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ABSTRACT

This work deals with the production of cellulase-system by using lignocelluloses as a source of carbon.

Three different mutants of Trichoderma reesei (Rut-C30, QM9414, and QMY-1) were compared by their enzyme productions. QMY-1 appeared to have as good cellulase system as Rut-C30.

The use of variously pretreated aspen wood revealed that the Chemithermomechanical pulp (CTMP) was a better substrate for cellulase production than sodium hydroxide treated wood. The cellulase yield (283 IU/g cellulose supplied) by growing T. reesei QMY-1 on CTMP1 was higher than that obtained by others from Rut-C30 grown on various substrates.

The substrate concentrations more than 2% in conventional fermenter forms a thick slurry. Because of poor mass transfer poor cellulase production was recorded when the concentration of CTMP was more than 2%.

The experiments on the effect of pH on different substrates revealed that the best pH-levels for cellulase production on pure cellulose (Alpha Cellulose) and ligno- cellulose (CTMP1) were 5 and 6, respectively.

By collecting samples at shorter time intervals two-phase cellulase production was noticed on Alpha-Cellulose and CTMP1. It may be due to the fact that cellulose in both of the substrates contained amorphous and crystalline cellulose; the former being easily utilized than the latter. The first phase of cellulase production was due to the growth of organism on amorphous cellulose and the second phase started after a lag of a few hours of adaptation of the organism on crystalline cellulose for cellulase production. The two phase cellulase confirmed by repeated experiments.

A documented fermentation run was performed using 1% CTMP1 in which filter paper, β-glucosidase, cotton cellulase (C_1) and xylanase activities were determined at short time intervals along with the residual cellulose and protein contents of the fermentation broth; acid/base consumption and dissolved oxygen (DO) were monitored; and Qo_2 and K_1a values were calculated.

ACKNOWLEDGEMENT

I am indebted to my thesis Supervisor, Professor Gérald André for his indispensable advise and suggestions and Professor Devinder Singh Chahal for his guidance in conducting this work at his laboratory (Applied Microbiology Research Centre, Institut Armand Frappier, Laval, Quebec).

I would like to thank Drs S.N. Lo and J.L. Valade of University of Quebec, Trois-Rivieres, Quebec, for supplying Chemithermomechanical pulp (CTMP).

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NOMENCLATURE

a	Interfacial surface area for mass transfer (m^2/m^3).
°A	Angstrom.
°C	Degree Centigrade or Celsius.
C_*	Concentration of Dissolved Oxygen (mg/l).
C^*	Concentration of Dissolved Oxygen at Saturation (mg/l).
CTMP	Chemithermomechanical pulp.
d	day.
dC/dt	Rate of change in Dissolved Oxygen Concentration (mg/l/s) or (mg/l/h).
DIP	Decrease In enzyme Production.
dN/dS	Amount of sodium hydroxide (N in g) required per 100 g of wood for every increase in percent solubilization (S).
DO	Dissolved Oxygen (mg/l) or (m mole/l).
FPA	Filter Paper Activity (IU/ml).
g	gram.
h	hour.
IU	International Unit = (µ mole of glucose produced/min).
k	Temperature in °C.
kg	kilogram.
K_l	Mass transfer coefficient, (m/s) or (m/h).
l	(or L) liter.
m	meter.
min	minute.
ml	milliliter
M t	Million ton.
P	Barometric Pressure (mm Hg).
P'	Saturation vapour pressure of water (mm Hg).
Qo_2	Specific Oxygen Uptake rate, (mg/g protein/s) or (mg/g protein/h).
rpm	(or RPM) Agitation speed (round/min).
s	second.
t	time (in second).
v/v	Volume by Volume.
vol	Volume.
wt	Weight.
x	Biomass Expressed as Protein Concentration (g/l).
µm	10^{-6} meter.
µ	Specific Growth Rate (h^{-1}).

1. INTRODUCTION.

As the supply of fuel reserves and food available dwindled and their respective prices augmented over the past several years, the use of ethanol as an alternative liquid fuel source and single cell protein (SCP) as an alternative food source has attracted more interest. It is believed that these alternative sources can be successful only if renewable materials are used for their production. Literature survey indicates that energy provided by sun is preserved efficiently through photosynthesis by plants on the planet Earth. Therefore, the crop residues and forest wastes, which are produced every year, can be used to educe the energy stored in them. There is a large availability of lignocelluloses (wood) in Canada, about 2.5 times than that of USA (Table 1.1) (Lowe and Dolenko, 1981; US Department of Energy, 1979). Wood and crop residues, the renewable wealth in Canada, have been considered to be the ultimate choice for production of ethanol, SCP, and other useful products (Fig. 1.1).

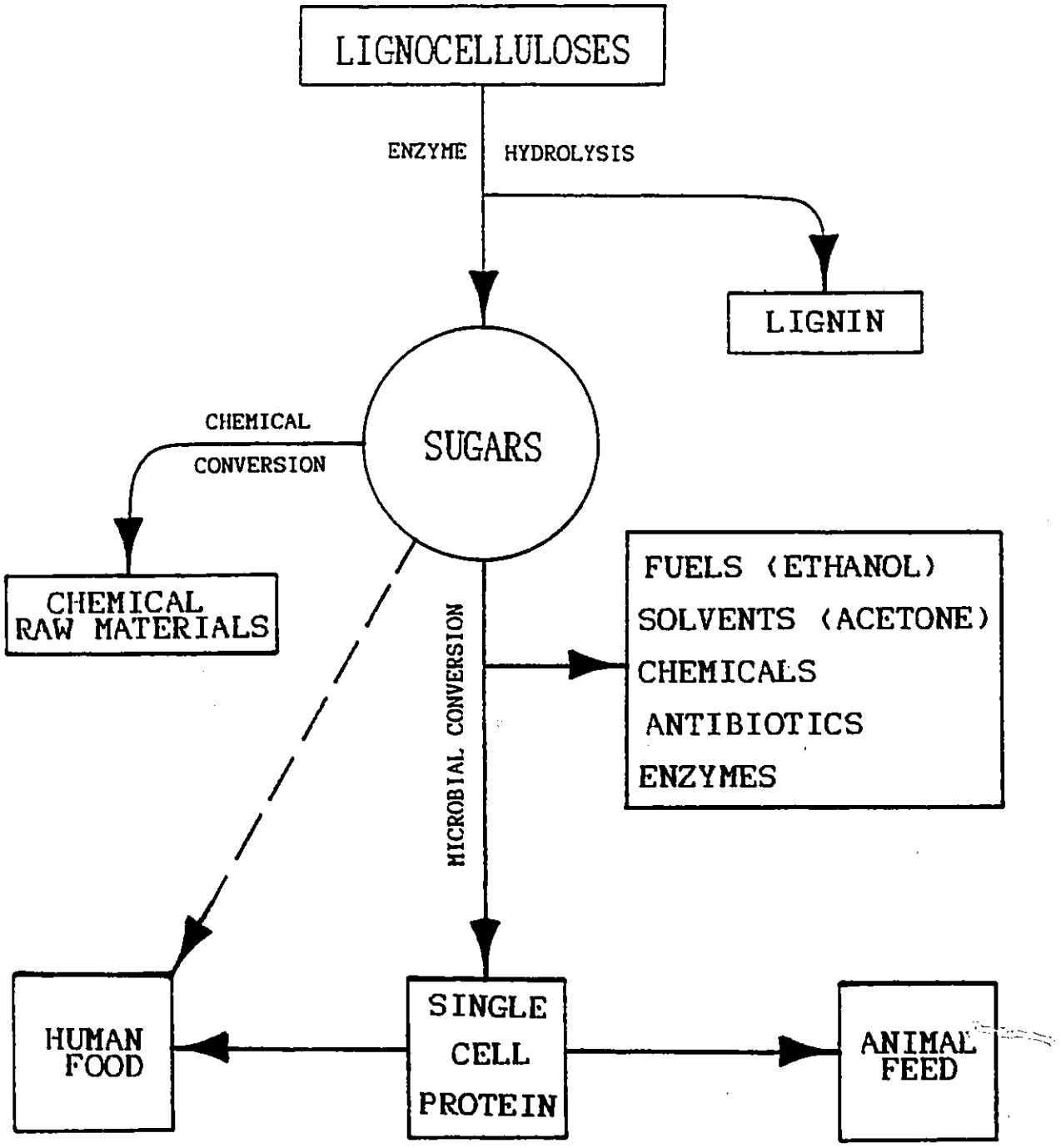
The US gasohol (a blend of 17% ethanol in gasoline) program shows that, whatever the future may hold, the biomass-derived liquid fuel of today is

TABLE 1.1. AVAILABILITY OF LIGNOCELLULOSES IN CANADA AND USA IN 1980.

TYPE OF LIGNOCELLULOSES:	QUANTITY M t / year Canada	USA *
Forest biomass	1818.0	449.0
Agricultural residues	36.0	193.0
Municipal wastes	15.5	86.0
TOTAL	1869.5	728.0
Reference	Lowe and Dolenko, 1981	U.S. Department of Energy 1979.

Canada has about 2.5 times more lignocelluloses than that of USA.

* Projected maximum US Resources available.


LIGNOCELLULOSES
ENZYME HYDROLYSIS
LIGNIN
CHEMICAL
CONVERSION
SUGARS
CHEMICAL
RAW MATERIALS
FUELS (ETHANOL)
SOLVENTS (ACETONE)
CHEMICALS
ANTIBIOTICS
ENZYMES
MICROBIAL CONVERSION
HUMAN
FOOD
SINGLE
CELL
PROTEIN
ANIMAL
FEED

ethanol (Stone and Marshall, 1980). To get ethanol, sugar must be produced from lignocelluloses by acid or enzymatic hydrolysis. It has been reported by Converse and Grethlein (1979), that the sugar yield of 50-57% of the weight of cellulose can be achieved by acid hydrolysis. Recently, Burton (1982) reported 70% monosaccharides recovery from aspen wood by acid hydrolysis. Due to high operating temperature (170°C) and high acid concentrations, expensive corrosion-proof reactors which can also tolerate high pressure are required for acid hydrolysis. On the other hand, glucose yield of 100% of the cellulose has been obtained by Spano et al. (1975) in 48 h at relatively moderate temperature (45-50°C) and pressure (1 atm).

The survey of the literature indicated that very little work has been done on enzyme production from forest biomass (a crude source of cellulose). It might be due to the fact that the forest biomass is more compact and heavily lignified and it is very difficult to prepare it for its utilization to produce cellulase. Most of the work conducted so far on cellulase production was by using pure cellulose as a source of carbon. Ryu et al. (1980), Tangnu et al. (1981), Hendy et al. (1982), Saddler et al. (1982), Watson and Nelligan (1983) and Duff et al. (1985) achieved

cellulase yield in the range of 203 to 280 IU (International Units: measured in Filter Paper units (FP units)) / g substrate by growing Rutgers C-30 strain of Trichoderma reesei on pure cellulose. It has been pointed out by Chahal (1982 a) that at present there is a very little possibility to increase the yield of cellulase (in terms of FP units / g of cellulose supplied) until a 'super bug' is produced. However, concentration of enzyme per unit volume (solution) can be increased by increasing the concentration of cellulose in the fermentation medium. Duff et al. (1985), Saddler et al. (1982), Tangnu at al. (1981), Watson and Nelligan (1983) and Ryu et al. (1980) reported 2.5, 4.4, 5.2, 12.0 and 14.0 IU (FP units)/ml of enzyme activity by using culture media containing 1, 2, 2.5, 5 and 6 % cellulose respectively, but their hydrolytic potential has not been mentioned. Hydrolytic potential is measured by hydrolyzing cellulose with cellulase in concentration of 20 IU/g cellulose and the amounts of glucose and cellobiose produced during the course of hydrolysis are recorded. High glucose and low cellobiose contents at a given time (usually 48-96 h) show higher hydrolytic potential of the enzyme system. Therefore, it is necessary to develop a process in which high amount of cellulase per unit volume (yield) and per unit time (productivity) can be produced by

using more realistic source of lignocelluloses (cheep source of cellulose).

The present study was taken up for the production of cellulases on lignocelluloses with the following objectives:

1. Select a suitable mutant of Trichoderma reesei which can grow and produce cellulase in higher concentration as well as in less time, when lignocelluloses will be used as a source of carbon.
2. Evaluate the hydrolytic potential of the cellulase system produced on lignocelluloses by the selected mutants.
3. The major hinderance in the use of lignocelluloses for enzyme production is the presence of lignin. Lignin in the cell wall encrusts the cellulose (Higuchi, 1971), and therefore, removal of lignin to expose carbohydrates (cellulose) is a must before one can use lignocelluloses as a substrate for cellulase production. Therefore, development of optimal conditions for pretreatment of lignocelluloses with sodium hydroxide to make them suitable for cellulase production and its comparison with the use of Chemithermomechanical pulp (CTMP) as substrate for cellulase production was also one of the objectives of

this study.

4. Development of optimal culture conditions (pH, addition of Tween 80, substrate concentration and type of the substrate) for cellulase production.

During this study, the time interval in collecting samples has been shortened in order to investigate the profile of enzyme production. The profile of enzyme production shows a decrease in enzyme production which resumes after a few hours (depending upon the culture conditions). This phenomenon has been labeled as 'DIP' in this study. Attempts were made to understand and to minimize the 'DIP' in order to achieve high yields of cellulase of high hydrolytic potential by utilizing lignocelluloses as a source of carbon.

2. LITERATURE SURVEY

In order to gain understanding about the production of a cellulase-system, a number of different subject areas has been scrutinized. It would be impossible to review all the available information on this subject but an attempt has been made to present an overall view of the production of the cellulase- system with special emphasis on later trends. Thus, literature has been reviewed under the following headings:

2.1. Lignocelluloses.

- 2.1.1. Structure of Plant Cell Wall.
- 2.1.2. Cellulose Availability from Lignocelluloses.

2.2. Biodegradation of Lignocelluloses.

- 2.2.1. Cellulolytic Microorganisms.
- 2.2.2. Cellulase System.
- 2.2.3. An Updated Complete Picture of Cellulase-System.

2.3. Cellulase Production.

2.1. LIGNOCELLULOSES

Cellulose is widely distributed in plants, the two principal types being: pectocelluloses and lignocelluloses. Pectocelluloses of flax, hemp and ramie etc., contain 82-85% cellulose. Lignocelluloses of cereal straws, wood and jute etc., contain 30-75% cellulose. The composition of various lignocelluloses is given in Table 2.1 Wood has high cellulose content while crop residues have low. On the other hand, the hemicelluloses content depends on the type of the material. The lignin content in general is higher in woody materials than in crop residues. About 25-60% of the lignocelluloses consist of hemicelluloses, lignin, protein and various extraneous materials.

The use of pectocelluloses is limited because of their scarcity. On the other hand, lignocelluloses are in aboundance and in certain cases they are cheap. Therefore, it has gained a favour among many researchers (Chahal, 1985; Deschamps *et al.*,1985; Peitersen, 1975; Sukan *et al.*, 1985; Tangnu *et al.*, 1981). It was observed that residues from cereal crops were often chosen as a substrate for cellulase, ethanol or single-cell protein (SCP) production. The woody materials have been tried by a few workers (Chahal *et*

TABLE 2.1. COMPOSITION OF LIGNOCELLULOSES (% dry wt).

TYPE OF LIGNO-CELLULOSES	CELLULOSE %	HEMI-CELLULOSES %	LIGNIN %	REF #
FOREST RESIDUES				
Aspen *	56.8	22.9	20.5	Bernier et al., 1983.
Aspen *	53.0	31.0	16.0	Timel, 1957.
Aspen (Average of *)	55.0	27.0	18.0	
Birch (angiosperm)	46.1	24.6	26.3	Cowling, 1975.
Spruce (gymnosperm)	46.1	24.6	26.3	" "
Wood (angiosperm)	40-55	24-40	18-25	Cowling and Kirk, 1976.
Wood (gymnosperm)	45-50	25-35	25-35	" "
Wood (angiosperm)	49	23	28	" "
CTMP1	60	NA	NA	This work
CROP RESIDUES				
Sugarcane Bagasse	33.4	30.0	18.9	Clark, 1969.
Wheat straw	30.5	28.4	18.0	" "
Wheat straw	34.7	20.2	14.5	Wilke, 1981.
Rice straw	32.1	24.0	12.5	Clark, 1969.
Rice straw	39.0	18.2	9.9	Wilke, 1981.
Barley straw	37.5	19.0	13.8	" "
Corn stover	37.0	16.9	15.1	" "
Grass, Bamboo, Wheat & Rice straw, & Sugarcane bagasse	25-40	25-50	10-30	Cowling and Kirk, 1976.
Municipal Solid Wastes (Cellulose remaining after removal of plastic and metal from the solid waste)	61	22	9	Emert and Katzen, 1979.

NA Not Analyzed.

al., 1982, 1984, 1985, 1986; Forintek Canada Corp. ENFOR Project 1983; Khan et al., 1984) for the same purpose. The high contents of lignin and the complex structure of woody material could be the reason that it did not gain favourable interest among the scientists to use it as a substrate for cellulase production. In order to better understand and study the utilization of lignocelluloses for cellulase production, their structure must be known. Understanding the nature of orientation of cellulose fibrils and the presence of hemicelluloses, lignin and other substances in the lignocelluloses, would help in studying the utilization of lignocelluloses by the organism to produce cellulase and also in studying the hydrolysis of lignocelluloses.

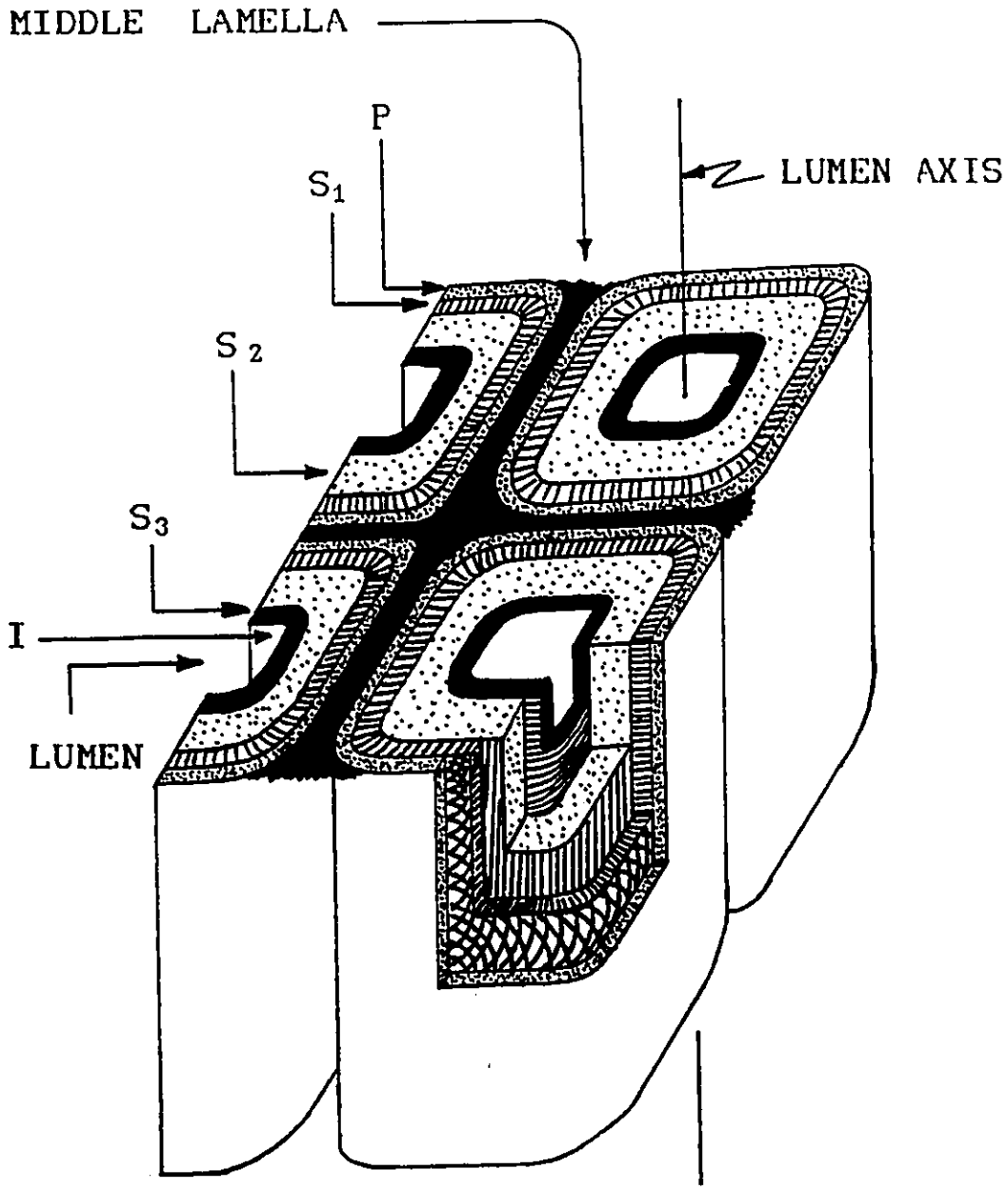
2.1.1 Structure of Plant Cell Wall

The cellulose in cotton and wood is very similar in molecular structure. The cotton fibre is unicellular and is an outgrowth on the seed of the cotton plant and it is the purest cellulose found in nature. But, the cellulose of lignocelluloses (wood and crop residues) is intimately mixed with lignin and hemicelluloses and is a part of the cell wall of the plant tissues. Cotton fibres are formed independently and thus contain no intercellular substance. Wood

fibres, on the other hand, form a cohesive three dimensional structure (Brown et al., 1949), the integrity of which is assured by large amounts of intercellular substances (Fig. 2.1) (Hagglund, 1949). Both cotton and wood cells have a thin primary wall that surrounds the relatively thick secondary wall. The primary wall (0.1 to 0.2 μm thick) contains randomly and loosely organized network of cellulose microfibrils. The outer layer of secondary wall, S1 has a crossed fibril structure. In the S2 layer, the main portion of secondary wall (1-5μm thick), the microfibrils are oriented almost parallel to the lumen axis. In the thin S3 layer (0.1 μm) the microfibrils form a flat helix. The innermost portion 'I' of the cell wall consists of the so-called warty layer, probably formed from protoplasmic debris. The central empty portion, formed after the disintegration of protoplasm at the time of aging, is called lumen. The primary wall is mostly composed of cellulose and pectic compounds. The primary walls of two adjacent cells of lignocelluloses are cemented together with pectic compounds and lignin. The space between two adjacent cells is called middle lamella.

Figure 2.1. Structure of wood cell.

P	-Primary wall. -Loosely organized network of cellulose microfibrils.
S_1	-Secondary Wall$_1$. -Crossed fibril structure.
S_2	-Secondary Wall$_2$. -Microfibrils almost parallel to the lumen axis.
S_3	-Secondary Wall$_3$. -Microfibrils form a flat helix.
I	-Warty layer formed from protoplasmic debris.
Lumen	-Central empty portion formed after the disintegration of protoplasm at the time of aging.


MIDDLE LAMELLA
P
S1
LUMEN AXIS
S2
S3
I
LUMEN

2.1.1.1. Cellulose

Within each layer of secondary wall, the cellulose occurs as long, slender bundles composed of long chains of anhydroglucose residues held together by β-1,4 linkages, called elementary fibrils, with diameter of 35 °A. The length of cellulose molecule in elementary fibril is in order of 10 000 residues of anhydrous glucose units. Each residue is placed at 180 rotation about the main axis with respect to its neighbouring residues. The cellulose in "arm chair" configuration built up of the basic recurring unit of cellobiose can be represented as in Fig. 2.2.

A number of elementary fibrils, when joined laterally form microfibrils. A few recent concepts about the structure of microfibril are discussed as follows:

According to Preston and Cronshaw (1958), the microfibril is about 50 X 100 Å in cross section and consists of a crystalline core of highly ordered cellulose surrounded by a sheath which in cotton contains mainly amorphous cellulose molecules but in wood also contains hemicelluloses and lignin molecules (Fig. 2.3). In this figure, the solid strokes

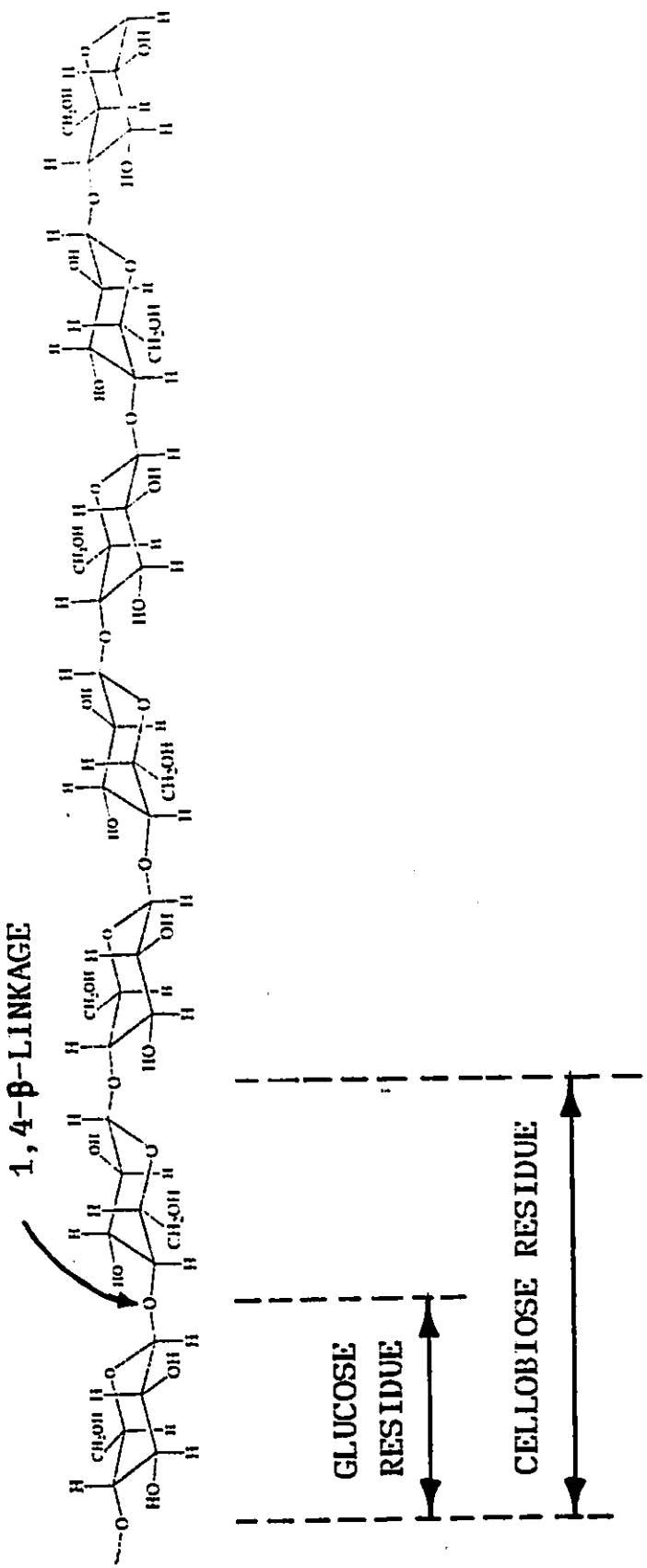


Figure 2.2. Structure of Cellulose.

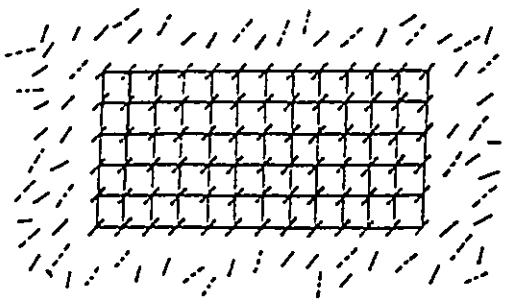


Figure 2.3. Structure of Microfibrils according to Preston and Cronshaw, 1958.

Diagramatic representation of a cellulose microfibril about 100°A wide and about 50°A thick. The solid strokes represent the planes of the glucose residues in the cellulose chain molecule and the broken strokes the planes of the other sugars or sugar derivatives in non-cellulosic molecular chains. The area joined into lattice represents the solid central core.

represent the planes of the glucose residues in the cellulose chain molecules and the broken strokes the planes of other sugars or sugar derivatives in non cellulosic molecular chains. The area joined into a lattice represents the solid central core.

Preston and Cronshaw (1958) also reported that the central crystalline core does not, however, run uninteruptedly along the whole length of a microfibril, and that the microfibril is, therefore, hetrogeneous along its length. It was reported that, there are regions of weakness, irregularly distributed along the length of the microfibrils, therefore, presumably the crystallinity is disturbed in some way.

Manley (1964) reported that the microfibril is composed of a flat ribbon of cellulose molecules wound in the form of a tight helix (Fig. 2.4). But, his theory was not supported by any proof to show the existance of this structure. It was mentioned by him that "other methods of proving the structure are at present under investigation". However, Cowling (1975) accepted Manley's above concept of microfibrilar structure.

According to Rowland and Roberts (1972), the

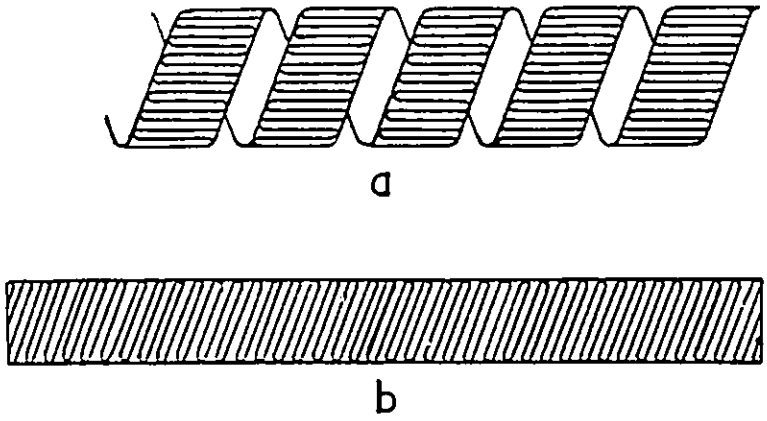


Figure 2.4. Structure of microfibrils according to Manley, 1964.

Microfibril composed of a flat ribbon (b) of cellulose molecules wound in the form of tight helix (a).

microfibrils at certain length contain strain-distorted tilt and twisted regions which are easily accessible for hydrolysis (Fig. 2.5).

Figures 2.6 and 2.7 represent the structures of microfibrils of cellulose, proposed by Gardner and Blackwell (1974) in the planes parallel to and perpendicular to the fiber axis respectively. Fig. 2.8 shows that each glucose residue form two intramolecular bonds (O3-H---O5' and O6---H-O2') and one inter- molecular bond (O6-H---O3) {Nomenclature defined in Fig. 2.8.}. The intramolecular bonds help to maintain the rigidity of the cellulose chain, whereas, the intermolecular bonds keep the cellulose chains in a tight and closely packed arrangement. The tight and closely packed arrangement strictly refers to the crystalline portion of the cellulose.

Whatever the different opinions about the structure of microfibril may be, it is certain that there are some crystalline and some amorphous regions. The regions that contain highly oriented molecules are called crystalline or micelles; those of lesser order, in which molecules are randomly oriented are called amorphous or paracrystalline regions.

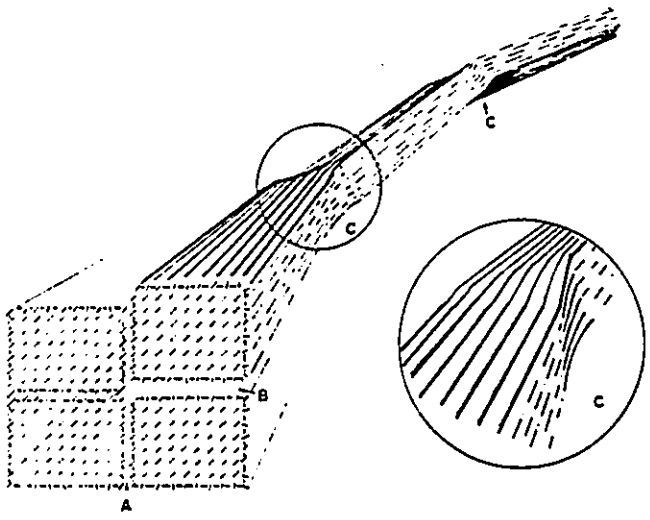


Figure 2.5. Structure of microfibrils according to Rowland and Roberts, 1972.

Schematic representation of the elementary fibril to illustrate the crystalline elementary fibril theory of the microstructure of cellulose and to show (A) coalesced surfaces of high order, (B) readily accessible slightly disordered surfaces, and (C) readily accessible surfaces of strain-distorted tilt and twist regions.

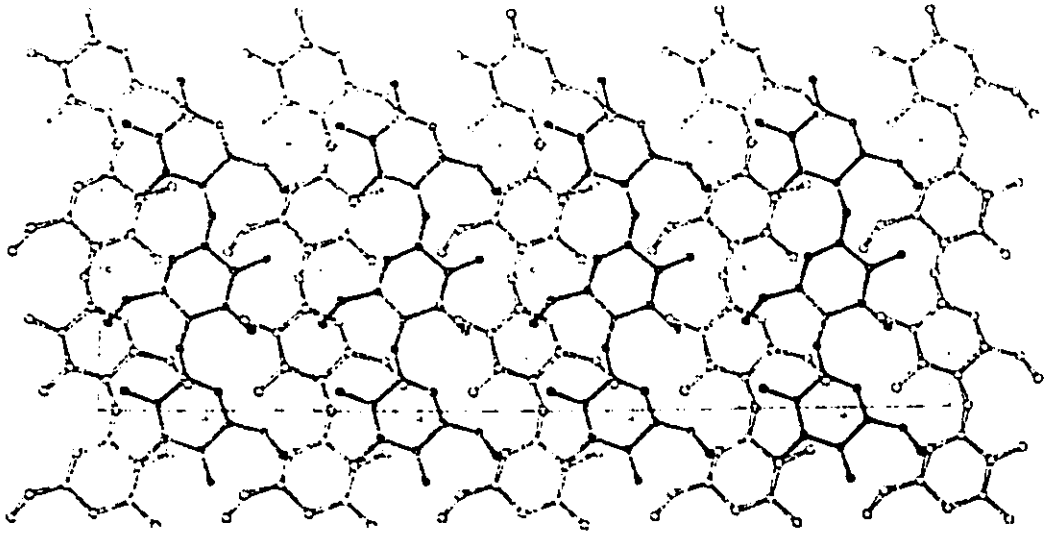


Figure 2.6. Structure of microfibrils (a) according to Gardner and Blackwell, 1974.

Projection of the proposed parallel chain model for cellulose. The unit cell is viewed parallel to the fiber axis (through ac plane).

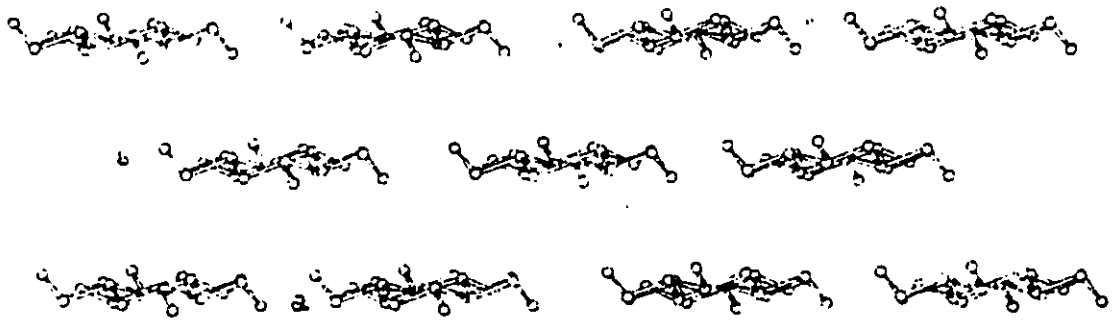


Figure 2.7. Structure of microfibrils (b) according to Gardner and Blackwell, 1974.

Projection of the proposed parallel chain model for cellulose. The unit cell is viewed perpendicular to the fiber axis (through ab plane)

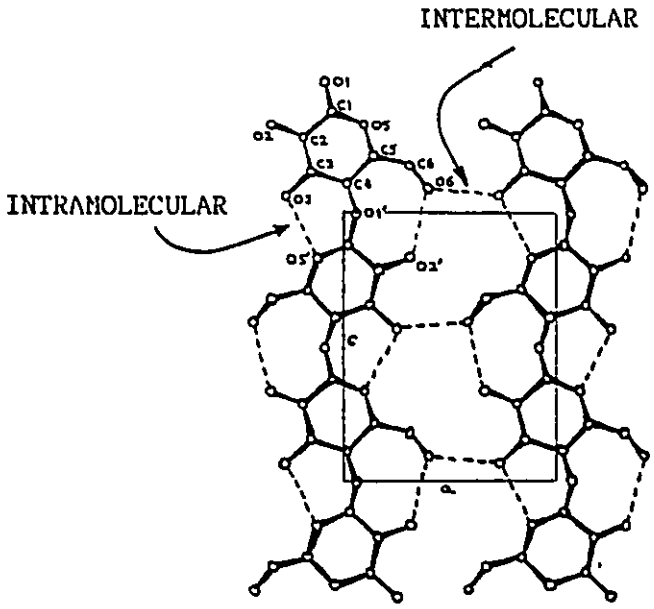


Figure 2.8. Intermolecular and Intramolecular bonds in cellulose.

-Projection of the parallel chain model for cellulose showing the hydrogen bonding network and the numbering of the atoms.

-Superscript (') refers to the atom number of the adjacent glucose molecule in the same chain.

-O, H and C refers to Oxygen, Hydrogen and Carbon atoms, respectively.

-Each glucose residue form one intermolecular bond (O6 - H --- O3).

-Each glucose residue form two intramolecular bonds (O3 - H --- O5' and O6 --- H - O2').

Lange (1958), Meier (1958), Sultze (1957) and Kalmes (1959) contributed much to the understanding of the distribution of cellulose across the cell wall which has been summerized as follows:

Crystalline material is in highest concentration near the lumen and diminishes toward the primary wall. The crystalline regions consist almost wholly of true cellulose. Because of very close packing and the strong forces of attraction between cellulose molecules in these regions, crystalline cellulose is more resistant to enzymatic or acidic hydrolysis than that of amorphous region.

2.1.1.2. Hemicelluloses

The hemicelluloses form one of the major constituents of the plant cell. The hemicelluloses include all the cell wall polysaccharides, which are removable by extraction with hot or cold dilute alkali. These can also be hydrolyzed by dilute acids to give the constituent monosaccharide units. The hemicelluloses are built up of linear and branched heteropolymers of D-xylose, L-arabinose, D-glucose, D-mannose; in addition to these, L-fucose and L-rhamnose are also present. The individual sugars may

be methylated or acetylated (Whistler et al., 1970). Hemicelluloses are commonly composed of two to six different sugar residues with a degree of polymerization (DP) of approximately 200. A D-xylose backbone with L-Arabinose side chains is the most common arrangement.

According to Timell (1967), the complete formula of hardwood xylan (O-acetyl-4-O-methylglucurono-xylan) is given in Fig. 2.9. The polysaccharide framework consists of approximately 200 β-D-xylopyranose residues, linked together by (1-4)-glucosidic bonds. Some of the xylose units carry a single terminal side chain consisting of a 4-O-methyl-α-D-glucuronic acid residue, attached directly to the 2-position of the xylose. Seven out of 10 xylose residues contain an O-acetyl group at C-2 or more frequently at C-3. The basic framework of softwood xylan (arabino-4-O-methyl-glucurono-xylan) is same as that of the hardwood. However, the softwood xylan also contains α-L-arabino-furanose residues directly linked to C-3 of the xylose.

Mannan (galacto-glucomannan) is a major component in softwood. The framework consists of (1-4)-linked β-D-glucopyranose and β-D-mannopyranose residues distributed at random (Fig. 2.10). Some of the hexose

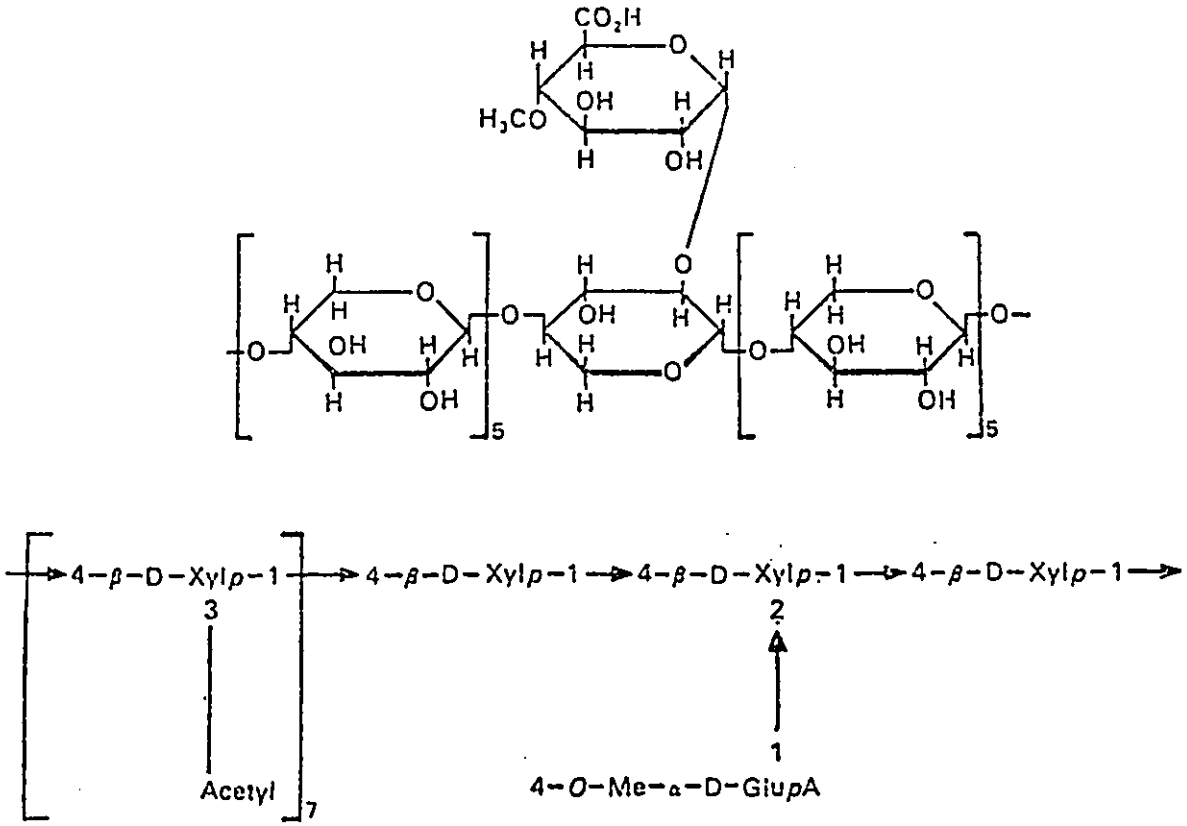


Figure 2.9. Structure and framework of Xylan.

The complete formula of Xylan (O-acetyl-4-O-methylglucurono-xylan) is shown above. The polysaccharide framework consists of approximately 200 β-D-xylopyranose residues, linked together (1->4)-glucosidic bonds. Some of xylose unit carry a single, terminal side chain consisting of a 4-O-methyl-α-D-glucuronic acid residue attached directly to 2-position of the xylose. Seven out of ten xylose residues contain an O-acetyl group at C-2 or C-3 position (Timell, 1967).

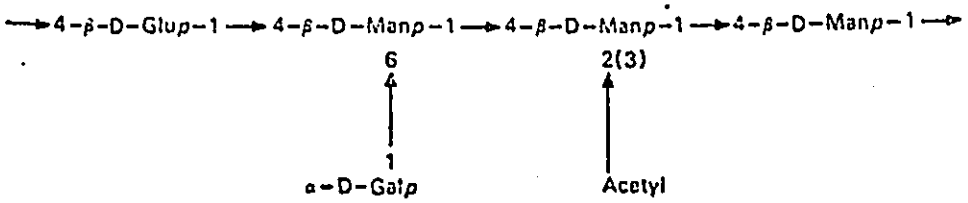


Figure 2.10. Framework of Mannan.

The framework of Mannan consists of (1->4)-linked β-D-glucopyranose and β-D-manopyranose residues distributed at random. Some of hexose units carry terminal residues distributed at α-D-galactopyranose attached to C-6 position. The acetyl groups are attached to mannose residues (Timell, 1967).

units carry a terminal residue of α-D-galactopyranose attached to C-6 (Timell, 1967). It is probable that all the galacto-glucomannans are acetylated in their native state. The acetyl groups are attached to the mannose residues. Reports of Kalmes (1959), Lange (1958), Meier (1958), and Sultze (1957) concluded that the concentration of holocelluloses (cellulose + hemicelluloses) is approximately uniform across the cell wall of cotton from the lumen through the primary wall but decreases from the lumen toward the middle lamella in wood fibers. In both types of fibers, hemicelluloses predominate in the primary wall and diminish in concentration toward the lumen.

In most of the studies pure cellulose has been used as a substrate for cellulase production. In the present study the role of hemicelluloses in cellulase production has been studied. The study on the role of hemicelluloses remained neglected in the past.

2.1.1.3. Lignin

Approximately 25% of most woods is lignin (Table 2.1). Lignin is a complex three-dimensional polymer formed from coniferyl alcohol units in the case of gymnosperms and both coniferyl and syringyl alcohol

units in the case of angiosperms. Lignin is concentrated mainly in the spaces between the cells (middle lamella) and in S2-layer of the cell wall where it is deposited during the lignification of the plant tissue although some lignin is also found in other layers. Lignin in the cell wall not only encrusts the cellulose microfibrils in a sheath-like manner but also is bonded physically and chemically to the plant polysaccharides (Higuchi, 1971).

The summarized information about lignin as reported by Kalmes (1959), Lange (1954, 1958), Meier (1958), Sultze (1957) and Wardrop (1957) is as follows: The lignin is concentrated primarily in the compound middle lamella of the wood cells and decreases in concentration towards the lumen. The amount of lignin in the secondary walls of coniferous woods is considerably higher than in angiospermous woods. The hemicelluloses and lignin form a matrix surrounding the cellulose. Within the microfibril, lignin and hemicelluloses may penetrate the spaces between cellulose molecules in the amorphous regions (Manley, 1964) providing rigidity to the fiberous wood structure.

As cellulose fibres are surrounded by

hemicelluloses and lignin, therefore, it becomes important to expose the cellulose by various physical and/or chemical treatments before it can be used for cellulase production or for hydrolysis. Vohra et al., (1980) reported that lignin and certain lignin components repress cellulase production in strain QM9414, but Natick Laboratories (Mandels, 1982) could not duplicate it.

2.1.1.4. Protein

Proteinaceous materials are the residues of the protoplast of the cell. Although the amount is quite small (0.5%) particularly in wood fibers (Reese, 1963), it is good for the growth of the microorganism.

2.1.1.5. Extraneous Materials

The small quantity of extraneous materials deposited in the capillaries of the cell wall include waxes, fats, essential oils, tannins, resin and fatty acids, terpenes, alkaloids, starch, soluble saccharides, and various cytoplasmic constituents (Cowling and Merrill, 1966; Hillis, 1962). The extraneous materials are in part deposited on the fiber surface, and in part with in the fiber wall. In cotton

most of these substances are a part of the primary wall. In wood they occur in lumen and within both the primary and secondary walls of the wood fibers (Kalmes 1959; Lange, 1958; Meier, 1958; and Sultze, 1957).

The presence of extraneous materials such as tannins, resins, terpenes, waxes and alkaloids may be harmful for the growth of the microorganism used to produce cellulase on lignocelluloses.

2.1.2. Cellulose Availability from Lignocelluloses

Most of the research work on enzyme production and hydrolysis has been conducted on pure cellulose, where isolation of pure cellulose from lignocelluloses is not an economical process for enzyme production. However, now there are many physical and chemical pretreatments available to make lignocelluloses suitable for enzyme production and hydrolysis. Some pretreatments (Steam, alkali, mild acid) make it possible to separate all the components (cellulose, hemicelluloses and lignin) while the other pretreatments (grinding, irradiation, low or high temperatures) do not.

Various pretreatments to increase the availability of carbohydrates from lignocellulosic materials as feedstock for animals, saccharification, and microbial protein production have been discussed in detail by Tarkow and Feist (1969) and Millett et al. (1975). The most common pretreatments to make the cellulose easily available for hydrolysis are described as follows.

a) Biological Removal of Lignin:

White-rot fungi (Basidiomycetes) are known to utilize lignin as well as cellulose, but they are

slow-growing organism (Cochrane, 1958). Phanerochaete chrysosporium Bursdal, a basidiomycete, has been shown to possess lignin-degrading enzymes (Eriksson, 1977) and is being tried to remove lignin from aspen wood or other lignocellulosic materials. About 50% removal of lignin from aspen wood was reported by growing P. chrysosporium for 3 d (Reid, 1979). The studies on degradation of lignin from fibres present in manure by Chrysosporium pruinosum (Rosenberg, 1980) and in wheat straw by Pleurotus ostreatus (Detroy and Rhodes, 1980) indicated that it took a long time (30-60 d) to obtain about 40% degradation of lignin.

Biological degradation of lignin does not hold any promise as a pretreatment because a cosiderable amount of carbohydrates (a valuable carbon source) is lost and the incubation time is very long.

b) Grinding/Ball Milling:

Grinding of lignocelluloses to a very small particle size yields a product very susceptible to enzymatic hydrolysis. Vibratory ball milling is the most effective method to decrease particle size, but it has been reported (Millett et al., 1970) that ball milling is species selective. With 140 min of milling

all woods more or less attain an *in vitro* digestibility plateau. However, the plateau is widely different for the different species, ranging from about 80% for aspen and sweetgum to only 20% for red alder. Softwoods are even less responsive than red alder. It has been reported that a long time is required to obtain a considerable decrease in crystallinity. It took 95 h to reduce the crystallinity index of 88.8 of Sigmacell to 36.5 (Fan *et al.*, 1980). Such a long period of ball milling may not be feasible from an economic point of view.

c) Alkali:

Sodium hydroxide and aqueous or gaseous ammonia cause extensive swelling and separation of structural elements and lead to the formation of cellulose II, which differs considerably from cellulose I when X-ray patterns are compared. Five to six grams of NaOH per 100 g of wood seems to be necessary for maximum effect (Feist *et al.*, 1970; Millett *et al.*, 1975). This level of alkali is essentially equivalent to the combine acetyl and carbonyl contents of wood. This leads to the postulate that the main cosequence of alkali treatment is the saponification of intermolecular ester bonds, thus promoting the swelling of wood beyond

water-swollen dimensions and favouring increased enzymatic hydrolysis (Tarkow and Feist, 1969).

The earliest patent issued for treatment of straw with aqueous ammonia under pressure was to F. Lehmann in 1905 (Lehmann, 1905). Another patent for a two-stage treatment was issued to Oehme and Koln-Rath in 1943 (Oehme and Koln-Rath, 1943). In this process wood meal is first reacted with NH_3 at 130 C and then oxidized with air under 10 atm pressure. In 1972, a new method of ammonia application was devised by Waiss et al. (1972). In this process dried straw is treated with 5% NH_3 and 30% water at room temperature in an airtight container for 30 d.

