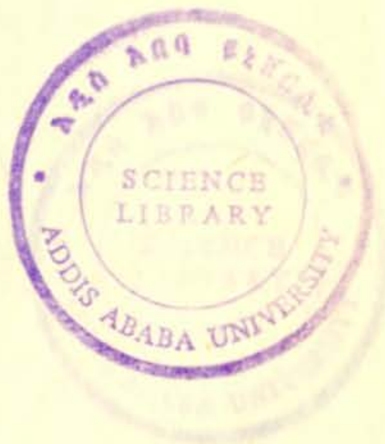
THE EFFECT OF SOME PHYSICO-CHEMICAL FACTORS ON THE PRODUCTION AND ACTIVITY OF CELLULASE AND β-GLUCOSIDASE FROM CLADOSPORIUM SP. BDCC-3



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A Thesis submitted in (part) fulfillment for the degree of Master of Science in Biology in the Addis Ababa University

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In Partial fulfillment
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Berhanu Abraha
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The Effect of Some Physico-Chemical Factors on the Production and Activity of Cellulases and β-Glucosidase from *Cladosporium* sp. BDCC-3

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ABSTRACT

Conditions for the production, release and activity of cellulases and β-Glucosidase from Cladosporium sp. BDCC-3 were investigated. Comparisons made between different carbon sources and their concentrations for maximum production of cellulases and β-Glucosidase depicted 1% CMC, 0.5% Avicel, 1.5% α-cellulose, 0.5% filter paper and 0.5% cotton to be optimum. From the nitrogen sources used, potassium nitrate was found to be the most utilizable by Cladosporium sp. BDCC-3. The optimum concentrations of nitrate for maximum production of the enzymes were 1%, 1%, 2%, 2% and 1% in CMC, Avical, α-cellulose, filter paper and cotton containing media, respectively. The most appropriate detergent for the release of cellulases and β-Glucosidase was 0.1% Tween-80. The enzymes had a wide range of pH. However, they were most active at pH 5.0 and 60°C. All enzymes lost activity when incubated at 70°C for 2 hours. Thus five different optimized media were formulated. These were:-

1. Optimized CMC medium 2(OCM-2) containing 1% CMC, 1% KNO_3 and 0.1% Tween - 80 plus the basal medium
2. Optimized Avicel medium 2(OAM-2) containing 0.5% Avicel, 1% KNO_3 and 0.1% Tween - 80 plus the basal medium.
3. Optimized α-Cellulose medium 2(OαM-2) containing 1.5% α-Cellulose, 2% KNO_3 and 0.1% Tween - 80 plus the basal medium.
4. Optimized filter paper medium 2(OFM-2) containing 0.5% FP, 2% KNO_3 and 0.1 Tween - 80 plus the basal medium.
5. Optimized cotton medium 2(OCOM - 2) containing 0.5% cotton, 1% KNO_3 and 0.1% Tween - 80 plus the basal medium.

The fungus produced maximum level of CMC-ase, Avicelase and α-celluslase after 6 days of incubation while Fp-ase, cottonase and β-Glucosidase were produced 3 to 6 days later. Cladosporium sp. BDCC-3 produced Cx(cmc-ase) much higher than C_1 (Avicelase, α-cellulase, FP-ase, cottonase) and β-Glucosidase. A comparative study of free, cytoplasmic and membrane bound enzymes showed that cellulases were extracellular while β-Glucosidase was cytoplasmic.

1. CELLULOLYTIC MICRO-ORGANISMS

Cellulose degrading micro-organisms are ubiquitous and are found in field and forest soils, in manure and on decaying plant tissues. These micro-organisms are involved in the decomposition of all types of cellulosic materials, contributing to the food and energy cycle of the living system. They regulate the dynamic equilibrium of carbondioxide in nature. Approximately $3x10^{10}$ tones of carbon in the form of carbondioxide is assimilated to plant material yearly (Norkrans, 1967). If microbial degradation of cellulose did not occur, dead vegetation would have piled up and suppressed new carbondioxide production, thereby disturbing its equilibrium in nature.

Cellulolytic micro-organisms, although very small in kind when compared to their benefit, are destructive to the raw materials and end products of wood, cotton, and other natural textile fibers. They cause damage to fruits and vegetables; and, many are phyto-pathogenic (Norkrans, 1967). Their physiological heterogeneity permits the degradation of cellulose to take place in habitats with or without oxygen, at acidic or alkaline pH, at low or high moisture levels and from temperatures just above freezing to the thermophilic range (Imshenetsky, 1968; Alexander, 1977).

These diverse group of micro-organisms can utilize cellulose as their carbon and energy source. Many of the cellulolytic micro-organisms are found amongst bacteria and fungi. But considerable species of Actinomycetes and protozoa are also involved in cellulose degradation.

The genera Aspergillus (King and Simbert, 1963; Li and king, 1963; Stewart and Parry, 1981), Pestalotiopsis (Mandels et al, 1974), Chaetomium (Fergus, 1969; Alexander, 1977; Chahal et. al., 1977), Trichoderma (Mandels and Reese, 1957 Wood and McCrae, 1972; Inglin et al., 1980; Robison, 1984; Kubicek, 1987; Kubicek et al. 1988; Khan et al., 1989; Estarda et al. 1988; Stutzenberger and Caws, 1988), Fusarium (Matsumo et al, 1974), Humicola (Macris, 1984), Penicillium, Curvularia, Memnonella, Phoma, Thielavia and Rhizopus (Alexander, 1977) contain strongly cellulolytic species amongst them. Furthermore, some species belonging to Cephalosporium, Geotrichum, Helminthosporium, Phythium, Stachybotrys and Zygorrhynchus are also cellulolytic (Fassil, 1982).

Of the bacterial species, cellulolytic members are found in genera Bacillus, Vibrio, Cellomonas Cytophaga, Sporocytophaga, Clostridium, Corynebacterium, Polyangium and Pseudomonas (Pardee, 1961: Berg et al, 1972; Priest, 1977; Alexander, 1977; Goksoyr and Erikson, 1980). The major cellulolytic Actinomycetes include some species in genus Nocardia, Streptosporangium, Streptomyces and Micromonospora (Alexander, 1977; Modoveanu et al., 1983).

Fungi are believed to be the main agents of cellulose degradation in humid soils. The filamentous ones have high metabolic rates and digest cellulosic wastes quite rapidly. This is partly due to their hyphal organization which gives them a penetrating capacity as well as a continuous cellulase releasing

phase. Because of the high temperature reached during decomposition of organic matter, composts serve as good sources of cellulolytic, specially thermophilic, micro-organisms (Norkrans, 1967; Stewart and Parry, 1981).

Cellulolytic bacteria are generally of greater significance in semi-arid localities. In anaerobic environments, as in guts of herbivores, in the digestive juices of invertebrates and in the rumen of cattle, bacteria are the major decomposers of cellulose (Norkrans, 1967; Alexander, 1977).

Different environmental factors influence cellulose metabolism by cellulolytic micro-organisms. The major parameters which govern cellulose metabolism are the level of available nitrogen, temperature, aeration, pH, the presence of other carbohydrates and the relative proportion of trace elements (Pardee, 1961; Alexander, 1977; Stewart and Parry, 1981).

2. THE SUBSTRATE : CELLULOSE

Cellulose is the most abundant of all naturally occurring organic compounds, probably comprising a third of all the vegetable matter on earth (Mandels et al., 1974; Hudson, 1980; Desai et al 1982; Estarda et al, 1988). It occurs in flowering and non-flowering plants, in the algae, in many of the fungi and in cysts of at least some Protozoa (Alexander, 1977). In addition, cellulose is the dominating waste material from agriculture in the form of stalks, stems, and also is one of the main waste products both in nature (dead vegetation such as forest litter, grasses, etc) and from man made activities (city waste, news papers, timber, textile products etc.) (Mandels et al, 1974; Goksoyr and Erikson, 1980;

stewart and Parry, 1981). This polysaccharide is mainly localized in the cell wall which in mature plant tissue is often associated with lignin and other polysaccharides (Norkrans, 1950; Hall 1976). The cellulose content of different cellulosic materials vary from 91% in cotton to 46-55% in Baggase (Goksoyr and Erikson, 1980).

Structurally, cellulose is a linear polymer of D-glucose, the configuration being that of a B(1-4) D-glucose which on complete hydrolysis yield glucose and on partial hydrolysis, the disaccharide cellobiose (Wood, 1960; Chochrane, 1958). The terminal group (C_6) lies alternately on one side of the chain and the other. This gives the appearance of a repeating cellobiose unit, though there is no difference in the linkage between pairs. Since all but reducing group of the glucose residues are involved in the glucosidic linkage, cellulose lacks detectable reducing ends. Its chief reactions are those of its three free hydroxyl groups (Norkrans 1950).

Cellulose is composed of 3,000 to 15,000 glucose units with molecular weight of 200,000 to almost 2.4 million (Alexander, 1977; Decon, 1980).

Besides its super-abundance, cellulose in its lignified form (wood) is also a great chemurgic crop; a crop grown for industrial use. It serves for construction purposes and as essentially pure cellulose in paper, fiber and textile industries. In addition, the decomposition of this carbohydrate has a special significance in the biological cycle of carbon. Furthermore, considerable attention is being given now to the possibilities of creating enzyme hydrolysis industries (Goksoyr and Erikson, 1980).

2. CATEGORY OF CELLULOSES BASED ON THEIR AVAILABILITY FOR CELLULASES

The development from cellobiose to cellulose fiber is a progression in chain length. As chain length increases, there is an increasing tendency for chains to associate. The hydrogen bonds involved are weak individually but strong collectively. As a result, segments of adjacent chains become so closely and regularly packed that their hydroxyl groups are no longer accessible for disruption of the hydrogen bonds by water. The micro fibrils in native cellulose are roughly 100Å in width. Because of this consideration, the insoluble substrates are divided into roughly three groups (whitaker, 1971). Group I belongs to dried, native fibers such as cotton fibers, filter paper, various ground celluloses as well as celluloses which though partly degraded are highly crystalline. These are slowly hydrolyzed by some cellulases and resist hydrolysis by others. Group II includes celluloses which though not highly degraded are extensively hydrated. Cellulose which has been swollen by cold phosphoric acid is one of the best substrates of this group. Group III consists of substrates which are extensively degraded and hydrated. Cellodextrins obtained by deacetylation of acetolyzed cellulose are examples of this type.

An average cellulase molecule has a diameter of 5nm (Goksoyr and Erikson, 1980). This means that the interior structure of crystalline fibrils (10nm wide) can not be penetrated by cellulases or other molecules of similar size. They can only act on the amorphous (less ordered) parts and on the surface of the microfibrils. Cellulose is believed to be composed of very rigid (crystalline) and amorphous regions (Goksoyr and Erikson, 1980).

The number of glucosidic bonds available for enzyme action will thus be to a large extent dependent on the degree of amorphosity (swelling) of the cellulose. Moreover, cellulolytic enzymes adsorb on the surface of cellulosic substrates and this increase degradation of the substrates (Sounders and Chandra; 1988; Stutzenberger and Caws; 1988).

2.2 EFFECT OF PRETEREATMENTS ON CELLULOSE HYDROLYSIS

Mandels et al (1974) working on T. viride and Pestalotiopsis westerdijki compared milling, boiling, and alkali treatments. All the treatment types increased the availability of cellulose to the cellulases of the respective micro-organisms. But alkali treatment which in addition to its swelling effect also solubilize other components of the substrate such as lignin (singh et al, 1988) increased susceptibility of the substrate to hydrolysis. Moreover, double treatments, for instance milling the substrate and swelling it, further increased its susceptibility to enzyme hydrolysis.

Milling reduce size of the cellulose fibers while the other two (boiling and alkali treatment) have swelling effects. They break the strong hydrogen bonds and penetrate into the crystalline areas. As a result, the crystalline areas swell and become available to enzyme action. Halliwell (1961) working on Myrothecium verrucaria has reported that both the rate and extent of breakdown of cotton fibers increased after swelling and complete solubilization (90%) was achieved in 22 hours on addition of enough amount of enzymes. This was similar to the result of Selby (1961) who used the same test organism.

3. CELLULOSE DEGRADING ENZYMES

Cellulases and B-Glucosidase are enzymes which catalyze the hydrolysis of glucosidic bonds of cellulose and cellobiose, respectively. The enzymes, sometimes, are viewed as a single complex (the cellulase complex) physically aggregated to form a functionally descrete entity which bring about the effective hydrolysis of cellulose to glucose (Griffin, 1981; Chochrane, 1958).

Cellulases and B-Glucosidase, like all other proteins, are synthesized in the cytoplasm. The synthesis of the enzymes is generally regulated by induction, repression and catabolite inhibition (Lehninger, 1973; 1977; Goksoyr & Erikson, 1980).

3 INDUCTION:- Although cellulases and B-Glucosidase appear to be constitutively synthesized in cellulolytic bacteria, they are inducible in fungi (Mandels and Reese, 1957; Rose, 1961; Whitaker, 1971; Kubicek, 1987). Since these enzymes are responsible for the hydrolysis of cellulose polymer into mono-, di-or Oligosaccharides, a B-14-glucan as an inducer is usually an essential component of the medium for their production. Carboxymethyl cellulose and hydroxyethyl cellulose can satisfy this condition (Whitaker, 1971). The disaccharide sophorose also is known to induce the enzymes (Inglin et al, 1980). But it is not efficient in some fungi including few strains of Trichoderma viride; and, this may be due to the insensitivity of the organisms under study to sophorose or a too rapid degradation of the disaccharide to carbondioxide and water (Rose, 1980; Goksoyr and Erikson 1980). The presence of free sophorose is usually required for continuous induction and production of cellulases.

A comparison of the relative inducing efficiencies of various celluloses is difficult because of differences in the surface areas, degrees of swelling (Hydration) and other factors which influence not only the availability of the substrate to the organism, but also the adsorption of cellulases to it (stewart et al, 1957).

Once the enzymes are induced, continued production is affected by many factors including the carbon, energy and nitrogen sources, mineral composition, pH of the medium and incubation temperature (Norkrans, 1950; Mandels and Reese, 1957: Stewart and Parry, 1981).

3.2 RELEASE FROM THE CELL

Enzymes of eukaryotic cells (fungi) are formed on the rough endoplasmic reticulum and associate with Golgi-equivalents that bud off cellulase containing vesicles. These vesicles release their contents into the free medium by exocytosis (Rose, 1980; Goksoyr and Erikson, 1980). Berg and Petterson (1977), in their study of cellulase production in a growing culture of T. viride, found that cellulases were first bound to particles (celluloses or mycelia). But when growth stopped sometime after cellulose has become depleted, there was a marked increase in free cellulases and a concomitant decrease in the bound activity. Thus cellulases, from an actively growing fungus, are either bound to the surface of the hyphae and act on the surface with which the hyphae are incontact and/or they are freely secreted into the environment diffusing away from the hyphae (cell free).

3.2.1 EFFECT OF DETERGENTS ON THE RELEASE OF CELLULOLYTIC ENZYMES

The extracellular libration of a product by a micro-organism requires either that the cell membrane is freely permeable to the product or that the product is actively transported out of the cell. In many Industrial Processes for the production of Metabolites, however, altering the permeability of the plasma membrane is a necessity (Niven et al. 1988; Demain and Birnbaum, 1968). This is achieved by the addition of detergents or by antibiotic treatment.

The interactions between detergents and cell membranes are very complex. They are dependent on many factors: pH, detergent concentration and chemical characterstics and membrane composition. (Niven et al, 1988).

3.3. MODE OF ACTION OF CELLULASES AND β-GLUCOSIDASE

A number of enzymes are involved in the degradation of native cellulose. One called 'C_1' releases individual chains (oligosaccharides and shorter chains of broken celluloses: Collodextrins) from native cellulose. The chains are then hydrolyzed by 'Cx' to cellobiose. Cellobiose is finally degraded to glucose by cellobiase: B-Glucosidase (Thomas and Whitaker, 1958; Wood, 1960; Goksoyr and Erikson, 1980). This is described schematically as follows (Norkrans, 1967):

$$\text{Cellulose} \xrightarrow{C_1} \underset{\text{(reactive cellulose)}}{\text{Oligosaccharides}} \xrightarrow{C_x} \text{Cellobiose} \xrightarrow{\text{B-Glucosidase}} \text{glucose}$$

Goksoyr and Erikson (1980) cancelled the terms C_1 and C_x to avoid confusion. Instead, they used the systematic names endoglucanase (1,4-β-D-glucan glucanohydrolase, EC 3.2.1.4) and Exoglucanase (1,4-β-D-glucan cellobiohydrolase, EC 3,2.1.91) for C_1 and C_x, respectively. The enzyme commission's name for β-Glucosidase is β-D-Glucoside glucohydrolase EC 3.2.1.21. (Rapp, 1989).

Endoglucanase breaks the cellulose ploymer in a random fashion to produce free chain ends while Exoglucanase cleaves cellobiose units from the non reducing chain ends. β-Glucosidase finally hydrolyzes cellobiose to 2 D-Glucose molecules (Hundson, 1980: Buchholz et al., 1983; Moldoveanu et al, 1983, Rapp, 1989; Bernier and Stutzenberger, 1989). These 3 enzymes when working synergestically bring about complete solubilization of native cellulose to glucose.

3.4 CHEMICAL AND PHYSICAL PROPERTIES OF CELLULASES AND β-GLUCOSIDASE

The chemical and physical properties of cellulases and β-Glucosidase vary from organism to organism. Even an enzyme from an organism has different isoenzymes (Decon, 1980; Griffin, 1981). This has been proven through electrophoretic fractionations of the components (Wood and Mccrae, 1972; Matsumo et al, 1974).

The cellulase system of T. viride was studied by Berghem et al (1976). The endoglucanase was found to be made up of several isoenzymes of which two were characterized. One had molecular weight of 12,500 of which 21% was carbohydrate. It is thus a

glycoprotein. The isoelectric point of this enzyme was 4.6. The second isoenzyme had molecular weight of 50,000 and it was also a glycoprotein containing 12% carbohydrate. Its isoelectric point was 3.39.

4.0 APPLICATION OF CELLULASES AND B-GLUCOSIDASE

Most of the out put of industrially produced cellulases is used as digestive tablets (Arima, 1964; Whitaker, 1971). Other current uses are related to the removal or softening of unwanted cellulose which interferes with extraction processes (Whitaker, 1971).

At present, Processes have already been developed to raise the protein content of straw to as high as 30% by growing cellulolytic molds on it (Steinkraus, 1978). This improves the nutritional quality of the straw as an animal feed. It is also possible to use lignocellulosics directly go to human food by growing mushrooms on them Mushrooms are reported to contain 2-5% protein on a fresh weight basis and 30% to 47% protein on dry weight basis (Steinkraus, 1978).

The use of cellulases in the preparation of protoplasts from plants with an ultimate goal of producing hybrids is a new development (Whitaker, 1971). This may prove to be of great practical importance in plant breeding. Cellulases together with proteases are also used to hydrolyze portions of the plant matrix to allow efficient dispersion and extraction of proteins (Childs et al., 1977).

Furthermore, the availability of C_6 sugars such as glucose is important in the development of biomass based chemical industries.

Pilot level studies have already shown that lignocellulosics have high potential in the production of biofertilizers, biochemicals, food, feeds and petrochemicals (Caripca et al., 1987). Cellulose, because of its low degradability, can not easily be converted to the above products with out first liberating the monosaccharides. Moreover, due to the use of high temperature in the chemical degradation of cellulose, large amount of by products are produced. Cellulases and B-Glucosidase catalyzed degradation of cellulose, however produce large amount of glucose at relatively lower temperature. Once the glucose units are produced, they can be converted to ethanol, n-butanol, acetaldehyde, acetone, glycerol, acetic acid, antibiotics, vitamins and etc. chemically and/or biologically. These products are extremely useful in the food, chemical and pharmaceutical industries. Thus, the role of cellulases in modern technology is great.

