CELLULOLYTIC ENZYMES OF TRICHODERMA LONGIBRACHIATUM Rifai

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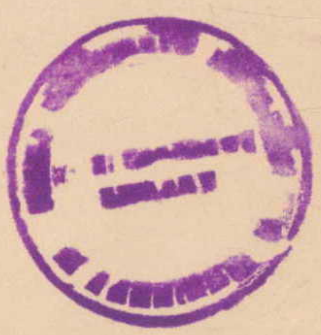

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CERTIFICATE

The work included in thesis entitled 'Cellulolytic enzymes of Trichoderma longibrachiatum Rifai' and submitted in partial fulfilment of the requirements for the degree of Master of Philosophy in Biology by Miss Maninder Kaur was carried out at the Department of Biology, Guru Nanak Dev University, Amritsar, under my supervision. This is an original piece of work and has not been submitted for any other degree/diploma at this or any other University/Institute.

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(Dr Mrs D.K. Sandhu)
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AFFECTIONATELY
TO MY
PARENTS

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INTRODUCTION

Cellulose is a most widely distributed natural polymer of β-1,4-linked D-glucose residues arranged in a supramolecular structure and constitutes about 50 per cent of the cell wall material of plants. It has a considerable potential as a source of chemicals, energy and microbial protein. Recently there has been much interest in the conversion of cellulosic materials to glucose. The high cost and problems connected with acid hydrolysis have directed attention to enzymic methods for the saccharification of cellulose. The structural features of cellulosic materials which determine its susceptibility to enzymic degradation are (a) degree of swelling by water (b) crystallinity (c) molecular arrangement (d) presence of substituent groups such as methyl, ethyl, carboxymethyl and hydroxymethyl. The addition of such groups makes cellulose less crystalline and more soluble in water.

Very effective hydrolytic enzymes for cellulose degradation are synthesized by different microbial species. The major enzymes of the cellulase complex are endo-β-1,4-glucanases (E.C. 3.2.1.4) CMCase, C_x; exo-β-1,4-glucanases (E.C. 3.2.1.91) FPA, C_1, cellobiohydrolase and

β-glucosidase (E.C. 3.2.1.21) cellobiase. Cooperative action of both exo- and endo splitting components produces high saccharification conversions of cellulose to glucose or cellobiose and finally cellobiase cleaves cellobiose to give glucose.

The cellulase enzymes have been shown to be located both in cell bound and cell free states, and their distribution is influenced by the carbon source. They are inducible and subject to catabolite repression and endproduct inhibition. Not many organisms have so far proved to be rich sources of cellulose and their industrial applications are at best uneconomic commercially. Search is continuing for cellulolytic fungi having adequate levels of all the cellulase components i.e. endoglucanase, exoglucanase and β-glucosidase.

The purpose of the present work has been to study the production and localization of cellulases and also the purification and physico-chemical properties of exo- and endoglucanases in Trichoderma longibrachiatum.

REVIEW OF LITERATURE

In its natural form, or as a waste material cellulose has considerable potential as a source of chemicals, energy and microbial protein. In order to make this goal economically feasible a thorough understanding of the microbial and enzymic processes is needed. The cellulase complex comprises three readily recognizable components, cellobiohydrolase, endoglucanase (CM-cellulase) and β-glucosidase or cellobiase. Although many fungi and bacteria degrade cellulose, only few microorganisms are known to produce highly active cellulase capable of extensively degrading insoluble cellulose to sugars in vitro. Of the different fungi studied for cellulolytic enzymes, Trichoderma reesei has gained much importance due to relatively high production, enzyme stability and efficient saccharification. In the past few years the production of cellulase has been raised by manipulating fermentation conditions and mutation programme.

SOURCES OF CELLULASE

Glucanases are synthesized by fungal and bacterial cells as extracellular, intracellular and cell associated

components. Few studies have been published on location of cellulases and they are mostly measured in the culture filtrate in terms of degradation of cellulose (Reese and Mandels, 1971). Cell lysis may also lead to the release of enzyme in the culture filtrate (Pollock, 1962).

Cellulolytic activity in Chaetomium species and Mycogone puccinoides was for the first time demonstrated by Iterson (1904). The above results were supported by Scales (1915) who reported that a number of Penicillium species tested showed the cellulose decomposing capacity. Otto (1916) reported the cellulolytic ability of Stemphylium, Botrytis, Mycogone, Cladosporium and Trichoderma. Ninteen species of Penicillium tested (Thaysen and Bunker, 1927) were found to be cellulase producing strains. A good number of fungal isolates including Acremonium, Brachysporium, Cephalosporium, Alternaria, Chaetomium, Fusarium, Hormodendron, Penicillium, Stemphylium, Stysanus, Thielavia, Stachybotrys, Metarhizium sp., Curvularia, Gliomastix, Helminthosporium, Humicola and Trichoderma were found to be cellulolytic and caused effective loss in strength of cotton fabric (Greathouse and Ames, 1945; Klemne et al., 1945; Marsh et al., 1949). Alternaria tenuis produced better CMCase on

pectin and cellulose than on carboxymethyl cellulose, filter paper and glucose (Pandey, 1965). Thom et al. (1934) pointed out that Chaetomium was the common cause of destruction of bags, shock covers, tentage etc. in the field. A number of Aspergillus species were also reported to be having cellulolytic activities (Basu, 1948; White et al., 1948; Jermyn, 1952). White et al. (1948) along with Aspergillus studied the cellulase enzymes of Penicillium, Fusarium, Humicola, Trichoderma viride, Myrothecium verrucaria, M. roridum, Chaetomium globosum and C. indicum. Garrett in 1962 found that Rhizoctonia solani decomposed cellulose in soil. He further (1963) reported the ability of Ophiobolus graminis, Cercosporella herpotrichoides, Fusarium colmorum and Helminthosporium sativum to grow on filter paper cellulose. Spalding (1963) showed the ability of Rhizopus stolonifer to produce cellulase in an artificial medium.

Carboxymethyl cellulase production by Choanophora cucurbitarum on galactose and other carbohydrates was reported by Gupta (1967). Mandels and Reese (1964), Halliwell (1965) and Li et al. (1965) observed the production of extracellular cellulase by Trichoderma viride and T. koningii which degraded cotton fibres. Further, Mandels and Reese (1964) treated dewaxed cotton fibres for 45 days with the culture filtrate of T. viride and found a weight

loss of 50 per cent. Trichoderma species were found to have the greatest ability to produce cellulolytic enzymes from peat and natural resources among the fungi tested including species of Penicillium, Aspergillus, Mucor, Domatium, Verticillium and Chaetomium (Lobanok and Shklyar, 1968). Trichoderma viride (ITCC 1433) produced high levels of enzyme when grown on different carbon sources particularly that of β-glucosidase (Herr, 1979). Chahal and Dhaliwal (1973) investigated the production of cellulase and utilization of cellulose by cellulolytic fungi, Rhizotonia solani, Trichoderma lignorum and Chaetomium globosum. Lacey in 1974 reported Allescheria terrestris, Chrysosporium pruinosum, Sporotrichum aureum and S. thermophile to be cellulolytic, isolated from bagasse. Aspergillus fumigatus and A. flavus were found to be cellulase producers by Sellars et al. (1976) and Olutiola (1976a).

Eriksen and Goksoyr (1976) studied the thermophilic mold, Chaetomium thermophile var. dissitum and found that it could grow on cotton, filter paper, cellulose powder and avicel. Studies on other thermophilic fungi include Humicola grisea var. thermoidea, H. insolens, Myriococcum albomyces, Sporotrichum thermophile and Torula thermophile (Fergus, 1969; Romanelli et al., 1975). Among phytopathogens are

Cercospora arachidicola which causes degradation of leaf tissues (Alabi and Naqvi, 1977), and the filtrates obtained after its growth on ground nut leaves causes loss in viscosity of carboxymethyl cellulose. However, little CMCase was synthesized when it was grown on pectate, pectin or glucose. In addition Ceratocystis paradoxa (Olutiola, 1976b), Aspergillus tamarii, A. sydowi, A. flavus (Olutiola and Cole, 1976, 1977), Pythium aphanidermatum (Winstead and McCombs, 1961) show restricted cellulolytic activity. Singh and Kunene (1980) found four isolates of Pyricularia which could degrade filter paper and carboxymethyl cellulose (CMC).

Berg and Pettersson (1977) and Berg (1978) reported the location and formation of cellulases and β-glucosidases in T. viride and Phialophora malorum on different substrates. The cellulases were found to be cell bound during active growth on cellulose, CMCellulose and cellobiose until substrate was utilized, although CMCase was cell free. Treatment of the mycelium with detergents hardly released any CMCase and β-glucosidase. Shewale and Sadana (1978, 1979) reported Sclerotium rolfsii a basidiomycete which could bring

about enzymatic hydrolysis of cellulosic materials. Eriksson and Hamp (1978) reported the absence of cell bound endoglucanases in Sporotrichum pulverulentum but in case of T. viride results were similar to those of Berg and Pettersson (1977). Sidhu and Sandhu (1979) isolated 7 fungi from decomposing bagasse out of which A. fumigatus among the Aspergilli showed highest cellulolytic activity. Vaheri et al. (1979) observed that filter paper activity and β-glucosidase were cell bound when grown on glycerol but in cellobiose the enzymes were released into the medium even at early stages of cultivation while β-glucosidase was mainly cell bound. ? Taniguchi et al. (1980) reported that Pellicularia filamentosa when grown under optimal conditions produces β-glucosidase 4 times as high as that of T. reesei 9414 mutant strain. Eupenicillium javanicum isolated from soil showed adequate cellulolytic activity towards crystalline cellulose and CMCellulase (Tanaka et al., 1980). 1,4-β-glucanases and 1,4-β-glucosidases produced by Penicillium janthinellum were found to be cell free with small amounts of β-glucosidase associated with cell walls as well as cytosol (Rapp et al., 1981). Halliwell and Lovelady (1981) reported that 99 per cent of endoglucanases were located extracellularly and one per cent of total activity was cell bound. Similar measurements on β-glucosidase showed that 90%

of this activity was cell associated at all times and 10% was extracellular in T. koningii.

Yeast cellulases were demonstrated by Dennis (1972) and Stevens and Payne (1977). They reported the ability of Trichosporon cutaneum and T. pullulans culture filtrates to cause 40 per cent solubilization when grown on ball milled filter paper. This was a little less than that shown by filtrates of T. viride and Mycothecium verrucaria. Bacterial cellulases are more firmly bound to cells or cellulose, since culture filtrates of bacteria do not always contain the cellulase enzymes. Saddler and Khan (1980) and Mackenzie and Bilous (1982) located the cellulose solubilizing and CMCase activities of Acetovibrio cellulolyticum in soluble fraction whereas β-glucosidase was cell associated. Common cellulase producers are Clostridium thermocellum (Forget et al., 1980), and Cellulomonas (Gray et al., 1980). Among the actinomycetes cellulase production has been studied in Thermomonospora (Phillips and Humphrey, 1980) and Streptomyces flavogriseum (Ishaque and Klupfel, 1980).

PURIFICATION OF THE CELLULASE COMPONENTS

The complex cellulase system consists of three main enzymatic components as follows:

1. Endo-β-(1-4)-glucan glucanases (CMCellulase, C_x enzyme) are present in several forms and are effective on cellulosic derivatives.

2. Exo-β-(1-4)-glucanases are present in two major forms (a) β-(1-4)-glucan cellobiohydrolase (CBH) removing cellobiose units from the non-reducing ends of the cellulose chain. This component is considered equivalent to C_1 enzyme by many workers, (b) β(1-4)-glucan glucohydrolase, which removes glucose from non-reducing end of the chain. Exoenzymes are fully active only when supplemented with other components of the cellulase complex.

(3) Cellobiase (β-1-4-glucosidase) cleaves cellobiose and other cellodextrins to yield glucose.

Fractionation of the cellulolytic components has been extensively studied on cellulases of Trichoderma reesei, T. koningii and Sporotrichum pulverulentum (Table I). The techniques involved in the purification process are solvent precipitation, ion exchange and molecular seive chromatography, isoelectric focussing and electrophoresis.

Li et al. (1963) and King (1965) separated a commercial enzyme preparation of Trichoderma viride into hydrocellulase

fraction which had high activity towards avicell, endoglucanase fraction with activity towards carboxymethyl cellulose (CMC) and cellobiose. Niwa et al. (1964, 1965) using the enzyme from the same source obtained by electrophoretic run, three to four fractions of cellulase with activity towards filter paper and carboxymethyl cellulose. Selby and Maitland (1967a) separated three major cellulolytic components on sephadex G-75. Further (1967b) they found that the enzyme from T. viride consisted of (a) cellobiase component without activity towards CMC or cotton (b) endoglucanase (C_x) with activity towards CMC only (c) a glycoprotein without activity towards any of the above substrates. Cellobiase along with glycoprotein accounted for 20 per cent of the activity towards cotton while endoglucanase and glycoprotein accounted for 35 per cent of the activity. All the three components together were responsible for complete activity towards cotton.

Initially T. viride was thought to produce a single exoglucanase referred to as cellobiohydrolase I (CBH) (Berghem and Pettersson, 1973) which had a molecular weight of 46,000 and degraded crystalline cellulose, swollen avicel and cellotetraose. However in 1979 Fagerstam and Pettersson identified a second cellobiohydrolase II. The specific activity of this new CBH II was 3-4 times higher than CBH I.

Both the cellobiohydrolases consisted of a number of isozymes which can be separated by isoelectric focussing. Gum and Brown (1977) purified four cellobiohydrolases from T. viride, which resembled one another in several properties but differed in composition of bonded carbohydrates.

Cell free culture filtrates from Trichoderma koningii were concentrated by precipitation with ammonium sulphate to 80 per cent saturation (Wood, 1968). Halliwell and Riaz (1971) working on cellulase of T. koningii fractionated it into four components cellobiase, endo β-1,4-glucanase, C_2 and C_1 which acted in a synergistic manner. The C_1 components from T. koningii was purified by Halliwell and Griffin (1973). This component alone could hydrolyse cellulose in acidic media of pH 5.0 to a disaccharide cellobiose. The degree of solubilization on addition of purified cellobiase increased by 20-80% since it degrades cellobiose to glucose and thus relieved the C_1 component from inhibition by cellobiose (Halliwell et al., 1972). Wood and Mcrae (1972) purified the C_1 enzyme from T. koningii and proposed it to be a β-1,4-glucan cellobiosylhydrolase. In 1978b they separated and purified four endoglucanase components of T. koningii. All the four hydro-

lyzed CMC, swollen cellulose, cellotetraose and cellopentaose, which differed in rate and mode of attack. Activity of all the components together accounts for effective breakdown of cellulose. Using Sigma cell-100, Ghai (1978) isolated CMCellulase, β-glucosidase, C_1 and C_2 enzymes from cellulase of T. koningii.

Shoemaker and Brown (1978 a,b) analysed the major enzymic components, endo and exo-glucanases. They revealed that each type of enzyme consists of a number of distinct enzyme species. Four different endoglucanases from commercial preparation of Trichoderma cellulase have been described each having unique substrate specificity. Selby et al. (1963) and Selby and Maitland (1965) suggested that Myrothecium verrucaria contained two enzymes that differ in rate and extent of attack on fibrous cellulose. Enzyme A was necessary for degradation of cotton while B had little effect on it and was called as CMCellulase. Aspergillus niger culture filtrate has been fractionated into eight partially purified components by ion exchange and adsorption chromatography (King and Smibert, 1963; Li and King, 1963). Using DEAE sephadex gel, Pettersson and Porath (1963, 1966) purified cellulase from Polyporus versicolor and Penicillium notatum. Whitney et al. (1969) showed three C_x components from enzyme of Verticillium albo-atrum.

Boretti et al. (1973) separated five fractions, three endo and two exoglucanases from Penicillium iriens which too showed a synergism in the attack of cotton fibres. Wood (1971) and Wood and McCrae (1977) separated the cellulase components of Fusarium solani cellulase on ultro gel ACA 54. The cellulase complex from Sporotrichum pulverulentum was fractionated into 5 endo-1,4-β-glucanases and one exo-1,4-β-glucanase which show a strong synergistic response towards degradation of dewaxed cotton and avicel (Streamer et al., 1975). A cellobiose quinone oxidoreductase was isolated which is important in cellulose and lignin breakdown. It oxidises cellulose during degradation and increases the extent of degradation in Sporotrichum pulverulentum (Eriksson et al., 1975b). Cellulase of Geotrichum candidum 3C was fractionated into five endoglucanases and one cellobiase (Rodionova et al., 1980). Ammonium sulphate fractionation and acrylamide gel electrophoresis was used by Vaidya et al. (1980) to purify one exoglucanase and two endocellulases from culture filtrate of Fusarium lini. Enari et al. (1980) working on A. niger and T. reesei isolated two cellulases and 2 β-glucosidases from T. reesei and 2 cellulases and one β-glucosidase from A. niger with the help of gel chromatography.

Molecular weights of the exo and endoglucanases vary between 12,000-145,000. β-glucosidases possess higher molecular weights and the highest reported so far is 400,000 (Wood, 1975). A comparative account of molecular weights of different cellulases is given in Table I.

MECHANISM OF ACTION

The ability of a variety of organisms to degrade cellulose is a long known phenomenon. Pringsheim (1912) proposed the theory of cellulase action by suggesting that two enzymes were involved in cellulose degradation. He observed that cellulolytic bacteria produced glucose and postulated that one enzyme (cellulase) had affinity for long chains of glucose and cleaved cellobiose from the chains while the second enzyme (cellobiase) splits cellobiose into 2 glucose molecules.

Reese et al. (1950) suggested that C_1 attacks crystalline cellulose, either producing short chains or decrystallizing the glucan chains so that the cellulose was susceptible to attack by the hydrolytic C_x enzymes. He reported that true cellulolytic organisms possess both C_1 and C_x enzymes. Mandels and Reese (1964) proposed a C_1-C_x system for the mode of action of cellulases. They succeeded in

separating the C_1, C_x and β-glucosidase components of cellulase and assumed that C_1 allows hydration of cellulose to make it accessible for the action of hydrolytic β-1,4-endoglucanase. This ability of C_1 component to attack highly ordered cellulose was supported by Li et al. (1965). C_1 and C_x acting randomly are referred to as endoenzymes and also at other times may be exoenzymes removing glucose or cellobiose from the non-reducing end of the cellulose molecule (King and Simibert, 1963).

Selby and Maitland (1967b) reported striking synergism between C_1 and C_x and proposed that C_1 makes cotton fibres susceptible to C_x action or C_1 is inhibited by its end products unless they are removed by action of C_x. Further, Halliwell and Griffin (1973) demonstrated that the weak action of C_1 component was not only promoted by C_x component but also by the removal of its inhibitor, cellobiose, which was hydrolysed by cellobiase to glucose. This purified C_1 enzyme was suggested to be a cellobiohydrolase acting as an exoglucanase against cellulose (Wood and McCrae, 1972; Halliwell and Griffin, 1973; Berghem and Pettersson, 1973).

