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Utilization of Low Concentrations of Starch by a *Flavobacterium* Species Isolated from Tap Water

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Experiments in well-cleaned glass flasks revealed that addition of starch in concentrations of 10 and 25 μg of substrate C per liter to the filtrate of slow sand filters stimulated the development of a yellow-pigmented bacterium which was identified as a *Flavobacterium* species. The isolate was able to multiply in tap water without substrates added, but addition of starch and glucose in amounts as low as 1 μg of substrate C per liter clearly enhanced growth. The substrate affinities of the *Flavobacterium* for these compounds were 3.9 μg of starch C and 3.3 μg of glucose C per liter. The results of this study indicate that microorganisms which rapidly utilize starch at a level of a few micrograms per liter commonly occur in water.

The ability of bacteria of aquatic habitats to utilize organic compounds at concentrations of a few micrograms per liter has frequently been demonstrated, particularly with low-molecular-weight compounds, such as glucose (9, 13, 23, 24, 26, 29), acetate (1, 19, 29), amino acids (5, 6, 8), some other organic acids (16), and synthetic organic chemicals (4). However, the utilization of biopolymers, such as proteins and polysaccharides, that can be taken up only after hydrolysis by extracellular enzymes has hardly been studied at such low concentrations.

Starch and starch-based compounds are being used as coagulant aids in water purification processes (15). Therefore, these compounds, when remaining at low concentrations in the final product, might contribute to the multiplication of microorganisms in tap water during distribution. For these reasons starch was selected to study the ability of bacteria to utilize high-molecular-weight compounds at the level of a few micrograms per liter.

MATERIALS AND METHODS

Glassware. The growth experiments were performed in calibrated, conical, glass-stoppered, Pyrex glass bottles with a volume of 1 liter. These bottles as well as the pipettes used were thoroughly cleaned as described previously (26).

Water. The cleaned bottles were filled with 600 ml of tap water produced by the Municipal Dune Waterworks of The Hague, The Netherlands. This tap water is prepared from dune-infiltrated river water by the successive steps of addition of powdered activated carbon, rapid sand filtration, and slow sand filtration. The dissolved organic carbon content of the final product varied between 2 and 3 mg of C per liter, whereas the pH was approximately 7.5.

Growth experiments. Growth experiments with the autochthonous bacterial population of the filtrate of the slow sand filters were performed by incubating the bottles at 15 ± 0.5°C directly after sampling. For pure culture studies, the vegetative cells of the autochthonous bacteria were killed by heating the bottles with the sampled water in a water bath at 60°C for 1 h followed by incubation at 60°C for 2 h. After cooling, an organic compound from a freshly prepared, separately heated solution was added as a source of carbon and energy. Subsequently the bottles were inoculated with an initial number of cells varying from 100 to 500 colony-forming units (CFU) per ml from a starving culture grown in tap water supplied with an assimilable carbon compound.

Growth of the autochthonous bacteria and of the pure culture was measured by colony counts. For this purpose, the spread plate technique was applied by inoculating (in triplicate) predried Lab-Lemco (Oxoid) agar plates with 0.05 ml from decimal dilutions of the water. These plates were incubated at 25°C until numbers of colonies on the plates no longer increased, i.e., 10 days for counts of autochthonous bacteria. With the obtained colony counts (N_t, in CFU per milliliter), the mean doubling times (G values, in hours) under the different experimental conditions were calculated by the equation:

$$G = \log 2 \cdot \Delta t / (\log N_{t+\Delta t} - \log N_t) \quad (1)$$

where Δt is the incubation period (hours) in which N_t increased to $N_{t+\Delta t}$. These calculations were only performed for that part of the growth curve during which the initial substrate concentration was not significantly reduced by the produced number of cells, i.e., when the colony counts were less than 10% of the maximum colony count (N_{max}, in CFU per milliliter).