5.0 OBJECTIVES

The explosive increase in world population calls for parallel development in food production to all people around the globe. The current emphasis on micro-organisms in the chemical, agricultural, medicinal, pharmaceutical and specially food industries reflects this point.

An efficient mode of conversion of cellulose to nutritive proteins, sugars and other substances involve the isolation, selection and/or genetic modification of appropriate micro-organisms which can grow on cellulosic matter.

In this research, attempts have been made to:

1. determine the effect of different carbon sources on growth of Cladosporium sp. BDCC-3 and elaboration of CMC-ase, Avicelase, α-Cellulase, FP-ase, cottonase and β-Glucosidase.
2. elucidate the optimum carbon and nitrogen concentrations for maximum production of cellulases (CMC-ase, Avicelase, α-cellulase, FP-ase, cottonase) and β-Glucosidase from Cladosporium sp. BDCC-3.
3. Check if adsorption of CMC-ase occurred on a carbon substrate (cotton) in the growth meida.
4. compare availability of different nitrogen sources to Cladosporium sp. BDCC-3 for maximum production of cellulases (CMC-ase, Avicelase, α-cellulase FP-ase cottonase) and β-Glucosidase.
5. assess the effect of detergents on the release of cellulases and β-Glucosidase in to the growth medium.
6. determine the optimum detergent concentration for maximum release of cellulases and β-Glucosidase to the culture filtrate of Cladosporium sp. BDCC-3.
7. find the optimum pH and temperature for maximum activity of cellulases and β-Glucosidase.
8. define the optimized growth media for Cladosporium sp. BDCC-3 and elaborate the production of cellulases and β-Glucosidase at the optimized media.
9. determine the location of cellulases and β-Glucosidase: extracellular, cytoplasmic and/or cellbound.

II. MATERIALS AND METHODS

1. ORGANISM:- The test organism used in this study was a Cladosporium. sp. identified as BDCC-3. It was obtained from the Biology Department Culture Collection, AAU.

Stock cultures were maintained on sabouraud dextrose agar (SDA) slants and on strips of filter paper partly immersed in Dubo's mineral salt solution contained in capped test tubes (16X50mm). The Dubo's salt solution contained the following (g/litre). Sodium nitrate 0.5; Dipotassium orthophosphate 1.0; Magnesium sulfate 0.5; Ferrous sulfate 0.02; potassium chloride, 0.2 and yeast extract 0.5. The final pH was 7.5 (Ramasay, et al, 1981)

2. CULTURAL CONDITIONS

2.1 Basal Medium Composition:- The basal medium (BM) contained (g/100 ml) dihydrogen potassium phosphate 0.62; Hydrated magnesium sulfate 0.05; Calcium chloride, 0.05; Ferrous sulfate 0.001, yeast extract (difco), 0.2 and trace minerals 2ml(Gashe, 1988).

The trace minerals consisted of (μg/100ml) Boric acid, 0.5; copper sulfate, 1.0; calcium carbonate, 10.0; Ferric ammonium sulfate, 2.0; Molybdic acid, 1.0 and zinc sulfate, 5.0. The final pH of the medium before sterilization was 5.0.

2.2. Inoculum Size:- A 7 days old SDA slant culture of Cladosporium sp. BDCC-3 was used as source of inocula. To each slant, about 10ml physiological saline solution (PSS) was added asceptically. Spores

nd mycelial fragments were dislodged using an inoculating needle. he resulting suspension was transferred to sterile test tubes ollowing aseptic techniques. This was adjusted to give an optical ensity of 1.0 at a wave length of 540 nm (Bausch and Lomb pectronic-21-spectrophotometer). One ml of this suspension was hen used to inoculate 100 ml of sterile growth medium contained n a 250 ml Erlenmyer flask. All incubations were carried out on n Orbital shaker, GallenKamp, model SGM-300 (100 rpm) at room emperature (20°C).

2.3 Carbon Sources

The following cellulosic materials were used as carbon/energy sources. Each source was used at different concentrations (g/100ml BM).

1. Carboxymethyl Cellulose, CMC (No-C-5013, high viscosity sodium salt from Sigma chemical company) at concentrations of 0.5, 1.0, 1.5 and 2.0.
2. Avicel (Art. 2330, cellulose microcrystalline from Merck). at concentrations of 0.5, 1.0 and 1.5.
3. Alpha - Cellulose (NO,C-8002, α- cellulose fiber, approx. 99.5% from sigma chemical company) used at concentrations of 0.5, 1.0 and 1.5.
4. Filter-paper (Whatman No. 1) and
5. Dried absorbant cotton, each used at concentrations of 0.1, 0.5 and 1.0.

The carbon/energy sources were introduced into the basal medium before sterilization. The last two (filter paper and cotton) were pulverized to smaller pieces using a philips Blender (type ML-3232).

Evaluations for the optimum carbon/energy source concentrations for maximum enzyme production were made statistically using the t-test.

2.3.1 Test for adsorption

To determine if adsorption of the enzymes occurred on to the cellulosics, two ml culture filtrate obtained from a medium containing cotton was passed gradually through a column packed with 0.05 gram cotton. The cotton which served in the column, 1 ml of the filtered fluid collected from the column and 1 ml of the cul ture filtrate were incubated with 2.5% carboxymethyl cellulose at 40°C for 3 1/2 hours. Then the amount of adsorbed CMC-ase was quantified.

2.4. Nitrogen Sources

To ascertain the most available nitrogen source for Cladosporium sp. BDCC-3, three different nitrogen sources at different concentrations (g/100 ml of basal medium) were compared. These were potassium nitrate at 0.1, 0.5, 1.0, and 2.0; ammonium chloride and urea, each at 0.5, 1.0, 2.0, 3.0 and 4.0.

The concentrations of each source was adjusted in terms of the percentage composition of nitrogen in each compound. Urea was filter sterilized and added to the sterile media asceptically.

Prior to the study of the alternative nitrogen sources, potassium nitrate at all concentrations indicated was used as a sole nitrogen source in the study of carbon sources. When carboxymethyl Cellulose was used as carbon source the concentration of KNO_3, was at 0.1, 0.5, 1.0 and 2.0%. On the other hand, when

Avicel α- cellulose, Filter paper and Cotton were studied its concentration was at 0.5, 1.0 and 2.0%. The carbon and nitrate combinations in the basal medium enabled us to determine the best media for maximum production of cellulases and B-Glucosidase.

The comparison amongst the nitrogen sources was, thus, conducted at the optimum concentrations of each carbon and energy source used.

2.5 Effect of detergents

In order to compare the effect of detergents on the release of cellulolytic enzymes, three non-ionic detergents (Tween-80, Triton-x-100 and Tergitol-7) were used. Tween - 80 (%, v/v) at 0.3, 0.2, 0.1, 0.05 and 0.01 was tested in all media containing the appropriate concentrations of carbon source and potassium nitrate for maximum production of cellulases and B-Glucosidase. The two other detergents (Triton-x-100 and Tergitol-7) were used at concentrations of 0.1%, 0.05% and 0.01% in media containing Avicel, α- cellulose, filter paper and cotton. These detergents in Media containing CMC were used at concentrations of 0.01%, 0.05%, 0.1%, 0.2% and 0.3%. The growth media were autoclaved at 121°C (15 lbs/sq in) for 15 minutes.

3. ENZYME ASSAYING CONDITIONS

3.1 Activities of extracellular cellulases and β-Glucosidase

Ten ml of culture filtrate was removed at periodic interval from the culture medium of Cladosporium sp. BDCC-3. This was centrifuged at 4000 rpm for 10 minutes using IEC Model CL clinical

centrifuge (USA) to remove the mycelia. The clear culture supernatant served as source of cellulases and β-Glucosidase.

Activity of the enzymes was determined employing the method of Chahal _et al_ (1977):

A. Carboxymethyl cellulase (CMC-ase) activity:- One ml of the supernatant was incubated with 1ml of 2,5% CMC in 2ml of 0.1 M acetate buffer, pH 5.0, for 30 minutes.

B. Avicelase activity:- One ml of the supernatant was incubated with 50 mg of avicel in 3 ml of 0.1M acetate buffer, pH 5.0, for 3 1/2 hours.

C. α- cellulase activity:- One ml of the supernatant was incubated with 50 mg of α- cellulose in 3 ml of 0.1 M acetate buffer, pH 5.0, for 3 1/2 hours.

D. Filter Paper activity (FP-ase):- One ml of the supernatant was incubated with 50 mg of filter paper strip (whatman No.1) in 3ml of 0.1 M acetate buffer, pH 5.0, for 3 1/2 hours.

E. Cottonase activity:- One ml of the supernatant was incubated with 50mg of dewaxed and ground absorbant cotton in 3ml of 0.1M acetate buffer, pH 5.0, for 4 hours.

F. B-Glucosidase activity:- One ml of the supernatant was incubated with 1ml of 0.2% salicin in 2ml of 0.1M acetate buffer, pH 5.0, for 4 hours.

The reaction mixture was then incubated in an incubator shaker (150 rev/min) at 40°C for the specified duration. The activity of the enzymes was measured by quantifying the amount of reducing sugars released into the suspension.

3.2. Unit of enzyme activity.

A unit of enzyme activity in this study was defined as the amount of reducing sugars (μg) Produced in one minute under the stated assay conditions.

3.3 Activity of cytoplasmic and cell bound cellulases and β-Glucosidase

One hundred ml of culture was centrifuged at 4000 rpm for 20 minutes in a centrifuge (model 200, Gallen kamp, uk). The residue (cell mass) was washed with distilled water to remove the unutilized substrates. This was then recentrifuged at 4000 rpm for 20 minutes.

The washed mycelial mass was suspended in 30 ml acetate buffer, pH 5.0. This was sonicated at 20 amplitude-microns for 3 minutes using a sonicater, (soniprep 150, MSE, West Germany). The disrupted cell suspension was then centrifuged, at 50,000xG for 15 minutes using a refrigerated Heraeus centrifuge West Germany.

The supernatant and the residue (cell debris) served as sources of cytoplasmic and membrane bound enzymes, respectively.

The cell bound enzyme source (fragmented mycelial residue) was suspended in 20ml acetate buffer, pH 5.0. After appropriate dilutions it served as source of the bound enzymes.

Activities of the cytoplasmic and cell bound cellulases (CMC-ase, Avicelase, α- cellulase, FP-ase, cottonase) and B-Glucosidase were determined following the method of Chahal et al (1977) as described previously for extracellular enzymes.

3.4. Effect of pH and temperature on the activity of cellulases and β-Glucosidase

The pH of the buffers used for this study were 4.0, 5.0, 6.0. 7.0 and 8.0. Acetate buffer was used for pHs of 4.0 and 5.0 and phosphate buffer for the others (6.0, 7.0 and 8.0). The buffers had a molarity of 0.1. To avoid variations in spectrophotometric reading due to ionic interactions (Phosphate and acetate), each buffer at the selected pH was calibrated with its own blank i.e. the effect of pH 4.0 was calibrated with a blank made from pH 4.0 and etc.

The temperatures considered were ambient temperature (20°C), 40°C, 50°C, 60°C and 70°C. The orbital incubator-shaker (Gallen Kamp, cat. No. INR 200-0110N)was used for Temperatures 40°C-60°C. A water bath (thermostirer-85, Gallen Kamp) was used for 70°C.

4.0 CHEMICAL ANALYSIS

4.1 Determination of reducing sugars

The amount of reducing sugars released during the enzyme-substrate interactions was quantified using Nelson's method as described in Clark and Switzer (1977). The reagents used were the following.

Reagent A

Sodium Carbonate, 12.5 g; sodium bicarbonate, 10g; Sodium sulfate, 100 g; potassium sodium tartrate, 12.5 g; and distilled water to make a final volume of 500 ml.

eagent B

Hydrated copper sulfate, 7.5 g; one drop of conc. sulfuric cid and distilled water; all together making 50 ml.

Arsenomolybdate reagent

25 g of ammonium molybdate was dissolved in 450ml distilled water and 21ml of concentrated sulfuric acid was added to the solution. Three grams of sodium arsenate in 25ml distilled water was separately prepared. The two solutions were finally combined and incubated at 37°C for 24 hours. This was stored in a dark bottle.

Reagent C

This is the working reagent composed of 12.5 ml of reagent A and 0.5ml of reagent B. This working reagent was always freshly prepared.

The amount of reducing sugars was determined as follows: At the end of the desired incubation period of the enzyme-substrate reaction mixture, 2 ml sample was removed and mixed with 1ml of the working reagent (Reagent C). This was thoroughly mixed with a vortex mixer (Gallen Kamp) and then immersed in boiling water for exactly 20 minutes. At the end of the 20 minutes, the samples in test tubes were dipped in water to cool them quickly. Then, 1 ml of Arsenomolybdate reagent was added and mixed. After 5 minutes, the final volume of the samples was adjusted to 10 ml using distilled water. To avoid suspended particles, samples were

centrifuged at 2000 rpm for 10 minutes and then the colour developed was read at 540nm using 1cm light path Cuvette (Bausch and Lomb, spectronic - 21 - spectrophotometer). A blank which contained all the buffer ingredients except the enzyme source was used to zero the spectrophotometer.

The optical density readings were then transformed to µg reducing sugars using glucose as a standard.

4.2 PROTEIN DETERMINATION

The method used for the quantification of proteins released in to the liquid medium was that of Lowery et al (1951). The reagents used were (%, v/v):

1. 2% sodium carbonate (Na_2Co_3) combined with 0.4g of 0.1 N sodium hydroxide (NaOH).

2. 1% sodium tartarate
3. 1% copper sulfate and
4. phenol reagent (BDH chemicals, U.K)

The working reagent was prepared by combining 1,2 and 3 in the ratio of 98:1:1 (always freshly prepared). Five ml of the combination was mixed with 1ml of the sample aliquot. Then, after 10 minutes 0.5 ml of phenol reagent was introduced into the sample and mixed thoroughly. This was left for 30 minutes at room temperature for colour to develop. This was read at 750nm using a spectronic - 21 - spectrophotometer (Bausch and Lomb). Bovine serum albumin served as standard.

III RESULTS

1. Effects of carbon and nitrogen sources on production of cellulases and B-Glucosidase.

In this study, attempt was made to determine the optimum concentrations of five different carbon sources namely, Carboxymethyl cellulose (CMC), Avicel, α- cellulose, filter paper and cotton, for maximum production of cellulases and B-Glucosidase. The Cellulosic substrates selected were of different types (crystalline, semi-crystalline and amorphous) varying in their availability to the action of cellulolytic enzymes. Because of the differences in the nature of the celluloses, it was found necessary to determine the optimum concentrations of the substrates for maximum production of cellulases and β-Glucosidase.

Potassium nitrate (KNO_3) at different concentrations was used as sole nitrogen source. This enabled us to determine the optimum carbon source and potassium nitrate combinations required in the basal medium for maximum production of the cellulolytic enzymes. In addition, a time course study was made to assess the optimum time for production and/or release of the enzymes into the culture filtrate.

1.1 Carboxymethyl cellulose (CMC) as carbon source

Time course studies in media containing 0.5% CMC as carbon source (Fig. 1) indicated that maximum production of carboxymethyl cellulase (CMC-ase), Avicelase, and alpha-cellulase occurred on the 4th day of incubation. However, Productions of filter paper activity (FP-ase), cottonase and β-Glucosidase was found to be maximum on the 7th day of incubation. C_1 (Cotton activity or

cottonase) was not detected in the culture filtrate early. It appeared only after the 6th day of growth and it was found to be greater than avicelase, α-cellulase and β-Glucosidase in production. The Peak in enzyme production was delayed by 1 to 4 days as a result of increment in CMC concentration from 0.5% to 2.0%. At 1% CMC (Fig. 2), maximum production was detected on the 5th day for CMC-ase, Avicelase, and α- cellulase and on the 6th to 8th days for Fp-ase, cottonase and β-Glucosidase. Production of all the enzymes in media containing 1.5% and 2% CMC (Fig. 3 and 4) also occurred at days 6 to 8 with the exceptions of cottonase and β-Glucosidase, at lower concentrations of potassium nitrate in 2% CMC which had their maximum activities on the 4th day of incubation. In most cases, CMC-ase, Avicelase and α- cellulase reached their peak earlier than FP-ase, cottonase and β-Glucosidase.

Higher enzyme production was obtained as the concentration of potassium nitrate increased from 0.1 to 2.0% in all CMC containing media. However, the rate of increment in enzymatic activity due to increases of nitrate from 1% to 2% in most of the media was not that significant. Eventhough 100% increment in nitrate concentration (from 1 to 2%) was made in the basal medium containing 1% CMC as carbon source, the enzyme production increased only by about 10%. Exceptions were those media containing 0.5% and 2% CMC for the production of FP-ase and 1.5% CMC for the production of β-Glucosidase. Thus, 1% potassium nitrate was considered as the optimum concentration for maximum production of cellulose degrading enzymes. As far as CMC as carbon sources was concerned, maximum production (units/ml) of CMC-ase (60), Avicelase (6-8), α- cellulase (6.0) FP-ase (4-6), cottonase (3.0) and β-Glucosidase

(4.0) occurred in media containing CMC at 1%, 1.5% and 2.0% concentrations. Since there was no increment in enzyme production beyond the 1% CMC concentration, it was considered as the appropriate concentration for maximum production of cellulases and B-Glucosidase.

Hence basal medium containing 1% CMC and 1% potassium nitrate was found to be the best medium for production of cellulases and β-Glucosidase when CMC and potassium nitrate were considered as carbon and nitrogen sources. This medium here-after unless otherwise stated will be referred to as CMC medium-1 (CM-1). Using this medium maximum production of CMC-ase, Avicelase and α-cellulase occurred on the 5th day of growth and Fp-ase, cottonase and β-Glucosidase on the 7th day of growth.

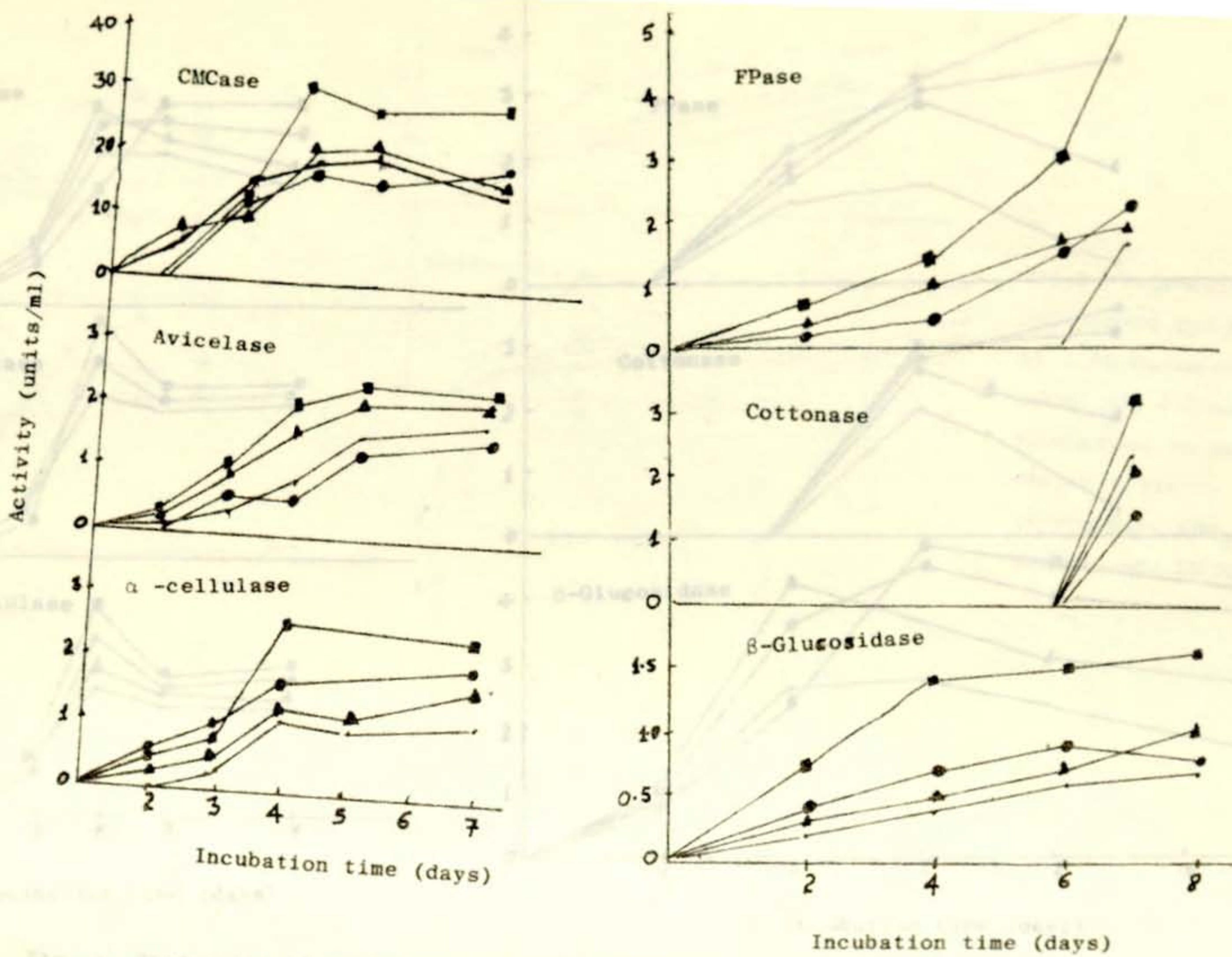


Fig.1. Production of cellusase and β-Blucosidase at 0.5% Carboxymethyl cellulose and 4 concentrations of KNO_3. Production is expressed in terms of enzyme activity. (——) 0.1% KNO_3, (▲——▲) 0.5% KNO_3, (●——●) 1% KNO_3, (■——■) 2% KNO_3.