Later, a number of reports appeared which contradicted the C_1-C_x concept of Reese and coworkers. Wood and McCrae (1972)

found a purified C_1 enzyme which in itself could only attack oligosaccharides such as cellotetraose, cellohexaose to produce cellobiose. Streamer et al. (1975) reported that the exo-glucanase degraded dewaxed cotton at a faster rate only if pretreated by endoglucanase. The above results support the other theory that C_x (endoglucanase) acts randomly over cellulose chain to open it, thus allowing further action by C_1 (exoglucanase) on exposed chain ends. Studies on purified components demonstrate that synergism among the components of a cellulase complex is necessary for efficient solubilization of native and derived form of cellulose e.g. as in T. koningii (Wood, 1975), T. reesei (Selby and Maitland, 1967 a,b), Fusarium solani (Wood and McCrae, 1977), Penicillium iriens (Boretti et al., 1973), Sporotrichum pulverulentum (Streamer et al., 1975).

The latest proposed scheme of mechanism of cellulase action is illustrated in Fig. I (Wood and McCrae, 1972, 1978a; Reese, 1975; Montenecourt et al., 1979b) where the endo-1,4-β-glucanase randomly cleaves internal glucosidic bonds within an unbroken glucan chain. The non-reducing ends of the chain thus become substrate for 1,4-β-glucan cellobiohydrolase, which cleaves cellobiase dimer from the chain end and releases them into the solution. The hydrolysis

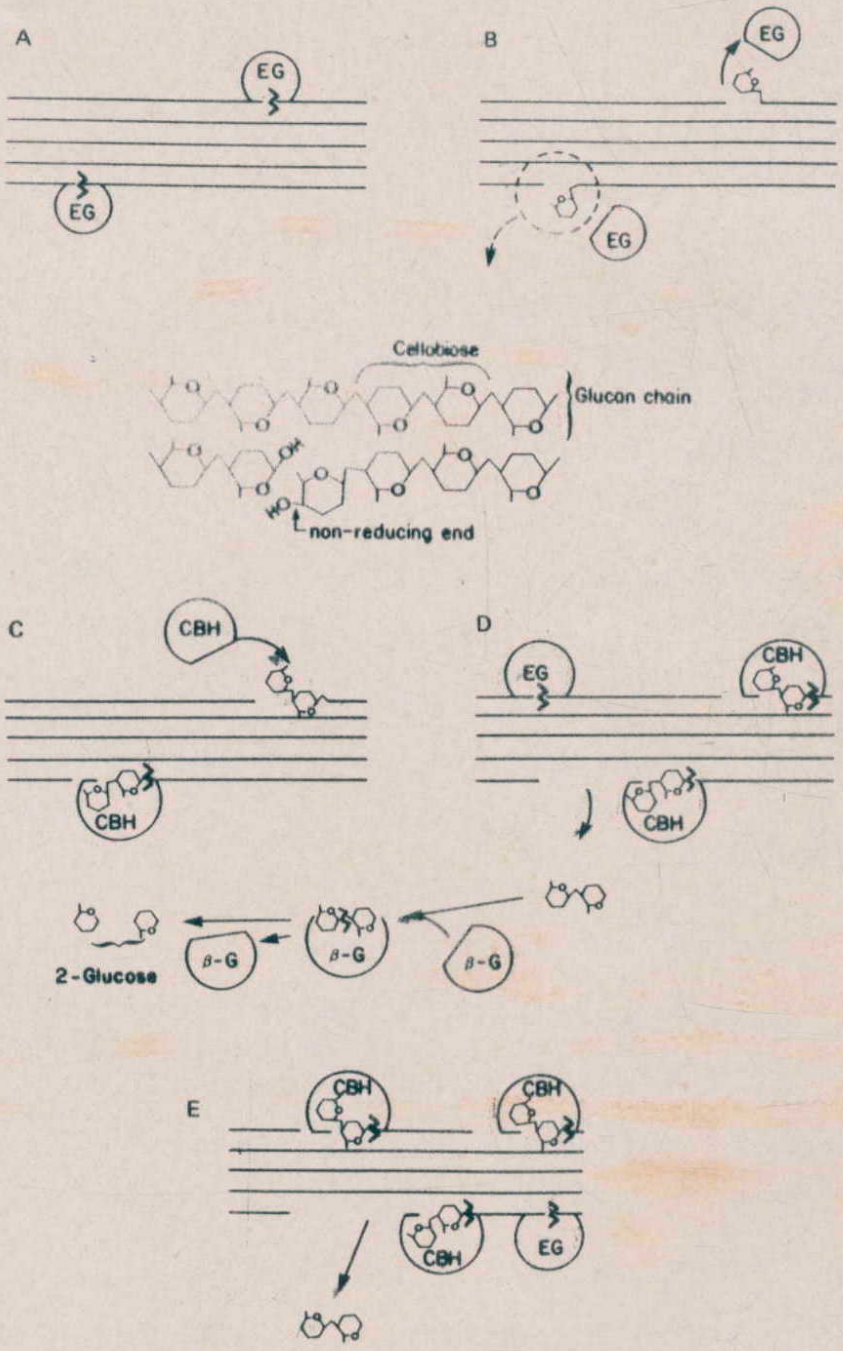

Fig.I. Schematic representation of cellulase action

of cellulose into glucose end-product is completed by β-glucosidase which splits cellobiose into glucose monomers. The creation of non-reducing glucan chain ends by endo-glucanase (EG) that are sites of catalytic action for cellobiohydrolase (CBH) leads to a synergism in the overall rate of cellulolysis.

CELLULASE SYNTHESIS AND ITS REGULATION

The regulation of the biosynthesis of the cellulase complex is under the control of mechanism of induction and catabolite repression (Montenecourt et al., 1979a). Cellulolytic enzymes are generally considered to be formed only in the presence of cellulose and repressed by the presence of glucose and other easily metabolizable sugars in the medium. Mandels and Reese (1960) observed that cellulase is an adaptive enzyme in fungi and is produced only when they are grown on cellulose or cellobiose. Other inducers for cellulase synthesis include cellulose derivatives, xylan, pectin, lactose, octaacetate and salicin. Sophorose has been reported to be one of the most potent inducers of CMCellulase in T. reesei (Mandels et al., 1962; Nisizawa et al., 1971; Sternberg and Mandels, 1979). Considering the production of CMCellulase along with C_1 enzyme the best natural inducers are cellulosic materials such as

cotton, wood, solca floc, filter paper, corn stover etc. (Mandels and Webber, 1969). Gupta and Heale (1971) stated that substrate must have a β-glucoside linkage for proper induction.

Horton and Keen (1966) studied the sugar repression in the case of Pyrenochaete terrestris and reported that on addition of glucose the cellulase synthesis was repressed to a basal level and on dilution of the basal medium cellulase formation commenced. So it was concluded that cellulase synthesis is regulated by products of cellulolysis through repressor inducer mechanisms. Acetate, butyrate, formate and propionate at a concentration of 10^{-3} M were reported to inhibit cellulase in T. viride (Mandels and Reese, 1960). Hulme and Stranks (1970) proposed that the rate of metabolism in an organism controls the production of cellulase when glucose is exhausted, the endogenous metabolism of the organism can support the production of cellulase even in the absence of cellulose. Observing similar results in Myrothecium verrucaria they confirmed that cellulase is a constitutive enzyme and its production may be partly controlled by catabolite repression (Hulme and Stranks, 1971). Not only glucose but other compounds such as succinate, malate, glutamate, glycerol, fructose and gluconate repressed cellulase induction but not secretion (Nisizawa et al., 1972).

Spalding et al. (1973) observed that production of CMCellulase by Penicillium expansum in 9 mineral salts medium was repressed by 0.1 M arabinose, mannose, galactose, glucose, sucrose, raffinose, galactouronic acid and glutamic acid. Component C_1 was found to be inhibited by cellobiose (its endproduct) to a larger extent than CMCellulase (Halliwell and Griffin, 1973).

Gong and Tsao (1979) have proposed a model for the cellulase regulation in fungi which shows that glucose in cytoplasm represses the transcription and translation of cellulases. The regulation of cellulase synthesis in part by catabolite repression and induced cellulase production in lactose pregrown cells of Aspergillus fumigatus in comparison to glucose pregrown cells, was reported by Trivedi and Rao (1980).

Table I

Organism	Cellulase components	Molecular weights
T. reesei (Pettersson, 1975)	Exoglucanase	42,000
	Endoglucanase I	12,000
	" II	50,000
	β-glucosidase	47,000
T. koningii (Wood, 1975)	Exoglucanase I	62,000
	" II	62,000
	Endoglucanases	
	Cx_1	13,000
	Cx_2	n.a.
	Cx_{3a}	38,000
	Cx_{3b}	38,000
	Cx_4	31,000
	Cx_5	n.a.
	β-glucosidase I	n.a.
	" II	n.a.
S. pulverulentum (Streamer et al., 1975)	Exoglucanase	48,600
	Endoglucanases	
	T_1	32,300
	T_{2a}	36,700
	T_{2b}	28,300
	T_{3a}	37,500
	T_{3b}	37,000
	β-glucosidase	n.a.

Contd.....

Table 1 contd.....

G. candidum 3C (Rodionova et al., 1980)	Exoglucanase	n.a.
	Endoglucanases	
	I	82,000
	II	145,000
	III	36,000
	IV	11,000
	V	67,000
	Cellobiase	200,000
F. solani (Wood, 1975)	Exoglucanase	45,000
	Endoglucanase	37,000
	β-glucosidase	400.000
F. lini (Vaidya et al., 1980)	Exoglucanase	56,000
	Endoglucanase I	47,000
	" II	27,000
Pellicularia filamentosa (Tanaka et al., 1977)	Endoglucanases	
	I-A-1	46,000
	I-A-2	68,000
	I-A-3	50,000
	I-A-4	46,000
	I-A-5	26,000

n.a. = not available

MATERIALS AND METHODS

The cellulase (exo- and endoglucanase along with β-glucosidase) has been worked out varying the cultural conditions like temperature of incubation, hydrogen ion concentration, shake and stable culture. A number of soluble and insoluble carbon sources including native celluloses were tested for enzyme production and the effect of substrate concentration was studied with respect to lactose and carboxymethyl cellulose (CMC). Different components of cellulase viz: filter paper activity (FPA), carboxymethyl cellulase (C_x) and β-glucosidase were estimated in the three fractions i.e. extracellular, cytosol and cell debris.

Filter paper activity and carboxymethyl cellulase after purification were characterized with respect to kinetic parameters.

MICROORGANISM

Trichoderma longibrachiatum Rifai (IMI 249401) used in the present study was isolated from degrading *Mangifera* wood. The stock culture was maintained in soil at 4°C and subcultured on Vogel's glucose agar whenever required.

PREPARATION OF INOCULUM

The fungus was grown on Vogel's glucose agar slants at 27°C. From a six day old culture a uniform suspension of spores was prepared with sterile distilled water which was filtered through layers of sterile gauze to make it free from mycelial debris. The suspension was appropriately diluted so that the final concentration in the inoculated medium was 5×10^6 spores per ml.

PRODUCTION OF CELLULASES

Twenty five ml of the Vogel's medium was dispensed in each of 100 ml conical flasks containing 250 mg of CMC as the carbon source. The flasks were autoclaved at 10 lbs pressure for 20 min and each was inoculated with standardized spore suspension. Incubation was done at 27°C as stationary cultures. Triplicate flasks were analysed daily for ten days. For dry weight determination the contents of the flasks were filtered through Whatman No. I filter paper, washed with distilled water and dried to a constant weight at 80°C.

For the estimation of enzymes the mycelium was separated by slow speed centrifugation. The culture filtrate free of mycelium was centrifuged again at 10,000 rpm for 15 min at 4°C. The resulting supernatant was assayed for extracellular enzymes. The above mycelial mats were washed thoroughly with

0.1 M acetate buffer (pH 5.0) and dried between the folds of filter paper for preparation of cytosol and cell debris fractions. Cytosol extracts were prepared by crushing the mycelium with macerating sand in a chilled pestle and mortar. This was suspended in 10 ml of acetate buffer and centrifuged at 16,000 rpm for 30 min at 4°C. The supernatant was collected and stored for further use. Mycelium for cell debris fraction was macerated without sand and the cell debris after centrifugation as above was suspended in 10 ml of acetate buffer for enzyme assay.

ESTIMATION OF ENZYMES

Cellulase was measured in terms of carboxymethyl cellulase, filter paper activity and β-glucosidase by the method of Mandels et al. (1976) as follows:

Filter paper activity (FPA): To 0.5 ml of diluted enzyme was added 0.5 ml of 0.1 M acetate buffer, pH 5.0 and 50 mg of Whatman No. I filter paper (1x6 cm strip). The reaction mixture was incubated at 50°C for 30 min.

Carboxymethyl cellulase (C_x): To 0.5 ml of appropriately diluted enzyme sample was added 0.5 ml of one per cent CMC solution and the reaction mixture was incubated at 50°C for 30 min.

β-glucosidase: To 0.5 ml of diluted enzyme was added 0.5 ml of one per cent salicin solution and the reaction mixture was incubated at 50°C for 30 min. The aryl-β-glucosidase was estimated with 5 mM p-nitrophenyl-β-D-glucopyranoside (PNPG) at 37°C for 30 min.

The amount of reducing sugars in each of the above reactions was estimated by dinitro-salicylic acid (DNS) reagent method (Miller, 1959), taking into consideration the reducing sugars already present in enzyme samples. The nitro-phenol released with aryl-β-glucosidase was read at 420 nm.

Culture filtrates of the media containing soluble sugars were dialyzed against acetate buffer for 24 h at 4°C before processing of the samples.

ENZYME UNIT

Extracellular enzyme activity has been expressed in international units (IU). One unit of activity has been defined as the amount of enzyme needed to release 1 μmol of glucose or its equivalent per ml per min. Cytosol and cell debris activities have been expressed in terms of specific activity i.e. IU per mg protein for cytosol and IU per mg dry weight for cell debris.

ESTIMATION OF PROTEIN

Soluble protein content was estimated as proposed by Lowry et al. (1951) and expressed as mg protein per ml of culture filtrate.

ESTIMATION OF CELLULOSE

Cellulose in the culture filtrate was determined with anthrone reagent (Updegraph, 1969).

To one ml of appropriately diluted culture filtrate was added 4 ml of freshly prepared chilled anthrone reagent and the mixture was kept in boiling water bath for 10 min. Absorbance was read at 620 nm after cooling with tap water.

EFFECT OF CULTURAL CONDITIONS ON THE PRODUCTION OF CELLULASE

Cellulase production under a range of temperature and pH and in shake/stable cultures was studied using one per cent carboxymethyl cellulose as carbon source in Vogel's medium. Flasks containing 25 ml of medium were inoculated with standardized spore suspension. The experiments were terminated on 5th day of incubation and the flasks were processed for determination of dry weight, extracellular, cytosol and cell debris enzymes.

Temperature: The incubation temperatures tested included 15°, 22°, 27°, 32°, 37° and $42^{\circ}C$.

pH: The pH of the different flasks containing basal medium was adjusted to 2.0, 3.0, 4.0, 4.5, 5.0, 5.5, 6.0, 7.0 and 8.0 after sterilization using 1N solutions of NaOH and HCl. After inoculation the flasks were incubated at 27°C.

Effect of shaking: The cultures were incubated on a shaker as well as stationary for 10 days. The samples were processed daily for substrate concentration and pH of the culture medium along with other parameters as mentioned above.

EFFECT OF VARIOUS SUBSTRATES ON CELLULASE PRODUCTION

a) Soluble carbohydrates: Carboxymethyl cellulose in Vogel's medium was substituted by lactose, maltose, sucrose and cellobiose separately as a sole source of carbon each at one per cent concentration. Sugars were sterilized by seitz filtration and added later to the autoclaved medium. Complex compounds like yeast and malt extract were also tried along with the above sources for cellulase production. The inoculated flasks were incubated at 27°C.

b) Insoluble native celluloses: The fungus was grown on Vogel's medium containing natural (untreated) and delignified sugar-cane bagasse, paddy and wheat straws including micro-crystalline cellulose (MCC) at a concentration of one per cent. Delignification of straws was carried out as proposed by

Sidhu and Sandhu (1980). Incubation was done on a rotary shaker at 27°C and flasks were analysed for extracellular and cell-substrate associated enzymes because it was difficult to separate cell mass from the unutilized substrate. For measuring cell-substrate associated enzymes the mycelium and unutilized straw were harvested by slow centrifugation and washed with acetate buffer. A uniform suspension of cell substrate complex was prepared by homogenizing the material in a chilled waring blender for 2 min. The final volume was made upto 25 ml and processed as in the case of cell debris. Reduction in dry weight of the straw was obtained by filtering the contents through Whatman filter paper and drying to a constant weight at 80°C. Observations were recorded on alternate days.

EFFECT OF SUBSTRATE CONCENTRATION ON ENZYME PRODUCTION

Among the various substrates tried lactose gave best results. Therefore different concentrations of lactose along with CMC were employed for production of cellulases. Vogel's medium was supplemented with 0.5, 1.0, 1.5, 2.0 and 2.5 per cent of CMC and lactose separately. The contents were analysed as usual after 5 days of incubation.

EFFECT OF GLUCOSE ON CELLULASE PRODUCTION

Two step cultures were used to study the effect of glucose on production of glucanases. Five hundred ml flasks

containing 100 ml of basal synthetic medium, supplemented with 2 per cent glucose were inoculated with spore suspension. The cultures were grown on a shaker at 27°C for 48 h. Actively growing mycelium was collected by filtration, washed with acetate buffer and finally transferred to 500 ml flasks containing similar medium with 2% glucose. The flasks were incubated on a shaker at 27°C and samples were taken at intervals for determination of dry weight, glucose concentration and glucanases.

PURIFICATION OF CELLULASE COMPLEX

For the production of enzyme in large amounts, five hundred ml conical flasks containing 100 ml of Vogel's medium with one per cent of delignified bagasse were inoculated with standardized spore suspension and incubated on a shaker at 27°C. The culture filtrate obtained after 10 days of growth was centrifuged at 10,000 rpm for 15 min at 4°C. All the operations were carried out at 4°C unless stated otherwise.

Ammonium sulphate precipitation: The clear supernatant was precipitated with ammonium sulphate between 40-80 per cent saturation. Before precipitation the enzyme was buffered to pH 5.0 with solid sodium acetate (0.1 M) and concentrated acetic acid. After 6 h the precipitate was collected by

centrifugation, dissolved in a small volume of buffer and dialyzed with several changes of the same buffer.

Ion-exchange chromatography:

(a) Preparation of DEAE (diethylaminoethyl) Sephadex A-50 and SE (sulphoethyl) Sephadex C-50: DEAE-sephadex was allowed to swell in distilled water and washed with 0.5 N NaOH on a buchner funnel until free of chloride. NaOH was removed by rinsing with distilled water. Then the ion exchange was washed with 0.5 N acetic acid. The excess of acid was removed with distilled water with a subsequent equilibration with 0.1 M acetate buffer at pH 5.0. SE-sephadex was prepared in a similar way but with a reverse order of washing procedures i.e. first with acid and then with base and the buffer used in this case was 0.01 M (pH 4.0).

(b) Preparation of column: A corning glass column (2.5x40 cm) was fixed in a vertical position on an iron stand. The column was prepared by pouring the equilibrated ion exchanger (70% v/v) into the column partially pre-filled with buffer. After setting of the adsorbant at the lower end of the column to about 2 cm thickness the buffer was allowed to flow down and a desired height (30 cm) was obtained. The column was then equilibrated with acetate buffer.

