BIOCONVERSION OF LIGNOCELLULOSIC SUBSTRATES
BY FUNGI

By

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The 1973 oil embargo and accompanying increase in oil prices led to worldwide interest in the development of alternative renewable fuels. The potential of global resources of lignocellulosic (LC) materials for conversion to fuel, feed and chemical has generated extensive efforts in developing indigenous technology in the past few decades. With an annual net production of 1.8x10^11 tons of biodegradable materials, 40% of which is cellulose, the LC reserves are by far larger in volume than any other carbohydrate source.

In the developing countries like Pakistan where economy is based on agriculture, large LC reserves are potential substrates for biotechnological implications. Our lands are blessed with the largest canal irrigation system and the Indus basin is a bowl, which can supply food to a greater part of this world. Due to the dearth of technology in the fields of agriculture biology our resources are not fully utilized. Apart from this third of the lands are badly stricken with the menace of salinity and water-logging. Added to this, the fuel resources on which economics of a country heavily resides, are bleak, especially for fuel oil. Gas resources are depleting every day. Taking these things into perspective, application of biotechnology in the area can play a vital role. Thus, it is imperative to evolve alternate indigenous technology. The marginal and salt affected lands can be utilized by evolving and growing such tolerant species which do not only ameliorate the soil, but also provide biomass of economic importance.
The LC material ought to be degraded into fermentable sugar for the yeast or bacteria to produce chemical solvents. But there are a few bottlenecks, which require the process development technology for an economic and energy efficient industrial operation. The LC material needs pretreatment (chemical or physical) for its effective degradation, which is otherwise the most stable biomolecule. The pretreatment renders the matrix components (lignin, cellulose and hemicellulose) to get loosened up and allow chemical or enzymatic treatment to be effective for conversion to monomeric sugars. The cost of chemicals and handling problems are immense and thus have paved way for enzymatic conversion. Nevertheless, the costs of enzymes takes 50-60% of total process. With the development of biotechnology employing fermentation processes like solid state fermentation and the vastly improved liquid fermentation or submerged culture and the advent of modern techniques like genetic engineering and protoplast fusion, the scope in this area has resurfaced. Gradually, information is being accumulated on the molecular biology of cellulose degrading organisms. There is ample possibility that with better understanding such microbial systems will be evolved which could make the process cost attractive as compared to the fossil fuels. The present work is based on similar lines and deals with the isolation of potent thermophilic cellulolytic microbes and the optimization of media components/environmental factors and the utilization of the enzymes produced for saccharification purpose. Apart from this, the effect of different pretreatments on LC substrates and their accessibility are also being reported.
ACKNOWLEDGEMENTS

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ABBREVIATIONS USED

LC       LIGNOCELLULOSE
LF       LIQUID FERMENTATION
SSF      SOLID STATE FERMENTATION
E & P    EGGINS AND PUGH
BMCA     BALL MILLED CELLULOSE AGAR
β        BETA
α        ALPHA
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SUMMARY

Potent thermophilic cellulolytic fungi were isolated from local habitats. These fungi, exhibited saccharifying ability showing clearance of cellulose medium in 5-6 days of incubation at 50°C. The isolates were namely: *Aspergillus fumigatus* (therm tolerant), *Chaetomium thermophile*, *Humincola grisea*, *Sporotrichum thermophile*, *Torula thermophila*, *Malbranchea pulchella* and *Mucor pusillus*. Rhizospheres of grasses like *Leptochloa fusca* (kaller grass) and *Cenchrus ciliaris*, were rich sources for the occurrence of these fungi.

Seven thermophilic and three mesophilic fungi were screened for cellulase/xylanase production. *A. fumigatus* produced great levels of 0.4, 2.4, 3.7 and 0.12 U/ml of FPase, CMCase, xylanase and β-xylosidase, respectively, when grown on 2% kaller grass. Glucosidase was produced to a higher extent (0.47 U/ml) by *thermophile*. *M. pulchella* and *M. pusillus* showed poor enzyme activities, whereas rest of the fungi showed moderate activity.

Among the mesophiles, *Chaetomium vergicophilum* exhibited better induction than *T. reesei RUT C-30* when grown on 2% kaller grass. The cellulase activity by the mesophiles was much lower than their thermophiles.

*S. thermophile* and *C. vergicophilum* because of their better overall enzyme activities were selected for further studies. Various lignocellulosic (LC) substrates were used for enzyme production in solid state fermentation (SSF) and liquid fermentation (LF). Quantitative cellulase production in general depended upon the particular inducer. In SSF, using *S. thermophile* great
level of avicelase, CMCase and xylanase induction took place when the substrate was rice straw, followed by *Sesbania aculeat* (dhancha) which induced β-glucosidase and β-xylosidase to greater extent. However, in case of *C. vergipehalum*, kollar grass induced more CMCase and xylanase, whereas dhancha was the substrate of choice for rest of the three enzymes. In LF wheat straw along with rice straw and kollar grass were the substrates of choice for *S. thermophile*, whereas dhancha showed better prospects instead of wheat straw for *C. vergipehalum*. Compared to SSF, the cellulase production, was substantially higher in case of LF. The production of β-glucosidase by *S. thermophile* was about 75 fold higher when the substrate was rice straw. It was found that *S. thermophile* produced higher cellulase titre in as compared to *C. vergipehalum*, whereas the latter produced marginally better enzyme titre than the former in SSF. *S. thermophile* was further selected for optimization studies in SSF a LF. In SSF, moisture (medium) level of 3:1 (v/w) favoured high cellulase production. Moreover, the mineral medium concentration when increased to 3-4 fold showed enhancement in cellulase activity.

In LF, vegetative inoculum size of 5-10% (v/v) grown for 72 hours was optimum for enzyme production in *S. thermophile*. ° medium pH set at 5.0-5.5 and a temperature of 45 °C was beneficial for higher yields. The fungus exhibited 4.6 and 8.3 times higher FPase and β-glucosidase activities when grown on LC substrates (untreated kollar grass) than on pure cellulose (α-cellulose). The order of induction by various substrates was; kollar gr (untreated) > kollar grass (treated with NaOH, 121 °C, 1h)
avicel > d-cellulose. Further studies enunciated that the fungus can tolerate high substrate concentration (10%). It produces maximal activities at 8% substrate (kellar grass) concentrations of 1.1 and 1.6 U/ml of FPase and β-glucosidase, whereas the extracellular protein and pH level were 3.1 mg/ml and 5.7, respectively after 9-11 days of incubation.

Medium used by Eggins and Pugh, E & P (1962), and Romanell et al (1975) induced higher cellulase and xylanase activity in S. thermophile. However, when each of the constituents in E & P medium was increased from zero concentration to four fold, there was no noticeable increase in the cellulase activity compared to the control. In fact, kellar grass (2%) resulted as a most suitable substrate with its high ash content fulfilling the mineral requirements of the media to quite an extent. Enhancement of nitrogen contents to two fold in the medium resulted in enhancement of cellulase activity. Various nitrogen sources tested alone and in combination, depicted the medium (E & P components of yeast extract, α-asparagine and (NH4)SO4) as most suitable for growth and enzyme production.

It was evaluated that ultrafiltration of crude enzyme from S. thermophile grown on 6% kellar grass, increased the FPase, β-glucosidase and extracellular protein activity by 4.3, 6.2 and 4.2 times, respectively, as compared to that of unconcentrated enzyme obtained from 2% kellar grass.

The characterization of cellulases revealed that pH level 5-5.5 was optimum for different enzymes. The optimum temperature for avicelase activity was 70°C, whereas CMCase, β-glucosida...
and xylanase showed maximum activity at 65 °C. The thermostabilit
of FPase at 60 and 70 °C was highest for Humicola grisea i.e., 8
and 60%, respectively, after 24 hours of incubation. Rest of the
thermophilic fungi were also found to retain about 70 and 45% of
FPase activity after incubation at above temperatures. Compare
to this, mesophilic strain of T. reesei Rut-30 showed 22 and 16
activity for FPase at 60 and 70 °C, whereas C. vergicepsal showed no activity.

Analysis of six untreated LC substrates showed that the
three salt tolerant plants had high ash (10-23%) and solub
sugar contents (9.6-12.9%). Out of the three pretreatments of
each substrate, NaOH (2%) with autoclaving (121 °C, 15 min
resulted in maximum enzymatic accessibility. It was followed
treatment with NaOH (2-3%) at room temperature, and steaming
high temperatures (200 °C). Among the different substrates the
straw and Panicum maximum resulted in maximum enzymatic accessi-
bility of 89 and 86%, respectively (based on pretreated fibre)
However, based on the raw materials, NaOH treatment at room
temperature was the method of choice. Mass balance studies show
that although harsher pretreatment conditions resulted in high
enzymatic accessibility because of greater delignification a
increase in polysaccharides along with structural modificatic
but at the expense of greater fibre losses. Steaming in gener
resulted in greater fibre recovery than NaOH with autoclaving in
poor delignification showed overall low hydrolysis yields. Bas
on the raw materials, wheat straw was enzymatically solubili-
up to 56, 53 and 31%, when the substrate was treated with 2% NaO
at room temperature. 2% NaOH along with autoclaving and steam.
at 190°C, respectively. Other substrates to follow the accessibility were in the order of Pannicium maximum > kallar grass bagasse > Atriplex amnicola > poplar.

Commercial cellulase preparation of Trichoderma reesei VT D-79125 with its FPase, supplemented with β-glucosidase from Aspergillus niger (Miles Kalli) in a ratio of 1:1.4 and at 16. 27 U/g substrate showed increase in saccharification of 2.5 a 10% kallar grass to 10.4, 6.3 and 6.0%, respectively. With 2 and 1.7 fold increase in these enzyme concentrations the overall yields attained were 67 and 60% from 5 and 10% kallar grass after 48 hours of hydrolysis. Compared to this enzyme filtrate obtained from 4% kallar grass by S. thermophile, yielded 60 to 33% reducing sugars. However, the estimated enzyme titre of FPase and β-glucosidase of 7.2:14 and 3.6:7.0 U/g of substrate respectively was substantially lower to that of supplemented reesei cellulase, for these substrate concentrations. This can attributed to better synergistic enzyme system as well as the stability of the cellulases of S. thermophile. The concentrate enzyme filtrate of S. thermophile after ultrafiltration, in a ratio of FPase : β-glucosidase of 24:80 U/g substrate, hydrolyses 5% kallar grass to 70% reducing sugars after 70 hours. However, maximum yields of 75% were attained by T. reesei, but only with the cellulase preparation was supplemented with β-glucosidase from A. niger in the ratio 33:54 U/gm of substrate. The contrast from S. thermophile (unconcentrated) and T. reesei with supplementation yielded 55 and 60% reducing sugars, respectively. The composition of sugars determined by HPLC showed high...
relative yield of glucose and oligosaccharides in the hydrolyzates from *S. thermophile*. The saccharification of filter paper was poor using enzyme filtrate of *S. thermophile*. However, because of potent β-glucosidase, the sugar composition showed almost 99% of glucose in the hydrolyzates. In order to elucidate the enzyme system from *S. thermophile* and *T. reesei*, freeze dried enzymes used at equivalent proportions of FPU/g of substrate resulted in only a slightly higher saccharification of 5% kallar grass by the latter preparation. However, the saccharification was much higher when the substrate was filter paper, in this case. HPLC of sugars from the hydrolysis of these substrates depicted higher relative glucose and oligosaccharides from kallar grass by *S. thermophile*, whereas higher xylose content resulted from *T. reesei*. The hydrolyzates from filter paper depicted apart from glucose and a small amount of oligosaccharide component, substantial amount of cellobiose in the hydrolyzate from the latter enzyme preparation. No cellobiose was formed by the *S. thermophile* preparation. The significance of these findings lies in the fact that in a fixed proportions of FPU's used, *T. reesei* enzyme system contains a strong endo-glucanase along with xylanase, whereas a greater level of β-glucosidase is present in *S. thermophile*. The optimum FPU/g of substrate yielding maximum saccharification results were 30 and 35 for *S. thermophile* and *T. reesei* enzyme sources.

Saccharifying ability of thermophilic fungi was evaluated by using culture filtrate obtained from 2% kallar grass. Among the thermophiles highest reducing sugar yield was obtained from *Chaetomium thermophile* (69.2%) from 5% kallar grass. All the te
fungi showed considerable saccharification rate up to 40 hours with the dilute enzymes. Freeze dried enzymes from T. reesei (mesophile) showed the maximum yield at 75% but at much higher enzyme concentrations. The HPLC elucidated the enzyme system of these fungi. Glucose sugar was obtained at higher concentration from C. thermophile > T. reesei > S. thermophile > H. grisea > T thermophila > A. fumigatus > M. pulchella. The potential of these fungi to carry hydrolysis at 60°C in relation to their thermostable enzyme system was studied. Enzymes from C. thermophile exhibited 58% of saccharification up to 40 hours, whereas rest of the fungal enzymes did not show any noteworthy increase after 2 hours of incubation. C. thermophile showed 1.4 times faster saccharification rate but could not increase the overall yield. In contrast T. reesei enzymes resulted in inactivation after 1 hour and depicted lowering in sugar yield of 46.5% reducing sugars, compared to that at 50°C. Whereas, C. thermophile showed an overall decline in yield of 11.2%, only.

The results have shown great merits for the microbial conversion of waste land plants into sugars, which can be fermented to various products. Use of thermophilic fungi for cellulase production and biomass conversion has generated interests in further elucidation of their behaviour and genetic structure.
INTRODUCTION

Lignocellulosic (LC) biomass reserve is the most abundant organic material (1.8x10^12 tons/year) available on this terrestrial globe, fixed through photosynthesis. This complex polymer material is composed of varying levels of cellulose (40-60%) hemicellulose (20-40%) and lignin (15-25%) in different plant (Spano et al, 1979).

The ever-increasing shortage of food and the onset of oil crisis in the 1970's brought with it the realization of the utilization of LC's as raw material for food, feed and fuel purposes. In order to utilize cellulosic substrates as feed stock its degradation to glucose, chemically or enzymatically is essential. However, because of their recalcitrant nature, LC's are the most stable biomolecules. Accordingly, their nature is more structural than functional. It is estimated that theoretically about 77 Kcal of incident radiation energy is stored in each gram of cellulose (Chose, 1977). Unfortunately majority of the processes utilizing cellulose, extract only a small portion of this energy due to their high energy consumption. Thus, only a small fraction of cellulosic resources are currently utilized. Some of the products manufactured are lumber, fuel, textiles, paper, plastics, films, foils, explosives, varnishes, thickeners and glue (Cassey, 1980). The possibility of using LC materials as inexpensive feed stock for the production of bulk chemicals such as ethanol and acetone are attractive but to date these have remained unrealised goals. Recently much of the research work has been aimed at enzymatic hydrolysis of cellulose to glucose pr:
to its conversion to other materials (Moo Young et al., 1987). The crystalline cellulosic substrates are pretreated prior to enzymatic attack in order to increase their accessibility. Despite problem areas, interest has been shown in the production of microbial cellulases. Fungal cellulases, because of their extracellular enzyme systems have received greater attention than bacterial cellulases. Nevertheless, the bacterial systems have the advantage of possessing fast growth rates. Mutagenesis and genetic engineering of microbes have surged as new hopes for possibilities of breaking the cost barriers for cheaper biomass conversion processes into energy.

THE CHEMISTRY AND STRUCTURAL MORPHOLOGY OF LC MATERIAL

The polymeric cellulose is composed of crystalline and amorphous regions. Cellulose is a linear homopolymer of hydroglucose units linked by β-(1,4)-glucosidic bonds (Fig. 1). These glucosidic bonds along with the hydroxyl groups are mainly responsible for its chemical properties.

![Cellulose](image)

**Fig. 1** The conformational form of cellulose.

The cellulosic molecules are joined to hemicellulose lignin by hydrogen and covalent bonds with incrustation of some of the components. The average degree of polymerisation (DP) of cellulose ranges between 1000-10,000 glucose units (Callihan Clemer, 1979). The cellulose molecule is more resistant
hydrolysis because of the secondary and tertiary configuratic
of its chain and its close association with other protective
polymeric structures such as lignin, starch, pectin, hemicelluloses, proteins and mineral elements present within the plant cell wall.

The hemicelluloses are heteropolymers of galactose, mannose,
xylene, arabinose and other sugars and their uronic acids which
form ester bonds with phenol groups of lignin. The chain lengths in hemicelluloses are short and seldom exceeds (DP) 200 of corresponding pentoses. Its role is to provide linkage between lignin and cellulose.

The lignin is another heteropolymer of phenyl propane units (trans-p-coumaryl alcohol, trans-p-coniferyl alcohol, and trans-sinapyl alcohol (Sarkanen et al, 1971; Adler, 1979). Lignin gives the structural rigidity by its cementing nature, holding the fibres of polysaccharides together.

The cell morphology shows (Fig. 2) multilayered cell walls. Each lamella (M), which is heavily lignified surrounding the cells. The primary wall (P) is relatively thin consisting of three layers of cellulose elementary fibrils. The next three layers consisting of the transition lamella (S1), secondary wall (S2) and inner secondary wall (S3) contain the majority of cellulose and are 1-10 μm in thickness. The lumen (L) contains intracellular material (Kosaric, 1984).

Individual cellulose molecules are linked together to form an elementary fibril or protofibril about 40 Å wide, 30 Å thick and 100 Å long. These polymeric chains are aligned in parallel
Fig. 2: Typical plant cell wall architecture. L, lumen; M, middle lamella; P, primary wall; S1, transition lamella; S2, secondary wall; S3, inner secondary wall. Source: Tsao (1978).

and are bound firmly together by numerous hydrogen bonds. The number of these elementary fibrils are aggregated to form microfibrils. Infact, different schematic models have been drawn to elucidate the structure of microfibrils (Ranby, 1969; Chang 1971; Fengel, 1970 and 1971; Cowling 1975). The molecular structure of cellulose and the structure of an elementary fibril and microfibril are important features, considering the hydrolytic degradation of cellulose. As postulated by Fengel (1970 and 1971) the elementary fibrils are cemented together by hemicelluloses (polyoses) to form microfibrils. This microfibril is surrounded by lignin and polyose layer. Pretreatments of cellulosic are aimed at degrading this protective layer so that rapid enzymatic hydrolysis could take place.
PRETREATMENTS

Structure of LC in the cell wall resembles that of reinforced concrete pillar with cellulose fibres being the metal rod and lignin the matrix cement. Biodegradation of native LC is very slow and yields not more than 20% of reducing sugars. To increase the susceptibility of cellulosic materials structural modifications, by means of various schemes is essentially based on the economics of the process. These pretreatments are broadly classified as physical, chemical and biological treatments according to their principle mode of action on the substrate. (Table 1) Some processes utilize a combination of one or more techniques applied in parallel and/or in sequence. Of the many pretreatments, some have been demonstrated to be effective in disrupting lignin-carbohydrate complex and others in disrupting highly crystalline cellulose itself.

Table 1: Methods used for pretreatment of lignocellulosic

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<td>Ball-milling</td>
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<td>Two-roll milling</td>
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<td>Pyrolysis</td>
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<td>Benzene-ethanol extraction</td>
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<td>Butanol-water extraction</td>
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CELLULOLYTIC ENZYMES

There are three major groups of cellulases: endo-β-1,4-glucanases, exo-β-1,4-glucanases, and β-1,4-glucosidases. The cellulase complex is found to contain three basic component which may be present in multiple forms, often as isoenzyme (Bisaria and Ghose, 1981). The composition of cellulase complexes in terms of relative content of individual components varies greatly from organism to organism. Similarly two major groups of hemicellulases are endo-β-1,4-xylanases and β-1,4-xylosidases. These groups of enzymes are classified as described under:

Endo-β-1,4-glucanases (EC 3.2.1.4)/CMC-ase:

This group consists of several components varying in the degree of randomness. The substrates for endo-glucanases are C and other soluble cellulose derivatives, Walseth cellulose a cellulodextrins. They are postulated to cleave β-(1,4)-glucosid linkage in native cellulose to smaller units. Purified endoglucanase has no activity on crystalline cellulose. It shows inhibition towards cellobiose above 0.01% and methyl cellulose.

Exo-β-1,4-glucanases (EC 3.2.1.24)/avicelase:

These enzymes consist of specifically two groups a) (1,4)-glucanase glucanohydrolase which removes glucose units from the non-reducing end of the chain. b) β-(1,4)-glucan cellobiohydrolase (EC 3.2.1.91) which removes cellobiose units from the non-reducing end of the chain. Cellulose with a low polymerisation like avicel or phosphoric acid swollen cellulose are used preferentially as substrates. They also show synergistic activity along with other cellulases. They have the ability to extensively...
hydrolyse less ordered substrates. Its main inhibitor is cellobiose.

\( \beta-1,4\text{-glucosidase} \ (EC\ 3.2.1.21)/\text{cellobiase}: \)

It hydrolyses the cellobiose and short chain oligosaccharides to glucose. The substrates for \( \beta\text{-D-glucosidase} \) is cellobiose. \( \beta\)-linked glucose dimers and cellodextrins and for aryl-\( \beta \) glucoside they are salicin and p-nitrophenyl \( \beta \)-glucosidase. has no action on cellulose. The dimers with B-glucosidase bond are (1,2 - 1,3 - 1,4 and 1,6) type and the product is glucos. Cellobiose is the substrate of choice, since aryl-\( \beta \)-glucosidase exists which does not hydrolyse cellobiose. \( \beta \)-glucosidase activity is determined following release of p-nitrophenol. Cellula systems lacking \( \beta \)-glucosidase or having low levels of this enzy have poor saccharification power and are prone to end produ inhibition.

The purified proteins of these enzymes show a high degree variation. Their molecular weights commonly range from 12,000 80,000 with \( \beta \)-glucosidase generally being the larger molecule. The enzymes may or may not be associated with the carbohydrate moities and are usually rich in acidic amino acid groups (Kar et al, 1980).

Cellulases are inducible enzymes and are produced in presence of cellulose, cellobiose, lactose, sophorose, or ot carbohydrate which contain \( \beta-1,4 \) linkages (Gritzali and Br. 1979). Moreover, cellulases are highly regulated by the product inhibition. Denaturation of cellulases occurs shearing, especially at air liquid interfaces (Kim et al. 19 which can be minimised by the addition of surfactants.
Hemicellulolytic/Xylanolytic Enzymes

There are two major groups of hemicellulases (xylanases) endo-\(\beta\)-1,4 xylan xylanohydroases and \(\beta\)-1,4-xylosidases. 
Endo-\(\beta\)-1,4 xylanase (3.2.1.8)/xylanase

It hydrolyses the short chained cellulosic and hemicellulosic substrate into sugars. In fact, xylanases act in a synergistic manner with endo-glucanase to rip open the cellulose porosity to about 20% higher level than when the latter are used only. Hydrolysis of pure cellulose is not affected by xylanase. The substrate mostly used for its assay is larchwood or beechwood xylan with a chain length of about 200.

\(\beta\)-1,4-xylosidase (3.2.1.37)/xylosidase

Like the \(\beta\)-glucosidase, it hydrolyses the oligomers (xylbiose, xylotriose, xylotetraose etc.) to xylose. The common used substrate is p-nitrophenyl \(\beta\)-xyloside.

ENZYMATIC HYDROLYSIS

Difficulties inherent in acid hydrolysis of cellulose have paved the way for enzymatic hydrolysis as the most viable process commercially for ethanol production. Enzymatic hydrolysis of cellulose accomplishes degradation of cellulose to glucose. The rate of the reaction is dependent upon the structural features of cellulose and mode of enzyme action. The hydrolysis of native untreated cellulose is at an extremely low rate, and thus needs a suitable pretreatment. Moreover, the use of a complex cellulase system which could completely hydrolyse the cellulosic substrates is most desirable. Fan et al (1980) reviewed the structural features of lignocellulosic materials which govern their susceptibility to enzymatic degradation. These include:
moisture content of the fiber, the size and diffusivity of th
cellulolytic enzymes and other reagent molecules to size an
surface properties of grown capillaries etc., degree of crystal
linity of cellulose, unit cell dimensions of cellulose, conforma
tion and steric rigidity of anhydroglucose units, degree c
polymerisation of the cellulose, nature of the substances t
which cellulose is attached and nature, concentrations and dis
tribution of substituted groups.

MECHANISM OF ENZYMATIC HYDROLYSIS

Specific theories on the mechanism of enzymatic hydrolys
have arisen since the original concept by Reese, (1950). Various
other workers in this regard include (Eriksson, 1969; Rees
1977; Wood, 1980). A number of review articles demonstrate t
mechanism/mode of cellulase attack on crystalline cellulono
(Ghose and Ghosh, 1978; Bisaria and Ghose, 1981; Chang et al

The mechanism involves the initial attack of endo-glucanases
on the glycosidic linkages to form short chains (Fig. 3). This
followed by synergistic attack of endo and exo-glucanases yie
ding oligosaccharides, cellobiose and glucose. This leaves t
B-glucosidase/cellobiase to convert them into monomeric glucose.

In a kinetic analysis, Rabinovich et al, (1979) showed t
the rate of glucose formation from cellobiose during CMC hydrol
sis could not account for the total glucose. In fact, it was e
ffect of exoglucosidase which was also rate limiting.

Considering the hydrolysis of LC as a whole, the hemicel
lases act synergistically along with the cellulases. The cre
ased assessibility because of the initial attack by xylan
Fig. 2. Mechanism of cellulases in a symmetrical hydrolysis of cellulose.

**Fig. 2**

- (DP = 1) Glucose
  - Mosty soluble
- (DP = 2) Cellobiose
  - Endocellulase
  - Exocellulase
  - Mostly soluble
- (DP = 5) Cellotriose
  - Endocellulase
  - Exocellulase
  - Mostly soluble
- (DP = 6) Cellotetraose
  - Endocellulase
  - Exocellulase
  - Mostly soluble

**Enzymatic Hydrolysis of Cellulose**
allows endo and exo-glucanase to be highly effective (Ghose and Bisaria, 1979). The substrates after suitable pretreatment become more susceptible to enzymatic accessibility and a complete cellulase/hemicellulase system can fully hydrolyse this substrate.

LIGNOCELLULOSIC RESERVE

Present supply of LC’s is in the form of agricultural, domestic and industrial reserves.

Agriculturally Derived Material

Crop and logging residues are produced in great abundance or global scale. This material does not have a zero value or possibly negative net value, because of collection costs, typically. $33-50/t in U.S.A. (Vervallin, 1980). Special cases exist where the residues are collected as part of harvesting but they are already put to valuable use; viz. sugar cane bagasse, wood chips and saw dust are used for process fuel or as fibre board components. Apart from this, global problems such as erosion (Gupta et al, 1979) use of economic fertilizers, pesticides and fuel for mechanization have increased the net costs. For increased profitability, growing plants, specifically for energy requires increased yield and lower costs per unit area in terms of both cash and energy inputs. Marginal lands, where agricultural productivity is not possible, can be utilized by growing adaptable varieties such as marine grass Spartina (Coombs, 1984) and river salt bush Atriplex amnicola. Sandhu and Malik (1975 have reported Leptochloa fusca (kellar grass) to grow profusely on highly saline sodic soils which covers about one third of land in Pakistan. Kellar grass along with other salt tolerant plant have been widely explored in these laboratories (Malik et al
1980, 1981, 1982). The authors elucidated in kellar grass, the presence of nitrogen fixation and C-4 photosynthetic pathways. This grass has shown the potential to ameliorate saline soil without the addition of fertilisers. Kollar grass can be harvested thrice in a year at 40 tons/ha/year (Malik, 1980). It has been shown to contain about 85% total carbohydrate with 55 polysaccharide contents which can be increased to 77% (based on pretreated fibre) after 2% NaOH pretreatment (Latif et al., 1988). This material has shown great potential for biotechnological applications (Malik et al., 1986). Our country has to pay about 50% of the foreign exchange for meeting demands for energy. Based on agricultural economy, there are lots of recyclable residues in Pakistan obtained from wheat, rice, sugar cane, sugar beet and cotton. These crops have yielded wheat straw, rice straw, sugar cane bagasse, sugar beet pulp up to a level of about 15.0, 8.0, 31.0 and 1.0 million tons, respectively, while the cotton seed production was 2.0 million tons in 1984-85 (Statistical Bulletin 1986). Apart from banana waste or reject, beet pulp, corn cobs are also some of the important wastes. National forest reserves produced 15.5 mil. cu. feet of timber and 16.5 mil. cu. feet of firewood in the year 1989-90 (Akhtar and Walters, 1990).

Domestically Derived Residues

In developed countries, municipal refuse is the greatest source of cellulosic wastes. Daily production of city municipal waste, cellulosic in character, are of the order of 28×10^6 in the US (Ghose and Ghosh, 1978). The urban waste generated in the cities contain about 45% cellulose. The constituents of the wastes (paper, plastic, polythene, metal, glass and garbage) a
recyclable. Cellulosic wastes can be generated from municipal garbage by air floatation. Due to its highly processed state, this cellulose component is easily hydrolysed.

Industrially Derived Effluents

Industrial effluents contain a high quantity of cellulosic material. The main industries are pulp and paper and food processing. The cellulose content from waste sludges varies from 25-45 (w/w) after primary clarification depending upon wood species pulping method, and bleaching agents (Pamment et al., 1979). The Kraft and sulphate process delignifies the material and cause severe disruption of cellulosics. Thus, the requirement of pretreatment is left to milling or even totally nullified (Andren and Nystrom, 1976). This waste material may be disadvantageous for ethanol fermentation due to the presence of inorganic matter which may influence fermentation kinetics (Pamment et al., 1979).

Characteristics of food industries make the large scale utilization of its wastes difficult because of seasonal production and high variability in composition and characteristics of both solid and liquid waste streams (Carroad and Wilke, 1978).

In Pakistan, the pulp, paper and food industries are not well developed but there is large potential for them since our economy is based on agriculture.

Considering the biomass situation in our country in perspective, there is a great potential of these residues. Apart from these resources, amelioration of salt affected lands growing salt tolerant plants have already shown to yield hu
biomass which can find access to biotechnological requirements.

The objectives of the present study are focused on bioutilization of LC substrates including biomass raised on saline soils. The objectives were as follows:

Isolation of potent thermophilic cellulolytic fungi. Use of existing mesophilic strains along with the thermophilic isolate for cellulase/xylanase production.

Production of cellulase/xylanase by fungi when grown on different lignocellulosic (LC) substrates in solid state fermentation (SSF) and liquid fermentation (LF).

Optimisation of culture conditions and enzymo parameters in SSF and LF.

Pretreatment of different LC substrates for accessibility to the enzymes.

Hydrolysis of lignocellulose (kallar grass).
REVIEW OF LITERATURE

MICROBIAL CELLULASES

Bisaria and Ghose (1981) listed 13 species of fungi and a few bacteria, capable of producing cellulases which can extensively break down insoluble cellulose in-vitro. The fungal species included *Trichoderma reesei*, *T. viride*, *T. koningii*, *T. lignorum*, *Penicillium funiculosum*, *Aspergillus wentii*, *Sclerotium rolfsi*, *Sporotrichum pulverulentum* and *Fusarium Solani*. While the bacterial species included *Cellulomonas* along with *Clostridium thermocellum*. The present review has been limited to fungal cellulases because of the nature of work being conducted.

Cellulolytic Mutants

*T. reesei* (QM6a) isolated from deteriorated cartridge be in New Guinea during World War II, was detected as a potent cellulolytic organism by Elwyn Reese, around 1950. Numerous mutants have been developed from it since then at Natick (Mandell et al., 1975; Andreotti et al., 1978; Mandel and Andreotti, 1977; Galo and Andreotti, 1979; Ryu and Mandel, 1980; Andreotti et al., 1980) and at Rutgers (Gallo, 1978, 1982; Montenecourt et al., 1981). These mutants produced higher levels of constitutive as well as inducible cellulases but showed catabolite repression which was later reduced for some of the mutants (Casebier et al., 1969; Sudo et al., 1976; Du Toit et al., 1984). Quantitative comparison among the mutants is difficult because fermentation conditions are not the same for all the strains. Depending upon conditions, best Natick and Rutgers strains produced 3-20 fold higher cellulase yield (Table 1) than the wild type QM6a.
<table>
<thead>
<tr>
<th>Process</th>
<th>$D$ (h$^{-1}$)</th>
<th>Productivity (IU L$^{-1}$ h$^{-1}$)</th>
<th>Concentration (IU mL$^{-1}$)</th>
<th>Strain</th>
<th>Particulars</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>11 (162 h)$^3$</td>
<td>1.85 (162 h)</td>
<td></td>
<td>1-2% cellulose</td>
<td>ANDREOTTI et al.</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>57 (192 h)</td>
<td>11 (192 h)</td>
<td>D1-6</td>
<td>6% cellulose</td>
<td>GHOULI et al.</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>42 (92 h)</td>
<td>3.9 (92 h)</td>
<td>MCG-77</td>
<td>2% cellulose</td>
<td>GALLO et al. (1983)</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>36 (67 h)</td>
<td>3.6 (164 h)</td>
<td>NG-14</td>
<td>2% cellulose</td>
<td>GALLO et al. (1983)</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>36 (163 h)</td>
<td>—</td>
<td>QM9414</td>
<td>2% cellulose</td>
<td>GALLO et al. (1983)</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>88 (120 h)</td>
<td>13.5 (120 h)</td>
<td>MCG-77</td>
<td>6% cellulose</td>
<td>GALLO (1982)</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>95 (120 h)</td>
<td>13.3 (120 h)</td>
<td>RUT-C30</td>
<td>6% cellulose, bi-level pH control/biotin supp.</td>
<td>GALLO (1982)</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>142 (96 h)</td>
<td>17.2 (96 h)</td>
<td>MCG-80</td>
<td>3% cellulose</td>
<td>GALLO (1982)</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>25 (198 h)</td>
<td>4.5 (80 h)</td>
<td>QM9414</td>
<td>3% cellulose</td>
<td>MUKhopadhyya (1980)</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>38.75</td>
<td>6.2</td>
<td>QM9414</td>
<td>3% cellulose, pH cycling</td>
<td>MUKhopadhyya (1980)</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>44</td>
<td>7.2</td>
<td>QM9414</td>
<td>3% cellulose, pH and temp. cycling</td>
<td>MUKhopadhyya (1980)</td>
</tr>
<tr>
<td>Continuous (1 stage)</td>
<td>0.03-0.08</td>
<td>5-13</td>
<td>0.33</td>
<td>—</td>
<td>0.4% - 1.1% cellulose feed</td>
<td>PETERSEN (197)</td>
</tr>
<tr>
<td></td>
<td>0.03-0.08</td>
<td>8.75</td>
<td>—</td>
<td>—</td>
<td></td>
<td>MANDELS et al. (1975)</td>
</tr>
<tr>
<td></td>
<td>0.03-0.08</td>
<td>21.2</td>
<td>—</td>
<td>—</td>
<td></td>
<td>SAKAI et al. (1971)</td>
</tr>
<tr>
<td>Continuous (2 stage)</td>
<td>0.015</td>
<td>2.6</td>
<td>0.12</td>
<td>—</td>
<td>1% glucose</td>
<td>MITRA et al. (1971)</td>
</tr>
<tr>
<td></td>
<td>0.015</td>
<td>1.2</td>
<td>0.08</td>
<td>—</td>
<td>1% glucose (1st stage) cellulose (2nd stage)</td>
<td>BROWN et al. (1978)</td>
</tr>
<tr>
<td></td>
<td>0.02</td>
<td>16</td>
<td>0.80</td>
<td>—</td>
<td>1% tophorase (1st stage) cellulose (2nd stage)</td>
<td>WILKE et al. (1978)</td>
</tr>
<tr>
<td></td>
<td>0.026-0.028</td>
<td>90</td>
<td>—</td>
<td>NCG-77</td>
<td>1% cellulose, feed semi-continuous 2 stage lactose induced</td>
<td>RYU et al. (1978)</td>
</tr>
<tr>
<td>Continuous (immobilized cells)</td>
<td>0.10</td>
<td>17-42</td>
<td>0.17-0.42</td>
<td>RUT-C30</td>
<td>[carrageenan support loading 1000 g L$^{-1}$ celite I support loading 40 g L$^{-1}$ celite II support loading 80 g L$^{-1}$]</td>
<td>MONTENECOUI (1981)</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>70</td>
<td>0.0-1.51</td>
<td>RUT-C30</td>
<td>lacrose induced</td>
<td>MONTENECOUI (1981)</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>134</td>
<td>0.0-1.93</td>
<td>RUT-C30</td>
<td>lacrose induced</td>
<td>MONTENECOUI (1981)</td>
</tr>
</tbody>
</table>

*Time in brackets indicates the time of optimal productivity and cellulose concentration for batch cultures.*
disadvantages of Trichoderma enzymes are i) the inability to metabolize lignin ii) the low specific activity of cellulase i) low levels of β-glucosidase (Mandels 1982). Here is yet another area where genetic engineering can play a vital role. There have been reports on cloning of cellobiohydrolase gene from T. reesei (Shoemaker et al., 1983; Montenecourt, 1983; Teeri et al., 1983) Escherichia coli but the specific activity remained unchanged. Same was reported of endoglucanase from Cellulomonas (Whittle et al., 1982, and Gilkes et al., 1984); Thermonospora (Collmer Wilson, 1983); Clostridium thermocellum (Cornet et al., 1984) and Scyzophillum comby (Faber et al., 1983); cellobioase from Agrobacterium (Wakarchuk et al., 1984) and xylanase from Bacillus polymyxa (Yang et al., 1985). Cellulolytic bacteria have been extensively studied for saccharifying ability of their cellulases (Choudhury et al., Lynd and Grethlein, 1987; Rajoka and Malik, 1984). Durand et al. (1988) reported mutant of T. reesei CL 847 to produce cellulase with increased specific activity. Schulein, (1988) has recently described the cellulase components from T. reesei. Cellulase families and their genes have been discussed by Knowles et al. (1987) and Glick and Pasternak (1989). UV induced mutant MC from T. reesei Rut C-30 showed almost similar activity as parent strains but could produce cellulases from different carbon sources other than cellulose (Allen and Andreotti 1982). Sad et al. (1987) compared T. reesei QM6a and its various mutants QM-9414, NG-14, Rut C-30, MCG-77, RL-P37, and CL-847. The authors reported increased protein content from 7 mg/ml to 22 mg/ml FPase productivity to 125 IU/L/h. Further improvement can
achieved by using novel fermentation processes (Turker and Mavituna, 1987).

Thermophilic Cellulolytic Fungi

Thermophilic species are well documented for their thermostable enzymes, high rate of cellulolysis, low amounts of enzyme required, non-asceptic conditions for saccharification (Margaritis and Merchant 1986a). Use of thermostable cellulase titres produce fermentable carbohydrates at elevated temperatures which make the saccharification process, cost attractive. Chahal, 198 Margaritis and Merchant, 1983 and 1986; Durand et al, 1984 a 1985; Merchant, 1984; Merchant et al, 1988. Cooney and Emers (1964) have reviewed thermophilic fungi to comprise a restrict number of species. These fungi grow at elevated temperatures of 50°C but not below 20°C. Their occurrence has been reported self heating piles of organic matter, often causing biodeterioration of agricultural (Cooney and Emerson, 1964; Flanningan, 196 and forest products (Tansey, 1970). Eggins and Malik, (196) reported the isolation of thermophilic fungi from grass, which showed the ability to breakdown ball milled cellulose (BMC) producing clearings in the solid medium. Similarly, Malik a Sandhu (1973) in these laboratories isolated thermophilic fungus which showed extensive breakdown of BMC. Various other report thermophilic species include: Chaetomium thermophile (Romane et al, 1975; Ericksen and Goksoyr, 1976 and 1977) Thiella terrestris (Skinner and Tokuyama, 1978 and Brueil et al, 198 Sporotrichum thermophile (Coutts and Smith, 1976; Canevascini al, 1979; Grajek, 1987a & b), Thermoascus aurantiacus (Romane et al, 1975; Yu et al, 1987 and Feldman et al, 1988). Talaromy
emersonii (Folan and Coughlan, 1979).

Other Potent Fungi

Another very well studied genus is Aspergillus. These are reported to yield endo-glucanases and β-glucosidases but lack substantial exo-glucanase activity. However, when mixed with Trichoderma cellulases the enzyme complex is reported to be very potent system. Duff et al, 1985 used a mixed culture of Trichoderma reesei Rut C-30 and Aspergillus phoenicis ATCC 329 in order to get a complete and balanced cellulase. Similarly, Pandit et al, (1987) used mixed culture of T. reesei D1-6 and A. wentii Pt 2804 to produce cellulases in high titres. Considering the economics of the process the authors used successive cultivation of selected fungi on rice straw and wheat bran for production of cellulases and xylanases after recycling the same substrate five times.