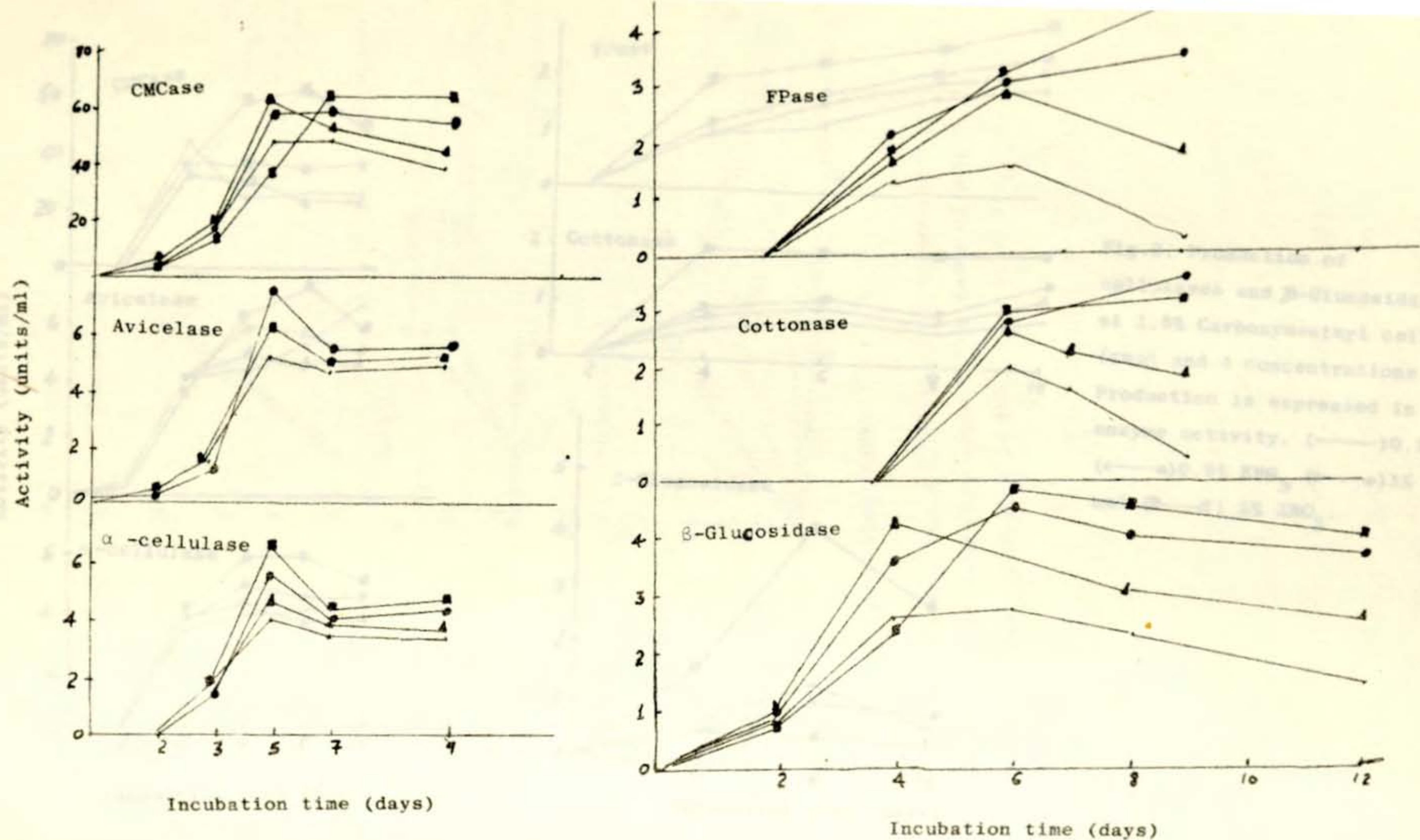
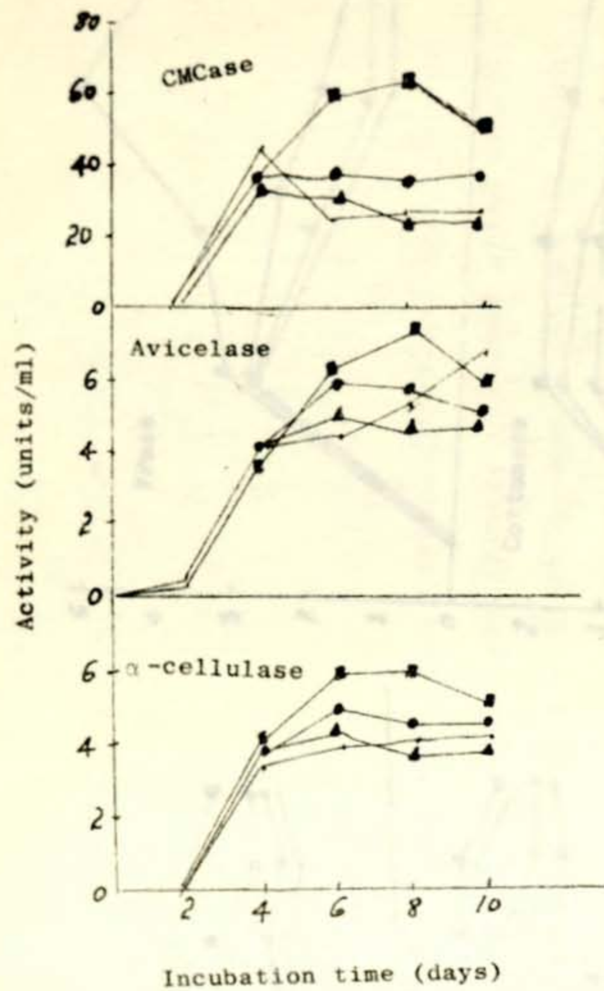


Fig. 2. Production of cellulases and β-Glucosidase at 1% Carboxymethyl cellulose (cmc) and 4 concentrations of KNO_3. Production is expressed in terms of enzyme activity. (——) 0.1% KNO_3, (▲—▲) 0.5% KNO_3, (●—●) 1% KNO_3 and (■—■) 2% KNO_3.



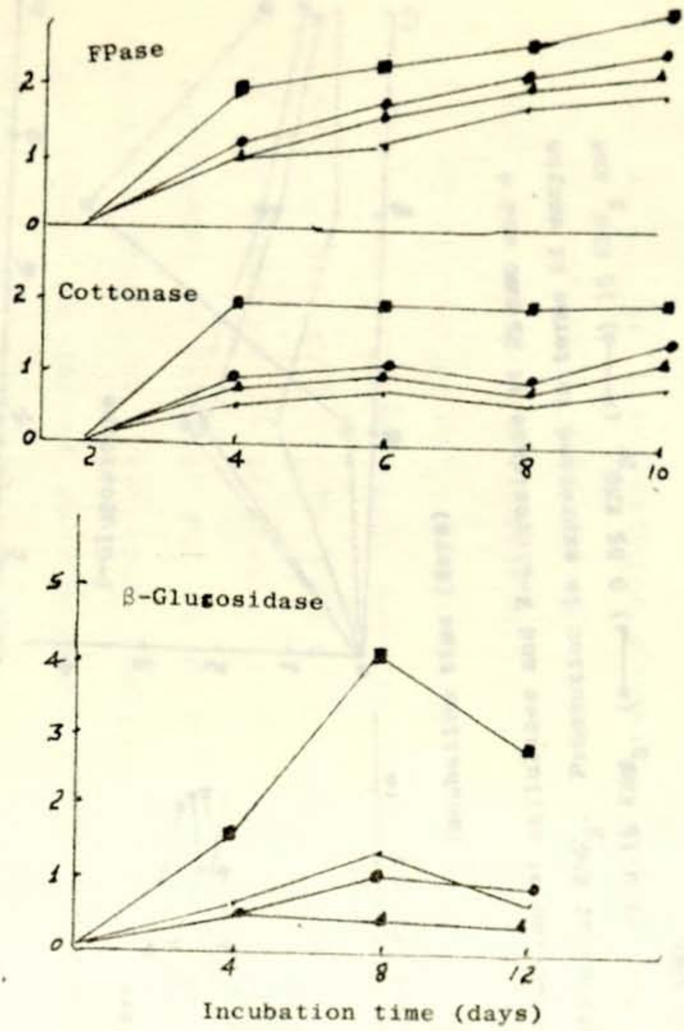


Fig.3. Production of cellulases and β-Glucosidase at 1.5% Carboxymenthyl cellul (cmc) and 4 concentrations of Production is expressed in te enzyme activity. (——)0.1% (▲—▲)0.5% KNO_3, (●—●)1% KN and (■—■) 2% KNO_3.

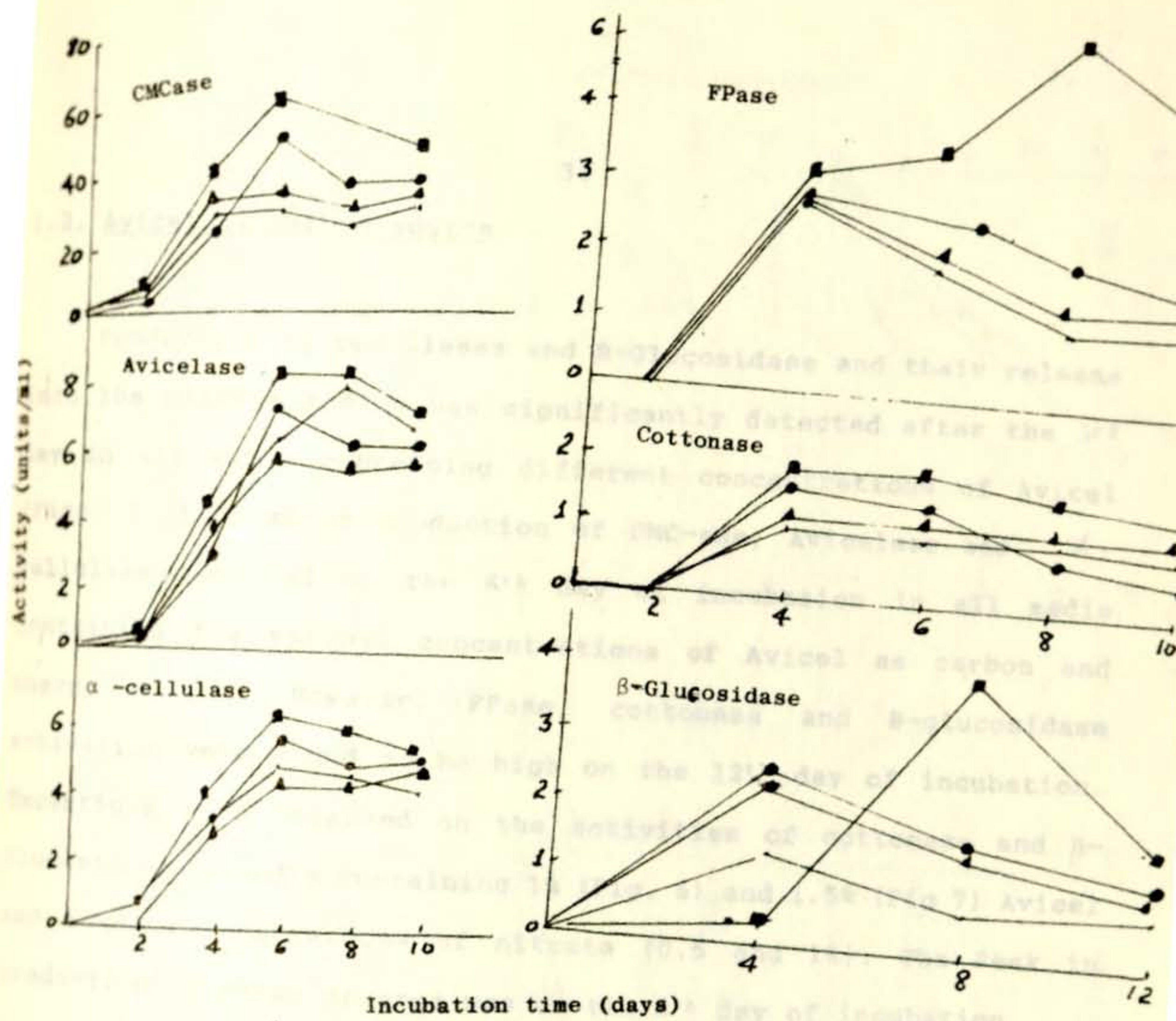


Fig.4. Production of cellulases and B-Glucosidase at 2% cmc and 4 concentrations of KNO_3. Production is expressed in terms of enzyme activity. (——) 0.1% KNO_3, (▲——▲) 0.5% KNO_3, (●——●) 1% KNO_3 and (■——■) 2% KNO_3.

1.2. Avicel as carbon source

Production of cellulases and β-Glucosidase and their release into the culture medium was significantly detected after the 3rd day in all media containing different concentrations of Avicel (Figs. 5-7). Maximum production of CMC-ase, Avicelase and α-cellulase occurred on the 6th day of incubation in all media containing the various concentrations of Avicel as carbon and energy source. However, FPase, cottonase and β-glucosidase activities were found to be high on the 12th day of incubation. Exceptions were observed on the activities of cottonase and β-Glucosidase in media containing 1% (Fig. 6) and 1.5% (Fig 7) Avicel and lower concentrations of nitrate (0.5 and 1%). The Peak in production of these enzymes was on the 8th day of incubation.

Production of all enzymes increased with increasing concentration of potassium nitrate. CMC-ase production was the most affected. Its activity increased progressively as the nitrate concentration was increased from 0.5% to 2%. Other activities did not respond as that of CMC-ase activity. The increments in activity in most media were less than 70% except in those containing 1.5% Avicel where double in activity was observed for CMC-ase, Fp-ase and cottonase. These elevated activities occurred as a result of the increment in concentration of potassium nitrate from 1% to 2%. Furthermore, maximum production (units/ml) of CMC-ase (8.4, 8.0, and 6.5), avicelase (1.4, 2.0 and 1.6), α- cellulase (0.5, 0.5

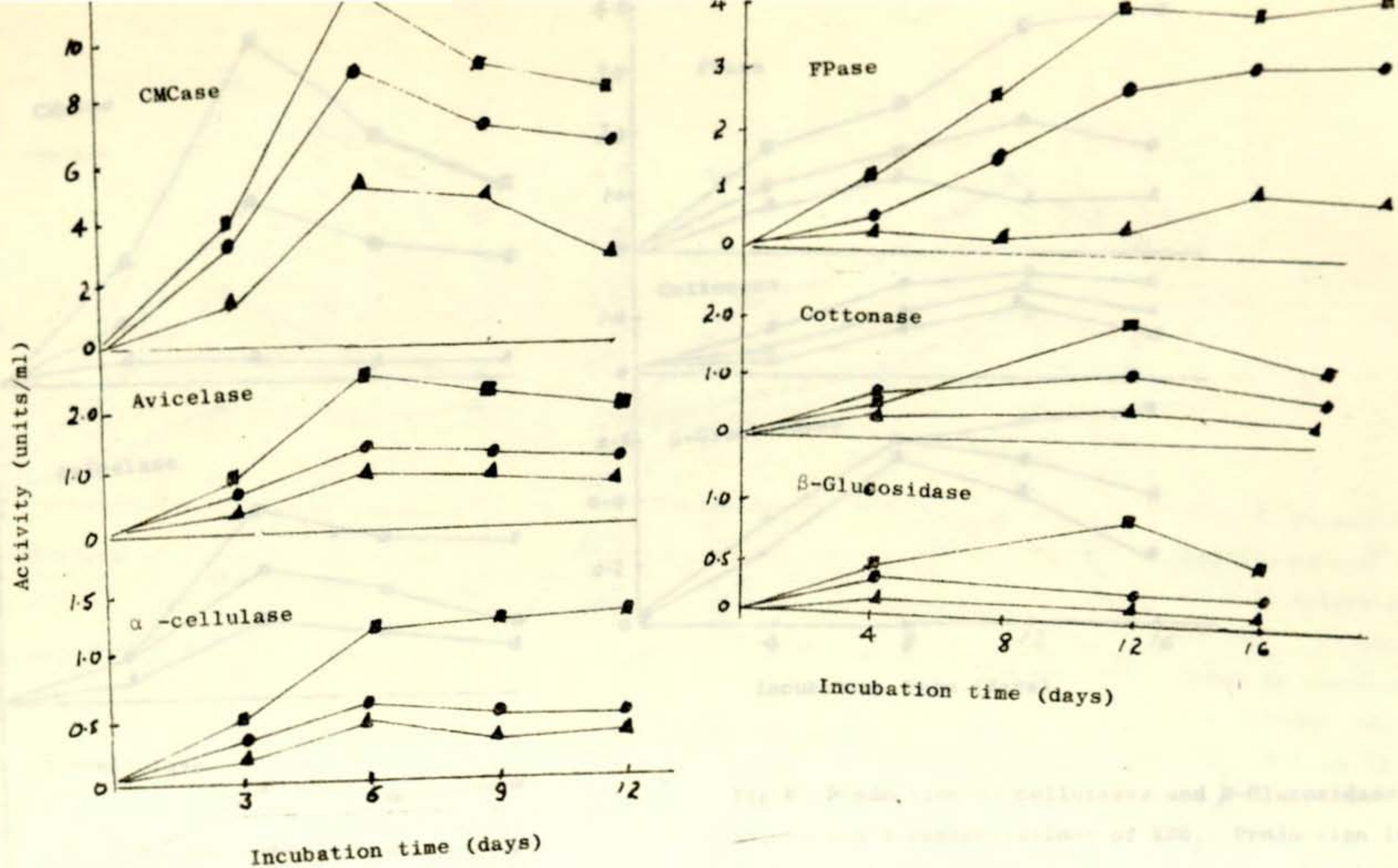
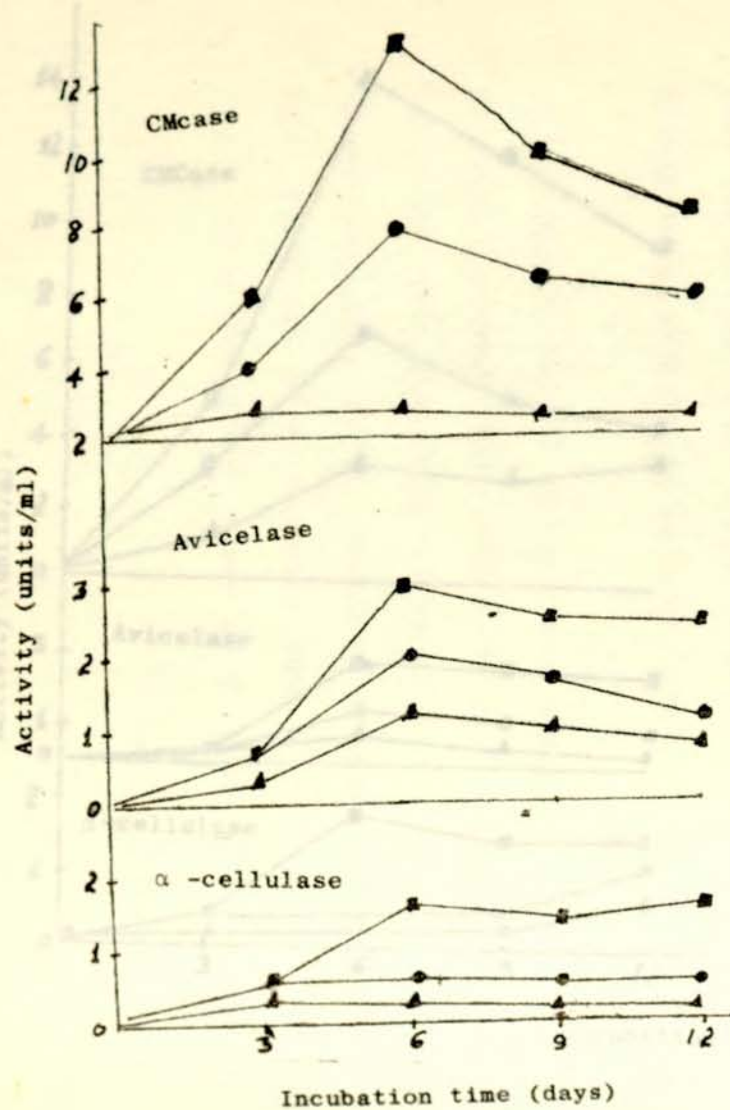


Fig.5. Production of cellulases and B-Glucosidase at 0.5% Avicel and 3 concentrations of KNO_3. Production is expressed in terms of enzyme activity. (▲—▲) 0.5% KNO_3; (●—●) 1% KNO_3 and (■—■) 2% KNO_3.



