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A comparative study of the decomposition of cellulosic substrates by selected bacterial strains.

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A COMPARATIVE STUDY OF THE DECOMPOSITION
OF CELLULOSIC SUBSTRATES BY
SELECTED BACTERIAL STRAINS

A dissertation presented

By

MARK CHARLES SEGAL

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INTRODUCTION

A limited number of quantitative tools are available
for the microbiologist attempting to assign relative roles
to cellulose decomposing bacteria in a variety of ecosystems.
Such limits are imposed, for the most part, by the criteria
commonly used for the determination of cellulolytic activity.
The definitions for the term "cellulolytic" bacteria and the
criteria on which they are based were developed during a
half-century period of investigations, and reflect the chang-
ing methodologies over this time span. Unfortunately, older
criteria have not been dropped or modified as newer tech-
niques were adopted, thus leaving a confusing assortment of
methodologies, criteria and definitions based thereon. A
perusal of the literature reveals that not all criteria can
be applied successfully to all bacterial species commonly
called cellulolytic, yet this is precisely what is needed for
the development of routine analysis procedures for these or-
ganisms.

These procedures have not been developed despite the
restrictive nature of cellulosic substrates. This is due,
in part, to the variability of the material called cellulose.
The definitions, and the criteria applied to those defini-
tions, for cellulolytic bacteria have varied with the form

of the substrate used. Investigations with fungal and bacterial cellulases have revealed, additionally, that differences exist among the known cellulase enzyme systems. Cellulases, rather than being single enzymes working on unique substrates, may be multiple enzyme systems, the components of which may attack a variety of subtrates, often with overlapping functions. Thus, in order to discuss cellulolytic activity, it is necessary to specify the form of the cellulosic substrate being attacked, thereby delimiting the function of the enzyme system under discussion. Until recently this was rarely done.

Not all bacteria capable of attacking one cellulosic substrate may be able to attack all substances considered to be cellulolytic. The lack of one enzyme in a cellulase system may prevent complete degradation of the cellulosic material. When this occurs the organism may be classified as non-cellulolytic. It is conceivable that cellulose could be degraded by microbial synergism in which none of the active species would be considered cellulolytic by "conventional" standards. Using less "conventional" standards any one of the participating organisms might be called cellulolytic because it participated in the destruction of cellulosic materials. At present there is no agreement as to which of

the two approaches is acceptable, nor is any compromise seen
to be agreeable to all parties working in the area.

What seems to have occurred in the development of know-
ledge about microbial cellulolysis is that great strides
have been made elucidating the function of certain cellulase
systems, while understanding of the manner in which the mi-
croorganisms that produce these enxymes function in their
native habitats has been barely advanced. It appears that,
with bacteria at least, one reason for this lack of progress
is the inability to detect all cellulolytic organisms either
in situ or when isolated from a given habitat. No clear cri-
teria are available to permit such detection.

In order to develop such criteria for bacteria, some
basic understanding of how cellulolytic bacteria differ from
non-cellulolytic bacteria is required. What the present
study attempts to do is draw together, from widespread seg-
ments of the literature, data pertinent to the formation of
such criteria and to support this with experimental evidence.

I. REVIEW OF LITERATURE

The chemistry of cellulose. Cellulose is one of the most abundant organic compounds found in nature. It occurs primarily in higher plants, in terrestrial environments, and in certain algae and higher plants in aquatic environments. All forms of cellulose are high linear polymers of β-1, 4-linked D-anhydroglucopyranose units. The cell walls of higher plants consist of fibrils of cellulose together with varying amounts of other components such as waxes, pectins, protein, hemicelluloses and lignin. It has been established that cotton seed hairs contain as much as 90% cellulose while fibers of most woody tissue from trees contain 40-60% cellulosic material(1, 2). Ramie and jute are other important sources of cellulose. Payen(3) proposed that the material remaining after the extraction of lignin from the fibrous tissue of young cells be called "cellulose".

As better analytical techniques were developed the term "holocellulose" was devised to represent that fraction of woody tissue remaining after extraneous materials (waxes, pectins, fats, proteins, etc.) and lignin are removed with minimal polysaccharide loss(2). Two such extraction procedures commonly used are called the chlorine-methanolamine method and the chlorite method(2). The "holocellulose" can

be further fractionated into α-cellulose and hemicelluloses
by the application of aqueous solutions of alkalies, e.g.
18% sodium hydroxide. The α-cellulose is primarily poly-β-1,
4-D-anhydroglucopyranose while the hemicelluloses recoverable
by acidification of the alkaline supernatant of the above
treatment are a mixture of polysaccharides, many of which
contain no glucose (4).

Whereas lignin is deposited primarily in the middle la-
mella of wood fibers, holocellulose is found in the various
layers of the cell wall. In general, alpha cellulose is
found in highest proportion in the layers nearest the lumen
with decreasing concentration toward the middle lamella,
while hemicelluloses are more concentrated in the primary
layer of the cell wall (1). Within each layer of the second-
ary wall, the cell wall constituents are aggregated into
bundles called microfibrils. The arrangement of these micro-
fibrils within the layers of the wall has been observed by
light and electron microscopy (1, 5). The fine structure of
microfibrils from various sources has also been revealed by
electron micrographs (5). Immergut (5) reviewed the informa-
tion concerning these fine structures and presented data and
photographs to illustrate that microfibrils from various
sources differed in length and diameter. The microfibrils

were believed to be of indefinite length and from 100 to 200-400 A in diameter.

The details of the molecular structure of cellulose have been subjects for investigation almost from the time Payen proposed a name for the substance. This is because many unusual properties which cellulose has are believed due to the structural organization of the large molecules. Many theories have been proposed to explain the insoluble nature of the material, the differences in ease of degradation of various forms of cellulosic material, the apparent imperfect crystallinity as revealed by X-ray diffraction studies, etc. These theories are adequately reviewed by Hearle (10) and Immergut (5) so that they shall be only briefly discussed here.

Naegli (7) was one of the first to propose a crystalline arrangement for plant fiber fine structure. He proposed a "brick and mortar" type of arrangement whereby crystalline areas, termed _micelles_, were interspersed with an indeterminate inter-micellar "mortar". This theory ultimately received support when X-ray diffraction studies appeared to show discrete crystalline structure (8). Controversy concerning the length of the micellar regions led to a proposal of a continuous crystalline polymer by Staudinger

(9) and others. He suggested that crystalline high polymers must consist of imperfect crystals in which the end-groups of the molecules appear as local distortions. Elements of both theories were incorporated into the "fringed micelle" concept which is currently favored by many. In this theory and its numerous variations (c.f.10), closely packed parallel oriented cellulose chains from crystallite areas where numerous opportunities for hydrogen bonding can take place. On occasion a certain proportion of the chains lose their parallel orientation, the chains having become less closely packed, forming a non-crystalline or "amorphous" region. The crystallite or micellar regions are believed to be much shorter than the lengths of the individual chains, which may be in excess of 2000 glucose units (11). Therefore most fringed micelle theories assume that individual chains run through several crystalline and amorphous regions. Those chains found in the amorphous regions would be more accessible to hydrolytic agents, since hydrogen bonding would be less of a factor.

Current fringed micelle concepts take into account the electron microscopic information showing that microfibrils can exist with about half the diameter previously proposed for them (6, 10). Other theories, entirely different from

those previously proposed, have been forwarded by Manley (12)
and Marx-Figni and Schulz (13). Manley proposed a unique
model invoking a helical arrangement to the microfibril,
with the chains so oriented that the lead of the helix causes
them to be generally parallel to the long direction of the
microfibril. During the process of the laying down of the
linear molecules reversals of directions would take place,
with bends in the chains accounting for noncrystalline prop-
erties. Marx-Figni and Schulz also suggest that the reversal
of direction of linear chains may account for amorphous re-
gions. They believed that the enzymatic apparatus for syn-
thesizing the chains was associated with microtubules observ-
able using electron microscopy. Unlike Manley's proposal, a
helical arrangement for cellulose microfibril components is
not necessary.

The occurrence of cellulases. Enzymes which degrade
cellulosic materials, cellulases, are produced by a wide
variety of organisms. Such enzymes have been found to be
associated with protozoa (14, 15, 16), invertebrate animals
(17, 18, 19, 20, 21, 22, 23, 24, 25), vertebrates (16, 26,
27, 28), and vascular plants (29, 30, 31, 32, 33, 34) as well
as bacteria and fungi. Protozoans frequently are found in
association with other animals, such as arthropods (15, 24),

mollusks (35), or ruminants (16). It has been difficult in the past to determine which of the organisms in such symbiotic or commensal relationships is responsible for cellulase production. Recently cellulases have been found to be produced directly by mollusks (17, 18, 20, 22) and arthropods (21, 25). Verterbrates are not believed to produce their own cellulases, but to rely on those produced by cellulolytic microorganisms housed within certain portions of the digestive tract (16, 27, 36). Celluases produced by vascular plants have been found to be important in growth (29, 30, 31), ripening of fruit (33) and abscission (32, 34).

The two most important groups of cellulolytic organisms are the fungi and bacteria. Fungi are important in well aerated terrestrial environments, especially when a low pH is found (81). Siu (38) compiled a list of almost 300 fungal species capable of decomposing cellulose. Among the genera represented were Aspergillus, Chaetomium, Fusarium, Memnoniella, Myrothecium, Polyporus, Stachybotrys and Trichoderma. Certain species have become frequently employed by those who wish to obtain large amounts of cellulase material for biochemical research. Some commonly used organisms are Chaetomium globosum (39), Myrothecium verrucarai (40, 41, 42), Trichoderma viride and T. koningi (42, 43, 44) and Stachybotrys atra (45, 46). Several companies in Europe, the

United States and Japan obtain commercial preparations of
cellulases from <u>Aspergillus</u> and <u>Trichoderma</u> spp.

Cellulose decomposing bacteria have been considered im-
portant organisms for quite a while. Mitscherlisch (47), in
1850, attributed cellulose degradation to vibrioid organisms.
Others, observing the decomposition of cellulose in plant
material by <u>Bacillus</u> <u>amylobacter</u>, included Popoff and Hoppe-
Seyler (48, 49). Omelianski (50) was concerned with anaerobic
bacteria which fermented cellulose. The organisms with which
he concerned himself were found to be in mixed rather than
axenic culture leading Omelianski to investigate the question
of associative action by two or more organisms on cellulose
(51, 52). Van Iterson (53) described aerobic cellulolytic
bacteria including a very small rod called <u>Bacillus</u> <u>ferrugi-</u>
<u>nus</u>. The latter was always associated with a large micro-
coccus that van Iterson believed stimulated the rod but did
not participate directly in cellulose degradation. Some now
believe that these organisms may have been <u>Sporocytophaga</u>
<u>myxococcoides</u> or a related organism, with the "micrococcus"
actually being the "sporoids" of the <u>Sporocytophaga</u>.

The early work of Karl Kellerman and his co-workers at
the U.S. Bureau of Plant Industry is of special notice.
Kellerman and his assistants, especially I. G. McBeth, F. M.

Scales and N. R. Smith, were responsible for first isolating and characterizing most of the species now included in the genus _Cellulomonas_ plus several others in different genera. They deviated from the previous limited successes of elective culture used by Omelianski and Van Iterson and devised plating methods for cellulolytic bacteria (67, 68).

The organism now called _Sporocytophaga myxococcoides_ was first obtained in pure culture by Hutchinson and Clayton (54). The detailed descriptions and photographs provided by these authors suggested to them that _Spirochaeta cytophaga_ (_S. myxococcoides_) possessed a life cycle passing through various phases. Current knowledge indicates that they actually observed both vegetative and resting cell forms of this organism.

The number of species shown to be able to decompose cellulose was increased considerably during the two decades following Hutchinson and Clayton's work. Winogradsky, within this period, made important contributions concerning cellulolysis by bacteria. In addition to perfecting a new method for isolating bacteria on solid cellulose media (the silica gel technique) and formulating important theories for the mode of action of some cellulases (the oxycellulose theory), he was responsible for naming and characterizing cellulolytic

species of the genera Cytophaga, Cellvibrio and Cellfalcicula
(55, 56). Cytophaga hutchinsonii, Winogradsky, was for many
years believed to be identical with the organism later named
Sporocytophaga myxococcoides (57). Although first accurately
described by Hutchinson and Clayton, S. myxococcoides was
later observed under various synonyms by several workers
(Krzemieniewska (58), Imsenecki and Solntzeva (59), Stapp and
Bortels (60)). Stanier (57) determined that C. hutchinsonii
differed from S. myxococcoides in one important respect, that
of not forming mycrocysts, and therefore has been accorded
separate recognition.

Some of the above authors found several vibrioid organ-
isms which were capable of decomposing cellulose. Winograd-
sky based his oxycellulose theories on the action of some
Cellvibrio species that he discovered. Dubos (37) reported
on some vibrioid cellulolytic bacteria which were later char-
acterized by Stapp and Bortels (60) and named Cellvibrio
fulvus and C. vulgaris. Gray and Chalmers (62) observed an
organism Microspira (Vibrio) agar liquifaciens capable of
degrading cellulose and agar. Several decades after these
early discoveries, Hulcher and King added the important spe-
cies Cellvibrio gilvus (63) and Kadota reported on numerous
cellulolytic marine vibrios (64).

Cellulolytic actinomycetes were reported by Krainsky (65) who included them in a general work on the genus Actinomyces. Rubentschik later observed some of these and other cellulolytic actinomycetes in lake bottom mud (66). Fuller and Norman (86) reported on several soil isolates, including species of Pseudomonas and Bacillus. Many of the Pseudomonas and Bacillus spp. found by Kellerman's group (67, 68) are now classified as Cellulomonas spp. As with Cytophaga, there has been some confusion surrounding the nomenclature of the genus Cellulomonas. Species in this genus have formerly been called Bacillus, Bacterium, Proteus and Corynebacterium. Clark (69) has clarified this situation removing many of the synonyms in the process.

Most of the above named organisms were aerobic or facultative anaerobic bacteria. Anaerobes have also been found to be important in the processes of cellulose degradation. Sporeformers such as Bacillus or Clostridium spp. may function in anaerobic environments. Thermophilic clostridia such as Cl. thermocellum (70, 71) and Cl. thermocellulaseum (72) have been shown to be especially important, the latter during such processes as composting. Another very important group has been the cellulolytic rumen bacteria. These organisms, in conjunction with the rumen methane bacteria (76),

are essential to the well being of ruminants feeding on materials with high cellulose content. Organisms such as Bacterioides succinogenes, Butyrivibrio fibrisolvens, Ruminococcus flavefaciens and Ruminobacter parvum (73, 74, 75, 77) were shown to degrade cellulose with the production of organic acids which are used by other rumen bacteria for the production of cell protein. The organisms which use the products of the cellulose fermentations produce volatile fatty acids from dietary protein. These fatty acids are required by some of the cellulolytic species (77). Each member of the rumen microflora may benefit from the presence of the others, and the ruminant has been found to benefit from the presence of the microflora by having its food partially degraded and having a ready source of usable protein.

Backgrounds of species used for experimental work. Of the species used for experiments herein, most were characterized initially by Kellerman and co-workers during the period 1912 to 1913. Cellulomonas fimi, C. gelida, C. flavigena, C. uda, Bacillus bibulus and Bacterium liquatum all emerged from that laboratory. C. flavigena (Bacillus flavigena) was obtained from one of Omelianski's cultures but found to differ from both his hydrogen and methane bacteria (78). Most of the above species were originally obtained from soils.

Unlike many of the strictly aerobic cellulolytic bacteria found by others later in the century, these organisms were facultative with respect to oxygen requirements. They were also found to be able to grow in a variety of artificial media, although prolonged subculture caused some to lose cellulose degradatory power. Organic nitrogen in the form of peptone may be used while inorganic nitrogen in the form of ammonium may be supplied to C. gelida, C. uda and C. flavigena with only the last of these unable to use nitrate (79). These and three other Cellulomonas species were found to exhibit typical coryneform morphology and seemed to form a unique group when examined for vitamin requirements and cell wall composition (80). Clark (69) considered B. bibulus and C. gelida to be synonyms and B. liquatum synonymous with C. fimi.

As indicated previously, Cytophaga butchinsonii was for a long time confused with the organism now called Sporocytophaga myxococcoides. Although differentiated by the ability of the sporocytophaga to form microcysts, these organisms and other cytophagas share many characteristics. It had long been thought that cellulolytic cytophagas were unable to use any sole carbon source other than cellulose. Stanier (83) showed that sugars autoclaved with media were found to produce toxic products while sugars sterilized by other means

were not toxic. Glucose was shown by Fahreus (82) to be tox-
ic at some concentrations even if prepared in the preferred
manners. Cytophagas exhibit elastotaxis, orienting themselves
parallel to the direction of cellulose fibril axes or other
surfaces, including agar (54, 57, 61, 83). Characteristics
such as flexible cell walls, gliding motility, the failure
to produce cell free zones of degradation (56) and elasto-
tactic orientation have led many to believe that a prerequ-
uisite for the degradation of insoluble cellulosic substrates
by cytophagas is direct contact between cell surface and
substrate. Finally, cytophagas produced surface mucilages
which, in the case of S. myxococcoides, have been found to
be direct products of cellulose degradation, formed by the
process of transglycosylation (89).

The cellvibrios first described by Winogradsky (55) and
Stapp and Bortels (60) had less unusual morphological fea-
tures than the pleomorphic Cellulomonas or the sinuous,
flexible Cytophaga. Stapp and Bortels described Cellvibrio
fulvus (Culture Y, Dubos (37)) and Cv. vulgaris (Culture Co,
Dubos (37)) as standard vibrios distinguished by their abil-
ity to utilize cellulose as the sole carbon source. Cv.
fulvus, as with most cellvibrios, grew poorly on all carbo-
hydrates except cellulose, lactose and starch, but Cv.

vulgaris grew well with most carbohydrates. Nutrient broth was a poor medium for both. Cellulose decomposition was inhibited by high concentrations of celloligosaccharides but not dextrins. A preference for cellobiose was found by Hulcher and King (63, 84) for _Cv. gilvus_. They demonstrated that utilization of this sugar was accomplished using a cellobiose phosphorylase system. Since glucose was converted directly to gluconic acid it yielded less energy per mole than the hexoses derived from cellobiose. _Cellvibrio polyoltrophicus_ (85) the last of the cellvibrios used in this report, was a provisional designation for an organism isolated from soils which was found to utilize various polyhydric alcohols and grow on cellobiose as sole carbon source.

Bacillus aporrheus was one of the soil organisms isolated by Fuller and Norman (86) which they used for their studies on the effect of non-cellulosic material on the decomposition of "natural" celluloses. It was one of the least active species used in these studies. It is currently the only member of the genus _Bacillus_ available from the American Type Culture Collection which was originally distinguished as being cellulolytic. Xylan was found to be important to decomposition of cellulose from cornstalk or jute by _B. aporrheus_ (95).

A variety of the common _Pseudomonas fluorescens_ was
used by Japanese authors (87) to examine the effect of car-
bon sources on the formation of cellulases. _Ps. fluorescens_
var. _cellulosa_ formed small amounts of cellulase constitua-
tively but an active multicomponent extracellular cellulase
was induced by highly polymerized cellulosic substrates,
sophorose and very low concentrations of cellobiose. A cell
bound fraction was found to exhibit transglycosylation simi-
lar to that shown for _S. myxococcoides_ (88, 89).

Cellulases and cellulolysis. The biological degradation
of cellulose is mediated by cellulase enzymes. Unienzymic
cellulases have been proposed for certain fungi (40), but
evidence now seems to favor multicomponent enzyme systems
operating on somewhat distinct substrates (91). A model for
such systems was proposed by Reese et al. (92) after observa-
tions that some non-cellulolytic organisms were able to de-
grade soluble cellulosic materials (carboxymethyl-, hydro-
xyethyl- and methyl cellulose) at rates similar to some cellu-
lolytic organisms. Culture filtrates from non-cellulolytic
species were found to contain enzymes very similar to "cellu-
lases" produced by cellulolytic forms. This model, with some
later modifications is indicated below as follows:

Native ⟶ Shorter linear ⟶ cellobiose
Cellulose   poly AHG

C_1 C_X B-gluco-
                                                              sidase

                                                        glucose
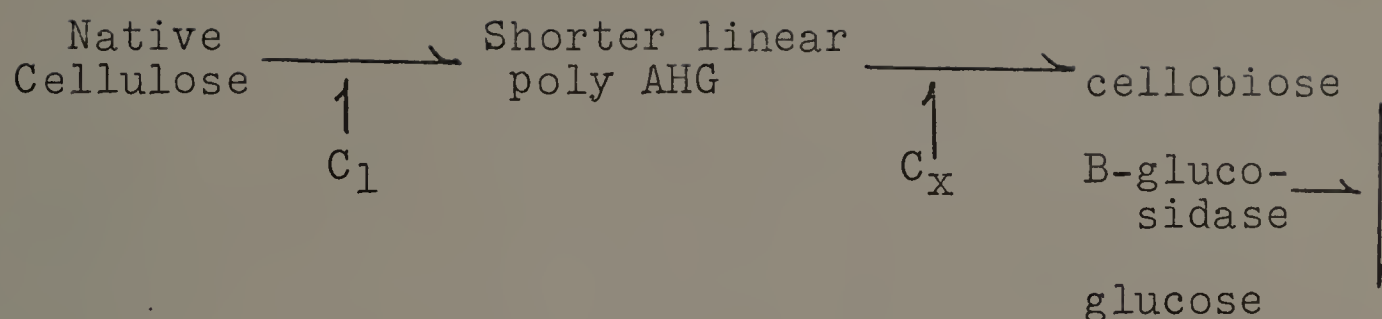
The first component (C_1) is believed to cause the separation of the crystalline micelles releasing individual poly-β- 1, 4 - anhydroglucopyranose (poly AHG) molecules. This may not necessarily cause a change in degree of polymerization (D.P.). Sui (93) first suggested what has been called a "hydrogen-bondase" for C_1. He later indicated that this was suggested in semi-jest (94), but recently this view has received some serious consideration (95). Mandels and Reese (96) showed that C_1 acted so as to permit an increase in moisture uptake, thus hydrating the cellulose and separating the linear molecular chains. The major linkages were thus exposed and accessable for C_X hydrolysis. Li et al. (44) using Avicel or hydrocellulose, believed that the key to C_1 is its ability to attack highly crystalline cellulose. Halliwell (43) showed that perioxide formation may accelerate cellulose degradation, thus allowing for the suggestion of a biogen hydrogen peroxide as C_1 factor (93).

The C_X components apparently attack amorphous areas preferentially and split linear chains into smaller units.

These enzymes should be called β- 1, 4-D- glucan 4 glucano-
hydrolases (E.C. 3.2.1.4.) (44, 93, 97).

Cellobiase is the last major component of cellulase sys-
tems in the sequence. This β-glucosidase cleaves oligosac-
charides of up to six glucose molecules and, in some organisms,
may have transglycosylase activity (98). Charpentier (89)
confirmed the synthetic function of the "cellulase" of cyto-
phagas originally suggested by Winogradsky (56) by demonstrat-
ing transglycosylation in the formation of the mucilage of
Sporocytophaga myxococcoides.

Enzymes with C_1 or C_x activity have been at least parti-
ally characterized after isolation from Aspergillus niger,
Penicillium notalum, Trichoderma viride and T. koningi,
Myrothecium verrucaria, Polyporus versicolor, Streptomyces
gilvus, Cellvibrio gilvus, Pseudomonas fluorescens var.
cellulosa, and Sporocytophaga myxococcoides (87, 88, 89, 91,
98, 99). Most appear to be endoglucanases, with exoglucanases
apparently identified from Aspergillus. spp, Trichoderma
viride and Cellvibrio gilvus (91, 98).

Until recently only a few papers concerning bacterial
cellulases had been published. This may have been due to
the greater importance of fungi in deterioration of fabrics
and foodstuffs and the greater ease in obtaining enzyme

preparations in large quantities from fungal sources.

In discussing bacterial cellulases it is important to consider the mode of attack of the organism on cellulosic substrates, especially filter paper like substrates. Terui and Fujiwara (100) proposed the term "weathering" to describe the action of Bacillus hydrolyticus on filter paper. Weathering results in the physical separation of the fibers of filter paper without the production of large amounts of reducing sugars. "hydrolytic" attack involves the production of much reducing sugars while the physical state of the residual cellulose is only slightly altered. Okamoto and Asai (101) considered the type of decomposition shown by Ps. fluorescens var. cellulosa to be "hydrolytic" also. Nisizawa (102) associated the terms "weathering and hydrolytic" with "random" and "endwise" cleavage, respectively, of cellulase chains. Components of the Pseudomonas fluorescens va. cellulosa system were considered to be of the "random" cleavage type, though it was suggested that a better terminology for that cellulase system might be "less random" (versus endwise) (102). A true endwise component has been suggested for Cellvibrio gilvus (98).

Although it has been considered that most cellulase systems were inducible, considerable evidence has been obtained to indicate that constitutive components exist in

the cellulases of <u>Cv</u>. <u>gilvus</u> (98), <u>Ps</u>. <u>fluorescens</u> var. <u>cel-
lulosa</u> (87, 88), <u>Clostridium thermocellum</u> and <u>Cellulomonas
spp</u>. (103). The constitutive component of the pseudomonad
was a minor component, however, when the organism was grown
on cellulosic materials or non-cellulosic inducers such as
sophorose and gentiobiose (87).

The question of the location of cellulases in bacteria
has been a topic of discussion for some time. Whereas wide
zones of decomposition surrounding <u>Cellvibrio</u> colonies in
solid media indicated extracellular enzymes, other bacterial
cellulases were believed to be associated with the cell enve-
lope. Tightly bound cellulases have been found in <u>Sporocyto-
phaga myxococcoides</u> (89) and <u>Pseudomonas fluorescens</u> var <u>cel-
lulosa</u> (87), though the latter also produces extracellular
components. Rahn and Leet had suggested that the cellulases
of <u>Cellulomonas</u> species were on or in the membrane (105), but
Porter (105) found that trypinization of the cells had only
limited effect on observed activity concluding that the cel-
lulases of <u>Cellulomonas flavigena</u> were not membrane bound.

Multiple components for cellulases have been demon-
strated for <u>Sporocytophaga myxococcoides</u>, <u>Cellvibrio gilvus</u>
(98), <u>Pseudomonas fluorescens</u> var. <u>cellulosa</u> (87, 88) and
<u>Streptomyces gilvus</u> (99).

Finally, Youatt (106) after finding much difference be-
tween the activities of two rapid bacterial cellulose decom-
posers, S. myxococcoides and Cellulomonas biazotea, on the
same substrates, cautioned about the danger of attempting
"to deduce a composite theory of cellulose breakdown by the
combination of results with different organisms and differ-
ent substrates."

Criteria. Although there have been many reports of the
isolation of bacteria from various habitats which possess
cellulolytic enzymes, there does not seem to be one set of
criteria for the demonstration that these isolates indeed
function as cellulose decomposers in their natural habitats.
Instead, there are almost as many criteria as reports. The
most common single criterion for the establishment that a
culture is cellulolytic is the demonstration of filter paper
destruction by the culture. Loss of integrity of a strip of
filter paper upon slight agitation of a test tube was an ac-
cepted sign of decomposition, especially if the strip sepa-
rated at the air-liquid interface when aerobic cultures were
tested. Popoff (48) used filter paper when investigating
cellulose fermentation by sewage slime. Omelianski (50) saw
filter paper change to a yellowish gelatinous mass when in-
oculated with slime from the river Neva. Van Iterson (53)

used filter paper as did Hutchinson and Clayton (54), Wino-
gradsky (55), Stapp and Bortels (60) as well as many later
workers too numerous to mention. Instead of using strips
immersed in tubes of liquid, some authors used variations of
the technique, such as embedding filter paper in agar (as
practiced by Dubos (37), or silica gell as used by Winogradsky
(56) and Imsenecki and Solntseva (59). Using filter paper,
cytophagas usually could be distinguished macroscopically by
the formation of translucent "windows" in the paper. These
were areas where the paper seemed to disappear to be replaced
by a mucilage in which was imbedded the vegetative cells (and
microcysts in the case of Sporocytophaga) of the active or-
ganisms.