The cost of enzymes at present take a fair share of 50-60% of lignocellulosic (LC) hydrolysis. About 30% is spent for pretreatment and the rest is value of the substrate, etc. Thus an ideal organism could be realized as the one that produces a high titre of a complete cellulase, utilizes cheap carbon and nitrogen sources without showing catabolite repression. The enzyme should have high specific activity and stability at high temperature; remain insensitive to end product inhibition and to shear stress effects.

CELLULASE PRODUCTION BY FUNGI

Cellulases are constitutive as well as inducible enzyme. Their biosynthesis is repressed by the presence of soluble sugars or other easily metabolizable substrates. Some of the cellula
producing microorganisms have shown to induce more enzyme activities from pure cellulosics, others from untreated or treated L while still others from different carbon sources. It is difficul
to make a conclusive comparison in the assessment of the various strains as to their efficiency of cellulase productivity and degradation. Table 1 shows the filter paperase (FPase activity of a number of T. reesei strains under varying mode of fermentations. Productivity and concentration of the enzyme is quantified in terms of international unit defined as umol of reducing sugars released per minute from a filter paper substrate. The variation in activities may be due to various factors including the organism, type of inducer, type of growth conditions, methods of harvesting, concentration, as well as the method of fermentation. Many of the microbes are deficient in producing or the other component in the cellulase complex. Since the enzyme act in a synergistic fashion the presence of each one of them at optimal relative level would be most suitable (Herr, 198 Woodward and Wiseman, 1982).

Most of the microbes produce maximal cellulase activity when grown on pure cellulose as in the case of Trichoderma s (Table 1). But the cost of pure substrate and the option utilizing cheap available LC provokes one to find better alternate. This includes the isolations and development of strains (mutagenesis and/or genetic engineering) which can grow profuse on the waste LC substrates, to produce concentrated enzyme titr and the use of optimal fermentation conditions in solid state fermentation (SSF) and liquid fermentation (LF).
Cellulase Production By SSF

SSF is similar to the Koji process of Toyama (1976) and is an attractive alternate to use LC substrates than costly cellulolytic substrates in higher than 6% concentrations (Chahal, 1982 and 1985; Shamala and Sreekantiah, 1986). SSF has been greatly exploited by many other workers (Rao et al, 1983; Duff et al, 1986; Gibbons and Westby, 1986). Chahal (1985) described SSF as a most practical and comparatively cheaper process for the production of complete cellulase complex in reesel using LC substrates. Wheat straw as a substrate yielded enzyme activities of 17.2, 21.2 and 540 IU/ml of cellulases, β-glucosidases and xylanases, respectively. SSF has been reported by many workers: Sternberg et al, (1976) used wheat bran to get a yield of 200 U/g of β-glucosidase in A. phoenicis. Deschamps and Huet (1984) reported potato starch and sugar beet pulp to induce β-glucosidase to 66 and 550 IU/g, respectively after 4 days of incubation. Kim et al (1985) used a combination of starch and wheat bran to obtain higher CMC-ase activity of 60 U/ml by T. reesel QM 9414 than tha of 50U/ml by S. cellulophilum after 50-70 hours of cultivation Shamala and Sreekantiah, (1986) used rice straw (RS) and wheat bran (WB) to induce enzyme activities in various fungi under optimal conditions. A. ustus gave the highest β-glucosidase activity of 60 U/g WB and a xylanase activity of 740 U/g RS after a incubation of 5 days. A mixture of seven parts of RS and three parts of WB, mixed with 40 parts of Toyama’s mineral solution yielded 6U FP-ase, 40U β-glucosidase, 12U CMC-ase and 650U xylanase per gram substrate. Orajek (1987a) used a combination of sugar beet pulp and cellulose powder (8:2) and induced maxim
activity of 1.3, 5.3 and 8.9 U/ml for exo-glucanase, endo-glucanase and β-glucosidase after an incubation of 3-5 days by *Thermosascus aurantiacus*. Sanyal et al. (1988) in *Aspergillus japonicus* have shown that barley husk induced specific activity of CMCase 8.0 and β-glucosidase 2.0 U/ml, whereas, wheat bran induce 2.0 and 0.48 U/ml, respectively after six days of growth. Madanwar et al. (1989) found 5M NaOH treated bagasse as the best substrate yielding maximal enzyme activities of 12.1, 21.5, 7.0 of CMCase, FPase and β-glucosidase, respectively. Other treated substrates induced in the order computer cards > corn cobs > sa dust for cellulase production. Apart from the advantages in SE there are a few bottlenecks. Although, the enzyme is concentrated in SSF but the enzyme activity per gram of substrate is significantly higher in LF process in most of the strains. This might be due to problem areas in SSF such as aeration, agitator and mixing, heat dissipation, pH control, etc.

**Cellulase Production BY LF**

To date LF is the method of choice for the production of most of the industrial enzymes because of the considerable advances being made in this area especially in the designing of fermentors. The fermentations can be batch, fed-batch, semi-continuous and continuous. An advantage of fed-batch is that the addition of substrate and nutrients can be added as per requirement. Hendy et al. (1984) in *T. reesei* Rut C-30 exhibited optimum productivity of 130 U/L/H in a fed-batch cultivation at a feed rate of 1.7 solka floc, while the enzyme activity of 26.0 U/ml of cellobioh drolase as compared to 8.4 U/ml in batch culture was obtained.
Semi-continuous and continuous process for cellulase production are less common because of insoluble nature of the substrate. A mutant of *T. reesei* CL-847 has been able to produce β-glucosidase constitutively using glucose or lactose in a continuous culture (Durand et al., 1988). Allan and Andreotti (1982) reported cellulase productivity of 168 IU/L/H in fed batch culture by *T. reesei* MC-G-80 while batch culture showed 101 IU/L/H. Watson et al. (1984) using *T. reesei* Rut C-30 produced a maximal level of 57 U/ml for cellobiohydrolase at a productivity rate of 201 IU/L/HR. Some of the other reported work in LF on cellulase production from cellulosic substrates include: Enari et al. (1975) in *T. viride* using 2% solka floc plus 1% glucose among different cellulosic substrates induced a maximal of 8.8 and 11.0 U/ml of endoglucanase and cellobiohydrolase after 6 days of fermentor cultivation, respectively. Cottte and Smith (1975) in similar studies using 1% solka floc induced in *S. thermophile* almost similar levels of cellulolytic activity in less than one fourth the time as in *T. viride*. Skinner and Tokuyama (1978) reported 1% cellulose powder to yield a maximal cellulase activity *T. terrestris* releasing 12.0 and 59.0 mg/ml of glucose for FP-ase and CMC-ase, respectively, after 48 hours of incubation only. Other substrates followed the order of enzyme induction of newsprint > bagasse > cotton gin trash > wheat bran. Ryu and Mandels (1980) using 6% two roll milled cotton obtained a maximal of 150 U/ml of endoglucanase from *T. reesei* Rut C-30, while the mutant NG-14 yielded a maximal of 15, 0.6 and 21 U/ml of cellobiohydrolase, β-glucosidase and protein, respectively after 12 days of fermentor cultivation. Dashpande et al. (1984) using 10% cellulose obtained 20.0, 2.0
and 0.5 U/ml of endoglucanase, cellbiohydrolase and \( \beta \)-glucosidase activity by *Penicillium janthinellum* after 11 days of shake flask incubation. Breuil (1986) revealed that *T. terrestris* C 464 grown on 2% avicel yielded the highest combined \( \beta \)-glucosidase (intracellular and extracellular) activity of 32.0 U/ml and a rather low cellbiohydrolase of 0.55 U/ml after 7 days of incubation.

**Cellulosic And LC Substrates**

LC's hold much more promise than pure cellulosics as far as their abundance and economics is concerned, however, only a few organisms in LF have shown high productivity, on them. Gokhale (1984) using a combination of 3% wheat bran and 2% cellulose obtained a maximum of 10, and 22.6 IU/ml for \( \beta \)-glucosidase and cellobiase, respectively. Khan and Lamb (1984) reported almost a similar level of enzyme activities produced from steam exploded aspen wood when 10-20% of pure cellulose was added to it than compared to pure cellulose, only. Hoffman and Wood (1985) showed that 1% hammer milled barley straw induced the enzyme activity in *P. funiculosum* IMI 87160 to a level of 0.31 and 1.87 U/ml of cellbiohydrolase and xylanase, respectively, while the soluble protein was 0.61 mg/ml. Saddler et al. (1985) reported steam exploded water extracted aspen wood (SEAW) as more suitable for enzyme induction in *T. harzianum* yielding 66, 2.3, 2.8 and 45 U/ml for endo-glucanase, \( \beta \)-glucosiase, cellbiohydrolase and xylanase, respectively. In comparison *T. reesei* Rut C-30 showed an activity of 73, 0.2, 4.5 and 140 U/ml, respectively, for these enzymes, respectively after 6 days of shake flask incubation. SEAW was followed in the order of induction by solka floc. S
aspenwood xylan. The last substrate yielded maximum xylanase activity. Bhat (1987) using blotting paper from bamboo shoots, obtained enzyme activities of 0.1, 1.2, 0.18 and 1.65 U/ml of FP-ase, endo-glucanase, exo-glucanase and β-glucosidase, respectively, in a local strain of S.thermophile. Brown et al, (1987a isolated a number of over-producing strains from P.piniophily 871600iii after u.v. mutagenesis and/or chemical treatment. The mutant MTG III/6 which also showed higher Vmax value from the kinetics, induced the cellulase activity from 1% hammer milled barley straw up to 1.5, 16.2, 6.9 and 4.6 U/ml for FP-ase, CMC-ase, xylanase and β-glucosidase, respectively while 2.4 mg/ml of protein activity was obtained after 10-14 days of incubation Steiner et al (1987) in Schizoschyzum commune using 4% avicel obtained 5.0, 1244, 108 and 65 U/ml of FP-ase, xylanase, β-glucosidase and CMC-ase, respectively, after 11 days of shake culture fermentation. In a laboratory fermentor the respective activities were 4.5, 200, 100, and 60 U/ml. Yu et al, (1987 screening twenty one strains of thermophilic fungi found maximum production of xylanase activity (575.9 U/ml) in Thermoascus aurantiacus strain C 436 from 1% solka floc after 7 days in shake flasks. Cellulase activities of 10.1, 0.16 and 6.74 U/ml were obtained for endo-glucanase, FP-ase and β-glucosidase, respectively. Solka floc was followed by ball milled saw dust, steamed exploded water insoluble aspen SEA-W1 and untreated saw dust for cellulase and xylanase activities. Acebal et al (1988) reports maximal FPase production of 1.25 IU/ml by T.reesei QM9414 when grown on 1% wheat straw pretreated with 1% NaOH, 120 C, 2atm. The addition of raw material elongated the exponential phase, whil
the activities remained same. Mes Hartree et al (1986) steam pretreated (240°C, 180 sec) aspen wood samples to yield a maximum of 59.2, 2.6 and 1.5 U/ml of endo-glucanase, FP-ase and \( \beta \)-glucosidase in \text{\textit{T. harzianum}} after 4 days of incubation. Acid impregnated steam treated samples could not enhance enzyme activity to similar extent. The pretreated substrate yielded higher enzyme activities than the pure substrate. Jan and Tiraby (1987) using 3% paper pulp and 15% wheat bran for enzyme production in \text{\textit{Talaromyces}} sp. CL 240 and \text{\textit{T. reesei}} CL 487 obtained increased enzyme activities in all except FP-ase from the former substrate. The FP-ase activity at 65°C showed even higher activity for \text{\textit{Talaromyces}} sp. because of the thermostable enzymes.

**OPTIMUM CULTURE CONDITIONS FOR CELLULASE PRODUCTION**

Environmental factors have a fundamental role in the growth and product formation of the microbe. These factors include, apart from a carbon source, nitrogen source, ionic concentration, pH, temperature, incubation period, stirring speed and aeration, etc.

**Nitrogen Source**

Medium composition and growth factors need to be optimised for exploiting the microbial system for getting maximum possible yield. Along with the carbon source, the choice of the nitrogen source is of great importance. The use of well known and expensive nitrogen sources might be excellent for laboratory optimisation studies but not for the industrial use. The nature and concentration of both nitrogen and carbon source affect the pH profile during a cultivation process without pH control. \text{\textit{T. reesei}} strains grow rapidly on a rich organic nitrogen source.
like peptone, inorganic nitrogen sources of ammonium sulphate, ammonium phosphate and ammonia. But it is unable to utilize nitrate. Complex nitrogen sources containing amino acids stimulated growth (Montenecourt and Eveleigh, 1977; Ryu and Mandels, 1980). Pourquie and Vandecasteele (1984) reported a selected strain of T. reesei CL347 which surpassed the previous best results when grown in a mineral medium containing in g/l: lactose 60 and yeast extract 1 in liquid fermentation (LF). In SSF, the addition of nitrogen and mineral requirements decreases with decrease in liquid volume. Kim et al. (1985) in SSF used only wheat bran with a suitable moisture content in a fermentor adequately supplied with air. Shamaia and Sreekantiah (1986) in SS used Toyama's mineral medium containing (NH₄)₂SO₄ (0.1%) only a nitrogen source. Apart from this the authors also used Rees and Mandels medium which contained (NH₄)₂SO₄ (0.14%), urea (0.03%), yeast extract (0.01%) and peptone (0.025%). Toyama's mineral medium mixed with rice straw was found to be better for cellulase and xylanase production. Brown et al. (1986b) in I using a mutant of Penicillium pinophilum NTG III/6 obtain optimum activity using 6% solka floc and/or hammer milled barley straw along with nitrogen sources in g/l of (NH₄)₂SO₄ 1.4, urea 42 4, 0.3 and proteose peptone 1.0 in Mandels and Weber medium using an instrumental fermentor.

Romanelli et al. (1975) working on thermophilic fungi found that addition in g/l of organic carbon 15 and nitrogen (peptone 0.75 and urea 0.3) in a mineral medium markedly increased the cellulose degradation. Among the tested fungi Sporotrichum thermophile showed the highest rate of cellulose utilization follow
by Chaetomium thermophile and Thermosascus aurantiacus. Skinner and Tokuyama, (1978) reporting optimum conditions in their patent on Thielavia terrestris used the nitrogen content in g/l of Corn steep liquor 2.0 and peptone 1.5 along with cellulose powder 10, in the nutrient medium. Coutts and Smith (1976) working on S. thermophile reported maximum production of cellulases when NaN 3 or urea were used as sources of nitrogen. However, when used at varying concentration of 0.05-0.4% it had little effect on increase in cellulase activity. Working on this organism, Canevaccini et al, (1979) used Eggins ad Pugh (1962) medium but without α-asparagine. They replaced it with iron ammonium citrate and also added trace mineral elements. While in another composition the authors replaced ammonium sulphate with cas-amino acids. Grajek (1987b) working on the same organism used in g/l: Carbon source 20 along with yeast extract 0.1 and (NH 4 ) 2 SO 4 2 4
Among the various supplemented nitrogen sources: ammonium sulphate, ammonium chloride and potassium nitrate appeared to be the best for β-glucosidase production. The amount of supplemented nitrogen was calculated at 40 g of total nitrogen per kilogram of carbohydrates.

Ionic Strength

The addition of proper and adequate mineral contents in the basal medium is a basic requirement for optimum growth and product recovery from the microbe. Various scientists have used mineral contents in the medium after appropriate designing base on the specific requirement of the microbe. The nutritional requirements increases with the increase of carbon source to certain extent above which osmotic/inhibitory effects are bein
experienced (Wase et al., 1985). Eggins and Pugh (1962) designed a medium for thermophilic fungi which contained the mineral content in g/l: KHPO 1.0, NH4SO 0.5, KCl 0.5, MgSO 0.2, CaCl 0.1, along with organic nitrogen source of L-asparagine 0.5 and yeast extract 0.5. Romanelli et al. (1975) also designed a medium composition for thermophilic fungi which contained in g/l KHPO 2.0, (NH4)2SO4 1.4, CaCl2 2H2O 0.3, MgSO4 7H2O 0.03, along with peptone 0.75, urea 0.3 and Tween-80 0.5. Coutts and Smith (1975) formulated medium for S. thermophile which contained in g/l: NH4HPO4 2.0, KHPO4 0.6, KH2PO4 0.4, MgSO4 7H2O 0.5, yeast extract 1.0, trace mineral solution and thiamine HCl 100 μg/l. Wase et al., 1985 working on Aspergillus fumigatus used elementary statistical techniques. The endo-glucanase yields were enhanced to 28% when the carbon source along with potassium phosphate and ammonium sulphate were increased to five fold. However, the increase in peptone and MgSO4 had negative effect. Brown et al. (1986b) optimising conditions for Penicillium pinophilum mutant NTG III/6 used Mandels and Weber (1969) medium. This consisted in (g/l) of (NH4)2SO4 1.4, urea 0.3, proteose peptone 1.0, KHPO4 2.0, CaCl2 2H2O 2.4, MgSO4 7H2O 0.3 and Trace element solution. Ball milled barley straw 6% yielded the maximum enzyme yields as compared to solka floc.

pH

The optimum pH for growth rate may be different from that of growth yield and entirely different from the optimum for product formation. The ammonium salts supplied to a culture as sole nitrogen source leads to ammonium uptake and a decrease in pH. By the use of nitrate ion and ammonium ions simultaneously the hydrogen
concentration can be kept balanced (Porage et al., 1964). The use of \textit{NaNO}\textsubscript{3} to keep the pH at a higher level is also reported by Umezurike (1970) and Hulme and Stranks (1971). However, highly cellulolytic culture filtrates produced under acidic conditions have been reported by Mandels and Weber, 1969 and Neudorffer and Smith, 1970. Ryu et al (1982) working on the cellulase biosynthesis, separated the growth phase which was optimised at pH 4.5, temperature 32°C and high dilution rate. The enzyme production phase was optimised with pH 3.5, temperature 25°C and a low dilution rate. This initial lowering in pH goes well with most of the \textit{Trichoderma} strains (Nystrom and Luca, 1977; Ryu and Mandels, 1980; Galo, 1981; Watson et al 1984. In fact, \textit{Trichoderma} strains lower the medium pH to acidic when left uncontrolled to a level where enzyme synthesis stops (Allen and Mortensen, 1980; Mukhopadhyay and Malik, 1980; Gaur and Neelakantan, 1982). The latter strains such as Rut C-30 (Tangnu et al., 1981), showed appropriate results at pH 5.0 and 23°C. Shulz and Hirte (1989) working on \textit{Penicillium janthinellum} reported sudden fall in the pH level with increasing substrate concentration when the pH was no controlled. This resulted in very low cellulase activity of the culture filtrate.

Romanelli et al (1975) working on thermophilic fungi revealed that addition of small quantity of organic nitrogen such as peptone does not allow the pH to fall below 4.0. This effect allows the enhancement in cellulase activity. These results were found to be more pronounced by Coutts and Smith (1976) while working on \textit{S. thermophile}. They found that the organism grew well at higher pH values and produced greater yield of cell
lases. It has been suggested, that in thermophilic fungi two
distinct groups based on pH tolerance exist. The one that grows
well at alkaline pH is commonly associated with composts and the
other associated with soil, growing best below pH 6.0. S. thermophile
belongs to the former group (Coutts and Smith, 1976). The
same is also reported for Chaetomium thermophile (Eriksson and
Goksoyr, 1976). Skinner and Tokuyama (1978) reported pH 5.5-5.5
as the optimum for cellulase production Thielavia terrestris
Grajek (1987b) reported pH 6.5 as optimum for S. thermophile.

Cultivation Temperature

Most of the well known mesophilic species of Trichoderma
Penicillium and Aspergillus grow best at 28-30 C (Ryu and Mandel
1980; Mandels, 1982; Gokhale et al. 1984; Durand et al. 1984)

The increased cultivation temperature of 45-52 C adopted by th
thermophiles for growth could be beneficial in reducing the ris
of microbial contamination, cooling requirements for the ferme
tation process, decrease the cultivation time with concomitan
increase in enzyme kinetics (Margaritis and Merchant, 1983 an
1986; Yu et al, 1987). These high temperature are exhibited b
various thermophilic fungi. S. thermophile (Coutts and Smit
1976); G. thermophile (Eriksson and Goksoyr, var. dissitum 1976)
A. fumigatus (Vandamme et al, 1982); Myceliophthora thermophil
(Sen et al 1981); Talaromyces emersonii (Folan and Coughla
1979); Humicola insolens (Yoshioka and Hayashida, 1980); Humicol
grisea var. thermoidea (Yoshioka et al, 1982). Thielavia terrestris
(Margaritis and Merchant 1986); Thermascus aurantiacus (Y
et al 1987); Different thermophilic fungi (Grajek 1987a).
Cultivation Time

Incubation time for optimum enzyme production is an important parameter as far as the economics of the process are concerned. The shake flask studies with the Trichoderma reesei QM 9414 and Aspergillus sp. yielded maximum cellulase production after 12-14 days of incubation (Ryu and Mandels 1980; Gokhale et al., 1984). However, the strain MCG-77 showed maximum cellulase production in six days. Similar results were obtained from T. reesei CL847 in fed-batch fermentation (Pourquie and Vandecasteel 1984). Tanase (1971) found that the cellulolytic rate of some thermophilic fungi was two to three times that of Trichoderma spp. RomaneIl et al. (1975) found that S. thermophile degraded cellulose faster in liquid culture. Mandels (1975) also noted that thermophilic fungi degraded cellulose rapidly but produced cellulase activity in low titres. This fact was also confirmed by other scientists (Coutts and Smith, 1976; Bhat and Maheshwari, 1987. Skinner an Tokuyama, 1978) reported that T. terrestris (NRRL 8126) yields maximum cellulase activity only after 48 hours at 48°C. Durand et al. (1984) working on two thermophiles and two mesophiles species, reported maximum cellulase yields for the former after days whereas it was after 4 days for the latter. Grajek (1987) found that maximum β-glucosidase from S. thermophile was attained after six days of incubation in fermentor, although the maximum value obtained after 3 days was only a bit lower. Grajek (1987, working on different thermophilic fungi obtained the cellula yields after 3 days in LF while in SSF the incubation were carried out for 3-5 days.
Agitation and Aeration

In the conventional shake flask culture the aeration is limiting factor even if the agitation is increased. Yoshida et al (1968) reported that oxygen penetrated the peripheral 4 mm o the pellets while Kobayashi et al (1973) enunciated that the central biomass of pellets of *Aspergillus niger* becomes oxygen limited before they attain a radius of 250 μm. Nevertheless, the difference in mode of growth and aeration requirements vary from organism to organism. Ryu and Mandels (1982) suggested an adequate oxygen transfer rate in the fermentation broth during growth and enzyme production. In order to reduce the lag phase with a concomitant increase in the exponential phase for a greater period of time, fed-batch or continuous culture mode was adopted. Ryu and Mandels (1980) used a 10 litre submerged culture, maintained by aeration set at 2 litres/min at 7 psig pressure with varying agitation between 300 to 500 rpm. Watson et al (1984) working on *T. reesei* Rut C-30 used a laboratory ferment (11 L working volume) to control the agitation and aeration to maintain a dissolved oxygen content of not less than 20% of a saturation. They also maintained a cellulose feed rate of 1 g/l/h supplemented with pure oxygen at certain stages of fermentation to achieve a productivity of 201 IU/l/h. Grajek (198 working on *S. thermophile*, maintained an aeration of 60 l/l/h and agitation of 250 rpm in an 8 litre working volume fermentor for cellulase production. Very short lag phase period, limited to hour, and the rapid exponential growth accompanied by large oxygen consumption were noticed.
ENZYME CHARACTERISTICS

pH

The pH optima for cellulase activities in Trichoderma sp has been found to be 4.8 using 0.05 M citrate buffer (Mandels al. 1975; Sternberg et al., 1976; Ryu and Mandels, 1980). Other workers have used acetate buffer, 20 mM, (Pourquié and Vandersteel, 1984; Mes-Hartree, 1988). The optimum pH found for activity of A. phoenicis \( \beta \)-glucosidase was about 4.3. Skinner and Tokuyama (1978) used 0.5 M acetate buffer at pH 5.0. Grajek (1976b) showed that \( S. \) thermophile and Thermoascus aurantiacus produced optimum activities at pH 4.5-5.5. Grajek reported optimal pH in the range of 5.0-6.3 using 0.05 M McIlvaine's buffer. Although, \( S. \) thermophile cellulases are produced optimum at alkaline pH, they function most efficiently at acidic pH 5.0 (citrate or phosphate buffer). Durand et al. (1984) working on mesophilic and thermophilic fungi found that pH 5.0 was optimum for all the enzymes tested. Endoglucanase from all 4 strains and xylosidase from \( S. \) cepae had a more acidic pH, while \( S. \) cellulosphilum enzymes slightly more alkaline pH. Grajek (1986) listing the effects of pH on enzyme activity found that the most acid stable enzymes were cellulases of Aspergillus fumigatus specially endo-glucanases (pH 4.0). The enzyme obtained from SSF were more stable than in LF.

Temperature

The temperature up to 50°C increases the enzyme adsorption to a maximum above which the activity falls (Bisaria and Ghc 1978). Trichoderma strains show the highest activity up to 50°C in all the enzyme assays Schulz and Hirth, 1989). Thermophilic
fungi have a high rate of cellulolysis and show increased activity at 60°C. Similarly, other workers have reported different optimum temperatures for cellulases from various organisms. The optimum temperature reported for various organisms for cellulases are: \textit{T. viride} β-glucosidase, 50°C; \textit{A. niger} QM 877, cellobiase at 50°C (Sternberg et al., 1977); Basidiomyceta sp cellobiase 65°C (Shewale and Sadana, 1978); \textit{T. viride} β-glucosidase, 40°C (Herr, 1979); \textit{Sclerotium rolfsii} (UV-8) cellobiase, 65°C and \textit{Aspergillus} sp. cellobiase and β-glucosidase, 65°C (Gokhale et al., 1984). Durand et al., (1984) reported that among the thermophilic and mesophilic species, \textit{Thielavia terrestris} displays optimum temperature above 60°C for all the cellulases except cotton activity which was maximum at 55°C. The maximum was four for endo-glucanase at 75°C. Next in the order were \textit{Sporotrichum cellulophilum}, \textit{Trichoderma reesii} and \textit{Penicillium} spp., respectively (Durand et al., 1984). Macris and Panayatou (1986) from their mutants of \textit{Trichoderma harzianum} M5 showed optimal exo-cellobiohydrolase activity at 50-60°C. The CMC-ase and β-glucosidase from the same fungi had their optimum at 55-60 and 65°C, respectively. For \textit{Aspergillus ustus} M35 the optimum temperature of enzyme activities was at 60, 65 and 70°C for exo-cellobiohydrolase, β-D-glucosidase and CMC-ase, respectively. The optimum temperature for the latter enzyme is one of the highest reported in mesophilic and thermophilic organisms.

Thermotolerance

Linko (1977) reported two of the major limitations of cellulases obtained from mesophilic fungi to be their relative poor temperature stability characteristics and low rates of cellu
lulose hydrolysis. The best known cellulases such as those produced by Trichoderma, Penicillium and Aspergillus species are stable at 50°C (Mandels, 1975). At this temperature enzyme reactors are frequently contaminated. Increasing the reaction temperature would give a dual benefit by increasing hydrolysis rates and inhibiting microbial growth (Hagerdal et al, 1980; Durrand al, 1984). Thermophilic microorganisms are in general more active at high temperatures and more thermostable than those produced by mesophilic species (Zeikus, 1979). In fact, there is a direct correlation between temperature optima for growth and thermostability of the enzyme.

Cellulases derived from a few strains of Thielavia terrestris have been reported to be exceedingly thermostable. Skini and Tokuyama (1978) reported that 20% of the original enzyme activity still remained when culture filtrate was exposed to temperatures of 100°C for a period of 3 hours. These characteristics have also been reported by (Margaritis and Merchant, 1984). Durand et al (1984) working on a couple of mesophilic and thermophilic fungi confirmed that T. terrestris was the most promising source of thermostable enzymes. Feldman et al (1988) reported in Thermoascus aurantiacus the stability of cellulase system for at least 24 h at 70°C.

PRETREATMENT OF LC SUBSTRATES

The literature on various pretreatments applied is voluminous. Several review articles on this subject are available (Mandels et al, 1974; Millet et al, 1975; Cowling and Kirk, 1982; Dunlap et al, 1976; Millet et al, 1976; Ladisch, 1979; Dunlap...
Chiang, 1980; Horton et al., 1980; Chang et al., 1981; Lin et al., 1981; Fan et al., 1982; Ledisch et al., 1983; Marsden, 1986). These pretreatments are broadly classified into physical, chemical and biological pretreatments according to their principle mode of action on the substrate. Some processes are combinations of two or more pretreatment techniques applied in parallel or in sequence.

**Physical Treatments**

Ball milling reduces crystallinity and particle size, whereas it increases specific surface area, bulk density and water soluble fraction. Ball milling has proved to be very efficient pretreatment for cellulosic materials (Millet et al., 1979; 1969; Fan et al., 1980 and 1981; Asenjo, 1983; Lee et al., 1983). However, as commented by Andre and Nyström (1976) and Wilke et al. (1976) it is costly and unable to remove non cellulosic substances, probably due to lignin (Neilson et al., 1982). Most of the workers have used a combination of two or more pretreatments with milling to delignify the substrate. Millet et al. (1975) noted that effectiveness of ball milling varied from material to material. Soft wood showed the least response. These results were in agreement with Matsumura et al. (1977) who found that while vibratory ball milling of both softwood and hardwood decrease particle size and crystallinity, it was treatment with NaOH (12.5%) which enabled the I. viride (Meicelase) to produce up to 75-80% and 25-35% reducing sugars for these substrates, respectively. Shimizu and Usami (1978) used bantam milling along with exhaustive extraction by methanol and air drying of hardwoods and softwoods. Maximum delignification (90%) of hardwood Buna Fagus crenata was attained with 50% methanol containing 0.1% HC.
(160 C, 45 ml) allowed complete degradation of cellulose portion by the Meicelase. Sudo et al (1976) used a combination of bantam milling with acid chloride for pretreatment of hardwoods and softwoods. Enzymatic accessibility of these substrates (2%) increased to 70-80% and 90% as the delignification approached 60% and 50%, respectively. Millet et al (1979) compared the effects of vibratory milling on enzymatic accessibility which depended upon the substrate type and milling time. Cotton linters were totally hydrolysed after 60 minutes of milling. It was followed by red oak (240 m, milling). News print and douglas fir required greater milling time for total enzymatic accessibility. Tassinari et al (1977) subjected a wide range of substrates to two-roll milling. The susceptibility of hard woods and agricultural residues was more than soft woods.

Steaming conducted at high pressures (5-20 bars) and high temperatures (180-200 C) for 5-30 minutes has been used by many workers. Spano et al (1979) studying the effect of steaming pre-treatment on the enzymatic hydrolysis suggested increased accessibility for hardwoods and agricultural residues, whereas it decreased for softwoods and urban wastes. Puri and Maners (1983) used milled bagasse, wheat straw, along with eucalyptus chips for autohydrolysis (200 C, 15 m). The corresponding lignin content was 23.6, 15.6 and 20.3%, respectively, whereas the enzymatic digestibility (Onazuka 3S) showed 78, 81 and 75% reducing sugars after 48 hours. Dekker et al (1983) used a shorter time duration (4m) but subjected bagasse to further explosive defibration, resulting in solubilization of 90% of hemicellulose. The treated material was susceptible up to 80% after 24 hours when supplemen-
ted with \( \beta \)-glucosidase. Deshpande and Eriksson (1984) reported that steam explosion of wheat straw led to similar effects as above. The rapid saccharification level slowed up considerably because of lignin, after initial high rate. The lignin is easily removed by \( \text{NaOH} \) and ethanol. Vallander and Eriksson (1985) indicated that out of three tested pretreatments, steam explosion was followed by treatment with \( \text{H}_2 \) and defibration. The enzymatic accessibility produced 74, 56 and 29% of reducing sugars, respectively by \( T. \) reesei cellulases (Alko Company). Puls et al (1984) used defibration (30 s) along with optimum steaming at 200 \( ^\circ \) C on chopped wheat straw which resulted in 51.8 and 58.5 saccharification, respectively, by \( T. \) reesei cellulase supplemented with \textit{Aspergillus niger} \( \beta \)-glucosidase. The pretreatment increased the carbohydrate and lignin level to 76.6 and 22% whereas the fibre yield decreased considerably at 210 \( ^\circ \) C. Poutanen and Pulst (1985) further elaborated steaming process and enumerated separate processing of fibre fraction (lignin and cellulose) and the aqueous extract, hemicellulose. They optimised pretreatment conditions on birch wood. This material was hydrolysed to 71% theoretical yield by (40 FPU/gm) \( T. \) reesei cellulase supplemented with (2.7%) \( A. \) niger \( \beta \)-glucosidase after 24 hours. Vallander and Eriksson (1987) steam exploded aspen wood chips (234 \( ^\circ \) C, 48 s) and wheat straw (235 \( ^\circ \) C, 60 s) which resulted in material loss mainly in the form of hemicellulose, accumulative to 3-6% a 12%, respectively. The enzymatic accessibility for these substrates (6%) was increased to the effect of 53 and 49%, respectively. Nes-Hartree et al (1987) used the Iotec process for aspen wood (240 \( ^\circ \) C, 80 s) and subsequent wash for removal of solut
pentosans and inhibitors with water. The 5% substrate was saccharified up to a level of 73% using Trichoderma harzianum E58 in a combined cellulase hydrolysis and fermentation process. The addition of small amount H2SO4 (up to 4%) produced an even more susceptible substrate from aspen wood than the autohydrolysis (Nes-Hartree and Saddler, 1983). The explosive decompression is important with substrates such as wood chips but for agricultural wastes (bagasse) the increase was low. The lignin from Iotec process is superior to autohydrolysis treatment (Marchessault, 1984) and the cellulose-lignin binding was broken down without significant change to either component. The failure to achieve 100% conversion was attributed to enzyme factors (inactivation and end-product inhibition) rather than the substrate factor such as crystallinity and lignin (Dekker and Wallis, 1983). Ando et al (1988) working on cedar wood suggested that per-acetic acid combined with steaming was effective as an acid catalyst an initiator of radical reactions in lignin side chain. The lignin was decomposed, repolymerised and melted accompanied by hemicellulose hydrolysis. Treated substrate (4%) was saccharified up to 75% by Meicelase. Latif et al (1988) compared steaming of salt tolerant plants with alkali pretreatments. The results with steaming indicated lower hydrolytic yields, because of increase in overall lignin content in the fibre due to subsequent loss of hemicellulose extracted in wash liquor. The increased temperatures favoured accessibility but the fibre yields were also lowered at the same time. The maximum accessibility by I reser cellulase and β-glucosidase from Aspergillus niger yielded reducing sugars of 42, 51 and 32% for Leptochloa fusca (kollar gras
Paninum maximum and Atriplex amnicola. However, the highest yields were achieved after alkali pretreatments. As reported earlier the steaming pretreatment in combination is most effective (Wong et al., 1988). These workers used 50% impregnation of wood chips which was followed by steaming. The resulting material was solubilised by T. reesei cellulase plus A. niger B-glucosidase to nearly 100%. This was mainly attributed to increase in pore size. The harsher treatments to a certain extent increased pore size but at the same time fibre losses were observed. The increase in accessibility was as a result of hemicellulose extraction and lignin redistribution. These results were further strengthened by the work of Burns et al. (1989). It was stated that after acid hydrolysis of hard woods the total surface area of the substrates pores was too small (50-90 Å) to be accessible to the enzyme. Steaming at 200°C increased the surface area further but the large pore size diminished rapidly as was observed after initial rapid hydrolysis. There was a subsequently low hydrolysis due to increase in number of small pore size.

Chemical Pretreatments

Among the alkali pretreatments NaOH has been more extensively used for ruminant feed, rather than for hydrolysis purposes (Wilson, 1977; Kerley et al., 1983; Deschard et al., 1984). Various substrates respond differently to NaOH treatment. Feist et al. (1970) observed that the digestibility of soft woods with high lignin content increased slightly with NaOH pretreatment, while the digestibility of some hard woods and agriculture waste, with low lignin content, increased significantly upon NaOH treatment. On the other hand, NaOH showed little effect on the digestibilit
of cotton. It was inferred that the major effect of the alkali was the hydrolysis of alkali labile linkages (ester type) between the carbohydrate and lignin components (Fiest et al., 1970; Chesson, 1981). Soft woods and legumes contain few of these linkages in contrast to graminae species and hard woods and thus show lesser susceptibility to enzyme attack (Matsumura et al., 1970; Chesson 1981; Evan, 1979). The swelling effect of alkali has been discussed by Tarkow and Feist (1969) who showed that alkali treatment of hard woods increased the estimated protecritical molecular weight which could enter pore spaces from 30,000 to 50,000. Taniguchi et al. (1982) treated rice straw with 0.025 N NaOH, along with 20% peracetic acid and twice with sodium chlorite. The degree of enzymatic solubilization with the culture filtrate by Trichoderma reesei QM9414 was 69, 42 and 50%, respectively, based on residual straw, whereas it was 30, 32 and 37 respectively, based on untreated raw material. Gharpuray et al. (1983) found that the optimum conditions for alkali pretreatment of wheat straw were NaOH (0.1 g/g substrate) and autoclaving (129°C, 2.57 atm, 1.5 h) which resulted in increased enzymatic accessibility up to 71%. It was observed that above 30% delignification, the hydrolysis rate increased sharply up to about 3 delignification. It was indicated that up to 30% delignification the lignin in the middle lamella is removed. Beyond this level, lignin present in the fibre is removed resulting in exposure of cellulose. Tanaka et al. (1988b) reported pretreatment of rice straw with caustic soda (1%, 121°C, 1 h). The cellulase preparation (1.2 mg/ml) from Onozuka R-10 solubilised the holocellulose (2% rice straw) to 100% after 24 hours. The rice straw af-
pretreatment consisted of 95% holocellulose including 77% cellulose and 5% lignin including ash. Hamilton et al (1984) treated lignocellulosic (corn residue) with dilute acid hydrolysis (5% H$_2$SO$_4$, 90°C). Moreover, it was treated with ferric sodium tartrate complex in 1.5N NaOH. The resulting substrate (1%) was enzymatically hydrolysed up to 90%. The increasing concentration of T. reesei cellulase (1.74-7.71 IU/gm substrate) resulted in almost 50% increased cellulose conversion. Pretreatment with NaOH resulted in 25-30% lower conversion, but at high enzyme levels, NaOH pretreatment was as effective as the cellulose solvent.

Apart from alkali, other chemical pretreatments, such as solvent pretreatment were reported by Binder and Fiechter, (1979). They treated avicel and wheat straw with EWNN (alk sol of sodium tartrate and ferric chloride) to get a saccharification of 95 and 75%, respectively. Cellulase preparation of T. reesei QM 9414 (12 IU/g straw) was used in each case. In comparison and alkali treated wheat straw was saccharified to respectively. The fibre losses during the pretreatment, EWNN and ozone were 50, 30 and 0%, respectively. Chang (1985) treated rice straw with 1% (w/w) H$_2$O$_2$ and 8 h) which resulted in 60% delignification, 40% and a five-fold increase in the accessibility for one times increase in the water holding capacity but a decrease in the crystallinity and enzymatic access 53.2% was achieved. A weight ratio of hydrogen peroxide (0.25 g H$_2$O$_2$/g straw) in alkaline H$_2$O$_2$ solution (w/w) at 32°C, improved the structural features
Shambe (1984) evaluated pretreatment of wheat straw with LiCl in 1M HCl (27 °C, 24 hr). T. reesei (MVA 1284) cellulase (0.2-0.4% hydrolysed 4% wheat straw to give 57% (82-95.4%) monosaccharides. Pretreated sample contained 18.3% lignin and 73.3% total carbohydrate. Holtzapple and Humphrey (1984) used organosolv (alcohol/water/catalyst) for treatment of poplar in steam injected pressur vessels. The acetone washed material (3%) was treated with enzym (FP-ase 0.08 uM glucose/ml) preparation from Thermomonospor specie supplemented with concentrated B-glucosidase. The pretreatment removes the lignin intact, accompanied by a similar quantity of hemicellulose. Dissolved NaOH in ethanol increased the enzymatic susceptibility to greater extent. Gould (1984) treated wheat straw and other perennial grasses with 1% (w/v) H2O 25 C. The 6 and 24 hour treated straw was solubilized up to 1 and 87% with cellulase preparation. Pretreated grasses (monocot; such as big blue stem, Indian grass and phragmites were most susceptible with saccharification efficiencies > 90% of theoretical.

