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A STUDY OF THE AEROBIC DECOMPOSITION OF CHITIN BY MICROORGANISMS

WAARNEMINGEN OVER DE MICROBIËLE AFBRAAK VAN CHITINE ONDER AEROBE OMSTANDIGHEDEN

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CHAPTER 1

INTRODUCTION

1. SOME REMARKS ON CHITIN

Chitin belongs to the structural polysaccharides which form materials of considerable mechanical strength. There is general agreement that chitin, $(C_8H_9O_4.NH.CO.CH_3)_n$, is a straight chain polymer of N-acetylglucosamine (N-acetyl-2-amino-2-deoxy-D-glucose) units, joined to one another by 1,4-β-glucosidic bonds.

The N-acetylglucosamine was isolated for the first time in 1902 by Fraenkel, and the corresponding disaccharide chitobiose was not isolated until 1931 (BERGMANN, ZERVAS and SILBERKWEIT; ZECHMEISTER and TOTH).

Chitin closely resembles cellulose; cellulose might theoretically be converted to chitin by replacing the -OH group on each 2-carbon by an $-NH.CO.CH_3$ group. The structure of chitin as given in fig. 1 was firstly proposed by MEYER and MARK (1928)

Though N-acetylglucosamine is present in several other polysaccharides (hyaluronic acid, blood group A substance), chitin is the only polysaccharide built up exclusively of N-acetylglucosamine units. It is insoluble in water, dilute acids, dilute and concentrated alkalis, but soluble in concentrated mineral acids, whereby shortening of the average chain length occurs; the degradation product can be reprecipitated by diluting the acid with water. Treatment of chitin with hot concentrated alkali results in formation of the deacetylated chitin derivative known as chitosan. This substance, which cannot be regarded as a chemical entity, is soluble in dilute acids.

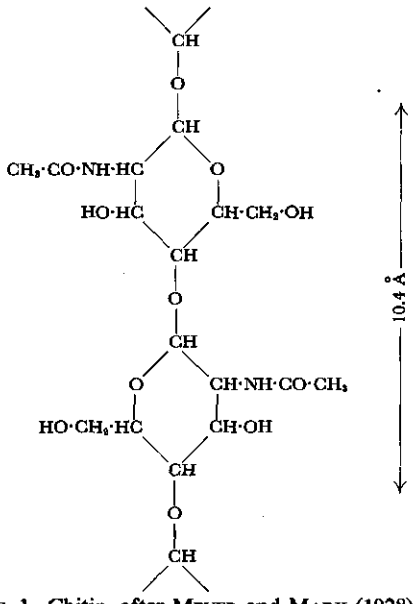

FIG. 1. Chitin, after MEYER and MARK (1928).

Excellent reviews on structure, properties, distribution, and decomposition of chitin are given by ZECHMEISTER and TOTH (1939) and RICHARDS (1951). Tests for chitin and its degradation products are reviewed by TRACEY (1955).

Chitin is widely distributed in nature and has been found in both the animal and plant kingdoms. No essential differences have been found in chitin from animal or vegetable sources. Chitin occurs in the cell walls of all fungi, with the exception of Oomycetes and Monoblepharidales in the Phycomycetes and Laboulbeniales in the Ascomycetes (cf. FOSTER 1949). There is still some controversy as to the presence of chitin in the Saccharomycetes. ROELOFSEN and HOETTE (1951) reported the presence of chitin in the cell walls of yeasts (ascogonous as well as non-ascogonous), but NORTHCOTE and HORNE (1952) who studied the chemical composition and structure of the cell wall of bakers' yeast do not mention the presence of chitin. As far as is known, chitin is absent from bacteria, actinomycetes and most Myxomycetes.

In the animal kingdom, chitin has been found in the Coelenterata (Hydromedusae), Annelida (Polychaeta, Oligochaeta), Mollusca (Cephalopoda, Gasteropoda) and Arthropoda (cf. RICHARDS 1951). The greater part of the chitin deposited in soil and in natural waters probably originates from dead Arthropoda.

2. PREPARATION OF CHITIN

Chitin, as it occurs in natural materials is not present in a pure condition. The raw material has to be purified, which is generally done by treatment with dilute acid and alkali. Our chitin preparations were made from the exoskeletons of marine shrimps (*Crangon*), following generally the procedure described by BENTON (1935).[1])

The skeletons were soaked in 2 % KOH for two days; the material was then

[1]) The author is indebted to Fa F. JANSEN, shrimp dealers in Stellendam (the Netherlands) for a regular supply of shrimp exoskeletons.

washed and dried at 80 °C. In most cases, the stomachs which cannot be removed from the skeletons by washing, were removed by hand. The dry material was ground in a hammer mill and subsequently decalcified in 1 % HCl. The resulting powder, washed free of HCl, was suspended in 2 % KOH and kept in alkali for 2–3 weeks. Several times during this period the suspension was brought to 90 °C and stirred mechanically. After cooling, the insoluble material was filtered, washed and resuspended in 2 % KOH. The alkali treatment was continued until no yellow pigment was extracted from the powder on heating. The material was once again treated with 1 % HCl and then filtered, washed until HCl-free, and dried at 80°C. Finally the powder was Soxhlet extracted with 96 % alcohol for 24 hours. The chitin thus prepared was almost white and showed the characteric X-ray diffraction pattern.[1]) The nitrogen content of different preparations varied from 6.6 to 6.9 % (calculated N = 6.89 %) and ash contents varied from 0.5 to 1.4 %. Before being used for the preparation of culture media, the powdered chitin was ground. A small amount of water was added to a known amount of chitin which was subsequently ground in a porcelain ball-mill until the diameter of the particles varied from 5 to 80 microns. The finely ground chitin was then washed into a flask and water added to make a 1 % chitin suspension. For the preparation of chitin agar, the following substances were added to 1000 ml of stock suspension: K_2HPO_4 1 g, $MgSO_4$ 1 g and agar 20 g. Chitin agar plates were made by pouring a thin layer of melted chitin agar on solidified tap water agar. This serves purposes of economy and makes microbial chitin decomposition more easily detectable.

CHAPTER 2

TAXONOMY AND OCCURRENCE OF CHITIN-DECOMPOSING MICROORGANISMS

1. LITERATURE

Although chitin is a highly durable substance (it has been detected even in fossils; cf. RICHARDS 1951), it apparently does not accumulate in soil or marine sediments despite the large amounts produced by innumerable organisms. Undoubtedly the greater part of this chitin from dead animals and fungi is decomposed by microorganisms.

BENECKE (1905) was the first to report the isolation of a chitin-decomposing bacterium. The organism, a Gram-negative, aerobic, asporogenous, motile rod was named *Bacillus chitinovorus* and was isolated from rotting plankton from Kiel harbour. STÖRMER (1908) mentions chitin decomposition brought about by a *Streptomyces*. FOLPMERS (1921) isolated two chitinovorous bacteria from water of the harbour of Bergen op Zoom. One of the strains had the same characteristics as *Bacillus chitinovorus* BENECKE and the other differed only slightly from this species. FOLPMERS mentions, moreover, the presence of chitin-decomposing actinomycetes in soil. RAMMELBERG (1931) isolated a chitinovorous bacterium from manured garden soil which differed only slightly from the *Bacillus chitinovorus* of BENECKE. STEINER (1931) demonstrated the presence of bacteria capable of decomposing chitin in lake water and showed that chitin

[1]) The author is indebted to Dr D. KREGER for the X-ray analysis of chitin preparations.

was decomposed aerobically as well as anaerobically by his raw cultures. JOHNSON (1931) isolated a Myxococcus, antibiotic to several *Ustilago* species, which also was capable of digesting chitin. The same author (1932) also mentions the isolation of chitin-decomposing microorganisms from decaying exoskeletons of the hard shell crab (*Cancer magister*). Some of these organisms were typical representatives of the genus *Myxococcus*, while others may have belonged to the genera *Sporocytophaga* and *Cytophaga* (cf. STANIER 1947). All of the cultures lost their chitin-destroying property when cultivated for a time on media without chitin. JENSEN (1932) reported a considerable increase of bacteria and actinomycetes in soil to which chitin was added. Among the fungi in this soil, *Mycogone nigra* and a *Fusarium* species were prevalent. These fungi as well as two actinomycetes were isolated and appeared to be able to grow on chitin with the concomitant production of ammonia. BERTEL (1935) observed the presence of chitinovorous bacteria in rotting plankton from the sea near Monaco. BENTON (1935) isolated 250 bacteria capable of chitin decomposition, and classified these into 17 types. All of these bacteria were motile aerobes and most were Gram-negative (15 types). Only part of the strains were able to attack cellulose and/or starch. As an example of the great variety of sources from which chitinovorous bacteria may be isolated, those listed by BENTON may well be mentioned here: intestines of frogs, bats, snipes and speckled trout; mud from stagnant pools and sand under running water; soil, compost heaps, and the water of different lakes; decaying crayfish and Mayfly nymph shells.

BUCHERER (1935) described two chitin-decomposing bacteria, isolated from soil. One of them, a sporeforming rod, he called *Bacillus chitinobacter*. Several strains of chitinovorous actinomycetes were isolated by BUCHERER and identified by WAKSMAN as *Streptomyces griseolus* WAKSMAN, *Str. exfoliatus* WAKSMAN and CURTIS, *Str. fradiae* WAKSMAN and CURTIS, *Str. aureus* WAKSMAN and CURTIS, and *Str. griseus* (KRAINSKY) WAKSMAN and CURTIS.

From solar salts of different parts of the world, eight halophilic chitinovorous bacteria were isolated by STUART (1936); five of them resembled *Serratia salinaria* HARRISON and KENNEDY, whereas the other three strains were similar to *Sarcina littoralis* PAULSEN. SKINNER and DRAVIS (1937) studied the abundance of chitinovorous microorganisms in different soils (cf. p. 135) and mention the isolation of 42 strains of fungi capable of decomposing chitin. The following genera were identified: *Aspergillus*, *Mucor*, *Penicillium*, *Absidia*, *Trichoderma*, *Fusarium*, *Gliacladium* and *Thamnidium*. Chitinovorous bacteria were isolated by HESS (1937) from live lobsters (*Homarus americanus*) that showed a disease of the exoskeleton. These bacteria were facultatively aerobic and did not digest cellulose and agar. ALESHINA (1938) reports the decomposition of chitin by a stable mixture of microorganisms which reduce sulphate. This anaerobic chitin decomposition was also accompanied by the liberation of ammonia.

ZOBELL and RITTENBERG (1938) isolated (but did not name) 31 strains of chitin-decomposing bacteria from marine sources. Most of the strains were Gram-negative rods; one coccus and two species of *Vibrio* were also present. Many of these strains liberated ammonia or acid during chitin decomposition; in some cases reducing sugars were produced. None of the strains could attack cellulose and a number of them were also unable to utilize glucose, although some could decompose starch with acid production. The authors observed the occurrence of bacteria which derive their nitrogen from chitin when other energy sources were available, but no non-nitrogenous breakdown product of

chitin was isolated. Other bacteria were found to digest chitin only in the presence of other carbon and nitrogen sources.

A wide variety of microorganisms pathogenic to man and animals were inoculated on chitin agar by SCHMIDT-LANGE and BUCHERER (1938). All of the chitin-decomposing organisms were actinomycetes. HOCK (1941) describes two chitinovorous bacteria isolated from marine sources. Both strains, *Bacterium chitinophilum* and *Bacterium chitinochroma*, are aerobic, Gram-negative, asporogenous, motile rods, and neither is able to digest cellulose. Ammonia, reducing substances, and organic acids could be demonstrated during the decomposition of purified chitin by these organisms.

ERIKSON (1941) studied 10 strains of actinomycetes of the species group *Micromonospora chalceae* (FOULERTON) ØRSKOV, isolated from lake mud. Nine of these strains were capable of decomposing cellulose as well as chitin.

KARLING (1945) mentions the isolation of species of two new genera, *Rhopalophlyctis* and *Chytriomyces*, belonging to the Chytridiales. These fungi, which appeared to be capable of chitin decomposition, were isolated from the exuviae of insects, the larval stages of which develop in fresh water.

STANIER (1947) isolated a number of chitin-decomposing bacteria from enrichment cultures in order to study the occurrence of chitinovorous *Myxobacteria*. These were apparently rather common in soil. All the isolated strains proved to be nonfruiting and amicrocystogenous. They showed such slight differences that they were all designated *Cytophaga johnsonae*. One of the strains was distinguished as *Cytophaga johnsonae* var. *denitrificans* because of its ability to grow anaerobically in the presence of nitrate with vigorous gas production.

CAMPBELL and WILLIAMS (1951) isolated 20 strains of chitin-decomposing bacteria from marine mud. Eight of them were described as new species as follows: *Achromabacter labrum*, *Achromabacter ureasporum*, *Achromabacter lipophagum*, *Achromobacter hyperopticum*, *Pseudomonas subrubra*, *Pseudomonas eryothasia*, *Flavobacterium indoltheticum* and *Micrococcus colpogenes*. All strains were able to liberate ammonia and reducing sugar from chitin.

This survey of the literature dealing with chitin-decomposing microorganisms indicates that these organisms are distributed over a number of known genera and that they may be isolated from a wide variety of habitats.

2. TAXONOMIC OBSERVATIONS MADE IN THE COURSE OF THE PRESENT INVESTIGATIONS

Among 50 chitin-decomposing bacterial strains which have been isolated from soils in the course of the present study, the following genera were represented: *Achromobacter*, *Flavobacterium*, *Chromobacterium*, *Bacillus*, *Cytophaga* and *Pseudomonas*; several isolates of nonsporeforming Gram-positive rods have not yet been identified. It may be that some of them are coryneform bacteria. 23 Strains of chitin-decomposing actinomycetes have been deposited in the collection of the 'Centraal Bureau voor Schimmelcultures' at Baarn in the Netherlands. Twelve strains belong to the genus *Streptomyces* (cf. Plate 1, fig, 2), two strains belong to the genus *Nocardia*, whereas nine strains are representatives of the genus *Micromonospora* (cf. Plate 1, fig. 3). The following chitinovorous fungi were isolated from soil: *Mortierella elongata* LINNEMAN, *M. alpina* PEYRONEL, *M. zychae* LINNEMAN, and *Aspergillus fumigatus* FRES. All strains have been included in the above mentioned collection.[1])

[1]) The author is indebted to Miss A. L. VAN BEVERWIJK and to Dr G. A. DE VRIES for their determinations of fungi and actinomycetes.

A description of the two chitinovorous bacterial strains which were used for our investigations on chitin decomposition is given below. The strains were isolated from enrichment cultures made by adding powdered chitin to manured garden soil and then incubating this mixture in a moist condition at 30 °C for 6 days. A suspension of this soil was plated out on mineral chitin agar (composition mentioned on p. 130). After an incubation of several days, those organisms that showed a clear zone underneath and around their colonies were isolated and grown further on yeast extract-glucose agar. One of the strains, though not in all respects identical with *Cytophaga johnsonae* STANIER, (cf. STANIER 1947), undoubtedly belongs to this species group, as may be seen from the following description.

Morphology: Thin rods of variable length were observed on agar media (Plate 2, fig. 6)[1]) Mean length of the cells 2–3 microns. In young liquid cultures (less than two days), long cells (up to 20 microns) are very common (Plate 3, fig. 7). These rods show definite flexing movements. In older cultures, short cells predominate. Gram negative. Flagella are absent.

Difco Bacto tryptone agar plate: Appearance of colonies depends on concentration of agar and tryptone. On a medium containing 2 % agar and 2 % tryptone, colonies are smooth, glistening, translucent, convex, pale yellow, and do not show lateral swarming. When the concentration of tryptone is lowered to 0.25 %, colonies are surrounded by a very thin layer of cells which is easily detected in reflected light. The creeping movement was studied by the method described by STANIER (1947). A thin layer of agar (1 % agar, 0.25 % tryptone) was spread on a sterile cover slip and inoculated at several spots with a mass of young cells. The cover slip was placed in a moist chamber at 30 °C. After 8 hours' incubation, the artificial colony was surrounded by a thin layer of cells, the swarming edge being at a distance of 400–500 microns from the artificial microcolony. A microscopic examination showed that just behind the advancing edge the cells lie at a considerable distance from one another.

Mineral chitin agar colonies: Flat, translucent, nearly colourless; edge of colonies extends beyond the zone of decomposed chitin (Plate 1, fig. 5).

Pepton gelatin stab: Rapid infundibuliform liquefaction.

Milk: Peptonized.

Carbon utilization: Growth has been observed in test tubes containing the basal medium described by SMITH et al. (1952) to which 1 % of the following substances was added: glycerol, rhamnose, arabinose (growth weak), glucose, fructose (growth weak), mannose, lactose, galactose, maltose, sucrose, raffinose, mannitol. On standing for one week, the medium containing glucose becomes slightly acid. The strain tends to form yellow pellicles on the liquids, part of which fall to the bottom of the tubes.

Cellulose and starch are not attacked.

Chitin is hydrolysed with formation of N-acetylglucosamine, glucosamine, acetic acid and ammonia (cf. Chapter 6).

Utilizable nitrogen sources: Nitrate, ammonia and peptones.

Nitrites are not produced from nitrates.

Catalase is produced.

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[1]) The author is indebted to Miss dra C. VAN DER SCHEER who operated the electron microscope.

Indol is not formed.
Aerobic.
Optimum temperature: 25–30 °C.
Source: Soil.
Habitat: Soil, rather common.
The strain has been deposited in 'The National Collection of Industrial bacteria' at the Chemical Research Laboratory, Teddington, Middlesex, England. Here it is designated as No. 8501.
On one occasion a mutant of the original strain was isolated which forms coulourless colonies on yeast extract-glucose agar, whereas the parental strain forms yellow colonies on this medium.

The name *Pseudomonas chitinovorans*, n.sp., is proposed for the second strain, despite the fact that occasionally Gram-positive cells have been encountered.
Morphology: Small rods, 0.3–0.5 by 0.8–1.5 microns. On the following medium: Difco yeast extract 0.7 %, glucose 1 %, K_2HPO_4 0.1 %, $MgSO_4$ 0.1 %, $CaCO_3$ 1 %, and agar 2 %, the cells, often occurring in pairs, have a length of 1–6 microns and are typically spindle-shaped (Plate 4, fig. 8). Especially on this medium, Gram-positive cells may occur among Gram-negative ones when stained with Hucker's modification of the Gram stain.[1]) On other media, Gram-positive cells are less common and the Gram-negative character of the strain is predominant. Motile (especially in liquid media), with one polar flagellum (Plate 5, fig. 9).
No fluorescent pigment is formed.
Difco Bacto tryptone agar plate: Colonies are circular, convex, smooth, entire.
On the above mentioned yeast extract-glucose medium, colonies become raised with concave bevelled edge, undulate. Acid is formed on this medium.
Mineral chitin agar: Colonies are circular, flat, smooth, translucent. A clear zone of decomposed chitin forms around each colony and extends well beyond the edge of the cell mass (Plate 1, fig. 4).
Pepton gelatin stab: Very slow liquefaction.
Milk with bromcresolpurple-o-cresolred indicator mixture: Slightly alkaline.
Carbon utilization: Growth has been observed in test tubes containing the basal medium described by SMITH et al. (1952) to which 1 % of the following substances were added: glycerol, rhamnose, arabinose, glucose, fructose, mannose, lactose, galactose, maltose, sucrose, raffinose and mannitol. Acid is formed from glucose, fructose, and mannose, but not from disaccharides yielding one of these sugars on hydrolysis. Pyruvic acid is formed from glucose. (cf. Chapter 7). No pellicle is formed on the liquid media.
Cellulose and starch are not attacked.
Chitin is hydrolysed with formation of N-acetylglucosamine, glucosamine, acetic acid and ammonia (cf. Chapter 6).
Utilizable nitrogen sources: Nitrate, ammonia and peptones.
Nitrites are produced from nitrates.
Catalase is produced.