Cotton has been used often as a substrate for cellulo-
lytic organisms, but not usually for the purpose of establish-
ing criteria for cellulose degradation. Most often, cotton
is used for special purposes such as providing a natural
matrix on which to observe morphology (56). Defatted cotton
was used as one of several substrates for quantitative analy-
ses of cellulose degradation by Berg et al. (107).

Cellophane was used by Stapp and Bortels (60) as an al-
ternative to filter paper. Went (108, 109) and Thomas (112)
used cellophane for microscopic analyses, since any change in

substrate is easily demonstrable under polarized light. However, using mixed cultures, individual species could not be identified and only vegetative cytophaga cells could be recognized. (109).

Cellulose agars employing ground up filter paper or various precipitated celluloses have been used as bases for the determination of cellulolytic capability. The criterion chosen with these substrates has been the formation of zones of clearing surrounding each cellulolytic colony (78). Winogradsky (56), using the same basic method, preferred silca gel to agar. Though many species produce clearing zones by elaborating extracellular cellulases, others have more tightly bound enzymes and do not exhibit this reaction (56). Since many species are strict aerobes it is usually necessary to provide a thin cellulose agar layer above clear agar in order to observe the zones (110).

The use of soluble cellulose derivatives has found increasing consideration as an aid in determining the mode of action of cellulases. Reese et al. (92) used the production of reducing sugars by culture filtrates acting on various derivatives (carboxymethyl-, hydroxyethyl-, and methyl cellulose) to indicate degradation. They considered "cellulolytic" to mean capable of attack on "native" cellulose (filter

paper, cotton) while cleavage of modified celluloses was considered C_x activity (c.f.). The C_1-C_x scheme was proposed to account for observed differences in the production of 1, 4-β-glucanohydrolases by diffrent organisms. Action toward both "native" and modified cellulosic substances by "cellulolytic" organisms indicated the presence of both C_1 and C_x activity, while "non-cellulolytic" species could still produce C_x activity. This separation of cellulolytic from non-cellulolytic was, however, not clearcut. Non-cellulolytic bacteria did not produce evidence of C_x, but some non-cellulolytic fungi did. Cellulolytic bacteria such as <u>Cellulomonas</u> and <u>Sporocytophaga</u> did not produce large amounts of reducing sugars, though cellulolytic fungi generally did. These authors also used a non-quantitative criterion for cellulase production, that of liquifaction of extremely viscous preparations of soluble derivatives.

An alternate technique involving the use of soluble cellulose derivatives for the determination of cellulase activity is viscometry. Those who have developed this method (111, 112, 113) have generally utilized cellulase preparations from fungi, often supplied by commercial sources. The use of living bacterial cultures has not been reported. Cellulolytic ability is determined by a change in viscosity of the

substrate caused by a shortening of the linear anhydroglu-
copyranose chains (111).

No consistent preference has been shown for a particu-
lar cellulose derivative by those who have used more than
one such material in their research. Reese et al. (92)
seemed to favor carboxymethyl cellulose (CMC) because both
degree of substitution and degree of polymerization could be
varied and controlled more easily than hydroxyethyl cellu-
lose (HEC). CMC was also stable when autoclaved at varying
pH values. Wirick (113) stated, however, that CMC at a given
substitution index (S.I.) levels offered greater protection
to viscosity loss (was less readily degradable) than exam-
ples of other water soluble cellulose ethers including HEC,
hydroxypropyl cellulose (HPC) and methyl cellulose. Unfor-
tunately, the latter ethers were not usually available at
D.S. values as low as some CMC ethers. Thus, the least sub-
stituted HEC tested had approximately 18% unsubstituted an-
hydroglucose (AHG) units and two or more adjacent AHG units
were required for rapid chain scission. Two CMC samples had
62% and 30% unsubstituted AHG but required three or more un-
substituted AHG units for chain breakage. It would seem
from this data that some of the less substituted CMC might
allow hydrolysis as well as most HEC which are of higher S.I.

Another criterion for the determination of cellulolytic activity is that of loss of tensile strength of cellulose textiles (114,115). The technique generally involved the placement of twisted cotton or rayon cord under plots of soil or bottom deposit and removal after periods of incubation for machine testing (116, 117). Went (108) has found that, even with non-twisted regenerated cellulose, reproducible results were difficult to obtain.

Other less direct techniques have been used to determine activity. Porter et al. (105) monitored activity using manometric techniques devised by Umbreit (119). Norkrans (104) used a turbidometric method to determine cellulose decomposition. Using cellulose sol precipitated from cuprammonium solution, extinction values were determined for several preparations using the Lambert-Beer formulae. A decrease in extinction value of the cellulose preparations, due to the action of cell-free preparations of celluloses, was compared with heat inactivated controls, and taken as a measure of cellulolytic activity. However, since the average size of the cellulose particles causing the extinction varied with the D.P., no correlation between dry matter and extinction value was found to exist.

Other substrates for cellulase determination have included oligoglucosides not necessarily of β -1, 4-linkage.

Lichenin has been used by Fahreus (120), though the β-1, 3
linkages were not clearly established at that time. Whit-
aker (121) used β-1, 4 oligoglucosides for his analyses.
The current versions of the model for cellulase systems
originally proposed by Reese et al. (92) now include pro-
visions for so called 'cellobiases' which can cleave oligoglu-
cosides higher than two AHG units (89, 98). However, it is
probably a bit extreme to classify an organism in a cellu-
lolytic genus solely on the basis of cellobiase utilization.
This appears to have been the case of <u>Cellvibrio polyoltro-
phicus</u> (85).

Criteria for the characterization of cellulases have
been proposed in detail by Whitaker (122). Methods for de-
termining specificity, rates of activity, effects of various
parameters of these rates, sites of attack on different sub-
strate forms and mechanisms of hydrolysis were reviewed.
All of these discussions assumed at least partial purifica-
tion of the enzyme preparations. These criteria were not
established to permit recognition that a particular culture
was cellulolytic, but to determine the characteristics of the
enzymes produced by previously recognized cellulolytic organ-
isms.

Criteria for the establishment of cultural purity of
cellulolytic bacteria have been indicated by Hungate (74).

While proposed specifically for anaerobic bacteria, they may
be applied to aerobic cultures. The criteria were based on
the ability of cultures to produce clearing zones in agar
media containing finely divided cellulose. Cells from such
colonies were diluted and subcultured on solid cellulose
media with the numbers of new colonies expected to corres-
pond to the size of the inoculum and dilution factors. Con-
sistency of colonial morphology was checked by subculture
using clear sugar agar media. Resultant colonies were ex-
pected to give rise to appropriate numbers of cellulose clear-
ing colonies when transferred back to cellulose agar. These
criteria assumed that all cellulolytic bacteria tested would
grow and produce clearing zones, would produce one colony
forming unit per cell on cellulose agar, would do so on sugar
agar without losing the ability to produce clearing when re-
transferred to cellulose.

Three difficulties arise with this system. First, not
all cellulolytic species grow well on agar media, though re-
duction of agar content seems to produce acceptable growth
with most cultures. Secondly, not all may produce diffusable
extracellular enzymes needed to give the effect of clearing.
Finally, most cellulases seem to be inducible and their pro-
duction may be reduced if soluble sugars which can be

metabolized are introduced in the medium. Transfer back to cellulose may give fewer than expected numbers if some cells are unable to readapt to cellulase production, thus giving the appearance of impurity.

Most of the above methods and criteria have been applied to cell free systems derived from terrestrial organisms. Applications to aquatic situations are lacking. Several investigators have discussed the role of cellulolytic bacteria in water (e.g. 64, 66, 123, 124, 125, 126), but most have relied on the decomposition of filter paper strips as sole criterion for cellulolytic activity. Other substrates and methodologies have been used infrequently (116, 117).

Though a variety of criteria exist, no set adequately defines the cellulose decomposing bacterial culture. What is needed is a consistency with respect to the definition of what is meant by "cellulose" and a consistency with respect to the nature of the alteration of the cellulose substrate which is accepted as "cellulolysis".

PRELIMINARY RESULTS AND RATIONALE

The initial aim of this study was to correlate the techniques used and information gained by those working with cellulolytic organisms in such a manner that this information might aid the detection of cellulolytic aerobic bacteria in various environmental situations. Rather than determining the specific characteristics of a species enzyme complement, a microbial ecologist is interested in the frequency of this organism's presence in a specific environmental situation. Enzymological data helps provide an indication of the functional capability of the species, provided that species is found sufficiently frequently in the total microbial population of a particular environment to produce significant effects. The problem now exists that the ecologist does not have as advanced a methodology for the determination of the presence of a cellulolytic species in particular habitats, as is possessed by the biochemist for determining that which the organism might accomplish if it were present.

Some of the techniques used by biochemists were examined to see if they could be modified or used outright to enable the microbiologist to observe cellulolytic bacteria obtained from field sources. Of most interest were characteristics observable in the large majority of cellulolytic bacteria

likely to be encountered. Techniques were wanted which, initially, were non-discriminatory with respect to certain qualitative aspects of cellulose-decomposing ability. It was not desired to separate immediately those organisms which possessed "C_1" from those which did not, as long as both exhibited "C_x". Methods were needed which did not require excessive manipulation of the viable culture. Finally, it was hoped that any methods which were adopted would retain the relative speed and accuracy inherent in many of the biochemical techniques.

One device which initially seemed promising was the use of Cellulose Azure (Calbiochem) which consists of an insoluble cellulosic substrate to which has been coupled a blue dye that is released when the cellulose is attacked by microorganisms. This is believed to be an indication that the cellulose chains have been sufficiently hydrolyzed to become soluble. Some of the cellulolytic cultures tested in this study proved incapable of causing dye release, while others caused release quite readily. Questions have been raised concerning the possible preferential hydrolysis of the dye linkage instead of the cellulose proper. The questions raised during those preliminary tests, suggested that this technique could not be pursued profitably for the purposes of this study.

A second method investigated was the Glucostat (Worthington Biochemicals). This technique employs glucose oxidase coupled to an indicator system to provide a colorimetric analysis for glucose in solution. It was believed that, since glucose may be produced from the hydrolysis of cellulose, it could be measured as an indicator of cellulase activity. This had been done previously using fungal enzyme sources (127). Barely detectable amounts of glucose have been obtained from the organisms in preliminary tests. It is believed that this was due either to the production of higher homologs, such as cellobiose or cellotriose, to which Glucostat will not respond, or to the immediate uptake of glucose from the hydrolyzed cellulose by viable cells. Both mechanisms may have been operative.

Another technique which was examined was similar to one used by soil microbiologists. An attempt was made to devise a workable perfusion system for the investigation of cellulolysis by bacteria. Such systems might provide an artificial environment more like that found in aquatic situations than ordinary laboratory culture methods. Except for the use of acid cleaned pure quartz sand, the system was otherwise similar to those used for soil perfusion (128). In the system used, a predetermined amount of cellulosic material was ground and mixed with the sand and placed in the per-

fusion column. The entire unit was sealed and all outlets plugged with cotton. Media was autoclaved in the separatory funnel portion of the assembled apparatus or was sterilized separately and added to the system aseptically, depending on the medium required.

The technique was designed such that two separate indicators of cellulolytic activity might be obtained with one setup. The fluid percolating through the cellulose-sand mix would contain hydrolysis products which might be detected by reducing sugar or glucose analyses. Gravimetric analysis of the column material would give another means of determining decomposition.

Bacteria were inoculated via syringe and eventually were believed to become located primarily within the column portion where places of attachment existed similar to those found in aquatic habitats (sand grains, detritus, etc.). Membrane filtration of the reducing sugar aliquot removed unwanted cells remaining in suspension.

The technique failed for a multitude of reasons. First, it was too easy to contaminate the apparatus. Fluctuations in the vacuum source caused overflows at the sample outlet thus providing an entry portal for contaminants. Partial disassembly of the apparatus for any reason could produce contamination, even with the greatest of care. The time

involved in operation of the system enhanced the difficulty of monitoring the fluid levels and could allow contaminants to overwhelm the inoculated species.

It was not possible to get a workable system for measuring reducing sugars when growth media were present. Either the media chosen did not support a sufficient proportion of the cellulolytic species chosen, or they interfered with the Somogyi-Nelson (129, 130) analysis procedure used during these preliminary studies. Since the interference problem was later corrected by modification of the colorimetric analysis procedure it is possible that medium problems might eventually have been solved, but they were not pursued due to the combined effects of all the difficulties encountered with this analysis system.

Finally, cellulose did not remain in the column even when plugged with glass fiber. Filters, which might have been used to prevent the migration of cellulose particles, would have entrapped bacteria as well, clogging, and causing failure in the circulation of the system. Removal of the unwanted weight of bacterial cells would have been difficult. Thus, errors would be introduced due to loss of cellulose in suspension, if unfiltered, or addition of microbial weight whether filtered or not. It was not possible to solve enough of the above, and other problems to make the perfusion

system workable although, it is felt that such a system might
be perfected which would provide valuable information on
rates of decomposition or total decomposition of cellulosic
materials as affected by various parameters.

During the process of attempting to develop the above
methods, it was recognized that each one seemed to be based
on a different criterion for cellulolytic activity and could
be applied only to those organisms which satisfied that par-
ticular criterion. There did not seem to be a consistent
set of criteria for all aerobic mesophilic cellulolytic bac-
teria. Yet, it seemed that this group of organisms should
have a great deal in common. Therefore, it was decided to
concentrate on finding these commonalities, if they existed.

The problem was approached in the following manner. Of-
ten, these species are placed in genera which are almost ex-
clusively cellulolytic. Therefore, it was thought that by
choosing among these active species and by comparing their
activities toward various cellulosic substrates under similar
conditions with the activity, or lack thereof, exhibited by
related forms not generally recognized as cellulolytic, one
could discover some areas of similarity which might provide
bases for development of uniform criteria. Of interest would
be the activity of these organisms as they might be observed
by a researcher attempting to discriminate between

cellulolytic and non-cellulolytic forms newly obtained from certain environments. Therefore, maximum biochemical activity of enzyme preparations was not of greatest concern since they would not necessarily reflect the function of these organisms in the particular environmental situations considered.

Due to restrictions resulting from a decision to employ viable cultures, relatively few biochemical techniques were found to be useful for these studies. The use of techniques which measure specific end products would have limited value due to potential uptake of these products. It was decided to concentrate on changes in the physical properties of the substrate as focal points for analyses. Techniques requiring specialized equipment, such as X-ray diffraction apparatus, which would not likely find routine use for ecological studies, were considered impractical and were not investigated. The availability of adequate substrate material was found lacking for tensile strength analyses (108). Although relatively imprecise, gravimetric methods seemed to give consistent data over the years. Viscometric techniques for soluble forms of cellulose seem to be gaining favor recently and appeared to be readily adaptable to viable cultures.

On the basis of the above considerations, a variety of aerobic mesophilic bacteria, reported to be cellulolytic,

and some related non-cellulolytic species were tested versus a variety of cellulosic materials using four techniques: gravimetry, viscometry, respirometry, and reducing sugar analyses. Responses by the various organisms to the test procedures were observed and categorization of the test cultures based on these results was suggested.

MATERIALS AND METHODS

Test strains. Cultures of the following atrains were

employed for these investigations. The majority of them were

obtained from the American Type Culture Collection (ATCC)

and are so designated by ATCC identification numbers:

Arthrobacter globiformis (Dr. R. Walker, Univ. of Mass.)
Bacillus aporrheus ATCC 9500
Bacillus cereus (Dr. H. Gunner, Univ. of Mass.)
Bacterium bibulum ATCC 483
Bacterium liquatum ATCC 485
Cellulomonas fimi ATCC 484
Cellulomonas flavigena ATCC 482
Cellulomonas gelida ATCC 488
Cellulomonas uda ATCC 491
Cellvibrio fulvus ATCC 12120
Cellvibrio gilvus ATCC 13127
Cellvibrio polyoltrophicus ATCC 14774
Cellvibrio vulgaris ATCC 12209
Cytophaga hutchinsonii (Dr. R. Walker, Univ. of Mass.)
Cytophaga johnsonii ATCC 17061
Pseudomonas fluorescens ATCC 11250
Pseudomonas fluorescens var. cellulosa ATCC 13042T

The nomenclature of several of these species is subject

to question. The Bacterium spp. are no longer considered to

be distinct entities (69) but are still listed as separate

from the corresponding Cellulomonas species in the 1970 edi-

tion of the ATCC catalog. Bacterium bibulum is believed to

be a variety of Cellulomonas gelida while C. fimi and Bact.

liquatum are considered synonymous. Cellvibrio polyoltrophi-

cus is a provisional name for a soil vibrio which uses cel-

lobiose as well as various polyhydric alcohols as carbon

sources (85). R.Hugh has suggested that _Pseudomonas fluorescens_ subsp. _cellulosae_ may be an _Alcaligenes_ sp. (132). This organism appears variously in the literature as a subspecies or variety with corresponding endings for "cellulosa(e)". Similarly the specific names for the cytophagas used may have an "ii" or "iae" ending.

ATCC cultures were revived according to instructions supplied with the cultures. However, to ensure that an entire culture would not be lost if an initial attempt failed, part of the pellet provided was not rehydrated immediately. Frequently, media suggested for rehydration were not appropriate for development of cellulolytic activity. Subsequent transfer to the more appropriate medium often resulted in failure of the culture. Therefore, it was found feasible to start cultures using media designed for development of cellulolytic ability slightly modified with 0.05% cellobiose in addition to filter paper. Since, with the exceptions of the cytophagas, all the ATCC cultures were supplied as rather large pellets contained in double vials, the dried cultures could be fragmented by agitation of the stoppered inner vial or manipulation with a sterile inoculating loop, each fragment being more than sufficient to start a culture. Restarting was done frequently when cultures "died" out or when cultural purity was in question.

The following growth media were employed. Cultural

specificity is indicated:

Medium #1 Peptone Broth (ATCC medium 31)

Peptone (Bacto)   5 g
Distilled water   1 liter

A strip of filter paper or Hercules Chemical Cotton

is immersed in a container (tube or flask) such that

a portion of the cellulose is always above the air

liquid interface.

Medium 2 Cytophaga medium (modified Bolds basal medium)

		Trace elements	
$NaNO_3$	75.0 g	$ZnSO_4.7H_2O$	8.82g
$CaCl_2$	3.32g	$MnCl_2.4H_2O$	1.44g
$MgSO_4.7H_2O$	7.5 g	MoO_3	0.71g
$KoHPO_4$	7.5 g	$CuSO_4.5H_2O$	1.57g
NaCl	2.5 g	$CoNO_3.6H_2O$	0.49
Trishydroxylmethyl-		the above dissolved	
aminomethane, pH7.6	50.0 g	in 1 liter H_2O	
H_2O	1 liter		
EDTA (50 g) and KOH			
(31g) in 1 liter H_2O	1ml		

$FeSO_4. 7H_2O$(4.98g) in
1 liter H_2O(acidified
1 ml H_2SO_4/999ml H_2O 1ml

H_3BO_3(11.42 gm/liter) 1ml
Trace elements   1ml
cellobiose   0.005g

1 strip cellulosic substrate as in Medium #1

Medium #3 Yeast Extract Medium (ATCC medium #112)

K_2HPO_4	1.0 g
$MgSO_4$	0.5 g
Yeast Extract (Difco)	10.0 g
Tap water	1 liter (for Cyt. johnsonii)

Medium #4 Morris' medium (modified) (138)

K_2HPO_4	7 g
KH_2PO_4	3 g
KNO_3	4 g
$MgSO_4.7H_2O$	0.2 g
$FeCl_3$	0.01g
$CaCl_2$	0.01g
H_3BO_3	5.6 mg
$MnCl_4.4H_2O$	3.7 mg
$CuSO_4.5H_2O$	0.4 mg
$NaMoO_4.2H_2O$	1.5 mg
$(Co(NH_3)_6) Cl_2$	0.6 mg
$ZnSO_4.2H_2O$	0.5 mg
cellobiose	2 gm
H_2O	1 liter

cellobiose is replaced by cellulose strip for
decompositio studies (for A. globiformis)

Medium #5 Qwens and Keddies' medium (79)

		Trace minerals	
$(NH_4)_2SO_4$	0.5 g		
K_2HPO_4	1.04g	EDTA	5.0 g
KH_2PO_4	0.75g	$ZnSO_4.7H_2O$	2.2 g
$CaCl_2$	0.025g	$MnSO_4.4H_2O$	0.57g
$MgSO_4.7H_2O$	0.2 g	$FeSO_4 17H_2O$	0.5 g
NaCl	0.1 g	$CoCl_2.6H_2O$	0.161g
Trace minerals	3 ml	$CuSO_4.5H_2O$	0.157g
		$NaMoO_4.2H_2O$	0.151g
Substrate and Growth Factors[1]		H_2O	1 liter
cellobiose	2.0 g	pH	6.8
Thiamine HC_1	500 g		
Ca pantothenate	500 g		
nicotinic acid	500 g		
vitamin B 12	2 g		
biotin	1 g		

(for Cellulomonas spp. but served well for all but Cv.
fulvus, Cv. polyoltrophicus and pseudomonads)

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[1]These six were added aseptically after autoclaving.

Medium #6 Dubos' medium (37

$NaNO_3$	0.50 g
K_2HPO_4	1.0 g
$MgSO_4 \cdot 7H_2O$	0.50 g
KCl	0.50 g
$Fe_2(SO_4)_3 \cdot 9H_2O$	0.01 g
distilled H_2O	1 liter

Medium #7 Fuller and Norman's (133)

$NaNO_3$	1	g
K_2HPO_4	1	g
KCl	0.5	g
$MgCl_2 6H_2O$	0.5	g
Yeast Extract (10g/100ml)	10	ml
H_2O	1	liter

(for B. aporrheus)

Medium #8 Hulcher and King's (63)

$NaNO_3$	0.5	g
KCl	0.25	g
K_2HPO_4	0.25	g
$MgSO_4$	0.5	g
H_2O	1	liter

cellobiose	2.0	g
carboxymethyl cellulose	4.4	g
Yeast extract	1.0	g
enzymic casein hydrolyzate	5.0	g

The cellobiose, yeast extract and casein hydrolyzate were
added aseptically as solutions.
(for Cellvibrio gilvus)

In all the above media cellulose strips or carboxymethyl
cellulose were the prime carbon sources with cellobuose
added to help start or maintain cultures. When used for

viscometric assays, the media contained 0.2% CMC. For gravi-
metric studies the amount of cellulosic substrate varied
from 0.3-1.0 grams per 100 milliliters. Since these were
water insoluble materials the amount of available cellulosic
matter varied with the decomposing ability of each test or-
ganism.

After successfully starting and subculturing the ATCC
organisms in suggested media, cultures were transferred to
media with the sole carbon source being a cellulose strip.
The cellvibrios require organic nitrogen or vitamin supple-
ments either of which probably contained some non-cellulosic
carbon. These species did not grow well in the medium fa-
vored by the majority of the other species, Medium #5. Me-
dia #1, #7 or #8 were therefore used for Cellvibrio spp.
Pseudomonas species preferred Dubos' (#6) or peptone (#1)
to medium #5 but were viable in the last medium. Bacillus
aporrheus did not grow well in any medium including that
which was used for its original isolation (#7).

Reference cultures were maintained in test tubes con-
taining a cellulose strip and the most favorable medium. No
soluble carbon source was intentionally provided during main-
tenance of a period of months. Storage temperature was 4C
and incubation temperature was 26C.

Substrates. The following cellulosic substrates were
used for these studies:

Insoluble.

Raw cotton linters (Hercules Inc.)
Solka Floc   (Brown Co.)
Hercules Chemical
    Cotton   (Hercules Inc.)
        Grade PS33   (D.P.   600)   P1HS-43 (D.S. 0.4-0.16)
              PS14   (D.P. 1000)
              PS21   (D.P. 1500
    Filter paper (Whatman)

Soluble.

Hercules Cellulose Gum (Hercules Inc.)
    Grade CMC 4H1   (D.P. 1000   D.S. 0.4)
          CMC 7H   (D.P. 1000   D.S. 0.7)
          CMC 7L   (D.P. 300   D.S. 0.7)
          CMC 7M   (D.P. 500   D.S. 0.7)
          CMC 9M8   (D.P. 500   D.S. 0.9

The list of insoluble substrates includes both wood

(Whatman filter paper and Solka Floc) and cotton celluloses

(Hercules Chemical Cotton and raw linters). Approximate

polymerization values for three of the substrates were ob-

tained from the manufacturers and are indicated above. Raw

linters are shorter than natural cotton fibers, but the D.P.

values are larger than the processed celluloses though no

data is available since D.P. is variable. No D.P. estimates

have been obtained for the wood celluloses. A substituted

ethylhydroxyethyl cellulose was also used. Substitute val-

ues are shown above.

Soluble carboxymethyl cellulose substrates covered a wide range of D.P. and D.S. values. Preferential utilization of substrates with respect to either or both of the above variables was possible using a series of substitutes. Hercules Inc. provided extensive data concerning physical characteristics of its CMC materials, thus aiding in the selection of materials for this study (131).

Viscometric determinations. The viscometric technique is most commonly employed for use with soluble cellulose substrates. It relates the reduction in viscosity of a cellulosic solution with the reduction of chain length (D.P.) due to hydrolytic action of cellulases on the cellulosic substrate. The usual technique employed involves the extraction of the cellulolytic enzymes from their source and their introduction to a solution of cellulose derivative in a supporting medium. The reaction usually is allowed to take place in the reservoir of a viscometer such as those of the Ostwald or Cannon types. These instruments measure viscosity as a function of the efflux time for the meniscus of the fluid. The viscosity of a sample can be computed by the comparison with a standard solution under identical conditions.

For these studies, quantities of growth media appropriate to the species being investigated, were prepared using

one of the carboxymethyl celluloses added as sole carbon
sources at 0.2% concentration (W/V). Two hundred ml. of media
were distributed into a 300 ml. Ehrlenmeyer flask and ster-
ilized by autoclaving. Replicate flasks were prepared to
allow for at least one uninoculated control per batch of
fresh media as well as additional inoculated flasks.

Capillary viscometers of the Ostwald or Cannon-Fenske
type were used. Five ml. aliquots were used for viscosity
determinations. After the fluid level had been raised above
the upper reservoir by the application of suction, the efflux
time for each aliquot was determined as the time required for
the meniscus to pass by two engraved marks delimiting the
ends of the upper reservoir. Temperature was maintained at
26C which gave a value of approximately 50 seconds for the
efflux time for water or mineral solution in each of the
three viscometers employed.

After determination of the initial viscosity of uninocu-
lated samples, the remainder of each was inoculated with 2
ml. of a six-day liquid culture of the strain to be observed.
The inoculated sample was immediately tested for any change
in efflux time. After each determination the viscometer was
emptied and cleaned in a solution of 5% NaOH in 95% ethanol.
Because of the time required to ensure cleanliness of the ap-
paratus, readings were separated by at least eight hours,

since the same instrument had to be used with a particular
sample flask. If a rapid early viscosity decrease were ob-
served greater than could be accounted for by the addition
of less viscous fluid during inoculation, readings of the
same aliquot were made on a continuous basis for up to four
hours, after which readings were discontinued until the in-
strument had been thoroughly cleaned. Otherwise, readings
were taken daily, when possible, using fresh aliquots for
each determination, until little or no positive change was
noted on two successive daily operations. Readings were con-
tinued if such observations were noted within the initial
three day period since lags or other fluctuations were found
to be occasional during preliminary testing of the system.

Change in viscosity of a sample was calculated in the
following ways:

Reciprocal Specific Viscosity $(1/\eta_{sp})$. The calcula-
tions for this value were:

$$1/\eta_{sp} = T_o / (T-T_o),$$

T_o = the efflux time of the solvent, which was water,
at time t

T (or T_e) = the efflux time of the solution at time t

Percent Change in Efflux Time ($\%\Delta T$). The calcula-
tions for this value were:

$$\%\Delta T = (T_i-T_e)/(T_i-T_o) \times 100$$

T_o = same as above

T_e = same as above

T_i = initial efflux time of the solution (or T_e when

$t = t_o$)

Reducing sugars determination. Methods exist which measure properties which indirectly relate to the decomposition of the substrate. The reducing sugar analysis is a frequently employed technique which uses this approach. The rate of increase in reducing ends can be related to the rate of increase in cleavage of polyanhydroglucose chains.

The analytical procedure chosen for this study was a modification of the method of Nelson (129) as modified by Somogyi (130) for colorimetric analysis. Interference by unknown components of the various growth media used was observed when initial attempts were made to use the basic Somogyi-Nelson technique. The modifications indicated below were attempts to eliminate the effects of such interference.

