraak plaats vindt van de NO-reduktase en de N_2O -reduktase hetgeen een ophoping van NO en N_2O tot gevolg heeft tijdens de daarop volgende anaërobe nitraatrespiratie. Bij reaëratie van deze cellen blijkt dan dat zelfs zeer kleine hoeveelheden van het gevormde NO voldoende zijn om de zuurstofopname te remmen waardoor aërobe dissimilatorische nitraatreductie mogelijk is. Aërobe dissimilatorische nitraatreductie van het type gevonden bij stam 15 bleek ook mogelijk te zijn bij een aantal andere uit actief slib geïsoleerde denitrificeerders. Bij actief slib was het niet

mogelijk de zuurstofopname lange tijd te remmen door toevoeging van NO, omdat in het slib veel bacteriën voorkomen die in staat zijn het toegevoegde NO snel te reduceren tot gasvormige stikstof (hoofdstuk V).

In de aëratietank van een actief-slib-installatie kan de zuurstofconcentratie tijdelijk tot zo'n laag niveau dalen dat het inwendige van vlokken anaëroob wordt. In dit anaërobe deel van de vlokken kan dan denitrifikatie optreden indien de aanwezige denitrificerende bacteriën de enzymen bezitten voor de opeenvolgende reducties van nitraat. In hoofdstuk VI wordt aangetoond dat een aantal van dergelijke denitrificeerders in staat is de dissimilatorische nitraatreduktase te syntetiseren in aanwezigheid van zuurstof en ammoniumionen. Dit houdt in dat deze bacteriën direkt beginnen met dissimilatorische nitraatreduktie zodra alle zuurstof is opgenomen.

Het bleek dat de syntese van de dissimilatorische nitraatreduktase bij stam 15 sterk gerepresseerd wordt door een oververzadiging van het groeimedium met zuurstof of door toevoeging van chloraat aan het groeimedium. Het represserend effect van chloraat werd verklaard door de redox-verhogende werking van deze stof aannemende dat de redox-potentiaal en niet zuurstof de regulerende faktor is bij de syntese van de dissimilatorische nitraatreduktase. Bij een andere denitrificerende bacterie, stam N4, kon de syntese van de dissimilatorische nitraatreduktase nauwelijks of niet worden onderdrukt door chloraat en/of zuurstof. In dit geval lijkt de syntese konstitutief te verlopen.

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