National Library
of Canada

Bibliothèque nationale
du Canada

Canadian Theses Service

Services des thèses canadiennes

Ottawa, Canada
K1A 0N4

CANADIAN THESES

THÈSES CANADIENNES

NOTICE

The quality of this microfiche is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Previously copyrighted materials (journal articles, published tests, etc.) are not filmed.

Reproduction in full or in part of this film is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30.

AVIS

La qualité de cette microfiche dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

Les documents qui font déjà l'objet d'un droit d'auteur (articles de revue, examens publiés, etc.) ne sont pas microfilmés.

La reproduction, même partielle, de ce microfilm est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30.

THIS DISSERTATION
HAS BEEN MICROFILMED
EXACTLY AS RECEIVED

LA THÈSE A ÉTÉ
MICROFILMÉE TELLE QUE
NOUS L'AVONS REÇUE

NL-339 (r. 86/06)

Canada

ISBN 0-315-30959-8

TABLE OF CONTENTS

INTRODUCTION

METHODS

RESULTS

Acknowledgements

The work presented in this thesis was done in the Department of Biology, University of Ottawa, under the supervision of Professor Donn J. Kushner.

I like to take this opportunity to thank Dr. D.J. Kushner for his advice and supervision of this research project.

I also would like to thank Dr. J. Saddler, Dr. D. Hickey and Dr. H. Yamazaki for accepting to be part of my thesis committee and for their helpful suggestions.

I wish to express my thanks to Dr. T. Kauri and to all my co-workers in Dr. D.J. Kushner's laboratory for their support and advice.

I am indebted to Dr. C. Breuil for translating the abstract of this thesis into French.

Finally I would like to thank my family for their patience, help and encouragement.

Abstract

Cellulomonas sp. (NRCC 2406) was grown on a basal medium or in a complex medium, with the addition of different celluloses (solka floc, avicel, CF 11 cellulose, Whatman no.1 filter paper and carboxymethyl cellulose) and/or glucose. When grown in the basal medium with cellulose addition only endocellulase production was observed, however when grown in complex medium with cellulose addition cultures produced endo and exo-cellulases and very little β-glucosidase. Addition of cellulose to both media stimulated growth as measured by cellular protein and also stimulated the production of cellulases. Higher endocellulase activity was obtained from whole cells than from the extracellular fraction suggesting that some of the endocellulases remain associated with the cells or with the cellulose fibers. β-Glucosidase was found to be a membrane bound enzyme. Addition of glucose in the presence of cellulose inhibited growth and cellulose breakdown. The proteins of the supernatants obtained from cultures grown in complex medium containing different kinds of cellulose fibers were examined by polyacrylamide gel electrophoresis. The supernatants contained a number of different proteins considerably more if the cells were grown in the presence of cellulose and the absence of glucose. Cellulolytic enzymes were identified by transferring the proteins from the polyacrylamide gels to agar gels containing carboxymethyl cellulose and stained with congo red. Experiments with non-denaturing gels showed up to 5 bands of

activity.

The potential utilization of cellulose requires microorganisms which have a high cellulase activity and/or are no longer under repressive control. It was found impossible to obtain mutants of *Cellulomonas* 2406 when using conventional mutagenic agents such as: ultraviolet light, methyl methanosulfonate and N-Methyl-N'-nitro-N-nitrosoguanidine.

Electron microscope studies showed that *Cellulumonas* 2406 adhered to the cellulose fibers by producing a polysaccharide material. This thick outer layer was not present when cells were grown in the complex medium without cellulose. This polysaccharide layer probably helps in the adhesion process but whether the contact between the cells and the cellulose fibers is necessary for cellulase induction is yet to be resolved.

Résumé

L'espèce *Cellulomonas* (NRCC 2406) croît sur milieu minéral ou sur milieu complexe contenant différentes sources de cellulose (cellulose CF 11, papier filtre Whatman no. 1, solka floc, avicel et cellulose carboxy-méthylée) et/ou du glucose. Lorsque la bactérie croît sur le milieu minéral avec la cellulose, seule l'endocellulase est synthétisée; lorsque la bactérie se développe sur milieu complexe avec cellulose, les cellules produisent de l'endo et de l'exocellulase et une très faible quantité de β-glucosidase. L'addition de cellulose aux deux milieux stimulent la croissance (mesurée par le contenu cellulaire protéique) et la production de cellulases. Les cellules entières produisent plus d'endocellulases que le surnageant, ce qui permet de penser qu'une partie de l'endocellulase reste associée aux cellules ou aux fibres de cellulose. La β-glucosidase est plutôt liée aux membranes. En présence de cellulose, l'addition de glucose inhibe la croissance et la dégradation de la cellulose. Les protéines du surnageant obtenues à partir des cultures développées sur milieu complexe avec différentes fibres de cellulose, ont été examinées par électrophorèse sur gel de polyacrylamide. Les surnageants provenant de cellules s'étant développées en présence de cellulose contiennent un nombre plus élevé de protéines que ceux des cellules s'étant développées sur glucose. Les enzymes cellulolytiques ont été identifiées après le transfert des protéines du gel de polyacrylamide à la gélose contenant de la cellulose carboxy-méthylée et après

coloration par le rouge congo. Les expériences faites avec les gels non dénaturés montrent 5 bandes ayant une activité cellulolytique.

L'utilisation effective de la cellulose nécessite des organismes qui possèdent une bonne activité cellulolytique et/ou des organismes qui ne sont pas soumis à un contrôle catabolique. L'utilisation d'agents mutagéniques conventionnels tels que les radiations ultra-violettes, le méthyl méthane sulfonate, et la N-méthyl-N-nitrosoguanidine, ne nous a pas permis d'obtenir des mutants de *Cellulomonas* 2406.

Des études au microscope électronique montrent que la *Cellulomonas* 2406 adhère aux fibres de cellulose par le biais d'une couche de polysaccharides. Cette couche externe épaisse n'est pas présente lorsque les cellules se sont développées dans un milieu complexe sans cellulose. Cette couche de polysaccharides est probablement nécessaire pour le processus d'adhésion; par contre, on ne sait pas si le contact entre les cellules et les fibres de cellulose est indispensable pour qu'il y ait induction des cellulases.

List of Tables

List of Figures

INTRODUCTION

1. Introduction

Cellulose is the most abundant natural polymer, comprising more than 50% of all carbon in vegetation. It is a major component of municipal waste and it is also produced in vast quantities from activities such as food processing, lumbering, paper making and cereal harvesting. In recent years its utilization as a source of fuel and chemicals became of even greater importance since it is rapidly renewable. Plant material such as cellulose can be used as feed stock for fuels and chemicals (Table 1). Cellulose may exist in relatively pure form such as cotton or in association with other compounds such as hemicellulose and lignin. Table 2 lists the cellulose contents of some agricultural and industrial materials. Most of the cellulose in such materials is converted to carbon dioxide by biological oxidation or combustion and returned to the atmosphere.

Microbial degradation is a major cause of cellulose conversion. However, attempts to utilize cellulose as an important source for energy have been disappointing. At present, mineral acid hydrolysis is the only technology used to release sugar from cellulolytic waste. However, acid hydrolysis is not an economically feasible process.

Table 1. Utilization of plant material*

Plant material	Conversion process	Products	References
Cellulose	Fermentation by *Zymomonas mobilis*	ethanol	Arcuri (1982) Flickinger (1980)
Cellulose	Fermentation by *Clostridium thermocellum*	ethanol	Cooney (1978)
Xylan	Fermentation by yeasts	ethanol butanol	Dekker (1982) Maddox (1982)
Lignin	Microbial degradation by fungi	low molecular weight phenolic compounds	Kirk & Chang (1981) Crawford (1981)

* Table from Woodward, 1984

Table 2. Cellulose content of different materials*

Material	Percent cellulose	Reference
Cotton	91	Gascoigne & Gascoigne (1960)
Wood (pine)	41	Virkola (1975)
(birch)	40	
Pulp	41	
Wheat	30.5	
Oat	42.8	Donefer et al. (1969)
Bagasse	46-55	Sricivasan & Han (1969)
Newspaper	40-80	Updergraff (1971)

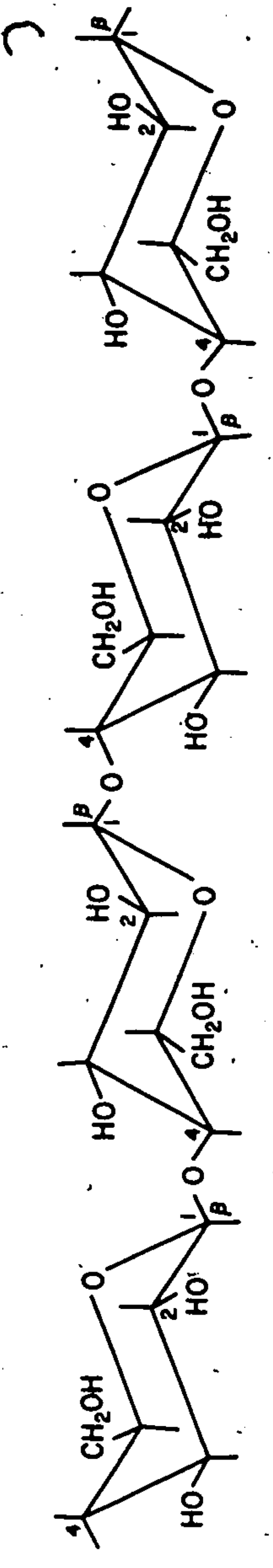
* Table from Goksøyer and Eriksen, 1980

Cellulose is a linear polymer of D-glucose linked by β-1, 4-glucosidic bonds (Fig. 1). The number of glucose units in a cellulose molecule was found to vary between 15 and 14,000 (Sihtola & Neimo, 1975). Natural cellulose was found to contain more than 10,000 glucose units, giving it a molecular weight of approximately 1.5 million. The length of a glucose molecule is 0.15 nm, which would make the length of natural cellulose molecule 5 µm.

The rate of hydrolysis of cellulose depends on the cellulase enzymes produced by different organisms and on the ability of the enzymes to attack cellulose fibers. It is now known that cellulose fibers are made of two different distinguishable parts; an easily hydrolyzable portion called «amorphous» cellulose and a resistant portion called «crystalline» cellulose (Fig. 2). Cellulase enzymes are known to attack the amorphous portion of the cellulose fibers more readily. Pretreatment of the cellulose fibers, usually with an acid, is supposed to expose more of the amorphous regions of the cellulose fibers thus making them more readily degradable (Chang, 1971).

Some prokaryotic and eukaryotic organisms possess the necessary enzymes for cellulose degradation. Most of the cellulolytic organisms produce enzymes which enable them to hydrolyse cellulose to soluble sugars, which in turn they can utilize as carbon sources. In some organisms the function of these enzymes is to regulate cell growth or germination (Gong & Tsao, 1979). In some fungi it has been shown that

Fig. 1. The structure of cellulose



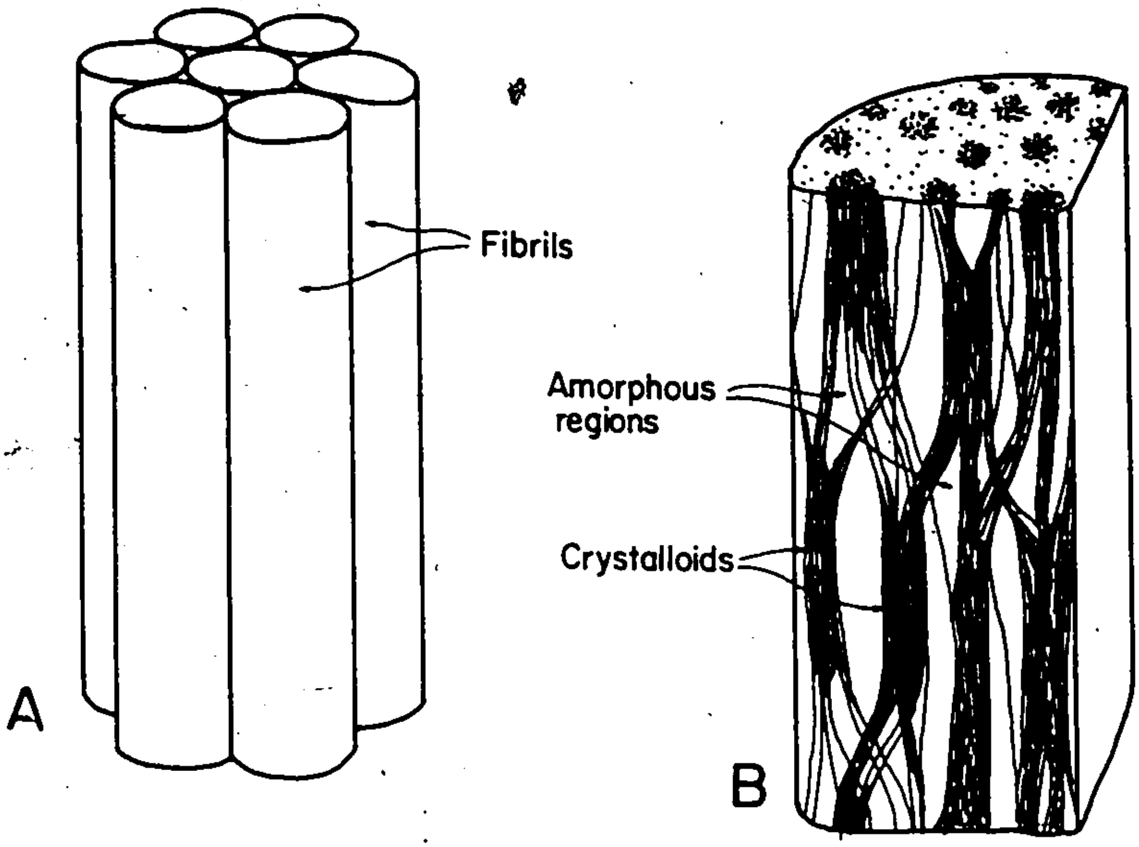
C
β
1
HO
2
O
CH$_2$OH
HO
4
O

Fig. 2. Alignment and composition of elementary fibrils of cellulose.

A. Bundle of parallel fibrils held together by hydrogen bonds

B. Lateral section view of one fibril

From Sihtola and Neimo, 1975.


Fibrils
A
Amorphous regions
Crystalloids
B

cellulases are necessary for spore germination (Jones et al., 1979).

The true cellulolytic microorganisms produce a «cellulase complex» capable of hydrolysing cellulose to soluble sugars. Three major enzymes in the cellulase complex can be identified. They are exo-β-1, 4-glucanase commonly called exocellulase, endo-β-1, 4-glucanase commonly called endocellulase and β-1, 4-glucosidase commonly called cellobiase.

2. Cellulase enzymes

Exocellulases, also called the C_1 component, were believed to initiate the attack on cellulose (Reese et al., 1950). It was initially thought that the C_1 component had a role in rearranging the cellulose fibers in a way that they would become more readily available for degradation by other enzymes. This concept led to the belief that C_1 component had no activity on its own (Li et al., 1965; Mandels & Reese, 1964; Selby & Maitland, 1967; Wood, 1968). But fractionation studies of the *Trichoderma koningii* cellulase (Wood, 1975) have shown this hypothesis to be wrong and that the C_1 component was active.

Exocellulases are now known to attack cellulose fibers in an endwise fashion, removing cellobiose and sometimes glucose units (Wood & McCrae, 1977; Gong & Tsao, 1979). Once the exoglucanase had been purified from different organisms it was evident that its activity varied from organism to organism. Wood (1975) showed that *Trichoderma koningii* had little activity towards highly ordered cellulose. Also it was shown that some microorganisms such as *Penicillum funiculosum* possessed more than one active exoglucanase (Wood & McCrae, 1977; Selby, 1969). Wood also conducted studies on the substrate specificity of exocellulases. His work on purified enzymes from *Trichoderma koningii*, *Penicillum funiculosum* and *Fusarium solani* has shown that the enzymes have similar substrate specificities, namely that they are all free of carboxymethyl cellulase activity, are unable to hydrolyse cotton cellulose but can hydrolyse avicel. In this case 6% of the original amount of avicel was hydrolysed. These results contradicted the findings of Berghem et al. (1975) who, working on purified exocellulases from *Trichoderma viride*, showed that 45% of the original amount of avicel could be hydrolysed. These results show that different organisms can produce exocellulases with quite different activities towards the same substrate. The three exocellulases isolated by Wood & McCrae (1977) were able to hydrolyse H_3PO_4-swollen cellulose suggesting that these enzymes could degrade cellulose substrates when they are present in a more accessible form. In the case of carboxymethyl cellulose, Wood suggested that although very accessible, carboxymethyl cellulose was too highly substituted for enzyme binding to occur. Therefore, exocellulases could exhibit activity towards crystalline cellulose but showed limited activity towards carboxymethyl cellulose (Gong & Tsao, 1979).

Endocellulases, also called the C_x enzymes (Reese et al., 1950), are capable of acting on highly ordered cellulose by attacking the cellulose polymer internally. By the action of these enzymes cellobiose, glucose and other soluble cellodextrins can be released from the cellulose fibers (Gong & Tsao, 1979).

Endocellulases are probably the most studied of the cellulase enzymes since all cellulolytic microorganisms produce them and they are usually produced in large amounts in the culture supernatant. Most intensive studies were done on *Trichoderma koningii* (Okada et al., 1968; Emer et al., 1974;

Berghem & Pettersson, 1973; Halliwell & Griffen, 1973) whose cellulase enzymes have been fractionated to reveal more than one endocellulase (Table 3). Studies on the thermophilic anaerobe *Clostridium thermocellum* have shown that this microorganism has only one endocellulase (Petre et al., 1981).

β-*glucosidase*: β-glucosidase occurs naturally in many plants, plant seeds, yeasts and microorganisms (Gascoigne & Gascoigne, 1960; Baruch & Swain, 1957; Duncan et al., 1956). β-glucosidase is involved in splitting the cellobiose released from the action of endo and exo-cellulases into glucose which can then be utilized as a carbon source for the microorganism.

There are two types of β-glucosidade: aryl-β-glucosidase and β-glucosidase. Aryl-β-glucosidase was isolated from microorganisms such as *Schizophyllum commune* (Wilson & Niederpruen, 1967) and *Chaetomium* (Louis & Becker, 1973) and shown to have action on p-nitrophenyl-β-D-glucoside (PNPG) but not on cellobiose.

β-Glucosidase isolated from cellulolytic fungi was shown to break down cellobiose (Gong & Tsao, 1979). This enzyme was also called cellobiase to distinguish it from aryl-β-glucosidase.

Studies done on the extracellular fluids obtained from liquid cultures of cellulolytic microorganisms have shown that usually very little cellobiase is present in this fraction, even when large amounts of cellulases are detected (Berghem & Pettersson, 1974). In *Trichoderma* this enzyme is usually intracellular (Berg & Pettersson, 1977). Cellobiase isolated from

Table 3. A list of the different molecular weights of cellulase enzymes obtained from different microorganisms.

Organism	Cellulase components	Molecular weight	Reference
Trichoderma reesei	exo-glucanase	42 000	Pettersson (1975)
	endo-glucanase 1	12 500	
	2	50 000	
	β-glucosidase	47 000	
Trichoderma koningii	exo-glucanase	62 000	Wood (1975)
	endo-glucanase		
	C_x1	13 000	
	C_x3	38 000	
	C_x4	31 000	
Sporotrichum pulverulentum	exo-glucanase	48 600	Eriksson (1975)
	endo-glucanase	32 300	
Fusarium solani	exo-glucanase	45 000	Wood (1975)
	endo-glucanase	37 000	
	β-glucosidase	400 000	
Sporotrichum thermophile	endo-glucanase	65 000	Magaritis & Merchant (1983)
	avicelase	84 000	
	β-glucosidase	440 000	Meyers & Canevascini (1981)
	aryl-β-glucosidase	40 000	
Clostridium thermocellum	β-glucosidase	43 000	Ait et al. (1982)
	endo-glucanase	56 000	Petre et al. (1981)
		94 000	Ng & Zeikus (1981)
Trichoderma viride	endo-glucanase 1	12 500	Berghem et al. (1975)
	2	50 000	
Aspargillus fumigatus	endo-glucanase	12 500	Perry et al. (1983)
Cellulomonas uda	endo-cellulase	66 000	Nakamura & Kitamura (1983)

bacteria was found to be mainly intracellular (i.e. located within the cell) or cell bound (i.e. associated with the plasma membrane) depending on the organism (Gong & Tsao, 1979). As with the other two cellulase enzymes it has been shown that more than one cellobiase can be produced by one organism. For *Botryodiplodia theobomae*, Umezurike (1979) was able to show that four different cellobiases were present, whereas for *Sporotrichum pulverulentum* five such enzymes have been described (Deshpande et al., 1978).

From the above discussion it is clear that the components of the cellulase complex are in themselves made up of multiple forms. This multiplicity is more evident for endocellulases than for exocellulases and cellobiase (Table 3). The multiplicity of endocellulases could be due to proteolysis, whereby an endocellulase is nicked into two different polypeptide chains with higher cellulase activity than the parent protein (Gong & Tsao, 1979; Bisaria & Ghose, 1981).

3. Mode of action of cellulases and the regulation of cellulase biosynthesis

The degradation of cellulose has been intensely studied but the exact mode of action of the cellulases involved in this process still remains uncertain. Over the years a number of models were proposed based on measurements of the cellulases produced by different microorganisms.

Reese et al. (1950) were first to postulate that cellulose was broken down in a «two step» sequential process.

$$\text{crystalline cellulose} \xrightarrow{C_1} \text{reactive cellulose} \xrightarrow{C_x} \text{soluble cellulose} \xrightarrow{\text{cellobiase}} \text{glucose}$$

In his model Reese suggested that the C_1 (exo-glucanase) enzyme can produce shorter cellulose chains from the native cellulose, thus initiating the process of cellulose degradation. The smaller chains would then be hydrolysed by C_x (endo-glucanase).

Work done by Wood & McCrae (1972) contradicted the hypothesis that C_1 initiated the attack on crystalline cellulose. Wood & McCrae (1972), Pettersson (1975) and Nisizawa et al. (1972) have all shown that C_1 has no or very little activity towards ordered cellulose; when acting alone it attacks oligosaccharides releasing cellobiose as a sole product. Their observation lead to a new concept, whereby C_x was the enzyme initiating cellulose degradation followed by the action of C_1 enzyme. Selby & Maitland (1967) and Wood (1975) showed that a synergism exists between the C_x and C_1 enzymes. The C_1 fraction was not able to degrade cellulose by itself, but it was active when it was present together with the C_x component. This evidence for synergism supported the model which was first suggested by Eriksson in 1969 and described by Pettersson in 1975 (Fig. 3). In this model endocellulases attack regions of low crystallinity creating free ends on which the exocellulases could start hydrolysis, removing cellobiose units. This model is exactly the opposite of Reese's hypothesis. In light of the new evidence Reese modified his model which originally maintained that the C_1 is somehow associated with the disruption of the hydrogen bonds thus producing a swelling action (Reese, 1977). In his new model the C_1 enzyme becomes a member of the endocellulases (C_x) but still maintains properties not present in most endocellulases. Reese also incorporated the discoveries on synergism in his model.

Regulation of the cellulases involves induction, end product inhibition and catabolite repression. Endocellulases and exocellulases can be induced by cellulose powder, carboxymethyl cellulose, acid swollen cellulose, glycerol and/or sophorose (Haliwell, 1961; Reese et al., 1969; Hulme & Stranks, 1971; Sternberg & Mandels, 1979) depending on the strain. Cellulose, cellobiose, sophorose, acid swollen cellulose and/or laminaribiose may act as inducers of β-glucosidase (Conevascini & Meyer, 1979).

Fig. 3. Schematic representation of the mode of action of cellulase enzymes on cellulose fibers *

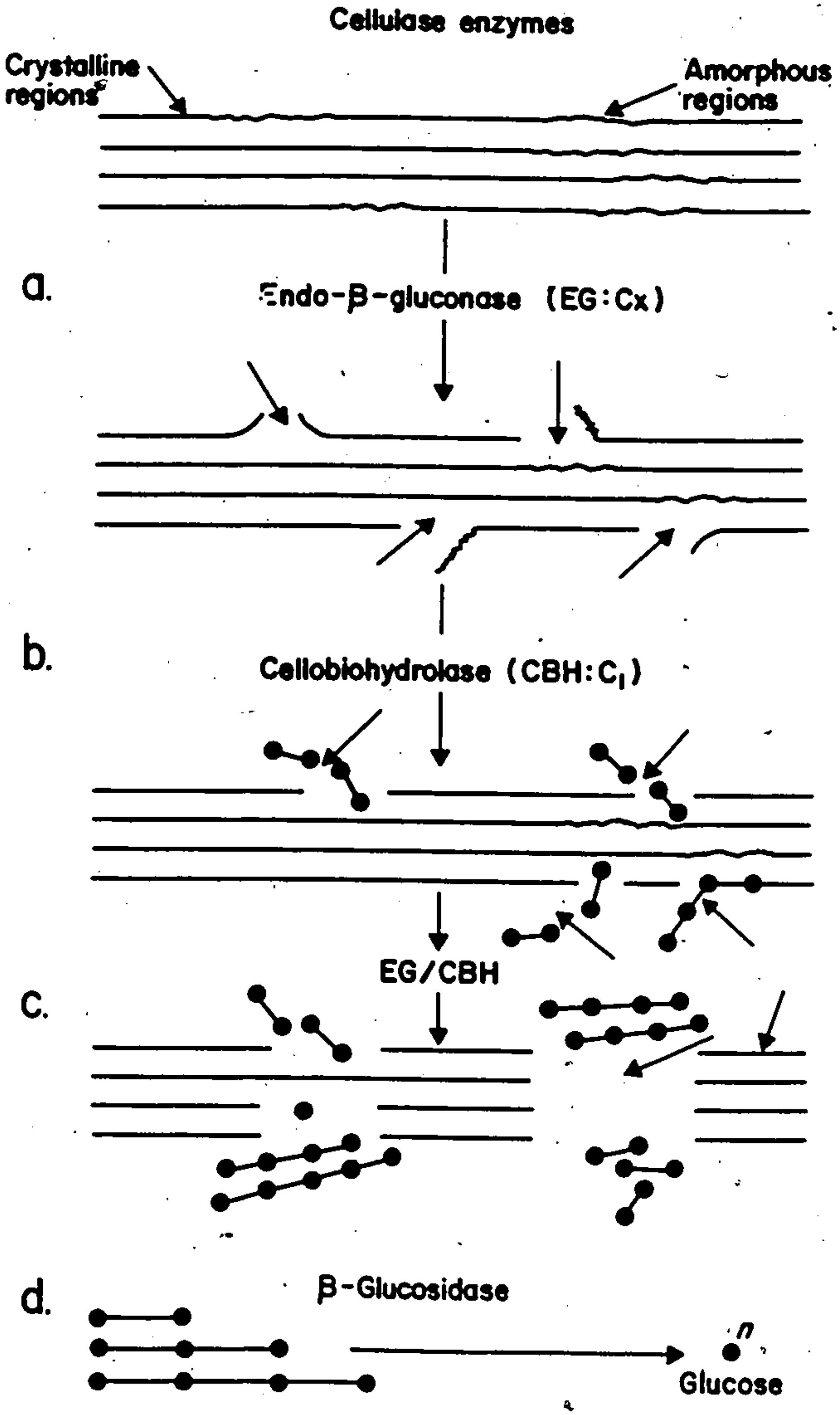
a. Endocellulases attack the amorphous regions of the cellulose fiber

b. Exocellulases attack the cellulose fiber in an endwise fashion releasing cellobiose units

c. Soluble oligosaccharides, cellobiose and a small amount of glucose is released by the action of endo and exo cellulases

d. Glucose units produced by the action of ß-glucosidase enzyme on cellobiose units

* From Bisaria & Ghose, 1981


Cellulase enzymes
Crystalline regions
Amorphous regions
a.
Endo-β-gluconase (EG:Cx)
b.
Cellobiohydrolase (CBH:C1)
c.
EG/CBH
d.
β-Glucosidase
n
Glucose

It is not clear if fungi and bacteria actually produce low amounts of constitutive enzymes (synthesized at a constant rate by the microorganism) which then act on the cellulose, releasing cellobiose, which in turn can penetrate the cell and stimulate greater production of cellulases (Gong & Tsao, 1979). During end product inhibition the accumulation of glucose, for example, in the media could lead to the inhibition of the enzymatic reactions that lead to its formation (Lehninger, 1978).

Enzyme synthesis is repressed by glucose or catabolites of glucose. Such repression of enzyme synthesis is present in a variety of microorganisms and was first described as the glucose effect (Epps & Gale, 1942) and later called catabolite repression (Magasanik, 1961). In the presence of high glucose concentrations a number of catabolic pathways are repressed. Catabolite repression as described for the lactose metabolism in *Escherichia coli* acts via the promotor region of DNA. Enzyme synthesis involves the presence of a catabolite activator protein which helps in the binding of RNA polymerase to the promotor region. The catabolite activator protein in turn must be bound to cyclic AMP before it can bind to the promotor region. When glucose accumulates in the media the levels of cyclic AMP decrease, the catabolite activator protein does not bind to the promotor region and in turn RNA polymerase can not bind to the catabolite repressible promotor and therefore the synthesis of enzymes capable of utilizing other energy sources ceases

(Lehninger, 1978; Atlas, 1984). Suzuki (1975) had found that when cyclic AMP was added to a culture of *Pseudomonas fluorescens* containing glucose or sophorose, the cellulase activity did not increase as expected if cyclic AMP catabolite repression would be regulating cellulase synthesis. However cellulase activity increased if ATP or other nucleotides were added to the culture. Similar results were found for *Pseudomonas aerogenosa* by Kight-Olliff and Fitzgerald (1978) when studying the induction of alkylsulphatase. This led to the suggestion that ATP rather than cyclic AMP might be regulating catabolite repression in these microorganisms. Catabolite repression was more extensively studied in fungi such as *Trichoderma viride* (Nisizawa et al., 1972). For *T. viride* it was found that ATP repressed cellulase induction but not the secretion of the enzyme, suggesting that catabolite repression was not caused directly by glucose or other related substrates but by some common metabolite that would act on the metabolic pathway in the fungus.

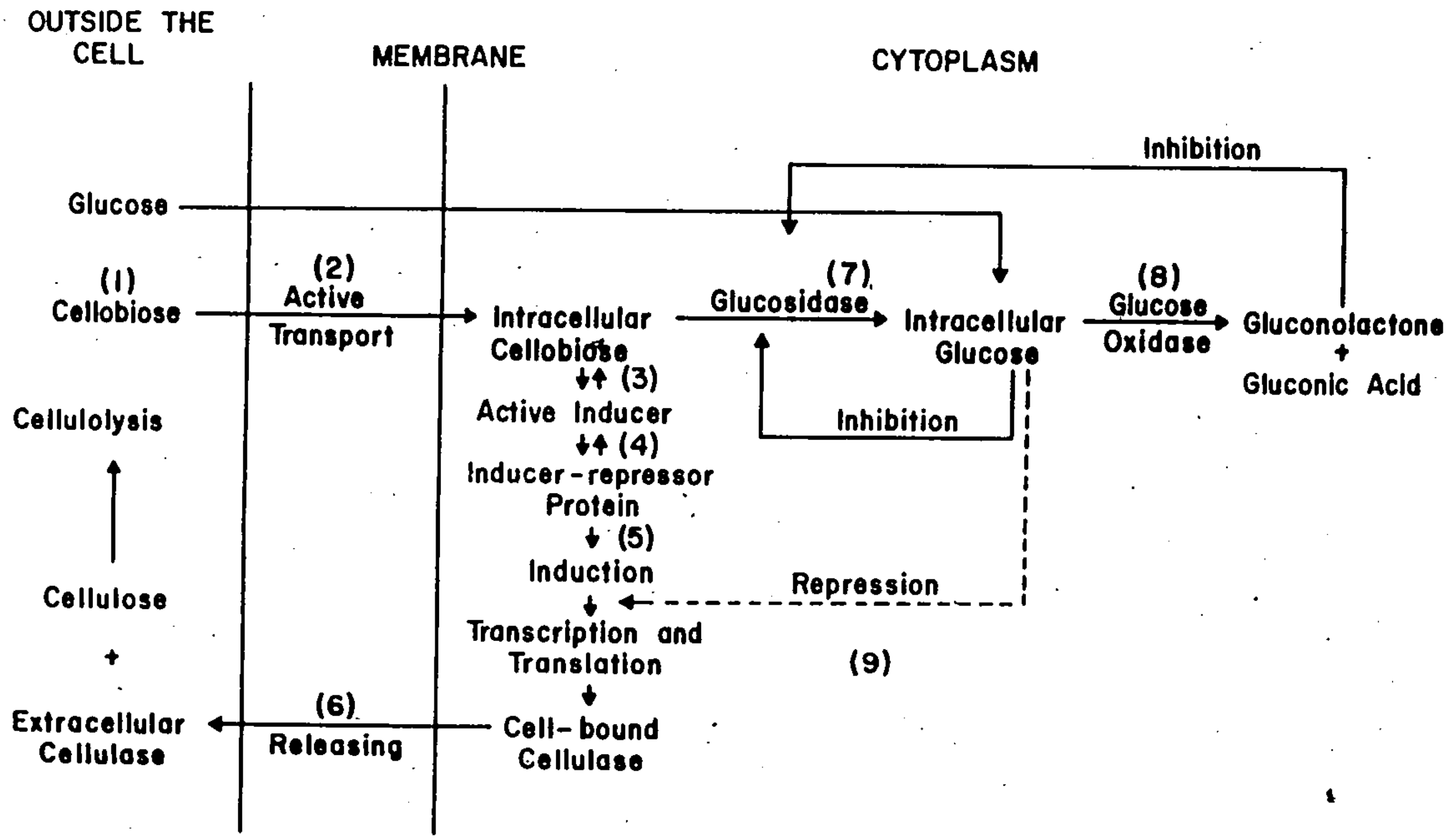
All cellulases are inhibited by glucose which is the product of cellobiose breakdown and some β-glucosidases are also inhibited by gluco-(1→5)-lactone (Levvy et al., 1964; Conche et al., 1967). Cellobiase isolated from a yeast, *Dekkera intermediata* Van der Walt, was inhibited by glucose, gluconolactone and p-chloromercurybenzoate (Blondin et al., 1983). In other cases such as cellobiase from *Sporotrichum thermophile* Apinis glucose did not inhibit cellobiase activity (Canevascine & Meyer, 1979). Cellobiase is not only influenced by the substrates used or by the products produced during hydrolysis. It can also be greatly influenced by yeast extract which is often added to the growth media of cellulolytic microorganisms (Woodward et al., 1983).