Two ml. of copper reagent were added to 5 ml. of test sample, media blank and reference sample containing glucose at a concentration similar to that expected in the test sample. The tubes were mixed and immersed in a boiling water bath for ten minutes after which they were removed and immersed in a room temperature water bath and allowed to cool. Two ml. of arsenomolybdate color reagent were added to each tube. The tubes were mixed, water was added to bring the

final volume to 25 ml. and the tubes were allowed to stand for 15 to 40 minutes as in the basic method. A precipitate generally formed in each tube after mixing and settled to the bottom within a few minutes. After color development was complete an aliquot from the surface of each tube was removed for determination of optical density at 500 nanometers. Alternatively, the contents of each tube were filtered through glass fiber filters of one micron approximate pore diameter. The latter method had the advantage of giving a larger number of usable aliquots per 25 ml. sample but required more equipment and greater time. Both techniques appeared to result in the same values of optical density for reference samples.

Another reducing sugar analysis technique which was briefly employed was that of the Glucostat (Worthington Biochemical Corporation). As mentioned earlier (p. 34) this method measures only glucose (with certain special exceptions). The Glucostat system is a coupled colorimetric enzymatic assay for β-D-glucose using glucose oxidase and peroxidase as the enzyme system as follows:

$$\text{glucose} + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} H_2O_2 + \text{gluconic acid}$$

$$H_2O_2 + \text{reduced chromogen} \xrightarrow{\text{peroxidase}} \text{oxidized chromogen}$$
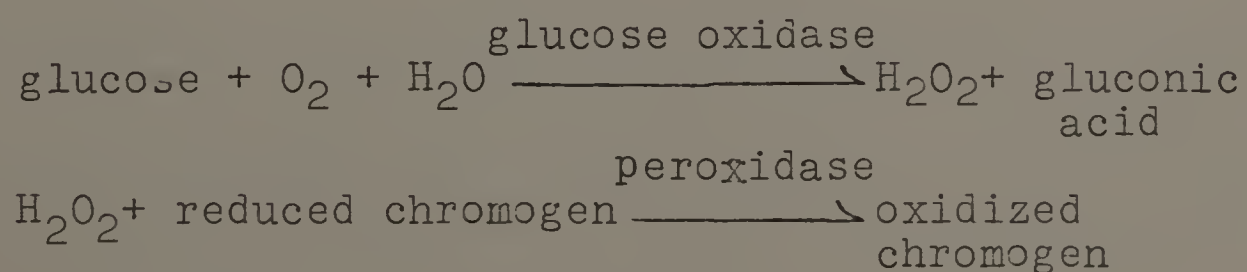
The technique used was that described in the instructions accompanying the Special Glucostat as used by Meyers et al. (116) when they examined marine fungal cellulolytic activity. No modifications were made to this method. As indicated previously, little glucose was detected, and this method was not used after preliminary testing was complete.

Gravimetric determination. The gravimetric method is a basic technique for measuring decomposition of insoluble cellulosic substrates which has been favored for quantitative analyses over the years since it involves a direct determination of change in the physical state of the substrate.

For these studies cellulosic substrates were ground to a powder form in a Wiley mill, were used as strips or were used in a "natural" state (cotton linters).

Substrates and filters were dried and tared in aluminum weighing dishes. Reeve Angel 934 AH glass fiber filters having approximate pore diameters of one micron were used. Substrates were placed in flasks, autoclaved and 200 ml. of appropriate sterile growth media were added aseptically. The flasks were then inoculated with the test cultures and incubated at 26C with rotary shaking. Zero time and sterility controls were uninoculated. Zero time controls provided an indication of filtration efficiency and any loss in dry weight which occurred was subtracted from the final dry

weight change for each set of flasks, thus giving a baseline
reading of zero.

Following incubation the contents of each flask was re-
moved and centrifuged at 3000 r.p.m. (International centri-
fuge) for ten minues. The supernatant was decanted with
care to avoid loss of residue which was repeatedly washed by
centrifugation until the supernatant was clear. The residue
was collected by filtration through the glass fiber filters.
It was washed until few bacteria could be detedted in the
filtrate microscopically at which time both filter and resi-
due were returned to the original weighing dishes and dried
overnight at 85C. After being allowed to cool to a constant
weight, final determinations were made using a Mettler bal-
ance. Percent change in dry weight was determined in the
following manner:

$(W_f - W_o)/ W_o \times 100 = \% W$

W = dry weight, o = original, f = final

<u>Manometric determination</u>. The uptake of oxygen or re-
lease of carbon dioxide have been used to monitor respira-
tion and, indirectly, enzyme activity of microbial prepara-
tions for some time (119). Porter et al. (105) used res-
pirometry to help measure the effect of trypsinization on
membranes of cellulolytic bacteria in order to locate the
cellulases. In the studies presented here, manometry was

used in conjunction with viscometric analysis to observe cel-
lulolytic activity.

A Gilson Differential Respirometer was used for all
manometric studies. Operating techniques were similar to
those of other respirometric apparatus except that flasks
and manometers were interchangeable between experiments with
no flask constant computations required.

Cultures were maintained in a solution of the substrate
to be used for respirometric studies for a period of six
days. For ease of handling and relative ease of observation
of viscosity change over a short period of time, Hercules
Cellulose Gum 7L was chosen. An 0.2% solution of this sub-
strate in Owens and Keddie's (79) mineral medium was used to
grow cultures which were harvested and washed by centrifuga-
tion. Cells were resuspended in M/50 phosphate buffer at a
concentration of about 10 mg per ml. (dry weight base).

Inoculated flasks contained either growth medium of buf-
fer in one side arm, and KOH or no KOH in the center well
for carbon dioxide absorption. One flask was uninoculated
but had growth medium. A sixth flask was used in one experi-
ment in which a nitrogen free medium was substituted for
standard Owens and Keddie's mineral solution in an attempt
to gain some information concerning the formation of new

protein, i.e. the possibility of induction of cellulases.

After equilibration at 26C the reaction in each flask was started by allowing the culture to mix with growth media or buffer, and frequent readings were made over periods of time up to nine hours. After the first readings, a visco-metric experiment was begun using the same culture as the respirometric experiment and designed to run simultaneously with it.

RESULTS

<u>Soluble subtrates</u>. Data obtained from viscometric analyses of the decomposition of carboxymethyl cellulose (Hercules Cellulose Gum) by species of aerobic bacteria are presented in Tables 1 through 5. Table 1 and Figures 1 through 13 show the results obtained using coryneform bacterial species. Six genera are represented; three <u>Cellulomonas</u>, two <u>Bacterium</u>, and one <u>Arthrobacter</u> species. The <u>Arthrobacter</u> sp. is the only one not commonly considered cellulolytic. The <u>Bacterium</u> spp. are probably variants of two <u>Cellulomonas</u> spp. (69).

Figures 1 through 3 show the effect of degree of substitution (D.S.) and degree of polymerization (D.P.) of the decomposition of several carboxymethylcellulose preparations by three <u>Cellulomonas</u> species. The data is presented as reciprocal specific viscosity versus time in days. The slope of the curve in its steepest region represents the maximum rate of change of the reciprocal specific viscosity ($1/\eta_{sp}$). This value ($d(1/\eta_{sp})dt$) has been computed for all cultures and is shown in Table 6. It was obtained from numerical data by dividing the difference in values for $1/\eta_{sp}$ found to be in the steepest segment of the curve appropriate for each organism-substrate combination by the time differential involved. Not all curves for all test organism-substrate combinations can be demonstrated to be linear over significant

lengths of the steepest portions of these curves. Therefore, these $d(1/\eta_{sp})/dt$ calculations must be considered approximate values in many cases.

Figure 1 shows these results for C.[1]flavigena. There appears to be little difference among the curves for the substrates having D.S. of 0.7 either for rate of change or maximum change. In a similar manner data presented as percent change in efflux time ($\%\Delta T$) shows little difference in rates of change and minor differences in total change of this value for 0.7 D.S. carboxymethyl cellulose. (Fig. 4) Carboxymethyl cellulose with a D.S. of 0.4 appeared to be decomposed at a rate somewhat faster than other carboxymethyl celluloses used by this organism when reciprocal specific viscosity was used as a measure, but this difference was deemphasized when $\%\Delta T$ was plotted. Both methods showed CMC 9M8 to be changed less rapidly and completely than the other substrates. Lags in the rate of decomposition for substrates 7L and 4H1 appear with both types of computation, but other lags shown in Fig. 1 may be reflections of the differences in emphasis of the two methods used. Reciprocal specific viscosity curves tend to emphasize differences in large values while $\%\Delta T$ emphasizes differences in small values.

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[1]Cellulomonas.

Figure 2 diagrams the viscosity change data for C. gelida. The graph for $1/\eta_{sp}$ seems to indicate little difference among the rates of decomposition or final values for the three substrates with 0.7 D.S. An exception is the rapid change with CMC 7H as substrate noted after four days. This change is suggested in the plot for $\%\triangle T$ (Fig. 5) but is deemphasized there. The change represents only a few seconds decrease, and the drop noted in both figures is also rather small. The differences between CMC 4H1 and the other substrates are less dramatic for C. gelida than seen with C. flavigena. The graph for $1/\eta_{sp}$ emphasizes the difference between the change in CMC 9M8 and the other substrates. Lags are noted for most substrates, with negative values observed with CMC 4H1 and CMC 7L. The maximum rates of change with substrates 7L and 9M8 are not very different and in Fig. 5 the appearances of the curves for $\%\triangle T$ have seom similarities.

The data obtained with C. uda also shows the overall similarity of substrates with DS 0.7, especially Cellulose Gums 7H and 7M. Neither rate nor maximum change of specific viscosity for substrate 4H1 was very great (Fig. 3). This was also reflected in $\%\triangle T$ (Fig. 6). Substrate 9M8 was utilized better by this organism than by the other two cellulomonads (Figs. 3 and 6).

Figures 7 through 11 show direct comparisons of the three organisms previously mentioned. These date indicate that, with the exception of substrate 4H1, $\underline{C}$. $\underline{uda}$ decomposes carboxymethyl cellulose more completely than the other two organisms. This is shown by the higher final values for $1/\eta_{sp}$ found in Figs. 8 through 11 for $\underline{C}$. $\underline{uda}$. $\underline{Cellulomonas}$ $\underline{flavigena}$ produced somewhat higher values than $\underline{C}$. $\underline{gelida}$ for most substances. Figure 12, which chows values of $\%\triangle T$ for all coryneform bacteria and substrate 7H, indicates less obvious differences tha noted in Fig. 8 due to the exaggeration of high values by $1/\eta_{sp}$ plots.

On the basis of values found for both $\%\triangle T$ and $1/\eta_{sp}$ it was decided that the most appropriate single carboxymethyl cellulose substrate for comparison among organisms was 7H. Cellulose Gum 7M gave similar data, however, and might be an equally useful substrate for this purpose. Most of the following results for soluble substrates pertain to experiments using Cellulose Gum 7H.

Three other coryneform bacteria were examined for ability to decompose CMC 7H in addition to the $\underline{Cellulomonas}$ spp. Data for $\underline{Bacterium}$ $\underline{bibulum}$ and $\underline{Bact}$. $\underline{liquatum}$, as well as the non-cellulolytic $\underline{Arthrobacter}$ $\underline{globiformis}$ are shown in Figs. 12 and 13. $\underline{Cellulomonas}$ $\underline{gelida}$ is included in Fig. 13 as a comparison with the synonymous $\underline{Bact}$. $\underline{bibulum}$. The

<u>Bacterium</u> cultures do not appear to produce as rapid nor as complete a change in the substrate as the <u>Cellulomonas</u> cultures while <u>A</u>. <u>globiformis</u> does not appear to produce any change in the substrate at all.

Data for bacterial species belonging to the <u>Pseudomonadaceae</u> are presented in Tables 2 and 3. The graphs of these data are shown in Figs. 14 through 16. <u>Cellvibrio gilvus</u> appears to be as active as some of the <u>Cellulomonas</u> spp. Despite several attempts it was not possible to demonstrate activity toward CMC 7H by the other two cellvibrios much greater than that exhibited by the <u>Arthrobacter</u> sp. This is shown both in Figs. 14 and 16.

Two pseudomonads were examined and found to be nearly identical. Both have been assigned to the species <u>Pseudomonas fluorescens</u> but one is considered a subspecies or variety with special cellulolytic ability. Initial attempts to obtain decomposition by the cellulolytic variety were not very successful (Table 3) but by modifying the growth media and "training" the organism using low concentrations of cellobiose (87) and CMC 7H improved activity was found. Plots of the data obtained using the improved methodology for both varieties are shown in Figs. 14 and 15. The reciprocal specific viscosity graph shows little more than that for the poorly effective cellvibrios but the $\%\Delta T$ plot separates

these into two distinct groups. However, the data for these pseudomonads is not as indicative of active decomposition as that of the Cellulomonas or Cellvibrio gilvus presented in the same figures.

Table 4 and Figs. 17 and 18 presented the results of ciscosity experiments using two species of Bacillus. B. aporrheus is supposed to be a cellulolytic form and B. cereus a common non-cellulolytic species. The data show that B. aporrheus was less active than B. cereus though neither can be considered to have produced much change in the substrate.

Finally, Table 5 and Figs. 19 and 20 give data obtained using cultures of Cytophaga spp. Cytophaga hutchinsonii was the most active species used in these experiments. Substrates 4H1 and 7H were both decomposed rapidly. Though the two curves seem to be superimposed in Fig. 20, initial viscosity differences caused a separation in the graph of $1/\eta_{sp}$. Substrate CMC 7L was decomposed very rapidly but was followed for a very short time only so that completeness of decomposition is not known. CMC 9M8 was also decomposed rapidly at first but decomposition stopped after only one day. A non-cellulolytic but chitinolytic Cytophaga johnsonii was surprisingly effective versus CMC 7H. The rates of change for Cyt. hutchinsonii were the highest for any test organism regardless of substrate employed (Table 6). That for Cyt.

<u>johnsonii</u> was among the lower values found, but completeness of decomposition, as indicated by the highest values for $1/\eta_{sp}$, was better than for most species except <u>Cyt</u>. <u>hutchin-sonii</u>, <u>Cv</u>.[2] <u>gilvus</u> and the cellulomonads.

Attempts were made to correlate the data obtained from viscosity change measurements with other independent indicators of activity. One method involved the simultaneous analysis of viscosity change and the production of reducing sugars. A modified Somogyi-Nelson test was employed for reducing sugar analysis, and the standard curve is shown in Fig. 21. The range was somewhat shorter than the standard Somogyi-Nelson procedure and values varied somewhat more than when distilled water was used as the diluent. The precipitate which formed from the growth medium after the boiling stage did not appear to interfere with the accuracy of the test, except that extra steps were required to remove the precipitate before reading in the spectrophotometer.

When the analyses were performed a single aliquot was removed from the growth flask at the appropriate time and split; half being used for viscometry, half for reducing sugar analyses. Data for three organisms tested in this manner are shown in Fig. 22. The organisms chosen were

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[2]<u>Cellvibrio</u>.

Cv. gilvus, a very active culture, Bact. bibulum, a less ac-
tive one, and B. cereus, a relatively inactive organism. As
can be seen in this Figure, the correlation among the curves
is good when reciprocal specific viscosity and reducing
sugars are compared. A similar correlation was found when
percent change in efflux time was used as the viscosity mea-
sure.

Figure 23 shows another attempt at correlating viscosity
change with some other indicator of activity. It was decided
to determine if activity was directly related to the basic
metabolic activity of the organism in question as indicated
by uptake in oxygen. For this purpose, simultaneous experi-
ments involving respirometry and viscometry were performed.
Because of the nature of the methodologies involved, the data
shown in Fig. 23 represents two separate experiments per-
formed at the same time using cultural material and substrate
(CMC 7L) from the same sources, but not the same aliquot.

The Figure shows that respiration begins to decrease
very soon, even when substrate is present, suggesting the
early exhaustion of a critical nutrient. For the duration
of these experiments the viscosity change did not come to a
halt.

Insoluble substrates. Table 7 summarizes the data ob-
tained when the decomposition of insoluble cellulosic

substrates was examined. Observations were for periods of
at least two weeks. The data indicates that, for most of
the strains examined, there were ten to twenty percent weight
losses from most substrates tested. The three unsubstituted
Chemical Cotton products were decomposed to about the same
degree by most species, although when the three materials,
PS 14, PS 21 and PS 33 were directly compared, PS 14 was
usually found to be least degraded. The hydroxyethyl cellu-
lost sample P1HS-43 does not appear to have been favored by
any species nor did it appear to be very inhibitory. Raw
cotton linters produced high values from Cv. gilvus and Bact.
liquatum but not C. flavigena. Solka floc wood cellulose was
attacked in a similar manner to the cotton celluloses. Cv.
fulvus did not produce any decomposition the one time de-
compostion of insoluble materials was observed quantitatively
and the health of this culture became suspect when this data
was combined with that for soluble cellulose derivatives.

TABLE 1.
DECOMPOSITION OF CARBOXYMETHYLCELLULOSE 7H
BY AEROBIC BACTERIAL SPECIES

CORYNEFORM BACTERIA

Species	Time Elapsed	$\bar{T}_o$	$\bar{T}$ (seconds)	$\bar{T}_c$	$\frac{1}{\eta}_{sp}^{1}$	$\%\triangle T$	Reducing sugars $\mu g/5ml$
C.[2] flavigena	0 min	53.8	397.6	365.9	0.16		
	30 "		365.0		0.17	9.5	
	48 hrs		81.8		1.85	91.9	
	72 "		64.9		4.85	96.8	
	96 "		62.5		6.18	97.5	
	120 "		60.5		8.03	98.1	
	144 "		60.5		8.03	98.1	
C. gelida	0 hrs	49.9	371.7	324.3	0.16		
	48 "		176.0		0.40	60.8	
	72 "		87.5		1.33	88.3	
	96 "		71.2		2.34	93.4	
	120 "		54.9		9.98	98.4	
	144 "		66.4		3.02	94.9	
	168		65.2		3.26	95.2	
C. uda	0 hrs	54.3	328.9	330.1	0.20		
	48 "		209.0		0.35	43.7	
	72 "		176.9		0.44	55.3	
	96 "		103.6		1.09	82.0	
	120 "		61.6		7.44	97.3	
	144 "		58.0		14.68	98.6	
	168		57.3		18.10	98.9	
Bact.[3] bibulum	0 min	49.7	468.6	453.9	0.12		0.
	8 "		426.3		0.13	10.1	
	30 "		370.3		0.16	23.3	
	14 hrs		121.7		0.69	82.8	63
	38 "		84.8		1.41	91.6	135
	66 "		82.9		1.49	92.1	114
	112 "		77.3		1.80	93.4	127

[1] $T_o/T-T_o$ [2] Cellulomonas   [3] Bacterium

TABLE 1.
DECOMPOSITION OF CARBOXYMETHYLCELLULOSE 7H
BY AEROBIC BACTERIAL SPECIES

CORYNEFORM BACTERIA (Continued)

Species	Time Elapsed	$\overline{T}_o$	$\overline{T}$ (seconds)	$\overline{T}_c$	$\frac{1}{\eta_{sp}}$	$\%\triangle T$	Reducing sugars $\mu g/5ml$
Bact. bibulum	0 min	53.6	452.1	390.0	0.135		
	15 "		435.3		0.140	4.2	
	50 "		409.7		0.151	10.6	
	6 hrs		300.3		0.217	38.1	
	7 hrs		288.4		0.228	41.1	
	25 "		293.0		0.224	39.9	
	44		279.1		0.238	43.4	
	105		181.9		0.418	67.8	
	192		114.8		0.876	84.6	
	216		112.0		0.918	85.3	
	384		104.7		1.049	87.2	
Bact. liquatum	0 hrs	49.7	350.1	369.6	0.165		
	67 "		244.7		0.255	35.1	
	85 "		240.1		0.261	36.6	
	168		204.7		0.321	48.4	
	216		189.6		0.355	53.4	
	264		178.3		0.386	57.2	
	384		173.0		0.403	58.9	
Bact. liquatum	0 hrs	53.6	230.4	216.8	0.259		
	18 "		214.8		0.281	8.8	
	48 "		123.1		0.771	60.7	
	114 "		82.3		1.867	83.8	
	160 "		68.0		3.720	91.9	
A. globiformis[4]	0 hrs	53.6	389.5	400.1	0.160		
	24 "		386.4		0.162	0.8	
	110		393.8		0.158	-0.8	

[4] Arthrobacter

TABLE 2.

DECOMPOSITION OF CARBOXYMETHYLCELLULOSE 7H BY AEROBIC BACTERIAL SPECIES

VIBRIOID BACTERIA

Species	Time Elapsed	$\overline{T}_0$	$\overline{T}$	$\overline{T}_c$	$\dfrac{1}{\eta sp}$	$\%\Delta T$	Reducing sugars μg/5ml
Cv. fulvus[1]	0 hrs	49.7	216.8	221.3	0.252		
	0 "		220.1		0.249		
	18 "		221.1		0.247	-0.6	
	48 "		218.6		0.250	0.9	
	114 "		218.2		0.251	1.1	
Cv. gilvus	0 min	49.7	137.1	141.3	0.569		107
	15 "		130.2		0.617	7.9	132
	12 hrs		94.8		1.102	48.4	70-100
	60 "		54.5		10.354	94.5	
	84 "		54.1		11.295	95.0	
	114 "		54.0		11.558	95.0	
Cv. gilvus	0 hrs	53.7	645.8	646.7	0.091		17.5
	18 "		213.8		0.327	72.2	63-70
	76 "		70.6		3.177	97.1	245-255
	112 "		69.3		3.442	97.3	270-276
	160 "		67.9		3.782	97.6	254-271
Cv. polyoltrophicus	0 hrs	49.7	653.0	665.4	0.082		
	16 "		679.5		0.079	-4.4	
	114 "		659.2		0.081	-1.0	
	192		679.8		0.079	-4.4	
Cv. polyoltrophicus	0 hrs	49.7	222.1	225.0	0.288		
	22 "		220.3		0.291	1.0	
	48 "		219.1		0.293	1.7	
	144 "		213.5		0.303	5.0	
Cv. polyoltrophicus	0 hrs		223.9	225.9	0.315		
	20 "		220.2		0.322	2.1	
	44 "		209.5		0.344	8.4	
	68 "		210.7		0.341	7.8	
	120 "		209.6		0.343	8.4	

[1]Cellvibrio

TABLE 3.
DECOMPOSITION OF CARBOXYMETHYLCELLULOSE 7H
BY AEROBIC BACTERIAL SPECIES

PSEUDOMONAD BACTERIA

Species	Time Elapsed	$\bar{T}_O$	$\bar{T}$	$\bar{T}_c$	$\frac{1}{\eta_{sp}}$	$\%\Delta T$	Reducing sugars $\mu g/5ml$
Ps.[1] fluorescens var. cellulosa							
	0 hrs	53.7	451.7	388.5	0.135		
	6 "		423.7		0.145	7.0	
	30 "		413.9		0.149	9.4	
	70 "		416.7		0.148	8.8	
	125 "		400.9		0.155	12.8	
	150 "		382.9		0.163	17.3	
	180 "		378.6		0.166	18.4	
Ps. fluorescens var. cellulosa							
	0 hrs	53.6	397.2	577.0	0.156		
	24 "		362.8		0.173	10.0	
	52 "		289.3		0.227	31.4	
	94 "		246.5		0.278	43.8	
	120 "		222.3		0.318	50.9	
	146 "		206.3		0.351	56.6	
	170 "		193.4		0.383	59.3	
	222 "		177.7		0.432	63.9	
	312 "		162.3		0.493	68.4	
	366 "		151.4		0.548	71.5	
Ps. fluorescens							
	0 hrs	53.6	439.8	448.7	0.139		
	21 "		379.2		0.165	15.6	
	44 "		324.7		0.198	29.8	
	192 "		209.7		0.343	59.6	
	312 "		194.2		0.381	63.4	

[1]Pseudomonas

TABLE 4.
DECOMPOSITION OF CARBOXYMETHYLCELLULOSE 7H
BY AEROBIC BACTERIAL SPECIES

BACILLI

Species	Time Elapsed	$\bar{T}_0$	$\bar{T}$	$\bar{T}_c$	$\frac{1}{\eta_{sp}}$	$\%\triangle T$	Reducing sugars $\mu g/5ml$
B.[1] aporrheus							
	0 hrs	53.6	228.9	231.1	0.306		
	20 "		232.6		0.299	- 2.1	
	68 "		230.0		0.304	- 0.6	
B. aporrheus							
	0 hrs	53.6	280.3	284.5	0.236		
	24 "		274.3		0.243	3.2	
	48 "		271.9		0.245	3.7	
	72 "		265.6		0.279	6.5	
	96 "		270.0		0.248	4.5	
	216 "		262.6		0.283	7.8	
B. cereus	0 min	53.6	436.8	384.3	0.140		15μg
	10 "		367.3		0.171	18.1	
	17 "		364.3		0.173	18.9	
	25 "		360.5		0.175	19.9	
	32 "		360.0		0.175	20.0	
	14 hrs		306.7		0.220	33.9	20μg
	38 "		302.3		0.226	35.1	60μg
	62 "		305.4		0.222	34.3	∼ 0μg
	112 "		305.7		0.221	34.2	∼10μg

[1]Bacillus

TABLE 5.
DECOMPOSITION OF CARBOXYMETHYLCELLULOSE 7H
BY AEROBIC BACTERIAL SPECIES

CYTOPHAGA

Species	Time Elapsed	$\overline{T}_o$	$\overline{T}$	$\overline{T}_c$	$\frac{1}{\eta_{sp}}$	$\% \Delta T$
Cyt.[1] hutchinsonii						
	0 min	49.7	376.0	380.2	0.153	
	7 "		366.6		0.157	3.2
	13 "		363.0		0.159	4.0
	25 "		354.8		0.163	6.5
	1 hr 25 "		330.8		0.178	14.1
	8 hrs		215.8		0.300	49.1
	20 "		82.4		1.528	89.9
	68 "		59.3		5.242	97.2
	102 "		57.6		6.385	97.6
	117 "		57.5		6.467	97.6
Cyt. johnsonii	0 min	49.8	375.8	380.2	0.153	
	6 "		382.0		0.149	-2.1
	14 "		372.5		0.154	1.1
	25 "		370.8		0.155	1.5
	40 "		369.8		0.156	1.8
	24 "		251.7		0.247	38.1
	48 "		149.6		0.499	66.3
	80 "		97.6		1.042	79.5
	95 "		87.1		1.335	88.5
	118 "		81.8		1.556	93.2
	142 "		77.9		1.772	94.4
	172 "		75.0		1.976	95.3
	220 "		70.8		2.371	96.6
	262 "		69.4		2.541	97.0

[1]Cytophaga

TABLE 6.
CHANGE IN RECIPROCAL SPECIFIC VISCOSITY

Species	Substrate (Cellulose Gum)	$d(1/\eta_{sp})/dt$	Time range (hrs)
C. gelida	4H1	0.068	46
	7H	0.318	24
	7M	0.036	48
	7L	0.024	72
	9M8	0.023	24
C. flavigena	4H1	0.331	26
	7H	0.063	96
	7M	0.123	48
	7L	0.065	72
	9M8	0.005	216
C. uda	4H1	0.003	288
	7H	0.283	48
	7M	0.305	72
	7L	0.193	60
	9M8	0.059	24
Bact. bibulum	7H	0.041	14
Bact. liquatum	7H	0.040	24
A. globiformis	7H	0.000	24
Cyt. hutchinsonii	4H1	2.56	24
	7H	0.750	68
	9M8	0.439	72
	7L	0.337	9.25
Cyt. johnsonii	7H	0.016	172
Cv. fulvus	7H	0.000	48
Cv. gilvus	7H	0.163	60
Cv. polyoltrophicus	7H	0.001	44
B. aporrheus	7H	0.001	72
B. cereus	7H	0.006	14
Ps. fluorescens	7H	0.001	216
Ps. fluorescens var. cellulosa	7H	0.001	366