Conner (1980) pretreated cellulose pulp with aqueous SO2 0.2, 0, 20 g SO2 100 ml; 100, 115 and 130 C, 1-3 hour. However, T. viride (Orazuka cellulase) increased the digestibility to 1 times of untreated substrate, only.

Some of the workers have reported a comparison of different pretreatments. Dekker et al. (1983) observed that milled bagasse treated with alkali (0.25 M, 20 C, 2 hour) and washed to neutrality was similar in hydrolysis yields as steaming. Extraction autohydrolysed pulps with dilute alkali or aqueous ethanol resulted in lower saccharification yields. Rao et al (1983) treated bagasse (Wiley milled 20-25 mesh) in NaOH (85 C, 60 m) and ne
rallised with HCl, whereas the other portion of sample was steam treated (7 kgcm). These treatments resulted in 63 and 59% saccharification, respectively, in 48 hours. Hemicellulose was not lost in these pretreatments. With a 30% slurry of steam treated bagasse a semisolid of 14% sugar was obtained. Cunningham and Carr (1984) pretreated wheat straw by thermal hydropulping, dilute acid pulping, thermal hydropulping followed by alkali extraction, alkali extraction and alkaline hydrogen peroxide. The last treatment was found to yield the highest amounts of glucose (98%) enzymatically from the treated substrate containing 57% cellulose. Dilute acid pulping rendered xylose in the wash liquor, whereas liquors from other pretreatments contained xylan. Chidambareswaran et al (1986) in another comparison of different modes of alkali pretreatment found that the highest saccharification of 96%, within 6 hours was achieved by cellulases from *Pencillium funiculosum* (0.277 FPU) when the fresh substrate was kept in formalin (10%) until use. The NaOH treated wet samples yielded 80% reducing sugars, whereas alkali treated wet, washed and dried fibres, gave only 45% yield. Untreated normal fibres yielded the lowest (19%). These high yields were attributed to highly opened structural organization which allowed the penetration of enzymes. However, the resulting reducing sugars contained only about 20% monomeric glucose. The decrease in reducing sugar yield was attributed to conformational changes at the molecular level. Cotton plant stalks purified with 7N HNO3 and left at room temperature for 48 hours were alkali treated, washed and wet enzyme treated to yield 80% sugars.
Biological Pretreatments

A biological pretreatment utilizes wood attacking microbes that can degrade lignin. The microbes can be classified into three categories: brown rots, white rot and red rots. Brown rots attack cellulose whereas white rots and red rots attack both cellulose and lignin. Removal of lignin by specific microorganisms has been reviewed by Kirk, (1975); Cowling and Kirk, (1976) Ander and Eriksson, (1978); Kirk and Chang, (1981) and Fan et al (1982). Kirk and Harkin, (1973) reported that white rot fungi removed 42% of lignin, 3% of glucan (including cellulose), and 30% of hemicellulose from birch wood. Ander and Eriksson, (1978) enunciated that degradation of lignin by white rot fungi is a co oxidative process and consequently an accompanying carbon source is necessary, for e.g., cellulose and/or hemicellulose. Th fungal ligninases appear to attack the phenolic residues with demethylation and ring cleavage. Eriksson and Goddall (1974 achieved almost specific lignin degradation with cellulase-less mutants of white rot fungi. Detroy et al (1980) working on growth of Pleurotus ostreatus on wheat straw observed losses of lignin and cellulose to be 22 and 14% after 30 days and 40 and 32% respectively, after 70 days. The organism appeared to selectively degrade lignin during the first six days.

Biological delignification appears to be a promising technique but its low rate has limited its usage in industrial processes.

Hydrolysis

Today, there have been few microorganisms which have shown enough potential to produce an enzyme system, which can extents
vely degrade the cellulosic material. Bisaria and Ghose (1981) listed 13 species of fungi along with a few bacteria in this regard. For effective saccharification of cellulose, the high sugar yield per enzyme unit is essential (Szczodrak, 1987). Factors effecting this yield include pretreatment, inhibition of enzyme by heat or degradation products, enzyme and substrate concentration, adsorption of cellulase to cellulose, stability of enzyme, speed of enzyme action and degree of agitation. Study of all the above factors were out of the scope of this work thus only pertinent literature is mentioned.

Enzyme and Substrate Concentration

The ultimate objective in the hydrolysis of cellulosic substrates is their conversion into monomeric sugars for the biotitization to useful energy rich products. But the economics of the process, for instance ethanol production, suggests the requirement of high sugar syrup (about 15%). This leads to problems such as end product inhibition and stirring of bulk substrates. Markman and Eklund (1975) using cellulases (Trichoderma virid QMN414) obtained about 100% yields from hydrolysis of furfural process waste containing 40% cellulose. They further reveal that by manipulation of a slightly lower pH than 5.0 and use of concentrated enzymes could yield about 5% glucose in 4 days. The enzyme could not be used for higher than 7% substrate concentration since it was present in dilute form. The use of ultrafiltration technique failed to concentrate the enzyme for effective saccharification due to changes in the hydrolytic character of the enzyme. By manipulating the condition in a fermentor, th
above yields were obtained in 24 hours. Moreover, the addition of \( \beta \)-glucosidase from *Aspergillus niger* could not bring about a noticeable increase, hereby indicating the presence of a complete cellulase system in *T. viride*. In a similar study, Wilke and Mitra (1975) using acetone concentrated cellulases (4.4 IU FPA) from *T. viride* QM9414 successfully hydrolysed 10% news paper (shredded and milled) to a level of 65% reducing sugars. Detroy et al. (1980) using commercial cellulase (Miles lab.) preparation (1.0 IU/g substrate) from Miles lab. were able to hydrolyse ED treated wheat straw (4%) to 70% glucose yield. Further modification by disc-milling EDA treatment yielded 83% of glucose. Moreover, they showed that after biological pretreatment (40-50 days) of *Pleurotus ostreatus* the conversion of cellulose to glucose increased 4 to 5 fold, yielding 72% glucose. Chahal (1984) using cellulase produced in solid state fermentation by a mutant QMY-developed from *T. reesei* QM9414 resulted in 99.75 g sugars/l from 100 g of delignified wheat straw. Very little cellobiose accumulated in the hydrolysate up to 20 hours, whereas its level further decreased after 96 hours. The hydrolyzates analysed at HPI contained in g/l: cellobiose 3, glucose 68, xylose 26.7, arabinose 1.7. McCrae et al. (1989) found enzyme productivity values much higher in the mutant strain of *Penicillium pinophilum* (MTG III/6) than those normally recorded for *T. reesei* mutant 30 (thought to be the best cellulase producer). The cellulase FPU/ml was very effective in hydrolysing 10% solka floc to 8% sugar solution. The hydrolysis of untreated straw from 30-35% w/w increased to a level of 92-98% when used after pretreatment with H2O at room temperature (24 h, pH 11.8). Most of the mutat
derived from T. reesei although had a potent cellulase but were devoid of a high β-glucosidase. Thus the deficient strains with β-glucosidase were prone to greater end product inhibition due to the presence of cellobiose. The presence of 1% cellobiose is as much inhibitory as 25% glucose (Morisset and Khan, 1984). Khan et al (1984) further elaborated the use of additional cellobiose from Aspergillus phoenicis along with the cellulase of T. reesei in the ratio of (0.9 : 1.0) in order to restore the effects of end product inhibition. By the addition of cellobiose to cellulase (30-35 FPU) of T. reesei the saccharification yields of 5% cellulose were enhanced from 56% sugars (86% glucose) to 80% (99% glucose). Dekker and Wallis (1983) increased the saccharification of 10% bagasse from 50% to 80% in 24 hours when B-glucosidase from Aspergillus niger was added to the T. reesei cellulase (2 FPU/gm substrate) in the ratio of FPU to β-glucosidase of 1:1.25. The workers used enzymes preparations from T. reesei C-30 an QM9414 and also Micelase (commercial enzyme), all of which varied to some extent in the proportion of different enzyme components.

Sakamoto et al (1982) used 0.5% of crude cellulase from Aspergillus aculeatus which hydrolysed 10% alkali treated rice straw a 37°C in 3 days to 85% (8% sugar solution). When used with commercial (Micelase) the yields were obtained in one day. Rao an Seeta (1983) described that Penicillium funiculosum produced complete cellulase which hydrolysed cotton, alkali treated bagasse and steam treated bagasse to 97, 63, and 59% sugar yield respectively, in 48 hours. With a 30% slurry of steam treated bagasse a 14% sugar in a semi-solid mass was obtained. McCrae et al (1980) reported cellulase (7FPU/ml) induced from solka floc
mutant strain NTG III/6 of *P. pinophilum* hydrolysed 10% solka floc to 70% in 72 hours at 50 °C. The hydrolysis yield was increased to 90% when enzyme induced on barley straw was used. Under the same conditions the polysaccharide in (alkline H₂O₂ treated) barley, oat, and wheat straw at room temperature) were hydrolysed to 93, 100 and 92%, respectively. Sattler et al (1989) determined that the extent of hydrolysis at fixed time increases with increasing enzyme dosage in a hyperbolic function. They revealed that in the system of sigma cell-50 and celluclast, the easily and difficultly hydrolyzable components were 43.0 and 57.0%, respectively and the maximum digestibility at 94 hours was 82.6%. Poplar wood steam treated at 200, 220, 240 °C, showed digestibility to glucose of 43.9, 64.9 and 60.0%, respectively. The easily digestible substrate (amorphous) was hydrolysed with cellulase (5 FPU/g), whereas the crystalline region was dependent upon increasing dose of FPU (5-100) which increased the yields to over three fold. It was concluded from the kinetics experiments that it was, in fact, a good pretreatment leading to a high proportion of amorphous cellulose of prime importance along with the type of enzyme. In contrast time and increase of enzyme dose was much less effective. The three different enzyme systems tested for hydrolysis showed different hydrolytic yields. Another criteria for the effectiveness of an enzyme is the enzyme dosage (FPU/g) which gives the half maximal digestibility. In hydrolysates, the reaction rate is proportional to the amount of adsorbed enzyme on the cellulose surface (Bisaria and Ghose, 1979). Various kinetic models are based on the properties of the cellular enzyme and mass transfer in the reaction system (Suga et a
A theoretical derivation of a hydrolysis model is given by Okazaki and Moo-Young (1978). Some authors have developed semi-empirical models based on the assumption that the reaction between cellulase and cellulose can be described by a summation of pseudo-first order reaction (Van Dyke, 1972; Brandt et al, 1973). Other models are based on structural features of the substrate like pore size distribution, pore size, index of crystallinity and specific surface area (Fan et al, 1980; Grethlein, 1985). Ohmine et al (1983) concluded that fall off in hydrolysis rate was not only because of crystallinity and product inhibition, but also due to a rate retarding factor. Matsumoto et al (1989) suggested it as a reversible inactivation of the adsorbed enzyme due to diffusion into the cellulose fibrils. Chernogolovov et al (1988) found that six different types of endo-glucanase purified to homogeneity varied in the intensity of their binding to cellulose and varied in similar order to their binding to lignin. Endoglucanase was deactivated after adsorption on lignin. Steam exploded lignocellulosic material also showed similar adsorption effects but not of when they were acid treated.

pH effect plays vital role in the optimization studies for hydrolysis as was revealed by Markkanen and Eklund (1975). Slight decrease in pH level from 5 to 4.8 has been suggested by many authors for Trichoderma strains (Wilke and Mitra, 1975; Mandel, 1982; Morisset and Khan, 1984; Saddler et al, 1985). However, the mutant QMY-1 developed from QM9414 showed optimum saccharification at pH 6.7 which is also the original pH of the enzyme solution. Sporotrichum thermophile has been reported to produce enzyme at alkaline pH 6.5 (Grajek, 1987b) but for saccha
rification purpose it has been reported optimum at pH 5.6 (Bhat and Maheshwari, 1987).

The temperature plays important role in the cellulolysis of substrate. It is the intrinsic characteristics of different microorganism to produce thermostable cellulases. Thermophilic microorganisms are reported to produce enzymes which show thermo-stability at temperatures of 70-80°C (Margaritis and Merchant 1983 and 1986). However, there are only a few successful reports on saccharification at temperatures higher than at 50°C. Skinner and Tokuyama (1978) using Thielavia terrestris (NRRL 8126) at 60°C obtained the highest glucose yield of 52% from cellulose powder and lowest of 2.9% from bagasse, respectively. Durand et al. (1984) working on mesophilic and thermophilic fungi enunciated that difference in the thermostability of mesophilic and thermophilic fungi is not enough to compensate for the low enzyme titres produced by the latter. This was evident in the hydrolysis experiment at high temperatures which revealed that at 60°C there was a significant decrease in the rates of hydrolysis for all the tested fungi. This was unexpected specially for T. terrestris (NRRL 8126) claimed to be as one of the most thermostable cellulase producing system.
MATERIALS AND METHODS

FUNGAL SPECIES

Two Chaetomium species namely: C. vergicepsum and C. gl
bosm from existing culture collection at NIAB and Trichoder
raesel Rut C-30 from NRCC were studied as reference mesophil
cultures along with the thermophilic isolates. All the fungi we
subcultured from time to time on ball milled cellulose ag
(BWCA) at 45°C for 4-6 days. The slants were stored at 4°C in
refrigerator.

MEDIUM AND CARBON SOURCES FOR ISOLATION AND PRODUCTION
CELLULASES

The widely used medium during the isolation, and growth
fungi for production of cellulases was from Eggins and Pugh, 16
(E & F). The composition of the medium in g/l was: carbon sour
1-10 (depending upon conditions), KH2PO4 1.0, (NH4)2SO4 0.2,
KCl 0.5, MgSO4 0.2, CaCl2 0.1, α-asparagine 0.5 and yeast extra
0.5. Distilled water was used to make the volume up to 1 litr
pH of the medium was set at 5.0. The medium was autoclaved
121°C for 20 minutes. All the chemicals used were of analytic
grade. Ball milled cellulose (BMC) (1%) was used as a
enrichment carbon source medium for the isolation of thermophil
fungi. Ball milling was achieved by using filter paper str:
(Whatman No. 1) with water in the ratio of (1:3) in a ball mi
apparatus. The milling for 2-4 days converted the paper in
pulpy material. For solid substrate 2% agar was added in an
enrichment medium.

For growth and enzyme production the carbon sources w
were namely: Dried shoots of *Leptochloa fusca* (kallar grass), *Sesbania aculeata* (dhancha), *Panicum maximum* (green panic), *Atriplex amnicola* (river salt bush), *Oryza sativa* (rice straw), *Panicum aestivum* (wheat straw), wheat bran and bagasse. The substrates were obtained from NIAB fields, except kallar grass which was harvested from Bio-saline Research Station (BSRS) Lahore during summer. The substrates were dried at 80°C in a oven before milling to 0.5 mm particle size. The mill substrates were kept at room temperature for further use.

**ISOLATION AND TAXONOMY OF THERMOPHILIC FUNGI**

Samples from five different habitats included: root rhizosphere of *Cenchrus ciliaris* and kallar grass from NIAB field; compost of kallar also from NIAB, decomposing bagasse piles from Sitara Chemicals Ltd. Faisalabad, and poultry droppings from local farm.

The soil samples obtained from the rhizospheres were moistened and enriched with 4% (w/w) BMC and mixed to homogeneity. The enriched soil samples were placed in polythene bags and incubated at 50°C. These incubated soil samples (50-100mg) were taken at 3 days interval up to 9 days and plated in triplicate by Warcup's method (1950), on 1% BMCA containing rose bengal as an anti-bacterial agent. The plates were incubated at 50°C in observing any growth.

The compost samples were also harvested after 3, 6 and 9 days of development at increasing temperatures of 40-45, 50 and 60-65°C, respectively. This sample along with decomposed bagasse and poultry droppings was washed with sterile water and cut into 1-2 cm pieces. These were then directly plated on BMCA plates:
triplicate and incubated at 50°C till the appearance of fungi.

The percent frequency of occurrence was determined from (3, 6, and 9 days of incubated and other unincubated samples) recording the appearance of any species in triplicate BMCA plates after five days incubation at 50°C.

The fungi were purified from bacterial contaminants using combination of penicillin plus streptomycin (10 mg/l). The purified strains were maintained on BMCA medium in slants. Identifications were made as described by Cooney and Emerson (1964).

FERMENTATION METHODS

The Liquid Fermentation (LF) was carried out in 500 ml erlenmeyer flasks containing 100 ml of medium. Where required, 250 ml and 1 litre flasks were also used. The different carbon sources were used at 2% concentration (unless otherwise mentioned) in E & P medium, set at pH 5.0. The uninoculated flasks were autoclaved at 121°C for 15 minutes. A seed inoculum prepared from a loopful of spores was harvested after an incubation of 30 hours and was used at 5% (v/v). The flasks were incubated at 4°C for thermophilic and 30°C for mesophilic fungi, at 120 rpm in an orbital shaker (Gallenkamp Co. U.K). The aliquots from duplicate flasks were pooled after 2-7 days and if required up to 7 days. The aliquots were centrifuged at 10,000 rpm for 10 minutes and supernatant refrigerated for different enzyme assays.

LF used at Fermentor level for enzyme production was carried out in a 14 litre fermentor (New Brunswick Scientific Co Inc. US). The working volumes were kept at 10 litres while kollar grass was used as a substrate in E & P medium at pH 5. Seed inoculum 5% (v/v) after an incubation of 24 hours at 45°C...
was mixed to the autoclaved (1 hour at 121 C) medium under sterile conditions. The rest of the conditions were as follows: aeration 0.5 (v/v/min), agitation 250-300 rpm, temperature 0 incubation 45 C. The medium pH effect was studied when it was allowed to fluctuate and when the initial pH was maintained a 5.0 by the addition of 1N HCl or 1N NaOH. Foam was controlled by the addition of antifoam agent. The condenser was charged with tap water for keeping the exhaust air cool. The samples were harvested after every 8 hours for enzyme assays.

The Solid State Fermentation (SSF) was carried out in 50 mL flasks, with 2 grams of carbon substrate. This was moistened with 3 parts v/v; unless otherwise mentioned of E & P medium set a pH 5.0. Spore inoculum (0.2 ml) in the range of 10^7 spores/mL was used. The flasks were incubated at 45 C for thermophilic and at 30 C for mesophilic fungi. The moisture conditions were controlled by providing a trough filled with water. After 2-7 day duplicate flasks were taken out and 40 ml of distilled water was added into each. The flasks were stirred gently for 10 minutes for the release of enzyme. The suspension was squeezed through muslin cloth and centrifuged as above. The supernatant was refrigerated for enzyme assays.

**INOCULUM PREPARATION**

For LF a seed inoculum was prepared by suspending a loopful of spores in 0.5% (w/v) cellobiose in the medium. The flasks were incubated at 45 and 30 C for thermophilic and mesophilic fungi for 24 hours, respectively. 5% (v/v) seed inoculum was used (unless otherwise mentioned) in all the experiments.
In SSF a spore inoculum was prepared by suitably diluting the spore suspension to about 1x10^7 spores/ml using (Petrof: Hauser) cell counter. Tween-80 (1-2 drops) was added to give a uniform suspension of spores. 0.2 ml of this spore suspension was used to inoculate 2 grams of substrate.

ENZYME ASSAYS

Different enzyme assays for cellulases and hemicellulases were performed, using various substrates. These substrates included filter paper (Whatman No.1), Avicel, carboxymethyl cellulose (CMC), larchwood xylan, P-nitrophenyl β-glucoside and P-nitrophenyl β-xyloside. The released reducing sugars were estimated by dinitrosaliclylic acid (DNS) reagent. The above chemicals were purchased from Sigma Co., USA.

Filter Paper activity (FPase activity)

FPase was determined by the method of Mandel et al. (1976) with a slight modification from Saddler et al. (1985). The unit values were calculated in the dilution range where approximatively 0.3-0.6 mg/ml reducing sugars were detected at the end of one hour assay. Culture filtrate (0.5 ml) after appropriate dilutions was added to 1.0 ml of 0.05 M citrate buffer, pH 5.0. Roller filter paper strips, 1-6 cm were added as the substrate and the reaction was carried at 50°C and 120 rpm for 1 hour. The reducing sugars were estimated according to Miller (1959) adding 3 ml of DNS reagent to the reaction mix and placed in a boiling water bath for 10 minutes, cooled to room temperature and the absorbance read at 550 nm.

Endo-1,4-β-glucanase or CMCase activity (EC 3.2.1.4)

CMCase activity was determined by incubating 1.0 ml of
appropriately diluted enzyme filtrate with 1.0 ml of 0.05M citrate buffer, pH 5.0 and 1.0 ml of 1% carboxymethylcellulose, for 10 minutes at 50°C. The method was modified based on initial rate of reaction (Wood and Bhat 1988). Reducing sugars were estimated by DNS reagent as above.

Exo-1,4-β-glucanase or avicelase activity

Avicelase activity was determined by incubating 1 ml of appropriately diluted enzyme filtrate with 1.0 ml of 0.05 citrate buffer, pH 5.0 and 1.0 ml of 1% microcrystalline avicel (Sigmacell 50), for 10 minutes at 50°C as for CMC-ase assay. Reducing sugars were determined by DNS method as above.

1,4-β-glucosidase or p-nitrophenyl β-glucosidase activity (EC 3.2.1.21)

The assay conditions were according to Rajoka and Mali (1984) and consisted of 0.2 ml of suitably diluted enzyme with 0.2 ml of 0.05 M citrate buffer, pH 5.0 and 0.2 ml of 5mM nitrophenyl-β-glucoside. The reaction was carried out for 10 minutes at 50°C. The color was developed by adding 3 ml of 0.1% Na2CO3 solution to read the extinction at 400 nm.

Exo-1,4-β-xylanase or larchwood xylanase activity (EC 3.2.1.8)

Xylanase activity was determined by incubating 0.2 ml suitably diluted enzyme in 0.2 ml of 0.05 M citrate buffer 5.0, along with 0.2 ml of 2% Larchwood xylan as a substrate. The reaction was modified as for avicelase assay.

1,4-β-xylosidase or p-nitrophenyl-β-xylopyranosidase activity (EC 3.2.1.37)

The assay conditions were followed from (Rajoka and Mali...
1984). 0.2 ml of diluted enzyme was added to 0.2ml of 0.05M citrate buffer, pH 5.0 along with 0.2 ml of 5mM p-nitrophenyl \( \beta \)-xylosidase. The rest of the conditions were as for \( \beta \)-glucosidase assay. Standards were run in parallel for avicelase and CMC-ase using 0.1% glucose, while for FP-ase and xylanase, 0.2% glucose was used. For \( \beta \)-glucosidase and \( \beta \)-xylosidase 30mM of p-nitrophenol was used as standard.

One unit of each enzyme was defined as the amount of enzyme required to release 1 \( \mu \)M of glucose equivalents from its corresponding substrate per ml per minute.

Extracellular protein was measured after method from Lowry, (1951), using Bovine serum albumin as a standard.

OPTIMISATION OF CULTURE CONDITIONS:

In SSF, the effect of moisture (medium) used in the ratio of 1:1, 3:1 and 5:1 (v/w) was carried out. Apart from this the effect of increased mineral medium concentration from zero to five fold was also studied.

In LF, six different media formulations, used by various workers for cellulase production were used. The composition of these media, as reported in g/l, is as follows:

Eggins and Pugh (1962) medium as described earlier. Romaneli et al. (1975) KH PO\( _4 \), (NH\( _4 \)) SO\( _4 \) 2.4, CaCl\( _2 \) 2H\( _2 \)O 0.3, MgSO\( _4 \) 2H\( _2 \)O 0.3. Trace element solutions, proteose peptone 0.75, ure 2 0.3 and Tween-80 0.5.

Coutes and Smith, (1976) used (NH\( _4 \))\( _2 \)H PO\( _4 \), 2.0, KH PO\( _4 \) 0.6 4 2 4 2 4 K HPO\( _4 \) 0.4, MgSO\( _4 \) 7H\( _2 \)O 0.5, yeast extract 1.0, mineral solution 2 4 4 2 thiamine, HCl 100 \( \mu \)g/l.

Saddler (1982) g/l: NaC\( _4 \)H\( _8 \)O 2.5, KH PO 5.0, NH\( _4 \)NO\( _3 \) 2.0 3 3 2 4 4 3
(NH₄)₂SO₄ 4.0, MgSO₄ 7H₂O 0.2, CaCl₂ 0.1, Peptone 1.0 Yeast extract 2.0, trace element solution 10 mg/l, vitamin solution 5 ml/l.

Macris and Panayatou (1986) used the medium in g/l: Na₂HPO₄ 1.2, KH₂PO₄ 2.0, (NH₄)₂HPO₄ 7.0, MgSO₄ 7H₂O 0.3, CaCl₂ 0.3, Proteose peptone 0.25, yeast extract 0.1, Tween-80 0.3, Trace elements solution

Sribir et al. (1982) NaNO₃ KCl 0.5, MgSO₄ 7H₂O 0.5, KH₂PO₄ 1.0, FeSO₄ 7H₂O 0.01.

Some other culture studies included:

Use of medium salts and nitrogen contents at two-fold concentration.

Use of different nitrogen sources at 0.05% concentration along with ammonium sulphate. In another combination yeast extract and ammonium sulphate were added along with other nitrogen sources. These were namely: α-asparagine, corn steep liquor, cotton seed flour, cas-aminoacids and urea.

The effect of different carbon sources on cellulase production was studied. These sources included: untreated kallar grass, treated kallar grass (2% NaOH, 121°C, 1h), avicel and α-cellulose.

Similarly effect of substrate concentration (2-10%) was studied for enzyme production.

The pH level in the range of 4-6 was used in order to find the optimum. Moreover, citrate buffer (0.05, 0.1, 0.5M) pH 5.0 was used to maintain the pH level.

The optimum incubation temperature of 30, 35, 40 and 45°C was used to study the effect on enzyme induction.
Miscellaneous additives namely; tween-80, trace element solution and vitamins solution were also used.

Enzyme Parameters

For determination of optimum pH the enzyme assays were performed at different pH in a range of (4-7) using citrate buffer (0.05M).

Similarly temperature optima were found after incubating the corresponding enzyme assays at temperatures of 50, 60, and 70 °C. Thermostability characteristics were determined after incubating the enzyme filtrates at 40, 50, 60 and 70 °C) for 24 hours. The relative FPase activity after the incubation time was evaluated to that at 0 hours.

ULTRAFILTRATION OF CRUDE ENZYME

Crude enzyme filtrate (500 ml) obtained after centrifugation was passed through ultrafiltration membrane cut-off size 20,000 daltons from Amicon Co. USA, in an ultrafiltration assembly RA2000 also from Amicon. The enzyme filtrate was passed at about 20 lb/in back pressure, through the ultrafiltration membrane. The enzyme was concentrated up to 5 times by volume. Diafiltration was carried out in order to remove the excessive salts in the concentration. The concentrated enzyme after determination of enzyme activities and losses incurred upon by ultrafiltration was used as such and also after freeze drying for saccharification purpose.

PRETREATMENT OF LIGNOCELLULOSIC SUBSTRATES:

Six lignocellulosic substrates used in this study consist of three plants growing in saline soils namely Leptochloa fusc (kollar grass) Panicum maximum (green panic) and Atriplex amnicc
la (river salt bush), were obtained from NIAB fields, Faisalabad.

The other substrates were namely: Panicum aestivum (wheat straw) Saccarum officinarum (bagasse) and Populus alba (poplar). The former two were also obtained from NIAB fields, whereas the latter was obtained from Sitara Chemicals, Faisalabad. They were harvested at peak periods of growth in summer and transported to Hamburg, F.R. Germany at the Institute of Wood Chemistry and Chemical Technology of Wood where these were kept in climatised rooms at 20°C for pretreatment studies.

STEAM EXTRACTION

About 100 g of chopped substrate each was treated in a laboratory defibrator (Martin Busch, Schermbek, FRG). After passing saturated steam for 10 min, the samples were defibrated for 30 s. The steam was then blown off and the humid fibre material thus obtained was washed with about 6 parts of tap water. The fibres were collected by passing through cheese cloth and pressue to remove liquor. The fibre materials after climatisation were ground in an electric grinder for further work.

SODIUM HYDROXIDE PRETREATMENT

Alkali pretreatment were carried out using 0.5mm partic size of LC fibre material with different concentration of NaOH from 2 to 4% in 5:1 (v/w) ratios. Pretreatment was carried at room temperature for 24 hours. In another alkali treatment the sample material after mixing with the NaOH concentration was autoclaved at 121°C for 15 minutes. After pretreatment, the material was washed free of alkali to neutralization. The fibre was acclimatised at 20°C and 65% relative humidity.
ANALYSIS TOTAL OF POLYSACCHARIDES BY CHROMATOGRAPHY

Dried fibre material before and after pretreatment were quantified for mass balance studies. Hydrolysis was carried on 200 mg sample using 2 ml of 72% sulphuric acid for 1 hour at 30°C. The hydrolyzate thus obtained was diluted with 56 ml water and autoclaved for 1 hour at 120°C. The contents were cooled, diluted to 100 ml and filtered through glass filters to analyse the sugars. The sugars were quantified on borate ion-exchange chromatography (Sinner et al., 1975) using 2, 2-bicinchoninic acid as the reagent (Sinner and Puls, 1987). The lignin was determined as acid insoluble residue (Klason lignin).

ANALYSIS OF SOLUBLE SUGARS

Powdered substrate were extracted with methanol-water (3:1) and the filtrate after evaporation were diluted, and analysed by ion-exchange partition chromatography, for sucrose, glucose, and fructose using a Shodex Ionpak S-801 column (Showa Denko K.K., Japan).

ASH CONTENT

Samples (5 g each) were ashed in a muffle furnace at 575°C for 3 hours till the residues was clear.

ENZYMATIC ACCESSIBILITY

The enzymatic accessibility tests after the different pretreatments were carried out using freeze dried enzyme preparat from Trichoderma reesei VTT-D-79125 (Bailey and Nevalainen, 1979) supplemented with cellobiase from Novo, Bagsvaerd (Novozyme 188). The reaction mixture consisted of 200 mg of dry substrate in 1 ml of 0.05 M ammonium acetate buffer pH 4.7 and the enzyme powder: 25 mg of cellulases (3 filter paper units) and 2 mg
Determining of enzymatic accessibility was carried out at 46°C for 24 hours. Appropriate blanks were run along with the tests. After incubation hydrolysates were filtered through sintered-glass filters. The residue was washed thrice with water and dried to determine the degree of solubilization or enzymatic accessibility. Enzymatic accessibilities were calculated on dry weight and cellulose content basis.

**CELLULASE SOURCES FOR HYDROLYSIS**

Commercial enzyme preparations included freeze dried cellulases from *Trichoderma reesei* mutant strain VTT-D-79125 and glucoamylase from *Aspergillus niger*. These preparations were found to contain the following enzyme activities in units/mg of FP-α and β-glucosidase of 1.0 and 0.71 for the former and 0.15 and 1.67 for the latter, respectively.

The crude enzyme preparations used for hydrolysis were mainly from *S. thermophile*, which was screened out during the initial studies, containing a complete cellulase. The enzyme filtrate used for various studies varied in its enzyme unit values. The enzyme filtrate used was obtained from 4 and 6% kallar grass, which contained 0.44, 0.88 and 0.8, 1.3 U/ml, respectively of FP-α and β-glucosidase. Moreover, maximal crude enzyme activity obtained at 6% kallar grass was further concentrated by ultrafiltration as mentioned elsewhere. The concentrate with increased activity at 1.4 and 5.0 U/ml for the above enzymes was used for saccharification along with its control. Comparison was also made with commercial enzyme preparations.

Further hydrolysis studies were carried out after freeze-drying the crude concentrate enzyme. The freeze-dried enz
contained the activities in U/mg preparation.

Culture filtrates by thermophilic fungi were obtained from 2% kallar grass for saccharification of 5% kallar grass at 50 or 60°C. Enzyme preparation from T. reesei was also used as a comparison. The enzyme titres for these fungi of FP-ase and \( \beta \) glucosidase were: A. fumigatus 0.5, 0.65; M. pulchella 0.03, 0.05; S. thermophile 0.5, 0.68; T. thermophile 0.5, 0.64; H. grisea and C. thermophile.

**SUBSTRATE AND PRETREATMENT**

Kallar grass obtained from BSRS during peak periods of growth was harvested and used for saccharification purposes extensively. After drying at 80°C it was milled at 0.5 mm size. Sodium hydroxide (2%) treatment along with autoclaving, found to be optimum, was used. Apart from kallar grass, filter paper was studied as a pure substrate for comparison. The filter paper was cut into 4-6 cm strips at random size.

**ENZYMATIC HYDROLYSIS**

Different titres/dosages of predetermined enzyme activities from commercial as well as crude enzyme preparations were used. The crude enzyme was used as such in the diluted form. The pH of the enzyme filtrate was adjusted to 5.0. The citrate or acetate buffer 0.05 M (wherever mentioned) was used to make up the reaction volume. Freeze dried enzyme was solubilised in 0.05M citrate buffer pH 5.0. The reaction mixture volume was varied from 20-ml in 100 ml conical flask as per requirement. The substrates were used at 2.5 & 10% concentrations in different experiments, but the standard concentration was used at 5%. The hydrolysis was carried out for 48-70 hours as per experimental conditions.
SUGAR ANALYSIS BY CHEMICAL METHOD

At different intervals of time 0.5 ml of sample was withdrawn from the hydrolysates, taking care that the slurry density of the withdrawn samples was the same as that in the remaining sample. The samples in microfuge tubes were centrifuged for 15 minutes at 300 rpm. The reducing sugar concentrations were determined by Dinitrosalicylic acid (DNS reagent) while the glucose (where mentioned) was determined by glucose kit (Human Co., UK).

SUGAR ANALYSIS BY HPLC

The sugar composition was determined in most of the experiments on HPLC (Gilson Co., France) using cation exchange color of aminoex HPX-87H running 0.001N H SO₄ as eluent. The flow rat 2 4 was kept at 0.6 ml/min at a column temperature of 85 C and chart speed of 5 cm/sec. The sugars were detected on a refracti, index detector from Shimadzu Co., Japan, while the column oven was also from the same company. The peak recorder for various sugars also belonged to Gilson Co. The standard sugars for glucose, D-xylose and cellobiose (all GLC grade sugars) were run to standardize the condition. Samples after suitable dilutions were injected (25ul) through a rheodyne injection valve.

The saccharification yields were calculated using the equ
Saccharification (%) = Reducing sugars formed x 0.9 x 100
                            carbohydrates in kollar grass (0.7%)

Glucose Yield % (based on total polysaccharides) = Glucose formed x 100
                                                   carbohydrate in kollar grass (0.77)

Glucose Yield % (based on total cellulose) = Glucose formed x 100
                                            potential glucose in k. grass (0.66)
REVIEW OF LITERATURE

MICROBIAL CELLULASES

Bisaria and Ghose (1981) listed 13 species of fungi and bacteria, capable of producing cellulases which can extensively break down insoluble cellulose in-vitro. The fungal species included Trichoderma reesei, T. viride, T. koningii, T. lignorum, Penicillium funiculosum, Aspergillus wentii, Sclerotium rolfsi, Sporotrichum pulverulentum and Fusarium Solani while the bacterial species included Cellulomonas along with Clostridium thermocellum. The present review has been limited to fungal cellulases because of the nature of work being conducted.

Cellulolytic Mutants

T. reesei (QM6a) isolated from deteriorated cartridge in New Guinea during World War II, was detected as a potent cellulolytic organism by Elwyn Reese, around 1950. Numerous mutants have been developed from it since then at Natick (Mande et al., 1975; Andreotti et al., 1978; Mandels and Andreotti, 1976; Galo and Andreotti, 1979; Ryu and Mandel, 1980; Andreotti et al., 1980) and at Rutgers (Gallo, 1978, 1982; Montene court et al., 1981). These mutants produced higher levels of constitutive as well as inducible cellulases but showed catabolite repression which was later reduced for some of the mutants (Casebier et al., 1969; Sudo et al., 1976; Du Toit et al., 1984). Quantitative comparison among the mutants is difficult because fermentation conditions are not the same for all the strains. Depending upon conditions, best Natick and Rutgers strains produced 3-20 fold higher cellulase yield (Table 1) than the wild type QM6a.
Table 1: Characteristics of cellulase production from various *Trichoderma reesei* strains under batch and continuous modes of operation. Source: Kosaric et al (1983).

<table>
<thead>
<tr>
<th>Process</th>
<th>Productivity (IU L⁻¹ h⁻¹)</th>
<th>Concentration (IU mL⁻¹)</th>
<th>Strain</th>
<th>Particulars</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 11(162 h)³</td>
<td>1.85 (162 h)</td>
<td>—</td>
<td>D1-6</td>
<td>1-2% cellulose</td>
<td>ANDREOTTI et al.</td>
</tr>
<tr>
<td>0 57(192 h)</td>
<td>11 (192 h)</td>
<td>—</td>
<td>MCG-77</td>
<td>6% cellulose</td>
<td>GIOVANI et al. (1983)</td>
</tr>
<tr>
<td>0 42(92 h)</td>
<td>3.9 (92 h)</td>
<td>—</td>
<td>NG-14</td>
<td>2% cellulose</td>
<td>GIOVANI et al. (1983)</td>
</tr>
<tr>
<td>0 36(67 h)</td>
<td>3.6 (164 h)</td>
<td>—</td>
<td>QM 9414</td>
<td>2% cellulose</td>
<td>GIOVANI et al. (1983)</td>
</tr>
<tr>
<td>0 36(163 h)</td>
<td>—</td>
<td>11.5 (120 h)</td>
<td>MCG-77</td>
<td>6% cellulose bi-level pH control/biotin supp.</td>
<td>GALLO (1982)</td>
</tr>
<tr>
<td>0 85(120 h)</td>
<td>13.3 (120 h)</td>
<td>—</td>
<td>RUT-C30</td>
<td>6% cellulose bi-level pH control/biotin supp.</td>
<td>GALLO (1982)</td>
</tr>
<tr>
<td>0 95(120 h)</td>
<td>13.3 (120 h)</td>
<td>—</td>
<td>RUT-C30</td>
<td>3% cellulose</td>
<td>GALLO (1982)</td>
</tr>
<tr>
<td>0 142(96 h)</td>
<td>17.2 (96 h)</td>
<td>MCG-80</td>
<td></td>
<td>3% cellulose</td>
<td>MUKHPADHYA (1980)</td>
</tr>
<tr>
<td>0 25(180 h)</td>
<td>4.5 (80 h)</td>
<td>QM9414</td>
<td></td>
<td>3% cellulose</td>
<td>MUKHPADHYA (1980)</td>
</tr>
<tr>
<td>0 38.75</td>
<td>6.2</td>
<td>QM9414</td>
<td></td>
<td>3% cellulose pH cycling</td>
<td>MUKHPADHYA (1980)</td>
</tr>
<tr>
<td>0 44</td>
<td>7.2</td>
<td>QM9414</td>
<td></td>
<td>3% cellulose pH and temp. cycling</td>
<td>MUKHPADHYA (1980)</td>
</tr>
<tr>
<td>Continuous (1 stage)</td>
<td>0.03-0.08</td>
<td>5.13</td>
<td>0.33</td>
<td>0.4% - 1.1% cellulose feed</td>
<td>PETERSEN (1975)</td>
</tr>
<tr>
<td>Continuous (2 stage)</td>
<td>0.03-0.08</td>
<td>8.75</td>
<td>—</td>
<td></td>
<td>MANDERS et al. (1975)</td>
</tr>
<tr>
<td>Continuous (2 stage)</td>
<td>0.03-0.08</td>
<td>21.2</td>
<td>—</td>
<td></td>
<td>SAHI et al. (1975)</td>
</tr>
<tr>
<td>Continuous (recycle)</td>
<td>0.015</td>
<td>2.6</td>
<td>0.12</td>
<td>1% glucose (1st stage) cellulose (2nd stage)</td>
<td>MITRA et al. (1975)</td>
</tr>
<tr>
<td>Continuous (immobilized cells)</td>
<td>0.015</td>
<td>1.2</td>
<td>0.08</td>
<td>1% cellulose fed semicontinuous</td>
<td>BROWN et al. (1975)</td>
</tr>
<tr>
<td>Continuous (recycle)</td>
<td>0.02</td>
<td>16</td>
<td>0.30</td>
<td>1% cellulose support loading</td>
<td>WILKE et al. (1981)</td>
</tr>
<tr>
<td>Continuous (immobilized cells)</td>
<td>0.026-0.028</td>
<td>90</td>
<td>NCG-77</td>
<td>2 stage lactose induced</td>
<td>RYU et al. (1981)</td>
</tr>
<tr>
<td>Continuous (immobilized cells)</td>
<td>0.10</td>
<td>17.42</td>
<td>0.17-0.42</td>
<td>RUT-C30</td>
<td>MONTENECOU (1981)</td>
</tr>
<tr>
<td>Continuous (immobilized cells)</td>
<td>0.10</td>
<td>0.0-1.51</td>
<td>RUT-C30</td>
<td>Lactose induced</td>
<td>MONTENECOU (1981)</td>
</tr>
<tr>
<td>Continuous (immobilized cells)</td>
<td>0.10</td>
<td>0.0-1.93</td>
<td>RUT-C30</td>
<td>Lactose induced</td>
<td>MONTENECOU (1981)</td>
</tr>
</tbody>
</table>

Time in brackets indicates the time of optimal productivity and cellulase concentration for batch cultures.
disadvantages of Trichoderma enzymes are 1) the inability to metabolize lignin ii) the low specific activity of cellulase i) low levels of β-glucosidase (Mandels 1982). Here is yet another area where genetic engineering can play a vital role. There have been reports on cloning of cellbiohydrolase gene from T. reesei (Shoemaker et al., 1983; Montene court, 1983; Teeri et al., 1983) Escherichia coli but the specific activity remained unchanged. Same was reported of endoglucanase from Cellulosomata (Whittle al. 1982, and Gilkes et al., 1984); Thermonospora (Collmer Wilson, 1983); Clostridium thermocellum (Cornet et al., 1988) Bacillus strains (Hincliffe, 1984) and Scyzophillum commute (Faber et al., 1983); cellobiase from Agrobacterium (Wakarchuk al., 1984) and xylanase from Bacillus polymyxa (Yang et al., 1986). Cellulolytic bacteria have been extensively studied for saccharifying ability of their cellulases (Choudhury et al., 1987 Lynd and Greathlein, 1987; Rajoka and Malik, 1984). Durand et al., (1988) reported mutant of T. reesei CL 847 to produce cellulase with increased specific activity. Schulein, (1988) has recently described the cellulase components from T. reesei. Cellulase families and their genes have been discussed by Knowles et al., (1987) and Glick and Pasternak (1989). UV induced mutant MC1 from T. reesei Rut C-30 showed almost similar activity as parent strains but could produce cellulases from different carbon sources other than cellulose (Allen and Andreotti, 1982). Sadeh et al., (1987) compared T. reesei QM6a and its various mutant QM-9414, NG-14, Rut C-30, MCG-77, RL-P37, and CL-847. The authors reported increased protein content from 7 mg/ml to 22 mg/ml FPase productivity to 125 IU/L/h. Further improvement can
achieved by using novel fermentation processes (Turker and Mavtuna, 1987).