The enzyme sample (5 ml) after dialysis was applied to the column. At first the column was eluted with initial buffer 0.1 M acetate with 0.05 M NaCl until 30 fractions (4 ml each) were collected, and then by NaCl (0.05 - 0.5) gradient. Elution gradient was achieved by continuous addition of 0.1 M acetate buffer containing 1 M NaCl to 0.1 M acetate buffer with 0.05 M NaCl (100ml of acetate buffer with 0.05 M NaCl in a mixer and 100 ml of acetate buffer with 1 M NaCl in reservoir). At an elution rate of 20 ml per h a total of 60 fractions (4 ml each) were collected with a LKB fraction collector. All the fractions were analysed for protein, C_x, FPA and aryl-β-glucosidase activities.

SE-sephadex chromatography: The preparation of column was similar to that of DEAE-sephadex with the difference that the column was equilibrated with 0.01 M acetate buffer at pH 4.0. Fraction No. (4-11) rich in C_x and FP activity were pooled and precipitated by ammonium sulphate. The precipitates were dissolved in a minimal volume of buffer and dialysed against the same. The above enzyme (5 ml) was applied to the SE-sephadex column, which was eluted with pH 4.0 - 6.0 gradient. Twenty fractions of 4 ml each were collected at an elution rate of 20 ml per h. The fractions were processed as usual.

Gel chromatography on Sephadex G-200: The gel was allowed to swell in distilled water at 90°C for 5 h. The cooled gel suspension was poured into the vertically mounted column (1x90 cm) using a glass rod and equilibrated with 0.1 M acetate buffer, pH 5.0. Three ml of enzyme sample after SE-sephadex was applied to the column. Three ml fractions were collected at a flow rate of 15 ml per h. The fractions were processed for protein content and various enzyme activities.

PROPERTIES OF GLUCANASES

The various kinetic parameters studied with the purified enzyme are as follows:

Progress curve and effect of enzyme concentration: The reaction mixture was incubated at 50°C for different periods of time ranging from 0-2 h. Various concentrations of enzymes were incubated in a standard reaction mixture to see the effect of enzyme concentration on rate of reaction.

Enzyme activity and stability at different pH and temperatures: The enzyme with the substrate was incubated at different pH from 2 - 8 (0.1 M acetate buffer from pH 2.5 - 5.5 and 0.1 M phosphate buffer from pH 6 - 8) and temperatures (30 - 90°C) for obtaining the optimal conditions

of enzyme assay. For determining the stability fthe enzyme was preincubated without substrate in buffer at pH 2 - 8 for 30 min and temperature varying from 30 - 100°C for different intervals of time before the standard assay procedure.

Km values and effect of metal ions: The effects of different substrate concentrations on the reaction rate were determined. The Michaelis-Menton constant was calculated from simple Lineweaver-Burk plots. A number of metal ions affecting the enzyme activity were examined by using the following compounds: $AgNO_3$, $HgCl_2$, $CuSO_4$, $CoCl_2$, $ZnCl_2$, $MgCl_2$, $CaCl_2$, NaCl , EDTA (ethylene diamine tetra acetic acid) and KCl. The relative activity was expressed as percentage of enzyme activity in the absence of metal ions.

Molecular weight determination: Molecular weights were determined on 7% polyacrylamide gel by the method of Davis (1964). One gel was stained for protein with commossie blue and the other was cut into sections, which were extracted with 0.5 ml of 0.1 M acetate buffer (pH 5.0) and then assayed for C_x and FP activity. The standard proteins used as control included Memocyanin, egg albumin and tripsinogen.

CULTURE MEDIA

Vogel's medium

Vogel's salts solution (a), 20 ml; Vogel's vitamin solution (b), 1 ml; Reese and Mandel's trace

elements (c), 1 ml; peptone, 1 g; distilled water, 1000 ml, pH 5.0.

(a) Vogel's salts solution (50 x strength): Sodium citrate - 31.25 g, K_2HPO_4 - 31.250 g; NH_4NO_3 - 25 g; $MgSO_4.7H_2O$ - 2.5 g; $CaCl_2$ - 1.250 g; distilled water - 250 ml.

(b) Vogel's vitamin solution: Biotin - 0.5 mg; inositol - 200 mg; calcium pentothenate - 20 mg; pyridoxine HCl - 20 mg; thiamine - 20 mg; distilled water - 100 ml.

(c) Reese and Mandel's trace elements: Conc. HCl - 5 ml; $FeSO_4 7H_2O$ - 4.68 g; $MnSo_4 7H_2O$ - 0.98 g; $ZnSO_4 7H_2O$ - 1.76 g; $Co(NO_3)_2 6H_2O$ - 1.25 g; $CoCl_2 6H_2O$ - 1.83 g; distilled water - 95 ml.

Vogel's glucose agar:

The above medium was supplemented with one per cent glucose and solidified by 1.5% gagar.

CHEMICALS

All the chemicals used were of high purity (A.R. grade) marketted by B.D.H./E.Merck/SISCO/Loba (India). Carboxy-methyl cellulose, dinitrosalicylic acid, cyanogum, Coomassie brilliant blue and standard proteins for SDS electrophoresis were procured from Sigma. Diethyl aminoethyl sephadex A-50, sulphoethyl sephadex C-50, sephadex G-200 and dextran blue

were procured from Pharmacia Fine Chemicals, Sweden, Dialysis tubing was obtained from CSIR Centre for Biochemicals, Delhi.

REAGENTS

One per cent carboxymethyl cellulose: Ten g of CMC was dissolved in 900 ml of 0.05 M acetate buffer at pH 5.0 and the final volume was made to one litre. The solution was stored at 4°C.

One per cent salicin: Ten g of salicin was dissolved in 900 ml of 0.05 M acetate buffer at pH 5.0. The final volume was made to one litre and stored at 4°C.

Dinitrosalicylic acid reagent: 3,5-dinitrosalicylic acid, 20 g, phenol, 4 g, sodium potassium tartarate (rochelle salt), 400 g, sodium sulphite, 1 g; dissolved in one litre of one per cent NaOH. The final volume was made to 2 litres.

Anthrone reagent: 0.2 per cent of anthrone in concentrated sulphuric acid.

GLASSWARE

Corning glassware was used throughout the study, every time it was washed with detergent with final washings with distilled water.

OBSERVATIONS

Trichoderma longibrachiatum has been studied in both shake and stable cultures for the production of cellulase components in cell free as well as cell associated states, using carboxymethyl cellulose as a sole source of carbon. Cultural conditions for the maximum synthesis of cellulase have been standardized. Purification, fractionation and physico-chemical characterization of endo- and exo-glucanases has been done.

EFFECT OF INCUBATION PERIOD

Stable culture: The fungus was incubated for 10 days at 27°C in Vogel's medium supplemented with one per cent carboxymethyl cellulose. Maximum cellulolytic activities including filter paper activity (FPA), carboxymethyl cellulase (CMCase) and β-glucosidase were obtained between 4-7 days of incubation after which they started decreasing (Fig. 1-3). Highest values for FPA in extracellular (0.275 units), cytosol (0.15 units) and cell debris (0.0075 units) fractions were observed on 6th, 5th and 4th day respectively (Fig. 1 a,b,c). Filter paper activity in the cytosol fraction declined to zero value on the 8th day while the extracellular and cell debris fractions maintained their activity with a little decrease upto the last day of incubation. Carboxymethyl

cellulase had a similar profile as that of FPA with extracellular (1.64 units) and cytosol)(0.195 units) maxima on 6th and 5th day respectively while the cell debris 6.01 units) reached its maximum on 2nd day of incubation (Figs. 2 a,b,c). The peak value for β-glucosidase was delayed upto 7th day for extracellular (0.10) and cytosol fraction (0.20) was maximum on 5th day whereas cell debris on 3rd day (0.05) (Fig. 3 a,b,c).

Both FPA and CMCase were mainly present in the extracellular fractions as determined on 8th day of growth. Although β-glucosidase was higher in extracellular (60.73%) the cell debris fraction also contributed 35.56% of the activity with cytosol being the least (3.89%) (Table 2).

Maximum growth in terms of dry weight was obtained on 6th day (29.5 mg/flask) and till this time the utilization of substrate was regular, thereafter the residual substrate was hardly consumed (Fig. 4). The pH value of the culture filtrate showed a steady increase with initial decrease on 3rd and 4th day.

Shake culture: All the three activities: FPA (0.17), CMCase (1.00) and β-glucosidase (0.080) in the extracellular fraction appeared earlier and showed lower values than that

of stable culture. The enzymes in the cytosol reached their maximum on 5th day simultaneously (Figs. 1b, 2b) but for β-glucosidase which was produced earlier (Fig. 3b). Cell debris cellulase reached higher values but later than that of stable culture (Figs. 1 to 3c). The level of enzymes was almost same in cytosol and cell debris fractions. Although the dry weight recorded in stationary culture was slightly more but the substrate consumption was faster under shake conditions (Fig. 4). The initial decline in pH was earlier in shake than stable conditions (Fig. 4).

EFFECT OF pH ON PRODUCTION OF CELLULASE

Cellulase production was determined over a pH range of 2 to 8. Synthesis of cellulase increased with increase in pH upto 5.0 followed by a gradual decrease later (Fig. 5). Maximum cellulolytic activity, which includes all the components was recorded at a pH of 4.5 and 5.0 with the exception of intracellular CMCase which showed an optimal pH at 4 and 4.5 (Fig. 5b). The pH values lower than 4.0 and higher than 5.5 had an adverse effect on cellulase production. Cytosol and cell debris activities reached zero values at pH 3.0 and 7.0 whereas a little of extracellular was still present. Growth was maximum at pH 5.0 and completely absent at pH 2.0 (Fig. 5a).

EFFECT OF TEMPERATURE ON CELLULASE PRODUCTION

The effect of different temperatures from 15°C to 42°C on the production of cellulase enzymes was studied. The activities in all the three fractions were highest at 27°C but the growth was supported better at 32°C (Fig. 6) with reasonable amounts of enzymes also. At 15 and 42°C the extracellular and cytosol fractions maintained some values but FPA and CMCase in the cell debris fraction were almost absent (Fig. 6 a, b).

CELLULASE PRODUCTION ON SOLUBLE CARBON SOURCES

A number of soluble sources which include simple synthetic saccharides along with complex compounds were used in place of CMC, so as to select a specific carbon source for maximum production of enzymes. The cellulase enzyme was studied in terms of FPA, CMCase and β-glucosidase activity (Fig. 7).

Filter paper activity (FPA): The highest extracellular FPA was recorded in the case of lactose (0.60) followed by CMC (0.28) and malt extract (0.11 units) (Fig. 7a). Only a little of FPA appeared on maltose, cellobiose and sucrose. Maximum activity in the cytosol fraction was observed on malt extract (0.39) followed by 0.28 units in the case of malt

extract-yeast extract. Maltose produced 0.17 units of enzyme and in the rest of the carbon sources it varied from 0.037- 0.087 units. Cellobiose was found to be the best source for cell debris enzyme (0.009 units) among the substrates tested.

Carboxymethyl cellulase (CMCase): The peak value for CMCase in the extracellular fraction, as in the case of FPA was observed with lactose as the carbon source (8.1 units) followed by CMC (1.50) and malt extract (0.42). In other sources it varied from 0.03 - 0.34 units (Fig. 7b). The enzymes in the cytosol fraction were again found to be highest on malt extract (0.65 units) followed by malt extract-yeast extract (0.50). Lactose (0.35 units) gave better yields of CMCase than that for FPA. Activity in the cell debris fractions ranged from 0.001 - 0.01 units, with maximum being on cellobiose (Fig. 7b). There was no CMCase activity in all the three fractions when sucrose was used as the carbon source.

β-glucosidase: The extracellular fraction as in the case of above two enzymes was maximum (Fig. 7c) on lactose (0.13) followed by CMC (0.093) and malt extract (0.04). Maltose and CMC gave equal yields (0.20 units) of cytosol β-glucosidase and in the rest of the substrates, varied from 0.04 - 0.06

units (Fig. 7c). The cell debris fraction was highest on yeast extract (0.06 units) and least on sucrose, cellobiose and maltose (0.02 units).

Effect of substrate concentration on cellulase production: Of the different concentrations of CMC tested, 1.5 per cent gave highest yield of extracellular FPA (0.27) and CMCase (1.72) while higher concentrations were inhibitory (Fig. 8a,b). In the case of β-glucosidase the yield was almost equal on 1-2 per cent of CMC and concentrations above this were slightly inhibitory (Fig. 8c). There was a proportional increase in cytosol and cell debris enzymes with increase in substrate concentration.

With lactose as sole carbon source all the cellulase components in the extracellular, cytosol and cell debris fractions reached their plateau at 1 - 1.5 per cent of substrate concentration (Fig. 9), after which the values were almost constant except for cell debris, FPA and CMCase (Figs. 9 a,b), which showed a decline. Growth expressed as dry weight increased with increase in substrate concentration both in the case of CMC and lactose. The increase being relatively less after one per cent.

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CELLULASE PRODUCTION ON INSOLUBLE CARBON SOURCES

Utilization of various straws (delignified and untreated) along with production of cellulase which include FPA and CMCase only were studied over a period of 10 days. Microcrystalline cellulose as carbon source was taken as a control.

Microcrystalline cellulose: Production of enzymes, both extracellular and cell-substrate associated along with consumption of substrate reached their peak values steadily upto 10th day of incubation (Fig. 10). The maximum units of extracellular FPA and CMCase produced were 0.06 and 0.46 respectively (Fig. 10a).The cell substrate associated enzyme values were 0.135 for FPA and 0.40 for CMCase showing, thereby that an equal amount of CMCase and twice that of FPA was particle bound as compared to the extracellular.

Wheat straw: In the case of untreated straw the enzymes in the extracellular fraction reached maximum values on 8th day with FPA having 0.09 units and 0.31 units for CMCase (Fig.11a). On delignified wheat straw enzyme production was higher than that of untreated, attaining maximum values of 0.40 units for FPA and 4.0 units for CMCase (Fig. 12a). The cell and substrate associated activities increased gradually showing a similar pattern as that of extracellular but

the values on untreated straw were still increasing when the experiment was terminated on the 10th day.

Paddy straw: All the components of cellulase in extracellular and cell substrate bound fractions showed high activity on delignified paddy on 10th day of incubation (Fig. 14a). The highest values for FPA were 0.47 and 0.10 units while that for CMCase, these were 3.6 and 0.36 in the extracellular and cell and substrate associated fractions respectively. Enzyme production on untreated straw was much less with extracellular fraction reaching its maximum on 8th day (Fig. 13a).

Sugar-cane bagasse: Enzyme synthesis increased with increase in incubation period and as in the case of other straws it was higher on delignified (Fig. 16a) than untreated bagasse (Fig. 15a), being 0.53 and 4.0 units for FPA and CMCase respectively. The cell and substrate bound enzymes showed a similar pattern.

On the whole comparative production of extracellular enzymes was higher on delignified straws. FPA was found to be maximum on delignified bagasse whereas CMCase was recorded to be highest on both delignified bagasse and wheat (Fig. 17). The enzyme produced on microcrystalline cellulose was

comparable to that of all untreated straws. Protein secreted in the medium was giving highest values of 1.4 mg/ml on delignified bagasse followed by delignified wheat straw (1.2 mg/ml). Reduction in dry weight of substrate was observed to be maximum on delignified bagasse (67 per cent).

PRODUCTION OF CELLULASE ON GLUCOSE

Minimum amounts of cellulolytic activities were observed on glucose as compared to other substrates. The secretion of FPA and CMCase was very little in the first two days (upto 0.008 and 0.022units respectively) whereas a steady rise in both the activities was followed by a decrease in the case of CMCase on 5th day (Fig. 18 c,d). Activities in the cell debris fraction were negligible. The cytosol fraction showed a steady decline till 3rd and 2nd day for FPA and CMCase respectively followed by a gradual rise upto 6th day. There was a regular increase in dry weight till a value of 23 mg/ml with a decrease during the first three days upto 1.12 mg/ml with a decrease later on (Fig. 18b). Most of the substrate was utilized by the 2nd day and the pH of the medium decreased from 5.0 to 3.15 on the 2nd day with a regular increase after that (Fig. 18a).

PURIFICATION OF CELLULASE

The culture filtrate containing 288 mg total protein in 240 ml and having 900, 96 and 16.8 units of CMCase; FP activity and β-glucosidase respectively was precipitated between 40 - 80 per cent of $(NH_4)_2SO_4$ saturation. This precipitation recorded 196 mg of protein carrying 750.24 units of CMCase, 77.0 of FPA and 14.8 units of β-glucosidase. (Table 3). The dialysed sample (5 ml) was run on DEAE-sephadex A-50 column and different fractions both adsorbed and unadsorbed were assayed for various enzymes and protein content (Fig. 19). All the active fractions (4-11 unadsorbed, 42 - 49 adsorbed) were pooled and precipitated with 80% $(NH_4)_2SO_4$. The dialysed unadsorbed fraction (52.4 mg protein) recovered 600, 35.67 and 11.8 units of CMCase, FPA and β-glucosidase respectively with a degree of purification of 3.67 for CMCase and 2.06 for FPA, while the adsorbed fraction (23 mg protein) revealed 21.67 units of FPA without CMCase and β-glucosidase. A degree of purification of the order of 2.85 was achieved for FPA with a net yield of 22.57% (Table 3). The dialysed unadsorbed sample was subjected to SE-sephadex C-50 chromatography. Fractions collected were assayed as above (Fig. 20). The active fractions (4-10) were pooled and precipitated with 80% $(NH_4)_2SO_4$ and dialysed. A

recovery of 13.0 mg protein was obtained which carried 525, 30.11 and 0.2 units of CMCase, FPA and β-glucosidase respectively. A purification factor of 12.74 for CMCase and 6.91 for FPA was achieved. The above sample (3 ml) was loaded on a G-200 gel and the active fraction (14-21) were pooled (Fig. 21). The purified sample (7.3 mg protein) recovered 423.33 and 22.7 units of CMCase and FPA, while β-glucosidase was altogether absent. A net yield of 47.04 and 23.73 % with a degree of 18.59 and 9.46 purification was achieved for CMCase and FPA respectively.

KINETIC STUDIES ON ENZYME REACTION

The purified enzyme sample containing both carboxymethyl cellulase and filter paper activity was stored at 4°C and used for studying the effects of various enzymes and substrate concentrations, incubation period, temperature, pH and metal ions on the velocity of enzyme catalysed reactions.

Effect of enzyme concentration: The effect of different enzyme concentrations revealed a linear curve for the hydrolysis of the substrates (50 mg filter paper and 5 mg CMC) upto 16 ug of FPA and 1.33 ug for CMCase proteins (Fig. 22). At higher concentrations a deviation from linear hydrolysis was observed.

Effect of substrate concentration: The effect of varying concentrations of filter paper (20 - 100 mg/ml) and CMC (1.5 - 13.5 mg/ml) on the velocity of the enzyme reaction were studied. The Michaelis Menton constant of FP activity (unadsorbed and adsorbed) and CMCase was calculated according to the method of Lineweaver and Burk (1934). The Km values for FP activities both unadsorbed and adsorbed were 47.61 and 33.3 mg/ml respectively and that of CMCase was 4.0 mg/ml (Figs. 23, 24).

Effect of incubation period: A constant amount of filter paper activity (10 ug protein) and carboxymethyl cellulase (0.5 ug protein) was incubated for 2 and 1 h respectively. The reaction system was linear upto 60 min for FPA (Fig. 22a) and 20 min for CMCase (Fig. 22b) after which the rate decreased till the reaction was terminated.

