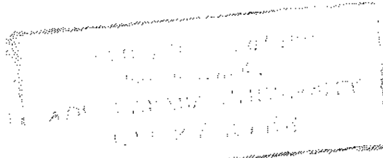
5e

ISOLATION AND CHARACTERIZATION OF AEROBIC CELLULOSE-DEGRADING MICROORGANISMS

A Thesis
Presented to
School of Graduate Studies
Addis Ababa University

In partial fulfillment
of the Requirements for the Degree of
Master of Science in Biology



by
Fassil Assefa

June 1983.

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my advisor, Dr. Berhanu A. Gashe for his relevant advice and strict supervision he gave towards the completion of this paper. My gratefulness also goes to Dr. Theodros Solomon, Head of the Chemistry Department, who was kind enough to supply me with the necessary chemicals for the completion of this work.

I am also indebted to Ato Dawit Abate for his generosity to give his fungal isolates as a reference for identification. I acknowledge Ato Assefa Areda, Ato Haile Dadi, Ato Adinew, Ato Admasu and Ato Abdu who provided the necessary apparatus for the work.

I am so much thankful to W/t Belaynesh Mengistu who helped me in typing the thesis. My deepest appreciation is extended to my friends, Zerihun, Wondwossen and Mulugeta Gebrewolde who encouraged me very much when I was preparing this dissertation.

Finally I am thankful to the Swedish Agency for Research Cooperation with Developing Countries (SAREC) for financial assistance towards the costs incurred in this research.

TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS....................................i

TABLE OF CONTENTS..................................ii

LIST OF TABLES.....................................iv

LIST OF FIGURES....................................vi

ABSTRACT..vii

CHAPTER

I INTRODUCTION.......................................1

Cellulolytic Microorganisms...............4

Cellulase.......................................8

Application....................................12

Objective......................................15

II MATERIALS AND METHODS...........................18

Screening of Cellulolytic

Microorganisms............ 18

Visual Determination of

Cellulolysis..................................19

Identification of Microorganism..........21

Effect of Carbon Source & pH

on Cellulase Induction...................22

Assay for Cellulase Activity.............24

Determination of Reducing Sugar..........24

Identification of Sugars Released by

Paper Chromatography.....................26

CHAPTER Page

III RESULTS.............................28

Isolation of Microorganisms..........28

Visual Determination of Rate of Growth............................30

Detection of Cellulolysis in Cellulose Agar.......................30

Effect of pH on Growth of the Fungi on Filter Paper (Stationary Culture).................32

Cell-free Cellulase Production.......32

Effect of Carbon Source on Cellulase Elaboration................37

Cellulase Activity on Different Substrates..........................41

Effect of Cellobiose and Glucose on Cellulase Activity................48

Identification of Sugars by Paper Chromatography.................48

IV DISCUSSION............................52

REFERENCES............................59

LIST OF TABLES

TABLE		Page
I	Cellulose-degrading Microorganisms Isolated from Different Sources...........	29
II	Rate of Growth of Cellulolytic Fungi on Filter Paper Immersed in Dubo's Salt Solution......................	31
III	Determination of Growth on Cellulose Agar	34
IV	Effect of pH on Growth of Fungi on Filter Paper (Stationary Culture).........	35
V	Effect of pH and period of Incubation on Extra-cellular Cellulase Production in Filter Paper Medium	36
VI	Effect of Carbon Source on Cellulase Elaboration..............................	40
VII.	Assay of Culture Filtrates Derived from Different Media on Various Cellulose-containing Substrates......	42-46
VIII	Cumulative Effect of Culture Filtrates Obtained from _Cephalosporium_ sp., _Verticillium_ sp., _Zygorrhynchus_ sp., on the Degradation of Cotton and Hay.........	47

TABLE | Page

IX The Effect of Glucose and Cellobiose on the *In Vitro* Activity of Cellulase from *Zygorrhynchus* sp. on Filter Paper..... 49

X. Chromatographic Identification of Reducing Sugars released by Cellulase in Culture Filtrates Obtained from Various organisms........................ 50

LIST OF FIGURES

FIGURES		Page
1	Free-Cellulase Activity of the Different fungi at pH 4.5	38
2.	Fre- -Cellulase Activity of the Dirrerent fungi at pH 7.2	39

ABSTRACT

Seventeen species of cellulose-degrading fungi that fall into fifteen genera were isolated from decomposing plant matter, garden soil, arable soil and manure. They were Alternaria sp., Aspergillus flavus, A. niger, Cephalosporium sp., Chaetomium sp., Curvularia sp., Fusarium sp., Geotrichum sp., Helminthosporium sp., Penicillium sp., Pythium sp., Rhizopus sp., Stachbotrys sp., Trichoderma sp. 1, Trichoderma sp. 2, Verticillium sp. and Zygorrhynchus sp.

Growth rate determination on filter paper showed that A. niger, Chaetomium sp., Curvularia sp., Helminthosporium sp., Rhizopus sp., Stachybotrys sp., Trichoderma sp. 1 and 2 and Zygorrhynchus sp., displayed abundant growth within 7-21 days at pH 4.5, 7.2 and 8.5.

Out of the fungi tested, only eight isolates namely Cephalosporium sp., Curvularia sp., Helminthosporium sp., Pythium sp., Stachybotrys sp., Trichoderma sp. 2., Verticillium sp., and Zygorrhynchus sp., were found to elaborate free cellulase in the culture medium.
High cellulase activity was found in Zygorrhynchus sp. at pH 7.2 and Stachybotrys sp., at pH 4.5. Low cellulase activity was shown by Cephalosporium sp., at pH 7.2 and Pythium sp., at pH 4.5

The effect of different cellulosic substrates on cellulase elaboration was tested on these organisms. The influence of cellobiose and glucose on the *in vitro* cellulase activity of *Zygorrhynchus* sp., was tested. The type and concentrations of reducing sugars released in the enzyme hydrolysates of the eight species were evaluated.

CHAPTER I

INTRODUCTION

Cellulose, the renewable carbonaceous constituent of plants, is the most abundant of all naturally occurring organic compounds (Alexander, 1964). It comprises at least a third of all the vegetable matter on earch, produced by photosynthetic processes to the tune of 100 billion tons per year (Han and Srivansan, 1968; Anonymous, 1974). Apart from the use of some cellulosic materials as constituent of wood, paper, cloth, rope and fillers, the bulk of it ends up in municipal trash, as animal feed lot, or agricultural, industrial and sewage wastes and residues (Anonymous, 1974; Ramasamy et al., 1981).

In structure, cellulose is a high polymer of β, 1-4 linked D-glucose residues bound together in a long and linear chain embedded in a non-cellulosic matrix such as hemicellulose and lignin. It shows a characteristic insolubility in water at even short chain lengths. The number of chain lengths ranges from 1400-10,000, with molecular weights 200,000 to almost 2 million (Norkrans, 1967; Srivansan, 1969).

The distribution of cellulose in higher plants is never fixed, and changes with age and type of plant. Chemical fractionation by means of dilute alkali revealed that soft and hard wood, with no secondary thickening, and the ones with secondary thickening, contain 20-35%

and 43% cellulose respectively (Roelofson, 1965). Similarily straw fibre, strands of jute, and cotton were found to have 40%, 70%, and 90% cellulose respectively (Lynch *et al.*, 1981).

Since a large part of the vegetation added to soil is cellulosic, the decomposition of this carbohydrate has a special significance in the biological cycle of carbon (Harper and Lynch, 1981).

To this effect, the degradation of cellulose by micro-organisms is a major component of the carbon cycle of and energy flux in soil. The products thus obtained contribute to the fertility of soils, and hence promote plant growth (Lynch, 1979; 1981).

Cellulose mineralization by microorganisms occurs in field and forest soils, in manure, and on decaying plant tissues. The physiological heterogeinity of the responsible microflora permits the transformation to take place in habitats with or without oxygen, at acidic or alkaline pH, low or high moisture levels, and from temperatures just above freezing to the extremes of the thermophilic range i.e. about 75°C (Alexander, 1964).

In nature, the rate at which cellulose is metabolized by microorganisms is governed by the above-mentioned environmental influences. These in turn, dictate the

number and species composition of the microflora, together with the cellulose-degrading activity of individual organisms. In laboratories, modification of the cellulose substrates with different chemicals was found to affect cellulose-degradation (Bhasu and Ghose, 1960; Han and Callihan, 1974; Gupta et al., 1981; Moo-Young et al., 1981; Susumu et al., 1981).

Cellulose degradation is not confined to arable and forest soils. A diverse group of aerobic and anaerobic bacteria, filamentous fungi, Basidiomycetes, Actinomycetes and certain protozoa with such ability are found elsewhere; in marine water, river beds (Arujo et al., 1981) waste-composting places (Stutzenberger, 1972), sewage-disposal systems (Ramasamy et al., 1981; Verachtert, 1982) in the guts of some invertebrates and in the rumen of cattle (Leatherwood, 1965; Groleau, 1981).

In nature, the distribution of anaerobic cellulose-decomposers is lower than that of the aerobic ones. Gray and Williams (1975) estimated the relative distribution of the aerobic and anaerobic microflora from the rhizosphere of Spring Wheat Soil, to be $9x10^3$ and $7x10^5$ per gram respectively.

The lower distribution of anaerobic cellulose-degraders; and the fact that the rate of cellulose metabolism in environments deficient in oxygen is much lower than that

of aerated habitats (Alexander, 1964) indicate the major part played by aerobic microorganisms in cellulose degradation. For these reasons, it is common to find numerous and diversified groups of aerobic cellulose degraders in nature.

Cellulolytic Microorganisms

Bacteria and fungi, with a few actinomycetes, are found to be the major cellulose-decomposers. The bacteria are represented by both aerobic and anaerobic microflora whereas the fungi and actinomycetes are predominantly aerobic, with a marked exception of *Merilius* and *Phoma* among the fungi and *Thermomonospora* among the Actinomycetes (Alexander, 1964; Stuzenberger, 1972).

The most important cellulose-fermenting anaerobic bacteria are found in the genera *Bacterioides*, *Ruminococcus* and *Clostridium*. The former two are found in the rumen of cattle and in the guts of some invertebrates. Extensive studies have been made on different aspects of these bacteria (Leatherwood, 1965; Groleau, 1981; Wood *et al.*, 1982). Members of the genus *Clostridium* are the dominant cellulose decomposers found elsewhere. They are found in soil, compost, manure and sewage systems (Anonymous, 1979). Another anaerobic species, *Bacillus polymaxa* was experimentally shown to have cellulolytic ability on wood by Greans and Leaver (Norkrans, 1967).

Aerobic cellulose-degrading bacteria are numerous and diversified in nature. They have been studied since the time of Omeliansky, 1895 (Imshensky, 1968). The pioneer organisms identified were members of the genera Cytophaga, Cellvibrio and Sporocytophaga. Later different organisms of the genera Achromobacter, Corynebacterium, Pseudomonas, Vibrio Mycobacterium, Bacillus, Cellulomonas and Xanthomonas have been extensively studied with respect to cellulose utilization (Alexander, 1964; Imshensky 1968; Anonymous, 1979).