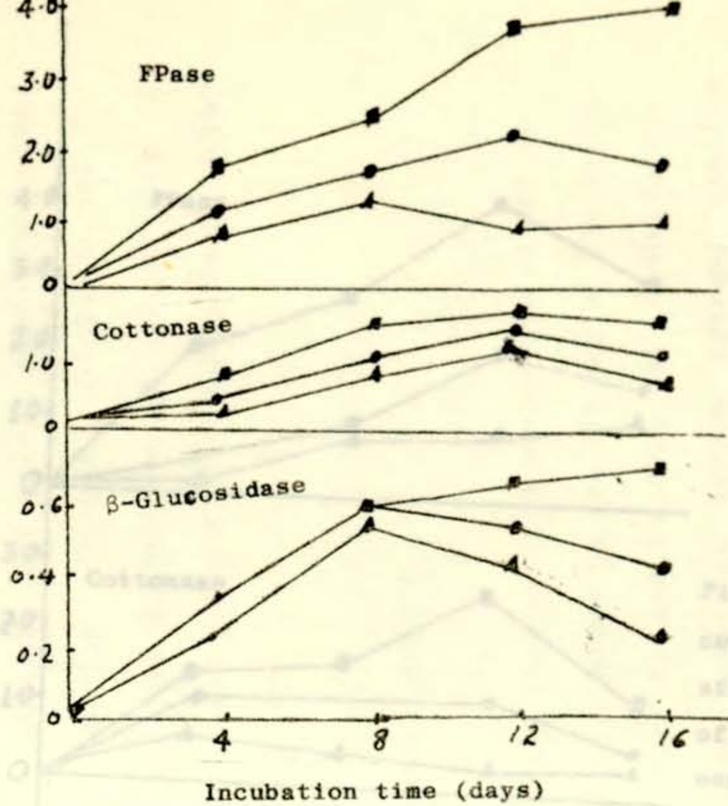
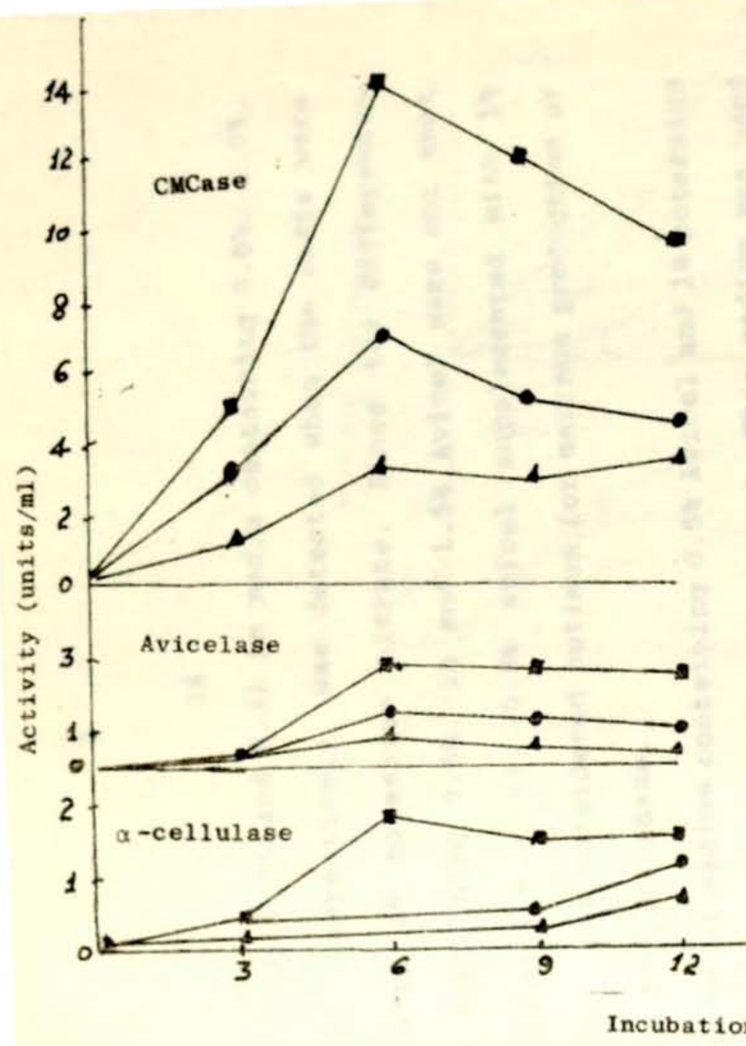


Fig.6. Production of cellulases and β-Glucosidase at 1% Avicel and 3 concentrations of KNO_3. Production is expresse in terms of the activity of the enzymes. (▲——▲)0.5% KNO_3, (●——●) %KNO_3 and (■——■) 2% KNO_3.



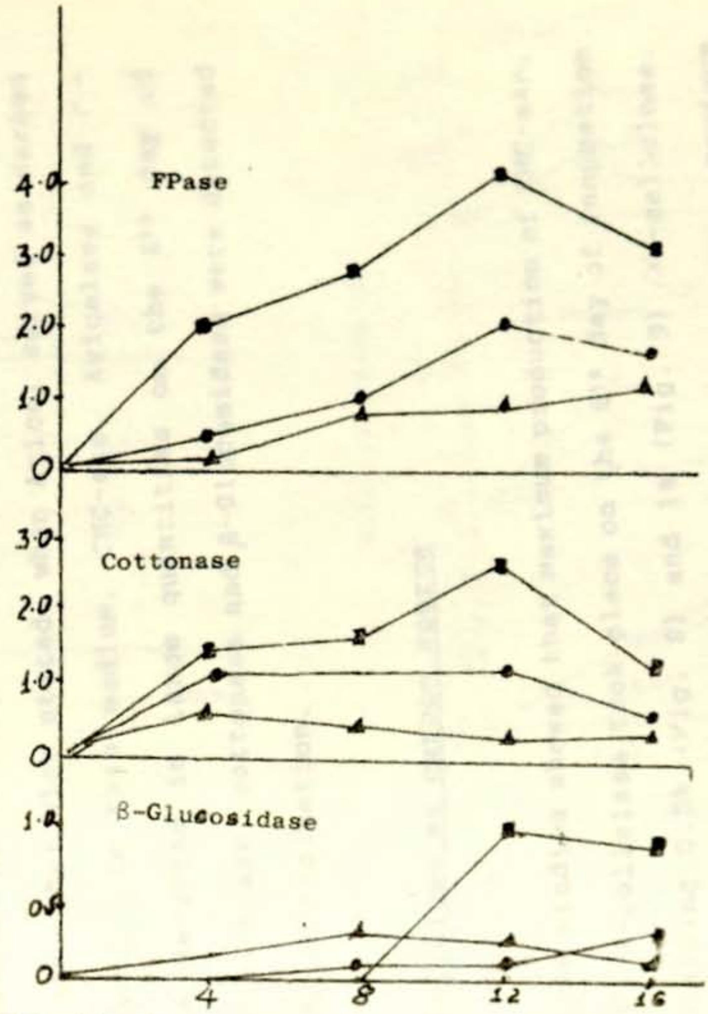


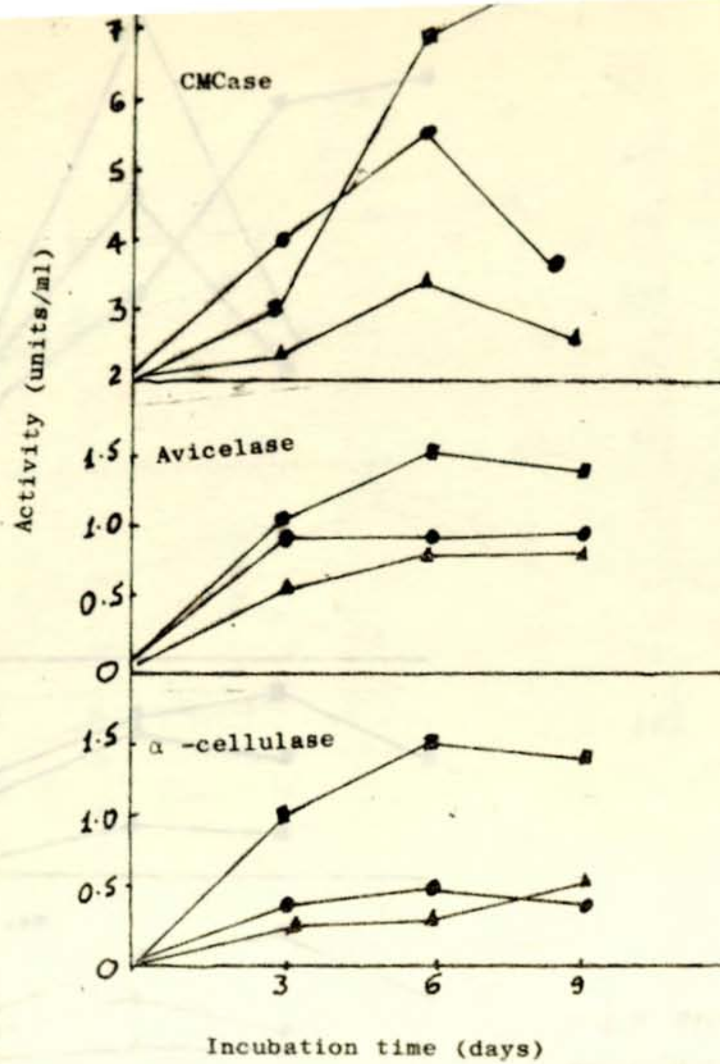
Fig. 7. Production of cellulases and β-Glucosi at 1.5% Avicel and 3 conc of KNO_3. Production is e essed in terms of activit the enzymes. (▲—▲) 0.5% KNO_3, (●—●) 1% KNO_3 and (■—■) 2% KNO_3.

β-Glucosidase (0.3, 0.56 and 0.3) in media containing 0.5%, 1.0%, and 1.5% Avicel, respectively, was detected when the media were supplemented with 1% potassium nitrate. Since the differences between media containing 0.5%, 1% and 1.5% Avicel were not that significant (appendix - T), 0.5% avicel supplemented with 1% potassium nitrate was considered optimum for maximum production of cellulases and β-Glucosidase.

Thus, the basal medium containing 0.5% Avicel and 1% potassium nitrate was named as Avicel medium-1 (Am-1). This medium was used hereafter, unless other wise stated, when Avicel served as carbon and energy source. In this medium, CMC-ase, Avicelase and α-cellulase were detected in large quantities on the 6th day of incubation while Fp-ase, cottonase and β-Glucosidase were detected on the 12th day of incubation.

1.3. <u>Alpha-cellulose as carbon source</u>

Time course studies showed that maximum production of CMC-ase, Avicelase and α-cellulase took place on the 6th day of incubation in media containing 0.5% (Fig. 8) and 1% (Fig. 9) α-cellulose. When 1.5% α-cellulose was used as carbon source, maximum production of these enzymes occurred on the 12th day of incubation (Fig. 10). Production of the other three enzymes (Fp-ase, cotton ase and B-Glucosidase) was the highest after 8 to 12 days of incubation in all media.



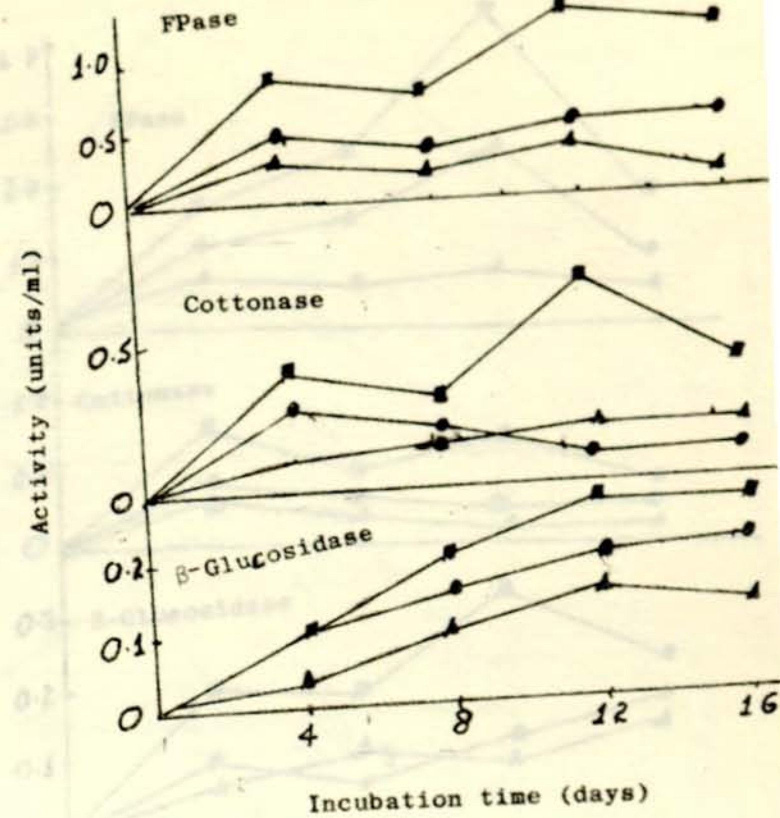


Fig.8. Production of cellulases and B-Glucosidease at 0.5% α-cellulose and 3 concentrations of KNO_3. Production is expressed in terms of activity of the enzymes (▲——▲) 0.5% KNO_3, (●——●) 1% KNO_3 and (■——■) 2% KNO_3

CMCase

Avicelase

α -cellulase

Activity (units/ml)

Incubation time (days)

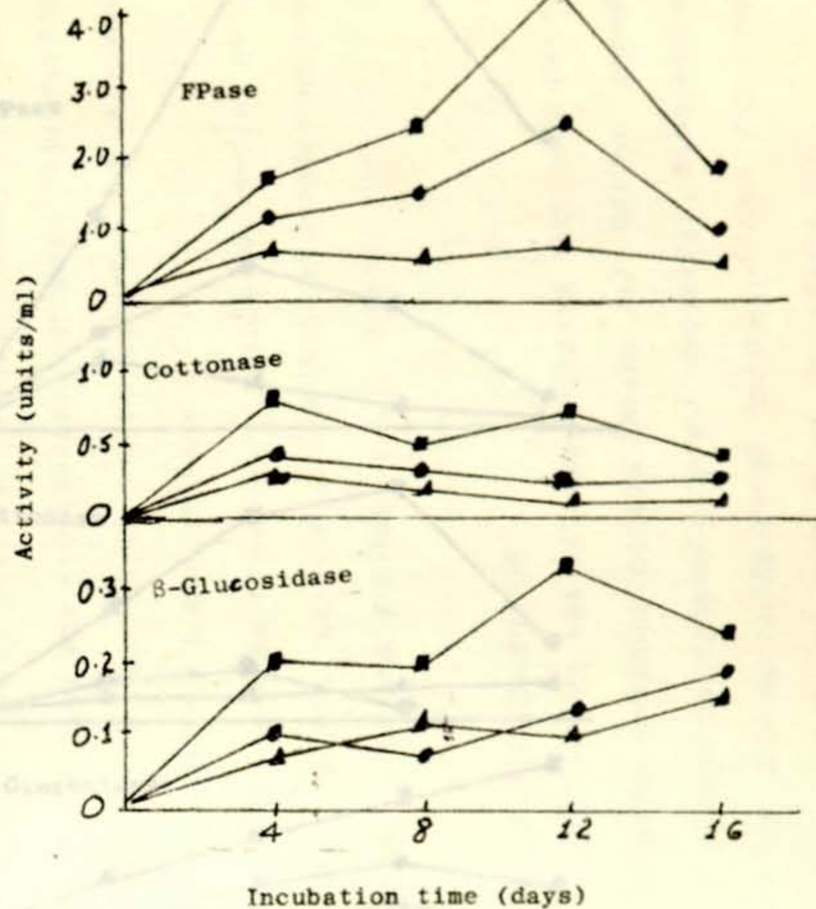


Fig.9. Production of cellulases and β-Glucosidase at 1% α-cellulose and 3 concentrations of KNO_3. Production is expressed in terms of activity of the enzymes. (▲——▲) 0.5% KNO_3, (●——●) 1% KNO_3 and (■——■) 2% KNO_3.