Effect of different pH on activity and stability of cellulase: The optimal pH for filter paper activity was 5.5 and it decreased a little at pH 5.0, while below 5.0 and above 5.5 the decline in activity was well marked (Fig. 25a). CMCase was active from pH 4.5 to 5.5 with 5.0 as the optimal pH (Fig. 25b). Both the enzymes i.e. FPA and CMCase were stable at pH 5.0 - 5.5 while FPA lost 68.69% and 80.1% of its activity at pH 4.5 and 6.0 respectively (Fig. 25). The CMCase

was comparatively stable and lost 27.[illegible]% at pH 4.5 and 44.40% of its activity at pH 6.0.

Effect of different temperatures on activity and stability of cellulase: Maximum hydrolysis of filter paper and carboxymethyl cellulose by the enzymes occurred at 60°C (Fig. 26, 27b). The FP activity was completely stable at 40°C upto 20 min of incubation (Fig. 26a) while it lost 24% of its activity in the next 10 min. There was a regular inactivation of enzyme at 50 - 70°C over a period of 30 min. Even at 80°C the enzyme could retain 26.6% of its activity after 10 min. Carboxymethyl cellulase as compared to FPA was relatively more stable (Fig. 27a). At 60°C it could retain 50% of its activity and at 90°C, 26.6% of the activity was still present over a period of 30 min.

Effect of various metal ions: Filter paper activity was found to be more sensitive to the metal ions tested (Table 4). At concentration of 1 mM Zn^{++}, Ca^{++} and Mg^{++} inhibited the activity upto 50%. EDTA and Cu^{++} with similar concentration inhibited 75% of the activity whereas with Hg^{++} and Ag^{++}, it was completely lost. Co^{++}, Na^{+} and K^{+} had no effect on the enzyme. The higher concentrations of EDTA, Mg^{++}, K^{+} i.e. 5 and 10 mM had little or no effect on the enzyme but that of Na^{+}, Co^{++}, Ca^{++} and Zn^{++} had a promotory effect (Table 4). Cu^{++},

Hg^{++} and Ag^{++} at higher concentrations led to complete loss of activity.

K^{+}, Cu^{++}, Hg^{++} and EDTA at 1 mM concentration inhibited the CMCase enzyme, and the inhibition was well marked only in case of Hg^{++} while with others it was negligible. Co^{++} and Ca^{++} were promotory at 1 mM concentration and this effect decreased with increase in concentration upto 10 mM. Higher concentration of Na^{+} raised the enzyme activity upto 29.2% while those of Cu^{++}, Hg^{++} and Ag^{++} caused complete inhibition of the enzyme. The effect of Zn^{++} and Mg^{++} on CMCase was insignificant.

Molecular weight: The molecular weights of filter paper activity and carboxymethyl cellulase as determined with SDS-gel polyacrylamide electrophoresis were found to be 44,000 and 55,000 respectively (Fig. 28).

Figs. 1-3 Production of enzymes on one per cent CMC 1) FP activity 2) CMCase 3) β-glucosidase

--------Shake culture, ———— Stationary culture

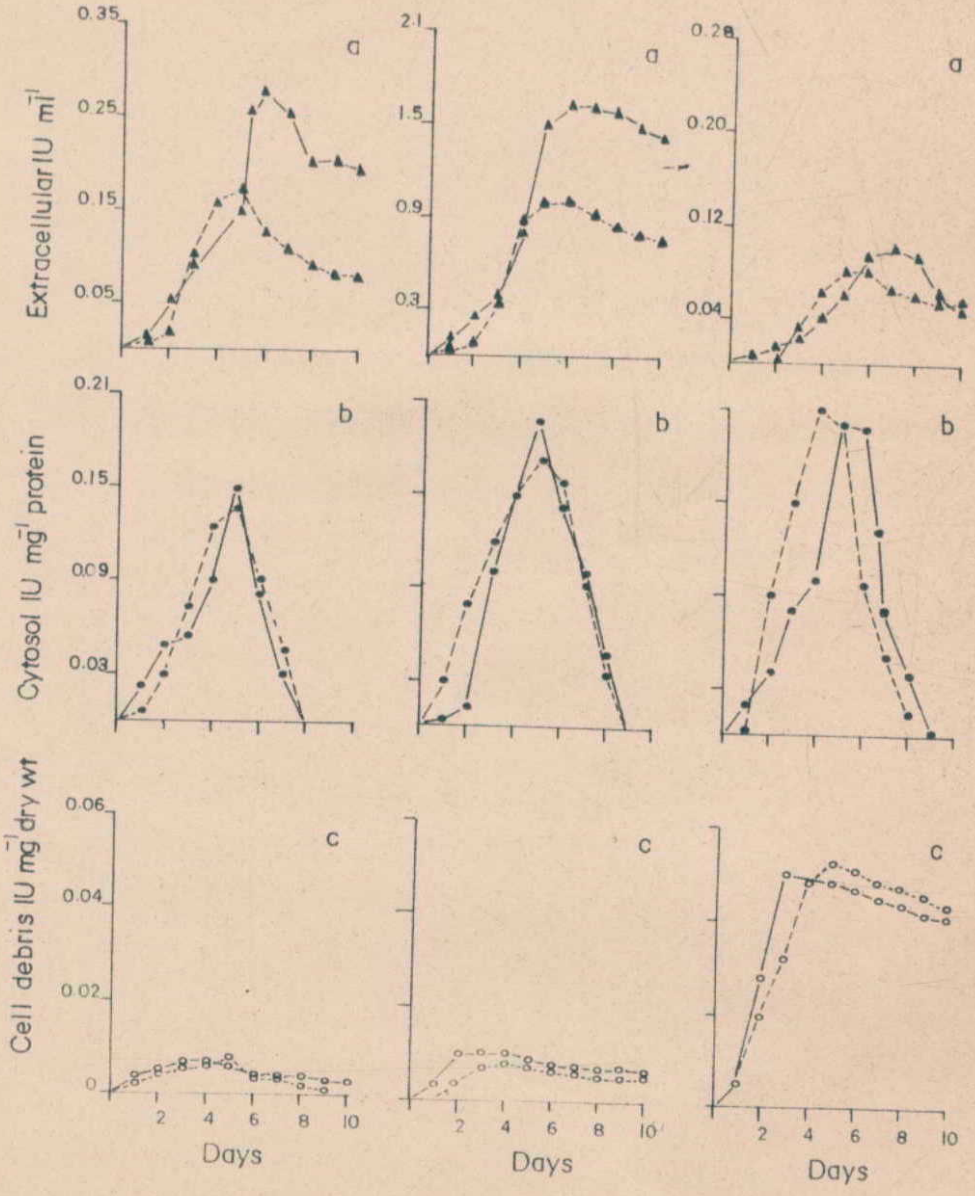

Fig.1 Fig.2 Fig.3

Fig. 4 Growth on CMC as carbon source a) pattern of substrate utilized and changes in pH of the medium b) growth in terms of dry weight per flask.--------Shake culture, ------ Stationary culture.

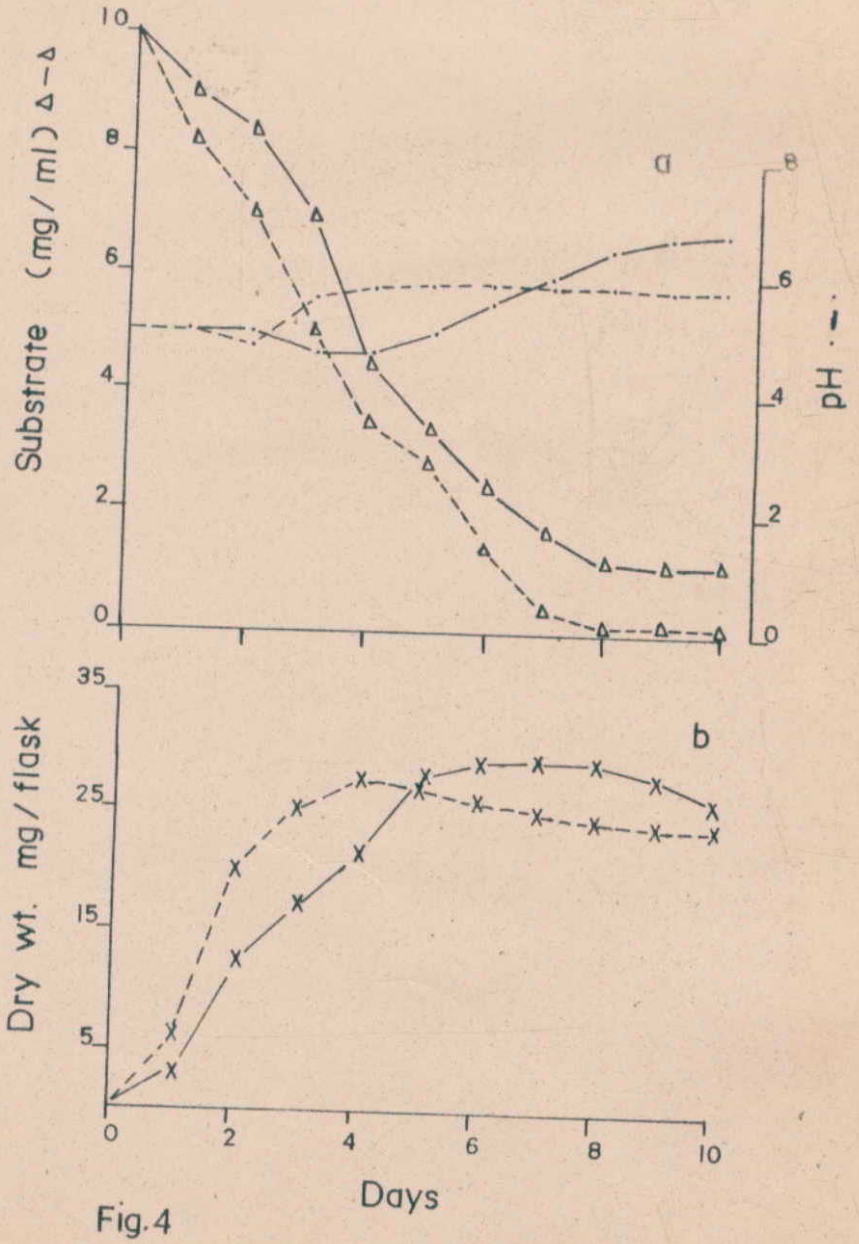

Fig. 4

Fig. 5 Production of enzyme and growth at different pH of the medium a) FP activity and growth b) CMCase c) β-glucosidase

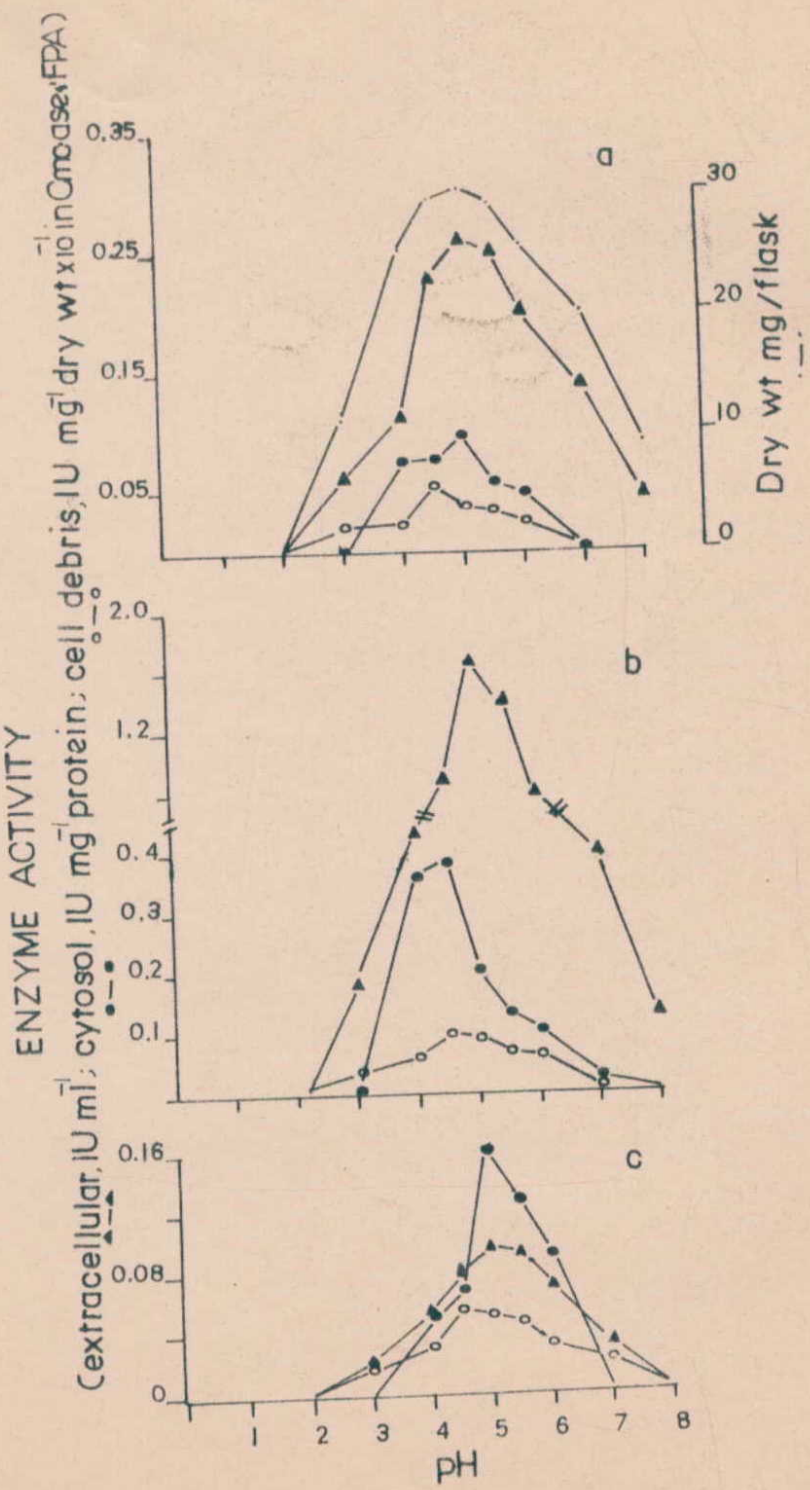
ENZYME ACTIVITY
Cextracellular, IU ml⁻¹; cytosol, IU mg⁻¹ protein; cell debris, IU mg⁻¹ dry wt x10 in Cmax (FPA)
Dry wt mg/flask
a
b
c
0.35
0.25
0.15
0.05
30
20
10
0
2.0
1.2
0.4
0.3
0.2
0.1
0.16
0.08
0
1
2
3
4
5
6
7
8
pH

Fig. 5

Fig 6: Production of enzyme and growth at different temperatures of incubation a) FP activity and growth b) CMCase c) β-glucosidase

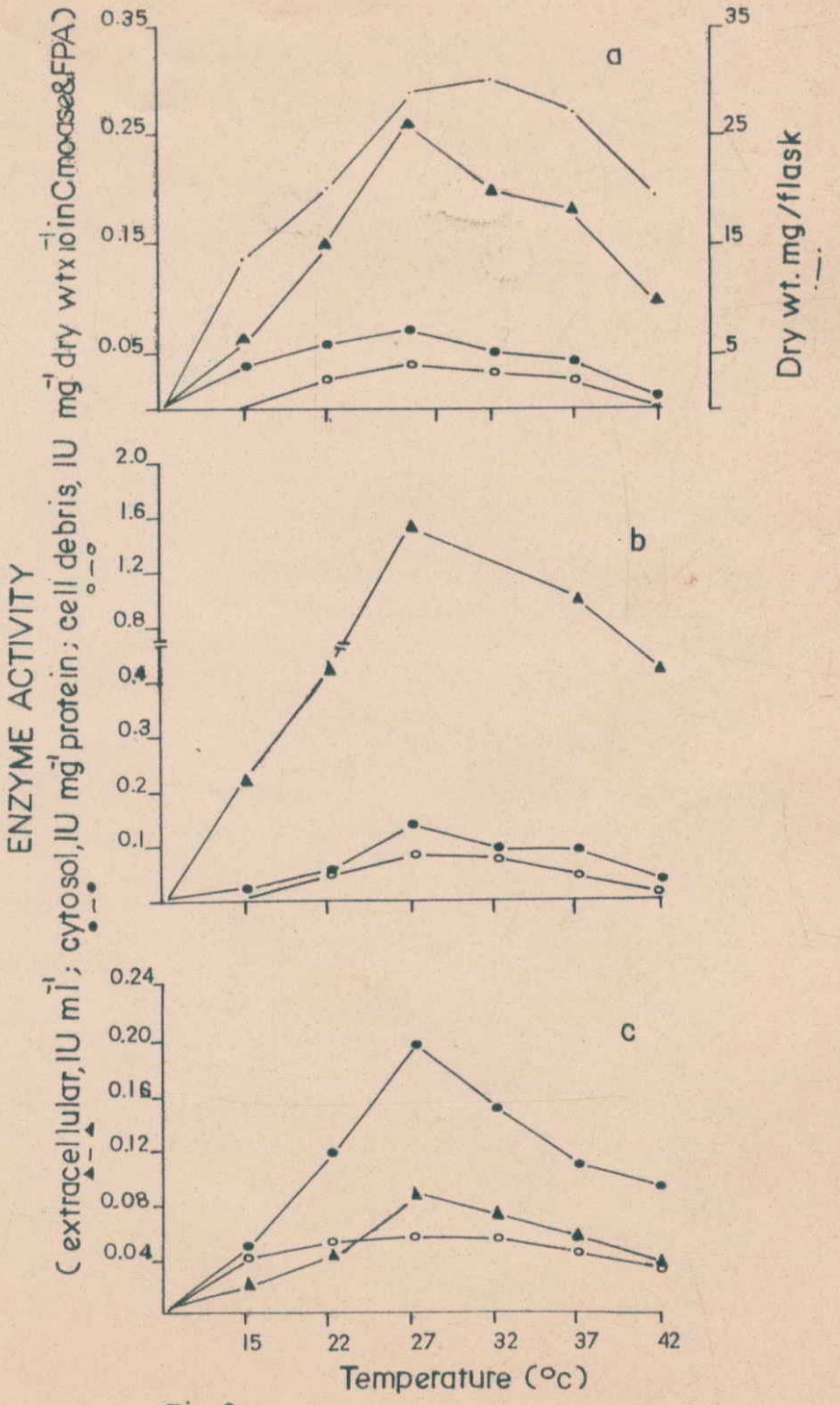
ENZYME ACTIVITY
(extracellular, IU ml⁻¹; cytosol, IU mg⁻¹ protein; cell debris, IU mg⁻¹ dry wt x 10 in Cmocase & FPA)
Dry wt. mg/flask
a
b
c
0.35
0.25
0.15
0.05
35
25
15
5
2.0
1.6
1.2
0.8
0.4
0.3
0.2
0.1
0.24
0.20
0.16
0.12
0.08
0.04
15
22
27
32
37
42
Temperature (°c)

Fig.6

Fig. 7 Production of enzyme and growth on various soluble substrates a) FP activity and growth b) CMCase c) β-glucosidase