The genera Cellulomonas and Pseudomonas are frequently found in soils receiving agricultural wastes and manures (Lynch, 1981), and in activated sludge (Ramasamy et. al., 1981;Verachtert et al., 1982). The potentiality of the genus Cellulomonas in the production of microbial proteins and other products has been studied in many laboratories (Srivansan, 1969; Anonymous, 1979; Khan et al., 1981).

The ability of cellulose-degradation by different bacterial species and Actinomycetes of the genera Streptomyces, Micromonospora, Streptosporangium, Norcardia, and Myxobacteria of the genus Sorangium shows that nearly every one of these major taxonomic groups contain representatives capable of cellulose decomposition (Alexander, 1964).

A diverse group of fungi are also involved in aerobic decomposition. Though the fungi are less in number and diversity than bacteria, they strongly attack cellulose because of their hyphal organization. This organization gives them a capacity for rapid penetration and decomposition of cellulose fibers as opposed to the bacteria (Norkrans, 1967).

It has been suggested that fungi are the main agents of cellulose-degradation in humid soils while bacteria are of greater significance in semi-arid conditions. Cellulolytic basidiomycetes are especially prominent in the destruction of forest litter, wood, and woody tissues (Alexander, 1964). Thus the fungi are important agents of different types of wood-rots and phytopathogenic spoilage of vegetables and cereals (Norkrans, 1967). Approximately 2000 species of Basidiomycetes have been classified as wood-decaying agents (Wagner and Davidson, 1954).

Many fungi seem able to decompose cellulose, in contrast to the bacteria, a group in which possession of the requisite enzymes is a comparative rarity. Bhasu and Ghose (1960) identified 124 fungal isolates, of which 119 were found to have cellulolytic ability, in mixed cellulosic cultures. In a successional study of 19 species of fungi, Sandhu and Arora (1981), reported that only one

species failed to utilize cellulose. El-Kady (1981) also isolated 37 spp., in 19 fungal genera, among cellulose-degraders from fifty wheat straw samples in Egypt.

Though most fungi have been shown to decompose cellulose, strongly cellulolytic fungi are represented by species of the genera Volvariella, Lentinus, Pleurotus, Aspergillus, Chaetomium, Curvularia, Fusarium, Trichoderma, Thielavia, Cladosporium, Rhizopus, Alternaria, Stachybotrys, Rhizoctonia and Penicillium (Alexander, 1964; Anonymous, 1979). Some yeasts are also shown to have cellulose-degrading ability (Stevens and Paynes, 1977; Arujo et al., 1981).

Different aspects of the enzyme kinetics of cellulose degradation; the influence of different biological, chemical and physical attributes involved, have been studied on different fungi. Nearly all successful works of this kind have been done on Myrothecium verrucaria, Trichoderma viride and Trichoderma koningii (Whitaker, 1971; Wood and McCrae, 1978; Halliwell, 1981).

Generally many different microorganisms have been found to use cellulose as their main carbon and energy source. Bacteria and fungi are prominent cellulose-degraders in nature. The ability of these organisms to metabolize cellulose stems from the fact that they

synthesize an enzyme-complex known as cellulase, which catalyzes the conversion of insoluble cellulose into simpler and water-soluble products.

Cellulases

Cellulase is an enzyme, or enzyme system, which hydrolyzes the cellulose polymer into mono, di- or oligosaccharides, an enzyme reaction characteristic of the entire cellulolytic flora. Cellulase is an induced or adaptive enzyme in most cellulose-decomposing fungi, i.e. it is synthesized only in the presence of its specific substrate; cellulose or a cellulose derivative. It appears to be constitutive in the cellulolytic bacteria (Rose, 1961).

Since cellulose under physiological conditions is insoluble and cannot permeate cell membranes, the microbial cellulase has been supposed to be extracellular (Norkrans, 1967). Extracellular cellulases are firmly bound to the cells, as in most bacteria or found in the free state in cultures as in many fungi (Whitaker *et al.*, 1963; Norkrans, 1967).

The cellulase enzyme system is found to contain many components. The degree of multiplicity and the role of each component have been a subject of discussion for many years. Reese *et al.*,(1950) postulated that two components namely "C_1" and "C_x" factors are responsible for the overall

degradative activity of the enzyme system. Accordingly, the non hydrolytic enzyme "C_1" is associated with the initial attack on native cellulose, whereas a hydrolase "C_x" is linked to complete the degradation to soluble sugars.

The hypothesis was subsequently modified by many workers (Mandels and Reese, 1964; Li _et al_., 1965; Selby and Maitland, 1965; 1966). They reported that cellulase in culture filtrates of _Myrothecium verrucaria_ and _Trichoderma viride_ can be fractionated into three components, each exhibiting different properties.

Recently, many workers have fractionated cellulases derived from different microorganisms, into 3-16 components (Wood and McCrae, 1972; 1978; Olutiola, 1976; Tanaka _et al_., 1981). However, much of the electrophoretic heterogeneity reported by some laboratories appears to have been a matter of dissociable complexes formed between enzymes and polysaccharides, which was earlier proposed by Li _et al_., (1965). Although it is now well established that cellulases found in culture filtrates are multienzymes, thier actual mode of action as well as their composition is still the subject of debate (Whitaker, 1981).

Whatever is the case, cellulase is a complex which carries out at least three distinct activities; Cellobiohydrolase (C_1), endo-and exo-glucanases (CM-cellulase) and β-glucosidase (Cellobiase) Halliwell, 1972, 1979).

The mode of action of these components on the substrate varies Endoglucanases form free reducing chain-ends and short-chain oligosaccharides by random cleavage of the β, 1-4 bonds of cellulose; exoglucanases form mainly cellobiose by endwise hydrolysis of the free reducing end of cellulose or cello-oligosaccharides. The β-glucosidase activity forms glucose by end-wise cleavage of cello-oligosaccharides (McCrae, 1972; 1978; Halliwell, 1979).

Each of these enzyme components exhibits a certain degree of multiplicity. McHale and Coughlan (1981) fractionated the exoglucanase, endoglucanase and β-glucosidase components of a fungus, Talaromyces emersonii, into five, two and three forms respectively. The amount of these components and their individual activity varies in different organisms. Wood et al.,(1982) showed that the endoglucanase component of Ruminococcus albus comprises 25% of all the activity.

The concerted action of all the components in the cellulose system leads to the complete solubilization of crystalline cellulose to glucose and/or cellobiose. But the ability to metabolize crystalline cellulose in nature and in the laboratory is limited to the relatively few microbial species which have acquired the whole spectrum of enzyme components in the system (Wood and McCrae, 1978; Halliwell, 1981; Wood et al., 1982).

The production of cellulases by microorganisms is affected by many factors, of which the following are mentioned; composition of culture medium (quantity and quality of cellulose used, and the amount of metal salts present), pH, temperature, and the adequacy of oxygen supply (Norkrans, 1963; 1967; Olutiola, 1976; 1977). The length of the incubation period was shown to affect the relative proportion of the enzyme components (Saddler and Khan, 1981).

Cellulases are induced in the presence of ß, 1-4 glucan substrates. As a result lactose, cellobiose, and sophorose have been shown to induce cellulase synthesis (Whitaker, 1971). However, cellulose-induced cellulase has been found to have more activity than cellobiose-induced cellulase (Groleau, 1981).

Many natural substances such as mercuric ion and phenolics suppress the synthesis of cellulase (Erikson and Peterson, 1967). But the most important inhibitors are the products derived from cellulose-degradation (Horton and Keen, 1966; Norkrans, 1967; Olutiola, 1976). Horton and Keen found that glucose concentration as low as 5×10^{-4}M is sufficient to repress cellulase synthesis by the plant pathogen _Pyrenochaeta terrestris_. Depending on the concentration, cellobiose can be both an inducer and a repressor for some organisms (Halliwell, 1981). _In vitro_ studies on the cellulase system of _Trichoderm viride_

showed that non-competitive inhibition by cellobiose dominates the reaction kinetics (Howell and Stuck, 1975).

Cellulases derived from different organisms have been shown to display different temperature and pH tolerance. Some were found to be strikingly stable at boiling temperature for a few minutes (Norkrans, 1967) and some remained stable four weeks at freezing temperature (Khan *et al.*, 1981). Many cellulases were reported to be acid-stable in the pH range of 2-4 in *Aspergillus tamarii* (Olutiola, 1977), 3-4 in *Aspergillus niger* (Paul *et al.* 1977), 2-5 in *Talaromyces emersonii* (McHale and Coughlan, 1981). A wide range of pH (4-8) tolerance in *Eupenicillium javanicum* was reported by Tanaka *et al.*, (1981).

Application

Cellulases derived from *Trichoderma viride*, *Myrothecium verrucaria*, and *Aspergillus* sp., have been used for different purposes (Arima, 1964; Jurasek *et al.*, 1967). The use of cellulase as a digestive aid is an old application, and is still produced for that purpose. Cellulases are also used to weaken or eliminate unwanted fibers in garment industries (Norkrans, 1967), to attack many physiological problems in botanical studies (Rose, 1961), to test the efficacy of biocides to protect starch-finished cloth

against bio-deterioration (Fermor and Eggins, 1981). The potentiality of cellulases as feed modifiers for cows and chickens has been studied, though large-scale production and application are found to have economic limitations (Khan et al., 1981; White et al., 1981).

The application of cellulase enzymes in the transformation of cellulose to useful products can be extended by growing the parent organisms on different lignocellulosic substrates. Cellulose can be converted to soluble sugars, particularly glucose by the strongly cellulolytic fungi; Trichoderma viride, Myrothecium verrucaria, and Fusarium sp. (Srivansan, 1969; Anonymous, 1974). The glucose recovered can be used as chemical feedstocks and substrates for different microorganisms to produce ethanol, butanol, acetone, single-cell protein and other products (Srivansan, 1969; Hartley and Walgate, 1980).

These syntheses, particularly single-cell protein and ethanol, have created renewed interest in the production of additional food and fuel with respect to the rising population and increasing costs of petroleum and coal, in many developing countries(Anonymous, 1979).

Many ambitious programmes for production of microbial protein and alcohol, from residues with a high sugar-content, have been undertaken in Brazil, India, and several other

countries (Anonymous, 1974; Imire, 1975; Malshe, 1975; Anonymous, 1979).

The production of ethanol and methane from cellulosic wastes by the thermophillic microbe, Clostridium thermocellum has been found to be promising for future development (Anonymous, 1979). C. thermocellum in pure culture, or in mixed culture with Methanobacterium thermoautotrophium, has been used to produce ethanol and biogas, respectively, in some pilot projects (Conney and Wise, 1975; Weimer et al., 1977; Weimer and Zeikus, 1979; Khan et al., 1981).