[1]) Due to the occurrence of Gram-positive cells and the description of motile Coryneform bacteria by several authors (cf. JENSEN 1952), the strain was originally thought to belong to this group of organisms. Dr H. L. JENSEN (Lyngby, Denmark) kindly studied the strain and found the cell shape too regular and the cells too motile for being a *Corynebacterium*. Moreover, angular growth was not observed.

Indol is not formed.
Aerobic.
Optimum temperature: 25–30 °C.
Source: Soil.
Habitat: Soil, not commonly found.

The strain has been deposited in 'The National Collection of Industrial Bacteria' at the Chemical Research Laboratory, Teddington, Middlesex, England. Here it is designated as No. 8500, which will be used as its reference number in this paper. Stock cultures of this strain have been kept for several years on a yeast extract-glucose medium of the following composition: glucose 1 g, yeast extract (Difco) 3 g, $CaCO_3$ 1 g, K_2HPO_4 1 g, $MgSO_4$ 1 g, agar 20 g per 1000 ml of soil extract. During this period, the strain has often been plated out on different media, and on several occasions mutants of the original strain have been found. These mutant strains differ slightly from the parental strain in colony type, chitin-decomposing capacity, or acid formation. One of these, a mutant which produces more acid from chitin than the original strain, has been used for further investigations (cf. Chapter 7). This strain, in all other respects identical with the strain originally isolated from soil, has also been deposited in the above mentioned collection, where it is designated as No. 8676. This number will be used as its reference number in this paper.

3. Summary

The literature on chitinovorous microorganisms shows that these organisms are distributed over a number of known genera and that they can be isolated from a wide variety of habitats.

Among chitin-decomposing bacteria isolated by us from soil, are representatives of the following genera: *Achromobacter*, *Flavobacterium*, *Chromobacterium*, *Bacillus*, *Cytophaga* and *Pseudomonas*. A description has been given of *Pseudomonas chitinovorans*, n.sp., and of a bacterium belonging to the species group *Cytophaga johnsonae* Stanier.

Chitin-decomposing actinomycetes have found to be very common in soil. Representatives of three genera: *Streptomyces*, *Micromonospora*, and *Nocardia*, have been isolated.

Aspergillus fumigatus Fres. and the following *Mortierella* species are among chitinovorous fungi which have been isolated from soil: *M. elongata* Linneman, *M. alpina* Peyronel, and *M. zychae* Linneman.

Chapter 3

A QUANTITATIVE DETERMINATION OF CHITIN-DECOMPOSING MICROORGANISMS IN DIFFERENT SOILS

1. Introduction

Reports on the numbers of chitinovorous microorganisms have been published by several authors. In the first of such studies, Skinner and Dravis (1937) have determined the numbers of these organisms in soil samples which were 'found in the laboratory and had been there for several years'. By means of the

dilution method, numbers of chitin-decomposing microorganisms were found, varying from about one hundred to several millions per gram of air-dried soil.

ZoBELL and RITTENBERG (1938) studied the occurrence of chitin-decomposing microorganisms in marine habitats. In samples of the upper layer of marine sediments, some of which were collected at a depth of 2000 meters, these organisms occur in numbers up to a thousand per gram. Up to a thousand per ml were found in stomach contents of the squid and other cephalopods.

In marine sand (littoral zone), mud (collected at a depth of 878 meters) and sea water HOCK (1940) found the following numbers of chitin-decomposing bacteria: 60,000 per g, 125 per g and 150 per ml, respectively.

In the present investigation, a study has been made of the occurrence of chitinovorous bacteria, actinomycetes and fungi in different soils.

2. METHODS

The soils were taken from the experimental plots (42 m^2 each) of the Laboratory of Microbiology at Wageningen, where every year for 33 years they have been treated, each in its particular way. The following soils have been used:

Soil No. 8. Sandy soil, annually receiving: 2 kg superphosphate, 1.5 kg Chilean salpeter and 1.25 kg potassium sulphate. The soil has never been limed. pH of this soil: 4.7.

Soil No. 23. The same type of soil as No. 8, receiving the same minerals. This soil, however, has been limed at regular intervals and ultimately (1949) received 60 kg of lime marl. pH of this soil: 6.8.

Soil No. 37. Same type of soil as soils No. 8 and No. 23, but treated with farmyard manure once every year; pH of this soil: 6.4.

Soil No. 33. A clay soil (originally from a brickyard near the river Rhine), treated with farmyard manure annually; pH 7.5.

The pH values of the soils were determined by shaking 20 g of soil with 100 ml of water. The suspension was allowed to stand overnight and the pH was subsequently measured with a glass electrode.

HARMSEN (1940) studied the influence of sampling and preparatory treatment of the samples on the enumeration of microorganisms in soils. He proposed a preparatory treatment in which large soil samples, suspended in water, were homogenized in a porcelain ball-mill. This method, with only slight modifications, was followed in our investigations. From each plot, 20 samples of 20 g were taken to a depth of 6 cm. The combined samples were placed in a porcelain ball-mill and two liters of water were added. After a rotation time of 20 minutes, two samples of 1 ml were taken from the suspension which was stirred mechanically during sampling. From each aliquot of the suspension, a dilution series was made. From the final dilution of one series, 10 samples of 0.1 ml were pipetted into Petri dishes containing solidified chitin agar (prepared as described on p. 130), whereas 10 0.1 ml samples, taken from an appropriate dilution of the other series were pipetted onto an unselective medium appropriate to meet the nutritional requirements of a relatively large number of microorganisms. This medium contained: casein 1g, Difco yeast extract 2g, glucose 5g, K_2HPO_4 1g, $MgSO_4$ 1g, $(NH_4)_2SO_4$ 1g and agar 20g per 1000 ml of soil extract; pH adjusted to 7. The casein (Difco Bacto-isoelectric casein) used for the preparation of this medium was kept as a sterile stock solution containing 10g casein per 160 ml of lime water. Stock solutions of soil extract were prepared by addition of 1 kg clay soil to 2 l tap water, followed by boiling, filtration and autoclaving.

The final dilutions were prepared so as to allow 100–300 colonies to develop on each plate (diam. 13 cm). Each inoculum was distributed over the agar surface by means of a sterile, bent glass rod. Subsequently the plates were inverted and a piece of filter paper, moistened with glycerol, was placed in the lid of each plate in order to obtain a quick drying agar surface and thus prevent the spread of bacteria. Petri dishes containing chitin agar were incubated for 9 days, those with enriched soil extract agar for 6 days at 28 °C. Subsequently the colonies of chitinovorous microorganisms (showing a clear zone of decomposed chitin around and/or underneath their colonies) and 'total' microorganisms were counted, and the number of microorganisms per gram of oven-dried soil was calculated.

The number of microorganisms found in an 0.1 ml aliquot of the final dilution is a sample of a Poisson distribution with expectation value m and standard deviation $\sqrt{m}$. The mean, $\bar{x}$,

of ten plate counts has expectation value m and standard deviation $\sqrt{m}/\sqrt{10}$. In all our cases $\bar{x}$ was greater than 100. Then from statistical considerations it follows that the number of microorganisms, Nm (N = dilution involved), may be expected to lie between 0.93 $N\bar{x}$ and 1.07 $N\bar{x}$, with level of significance 0.05.[1])

The determination of numbers of microorganisms was carried out several times. Although the numbers found during autumn were about ten times as large as those in spring, the ratio between the numbers found in different soils were approximately the same in all cases.

3. Results

From fig. 10 it may be seen that a larger number of 'total', as well as chitinovorous microorganisms, was found in a sandy soil (No. 37) treated with farmyard manure, compared with the same type of soil treated with mineral fertilizer (No's 8 and 23). The smallest number of both types of organisms were found in a sandy soil (No. 8) of acid pH. The highest numbers were found in a clay soil (No. 33) treated with farmyard manure, although the difference in the numbers of chitinovorous microorganisms in soils No. 33 and No. 37 is hardly significant.

The percentage of actinomycetes in 'total' microorganisms is rather small, whereas these organisms form the major part of the population of chitinovorous microorganisms. The latter observation is in contrast to that of Skinner and Dravis (1937), who found that 'the soil has a larger population of true bacteria than of molds or actinomycetes'. The difference between our results and those of Skinner and Dravis may be explained by the use of different methods for the enumeration of chitinovorous microorganisms. In our experiments, aliquots of diluted soil suspensions were plated out on chitin agar, whereas in the experiments of Skinner and Dravis, aliquots were added to test tubes containing

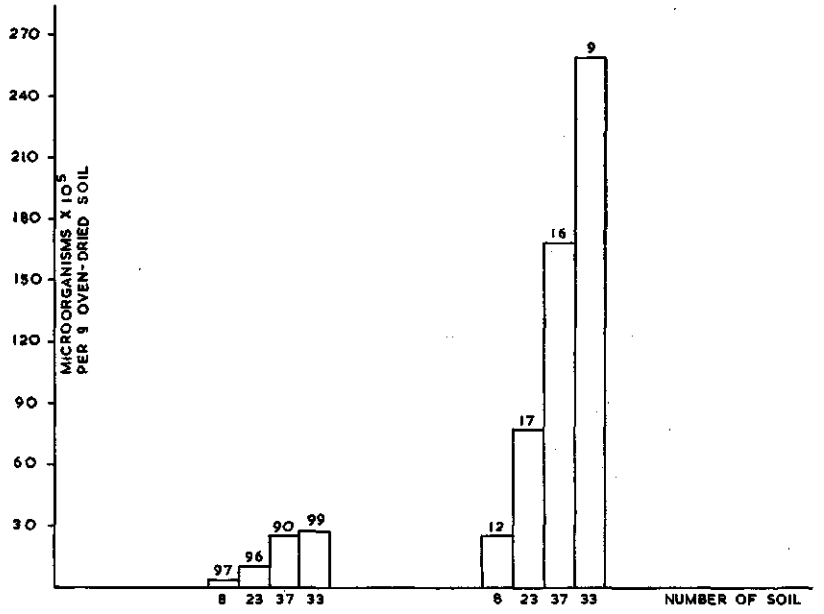

Fig. 10. Numbers of chitinovorous microrganisms (left) and 'total' microorganisms (right) in different soils. Figures above columns refer to percentage of actinomycetes.

[1]) The author is indebted to Prof. Dr N. H. Kuiper for advice in statistical questions.

chitin strips, partly submerged in a liquid mineral medium. Since it has been found that bacteria show a rapid multiplication in chitin-containing soils which are saturated with water, whereas actinomycetes do not develop in such soils (cf. Chapter 4), the low counts of actinomycetes found by SKINNER and DRAVIS may be explained simply by the fact that these authors used a liquid medium for their enumeration of chitinovorous microorganisms.

Chitin-decomposing fungi formed less than 1 % of the population of all chitinovorous microorganisms in the soils investigated.

4. SUMMARY

Large numbers of chitinovorous microorganisms were found in all soils investigated. The smallest numbers of these organisms ($3.5.10^5$ per g of oven-dried soil) was found in a sandy soil with acid pH.

Only a small part of the population of 'total' microorganisms in the soils is formed by actinomycetes, whereas these organisms form the greater part of the population of chitinovorous microorganisms. Bacteria formed 1–3 % and fungi less than 1 % of the latter population.

CHAPTER 4

CHANGES IN THE MICROBIAL POPULATION OF DIFFERENT SOILS AS A RESULT OF THE ADDITION OF CHITIN

1. INTRODUCTION

The dynamic equilibrium which soil microorganisms establish between themselves changes with every change in environment. The addition, for instance, of an organic substance to a soil may produce such a change. Generally, an increase may be observed of the microorganisms capable of decomposing this particular substance. This enrichment is often coupled with the production of breakdown products which can subsequently be utilized by other organisms. The nature of the population change depends not only on the chemical nature of the added substrate, but also on temperature, moisture content and aeration of the particular soil.

In the present investigations, the effects of adding chitin to various microbial soil populations have been studied. These experiments were carried out both with water saturated soils and with relatively dry soils at 28 °C. The soils were taken from the experimental plots No's 23, 37 and 33 (description see p. 136) of the Laboratory of Microbiology at Wageningen.

2. EXPERIMENTS WITH WATER SATURATED SOILS

a. Methods

In these experiments use was made of the soil perfusion technique, introduced by LEES and QUASTEL (1944). The function of their apparatus is 'to percolate a column of sieved soil automatically and regularly with small quantities of a well-aerated solution of metabolites and to return the percolated solution, now modified by the activities of the soil microflora, to the reservoir whence it came. As the circulatory process is continuous, metabolic activities in the soil are reflected as chemical changes in the reservoir solution and can be followed by analysis of it' (LEES 1947).

In our investigations chitin was added to columns of different soils which were percolated

with water in soil percolators (model 1) as described by JEFFERYS and SMITH (1951). In this percolator, use is made of a valve between soil column and the fluid reservoir above it which automatically controls the amount of percolate reaching the soil; this valve stops percolation when the soil approaches saturation and thus waterlogging of the soil is avoided. Between valve and soil surface, compressed air enters the soil tube and forces the water to pass through the soil column, and the percolate is subsequently carried by the airlift tube to the reservoir. When the resistance of the soil to the air flow has decreased sufficiently, a further quantity of percolation liquid passes into the soil tube.

It appeared that in our hands the perfusion technique had the disadvantage that the structure of the soil was disturbed soon after initiating an experiment, followed by clogging of the soil column. This was especially the case with clay soil. In order to prevent this phenomenon, soil aggregating substances were added to the soils.

Sampling and preparation of the soils were carried out as follows. 15 Soil samples to a depth of 6 cm were collected in plots No. 37 and No. 33 respectively. The combined samples of each soil were passed through a 4 mm sieve (clay soil was dried to some extent before sieving) and exposed to the air to dry. The dried soil was thoroughly mixed and 0.5 g of powdered chitin (particle diam. 80–300 microns) was added to a 40 g sample of each soil. Moreover, 40 mg of carboxymethylcellulose was added to the sandy soil and 40 mg of 'krilium' (Monsanto Chem. Comp., St. Louis, U.S.A.) was added to the clay soil. No chitin was added to those soil samples which were to serve as controls. Subsequently the soils were moistened (sandy soil to 10%; clay soil to 17%), screened through a 2 mm mesh and again air dried. The soils thus prepared were placed in the soil tubes with a packing of glass wool at each end of the tubes. The tubes were attached to the percolator units and subsequently compressed air was allowed to enter each unit; 100 ml of tap water was then added to the reservoir of each percolator. Every few minutes, a small amount of percolate is carried by the air lift tube to the reservoir and thus the composition of the fluid in the reservoir is kept in close approximation to that actually in contact with the soil.

Until now the percolation technique has been used chiefly for the analysis of chemical changes brought about by the soil microorganisms. In our investigations a microbiological analysis has been made of the reservoir fluids in order to investigate whether changes in the microbiological populations of the soils might be followed in this way. A comparison has been made of the numbers of chitinovorous microorganisms present in the reservoirs of units containing soils with and without added chitin.

At regular intervals a one ml sample was taken from the thoroughly stirred liquid of each reservoir and a dilution series made. From the final dilution of each series, four 0.1 ml samples were pipetted into sterile Petri dishes (diam. 13 cm) containing solidified chitin agar. Each inoculum was distributed over the agar surface by means of a sterile, bent glass rod. The plates were incubated for 9 days at 28 °C and subsequently the colonies showing chitin decomposition were counted. The average number of colonies per plate was then multiplied by the dilution involved and in this way the total amount of chitinovorous organisms per ml of reservoir fluid was determined. The number of microorganisms found in an 0.1 ml aliquot of the final dilution is a sample of a Poisson distribution with expectation value m and standard deviation $\sqrt{m}$. The mean, $\bar{x}$, of four plate counts has expectation value m and standard deviation $\sqrt{m}/\sqrt{4}$. In all our cases, $\bar{x}$ was greater than 100. Then from statistical considerations it follows that the number of bacteria per ml of reservoir fluid, Nm (N is dilution involved), may be expected to lie between $0.9\,N\bar{x}$ and $1.1\,N\bar{x}$, with level of significance 0.05.

The pH of the reservoir fluids was measured at regular intervals by means of a glass electrode.

These experiments were repeated several times and the results obtained showed a similar trend. Examples of typical experiments will be given below.

b. Results

The numbers of chitinovorous microorganisms per ml of liquid in the reservoirs of the percolators at various days after starting the experiment are given in fig. 11.

Apparently in percolators containing soils without chitin a gradual decrease of chitin-decomposing microorganisms occurred. In those with chitin-containing soils, however, a considerable increase of these organisms could be observed up to 2.5–3 weeks after the start of the experiment.

A remarkable feature of these experiments was the nearly complete absence

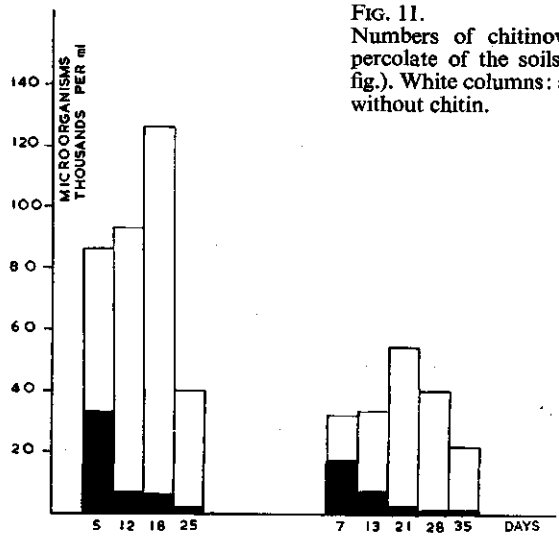

FIG. 11.
Numbers of chitinovorous microorganisms per ml of percolate of the soils No. 33 (left fig.) and No. 37 (right fig.). White columns: soil with chitin; black columns: soil without chitin.

of actinomycetes in the fluid reservoirs. Only bacterial colonies have been observed on the chitin agar inoculated with dilute samples of the reservoir fluids, whereas plate counts made from fresh soils showed that the population of chitinovorous microorganisms living under field conditions consists chiefly of actinomycetes (cf. Chapter 3). As the pH values of the reservoir fluids were about 7 throughout the experiments, they could not be responsible for the absense of actinomycetes. The bacterial colonies mentioned above represented only a few bacterial strains. In most cases no more than about 5 colony types (different for each soil) could be observed, indicating that only a few bacterial strains multiplied rapidly when chitin had been added to the soils.

Since no actinomycetes were found in the fluid reservoirs, the chitin-containing soils themselves were controlled on several occasions to exclude the possibility that the percolating water might simply not be able to remove spores or fragments of colonies from the soil. In these cases, the soil columns were removed from the percolators and added to Roux bottles containing 500 ml sterile tap water. After rotating the flasks for 15 min. (as described on p. 142), dilution series were made from the soil suspensions and Petri dishes containing solidified chitin agar were inoculated and incubated as described above. It appeared that in this case too, the percentage of actinomycete colonies was always less than 1 % of the total number of colonies found on the plates. The amount of chitinovorous bacteria in the soil columns, however, was about 1000 times the number of those present in 100 ml of reservoir fluid at the moment when the percolators had been running for 13 days. The colony types found on chitin agar inoculated with a diluted soil suspension were the same as those found on the plates inoculated with a diluted sample of reservoir fluid.