Both sodium hydroxide and ammonia treatments proved to be very useful in increasing the in vitro digestibility of lignocellulosic materials (Millett et al., 1975). As these treatments affect the crystallinity of cellulose, break the lignin and carbohydrate bonds, and increase the swelling power, they would greatly increase their susceptibility to enzymatic hydrolysis as well their utilization as carbon source for enzyme production by microorganisms.

d) Steam (Autohydrolysis)

Several years ago the Masonite Company developed, on a commercial scale, a process whereby wood chips were quickly heated with about 600 psi steam for about 10 min and just as quickly the pressure was released to atmospheric pressure by dumping the whole load into a large bin (Gallowy, 1975). The wood chips were exploded into large wool-like fibers. The fibers are washed to yield solubilized hemicelluloses, which are concentrated and sold as animal feed supplement under the trade name "Masonex". The fibers are used to fabricate press board.

Steam treatment under high pressure (autohydrolysis) makes the lignocellulosic materials easily accessible to hydrolytic enzymes (Alcohol Fuels Process R/D Newsletter, 1980; Bender, 1979; Casebier et al., 1969; Lora and Wayman, 1978; Noble, 1980). The basic principle of the steam treatment (Iotech, Masonite, or Stake Processes) is to pressurize the substrate (wood chips, straw, etc.) with steam in a pressure-tight reactor. Then the pressure is suddenly released and the cooked substrate is extruded through a nozzle. In the Iotech process the cooking time varies with the steam pressure used and the optimum operating

conditions are 500 - 550 psig, 40 - 50 s of cooking time, and 35% starting moisture content (Alcohol Fuels Process R/D Newsletter, 1980). Stake Technology Limited, Toronto, developed a patent Stake II continuous steam treatment system that is now in commercial operation for producing cattle feed. Steam consumption is typically 0.7 kg of steam per kilogram of dry matter processed (raw material contained 50% moisture). The steam consumption could be significantly reduced in a biomass-to-ethanol system through the employment of a heat recovery system (Taylor, 1980).

The effect of steam hydrolysis on various fractions of lignocellulosic materials can be represented by the following first-order reactions:

1. Cellulose	-->	Enzyme-accessible low DP cellulose	--> Degradation products (hydroxymethyl furfural)
2. Hemicelluloses	-->	Water soluble monomers and oligomers (mainly xylose)	--> Degradation products (furfural)

3. Lignin --> Low molecular weight reactive lignin --> High molecular weight condensed lignin

The chemical changes in steam-treated wood depend on the temperature, pressure, and time of exposure to steam. The hemicelluloses are hydrolyzed to soluble sugars by organic acids, mainly acetic acid derived from acetylated polysaccharides present in wood (Casebier et al., 1969). Under more drastic conditions, secondary reactions occur which result in the formation of furfural, hydroxymethyl furfural, and their precursors by dehydration of pentoses and hexoses. It has been reported (Campbell et al., 1973) that phenolic-like compounds increased from 0.43 to 5.3% in steam-pretreated bagasse at 27.6 bar (ca. 500 psig) for 45 min. Since phenolic-like compounds and furfurals are usually toxic to most microorganisms, such pretreated lignocelluloses may not be good substrates for enzyme production. However, the toxicity of these compounds on the activity of the cellulase-complex has not been reported so far. This necessitates determining the optimum temperature, pressure, and time combination for steam explosion of wood, so that minimum amounts of such toxic compounds

are produced.

Another process very similar to that of steam treatment was patented by Jelks (Jelks, 1976). In this process the lignocellulosic materials are oxidized in the presence of oxygen (oxygen gas or H_2O_2), acid (phosphoric, acetic, carbonic, hydrochloric, sulphuric, or sulphurous acids), and a catalyst (iron, manganese or derivations of either of these two metals) at 105 to 110°C and pressure of about 150 psig for 12-20 min during the first step of treatment. During this treatment the lignocellulosic bonds are broken. During the second step the oxidized material is subject to steam under high pressure (135 to 150 psig at 180°C) for partial hydrolysis. The material is dried to desired moisture content for storage and shipping. The product is in powder (fine fibers) form. This product is worth evaluating as a pretreatment for hydrolysis of lignocellulosic materials by T. reesei cellulase.

e) Sodium Chlorite ($NaClO_2$):

Sodium chlorite, a strong oxidizing agent, has long been used for removing lignin during the preparation of "holocellulose", the total carbohydrate

portion of lignocelluloses (Green, 1963). Goering and Van Soet (1968) demonstrated that *in vitro* digestibility of straws is increased with $NaClO_2$ treatment.

Chahal *et al*. (1979) reported that protein productivity increased considerably on delignified wheat straw when fermented with *Cochliobolus specifer* Nelson. Delignification of lignocelluloses by $NaClO_2$ proved to be useful for increasing the production of protein per unit weight of original substrate because hemicelluloses, a potential carbon source, remain intact with cellulose, and both the substrates (hemicelluloses and cellulose) can be hydrolyzed concurrently with the respective enzymes. Similarly an increased yield of total sugars (hexoses and pentoses) can be obtained on hydrolysis of $NaClO_2$-treated lignocellulosic materials, because most of the pentoses and some hexoses (of hemicelluloses) are removed during other delignification procedures. However, this process of delignification is not economically attractive because of the high cost of chemicals involved (Goering and Van Soet, 1968).

f) Selective Acid Hydrolysis:

During the enzymatic/acid hydrolysis of lignocelluloses a mixture of pentoses (xylose, arabinose) and hexoses is produced. Unless the pentoses are separated from this solution, it is not likely that the pentoses fraction will be utilized in further processes. A selective hydrolysis of lignocelluloses by mild acid has been developed to separate hemicellulose sugars (especially xylose) so that the residual material when hydrolyzed (enzymatically/acid) gives mostly glucose (Knappert et al., 1980; Lee et al., 1978). In one of the processes (Lee et al., 1978) it has been reported that hardwood treated with 0.2% H_2SO_4 at 170°C for 2 h gave optimum yield of hemicelluloses containing 90 % xylose. The residual material (cellulose and lignin) required 45 min for milling to collect 50% through 170 mesh. When such milled residual material was treated with Trichoderma viride cellulase a six-fold increase in hydrolysis over the untreated material was obtained.

In another such process (Knappert et al., 1980) the selective acid hydrolysis was carried out in a continuous flow reactor on oak, corn stover, newsprint,

and Solka Floc at temperatures ranging from 160 to 220 °C, concentrations ranging from 0 - 1.2%, and a fixed treatment time of 0.22 min. The resulting slurries and the washed solids were then hydrolyzed with T. reesei QM9414 cellulase. In the cases of newsprint and corn stover, 100% of potential glucose content of the substrates was converted to glucose after 24 h of enzymatic hydrolysis. In the cases of oak and Solka Floc 90% and 81% conversions were obtained after 48 h, respectively.

In conclusion: Grindig reduces the particle size to increase the surface area, and ball milling reduces the crystallinity. Alkali and steam treatments seem to be the most suitable pretreatments. During these treatments hemicelluloses are solubilized, lignin is solubilized or depolymerized or both, and almost all of the cellulose become available to the cellulases. The alkali treatment has an additional advantage over the steam treatment in that the crystallinity of cellulose is reduced due to swelling action whereas the crystallinity of steam treated cellulose remains unchanged. Selective acid hydrolysis seems to be another good pretreatment.

2.2. BIODEGRADATION OF LIGNOCELLULOSES

Lignocelluloses can be degraded or hydrolysed to soluble products by acids or enzymes. Processes for acid hydrolysis of cellulose exist and new technologies are being researched. When using acids, expensive corrosion-proof equipment is required. Moreover, the crystalline structure of cellulose makes it very resistant to acid thus the temperature and acid concentration needed to achieve hydrolysis also cause decomposition of the resulting sugars. Waste cellulose invariably contains impurities which will react with the acid thereby producing other unwanted by-products and reversion compounds. Therefore, drawbacks of the acid process include further degradation of the monosaccharides, interaction of acid with non-cellulosic materials in crude cellulosic materials (lignocelluloses or municipal wastes) and corrosion of equipment, resulting in low yields, impurities in the syrups and high capital costs (Ryu and Mandles, 1980). The monosaccharides recovery of approximately 50% of the weight of aspen wood was obtained by acid hydrolysis (Burton, 1982) indicating recovery of 70% of the theoretical yield. On the other hand, Spano et al. (1975) reported 70% hydrolysis of milled newspaper with cellulase. They further reported that newspaper

contained 30% lignin, thus his 70% hydrolysis represents total hydrolysis (100% of theoretical) of the polysaccharides (cellulose and hemicelluloses) of the newspaper. Chahal (1985) also reported 100% hydrolysis of delignified wheat straw with cellulase. Enzymatic hydrolysis is specific for cellulose and the enzyme does not react with the impurities present in the waste. In the long run an enzyme process should be prefered as has been the case for starch hydrolysis (Spano et al., 1975). The major cost consuming factor in enzymatic hydrolysis is the cost of enzyme production. Therefore, a need for a suitable microorganism, capable of producing a better cellulase system with high yield and productivity, is in high priority. Prior to discussing cellulase production it is necessary to understand some properties of cellulolytic microorganism and cellulase-system.

2.2.1. Cellulolytic Microorganisms

In the late 1940's, Dr Elwyn T. Reese (1950) and his co-workers at Natick Laboratories noticed that although many fungi degrade cellulose in nature, very few produce culture filtrate active against insoluble cellulose. They identified Trichoderma spp. which produced an active and well balanced cellulase complex.

Many other fungi (Aspergillus niger, Penicillium funiculosum, Sporotrichum pulverulentum and Chaetomium thermophilum) having cellulase activity, have been isolated and reported (Mandels et al., 1978) but most do not produce adequate levels of extracellular cellulase for practical use, i.e. extensive hydrolysis of high concentrations of cellulose. New mutants of Trichoderma viride [now recognized as Trichoderma reesei, (Simmons, 1977)] (Fig. 2.11) with high cellulase activities have been produced (Mandels et al., 1971b; A Report on Natick Program, 1981). The mutants of Trichoderma reesei: QM9123 and QM9414 (Mandels, 1975), MCG77 (Gallo et al., 1979), Rutgers C-30 and Rutgers NG14 (Montenecourt et al., 1977), presently in use are all descendants from QM6a, the parent strain (Mandels et al., 1969).

A new mutant QMY-1 of T. reesei, which can produce high cellulases on lignocelluloses in solid state and liquid state fermentation has recently been reported by Chahal (1985).

The advantage of T. reesei is that it produces a cellulase-system with all the components required for hydrolysis of crystalline cellulose, and this cellulase-system is stable in stirred tank reactors at

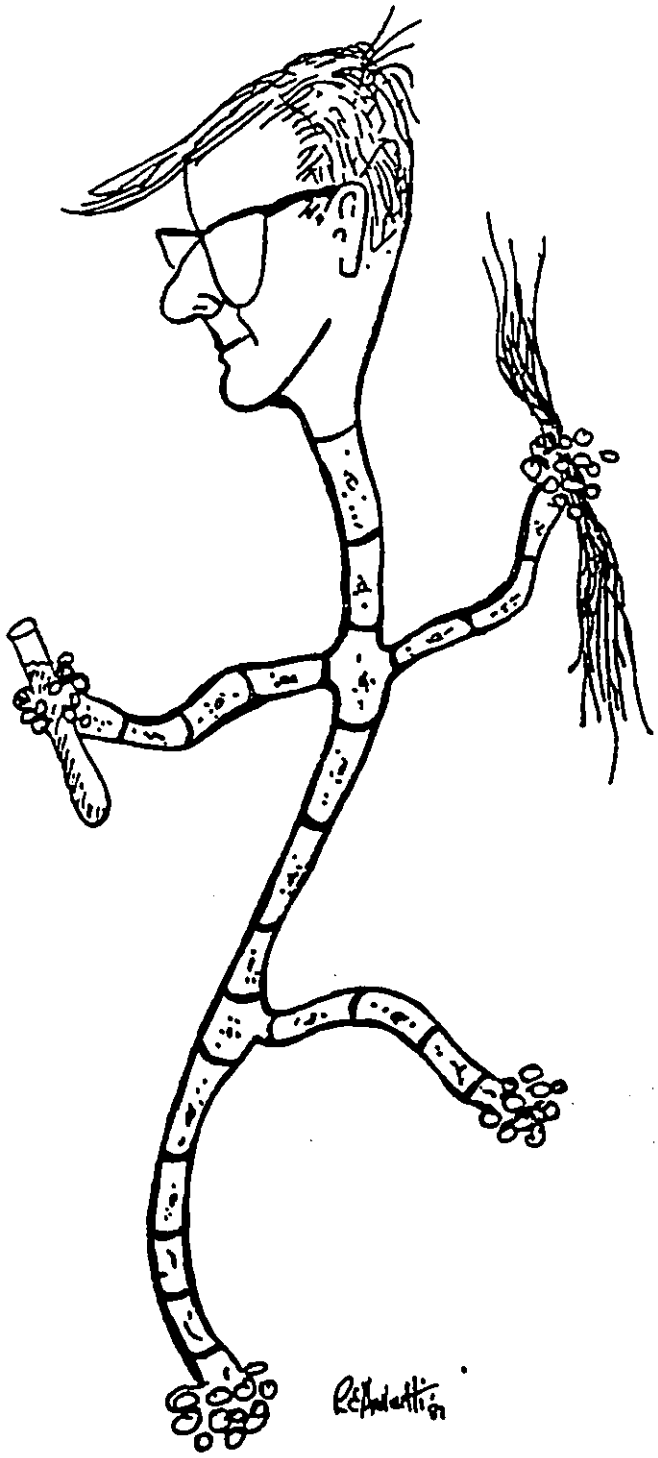


Figure 2.11. Diagramatic representation of fungus: Trichoderma reesei.

pH 4.8, 50°C for 48 h or longer (Mandels et al., 1965).

2.2.2. Cellulase-System

Presently, it is considered that the cellulase-system consists of three basic types of enzymes (i) Endo-glucanase: (EC 3.2.1.4) also known as 1,4-β-glucan glucanohydrolase (King and Vessal, 1969; Pettersson, 1975); (ii) Exo-glucanase or Cellobiohydrolase (EC 3.2.1.91) also known as 1,4-β-glucan cellobiohydrolase (Wood, 1972); and (iii) Cellobiase or β-glucosidase (EC 3.2.1.21) also known as β-D-glucoside glucohydrolase. The 'C_1' and 'C_x' enzymes as recognized by Dr Reese (Reese et al., 1950) about 40 years ago have been labelled as exo-glucanase (cellobiohydrolase) and endo-glucanase, respectively, by many workers now (Nisizawa et al., 1972; Petterson et al., 1972; Wood, 1972; Haliwell and Griffen, 1973; and Gilbert and Tsao, 1983). However, the C_1 is still being recoginized for its swelling action as originally assigned to it, but it is now accepted that this is accomplished through the action of 'any enzyme' which randomly splits the covalent bonds of cellulosic chains found at the crystalline surfaces (Reese, 1976). In his next paper (Reese, 1977) the 'any enzyme' has been mentioned as endo-glucanase. The 'C_1' has been

described in detail under sections 2.2.2.1. and 2.2.2.1.1.

In addition to the above enzymes in cellulase system, xylanase enzyme-system, referred to as 1,4-β-D-xylan-xylanohydrolase (EC 3.2.1.8), is required for hydrolysis of xylan in lignocelluloses. These enzymes are produced by many organisms when grown on pure xylan or lignocelluloses (Kluepfel and Ishaque, 1982; Margaritis and Merchant, 1983; Bernier et al., 1983; Robison, 1984; Chahal, 1985; Gokhale et al., 1986; Rapp and Wagner, 1986; and Wong et al., 1986).

Still another un-named enzyme, 'X' is required to separate cellulose and hemicelluloses from lignin in lignin-carbohydrate complex (L.C.C.) (Cowling, 1975). The physical and chemical bonds between lignin and carbohydrates in L.C.C. were reported by Higuchi (1971, 1978). The 'X' is quite different than those required for degradation of lignin. No such enzyme ('X') has been reported since it was hypothesized by Cowling (Higuchi, 1978).

Ligninases are required to solubilize lignin to expose cellulose and hemicelluloses for hydrolysis by cellulase and hemicellulases, respectively. Ligninases

have not been discussed here as it would form another big chapter (for details see Kirk et al., 1978).

2.2.2.1. Concept of C_1 - C_x System

The old concept of C_1 - C_x system developed by Dr Reese (1950) and his co-workers is that classical enzyme "Cellulase" (Pringsheim, 1912), presumably converting native cellulose to sugars, consists of at least two enzyme reactions. The first, which involves the action of C_1, occurs prior to the second, involving action of C_x. It was postulated that C_1 enzyme breaks down the "native cellulose" to shorter linear polyanhydrous glucose (cellodextrins) chains which are further broken down to soluble, small molecules capable of diffusion into the cell by the action of C_x enzyme. However, they could not observe the precise action of C_1. They labelled the organism "Cellulolytic" if it has both C_1 and C_x enzyme system and "Non-cellulolytic" if it has only C_x enzyme system.

The cellulase of T. reesei is much more effective in hydrolyzing native cellulose than are the cellulases of the other organisms (Mandels et al., 1964). It was reported that it is rich in C_1 factor. The C_1

activity is maximum at pH 4.0, and at 40 °C (Mandels et al., 1964).

Mandels and Reese (1964) concluded that C_1 is an induced enzyme which is a large molecule, pH and temperature dependent, and inactivated by heat. Cotton, filter paper (Mandels and Reese, 1964), cellulose sol (Petterson, 1963) and swollen cellulose (Walseth, 1952) have been used as substrate for C_1 production. It was reported that the swelling or pulping process has partially simulated the action of C_1 by disruption of the hydrogen bonds (Mandels and Reese, 1964).

2.2.2.1.1. Role of C_1:

Selby and Maitland (1967) reported that cellulase culture filtrates of Trichoderma viride had a much higher activity towards cotton and their assay was based on determinations of the solubilization of cotton fibers over a period of 7 days. Gel filtration of filtrate on Sephadex G-75, followed by ion-exchange on DEAE-Sephadex and SE-Sephadex gave three components which influenced the degradation of cotton: (a) a C_1 component with no measurable activity toward carboxymethyl cellulose or cellobiose (b) a component with high activity toward carboxymethyl cellulose and a

low activity towards cellobiose and (c) a component with a low activity towards carboxymethyl cellulose and high activity towards cellobiose. Individually none of these components gave appreciable solubilization of cotton but the combination (a) + (b) accounted for 35%, (a) + (c) for 20% and (a) + (b) + (c) for approximately 100 % of filtrate's activity. The molecular weights of all three were estimated by gel filtration to be in the range 48 000 - 62 000; the C_1 component was a glyco-protein with approximately 50% carbohydrate.

Wood extended the findings of Selby and Maitland (1967) with essentially similar data for culture filtrates of Trichoderma koningi (Wood, 1968) and of Fusarium solani (Wood, 1969). He reported that the C_1 component has little or no effect on either soluble derivatives of cellulose (such as CMC) or highly ordered substrates but acts synergistically with C_x enzyme(s) (Wood, 1968, 1969, 1971) and cellobiase is required to accomplish the conversion of native cellulose into water soluble products as reported by Mandels et al., (1964). Later, Wood and McCrae, (1972) concluded that the C_1 component is a 1,4-β-glucan-cellobiosylhydrolase which releases cellobiose from the new chain ends formed by the action of C_x enzymes. It (C_1) is most active in hydrolysis of cellulose in

combination with C_x. Wood (1972) reported that it is not immediately apparent why the presence of C_1 is essential? What is apparent, however, is that this mechanism assigns the role of initiating the attack on highly ordered forms of cellulose to C_x and not to C_1 as suggested by Reese et al. (1950). Henceforth, the C_1 has been wrongly assigned the name of cellobiosylhydrolase ignoring its original role of swelling of crystalline cellulose or breaking covalent bonds or physical bonds (van der Waals' forces) between the glucose chains in the crystalline cellulose as postulated by Reese and associates.

Whitaker's comments on C_1 factor are as following (Whitaker, 1971):

"At present then C_1 factor cannot be claimed to be an enzyme or to be operative on substrates other than dried fibers or to have a C_1 function in the sense that it initiates a degradation. It may be that it has catalytic bond breaking or exchange properties which remain to be detected but nonenzymic effects are also possible. For example, as one possibility, when adsorbed on cellulose it may distort the local structure sufficiently to allow water molecules to hydrate previously unexposed segments of chains or it

could be that C_1 factor simply protects the cellulase from denaturation or irreverible adsorption."

The above statement of Whitaker does not approve that C_1 factor (or enzyme) could be 1,4-β-glucan-cellobiosylhydrolase as reported by Wood (1972).

In 1977, Reese re-evaluated C_1-C_x concept and was not in the agreement that C_1 is cellobiohydrolase. Now the modified C_1-C_x concept of Dr Reese is as following (Reese, 1977):

"Instead of disruption of only hydrogen bonds by C_1, it is now believed that the covalent linkage (glucose-glucose β-1-4 bond) is first split by endo-glucanase and that this act is accompanied by splitting of hydrogen bonds by C_1. It is very special enzyme having properties not possessed by other enzymes in the cellulase system e.g. activity on crystalline cellulose; disruption of H-bonds; lack of measurable action on CMC, and inability to act on products of its own action (since it produces no soluble products from crystalline cellulose)."

Whitaker (1971) commented that C_1 may be required to hydrate unexposed segments of glucose

chains. Chahal (1982 , 1983) reported that the activity of C_1 is associated with the swelling of the crystalline cellulose (and separation of anhydrous glucose chains), whereas, C_x (containing exo- and endo- glucanase) is associated with the hydrolysis of soluble substrate (anhydrous glucose chains) or derivatives of cellulose (carboxymethyl cellulose) into glucose and cellobiose. The cellobiose is hydrolysed to glucose by β-glucosidase.

From the above discussion it appears that in C_1-C_x concept there is another very specific enzyme, 1,4-β-glucan-cellobiosylhydrolase, which cuts off cellobiose units from cellulose chains it is considered to be responsible for initiating hydrolysis of crystalline cellulose instead of C_1. However, this function does not make it C_1 because the role of C_1 is quite different as mentioned by Reese (1977).

2.2.2.1.2. Role of C_x Enzyme

Reese (1950) assumed that C_x, the enzyme hydrolyzing the 1,4-β-glucosidic linkage in CMC, is also the enzyme attacking the same linkage in cellulose. This belief was based on the presence of

C_x in the filtrates of cellulolytic organism grown on cellulose and he found out that C_x is not produced by the same organism in the absence of substances containing the β-1-4 bond. Thus no C_x was obtained when glycerol was substratum, though good growth took place. Reese reported that C_x is produced apparently in response to the presence of the β-glucosidic linkage, whether that linkage is in cellulose, in hydroxymethyl cellulose or in carboxymethyl cellulose, and it was not specific for the modified chain. It was believed that C_x has the ability to produce glucose as an end-product from the long-chain compounds but that it is unable to hydrolyze cellobiose. The hydrolytic enzyme, C_x, is unable to hydrolyze starch, pectic acid, alginic acid or bacterial dextran. The C_x activity is maximum at pH 4.8, and at 60 °C (Mandels et al., 1964).

Eriksson and Pettersson (1975) found exo-glucanase along with endo-glucanase in C_x mixture. The exo-glucanases had a molecular weight of 48 600 and was isoelectric at pH 4.3 and contained no carbohydrate. The weight ratio of endo-glucanase protein to exo-glucanase protein is approximately 1:1. Eriksson (1975) reported that endo-glucanases act randomly over the long chain of glucose monomers (cellulose) to form

shorter chains of glucose monomers, whereas, exo-glucanases act on the chain ends to form cellobiose and glucose.

The C_x enzyme exist in multiple forms in most cellulase systems. As a rule they vary in the degree of "randomness" of their attack on CM-cellulose. Eriksson and Petersson (1975) reported five endo-glucanases isolated from Sporotrichum pulveruleutum cellulase, whereas Ogawa and Toyama (1972) reported four from T. viride "Onozuka" cellulase, and only three from T. viride "Meicelase" have been reported by Okada et al. (1968). There are other reports of the fraction of multiple C_x components: P. funiculosum cellulase contains three and possibly four endo- glucanases (Wood et al., 1979) while T. koningii cellulase contains five (Wood et al., 1975) and later found to contain six (Wood et al., 1978).

Thus the subscript 'x' in C_x emphasizes the multi-component nature of this fraction. The C_x (or more specifically β-1-4-glucanases) enzymes are clearly of two types:

a) exo-β-1-4 glucanases, successively removing single glucose units from the non-reducing ends of the

cellulose chain;

b) endo-β-1-4-glucanases, with the action of a random nature, break the cellulose chain into smaller fragments, (the terminal linkages generally being less susceptible to hydrolysis than internal linkages).

2.2.2.2. β-glucosidase

Shewale (1982) and Woodward and Wiseman (1983) had reviewed β-glucosidase enzyme: it catalyzes removal of glucose from the non-reducing ends of cellodextrins up to cellohexaose and thus effects the cleavage of cellobiose. The later part is important in that it releaves inhibition of cellulase action caused by accumulation of cellobiose in the hydrolysate (Reese et al., 1952). β-glucosidase acts on variety of β-linked glucopyranosides, including cellobiose, trehalose, sophcrose, laminaribiose and gentiobiose but at different rates. Generally speaking the rate of hydrolysis decreases as chain length increases (Shewale, 1982).

Molecular weight values range from 35 000 to 440 000 (Meyer et al., 1981). Trichoderma reesei produces little extracellular β-glucosidase (Allen

et al., 1980) - in fact only 0.2% of the extracellular protein has such activity. This decreases the effectiveness of such filtrates in the saccharification of cellulosic substrates and neccessitates supplementation of reaction mixtures with β-glucosidase from other sources (Dekker et al., 1983). Aspergilli are among the better sources of β-glucosidase (Enari, 1983; Duff et al., 1987). Breuil et al. (1986) showed T. harzianum E.58 as another good source of β-glucosidase.

2.2.3. An Updated Complete Picture Of Cellulase-System

It may be necessary to name the various enzymes and their reactions before hydrolysis of lignocellulases in nature could be explained:

ENZYME/FACTOR	REACTION(S)	REFERENCES
Pectinases and Ligninases	To hydrolyze middle lamella to set free individual plant cells.	
Hypothetic 'X' enzyme	Breaks the bonds between lignin and carbohydrate (Higuchi, 1971, 1978) to release cellulose, hemicelluloses and lignin.	Cowling, 1958.
C_1 Factor	Releases the linear chains of glucose residues from crystalline cellulose by breaking H-bonds with the help of endoglucanase.	Reese, 1976, 1977.

Endo-glucanase (1,4-β-glucan glucanohydro-lase)	Breaks the long linear chain of glucose residues randomly into small linear chains (oligomers).	King and Vessal 1969.
Exo-glucanase (1,4-β-glucan glucanohydro-lase)	Cuts away single glucose units from the non-reducing end of the cellulose chain.	King and Vessal 1969.
Exo-glucanase (1,4-β-glucan cellobiosyl-hydrolase). (It has been named as C_1 by Wood, 1972)	Cuts away cellobiose from linear glucose chains.	Wood, 1972.
Cellobiase (β-glucosid-ase)	Hydrolyzes cellobiose into glucose units. It may act on short chain oligomers to cut away glucose units.	Shewale (1982); Woodward and Wiseman, 1983.

Hemicellu-	Hydrolyze hemicelluloses
lases	into its monomers
	(xylose, mannose,
	arabinose, galactose,
	etc.)

The latest representation of the breakdown of cellulose in lignocelluloses developed by Chahal and Overend (1982) has been slightly modified by the author, on the basis of literature surveyed. An attempt is made to summerize the complete picture of breakdown of lignocelluloses by "ligno- cellulase- system" of "lignocellulolytic organisms". The term "lignocellulolytic organisms" is used for the organisms that degrade not only crystalline cellulose but also the cellulose which is mixed with hemicelluloses and lignin as found in native state- the lignocelluloses (wood and agricultural residues).

The lignocelluloses are first ground to small particles to expose the surface area for reactions of various enzymes. The individual plant cells are released from the small particles by the action of ligninases and/or pectinases. To degrade the released individual plant cells the first step required is to break the lignin-carbohydrate bonds (Higuchi, 1971,

1978) to expose cellulose and hemicelluloses. Cowling (1958) reported a hypothetical enzyme 'X' for such reaction (to separate the cellulose and hemicelluloses from lignin). Nothing is known about the nature of the 'X' enzyme, although a lot of work is being done to understand the nature and action of ligninases (Kirk et al., 1978). The ligninase degrades the lignin to expose cellulose and hemicelluloses.

Figure 2.12 shows part of plant tissue (small particle) showing different types of cells and the action of ligninases and pectinases on middle lamella to release the individual plant cells. Some of the individual cells released show broken ends while others show some cracks in their cell walls. Broken ends and cracks in these cell walls were formed during grinding of lignocelluloses into small particles and are the first cites for attack by cellulases.

The C_1 enters into plant cell wall through the previously formed broken ends and cracks, and due to its swelling effect and breaking of H-bonds it releases macrofibrils as well as microfibrils. During this process, starting from primary wall towards lumen, hemicelluloses (which predominate in the primary wall and diminish in concentration toward the lumen,

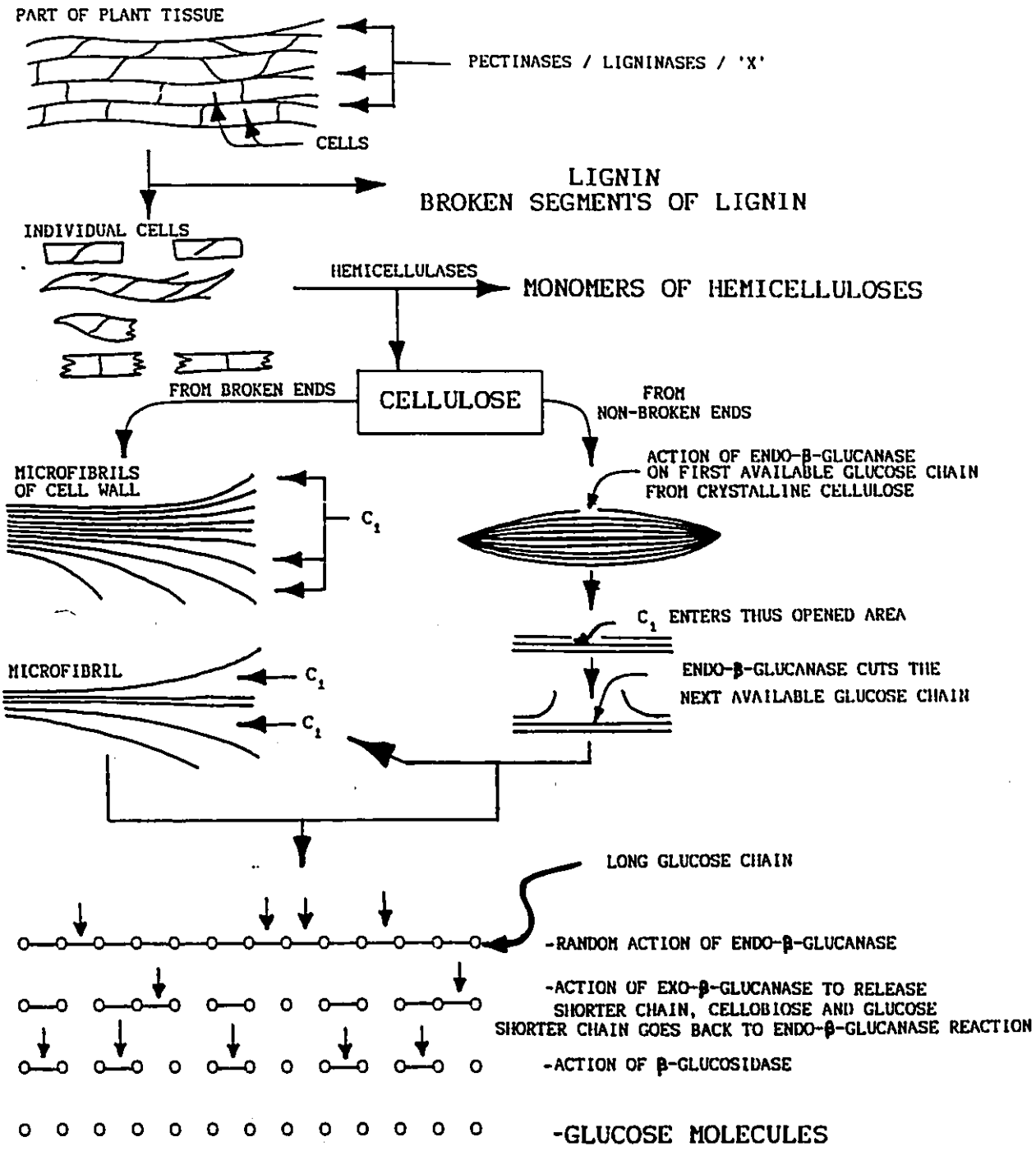


Figure 2.12. Representation of Breakdown of Lignocellulose by Lignocellulase-System.

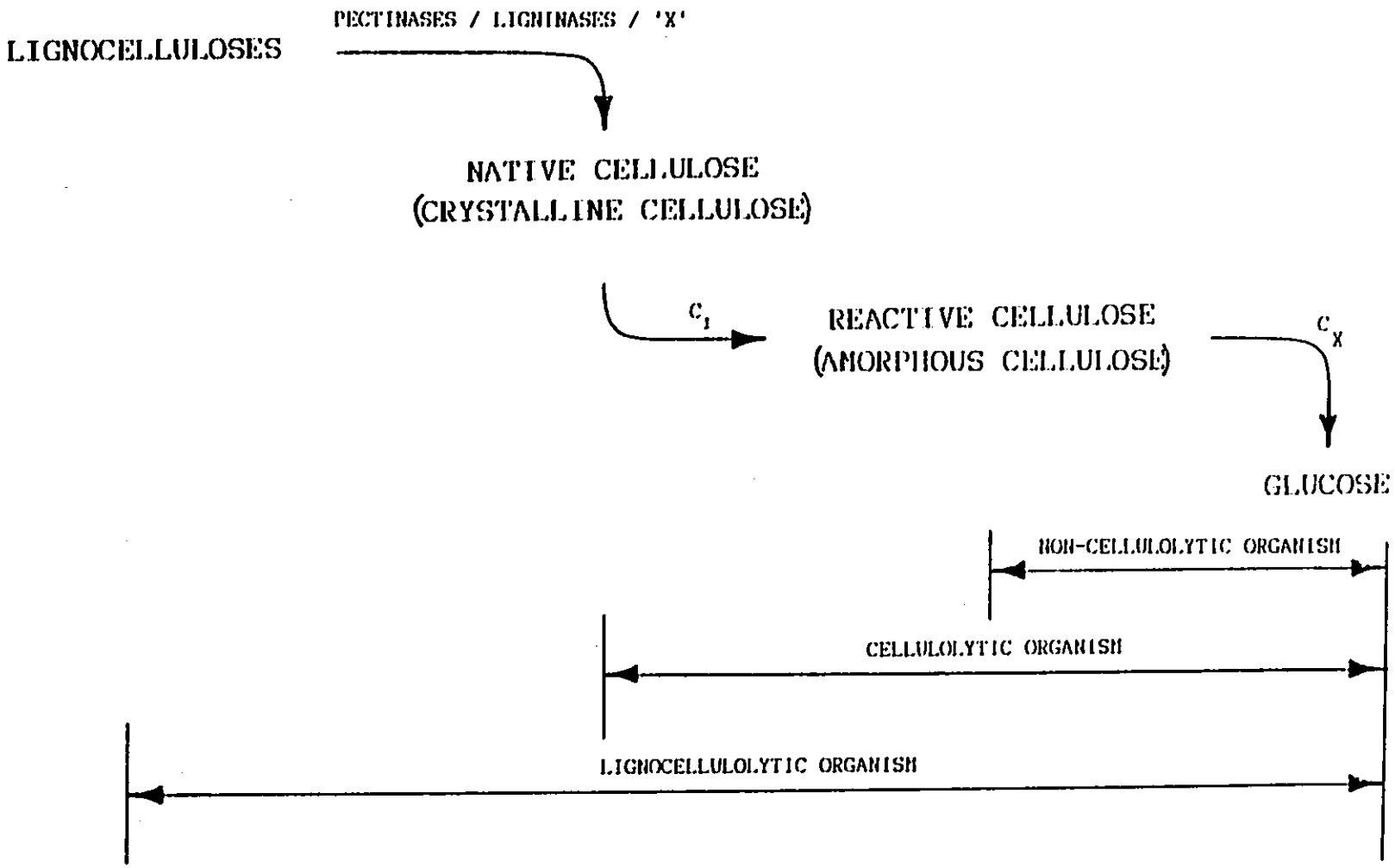
whereas, cellulose follows an opposite pattern of distribution) are released along with macro- and microfibrils. The C_1 continues to work from the open ends of the macrofibrils as well as on microfibrils by penetrating deep inside giving a "swelling" effect and releases more microfibrils, and single linear anhydrous glucose chains.

On linear anhydrous glucose chain, endo-glucanases act at random to release oligomers. During this reaction some glucose and cellobiose units are also released. Exo-glucanase acts on non-reducig ends of long linear anhydrous glucose chains and also on oligomers to release cellobiose and glucose units. β-glucosidase acts on cellobiose to release glucose. β-glucosidase can also release glucose from the oligosaccharides up to cellohexaose according to Shewale, (1982); Whitaker (1971) and Woodward et al. (1983). All these reactions occur simultaneously and also synergistically on cellulose to release glucose units.

Simultaneously, hemicellulases (such as xylanase, mannanase etc.) act on hemicelluloses and release their monosaccharide units (such as xylose, mannose etc., respectively).

Thus glucose is obtained along with monosaccharides of hemicelluloses (depending upon the type of hemicelluloses present), lignin and its solubilized components from the ligno- celluloses by the action of "ligno- cellulase- system". Therefore, ligninases, pectinases, 'X', hemicellulases, C_1, C_x, and β-glucosidase must be produced by an organism to be called as 'lignocellulolytic organism' (Fig. 2.13).

Figure 2.13. Characterization of Lignocellulolytic, Cellulolytic and Non-Cellulolytic Organism.


PECTINASES / LIGNINASES / 'X'
LIGNOCELLULOSES
NATIVE CELLULOSE
(CRYSTALLINE CELLULOSE)
C_1
REACTIVE CELLULOSE
(AMORPHOUS CELLULOSE)
C_X
GLUCOSE
NON-CELLULOLYTIC ORGANISM
CELLULOLYTIC ORGANISM
LIGNOCELLULOLYTIC ORGANISM

2.3. CELLULASE PRODUCTION

In the literature, a standardized commercial process for production of cellulase has not been reported yet; improvements are still being reported and such a commercial process may not be developed in the near future. However, enough technology is available from various laboratories to produce a large quantity of enzyme for entering a commercial enterprise. The economical production of cellulase depends on the selection of the right type of organism and further improvement of organism by mutation and genetic engineering, use of cheap and easily available cellulosic substrate, cheap media formulations, and modifications in fermentation technology. Most of the studies on cellulase production have used different mutants of T. reesei. The present state-of-the-art in cellulase production is given as follows:

Medium: The basic medium for cellulase production was developed by Mandels et al. (1969) at the U.S. Army Natick Laboratories, Natick, MA, U.S.A. The composition of the medium is given as following: (in g/l) $(NH_4)_2SO_4$, 1.4; KH_2PO_4, 2.0; Urea, 0.3; $CaCl_2$, 0.3; $MgSO_4.7H_2O$, 0.3; and

(in mg/l) $FeSO_4.7H_2O$, 5.0; $MnSO_4.H_2O$, 1.6; $ZnSO_4.7H_2O$, 1.4; $CoCl_2$, 2.0; Cellulose 0.75 - 1.0%, Proteose peptone 0.075 - 0.1%, and Tween-80 0.1 - 0.2%, at initial pH 5.0 - 6.0. There have been various changes in the constituents, especially in the protein source and surfactants like Tween-80 to reduce the cost of the medium as well as to increase the yields of cellulase.

Inducers: The carbon source is mainly cellulosic substrate because the enzyme is induced by cellulose, although, there are many other inducers. These inducers are glucans of mixed linkage including the β-linkange and a few other oligosaccharides (Mandels et al., 1957, 1962, 1964, 1969). The true inducers of cellulase for a fungus growing on cellulose are the soluble hydrolysis products of the cellulose, especially cellobiose (Mandels et al., 1960). The role of cellobiose is complex; it is an inducer at low concentrations but is a repressor at high concentrations (Reese et al., 1952). Concentrations of 0.5 - 1.0% of cellobiose or other rapidly metabolizable carbon sources such as glucose or glycerol strongly repress cellulase production (Mandels et al., 1960; Mandels et al., 1962) until the carbon sources are consumed by the organism. If these sugars are added at

these concentrations (0.5 - 1.0%) to a culture already producing cellulases, they inactivate the enzyme already formed (Mandels et al., 1960). If the culture is still in its logarithmic stage, the enzyme activity will reappear when these sugars are utilized and the fungus metabolism is shifted from soluble sugars to insoluble substrate, the cellulose.

Mandels and Weber (1969) reported that if rapid metabolism of the sugar (cellobiose) is slowed down by sub-optimum temperature and aeration, deficiencies of mineral nutrients such as calcium, magnesium, or trace metals, or excess of mineral such as cobalt (Mandels et al., 1960), T. reesei will produce as much cellulase as on cellulose. However, it has been noticed that new hypercellulase-producing mutant Rut-C30 gave about one-third cellulase even when grown on glucose as compared to that on cellulose under similar cultural conditions (Chahal et al., 1982a).

In addition of small amounts (0.1%) of readily available substrates such as glucose, glycerol, or peptone along with cellulose reduces the lag phase and increases the cellulase production (Mandels et al., 1957). Similar results were reported by Chahal and Gray (1970), that a small amount of readily available

sugars reduced the lag phase and stimulated the growth of various cellulolytic fungi on wood pulp. These findings were exploited to increase the cellulase productivity by Gallo et al. (1978). A new mutant of T. reesei MCG77 was first grown on glucose for mycelial growth. This mycelial broth, dilluted to its half concentration, was used to inoculate 4% roll-milled and ball-milled cellulose. A productivity of 72 filter paper units per litre per hour on 84-h batch cycle was obtained. A very high level of cotton activity (17 mg/ml at 84 h) was also achieved. Steam exploded wood (Noble, 1980; Reese et al., 1952) contains about 15-20% soluble sugars (mainly xylose, glucose, galactose, etc.). These soluble sugars, when retained in the fermention media, increased the cellulase productivity (Chahal et al., 1981a). It was recorded that when these sugars were retained in the medium of 1.4 - 1.9% cellulose from steam-exploded wood (H60 - Trade name of Iotech Corporation) a cellulase productivity of 37-45 filter paper units per litre per hour was obtained in 52-60 h of cyclic-batch fermentation with a new strain of T. reesei, Rut-C30. In this medium no proteose peptone was used. These results were much better than obtained by another improved mutant of T. reesei, MCG77, when compared in terms of quantity of cellulose used. Thus, keeping the soluble sugars in the

steam-exploded wood medium will greatly reduce the cost of cellulase production as compared to the use of pure cellulose, proteose peptone, and heavy inoculum grown on glucose.

Sophorose, a (β 1->2) glucoside, is a very powerful inducer of cellulase for T. reesei (Mandels et al., 1969; Sternberg et al., 1979). Lactose, (β 1->2) galactoside, is another cellulase inducer. These two sugars are the only known cellulase inducers that do not have a (β 1->4) glucosidic linkage.

Replacement of Proteose Peptone: Proteose peptone (as a growth factor) in small concentration is necessary to obtain high yields of cellulases, but it is the most costly constituent in the cellulase fermentation medium. Optimum peptone concentration is 0.1 to 0.2%, depending on the cellulose concentration; concentrations higher than 0.5% strongly inhibit the cellulase production whatever may be the cellulose concentration (Mandels et al., 1969). Peptone can be replaced with proflo (cotton seed flour), phyton, casein hydrolysate, yeast extract (Mandels et al., 1969). and corn steep liquor (Montenecourt et al., 1979) with slight decrease in cellulase production. The spent mycelium (0.25 - 0.30%) from a cellulase fermentation was also an

excellent replacement for proteose peptone (Andreotti et al., 1977). It was also found (Chahal et al., 1981) that retention of soluble sugars available in steam exploded wood medium did not need proteose peptone (as a growth factor) in cyclic-batch (semi-continuous) cellulase fermentation.

Effect of Surfactants: Surfactants are known to be useful in the fermentation industry for increasing growth rates and metabolite production. Addition of Tween-80 and Tween-40 doubled the yield of cellulase in T. reesei (Reese et al., 1971). The mechanism of enhancement in cellulase yield is not well understood, but may be related to an increase in the permeability of the cell membrane of the organism, allowing more rapid secretion of the enzymes.

pH: The initial pH of the medium at the time of inoculation is usually 5.6. In uncontrolled cultures, pH falls with the increase in growth and utilization of NH_4^+ ions from $(NH_4)_2SO_4$ (Chahal et al., 1978) by the organism and formation of H_2SO_4. The growth of T. reesei was most rapid when the culture was controlled at pH 5.0, somewhat slower in the cultures controlled at 4.5 and 4.0, and even slower in the culture that was controlled at 3.5. However, the

production of cellulase was in the reverse order (Andreotti et al., 1977).

Age and Volume of Inoculum: Spore inoculum (10 000 spores/ml in the fermentation medium) gave long lag phase and produced about half the amount of enzyme produced using 3-day-old mycelial inoculum. The level of inoculum, 1 or 5% v/v, had little effect on the yield of the cellulases (Andreotti et al., 1977), but most studies have shown that an inoculum level higher than 5% v/v was most suitable for cellulase production (Chahal et al., 1981; Gallo et al., 1978; Nystrom et al., 1976; Ryu et al., 1979). The use of inoculum by volume may not be a good criterion, as the amount of mycelial biomass produced varies with the concentration of carbon substrate in inoculum medium.

Temperature: Normally the fermentation is carried out at 29°C (optimum for enzyme production) while the growth rate continues to increase up to temperatures as high as 35°C. A considerable increase in cellulase production was recorded when the temperature of fermentation was maintained at 33-34°C in early phase of the growth (about 24 h), then reduced to 29°C throughout the rest of the fermentation (Nystrom et al., 1977).

Substrate: It is well known that the high cellulase yields are obtained by growing T. reesei on cellulose. The cellulase production depends on the nature of cellulosic substrate and its pretreatment. The interpretation of the data on cellulase production given by Mandels et al., (1974) indicated that the cellulosic substrates differed slightly when their filter paper activity was measured but on the other hand they differed a lot when C_1 activity was compared. In general, highest C_1 activity was noticed in the case of crude cellulosic substrate, newspaper. The crystalline cellulosic substrates, Avicel PH-105 and absorbent cotton, produced the lowest C_1 activity; but the Avicel PH-105 produced the highest filter paper activity. Pure cellulosic substrates (SW40, SW200, Sweco 270, Avicel PH-105, and absorbent cotton) produced the highest C_x activity as compared to the crude cellulosic substrates. Unfortunately the hydrolytic activity of these cellulase systems had not been evaluated. Had this been done the real role of C_1 activity in hydrolysis could have been determined.

There are some indications (Chahal et al., 1976, 1982 b) that the C_1 activity of T. reesei increased

with an increase in native crystallinity of the cellulosic substrate. It appeared that the native crystallinity found in wheat straw cellulose was more favourable for production of C_1 activity than that of 'Sigmacell', a crystalline cellulose, or 'Solka Floc', a purified form of cellulose with many amorphous portions. On the other hand, the crystalline substrates (wheat straw and Sigmacell) gave lower filter paper activity than on Solka Floc. The cellulase systems produced on wheat straw which had high C_1 activity were found to be more active in hydrolyzing the three substrates (wheat straw, Sigmacell, and Solka Floc). The ratio of C_1 and filter paper activity in this system was about 3:1. The cellulase system produced on Solka Floc with C_1 and filter paper activity of about 1:1 was poor in hydrolysis of all the three substrates. It is , therefore, inferred from the data available (Chahal, et al., 1976) that the potential of any cellulase-system to hydrolyze various cellulosic substrates depends on the nature of the substrate used to produce the enzyme system. It has been reported that the enzyme produced by Rut-C30 mutant of T. reesei on steam-exploded wood was faster and more effective in hydrolysis of wood than the commercial enzyme, Novo enzyme (Chahal et al., 1981). This might be due to the

fact that the enzyme was produced on wood with native crystallinity comparable to that of wheat straw (Chahal et al., 1976).

Concentration of Substrate: In most of the early studies the concentration of cellulose used for cellulase was 0.5 to 1% (Chahal et al., 1976; Mandels et al., 1969; Sternberg, 1976). A cellulose concentration of 0.75% was reported to be the optimum concentration for cellulase production by T. reesei; higher cellulose concentrations resulted in a marked decrease in the final enzyme level (Mandels et al., 1969). The decrease in enzyme production with increase in cellulose concentration was because there was no corresponding increase in the nutrient salts. This fact became very evident when the data of Sternberg (1976) were examined in the light of the above statement. He obtained 0.6 filter paper units with 0.75% cellulose and the filter paper units increased correspondingly to 1.6 units when the cellulose concentration was increased to 2% along with an increase in nutrient salts. The low enzyme yields obtained at 2% cellulose by Mandels and Weber (1969) have been explained by Sternberg (1976) as due to the fall of pH to 2.4; under this condition the organism cannot grow properly and also cellulase becomes inactivated. Sternberg claimed that the increase in

enzyme yield on 2% cellulose was mainly because of controlled pH of 3.0, but he failed to note that it was also due to an increase in nutrient salts. It is evident from the first principle of growth of an organism that more nutrients are required to produce more biomass or more metabolites when the carbon substrate concentration is increased. This fact has been confirmed by growing T. reesei in various cellulose concentrations (0.94, 2.55, and 5.04%) with a corresponding increase in nutrient salts (Nystrom, 1977). There was a corresponding increase in cellulase yields with the increase in cellulose concentration when the media were also enriched with salts.

Kinetics of Cellulase Production: A good understanding of the kinetics of cellulase production is the most important feature of the whole process of enzyme production on industrial scale. It is very difficult to determine the true kinetics of cellulase production because of the lack of complete information on all the parameters of growth of the organism. One study (Sternberg, 1976) showed that the cellulase production is growth related while others (Berg et al., 1977; Ghose et al., 1975) showed that it is just the reverse. In the latter case it has been reported by Ghose et al, (1975) that enzyme synthesis begins near or at the end

of the phase of cell growth. It was further suggested by them that the specific enzyme synthesis rate has a negative correlation with the specific growth rate of the organism.

The kinetics of growth and cellulase production of T. reesei described here by Chahal and Overend (1982 b) is based on the data obtained from Andreotti et al. (1977), Chahal et al. (1976, 1981, 1982), Gallo et al. (1978), Peitersen (1975) and Ryu and Mandels (1980). The kinetics of cellulase production given in Figure 2.14 are explained as follows (Chahal and Overend, 1982 b):

(i) Initial growth of the organism starts immediately on the soluble sugars or proteins (proteose peptone, etc.) present in the growth medium. This phase of growth is faster than the following stage. The soluble sugars or proteins are consumed by the organism within a short time (12-24 h), depending on their concentrations. During this time almost no cellulose is used.

