BIOCONVERSION OF LIGNOCELLULOSIC WASTE MATERIALS

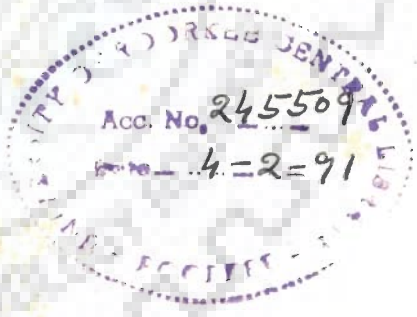
A THESIS

submitted in fulfilment of the requirements for the award of the degree of

DOCTOR OF PHILOSOPHY

By

ASHWANI KUMAR LAKHANI



DEPARTMENT OF BIOSCIENCES AND BIOTECHNOLOGY
UNIVERSITY OF ROORKEE
ROORKEE-247 667 (INDIA)

APRIL, 1990

DEDICATED

TO

MY GOD SATYA NARAYAN SWAMY

AND

MY PARENTS

CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **'BIOCONVERSION OF LIGNOCELLULOSIC WASTE MATERIALS'** in fulfilment of the requirement for the award of the Degree of Doctor of Philosophy submitted in the **Department of Biosciences & Biotechnology** of the University is an authentic record of my own work carried out during a period from March 1985 to Dec. 31 1989 under the supervision of **Prof.C.B.Sharma and Dr.G.S.Randhawa.**

The matter embodied in this thesis has not been submitted by me for the award of any other degree.

Ashwani Lakhani

(Ashwani Kumar Lakhani)

This is to certify that the above statement made by the candidate is correct to the best of our knowledge.

G. S. Randhawa

(Dr.G.S.Randhawa)
Dept of Biosciences &
Biotechnology,
University of Roorkee,
Roorkee-247 667 INDIA

C. B. Sharma 29/1/90

(Prof.C.B.Sharma)
Dept of Biosciences &
Biotechnology,
University of Roorkee,
Roorkee-247 667 INDIA

The candidate has passed the viva-voce examination held on at . The thesis is recommended for award of the Ph.D. Degree.

Signature of Guide(s)

Signature of External Examiner(s)

R. C. Bansal 30.4.90

Head
Department of Biosciences
and Biotechnology
University of Roorkee
ROORKEE-247 667

ABSTRACT

The purpose of this study was to determine the effect of high pressure steaming pretreatment followed by alkali treatment to make the lignocellulose waste material susceptible to enzymatic attack. The Mangifera indica saw dust was subjected at high pressure steaming (350 ± 15 psi) for 30 min. followed by alkali treatment at room temperature in attempt to improve extent of digestibility and both methods of treatment found to be ideal. The saw dust waste, mainly consists of approximately cellulose (53 - 58%), hemicellulose (18 - 21%) and lignin (18 - 22%) . The ternary complex cellulose-lignin hemicellulose, was broken and the hemicellulose and lignin components were rendered easily extractable with water and dilute sodium hydroxide solution at room temperature, respectively. The X-ray diffraction pattern before and after the pretreatment showed a marked decrease in the ordered crystalline structure of the cellulose fibre in the raw material. Trichoderma viride 1060 and Aspergillus wentii Pt. 2804 were found to be suitable culture for production of maximum amount of cellulase and β-glucosidase in 13 days at 30 C. Lignin free saw dust was nearly as good a substrate for production of extracellular cellulase and β-glucosidase by T. viride and A. wentii, as pure cellulose where as, untreated saw dust found to be the poor substrate due to the presence of admixtures binding the cellulose. The equi proportion of β-glucosidase and

cellulase, hydrolysed cellulose to about 80% in 48 h whereas, only with cellulase, 54.6% hydrolysis of cellulose was possible. The addition of β-glucosidase found to be an important step, both in terms of maximum saccharification and less incubation period. The pH 4.8 was an optimum because below and above this pH, there was and sharp decline in maximum percent saccharification. Similarly the temperature also played an important role in the hydrolysis of cellulose and 50°C found to be ideal temperature. The glucose obtained from the cellulose found to be an cheap and excellent substrate for single cell protein and citric acid production.

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ASHWANI K. LAKHANI

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CMC - Carboxymethyl cellulose

CBH - Cellobiohydrolase

pNPG - p-Nitrophenyl β-D-glucopyranoside

CHAPTER I

1.0 Introduction

The increasing demand of energy coupled with shortage of fossil fuel and variability of petroleum in most part of the world have renewed interest in the production of fermentable sugars from renewable lignocellulosic waste materials, which can be used as a feed stock for the production of biofuel, organic acids and animal feed.

Lignocellulosic materials (biomass) provide the world's most abundant renewable resources annually produced approximately 1.8 x 10^{12} tons (53) through massive photosynthesis. The production of biomass and its efficient utilisation is probably one of the best methods of harnessing solar energy for alleviating mankind's resources problem for chemicals, energy and food. Lignocellulosic materials derived from crop plants as agricultural residues are available in fairly large quantities. Most of the lignocellulosic materials comprises of three major components, namely cellulose, hemicellulose and lignin. The percentage of each varying from one form of materials to another. Generally the lignocellulosics contains 50% of cellulose, 25% of hemicellulose and 25% of lignin (31).

The available lignocellulosic raw materials in India are high cellulose-containing wastes such as wheat husk, wheat straw, rice husk, rice straw, bagasse and saw dust of wood. The major residues amount to approximately 39.0 million metric tons or approximately 18 - 20 million metric

tons of bio-degradable cellulose (56).

Cellulose is a linear homopolymer of anhydroglucose units linked by β-1-4 linkage and has crystalline nature in naturally occuring sources. These cellulosic wastes can be converted through enzymatic hydrolysis to enable additional supply of ethanol for the production of chemicals and other purposes at a predictable cost. The amount of ethanol which can be obtained from the estimated cellulosic wastes available in India amounts to approximately 8.0 million metric tons (57). If 80% is converted into energy rich chemicals, this will be able to provide India with an equivalent of petrochemicals products derived from about 7.2 million metric tons of naptha. Morever the lignin obtained as a by-product can be used as feed stock for a number of aromatic compounds and hence will increase the range of available products.

The characteristics of lignocellulosic substances such as crystallinity of cellulose, lignin content, pore size, moisture content and available surface area, all determine the suitability to enzymatic attack. Pretreatment methods must often be employed as a first step in the utilisation of such residues to enhance cellulose susceptibility to enzymatic attack. It is known that crystalline regions are less accessible to enzymatic attack due to transport limitations imposed by the closely ordered lattice of cellulose molecule (126). Several physical and chemical methods have been used to increase cellulose

susceptibility to enzymatic attack. Among the physical methods (3,99,114,115,121) are milling (ball, hammer, colloid, rotter mills) which are effective in reducing particle size but may operate at high energy consumption. The chemical methods include alkali treatment (90) and solvent pretreatment (141).

Once it is possible to pretreat lignocellulosic material, the immediate next step is to hydrolyze pretreated lignocellulosic material to produce fermentable sugars by cellulase enzyme. The rate and extent of cellulose saccharification depends on substrate characteristics, enzyme and substrate concentration ratio, product inhibition and enzyme stability. The biodegradation (enzymatic break down) of crystalline cellulose is complex process, requiring the participation of many enzyme components of cellulase such as (i) endo-(1-- 4) β-D-glucanase, (Ec 3.2; 1.4) which chops the cellulose polymer in a random way; (ii) cellobiohydrolase (Ec 3.2 ;1.91) which hydrolyses the terminal cellobiose groups, (iii) β-D-glucosidase (β-D-glucosidehydrolase, E.C. 3.2.1.21) which produces D-glucose by splitting cellobiose (112).

Mostly the cellulase enzyme has been produced by micro-organism Trichoderma reesei and other strains of Trichoderma and its mutants. They produce more stable and active extracellular cellulases. However, T.reesei enzyme complex has a very low level of β-glucosidase and is also inhibited severely by end product (59,62,67), leading to

accumulation of cellobiose. Cellobiose is also a strong inhibitor of other cellulase components, endo- and exo-glucanases (13,64,105). Thus, if the enzyme complex is deficient in β-glucosidase, the rate of cellulose hydrolysis will be reduced drastically. Furthermore, the predominant sugar in the cellulose hydrolysate would be cellobiose and not glucose. While glucose has varied applications, cellobiose, an unfermentable sugar, has not yet found major applications. Cellulose hydrolysis with Trichoderma culture cellulase, to be efficient and occur at a reasonably fast rate requires high amounts of the enzyme. The high cellulase requirement and its cost of production are the major barriers that must be overcome to make enzymatic saccharification of cellulose economical. Not only the cost of production of cellulases and cellulose saccharification, the cost of pretreatment of lignocellulosics can also be a key factor in making the whole process economically viable.

Next to cellulose, lignin is an important component of lignocellulosic material. Lignin is the major participant in the energy flows in the biosphere. More solar energy is stored/entraped in the lignin content than in any other component of plants. A unique property of lignin is, that it is very stable in nature and actually acts as a stabiliser towards various stresses to which the plant is subjected, for example as experienced by the inhibiting effect of lignin on the rate of enzymatic hydrolysis of wood. The enthalpic value of lignin is more in comparable to lignocellulosic material.

Thus, for the efficient bio-conversion of lignocellulosic material, the following aspects must be taken into account.

1. Pretreatment - The pretreatment should be simple and able to break the ternary complex releasing hemicellulose,lignin and cellulose components.

2. The crystalline structure of cellulose must be converted to amorphous state for efficient enzymatic degradation.

3. High specific activity cellulases: containing appropriate amounts of β-glucosidases.

4. Large scale production of high specific activity cellulase enzyme from a suitable source and finally the economic viability of the process including the use of by-products. Although sufficient information is available in literature on these aspects, there is a need to obtain more precise data in regard to particular raw material to be used for bioconversion. Keeping this in view the present study was undertaken to develop a suitable biodegradation process for production of fermentable sugars from saw dust of Mangifera indica, Eucalyptus and Cedrus deodar which are available in abundance at reasonably cheap price.

The specific objectives carried out in the present work have been as under :

1. To develop a suitable physical or chemical method for treatment of saw dust.

2. To produce large quantities of high specific activity cellulase enzyme from suitable strains of Trichoderma.

3. To prepare large amount of β-glucosidase from Aspergillus niger and Aspergillus wentii for supplementing the cellulases, for degradation of cellulose.

4. To study the kinetic parameters of enzymatic biodegradation.

5. To develop a bioreactor.

6. Utilisation of enzymatic hydrolysate for the production of single cell protein and citric acid.

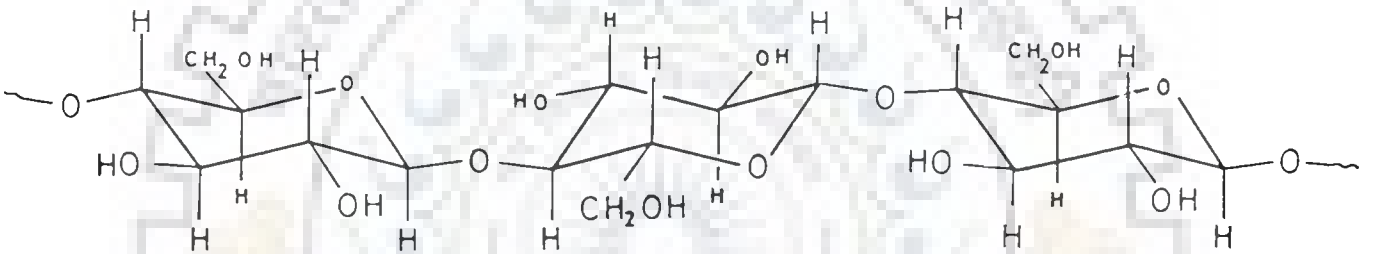
CHAPTER - II

2.0 Literature Review

Lignocellulosics are the main sources of the naturally occuring cellulose. Cellulose is the most widely distributed skeletal polysaccharide, amounting to approximately 50% of the cell wall material of wood and plant. Cellulose is a high molecular weight linear polymer of anhydroglucose units connected by 1, 4, β-glucosidic bonds. The number of glucose units per molecule (degree of polymerisation) ranges from as low as 15 to as high as 10,000 - 14,000 (31). In nature cellulose, upto 10,000 β-anhydroglucose residues are linked to form a long chain molecule. This means that molecular weight of native cellulose is above 1.5 million. The total length of cellulose molecule is about 5 μm.

In a cellulose chain molecule the anhydroglucose units adopt the chair configuration with the hydroxyl groups in the equatorial and the hydrogen atoms in the axial positions. The conformational formula (chair form) of cellulose (poly-β-1, 4,D-glucosan) is shown in Fig.1. Liang and Marchessault (92) have proposed that every other chain unit is rotated at 180^{o} around the main axis resulting in an unstrained linear configuration with minimum steric hinderance and that the hydroxyl group in the 3-position is bound by an intramolecular hydrogen bond to the ring oxygen atom of the next chain unit (Fig.2). The glycosidic linkage acts as a functional group that alongwith the hydrogen groups

Fig. 1 Conformational formula (Chair form) of cellulose (Poly - β - 1, 4 - D - glucosan).



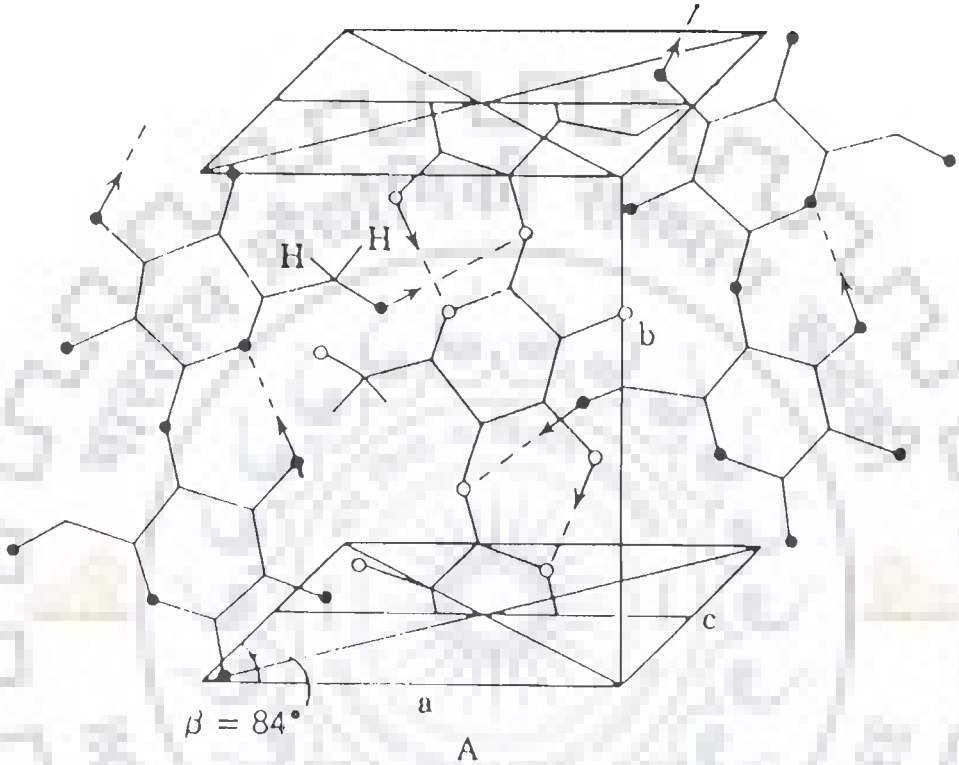
Cellulose

mainly determines the chemical properties of cellulose.

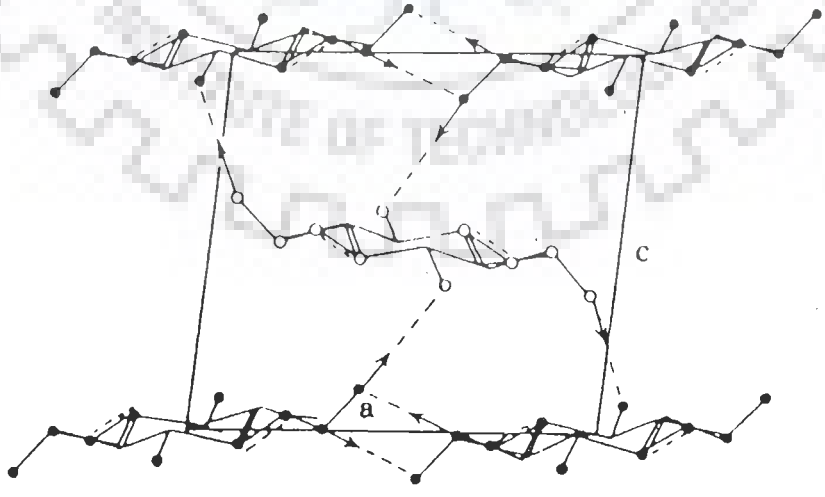
Cellulose molecules form a fibril, a thread like long bundle of molecules, which is stabilized laterally by hydrogen bonding between hydroxyl groups of adjacent molecules (Fig.2). The molecular arrangement of this fibrillar bundle is sufficiently regular that cellulose exhibits a crystalline X-ray diffraction pattern. The schematic model of the native cellulose lattice was proposed by Meyer, Mark and Mish (109,110,111). According to them the basic repeating unit of the crystal is the unit cell, as a monoclinic lattice with cellulose chains packed at the corners and the center of the cell. The spatial representation of the unit cell or crystal of cellulose was developed by Liang and Marchessault (92) as shown in Fig.2. According to this model each unit cell contains four glucose residues. These include two in the center of the figure and one fourth of each of the eight which are placed at the corners of a monoclinic cell. The corner residues are shared by each of the four unit cells which meet at the corners; The cellulose chain in the center of the unit cell runs in a direction opposite to that of edges (111).

The consequence of the high degree of order in native cellulose is that neither a water molecule nor an enzyme molecule can enter the structure (166). Therefore, the native cellulose is inert in the digestive tract. While the pretreated (i.e. acid or alkali swollen) cellulose is open and can readily be splitted by cellulases.

Fig. 2 Hydrogen bonding and the arrangement of cellulose molecules in native cellulose (92).

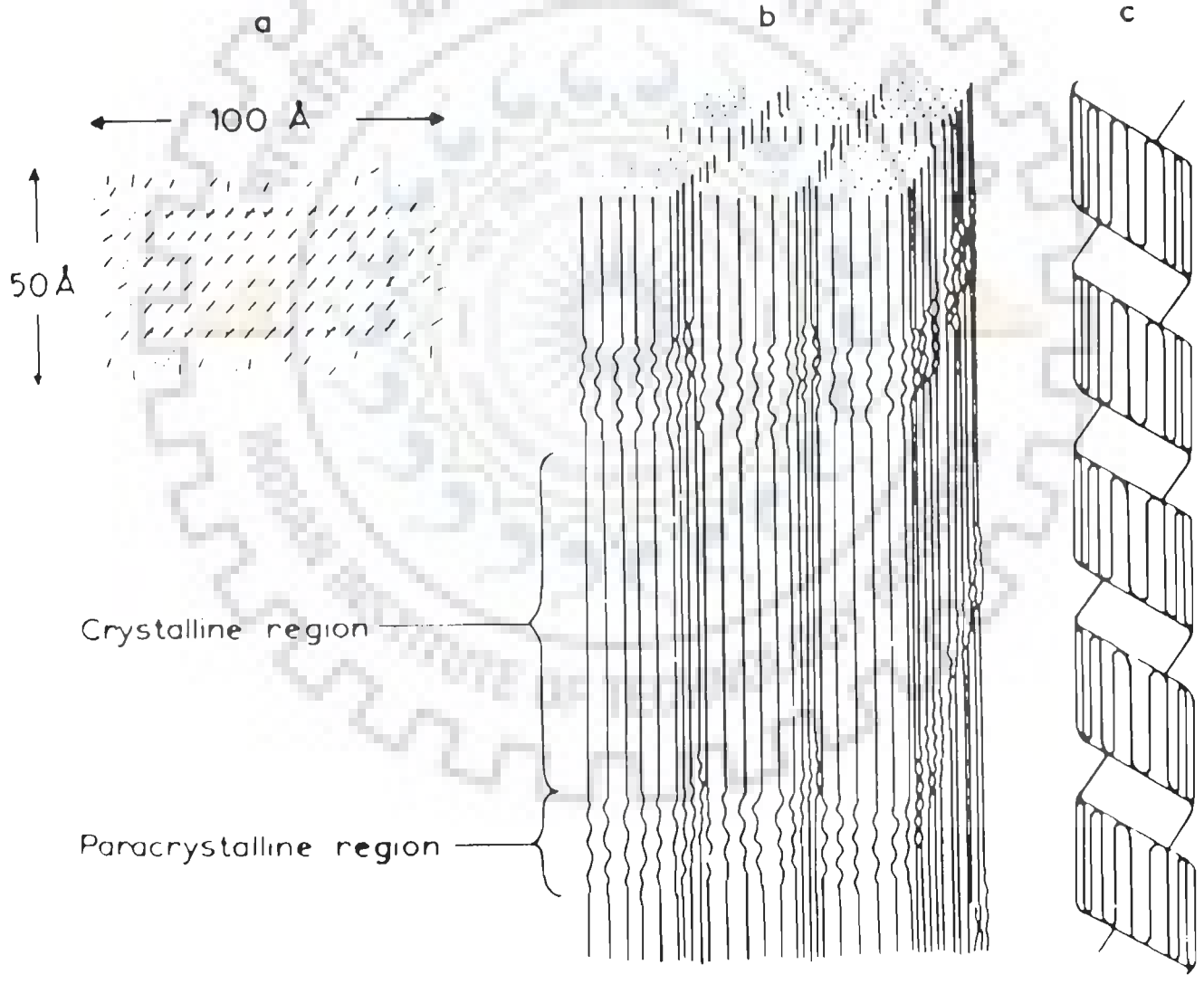


A



B

Fig. 3 Recent Concepts of the structure of cellulose microfibril (30).


a
b
c
100 Å
50 Å
Crystalline region
Paracrystalline region

2.1 Structure And Morphology Of Cellulose Fibers

Molecular structure is a key to determine or deduce, the chemical nature of cellulose, its physical, mechanical properties and its fibrillary structure. The individual cellulose molecules are linked together to form elementary fibrils or protofibrils, about 40 A^{O} wide, 30 A^{O} thick and 100 A^{O} long, in which the polymer chains are oriented in a parallel alignment and firmly bound together by numerous strong hydrogen bonds. The elementary fibril is the smallest structural unit of microfibrils and fibers, and a number of the elementary fibrils are aggregated into long slender bundles called microfibrils. The electron micrograph of the native cellulose shows that microfibrils generally are present in the form of bundles in lamellae which contain an extremely large number of fibrillar units (132).

The crystalline region is bounded by a layer of cellulose molecules that exhibit various degree of parallelism. The less ordered region is called the para crystalline or amorphous region. The amorphous region of microfibrils allows hydrolysis of cellulose into rod-like particles by aqueous, non swelling, strong acid. Amorphous region may be native one or formed by mechanical forces giving deformation beyond the limit of elastic recovery of the microfibrils. The length of the resultant particle after acid hydrolysis (micelles or micro crystals) corresponding to the levelling off the degree of polymerisation of the hydrocellulose varies with the extent of pretreatment of

native cellulose.

Cowling proposed and reviewed models of microfibrillar structure (30). The three observations can be made from the proposed models. (a) The microfibril is about 50 x 100 A^{o} in cross section and consists of a crystalline core of highly ordered cellulose molecules surrounded by a paracrystalline sheath (Fig.3). The sheath in cotton, contains mainly cellulose molecules, but in wood it also contains hemicellulose and lignin molecules. (b) The cellulose molecules are less ordered along the length of a microfibril. (c) The cellulose molecules may exist in a folded chain lattice formed as ribbon wound in a tight helix. Chang summarised the models on the various molecular arrangements of cellulose and proposed a folding chain model (25). The models are shown in the Fig. 4.

The two different types of models of the molecular arrangement of elementary fibrils are :

1. Folded chain models (c,d,e), 2. extended cellulose molecules without folding (models a and b). Chang concluded that model 'c' is supported by most of the known facts about cellulose (25).

Fengel postulated a model after incorporating Model 'C' into a high level structure (48). This shows the ultra-structural organisation of the cell wall components shown in Fig.5. The elementary fibrils are cemented together by polyoses such as hemicellulose to form a microfibril.

Fig. 4 Models of molecular arrangements in the protofibril. (a) Full extension and complete crystalline model. (b) Fibrillized micellar model. (c) Fibrillized models of Ellefsen. (d) Manley's planar Zig-Zag model. (e) Chair folding with fold length much smallar than LODP.