Thermophilic Cellulolytic Fungi

Thermophilic species are well documented for their thermoslabile enzymes, high rate of cellulolysis, low amounts of enzyme required, non-aseptic conditions for saccharification (Margaritis and Merchant 1986a). Use of thermostable cellulase titres produce fermentable carbohydrates at elevated temperatures making the saccharification process cost attractive. Chahal, 1983 and 1986; Durand et al, 1984 a 1985; Merchant, 1984; Merchant et al, 1988. Cooney and Emers (1964) have reviewed thermophilic fungi to comprise a restrict number of species. These fungi grow at elevated temperatures above 50°C but not below 20°C. Their occurrence has been reported self heating piles of organic matter, often causing biodeterioration of agricultural (Cooney and Emerson, 1964; Flanningan, 1964 and forest products (Tansey, 1970). Eggins and Malik, (1964) reported the isolation of thermophilic fungi from grass, which showed the ability to breakdown ball milled cellulose (BMC) producing clearings in the solid medium. Similarly, Malik et al, 1973 in these laboratories isolated thermophilic fur which showed extensive breakdown of BMC. Various other report thermophilic species include: Chaetomium, thermophile (Romane et al, 1975; Ericksen and Goksoyr, 1976 and 1977) Thiella

Other Potent Fungi

Another very well studied genus is *Aspergillus*. These are reported to yield endo-glucanases and B-glucosidases but lack substantial exo-glucanase activity. However, when mixed with *Trichoderma* cellulases the enzyme complex is reported to be a very potent system. Duff et al., 1985 used a mixed culture of *Trichoderma reesei* Rut C-30 and *Aspergillus phoenicis* ATCC 329 in order to get a complete and balanced cellulase. Similarly, Pand et al., (1987) used mixed culture of *T. reesei* D1-6 and *A. wenti* Pt 2804 to produce cellulases in high titres. Considering the economics of the process the authors used successive cultivation of selected fungi on rice straw and wheat bran for production of cellulases and xylanases after recycling the same substrate five times.

The cost of enzymes at present takes a fair share of 50-60% of lignocellulosic (LC) hydrolysis. About 30% is spent for pretreatment and the rest is value of the substrate, etc. Thus an ideal organism could be realized as the one that produces a high titre of a complete cellulase, utilizes cheap carbon and nitrogen sources without showing catabolite repression. The enzyme should have high specific activity and stability at high temperature and remain insensitive to end product inhibition and to sheer stress effects.

**CELLULASE PRODUCTION BY FUNGI**

Cellulases are constitutive as well as inducible enzymes. Their biosynthesis is repressed by the presence of soluble sugars or other easily metabolizable substrates. Some of the cellula
producing microorganisms have shown to induce more enzyme activities from pure celluloses, others from untreated or treated L while still others from different carbon sources. It is difficult to make a conclusive comparison in the assessment of these various strains as to their efficiency of cellulase production and degradation. Table 1 shows the filter paperase (FPase activity of a number of T. reesei strains under varying mode c fermentations. Productivity and concentration of the enzyme is quantified in terms of international unit defined as umol r reducing sugars released per minute from a filter paper substrate. The variation in activities may be due to various factors including the organism, type of inducer, type of growth conditions, methods of harvesting, concentration, as well the method of fermentation. Many of the microbes are deficient in producing one or the other component in the cellulase complex. Since the enzymes act in a synergistic fashion the presence of each one of them in optimal relative level would be most suitable (Herr, 198 Woodward and Wiseman, 1982).

Most of the microbes produce maximal cellulase activity when grown on pure cellulose as in the case of Trichoderma s (Table 1). But the cost of pure substrate and the option utilizing cheap available LC provokes one to find better alternatives. This includes the isolations and development of strains (mutagenesis and/or genetic engineering) which can grow profuse on the waste LC substrates, to produce concentrated enzyme titr and the use of optimal fermentation conditions in solid state fermentation (SSF) and liquid fermentation (LF).
Cellulase Production By SSF

SSF is similar to the Koji process of Toyama (1976) and is an attractive alternate to use LC substrates than costly cellulosic substrates in higher than 6% concentrations (Chahal, 1982 and 1985; Shamala and Sreekantiah, 1986). SSF has been greatly exploited by many other workers (Rao et al., 1983; Duff et al., 1986; Gibbons and Westby, 1986). Chahal (1985) described SSF as a most practical and comparatively cheaper process for the production of complete cellulase complex in reesei using LC substrates. Wheat straw as a substrate yielded enzyme activities of 17.2, 21.2 and 540 IU/ml of cellulases, β-glucosidases and xylanases, respectively. SSF has been reported by many workers: Sternberg et al., (1976) used wheat bran to get a yield of 200 U/g of β-glucosidase in A. phoenicis. Deschamps and Huet (1984) report potato starch and sugar beet pulp to induce β-glucosidase to 66 and 550 IU/g, respectively after 4 days of incubation. Kim et al. (1985) used a combination of starch and wheat bran to obtain higher CMCase activity of 60 U/ml by T. reesei QM 9414 than that of 50U/ml by S. cellulophilum after 50-70 hours of cultivation. Shamala and Sreekantiah, (1986) used rice straw (RS) and wheat bran (WB) to induce enzyme activities in various fungi under optimal conditions. A. ustus gave the highest β-glucosidase activity of 60 U/g WB and a xylanase activity of 740 U/g RS after a incubation of 5 days. A mixture of seven parts of RS and three parts of WB, mixed with 40 parts of Toyama's mineral solutic yielded 6U FP-ase, 40U β-glucosidase, 12U CMCase and 650U xylanase per gram substrate. Grajek (1987a) used a combination of sugar beet pulp and cellulose powder (8:2) and induced maxim
activity of 1.3, 5.3 and 8.9 U/ml for exo-glucanase, endo-glucanase and β-glucosidase after an incubation of 3-5 days by *Thermosascus aurantiacus*. Sanyal et al. (1988) in *Aspergillus japonicus* have shown that barley husk induced specific activity of CMCase 8.0 and β-glucosidase 2.0 U/ml, whereas, wheat bran induce 2.0 and 0.48 U/ml, respectively after six days of growth. Madanwar et al. (1989) found 5M NaOH treated bagasse as the best substrate yielding maximal enzyme activities of 12.1, 21.5, 7. of CMCase, FPase and β-glucosidase, respectively. Other treated substrates induced in the order computer cards > corn cobs > dust for cellulase production. Apart from the advantages in SSF there are a few bottlenecks. Although, the enzyme is concentrated in SSF but the enzyme activity per gram of substrate is significantly higher in LF process in most of the strains. This might be due to problem areas in SSF such as aeration, agitator and mixing, heat dissipation, pH control, etc.

**Cellulase Production BY LF**

To date LF is the method of choice for the production of most of the industrial enzymes because of the considerable advances being made in this area especially in the designing of fermentors. The fermentations can be batch, fed-batch, semi-continuous and continuous. An advantage of fed-batch is that the addition of substrate and nutrients can be added as per requirement. Hendy al. (1984) in *T. reesei* Rut C-30 exhibited optimum productivity 130 U/L/H in a fed-batch cultivation at a feed rate of 1.7 solka floc, while the enzyme activity of 26.0 U/ml of celllobioh drolase as compared to 8.4 U/ml in batch culture was obtained
Semi-continuous and continuous process for cellulase production are less common because of insoluble nature of the substrate. A mutant of *T. reesei* CL-847 has been able to produce β-glucosidase constitutively using glucose or lactose in a continuous culture (Durand et al, 1988). Allan and Andreotti (1982) reported cellulase productivity of 168 IU/L/H in fed batch culture by *T. reesei* MCG-80 while batch culture showed 101 IU/L/H. Watson et al (1984) using *T. reesei* Rut C-30 produced a maximal level of 57 U/ml for cellubiohydrolase at a productivity rate of 201 IU/L/HR. Some of the other reported work in LF on cellulase production from cellulosic substrates include: Enari et al, (1975) in *T. viride* using 2% solka floc plus 1% glucose among different cellulosic substrates induced a maximal of 8.8 and 11.0 U/ml of endoglucanase and cellubiohydrolase after 6 days of fermentor cultivation, respectively. Coutts and Smith (1975) in similar studies using 1% solka floc induced in *S. thermophile* almost similar levels of cellulolytic activity in less than one fourth the time as in *T. viride*. Skinner and Tokuyama (1978) reported 1% cellulose powder to yield a maximal cellulase activity *T. terrestris* releasing 12.0 and 89.0 mg/ml of glucose for FP-ase and CMC-ase, respectively, after 48 hours of incubation only. Other substrates followed the order of enzyme induction of newsprint > bagasse > cotton gin trash > wheat bran. Ryu and Mandels (1980) using 6% two roll milled cotton obtained a maximal of 150 U/ml of endoglucanase from *T. reesei* Rut C-30, while the mutant NG-14 yielded a maximal of 15, 0.6 and 21 U/ml of cellubiohydrolase, β-glucosidase and protein, respectively after 12 days of fermentor cultivation. Dashpande et al, (1984) using 10% cellulose obtained 20.0, 2.0
and 0.5 U/ml of endoglucanase, cellobiohydrolase and β-glucosidase activity by *Penicillium janthinellum* after 11 days of shake flask incubation. Breuil (1986) revealed that *T. terrestris* C 46 grown on 2% avicel yielded the highest combined β-glucosidase (intracellular and extracellular) activity of 32.0 U/ml and a rather low cellobiohydrolase of 0.55 U/ml after 7 days of incubation.

**Cellulosic And LC Substrates**

LC's hold much more promise than pure cellulosics as far as their abundance and economics is concerned, however, only a few organisms in LF have shown high productivity on them. Gokhale (1984) using a combination of 3% wheat bran and 2% cellulose obtained a maximum of 10, and 22.6 IU/ml for β-glucosidase and cellobiase, respectively. Khan and Lamb (1984) reported almost a similar level of enzyme activities produced from steam exploded aspen wood when 10-20% of pure cellulose was added to it that compared to pure cellulose, only. Hoffman and Wood (1985) showed that 1% hammer milled barley straw induced the enzyme activity in *P. funiculosum* IMI 87160 to a level of 0.31 and 1.87 U/ml of cellobiohydrolase and xylanase, respectively, while the soluble protein was 0.61 mg/ml. Saddler et al (1985) reported steam exploded water extracted aspen wood (SEAW) as more suitable for enzyme induction in *T. harzianum* yielding 66, 2.3, 2.8 and 45 U/ml for endo-glucanase, β-glucosidase, cellobiohydrolase and xylanase, respectively. In comparison *T. reesei* Rut C-30 showed an activity of 73, 0.2, 4.5 and 140 U/ml, respectively, for these enzymes, respectively after 5 days of shake flask incubation. SEAW was followed in the order of induction by solka floc > S
aspenwood xylan. The last substrate yielded maximum xylanase activity. Bhat (1987) using blotting paper from bamboo shoots, obtained enzyme activities of 0.1, 1.2, 0.18 and 1.65 U/ml of FPase, endo-glucanase, exo-glucanase and β-glucosidase, respectively, in a local strain of *S. thermophile*. Brown et al. (1987a) isolated a number of over-producing strains from *P. pinophilus* 87160111 after u.v. mutagenesis and/or chemical treatment. The mutant MTG III/6 which also showed higher Vmax value from the kinetics, induced the cellulase activity from 1% hammer milled barley straw up to 1.5, 16.2, 6.9 and 4.6 U/ml for FP-ase, CMC-ase, xylanase and β-glucosidase, respectively while 2.4 mg/ml of protein activity was obtained after 10-14 days of incubation. Steiner et al. (1987) in *Schizophyllum commune* using 4% avicel obtained 5.0, 1244, 108 and 65 U/ml of FP-ase, xylanase, β-glucosidase and CMC-ase, respectively, after 11 days of shake culture fermentation. In a laboratory fermentor the respective activities were 4.5, 200, 100, and 60 U/ml. Yu et al. (1987) screening twenty one strains of thermophilic fungi found maximum production of xylanase activity (575.9 U/ml) in *Thermoaerium amenta* strain C 436 from 1% solka flocc after 7 days in shake flasks. Cellulase activities of 10.1, 0.16 and 6.74 U/ml were obtained for endo-glucanase, FP-ase and β-glucosidase, respectively. Solka flocc was followed by ball milled saw dust, steamed exploded water insoluble aspen SEA-W1 and untreated saw dust for cellulase and xylanase activities. Acebal et al. (1988) reported maximal FPase production of 1.25 IU/ml by *T. reesei* QM9414 when grown on 1% wheat straw pretreated with 1% NaOH, 120 C, 2atm. The addition of raw material elongated the exponential phase, whi
the activities remained same. Mes Hartree et al (1988) steam pretreated (240 C, 180 sec) aspen wood samples to yield a maximum of 59.2, 2.6 and 1.5 U/ml of endo-glucanase, FP-ase and β-glucosidase in T. harzianum after 4 days of incubation. Acid impregnated steam treated samples could not enhance enzyme activity to similar extent. The pretreated substrate yielded higher enzyme activities than the pure substrate. Jan and Tiraby (1987) using 3% paper pulp and 15% wheat bran for enzyme production in Talaromyces sp. CL 240 and I. reesea CL 487 obtained increased enzyme activities in all except FP-ase from the former substrate. The FP-ase activity at 65 C showed even higher activity for Talaromyces sp. because of the thermostable enzymes.

OPTIMUM CULTURE CONDITIONS FOR CELLULASE PRODUCTION

Environmental factors have a fundamental role in the growth and product formation of the microbe. These factors include, apart from a carbon source, nitrogen source, ionic concentration, pH, temperature, incubation period, stirring speed and aeration, etc.

Nitrogen Source

Medium composition and growth factors need to be optimised for exploiting the microbial system for getting maximum possible yield. Along with the carbon source, the choice of the nitrogen source is of great importance. The use of well known and expensive nitrogen sources might be excellent for laboratory optimisation studies but not for the industrial use. The nature and concentration of both nitrogen and carbon source affect the profile during a cultivation process without pH control. I reesea strains grow rapidly on a rich organic nitrogen sourc
like peptone, inorganic nitrogen sources of ammonium sulphate, ammonium phosphate and ammonia. But it is unable to utilize nitrate. Complex nitrogen sources containing amino acids stimulated growth (Montenecourt and Eveleigh, 1977; Ryu and Mandels, 1980). Pourquie and Vandecasteele (1984) reported a selected strain of T. reesei CL547 which surpassed the previous best results when grown in a mineral medium containing in g/l: lactose 60 and yeast extract 1 in liquid fermentation (LF). In SSF, the addition of nitrogen and mineral requirements decreases with decrease in liquid volume. Kim et al. (1985) in SSF used only wheat bran with a suitable moisture content in a fermentor adequately supplied with air. Shamaia and Sreekantiah (1986) in SS used Toyama's mineral medium containing (NH₄)₂SO₄ (0.1%) only a nitrogen source. Apart from this the authors also used Rees and Mandels medium which contained (NH₄)₂SO₄ (0.14%), urea (0.03%), yeast extract (0.01%) and peptone (0.025%). Toyama's mineral medium mixed with rice straw was found to be better for cellulase and xylanase production. Brown et al (1986b) in 1 using a mutant of Penicillium pinophilum MTG III/6 obtain optimum activity using 6% solka floc and/or hammer milled barley straw along with nitrogen sources in g/l of (NH₄)₂SO₄ 1.4, urea 4.2, yeast extract 0.3 and proteose peptone 1.0 in Mandels and Weber medium using an instrumental fermentor.

Romanelli et al (1975) working on thermophilic fungi found that addition in g/l of organic carbon 15 and nitrogen (peptone 0.75 and urea 0.3) in a mineral medium markedly increased t cellulose degradation. Among the tested fungi Sporotrichum the mophile showed the highest rate of cellulose utilization follow
by *Chaetomium thermophile* and *Thermosascus aurantiacus*. Skinner and Tokuyama, (1978) reporting optimum conditions in their patent on *Thielavia terrestris* used the nitrogen content in g/l of Corn steep liquor 2.0 and peptone 1.5 along with cellulose powder 10, in the nutrient medium. Coutts and Smith (1976) working on *S. thermophile* reported maximum production of cellulases when NaNO$_3$ or urea were used as sources of nitrogen. However, when used at varying concentration of 0.05-0.4% it had little effect on increase in cellulase activity. Working on this organism, Canevascini et al, (1979) used Eggins ad Pugh (1962) medium but without α-asparagine. They replaced it with iron ammonium citrate and also added trace mineral elements. While in an another composition the authors replaced ammonium sulphate with cas-amino acids. Grajek (1987b) working on the same organism used in g/l: Carbon source 20 along with yeast extract 0.1 and $(NH_4)_2SO_4$ 1.0. Among the various supplemented nitrogen sources; ammonium sulphate, ammonium chloride and potassium nitrate appeared to be the best for β-glucosidase production. The amount of supplemented nitrogen was calculated at 40 g of total nitrogen per kilogram o carbohydrates.

**Ionic Strength**

The addition of proper and adequate mineral contents in the basal medium is a basic requirement for optimum growth and product recovery from the microbe. Various scientists have use mineral contents in the medium after appropriate designing base on the specific requirement of the microbe. The nutritional requirements increases with the increase of carbon source to certain extent above which osmotic/inhibitory effects are being
experienced (Wase et al, 1985). Eggnins and Pugh (1962) designed a medium for thermophilic fungi which contained the mineral content in g/l: KH PO 1.0, NH SO 0.5, KCl 0.5, MgSO 0.2, CaCl 0.1, along with organic nitrogen source of γ-asparagine 0.5 and yeast extract 0.5. Romanelli et al (1975) also designed a medium composition for thermophilic fungi which contained in g/l: KH PO 2.0, (NH) SO 1.4, CaCl 2H O 0.3, MgSO 4.0.03, along with peptone 0.75, urea 0.3 and Tween-80 0.5. Coutts and Smith (1976) formulated medium for S. thermophile which contained in g/l: NH4 HPO 2.0, KH PO 0.6, K HPO 0.4, MgSO 4.0.05, yeast extract 1.0, trace mineral solution and thiamine HCl 100 μg/l. Wase et al, 1985 working on Aspergillus fumigatus used elementary statistical techniques. The endo-glucanase yields were enhanced to 28% when the carbon source along with potassium phosphate and ammonium sulphate were increased to five fold. However, the increase in peptone and MgSO had negative effect. Brown et al (1966b) optimising conditions for Penicillium pinophilium mutant NTG III/6 used Mandels and Weber (1969) medium. This consisted in (g/l) of (NH) SO 1.4, urea 0.3, proteose peptone 1.0, KH PO 2.0, CaCl 2.4, 2.2.4, MgSO 4.2, 7H 0 0.3 and Trace element solution. Ball milled barley straw 6% yielded the maximum enzyme yields as compared to solka floc.

pH

The optimum pH for growth rate may be different from that of growth yield and entirely different from the optimum for product formation. The ammonium salts supplied to a culture as sole nitrogen source leads to ammonium uptake and a decrease in pH. By the use of nitrate ion and ammonium ions simultaneously the hydrogen
concentration can be kept balanced (Porage et al., 1964). The use of NaNO<sub>3</sub> to keep the pH at a higher level is also reported by Umezurike (1970) and Hulme and Stranks (1971). However, highly cellulolytic culture filtrates produced under acidic conditions have been reported by Mandels and Weber, 1969 and Neudoerffer and Smith, 1970. Ryu et al. (1982) working on the cellulase biosynthesis, separated the growth phase which was optimised at pH 4.5, temperature 32°C and a high dilution rate. The enzyme production phase was optimised with pH 3.5, temperature 25°C and a low dilution rate. This initial lowering in pH goes well with most of the Trichoderma strains (Nystrom and Luca, 1977; Ryu and Mandels, 1980; Galo, 1981; Watson et al., 1984. In fact, Trichoderma strains lower the medium pH to acidic when left uncontrolled to a level where enzyme synthesis stops (Allen and Mortensen, 1980; Mukhopadhyay and Malik, 1980; Garg and Neelakantan, 1982). The latter strains such as Rut C-30 (Tangnu et al., 1981), showed appropriate results at pH 5.0 and 23°C. Shulz and Hirte (1989) working on Penicillium janthinellum reported sudden fall in the pH level with increasing substrate concentration when the pH was not controlled. This resulted in very low cellulase activity of the culture filtrate.

Romanelli et al. (1975) working on thermophilic fungi revealed that addition of small quantity of organic nitrogen such as peptone does not allow the pH to fall below 4.0. This effect allows the enhancement in cellulase activity. These results were found to be more pronounced by Coutts and Smith (1976) while working on S. thermophila. They found that the organism grew well at higher pH values and produced greater yield of cell
lases. It has been suggested, that in thermophilic fungi two distinct groups based on pH tolerance exist. The one that grows well at alkaline pH is commonly associated with composts and the other associated with soil, growing best below pH 6.0. *S. thermophile* belongs to the former group (Coutts and Smith, 1975). The same is also reported for *Chaetomium thermophile* (Erickson and Goksoyr, 1976). Skinner and Tokuyama (1978) reported pH 5.5-5.5 as the optimum for cellulase production. *Thielavia terrestris* Grajek (1987b) reported pH 6.5 as optimum for *S. thermophile*.

**Cultivation Temperature**

Most of the well known mesophilic species of *Trichoderma*, *Penicillium* and *Aspergillus* grow best at 28-30°C (Ryu and Mandel 1980; Mandels, 1982; Gokhale et al. 1984; Durand et al., 1984). The increased cultivation temperature of 45-52°C adopted by thermophiles for growth could be beneficial in reducing the risk of microbial contamination, cooling requirements for the fermentation process, decrease the cultivation time with concomitant increase in enzyme kinetics (Margaritis and Merchant, 1983 and 1986; Yu et al., 1987). These high temperature are exhibited by various thermophilic fungi. *S. thermophile* (Coutts and Smith 1976); *C. thermophile* (Eriksson and Goksoyr, var. dissitum 1976) *A. fumigatus* (Vandamme et al., 1982); *Myceliophthora thermophila* (Sen et al., 1981); *Talaromyces emersonii* (Folan and Coughla 1979); *Humicola insolens* (Yoshioka and Hayashida, 1980); *Humicola grisea* var. thermoidea (Yoshioka et al., 1982). *Thielavia terrestris* (Margaritis and Merchant 1986); *Thermoascus aurantiacus* (Y et al., 1987); Different thermophilic fungi (Grajek 1987).
Cultivation Time

Incubation time for optimum enzyme production is important parameter as far as the economics of the process are concerned. The shake flask studies with the Trichoderma reesei QM 9414 and Aspergillus sp. yielded maximum cellulase production after 12-14 days of incubation (Ryu and Mandels 1980; Gokhale et al., 1984). However, the strain MCG-77 showed maximum cellulase production in six days. Similar results were obtained from T. reesei CL847 in fed-batch fermentation (Pourquie and Vandecasteel 1984). Tannse (1971) found that the cellulolytic rate of some thermophilic fungi was two to three times that of Trichoderma spp. Romanell et al. (1975) found that S. thermophile degraded cellulose faster in liquid culture. Mandels (1975) also noted that thermophilic fungi degraded cellulose rapidly but produced cellulase activity in low titres. This fact was also confirmed by other scientists (Coutts and Smith, 1976; Bhat and Maheshwari, 1987. Skinner and Tokuyama, 1978) reported that T. terrestris (NRRL 8126) yielded maximum cellulase activity only after 48 hours at 48°C. Durand et al. (1984) working on two thermophiles and two mesophiles species, reported maximum cellulase yields for the former after days whereas it was after 4 days for the latter. Grajek (1987) found that maximum β-glucosidase from S. thermophile was attained after six days of incubation in fermentor, although the maximum value obtained after 3 days was only a bit lower. Grajek (1987) working on different thermophilic fungi obtained the cellulase yields after 3 days in LF while in SSF the incubation were carried out for 3-5 days.
Agitation and Aeration

In the conventional shake flask culture the aeration is limiting factor; even if the agitation is increased. Yoshida et al. (1968) reported that oxygen penetrated the peripheral 4 mm of the pellets while Kobayashi et al. (1973) enunciated that the central biomass of pellets of *Aspergillus niger* becomes oxygen limited before they attain a radius of 250 μm. Nevertheless, the difference in mode of growth and aeration requirements vary from organism to organism. Ryu and Mandels (1982) suggested an adequate oxygen transfer rate in the fermentation broth during growth and enzyme production. In order to reduce the lag phase with a concomitant increase in the exponential phase for a greater period of time, fed-batch or continuous culture mode adopted. Ryu and Mandels (1980) used a 10 litre submerged culture, maintained by aeration set at 2 litres/min at 7 psig pressure with varying agitation between 300 to 500 rpm. Watson et al. (1984) working on *T. reesei* Rut C-30 used a laboratory fermentor (11 L working volume) to control the agitation and aeration to maintain a dissolved oxygen content of not less than 20% of saturation. They also maintained a cellulose feed rate of 1 g/l/h supplemented with pure oxygen at certain stages of fermentation to achieve a productivity of 201 IU/l/h. Grajek (1986) working on *S. thermophile*, maintained an aeration of 60 l/l/h and agitation of 250 rpm in an 8 litre working volume fermentor for cellulase production. Very short lag phase period, limited to one hour, and the rapid exponential growth accompanied by large oxygen consumption were noticed.
ENZYME CHARACTERISTICS

pH

The pH optima for cellulase activities in Trichoderma sp has been found to be 4.8 using 0.05 M citrate buffer (Mandels al. 1975; Sternberg et al. 1976; Ryu and Mandels, 1980. Other workers have used acetate buffer, 20 mM, (Pourquie and VandeCasteel, 1984; Mes-Hartree, 1988). The optimum pH found for activity of A. phoenicis, β-glucosidase was about 4.3. Skinner and Tokuyr (1978) used 0.5 M acetate buffer at pH 5.0. Grajek (1976b) showed that S. thermophile and Thermoascus aurantiacus produced optimal activities at pH 4.5-5.5. Grajek reported optimal pH in the range of 5.0-6.3 using 0.05 M McIlvaine's buffer. Although, S. thermophile cellulases are produced optimum at alkaline pH to function most efficiently at acidic pH 5.0 (citrate or phosphate buffer). Durand et al. (1984) working on mesophilic and thermophilic fungi found that pH 5.0 was optimum for all the enzymes tested. Endoglucanase from all 4 strains and xylosidase from reses had a more acidic pH, while S. cellulophilum enzymes slightly more alkaline pH. Grajek (1986) listing the effects pH on enzyme activity found that the most acid stable enzyme were cellulases of Aspergillus fumigatus specially endo-glucanases (pH 4.0). The enzyme obtained from SSF were more stable than in LF.

Temperature

The temperature up to 50°C increases the enzyme adorption a maximum above which the activity falls (Bisaria and Ghc 1976). Trichoderma strains show the highest activity up to in all the enzyme assays Schulz and Hirte, 1989). Thermophi
fungi have a high rate of cellulolysis and show increased activity at 60°C. Similarly, other workers have reported different optimum temperatures for cellulases from various organisms. The optimum temperature reported for various organisms for cellulase are: *T. viride* β-glucosidase, 50°C; *A. niger* QM 877, cellobiase at 50°C (Sternberg et al., 1977); *Basidiomycete* sp cellobiase 65°C (Shekhale and Sadana, 1978); *T. viride* β-glucosidase, 40°C (Herr, 1979); *Sclerotium rolfsii* (UV-8) cellobiase, 65°C an *Aspergillus* sp. cellobiase and β-glucosidase, 65°C (Gokhale et al., 1984). Durand et al., (1984) reported that among the thermophilic and mesophilic species, *Thielavia terrestris* displays optimum temperature above 60°C for all the cellulases except cotton activity which was maximum at 55°C. The maximum was four for endo-glucanase at 75°C. Next in the order were *Sporotrichum cellulophilum*, *Trichoderma reesei* and *Penicillium* spp., respectively (Durand et al., 1984). Macris and Panayatou (1986) from their mutants of *Trichoderma harzianum* M5 showed optimal exo-cellobiohydrolase activity at 50-60°C. The CMC-ase and β-glucosidase from the same fungi had their optimum at 55-60 and 65°C respectively. For *Aspergillus usitatus* M35 the optimum temperature of enzyme activities was at 60, 65 and 70°C for exo-cellobiohydrolase, β-D-glucosidase and CMC-ase, respectively. The optimum temperature for the latter enzyme is one of the highest report in mesophilic and thermophilic organism.

**Thermotolerance**

Linko (1977) reported two of the major limitations of cellulases obtained from mesophilic fungi to be their relative poor temperature stability characteristics and low rates of ce
lulose hydrolysis. The best known cellulases such as those produced by Trichoderma, Penicillium and Aspergillus species are stable at 50°C (Mandels, 1975). At this temperature enzyme reactors are frequently contaminated. Increasing the reaction temperature would give a dual benefit by increasing hydrolysis rate and inhibiting microbial growth (Hagerdal et al, 1980; Durrand et al, 1984). Thermophilic microorganisms are in general more active at high temperatures and more thermostable than those produced by mesophilic species (Zeikus, 1979). In fact, there is a direct correlation between temperature optima for growth and thermostability of the enzyme.

Cellulases derived from a few strains of Thielavia terrestris have been reported to be exceedingly thermostable. Skin and Tokuyama (1978) reported that 20% of the original enzyme activity still remained when culture filtrate was exposed to temperatures of 100°C for a period of 3 hours. These characteristics have also been reported by (Margaritis and Merchant, 1981; Merchant, 1984). Durand et al (1984) working on a couple of mesophilic and thermophilic fungi confirmed that T. terrestris was the most promising source of thermostable enzymes. Feldman et al (1988) reported in Thermoascus aurantius the stability of cellulase system for at least 24 h at 70°C.

PRETREATMENT OF LC SUBSTRATES

The literature on various pretreatments applied is voluminous. Several review articles on this subject are available (Mandels et al, 1974; Millet et al, 1975; Cowling and Kirk, 1976; Dunlap et al, 1976; Millet et al, 1976; Ladisch, 1979; Dunlap
Chiang, 1980; Morton et al, 1980; Chang et al, 1981; Lin et al, 1981; Fan et al, 1982; Lodish et al, 1983; Marsden, 1986). These pretreatments are broadly classified into physical, chemical and biological pretreatments according to their principle mode of action on the substrate. Some processes are combinations of two or more pretreatment techniques applied in parallel or in sequence.

**Physical Pretreatments**

Ball milling reduces crystallinity and particle size, whereas it increases specific surface area, bulk density and water soluble fraction. Ball milling has proved to be very efficient pretreatment for cellulosic materials (Millet et al, 1979; 1969; Fan et al, 1980 and 1981; Asensio, 1983; Lee et al, 1983). However, as commented by Andreassen and Nyström (1976) and Wilke et al (1976) it is costly and unable to remove non cellulosic substances, probably due to lignin (Neilson et al, 1982). Most of the workers have used a combination of two or more pretreatments with milling to delignify the substrate. Millet et al (1975) noted that effectiveness of ball milling varied from material to material. Softwood showed the least response. These results were in agreement with Matsumura et al. (1977) who found that while vibratory ball milling of both softwood and hardwood decrease particle size and crystallinity, it was treatment with NaCl (12.5%) which enabled the T. viride (Meisclase) to produce up to 75-80% and 25-35% reducing sugars for these substrates, respectively. Shimizu and Usami (1978) used bantam milling along with exhaustive extraction by methanol and air drying of hardwoods and softwoods. Maximum delignification (90%) of hardwood Buna Fagus crenata was attained with 50% methanol containing 0.1% HC
allowed complete degradation of cellulose portion by the Meicelase. Sudo et al (1976) used a combination of bantam milling with acid chloride for pretreatment of hardwoods and softwoods. Enzymatic accessibility of these substrates (2%) increased to 70-80% and 90% as the delignification approached 60% and 50%, respectively. Millet et al (1979) compared the effects of vibratory milling on enzymatic accessibility which depended upon the substrate type and milling time. Cotton linters were totally hydrolysed after 60 minutes of milling. It was followed by red oak (240 m, milling). News print and douglas fir required greater milling time for total enzymatic accessibility. Tassinari et al (1977) subjected a wide range of substrates to two-roll milling. The susceptibility of hard woods and agricultural residues was more than soft woods.

Steaming conducted at high pressures (5-20 bars) and high temperatures (180-200 °C) for 5-30 minutes has been used by many workers. Spano et al (1979) studying the effect of steaming pretreatment on the enzymatic hydrolysis suggested increased accessibility for hardwoods and agricultural residues, whereas it decreased for softwoods and urban wastes. Puri and Mamers (1983) used milled bagasse, wheat straw, along with eucalyptus chips for autohydrolysis (200 °C, 15 m). The corresponding lignin content was 23.6, 15.6 and 20.3%, respectively, whereas the enzymatic digestibility (Onazuka 3S) showed 78, 81 and 75% reducing sugars after 48 hours. Dekker et al (1983) used a shorter time duration (4m) but subjected bagasse to further explosive difibration, resulting in solubilization of 90% of hemicellulose. The treated material was susceptible up to 80% after 24 hours when supplemen-
ted with β-glucosidase. Deshpande and Eriksson (1984) reported that steam explosion of wheat straw led to similar effects as above. The rapid saccharification level slowed up considerably because of lignin, after initial high rate. The lignin is easily removed by NaOH and ethanol. Vallander and Eriksson (1985) indicated that out of three tested pretreatments, steam explosion was followed by treatment with H2O and defibration. The enzymatic accessibility produced 74, 56 and 29% of reducing sugars, respectively by T. reesei cellulases (alko company). Puls et al. (1984) used defibration (30 s) along with optimum steaming at 200 an 210 °C on chopped wheat straw which resulted in 51.8 and 58.5 saccharification, respectively, by T. reesei cellulase supplemented with Aspergillus niger β-glucosidase. The pretreatment increased the carbohydrate and lignin level to 76.6 and 22% whereas the fibre yield decreased considerably at 210 °C. Poutanen et al. Puls (1985) further elaborated steaming process and enumerated separate processing of fibre fraction (lignin and cellulose) as the aqueous extract, hemicellulose. They optimised pretreatment conditions on birch wood. This material was hydrolysed to 71% theoretical yield by (40 FPU/gm) T. reesei cellulase supplemented with (2.7%) A. niger β-glucosidase after 24 hours. Vallander and Eriksson (1987) steam exploded aspen wood chips (234 °C, 48 °C and wheat straw (235 °C, 60s) which resulted in material losses mainly in the form of hemicellulose, accumulative to 3-6% a 12%, respectively. The enzymatic accessibility for these substrates (6%) was increased to the effect of 53 and 49%, respectively. Nes-Hartree et al. (1987) used the Iotec process for aspen wood (240 °C, 80s) and subsequent wash for removal of solut
pentoses and inhibitors with water. The 5% substrate was saccharified up to a level of 73% using *Trichoderma harzianum* E58 in a combined cellulase hydrolysis and fermentation process. The addition of small amount H$_2$SO$_4$ (up to 4%) produced an even more susceptible substrate from aspen wood than the autohydrolysis (Mes-Hartree and Saddler, 1983). The explosive decompression is important with substrates such as wood chips but for agricultural wastes (bagasse) the increase was low. The lignin from Iotec process is superior to autohydrolysis treatment (Marchessault, 1984) and the cellulose-lignin binding was broken down without significant change to either component. The failure to achieve 100% conversion was attributed to enzyme factors (inactivation and end-product inhibition) rather than the substrate factor such as crystallinity and lignin (Dekker and Wallis, 1983). Ando et al (1988) working on cedar wood suggested that per-acetic acid combined with steaming was effective as an acid catalyst an initiator of radical reactions in lignin side chain. The lignin was decomposed, repolymerised and melted accompanied by hemicellulose hydrolysis. Treated substrate (4%) was saccharified up to 75% by Meicelase. Latif et al (1988) compared steaming of salt tolerant plants with alkali pretreatments. The results with steaming indicated lower hydrolytic yields, because of increase in overall lignin content in the fibre due to subsequent loss of hemicellulose extracted in wash liquor. The increased temperatures favoured accessibility but the fibre yields were also lowered at the same time. The maximum accessibility by T resea cellulase and B-glucosidase from *Aspergillus niger* yielded reducing sugars of 42, 51 and 32% for *Leptochloa fusca* (kallar grass).
Panicum maximum and Atriplex amnicola. However, the highest yields were achieved after alkali pretreatments. As reported earlier the steaming pretreatment in combination is most effective (Wong et al., 1988). These workers used 2% impregnation of wood chips which was followed by steaming. The resulting material was solubilised by \textit{T. reesei} cellulase plus \textit{A. niger} \( \beta \)-glucosidase to nearly 100%. This was mainly attributed to increase in pore size, the harsher treatments to a certain extent increased pore size but at the same time fibre losses were observed. The increase in accessibility was as a result of hemicellulose extraction and lignin redistribution. These results were further strengthened by the work of Burns et al. (1989). It was stated that after acid hydrolysis of hard woods the total surface area of the substrate pores was too small (50-90 Å) to be accessible to the enzyme. Steaming at 200°C increased the surface area further but the large pore size diminished rapidly as was observed after initial rapid hydrolysis. There was a subsequently low hydrolysis due to an increase in number of small pore size.