Conversion of cellulosic material, such as bagasse from sugar cane, to single-cell protein by bacteria of the genera Cellulomonas and Alcaligenes has been investigated on a laboratory and small-pilot plant scale (Srivansan, 1969). The direct conversion of cellulose into protein has been shown to be promising by a thermotolerant cellulolytic fungus, Cheatomium Cellulolyticus,(Anonymous, 1979; Moo-Young, 1981). A pilot-scale operation in Guatemala has shown that growing a cellulolytic fungus, Trichoderma sp., on coffee-processing wastes yields a protein product of interest for use in animal feed (Anonymous, 1979).

At present, many processes involve co-fermentation of cellulosic wastes by a cellulolytic organism and a fast-growing non-cellulolytic one. This has been found

feasible in a large-scale production of useful products, for it overcomes the barrier of the slow growth-rate of celluloytic organisms, which hampers the direct use of these organisms for biomass production (Molina _et al._, 1981; Moo-Young, 1981).

The prospect of microbial conversion of cellulosics to useful products is a promising approach to combat the food and fuel problem of the world. The facts that agricultural raw materials, mainly lignocellulosics are relatively free from toxic substances, and their year-round availability stimulate their use as substrate for the cellulose-fermenting microorganisms in the production of useful end-products. This has great economic significance for many less-developed countries, particularly those found in tropical and sub-tropical regions.

Objective

Ethiopia, one of the least developed countries in the tropics, has an economy which is dependent upon agricultural and agro-industrial economic systems. Consequently the country imports quite a large amount of petroleum, petroleum products and fertilizers which are used as raw materials for these industries.

The ever-increasing price of these commodities has already forced the country to spend almost half of its annual foreign currency earning. In order to avoid or

reduce its total dependence on these imported commodities, the potential of converting waste cellulosic material into these and other useful products cannot be overlooked.

The degradation of cellulosic materials can give rise to useful precursors for many synthetic products. Ethanol, produced from the broken-down cellulosic residues, either synthetically or by the fermentative action of the appropriate microorganisms, can be blended with gasoline. This serves as a means of offsetting the rising coasts of petroleum and its products.

Degraded cellulose can be used to grow both aerobic and anaerobic microflora, with the ultimum production of glycerol, butanol, acetone, ethanol, etc. which have wide industrial use. The growth of fungi, bacteria and yeasts on sugars released from cellulose produces additional biomass, in the form of single-cell protein, to be used as human food or animal feed. This will partly solve the protein probelm which is acute in many developing countries at present.

The generation of biogas using anaerobic microorganisms on cellulosic garbage is a prospective substitute for fuel which normally uses forest products. This will enable the nation to conserve its forest biomass which is being devastated at an alarming rate.

Cellulose conversion can be achieved by enzymatic and chemical means. Since chemical treatment of cellulosics is a capital-intensive process, enzymatic conversion through cellulose-degrading organisms is better emphasized. The biological conversion of cellulose calls for the selection of the proper microorganisms. An understanding of the growth pattern, conversion efficiency and the overall cellulolytic activity of the organisms, is a prerequisite for their large-scale utilization.

In this research an attempt has been made to isolate and characterize several aerobic cellulolytic microorganisms, especially those that are capable of secreting cellulase into the growth substratum. It is hoped that, based on these organisms and others that may be isolated in future, supercellulase-producing organisms can be either isolated, or created in the laboratory by genetic manipulations. This genetic modification, together with large-scale screening programmes will give strains which are capable of releasing cellulase in large quantities. These strains, in the long run, will be exploited to produce industrially useful products.

CHAPTER II

MATERIALS AND METHODS

Screening of Cellulolytic Microorganisms

The screening for cellulolytic organisms involved several techniques of which the most important are explained below.

1. Decomposing debris of plants, garments, and paper were taken and spread on filter paper strips moistened with Dubo's mineral salt solution in several petridishes. The solution contained the following per liter: Sodium nitrate 0.5 gm., dipotassium hydrogen orthophosphate (K_2HPO_4) 1.0 gm., hydrated magnesium sulphate ($MgSO_4.7H_2O$) 0.5 gm., hydrated ferrous sulphate ($FeSO_4.5H_2O$) 0.02 gm., potassium chloride 0.2 gm., yeast extract extract (DIFCO) 0.5 gm. The final pH was adjusted to 7.5 (Ramasamy, et al., 1981). This procedure allows cellulolytic microorganisms from the decomposing organic matter to grow on the filter paper strips.

2. Equal volumes of fresh or dried manure of cattle and water were mixed thoroughly to make a suspension. From the suspension, 1 ml. was inoculated on different petri-dishes overlayered with filter paper strips moistened with Dubo's mineral salt solution.

3. Soils from the surface layers of a garden and arable lands were taken in which filter paper strips were sand-witched in mud within petridishes. After three weeks, the decomposing paper strips were then soaked and shaken in water to form suspensions. 1 ml. of the suspension was sprinkled on circular filter paper discs, moistened with Dubo's salt solution. The diameter of the discs was slightly smaller than the bottom side of a 9x101 mm. petridish. All cultures were kept at 30°C and checked for growth periodically. Growth and degradation were observed by clearance or trans-parency of the filter paper discs. This was used as the criterion for classification of organisms as cellulolytic.

Visual Determination of Cellulolysis

In order to ascertain the cellulolytic nature of the organisms, the different colonies which grew on the filter paper discs and strips were transferred to another system similar to that used by Han and Srivansan (1968) for visual determination of cellulolysis. This involved inoculating organisms into sterilized, rubber-capped test tubes (18x180 mm) containing a 50 mm. strip of filter paper partially immersed in 3 ml. of Dubo's salt solution.

To determine at what pH optimum growth can be observed, the system of Han and Srivansan (1968) was prepared in triplicates at pH's 4.5, 7.2 and 8.5, and incubated at 30°C. Growth and digestion of the filter paper strips were checked every other day for well over a month. The visual determination was used as a preliminary screening test for the cellulolytic organisms. The tubes showing good growth and disintegration of the strips were selected and the organisms saved for future tests.

The different organisms obtained were then inoculated into petridishes containing Potato Dextrose Agar (PDA) and incubated at 30°C.

To recheck purity and cellulolytic ability, mycelial fragments and spores from the PDA-grown cultures were reinoculated into Han and Srivansan tubes (1968) for visual determination of cellulolysis.

PDA slants were used to keep the stocks of the truly cellulolytic microorganisms.

To further evaluate the visual screening of the microorganisms, they were allowed to grow on a solid cellulose medium as modified by Eggins and Pugh (1962). The medium had the following constituents per litre:

ammonium sulphate 0.5 gm., L-asparigine 0.5 gm., potassium dihydrogen phosphate 1.0 gm., potassium chloride 0.5 gm., magnesium sulphate 0.2 gm., calcium chloride 0.1 gm., yeast extract (DIFCO) 0.5 gm., agar (DIFCO) 20 gm., cellulose powder (Whatman No. 1) 10 gm. The final pH was adjusted to pH 7.5. Three standard loopfuls of mycelial and spore fragments were inoculated into the medium, using a needle with a loop diameter of approximately 2 mm. Comparison of the cellulose-decomposing ability of each of the organisms was made by measuring the extent and zone of clearing at intervals of two weeks.

Identification of Microorganisms

To identify and characterize the microorganisms (all were fungal isolates); the slide culture techniques, as described by Beneke and Rogers (1970), was used. The procedure involved pouring sterile plates with 2% PDA medium. After solidification, the medium was rapidly cut into 1 cm. square portions with a flamed dissecting knife. Petridishes, each containing a water-soaked cotton pad, and a slide placed on top of a bent glass rod, were sterilized at 121°C for 15 minutes at 1.055kg/cm^2. Using sterile technique, each 1 cm sq. agar block was placed on the slide in the sterile petridishes. The four sides of the agar

block were inoculated with mycelial and spore fragments of the respective organisms. Alcohol and heat-sterilized cover slips (25x32 mm.) were placed centrally upon each of the agar blocks. The cultures were then incubated at 30°C and periodically checked for growth and sporulation. After sporulation the culture were fumigated for about 30 min. with formaldehyde. Thereafter the agar blocks were carefully removed, and the coverslip as well as the slide which held the agar block, were stained with lactophenol-cotton blue, and then observed microscopically.

Microscopic characteristics, rate of growth on PDA, Sabouraud Dextrose Agar (SDA) and Malt Extract Agar (MEA), appearance of colony at the front and reverse side as well as coloration of mycelia and medium were observed, and information recorded. Based on such information, several manuals were consulted for the proper naming of the organisms to the genus and, when possible, to the species level (Bessey, 1950; Clements, 1964; Gilman, 1957; Gwyne-Vaughan and Barnes, 1965; Malloch and Cain, 1973; Smith, 1966; Webster, 1970).

Effect of Carbon Source and pH on Cellulose Induction

The method used for cellulase induction is the one used by Norkrans (1963), with minor modifications. The microorganisms to be tested were allowed to grow in 50 ml. of Dubo's salt solution containing 0.4 gm. either of pulverized Whatman filter paper No. 1, pulverized cotton, pulverized newspaper, pulverized hay or cellulose powder. The hay after pulverization was soaked in warm water to remove the reducing sugars which were initially present in a substantial amount. Spores and mycelial fragments from a region of 1 cm x 1 cm. of PDA-grown plates were each, separately inoculated into an 150 ml capacity Erlenmeyer flask containing the 0.4 gm. of substrate suspended in the 50 ml. of Dubo's salt solution. The system was prepared in triplicate to test the effect of pH at 4.5, 7.2 and 8.5 on the induction of cellulase. The culture were incubated on a reciprocal water-bath shaker (80 complete strokes/minute with a stroke length of 10 cm.). Maximum cellulase production for each organism was determined within a time range of four weeks, by taking samples every 7 days. Samples were centrifuged at 2000 xg for 10 minutes and the cell-free filtrates which contained the cellulase enzymes were saved for further examination, as described below.

Assay for Cellulase Activity

The method used for the assay of cellulase activity was that of Mandels and Weber (1969) with minor modifications. Two mililiters of the filtrate containing the cellulase enzymes, 0.2 gm. of the substrate in question (filter paper) suspended in 20 ml. of sodium acetate buffer at pH 5.2 were incubated at 40^{0}C in a 150 ml. Erlenmeyer flask. To avoid bacterial contamination and thus degradation of the proteins in the filtrate, the assay mixture was layered with toluene. Sample aliquots were removed every 12 hours for 48 hours for the determination of cellulase activity. The activity was measured by quantifying the amount of reducing sugars released in the suspension, as described below.

Cellulase assay was also made to evaluate the combination effect of cellulases derived from the selected isolates with high activity. Their culture filtrates which were combined in equal proportion, were assayed on pulverized cotton and hay.

Determination of Reducing Sugar

The quantity of reducing sugars released was determined using the method of Nelson (1944) as modified by Clark and Switzer (1977). The reagents contained the following:

Reagent A:sodium carbonate 12.5 gm., potassium sodium tartrate 12.5 gm., sodium bicarbonate 10 gm., sodium sulphate 100 gm., and distilled water 500 ml.