Characterization procedures. Isolates were characterized by the following tests: Gram stain, oxidase test (12), oxidation-fermentation test with glucose (11), arginine deiminase test (22), test for NO_2^- or N_2 production from NO_3^- (18), and urease activity (20). In addition, the isolates were tested for the ability to

hydrolyze proteins (casein, gelatin), starch, chitin, and Tween-80, using a standard agar medium either made turbid with casein or chitin or containing 2.5 g of gelatin, starch, or Tween-80 per liter. The media and procedures used for these tests have been described previously (25).

RESULTS

Isolation and characterization of a bacterium utilizing starch at low concentrations. Starch was added in concentrations (added substrate [ΔS]) of 10 and 25 μg of C per liter to duplicate bottles containing 600 ml of freshly sampled filtrate from slow sand filters. Two bottles received no starch. The development of the bacteria in these bottles, stored at 15°C, revealed that both N_{max} and G of the bacteria present in the water were strongly influenced by the low amounts of starch added (Fig. 1). Moreover, it was observed that in the logarithmic as well as in the stationary phases of growth, the population of all four bottles with added starch consisted almost completely of bacteria that formed similar yellow-pigmented colonies requiring 72 h of incubation before being visible. The bottles without starch contained bacteria which formed nonpigmented, transparent colonies.

Seven isolates of the yellow-pigmented bacteria of the starch-supplied cultures were obtained. The characterization procedures confirmed that one type of organism had become dominant, and from the properties of the organism (Table 1) it was concluded that it belonged to the genus *Flavobacterium*.

FIG. 1. *Growth of bacteria present in the filtrate of slow sand filters without added starch (★) or with 10 (○) or 25 (●) μg of starch C added per liter. Solid and broken lines indicate duplicate determinations.*

TABLE 1. *Some properties of the starch-utilizing Flavobacterium sp.*

Property	Isolate characteristic
Shape of cells	Rods
Gram stain	−
Motility	−
Pigment of colony	Yellow
Oxidase	+
Acid from glucose:	
Oxidation	−
Fermentation	−
Arginine deiminase	−
Urease	−
NO_2^- from NO_3^-	−
N_2 from NO_3^-	−
Casein hydrolysis	+
Gelatin hydrolysis	+
Starch hydrolysis	+
Chitin hydrolysis	−
Growth at 37°C	−

Growth on starch. To test the ability of the isolated organism to utilize starch at low concentrations, cells of one of the isolates (strain 166) grown on a Lab-Lemco agar slant were added (N_0 = 600 CFU/ml) to a bottle with heated tap water supplied with 5 μg of starch C per liter and to two bottles containing this heated tap water without added substrate. Growth of the organism in the presence of starch ($N_{max} = 1.1 \times 10^5$ CFU/ml, G = approximately 8 h) was much stronger than in the bottles without substrate added ($N_{max} = 3.2 \times 10^4$ CFU/ml, G = 30 to 38 h). These observations confirmed that the organism was able to utilize starch at the level of micrograms per liter.

Strain 166 was also grown in tap water supplied with starch at different concentrations (ΔS). The growth curves (Fig. 2) and the calculated mean doubling times (Table 2) clearly demonstrate that even addition of starch at initial concentrations below 5 μg of C per liter stimulated growth remarkably. The relationship between the N_{max} values observed and the different initial concentrations of starch (Fig. 3) revealed that at low concentrations of this substrate, the organism yielded 2.0×10^{10} CFU/mg of starch C. Using this value and the N_{max} of the blank, the natural substrate concentration (S_n) may be expressed in micrograms of starch C equivalents per liter. Thus, at an N_{max} of 2.7×10^4 CFU/ml, S_n = 1.4 μg of starch C equivalents per liter.