Fig. 7

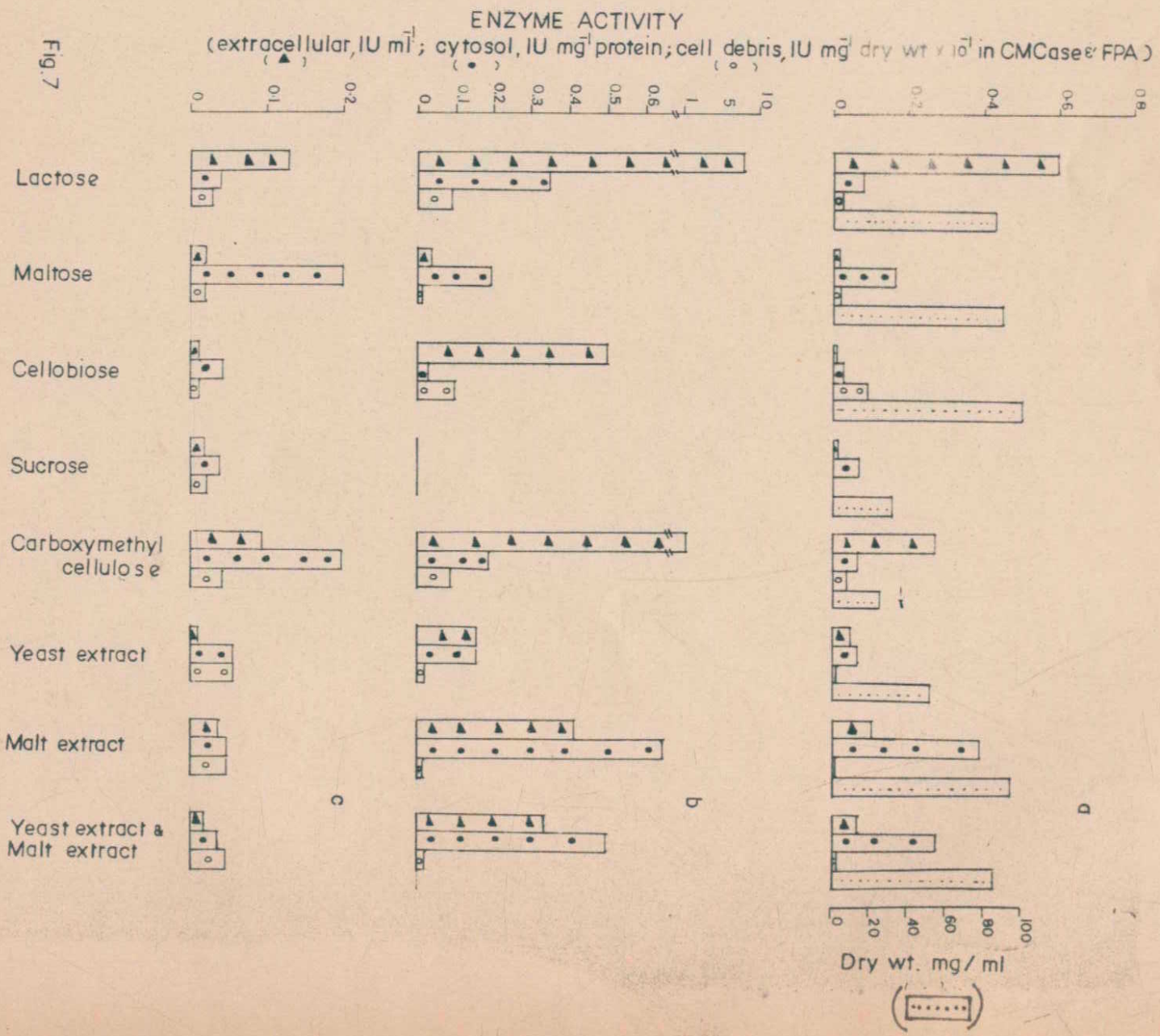
ENZYME ACTIVITY
(extracellular, IU ml⁻¹; cytosol, IU mg⁻¹ protein; cell debris, IU mg⁻¹ dry wt x 10⁻¹ in CMCase & FPA)
Lactose
Maltose
Cellobiose
Sucrose
Carboxymethyl cellulose
Yeast extract
Malt extract
Yeast extract & Malt extract
c
b
a
Dry wt. mg/ml

Fig. 8 Production of enzymes and growth at different concentrations of CMC a) FP activity and growth b) CMCase c) β-glucosidase

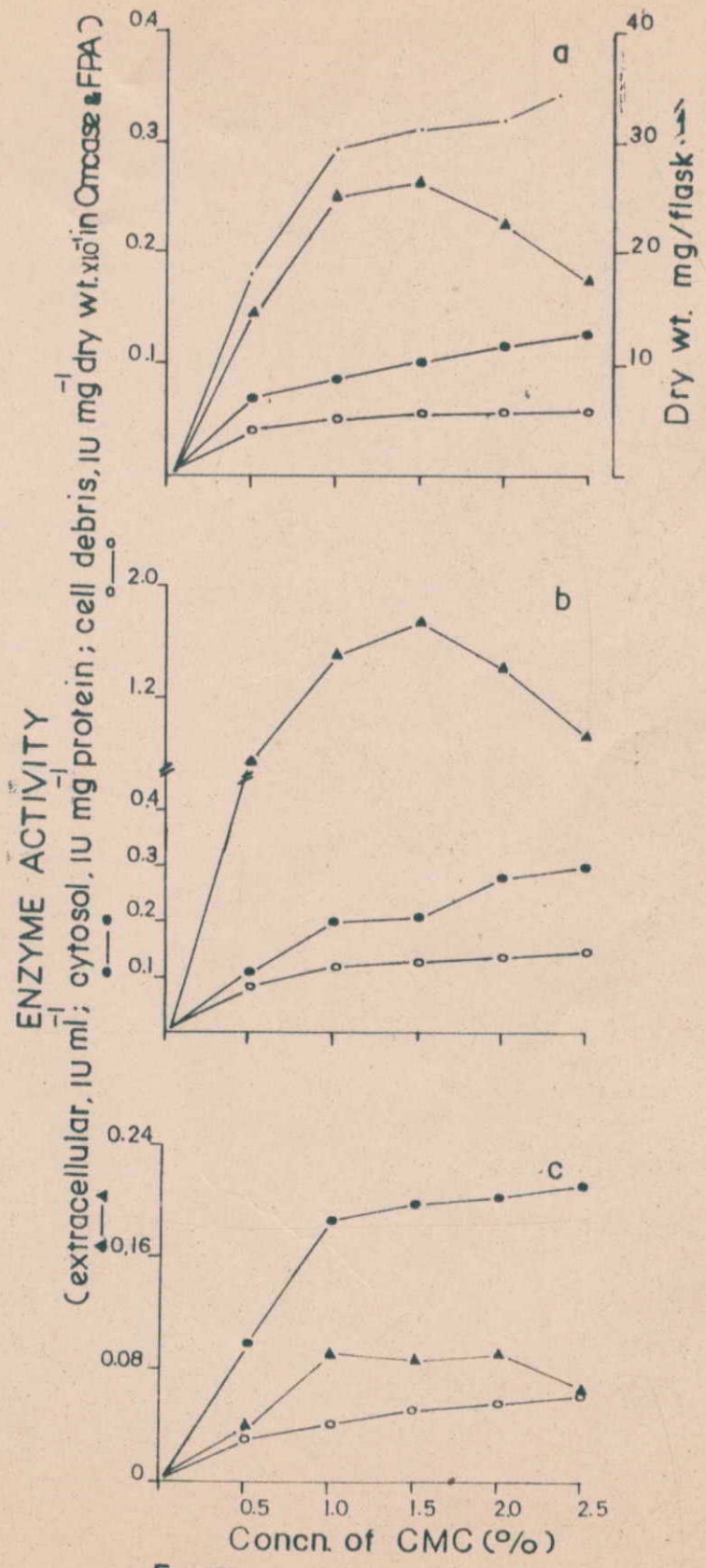
ENZYME ACTIVITY
(extracellular, IU ml⁻¹; cytosol, IU mg⁻¹ protein; cell debris, IU mg⁻¹ dry wt. x10⁻¹ in Cmcase & FPA)
Dry wt. mg/flask
a
b
c
0.4
0.3
0.2
0.1
40
30
20
10
2.0
1.2
0.4
0.3
0.2
0.1
0.24
0.16
0.08
0
0.5
1.0
1.5
2.0
2.5
Concn. of CMC (%)

Fig. 8

Fig. 9 Production of enzymes and growth at different concentrations of lactose a) FP activity and growth b) CMCase c) β-glucosidase

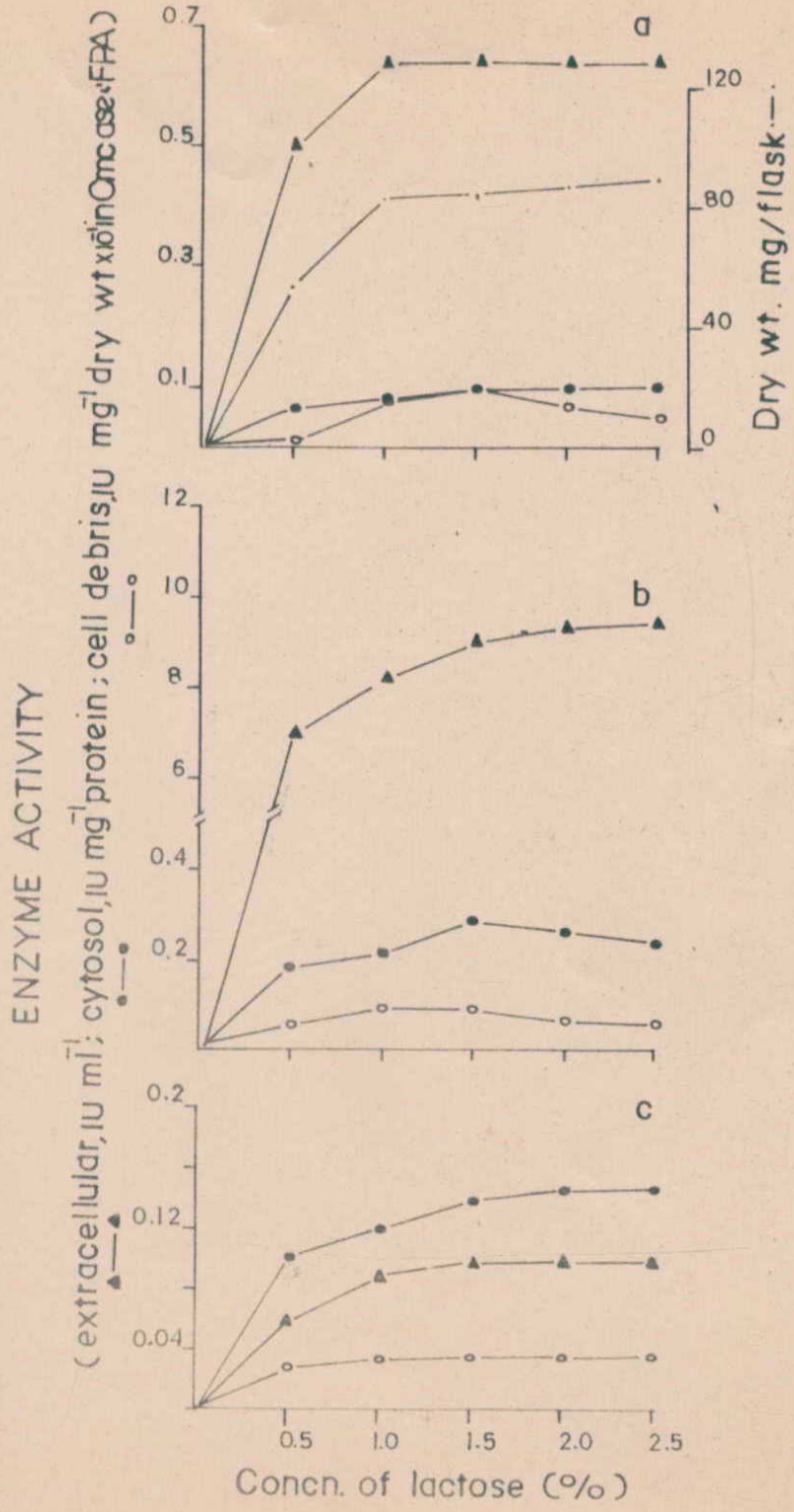
ENZYME ACTIVITY
(extracellular, IU ml⁻¹; cytosol, IU mg⁻¹ protein; cell debris, IU mg⁻¹ dry wt x 10³ in Omcase: FFA)
Dry wt. mg/flask
a
b
c
0.7
0.5
0.3
0.1
120
80
40
0
12
10
8
6
0.4
0.2
0.2
0.12
0.04
0.5
1.0
1.5
2.0
2.5
Concn. of lactose (%)

Fig.9

Fig. 10 Production of enzymes and growth on microcrystalline cellulose

a) CMCase -------; FP activity ————————

b) Utilization of substrate and production of extracellular protein

Enzyme activity
(extracellular, IU ml⁻¹; cell-subst associated, IU ml⁻¹)
(▲)
(●)
a
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
b
% reduction in dry wt
(▲)
00
20
40
60
80
100
Protein mg/ml
(■)
1.4
1.0
0.6
0.2
2
4
6
8
10
Days

Fig.10

Fig. 11 Production of enzymes and growth on untreated wheat straw

a) CMCase ---------; FP activity————————

b) Utilization of substrate and production of extracellular protein

Fig. 12 Production of enzymes and growth on delignified wheat straw

a) CMCase ---------; FP activity ————————

b) Utilization of substrate and production of extracellular protein

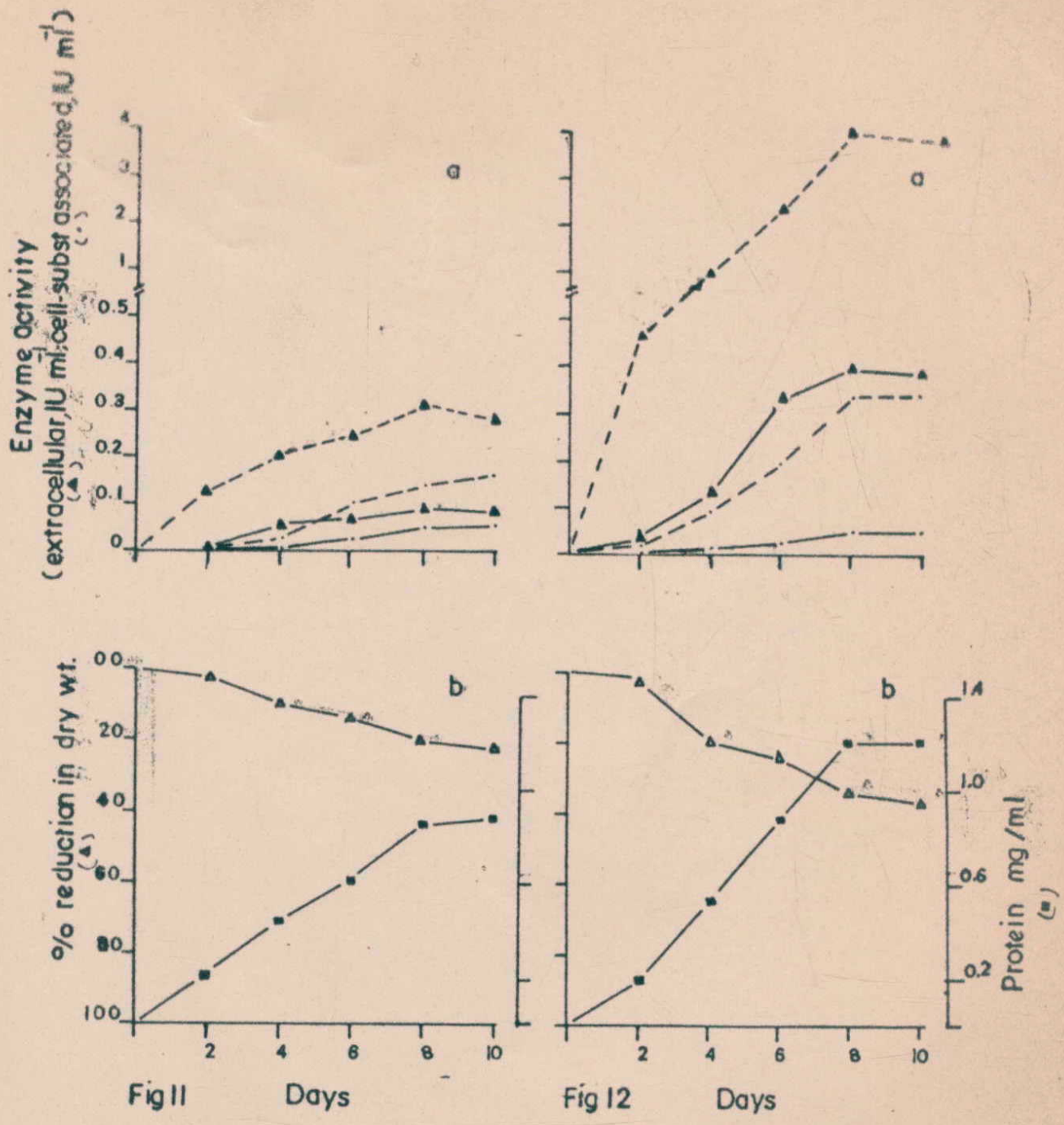
Enzyme activity
(extracellular, IU ml⁻¹; cell-subst associated, IU ml⁻¹)
(▲)
a
0
0.1
0.2
0.3
0.4
0.5
1
2
% reduction in dry wt.
(▲)
b
00
20
40
60
80
100
2
4
6
8
10
Days
Protein mg/ml
(■)
0.2
0.6
1.0
1.4