During the experiments with soil percolators, a number of chitin-decomposing bacterial strains were isolated and purified on chitin agar. It appeared that a high percentage of these strains lost their chitin-decomposing capacity after several transfers on yeast extract-glucose agar. Out of 15 different strains, 12 were unable to decompose chitin after several transfers. Attempts were made to

reestablish their chitin-decomposing ability by growing these strains in a liquid medium containing: 1 % chitin (finely ground), 0.5 % glucose and 0.1 % of $(NH_4)_2SO_4$, $MgSO_4$ and K_2HPO_4, and subsequently transferring the strains to media with gradual diminishing amounts of glucose, but these attempts were unsuccessful. Although the loss of chitin-decomposing capacity has also been observed with strains isolated from other sources, it was never so pronounced as in this case.

c. Summary

During experiments with water saturated soils, carried out with the soil perfusion technique, it became clear that changes in the microbial population of different soils resulting from chitin addition to these soils, are reflected in the reservoir fluids of the percolators. It appeared that in soils saturated with water, only chitinovorous bacteria achieve a rapid multiplication when chitin had been added to the soils. The numbers of these bacteria found in the reservoir fluids form only a small part of those present in the soil columns.

Chitin decomposing actinomycetes form less than 1 % of the total population of chitinovorous microorganisms found in the fluid reservoirs, as well as in the soil columns.

A high percentage of chitinovorous bacteria isolated from the percolators lost their chitin decomposing capacity after several transfers on yeast extract-glucose agar.

3. Experiments with relatively dry soils

Even though the actinomycetes have been found to form a large part of the population of chitin-decomposing microorganisms living under field conditions, no chitinovorous actinomycetes have been found in our laboratory experiments with water-saturated soils. It therefore seems likely that the moisture content has a marked influence on the composition of the chitin-decomposing flora. In order to investigate this further, experiments have been carried out with relatively dry soils.

a. Methods

Three soils were used for these investigations (No.'s 23, 37 and 33; cf. p. 136). Soil samples were collected to a depth of 6 cm in 15 areas of each plot. The combined samples of each soil (± 2 kg) were screened through a 4 mm mesh and air-dried. After drying, the samples were thoroughly mixed and passed through a 2 mm sieve. From each soil 16 portions of 60 g were taken. To 8 portions, 250 mg of powdered chitin (particle diam. 80–300 microns) was added and mixed through the dry soils; the remaining 8 portions were used for control experiments. Subsequently the soils were carefully moistened by means of a capillary pipette in order to prevent the formation of large crumbs. The moisture content of the sandy soils was adjusted to 10 %, that of the clay soil to 17 %. Then the samples were transferred to 500 ml Erlenmeyer flasks which were stoppered and incubated at 28 °C. At 3-day intervals, the flasks were aerated by means of compressed air, while the soils were gently shaken. The moisture content was kept constant during the experiments. At intervals of several days two flasks (containing soil samples with and without chitin, respectively) of each soil series were removed from the incubator and numbers of microorganisms were determined by the plate count method. During these experiments soil dilutions were not only plated on chitin agar, but also on a non-selective medium appropriate to meet the nutritional requirements of a relatively large number of microorganisms. The composition of this medium, an enriched soil extract agar, has been mentioned on p. 136.

A standard method was used for the determination of the numbers of microorganisms in the soils. Soil samples of 10 g were taken from appropriate Erlenmeyer flasks and added to Roux

flasks containing 500 ml of sterile tap water. The rubber-stoppered flasks were mounted on a wheel whose axis was inclined 45° from the vertical, rotating at 16 r.p.m. This motion imparted a gentle circulation to the flask contents. The flasks were allowed to rotate for 15 min. and dilution series were subsequently made from two replicate 1 ml samples of each soil suspension. From the final dilution of one series 4 samples of 0.1 ml were pipetted into Petri dishes containing solidified chitin agar, whereas four 0.1 ml samples taken from an appropriate dilution of the other series were pipetted onto enriched soil extract agar. The final dilutions were prepared so as to allow 100–300 colonies to develop on each plate (diam. 13 cm). Treatment of the plates was similar to that mentioned on p. 136. Statistical considerations have been given on p. 139.

Inasmuch as bacterial colonies generally developed more rapidly than the colonies of actinomycetes, it seems likely that even in cases in which colonies of actinomycetes were abundant on both media, the plate counts were not seriously affected by the presumably antibiotic activities of actinomycetes.

For various reasons it was impossible to take more than one sample at a time from each Erlenmeyer flask for the enumeration of microorganisms. On several occasions, however, control experiments have been carried out in which more samples were taken from each flask in order to determine the distribution of microorganisms in the soils. The results of a typical experiment, obtained with a sandy soil (No. 23) and a clay soil (No. 33) will be presented here.

Sixty g of each soil, prepared as described above and containing 250 mg powdered chitin, were incubated for 25 days. From each soil 3 samples of 10 g were taken; each sample was then added to a Roux flask containing 500 ml of sterile tap water. The flasks were rotated for 15 min. and subsequently two dilution series were made from each soil suspension, one for the inoculation of chitin agar, the other for that of enriched soil extract agar. Four replicate plates of each medium were prepared. The counts were as follows:

α. Sandy soil. Plate counts, enriched soil extract agar	Mean
230, 207, 211, 216	216
212, 225, 230, 222	222
207, 190, 217, 192	202
Plate counts, chitin agar	
162, 148, 143, 103	139
157, 158, 162, 156	158
148, 137, 146, 136	142
β. Clay soil. Plate counts, enriched soil extract agar	
199, 225, 218, 214	214
199, 187, 212, 191	197
214, 190, 200, 185	197
Plate counts, chitin agar	
160, 150, 147, 167	156
143, 150, 175, 140	152
156, 162, 148, 146	153

These figures indicate that the distribution of microorganisms in the soils showed a satisfactory homogeneity. The colonies of bacteria and actinomycetes were counted separately on both media to determine the relative incidence of both groups of microorganisms. Especially on chitin agar, the colonies of actinomycetes can be easily distinguished from those of bacteria. In most cases, the formation of an aerial mycelium by actinomycetes on this medium is more pronounced than on the enriched soil extract agar. Though on the latter medium the greater part of the actinomycetes form a typical, compact colony which is hard to disperse, some strains (*Nocardia*) form colonies which to the naked eye are not discernable from those of bacteria. The colony counts have been carried out with great care and with frequent microscopic controls.

In addition to the soil samples required for the enumeration of microorganisms, replicate samples were taken for the determination of dry weight, pH, nitrate and ammonia contents (cf. Chapter 5) of the soils. The pH of the soils was determined by shaking a 10 g sample of soil with 50 ml of distilled water. The suspension was allowed to stand overnight and the pH subsequently measured with a glass electrode.

These experiments were repeated three times in succession. The results of the three series showed the same trend. Results of a typical series of experiments will be given below.

b. Results

From figures 12, 13 and 14 it may be seen that the addition of chitin to different soils results in a considerable increase of the microbial population,

compared to relatively small changes in the population in soils without chitin. The numbers of microorganisms found on the first day of the experiment as given here are different from those present in the fresh soils (cf. fig. 10) because of the pretreatment of the soils.

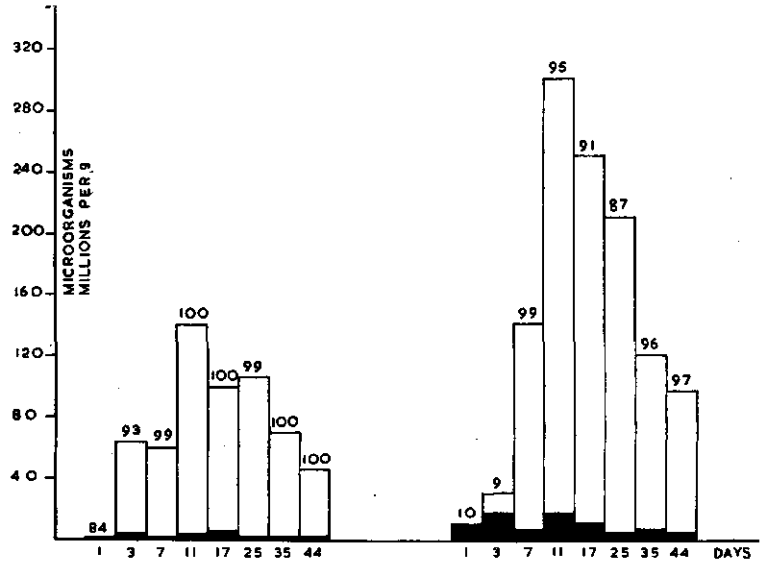

FIG. 12. Effect of chitin addition to soil No. 37. Left figure: chitinovorous microorganisms. Right figure: 'total' microorganisms. White columns: soil with chitin. Black columns: soil without chitin. Figures above white columns refer to percentage actinomycetes in soil with added chitin.

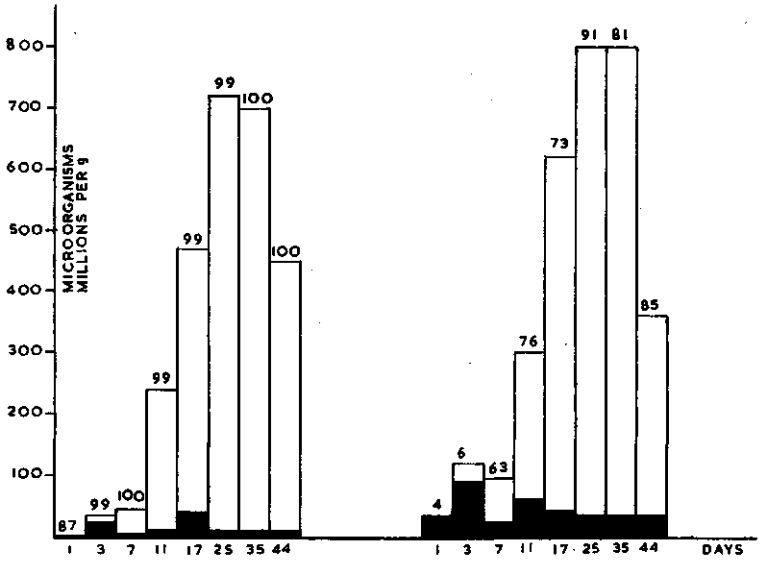

FIG. 13. Effect of chitin addition to soil No. 33. Left figure: chitinovorous microorganisms. Right figure: 'total' microorganisms. White columns: soil with chitin. Black columns: soil without chitin. Figures above white columns refer to percentage actinomycetes in soil with added chitin.

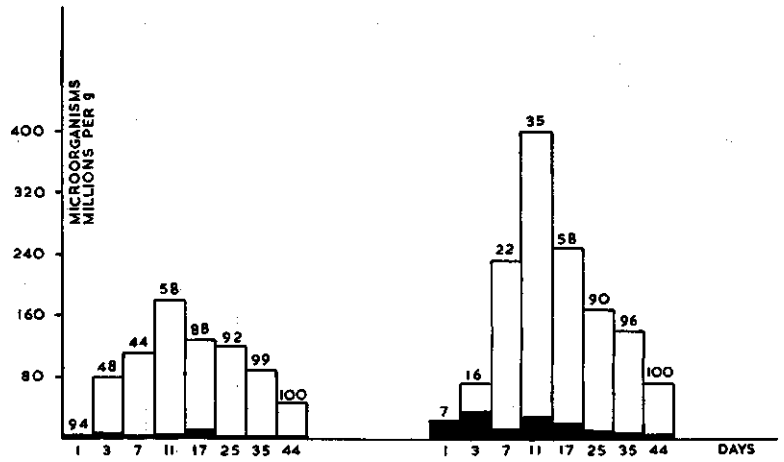

FIG. 14. Effect of chitin addition to soil No. 23. Left figure: chitinovorous microorganisms. Right figure: 'total' microorganisms. White columns: soil with chitin. Black columns: soil without chitin. Figures above white columns refer to percentage actinomycetes in soil with added chitin.

The maximum numbers of microorganisms are found 11 days after initiating the experiments with the sandy soils to which chitin had been added; for clay soil this interval is 25 days. In the latter soil, the maximum numbers of chitinovorous, as well as 'total' microorganisms, are considerably higher than those found for the sandy soils. In these soils the maximum number of total microorganisms, as found by plate counts from the enriched soil extract agar, are about twice the numbers of chitinovorous microorganisms. This was a surprising fact for soil No. 37, since it appeared that here nearly the whole soil population consisted of actinomycetes of the *Streptomyces* type. From fig. 12 it could be concluded that about half of the total number of actinomycetes in the soil could not attack chitin. In order to investigate this point further, a large number of actinomycete colonies, found on the enriched soil extract agar during the period between 11 and 44 days after beginning the experiment, were transferred to chitin agar. It appeared that 90–100 % of the transferred colonies were capable of chitin decomposition. Thus it must be concluded that about half of the actinomycete germs plated out on chitin agar, did not succeed in developing a colony. As it is well known that mycelial fragments of actinomycetes are less viable than spores, it seems likely that only the spores were able to germinate on chitin agar, whereas on the enriched soil extract agar containing a large variety of soluble nutrients, the spores as well as a large part of mycelial fragments were able to form colonies. It should be noted that this phenomenon was not observed in the first stage of the experiment, when the number of actinomycetes observed on chitin agar was greater than that on the enriched soil extract agar. Even though this phenomenon has not been studied in detail, it may be stated that a greater diversity of actinomycete colonies was observed on chitin agar during the first stage of the experiment. This suggests that perhaps part of these diverse actinomycetes was not able to grow on the enriched soil extract agar, contrasted to those which afterwards multiplied rapidly on this medium.

In the experiment with the clay soil (No. 33) too, actinomycetes form the

greater part of the microbial population, already during the early stages of the experiment. No great difference has been observed, however, in the numbers of actinomycetes on both media at the moment of maximal development of the microbial population. It must be noted, however, that in the experiment with the clay soil the composition of the actinomycete flora showed a remarkable change during the course of the experiment. When counting the colonies on the enriched soil extract agar plates during the first week of the experiment, it was observed that some *Nocardia* colonies were present on these plates. The white colonies were moist and could be easily taken up with a platinum loop. It could then be seen that part of the colony had been growing into the agar. This part of the colony showed the typical actinomycete hyphae, whereas the moist part of the colony consisted of coccoid cells. The numbers of this colony type were increasing gradually during the course of the experiment and at the moment when the maximum number of microorganisms in the clay soil was observed, they formed the greater part of the colonies observed on the enriched soil extract agar. When such colonies were transferred to chitin agar, decomposition of chitin could be observed after several days as a clear zone around the colonies. On chitin agar too, no aerial mycelium could be observed. The fact that no great difference has been found between the plate counts of chitin agar and enriched soil extract agar in this case may be at least partly due to the abundance of the *Nocardia* strain in the clay soil.[1]) A phenomenon common to both soils No. 33 and No. 37, however, was the increase of the microbial population as a result of chitin addition which was almost entirely due to multiplication of chitinovorous actinomycetes. At the end of the experiment, actinomycetes formed 30 % of 'total' microorganisms and 100 % of chitinovorous organisms in soil No. 33 to which no chitin had been added. For soil No. 37, these figures were 40 % and 100 %, respectively.

A different result has been obtained in the experiment carried out with soil No. 23 (fig. 14) which had been previously treated only with synthetic fertilizers. While as before, actinomycetes become the major part of the population of microorganisms when chitin had been added to the soil, the bacterial flora on the other hand remained an integral part of this population for a long time. When the soil had been incubated for 11 days, the maximum number of chitinovorous as well as 'total' microorganisms was found. At this time only 35 % of the colonies observed on the enriched soil extract agar appeared to be colonies of actinomycetes. When transferred from this medium to chitin agar, a considerable part of the bacterial colonies showed no chitin-decomposing ability. This indicates that the breakdown of chitin in this soil results in an increase of both chitinovorous and non-chitinovorous bacteria.

During the period of decrease in numbers of microorganisms, actinomycetes became the predominant part of the population. At this time, when the actinomycete colonies formed the major part of the colonies observed on the enriched soil extract agar, a large number of these colonies were transferred to chitin agar. It appeared that 90–100 % of these colonies showed chitin decomposition after 7 days' incubation.

In order to investigate whether antibiotic activities of the actinomycetes could have played a role in their interrelationships with bacteria, the following experi-

[1]) The strain was deposited in the collection of the 'Centraal Bureau voor Schimmelcultures', at Baarn in the Netherlands.

ment was carried out. At the time when actinomycetes formed the predominant part of the microbial population, a soil sample was plated out on an agar medium of the following composition: glucose 0.1 g, K_2HPO_4 0.05 g, $MgSO_4$ 0.05 g, $(NH_4)_2SO_4$ 0.05 g and agar 2 g, per 100 ml of soil extract; pH 7. The soil extract was prepared by boiling 50 g of the same soil with 100 ml of tap water, followed by filtration. After 4 days' incubation, a number of actinomycete colonies could be observed on the plates. These were then flooded with melted agar suspensions of the test organisms, separate series being tested with either *Escherichia coli*, *Bacillus subtilis*, or *Pseudomonas chitinovorans*. The agar suspensions were prepared by seeding beef agar with the appropriate cultures, grown for 18 hours in beef broth at 30 °C. On the plates inoculated with *Bac. subtilis*, a number of actinomycete colonies were surrounded by a transparant zone leaving the rest of the plate covered with bacterial growth. The effect on the growth of *E. coli* and *Ps. chitinovorans* was far less pronounced.

As the actinomycetes were grown on a medium approximating that present in the soil, it seems likely that antibiotic activities of a number of actinomycetes have been affecting their relations with at least part of the bacterial population.

The same experiment has been carried out with the soils No. 33 and No. 37 and with results similar to those obtained with soil No. 23.

In the experiments described in this chapter, a relatively large amount of finely powdered chitin was added to different soils and the temperature and moisture content of these soils were kept constant for a long period. Although these conditions might never occur in nature, the actinomycetes undoubtedly play an important role in the decomposition of chitin under natural conditions.

The chitin particles which are naturally deposited in the soil are often much larger than those used in our experiments, and thus the surface which can be attacked by microbial enzymes is much smaller, resulting in a very slow breakdown. This has been proved by placing *Carabus* elytra (length 1.5 cm) in different soils, 1 cm below the surface. The soils were incubated at 28 °C and the moisture contents (sandy soils 10 %, clay soil 17 %) were kept constant. Though the elytra became partly covered with colonies of actinomycetes, they were still completely intact after 6 months' incubation. The breakdown of such substrates under the continually changing natural conditions, often less optimal than those used in our experiments, would undoubtedly be still slower.

c. Summary

The changes in the composition and size of the microbial population of relatively dry soils, resulting from addition of powdered chitin to these soils, appear to be different for each soil. It may be stated, however, that in all the soils studied, the changes resulted in a predominance of actinomycetes over bacteria. In two soils (a clay soil and a sandy soil) which had previously been treated with farmyard manure, actinomycetes formed the major part of chitinovorous as well as 'total' microorganisms soon after the addition of chitin.

A sandy soil previously treated only with inorganic fertilizer salts, showed an increase of bacterial as well as actinomycete population when chitin was added. This was followed by a period of decrease of microorganisms, in which actinomycetes became the predominant part of the population.

A number of actinomycetes present in soils to which chitin was added showed antagonistic activities against *Bac. subtilis*, whereas the growth of *E. coli* and of *Ps. chitinovorans* was only slightly inhibited by some of these actinomycetes.

Carabus elytra placed in different soils under optimal conditions for the development of actinomycetes became partly covered with colonies of actinomycetes, but were still intact after a period of 6 months. This indicates that the debris of insects deposited in soil is only slowly decomposed.

CHAPTER 5

NITRIFICATION AS A RESULT OF CHITIN DECOMPOSITION IN SOIL

1. Introduction

Addition of nitrogenous substances to soil often results in the liberation of ammonia, followed by microbial conversion to nitrate. The fate of the nitrogen present in an organic substance in soil is at least partly determined by the C/N ratio of that substance. A rapid microbial decomposition of a carbon compound can only take place when enough nitrogen is available for microbial multiplication. Thus, when a rapid decomposition of a substance like straw (with C/N ratios as high as 200) is required, additional nitrogen is needed which enables microorganisms to multiply rapidly. On the other hand, when the C/N ratio of a substance is low, only part of the nitrogen of that substance is needed for microbial growth requirements, and possibilities for liberation of ammonia then are present.