The survival ability of the isolate after exhaustion of the added substrate was studied by measuring the number of viable cells in bottles originally containing 100 μg of starch C per liter during prolonged incubation at 15°C. From the results obtained (Fig. 4) it is clear that the colony

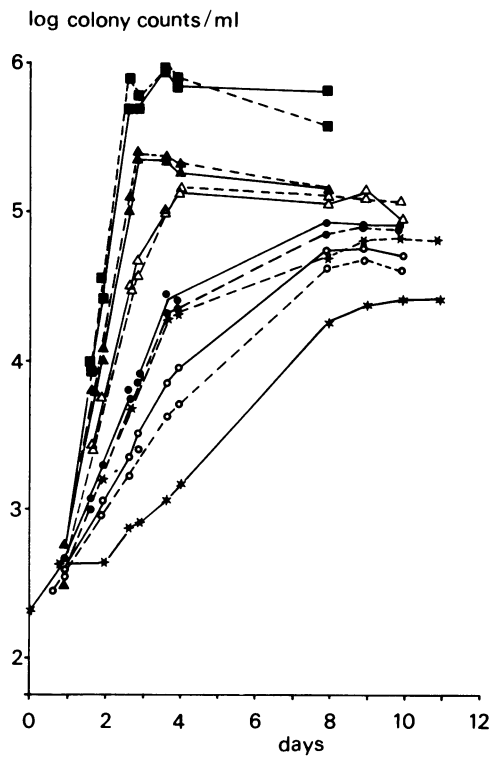

FIG. 2. *Growth of Flavobacterium strain 166 in the filtrate of slow sand filters without added starch (★) or with 1 (○), 3 (●), 5 (△), 10 (▲), or 100 (■) μg of starch C added per liter. Solid and broken lines indicate duplicate determinations.*

TABLE 2. *Growth (at 15 ± 0.5°C) of isolate 166 in tap water supplied with starch*[a]

Starch added (μg of C/liter)	Growth[b]			
	G (h)		N_{max} (CFU/ml)	
100	4.0	3.8	9.2×10^5	9.1×10^5
10	5.3	4.9	2.3×10^5	2.3×10^5
5	6.8	6.5	1.4×10^5	1.4×10^5
3	9.9	9.5	8.4×10^4	7.9×10^4
1	15.0	17.8	5.6×10^4	4.7×10^4
0	27.5	12.3[c]	2.7×10^4	6.6×10^4[c]

[a] Starving cells grown at an initial concentration of 5 μg of starch C per liter were used as inoculum.

[b] Data from duplicate flasks are presented in each pair of columns.

[c] Aberrant results, which were not used for calculations.

counts decreased rapidly; i.e., a 99% decrease was obtained after about 42 to 58 days of incubation.

Growth on glucose and some other substrates. Growth of isolate 166 was assessed in bottles with tap water to which D-glucose had been added in different concentrations. The results of this experiment are shown in Table 3, whereas the relationship between the N_{max} and ΔS values is presented in Fig. 3. Comparison of the observed mean doubling times at different glucose concentrations (Table 3) with those observed at different starch concentrations shows that the organism grew more rapidly in the presence of starch. The yield of strain 166 on glucose at low concentrations ($\Delta S \leq 10$ μg of glucose C per liter) was 1.6×10^{10} CFU/mg of glucose C; thus, S_n expressed in glucose C equivalents was 2.0 μg of glucose C per liter.

As it is unlikely that glucose or starch contributed significantly to the S_n of the tap water used, a number of compounds with relatively low molecular weights which might have contributed to the S_n were tested at low concentrations for their growth-stimulating properties for strain 166. These compounds were: acetate, DL-lactate, pyruvate, succinate, L-aspartate, L-glutamate, and L-alanine. Glycerol, maltose, and yeast extract were also tested. Growth of isolate 166 was stimulated strongly only by maltose and, to a much lesser extent, acetate (Table 4). The other substances tested did not affect the G or N_{max} values.