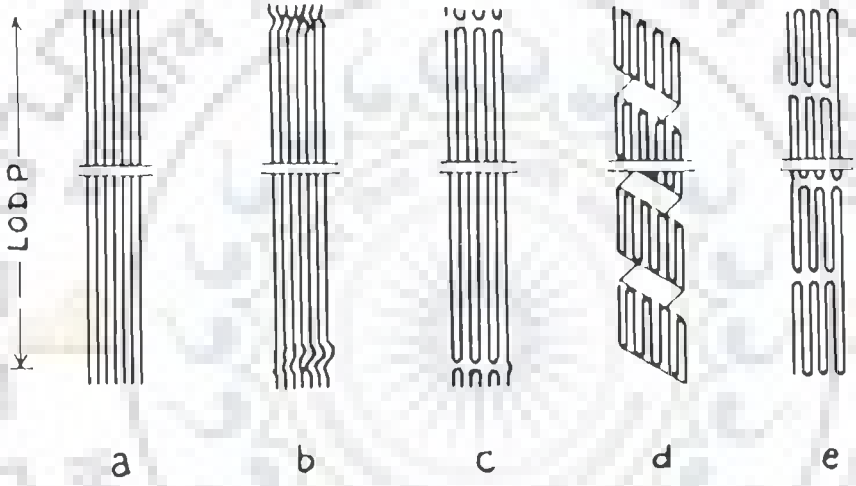
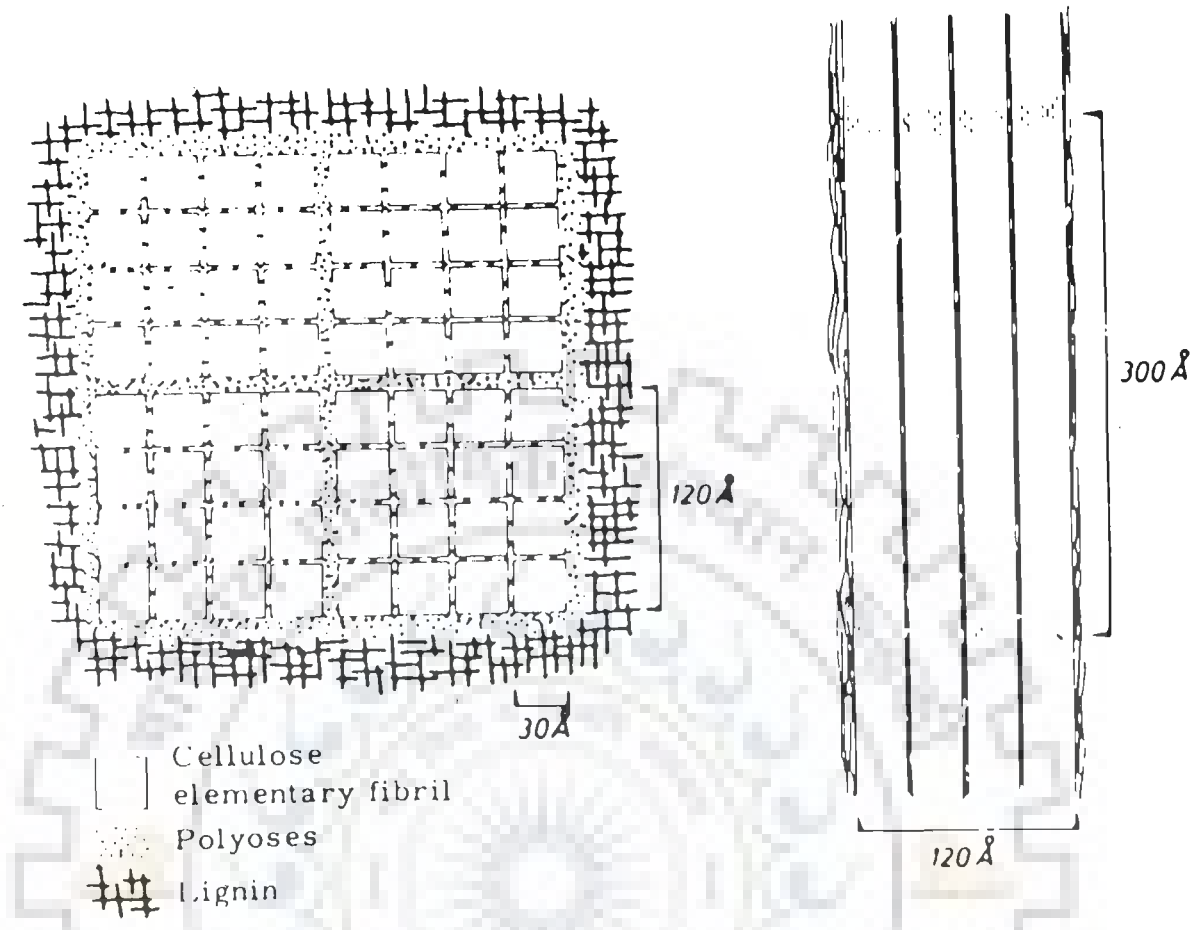
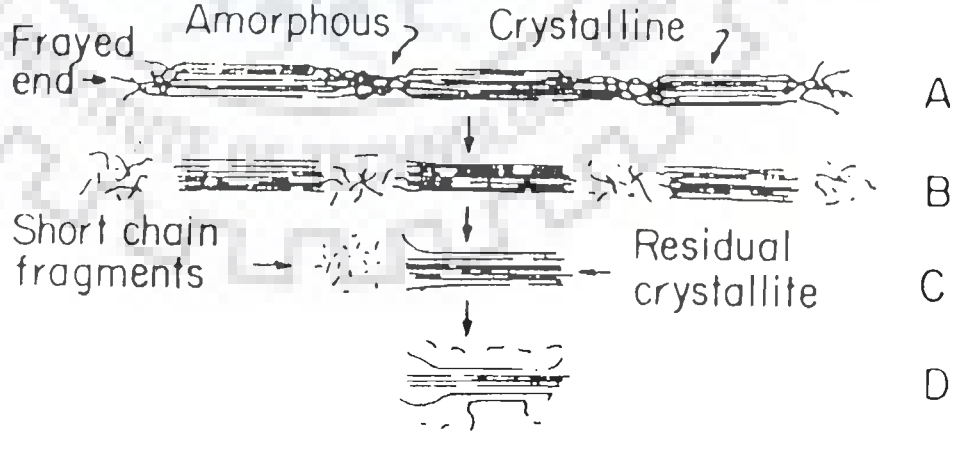

LOOP
a
b
c
d
e

Fig. 5A Model of ultrastructural organization of the cell wall components of wood.

Fig. 5B Schematic summary of initial stages of dissimilation of a partially crystalline cellulose fibril. (a) Original cellulose fibril. (b) Initial attack on amorphous region. (c) Residual crystallite and digestion of short chain fragments. (d) Attack on residual crystallite.


120 Å
30 Å
300 Å
120 Å
Cellulose elementary fibril
Polyoses
Lignin

5A


Frayed end
Amorphous
Crystalline
A
B
Short chain fragments
Residual crystallite
C
D

5B

This microfibril is surrounded by a lignin and a polyose layer which protects the microbril from the enzyme action. Pretreatment of lignocellulosic biomass is aimed at degrading these protective layers and to break crystalline structure and is essential to rapid enzymatic hydrolysis of cellulose is known to exist in various polymorphic forms (11). Cellulose I is the polymorphic form that occurs in naturally occuring materials, i.e., cotton, ramie and wood pulps. Cellulose-II is the crystalline modification which is representative of the regenerated forms of celletose, cotton and lignocellulosic treated with sodium hydroxide and also saponified cellulose derivatives. Rayon is a typical example of cellulose-II. Solvent pretreatment brings about a transformation in the cellulose crystalline structure from that of cellulose-I to cellulose-II (18,142). The changed crystalline structure of cellulose on solvent pretreatment and the resultant increase in rate of saccharification can be explained in terms of the folding chain model for the cellulose structure. In naturally occuring cellulose the molecule folds back and forth on itself with a fold length of DP = 160. On solvent pretreatment the width of the crystallite is increased due to swelling but the fold length is decreased from DP = 160 to DP = 30 (19).

2.2 Capillary Structure Of Cellulose Fibers

The susceptibility of cellulose to enzymatic hydrolysis is determined largely by its accessibilty to a cellulolytic enzyme. The physical contact between the enzyme

and the substrate molecules of cellulose is a prerequisite to enzymatic hydrolysis. This type of achievement can only be possible by diffusion of the enzyme into the complex structural matrix of the cellulose. It has been reported by Lee (91) that the most reactive regenerated cellulose is also the most crystalline, in contrast to most reports. This implies that the degree of swelling is the most important factor rather than crystallinity of the substrate, because a higher degree of swelling increases the pore size above a certain critical value. The increase in pore size is necessary, to allow enzyme molecule to penetrate the substrate. Stone et al.(151) reported that a highly amorphous substrate may be slow to hydrolyze because the amorphous fraction is accessible to water and hydrogen ions but is completely inaccessible to enzyme. These two examples emphasize the importance of capillary structure in relation to the enzymatic hydrolysis of cellulose.

It is reported that the hemicellulose and lignin occupy the spaces among the microfibrils and cellulose molecules in the amorphous region. If the physical or chemical treatments do not increase reactivity, it must be due to the changes within the cell wall itself rather than that of the size of the cell wall fragment. The dimensions of the cellulose capillaries have been determined by using the salute exclusion technique by Aggebrandth and Samuelson (1), Stone et al. (151), Nelson and Oliver(122)and Van Dyke (164). The minimum and maximum dimensions of cell wall capillaries are about 5 and 40 A^{o} for water swollen wood,

about 75 and 110 A^{o} for water swollen cotton, and about 45 and 110 A^{o} for water swollen wood pulp (29).

Cellulolytic enzyme molecules range from 24 to 77 A^{o} in diameter with an average of 59 A^{o} (30). Thus these molecules would be expected to diffuse readily within the gross capillaries having 200 A^{o} or more diameter and act on cellulose molecule exposed on the surface of these capillaries. However, only a small fraction of the cell wall capillaries in water swollen wood and cotton fibres is sufficiently large to permit penetration of most of the cellulolytic enzyme molecules. Because of this fact, it is likely that the cellulolytic enzymes are physically excluded from all but the largest cell wall capillaries. Stone et al.(151) measured the pore volume and calculated the surface area within the swollen fibres accessible to all the molecules. The substrate reacted with a commercial cellulase enzyme preparation and the initial rate of reaction compared with the accessability of the substrate to molecules of various sizes. A linear relationship was found between the initial reaction rate and the surface area within the cellulose gel, which was accessible to a molecule of 40 A^{o} diameter. The conclusion made by Stone et al. (151) was, that the digestibility of cellulose with different degrees of swelling is directly proportional to the acessibility of molecules of 20 - 40 A^{o} in diameter, which corresponds to the size of cellulose molecules.

2.3 Pretreatment Of Lignocellulosics

Due to protection of the lignin sheath and the crystalline structure, the cellulose in wood or other plant tissue is normally not degradable by extracellular enzymes to any appreciable extent. Pretreatment of the substrate is, therefore, carried out to increase its suscepibility to hydrolysis. It is an important variable which will affect not only the degree of saccharification but also the economics of the process. Hence the pretreatments are designed to achieve the following : loosening the highly crystalline structure and extending the amorphous area of the cellulose molecule and the removal of lignin and hemicellulose. There are several methods of pretreatment which are used such as solubilisation of cellulose in cadoxen, CMCS (Fe, tartrate, OH) solvent, cuprammonium or any strong acid (46,90,130,66,32) and subsequently reprecipitation and washing. No doubt the process is effective but because of economy and pollution control constraints the solvent must be recovered and this is formidable process. Furthermore, it is much easy to dissolve pure cellulose than to extract the cellulose from the lignocellulosic waste materials. Extraction by means of acid with removal of some hemicellulose (11,67,68,81) or swelling in dilute alkali (41,54) is more economical but the process requires lot of washing or pH adjustment before using it for saccharification.

Physical methods like ball or attrition milling

(116) and compression milling (157) reduce particle size, thereby increasing bulk density, available surface area and reactivity. All milling processes are effective for all substrates and permit the use of high concentration (20 - 30%) of slurries in the hydrolysis vessel. The wet milling pretreatment (82) during saccharification enhances the action of cellulases. The alkali explosion pretreatment of straw and bagasse was also done by Puri and Pearce (131) to increase the surface area and to reduce the degree of polymerisation. This resulted in enhanced rate of saccharification to a great extent. The use of low pressure steam autohydrolysis in the pretreatment of corn stover was found to be highly successful in removing hemicellulose and lignin almost completely and enhancing the rate of cellulose hydrolysis with a commercial cellulase preparation (79). Steam explosion (240^{o}C, 40 - 50 atm pressure, 20 - 80 sec.) by the Iotech process (80,93) is found to be economical and also very effective for hard woods and agricultural residues. Steam explosion (steaming) pretreatments of wood and some lignocellulosic materials are also reported recently to increase the susceptibility of lignocellulosics for enzymatic degradation and removal of hemicellulose and lignin, which otherwise are inhibitory to the growth of micro-organism and production of enzyme (20,21,81,106,108,33,158).

Microbiological methods like partial removal of lignin by white rot fungi (34,45,74,138,139,168) are slow processes and also cause the degradation of the residual carbohydrate. Some other processes include high energy

irradiation (6,7,10,91) and soluble cellulose derivatives (175).

2.4 Crystallinity Of Cellulose

The degree of crystallinity of cellulose is one of the most important stuctural parameters which affects the rate of enzymatic hydrolysis. The crystallinity of native cellulose was experimentally determined with X-ray diffractometer using the focussing and transmission techniques by Segal et al. (143). The intensity of the 002 interference and the amorphous scatter at $2\theta = 18^{\circ}$ measured; The fraction of crystalline material in the total cellulose was expressed in terms of an X-ray crystallinity index. The data reported have been reanalysed (36) to examine the relationship between the cellulose crystallinity and digestibility. Norkans (126) and Walseth (165) reported that the cellulase enzyme degrade the more readily accessible amorphous positions of regenerated cellulose, but are unable to attack the less accessible crystalline portions. They observed a significant increase in crystallinity during hydrolysis of cellulose with enzymes, thereby increasing resistance to further hydrolysis (134).

Caulfield and Moore (23) and Reese et al.(134) reported somewhat different results. They measured the degree of crystallinity of ball milled cellulose before and after partial hydrolysis and found that the mechanical action (ball milling) increases the susceptibility of both the

amorphous and crystalline components of cellulose. The conclusion made by them was that the overall increase in digestibility is apparently a result of decreased particle size and increased surface area rather than a result of reduced crystallinity. But this may not be fully true or a general phenomenon.

2.5 Factors Affecting Enzymatic Saccharification

The factors which affect the hydrolysis of cellulosic materials include : type of substrate, pretreatment, characteristics of the enzyme preparations, temperature, time, pH and enzyme substrate ratio. These factors are directly or indirectly related to the following:
(i) Adsorption and desorption
(ii) Enzyme diffusion, and (iii) Product inhibition.

2.5.1 Adsorption And Desorption

The most significant and important aspect of cellulose hydrolysis is the process of adsorption of the active components of cellulases. Halliwel (63) reported that the adsorption of cellulase enzyme is very fast and the extent of adsorption depended on the initial quantity of cellulose present and on the subsequent incubation time. Mandel et al. (98) also reported that cellulose strongly adsorbs cellulase under optimal conditions for enzyme actions and the extent of adsorption (i.e. uptake of enzyme by the insoluble substrate used) is proportional to the initial cellulose concentration. It was observed (98) that

approximately 70% of the enzyme was adsorbed from the concentrated enzyme solution. The enzyme adsorbed ranged from 0.005 to 0.64 mg protein per mg of cellulose. The maximum adsorption of enzyme per volume of cellulose was attained when the cellulose concentration was the lowest. Adsorption of the enzyme increased as the particle diameter decreased from 50 μm to 6.7 μm. Peitersen et al.(127) reported that the adsorption of enzyme protein from the solution is largely independent of pH but the maximum adsorbed enzyme decreased as the temperature increased. They used a langmuir adsorption isotherm type equation to relate the adsorbed enzyme concentration to the free enzyme concentration.

$$E_{ads} = \frac{Kp\ Eads'm\ Eo}{1 + K_p Eo}$$

Where, Eads, adsorbed protein

Eads'm = maximum adsorbed protein

Kp = Kinetic constant

Eo = Protein concertration in supernatant

Several studies of adsorption of cellulase components on cellulose were carried out (52,169). Mandel et al.(98) reported that component, which is active on filter paper was more strongly absorbed than component (C_x), displaying activity on CMC; Wilke and Mitra (173) observed similarity in adsorption of both C_1 and C_x activities at various concentration of milled newsprint. Kim (84) showed from his experiments on digestion of 6% ball milled solka floc that the enzyme which adsorbed on cellulose was

primarily responsible for cellulose hydrolysis. His conclusion, however, needs further verification because of removal of the adsorbed enzyme from the cellulose to the supernatant.

Ghose and Bisaria (54) reported that in 15 minutes of contact time 50% of C_1 and C_x enzyme were adsorbed on delignified bagasse at a low temperature at 5°C. Goel and Ramachandran (50) studied the adsorption of β-glucosidase on both rice straw and bagasse. They observed that in 15 min. of contact time 80% of the β-glucosidase activity was adsorbed. Studies of adsorption of enzyme on cellulose was also done by Klyosov and Rabinowitch (85) and Klyosov, et al. (88). These workers found a correlation between the adsorption of cellulases and the efficiency of enzymatic hydrolysis of cellulose.

2.5.2 Diffusion Of Enzymes

The formation of an enzyme-substrate complex is a pre-requisite to the enzymatic hydrolysis of native cellulose. Cellulose is an insoluble and structurally complex substrate. The formation of an enzyme-substrate complex can be achieved only by diffusion of enzymes into the complex structural matrix of cellulose. Furthermore, the hydrolysis of cellulose by cellulase enzyme is an heterogeneous reaction process. So the overall rate of reaction could be conceivably influenced by mass transfer resistances, such as the bulk phase resistance, the

resistance through the surface film around cellulose particles and the resistance through capillary pores of cellulosic particles. The bulk phase and film resistance depend on the size of cellulose particles, enzyme concentration and hydrodynamic conditions in the reactor such as the intensity of agitation and Reynolds number flow through the reactor. Van Dyke (164) reported that the bulk and film resistance can be made virtually nonexistent under certain experimental conditions, including adequate mixing,proper particle size of cellulose and proper enzyme concentration. Similar observations were also reported by Huang and Kim (72,84). These workers suggested that the enzyme molecule does not diffuse through the pores of cellulose particles because the size of the enzyme molecules is larger than most of the capillary pores of cellulose. There is no any experimental studies which have been reported on the pore diffusion inside the cellulose particles.

2.5.3 Product Inhibition

The accumulated end products in certain enzyme reactions usually inhibit the forward reaction. A comprehensive review on the inhibition of cellulase and β-glucosidase has been published by Mandel's and Reese (95). Cellulase acting on insoluble cellulose are strongly inhibited by cellobiose (13,64,105). The degree of inhibition depends on the relative affinities of the inhibitor and the susbstrate for the active site and on the ratio of inhibitor and substrate concentration. It was

observed that the extent of inhibition increases with increase in size of the product molecules (125), The another report by Ghose and Das (51) shows that enzymatic hydrolysis of cellulosic material is a competitive product inhibition system. They analysed their results on the basis of two products of hydrolysis, glucose and cellobiose, and concluded that the nature of inhibition can be represented by competitive product inhibition. The quantitative nature of glucose and cellobiose inhibition has also been described (51).

Stuck (153) and Howell and Stuck (71) suggested a mechanism that is different from Ghose and Das (51). They developed a product inhibition model and assumed that the non-competitive inhibition by cellulose dominates the reaction kinetics and reported that under various experimental conditions the model can predict the progress of the reaction for an extended time more adequately than a competitive product inhibition model. The extent of inhibition also depends on the nature of substrate, . Klyosov and Rabinowitch (85) reported recently that cellobiose is less inhibitory and may even be an activator when CMC is the substrate. In view of the contradictory findings it would be essential to investigate the product inhibition in more detail.

2.6 Mode Of Action

The native crystalline cellulose is water insoluble and its structure and complexity make it highly resistant to the hydrolytic action of enzymes. In most cellulolytic organisms several cellulase components form a cellulase complex that synergistically hydrolyzes cellulosic substrates. It has been reported from nearly last three and half decades that some fungi like Trichoderma viride (T. reesei ,126b) Trichoderma Koningii(65), Sclerotium rolfsi, Penicillium funiculosum (172), Fusarium solani(171), Sporotrichum pulverulentum, Trichoderma lignorum, Trichoderma harzianum, Penicillium pinophilum and some bacteria like Clostridium thrmocellum provide culture filterate that are highly active in degrading cellulose. There are two hypothesis regarding the enzymatic hydrolysis of cellulose. Reese,et al. (133) proposed that multiple enzymes are involved in the hydrolysis of native cellulose into fermentable sugars. Their proposal was based on some findings that some microorganism were only able to attack derivatives of cellulose but not native cellulose. So there must be some enzyme present in the former that is missing in the latter. A concept of C_1 - C_x was proposed by Reese etal. (133). The C_1 was found to be a prehydrolytic factor (147) producing an activated cellulose which was then capable of attack by C_x (hydrolytic) enzymes of the complex producing soluble sugars. The precise action of C_1 was not clear for no component of this type was isolated at that time. Enzymes named as C_x can hydrolyze amorphous cellulose and soluble or

partially degraded cellulose, producing cellooligosaccharides, cellotriose, cellobiose and glucose and are hydrolytic in nature. The C_1 - C_x concept of Reese (133) dominated the earlier thinking about cellulolytic enzymes Whitaker (167)in 1953 proposed that only one enzyme was involved in the breakdown of native cellulose to simple sugars. The multienzyme hypothesis (144,152,167) and the unienzyme hypothesis (16,38) were each supported by several group of researchers.

The action of cellulases on cellulose and the participating enzyme components have been the subject of numerous investigations (13). The mode of action of the cellulase complex as accepted to date is : (i) Endo-β-1, 4 glucan glucanases (formerly C_x enzymes) as designated by Reese (133) attacks at different sites along the length of the chain causing a rapid increase in the fludity of a CMC solution which slowly increases in reducing group production. The substrates that were preferred include CMC, H_3PO_4 swollen cellulose and cellodextrins. The rate of hydrolysis of cellooligosaccharide increased with degree of polymerisation within the limits of substrate solubility. There is a general requirement for adjacent unsubstituted units and the extent of hydrolysis is reduced as the number of substituents increases. The major end products of endo-glucanases are mixture of transient cellooligosaccharides, cellotriose, cellobiose and small quantities of glucose. Endo-glucanases act with retention of configuration. The specificity region of endo-glucanases appears to be five units in length.

Different endo-glucanases exhibit different mode of attack on cellodextrins. Cellobiose is not hydrolyzed while cellotriose is hydrolyzed extremely slowly by most of the endo-glucanases. Most endo-glucanases preferably cleave second bond from the nonreducing end of cellotetrose through bond one and bond three are also cleaved slowly. With cellopentose the second and/or third glucosidic bonds are cleaved yielding cellobiose and cellotriose, almost in equal amounts, as principal products. The products of cellopentaitol hydrolysis were cellobiose and cellotriose almost in equimolar amounts, indicating cleavage at bond 2 or bond three of the molecule; alternatively the endo-glucanase produced cellobiose and cellotriose from the reducing or non reducing end with equal ease (59).

(ii) Exo-β-1-4 glucan glucanases identified in two major forms : (a) β-1-4 -cellobiohydrolase and (b) β-1-4-glucan glucohydrolase, β-1-4-cellobiohydrolase (CBH) which removes cellobiose units one by one from the nonreducing end of the cellulose chain. CBH has the highest affinity for cellulose. All CBHs were found to hydrolyze H_3PO_4 - swollen cellulose to cellobiose, small amounts of glucose (3.7%) are also formed. CBH action is effectively blocked once the available unsubstituted residues are removed. Cellobiohydrolase show synergistic effect when acting in concert with endo-glucanases to solubilize crystalline cellulose. The CBH from C.gilvus and T. aurantiacus do not show synergism with endo-glucanases though the CBH from C.Sterocarium shows the synergistic effect. The

enzyme from T.reesei (26) shows an initial endo-type mode of action.

β-1, 4 glucan glucohydrolase (glucohydrolase) removes the terminal D-gluco pyranosyl units from the non reducing end of the chain. The enzyme has the greatest affinity for the cellooligosaccharides of four to seven units. This enzyme according to Wood and McCrae (145) does not act synergistically with endo-glucanases in solubilisation of cotton.

β-1, 4 glucosidase or cellobiase, its function is to convert cellodextrins and cellobiose to fermentable sugar (i.e. glucose). It is suggested (43,117,137,144) that endo-glucanases cause random nicks in the amorphous regional cellulose long chain polymer releasing new chain ends. This is followed by removal of cellobiose by cellobiohydrolase. Some kind of synergism is strongly indicated between endo- and exo-glucanases resulting in production of cellulose oligomers and cellobiose. In the last and the final step of cellulose hydrolysis β-glucosidase brings about the degradation of cellodextrins and cellobiose to glucose.

Hence, from these data it is clear that cellulose degradation to glucose requires a mixture of exo-β-1,4-glucan glucanases and β-glucosidase.

2.7 Sources Of Cellulases

The availability of active cellulase and β-glucosidase is the basis of successful process for bioconversion of lignocellulosic materials. This requires selection and improvement of suitable strains for production of enzymes in sufficient quantities. Although there are a number of microorganisms (fungi and bacteria) that have the ability to degrade cellulose to CO_2 and CH_4, only a very few microorganisms that produce cellulase enzyme in appreciable quantity are known. In addition, some microorganisms secrete only either endo-glucanase (C_1) or β-glucosidase, and are, therefore, unable to hydrolyze native crystalline cellulose. Only those organisms which produce cellulase containing required amount of endo-glucanase (C_1), would be able to effectively degrade the native cellulose. In 1950, Reese and coworkers(133) isolated and identified few strains of Trichoderma which produced an extracellular cellulase complex effecting the degradation of native cellulose. Since then many microorganisms have been isolated but only quite a few strains have been shown to produce adequate levels of extracellular cellulase for practical utilisation (103). As pointed out above all the components of extracellular cellulase enzyme are essential for the hydrolysis of cellulose. In general, β-glucosidase which catalyze the hydrolysis of cellobiose, a reaction product of cellulose degradation by endo- and exo-glucanases is either lacking or present in relatively small proportions in the extracellular cellulase complex. Thus, sugars decrease as cellobiose

inhibits the activity of endo- and exo-glucanases. One of the ways to meet this deficiency is to add β-glucosidase to the reaction mixture along with cellulases. Another approach can be to design a suitable bioreactor in which cellobiose is removed from the reaction mixture and treated in a separate reactor to obtain glucose. However, in the present studies only the former method has been used and results were found to be satisfactory.