(ii) As soon as about 80 - 90% of sugars or proteins are consumed the organism shifts its metabolism for utilization of cellulose. There may or

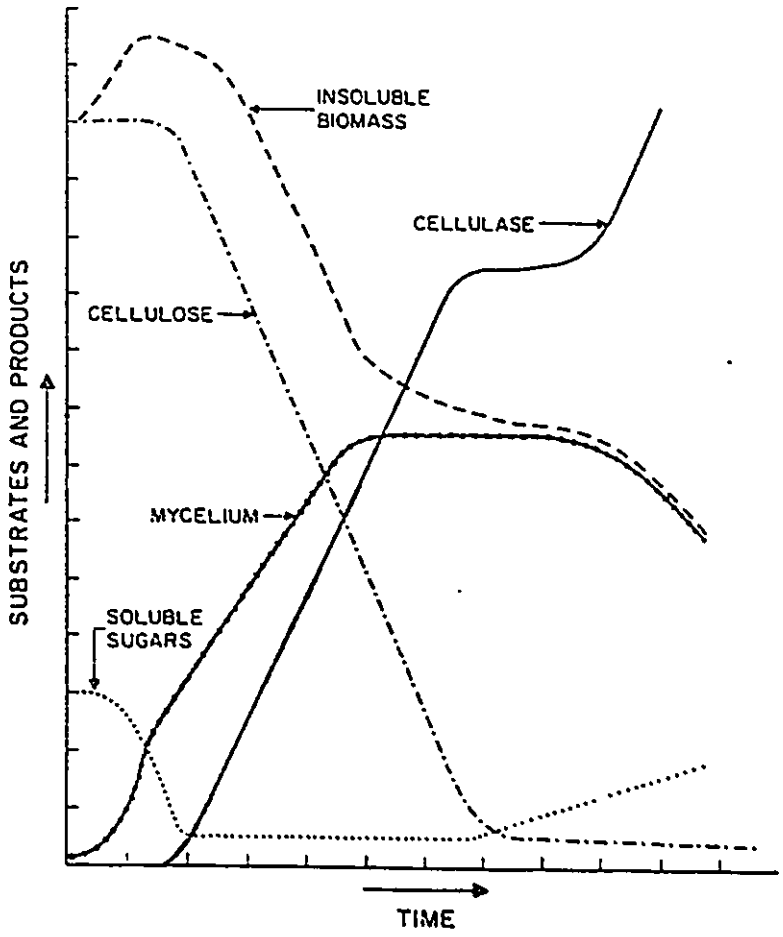


Figure 2.14. Kinetics of Growth and Cellulase Production of T. reesei.

—·—·—· Cellulose

— — — — Insoluble biomass (mixture of mycelium and unutilized cellulose)

..... Soluble sugars added or already present in the substrate (lignocelluloses) produced during pretreatment with acid at high pressure or with steam at high pressure.

—●—●—●— Mycelium. Calculated on the basis of insoluble protein synthesized by fungal biomass (mixed with unutilized cellulose).

——————— Cellulase as measured in the cell (biomass) free fermentation broth.

(Source: Chahal and Overend, 1982b).

may not be some signs of diauxic type of growth, depending on the crystallinity of the cellulose and particle size of cellulose. Thereafter, there is continuous consumption of cellulose until it reaches the lowest concentration and then it levels off.

(iii) It is difficult to estimate the amount of mycelium synthesized becauseit is mixed with cellulose. It is for this reason that most studies do not show growth parameters in cellulase fermentation, which makes it difficult to interpret the cellulase kinetics. Wherever such growth parameters were given they were based on indirect measurements - usually protein content of the total biomass (mycelium + unutilized cellulose). The growth curve of T. reesei given here is based on such estimations of biomass protein at certain times of its growth. There is a slight lag phase followed by fast exponential growth on soluble sugars and proteins. It is followed by another slow exponential growth on insoluble substrate, cellulose. Thereafter, there is a stationary phase. During this stationary phase the cellulose is still being consumed. This indicates that new mycelium is being synthesized while the older mycelium may be autolysing and so growth seems to be stationary, in other words, the growth rate is equal to the death rate of the organism.

The stationary phase is followed by a declining phase. It is because there is no more cellulose available for further growth and older mycelium is being autolyzed. Autolysis of mycelium is a common phenomenon in fungi (Cochrane, 1958).

(iv) The weight of insoluble biomass (mixture of mycelium and unutilized cellulose) increased a little during the first few hours of growth of the organism on soluble sugars and proteins. This increase in weight is due to the newly synthesized mycelium and the presence of insoluble cellulose which was not utilized during this stage. Thereafter, continuous decline in weight of insoluble biomass is seen because of about half of the mass of cellulose used is converted into mycelial biomass while the other half is consumed as a source of energy for various metabolic processes of the organism and synthesis of cellulase. Then there is a leveling of the curve just parallel to the stationary phase of growth. Thereafter, the decline in weight of insoluble biomass begins again, due to the autolysis of the mycelium of the organism.

(v) Cellulase activity is not noticed until almost all the soluble sugars and proteins are consumed by the organism and the growth is started on cellulose. There

might be some cellulase activity during the initial growth of the organism on soluble sugars or proteins and some amorphous portion of cellulose substrate, but it is not detected in the fermentation broth because the synthesized cellulase is adsorbed on the residual substrate, cellulose. Soon the quantity of cellulase synthesized is continuously increased and runs almost parallel to the exponential growth phase. Cellulase production continues even during the stationary phase when some cellulose is also being consumed. The cellulase production levels off when the substrate cellulose is completely consumed. Thereafter, in most of the fermentations more cellulase appears in the broth even when there is no growth of the organism and no more cellulose is present in the fermentation medium. This increase in cellulase at the end of the fermentation is mainly due to the release of cellulase from the mycelial cells being autolyzed but not due to de novo synthesis as postulated by Ghose (1977). The autolysis of the mycelium is indicated by the decline in the weight of insoluble biomass, mycelial biomass, and an increase in soluble sugars released from the disintegration of protoplasm during autolysis. The specific enzyme activity will be low at this stage because some of the protein released from the autolyzing cells is of non-enzymatic nature. The

specific activity of the enzyme at this stage will have insignificant value determining its hydrolytic potential.

Batch, Cyclic-Batch, Fed-Batch and Continuous Fermentation: The enzyme production is the major cost in the whole process of enzymatic hydrolysis of cellulose (Ghose et al., 1979; Spano et al., 1978). Continuous enzyme production, where constant product is realized, is very attractive from both a capital and an operating cost basis. Ideally the process can be designed to incur less capital cost and to have lower overall operating cost. The lower operating cost is due to less down time when compared to a batch type where the vessels must be cleaned, sterilized, inoculated, maintained, and harvested in a cyclic manner. Additionally, productivity per unit time is actually greater as compared to a batch process because of the lowered residence time, although the actual product yield may be less (Wang et al., 1979).

In an early attempt (Mandels et al., 1969) on semi-continuous fermentation the cellulase activity of 1.0-1.7 units/ml was recorded as compared to that 3.5 - 4.0 units/ml in batch fermentation under similar conditions. Bevernitze et al. (1982) have also

reported that in a batch fermentation 9-10 mg of extracellular cellulase protein per millilitre was achieved in 5 days whereas, in continuous fermentation the yield fell to 3.5 - 4.5 mg/ml with residence time of 2 days. Other studies (Montenecourt et al., 1981; Ryu et al., 1979) on continuous fermentation report cellulase activity varying from 0.31 to 2.6 cellulase units/ml with productivity varying from 40 to 151 units/l/h, but there was no comparison with the results of batch fermentation under similar conditions. From the small amount of data available it is clear that continuous fermentation gives an enzyme system with a very high productivity per unit of time but low cellulase activity per unit volume. Although the cost of enzyme production will be greatly reduced, the enzyme system produced under such conditions will be uneconomical for hydrolysis of cellulose as it would yield hydrolysate with very low concentration of glucose. It would have to be concentrated.

On the other hand, cyclic-batch seems to be the solution of all these problems. It has been reported by Chahal et al. (1981) that high productivity (37 - 45 units/l/h) as well as high cellulase activity (2.3 - 2.7 units/ml) were obtained in cyclic-batch of 50-66 h of residence time with about 2% cellulose and T. reesei

C30. The cellulase activity obtained in cyclic-batch fermentation was as good as in the batch fermentation (2.6 units/ml) under similar conditions. Remembering the importance of high cellulase activity per unit of volume, the results seem to be much better than those achieved under continuous fermentation by other workers (Montencourt et al., 1981; Ryu et al., 1979). Moreover, the cyclic-batch fermentation gives all the advantages of continuous fermentation.

Hendy et al. (1982), Watson et al. (1984) and Mclean and Podruzny (1985) used fedbatch fermentation for cellulase production using T. reesei Rut-C30 . Although, it was claimed as fed-batch by Mclean and Podruzy (1985) it was actually a cyclic-batch (semi-continuous) by definition, because when a certain volume was added, an equal volume was harvested at the same time to keep the total volume for fermentation constant. However, they obtained a yield of 389 IU/g cellulose with cellulase activity of 31.1 IU/ml. Hendy et al. (1982) achieved cellulase activity of 30 IU/ml with a yield of 200 IU/g cellulose. Watson et al. (1984) achieved the highest cellulase activity of 57 IU/ml and a yield of 226 IU/g cellulose with cellulose feed rate of 1 g/l/h.

Favourable cost benefits realized in continuous fermentation, therefore, can be offset if the product realized (i.e. enzyme-system) is of significant lower activity. The process would then require more vessel capacity or an extended residence time in the vessel, either of which lessens the economic advantages. Great care must be taken against the contamination problems which can nullify all the advantages of continuous fermentation. However, there is much to be done to improve the continuous or cyclic-batch fermentation to produce a cellulase system with high activity per unit of volume.

3. MATERIALS AND METHODS

3.1. MICROORGANISMS

The microorganisms (fungi) used in this study are mutants of Trichoderma reesei (i) QMY-1, developed by Dr D.S.Chahal, Institut Armand-Frappier, University of Quebec, Laval, Quebec, Canada, (ii) Rut-C30, procured from Dr J.J. Ellis, Northern Regional Research Centre, Peoria, Illinois, U.S.A. and (iii) QM9414, procured from Dr W.A. Khan, National Research Council of Canada, Ottawa, Canada.

The mutants were maintained at 30°C for 2 or 3 weeks in test tubes or petri plates, respectively, and then stored at 4°C (for not more than 2 months). The frequency of transfer varied from twice a month to once a month. The medium used in test tubes or petriplates was composed of Mandels' medium (see section 3.2.1) with addition of 1% agar and replacement of glucose with delignified wheat straw.

3.2. CULTURE CONDITIONS

3.2.1. Medium

The composition of the medium for 10 g/l glucose (or its equivalent) given by Mandels and Weber (1969) is as following: (in g/l) KH_2PO_4, 2.0; $(NH_4)_2SO_4$, 1.4; Urea, 0.3; $MgSO_4 \cdot 7H_2O$, 0.3; $CaCl_2$, 0.3; Yeast extract, 0.5; and (in mg/l) $FeSO_4 \cdot 7H_2O$, 5.0; $MnSO_4 \cdot 4H_2O$, 2.23; $ZnSO_4 \cdot 7H_2O$, 2.76; $CoCl_2 \cdot 6H_2O$, 3.66. Proteose peptone as used in original medium was replaced with yeast extract (Difco).

3.2.2. Sterilization

In 250 ml Erlenmeyer flasks, 100 ml volume of medium was used. The flasks were then autoclaved at 121 C for 25 minutes.

In 14- or 16-L fermenter, 9- or 11-L of medium was used and sterilized at 121 C for 40 minutes when glucose was used, but for 90 minutes when wood served as a substrate. The fermenter with wood as a substrate was left overnight with 0.1 vvm (volume of air per volume of liquid per minute) aeration at 30 C after

first sterilization. It was again sterilized for 40 minutes at 121 C to secure complete destruction of bacterial spores.

3.2.3. Inoculum

Spores of the three mutants of T. reesei were grown on 1% glucose solution in Mandels' medium (section 3.2.1) at 30 C for 36 h on shaker at 200 rpm. This will be referred to as pre-inoculum in the text. After 36 hours of growth it was used to inoculate other flasks containing 1% glucose solution in Mandels' medium (with pre-inoculum size of 10% v/v). The inoculated flasks were incubated on shaker at 200 rpm for 24 h where mycelium reached its exponential growth phase. This will be referred to as inoculum in the text. The inoculum is added to the fermenter or to other flasks in such a way that it will make 10% of the final volume of the fermentation broth.

3.2.4. Antifoam

In the fermenter, the foam build up was controlled automatically or manually by the addition of 'Antifoam-A Emulsion' (Sigma Chemical Co). Antifoam was not used in the shake flask experiments.

3.2.5. Temperature

Temperature in the fermenter and in the incubator for rotory shake flasks, test tubes and petriplates was maintained at 30 °C.

3.2.6. pH:

The pH values of 3, 4, 5, 6 and 7 has been used in this study for cellulase production. The pH used for the particular run has been mentioned at its appropriate place in the text.

In the shake flasks, the pH was adjusted by NaOH (10% wt/vol) or H_2SO_4 (10% wt/vol) to the desired pH after every 6, 12 or 24 hours of interval depending upon the type of the substrate used and how fast the pH change occured.

In the fermenter, the pH was adjusted automatically (see section 3.5) . The solutions of sodium hydroxide and sulphuric acid were used to maintain the desired pH.

3.2.7. Solid State Fermentation

From our past experience it was observed that solid state fermentation produced β-glucosidase to filter paper activity ratio of about 1:1. Thus higher β-glucosidase activity is obtained per filter paper activity. The solid state fermentation was performed in this study only for comparing the enzyme system produced by different mutants of T. reesei for their hydrolytic potential. The solid state fermentation was performed as following:

Five grams of wheat straw (WS) was treated with 4% sodium hydroxide (wt/wt of WS) with water:WS ratio of 2:1 for one hour at 121°C. Five ml of concentrated Mandels' medium based on 70% carbohydrates in wheat straw was added to the solids and pH was adjusted to 6.0 with sulphuric acid. Five ml of 24-hour old inoculum of each of mutant Rut-C 30 and QMY-1 were added severely to thus prepared sterilized medium. The samples after 14 days were taken and 80 ml of water was added to bring the total volume to 100 ml and substrate concentration to 5% (wt/vol). The enzymes of Rut-C30 and QMY-1 were collected by centrifuging the fermentation broth at 14 000 rpm in Beckman's Model J-21B Centrifuge at 4°C for 30 minutes. The

supernatants were stored at 4°C with 0.003% streptomycin, 0.1% sodium azide and 0.1% thimerosal (to stop bacterial or fungal growth). The cellulase activity was determined for the both enzyme systems.

3.3. SUBSTRATES

3.3.1. Glucose

Glucose was used as a substrate for growing pre-inoculum and inoculum.

3.3.2. Cellulose

Alpha-Cellulose (40 mesh size) of Sigma Chemical Co., was used as a substrate for cellulase production. It is 99.9% pure cellulose and is of fibrous form.

3.3.3. Agricultural Residues

Wheat straw was collected from Macdonald Campus, McGill University, Montreal. It was ground to 20 mesh and was pretreated as explained in section 3.4.

3.3.4. Wood

Aspen (Populus tremuloides) as wood powder of 40 and 20 mesh size were obtained from a local saw mill, and were pretreated as explained in section 3.4.

Chemithermomechanical pulp (CTMP), produced from aspen was obtained from Dr S.N.Lo and Dr J.L.Valade, University of Quebec, Trois- Rivieres, Quebec, Canada.

3.4. PRETREATMENTS

Two pretreatments, sodium hydroxide and chemithermomechanical pulping were used for the cellulase production.

3.4.1. Pretreatment I: Sodium Hydroxide Treatment

3.4.1.1. Conditions

a) Wood: The cellulose content of the aspen wood is about 50% cellulose. Five gram (dry weight basis) of wood (3.83% moisture) was used as a basis for this treatment.

b) Sodium Hydroxide Concentrations: Wood was mixed with sodium hydroxide at application ratios ranging from 0-104% wt/wt of wood. Sodium hydroxide solutions were made in such concentrations that the required ratios were obtained with 10 ml sodium hydroxide solution. Two other sets were made in order to check the effect of water concentration, therefore, additional water was added to the flasks to get three different water contents. By doing this, application ratio of sodium hydroxide with respect to wood remained

same but the sodium hydroxide concentration wt/vol decreased.

c) Water Contents: In order to check the effect of different water contents on the solubilization of hemicelluloses and lignin in addition to the required sodium hydroxide application ratio, extra water was added as following:

SET A: 10 ml NaOH solution of given application ratio (200% solution vol/wt of wood)

SET B: As in SET A + 5 ml water (300% solution vol/wt of wood)

SET C: As in SET A + 10 ml water (400% solution vol/wt of wood)

d) Soaking Time: The contents in the flasks (wood and sodium hydroxide solution) were mixed as quickly as possible (within 30-40 seconds / flask) with stainless steel spatula. The flasks were left at room temperature for various time period: 1.5, 6 and 24 h. From first few experiments, by observing the solubilization, the optimal soaking time was calculated and then the rest of the experiments were performed by using thus found optimal soaking time.

e) Heating Time: Heating time after soaking the substrate at room temperature was fixed for one hour and was provided by autoclaving at 121°C at fast exhaust setting. The heat-up time for all the sets in this treatment was constant, therefore, it was not taken into account for its effect on the solubilization.

f) Procedure: The flasks given the fixed soaking time and heat treatment after mixing the sodium hydroxide solution to wood were cooled down to room temperature (forced cooling was not performed). The contents of each flask were filtered by using the tared Whatman filter papers (grade 802) and washed four times with distilled water. The contents on the filter paper were dried in oven at 80°C overnight and percentage weight loss of wood was calculated on dry weight basis.

3.4.1.2. Cellulase Production on NaOH Treated Wood

Four sets of experiments were performed using 1% aspen (dry wt basis) in Mandels' medium at pH 6.0 and 30°C.

Experiment 1: Aspen as such (no treatment) in shake flasks.

Experiment 2: Aspen treated with 20% NaOH, heated at 121°C for 1 h in shake flasks, water:wood ratio = 2:1.

Experiment 3: Aspen treated with 20% NaOH, by boiling on hot plate for 3 h, water:wood ratio = 20:1, in shake flasks. The volume of the solution was reduced to half by evaporation.

Experiment 4: Same as Experiment 3 but, in 14 - 1 fermenter.

The pH was adjusted to initial value of 6.0 after every 24 h in the shake flasks, whereas, pH was automatically maintained at 6.0 in the fermenter by pH controller unit of New Brunswick Inc., with the addition of sodium hydroxide or sulphuric acid solutions.

3.4.2. Pretreatment II: Chemithermomechanical Pulp (CTMP)

The CTMP was prepared by Dr S.N. Lo and Dr J.L. Valade, University of Quebec, Trois-Rivieres, Quebec,

Canada, and the following procedure was followed:

Regular size (2-4 cm wide and 0.5-1.0 cm thick) industrial wood chips of aspen from the Lac St. Jean region of Quebec, Canada, were used in preparation of chemithermomechanical pulps (Law et al., 1983). All chips were screened and washed with water before use. Pulping was carried out in a Sunds Defibrator pilot plant unit, type 300 CD, using single stage pressurized refining (Fig. 3.1).

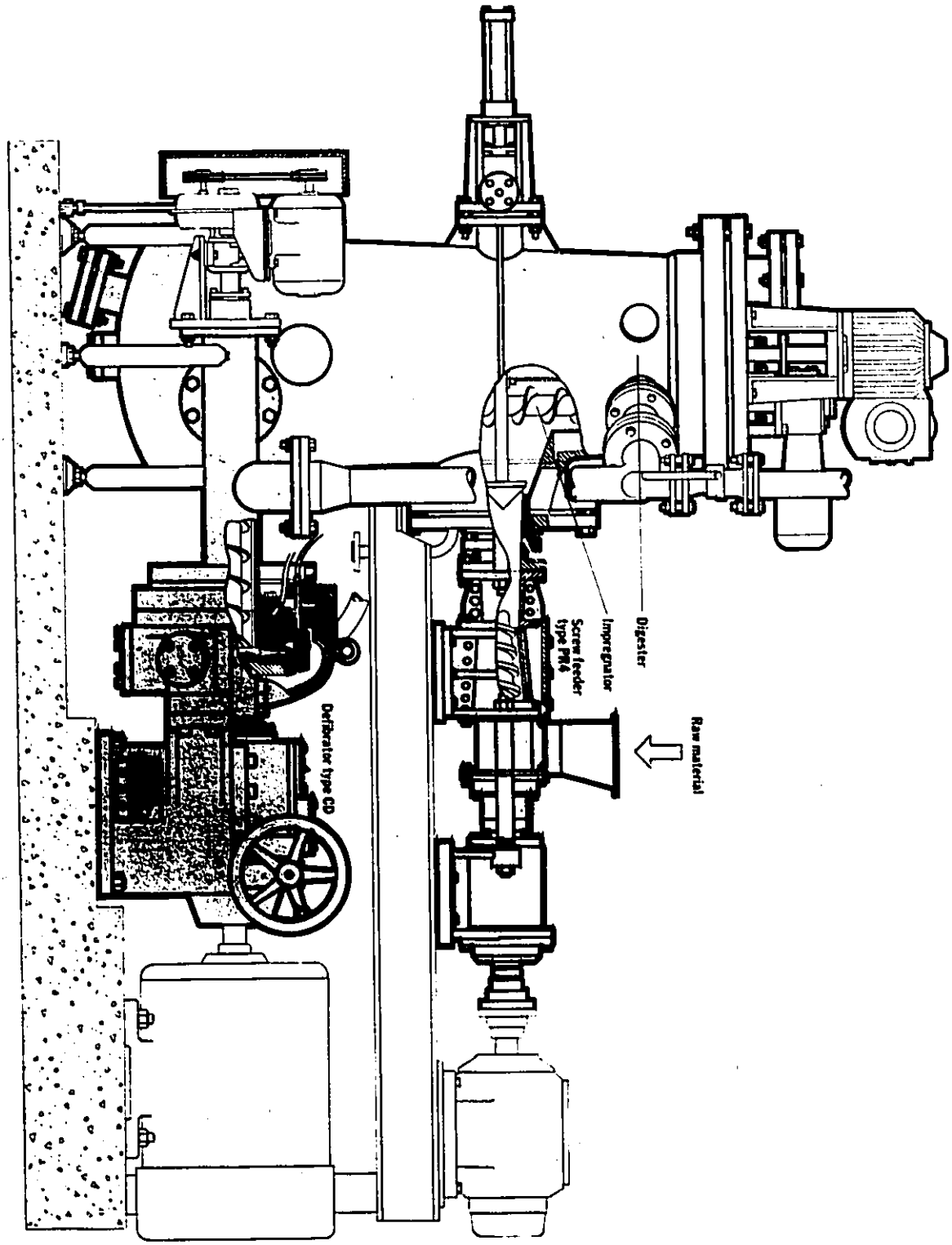
Wood chips were chemically treated in an impregnator built inside the pilot plant unit. Thus chemically treated chips were then steamed in the preheater of the unit for five minutes after which they were fed to the refiner for refining. The conditions used in the pulping process were as follows:

1) the input of the chips to the pilot plant was 0.8 kg (on oven dry weight)/minute;

2) the chemicals charged for the treatment of the chips, on oven dry weight of the wood were a) 10% NaOH, b) 5% NaOH and 5% Na_2SO_3;

3) the ratio of chemical solution fed to the impregnator to the oven dry weight of the chips fed to the unit was 1:1 (wt/wt);

Figure 3.1. Sunds Defibrator Pilot Plant Unit.


Defibrator type CD
Screw feeder type PR4
Impregnator
Digester
Raw material

4) the steam pressure in the preheater was 238 kPa (20 psig) which corresponds to 126°C;

5) the steam pressure for refining was 260 kPa (23 psig) which corresponds to 129°C;

6) the clearance between the two flat refining discs was 0.2 mm;

7) the input of water to flat disc refining zone was 2.3 l/min;

8) the clearance between the two cone refining discs was 0.1 mm and

9) the input of water to the cone refining zone was 0.38 l/min.

Under the above conditions, the pulps produced had a consistency of about 25%. The yield of pulp was 90% and the resulting pulp had a Canadian standard freeness of 100 ml (Law et al., 1983). The CTMP thus produced contains about 60% cellulose (determined as explained in section 3.6.2).

3.5. REACTORS: SHAKE FLASKS / FERMENTERS

Erlenmeyer flasks of 250 ml capacity were used with working volume of 100 ml. Preliminary growth experiments were carried out in the shake flasks because of the following advantages: initial substrate concentration, fixed volume, temperature and agitation can be controlled for each substrate or medium and is quite convenient in investigating the growth characteristics of different microorganisms. However, there is a disadvantage in controlling the desired pH. This problem was overcome to some extent by adjusting pH at a short time intervals (6, 12 or 24 h), depending on the type of substrate and the organism.

Microferm Fermenter of New Brunswick Scientific Co. Inc., Edison, N.J. U.S.A. of 14- and 16- litre capacity were used. Their working capacity is 10 and 12 litres, respectively. The pH was controlled by pH Controller model pH22 with acid or base, and dissolved oxygen was monitored by D.O. Controller for Automatic Regulation and Recording Model DO-81 of New Brunswick Scientific Co., Inc. Edison, N.J., U.S.A. The electrode used with DO-81 instrument was steam sterilizable Galvanic-Type Dissolved Oxygen electrode with replaceable membrane Model 900 DO of New Brunswick

Scientific Co., Inc. The electrode contained lead anode, a replaceable platinum cathode and fluorinated ethylene propylene (FEP) membrane of 51 μ thickness. Temperature was maintained automatically by using cooling water or heating it with 750 Watt heater. The agitation was also maintained automatically at the desired value (in the range of 150 - 400 rpm).

3.6. ANALYTICAL PROCEDURES

3.6.1. Dry Weight Determination

Dry weight was determined by filtering 100 ml of fermentation broth through tared Whatman filter paper No.1. The broth was then washed at least three times with distilled water on the filter paper . The solids were dried at 80 C overnight to determine the total dry weight of the fermentation broth. The solids were reweighed after additional 4 hours of drying at 80°C for the consistancy of the results. Dry weight thus determined contained mycelium, insoluble salts used in the medium, and unutilized substrate (if insoluble eg. wood).

3.6.2. Cellulose Determination

Cellulose was determined by the method described by Updegraff (1969). Approximately 30 mg of dried sample was boiled for 30 minutes with 3 ml of acetic / nitric acid reagent (15 ml concentrated nitric acid in 150 ml 80% acetic acid) (Crampton et al., 1938) for removing lignin and hemicelluloses. The remaining

sample was centrifuged and washed three times with 5 ml of deionized water at 12 000 rpm for 10 minutes. The supernatant was discarded and 3 ml of 67% w/v sulphuric acid was added to the residuals (cellulose) and left at room temperature for 60 minutes. The hydrolysis of cellulose was achieved by this reaction and glucose was produced. The solution was then dilluted to 200 ml with deionized water. Five ml of cold 0.2% anthrone (0.2 g anthrone in 100 ml of concentrated sulphuric acid, cooled for at least 2 hours at 4 C, prepared fresh) (Scott et al., 1953), was added to 1 ml of diluted sample, mixed and boiled for 20 minutes. After cooling and bringing to room temperature (5-10 minutes), the absorbance was read at 620 nm in a Unicam SP-1800 Ultraviolet Spectrophotometer (slit width of 0.3mm and band width of 0.9 nm). The standard curve was prepared using alpha cellulose (99.9% pure cellulose; Sigma Chemical Co).

3.6.3. Nitrogen / Crude Protein Estimation

Total nitrogen was determined by the micro-kjeldahl method of AOAC (1970). Fifty to 100 mg of dried sample was digested in a digestion mixture

(mercuric oxide 0.04 g, potassium sulphate 1.3 g and concentrated sulphuric acid 2.0 ml) for 2 h at medium to high heat. The sample was cooled and 5 ml of deionized water was added. The sample was added to the "Rapid Distillation Unit" of Labconco, by rinsing 3 times with water (total water to be added ca 30 ml). Eight ml of sodium hydroxide-sodium thiosulphate solution (solution A: sodium hydroxide 100 g and water 130 ml; solution B: sodium thiosulphate 10g and water 30 ml; solutions A and B were mixed when cold, then brought to the volume of 200 ml, kept in dark) was added to the sample solution. Distilled for five minutes, starting from the first drop from the condenser of the "Rapid Distillation Unit", and the condensate is recovered in five ml of 4% boric acid (prepared in deionized water, pH adjusted to 4.0 with 2% NaOH) containing 4 drops of mixed indicator. The boric acid solution absorbed ammonia, which was determined by back titrating with 0.1 N sulphuric acid solution. Casein (100 mg) was used to construct a standard curve for amount of sulphuric acid required for titration vs amount of protein in the sample. Casein (Sigma Chemical Co) contains 90% protein. Total nitrogen was calculated by using the formula given in Appendix I.

The crude protein was calculated as 6.25 X total nitrogen.

3.6.4. Sugar Estimation

Sugars (glucose, cellobiose, xylose and arabinose) were estimated by using a Beckman 344 gradient high-pressure liquid chromatograph equiped with an Altex 156 Refractive Index detector and Spherogel 7.5% Carbohydrate column with a flow rate of 0.3-0.5 ml/min in the mobile phase of deionized and degassed water at 90°C. The sugar samples (25 µl) were appropriately diluted before injection. Calibration was based on the peak heights of the sugars on the chromatogram.

Reducing sugars were estimated by the method described by Mandels et al. (Mandels et al., 1976) by using dinitrosalicylic (DNS) acid, expressed as glucose equivalents.

Dintrosalicylic (DNS) reagent used in this study was prepared by dissolving, first, 16 g NaOH and 10 g 3,5-dinitrosalicylic (DNS) acid in 500 ml deionized water. When DNS was completely dissolved, 300g of

sodium-potassium tartrate was added and dissolved. The solution was made to one litre with deionized water. Glucose 0.1 g and thimerosal 0.1 g were added to the solution and kept in dark at 4°C.

3.6.5. Enzyme Activity Estimation

The samples were stored at 4°C. Tween 80 in 0.1% concentration was added to the sample of about 100 ml volume, and was placed on the rotary shaker at 200 rpm for half an hour to release the enzyme adsorbed onto the substrate. Castanon and Wilke (1980) reported that bulk of enzyme including total protein is adsorbed almost instantaneously on the cellulosic matrix. Tween 80, being a good surfactant, (Dennis et al., 1979), contributes to release the adsorbed enzyme on the residual cellulose into the broth. The solution was then centrifuged at 14 000 rpm at 4°C for 30 minutes. Supernatant was stored in the test tubes for enzyme assays.

Cellulase:

Cellulase was measured by the method described by Mandels et al. (1976). With appropriately diluted

(dilutions in halves e.g. 1/2, 1/4 etc., higher dilution was required for concentrated enzyme) 1 ml enzyme in 1 ml of 0.1 M citrate buffer solution, pH 4.8, 50 mg Whatman No. 1 filter paper strip was incubated for 60 minutes at 50°C. The reaction was then stopped by addition of 3 ml dinitrosalicylic (DNS) acid solution and boiling for 10 minutes. The intesity of colour (representing amount of glucose produced) was read at 550 nm using SP-1800 spectrophotometer. Glucose was used as a standard. The results were reported in International Units (IU) per millilitre which referred to as the filter paper activity (FPA) or cellulase activity.

1 IU = 1 μ mole of glucose produced / min
(based on 1 h of reaction)

β-glucosidase:

β-glucosidase was measured by the following method: With appropriate diluted (as for cellulase) 0.5 ml enzyme and 0.5 ml of 1% Salicin (2-[hydroxy-methyl] phenyl - β - D - glucopyranoside) solution (prepared in 0.05M Citrate buffer, pH 4.8) was

incubated for 30 minutes at 50°C. Blank consisting of 0.5 ml of buffer and 0.5 ml of salicin solution was incubated concurrently with the test. The reaction was stopped by the addition of 2 ml dinitrosalicylic (DNS) acid and then boiled for ten minutes. Glucose was used as a standard. The intensity of colour produced was read at 550 nm on SP-1800 spectrophotometer. The results were reported in International Units (IU) per millilitre:

1 IU = 1 μ mole of glucose produced / minute
(based on 30 minutes of reaction)

Cotton Cellulase Activity (C_1):

Cotton cellulase was measured by adding appropriately diluted (as for cellulase) 1 ml enzyme to 50 mg of absorbent cotton. Five ml of 0.05M Citrate buffer, pH 4.8, was added, mixed and incubated for 24 h at 50°C. After incubation, two ml of this solution was mixed with 3 ml of DNS reagent to stop the reaction. The intensity of the colour produced by boiling for 10 minutes, and cooled to room temperature, was read at 550 nm on SP-1800 spectrophotometer, representing the

amount of reducing sugars produced. Glucose was used as a standard. The cotton cellulase activity was expressed as Units/ml (mg of reducing sugars produced/ ml of undiluted enzyme in 24 h at 50°C).

Xylanase:

The activity of xylanase was measured by following the release of reducing sugars from xylan. The assay mixture containing 1 ml of appropriately diluted (as for cellulase) enzyme preparation and 1 ml of 1% xylan suspension in McIlvaine citrate buffer, pH 6.5, was incubated at 50°C for 10 minutes. Blanks consisting of 1 ml of buffer and 1 ml of xylan suspension were incubated concurrently with the test. The reaction was terminated by the addition of 2 ml of dinitrosalycilic (DNS) acid reagent and boiling for ten minutes. The released reducing sugars were determined by the intensity of the colour read at 550 nm using SP-1800 spectrophotometer. D-xylose was used as a standard. One unit of xylanase was defined as the release of one micro-mole of reducing sugar as xylose produced in one minute (based on 10 minutes of reaction) by 1 ml of enzyme which corresponds to one international unit (IU).

Corrections were made for reducing sugars initally present in the broth, by substracting sugars present in the blanks (i.e. enzyme solution without addition of any filter paper, salicin or xylan).

3.7. DISSOLVED OXYGEN DETERMINATION

In order to determine Qo_2X and K_1a values by 'dynamic' method of Bandopadhyay et al., (1967), dissolved oxygen (DO) was monitored throughout the fermentation in the fermenter. The DO was monitored to keep dissolved oxygen above critical (20% of saturation) level. The Qo_2 values were used to study the rate of oxygen consumption by the microorganism during the fermentation period. The K_1a values were calculated to observe if the properties of the broth has been changed and also how fast oxygen was being dissolved in the broth during the fermentation period. The calibration of DO probe was necessary in order to know the precise value of dissolved oxygen in the fermentation broth. Reproducibility of the probe was within 1% at constant temperature.

3.7.1. Calibration of DO Probe

In distilled water at 30°C, air was sparged for at least 10 minutes in the fermenter. When dissolved oxygen reading by DO probe was stabilized, barometric pressure was recorded (since no barometer was available

in our laboratory, the barometric pressure was obtained from the Environment Canada at Montreal Airport, Dorval at the time of the reading). Using the following Equation for temperature range 0-30°C (Standard Methods for Examination of Water and Waste Water, 1976) the dissolved oxygen in mg/l was calculated:

$$DO \ (mg/l) = \frac{(P-P') \cdot X \cdot 0.678}{35 + k}$$

where P = barometric pressure, in mm Hg,

P' = saturation vapour pressure of water (obtained from Table 1.3 of Handbook of Tables for Applied Engineering Science (CRC)), and

k = temperature in °C.

The meter reading was then set to the DO value obtained times 10 (because DO meter scale range was 0 to 100 % saturation and DO values were usually in the range of 0-10 mg/l). The nutrients and substrate were added to the water at the same temperature and was mixed for at least 15 minutes with air being continuously sparged through the solution. When DO value was stabilized the value of dissolved oxygen was recorded (Reading A). The fermenter was then

sterilized. As recommended by Johnson et al. (1964) the DO probe was short circuited during the sterilization and the recorder was connected to the cold probe. After the sterilization, the temperature was maintained at 30°C for more that 30 minutes. Air was sparged again through the solution at the same agitation as initially set prior to sterilization. The DO value was read when DO reading was stabilized (Reading B). A slight change in reading was observed due to sterilization (e.g. before and after sterilization the DO values recorded in one experiment were 6.5 and 6.2 mg/l, respectively). The new DO reading (Reading B) was brought back to the correct value (Reading A), assuming that sterilization did not bring any physical or chemical changes in the medium. Thus the DO meter reading divided by ten gave the dissolved oxygen concentration in mg of O_2/l.

3.7.2. Qo_2X and K_1a Determination

The Qo_2X and K_1a values were determined by "dynamic" method of Bandopadhyay et al., (1967). The Qo_2X value represents the rate at which oxygen is being consumed by the organism at a given time, wheras,

K_1a (the oxygen transfer coefficient) represents how fast oxygen is being dissolved into the fermentation broth from the supplied air. The value of K_1a depends upon the impeller speed, number of impellers, the size and type of impellers, the size of the fermenter, air flow rate, the composition of the medium, properties of broth, temperature and pH.

The dynamic method is based on following the dissolved oxygen concentration during brief interruption of the aeration of the fermenting system. In this procedure, air is turned off (referred to as "Non-Gassing Situation"), and at the same time the surface of the fermentation broth is saturated with helium gas. This prevents the back mixing of the oxygen in the fermentation broth by the vortex created by the impeller. The air is kept off untill the dissolved oxygen reaches about 30% saturation. The rate at which dissolved oxygen is dropped is represented by:

$$dC/dt = - Qo_2X \qquad (1)$$

where C is the concentration of dissolved oxygen (mg O_2/l)

t is the time (s)

Qo_2 is the specific oxygen uptake rate (mg O_2/g protein/s)

and X is the biomass expressed as protein concentration (g/l).

A straight line is obtained (Appendix II). The absolute value of the slope on C vs t plot gives the value of Qo_2X.

Next, the aeration is resumed (referred to as "Gassing Situation"). In this situation the dissolved oxygen reaches its saturation value. This curve includes both the rate at which microorganism is consuming the oxygen and the rate at which the oxygen is being dissolved into the fermentation broth. The rate of change of dissolved oxygen in this case is represented by the following Equation:

$$dC/dt = K_1a(C^*-C) - Qo_2X \qquad (2)$$

where K_1 is the mass transfer coefficient, (m/h)

a is the interfacial surface area for mass transfer, (m^2/m^3)

C^* is the concentration of dissolved oxygen at saturation (mg O_2/l)

C is the concentration of dissolved oxygen at any time (mg O_2/l)

Rearranging the Eq. 2 and solving for C, following Eq. 3 is obtained:

$$C = - (1/K_1a) (dC/dt + Qo_2X) + C^* \qquad (3)$$

and by plotting C vs ($dC/dt + Qo_2X$), the value of K_1a can be calculated as an inverse of the negative slope, and C intercept will represent the C^* value. A typical data obtained for determining Qo_2X and K_1a has been shown in Appendix II.

By knowing the protein content in the fermentation broth at the time when Qo_2X value was determined, the value of Qo_2 can be calculated.

4. RESULTS AND DISCUSSION:

To date, the enzymatic hydrolysis of lignocellulosic materials have not been proven economical because of the high cost of enzyme production, low cellulase activity per unit volume and low concentration of sugar in the hydrolysate. The low concentration of sugar in the fermentation broth further adds cost to the ethanol distillation. Therefore, there is a need for a better mutant of Trichoderma reesei, an appropriate substrate (preferentially lignocellulose, to avoid pre-separation of cellulose from lignin), requiring minimum pretreatment (to make substrate easily excessible to the organism) and optimal pH. These needs were taken as basis of this study, to optimize the cellulase production per unit volume and maximize the cellulase yield per unit time.

4.1. CHOICE OF MICROORGANISM

4.1.1. Comparison of Mutants of T. reesei

Test tubes containing 5 ml of 1% delignified wheat straw in Mandels' medium and 1% agar were prepared. The three mutants when grown in these test tubes produced a clear zone in order of increasing thickness:QM9414, Rut-C30, QMY-1, respectively. QMY-1 showed that the cellulase enzyme penetrated deeper in the agar than other mutants at any given time. Similar results were observed when mutants were grown on agar-delignified wheat straw (in Mandels' medium) in Petri plates. QMY-1 imparted a characteristic yellow colour to agar whereas mutants Rut-C30 and QM9414 were both whitish or whitish grey in colour (Figure 4.5).

4.1.2. Enzyme Production by Mutants of T. reesei on Pure Cellulose

The first comparison was performed by using 1%, alpha-cellulose in Mandels' medium. In Erlenmeyer flasks, 90 ml of this medium was inoculated with 10 ml of 24 h old inocula of Rut-C30, QM9414, and QMY-1 in three different sets A, B and C respectively. The pH was adjusted to 6.0 after every 24 h with sodium

hydroxide or sulphuric acid solutions.

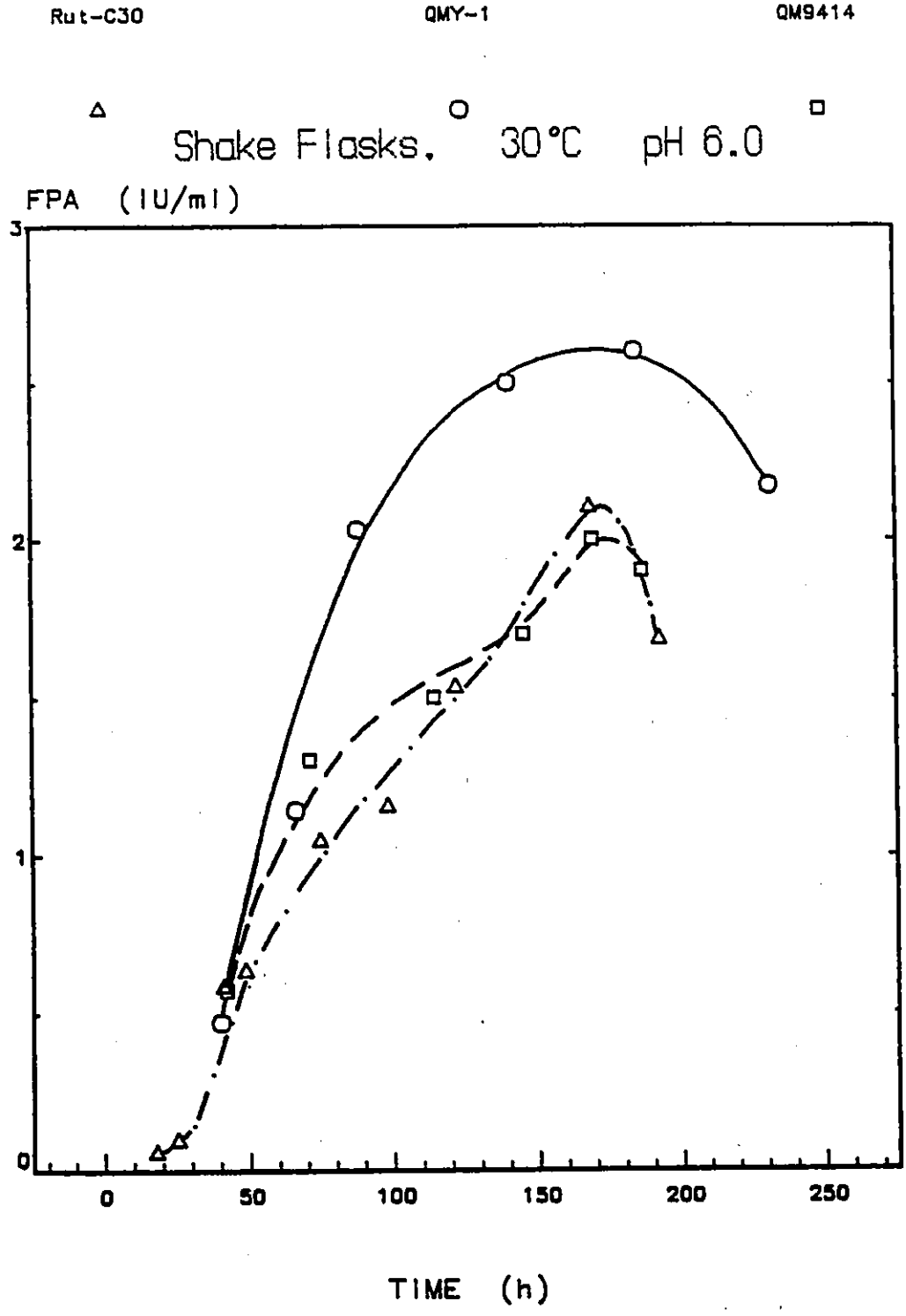
Cellulase activities of 2.1, 2.0 and 2.6 IU/ml were obtained with Rut-C30, QM9414 and QMY-1 with a cellulase yield of 210, 200 and 260 IU/g cellulose supplied after 168, 169 and 184 h of incubation, respectively, on rotary shaker at 30°C. (Fig. 4.1 and Table 4.1). The cellulase yield is defined as following (see Appendix III for sample calculations):

$$\text{Cellulase yield} = \frac{\text{Total cellulase produced (IU/L)}}{\text{Total cellulose supplied (g/L)}}$$

The cellulase activity of Rut-C30 and QM9414 were almost the same (2.1 and 2.0 IU/ml respectively), but, QMY-1 yielded a relatively higher cellulase activity (2.6 IU/ml). It was noticed that QMY-1 took 184 h to achieve this high value whereas maximum of Rut-C30 and QM9414 was achieved in 168 and 169 h, respectively. In the fermenter, the cellulase activity of 4.0 IU/ml (yield of 400 IU/g cellulose) was achieved with QMY-1 when grown on 1% alpha-cellulose and the time was significantly reduced to 91 h from 184 h (in the case of the flasks). (Fig. 4.2 and Table 4.1). This could be due to better aeration and constant pH maintained in the fermenter.

Fig. 4.1. CELLULASE PRODUCTION ON PURE CELLULOSE

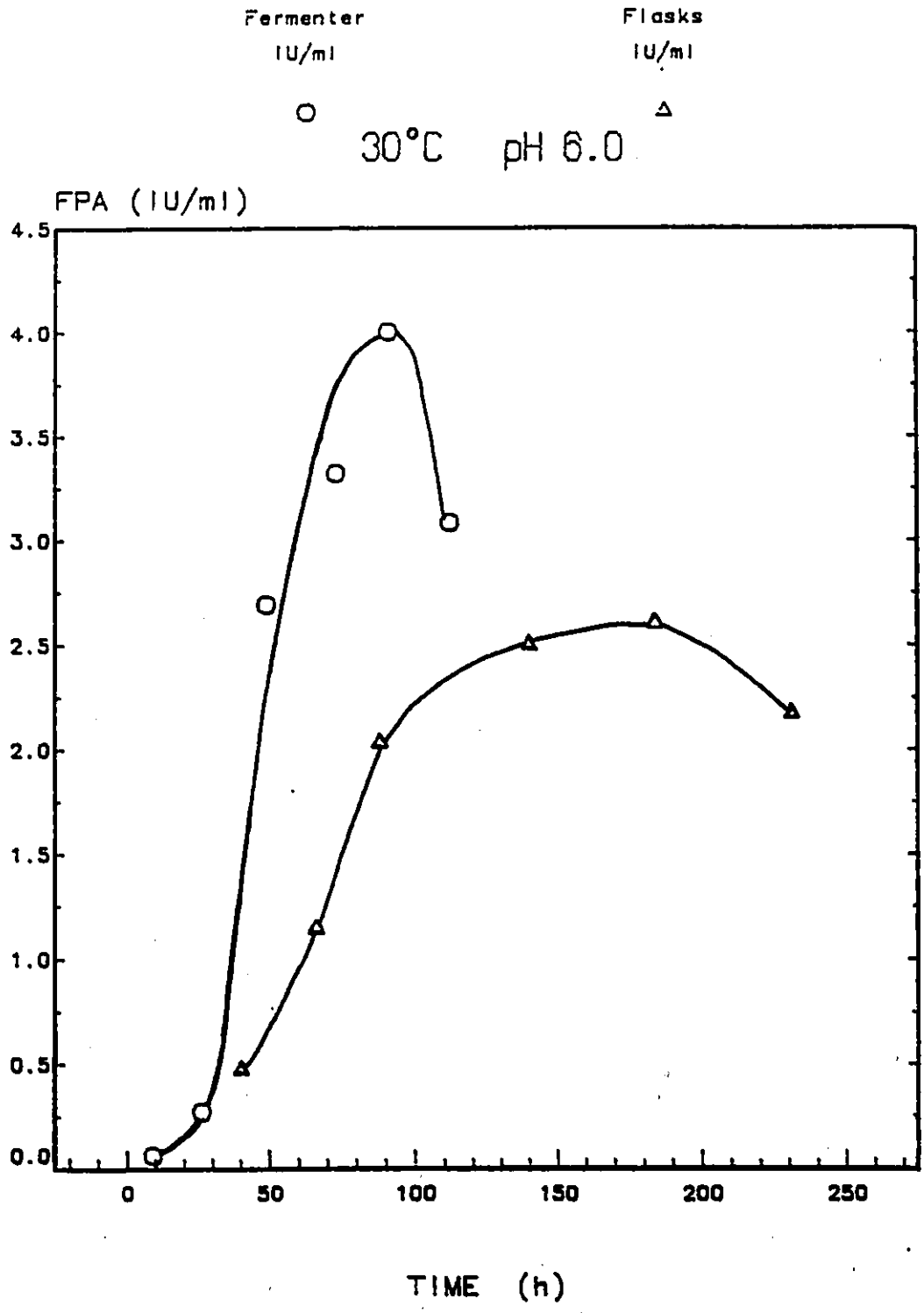
by mutants of T. reesei: Rut-C30, QMY-1 and QM9414



Data in Table 4.1

Fig. 4.2. CELLULASE PRODUCTION ON PURE CELLULOSE

by QMY-1, in Fermenter and in Shake Flasks



Data in Table 4.1.

Mitra and Wilke (1975) and Andreotti et al., (1977) achieved cellulase yield of 130 and 170 IU/g cellulose supplied (in the fermenter) on 1% alpha cellulose, respectively, by using mutant QM9414. Chahal et al., (1982) and Duff et al., (1985) achieved cellulase yield of 102 and 250 IU/g cellulose supplied (in the fermenter) on 1% alpha cellulose respectively by using mutant Rut-C30. In the literature, the maximum cellulase yield of Rut-C30 and QM9414 is in the range of 39 - 450 IU/g cellulose supplied (Table 4.2). The high values were obtained (i) by adding Alon (Duff et al., 1985) or sophorose [2-O-β-D-glucopyranosyl-D-glucose] (Webb et al., 1986), and (ii) by performing fed-batch fermentation (Gottvaldova et al., 1982; Hendy et al., 1982; McLean and Podruzny 1985; Watson et al., 1984).

From this data, QMY-1 appears to be a better cellulase producer than Rut-C30 and QM9414 mutants of Trichoderma reesei.