DISCUSSION

The above described experiments reveal that biodegradation of certain compounds at levels of ecological significance, as well as the microor-

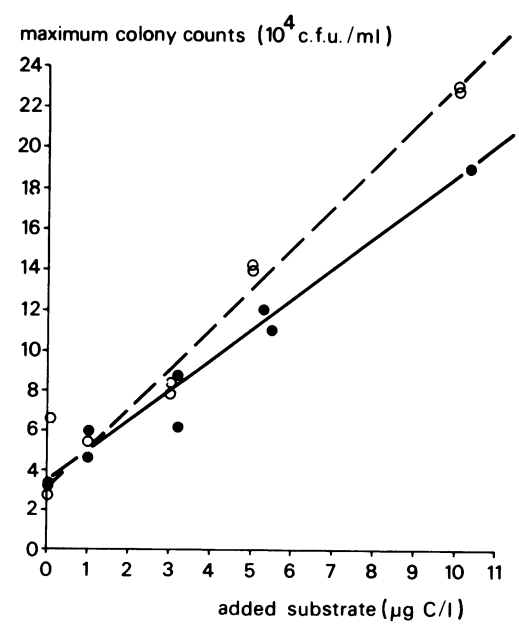

FIG. 3. *Maximum colony counts of Flavobacterium strain 166 in relation to the amounts of starch C (○) and glucose C (●) added to the filtrate of slow sand filters.*

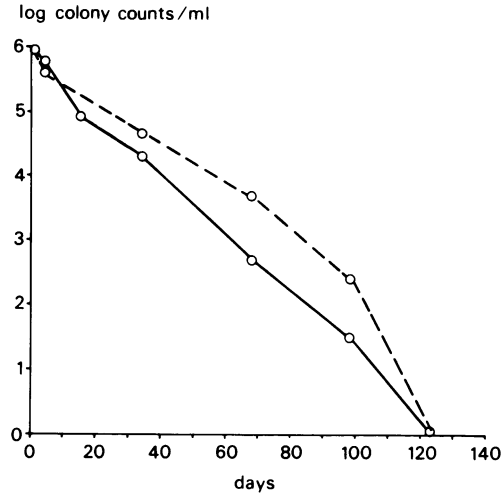

FIG. 4. *Colony counts of Flavobacterium strain 166 during prolonged incubation at 15 ± 0.5°C (values of duplicate flasks).*

TABLE 3. *Growth (at 15 ± 0.5°C) of isolate 166 in tap water supplied with glucose*[a]

Glucose added (μg of C/liter)	Growth[b]			
	G (h)		N_{max} (CFU/ml)	
100	5.0	4.8	4.4×10^5	4.7×10^5
10.3	6.1	6.5	1.9×10^5	1.9×10^5
5.4	9.5	8.9	1.2×10^5	1.1×10^5
3.2	11.2	11.7	8.7×10^4	6.1×10^4
1	14.0	14.4	4.6×10^4	5.9×10^4
0	28.2	30.5	3.2×10^4	3.3×10^4

[a] Starving cells grown at an initial concentration of 100 μg of starch C per liter were used as inoculum.

[b] Data from duplicate flasks are presented in each pair of columns.

ganisms responsible for this degradation, may be studied by very simple experiments, i.e., without the use of radioactive substrates and without laborious continuous culture experiments. However, such experiments cannot be performed with water in which the S_n enables the autochthonous microorganisms or the pure culture to multiply to high colony counts or in which the N_0 (autochthonous bacteria) is high as compared with the N_{max} that may be expected on a ΔS of a few micrograms. The application of the above-described method may also be limited by the presence or growth in the water of microorganisms which utilize the added substrate, but which do not grow on a solid medium.