TABLE 7.
DECOMPOSITION OF INSOLUBLE SUBSTRATES
BY AEROBIC BACTERIAL SPECIES

Species	Substrate	Weight loss mg.	% Weight loss	Days
C. flavigena	PS14	112.7	11.3	15
	PS14	39.0	7.8	14
	PS21	96.1	18.9	14
	PS33	82.2	16.4	20
	PS33	94.2	18.7	14
	P1HS-43	39.0	7.7	14
	Solka Floc	88.0	17.6	14
	raw linters	55.1	10.9	14
C. uda	PS33	68.2	13.6	20
B. liquatum	PS14	55.0	10.8	14
	PS21	105.1	20.8	14
	PS33	55.1	10.9	14
	P1HS-43	87.3	17.1	14
	Solka Floc	88.0	17.6	14
	raw linters	370.4	48.8	14
Cv. gilvus	PS14	42.2	8.4	14
	PS21	127.0	25.4	14
	PS33	184.9	37.2	14
	P1HS-43	77.0	15.4	14
	Solka Floc	88.0	17.6	14
	raw linters	156.9	31.4	14
Cv. fulvus	P1HS-43	-7.1	-2.0	14
Cyt. hutchinsonii	P1HS-43	86.0	25.7	14
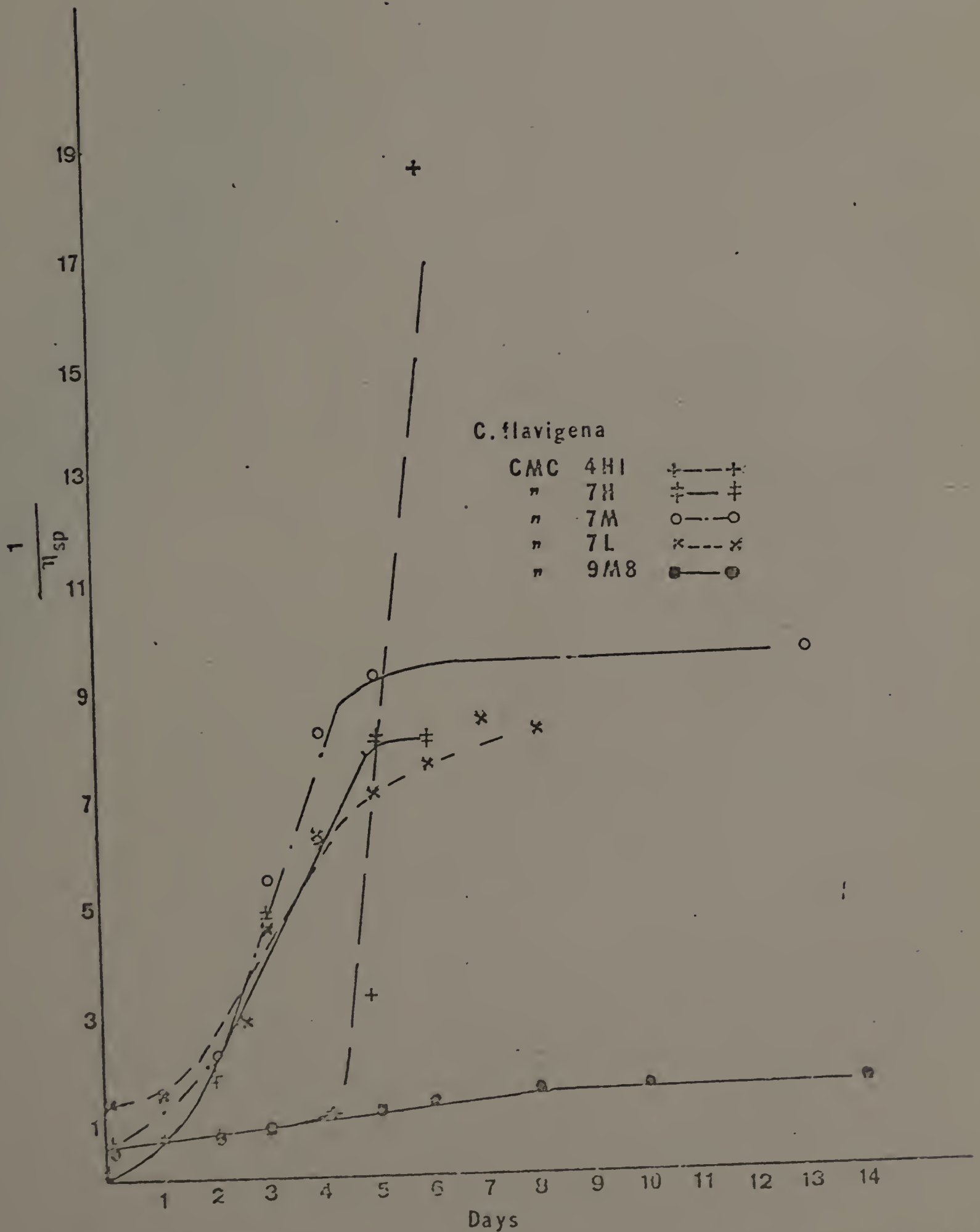
Fig. 1 Decomposition of Carboxymethyl Cellulose Substrates by *Cellulomonas flavigena*. Reciprocal Specific Viscosity.
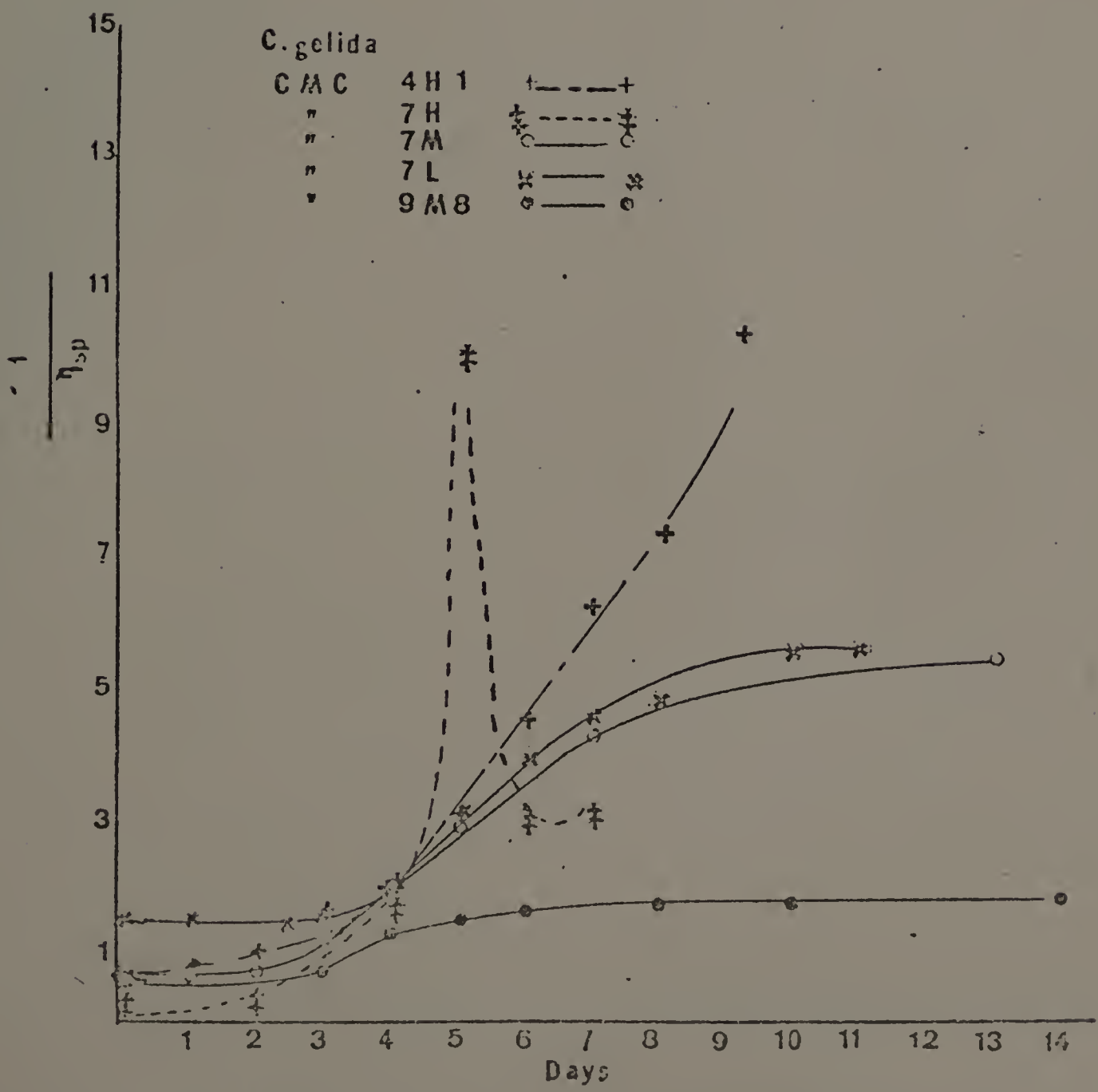
Fig. 2. Decomposition of Carboxymethyl Cellulose Substrates by _Cellulomonas Gelida_. Reciprocal Specific Viscosity.
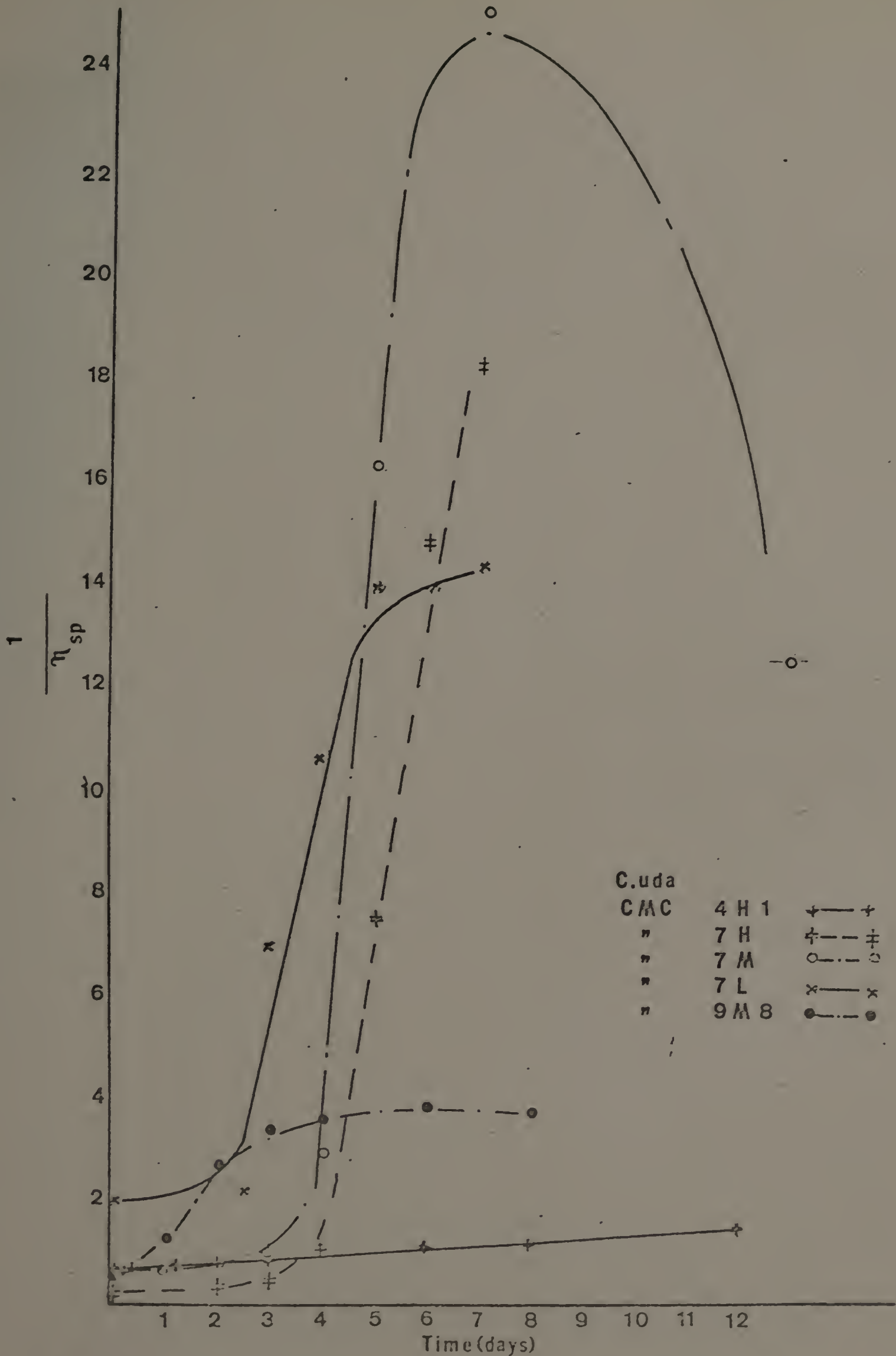
Fig. 3. Decomposition of Carboxymethyl Cellulose Substrates by *Cellulomonas sp.* Reciprocal Specific Viscosity.
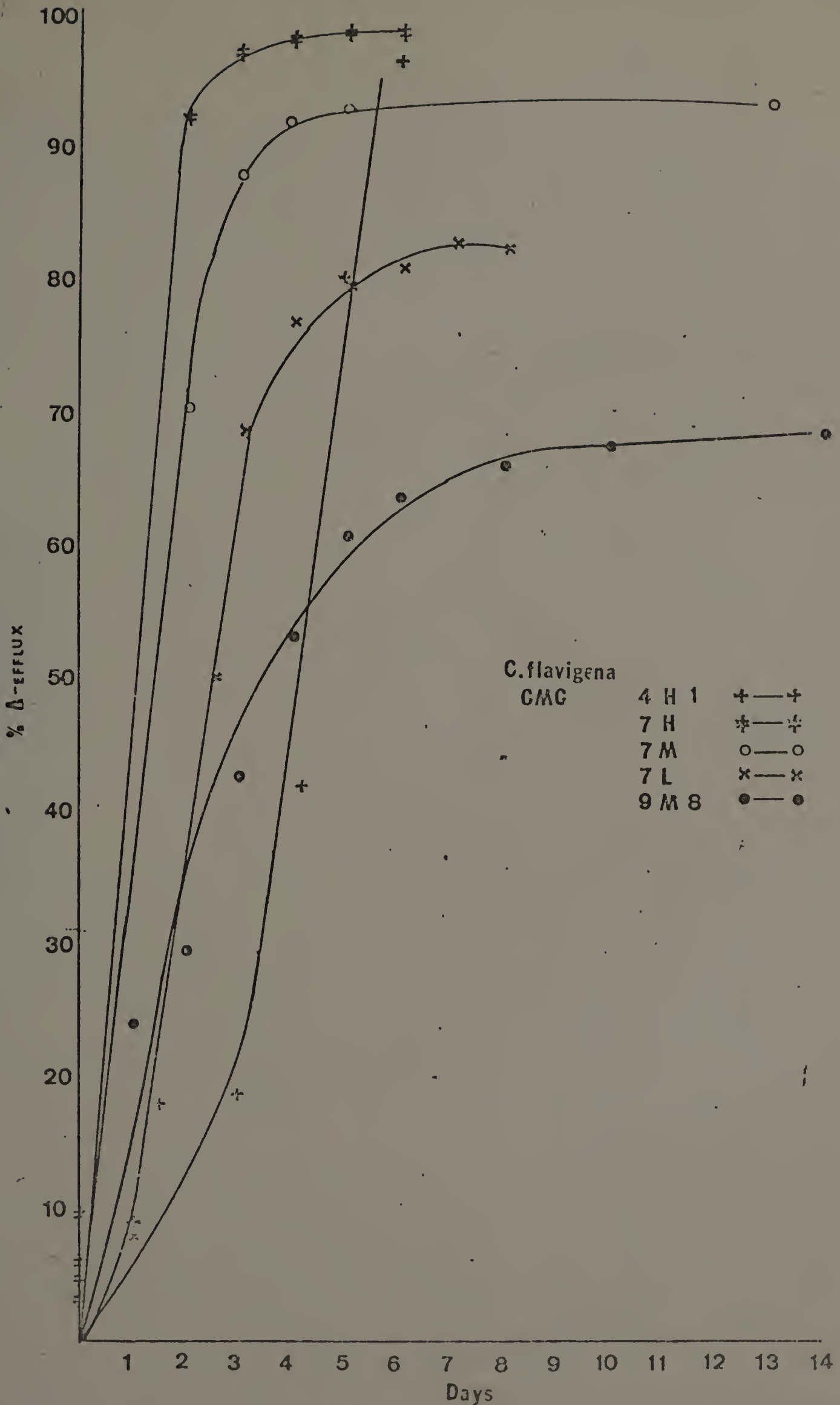
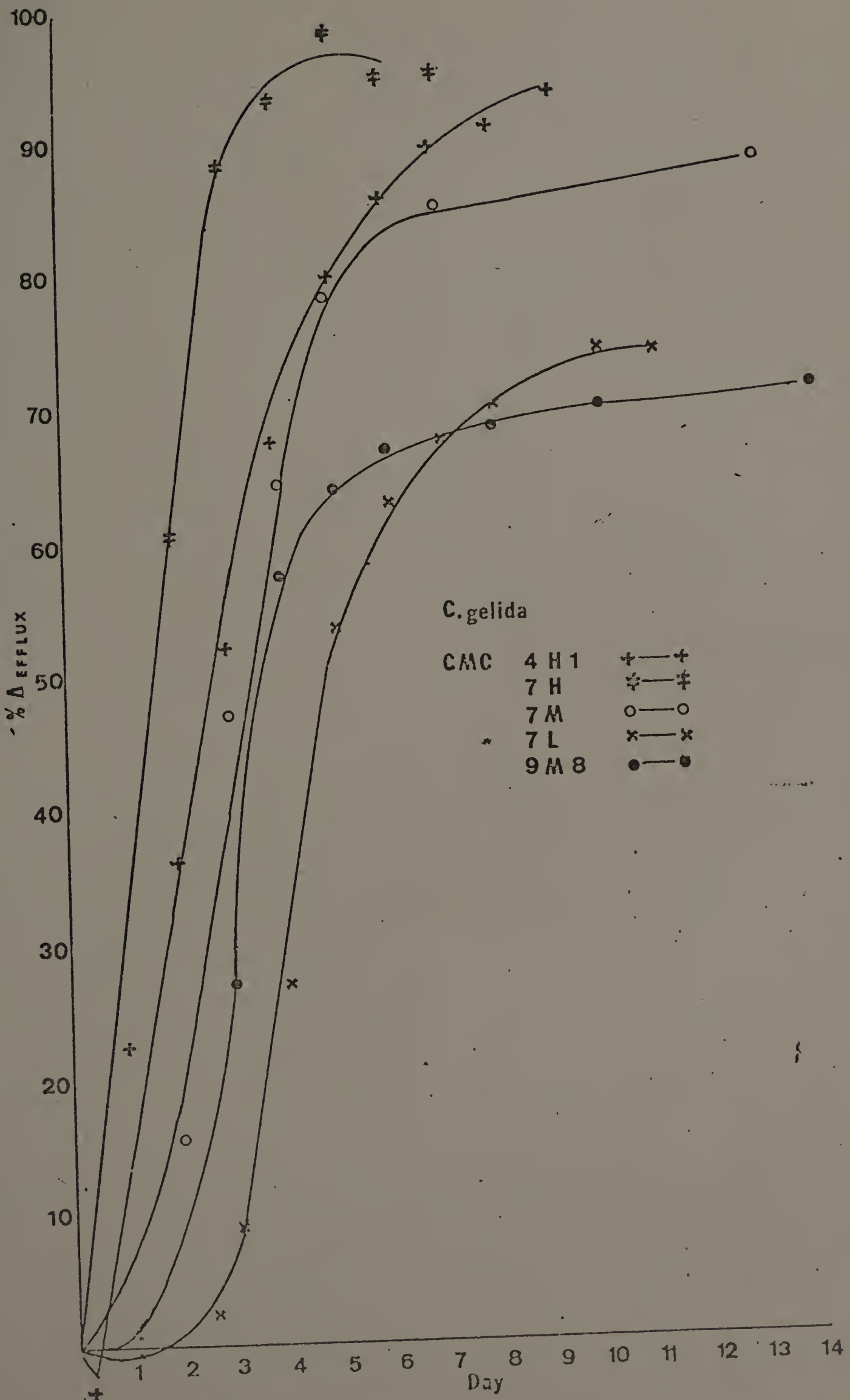Fig. 4. Decomposition of Carboxymethyl Cellulose Substrates by Cellulomonas flavigena. Percent change in Efflux Time.