On mixing 18 different amino acids with soil, Owen et al. (1950) observed a highly significant correlation between the C/N ratio and the formation of both ammonia and nitrate. From an amino acid with a C/N ratio of about 8, only 60 % of the nitrogen could be recovered as nitrate, whereas the decomposition of amino acids with C/N ratio of about 2 resulted in the formation of nitrate containing nearly 90 % of the nitrogen originally present in the amino acids.

As the C/N ratio of chitin is 6.8, it might be expected that decomposition of chitin in soil would result in the liberation of a considerable part of the chitin-N as ammonia which could subsequently be nitrified. This has been confirmed by investigations of Jensen (1932) and Bremner and Shaw (1954) who found that about half of the nitrogen originally present in chitin could be recovered as nitrate. We have obtained similar results which will therefore be presented only briefly.

2. Methods

The details of our experiments with water saturated and relatively dry soils have been described in Chapter 4. During experiments with soil percolators, samples were taken at regular intervals from the reservoir fluids in order to determine the nitrate and ammonia content. In experiments with the relatively dry soils, 30 g soil samples were extracted with 100 ml of an 1 N K_2SO_4 solution containing sufficient H_2SO_4 to give a reaction of pH 1.0–1.5 after interaction with the soil. The soil samples were then further washed with two 50 ml portions of the same solution, and aliquots of the combined and filtered extract were assayed for ammonia and nitrate.

a. Determination of ammonia

The microdiffusion method (Conway 1950) was used for this determination; 1 ml of boric acid (1 % sol. containing bromcresolgreen-methylred indicator, brought to the desired end-point colour) was introduced in the central chamber of a Conway unit as modified by Lips

(1937). In the outer chamber were run 1 ml of soil extract and 1 ml of 40% KOH, respectively. After applying the fixative described by BONTING (1951) to the lid, the unit was closed and soil extract and alkali were tipped together. After the units had remained overnight at 25 °C, the fluid in the central chamber was titrated with 0.02 N HCl.

Known amounts of ammonia added to soil extracts (up to 200 micrograms per ml) could be easily recovered within two per cent accuracy.

b. Determination of nitrate

This determination was carried out according to the method of BLOM and TRESCHOW (1929) as extensively described by GALESLOOT (1946). The method depends on nitrating 2,4-xylenol to o-nitro-xylenol which is distilled into a NaOH solution. The intensity of the yellow colour which develops in this solution is determined colorimetrically. When plotting nitrate concentrations against extinction values, a straight line is obtained up to concentrations of about 1 mg KNO_3 (fig. 15).

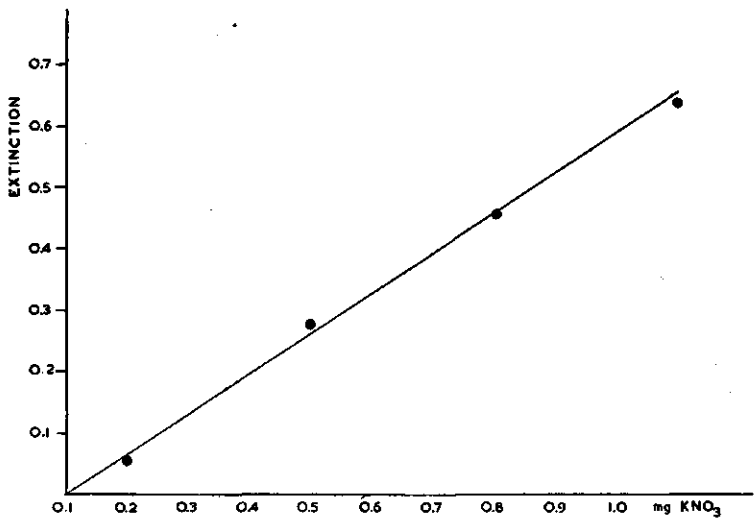

FIG. 15. Standard curve for nitrate determinations.

The nitrating reaction does not go to completion, and the extent of the reaction may vary from one xylenol preparation to another. It was therefore recommended that the same preparation be used throughout a series of experiments.

Determinations were carried out as follows: To a 500 ml flask were added: 10 ml of the acid soil extract or reservoir fluid (when 10 ml contained more than 140 μg NO_3-N, smaller samples were taken and distilled water added up to 10 ml), 50 ml 78% H_2SO_4 and 0.1 ml xylenol (10% sol. (w/v) in 50% alcohol). The flask was stoppered, shaken, and placed in an incubator at 30 °C for 60 min. Then 150 ml of distilled water was added and the o-nitroxylenol distilled into a 100 ml volumetric flask containing 25 ml of 0.2 N NaOH. About 70 ml of distillate was collected and made up to 100 ml with distilled water. The flasks were allowed to stand for 30 min. and subsequently the extinction of the liquid was measured in a Kipp colorimeter, using 3 cm cuvettes and an S 47 filter. The amount of nitrate originally present in the soil extract sample could then be determined with reference to the standard curve.

Nitrite was present in the soil extracts in only negligible amounts and was not therefore removed before the nitrate determinations.

Known amounts of KNO_3, added to extracts of different soils could be recovered with about two percent accuracy.

Before calculating the percentage of chitin-N recovered as nitrate, the amounts of nitrate found in control experiments with soils containing no chitin were subtracted from those found in experiments in which chitin was added.

3. Results

Results obtained with water saturated soils and relatively dry soils are represented in figures 16 and 17. The data given in figure 17 are obtained from the same experiment as those of figures 12, 13 and 14.

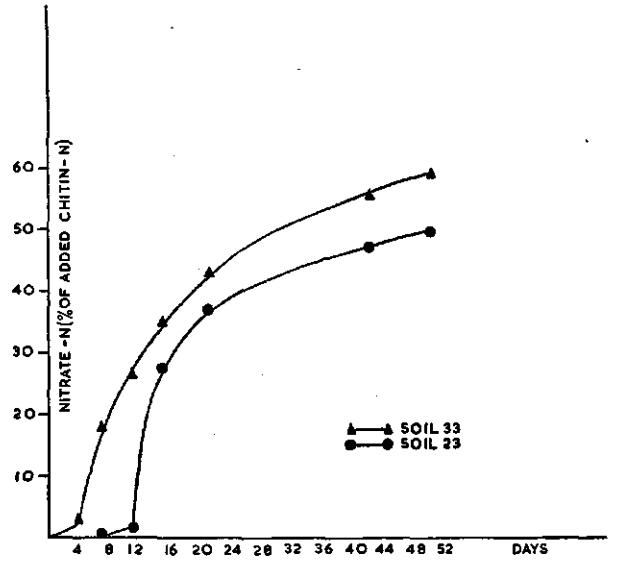

Fig. 16. Percentage recoveries of added chitin-N as nitrate-N after various periods of incubation of the water saturated soils No. 33 and No. 23.

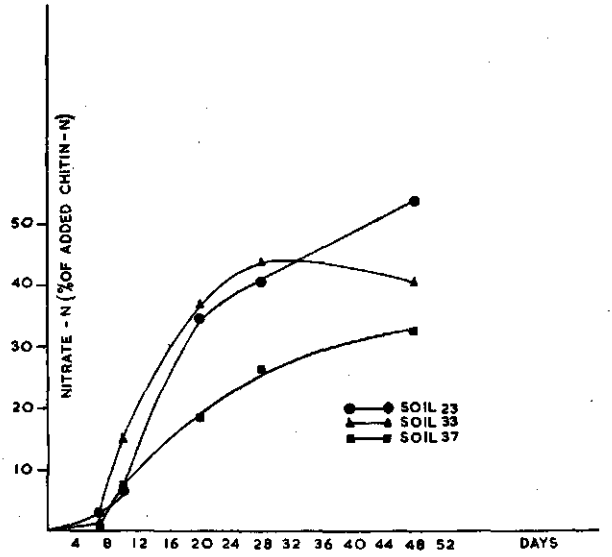

Fig. 17. Percentage recoveries of added chitin-N as nitrate-N after various periods of incubation of the relatively dry soils No. 23, No. 33 and No. 37.

From figures 16 and 17 it may be seen that a considerable part of the nitrogen originally present in chitin could be recovered as nitrate. A relatively rapid accumulation of nitrate could generally be observed up to about 3 weeks after the start of the experiment, followed by a period of slower nitrate accumulation. As during the latter period a rapid fall in numbers of microorganisms could be observed, it seems likely that the nitrate formed during this period originated from the oxidation of ammonia liberated during decomposition of cell proteins. Thus this part of the inorganic nitrogen was presumably derived indirectly from chitin-N. During experiments with the relatively dry clay soil (No. 33), a slight decrease in the nitrate content of the soil could be observed towards the end of the experiment, whereas the maximum nitrate content was associated with maximum development of the microbial population (cf. fig. 13, Chapter 4).

Relatively little chitin-N could be recovered as nitrate in experiments with soil No. 37. During experiments carried out with the soil perfusion technique, not more than 1–5 % of the nitrogen originally present in chitin could be recovered as nitrate, even though a rapid development of chitinovorous bacteria could be observed for about 3 weeks after starting the experiment. Though a greater accumulation of inorganic N could be observed in soil No. 37 when it was dry, even this accumulation was less than that observed in experiments with other soils.

Accumulation of ammonia during chitin decomposition has been observed neither in the reservoir fluids of the percolators, nor in the relatively dry soils.

4. Summary

Decomposition of powdered chitin in different soils results in an accumulation of nitrate. The percentage of chitin-N which can be recovered as nitrate is determined by type of soil and by soil conditions. Up to 60 % of the nitrogen originally present in chitin has been recovered as nitrate-N.

CHAPTER 6

HYDROLYSIS OF CHITIN BY BACTERIAL ENZYMES

1. Introduction

In our studies of chitin decomposition in soil, merely indirect evidence as to this decomposition has been collected. Toward a more direct analysis of chitin breakdown and its intermediary products, experiments have been carried out with pure cultures of chitinovorous microorganisms.

An extensive literature exists on the microbial degradation of cellulose, which is closely related to chitin. It appears that hydrolysis is the primary stage in microbial cellulose breakdown (cf. Nord and Vitucci, 1948). Evidence has been obtained that cellulose is hydrolysed in two stages with cellobiose as a first intermediate, subsequently split to glucose. In view of these results it might be expected that the degradation of chitin by microorganisms might involve a sequence of hydrolyses resulting in the ultimate formation of N-acetylglucosamine. This seemed particularly likely since chitinovorous microorganisms grown on chitin agar show a clear zone around their colonies, indicating a breakdown of chitin by extracellular enzymes.

At the time when our investigations were started, no information was available about intermediary products in microbial chitin breakdown. It might be mentioned, however, that KARRER and HOFMANN (1929) working with hepatopancreas juice of the vineyard snail (*Helix pomatia*) succeeded in hydrolysing chitin and obtained a high yield of N-acetylglucosamine. This observation has recently been confirmed by HACKMAN (1954). ZECHMEISTER et al. (1939) showed that hepatopancreas extracts of *Helix pomatia* contained a chitinase as well as a chitobiase.

Our investigations have been carried out with two bacterial strains, *Pseudomonas chitinovorans*, strain 8500 (cf. p. 135), and *Cytophaga johnsonae* STANIER, both of which have been grown in a medium containing chitin as the sole source of C and N.

2. METHODS

Each of seven 1 l Erlenmeyer flasks containing 100 ml of the following medium: chitin (finely ground; particle diam. 5–80 microns) 1 g, K_2HPO_4 0.1 g, $MgSO_4$ 0.1 g; pH 7) were inoculated with 1 ml of a cell suspension of one of the strains. This suspension was prepared by washing cells from agar slants (composition see Chapter 2, p. 135) with sterile water. The cells were harvested after 24 hours' incubation at 30 °C. The Erlenmeyer flasks were placed at 30 °C on a reciprocating shaker run at 92 cycles per minute. The chitin particles tend to adhere to the walls of the flasks and were resuspended at several hour' intervals. At successive stages of chitin decomposition, a flask was removed from the shaker in order to analyse the culture liquid. Viable cell counts were made as described in Chapter 7 (p. 155). The pH of the liquids was determined using a glass-electrode potentiometer and for chromatographic analysis the liquids were filtered through a Jena 17G5 bacterial filter and concentrated *in vacuo* down to 4–5 ml. Paper chromatograms were made using Whatman No. 1 filter paper and collidine (for separation of amino sugars) and phenol (chiefly used in search for glucose) as solvents. The solvents were distilled before use; phenol distillations were carried out as described by DRAPER and POLLARD (1949) and subsequently the water content was adjusted to 25% (w/w) Collidine was saturated with water at room temperature. Phenol runs were carried out in an atmosphere containing NH_3 and HCN. For this purpose the aqueous layer in the bottom of the chamber was brought to a 1% (w/v) concentration with NH_3 solution and several crystals of KCN were added. For purposes of comparison, N-acetylglucosamine, glucosamine-HCl and glucose were included on each chromatogram. Use was made of the following spotting reagents: ninhydrin (0.1% sol. in chloroform), aniline hydrogen phtalate (PARTRIDGE 1949) and the hexosamine reagents (PARTRIDGE 1948). The latter reagents consist of the alkaline acetylacetone reagent and the p-dimethylaminobenzaldehyde reagent. They were prepared and used as described by PARTRIDGE. Sprayed with these reagents, glucosamine and N-acetylglucosamine gave pink and violet spots, respectively. Spots of N-acetylglucosamine could also be revealed by spraying with the p-dimethylaminobenzaldehyde reagent alone; this was used as a confirmatory test for N-acetylglucosamine. The colour of the spots, however, was more pronounced when the paper was sprayed with the alkaline acetylacetone reagent before treatment with the p-dimethylaminobenzaldehyde reagent. This appeared to be due to the presence of alkali and not to that of acetylacetone in the former reagent.

Acetic acid was determined qualitatively only. On several occasions part of the culture liquid was steam distilled. A spot test was carried out on the distillate with lanthanum nitrate and iodine as described by FEIGL (1947). Moreover, acetic acid was determined by chromatographic analysis of the steam distillate by the method of NIJKAMP (1951). According to this method, fatty acids may be separated on a column of silica gel, using methanol as the immobile solvent, iso-octane as the mobile solvent, and bromcresol green as the indicator.[1])

Qualitative tests for the presence of ammonia have been carried out on destillates of culture liquids. After removal of bacteria by filtration MgO was added and the ammonia distilled off *in vacuo* at 40 °C. Nessler's reagent was used for the ammonia assay according to FEIGL (1947).

Manometric techniques. Manometric experiments have been carried out in order to study the oxidation of glucose, N-acetylglucosamine and acetic acid by *Pseudomonas chitinovorans*,

[1]) The author is indebted to Ir W. BEYER, who carried out the determinations of acetic acid by this method.

strain 8500. The conventional Warburg apparatus was used at a temperature of 30 °C. The Warburg flask ingredients were: cell suspension 3 ml, substrate 0.5 ml and KOH (40%) 0.15 ml in the center cup. The cells used were grown in 6 l Erlenmeyer flasks containing 1000 ml medium of the following composition: yeast extract Difco 3.5 g, glucose 1 g, $MgSO_4$ 1 g, Na_2HPO_4 8.31 g and KH_2PO_4 2.72 g per liter. The high concentration of phosphate appeared to reduce the endogenous respiration of the cells. The cultures were incubated for 24 hours at 30 °C with continuous shaking. The cells were then harvested by centrifugation (Cepa supercentrifuge; 150,000 × G), washed twice and resuspended in M/15 phosphate buffer, pH 7.0, to a standard turbidity; 1 ml of suspension added to 9 ml of water gave 40% transmittance in a Kipp colorimeter at 420 mμ against a distilled water blank.

3. Results

From Tables 1 and 2 it may be seen that N-acetylglucosamine and glucosamine are to be found in the culture liquid concentrates of both strains up to the end of the logarithmic growth phase.

TABLE 1

Chitin decomposition by *Pseudomonas chitinovorans*, strain 8500.
NAG = N-acetylglucosamine; GA = glucosamine.

Incubation time, hours	pH of medium	Numbers of microorganisms	Result of chromatogr. analysis
0	7.2	$1.0 \cdot 10^7$	
8	7.2	$3.4 \cdot 10^8$	NAG
13	6.9	$1.3 \cdot 10^{10}$	NAG
20	6.0	$3.1 \cdot 10^{11}$	NAG, GA
26	6.3	$6.0 \cdot 10^{11}$	NAG, GA
37	6.7	$3.3 \cdot 10^{12}$	NAG, GA
48	7.0	$3.0 \cdot 10^{11}$	

TABLE 2

Chitin decomposition by *Cytophaga johnsonae* Stanier.
NAG = N-acetylglucosamine; GA = glucosamine.

Incubation time, days	pH of medium	Numbers of microorganisms	Result of chromatogr. analysis
0	7.2	$3.6 \cdot 10^8$	
2	7.2	$7.0 \cdot 10^{10}$	NAG, GA
4	7.6	$1.7 \cdot 10^{12}$	NAG, GA
6	8.7	$2.7 \cdot 10^{10}$	

N-acetylglucosamine as well as glucosamine could be easily detected by paper chromatographic analysis, even though the concentrations (especially of glucosamine) were very low as shown by the colour intensity and dimensions of the spots. An accumulation of both substances in the culture liquids could be obtained by checking bacterial metabolism in the logarithmic growth phase. During this phase, cultures were removed from the shaker and placed under anaerobic conditions (nitrogen atmosphere), or at a temperature of 45 °C. The flasks were allowed to stand for 24 hours and then tested for the presence of N-acetylglucosamine and glucosamine. It appeared that both substances had accumulated, indicating that hydrolysis of chitin and subsequent deacetylation of N-acetylglucosamine had continued under conditions in which the bacterial metabolism was inhibited. The same result could be obtained still more easily by

removal of the cultures from the shaker and letting them stand at room temperature for 24 hours.

Tests for the presence of acetic acid in culture liquids containing N-acetylglucosamine and glucosamine were positive, indicating that N-acetylglucosamine is deacetylated to yield glucosamine and acetic acid.

Manometric experiments showed that addition of glucose, as well as N-acetylglucosamine and acetic acid, to suspensions of glucose grown cells, results in a rapid oxygen uptake, the initial rate of uptake being nearly the same for the three substrates (fig. 18).

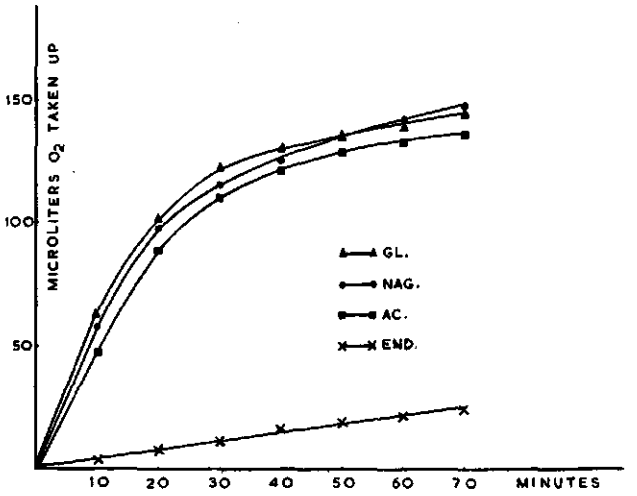

FIG. 18. Respiration curves for glucose-grown cells of *Pseudomonas chitinovorans*, strain 8500. Substrates: 0.5 ml 0.008 M acetic acid, 0.5 ml 0.004 M of both glucose and N-acetylglucosamine were added to 3 ml of bacterial suspension.

The amounts of oxygen consumed by *Pseudomonas chitinovorans* in the manometric experiment were 32 %, 38 % and 58 % of the theoretical amount for the complete oxidation of N-acetylglucosamine, glucose and acetic acid, respectively.