The whole picture on the regulation of cellulases is far from clear; possibly different genera of microorganisms possess different regulatory mechanisms. A model for the regulation of cellulases was suggested by Gong & Tsao (1979) (Fig. 4). Their model tries to take in account the importance of all cellulases and the wide spectrum of substrate specificities. In this model it is assumed that a low level of constitutive cellulases is produced which in turn could release cellobiose from the cellulose fibers. This cellobiose could be the initial inducer which could enter the cell and stimulate further production of cellulases. This postulated regulatory mechanism also includes: 1) the role of β-1,4-glucanase in the regulation of glucose and cellobiose levels within the cell 2) interaction of induction and repression 3) regulation of the amount of extracellular enzymes 4) the role of proteolysis in the modification of cellulases 5) coordinate gene expression for both endo and exo-cellulases. This model can explain a diversity of responses of cellulolytic organisms to various conditions but it has not been proven.

Fig. 4. Proposed model of regulation of cellulase biosynthesis developed by Gong & Tsao, 1979 *

1. Glucose and cellobiose are the product of a basal level of cellulases. Cellobiose is considered to be the «potential» inducer
2. Active transport of cellobiose into the cell
3. Cellobiose becomes the active inducer
4. Cellobiose reacts with the repressor protein
5. Induction of cellulase synthesis (it is assumed that the expression of both endo and exo cellulases is controlled by the same gene)
6. The cellulases produced become cell bound and their release into the extracellular medium is regulated by a specific releasing mechanism
7. Intracellular glucosidase might hydrolyse cellobiose into glucose
8. Glucose oxidase may be produced by certain microorganisms in response to an intracellular accumulation of glucose
9. Intracellular glucose may repress cellulase formation

* From Gong & Tsao, 1979


OUTSIDE THE CELL
MEMBRANE
CYTOPLASM
Glucose
(1) Cellobiose
(2) Active Transport
Intracellular Cellobiose
(3)
Active Inducer
(4)
Inducer-repressor Protein
(5)
Induction
Transcription and Translation
Cell-bound Cellulase
(6) Releasing
Extracellular Cellulase
Cellulose
+
Cellulolysis
(7) Glucosidase
Intracellular Glucose
Inhibition
(8) Glucose Oxidase
Gluconolactone
+
Gluconic Acid
Inhibition
Repression
(9)

4. Bacterial cellulases

From the previous account of cellulase enzymes involved in the degradation of cellulose it is obvious that most research was done on cellulase enzymes from fungi. Fungi generally produce much higher levels of cellulases than bacteria and have been considered the most important cellulose decomposers in soil (Satchell, 1971). Bacteria, however, are also active degraders of cellulosic materials, and some now consider them as important as fungi in the initial decomposition of organic materials (Thayer & Murray, 1977; Sreenath et al., 1978; Deschamps et al., 1980).

The studies done on bacteria showed that not all cellulolytic bacteria possess endo and exo-cellulases. Lee and Blackburn (1975) suggested that one enzyme was responsible for the activities of both endo and exo-cellulases in *Clostridium thermocellum* since they were both produced at the same rate. *Cytophaga* species were shown to actually possess both cellulases (Chang & Thayer, 1977).

In bacteria, cellulases have been found to be only cell-bound or intracellular, as in *Cytophaga*, (Chang, 1977), or both cell bound and cell-free, as in *Pseudomonas* (Yamane et al., 1970 & 1971) and *Cellvibrio fulvus* (Berg, 1975; Berg et al., 1972a). Berg (1975) has shown that *Cellvibrio fulvus* has endocellulases which were partially cell-bound when the bacteria were grown in glucose or cellobiose and found in the periplasm and membrane-bound when grown on cellulose. This is in contrast to the endocellulases of *Cellvibrio vulgaris* which were actively secreted from cells grown on cellulose (Oberkotter & Rosenberg, 1978).

It has not been established if the cellulase enzymes present in the culture fluid have been released extracellularly by an active mechanism or released due to cell lysis. Berg et al. (1972a) attributed the release of cellulases from *Cellvibrio fulvus* to cell lysis whereas Oberkotter and Rosenberg (1978) attributed the release of cellulases from *Cellvibrio vulgaris* to an active mechanism.

Much research on bacterial cellulases has been done on *Clostridium thermocellum*, an anaerobic microorganism capable of producing ethanol as a final hydrolysis product. Other research has been done on anaerobic cellulolytic bacteria present in the rumen of cows, bacteria which help to break down straw and grass.

5. The genus *Cellulomonas*

The genus *Cellulomonas* was established by a committee in 1923 and it contained a group of 31 species of bacteria previously described as aerobic cellulose degraders, isolated from soil, gram-negative, motile or non-motile and rod shaped. All these species were previously isolated from soil by Kellerman (1912, 1913) and his co-workers (McBeth, 1916). In 1934 Jensen had reported that one of the previously described microorganisms, *Cellulomonas fimi*, was weakly gram-positive, not gram-negative. In 1953 Clarke reclassified the genus *Cellulomonas* and included it with the family Corynebacteriaceae. On the basis of his studies he recognized ten *Cellulomonas* species. Following Jensen's and Clarke's observations *Cellulomonas* was referred to as a gram variable microorganism (Keddie, 1974). It is now well established that *Cellulomonas* is a gram positive microorganism which is very easily destained under the gram stain procedure. *Cellulomonas* is a rod shaped bacterium in exponential cultures. As the culture becomes older more and more coccoid cells or short rods are present. However *Cellulomonas* does not show the rod-coccus cycle of the genus *Arthrobacter* (Keddie & Jones, 1981). Bacteria of the genus *Cellulomonas* can be non-motile or motile using one or a few flagella (Keddie & Jones, 1981).

In the eighth edition of Bergey's manual (1974) only one species *Cellulomonas* is recognized, namely *Cellulomonas flavigena*. In 1976 Braden and Thayer showed that two *Cellulomonas flavigena* isolates, though similar in many characteristics, were not serologically identical; they questioned Bergey's classification. Identification of *Cellulomonas* to species level is not easy since the members of the genus form a phenotypically homogeneous group (Keddie, 1974; Stackebrandt and Kandler, 1979). Only in 1979 was DNA-DNA homology conducted by Stackebrandt and Kandler who proposed that seven species of *Cellulomonas* should be recognized.

The main habitat of *Cellulomonas* was always considered to be the soil but no attempts were made to determine the numbers, distribution or role of these bacteria in different soils (Keddie & Jones, 1981).

Most of the research conducted with regards to cellulose decomposition was done on *Cellulomonas flavigena* which is still the only recognized species in Bergey's manual (Buchanan & Gibbons, 1974).

6. The cellulases of *Cellulomonas*

Studies conducted on different *Cellulomonas* isolates have shown that these bacteria possess all three major enzymes involved in cellulose degradation; exocellulases, endocellulases, and β-glucosidase (Choi et al., 1978; Kim & Wimpenny, 1981; Langsford et al., 1984; Stoppok et al., 1972; Thayer et al., 1984).

Experiments conducted by Beguin & Eisen (1978) showed that *Cellulomonas* 11 bc has three extracellular cellulases, all behaving as endocellulases towards carboxymethyl cellulose. Nakamura and Kitamura (1983) have isolated a cellulase from the culture filtrate of *Cellulomonas uda* CB4. This enzyme was found to have a molecular weight of 66,000. It hydrolyzed crystalline cellulose producing cellobiose but showed little activity towards carboxymethyl cellulose.

Much work was done on the characterization of β-glucosidase enzyme from *Cellulomonas*. Wakarchuk et al. (1984) have shown that β-glucosidase of *Cellulomonas fimi* (ATCC 484) is located strictly internally. In contrast to Wakarchuk's findings, the β-glucosidase of *Cellulomonas flavigena* was shown to be cell-bound; any activity detected in the culture fluid was thought due to cell lysis (Antheunisse, 1984). Similar results were found for *Cellulomonas uda* by Stoppok et al. (1982), which showed that in this bacterium β-glucosidase was cell-bound and constitutive.

Schimz et al. (1983) showed that *Cellulomonas* spec. (DSM 20108) possessed another enzyme called cellobiose phosphorylase capable of converting cellobiose to D-glucose and α-D-glucose-1-phosphate.

Inducers of cellulases in bacteria such as *Cellulomonas* are the same as the inducers in fungi. Cellulose and cellobiose are the most common inducers (Rajaka & Malik, 1984). For *Cellulomonas flavigena* NIAB 441 the best inducer was found to be Kallar grass which also induced the production of hemicellulase (Rojaka & Malik, 1984). The production of cellulases is regulated by the same mechanisms found for fungi, namely induction, catabolite repression and end product inhibition.

Since none of these *Cellulomonas* species showed very high levels of cellulases, researchers have tried to isolate mutants which might be more powerful degraders, especially mutants whose production of cellulases would not be inhibited by the end products of cellulose hydrolysis. Stewart and Leatherwood (1976) were able to isolate a mutant of a new *Cellulomonas* strain from soil that no longer showed catabolite repression but the cellulase activity produced was still under the influence of end product inhibition and induction.

Choi et al. (1978) and Haggett et al. (1978) also mutated *Cellulomonas* CSI-1 and their new strain was able to degrade crystalline cellulose more efficiently than the parent strain.

Cloning of cellulase genes is another approach at enhancing cellulase production. Cellulase genes from *Cellulomonas fimi* were cloned into *Escherichia coli*. The clones fell into three different groups; two had low carboxymethyl cellulase activity and one contained high levels of carboxymethyl cellulase activity. The carboxymethyl cellulase activity) of these individual clones was still much lower than the activity found in the culture supernatant, suggesting that cloning would be of great advantage if one could clone all components of the cellulase system and reconstruct the synergism between the various products of the cloned genes (Gilkes et al., 1984).

7. Assays for the determination of cellulase enzymes activities

There is no single assay for determining cellulase enzyme activities, probably due to the variety of suitable substrates which can be used.

Table 4 summarizes some of the assays which have been developed and used to determine the activities of the different cellulolytic enzymes. None of the methods for determining cellulase activities are perfect. For example the filter paper assay developed by Mandels and Weber (1969) and later modified by Mandels et al. (1976) was found to measure both endocellulases and exocellulases activities. Most purified exocellulases still possess some carboxymethyl cellulase (endocellulase) activity.

The assay for carboxymethyl cellulase activity has its own problems, due to the nature of the substrate and the enzyme (Lindner et al., 1983). Enzyme dilution curves were found to be not linear at times and not to pass through the origin. Also the carboxymethyl cellulase assay does not distinguish between different endocellulases that could be present in one system since all endocellulases would act on the same site (Lindner et al., 1983). The essay also measures the presence of exocellulases, to a small extent. One method used to determine endocellulase activity is by the reducing end groups formed by hydrolysis. The different ways one can measure reducing sugars also adds to the confusion (Table 4), since each method gives a different result when measurements are done

Table 4. Cellulase assays

Enzyme	Substrate	Product
Cellobiase (β-glucosidase)	cellobiose cellodextrins	Glucose
	salicin	Saligenin
	p-Nitrophenyl β-glucoside	p-Nitrophenol
endo β-1,4-glucanase	CMC* cellodextrins	reducing sugar** and/or loss of viscosity
exo β-1,4-glucanase	amorphous cellulose crystalline cellulose avicel cellodextrins	glucose cellobiose
cellulase	dyed cellulose filter paper	release of dye reducing sugar** loss of weight
avicelase	avicel	reducing sugar**
swelling factor	cotton	uptake of alkali

* CMC = carboxymethyl cellulose

** Reducing sugars can be measured by three different methods:
1. Dinitrosalicylic (Mandels et al., 1976)
2. Nelson-Somogyi method (Nelson, 1944; Somogyi, 1952)
3. Tetrazonium blue reducing sugar assay (Mullings & Parish, 1984)

in the presence of carboxymethyl cellulose. Therefore comparing enzyme activities between different bacteria of the same genus and comparing results obtained by different researchers for the same microorganism is very difficult (Marais et al., 1966; Mullings & Parish, 1984).

Viscometric measurements can also be done to determine endocellulase activity. These measurements are more sensitive than the measurements of reducing sugars since they require a shorter incubation time (Thomas, 1956; Osmusvag & Goksøyr, 1975; Eriksen & Goksøyr, 1976). However viscometric measurements are not favorable because they require careful chemical analysis of the carboxymethyl cellulose used (Almin & Eriksson, 1967). Carboxymethyl cellulose should be of low viscosity (D.S. 0.7) but the essay is still influenced by the PH and the ionic strength of the solvent.

It is evident that to obtain all the different cellulolytic components in the culture fluid, more than one of the tests should be performed. To be able to compare cellulase activities from one microorganism to another, similar substrates and similar nomenclature have to be used. Since carboxymethyl cellulose, the most universal substrate (Rose, 1980), can be affected by the assay conditions, a compound such as hydroxymethylcellulose might be preferable as a substrate (Child et al., 1973). The problem of comparing the values obtained by different researchers is that very few report on the viscosity of the substrate used; and hence on the degree of substitution, which can influence measurements of reducing sugars (Lindner et al., 1983). Viscometric measurements can not be compared to the measurements of reducing sugars since there is no accurate formula which can convert specific viscosity to glucose equivalents. Different tests for cellulases of fungi and bacteria should be used. Viscometric measurements might be more favorable for bacteria since bacteria usually produce much smaller cellulase activities. The tests which were mostly designed for fungal cellulases might not be sufficiently sensitive.

8. Goals of thesis

The goal of this thesis was to investigate the potential of *Cellulomonas* 2406 as a cellulose degrader. The first step in achieving this goal was to look at the nutritional requirements of the bacteria and then measure the activities of the cellulase enzymes released by *Cellulomonas* 2406. Secondly, the location of some of the cellulase enzymes was also investigated.

Also examined was the possibility of producing mutants which would produce more cellulase enzymes and continue cellulose breakdown even in the presence of inhibitors such as glucose.

The first barrier in the process of cellulose breakdown is the size of the cellulose molecule which is too big to enter the cell and stimulate enzyme production. Therefore the possibility that contact between the cellulose fibers and cells might initiate enzyme production was studied.

METHODS

1. Growth conditions

Cellulomonas 2406 was obtained from Dr. R. Latta from the National Research Council of Canada. The organism was kept on PTYE agar slants at 4°C.

Cellulomonas 2406 was grown on a basal medium containing $NaNO_3$ 0.1%; K_2HPO_4 0.1%; KCl 0.05%; $MgSO_4.H_2O$ 0.05%; and yeast extract 0.05%, with the addition of 1% of different carbon sources, usually cellulose fibers (solka floc, avicel, CF 11 cellulose and Whatman no. 1 filter paper) or carboxymethyl cellulose. Before sterilization the pH was adjusted to 7.0-7.2. When glucose or cellobiose were added as a carbon source they were filter sterilized and added aseptically. A complex medium containing peptone 0.5%, tryptone 0.5% and yeast extract 0.5%, called PTYE medium was also used. Different cellulose fibers were added to the medium at a concentration of 1%.

Growth was carried out in Erlenmeyer flasks containing 1/5 volume of liquid, agitated on a reciprocal shaker for adequate aeration at 30°C, the optimum growth temperature of *Cellulomonas* 2406. A day three culture was used as a preculture from which 1 ml was transferred to 100 ml media as an inoculum. When a soluble carbon source was used growth was monitored by following absorbance at 550 nm, in 10 x 10 x 45 mm cuvettes, with an LKB spectrophotometer. When an insoluble carbon source was used growth was measured by protein and/or DNA determination(s).

2. Viable count

Culture samples were diluted in 0.1 M sodium phosphate buffer, pH 7, before 0.1 ml was plated onto PTYE plates, for counting by the spread plate method (Kauri, 1980).

3. Protein determination

Proteins were measured by the method of Lowry et al. (1951). A standard curve was prepared each time the test was run using bovine serum albumin (Sigma). When cellulose was present in the samples, the samples were centrifuged at 5 000 x g for 5 min before reading their absorbance in the LKB spectrophotometer.

4. DNA determination

For DNA determination the culture was centrifuged and the pellet was resuspended in 2 ml distilled water to which 2 ml 0.5 $HClO_4$ was added. After 20 min at 0°C the mixture was centrifuged at 10 000 x g (4°C) for 15 min. The pellet was resuspended in 2 ml distilled water plus 1 ml of 1.5 N $HClO_4$, incubated at 70°C for 15 min., and centrifuged as before. The extraction was repeated two more times. The three extracts were combined and their DNA content measured by a modification of the Dische diphenylamine-acetic acid-sulfuric acid method (Dische 1930) developed by Burton (1956). In this method the nucleic acid extracts were diluted so as to have between 5 and 75 μgm DNA/ml. A measured volume was put in the test tube and twice the volume of diphenylamine reagent added. Colour was developed by incubating the tubes for 16-20 hours at 30°C. Optical density was measured against a blank containing 0.5 N $HClO_4$. A highly polymerized calf thymus DNA (Sigma) was employed as a standard.

5. Determination of residual cellulose

This method is a modification of the gravimetric method, used by Fahraeus (1947), developed by Berg et al. (1972). The culture fluid was acidified with HCl and filtered through a sintered glass filter which retained, the cellulose but not the cells. In order to remove the cells that might be bound to the cellulose fibers, the filtered cellulose was treated with 100 ml each of 12% HCl, distilled water, 5% NH_4OH, distilled water, ethanol and ether, consecutively. The residual cellulose was dried at 50°C for one hour then at 105°C for another hour and cooled in a dessicator before weighing.

6. DNA extraction for measuring G + C contents

DNA extraction of *Cellulomonas* 2406 was done by the procedure of Marmur (1961).

Two liters of *Cellulomonas* 2406 culture grown in PTYE medium were centrifuged at 10 000 x g (4°C) for 15 min. The pellet was resuspended in 0.025 M Tris buffer (pH 8) containing 5 mM EDTA, 0.5 M sucrose and lysozyme (0.1 mg/ml of cell suspension) and incubated 30 min at 30°C after which an equal volume of 0.1 M EDTA (ethylenediaminetetraacetic acid)-0.15 M sodium chloride, pH 8, was added together with sodium perchloride to give a final concentration of 1 M. An equal volume of chloroform-isoamylalcohol (24:1) was added, the mixture was shaken vigorously for 30 min, then centrifuged at 3 000 x g for 5 min and the upper aqueous phase retained. The DNA fibers present in this aqueous phase were precipitated and collected with two volumes of 95% ethanol and resuspended in 10 ml SSC (0.15 M NaCl- 0.015 M sodium citrate pH 7). This was accomplished by first resuspending the fibers in 9 ml dilute SSC (1/10) and then adding 1 ml of concentrated SSC (10x) to obtain the final SSC concentration. This suspension was again treated with chloroform-isoamylalcohol (24:1) and the extraction of DNA fibers was done as before until only a thin layer of protein was obtained at the interface after centrifugation. Once this stage was reached the fibers were again collected with 3 volumes ethanol and resuspended in SSC. To this suspension ribonuclease (50 μgm/ml) was added and the mixture was agitated at 37°C for 30 min, after which the extraction of the DNA fibers was repeated. The fibers were precipitated with 3 volumes ethanol and resuspended in 9 ml dilute SSC (1/10) to which 1 ml sodium acetate (3 M) plus 0.001 M EDTA pH 7 was added. To separate the DNA and RNA fibers isopropanol (5.4 ml) was added drop by drop to the above suspension while the DNA fibers were collected again and resuspended in 0.1 N acetic acid (pH 3). To measure the G + C content the spectrophotometric method developed by Fredericq et al. (1961) was used, which measures the ratios of absorbance of DNA at 260 and 280 nm to determine the molar proportions of the bases. A LKB spectrophotometer was used.

7. Preparation of cell extracts

For this procedure cells were grown in two liter flasks containing 500 ml PTYE medium. The extracellular fraction was the supernatant obtained after centrifuging the whole culture at 10 000 x g (4°C) for 15 min. One half by weight of the pellet was the source of the cell-bound fraction and the other of the intracellular fraction.

To obtain the cell bound fraction (bound to the cell wall) the pellet was resuspended in 50 ml of lysozyme (0.05 mg/ml of cell suspension) in 0.025 M Tris (hydroxymethyl-aminomethane) hydrochloride buffer, pH 8, containing 5 mM EDTA and 0.5 M sucrose. The suspension was agitated for 30 min. The pellet obtained by centrifuging the suspension at 10 000 x g (4°C) was resuspended in 80 ml cold $MgCl_2$ (5×10^{-4}M) solution, agitated for 10 min and centrifuged as before. The supernatant was considered to contain the cell bound fraction (Oberkotter and Rosenberg, 1978; Berg, 1975).

To obtain the intracellular fraction half of the original pellet containing whole cells was mixed with twice its weight with aluminum oxide and ground for 15 min on ice. The mixture was resuspended in 80 ml of sodium phosphate buffer (0.05 M, pH7) and then centrifuged at 10 000 x g (4°C) for 20 min. The supernatant was retained as the intracellular fraction. It is assumed in this procedure that the cell-bound fraction would contain mainly cell-bound enzymes and little intracellular enzymes and that the intracellular fraction would contain mainly intracellular enzymes and only a few cell-bound ones.

8. Enzyme assays

Three major enzymes are involved in cellulose degradation: endocellulases, exocellulases and β-glucosidase. When *Cellulomonas* 2406 was grown in PTYE medium with or without cellulose the following enzyme assays were run to measure cellulase and β-glucosidase activities.

β-Glucosidase activity was measured by the method of Whealan (1964). The enzyme solution (0.5 ml) was mixed with 0.5 ml of 1% salicin solution in 0.05 M citrate buffer pH 6.2. After 30 min incubation at 50°C, 3 ml dinitrosalicylic acid solution (DNS) was added and the mixture was heated in boiling water for 15 min (Miller et al., 1960). After cooling, the absorbance was measured at 550 nm. One unit of enzyme activity liberates 1 μgm of reducing sugar, expressed as glucose, per minute per milliliter of culture.

Endocellulase activity was measured by the method of Mandels et al. (1976). The enzyme solution (0.5 ml) was mixed with 0.5 ml of 1% carboxymethyl cellulose solution in 0.05 M citrate buffer pH 6.2. The mixture was incubated at 50°C for 30 min, after which 3 ml DNS reagent was added to it. The entire sample was boiled for 15 min, cooled, and the absorbance measured at 550 nm. One unit of activity was defined as one microgram of reducing sugar (relative to a glucose standard) per minute per milliliter of culture.

Filter paper activity: unfortunately there is no method for measuring exocellulase activity specifically as there is for β-glucosidase. The filter paper assay developed by Mandels et al. (1976) actually measures total cellulase activity rather than exocellulase activity alone. Filter paper, like other insoluble celluloses, is a multiple substrate varying from free ends and amorphous regions to crystalline fibers; therefore, both endo and exocellulases are expected to act on it. The filter paper assay procedure (Fig. 5) involves the incubation of 0.5 ml of enzyme with 1 ml citrate buffer with 1 x 6 cm strip (50 mg) Whatman no. 1 filter paper, for one hour at 50°C. Three ml of DNS reagent was added to stop the reaction, placed in boiling water for 15 min and absorbance read at 550 nm.

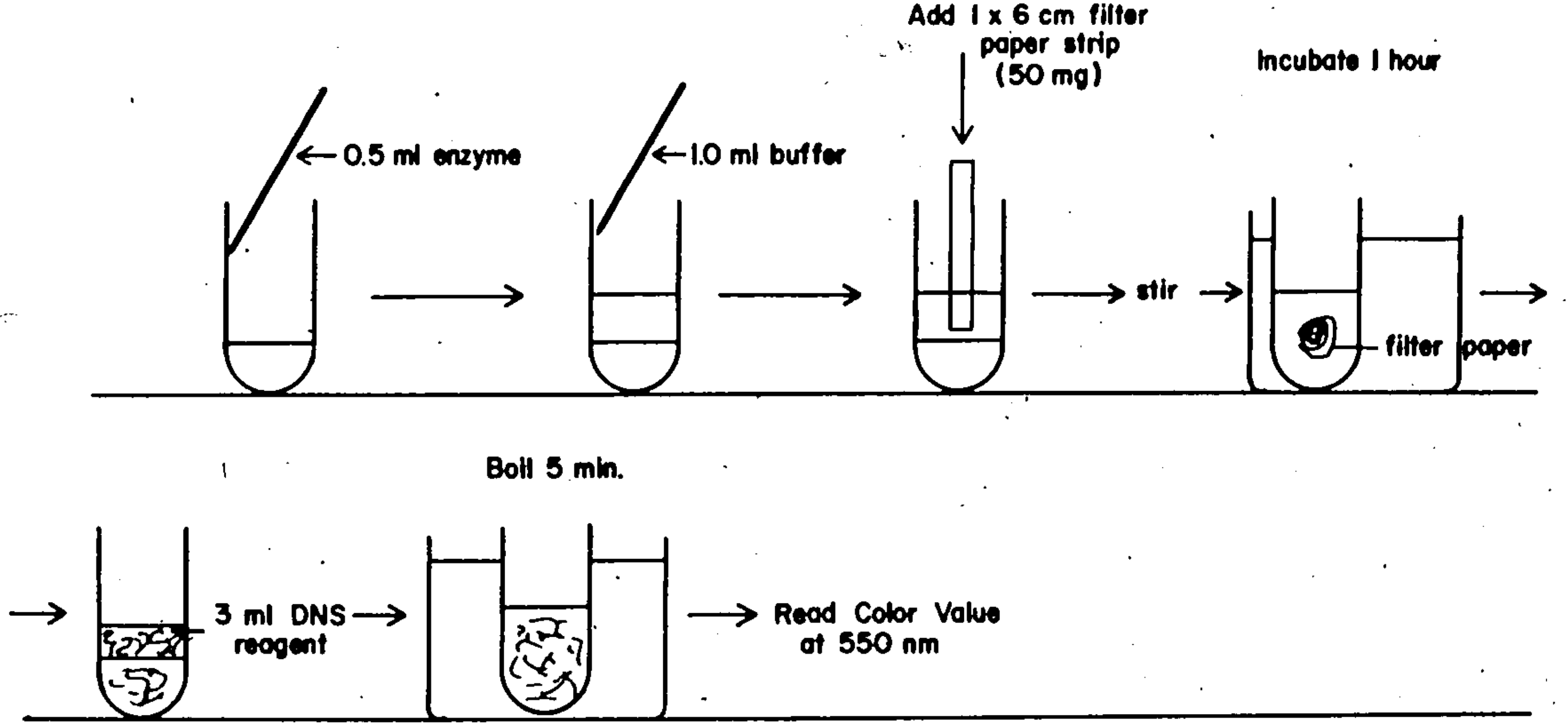
When the assays were run for cultures grown in simple media the same above procedures were used but instead of citrate buffer, 0.05 M sodium phosphate buffer pH 7 was used. Sodium phosphate buffer (0.05 M, pH 7) was also used when measuring β-glucosidase localization.

9. Gas chromatography

Gas chromatography was performed on supernatants obtained from *Cellulomonas* 2406 grown in complex medium (PTYE) containing different cellulose fibers, glucose, cellobiose or carboxymethyl cellulose. Gas chromatography was done on a

Fig. 5 Filter paper assay procedure

From Mandels et al., 1976



Filter Paper Assay Procedure

GOW-MAC gas chromatography with the help of V. Laube from the National Research Council. The internal standard used was 1,2-butanediol (0.05% w/v) and 4 µl of sample was injected into the gas chromatograph.

10. Contact experiment

This is a simple method which was first described by Kilbertus et al. (1973) for the study of cellulose degradation (Fig. 6). The cellulose fibers were introduced into a porcelain filter (Fisher) which was then placed in a 250 ml Erlenmeyer flask containing 50 ml of PTYE medium. The entire system was steam sterilized, cooled and the medium was inoculated. In this manner, cells and cellulose were kept separate and the production of cellulase enzymes in the extracellular fraction (i.e. supernatant) was monitored.

11. Glucose determination

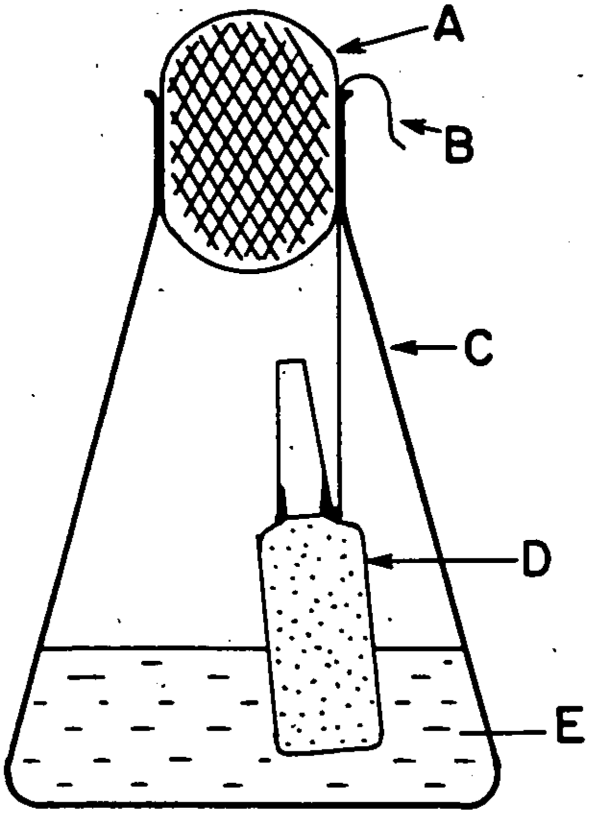
Glucose was measured with the aid of Glucostat kit from Sigma Chemicals, developed after the procedure of Raabo and Terkildsen (1960).

12. Treatment of CF 11 cellulose

CF 11 cellulose was dissolved in orthophosphoric acid and then precipitated in ice cold water. The precipitate was washed several times until the pH came back to neutral. This method of pretreating the cellulose was previously

Fig. 6. Flask and filter candle assembly used for contact experiment

From Kilbertus et al., 1973



A – cotton plug
B – nylon thread
C – erlenmeyer flask
D – porcelain filter containing cellulose
E – PTYE medium

described by Kauri (1980) for the treatment of J.H. Munktell's cellulose powder no. 400. This material was added to agar plates at a concentration of 0.25% or 0.5% to demonstrate cellulose degradation as described by Wood (1980). If CF 11 cellulose was not pretreated haloes of degradation were not readily observed.

13. Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sodium dodecyl sulfate (SDS) dissociates proteins into their constituent polypeptide chains. The polypeptide chains are then separated according to their molecular weight by SDS polyacrylamide gel electrophoresis (Weber & Osborn, 1975). The running gel contained 14 ml of a 40% acrylamide monomer solution, 12 ml of a 1.5 M Tris HCl buffer solution, 20 ml distilled water, 0.02 gm ammonium persulfate and 11.5 µl tetramethylethylendiamin (TEMED). The spacer gel contained 4 ml of the 40% acrylamide monomer solution, 10 ml of 0.25 M Tris HCl buffer solution, 23 ml distilled water, 0.02 gm ammonium persulfate and 100 µl TEMED. The spacer gel was loaded with 20-200 µg protein. Separation of proteins was carried out in a 0.3% Tris (hydroxymethyl) amino-methane-1.44% glycine electrode buffer (pH 8.6-8.8). By applying an initial current of 50-60 mA the proteins were driven from the spacer gel into the running gel. The current was then lowered to 15-20 mA and the gel was run overnight. The separation of proteins was complete in 14-16 hours.

14. Non denaturing polyacrylamide gels

A 5% non denaturing polyacrylamide slab gel in 0.1 M Tris Borate buffer pH 8.9 was prepared using the method of Prakash et al. (1969). Electrophoresis at 200 V, 75 mA was performed for 3 to 4 hours. Gels were kept cool during electrophoresis by having a cooling coil directly in the main buffer vessel. The samples contained 5% sucrose and bromophenol blue as indicator, and 30 µl samples were loaded into the gel pockets.

15. Protein and glycoprotein staining

For proteins the gels were stained at room temperature with 0.5% Coomassie blue R-250 dissolved in a mixture of 40% methanol and 7% acetic acid, for 30 min. The excess stain was removed by soaking the gel in 5% methanol containing 7.5% acetic acid.

For glycoprotein staining the gels were first treated with 10% acetic acid to remove any detergent or fixative, after which they were treated with 0.5% periodic acid for 2 hours. The periodic acid was removed by incubating the gels twice for 30 min in 0.5% sodium arsenite in 5.5% acetic acid, and three times for 20 min in 5% acetic acid. After these repeated washes the gels were stained with the Schiff reagent overnight, and then washed repeatedly with 0.1% sodium metabisulfite in 0.01 M HCl until the washes no longer turned pink on the addition of formaldehyde. The gels were then stored in water (Maddya, 1976).