Fig. 5. Decomposition of Carboxymethyl Cellulose Substrates by Cellulomonas species. Percent change in Efflux Time.
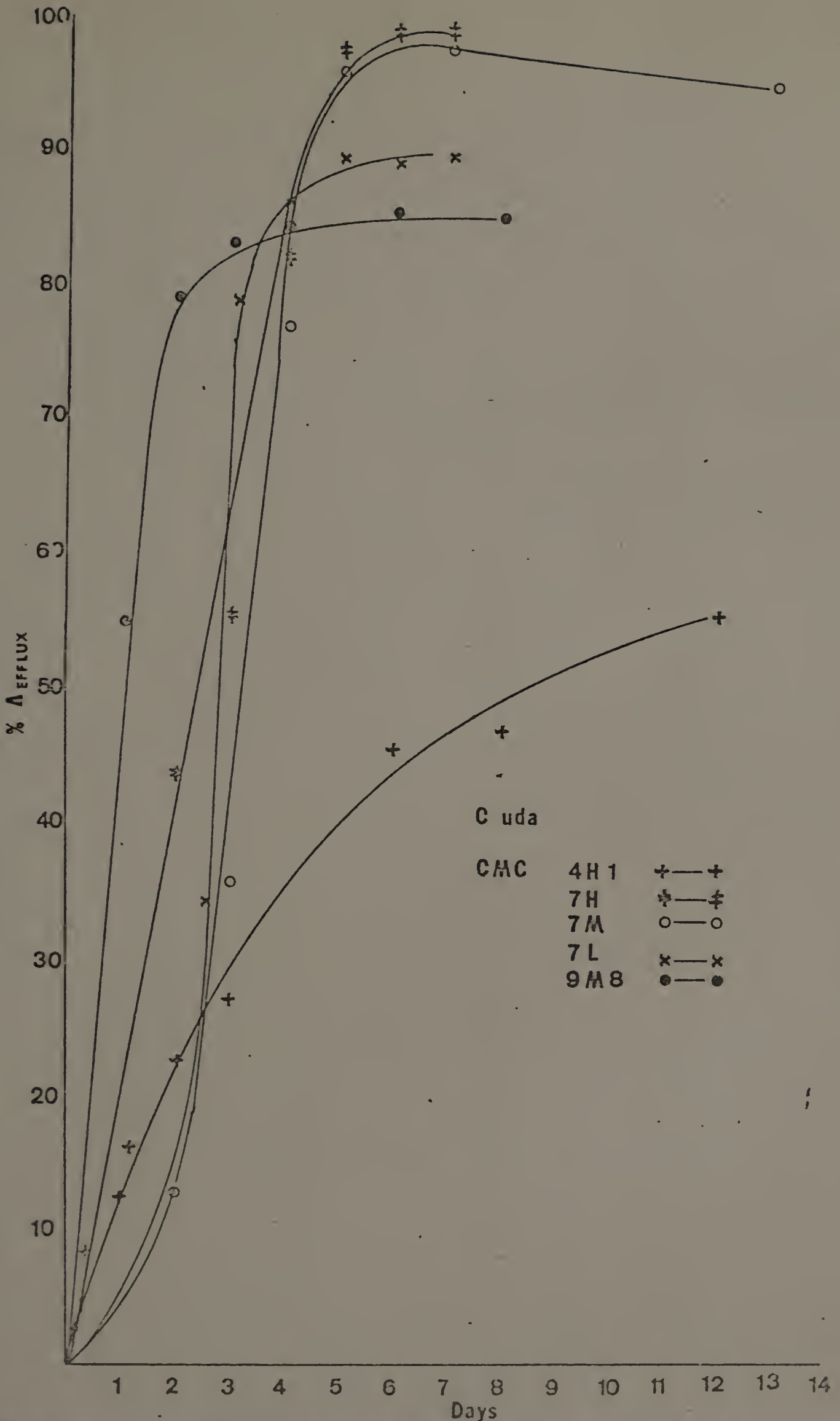
Fig. 6. Decomposition of Carboxymethyl Cellulose Substrates by Cellulomonas uda. Percent Change in Efflux Time.
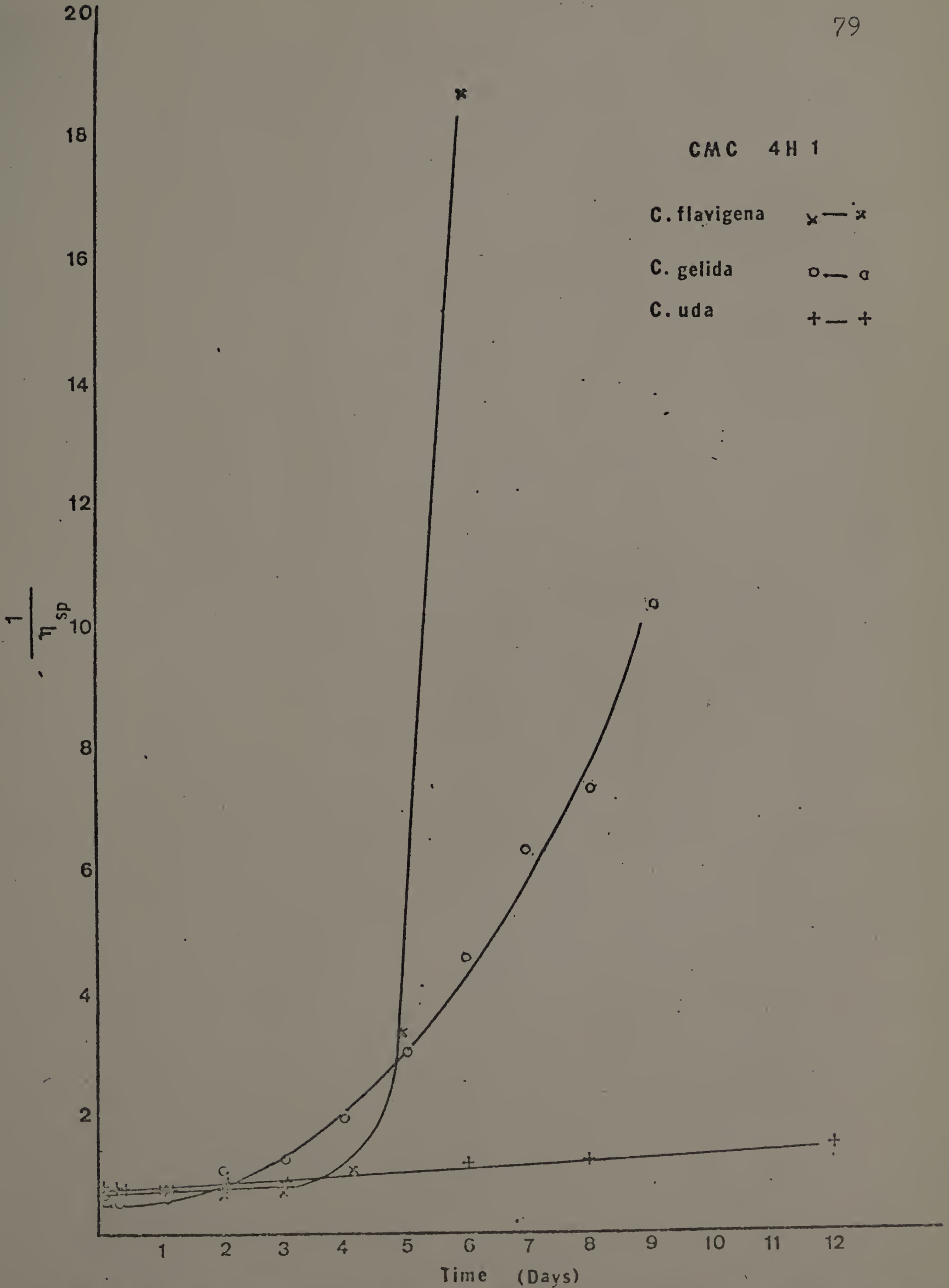
Fig. 7. Decomposition of substrate CMC 4H1 by _Cellulomonas_ spp.
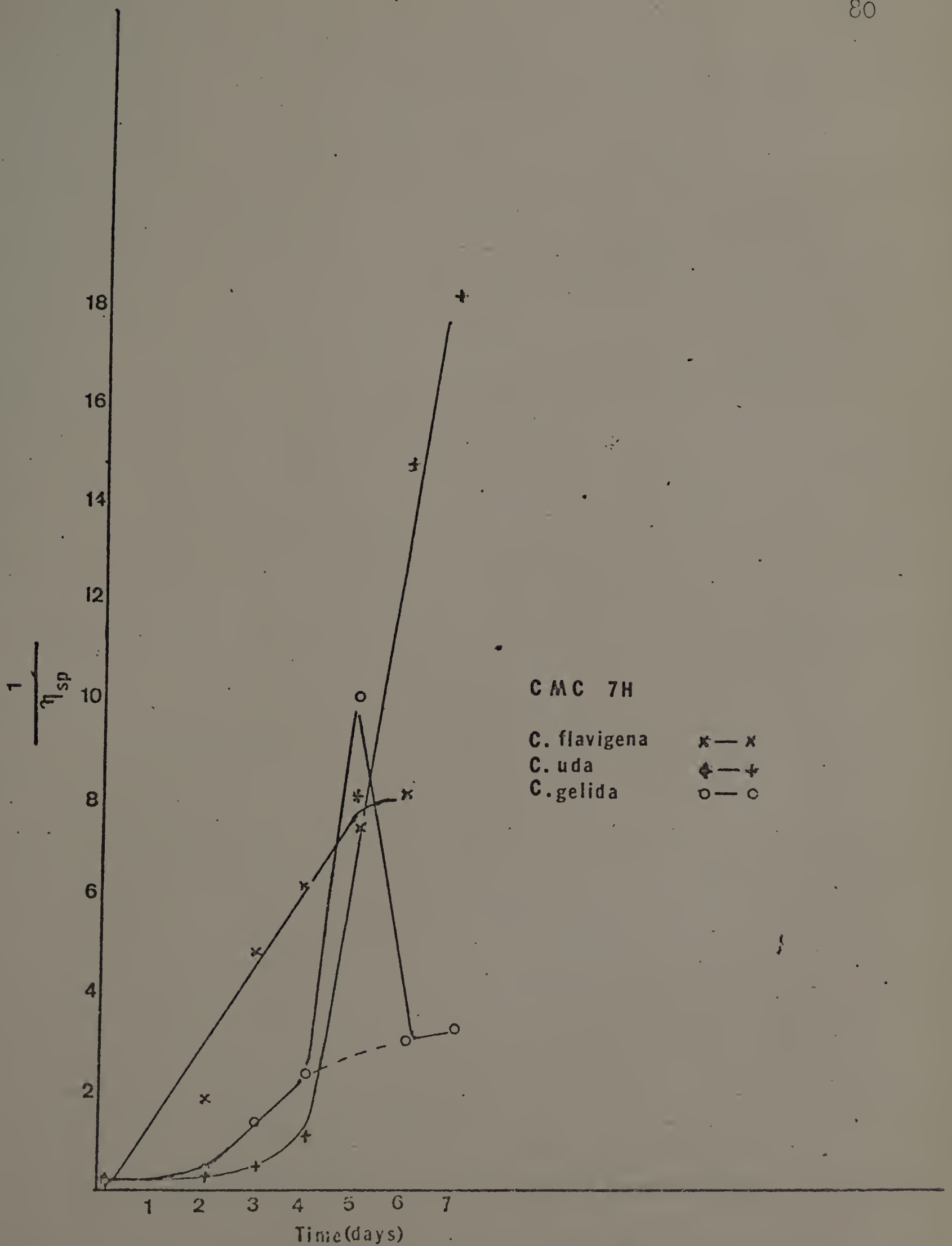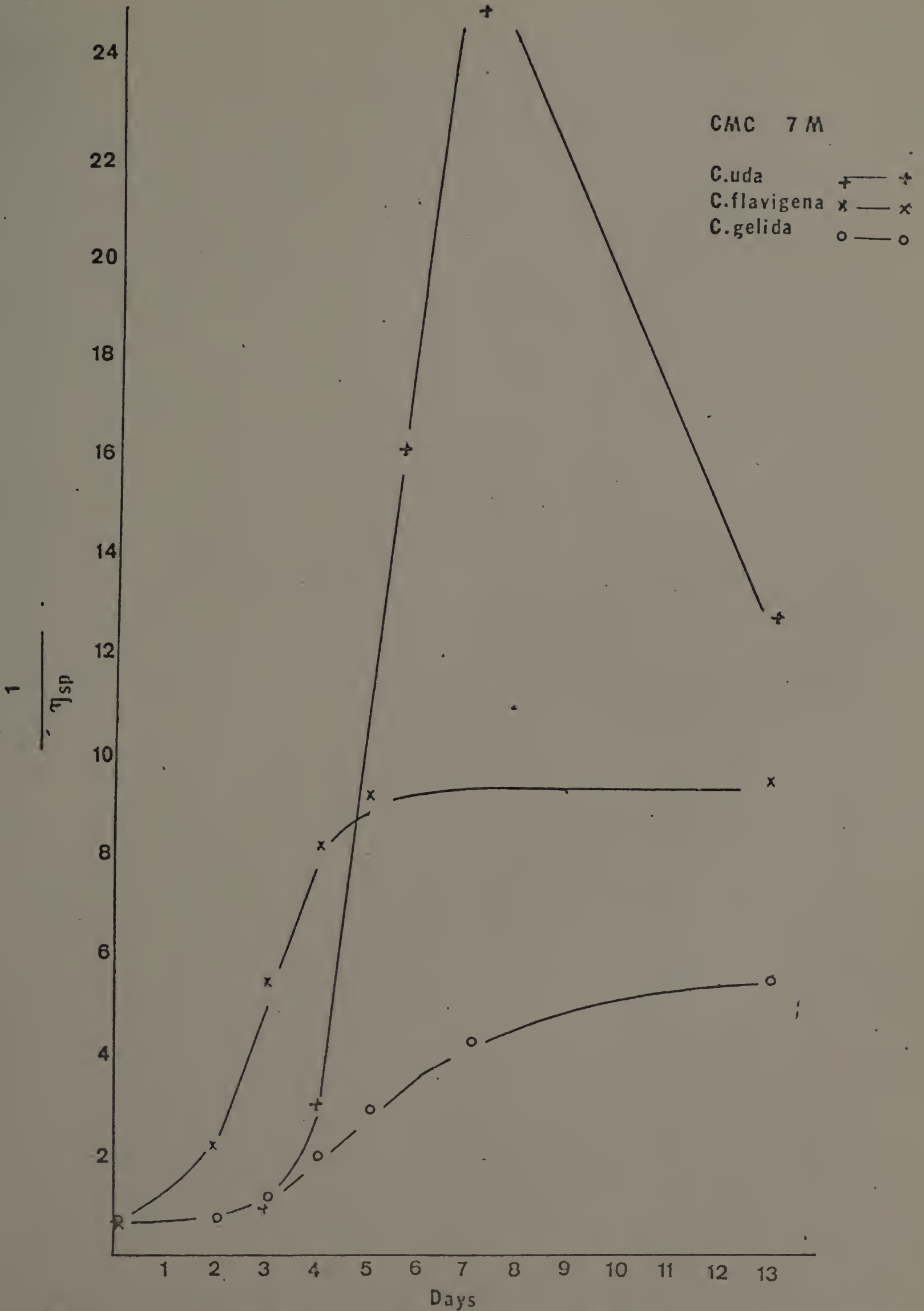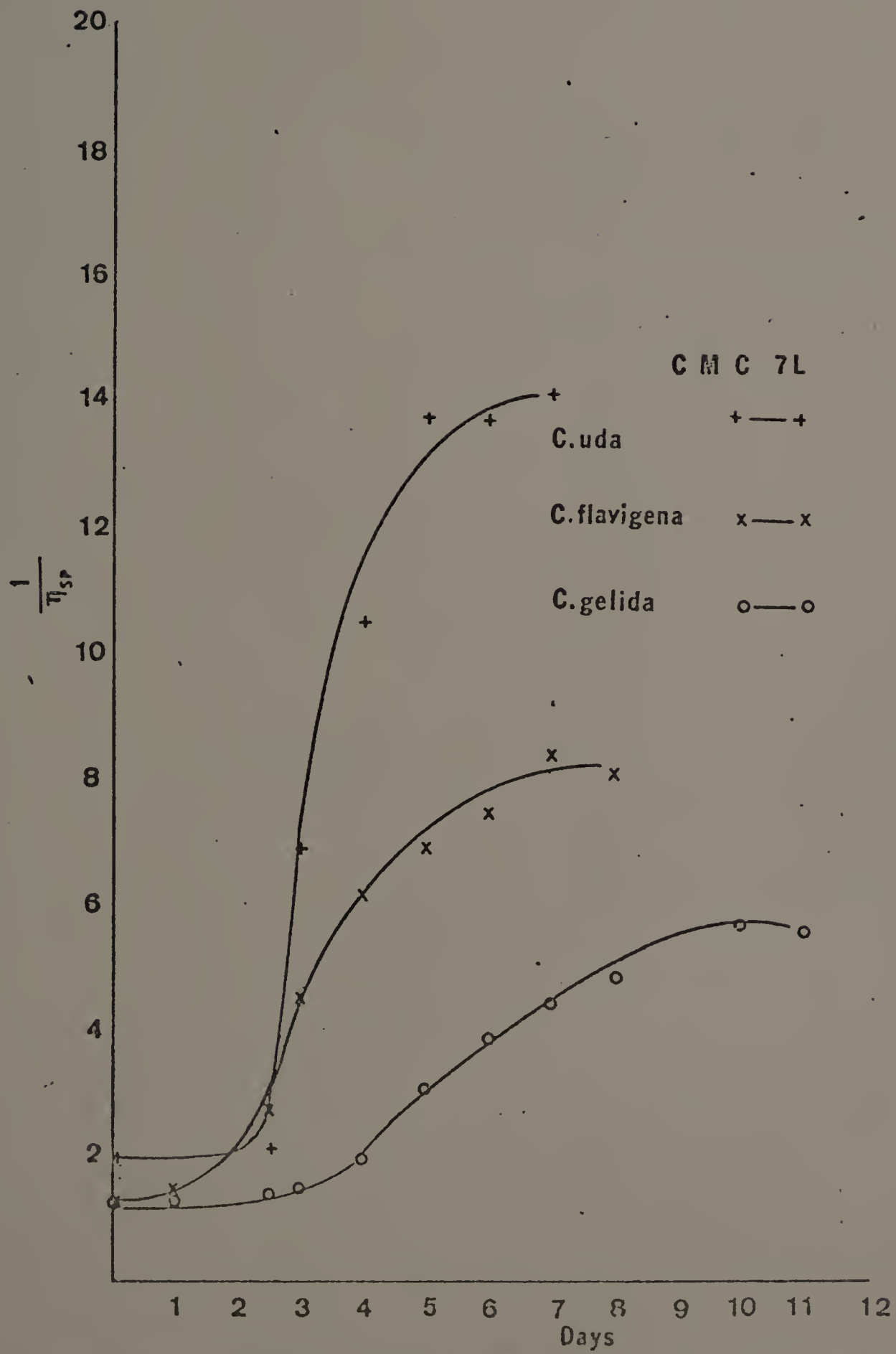
Fig. 8. Decomposition of substrate CMC 7H by *Cellulomonas* spp.

Fig. 9. Decomposition of substrate CMC 7M by Cellulomonas spp.

Fig. 10. Decomposition of substrate CMC 7L by *Cellulomonas* spp.
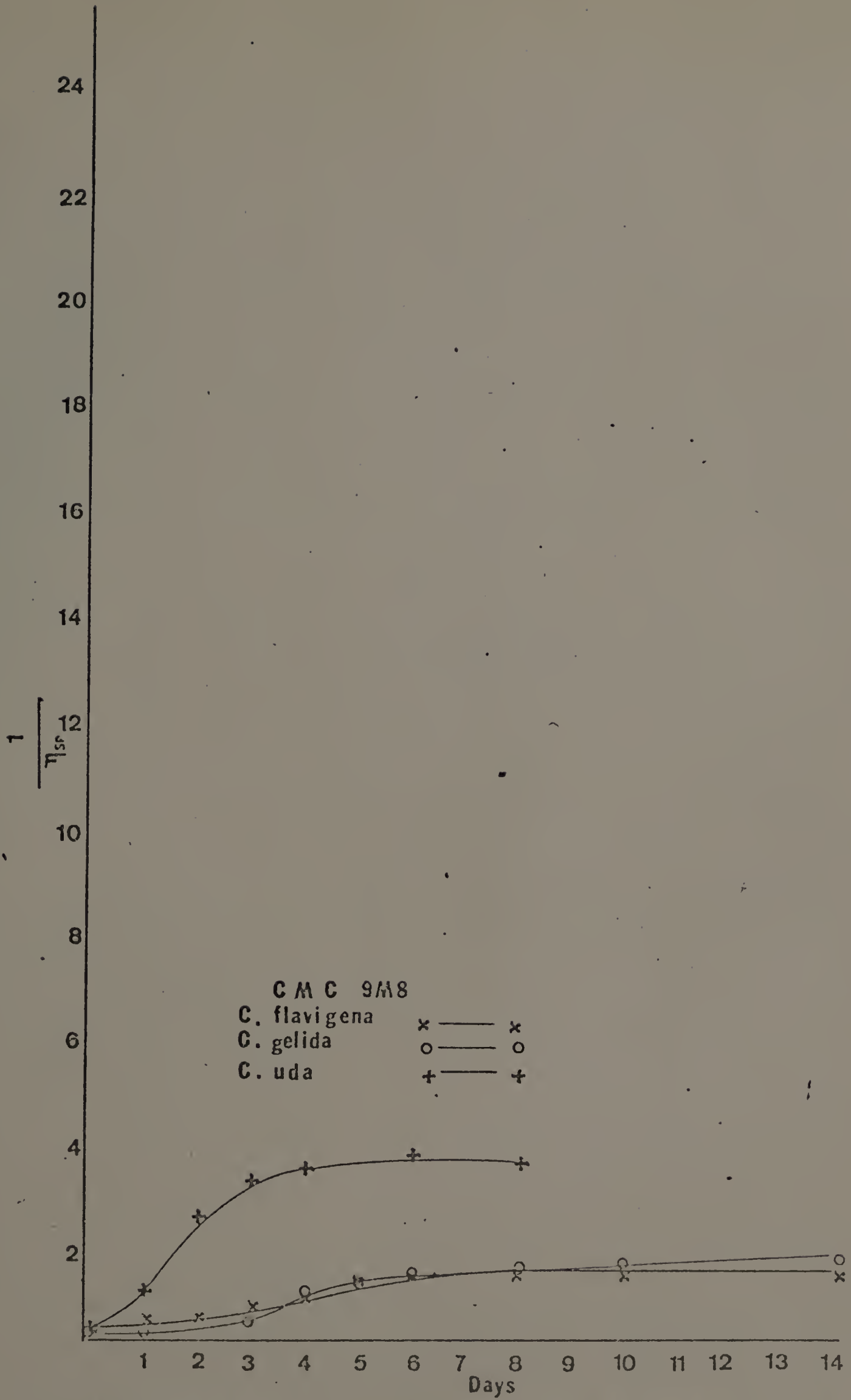
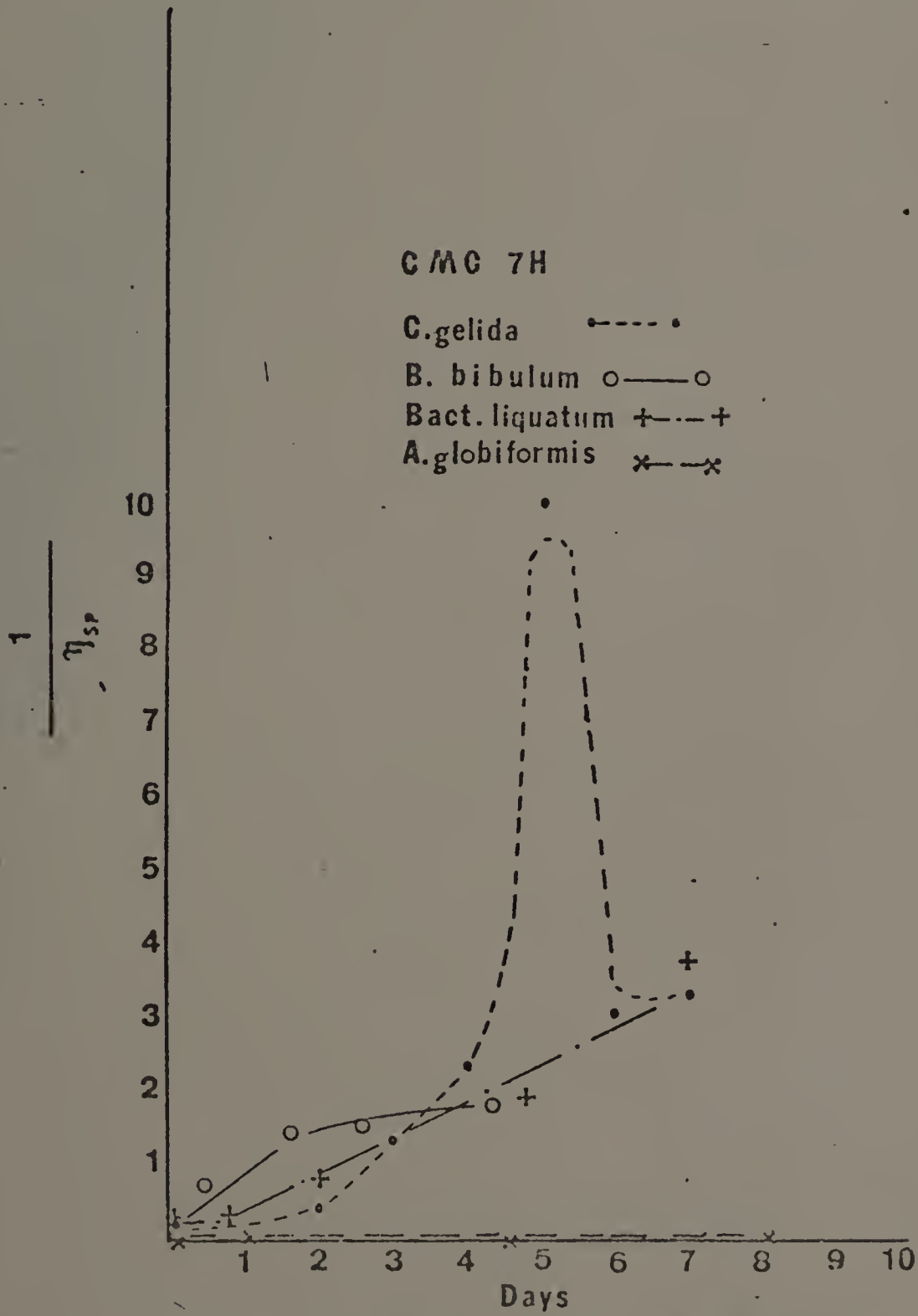Fig. 11.   Decomposition of substrate CMC 9M8 by Cellu-
lomonas spp.

Fig. 12. Decomposition of CMC 7H by coryneform bacteria. Reciprocal Specific Viscosity.
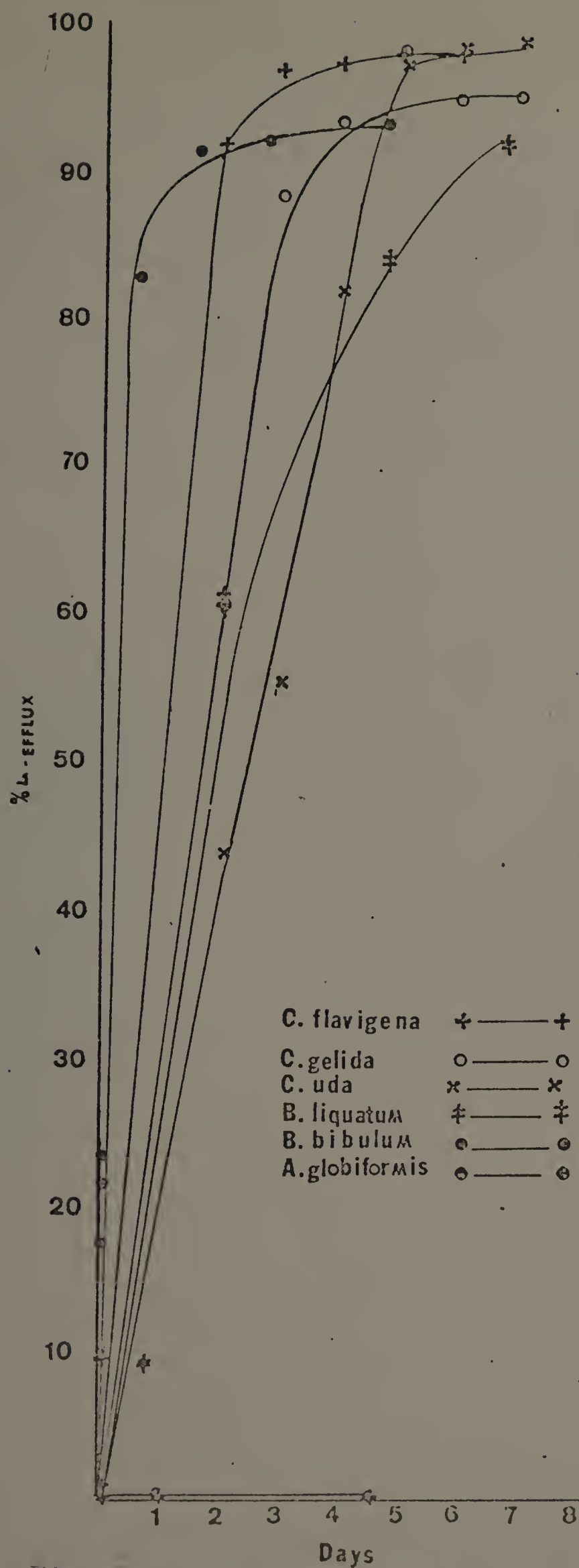
Fig. 13. Decomposition of CMC ? by coryneform bacteria. Percent change in Efflux Time.
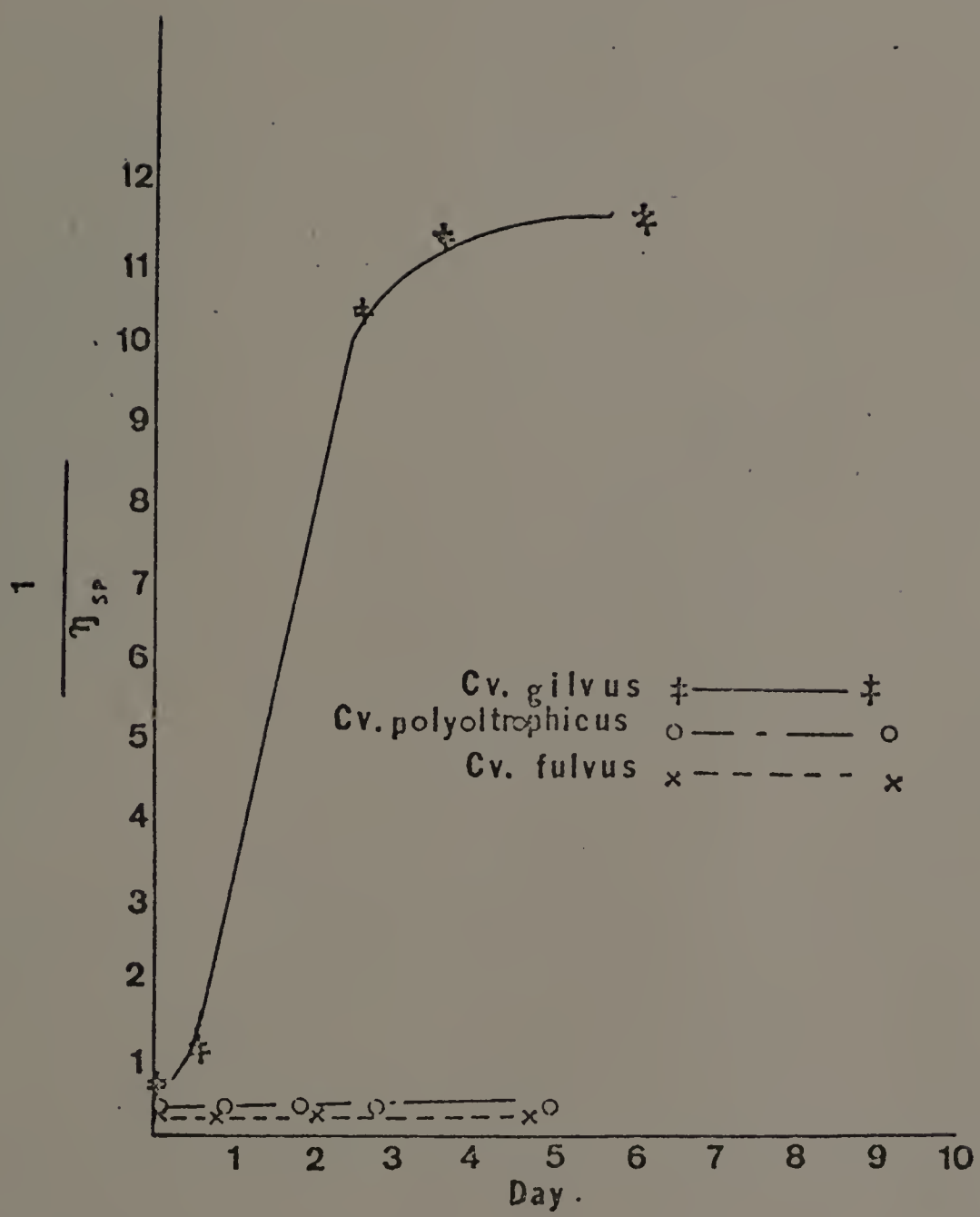
Fig. 14. Decomposition of substrate CMC 7H by *Cellvibrio* spp. Reciprocal Specific Viscosity.