Reagent B:hydrated copper sulphate ($CuSO_4.5H_2O$) 7.5 gm., 1 drop concentrated sulphuric acid and 50 ml. of distilled water.

Arsenomolybdate Reagent:ammonium molybdate 25 gm., concentrated sulphuric acid 21 ml., distilled water 450 ml., mixed with a separately prepared sodium arsenate 3.0 gm., and 25 ml. distilled water.
The working reagent is composed of 12.5 ml. of Reagent A and 0.5 ml. of Reagent B.

One ml. of the sample and 1 ml. of the working reagent were thoroughly mixed with a vortex mixer (Gallenkamp), and then immersed in boiling water for 20 minutes. At the end of the 20 minutes, the samples were immersed in running water to cool them down quickly. Thereafter 1 ml. of Arsenomolybdate Reagent was introduced into the samples and vortex-mixed. The final volume of sample was then adjusted to 10 ml. using distilled water. The color produced was read on a spectronic-20-spectrophotometer (Bausch and Lamb) at 540 nm using a 1 cm. light-path cuvette, against a blank which contains all the buffer ingredients except the enzyme source.

The readings were then compared to a glucose standard curve, to extrapolate the amount of reducing sugars released. During these determinations, standard samples of glucose solutions were run to ensure reproducibility of color tests.

A unit of enzyme activity here is defined as the amount of nanomoles of reducing sugar released from 1 mg. of cellulosic substrate in one hour at pH 5.2 and 40°C.

Identification of Sugar Released by Paper Chromatography

In order to identify the different reducing sugars released by the cellulases derived from the different microorganisms, Ascending Chromatographic experiment was run. The technique was that used by Smith and Seakins (1976).

A 22x22 cm Whatman's Filter Paper No.1 was spread out on the table. Sample aliquots were taken from the mixture where enzyme activity of each organism had been determined. Each sample was placed on different spots on the starting line, 1 cm. above the lower edge of the paper. This was made by alternate spotting and drying using a 10 µl hypodermic syringe (Hamilton). The process continued until 0.2 ml. of the mixture was deposited without increasing the size of the spot (1 cm). Standard glucose and cellobiose were run alongside for reference. The paper was then allowed to air-dry.

The paper was held in cylindrical shape by fastening with staples and put in a glass-stoppered jar 25 cm in diameter and 25 cm. high, in which 100 ml. of the developing solution (n-butanol, acetic acid and water; 60:15:25) was poured. The solvent was allowed to travel upwards through the paper, 20 cm. beyond the starting line. The paper was then dried in air. The location of the sugar spots on the paper was revealed by spraying with the aniline-diphenyl-amine developer just heavily enough to wet the paper. Aniline-dephenyl amine developer consists: Aniline 4 ml., diphenyl-amine 4 gm., and acetone 200 ml. The paper was then heated in an oven for about 5 minutes at about $80^{\circ}C$. The sugars were identified by their relative positions and specific color production and compared with the relative positions of cellobiose and glucose on the same chromatographic paper.

CHAPTER III

RESULTS

Isolation of Microorganisms: Several bacterial and fungal cellulose-decomposers were isolated from various sources. However, because of their known capability to release extracellular cellulases into the growth media , fungi were chosen for this research. Seventeen species of fungi which fall into fifteen genera were isolated on filter paper baits from decomposing plant and garment, garden soil, arable soil and manure. The organisms and the sources from which they were isolated are tabulated in TABLE I.

Two species each of the genera Trichoderma and *Aspergillus*, and one species from the genus *Curvularia* were isolated from all sources. *Cephalosporium* sp. was isolated from the decomposing plant and garment, garden and arable soils. Even though *Chaetomium* sp. was not isolated from garden soil, it was present in all other sources. *Rhizopus* sp. was also isolated from all sources except the decomposing plant and garment samples.

As shown in TABLE I, the majority of the fungi were isolated from arable and garden soils. The species isolated from these two sources represented the following genera, *Helminthosporium*, *Penicillium*, *Pythium*, *Stachybotrys*, *Verticillium* and *Zygorrhynchus*. *Alternaria* sp. and *Fusarium* sp. were isolated from decomposing plant matter and arable soil, whereas *Geotrichum* sp. was found in only arable soil.

TABLE I

Cellulose-degrading microorganisms isolated from different sources

Name of Fungi	SOURCE			
	Decomposing plant and garment	Garden soil	Arable soil	Manure
Alternaria sp.	+	-	+	-
Aspergillus flavus	+	+	+	+
Aspergillus niger	+	+	+	+
Cephalosproium sp.	+	+	+	-
Chaetomium sp.	+	-	+	+
Curvularia sp.	+	+	+	-
Fusarium sp.	+	-	+	-
Geotrichum sp.	-	-	+	-
Helminthosporium sp.	-	+	+	-
Penicillium sp.	-	+	+	-
Pythium sp,	-	+	+	+
Rhizopus sp.	-	-	+	-
Stachybotrys sp.	-	+	+	-
Trichoderma sp. 1	+	+	+	+
Trichoderma sp. 2	+	+	+	+
Verticillium sp.	-	+	+	-
Zygorrhynchus sp.	-	+	+	-

Visual Determination of Rate of Growth:The fungal isolates were grown on filter paper strips to determine their growth rates. As shown in TABLE II, each organism displayed different growth rates within a time lapse of 7-21 days. The rate of growth was scored as 4 when growth was abundant, and 1 when growth was found to be minimum.

Growth rate determination showed that *Curvularia* sp., *Helminthosporium* sp., the two species of *Trichoderma*, *Chaetomium* sp., *Stachybotrys* sp., and *Zygorrhynchus* sp. had abundant growth in 14 days. *Aspergillus niger*, and *Rhizopus* sp. displayed a good growth pattern in the same time period. Species of the genera *Alternaria*, *Cephalosporium*, *Penicillium*, *Pythium* and *Verticillium* grew slowly. However, upon continued incubation they also displayed fairly moderate growth on the filter paper strips.

Growth of *Aspergillus flavus*, *Fusarium* sp., and species of *Geotrichum* was initially very low; nevertheless upon prolonged incubation (>14 days), they also showed detectable growth.

Detection of Cellulolysis in Cellulose Agar:Most of the fungi grew well and produced clear zones on the cellulose agar medium indicating that the cellulose has been degraded to soluble products. The extent and degree of

TABLE II

Rate of growth of Cellulolytic fungi on filter paper Immersed in Dubo's slat solution at pH 7.2 (Stationary Culture)

Name of Fungus	Days of Incubation of 30° C		
	7 days	14 days	21 days
Alternaria sp.	1	2	2
Aspergillus flavus	1	1	2
Aspergillus niger	2	3	4
Cephalosporium sp.	1	2	2
Chaetomium sp.	3	3	4
Curvularia sp.	3	4	4
Fusarium sp.	-	1	2
Geotrichum sp.	-	1	1
Helminthosporium sp.	3	4	4
Penicillium sp.	1	2	3
Pythium sp.	2	2	3
Rhizopus sp.	2	3	3
Stachybotrys sp.	3	4	4
Trichoderma sp. 1	3	3	4
Trichoderma sp. 2	3	4	4
Verticillium sp.	1	2	3
Zygorrhynchus sp.	3	4	4

- No growth
1 Slight Growth
2 Moderate Growth
3 Good Growth
4 Abundant Growth

cellulose degradation was then estimated by measuring the zone of clearance around each inoculum site at days 7 and 14 (TABLE III).

Effect of pH on growth of the fungi on Filter Paper in Stationary Culture: Growth of the fungal isolates at acidic near-neutral and basic pHs was determined at various incubation periods on filter paper strips immersed in Dubo's salt solution (TABLE IV). Generally all of them were found to grow well at the near-neutral pH (7.2).

Seven of the species isolated exhibited good growth at all of the pH values. These were: Aspergillus niger, Cephalosporium sp., Curvularia sp., Pythium sp., Rhizopus sp., Stachybotrys sp., and Zygorrhynchus sp. At pH 4.5 and 7.2, the two species of Trichoderma ., Aspergillus flavus, Helminthosporium sp., Penicillium sp., and Verticillium sp. were shown to grow well. The slow-growers, represented by Fusarium sp., and Geotrichum sp., grew only at pH 7.2 after 14 days of incubation.

Cell-free Cellulase Production: To determine the capacity and efficiency of cellulase production by the fungal isolates, all of them were inoculated into filter paper medium at pHs 4.5, 7.2 and 8.5. They were incubated at 30°C in shake cultures for four weeks. Culture filtrates were then used as enzyme source. Samples from

TABLE III

Determination of growth on cellulose agar (pH 7.2)

Name of Fungus	Zone of Clearance (cm)	
	7 days	14 days
Alternaria sp.	3	6
Aspergillus flavus	6	9
Aspergillus niger	9	9
Cephalosporium sp.	2	6
Chaetomium sp.	7	9
Curvularia sp.	7	9
Fusarium sp.	3	5
Geotrichum sp.	2	4
Helminthosporium sp.	8.3	9
Penicillium sp.	2	4.5
Pythium sp.	5.2	7.8
Rhizopus sp.	9	9
Stachybotrys sp.	9	7
Trichoderma sp. 1	9	9
Trichoderma sp. 2	9	9
Verticillium sp.	4.5	6.9
Zygorrhynchus sp.	9	9

TABLE III

Determination of growth on cellulose agar (pH 7.2)

Name of Fungus	Zone of Clearance (cm)	
	7 days	14 days
Alternaria sp.	3	6
Aspergillus flavus	6	9
Aspergillus niger	9	9
Cephalosporium sp.	2	6
Chaetomium sp.	7	9
Curvularia sp.	7	9
Fusarium sp.	3	5
Geotrichum sp.	2	4
Helminthosporium sp.	8.3	9
Penicillium sp.	2	4.5
Pythium sp.	5.2	7.8
Rhizopus sp.	9	9
Stachybotrys sp.	9	7
Trichoderma sp. 1	9	9
Trichoderma sp. 2	9	9
Verticillium sp.	4.5	6.9
Zygorrhynchus sp.	9	9

culture filtrates were taken and assayed on the substrate suspended in sodium acetate buffer at pH 5.2 and 40°C. They were incubated for 48 hours taking samples every 12 hours, to detect the maximum activity of the enzyme in the culture filtrates with respect to the release of the reducing sugars.

The fungi capable of releasing cell-free cellulase in the medium at different pHs, and the enzyme activity found in their respective culture filtrates are shown in TABLE V, Fig. 1, 2.

Of the organisms tested, only eight were shown to elaborate cell-free cellulase enzymes in the culture. Only one, Cephalosporium sp. was found to release cell-free enzyme at all of the pH values tested, with the maximum excretion at 8.5, Pythium sp., and Zygorrhynchus sp. were found to produce cell-free cellulase at pHs 4.5 and 7.2. The other three namely Curvularia sp., Helminthosporium sp., and Stachybotrys sp., were found to be restricted to pH 4.5 in the excretion of the enzyme in the medium. The two isolates of the genera Trichoderma and Verticillium were shown to actively elaborate cell-free cellulase at pH 7.2. This difference showed that pH had an effect on cellulase elaboration as it had on growth of the fungal isolates on filter paper (TABLE IV and V).