16. Preparation of the SDS polyacrylamide gel for activity staining

Activity staining of enzymes after SDS gel electrophoresis requires the removal of SDS. To accomplish this the gels were incubated for 30 min in 50 mM disodium hydrogen phosphate plus 12.5 mM citric acid, pH 6.3. The first two washes also contained 25% isopropanol. These incubations may permit renaturation of most protein molecules from the SDS polyacrylamide gels (Beguin, 1983).

17. Preparation of an agar replica for activity staining

A thin (0.8 mm) agar replica was prepared containing 2% agar, 0.1% carboxymethyl cellulose, 50 mM citric acid, pH 6.3. The whole gel assembly was steam heated to prevent premature gel hardening and air bubbles, then stored at 4°C. Before use the glass plates were disassembled and air bubbles between the gel and the bottom glass were removed by passing a test tube over the gel. Excess moisture which would smear the activity bands was removed by placing the gel at 50°C for 15 min. This procedure was developed by Beguin (1983).

18. Replication of the polyacrylamide gel and its staining

Excess moisture from the polyacrylamide gel was removed in the same manner as for the agar gel. Once the gels were ready the washed polyacrylamide gel was placed on top of the agar gel. The air bubbles between the two gels were removed by passing a test tube over the top gel. The assembly was then wrapped in Saran Wrap to prevent drying (Beguin, 1983). The optimum temperature for the cellulases of *Cellulomonas* 2406 was previously found to be 50°C and therefore carboxymethyl cellulose hydrolysis was allowed to take place for 12-16 hours at 50°C. For identification of activity bands the polyacrylamide gel was removed and the agar replica was stained with 0.1% Congo red for 30 min. The stain was then poured off and the excess was removed by incubating the gel in 0.1 M sodium chloride. Congo red is known to bind to polysaccharides containing continuous β-1-4 linked D-glucopyranosyl units (Theater & Wood, 1982), and it will not bind where carboxymethyl cellulose was degraded. Thus regions of endocellulase activity were identified. When non-denaturing polyacrylamide gels were used, carboxymethyl cellulose hydrolysis was allowed to take place for two hours at 50°C.

19. Mutagenesis

Different methods were used to try to mutate *Cellulomonas* 2406 in order to find derepressed mutants which could grow in high concentrations of glucose and still produce high levels of cellulolytic enzymes.

NTG (N-Methyl-N'-nitro-N-nitrosoguanidine) mutagenesis was done as described by Stewart & Leatherwood (1976). Exponential cultures of *Cellulomonas* 2406 were centrifuged at

10 000 x g (4°C) for 10 min and washed twice in 5 ml of 0.1 M citrate buffer. The NTG was dissolved in 0.1 M citrate buffer and then added to the cells at a final concentration of 10, 100, 250, 300 and 500 μgm/ml. The mixture was incubated at 30°C for 30 min. The cells were washed twice in sodium phosphate buffer, 0.1 M, pH 7, after incubation and then plated on PTYE plates. The plates were incubated at 30°C and cultures were counted. The NTG treated cells were also plated onto PTYE plates containing 0.3% glucose and 0.5% pretreated cellulose, incubated at 30°C in the hope that haloes of degradation would appear, indicating the presence of mutants.

MMS (methyl methanosulfonate) mutagenesis was done following the same procedure as for NTG mutagenesis. Washed cells suspended in up to 1% MMS in sodium phosphate buffer, pH 7, were incubated at 30°C for 30 min, washed once in phosphate buffer and resuspended in 5 ml of the same buffer. Dilutions were plated out on PTYE plates.

U.V. mutagenesis: ultraviolet light which induces a wide range of mutations was used in the hope of obtaining derepressed mutants of *Cellulomonas* 2406. The procedure followed was that of Miller (1972). Exponential cultures were washed twice with 0.1 M magnesium sulfate and resuspended in 0.1 M magnesium sulfate. Five milliliters of the suspension was exposed to U.V. light for various lengths of time under constant agitation, then plated out on PTYE medium. In variation of this method U.V. treated cells were added to a liquid basal medium containing 1% glucose and 0.25% treated cellulose. The flasks were then incubated at 30°C in the dark and at various time intervals samples were plated out for viable count on PTYE plates containing 0.3% glucose and 0.5% treated cellulose. This variation was made to give the derepressed mutants a selective advantage of growth since in the dark the mutated cells could not undergo photoreactivation.

20. Electron microscopy

One tenth volume of 5% (v/v) glutaraldehyde in 0.01 M cacodylate buffer (pH 7.2) was added to cells in media for 20 min at room temperature. The pre-fixed cells were allowed to settle in a conical centrifuge tube and the pellets resuspended and fixed in 5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer for 1.5 hours at room temperature. The fixed cells were then allowed to settle again and resuspended in warm 4% Noble agar (Difco) and drawn into a Pasteur pipette. After the agar gelled, the gel was extruded and cut into 5 mm cores containing the cells. These cores were subsequently washed 5 times for 10 min each with 0.1 M cacodylate buffer. Cells were post fixed with 2% osmium tetroxide in 0.1 M cacodylate buffer containing 7×10^{-3} M $MgCl_2$ for 2 hours at room temperature, washed 5 times at 10 min intervals with 0.1 M cacodylate buffer and dehydrated with a water-acetone dehydration series, the cores being exposed to 30, 50, 70, 90, 100% acetone for 30 min each. The acetone-dehydrated cores were then immersed twice in propylene oxide for 20 min at room temperature and placed in a 3:1 mixture of propylene oxide and resin and allowed to stand overnight in a fume hood. After the propylene oxide had evaporated, the cores were suspended in fresh resin for 4 hours for further infiltration and finally embedded in fresh resin and polymerized at 60°C for 8 hours. The resin utilized contained 9.85 gm Vestopal W, 0.1 gm tertbutyl perbenzoate as catalyst, and 0.05 gm cobalt napthenate (Polyscience Inc.). Thin sections (about 0.02 µm) of the embedded specimens were cut on an LKB-ultratome 111 (LKB-Producter AB) with glass knives. The sections were mounted on 200 mesh, 3 mm copper grids, stained with 1% uranyl acetate (pH 5) and lead citrate (Reynolds, 1963) and subsequently carbon backed using a Balzer BA 360 Freeze-etching Device. Electron microscopy was carried out on a Phillips electron microscope (201) at an accelerating voltage of 60 kV and photographed on a Kodak Electron Microscope Film.

21. Ruthenium red staining

Ruthenium red (Luft, 1971) was utilized to visualize the anionic exopolysaccharide produced by the bacteria. The entire procedure was identical with routine embedding procedures previously described except that 0.15% (w/v) ruthenium red (B.D.H. Chemicals) was present in the fixation buffer and that 0.05% (w/v) ruthenium red was present in all solutions until the 50% acetone step. The thin sections of the specimen were stained with uranyl acetate and lead citrate, carbon backed, and examined under a Phillips 201 electron microscope.

22. Light microscopy

A Zeiss microscope with phase contrast optics was used. Photographs were taken using Kodak film, using wet mounts with Congo red for a better contrast.

23. Source of materials

Microcrystalline Avicel PH-101 from FMC Corporation, Philadelphia U.S.A.; Solka floc BW-300 from Lee Chemicals Toronto, Ontario; CF 11 cellulose from Fisher Chemicals, Whitney, Ontario. Materials such as: Tris, lysozyme, Schiff reagent, cellobiose and carboxymethyl cellulose were purchased from Sigma Chemicals. Bacto-peptone was obtained from Difco Laboratories, Detroit, Mich., tryptone and yeast extract from Oxoid Canada Inc., acrylamide, TEMED (N,N'-tetramethyl ethylenediamine) and ammonium persulfate from Bio Rad Laboratories, Richmond, California; molecular weight standards (14 000-100 000 daltons) and sodium dodecyl sulfate (SDS) for SDS gel electrophoresis from B.D.H. Chemicals Ltd., England. Amicon filters: Amicon, Danvers, Ma., U.S.A. Films for light microscopy and electron microscopy were obtained from Hall Photographics, Quebec. All other chemicals were of analytical grade.

Note: All the experiments presented in Results were repeated at least twice.

RESULTS

1. The nutritional preference of the aerobic cellulolytic bacterium *Cellulomonas* 2406

Cellulomonas 2406 was isolated from rotting sugar cane and the adjacent soil mixture by Han and Srinivasan (1968) and obtained from the National Research Council of Canada.

For any microorganism to be able to grow it requires a usable form of carbon and nitrogen. *Cellulomonas* 2406 did not grow in nutrient broth (peptone 0.5% plus beef extract 0.3%) or glucose broth (nutrient broth plus glucose 0.5%). It grew best in yeast extract broth. It was also able to use a variety of carbon sources such as mannose, sucrose and glycerol, but it grew poorly in media containing ethanol (Table 5). It could not utilize urea or uric acid as a nitrogen source, but it did use ammonium phosphate and sodium nitrate.

Cellulomonas 2406 possesses a multitude of enzymes enabling it to degrade different materials other than cellulose, including starch and gelatin (Table 5). Characteristics which help in distinguishing *Cellulomonas* 2406 from other microorganisms of the same genus with which it has been classified in Bergey's manual, are listed in Table 5. For example, *Cellulomonas* CSI-1 isolated from cotton wool is non-motile (Choi et al., 1979) whereas our strain is capable of movement. Also the G + C content of our *Cellulomonas* strain is within the range as measured by Kellerman & McBeth (1983) for *Cellulomonas flavigena* and reported in Bergey's manual (Buchanan & Gibbons, 1974). Other characteristics distinguish *Cellulomonas* 2406 from microorganisms from the same family, for example, starch

Table 5. Differential Characteristics of the genus *Cellulomonas*

	Cellulomonas 2406	*Cellulomonas* CSI-1 (1)	*Cellulomonas flavigena* KIST 321 (2)	*Cellulomonas flavigena* ATCC 482 (3)	*Cellulomonas flavigena* NCTC 7547 (4)	*Cellulomonas* strain 11bc (5)	*Cellulomonas uda* (6)
Biotin requirement	-	-	-	+	+	+	
Thiamine requirement	-	-	+	+	+	+	-
Motility	+	-			+	-	-
Gram stain	variable	positive			variable	variable	positive
Anaerobic growth	poor	poor		poor			
Catalase	+	+	+				
Nitrate → Nitrite	+		+				-
Cellulose attacked	+	+	+	+	+	+	+
Starch hydrolysis	+			+	+	+	
Gelatin hydrolysis	+		±**	±**	±**		
Growth on glucose	+	+		+	+	+	+
cellobiose	+				+	+	+
sucrose	+						
mannose	+				+	+	
glycerol	+				+	+	
fructose	±*				+	+	
maltose	±*				+	+	+
ethanol	-			-			
Yellow pigment	+	+	+	+	+	+	+
Growth on nutrient broth	-	+					+
Decarboxylation of lysine	-				-	-	
argenine	-				-	-	
ornithine	-				-	-	
Yeast extract requirement	+	+					+
G + C content	71-72%			71.1-72.7%			72%

* very poor growth
** slow hydrolysis

(1) from Choi et al., 1978
(2) from Kim & Wimpenny, 1981
(3) from Bergey's manual, Buchanan & Gibbons, 1974
(4) from Beguin & Eisen, 1977
(5) from Beguin & Eisen, 1977
(6) from Stoppok et al., 1982

could not be hydrolysed by *Arthrobacter* species and those belonging to the genus *Corynebacterium* do not have yellow pigment, are catalase negative and grow faster than *Cellulomonas*.

2. Induction of cellulase enzymes by different cellulose substrates in *Cellulomonas* 2406 grown in simple media

Four types of media (Table 6) were tested for obtaining a good growth for *Cellulomonas* 2406, but only one medium (no. 4) supported the best growth. The composition of this and other media tested are given in Table 6. Yeast extract is essential for the growth of *Cellulomonas* 2406. Other *Cellulomonas* species can grow in simple media containing only peptone (Thayer et al., 1984), but not *Cellulomonas* 2406 (medium 1, Table 6). Also no growth was obtained in medium 2 (Table 6) which contained neither peptone nor yeast extract. In medium 3, less growth was obtained than in medium 4 (Table 6).

Since best growth was obtained in medium no. 4, growth of *Cellulomonas* 2406 and the formation of endocellulase activity were monitored in this medium (Table 7). The addition of cellulose stimulated the growth of the bacteria as well as cellulase enzyme production (Table 7, exp. 2 & 3). Soluble cellulose, as carboxymethyl cellulose, also stimulated growth and cellulase activity, but to a lesser extent than did the insoluble cellulose (Table 7, exp. 4 & 5). Cellobiose, glucose and glycerol supported growth of *Cellulomonas* 2406, but also inhibited endocellulase production when added to the medium containing filter paper (Table 7, exp. 10, 12, 14, 15). Glycerol inhibited growth slightly when added to filter paper. When carboxymethyl cellulose was added together with filter paper to the medium, smaller amounts of endocellulase were formed (Table 7, exp. 6 & 7).

Table 6. Different basal media which were tested as growth media for *Cellulomonas* 2406. Glucose was added as a carbon source at a concentration of 1%.

Medium 1 content		Growth measured as protein (mg/ml)	
$NH_4H_2PO_4$	0.1%	Day 1	0
KCl	0.1%	2	0
$MgSO_4$	0.1%	3	0
peptone	1.0%	4	0

Medium 2 content		Growth measured as protein (mg/ml)	
NH_4NO_3	1.00mg/l	Day 1	0
NaH_2PO_4	1.18 "		
KH_2PO_4	0.90 "	2	0
$MgSO_4 \cdot 7H_2O$	0.50 "		
KCl	0.25 "	3	0
NaCl	0.25 "	6	0

Medium 3 content		Growth measured as protein (mg/ml)	
NH_4NO_3	0.1%	Day 1	0.050
K_2HPO_4	0.05%	2	0.250
$MgSO_4 \cdot 7H_2O$	0.02%	3	0.280
$CaCl_2 \cdot 2H_2O$	0.002%	6	0.320
$FeSO_4 \cdot 7H_2O$	0.002%		
$MnSO_4 \cdot 7H_2O$	0.002%		
peptone	0.02%		
yeast ext.	0.02%		

Medium 4 content		Growth measured as protein (mg/ml)	
$NaNO_3$	0.1%	Day 1	0.200
K_2HPO_4	0.1%		
$MgSO_4 \cdot 7H_2O$	0.05%	2	0.350
KCl	0.05%		
yeast ext.	0.05%	3	0.575
		6	0.530

Table 7. Effect of additions to medium no. 4 on growth and endoglucanase formation by *Cellulomonas* 2406.

Experiment	Addition	Hours	Growth (protein mg/ml)	Endoglucanase activity (units/ml)
1.	Nil	24	0.015	0
		48	0.018	0
		96	0.020	0
		168	0.070	0
2.	Filter paper (1%)	24	0.050	0
		48	0.200	8.13
		96	0.530	15.68
		168	0.665	12.70
3.	Filter paper (0.5%)	24	0.070	0
		48	0.257	9.80
		96	0.355	8.90
		168	0.450	8.50
4.	Carboxymethyl cellulose (1%)	24	0.015	8.60
		48	0.015	11.60
		96	0.150	10.20
		168	0.200	7.83
5.	Carboxymethyl cellulose (0.5%)	24	0.067	6.70
		48	0.085	10.00
		96	0.110	9.00
		168	0.145	4.54
6.	Filter paper (1%) plus carboxymethyl cellulose (1%)	24	0.025	0
		48	0.127	3.00
		96	0.210	10.00
		168	0.400	7.67
7.	Filter paper (0.1%) plus carboxymethyl cellulose (0.1%)	24	0.030	3.77
		48	0.058	3.33
		96	0.150	10.00
		168	0.430	12.33
8.	Glycerol (0.5%)	24	0.025	0
		48	0.070	2.87
		96	0.135	2.96
		168	0.235	2.00

Table 7 cont'd

9. Glycerol (1%)	24	0.050	0
	48	0.080	2.33
	96	0.215	2.96
	168	0.315	2.50
10. Glycerol (1%) plus filter paper (1%)	24	0.090	0
	48	0.265	2.90
	96	0.365	2.83
	168	0.400	3.00
11. Cellobiose (1%)	24	0.025	0
	48	0.120	0
	96	0.214	1.50
	168	0.350	3.00
12. Cellobiose (0.5%) plus filter paper (0.5%)	24	0.115	0
	48	0.215	3.00
	96	0.655	4.65
	168	0.700	5.60
13. Glucose (0.5%)	24	0.189	2.67
	48	0.335	3.30
	96	0.585	3.33
	168	0.590	4.27
14. Glucose (0.5%) plus filter paper (0.5%)	24	0.015	0
	48	0.085	2.70
	96	0.360	4.30
	168	0.500	4.83
15. Glucose (0.1%) plus filter paper (1.0%)	24	0.040	2.17
	48	0.155	6.33
	96	0.405	10.67
	168	0.550	11.13

Lower amounts of glucose in the medium containing filter paper caused lower inhibition of endocellulase (Table 7, exp. 14 & 15).

Table 8 shows the effect of adding other cellulose substrates (1%) than filter paper to the medium no. 4. The production of endocellulase, β-glucosidase, filter paper activity and growth were measured. Almost no cellulase activity was obtained at day one in all cultures containing cellulose (Table 8). Extracellular β-glucosidase and filter paper activity were not detected in any of the cultures. Growth and endocellulase activity were similar in all four cultures, except when CF 11 cellulose was present the highest endocellulase activity was obtained at day two, four days earlier than in the other cultures.

When grown in medium no. 4 *Cellulomonas* 2406 degraded cellulose quite slowly and incompletely. Even after 26 days less than 70% of the cellulose was degraded (Fig. 7). Fig. 7 also shows that CF 11 cellulose was degraded the least whereas solka floc and filter paper were degraded at about the same rate. When glucose (1%) was present in the medium containing cellulose fibers no degradation was observed.

As compared to growth on cellulose, when *Cellulomonas* 2406 was grown on glucose, it utilized almost 80% of the glucose present in the medium within the first two days. When grown on cellobiose or soluble cellulose such as carboxymethyl cellulose, very little glucose was detected in the culture

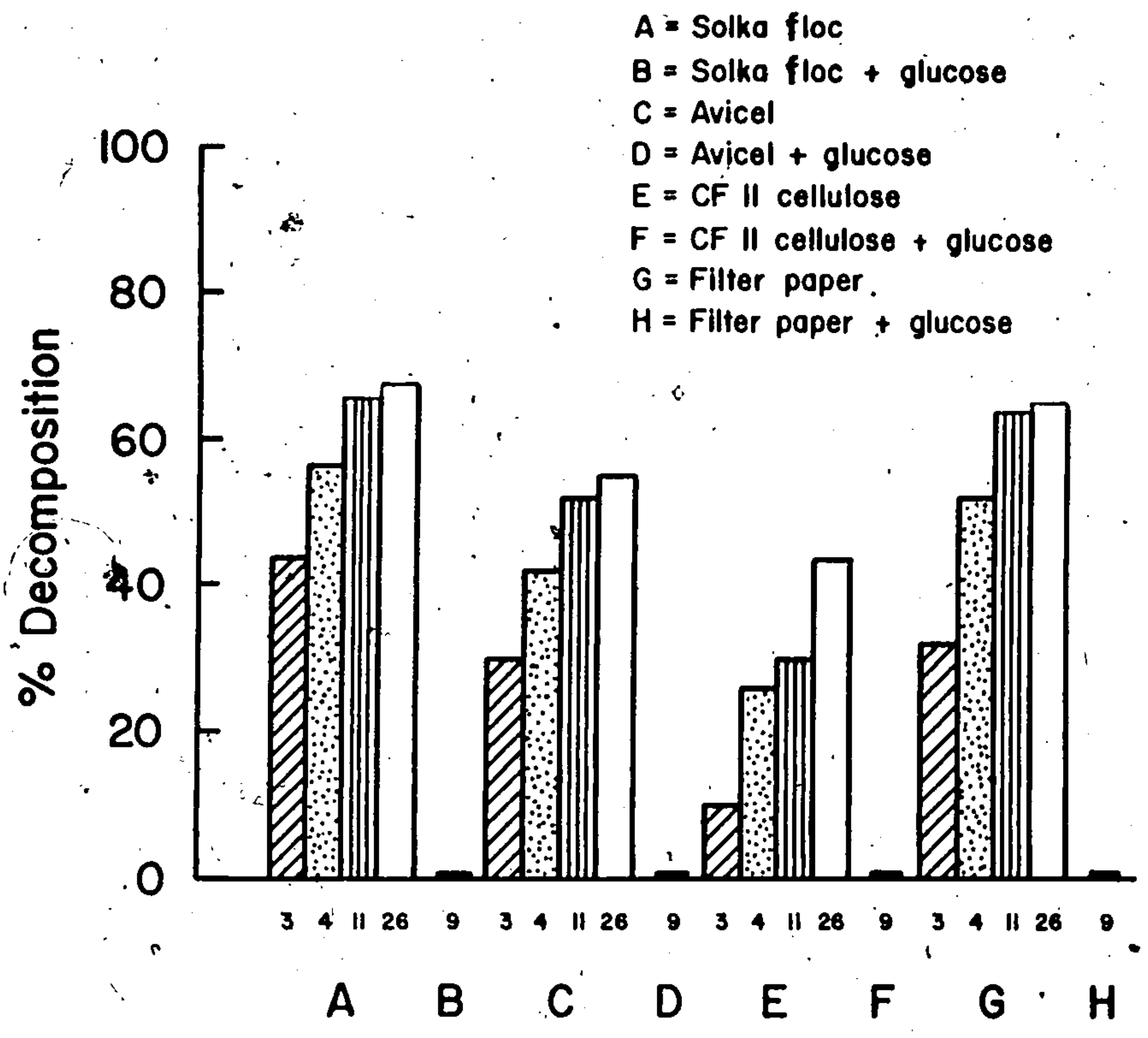
Table 8. Cellulase enzyme activities obtained from *Cellulomonas* 2406 growing in medium no. 4 containing different cellulolytic substrates.

Time	Addition	Cellulase activity*			Protein
days	1%	endoglucanase	β-glucosidase	filter paper activity	mg/ml
1.	Solka floc	0.50	0	0	0.120
2.	''	11.00	0	0	0.260
3.	''	13.33	0	0	-
6.	''	13.80	0	0	0.600
1.	Avicel	0.50	0	0	0.200
2.	''	3.70	0	0	0.450
3.	''	12.30	0	0	0.575
6.	''	10.85	0	0	0.630
1.	CF 11 cellulose	0.50	0	0	0.107
2.	''	7.17	0	0	0.200
3.	''	6.60	0	0	0.340
6.	''	3.50	0	0	0.420
1.	Filter paper	0.50	0	0	0.125
2.	''	8.00	0	0	0.190
3.	''	10.70	0	0	0.400
6.	''	5.75	0	0	0.650

* A unit of activity is defined as micrograms of reducing sugar (relative to a glucose standard) per minute per millilitre of culture.

Fig. 7. Decomposition of different types of celluloses by *Cellulomonas* 2406 grown in basal media, by determination of residual cellulose.

(The number of days of incubation is indicated under each bar.)


A = Solka floc
B = Solka floc + glucose
C = Avicel
D = Avicel + glucose
E = CF II cellulose
F = CF II cellulose + glucose
G = Filter paper
H = Filter paper + glucose
% Decomposition
100
80
60
40
20
0
3
4
11
26
9
A
B
C
D
E
F
G
H

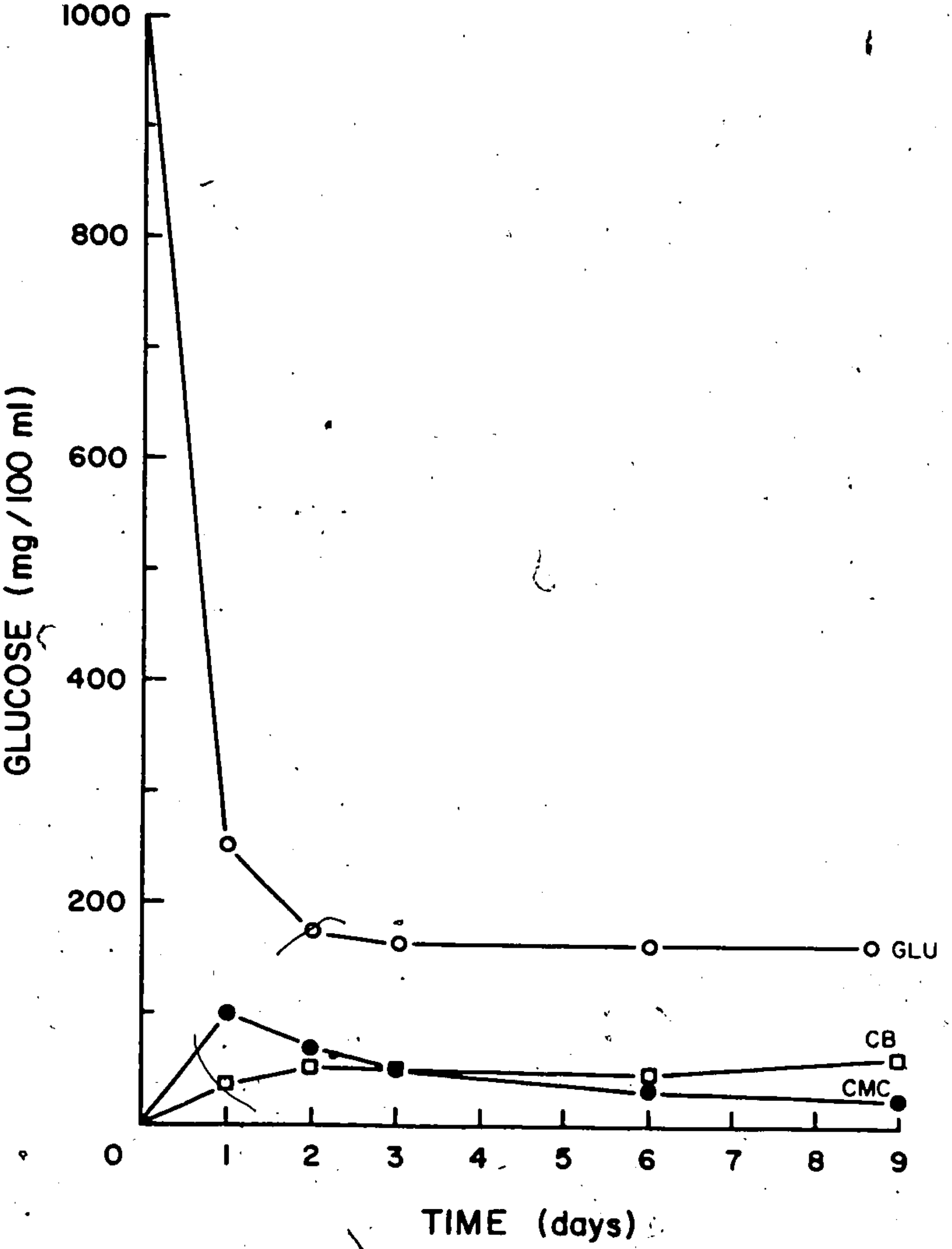
media (Fig. 8). When cells were grown on insoluble cellulose one-tenth as much glucose was detected in the culture media as when cells were grown on cellobiose or carboxymethyl cellulose (Fig. 9). These results indicate that as soon as glucose is released from the cellulose fibers the bacteria utilize it.

Fig. 8. The amount of glucose produced or utilized by *Cellulomonas* 2406 grown in basal media containing

a) glucose (GLU)

b) cellobiose (CB)

c) carboxymethyl cellulose (CMC).



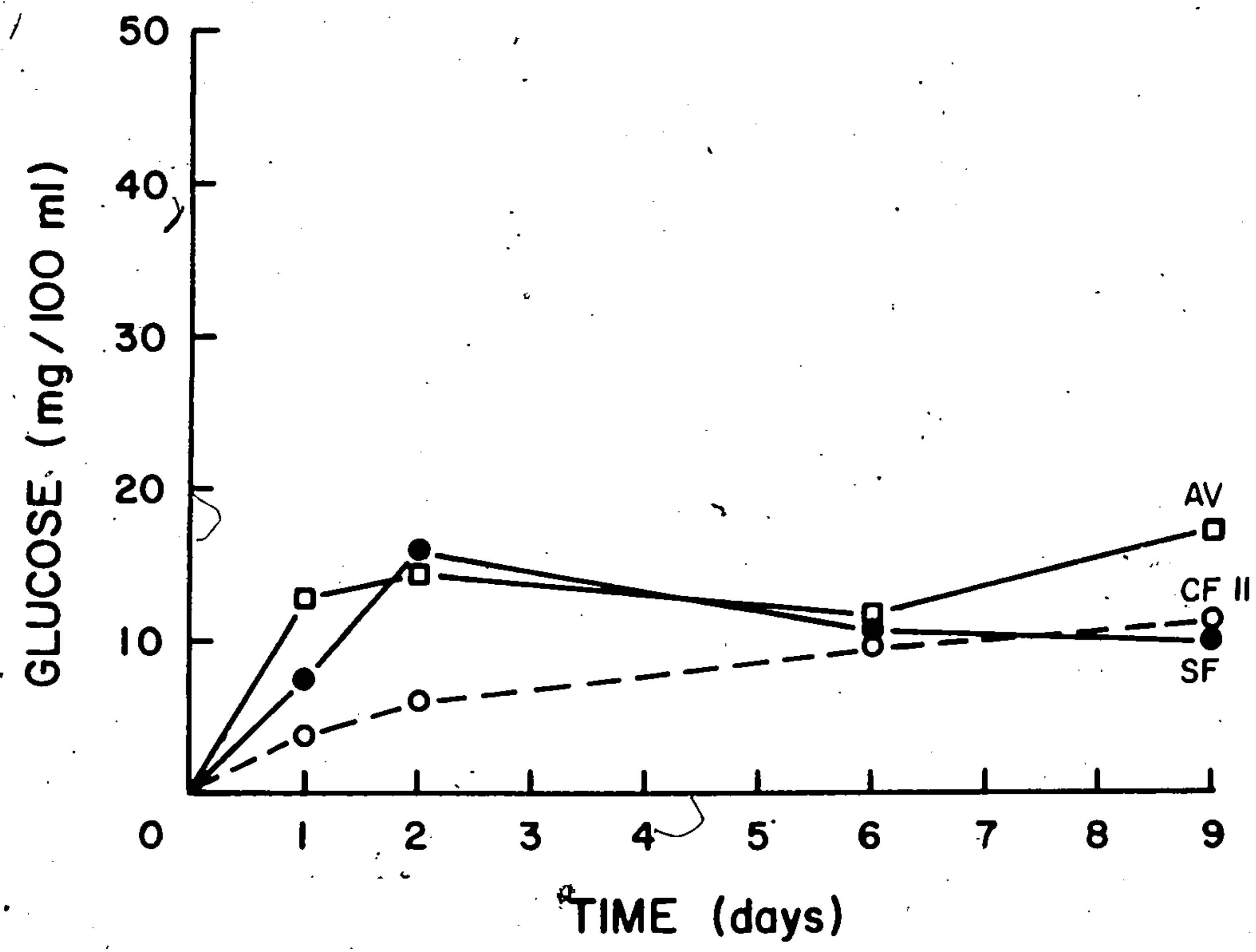
1000
800
600
400
200
0
GLUCOSE (mg/100 ml)
1
2
3
4
5
6
7
8
9
TIME (days)
GLU
CB
CMC

Fig. 9. The amount of glucose released into the media by *Cellulomonas* 2406 grown in basal media containing different celluloses as substrate:

a) AV = avicel

b) CF 11 = CF 11 cellulose

c) SF = solka floc.


GLUCOSE (mg/100 ml)
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
TIME (days)
AV
CF II
SF

3. Growth and cellulase enzyme activity of *Cellulomonas* 2406 grown in the complex medium PTYE

As seen from the previous results in cultures of *Cellulomonas* 2406 in medium no. 4 containing different celluloses, only endocellulase activity was detected. In complex medium (PTYE) containing different cellulosic substrates β-glucosidase and exocellulase activities could also be detected.

When endocellulase activity in PTYE medium containing different amounts of avicel was measured it was found that 1% avicel produced the highest endocellulase activity (Fig. 10). Therefore all experiments from this point on were conducted in medium containing 1% cellulose.

Cellulase enzyme activities in the extracellular fraction were very similar when *Cellulomonas* 2406 was grown on different cellulose substrates: solka floc, avicel, CF 11 cellulose and filter paper (Fig. 11 a, b, c, d). β-glucosidase and filter paper activities were very low as compared with the activity of endocellulase.

Even though the β-glucosidase and filter paper activities were similar for all four cellulose substrates the appearance of endocellulase activity varied from one cellulose substrate to another. When the cells were grown in PTYE containing CF 11 cellulose the maximum activity for endocellulases was reached within three days after which it steadily declined (Fig. 11 c). When cells were grown on the other cellulose

Fig. 10. Endocellulase activity obtained from *Cellulomonas* 2406 grown in PTYE medium containing avicel.