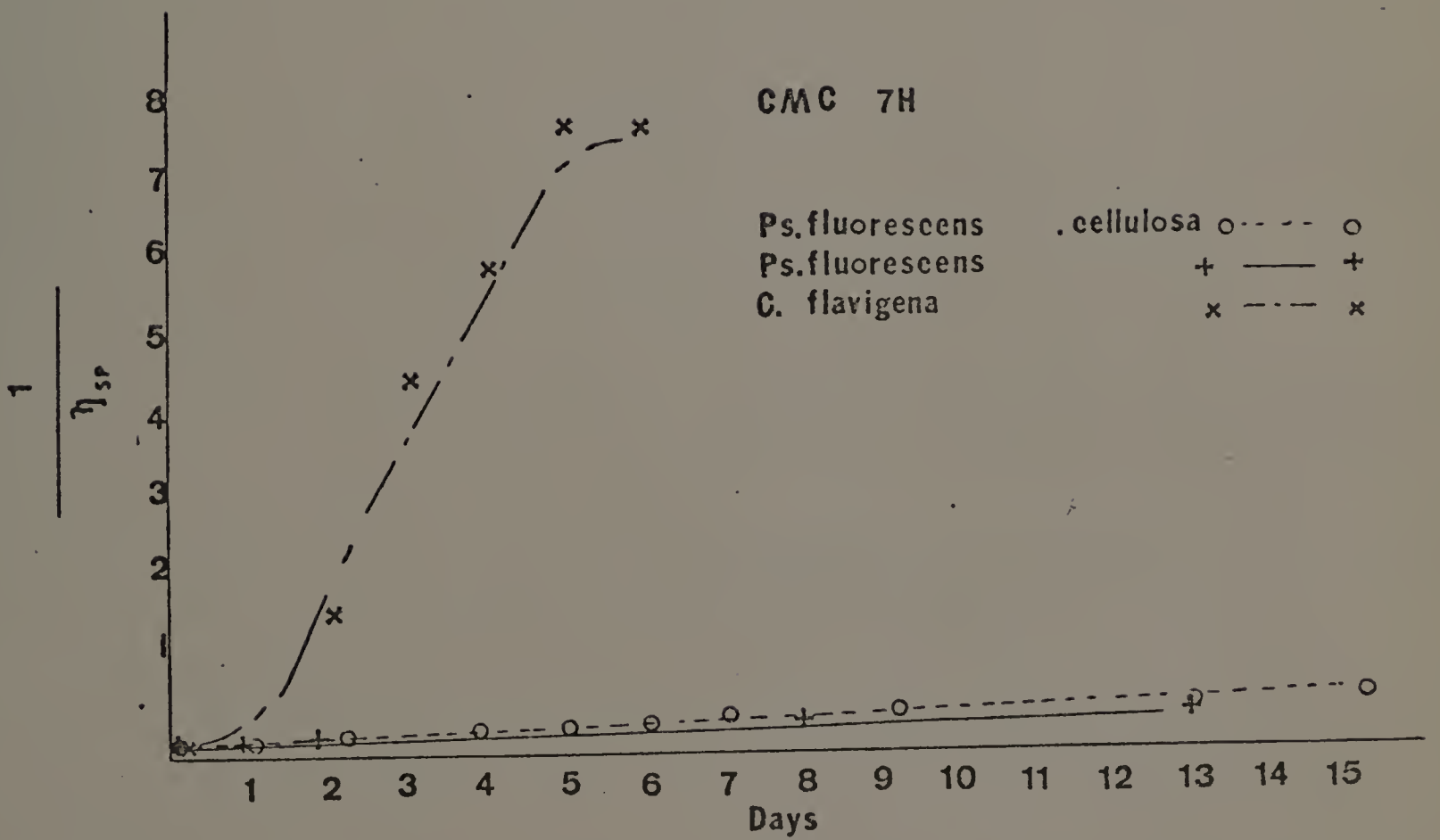Fig. 15. Decomposition of substrate CMC 7H by Pseudomonas spp. and C. flavigena.
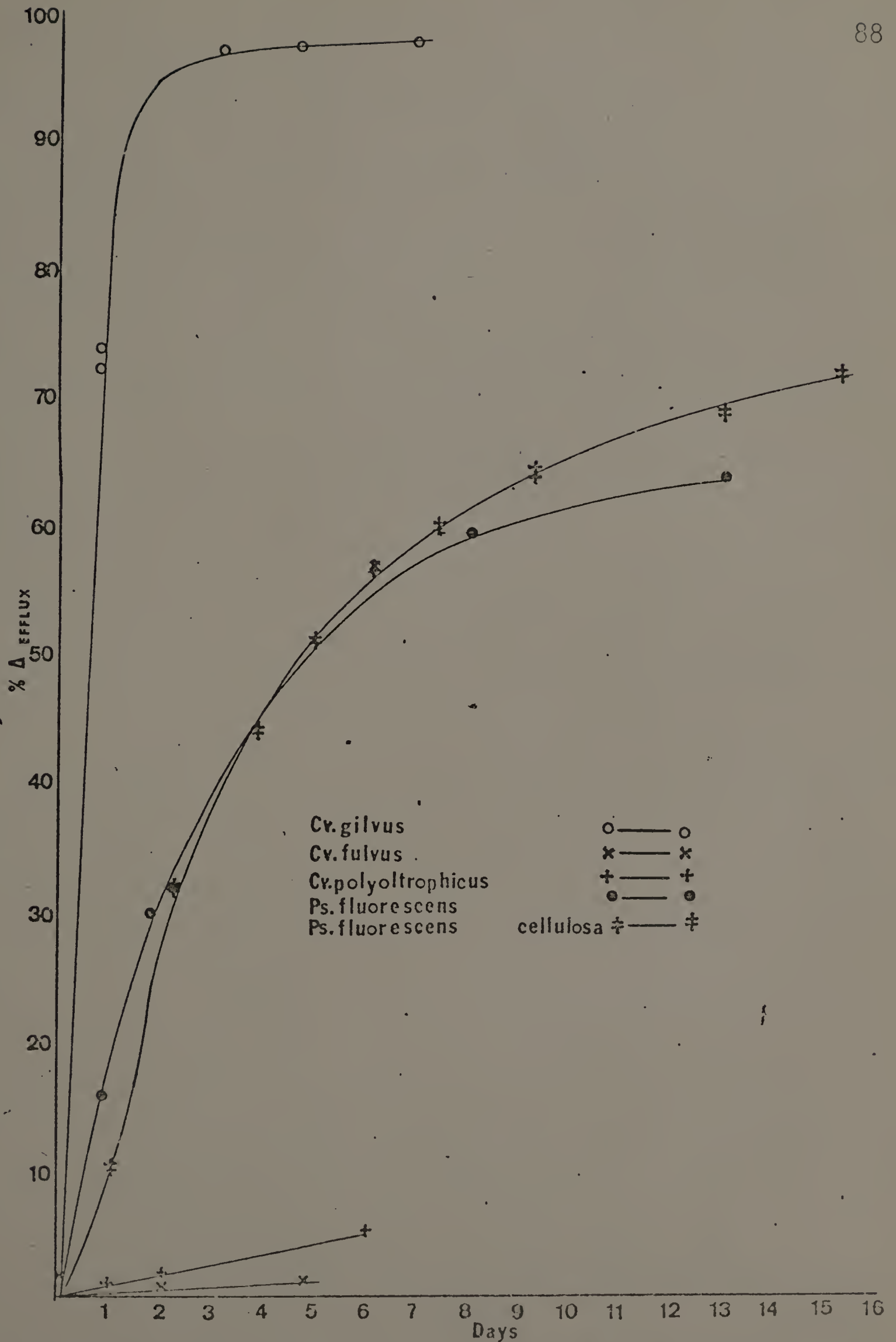
Fig. 16. Decomposition of substrate CMC 71 by Pseudo-monadaceae. Percent Change In Efflux Time.

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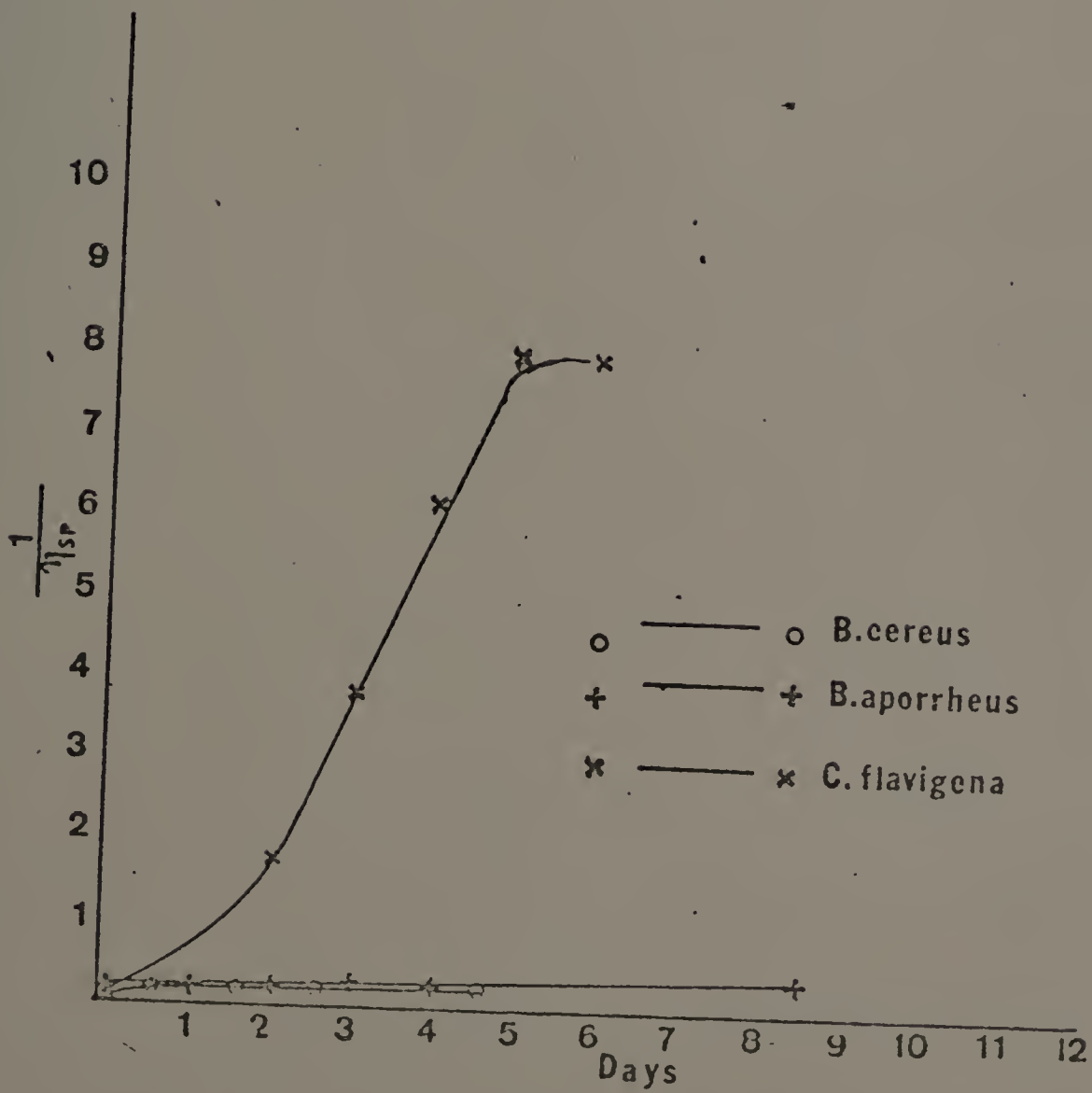
Fig. 17. Decomposition of substrate C.C 71 by Bacillus
spp. and C. flavigena. Reciprocal Specific Viscosity.
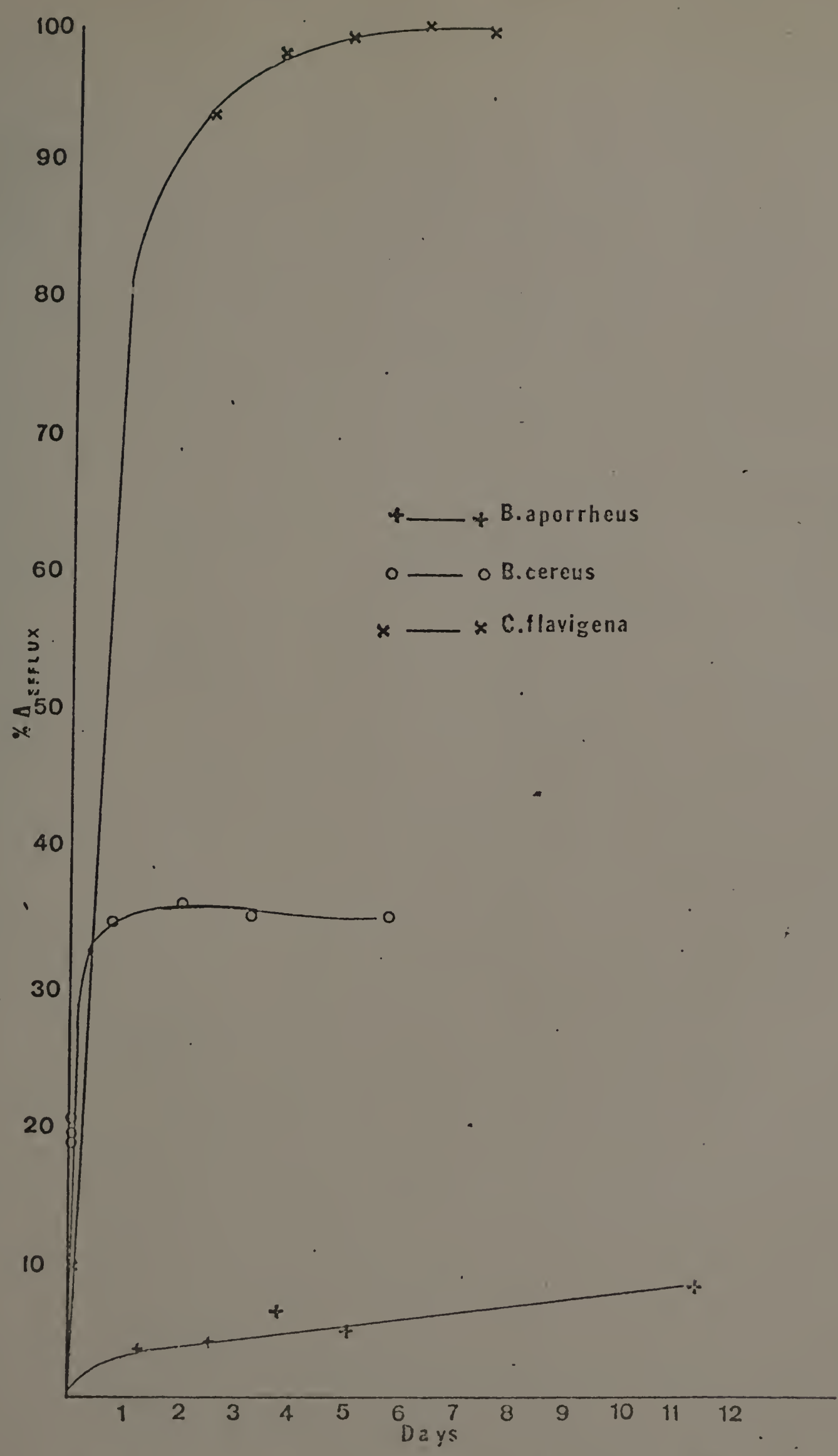
Fig. 16. Decomposition of substrate CMC 70 by _Bacillus_
spp. and C. flavigena. Reciprocal Specific Viscosity.
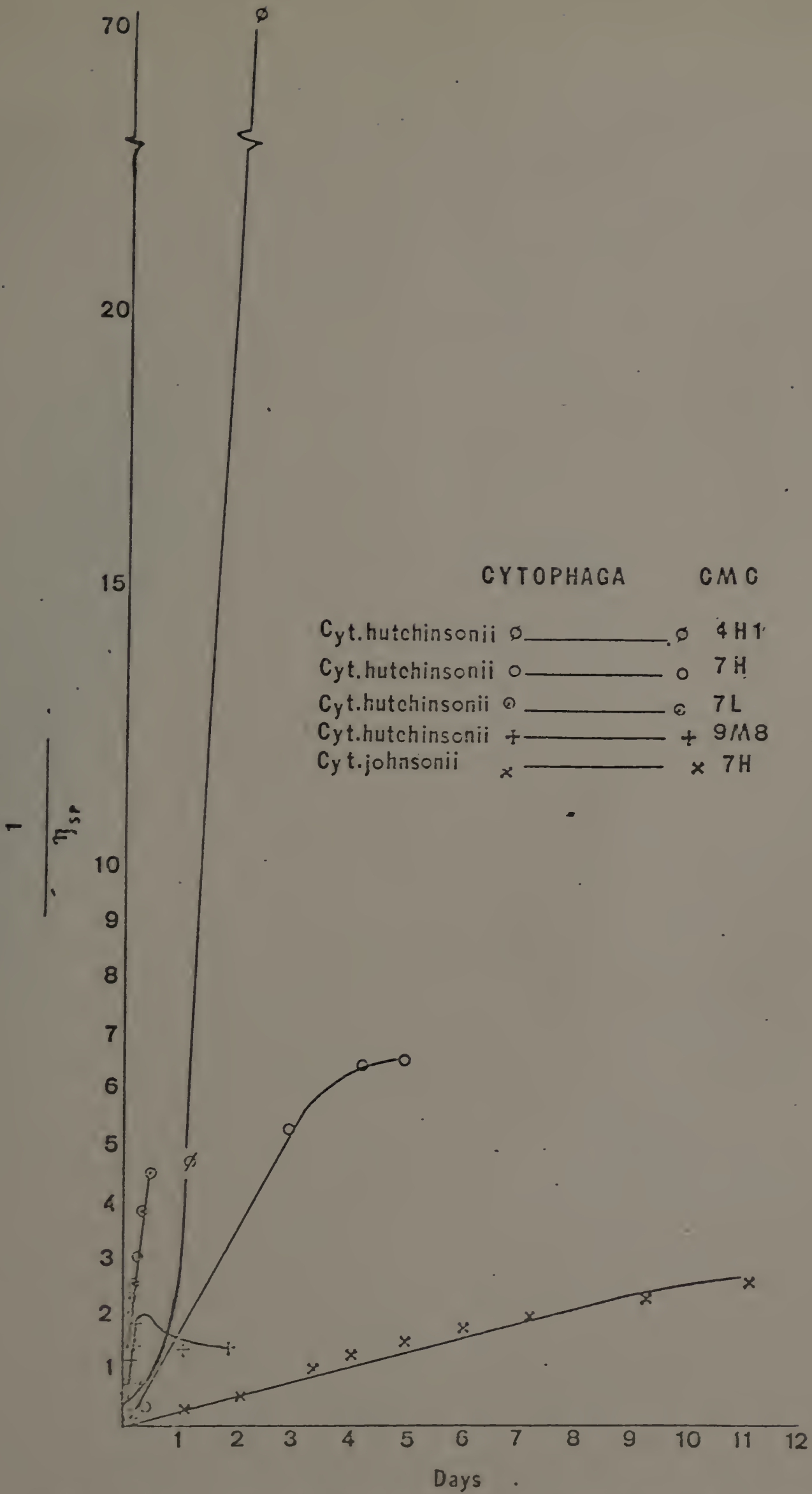
Fig. 19. Decomposition of Carboxymethyl Cellulose Substrates by Cytophaga spp. Reciprocal Specific Viscosity.

Fig. 20. Decomposition of Carboxymethyl Cellulose Substrates by Cytophaga spp. Percent Change in Efflux time.

Fig. 21. Standard Curve for Reducing Sugar Analyses.
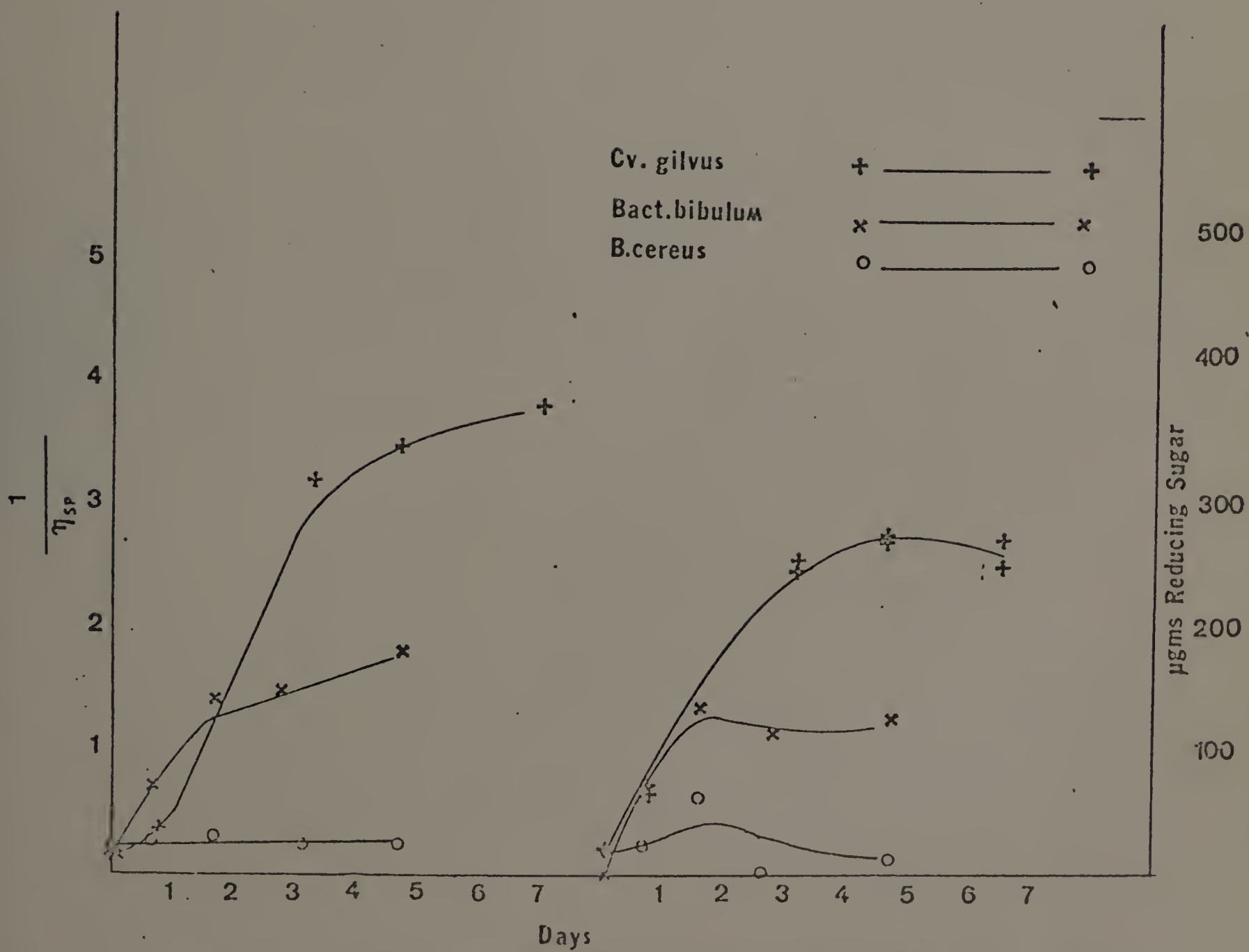
Fig. 22. Comparison of Viscometric and Reducing Sugar Analyses for Three Species.
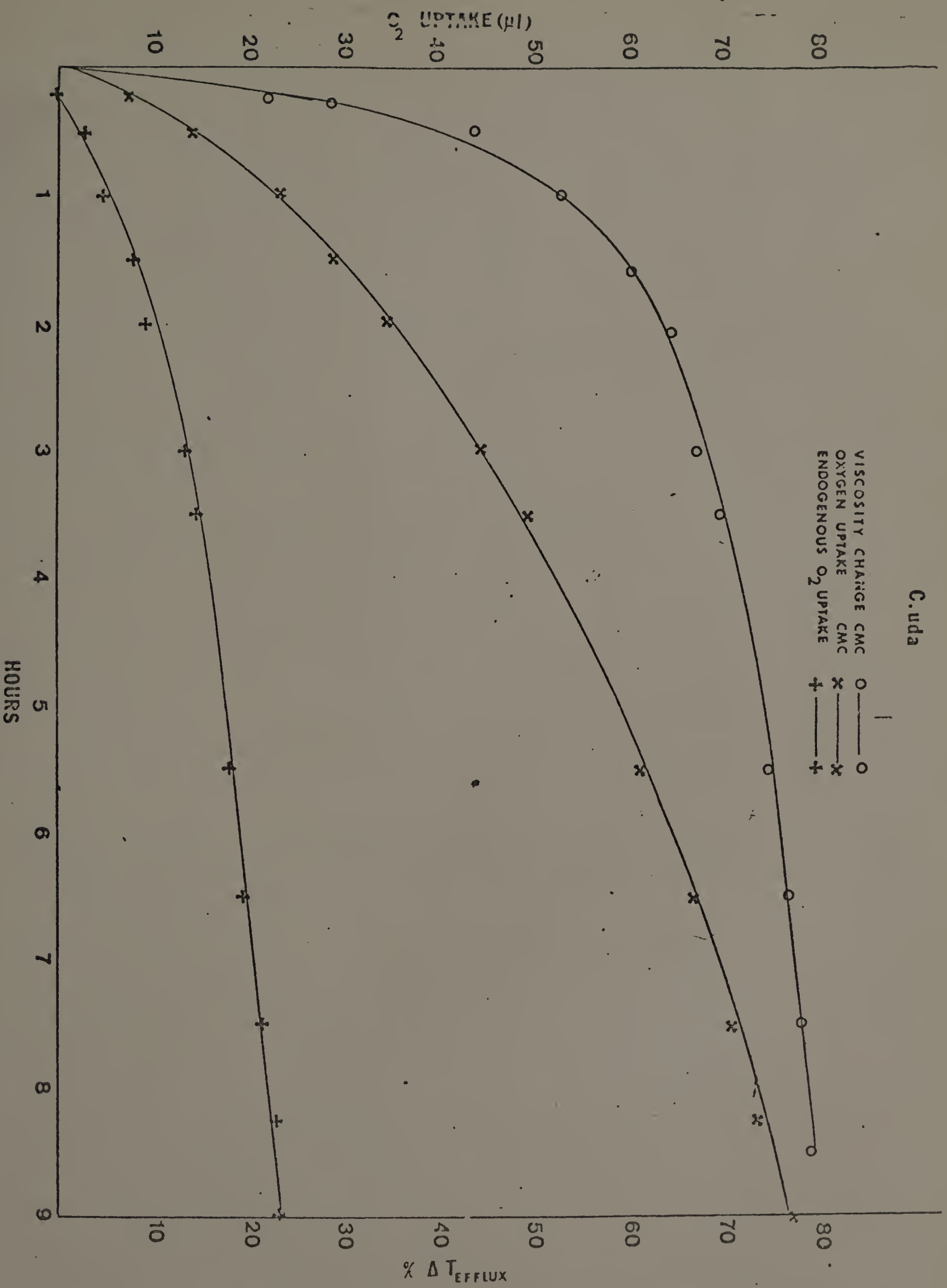
Fig. 23. Viscosity Change and oxygen uptake by Cellu-
lomonas uda using ClC 7L.