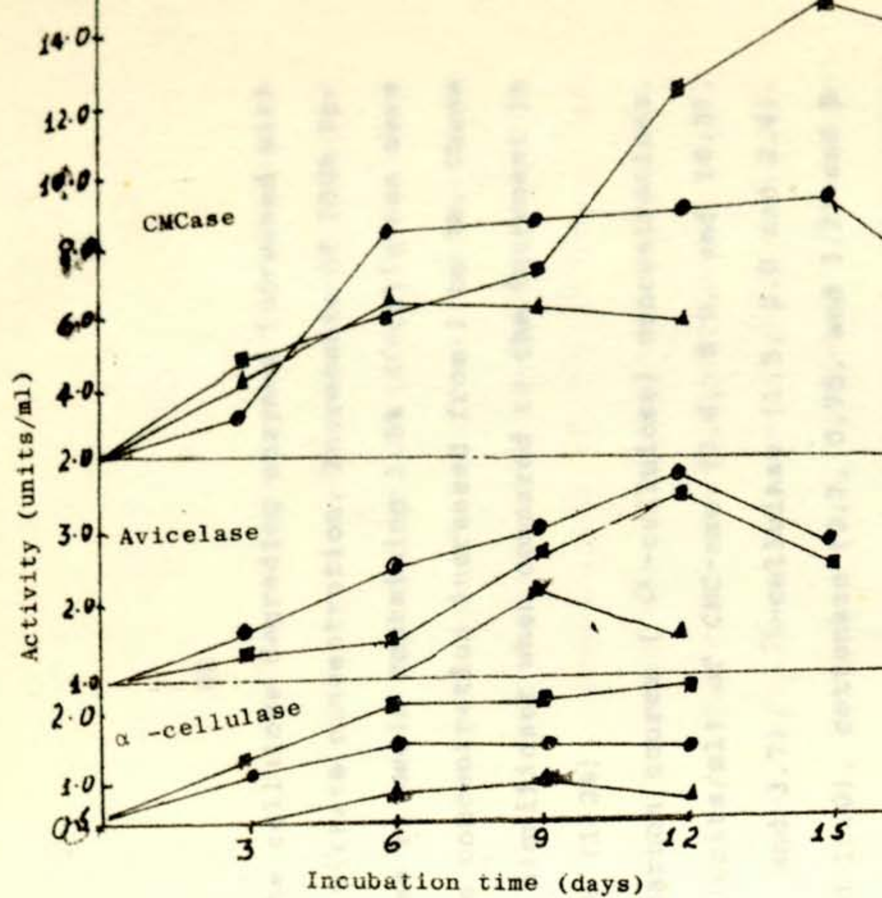


Fig.10. Production of cellulases and β -Glucosidase at 1.5% α-cellulose and 3 concentrations of KNO_3. Production is expressed in terms of activity of the enzymes. (▲—▲)0.5% KNO_3, (●—●) 1% KNO_3 and (■—■)2% KNO_3

Production of the cellulose degrading enzymes increased with increasing potassium nitrate concentration. Increments of 200% Fp-ase and 500% cottonase in media containing 1.5% α-cellulose were recorded when nitrate concentration increased from 1 to 2%. These increments are quite significant when compared to the increment in nitrate concentration (1-2%).

Regarding the carbon source (α-cellulose) concentrations, maximum production (units/ml) of CMC-ase (7.6, 8.9, and 14.3), Avicelase (1.5, 2.5, and 3.7), α-cellulase (1.5, 2.0 and 2.4), Fp-ase (1.4, 4.5, and 10.0), cottonase (0.7, 0.75, and 3.2) and β-Glucosidase (0.3, 0.31 and 0.6) were detected in media containing 0.5, 1.0 and 1.5% α-cellulose, respectively. Thus, the rate of increment in enzyme production from 1% to 1.5% α-cellulose when supplemented with 2% nitrate was significantly higher. This carbon and nitrogen combination was found to be optimum for maximum production of cellulases and β-Glucosidase.

The medium formulated was named as α-cellulose medium -1 (αm-1). Using this medium, maximum production of cellulases and β-Glucosidase occurred on the 9th day of incubation.

1.4. Filter paper as carbon source

The enzymes detected in the culture filtrate were very low in all media containing various concentration of filter paper. Nevertheless, there was considerable growth (biomass). Time course studies (carried out for up to 20 days) indicated that there was no significant increment in production of cellulases and

β-Glucosidase through time in media containing 0.1% Filter paper (Fig. 11). Production of CMC-ase, Avicelase and α-cellulase in media containing 0.5% filter paper though better than 0.1% was also scanty. However, Fp-ase, cottonase and β-Glucosidase were produced in relatively greater quantities in these media. Better production of all enzymes occurred as concentration of filter paper was increased to 1%. The highest production of cellulases was detected on the 10th day of incubation. But, maximum production of β-Glucosidase appeared at the later growth stage of the fungus. Fp-ase activity was higher than that of CMC-ase, Avicelase, α-cellulase, Cottonase and β-Glucosidase in media containing 0.5% and 1% filter paper. Its production reached 1.79 units/ml in medium containing 1% filter paper and 2% potassium nitrate on the 10th day of growth. The other enzymes had activities less than one in all media containing filter paper.

Generally, production of cellulases and β-Glucosidase in media containg 0.1% filter paper was significantly less ((tcal=1.5) than those media containing 1% filter paper. Since enzyme production in the latter was not significantly higher than in media containg 0.5 filter paper, 0.5% filter paper supplemented with 1% potassium nitrate was selected as best carbon and nitrogen combination for the production of cellulases and β-Glucosidase. This medium was identified as filter paper medium -1(FM-1). In this medium, maximum production of all the enzymes occurred on the 10th day of incubation. In all subsequent works when filter paper is used as

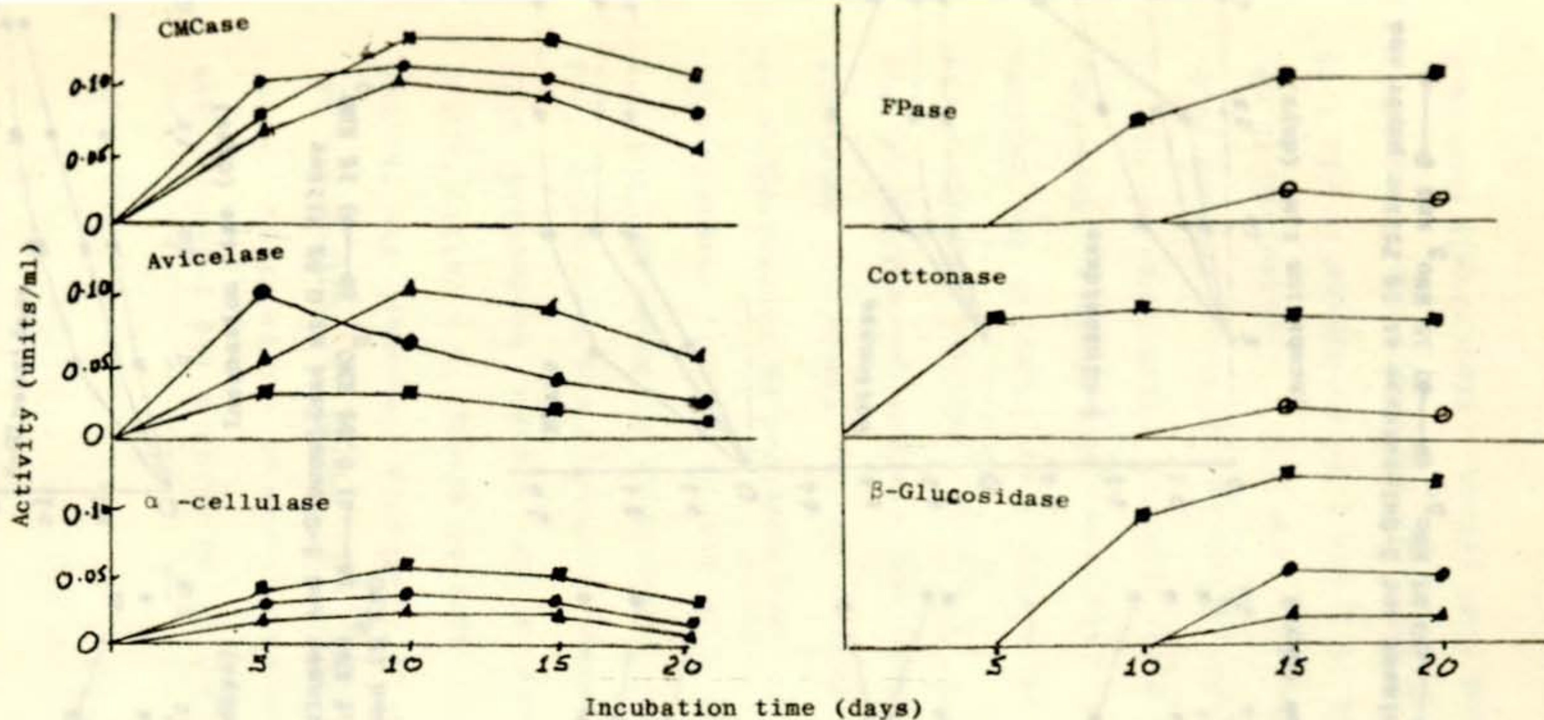


Fig. 11. Production of cellulases and β-Glucosidase at 0.1% Filter paper and 3 concentrations of KNO_3. Production is expressed in terms of activity of the enzymes. (▲—▲) 0.5% KNO_3, (●—●) 1% KNO_3, (■—■) 2% KNO_3, and (⊖—⊖) 0.5% and 1% KNO_3.

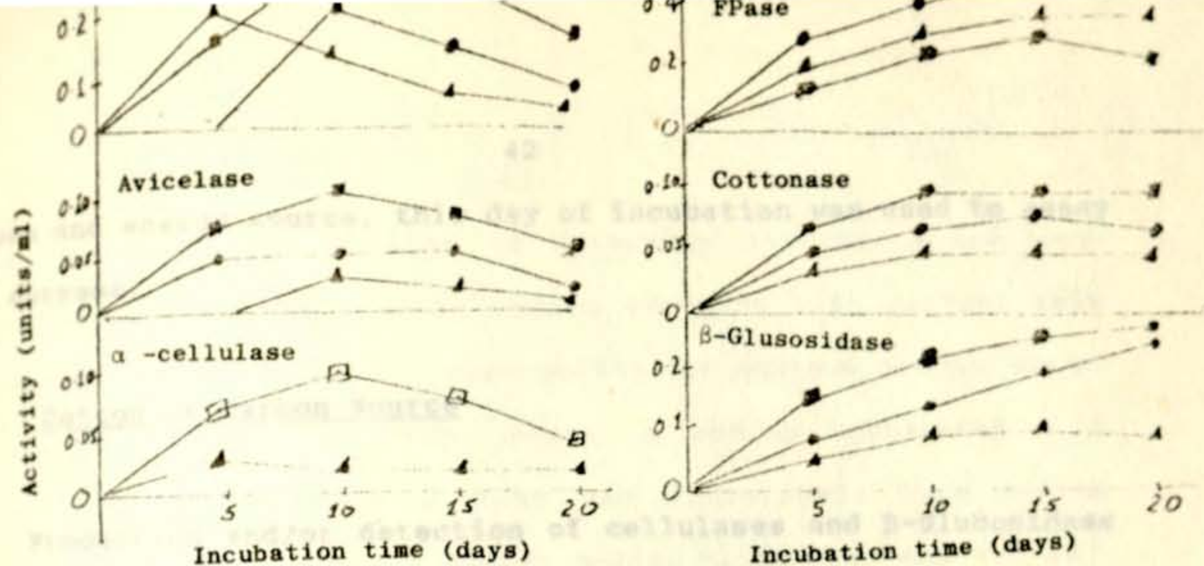


Fig. 12. Production of cellulases and β-Glucosidase at 0.5% Filter paper and 3 concentrations of KNO_3. (▲——▲) 0.5% KNO_3, (●——●) 1% KNO_3, (■——■) 2% KNO_3, (□——□) 1% and 2% KNO_3.

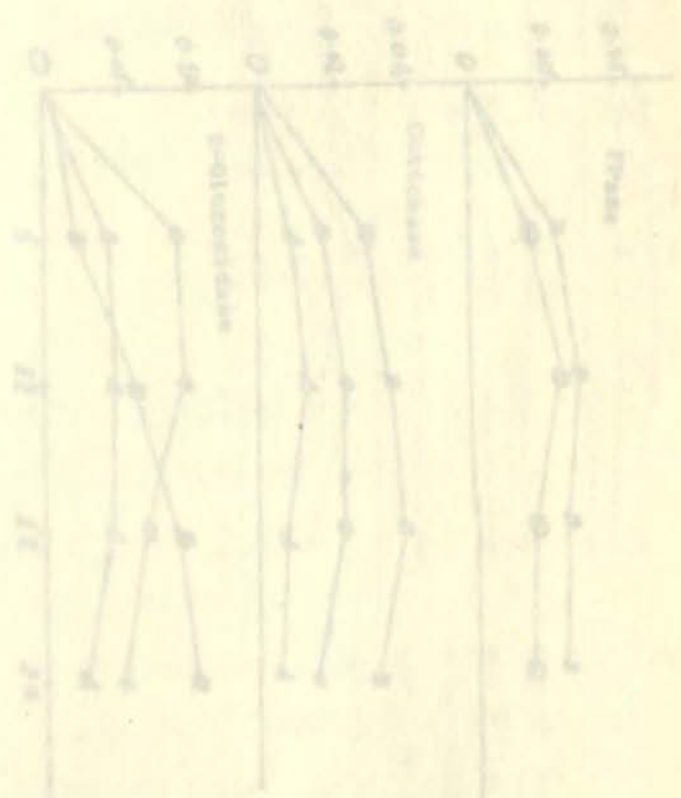
Fig.13. Production of cellulases and β-Gulcosidase at 1% Filter paper and 3 concentrations of KNo_3 (▲——▲) 0.5% KNo_3, (●——●) 1% KNo_3 and (●——●) 2% KNo_3.

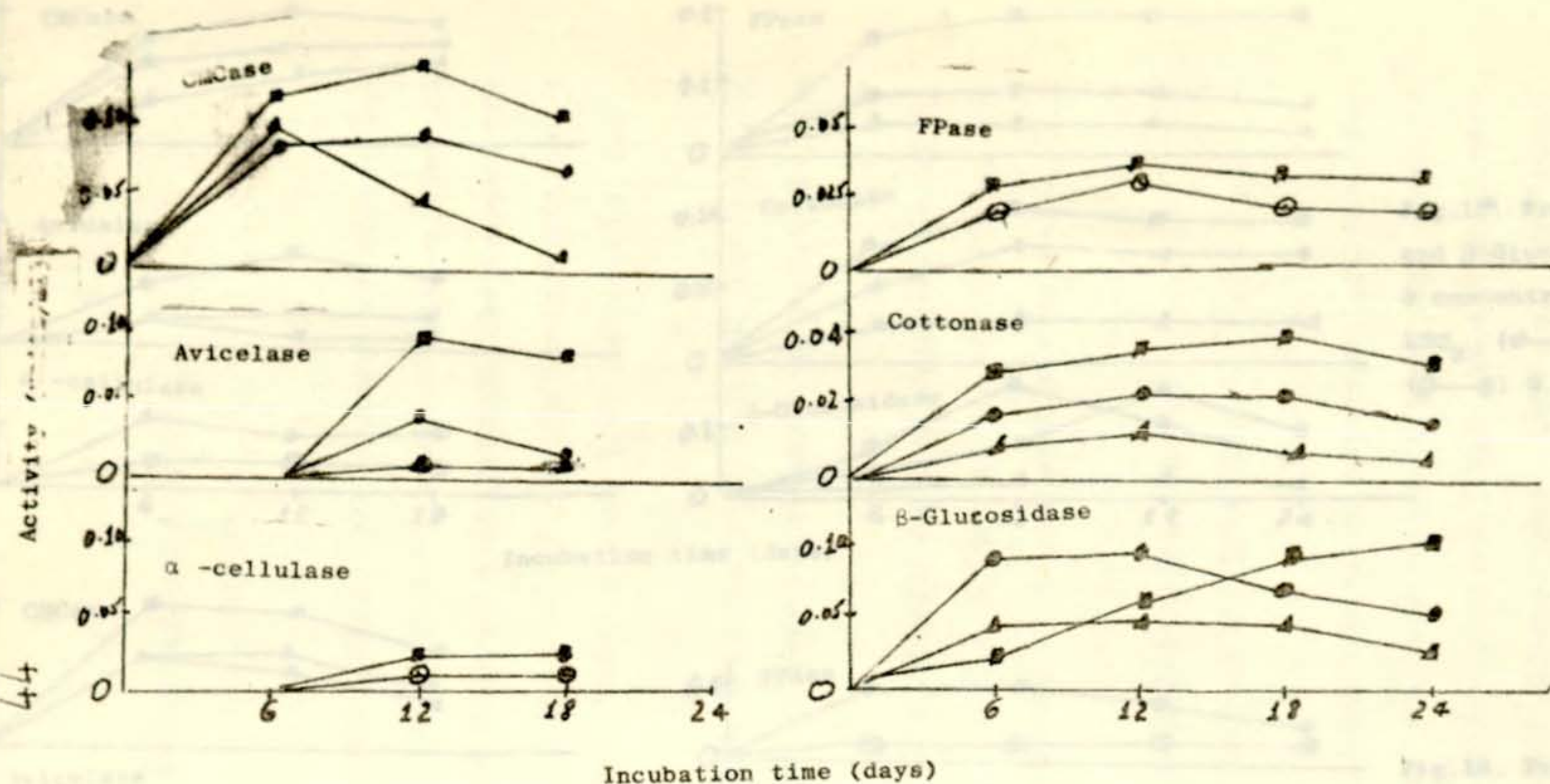
carbon and energy source, this day of incubation was used to assay the enzymes.

1.5. Cotton as Carbon Source

Production and/or detection of cellulases and β-Glucosidase in media containing various concentrations of cotton (Figs. 14-16) was very poor. Significant increment of enzyme production was not detected through time in most of the media. However, the enzymes were detected on the 9th to 12th days of growth in a fairly better quantities. Likewise, increments in enzyme production with increasing concentration of potassium nitrate was very small (less than 50%). Exceptions were for productions of Avicelase and α-cellulase in media containing 0.1% and 1% cotton, respectively, where more than 100% increments were observed as concentration of potassium nitrate increased from 1 to 2%. Hence, 1% potassium nitrate was found to be optimum for maximum production of the enzymes. In media containing 0.1% cotton, CMC-ase, Avicelase and β-Glucosidase were detected in larger quantities in the culture filtrate than α-cellulase, cottonase and Fp-ase. Moreover, Avicelase and α-cellulase were detected after the 6th day of incubation (growth). Maximum production (units/ml) of CMC-ase (0.1, 0.6, and 0.6), Avicelase (0.04, 0.3, and 0.1), α-cellulase (0.02, 0.20, and 0.10), Fp-ase (0.03, 0.1 and 0.4) cottonase (0.03, 0.08 and 0.05) and β-Glucosidase (0.10, 0.25 and 0.20) was detected in media containing 0.1%, 0.5% and 1.0% cotton, respectively when each

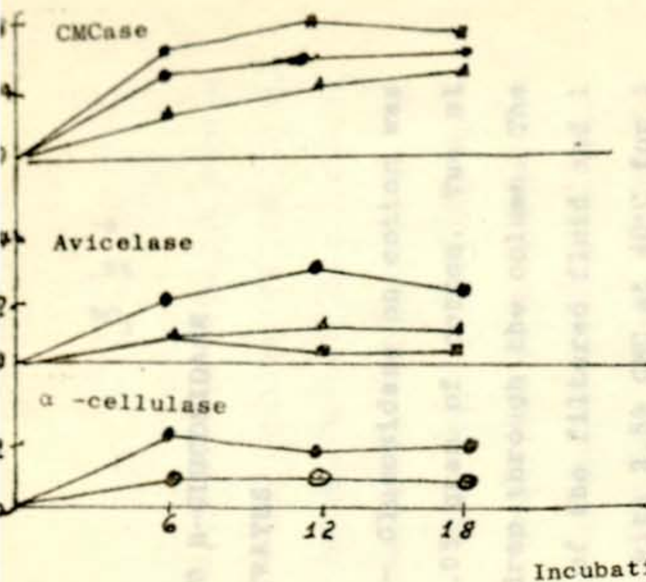
medium was supplemented with 1% potassium nitrate. Since each enzyme was better detected in medium containg 0.5% cotton, this concentration was considered appropriate for maximum production of cellulases and B-Glucosidase. Hence, a medium containing 0.5% cotton and 1% potassium nitrate was formulated. This medium hereafter is referred to as cotton medium -1 (COM-1) and the 12th day of incubation was considered optimum for maximum expression of the enzymes.