Fig 11

Fig 12

Fig. 13 Production of enzymes and growth on untreated paddy straw

a) CMCase ---------; FP activity ————————

b) Utilization of substrate and production of extracellular protein

Fig. 14 Production of enzymes and growth on delignified paddy straw

a) CMCase --------; FP activity ————————

b) Utilization of substrate and production of extracellular protein

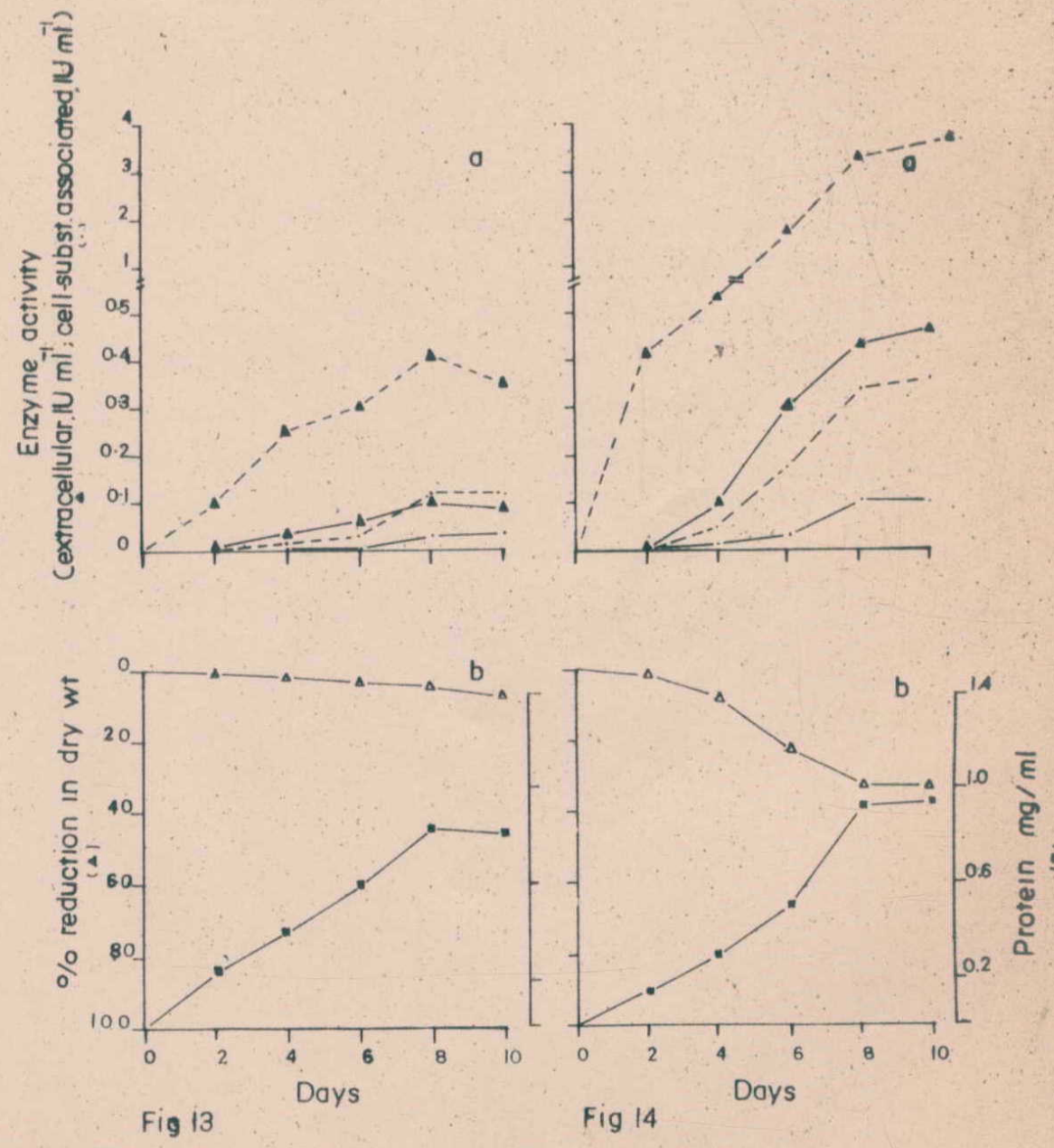
Enzyme activity
(extracellular, IU ml⁻¹; cell-subst. associated, IU ml⁻¹)
(▲)
a
4
3
2
1
0·5
0·4
0·3
0·2
0·1
0
% reduction in dry wt
(▲)
b
0
20
40
60
80
100
0
2
4
6
8
10
Days
Protein mg/ml
(■)
1·4
1·0
0·6
0·2

Fig 13

Fig 14

Fig. 15 Production of enzymes and growth on untreated sugar-cane bagasse

a) CMCase --------; FP activity ————————

b) Utilization of substrate and production of extracellular protein

Fig. 16 Production of enzymes and growth on delignified sugar-cane bagasse

a) CMCase --------; FP activity ————————

b) Utilization of substrate and production of extracellular protein

Enzyme activity
(extracellular IU ml⁻¹; cell subst. associated IU ml⁻¹)
(▲)
(•)
a
b
% reduction in dry wt.
(▲)
Protein mg/ml
(■)
Days

Fig.15

Fig.16

Fig. 17 Comparative production of enzymes and growth on various strains after 10 days a) Circles represent FP activity and triangles CMCase

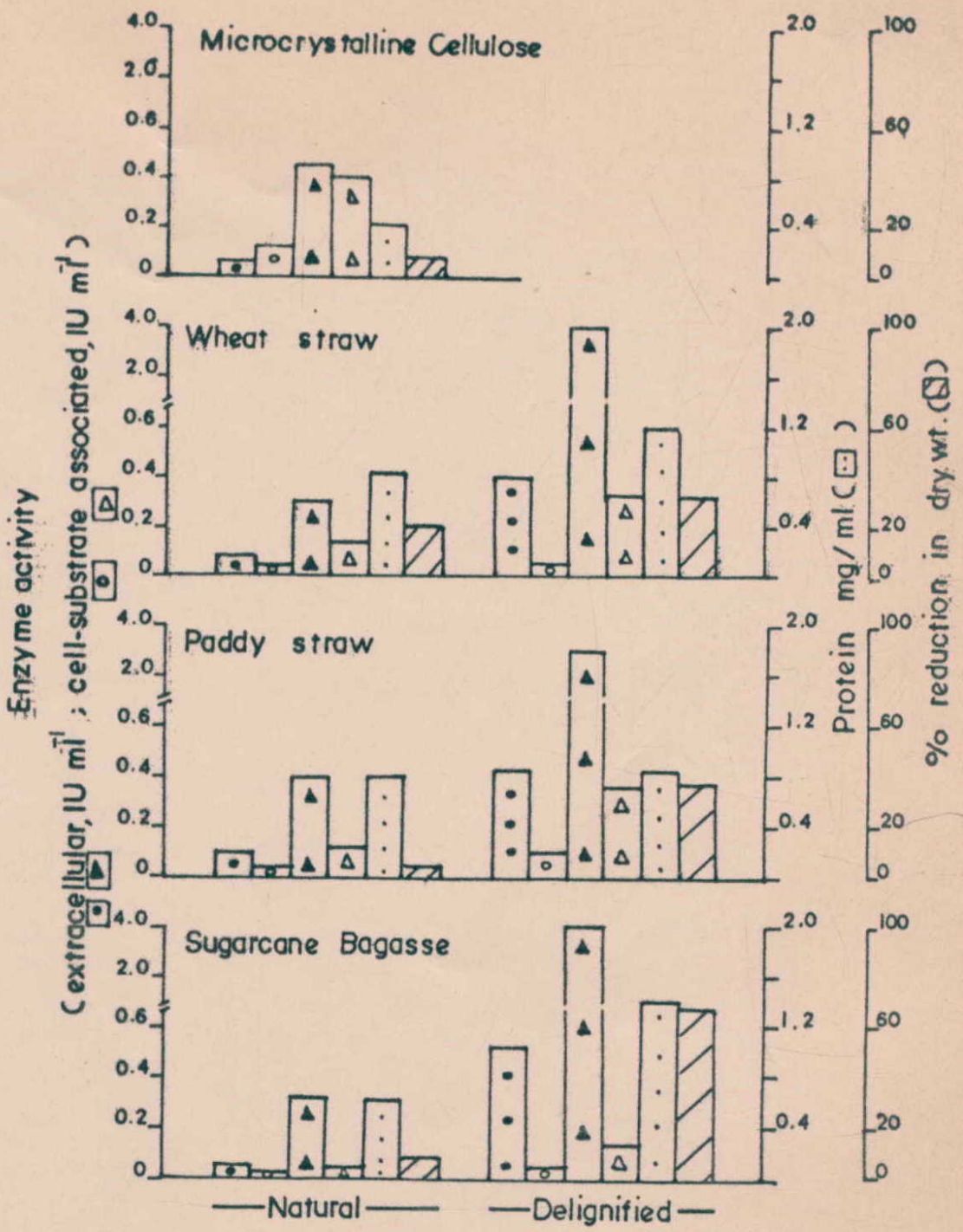
Microcrystalline Cellulose
Wheat straw
Paddy straw
Sugarcane Bagasse
Enzyme activity
(extracellular, IU ml⁻¹ ; cell-substrate associated, IU ml⁻¹)
Protein mg/ml (□)
% reduction in dry wt. (⊠)
Natural
Delignified

Fig.17

Fig. 18 Growth and production of enzymes on 2% glucose of glucose grown mycelium

a) substrate utilization and pH of the medium;
b) Growth as dry wt. and extracellular protein;
c) FP activity;
d) CMCase.

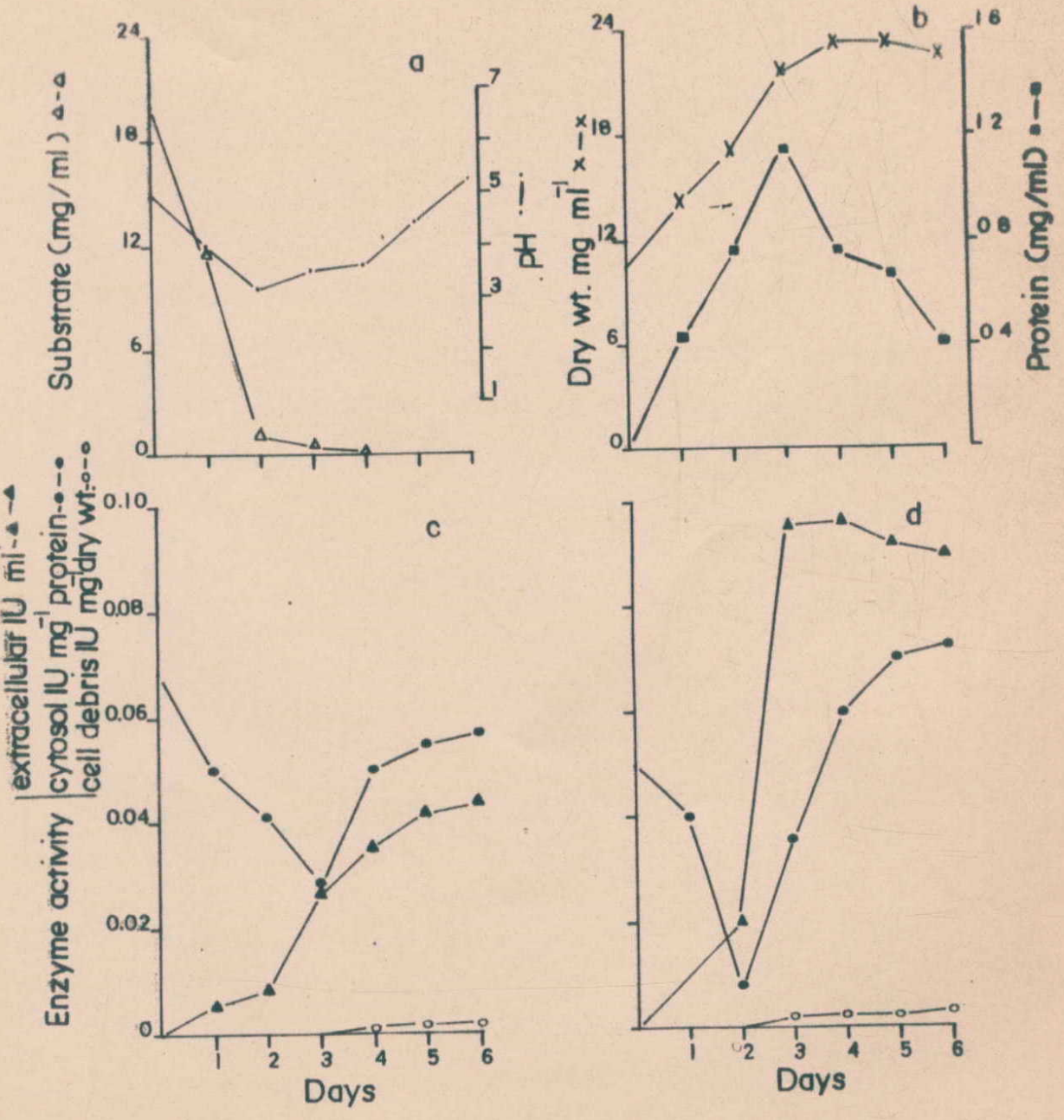
a
Substrate (mg/ml) Δ-Δ
pH
b
Dry wt. mg ml⁻¹ x-x
Protein (mg/ml) ■-■
c
Enzyme activity
extracellular IU ml⁻¹ Δ-Δ
cytosol IU mg⁻¹ protein ●-●
cell debris IU mg⁻¹ dry wt. o-o
Days
d
Days

Fig.18

Fig. 19 Ion exchange chromatography on DEAE-Sephadex A-50. Values plotted as absorbance of the product per ml of the enzyme. FP activity assay at 50°C for 10 min; CMCase assay at 50°C for 10 min; β-glucosidase assay at 37°C for 30 min

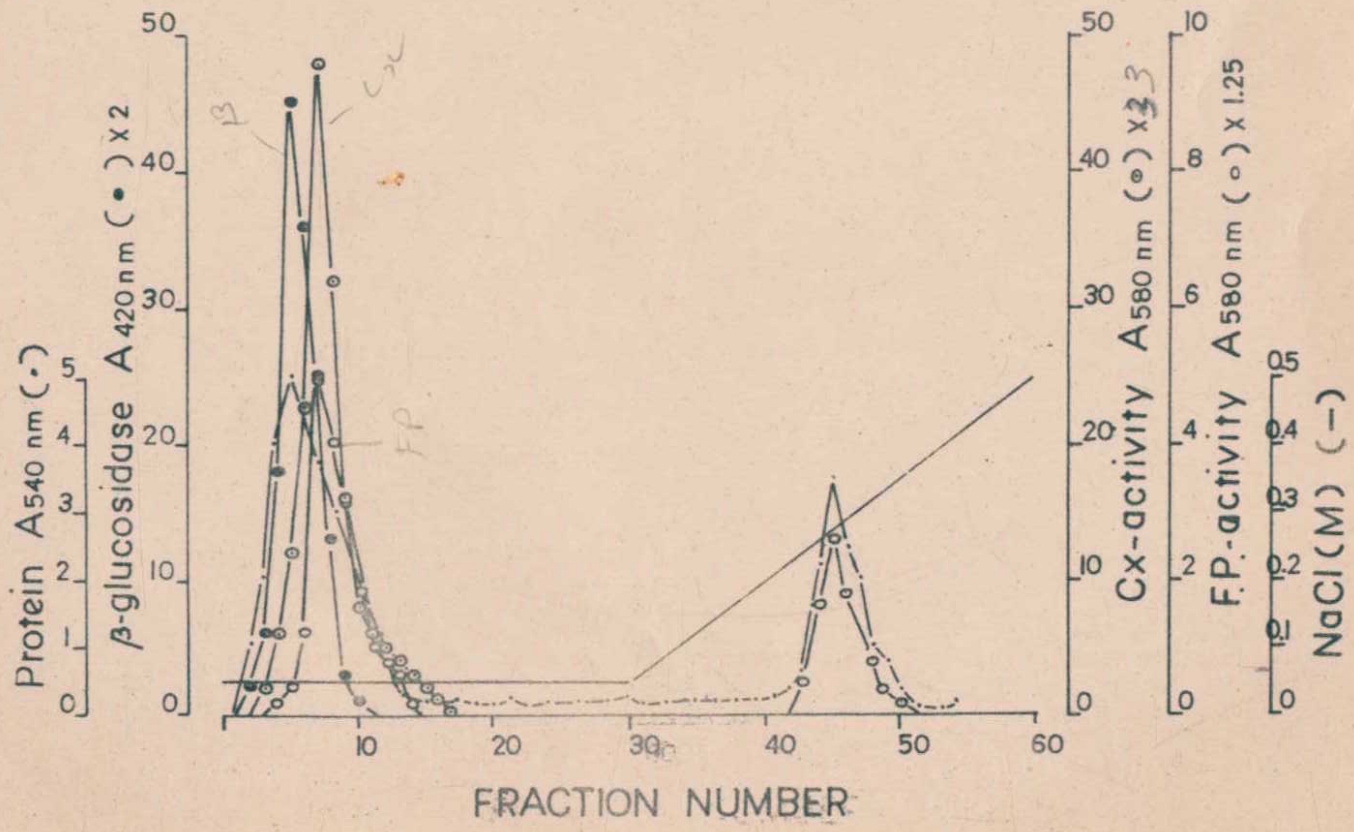
Protein A540 nm (·)
β-glucosidase A420 nm (•) X 2
Cx-activity A580 nm (○) X 3
F.P.-activity A580 nm (○) X 1.25
NaCl (M) (—)
FRACTION NUMBER

Fig 19

Fig. 20 Ion exchange chromatography on SE sephadex C-50.
Values as in Fig. 19.

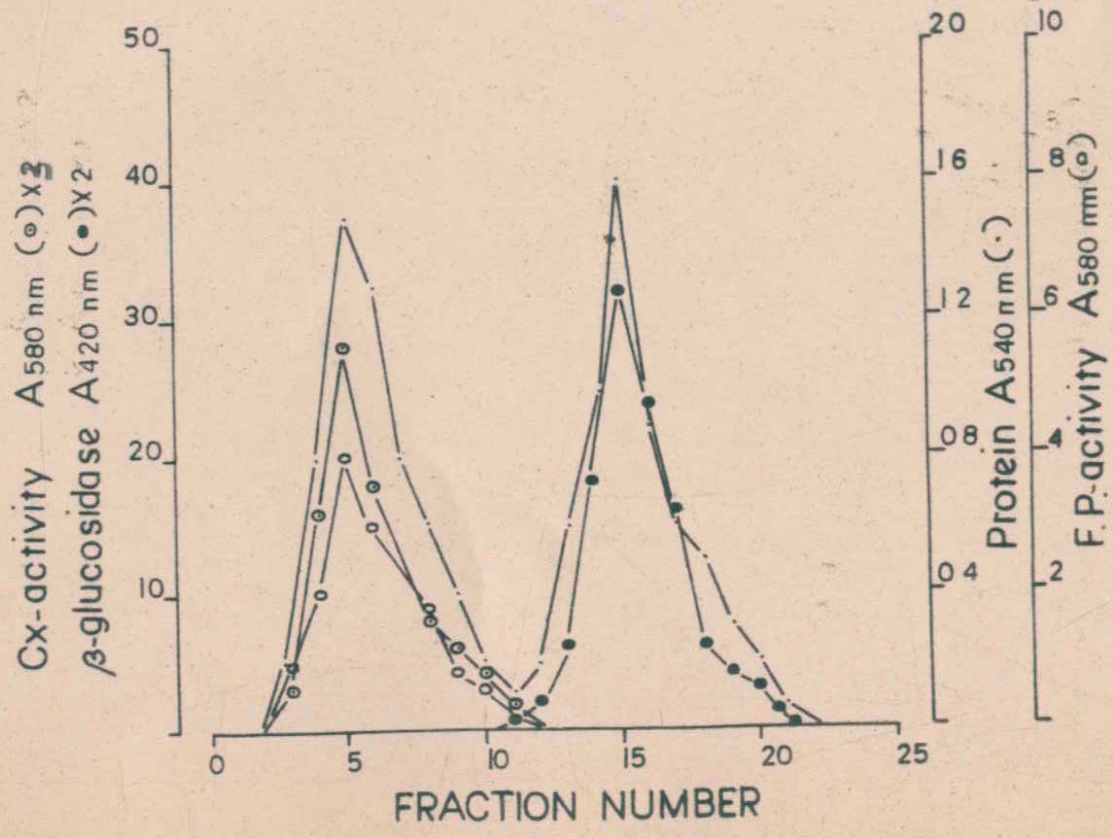
Cx-activity A580 nm (o) X2
β-glucosidase A420 nm (•) X2
Protein A540 nm (·)
F.P.-activity A580 nm (o)
FRACTION NUMBER
0
5
10
15
20
25
10
20
30
40
50
0.4
0.8
1.2
1.6
2.0
2
4
6
8
10

Fig 20

Fig. 21 Gel chromatography on Sephadex G-200. Values as in Fig. 19.