TABLE IV

Effect of pH on Growth of Microorganisms on Filter Paper (Stationay Cul ture) at 30°C

Name of Fungus	7 days			14 days			21 days		
	pH			pH			pH		
	4.5	7.2	8.5	4.5	7.2	8.5	4.5	7.2	8.5
Alternaria sp.	-	+	-	-	+	-	-	+	-
Aspergillus flavus	+	+	-	+	+	-	+	+	-
Aspergillus niger	+	+	+	+	+	+	+	+	+
Chaetomium sp.	-	+	-	-	+	-	-	+	-
Curvularia sp.	+	+	+	+	+	+	+	+	+
Fusarium sp.	-	-	-	+	-	-	-	+	-
Geotrichum sp.	-	-	-	-	+	-	-	+	-
Helminthosporium sp.	+	+	-	+	+	-	+	+	-
Penicillium sp.	+	+	-	+	+	-	+	+	-
Pythium sp.	+	+	+	+	+	+	+	+	+
Rhizopus sp.	+	+	+	+	+	+	+	+	+
Stachybotrys sp.	+	+	+	+	+	+	+	+	+
Trichoderma sp. 1	+	+	-	+	+	-	+	+	-
Trichoderma sp. 2	+	+	-	+	+	-	+	+	-
Verticillium sp.	+	+	-	+	+	-	+	+	-
Zygorrhynchus sp.	+	+	+	+	+	+	+	+	+

+ = Growth - = No Growth

TABLE V

Effect of pH and period of incubation on extra-cellular cellulase production in filter paper medium

Name of Fungus	7 days			14 days			21 days			28 days		
	pH			pH			pH			pH		
	4.5	7.2	8.5	4.5	7.2	8.5	4.5	7.2	8.5	4.5	7.2	8.5
Alternaria sp.	-	-	-	-	-	-	-	-	-	-	-	-
Aspergillus flavus	-	-	-	-	-	-	-	-	-	-	-	-
Aspergillus niger	-	-	-	-	-	-	-	-	-	-	-	-
Cephalosporium sp.	+	+	+	+	+	+	+	+	+	+	+	+
Chaetomium sp.	-	-	-	-	-	-	-	-	-	-	-	-
Curvularia sp.	+	-	-	+	-	-	+	-	-	-	-	-
Fusarium sp.	-	-	-	-	-	-	-	-	-	-	-	-
Geotrichum sp.	-	-	-	-	-	-	-	-	-	-	-	-
Helminthosporium sp.	-	-	-	+	-	-	+	-	-	-	-	-
Pencillium sp.	-	-	-	-	-	-	-	-	-	-	-	-
Pythium sp.	+	+	-	+	+	-	+	+	-	+	+	-
Rhizopus sp.	-	-	-	-	-	-	-	-	-	-	-	-
Stachybotrys sp.	+	-	-	+	-	-	+	-	-	-	-	-
Trichoderma sp. 1	-	-	-	-	-	-	-	-	-	-	-	-
Trichoderma sp. 2	-	+	-	-	+	-	-	+	-	-	+	-
Verticillium sp.	-	+	-	-	+	-	-	+	-	-	+	-
Zygorrhynchus sp.	+	+	-	+	+	-	+	+	-	+	+	-

The time at which cellulase elaboration starts and declines in each fungus was determined at weekly interval for four weeks. As shown in Fig. 1 and 2, each isolate was shown to display its own cellulase elaboration periods. The time of active cellulase elaboration lay between 14-21 days for almost all organisms.

The highest production of cellulase was shown by Verticillium sp. and Zygorrhynchus sp. at pH 7.2 and, Helminthosporium sp. and Stachybotrys sp. at pH 4.5. Low activity (production) was found in Cephalosporium sp. and Trichoderma sp. 2 at pH 7.2, and Cephalosporium sp. Pythium sp. at pH 4.5, respectively. The overall pattern showed higher cellulase elaboration when the organisms were grown at pH 7.2 rather than 4.5 or 8.5.

Effect of Carbon Source on Cellulase Elaboration: Based on types of the cellulosic material, cellulase activity in the culture filtrates of the isolates was found to vary. Only Cephalosporium sp., Trichoderma sp. 2, Verticillium sp. and Zygorrhynchus sp. were found to show activity on cotton and cellulose powder, which is a characteristic of the true cellulolytic microorganisms (TABLE VI).

These isolates were also tested on different substrates and found to display different activities. However, maximum activity of cellulase from all culture

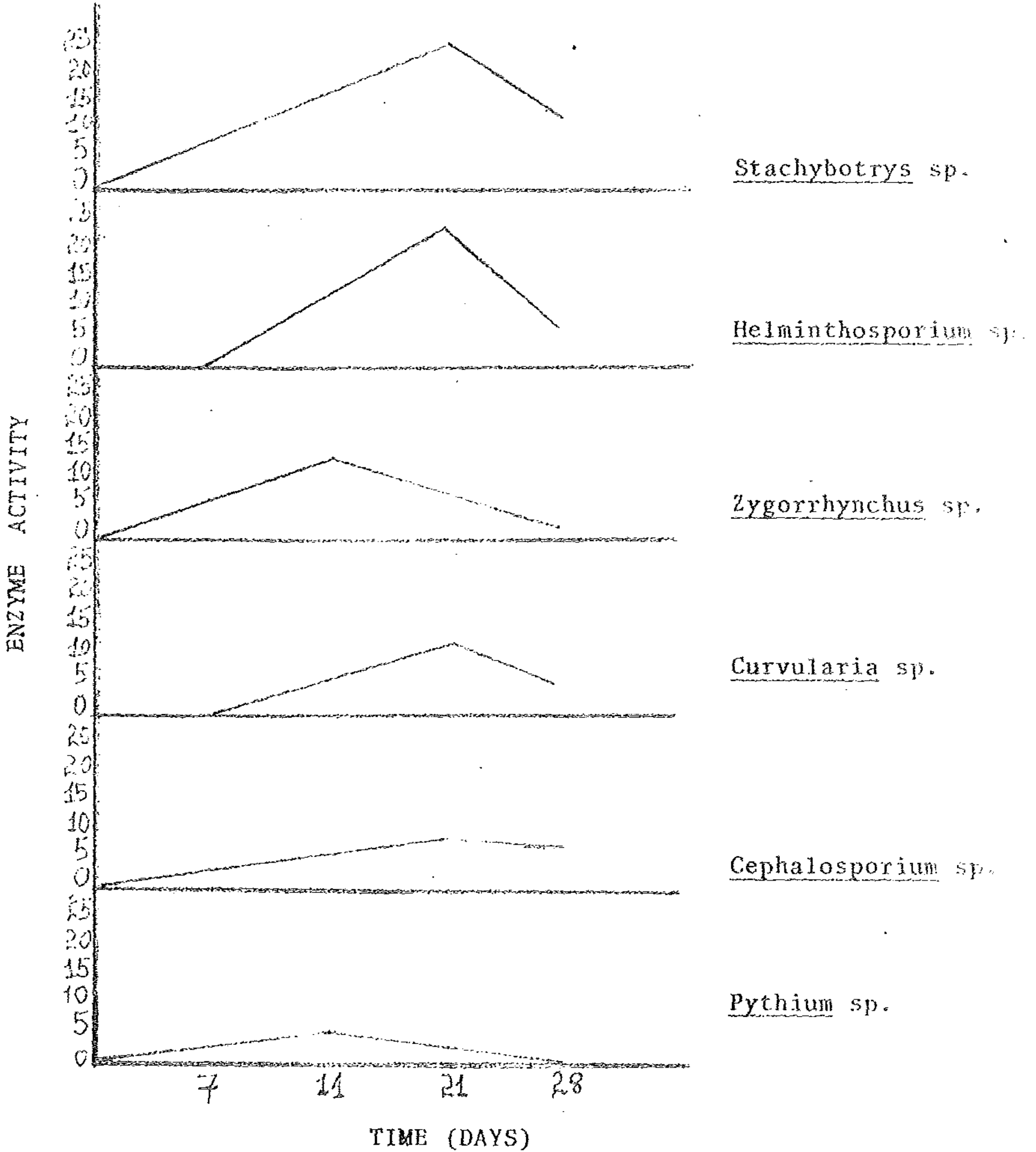


Fig. 1 Free cellulase activity of the different fungi at pH 4.5

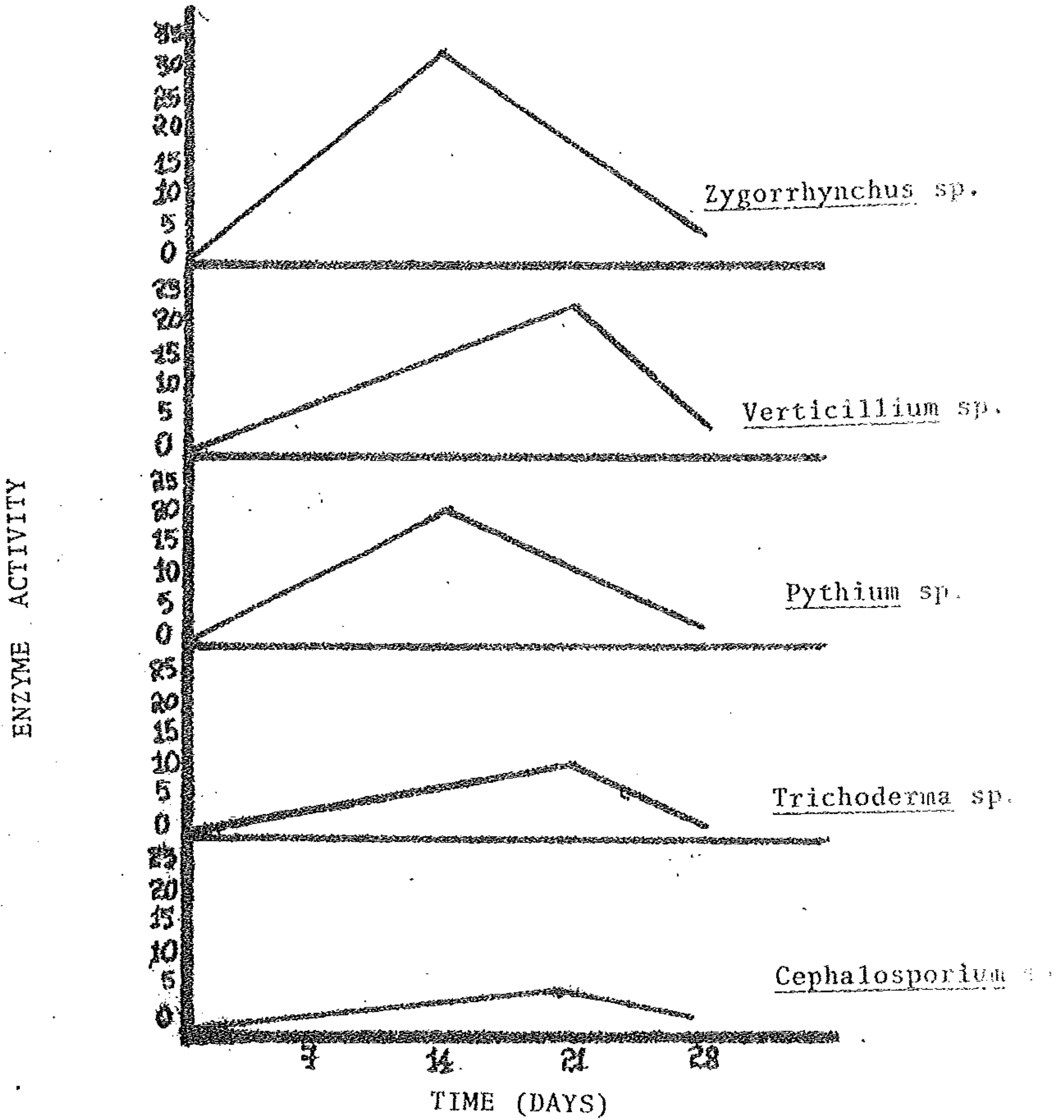


Fig. 2 Free cellulase activity of the different fungi at pH 7.2.