DISCUSSION

There is no operating definition of a cellulolytic bac-
terium that can be applied to all strains reported to be cel-
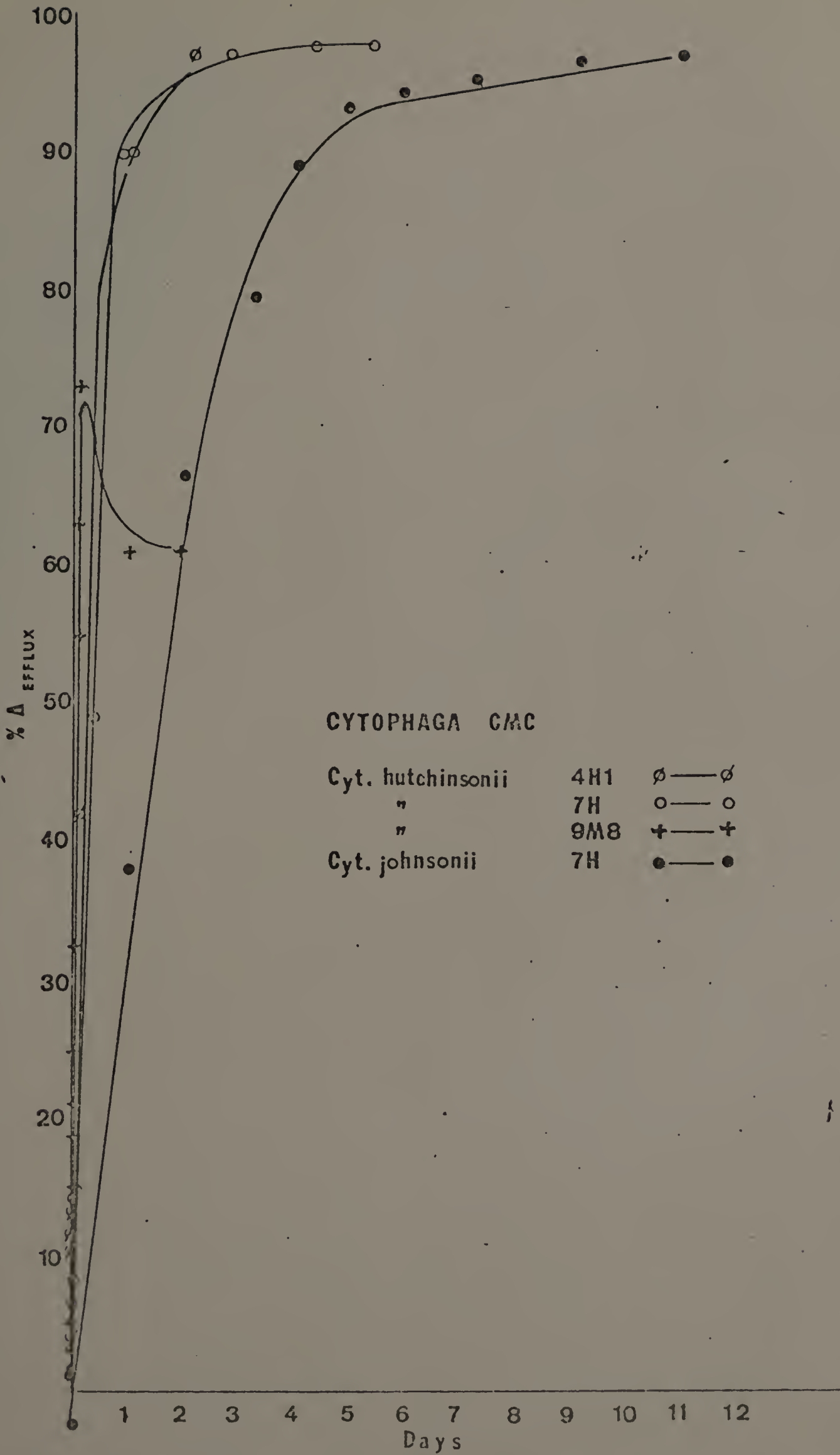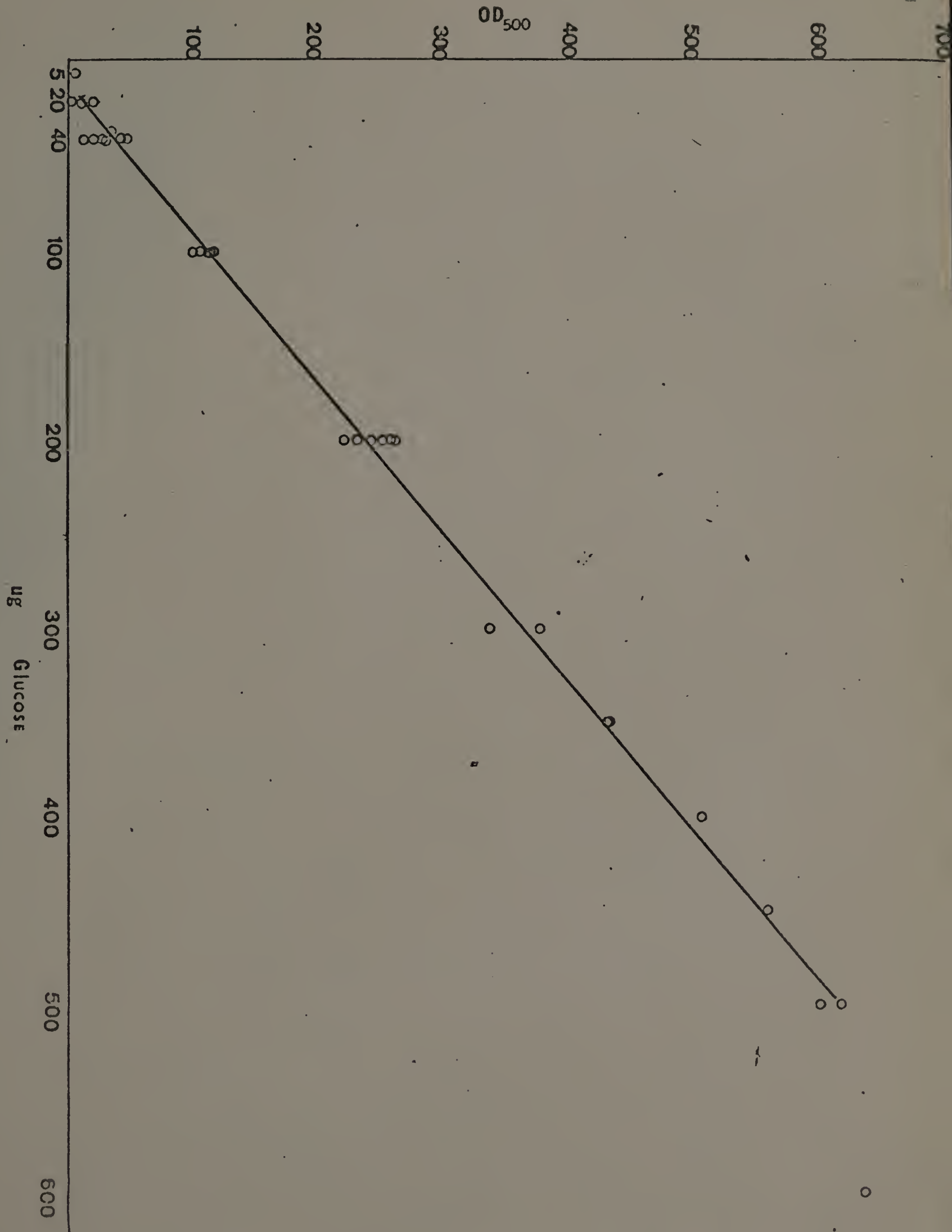lulolytic. There appear to be several reasons for this dif-
ficulty. To a large extent, the lack of clarity surrounding
the nature of the substrate hinders further progress in for-
mulating an all encompassing definition for cellulolytic bac-
teria. Such a wide variety of substances have been called
"cellulose" and used as substrates for the study of cellulo-
lysis that the word cellulose has lost almost all meaning.
It is necessary to refer to "cellulosic materials" and des-
cribe in sufficient detail cellulosic substrated before one
can make judgements on experimental research concerning the
process of cellulolysis. Some have suggested (92) that a
uniform standard substrate be established to aid research,
but current knowledge does not seem to permit such a choice.
Does one use the same substrate to study cellulolytic fungi
as well as cellulolytic bacteria or invertebrates? Should
aerobic and anaerobic species be treated the same? Should
natural habitat be considered in the selection? How does a
refined substrate such as filter paper relate to the form of
cellulose likely to be found in the A horizons of soil? Is
cotton an "abnormal" substrate for species not found near

cotton plantations? How "unnatural" are the substituted

soluble cellulosic materials? These are some of the ques-

tions which must be answered before the selection of standard

cellulosic substrates can be accomplished.

As implied above, the cellulase systems of various or-

ganisms are different. Most systems which have been isolated

and characterized seem to have the basic components of the

C_1-C_x model proposed by Reese et al. (92). However, suffi-

cient differences have been observed in these systems to in-

dicate that Youatt's (46) caution against the formulation of

a composite model for all cellulase systems should be heeded.

For example, the activities of the majority of well des-

cribed cellulase systems seem to involve a random internal

cleavage of the linear anhydroglucose chains of the substrate;

but the enzyme system of at least one species, Cellvibrio

gilvus, seems to have a component which has a specificity for

endwise attack (98). Care should be taken in categorizing

the differences between types of cellulose systems by the

association of the terms "weathering" and "hydrolytic" with

those of "random" and "endwise", since the former refer to

observations obtained with whole cells and the latter refer

to characteristics of enzymes in cell free systems in which

components within the systems may differ in mode of attack.

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The bulk of the current literature seems to be directed toward examination of the characteristics of an increasing number of cell free cellulase systems. Such an approach is helpful because, as the number of different cellulolytic organisms examined increases, the intricacies and subtle differences among the many systems will be revealed. However, the information gathered will be of limited immediate usefulness to microbial ecologists since there is no necessary correlation between the activity of the enzyme extracts of cellulolytic species and their performance in native habitats. Some cellulases may not function in cell free systems, since many of bacterial origin appear to be tightly bound to or part of the cell envelope (88, 103, 135). Conversely, the activity of crude extracts is almost always lower than that of purified enzymes. The influence of environmental factors on the cellulolytic capability of an organism cannot be completely deduced from data obtained using the cell free enzymes derived from that organism.

Although both the cell free and viable culture approaches are necessary to elucidate the role of cellulose decomposing bacteria in nature, the literature seems to be lacking in reports of the latter approach as a quantitative tool. There are a variety of reasons for this. Probably the most important one is that by the use of enzyme preparations

more parameters can be held constant than when viable cultures are used. In addition to allowing more confidence in interpretation of results it permits the development of more precise assay systems. But the methodologies developed for the biochemist need not be restricted to that approach. Some of the techniques which measure changes in the substrate and are not affected by the state of the products of decomposition should be generally applicable to viable culture as well as cell free systems. A few have been examined in this study in an effort to determine if meaningful information can be gathered utilizing living systems similarly to cell free ones.

A cellulosic substrate traditionally used by microbiologists is filter paper. When degraded rapidly the process is relatively easy to observe since the paper begins to lose its integrity and either flake apart or separate into two strips at the air-liquid interface. Early workers assumed this meant that a large proportion of the insoluble substrate had been solubilized, but when quantitative analyses were performed by gravimetric means, only a small percentage of substrate was seen to have been lost in the process.

The data presented in Table 7 of this report confirms results shown by other workers that indicate that relatively little insoluble cellulose is solubilized by cellulolytic

bacteria. Bradley (136), Fuller and Norman (86) and Berg
et al. (107) employing different genera have given evidence
that cellulose-decomposing bacteria do not often cause more
than 30% of the insoluble substrate to disappear when ob-
served gravimetrically. Bradley, using cultures now con-
sidered to be synonymous with <u>Cellulomonas</u> observed 10% to
15% weight loss after 15 days. Fuller and Norman, observing
a variety of species, including <u>Sporocytophaga</u> and <u>Bacillus</u>
species, observed losses of less than 5% to 30-40% for fil-
ter paper. The lowest values were obtained using <u>Bacillus</u>
<u>aporrheus</u> and the highest from the cytophaga. Cornstalk cel-
lulose, a natural substrate, increased values to from 30% to
nearly 90% weight loss, with the most marked effect being on
the <u>Bacillus</u>. These writers concluded that on the basis of
what they accomplish using filter paper, most aerobic cellu-
lolytic bacteria cannot be regarded as being particularly
vigorous. Berg et al., in their recent note, have shown 15-
30% decomposition by cellvibrios using cotton cellulose.

The data presented in Table 7 is very similar to that
found by the above authors. In only three cases were values
higher than 30% found. In two of the cases, with the cellu-
lomonad <u>Bacterium liquatum</u> and <u>Cellvibrio gilvus</u>, the sub-
strate was a natural one, raw cotton linters. Most values
were less than twenty percent. Whether the substrate is a

natural one, or refined wood cellulose wuch as Solka Floc, or cotton cellulose as the various Chemical Cottons of the PS series, or the ethylhydroxyethel cellulose--none of the results indicate extensive solubilization.

This study also confirmed findings of earlier reports that gravimetry when applied to cellulolysis has another liability. This technique is not precise. Fuller and Norman showed values varying as much as 50%. It was originally our intention to attempt to observe variation with respect to chain length but our first attempts varied sufficiently to prohibit conclusions about any effect of chain length even though our data did show that values for the shorter PS 14 substrate were usually lower than other Chemical Cottons. Any values obtained from gravimetric techniques concerning cellulolysis must be considered approximate.

Although used frequently with cell-free enzyme systems derived from molds and bacteria, substrates such as carboxymethyl or hydroxyethyl cellulose have not often been used in a quantitative manner with living bacterial cultures as sources of cellulase activity. Viscometric methods provide a reasonable potential means for utilizing these substrates with whole cells. Much of the collected data in this study is concerned with the possible usefulness of this methodology.

The most basic question concerning the observation of decomposition of soluble materials such as carboxymethyl cellulose by viscometric methods was whether quantitative data could be obtained using whole viable cells instead of cell free enzymes. The data in Tables 1 through 5 and the accompanying Figures indicate that, though rates were lower than found for the purified enzymes reported in the literature (112, 42) significant alteration of the substrates resulted. The results showed that strains differed in their ability to degrade carboxymethyl cellulose substrates even though closely related to one another. But they also showed that the strains tended to be grouped into active or inactive ones in much the same way they might have been classified by other means. There were notable exceptions.

A spectrum of relative activity toward the substrates was found to exist among the organisms tested. Certain organisms such as the cellulomonads, Cellvibrio gilvus and Cytophaga hutchinsonii were sufficiently active so that there was no question about their cellulolytic ability. Others, such as the Pseudomonas fluorescens cultures, the Bacterium "species", Cytophaga johnsonii and possibly Bacillus cereus, produced less change than the active organisms but enough to warrant consideration as cellulolytic. A third grouping included Cellvibrio fulvus, Cv. polyoltrophicus, Bacillus

aporrheus and Arthrobacter globiformis for which little or no discernible activity was detectable.

No matter which way the data was presented, either as percentage change in efflux time ($\%\Delta T$) or reciprocal specific viscosity ($1/\eta_{sp}$), the active cultures appeared as one group. Certain of the Group II species appeared to behave similarly to Group I organisms when the data was displayed one way while appearing inactive when the other method was used. The inactive third group was such no matter which method of presentation was used.

Those which fell into Group I are generally acknowledged to be active aerobic cellulolytic bacteria (69, 138, 56). They produced the highest rates ($d(1/\eta_{sp})/dt$) of the organisms tested (Table 5) and the highest final values ($1/\eta_{sp}$ or $\%\Delta T$) (Tables 1-4). Rates of 0.100 or better were considered very high for the species tested under the conditions used. All from Group I but C. flavigena gave such values for substrate 7H and the latter gave high values from two other substrates.

Most of those which fell into the first grouping were examined for substrate preference. The data in Table 5 and Figs. 1-6, 19 and 20 show comparative rates of utilization of the five carboxymethyl substrates by these organisms. Both DP and DS were compared. Substrates 7H, 7M and 7L form

a series with the same D.S. and D.P.s of 1000, 500, and 300 respectively (140). When the results for the three Cellulomonas species were compared little difference was found among the three substrates with D.S. 0.7. Although less obvious when maximum rates (Table 5) were compared, final values and patterns of overall change (Figs. 1-6) indicated the similarities. The decomposition of substrate 4H1, which has a lower D.S. (0.4) than the first three and approximately the same D.P. as substrate 7H, was the same as or slightly faster than the 0.7 D.P. substrates except for Cms. uda (Figs. 5 and 6). Substrate 9M8 was uniformly not preferred by the cellulomonads. All four substrates tested with Cytophaga hutchinsonii were rapidly utilized but decomposition of 9M8 was halted after less than a day. Further testing of this organism will be required to determine substrate preference. Although Fig. 19 seems to indicate 7H as less desirable than 4H1 these differences are not indicated when %ΔT is plotted (Fig. 20). Substrate 7L was degraded very rapidly early on, but was not observed long enough to determine if this change would be sustained. These data confirm what has been suggested frequently starting with Reese et al. (92), that degree of polymerization makes little difference in decomposition rate.

Degree of substitution did seem to make some difference however. Cellulose Gum 9M8 (D.S. 0.9) was never preferred and distinct suggestions of inhibition after some time was found with Cytophaga hutchinsonii and the Cellulomas species. With the exception of C. uda, substrate 4H1 (D.S. 0.4) was one of the two most readily degraded substrates for each of the above four species. Wirick (113) has indicated that, using cell free systems, some celluloses seem to require three adjacent unsubstituted glucose units in order to cleave carboxymethyl cellulose. The probability of three or more adjacent AHG units occurring when the D.S. is 0.9 or more is quite low and increases as the D.S. decreases. Our data seems to suggest that, initially, both 0.4 and 0.7 D.S. are sufficiently low so as not to inhibit cleavages of these substrates by living cells but nine carboxymethyl substitutions in ten anhydroglucose units soon becomes inhibitory.

The organisms which were assigned to Group II were a mixed group of bacteria. Some were species for which evidence for cellulase activity had been found earlier but which did not perform as well as expected. These included the two Bacterium species, and Pseudomonas fluorescens var. cellulosa. Three were species originally believed to possess no cellulolytic capability; Cytophaga johnsonii, Pseudomonas fluorescens (ATCC 11250) and Bacillus cereus.

High maximum rates (Table 6) were not obtained from
Bacterium bibulum or Bacterium liquatum and the data plotted
as $1/\eta_{sp}$ was not the same as that of the other cellulomonads
(Figs. 8 and 12). However, the maximum slope (Table 6) and
final values (Table 1) were higher than found with any or-
ganism not included in Group I, and the %ΔT plots for the
Bacterium cultures were very similar to such curves for Group
I organisms (Fig. 13).

Pseudomonas fluorescens var. cellulosa is an organism
which has been actively investigated for cellulase production
by the Japanese fermentation industry. Data of Yamane et al.
(87, 88) indicate it to be most active. This was not con-
firmed by the studies presented here. It was found to be no
more cellulolytic than another strain of this same species
not noted for cellulase production (Figs. 14 and 16). The
Japanese authors have indicated that the cellulolytic strain
does exhibit a certain low constitu tive activity and that
greater cellulase production may be induced (88). Taking
into account this last observation there are several possible
ways to reconcile the differences between our data and those
of the Japanese. One is that only the constitu tive frac-
tion was detected and that efforts to induce high activity
failed. This constitu tive activity may be a feature of sev-
eral strains of Ps. fluorescens including ATCC 11250 and

would account for the similarity of the two strains employed in this study. Iamane (88) reported that the constitutive fraction was cell bound and comprised only 10% of the overall maximum activity. Although, by the use of inducing substrates used by the above authors (87, 88), decomposition rates were somewhat increased, it is this cell bound cellulase which may have been the predominant one detected in both cultures.

Cytophaga johnsonii does not decompose insoluble cellulosic substrates and is generally not considered cellulolytic (81). Preliminary experiments with this organism indicated that no change in a strip of filter paper or Chemical Cotton could be produced. Yet this organism produced definite change in the viscosity of carboxymethyl cellulose, though at a slow rate (Figs. 19 and 20). Since this is a chitinolytic species, it is conceivable that the carboxymethyl substituent is sufficiently similar to the N-acetyl of chitin to permit this organism to hydrolyze CMC.

Bacillus cereus has not been considered to be a cellulolytic organism, yet initial rates versus substrate 7H were similar to those found during rapid decomposition by the active organisms. The decomposition was not long lived and overall very little change occurred. Since B. cereus is a sporeformer and did sporulate during decomposition

experiments it is possible that higher values might have been obtained if sporulation could have been delayed (Figs. 17 and 18). This organism was chosen as the non-cellulolytic counterpart to B. aporrheus and is not indicated to possess either cellulase or chitinase activity by Bergey's Manual (137). It might be reasonable to further investigate both possibilities in light of the data for Cyt. johnsonii.

The organisms in Group III produced little or no change in carboxymethyl substrates. There is no data in the literature indicating cellulolytic ability for Arthrobacter globiformis or Cellvibrio polyoltrophicus. Bacillus aporrheus was the least active of the species observed by Fuller and Norman (86). Cellvibrio fulvus was expected to have been an active organism. Bacillus cereus, previously discussed, is borderline between Groups II and III.

Arthrobacter globiformis, chosen as a non-cellulolytic coryneform to compare with the cellulomonads was completely inactive (Table 1, Figs. 12 and 13). B. aporrheus produced about 5% change with filter paper when tested by Fuller and Norman (86) and did about the same with substrate 7H in these studies. Despite repeated attempts, almost no decomposition was observed for Cellvibrio polyoltrophicus. Although this species produced a great deal of turbidity, no significant change in viscosity was observed (Table 2,

Figs. 15 and 16). Although tested with a variety of media
Cellvibrio fulvus did not produce strong turbid growth when
introduced into carboxymethyl cellulose media and did not
cause a change in viscosity. Maintenance of viable cultures
of a similar organism Cv. vulgaris for more than a short
period of time after rehydration from ATCC pellets was not
possible. The failure of active decomposition of both these
cultures may have more to do with the relative overall health
of the culture than do inhibition by the substrate of normal
inability to produce carboxymethyl cellulase enzymes.

The usefulness of viscometry with whole cells was rein-
forced by experiments summarized in Figure 21. Viscometric
and reducing sugar analyses were performed simultaneously
with three test organisms. Very active, moderately active
and relatively inactive cultures, Cellvibrio gilvus, Bacter-
ium and Bacillus cereus respectively, were chosen for this
comparison. The relative activity of each organism is basic-
ally the same whether determined by viscometry or by analysis
of products. The distinct separation of these three organ-
isms is readily observed. More data using larger numbers of
species is required, but it is expected that members of each
of the three groupings previously mentioned would form
similarly separated categories.

It appears from the results presented that it is not necessary to extract cellulase enzymes from active bacterial cultures to determine if they are present. Crude estimates of relative cellulolytic potential can be obtained with viable cultures using the viscometric methodology with adequate confirmation obtained by coupling to reducing sugar analyses. However, it is required that one accept that carboxymethyl cellulase activity (or any other substituted cellulase activity) is at least indicative of some form of "eucellulase" function. Carboxymethyl cellulase production is frequently used (42, 93, 112, 113, 122) to indicate hydrolytic activity versus poly β-1, 4-AHG, the so called C_X. We believe that CMCase activity is a legitimate indicator, of, but is not necessarily identical to, C_X activity.

In a strict sense, organisms are considered cellulolytic if, as the result of the possession of all components of a cellulase system required to degrade an insoluble substrate, they can reduce the substrate to soluble end products such as glucose or cellobiose. However, not all organisms which are called cellulolytic can perform this task. In an attempt to clarify this situation Charpentier (90) described two categories of microorganisms involved in cellulolysis:

> Microorganisms which can complete this transformation "true cellulolytic" or "cellulophage", possess enzymatic cellulase and glucosidase equipment: the

cellulases are comprised of two factors, one of solubilization, called C_1, the other of depolymerization and formation of oligosaccharides, called C_x.

Certain microorganisms which do not possess the C_1 factor, can only metabolize already solubilized cellulose; they are less specialized but more numerous in soil, and one might categorize them as "polyphages" as opposed to "cellulophages".

This type of categorization, forming two classes separated by the possession of C_1, is attractive to microbiogists for several reasons. Because most bacterial species heretofore employed for the investigation of cellulolysis probably would have to be characterized as cellulophage types, many species with the capability to produce change in cellulosic substrates in natural environments but, which would be classified as polyphages, have been ignored. If one's aim is to characterize an enzyme system, it is natural to choose, as sources, bacteria which produce relatively large amounts of enzymes with great activity rather than those which produce small amounts or which elaborate less active enzymes. Yet, if the latter group is much more numerous in a natural habitat than members of the former, the overall effect may be that the polyphage types are responsible for as much cellulose degradation as the cellulophage types. The new classification system makes the polyphage generalist at least as important as the cellulophage specialist.

The cellulophage-polyphage nomenclature also provides for an approach to the problem of synergistic degradation of cellulose. Polyphages may be understood to be lacking in certain parts of the cellulolytic complement possessed by cellulophages. Charpentier has indicated that the polyphages lack C_1. If the C_1 function were restored the polyphage might appear to be a cellulophage. This might be done chemically or enzymatically in an artificial environment. In a natural situation, the C_1 function might be supplied by another organism. The second organism would not necessarily be required to possess any of the remainder of a cellulase system besides C_1 to allow complete degradation of cellulose by the combined efforts of both microbes. All that would be required is the complementation of the enzyme systems of the two organisms and the overall compatibility of both (e.g. range, temperature tolerance, lack of antagonistic responses, etc.). Synergistic cellulolysis has been assumed to exist but has been found difficult to prove. Possibly the elimination of the bias toward cellulolysis in sensu stricto will permit the establishment of means to investigate this possibility.

Since most schemes for isolating or enumerating cellulolytic bacteria from natural habitats are predicated on the ability of the isolate to degrade an insoluble material,

generally filter paper, in order to be detected, freshly iso-
lated cellulolytic cultures generally possess some initial
C_1 activity. Because of the bias against the polyphage type
numbers of cellulolytic bacteria observed are probably equiv-
alent to numbers of cellulophage bacteria detectable. It
would seem that in order to determine the extent of the total
aerobic cellulolytic bacterial population in a certain habi-
tat, some criteria must be established for them which do not
automatically rule out polyphages.

The polyphage-cellulophage concept is a model system
for which little evidence for its existence has been found.
Nevertheless some of the data presented herein are suggestive
of support for this hypothesis. The groupings for the var-
ious test species were determined from performance obtained
using substrate 7H. Those species which formed Group I were
all species regarded as active cellulose decomposers by most
authors. Those which formed Group II, though producing some
measurable change in the substrate, were not primarily organ-
isms previously noted as cellulose decomposers. Only the
two organisms originally named "Bacterium" and later classi-
fied as cellulomonads and the variety of Pseudomonas fluores-
cens ATCC 13042T have been mentioned previously as having
cellulolytic capability. Half the cultures, the pseudomonads
and Bacillus cereus are basically "generalist" soil organisms.

Only the <u>Cytophaga johnsonii</u> was noted as a specialist, that specialty being chitinolysis.

It is tempting to call those belonging to Group I cellulophage type, and those in Group II polyphage type but, obviously, such a brief study, which was not intended to pursue the cellulophage-polyphage hypothesis, is insufficient to allow such a classification. This study could be extended, however, to include a larger number of "garden variety" bacteria known to possess hydrolytic capability toward such substrates as starch or chitin. The existence of large numbers of common bacteria possessing C_X activity would suggest an important role for the polyphage group. Conversely the lack of C_X by large numbers of non-specialist bacteria would indicate that the cellulophage population was indeed the dominant factor in cellulose decomposition in those situations where bacterial cellulolysis was predominant. The polyphage would then become the occasional opportunist which could fill a vacant niche until the cellulophage population became re-established.

The information so far presented may lead to various applications. The impetus for this study resulted from an inability to justify the accuracy of numbers of cellulolytic bacteria from aquatic situations obtained in previous work. Most data pertaining to numbers of bacteria have been

obtained by the use of MPN techniques using filter paper or some similar substrate. But this traditional method has many limitations. The MPN basic technique is predicated on the ability of a single cell to give rise to a culture capable of producing a detectable reaction, such as gas from lactose in tests for sanitary significant bacteria. But it is highly unlikely that a single cell can initiate decomposition of filter paper cellulose. A critical population is probably needed to provide sufficient enzymatic activity to partially degrade the original substrate thus giving the survivors of the initial population a favorable energy balance to permit further enzyme production and cell reproduction. Fuller and Norman suggested that the hemicelluloses in natural cellulose are used as substrates to enable cellulolytic forms to establish the critical populations (86). Thus, numbers detected by the traditional method are probably lower than real values.

The use of filter paper also tends to reduce the number of species detected to those possessing strong C_1 activity. As indicated previously, even very active cellulolytic bacteria have difficulty decomposing filter paper. However, hydrolysis of cellulosic substrates can be accomplished by those not able to use filter paper. These would not be detected by the usual MPN methods.

The selection of the most active organisms in a particular habitat may be very desirable in many circumstances, and the filter paper-in-liquid techniques work well for those purposes. But an accurate estimation of the total cellulose hydrolytic potential requires a means of detecting those which possess C_X but not C_1. The use of a soluble substrate such as carboxymethyl cellulose may be advisable. It was indicated here that most acknowledged cellulolytic bacteria tested did produce decomposition of these substrates. Detection of the decomposition was relatively easy and first signs occurred within three days or less. Detection of decomposition of filter paper-in-liquid media may take as long as a month. If one accepts that CMCase activity is indicative of C_X potential then the use of CMC or similar substrates should be encouraged as part of any comprehensive enumeration scheme.

A simple application might involve the routine inoculation of a series of flasks of soluble cellulosic substrate from the same batch with aliquots from a sample which was also used to inoculate an MPN series. A comparison between the two series would indicate numbers of organisms possessing or lacking C_1 but having C_X. A reduction of $50\% \triangle T$ or its equivalent $1/\eta_{sp}$ value within 3-5 days could be construed as a positive sign of decomposition. Subculture of the organisms in the flasks using both soluble and insoluble

substrates might give an indication of the numbers of "cellu-
lophage" types found in both series, or conversely, numbers
of "polyphage" types not detected by the MPN series.

The results of this study indicate several research
directions which may profitably be pursued. It has been
shown that the biochemical tool of viscometry may be readily
adaptable to the use of viable cultures. Using a modified
viscometric technique, a distinction between very active
generally recognized cellulolytic forms and less active or
inactive forms not generally considered to be cellulolytic
can be demonstrated. Some of the previously considered non-
cellulolytic forms did provide some detectable carboxymethyl
cellulase activity, however. It was suggested that further
investigations of the cellulolytic potential of ordinary
soil and water organisms not previously tested for cellulase
activity or considered non-cellulolytic because they fail to
degrade filter paper might be profitable to be pursued. The
substrates chosen for these studies have been carboxymethyl
cellulases but since some studies have suggested that hydro-
xyethyl or other similar soluble substituted cellulosic
polymers may be more suitable, it would be wise to expand
the type of study initiated herein to include these sub-
strates. Filter paper does not appear to be an ideal sub-
strate for revealing the cellulolytic potential of bacteria.