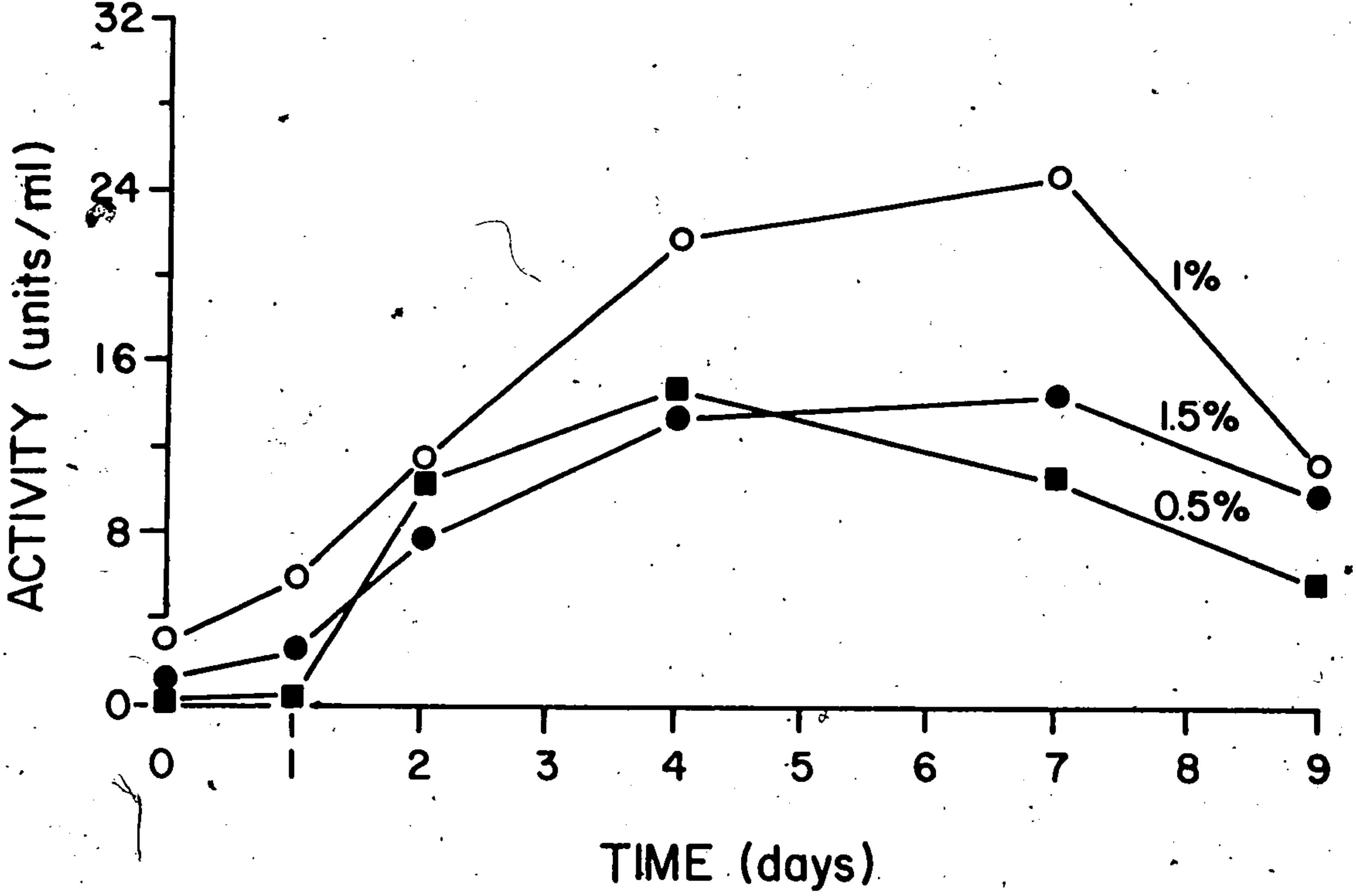

32
24
16
8
0
ACTIVITY (units/ml)
0
1
2
3
4
5
6
7
8
9
TIME (days)
1%
1.5%
0.5%

Fig. 11. Cellulase enzyme activities obtained from *Cellulomonas* 2406 growing in complex medium (PTYE) containing different cellulolytic substrates.

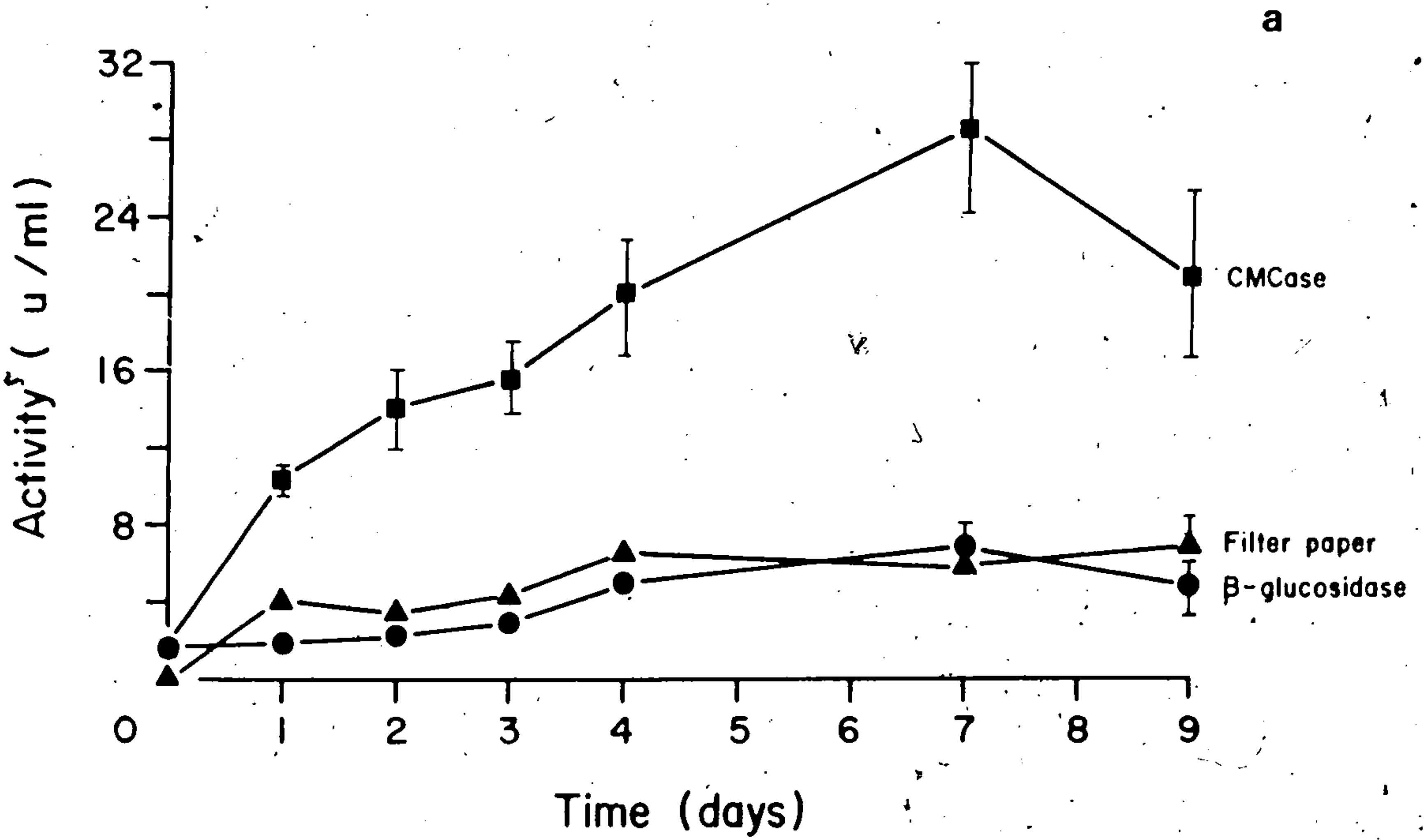
a) Solka floc

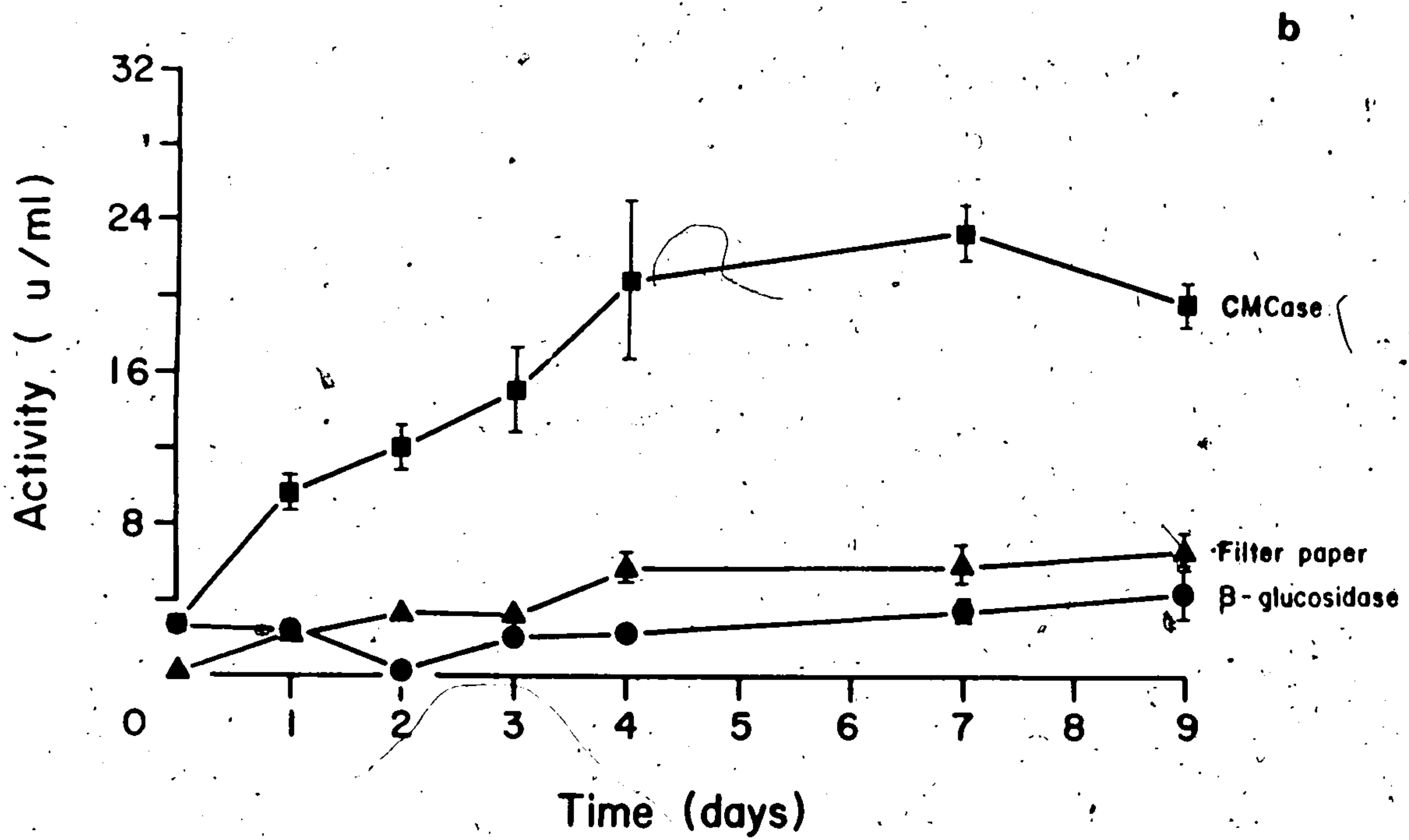
b) Avicel

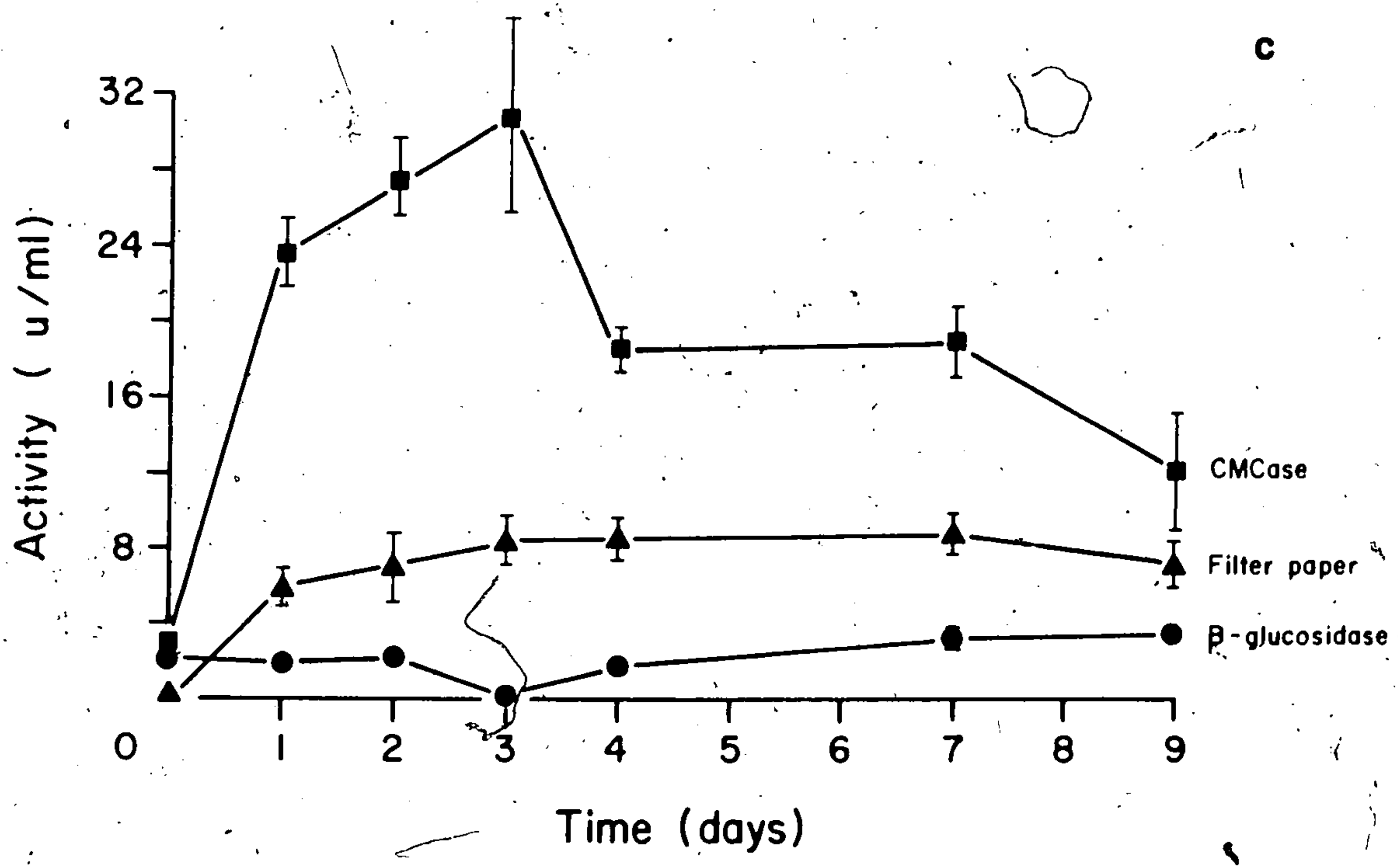
c) CF 11 cellulose

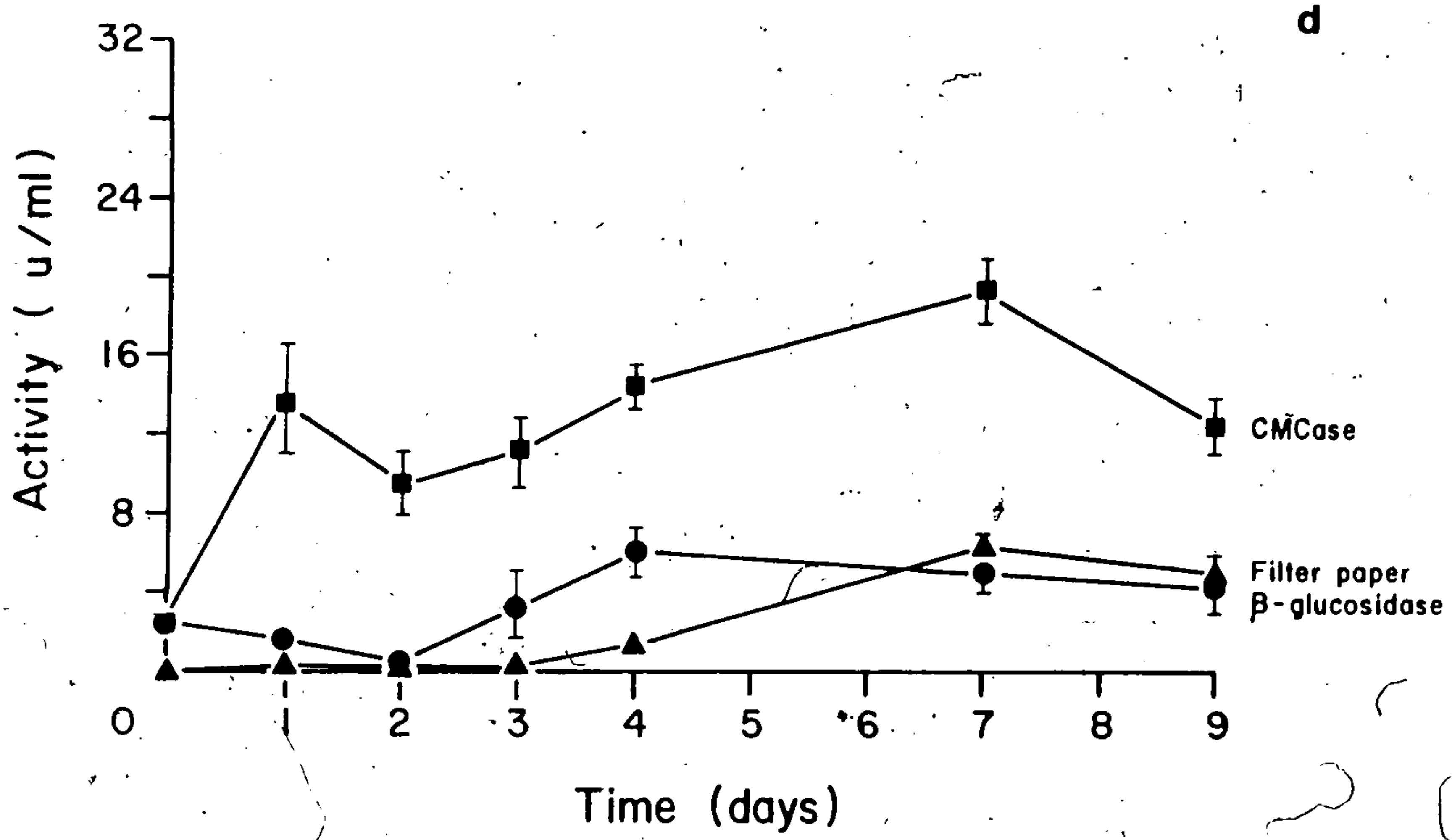
d) Filter paper

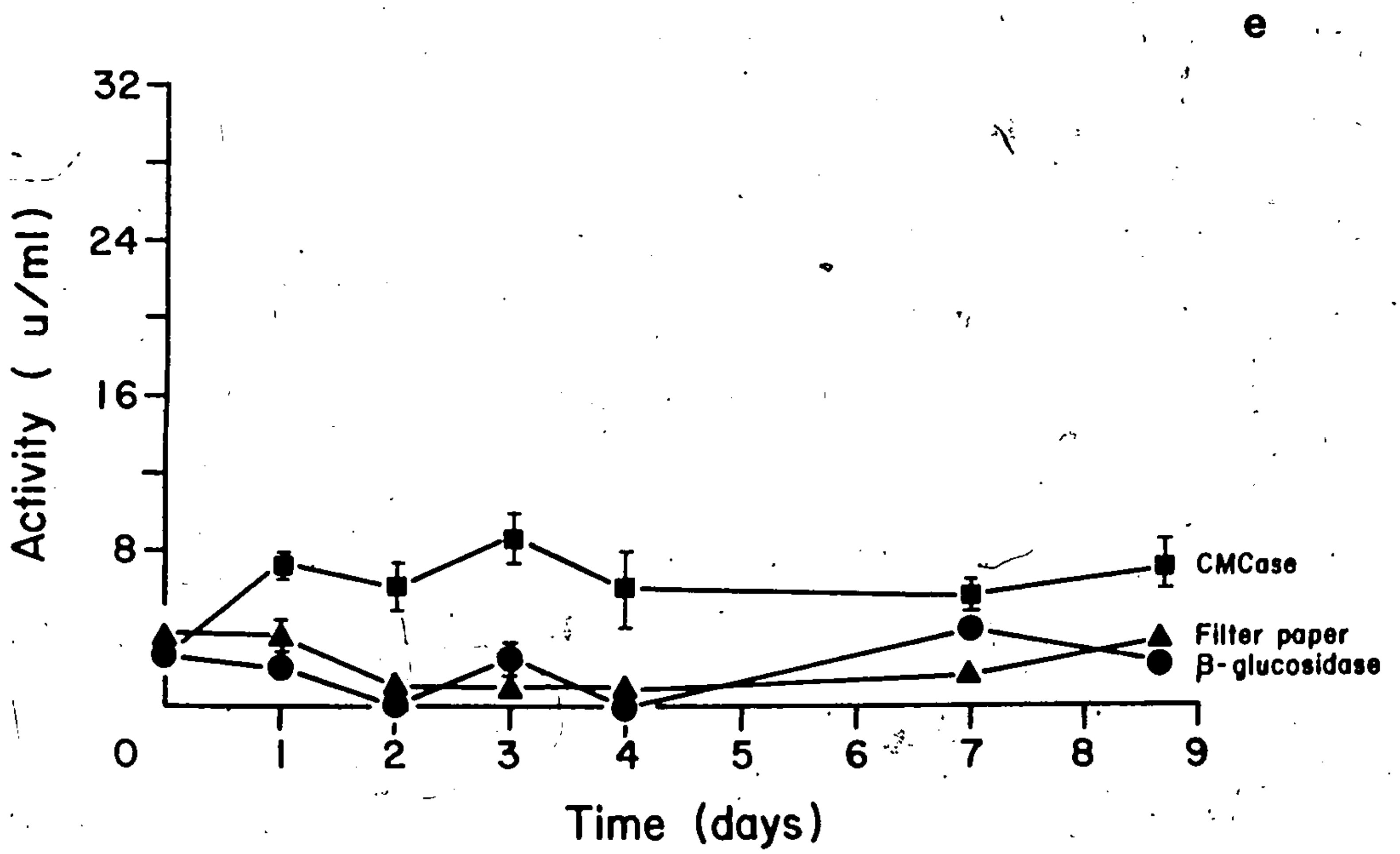
e) Complex medium without cellulose substrates


a
Activity^{s} (u / ml)
32
24
16
8
0
1
2
3
4
5
6
7
8
9
Time (days)
CMCase
Filter paper
β-glucosidase


b
Activity (u/ml)
32
24
16
8
0
1
2
3
4
5
6
7
8
9
Time (days)
CMCase
Filter paper
β-glucosidase


c
Activity (u/ml)
32
24
16
8
0
1
2
3
4
5
6
7
8
9
Time (days)
CMCase
Filter paper
β-glucosidase


d
Activity (u/ml)
32
24
16
8
0
1
2
3
4
5
6
7
8
9
Time (days)
CMCase
Filter paper
β-glucosidase


e
Activity (u/ml)
32
24
16
8
0
1
2
3
4
5
6
7
8
9
Time (days)
CMCase
Filter paper
β-glucosidase

substrates maximum activity was reached between days 6 and 7. The highest endocellulase activity was obtained when the cells were grown on CF 11 cellulose.

When *Cellulomonas* 2406 was grown in complex medium PTYE alone (Fig. 11 e), low amounts of cellulase enzymes were produced.

Endocellulase is an induced enzyme in *Cellulomonas* 2406. When *Cellulomonas* 2406 was grown in PTYE medium containing carboxymethyl cellulose the endocellulase activity obtained was similar to that obtained when solka floc was present (results not shown).

Cellulose is a porous material and a surface to which enzymes might remain attached. Fig. 12 shows both the activity of endocellulase enzyme in whole culture and in extracellular fraction for cells grown on three of the cellulose substrates under study. In all three cases one can see that the whole culture had a little higher activity than the extracellular fraction. This difference is probably due to the fact that some of the enzymes remain associated with the cellulose or the actual cells themselves.

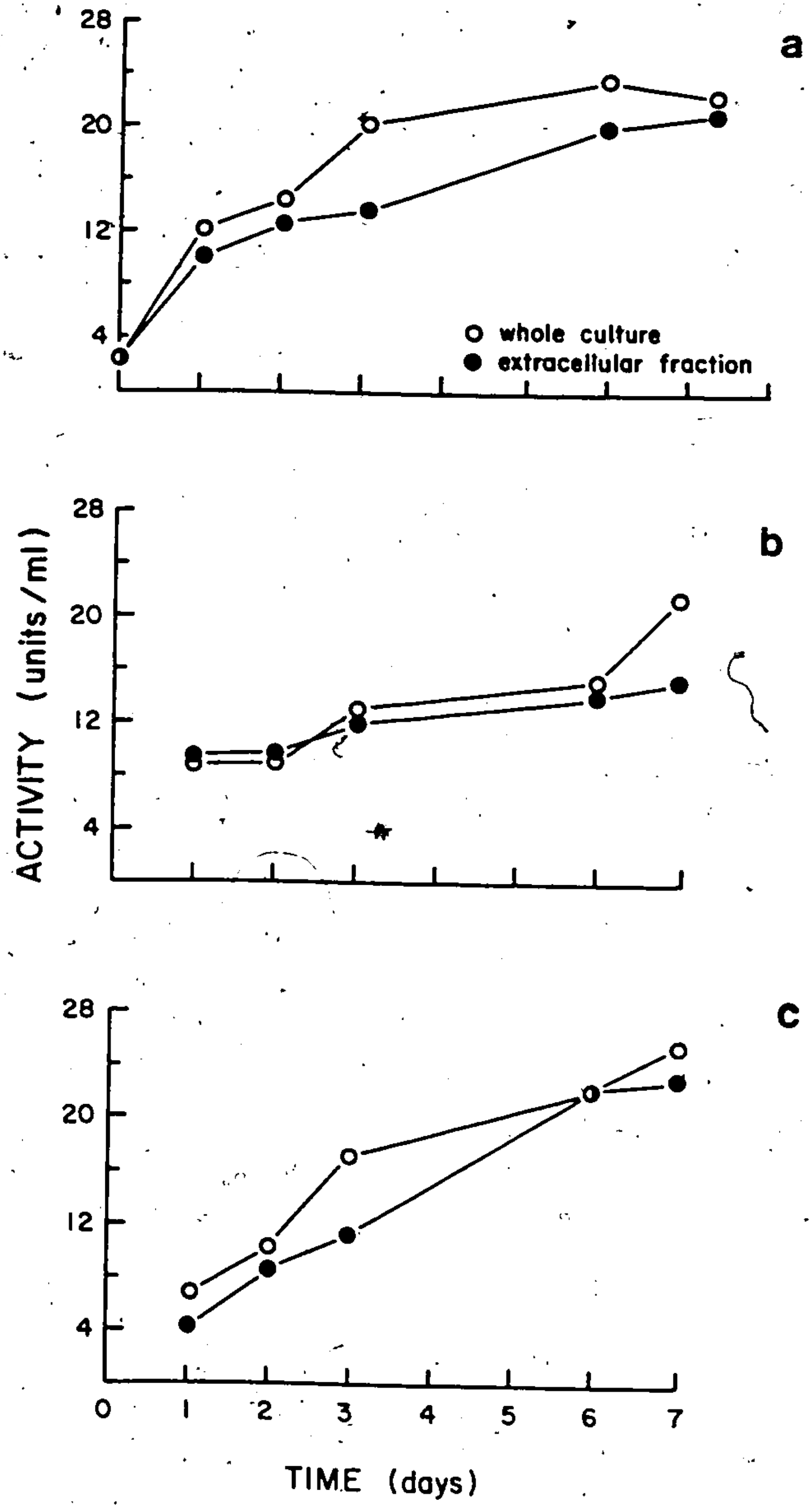
Three different methods for measuring growth (protein, DNA and viable counts) of *Cellulomonas* 2406 were tried out to see which one is best correlated with the cellulase enzyme activities. Growth measured by DNA determination showed a similar response as growth measured by protein (Table 9 & 10). The cells grew better if cellulose was present (Table 9 & 10). Glucose stimulated growth of *Cellulomonas* 2406 in PTYE medium, but the addition of glucose to PTYE medium containing cellulose generally depressed growth compared to PTYE medium with only cellulose added (Table 9).

Fig. 12. Endocellulase activity obtained from whole cultures (——0——) and from extracellular fraction (——●——) of *Cellulomonas* 2406 grown in PTYE medium containing different cellulose substrates:

a) Solka floc

b) Filter paper

c) Avicel


a
b
c
28
20
12
4
○ whole culture
● extracellular fraction
ACTIVITY (units/ml)
0
1
2
3
4
5
6
7
TIME (days)

These experiments show that activity started to appear at the beginning of the stationary phase and increased thereafter, except when grown on CF 11 cellulose when activity decreased during stationary phase.

When growth was measured by viable counts quite a different picture emerged from that obtained from measurements of growth by protein or DNA. At the end of first day the viable counts in the complex medium PTYE with or without cellulose were the same (Fig. 13 a, b, c, d). In the cultures containing cellulose the viable counts dropped 10 fold at day two and lumps were observed in the media. This was probably due to an association of the cells with the cellulose fibers. To try to separate the cells from the cellulose fibers the cultures were treated with either 1% tween or 1% triton which have been shown not to kill the cells. This treatment did not increase the viable counts and the lumps were still visible, suggesting that the cell-cellulose association was unchanged. The presence of glucose in the cellulose-containing medium prevented the formation of lumps and the abrupt fall in the viable counts at day two (Fig. 13). After day two the viable counts started to increase in the cultures containing cellulose and then slowly decreased. In the cultures containing glucose and cellulose after day two the viable counts were lower than those in PTYE with cellulose and no glucose present.

As was previously done for the growth of the *Cellulomonas* 2406 in basal medium, no. 4, residual cellulose was

Table 9. Growth of *Cellulomonas* 2406 measured as protein concentration in a complex medium (PTYE) containing different cellulolytic substrates.

Substrates added (1%)	Protein (mg/ml)						
Day	0	1	2	3	4	7	9
None	0.09	0.11±0.01	0.32±0.63	0.31±0.01	0.39±0.01	0.51±0.01	0.49±0.01
Solka floc	0.20±0.02	0.86±0.03	0.92±0.04	1.04±0.10	1.55±0.15	1.00±0.20	1.28±0.01
Solka floc + Glucose	0.22±0.02	0.80±0.10	0.91±0.10	0.99±0.14	1.06±0.20	1.06±0.20	0.68±0.02
Avicel	0.23±0.02	0.77±0.02	0.92±0.03	1.23±0.17	1.61±0.19	1.41±0.30	1.33±0.20
Avicel + Glucose	0.18±0.04	0.89±0.02	0.90±0.10	0.85±0.10	1.04±0.20	1.20±0.25	0.76±0.05
CF 11 Cellulose	0.24±0.01	0.68±0.15	1.26±0.32	1.22±0.03	1.21±0.04	0.96±0.02	1.02±0.20
CF 11 Cellulose + Glucose	0.18±0.05	0.74±0.09	1.10±0.40	1.04±0.10	1.05±0.04	1.16±0.20	0.78±0.15
Filter paper	0.10±0.04	0.86±0.10	1.11±0.02	1.02±0.08	1.18±0.02	1.24±0.01	1.02±0.02
Filter paper + Glucose	0.12±0.02	0.66±0.07	0.90±0.10	----	1.02±0.10	1.08±0.10	0.76±0.09
Glucose	0.11	0.22±0.03	0.40	0.41±0.02	0.62±0.01	0.73±0.03	0.54±0.04

* Figures show $\overline{X}$ ± S.E of four observations

Table 10. Growth of *Cellulomonas* 2406 measured as DNA concentration in a complex media (PTYE) containing different cellulolytic substrates.

Substrates added (1%)		DNA (μg/ml)					
Day:	0	1	2	3	4	7	6
None	0	60.0±3.0	84.0±5.2	76.5±0.1	75.0±2.7	76.0±0.1	67.5±1.8
Solka floc	0	91.5±0.1	-	84.0±0.8	93.5±3.4	150.0±6.5	120.0±0.9
Avicel	0	91.0±2.8	70.5±10.5	117.0±3.2	108.0±1.1	96.0±0.2	96.0±0.4
CF 11 cellulose	0	95.5±5.8	74.5±2.4	86.5±0.1	64.5±2.6	87.5±7.0	135.0±13.4
Filter paper	0	99.5±0.0	105.0±0.1	129.0±3.7	97.5±0.0	108.0±12.0	102.0±6.2

* Figures show $\overline{X}$ ± S E of duplicate observations.