**Chemical Pretreatments**

Among the alkali pretreatments NaOH has been more extensively used for ruminant feed, rather than for hydrolysis purposes (Wilson, 1977; Kerley et al., 1983; Deschard et al., 1984). Various substrates respond differently to NaOH treatment. Feist et al. (1970) observed that the digestibility of soft woods with high lignin content increased slightly with NaOH pretreatment, while the digestibility of some hard woods and agriculture waste, with low lignin content, increased significantly upon NaOH treatment. On the other hand, NaOH showed little effect on the digestibilit
of cotton. It was inferred that the major effect of the alkali was the hydrolysis of alkali labile linkages (ester type) between the carbohydrate and lignin components (Fiest et al, 1970; Chesson, 1981. Soft woods and legumes contain few of these linkages in contrast to graminae species and hard woods and thus show lesser susceptibility to enzyme attack (Matsumura et al 1970; Chesson 1981; Evan, 1979). The swelling effect of alkali has been discussed by Tarkow and Feist (1969) who showed that alkali treatment of hard woods increased the estimated protein critical molecular weight which could enter pore spaces from 30,000 to 50,000. Taniguchi et al (1982) treated rice straw with 0.025 M NaOH, along with 20% peracetic acid and twice with sodic chlorite. The degree of enzymatic solubilization with the culture filtrate by Trichoderma reesei QM9414 was 69, 42 and 50%, respectively, based on residual straw, whereas it was 30, 32 and 37 respectively, based on untreated raw material. Gharpuray et al (1983) found that the optimum conditions for alkali pretreatment of wheat straw were NaOH (0.1 g/g substrate) and autoclave (129°C, 2.57 atm, 1.5 h) which resulted in increased enzymatic accessibility up to 71%. It was observed that above 30% delignification, the hydrolysis rate increased sharply up to about 3 delignification. It was indicated that up to 30% delignification the lignin in the middle lamella is removed. Beyond this level lignin present in the fibre is removed resulting in exposure cellulose. Tanaka et al (1988b) reported pretreatment of rice straw with caustic soda (1%, 121°C, 1 h). The cellulase preparation (1.2 mg/ml) from Onozuka R-10 solubilised the holocellulose (2% rice straw) to 100% after 24 hours. The rice straw af-
pretreatment consisted of 95% holocellulose including 77% cellulose and 5% lignin including ash. Hamilton et al (1984) treated lignocellulosic (corn residue) with dilute acid hydrolysis (5% H \text{SO}_4, 90 \degree \text{C}). Moreover, it was treated with ferric sodium tartarate complex in 1.5N NaOH. The resulting substrate (1%) was enzymatically hydrolysed up to 90%. The increasing concentration of \text{T. reesei} cellulase (1.74-7.71 IU/gm substrate) resulted in almost 50% increased cellulose conversion. Pretreatment with NaOH resulted in 25-30% lower conversion, but at high enzyme levels, NaOH pretreatment was as effective as the cellulose solvent.

Apart from alkali, other chemical pretreatments, such as solvent pretreatment were reported by Binder and Fiechter, (1979). They treated avicel and wheat straw with EWNN (alk soln of sodium tartarate and ferric chloride) to get a saccharification of 9% and 75%, respectively. Cellulase preparation of \text{T. reesei QM 1414} (12 IU/g straw) was used in each case. In comparison, and alkali treated wheat straw was saccharified to respectively. The fibre losses during the pretreatment, EWNN and ozone were 50, 30 and 0%, respectively. Chang (1985) treated rice straw with 1% (w/w) H \text{O}_2 and 22 h which resulted in 60% delignification, 40\degree, and a five-fold increase in the accessibility for the times increase in the water holding capacity but decrease in the crystallinity and enzymatic access 53.2% was achieved. A weight ratio of hydrogen pero straw (0.25 g H \text{O}_2 /g straw) in alkaline H \text{O}_2 solution 22 2 \text{OH} (w/w) at 32 \degree \text{C}, improved the structural features
Shambe (1984) evaluated pretreatment of wheat straw with LiCl in 1M HCl (27 C, 24 hr). T. reesei (MVA 1284) cellulase (0.2-0.4% hydrolysed 4% wheat straw to give 57% (82-95.4%) monosaccharides. Pretreated sample contained 18.3% lignin and 73.3% total carbohydrate. Holtzapple and Humphrey (1984) used organosolv (alcohol/water/catalyst) for treatment of poplar in steam injected pressurized vessels. The acetone washed material (3%) was treated with enzym (FP-ase 0.08 uM glucose/ml) preparation from Thermomonomospora species supplemented with concentrated B-glucosidase. The pretreatment removes the lignin intact, accompanied by a similar quantity of hemicellulose. Dissolved NaOH in ethanol increased the enzymatic susceptibility to greater extent. Gould (1984) treated wheat straw and other perennial grasses with 1% (w/v) H O 22 ° C 28°C. The 6 and 24 hour treated straw was solubilized up to 4 and 87% with cellulase preparation. Pretreated grasses (monocots such as big blue stem, Indian grass and phragmites were more susceptible with saccharification efficiencies > 90% of theoretical. Conner (1980) pretreated cellulose pulp with aqueous SO 2 10, 20 g SO 2 100 ml; 100, 115 and 130 C, 1-3 hour). However, T. viride (Orazuka cellulase) increased the digestibility to 1 times of untreated substrate, only.

Some of the workers have reported a comparison of different pretreatments. Dekker et al. (1983) observed that milled bagasse treated with alkali (0.25 M, 20 C, 2 hour) and washed to neutrality was similar in hydrolysis yields as steaming. Extraction autohydrolysed pulps with dilute alkali or aqueous ethanol resulted in lower saccharification yields. Rao et al. (1983) treated bagasse (Wiley milled 20-25 mesh) in NaOH (85 C, 60 m) and ne
ralised with HCl, whereas the other portion of sample was steam treated (7 kgcm). These treatments resulted in 63 and 59% saccharification, respectively, in 48 hours. Hemicellulose was not lost in these pretreatments. With a 30% slurry of steam treated bagasse a semisolid of 14% sugar was obtained. Cunnigham and Carr (1984) pretreated wheat straw by thermal hydropulping, dilute acid pulping, thermal hydropulping followed by alkali extraction, alkali extraction and alkaline hydrogen peroxide. The last treatment was found to yield the highest amounts of glucose (98%) enzymatically from the treated substrate containing 57% cellulose. Dilute acid pulping rendered xylose in the wash liquor, whereas liquors from other pretreatments contained xylan. Chidambareswaran et al (1986) in another comparison of different modes of alkali pretreatment found that the highest saccharification of 96%, within 6 hours was achieved by cellulases from Pencillium funiculosum (0.277 FPU) when the fresh substrate was kept in formalin (10%) until use. The NaOH treated wet samples yielded 80% reducing sugars, whereas alkali treated wet, washed and dried fibres, gave only 45% yield. Untreated normal fibres yielded the lowest (19%). These high yields were attributed to highly open structural organization which allowed the penetration of enzymes. However, the resulting reducing sugars contained only about 20% monomeric glucose. The decrease in reducing sugar yield was attributed to conformational changes at the molecular level. Cotton plant stalks purified with 7N HNO and left at room temperature for 48 hours were alkali treated, washed and wet enzyme treated to yield 80% sugars.
Biological Pretreatments

A biological pretreatment utilizes wood attacking microbes that can degrade lignin. The microbes can be classified into three categories: brown rots, white rot and red rots. Brown rots attack cellulose whereas white rots and red rots attack both cellulose and lignin. Removal of lignin by specific microorganisms has been reviewed by Kirk, (1975); Cowling and Kirk, (1976); Ander and Eriksson, (1978); Kirk and Chang, (1981) and Fan et al (1982). Kirk and Harkins, (1973) reported that white rot fungus removed 42% of lignin, 3% of glucan (including cellulose), and 30% of hemicellulose from birch wood. Ander and Eriksson, (1978) enunciated that degradation of lignin by white rot fungi is a co-oxidative process and consequently an accompanying carbon source is necessary, for e.g., cellulose and/or hemicellulose. Th fungi ligninases appear to attack the phenolic residues with demethylation and ring cleavage. Eriksson and Goddall (1974) achieved almost specific lignin degradation with cellulase-less mutants of white rot fungi. Detry et al (1980) working on growth of Pleurotus ostreatus on wheat straw observed losses of lignin and cellulose to be 22 and 14% after 30 days and 40 and 32% respectively, after 70 days. The organism appeared to selectively degrade lignin during the first six days.

Biological delignification appears to be a promising technique but its low rate has limited its usage in industrial processes.

Hydrolysis

Today, there have been few microorganisms which have shown enough potential to produce an enzyme system, which can extend
ably degrade the cellulosic material. Bisaria and Ghose (1981) listed 13 species of fungi along with a few bacteria in this regard. For effective saccharification of cellulose, the high sugar yield per enzyme unit is essential (Szczodrak, 1987). Factors effecting this yield include pretreatment, inhibition of enzyme by heat or degradation products, enzyme and substrate concentration, adsorption of cellulase to cellulose, stability of enzyme, speed of enzyme action and degree of agitation. Study of all the above factors were out of the scope of this work thus only pertinent literature is mentioned.

Enzyme and Substrate Concentration

The ultimate objective in the hydrolysis of cellulosic substrates is their conversion into monomeric sugars for the bioutilization to useful energy rich products. But the economics of the process, for instance ethanol production, suggests the requirement of high sugar syrup (about 15%). This leads to problems such as end product inhibition and stirring of bulk substrates. Markannen and Eklund (1975) using cellulases (Trichoderma virid QM9414) obtained about 100% yields from hydrolysis of fufura process waste containing 40% cellulose. They further reveals that by manipulation of a slightly lower pH than 5.0 and use of concentrated enzyme could yield about 5% glucose in 4 days. The enzyme could not be used for higher than 7% substrate concentration since it was present in dilute form. The use of ultrafiltration technique failed to concentrate the enzyme for effective saccharification due to changes in the hydrolytic character of the enzymes. By manipulating the condition in a fermentor, th
above yields were obtained in 24 hours. Moreover, the addition of
β-glucosidase from *Aspergillus niger* could not bring about a
noticeable increase, hereby indicating the presence of a complete
cellulase system in *T. viride*. In a similar study, Wilke and
Mitra (1975) using acetone concentrated cellulases (4.4 IU FPA)
from *T. viride* QM9414 successfully hydrolysed 10% news paper
(shredded and milled) to a level of 65% reducing sugars. Detroy et
al. (1980) using commercial cellulase (Miles lab.) preparation
(1.0 IU/g substrate) from Miles lab. were able to hydrolyse ED
treated wheat straw (4%) to 70% glucose yield. Further improve
by disc-milling EDA treatment yielded 83% of glucose. Moreover
they showed that after biological pretreatment (40-50 days) of
*Pleurotus ostreatus* the conversion of cellulose to glucose in
creased 4 to 5 fold, yielding 72% glucose. Chahal (1984) using
cellulase produced in solid state fermentation by a mutant QMY-
developed from *T. reesei* QM9414 resulted in 99.75 g sugars/l from
100 g of delignified wheat straw. Very little cellulbiose accumu-
lated in the hydrolysate up to 20 hours, whereas its level fur-
ther decreased after 96 hours. The hydrolyzates analysed at HPI
contained in g/l: cellulbiose 3, glucose 68, xylose 26.7 and
arabinose 1.7. McCrae et al (1989) found enzyme productivity
values much higher in the mutant strain of *Penicillium pinophil*
(WTG III/6) than those normally recorded for *T. reesei* mutant
30 (thought to be the best cellulase producer). The cellulase
FPU/ml) was very effective in hydrolysing 10% solka floc to 8
sugar solution. The hydrolysis of untreated straw from 30-35% w
increased to a level of 92-98% when used after pretreatment wi
H₂O at room temperature (24 h, pH 11.8). Most of the mutat
derived from T. reesei although had a potent cellulase but were devoid of a high β-glucosidase. Thus the deficient strains with β-glucosidase were prone to greater end product inhibition due to the presence of cellobiose. The presence of 1% cellobiose is as much inhibitory as 25% glucose (Morisset and Khan, 1984). Khan et al (1984) further elaborated the use of additional cellobiose from Aspergillus phoenicis along with the cellulase of T. reesei in the ratio of (0.9 : 1.0) in order to restore the effects of end product inhibition. By the addition of cellobiose to cellulase (30-35 FPU) of T. reesei the saccharification yields of 5% cellulose were enhanced from 56% sugars (86% glucose) to 80% (99% glucose). Dekker and Wallis (1983) increased the saccharification of 10% bagasse from 50% to 80% in 24 hours when β-glucosidase from Aspergillus niger was added to the T. reesei cellulase (20 FPU/gm substrate) in the ratio of FPU to β-glucosidase of 1:1.25. The workers used enzymes preparations from T. reesei C-30 and QM9414 and also Micelase (commercial enzyme), all of which varied to some extent in the proportion of different enzyme components.

Sakamoto et al (1982) used 0.5% of crude cellulase from Aspergillus aculeatus which hydrolysed 10% alkali treated rice straw at 37°C in 3 days to 85% (8% sugar solution). When used with commercial (Micelase) the yields were obtained in one day. Rao and Seeta (1983) described that Penicillium funiculosum produced complete cellulase which hydrolysed cotton, alkali treated bagasse and steam treated bagasse to 97, 63, and 59% sugar yield respectively, in 48 hours. With a 30% slurry of steam treated bagasse a 14% sugar in a semi-solid mass was obtained. McCrae et al (1980) reported cellulase (7 FPU/ml) induced from solka flc
mutant strain NTG III/6 of *P. pinophilum* hydrolysed 10% solka floc to 70% in 72 hours at 50 °C. The hydrolysis yield was increased to 90% when enzyme induced on barley straw was used. Under the same conditions the polysaccharide in (alkline H₂O treated) barley, oat, and wheat straw at room temperature were hydrolysed to 93, 100 and 92%, respectively. Sattler et al (1989) determined that the extent of hydrolysis at fixed time increases with increasing enzyme dosage in a hyperbolic function. They revealed that in the system of sigma cellulase and cellulast, the easily and difficultly hydrolyzable components were 43.0 and 57.0%, respectively and the maximum digestibility at 94 hours was 82.6%. Poplar wood steam treated at 200, 220, 240 °C, showed digestibility to glucose of 43.9, 64.9 and 60.0%, respectively. The easily digestible substrate (amorphous) was hydrolysed with cellulase (5 FPU/g), whereas the crystalline region was dependent upon increasing dose of FPU (5-100) which increased the yield threefold. It was concluded from the kinetics experiment that it was, in fact, a good pretreatment leading to a high proportion of amorphous cellulose of prime importance along with the type of enzyme. In contrast time and increase of enzyme dosage was much less effective. The three different enzyme system tested for hydrolysis showed different hydrolytic yields. Another criteria for the effectiveness of an enzyme is the enzyme dosage (FPU/g) which gives the half maximal digestibility. In hydrolysis: the reaction rate is proportional to the amount of adsorb enzyme on the cellulose surface (Bisaria and Ghose, 1979). Various kinetic models are based on the properties of the cellulase enzyme and mass transfer in the reaction system (Suga et a
1975). A theoretical derivation of a hydrolysis model is given by Okazaki and Moo-Young (1978). Some authors have developed semi-empirical models based on the assumption that the reaction between cellulase and cellulose can be described by a summation of pseudo-first order reaction (Van Dyke, 1972; Brandt et al, 1973). Other models are based on structural features of the substrate like pore size distribution, pore size, index of crystallinity and specific surface area (Fan et al, 1980; Grethlein, 1985). Ohmine et al (1983) concluded that fall off in hydrolysis rate was not only because of crystallinity and product inhibition, but also due to a rate retarding factor. Matsumo et al (1989) suggested it as a reversible inactivation of the adsorbed enzyme due to diffusion into the cellulose fibrils. Chernoglasov et al (1988) found that six different types of endo-glucanase purified to homogeneity varied in the intensity of their binding to cellulose and varied in similar order to their binding to lignin Endoglucanase was deactivated after adsorption on lignin. Steam exploded lignocellulosic material also showed similar adsorption effects but not of when they were acid treated.

pH effect plays vital role in the optimization studies for hydrolysis as was revealed by Markanen and Eklund (1975). A slight decrease in pH level from 5 to 4.8 has been suggested by many authors for Trichoderma strains (Wilke and Mitra, 1975; Mandels, 1980; Morisset and Khan, 1984; Saddler et al, 1985). However, the mutant QMY-1 developed from QM9414 showed optimum saccharification at pH 6.7 which is also the original pH of the enzyme solution. Sporotichum thermophile has been reported to produce enzyme at alkaline pH 6.5 (Grajek, 1987b) but for saccha
purification purpose it has been reported optimum at pH 5.6 (Bhat and Maheshwari, 1987).

The temperature plays important role in the cellulolysis of substrate. It is the intrinsic characteristics of different microorganism to produce thermostable cellulases. Thermophilic microorganisms are reported to produce enzymes which show thermostability at temperatures of 70-80 C (Margaritis and Merchant 1983 and 1986). However, there are only a few successful reports on saccharification at temperatures higher than at 50 C. Skinner and Tokuyama (1978) using Thielavia terrestris (NRRL 8126) at 60 C obtained the highest glucose yield of 52 from cellulose powder and lowest of 2.9% from bagasse, respectively. Durand et al (1984) working on mesophilic and thermophilic fungi enunciated that difference in the thermostability of mesophilic and thermophilic fungi is not enough to compensate for the low enzyme titres produced by the latter. This was evident in the hydrolysis experiment at high temperatures which revealed that at 60 C there was a significant decrease in the rates of hydrolysis for all the tested fungi. This was unexpected specially for T. terrestris (NRRL 8126) claimed to be as one of the most thermostable cellulase producing system.
RESULTS

ISOLATION OF THERMOPHILIC FUNGI

Thermophilic fungi were isolated from different habitats including rhizosphere of Cenchrus ciliaris and Leptochloa fusa (kollar grass), kollar grass compost, decomposing bagasse and poultry droppings. Percent frequency of occurrence of various fungi (Table 1) was recorded on ball milled cellulose agar (BMCA) after 5 days incubation at 50°C. Humicola grisea and Aspergillus fumigatus were found common in different habitats. A. fumigatus showed 100% frequency of occurrence from 0, 3 and 6 days enriched soil incubated at 50°C. The fungus also sporulated earlier than the other fungi on BMCA. Likewise, H. grisea was also dominant and present at 100% frequency of occurrence after three days incubation. Apart from these two fungi Chaetomium thermophile was also isolated from both the rhizospheres, while Sporotrich thermophile was isolated from the rhizosphere of kollar grass only. Both of these fungi showed dominance from the enriched soil at later stages of incubation. Low percentage of frequency accompanied slow mode of growth specially in case of thermophile.

Table 1 shows that kollar grass compost samples obtained after 9 days at temperatures of 60-65°C harboured Torula thermophile, H. grisea and A. fumigatus at 100 percent frequency of occurrence.

From decomposing bagasse, again a number of repeat species were obtained (Table 1). Only Mucor pusillus was obtained as new isolate in addition to the existing ones.
### Table 1: Thermophilic fungal isolates from different habitats

Percentage frequency of occurrence was determined after incubating the BMCA plates for 5 days at 50°C.

<table>
<thead>
<tr>
<th>Habitat</th>
<th>Fungal isolate</th>
<th>Percentage frequency of occurrence after initial enrichment at 50°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0hrs</td>
</tr>
<tr>
<td>Rhizosphere of <em>C. ciliaris</em></td>
<td><em>H. grisea</em></td>
<td>66</td>
</tr>
<tr>
<td></td>
<td><em>C. thermophila</em></td>
<td>33</td>
</tr>
<tr>
<td></td>
<td><em>A. fumigatus</em></td>
<td>100</td>
</tr>
<tr>
<td>Rhizosphere of kollar grass</td>
<td><em>H. grisea</em></td>
<td>66</td>
</tr>
<tr>
<td></td>
<td><em>C. thermophila</em></td>
<td>33</td>
</tr>
<tr>
<td></td>
<td><em>S. thermophila</em></td>
<td>33</td>
</tr>
<tr>
<td></td>
<td><em>A. fumigatus</em></td>
<td>100</td>
</tr>
<tr>
<td>Kollar grass compost</td>
<td><em>H. grisea</em></td>
<td>--</td>
</tr>
<tr>
<td></td>
<td><em>T. thermophila</em></td>
<td>--</td>
</tr>
<tr>
<td></td>
<td><em>A. fumigatus</em></td>
<td>--</td>
</tr>
<tr>
<td>Decomposing bagasse</td>
<td><em>M. pusillus</em></td>
<td>66</td>
</tr>
<tr>
<td></td>
<td><em>H. grisea</em></td>
<td>100</td>
</tr>
<tr>
<td></td>
<td><em>A. fumigatus</em></td>
<td>100</td>
</tr>
<tr>
<td></td>
<td><em>T. thermophila</em></td>
<td>66</td>
</tr>
<tr>
<td>Poultry dropping</td>
<td><em>M. pulchella</em></td>
<td>66</td>
</tr>
<tr>
<td></td>
<td><em>A. fumigatus</em></td>
<td>100</td>
</tr>
</tbody>
</table>

* Nomenclature used by Cooney & Emerson (1964) was followed after microscopic examination of the fungal isolates.

Frequency of occurrence determined from triplicate plates:

- **a)** After initial enrichment of samples with BMG (materials & methods) the isolations were made by Varcup's method.
- **b)** By using 3, 6 and 9 days old compost at temperatures of 45 and 60-65°C, respectively.
- **c)** By direct plating.

Poultry droppings yielded yet another new isolate named *Malbranchea pulchella* along with *A. fumigatus*.

All the above isolated species showed profuse growth and clearance on BMCA at 50°C. The isolated fungi were mostly contaminated with other fungi and bacteria. Initially rose bengal w
used as an antibacterial agent for the isolation studies. Finally, the use of antibiotics including penicillin and streptomycin along with rose bengal helped to eradicate the bacterial contaminations. The fungi were separated, where required by dilute plate method.

CELLULASE AND XYLANASE PRODUCTION BY THERMOPHILIC AND MESOPHILIC FUNGI

The ability of different thermophilic fungi for the biosynthesis of cellulases and xylanases, when grown on 2% kellar grass at 45°C, was measured in shake flasks. All the duplicate isolates were screened in a preliminary test for enzyme productions. The duplicates showed no significant difference in enzyme activities. The production of cellulases and xylanases by thermophilic fungi from kellar grass showed maximal activities after 5-7 days of incubation, as shown in Fig. 1. All the tested thermophilic exhibited a short lag phase, and the enzyme activities increased appreciably after 2 days. A. fumigatus showed maximal activities of 0.4, 2.4, 3.7 and 0.12 U/ml for Fase, CMCase, xylanase and β-xylosidase, respectively (Table 2). S. thermophile produced a maximal level of 0.47 U/ml for β-glucosidase after 5 days. C. thermophile also showed maximal CMCase activity of 2.4 U/ml, besides A. fumigatus. The former also exhibited high xylanase activity after the latter. Among the isolates, grisea and T. thermophila showed moderate enzymatic yields, while M. pulchella and M. pusillus exhibited lower titres. β-xylosidase was only induced to a small extent in A. fumigatus (Fig. 1e).
Fig. 1. Screening of thermophilic fungi for cellulase and xylanase production when grown on 2% kah-llar grass in shake flask cultures, at 45°C.

a) FPase, b) CMCase, c) xylanase, d) β-glucosidase, and e) β-xylosidase.
Table 2: Maximal cellulase and xylanase production by thermophilic fungi when grown on 2% kellar grass. The fungi were inoculated in shake flasks at 45°C for 7 days.

<table>
<thead>
<tr>
<th>Fungal isolates</th>
<th>FPase</th>
<th>CMCase</th>
<th>β-Glucosidase</th>
<th>Xylanase</th>
<th>β-Xylosidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. fumigatus</td>
<td>0.40</td>
<td>2.4</td>
<td>0.38</td>
<td>3.7</td>
<td>0.12</td>
</tr>
<tr>
<td>C. thermophile</td>
<td>0.30</td>
<td>2.4</td>
<td>0.20</td>
<td>3.6</td>
<td>0.07</td>
</tr>
<tr>
<td>H. grisea</td>
<td>0.28</td>
<td>1.4</td>
<td>0.30</td>
<td>2.6</td>
<td>0.04</td>
</tr>
<tr>
<td>M. pulchella</td>
<td>0.14</td>
<td>0.5</td>
<td>0.04</td>
<td>2.5</td>
<td>0.04</td>
</tr>
<tr>
<td>M. pusillus</td>
<td>0.10</td>
<td>0.2</td>
<td>0.05</td>
<td>1.0</td>
<td>0.04</td>
</tr>
<tr>
<td>S. thermophila</td>
<td>0.33</td>
<td>1.8</td>
<td>0.47</td>
<td>2.5</td>
<td>0.07</td>
</tr>
<tr>
<td>T. thermophila</td>
<td>0.25</td>
<td>1.8</td>
<td>0.20</td>
<td>2.2</td>
<td>0.06</td>
</tr>
</tbody>
</table>

During growth all the test fungi affected the initial pH of the growth medium. The response on pH varied with the test fungi (Fig. 2a). Initial pH set at 5.0 increased up to 6.9, 6.6, 5.6, 6.9, 6.9, 6.8, 6.9 for A. fumigatus, C. thermophile, H. grisea, S. thermophila, T. thermophila, M. pulchella and M. pusillus respectively (Fig. 2a). The increased pH level steadied after 5 days and even showed a slight decline during fermentation. Similarly the extracellular protein level increased up to 1.3, 1.1, 1.3, 1.3, 1.5, 2.2 and 1.0 mg/ml, respectively for the abovementioned fungi (Fig. 2b). The extracellular proteins declined after 5 days which also emphasizes the optimum enzyme production between 5-7 days.
**Fig. 2.** Effect of growth on a) pH and b) production of extracellular protein, in thermophilic fungi.

The culture conditions for mesophilic fungi were in parallel with those of thermophiles, except that the incubation temperature was adjusted to 30 C. Among the tested mesophiles, *C. vergicephalum* exhibited maximal yields of 0.44, 1.4, 2.4, 0.23 am 0.04 U/ml for FPase, CMCase, xylanase, β-glucosidase and β-xylosidase, respectively (Table 3). The maximal enzyme activities:

**Table 3:** Maximal Cellulase and Xylanase production by mesophilic fungi when grown on 2% kallar grass. The fungi were inoculated in shake flasks at 30°C for 9 days.

<table>
<thead>
<tr>
<th>Fungal isolates</th>
<th>Enzyme activities (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FPase</td>
</tr>
<tr>
<td><em>C. globosum</em></td>
<td>0.18</td>
</tr>
<tr>
<td><em>C. vergicephalum</em></td>
<td>0.44</td>
</tr>
<tr>
<td><em>T. reessi</em> Rut C-30</td>
<td>0.23</td>
</tr>
</tbody>
</table>
Fig. 3. Screening of mesophilic fungi for cellulase and xylanase production when grown on 2% kale grass in shake flask culture at 30°C.

a) FPase, b) CMCase, c) xylanase, d) B-glucosidase and e) B-xylosidase.
were obtained after 7-9 days of incubation (Fig. 2). *T. reesei* Rut C-30, showed poor yield from kollar grass. In fact, *T. reesei* and *C. globoseum* showed no significant increase in activity after 7 days.

Initial pH of 5.0 of the medium increased due to growth of the fungi. The pH increase reached a maximal level of 6.9, 6.45 and 7.0 for *C. globosum*, *C. vergiccephalum* and *T. reesei*, respectively. Similarly the extracellular protein level increased up to 0.8, 1.2 and 1.1 mg/ml for the above fungi, respectively (Fig. 4b).

![Graphs showing pH and extracellular protein production](image)

**Fig. 4.** Effect of growth on a) pH and b) production of extracellular protein in mesophilic fungi.

**CELLULASE AND Xylanase Induction by Lignocellulosic (LC) Substrates**

Two fermentation modes were adopted for cellulase and xylanase induction by LC substrates by *S. thermophile* and *C. vergiccephalum*.

The fermentation modes were namely: Solid state fermentation (GSP) and Liquid fermentation (LF).
SSF by *S. thermophile*

Different LC substrates were used with moisture (medium) level in the ratio of 1:3 (w/v) for cellulase induction in SSF at 45°C. Fig. 5 gives the profile for different enzymes induced from the LC substrates during 2-7 days. Table 4 shows maximal enzyme induction in general by rice straw which produced avicelase, CMCase and xylanase to a greater level of 1.3, 7.2 and 16 U/ml, respectively. Fig. 5d,e depicts *Sesbania aculeata* (dhancha)

Table 4: Maximal cellulase and xylanase induced in *S. thermophile* by different lignocellulosic substrates in solid state fermentation.

<table>
<thead>
<tr>
<th>Growth substrate</th>
<th>Enzyme activities (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Avicelase</td>
</tr>
<tr>
<td>Kellar Grass</td>
<td>1.1</td>
</tr>
<tr>
<td>Rice Straw</td>
<td>1.3</td>
</tr>
<tr>
<td>Wheat Straw</td>
<td>0.6</td>
</tr>
<tr>
<td>Bagasse</td>
<td>0.9</td>
</tr>
<tr>
<td>Dhancha</td>
<td>0.8</td>
</tr>
</tbody>
</table>

as a potent inducer among the tested substrates resulting in higher induction for β-glucosidase and β-xylosidase of 0.43 and 0.22 U/ml, respectively. The profiles from Fig. 5 shows that apart from rice straw and dhancha, kellar grass also exhibited moderate induction level. In fact, it was the substrate of choice after rice straw for avicelase and CMCase activities, whereas wheat straw showed higher induction of xylanase activity than kellar grass. Bagasse exhibited least influence on induction.
Fig. 5. Effect of different 1 no-celullulosic substrates on induction of cellulases and xy
mases in S. thermophile in so state fermentation at 45°C.

a) avicelase, b) CMCase, c) xylanase, d) β-glucosidase
and e) β-xylosidase.
SSF by *C. verrucosephalum*

*C. verrucosephalum* was also cultivated under similar conditions by SSF as mentioned above except that the incubation temperature was maintained at 30°C. The enzymes harvested during 2-days showed that kallar grass induced CMCase, xylanase and xyllosidase to a greater extent of 9.1, 20.7 and 0.28 U/ml, while dhancha induced higher levels of avicelase and β-glucosidase to 1.2 and 1.5 U/ml, respectively (Table 5). Maximal enzyme activities were observed during 5-7 days of incubation.

**Table 5:** Maximal cellulase and xylanase induced in *C. verrucosephalum* by different lignocellulosic substrates solid state fermentation.

<table>
<thead>
<tr>
<th>Growth substrate</th>
<th>Enzyme activities (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Avicelase</td>
</tr>
<tr>
<td>Kallar Grass</td>
<td>0.78</td>
</tr>
<tr>
<td>Rice Straw</td>
<td>0.75</td>
</tr>
<tr>
<td>Wheat Straw</td>
<td>0.36</td>
</tr>
<tr>
<td>Bagasse</td>
<td>0.43</td>
</tr>
<tr>
<td>Dhancha</td>
<td>1.20</td>
</tr>
</tbody>
</table>

Fig. 6 shows that other substrates which followed kall grass and dhancha for induction of various enzymes were rice straw, wheat straw and bagasse. In fact, CMCase and xylanase were induced to a greater extent by rice straw after kall grass, than the other substrates. Xylanase activity was high induced by kallar grass followed by rice straw > dhancha > wheat straw. The induction of β-glucosidase by dhancha resulted
Fig. 6. Effect of different lignocellulosic substrates on the induction of cellulases and xylanases in C. vergicepscalum in solid state fermentation at 30°C.

a) Avicelase, b) CMCase, c) Xylose,
d) β-glucosidase, and e) β-xylosidase.
almost 3 times more activity than the next best substrate i. kellar grass. Fig. 6e gives the profile for $\beta$-xylosidase which shows higher induction level by kellar grass followed by dhanch > rice straw > wheat straw and bagasse. The overall enzyme induction by bagasse was lowest.

**LF by S. thermophile**

Eight LC substrates (2%) were used for cellulase and xylarase induction during the course of studies for 2-7 days at 45°C in shake flasks. Maximal enzyme activities for *S. thermophile* were obtained after 5-7 days of incubation (Table 6). The induc

<table>
<thead>
<tr>
<th>Growth substrate</th>
<th>Enzyme activities (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FPase</td>
</tr>
<tr>
<td>A. amnicola</td>
<td>0.20</td>
</tr>
<tr>
<td>Bagasse</td>
<td>0.20</td>
</tr>
<tr>
<td>Kellar Grass</td>
<td>0.32</td>
</tr>
<tr>
<td><em>F. maximum</em></td>
<td>0.24</td>
</tr>
<tr>
<td>Rice Straw</td>
<td>0.38</td>
</tr>
<tr>
<td>Dhancha</td>
<td>0.22</td>
</tr>
<tr>
<td>Wheat Broa</td>
<td>0.20</td>
</tr>
<tr>
<td>Wheat Straw</td>
<td>0.40</td>
</tr>
</tbody>
</table>

Induction profile from various substrates exhibited a short lag nature in enzymes (Fig. 7). Wheat straw in general induced FPase and CMCase.
Enzyme activity at high temperature can enhance the rate of reaction and thus requires low enzyme titres. The temperature optimum studies revealed that avicelase activity showed appreciable increase at 70°C over that 50°C (Fig. 19). The CMCase, xylanase and β-glucosidase registered maximal increase at 65°C. Skinner and Tokuyama (1978) used 60°C while Fahnrich and Irrgang (1982) reported 55°C to be optimum for cellulase activity from thermophilic fungi. However, Durand et al. (1984) found that there is a difference of only 10°C for cellulase activities in mesophilic and thermophilic fungi.

The stability of FFase (taken as a complete cellulase) revealed that H. grisea possesses the most stable enzyme showing tolerance of up to 85 and 65% for its activity after incubation at 60°C and 70°C for 24 hours (Fig. 20). All the thermophiles showed considerably higher stability for FFase, as compared to the mesophiles. In fact, T. reesei RUT C-30 showed 22 and 10% stability for its FFase, whereas, Q. virgicophalum cellulase was not stable at the above temperatures. Both the mesophilic fungi showed relative stability up to 50°C only. Skinner and Tokuyama (1978) reported 20% relative FFase activity by Thielavia terrestris even after an incubation of 3 hours at 100°C. Fahnrich and Irrgang (1982) reported that CMCase from Q. cellulolyticum is stable at 50°C for 2 hours but FFase is stable up to 20% only while β-glucosidase got inactivated rapidly. Durand et al. (1984) found that T. terrestris cellulase is the most thermostable at 60°C whereas it is completely unstable at 70°C contrary to claims made by Skinner and Tokuyama (1978).
activities to a higher level than the other substrates with 0. and 3.7 U/ml; rice straw induced xylanase and β-glucosidase activities up to 3.0 and 0.80 U/ml, respectively. None of the substrates effectively induced β-xylanosidase more than 0.1 U/ml.

Fig. 7 shows that apart from wheat straw and rice straw, kallar grass produced all the enzymes effectively. Followed this substrate were bagasse > P. maximum > dhancha > wheat bran A. amnicola.

LF by C. vergisephalum

Experimental conditions for the present studies were similar as in the previous one, except that the incubation temperature was kept at 30°C. Table 7 and Fig. 8 shows the maximal enzyme activities obtained during 5-7 days by C. vergisephalum.

**Table 7:** Maximal cellulase and xylanase induced by different lignocellulosic substrates (2%) in C. vergisephalum in liquid fermentation. Optimum enzyme activities were determined after 5-7 days of growth.

<table>
<thead>
<tr>
<th>Growth substrate</th>
<th>Enzyme activities (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FPase</td>
</tr>
<tr>
<td>A. amnicola</td>
<td>0.16</td>
</tr>
<tr>
<td>Bagasse</td>
<td>0.11</td>
</tr>
<tr>
<td>Kallar Grass</td>
<td>0.25</td>
</tr>
<tr>
<td>P. maximum</td>
<td>0.15</td>
</tr>
<tr>
<td>Rice Straw</td>
<td>0.24</td>
</tr>
<tr>
<td>Dhancha</td>
<td>0.28</td>
</tr>
<tr>
<td>Wheat Bran</td>
<td>0.13</td>
</tr>
<tr>
<td>Wheat Straw</td>
<td>0.20</td>
</tr>
</tbody>
</table>
Fig. 8. Effect of different lignocellulosic substrates on the induction of cellulases and xylanases by *C. verginipes* liquid fermentation at 30°C.

a) FPase, b) CMCase, c) xylanase, d) β-glucosidase, and e) β-xylanosidase.
Fig. 8a, d, e shows that dancha induced somewhat higher FPase, glucosidase and β-xylosidase activities of 0.28, 0.36 and 0. U/ml, respectively, while rice straw induced CMCase up to 1 U/ml (Fig. 8b). Kallar grass induced xylanase up to 3.5 U/ (Fig. 8c) and was the next substrate of choice with comparative higher activities than the rest of the substrates except wheat bran which induced more β-glucosidase activity. After kall grass the order of induction, in general was, wheat straw > wheat bran > A. amnicola > bagasse > P. maximum.

Comparison of SSF with LF in S. thermophile

The SSF and LF (shake flasks) modes were operated at laboratory scale. Preliminary experiments for these fermentations were carried out in 50 and 500 ml Erlenmeyer flasks, respectively using 2.0 gm substrate. The moisture (medium) level was maintained at 3 parts (v/w) and 50 parts (v/w) for the former and latter process at 45 C. Five LC substrates (found common in the process studied) were compared for CMCase, xylanase and glucosidase induction per gram of substrate.

Fig. 9 depicts an overall much higher yield for enzymes produced in LF per gram of substrate than SSF. The induction pattern of different enzymes is as follows; rice straw induced more CMCase and xylanase titre, whereas dancha produced high amounts of β-glucosidase in SSF. In case of LF wheat straw induced higher levels of CMCase while rice straw induced more xylanase and β-glucosidase.

Fig. 9 compares the increase in the overall activity 1 gram of substrate for different enzymes by LF over SSF. CMCase was induced up to a higher level of 20.6, 9.3, 7.6, 7.1 and 6.
Fig. 9. Comparison of enzyme production and induction in SSF and LF by various lignocellulosic substrates in *S. thermophile*. BG-bagasse, DC-dhancha, KG-kallar grass, RC-rice straw, WS-wheat straw.
a) CMCase, b) xylanase
and c) β-glucosidase.

Fig. 10. Comparison of enzyme and induction in SSF and LF by various lignocellulosic substrates in *C. vergicepsallum*.
a) CMCase, b) xylanase
and c) β-glucosidase.
times by wheat straw, bagasse, rice straw, kallar grass a
dhancha, respectively (Fig. 9a). Similarly for xylanase the in
creased level was 3.0, 5.9, 2.6, 3.7 and 3.5 times, respective
(Fig. 9b), while for \( \beta \)-glucosidase it was 59.0, 61.7, 63.6, 66
and 14.0 times, respectively. The order of increase for t
zymes was not same for different substrates.

Comparison of SSF with LF in \( C. \ vergicepsalum \)

The enzyme production in \( C. \ vergicepsalum \) was carried out
under similar conditions as mentioned previously except that a
incubation temperature was maintained at 30 C. In this case, a
produced much higher enzyme titre per gram of LC substrates th
in SSF. Considering the effect of various substrates, Fig. 10
shows that in SSF, kallar grass induced higher CMCase and xylan
ase while dhancha induced more \( \beta \)-glucosidase activities. In
rice straw and kallar grass induced more CMCase and xylan
activities, respectively, while dhancha yielded maximal level
\( \beta \)-glucosidase.

Comparing the induction level per gram of LC substrates
LF over SSF shows that CMCase was induced up to 5.6, 4.8, 3.1,
and 1.8 times by dhancha, bagasse, rice straw, wheat straw,
kallar grass, respectively (Fig. 10a). Similarly, the in cre
in xylanase and \( \beta \)-glucosidase production by the above substra
was 3.8, 5.1, 4.1, 6.8 and 2.6 and 4.2, 14.7, 10.0, 7.1 and
times, respectively (Fig. 10b,c). The three enzymes exhibi
different order of induction by various substrates.
OPTIMIZATION OF CULTURE CONDITIONS IN SSF BY S. THERMOPHILE

Moisture Level

*S. thermophilus* was cultured on 2 gm of kollar grass in SS. The moisture content adjusted by Eggins and Pugh (E & P) media at 1, 3 and 5 times (v/w) of substrate. The flasks were incubated for 7 days to determine the respective CMCase activities. Tab 8 indicates that, although the enzyme activity was greater when 3 times (v/w) moisture level was used, but the estimates of total CMCase units per gram of substrate, were much higher for 5 times (v/w) moisture level.