2.7.1 Cellulase Producing Microbes

Apart from cellulolytic fungi Trichoderma viride there are also many fungi and bacteria that produce cellulases which degrade pretreated cellulose or soluble cellulose derivatives as for eg. carboxy methyl cellulose (CMC) but not crystalline cellulose (154,40,96,97). Some thermophillic organisms suchas Actomycete Chaetomium thermophile var dissitium (60) Humicola (27)and Thermonospora. sp (69) also produce cellulase enzyme which can degrade native cellulose. Such thermophillic organisms may be valuable sources of thermostable cellulase like that of cellulases from mesophiles (100). The cellulase production by some other microorganism such as Bortryodiplodia theobromae (162), Geotrichum candidum (26), Pellicularia filamentosa (156), Neurospora crassa (38), Aspergillus niger (87). Aspergillus fumigatus (161) and Aspergillus niger (41) have also been reported. The cellulases from Aspergillus have usually high β-glucosidase activity but low endo- and exo-glucanases activity. So they

are limited in their ability to hydrolyse crystalline cellulose.

Strains of Trichoderma viride (Trichoderma reesei) which provide fairly active cellulase have been identified (10,58,124,146). A mutant D1 -6 from T.reesei Qm 9414 was developed by Ghosh and his co-workers (58). The other strain of T.reesei (VTT - D78085) is also reported by Herr (68) which was capable of producing 2 - 3 fold increase in cellulase activity and 6 fold in β-glucosidase activity. The T.reesei Qm 9414 has been widely used in process development research (22,37,39,54,55,120). The mutants of T.reesei (cellulase producing) from Natick and Rutgers have been compared for growth, enzyme production and properties of the cellulase enzyme (5,16).

Currently cellulases from Trichoderma viride are preferred for use in process development research for its better efficiency in hydrolysing cellulose and greater stability. The Trichoderma viride cellulases also suffer from several shortcomings such as (i) its inability to digest or degrade polyphenolic component lignin present in the native cellulose hence it acts as a barrier for T.viride cellulases to attack freely on cellulose, and (ii) cellulase complex is lacking cellobiases which is essential for the degradation of cellobiose, which otherwise acts as an inhibitor of cellulase.

In summary, the literature review has revealed the following :

1. Lignocellulosic waste material is a renewable energy resource which with the help of proper technology/ biotechnology could be used as a feed stock for the production of fermentable sugars.

2. The process development based on following considerations is essential.

To break the ternary lignin-hemicellulose-cellulose complex and to damage as much as possible the crystalline structure of the cellulose fibre by the pretreatment. High pressure steaming seems to be the obvious method of choice because it renders the hemicellulose water soluble and makes extraction of lignin by dilute alkali simple and easy.

3. The cellulose fibre after pretreatment can be easily digested with extracellular cellulase complex produced by strains of Trichoderma viride.

4. The high specific activity cellulases and β-glucosidases can be obtained by proper selection of Trichoderma strains and experimental conditions.

5. To prevent inhibition of cellulase by cellobiose, two methods can be utilized one, a two stages bioreacter can be designed to achieve efficient digestion of pretreated lignocellulosic waste material. In the

first stage the digestion of the cellulosic material can be done with cellulase and the reaction product containing cellobiose then passed through the 2nd stage reactor containing immobilized β-glucosidase.Secondly, the cellulase can be supplemented with β-glucosidase which would degrade cellobiose.

6. Cellulase production on large scale must be achieved.

7. Large scale production of β-glucosidase is essential for supplementing the cellulase from fungal strains.

8. Finally the hydrolysate must be utilized as a substrate for the production of single cell protein using yeast cells and citric acid using Aspergillus niger as fermenting microorganism.

The present study was, therefore, undertaken to obtain sufficient data on some of the above mentioned aspects using saw dust as the cheap lignocellulosic materials.

CHAPTER - III

3.0 Materials And Methods

3.1 Materials

Saw dusts of Mangifera indica, Eucalyptus and Cedrus deodar were obtained from local market and washed with water to remove soluble impurities. Pure cellulose powder was purchased from CSIR centre for Biochemicals, V.P.Chest Institute, Delhi, India. Glucose, sucrose, lactose and soluble starch were purchased from Glaxo laboratories, India. Peptone (bacteriological and mycological grade) was purchased from Difco laboratories, Tween-80, (Poly oxyethylene (20 sorbitan monooleate), was obtained from Loba chemicals, India. Carboxy methyl cellulose, p-nitro-phenyl-β-D-glucopyranoside (pNPG), cellobiose and enzymes oxidase and peroxidase were purchased from Sigma Chemical Company, U.S.A. Whatmann No.1 filter paper used as substrate was purchased from standard company.

All other chemicals were of reagent grade and purchased from various commercial sources.

3.1.1 Microorganisms For Cellulase And β-Glucosidase Production

Tricoderma viride 1060, Trichoderma viride 1051 and Aspergillus niger 683 were obtained from National Chemical Laboratory, Pune, India and Aspergillus wentii Pt 2804 was a

generous gift from Prof. S. N. Mukhopadhyay, Biochemical Engineering Research Centre, Indian Institute of Technology, Delhi, India. The microorganisms were maintained on PDA slants (2% Dextrose + 2.5% Agar in Potato starch extract) stored at 4°C in a cold room. The microorganisms were subcultured after one month to maintain its potency and metabolic activities for cellulase and β-glucosidase production.

3.2 Methods

3.2.1 Pretreatment Of Lignocellulosic Material

Saw dust from Mangifera indica, Eucalyptus and Cedrus deodar were screened through sieves of different pore sizes to obtain particles of uniform size viz. 40, 60, 80 and 100 mesh. The saw dust was washed repeatedly with water to remove water soluble impurities like dust particles. The heavy insoluble impurities which settle down at the bottom of the container werealso removed and rejected. The floating cake was removed, rewashed with water and dried at 80 - 90°C overnight. The saw dust with particle size in the range 20 mesh or less were reduced by ball milling for 12 to 14 h and screened through the nylon nets of 150, 200, 250 and 300 mesh sizes.

3.2.1.1 High Pressure Steaming And Alkali Pretreatment

The saw dust of known mesh size was suspended in water as to obtained 10 - 15% suspension and subjected tohigh pressure steaming at 350 psi (21 atmospheric pressure) for varying time intervals from 30 to 60 min. to break the ternary complex of hemicellulose, lignin and cellulose.

During this treatment autohydrolysis occurs as a result of formation of acetic acid on account of hydrolysis of acetylated (128) sugars. The hemicellulose component of the ternary complex of natural cellulose is rendered water soluble. This was removed by pressure filteration. The residue was washed twice with equal volume of water and dried at 110^{o}C in an hot air oven until a constant weight was obtained. The difference between the weights of the starting materials and the dried residue after washing gave the measure of amount of hemicellulose component in the cellulose complex. The hemicellulose free residue was then subjected to aqueous alkali treatment. Approximately 100 gm residue was treated with 1 litre of 2% sodium hydroxide solution for overnight with constant shaking at room temperature. The insoluble material was removed by filteration under vaccum, washed with water until free from alkali, dried and weighed. The weight of the dried mass gave the amount of pure cellulose in the lignocellulosic materials. The alkaline filterate was adjusted to pH 7 to precipitate lignin. The dark brown precipitate of lignin was washed with water several times to remove alkali. The lignin was dried under vaccum and its weight was determined. Average composition of hemicellulose, lignin and cellulose present in the lignocellulosic material was determined on the basis of atleast three separate determinations.

3.2.2 Estimation Of Total Cellulose Contents

Total cellulose content was measured by Updegraph method (163). To 25 mg of dried sample containing cellulose, 2.5 ml of 72% H_2SO_4 was added. The mixture was mixed on vortex mixture for about 40 to 60 min. by heating gently for complete solubilisation. The volume of the above sample was made upto 50 ml by adding distilled water and filtered to remove the residual matter. 0.02 ml of the filterate was made to 1 ml by adding distilled water and tubes placed in ice bath. 4 ml of ice cold 2% anthrone reagent (prepared in 98% AR grade sulphuric acid) was added slowly and mixed well on a vortex mixer. The tubes with marble on the top were incubated in a boiling water bath for 10 min. and cooled under running tap water. The absorbance of the dark green colour was measured at 620 nm against the blank, prepared without sample. Amount of total cellulose contents were computed from the standard curve of cellulose prepared under similar conditions.

3.2.3 Estimation Of Lignin Contents

Total lignin content was measured by acetyl bromide method of Morrison (119). To 2 mg sample 5 ml of 25% acetyl bromide (prepared in glacial acetic acid) was added in stoppered test tubes. The mixture was incubated at 70°C for 30 min, cooled under running tap water and transferred to 250 ml volumetric flask containing 4.5 ml of 2N NaOH solution (prepared in 25 ml of acetic acid). The volume of the above solution was made up to 200 ml approximately and 8 ml of 0.5M

hydroxylamine hydrochloride was added. The volume was made upto 250 ml by adding acetic acid. The contents were shaken well and allowed to stand for one hour for settling down any undissolved matter. The absorbance was measured at 200nm against the blank prepared without sample. Amount of lignin content was determined from the standard curve of lignin, prepared under identical conditions.

3.2.4 X-Ray Crystallography of Lignocellulosic Samples

3.2.4.1 X-Ray Spectroscopy

X-ray spectroscopy is a non destructive technique used to determine the elements present in a particular substrate and to confirm the nature of material (crystalline or amorphous). A beam of X-ray is directed on to sample of the substance causing secondary or fluorescent X-ray to be emitted. The secondary radiations have lengths that are characteristics of each element present. High energy electrons colloide with the anode material and a small fraction of their energy is converted into primary X-ray radiations.

3.2.4.2 Determination Of Crystallinity ByX-Ray Crystallography

Pretreated and untreated lignocellulosic material was vaccum dried in a dessicator to remove water completely from the sample. The crystallinity was measured by the method of X-ray diffraction (122) using a Phillips X-ray diffractometer. The sample was mounted horizontally while

the Geiger Counter moved in vertical arc. A Cu target with a Nickel filter was used. The samples were scanned in the range of 0 to 40^{o} and 0 to 60^{o} . The method of Segal et al.(122) was used for calculating the crystallinity index using the following expression.

$$Cr\ I = \frac{(I_{002} - I_{am}) \times 100}{I_{002}}$$

Where I_{002} is the intensity of peak (002) at about 2 (2 equals to 22^{o} which corresponds to crystalline fraction; I_{am} is the intensity of the peak at 2θ (2θ equals to 18^{o}) Corresponding to amorphous fraction and CrI is crystallinity index.

3.2.5 Production Of Cellulase And β-Glucosidase Enzymes

3.2.5.1 Culture Medium For Cellulase And β-Glucosidase Producing Strains Of Trichoderma And Aspergillus

The culture medium of the following composition was used for growing the cultures of Trichoderma and Aspergillus strains for production of cellulase and β-glucosidase, respectively.

One litre medium contained the following :

Cellulose	10 gm	1 % (w/v)
$(NH_4)_2SO_4$	1.4 gm	0.14 % (w/v)
Urea	0.3 gm	0.03 % (w/v)
$MgSO_4.7H_2O$	0.3 gm	0.03 % (w/v)
KH_2PO_4	2.0 gm	0.20 % (w/v)
Peptone	1.0 gm	0.10 % (w/v)

$CaCl_2$	0.3 gm	0.03 % (w/v)
*Trace metal solution	1.0 ml	0.01 % (w/v)
Tween-80	2.0 ml	0.02 % (w/v)
pH		4.8

* Trace metal solution

* Trace Metal Solution

2.50 gm, $FeSO_4$; 1.76 gm, $ZnSO_4$; 0.98 gm, $MnSO_4$; and 1.0 gm, Cobalt chloride were dissolved in 495 ml of distilled water followed by addition of 5 ml conc. HCl to the aqueous solution.

3.2.5.2 Preparation of Inoculum

For inoculum preparation, the culture on the potato Dextrose Agar (PDA) slant was first activated by incubating at 30°C for 72 h The activated spores were removed with the help of a platinum loop and suspended in 5 ml of sterile distilled water. The medium was prepared as described above but with 1% glucose as a carbon source instead of cellulose and peptone. The pH of medium was adjusted to 4.8 and sterilized in an autoclave at 15 psi for 20 min. 2 ml of spore suspension (2×10^5 to 2×10^6 spores) was added to sterile 50 ml of sterile medium and incubated at 30°C for 24 h with continuous shaking in a rotatory shaker at 150 rpm.

3.2.5.3 Cellulase And β-Glucosidase Production In Shake Flasks And Media Optimisation Studies.

Selection of suitable cultures was done on the basis of yields of enzymes cellulase and β-glucosidase. The cultures which were selected were Trichoderma viride 1060 and

Aspergillus wentii Pt. 2804 for cellulase and β-glucosidase production.The effect of cellulose concentration on cellulase production (Fig.7) and pH was determined during the fermentation period (Fig.8). As pH below 3 and 5 is not ideal for enzymes production, so for the pH control, growth medium was buffered with 0.1M sod.citrate buffer [(pH 5.0),(Fig. 9 & 10)]. The untreated and pretreated Mangifera indica saw dust were used as a carbon source for cellulase and β-glucosidase production (Table V).Addition to this starch, sucrose and lactose were employed as a carbon source in the growth medium. The results which were obtained from theabove mentioned studies, were used in the further studies. Effect of different levels of Mg^{+} (Table VII)and surfactant Tween-80was observed. The effect of type, age and level of inoculum were observed and the optimal conditions are described in Table IX.

3.2.5.4 Production Of Cellulase And β-Glucosidase In 9 litre Fermenter.

Operating volumes upto 5 litre were used in Bioengineering fermenter. Temperature of the cultivation vessel was maintained at $30^{o}C$ and pH was controlled automatically by the addition of 1N NaOH/HCl. Aeration rate 1 vvm and agitation 250 rpm was maintained. The impeller speed broke down the mycelium to very small aggregates generating homogeneous culture solution. The fermenter was started up as a batch process using 2% cellulose for cellulase production whereas 4% starch was used as carbon source for β-glucosidase production. The 5% and 7% inoculum

for cellulase and β-glucosidase production respectively in a fermenter was prepared from cells grown in shake flasks in Mandel's basal medium, containing 1% glucose as carbon source and other nutrients but devoid of bactopeptone and tween-80, for 48 h. The pH was allowed to have a natural fall from 4.8 to 3.0, it was then controlled not to go below this value. The fermentation was studied up to the indicated period in results. These conditions were maintained in the results, reported in this section unless mentioned otherwise. The values obtained represent data from two runs.

3.2.5.5 Enzyme Assays

3.2.5.5.1Cellulase Activity

Cellulase activity was assayed using Whatmann No.1 filter paper and/or carboxymethyl cellulose (CMC) by the method of Mandels et al. (102). The standard reaction mixture unless stated otherwise contained the following in a total volume of 2 ml; 50 mg filter paper (1x6 cm strip), 50 mM sodium citrate buffer, pH 4.8 and 0.5 ml of enzyme (0.5 mg protein). The reaction mixture was thoroughly mixed and incubated at 50°C for 60 min. The enzyme activity was terminated by adding 3 ml of Dinitrosalicylic acid reagent and the mixture was heated in a boiling water bath for 5 min. The reaction mixture was cooled under running tap water and centrifuged at 3000 g for 15 min. to remove the insoluble material. The clear supernatant was separated and the absorbance was measured at 600 nm against the enzyme and substrate blank prepared concurrently in the same manner. The amount of reducing sugar was determined by refering to a

glucose standard curve prepared under identical experimental conditions. The cellulase activity was expressed as International Units (IU). One international unit is equal to the number of micro moles of reducing sugar as glucose released per min. per ml of enzyme.

3.2.5.5.2 β-Glucosidase Activity

β-glucosidase activity was assayed using both p-nitro-phenyl-β-D-glucopyranoside and cellobiose as substrate described by Bailey et al.(8). When p-nitro-phenyl-β-D-glucopyranoside (pNPG) was used as substrate the following reaction mixture was used : 0.9 mM susbstrate, 50 mM citrate buffer, pH 4.8, and 0.1 ml enzyme (0.1 mg) in a total volume of 1 ml. The incubation was carried out at 50°C for 10 min. and the reaction was stopped by adding 1.5 ml of 4% Na_2CO_3 solution. The absorbance of yellow colour of p-nitrophenol released during the reaction was measured at 400 nm. The control was prepared simultaneously using the reaction mixture without enzyme. The β-glucosidase activity was expressed as International Units (IU) per ml i.e. number of micromoles of p-nitrophenol released per min per ml of enzyme.

When cellobiose was used, 1 ml reaction mixture contained the following : 50 mM citrate buffer pH 4.8, 5 mM cellobiose solution and 0.1 mg enzyme (0.5 ml). Incubation was carried out at 50°C for 30 min. and the reaction was stopped by placing the reaction mixture in a boiling water bath for 10 min. The mixture was cooled under running tap

water and 1 ml of Nelson reagent (123) was added and the absorbance was read at 540 nm. The glucose standard was also prepared using same method.

The activity of β-glucosidase against cellobiose was also estimated by the method of Bergmeyer and Bernt (13a). The reaction mixture (1 ml) consisted of an aliquot of the enzyme in 0.05M citrate buffer at pH 4.8 and 0.5 ml of 2 mM cellobiose solution. The reaction was carried out at 50°C for 30 min. and stopped by boiling the tubes in a bath for 10 min. Glucose released was estimated by the glucose- oxidase (GOD - POD) method of Bergmeyer and Bernt (13a). One unit of β-glucosidase activity is the amount of enzymes which liberate one μ mole glucose per min. at 50°C.

3.2.5.6 Protein Estimation

Protein was estimated by the method of Lowry et al. 94) using BSA (10 - 100 ug) as standard. The colour was developed with Folin and Ciocalteu reagent (1:2 dilution v/v) and the colour density was measured at 690 nm. The amount of protein in the enzyme samples was computed by referring to standard protein curve prepared concurrently.

3.2.5.7 Estimation Of Total Reducing Sugars As Glucose

Total reducing sugars were measured by Dinitrosalicylic acid method of Miller (113). To 1 ml of sample containing 0.2 to 2.0 mg of reducing sugar (glucose), 1 ml of citrate buffer (0.05 pH 4.8) and 3 ml of DNS reagent were added, followed by quick stirring on vortex mixer. The

mixture was incubated for 5 min. in the boiling water bath, cooled under running tap water and diluted to 10 ml by adding distilled water. The absorbance of the dark yellow colours was measured at 600 nm against the blank prepared without sample.Amount of reducing sugar as glucose was computed from the standard curve of glucose, prepared under identical conditions.

3.2.6 Enzymatic Hydrolysis Of Cellulose

The enzymatic hydrolysis of cellulose was performed in 250 ml Erlenmeyer flask. 2.5 gm, 5.0gm and 10.0gm cellulose material were suspended separately in 100 ml of 50 mM citrate buffer, pH 4.8 containing 100 units of partially purified cellulase enzyme. The suspension was incubated for varying intervals of time from 0 to 48 h at 50^{o}C with constant shaking at the rate of 150 rpm. In order to prevent bacterial contamination 0.01%(v/v) toluene was added. At indicated time intervals samples were drawn and immersed in a boiling water bath for few min. to stop the enzyme action. The residual material was removed by centrifugation for 10 min. at 5,000 rpm. The supernatant was decanted off and reducing sugar was expressed as glucose. Whenever more amount was to be hydrolysed, multiple incubations were carried out or procedure was scaled upto 5 litre. Effect of different concentration of cellulose (Fig.20), supplementation of β-glucosidase to cellulase (Table X), effect of various pH (Fig. 23) and the effect of different temperature (Table XI) on hydrolysis of Mangifera indica saw dust were studied.

3.2.7 Cultivation Of Yeast On Enzymatic Hydrolyzate Of *Mangifera indica* Saw Dust.

The hydrolysate was added to Carbon free Czapex Dox medium in 250 ml Erlenmeyer flask, where the sugar concentration was adjusted to 2%. It was supplemented with 0.1% peptone. The medium was sterilised at 121°C and 15 psi for 20 min.and inoculated with yeast cells (*Saccharomyces cerevesiae*) for production of biomass. 10 ml yeast (O.D. 578 nm) suspension was added to 90 ml of the above medium in each flask and incubated at 30°C from 12 to 48 h on a rotatory shaker at 150 rpm. At the end of incubation the cell mass was separated by centrifugation, washed with water and dried at 80°C for 10-12 h or by freeze drying .

3.2.8 Production Of Citric Acid

3.2.8.1 Culture Conditions

The *Aspergillus niger* microorganism was isolated from biogas digester. In order to produce fresh spores, the isolated culture was allowed to grow on potato dextrose agar slants, at 30°C for 5 days. The spores were aseptically harvested with 100 ml of sterile distilled water. 2 ml of well dispersed spore suspension containing about $2x10^5$ to $2x10^6$ spores was used as inoculum. Inoculation was done for 50 ml of fermentation medium contained in sterile 250 ml Erlenmeyer flasks. Incubation was carried out at 30°C for 14 days.

3.2.8.2 Fermentation Medium For Citric Acid Production

The medium used for the growth of A.niger and citric acid production was composed of enzymatic hydrolysate of Mangifera indica saw dust. The concentration of glucose (present in enzymatic hydrolysate) varied from 4 to 14% (wt/v). The fermentation medium also contained NH_4NO_3 (0.223%), KH_2PO_4(0.1%) and $MgSO_4.7H_2O$(0.023%). The initial pH of the medium was set to 2.2 with 1N HCl. 10% concentration of glucose in the fermentation medium was found to be optimum for the maximum yield of citric acid. Further studies for citric acid production were carried out, using 10%.

3.2.8.3 Estimation Of Citric Acid

Total citric acid was measured by Marier and Boulet method (107). 0.1 ml aliquot from the fermented sample was taken and volume was made to 1.0 ml with distilled water. This was followed by the addition of 1 ml pyridine and 5 ml acetic anhydride. The mixture was incubated at 30°C for 30 min. and their optical density was measured at 420 nm against the blank prepared without sample. The amount of citric acid formed was determined with a standard curve prepared under identical conditions with commercially available citric acid.

3.2.8.4 Estimation OfResidual Sugar

Residual sugar contents were determined colorimetrically using the phenol-sulphuric acid method of

Dubois et al.(35). Required amount of sample aliquot wastaken and its volume was made up to 1 ml with distilled water, followed by the addition of 1 ml of 5% Phenol solution and 5 ml of concentrated sulphuric acid. The mixture was further, incubated at 30°C for 30 min and their optical density measured spectrophotometrically at 490 nm wavelength. Carbohydrate as glucose contents were computed from a glucose standard curve prepared in the same way.

3.2.8.5 Estimation Of Cell Mass

The cells were separated from the fermentation medium by filteration. The separated cells were then blotted, dried at 110°C overnight in an electric oven, cooled and weighed.

CHAPTER - IV

4.0 RESULTS

4.1 Pretreatment Of Lignocellulosic Material

Enzymatic hydrolysis is a promising means of converting waste celluose that can be used as a cheap and easily available substrate for production of fermentable sugars for power alcohol production. However, enzymatic hydrolysis of cellulose is restricted by crystalline structure of cellulose microfibrils that are aggregated and embedded within the lignified cell wall matrix. In order to increase enzymatic susceptibility, the cellulosic material was subjected to various pretreatments to expose and to separate elementary cellulose microfibrils.

4.1.1. High Pressure Steaming and Alkali Pretreatment

Table 1 shows the percent composition of cellulose, hemicellulose and lignin in the saw dusts of Mangifera indica, Cedrus deodar and Eucalyptus, obtained after high pressure steaming at 21 atmospheric pressure (350 ± 15 psi) for 30 min and alkali pretreatment. The data show that the contents of cellulose, hemicellulose and lignin are comparable in all three sources. Mangifera indica which is available in large abundance in India, was used for further studies. Table II shows the effect of high pressure steaming on release of hemicellulose as water soluble component and extraction of lignin by alkali from the saw dust as a function of time of steaming. It was found that 30 min

Fig. 6 X-ray diffractograph of *Mangifera indica* saw dust (60 - 80 mesh).

1. Untreated.
2. After high pressure steaming (350 ± 15 psi).
3. After alkali treatment.

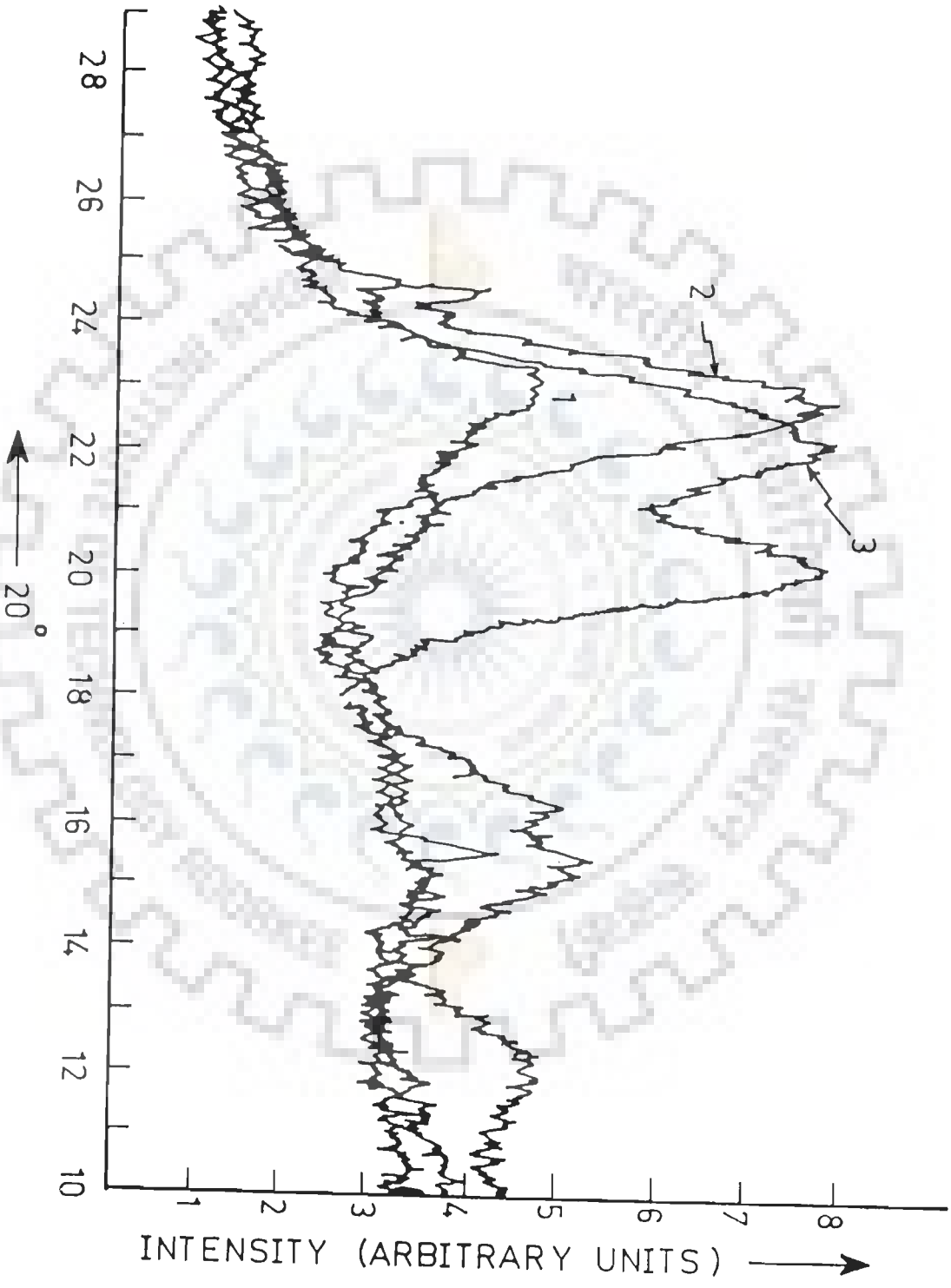

28
26
24
22
20
18
16
14
12
10
20°
1
2
3
1
2
3
4
5
6
7
8
INTENSITY (ARBITRARY UNITS)

TABLE I : SEPARATION OF HEMICELLULOSE, LIGNIN AND CELLULOSE FROM SAW DUST OF EUCALYPTUS, MANGIFERA INDICA AND CEDRUS DEODAR.