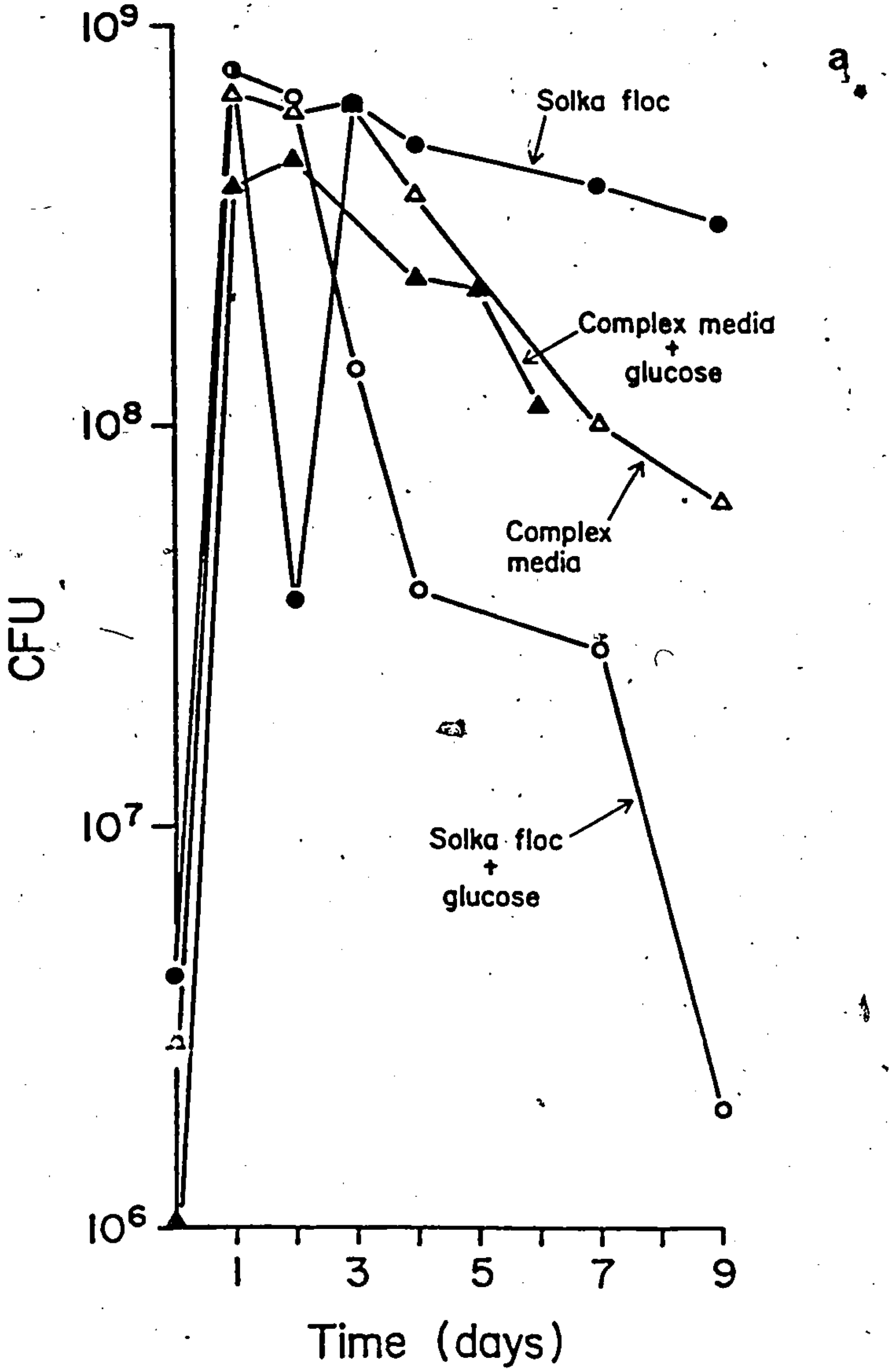
Fig. 13. Growth measured by viable counts.

a) Growth of *Cellulomonas* 2406 in complex medium (PTYE) (——△——); complex medium plus glucose (1%) (——▲——); complex medium plus solka floc (1%) (——●——); complex medium plus solka floc (1%) plus glucose (1%) (——○——).

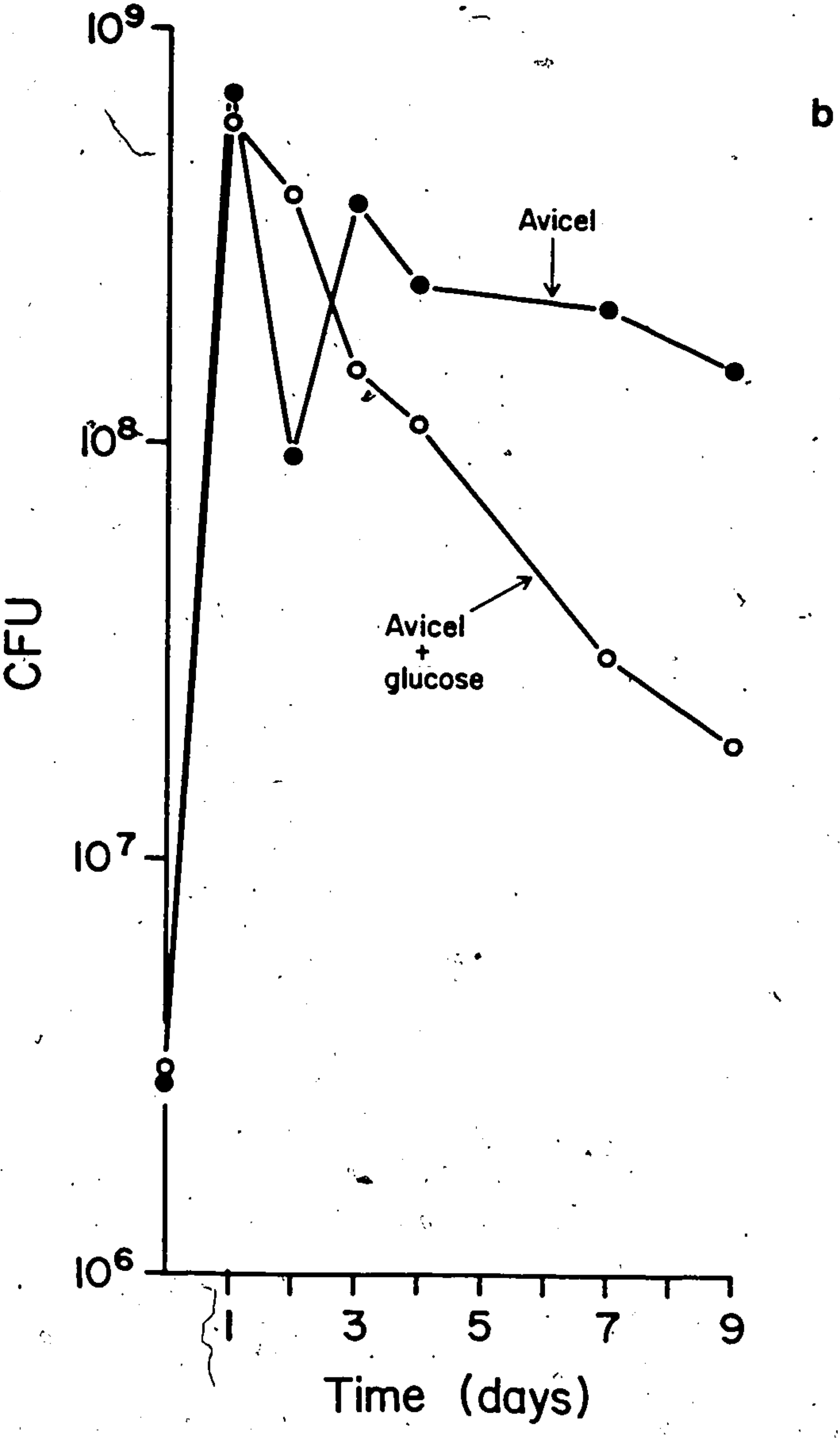
b) Growth of *Cellulomonas* 2406 in complex medium PTYE) containing avicel (1%) (——●——) or containing avicel (1%) plus glucose (1%) (——○——).

c) Growth of *Cellulomonas* 2406 in complex medium (PTYE) containing CF 11 cellulose (1%) (——●——) or containing CF 11 cellulose (1%) plus glucose (1%) (——○——).

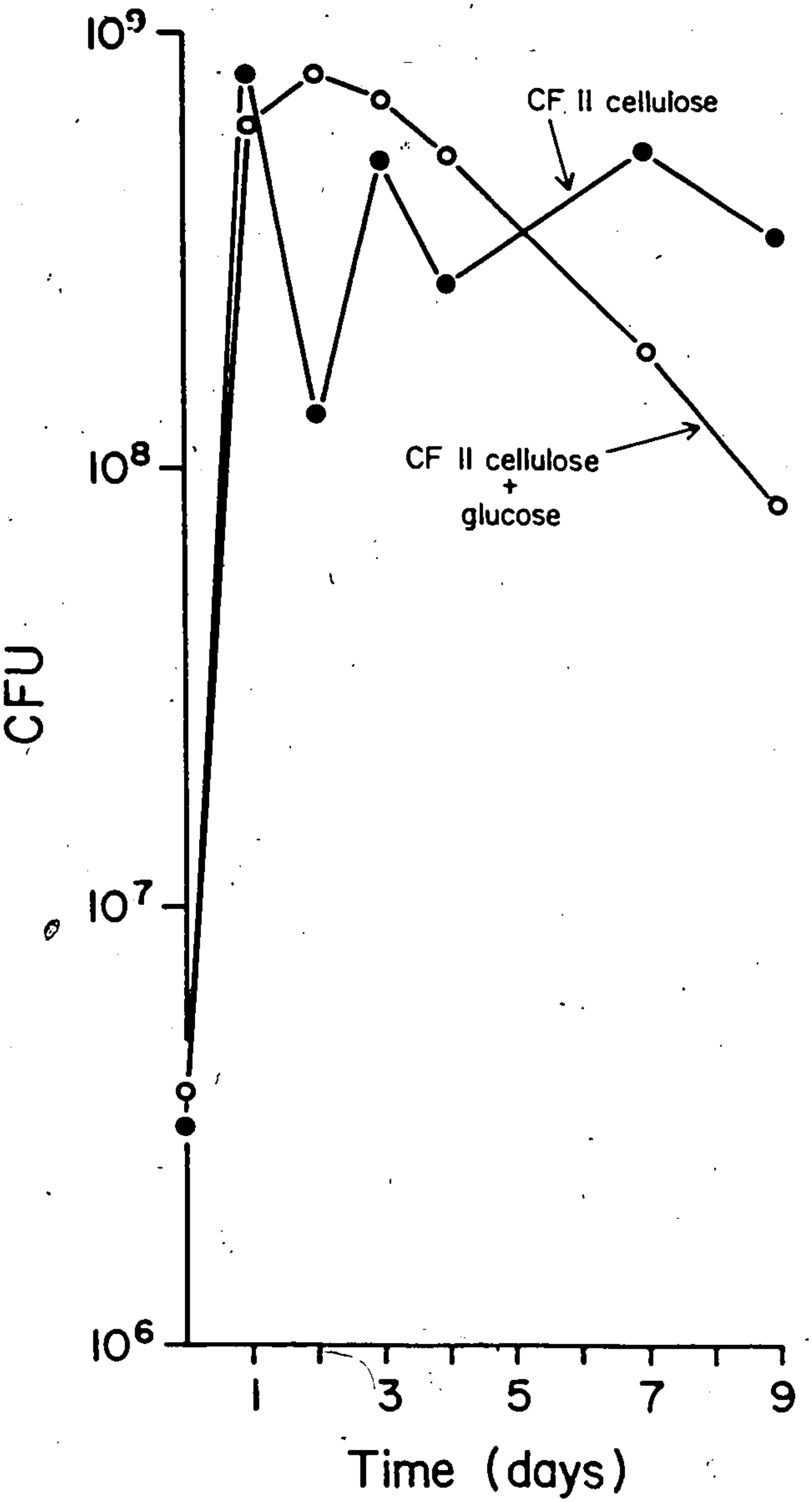
d) Growth of *Cellulomonas* 2406 in complex medium (PTYE) containing filter paper (1%) (——●——) or containing filter paper (1%) plus glucose (1%) (——○——).

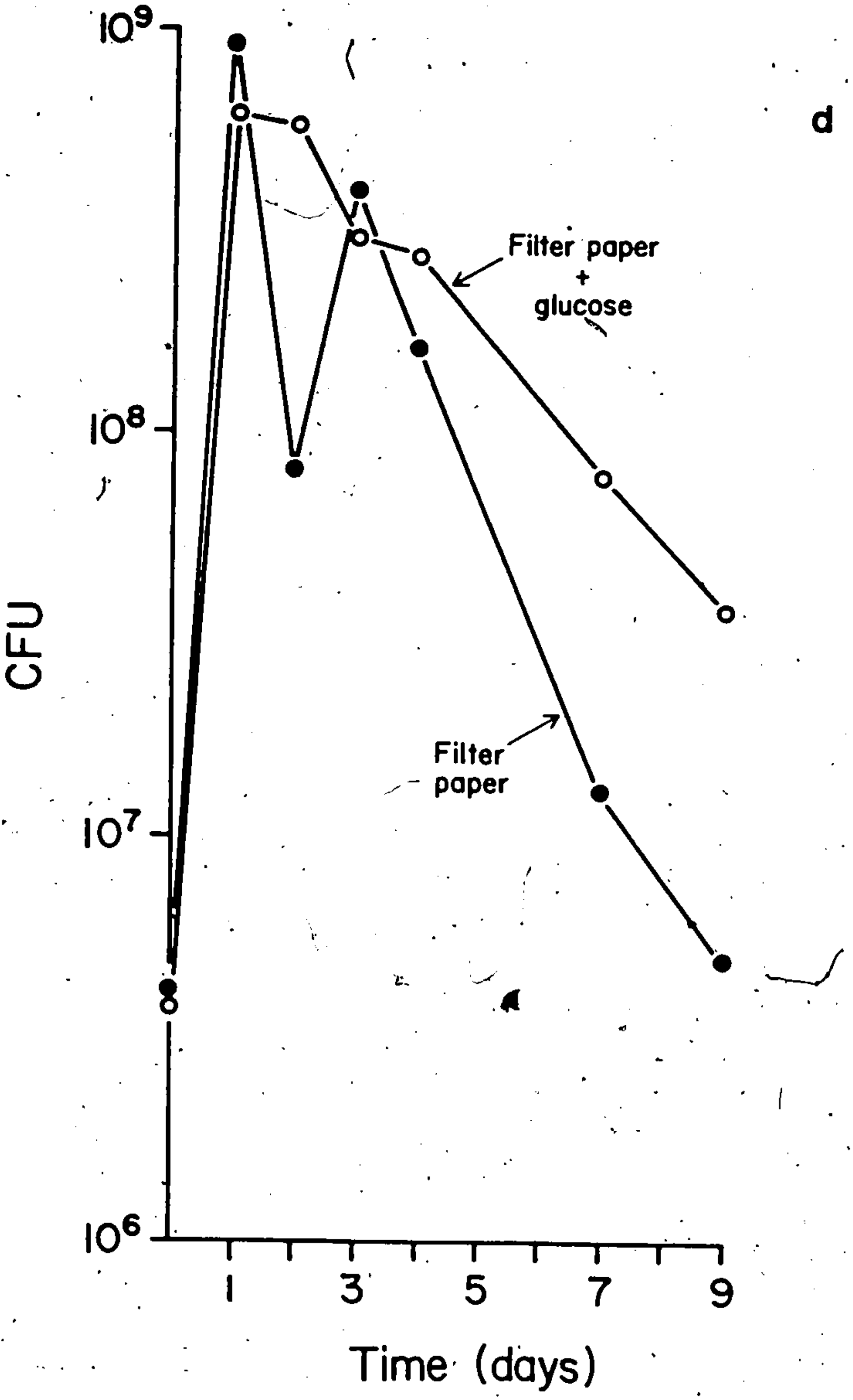


a
10^9
10^8
10^7
10^6
CFU
1
3
5
7
9
Time (days)
Solka floc
Complex media + glucose
Complex media
Solka floc + glucose



b
10^9
10^8
10^7
10^6
CFU
Avicel
Avicel + glucose
1
3
5
7
9
Time (days)


c
CF II cellulose
CF II cellulose + glucose
CFU
10^9
10^8
10^7
10^6
1
3
5
7
9
Time (days)


d
CFU
10^9
10^8
10^7
10^6
1
3
5
7
9
Time (days)
Filter paper + glucose
Filter paper

also monitored when grown in complex medium (PTYE). It was found that almost 90% of the cellulose present in the PTYE medium was degraded after 26 days (Fig. 14). This is almost 20% higher than the degradation in the basal medium. This difference could be due to the better growth of *Cellulomonas* 2406 in the PTYE medium. Comparing the degradation of the different cellulolytic substrates (Fig. 14), one can see that solka floc was degraded the fastest whereas CF 11 cellulose was degraded the slowest. At the same time the highest cellulase ~~enzyme~~ activity was obtained when cells were grown on CF 11 cellulose (Fig. 11 c). When glucose was present in the medium (PTYE) already containing cellulose very little degradation was observed (Fig. 14 B, D, F, H) suggesting that no cellulase enzymes were produced. By the use of plates which contained pretreated cellulose, one can see that when glucose was present in the medium (PTYE) no cellulase enzymes were produced since no zones of degradation appeared (Fig. 15).

Fig. 14. Decomposition of different types of celluloses by *Cellulomonas* 2406 measured by determination of residual cellulose.

(The number of days of incubation is indicated under each bar.)

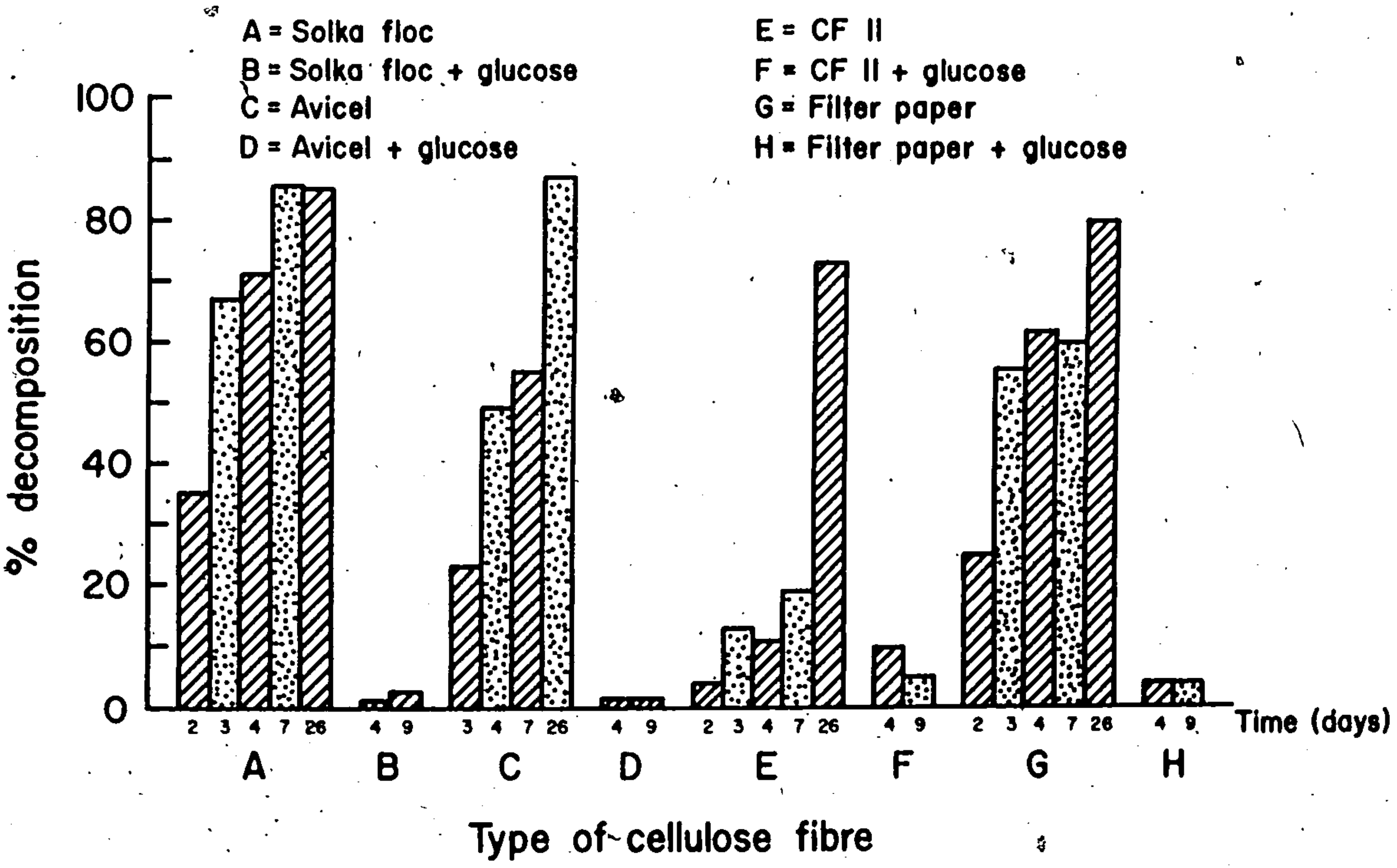

A = Solka floc
B = Solka floc + glucose
C = Avicel
D = Avicel + glucose
E = CF II
F = CF II + glucose
G = Filter paper
H = Filter paper + glucose
% decomposition
100
80
60
40
20
0
2 3 4 7 26
4 9
3 4 7 26
4 9
2 3 4 7 26
4 9
2 3 4 7 26
4 9
Time (days)
A
B
C
D
E
F
G
H
Type of cellulose fibre

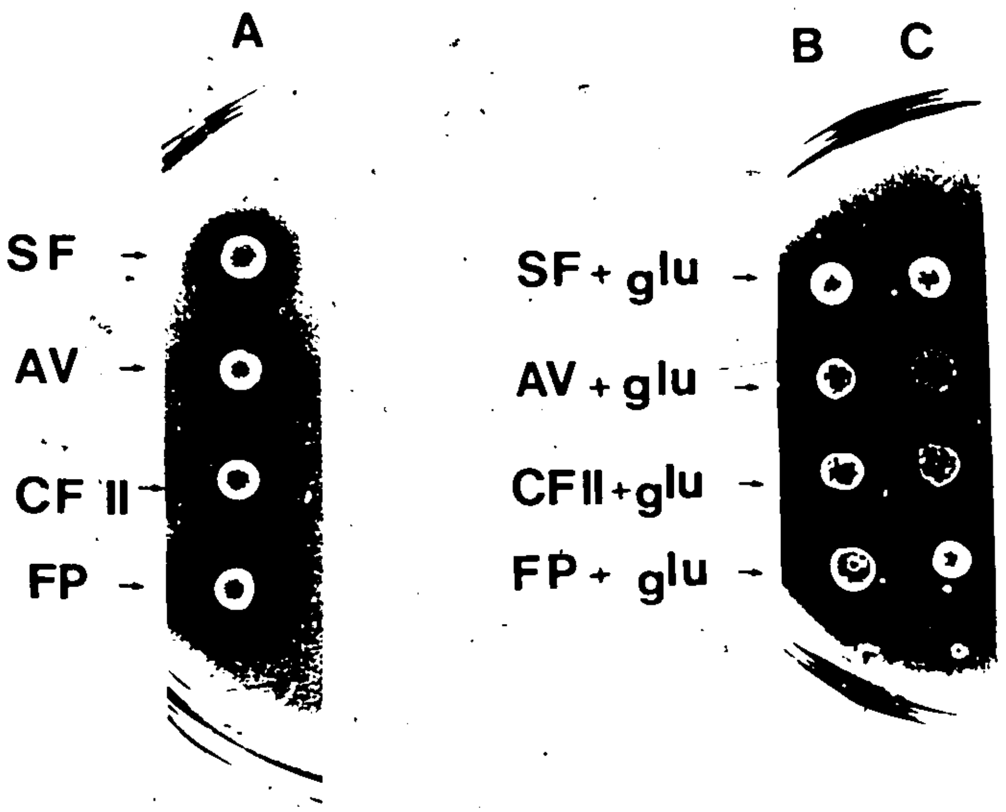
Fig. 15. Zones of degradation produced by cellulase enzymes of *Cellulomonas* 2406 on plates containing pretreated cellulose:

a) zones of degradation produced by cells grown in PTYE medium containing different celluloses

b) zones of degradation produced by cells grown in PTYE medium + glucose + different celluloses

c) zones of degradation produced by cells grown in PTYE medium containing different celluloses to which glucose (1%) was added just before plating.

Note: Both glucose and celluloses were added at a concentration of 1%.


A
B
C
SF
AV
CF II
FP
SF + glu
AV + glu
CFII + glu
FP + glu

4. End products produced by *Cellulomonas* 2406 grown in complex medium PTYE

Cellulomonas 2406 grew well in PTYE medium but grew slightly better when glucose was present in the medium (Table 9). When measuring the amount of glucose in the medium it was observed that 60% of the glucose present was utilized within three to four days (Fig. 16 A) and acetate (1.4 g/ℓ) and ethanol (0.2 g/ℓ) were produced as end products as shown by gas chromatography (Fig. 16 B). The utilization of glucose from the PTYE medium stopped at day four, probably because at that time *Cellulomonas* 2406 had entered stationary phase.

When cells were grown in PTYE medium containing different cellulolytic substrates (Fig. 17), cellobiose or carboxymethyl cellulose (Fig. 16 A), very little glucose was detected in the medium and no acetate or ethanol were detected by gas chromatography. These results suggest that when the cells were grown in PTYE medium containing cellulose the glucose that was produced was immediately utilized but the amounts of acetate and ethanol were too small to be detected. Furthermore, since only very little glucose was produced when *Cellulomonas* 2406 was grown on PTYE medium containing carboxymethyl cellulose (Fig. 16A) it suggests that cellobiose rather than glucose was probably the main end product of cellulose degradation at the time of sampling.

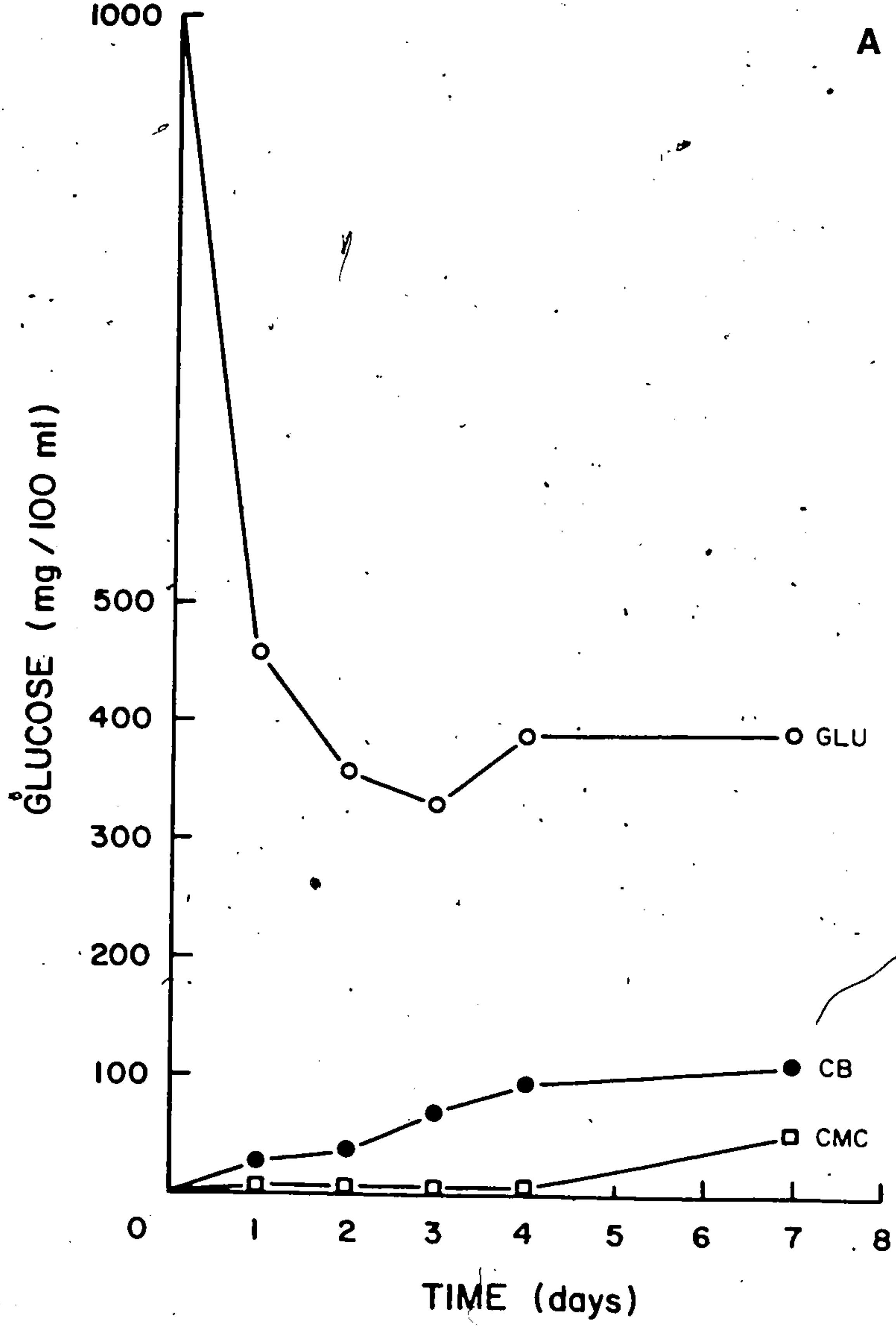
Fig. 16. A) The amount of glucose produced or utilized by *Cellulomonas* 2406 grown in PTYE medium containing:

a) glucose (GLU)

b) cellobiose (CB)

c) carboxymethyl cellulose (CMC)

B) Gas chromatography of a culture medium (day 4) obtained from *Cellulomonas* 2406 grown on PTYE medium plus glucose (1%).