Table 8: Effect of different levels of moisture (medium) CMCase activity and the enzyme produced per gram substrate by *S. thermophilus* in SSF.

<table>
<thead>
<tr>
<th>Moisture Level (ml/gm)</th>
<th>CMCase Activity (U/ml)</th>
<th>(U/gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.5</td>
<td>4.5</td>
</tr>
<tr>
<td>3</td>
<td>5.2</td>
<td>15.6</td>
</tr>
<tr>
<td>5</td>
<td>4.9</td>
<td>24.6</td>
</tr>
</tbody>
</table>

Nutrient Concentration

For SSF mode E & P medium was used at 0 to 5 fold nutrient concentration. Table 9 and Fig. 11, indicates that 4 fold nutrient concentration over the control (without salts) showed increase of up to 1.4, 2.1, 3.0 and 1.4 times for PPase, CMCase β-glucosidase and xylanase activity, respectively. It was found that substrate with and without mineral medium at high concent
11. Effect of five fold increase in nutrient concentration on the production of cellulase and xylanase activities in SSF.

a) Cellulase, b) CMCase, c) xylanase, d) B-glucosidase.
### Table 9:

<table>
<thead>
<tr>
<th>Mineral conc. (fold)</th>
<th>Enzyme Activities (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FPase</td>
</tr>
<tr>
<td>0</td>
<td>0.42</td>
</tr>
<tr>
<td>1</td>
<td>0.50</td>
</tr>
<tr>
<td>2</td>
<td>0.52</td>
</tr>
<tr>
<td>3</td>
<td>0.56</td>
</tr>
<tr>
<td>4</td>
<td>0.60</td>
</tr>
<tr>
<td>5</td>
<td>0.12</td>
</tr>
</tbody>
</table>

### OPTIMIZATION OF ENVIRONMENTAL PARAMETERS IN LF BY S. THERMOPHII

#### Size and Age of Inoculum

A loopful of spores from S. thermophile were inoculated into E & P medium with 0.5% celllobiose as an inducer for growth. Periodically, 5% (v/v) vegetative inoculum was taken out and inoculated into shake flasks containing 2% kallar grass. Enzyme production (Table 10). The results indicate that...

### Table 10:

<table>
<thead>
<tr>
<th>Inoculum Age (h)</th>
<th>FPase U/ml</th>
<th>β-Glucosidase U/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>0.27</td>
<td>0.37</td>
</tr>
<tr>
<td>24</td>
<td>0.27</td>
<td>0.42</td>
</tr>
<tr>
<td>36</td>
<td>0.30</td>
<td>0.39</td>
</tr>
</tbody>
</table>
inoculum after 12 hours of growth was potent enough, although 24 hours of incubation seemed optimum.

Size of the 24 hours vegetative inoculum varied from 2-10\% (v/v) indicated that 5-10\% (v/v) was optimum for enzyme production (Table 11).

Table 11: Effect of inoculum size on cellulase activities and extracellular protein of \textit{S. thermophile} after 5 days of incubation.

<table>
<thead>
<tr>
<th>Inoculum size% (v/v)</th>
<th>FFase U/ml</th>
<th>(\beta)-Glucosidase U/ml</th>
<th>Protein mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.33</td>
<td>0.44</td>
<td>1.34</td>
</tr>
<tr>
<td>5</td>
<td>0.32</td>
<td>0.50</td>
<td>1.42</td>
</tr>
<tr>
<td>10</td>
<td>0.33</td>
<td>0.51</td>
<td>1.64</td>
</tr>
</tbody>
</table>

Temperature

The effect of increasing incubation temperature on cellulase activity is shown in Fig. 12. The temperature was raised from 30 to 45 \(\text{C}\). The increased cellulase production with tempera

![Fig. 12. Effect of increase in incubation temperature on cellulase activities of \textit{S. thermophile} in SSF.](image)
ture was characteristic of thermophilic fungi, which mostly produce active enzyme at 45°C. The FPase and β-glucosidase activity increased to almost double, when temperature of incubation was raised from 30 to 45°C.

**pH**

The initial pH level was set in the range of (4 to 6) in the growth medium. The pH was then allowed to fluctuate and the enzyme activities recorded after 7 days. Initial pH of 4.5 to 5.5 in general seemed beneficial for enzyme activity (Table 12). The highest FPase and β-glucosidase activities of 0.30 and 0.65 U/ml, respectively were recorded at initial pH of 5.0. However, the initial pH value (5.0) kept on varying and reached to a level of about 6.7 – 6.9 during the course of study. The pH fluctuation above this level showed lesser enzyme activity. Thus, for further experiments the initial pH of 5.0 was used in the cultivation medium.

<table>
<thead>
<tr>
<th>Initial pH</th>
<th>FPase (U/ml)</th>
<th>β-Glucosidase (U/ml)</th>
<th>Final pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.0</td>
<td>0.19</td>
<td>0.49</td>
<td>6.3</td>
</tr>
<tr>
<td>4.5</td>
<td>0.25</td>
<td>0.59</td>
<td>6.4</td>
</tr>
<tr>
<td>5.0</td>
<td>0.30</td>
<td>0.65</td>
<td>6.4</td>
</tr>
<tr>
<td>5.5</td>
<td>0.24</td>
<td>0.62</td>
<td>6.5</td>
</tr>
<tr>
<td>6.0</td>
<td>0.20</td>
<td>0.21</td>
<td>6.7</td>
</tr>
</tbody>
</table>
Sodium citrate buffers (pH 5.0) of different concentrations (0.05, 0.1 and 0.5 M) were used in the medium in order to keep the pH stable. Table 13 shows that 0.05 M citrate buffer maintained the pH level up to 5.4 after 3 days of incubation. The other two molar strengths used resulted in lowering of pH with a decrease of β-glucosidase activity after 2 days of incubation. Thus only 0.05 M buffer seemed to have a greater buffering capacity. However, the control without buffer showed higher enzyme titres for β-glucosidase.

Table 13: Effect of different strengths of citrate buffer pH 5. for the maintenance of pH in the growth medium on cellulase activity by S. thermophile. A control without buffer was also run.

<table>
<thead>
<tr>
<th>Buffer strength</th>
<th>pH</th>
<th>FPass (U/ml)</th>
<th>β-Glucosidase (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
<td>3</td>
<td>2  3</td>
</tr>
<tr>
<td>Control</td>
<td>6.0</td>
<td>6.7</td>
<td>0.27 0.30</td>
</tr>
<tr>
<td>0.05</td>
<td>5.2</td>
<td>5.4</td>
<td>0.27 0.30</td>
</tr>
<tr>
<td>0.10</td>
<td>5.1</td>
<td>4.8</td>
<td>0.20 0.21</td>
</tr>
<tr>
<td>0.50</td>
<td>5.1</td>
<td>4.7</td>
<td>0.19 0.18</td>
</tr>
</tbody>
</table>

Cellulose or LC substrate

Effect of cellulase induction by 2% cellulose, LC treated LC substrate was determined after incubating S. theri for 5 days in the shaker. Table 14 shows that untreated kaller grass induced the FPass and β-glucosidase to the highest level of about 4.6 and 8.3 times over α-cellulose; 2.1 and 4 times over avicel and 1.6 and 2.0 times over treated (2% M
with autoclaving) kallar grass.

Table 14: Effect of cellulosic and lignocellulosic substrates on cellulase production by \textit{S. thermophile} after 5 days of incubation.

<table>
<thead>
<tr>
<th>Substrate (2%)</th>
<th>Enzyme Activities (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FPase</td>
</tr>
<tr>
<td>Avicel</td>
<td>0.15</td>
</tr>
<tr>
<td>α-cellulose</td>
<td>0.07</td>
</tr>
<tr>
<td>Kallar Grass (Treated)</td>
<td>0.20</td>
</tr>
<tr>
<td>Kallar Grass (Untreated)</td>
<td>0.32</td>
</tr>
</tbody>
</table>

Substrate Concentration

Increase in substrate (kallar grass) concentration from 2 to 10% resulted in an overall doubling of FPase and β-glucosidase activities after 3 days of incubation (Fig. 13 a, b). The maximal activities for the cellulases were attained during 7-9 days from 2-4% kallar grass, while from 6-10% substrate concentration the maximal activities were generally obtained during 9-11 days. The highest activities were obtained from 8% kallar grass for FPase and glucosidase of 1.1 and 1.6 U/ml, respectively (Table 15). At and 10% kallar grass concentration the FPase became almost constant after 5 days while β-glucosidase increased up to 11 days. Fig. 14 shows the study of growth parameters including extracellular protein which increased up to a certain level and thereafter, lowered to some extent or got stationary.
Fig. 13. Effect of increase in substrate (2-10% kallar grass) concentration on the production of cellulase by S. thermophile.

a) Ffase and b) B-glucosidase.

Fig. 14. Effect of increase substrate (2-10% kallar grass) concentration on growth parameters.

a) pH and b) Extracellular proteins.
Table 15: Maximal cellulase activities, extracellular protein at pH changes by *S. thermophiles* when grown at increased concentration of substrate (kollar grass).

<table>
<thead>
<tr>
<th>Substrate conc. (%)</th>
<th>FPass U/ml</th>
<th>β-Glucosidase U/ml</th>
<th>Ext. protein mg/ml</th>
<th>Medium pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.40</td>
<td>0.65</td>
<td>1.60</td>
<td>6.5</td>
</tr>
<tr>
<td>4</td>
<td>0.75</td>
<td>1.20</td>
<td>2.40</td>
<td>6.4</td>
</tr>
<tr>
<td>6</td>
<td>0.90</td>
<td>1.50</td>
<td>2.90</td>
<td>6.4</td>
</tr>
<tr>
<td>8</td>
<td>1.10</td>
<td>1.60</td>
<td>3.00</td>
<td>5.8</td>
</tr>
<tr>
<td>10</td>
<td>0.98</td>
<td>1.38</td>
<td>3.10</td>
<td>5.7</td>
</tr>
</tbody>
</table>

pH decreased with increase in substrate concentration. The values attained after 11 days were 6.6, 6.4, 6.1, 5.6 and 5.6 for 2, 4, 6, 8 and 10% substrate concentration, respectively. The maximum extracellular protein values obtained after 9 days were 1.4, 1.8, 2.5, 2.9, and 3.1 mg/ml for the above substrate concentrations, respectively. This showed an increase of 2.1 for extracellular protein for the enzyme filtrate obtained at 10% compared with 2% substrate concentration. This was in accord with enzyme activities obtained at high substrate concentration.

**Synthetic Media**

Six different media compositions used by different works were compared for enzyme yields after 5 days of incubation. Among the various media, the one used by Eggins and Pugh (1962), Romanelli et al (1975) was found to be more effective than others (Table 16). The major difference in composition of the two media were in their nitrogen sources. Yeast extract, ammonium sulphate and asparagine at (0.05%) were present in the for
Table 16: Effect of different mineral media composition on cell
ulase, xylanase and extracellular protein productic
in S. thermophile when grown on 2% kallar grass. Th
enzyme activities were determined after 5 days +
incubation.

<table>
<thead>
<tr>
<th>Media</th>
<th>Ext. Protein mg/ml</th>
<th>Enzyme activities (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>FP-ase</td>
</tr>
<tr>
<td>A</td>
<td>0.9</td>
<td>0.24</td>
</tr>
<tr>
<td>B</td>
<td>0.9</td>
<td>0.25</td>
</tr>
<tr>
<td>C</td>
<td>0.6</td>
<td>0.20</td>
</tr>
<tr>
<td>D</td>
<td>0.8</td>
<td>0.12</td>
</tr>
<tr>
<td>E</td>
<td>0.7</td>
<td>0.17</td>
</tr>
<tr>
<td>F</td>
<td>0.7</td>
<td>0.18</td>
</tr>
</tbody>
</table>

* For the composition of the media consult Materials and Methods:

A = Eggins & Pugh, 1952
B = Romaneli et al, 1975
C = Coutts & Smith, 1976
D = Saddler et al, 1982
E = Gribir et al, 1982
F = Macris & Galiothou-Panayotou, 1986

While Proteose Peptone (0.075%), urea (0.03%) were present in tl
latter. The rest of the media's used, showed lesser enzyme act:
ivities. For convenience E & P medium was adopted for furthe
optimization studies, since it had been used in the earli:
studies for isolation of fungi and production of cellulases.

Mineral Salts and Organic Nitrogen:

In a scheme, each of the mineral salts and organic nitrog
constituents present in the E & P medium was used at zero
increasing concentration of 4 fold, compared to the origin
centration (1 fold). One constituent was varied at a tim
while keeping the rest at the original concentration. The enzyme activities were obtained, using 2% kollar grass as carbon source after 5 days of incubation. The results from Table 17 show th

**Table 17:** Effect of medium (E & P) constituents (0-4 fold) on the cellulase activity of *S. thermophile*. Kollar grass (2%) was used as carbon source while the enzyme activities were determined after 5 days of incubation.

<table>
<thead>
<tr>
<th>Selective medium constituents</th>
<th>FPase (U/ml)</th>
<th>(\beta)-Glucosidase (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0X 1X 2X 4X</td>
<td>0X 1X 2X 4X</td>
</tr>
<tr>
<td>KH PO 2 4</td>
<td>0.21 -- 0.53 0.50</td>
<td>0.40 -- 0.58 0.58</td>
</tr>
<tr>
<td>((NH_4)SO_4) 4 2 4</td>
<td>0.24 -- 0.42 0.33</td>
<td>0.56 -- 0.58 0.60</td>
</tr>
<tr>
<td>KCl</td>
<td>0.35 -- 0.38 0.32</td>
<td>0.57 -- 0.55 0.55</td>
</tr>
<tr>
<td>MgSO_4 4</td>
<td>0.24 -- 0.23 0.22</td>
<td>0.61 -- 0.58 0.53</td>
</tr>
<tr>
<td>CaCl_2</td>
<td>0.40 -- 0.42 0.44</td>
<td>0.57 -- 0.52 0.40</td>
</tr>
<tr>
<td>a-asparagine</td>
<td>0.25 -- 0.24 0.22</td>
<td>0.43 -- 0.55 0.53</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>0.27 -- 0.23 0.24</td>
<td>0.50 -- 0.59 0.62</td>
</tr>
<tr>
<td>Control</td>
<td>-- 0.40 -- --</td>
<td>-- 0.62 -- --</td>
</tr>
</tbody>
</table>

* Medium constituent used at concentration (X) of:
  0X: zero
  1X: original
  2X: 2 times
  4X: 4 times

removal of mineral salts declined the FPase activity and extracellular protein in general while the \(\beta\)-glucosidase was little affected when compared to the control. On the other hand decrease in nitrogen content effected both the enzymes by lowering the activity. The increase of mineral and nitrogen content also could not bring about any noticeable increase in the enzyme activity. Thus the normal composition of the medium was adopted.
Effect of doubling of nutrients and nitrogen contents separately in the E & P medium supplied by 2% kollar grass enhance the cellulase activity, determined after six days of incubation. Table 18 shows that the enhancement of FP-ase and β-glucosidase was of the order of 1.1 and 1.2 times for the former and 1.2 and 1.6 times for latter. The pH level also increased with the increase of salts and nitrogen content.

Table 18: Effect of two fold increase in salts and nitrogen content on the production of enzymes by S. thermophile after 7 days of incubation.

<table>
<thead>
<tr>
<th>Medium contents (x fold)</th>
<th>pH</th>
<th>Enzyme Activities (U/ml)</th>
<th>FP-ase</th>
<th>β-glucosidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.0</td>
<td>0.55</td>
<td>0.67</td>
<td></td>
</tr>
<tr>
<td>Minerals (x 2)</td>
<td>6.6</td>
<td>0.68</td>
<td>0.80</td>
<td></td>
</tr>
<tr>
<td>Nitrogen (x 2)</td>
<td>6.95</td>
<td>0.65</td>
<td>1.10</td>
<td></td>
</tr>
</tbody>
</table>

Different nitrogen sources at 0.05% concentrations each were used along with (NH₄)SO₄ (0.05%) present as a common nitrogen source in all the formulations. The nitrogen sources were also used in another combination of yeast extract and ammonium sulphate at 0.05% each and were used commonly along with seven different nitrogen sources. The enzyme activities were determined after an incubation of 5 days. Table 19 shows that ammonium sulphate used alone produced low enzymatic yields concomitant with lowering of pH. Out of seven nitrogen sources tested combination with ammonium sulphate α-asparagine and cotr...
seed flour had a greater influence on FP\textit{ase} and $\beta$-glucosidase activity. The pH for different nitrogen sources ranged between 5-6.2 except for urea where pH level increased up to 9.5. The high pH was also accompanied by lower yields.

\textbf{Table 19:} Effect of different nitrogen sources (0.05% each) on cellulase activity of \textit{S. thermophile} when used singly with ammonium sulphate and in combination with yeast extract. The enzyme activities were determined after 10 days of incubation.

<table>
<thead>
<tr>
<th>Nitrogen Sources</th>
<th>FP\textit{ase} U/ml</th>
<th>$\beta$-Glucosidase U/ml</th>
<th>Medium pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium Sulphate</td>
<td>0.12</td>
<td>0.13</td>
<td>4.7</td>
</tr>
<tr>
<td>Yeast Extract*</td>
<td>0.21</td>
<td>0.26</td>
<td>5.7</td>
</tr>
<tr>
<td>$\alpha$-asparagine*</td>
<td>0.22</td>
<td>0.40</td>
<td>6.2</td>
</tr>
<tr>
<td>Urea*</td>
<td>0.19</td>
<td>0.25</td>
<td>9.5</td>
</tr>
<tr>
<td>Peptone*</td>
<td>0.21</td>
<td>0.24</td>
<td>5.1</td>
</tr>
<tr>
<td>Corn Steep Liquor*</td>
<td>0.16</td>
<td>0.22</td>
<td>5.0</td>
</tr>
<tr>
<td>Cotton Seed Flour*</td>
<td>0.22</td>
<td>0.33</td>
<td>5.4</td>
</tr>
<tr>
<td>Cas-amino acid*</td>
<td>0.21</td>
<td>0.28</td>
<td>5.2</td>
</tr>
<tr>
<td>$\alpha$-asparagine**</td>
<td>0.37</td>
<td>0.50</td>
<td>6.5</td>
</tr>
<tr>
<td>Urea**</td>
<td>0.22</td>
<td>0.24</td>
<td>10.1</td>
</tr>
<tr>
<td>Peptone**</td>
<td>0.24</td>
<td>0.33</td>
<td>5.9</td>
</tr>
<tr>
<td>Corn Steep Liquor**</td>
<td>0.19</td>
<td>0.19</td>
<td>5.1</td>
</tr>
<tr>
<td>Cotton Seed Flour**</td>
<td>0.25</td>
<td>0.40</td>
<td>6.1</td>
</tr>
<tr>
<td>Cas-amino acid**</td>
<td>0.30</td>
<td>0.40</td>
<td>5.9</td>
</tr>
</tbody>
</table>

* Ammonium Sulphate added  
** Ammonium Sulphate plus Yeast Extract added

The second formulation from Table 19, indicates the combination of ammonium sulphate and yeast extract along with $\alpha$-asparagine as most suitable for enzyme production. This was incidentally also the composition of E & P medium. A pH value of 6.5 was found to be optimum. Apart from this, combination with cas-amino acids cotton-seed flour was also effective for good yields.
Miscellaneous Additives

The FPase is mostly taken as a complete cellulase and thus a representative of the cellulase system. In this experiment FPase was determined after 5 days, using different sources in medium including trace elements, vitamin solution and tween separately and in combined form.

Table 20 gives an overview of the fact that none of the sources had any significant effect on FPase activity. Kall grass in E & P medium was taken as a complete medium fulfill the requirements to a greater extent in S. thermophile.

<table>
<thead>
<tr>
<th>Medium additives</th>
<th>FPase U/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium only</td>
<td>0.40</td>
</tr>
<tr>
<td>Trace min. soln.</td>
<td>0.36</td>
</tr>
<tr>
<td>Vitamin soln.</td>
<td>0.38</td>
</tr>
<tr>
<td>Tween-80</td>
<td>0.40</td>
</tr>
<tr>
<td>Medium + all above</td>
<td>0.46</td>
</tr>
</tbody>
</table>

CONCENTRATION OF CELLULASES BY ULTRAFILTRATION

Enzyme filtrate from (2, 4 & 6%) kollar grass was passed through ultrafiltration membrane having a molecular weight cut off size of 20,000 daltons. As also previously reported, higher enzymatic titres were obtained when substrate (kollar grass concentrations were increased. Table 21 enumerates the enzy.
titres which were further enhanced when the enzyme filtrate from 2% kollar grass was concentrated five times to yield 3.0, 4.0 and 2.6 fold increase for FPase, β-glucosidase and extracellular proteins, respectively. Similarly when enzyme filtrate from 4% and 6% kollar grass was ultrafiltered the enzyme was concentrate further. However, the increase was obtained to a relative lower extent, in general. It was estimated that after increasing

Table 21 Effect of ultrafiltration of crude enzyme obtained from different concentrations of kollar grass by thermophile, on cellulase activities and extracellular protein.

<table>
<thead>
<tr>
<th>Substrate conc. (%)</th>
<th>FPase U/ml</th>
<th>β-Glucosidase U/ml</th>
<th>Ext. Protein mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>2(a)</td>
<td>0.35</td>
<td>0.60</td>
<td>1.4</td>
</tr>
<tr>
<td>2(b)</td>
<td>1.00</td>
<td>2.40</td>
<td>4.0</td>
</tr>
<tr>
<td>2(c)</td>
<td>0.15</td>
<td>0.12</td>
<td>0.3</td>
</tr>
<tr>
<td>4(a)</td>
<td>0.65</td>
<td>1.10</td>
<td>2.0</td>
</tr>
<tr>
<td>4(b)</td>
<td>1.56</td>
<td>3.90</td>
<td>5.0</td>
</tr>
<tr>
<td>4(c)</td>
<td>0.18</td>
<td>0.17</td>
<td>0.3</td>
</tr>
<tr>
<td>6(a)</td>
<td>0.70</td>
<td>1.30</td>
<td>2.4</td>
</tr>
<tr>
<td>6(b)</td>
<td>1.50</td>
<td>3.70</td>
<td>6.0</td>
</tr>
<tr>
<td>6(c)</td>
<td>0.23</td>
<td>0.10</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Total volume used:
(a) Control - 500ml
(b) Concentrate - 100ml
(c) Filtrate - 400ml

the kollar grass concentration from 2 to 6%, the FPase, glucosidase and extracellular protein increased to 2.0, 2.2 a 1.7 folds in the crude extract while processing it further through ultrafiltration it was enhanced to 4.3, 6.2 and 4 folds, respectively. However, the enzyme activity in the concentrate compared with the dilute enzyme did not increase to t
same extent, based on the 5-fold concentration, volumetrically.

Fig. 15a shows that the recovery of the enzyme activities (based on the total titre) in the concentrate decreased for the crude enzyme filtrate obtained at increased kallar grass concentration. However, Fig. 15b indicates that the filtrate from ultrafiltration also contained lower enzyme activities for the enzyme filtrate obtained at higher substrate concentration. This:

Fig. 15. Percent recovery (a) and losses (b,c) for the cellulosic and extracellular proteins, incurred upon concentration of crude enzyme filtrate obtained from 4% and 6% kallar grass.

a) concentrate, b) filtrate, c) due to adsorption.
analogy from Fig. 15a and b was attributed to loss of a proportion of enzymes obtained at 4 and 6% kollar grass, due to adsorption on the ultrafiltration membrane (Fig. 15c). There was more loss of FPase than β-glucosidase as observed in the concentrate.

CELLULASE PRODUCTION IN A FERMENTOR

The fermentor, 14 litre capacity was used in order to enable conditions in which better growth and enzyme production could take place. Kollar grass (2%) was inoculated with 5% (v/v) vegetative inoculum grown for 24 hours at 45 C. The time course study after every 8 hours was carried out under sterilized conditions. Fig. 16a gives the profile for pH which fluctuated and rose up to a level of 6.9 up to 16 hours. The enzymes were induced to some extent after 8 hours indicating a short lag. The maximal activity range was about 32 to 88 hours, yielding a maximal 0.37 and 0.55 U/ml for FPase and β-glucosidase, respectively (Fig. 16b,c). The extracellular protein level increased up to 5 mg/ml after 40 hours of growth. The thick mycelial slurry which grew very dense after 40-50 hours started to lose its filamentous nature while the enzyme production level increased through this stage to a maximum.

In another experiment under similar conditions, the pH of the medium was controlled at 5.0 in the fermentor. The lag per increased from 8-12 hours. The maximal growth and enzyme activities of 0.40 and 0.84 U/ml and 1.2 mg/ml were obtained for FPase, β-glucosidase and extracellular protein, after 40 to 80 hours respectively (Fig. 16b,c).
Fig. 18. Batch growth in an instrumented fermentor by S. thermophile on 2% kollar grass, when the pH was allowed to fluctuate.

a) Cellulase activity, b) pH fluctuations and c) Extracellular proteins.

Fig. 17. Batch growth in a instrumented fermentor by S. thermophile when pH was controlled at 5.0.

a) Cellulase activity.
b) Extracellular proteins.
ENZYME PARAMETERS

pH

In order to determine the exact pH values for the different enzymes from *S. thermophile*, 0.05 M citrate buffer in the range of pH 3 to 7 was used. Crude enzyme preparation obtained from kallar grass by SSF showed that a pH range of 5.0 to 5.5 was most suitable for all the enzymes tested (Fig. 17). Avicelase, CMC and B-glucosidase showed higher activities at pH 5.0, whi

![Graph](image)

**Fig. 18.** Effect of pH in the range of 3.0-7.0 on enzyme activities. The citrate buffer (0.05M) was used.

a) avicelase/B-glucosidase and b) CMCase/xylanase.

Xylanase showed a maximum rise in activity at pH 5.5. Poor enzymatic activities were obtained at pH < 4 and > 6.

Temperature

The crude extract obtained from SSF by *S. thermophile* was used to determine the effect of temperature in the range of 0-70°C on different enzyme activities. Fig. 19 shows that optimum temperature for enzyme activities was 65-70°C. Avicelase exhibited optimum activity at 70°C, whereas CMCase, CMC-ase
xylanase were found to be optimum at 65°C.

Fig. 19. Effect of temperature in the range of 40-70°C on enzyme activities.

a) avicelase/β-glucosidase and b) CMCase/xylanase.

Thermostability:

The enzyme filtrates from different thermophilic and two mesophilic fungi were subjected to incubation temperatures of (40-70°C) for 24 hours. The FPase activities of thermophilic fungi showed 100% stability at 50°C (Fig. 20). H. grisea exhibited maximum stability of about 85% for FPase at 60°C, while the rest excluding T. thermophila showed about 70% stability. The thermostability at 70°C was recorded at about 60% for C. thermophila and H. krisae. Rest of the thermophiles showed about 45-48% of stability at 70°C. In comparison, enzymes from mesophilic strains of T. reesei VIT-D-79125 and C. vergicephalum showed 100% and 10% FPase at 50°C, respectively, but the former lost more than 78% and 94% of its FPase activity at 60 and 70°C, while the latter did not retain any activity at these temperatures.
Fig. 20 Stability of relative FPase activity in the culture filtrate of thermophilic and mesophilic fungi, incubated at 40-70°C for 24 hours.

PRETREATMENT OF LC SUBSTRATES

Six LC substrates were analysed for ash, soluble sugars Klassen lignin and total polysaccharides. The total polysaccharid content of LC's was determined by acid hydrolysis of the substrate and subjecting the hydrolysate to borate ion exchange chromatography system using using 2-2 bicinchoninate reagent (see materials and methods). These substrates were pretreated with steaming at high temperature of 190 and 200°C, NaOH (2, 3 or 4%; 5 times v/w) at room temperature for 24 hours and NaOH (2, 4% and 4%; 5 times v/w) with autoclaving at 121°C for one hour. with autoclaving. The pretreated substrates were analysed again for the mass balances. These pretreated fibre material after climatication at 10°C were treated with commercial enzyme preparation of T. reesei (15 FPU/g substrate) along with the addition of \( \beta \)-glucosidase for determining the accessibility.

Kellar Grass

Mass balance studies carried out on untreated kellar grass showed that it contained 10.7% ash. The total carbohydrate
fraction of untreated sample also contained 9.6% of soluble sugars. At the first site sodium hydroxide pretreatment with autoclaving caused maximum enzymatic accessibility (Table 22).

Table 22: Pretreatment analysis of Leptochloa fusca (kollar grass) and its subsequent enzymatic accessibility

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fibre Yield (%)</th>
<th>Klasson Lignin (%)</th>
<th>Cellulose &amp; hemicellulose sugars after analytical hydrolysis (%)</th>
<th>Enzymatic accessibility (%)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated*</td>
<td>100</td>
<td>19.7</td>
<td>55.7</td>
<td>33.3</td>
</tr>
<tr>
<td>Steaming</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>190oC</td>
<td>53.2</td>
<td>27.7</td>
<td>65.2</td>
<td>52.0</td>
</tr>
<tr>
<td>200oC</td>
<td>46.6</td>
<td>22.9</td>
<td>72.5</td>
<td>51.6</td>
</tr>
<tr>
<td>NaOH at Room Temperature</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2%</td>
<td>70.4</td>
<td>18.4</td>
<td>70.0</td>
<td>58.6</td>
</tr>
<tr>
<td>3%</td>
<td>66.6</td>
<td>16.4</td>
<td>73.0</td>
<td>61.9</td>
</tr>
<tr>
<td>4%</td>
<td>51.7</td>
<td>15.9</td>
<td>72.0</td>
<td>60.2</td>
</tr>
<tr>
<td>NaOH with Autoclaving</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2%</td>
<td>57.0</td>
<td>10.3</td>
<td>77.0</td>
<td>66.5</td>
</tr>
<tr>
<td>3%</td>
<td>51.0</td>
<td>10.3</td>
<td>78.0</td>
<td>69.2</td>
</tr>
<tr>
<td>4%</td>
<td>45.0</td>
<td>10.1</td>
<td>75.0</td>
<td>69.5</td>
</tr>
</tbody>
</table>

* Ash content in the untreated substrate was 10.7%. Soluble sugars in the same substrate were 9.6%.
** Based on percent of dry treated fibre, values in brackets are based on untreated raw material.

For untreated Kollar grass, the enzymatic accessibility was 11.2% which increased to 42.3, 66.4 and 77.2% on steaming-extraction, pretreatment with NaOH at room temperature and pretreatment with NaOH along with autoclaving respectively. The results show that 3% alkali pretreatment with autoclaving give the maximum enzymatic accessibility based on the treated substrate. However, due
to higher fibre losses during autoclaving at 3 and 4% NaOH concentration, autoclaving with 2% NaOH gave higher enzymatic accessibility based on raw material (40.3%). Sodium hydroxide and autoclaving caused higher lignin losses than others. Alkali pretreatment at room temperature rendered Kallar grass more accessible enzymatic degradation as compared to steaming. Due to moderate weight losses of the raw material during treatment of kallang with NaOH at room temperature 3% NaOH turned out to optimal. 43.2% (based on raw material) was solubilized by 15 FPU fibre.

**Panicum maximum**

*P. maximum* showed both high ash (13%) and soluble sugar 12.9%. Enzymatic accessibility of >85% was obtained after pretreatment with 2% NaOH followed by autoclaving. The enzymatic accessibilities of samples pretreated by steaming, pretreatment with NaOH at room temperature and NaOH with autoclaving were 51.2, 72.2, and 85.9% respectively (Table 23). Sample pretreatment with 2% NaOH at room temperature showed higher enzymatic accessibility than for steaming. The mass balance studies showed that NaOH pretreatment followed by autoclaving removed a considerable part of the initial lignin content. However, large fibre loss at 3% and 4% NaOH concentrations indicate carbohydrate solubilization during pretreatment. Although enzymatic accessibility was highest for autoclaved fibres, soaking of *P. maximum* with 2% NaOH at room temperature was more favourable with regard to optimal carbohydrate recovery (based on raw material) than any other treatment. The amount of the original plant materials solubilized: 116
Table 21: Pretreatment analysis of Panicum maximum and its subsequent enzymatic accessibility.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fibre Yield (%)</th>
<th>Klason Lignin (%)</th>
<th>Cellulose &amp; hemicellulose (%)</th>
<th>Enzymatic sugars after analytical hydrolysis (%)</th>
<th>Enzymatic accessibility (%)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>U n t r e a t e d*</td>
<td>100</td>
<td>18.2</td>
<td>51.6</td>
<td>29.8</td>
<td>16.9</td>
</tr>
<tr>
<td>Steaming</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>190°C</td>
<td>75.8</td>
<td>24.0</td>
<td>63.4</td>
<td>48.6</td>
<td>13.5</td>
</tr>
<tr>
<td>200°C</td>
<td>72.3</td>
<td>22.4</td>
<td>64.6</td>
<td>50.3</td>
<td>13.3</td>
</tr>
<tr>
<td>NaOH at Room Temperature</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2%</td>
<td>68.9</td>
<td>17.2</td>
<td>72.2</td>
<td>45.1</td>
<td>21.6</td>
</tr>
<tr>
<td>3%</td>
<td>70.6</td>
<td>13.6</td>
<td>79.8</td>
<td>53.3</td>
<td>20.0</td>
</tr>
<tr>
<td>4%</td>
<td>61.0</td>
<td>14.0</td>
<td>76.0</td>
<td>53.0</td>
<td>18.1</td>
</tr>
<tr>
<td>NaOH with Autoclaving</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2%</td>
<td>60.0</td>
<td>9.1</td>
<td>80.6</td>
<td>56.0</td>
<td>21.1</td>
</tr>
<tr>
<td>3%</td>
<td>45.6</td>
<td>8.9</td>
<td>83.8</td>
<td>60.5</td>
<td>20.1</td>
</tr>
<tr>
<td>4%</td>
<td>38.9</td>
<td>8.2</td>
<td>77.7</td>
<td>61.1</td>
<td>16.6</td>
</tr>
</tbody>
</table>

* Ash content in the untreated substrate was 13.0%. Solubilized sugars in the same substrate were 12.9%.
** Based on per cent of dry treated fibre, values in brackets are based on untreated raw material.

was 54.7%. This value is higher than the amount of cellulose polysaccharides which indicates the solubilization of some lignin.

**Aegilops amnicola**

Raw material analysis of A. amnicola revealed 23.3% as 18.4% lignin, 34.8% cell wall polysaccharides and 11.7% solubilized sugars (Table 24). The amount of ash and lignin was higher than the amount of cell wall polysaccharides. A. amnicola without a pretreatment was only 5.7% accessible to enzymatic degradation which increased as a result of steaming, pretreatment with Na
Table 24: Pretreatment analysis of *Atriplex amnicola* and its subsequent enzymatic accessibility

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fibre Yield (%)</th>
<th>Klason Lignin (%)</th>
<th>Cellulose &amp; hemicellulose (%)</th>
<th>Enzymatic sugars after analytical accessibility</th>
<th>Enzymatic hydrolysis (%)**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated*</td>
<td>100</td>
<td>18.4</td>
<td>34.8</td>
<td>19.7</td>
<td>7.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5.7 (5.7)</td>
</tr>
<tr>
<td>Steaming</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>190°C</td>
<td>49.2</td>
<td>36.0</td>
<td>49.8</td>
<td>43.1</td>
<td>5.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>28.9 (14.2)</td>
</tr>
<tr>
<td>200°C</td>
<td>46.4</td>
<td>35.6</td>
<td>51.6</td>
<td>44.2</td>
<td>5.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>31.6 (14.7)</td>
</tr>
<tr>
<td>NaOH at Room Temperature</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2%</td>
<td>64.4</td>
<td>22.9</td>
<td>44.9</td>
<td>27.1</td>
<td>9.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>27.4 (17.6)</td>
</tr>
<tr>
<td>3%</td>
<td>60.0</td>
<td>21.8</td>
<td>51.3</td>
<td>31.3</td>
<td>10.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>32.3 (19.4)</td>
</tr>
<tr>
<td>4%</td>
<td>54.4</td>
<td>19.7</td>
<td>52.4</td>
<td>32.9</td>
<td>7.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>33.0 (17.9)</td>
</tr>
<tr>
<td>NaOH with Autoclaving</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2%</td>
<td>39.6</td>
<td>24.9</td>
<td>58.8</td>
<td>40.9</td>
<td>13.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>45.4 (18.0)</td>
</tr>
<tr>
<td>3%</td>
<td>37.8</td>
<td>23.2</td>
<td>55.4</td>
<td>45.1</td>
<td>14.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>46.9 (17.7)</td>
</tr>
<tr>
<td>4%</td>
<td>36.7</td>
<td>23.4</td>
<td>60.6</td>
<td>45.8</td>
<td>12.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>48.1 (17.6)</td>
</tr>
</tbody>
</table>

* Ash content in the untreated substrate was 23.3%. Soluble sugars in the same substrate were 11.7%.

** Based on per cent of dry treated fibre, values in brackets are based on untreated raw material.

at room temperature and pretreatment with NaOH with autoclaving to 31.6, 33.0 and 46.1, respectively (values based on pretreated fibres). For *A. amnicola*, a pretreatment using 2% NaOH with autoclaving gave near maximum enzymatic accessibility. Moreover with increasing concentration of NaOH, the fibre yield decrease considerably which reduced the accessibility to 18% based on raw material. Best utilization of *A. amnicola* was obtained by enzymatic hydrolysis of 3% NaOH treated material at room temperature.
Bagasse

Untreated bagasse as compared to the salt tolerant plant contained only 1.4% ash, whereas no soluble sugars were obtained. Alkali (3%) with autoclaving caused maximum digestibility. The enzymatic accessibility of untreated bagasse increased from 9.0% to 35.5, 64.0 and 68.3% as a result of following pretreatments: steaming, NaOH at room temperature and NaOH with autoclaving, respectively (Table 25). Higher fiber losses incurred due to the harsh treatments and the mass balance studies indicated that there were not only losses in lignin contents but also in cellulose.

Table 25: Pretreatment analysis of Bagasse and its subsequent enzymatic accessibility.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fibre Yield (%)</th>
<th>Klason Lignin (%)</th>
<th>Cellulose &amp; Hemicellulose sugars after analytical digestibility (%)</th>
<th>Enzymatic hydrolysis (%)</th>
<th>Total (%)</th>
<th>Glucose (%)</th>
<th>Xylose (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated*</td>
<td>100</td>
<td>17.6</td>
<td>73.8</td>
<td>46.7</td>
<td>24.4</td>
<td>9.0</td>
<td>(9.1)</td>
</tr>
<tr>
<td>Steaming</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>190°C</td>
<td>66.4</td>
<td>22.3</td>
<td>73.9</td>
<td>62.7</td>
<td>11.2</td>
<td>30.5</td>
<td>(20.3)</td>
</tr>
<tr>
<td>200°C</td>
<td>64.0</td>
<td>20.0</td>
<td>77.5</td>
<td>62.4</td>
<td>15.1</td>
<td>35.5</td>
<td>(22.7)</td>
</tr>
<tr>
<td>NaOH at Room Temperature</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2%</td>
<td>80.9</td>
<td>13.0</td>
<td>80.3</td>
<td>54.5</td>
<td>23.8</td>
<td>42.9</td>
<td>(34.5)</td>
</tr>
<tr>
<td>3%</td>
<td>74.2</td>
<td>11.7</td>
<td>79.3</td>
<td>56.3</td>
<td>22.3</td>
<td>64.0</td>
<td>(47.5)</td>
</tr>
<tr>
<td>4%</td>
<td>73.3</td>
<td>12.8</td>
<td>80.1</td>
<td>55.0</td>
<td>22.7</td>
<td>60.0</td>
<td>(43.8)</td>
</tr>
<tr>
<td>NaOH with Autoclaving</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2%</td>
<td>69.7</td>
<td>12.6</td>
<td>87.5</td>
<td>58.0</td>
<td>27.7</td>
<td>56.7</td>
<td>(39)</td>
</tr>
<tr>
<td>3%</td>
<td>62.5</td>
<td>8.7</td>
<td>92.9</td>
<td>64.9</td>
<td>26.2</td>
<td>68.3</td>
<td>(42)</td>
</tr>
<tr>
<td>4%</td>
<td>63.2</td>
<td>8.7</td>
<td>90.2</td>
<td>64.7</td>
<td>23.4</td>
<td>66.0</td>
<td>(41)</td>
</tr>
</tbody>
</table>

* Ash content in the untreated substrate was 1.4%. Soluble sugars in the same substrate were absent.
** Based on per cent of dry treated fibre, values in brackets based on untreated raw material.
also in the total carbohydrates. Maximum fibre recovery (80.9%) for total polysaccharides was obtained after alkali (2%) treatment at room temperature. However, the enzymatic accessibility (on raw material basis) was maximum at 47.5% with 3% alkali treatment at room temperature. Steaming at high temperatures produce significantly lower yields as compared to alkali pretreatments.