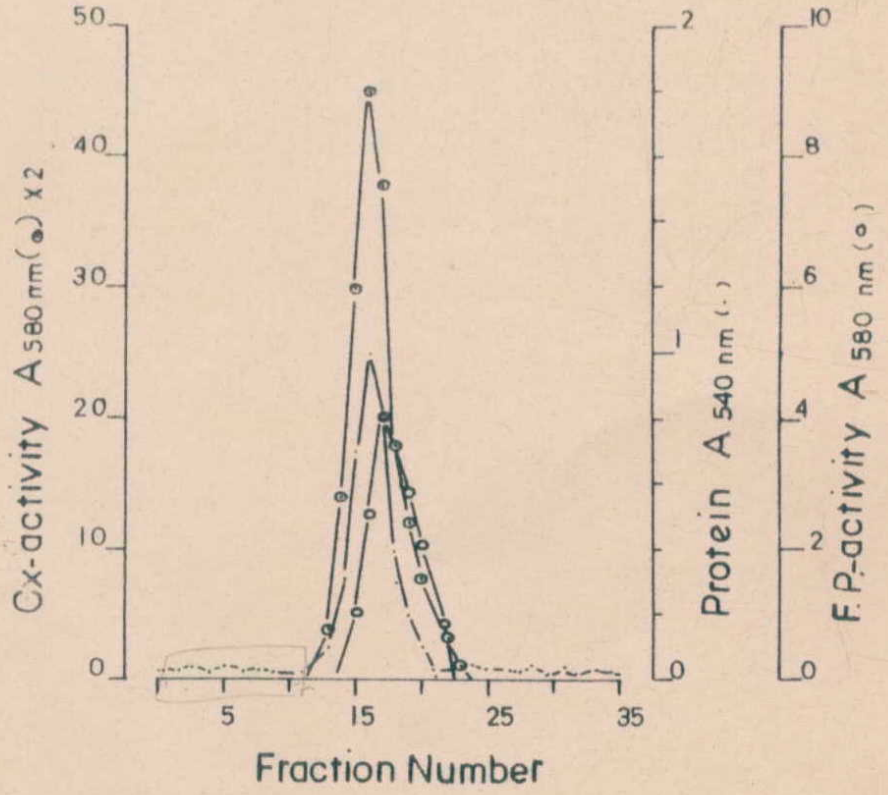
Cx-activity A 580 nm (●) x 2
50
40
30
20
10
0
Protein A 540 nm (·)
2
1
0
F.P.-activity A 580 nm (○)
10
8
6
4
2
0
5
15
25
35
Fraction Number

Fig. 21

Fig. 22 Progress curve and effect of enzyme concentration on the ra
of the reaction a) FP activity b) CMCase

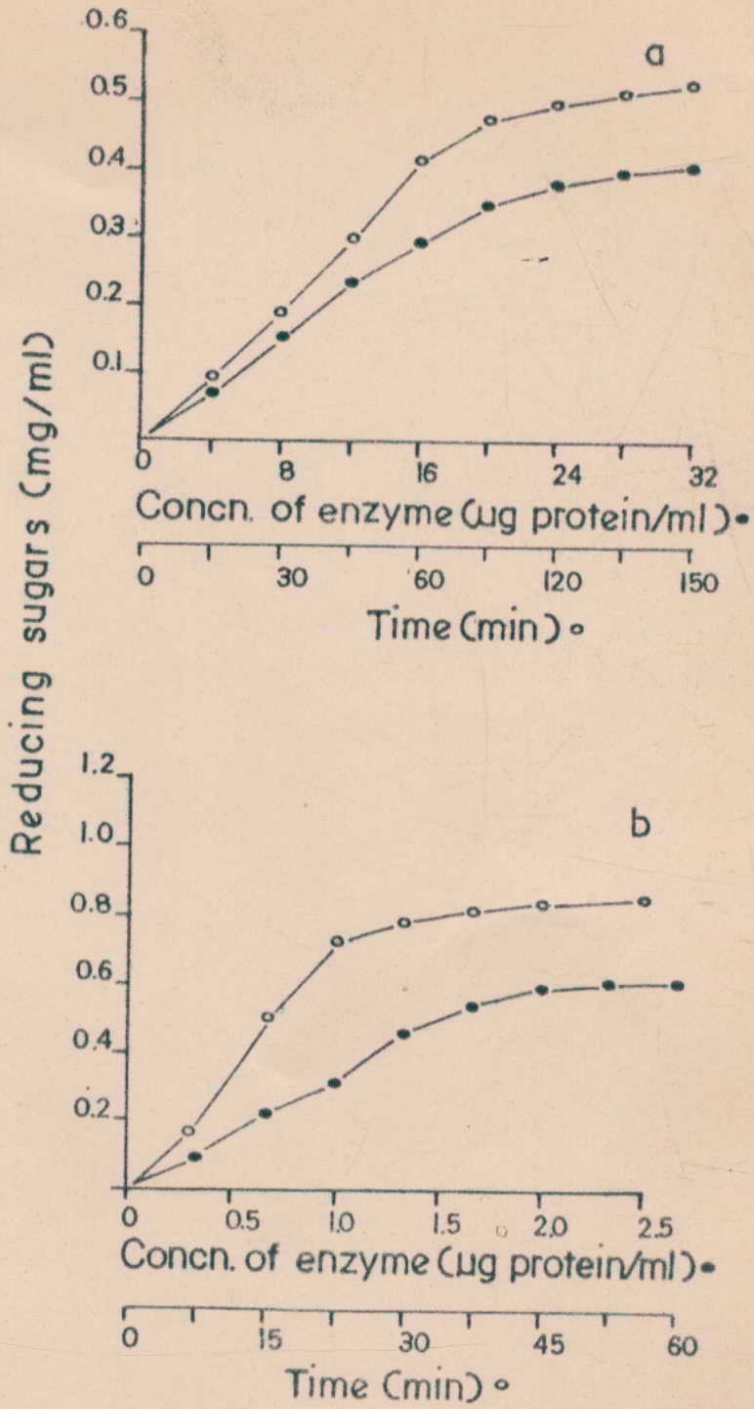
a
0.6
0.5
0.4
0.3
0.2
0.1
0
8
16
24
32
Concn. of enzyme (ug protein/ml)
0
30
60
120
150
Time (min)
Reducing sugars (mg/ml)
b
1.2
1.0
0.8
0.6
0.4
0.2
0
0.5
1.0
1.5
2.0
2.5
Concn. of enzyme (ug protein/ml)
0
15
30
45
60
Time (min)

Fig. 22

Fig. 23 Lineweaver-Burk plot. a) adsorbed FP activity
b) unadsorbed FP activity

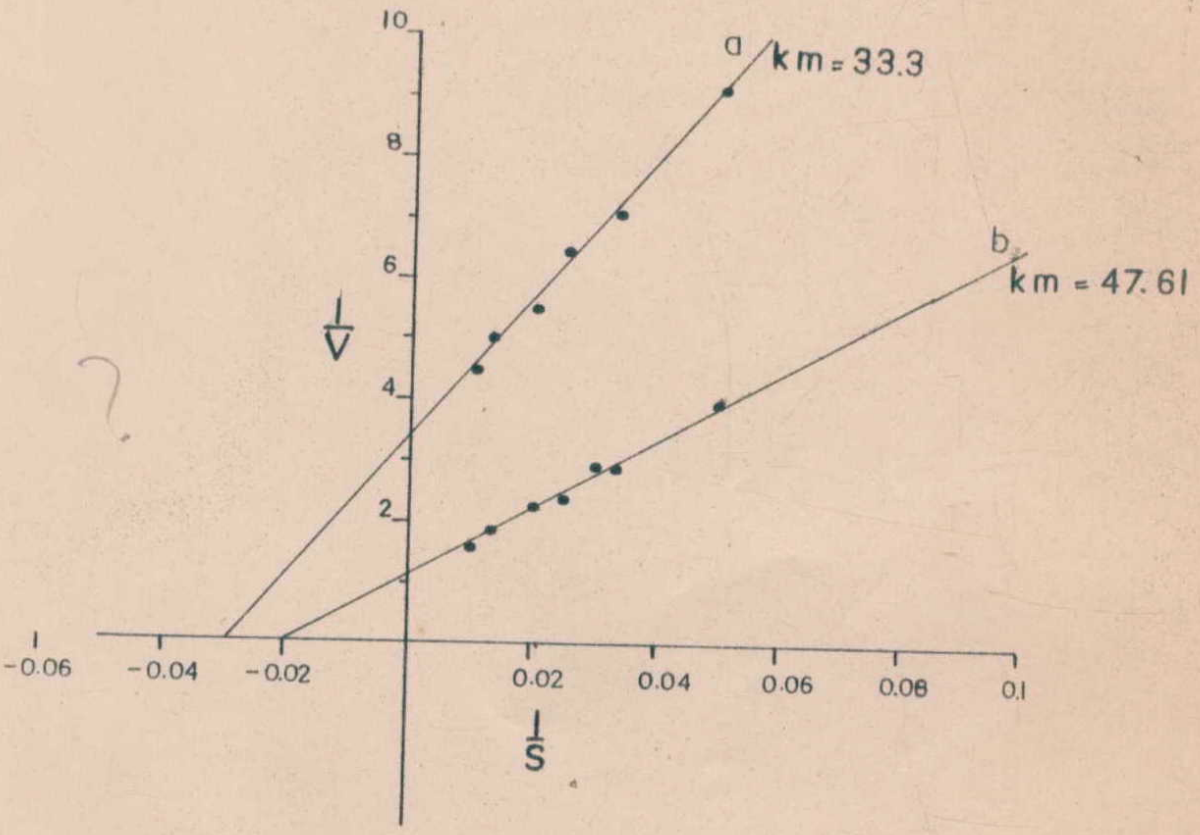

Fig. 23

Fig. 24 Lineweaver-Burk plot of CMCase

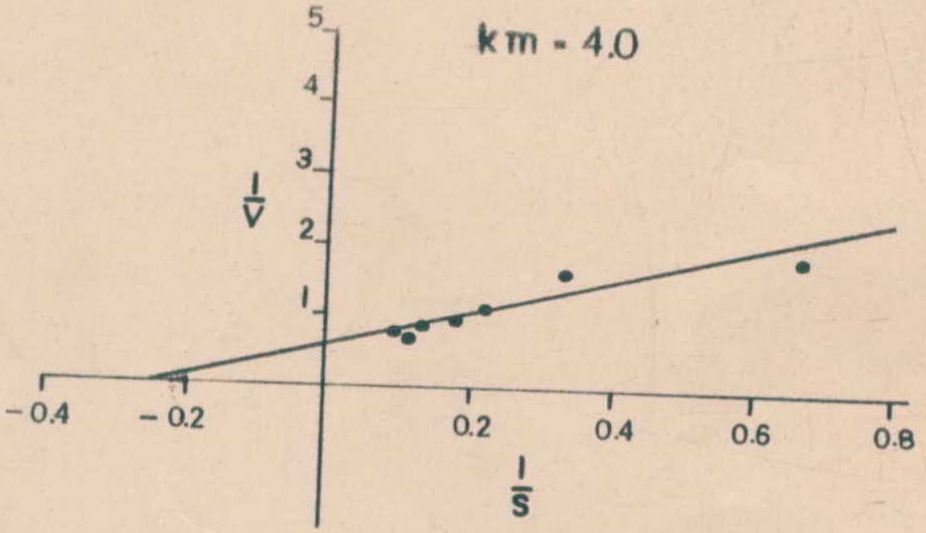

Fig.24

Fig. 25 Enzyme stability (•) and optimal activity (o) at different pH values a) FP activity b) CMCase

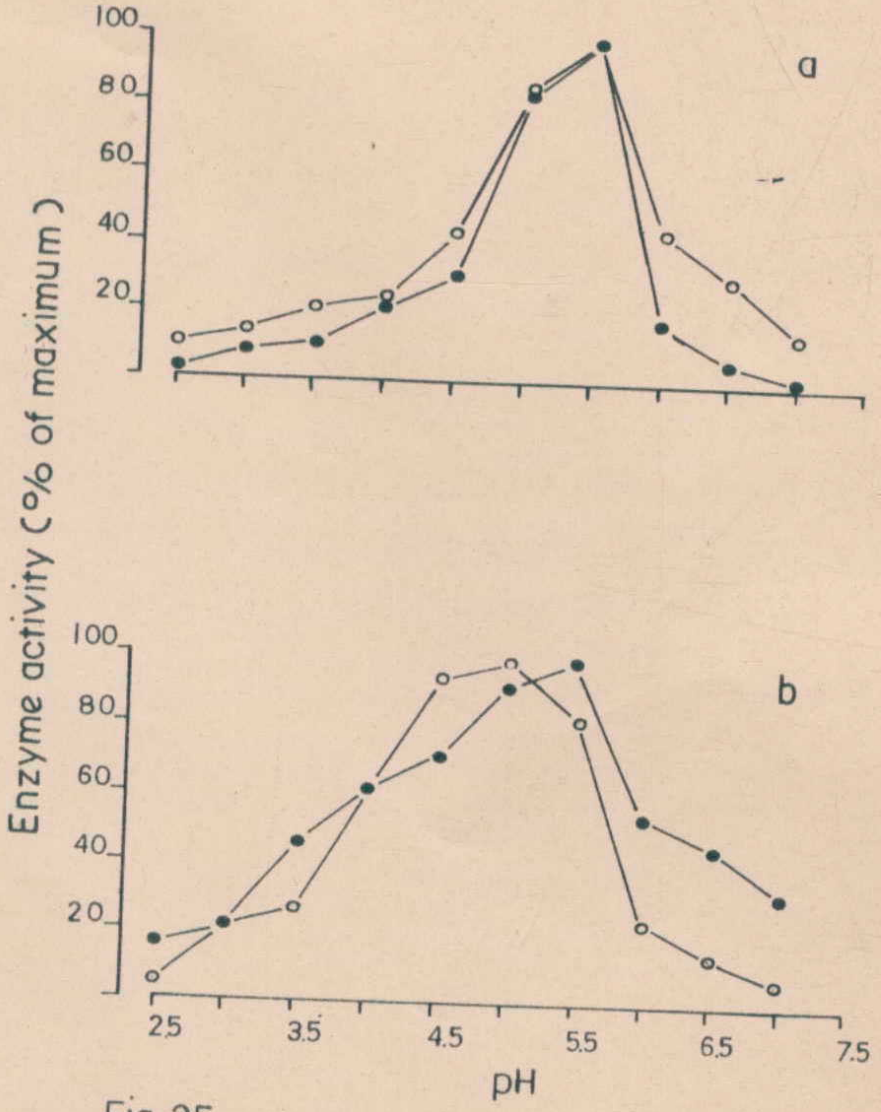
Enzyme activity (% of maximum)
100
80
60
40
20
a
b
2.5
3.5
4.5
5.5
6.5
7.5
pH

Fig. 25

Fig. 26 Filter paper activity a) stability at different temperatures b) optimal temperature

Fig. 27 Carboxymethyl cellulase a) stability at different temperatures b) optimal temperature

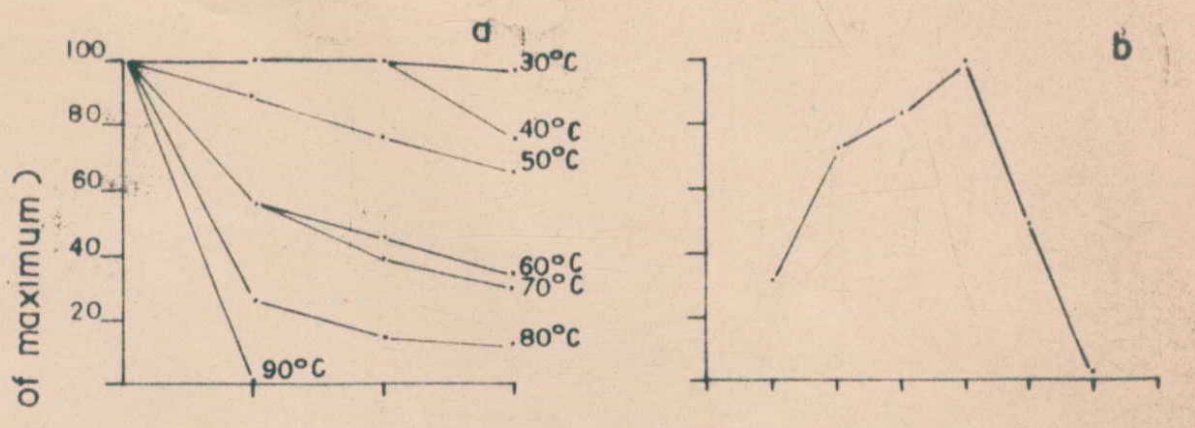
a
b
100
80
60
40
20
30°C
40°C
50°C
60°C
70°C
80°C
90°C

Fig. 26

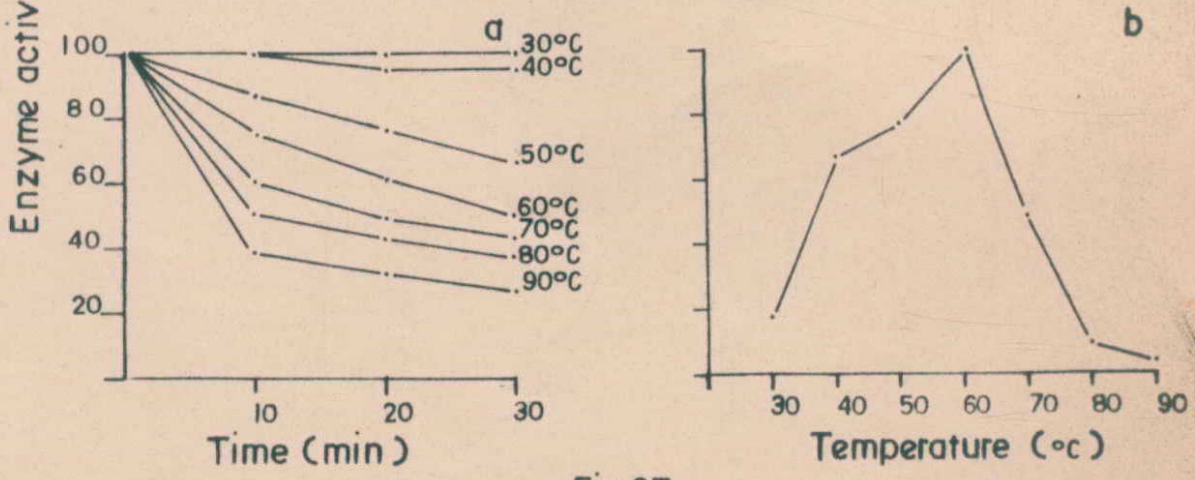
a
b
Enzyme activity (% of maximum)
100
80
60
40
20
30°C
40°C
50°C
60°C
70°C
80°C
90°C
10
20
30
Time (min)
30
40
50
60
70
80
90
Temperature (°c)

Fig. 27

Fig. 28 Molecular weight determination of purified FP activity (C_1) and CMCase (C_x) Standard proteins

1 Tripsinogen (24,000 2 egg albumin (45,000)

3 Memocyanin (67,000)

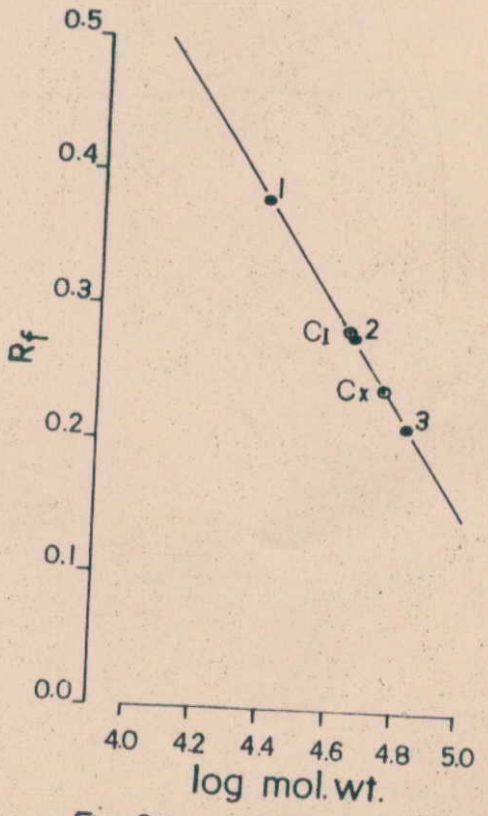

Fig 28

Table 2: Per cent distribution of activities in the three fractions on 1st and 6th day of growth