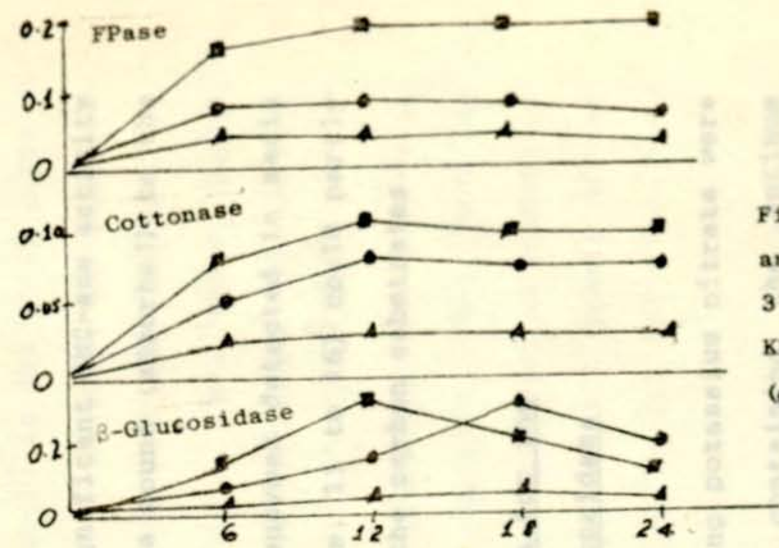




Incubation time (days)

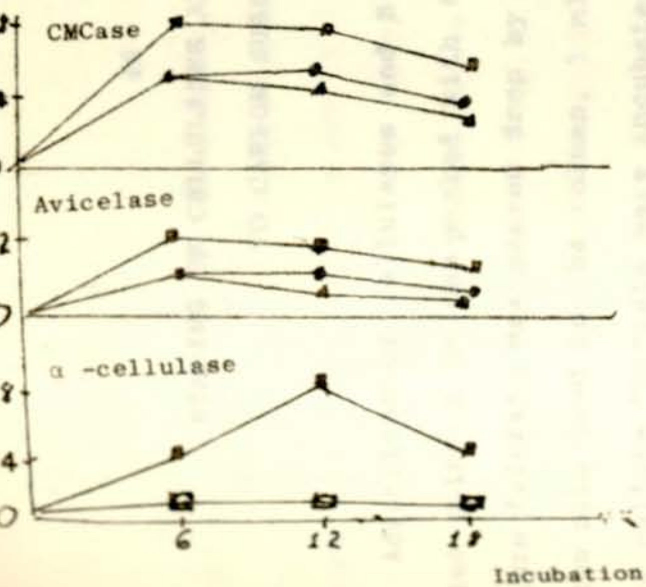
Fig.14. Production of cellulases and B-Glucosidase at 0.1% cotton and 3 concentrations of KNO_3. (▲—▲) 0.5% KNO_3, (●—●) 1% KNO_3, (■—■) 2% KNO_3 and (⊖—⊖) 0.5% and 1% KNO_3.

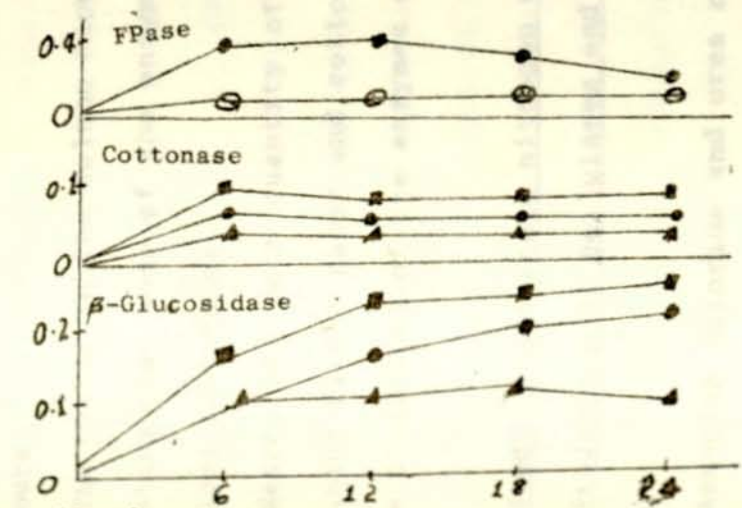




Incubation time (days)

Fig.15. Production of cellulas
and β-Glucosidase at 0.5% cott
3 concentrations of KNO_3 (▲—
KNO_3 (●—●)1% KNO_3, (■—■)
(O—O) 0.5% and 2% KNO_3





Incubation time (days)

Fig.16. Production of celluls
and β-Glucosidase at 1% cotto
3 concentrations of KNO_3. (▲—
0.5% KNO_3, (●—●) 1% KNO_3,
2% KNO_3, (□—□) 0.5% and 1%
(O—O) 0.5% and 2% KNO_3.

BINDING OF CELLULASES AND β-GLUCOSIDASE TO CARBON SUBSTRATES

Adsorption of cellulases and β - Glucosidase on cotton was tested using a column packed with 0.05 gram of cotton. Two ml culture filtrate was passed drop by drop through the column. The cotton plug used in the column, 1 ml of the filtered fluid and 1 ml of culture filtrate were incubated with 2.5% CMC at 40°C for 3 1/2 hours.

The cotton in the column showed significant CMC-ase activity indicating that some of the enzymes were bound (adsorbed) to the cellulose substrates.

Hence, the poor quantity of all enzymes detected in media containing filter paper and cotton (Figs. 11 to 16) could partly be due to binding of the enzymes on to the carbon substrates.

2.0 <u>Study of alternate nitrogen sources for the Production of cellulases and β-Glucosidase</u>

Ammonium chloride and urea replacing potassium nitrate were used as nitrogen sources in media containing the optimum concentration of the five carbon sources (CM-1, AM-1 αM-1, FM-1 and COM-1). Each nitrogen source was used at different concentrations in each medium.

As can be seen in Table - I, Production of cellulases and β-Glucosidase increased at lower concentrations of ammonium chloride

(0.5%) in CM-1. Increasing concentrations of the nitrogen source resulted in decreased enzyme production. Unlike in CM-1, an increasing trend in production of Avicelase, α-cellulase, Fp-ase and cottonase was observed with increasing concentration of ammonium chloride in Am-1. However, activity of β-Glucosidase remained similar to that of CM-1, i.e. Production of β-Glucosidase decreased with increasing concentration of Ammonium chloride. CMC-ase production was relatively higher at the lower and higher concentrations of ammonium chloride in Am-1. In the other media (αm-1, Fm-1 and COM-1) all concentrations of ammonium chloride had similar effect on production of the cellulolytic enzymes. An exception was production of CMC-ase in αm-1 where higher production of the enzyme was detected at lower and higher concentrations of the nitrogen source.

Production of cellulases and β-Glucosidase decreased with increasing concentration of urea in Cm-1. In Am-1, αm-1, FM-1 and Com-1, all concentrations of urea had similar effect on production of the enzymes. Generally, urea supported little growth of the fungus and as a result, enzyme production in all media was very law.

Potassium nitrate at all concentrations in all media except in com-1 produced quite considerable amount of cellulases and β-Glucosidase. Production increased with increasing concentration of potassium nitrate in Cm-1, Am-1, αm-1 and Fm-1. However, the rate of increments were not that great when compared to the increasing concentration of potassium nitrate used (double concentration).

Exceptions were production of Cmc-ase. Avicelase and [illegible]-cellulase in Am-1 where more than double activities were detected as the nitrate concentrations moved from 0.5% to 1% and then to 2%. In Com-1, Production of CMC-ase and Avicelase was higher at 1% nitrate while higher production of all others was in medium containing 2% potassium nitrate.

Generally the best levels of ammonium chloride which gave high production of cellulases and B-Glucosidase were the lowest concentrations used (0.5% in Cm-1, Am-1, Com-1 and 1% in [illegible]m-1 and Fm-1). Although lower than ammonium chloride, similar concentrations of urea were also optimum for production of the cellulolytic enzymes. Enzyme production at the optimum levels of ammonium chloride and urea were low when compared to the same concentrations of potassium nitrate. Thus, potassium nitrate was the most preferred nitrogen source by Cladosporium sp. BDCC-3 from the alternate sources studied. Urea is the least utilizable nitrogen source.

Since potassium nitrate was the superior nitrogen source than ammonium chloride and urea, the code of each medium remained unchanged (Cm-1, Am-1, [illegible]m-1, Fm-1 and COM-1).

TABLE 1. Production of cellulases and β-Glucosidase at different nitrogen sources in media-1. Production is expressed in terms of activity of the enzymes.

Carbon Source	Nitrogen Source	Concentration of the N-source	Activity (Units/ml)					
			cellulases					β-Glucosidese
			cmc-ase	Avicelase	α-cellulase	Fp-ase	Cottonase	
1% cmc (CM-1)	NH_4Cl	0.5%	48.8	4.6	2.9	2.1	2.3	3.4
		1%	21.6	2.5	1.7	2.8	2.3	1.3
		2%	19.7	2.0	2.0	2.7	2.4	1.0
		3%	17.9	1.8	1.3	2.5	2.3	0.9
	Urea	0.5%	10.90	0.85	1.12	1.30	0.53	0.52
		1%	8.2	0.85	0.79	1.50	1.0	0.72
		2%	6.0	0.15	0.58	0.22	1.1	0.17
		3%	4.6	0.12	0.47	0.60	0	0.14
	KNO_3	0.5%	49.9	4.8	3.7	2.7	2.6	4.2
		1%	58.6	4.9	3.9	3.0	2.8	4.5
		2%	63.6	4.8	4.0	4.0	3.0	4.7
0.5% Avicel (AM-1)	NH_4Cl	0.5%	6.7	0.4	0.2	0.2	0.1	0.4
		1%	4.9	0.4	0.3	0.3	0.2	0.3
		2%	5.0	0.9	0.7	0.9	0.5	0.1
		3%	6.8	1.6	1.0	1.6	0.7	0.2
	Urea	0.5%	0.27	0.18	0.11	0.01	0.008	0.18
		1%	0.44	0.14	0.08	0.001	0.07	0.01
		2%	0.44	0.13	0.14	0.07	0.04	0.11
		3%	0.44	0.13	0.16	0.07	0.04	0.13
	KNO_3	0.5%	1.1	0.4	0.2	1.2	0.8	0.50
		1%	3.0	0.7	0.5	2.1	1.2	0.52
		2%	6.1	1.9	1.4	3.5	2.0	0.58
1.5% α-cellulose (α m-1)	NH_4Cl	1%	5.8	0.8	0.6	0.7	0.5	0.2
		2%	3.5	1.1	0.8	1.1	0.6	0.2
		3%	3.6	1.2	0.8	0.9	0.6	0.2
		4%	5.6	1.2	1.0	1.2	0.8	0.4
	Urea	1%	0.53	0.17	0.20	0.11	1.40	0.27
		2%	0.40	0.16	0.25	0.07	0.09	0.21
		3%	0.49	0.16	0.21	0.10	0.10	0.16
		4%	0.87	0.15	0.20	0.11	0.07	0.17
	KNO_3	0.5%	3.0	1.0	0.40	0.40	0.20	0.20
		1%	5.5	1.0	0.50	1.0	0.23	0.20
		2%	7.3	1.4	1.4	1.4	0.43	0.30
0.5% Filter paper (FM-1)	NH_4Cl	1%	1.6	0.40	0.30	0.90	0.40	0.10
		2%	1.7	0.60	0.50	0.70	0.30	0.20
		3%	1.4	0.70	0.60	0.60	0.40	0.20
		4%	2.0	0.50	0.40	1.0	0.30	0.20
	Urea	1%	4.8	0.13	0.11	0.08	0.08	0.27
		2%	6.7	0.11	0.11	0.08	0.08	0.15
		3%	4.8	0.13	0.10	0.06	0.08	0.11
		4%	3.8	0.11	0.10	0.06	0.08	0.11
	KNO_3	0.5%	8.10	0.30	0.09	0.36	0.11	0.16
		1%	11.60	0.60	0.40	0.48	0.50	0.23
		2%	12.30	0.89	0.46	1.26	0.42	0.37

Table 1. Cont'd

Carbon Source	Nitrogen Source	Concentration of the N-source	Activity (Units/ml) cellulases					B-Glucosidese
			cmc-ase	Avicelase	α-cellulase	Fp-ase	Cottonase	
0.5% Cotton (COM-1)	NH_4Cl	0.5%	0.20	0.09	0.07	0.03	0.03	0.07
		1%	0.40	0.10	0.12	0.06	0.06	0.03
		2%	0.40	0.10	0.07	0.03	0.03	0.03
		3%	0.20	0.10	0.13	0.06	0.06	0.09
	Urea	0.5%	0.03	0.05	0.09	0.03	0.03	0.03
		1%	0.13	0.09	0.07	0.04	0.03	0.09
		2%	0.13	0.10	0.05	0.05	0.04	0.07
		3%	0.21	0.13	0.08	0.04	0.05	0.07
	KNO_3	0.5%	0.35	0.06	0.08	0.01	0.04	0.03
		1%	0.50	0.20	0.03	0.03	0.05	0.03
		2%	0.30	0.05	0.06	0.08	0.75	0.08

3.0 Effect of detergents on the release of cellulases and B-Glucosidase

The effect of three detergents namely Tween-80, Triton*100 and Tergitol-7 on the release of cellulases and β-Glucosidase from Cladosporium sp. BDCC-3 into the culture medium was studied using CM-1, AM-1, αM-1, FM-1 and COM-1 (media containing optimum concentrations of carbon and nitrogen sources).

Release of cellulases and β-Glucosidase increased with increasing concentration of Tween-80 until it reached 0.1% where maximum production of the enzymes was detected in all media (Table-II). Release of all enzymes except Fp-ase in Cm-1 declined beyond the 0.1% concentration of Tween-80. Higher activity of Fp-ase was expressed at higher Tween-80 levels (0.2 and 0.3%) in Cm-1.

The effect of Trition-x-100 on the release of cellulases and β-Glucosidase increased with increasing concentration of the

detergent upto 0.05%. Then the quantity of enzymes in the culture filtrate started to decline as concentration increased further. An exception was observed in the release of B-Glucosidase where higher proportion of the enzyme was detected at the lowest concentration (0.01%) of Trition-x-100 in all media. Higher concentrations of Trition-x-100 retarded growth of the fungus in Cm-1.

Cellulases and β-Glucosidase were released least when the detergent used was Tergitol-7. Production of all enzymes in media containing this detergent was much lower than even those produced in media with out detergents.

However, Tergitol-7 at 0.01% Produced relatively higher enzymes than 0.05% and 0.1%, because as concentration of the detergent decreased, toxicity also was decreased proportionally. Higher concentration of this detergent (0.2 and 0.3%) inhibited growth of <u>Cladosporium</u> sp.BDCC. 3 in Cm-1.

In general terms, the most effective detergent at comparable concentrations was Trition-x-100. Higher proportion of Enzymes were released in media supplemented with Trition x-100 at considerably lower concentration (0.05%). However, it is also important to note that Tween-80 at 0.1% was effective in releasing cellulses and β-Glucosidase to the culture media. Both detergents increased release of the enzymes up to more than 100% when compared to enzyme production with out detergents (Table-II).

In this study, although Triton-x-100 was the most effective detergent in releasing the cellulolytic enzymes into the culture media, Tween-80 at 0.1% was a surfactant of choice. This decision

was made due to the abnormal growth characteristic of Cladosporium sp. BDCC-3 observed when the growth media were supplemented with Trition-x-100. The increased release of enzymes may be due to the release of cell protein as a result of membrane disintegration. Thus, the incorporation of Tween-80 with M-1 (CM-1, AM-1, αM-1, FM-1 and COM-1) formed a modified media-2 (CM-2, AM-2, αM-2, FM-2 and COM-2) for subsequent works.

TABLE II. The effect of detergents on the release of cellulases and β-Glucosidase from Cladosporium sp. BDCC-3 to the culture medium Quantity of enzymes in the culture filtrate was determined interms of their activity
* SG = Small growth * GI = Growth inhibited

Medium	Detergent	concentration of detergent	Activity (Units/ml) cellulases cmc-ase	Avicelase	α-cellulase	Fp-ase	Cottonase	β-Glucosidese
CM-1	Tween-80	0.01%	64.3	5.2	3.5	2.7	2.7	5.1
		0.05%	78.9	6.7	3.9	3.0	2.8	5.2
		0.1%	89.9	7.5	4.7	3.5	3.4	20.5
		0.2%	72.9	7.4	4.8	3.5	3.4	22.5
		0.3%	72.3	7.4	4.2	4.1	3.9	21.7
	Triton-x-100	0.01%	53.3	5.4	4.0	2.6	2.6	10.1
		0.05%	50.8	7.4	5.2	4.4	3.9	9.6
		0.1%	53.3	6.9	4.4	2.9	2.8	8.7
		0.2%	SG	SG	SG	SG	SG	SG
		0.3%	SG	SG	SG	SG	SG	SG
	Tergitol-7	0.01%	4.1	0.5	0.4	0.4	0.5	0.4
		0.05%	1.8	0.9	0.3	0.6	0.7	0.5
		0.10%	0.14	0.1	0.01	0.09	0.04	0.03
		0.2%	GI	GI	GI	GI	GI	GI
		0.3%	GI	GI	GI	GI	GI	GI
	None	—	58.6	4.9	3.9	3.0	2.8	4.5
AM-1	Tween-80	0.01%	11.2	5.0	0.6	3.2	1.2	1.6
		0.05	10.3	5.0	0.8	7.5	0.9	3.2
		0.1%	13.0	10.5	3.7	7.8	6.3	4.0
		0.2%	8.3	6.3	1.8	6.3	2.6	2.4
		0.3%	7.8	6.3	2.0	4.8	3.7	1.4
	Triton-x-100	0.01%	12.8	12.5	3.0	9.3	3.7	1.0
		0.05%	17.2	14.0	5.8	12.2	4.0	0.8
		0.10%	10.4	13.3	4.8	0.5	3.7	0.4

Cont'd Table II

Medium	Detergent	concentration of detergent	Activity (Units/ml) cellulases cmc-ase	Avicelase	α-cellulase	Fp-ase	Cottonase	B-Glucosidese
	Tergitol-7	0.01%	0.9	0.8	0.24	0.2	0.3	0.2
		0.05%	1.0	0.5	0.24	0.2	0.26	0.1
AM-1		0.1%	0.2	0.08	0.06	0.02	0	0
	None	—	7.8	2.0	0.6	2.0	1.2	0.6
	Tween-80	0.01%	34.8	10.1	6.0	5.9	4.7	5.5
		0.05%	35.5	8.9	7.0	9.5	4.1	5.0
M-1		0.1%	35.3	11.2	7.1	9.2	5.1	7.4
		0.2%	26.1	9.5	5.6	6.5	2.7	5.5
		0.3%	23.6	6.9	5.3	6.0	2.5	3.7
	Triton-x-100	0.01%	36.1	9.7	10.4	16.9	6.9	5.3
		0.05%	48.8	16.3	11.9	17.1	6.3	2.7
		0.1%	46.1	20.7	14.4	24.1	6.9	0.4
	Tergitol-7	0.01%	18.7	4.1	2.7	2.2	1.5	0.13
		0.05%	0.9	0.3	0.01	0.11	1.1	0.80
		0.10%	0.5	0.2	0.01	0.11	0.7	0.08
	None	—	27.4	5.1	4.1	4.1	2.5	2.1
	Tween-80	0.01%	33.8	3.2	1.6	4.0	1.7	1.8
		0.05%	35.7	2.8	1.6	4.7	1.7	1.8
FM-1		0.10%	38.3	3.8	2.7	4.5	2.0	2.5
		0.2%	22.9	3.2	2.0	3.5	1.4	2.5
		0.3%	18.3	3.2	1.7	3.3	1.4	2.0
	Triten-x-100	0.01%	34.9	7.0	2.0	13.3	3.2	3.7
		0.05%	44.0	13.3	7.5	16.9	4.8	3.3
		0.10%	31.4	8.5	5.0	8.8	2.3	3.0
	Tergitol-7	0.01%	1.7	1.2	0.4	0.08	0.08	0.08
		0.05%	0.9	0.3	0.11	0.07	0.04	0.09
		0.10%	0.8	0.2	0.09	0.04	0.03	0.04
	None	—	37.7	2.7	1.5	3.3	1.2	1.8
	Tween-80	0.01%	0.80	0.23	0.23	0.03	0.09	0.5
		0.05%	2.0	0.40	0.20	0.50	0.20	0.3
		0.1%	2.3	0.50	0.30	0.30	0.20	0.3
		0.2%	0.8	0.20	0.15	0.01	0.05	0.9
CCM-1		0.3%	0.7	0.20	0.15	0.03	0.05	0.9
	Triton-x-100	0.01%	3.9	1.4	1.4	1.5	0.63	1.94
		0.05%	7.3	1.8	2.0	2.5	1.34	1.50
		0.1%	0.7	0.16	0.40	0.1	0.05	1.2
	Tergitol-7	0.01%	1.1	0.16	0.14	0.05	0.05	0.50
		0.05%	0.9	0.15	0.12	0.03	0.05	0.31
		0.10%	0.4	0.20	0.04	0.03	0.05	0
	None	—	1.7	0.23	0.23	0.14	0.09	0.30

4.0 Effect of assaying pH and temperature on the activity of Cellulases and β-Glucosidase

4.1 Assaying pH

The media employed for growth and production of cellulases and β-Glucosidase in this study were media-2. The effects of varying pH values on the activities of cellulolytic enzymes were studied using culture filtrate obtained from the above media (within the pH range of 4.0 to 8.0). One ml culture filtrate was added to the substrates suspended in acetate buffer for pHs of 4.0 and 5.0 and phosphate buffer for pHs 6.0, 7.0 and 8.0.