The experiments also showed that an organism, tentatively identified as a *Flavobacterium* sp., utilized starch at low concentrations at least as rapidly as it did low concentrations of the monomer glucose. None of the species of the genera *Flavobacterium* and *Cytophaga* described in *Bergey's Manual* (27) and by Hayes (10) were similar to the isolated bacterium. However, the ability to utilize starch as a source of carbon and energy seems to be a common property of bacteria belonging to these genera. Moreover, *Flavobacterium* spp. have been found to dominate in activated sludge fed with starch (J. M. A. Janssen, Ph.D. thesis, Agricultural University, Wageningen, The Netherlands, 1979). Therefore, the ability of the isolate studied to grow at concentrations of a few micrograms of either starch, glucose, or maltose per liter of water may contribute to explaining the widespread occurrence of bacteria that form yellow-pigmented colonies in surface water (2, 21), groundwater (28), and tap water (3, 7, 14, 17).

The yields (CFU per milligram of substrate C) of *Flavobacterium* strain 166 on glucose and on starch, respectively, were approximately five times larger than the yields of an *Aeromonas hydrophila* strain on these compounds (26), suggesting that cells of the *Flavobacterium* isolate were five times smaller than those of the aeromonad. Calculation based on the assumptions that 50% of the substrate C is assimilated and that dry weight is 20% of total weight reveals that the volume of the *Flavobacterium* cells grown on starch and on glucose approximated 0.25 to 0.3 μm^3, which is very small. This small cell volume is expected to be advantageous at low substrate concentrations.

The N_{max} values of isolate 166 observed in the experiments with starch or glucose in tap water resulted from the utilization of both the added substrate and some unknown compounds present in the filtrate of slow sand filters (Fig. 3). These unknown compounds (S_n available to iso-

TABLE 4. *Growth (at 15 ± 0.5°C) of isolate 166 in tap water supplied with different organic carbon compounds*[a]

Substrate added	Concn (μg of C/liter)	Lag period (days)	*G* (h)	N_{max} (CFU/ml)
None		8	29.6	4.9×10^4
None		8	19.3	5.4×10^4
Glycerol	5.5	2.5	22.5	4.3×10^4
Maltose	5	1.5	5.8	9.6×10^4
L-Alanine	5	10	25.9	5.2×10^4
L-Aspartate	5	9	24.1	5.0×10^4
L-Glutamate	5	9	24.0	5.4×10^4
Yeast extract	4.2	1.5	23.0	5.1×10^4
Acetate	5	3.5	21.3	1.4×10^5
DL-Lactate	8.2	8	22.7	4.9×10^4
Pyruvate	5	9	23.9	6.8×10^4
Succinate	5	8.5	24.2	4.3×10^4

[a] Starving cells grown at an initial concentration of 100 μg of starch C per liter were used as inoculum.

late 166) amounted only to a few micrograms of carbon per liter, which was about 0.1% of the dissolved organic carbon content of the water. The nature of the unknown compounds utilized by the organism was not elucidated, although it may be concluded from Table 4 that alanine, aspartate, glutamate, lactate, pyruvate, and succinate did not contribute to S_n. Nevertheless, these compounds may have been present in the filtrate used.

The G values of isolate 166 observed in the experiments with different concentrations of starch and glucose may be used to obtain the substrate affinities (K_s, micrograms of C per liter) of the isolate for these compounds. For this purpose, the following slightly adapted Lineweaver-Burk modification of the Monod equation was used:

$$G = G_{min} + (G_{min} \cdot K_s)(1/S) \quad (2)$$

In this equation G and G_{min} are the real and the minimum mean generation times (hours), respectively, S is the concentration of the growth-limiting substrate, and K_s is the substrate affinity constant. The use of equation 2 is complicated by the fact that in the experiments described above, S is composed of a known amount of the added compound (ΔS) and unknown amounts of other utilized compounds (S_n). The pronounced and immediate stimulation of growth as observed after the addition of starch or glucose suggested that these compounds were utilized preferentially. Plotting the observed G values (cf. Tables 2 and 3) against the reciprocal values of ΔS shows that in the starch experiment the G values were linearly related with $1/\Delta S$ when ΔS was ≥ 5 μg of starch C per liter (Fig. 5). Calculation of this relationship gave:

$$G = 3.7 + (14.5)(1/\Delta S) \quad (3)$$

thus, G_{min} was 3.7 h and K_s was 3.9 μg of starch C per liter. In the experiment with glucose, deviating generation times were observed when ΔS was ≤ 5 μg of glucose C per liter. Calculation of the linear relationship shown in Fig. 5 gave:

$$G = 4.7 + (15.5)(1/\Delta S) \quad (4)$$

thus, G_{min} was 4.7 h and K_s was 3.3 μg of glucose C per liter.