Saw dust from different sources was subjected to high pressure steaming to separate hemicellulose as water soluble component. After removal of hemicellulose, the residue was extracted with NaOH to separate lignin from the cellulose. The residue after separation of hemicellulose and lignin reperesented as cellulose. Results are average of at least three separate experiments and expressed on dry weight basis.

Saw dust Source	Hemicellulose (%)	Lignin (%)	cellulose (%)
Eucalyptus	22.7 ± 3	20.0 ± 3	52.6 ± 4
Mangifera indica	20.0 ± 3	21.0 ± 25	56.0 ± 5
Cedrus deodar	21.5 ± 3	22.5 ± 3	54.0 ± 4.5

TABLE II : EFFECT OF HIGH PRESSURE STEAMING ON THE SEPARATION OF HEMICELLULOSE AND LIGNIN FROM MANGIFERA INDICA SAW DUST (60 - 80 MESH)

Mangifera indica saw dust was subjected to high pressure (350 ± 15 psi) steaming for the indicated periods. The water soluble component represented hemicellulose, NaOH soluble fraction after water extraction, and the residue after NaOH extraction were lignin and cellulose components, respectively. Results are average of three separate experiments.

Period for autoclaving (min.)	Hemicellulose (%)	Lignin (%)	Cellulose fiber (%)
00 (Control)	0.0	20.0 - 25.0	75.0 - 80.0
30	19.0 - 21.0	20.0 - 22.5	52.6 - 55.6
45	20.0 - 21.2	18.0 - 20.0	56.8 - 58.0
60	18.0 - 21.0	18.6 - 21.0	55.0 - 57.0

treatment at 350 ± 15 psi was sufficient to break the ternary complex of hemicellulose, lignin and cellulose in the native lignocellulosic material. Mangifera indica saw dust contained on an average 18 - 21% hemicellulose and equal amount of lignin. The cellulose fiber constituted 53 - 58% of the total dry weight of saw dust. Estimation of cellulose by Updegraph method (163) also yielded similar results.

Figure 6 shows the X-ray diffractogram of the Mangifera indica saw dust before and after pretreatments. Peak at $2\theta = 23^{o}$ shows the degree of crystallinity in the untreated lignocellulosic material. A significant increase in the size of this peak was noticed in the case of saw dust which was pretreated with high pressure steaming indicating that the crystalline structure of the cellulose was also damaged which would enhance the digestibility of the cellulose fibre. Removal of lignin by alkali treatment of the high pressure steam treated saw dust showed an additional peak of almost equal intensity at $2\theta = 20^{o}$ which may be due to exposure of new amorphous regions in the cellulose fibre obtained from saw dust. As such alkali treatment would further enhance the digestibility of cellulose fibre.

Table III shows the effect of particle size of the lignocellulosic on the saccharification of saw dust by cellulase enzyme. It was found that the particle size has a profound effect on the enzymatic digestibility of the untreated saw dust. For instance, compared to 40 mesh saw dust, the digestibility of the 300 mesh size saw dust by

TABLE III : EFFECT OF PARTICLE SIZE AND PRETREATMENT ON THE ENZYMATIC HYDROLYSIS OF *MANGIFERA INDICA* SAW DUST.

Saw dust of *Mangifera indica* was subjected to ball milling to obtain saw dust particles of various sizes. Both untreated and pretreated saw dust samples were digested with a mixture of *Trichoderma viride* cellulase (1.0 iu/ml) and *Aspergillus wentii* β-glucosidase (1.2 iu/ml) as described in methods.

Treatment	Saccharification, %			
	Particle size, mesh			
	40	100	200	300
None (Control)	16.8	26.5	29.1	33.5
High pressure steaming	36.2	–	–	–
High pressure steaming and alkali extraction	80.0	–	–	87.6

cellulase was doubled. However, if the dust of 40 mesh size was pretreated with high pressure steaming followed by NaOH extraction, the enzymatic hydrolysis of cellulosic material was nearly comparable with that of 300 mesh. Thus particle size of saw dust above 40 mesh (normal size of saw dust available commercially as by product) was unnecessary, since pretreatment, high pressure steaming and NaOH extraction to remove hemicellulose and lignin is definitely required for the efficient enzymatic saccharification of saw dust. Elimination of ball milling also reduced the cost of process. Hence in most experiments saw dust of 40 - 80 mesh was used as substrate in saccharification studies.

4.2. Production Of Cellulase And β-glucosidase

4.2.1 Selection of Suitable Cultures for Large Scale Production of Cellulase and β-glucosidase.

Four fungi Viz. Trichoderma viride 1060, Trichoderma viride 1051, Aspergillus niger 683 and Aspergillus wentii Pt. 2804 were screened for large scale cellulase and β-glucosidase production. The cellulase and β-glucosidase were produced under submerged fermentation conditions using Mandel's basal medium supplemented with one percent cellulose. The results are presented in Table IV. The extracellular enzymes were assayed using filter paper, Carboxy methyl cellulose, cellobiose and pNPG. These results showed that the enzymes secreted by Aspergillus wentii had the highest activity against cellobiose and p-nitrophenyl β-D-pyranoside (pNPG) followed by Aspergillus niger 683.

TABLE IV : PRODUCTION OF EXTRACELLULAR CELLULASE DEGRADING ENZYMES BY DIFFERENT STRAINS OF *TRICHODERMA VIRIDE* AND *ASPERGILLUS* FUNGI.

Cultures were grown in Mandels basal medium, supplemented with 1% cellulose, under submerged fermentation conditions. After indicated incubation periods, cells were harvested by centrifugation and the supernatant was brought to 100% saturation with $(NH_4)_2SO_4$. The protein precipitate was dissolved in reaction buffer, dialysed overnight at 4^oC against 100 volumes of the same buffer, and the enzyme activity was assayed using fliter paper (FP), Carboxy methyl cellulose (CMC), p-nitrophenyl β-pyranoside (pNPG), and cellobiose (cb) as substrates as described under methods. Enzyme activity was expressed as iu/ml of original medium.

Organism	Incubation period (days)	Enzyme activity(iu/ml) and substrate used FP	CMC	pNPG	Cb
Trichoderma viride 1060	4	0.16	0.24	0.09	0.03
	6	0.52	0.61	0.29	0.10
	9	0.61	0.99	0.38	0.13
	13	0.87	1.07	0.51	0.39
Trichoderma viride 1051	4	0.11	0.15	0.06	0.02
	6	0.47	0.59	0.25	0.04
	9	0.58	0.89	0.34	0.09
	13	0.81	1.02	0.49	0.32
Aspergillus wentii Pt.2804	4	0.00	0.14	0.37	0.56
	6	0.01	0.36	0.86	1.68
	9	0.03	0.57	1.20	2.92
	13	0.04	0.63	2.55	6.23
Aspergillus niger 683	4	0.00	0.07	0.26	0.26
	6	0.00	0.26	0.81	1.43
	9	0.02	0.43	1.04	2.23
	13	0.08	0.54	2.08	5.67

Cellulase enzyme secreted by Trichoderma viride 1060 showed highest activity against Whatmann No.1 filter paper and carboxymethyl cellulose followed by Trichoderma viride 1051. The enzyme from Trichoderma viride 1060 and Trichoderma viride 1051 was much less active against p-nitrophenyl-β-D-pyranoside and cellobiose indicating that the strains of Trichoderma viride are a poor source of β-glucosidase enzyme. The low activity of enzyme from Aspergillus wentii against filter paper as compared to Trichoderma viride 1060 indicated that the enzyme obtained from Aspergillus wentii source contained less amount of endo- and exo-glucanases involved in cellulose degradation.

The relative concentration of both cellulase and β-glucosidase increased with incubation period (Table IV). It was observed that Trichoderma and Aspergillus produced maximum amount of enzyme in 13 days at 30°C. Hence for the large scale production of cellulase as well as β-glucosidase, 13 days incubation at 30°C were carried out using Trichoderma viride 1060 and Aspergillus wentii Pt 2804.

4.2.2 Effect of Cellulose Concentration On The Production of Cellulase Enzyme.

The results of cellulase production at different cellulose levels in the growth medium are shown in Fig.7. The results show that the enzyme activity increased with the increase in concentration of cellulose from 0.5% (w/v) to

Fig. 7 Effect of Avicel microcrystalline cellulose concentration on the production of cellulase by Trichoderma viride grown in shake flasks for 13 days. (o-----o) Filter paper as substrate. (●——●) CMC as substrate.

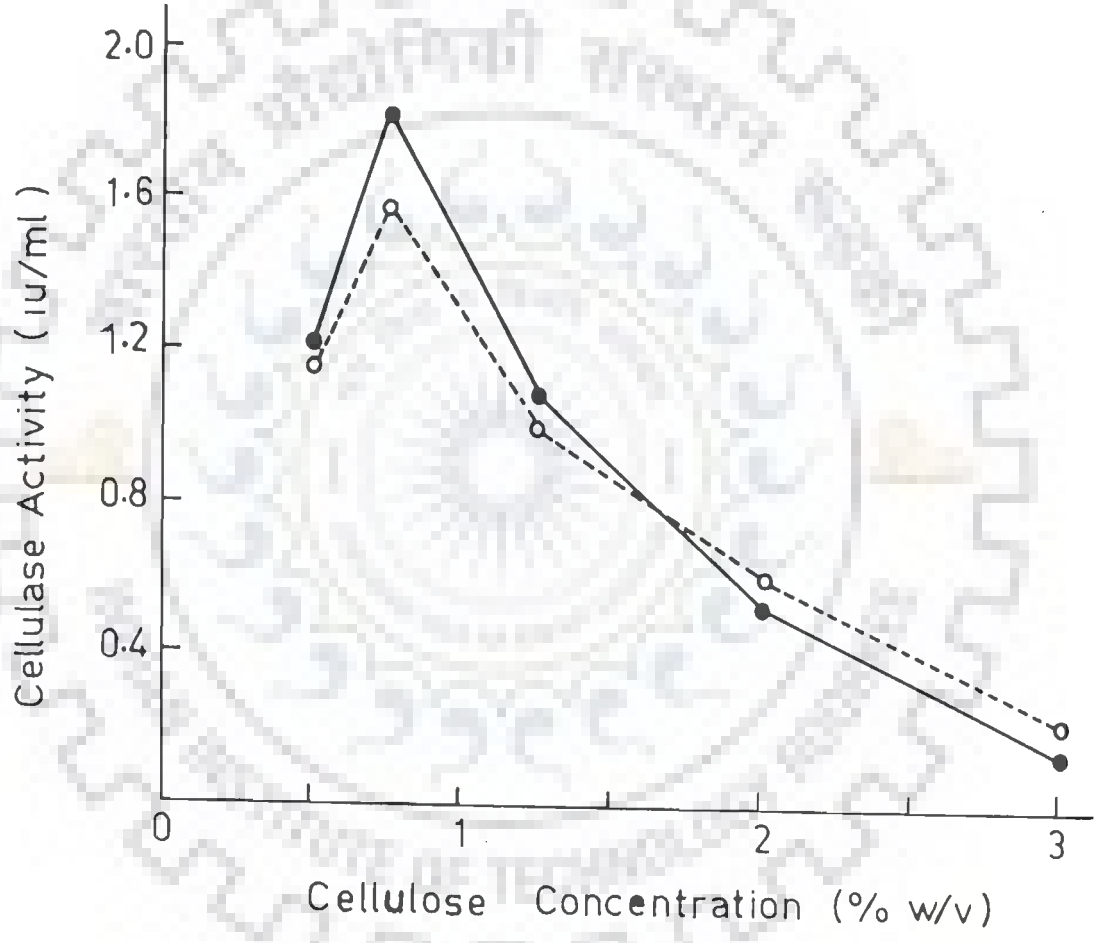

2·0
1·6
1·2
0·8
0·4
0
1
2
3
Cellulase Activity (iu/ml)
Cellulose Concentration (% w/v)

Fig. 8 Change in pH of medium during incubation of Trichoderma viride in the presence of 2 percent Avicel microcrystalline cellulose as substrate.

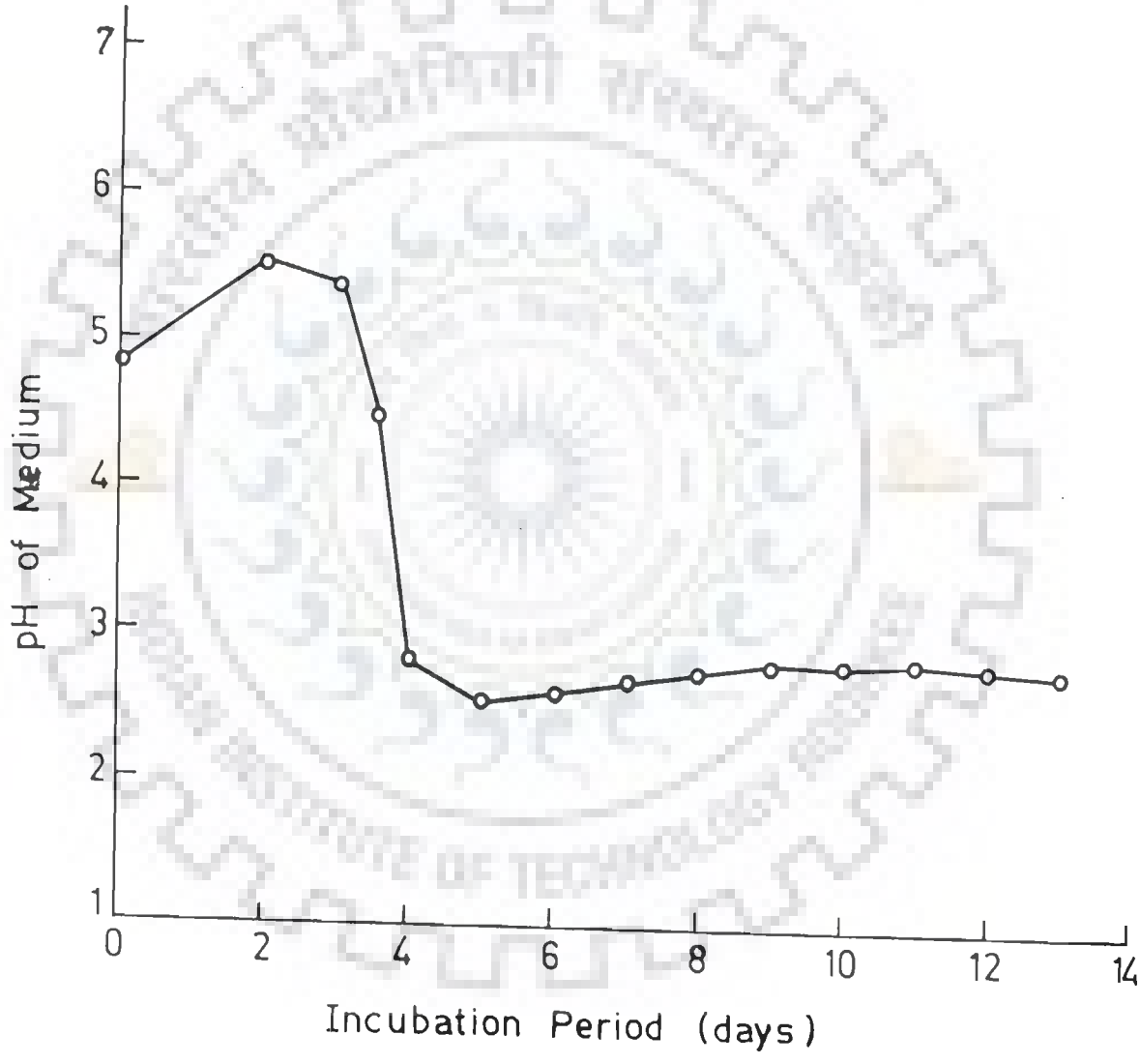

pH of Medium
7
6
5
4
3
2
1
0
2
4
6
8
10
12
14
Incubation Period (days)

Fig. 9 Production of extracellular cellulase in shake flask cultures of Trichoderma viride in the presence of 2% Avicel microcrystalline cellulose without (●--------●) and with (O——O) pH control. Enzyme activity as assayed using carboxy methyl cellulose as described under methods. pH of broth during incubation was maintained by the addition of 0.1M sodium citrate buffer pH 5.0.

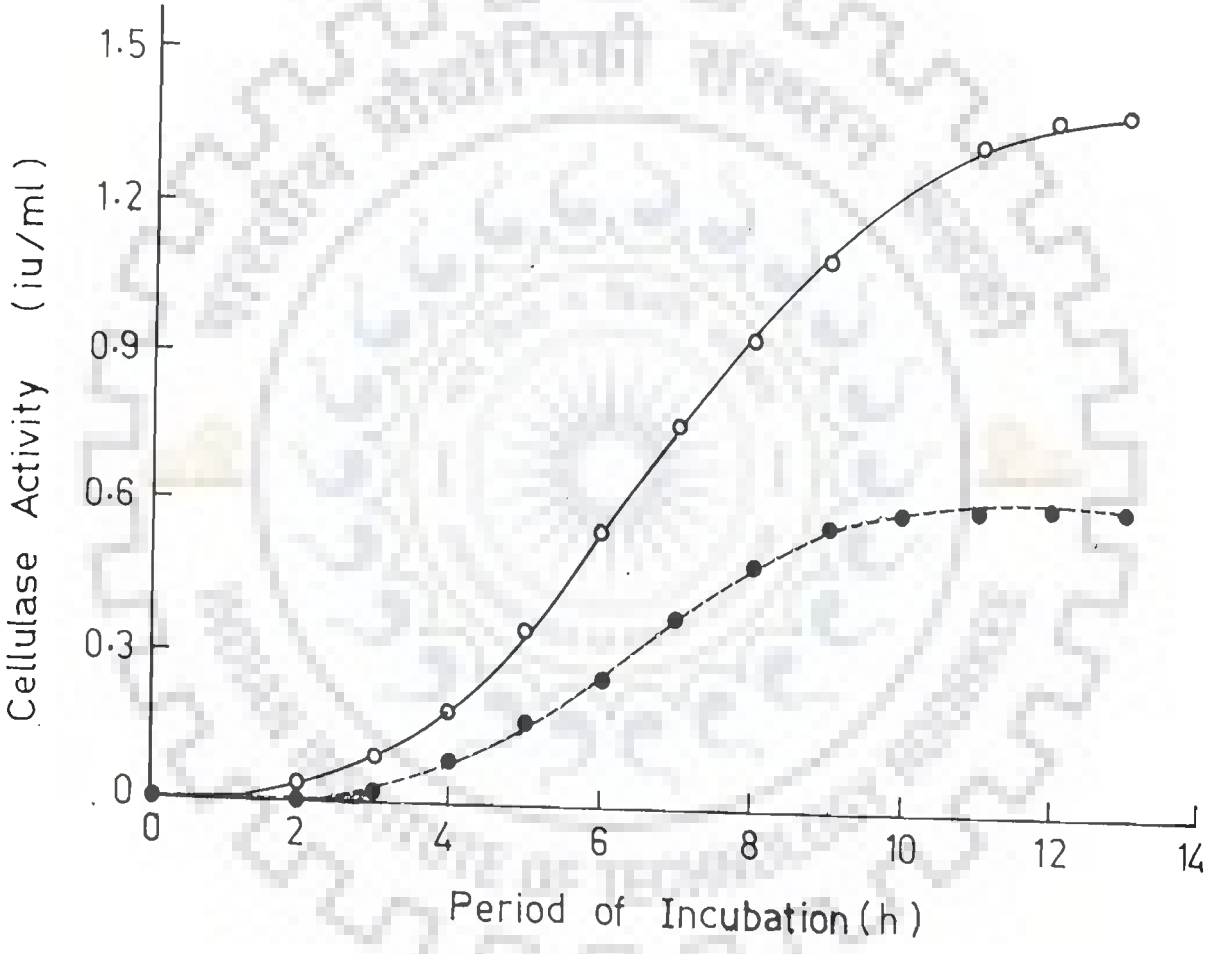
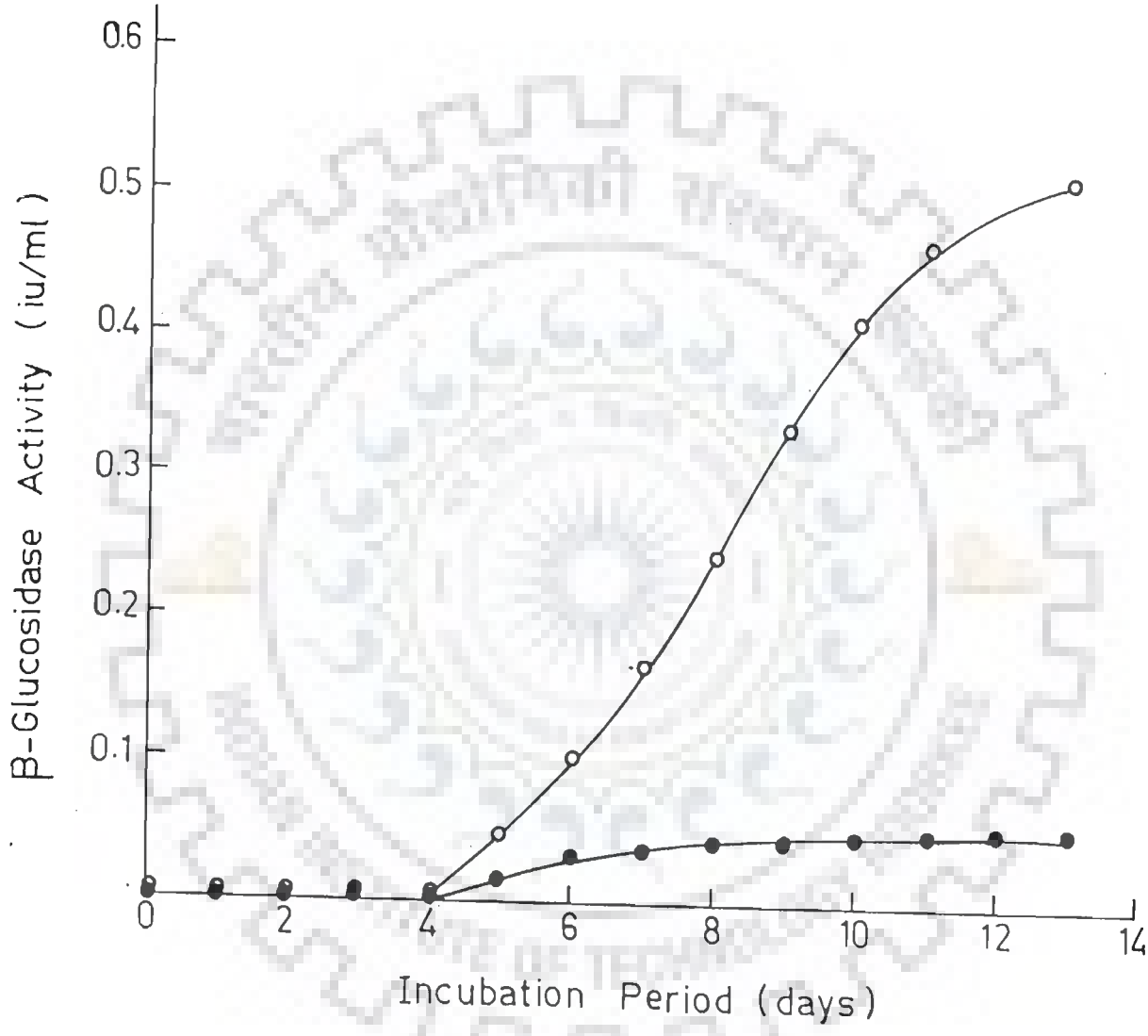

Cellulase Activity (iu/ml)
1.5
1.2
0.9
0.6
0.3
0
0
2
4
6
8
10
12
14
Period of Incubation (h)

Fig. 10 Production of β-glucosidase

Trichoderma viride cells were grown in a standard growth medium containing 2% Avicel (microcrystalline cellulose) with and without pH control. The activity of extracelluiar β-glucosidase was assayed after indicated periods as described under. Methods, (○——○), under controlled pH, (●——●), under uncontrolled pH incubations.