Enzyme fraction	FPA		CMCase		β-glucosidase	
	Day		Day		Day	
	1	6	1	6	1	6
Extracellular	76	97.40	98	99.1	51	60.73
Cytosol	67	0.86	0.19	0.24	7.4	3.89
Cell debris	17	1.67	1.6	0.5	41	35.56

Table 3 Purification of CMCase and FPA

Steps of purification	Total protein (mg)	Total activity 1U		Specific activity		Degree of Purification		Yield(%)	
		CMCase	FPA	CMCase	FPA	CMCase	FPA	CMCase	FPA
Culture filtrate 240 ml	288	900	96	3.12	0.33	1.0	1.0	100	100
$(NH_4)_2SO_4$ 40-80% saturation	196	750.24	77.0	3.825	0.39	1.22	1.18	83.36	80.21
DEAE-Sephadex A-50 (ionic strength gradient)	52.4 / 23*	600	35.67 / 21.67*	11.44	0.68 / 0.94*	3.67	2.06 / 2.85*	66.67	37.15 / 22.57*
SE-Sephadex C-50 (pH gradient)	13.2	525	30.11	39.76	2.28	12.74	6.91	58.33	31.36
Sephadex G-200	7.3	423.33	22.78	57.99	3.12	18.59	9.46	47.04	23.73

*Values refer to adsorbed fraction of FP activity

Table 4: Effect of metal ions on FPA and CMCase activity

Metal ions	FPA Concentration (mM)			CMCase Concentration (mM)		
	1	5	10	1	5	10
Zn^{++}	57.5	140	102.5	102	102	102
Ca^{++}	50.5	90	150	117.6	100	100
Co^{++}	102.5	102.5	125	159.2	135.3	129.2
Na^{+}	100	105	120	100	129.2	126.5
K^{+}	100	102.5	102.5	97.3	98.0	98.0
Mg^{++}	70	72.5	52.5	106	100	91.1
Cu^{++}	27.5	0	0	88.4	0	0
Hg^{++}	0	0	0	33.6	0	0
Ag^{++}	0	0	0	100	0	0
EDTA	25	50	43.7	84.9	100	100

Table 5 Relative occurrence of enzymes in total units on various soluble substrates

Substrates	Extracellular			Cytosol			Cell debris		
	FPA	CMCase	β-glucosidase	FPA	CMCase	β-glucosidase	FPA	CMCase	β-glucosidase
Lactose	16.5	202.5	3.25	0.096	0.42	0.0048	0.215	0.774	2.66
Maltose	0.375	0.75	0.5	0.221	0.247	0.26	0.18	0.09	1.8
Cellobiose	0.25	12.5	0.3125	0.037	0.033	0.046	0.9	1.0	2.0
Sucrose	0.35	0	0.5	0.035	0	0.02	0	0	0.80
CMCellulose	7.0	37.5	2.325	0.045	0.115	0.12	0.108	0.243	1.2
Yeast extract	0.75	4.12	0.375	0.147	0.336	0.126	0.053	0.106	3.18
Malt extract	2.75	10.5	1.0	0.702	1.17	0.09	0.095	0.19	4.75
Yeast extract-malt extract	1.75	8.5	0.50	0.78	1.4	0.112	0.086	0.172	3.87

Table 6 Per cent of enzyme activity expressed in 2% glucose as compared to 2% CMC

Enzyme	Extracellular	Cytosol	Cell debris
FPA	19.11	47.5	30
CMCase	6.43	30	15.38

Table 7 Physico-chemical properties of enzymes

Property	FP activity	CMCase
Km	33.3 (adsorbed) 47.61(unadsorbed)	4.0
pH optimum	5.5	5.0
Temperature optimum	60°C	60°C
Molecular weight	44,000	55,000

DISCUSSION

Production of various cellulase components which included filter paper activity (FPA), carboxymethyl cellulase (CMCase) and β-glucosidase on carboxymethyl cellulose (CMC) revealed a regular pattern of three fractions localized in extracellular cytosol and cell debris of the culture. The extracellular enzyme production was linear with growth and the maximum values for the enzyme and dry weight were observed on 6-7th day by which time most of the substrate was also utilized. A similar pattern has been reported earlier for Penicillium iriens, Lenzites trabea, Poria monticola and Trichoderma viride (Greaves, 1971; Boretti et al., 1973; D'Souza and Furtado, 1977). The cytosol and cell debris fractions contributed minor proportions of FPA and CMCase but reasonable amounts of β-glucosidase. These showed a similar profile of enzyme production reaching their maximum values earlier than extracellular enzymes. However, relative proportions of the three fractions varied during the growth phase as cell associated (cytosol and cell debris) a was more in the early phase as compared to the late growth phase (Table 2), thus indicating the synthesis and release of these enzymes into the medium which added to the concentration of extracellular enzymes (Pollock, 1962; Berg and Hofsten, 1976; Berg and Pettersson, 1977).

Regarding distribution of the enzyme in the three fractions during late exponential growth phase, most of the FPA and CMCase (97.4, 99.1 on 6th day) was located extracellularly (Table 2). Measurements of β-glucosidase showed upto 40% of the total activity to be associated with the cells throughout the growth period. Recently, Halliwell and Lovelady (1981) have reported that 99.0% of CMCase in T. koningii is extracellular, whereas 90% of β-glucosidase is associated with the cells. Other reports concerning localization of cellulase enzymes in Trichoderma are variable. Berg and Pettersson (1977) showed that much of the enzyme in T. viride was cell bound during active growth and released into the medium only when substrate was consumed or fungus was starving. Eriksson and Hamp (1978) could not detect extracellular endoglucanases while studying the sophorose induction of cellulase system in T. viride. Sternberg and Mandels (1979) contradicted the earlier reports on the existence of cellulase in mycelial bound state which has been supported by Vaheri et al. (1979) by showing active release of these enzymes in the medium during early stages of cultivation. Studies on fungi other than Trichoderma which include Penicillium janthenellum and Sporotrichum pulverulentum have revealed that β-1,4-glucosidases are predominantly

cell free with small amounts associated with cells. In the case of S. pulverulentum only cell wall bound β-glucosidases were formed when cellobiose was used as the sole carbon source. The location of cellulases in solution or bound to the cells may also depend upon the carbon source for growth (Suzuki et al. 1969; Berg, 1975).

A comparison of stationary and shake cultures on CMC revealed that the extracellular enzymes reach their maximum values earlier in shake but the activity is low as compared to the stable cultures. This may be due to the inactivation of enzymes by shaking as reported earlier (Reese and Mandels, 1980). The shake culture does not appear to be suitable for biomass or enzyme production as for both, the values were lower in the case of shake culture, although more of the substrate was utilized. The final pH values of the medium did not decline below 4.5 and thus inactivation of enzymes was prevented as reported in T. reesei (Halliwell, 1979).

The effect of initial pH on cellulase production showed a regular increase in synthesis of all cellulase components with increase in pH, and the optimum production of extracellular and cytosol enzymes at pH 5.0 except for CMCase which along with all cell debris activities showed a pH

optima at 4.5. The growth and enzyme production were markedly inhibited when the initial pH was below 4.0 and above 6.0. These optima for enzyme synthesis and growth are in agreement with the pH optima of different fungi reported earlier (Shewale and Sadana, 1978; Ghosh et al. 1980; Mukhpadhyay and Malik, 1980; Taniguchi et al. 1980; D'Souza and Volfova, 1982). However, Griffin (1974), Vilela et al. (1977) and Andreotti et al. (1980) reported optimal production of different enzymes at an initial pH of 3.0 - 4.0 in some other fungi.

As reported in studies on T. viride, production of maximum cell mass will not necessarily produce maximum cellulase yield (Andreotti et al. 1977; 1980; Mukhpadhyay, 1982). The observations made in the present study also show similar characteristics. Here the growth was maximum at 32°C while optimal production of all cellulase components in all the three fractions was at 27°C.

The growth of T. longibrachiatum on lactose gave the highest yield of cellulase in the extracellular medium. The production was even higher than that on native straws (natural and delignified) and microcrystalline cellulose (MCC). This property differentiates this species from T. viride in which lactose gave good yield of cellulase also but it

was less than that on cellulose as substrate (Mandels and Reese, 1960; Andreotti et al., 1980). Lactose as a good inducer of β-glucosidase but giving low yields of other cellulase components has been reported in Penicillium sps (Lakshmikanthan and Jaganathan, 1980). In addition to lactose, CMC, malt extract and cellobiose could also support cellulase production but other soluble sources gave negligible amounts of cell free enzymes. This report on the repression of cellulase activity with sucrose and maltose agrees with similar results obtained by different workers (Whitney et al. 1969; Mathur and Dube, 1978). Carboxymethyl cellulose as a poor inducer has been indicated in T. reesei (Herr, 1979). This may be due to the release of high levels of reducing sugars in the culture broth which is a known repressor of cellulase.

There was a differential induction of cytosol and cell bound cellulolytic enzymes. Malt in combination with yeast extract gave high yields of total FPA and CMCase in the cytosol fraction, while giving low amounts of cell debris enzymes (Table 5). On the other hand cellobiose produced high levels of cell debris FPA and CMCase but quite low amounts of cytosol as well as extracellular enzymes. Cell debris and cytosolic β-glucosidase was highest on malt extract and

maltose respectively. The higher production of cell free and cell associated enzymes when grown on CMC and cellobiose respectively is in accordance with earlier studies on T. viride and Sporotrichum pulverulentum where presence of cellulose in the medium induced extracellular enzymes preferentially (Berg and Pettersson, 1977; Deshpande et al., 1978). The relative occurrence of cytosol and cell debris cellulase enzymes with cellobiose as substrate are similar to the studies on Penicillium janthenellum (Rapp et al., 1981), where β-glucosidase was mainly found in cell bound state and less in cytosol. The induction pattern of extracellular and cell associated enzymes by various carbon sources suggests that a different control mechanism is operative in synthesis of each enzyme form.

Using different concentrations of CMC as carbon source, 1.5% of the substrate was found to produce maximum amounts of cellulase enzymes while concentrations higher than this showed lower yields. The decreased enzyme activity can be due to its adsorption on unutilized substrate which is excluded from estimation by higher speed centrifugation or the saccharification of CMC by the enzyme would yield glucose which represses cellulase production. The cell associated enzymes showed maximum production on highest

concentration of substrate used and this also corresponds to the maximum fungal mass. Production of all the enzymes was maximum at 1% concentration of lactose and remained at the same level later on except for β-glucosidase which showed a little increase with higher concentrations.

Production of different enzymes on insoluble substrates showed a marked effect of delignification on enzyme synthesis. Perhaps the hemicellulose contents of the delignified strains contribute to the overall high induction of cellulase (Sidhu and Sandhu, 1980). With these strains the secretion of soluble protein into the culture broth was significantly high which may correspond to the increased cellulolytic activities (Herr, 1979). The effect of various pretreatments of natural cellulosic wastes for the better susceptibility to microbial attack and synthesis of cellulase enzymes has been studied extensively by different workers (Mandels et al., 1974; Peitersson, 1975; Nesse et al., 1977). The cell and substrate associated enzymes on various strains and microcrystalline cellulose showed that there is differential enzyme adsorption on natural polymers. Microcrystalline cellulose seems most favourable substrate for adsorption while delignified strains came the next followed by untreated ones.

Growth of the repressed mycelium of T. longibrachiatum on 2% glucose gave very low yields of cellulase enzymes in all the three fractions as compared to CMC. Due to glucose exhaustion after two days of incubation there was a regular increase in the enzymes even in the absence of the inducer. The basal synthesis of these enzymes was very low being 19.11 and 6.43% for extracellular FPA and CMCase respectively as compared to that produced in the presence of CMC (Table 6). The cytosol and cell debris enzymes were repressed too but to a lesser extent than the extracellular. Similar partial inhibition of enzymes has been reported in S. pulverulentum (Eriksson and Hamp, 1978) and T. viride (Mandels and Reese, 1960; Mandels et al., 1962; Nisizawa et al., 1971, 1972; Lowenberg and Chapman, 1977). However, Berg and Pettersson (1977) showed complete inhibition of CMCase with glucose as the substrate.

The fractionation of extracellular cellulase (FPA and CMCase) with DEAE-sephadex A-50 did not achieve higher degree of purification (Table 3) but could separate all the pigments of the culture filtrate and also separated filter paper activity into unadsorbed and adsorbed fractions latter being eluted at 0.25 M of NaCl concentration. Similar adsorption of FPA/C_1 has been reported in T. viride and

T. koningii (Mandels and Reese, 1964; Selby and Maitland, 1967ab; Wood, 1968; Wood and Mcrae, 1972; Berghem and Pettersson, 1973; Berg and Pettersson, 1977). SE-sephadex C-50 could attain a reasonable amount of purification along with separation of β-glucosidase from FPA and CMCase. The separation of β-glucosidase from other components has also been achieved in T. viride (Selby and Maitland, 1967ab, and Geotrichum candidum (Rodionona et al., 1980). Biospecific absorption has also helped in resolving cellulase complex into different components through cellulose (Enari et al., 1981). The final purification through gel chromatography G-200 of the combined CMCase and FPA fractions achieved 18.59 and 9.46 degree of purification with 47.04 and 23.73 per cent recovery of the two enzymes respectively. In T. viride a recovery of 0.144% for C_1 enzyme with very high purification was achieved after four steps of purification which included isoelectric focussing also (Berghem and Pettersson, 1973).

The effect of enzyme concentration and time of incubation on cellulase enzymes showed an optimal concentration of 16 and 1.33 ug protein along with 60 and 20 min of incubation period for FPA and CMCase respectively. This is in agreement with the results of Denison and Koehn (1977).

Further increase in the enzyme concentration or time of incubation resulted in end product inhibition of the enzymes. The Km values given in the table 7 for FPA in the adsorbed and unadsorbed fractions with filter paper as substrate were 33.3 and 47.61 mg/ml and that of CMCase was 4.0 mg/ml. Km values for endo-1,4-β-glucanases from different sources and of different purity with CMC are reported to vary from 0.07 mg/ml to 44 mg/ml (Jermyn, 1952; Halliwell, 1961; Almin et al., 1967; Eriksson and Hollmark, 1969; Gupta and Gupta, 1979). While working on purified C_1 component of T. koningii, Halliwell and Griffin (1973) found a Km value of 0.05 mg of cellulose/ml. Km value for FPA in case of Aspergillus terreus was shown to be 80 mg/ml (Garg and Neelakantan, 1982).

The optimal pH for estimation of FPA and CMCase was found to be 5.5 and 5.0 respectively with optimal temperature being 60°C in both the cases (Table 7). The optimal conditions of cellulase activity vary from pH 4 - 5.0 and temperature assay being 50 - 55°C as shown in Trichoderma sp. (Halliwell, 1970; Okada, 1975; Gupta and Gupta, 1979), Sclerotium rolfsii (Sadana et al., 1980), Pellicularia filamentosa (Tanaka et al., 1977) and Aspergillus terreus (Garg and Neelakantan,

1982). Both the activities were stable at pH 5.0 - 5.5, the CMCase being relatively more stable. The better stability of CMCase to higher temperature was also apparent as it could retain 50% of its activity as compared to 34% for FPA at 60°C. However, further increase in temperature resulted in loss of both the activities.

It was observed that higher concentrations of Zn^{++}, Ca^{++}, Co^{++} and Na^{+} stimulated the FPA whereas inhibition was 100% with higher concentrations of Cu^{++} and all concentrations of Hg^{++} and Ag^{++} (Table 4). On the other hand CMCase was stimulated by Na^{+} and Co^{++} but the stimulatory effect of Co^{++} decreases with increase in concentration. Ca^{++} promoted the CMCase activity only at a lower concentration of 1 mM. This effect of Ca^{++} at 1 mM concentration and at higher concentration of 10 mM for CMCase has been reported in Acetovibrio cellulolyticus (Mackenzie and Bilous, 1982). CMCase was more stable than FPA with heavy metals like Ag^{++}, Hg^{++} and Cu^{++} at lower concentrations but higher concentrations inhibited the activity. EDTA resulted in 60% loss of FPA whereas CMCase was resistant to it. Similar stability of endoglucanases has been demonostrated in the case of T. reesei (Reese and Mandels, 1980). The inhibitory effect of heavy metals has been reported in T. koningii,

T. viride and Aspergillus terreus (Halliwell and Griffin, 1973; Gupta and Gupta, 1979; Garg and Neelakantan, 1982).

Polyacrylamide gel electrophoresis revealed molecular weights of 44,000 and 55,000 for FPA and CMCase respectively (Table 7), whereas molecular weights of different endoglucanases in Trichoderma sps have been reported to vary from 13,000 to 50,000 and that of exoglucanases have a range of 40,000 to 62,000 (Bisaria and Ghose, 1981).

SUMMARY

Production of cellulase components which include filter paper activity (FPA), carboxymethyl cellulase (CMCase) and β-glucosidase on CMC has revealed the occurrence of all the activities in both cell free and cell associated states. The relative distribution of these activities varies with the age of the culture. Extracellular fraction contributes the major portion of the total enzymes but for β-glucosidase where cytosol and cell debris retain reasonable amount of enzyme. The shake culture conditions gave comparatively low yields of the enzymes as compared to stable cultures.

The optimal pH of the medium for the synthesis of the enzymes at different localizations varied from 4.5 - 5.0 with the optimal temperature being 27°C. The growth was maximum at 32°C. The enzyme production was highest on lactose when different soluble and insoluble (both delignified and untreated straws) carbon sources were compared. There was differential induction of cell debris and cytosol enzymes with different carbon sources. Malt extract-yeast extract and cellobiose induced high yields of cell associated FPA and CMCase. There was no significant increase in the enzymes with increase in substrate concentration (CMC and lactose). Glucose caused a marked inhibition of cellulase enzymes although the repression was more in the case of extracellular than cell associated enzymes.

The purification of FPA and CMCase through ammonium sulphate precipitation, ion exchange and gel chromatography gave a yield of 23.73 and 47.04% with a purification factor of 9.46 and 18.59 for the above two enzymes respectively. The purified enzyme showed an optimal enzyme concentration of 16 and 1.33 ug protein with 60 and 20 min of incubation as an optimal time for FPA and CMCase respectively. The optimal pH and temperature for FPA and CMCase were 5.5 and 5.0 at 60°C for both the enzymes. Carboxymethyl cellulase was relatively more stable at different pH and temperatures of incubation. The effect of substrate concentration revealed the Km values to be 33.3 and 47.61 mg/ml respectively for adsorbed and unadsorbed FPA and 4.0 mg/ml for CMCase. Of the different metal ions tested Ag^{++}, Hg^{++} and Cu^{++} were found to be inhibitory while Ca^{++}, Co^{++}, Na^{+} and Zn^{++} were promotory. The molecular weight of FPA and CMCase has been found to be 44,000 and 55,000 as determined with polyacrylamide gel electrophoresis.

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