4.1.3. Effect of Hemicelluloses on Enzyme Production:

An alkaline extract (which included lignin, hemicelluloses and extraneous matters) was prepared by

TABLE 4.2. CELLULASE PRODUCTION OF MUTANTS OF T. reesei

No.	SUBSTRATE	CONC. %	MUTANT	CELLULASE IU/ml	CELLULASE IU/g*	REFERENCE
1.	Pure Cellulose	1.0	Rut-C30	1.02	102	Chahal et al., 1981
2.	" "	"	"	2.5	250	Duff, et al., 1985
3.	" "	2.0	"	8.0	400**	Mclean & Podruzny 1985
4.	" "	"	"	4.4	220	Saddler, et al., 1982
5.	" "	"	"	4.2	210	Hendy et al., 1982
6.	" "	"	"	5.9	295	Duff et al., 1985
7.	" "	2.5	"	3.5	140	Chahal et al.1981
8.	" "	"	"	5.2	208	Tangnu et al.,1981
9.	" "	3.0	"	7.1	237	Duff et al., 1985
10.	" "	4.0	"	4.1	102	Khan and Lamb,1984
11.	" "	"	"	5.56	139	Forintek Canada Corp.1983
12.	" "	"	"	4.0	100	Morisset and Khan 1984
13.	" "	5.0	"	12.0	240	Watson & Nelligan 1983
14.	" "	"	"	14.0	280	Tangnu et al., 1981
15.	" "	"	"	10.0	200**	Hendy et al.,1982
16.	" "	"	"	8.0	160	"
17.	" "	6.0	"	6.7	112	Mandels et al., 1981
18.	" "	"	"	14.0	233	Ryu and Mandels, 1980
19.	" "	7.5	"	8.4	112	Hendy et al., 1982
20.	" "	8.0	"	31.1	389**	Mclean & Podruzny, 1985

21.	" "	10.0	"	8.0	80	Hendy et al., 1982
22.	" "	"	"	20.0	200**	"
23.	" "	15.0	"	30.4	203**	"
24.	" "	25.2	"	57.0	226**	Watson et al., 1984
25.	Cellulose + ALON	1.0	"	4.5	450	Duff et al., 1985
26.	" "	2.0	"	7.6	380	"
27.	" "	3.0	"	9.5	317	"
28.	Lactose	1.0	"	1.5	-	"
29.	"	6.0	"	1.1	-	Mandels et al., 1981
30.	Steam exploded wood (56V water washed), cellulose conc=1%	1.0	"	1.2	120	Chahal et al. 1981
31.	" "	2.2	"	3.7	168	"
32.	Steam exploded wood (SED)	4.0	"	1.6	40	Khan and Lamb, 1984
33.	SED+20% cellulose	4.0	"	3.9	97.5	"
34.	SE wood	5.0	"	1.57	57.1	Forintek Canada Corp. 1983
35.	SE water extracted	5.0	"	2.1	46.1	"
36.	Pure cellulose	0.5	QM9414	1.2	240***	Sahai & Ghose, 1977
37.	" "	"	"	1.8	360	Joglekar & Karanth 1984
38.	" "	0.75	"	0.6	80	Sternberg 1976a
39.	" "	1.0	"	1.7	170	Andreotti et al., 1977
40.	" "	"	"	1.3	130	Mitra and Wilke, 1975
41.	" "	"	"	0.6	60	Labudova & Farkas 1983
42.	" "	2.0	"	1.6	80	Sternberg 1976a

43.	" "	"	"	4.5	225	Saddler et al., 1982
44.	" "	2.5	"	4.3	172	Tangnu et al.,1981
45.	" "	5.0	"	5.1	102	"
46.	" "	6.0	"	3.0	50	Mandels et al., 1981
47.	Pure cellulose + 0.1% peptone.	6.0	"	5.1	85	"
48.	Cotton	6.0	"	10.0	167	Ryu and Mandels 1980
49.	Steam exploded wood	5.0	"	0.7	25.4	Forintek Canada Corp.1983
50.	Stean exploded wood, water extracted & alkali treated.	5.0	"	0.96	21.1	"
51.	Lactose	6.0	"	0.5	-	Mandels et al., 1981
52.	Feedlot wastes	2.5	QM9123	5.5	220	Griffin et al., 1976
53.	Barley straw NaOH treated, unwashed, + 0.05% Peptone	1.0	"	1.4	350	Peitersen 1975
54.	Barley straw NaOH treated, unwashed, + 0.1% Peptone	"	"	1.3	325	"
55.	Barley straw untreated, + 0.05% Peptone.	"	"	0.8	200	"
56.	Cotton	6.0	"	5.0	83	Ryu and Mandels 1980

57. Barley straw NaOH treated, unwashed, + 0.05% Peptone	1.0	"	1.22	305	Peitersen 1975
58. Barley straw NaOH treated, unwashed, + 0.1% Peptone	"	"	0.5	125	"
59. Barley straw untreated, + 0.05% Peptone.	"	"	0.38	95	"
60. Pure Cellulose	2.0	E-58	3.5	175	Sadler et al., 1982
61. Pure Cellulose	6.0	MCG-77	5.7	95	Mandels et al., 1981
62. Cotton	6.0	"	11.0	183	Ryu and Mandels, 1980
63. Lactose	"	"	1.1	-	Mandels et al., 1981
64. Cotton	6.0	NG-14	15.0	250	Ryu and Mandels, 1980
65. Pure Cellulose	2.5	P.Puni-culosum UV-49	3.3	132	Joglekar & Karanth 1984

* IU/g = (Total IU produced /L)/(g cellulose supplied/L)

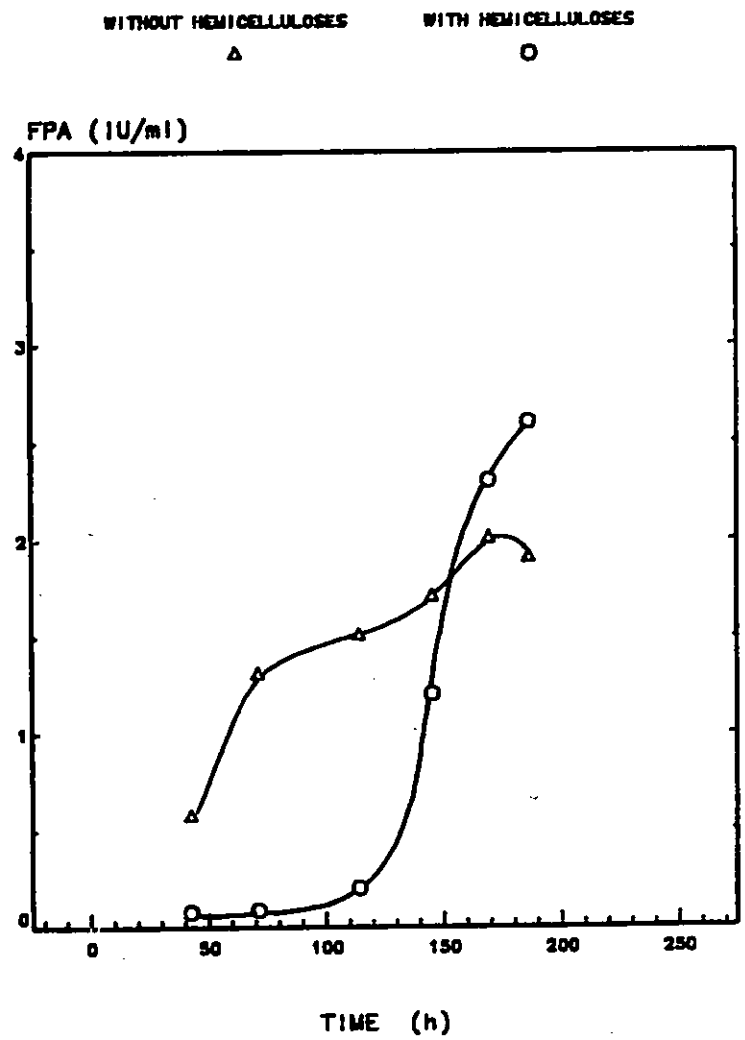
** Fed-Batch Fermentation

*** Continuous Fermentation

treating wheat straw (WS) for one hour at 121°C with 20% sodium hydroxide application ratio (wt/wt of wheat straw) and 2 to 1 liquid to solid ratio. This extract (obtained by squeezing through four layers of cheese cloth) was added to 1% alpha cellulose solution in Mandels' medium in 30% ratio to alpha cellulose (to simulate wood composition). This medium was inoculated with QM9414 and QMY-1 and cellulase activity of 2.6 and 3.4 IU/ml was achieved with cellulase yield of 260 and 340 IU/g cellulose supplied in 186 and 184 h, respectively (Fig. 4.3A and 4.3B). There was a long delay in cellulase production when the alkaline extract was added in the medium. The reason for the lag phase in cellulase production was that when hemicelluloses were being utilized by the organism during the early phase of growth, cellulase was not produced. The lag phase could also be due to the presence of some toxic materials produced (lignin degradation products) during alkali treatment. But, the absence of lag phase in cellulase profile, by QMY-1, may be due to the microorganism's ability to withstand the toxicity at the level present in the medium. Since, the biomass increased during the early phase of fermentation, by growing on hemicelluloses (probably), therefore, there might be more mycelium to convert cellulose to cellulase in the later phase of fermentation. The

Fig. 4.3A. EFFECT OF HEMICELLULOSES ON CELLULASE PRODUCTION

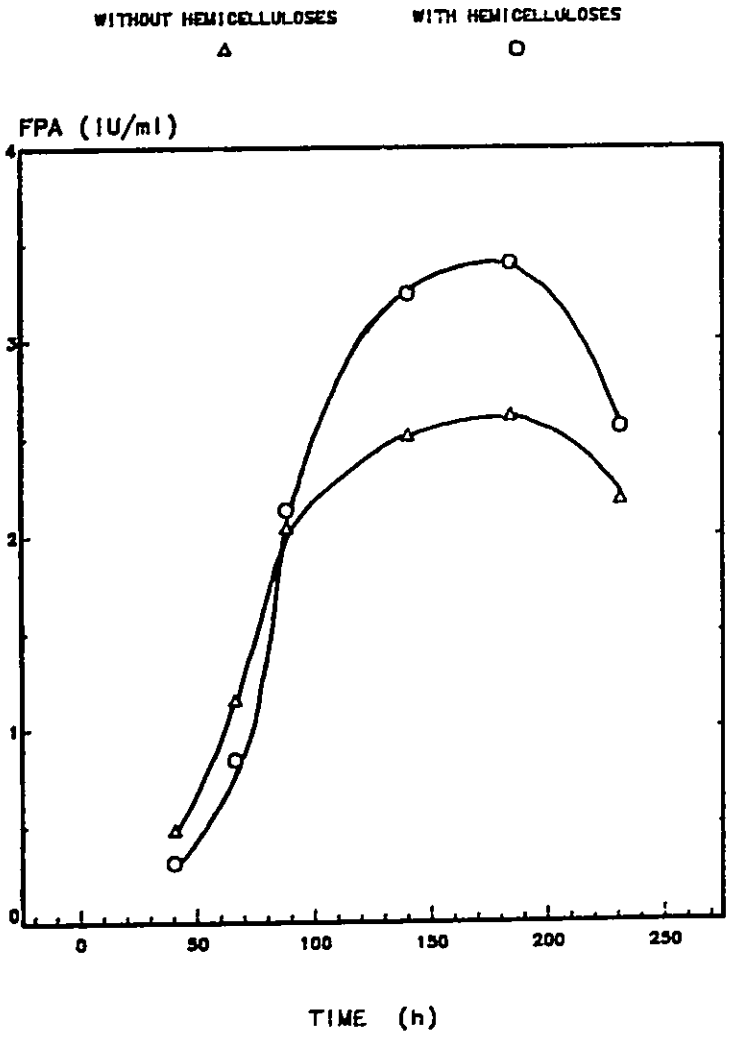
On 1% Alpha-Cellulose, With QM9414



Data in Tables 4.1 and 4.3.

Fig. 4.3B. EFFECT OF HEMICELLULOSES ON CELLULASE PRODUCTION

On 1% Alpha-Cellulose, With QMY-1



Data in Tables 4.1 and 4.3.

increase in cellulase production was significant when compared to that without alkaline extract (Fig. 4.3A and 4.3B).

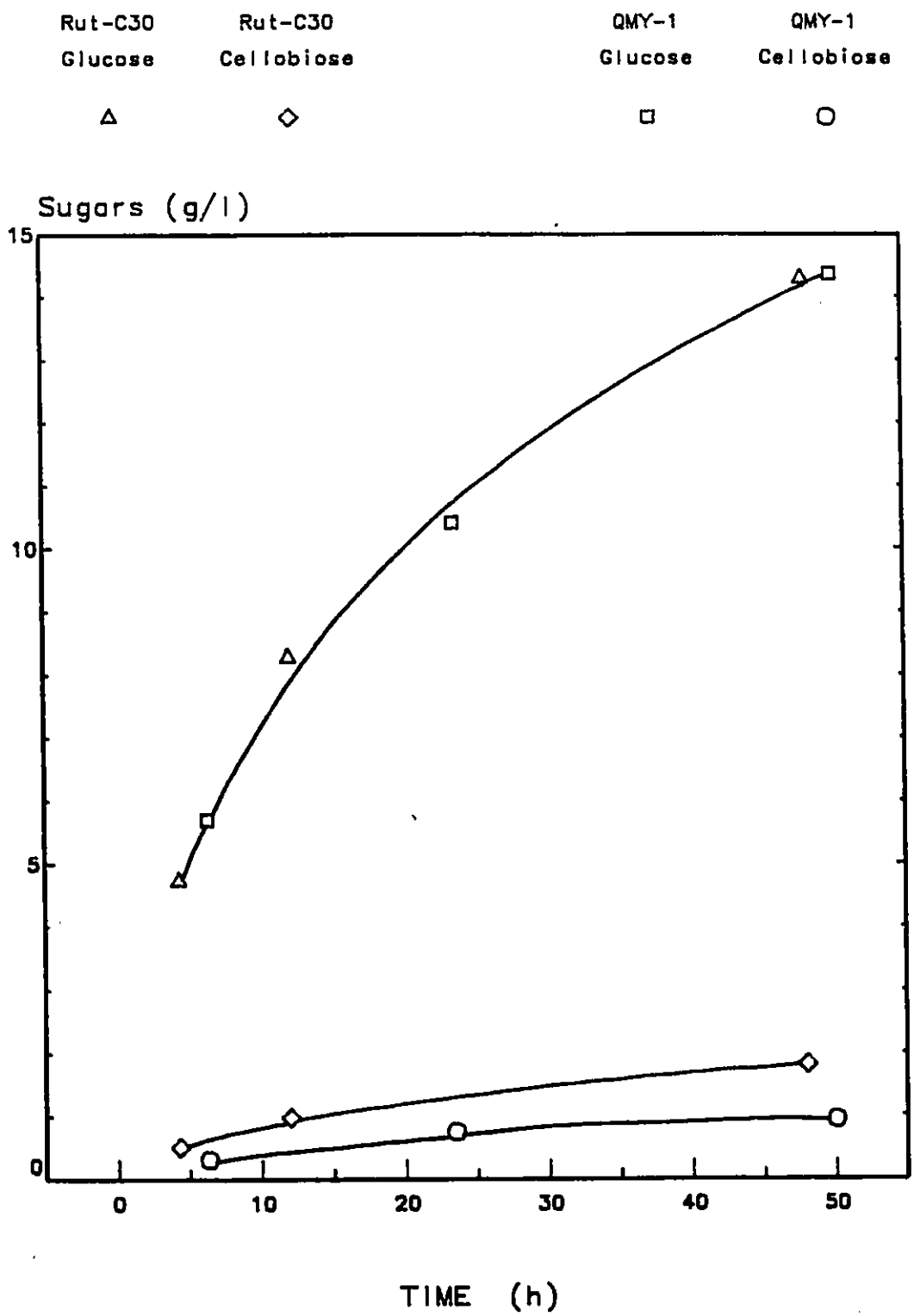
It clearly indicated that by the addition of hemicelluloses the cellulase production was increased and maximum of 3.4 IU/ml was achieved by QMY-1 with a cellulase yield of 340 IU/g cellulose supplied.

4.1.4. Hydrolytic Potential: Rut-C30 vs. QMY-1

Enzymes of Rut-C 30 and QMY-1 were produced in solid state fermentation on wheat straw (section 3.2.7) and 20 IU of enzyme were added to a 5% alpha cellulose solution in citrate buffer at pH 4.8. The β-glucosidase activities of 21 and 22 IU were present in the enzyme systems of Rut-C30 and QMY-1, respectively. The flasks were placed in water bath at 45 C and shaken slowly to keep the solution moving. The glucose produced in 50 h of hydrolysis was almost the same for both enzyme systems, but almost twice as much cellobiose was accumulated by Rut-C30 enzyme system as compared to QMY-1 enzyme (1.81 and 0.94 g/l respectively) (Fig. 4.4). Although β-glucosidase activities were same in both the enzyme-system, it is not understood why more cellobiose accumulated in the case of the hydrolysate

Fig.4.4. HYDROLYTIC POTENTIAL OF ENZYMES

Produced by Rut-C30 and QMY-1



Data in Table 4.4.

produced by Rut-C30. It may be due to poor resolving power of HPLC at low concentration of cellobiose.

The cellobiose has an inhibitory effect on components of the cellulase system (Reese et al., 1952), therefore, an enzyme system with better β-glucosidase is required to reduce the accumulation of cellobiose.

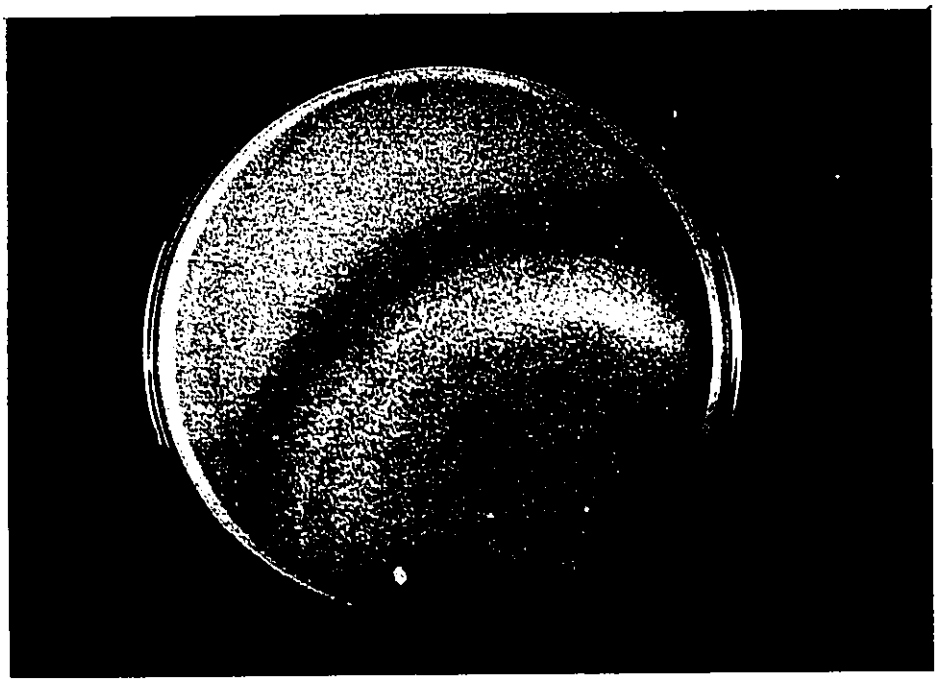
From this data, QMY-1 appears to have as good cellulase system as Rut-C30 from hydrolytic potential point of view.

4.1.5. The Yellow Colour in QMY-1

The mutant QMY-1 of Trichoderma reesei is yellow in colour when grown on glucose, alpha-cellulose or any other lignocellulosic material (eg. crop residues or wood). This yellow colour seems to be strongly associated with its hydrolytic potential. As it was observed in petri plates during isolation of this mutant that colourless colonies produced by single spores cleared less area compared to that of the yellow colonies, when grown on 1% delignified wheat straw (20 mesh) agar plates containing Mandels' medium. Therefore, only yellow coloured and fast growing

colonies capable of producing large clear areas were selected for further use. (Fig. 4.5A). The yellow colour has also been linked to enzymatic activity in the cellulolytic bacteria by Ljungdahl et al. (1983). Figure 4.5B shows the characteristic yellow colour of QMY-1 as compared to the greyish white colour of Rut-C30.

Figure 4.5A. Clear zones produced by QMY-1 when grown on delignified wheat straw in petriplates Top and Bottom views.



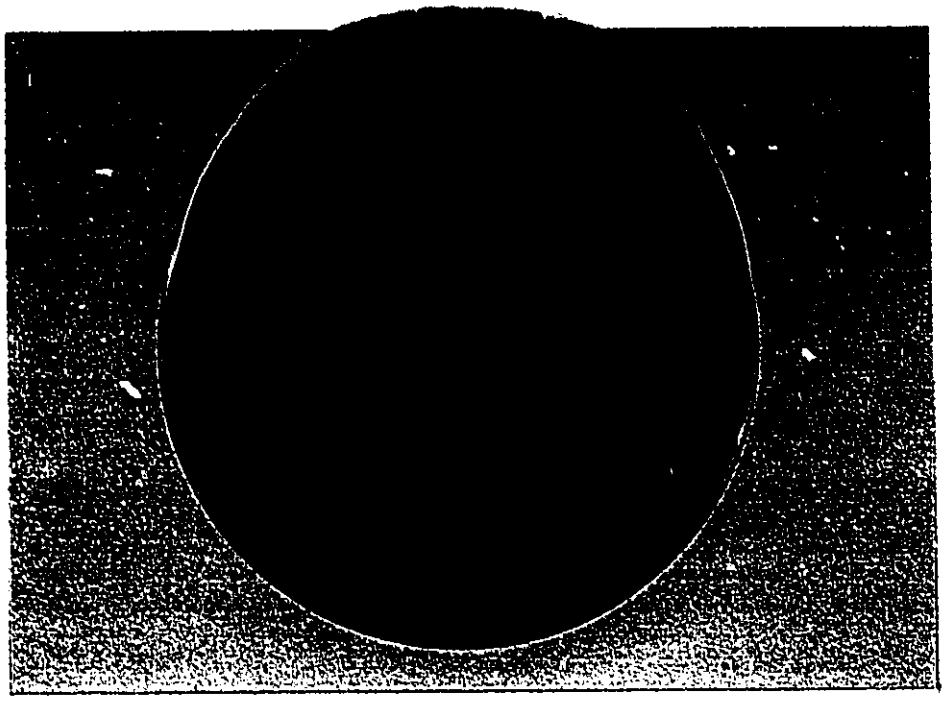
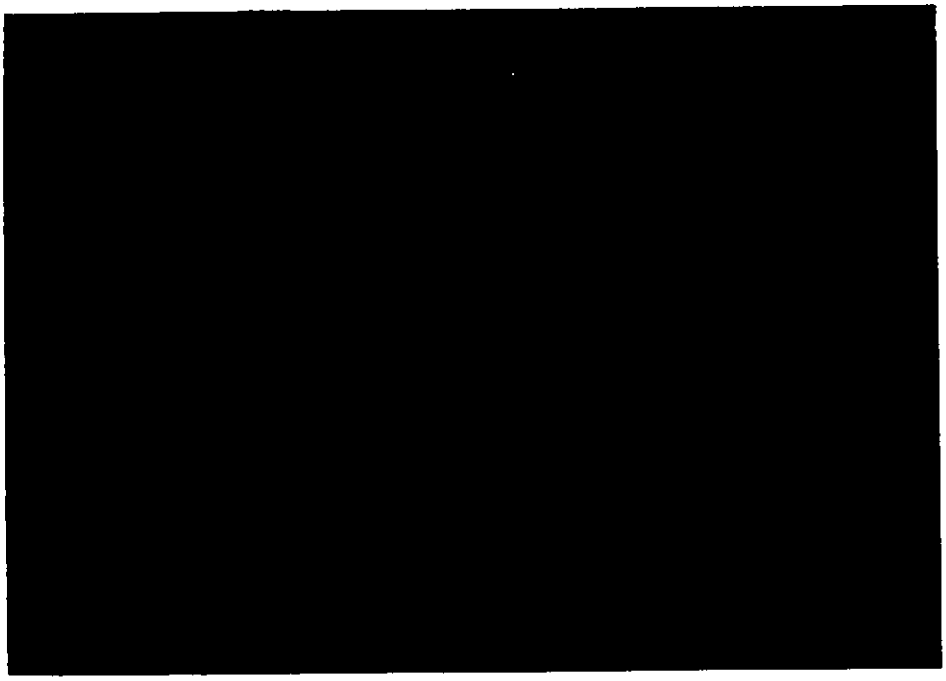


Figure 4.5B. Characteristic yellow colour of QMY-1 compared to greyish white colour of Rut-C30.



4.2. CHOICE OF SUBSTRATE

Most of the published research has been done using pure cellulose (such as alpha-cellulose or Solka Floc) for the production of cellulase. Very little work has been reported on the production of cellulase by using crude source of cellulose such as wood or crop residues (lignocelluloses). In this study, aspen wood has been chosen for cellulase production because of its vast availability in Canada and its fast growing capacity. Aspen wood contains high percentage of lignin (23%), which interferes with the utilization of hemicelluloses and cellulose for the cellulase production as well as for the hydrolysis by the enzyme. To solubilize or depolymerize lignin, pretreatment is necessary.

4.2.1. Choice of Pretreatment

In recent years, much of the attention has been given to the modification of lignocellulosic materials for enzyme production as well as for its hydrolysis. Presence of lignin is the main hinderance for the utilization of cellulose from the lignocellulosic materials. Lignin can be depolymerized or solubilized by various physico-chemical treatments. Lignin in the cell wall of lignocelluloses is bonded physically and

chemically to the plant polysaccharides (Higuchi, 1971, 1978), therefore, limited action is performed by microbial hemicellulases and cellulase-complex due to metabolic blocks of lignin-carbohydrate bonds. This barrier suppresses the penetration by polysaccharide-digesting enzyme (Kirk et al., 1973), hence, the lignin has to be solubilized or partially removed in order to let cellulase- complex and hemicellulases hydrolyze thus exposed cellulose and hemicelluloses. Since, lignin content in general is higher in woody material than in crop residues (Table 2.1), the former are more difficult to hydrolyze. Hence, pretreatment of lignocelluloses is necessary in order to get an efficient cellulose conversion into soluble sugars (Chahal, 1982).

There are many pretreatments available to make lignocelluloses suitable for enzyme production as well as for their hydrolysis. Grinding / ball milling (Millett et al., 1970), mild acid (Knappert et al., 1980; Lee et al., 1978), or alkali (Chahal et al., 1981, 1986; Fiest et al., 1970; Millett et al., 1975; Tarkow et al., 1969) treatment, steam or autohydrolysis (Lora et al., 1978; Noble, 1980; Taylor, 1980; Taylor et al., 1980) ozonation (Puri, 1983), sulphur dioxide (Miron et al., 1982), ammonia treatment (Tarkow et al.,

1969; Waiss et al., 1972), sodium chlorite (Goering et al., 1968) and irradiation (Lawton et al., 1951; Millet et al., 1970; Pritchard et al., 1962) are few common treatments available to achieve the necessary modifications. However, the alkali treatment is the most commonly used pretreatment because, it is practicle and simple.

In this study sodium hydroxide (alkali) treatment was studied in detail (for example: sodium hydroxide concentration, water content level, soaking and heating time) to find the optimal conditions for solubilization of wood (mainly hemicelluloses, lignin, and extraneous materials) in order to cut down the cost of pretreatment. Another pretreatment, chemithermomechanical pulping, was also used to prepare a pulp for enzyme production.

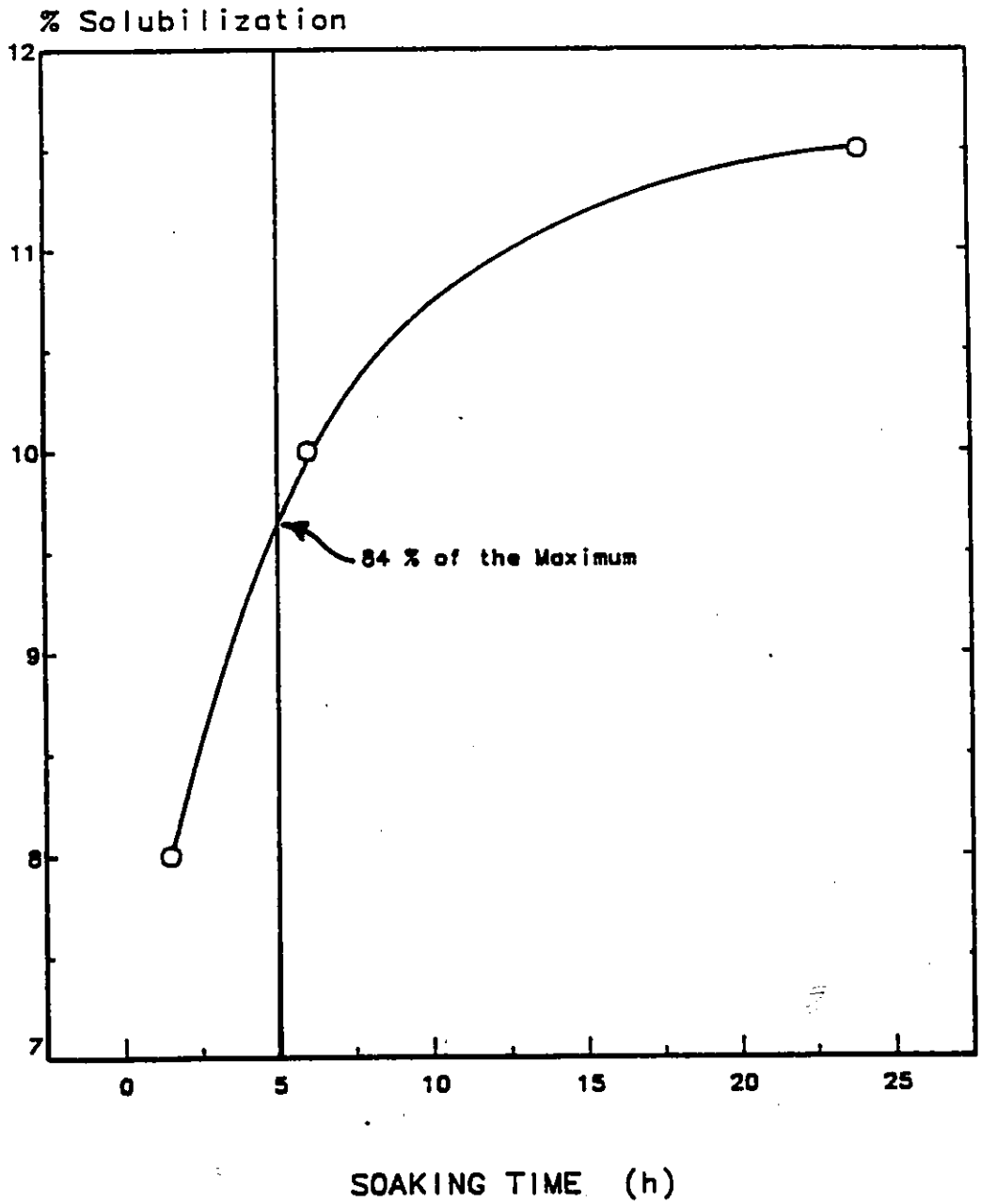
4.2.1.1. Pretreatment I: Sodium Hydroxide Treatment

a) Effect of Soaking Time: Aspen wood (20 mesh) was mixed with sodium hydroxide application ratio (wt of NaOH/wt of wood) with solution to water ratio of 2:1 at room temperature (22°C). The samples were picked up after 1.5, 6 and 24 h and solubilization was determined. The extent of solubilization of wood increased with an increase in soaking time and reached 11.5% in 24 h. In 6 h, 10% solubilization was obtained

(Fig. 4.6) . It was observed that 9.3 to 10% solubilization [that is, 80 to 87% of maximum solubilization (11.5%) obtained in 24 h], was attained in 4 to 6 h of soaking time, respectively. Therefore, 5 h of soaking time was considered to be a reasonable soaking time before giving the heat treatment.

Millett et al. (1970) reported only 2% solubilization of aspen wood (40 mesh) with 4% sodium hydroxide application with water (solution) to wood ratio of 8:1 in 2 h at room temperature. In the present study 8% solubilization of aspen wood of 20 mesh (bigger particles than 40 mesh) was achieved with the same concentration of sodium hydroxide in less than 2 h with solution:wood ratio of 2:1. High solubilization achieved here was due to the fact that by reducing the water content from 8 to 2 parts / part of wood, the concentration of sodium hydroxide in soaking solution increased from 0.5 to 2%. It is plausible that the high concentration of sodium hydroxide results in a more extensive solubilization. Therefore, sodium hydroxide treatment with minimum water content was studied in detail. The need for minimum water is also necessary in conducting experiments in solid state fermentation, where free water is not desirable.

Fig. 4.6. EFFECT OF SOAKING TIME ON SOLUBILIZATION of Wood at Room Temperature (22°C)



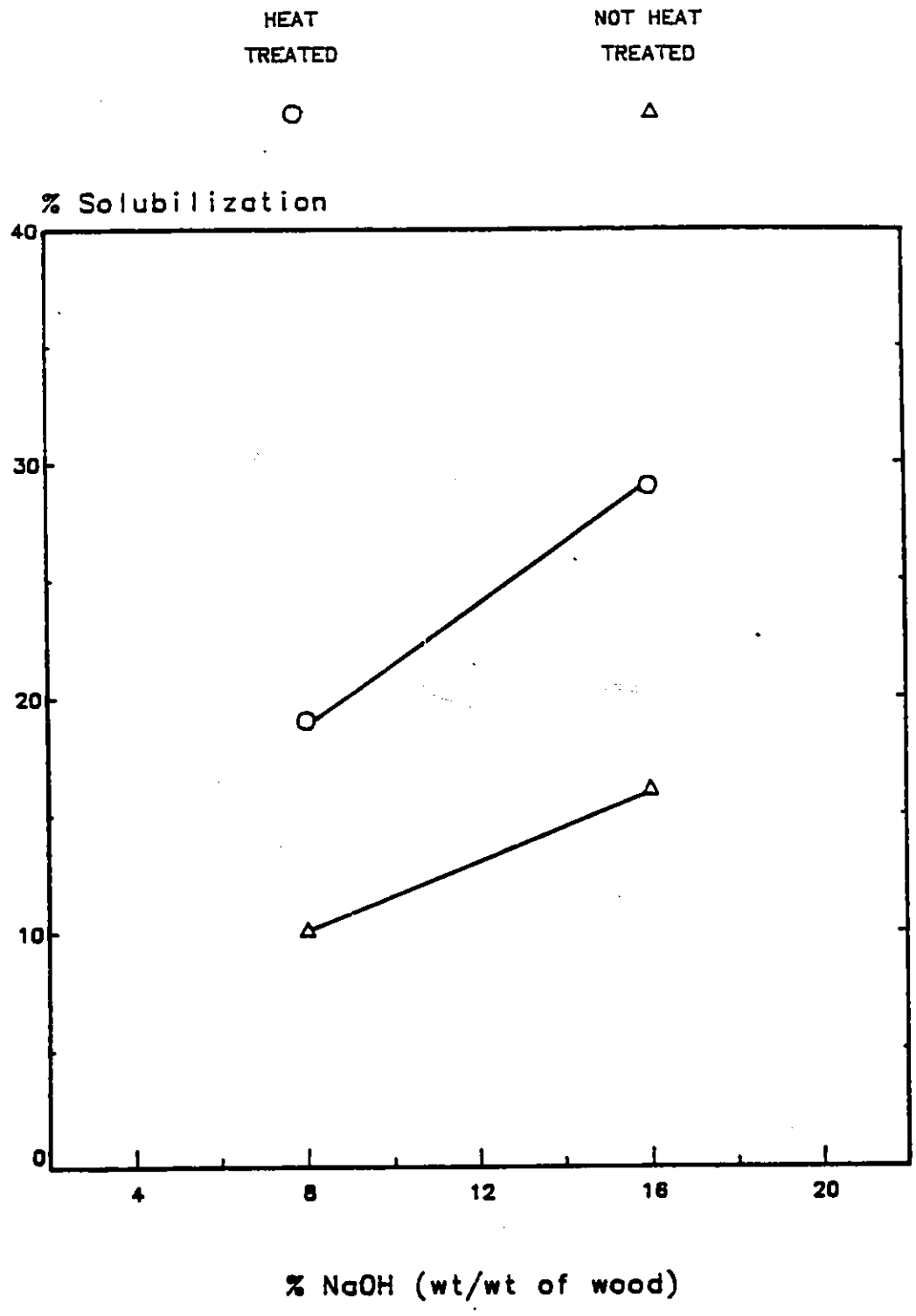
Data in Table 4.5.

b) Effect of Heating: Sodium hydroxide was added to two sets of flasks containing 5 g wood each in such a way that 8 and 16 % application ratios of sodium hydroxide could be achieved in set I and II, respectively, keeping same solution:wood ratio of 2:1. After keeping the flasks at room temperature for 5 h, two flasks of each set were treated at 121°C (autoclaving) for one hour and two other flasks of each set were kept at room temperature for the same duration of time as required for heat treatment.

Heat treatment doubled the solubilization of soluble wood components at both application ratios of sodium hydroxide (Fig. 4.7), indicating that 8% sodium hydroxide application ratio can achieve more solubilization with heat than that what the twice the amount of sodium hydroxide application ratio (16%) could achieve at room temperature. Solubilization of aspen wood increased from 10 to 16% (when no heat treatment was performed) and 19 to 29% (when heat treatment was performed) with an increase in sodium hydroxide concentration from 8 to 16%, respectively.

c) Effect of NaOH and Water Contents: Three sets (A,B and C) of experiments (as explained in section 3.4.1.1.c) were performed for definite NaOH application

Fig. 4.7. EFFECT OF HEAT TREATMENT ON SOLUBILIZATION of Wood Treated with Different NaOH Conc.

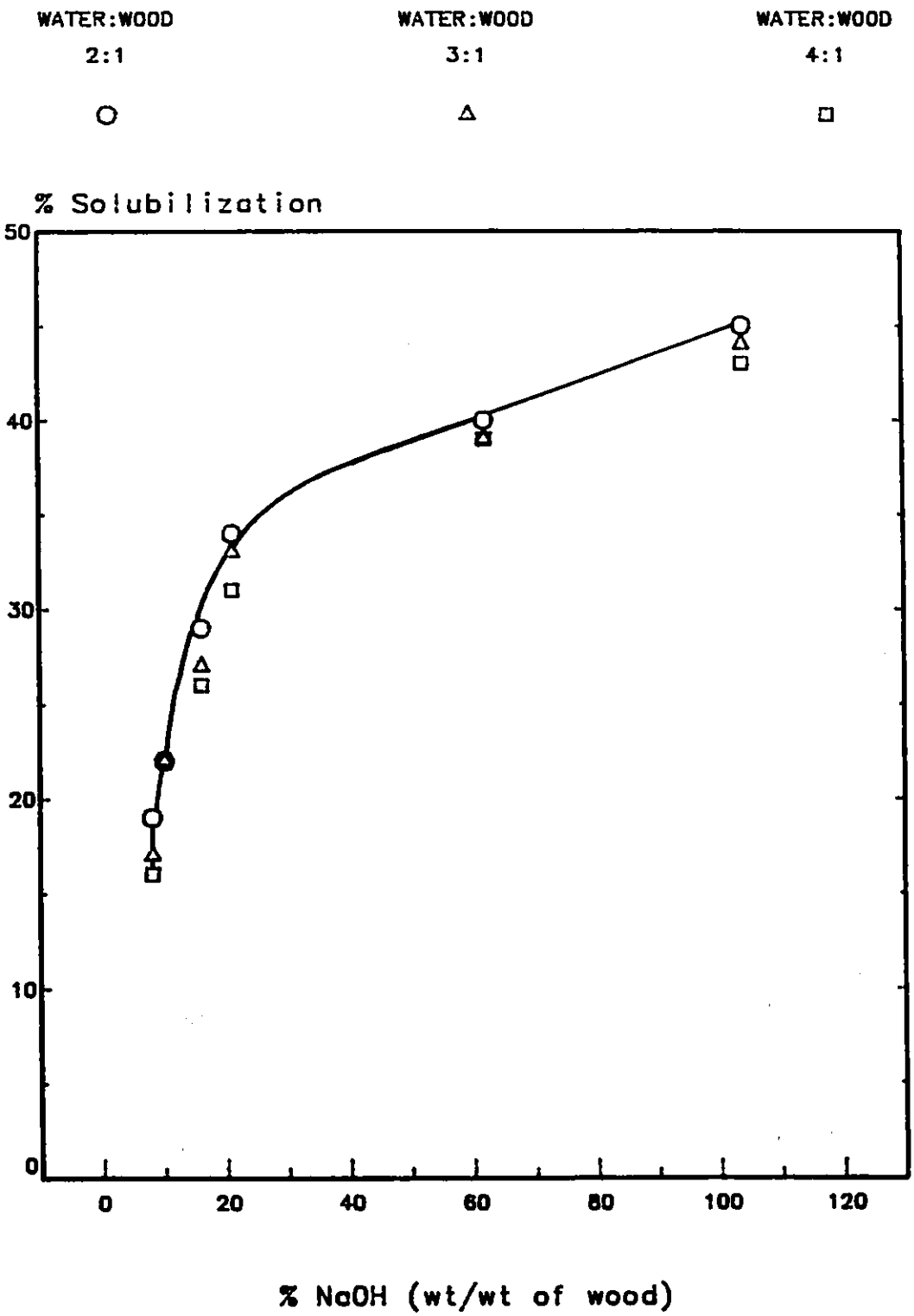


Data in Table 4.6.

ratios (ranging from 8 to 104%) varying the solution to wood ratio (2:1, 3:1 and 4:1 for sets A, B and C, respectively). The samples were left at room temperature for 5 h and were treated at 121 C for one hour. Figure 4.8 (constructed from values in Table 4.7) shows that there is an increase in solubilization of soluble wood components with an increase in sodium hydroxide concentrations . The extent of solubilization did not increase significantly beyond 20% sodium hydroxide application ratio (Fig. 4.8). Increase in water content decreased the solubilization for every fixed amount of sodium hydroxide application to a limited extent and in some cases there was very little decrease (Table 4.7).

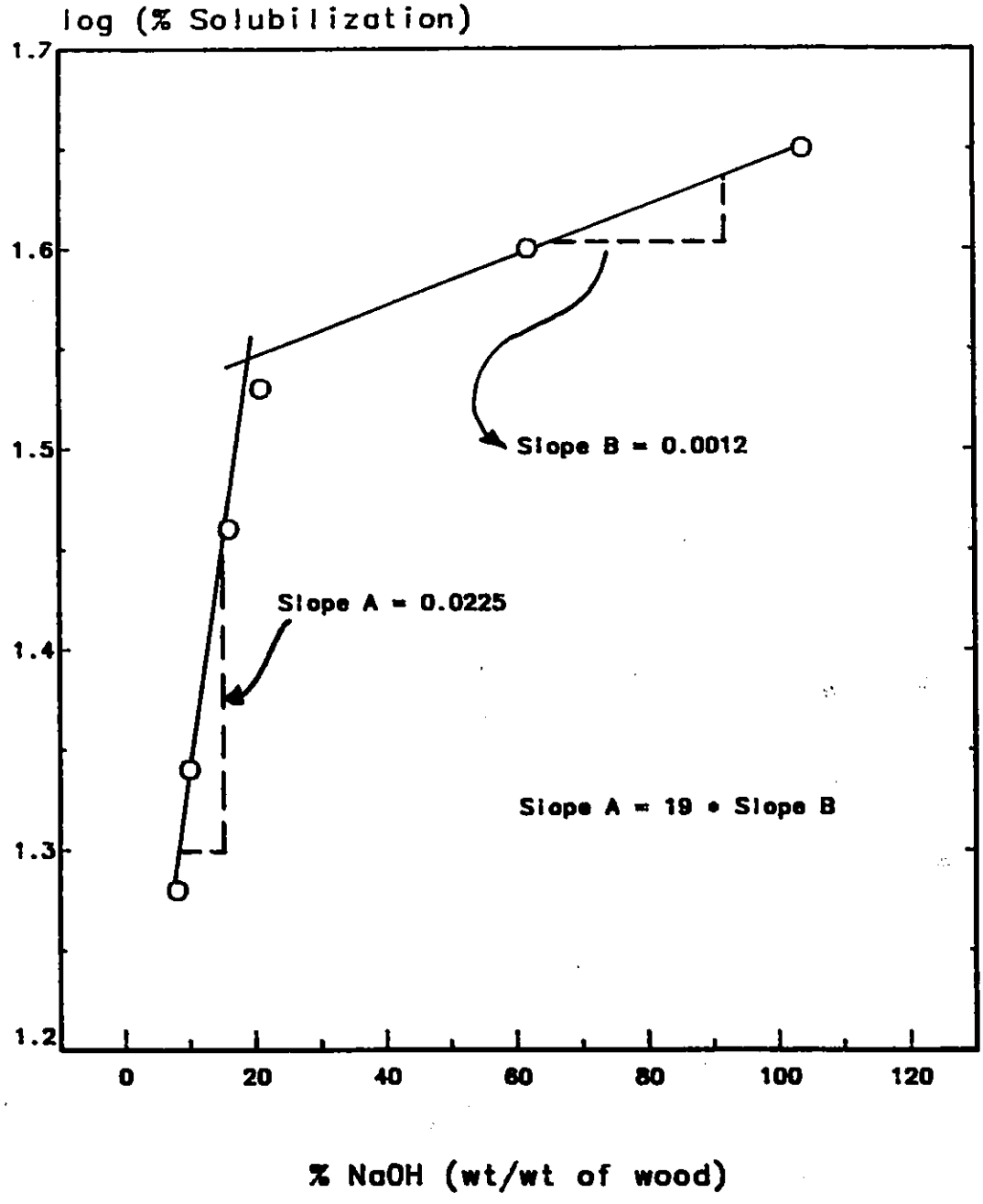
Figure 4.9 constructed from the data of Set A from Table 4.7 on a semi-log plot (Table 4.8) showed that 20% NaOH application ratio could be considered as a cut off point, because, the increase in solubilization per unit increase in NaOH application ratio is significantly less beyond that application ratio. Slope of 0.0225 decreased to 0.0012 (19 times less steeper) after 20% NaOH concentration. Hence, from these results it was concluded that 20% could be considered as a reasonable NaOH application ratio for optimal solubilization of about 33%.

Fig 4.8. EFFECT OF NaOH CONC. ON SOLUBILIZATION OF WOOD



Data in Table 4.7.

Fig. 4.9. SOLUBILIZATION (log %) vs. NaOH CONCENTRATION

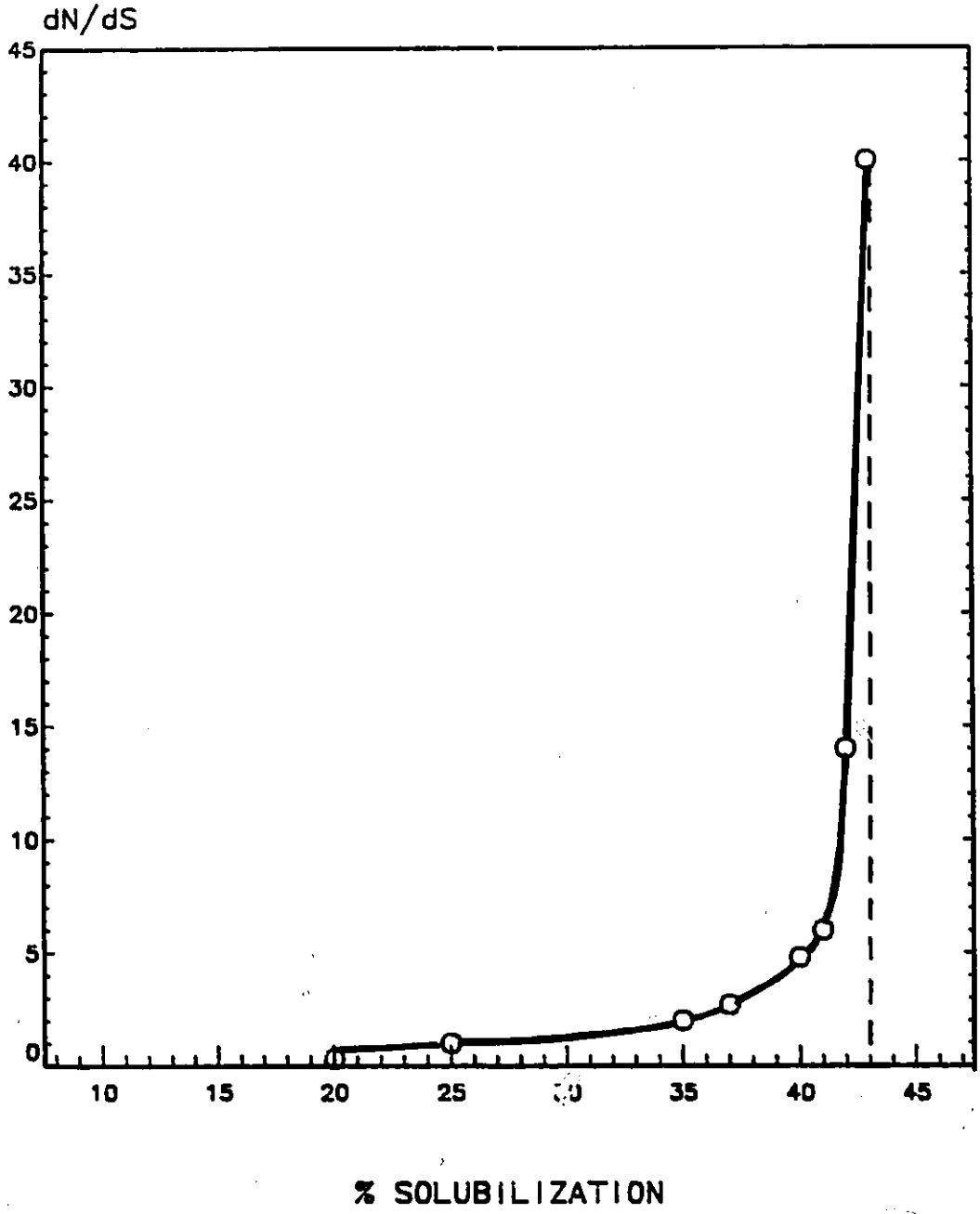


Data in Table 4.8.

Figure 4.10 was constructed for dN/dS vs percent solubilisation, where dN/dS is the amount of sodium hydroxide required per 100 g wood for every increase in percent solubilization (S). It can be seen that the requirement of NaOH increases drastically for every 1% increase in solubility, after 35% solubilization. For example, to achieve 44% solubilization from 43%, 40 g more NaOH is required. Interpretation of the data in Fig. 4.10 confirms the cut off point at 20 g NaOH for 100 g of wood to achieve 33% solubilization. Based on 45% total soluble materials from aspen wood, equivalent of about 75% solubilization has been obtained. The solubilization of 45% of aspen wood is based on the fact that aspen wood contains an average of 27% hemicelluloses and 18% lignin which can be solubilized completely by an alkali treatment at high temperature and pressure (Table 2.1). The exact values of hemicelluloses and lignin were not analyzed in this work.

Macdonald et al. (1983) have obtained about 86.6% solubilization of soluble components from corn stover by treating it with 20% NaOH (wt/wt of corn stover) at 125 C for 35 minutes. Corn stover contains almost same amount of lignin as aspen wood (Table 2.1), but is

Fig. 4.10. RATE AT WHICH SODIUM HYDROXIDE (N) IS REQUIRED for 100 g Wood for every Increase in % Solubilization (S)



Data in Table 4.9.