TABLE VI

Effect of carbon source on cellulase elaboration

Name of Fungus	Unit of Enzyme Activity				
	Cotton	Cellulose powder	Filter paper	Newspaper	Hay
Cephalosporium sp.	1.0	1.2	5.6	5.3	7.6
Trichoderma sp. 2	0.8	0.87	11.0	5.6	6.1
Verticillium sp.	1.6	2.2	24.0	5.6	5.7
Zygorrhynchus sp.	1.2	1.2	33.0	3.0	6.6

filtrates was shown on filter paper (TABLE VI). Relatively moderate activity was shown on newspaper and hay, whereas weak cellulolytic activity was found on cotton and cellulose powder in most cases.

Cellulase Activity on Different Substrates: The activity of cellulases derived from various substrates was assayed on each substrate by taking the most cellulolytic organisms (TABLE VII). In all cases, the culture filtrates derived from all the substrates showed weak activities on cotton and cellulose powder. However, relatively high activity of the enzyme was often exhibited when a culture filtrate was assayed on the parent substrate on which the fungus was previously induced to produce the enzyme.

The cumulative effect of cellulase components derived from the different cellulolytic fungi was determined by taking culture filtrates of *Cephalosporium* sp., *Verticillium* sp., and *Zygorrhynchus* sp. The combined effect of paired culture filtrates from these organisms was compared to the cellulase activities of the individual species on cotton and hay (TABLE VIII).

As a result, paired culture filtrates of *Verticillium* sp. and *Zygorrhynchus* sp. showed a higher activity on both substrates than the individual culture filtrates of the organisms. This was also shown by the combined action of culture filtrates of Cephalosporium sp. and *Zygorrhynchus*

Assay of culture filtrates derived from different media on various cellulose containing substrates

Organism	Growth medium	Assay substrate	Unit of enzyme activity
Cephalosporium	Cellulose powder	Cellulose powder	1.2
		Cotton	1.0
		Newspaper	3.0
		Hay	4.0
		Filter paper	4.2
	Cotton	Cellulose powder	0.8
		Cotton	0.78
		Newspaper	2.5
		Hay	12.0
		Filter paper	12.5
	Newspaper	Cellulose powder	2.3
		Cotton	2.0
		Newspaper	5.3
		Hay	6.1
		Filter paper	4.8
	Hay	Cellulose powder	3.2
		Cotton	3.0
		Newspaper	4.8
		Hay	7.6
		Filter paper	5.6

TABLE VII (Cont'd)

Organism	Growth medium	Assay Substrate	Unit of Enzyme activity
Cephalosporium sp.	Filter paper	Cellulose powder	1.4
		Cotton	1.1
		Newspaper	3.3
		Hay	5.1
		Filter paper	5.6
Trichoderma sp. 2	Cellulose powder	Cellulose powder	0.78
		Cotton	0.8
		Newspaper	4.2
		Hay	5.2
		Filter paper	8.0
	Cotton	Cellulose powder	1.1
		Cotton	0.8
		Newspaper	2.1
		Hay	5.2
		Filter paper	6.5
	Newspaper	Cellulose powder	-
		Cotton	-
		Newspaper	5.6
		Hay	5.0
		Filter paper	4.8
	Hay	Cellulose powder	1.0
		Cotton	0.7
		Newspaper	2.5
		Hay	6.1
		Filter paper	7.8

TABLE VII (Cont'd)

Organism	Growth medium	Assay substrate	Unit of enzyme activity
Trichoderma sp. 2	Filter paper	Cellulose powder	0.7
		Cotton	-
		Newspaper	1.0
		Hay	1.2
		Filter paper	11.0
Verticillium sp.	Cellulose powder	Cellulose powder	2.2
		Cotton	1.8
		Newspaper	3.8
		Hay	4.7
		Filter paper	9.6
	Cotton	Cellulose powder	1.2
		Cotton	1.6
		Newspaper	1.6
		Hay	5.5
		Filter paper	8.6
	Newspaper	Cellulose powder	1.3
		Cotton	1.5
		Newspaper	3.7
		Hay	3.7
		Filter paper	2.31
	Hay	Cellulose powder	3.8
		Cotton	0.5
		Newspaper	2.5
		Hay	2.9
		Filter paper	2.3

TABLE VII (Cont'd)

Organism	Growth medium	Assay substrate	Unit of enzyme activity
Verticillium sp.	Filter paper	Cellulose powder	2.8
		Cotton	5.6
		Newspaper	10.5
		Hay	16.2
		Filter paper	24.1
Zygorrhynchus sp.	Cellulose powder	Cellulose powder	1.2
		Cotton	0.5
		Newspaper	1.8
		Hay	1.3
		Filter paper	2.5
	Cotton	Cellulose powder	2.3
		Cotton	1.2
		Newspaper	1.0
		Hay	2.9
		Filter paper	2.5
	Newspaper	Cellulose powder	1.8
		Cotton	-
		Newspaper	3.0
		Hay	5.3
		Filter paper	2.5

TABLE VII (Cont'd)

Organism	Growth medium	Assay substrate	Unit of enzyme activity
Zygorrhynchus sp.	Hay	Cellulose powder	1.8
		Cotton	1.0
		Newspaper	1.3
		Hay	6.6
		Filter paper	5.8
	Filter paper	Cellulose powder	9.1
		Cotton	8.2
		Newspaper	16.3
		Hay	25.2
		Filter paper	33

TABLE VIII

Cummulative effect of culture filtrates obtained from *Cephalosporium* sp., *Verticillium* sp., and *Zygorrhynchus* sp. on the degradation of cotton & hay

Name of Fungi	Unit of enzyme activity	
	Cotton	Hay
Cephalosporium sp.	0.78	7.6
Verticillium sp.	0.5	5.7
Zygorrhynchus sp.	1.2	5.7
Cephalosporium sp. + *Verticillium* sp.	1.5	7.4
Cephalosporium sp. + *Zygorrhynchus* sp.	1.7	7.8
Verticillium sp. + *Zygorrhynchus* sp.	1.8	7.8
Cephalosporium sp. + *Verticillium* sp. + *Zygorrhynchus* sp.	1.0	5.6

sp. on one hand, and that of *Cephalosporium* sp. and *Verticillium* sp. on the other.

The cumulative effect of culture filtrates derived from all of the three isolates did not show an increase in the activity. On the contrary, less activity was exhibited than the paired ones.

Effect of Glucose and Cellobiose on Cellulase Activity: The concentrations at which cellulase from *Zygorrhynchus* sp. could be inhibited by glucose and cellobiose, was tested *in vitro* taking sugar concentrations of 50, 100, and 200μg/ml. In all cases, cellulase was not inhibited. A further attempt was made by increasing the concentrations. However, the results with increased concentrations were conflicting, either due to the masking effect of the high concentration added to detect the smaller changes in the activity, or the inhibitory effect (TABLE IX).

Identification of Sugars by Chromatography: All of the samples were found to contain cellobiose and glucose in different proportions (TABLE X). Based on the diameters of the spots of each sugar on the paper, it was found that the concentrations vary as to the activity of the enzyme derived from the culture filtrates of the different isolates. The result showed that *Stachybotrys* sp. and *Verticillium* sp.

TABLE IX

The effect of glucose and cellobiose on the in vitro activity of cellulase from Zygorrhynchus sp. on filter PAPER

Culture filtrates (C.F.)	µg reducing sugar released/mg substrate at various incubation periods (hours)			
	12	24	36	48
CF	6.1	14.6	15.0	15.1
CF + 50µg Glucose	6.2	14.0	15.5	15.8
CF + 100µg "	5.8	13.9	14.5	16.0
CF + 500µg "	5.9	14.1	14.3	16.0
CF + 1000µg "	-	-	-	-
CF + 2000µg "	-	-	-	-
CF + 50µg "	5.8	13.2	13.4	14.2
CF + 100µg "	6.1	14.3	14.6	15.1
CF + 500µg "	6.0	14.4	15.3	14.0
CF + 1000µg "	-	-	-	-
CF + 2000µg "	-	-	-	-

TABLE X

Chromatographic identification of reducing sugars released by cellulase in culture filtrates obtained from various organisms

Name of Organism	Type of Sugar	
	Cellobiose	Glucose
Cephalosproium sp.	++	+
Curvularia sp.	+	++
Helminthosprium sp.	+	++
Pythium sp.	+	++
Stachybotrys sp.	++	++
Trichoderma sp. 2	+	++
Verticillium sp.	++	++
Zygorrhynchus sp.	+	+++

contained both sugars in equal proportions. Glucose was the dominating sugar in the remaining fungal isolates except *Cephalosporium* sp., where the concentration of cellobiose was higher than that of Glucose.

CHAPTER IV

DISCUSSION

Generally, isolation of cellulose-degrading microorganisms from the soil,or any decaying cellulosic substrata is not an easy task. This is mainly due to lack of strict standard procedures in the cultivation of most of the organisms (Warcup, 1967). Even though baiting methods are normally applied for the isolation of these organisms, no single baiting technique exists to cover all of them (Willoughby, 1960).

The distribution of microorganisms is affected by many ecological factors. Of these factors, the nature and condition of the substrata play a major role. The conditions of the substrata govern the pattern of colony succession. This, in turn, dictates the type and number of microorganisms which can be isolated under laboratory conditions.

Exhaustive search for cellulolytic microorganisms from different sources of the environment was not carried out in this research. However, the few sample sources from which these organisms were isolated do seem to indicate that they are widespread in nature (TABLE I).