0.6
0.5
0.4
0.3
0.2
0.1
β-Glucosidase Activity (iu/ml)
0
2
4
6
8
10
12
14
Incubation Period (days)

0.75 (w/v). After 0.75 % cellulose concentration the enzyme activity gradually decreased and at 3% (w/v) cellulose concentration the enzyme activity was nearly completely inhibited . During incubation it was noticed that whereas at a cellulose concentration of 0.75% (w/v) or less the pH of the broth remained more or less unchanged, at higher concentrations of cellulose the broth pH decreased from 4.8 to 2.5 (Fig.8) and the production of cellulase enzyme was reduced to less than half compared to the yield obtained when the pH of the broth was maintained around 4.8 $\pm$ 1 with the help of 0.1 M sodium citrate buffer pH 5.0 (Fig.9).

As shown in Fig.10 the drop in production of extracellular β-glucosidase in uncontrolled pH condition of incubation of T.viride cells was even more pronounced. Thus, for optimum production of extracellular cellulase and as well as β-glucosidase it was necessary to maintain broth pH around 4.8 $\pm$ 1 during the entire period of incubation. It may be pointed out here that β-glucosidase catalyzes the hydrolysis of cellobiose produced by the action of cellulase enzyme on cellulose, which otherwise is inhibitory to cellulase action. Hence, conditions that favoured production of both cellulase and exo-β-glucosidase by T.viride were used.

Both treated and untreated saw dust were also used for production of cellulase and β-glucosidase by Trichoderma viride 1060 and Aspergillus wentii, respectively. Results are summarized in Table V. It was found that the lignin free Mangifera indica saw dust was nearly as good a substrate for

TABLE V : PRODUCTION OF EXTRACELLULAR CELLULASE AND β-GLUCOSIDASE USING TRICHODERMA VIRIDE AND ASPERGILLUS WENTII CULTURES GROWN ON MANGIFERA INDICA SAW DUST.

Erlenmeyer flasks (250 ml capacity) containing 100 ml medium and 1% (w/v) Mangifera indica saw dust were inoculated with appropriate amount of inoculum (24h old cells grown in a medium containing glucose) and incubated at 30°C. After the indicated periods, the enzymes activity in the medium was assayed as described in methods.

Saw dust	T.viride cellulase[a], iu/ml				A.wentii β-glucosidase[b], iu/ml			
	Incubation Period days				Incubation Period days			
	4	6	9	13	4	6	9	13
Untreated	0.06	0.19	0.24	0.33	0.08	0.31	0.59	1.26
Pretreated[c]	0.20	0.55	0.81	0.98	0.39	1.31	2.42	5.87

a : Cellulase activity assayed with carboxy methyl cellulose (CMC) as substrate.

b : β-glucosidase activity assayed with cellobiose as substrate.

c : After high pressure steaming followed by dilute alkali extraction.

production of extracellular cellulase and β-glucosidase by T. viride and A. wentii as the Avicel microcrystalline cellulose. Untreated lignin containing saw dust was a poor substrate. Since use of saw dust for producing enzymes on large scale would be more appropriate, Mangifera indica saw dust after high pressure steaming and alkali extraction was used in the incubation broth instead of Avicel.

4.2.3 Effect Of Different Carbon Sources On The Production of Extracellular Cellulase And β-glucosidase.

In an attempt to increase the specific activity of cellulase and β-glucosidase produced by Trichoderma viride and Apergillus wentii, cultures were grown on different carbon sources. The results are summarized in Table VI. It was found that the specific activity of enzymes produced by both T. viride and A. wentii was significantly enhanced when cellulose (delignified Mangifera indica saw dust) mixed with one-fourth amount of glucose was used as the carbon source. Sucrose and lactose were also found to be good carbon source for production of cellulase by Trichoderma viride or β-glucosidase by Aspergillus wentii. Interestingly, production of cellulase by T. viride cells was also enhanced when starch was used as the source of carbon in the growth medium. However, there was no effect on the cellulase production by A. wentii. Since β-glucosidase is an essential component of the cellulase enzyme complex involved in the degradation of cellulose, the relative effect of different carbon sources on β-glucosidase specific activity was an

TABLE VI : EFFECT CARBON SOURCE ON THE PRODUCTION OF EXTRACELLULAR CELLULASE AND β-GLUCOSIDASE BY TRICHODERMA VIRIDE 1060 AND ASPERGILLUS WENTII Pt. 2804.

Conditions of incubation were those as described in Table V. Incubation were carried out after 13 days at 30°C.

Carbon source	Conc. (%)	Extracellular cellulase[a]		Extracellular β-glucosidase[b]	
		T.viride (iu/ml)	A.wentii (iu/ml)	T.viride (iu/ml)	A.wentii (iu/ml)
Saw dust	2	1.37	0.87	0.59	6.23
Saw dust +glucose (3 : 1)	2	1.68	0.96	0.71	9.87
Sucrose	2	1.08	0.62	0.46	8.56
Starch	2	1.59	0.89	0.61	12.89
Lactose	2	0.93	0.57	0.37	4.97

a : Cellulase activity was assayed using CMC as substrate.

b : β-glucosidase activity was assayed using cellobiose as substrate.

c : Mangifera saw dust pretreated with high pressure steaming followed by dilute alkali extraction.

important consideration in the selection of carbon source. From the results given in the Table VI, it is clear that cellulose glucose (3:1 w/v) mixture followed by starch provided the best carbon source for the production of extracellular cellulase and β-glucosidase of Trichoderma viride. However, if cost factors were to be taken into consideration, Mangifera indica saw dust may be a good and easily available carbon source of choice.

Compared to Trichoderma viride, Aspergillus wentii is a poor source of the extracellular cellulase enzyme. In contrast, however, Aspergillus wentii is an excellent source of β-glucosidase, where as T. viride is a poor source of β-glucosidase (Table VI). Thus, Cellulase preparation from Trichoderma viride together with the β-glucosidase preparation from Aspergillus wentii, as will be shown later, provided a potential system for the cellulose degradation. The β-glucosidase production by A. wentii , when grown on starch as carbon source in the growth medium was increased two fold. Therefore, unless stated otherwise Trichoderma viride and Aspergillus wentii were grown on a medium containing saw dust-glucose (3:1) mixture and starch at 2% level as source of carbon.

4.2.4 Effect of Mg^{++} And Tween - 80 On The Production Of Cellulase and β-glucosidase.

Table VII shows the effect of Mg^{++} on the production of extracellular cellulase and β-glucosidase by

TABLE VII : EFFECT OF Mg^{2+} ON CELLULASE PRODUCTION BY *TRICHODERMA VIRIDE* AND β-GLUCOSIDASE BY *ASPERGILLUS WENTII*.

For cellulase production, *T.viride* cultures were grown in a buffered medium, pH 4.8, containing 0.75% cellulose and 0.25% glucose and the indicated amounts of $MgSO_4$. For the production of β-glucosidase, *Aspergillus wentii* cells were grown on 2% starch. After 13 days incubation at 30°C, cells were removed by centrifugation. The supernatant was brought to 100% saturation with $(NH_4)_2SO_4$ and the protein precipitate was separated by centrifugation redissolved in citrate buffer, pH 4.8 and activity of cellulase and β-glucosidase were measured using carboxymethyl cellulose and cellobiose as substrate, respectively.

$MgSO_4$ (%)	*T.viride* cellulase activity iu/ml		*A.wentii* β-glucosidase activity iu/ml	
0.00	0.83	(0.74)[a]	5.2	(2.73)[b]
0.01	1.27	(1.29)	5.93	(3.90)
0.02	1.49	(1.36)	11.32	(4.89)
0.03	1.68	(1.57)	12.93	(4.93)
0.04	1.62	(1.49)	11.12	(3.87)
0.05	1.59	(1.47)	10.83	(3.50)

a : Values in parentheses show the cellulase activity towards filter paper.

b : Value in parentheses show the β-glucosidase activity towards p-nitrophenyl β-glucoside.

Trichoderma viride and Aspergillus wentii , respectively. Addition of Mg^{++} to culture medium increased the production of cellulase by T. viride two fold and approximately 2.5 fold that of β-glucosidase by Aspergillus wentii. In both cases 0.03% Mg^{2+} concentration appears to be optimum.

Addition of surfactant Tween-80 to the culture medium was also found to enhance the yield of both cellulase and β-glucosidase. At optimum concentration of Tween-80 (0.25 to 0.3% v/v) the production of extracellular T. viride cellulase and A. wentii β-glucosidase (cellobiase) was increased 2 and 2.5 fold respectively.

4.2.5 Yield Of Extracellular Cellulase And β-glucosidase As a Function Of The Amount And The Age Of The Cells.

Both amount and age of inoculum are important for optimum production of high specific activity extracellular cellulase and β-glucosidase. Fig.11 and Fig.12 show the effect of inoculum level on the production of cellulase and β-glucosidase, respectively. For the cellulase production by Trichoderma viride, the optimum level of inoculum was 5% and that for β-glucosidase production by Aspergillus wentii was 7% . Further increase in the level of inoculum did not increase the enzyme production. In fact, on the contrary the yield of both enzymes was reduced. Figures 13 and 14 show the effect of age of inoculum on the production of cellulase and β-glucosidase by Trichoderma viride and Aspergillus wentii, respectively. Trichoderma viride that were grown for 24 h

TABLE VIII : EFFECT OF TWEEN-80 ON PRODUCTION OF EXTRACELLULAR CELLULASE BY *TRICHODERMA VIRIDE* AND EXTRACELLULAR β-GLUCOSIDASE BY *ASPERGILLUS WENTII*.

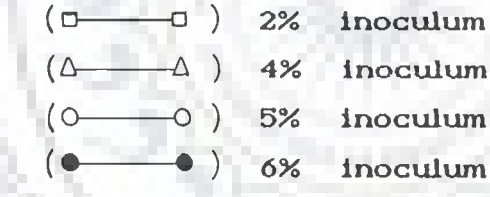
Incubation conditions were same as described in Table VI and VII

Tween-80 (%)	*T.viride* cellulase,iu/ml		*A.wentii* β-glucosidase,iu/ml	
	CMC[a]	Filter paper	Cellobiose	pNPG[b]
0.00	0.87	0.62	5.32	2.66
0.10	1.12	1.02	10.96	3.87
0.20	1.37	1.41	12.63	4.79
0.25	1.67	1.55	12.95	4.96
0.30	1.53	1.48	12.84	4.88
0.35	1.50	1.46	12.78	4.81

CMC[a] = Carboxy methyl cellulose

pNPG[b] = P-nitrophenyl β-D-pyranoside

Fig. 11 Effect of Inoculum level on cellulase production by Trichoderma viride.



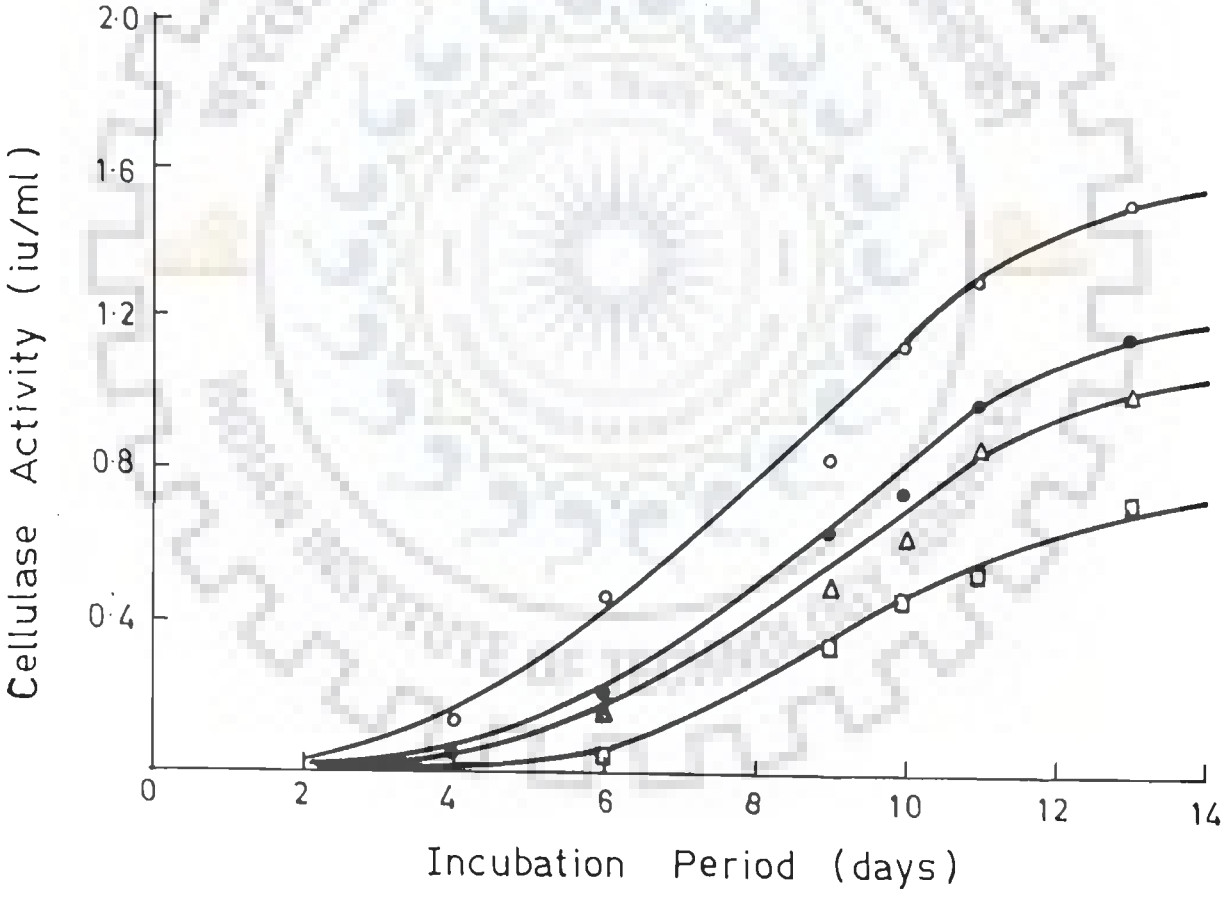
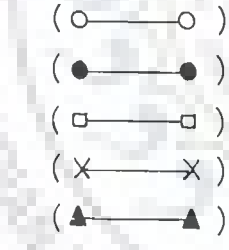

Cellulase Activity (iu/ml)
2·0
1·6
1·2
0·8
0·4
0
2
4
6
8
10
12
14
Incubation Period (days)

Fig. 12 Effect of inoculum level on the β-glucosidase production by Aspergillus wentii.



, 2% inoculum

, 4% inoculum

, 6% inoculum

, 7% inoculum

, 8% inoculum

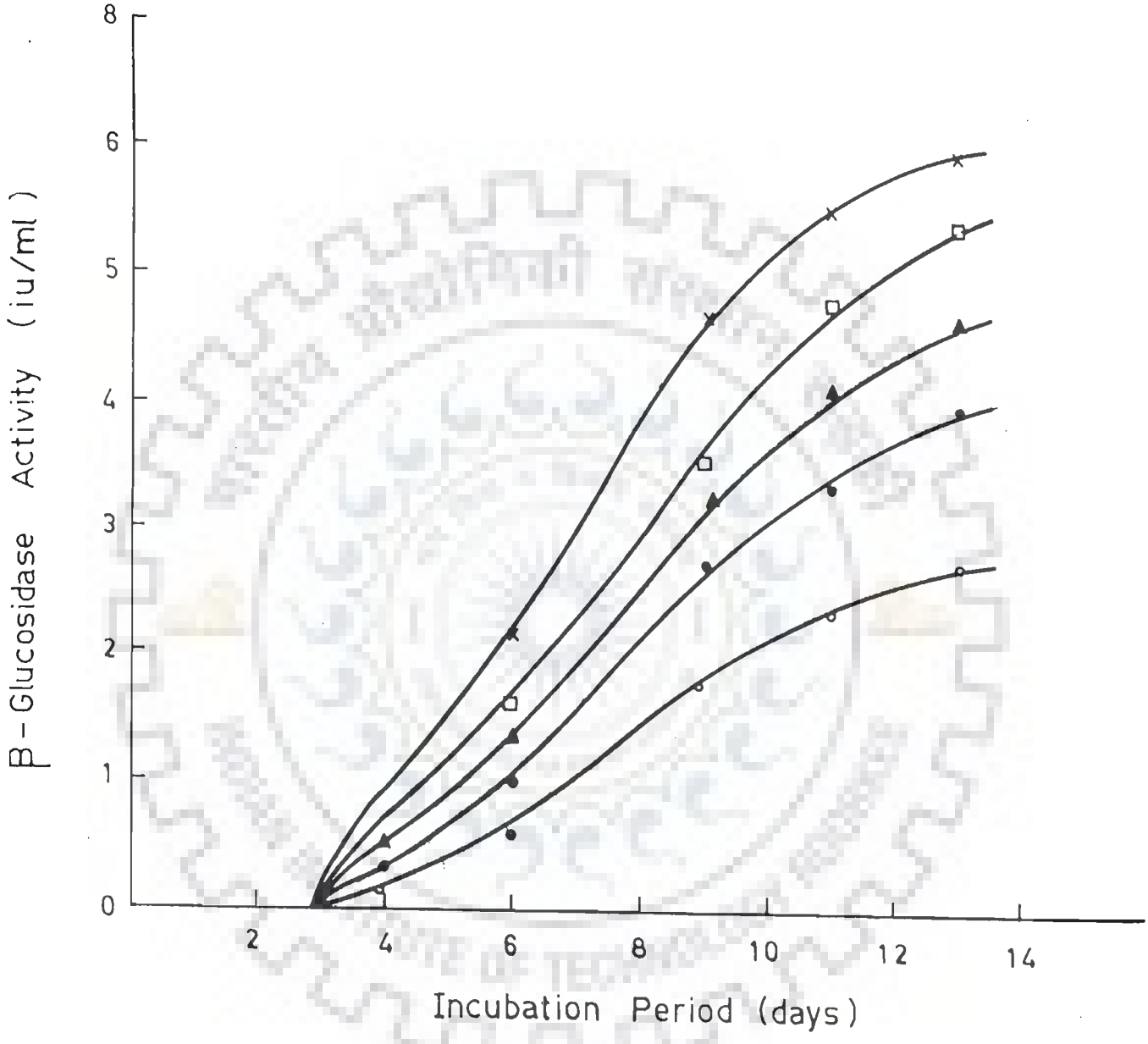
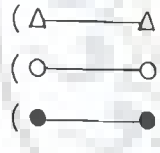

β-Glucosidase Activity (iu/ml)
0
1
2
3
4
5
6
8
2
4
6
8
10
12
14
Incubation Period (days)

Fig. 13 Effect of Inoculum age on the cellulase production by Trichoderma viride.



(△——△) 16h grown cells

(○——○) 24h grown cells

(●——●) 32h grown cells

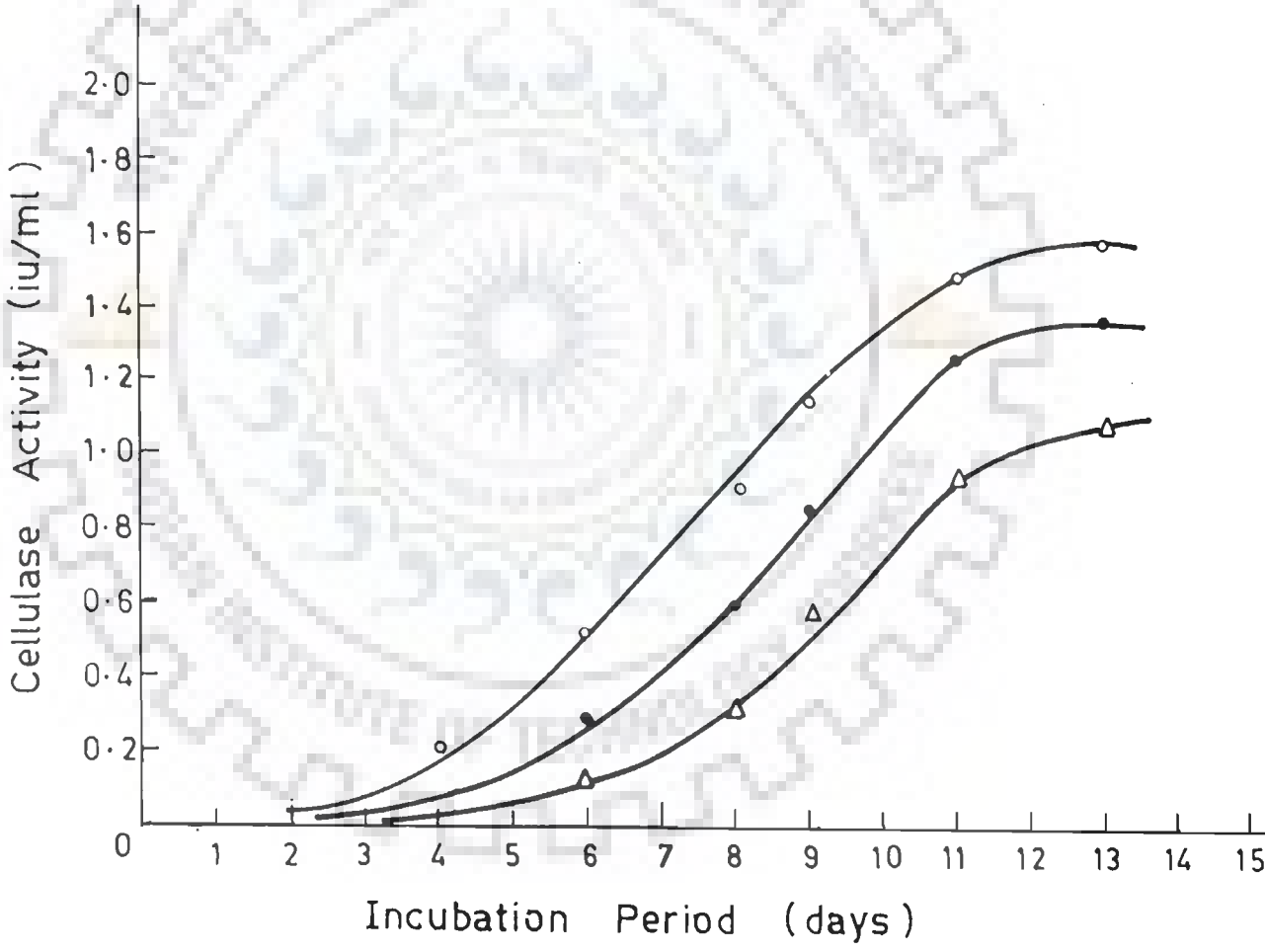

Cellulase Activity (iu/ml)
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
Incubation Period (days)

Fig. 14 Effect of Inoculum age on β-glucosidase production by Aspergillus wentii.

(✕——✕) , 8h grown cells

(▲——▲) , 16h grown cells

(○——○) , 24h grown cells

(●——●) , 32h grown cells

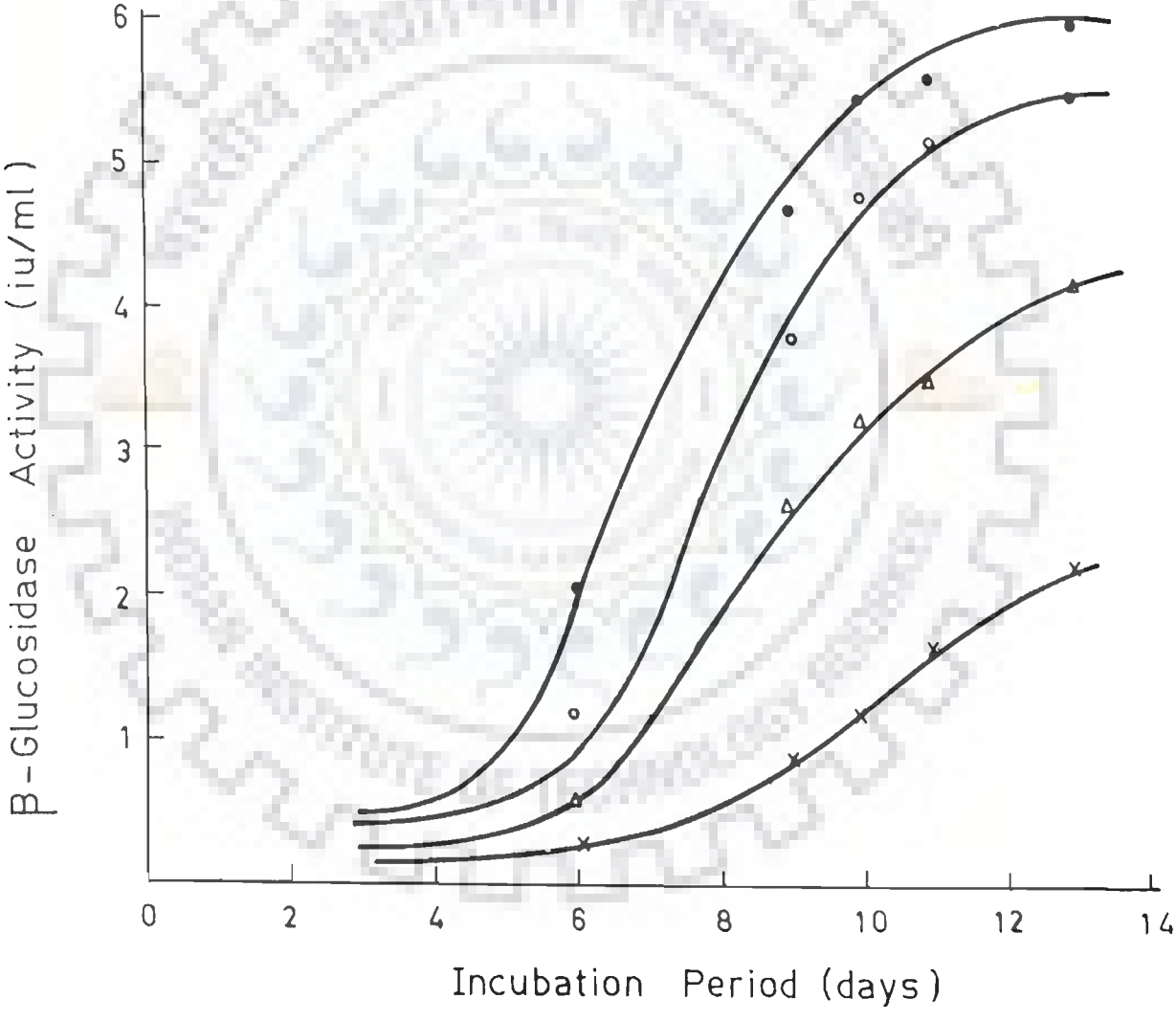

B-Glucosidase Activity (iu/ml)
6
5
4
3
2
1
0
2
4
6
8
10
12
14
Incubation Period (days)

Fig. 15 Effect of cellulose and glucose grown inoculum on cellulase production by T. viride.