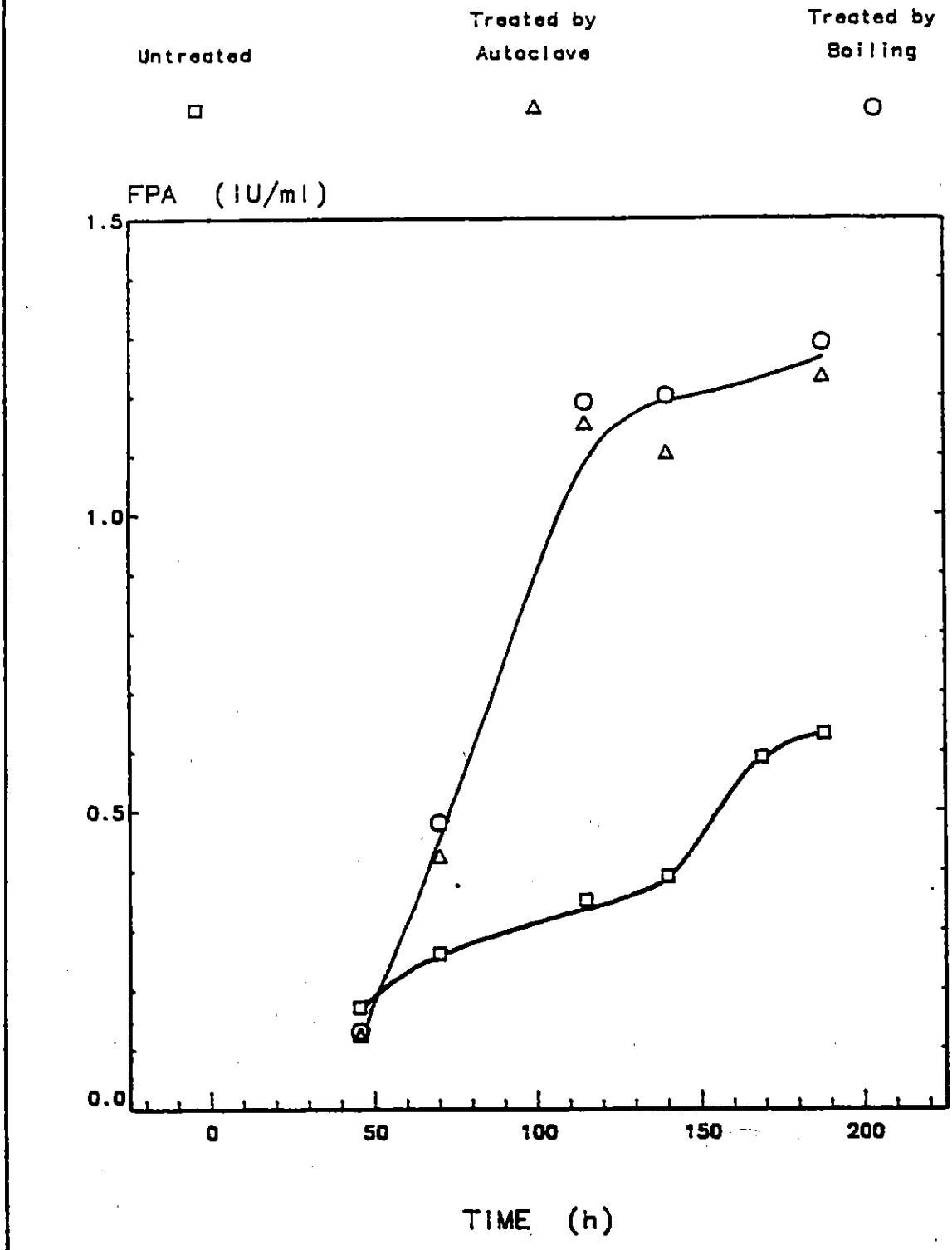
structurally less compact than wood. Therefore, corn stover is easily solubilized as compared to wood. On the other hand, very high application ratios of NaOH (50-100% wt/wt) were used by Han (1963) to solubilize hemicelluloses and lignin from sugarcane bagasse containing 14.9% lignin. About 33% of aspen wood was dissolved with the procedure developed in this work by treating wood with 20% sodium hydroxide application ratio (wt/wt of wood) with water to wood ratio of 2:1 to 4:1 at 121°C for one hour.

4.2.1.2. Cellulase Production on Pretreated Wood

4.2.1.2.1. On NaOH Pretreated Wood

The experiment was performed as outlined in section 3.4.1.2. Aspen without treatment gave very little filter paper activity (0.63 IU/ml, Experiment 1) whereas, in the Experiment 2 and 3 relatively higher activity was achieved (1.23 and 1.29 IU/ml, respectively) in 188 h (Fig. 4.11). It indicated that autoclaving at 121°C for 1 h and boiling for 3 h did not show significant difference to make aspen wood suitable for cellulase production. In the Experiment 4 when the aspen treated as in the Experiment 3 was used in the fermenter, the filter paper activity of 1.87

Fig. 4.11. CELLULASE PRODUCTION WITH QMY-1 ON ASPEN Pretreated by Various Methods.



Data in Table 4.10.

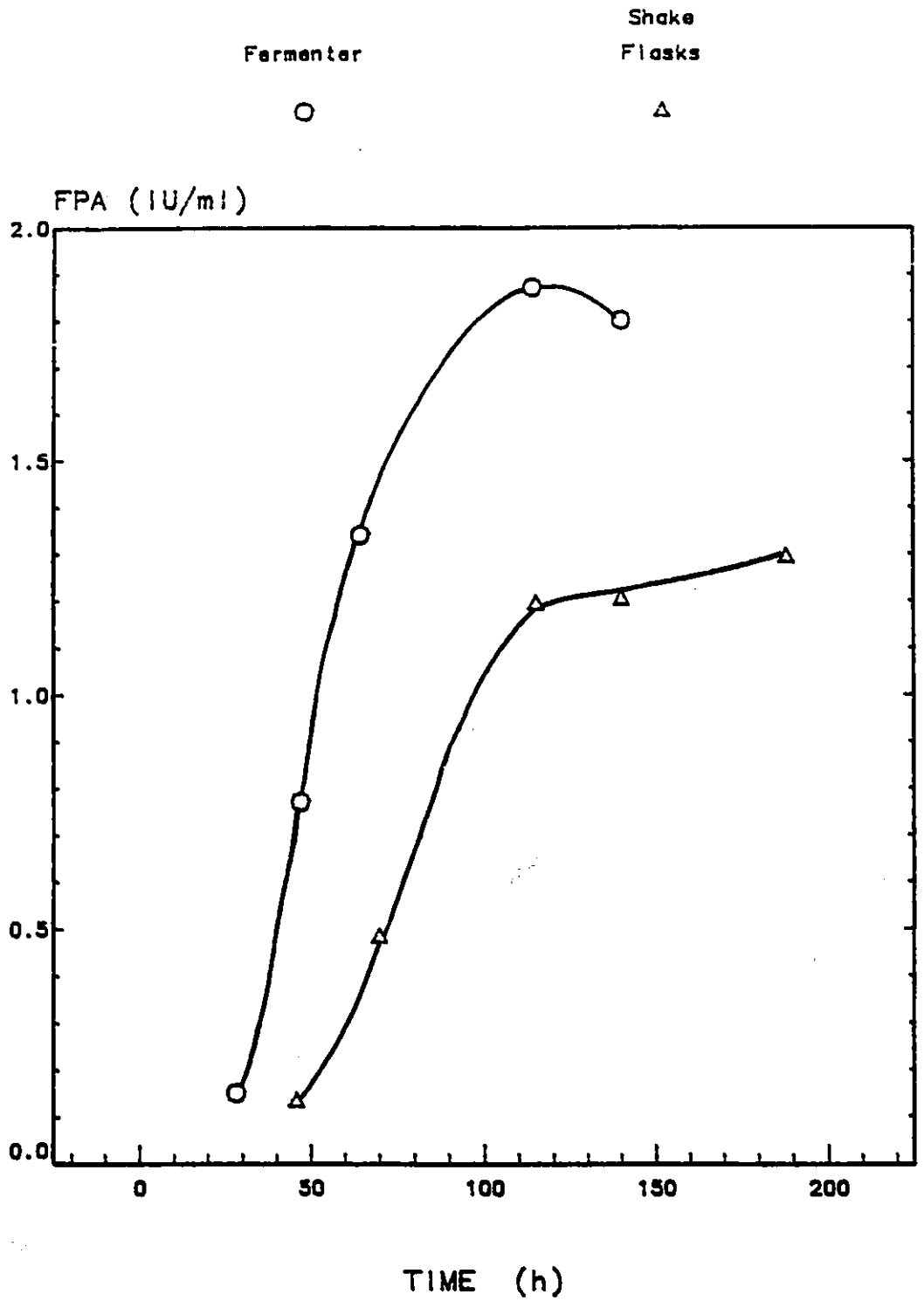
IU/ml was achieved in relatively much less time (114 h) (Fig. 4.12).

Almost double cellulase activity was achieved by treating aspen with 20% NaOH (wt/wt of wood) at 121°C or at boiling temperature (100°C) when compared to non-treated aspen. With NaOH heat treatment the lignin and hemicelluloses are solubilized to expose cellulose for microbial degradation. This treatment also converts crystalline cellulose to a more easily utilizable form by swelling it which in fact makes it more accessible to microbial degradation cellulase production because more cellulose was utilized by the organism.

4.2.1.2.2. On Chemithermomechanical Pulp (CTMP)

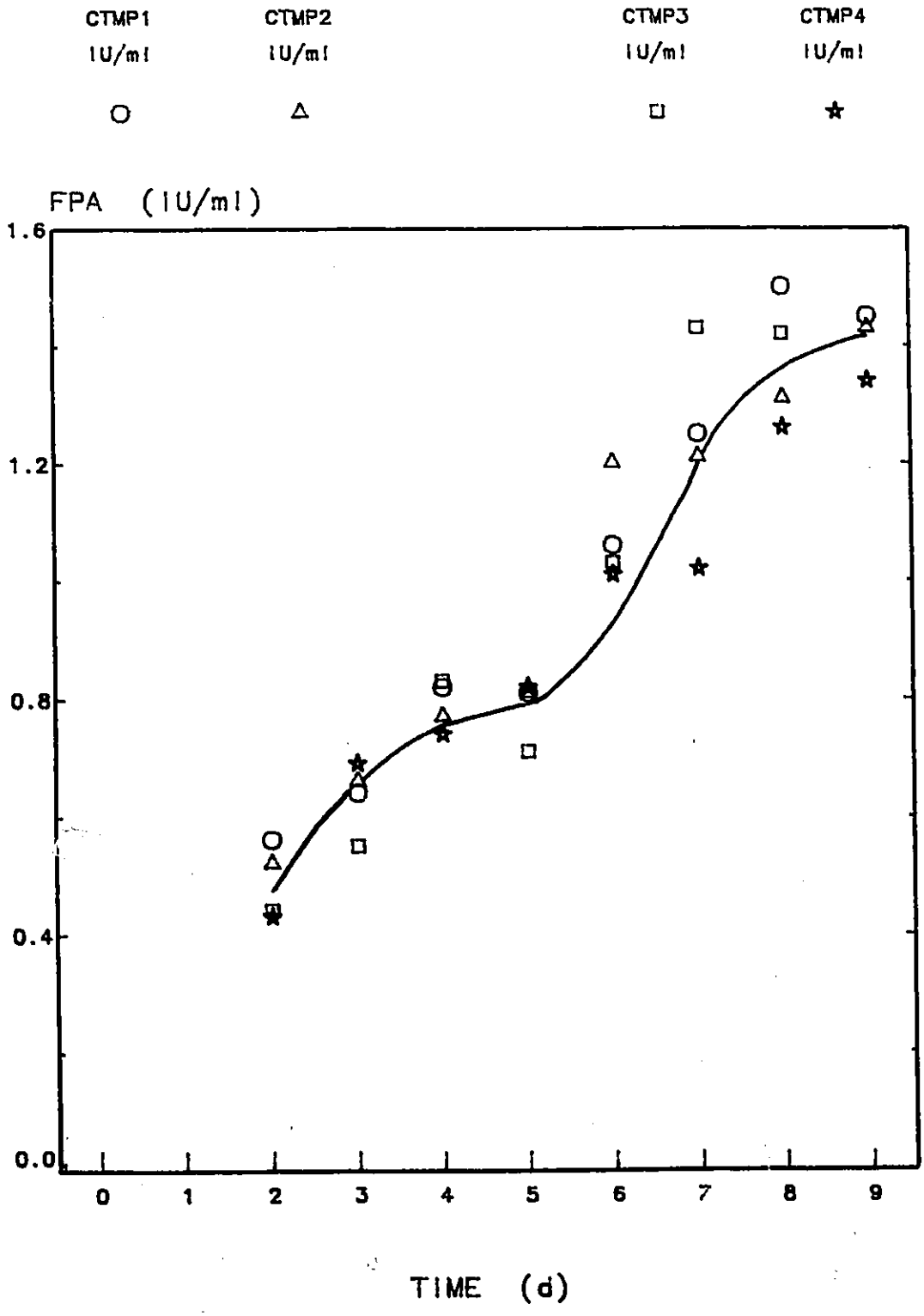
Chemithermomechanical pulp prepared by four different types of chemical treatments: CTMP1 (5% NaOH and 5% Na_2SO_3, washed), CTMP2 (5% NaOH and 5% Na_2SO_3, not washed), CTMP3 (10% NaOH, washed) and CTMP4 (10% NaOH, not washed), were used as the substrate for cellulase production in the shake flasks at 30 C, pH 6.0. The cellulase production was not significantly affected when sodium hydroxide was used alone or in combination with sodium sulphite for preparation of CTMP (Fig. 4.13). There was a trend of slowing of cellulase production in all

Fig. 4.12. CELLULASE PRODUCTION WITH QMY-1 ON ASPEN Pretreated by Boiling with NaOH.



Data in Table 4.10 and 4.11

Fig. 4.13. CELLULASE PRODUCTION ON 1% CHEMITHERMOMECHANICAL PULP (CTMP) by QMY-1, in Shake Flasks



Data in Table 4.12.

substrates after 4th day. During the period of first 4 days the organism was utilizing hemicelluloses; it was also utilizing cellulose which was exposed after the utilization of hemicelluloses from the loosened up portion of CTMP1 (produced during its preparation). Cellulase production resumed after 5th day, after adaptation by the organism to the residual portion of CTMP1 which consisted structurally more compact portion of cellulose. Cellulase activity of about 1.5 IU/ml was achieved on all types of CTMP in 192 h with the cellulase yield of 250 IU/g cellulose supplied (the later studies will show that hemicelluloses do not produce cellulase, therefore, the basis of per gram of cellulose supplied can be used here). Sample calculations for determining cellulase yield are shown in Appendix III.

4.2.2. Comparison of Cellulase Production on Different Substrates

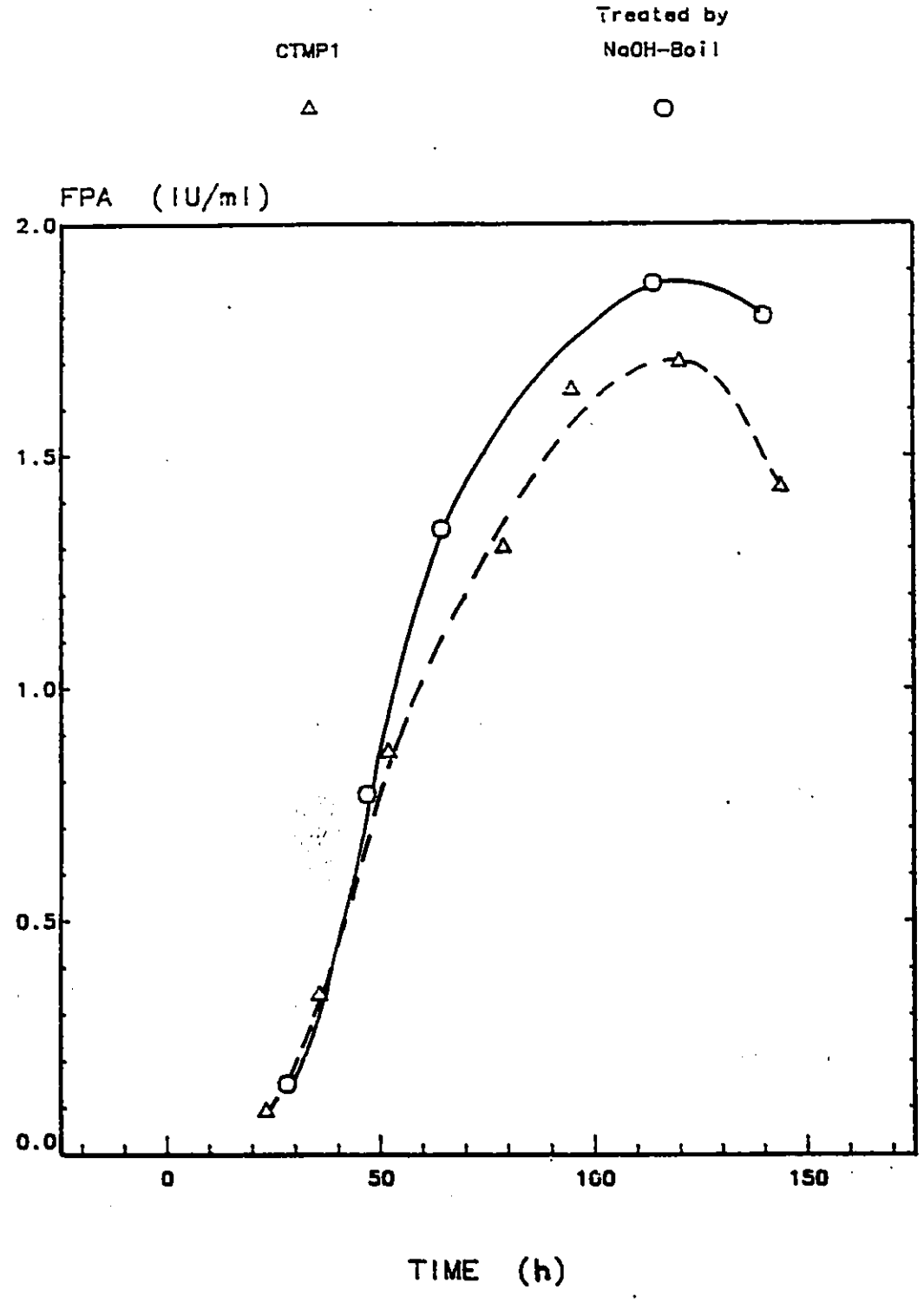
The best pretreated-substrate from each pretreatment was tested for cellulase production by mutant QMY-1, in 14-l fermenter at 30°C and pH 6.0. Aspen wood treated with 20% NaOH wt/wt in solid to liquid ratio of 1:20 by boiling for 3 h was chosen from Pretreatment I as autoclaving and boiling did not show

any significant difference in cellulase production and it was assumed that boiling might be producing less toxic compounds because, the treatment is at lower temperature. Whereas, CTMP1 (5% NaOH and 5% Na_2SO_3 treated and washed) was chosen from Pretreatment II.

The cellulase production (1.7 IU/ml) on CTMP1 was almost equal to that of aspen wood (1.87 IU/ml) treated with NaOH (20% wt/wt of wood) (Fig. 4.14).

The cellulase production on 1% concentration of both the substrate was higher and faster in the fermenter than in the shake flasks (Fig. 4.11 and 4.13). In the preparation of CTMP1 only 5g NaOH and 5g Na_2SO_3 per 100 g of wood were used , whereas 20 g NaOH was used for the same amount of wood in Pretreatment I. Since, large amount of NaOH was used per gram of wood and wood has to be ground to fine powder in Pretreatment I, therefore it may be an expensive method of treatment than CTMP. The cost comparison of both of the methods is not available yet. The CTMP1 was chosen as the pretreated-substrate for cellulase production by T. reesei QMY-1 for further study as it was produced in large quantity in one lot to keep the uniformity of the substrate.

Fig. 4.14. CELLULASE PRODUCTION WITH QMY-1 On CTMP1 and NaOH Treated Aspen.



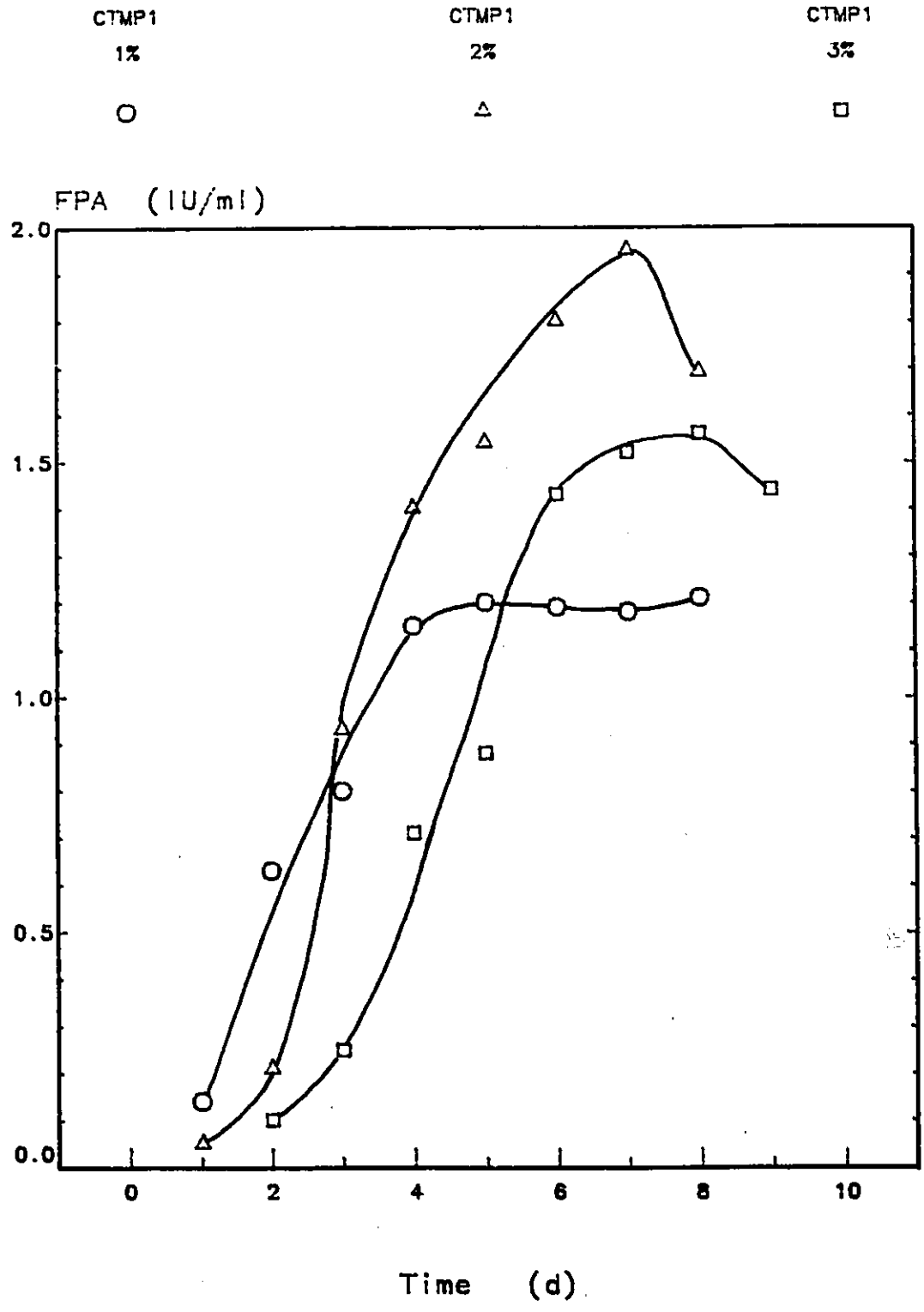
Data in Tables 4.11 and 4.13.

4.3. EFFECT OF SUBSTRATE CONCENTRATION

The CTMP1 was used for cellulase production to check the effect of different concentrations (1, 2 and 3%) of substrate in the shake flasks at 30°C, pH 6.0. It was difficult to get a good mixing for mass transfer in the shake flasks or in the conventional fermenter for CTMP concentrations higher than 3%, because of its rheological properties: density and viscosity. Sternberg (1976 a) also observed that 4% cellulose concentrations cannot be used in the fermenter, because the conventional fermenter design failed to agitate the thick slurry of the cellulose efficiently. The cellulase activity increased from 1.2 to 1.95 IU/ml by increasing the substrate concentration from 1 to 2% (wt/vol) but it took longer time (6 days versus 4 days) to achieve this high value (Fig. 4.15). Further increase in the substrate concentration to 3% level did not give corresponding increase in activity (1.56 IU/ml). The poor aeration and poor mixing of the substrate (since substrate concentration of 2% or more formed a thick slurry because of low density) could be responsible for the low cellulase activity. Higher concentrations may not be responsible for inhibitory effect because Solka Floc which does not make thick slurry as CTMP has been used in high concentrations by

Fig. 4.15. EFFECT OF SUBSTRATE CONCENTRATION

On Cellulase Production, by QMY-1, in Shake Flasks.



Data in Table 4.14.

many workers (Table 4.2) for cellulase production.

Although, all the culture conditions (nutrients, pH, temperature, rpm of the shaker, size and origin of inoculum) were identical at all these levels of substrate concentration, it seems that the only factor responsible for lower yield of cellulases could be the problem of oxygen transfer in the thick slurry of 3% substrate concentration. It is always possible to adopt fed-batch fermentation in order to achieve high cellulase activity in the fermentation broth, instead of using batch fermentation. High cellulase activity per unit volume have been reported in the fed-batch fermentation (Gottvaldova et al., 1982; Hendy et al., 1982; Mclean et al., 1985). Since doubling the substrate did not double the cellulase activity, therefore, one percent substrate concentration was chosen to be more suitable concentration from practical point of view for further experimental studies.

4.4. pH OPTIMIZATION

In order to increase the cellulase activity, it was necessary to find the optimal pH for cellulase production for the mutant QMY-1 of T. reesei. Two different substrate: (i) pure cellulose (alpha-cellulose) and (ii) crude cellulose (lignocellulosic material: CTMP1) were used.

4.4.1. On Pure Cellulose (alpha-cellulose)

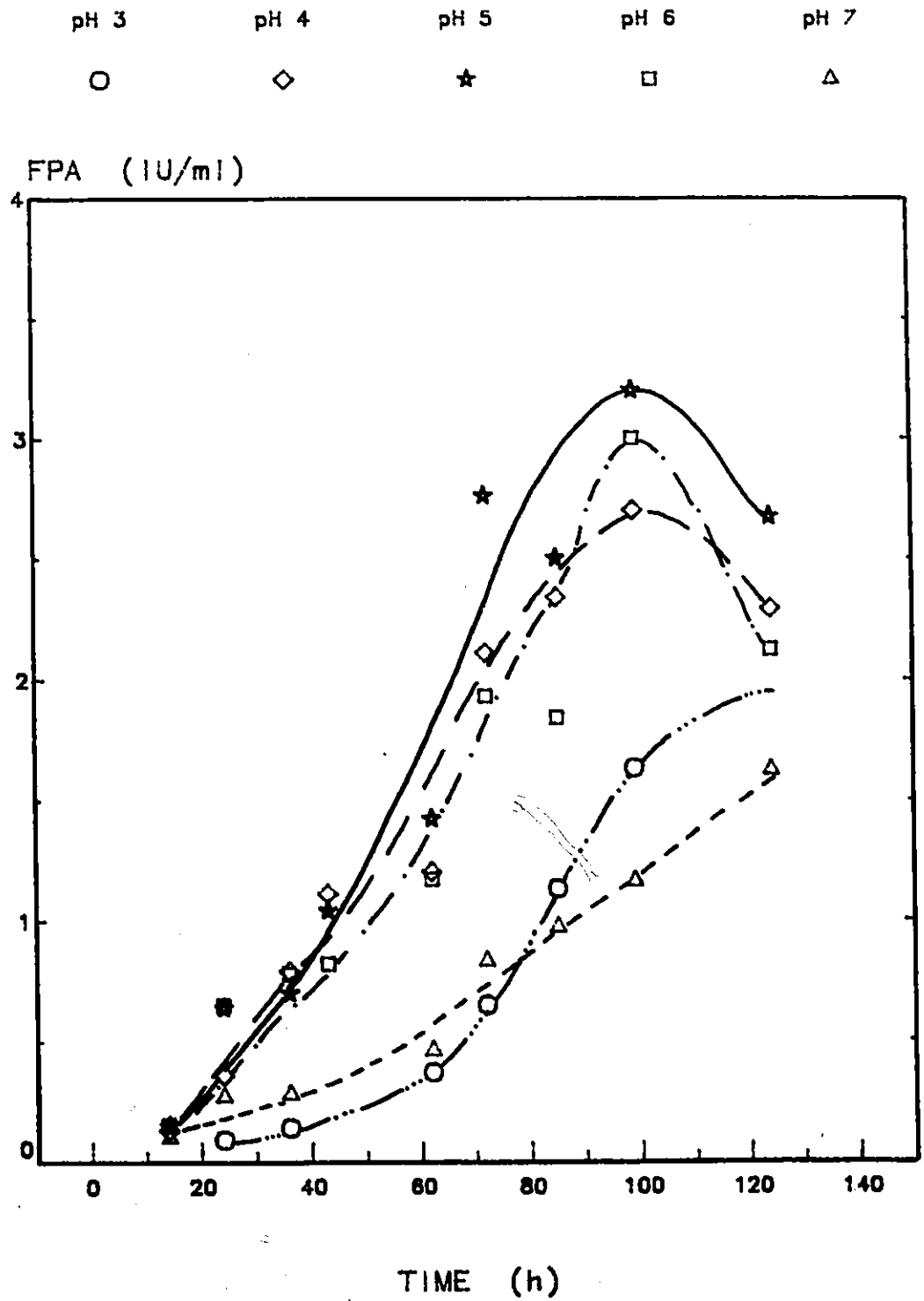
Alpha-cellulose is about 99.9% cellulose and is fiberous in nature. It contains both amorphous and crystalline portions of cellulose.

The effect of different pH (3,4,5,6 and 7) on cellulase production was tested with 1% alpha cellulose with T. reesei QMY-1 in shake flasks. The pH was adjusted close to initial pH (±0.1) by adding sulphuric acid or sodium hydroxide after every (about) 12 h of interval. The fluctuation in pH is given in Table 4.15 and cellulase activities (as filter paper activity = FPA) for different pH levels in the medium are presented in Fig. 4.16 (Table 4.16).

Low cellulase production was obtained when QMY-1

Fig. 4.16. EFFECT OF pH ON CELLULASE PRODUCTION

1% Alpha-Cellulose, in Shake Flasks, QMY-1



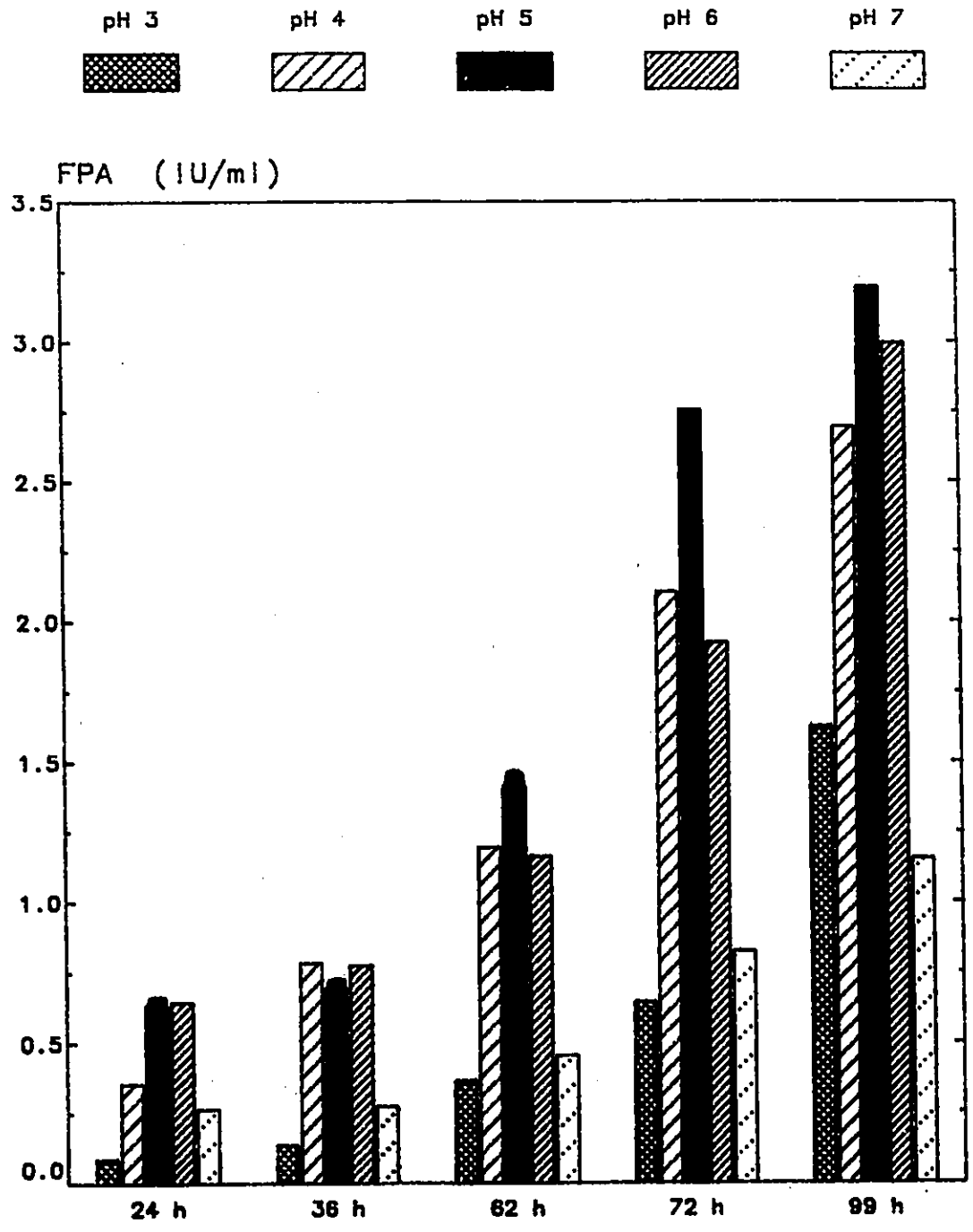
Data in Table 4.16.

was grown at pH 3 and 7. However, in the literature, the use of pH 3.0 (Andreotti et al., 1977; Duff et al., 1985; Mandels et al., 1981; Tangnu et al., 1981), pH 4.0 (Dekker, 1983; Mclean et al., 1981; Peitersen, 1975; Sternberg, 1976; Watson et al., 1983) and pH 5.0 (Duff et al., 1985; Griffin et al., 1974; Hendy et al., 1982; Mitra et al., 1975; Tangnu et al., 1981) have been reported for cellulase production with T. reesei. Table 4.2 shows the filter paper activities obtained by the above mentioned workers.

Filter paper activity was plotted for different pH at different times of fermentation (represented by bars) in Fig. 4.17. In this figure it was noticed that pH 5.0 and 6.0 seems to be more suitable than other pH levels for cellulase production where filter paper activity of 3.2 and 3.1 IU/ml was obtained, respectively, after 98 h of fermentation. It gave the productivity of about 32 IU/l/h and a yield of 320 IU/g cellulose supplied at pH 5 and 6. In our previous experiments the samples were taken at 24-h intervals and the pH was adjusted to the 'set value' at that time. But in this particular set of runs pH was adjusted more frequently (at about 12-h intervals), therefore, higher cellulase activity was achieved.

Fig. 4.17. EFFECT OF pH ON CELLULASE PRODUCTION

On 1% Alpha-Cellulose, in Shake Flasks, QMY-1

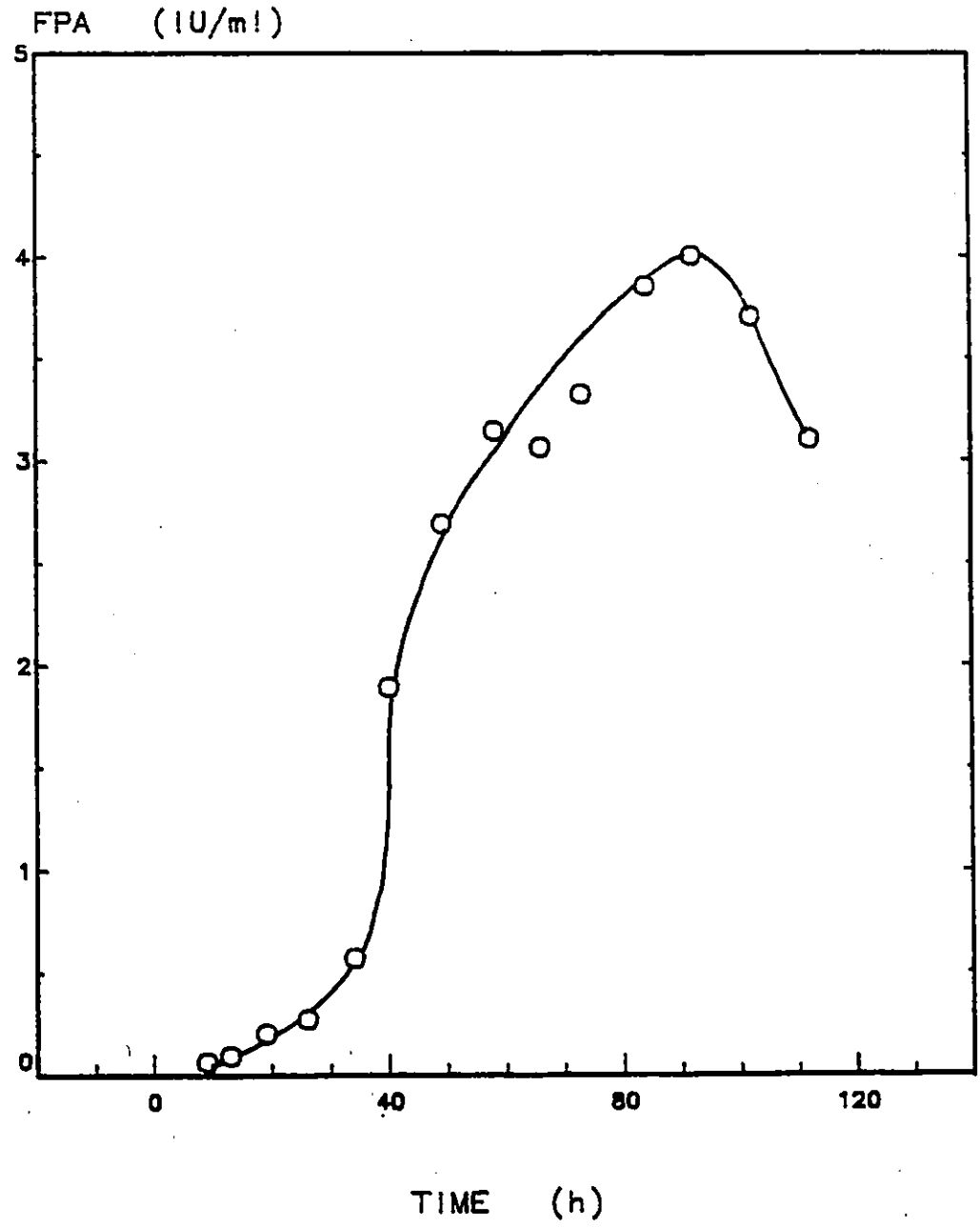


Data in Table 4.16.

Since, low cellulase activity was obtained at pH 3 and it was noticed (Table 4.15) that in the case of pH 5.0 set of experiment, the drop in pH was more (3.1) as compared to that of pH 6.0 set of experiment, thus dropping of pH close to 3.0 in the medium for a long time might have retarded the cellulase production. Therefore, it was believed that at a constant pH of 5.0, the cellulase activity could be still higher than that obtained. Hence, experiment was performed in the fermenter using 1% alpha cellulose with mutant QMY-1 at pH 5.0. The pH was maintained at 5.0$\pm$.05 automatically by 10% solutions of sodium hydroxide and sulphuric acid. The cellulase activity obtained has been presented in Fig. 4.18. Figure 4.19 compares the results of fermenter and the shake flasks at the same pH. The pattern of cellulase production in the fermenter was very similar to that obtained in shake flasks. However, much higher cellulase activity (4.0 IU/ml) was obtained after 91 h of fermentation in the fermenter. This is due to the controlled pH, better mixing and high oxygen mass transfer. The productivity of 43.5 IU/l/h and yield of 400 IU/g of cellulose supplied was obtained. The cellulase yield of mutant QMY-1 seems to be high as compared to the other mutants of T. reesei (Table 4.2).

Fig. 4.18. CELLULASE PRODUCTION ON 1% ALPHA-CELLULOSE

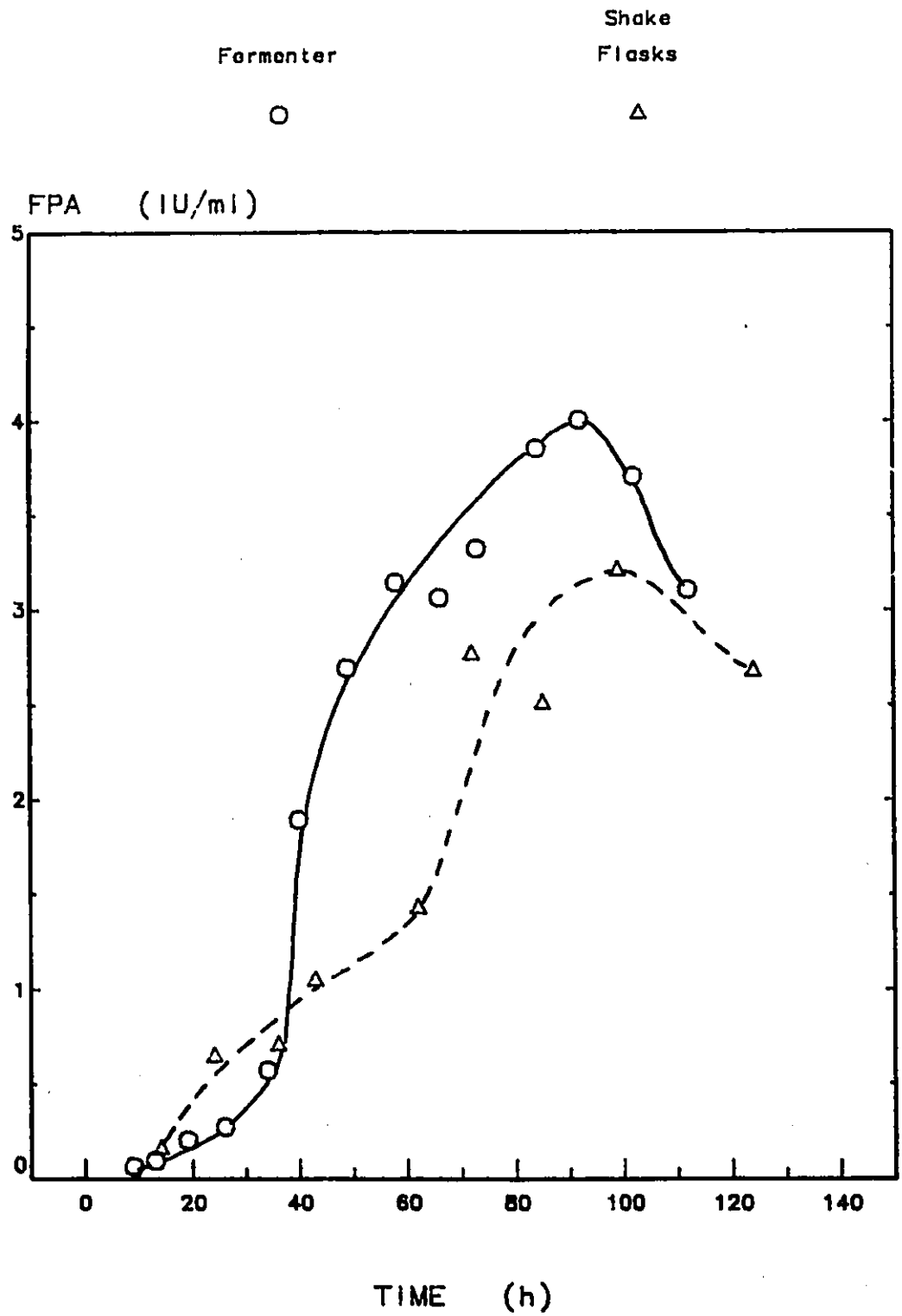
At Optimal pH 5.0, in the Fermenter, With QMY-1



Data in Table 4.17.

Fig. 4.19. CELLULASE PRODUCTION ON 1% ALPHA-CELLULOSE

At Optimal pH 5.0, With QMY-1



Data in Tables 4.16 and 4.17.

The pH 5.0 was chosen to be the optimal pH when 1% alpha cellulose (pure cellulose) was used for the cellulase production in the shake flasks or in the fermenter.

4.4.2. On Crude Cellulose (CTMP1)

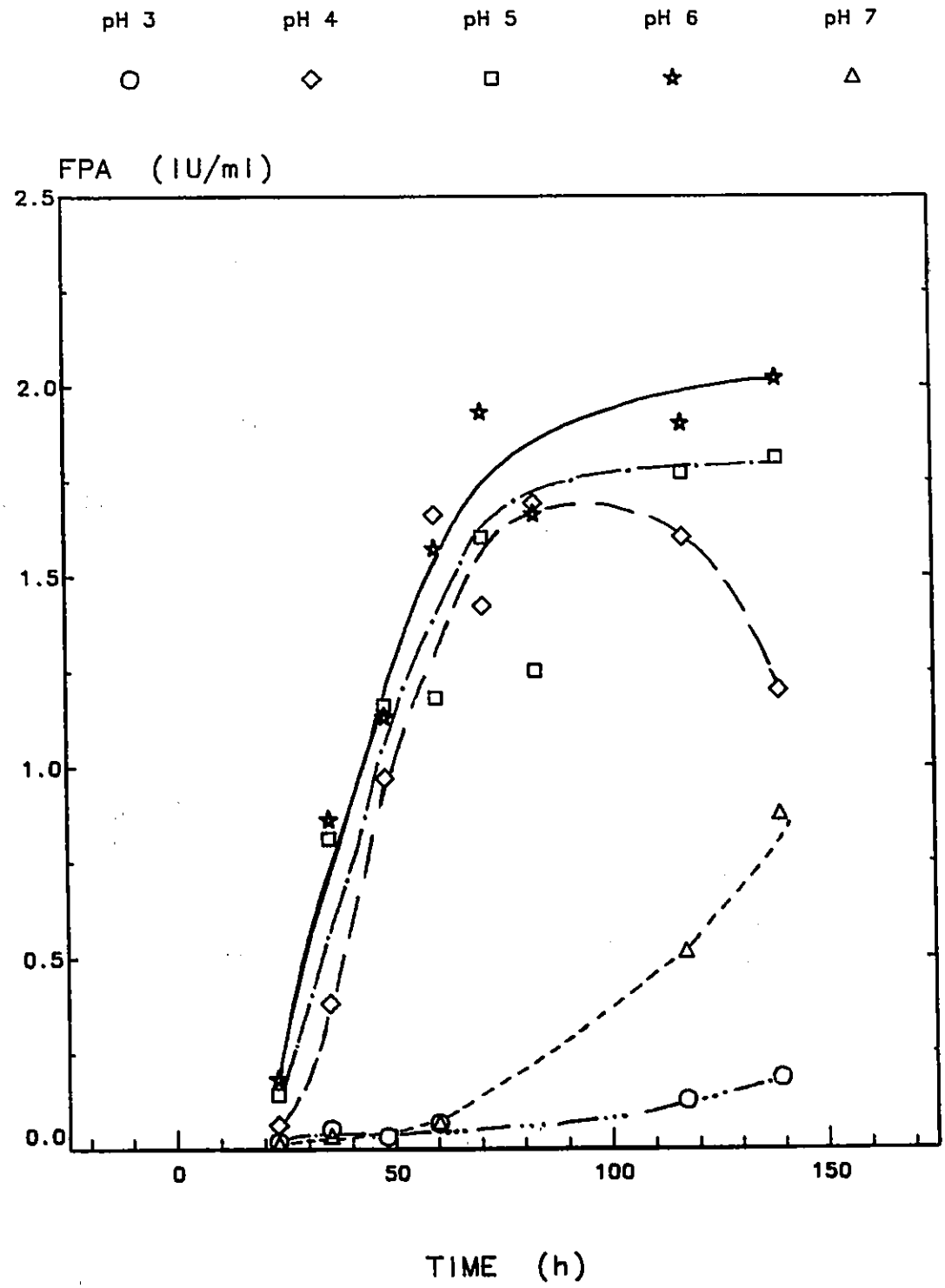
Chemithermomechanical pulp (CTMP1) was used as a crude source of cellulose, lignocelluloses. The cellulose contentant in CTMP1 was 60%, which was determined by the method described by Updegraff (1969) (Table 2.1). The CTMP1 contains both amorphous and crystalline cellulose.

The effect of different pH (3,4,5,6 and 7) on cellulase production was tested with 1% CTMP1 as described for pure cellulose (section 4.4.1). The fluctuation in pH is given in Table 4.18 and cellulase activities (as filter paper activity = FPA) for different pH levels in the medium are presented in Fig. 4.20 (Table 4.19).

The pH levels of 3 and 7 also proved to be poor for cellulase production for CTMP1. Filter paper activity was plotted for different pH at different times of fermentation (represented by bars) in Fig.