A
1000
500
400
300
200
100
0
GLUCOSE (mg/100 ml)
1
2
3
4
5
6
7
8
TIME (days)
GLU
CB
CMC

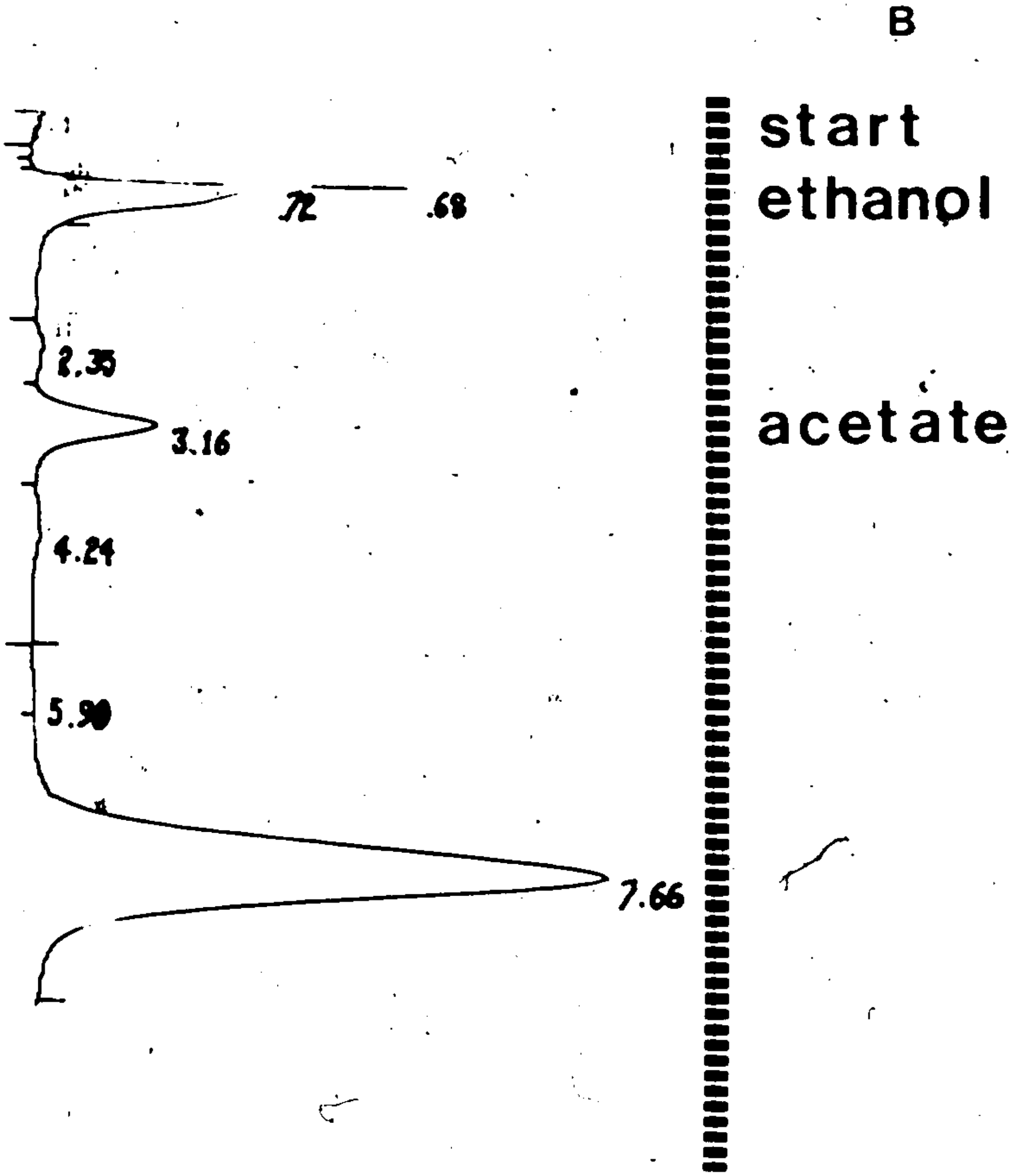
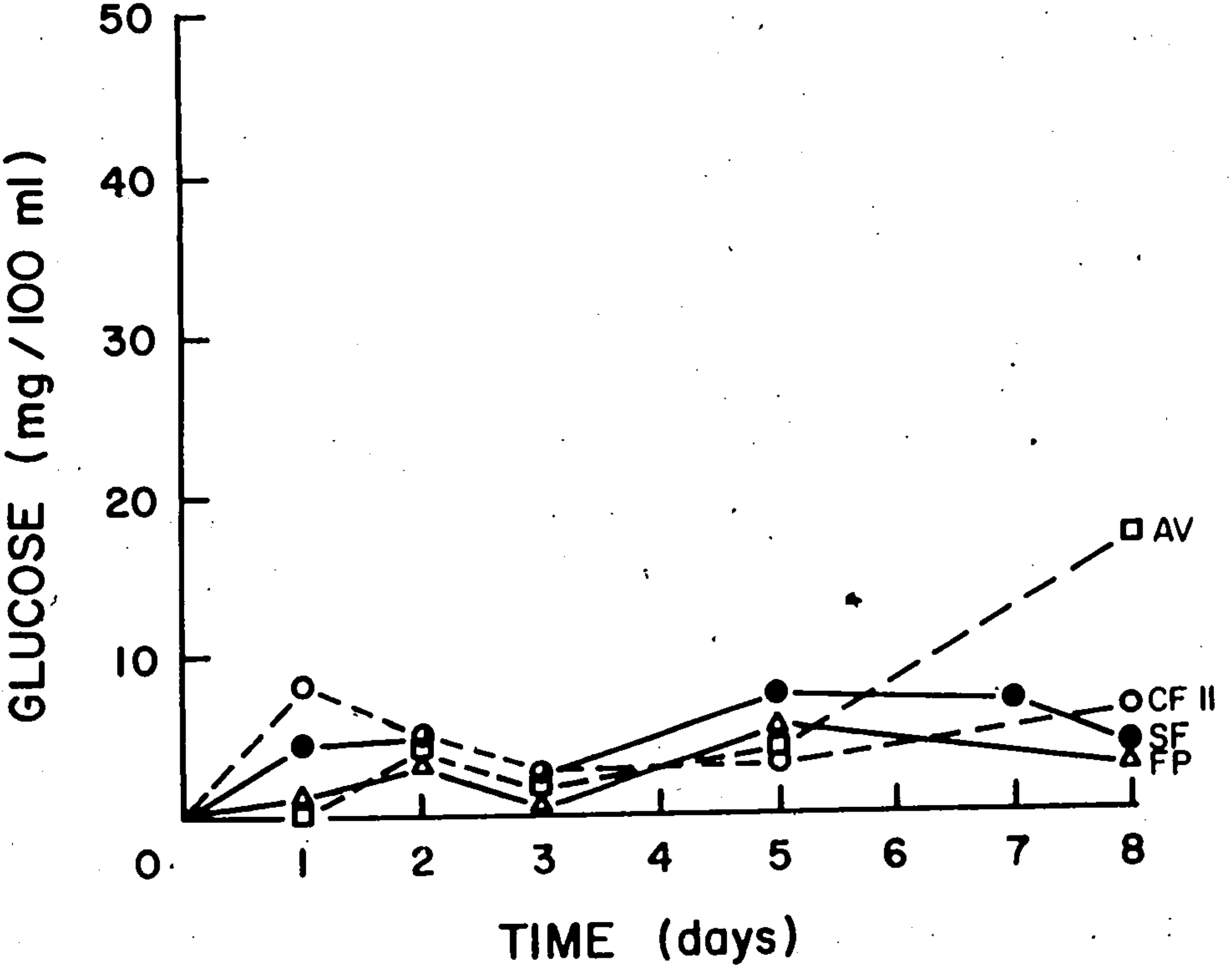

B
start
ethanol
acetate
.72
.68
2.35
3.16
4.24
5.90
7.66

Fig. 17. The amount of glucose released into the media by *Cellulomonas* 2406 grown in PTYE medium containing different culluloses as substrate:

a) AV = avicel
b) CF 11 = CF 11 cellulose
c) SF = solka floc
d) FP = filter paper


GLUCOSE (mg / 100 ml)
50
40
30
20
10
0
1
2
3
4
5
6
7
8
TIME (days)
AV
CF II
SF
FP

5. Location of cellulase enzymes in *Cellulomonas* 2406

a. Location of endo-β-1,4 glucanase (endocellulase)

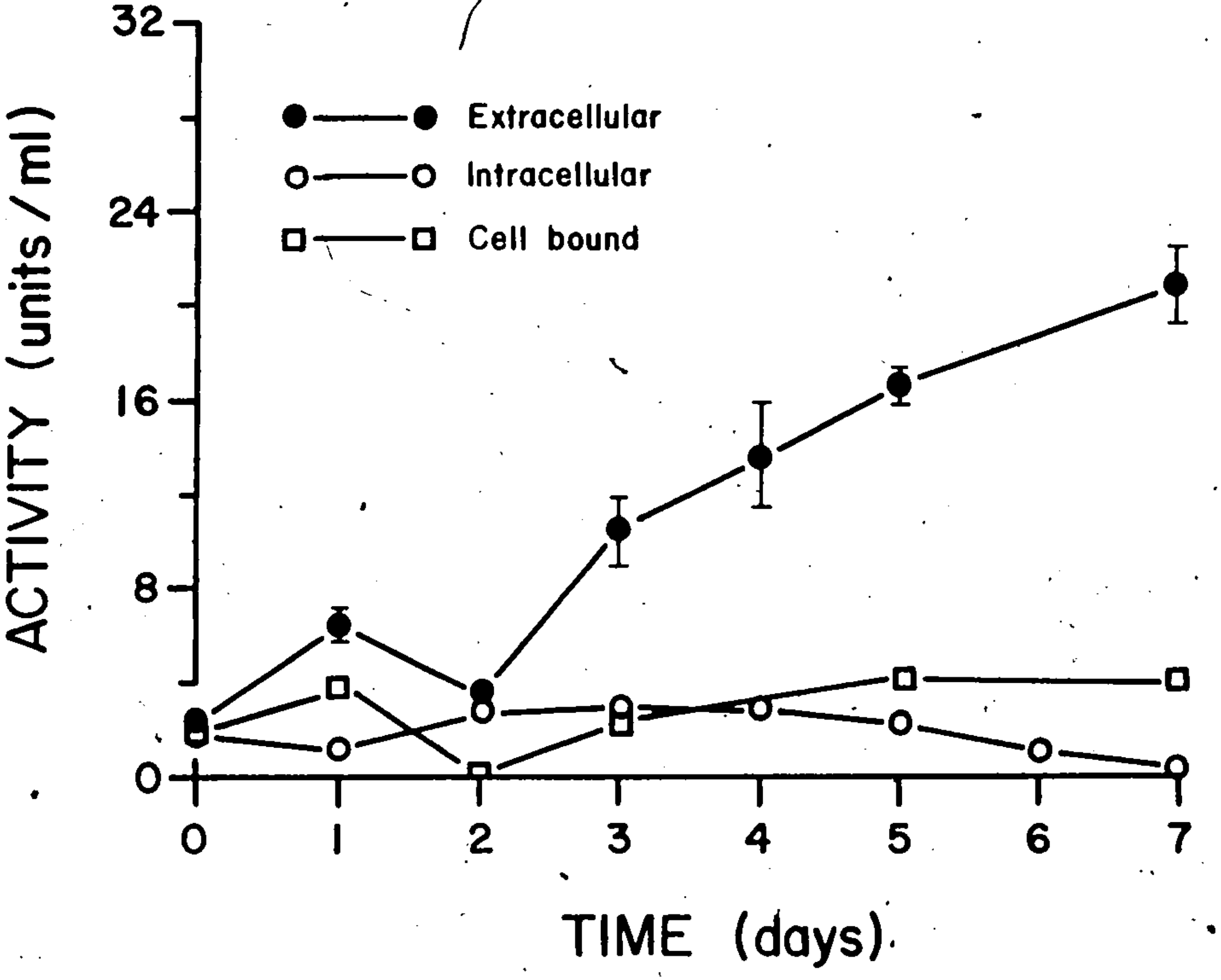
b. Location of β-glucosidase

Bacterial enzymes may be restricted to particular regions in the cells or secreted into the medium. The methods used to measure cellulase activities permitted one to investigate the location of the endocellulases and β-glucosidase enzymes. Unfortunately, there is no method for measuring exocellulase activity alone and therefore its location cannot be determined.

a. Extracellular location of endo-β-1,4 glucanase (endocellulase)

Fig. 18 shows the endocellulase activity in the intracellular, extracellular and cell-bound fractions of a culture of *Cellulomonas* 2406 grown in PTYE medium plus filter paper (1%). Extracellular activity was always higher than that associated with the intracellular or cell-bound fractions. Endocellulases were found to be located extracellularly in other bacteria such as *Cellvibrio vulgaris* (Oberkotter & Rosenberg, 1978), *Cellulomonas uda* (Nakamura & Kitamura, 1983) and a variety of *Bacillus* species (Priest, 1977). As can be seen from Fig. 18, the intracellular and cell-bound fractions contained similar and very small levels of endocellulases. This would indicate that the cell-bound and intracellular fractions were the same. These results also indicate that the level of cell associated enzyme is always very small, the enzyme being released extracellularly once formed.

Fig. 18. Location of endocellulase activity from *Cellulomonas* 2406 grown on filter paper. The activity was expressed as micrograms of reducing sugars (relative to glucose standard) per minute per milliliter of culture.



32
24
16
8
0
ACTIVITY (units / ml)
● Extracellular
○ Intracellular
□ Cell bound
0
1
2
3
4
5
6
7
TIME (days)

Cellulose is completely insoluble and cellulolytic microorganisms must therefore either form cell-free cellulases or have such enzymes located on the outside of the cell. It was important to show whether an enzyme found in the culture media was secreted by growing cells or passively released as a result of cell lysis. Experiments done on *Cellvibrio fulvus* suggested that the enzyme accumulates in the cells and it is then released after growth ceases during the process of cell lysis (Berg et al., 1972; Berg, 1975). *Cellulomonas* 2406 was found to be sensitive to a variety of antibiotics including chloramphenicol which inhibits protein synthesis by the 70s ribosomes (Lehninger, 1972). If the cells accumulate the enzyme it would be expected that the secretion phase would not be affected by the effect of chloramphenicol on protein synthesis. Fig. 19 shows that the synthesis was prevented by addition of chloramphenicol regardless of the time of addition, suggesting that de novo protein synthesis is required for continued secretion of endocellulases.

b. Location of β-glucosidase

In all previous experiments β-glucosidase was found to be low in the extracellular fraction. In the experiments performed on *Cellulomonas* 2406 it was found that β-glucosidase was cell-bound when cells were grown in medium containing insoluble cellulose (Table 11). When cells were grown in medium containing soluble cellulose most of the β-glucosidase was cell-bound except at day one when a larger amount of this enzyme was extracellular (Table 12).

Fig. 19. Effect of chloramphenicol (CAP) on synthesis and/or secretion of endocellulases.

Cellulomonas 2406 was grown in complex medium plus filter paper (1%) and at the assigned times CAP was added aseptically to a final concentration of 10 μg/ml. Flasks were incubated at 30°C.

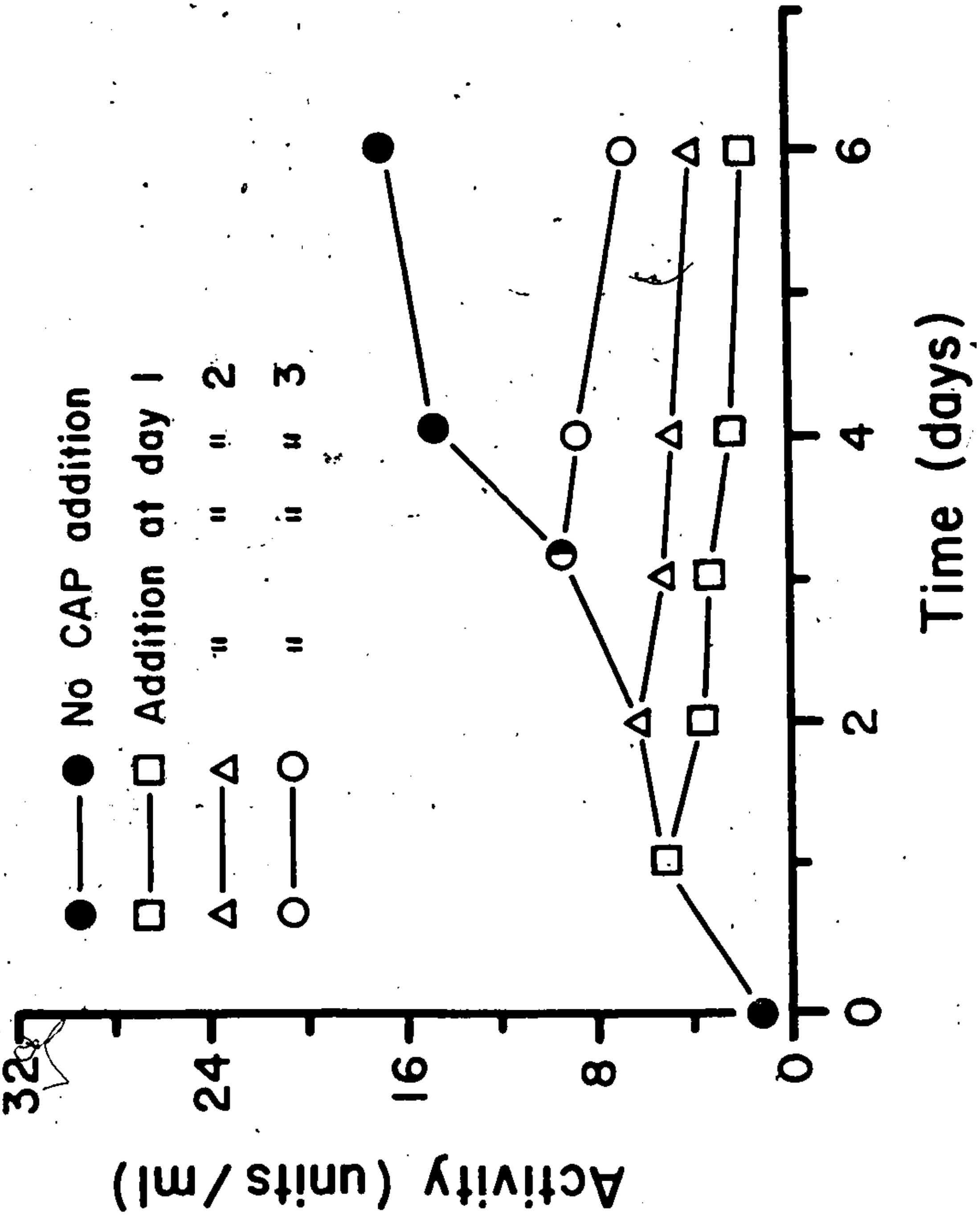

●—● No CAP addition
□—□ Addition at day 1
△—△ " " " 2
○—○ " " " 3
Activity (units / ml)
0
8
16
24
32
Time (days)
0
2
4
6

Table 11. β-glucosidase activity* in *Cellulomonas* 2406 grown in complex medium (PTYE) containing filter paper

Fractions	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Extracellular	0	0	1.50	0	1.2	1.8
Intracellular	0	0	–	0.5	0.55	0.60
Membrane bound	2.0	1.9	2.20	0.5	0	0

Table 12. β-glucosidase activity* in *Cellulomonas* 2406 grown in complex medium (PTYE) containing carboxymethyl cellulose

Fractions	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Extracellular	2.67	3	0	–	0	0
Intracellular	1.20	1.32	1.47	–	0	0
Membrane bound	0.73	3.43	2.50	–	0.60	0.53

*A unit of activity is defined as micrograms of reducing sugar (relative to a glucose standard) per minute per milliliter of culture.

6. Mutagenesis

Attempts were made to increase the rate of cellulose breakdown by *Cellulomonas* 2406 by producing mutants whose ability to degrade cellulose would not be inhibited by the end products (such as glucose) of cellulose degradation. The following mutagenic agents were tested: ultraviolet light (U.V.), methyl methanosulfonate (MMS) and N-Methyl-N'-nitro-N-nitrosoguanidine (NTG).

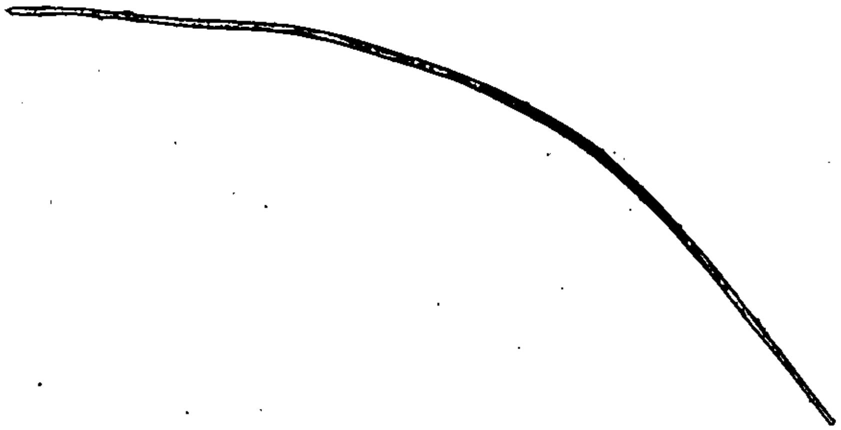
U.V. mutagenesis

U.V. has a killing effect on the *Cellulomonas* 2406 as can be seen from Fig. 20. *Cellulomonas* 2406 was grown in PTYE medium and was then exposed to U.V. light aiming for a 99% killing (5500 ergs/mm²). The irradiated cells were plated on a growth medium containing both cellulose and glucose after the U.V. treatment, or the irradiated cells were allowed to grow overnight and then plated as above. Even though U.V. had the potential to kill *Cellulomonas* 2406 cells, it did not mutate them in such a way as to obtain cellulose degradation in the presence of glucose (Table 13). The U.V. mutagenesis was repeated many times and no derepressed mutants were found.

Methyl methanosulfonate (MMS) mutagenesis

MMS spontaneously transfers alkyl groups to ring nitrogen atoms of the bases in the DNA (Lehninger, 1972). As can be seen in Table 14 no killing of *Cellulomonas* 2406 was observed.

Fig. 20. Survival curve for *Cellulomonas* 2406 using U.V. irradiation.



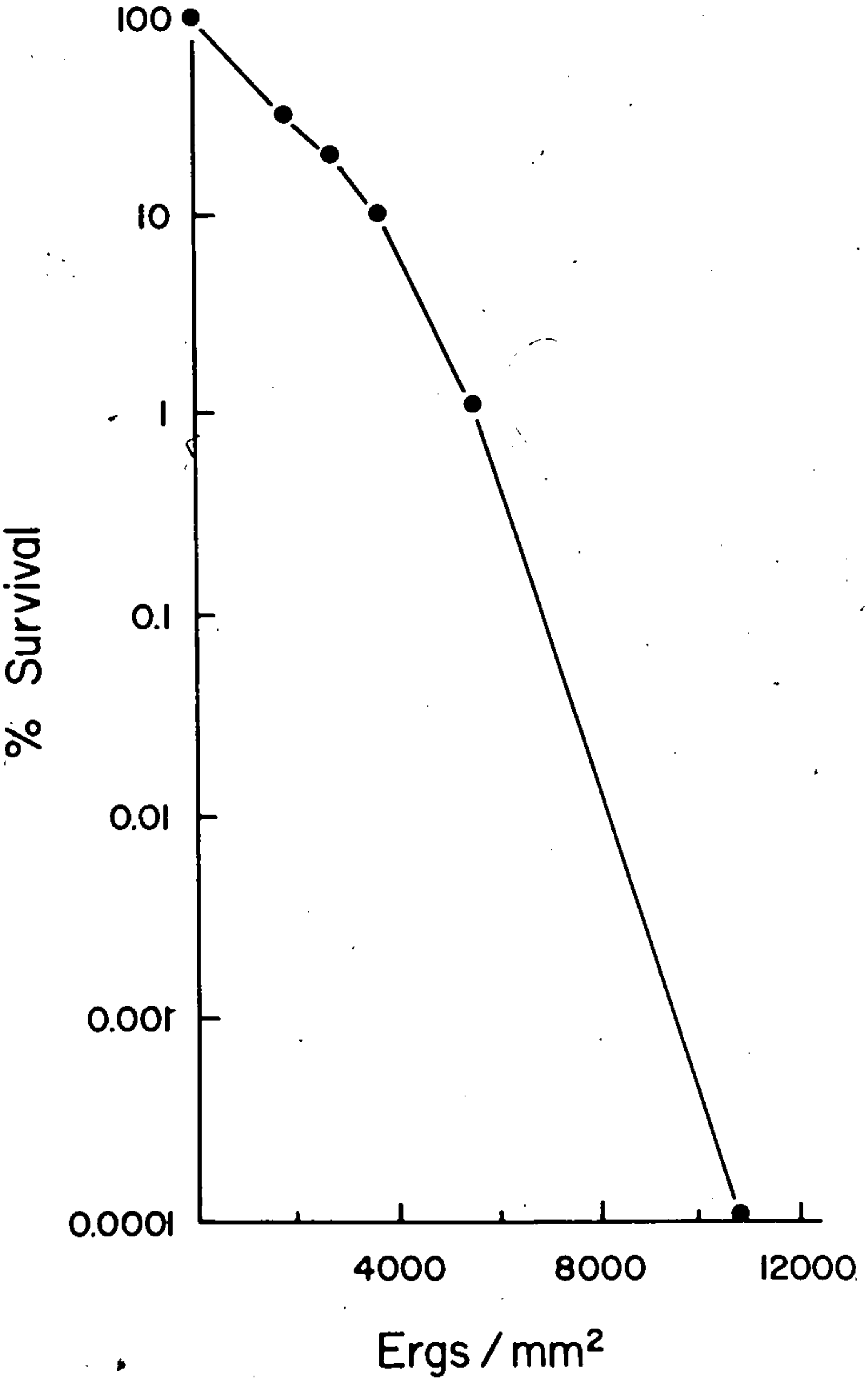

100
10
1
0.1
0.01
0.001
0.0001
% Survival
4000
8000
12000
Ergs / mm²

Table 13. The effect of ultraviolet light (U.V.) on the survival of *Cellulomonas* 2406.

Time U.V. exposure	Viable counts (cells/ml)			Colonies showing degradation
	Cells grown on PTYE plates	Cells grown on plates containing cellulose* after U.V. mutagenesis	Cells grown on cellulose* plus glucose (0.3%) plates after mutagenesis	
no U.V.	4.4×10^9			
60 sec.	5.3×10^7	8.5×10^7	8.0×10^7	0
75 sec.	1.7×10^6	4.1×10^6	4.7×10^6	0

* The cellulose used in these plates was pretreated CF 11 cellulose.

Table 14. The effect of MMS on the survival of *Cellulomonas* 2406.

MMS concentration (%)	Viable counts (cells/ml) *Cellulomonas* 2406
0	1.7×10^7
0.001	2.1×10^7
0.01	1.8×10^7
0.1	1.3×10^7

N-Methyl-N'-nitro-N-nitrosoguanidine (NTG) mutagenesis

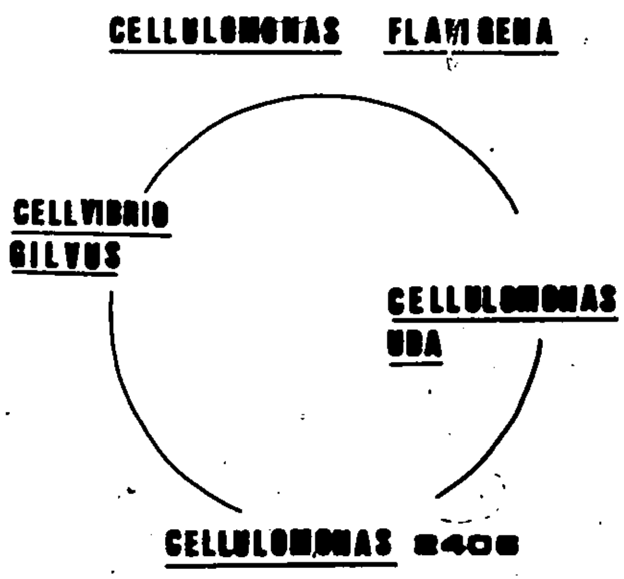
NTG is one of the most powerful mutagens known, and the most used for obtaining a variety of mutants. As can be seem in Table 15 NTG killed *E. coli* but not *Cellulomonas* 2406. Since we were not able to produce mutants in the usual manner with NTG (500 µgm/ml) an attempt was made to see what concentration of NTG would kill or prevent growth of *Cellulomonas* 2406. For this another method was used which involved making plates of PTYE medium containing NTG at different concentrations. Three different strains of *Cellulomonas* were plated out. As can be seen in Fig. 21 all strains grew at 0 and 10 µgm/ml NTG. At 100 µgm/ml NTG only *Cellulomonas uda* and *Cellvibrio gilvus* grew whereas at 250 µgm/ml NTG, only *Cellulomonas uda* showed any growth. At 500 µgm/ml NTG, no growth was obtained. Thus, although 30 min exposure to NTG did not kill *Cellulomonas* 2406, continuous exposure killed or inhibited the growth of these cells.

Table 15. The effect of NTG on the survival of *Cellulomonas* 2406 and *Escherichia coli*.

NTG concentration* (μg/ml)	Viable counts (cells/ml)	
	Cellulomonas 2406	*Escherichia coli*
0	8.2×10^7	6.8×10^7
100	7.3×10^7	2.9×10^6
300	7.0×10^7	-
500	7.5×10^7	1.7×10^5

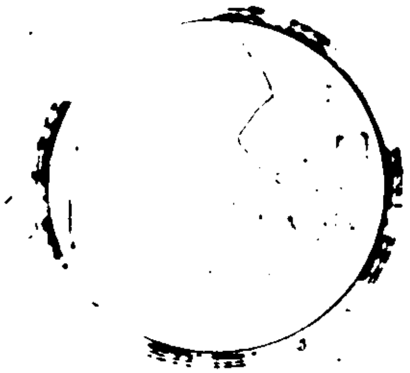
* The cells were exposed to each concentration of NTG for 30 minutes.

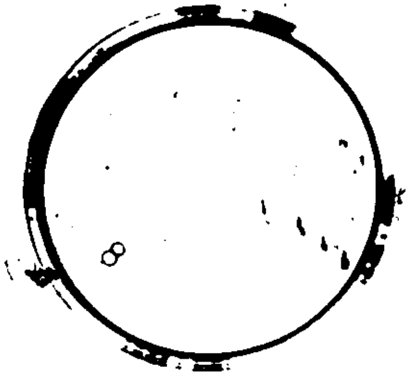
Fig. 21. The effect of NTG on the growth of three different strains of *Cellulomonas* and *Cellvibrio gilvus*.




0 µg/ml NTG


10 µg/ml NTG


100 µg/ml NTG


250 µg/ml NTG


500 µg/ml NTG

7. Detection and differentiation of cellulase components in polyacrylamide gels

The extracellular fluid from cells growing on PTYE medium with or without cellulose was concentrated by passage through an AMICOM filter and subjected to SDS gel electrophoresis.

When grown on cellulose as a carbon source, *Cellulomonas* 2406 secreted a set of enzymes into the culture medium, some of which are involved in cellulose hydrolysis. The different protein species were separated into several (more than 20) distinctly detectable bands (Fig. 22), a few of which were of glycoprotein character as observed by the Schiff staining procedure (Maddya, 1976). When the cells were grown in PTYE medium alone and the extracellular fluid was concentrated and then run on the gel fewer bands appeared, all of which were of high molecular weight (> 43000)[illegible] (Fig. 22). When the cells were grown in PTYE medium plus cellulose the bands varied between 24350 to over 94000 molecular weight. When the cells were grown in PTYE medium containing both cellulose and glucose fewer bands appeared, some of which were the same as observed from the cells grown in PTYE medium plus cellulose alone (Fig. 23). This suggests that the presence of glucose in the media containing cellulose represses the production of some proteins. Activity staining was performed after SDS was removed from the gels. This was achieved by incubating the gel in a buffer which is sufficient to renature some protein molecules (Beguin, 1983). Upon staining the replica gels with

Fig. 22. Protein stain of *Cellulomonas* 2406

Concentrated extracellular enzymes obtained from cultures of *Cellulomonas* 2406 grown in PTYE medium, were submitted to electrophoresis in the presence of SDS on a 15% polyacrylamide gel followed by staining for total protein.

Migration was from top to bottom.

Lane 1 Complex medium alone (no cellulose or cells added)

2 *Cellulomonas* 2406 grown in complex medium alone

3 *Cellulomonas* 2406 grown in complex medium containing 1% filter paper

4 *Cellulomonas* 2406 grown in complex medium containing 1% CF 11 cellulose

5 *Cellulomonas* 2406 grown in complex medium containing 1% avicel

6 *Cellulomonas* 2406 grown in complex medium containing 1% solka floc

7 Set of molecular weight markers (phosphorylase M.W.92500; bovine serum albumin M.W.68000; ovalbumin M.W.45000; carbonic anhydrase M.W.31000; soybean trypsin inhibitor M.W.21500; lysozyme M.W.14400) migrated together with the dye front.

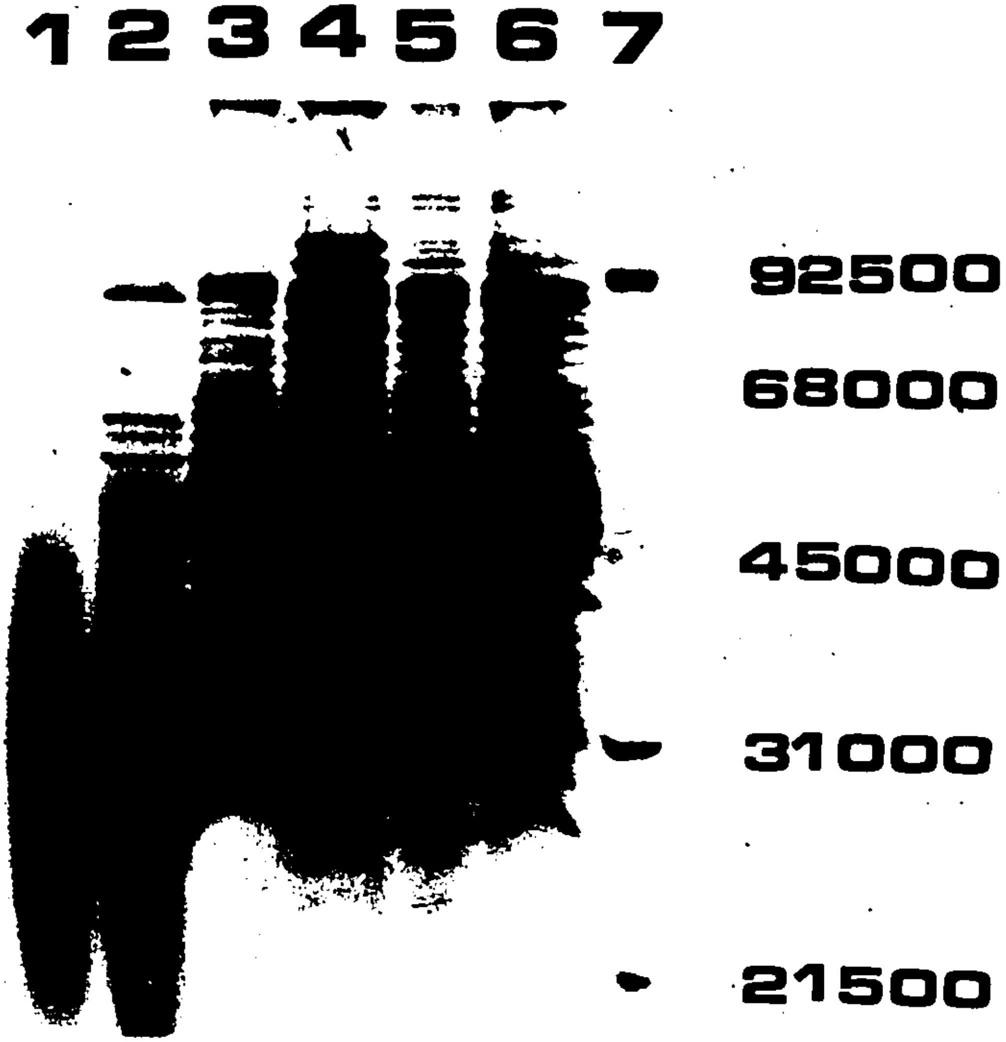

1 2 3 4 5 6 7
92500
68000
45000
31000
21500

Fig. 23. Protein stain of *Cellulomonas* 2406.

Concentrated extracellular enzymes obtained from cultures of *Cellulomonas* 2406 grown in PTYE medium containing cellulose and also from cells grown in complex medium plus cellulose supplemented with glucose (1%).