(○——○) , cells grown on glucose

(●——●) , cells grown on cellulose

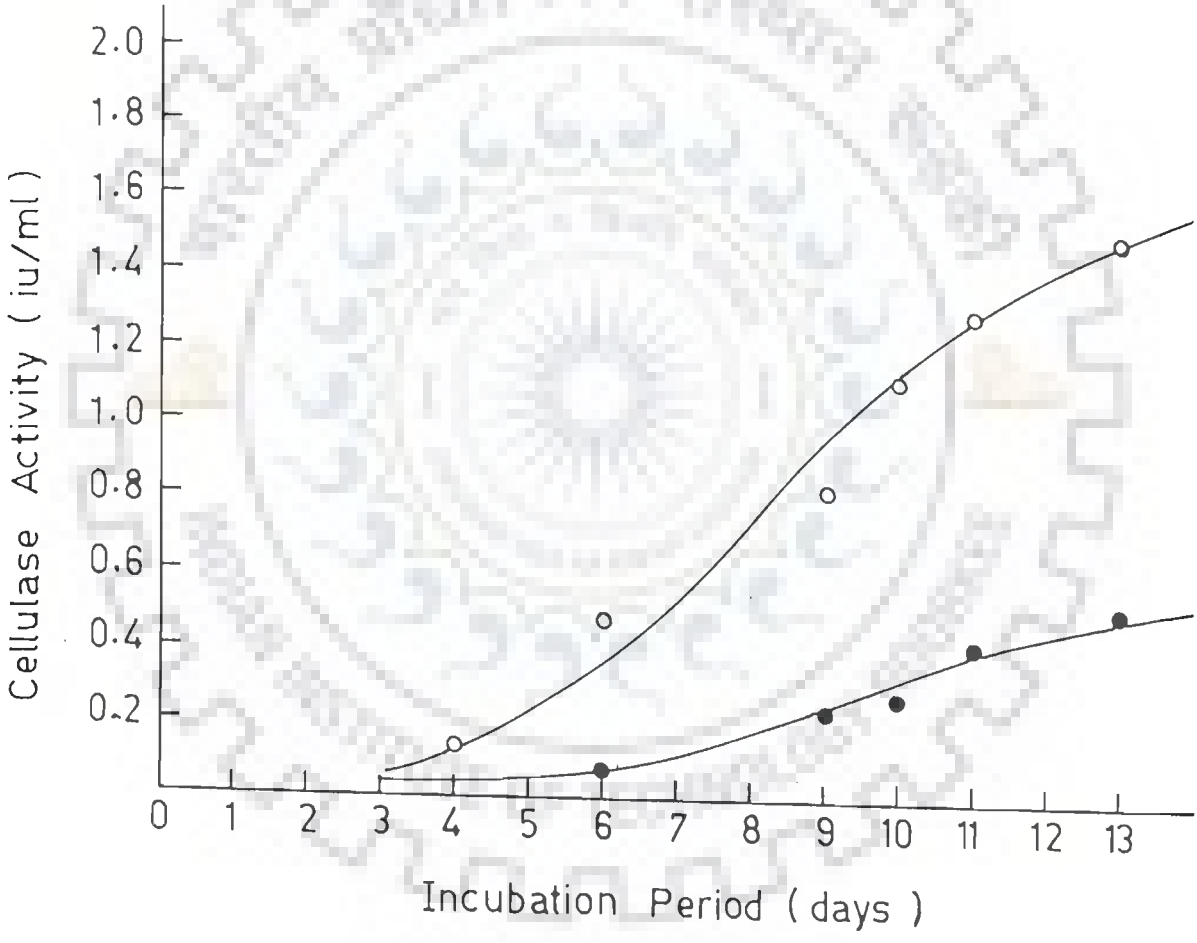
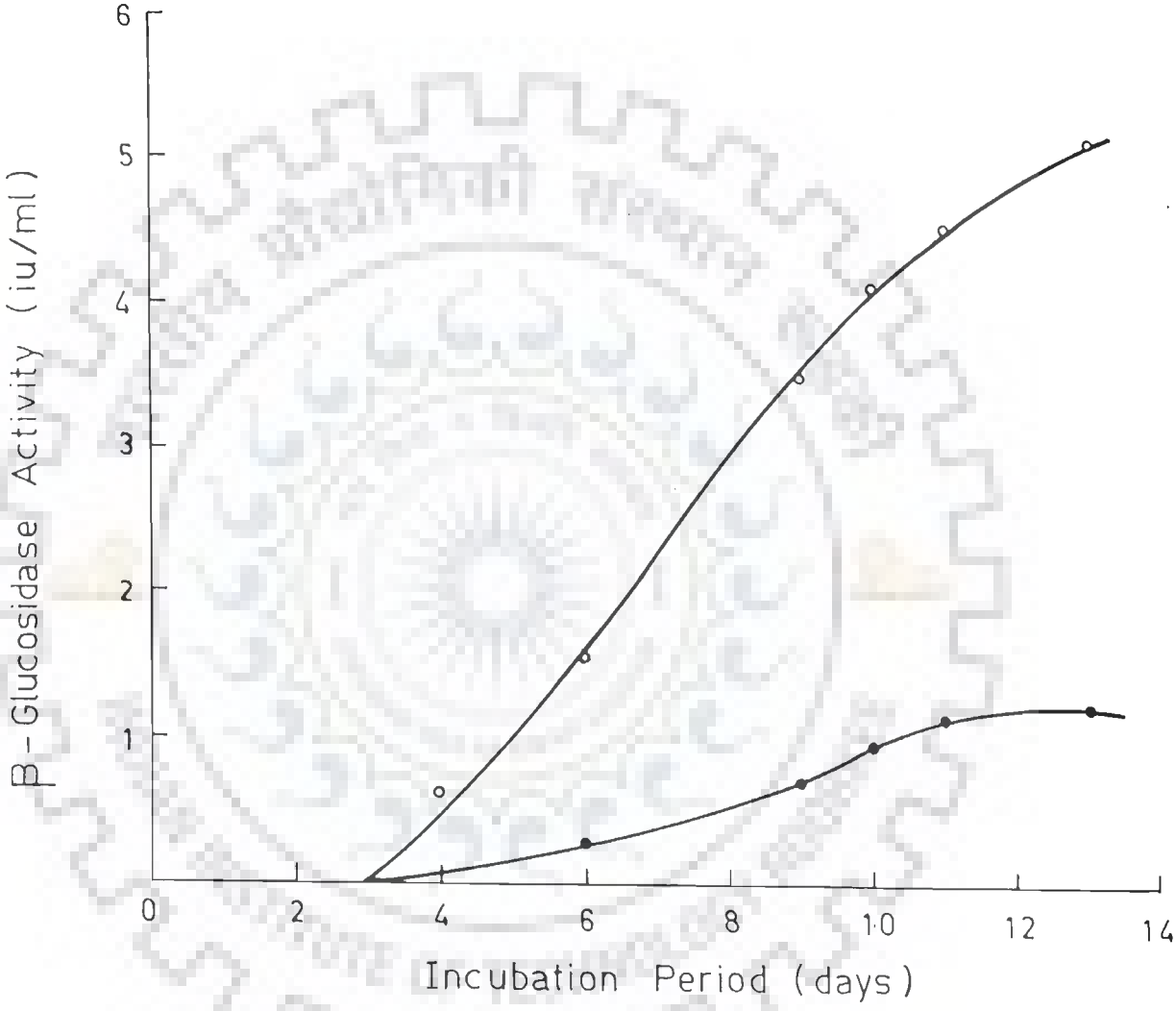

Cellulase Activity (iu/ml)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0 1 2 3 4 5 6 7 8 9 10 11 12 13
Incubation Period (days)

Fig. 16 Effect of cellulose and glucose grown inoculum on β-glucosidase production by Aspergillus wentii. (○———○) 1% Glucose grown inoculum. (●———●) 1% Cellulose grown inoculum.


6
5
4
3
2
1
0
2
4
6
8
10
12
14
β-Glucosidase Activity (iu/ml)
Incubation Period (days)

in a medium containing 1% glucose and then inoculated in a medium containing a 2% mixture of cellulose and glucose (3:1,w/w) gave maximum yield of extracellular cellulase (Fig.13). For the Aspergillus wentii 32 h old cells produced maximum amount of extracellular β-glucosidase (Fig.14). However, if T. viride or A. wentii cells were grown on 1% cellulose instead of 1% glucose the level of cellulase (Fig.15) and β-glucosidase (Fig.16) after 13 days incubation in the standard growth medium was only less than one fourth. Thus inocula of T. viride and A. wentii were prepared by growing the cells in a medium containing 1% glucose instead of 1% cellulose.

Based on the results described above optimum conditions for production of extracellular cellulase by Trichoderma viride and β-glucosidase by Aspergillus wentii are summarized in Table IX.

For large scale production of cellulase by Trichoderma viride 1060, pretreated Mangifera indica saw dust was used as the carbon source in the growth medium and glucose, unless stated otherwise was omitted to make process less expensive. Under these conditions cellulase and β-glucosidase were produced in maximum yield by Trichoderma viride and Aspergillus wentii respectively.

TABLE IX : OPTIMUM CONDITIONS FOR THE LARGE SCALE PRODUCTION OF EXTRACELLULAR CELLULASE AND β-GLUCOSIDASE (CELLOBIASE) ENZYMES FOR CELLULOSE DIGESTION.

Conditions	Enzyme	
	Cellulase	β-glucosidase
Culture strain	Trichoderma viride-1060	Aspergillus wentii Pt-2804
Carbon source	Pretreated cellulose[a]-glucose[b] (3:1), 2%	Starch, 2%
Incubation period	13 - 14 days	13 - 14 days
Incubation temp.	30°C	30°C
pH	4.8 - 5.0	4.8 - 5.0
$MgSO_4$	0.03%	0.02 - 0.03%
Inoculum age and level	Cells grown for 24h on glucose containing medium; 5%	Cells grown for 24h on glucose containing medium; 7%
Tween-80	0.03 - 0.04%	0.03 - 0.04%

a : Mangifera indica saw dust was subjected to high pressure steaming followed by dilute sodium hydroxide extraction.

b : Since pretreated cellulose alone also is an excellent carbon source for production of extracellular cellulase, in large scale production only cellulose was used for cellulase production, unless stated otherwise.

4.3 Large Scale Production Of Cellulase And β -Glucosidase (Cellobiase).

Using the optimum conditions for the production of cellulase and β-glucosidase (Cellobiase) (Table IX), by Trichoderma viride and Aspergillus wentii, respectively, the large scale preparations of these enzymes were attempted in a 9 litre fermenter under strict control of pH (pH 3.5 to 5.0) and temperature (30°C). Fig.17 shows the kinetics of cellulase production during 10 days of incubation in a medium containing 2% cellulose fibre as the carbon source. It was observed that cells start to release the enzyme into the medium after a lag period of 2h. Following the lag period the concentration of cellulase in the medium increases markably, reaching a maximum level (1.8 iu/ml) in 10 days, It is interesting to note that during first 6 days of incubation period nearly 85 to 90% cellulose was degraded very rapidly, but during this period the rate of production of cellulase was relatively low. After this period however, cellulase production rate increased rapidly. The overall production of the enzyme seems to follow the sigmoid type of kinetics. It is likely that the cellulase produced during the first phase helps to degrade cellulose and thereby helps in growth of Trichoderma viride cells, which in turn during the final stage of growth period produce more cellulase enzyme. This observation is in agreement with the result that addition of glucose to cellulose containing medium enhanced the cellulase production by Trichoderma viride.

Fig. 17 Production of cellulase in a 9 liter fermenter. Medium contained 2% cellulase fibreas carbon source. (○——○), activity against CMC; (●——●), activity against filter paper. (△——△), percent cellulose remaining.

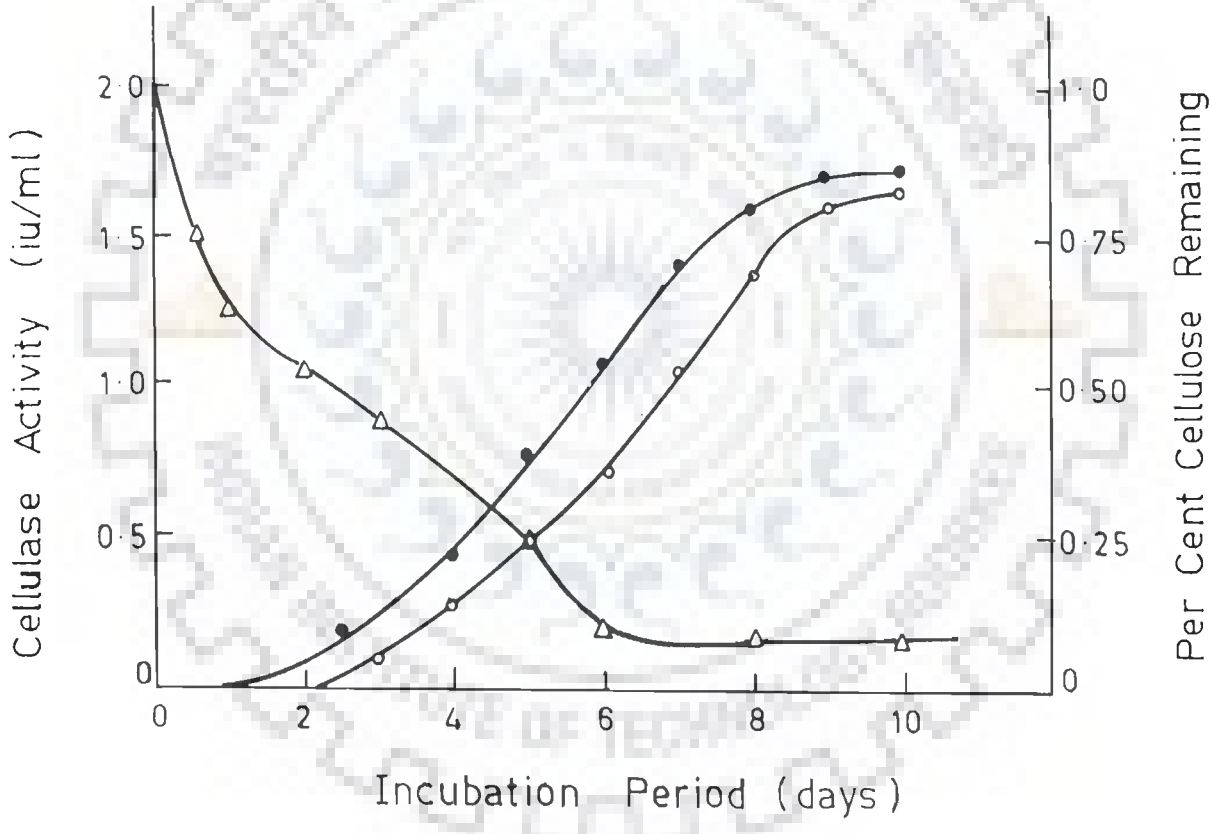

Cellulase Activity (iu/ml)
2·0
1·5
1·0
0·5
0
1·0
0·75
0·50
0·25
0
0
2
4
6
8
10
Incubation Period (days)
Per Cent Cellulose Remaining

Fig. 18 Scaled - up production of extracellular β-glucosidase. Aspergillus wentii cells were grown on a medium containing 4% starch as carbon source in a 9 liter fermenter. (○——○) , enzyme activity against pNpG and (●——●) , enzyme activity against cellobiose; (×——×) , starch concentration remaining (g/lit); (□——□) , Mycelium dry weight (g/lit).

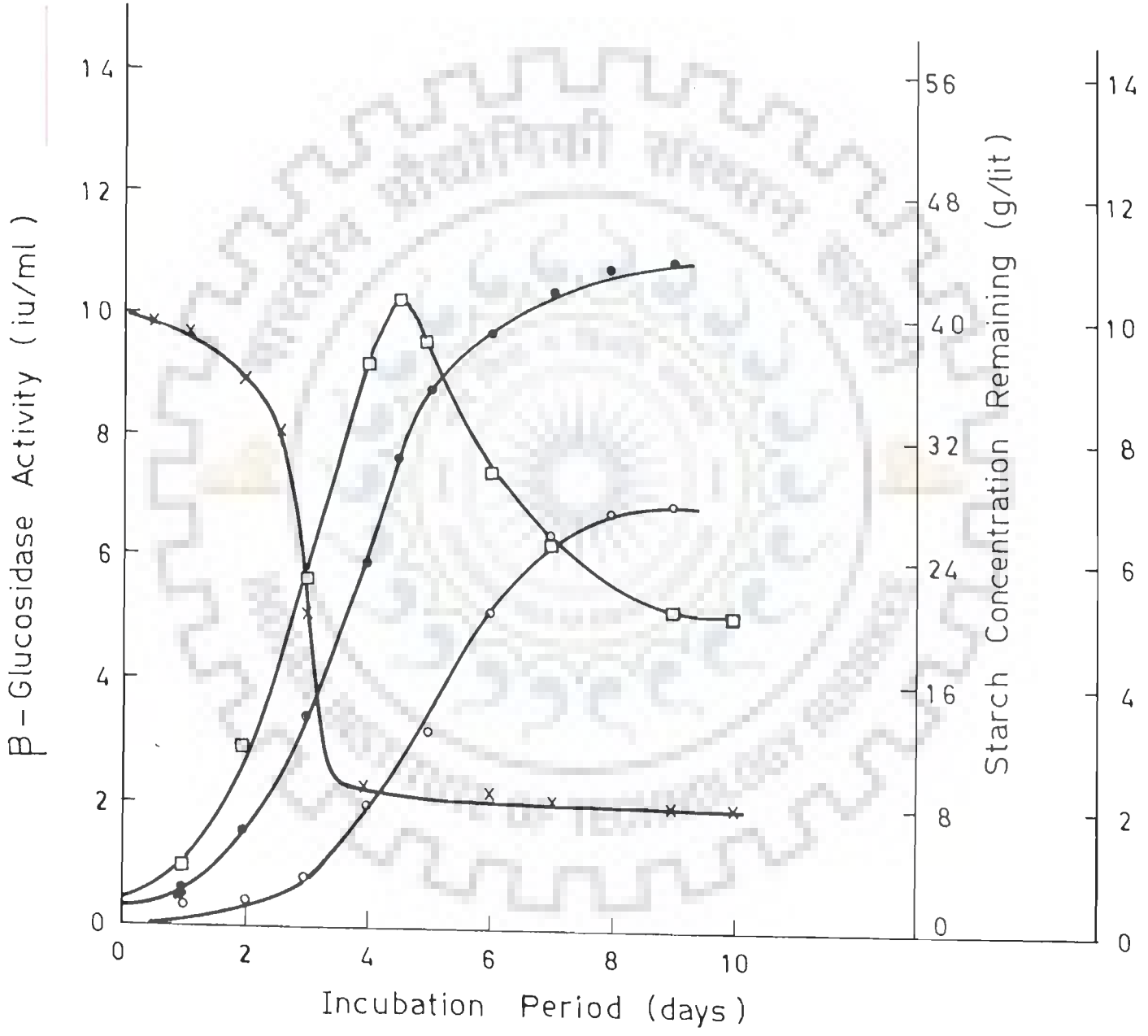

β-Glucosidase Activity (iu/ml)
Starch Concentration Remaining (g/lit)
Mycelium Dry Weight (g/lit)
Incubation Period (days)
0
2
4
6
8
10
12
14
16
24
32
40
48
56

Fig.18 shows the scaled up production of cellobiase by Aspergillus wentii, grown on a medium containing 4% starch as carbon source, in a 9 liter fermenter. It was found that the utilization of starch in the first phase of growth, 12 to 100 h resulted in a parallel increase of the cellobiase activity. The cellobiase production reached a maximum level after 8 days incubation. In fact, maximum production of cellobiase occurred from 6 to 10 days incubation. During this period, surprisingly enough, the concentration of the residual starch remained unchanged at a level of 8 mg/ml and the dry weight of the Aspergillus wentii cells in the medium decreased indicating that the cells were being lysed and growth was arrested. The kineticsof the cellobiase production, like that of cellulase was clearly sigmoid. Perhaps the reaction products enhance the cellobiase production.

The results of the scaling up experiments show that sufficient quantity of both cellulase and cellobiase can be produced at relatively low cost inputs for use in enzymatic hydrolysis of cellulosic materials.

4.4 Enzymatic Hydrolysis Of Cellulosic Material

The cellulase enzyme prepared from Trichoderma viride was used to study the saccharification of the cellulose fiber from Mangifera indica saw dust. The latter was subjected to high pressure steaming to break the ternary complex of lignin, hemicellulose and native cellulose

Fig. 19. Hydrolysis of pretreated Mangifera indica saw dust by Trichoderma viride cellulase. (●——●) , 2.5% cellulose; (○——○) 5.0% cellulose and (△——△) , 10% cellulose.

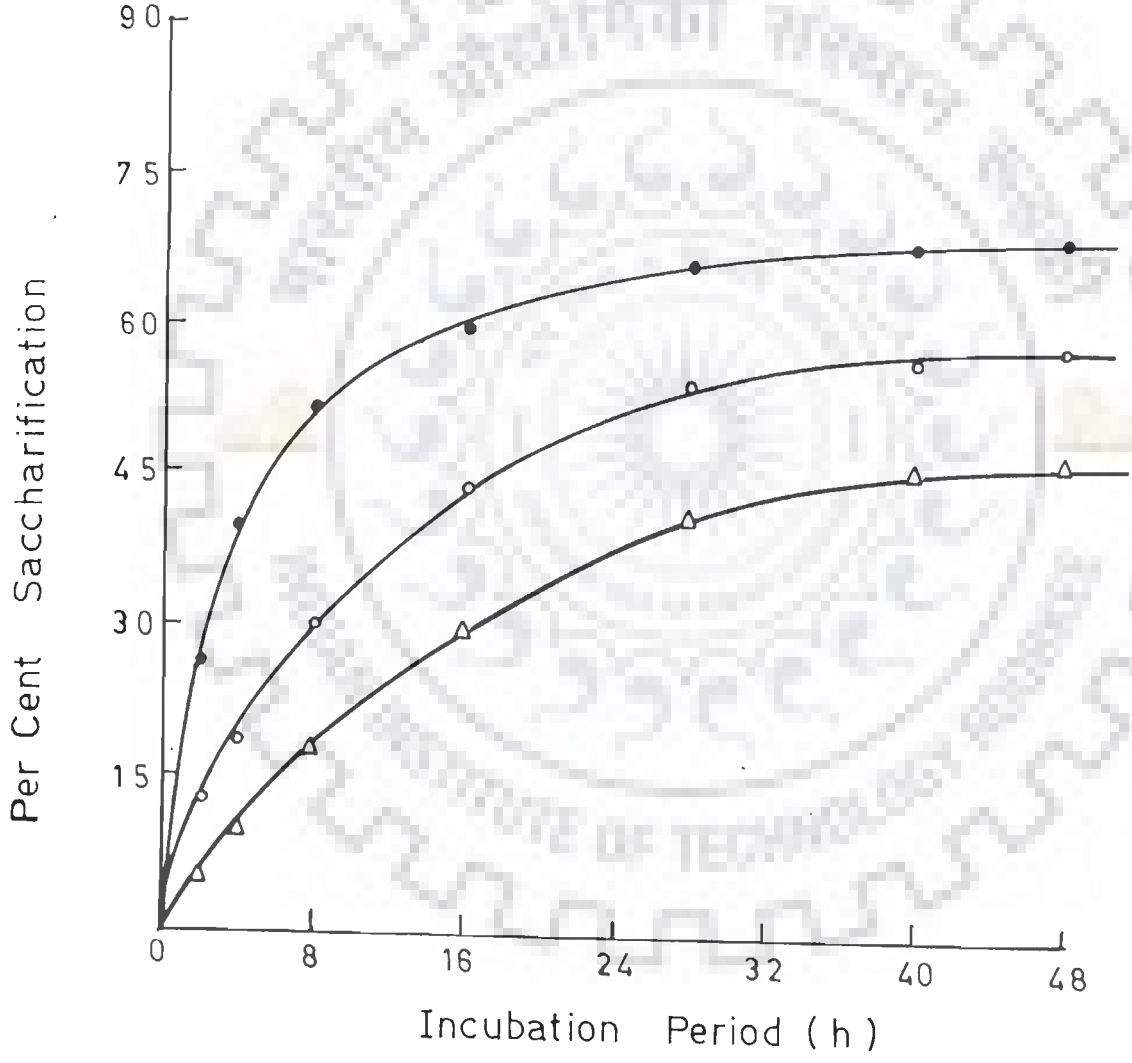

90
75
60
45
30
15
Per Cent Saccharification
0
8
16
24
32
40
48
Incubation Period (h)

Fig. 20 Hydrolysis of pretreated Mangifera indica saw dust (cellulose fiber) as a function cellulase concentration. 2.5% cellulose fiber was used and incubations were carried out for indicated periods using 16, 32, 48 and 64 units of Trichoderma viride cellulase. (●——●) , 8h incubation; (○——○) , 16h incubation; (△——△) ,32h incubation and (X——X) , 48h incubation.