The enzymes were active in all pHs studied. But, the activities were low at pH 4.0 and increased rapidly when the pH of the reaction mixture was shifted from pH 4.0 to pH 5.0 (Fig. 17) Maximum activity was observed at pH 5.0. The activity started to decline at around pH 6.0 and then increased when the pH shifted from 6.0 to 7.0. However, the rate of increment from 6.0 to 7.0 was much lower than that observed from pH 4.0 to 5.0. An exception was the activity of β-Glucosidase in Fm-2 where the peak was found to be at pH 6.0. All enzymes showed a drop in activity when the pH increased from 7.0 to 8.0. The decline was sharper in Fm-2 for the activity of all enzymes except CMC-ase. Generally, cellulases and β-Glucosidase from *Cladosporium* sp BDCC-3 were found to be most active at pH 5.0 and 7.0. Since the major peak was at pH 5.0, all subsequent works were conducted at this optimum pH for activity of the enzymes.

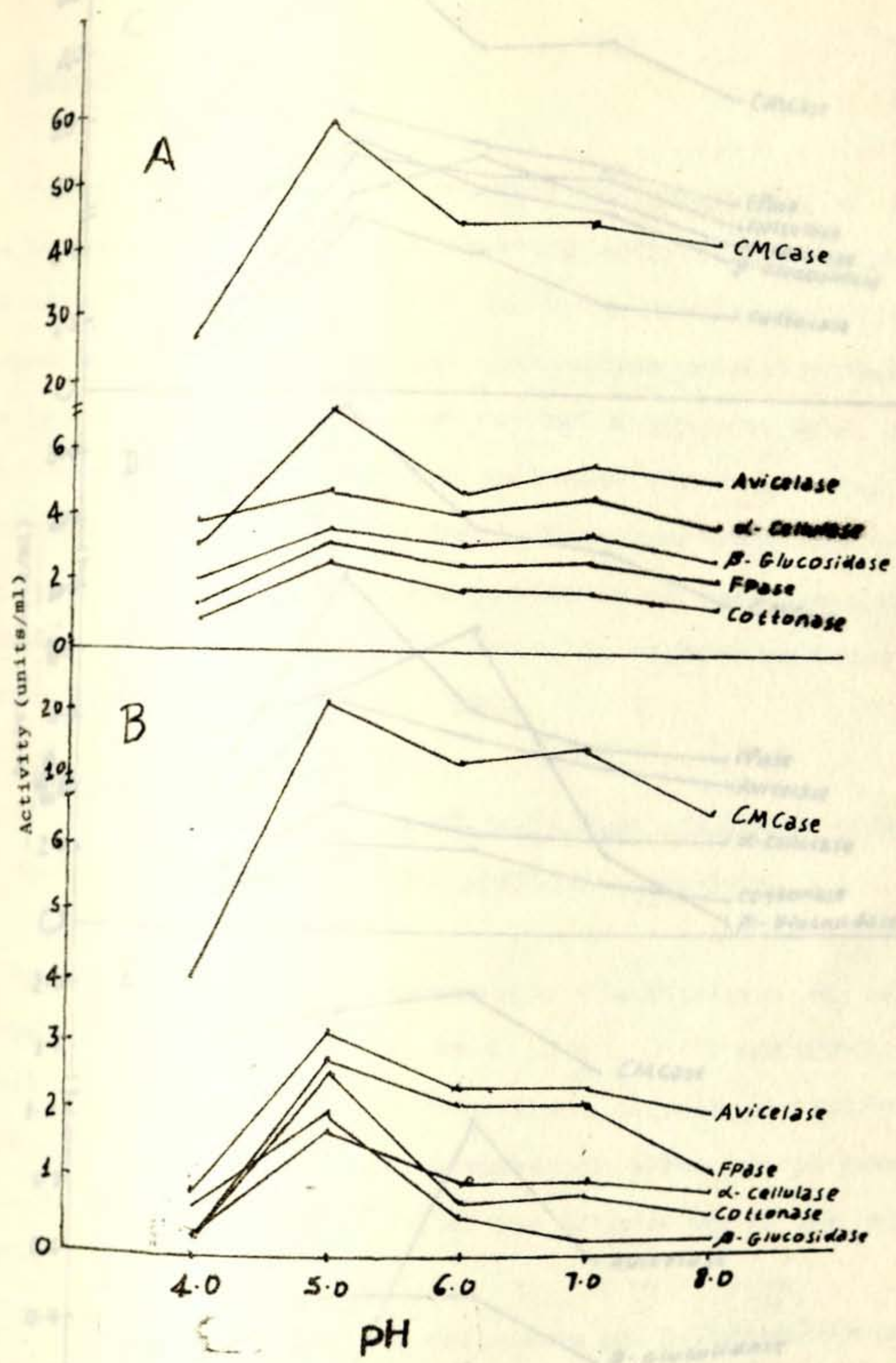


Fig.17. Effect of pH on the activity of cellulases and β-Glucosidase. Growth media employed were A) CM-2 B) AM-2

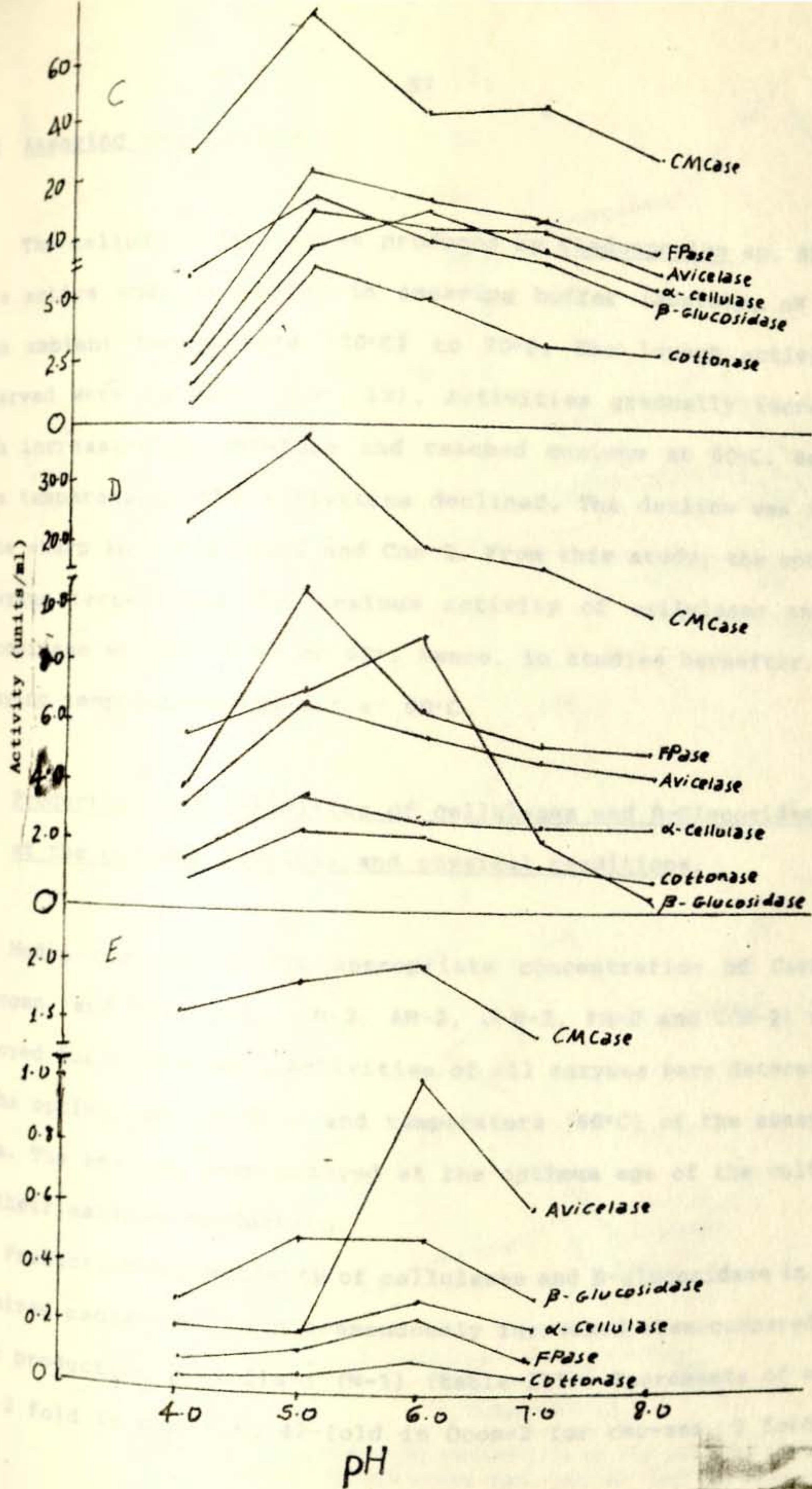


Fig.17. Cont'd. Effect of pH on the activity of cellulases and β-Glucosidase. Growth media used were C) αm-2 D) FM-2 E) CoM-2

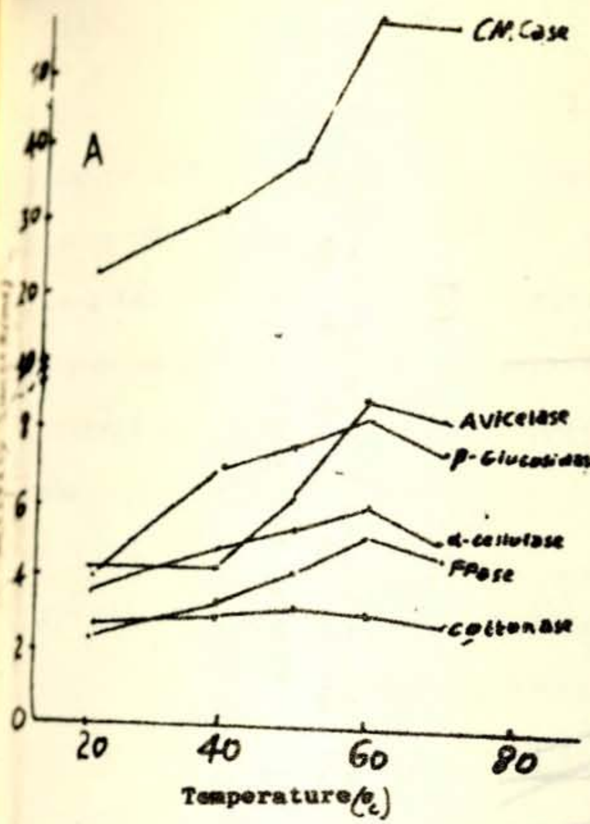
4.2 Assaying temperature

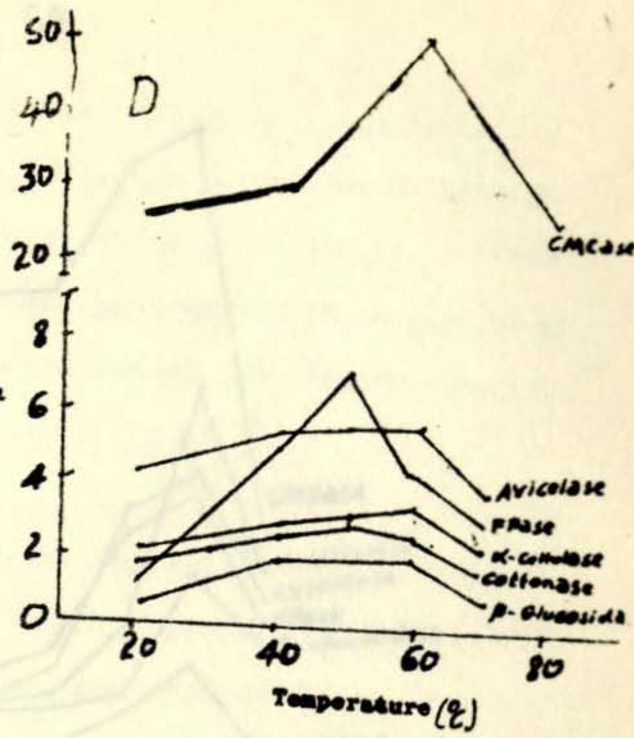
The cellulolytic enzymes produced by Cladosporium sp. BDCC-3 were active when incubated in assaying buffer (acetate, pH 5.0) from ambient temperature (20°C) to 70°C. The lowest activities observed were at 20°C (Fig. 18). Activities gradually increased with increasing temperature and reached maximum at 60°C. Beyond this temperature, the activities declined. The decline was found to be sharp in ..m-2, Fm-2 and Com-2. From this study, the optimum assaying temperature for maximum activity of cellulases and β-Glucosidase was found to be 60°. Hence, in studies hereafter, the assaying temperature was set at 60°C.

5.0 Production and activities of cellulases and β-Glucosidase at the optimum chemical and physical conditions.

Media containing the appropriate concentration of Carbon, nitrogen, and detergent (CM-2, AM-2, ..M-2, FM-2 and COM-2) were employed for this study. Activities of all enzymes were determined at the optimum pH (pH 5.0) and temperature (60°C) of the assaying media. The enzymes were assayed at the optimum age of the culture for their maximum production.

Production and activity of cellulases and β-glucosidase in the optimized media (OM) have tremendously increased when compared to their production in Media-1 (M-1) (table-III). Increments of more than 2 fold in Ocm-2 to 42-fold in Ocom-2 for cmc-ase, 2 fold in





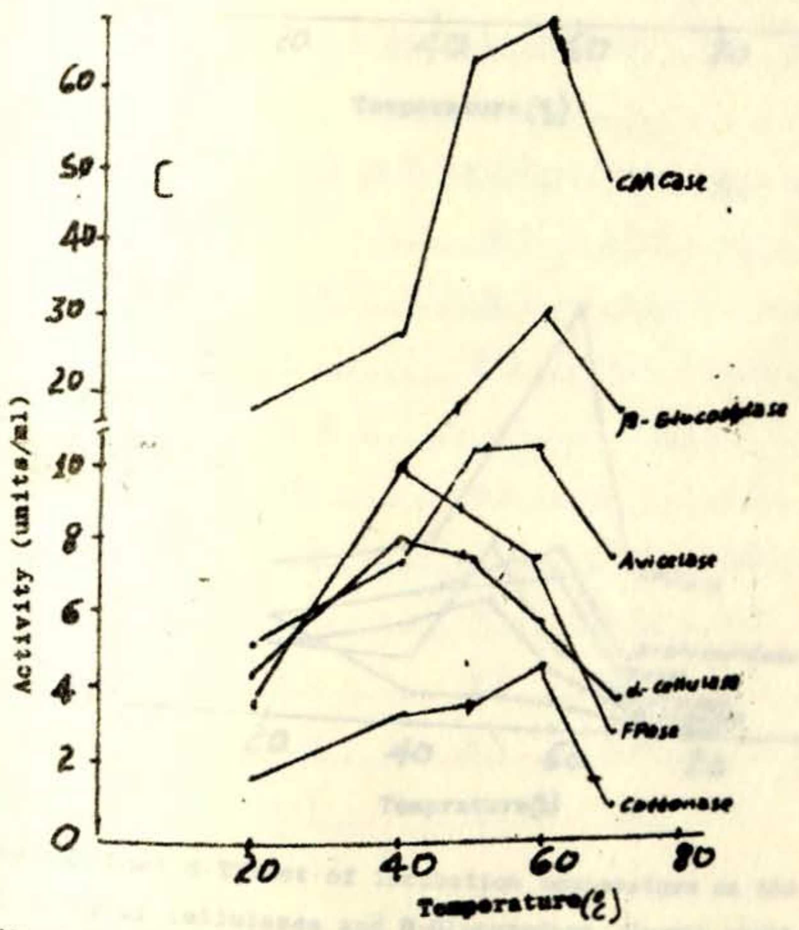
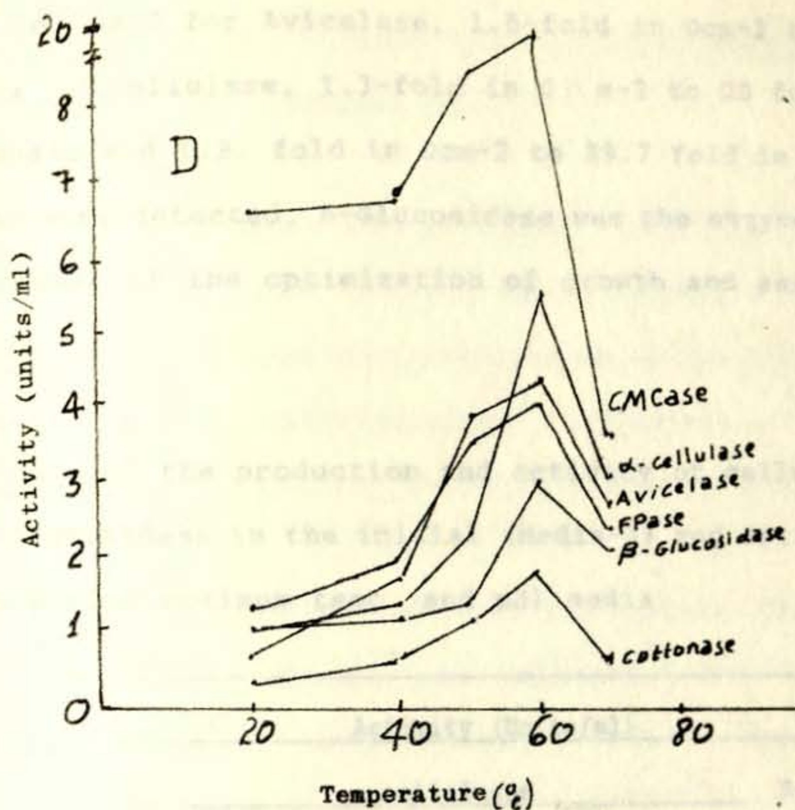


Fig.18. Effect of incubation temperature on the activity of cellulases and β-Glucosidase. Growth media used were A) CM-2 B) AM-β C) [illegible]-2



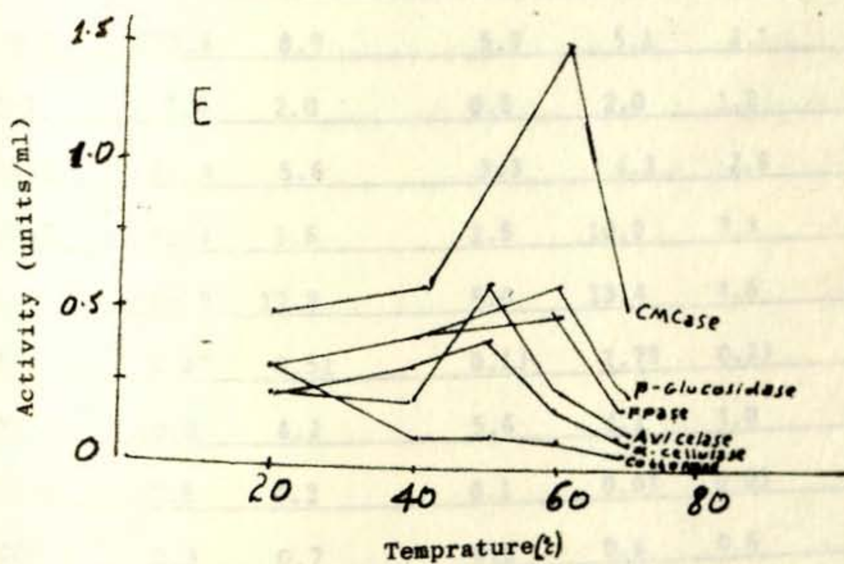


Fig.18. Cont'd. Effect of incubation temperature on the activity of cellulases and β-Glucosedese. Growth media used were D) FM-2 E) CoM-2

Ocm-2 to 8-fold in OFm-2 for Avicelase, 1.5-fold in Ocm-2 to 43-fold in OFm-2 for α-cellulase, 1.3-fold in Oαm-2 to 20 fold in Ocom-2 for cottonase and 1.9. fold in Ocm-2 to 89.7 fold in OαM-2 for B-Glucosidase were detected. B-Glucosidase was the enzyme more increased as a result of the optimization of growth and assaying media.