**Wheat Straw**

Wheat straw contained little ash content (4.8%), while the soluble sugars were only 1.1%. The enzymatic solubility of untreated wheat straw was 13.0% (Table 26) which increased to 45.1%

**Table 26:** Pretreatment analysis of wheat straw and its subsequent enzymatic accessibility

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fibre Yield (%)</th>
<th>Klason Lignin (%)</th>
<th>Cellulose &amp; hemicellulose sugars after analytical accessibility (%)</th>
<th>Enzymatic hydrolysis (%)</th>
<th>(%)**</th>
<th>Total</th>
<th>Glucose</th>
<th>Xylose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated*</td>
<td>100</td>
<td>19.6</td>
<td>70.8</td>
<td>44.0</td>
<td>22.7</td>
<td>13.0</td>
<td>(13.0)</td>
<td></td>
</tr>
<tr>
<td>Steaming 190°C</td>
<td>69.5</td>
<td>21.0</td>
<td>58.4</td>
<td>45.6</td>
<td>12.8</td>
<td>44.1</td>
<td>(30.6)</td>
<td></td>
</tr>
<tr>
<td>200°C</td>
<td>66.6</td>
<td>19.5</td>
<td>68.1</td>
<td>51.1</td>
<td>15.7</td>
<td>45.0</td>
<td>(30.0)</td>
<td></td>
</tr>
<tr>
<td>NaOH at Room Temperature 2%</td>
<td>77.2</td>
<td>14.7</td>
<td>70.0</td>
<td>50.0</td>
<td>16.2</td>
<td>72.8</td>
<td>(56.2)</td>
<td></td>
</tr>
<tr>
<td>3%</td>
<td>72.8</td>
<td>14.3</td>
<td>73.0</td>
<td>52.5</td>
<td>16.0</td>
<td>72.7</td>
<td>(52.9)</td>
<td></td>
</tr>
<tr>
<td>4%</td>
<td>69.6</td>
<td>13.8</td>
<td>74.0</td>
<td>53.0</td>
<td>16.0</td>
<td>73.0</td>
<td>(50.8)</td>
<td></td>
</tr>
<tr>
<td>NaOH with Autoclaving 2%</td>
<td>59.8</td>
<td>7.8</td>
<td>80.0</td>
<td>60.0</td>
<td>16.5</td>
<td>89.2</td>
<td>(53.3)</td>
<td></td>
</tr>
<tr>
<td>3%</td>
<td>55.4</td>
<td>7.4</td>
<td>82.0</td>
<td>62.0</td>
<td>17.0</td>
<td>89.0</td>
<td>(49.3)</td>
<td></td>
</tr>
<tr>
<td>4%</td>
<td>52.2</td>
<td>6.7</td>
<td>86.0</td>
<td>66.0</td>
<td>20.0</td>
<td>89.4</td>
<td>(46.7)</td>
<td></td>
</tr>
</tbody>
</table>

* Ash content in the untreated substrate was 4.8%. Soluble sugars in the same substrate were 1.1%.
** Based on per cent of dry treated fibre, values in brackets are based on untreated raw material.
73.0 and 89.2% after steaming extraction, pretreatments with alkali at room temperature and alkali pretreatment with autoclaving, respectively. Considering on raw material basis 2% NaOH treatment at room temperature yielded 77.2% fibre with the maximum enzymatic accessibility of 56.2%. Steaming extraction although showed better fibre yields than alkali with autoclaving but depicted poor enzymatic accessibility than the latter. This alkali treatment showed maximum delignification along with concomitant increase in total polysaccharides. The raw material losses, although indicated some polysaccharide solubilisation as well.

Poplar

This substrate contained, only 1.9 and 0.9% ash and soluble sugar contents, respectively. The enzymatic accessibility showed poor response even after the pretreatments. Alkali with autoclaving showed the overall best results based on pretreatment fibre and raw material basis. Enzymatic accessibility increased from 8.0% to 24.8, 18.0 and 32.2% after treatment with steaming, alkali at room temperature and alkali with autoclaving, respectively. (Table 27). On raw materials basis 2% NaOH with autoclaving yielded a maximum of 22.3% enzymatic solubilization. Steaming as a pretreatment for poplar was slightly better than NaOH treatment at room temperature which was found to be too mild for the hardwood. The lignin content did not decrease to a greater extent as in the previous substrates.
Table 27: Pretreatment analysis of Poplar and its subsequent er tic accessibility.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fibre Yield (%)</th>
<th>Klason Lignin (%)</th>
<th>Cellulose &amp; hemicellulose sugars after analytical accessibility hydrolysis (%) Total</th>
<th>Enzymatic accessibility (%)** Total Glucose Xylose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated*</td>
<td>100</td>
<td>22.6</td>
<td>63.6</td>
<td>45.7</td>
</tr>
<tr>
<td>Steaming</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>190oC</td>
<td>75.3</td>
<td>28.1</td>
<td>63.0</td>
<td>55.0</td>
</tr>
<tr>
<td>200oC</td>
<td>74.2</td>
<td>25.6</td>
<td>69.4</td>
<td>57.4</td>
</tr>
<tr>
<td>NaOH at Room Temperature</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2%</td>
<td>91.9</td>
<td>23.7</td>
<td>61.6</td>
<td>45.8</td>
</tr>
<tr>
<td>3%</td>
<td>86.4</td>
<td>24.4</td>
<td>65.3</td>
<td>50.0</td>
</tr>
<tr>
<td>4%</td>
<td>81.1</td>
<td>23.5</td>
<td>64.8</td>
<td>51.2</td>
</tr>
<tr>
<td>NaOH with Autoclaving</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2%</td>
<td>74.2</td>
<td>23.4</td>
<td>71.2</td>
<td>56.3</td>
</tr>
<tr>
<td>3%</td>
<td>68.8</td>
<td>21.7</td>
<td>71.0</td>
<td>57.0</td>
</tr>
<tr>
<td>4%</td>
<td>67.6</td>
<td>23.6</td>
<td>69.6</td>
<td>57.7</td>
</tr>
</tbody>
</table>

* Ash content in the untreated substrate was 1.9%. Soluble sugars in the same substrate were 0.9%.
** Based on per cent of dry treated fibre, values in brackets are based on untreated raw material.

Comparison of Untreated Substrates

Different LC substrates (untreated), when compared, showed that for all the salt tolerant plants, ash and soluble sugar contents were significantly higher than the other substrates used. A. amnicola was found to contain greater ash contents while P. maximum showed slightly higher amounts of soluble sugars (Table 28). Klason lignin content was found to be in the range of (17.6-22.6%), with P. maximum exhibiting the lowest and popula the highest values. Total carbohydrate level was found to be lesser for salt tolerant plants than the other 3 substrate.
Table 28: Analysis of untreated lignocellulosic substrates.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Ash (%)</th>
<th>Soluble sugars (%)</th>
<th>Klason Lignin (%)</th>
<th>Cellulose &amp; Hemicellulose sugars after analytical hydrolysis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Total Glucose Xylose</td>
</tr>
<tr>
<td>Kaller grass</td>
<td>10.7</td>
<td>9.6</td>
<td>19.7</td>
<td>55.7 33.3 19.6</td>
</tr>
<tr>
<td>P. maximum</td>
<td>13.0</td>
<td>12.9</td>
<td>18.2</td>
<td>51.6 29.8 16.9</td>
</tr>
<tr>
<td>A. amnicola</td>
<td>23.3</td>
<td>11.7</td>
<td>18.4</td>
<td>34.8 19.7 7.5</td>
</tr>
<tr>
<td>Bagasse</td>
<td>1.4</td>
<td>0.0</td>
<td>17.6</td>
<td>73.8 46.7 24.4</td>
</tr>
<tr>
<td>Wheat Straw</td>
<td>4.8</td>
<td>1.1</td>
<td>19.6</td>
<td>70.8 44.0 22.7</td>
</tr>
<tr>
<td>Poplar</td>
<td>1.9</td>
<td>0.9</td>
<td>22.6</td>
<td>63.6 45.5 14.1</td>
</tr>
</tbody>
</table>

(Table 22-27). The maximum cellulose level was found in bagasse (73.8%) and the least obtained from A. amnicola (34.8%). Among the different substrates bagasse contained the highest level polysaccharide (73.8%) whereas A. amnicola showed the lowest level (34.8%). The order of total polysaccharide content in the substrates was bagasse > wheat straw > poplar > kaller grass > P. maximum > A. amnicola. Almost similar order was found for glucose (cellulose) potential and xylose (xylan) potential except: Poplar which exhibited the lowest level of xylose. The low overall carbohydrate level in salt tolerant plants was attributed high ash content.

Comparison of Different Substrates after Pretreatments

Steaming at High Temperatures

The results in (Table 22-27 and Fig. 21a) shows that in general a steaming temperature of 200°C proved harsher than 190°C especially for the salt tolerant plants. The maximum fibre yi
Fig. 21  Comparison of different lignocellulosic substrates after steaming pretreatment at high temperatures.

a) Fibre yield, b) Klason lignin, c) Total polysaccharide, d) Glucose and e) Xylose.

Kg, Kollar grass; PM, Panicle maximum; AA, Atriplex amnic; WS, Wheat straw; BG, Bagasse; PL, Poplar.
of about 75.0% was obtained for P. maximum and poplar each whereas the minimum of about 46.5% resulted for kollar grass an A. amnicola each after steaming at 190 and 200 C, respectively. The Klason lignin content indicated an increase in overall lignin content even after steaming at 200 C for all the substrate except wheat straw, in the pretreated fibre. The maximum and minimum values of 35.6 and 19.5% lignin content were attained by A. amnicola and wheat straw after steaming at 190 and 200 C respectively (Fig. 21b).

Results from (Table 22-27 and Fig. 21c) shows a maximum polysaccharide content of 77.5% for bagasse while the minimum value of 51.6% was found in A. amnicola after steaming treatments at 200 C. However, the maximal increase in polysaccharides over the untreated substrate was obtained from A. amnicola resulting in an increase of 16.5%. Fig. 21c, also indicates that among the various substrates wheat straw lost a small amount of polysaccharides during the harsh steaming pretreatments, based on pretreated fiber material. Fig. 21d gives the profile of glucose (cellulose) potential in bagasse which increased to a maximum concentration of 63.0%. The xylose (Xylan) potential in kollar grass decreased to level of 18.6% for bagasse. However, A. amnicola resulted in a maximal increase of 24.3% glucose potential over its untreated control. The xylose (xylan) potential counter decreased for all the substrates in the extraction liquor after treatment but kollar grass showed the maximal increase of xylose potential content of 18.6% (Fig. 21e).

The enzymatic accessibility attained after pretreatment at 200 C, was highest for P. maximum at 51.2% and lowest for poplar.
Fig. 22. Enzymatic accessibility of different lignocellulosic substrates after steaming at high temperature.

Based on
a) Pretreated material and b) Raw material.

at 24.6% over their respective controls at 13.2 and 8.0%. Likewise, P. maximum also exhibited maximum enzymatic accessibility of 37% based on raw materials but A. ammonicola resulted in the minimum values of 14.2% because of greater fibre losses. (Figs 22a, b and Table 22-27). The yields for the latter were obtained as a result of pretreatment at 190 and 200°C.

Sodium Hydroxide at Room Temperature

Table 22-27 and Fig. 23a indicates that maximum fibre yield of 91.9 and 86.9% was obtained for poplar and P. maximum, respectively, whereas the minimum of 44.4% was obtained from A. ammonicola after 2 and 4% alkali treatments. In general, the fibre yield for lignin and xylose (Xylan) potential lowered with the increase in the alkali concentration, while the polysaccharide level especially glucose (cellulose) potential increased after the pretreatment.
Fig. 23. Comparison of different lignocellulosic substrates after alkali pretreatment at room temperature.

a) Fibre yield, b) Klason lignin, c) Total polysaccharide, d) glucose and e) xylose.
Fig. 23. Comparison of different lignocellulosic substrates after alkali pretreatment at room temperature.

a) Fibre yield, b) Klason lignin, c) Total polysaccharide, d) glucose and e) xylose.
Except for *A. amnicola* and poplar all the substrates show a decrease in lignin content after pretreatment. The maximum lowering in lignin content resulted in 11.7% (Klassen lignin) from bagasse with an overall decrease of 5.9% from its control with 3% NaOH treatment, while on the converse, maximum lignin 23.5% resulted for poplar even after 4% alkali treatment (Fig. 23b). The increase in total polysaccharides in general reached maximum or near maximum values at 2-3% alkali treatment. With alkali, fractional higher values were obtained for a few substrates, but at the expense of greater fibre losses. Maximum values of 80.3 and 79.8% for bagasse and *P. maximum* were obtained from 2 and 3% alkali pretreatment, respectively (Fig. 22c). However, the maximum overall increase in polysaccharides over control was favoured for *P. maximum* and kollar grass showing 21 and 17.3% increase, respectively. There were losses of 2.0 ± 0.8% for poplar and wheat straw, respectively, after similar pretreatments. The lowest level of polysaccharides were attained for *A. amnicola* (52.4%) even after 4% alkali concentration. The increase in glucose (cellulose) potential obtained was much higher in salt tolerant plants after the pretreatment. The maximum level of glucose potential was attained in kollar grass with 61.9%, whereas the minimum was found in *A. amnicola* with 32 after 3 and 4% alkali treatments (Fig. 23d). The xylose (xyl) potential contents decreased for all the substrates except *maximum* and *A. amnicola*. The maximum and minimum values of 21 and 8.9% were found in bagasse and kollar grass after 2 and alkali treatments, respectively (Fig. 23e).
Maximum enzymatic accessibility was observed for wheat straw which increased from 13.0 to 72.8%, whereas it was 56.2% on raw material basis after 2% alkali treatment (Fig. 24a,b). The results indicate that poplar was least modified and showed 15.1 and 14.8% accessibility, only. Other substrates followed in the order of P. maximun > kellar grass > bagasse > A. amnicola > poplar.

**Fig. 24.** Enzymatic accessibility of different lignocellulosic substrates after NaOH treatment at room temperature.

Based on
a) Pretreated material and b) Raw material.

**Sodium Hydroxide with Autoclaving**

Table 22-27 and Fig. 25a compares the six LC substrates for percent fibre yield after alkali pretreatment with autoclaving which showed decrease with increasing alkali concentration. The highest and the lowest fibre yield of 74.2 and 36.7% was obtained for poplar and A. amnicola after 2 and 4% alkali treatments, respectively. The lignin profile from Fig. 25b shows decrease in lignin content for all substrates except A. amnicola and poplar. The increase in delignification, concomitant wi
**Fig. 25** Comparison of different lignocellulosic substrates after alkali pretreatment along with autoclaving.

- a) Fibre yield
- b) Klasson lignin
- c) Total polysaccharide
- d) Glucose
- e) Xylose
total polysaccharides was enhanced by higher alkali concentrations, although 2-3% seemed optimum considering fibre losses. The maximum lowering in lignin content resulted in 6.7% Klason lignin for wheat straw, with an overall decrease of 12.9% over it control after 4% alkali treatment (Fig. 25b). On the other hand, maximum lignin attained was found at 23.6 and 23.4% for poplar and A. amnicola hereby, showing an increase of 1.0 and 5.0% over the control values, respectively. The highest level of total polysaccharides up to 86.0% and the lowest at 55.4%, was attained for wheat straw and A. amnicola after pretreatment with 4% alkali. However, the maximum increase of 32.2% and a minimum of 7.6 resulted for P. maximum and bagasse over their respective controls (Fig. 25c). Kaller grass attained 69.5% glucose (cellulose potential with an overall maximum increase of 36.2% over it control (Fig. 25d). A. amnicola registered the lowest glucose (cellulose) value of 45.8% with an increase of 26.1% over it control after 4% alkali treatment. However, the minimum increase of 12% over its control resulted from poplar, even after 4 alkali treatment (Fig. 25c). In contrast to glucose potential, xylene potential from kaller grass showed the lowest value (4.9%) whereas the highest (27.7%) was found in bagasse after 4 and 2 alkali treatment, respectively (Fig. 25e). These substrates also depicted maximum overall increase and decrease in xylose (xylar potential) content over their respective controls.

All the substrates showed maximum enzymatic accessibility in 2-3% NaOH treatment. Wheat straw resulted in 89.0% solubilization closely contested by P. maximum with 86.0% after 2 and 4% alkali treatment (Table 22-27 and Fig 25 a,b). Other substrates
Fig. 26. Enzymatic accessibility (a, b) of different lignocellulosic substrate after NaOH treatment along with autoclaving

Based on
  a) Pretreated material and b) Raw material.

followed the order of accessibility for kallar grass > bagasse A. amnicola > poplar. The respective yields were reduced to 53. and 51.5%, on raw material basis with 2% alkali treatment because of the fiber losses (Fig. 26b).

SACCHARIFICATION OF KALLAR GRASS BY COMMERCIAL ENZYMES.

T. reesei Cellulase Supplemented with β-glucosidase

Table 29 shows that kallar grass (2, 5 & 10%) was saccharified with known FPase to β-glucosidase ratio of 16:27 units/substrate, using the enzymatic combination of commercial T. reesei (VIT-D-79125) and Aspergillus niger (Miles Kalli). When T. reesei was used as such, the FPase and β-glucosidase ratio was 15:10 U/gm substrate. The saccharification was carried out for 48 hours and the reducing sugars were estimated by DNS method. The saccharification of 2, 5 and 10% kallar grass, resulted in
Table 29: Effect of commercial cellulase (FPase) preparations from T. reesei alone and in supplementation with β-glucosidase from A. niger on saccharification of 2, 5 and 10% mallar grass.

<table>
<thead>
<tr>
<th>Substrate (%)</th>
<th>Saccharification Yield (%) /gm fibre</th>
<th>/gm polysaccharides</th>
</tr>
</thead>
<tbody>
<tr>
<td>2a</td>
<td>45.0</td>
<td>52.2</td>
</tr>
<tr>
<td>2b</td>
<td>54.0</td>
<td>62.6</td>
</tr>
<tr>
<td>5a</td>
<td>39.6</td>
<td>45.9</td>
</tr>
<tr>
<td>5b</td>
<td>45.0</td>
<td>52.2</td>
</tr>
<tr>
<td>10a</td>
<td>27.9</td>
<td>32.4</td>
</tr>
<tr>
<td>10b</td>
<td>33.3</td>
<td>38.6</td>
</tr>
</tbody>
</table>

FPase : β-glucosidase units/gm of substrate:
- a) T. reesei (15:10)
- b) T. reesei + A. niger (16.5:27)

Maximal yield of reducing sugars i.e. 62.6, 52.2 and 33.6% respectively (based on total polysaccharides), when β-glucosidase from A. niger was supplemented with T. reesei cellulase. Table 29 also gives the saccharification yield on the basis of total fibres in the substrate. Because the saccharification is related to the polysaccharides, therefore, for rest of the studies, only theoretical yields are mentioned.

Increased Enzyme Concentration

Enzyme concentration from T. reesei and A. niger was increased to two folds in four increments for FPase and β-glucosidase, in ratio of 1:1.6. The saccharification was carried out
for 48 hours. Reducing sugars were estimated by DNS method and glucose was determined by glucose oxidase/peroxidase method. Table 30 shows that 5 and 10% kollar grass was saccharified (on theoretical basis) to a maximal level of 67 and 60% reducing sugars; 42 and 39% glucose, respectively, with 2.0 and 1.66 fold enhanced enzyme concentration. The increase in saccharification

Table 30: Effect of increase in enzyme titre from cellulase preparation of T. reesei supplemented with β-glucosidase from A. niger on saccharification of 5 and 10% kollar grass.

<table>
<thead>
<tr>
<th>Substrate (%)</th>
<th>FFase:β-Glucosidase</th>
<th>Saccharification</th>
<th>Glucose (Yield %)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>U/gm substrate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>17 : 27</td>
<td>54</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>22 : 36</td>
<td>56</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>27 : 45</td>
<td>59</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>33 : 54</td>
<td>67</td>
<td>42</td>
</tr>
<tr>
<td>10</td>
<td>33 : 54</td>
<td>47</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>44 : 72</td>
<td>46</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>55 : 90</td>
<td>60</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>66 : 108</td>
<td>60</td>
<td>39</td>
</tr>
</tbody>
</table>

and glucose yield decreased with concomitant increase in substrate concentration. A two fold increase in enzyme concentration could not saccharify 10% kollar grass to the similar extent as compared to 5%. The latter produced 7 and 3% higher yields of reducing sugars and glucose, respectively, at 10% substrate.

SACCHARIFICATION OF KALLAR GRASS BY CRUDE ENZYME

Increased Enzyme Titre By S. thermophile

The enzyme filtrate obtained from 4% kollar grass having enzyme activity for FFase and β-glucosidase of 0.44 and 0.88 U/ml was used. This enzyme filtrate in the range of 10 to 40 ml
was used to saccharify 1, 2, 5 and 10% kallar grass for 48 hour in a 50 ml reaction mixture. Results (Fig. 27) showed that maximum saccharification of 75, 68, 60 and 33% for 1, 2, 5 and 10 substrate concentrations was obtained when the maximum enzyme concentration (40 ml) was used. This produced an increase yield of 34, 30, 24 and 7% as compared to 10ml enzyme concentration. When the substrate concentration was increased, the overa reducing sugars yield decreased per gram of substrate. Furthe increase in enzyme titre was not possible, since it was in dill form in crude extract. Moreover, Fig. 27a shows that for 1 and

![Graph](image)

**Fig. 27.** Effect of enzyme and substrate concentration on percent saccharification from 1, 2, 5 and 10% kallar grass. Enzyme titre by *S. thermophile* was produced from 4% kallar grass.

a) 24 hours and b) 48 hours.

The FPase to β-glucosidase U/g substrate (1, 2, 5 and when the enzyme titre was:

<table>
<thead>
<tr>
<th></th>
<th>1%</th>
<th>2%</th>
<th>5%</th>
<th>10%</th>
</tr>
</thead>
<tbody>
<tr>
<td>10ml</td>
<td>8.8:17.6</td>
<td>4.4:8.8</td>
<td>1.8:3.5</td>
<td>0.9:1.8</td>
</tr>
<tr>
<td>20ml</td>
<td>17.6:35.2</td>
<td>8.8:17.6</td>
<td>3.6:7.0</td>
<td>1.8:3.5</td>
</tr>
<tr>
<td>30ml</td>
<td>26.2:52.8</td>
<td>13.2:26.4</td>
<td>5.4:10.5</td>
<td>2.7:5.3</td>
</tr>
<tr>
<td>40ml</td>
<td>35.2:70.4</td>
<td>17.6:35.2</td>
<td>7.2:14.0</td>
<td>3.6:7.0</td>
</tr>
</tbody>
</table>

kallar grass the saccharification was rapid up to 24 hours after which it slowed down considerably up to 48 hours. However, in case of 5 and 10% kallar grass there was a comparatively stea
increase after 24 hours (Fig. 27a, b).

Saccharification of Kollar Grass and Filter Paper

In order to elucidate the potential of enzyme complex from *S. thermophile*, pure substrate like filter paper (2 & 5%) along with kollar grass (2, 5 & 10%) was used for saccharification. Enzyme concentrations (20 to 40 ml) in a 50 ml reaction mixture with enzyme titre of 0.8 and 1.1 U/ml for FFase and B-glucosidase (obtained from 4% kollar grass) respectively, were used. The hydrolysis was carried out for 48h. The saccharified sugars were

<table>
<thead>
<tr>
<th>Substrate conc. (%)</th>
<th>Enzyme Titre (ml)</th>
<th>Saccharification</th>
<th>Glucose (Yield %)</th>
<th>Glucose Potential</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>49.0</td>
<td>27.0</td>
<td>30.6</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>61.0</td>
<td>38.2</td>
<td>44.4</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>94.0</td>
<td>45.0</td>
<td>52.2</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>30.6</td>
<td>14.8</td>
<td>17.6</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>36.1</td>
<td>17.9</td>
<td>20.1</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>42.6</td>
<td>21.2</td>
<td>24.6</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>27.5</td>
<td>11.0</td>
<td>12.1</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>29.3</td>
<td>12.8</td>
<td>14.1</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>35.6</td>
<td>15.1</td>
<td>17.1</td>
</tr>
</tbody>
</table>

Glucose Potential = Yield based on total cellulose.

The cellulase units per gram of substrate (2, 5 & 10%) when the enzyme titre was:

<table>
<thead>
<tr>
<th></th>
<th>2%</th>
<th>5%</th>
<th>10%</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 ml</td>
<td>16.0:22.0</td>
<td>6.4:8.8</td>
<td>3.2:4.4</td>
</tr>
<tr>
<td>30 ml</td>
<td>24.0:33.0</td>
<td>9.6:13.2</td>
<td>4.8:6.6</td>
</tr>
<tr>
<td>40 ml</td>
<td>32.0:44.0</td>
<td>12.6:17.6</td>
<td>6.4:8.8</td>
</tr>
</tbody>
</table>
estimated by DNS method, whereas the sugar composition was analysed by HPLC. Table 31 shows the enzyme used at a maximum titre of 40 ml for FPase and β-glucosidase of 32 : 44 units yielded 94, 42.6, and 35.6% reducing sugars from 2, 5 and 10% kollar grass, respectively. The results by HPLC indicated that two fold enzyme concentration increased the amount of reducing sugar and glucose from 2 and 5% substrate concentration to a level of 45, 12% and 18, 6.4%, respectively, whereas at 10% concentration an increase of only, 8.1 and 4.1 was obtained. The same enzyme titre yielded only 13.3 and 8.4% of sugars from 2 and 5% filter paper, respectively (Table 32). The increase in enzyme titre had almost a proportional effect on saccharification of 2 and 5% kollar grass which was doubled with the two fold enzyme titre.

**Table 32:** Effect of increased enzyme concentration from *S. thermophile* on saccharification and glucose yield for 2 and 5% filter paper.

<table>
<thead>
<tr>
<th>Substrate Conc. (%) (ml)</th>
<th>Enzyme Titre</th>
<th>Saccharification (Yield %)</th>
<th>Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>20</td>
<td>10.1</td>
<td>9.7</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>13.7</td>
<td>12.8</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>13.3</td>
<td>12.8</td>
</tr>
<tr>
<td>5</td>
<td>20</td>
<td>5.0</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>8.6</td>
<td>8.3</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>8.4</td>
<td>7.5</td>
</tr>
</tbody>
</table>

**Fig. 38** shows the composition of sugars as determined by HPLC. Kollar grass hydrolysates contained glucose, xylose and oligomeric sugars as the major components. Apart from this a negligible amount of cellobiose level suggested a potent β-glucosidase com-
Fig. 28. Effect of increasing enzyme concentration on the saccharification yield of different sugars from kallar grass.

a) 2%, b) 5% and c) 10%.

Fig. 29. Saccharifying ability of S. thermophile cellulases based on percent relative yield of different sugars in hydrolysates from kallar grass.

a) 2%, b) 5% and c) 10%.
ponent present in the enzyme titre. Table 31 and Fig. 28 shows that with the increase in enzyme and substrate concentration there was a concomitant over all increase in the yield of reducing sugars from koller grass. There was a greater increase in oligomeric sugar level than glucose and xylose contents. This behaviour was more pronounced at 5 and 10% koller grass, concentrations. Similar results were obtained from filter paper saccha-

Fig. 30. Effect of increasing enzyme concentration on the yield of different sugars from filter paper.

a) 2% and b) 5%

Fig. 31. Saccharifying ability of S. thermophile cellulases, based on percent relative yield of different sugars in the hydrolyzates from filter paper.

a) 2% and b) 5%.
rification, where glucose sugar was predominant (Table 32 and Fig. 30). The saccharifying ability of cellulases was elucidated (Fig. 29 and 31) considering the relative percent of different sugars in the hydrolysates. At increased enzyme titre the reducing sugars increased in general with a consequent increase in the oligomeric sugars and a small level of celllobiose (Fig. 29). However, 10% kollar grass exhibited an increase in glucose and xylose with concomitant decrease in oligomeric sugars. The relative sugar yield present in the hydrolysates from filter paper, suggested about 95% glucose from 2 and 5% filter paper (Fig. 31). These results indicated an overall dilute enzyme titre from \textit{S. thermophile}. However, a high relative percent glucose suggested a balanced celllobiohydrolase and \(\beta\)-glucosidase combination.

Use of Concentrated Enzyme

The crude enzyme extract obtained from 4% kollar grass was concentrated by ultrafiltration membrane (cut out size 20,000 D). Concentrated enzyme was adjusted to pH 5.0 and was used to saccharify 5% kollar grass. Enzyme preparations from \textit{T. reesei} VTT-D-78125 alone and supplemented with \(\beta\)-glucosidase from \textit{A. niger} (Niles Kalli) were also used. The enzyme concentration from different sources were calculated in units per gram of substrate for FFase and \(\beta\)-glucosidase (see legend to Table 33). Maximum saccharification yield of 74.8% for reducing sugars was obtained from the supplemented \textit{T. reesei} after 70 hours. Th sugars were produced at a rapid rate up to 20 hours after which the pace slowed down (Fig. 32). It was followed by the concentrated cellulase preparation from \textit{S. thermophile} (70.7%) which also showed an increase of about 15% yield over its control (Fig
Table 33: Saccharification of 5% kallar grass by dilute and concentrated enzyme preparation from *S. thermophile*. Commercial enzyme preparation from *T. reesel* alone and in supplementation with *A. niger* was also used.

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>Saccharification</th>
<th>Glucose</th>
<th>Relative glucose Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (<em>S. thermophile</em>)</td>
<td>55.5</td>
<td>28.2</td>
<td>50.8</td>
</tr>
<tr>
<td>Concentrate (<em>S. thermophile</em>)</td>
<td>70.7</td>
<td>37.2</td>
<td>52.6</td>
</tr>
<tr>
<td><em>T. reesel</em></td>
<td>60.0</td>
<td>28.5</td>
<td>47.5</td>
</tr>
<tr>
<td><em>T. reesel</em> + <em>A. niger</em></td>
<td>74.8</td>
<td>36.7</td>
<td>49.1</td>
</tr>
</tbody>
</table>

*Cellulase units per gm of substrate were:

*S. thermophile*
- Control, 10:28
- Concentrate, 24:80

*T. reesel*
- Without supplementation 31:27
- With supplementation 33:54

Fig. 32. Time course saccharification of 5% kallar grass enzyme preparations of *S. thermophile* and *T. reesel*. 
Considering the percent relative yield of different sugars in the hydrolyzates (Fig. 34) the cellulase from control and concentrate of S. thermophile revealed much higher level of glucose yield in contrast to that from T. reesei cellulase alone and with supplementation. These results depict the potential of enzyme components present in the enzyme system of the former source.

Fig. 33. Effect of cellulase preparations from S. thermophile and T. reesei on percent saccharification of 5% kellar grass. For enzyme dosage see legend to Table 33.

Fig. 34. Saccharifying ability of different cellulase preparations based on percent relative yield of sugars in the hydrolysates of 5% kellar grass.

The sugar composition by HPLC (Fig. 35) revealed the presence of 4-6 sugars in the hydrolysates from these sources. The glucose yield by the concentrate (38.4%) was a little higher than the supplemented T. reesei at 36.7% (Fig. 35b, d). In contrast to this the hydrolyzates by the supplemented T. reesei contain higher amounts of xylose and to some extent oligosaccharide contents (Fig. 35b, c). Cellobiose was obtained at a low level in the hydrolysate by S. thermophile, control (Fig. 35a).
Fig. 35. Separation of sugars by HPLC after hydrolysis.

a) S. thermophile (control), b) S. thermophile (concentrate),
c) T. reesei (without supplementation) and d) T. reesei (with supplementation from A. niger).

Conditions for HPLC were as follows:
Column: Aminex HPX-87H cation exchanger; Mobile phase: 0.001 N H2SO4; Column temperature: 85°C; Flow rate 0.6 ml/min; Injection volume: 20μl; Attenuation ×16
COMPARISON OF CELLULASES FROM _S. thermophile_ AND _T. reesei_.

Use of Equal Filter Paper Units (FPU's)

Freeze dried enzyme preparation from _S. thermophile_ was equilibrated for FPU per gram of substrate with commercial _T. reesei_ cellulase for saccharification of 5% kollar grass and filter paper. The sugars were estimated by DNS method while the composition of sugars was determined by HPLC. Fig. 36 and Table 34 indicates that enhanced enzyme dosage from 15 to 20 FPU's increased the saccharification and glucose yield appreciably. Using 20 FPU's the overall yield for reducing sugars of 53.4 and 52.4% from kollar grass was slightly higher by the enzyme preparation from _T. reesei_. The yields for other sugars revealed that xylose was produced substantially higher by the enzyme preparation by _T. reesei_, whereas more oligomeric sugars were formed by _S. thermophile_ in the kollar grass hydrolysates.

**Table 34:** Effect of _S. thermophile_ and _T. reesei_ cellulase titres on saccharification of 5% kollar grass and filter paper. The hydrolysis was carried out for 48 h. The reducing sugars were analysed by HPLC.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme Preparation</th>
<th>Enzyme conc. FPU/gm</th>
<th>Saccharification</th>
<th>Glucose Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kollar Grass</td>
<td><em>S. thermophile</em></td>
<td>15</td>
<td>45.0</td>
<td>24.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>52.4</td>
<td>26.4</td>
</tr>
<tr>
<td></td>
<td><em>T. reesei</em></td>
<td>15</td>
<td>50.0</td>
<td>26.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>53.4</td>
<td>28.6</td>
</tr>
<tr>
<td>Filter Paper</td>
<td><em>S. thermophile</em></td>
<td>15</td>
<td>14.3</td>
<td>12.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>17.2</td>
<td>14.5</td>
</tr>
<tr>
<td></td>
<td><em>T. reesei</em></td>
<td>15</td>
<td>23.0</td>
<td>14.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>27.0</td>
<td>18.4</td>
</tr>
</tbody>
</table>
When the substrate was filter paper, the difference in saccharification yield was considerable at 27.0% as compared to 17.2% for T. reesei and S. thermophile enzymes, respectively. However, filter paper the former also showed a considerable amount of cellulobiose, absent in latter.

Fig. 36. Effect of cellulase preparations from S. thermophile and T. reesei at 15 and 20 FPU (per gram substrate) on percent saccharification of 5% substrate.

a) Kollar grass, b) Filter paper.

Fig. 37. Effect of cellulase concentration (FPU/g substrate) of S. thermophile and T. reesei in time course studies for percent saccharification of 5% substrate concentration.

a) Kollar grass, b) Filter paper.
The time course saccharification revealed a higher rate up to 10 hours after which it slowed down considerably (Fig. 37). In fact the saccharification rate resulted in a marginal increase after 10 hours when the substrate was filter paper.

Fig. 38 shows the HPLC chromatograms obtained for the hydrolyzates in a time course study after 8, 24, and 48 hours. Glucose, xylose and cellobiose sugars were identified, whereas the rest of the peaks were pooled for oligosaccharides. These chromatograms elucidated the enzyme system from both the fungal sources to some extent. The composition of sugars from the peak showed a small amount of cellobiose produced up to 24 hours from T. reesei along with other sugars which diminished to zero after 48 hours when kellar grass was the substrate (Fig. 38 and 40a,b). Moreover, the cellobiose content was present at substantial amounts when filter paper was the substrate. The cellobiose lowered with the time period to some extent (Fig. 39). There was no cellobiose formed in the hydrolysates from S. thermophile.

From Fig. 40a,b the relative yield as determined by the time course study also revealed an increase of glucose up to 24 and 48 hours from S. thermophile and T. reesei, respectively when the substrate was kellar grass. The slight decrease in glucose at xylose concentrations corresponded with increase in oligomer sugars, by the enzyme preparations of S. thermophile up to 24 hours. On the contrary, slight increase in glucose and oligomer sugars resulted with a decline in xylose contents by T. reesei enzyme. Similar results were obtained when the substrate was filter paper (Fig. 41a,b). The glucose content decreased with increase in oligo-sugars by the former, whereas increase in gl
Fig. 33. HPLC analysis of sugars released from 5% filter paper time intervals of 8, 24 and 48 hours in the hydrolysates by S. thermophile; b) T. reesei. Conditions as mentioned earlier.
Fig. 40. Saccharifying ability of cellulase preparations in a time course study from S. thermophile and T. reesei, based on percent relative yield of different sugars in the hydrolysates from kallar grass.

FPU/gm substrate: a) 15 and b) 20.

Fig. 41. Saccharifying ability of cellulase preparations in a time course study from S. thermophile and T. reesei, based on percent relative yield of different sugars in the hydrolysates from 5%. Filter paper.

FPU/gm substrate: a) 15, b) 20.
cose with decrease in oligo-sugars and cellobiose accompanied the latter enzyme preparation up to 48 hours. The increase in enzyme dosage from 15 to 20 FPU enhanced the glucose level generally with a relative decrease in only, oligo-sugars by the former preparation and cellobiose plus oligo-sugars by the latter.

Optimum Enzyme Concentration (FPU’s per gram substrate)

The enzyme titre (20-40 FPU/gm substrate) was used from thermophile and T. reesei for saccharification of 5% Kali grass. The maximum yield for reducing sugars was 56.6 and 68. with 30 and 35 FPU from the former and latter, respectively after 60 hours of incubation (Table 35). The maximum glucose yield 31.5 and 43.7% was attained from 30 and 35 FPU from the former and latter enzyme preparations, respectively. Time course study from Fig. 42 shows a rapid saccharification yield up to 10 hour specially for T. reesei, but it slowed up after that as compar

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Enzyme conc. (FPU/gm)</th>
<th>Saccharification Yield (%)</th>
<th>Glucose Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. thermophile</td>
<td>20</td>
<td>52.2</td>
<td>30.4</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>53.0</td>
<td>30.6</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>56.6</td>
<td>31.5</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>52.3</td>
<td>32.0</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>55.2</td>
<td>31.0</td>
</tr>
<tr>
<td>T. reesei</td>
<td>20</td>
<td>55.2</td>
<td>34.2</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>60.5</td>
<td>35.8</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>65.0</td>
<td>39.4</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>68.5</td>
<td>43.7</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>68.3</td>
<td>41.2</td>
</tr>
</tbody>
</table>
Fig. 42. Time course saccharification of 5% Kallar grass by optimum enzyme dosage from a) *S. thermophile* b) *T. reesei*.

to *S. thermophile* which kept a reasonable increase up to hours. The composition of sugars obtained by HPLC (Fig. 43a.

Fig. 43. Optimum cellulase dosage (FPU/g substrate) for saccharification of 5% kallar grass.

a) *S. thermophile*, b) *T. reesei*. 
showed the presence of five different sugars. Only glucose and xylose were detected from the standards and no cellobiose was present in the hydrolysates. The rest of the 3 peaks were pool as oligo-saccharides. The results indicate a small increase in yield when enzyme titre was enhanced from 20-40 FPU/g substrate. In general, increased titre of up to 30-35 FPU/gm and incubation period of 60 hours or more was found to be the optimum.

SACCHARIFICATION BY THERMOPHILIC FUNGI

Hydrolysis at 50°C

Culture filtrate from thermophilic fungi for saccharification purpose were obtained from 2% kollar grass after 6 days incubation. The dilute enzyme filtrates were set at pH 5.0. Reaction conditions and sugar estimations are as described in the legend to Table 36. Fig. 44 presents a time course study, which depicts increased yield for sugars during the first 10 hr.
During 10 to 30 hours there was a slower increase, after which the increase was minimal except in case of C. thermophile, which showed consistent saccharification ability up to 70 hours. The highest saccharification and glucose yields of 69.2 and 31.6% were obtained from C. thermophile. It was followed by T. reesei, H. grisea > S. thermophile > T. thermophila > A. fumigatus > M. pulchella.