The presence of ammonia in the culture liquids during chitin decomposition makes it probable that glucosamine is deaminated, but attempts to detect glucose in the culture liquids failed. The search for glucose was extended to experiments with suspensions of glucose-grown cells (preparation see p. 152) of both strains, to which glucosamine hydrochloride was added; 0.1 g of this substance was added to 100 ml Erlenmeyer flasks containing 10 ml of bacterial suspension. These flasks were placed at 30 °C on a reciprocating shaker run at 92 cycles per minute. Although a considerable amount of ammonia was formed in the culture liquids during several hours' incubation, no glucose could be detected in liquids, freed from bacteria by filtration and concentrated *in vacuo*.

Thus, from our experiments it cannot be determined whether glucosamine is deaminated directly, or whether the glucosamine undergoes other changes before deamination. Evidence for the latter possibility was obtained by LUTWAK-MANN (1941), who found that oxidation preceded deamination during the breakdown of amino sugars by bacteria, yeast and enzymes from animal tissues.

The breakdown of N-acetylglucosamine by streptococci was studied by ROGERS (1949). These organisms yielded approximately 1 mol. of ammonia, 1 equivalent acetic acid and from 1.2–1.7 mol. of lactic acid per mol. sugar. The amount of lactic acid produced from glucose and N-acetylglucosamine appeared to be closely similar for the two substrates. Evidence was obtained that glycolysis and deamination were closely related.

Another unsolved problem must be mentioned. On several occasions chromatographic analysis of concentrated culture liquids showed the presence of 4 substances; two of them could be identified as N-acetylglucosamine and glucosamine, the others remained unidentified. The latter spots moved faster than those of N-acetylglucosamine and glucosamine (Rf values of unknown spots 0.73 and 0.88, using collidine as the solvent) and showed the same colour reactions; one spot being pink like that of glucosamine, the other mauve coloured like N-acetylglucosamine, when sprayed with the hexosamine reagents. Presumably the fast moving substances are products of chitin breakdown other than N-acetylglucosamine and glucosamine, one of them deacetylated, the other not. Both unknown substances could be traced in cultures of *Pseudomonas chitinovorans* as well as in cultures of *Cytophaga johnsonae* STANIER.

The above mentioned results have already been published in brief (VELDKAMP 1952). A recent paper by REYNOLDS (1954) describes the breakdown of chitin brought about by a concentrate of extracellular enzymes from a *Streptomyces* culture. The products of this enzymic degradation of chitin were identified as N-acetylglucosamine and its corresponding disaccharide N.N-diacetylchitobiose, indicating the presence of a chitobiase as well as a chitinase. Chitin hydrolysing enzymes could only be obtained from cultures grown in a chitin medium; the presence of chitin apparently induced the formation of these enzymes.

4. SUMMARY

In chitin cultures of *Pseudomonas chitinovorans*, strain 8500, and *Cytophaga johnsonae* STANIER, N-acetylglucosamine as well as glucosamine, acetic acid, and ammonia could be traced. This indicates that chitin is hydrolysed by bacterial enzymes, the resulting N-acetylglucosamine being subsequently deacetylated with the formation of glucosamine and acetic acid. The question of whether the ammonia found in the cultures was derived from glucosamine directly or from a breakdown product of glucosamine remained unsolved. Two unknown breakdown products of chitin were detected chromatographically in culture liquids of both strains, and their Rf values characterized.

CHAPTER 7

AEROBIC BREAKDOWN OF CHITIN AND GLUCOSE BY *PSEUDOMONAS CHITINOVORANS*, STRAIN 8676

1. INTRODUCTION

During preliminary experiments with *Pseudomonas chitinovorans*, a mutant of the original strain was isolated (cf. Chapter 2). This mutant differs from the parental strain in that it produces considerably more acid from chitin. When the

original strain was seeded into a liquid medium containing 1 % chitin (finely ground), 0.1 % K_2HPO_4 and 0.1 % $MgSO_4$, rapid growth followed and the pH fell to 6.0 (initial pH 7.2); subsequently a rise of pH to about 7, and a complete disappearance of chitin could be observed.

With the mutant strain, however, a fall of pH to 4.0 was observed within 20 hours after inoculation. Subsequently bacterial growth and CO_2 production ceased. The bacteria which in the beginning were actively motile, became non-motile at this low pH. A considerable amount of chitin was still present at this time and an accumulation of acetylglucosamine could be observed. Apparently bacterial activities were inhibited by the acid accumulation. Since it was thought interesting to identify the acid(s), this mutant strain was used for more detailed investigations.

Since the bacterial breakdown of chitin involves the production of intermediates closely related to glucose, the aerobic breakdown of chitin will be compared with that of glucose.

2. Cultural methods

Three media were used in the study of glucose and chitin breakdown.

The chitin medium contained: chitin 1 g (finely ground; particle diam. 5–80 microns; N content 6.9 %), K_2HPO_4 0.1 g, $MgSO_4$ 0.1 g and $CaCO_3$ 1 g per 100 ml.

The first glucose medium had the following composition: glucose 1 g, $CaCO_3$ 1 g, K_2HPO_4 0.1 g, $MgSO_4$ 0.1 g and $(NH_4)_2SO_4$ 0.35 g per 100 ml.

The second glucose medium differed from the first only in the concentration of ammonium sulphate, i.e., 0.1 g per 100 ml in the second medium. One liter of culture medium (complete except $CaCO_3$) was prepared for every experiment, and 100 ml of this was added to each of nine 1 l Erlenmeyer flasks. Then exactly 1 g $CaCO_3$ was added to each flask and the media were sterilized for 20 min. at 110 °C. Just before inoculation the temperature of the media was brought to 30 °C. Each of the nine Erlenmeyer flasks was inoculated with 1 ml of bacterial suspension. This suspension was prepared by washing bacterial cells with sterile tap water from two agar slants (composition mentioned on p. 135). The cells were harvested after 16 hours' incubation at 30 °C. As these young cultures were easily washed into the water, a homogeneous suspension ($\pm$ 12 ml) could be obtained in this way. Furthermore, it appeared that the numbers of cells added to the Erlenmeyer flasks were approximately the same from experiment to experiment; thus in the determinations of the initial number of viable cells, the same dilution series could be used in all experiments.

One Erlenmeyer flask was used for blank analysis: determination of the exact amount of glucose or chitin, $CaCO_3$ and carbon dioxide dissolved in culture liquid, initial number of viable cells, and liquid volume after sterilisation. The other flasks were placed at 30 °C on a reciprocating shaker run at 92 cycles per minute. The rubber stoppers of the flasks were provided with two cotton plugged glass tubes by means of which each flask could be connected into an 'aeration' train. The inlet tube reached the liquid surface; the outlet tube opened close under the stopper. Sterile, carbon dioxide-free air was carried over the culture liquids at a rate of 25 ml per minute. The air entering the culture flasks was freed from microorganisms by filtration through cotton and freed from carbon dioxide by consecutive passage through two spiral gas-washing bottles, filled with 30 % KOH and saturated $Ba(OH)_2$ solution respectively.

The air leaving the culture flasks carried the carbon dioxide formed in the utilization of glucose or chitin through a Drechsel gas-washing bottle filled with concentrated H_2SO_4 to remove moisture, and then the carbon dioxide was collected in an ascarite tube. The first half of this tube contained ascarite (mixed up with pumice stone to prevent clogging), the second half was filled with $CaCl_2$. Finally the air passed through an U-tube partly filled with concentrated H_2SO_4 and functioning as a bubble counter. At intervals of several hours, a culture flask was removed from the shaker in order to analyse the culture liquid.

Determination of the number of viable cells

This determination was carried out by the plate-count method. Before starting the chemical analysis of the culture-liquids, a 1 ml sample was taken from each culture liquid and from

this sample a dilution series was made; four 0.1 ml samples (giving 100–300 colonies per plate) were taken from the final dilution and pipetted with the same 0.1 ml pipette into sterile Petri dishes (diam. 13 cm) containing 25 ml of a solidified nutrient medium. This medium had the following percentage composition: glucose 1, yeast extract (Difco) 0.7, $CaCO_3$ 1, K_2HPO_4 0.1, $MgSO_4$ 0.1, agar 2. Each inoculum was distributed over the entire surface of the plate by means of a sterile, bent glass rod. Colonies were counted after two days incubation at 30 °C. Statistical considerations are similar to those mentioned on p. 139.

3. Chemical methods

a. Filter-paper partition chromatography

Qualitative analysis of organic acids and hydrazones of keto acids were carried out with the aid of single-dimensional paper chromatography. A chamber suitable for either one- or two-dimensional chromatography as described by Ylstra (1953) was used for this purpose. Whatman No. 1 filter paper has been used exclusively.

For the analysis of organic acids one of the solvents described by Cheftel, Munier and Macheboeuf (1951; cf. Munier 1952) was used. It consists of a mixture of n-butanol, methylbenzoate and formic acid in the following proportions: 50 ml – 50 ml – 20 ml respectively. To each 120 ml of this mixture 1.5 ml distilled water was added.

Due to the presence of methylbenzoate in the solvent mixture the papers could easily be freed from solvent, including formic acid, by drying them overnight at 60–70 °C. The dried strips were first sprayed with bromcresolgreen solution (40 mg per 100 ml ethanol 96%) in order to find total acids; after marking the spots, the papers were subsequently sprayed with a solution of 2,4-dinitrophenylhydrazine (0.1% in 2 N HCl) to detect the keto acids. Chromatograms for Rf reference were always run with one or more of the following known test acids: α-ketoglutaric acid, pyruvic acid and lactic acid. When a mixture of these acids is present, the spots are well-separated only when the length of the paper is at least 57 cm and the solvent has been running to the end of the paper.

The separation of 2,4-dinitrophenylhydrazones of α-keto acids was carried out as follows. A small amount of the hydrazone was dissolved in acetone and the yellow solution was transferred to the paper with a Pasteur pipette. The hydrazones of α-ketoglutaric acid and pyruvic acid were used as reference substances. It appeared to be very easy to separate a mixture of both hydrazones using n-butanol as a solvent (Cavallini, Frontali, Toschi, 1949), the spot of α-ketoglutaric acid-hydrazone moving much slower than the pyruvic acid-hydrazone. The latter resolved into two spots, one being very faint, the other always present in high concentration. As pointed out by Isherwood and Cruickshank (1954), this is due to the fact that each α-keto acid can give rise to two stereoisomeric hydrazones.

b. Determination of diacetyl

This substance never was produced in more than very small amounts, and was therefore determined only qualitatively, according to the method described by van Niel (1927).

Part of the culture liquid was slightly acidified with acetic acid and three fifths of this solution distilled off. The distillate was collected on a solution of diacetyl reagent, consisting of 2 ml of 20% hydroxylamine hydrochloride (neutralized), 2 ml of 20% sodium acetate and 1 ml of 10% nickelchloride. On standing, red crystalline needles of nickeldimethylglyoxime were formed whenever diacetyl was present in the culture liquid.

c. Determination of acetylmethylcarbinol

A method of determining acetylmethylcarbinol, depending upon its oxidation to diacetyl, has also been described by van Niel (1927), using the same diacetyl determination as described above. When both diacetyl and acetylmethylcarbinol were present in the culture liquid, a comparison was made of the amounts of the nickeldimethylglyoxime precipitates in oxidized and unoxidized samples. The van Niel method was used only as a qualitative test for acetylmethylcarbinol. Quantitative determinations were made according to the method of Langlykke and Peterson (1937). This method is based on the following properties of acetylmethylcarbinol: it can be distilled from aqueous solution at a definite rate which is independent of the concentration, and it reacts quantitatively with alkaline iodine to form iodoform.

Distilled water was added to an appropriate sample of culture liquid to bring the sample volume up to 100 ml. To the third quarter of distillate from this solution, 15 ml 1 N NaOH and 5 ml 0.2 N iodine were added. When acetylmethylcarbinol was present in the culture

liquid, iodoform precipitated, and after standing for 10–15 min., the excess iodine was liberated by the addition of 20 ml 1 N sulfuric acid and titrated with 0.1 N sodium thiosulphate solution.

d. Determination of lactic acid

Lactic acid was determined qualitatively by paper chromatography (cf. p. 156). Quantitative determinations were made according to the method of FRIEDEMANN and GRAESER (1933). Determinations were carried out on an aqueous dilution of an ether extract of an acidified sample from the culture liquid. The method depends on the oxidation of lactic acid to acetaldehyde which can be subsequently determined by one of the conventional aldehyde assays. The oxidation was carried out with colloidal 0.1 N MnO_2 in the presence of phosphoric acid – manganese sulphate reagent, prepared as described by FRIEDEMANN and GRAESER. The resulting acetaldehyde was distilled into a sodium bisulfite solution; when the distillation was complete, excess bisulfite was removed by a strong iodine-KI solution. The bound bisulfite was then liberated by alkali and titrated with standard iodine (0.01 N) to a starch end point.

Control determinations were carried out in distilled water to which small amounts of pyruvic acid (and, if necessary, of α-ketoglutaric acid) were added, approximating those likely to be present in the ether extracts of the culture liquids. Since the reaction of pyruvic acid with collodial MnO_2 gave only very small amounts of bisulfite-addition products, the control titrations remained very small. These control titrations were always subtracted before the results were calculated.

e. Determination of keto acids

Qualitative determinations

Three types of qualitative determinations were carried out.

1. Paper chromatography of the free acids: an acidified aliquot of culture liquid was extracted with ether (sodium distilled) in a Kutscher-Steudel continuous extractor for 18 hours. An aqueous solution of the extracts was analyzed by paper chromatography as described on p. 156.

2. Paper chromatography of the corresponding 2,4-dinitrophenylhydrazones: to the above mentioned aqueous extract 2,4-dinitrophenylhydrazine (sat. sol. in 2 N HCl) was added in excess. Two hours after the addition of the hydrazine, the precipitate was filtered on a Jena 1G4 crucible, washed with 2 N HCl and distilled water (till HCl-free) and dried. The paper chromatographic analysis of the precipitate was carried out as described above.

3. Separation and identification of the 2,4-dinitrophenylhydrazones: when pyruvic acid was the only detectable keto acid present in the culture liquid, its hydrazone could be prepared either in an aqueous solution of an ether extract of acidified culture liquid or in the culture liquid directly. In the latter case, 2 ml of concentrated HCl was added to 25 ml of culture liquid and the liquid was filtered through a Jena 17G5 bacterial filter. An excess of 2,4-dinitrophenylhydrazine (sat. sol. in 2 N HCl) was added to the clear filtrate. The solution was allowed to stand for two hours and the precipitate then filtered on a Jena 1G4 crucible, washed with 2 N HCl and distilled water (till HCl-free), dried and dissolved in 10% Na_2CO_3 solution. Then 2 N HCl was added and the precipitate washed free of HCl and dried. If necessary the hydrazone was again dissolved in 10% Na_2CO_3 solution and reprecipitated with 2 N HCl but in most cases only one recrystallisation appeared to be sufficient to obtain the pure hydrazone. The melting point of the hydrazone thus prepared was 217 °C (decomposition). The melting point of the 2,4-dinitrophenylhydrazone of the synthetic acid (217 °C) was not depressed by admixture with the derivative of the natural acid.

In only one experiment, both pyruvic acid and α-ketoglutaric acid were produced. In this case the pyruvic acid-hydrazone was isolated (as described above) when α-ketoglutaric acid was not yet present in the culture liquid. The hydrazone of α-ketoglutaric acid was prepared at the end of the experiment after pyruvic acid had disappeared from the culture liquid (cf. fig. 20 p. 162).

The preparation and isolation of the 2,4-dinitrophenylhydrazone of α-ketoglutaric acid was carried out as follows. The liquids of ten 100 ml cultures were concentrated *in vacuo*, acidified to pH 2 and extracted with ether (sodium distilled) in the Kutscher-Steudel continuous extractor for 18 hours. The hydrazone was prepared in the usual way and further purified as follows. After recrystallisation from water, the hydrazone was dissolved in a 10% Na_2CO_3 solution and reprecipitated with 2 N HCl. The precipitate (washed free of HCl) then was recrystallized from methanol-water. The hydrazone was subsequently dissolved in acetone and the liquid evaporated at 30 °C in a microcrucible. After evaporation, the residue adhering to the walls appeared as a broad layer of lemon yellow crystalline needles. These could easily be

separated from an orange-coloured impurity which formed a narrow zone on the upper parts of the wall. The crystals thus separated were treated in the same way several times and then finally recrystallized again from methanol-water. The melting point of this hydrazone was 220–221 °C (decomposition); the melting point of the 2,4-dinitrophenylhydrazone of pure α-ketoglutaric acid (221 °C) was not depressed by admixture with the derivative of the natural acid. Paper chromatographic analysis (cf. p. 156) showed that the hydrazone isolated from the culture liquid had the same Rf value as the hydrazone prepared from pure α-ketoglutaric acid.

Quantitative determinations

Total keto acids were determined by preparation and weighing of their 2,4-dinitrophenylhydrazones. Pyruvic acid and α-ketoglutaric acid were quantitatively converted into the corresponding 2,4-dinitrophenylhydrazones by reacting with an excess of 2,4-dinitrophenylhydrazine for 2 hours at room temperature. Glucose in the amounts used in our experiments gives no precipitate during this time. A correction had to be applied when acetoin was present in the culture liquid, but the calculated amounts of the 2,4-dinitrophenylhydrazone of acetoin were always very small.

In most cases the hydrazones were prepared directly in the bacteria-free culture liquid; 2 ml of concentrated HCl was added to a 25 ml sample of culture liquid which had been vigorously shaken before sampling in order to suspend the $CaCO_3$ precipitate. This precipitate contained small amounts of Ca-ketoglutarate whenever α-ketoglutaric acid was present in the culture liquid. The acidified liquid was filtered through a bacterial filter (Jena 17G5). Two hours after the addition of excess 2,4-dinitrophenylhydrazine (sat. sol. in 2 N HCl) to the clear filtrate, the hydrazone precipitate was filtered on a Jena 1G4 crucible, washed with 2 N HCl and distilled water (till HCl-free), dried at 100 °C and weighed. The results obtained in this way were nearly equal to those obtained in other experiments in which the acidified culture liquid was extracted with peroxide-free ether in a Kutscher-Steudel extractor, followed by preparation of the hydrazone in an aqueous solution of the ether extract.

In most experiments qualitative analysis showed that pyruvic acid was the only keto acid present in the culture liquid. When both pyruvic acid and α-ketoglutaric acid were present, the latter acid was determined separately by the method of KREBS (1938). The calculated weight of the corresponding 2,4-dinitrophenylhydrazone could subsequently be subtracted from total hydrazone-weight and thus the amount of pyruvic acid computed. The method of KREBS for the quantitative determination of α-ketoglutaric acid depends on the oxidation of the acid (or its 2,4-dinitrophenylhydrazone) with acid permanganate to succinic acid which can be subsequently determined in one of the conventional ways. In our experiments, the resulting succinic acid was determined quantitatively by the method of PHELPS et al. (1939) in which the acid is converted to Ag-succinate, followed by titration with KCNS.

Preparation and permanganate oxidation of the hydrazone were carried out as follows. A 25 ml sample of thoroughly shaken culture liquid was acidified and extracted with ether (sodium distilled) in the Kutscher-Steudel continuous extractor for 18 hours. Excess of 2,4-dinitrophenylhydrazine (sat. sol. in 2 N HCl) was added to an aqueous solution of the extract and the solution was allowed to stand for 30 min. Subsequently the hydrazone was extracted by shaking the solution twice with 1/5 vol. ether. The etherial solution was evaporated and the residue, containing hydrazone(s) and excess hydrazine was dissolved in ± 5 ml 2 N NaOH. The solution was washed quantitatively into the Kutscher-Steudel extractor and acidified with 50 % H_2SO_4 to about N; 4 ml 3 % $KMnO_4$ were then added.