Fig. 4.20. EFFECT OF pH ON CELLULASE PRODUCTION

1% Chemithermomechanical Pulp (CTMP1), in Shake Flasks



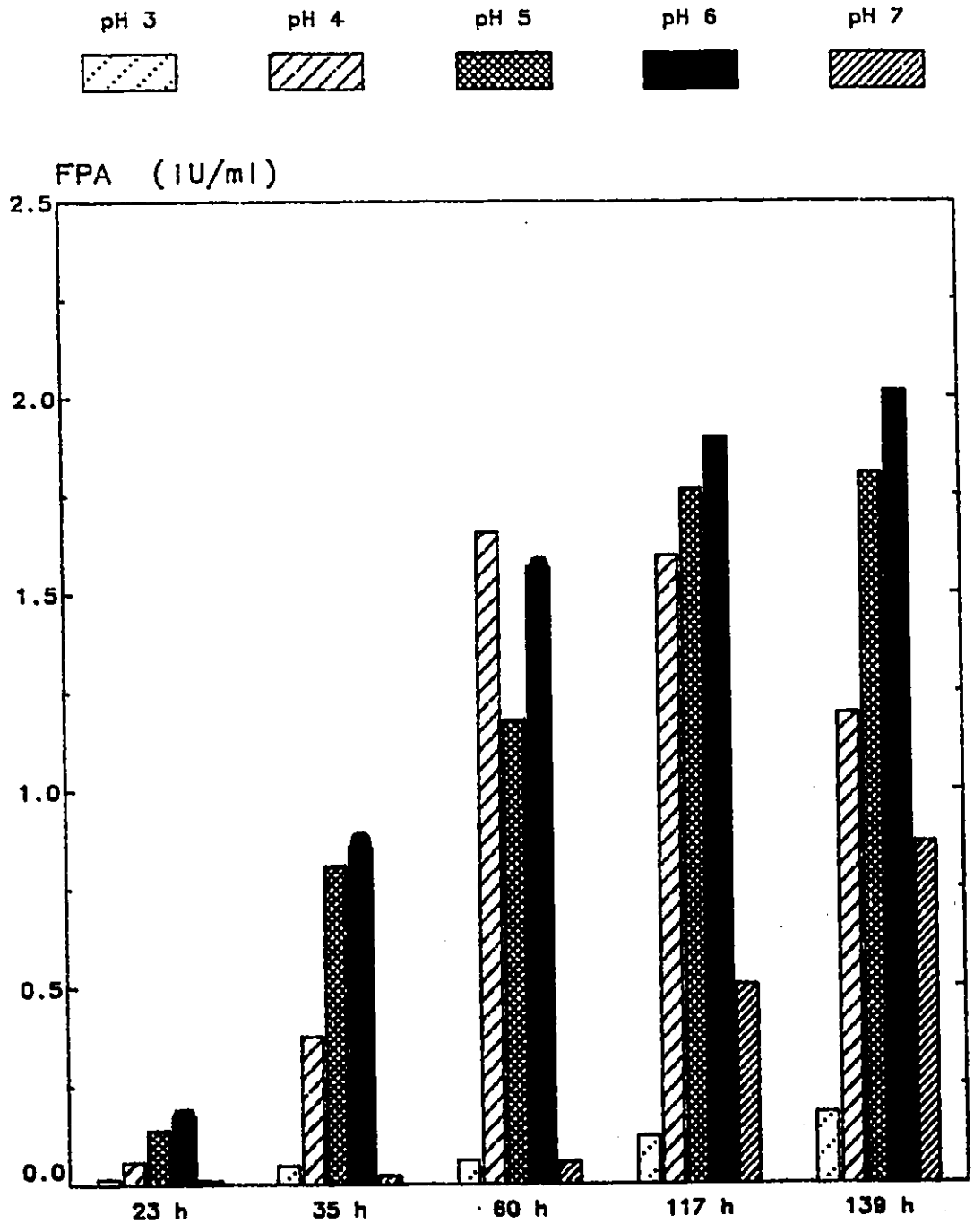
Data in Table 4.19.

4.21. In this figure it was noticed that pH 6.0 was optimal for cellulase production. The filter paper activity of 2.02 IU/ml at pH 6.0 and 1.87 IU/ml at pH 5.0 was obtained after 139 and 117 h with productivity of 14.5 and 16.0 IU/l/h on 1% CTMP1, respectively. The yield of 202 IU/g CTMP1 supplied (337 IU/g cellulose supplied) and 187 IU/g CTMP1 supplied (312 IU/g cellulose supplied) was obtained at pH 6 and 5, respectively. As mentioned earlier, better cellulase activities were achieved in these experiments (sets of pH 5 and 6), since the pH was adjusted close to 12-h interval. The reason for high pH preference on CTMP1 may be due to its different chemical composition (CTMP1 contains cellulose, hemicelluloses and lignin) as compared to Solka Floc (which is pure cellulose).

The experiment was repeated in a fermenter at an optimal pH 6.0 as recorded in shake flasks. Although, pH 5 and pH 6 did not show any significant difference in cellulase production; however pH 6 produced higher cellulase activity with CTMP1. The pH was maintained at 6.0$\pm$.05 automatically by 10% solutions of sodium hydroxide and sulphuric acid. The cellulase activity obtained has been presented in Fig. 4.22. Figure 4.23 compares the results of fermenter with the shake flasks at the same pH. The pattern of cellulase production in

Fig. 4.21. EFFECT OF pH ON CELLULASE PRODUCTION

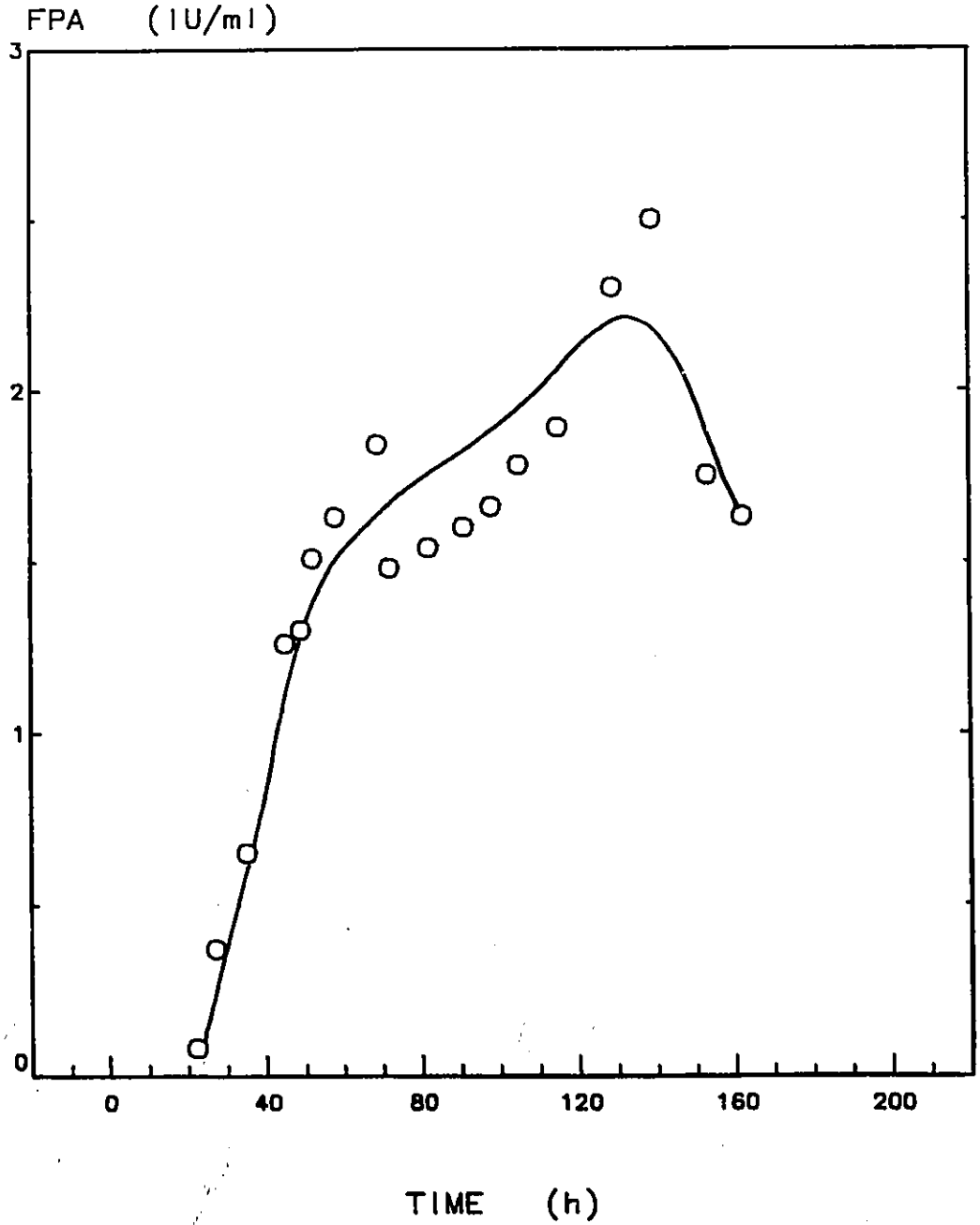
1% Chemithermomechanical Pulp (CTMP1), Shake Flasks



Data in Table 4.19.

Fig. 4.22. CELLULASE PRODUCTION ON 1% CTMP1

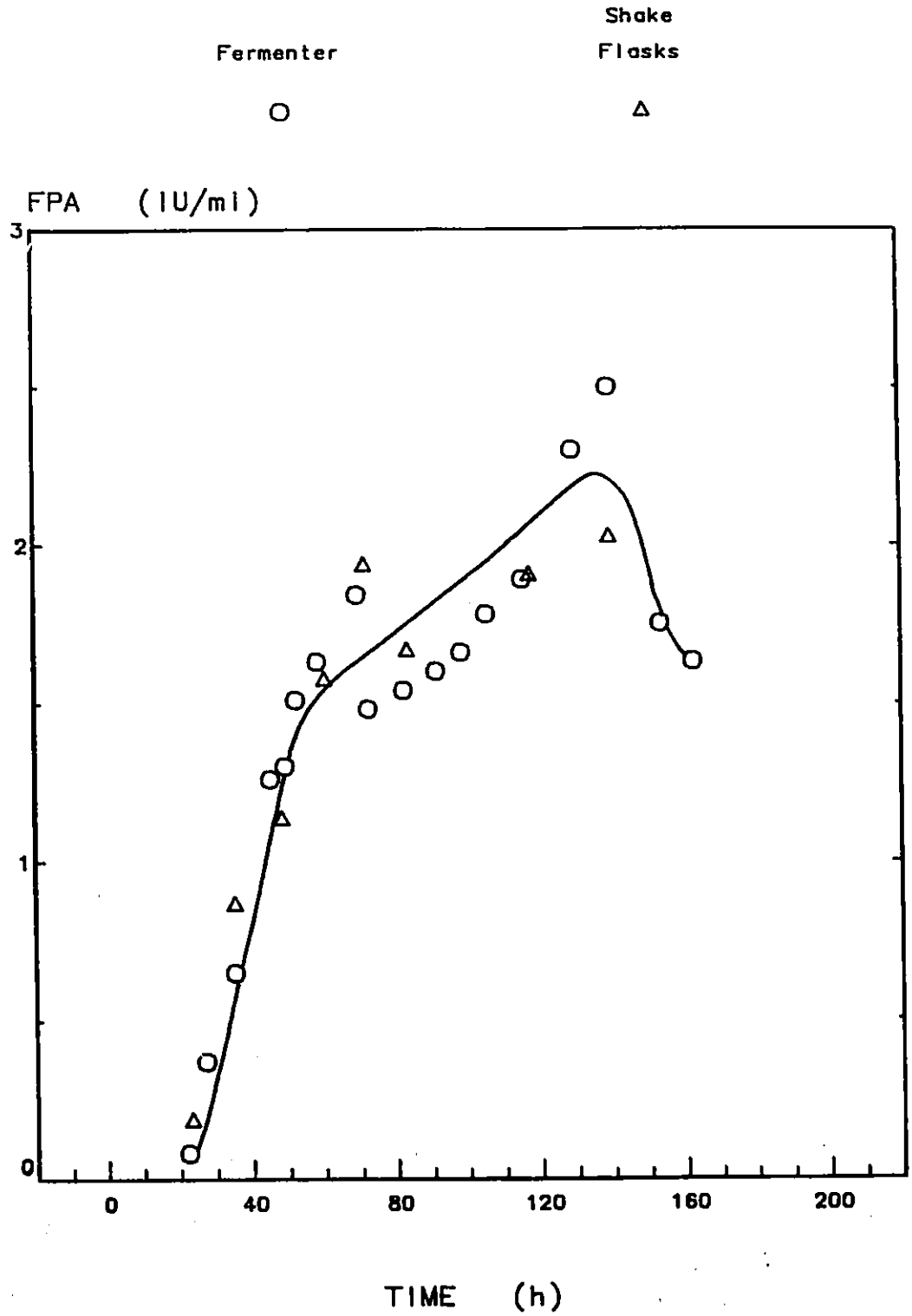
At Optimal pH 6.0, in the Fermenter, With QMY-1



Data in Table 4.20.

Fig. 4.23. CELLULASE PRODUCTION ON 1% CTMP1

At Optimal pH 6.0, in Fermenter and Shake Flasks



Data in Tables 4.19 and 4.20.

the fermenter was very similar to that obtained in the shake flasks. However, higher cellulase activity of 2.5 IU/ml was achieved in 139 h of fermentation in the fermenter. The productivity of 18 IU/l/h and yield of 250 IU/g CTMPl supplied and 417 IU/g cellulose supplied was obtained. The high cellulase activity in the fermenter was obtained due to the controlled pH, better mixing and high oxygen mass transfer.

The pH 6.0 was chosen to be the optimal pH when 1% CTMPl (lignocellulose) was used for cellulase production in the shake flasks or in the fermenter.

4.5. IMPORTANCE OF FREQUENT SAMPLING

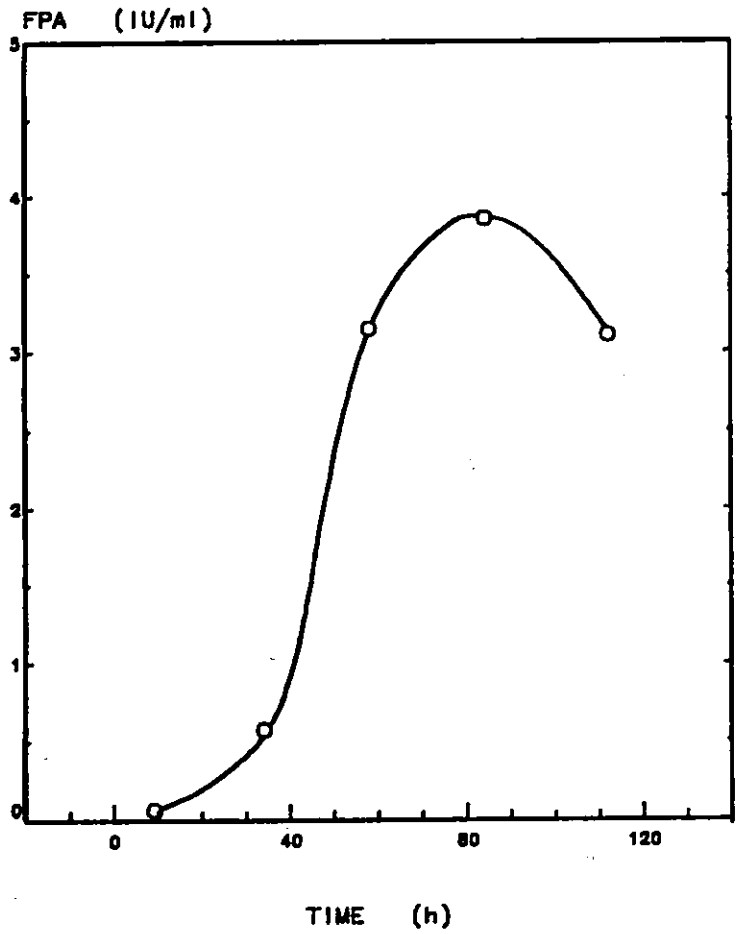
In general for cellulase production there is a trend to take samples after every 24-h (Andreotti, et al., 1977; Dekker, 1983; Duff et al., 1985; Kluepfel et al., 1982; Mclean et al., 1981; Sahai et al., 1977; Tangnu et al., 1981; Vanzyl, 1985), but in some cases 12-h (Sukan, 1985; Watson et al., 1983) sampling times have also been recorded. In our study samples were taken at about 6-h of interval. In Fig. 4.24, the filter paper activity obtained using 1% alpha cellulose, after (about) 24-h only, has been plotted. In general, a mean average curve will be drawn through the data points. In most of the research work it has been reported that the filter paper activity follows the curve similar to that shown in Fig. 4.24. When samples taken after 12-h interval were plotted for their cellulase activity (FPA), again a mean average curve will be drawn through the data points as shown in Fig. 4.25. But, when a 6-h interval was used for collecting samples, the filter paper activity was plotted as shown in Figure 4.26. It leaves no doubt to follow the data points rather than drawing a mean average curve through them. By examining this curve (Fig. 4.26), two phases in enzyme production were observed (compare with Fig. 4.18 in which no 'DIP' was

Fig. 4.24. CELLULASE PRODUCTION ON 1% ALPHA-CELLULOSE

At Optimal pH 5.0, in the Fermenter, With QMY-1

Sampling
24 hours

o



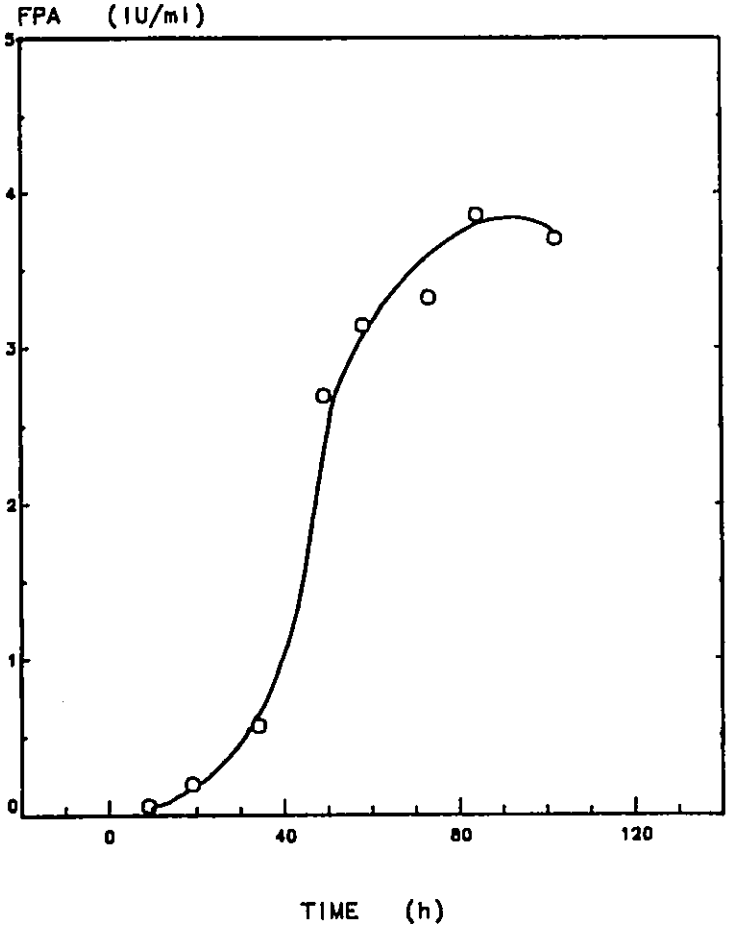
Data in Table 4.17.

Fig. 4.25. CELLULASE PRODUCTION ON 1% ALPHA-CELLULOSE

At Optimal pH 5.0, in the Fermenter, With QMY-1

Sampling
12 hours

o



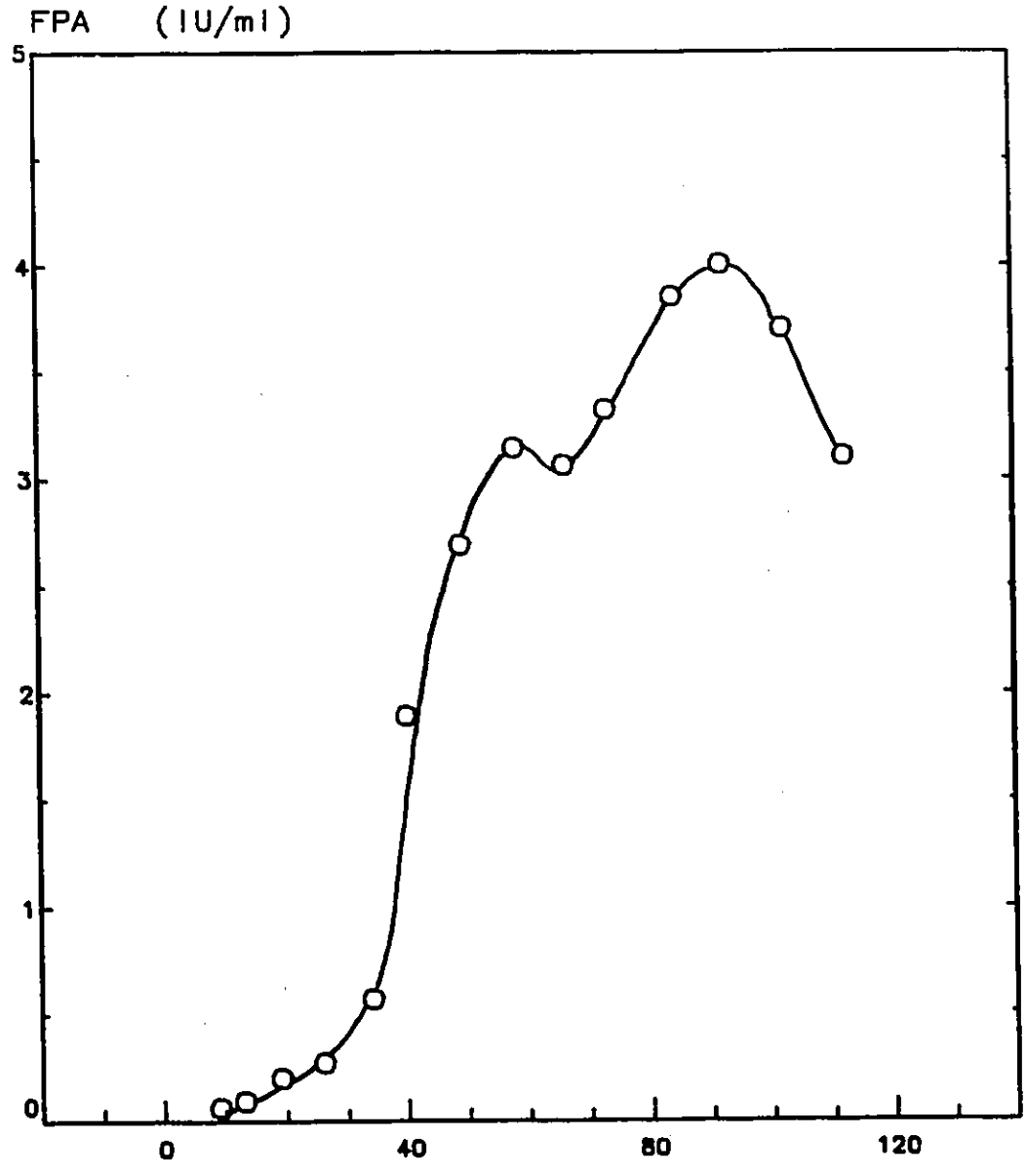
Data in Table 4.17.

Fig. 4.26. CELLULASE PRODUCTION ON 1% ALPHA-CELLULOSE

At Optimal pH 5.0, in the Fermenter, With QMY-1

Sampling
6 hours

O



Data in Table 4.17.

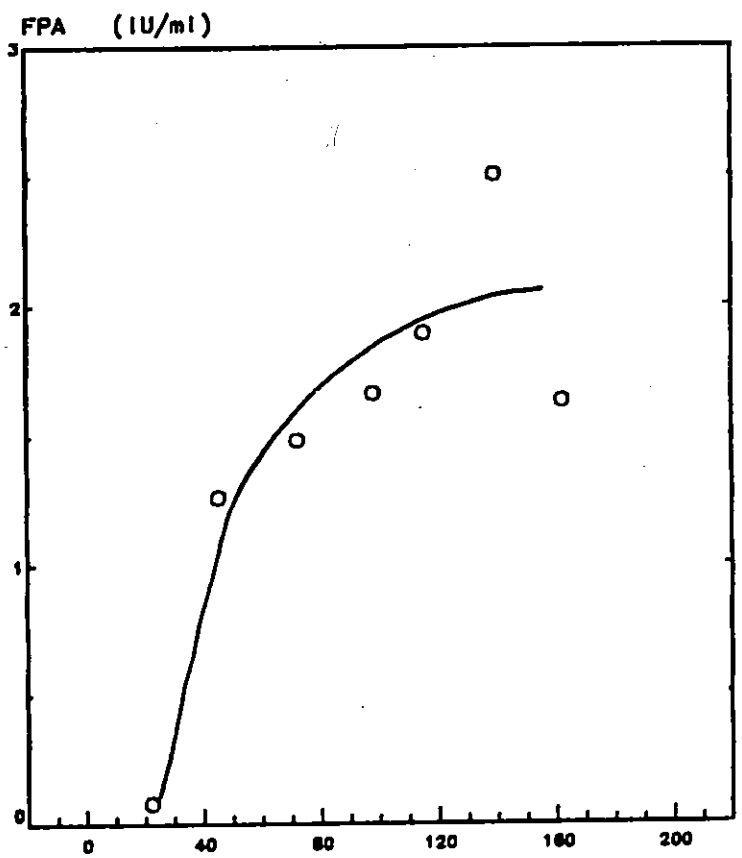
shown). The first phase lasted for about 60 h and the second phase from 60 h to end of run. It was assumed that during the first phase amorphous portion of the cellulose was consumed, whereas, in the second phase crystalline portion (hard to be utilized) of the cellulose was consumed.

A similar trend was observed when 1 % CTMP1 was used as a substrate and 6-h interval in taking sample was used. Figures 4.27 and 4.28 represent the 24-h and 12-h sampling intervals. It was noticed that the general trend of the investigator would be to stop the experiment around 80 h of fermentation because, it is generally assumed that, since, there is no significant increase in celulase production, therefore, there is no need to continue fermentation beyond 80 h. But when the samples were taken at 6-h interval, the filter paper activities analyzed for those samples were plotted against time, the curve represented in Fig. 4.29 resulted (compare with Fig. 4.22 in which no 'DIP' was shown). Here again during the first phase the organism was utilizing hemicelluloses; it was also utilizing cellulose which was exposed after the utilization of hemicelluloses from the loosened up portion of CTMP1 (produced during its preparation). Whereas, in the second phase compact cellulose

Fig. 4.27. CELLULASE PRODUCTION ON 1% CTMP1

At Optimal pH 6.0, in the Fermenter, With QMY-1

Sampling
24 hours

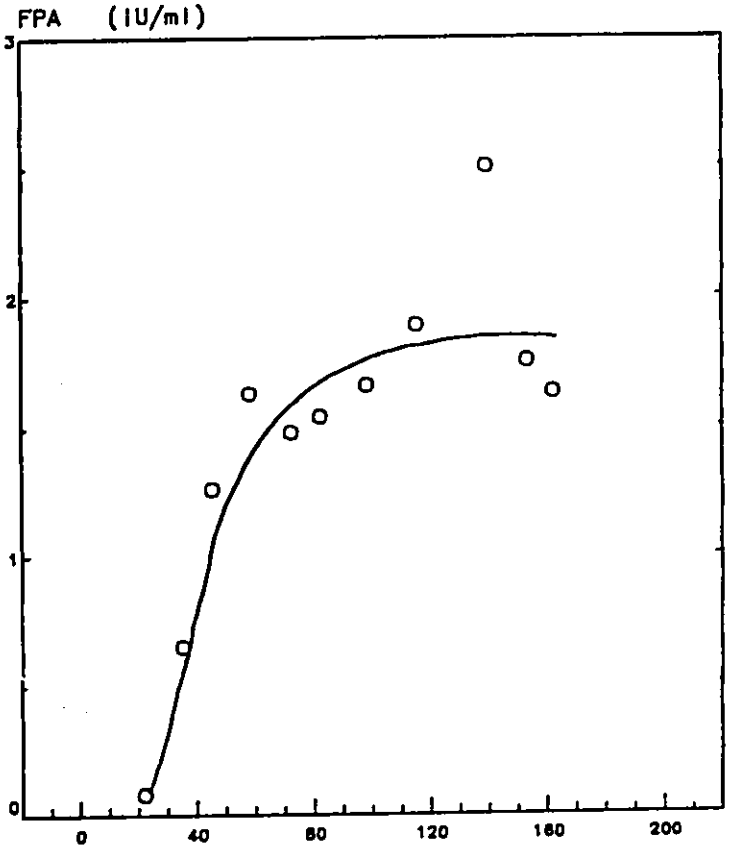


Data in Table 4.20.

Fig. 4.28. CELLULASE PRODUCTION ON 1% CTMP1

At Optimal pH 6.0, in the Fermenter, With QMY-1

Sampling
12 hours



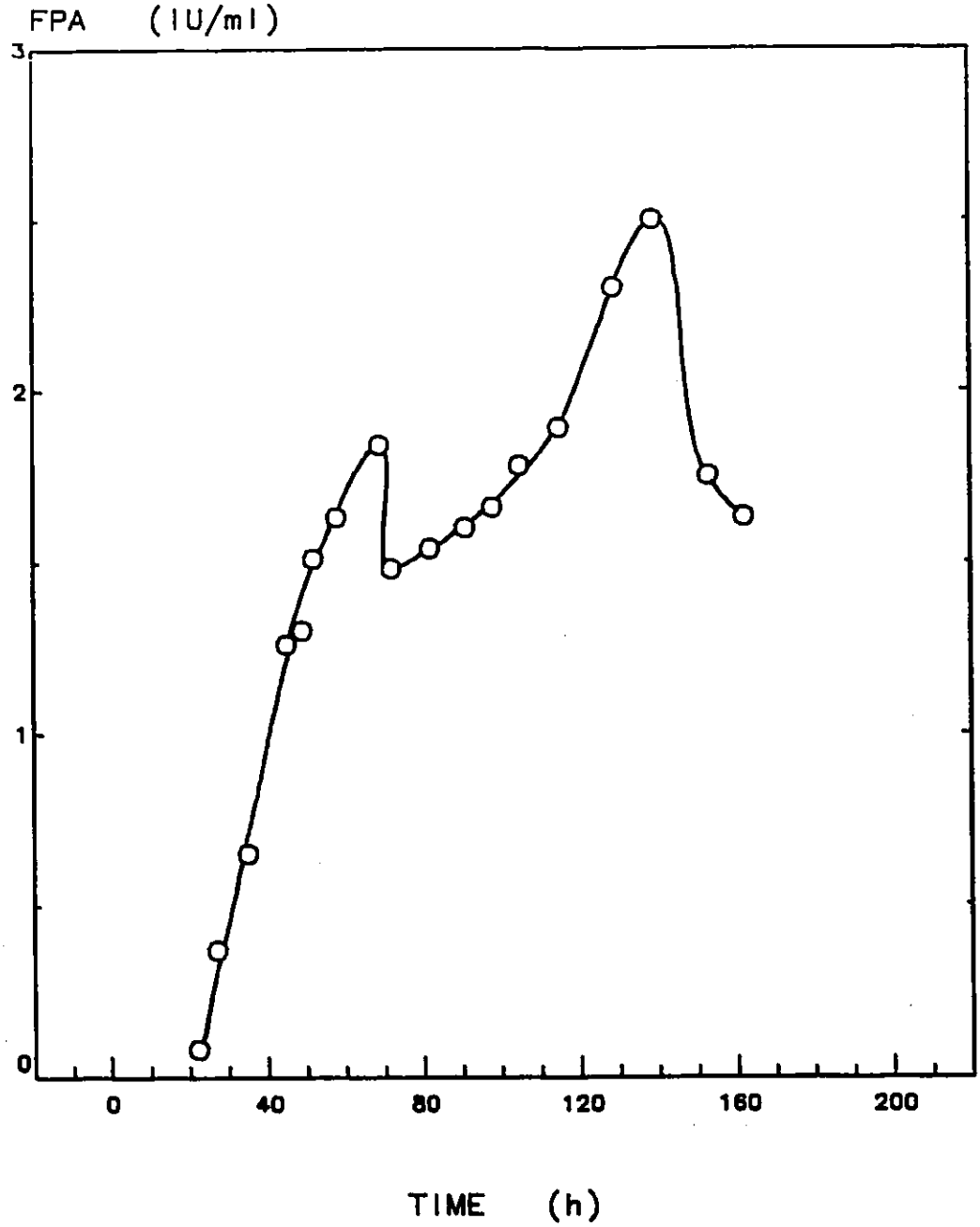
Data in Table 4.20.

Fig. 4.29. CELLULASE PRODUCTION ON 1% CTMP1

At Optimal pH 6.0, in the Fermenter, With QMY-1

Sampling
6 hours

O



Data in Table 4.20.

(crystalline portion) was consumed for cellulase production. The first phase in the case of CTMP1 lasted for 70 h and the second phase from 70 h to end of run.

It was noticed that in the case of 1% CTMP1 there was a large drop in filter paper activity at around 70th hour, when compared to 1% alpha cellulose at about 60th hour. This drop in filter paper activity, between the two phases of cellulase production, was termed as 'DIP'.

The 'DIP' in cellulase production during the period of fermentation could be attributed due to the following factors:

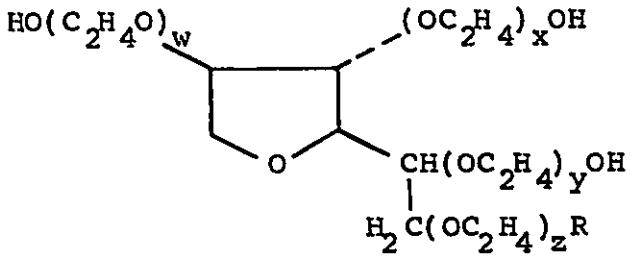
(i) substrate was not easily available after 70 h of fermentation, therefore, shifting from amorphous to crystalline portion, needed more energy for maintainance than cellulase production; (ii) the rate of growth on amorphous cellulose and hemicelluloses is much higher than crystalline cellulose and when hemicelluloses and amorphous cellulose depleted suddenly, the cellulase production stopped; and (iii) since, the cellulase production is extremely slow, the agitation destroyed some of the accumulated cellulase.

As soon as the organism adapted itself on crystalline portion the rate of cellulase production increased, more than the rate of decay of the enzyme, thus again accumulation of cellulase activity observed.

Frequent sampling showed a 'DIP' in cellulase enzyme production profile. However, it was of an interest to make a check if the 'DIP' also exists in the other enzymes (such as β-glucosidase and xylanase).

4.5.1. 'DIP' in Cellulase, β-Glucosidase and Xylanase Production Profiles With or Without the Addition of Tween 80

Before, going to do further experiments regarding the study of the 'DIP' it was necessary to know the effect of Tween 80 on enzyme production. Tween 80 [polysorbate 80: (z)-sorbitan mono-9-octadecenoate poly (oxy-1, 2-ethanediyl) derivs] is an oleate ester of sorbitol.



[sum of w, x, y, z is 20; and R is $(C_{17}H_{33})COO$]

It is an emulsifier and dispersing agent, and is used by many researchers (Andreotti et al., 1977; Duff et al., 1985; Griffin et al., 1974; Ikediobi et al., 1985; Mandels et al., 1981; Sahai et al., 1977; Tangnu et al., 1981) in cellulase production.

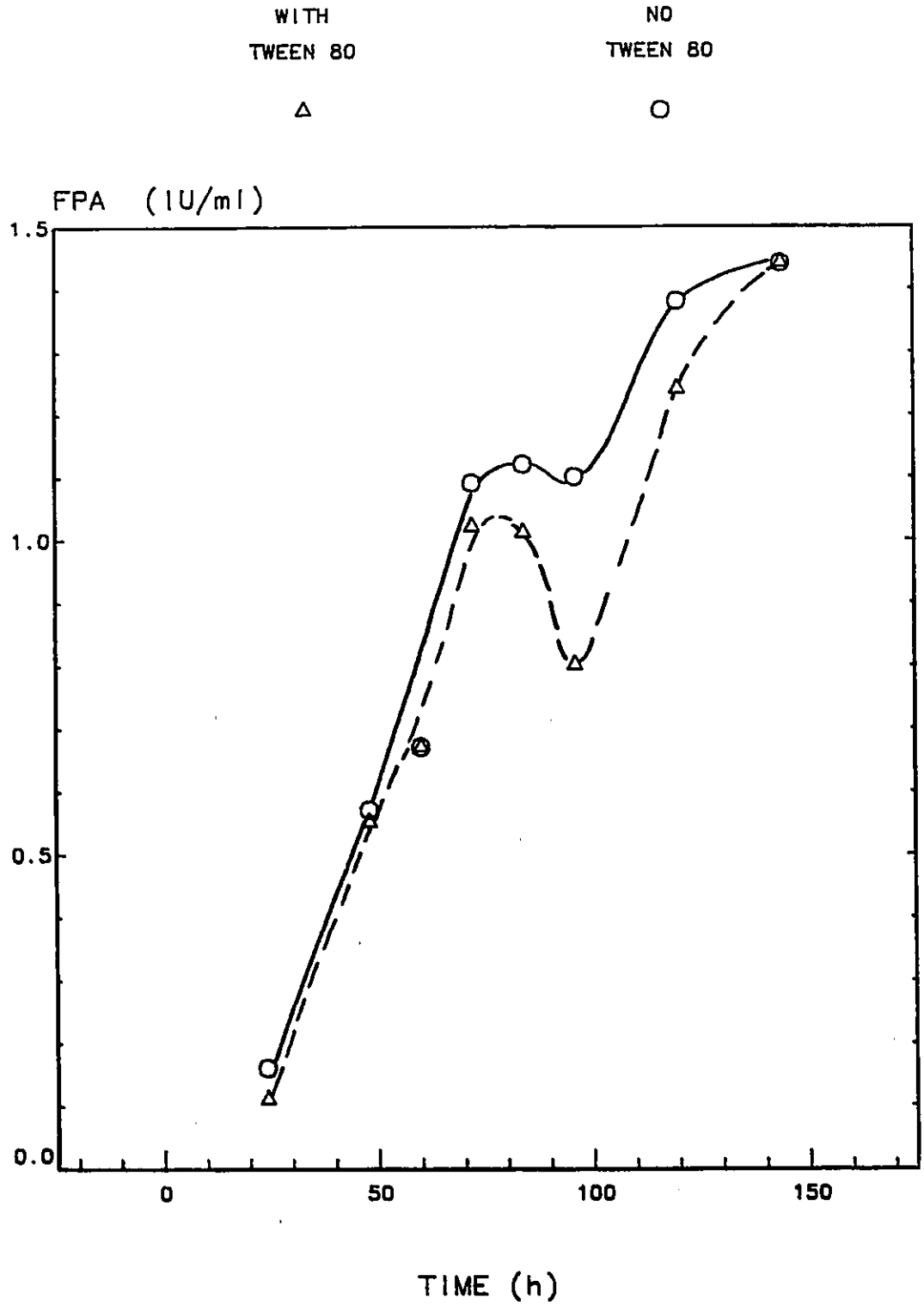
To check the effect of Tween 80 on 1% (wt/vol) CTMP1 in Mandels' medium at pH 6.0, 30°C, Tween 80 in 0.1% concentration was added to the medium (before sterilization) in one set and in other set no Tween 80 was used to serve as a control. The data obtained are presented in Figure 4.30, 4.31 and 4.32 for filter paper, β-glucosidase and xylanase activity, respectively (Table 4.21).

Tween 80 did not alter the yields of filter paper, β-glucosidase or xylanase activity. However, the values of filter paper activity were lower during the second phase of enzyme production. On the other hand 150% increase in enzyme activity was reported by the addition of 0.1% Tween 80 by Griffin et al. (1974).

Drop in activity was noticed after 60, 72 and 84 h in xylanase, filter paper and β-glucosidase activities, respectively, in both cases: with or without the addition of Tween 80.

Fig. 4.30. EFFECT OF TWEEN 80 ON CELLULASE PRODUCTION

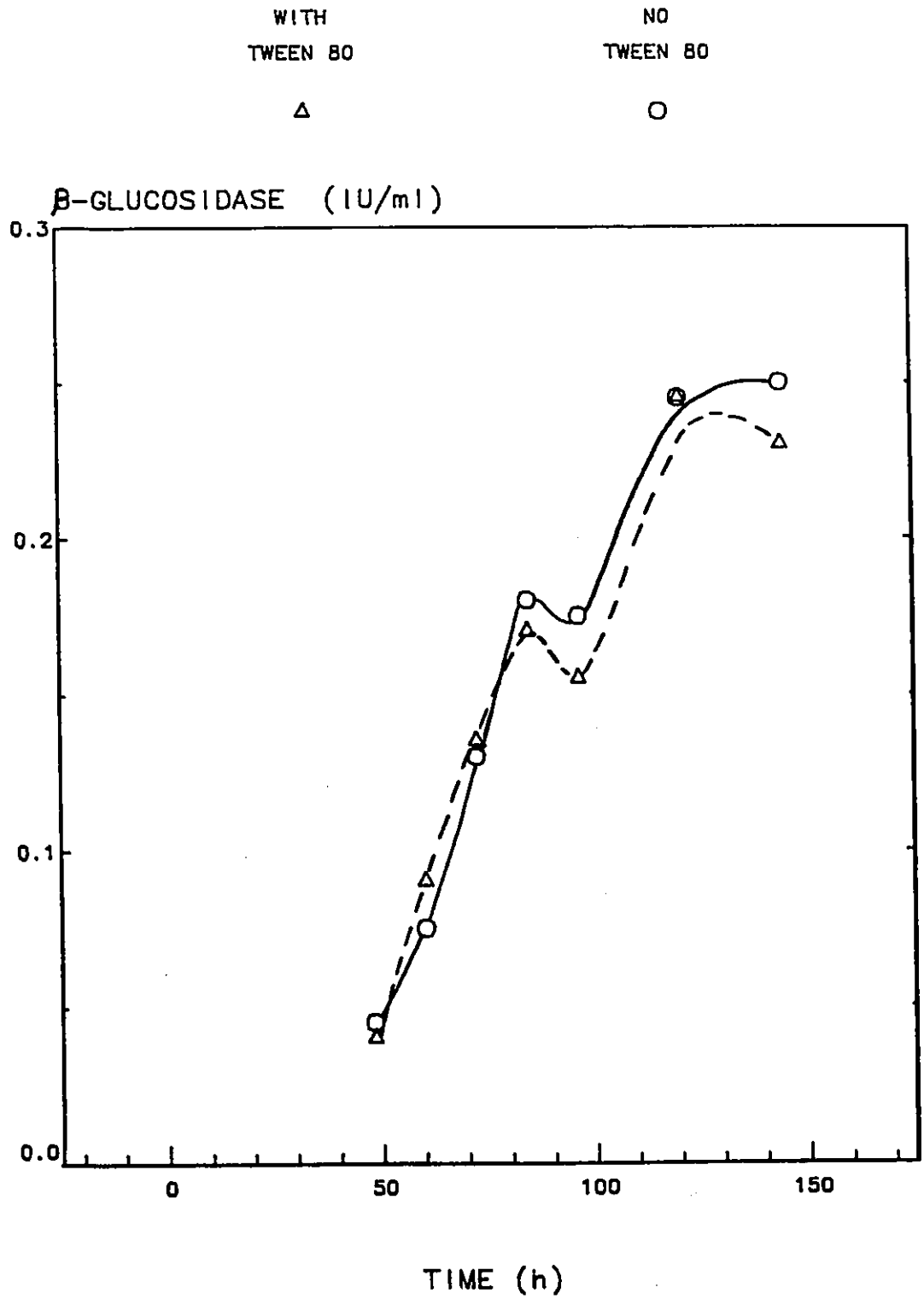
1% CTMP1, at pH 6.0, with QMY-1



Data in Table 4.21.

Fig. 4.31. EFFECT OF TWEEN 80 ON β-GLUCOSIDASE PRODUCTION

1% CTMP1, at pH 6.0, with QMY-1



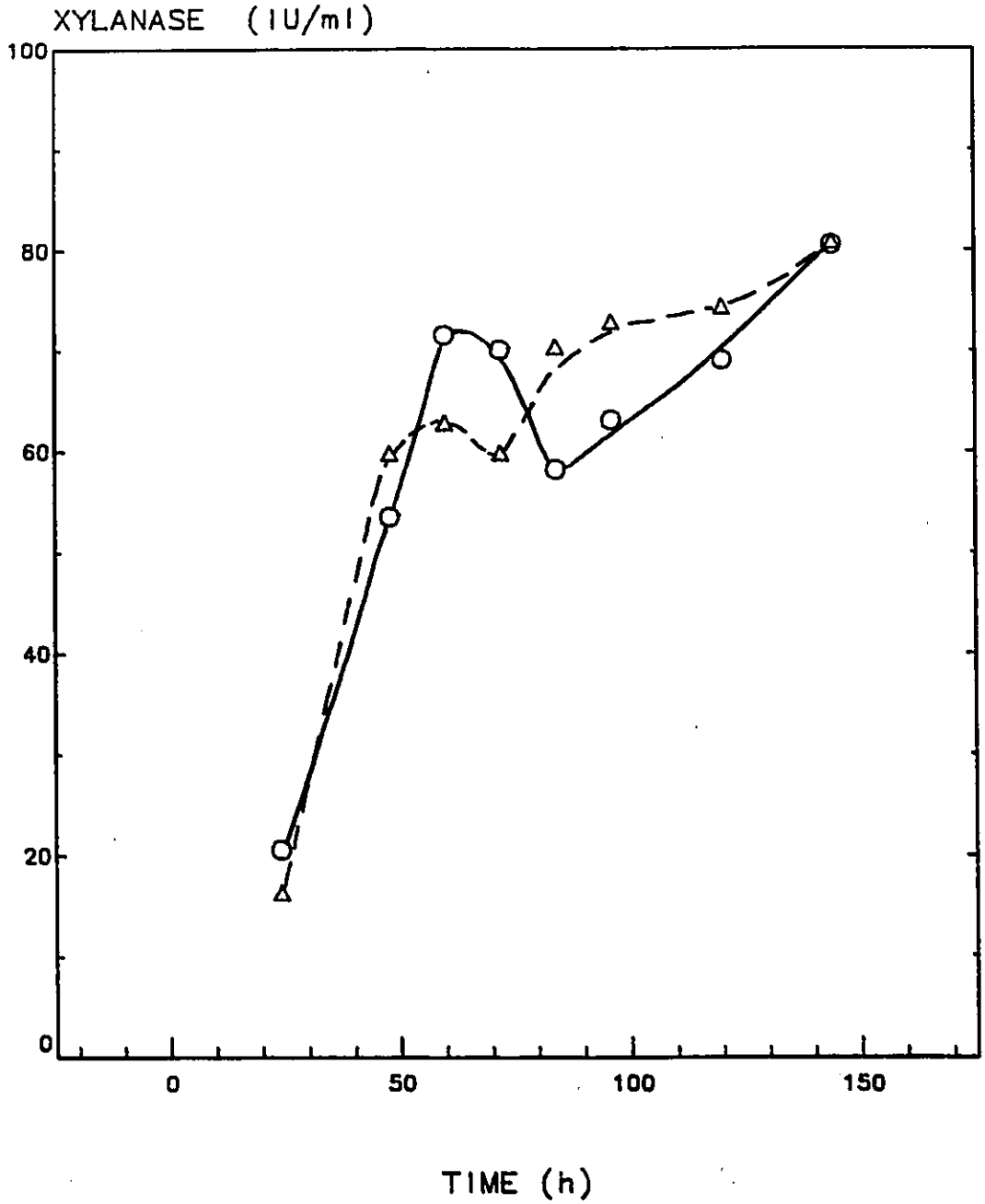
Data in Table 4.21.

Fig. 4.32. EFFECT OF TWEEN 80 ON XYLANASE PRODUCTION

1% CTMP1, at pH 6.0, th QMY-1

WITH TWEEN 80 △

NO TWEEN 80 ○



Data in Table 4.21.

Since the experiments were performed in shake flasks the enzyme activities were lower. Maximum cellulase activity of 1.44 IU/ml, xylanase activity of 80.5 IU/ml and β-glucosidase activity of 0.25 IU/ml was achieved in 144 h without adding Tween 80.

Elimination of Tween 80 from the medium would reduce the cost of cellulase production to a great extent. No Tween 80 was used in any of the previous experiments for cellulase production. However, it was used at the time of recovery of the enzyme from the broth. But it can be concluded from the above experiment that Tween 80 did not effect the cellulase production but retards to some extent when added in the medium.

4.6. INVESTIGATION OF THE CELLULASE PRODUCTION PROFILE

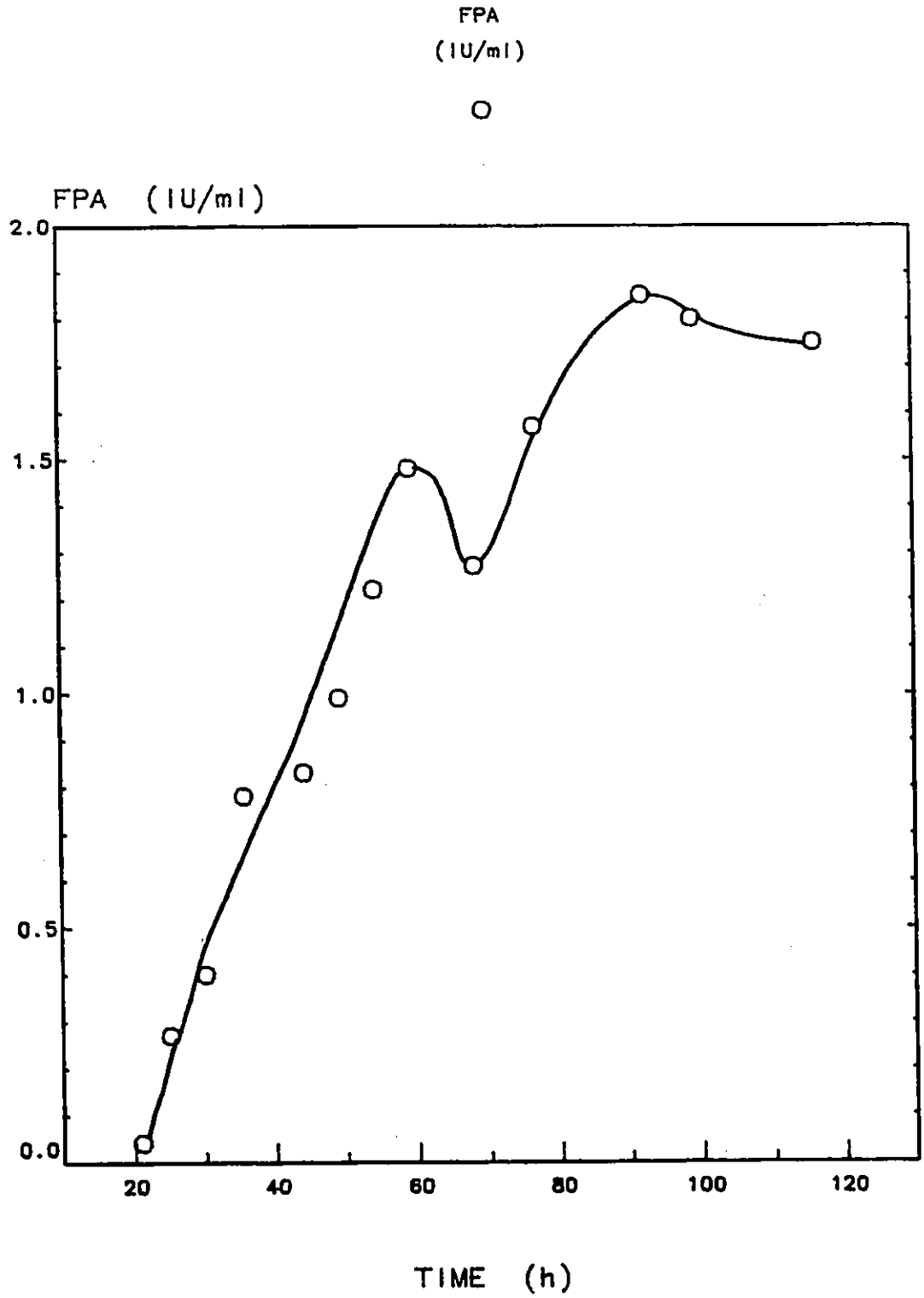
4.6.1. Preliminary Experiments

The 'DIP' in cellulase production curve was observed not only in QMY-1 but also in QM9414 (Fig. 4.33). The drop of 14% in cellulase production was observed at 59th hour and maximum cellulase activity of 1.85 IU/ml was achieved in 92 h of fermentation when QM9414 was grown on 1% alpha-cellulose at pH 5.0 and at 30 C in the shake flasks. The mutant QMY-1 will be used for further experimentation in order to understand the cause of the 'DIP' and to reduce it.