Its continued use as a basis for criteria for that purpose should be questioned. Its use as a substrate for the selection of organisms possessing high C_1 activity is probably quite valid. The use of other insoluble substrates may be better than ordinary filter paper. Avicel is a commercial product which was not tested in this study and has been found to stimulate cellulase production in Pseudomonas fluorescens (87, 88). It is possible that this or other insoluble substrates may be favorably combined with techniques other than gravimetry to demonstrate cellulase activity by a wider variety of organisms than has been found using filter paper.

The cellulophage-polyphage hypothesis also provides a departure point for much future research in the microbial ecology of cellulose decomposition. With proper methodological development, basic field investigations can provide much information concerning the role of the polyphage. The sharp distinctions between the two categories may not exist but could be of degree rather than kind. Biochemical research of the variety currently being pursued should reveal whether those which seem to possess only C_x may also produce small amounts of C_1. It is important, in any case, that microbial ecologists recognize that cellulose degradation in natural situations may involve more than the organism classified as cellulophages and that the experimental design take into account this possibility.

Understanding of the biochemical nature of cellulolysis has been advanced considerably in recent years, but much of this information does not seem to have filtered down to the microbiologist who continues to rely on the limited traditional methodologies. This study has indicated that some of the biochemical techniques can be readily adapted to the use of viable cultures with which an ecologist may prefer to work. It has shown that cellulose in modified form may be degraded by organisms not usually considered capable of this effort. Since cellulose in natural habitats is probably found as a partially degraded polymer as a result of the activities of certain organisms, these organisms may have an important function in the completion of degradation of cellulosic material. This function is implied in the cellulophage-polyphage hypothesis and merits further consideration. The application of traditional criteria based on observations obtained with organisms which would be classified cellulophage types inhibits such considerations and those criteria should be modified.

SUMMARY AND CONCLUSIONS

1) In recent decades great strides have been made in understanding the enzmyology of cellulose decomposition by specific microorganisms. However, this progress does not appear to have produced a similar response from microbiologists concerned with the detection of cellulose-decomposition by bacteria in natural systems. The various criteria for the determination of cellulolytic action in simplified cell free systems are not uniformly applicable to the complex living systems with which an ecologist must deal.

2) In categorizing species on the basis of cellulolytic capability, the choice of substrate which can be accepted as "cellulose" is very important. Some consider as acceptable only those very purified, highly polymeric, crystalline unsubstituted materials such as filter papers. However, early research, supported by data presented herein, indicate that these materials are very restrictive for bacterial species including those commonly called cellulolytic.

3) Viscometry is one of several methodologies found to be very useful in enzymological studies of cellulolysis. It was shown that this technique is readily adaptable for quantitative determination of cellulolytic potential (C_X hydrolase activity) by living cultures of aerobic mesophilic bacteria.

4) Fifteen cultures of aerobic mesophilic bacterial species were tested using viscometric techniques employing several carboxymethyl cellulose substrates for C_x (CMCase) potential. On the bases of these tests three relative groupings were observed. The most active group included three Cellulomonas species, Cytophaga hutchinsonii, and Cellvibrio gilvus. Species less active than the first group but possessing some decompositional capability included two cellulomonad Bacterium species, two varieties of Pseudomonas fluorescens, Cytophaga johnsonii and a Bacillus cereus culture. Organisms for which no significant change in the substrates could be observed included Bacillus aporrheus, Arthrobacter globiformis, Cellvibrio polyoltrophicus and Cellvibrio fulvus.

Several of the organisms found capable of producing low to moderate change in cellulosic substrates were common soil organisms, and had not been previously classified as cellulolytic species. On the basis of the findings concerning those organisms comprising the second grouping, it was recommended that these studies be extended to other common soil and water bacteria previously untested for C_x activity, especially those possessing known chitinase activity.

5) Until more information is obtained with respect to the capabilities of the majority of soil and water organisms

possessing β -1, 4 glucan-glucanohydrolases it would behoove the microbiologist to use a less strict rather than a more strict interpretation of "cellulose" during ecological investigation of bacterial cellulolysis.

LITERATURE CITED

1. Cowling, E. B. 1963. Structural features of cellulose that influence its susceptibility to enzymatic hydrolysis. In: E. T. Reese (Ed.). Enzymic Hydrolysis of Cellulose and Related Materials. Pergamon Press, New York, pp. 1-32.

2. Browning, B. L. 1963. The composition and chemical reactions of wood. In: Browning, B. L. (Ed.). The Chemistry of Wood. John Wiley & Sons, New York. pp. 57-102.

3. Payen, A. 1838. Compt. Rend. 7:1052, 1125 (As cited in (5)).

4. Schuerch, C. 1963. The hemicelluloses. In: Browning, B. L. (Ed.). The Chemistry of Wood. John Wiley & Sons, New York. pp. 191-243.

5. Immergut, E. H. 1963. Cellulose. In: Browning, B. L. (Ed.). The Chemistry of Wood. John Wiley & Sons, New York. pp. 103-190.

6. Ohad, I. and D. Mejzler. 1965. On the ultrastructure of cellulose microfibrils. J. Polymer Sci. 3:339-406.

7. Naegli, C. Micellartheorie. In: A. Frey (Ed.). Ostwalds Klassiker. No. 227, Leipzig, 1928 (As cited in (10)).

8. Meyer, K. H. 1930. Raumliche Vorstellungen uber den Bau der Kohlenstoffverbindungen und ihre Verwendung in der Chemie der Hochpolymeren. Kolloid Zeitschrift 53:8-19.

9. Staudinger, H. 1933. Viscosity investigations for the examination of the constitution of natural products of high molecular weight and of rubber and cellulose. Trans. Faraday Soc. 29:18-32.

10. Hearle, J. W. S. 1963. The development of ideas of fine structure. In: Hearle, J. W. S. and R. M. Peters. Fibre Structure. Butterworth, Washington. pp. 209-234.

11. Gralen, N. and T. Svedberg. 1943. Molecular weight of native cellulose. Nature(London) 152:625.

12. Manley, R. St. J. 1964. Fine structure of native cel-
     lulose microfibrils. Nature(London) 204:1155-1157.

13. Marx-Figni, M. and G. U. Schulz. 1966. Uber die Kinet-
     ik und den Mekanismus der Biosynthese der Cellulose in
     den Hoheren Pflanzen (Nach Versuchen an den Samenhaaren
     der Bau Wolle). Biochem. Biophys. Acta. 112:81-100.

14. Trager, W. 1932. A cellulase from the symbiotic in-
     testinal flagellates of termites and the roach, Crypto-
     cercus punctutatus. Biochem. J. 26:1763-1771.

15. Hungate, R. E. 1938. Studies on the nutrition of Zo-
     otermopsis. II. The relative importance of the termite
     and the protozoon in wood digestion. Ecology 19:1-25.

16. Bryant, M. P. 1964. Some aspects of the bacteriology
     of the rumen, p. 366-393. In: H. Heukelikian and M.
     Dondero (Eds.). Principles and Applications in Aquatic
     Microbiology. John Wiley & Sons, Inc., New York.

17. Ray, D. L. 1959. Some properties of cellulase from
     Limnoria. In: Ray, D. L. (Ed.). Marine Boring and
     Fouling Organisms. University of Washington Press,
     Seattle. pp. 372-396.

18. Salum, J. 1968. Cellulolytic activity in the digestive
     gland of Strophocheilus (Megabulimus) ovatus (Muller
     1774). Rev. Brasil Pesqui. Med. Biol. 1:227-234.

19. Dropkin, V. 1963. Cellulase in phytoparasitic nema-
     todes. Nematologia 9:444-454.

20. Meyers, F. L. and D. H. Nothcote. 1959. Partial puri-
     fication and some properties of a cellulase from Helix
     pomatia. Biochem. J. 71:749-756.

21. Tracey, M. V. and G. Youatt. 1958. Cellulase and chit-
     inase from two species of Australian termites. Enzymol-
     ogia. 19:70-72.

22. Rebeyrotte, P., F. Dupin and M. Jayle. 1967. Contribu-
     tion a l'etude d'une cellulase extraite du suc digestif
     d'Helix pomatia. Bull. Soc. Chim. Biol. 49:1247-1256.

23. Yamanouchi, J. and S. Horiuchi. 1968. Cellulase activ-
     ity in the hepatopancreas of a sea-hare, Aplysia kurodai
     (Opisthobranchia). Zool. Mag. 77:157-59 (Japanese).

24. Brooks, M. A. 1963. Symbiosis and aposymbiosis in arthropods. In: Symbiotic Associations. Cambridge, England. pp. 200-231.

25. Lasker, R. and A. C. Giese. 1958. Cellulose digestion by the silverfish Ctenolepisma lineata. J. Exptl. Biol. 33:542-553.

26. Khouvine, Y. 1923. Digestion de la cellulose par la flore intestinale de l'homme. B. cellulosae dissolvens n. sp. Ann. Inst. Pasteur. 37:711-752.

27. Hungate, R. E. 1946. The cellulose-decomposing bacteria in the rumen of cattle. J. Bacteriol. 51:589.

28. Hungate, R. E. 1963. Symbiotic associations: the rumen bacteria. In: P. S. Nutman and B. Mosse (Eds.). Symbiotic Associations, Cambridge Univ. Press. Cambridge, England. pp. 266-297.

29. Matchett, W. H. and J. F. Nance. 1962. Cell-wall breakdown and growth in pea seedling stems. Am. J. Botany 49:311-319.

30. MacLachlan, G. A. and J. Perrault. 1964. Cellulose from pea epicotyls. Nature(London) 204:81-82.

31. Frey-Wyssling, A. 1950. Physiology of cell wall growth. Ann Rev. Plant Physiol. 1:169-182.

32. Abeles, F. B. 1969. Abscission: role of cellulase. Plant Physiol. 44:447-452.

33. Hobson, G. E. 1968. Cellulase activity during the maturation and ripening of tomato fruit. J. Food Sci. 33:588-592.

34. Horton, R. F. and D. J. Osborne. 1967. Senescence, abscission and cellulase in Phaseolus vulgaris. Nature (London). 214:1086-1088.

35. Greenfield, L. J. and C. E. Lane. 1953. Cellulose digestion in Teredo. J. Biol. Chem. 204:669-672.

36. Brock, T. D. 1966. Principles of Microbial Ecology. Prentice Hall, Englewood Cliffs, N. J. pp. 198-204.

37. Dubos, R. J. 1928. The decomposition of cellulose by aerobic bacteria. J. Bacteriol. 15:223-234.

38. Siu, R. G. H. 1951. Microbial Decomposition of Cellulose Reinhold Pub. Co., New York. 531 pp

39. Greathouse, G. A. and L. M. Ames. 1945. Fabric deterioration by thirteen described and three new species of Chaetomium. Mycologia 37:138-155.

40. Whitaker, D. R. 1953. Purification of Myrothecium verrucaria cellulase. Arch. Biochem. Biophys. 43:253-268.

41. Datta, P. K., K. R. Hanson and D. R. Whitaker. 1963. Improved procedures for preparation and characterization of Myrothecium cellulase. Part. 3. Molecular weight, amino acid composition, terminal residues and other properties. Can. J. Biochem. Physiol. 41:697-706.

42. W. Klop and P. Kooiman. 1965. The action of cellulolytic enzymes on substituted celluloses. Biochem. Biophys. Acta 99:102-120.

43. Halliwell, G. and M. Riaz. 1970. The formation of short fibres from native cellulose by components of Trichoderma koningii cellulase. Biochem. j. 116:35-42.

44. Eriksson, K-E. and B. H. Hollmark. 1969. Kinetic studies of the action of cellulase upon sodium carboxymethyl-cellulose. Arch. Biochem. Biophys. 133:233-237.

45. Jermyn, M. A. 1955. Fungal cellulases. IV. Production and purification of an extracellular B-glucosidase of Stachybotrys atra. Austral. J. Biol. Sci. 8:541-562.

46. Youatt, G. 1958. Fungal cellulases. IX. Growth of Stachybotrys atra on cellulose and production of a B-glucosidase hydrolysing cellobiose. Austral. J. Biol. Sci. 11:209-217.

47. Mitscherlich. 1850. Zussammensetzung der wand der Pflanzenzelle. Bericht uber die zur Bekannemachung geeigneten Verhandlungen der Koniglichen Preussischen Akademie der Wissenschaften zu Berlin. pp. 102-110. (As cited in 67)

48. Popoff, L. 1875. Ueber die Sumpfgasgagrung. Pfluger's Archiv. 10:113-146.

49. Hoppe-Seyler, F. 1883. Garung der cellulose. Berichte Deutsch. Chem. Gesellschaft. 16:122-123.

50. Omelianski, W. 1895. Sur la fermentation de la cellulose. Comptes Rendus. 121:653-655.

51. Omelianski, W. 1897. Sur un ferment de la cellulose. Comptes Rendus. 125:970-973.

52. Omelianski, W. 1899. Sur la fermentation de la cellulose. Arch. Sci. Biol. 7:411-434.

53. van Iterson, G., Jr. 1904. Die Zersetzung von Cellulose durch Aerobe Mikrooganismen. Zentr. f. Bakteriol. Abt. 2. 11:689-698.

54. Hutchinson, H. B. and J. Clayton. 1919. On the decomposition of cellulose by an aerobic bacterium (Spirochaeta cytophaga, n. sp.) J. Agri. Sci. 9:143-173.

55. Winogradsky, S. 1929. Etudes sur la microbiologie du sol. 4e memoire: Sur la degradation de la cellulose sans le sol. Ann. Inst. Pasteur. 43:549-596.

56. Winogradsky, S. 1932. Sur la decomposition de la cellulose par les bacteries. Bull. Inst. Pasteur. 30:369-376.

57. Stanier, R. Y. 1940. Studies on the cytophagas. J. Bacteriol. 40:619-635.

58. Krzemieniewska, H. 1933. Contribution a l'etude du genre Cytophaga (Winogradsky). Arch. f. Mikrobiol. 4:394-408.

59. Imsenecki, A. and L. Solntzeva. 1936. On aerobic cellulose-decomposing bacteria. Akad. Nauk SSSR Isvest. Serv. Biol. 6:1115-1172.

60. Stapp, C. and H. Bortels. 1934. Mikrobiologische Untersuchungen uber die Zersetzung von Waldstreu. Zentr. f. Bakt. Abt. II. 90:28-67.

61. Stanier, R. Y. 1942. The Cytophaga group. A contribution to the biology of Myxobacteria. Bac. Rev. 6:143-196.

62. Gray, P. H. H. and C. H. Chalmers. 1924. On the stimu-
    lating action of certain organic compounds on cellu-
    lose decomposition by means of a new aerobic microor-
    ganism that attacks both cellulose and agar. Ann. Appl.
    Biol. 11:324-338.

63. Hulcher, F. H. and K. W. King. 1958. Disaccharide
    preference of an aerobic cellulolytic bacterium, Cell-
    vibrio gilvus, n. sp. J. Bacteriol. 77:565-570.

64. Kadota, H. 1956. A study on the marine aerobic cellu-
    lose-decomposing bacteria. Mem. Coll. Agri., Kyoto
    Univ. #74, Fisheries Ser. 6:1-127.

65. Krainsky, A. 1914. Die Aktinomyceten und ihre Bedeu-
    tung in der Natur. Zentr. f. Bakt. Abt. II. 41:649-688.

66. Rubentschik, L. 1933. Zur anaeroben Zellulosezersət-
    zung in Salzeen. Zentr. f. Bakt. Abt. II. 88:182-186.

67. McBeth, I.G. and F. M. Scales. 1913. The destruction
    of cellulose by bacteria and filamentous fungi. U.S.D.A.
    Bur. Plant Ind. Bull. No. 266. 52pp.

68. Kellerman, K. F., I. G. McBeth, F. M. Scales and N. R.
    Smith. 1913. Identification and classification of cel-
    lulose-dissolving bacteria. Zentr. f. Bakt. Abt. II.
    39:502-522.

69. Clark, F. E. 1953. Criteria suitable for species dif-
    ferentiation in Cellulomonas and a revision of the
    genus. Int. Bull. Bacteriol. Nomen. Tax. 4:179-199.

70. Viljoen, J. A., E. B. Fred and W. H. Peterson. 1926.
    The fermentation of cellulose by thermophilic bacteria.
    J. Agri. Sci. 16:1-17.

71. McBee, R. H. 1950. The anaerobic thermophilic cellu-
    lolytic bacteria. Bacteriol. Revs. 14:51-63.

72. Enebo, L. 1951. On three bacteria connected with
    thermophilic cellulose fermentation. Physiol. Plant.
    4:652-666.

73. Hungate, R. E. 1946. The cellulose-decomposing bac-
    teria in the rumen of cattle. J. Bacteriol. 51:589.

74. Hungate, R. E. 1950. The anaerobic mesophilic cellu-
    lolytic bacteria. Bacteriol. Rev. 14:1-49.

75. Sijpesteijn, A. K. 1948. Cellulose-decomposition by
    bacteria in the rumen. Dissertation, Univ. Leiden.

76. Smith, P. H. and R. E. Hungate. 1958. Isolation and
    Characterization of Methanobacterium ruminatum, n. sp.
    J. Bacteriol. 75:713-718.

77. Allison, M. J., M. P. Bryant, and R. N. Doetsch. 1958.
    A volatile fatty acid growth factor for cellulolytic
    cocci of the bovine rumen. Science 128:474-475.

78. Waksman, S. A. and C. E. Skinner. 1926. M croorganisms
    concerned in the decomposition of cellulose in soil.
    J. Bacteriol. 12:57-84.

79. Keddie, R. M., B. G. S. Leask and J. M. Grainger. 1966.
    A comparison of coryneform bacteria from soil and
    herbage: cell wall composition and nutrition. J.
    Appl. Bact. 29:17-43

80. Owens, J. D. and R. M. Keddie. 1969. The nitrogen
    nutrition of soil and herbage cornyeform bacteria. J.
    Appl. Bacteriol. 32:338-347.

81. Stanier, R. Y. 1947. Studies on non-fruiting Myxo-
    bacteria I. Cytophaga johnsonae, n. sp. a chitin-
    decomposing Myxobacterium. J. Bacteriol. 53:297-315.

82. Fahreus, G. 1947. Studies in the cellulose decomposi-
    tion by Cytophaga. Symbolae Botan. Upsaliensis. 9:1-
    128.

83. Stanier, R. Y. 1942. Are there obligate cellulose de-
    composing bacteria? Soil Science. 53:479-480.

84. Hulcher, F. H. and K. W. King. 1958. Disaccharide
    preference of an aerobic cellulolytic bacterium. Cell-
    vibrio gilvus, n. sp. J. Bacteriol. 76:565-570.

85. Scolnick, E. M. and E. C. C. Lin. 1962. Parallel in-
    duction of D-arabitol and D-sorbitol dehydrogenases.
    J. Bacteriol. 84:631-637.

86. Fuller, W. H. and A. G. Norman. 1943. Cellulose de-
    composition by aerobic mesophilic bacteria from soil.
    I. Isolation and description of organisms. J. Bac-
    teriol. 46:281-289.

87. Yamane, K., H. Suzuki, M. Hirotani, H. Ozawa and K.
    Nisuzawa. 1970. Effect of nature and supply of carbon
    sources on cellulase formation in Pseudomonas fluores-
    cens var. cellulosa. J. Biochem. 67:9-18.

88. Yamane, K., H. Suzuki, and K. Nisizawa. 1970. Purifi-
    cation and properties of extracellular and cell-bound
    cellulase components of Pseudomonas fluorescens var.
    cellulosa. J. Biochem. 67:19-35.

89. Charpentier, M. 1965. Etude de l'activite cellulolytique
    de Sporocytophaga myxococcoides. Ann. Inst. Pasteur.
    109:771-797.

90. Charpentier, M. 1968. Rapport general. Ann. Inst.
    Pasteur. 115:497-537.

91. Jurasek, L., J. R. Colvin, and D. R. Whitaker. 1967.
    Microbiological aspects of the formation and degrada-
    tion of cellulosic fibers. Adv. Appl. Microbiol. 9:
    131-170.

92. Reese, E. T., R. G. H. Siu, and H. S. Levinson. 1950.
    The biological degradation of soluble cellulose deriv-
    atives and its relationship to the mechanism of cellu-
    lose hydrolysis. J. Bacteriol. 59:485-498.

93. Norkrans, B. 1967. Cellulose and cellulolysis. Adv.
    Appl. Microbiol. 9:91-130.

94. Siu, R. G. H. 1963. The state of the art. In: E. T.
    Reese (Ed.). Advances in Enzymic Hydrolysis of Cellu-
    lose and Related Materials. Pergamon Press, New York.
    pp. 257-259.

95. Fuller, W. H. and A. G. Norman. 1943. Cellulose de-
    composition by aerobic mesophilic bacteria from soil.
    II. Biochemical studies on filter paper and cellulose
    preparations. J. Bacteriol. 46:281-289.

96. Li, L.-H., R. M. Flora and K. W. King. 1965. Arch.
    Biochem. Biophys. 111:439-447.

97. _Enzyme Nomenclature, Recommendations 1964 of the International Union of Biochemistry_, Elsevier, Amsterdam, 1965.

98. Cole, F. E. and K. W. King. 1964. Site of hydrolysis of cellulodextrins and reduced cellulodextrins by purified cellulase components. Biochem. Biophys. Acta. 81:122-129.

99. Enger, M. D. and B. P. Sleeper. 1965. Multiple cellulase system from _Streptomyces antibioticus_. J. Bacteriol. 89:23-27.

100. Terui, G. and H. Fujiwara. 1949. Behavior of _Bacillus hydrolyticus_ toward celluloses. J. Ferment. Technol. (Japanese). 27:10-15.

101. Okamoto, T. and T. Asai. 1952. Studies on the aerobic mesophilic cellulose-decomposing bacteria in Japan. VI. On the bacterial cellulases. J. Agri. Chem. Soc. (Japan). 26:137-140. (As cited in 102)).

102. Nisizawa, K., Y. Hashimoto, and Y. Shibata. 1963. Specificicities of some cellulases of the "random" type. _In_: E. T. Reese (Ed.). Advances in Enzymic Hydrolysis of Cellulose and Related Materials. Pergamon Press, New York. pp. 171-184.

103. Hammerstrom, R. A., K. D. Claus, J. W. Coghlan and R. H. McBee. 1955. The Constituative nature of bacterial cellulases. Arch. Biochem. Biophys. 56:123-129.

104. Norkrans, B. 1950. Studies in growth and cellulolytic enzymes of _Tricholoma_. Symbolae Botan. Upsaliensis.

105. Porter, F. E., and H. H. Weiser and W. D. Gray. 1955. Studies on bacterial interaction: I. The effect of trypsin on the cellulase activity of _Cellulomonas flavigena_. Ohio J. Sci. 55:335-339.

106. Youatt, G. 1960. Some factors influencing the breakdown of cellulose by bacteria. Aust. J. Biol. Sciences. 13:188-195.

107. Berg, B., B. v. Hofsten and B. Goransson. 1968. Some properties of cellulolytic _Cellvibrio_ strains from polluted water. Appl. Microbiol. 16:1424-1425.

108. Went, J. D. 1959. Cellophane as a medium to study the cellulose decomposition in forest soils. Acta Botan. Neerl. 8:490-491.

109. Went, J. C. and F. de Joong. 1966. Decomposition of cellulose in soils. Antoinie v. Leeuwen. 32:39-56.

110. Tetreault, P. A. 1930. The fermentation of cellulose at high temperatures. Zentr. f. Bakt. Abt. II. 81:28-45.

111. Joos, P., W. Sierens and R. Ruyssen. 1969. The determination of cellulase activity by viscometry. J. Pharm. Pharmac. 21:848-853.

112. Thomas, R. 1956. Fungal cellulases. VII. Stachybotrys atra: Production and properties of the cellulolytic enzyme. Austral. J. Biol. Science. 9:559-562.

113. Wirick, M. G. 1968. A study of the enzymic degradation of CMC and other cellulose ethers. J. Poly. Science Pt. A-1. 6:1965-1974.

114. Richard, F. 1945. Der Biologische Abbau von Zellulose und Eiweiss ihren im Boden von Walt und der Razen Gesellschaft. Mitt. Schweiz. Anst. Forstl. Versuchsw. 24:297-397.

115. Marsh, P. B., G. A. Greathouse, M. L. Butler and K. Bollerbacher. 1945. USDA Tech. Bull. #892. (As cited by (116)).

116. Meyers, S. P., Prindle, B., K. Kamp and J. U. Levy. 1963. Degradation of manila cordage by marine fungi: Analysis of breaking strength tests. TAPPI. 46:164-167.

117. Laurent, M. 1969. Experimental investigations of cellulolysis in mud. In: H. Jenkins (ed.). Advances in Water Pollution Research. Pergamon Press, London. pp. 917-925.

118. Bailey, P. J., Liese, W., R. Roesch, G. Keilich and E. G. Arting. 1969. Cellulase (B-1, 4-glucan 4-glucanohydrolase) from the wood-degrading fungus Polyporus schweinitzii Fr. I. Purification. Biochem. Biophys. Acta. 185:381-391.

119. Umbreit, W. W., R. H. Burris and J. F. Stauffer. 1957. Manometric Techniques. Burgess Publ. Co., Minneapolis. 338 pp.

120. Monreal, J. and E. T. Reese. 1968. The chitinase of Serratia marcescens. Can J. Micro. 15:689-696.

121. Whitaker, D. R. 1954. Hydrolysis of a series of B-1, 4"- oligoglucosides by Myrothecium verrucaris cellulase. Arch. Biochem. Biophys. 53:436-438.

122. Whitaker, D. R. 1963. Criteria for characterizing cellulases. In: E. T. Reese (Ed.). Advances in Enzymic Hydrolysis of Cellulose and Related Materials. Pergamon Press, N. Y. pp. 51-70.

123. Kadota, H. 1959. Cellulose decomposing bacteria in the sea. In: D. L. Ray (Ed.). Marine Boring and Fouling Organisms. Univ. Washington Press, Seattle.

124. Waksman, S. A., C. Carey and H. W. Reuszer. 1933. Maine bacteria and their role in the cycle of life in the sea. I. Decomposition of marine plant and animal residues by bacteria. Biol. Bull. 65:57-79.

125. Rodina, A. G. and N. K. Kuz'mtskaya. 1964. Species composition of the heterotrophic microorganisms of Lake Ladoga. Microbiology. 33:894-900. (Transl. of Mikrobiologiya).

126. Graf, W. and P. Sturzenhofecker. 1967. Uber die Okologie von Wassermxyobakterien (Sporocytophaga cauliformis) im Bodensee. Arch. Hyg. 149:256-265.

127. Meyers, S. P., B. Prindle and E. S. Reynolds. 1960. Cellulolytic activity of marine fungi. TAPPI. 43: 534-539.

128. Alexander M. 1961. Introduction to Soil Microbiology. John Wiley and Sons, New York. 472 pp.

129. Nelson, N. 1944. A photometric adaptation of Somogyi method for the determination of glucose. J. Biol. Chem. 153:375-380.

130. Somogyi, M. 1952. Notes on sugar determinations. J. Biol. Chem. 195:19-23.

131. Hercules Incorporated. 1968. Hercules Cellulose Gum.

132. H. Hatt. personal communication, Oct. 22, 1969

133. Fuller, W. H. and A. G. Norman. 1942. A cellulose-
     dextrin medium for identifying cellulose organisms in
     soil. Soil. Sci. Soc. Am., Proc. 7:243-246.

134. Ueda, K., S. Ishikawa, T. Itami and T. Asai. 1952.
     Studies on the aerobic mesophilic cellulose-
     decomposing bacteria in Japan (V): Sporocytophaga
     and Pseudomonas. J. Agric. Chem. Soc.Japan. 26:35-
     41.

135. Norkrans, B. 1963. Degradation of cellulose. Ann.
     Rev. Phytopath. 1:325-350.

136. Bradley, L. A. 1925. The decomposition of cellulose
     by aerobic bacteria. Ph.D. Dissertation, Yale.

137. Breed, R. S., E. G. D. Murray and N. R. Smith (Eds.).
     1957. Bergey's Manual of Determinative Bacteriology,
     7th Ed. Williams and Wilkins Co., Baltimore.

138. Morris, J. G. 1960. Studies on the metabolism of
     Arthrobacter globiformis. J. Gen. Microbiol. 22:564-
     582.