The calculated linear functions of equations 3 and 4 represent the kinetic behavior of isolate 166 towards starch and glucose when sequential uptake of substrates occurred (i.e., the added substrates were preferentially used) and when S_n did not contain significant amounts of either starch or glucose. The mechanisms responsible for the sequential uptake would have been most effective at the highest ΔS values. As the water

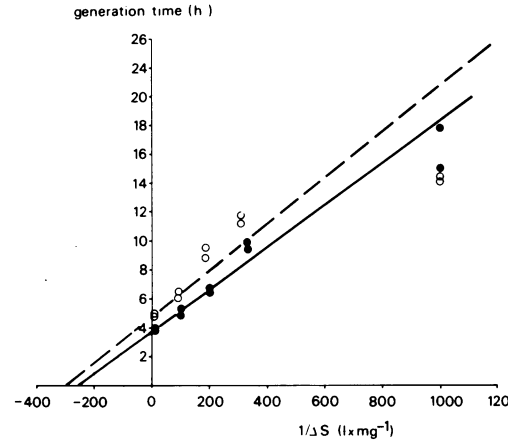

FIG. 5. *Lineweaver-Burk plots of Flavobacterium strain 166 grown on starch (●) or glucose (○) added to the filtrate of slow sand filters.*

used in the experiments was prepared without the addition of starch or starchlike compounds in any treatment stage and previous experiments with water from the same location revealed that the natural glucose concentration was extremely low (26), the contribution of naturally occurring starch and glucose to S_n is assumed to have been negligible, at least at the ΔS values used to calculate linear functions. Hence, equations 3 and 4 indeed represent the kinetic behavior of isolate 166 towards starch and glucose.

The use of substrate for maintenance processes did not clearly influence the yield of the *Flavobacterium* on the added substrates (cf. Fig. 3), as was observed previously (26) for an *A. hydrophila* strain when $S < K_s$. The ability of isolate 166 to use natural substrates in addition to the added substrates is thought to be responsible for this difference in behavior. This uptake of natural substrates may also explain the positive deviations from the linear functions at a ΔS of 1.0 μg of C per liter (Fig. 5).

K_s values as found in the present study have frequently been reported for glucose. By using radioactive substrates and pure cultures, transport constants (K_t) of between 2 and 6 μg of glucose C per liter were obtained by Wright and Hobbie (29) for a *Pseudomonas* sp. obtained from lake water; by Hamilton et al. (9) for gram-negative, oxidase-positive marine isolates; and by Vaccaro and Jannasch (24) for *Achromobacter aquamarinus* (*Alcaligenes aquamarinus*). However, these transport constants, which had been estimated at 5 to 6°C, were all considerably higher at 15°C. Nevertheless, in nature many microorganisms must have very low K_t values for glucose, as may be concluded from the $K_t + S_n$ values of glucose in various water types, in-

cluding seawater and freshwaters, being usually as low as a few micrograms of glucose C per liter (13, 23, 24, 29).

A previous investigation (26) showed that growing cells of an *A. hydrophila* isolate were able to use starch at concentrations of a few micrograms per liter. However, bacteria with a low K_s for starch, as described herein, seem to be scarcely studied. Still, the presence of the described *Flavobacterium* sp. in tap water, produced without the use of starch or starchlike compounds in any of the treatment stages, indicates that such bacteria are common components of the bacterial flora of water.

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