The 'DIP' in the cellulase production was more when CTMP1 was used as a substrate (20% drop) with QMY-1, than that when alpha-cellulose (2.5% drop). Therefore, to examine the causes of this drop ('DIP') in cellulase production further studies were performed on CTMP1. An experiment was performed using 1% CTMP as substrate for cellulase production, in which pH was maintained at 5.0 (similar to the optimal pH obtained for alpha cellulose, where a small 'DIP' was produced). It was noticed that the 'DIP' at pH 5.0 was much larger (40% drop) than that at pH 6.0 (20% drop) on 1% CTMP1 (Figs. 4.34 and 4.29, respectively), but the maximum

Fig.4.33. CELLULASE PRODUCTION ON 1% ALPHA-CELLULOSE

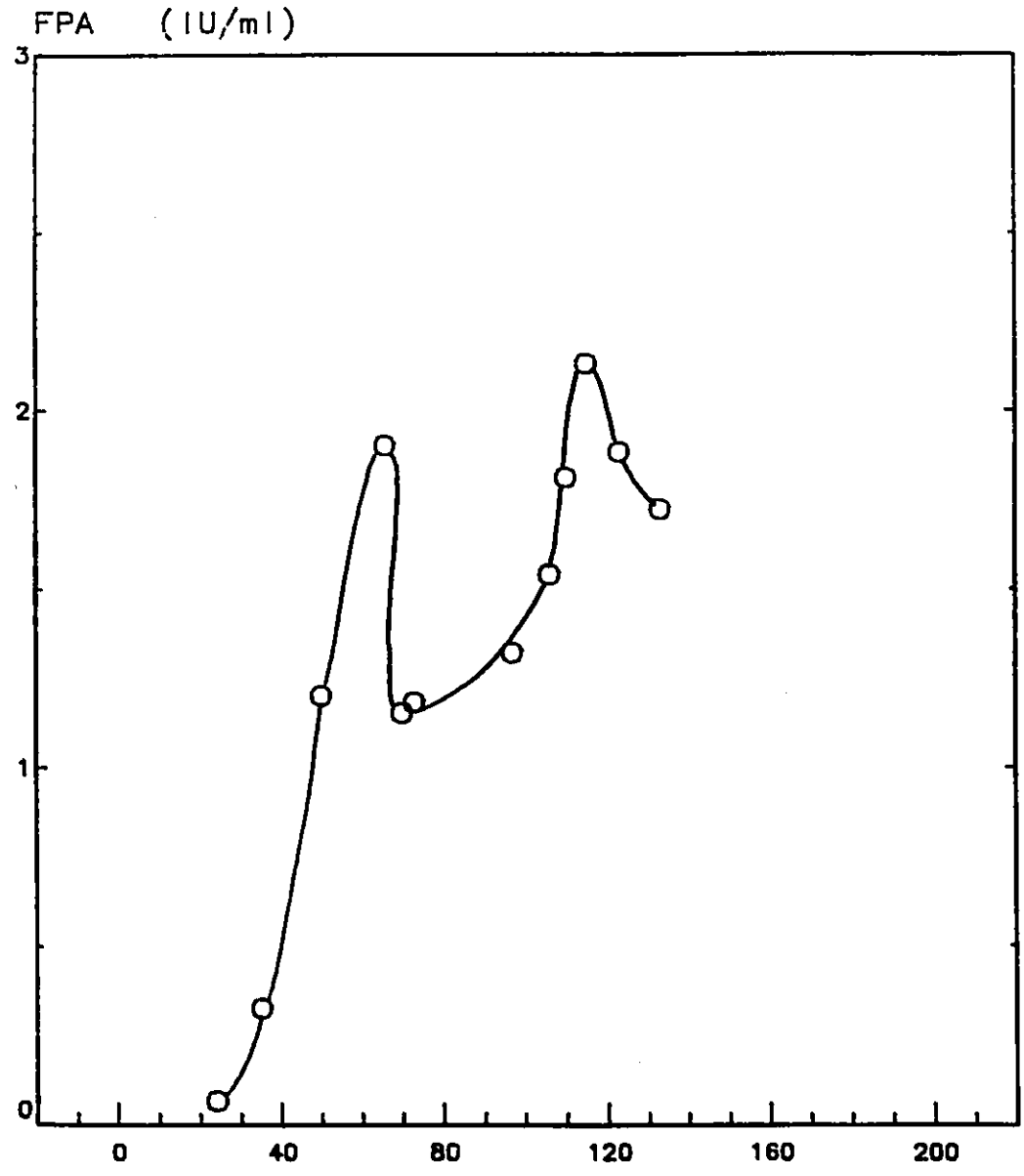
With QM9414, at pH 5.0, in Shake Flasks



Data in Table 4.22.

Fig. 4.34. CELLULASE PRODUCTION ON 1% CTMP1

At pH 5.0, in the Fermenter, With QMY-1



Data in Table 4.23.

cellulase activity was obtained in much shorter time (2.13 IU/ml in 115 h at pH 5.0; 2.5 IU/ml in 139 h at pH 6.0).

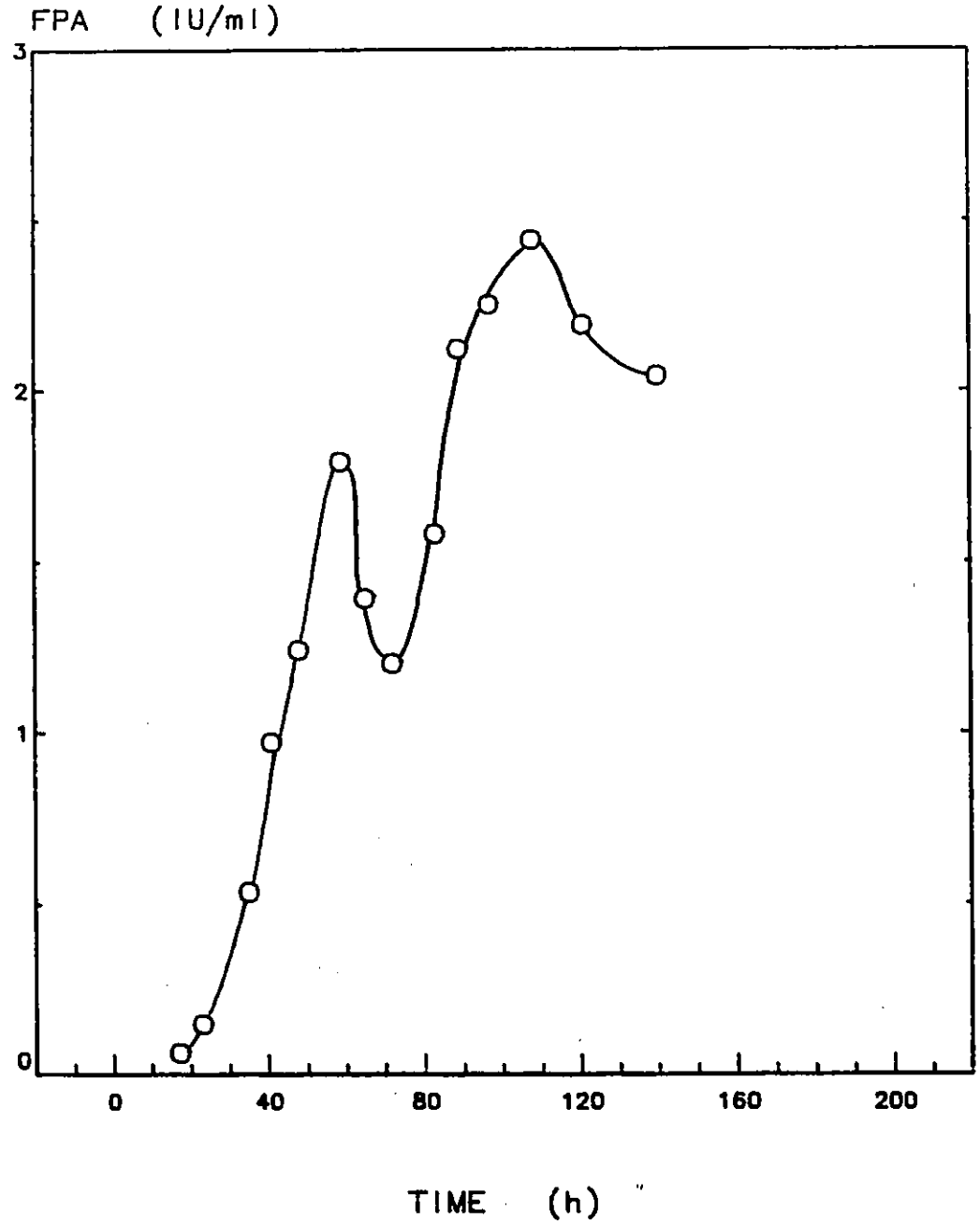
It was assumed that in the first phase hemicelluloses and amorphous cellulose were being consumed by the microorganism, whereas, in the second phase crystalline cellulose was consumed. Therefore, the first phase of cellulase production was linked to the 'optimal pH experiment for CTMP1' (which is at pH 6.0) and the second phase was linked to the 'optimal pH experiment for alpha-cellulose' (which is at pH 5.0). Another experiment was set-up in which for the first 65 h pH was maintained at 6.0 and thereafter, the pH was dropped to 5.0 and was maintained at that level for the rest of the fermentation time. The pH-drop from 6 to 5 was achieved in 50 minutes (Fig. 4.35). The maximum cellulase activity of 2.44 IU/ml was obtained in 108 h of fermentation and the 'DIP' was about 33%. The yield of 244 IU/g CTMP1 (407 IU/g cellulose) was obtained in 108 h.

The second phase had a more suitable pH (5.0) for enzyme production and therefore, the maximum cellulase activity was achieved in less time; higher cellulase productivity (25.6 IU/l/h) by shifting pH from 6 to 5

Fig. 4.35. CELLULASE PRODUCTION ON 1% CTMP1

0 to 65 h = pH 6.0

65 to - h = pH 5.0



Data in Table 4.24.

(compare the lengths of second phase in Figures 4.34 and 4.29). Whereas, productivity of only 18.5 and 18.0 IU/l/h was achieved when pH was maintained at 5 and 6, respectively. The corresponding 'DIP' of 20% and 40% was observed at pH 6 and 5, respectively. But by shifting pH from 6 to 5, the 'DIP' had an intermediate value of 33%.

4.6.2. Documented Fermentation Run

In order to better understand the cellulose consumption, growth of the microorganism, and types of enzymes produced and the particular profile observed ('DIP') in cellulase production, a well-documented fermentation run was performed using 1% CTMP1. Filter paper, β-glucosidase, cotton cellulase (C_1) and xylanase activities were determined at short time-intervals (4 h) along with, the residual cellulose and protein contents of the fermentation broth. The pH in the first phase was maintained at 6.0. The experiment was run such that when growth rate slowed down (indicated by very slow consumption of alkali), pH set point was shifted from 6 to 5. The reason for doing so was that when growth slows down, it means that the residual substrate is not being used as quickly by the organism, in other words the easily available hemicelluloses and amorphous cellulose have been depleted and the microorganism is adapting itself for the utilization of the crystalline cellulose. The pH was monitored with the pH recorder and each addition of the acid or alkali was represented by a small stroke on the chart of the recorder. The number of strokes per hour was the guide to know the rate of consumption of

the acid or alkali. The data obtained are presented in Fig. 4.36 and Table 4.24. It was noticed that the alkali consumption rate was significantly lower after 43 h, therefore, the pH set point was adjusted to 5 at 48th hour.

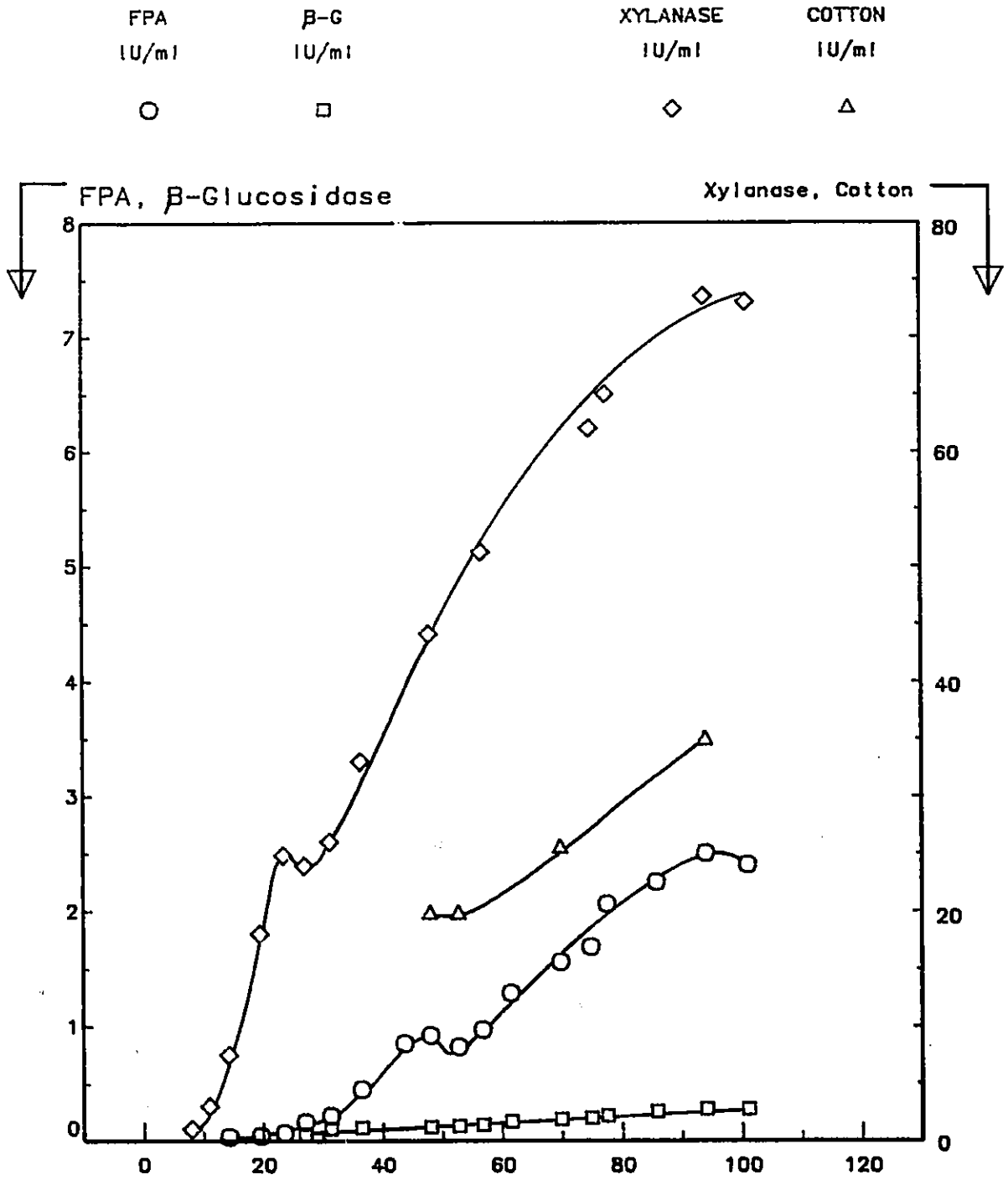
4.6.2.1. Results of Fermentation Run

a) Xylanase Activity

The xylanase production started right at the begining of the fermentation (1.0 IU/ml at 8 h). The xylanase activity increased exponentially upto 23.5 h and then a small reduction of 3.6% was observed. The second phase of exponential production of xylanase was observed after 27 h and the maximum of 73.5 IU/ml of xylanase activity was reached at 94 h. This corrosponds to a yield of 7 350 IU/g CTMP1 or 36 750 IU/g hemicelluloses (based on 20% hemicelluloses in CTMP1) and productivity of 782 IU/l/h. Kluepfel and Ishaque (1982), Rapp and Wagner (1986) and Gokhale et al. (1986) grew Streptomyces flavogriseus, Cellulomanas uda and Aspergillus niger, respectively, on xylan for the xylanase production. The xylanase activities of 45 IU/ml (Kluepfel and Ishaque, 1982) and 3.2 IU/ml (Gokhale et al., 1986) were obtained on 1%

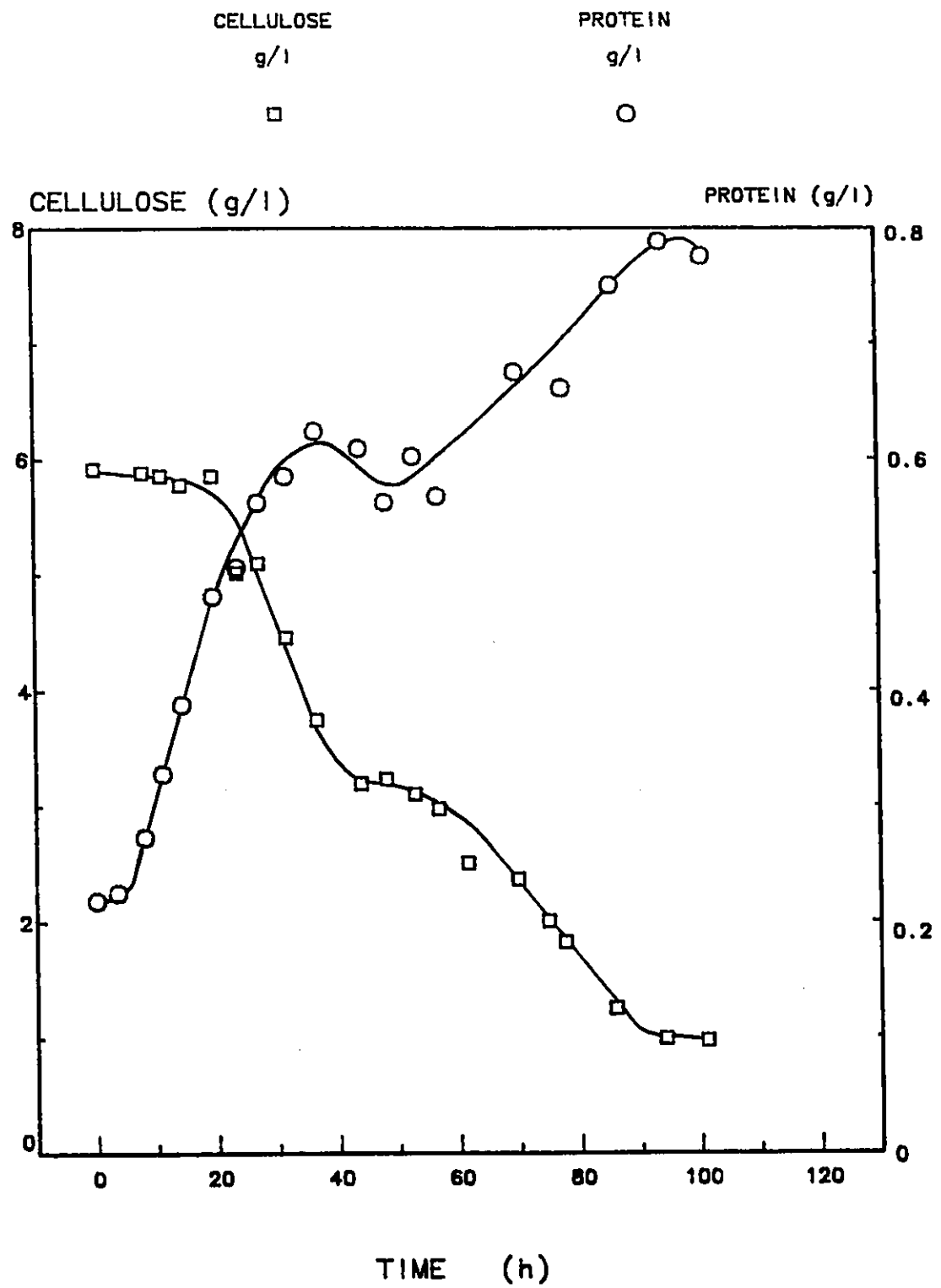
Fig. 4.36 A. ENZYME SYSTEM OF T. reesei QMY-1

Grown on 1% CTMP1



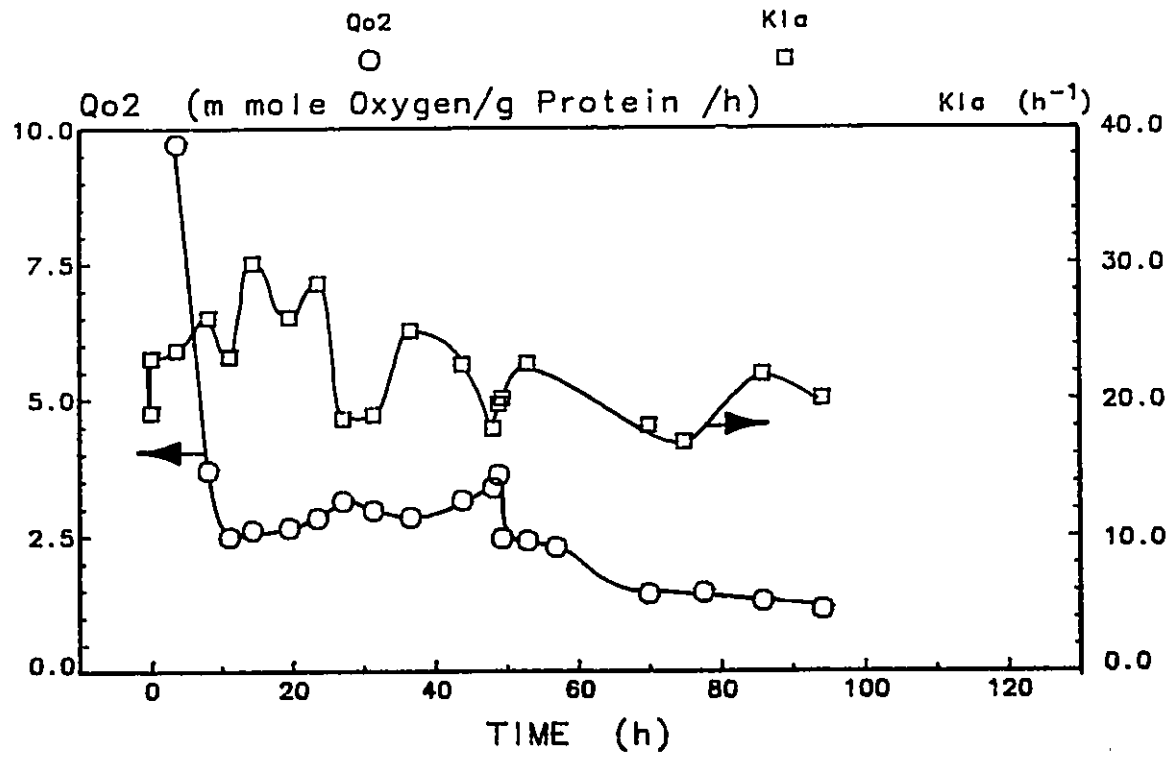
Data in Table 4.36.

Fig 4.36 B. GROWTH AND RESIDUAL CELLULOSE



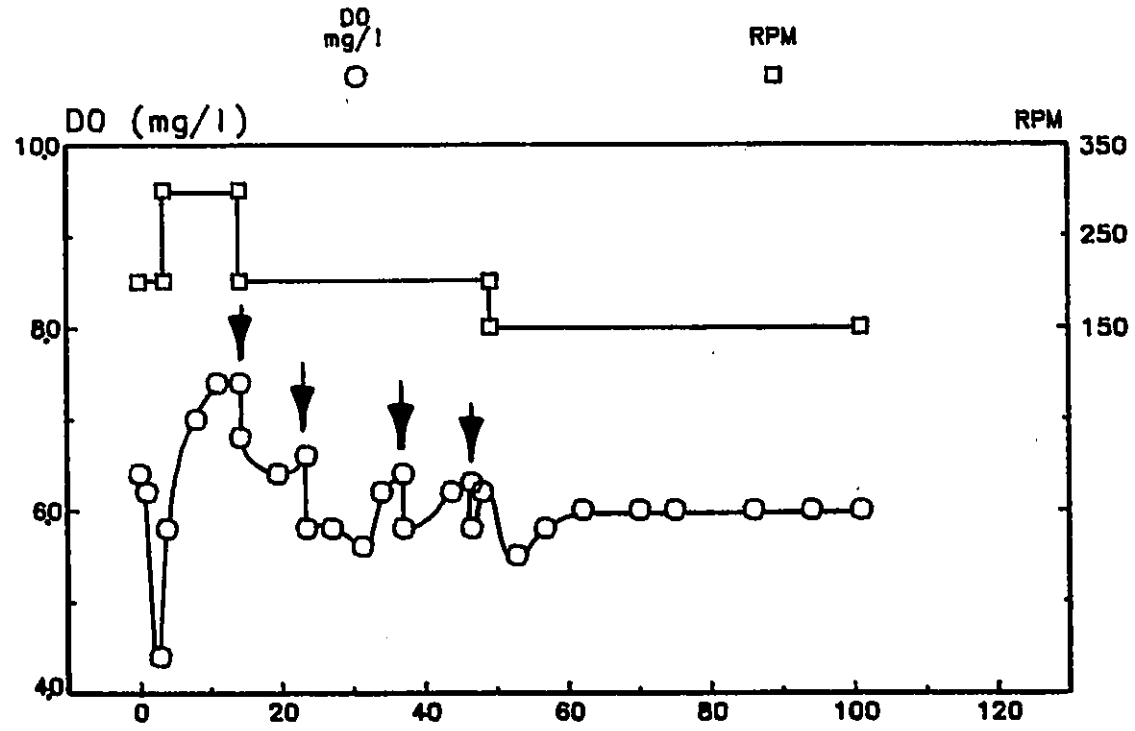
Data in Table 4.25.

Fig 4.36 C. THE Qo2 AND Kla VALUES



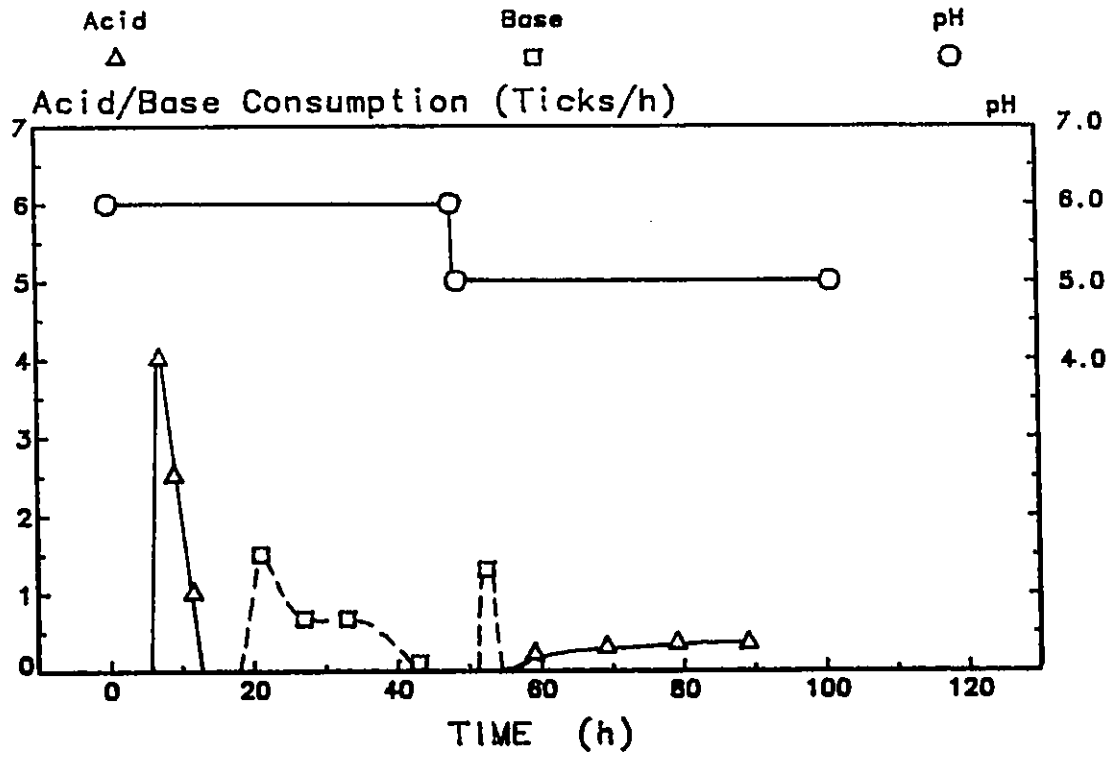
Data in Table 4.25.

Fig. 4.36 D. DISSOLVED OXYGEN (DO) AND AGITATION SPEED (RPM)



Data in Table 4.25.

Fig. 4.36 E. THE pH AND ACID/BASE CONSUMPTION



Data in Table 4.25.

xylan, whereas, 16 IU/ml (Rapp and Wagner, 1986) were obtained on 2% xylan.

b) Cellulase Production

The cellulase production started somewhat later than xylanase production. The lag phase in cellulase production was approximately 24 h. The first exponential phase of cellulase production lasted from 24 to 48 h and the second exponential phase lasted from 52.75 to 94 h. A drop ('DIP') of 11% in cellulase production was observed during 48 to 52.75 h period. The maximum of cellulase activity of 2.5 IU/ml was achieved in 94 h with a yield of 250 IU/g CTMP1 (423 IU/g cellulose) and a productivity of 26.6 IU/l/h. The values obtained by other workers have been reported in Table 4.2.

c) β-glucosidase Activity

The β-glucosidase activity was low and a maximum of 0.28 IU/ml was obtained in 94 h of fermentation. The yield of 28 IU/g CTMP1 (47.4 IU/g cellulose and productivity of 3.0 IU/l/h was obtained. The 'DIP' was not observed in β-glucosidase activity. However, in the experiment where Tween 80 was used (Fig. 4.31) the

'DIP' was noticed in β-glucosidase activity.

Duff et al. (1987) used Aspergillus phoenicis along with T. reesei Rut-C30 (as mixed cultures) to get higher β-glucosidase activity in the cellulase system produced, since Aspergillii are among the better sources of β-glucosidase (Enari, 1983). Duff et al. (1987) achieved 1.1 U of β-glucosidase which was assayed according to Tangnu et al. (1981). This assay was based on cellobiose hydrolysis instead of salicin hydrolysis (salicin was used in author's work). It is noteworthy that one molecule of salicin releases only one molecule of glucose, whereas one molecule of cellobiose releases two molecules of glucose. Breuil et al. (1986) achieved 0.73 U/ml of β-glucosidase activity with T. harzianum E58 using 1% Solka Floc as a substrate, whereas in author's work 1% CTMP1 was used containing 60% cellulose.

d) Cotton Cellulase Activity (C_1)

The cotton cellulase activity of 19.65 U/ml was obtained at 48 h under the assay conditions. The maximum cotton activity of 34.8 U/ml was acheived at 96 h of fermentation. Ishaque and Kluepfel, (1980) obtained 0.7 U/ml (0.7 mg reducing sugars) of C_1

activity with the enzyme broth of Streptomyces flavogriseus 45-CD, grown on Avicel PH 105 as a substrate. Mandels and Sternberg (1976) reported C_1 activity of 8.6 and 7.6 U/ml on 1% Swecc- and Jay bee-newspapers, respectively, by T. viride QM9414. They also reported that C_1 activities of QM9414 when grown on other substrates were less than 7.6 U/ml (values range from: 2.4 U/ml for BW200 to 7.6 U/ml for SW40).

e) Residual Cellulose

The cellulose content remained almost constant for the first 20 hours and thereafter a sharp drop in cellulose content was observed (Fig 4.36B). About 46% of the initial cellulose was consumed during 20 to 43.75 h of fermentation. A plateau in the cellulose consumption was recorded from 43.75 to 52.75 h of fermentation. Thereafter, drop in cellulose was observed again during 52.75 to 94 h of fermentation but at a relatively slower rate. Total of 83.5% of the initial cellulose was consumed in 101 h of fermentation.

f) Mycelial Growth

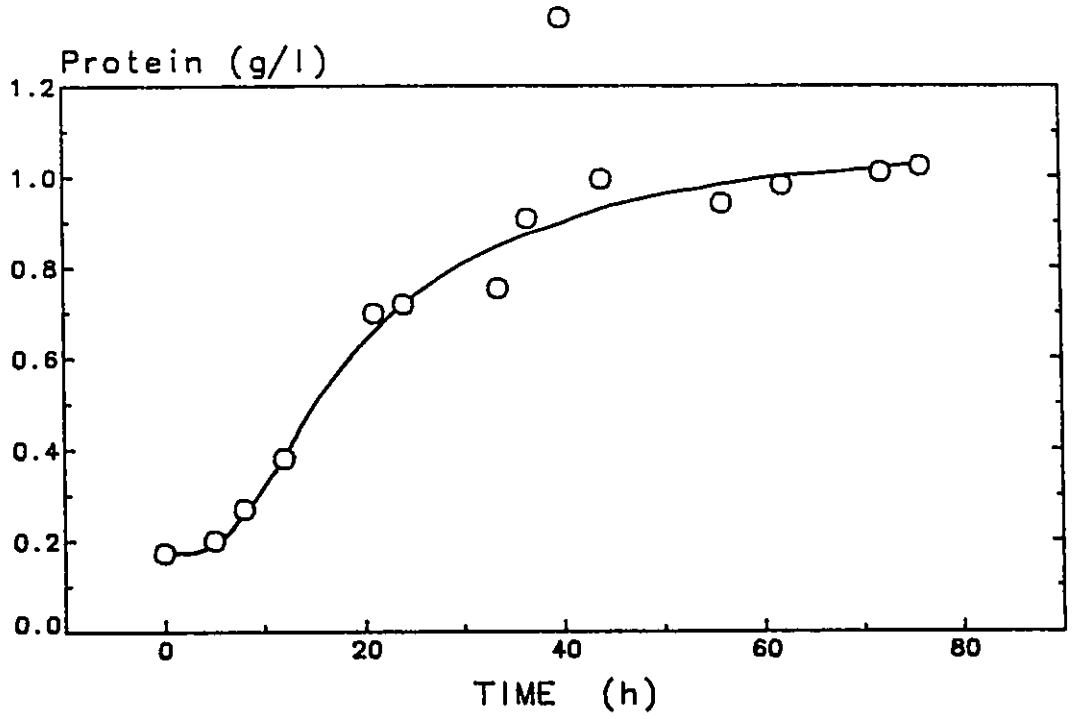
The growth was measured as the amount of protein

synthesized in the mycelial biomass (Fig 4.36B). The lag phase in mycelial growth was about 3.0 h. The first exponential phase of growth related to the growth on hemicelluloses lasted upto 15 h of fermentation because during this time no cellulose was consumed (Fig 4.36B). A slower rate of growth was observed during 15-40 hours. This was because the growth was shifted to the utilization of cellulose (see cellulose curve, Fig. 4.36B). During 40 to 53 h the rate of death or autolysis of mycilial cells was more than the rate of growth, therefore, decrease in growth rate was observed. During this period cellulose consumption was negligible. The rate of growth increased again during 53 to 101 h period. During this period, growth was on residual crystalline cellulose. The specific growth rate of 0.051, 0.018 and 0.008 h^{-1} was recorded for the period of 3-15, 15-40 and 53-94 h, respectively. The corresponding doubling time of 14, 38 and 88 h was obtained during these three periods of growth, respectively (Fig. 4.39).

The maximum biomass yield, $Y_{x/s}$= .116 g protein/ g cellulose consumed, was based on cellulose only, because hemicelluloses and lignin were not assayed. The maximum protein concentration of 0.788 g/l was obtained in 94 h of fermentation.

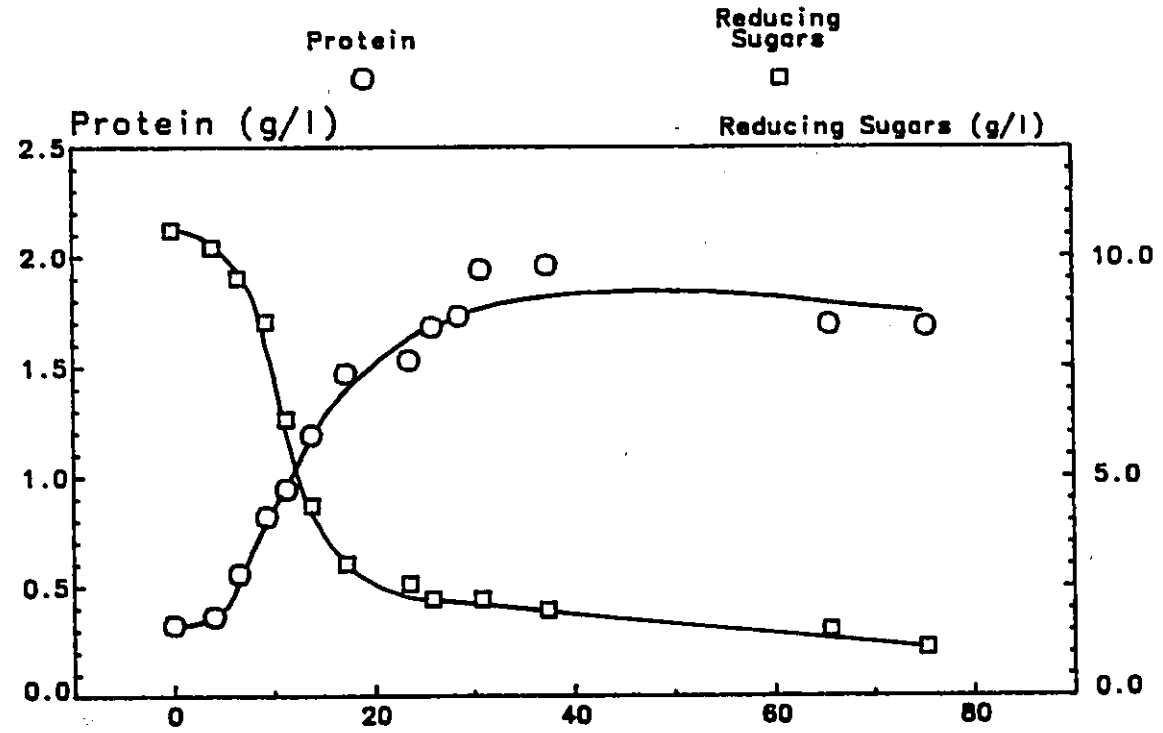
The growth of QMY-1 in 1% glucose at pH 6.0 was monitored in the fermenter (Fig. 4.37) to compare the growth parameters with those when 1% CTMP1 was used. The specific growth rate of 0.116 h^{-1} was obtained with doubling time of 6.0 h. Since the growth was very fast and 4 h sampling interval was also too long, therefore, a good growth curve was not obtained by this experiment. Also, some of the mycelium stuck to the walls of the fermenter, therefore, next experiment was performed in the shake flasks. The samples in this case were taken after 2-h intervals. A good growth curve was obtained (Fig. 4.38). The residual glucose (reducing sugars) was also determined. It was observed that 97% of the sugar was consumed within 24 h with a lag phase of about 2 h. The specific growth rate of 0.123 h^{-1} (doubling time of 5.6 h) was obtained. Specific growth rates obtained by other workers have been tabulated in Table 4.29. The maximum protein concentration of 1.96 g/l was obtained in 37.33 h (Table 4.27), with the maximum yield of 0.157 g protein produced / g of sugar consumed. The dry weight at 37.33 h was 3.89 g/l which contains only biomass, and therefore, contains 50.4% protein [(1.96 g protein/l)/(3.89 g Biomass/l)].

Fig 4.37. GROWTH OF QMY-1 ON 1% GLUCOSE IN FERMENTER, AT pH 6.0



Data in Table 4.26.

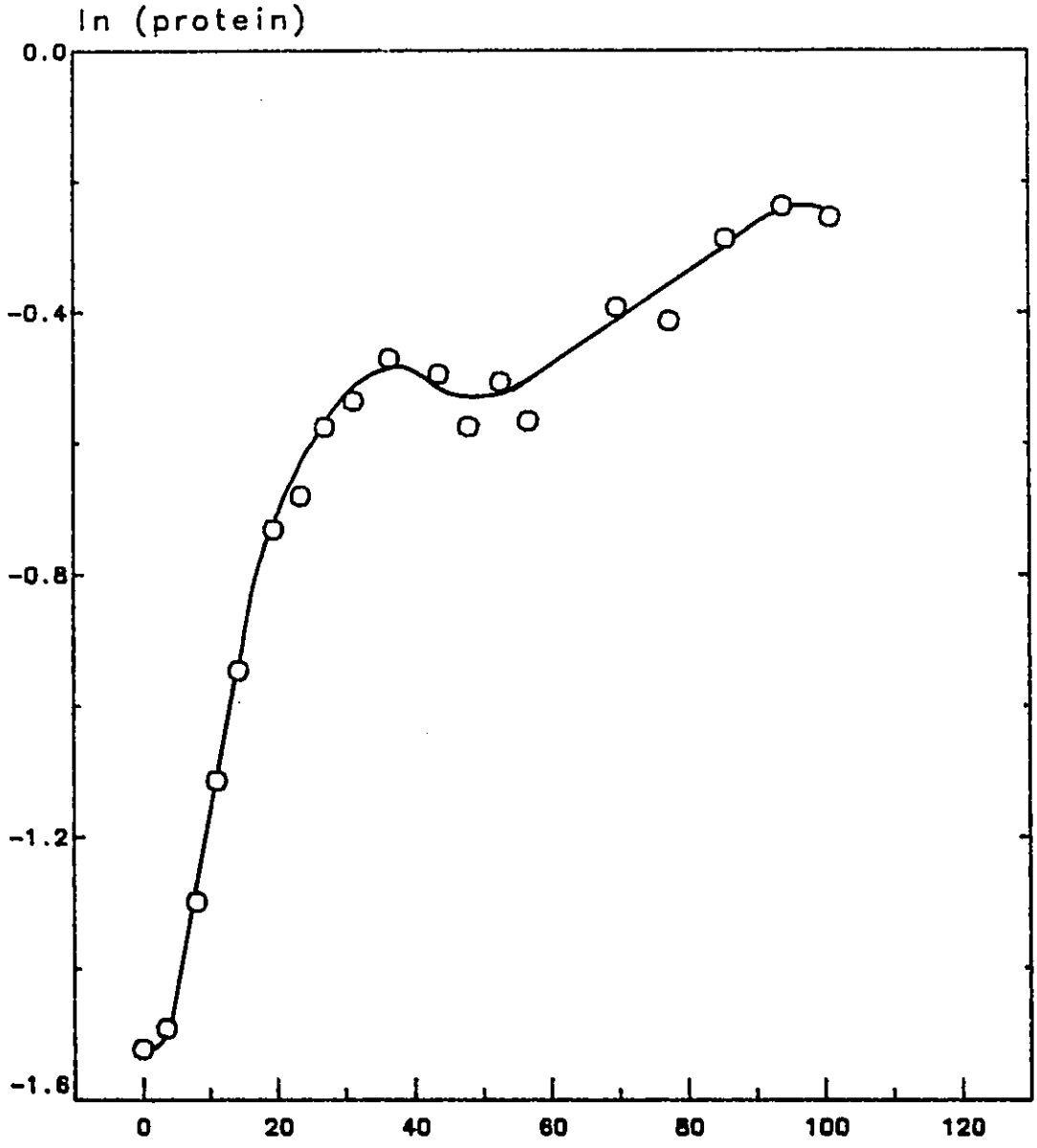
Fig 4.38. GROWTH OF QMY-1 ON 1% GLUCOSE IN SHAKE FLASKS, AT pH 6.0



Data in Table 4.27.

Fig. 4.39. THE GROWTH CURVE ON ln SCALE

QMY-1 ON 1% CTMP1



Data in Table 4.28.

TABLE 4.29. SPECIFIC GROWTH RATES OF T. reesei REPORTED BY SEVERAL WORKERS

SUBSTRATE	ORGANISM	SPECIFIC GROWTH RATE (μ) (h^{-1})	REFERENCE
Glucose	T. reesei	0.104	Nihtila et al 1977
	T. reesei QM9414	0.294	Mitra and Wilke, 1975
	T. reesei QM9414	0.1-0.25	Ryu et al. 1980
	T. reesei QM9414	0.21	Sahai and Ghose, 1977
	T. reesei QM9123	0.097	Brown et al. 1975
Cellulose	T. reesei QM9414	0.10	Nagai et al. 1976
	T. reesei QM9414	0.17	Peitersen et al. 1977
	T. reesei QM9414	0.0317	Sahai and Ghose, 1977

g) Dissolved Oxygen

The dissolved oxygen (DO) was monitored and has been plotted in Fig. 4.36D. A sharp drop in DO value was observed during the first 2.75 hours. Addition of antifoam reduced the foam build up during fermentation by reducing the surface tension of foam. Antifoam also increases the interfacial resistances to mass transfer (Bailey and Ollis, 1977), therefore, drop in DO value was observed whenever the antifoam was added to the fermentation broth. The arrows represents the antifoam addition on DO curve (Fig. 4.36D). To maintain proper mixing (with minimum shearing) the agitation was increased or decreased (150-300 rpm). From 3.5 to 14.25 h the agitation was maintained at 300 rpm, because the fermentation broth was thick. The thickness was due to the (i) presence of the viscous slurry of CTMP1 and (ii) the production of mycelial biomass. Around 14.25 h there was a build up of foam, therefore, the agitation was reduced to 200 rpm and antifoam was added to control the foam build up. At 200 rpm the broth was properly mixing, but the DO value was reduced from 7.4 to 6.8 mg/l. The DO value stabilized at 6 mg/l after 60 h of fermentation. Since, the growth (or oxygen consumption) rate was slow

after 60 h, and the air flow rate of 1 vvm was kept constant for the whole duration of fermentation, therefore, the maximum dissolved oxygen concentration stayed at 6 mg/l level.

h) $\underline{K_1a}$ values

The K_1a values stayed almost uniform in the range of 20-26 h^{-1}. However the value of K_1a as low as 16.8 h^{-1} (at 74.75 h) and as high as 30 h^{-1} (at 14.25 h) were also obtained. The addition of antifoam reduces K_1a to some extent by increasing the interfacial resistances to mass transfer (Bailey and Ollis, 1977), as seen in Fig. 4.36C. The K_1a values were determined prior to the antifoam addition. The dissolved oxygen just before the inoculation (0 h) was 7.9 mg/l which dropped to 6.4 mg/l at the time of inoculation. Assuming, that the critical value of dissolved oxygen concentration is 20% of saturation, (1.58 mg O_2/l), therefore, the maximum driving force for mass transfer will be (7.9-1.58) mg O_2/l = 6.32 mg O_2/l (or = 0.1975 m mole O_2/l). The value of Qo_2X in the fermenter varied from as high as 2.19 to as low as 0.90 m mole O_2/l/h. Therefore, the K_1a value should be at least 11 h^{-1}, corresponding to the maximum rate of oxygen utilization. The values of

K_1a obtained in this experiment were slightly higher than 11 h^{-1} as found by the above calculation. The reason for high K_1a is that in this fermenter three impellers were used along with a sintered steel sparger, hence better mixing and oxygen transfer took place.

i) $\underline{Qo_2X \text{ and } Qo_2}$

The Qo_2X values were calculated and have been reported in Table 4.25C. The value of Qo_2 was calculated by dividing Qo_2X by the mycelial protein (X). The Qo_2 values have been plotted in Fig. 4.36C. A very high value of 9.75 m mole O_2/g mycelial protein/h was observed at 3.5 h of fermentation. The value dropped to 2.6 m mole O_2/g mycelial protein/h at 14.25 h and then rose to 3.6 m mole O_2/g mycelial protein/h during 14.25 to 49.25 h of fermentation. After 50 h the Qo_2 value started to drop till 94 h from 2.42 to 1.14 m mole O_2/g mycelial protein/h.

Mitra and Wilke (1975) reported Qo_2 value of 1 m mole O_2/g mycelial dry weight/h on glucose for QM9414. However, Qo_2 values of 2.6, 1.66 and 0.74 m mole O_2/g cells/h have been reported by André (1982) in 14-litre fermenter by using corn stover as a

substrate, for Chaetomium cellulolyticum at 18.5, 21.5 and 27.5 h of fermentation. Swan (1977) reported a significant decrease in the Qo values with time for Chaetomium cellulolyticum when grown on 1% Solka-Floc; the values of Qo_2 based on material balances performed on oxygen were 2.7, 1.9 and 0.5 m mole O_2/g cells/h for time values of 6, 12 and 21 hours, respectively. The corrosponding values calculated for g mycelial dry weight (based on that there is 50.4% protein in the mycelium dry weight when QMY-1 was grown on 1% glucose solution) are as follows: 4.91, 1.31, 1.23 and 0.57 m mole O_2/g mycelial dry weight/h at 3.5, 14.25, 49.25 and 94 h, respectively. The values of Qo_2 are within the range of the values obtained by other workers. During active growth, the oxygen requirements (Qo_2) were high whereas, relatively low values were recorded in the later stage. In the later stage of the fermentation, where the growth was almost stopped (ie. close to stationary phase), the Qo_2 value is probably related to the maintenance requirement for oxygen, but in the earlier stage of fermentation Qo_2 value included both growth and maintenance requirements. Therefore, maintenance (m_{o2}) can be equated to 0.57 m mole O_2 / g mycelial dry weight / h (recorded at 94 h). The m_{o2} values of 0.74 m mole O_2/g cell/h was determined by Righelato

et al. (1968) for Penicillium chrysogenum.

4.6.2.2. Discussion of Fermentation Curves

The interpretation of Fig.4.36 is as follows:

At the time of inoculation (0 h) the dissolved oxygen (DO) monitored was 6.4 mg O_2/l and it dropped to 4.4 mg O_2/l at 2.75 h. A very high Qo_2 value (9.75 m mole O_2/g mycelial protein/h) was observed at the same time. Since, inoculum was grown on 1% glucose solution, therefore, the microorganism was still growing on the residual glucose (about 0.2 g/l) carried along with the inoculum into the fermenter broth, therefore, that could be the reason why such a high Qo_2 value was observed during this time. There was a lag of 3 h, because there was no significant increase in biomass; however, the organism was growing as indicated by the utilization of O_2 curve. As, in about 3 h, the glucose depleted, the microorganism started to adapt itself for further growth on hemicelluloses (more excessible than cellulose). It seems that the hemicelluloses were utilized from 3 to 20 h, because, no cellulose was consumed during this period (Fig. 4.36B), but, an increase in mycelial protein was observed along with a substantial increase

in xylanase production. The growth curve shows that from 3 to 10 h there was an exponential growth of the organism on hemicelluloses with the specific growth rate of 0.051 h^{-1} (doubling time of 14 h). During this time the DO concentration also started rising, because the microorganism did not require oxygen at the same rate as the first phase corresponding to glucose assimilation. At about 8 h, the xylanase activity of 1.0 IU/ml was noticed in the broth which started increasing exponentially and reached 24.8 IU/ml at 23.5 h. At the same time the Qo_2 value started to increase somewhat. The oxygen was being utilized for growth, maintainance and formation of the products.