One hour after the addition of permanganate, the succinic acid was extracted with ether in the continuous extractor for 18 hours. From control experiments several points were noted:

1. The hydrazones prepared from the culture liquids apparently were derivatives of either pyruvic acid or a mixture of this acid and α-ketoglutaric acid. Only the oxidation of the hydrazone of α-ketoglutaric acid yields succinic acid. A paper chromatographic control showed that succinic acid was the only acid present in the ether extract of the permanganate treated samples.

2. No succinic acid or ether soluble substances yielding succinic acid on permanganate oxidation, other than the hydrazone of α-ketoglutaric acid, were present in the culture liquids.

Therefore the shortened procedure described by KREBS was tried as follows. A 25 ml sample of thoroughly shaken culture liquid was acidified with 2 ml H_2SO_4 50%. The liquid was filtered through a bacterial filter (Jena 17 G 5) and washed quantitatively in a Kutscher-Steudel extractor; then 4 ml 3 % $KMnO_4$ were added. One hour after the addition of permanganate the liquid was extracted with ether for 18 hours.

Since the yield of succinic acid by this method was the same as that obtained by the oxidation of the hydrazone, the shortened procedure was used for all further determinations of α-

ketoglutaric acid. The succinic acid extracted from the permanganate treated culture liquids was determined qualitatively by paper chromatography (cf. p. 156) and quantitatively by the method of PHELPS et al. (1939), as follows. To an aqueous solution (about 15 ml) of the ether extract, 5 ml of 10% $AgNO_3$ were added; this solution was adjusted to pH 6.0–6.5 with 0.5 N NH_4OH and bromcresolpurple. The white precipitate of Ag succinate was filtered on a Jena 1G4 crucible and washed with four or five 3 ml portions of 50% alcohol. The precipitate was then dissolved in hot 1:4 HNO_3, and the solution cooled and titrated with 0.007 N KCNS and saturated ferric alum indicator.

Lactic acid does not interfere in the succinic acid determination because it is destroyed by permanganate oxidation.

f. Determination of cellular carbon

In order to determine the amount of carbon assimilated by the cells, cellular carbon determinations were carried out when the substrate (glucose or chitin) had been consumed.

To a centrifuge-combustion tube were added 1 ml 5 N H_2SO_4, 1 ml 10% Na-tungstate and 1 ml culture liquid. The contents of the tube were centrifuged at 3,000 rpm for 30 minutes. The clear supernatant was carefully removed and the firmly packed bacterial precipitate was combusted according to the method of VAN SLYKE and FOLCH (1940). By this method carbon compounds are oxidized in a mixture of chromic, iodic, sulphuric and phosphoric acids. The resulting carbon dioxide was absorbed in alkali solution in the VAN SLYKE-NEILL apparatus and subsequently determined manometrically, following the extensive instructions of VAN SLYKE and FOLCH. The carbon content of bacteria could then be calculated. A blank determination was carried out to determine the carbon content of the reagents used.

At the end of several experiments with glucose as substrate, the cellular carbon as well as the dry weight of the cells were determined. The dry weight was obtained as follows: 50 ml of culture liquid were centrifuged for several minutes to remove inorganic precipitates. The supernatant was then centrifuged at 3,000 rpm for one hour. The cell sediment was washed with distilled water, recentrifuged, dried under vacuum at 45 °C and weighed. It appeared that the carbon content as determined by the combustion method differed by less than 2% from one half the dry weight value.

g. Determination of glucose

A sample of culture liquid was filtered through a Jena 17G5 filter and the bacteria-free filtrate used for the determination of glucose by the method of LUFF as described by SCHOORL (1929).

The LUFF reagent is reduced only by substances containing one or more hydroxyl groups adjacent to the carbonyl group, and thus pyruvic acid (often present in considerable amounts in the culture medium) did not interfere in the glucose determinations. These were carried out as follows. To a 300 ml Erlenmeyer flask were added: 25 ml LUFF solution, 5 ml bacteria-free culture liquid (maximum glucose content 50 mg) and 20 ml distilled water. The solution was boiled for 10 min. (with reflux condensor) and cooled under the tap. Then 3 g KI and 25 ml 25% H_2SO_4 were added and the solution was titrated with 0.1 N thiosulphate to a starch endpoint.

A blank determination (25 ml LUFF solution + 25 ml distilled water) was carried out in the same way. The difference between the titration values was calculated and the corresponding amount of glucose could be found using the table given by SCHOORL.

h. Determination of chitin

The amount of chitin initially present in the culture medium was determined as follows. A 25 ml sample of thoroughly shaken culture liquid was acidified with 2 N HCl in order to remove $CaCO_3$. The medium was filtered on a Jena 1 G 4 crucible, washed with distilled water (till HCl-free), dried at 100 °C and weighed. No chitin determinations were carried out during the course of bacterial chitin decomposition.

i. Determination of carbon dioxide

The carbon dioxide formed by the cultures is a product of several processes. Part of it is produced as an end product of the aerobic dissimilation of chitin or glucose. Another part originates from the reaction between $CaCO_3$ and acids formed in the culture liquid. Not only are organic acids formed in the bacterial breakdown of the substrates but also the utilization of $(NH_4)_2SO_4$ (in experiments with glucose as substrate) leads to the formation of H_2SO_4.

The carbon dioxide formed in these different ways was collected for the most part in the previously mentioned ascarite tube (cf. p. 155) and determined gravimetrically. The carbon dioxide still dissolved in the culture liquid and the carbon dioxide present in the $CaCO_3$ was subsequently determined by acidification of an aliquot from the culture liquid and collection of the resulting carbon dioxide in another ascarite tube. Thus total carbon dioxide was determined for each culture flask during the course of substrate decomposition. It must be noted, however, that a very small amount of carbon dioxide, present in the air between culture liquid and ascarite tube at the moment of removing the culture flask from the aeration train, escaped determination.

The amount of carbon dioxide formed as an end product of substrate utilization could be calculated by subtracting the total carbon dioxide found in a blank determination from the total carbon dioxide values found in the various stages of substrate breakdown. The determination of the carbon dioxide content of the culture liquids was carried out as follows.

From a constantly shaken culture liquid (with $CaCO_3$ homogeneously suspended) 20 ml were taken and pipetted into a wide-mouth bottle of 100 ml capacity. Subsequently a few drops of silicone antifoam were added. The bottle was fitted with a stopper containing the stem of a separatory funnel and containing also an air inlet reaching the bottom of the bottle (and provided with a sintered glass diffuser) as well as an air outlet opening close under the stopper.

The bottle was placed on a magnetic stirrer and a stream of carbon dioxide-free air was passed through the liquid while 2 N HCl was added from the separatory funnel. The outcoming air was passed through concentrated sulphuric acid to remove moisture and the carbon dioxide was subsequently collected in an ascarite tube and determined gravimetrically. Aeration was continued for 45 minutes after acidification of the medium to remove dissolved carbon dioxide.

4. Results

a. Experiments with glucose as the substrate

The first experiments were carried out with a 1% glucose-0.35% ammonium sulphate medium (cf. p. 155). The results of a typical experiment are given in fig. 19.

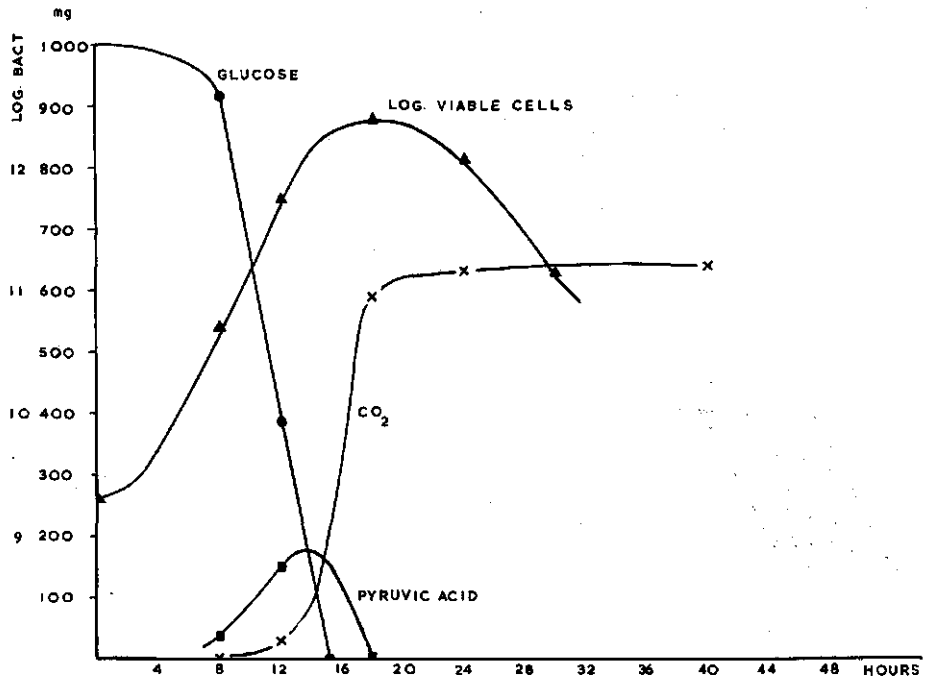

FIG. 19. Glucose breakdown by *Ps. chitinovorans*, strain 8676. Composition of medium: glucose 1 g, $(NH_4)_2SO_4$ 0.35 g, $CaCO_3$ 1 g, K_2HPO_4 0.1 g, $MgSO_4$ 0.1 g per 100 ml of water.

As may be seen from the bacterial growth curve, the logarithmic phase begins shortly after inoculation. A considerable increase in the number of bacteria was observed before disappearance of glucose and production of carbon dioxide could be observed. A rapid evolution of carbon dioxide follows at the end of the logarithmic growth phase and the maximum number of bacteria is observed at the time of complete disappearance of glucose from the medium. The carbohydrate supply apparently becomes a limiting factor for further multiplication.

Between 8 and 12 hours after inoculation, a rapid production of pyruvic acid was observed, whereas the production of carbon dioxide at this time was still limited. The maximum pyruvic acid content of the medium was observed at the moment of complete disappearance of glucose. Subsequently this acid disappeared about as rapidly as it had been produced.[1]) The carbon dioxide produced after the disappearance of pyruvic acid is apparently due to endogenous respiration only.

In most experiments small amounts of lactic acid have been found at the same time that pyruvic acid was present in the medium. The maximum amount of this acid, however, was more variable than that of pyruvic acid.

TABLE 3. Carbon balance of the experiment represented in fig. 19.

	mg C	% C
Carbon added as glucose	400	100
Carbon recovered, 24 h. after inoculation, as:		
Carbon dioxide	173	43.2
Bacteria	209	52.3
Acetoin	2	0.5
Total C recovered	384	96.0

A carbon balance (table 3) shows that nearly all the carbon initially present in glucose could be recovered at the end of the experiment as cell material and carbon dioxide. A small amount of acetoin was always present in the culture medium at the end of the experiment.

A second experiment was carried out with the 1 % glucose–0.1 % $(NH_4)_2SO_4$ medium (cf. p. 155). In this case, the nitrogen supply becomes a limiting factor for growth, 12 hours after inoculation, as is evident from fig. 20.

Further evidence of the disappearance of nitrogen from the medium is apparent from the following observations. From CO_2 determinations in the culture medium, carried out after 12 hours' incubation, it appeared that more CO_2 was lost from $CaCO_3$ than could be expected through the reaction between $CaCO_3$ and organic acids alone. However, it appeared that the additional CO_2 that disappeared from the medium was close to that calculated from the reaction between H_2SO_4 and $CaCO_3$, if it were supposed that all $(NH_4)_2SO_4$ had been converted to H_2SO_4, and that the CO_2 formed had been carried away with the stream of air that continually passed the culture liquid.

A small increase in the number of viable cells was observed at the end of the experiment; this is possibly due to autolysis of dead bacteria and the consequent reoccurrence of nitrogen-containing substances in the medium. This observation, like all others in this experiment, was reproducible.

[1]) Preliminary experiments with *Ps. chitinovorans*, strain 8500, showed that this strain too is able to produce pyruvic acid from glucose.

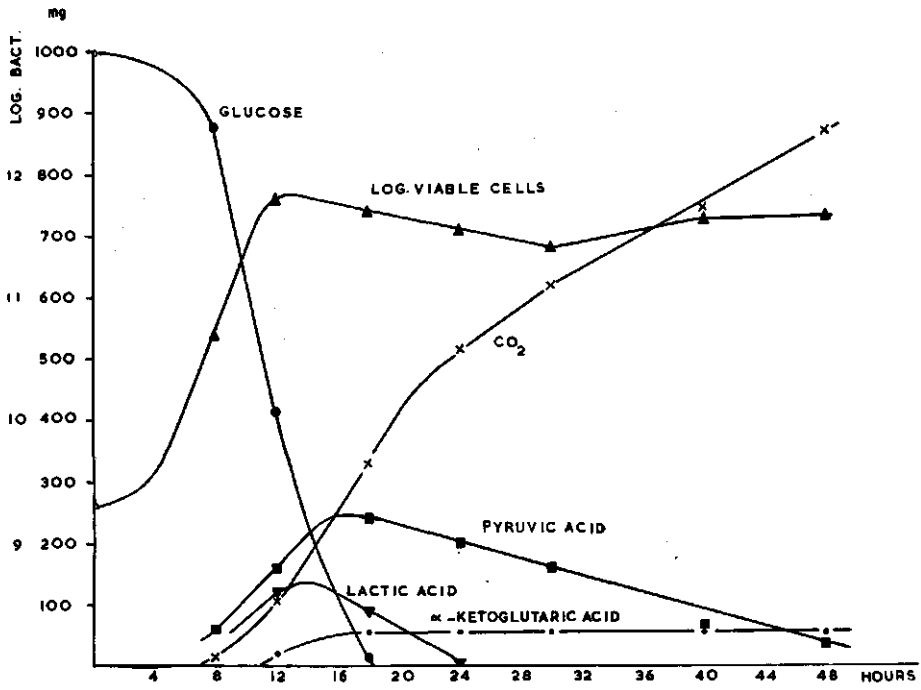

FIG. 20. Glucose breakdown by *Ps. chitinovorans* strain 8676. Composition of medium: glucose 1 g, $(NH_4)_2SO_4$ 0.1 g, $CaCO_3$ 1 g, K_2HPO_4 0.1 g, $MgSO_4$ 0.1 g per 100 ml of water.

The cessation of bacterial multiplication 12 hours after inoculation was coincident with a continued production of pyruvic acid and a sudden production of α-ketoglutaric acid. Presumably, the latter acid was present as such in the cells as a key intermediate in the synthesis of bacterial protoplasm and was released when further multiplication became impossible. Contrasted with pyruvic acid, α-ketoglutaric acid was not further used by the bacterial cells. This observation was in accordance with Warburg experiments (carried out with resting cells) in which the acids of the tricarboxylic acid cycle were used as substrates for oxidative dissimilation. In this case too, α-ketoglutaric acid belonged to the substances which showed hardly any respiration promoting activity. Even in experiments carried out with cell-free suspensions, (prepared by grinding washed cells with ground glass wool, followed by centrifugation) the addition of α-ketoglutaric acid did not alter the rate of oxygen uptake, whereas, for instance, the addition of acetate was followed by a rapid increase of oxygen consumption. As α-ketoglutaric acid apparently plays a role in the metabolism of *Pseudomonas chitinovorans*, these observations may serve as an example of the caution with which the results of Warburg experiments must be interpreted.

The production of pyruvic acid continued up to the moment of complete disappearance of glucose from the medium. At this time, a change in the rate of carbon dioxide production could be noticed; this rate becoming smaller when pyruvic acid is the only substrate.

As can be seen from fig. 20, appearance and disappearance of lactic acid show a similarity to those of pyruvic acid. Apparently part of the pyruvic acid is reduced to lactic acid before being released by the cells.

The carbon balance of this experiment is shown in table 4.

TABLE 4. Carbon balance of the experiment represented in fig. 20.

	mg C	% C
Carbon added as glucose	400	100
Carbon recovered, 48 h. after inoculation, as:		
Carbon dioxide	237	59.3
Bacteria	104	26.0
a-Ketoglutaric acid	25	6.2
Pyruvic acid	12	3.0
Acetoin	5	1.2
Total C recovered	383	95.7

Again the greater part of the carbon recovered at the end of the experiment could be found in cell material and carbon dioxide. Less carbon has been recovered as cell material and more as carbon dioxide compared to the foregoing experiment in which excess ammonium sulphate was used. A small amount of acetoin and a trace of diacetyl were present in the culture liquid at the end of the experiment.

b. Experiments with chitin as the substrate

The composition of the medium used has been mentioned above (cf. p. 155). A stock suspension of finely ground chitin was prepared, portions of which were used for various experiments. As was mentioned above, 1 liter Erlenmeyer flasks containing 100 ml of culture medium were used throughout the experiments. When these were placed on the reciprocating shaker, it appeared that chitin particles tended to adhere to the walls of the flasks just above the liquid surface. The adhering chitin could be easily removed from the walls by shaking the flasks by hand. From preliminary experiments it became clear that the frequency with which the adhering chitin was thus removed had a marked influence on the course of chitin decomposition. In view of this observation, two types of experiments were carried out. In the first one, the chitin adhering to the walls was washed into the culture liquid every hour; in the second one, the adhering chitin was removed from the walls only 16 hours after inoculation or was not resuspended at all. Experiments of both types were carried out several times and the results appeared to be reproducible.

When all chitin is kept continuously in the culture liquid (first experiment; fig. 21), the curve of bacterial multiplication shows a relatively short lag phase.

The maximum number of bacteria is observed at about 18 hours after inoculation. At this time the amount of carbon dioxide produced is about half of the total amount produced during this experiment. Since the nitrogen needed for growth must come from the chitin itself, and since there is still a large amount of carbon dioxide produced after the time of maximal population, there must be a large amount of some nitrogen-free intermediate of chitin breakdown present at about 18 hours after inoculation. As to the nature of this substance, no data are available at this time.

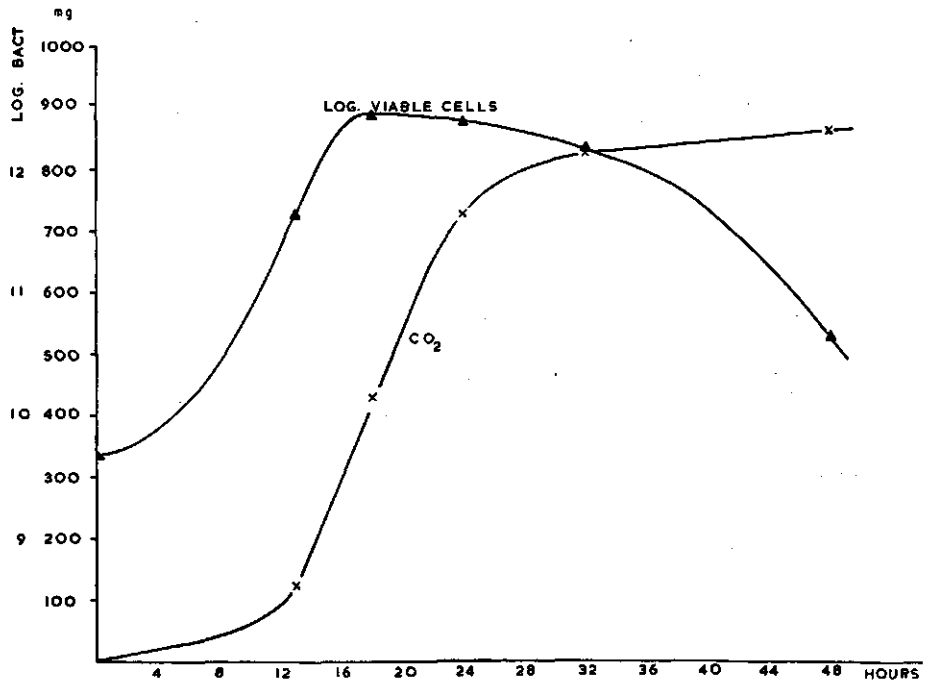

FIG. 21. Chitin breakdown by *Ps. chitinovorans*, strain 8676. Composition of medium: Chitin (finely ground) 1 g, $CaCO_3$ 1 g, K_2HPO_4 0.1 g, $MgSO_4$ 0.1 g per 100 ml of water.