Cellulolytic members of the genera Aspergillus Trichoderma, Cephalosporium, and Curvularia were isolated from most of the cellulosic source i.e. decomposing plant debris, garden soil, arable soil and manure (TABLE I). The capacity to produce large quantities of spores, rapid production of mycelia, ability to elaborate inhibitory substances to avoid competition, and tolerance of many metabolic waste products in the substrata enable these organisms to colonize various cellulosic sources (Barton, 1960, Garret, 1962; Alexander, 1964; Thomas and Dietz, 1966; Smith and Long, 1981).

The isolation of Helminthosporium sp., Pythium sp., and Stachybotrys sp.from mainly garden and arable soils results from the fact that these fungi are active members of the soil microflora, and exhibit co-dominance with the highly sporulating fungi such as Trichoderma and Aspergillus species (Saito, 1955; Nicot, 1960; Tribe, 1960). Though Chaetomium and Fusarium species were not isolated from all sources in this research, they were frequently recorded from different cellulosic substrate by others (Webster, 1970). The isolation of Chaetomium sp., and Rhizopus sp., from manure shows that these caprophilic fungi are the pioneer colonizers of dung and other animal excreta which are often rich in cellulosic substances (Gwynne-Vaughen and Barnes, 1965).

The rate of growth of cellulolytic organisms was found to vary on the filter paper due to the inherent capacity of the organisms to produce the cellulase enzyme system. (Norkrans, 1967). Consequently, *Trichoderma* sp. 1 and 2, *Aspergillus niger*, *Curvularia* sp., *Helminthosporium* sp., *Rhyzopus* sp., *Stachybotrys* sp., *Chaetomium* sp., and *Zygorrhynchus* sp., showed faster growth rates (TABLE II and TABLE III).

Although growth of the organisms on the suhstrates is dicatated by the induction of cellulase enzyme, this does not necessarily imply the elaboration of the enzyme into the culture medium. Out of the organisms that exhibited high growth rates on filter paper, only *Cephalosporium* sp. *Helminthosporium* sp., *Pythium* sp., *Stachybotrys* sp., *Trichoderma* sp. 2, *Verticillium* sp. and *Zygorrhynchus* sp., were shown to display cellulase activity in their culture filtrates (TABLE V).

In this study, the isolates of the commonly cellulolytic members of the genera *Alternaria*, *Chaetomium* and *Fusarium* were not shown to elaborate cell-free cellulase in the medium. Even the isolates from the famous genus *Aspergillus*, whose cultures have been used as normal source of cellulase for industrial use (Lynch *et al.*, 1981), did not exhibit cellulase activity in their culture filtrates (TABLE V).

The difference between detection of growth on filter paper in stationary culture and cellulase elaboration in shake culture emanates from various factors. The nature of the enzyme system, and whether the different components in the system are cell-bound or not, the adsorption and release of the enzyme from the substrate, inactivation during mechanical shaking and foaming during agitation are the major factors governing the detection of cellulase activity in culture filtrates (Norkrans, 1963; Kudryashova *et al.*, 1974).

The gwowth of cellulose-decomposing fungi; their elaboration of cellulases in the medium, and the rate at which they degrade the cellulosic substrate on which they grow were found to be influenced by the origin and nature of the cellulosic material (TABLE VI). This difference is as a result of the degree of polymerization and the content of non-cellulosic material found in the substrate which, not only dicate the quality and quantity of the enzyme to be produced, but also influence the kinetics of the enzyme on them(Halliwell, 1966; Lynch *et al.*, 1981).

Only *Cephalosporium* sp., *Trichoderma* sp., *Verticillium* sp., and *Zygorrhynchus* sp. exhibited growth and cell-free cellulase activity on high-ordered cellulose namely, cotton

and cellulose powder (TABLE VI). Activity on cotton is attributed to the presence of the major components, particularly C_1 in the enzyme system of the organism. The concerted action of C_1 and the other components results in the solubilization of cotton and native celluloses (Mandels and Reese, 1964; Halliwell, 1981).

Cellulases derived from culture filtrates of Cephalosporium sp., Trichoderma sp., Verticillium sp., and Zygorrhynchus sp., grown on different substrates showed different activities (TABLE VI). Because the best growth and cellulase elaboration was displayed on filter paper, this indicates that these organisms favour highly-modified cellulosic substances more than crystalline and native celluloses (Mandels and Reese, 1964). Good growth and cellulase elaboration shown by some of these fungi on hay signifies the point that non-cellulosic components of this substrate play an important role for the induction and activity of the enzyme system (Babiskaya, and Stakeen, 1981, Moo-Young et al., 1981).

Cellulases induced either on filter paper, cotton, hay or newspaper displayed their optimum activity when assayed on the same substrate in which growth of the organism was initiated. Hence the use of an assay substrate different from that of the growth medium invariably yields different results (TABLE VII).

This shows that the substrate on which the organisms grow dictates the type and amount of cellulase that can be produced.

The cumulative activity of culture filtrates derived from the different paired organisms on hay and cotton was shown to be higher than the individual activity from each organism (TABLE VIII). This is mainly due to the fact that all organisms do not produce all the components evenly in their cellulase system . The addition of different culture filtrates therefore, induces higher activity as a result of the combined action of the components derived from the individual enzyme system (Desrochers and Jurasek, 1981).

Apart from the nature of the substrate which dictates the production of the cellulase enzyme, pH was also shown to affect growth rates and cellulase elaboration. The different results obtained from the stationay and shake cultures showed that the fungi favour acidic and near-neutral pH values for their growth and for the excretion of cellulase into the culture medium (TABLE IV, Fig. 1 & 2).

This finding agrres with previous works which showed the wide range of pH tolerance displayed by cellulose-decomposers (Bhasu & Ghose, 1960; Alexander, 1964; Halliwell, 1971). However, cellulase elaboration exhibited by 6 of the 8 organisms at low pH results from the fact

that low pH causes lysis of the mycelial cells. The disruption of the cells by lysis releases the cell-bound cellulase into the culture medium which, in turn results in the activity of the enzyme in the culture filtrates (Norkrans, 1963).

The inhibition of cellulase activity in vitro by cellobiose and glucose on the culture filtrate of Zygorrhynchus sp. was not achieved at concentrations of 50, 100 and 200 μg/ml. However, glucose and cellobiose have been shown to reduce the percentage activity of cellulase obtained from Trichoderma viride at concentrations of 0.036-5.2 mg/ml. (Wood, 1971).

The reducing sugars released during cellulose hydrolysis by the fungi tested were glucose and cellobiose (TABLE X). The difference in the concentration of the two sugars released by cellulolysis is one indication of the type and concentration of the enzyme components induced by the substrate. The high concentration of cellobiose in the assay mixture implies the higher activity of the carboxymethyl cellulase (C_x) component in the system, whereas the higher glucose content in the enzyme hydrolysate is the manifestation of the high activity of β-glucosidase

REFERENCES

Alexander, M. (1964). Introduction to Soil Microbiology. London, New York, John Wiley and Sons. 472 pp.

Anonymous (1974). Making sugar and protein from trash. Environ. Sci. Tech. 8(9): 784-785.

Arima, K. (1964). Microbial enzyme production. In: Global Impacts of Applied Microbiology. pp 420-422. Edited by S.D. Starr., Wiksells: Uppsala, Sweden.

Arujo, A., Alberto, A., Douza, J., and Karrande, C. (1981) Studies on fungi and yeasts from the west coast of India. In: Biol. Abst. 73(9): 59846, p. 6214.

Babiskaya, V.G., and Stakeen, I.V. (1981). Single-cell protein from fungi. In:Biol. Abst. 73(3): 16975, p. 1753.

Barton, R. (1960). Antagonism amongest some sugar fungi. In: The Ecology of Soil Fungi.pp. 160-167. Edited by D.E. Parkinson and L.T. Waid. Liverpool University Press: Liverpool.

Beneke, E.S., Rogers, A.L. (1970). Slide culture method. In: Medical Mycology Manual. 3rd ed. Burgers Publishing Company: Minnesota.

Bessey, E.A. (1950). Morphology and Taxonomy of Fungi Philadelphia, Toronto: Blakiston. 791 pp.

Bhasu, S.N., and Ghose, S.N. (1960). The production of cellulase by fungi on mixed cellulosic substrates. Can. J. Microbiol. 6, 265-281.

Clark, M.J. and Swittzer, L.R. (1977). Experimental Biochemistry. 2nd ed. Freeman: San Francisco

Clements, F.W. (1964). The Genera of Fungi. 4th ed. . Hafner: New York. 496 pp.

Cooney, C.L., and Wise, D.L. (1975). Thermophilic anaerobic digestion of solid waste for fuel gas production. Biotech. Bioeng. 17, 1119-1135.

Desrochers, M., Jurasek, M.G. (1981). High production of β-glucosidase in Schizophylum commune, Trans. Br. Mycol. Soc. 75(3): 451-454.

El-Kady, I.A. (1981). Survey on the cellulose-decomposing fungi of wheat in Egypt. Mycopathologia. 76(1) 59-64.

Eggins, H.W.W., and Pugh, G.J.F.(1962). Isolation of cellulose-decompasing fungi from the soil. Nature, London. 193, 94-95.

Erikson, K.E. and Petterson, G. (1967). Resistance of cellulose to enzyme attack. Arch. Biochem. Biophys. 21, 125-136.

Fermor, T.R., and Eggins, H.O.W. (1981). Use of Actinomycetes in textile biocide testing. In: Biol. Abst. 72(3): 66028, p. 6810.

Garret, S.D. (1962). Ecological groups of soil fungi· a survey of substrate relationships. New Phytol. 60, 149-166.

Gilman, J.C. (1957). A manual of Soil Fungi. 2nd ed. 495 pp. Imes, Iowa State College Press: Iowa.

Gray, T.R.G. and Williams, G. (1975). Soil Microorganisms. Longman: New York p. 144.

Groleau, D. (1981). Cellulolytic activity of rumen bacterium Bacterioides succinogenes. J. Gen. Microbiol. 123(2): 287-296.

Gupta, J.K., Shirkot, C.K., and Hawan, S.D. (1981). Growth of bacteria and yeast on enzymatically degraded rice and straw. Acta. Microbiologia Acad. Sci. Hun. 28(2): 131-140.

Gwyne-Vaughan, H.C.I., and Barnes, B. (1965). The Structure and Development of the Fungi. 2nd ed. 479 pp. Cambridge University Press. Cambridge.

Halliwell, G. (1966). Solubilization of native and derived forms of cellulose by cell-free microbial enzymes. Biochem. J. 100, 315-318.

Halliwell, G., (1972). The role of component C_1 in cellulolytic systems. Biochem. J. 127. 43-45.

Halliwell, G., (1978). Affinity chromatography of the cellulase system of Trichoderma koningii. Biochem. J. 169, 713-715.

Halliwell, G., (1981). The action on cellulose and its derivatives of a purified 1, 4-ß-glucosidase from Trichoderma koningii. Biochem. J. 199, 409-417.