At about 20 h the cellulose consumption started as seen from cellulose curve (Fig. 4.36B). At the same time the cellulase production started to increase and continued upto 48.8 h (when the cellulose consumption almost stopped momentarily) (Fig. 4.36A). The amorphous cellulose is easily utilized by the microorganism than crystalline cellulose, therefore, it is reasonable to assume that the amorphous cellulose was consumed first i.e. until 40 hours. As amorphous cellulose was being consumed, small quantity of hemicelluloses (mixed with amorphous cellulose) were also released into the solution, therefore, the growth

during this period (20-48 h) was mainly on amorphous cellulose with the specific growth rate of 0.018 h^{-1} (doubling time of 38 h). As the consumption of amorphous cellulose stopped, the'DIP' in the cellulase production was observed during 48 to 52.75 h. As the hemicelluloses (released from the amorphous portion of cellulose) were still available (although in small quantity), therefore, xylanase was still being produced during this period. From 55 h onwards the cellulose consumption started again and continued till 94 h where residual cellulose, mainly crystalline cellulose, was consumed along with residual amorphous cellulose and possibly residual hemicelluloses. During this period (55 to 94 h) xylanase production continued by the organism. It could be possible that once the xylanase production was triggered, the microorganism kept on producing xylanase without hemicelluloses in the medium. Margaritis et al. (1983) obtained xylanase activity of 25.5 IU/ml by growing Thielavia terrestris on 1% Solka Floc (pure cellulose). The xylanase production was also reported by Tangnu et al., (1981) when T. reesei was grown on pure cellulose. Therefore, it could be possible that there was no or very little residual hemicelluloses during the period of 50 to 101 h in the fermentation broth. Since, hemicelluloses were not analyzed, therefore, the above

statement is not based on solid evidence. The specific growth rate of 0.008 h^{-1} (doubling time of 88 h) on crystalline cellulose was obtained during this period. The DO value remained constant during the period from 50 to 94 h, but the Qo_2 value kept on dropping because of lower requirements expected from a maturing culture.

The period (48 to 52.75 h) where the 'DIP' in the cellulase production was observed, the cotton cellulase activity (C_1) of 19.65 U/ml was obtained. The cotton cellulose activity increased to 34.8 U/ml at 94 h, the later phase of fermentation which was mainly on crystalline cellulose.

The present study shows that in 20 h (from 20 to 40 h of fermentation), amorphous cellulose was consumed. The amount of cellulose (amorphous) consumed during this period was about 46% of the initial cellulose. Hendy et al. (1982) used fed-batch cultivation of T. reesei Rut-C30 and used 20 g/l solka floc as initial concentration for 48 hours. The cellulose consumption or the residual cellulose was not reported. The cellulose concentration was raised to more than 20 g/l at interval of 10 to 20 hours, when the growth rate slowed down. They observed the

decrease in growth by the decrease in rate of alkali used to maintain the pH. Both of the above observations, when compared to the present study (Fig. 4.36), it can be concluded that only amorphous cellulose was consumed in the experiment of Hendy et al. (1982). Therefore, the enzyme-system produced by them might have been deficient in cotton cellulase (C_1) activity, which is usually activated by the presence of crystalline cellulose. The cellulase-system deficient in C_1 activity gives poor hydrolysis of cellulose, therefore, that could be the reason that no hydrolytic potential of the enzyme-system produced by them was reported, although, very high filter paper activity (30.4 IU/ml) was achieved in 288 h using 15% substrate in a fed-batch mode. Moreover, the yield of 202 IU/g cellulose achieved by them was also about one-half of the yield (423 IU/g cellulose) reported in this study. Similarly, Mclean and Podruzny (1985) have achieved filter paper activity of 31 IU/ml in 240 h on 8% cellulose in fed-batch mode, but its hydrolytic potential was not reported, although, the yield of 389 IU/g cellulose obtained was close to the yield obtained in the present study (423 IU/g cellulose).

4.6.2.3. Hydrolytic Potential

Hydrolysis of delignified wheat straw and Alpha Cellulose was performed by supplying 20 IU of FPA. The amount of total reducing sugars produced during the hydrolysis has been reported in Table 4.30.

Delignified wheat straw was completely hydrolyzed (100%) to its monomer sugars within 48 hours, whereas, Alpha Cellulose was 76.8% hydrolyzed in 72 hours. The rate and extent of hydrolysis depends on the the nature and pretreatment of the substrate (Spano et al., 1975). During the process of delignification, wheat straw was swollen up, therefore, more of the cellulose was exposed to the enzyme. But, in the case of Alpha Cellulose, no pretreatment was performed, therefore, relatively less hydrolysis was obtained. Spano et al., (1975) reported that the total hydrolysis in forty-eight hours ranged from 6% for fibrous cotton to over 90% for milled pulp, SWECO 270 with QM9414 enzyme system. Chahal (1985) obtained 90% hydrolysis of delignified wheat straw (10% concentration) in 96 hours with T. reesei QMY-1 cellulase system. Sidi et al., (1984) also reported 90% hydrolysis of sugar beet pulp,

TABLE 4.30. HYDROLYSIS OF DELIGNIFIED WHEAT STRAW AND ALPHA CELLULOSE BY T. reesei QMY-1 ENZYME SYSTEM.

Conditions for Enzyme Production : See Table 4.25A.

Substrate Concentration = 5% in Enzyme broth and 0.05M Citrate buffer.

Hydrolysis Conditions:

Enzyme/Substrate ratio	= 20 IU FPA/g Substrate
pH	= 4.8
Temperature	= 45 °C

A) Delignified Wheat Straw:

Time (h)	Reducing Sugars (g/l)		
	#1	#2	Average
0	4.8	4.8	4.8
24	41.8	42.3	42.1
48	53.4	53.4	53.4

B) Alpha Cellulose:

Time (h)	Reducing Sugars (g/l)		
	#1	#2	Average
0	1.0	1.0	1.0
24	15.4	15.9	15.7
48	22.5	22.3	22.4
72	38.6	38.2	38.4

pretreated by heat at 120°C for 30 minutes in 48 h.

From the data presented it seems that total hydrolysis can be achieved by the mutant QMY-1 in a short time (48 h).

CONCLUSIONS

1. Preliminary experiments showed that QMY-1 is a better cellulase producer than Rut-C30 and QM9414 mutants of Trichoderma reesei.

2. Addition of hemicelluloses increased the cellulase production.

3. QMY-1 appeared to produce as good cellulase as Rut-C30 from hydrolytic point of view.

4. The cellulase activity using aspen, pretreated with 20% NaOH (wt/wt of wood) at 121°C or at boiling temperature (100°C), was almost twice as much as using non-treated aspen.

5. CTMP prepared by four different types of chemical treatments when used for enzyme production showed almost similar profiles of cellulase production.

6. The cellulase production on CTMP1 (1.7 IU/ml) was almost equal to that on aspen wood (1.87 IU/ml) treated with NaOH (20% wt/wt of wood). CTMP1 was chosen as the pretreated substrate for cellulase production by T. reesei QMY-1 for further study as it was produced in large quantity in one lot to keep the uniformity of the substrate.

7. Further increase in the substrate (CTMP1) concentration from 1% level to 3% level did not give corresponding increase in the cellulase activity (1, 2 and 3% substrate concentrations were studied). The substrate concentration was fixed to 1% CTMP1 for the cellulase production in further experiments.

8. The pH 5.0 was chosen to be the best pH when 1% Alpha Cellulose (pure cellulose) was used for the cellulase production in the shake flasks or in the fermenter.

9. The pH 6.0 was chosen to be the best pH when 1% CTMP1 (lignocellulose) was used for cellulase production in the shake flasks or in the fermenter.

10. Frequent sampling showed a 'DIP' in cellulase, β-glucosidase and xylanase production profiles.

11. Tween 80 did not alter the yields of filter paper, β-glucosidase or xylanase activities, but somewhat retarded the enzyme production to some extent.

12. Not only QMY-1 but QM9414 also showed a 'DIP' in cellulase production profile.

13. Shift in pH set point from 6.0 to 5.0 during fermentation increased the cellulase productivity to 25.6 IU/l/h, when compared with the cellulase productivities of 18 and 18.5 IU/l/h at pH 6.0 and 5.0 alone, respectively.

14. The reason for 'DIP' is unclear, but, a second lag phase, shift from amorphous cellulose to crystalline cellulose and effect of temperature and agitation, has been considered.

15. The results of the documented fermentation run are as follow:

Enzyme Activities and Yields:

Xylanase	73.5	IU/ml	36750	IU/g Hemicellulose
Filter paper	2.5	IU/ml	423	IU/g Cellulose
b Glucosidase	0.28	IU/ml	47.3	IU/g Cellulose
Cotton (C_1)	34.8	U/ml	58	U/g Cellulose

Cellulase Productivity 4.5 IU/g Cellulose/ h.

Cellulose Consumption: 83.5%

Three Growth Phases Observed:

Growth on	μ (h^{-1})	Doubling Time (h)
Hemicelluloses	0.051	14
Amorphous Cellulose	0.018	38
Crystalline Cellulose	0.008	88

Maximum Protein Conc. 0.788 g/l

Maximum Protein Yield 0.116 g Protein/g Cellulose

Protein Content in Mycelial Dry Weight: 50.4%

K_1a Value Range 16.8 - 30 h^{-1}

Qo_2X Value Range 9.76 - 1.14 $\frac{\text{m mole } O_2}{\text{g Protein. h.}}$

Maintenance (m_{O2}) 0.57 $\frac{\text{m mole } O_2}{\text{g mycelial dry wt. h.}}$

RECOMMENDATIONS

1. It is recommended to use lignocelluloses as a source of cellulose, because the presence of hemicelluloses has shown higher cellulase activity.

2. Elimination of Tween 80 from the medium is recommended since it did not increase the enzyme activity, but retarded the enzyme production to some extent.

3. It is recommended to take samples more frequently to gain a better understanding of the enzyme and biomass production and the substrate utilization profiles.

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APPENDIX I

TOTAL NITROGEN CALCULATIONS:

Titration of ammonia (in the form of ammonium hydroxide) with 0.1N H_2SO_4:

$$2NH_4OH + H_2SO_4 = (NH_4)_2SO_4 + 2H_2O$$

1 mole H_2SO_4 = 2 moles NH_4OH

Therefore, calculate the amount of Nitrogen (in gram) titrated with 1 ml of 0.1N H_2SO_4 solution.

$$0.1N\ H_2SO_4 = 4.98\ g\ H_2SO_4 \text{ / } 1000\ ml\ H_2SO_4\ solution$$

98 g H_2SO_4 = 70 g NH_4OH

35 g NH_4OH = 14 g N

Therefore, 1 ml of 0.1N H_2SO_4 solution will titrate 0.0014 g N.

Total Nitrogen in the sample: N =

$$\frac{\left\{\begin{array}{l}\text{ml of } H_2SO_4 \\ \text{for sample}\end{array} - \begin{array}{l}\text{ml of } H_2SO_4 \\ \text{for blank}\end{array}\right\} \times\ 0.0014}{\text{g sample}}$$

(units for N: g N / g sample).

APPENDIX II

Qo_2X measurement during the growth of T. reesei QMY-1 on 1% CTMP1

-Sample Calculations

-See Figures IIa and IIc
-Oxygen absorption /desorption experiments

Fermentation Conditions: See Table 4.25A.		
Time since air has been turned off (second)	Dissolved Oxygen (DO) mg/l	
	3.5 h of fermentation	11 h of fermentation
0	5.76	7.48
20	5.54	7.48
40	5.20	7.39
60	4.92 (a)	7.30
80	4.51	7.26
100	4.14	7.04
120	3.74	6.95 (a)
140	3.34	
160	2.97 (b)	6.69
200		6.40
240		6.07
300		5.65
340		5.37
400		4.93
460		4.49
540		3.92 (b)
Qo_2X (mg O_2/l/h)	70.2	25.97
(m mole O_2/l/h)	2.19	0.81

(a) - (b) the range in which the slope was taken.

K_1a measurement during the growth of T. reesei QMY-1 on 1% CTMP1

-Sample Calculations for 13.5 h of fermentation
-See Figures IIb
-Oxygen absorption /desorption experiments

Fermentation Conditions: See Table 4.25A.

Time since air has been turned on (second)	C*	dC/dt **	dC/dt + Qo_2X **
0	2.99		
20	3.41	.0210	.0405
40	3.70	.0145	.0340
60	3.96	.0130	.0325
80	4.17	.0105	.0300
100	4.37	.0095	.0290
120	4.53	.0085	.0280
140	4.71	.0090	.0285
160	4.85	.0070	.0265
180	4.97	.0060	.0255
200	5.10		
220	5.17	.0035	.0230
240	5.24	.0035	.0230
260	5.29	.0025	.0220

Slope = $-1/K_1a$ = .0423 h

$K_1a = 23.6\ h^{-1}$

* Dissolved Oxygen (mg O_2/l)
** units: mg O_2/l/sec.

K_1a measurement during the growth of T. reesei QMY-1 on 1% CTMP1

-Sample Calculations for 11 h of fermentation
-See Figures IId
-Oxygen absorption /desorption experiments

Fermentation Conditions: See Table 4.25A.

Time since air has been turned on (second)	C*	dC/dt **	dC/dt + Qo_2X **
0	3.92		
20	4.02		
40	4.40	.0205	.0277
60	4.81	.0170	.0242
80	5.15	.0160	.0232
100	5.47	.0125	.0197
120	5.72	.0130	.0200
140	5.98	.0090	.0160
160	6.16	.0085	.0157
180	6.33	.0090	.0162
200	6.51	.0055	.0127
220	6.62	.0075	.0147
240	6.77	.0045	.0117
260	6.86	.0045	.0117
280	6.94	.0040	.0112
300	7.04	.0050	.0122
320	7.08	.0025	.0097
340	7.13	-	-
360	7.26	.0020	.0092
380	7.30		
400	7.37		

Slope = $-1/K_1a$ = .0432 h

$K_1a = 23.11\ h^{-1}$

* Dissolved Oxygen (mg O_2/l)
** units: mg O_2/l/sec.

Figure IIa. Oxygen Absorption/Desorption Experiments.
3.5 hour of Fermentation

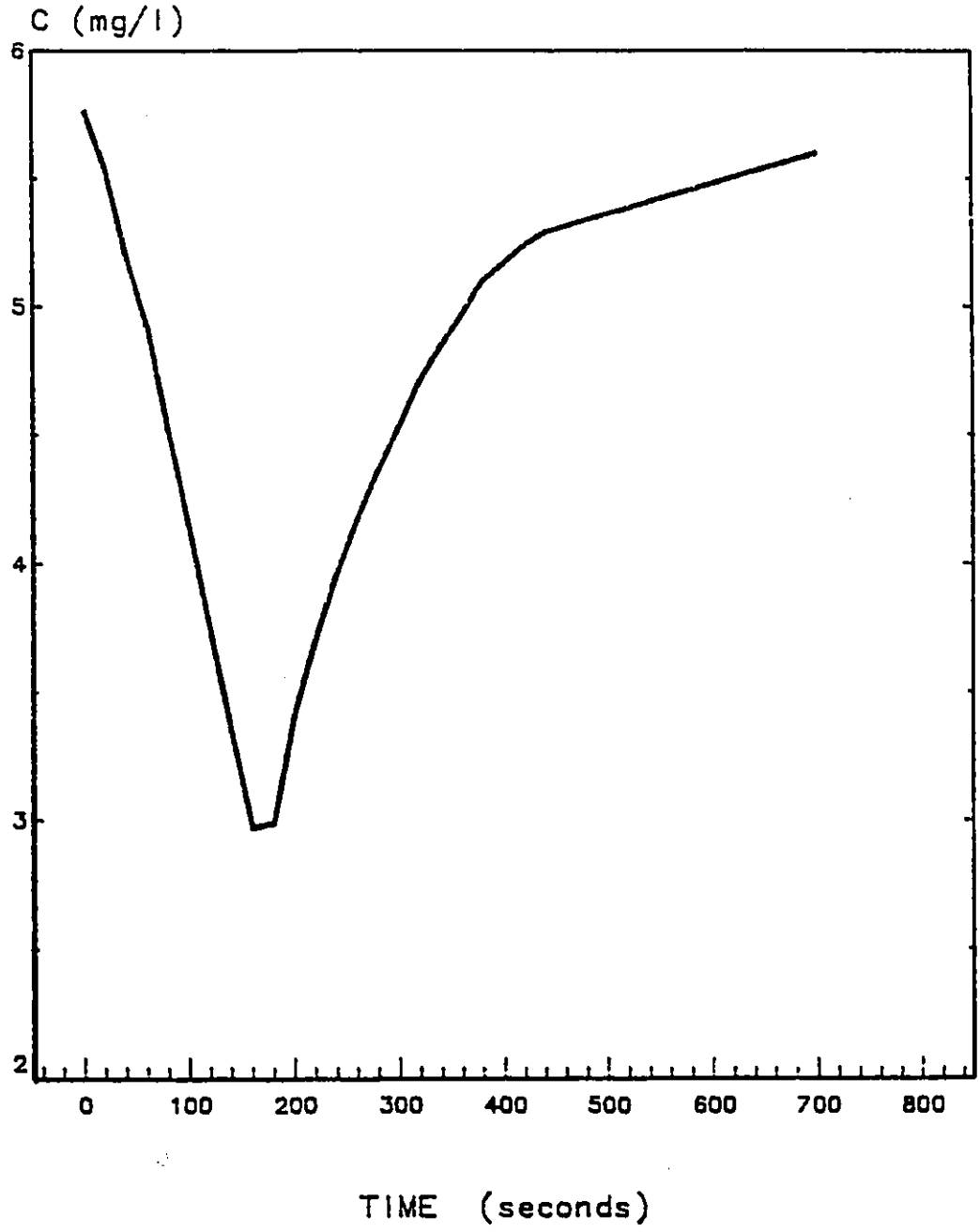


Figure IIb. Oxygen Absorption/Desorption Experiments.
3.5 hour of Fermentation
K_1a measurement.

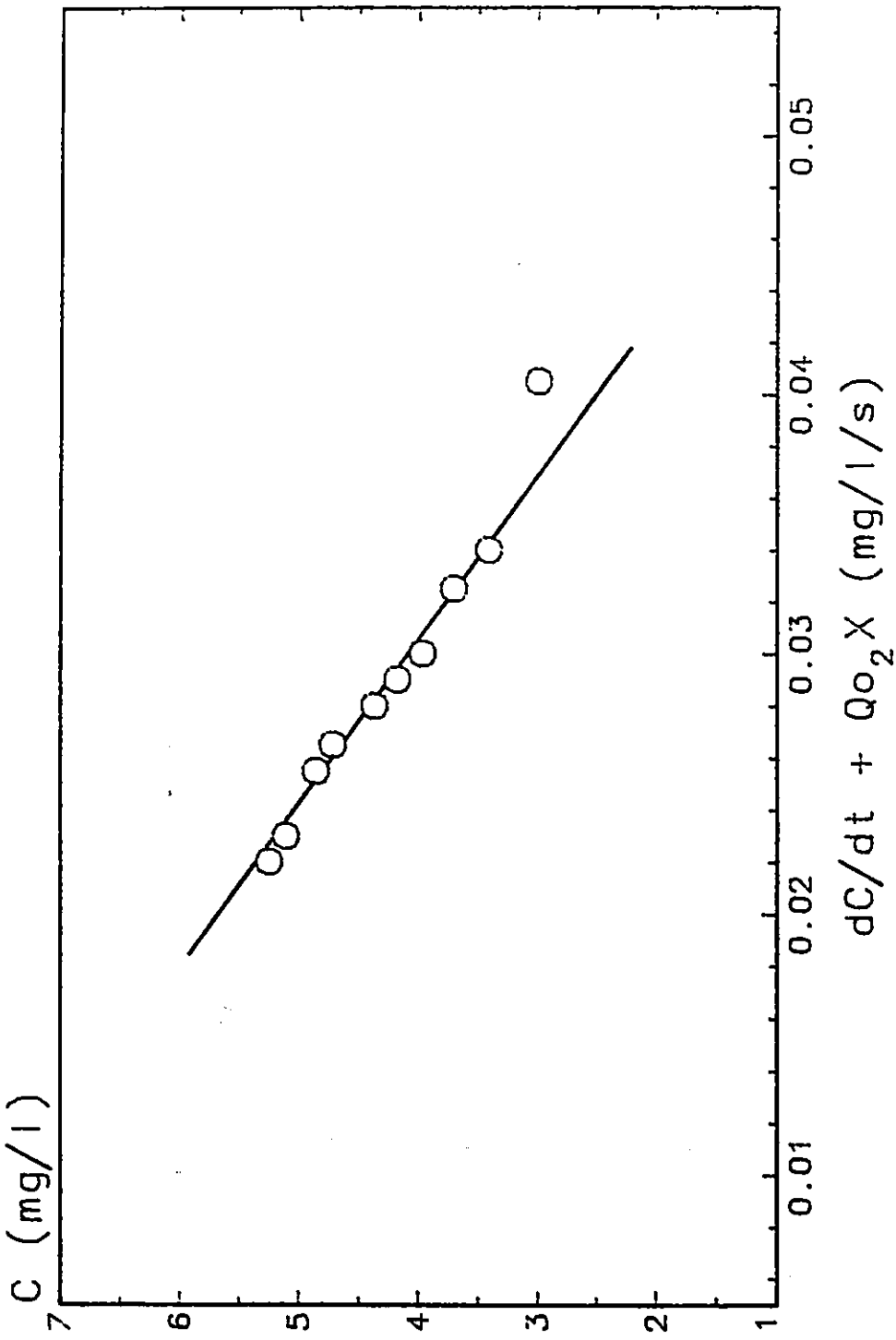


Figure IIc. Oxygen Absorption/Desorption Experiments.
11 hour of Fermentation

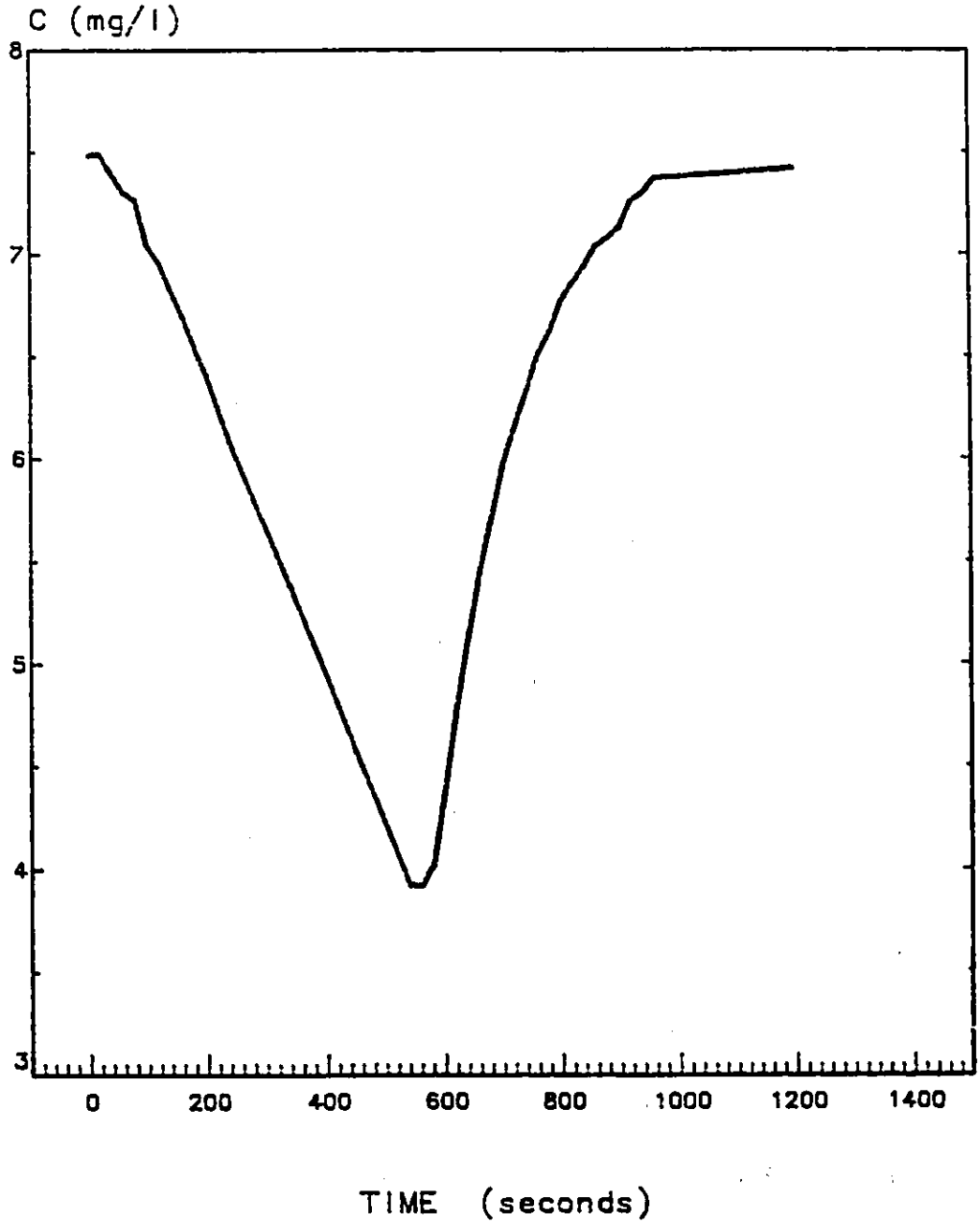
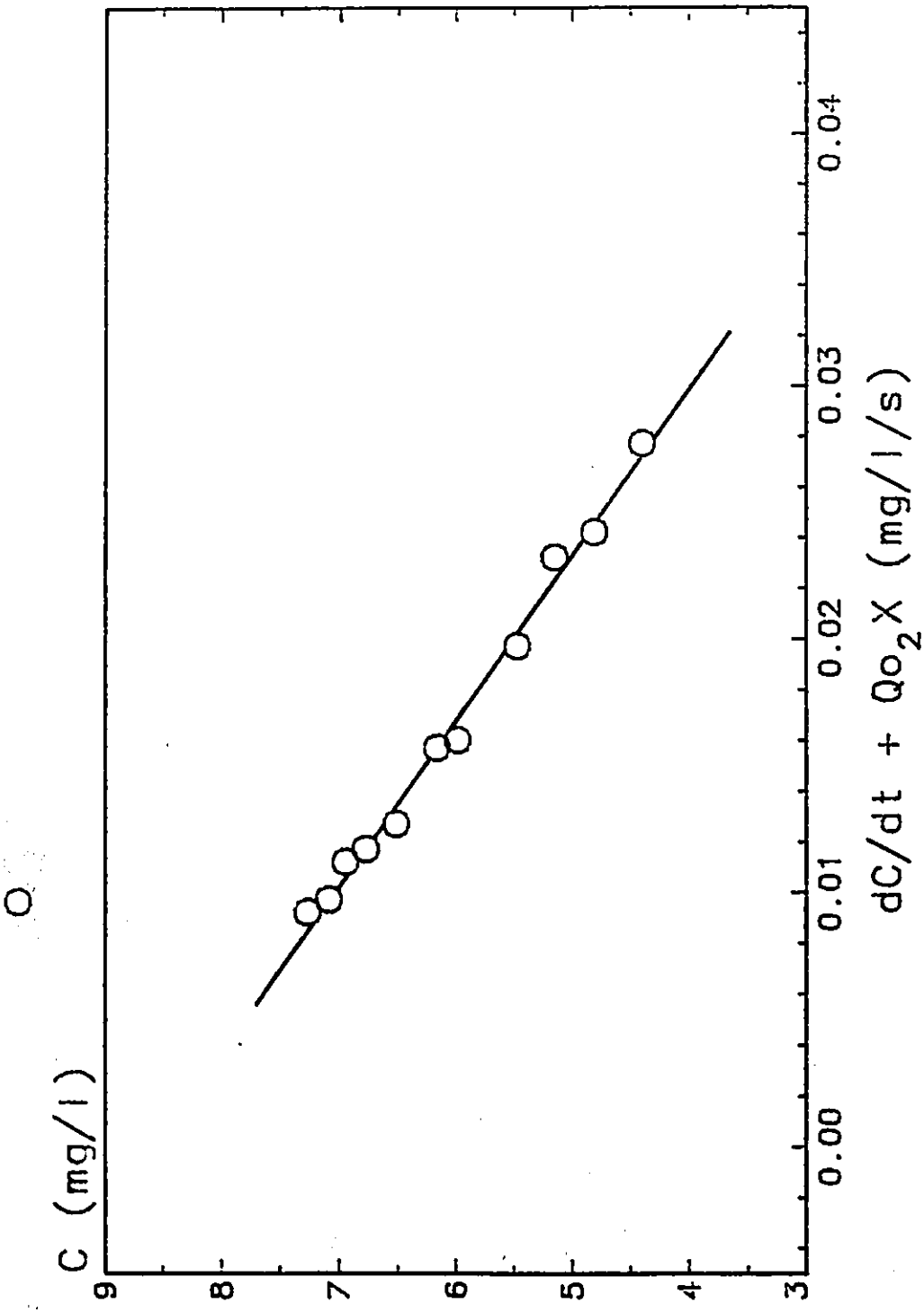


Figure IId. Oxygen Absorption/Desorption Experiments.
11 hour of Fermentation
K_1a measurement.



APPENDIX III

SAMPLE CALCULATIONS FOR DETERMINING CELLULASE YIELD:

For, Cellulase activity = 1.5 IU/ml

Initial CTMP concentration = 1.0 g/100 ml

Cellulose in CTMP = 60 %

Initial Cellulose conc. = 0.6 X 1 g/100 ml

= 0.6 g/ 100 ml

$$\text{Cellulase yield} = \frac{\text{Cellulase Activity}}{\text{Initial Cellulose Concentration}}$$

$$= \frac{1.5 \text{ IU/ml}}{0.6 \text{ g Cellulose/ 100 ml}}$$

$$= 250 \text{ IU / g Cellulose}$$

APPENDIX IV

TABLES

TABLE 4.1. CELLULASE PRODUCTION ON PURE CELLULOSE.

Substrate	1% Alpha Cellulose
Organism	Rut-C 30 QM9414 QMY-1
Mode	a) Shake flasks (Rut-C30, QM9414, and QMY-1) b) Fermenter
Temperature	30 °C
pH	6.0

Filter Paper Activity							
Shake Flasks						Fermenter	
Time h	SET A Rut-C30 IU/ml	Time h	SET B QM9414 IU/ml	Time h	SET C QMY-1 IU/ml	Time h	QMY-1 IU/ml
17.5	0.056	42	0.57	40	0.47	9	0.06
25	0.094	71	1.30	66	1.14	26	0.27
41	0.58	114	1.50	88	2.03	49	2.69
48.5	0.63	145	1.70	140	2.50	73	3.32
74.5	1.04	169	2.00	184	2.60	91	4.00
98	1.15	186	1.90	231	2.17	112	3.08
121.5	1.53						
168	2.10						
192	1.68						

TABLE 4.3. CELLULASE PRODUCTION ON PURE CELLULOSE WITH ADDITION OF HEMICELLULOSES AND LIGNIN EXTRACTED FROM WHEAT STRAW.

Substrate	1% Alpha Cellulose + Alkaline extract
Organism	QM9414 QMY-1
Mode	Shake flasks.
Temperature	30 °C
pH	6.0

Filter Paper Activity			
Time h	QM9414 IU/ml	Time h	QMY-1 IU/ml
42	0.08	40	0.31
71	0.09	66	0.84
114	0.20	88	2.13
145	1.20	140	3.24
169	2.3	184	3.40
186	2.6	231	2.55

TABLE 4.4. HYDROLYTIC POWER OF Rut-C30 AND QMY-1.

Substrate	5% Alpha cellulose (fiberous) in Citrate buffer, pH 4.8
Enzyme	a) Recovered from Rut-C 30 b) Recovered from QMY-1
Enzyme Units	20 IU of FPA / g substrate

Products of Hydrolysis by Enzyme of Rut-C 30		
Time h	Glucose g / l	Cellobiose g / l
4.3	4.71	0.50
12.0	8.26	0.97
48.0	14.27	1.81

Products of Hydrolysis by Enzyme of QMY-1		
Time h	Glucose g / l	Cellobiose g / l
6.3	5.69	0.30
23.5	10.40	0.74
50.0	14.35	0.94

TABLE 4.5. EFFECT OF SOAKING TIME ON SOLUBILIZATION OF WOOD AT ROOM TEMPERATURE (22 °C)

Soaking Time h	Solubilization %
1.5	8.0
6.0	10.0
24.0	11.5

TABLE 4.6. EFFECT OF HEAT TREATMENT ON SOLUBILIZATION OF WOOD FOR DIFFERENT SODIUM HYDROXIDE APPLICATION RATIOS.

% NaOH	% Solubilization	
(wt/wt of wood)	Not Autoclaved	Autoclaved
8	10	19
16	16	29

TABLE 4.7. EFFECT OF % NaOH (wt/wt of wood) ON SOLUBILIZATION OF WOOD FOR DIFFERENT WATER:WOOD RATIOS

% NaOH (wt/wt of wood)	Solubilization (%)		
	Set A Water:Wood 2:1	Set B Water:Wood 3:1	Set C Water:Wood 4:1
8	19	17	16
10	22	22	22
16	29	27	26
21	34	33	31
62	40	39	39
104	45	44	43

TABLE 4.8. SOLUBILIZATION (log%) vs % NaOH (wt/wt of wood), SET A from Table 4.7.

% NaOH (wt/wt of wood)	% Solubilization (log %)
8	1.28
10	1.34
16	1.46
21	1.53
62	1.60
104	1.65

TABLE 4.9. RATE AT WHICH SODIUM HYDROXIDE (N) IS REQUIRED PER 100 g WOOD FOR EVERY INCREASE IN PERCENT SOLUBILIZATION (S).

% Solubilization	dN/dS
20	0.3
25	1.0
35	2.0
37	2.7
40	4.8
41	6.0
42	14.0
43	40.0

TABLE 4.10. CELLULASE PRODUCTION ON 1% ASPEN.

Substrate	Experiment 1: Aspen as such no pretreatment. Experiment 2: Aspen treated with NaOH by autoclaving at 121 °C for 1 hour. Experiment 3: Aspen treated with NaOH by boiling at 100 °C for 3 hours.
Organism	QMY-1
Mode	Shake flasks
Temperature	30 °C
pH	6.0

Time h	Filter Paper Activity IU/ml		
	Exp 1	Exp 2	Exp 3
45.5	0.17	0.12	0.13
70.0	0.26	0.42	0.48
115.0	0.35	1.15	1.19
140.0	0.39	1.10	1.20
169.0	0.59	--	--
188.0	0.63	1.23	1.29

TABLE 4.11. CELLULASE PRODUCTION ON 1% PRETREATED ASPEN WOOD BY BOILING WITH 20% NaOH (wt/wt of wood) IN FERMENTER.

SUBSTRATE	Experiment 4: NaOH pretreated Aspen by Boiling for 3 hours Similar to Experiment 3, as in TABLE 4.11.
Organism	QMY-1
Mode	Fermenter
Temperature	30 °C
pH	6.0

Time h	FPA IU/ml
28	0.15
47	0.77
64.5	1.34
114	1.87
140	1.80

TABLE 4.12. CELLULASE PRODUCTION ON CTMP IN SHAKE FLASKS

Substrate	1% Chemithermomechanical pulp
Organism	QMY-1
Mode	Shake Flasks
Temperature	30 °C
pH	6.0

	Filter Paper Activity			
Time days	CTMP1 IU/ml	CTMP2 IU/ml	CTMP3 IU/ml	CTMP4 IU/ml
2	0.56	0.52	0.44	0.43
3	0.64	0.66	0.55	0.69
4	0.82	0.77	0.83	0.74
5	0.81	0.81	0.71	0.82
6	1.06	1.20	1.03	1.01
7	1.25	1.21	1.43	1.02
8	1.50	1.31	1.42	1.26
9	1.45	1.43	--	1.34

TABLE 4.13. CELLULASE PRODUCTION ON CTMP1 IN FERMENTER.

Substrate	1% CTMP1
Organism	QMY-1
Mode	Fermenter
Temperature	30 °C
pH	6.0

Time h	FPA IU / ml
23	0.09
35.5	0.34
52	0.86
79	1.30
95	1.64
120	1.70
144	1.43

TABLE 4.14. EFFECT OF SUBSTRATE CONCENTRATION ON CELLULASE PRODUCTION

Substrate	1%, 2% and 3% CTMP1
Organism	QMY-1
Mode	Shake Flasks
Temperature	30°C
pH	6.0

Time days	Filter Paper Activity (IU/ml)		
	1% CTMP1	2% CTMP1	3% CTMP1
1	0.14	0.05	
2	0.63	0.21	0.10
3	0.80	0.93	0.25
4	1.15	1.40	0.71
5	1.20	1.54	0.88
6	1.19	1.80	1.43
7	1.18	1.95	1.52
8	1.21	1.69	1.56
9	--	--	1.44

TABLE 4.15. CHANGE IN pH DURING FERMENTATION WITH *T. reesei*, QMY-1 IN 1% ALPHA-CELLULOSE.

Substrate	1% Alpha cellulose
Organism	QMY-1
Mode	Shake Flasks
Temperature	30°C

Time h	Change in pH value				
	pH 3	pH 4	pH 5	pH 6	pH 7
14	2.90	5.70	5.90	6.65	7.75
24	2.60	3.10	3.60	5.45	7.05
36	5.65	3.00	3.30	5.00	6.75
43	2.75	5.00	4.35	6.40	7.50
62	2.65	2.90	3.10	4.60	5.50
72	3.05	5.25	5.10	6.30	7.90
85	2.70	4.10	5.20	4.90	7.10
99	2.65	5.20	5.80	6.50	5.50
124	-	5.80	5.90	6.50	7.30

TABLE 4.16. CELLULASE PRODUCTION BY QMY-1 ON ALPHA-CELLULOSE AT DIFFERENT pH-LEVELS.

Substrate	1% Alpha cellulose
Organism	QMY-1
Mode	Shake Flasks
Temperature	30 °C

Time	Filter Paper Activity (IU/ml)				
h	pH 3	pH 4	pH 5	pH 6	pH 7
14	-	0.13	0.15	0.15	0.10
24	0.09	0.36	0.64	0.65	0.27
36	0.14	0.79	0.70	0.78	0.28
43	-	1.11	1.04	0.82	-
62	0.37	1.20	1.42	1.17	0.46
72	0.65	2.11	2.76	1.93	0.83
85	1.13	2.34	2.50	1.84	0.97
99	1.63	2.70	3.20	3.00	1.16
124	-	2.29	2.67	2.12	1.62

TABLE 4.17. CELLULASE PRODUCTION ON 1% ALPHA-CELLULOSE IN FERMENTER, AT pH 5.0

Substrate	1% Alpha cellulose
Organism	QMY-1
Mode	FERMENTER
Temperature	30 °C

Time (h)	Filter Paper Activity (IU/ml) Sampling Time Intervals		
	6 h	12 h	24 h
9	0.06	0.06	0.06
13	0.09		
19	0.20	0.20	
26	0.27		
34	0.57	0.57	0.57
40	1.89		
49	2.69	2.69	
58	3.14	3.14	3.14
66	3.06		
73	3.32	3.32	
84	3.85	3.85	3.85
92	4.00		
102	3.70	3.70	
112	3.10		3.10

TABLE 4.18. CHANGE IN pH DURING FERMENTATION WITH *T. reesei*, QMY-1 ON 1% CTMP1 AT DIFFERENT LEVELS OF pH, IN SHAKE FLASKS.

Substrate	1% Chemithermomechanical Pulp (CTMP1)
Organism	QMY-1
Mode	Shake Flasks
Temperature	30 °C

Time	Change in pH value				
h	pH 3	pH 4	pH 5	pH 6	pH 7
12	2.80	5.40	5.50	6.45	7.35
23	3.30	3.75	3.90	4.80	7.65
35	3.10	3.30	3.80	6.10	7.10
48	3.00	4.25	5.65	6.20	6.50
60	2.80	5.10	5.40	6.20	7.50
71	2.80	3.70	5.20	6.10	6.50
83	2.85	5.50	5.40	6.60	6.80
117	3.00	6.20	5.60	6.00	7.60
139	3.00	3.30	4.80	6.00	6.50

TABLE 4.19. CELLULASE PRODUCTION BY QMY-1 ON CTMP1 AT DIFFERENT pH-LEVELS.

Substrate	1% Chemithermomechanical Pulp (CTMP1)
Organism	QMY-1
Mode	Shake Flasks
Temperature	30°C

Time	Filter Paper Activity (IU/ml)				
h	pH 3	pH 4	pH 5	pH 6	pH 7
23	0.016	0.06	0.14	0.18	0.006
35	0.05	0.38	0.81	0.86	0.025
48	0.03	0.97	1.16	1.13	-
60	0.065	1.66	1.18	1.57	0.06
71	-	1.42	1.60	1.93	-
83	-	1.69	1.25	1.66	-
117	0.125	1.60	1.77	1.90	0.51
139	0.185	1.20	1.81	2.02	0.87

TABLE 4.20. CELLULASE PRODUCTION ON 1% CTMP1 IN FERMENTER, AT pH 6.0

Substrate	1% CTMP1
Organism	QMY-1
Mode	FERMENTER
Temperature	30 °C
pH	6.0

Time (h)	Filter Paper Activity (IU/ml)		
	Sampling Time Intervals		
	6 h	12 h	24 h
22	0.08	0.08	0.08
27	0.37		
35	0.65	0.65	
45	1.26	1.26	1.26
49	1.30		
52	1.51		
58	1.63	1.63	
69	1.84		
72	1.48	1.48	1.48
82	1.54	1.54	
91	1.60		
98	1.66	1.66	1.66
105	1.78		
115	1.89	1.89	1.89
129	2.30		
139	2.50	2.50	2.50
153	1.75	1.75	
162	1.63	1.63	1.63

TABLE 4.21. EFFECT OF TWEEN 80 ON CELLULASE, b-GLUCOSIDASE AND XYLANASE PRODUCTION.

Substrate	1% CTMP1, with 0.1% Tween 80. 1% CTMP1, without Tween 80
Organism	QMY-1
Mode	Shake Flasks
Temperature	30 °C

Time	FPA		β-Glucosidase		Xylanase	
	Tween	No Tween	Tween	No Tween	Tween	No Tween
h	IU/ml	IU/ml	IU/ml	IU/ml	IU/ml	IU/ml
24	0.11	0.16	-	-	16.0	20.5
48	0.55	0.57	0.040	0.045	59.5	53.5
60	0.67	0.67	0.090	0.075	62.5	71.5
72	1.02	1.09	0.135	0.130	59.5	70.0
84	1.01	1.12	0.170	0.180	70.0	58.0
96	0.80	1.10	0.155	0.175	72.5	63.0
120	1.24	1.38	0.245	0.245	74.0	69.0
144	1.44	1.44	0.230	0.250	80.5	80.5

TABLE 4.22. CELLULASE PRODUCTION ON 1% ALPHA-CELLULOSE BY QM9414, AT pH 5.0

Substrate	1% Alpha-Cellulose
Organism	QM9414
Mode	Shake Flasks
Temperature	30°C
pH	5.0

Time h	FPA (IU/ml)
21	0.04
25	0.27
30	0.40
35.5	0.78
44	0.83
49	0.99
54	1.22
59	1.48
68	1.27
76.5	1.57
92	1.85
99	1.80
116	1.75

TABLE 4.23. CELLULASE PRODUCTION ON 1% CTMP1 IN FERMENTER, AT pH 5.0

Substrate	1% CTMP1
Organism	QMY-1
Mode	FERMENTER
Temperature	30 °C
pH	5.0

Time h	FPA (IU/ml)
24	0.065
35	0.325
50	1.20
66	1.90
70	1.15
73	1.18
97	1.32
106	1.54
110	1.81
115	2.13
123	1.88
133	1.72

TABLE 4.24. EFFECT OF CHANGING pH DURING FERMENTATION

Substrate	1% CTMP1
Organism	QMY-1
Mode	Fermentation
Temperature	30 °C
pH	for 0 to 65 h = pH 6.0 for 65 to 140 h = pH 5.0

Time h	FPA (IU/ml)
17	0.063
23	0.148
35	0.534
41	0.97
48	1.24
59	1.79
65	1.39
72	1.20
83	1.58
89	2.12
97	2.25
108	2.44
121	2.19
140	2.04

TABLE 4.25 A. CELLULASE PRODUCTION BY QMY-1 ON CTMP1 RESIDUAL CELLULOSE AND PROTEIN.

Substrate	1% CTMP1
Organism	QMY-1
Mode	Fermenter
Temperature	30°C
pH	for 0 to 48 h = pH 6.0 for 48 to - h = pH 5.0

Time h	Residual Cellulose g/l	Protein g/l
0	5.91	0.218
3.5	-	0.225
8.0	5.88	0.273
11.0	5.85	0.328
14.25	5.77	0.388
19.5	5.85	0.481
23.5	5.01	0.506
27.0	5.09	0.562
31.25	4.45	0.585
36.5	3.74	0.624
43.75	3.19	0.609
48.0	3.23	0.562
52.75	3.10	0.602
56.75	2.97	0.567
61.5	2.50	-
69.8	2.36	0.675
74.75	2.00	-
77.5	1.82	0.661
85.75	1.25	0.750
94.0	0.99	0.788
101.0	0.97	0.775

TABLE 4.25 B. CELLULASE PRODUCTION BY QMY-1 ON CTMP1 FPA, b-GLUCOSIDASE, XYLANASE and COTTON-CELLULASE ACTIVITIES.

Conditions Same as in Table 4.25 A.

Time h	FPA IU/ml	β-Glucosidase IU/ml	Xylanase IU/ml	Cotton Cellulase U/ml
0	-	-	-	-
3.5	-	-	-	-
8.0	-	-	1.0	-
11.0	-	-	3.0	-
14.25	0.035	-	7.5	-
19.5	0.045	-	18.0	-
23.5	0.068	-	24.8	-
27.0	0.165	0.055	23.9	-
31.25	0.220	0.105	26.0	-
36.5	0.45	0.115	33.0	-
43.75	0.85	-	-	-
48.0	0.92	0.120	44.1	19.65
52.75	0.82	0.13	-	19.65
56.75	0.97	0.14	51.2	-
61.5	1.29	0.17	-	-
69.8	1.56	0.19	-	25.40
74.75	1.69	0.20	62.0	-
77.5	2.06	0.22	65.0	-
85.75	2.25	0.26	-	-
94.0	2.50	0.28	73.5	34.8
101.0	2.40	0.28	73.0	-

TABLE 4.25 C. CELLULASE PRODUCTION BY QMY-1 ON CTMP1 pH, RPM, Qo_2X, Qo_2, AND K_1a.

Conditions Same as in Table 4.25 A.

Time h	pH	rpm	Qo_2X $\frac{\text{m mole } O_2}{\text{l h}}$	Qo_2 $\frac{\text{m mole } O_2}{\text{g protein. h}}$	K_1a h^{-1}
0^-	-	-	-	-	19.0
0^+	6	200	-	-	23.0
3.5	6	300	2.19	9.75	23.6
8.0	"	"	1.01	3.69	26.0
11.0	"	"	0.81	2.48	23.1
14.25	"	200	1.07	2.60	30.0
19.5	"	"	1.27	2.64	26.0
23.5	"	"	1.43	2.82	28.5
27.0	"	"	1.76	3.13	18.5
31.25	"	"	1.73	2.96	18.8
36.5	"	"	1.77	2.83	25.0
43.75	"	"	1.91	3.14	22.5
48.0	"	"	1.89	3.37	17.8
48.8	5	200	2.13	3.61	19.6
49.25	"	150	1.96	2.44	20.0
52.75	"	"	1.44	2.40	22.6
56.75	"	"	1.29	2.28	-
61.5	"	"	1.11	-	-
69.8	"	"	0.96	1.42	18.0
74.75	"	"	-	-	16.8
77.5	"	"	0.96	1.45	-
85.75	"	"	0.97	1.29	21.8
94.0	"	"	0.90	1.14	20.0
101.0	"	"	-	-	-

TABLE 4.25 D. CELLULASE PRODUCTION BY QMY-1 ON CTMP1 ACID/BASE CONSUMPTION RATE.

Conditions Same as in Table 4.25 A.

Time h	Ticks / h	
	Acid	Base
6 - 8	4.0	-
8 - 10	2.5	-
10 - 13	1.0	-
13 - 18	-	-
18 - 24	-	1.50
24 - 30	-	0.67
30 - 36	-	0.67
36 - 43	-	0.10
48 - 48.8	pH shifted to 5 from 6	
48.8- 51	-	-
51 - 54	-	1.33
54 - 65	0.21	-
65 - 75	0.30	-
75 - 85	0.35	-
85 - 95	0.35	-

TABLE 4.25 E. CELLULASE PRODUCTION BY QMY-1 ON CTMP1 MONITORING DISSOLVED OXYGEN.

Conditions Same as in Table 4.25 A.

Time h	Dissolved Oxygen mg / l
0	6.4
1.0	6.2
2.75	4.4
3.75	5.8
8.0	7.0
11.0	7.4
14.25	7.4 -> 6.8
19.5	6.4
23.5	6.6 -> 5.8
27.0	6.0
31.25	5.6
34.0	6.2
37.0	6.4 -> 5.8
43.75	6.2
46.5	6.3 -> 5.8
48.0	6.2
52.75	5.5
56.75	5.8
62.0	6.0
70.0	6.0
75.0	6.0
86.0	6.0
94.0	6.0
101.0	6.0

TABLE 4.26. GROWTH OF T. reesei QMY-1 ON GLUCOSE IN FERMENTER.

Substrate	1% Glucose
Organism	QMY-1
Mode	Fermenter
Temperature	30 °C
pH	6.0

Time h	Protein (g/l)
0	0.172
5	0.200
8	0.269
12	0.380
21	0.698
24	0.718
33.5	0.753
36.5	0.907
44	0.994
56	0.941
62	0.981
72	1.009
76	1.023

TABLE 4.27. GROWTH OF T. reesei QMY-1 ON GLUCOSE IN SHAKE FLASKS.

Substrate	1% Glucose
Organism	QMY-1
Mode	Shake Flasks
Temperature	30 °C
pH	6.0

Time h	Protein (g/l)	Reducing Sugars (g/l)
0	0.324	10.60
4	0.363	10.20
6.5	0.556	9.45
9.30	0.818	8.50
11.25	0.944	6.30
13.75	1.190	4.33
17.20	1.47	3.000
23.50	1.53	0.255
25.75	1.68	0.220
28.50	1.73	-
30.75	1.94	0.220
37.33	1.96	0.195
65.50	1.69	0.150
75.25	1.68	0.110

TABLE 4.28. GROWTH OF QMY-1 ON CTMP1
Time vs. ln [Protein (g/l)].

Conditions Same as explained in Table 4.25A

TIME h	ln [Protein (g/l)]
0	-1.52326
3.5	-1.49166
8.0	-1.29828
11.0	-1.11474
14.25	-0.94675
19.5	-0.73189
23.5	-0.68122
27.0	-0.57625
31.25	-0.53614
36.5	-0.47160
43.75	-0.49594
48.0	-0.57625
52.75	-0.50750
56.75	-0.56740
69.8	-0.39304
77.5	-0.41400
85.75	-0.28768
94.0	-0.23826
101.0	-0.25489

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