There is only a slight decrease in the number of viable cells up to the moment when carbon dioxide production reaches the level of endogenous respiration (about 32 hours after inoculation). After that time a rapid decrease of viable cells could be observed.

The major part of the carbon initially present in chitin could be recovered as cell material and carbon dioxide at the end of the experiment (table 5).

TABLE 5. Carbon balance of the experiment represented in fig. 21.

	mg C	% C
Carbon added as chitin	486	100
Carbon recovered, 32 h. after inoculation, as:		
Carbon dioxide	226	46.5
Bacteria	231	47.5
Acetoin	1	0.2
Total C recovered	458	94.2

A remarkable feature of this experiment is the absence of pyruvic acid in the culture medium during the decomposition of chitin. Pyruvic acid determinations were carried out between 12 and 32 hours after inoculation. Whereas a large amount of pyruvic acid is produced during the period of logarithmic growth in glucose decomposition (fig. 19), this was not the case in this experiment with chitin.

In the second type of experiment, chitin particles adhering to the walls were resuspended 16 hours after inoculation, but even when this was not done, the adhering particles disappeared rather rapidly from the walls after 16 hours' incubation. In both cases, pyruvic acid was present in the culture liquids throughout a period of 18–25 hours after inoculation. The maximum amounts of pyruvic acid found during this period in various experiments, varied from 122 to 250 mg.[1])

A carbon balance made of an experiment of the second type (Table 6) shows that more carbon has been recovered as carbon dioxide and less as cell material in comparison with the foregoing experiment.

TABLE 6. Explanation see text.

	mg C	% C
Carbon added as chitin	486	100
Carbon recovered, 40 h. after inoculation, as:		
Carbon dioxide	289	59.5
Bacteria	170	34.9
Acetoin	3	0.6
Total C recovered	462	95.0

The different results obtained with the two types of experiments of chitin breakdown might be explained by the following considerations. In the experiment in which chitin was continually kept in the liquid, the products of hydrolysis are formed regularly in small amounts. In this medium with low concentrations of N-acetylglucosamine and glucosamine (cf. Chapter 6), pyruvic acid is formed in the cells at a comparatively slow rate and can be completely utilized in terminal respiration and synthetic processes.

In the second type of experiment, however, a large amount of chitin is added to the medium at a moment when a relatively high concentration of extracellular enzymes is present in the medium. Thus it might be expected that products of chitin hydrolysis are rapidly formed, and this would enable the cells to form pyruvic acid at a rapid rate. Since the bacteria are unable to utilize this acid as fast as it is formed, the acid is released into the medium.

The following experiment proves that pyruvic acid is actually formed in a medium containing a high concentration of N-acetylglucosamine. When cells were inoculated in the following medium: N-acetylglucosamine 1 g, K_2HPO_4 0.1 g, $MgSO_4$ 0.1 g and $CaCO_3$ 1 g per 100 ml of water (N-acetylglucosamine was sterilized separately by filtration), pyruvic acid could be found in 12 hour cultures in amounts close to those found in the glucose-0.35 % $(NH_4)_2SO_4$ medium (cf. fig. 19). Although it is still uncertain whether glucose is an intermediate in the breakdown of N-acetylglucosamine, the formation of pyruvic acid from both substrates by *Pseudomonas chitinovorans*, strain 8676, indicates that the terminal respiration pattern is the same for both substrates.

Preliminary experiments to investigate the conditions responsible for the production of pyruvic acid from chitin and glucose showed that addition of

[1]) In experiments with *Pseudomonas chitinovorans*, strain 8500, only traces of pyruvic acid have been found during chitin decomposition.

thiamine hydrochloride or thiamine pyrophosphate (cocarboxylase) up to concentrations of 200 μg per ml of culture medium did not prevent pyruvic acid production.[1]) Neither did the addition of 0.1 % yeast extract (Difco) to the culture medium.

5. Summary

The oxidative breakdown of glucose and chitin by *Pseudomonas chitinovorans*, strain 8676, results in the production of carbon dioxide and cell material. Small amounts of acetoin are produced from both substrates.

Pyruvic acid production may be observed during the breakdown of both glucose and chitin, but with either substrate this acid is eventually consumed. This is not the case with the small amount of α-ketoglutaric acid produced during the breakdown of glucose with a limited nitrogen supply.

GENERAL SUMMARY

It has been an attractive problem to try to further the understanding of chitin decomposition. Chitin is a compound of such wide occurrence in nature, that there must be some extremely effective processes of chitin breakdown that prevents its staggering accumulation. This natural decomposition is all the more remarkable because chitin is particularly resistant against simple chemical attack. Indeed it is possible to prepare a very pure chitin by treatment of the skeletons of shrimps alternately with dilute hydrochloric acid and dilute sodium hydroxide by which all the other constituents of the shell are removed. This method is described in detail in Chapter 1, where also a few data are presented on the chemical composition of chitin, which give some indication as to the probable direction of its biological breakdown.

The literature on microbial chitin decomposition (reviewed in Chapter 2) consists chiefly of reports on the isolation of chitinovorous microorganisms. From these reports it appears that these organisms are distributed over a number of known genera and that they occur in a wide variety of habitats.

In the course of the present investigations, 50 strains of chitin-decomposing bacteria have been isolated from soil; among these organisms are representatives of the following genera: *Achromobacter*, *Flavobacterium*, *Chromobacterium*, *Bacillus*, *Cytophaga* and *Pseudomonas*. Two strains, *Pseudomonas chitinovorans* n. sp., and a bacterium belonging to the species group *Cytophaga johnsonae* Stanier, which were used for further investigations on chitin decomposition, are described in detail in Chapter 2. Among 23 chitinovorous actinomycetes, isolated from soil, representatives of the following genera have been found: *Streptomyces*, *Micromonospora* and *Nocardia*. Several chitin-decomposing fungi have been isolated from soil; one of them could be identified as *Aspergillus fumigatus* Fres., others were representatives of the genus *Mortierella*.

A quantitative determination of the number of chitin-decomposing microorganisms in different soils (cf. Chapter 3) showed that in all soils investigated, these organisms occur in great numbers. The smallest number ($3.5 \cdot 10^5$ per g) was found in a sandy soil with acid pH. In all soils, actinomycetes formed the major part of the population of chitin-decomposing microorganisms, whereas

[1]) The author is indebted to Prof. Dr H. G. K. Westenbrink for providing thiamine pyrophosphate.

the percentage of actinomycetes in 'total' microorganisms (as determined on an enriched soil extract agar) was relatively small. Bacteria formed 1–3 % and fungi less than 1 % of the population of chitin-decomposing microorganisms.

Investigations on the changes in the microbial population of different soils as a result of the addition of powdered chitin, are described in Chapter 4. These investigations have been carried out with water saturated soils and with relatively dry soils, respectively. During experiments with water saturated soils, carried out with the soil perfusion technique, it became evident that changes in the microbial population of different soils, resulting from chitin addition to these soils, are reflected in the reservoir fluid of the percolators. It appeared that in soils saturated with water, only chitinovorous bacteria achieve a rapid multiplication, whereas actinomycetes do not develop in such soils. These organisms form less than 1 % of the population of chitinovorous microorganisms found in the reservoir fluids as well as in the soil columns. A high percentage of chitin-decomposing bacteria isolated from the percolators lost their chitin-decomposing capacity after several transfers on yeast extract-glucose agar.

During experiments with the relatively dry soils, soil dilutions were plated out not only on mineral chitin agar, but also on a non-selective medium (an enriched soil extract agar), appropriate to meet the nutritional requirements of a relatively large number of microorganisms. In all soils to which chitin was added, the changes in the microbial population resulted in a predominance of actinomycetes over bacteria. In two soils (a clay soil and a sandy soil) which had previously been treated with farmyard manure, actinomycetes formed the major part of the chitinovorous as well as 'total' microorganisms soon after the addition of chitin. A sandy soil, previously treated only with mineral fertilizer salts, showed an increase of bacteria as well as actinomycetes when chitin was added. This was followed by a period of decrease in the number of microorganisms, in which actinomycetes became the predominant part of chitinovorous as well as 'total' microorganisms.

A large number of actinomycetes present in soils to which chitin was added showed antagonistic activities against *Bac. subtilis*, whereas the growth of *E. coli* and *Ps. chitinovorans* was only slightly inhibited by some of these actinomycetes.

In the experiments with relatively dry soils, a considerable amount of finely powdered, purified chitin was added to different soils and the temperature and moisture content of these soils was kept constant for a long period. Although these conditions might never occur in nature, the actinomycetes undoubtedly play an important role in the decomposition of chitin under natural conditions.

When *Carabus* elytra were placed in different soils under conditions optimal for the development of actinomycetes they became partly covered with colonies of these organisms, but were still intact after a period of 6 months. The breakdown of such substrates under the continually changing natural conditions, often less optimal than those used in our experiments, would undoubtedly be still slower.

In Chapter 5, observations of nitrification as a result of chitin-decomposition in different soils are described. A relatively rapid accumulation of nitrate could generally be observed up to about 3 weeks after chitin addition to water saturated or relatively dry soils; this was followed by a period of slower nitrate accumulation, accompanied by a rapid fall in the numbers of microorganisms. It seems likely that the nitrate formed during the latter period originated from the oxidation of ammonia liberated during decomposition of cell proteins and

thus was derived merely indirectly from chitin-N. Up to 60 % of the nitrogen originally present in chitin has been recovered as nitrate.

Toward a more direct analysis of chitin breakdown and its intermediary products, experiments have been carried out with pure cultures of chitin-decomposing bacteria. These experiments are described in Chapters 6 and 7.

In Chapter 6 investigations on hydrolysis of chitin by bacterial enzymes have been described. These experiments were carried out with *Pseudomonas chitinovorans*, strain 8500, and *Cytophaga johnsonae* STANIER. It appeared that in cultures of both strains, containing chitin as the sole source of C and N, N-acetylglucosamine as well as glucosamine could be traced, even though concentrations were very low. An accumulation of both substances in the culture liquids could be obtained by checking bacterial metabolism in the logarithmic growth phase, while hydrolysis of chitin by extracellular enzymes continued.

Acetic acid has been found during chitin decomposition by both strains, indicating that N-acetylglucosamine is deacetylated to yield glucosamine. Tests for the presence of glucose were negative and the question of whether the ammonia found in the cultures was derived from glucosamine directly or from a breakdown product of glucosamine remained unsolved.

Two unknown hydrolysis products of chitin were detected chromatographically in culture liquids of both strains, and their R_f values characterized.

Chapter 7 describes investigations of the aerobic breakdown of chitin and glucose by *Pseudomonas chitinovorans*, strain 8676. This strain forms more acid from chitin than *Pseudomonas chitinovorans*, strain 8500, and it was the aim of these experiments to identify the acid(s) and to compare the total breakdown of chitin with that of glucose, since this substance is closely related to intermediates of chitin decomposition.

Experiments on the breakdown of glucose were carried out in a mineral medium containing 1 % glucose and 0.35 % or 0.1 % $(NH_4)_2SO_4$.

In the medium containing 0.35 % $(NH_4)_2SO_4$, the maximum number of viable cells was observed at the moment of complete disappearance of glucose. During the period of logarithmic growth, lactic acid and pyruvic acid have been found in the medium; these acids were eventually consumed.

In the medium containing 0.1 % $(NH_4)_2SO_4$, the nitrogen supply becomes the limiting factor for growth, 12 hours after inoculation. The cessation of bacterial multiplication was coincident with a continued production of pyruvic acid and a sudden production of α-ketoglutaric acid. As contrasted with pyruvic acid, α-ketoglutaric acid was not used further by the bacterial cells.

Carbon balances of both experiments with glucose as the substrate showed that the major part of the carbon recovered at the end of the experiments could be found in cell material and carbon dioxide. A small amount of acetoin was present in the culture liquids at the end of the experiments.

In experiments with chitin as the substrate it appeared that chitin particles tended to adhere to the walls of the flasks just above the liquid surface of the shake cultures. The frequency with which this chitin was resuspended had a marked influence on the course of chitin decomposition. Pyruvic acid has not been found in experiments in which chitin was continually kept in the medium, whereas this acid was always present during a period of 18–25 hours after inoculation, when the adhering chitin was resuspended after 16 hours' incubation.

The results of different experiments using chitin as the substrate are discussed;

it is proposed that the formation of pyruvic acid from chitin depends on the concentration of the products of hydrolysis in the medium.

Carbon balances made in experiments with chitin as the substrate showed that the major part of the carbon initially present in chitin could be recovered as cell material and carbon dioxide at the end of the experiments.

When N-acetylglucosamine was used as the substrate, production of pyruvic acid was always observed. Although it is still uncertain whether glucose is an intermediate in the breakdown of N-acetylglucosamine, the formation of pyruvic acid from both substances by *Pseudomonas chitinovorans*, strain 8676, indicates that the terminal respiration pattern is the same for both substrates. Addition of yeast extract or cocarboxylase did not prevent the production of pyruvic acid from both substrates.[1])

SAMENVATTING

Het was een aantrekkelijke opgaaf om te trachten, iets te weten te komen over de wegen der natuurlijke afbraak van chitine, een stof die zo sterk verbreid in de natuur voorkomt. Het is zonder meer duidelijk, dat naast een zo uitgebreide vorming van chitine in de levende wereld een snelle natuurlijke afbraak het evenwicht bewaren moet. Deze biologische afbraak is des te merkwaardiger, omdat chitine tegen allerlei chemische inwerkingen bijzonder resistent is. Het bleek dan ook mogelijk uit cuticulae van garnalen zuivere chitine te verkrijgen door afwisselende behandeling met verdund zuur en alkali, welke methode uitvoerig in Hoofdstuk 1 beschreven is. Men vindt daarin tevens enige gegevens over de chemische samenstelling van chitine, die alreeds een aanduiding geeft van de richting waarin de biologische afbraak kan plaats hebben.

De literatuur over de microbiële chitine afbraak, waarvan in Hoofdstuk 2 een overzicht gegeven is, bestaat voornamelijk uit gegevens betreffende isolatie van chitine-aantastende microorganismen. Uit deze gegevens blijkt, dat genoemde organismen tot een aantal bekende geslachten behoren en dat zij uit zeer uiteenlopende biotopen geïsoleerd kunnen worden.

Tijdens het eigen onderzoek werden 50 chitine-aantastende bacteriën uit grond geïsoleerd; hieronder bleken vertegenwoordigers van de volgende geslachten voor te komen: *Achromobacter*, *Flavobacterium*, *Chromobacterium*, *Bacillus*, *Cytophaga* en *Pseudomonas*. Twee stammen, *Pseudomonas chitinovorans*, n. sp., en een organisme, dat in grote trekken overeenkomt met *Cytophaga johnsonae* STANIER, werden gebruikt voor verder onderzoek over chitine-afbraak en zijn in Hoofdstuk 2 uitvoerig beschreven. Bij 23 uit grond geïsoleerde chitine-afbrekende actinomyceten bleken de volgende geslachten vertegenwoordigd te zijn: *Streptomyces*, *Micromonospora* en *Nocardia*. Tot enkele uit grond geïsoleerde chitine-aantastende schimmels behoorden *Aspergillus fumigatus* FRES. en enige vertegenwoordigers van het geslacht *Mortierella*.

Een quantitatieve bepaling van het aantal chitine-aantastende organismen in verschillende gronden werd in Hoofdstuk 3 beschreven; het bleek, dat deze organismen in alle onderzochte gronden zeer talrijk waren. Het kleinste aantal ($3.5 \cdot 10^5$ per g) werd aangetroffen in een zure zandgrond. In alle gronden bestond de populatie der chitine-aantastende microorganismen grotendeels uit actinomyceten, terwijl deze organismen slechts een gering gedeelte van het

[1]) The author is indebted to Dr R. S. PLATT who corrected the english manuscript.

'totale' aantal microorganismen bleken uit te maken. Bacterien vormden 1–3 % en schimmels altijd minder dan 1 % van het aantal chitine-aantastende microorganismen in verschillende gronden.

Onderzoekingen over de veranderingen welke in de microbiële populatie optreden als gevolg van de toevoeging van fijngemalen chitine aan verschillende gronden, werden in Hoofdstuk 4 beschreven. De onderzoekingen werden uitgevoerd in met water verzadigde en in relatief droge gronden. Tijdens experimenten met eerstgenoemde gronden, welke met behulp van de percolatortechniek werden uitgevoerd, bleken de veranderingen in de microbiële populaties tengevolge van de toevoeging van chitine weerspiegeld te worden in de vloeistofreservoirs der percolators. Het bleek, dat in met water verzadigde gronden slechts chitine aantastende bacteriën een snelle vermeerdering vertoonden, terwijl actinomyceten zich in dergelijke gronden niet ontwikkelden. Deze organismen vormden minder dan 1 % van het aantal chitine-aantastende microorganismen, zowel in de vloeistofreservoirs als in de grondkolommen. Een hoog percentage van de chitine-aantastende bacteriën, welke uit de percolators geïsoleerd werden, bleek het vermogen om chitine af te breken te verliezen wanneer ze gedurende enige tijd op een gistextract-glucose-agar gekweekt werden.

Tijdens de experimenten met de relatief droge gronden, werden zowel de veranderingen in het aantal chitine-aantastende als die van het 'totaal' aantal microorganismen nagegaan. Deze aantallen werden bepaald met behulp van telplaten op chitine-agar en een niet selectieve grondextract-agar. In alle gronden waaraan chitine toegevoegd werd trad een overheersende groei van actinomyceten op. In twee gronden (een kleigrond en een zandgrond) die voordien met stalmest behandeld waren, vormden actinomyceten al zeer snel na de toevoeging van chitine niet alleen het grootste deel van het aantal chitine-aantasters, maar ook van het 'totaal' aantal microorganismen. In een zandgrond welke voordien met kunstmest behandeld was, kwamen zowel bacteriën als actinomyceten tot ontwikkeling nadat chitine toegevoegd was. In de daarop volgende periode van vermindering van het aantal microorganismen vormden echter ook hier actinomyceten het grootste deel, zowel van de populatie der chitine-aantasters als van het 'totaal' aantal microorganismen.

Een groot aantal van de actinomyceten, welke aangetroffen werden in de gronden waaraan chitine toegevoegd was, bleek een antibiotische werking op *Bac. subtilis* uit te oefenen, terwijl daarentegen slechts een klein aantal antibiotisch actief was tegen *E. coli* en *Ps. chitinovorans*.

In de experimenten met de betrekkelijk droge gronden werd aan deze een vrij grote hoeveelheid fijngemalen chitine toegevoegd, terwijl de temperatuur en het vochtgehalte gedurende lange tijd constant gehouden werden. Hoewel deze omstandigheden in de natuur nooit voorkomen, is het toch aan geen twijfel onderhevig, dat actinomyceten een belangrijke rol spelen bij de chitine afbraak onder natuurlijke omstandigheden. Dekschilden van *Carabus*, welke geplaatst werden in verschillende gronden, bij omstandigheden welke optimaal zijn voor de ontwikkeling van actinomyceten, werden weliswaar gedeeltelijk bedekt met actinomyceten-kolonies, doch waren na een half jaar nog volledig intact. De afbraak van dergelijk materiaal onder de steeds wisselende natuurlijke omstandigheden, welke vaak minder optimaal zijn dan die welke in dit experiment toegepast werden, zal ongetwijfeld nog langzamer gaan.