Lane 1 *Cellulomonas* 2406 grown in complex medium containing filter paper 1% and glucose 1%

2 *Cellulomonas* 2406 grown in complex medium containing filter paper

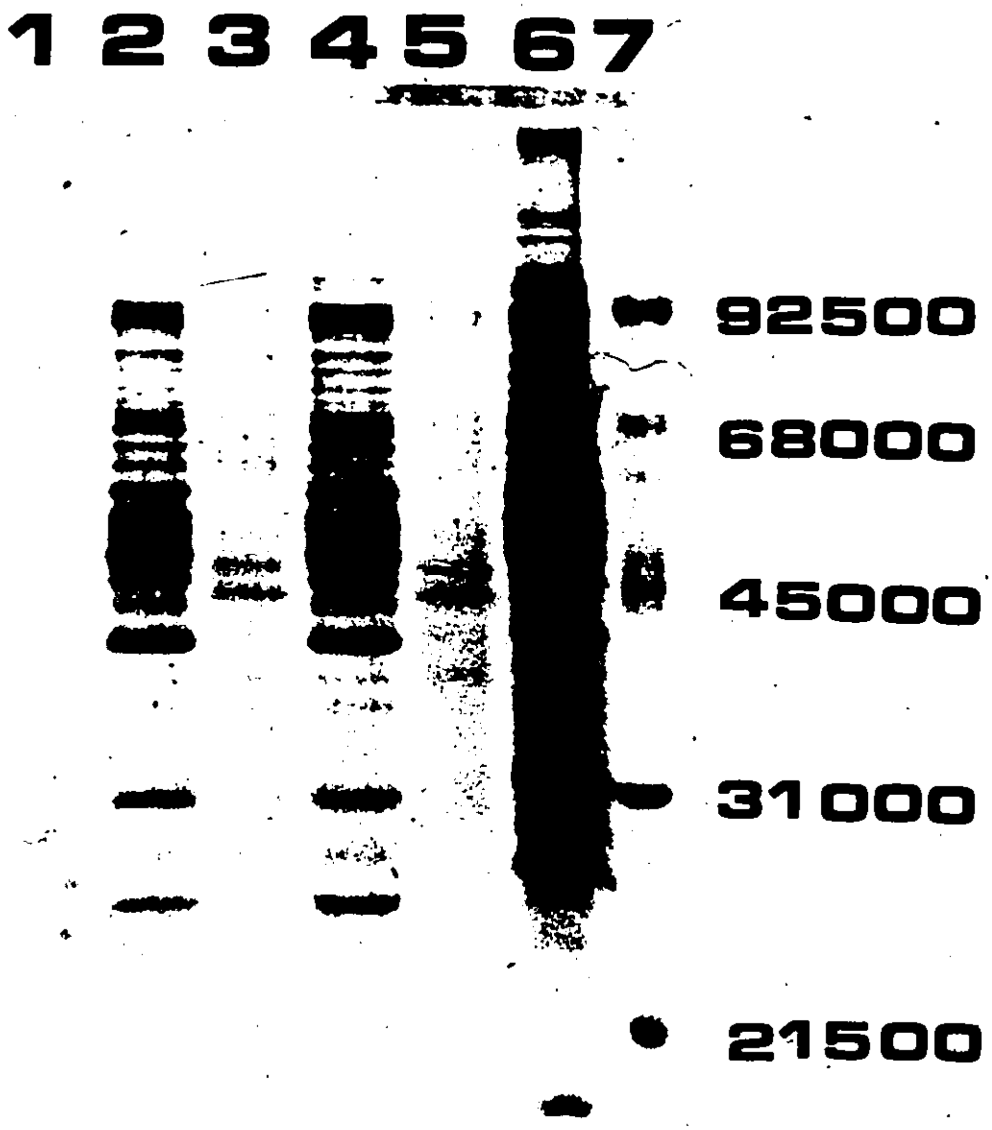
3 *Cellulomonas* 2406 grown in complex medium containing avicel 1% and glucose 1%

4 *Cellulomonas* 2406 grown in complex medium plus avicel

5 *Cellulomonas* 2406 grown in complex medium containing solka floc 1% and glucose 1%

6 *Cellulomonas* 2406 grown in complex medium containing solka floc

7 Set of molecular weight markers (same as in Fig. 18)


1 2 3 4 5 6 7
92500
68000
45000
31000
21500

Congo red, bands of degradation were observed. The activity profiles of these gels showed three bands which probably correspond to three endocellulases (Fig. 24). This test is specific for endocellulases due to the substrate that was used, carboxymethyl cellulose. The number of bands corresponding to enzymes involved in the degradation of cellulose is probably the minimum number since it is always possible that not all proteins renatured. Contamination by hexadecyl and tetradecyl sulfates in the SDS itself as well as the type of SDS being used can inhibit renaturation (Lack et al., 1979; Blank et al., 1982). The intensity of the activity bands varied, probably due to the efficiency of renaturation. Because of the question of renaturation of proteins in the SDS gel, non-denaturing gels were run. Fig. 25 shows the results obtained from such a gel. The activity band shows that there are at least five endocellulases present in the extracellular fraction. This confirmed the suspicion that not all proteins were renatured from the SDS gels. The non-denatured gels were also stained with 4-methylumbelliferyl-β-D-glucoside (MUG) which helps to visualize bands of β-glucosidase activity. Under shortwave U.V. light, one band of weak activity was seen. This suggests that there is a small amount of β-glucosidase enzyme in the extracellular fraction.

Fig. 24. Activity staining of *Cellulomonas* 2406 cellulases. *Cellulomonas* 2406 was grown in PTYE medium containing solka floc (1%).

Lane 1 Activity staining for day 4 culture
2 Activity staining for day 7 culture
3 Activity staining for day 9 culture

(Each sample was run three times)

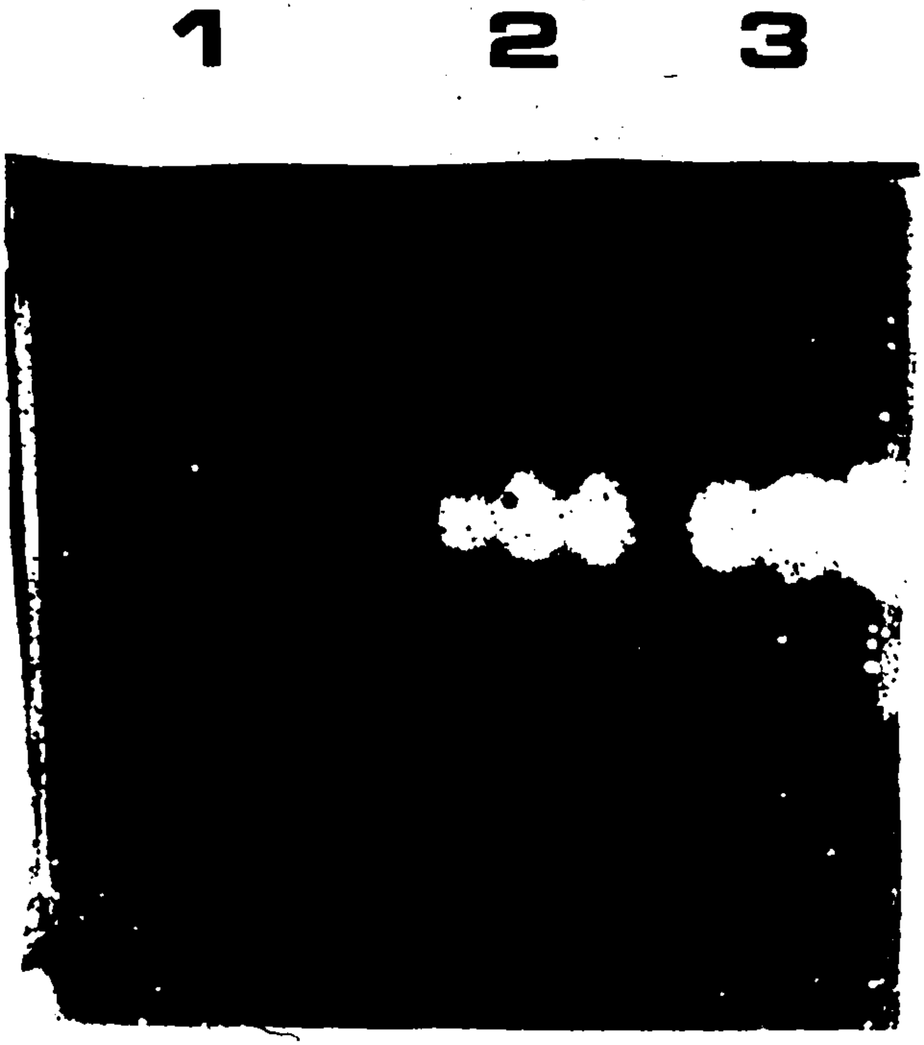

1
2
3

Fig. 25. Protein and activity stain of *Cellulomonas* 2406 cellulases. Cells were grown in PTYE medium containing different cellulose substrates.

Lane 1 Activity staining for cells grown in PTYE plus solka floc 1%

2 Protein staining for cells grown in PTYE plus solka floc 1%

3 Protein staining for cells grown in PTYE plus avicel 1%

4 Protein staining for cells grown in PTYE plus CF 11 cellulose 1%

5 Protein staining for cells grown in PTYE plus filter paper 1%.


1
2
3
4
5

8. Adhesion of cells to cellulose

When cells were grown in media containing cellulose, clumping was evident. As already pointed out, this clumping was associated with a decrease in viable counts during growth in the presence of cellulose. To determine if such clumping was due to attachment of cells to cellulose and to determine if such attachment is necessary for the onset of cellulase production, several experiments were performed.

Initially a system using a filter candle was used to separate cells from the cellulose fibers and endocellulase activity was measured in the culture media. Under these conditions endocellulase activity was very low as compared to the activity found when cells and cellulose were not separated (Table 16), suggesting that contact between the cells and cellulose fibers might be necessary for induction of cellulases. Further studies were conducted by electron microscopy. When cells were grown in PTYE medium containing cellulose one can see in the electron micrograph that a fibrillar material is present between the cells and the cellulose and between the cells themselves (Fig. 26). Ruthenium red staining suggests that the fibrous material associated with the bacteria is composed of acid mucopolysaccharide. Many rumen bacteria are known to have a thick surface coating when they are involved in cellulose degradation (Dinsdale et al., 1978). Also this thick region around the cells was seen in cultures of *Clostridium thermocellum* (Wiegel and Dykstra, 1984). When cells were grown in PTYE medium alone, this thick coating was not

Table 16. Endocellulase activity in the supernatant of 7 day cultures of *Cellulomonas* 2406 when cells were in contact or were not in contact with the cellulose fibers.

Media	endocellulase activity (units/ml)
PTYE + SF*	21.33
PTYE + AV*	19.07
PTYE + SF**	5.34
PTYE + AV**	6.20
PTYE***	5.80

* Cells grown in PTYE medium containing solka floc or avicel

** Cells grown in PTYE medium with solka floc or avicel contained in a porcelain candle filter

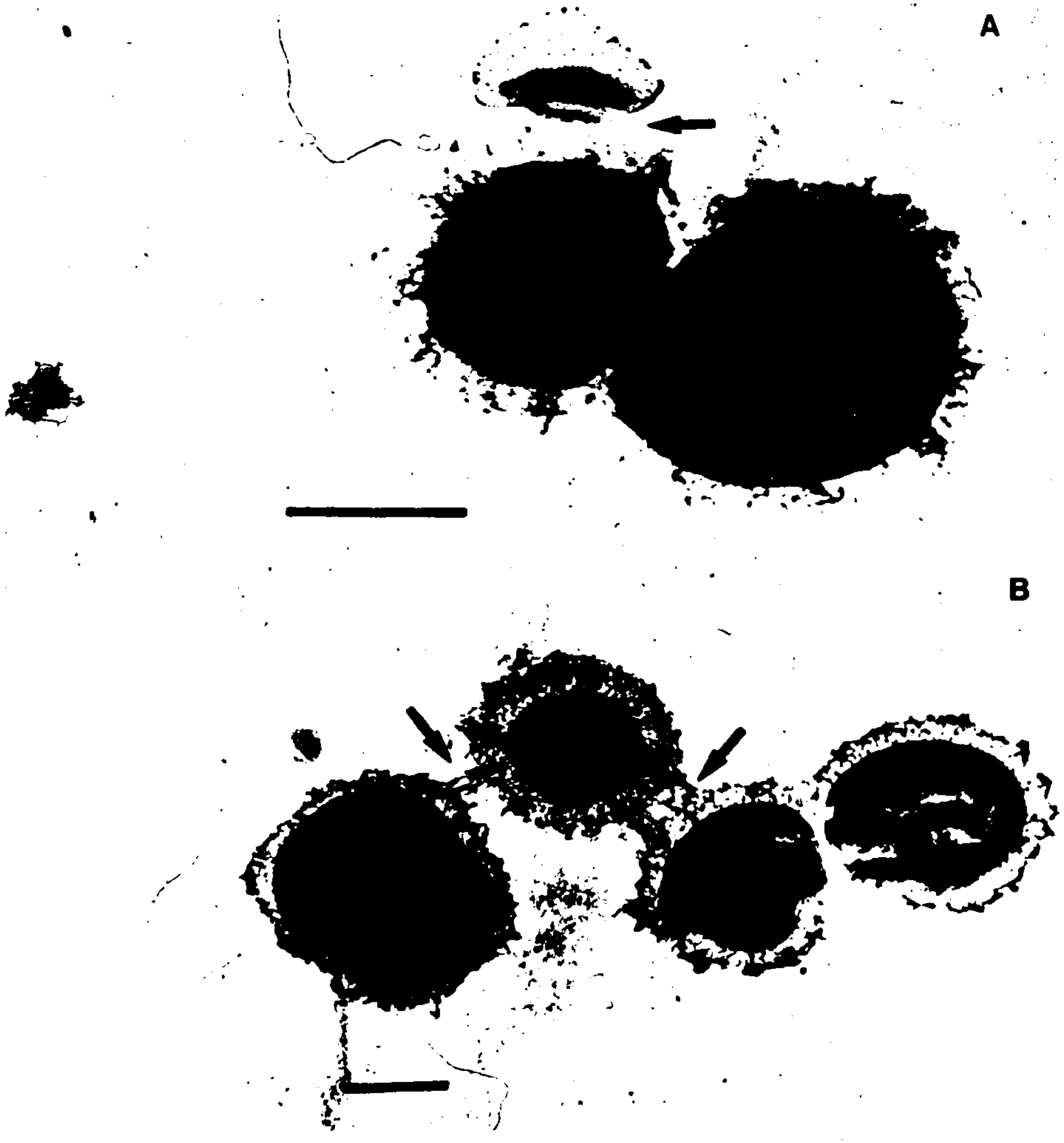
*** Cells grown in PTYE medium without cellulose or filter candle.

Fig. 26. Electron micrograph of *Cellulomonas* 2406 grown in PTYE medium containing filter paper. Arrow points

A) at the fibrous material between cell and the cellulose fiber

B) at the fibrous material between the cells. Preparation stained with ruthenium red.

The bars represent 0.5 µm.


A
B

present and the cells were mainly single (Fig. 27). The association of cells and cellulose fibers was also seen in light microscopy studies (Fig. 28). Cells grown in PTYE medium containing CF 11 cellulose, avicel or filter paper appeared inside the cellulose fibers (Fig. 28). Light microscopy studies also showed that cells grown in PTYE medium containing cellulose aggregated together forming a network with the cellulose fibers trapped inside (Fig. 29). This network is probably formed by the cells attaching to each other.

When the cells were grown in cultures containing cellulose plus glucose no clumps were seen in the media. Cells grown under these conditions did not form such a network but rather formed small groups of cells or remained as single cells in the media (Fig. 30). Light micrographs also showed that no degradation of cellulose took place when glucose was present. The size of the fibers of avicel remained large after 4 days of growing cells in PTYE medium containing avicel plus glucose compared to the size of the fibers of avicel after 4 days of growing in PTYE medium plus avicel alone (Fig. 31).

Another way to study if contact between cells and cellulose was necessary for cellulose degradation was by growing the cells on plates containing cellulose overlayed with a thin layer of pure agar which kept cells and the cellulose apart and looking for the appearance of degradation haloes (Kauri & Kushner, 1985). The degradation zones of these plates were actually larger than those obtained when no agar layer was present (Fig. 32 a, c and d, e).

Fig. 27. Electron micrograph of *Cellulomonas* 2406 grown in PTYE medium alone. Arrow points to the cell wall. Notice the absence of this fibrous material around the cell. This preparation was stained with ruthenium red. The bar represents 0.5 μm.

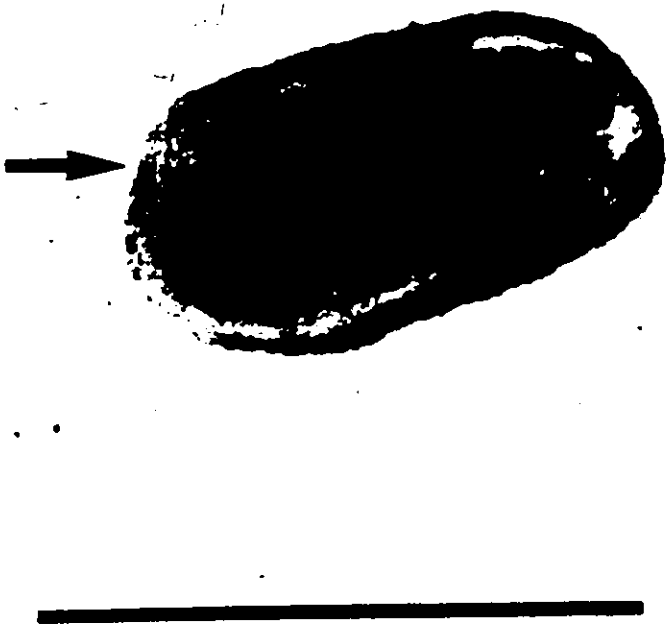


Fig. 28. Light micrograph of cellulose fibers with adherent *Cellulomonas* 2406.

A) cells adherent to CF 11 cellulose fibers

B) cells adherent to avicel fibers

C) cells adherent to filter paper fiber.

Magnification 40x.


A
B
C

Fig. 29. Light micrograph of *Cellulomonas* 2406 grown in PTYE medium containing CF 11 cellulose.

Note the long strands of cells entrapping the cellulose fibers.

A) 40x magnification

B) 100x magnification.


A
B

Fig. 30. Light micrograph of *Cellulomonas* 2406 grown in PTYE medium containing CF 11 cellulose (1%) and glucose (1%).

Magnification 40x.

Fig. 31. Light micrograph of *Cellulomonas* 2406 grown in
A) PTYE medium plus glucose (1%) plus avicel (1%)
B) PTYE medium plus avicel (1%)
Pictures were taken at day 4 at 40x magnification.

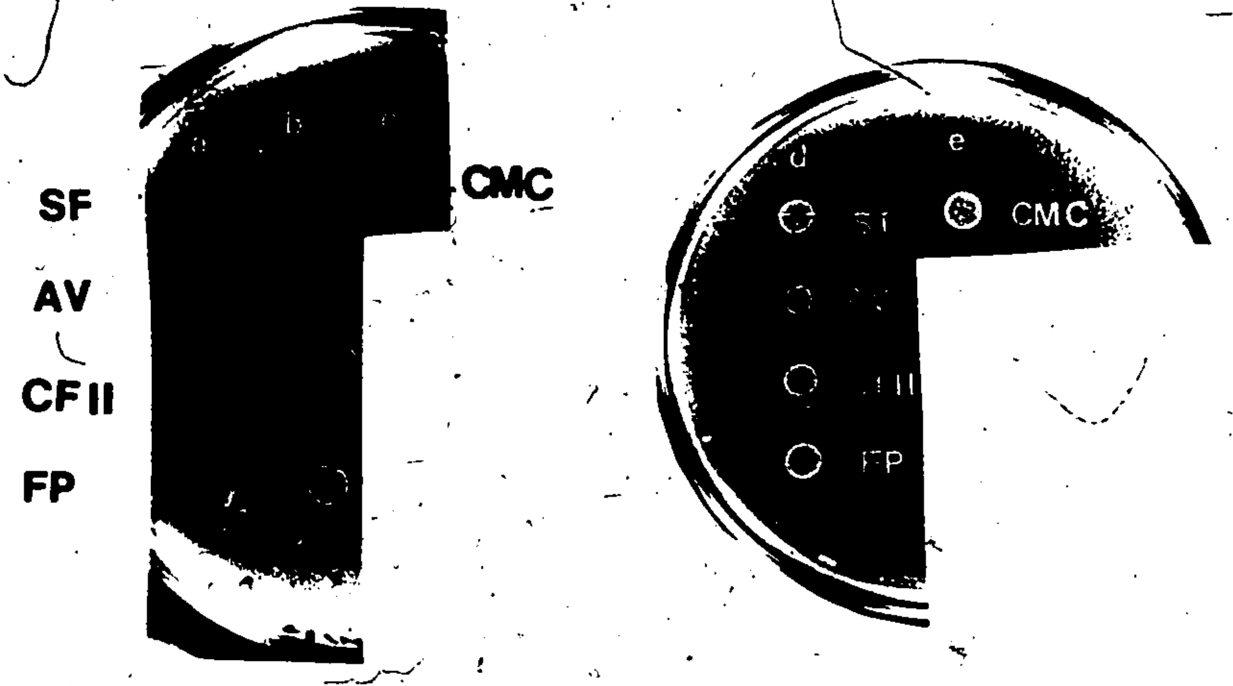

A
B

Fig. 32. Zones of degradation produced by *Cellulomonas* 2406 grown on PTYE medium containing different cellulose substrates: SF = solka floc, AV = avicel, CF 11 = CF 11 cellulose, FP = filter paper.

a) & c) Degradation zones produced on a plate containing pretreated cellulose by cells grown on PTYE medium containing different celluloses.

b) Degradation zones produced on a plate containing pretreated cellulose by cells grown on PTYE medium containing different celluloses (1%) and glucose (1%).

d) & e) Degradation zones produced on a plate containing pretreated cellulose overlayed with pure agar by cells grown on PTYE medium plus different celluloses.


a
b
c
SF
AV
CF II
FP
CMC
d
e
SF
CMC
CF II
FP

GENERAL DISCUSSION

Cellulomonas 2406 is a cellulose degrader capable of producing all the enzymes involved in the cellulase complex. For growth it required yeast extract as a supplement to the minimal media used. A similar result was found by Choi et al., (1978) for *Cellulomonas* CSI-1 and by Antheunisse (1984) for *Cellulomonas flavigena* ATCC 484. Rajoka and Malik (1984) also grew *Cellulomonas flavigena* NIAB 441 in minimal media containing yeast extract. The yeast extract concentration did not interfere with the determination of growth responses or with the determination of cellulase activities (Choi et al., 1978).

When grown in basal medium no. 4, *Cellulomonas* 2406 produced only endocellulases in measurable amounts as previously shown for *Cellulomonas* CSI-1 by Choi et al. (1978). *Cellulomonas* 2406 was also able to grow when the basal medium was supplemented with glucose, cellobiose or glycerol but grew best when cellulose was added to the medium. When grown in basal medium no. 4 plus solka floc, *Cellulomonas* 2406 exhibited the highest endocellulase activity. This was probably due to the fact that solka floc is the least crystalline cellulose fiber which was used. When residual cellulose was measured in the basal medium, solka floc and filter paper were degraded more quickly whereas avicel and CF 11 cellulose were degraded more slowly. When glucose was added to the medium containing cellulose very little endocellulase activity was detected (Table 7). To explain a similar phenomenon in *Cellulomonas flavigena* ATCC 482, Thayer et al. (1984) have suggested that when glucose was added to the medium, the lack of endocellulase activity was not due to the inhibition of endocellulases but rather to the inhibition of β-glucosidase by glucose which in turn could produce a high level of cellobiose in the medium which either inhibits or possibly represses the synthesis of endocellulases. Experiments conducted in the basal medium no. 4 have shown that β-glucosidase was not present at all in the culture medium (Table 8) as was also found for *Cellulomonas* CSI-1 (Choi et al., 1978). *Cellulomonas* 2406 was able to grow in the basal medium containing cellobiose or glucose and a small amount of glucose was detected in the medium when cellobiose was the only source of carbon. The endocellulase activity obtained for *Cellulomonas* 2406 in the basal medium no. 4 plus different celluloses were lower than those obtained when grown in PTYE and various celluloses. This could be probably due to the difference in the amount of growth. The PTYE medium was considered a better medium for growth of *Cellulomonas* 2406 as well as for the production of cellulases and thus further experiments were conducted using this medium. Growth of *Cellulomonas* 2406 was measured up to 9 days by protein determination (Table 9) in the PTYE medium alone or with the addition of different cellulose substrates with or without glucose. When cellulose was present in the PTYE medium *Cellulomonas* 2406 grew better than on the PTYE medium alone. When glucose was added to the PTYE medium containing cellulose, growth was slightly depressed even though *Cellulomonas* 2406 grew better in PTYE medium plus glucose than it did in PTYE medium alone. Growth measured by DNA determination showed a similar response to different culture conditions as did growth measured by protein. For all cellulose substrates when cells were grown in PTYE medium, endocellulase activity was the highest followed by exocellulase (filter paper activity) and β-glucosidase. The highest endocellulase activity was obtained when the cells were grown on CF 11 cellulose and the lowest on filter paper. Our experiments have shown that all cellulosic substrates tested were degraded but the rate of degradation differed from one substrate to another. Cultures grown on CF 11 cellulose had reached the highest endocellulase activity at day 3 rather than at day 6 or 7 as was found for all other cellulosic substrates. CF 11 cellulose was degraded the least. CF 11 cellulose degradation seems to be different probably due to a different crystalline structure. Comparing the figures for growth with the cellulase activities obtained, one can see that activity of endocellulases increased after the phase of exponential growth and even during stationary phase of growth, with the exception of cells grown on CF 11 cellulose, whose endocellulase activity decreased during stationary phase. Measurements of residual cellulose (Fig. 15 and Fig. 8) showed that glucose, a catabolite repressor and an end product inhibitor, prevented cellulose breakdown both in PTYE and basal media.

When cells were grown in PTYE medium little activity was found in the intracellular and membrane bound fractions of broken cells. β-Glucosidase was highest in the cell-bound fraction while endocellulase activity was highest in the extracellular fraction. Since a very low cell-bound endocellulase activity was found, it was considered to be the basal endocellulase level of the cells (Berg, 1975). β-Glucosidase of other organisms was found to be cell-bound; *Cellulomonas uda* and *Cellulomonas flavigena* have cell-bound β-glucosidases (Stoppok et al., 1982; Rajoka & Malik, 1984; Antheunisse, 1984). Other microorganisms such as *Cellulomonas fimi* have a strictly intracellular β-glucosidase (Wakarchuk et al., 1984). The cell-bound β-glucosidase activity was quite low, and this leaves open the possibility that other enzymes such as cellobiose phosphorylase was found to be present in *Cellulomonas flavigena* DSM involved in the breakdown of cellobiose to glucose. Cellobiose phosphorylase was found present in *Cellulomonas flavigena* DSM 20109 (Schmiz et al., 1983). Cellodextrin phosphorylase was found in *Clostridium thermocellum* (Alexander, 1972, Alexander, 1968). In comparison with *Cellulomonas* species other microorganisms have cytoplasmic β-glucosidases (Hwang & Suzuki, 1976; Ramasamy & Verachtest, 1980; Yamane et al., 1971; Ait et al., 1979). The low extracellular β-glucosidase value was probably due to the release of enzymes into the medium during cell autolysis.

It was also found that the whole culture had 20-40% more endocellulase activity than the supernatant, depending on the time of growth. The difference may be due both to enzyme absorbed (Lee et al., 1982) on the cellulose and enzyme associated with the cells. The smallest difference between the endocellulase activity in whole cultures and the endocellulase activity of the extracellular fraction was observed when *Cellulomonas* 2406 was grown in PTYE medium plus filter paper (Fig. 13). This might be due to the fact that filter paper is more crystalline than solka floc and thus less enzyme could be absorbed onto it since cellulases usually prefer to be associated within the amorphous regions of the fibers (Paralikar & Bhatawdekar, 1984; Weimer & Weston, 1985).

When *Cellulomonas* 2406 was grown in PTYE medium plus glucose, ethanol and acetate were produced as end products, but only glucose was produced when *Cellulomonas* 2406 was grown in PTYE medium or in basal medium plus different celluloses. Since the amount of glucose produced was low when *Cellulomonas* 2406 was grown in PTYE medium plus carboxymethyl cellulose, this suggests that cellobiose rather than glucose was the main end product of cellulose degradation. This would not be surprising since Stoppok et al. (1982) had also found that cellobiose was the major end product of *Cellulomonas uda*. Kilian et al. (1983) showed that *Candida wickrhamii* utilized first glucose and then cellobiose from the medium. A similar diauxic utilization of glucose-cellobiose could be employed by *Cellulomonas* 2406 since the amounts of glucose measured in the PTYE medium plus different celluloses were always low. Such mechanism would ensure that glucose is used preferentially once produced. Since the amount of cellobiose present in the PTYE medium at the time was not measured, this phenomenon has not been actually demonstrated for our bacteria.

Induction of cellulase formation was studied in *Cellulomonas* 2406 and it was found that all celluloses tested induced the production of endocellulases even when present in an already nutritionally rich medium. The endocellulases present in the PTYE medium are induced enzymes as was previously shown also for *Cellvibrio gilvus* and other cellulolytic microorganisms (Breuil & Kushner, 1976; Chang & Thayer, 1977; Gong & Tsao, 1979). It has been postulated that the induction of cellulases is brought about by a constitutive enzyme (Mandels, 1982) which would split off small units from the cellulose chain which in turn would enter the cell and stimulate the production of more enzymes (Gong & Tsao, 1979). If this were true the constitutive enzymes would be an endocellulase since it was found membrane bound and intracellularly in almost constant amounts throughout the experiment.

Catabolite repression of inducible and/or constitutive enzymes plays a major role in the regulation of cellulases. Endocellulases were repressed by glucose and cellobiose as previously observed for other *Cellulomonas* species (Choi et al., 1978; Beguin & Eisen, 1977; Stewart & Leatherwood, 1976) but the mechanism of induction or catabolite repression still remains unclear (Gong & Tsao, 1979).

The endocellulases were further investigated by using SDS-polyacrylamide gel electrophoresis. When *Cellulomonas* 2406 was grown in PTYE medium containing cellulose more than 20 distinctly detectable bands appeared on the SDS polyacrylamide gels. When the cells were grown in PTYE medium containing both cellulose and glucose fewer bands appeared, some of which were the same as observed for the cells grown in PTYE medium plus cellulose alone. Therefore the presence of glucose repressed the production and/or secretion of many proteins. Activity staining of renatured proteins from the SDS polyacrylamide gels have shown that some of these proteins were endocellulases. The major activity bands were produced by proteins with molecular weights around 68000, 43000 and 37000. Our results were similar to those obtained by Thayer et al. (1984) who partially purified an endocellulase from *Cellulomonas flavigena* ATCC 484 with a molecular weight of 40000, and to the results obtained by Nakamura and Kitamura (1983) who purified an endocellulase with a molecular weight of 66000. It is not clear if our enzymes or the ones previously described are different endocellulases or if they are derived from a common precursor. Activity staining of non-denaturing gels had shown that more endocellulase bands were present but their molecular weights have not been determined. To determine the molecular weight of biologically active endocellulases one would have to run an acetyltrimethylammonium bromide-polyacrylamide gel electrophoresis by which both molecular weight and visualization of activity would be obtained (Akin et al., 1985).

Cellulomonas 2406 was found to be quite a slow degrader of cellulose and therefore it was desired to increase the rate of cellulose breakdown by this microorganism. Production of mutants, the formation of whose cellulases would not be inhibited by the end products of cellulose hydrolysis, was attempted. Choi et al. (1978) and Haggett et al. (1979) were able to obtain such mutants of *Cellulomonas* CSI-1 by using NTG and ultraviolet irradiation. Mutants obtained by treating the parent strain with 150 μg/ml NTG degraded avicel much faster. Upon treating these mutants with ultraviolet light they obtained even faster degradation of avicel. Stewart & Leatherwood (1976) also obtained mutants of *Cellulomonas* sp. by treating the parent strain with 300 μg/ml NTG. Their mutants were able to degrade cellulose in the presence of higher concentrations of glucose than could the parent strain. Similar treatment of *Cellulomonas* 2406 with NTG at concentrations up to 500 μg/ml did not affect the bacteria at all. NTG only killed or inhibited growth of *Cellulomonas* 2406 on prolonged exposure.

Ultraviolet treatment also did not produce any mutants. Our results suggest that *Cellulomonas* 2406 is more resistant to conventional mutagenic agents. Possibly a combination of NTG and ultraviolet light treatment could have been more effective in obtaining mutants. Successful mutagenesis has been done on fungi such as *Sporotrichum pulverulentum* whose cellulase activities were made to reach and surpass those of *Trichoderma reesei* QM 9414 (Eriksson & Johnsrud, 1983). Mutants of *Alternaria alternata* were also produced. These mutants were able to grow and produce increasing yields of endocellulases and β-glucosidase activity when growing in glucose broth (1% glucose) supplemented with 2% (w/v) wheat bran (Macris, 1984). None of the mutants obtained from the parental strains of the genus *Cellulomonas* were capable of producing cellulases as high as those reported for fungi.

When cells were grown in PTYE medium containing different celluloses, clumping was evident, and this phenomenon interfered with the viable counts. Upon examination by electron microscopy it became evident that these clumps were formed due to the association of the cells with the cellulose fibers. These studies showed that *Cellulomonas* 2406 produces a fibrous material composed most probably of acid mucopolysaccharide. This fibrous material was also seen to connect cells to each other. Many rumen bacteria are known to have a thick surface coating when involved in cellulose degradation (Lathamen et al., 1978; Dinsdale, 1978). In the case of rumen bacteria, attachment is important in achieving an efficient digestion (Akin, 1976). Bacteria such as *Cellvibrio fulvus* would only digest cotton fibers within the rumen when the bacteria became physically trapped (Akin, 1976). When *Cellulomonas* 2406 was grown in PTYE medium without cellulose, no fibrous material was present suggesting that the bacteria might lose the glycocalyx in the absence of cellulose and the presence of a rich medium such as PTYE. Many microorganisms are known to attach to the nutritive substrate by means of their glycocalices (Akin & Amos, 1975; Patterson et al., 1975; Hungate, 1966). Whether the attachment of cells to cellulose fibers is necessary for cellulase production is not yet clear. Viable counts would suggest that a large number of cells attach to cellulose or at least clump together during the first 24 hours of growth.

Light microscopy studies showed that cells actually attach to each other forming large masses and trapping the cellulose fibers. They also show that cells attach to the cellulose fibers. The study done by Berg et al. (1972b) suggests that contact between the cells and cellulose is necessary for cellulase induction. Our study shows that even though cells attach to the cellulose, the majority remain connected to each other suggesting that these cells are probably induced to produce cellulases by small oligosaccharides released from the cellulose chain by previously induced cells. The previously induced cells could be those which were found attached to the cellulose fibers. When glucose was present in the medium together with cellulose, no strand formation was evident suggesting that glucose might also interfere with the formation of the fibrous material by which the cells attach themselves to each other. It is not clear if attachment is necessary for growth stimulation, but it is well known that many microorganisms attach to surfaces and to each other to be better able to concentrate nutrients (Marshal, 1980; Burchard, 1981; Costerton et al., 1981). Attachment of other bacteria to cellulose fibers has been reported in *Clostridium thermocellum* (Wiegel & Dykstra, 1984), *Cellvibrio fulvus* (Berg et al., 1972b), *Sporocytophaga myxococcoides* (Eriksson & Hamp, 1978), *Mycobacterium* sp. strain BT2-4 (Ridgway et al., 1984) and many rumen bacteria (Akin, 1976; Latham et al., 1978; Minato et al., 1966; Patterson et al., 1975; McCowan et al., 1980).