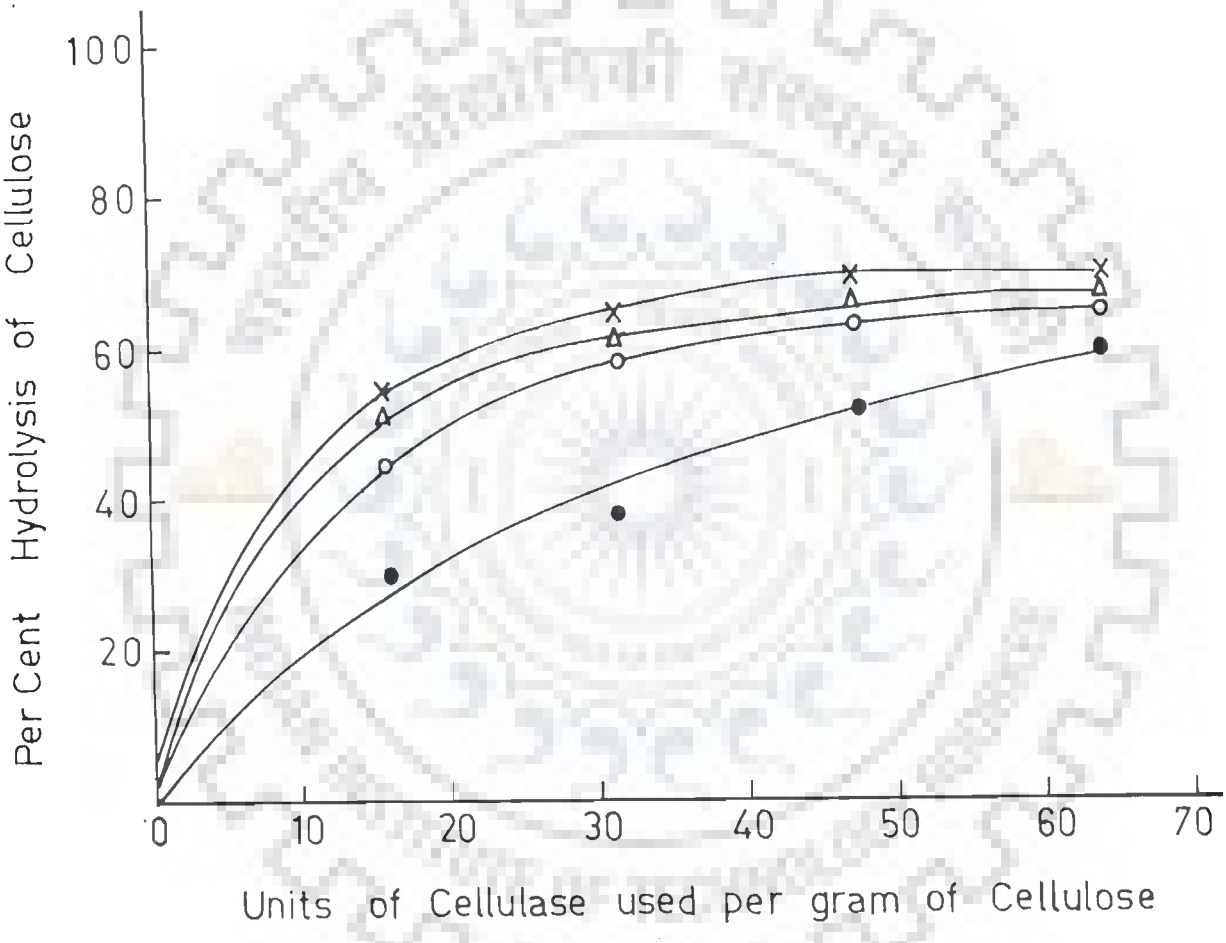

Per Cent Hydrolysis of Cellulose
100
80
60
40
20
0
10
20
30
40
50
60
70
Units of Cellulase used per gram of Cellulose

Fig. 21 Hydrolysis of Mangifera indica saw dust, pretreated with high pressure steaming followed by alkali extraction, by Trichoderma viride cellulase. 2.5% cellulose fibre was subjected to digestion with various concentrations of cellulase. Percent saccharifications was measured at indicated times. (○——○) , 16 units/g cellulose; (△——△), 32 units/g cellulose; (□——□) ,48 units/g cellulose and (●——●) , 64 units of cellulase/g cellulose.

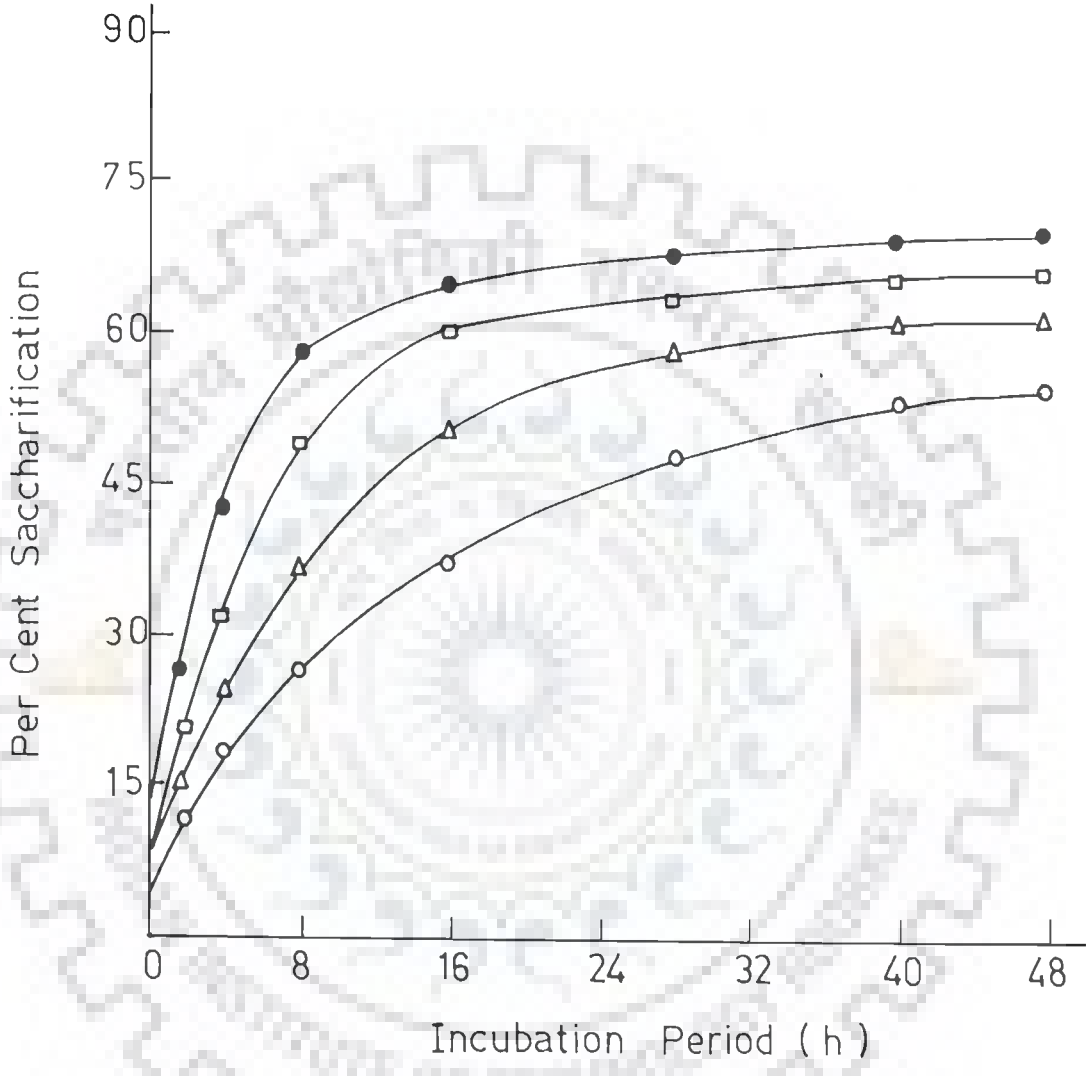
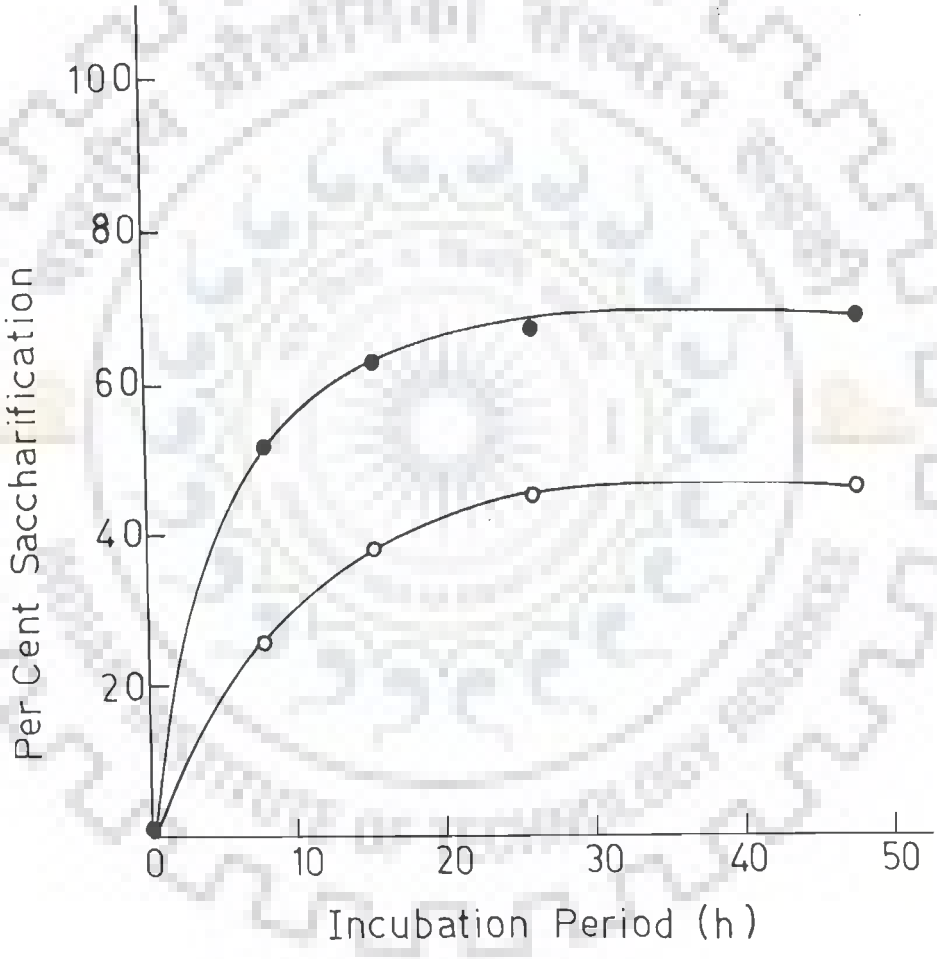

90
75
60
45
30
15
Per Cent Saccharification
0
8
16
24
32
40
48
Incubation Period (h)

Fig. 22 A comparison of the enzymatic hydrolysis of pretreated high pressure steaming followed by alkali extraction . Mangifera saw dust and Avicel microcrystalline cellulose under optimal conditions :(●——●) Mangifera saw dust and (○———○) Avicel microcrystalline cellulose powder.


Per Cent Saccharification
100
80
60
40
20
0
10
20
30
40
50
Incubation Period (h)

followed by dilute alkali extraction of lignin. Cellulose fibers thus obtained were virtually free of lignin and hemicellulose components and served as a suitable substrate for the cellulase enzyme. Fig.18 shows the saccharification of Mangifera indica saw dust by cellulase enzyme over a period of 48 h. Three different concentrations of pretreated saw dust (2.5%, 5% and 10%) were used. It was found that the rate of saccharification reaches to a maximum in 40h regardless the substrate concentration. However, percent saccharification is dependent on the substrate concentration. For instance, at 2.5% level of cellulose the saccharification was nearly 70% in 48 h, whereas the percent saccharification of Mangifera indica dust at 5 and 10% concentration was around 60 and 47 respectively. Thus, at higher cellulose concentration the saccharification of cellulose was lower. These results suggest that the substrate: enzyme ratio may play an important role in the efficient degradation of cellulose. Results in Fig.19 show the percent saccharification of Mangifera indica saw dust as a function of cellulose to cellulase ratio. It was found that in the first 8 h of digestion the enzyme: cellulose ratio has a profound effect on the rate of hydrolysis of cellulose and it required large amount of enzyme (over 70 units/g cellulose) to achieve 60% saccharificaton in 8 hours. On the other hand if the incubation is carried out for 16 h or longer period, 20 - 30 units of cellulase per gram of cellulose fiber were sufficient for 65 - 67% saccharification of cellulose. Thus by increasing the enzyme concentration from 30 units/g cellulose to 70 units/g cellulose, the time required for

achieving maximum percent saccharification (65 - 70%) was reduced from 48 h to just 16h (Fig.20). Since the amount of cellulase is a limiting factor, incubation for 48 h using 20 - 30 units of the enzyme was preferred. Under these conditions the hydrolysis of pretreated Mangifera saw dust was about 70% compared to only 46% hydrolysis of the avicel microcrystalline cellulose (Fig.21). It is however, not surprising since crystallinity of the Mangifera indica saw dust was greatly reduced after the high pressure steaming followed by delignification by NaOH extraction (Fig.6).

4.4.1 Effect Of β-glucosidase On The Hydrolysis Of Mangifera Indica Saw Dust By Trichoderma Viride Cellulase

Cellobiose is produced during the digestion of cellulose by the action of cellulase and as the amount of cellobiose increases in the medium the cellulase activity is greatly inhibited due to the inhibitory activity of cellobiose. Thus, for efficient enzymatic digestion of cellulose it is essential that the level of cellobiose is kept as low as possible. One way of doing it is to supplement the cellulase with high specific activity β-glucosidase which would hydrolyze the cellobiose to glucose. We have used Aspergillus wentii β-glucosidase (0-1.2 units/ml) along with the Trichoderma viride cellulase to hydrolyse the cellulose fiber. Results shown in Table X clearly demonstrate that the supplementation of cellulase with β-glucosidase (1.2 units/ml) has markedly increased the percent hydrolysis of cellulose as well as has greatly

TABLE X : EFFECT OF β-GLUCOSIDASE SUPPLEMENTATION ON THE HYDROLYSIS OF CELLULOSE BY TRICHODERMA VIRIDE CELLULASE.

Cellulose (2.5g) was suspended in 100ml of 50mM citrate buffer, pH 4.8 and incubated with cellulase (1.0 unit/ml) and varying amounts (0 to 1.2 unit/ml) of Aspergillus wentii β-glucosidase at 50°C for the indicated periods with a constant shaking at 150 r.p.m. in an orbital shaker. The amount of reducing sugar (R.S.) was determined and percent saccharification calculated.

β-glucosidase concentration Units/ml	Incubation Period and Saccharification							
	8h		16h		24h		48h	
	R.S' mg/ml	Saccharification (%)	R.S' mg/ml	Saccharification (%)	R.S.' mg/ml	Saccharification (%)	R.S' mg/ml	Saccharification (%)
0.0	6.5	23.4	10.16	36.6	12.6	45.6	15.16	54.6
0.4	9.0	32.4	13.0	46.8	16.3	58.8	18.50	66.6
0.8	11.0	39.6	15.3	55.2	18.0	64.8	21.56	77.6
1.2	12.3	44.4	17.33	62.4	20.16	72.6	22.30	80.4

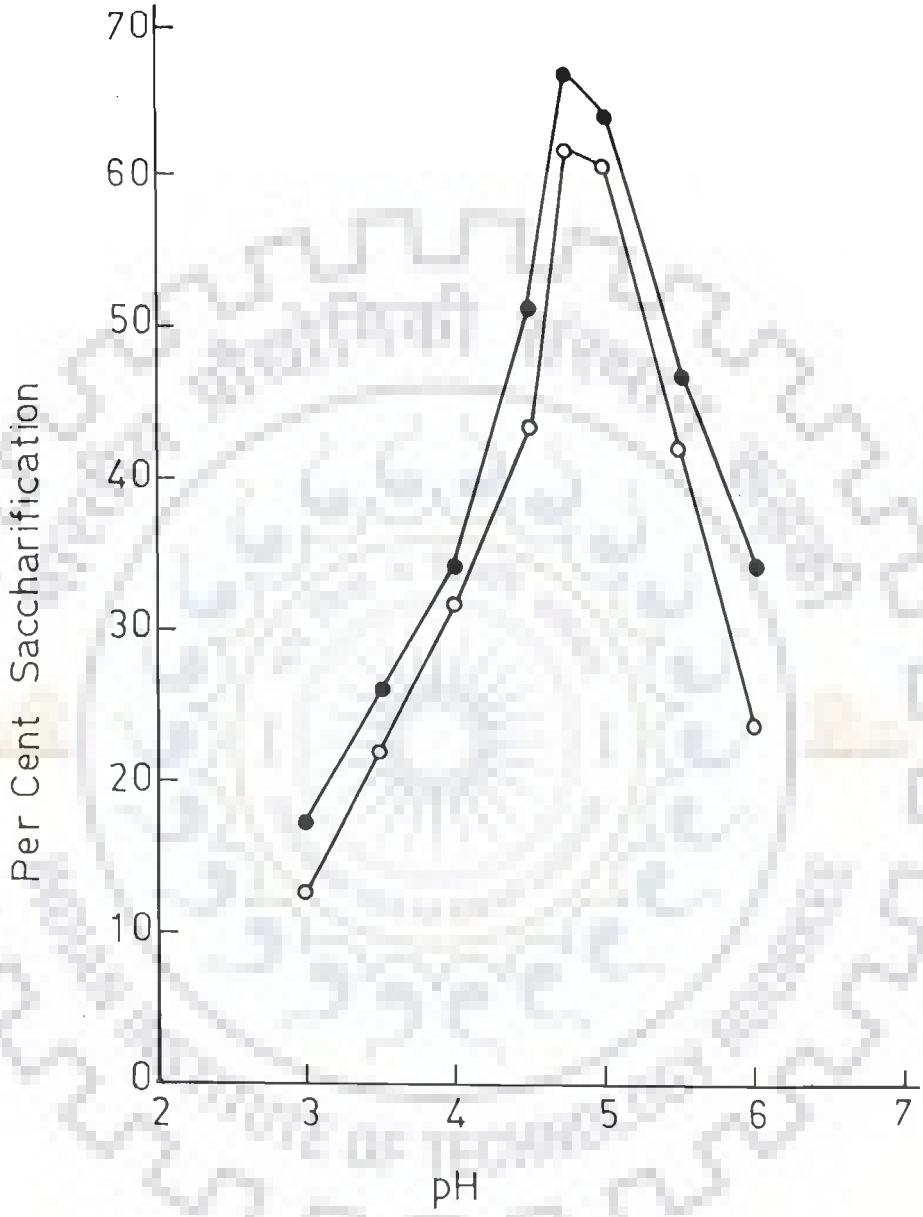
shortend the incubation period. For example in the presence of β-glucosidase nearly 80% cellulose was hydrolyzed by Trichoderma cellulase in 48 h compared to only 54.6% without β-glucosidase under identical conditions. In addition 62.4% hydrolysis of cellulose was achieved by the cellulase in presence of β-glucosidase (1.2 unit/ml) in just 16 h incubation whereas without β-glucosidase it required about 48 h to achieve 54% hydrolysis. Thus, supplementing cellulase with β-glucosidase (1.2 - 2.0 units/ml) was an important step in the enzymatic hydrolysis of cellulose to glucose, both in terms of percent saccharification (hydrolysis) and period of incubation required for obtaining maximum digestion. These parameters are also important from the point of commercial viability of the process.

4.4.2 Effect Of pH On The Enzymatic Hydrolysis Of Cellulose.

The saccharification of the cellulose fiber of Mangifera indica, prepared by treatment of the saw dust with high pressure steaming followed by extraction with dilute alkali to remove hemicellulose and lignin components, was carried out at a pH range of 3.0 to 6.0. The results are shown in Fig.22. It can be seen that the maximum enzymatic hydrolysis of cellulose fiber by Trichoderma viride cellulase occurred at pH 4.8. Below and above this pH, the percent hydrolysis of cellulose declined sharply. It was also observed that after 20 h digestion there was only a marginal increase in the overall saccharification of cellulose. It may be pointed out that the incubations were carried out

Fig. 23 Effect of pH on saccharification of the pretreated Mangifera indica saw dust by Trichoderma viride cellulase.

Cellulose fiber from Mangifera indica saw dust (2.5g) was suspended in 100ml of 50mM citrate buffer at pH values ranging from 3 to 6 and incubated with T. viride cellulase (1.5 units/ml) at 50°C for 20h (o——o) and 36h (●——●) with shaking at 150 rpm. The percent saccharification was measured as described in methods.


70
60
50
40
30
20
10
0
Per Cent Saccharification
2
3
4
5
6
7
pH

without supplementing β-glucosidase.

4.4.3 Effect Of Temperature On The Cellulose Hydrolysis By *Trichoderma viride* Cellulase.

Table XI summarizes the hydrolysis of cellulose from *Mangifera indica* by *Trichoderma viride* cellulase as a function of temperature. The results indicate the optimum temperature for the enzymatic hydrolysis of cellulose to be 50°C. Like the effect of pH, only a marginal increase in the percentage of cellulose hydrolysis was noticed when incubations longer than 20 h were performed.

Thus, from the results described above the optimum conditions for the enzymatic hydrolysis of *Mangifera indica* cellulose fiber were found to be as following:

Cellulose fiber concentration	2.5 to 5%
pH of incubation mixture	4.8
Temperature	50°C
Cellulase concentration	1.2 to 2.0 units/ml
β-glucosidase supplementation	1.5 to 2.0 unit/ml
Incubation period	20 to 36 h

Under these conditions about 80% cellulose was hydrolyzed to glucose by *Trichoderma viride* extracellular cellulase when used in combination with equal amount of *Aspergillus wentii* β-glucosidase.

TABLE XI : EFFECT OF TEMPERATURE ON THE HYDROLYSIS OF PRETREATED *MANGIFERA INDICA* SAW DUST BY *TRICHODERMA VIRIDE* CELLULASE.

Cellulose fiber (2.5g), prepared from *Mangifera indica* saw dust after treatment with high pressure steaming followed by delignification by dilute alkali, were suspended in 100 ml of 50mM citrate buffer, pH 4.8, and incubated with *T.viride* cellulase (1.5 units/ml) at different temperatures for 8, 20, 36 and 46h. The percent saccarification was measured as described under methods.

Temperature °C	Incubation period, h			
	8	20	36	48
	Saccharification (%)	Saccharification (%)	Saccharification (%)	Saccharification (%)
40	27.4	33.1	36.2	39.7
45	43.9	52.1	58.4	64.1
50	51.7	63.0	67.5	69.0
55	45.1	49.1	56.2	61.4
60	25.1	32.2	37.3	42.2

4.5 Some Applications

4.5.1 Use Of Cellulose Hydrolyzate As Carbon Source For The Production Of Bakers Yeast.

The enzymatic hydrolyzate of Mangifera indica saw dust was used as a carbon source for growing yeast in Carbon free medium (Czapex DOX medium) supplemented with 0.1% peptone. The final concentration of reducing sugars, measured as glucose, in the hydrolyzate was adjusted to 2% . Medium containing 2% dextrose was used as control. Yeast was grown in 1 liter Erlenmeyer flasks containing 300 ml of growth medium on a rotatory shaker at 150 rpm for 12, 24 and 48 hours at 30^{o}C.

At indicated periods, the cells were harvested , washed thrice with water and then freeze dried overnight. The results of triplicate experiments are given in Table XII. It can be seen that the production of yeast on enzymatic hydrolyzate of Mangifera indica saw dust is very comparable to that of the control in which 2% dextrose is the carbon source. The process was scaled upto 10 litres to get about 1 Kg yeast in 24 h. Thus use of enzymatic hydrolyzate of Mangifera indica saw dust appears to be promising for yeast growth and the process could be extended to pilot plant production.

TABLE XII : CULTIVATION OF SACCHAROMYCES CEREVISIAE ON HYDROLYZATE OF MANGIFERA INDICA SAW DUST.

Bakers yeast (Saccharomyces cerevisiae) was grown in enzymatic hydrolyzate with reducing sugars adjusted to 2% glucose equivalent in a carbon free Czapex Dox medium. The medium was supplemented with 0.1 % peptone. Cells were harvested, freeze dried and weighed. Cells grown in a medium containing 2% dextrose served as control. The 1% inoculum ($O.D_{578}$ 4-5) was added to the medium.