Waarnemingen betreffende nitrificatie tengevolge van chitine-afbraak in verschillende gronden zijn in Hoofdstuk 5 vermeld. In het algemeen werd een

betrekkelijk snelle accumulatie van nitraat waargenomen gedurende drie weken na toevoeging van chitine, zowel in met water verzadigde als in relatief droge gronden. Hierop volgde een periode van langzame nitraatvermeerdering, welke gepaard ging met een snelle vermindering van het aantal microorganismen. Vermoedelijk vond het gedurende deze laatste periode gevormde nitraat zijn oorsprong in ammoniak, dat afkomstig was van de afbraak van de cellen van microorganismen en was dus slechts indirect van chitine afkomstig. Tot 60 % van de oorspronkelijk in chitine aanwezige stikstof kon als nitraatstikstof teruggevonden worden.

Teneinde een meer directe indruk te krijgen van de microbiële chitine-afbraak en de tussenproducten welke daarbij ontstaan, werden experimenten uitgevoerd met reincultures van chitine-aantastende bacteriën. Deze experimenten zijn in de Hoofdstukken 6 en 7 beschreven. In Hoofdstuk 6 vindt men onderzoekingen over de hydrolyse van chitine door *Pseudomonas chitinovorans*, stam 8500, en *Cytophaga johnsonae* STANIER. Het bleek, dat in cultures van beide stammen, welke chitine als enige C en N bron bevatten, N-acetylglucosamine en glucosamine aangetoond konden worden, ondanks de zeer geringe concentraties der genoemde stoffen. Een ophoping van beide tussenproducten kon verkregen worden door de bacteriële stofwisseling tijdens de logarithmische groeiphase te remmen, onder omstandigheden waarbij de hydrolyse van chitine door exoenzymen voortgang vond.

Ook azijnzuur is gevonden tijdens de chitine-afbraak door beide bacteriën, hetgeen er op wijst, dat N-acetylglucosamine gedeacetyleerd wordt, waarbij glucosamine en azijnzuur ontstaan. Glucose werd niet in de chitinecultures aangetroffen en de vraag of het ammoniak, dat tijdens de chitine-afbraak door beide stammen gevormd wordt, direct van glucosamine afkomstig was, of van een afbraakproduct hiervan, bleef onbeantwoord.

Twee onbekende hydrolyse-producten van chitine werden aangetoond met behulp van papierchromatografie en enkele bijzonderheden van deze stoffen werden beschreven.

Onderzoekingen over de afbraak van chitine en glucose door *Pseudomonas chitinovorans*, stam 8676, werden in Hoofdstuk 7 beschreven. Deze stam vormt meer zuur uit chitine dan *Pseudomonas chitinovorans*, stam 8500, en het doel van deze onderzoekingen was, het gevormde zuur nader te identificeren en tevens de afbraak van chitine met die van glucose te vergelijken, daar glucose nauw verwant is aan de hydrolyseproducten van chitine.

Experimenten over de glucose-afbraak werden uitgevoerd in een anorganisch medium, dat 1 % glucose en 0.35 % of 0.1 % ammonium sulfaat bevatte. In het medium met 0.35 % ammonium sulfaat werd het maximum aantal bacteriën waargenomen op het moment, dat glucose volledig verbruikt was. Tijdens de periode van logarithmische groei werden melkzuur en pyrodruivenzuur in het medium aangetroffen; deze zuren werden in een later stadium weer door de bacteriën verbruikt. In het glucose-medium met 0.1 % ammonium sulfaat werd 12 uur na enting de stikstofvoorziening beperkend voor verdere groei; dit ging gepaard met voortzetting van de vorming van pyrodruivenzuur, terwijl tevens plotseling α-ketoglutaarzuur in het medium werd afgescheiden. Het pyrodruivenzuur werd later weer door de bacteriën verbruikt, hetgeen niet het geval was met α-ketoglutaarzuur.

Een koolstofbalans, opgemaakt in beide experimenten met glucose als substraat, doet zien, dat vrijwel alle koolstof, oorspronkelijk in glucose aan-

wezig, uiteindelijk in celmateriaal en koolzuur teruggevonden kon worden.

In experimenten waarin chitine als substraat gebruikt werd bleek, dat de chitinedeeltjes de neiging vertoonden zich aan de wand van de kolven te hechten, juist boven het vloeistofoppervlak van de schudcultures. Het bleek, dat de frequentie waarmee deze deeltjes weer in de vloeistof gebracht werden van grote invloed was op het verloop van de chitine-afbraak. Pyrodruivenzuur werd niet gevormd in media, waarin de chitine permanent in de vloeistof gehouden werd, terwijl dit zuur daarentegen altijd 18–25 uur na enting aangetroffen werd in de cultuurvloeistof, indien de aan de wand klevende chitine deeltjes niet eerder dan na 16 uur cultiveren in het medium teruggebracht werden. De resultaten der verschillende experimenten werden besproken, waarbij de veronderstelling geopperd werd, dat de vorming van pyrodruivenzuur uit chitine afhankelijk is van de concentratie der hydrolyseproducten in het medium.

Een koolstofbalans, opgemaakt in verschillende experimenten met chitine als substraat wees uit, dat vrijwel alle koolstof oorspronkelijk aanwezig in chitine aan het einde der experimenten in celmateriaal en koolzuur teruggevonden kon worden.

In experimenten waarbij N-acetylglucosamine als substraat aanwezig was, werd zonder uitzondering vorming van pyrodruivenzuur waargenomen, terwijl ook hier dit zuur later weer door de bacteriën verbruikt werd.

Hoewel het nog onzeker is of glucose tussenproduct is bij de afbraak van N-acetylglucosamine, wijst de vorming van pyrodruivenzuur uit beide substraten door *Pseudomonas chitinovorans*, stam 8676, erop, dat de eindprocessen van de afbraak van beide substraten dezelfde zijn. Toevoeging van gistextract of cocarboxylase verhinderde de vorming van pyrodruivenzuur uit beide substraten niet.

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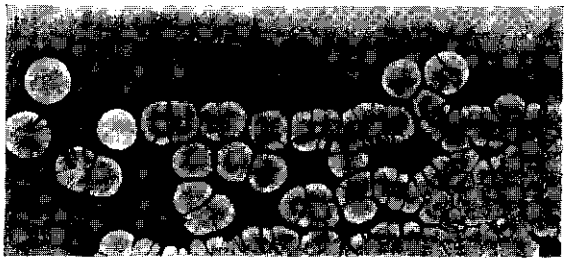

Fig. 2

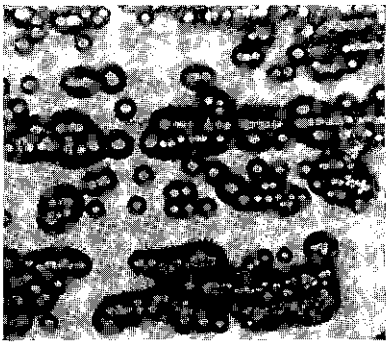

Fig. 4

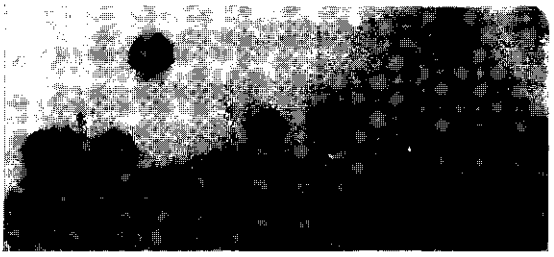

Fig. 3

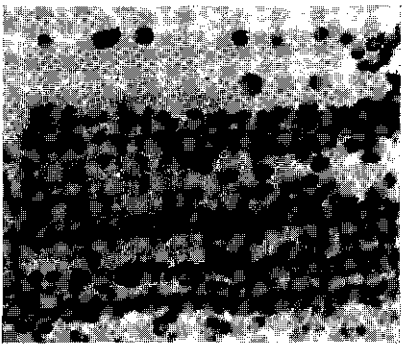

Fig. 5

FIG. 2. *Streptomyces* strains grown on mineral chitin agar often show a clear zone of decomposed chitin only underneath their colonies, as may be seen in this figure. Aerial mycelia of three colonies have been removed.

FIG. 3. Strain of *Micromonospora* grown on mineral chitin agar, showing zone of decomposed chitin around the colonies.

FIG. 4. *Pseudomonas chitinovorans*, n. sp., grown for four days on mineral chitin agar. Colonies surrounded by clear zone of decomposed chitin.

FIG. 5. Bacterium of species group *Cytophaga johnsonae* STANIER grown for seven days on mineral chitin agar. Vague appearance of zone of decomposed chitin is due to bacterial growth. Swarming edge of colonies extends beyond zone of decomposed chitin.

PLATE 2

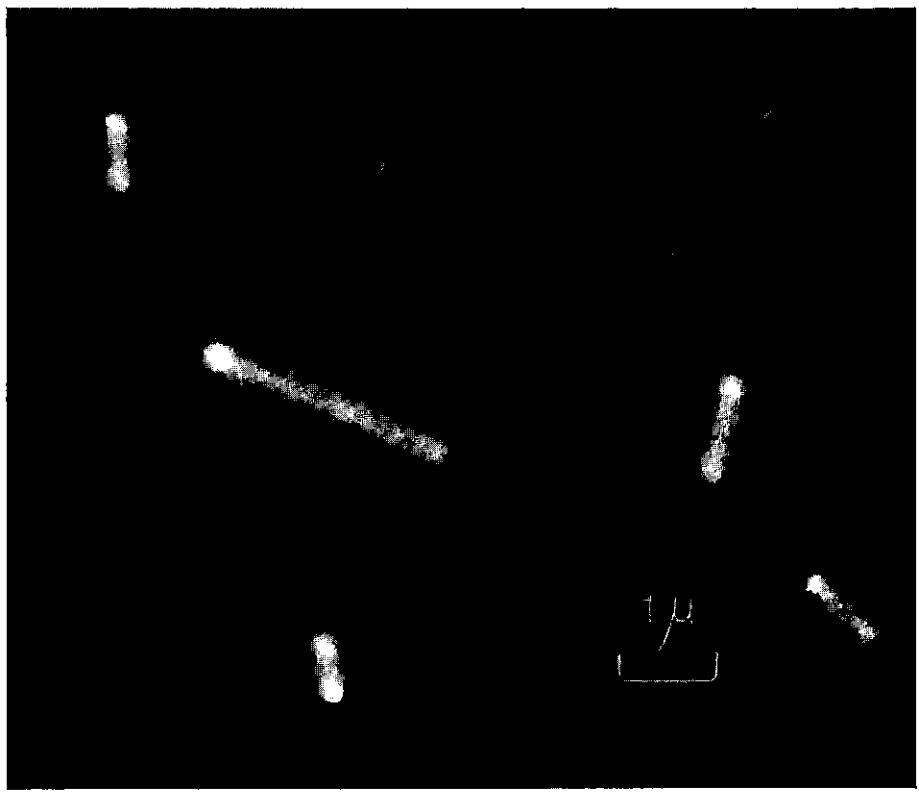

FIG. 6. Short cells of bacterium belonging to species group *Cytophaga johnsonae* STANIER.

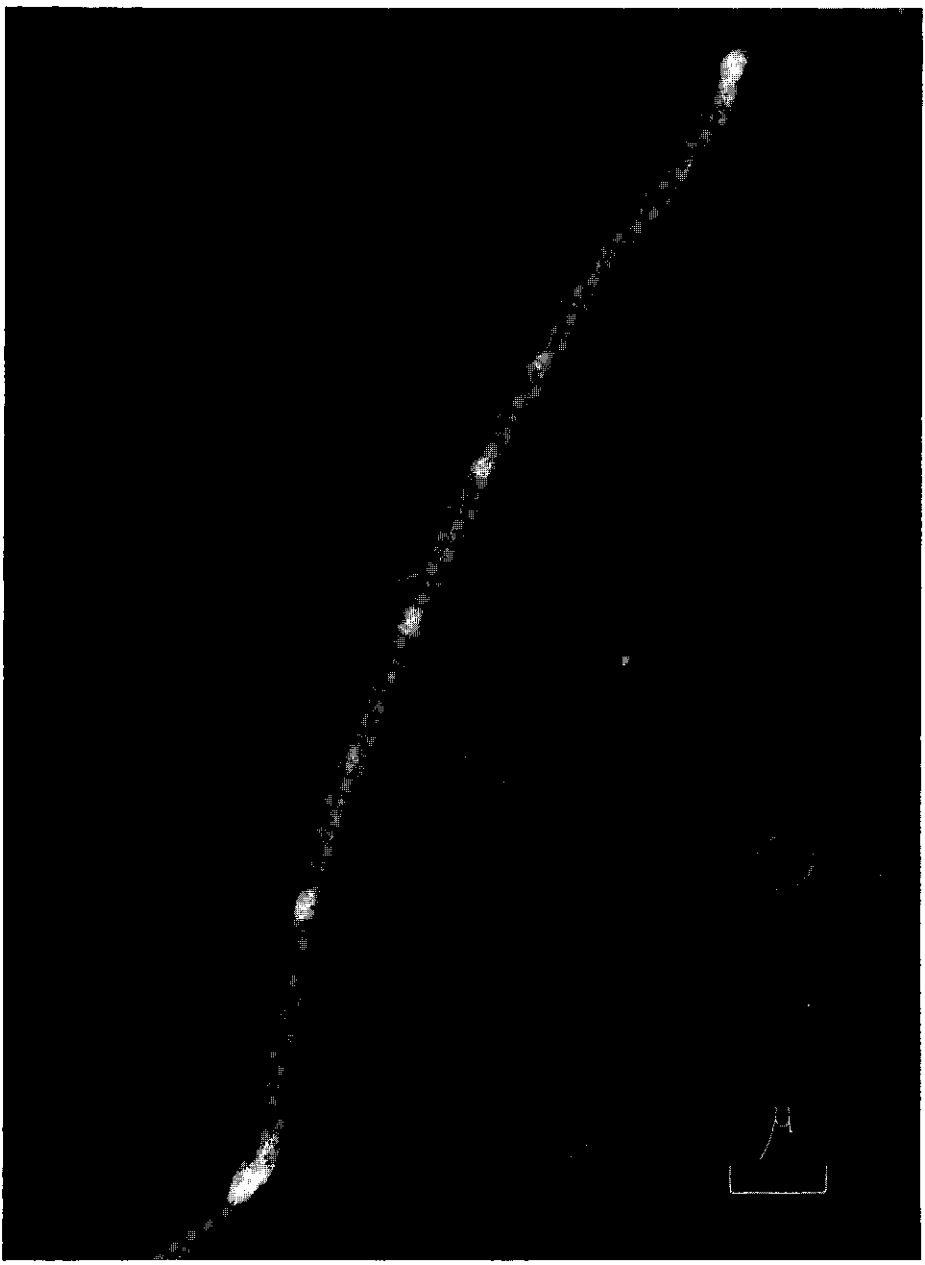

FIG. 7. Long cell of bacterium belonging to species group *Cytophaga johnsonae* STANIER.

PLATE 4

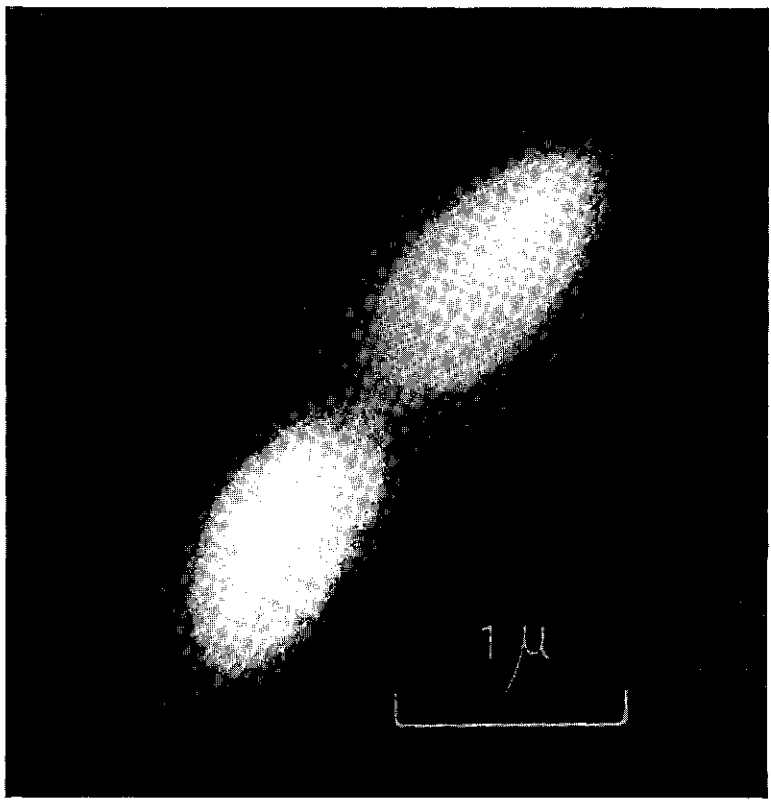

FIG. 8. Spindle shaped cells of *Pseudomonas chitinovorans*, n. sp., grown on yeast extract-glucose agar.

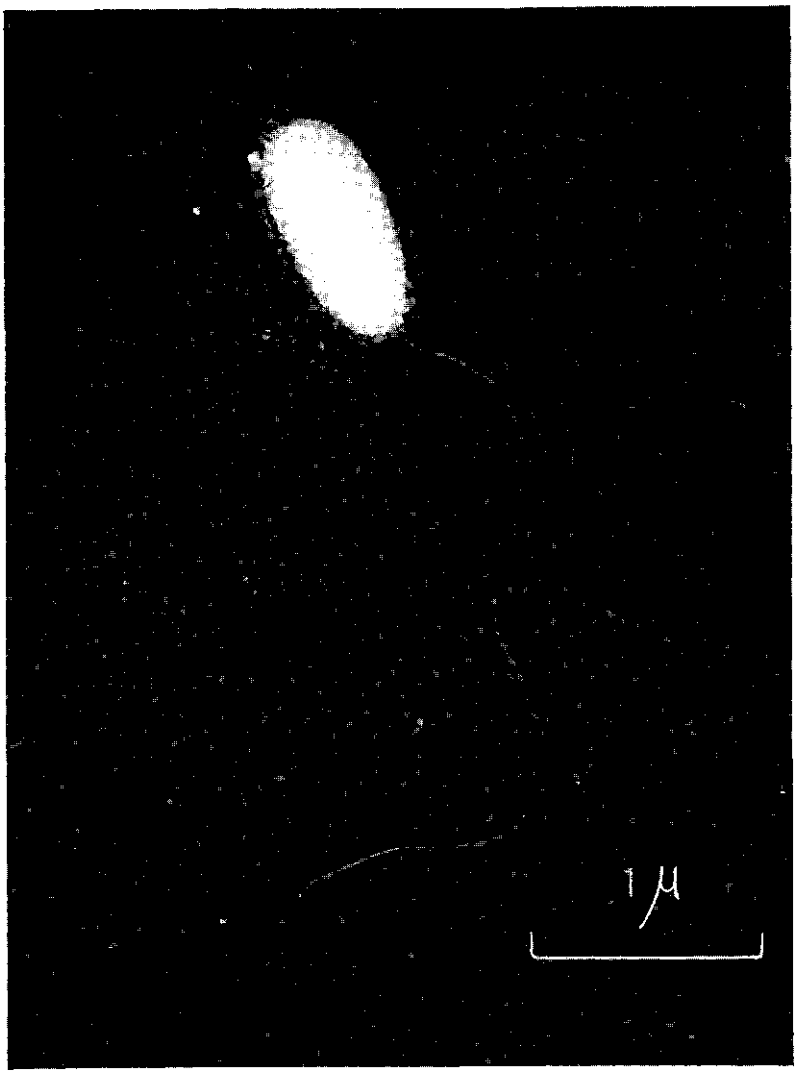

FIG. 9. *Pseudomonas chitinovorans*, n. sp., grown in liquid culture.