It was recently observed that when *Cellulomonas* 2406 was grown on cellulose plates overlayed with pure agar larger degradation zones were observed than when grown directly on the cellulose plates. T. Kauri & D.J. Kushner (1985, in press) had suggested that the larger zones were observed since the agar layer decreased the effect of catabolite repression and inhibition by having the end products diluted out so that smaller amounts reach the cells.

Production of cellulases in *Cellulomonas* 2406 appears to be regulated by induction and catabolite repression. Its activity may be regulated by end product inhibition. Since the cellulase activities were lower than those of fungi, this microorganism would not be suitable for use in the industrial enzymatic hydrolysis of cellulose. In order to ferment cellulose to ethanol it is first necessary to convert it to glucose. Our results showed that the major product of cellulose degradation by *Cellulomonas* 2406 is probably cellobiose, not glucose; thus to obtain ethanol from cellulose co-cultures might be necessary. This study has put a new light on the subject of contact between cells and the cellulose fibers and their role in cellulase induction. *Cellulomonas* 2406 produces a fibrous material around itself when grown on cellulose. The fibrous material is probably acid mucopolysaccharide in nature and is probably involved in the contact that takes place between cells and the cellulose fibers as well as between the cells themselves. The results suggest that contact between cells and cellulose takes place in the first 24 hours of growth.

During this time the attached cells could be induced to produce cellulases which in turn would break down cellulose (Gong & Tsao, 1979). Small oligosaccharides produced in this way would then be responsible for the induction of cellulases in the cells which were not attached to the cellulose. Cells no longer need to be attached to the cellulose; rather they attach to each other in this way concentrating the nutrients. Whether this contact between cells and cellulose is necessary for cellulose degradation awaits further investigation.

References

Ait N., Creuzet N. and Cattanéo J., 1979; Characterization and purification of thermostable β-glucosidase from *Clostridium thermocellum*. Biochem. Biophys. Res. Common. 90:537-546.

Ait N., Creuzet N. and Cattanéo J., 1982, Properties of β-glucosidase purified from *Clostridium thermocellum*. J. Gen. Microbiol. 128:569-577.

Akin D.E. and Amos H.E., 1975, Rumen bacterial degradation of forage cell walls investigated by electron microscopy. Appl. Microbiol. 29:692-701.

Akin D.E., 1976, Ultrastructure of rumen bacterial attachment to forage cell walls. Appl. Environ. Microbiol. 31:562-568.

Akin D.T., Shapira R. and Kinkade J.M. Jr., 1985, The determination of molecular weights of biologically active proteins by cetyltrimethlylammonium bromide-polyacrylamide gel electrophoresis. Anal. Biochem. 145:170-176.

Alexander J.K., 1968, Purification and specificity of cellobiose phosphorylase from *Clostridium thermocellum*. J. Biol. Chem. 243:2899-2904.

Almin K.E. and Eriksson K.E., 1967, Enzymatic degradation of polymers. I. Viscometric method for the determination of enzymatic activity (enzyme A from *Penicillium chrysogenum notatum*). Biochem. Biophys. ACTA. 139(2):238-247.

Alexander J.K., 1972, Cellobiose phosphorylase from *Clostridium thermocellum*. In: Colowick S.P., Kaplan N.O. (eds in chief) Methods in enzymology vol. 28 Academic Press, New York, pp. 944-948.

Antheunisse J., 1984, Free and cell bound cellulase activity of *Cellulomonas flavigena*. Antonie van Leeuwenhoek 50: 7-15.

Arcuri E.J., 1982, Continuous ethanol production and cell growth in an immobilized cell bioreactor employing *Zymomonas mobilis*. Biotechnol. Bioeng. 24:595-604.

Atlas R.M., 1984, Microbiology, fundamentals and applications. MacMillan Publishing Company, New York, pp. 236-238.

Baruch P. and Swain T., 1957, β-gluco[illegible]ase of potato. Biochem. J. 66:321-323.

Beguin P., 1983, Detection of cellulase activity in polyacrylamide gels using congo red stained agar replicas. Anal. Biochem. 131:333-336.

Beguin P. and Eisen H., 1977, Free and cellulose-bound cellulases in a *Cellulomonas* sp. J. Gen. Microbiol. 101:191-196.

Beguin P. and Eisen H., 1978, Purification and partial characterization of three extracellular cellulases from *Cellulomonas* sp. J. Biochem. 87:525-531.

Berg B., 1975, Cellulase location in *Cellvibrio fulvus*. Can. J. Microbiol. 21:51-57.

Berg B., Hofsten B.V. and Pettersson G., 1972a. Growth and cellulase formation by *Cellvibrio fulvus*. J. Appl. Bact. 35:201-214.

Berg B., Hofsten B.V. and Pettersson G., 1972b. Electronmicroscopic observations on the degradation of cellulose fibers by *Cellvibrio fulvus* and *Sporocytophaga myxococcoides*. J. Appl. Bact. 35:215-219.

Berg B. and Pettersson L.G., 1977, Location and formation of cellulases in *Trichoderma viride*. J. Appl. Bacteriol. 42:65-75.

Berghem L.E.R. and Pettersson L.G., 1973, The mechanism of enzymatic cellulose degradation. Purification of a cellulolytic enzyme from *Trichoderma viride* active on highly ordered cellulose. Eur. J. Biochem. 37:21-30.

Berghem L.E.R. and Pettersson L.G. 1974, The mechanism of enzymatic cellulose degradation. Isolation and some properties of a β-glucosidase from *Trichoderma viride*. Eur. J. Biochem. 46:295-305.

Berghem L.E.R., Pettersson L.G., Axiö-Fredriksson U.B., 1975, The mechanism of enzymatic cellulose degradation. Characterization and enzymatic properties of a β-1,4-glucan cellobiohydrolase from *Trichoderma viride*. Eur. J. Biochem. 53:55-62.

Bisaria V.S. and Ghose T.K., 1981, Biodegradation of cellulosic materials: substrates, microorganisms, enzymes and products. Enz. Microb. Technol. 3:90-104.

Blank A., Sugiama R.H. and Dekker C.A., 1982, Activity staining of nucleic enzymes after sodium dodecyl sulfate polyacrylamide gel electrophoresis. Use of aqueous isopropanol to remove detergent from gels. Anal. Biochem. 120:267-275.

Blondin B., Ratomahenia R., Arnaud A. and Galzy P., 1983, Purification and properties of the β-glucosidase of a yeast capable of fermenting cellobiose to ethanol: Dekkera intermedia Van der Walt. Eur. J. Appl. Microbiol. Biotechnol. 17:1-6.

Branden A.R. and Thayer D.W., 1976, Serological study of *Cellulomonas*. International Journal of Systematic Bacteriology 26:123-126.

Breuil C. and Kushner D.J., 1976, Cellulase induction and the use of cellulose as a preferred growth substrate by *Cellvibrio gilvus*. Can. J. Microbiol. 22:1776-1781.

Buchanan R.E. and Gibbons N.E. (eds) Bergey's manual of determinative bacteriology. 8th edition. Baltimore, Williams & Wilkins.

Burchard R.P. 1981, Gliding motility of prokaryotes; ultrastructure, physiology and genetics. Ann. Rev. Microbiol. 35:497-529.

Burton K., 1956, A study of the conditions and mechanisms of diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. 62:315-323.

Chang M.J., 1971, Folding chain model and annealing of cellulose. Journal of Polymer Science part C polymer Symposia, 36: 343-362.

Chang W.T.H. and Thayer D.W., 1977, The cellulase system of a *Cytophaga* species. Can. J. Microbiol. 23:1285-1292.

Child J.J., Eveleigh D.E. and Sieben A.S., 1973, Determination of cellulase activity using hydroxymethylcellulose as substrate. Can. J. Biochem. 51:39-43.

Choi W.Y., Haggett K.D. and Dunn N.W., 1978, Isolation of a cotton wool degrading strain of *Cellulomonas*: Mutants with altered ability to degrade cotton wool. Aust. J. Biol. Sci. 31:553-564.

Clarke F.E., 1953, Criteria suitable for species differentiation in *Cellulomonas* and a revision of the genus. International Bulletin of Bacteriological Nomenclature and Taxonomy 31: 179-199.

Conche J., Hay A.J., Strachan I. and Levvy G.A., 1967, Inhibition of glucosidase by aldonolactones of corresponding configuration. Preparation of (1→5) lactones by catalytic oxidation of pyranoses and study of their inhibitory properties. Biochem. J. 102-929-941.

Conevascini G. and Meyer H.P., 1979, β-glucosidase in the cellulolytic fungus *Sporitrichum thermophile* Apinis. Experimental Mycology 3:203-214.

Cooney C.L., Wang D.I.C., Wang S.D., Gordon J. and Jiminez M., 1978, Simultaneous cellulose hydrolysis and ethanol production by a cellulolytic anaerobic bacterium. Biotechnol. Bioeng. Symp. 8:103-114.

Costerton J.W., Irvin R. and Cheng K.J., 1981, The bacterial glycocalyx in nature and disease. Ann. Rev. Microbiol. 35:299-324.

Crowford D.L., 1981, Microbial conversions of lignin to useful chemicals using a lignin degrading *Streptomyces*. Biotechnol. Bioeng. Symp. 11:275-291.

Dechamps A.M., Mahoudeau G., Leulliette L. and Lebeault J.M., 1980, Isolation and identification of bark decaying and utilizing bacteria of various origins. Revue d'Ecologie et de Biologie du Sol 17:577-581.

Dekker R.F.H., 1982, Ethanol production from D-xylose and other sugars by the yeast *Pochysolen tannophilus*. Biotechnol. Lett. 4:411-416.

Deshpande V., Eriksson K.E. and Pettersson D., 1978, Production, purification and partial characterization of 1,4 β-glucosidase enzymes from *Sporitrichum pulverulentum*. Eur. J. Biochem. 90:191-198.

Dinsdale D., Morris E.J. and Bacon J.S.D., 1978, Electron microscopy of the microbial populations present and their modes of attack on various cellulosic substrates undergoing digestion in the sheep rumen. Appl. Environ. Microbiol. 36:160-168.

Dische Z., 1930, Uber einige neue charakteristiche farbreaktionen der thymonukleinsaure und une mikromethod zur bestimmung dersenlben in tierischen organen mit hilfe dieser reaktionen. Mikrochemie 8:4-32.

Donefer E., Adeleye I.O.A. and Jones T.A.O.C., 1969, Effect of urea supplementation on the nutritive value of NaOH-treated oat-straw. Adv. Chem. Ser. 95:328-342.

Duncan W.A.M., Manners D.J. and Ross A.G., 1956, Enzyme synthesis in marine algae. Biochem. J. 63:44-51.

Emert G.K., Lang J.A., Liu T.[illegible] and Brown D.R., 1974, Cellulases. Adv. Chem. Ser. 136-79-100.

Epps H.M.R. and Gale E.F., 1942, The influence of the presence of glucose during growth on the enzymatic activities of *Escherichia coli*: comparison of the effect with that produced by fermentation acids. Biochem. J. 26:249-256.

Eriksen J. and Goksøyr, 1976, The effect on growth and cellulase (β-1,4-endoglucanase) production on the compost fungus *Chaetomium thermophile* var. *dissitum*. Archv. Microbiol. 110:233-238.

Eriksson K.E., 1969, Discussion to J.M. Leatherwood; Cellulase complex of *Ruminoccus* and a new mechanism of cellulase degradation. Adv. Chem. Ser. 95: pp. 58.

Eriksson K.E., 1975, Enzyme mechanism involved in the degradation of wood components. In Proc. Symp. Enz. Hydrol. Cellulose. (Bailey M., Enari T.M., Linko M. eds) Aulanko Finland pp. 263-280.

Eriksson K.E. and Hamp , 1978, Regulation of endo-k,4-β-glucanase production in *Sporotrichum pulverulentum*. Eur. J. Biochem. 90:183-190.

Fahraeus G., 1947, Studies in the cellulose decomposition by *Cytophaga*. Symb. Bot. Ups. 9:1-28.

Flickinger M.C., 1980, Current biological research in conversion of cellulosic carbohydrates into liquid fuels; how far have we come? Biotechnol. Bioeng. 22 (suppl1), 27-48.

Fredericq E. and Oth. A. yFontaine, 1961, The ultraviolet spectrum of deoxyribonucleic acids and their constituents. J. Mol. Biol. 3:11-77.

Gascoigne J.A. and Gascoigne M.M., 1960. Biological degradation of cellulose. Buttler Worth ed. London.

Gilkes N.R., Kilburn D.G., Langsford M.L., Miller R.C. Jr., Wakarchuk W.W., Warren R.A.J., Whittle D.J. and Wong K.R., 1984, Isolation and characterization of *Escherichia coli* clones expressing cellulase genes from *Cellulomonas fimi*. J. Gen. Microbiol. 130:1377-1384.

Goksøyr J. and Eriksen J., 1980, Cellulases in economic microbiology. Vol. 5 in: Microbial enzymes and bioconversions. A.H. Rose ed., Academic Press.

Gong C.S. and Tsao G.T., 1979, Cellulase and biosynthesis regulation. Annual reports on Fermentation Processes 3:111-140.

Haggett K.D., Gray P.P. and Dunn N.W., 1979, Crystalline cellulose degradation by a strain of *Cellulomonas* and its mutant derivatives. European J. Appl. Microbiol. Biotechnol. 8:183-190.

Haggett K.D., Choi W.Y. and Dunn N.W., 1978, Mutants of *Cellulomonas* which produce increased levels of β-glucosidase. Eur. J. Appl. Microbiol. Biotechnol. 6:189-191.

Halliwell G., 1961, The action of cellulolytic enzymes from *Myrothecium verrucaria*. Biochem. J. 79:185-192.

Halliwell G. and Griffen M., 1973, The nature and mode of action of the cellulolytic component C_1 of *Trichoderma konongii* on native cellulose. Biochem. J. 135:587-594.

Han Y.W. and Srinivasan V.R., 1968, Isolation and characterization of a cellulose utilizing bacterium. Appl. Microbiol. 16:1140-1145.

Hulme M.A. and Stranks D.W., 1971, Regulation of cellulase production by *Myrothecium verrucaria* grown on non cellulosic substrates. J. Gen. Microbiol. 69:145-155.

Hungate R.E., 1966, The rumen and its microbes. New York: Academic, pp. 36-59.

Hwang J.T. and Suzuki H., 1976, Intracellular distribution and some properties of β-glucosidase of a cellulolytic *Pseudomonad*. Agricultural and Biological Chemistry 40:2169-2175.

Jensen H.L., 1934, Studies on saprophytic mycobacteria and corynebacteria. Proceedings of the Linnean Society of New South Wales 59:19-61.

Jones T.H.D., Renobales M. and Pon N., 1979, Cellulases released during germination of *Dictyostellum discoideum* spores. J. Bacteriology 137:752-757.

Kauri T., 1980, Rapid multipoint method for quantification of various physiological groups of bacteria in soil. Soil Biol. Biochem. 12:125-130.

Kauri T. and Kushner D.J., 1985, Role of contact in bacterial degradation of cellulose, FEMS Microbiology Ecology (in press).

Keddie R.M., 1974, *Cellulomonas*. pp. 629-631. In: Buchanan R.E.; Gibbons N.E. (eds) Bergey's manual of determinative bacteriology. 8th edition. Baltimore, Williams & Wilkins.

Keddie R.M. and Jones D., 1981, Soprophytic, anaerobic coryneform bacteria. pp. 1838-1878 in: The Prokaryotes. A handbook on habitats, isolation and identification of bacteria. M.P. Starr, H. Stolp, H.G. Truper, A. Balows and H.G. Schlegel eds. Springer-Verlar, New York.

Kellerman K.F. and McBeth I.G., 1912, The fermentation of cellulose. Zentralbalatt fur Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene, Abt. 2 Orig. 34:485-494.

Kellerman K.F., McBeth I.G., Scales F.M. and Smith N.R., 1913, Identification and classification of cellulose dissolving bacteria. Zentralbalatt fur Bakteriologie, Parasitenkunde, Infektionskrantheiten und Hygiene, Abt. 2 Orig. 39:502-522.

Kight-Olliff L.C. and Fitzgerald J.W., 1978, Inhibition of enzyme induction in *Psudomonas aeruginosa* by exogenous nucleotides. Can. J. Microbiol. 24:811-817.

Kilbetus G., Mangenot F. and Reisinger O., 1973, Cellulolyse in vitro. Technique d'étude et vérification électronique. Mycopathologia et mycologia applicata 49:101-107.

Kilian S.G., Prior B.A. and Lategan P.M., 1983, Diauxic utilization of glucose-cellobiose mixtures by *Candida wickeihamii*. Eur. J. Appl. Microbiol., Biotechnol. 18:369-373.

Kim B.H. and Wimpenny J.W.T., 1981, Growth and cellulolytic activity of *Cellulomonas flavigena*. Can. J. Microbiol. 27:1260-1266.

Kirk T.K. and Chang H.M., 1981, Potential applications of bioligninololytic systems.

Lack S.A., Springhorn S.S. and Rosenthal A.L., 1979, Effect on the composition of sodium dodecyl sulfate preparations on the renaturation of enzymes after polyacrylamide gel electrophoresis. Anal. Biochem. 100:357-363.

Langsford M.L., Gilkes N.R., Wakarchuk W.W., Kilburn D.G., Miller R.C. Jr., and Warren R.A.J., 1984, The cellulase system of *Cellulomonas fimi*. J. Gen. Microbiol. 130:1367-1376.

Latham M.J., Brooker B.E., Pettipher G.L. and Harris P.J., 1978, Adhesion of *Bacteroides succinogenes* in pure culture and in the presence of *Ruminococcus flavefaciens* to cells walls in leaves of perennial ryegrass (Lolium peienne). Appl. Environ. Microbiol. 35:1166-1173.

Lee B.H. and Blackburn T.H., 1975, Cellulase production by a thermophilic *Clostridium* species. Appl. Microbiol. 30:346-353.

Lee S.B., Shin H.S. and Ryu D.D.Y., 1982, Adsorption of cellulase on cellulose; effect of physicochemical properties of cellulose on adsorption and rate of hydrolysis. Biotechnol. Bioeng. 24:2137-2353.

Lehninger A.L., 1972, Biochemistry, the molecular basis of cell structure and function. Worth Publishers Inc.

Lehninger A.L., 1978, Biochemistry, the molecular basis of cell structure and function. Worth Publishers Inc.

Levvy G.A., Hay A.J. and Conchie J., 1964, Inhibition of glucosidases by aldonolactones of corresponding configuration. Inhibition of mannosidase and glucosidase. Biochem. J. 91:378-384.

Li L.H., Flora R.M. and King K.W., 1965, Individual roles of cellulase components derived from *Trichoderma viride*. Arch. Biochem. Biophys. 111:439-447.

Lindner W.A., Dennison C. and Quicke G.V., 1983, Pitfalls in the assay of carboxymethyl cellulase activity. Biotechnol. and Bioeng. 25:377-385.

Louis A.J. and Becker R.R., 1973, The β-glucosidase system of the thermophilic fungus *Chaetomoin thermophile* var. *Coprophilen* var. Biochem. Biophys. Acta 329:5-16.

Lowry O.H., Roseborough N.Y., Farrand A.L. and Randall R.Y., 1951, Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265-275.

Luft J.H., 1971, Ruthenium red and violet; Chemistry, purification, methods of use for electron microscopy and mechanism of action. Anat. Rec. 171:347.

Macris B.J., 1984, Enhanced cellulase and β-glucosidase production by a mutant of *Alternaria alternata*. Biotechnol. Bioeng. 26:194-196.

Maddox 1982, Production of ethanol and n-buthanol from hexose/pentose mixtures using consecutive fermentations with *Saccharomyces cerevisiae* and *Clostridium acetobutylicum*. Biotechnol. Lett. 4:23-28.

Maddya H., 1976, Biochemical analysis of membranes. John Wiley and Sons Inc. N.Y. p. 236.

Magasanik B., 1961, Catabolite repression. Cold Spring Harbor Symposium on Quantitative Biology 26:249-256.

Mandels M., 1982, Cellulases. Annual Rep. Ferment. Processes 5: 35-78.

Mandels M., Andreotti R. and Roche C., 1976, Measurement of saccharifying cellulase. Biotechnol. Bioeng. Symp. 6: 21-33.

Mandels M. and Reese E.T., 1964, Fungal cellulases and the microbial decomposition of cellulosic fabric. Develop. Ind. Microbiol. 5:5-20.

Mandels M. and Weber J., 1969, The production of cellulases. Adv. Chem. Ser. 95:391-414.

Marais J.P., de Wit J.L. and Quicke G.V., 1966, A critical examination of the Nelson-Somogyi method for the determination of reducing sugars. Anal. Biochem. 15(3):373-381.

Margaritis A. and Merchant R., 1983, Xylanase, CM-cellulose and avicelase production by the thermophilic fungus *Sporotrichum thermophile*. Biotechnol. Lett. 5:265-270.

Marmur J., 1961, A procedure for the isolation of deoxyribonucleic acid from microorganisms. J. Mol. Biol. 3:208-218.

Marshall K.C., 1980, Reactions of microorganisms, ions and macromolecules at interfaces. In: Contemporary Microbial Ecology (D.C. Ellwood, J.N. Hedger, M.J. Latham, J.M. Lynch and J.H. Slater, editors), pp. 93-106, Academic Press.

Mayers H.P. and Canevascini G., 1981, Separation and some properties of two intracellular β-glucosidase of *Sporitrichum (hrysosporium) thermophile*. Appl. and Env. Microbiol. 41: 924-931.

McBeth I.G., 1916, Studies on the decomposition of cellulose in soils. Soil Science 1:437-487.

McCowan R.P., Cheng K.J. and Costerton J.W., 1980, Adherent bacterial populations on the bovine rumen wall; distribution patterns of adherent bacteria. Appl. Env. Microbiol. 39:233-241.

Miller G.L., Blum R., Geennon W.E. and Burton A.L., 1960, Measurement of carboxymethyl cellulase activity. Anal. Biochem. 2:127-132.

Miller J.H., 1972, Experiments in molecular genetics. Cold Spring Harbor Laboratory.

Minato H., Endo A., Higuchi M., Ootomo Y. and Uemura T., 1966, Ecological treatise on the rumen fermentation. I. The fractionation of bacteria attached to the rumen digest solids. J. Gen. Appl. Microbiol. 12:39-52.

Mullings R. and Perish J.H., 1984, New reducing sugar assay for the study of cellulases. Enzyme Microb. Technol. 6: 491-496.

Nakamura K. and Kitamura K., 1983, Purification and some properties of a cellulase active on crystalline cellulose from *Cellulomonas uda*. J. Ferment. Technol. 61:379-382.

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

Nisizawa T., Suzuki H. and Nisizawa K., 1972, Catabolite repression of cellulase formation in *Trichoderma viride*. J. Biochem. 71:999-1007.

Nisizawa K., Tamita Y., Kanda T., Suzuki H. and Wakabayashi K., 1972, Substrate specificity of C_1 and C_x cellulase components from fungi. Proc. 4th Int. Ferment. Symp. (Terui G. editor) 719-725.

Ng T.K. and Zeikus J.G., 1981, Purification and characterization of an endoglucanase (1,4-β-D-glucan glucohydrolase) from *clostridium thermocellum*. Biochem. J. 199:341-350.

Oberkotter L.V. and Rosenberg F.A., 1978, Extracellular endo β-1, 4-glucanase in *Cellvibrio vulgaris*. Appl. and Env. Microbiol. 36:205-209.

Okada G., Nisizawa K. and Suzuki H., 1968, Cellulase components from *Trichoderma viride*. J. Biochem. (Tokyo) 63:591-607.

Osmunsvang K. and Goksøyr J., 1975, Cellulases from *Sporocytophaga myxococcoides*. Eur. J. Biochem. 57:405-409.

Paralikar K.M. and Bhatawdekar S.P., 1984, Hydrolysis of cotton fibers by cellulase enzymes. J. Appl. Polymer Sci. 29: 2573-2580.

Patterson H., Irvin R., Costerton J.W. and Cheng K.J., 1975, Ultrastructure and adhesion properties of *Ruminococcus albus*. J. Bacteriol. 122:278-287.

Perry J.B., Stewart J.C. and Heptistall J., 1983, Purification of the major endoglucanase from *Aspargillus fumigatus* Fresenius. Biochem. J. 213:437-444.

Petre J., Longin R. and Millet J., 1981, Purification and properties of an endo-β-1,4 glucanase from *Clostridium thermocellum*. Chimie 63: 629-639.

Pettersson L.G., 1975, The mechanism of enzymatic hydrolysis by *Trichoderma viride*. In Proc. Symp. Enz. Hydrol. Cellulose. (Bailey M., Enari T.M., Linko M. eds.) Aulanko Finland pp. 255-262.

Prakash S., Lewontin R.C. and Hubby J.L., 1969, A molecular approach to the study of genic heterozygocity in natural populations IV. Patterns of genic variation in central, marginal and isolated populations of *Drosophila pseudoobscura*. Genetics 61:841-858.

Priest, F.G., 1977, Extracellular enzyme synthesis in the genus *Bacillus*. Bacteriol. Rev. 41:711-753.

Raabo E. and Terikidsen T.C., 1960, On the enzymatic determination of blood glucose. Scand. J. Clin. Lab. Invest. 12: 402.

Rajaka M.I. and Malik L.A., 1984, Cellulase and hemicellulase production by *Cellulomonas flavigena* NIAB 441. Biotechnol. Lett. 6:597-600.

Ramsamy K. and Verachtest H., 1980, Location of cellulase components in *Pseudomonas* sp. isolated from activated sluge. J. Gen. Microbiol. 117:181-191.

Reese E.T., 1977, Degradation of polyneric carbohydrates by microbial enzymes. In: Recent advances in phytochemistry. (Loewvs F.A. and Runeckles V.C. eds.) Vol. 11 pp. 311-367.

Reese E.T., Lola J.E. and Parrish F.W., 1969, Modified substrates and modified products as inducers of carbohydrates. J. Bacteriol. 100:1151-1154.

Reese E.T., Siu R.G.H. and Levinson H.S., 1950, The biological degradation of soluble cellulose derivatives and its relationship to the mechanism of cellulose hydrolysis. J. Bacteriol. 59:485-497.

Reynolds E.S., 1963, The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell. Biol. 17:208-213.

Ridgway H.F., Rigby M.G. and Argo D.G., 1984, Adhesion of a *Mycobacterium* sp. to cellulose diacetate membranes used in reverse osmosis. Appl. Env. Microbiol. 47:61-67.

Rose A.H. (ed.), 1980, Microbial enzymes and bioconversions. Economic Microbiology Vol. 5., pp. 283-326. Academic Press Inc. (London) Ltd.

Satchell J.E., 1971, Feasibility study of an energy budget for Meathop Wood. In: Productivity of Forest Ecosystems No. 4, Proceedings of the Brussels Symposium (P. Duvigneaud ed.), Paris, UNESCO.

Schmiz K.L., Broll B. and John B., 1983, Cellobiose phosphorylase (EC 2.41.20) of *Cellulomonas*: occurrence, induction and its role in cellobiose metabolism. Arch. Microbiol. 135:241-249.

Selby K., 1969, The purification and properties of the C_1 component of the cellulase complex. Adv. Chem. Ser. 95:34-50.

Selby K. and Maitland C.C., 1967, The cellulases of *Trichoderma viride*. Biochem. J. 10[illegible]6-724.

Sihtola H. and Neimo L., 1975, The structure and properties of cellulose. Symposium on enzymatic hydrolysis on cellulose. pp. 9-21. Sitra Helsinki.

Somogyi M., 1952, Notes on sugar determination. J. Biol. Chem. 195-19-23.

Sreenath H.K., Joseph R. and Murthy U.S., 1978, Studies on xylan hydrolases from different strains of *Streptomyces* and their mutual influences in the breakdown of xylan. Folia Microbiologica 23:299-303.

Srivivasan V.R. and Han Y.W., 1969, Utilization of bagasse. Adv. Chem. Ser. 95:447-460.

Stackebrandt E. and Kandler O., 1979, Taxonomy of the genus *Cellulomonas* based on phenotypic characters and deoxyribonucleic acid-deoxyribonucleic acid homology, and proposal of seven neotype strains. International Journal of Systematic Bacteriology 29:273-282.

Sternberg D. and Mandels G.R., 1979, Induction of cellulosic enzymes in *Trichoderma reesei* by sophorose. J. Bacteriol. 139:761-769.

Stewart B.J. and Leatherwood J.M., 1976, Derepressed synthesis of cellulase by *Cellulomonas*. J. Bacteriol. 128:609-615.

Stoppok W., Rapp P. and Wagner F., 1982, Formation, location and regulation of endo-1,4-β-glucanases and β-glucosidases from *Cellulomonas uda*. Appl. and Env. Microbiol. 44:44-53.

Suzuki H., 1975, Cellulase formation in *Pseudomonas fluorescens* var. *cellulosa*. pp. 155-169 in: M. Baily, T.M. Enari and M. Linko (ed.). Symposium on Enzymatic Hydrolysis of Cellulose, Helsinki, Finland.

Thayer D.W., Lowther S.V. and Phillips J.G., 1984, Cellulolytic activities of strains of the genus *Cellulomonas*. International Journal of Systematic Bacteriology 34:432-438.

Thayer D.W. and Murray J.O., 1977, Physiological, biochemical and morphological characteristics of mosquite wood-digesting bacteria. J. Gen. Microbiol. 101:71-77.

Theater R.M. and Wood P.J., 1982, Use of congo red polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. Appl. Environ. Microbiol. 43:777-780.

Thomas R., 1956, Fungal cellulases VII: *Stachybotrys atra*: production and properties of the cellulolytic enzyme. Australian J. Biol. Sci. 9:159-183.

Umerzurike G.M., 1979, The cellulolytic enzymes of *Botryodiplodia theobromae* Pat. Separation and characterization of cellulases and β-glucosidase. Biochem. J. 177:9-19.

Updergraff D.M., 1971, Utilization of cellulose from waste paper by Myrotheum verrucaria. Biotechnol. and Bioeng. 13:77-97.

Vircola N.E., 1975, Available Cellulosic Materials, Symposium on Enzymatic Hydrolysis of Cellulose, pp. 23-40.

Wakarchuk W.W., Kilburn D.G., Miller R.C. Jr. and Warren R.A.J., 1984, The preliminary characterization of the β-glucosidase of *Cellulomonas fimi*. J. Gen. Microbiol. 130: 1385-1389.

Weber K. and Osborn M., 1975, Protein and sodium dodecyl sulfate; molecular weight determination on polyacrylamide gels and related procedures. Proteins 1:179-223.

Weimer P.J. and Weston W.M., 1985, Relationship between the fine structure of native cellulose and cellulose degradability by the cellulase complexes of *Trichoderma reesei* and *Clostridium thermocellum* (to be published).

Whealan W.J., 1964, Hydrolysis with α-amylase. pp. 252-260 in: R.L. Whistler (ed.), Methods in Carbohydrate Chemistry Vol. 4, Academic Press Inc. New York.

Wiegel J. and Dykstra M., 1984, *Clostridium thermocellum:* Adhesion and sporulation while adhered to cellulose and hemicellulose. Appl. Microbiol. Biotechnol. 20:59-65.

Wilson W.R. and Niederpruem D.J., 1967, Control of β-glucosidase in *Schizophyllum commune*. Can. J. Microbiol. 13:1009-1020.

Wood P.J., 1980, Specificity in the interaction of direct dyes with polysaccharides. Carbohydr. Res. 85:271-287.

Wood T.W., 1968, Cellulolytic enzyme system of *Trichoderma koningii*. Biochem. J. 109:217-227.

Wood T.W., 1975, Properties and mode of action of cellulases. Biotechnol. and Bioeng. Symp. 5:111-137.

Wood T.W. and McCrae S.I., 1972, The purification and properties of the C_1 component of *Trichoderma koningii* cellulase. Biochem. J. 128:1183-1192.

Wood T.W. and McCrae S.I., 1977, The mechanism of cellulase action with particular reference to the C_1 component. Proc. Bioconversion Symp. IIT Delhi, pp. 111-141.

Wood T.W. and McCrae S.I., 1979, Synergism between enzymes involved in the solubilization of native cellulose. Adv. Chem. Ser. 181:181-209.

Woodward J., 1984, Xylanases: Functions, properties and applications. In: Topics in Enzyme and Fermentation Biotechnology 8. A. Wiseman ed. Halsted Press.

Woodward J., Lee J.M. and Zachry G.S., 1983, The inhibition of β-D-glucosidase (*Aspargillus niger*) by a factor in the commercial yeast extract. Biotechnol. Letters 57:469-474.

Yamane K., Suzuki H., Hiratani M., Ozawa H. and Nisizawa K., 1970, Effect of nature and supply of carbon sources on cellulase formation in *Pseudomonas fluorescens* var. *cellulosa*. J. Biochem. 67:9-18.

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

Yamane K., Yoshikawa T., Suzuki H. and Nisizawa K., 1971, Localization of cellulase components in *Pseudomonas fluorescens* var. *cellulosa*. J. Biochem. 69:771-780.