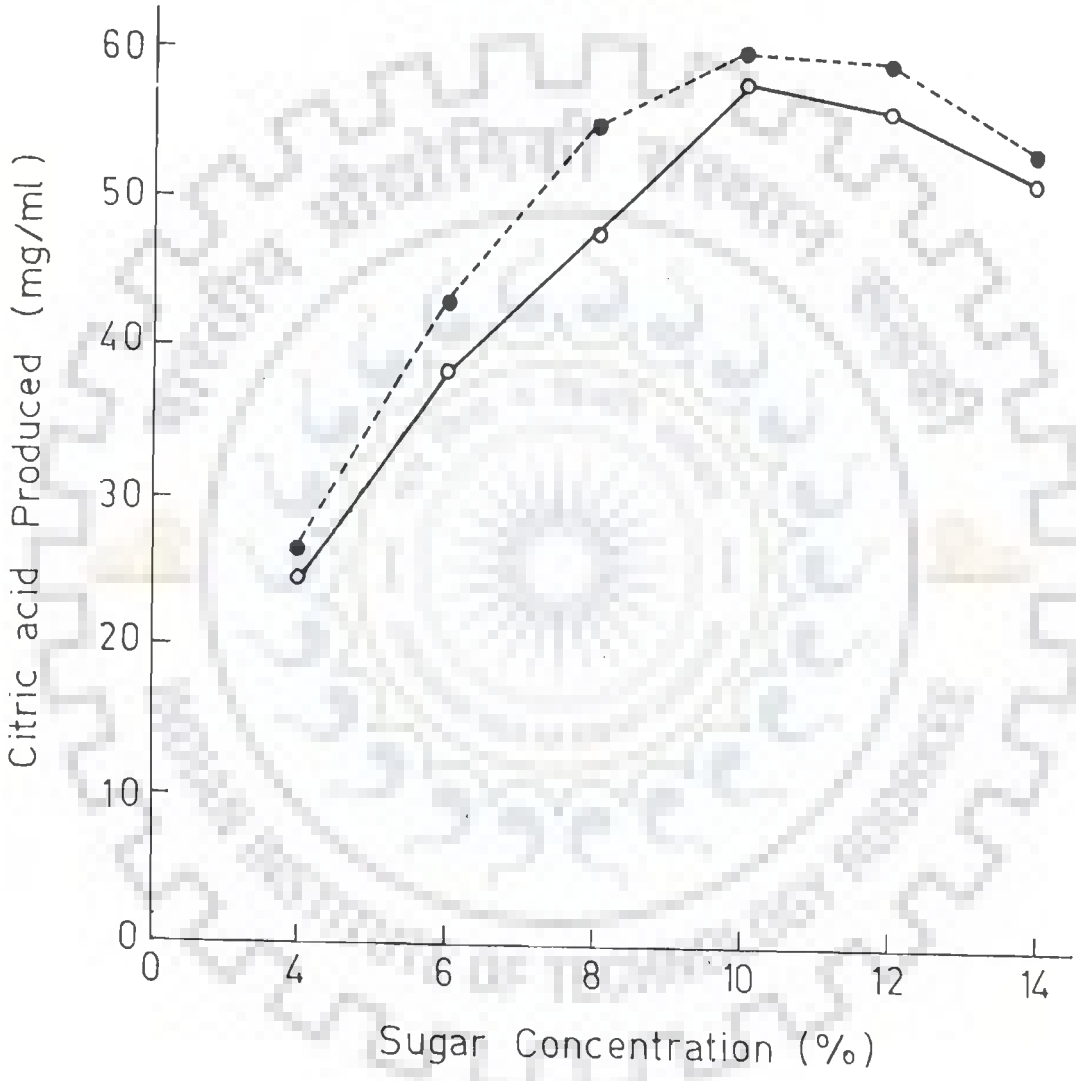
Carbon Source	Concentration of reducing sugars (%)	Dry weight of cells[2] (g/100 ml medium) 12h	24h	48h
Enzymatic hydrolyzate of Mangifera saw dust	2	1.7 ± 0.3	10.9 ± 0.3	15.4 ± 1.0
Dextrose	2	1.8 ± 0.3	12.2 ± 0.3	16.8 ± 1.0

1. Concentration is given in terms of glucose
2. Average of three experiments

4.5.2 Use Of Cellulose Hydrolyzate As A Substrate For Citric Acid Fermentation.

The hydrolyzate, prepared by digestion of Mangifera indica saw dust with cellulase in the presence of β-glucosidase, was used as a substrate for the production of citric acid by Aspergillus niger spores. In a typical experiment the fermentation broth was prepared as follows : to the hydrolyzate containing 4 to 14% glucose (reducing sugars) were added 0.22% NH_4NO_3, 0.1% KH_2PO_4, 0.02% $MgSO_4$, $7H_2O$ and 1N HCl to adjust the pH 2.2. Fermentation was started by adding Aspergillus niger spores ($2x10^5$ - $2x10^6$), which were produced by growing the Aspergillus niger cells on potato dextrose agar plates for 5 days. After 10 days fermentation period, the concentration of citric acid excreted into the medium was measured by the method of Marier and Boulet (107). The results of these experiments are shown in Fig.23. It was found that sugar was converted to citric acid and the maximum production (58mg/ml) of citric acid occurred at 10%(w/v) glucose concentration. In addition, the profile of citric acid production with glucose was found to be comparable with glucose as a substrate for the citric acid production. The results clearly show that the sugars produced by the enzymatic hydrolysis of Mangifera indica saw dust may be used as a cheap substrate for citric acid fermentation and require no pretreatment as required in the case of molasses. However, further investigations will be required to develop the complete process.

Fig. 24 Utilization of enzymatic hydrolysate of Mangifera indica saw dust for citric acid fermentation using Aspergillus niger. Submerged fermentation was carried for 10 days at 30°C as described under methods. (●------●) , glucose as substrate and (○——○) , enzymatic hydrolysate of Mangifera indica saw dust.


Citric acid Produced (mg/ml)
60
50
40
30
20
10
0
0
4
6
8
10
12
14
Sugar Concentration (%)

CHAPTER - V

5.0 DISCUSSION

5.1 Pretreatment Of LignoCellulosic Material Is Essential For Enzymatic Hydrolysis.

The purpose of the present study was to explore the possibility of using lignocellulosic wastes for production of fermentable sugars which in turn would provide cheap subsrates for production of liquid fuel (ethanol) and other useful chemicals, single cell protein, etc. Mangifera indica saw dust is produced in huge quantities as a by product of timber industry in North India. Currently, it is being used in very wasteful manner as a low energy fuel. One way to utilize this useful biomass in more efficient manner is to convert it into fermentable sugars and use the hydrolyzate as a substrate for production of feed supplement in the form of single cell proteins, chemicals like citric acid and of course, the liquid fuel (ethanol).

The native cellulose of Mangifera indica saw dust, like any other classical lignocellulosic material, is present as a ternary complex composed of approximately 25% hemicellulose, 25% lignin and 50% cellulose. In addition, native cellulose is present in a highly crystalline form which together with lignin component makes it resistant to the enzymic hydrolysis. Thus, in order to render the native

cellulose maximally susceptible to attack to the polysaccharide degrading enzymes, the Mangifera indica saw dust was subjected to various pretreatments that would result in breaking the ternary complex and reduce the crystallinity of cellulose. Of the different methods tried for the pretreatment of Mangifera indica saw dust, the high pressure steaming followed by alkali extraction at room temperature was found to be the most efficient method. The high pressure steaming method used here was slightly different than the one used by Ioteck (80,93). In the latter process the material is rapidly heated to 243° - 249°C by high pressure steam and after 1 min is exploded by a rapid release of the pressure, while in our process the temperature was lower (180°- 190°C) and time of steaming was about 30 min. Under these conditions the ternary complex of hemicellulose-lignin-cellulose was broken, the crystallinity of the cellulose molecule was damaged considerably (Fig.6) and the cellulosic residue was rendered susceptible to cellulase degradation. As reported by Tanahashi et al. (155) lignin and hemicellulose are perhaps hydrolyzed to low molecular weight products during high pressure steaming of the lignocellulosic material. It may also be pointed out that after high pressure steaming the hemicellulose and lignin components were readily extracted with water and dilute NaOH, respectively, indicating the breaking of the lignocellulosic complex.

Alkali treatment followed by steaming has been reported by various Workers (73) to increase the digestibility of lignocellulosic material. Similar results

were obtained in our studies even though steaming of lignocellulosic material was carried out prior to alkali treatment at room temperature. The explosion method involves basically the use of steam and high pressure to effect rapid hydrolysis. The release of pressure defibrate the lignocellulosic material with a considerable increase in surface area (131). Reducing lignocellulose to single fibers and damage to small fiber contribute to increased accessability and hence to higher reaction rates during enzymatic saccharification. Applying alkali at a higher tempreature (200^{o}C) causes various structural changes. The three major components of lignocellulose undergo hydrolytic or other degradative reactions with hot alkali. The extent of lignin solubility is largely dependent on the concentration of alkali, temperatures, pressure,source and nature of the lignocellulose. Degradation and dissolution of lignin occur mainly through reactions and its phenolic groups including rupture of phenolic ether bonds.

At higher temperatures, hemicellulose depolymerizes and solvation occurs mainly through end group "Peeling reactions" (78). Cellulose also degrades through alkaline peeling reactions following enolisation of carbonyl groups, isomerisation to β-alkoxy-carbomyl configurations, and finally decomposition of the end unit. These reactions, however, may be followed by "stopping reactions" which limit the degradation of cellulose (2). These types of reactions are expected to occur in the explosion digester during alkali treatment. The significant loss of solid material is due to

the degradation and dissolution of hemicelluloses and the solubilization of part of lignin. Since, in the present study the high pressure steaming was carried out before the alkali treatment and since alkali treatment was done at room temperature, there was little or no possibility of peeling reactions. As such there was no loss of cellulose during the pretreatment process.

The maintenance of alkalinity in the digester is also an important factor as a drop in alkalinity was associated with lower digestibility values (49). The significance of some of the factors that are thought to impede enzymatic saccharification such as various associations between lignin, hemicellulose and cellulose through hydrogen and covalent bonds, low surface area and high degree of polymerization of cellulose (131) may be considered on the basis of these reactions. For example, alkali explosion provides a pulp with a greatly increased surface area. The cellulose fiber that we got after high pressure steaming followed by alkali treatment was found to be highly susceptible to enzymic hydrolysis (Fig.19). As was judged from the X-ray diffractograph (Fig.6) of the Mangifera indica saw dust before and after the treatment, a significant damage of the crystalline structure of the native cellulose had occurred as a result of pretreatment by high pressure steaming and extraction with alkali at room temperature. Thus in agreement to the findings of Fan et al.(47) it seems that crystallinity of cellulose is atleast partly responsible for the poor enzymatic saccharification of

native cellulose.

Besides the crystallinity of cellulose the presence of O-acetyl groups in Xylan has also been suggested to impede enzymatic hydrolysis of cellulose. The high pressure steaming, however, initiates autohydrolysis splitting the acetyl groups, hence eliminating any barrier imposed by these groups (118).

Post pretreatment of cellulose fiber is also very important factor as far as the enzymic hydrolysis of cellulose is concerned, since Yin and Brown (174) reported reduced cellulose activity by drying in the nitration of cotton linters. Presumably, the presence of water in the fiber capillaries maintains dilation of the fiber wall and may reduce interchain associations through a cellulose-HOH-cellulose hydrogen bonding arrangement. Drying would draw fibers and microfibrils into close contact resulting in the formation of stronger interfiber and interfibril hydrogen bonding, and in the collapse of the lumen. These effects in turn would reduce the accessibility to the enzyme attack. In view of the above we did not dry the cellulose fiber obtained after the pretreatment and was suspended in citrate buffer for enzymic hydrolysis. This step was also cost effective.

Although, high pressure steaming followed by alkali treatment is an effective method for increasing the susceptibility of lignocellulosic material to cellulolytic

enzymes (129), the high energy demand for the high pressure technique and also cost of alkali may be major factors in deciding the economic viability of utilization of lignocellulosic material. Nonetheless the autohydrolysis explosion may be a useful pretreatment process for preparing feed stock for production of single cell protein, microbial oil, higher alcohols, etc. The over all economics would also depend on the effective utilization of lignin and hemicellulose which constitute about 50% of the total biomass.

5.2 Large Scale Production Of Cellulase Complex

One of the bottle neck in the enzymic hydrolysis of cellulose is the preparation of sufficient amount of high specific activity cellulase complex that would efficiently hydrolyze cellulose into glucose. Although celluloytic enzymes are synthesized by large number of microorganisms, it is only the fungi which appears to excrete large amounts of cellulase enzymes in active form into the culture media [wood, T.M. (1985). Properties of cellulolytic enzyme systems, Biochemical Soc. Transactions.13, 407 - 410]. In addition, only a few cellulases are known which have the capacity of hydrolyzing native cellulose which has high degree of crystalline structure. Trichoderma viride [Ogawa, K. and Toyama, N. (1972) J.Fermentation Technology, 50, 236] and Trichoderma reesei (Mandels, M. and Reese, E.T., 1964, Dev. Indust. Microbiol 5 , 5-20) have been reported to be of industrial potential. In the present study we have used Trichoderma viride strains to improve the production of

extracellular cellulase complex. The cellulase complex is composed of three different hydrolytic activities: endo-1, 4, β-D-glucanase (E C 3.2.1.4) which attacks the amorphous cellulose, but does not hydrolyze the crystalline cellulose; cellobiohydrolase (EC 3.2.1.91) which hydrolyzes both amorphous and crystalline cellulose; and cellobiase, (EC 3.2.1.21) which hydrolyzes cellodextrins and cellobiose into glucose. Thus, it seems necessary that for the efficient solubilization of cellulose three components of cellulase complex be present in appropriate concentration. We have approached this problem to grow Trichoderma viride cell cultures under such conditions which secrete large amount of cellulase complex or by supplementing the enzyme components, as for example by addition of appropriate amount of cellobiase enzyme.

Results reported here indicate that if Trichoderma viride strains 1051 and 1061 are grown in the presence of 1% cellulose in submerged fermentation for 13 days at 30°C, cellulase which is sufficiently active towards filter paper (crystalline form of cellulose) and carboxy methyl cellulose, was secreted into the medium in fairly large concentration, 1 iu/ml. But unfortunately, it was extremely poor in cellobiase activity. However, under identical conditions of submerged fermentation Aspergillus niger 683 and Aspergillus wentii Pt-2804 produced at least 6 times higher cellobiase compared to the Trichoderma strains, but the amount of cellulase activity against filter paper or CMC was only one-tenth of the celluloytic activity secreted by the

Trichoderma viride strains. Thus, we supplemented the cellulase produced by Trichoderma viride 1060 with necessary amount of cellobiase. By doing so it was possible for us to achieve up to 80% hydrolysis of Mangifera indica cellulose fiber.

The cellulose concentration used to induce the cellulase enzyme in Trichoderma viride appeared to be critical for the production of extracellular cellulase enzyme complex. When concentration of cellulose in the medium was greater than 0.75% the overall activity of extracellular cellulase was declined. In fact at 3% cellulose concentration the activity of cellulase in the medium was declined 3 fold. The possible reason for this decline in the yield is probably due to the fall in pH of medium containing higher cellulose concentration during the metabolism. For instance at 3% cellulose the pH of medium in 48h became 2.5. This was further supported by the fact that when the pH of the medium was controlled with the help of 0.1M sodium citrate buffer higher yield of cellulase was obtained even with 2% cellulose in the growth medium. It has been argued that the fall in pH of medium during the incubation was not due to secretion of organic acids, but was caused by the consumption of NH_4^+. With the uptake of NH_3 there is a concomitant accumulation of H^+ in the medium. The low pH levels which develop under these circumstances are at least partly responsible for the loss in enzyme activities. It is also apparent from the studies that Trichoderma viride also contains low levels of β-glucosidase. The reason of

exhibiting less activity against p-nitrophenyl β-D-glucopyranoside is the acid conditions that develop in the medium during the time of maximum cellulase production. When the medium is buffered to about 4.8, extracellular β-glucosidase activity increased by ten fold as compared to the enzyme produced in unbuffered medium (Fig.10). Mandel et al.(101) also predicted the same reason of not getting the promising yields of β-glucosidase. It was also observed that high pressure steam followed by alkali treated saw dust produced comparatively more cellulase and β-glucosidase than untreated saw dust at 1% concentration (w/v). The present findings agree with the work of Chahal (24) who also reported pure cellulose as a better inducer of cellulase complex than lignocellulosic substrates. The low yield of cellulase complex was not unexpected, however, since in untreated saw dust cellulose was present as a ternary complex with hemicellulose and lignin. It was found that when glucose and cellulose were used in the ratio of 1:3 as carbon source, the cellulase activity in the medium was enhanced substantially. These results suggest that presence of glucose in the medium along with cellulose helped cell growth in the initial stage in particular to give necessary cell mass which was then induced by cellulose to produce cellulase enzyme. In the case of β-glucosidase synthesis by Aspergillus wentii the maximum yield of the enzyme was obtained when the cells were grown on 2% starch and the lowest yield was when cellulose was used as the carbon source. It is, therefore, apparent that the induction of two enzymes occurs differently and Trichoderma viride or Aspergillus wentii alone may not be

used to prepare high specific activity cellulase complex. The feasible alternative is to prepare extracellular cellulase by Trichoderma viride and supplement this with the β-glucosidase prepared separately by cultures of Aspergillus wentii.

The production of cellulase complex on soluble sugars indicates that the enzymes in cellulase complex are consitutive in T. viride and A. wentii. Considering the importance of Mg_2^+ in tricarboxylic acid cycle (TCA) effect of Mg_2^+ ion on the cellulase production by T. viride and β-glucosidase production by A. wentii was investigated. As expected Mg^{2+} enhance the overall production of extracellular cellulase. In addition to carbon source and Mg^{2+} some surfactants have also been reported to increase the production of extracellular cellulase enzymes (14,136). We have also found that use of 0.25% Tween-80 increased the level of cellulase activity in the medium. The effect of Tween-80 has been attributed to its action in causing an increase in membrane permeability, partly to inducer formation and partly to promotion of enzyme release into the medium.

Preparation of inoculum was also found to be an important factor for production of extracellular cellulase. We found that cells of Trichoderma viride that were cultured for 24 h in 1% glucose containing medium gave a very active inoculum for production of cellulase. These results were in agreement with the findings of Smith and Berry (148). Thus,

by modifying the medium and growth conditions for T. viride it was possible to obtain sufficient amount of cellulase enzyme with reasonably high specific activity for hydrolyzing cellulose in a small bioreactor of 5 litres capacity.

5.3 Enzymic Hydrolysis Of Mangifera Indica Saw Dust

So far it has been shown that high pressure steaming followed by NaOH extraction was necessary to render the lignocellulosic mass susceptible to the enzymatic hydrolysis. Trichoderma viride cellulase used for enzymic hydrolysis was found to exhibit endo- and exo - β 1,4-glucanase activities, but was highly deficient in cellobiase activity. Thus, supplementation of Trichoderma viride cellulase with Aspergillus wentii β-glucosidase was found to be extremely useful in achieving upto 80% hydrolysis of Mangifera indica saw dust cellulose, which may be considered extremely good and makes the enzymic digestion of cellulose very attractive.

The results of the hydrolysis of the Mangifera indica saw dust by cellulase complex are in complete agreement with the currently accepted model for the enzymic hydrolysis of cellulose [Wood, T.M.(1985). Biochemical Soc. of transactions. 13, 407 - 410]. This model envisages that endo-glucanase initiates the attack and the newly formed non-reducing chain ends are then attacked by the exoglucanase (Cellobiohydrolase) to yield cellobiose. The accumulation of the latter product becomes inhibitory to the cellulase

activity. Hence, simultaneous hydrolysis of cellobiose by cellobiase results in increased rate of the cellulose digestion. Compared to the saccharification of pretreated Mangifera indica saw dust cellulose fiber, the Avicel microcrystalline cellulose was poorly hydrolyzed by the Trichoderma viride cellulase complex, indicating a lesser degree of crystallinity in the former.

Concentration of cellulose was found to have a profound influence on the overall hydrolysis of cellulose. In the case of Mangifera indica saw dust maximum hydrolysis was achieved at 2.5% cellulose concentration, whereas at 10% cellulose concentration the saccharification of cellulose was decreased by 30-35%. This indicates that at lower concentrations the substrate is easily accessible to the enzyme than at the higher substrate concentration. This is further supported by the fact that at 2.5% cellulose concentration maximal hydrolysis was attained in less than 18 h compared to 30 h or more at higher concentration. The decrease in percent enzymic saccharification of cellulose with the increasing concentration of cellulose was also reported by Nozinic and Drazic (127). From economics point of view, however, it may be recommended to use 10% cellulose rather than 2.5%, since it would allow to process 4 times more material with little difference in overall yield and this would be in line with the recommendations of Toyama and Ogawa (159) who found 8.5% substrate concentration economical for enzymatic hydrolysis.

The pH 4.8 found optimum for maximum saccharification of Mangifera indica saw dust by Trichoderma viride cellulase is in complete agreement with Erikson (44),Mandel's(101), Toyama(160) and Ismail et al. (75) used pH 5.0 for the saccharification of bagasse. Sadana et al.(140) and Herr(68) reported an optimum pH of 4.5 for the saccharification of cellulose with the cellulases of S. rolfsii and T. viride ITCC - 1433. From these data it seems that optimum pH for the enzymic saccharification of cellulose is between 4.5 and 5.0.

The data of enzymic hydrolysis are highly promising and strongly suggest for pilot plant studies.We have successfully scaled up the process upto 10 litres and the hydrolyzate obtained was used directly for the production of single cell protein (Bakers yeast) and also for the production of citric acid by fermentation using Aspergillus niger. The results were highly comparable with those when glucose and sucrose were used as substrates for production of yeast and citric acid, respectively. In fact, enzymic hydrolyzate of Mangifera indica saw dust is a better substrate than molasses, as unlike the latter, it can be directly used for without pretreatment. Work on establishing a pilot plant for citric acid production from enzymatic hydrolysis of Mangifera indica saw dust is being undertaken.

CHAPTER VI

Summary And Conclusion :

The purpose of the present study was to explore the possibility of using lignocellulosic wastes for production of fermentable sugars which in turn would provide cheap subsrates for production of liquid fuel (ethanol) and the other useful chemical, single cell protein, etc. Mangifera india saw dust is produced in huge quantities as a by product of timber industry in North India. The native cellulose of Mangifera indica saw dust, like any other classical lignocellulosic material is present as a ternary complex composed of approximately 25% hemicellulose, 25% lignin and 50% cellulose. In addition, native cellulose is present in a highly crystalline form which together with lignin component makes it resistant to the enzymatic hydrolysis. Of the different methods of pretreatment, the treatment of high pressure steaming at 350 $\pm$ 15 psi for 30 min. followed by alkali extraction at room tempreature was found to be the most efficient method. This method broken the saw dust, a ternary complex of hemicellulose, lignin and cellulose. The generated cellulose was found to be susceptible for cellulolytic enzymes which was further conformed by comparing the X-ray diffractograph of untreated and treated saw dust.

The microorganisms Trichoderma viride 1060 and Aspergillus wentii were found to be suitable strain for production of cellulase and β-glucosidase enzymes on large scale. The pH below 2.5 and above 5.0 were responsible for the loss in enzyme activities during enzymes production so the medium were buffered with 0.1 M sodium citrate buffer (in shake flasks), prior to the start of enzyme production. In case of buffered medium the level of β-glucosidase in cellulase (T. viride) increased by ten fold as compared to the enzyme produced in unbuffered medium (Fig.10). The lignin and hemicellulose free Mangifera indica saw dust after high pressure and alkali pretreatment, was nearly as good carbon source for production of extracellular cellulase and β-glucosidase as the Avicel microcrystalline cellulose. Untreated lignin containing saw dust found to be a poor carbon source. The present findings were in agreement with the studies of Chahal (24) that cellulose is a better inducer of cellulase complex than lignocellulosic material. The β-glucosidase production by A.wentii found to be maximum, when grown on starch as carbon source. The optimum conditions for production of extracellular cellulase by Trichoderma viride and β-glucosidase by Aspergillus wentii are summarized in the table given in the following table.

OPTIMUM CONDITIONS FOR THE LARGE SCALE PRODUCTION OF EXTRACELLULAR CELLULASE AND β-GLUCOSIDASE (CELLOBIASE) ENZYMES FOR CELLULOSE DIGESTION.

Conditions	Enzyme	
	Cellulase	β-glucosidase
Culture strain	Trichoderma viride-1060	Aspergillus wentii Pt-2804
Carbon source	Pretreated cellulose[a]-glucose[b] (3:1), 2%	Starch, 2%
Incubation period	13 - 14 days	13 - 14 days
Incubation temp.	30°C	30°C
pH	4.8 - 5.0	4.8 - 5.0
$MgSO_4$	0.03%	0.02 - 0.03%
Inoculum age and level	Cells grown for 24h on glucose containing medium; 5%	Cells grown for 24h on glucose containing medium; 7%
Tween-80	0.03 - 0.04%	0.03 - 0.04%

a : Mangifera indica saw dust was subjected to high pressure steaming followed by dilute sodium hydroxide extraction.

b : Since pretreated cellulose alone also is an excellent carbon source for production of extracellular cellulase, in large scale production only cellulose was used for cellulase production, unless stated otherwise.

The above conditions were further transferred for large scale production of enzymes in 9 litre fermenter and also found to be the optimum.

Enzymatic hydrolysis of cellulose obtained from saw dust after high pressure steaming and alkali treatment, was carried out by the partiallypurified cellulase and β-glucosidase prepared in the present studies. The incubation period 48 hours and 20-30 units of Trichoderma viride cellulase complex per gram of cellulose fiber were sufficient for 65-67% saccharification of cellulose where as only 46% hydrolysis of Avicel microcrystalline cellulose was possible (Fig.21). In the presence of β-glucosidase nearly 80% cellulose was hydrolyzed by Trichoderma cellulase in 48 hours compared to only 54.6% without β-glucosidase under identical condition (Table X). Below and above pH 4.8 the percent hydrolysis of cellulose declined sharply. 50 C temperature was an required temperature to achieve 80% hydrolysis of pretreated saw dust of Mangifera indica. The optimum conditions for the enzymatic hydrolysis of Mangifera indica cellulose fiber are given as under.

Cellulose fiber concentration	2.5 to 5 % (w/v)
pH of incubation mixture	4.8
Temperature	50 C
Cellulase concentration	1.2 to 2.0 units/ml
β-glucosidase supplementation	1.5 to 2.0 units/ml
Incubation period	20 to 36 h

Under these conditions about 80% cellulose was hydrolyzed to glucose by Trichoderma viride extracellular cellulase when used in combination with equal amount of Aspergillus wentii β-glucosidase. On the basis of above optimum conditions a reactor of 5 litre capacity was designed and the results were in agreement with the results obtained above.

The production of yeast on enzymatic hydrolyzate of Mangifera indica found to be comparable to that of the control in which 2% dextrose (w/v) used as a carbon source.

The process was scaled up to 10 litres capacity; to produce 1 Kg of yeast in 24 hours. The enzymatic hydrolysate of Mangifera indica saw dust appears to be promising for yeast growth and the process can be extended to pilot plant production.

The hydrolyzate prepared by digestion of Mangifera indica saw dust with cellulase in the presence of β-glucosidase, found to be cheap substrate for citric acid production. This substrate did not require any pretreatment as in case of molasses. The citric acid production (58 mg/ml) found to be possible at 10% (w/v)glucose concentration. Further investigations will be required to develop the complete process.

The overall economic feasibility is certainly dependent on the efficient utility of all main components of the lignocellulosic waste materials i.e. cellulose, hemicellulose and lignin. The economy is not great enough to allow any of these to be disregarded. The present status of feasibility studies is under process. The only way to get rid off expensive technology, is to design such a technology, considering all the important features of the utilization of lignocellulosic materials an enormous and renewable resources.

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