TABLE III Comparison of the production and activity of cellulases and B-Glucosidase in the initial (Media-1) and optimized (Media-2 plus optimum temp. and pH) media

		Activity (Units/ml)					
		cellulases					B-Gluco-
Substrate	Media	CMC-ase	Avicelase	α-cellulase	Fp-ase	Cottonase	sidese
CMC	CM-1	58.6	4.9	3.9	3.0	2.8	4.5
	OCM-2	107.8	8.9	5.9	5.1	3.1	8.6
Avicel	AM-1	7.8	2.0	0.5	2.0	1.2	0.59
	OAM-2	50.4	5.6	3.3	4.2	2.6	2.0
α-Cellulase	αM-2	12.3	3.6	2.5	10.0	3.3	0.37
	OαM-2	69.0	12.8	6.0	13.4	4.6	33.2
Filter paper	FM-1	0.47	0.51	0.13	1.79	0.23	0.15
	OFM-2	20.0	4.2	5.6	4.1	1.0	3.0
Cotton	COM-1	0.5	0.2	0.1	0.03	0.03	0.05
	OCOM-2	2.3	0.7	0.5	0.6	0.6	1.0

6.0 Study on the location of cellulases and β-Glucosidase of Cladosporium sp. BDCC-3

In this study, the presence of cellulases and β-Glucosidase enzymes in the culture filtrate (supernatant), Cytoplasm of the test organism and the cell debris were compared using the optimized CMC medium (CM-2 plus optimum assaying pH and temperature). The CMC medium was preferred because it was easy to separate the mycelia from the carbon substrate.

The results (Table - IV) showed that cellulases (CMC-ase, Avicelase, α - cellulase, Fp-ase and cottonase) but not B-Glucosidase were largely extracellular. The unreleased (cytoplasmic) cellulases were less than one-third of those found in the free medium. β-Glucosidase was considerably higher (60.2 units per mg protein) in the cytoplasm. The membrane bound enzymes of Cladosporium sp. BDCC-3 were much less than the extracellular ones. But the cytoplasmic portion of Fp-ase and cottonase were greater than 25%. The least enzyme found associated with the cell debris was CMC-ase (3.1%)

TABLE IV Comparative study of extracellular and intracellular cellulases and β-Glucosidase from cladosporium sp. BDCC-3. Figures in bracket are percent distribution of each enzyme.

Substrate	Source of enzyme	Activity (Units/ml)					
		cellulases					B-Glucosidase
		cmc-ase	Avicelase	α-cellulase	Fp-ase	Cottonase	
CMC	culture filtrate	350 (85.1)	85.7 (69.4)	51.9 (71.3)	42.3 (59.2)	35.8 (62.8)	41.8 (33.3)
	Cytoplasm	48.9 (11.8)	24.5 (19.4)	10.4 (14.3)	5.5 (7.7)	6.2 (10.9)	60.2 (48)
	Cell debris	12.6 (3.1)	13.2 (10.2)	10.5 (14.4)	23.7 (33.1)	15.0 (26.3)	23.3 (18.6)

DISCUSSION

In all of the five carbon and energy sources used in this study, as the concentration of the cellulose in the medium increased to a certain extent production of enzymes also increased proportionally (Figs 1-16). However, the rates of increment in cellulases and β-Glucosidase were low as concentration of substrates increased further. Usually 2% available carbohydrate in liquid medium is believed to be an upper limit for ample growth of micro-organisms (Chochrane, 1958; Stewart and Parry 1981: Desai et al, 1982; Macris, 1984). The lower concentration of enzymes in the culture filtrate, containing higher cellulose concentrations (in the presence of excess substrates) may possibly be due to depression of synthesis and/or adsorption of the enzymes on to the substrates. CMC-ase was found to adsorb on Avicel to the extent of 22% (Sounders and Chandra, 1988). Specially, the latter (adsorption) was observed on the scanty enzyme production from filter paper and cotton celluloses (figs. 11 to 16).

More enzymes were produced in the soluble carbon source than the crystalline ones. This was indicated by the higher production of the enzymes towards CMC than all other celluloses. Carboxymethyl cellulose (CMC) is soluble derivative of cellulose while all others are crystalline that range from the partially treated microcrystalline Avicel to the highly crystalline (about 90%) cotton. CMC-ase was also produced in considerable quantity in the Avicel medium. Production of Fp-ase was higher than all other

enzymes in the filter paper medium (Appendix-II A). This has explained the presence of more induction and production of Fp-ase on its own substrate. The least production was detected in media containing cotton followed by filter paper except for Fp-ase in the latter. These celluloses are more crystalline as compared to the other celluloses used.

The production peak of cellulases (CMC-ase, Avicelase, α-cellulase, Fp-ase, cottonase) and β-glucosidase vary with concentration of the carbon sources of the growth medium used, age of culture and concentration of nitrogen utilized. CMC-ase, Avicelase, and α-cellulase were expressed in the culture filtrate earlier than Fp-ase, cottonase and β-Glucosidase (Figs. 1 to 10). Usually C_1 or cottonase is expressed later as it breaks the substrates into short chained celluloses with a low increase in reducing groups (Desai, <u>et al</u>, 1982). The amount of CMC-ase exceeded by far the other five enzymes in all media employed. This probably may be a good indicator for the presence of higher Cx (CMC-ase) than C_1 (Avicelase, α-cellulase, Fp-ase, cottonase) and β-Glucosidase in <u>Cladosporium</u> sp. BDCC-3, Cx is an enzyme component which hydrolyze the linear reactive and pretreated cellulose while C_1 is believed to be that responsible for the degradation of crystalline cellulose (Jurasek <u>et al</u>., 1967; Fergus, 1969; Hudson, 1980). β-Glucosidase was excreted to the culture medium in less quantities than CMC-ase, Avicelase and α-cellulase. This enzyme is responsible for the degradation of cellobiose to glucose. Apart from cellobiose, the enzyme also hydrolyze aryl-β-Glucoside

(Todorovic et al., 1989). The activity of cellulolytic enzymes is significantly inhibited by the reaction products: Cellobiose and glucose. Thus, β-Glucosidase plays a major role in the hydrolysis of cellulose as its action suppress the inhibitory effect of cellobiose.

The decline in the amount of free enzymes (Figs. 1 to 10) could partly (at least in some media) be due to substrate limitations and/or cultural conditions: accumulation of toxic substances and aeration due to increased mass of the fungus (Pelczar and Reid, 1965). Production of proteolytic enzymes could also be another possible reason. There was production of proteases at the later stage of growth of the test organism in all media used in this study.

Ammonium chloride at higher concentration depressed enzyme production (Table -I). Ammonia can be utilized by many fungi including Cladosporium (Pateman and knghorn, 1976; Burnett, 1976). The form in which the ammonia is supplied, however, is important. Pateman and kinghorn (1976) believed that ammonium salts (NH_4Cl, NH_4NO_3 and $(NH_4)_2$ SO_4) are utilized poorly or not at all by some fungi. Such depressed enzyme production in the presence of ammonium salts from Scytalidium lignicola was also reported by Desai et al (1982). Since plasma membranes of some fungi are leaky to ammonia (Chochrane, 1958), back movement from cells to the medium can also be another possible reason for the decline in its use.

Most fungi can use nitrate as sole source of nitrogen (Pateman and Kinghorn, 1976). Cladosporium sp. BDCC-3 utilize potassium

nitrate better than the other nitrogen sources tested (table-I). This effective utilization of nitrate proved the presence of enzymes used in the reduction of nitrate to ammonia (Nitrate reductase, Nitrite reductase and Hydroxylamine reductase) in Cladosporium sp. BDCC-3.

Urea is generally recognized as utilizable nitrogen source (Chochrane, 1958). But it supported minimum production of enzymes from Cladosporium sp. BDCC-3. Such least effect of urea in production of cellulases from Aspergillus fumigatus was also reported by Stewart and Parry (1981). Furthermore, it has already been shown in Aspergillus niduelans and Neurospora crassa that mutant strains which lack urease can not utilize urea as nitrogen source (Pateman and kinghorn, 1976). Although it seemed small in quantity Cladosporium sp. BDCC-3 produced urease. Urease production was tested in Christensen's medium (Wilkinson, 1969) and it was detected after two days of incubation at 40°C. urease, the enzyme hydrolyzing urea to carbondioxide and ammonia, is well known in higher plants, Bacteria and fungi (Chochrane, 1958.)

Release of cellulases and β-Glucosidase from Cladosporium sp. BCDD-3 to the culture medium increased after addition of detergents (Table-II). Trition-x-100 at lower concentrations and Tween-80 but not Tergitol-7 were effective in releasing all enzymes. Although higher enzymes were produced at 0.05% Triton-x-100 in Am_1, Cm_1 and Fm_1, Tween - 80 at 0.1% was chosen to be the optimum detergent concentration for maximum release of the enzymes. This was due to the abnormal growth characteristics of the fungus in the presence

of Triton-x-100 and Tergitol 7. Cladosporium sp. BDCC-3 in the liquid medium is first olive-green and then black (dark green). But, there appeared depigmentation and/or retarded growth in the presence of Trition-x-100 and Tersitol 7. It is logical to believe that the detergents might have had membrane rupturing and cell lysis effect. The increased release of enzymes in Triton-x-100 may thus partly be due to the release of the protein of cells as a result of membrane disintegration. Demain and Birnbaum (1968), observed loss of membrane phospholipids after addition of detergents in Corynebacterium glutamicum medium.

From the three detergents tested (Tween-80, Triton-x-100 and Tergitol-7), Tergitol-7 was the most toxic. This was visualized from the very poor fungal growth and very scanty enzyme production in the Tergitol-7 containing media. Usually, detergents vary in their chemical characteristics and mode of action on membranes (Chochrane, 1958).

Production and release of cellulases and β-Glucosidase in media-2 (optimized media) was significantly increased (Table-III). The increment of each enzyme in most media was greater than or equal to 2-fold, but that of β-Glucosidase had reached to about 89-fold, in the α-cellulose medium. This explained the role of nutritional and cultural conditions on the production, release and activity of the enzymes. Incubation temperature was found to have the greatest role in increasing the activity of enzymes. Cellulases and β-Glucosidase from Cladosporium sp BDCC-3 were active at 60°C (Fig. 18) which is higher than those observed for the cellulases

from Aspergillus fumigatus and Talaromyces emersoni (Oso, 1978; Stewart and Parry, 1981). Temperture, usually, affects all activities (growth, spore germination and almost all metabolic activities) of a fungus (Chochrane, 1958). Thus, the data obtained (Table-III) leads to a conclusion that cultural conditions play an important role in the production and activity of cellulases and β-Glucosidase.

A study for the location of cellulases and β-Glucosidase in Cladosporium sp. BDCC-3 (Table IV) revealed that CMC-ase, Avicelase, [illegible]-cellulase, Fp-ase and cottonase were extracellular while β-Glucosidase was cytoplasmic. This probably may be related to the difference in molecular weight of the enzymes. In a study of production and characterization of cellulases and β-Glucosidase, the molecular weight of Cx from Alternaria alternata was 13,000 while that of β-Glucosidase was 45,000 (Macris, 1984). Thus, although further study on characterization of the cellulolytic enzymes from Cladosporium sp. BDCC-3 may be essential, the highly probable justification for the variation between cytoplasmic and cell free enzymes appeared to be the molecular weight difference of the enzymes.

Production of CMC-ase and β-Glucosidase by Cladosporium sp. BDCC-3 and few other fungi was compared (Appendix IIB). CMC-ase production by Cladosporium sp. BDCC-3 when grown on CMC was lower than Trichoderma reesi Qm 9424 and another species of Trichoderma (Desai et al. 1982). Production of this enzyme when the growth medium carbon source is filter paper was also less than that

produced by Trichoderma sp., Cladosporium sp. and Myrothecium roredium ATCC-28814 (Gashe, 1988). On the other hand, higher CMC-ase production was obtained from Cladosporium sp. BDCC-3 grown on CMC when compared to that produced by Scytalidium lignicoca, Trichoderam reesi Qm 6a and Qm 9123 and Myrothecium roredium ATCC-28814 (Desai et al., 1982; Gashe, 1988). Production of cell free β-Glucosidase by Cladosporium sp. BDCC-3 was lower than that produced by Trichoderma sp. but higher than that produced by Scytaldium lignicola, Trichoderma reesi, Aspergillus fumigatus and a species of Cladosporium (Desai et al., 1982 : Gashe, 1988).

In general Cladosporium sp. BDCC-3 seems a moderately efficient fungus in degrading both soluble and insoluble cellulosic substrates.

CONCLUSION

The study of production and release of cellulases and β-Glucosidase from Cladosporium sp. BDCC-3 and their activity revealed the following concluding remarks.

1. Although higher carbon and nitrogen concentrations seemed to yield higher proportion of enzymes, production and/or release of the enzymes into the culture filtrate was at a decreasing rate as nutrient concentration increased beyond a certain limit: usually from 1 to 1.5% cellulose.

2. In all media, CMC-ase was produced in higher concentration than all the others. This explained that Cladosporium sp. BDCC-3 produced more Cx than C_1 and B-Glucosidase. Moreover, Cmc-ase, Avicelase and α-cellulase were expressed earlier in the culture filtrate than FP-ase, cottonase and β-Glucosidase.

3. Cladosporium sp. BDCC-3 prefer nitrate than ammonium chloride or urea as nitrogen sources.

4. Production of proteases and their release into the culture filtrate of cladosporium sp. BDCC-3 may have contributed to the early decline of cell-free cellulases and β-Glucosidase. This calls for further work on proteases from the test organism.

5. Detergents increase permeability of the cell membrane of *Cladosporium* sp. BDCC-3 which facilitate release of the cellulolytic enzymes.

6. The activity of cellulases and B-Glucosidase was highly influenced by temperature. 60°C was optimum for maximum activity of all enzymes.

7. The study for location of cellulases and β-Glucosidase revealed that all except B-Glucosidase are extracellular.

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APPENDIX - I

Statistical test for the presence of significant difference in enzyme production between different concentrations of carbon sources at t = 0.1 significance level.

Carbon Source	Carbon source Concentration (%)	t-calc-Ulated	Remark	Optimum conc. obtained
Carboxy-methyl cellose (CMC)	0.5 and 1.0	2.04	(+)	1%cmc
	0.5 and 1.5	0.99	(-)	
	0.5 and 2.0	1.09	(-)	
	1.0 and 1.5	0.94	(-)	
	1.0 and 2.0	0.79	(-)	
	1.5 and 2.0	0.13	(-)	
Avicel	0.5 and 1.0	0.036	(-)	
	0.5 and 1.5	0.65	(-)	0.5%Avicel
	1.0 and 1.5	0.61	(-)	
α-cellulose	0.5 and 1.0	0.54	(-)	
	0.5 and 1.5	8.74	(+)	1.5%-cellulose
	1.0 and 1.5	9.17	(+)	
Filter paper	0.1 and 0.5	0.65	(-)	
	0.1 and 1.0	1.50	(+)	0.5% FP
	0.5 and 1.0	0.61	(-)	
Cotton	0.1 and 0.5	1.6	(+)	
	0.1 and 1.0	0.0	(-)	0.5 % cotton
	0.5 and 1.0	1.43	(+)	

$t_{(23)} = 1.33$ $t_{(17)} = 1.32$

(+) indicates presence of significant difference while (-) show that the compared concentrations are not significant.

APPENDIX - II

A. Comparison of carbon sources for their degradability by cellulolytic enzymes of Cladosporium sp. BDCC-3. All comparisons were made at the optimum concentrations of carbon and nitrogen sources. Production of enzymes was expressed as activity per mg protein.

Optimum carbon concentration	Optimum Nitrogen concentration	Activity (units/mg protein) Cellulases					B-Glucosidese
		cmc-ase	Avicelase	α-cellulase	Fp-ase	Cottonase	
1% cmc	1% NO_3^-	651.0	53.3	63.3	48.8	17.8	25.0
0.5% Avicel	1% NO_3^-	102.6	26.3	7.9	2.9	2.9	0.7
1.5% α-cellulose	2% NO_3^-	41.3	16.7	12.7	37.2	16.9	3.5
0.5 Fp	2% NO_3^-	1.2	0.6	0.6	12.8	1.6	1.1
0.5% cotton	1% NO_3^-	2.8	0.5	0.8	0.1	0.1	0.5

APPENDIX - II.B.

Comparison for the production of cmc-ase and B-Glucosidase by Cladosporium sp. BDCC-3 and other fungi. A unit of activity is defined as the amount of μ mole glucose or its equivalents produced in one minute from 1ml culture filtrate.

Organism	Substrate Concentration	Cellulase (cx)	β-Glucosidase	Reference
Cladosparium sp. BDCC-3	1% cmc	150	11.9	Test Organism
	0.5% Avicel	80	2.8	
	1.5% -cellulose	96	46.1	
	0.5% Filter paper	27.8	4.16	
	0.5% Cotton	3.9	0.07	
Scytalidium Lignicola	1-1.75% cellulose	17.23	10.3	Desai et al, 1982
Trichoderma reesi				
Qm 6a	#	18.0	-	
Qm 9424	#	152.0	0.48	
Qm 9123	#	59.0	0.15	
Pencillium sp.	#	-	10.18	
Aspergillus fumigatus	#	-	5.0	
Trichoderma sp.	Filter paper	790.0	158.0	Gashe, 1988.
Cladosparium sp.	Filter paper	135.0	3.0	
Myrothecium roredium ATCC-28814	Fitler paper	53.0	9.0	

DECLARATION

I, the undersigned, declare that this thesis is my work and that all sources of materials used for the thesis have been duly acknowledged.

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This thesis has been submitted for examination with my approval as university advisor

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