Han, Y.M. and Callihan, C.D. (1974). Cellulose fermentation: effect of substrate pretreatment of microbial growth. Appl. Microbiol. 27, 1-10.

Han, Y.W., and Srivansan, V.R. (1968). Isolation and characterization of a cellulose-utilizing bacterium. Appl. Microbiol. 16(8): 1140-1145.

Harper, S.H.T., and Lynch, J.M. (1981). The kinetics of straw decomposition in soil. Journal of Soil Science. 32, 169-172.

Hartley, B., and Walgate, R. (1980). The biology business. Nature, London. 283, 122-123.

Horton, J.C., and Keen, N.T. (1966). Effect of glucose on cellulase induction. Can. J. Microbiol. 12, 209-220.

Howell, J.A., and Stuck, J.D. (1975). Kinetics of solka floc cellulose hydrolysis by Trichoderma viride cellulase. Biotech. Bioeng. 17(6): 873-894.

Imire, F. (1975). Single-cell protein from agricultural wastes. New Scientist 66, 458-460.

Imshensky, A.A. (1968). Cellulose degradation by bacteria. In: The Ecology of Soil Bacteria. pp. 130-140. Edited by Gray. S.D., and Parkinson: University of Toronto: Toronto.

Jurasek L., Colvin, R.J., and Whitaker, D.R. (1967). Microbiological aspects of the formation and degradation of cellulosic fibers. Adv. Appl. Microbiol. 9, 131-166.

Khan, A.W., Duncan, W. J.N., and Vanderberg. L. (1981). Fermentative conversion of cellulose to acetic acid. Appl. Environ. Microbiol. 41(5): 1214-1218.

Kudryashova, T.I. Sidorova, I.I., and Feniksova, R.V. (1974). Cellulase of molds from tropical countries. In Biol. Abst. 61(12): 66330, p. 6888.

Leatherwood, J.M. (1965). Cellulase from Ruminococcus albus. Appl. Microbiol. 13, 771-775.

Li, L.H., Flora, R.M., Peter, T.W., and King, K.W. (1965). Individual roles of cellulase components derived from Trichoderma viride. Arch. Biochem. Biophy. 111, 439-445.

Lynch, J.M. (1979). The degradation of lignocelluloses in soil. Journal of Soil Science 30, 165-176.

Lynch, J.M., (1981). Cellulose decomposition in arable soils. Journal of Soil Science 32, 275-279.

Lynch, J. M., Slater, J.H. Benneth, J., and Harper S.H.T. (1981). Cellulase activities of some aerobic microorganisms isolated from soil. J. Gen. Microbiol. 5, 5-20.

Malloch, D. and Cain, R.F. (1973). The genus Thielavia Mycologia 65, 1055-1057.

Malshe, V.C. (1975). Protein from cellulose. Science Today. 6, 51-52.

Mandels, M. and Reese, E.T. (1964). Comparison of attack of substrate by T. viride. Develop. Ind. Microbiol. 5, 5-20.

Mandels, M., and Weber, J. (1969). The production of cellulases. Adv. Chem. Ser. 95, 391-4141.

McCrae, S.I. (1972). The action of Endoglucanase on cellulose by Trichoderma viride Biochem. J. 106, 121-123.

McCrae, S.I. (1978). The activity of the different components of cellulase on native cellulose. J. Gen. Microbiol. 3, 120-125.

McHale, A., and Coughlan, M. P. (1981). The cellulolytic system of Talaromyces emersonii Biochem Biophys. Acta. 662, 145-151.

Molina, O.E., Dandey, A.S., Calluri, T., and DeGalvez, N.P. (1981). Production of bacterial protein from mixed culture of cellulolytic bacteria. In: Biol. Abst. 73(4): 16523, p. 2456.

Moo-Young, M. (1981). Effect of physical and physiochemical pretreatment of food for single-cell protein production with Chaetomium cellulolyticus. Biotech. Bioeng. 23(11): 2417-2420.

Moo-Young, M., Douglas, M., McDonald, G., and Ling, A. (1981). Pollution control of swine manure and straw conversion by Chaetomium cellulolyticum, single-cell protein. Biotech. Bioeng. 23(11): 2417-2419.

Anonymous (1979) In: National Academy of Sciences. Microbial Processes: Promising Technologies for Developing Countries. Report of an ad. hoc. panel of the Advisory committee on Technology, Innovation, Board on Science and Technology for International Development, Commission on International Relations. Washington, D.C: National Academy of Science. pp.131.

Nicot, J. (1966). Some characteristic of the micro-flora in desert soils. In the Ecology of Soil Fungi pp. 125-132. Edited by D.E. Parkinson and L.T. Waid. Liverpool University Press: Liverpool.

Norkrans, B. (1963). Influence of some culture conditions on fungal cellulase production. Physiol. Plant. 16, 1-10.

Norkrans, B. (1967). Cellulose and cellulolysis. Advan. Appl. Microbiol. 9, 91-130.

Olutiola, P.O. (1976). A cellulase complex in culture filtrates of Penicillium citrinum. Physiol. Plant. 37, 313-316.

Olutiola, P.O. (1977). Cellulolytic enzymes in culture filtrates of Aspergillus clavatus. J. Gen. Microbiol. 102, 27-33.

Paul, L.H., Nielson, J., Sullivan, A.P., and Shepherd, M.G. (1977). Purification and properties of a cellulase from Aspergillus niger, Biochem. J. 165, 33-41.

Ramasamy, K., Meyers, M., Bevers, J., and Verachtert, H. (1981). Isolation and characterization of cellulolytic bacteria from activated sludege. J. Appl. Bacteriol. 51, 457-481

Reese, E.T., Siu., R.G.H., Li, M., and Levinson, H.S. (1950). Mechanism of cellulose degradation J. Bacteriol. 50, 485-497.

Roelofsen, P.A. (1965). Cellulose component of different fibers. Botan. Res., 2, 69-149.

Rose, H.A. (1961). Cellulase production by Micro-organisms. In: Industrial Microbiology 4th ed. p. 136-152, Butherworths London.

Saddler, J.N., Khan, G.L. (1981). Conversion of Cellulose to ethanol. Biotech. Lett. 3(6): 321-326.

Saito, T. (1955). The significance of plate count of soil fungi and the detection of their mycelia. Ecol. Rev. 14, 69-74.

Sandhu, J.N., and Arora, K.W. (1981). Cellulolytic enzyme system of Acetilvibrio cellulolyticus. Can. J. Microbiol. 27(3): 288-294.

Selby, K., and Maitland, C.C. (1965). The fractionation of cellulase of Trichoderma viride by gel filtration. Biochem. J. 94, 578-583.

Selby, K., (1966). Components of Trichoderma viride cellulase. Arch. Biochem. Biophys. 118, 254-256.

Smith, H. (1966). An introduction to Fungi. 2nd ed. Hutchinson University Library: London. 424 pp.

Smith, I., and Seakins, J.W.T.(1976). Chromatographic and electrophoretic techniques. In Paper I: Layer Chromatography. 4th ed. pp. 236. Medical Book Publication: Chicago.

Smith, R.N., and Long, P.A. (1981). The effect of two fungicides on a population of cellulolytic fungi in soil and in pure culture. In: Biol-Abst. 72(10) 70365, p. 7238.

Srivansan, V.R. (1969). Microbial Conversion of waste cellulose into single-cell protein. In: Global Impacts of Applied Microbiology. pp. 476-485. Edited by Freitas, H., Fernandes, G. Chidambaran: Bombay, India.

Stevens, B.J.H., and Paynes, J. (1977). Cellulase and Xylanase production by yeasts of the genus Trichosphoron. J. Gen. Microbiol. 100, 381-393.

Stuzenberger, J.F. (1972). Cellulase production by Thermomonospora curvata isolated from municipal solid waste. Appl. Microbiol. 22, 147-152.

Susumu, O., Tanaka, T. Tokehito, O., and Yamamoto (1981). Methane fermentation of coffee. Agric. Biol. Chem. 45(4): 871-878.

Tanaka, M., Masayuki, T., Mastsuno, R., and Kamikubo, T. (1981). Purification and properties of cellulases from Eupenicillium javanicum. J. Ferment. Technol. 59(3): 177-184.

Thomas, R.P., and Dietz, A. (1966). Biological activity of Trichoderma viride. Appl. Microbiol. 14, 506-510.

Tribe, H.T. (1960). Decomposition of buried cellulose film in soil. In: The Ecology of Soil Fungi pp. 117-125. Edited by D.E. Parkinson and L.T. Waid, University Press: Liverpool.

Verachtert, H., Ramasamy, K., Meyers, M., Beavers, J. (1982). Investigation on biodegradation in activated sludge plant. J. appl. Bacteriol. 52, 185-190.

Warcup, J.H. (1967). Fungi in Soil. In: Soil Biology. pp. 51-102. Edited by Burges, A., and Raw, F. Academic Press. London and New York.

Wagner, W.W., and Davidson, R.W. (1954). Lignocellulosic component of hay. Botan. Rev. 28, 241-285.

Webster, J. (1970). Introduction to Fungi. Cambridge University Press: Cambridge.

Weimer, P.J.P., and Zeikus, J.G. (1979). Fermentation of cellulose and cellobiose by Chaetomium thermocellum. Appl. Env. Microbiol. 333, 289-297.

Weimer, P.J.P., Peter, P., Zeikus, J.G., and White, P. (1977). Fermentation of swine waste-corn mixture for animal feed. Europ. J. Appl. Microbiol. 4, 59-65.

Whitaker, D.R. (1971). Cellulases. In: The Enzymes. 3rd ed. pp. 273-283. Edited by Boyer, D. Academic Press: New York and London.

Whitaker, D.R. (1981). The activity of Cellobiohydrolase C_1 derived from Trichoderma viride. Biochem. Biophys. Acta. 662(1): 152-159.

Whitaker, D.R., Datta, Hanson, K.R., and Brikson, K.E. (1963). Fractionation of the cellulase from Myrothecium verrucaria. Can. J. Biochem. Physiol. 41, 671-673.

White, W. Bird, B.H.R., Sunde, M.L., Prentice, H., Burger, W.C., and Marlett, J.A. (1981). The Viscosity interaction of barley-glucan with T. viride. Poult. Sci. 60(5): 1043-1048.

Willoughby, L.G. (1960). The occurrence of some lower fungi in lake muds and soil. In: The Ecology of Soil Fungi. pp. 29-32. Edited by D.P. Parkinsonand L.T. Waid. Liverpool University Press: Liverpool.

Wood, T.M. (1971). Cellulase activity in Trichoderma viride Biochem. J. 121, 353-362.

Wood, T.M., 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.M., and McCrae, S.I. (1978). The cellulase of Trichoderma knoningii Biochem. J. 171, 61-72.

Wood, T.P., Wilson, A.C., Hency, F. and Stewart, S.C. (1982). Preparation of the cellulase from Ruminococcus albus and its release from the bacterial cell wall. Biochem. J. 205, 129-137.