Table 36: Comparison of saccharification yield from 5% kalar grass by various thermophilic fungi and T. reesei (mesophile) cellulase. The reaction was carried out at 50°C for 70 h. Composition of sugars was determined by HPLC.

<table>
<thead>
<tr>
<th>Enzyme Source</th>
<th>Sugar Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Red. Sugars</td>
</tr>
<tr>
<td>A. fumigatus</td>
<td>36.5</td>
</tr>
<tr>
<td>C. thermophile</td>
<td>69.2</td>
</tr>
<tr>
<td>H. grisea</td>
<td>58.4</td>
</tr>
<tr>
<td>M. pulchella</td>
<td>18.7</td>
</tr>
<tr>
<td>S. thermophile</td>
<td>51.4</td>
</tr>
<tr>
<td>T. thermophila</td>
<td>50.3</td>
</tr>
<tr>
<td>T. reesei</td>
<td>60.0</td>
</tr>
</tbody>
</table>

The enzyme filtrates, 20 ml each, set at pH 5.0, were used with kalar grass in conical flasks. The enzyme titre for each of the above fungi in the ratio of FPase:β-glucosidase 1:1 per gm substrate was as follows:

- a) 10.0:13.0
- b) 6.4:10.4
- c) 4.6:12.8
- d) 0.6:10.0
- e) 10.0:13.6
- f) 10.0:12.8
- g) 30.0:21.0

The composition of sugars in the hydrolyzates as obtained by HPLC (Fig. 45 and Table 36) shows various components, of which...
Fig. 45. HPLC analysis of sugars released from 5% Stallar grass by various thermophilic fungi. The saccharification was carried out at 50°C. Conditions as reported previously.

4. Cellobiose
6. Glucose
7. Xylose
1, 2, 3, 5 and 8. Oligosaccharides and other sugars.
three main sugar components were detected as glucose, cellobiose, and xylose while rest of the 3 peaks were pooled as oligosaccharides. In the hydrolyzates of these fungi, glucose was present as the main sugar component, excluding M. pulchella which showed higher xylose content. The highest yield for glucose from C. thermophile was followed by T. reesei (mesophile) > I. grisea > S. thermophile > T. thermophila > A. fumigatus > M. pulchella. Xylose was present in considerable amounts in all these tested fungi except S. thermophile and A. fumigatus which showed greater amounts of oligosaccharides dominant in these fungi along with T. reesei. Apart from M. pulchella and T. reesei, all the other fungi also exhibited a small proportion of cellobiose content. The above facts are well illustrated by Fig. showing percentage relative yields for sugars from different fungi. T. reesei showed a relative high yield for glucose in the hydrolyzates from 5% kall grass.

Fig. 46. Saccharifying ability of different thermophilic fungi at 50°C based on percent relative yield of different sugars in the hydrolyzates from 5% kall grass.
hydrolysates, while S. thermophile showed the highest oligosaccharide contents. The relative xylose and cellubiose were maximal for H. grisea and A. fumigatus, respectively.

Use of Enzyme Filtrate at High Temperature

Enzyme filtrates (as from the previous experiment) were used to saccharify 5% Kellar grass at 60°C, (for reaction conditions see legend to Table 36). A time course study revealed that at a high temperature of 60°C the rate of reaction was maximum up to 20 hours, after which it became stationary or declined (Fig. 47). Maximum yield was obtained from C. thermophile (58%). In fact, it was the only organism to show increase in percent yield after 40 hours. Moreover, C. thermophile achieved maximal yield about half the time at 60°C as compared to that at 50°C (Table 37). T. reesei (mesophile) showed about one third the yield at 60°C than at 50°C. The lowering in yield at 60°C from thermophilic fungi was to a much lower extent than that of T. reesei.

Fig. 47. Time course saccharification of 5% Kellar grass with cellulases of different thermophilic fungi at 60°C. For cellase concentration per gm substrate see legend to Table 3.

Fig. 48 and Table 37 shows the composition and yield of different sugar components as elucidated by HPLC. The three major peaks (glucose, xylose and cellubiose) were determined from t
Fig. 48. HPLC analysis of sugars released from saccharification of 5% kellar grass by various thermophilic fungal cellulases at 60°C. Conditions as mentioned earlier.

2- Cellobiose
4- Glucose
5- Xylose
1, 3 & 6 Oligosaccharide other sugars.
standards. The maximum yield for glucose was obtained from \( C. \) thermophile followed by \( M. \) grisea > \( T. \) reesei > \( T. \) thermophil > \( S. \) thermophile > \( M. \) pulchella > \( M. \) pussilus. The other main components were xylose, oligosaccharides and cellobiose as also found in the previous experiment at 50°C.

**Table 37:** Comparison of saccharification yield from 5% kalla grass at 60°C by various thermophilic fungi \( T. \) reesei (mesophile) cellulase. Composition of sugars was determined by HPLC.

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Sugars Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Red.Sugars</td>
</tr>
<tr>
<td>( A. ) fumigatus</td>
<td>15.9</td>
</tr>
<tr>
<td>( C. ) thermophile</td>
<td>58.0</td>
</tr>
<tr>
<td>( M. ) grisea</td>
<td>46.0</td>
</tr>
<tr>
<td>( M. ) pulchella</td>
<td>10.5</td>
</tr>
<tr>
<td>( S. ) thermophile</td>
<td>29.5</td>
</tr>
<tr>
<td>( T. ) thermophila</td>
<td>38.5</td>
</tr>
<tr>
<td>( T. ) reesei</td>
<td>26.0</td>
</tr>
</tbody>
</table>

* The reaction conditions were same as for Table 34.

The percent relative yield for different sugars is shown (Fig. 49). Although \( T. \) reesei showed lower yields, still depicted greater relative glucose in the hydrolyzates. The relative xylose, and cellobiose content were highest from \( C. \) thermophile, whereas oligo-saccharides were found to be at maximum level from \( S. \) thermophile.
Fig. 49. Saccharifying ability of different thermophilic fungi at 60°C based on percent relative yield of different sugars in the hydrolysates from kollar grass.
DISCUSSION

ISOLATION AND SCREENING OF THERMOPHILIC CELLULOLYTIC FUNGI ON LIGNOCELLULOSE (LC)

LC find diverse applications in chemical feed, pulp and paper and bioenergy industries. They possess an immense potential for fulfilling the ever-rising energy demands of the present world. The developments in the LC bioconversion are dependent on the use of cheap raw materials, a potent microbe (mutant or genetically engineered), economically designed pretreatment efficient recovery processes and recycling of enzymes, etc. In Pakistan, we are short of fuel reserves and deprived of about one-third of our cultivable lands due to salinity and water logging. The use of such lands for growing perennial grasses like Leptoc bula fusca (kollar grass) even under water-logged conditions has shown much promise for the reclamation of these soils (Malik et al., 1986). Moreover, biomass (kollar grass) raised from these lands apart from other economic utilizations has exhibited itself as a suitable substrate for biofuel production (Rajoka and Malik 1985). Kollar grass, other salt-tolerant plants and waste L materials were used in these studies for pretreatments, enzyme production and enzymatic saccharification.

Efforts were made to isolate thermophilic fungi from habitats where thermogenesis was possible. From the five habitats tested (Table 1) seven different thermophilic fungi were isolate on ball milled cellulose agar (BMCA). Rhizospheres of grass like kollar grass and Cenchrus ciliaris were rich sources of thermophilic microbes. Sporotrichum thermophile, Humincola grisea, Chaetomium thermophile, Torula thermophila and thermotoleran
Aspergillus fumigatus showed rapid clearance of BMC medium at 50°C in 5-6 days. The cellulolytic ability of these thermophilic fungi was in congruence with Tansey (1970). Similar reports for the isolation of thermophilic fungi have been made by Eggins and Malik (1969); Malik and Sandhu (1978). A. fumigatus and H. grisea were found in all the tested samples from various habitats thus indicating their abundance in nature. Use of antibiotics with rose bengal enabled the selective isolation of these fungi.

Thermophilic fungi were screened for cellulase and xylanase production by growing them on untreated kallar grass (2%) at 45°C in shake flask cultures (SFC). A. fumigatus produced all the five tested enzymes (Table 2 and Fig. 1). Along with other cellulases, S. thermophile induced a higher level of β-glucosidase required for the ultimate conversion of cellulose to monomeric glucose. Duff (1986) obtained 2-fold cellulolysis by the addition of β-glucosidase from Aspergillus to the cellulase of T. reesei, which has a poor specific activity for this enzyme. M. pussillus and M. pulchella showed lesser enzyme yields. The initial pH 5.0 of the fermentation medium rose to 6.6 or above for all the fungi except H. grisea, which stayed at 5.6 (Fig. 2). The increase in pH level was attributed to the release of NH₃ from asparagine and yeast extract used in the medium. pH rise towards alkalinity in thermophilic fungi during growth is well documented (Romanelli et al 1975); Coutts and Smith, 1976; Ericksen and Goksoyr, 1976 and 1977; Grajek, 1987). The extracellular proteins were in the range of 1.0-1.6 mg/ml (Fig 2).

In contrast to thermophilic fungi, mesophilic species of C. verricinubalum showed good induction of cellulase. However, T
C-30, which has been reported (Ryu and Mandels; 1982) as one of the most potent organism for cellulase production could not induce this enzyme by kalar grass (Table 3 and Fig 3). The overall enzyme titres from the thermophilic isolates were lower as compared to some of the other potent wild type 

*Trichoderma*

strains. However, latter strains show high activity on pure cellulose (Linko, 1978; Pourquie and Vandecasteele, 1984) as compared to untreated LC substrate. In fact, for enzyme inductive LC substrates have been more successfully used after pretreatment (Rajoka and Malik, 1984; Acebal et al, 1988; Madamwar et al 1989).

*S. thermophile* was selected among the thermophilic fungi for optimization studies due to its higher \(\beta\)-glucosidase component as also reported by Grajek (1987b). Romanelli et al (1975) also reported its higher cellulose degrading ability as compared to *thermophile* and *Thermoascus aurantiacus*. *S. thermophile* and *V. verrucophalum* were grown on different LC substrates using solid state fermentation (SSF) and liquid fermentation (LF). LC substrates are well documented for their overall potential as substrates of choice for making the bioconversion process economical.

Cellulase production by SSF showed that quantity of different enzymes depends on the particular inducer. In *S. thermophile*, rice straw induced more avicelase, CMCase and xylanase, while *Sambhania aculeata* (dhanicha) another salt tolerant plant induced greater level of \(\beta\)-glucosidase and \(\beta\)-xylosidase. In fact dhanicha induced two fold higher activity for these enzymes (Tab 4 and Fig. 5). Kallar grass was found to be the next substrate choice as reported by Rajoka and Malik (1984) for induction
cellulases. In C. vergicrinalum, kallar grass induced more CMCase and xylanase while dhancha induced rest of the three enzymes to a greater extent (Table and Fig. 6). Rice straw was found to be the next best inducer. Deschamps and Huet (1984) found sugar beet pulp among the LC's as the best substrate for β-glucosidas activity. Shamala and Sreekantiah (1986) reported rice straw to be a better substrate for induction of cellulase and xylanase while wheat bran for β-glucosidase.

Similar studies for the above two species were carried out in LF employing Shake flasks. Wheat straw, rice straw and kallar grass among the eight LC substrates showed higher induction for all the enzymes in S. thermodon (Table 6 and Fig. 7). Dhancha could not induce the enzymes to similar effect in S. thermodon as in SSF. C. vergicrinalum, showed more promise for dhancha, rice straw and kallar grass than the rest of the substrate (Table 7 and Fig. 8). Skinner and Tokuyama (1978) found the newsprint followed by bagasse produces higher FPase than wheat bran and cotton gin trash in Thielavia terrestris. Saddler et al. (1985) found steam treated aspenwood and subsequently, washed material to be as good as solka floc-cellulose. However, Brown et al. (1986) revealed higher cellulase spectrum with ball mill barley straw than with solka floc in the mutant strain of Penicillium pinophilum. Margaritis and Merchant (1985a,b) found that pretreated wheat straw is more potent than barley straw and other cellulosic substrates for cellulase induction. Rajoka and Mal (1984 and 1986) used treated kallar grass and dhancha a obtained higher cellulase activity as compared to pure cellulos.
xylanases suggests that the synthesis of individual enzyme independently regulated (Montenecourt and Eveleigh, 1977). S often used for cellulase and single cell protein production w compared with LF. The results revealed that in LF the enzy titre produced per gram of substrate was much higher than in S (Fig. 9 and 10). However, comparing the enzyme titre on volumetric basis the yields were marginally higher by SSF, except for glucosidase and β-xylosidase, but about 15 times more substrate concentration was required. The low enzyme titres obtained fr SSF than LF is attributed to better controlled conditions including aeration, agitation, pH control and release of extracellular enzymes in the latter. But on the contrary, some of the repo emphasize SSF as a potent and cheaper technology, possibly depending upon the organism and maintenance of conditions (Sternberg et al. 1976; Kim et al. 1985; Deschamps 1984; Grajek, 1987; Madamwar et al. 1989).

OPTIMIZATION OF CULTURE CONDITIONS AND ENZYME CHARACTERISATION

In SSF, the optimum moisture level required for high enzyme activity was found to be three parts water (mineral medium) to one part of substrate (kollar grass) by S. thermophilus (Table 8). At high temperature (45°C), in case of S. thermophilus evaporation losses were curtailed by employing a water fill tray in the incubator. Sternberg et al (1976) reported, two part moisture to one part wheat bran for Aspergillus strai Deschamps and Huet (1984) used three parts (v/w) moisture corn-breaks and potato starch. Grajek (1986) used two part moisture to one part of starch. Increase in strength of Egi and Pugh (E & P. 1964) medium up to 3-4 fold enhanced the en:
activity to more than double in SSF (Table 9 and Fig. 11). The decrease in enzyme activity at zero and five fold medium strength could be attributed to non-availability of nitrogen contents and increase of mineral and nitrogen level, respectively, to an extent where osmotic interactions lowered the production of enzyme activity. However, Shamala and Sreekantiah (1986) found a five fold concentration from Toyama’s mineral medium to yield maximum enzyme activity.

LD studies regarding age and size of inoculum suggested a seed culture of 16-30 hours, to be effective when used at 5° (v/v) (Table 10 and 11). Grajek (1986b) prepared the inoculum for thermophilic fungi after incubation for 3-5 days. An inoculum size of 10% (v/v) was used in this case.

In LD Optimum culture conditions for incubation temperature and initial pH were found to be 45° C and 5.0, respectively (Fig 12 and table 12). The FFase and β-glucosidase activity increased up to 2 and 1.5 fold, respectively over an increase in incubation temperature from 30 to 45° C. This phenomenon can be attributed to thermophilic characteristics of the fungus. The medium pH value of 5.0 fluctuated to rise up to 6.4-6.6 after which enzyme production reached the maximum level. The rise in pH could be due to release of ammonia from the β-asparaginase and yeast extracts used as nitrogen sources. Coutts and Smith (1976) and Grajek (1987a) found S. thermophile to grow profusely at alkaline pH while the latter also used an incubation temperature of 45°C for the thermophile. In order to maintain the pH in the medium citrate buffer of different strengths were used. Results from Table 13 indicate that a low molarity (0.05 M) buffer was mor
suitable for maintenance of pH and enzyme activity. However, the control run without buffer showed a rise in pH resulting in higher β-glucosidase activity. This was in agreement with Graje (1987b) who reported control of pH with buffer at pH 6.5 which resulted in optimum β-glucosidase activity for S. thermophile. Durand et al. (1984) used potassium phthalate buffer in the medium. pH level was allowed to fall from 5.5 to 5.0 at which it was maintained for rest of the batch culture.

The cost of enzyme production accounts for about 50% of the total process, therefore considerable effort is being made to attain process profitability. Use of cheap available raw materials can greatly affect the costs of enzyme rather than using costly cellulosic materials. In the present study S. thermophile showed the highest activities for FPase and β-glucosidase when grown on untreated kallah grass (Table 14). Other substrates followed in order of induction of alkali treated kallah grass > avicel > cellulose. These results are unique since most of the scientists have reported higher yields after applying suitable pretreatment to various LC materials in order to enhance cellulose susceptibility, especially in LF (Millet and Baker, 1975; Brown et al., 1988; Acebal et al., 1988; Szczodrak, 1988; Shulz and Hirte, 1989). Kallah grass used as a cellulose substrate showed an increase up to 1.6 fold FPase and β-glucosidase activity at 8% substrate concentration over that at 2% (Table 15). At higher substrate concentration, the incubation periods increased up to 9-11 da for maximal activity of 1.1 and 1.6 IU/ml of FPase and β-glucosidase, respectively (Fig. 13). It is in fact, the cessation growth followed by sporulation and enzyme production which star
simultaneously (Hulme and Strank 1971; Coutts and Smith 1976 Grajek 1987b). At 10% substrate concentration, there was decline observed in FPase, after an initial increase up to days, while β-glucosidase maintained a steady increase. The slow initial growth rate and enzyme activities at higher substrate concentration were because of agitation and aeration difficulties. In fact, cellulase production on untreated LC substrate at such high concentrations has not been recorded in LF. These findings are in agreement with Sternberg and Dorval (1979) Gokhale et al (1984); Schulz and Hirte (1989). The growth parameters, including pH level in general increased. However, it recorded a lower increase at higher concentrations of substrate (Fig 14). Increase at higher concentrations inflicts decrease in r level, concomitant with the mycelium increase due to high rate of nitrogen assimilation (Mandels et al, 1976; Acebal et al, 1988) This fact was also affirmed by Schulz and Hirte (1989). The extracellular protein (3.1 mg/ml) increased up to 10% substrate concentration, with an estimated increase of 2.1 times over 2 substrate.

Optimization of mineral medium of different formulations used by various workers revealed that medium used by Romanelli et al (1976) followed by Eggins and Pugh (1964) yielded best result (Table 16). Both of these media have been used for cellulase production in thermophilic fungi. The basic difference in these media lies in the nitrogen sources (proteose peptone, urea) along with trace mineral solution and tween 80 in the former and use of yeast extract, α-asparagine and ammonium sulphate in the latter medium. Rest of the four media showed lower enzyme activiti
possibly due to poor nitrogen sources. In another study with 2% kellar grass, selective increase of each (E & P) medium component did not show any noticeable increase in enzyme activity compared to that of the normal medium concentration (Table 17). The presence of high ash contents in kellar grass (Latif et al. 1988) might have supplemented the mineral requirements at zero level in the medium since P-glucosidase level was steady even in the absence of mineral components in the medium. In yet another medium design, the salts and nitrogen level was doubled separately for 2% kellar grass. The doubling of latter in the medium induced the cellulase activity to a greater extent than that of the former (Table 18). Hendy et al. (1984) pointed out the use of increased nutrients to be effective at higher cellulose concentrations. Wase et al. (1985) used a standard factorial design for the increase in mineral salts and cellulose concentration for enhanced cellulase activity. In another study with seven different nitrogen sources (0.05% each) used separately with ammonium sulphate and also in combination with ammonium sulphate and yeast extract, showed that the E & P medium combination of (NH)SO₄ ₄H₂O, asparagine and yeast extract at 0.05% concentration was optimum (Table 19). Other nitrogen sources which showed promise were case amino acids and cotton seed flour. Urea, separately as well as in combination showed highly alkaline pH of 10.0 which might be due to the non-utilization of ammonia, released in the medium. Effect of nitrogen source like the carbon source varies from organism to organism for enzyme production. Canevascini et al. (1979) used E P medium effectively for cellulase production with some modification using S. thermophile. Steiner et al. (1987) reported Bact
peptone to be most effective substrate of a number of caseins, mycological and meat peptone for enzyme production in *Schizophyllum commune*. Grajek (1986b) enunciated ammonium sulphate, ammonium chloride and potassium nitrate as the best inducers for β-glucosidase activity in *S. thermophile*.

Addition of trace element solution, vitamin solution and tween-80 could not produce any noteworthy increase in enzyme activity (Table 20). This supports our previous assumption that kallar grass because of high ash content is a good substitute for trace mineral salts. This was further affirmed by Mandels and Weber (1969), Skinner and Tokuyama (1978) and Wase et al (1985) who used tween-80 and trace element solution effectively with pure cellulose. From the optimisation studies it was concluded that for 2% kallar grass as a carbon source, E & P mineral medium composition is most suitable. However, two fold addition of nitrogen sources in the medium was found to have a greater influence on enzyme activity.

The ultrafiltration technique was adopted for concentrating the enzyme filtrate for use at higher strengths against increased amounts of substrate. The extent of increase in the enzyme titre after ultrafiltration decreased for the crude enzyme filtrate obtained at higher substrate concentration (Table 21 and Fig. 15). However, the overall increase for ultrafiltration of enzyme filtrate obtained from 6% kallar grass over 2% kallar grass (without ultrafiltration) was up to 4.3, 6.2 and 4.3 times for FPase, β-glucosidase and extracellular protein, respectively. The recovery of the enzyme from ultrafiltration suggested enzym losses due to adsorption of some part of the enzyme on to th-
membrane during processing. The increased adsorption for the culture filtrate obtained at higher substrate concentration, was ascribed to viscosity and pigmentation from kallar grass. There was in fact appearance of a gummy substance which possibly blocked the membrane pores and thus, about 30-35% cellulase was lost. Markanen and Eklund (1975) found that the culture filtrate from T. viride was denatured when passed through ultrafiltration membrane (cut off size, 10,000). However, Fahnrich and Irrgang (1982) concentrated the enzyme by ultrafiltration for effective saccharification.

Studies at fermentor level (14 L capacity) resulted in rapid growth taking place after a lag of 6-8 hours with a concomitant increase in pH level, characteristic of S. thermophilus (Grajek, 1987b). A short lag and an exponential phase between 3-88 hours (Fig. 16) suggested the production rate to be higher than most potent strains of T. reesei which takes around 120-140 hours for optimum activity in batch culture. But the time factor is dependent upon the substrate concentration at the same time (Hendy et al., 1984; Durand and Clanet, 1988). When the pH of the batch fermentation was kept constant at 5.0, the lag period increased up to 16 hours (Fig. 17). The enzyme activities were induced after the cessation of growth after about 40 hours to a slightly higher level than when the pH was not controlled.

Enzymatic properties showed a pH stability in the range of 4.0-6.0 (Fig. 18). The optimum pH was found to be at 5.0 for avicelase, CMC-ase and β-glucosidase, while for xylanase a level of 5.5 was more appropriate. These findings are in agreement with the previous works (Brueil et al., 1986 and Grajek...)
PRETREATMENT OF LC SUBSTRATES

Effect of three different pretreatments on six LC substrates was distinguished from mass balances and enzymatic accessibility. The three pretreatments were namely: Steaming, alkaline pretreatment at room temperature and alkali pretreatment with autoclaving.

Steaming at 200°C resulted in greater fibre losses, especially in case of salt tolerant plants, excluding P. maximum (Table 22-27 and Fig. 21a). This was mainly because of higher amounts of soluble sugars and ash contents being washed out in the liquors apart from the hemicellulose contents. In fact, P. maximum an poplar showed the highest fibre recovery of 75%. The latter, hardwood, is reported to yield high fibre recovery followed by steaming (Mes Hartree et al, 1987) contrary to straw material (Puri and Mamers, 1983; Puri, 1984). The lignin contents increased for all the substrates except wheat straw (Fig. 21b). In fact, steaming results in redistribution of lignin, which contributes to increase in enzymatic accessibility (Fuls et al, 1985; Wong et al, 1988). It is suggested that lignin holocellulose relationship rather than the lignin concentration (Stranks, 1961), its location or distribution in the fibre (Dekker and Wallis, 1983; Saddler et al, 1982) and its effect on the surface for hydrolysis are important parameters (Gharpuray et al, 1983).

With steaming, there was a resultant increase in total polysaccharides (glucose potential) except in wheat straw because of a greater extraction of hemicellulose and specifically the solubles and ash. This phenomenon was even more pronounced
for the salt tolerant plants. This rendered increase in fibr porosity corresponding to increased digestibility (Fig. 21d,e). These results are in agreement with Cowling and Kirk (1976); Pul et al (1984) and (1985); Burns et al (1988); Wong et al (1988). Bagasse showed the highest polysaccharide level even before an after the pretreatment, while A. amnicola showed the lowes levels. In fact, the latter showed a higher lignin and as content than the cell wall polysaccharides in the original ra materials. Pretreated at 200 C, P. maximum resulted in the hig hest enzymatic accessibility apart from the fact that bagasse as wheat straw showed lesser lignin values and higher xylose losse in the pretreated fiber (Fig. 22b). The susceptibility of th celluloseic substrates to enzymatic hydrolysis is based on th degree of crystallinity, nature of associated substances ar surface area (Fan et al, 1982) unit cell dimensions of th cellulose crystallites, moisture contents of the fibres, DF conformation and steric rigidity of anhydroglucose units ar natural concentration and distribution of substituent grou (Cowling and Brown, 1969; Cowling, 1975; Cowling and Kirk, 1976 Millet et al, 1975; Matsumura et al, 1977; Tsao et al, 1978). Th fact that poplar which showed high polysaccharide level was leas accessible is further strengthened by the above statements. Th enzymatic accessibility based on raw materials suggested P. maxi mum and A. amnicola at the maximum and minimum levels, respecti vely, because of fibre losses (Fig. 22b).

Alkali (NaOH 2%) treatment at room temperature for 24 hour resulted in maximal fibre recovery of 92.0 and 89.0% for popl and P. maximum as was the case for steaming. At higher alkal
concentration the fibre yield decreased because of the delignification and hemicellulose losses with an over all increase in the polysaccharides (glucose potential) and thus accessibility (Fig. 23). It was revealed by Klasson lignin content that apart from A. amnicola and poplar all the substrates showed considerable delignification (Fig. 23b). A maximum delignification of 33.5% for bagasse over its control was concomitant with maximal increase in level of total polysaccharides (77.5%), in close proximity with P. maximum. The alkali acts by disrupting the rigid lignin structure and ester bonds removing a portion of lignin and hemicellulose, swelling in the fibres and in some cases decreasing the crystallinity of cellulose (Wilson and Brigo, 1977; Gharapuray et al, 1983). Kallar grass showed maximal glucose potential (cellulose) after 3% NaOH treatment because of greater xylose losses in the black wash liquor. Wheat straw because of moderate delignification and higher xylose losses rendered the fibres material more accessible than P. maximum which showed slight lesser yields with 2 and 3% alkali treatment, respectively (Fig. 23b, c and 24a, b). The enzymatic accessibility was in the order: wheat straw > P. maximum > kallar grass > bagasse > A. amnicola poplar. Bagasse due to better fibre recovery, showed better results than kallar grass when the raw materials were considered (Fig. 24b). The results for increased accessibility from 2-NaOH are in agreement with many workers. The most commonly used alkali pretreatments have been employed using 0.05 to 1.0g Na per gram of substrate. The temperatures used, vary from room temperature to 160° C with time from 30 minutes to several days (Toyama and Ogawa, 1975; Ohlson et al, 1984; Marsden and Gra
1986). Gharpuray et al (1983) showed that 0.1g NaOH/g wheat straw at room temperature delignifies to a level of 8.6% hereby increasing the accessibility to 4 times.

Alkali (NaOH 2%) pretreatment with autoclaving resulted in higher fibre losses than the above two treatments. The fibre losses were enhanced by the increase in alkali concentration, especially for salt tolerant plants as also found from previous treatments. A maximum fibre recovery of 74.2% for poplar a minimum of 39% for A. amnicola suggested their inherent structural capacity (Fig. 25). Alkali (4%) resulted in alarming level delignification of about 66% from wheat straw. However, A. amnicola and poplar revealed a small increase in lignin in the pretreated fibre. Gharpuray et al (1983) enunciated that about 3 of delignification is required for maximal enzymatic hydrolysis.

Treatment with 2-3% alkali also resulted in appreciable lignin and hemicellulose losses. There was a concomitant increase in total polysaccharides exhibiting a maximum level for wheat straw in close proximity with P. maximum. A. amnicola ranked at the lowest, apart from showing appreciable increase in its polysaccharide content after pretreatment. Kellar grass because of greater hemicellulose losses, was accompanied by a higher glucose potential contents (Fig. 25c). The increase in xylose from maximum and bagasse could not be accounted for, in the pretreated fibre.

The maximum enzymatic accessibility attained for wheat straw was understandable from its analysis. Other substrates follow in the order of P. maximum > kellar grass > bagasse > A. amnicola poplar (Fig. 26a). In general, the increase in accessibility
mainly suggestive of higher delignification, accompanied by cellulose losses in the extract. But as mentioned earlier, other factors are included which directly or indirectly play role in increased accessibility (Millet et al, 1975; Matsumura et al, 1977; Tsao et al, 1978; Fan et al, 1982). Based on the materials, bagasse showed better fibre recovery to edge kallar grass for accessibility (Fig. 26b).

Taking the three pretreatments into perspective, all have different level of effectiveness on the substrates. P. maxim and wheat straw in particular, showed maximum accessibility of treatments from alkali with autoclaving, alkali at room temperature and steaming at high temperature (Table 22-25, Fig. 22, and 26). More severe pretreatment conditions resulted in excessive raw material losses including part of cellulose, although enzymatic digestibility enhanced to some extent. Thus consideration for raw material recovery besides enzymatic accessibility was found to be an important index, since economics of process is the key for its success. Among the three pretreatments based on raw materials, alkali (3%) treatment at room temperature was figured out as the treatment of choice, because of better fibre yields. Although steaming at 200°C showed greater fibre recovery than alkali treatment with autoclaving, however, because of poor delignification and modification the enzymatic solubilization was low. Steaming is more effective when used in combination with chemicals (Ando et al, 1988) and is also a better alternate for the recovery of hemicellulose in the wash liquor (Fuls et al., 1984; 1985). Alkali treatment best suited enzymatic accessibility, at large scale posses disposal prob.
of the resulting black wash liquor containing a lot of salts in it (Gharpuray et al, 1983).

**SACCHARIFICATION OF KG BY FUNGAL ENZYME PREPARATIONS**

Initial studies employing commercial enzyme preparations showed that *T. reesei* VTT-D-79125 when supplemented with \( \beta \)-glucosidase from *A. niger* (Miles Kalli) produced an increase of 10.4, 6.3 and 6.2% saccharification yields from 2, 5 and 10% kellar grass, respectively (Table 29). Enhancement in the above yields were materialized when cellulase in the ratio of 15:10 U/, substrate of FPase and \( \beta \)-glucosidase were increased to 16.5:2 U/g. Use of external \( \beta \)-glucosidase has been reported to enhance the saccharification rate and remove end-product inhibition caused by the production of cellobiose (Sternberg et al, 1976 Ladisch et al, 1977; Forchak et al, 1980; Wong et al, 1981 Gusakov et al, 1984; Morisset and Khan, 1984; Khan et al, 1985).

Moreover, it was observed that doubling of above enzyme dose improved the yields further. However, the enhancement decrease with the increase of kellar grass concentration (Table 30).

In a similar study, a 4 times (10 to 40ml) increased enzyme titre by *S. thermophile* of 0.44 and 0.88 U/ml of FPase and \( \beta \)glucosidase resulted in increased saccharification yield of 34 30, 20 and 7% for 1,2,5 and 10% kellar grass, respectively (Tab 31 and Fig. 26). The dilute enzyme titre from *S. thermophil* estimated per gram of substrate showed significantly high saccharification yields when compared with that of commercial enzyme preparations (see legend Fig. 26). The cellulase titre 7.2:14.0 U/g of FPase: \( \beta \)-glucosidase were found to give almost similar saccharification yield from 5% kellar grass. This can
attributed to a better synergistic behaviour and thermostable nature of the enzyme system in S. thermophile. The dilute enzyme titre by S. thermophile saccharified 10% kollar grass to a much lower extent and thus made it inevitable to use concentrated enzyme at higher substrate concentrations. S. thermophile has been reported by many workers as a most rapidly growing organism cellulolytic substrates. The enzyme system although low in titre has shown to degrade cellulose faster than hyperproducing mutants of T. reesei and Clostridium thermocellum (Coutts and Smit 1976; Bhat and Maheshwari, 1987; Ng and Zeikus, 1981). Properties of the enzyme system were further elucidated by using filter paper strips as a pure substrate along with kollar grass.

A maximal of 3 fold enhanced dosage (30ml) of 0.8 and 0.4 U/ml of FPase and B-glucosidase resulted in an increase of 35, and 6.3% reducing sugars from 2, 5 and 10% kollar grass, respectively (Table 31 and Fig. 28). A decrease in reducing sugar content from 5 and 10% kollar grass as compared to previous results (Table 29-30) was possibly due to some variations in analysis of sugars carried out by DNS and HPLC for the former and latter experiments. Compared to kollar grass 2 and 5% filter paper resulted in an increase in yield of 3.7% each, only. It was well documented that fibre porosity is a unifying characteris which relates digestibility with pretreatment (Cowling and Ki 1976; Grethlein et al, 1984; Grethlein, 1985). In general digestibility has been related with crystallinity, nature associated substrate, and surface area (Cowling and Kirk, 1977; Matsumu et al, 1977; Fan et al, 1982; Lee et al, 1988). Pretreated kollar grass (2% NaOH, 121°C, 1h) showed much hig
accessibility because of higher delignification, increase surface area and thus porosity. Apart from this initial attack, xylanase allows the endo-glucanase and exo-glucanase to be highly effective (Ghose and Bisaria, 1979; Taniguchi, 1982; Gharpuray et al, 1983).

The lowering in hydrolysis rate at increased substrate concentration was because of hydrodynamic instability, improper mixing and suspension of slurry (Lee et al, 1982; 1983). Other contributing factors included synergistic effect and concentration of endo-glucanase and cellulbiohydrolase, kinetic parameter and product inhibition (Okazaki and Moo-Young, 1978). Adsorptive of LC components (cellulose, lignin and hemicellulose) also effects increased digestibility. Especially, lignin is known to hinder the enzymes by steric and uncompetitive inhibition (Converse et al, 1987; Chernoglazov, 1988). Filter paper used as substrate has an amorphous and crystalline region. The slow saccharification rate and low conversion can be attributed to levelling off after initial solubilization of the former region. The latter region due to small pore size hinders the cellulbiohydrolase to enter and attack. In fact, there is only surface activity and some of the enzyme which travel in are trapped (Ohmine et al, 1983; Matsuno et al, 1983; Tanaka et al, 1986; Sattler et al, 1989). The results from HPLC indicated that enhanced enzyme dosage corresponded with increase in glucose and oligosaccharide yield, in contrast to xylose which showed slight enhancement in the case of kallar grass (Fig.28). This fact is supportive of a strong β-glucosidase with a relatively low xylanase in S. thermophile. However, at increased enzy:
titre the percent relative sugar yield (composition) in the hydrolyzates elucidated decrease in glucose and xylose level concomitant with increase in oligosaccharide content (Fig. 29). Apart from showing low yields from filter paper, the hydrolyzate showed about 96-98% glucose. The composition of sugars at high substrate concentration showed a substantial increase of oligosaccharide content, whereas decrease in glucose level suggested incomplete conversion (Fig. 30 and 31). This might be as a result of dilute enzyme apart from the facts described above.

Use of enzyme filtrate by S. thermophile obtained from 4' kollar grass and further concentrated by ultrafiltration membrane, yielded higher level of glucose as compared to T. reesei cellulase supplemented with β-glucosidase from A. niger (Table 33 Fig. 33 and 34). This was attributed to a concentrate enzyme with a potent β-glucosidase component as also reported by (Draijer. 1987b). T. reesei cellulase used alone showed higher reducing sugar yields than the control from S. thermophile, however, the glucose content was of similar level. Analysis by HPL revealed that none of the hydrolysates contained celllobiose except a negligible amount formed in the control of S. thermophil (Fig. 35). This preparation from T. reesei contained FPase: β-glucosidase in the ratio of 1:0.66 and thus along with a potent endo-glucanase and xylanase it formed a complete cellulase system and showed the ability to saccharify treated LC substrate into monomeric sugars. These results were in agreement with Sternberg et al (1976), Rabinovich et al (1979) and Sinitsyn et al (1982).

In order to better define the conditions, the concentrate
enzyme from S. thermophila was desalted by diafiltration and freeze dried to use at equal proportions of FPU per gram of substrate with T. reesei. Kellar grass (5%) was saccharified to a slightly higher degree by the latter enzyme preparation of 15 and 20 FPU/g substrate (Table 34, Fig.36a and 37a). However, 5% filter paper was saccharified to a much higher level by the latter (Fig. 36b) although, the overall yields were still very low. These results reveal the synergistic action of the endoglucanase and cellobiohydrolase from T. reesei (Okazaki and Moo-Young 1978; Ladisch, 1979). These results were attributed to microcrystalline portion of filter paper and the low overall surface area because of the strips. These results are consistent with Burns et al (1988).

Interesting results from HPLC were obtained which showed the presence of cellobiose in low concentrations up to 24 hours from the latter enzyme preparation which ultimately diminished after 48 hours (Fig.38 and 40). This is in agreement with Sternberg et al (1976) who also proposed a non-cellobiose route undergoing conversion to glucose by other route rather than cellobiose. This non-cellobiose route increased with the decreasing crystallinity of the substrate and was depicted by pretreated kellar grass as compared to filter paper (Fig. 38). On the converse filter paper, though with the increase of enzyme titre from 15 to 20 FPU's by T. reesei lowered the cellobiose level to some extent only, since it was present at the end of saccharification time at about 27% concentration (Fig.39 and 41). On the other hand, S. thermophila showed almost 90-100% of glucose sugar, which was attributed to its strong β-glucosidase component
(Grajek, 1987a,b). In general, for both the substrates there was a relative increase of glucose and oligo-sugars with time while xylose showed a partial levelling off after 8 hours when substrate was kallar grass (Fig. 38).

An optimum level of 30 and 35 FPU per gram of substrate (5% kallar grass) from *S. thermophilus* and *T. reesei* showed maximum saccharification (Table 35 and Fig. 43). The composition of sugars was the same as shown previously. However, the overall saccharification did not exceed to higher extent even at greater enzyme concentrations which could not be accounted for. It was illustrated from Fig. 42 that *T. reesei* showed a rapid hydrolysis up to 10 hours after which it slowed down considerably up to 4 hours after which the rate became stationary. This is in agreement with work of other investigators that suggests a strong synergistic behaviour of their endo- and exo-glucanases, apart from xylanase which increases, accessibility during the initial stages (Wood and McCrae 1975; Okazaki and Moo-Young 1978). In contrast, *S. thermophilus* showed a more consistent saccharification rate which can be enunciated from its low endoglucanase and xylanase activity but a strong cellobiohydrolase and β-glucosidase. The former two enzymes are required at higher concentrations during the initial stages while the latter two are required as the hydrolysis proceeds (Okazaki and Moo-Young 1978.)

Contrary to our previous results, the glucose yield was lower, especially in the case of *S. thermophilus*, which might be due to partial denaturing of its β-glucosidase during ultrafiltration and/or freeze drying. However, the composition of sugar suggested slightly higher level of relative glucose in th
hydrolyzates (Fig. 40) from that of T. reesei. Saccharification of 5% kollar grass using culture filtrate from thermophilic fungi when compared, showed a maximal saccharification of 69.2% from C. thermophile (Table 36 and Fig. 44). All the test fungi revealed considerable saccharification with dilute enzymes up to 40 hours. The rate slowed down after 10 hours because of the increase in the degree of polymerization and crystallinity of the substrate. This is consistent with Chang et al., 1981; Van Dyke 1972. In a detailed review Van Dyke (1972) concluded the rates a first order with respect to amorphous, crystalline and resistant substrates. The enzyme system for various fungi was elucidated to some extent from the composition of sugars by HPLC. All the thermophiles except S. thermophile and M. pulchella showed higher amounts of cellobiose and xylose. In contrast, high oligo-sugars were found for S. thermophile, T. reesei and A. fumigatus (Table 3 and Fig. 45). Glucose was obtained at higher concentrations in the order of C. thermophile > T. reesei > S. thermophile > H. grisea > L. thermophila > A. fumigatus > M. pulchella. The glucose level from the composition of various sugars in the hydrolyzates was in the order of S. thermophile > T. reesei > C. thermophile > H grisea > L. thermophila > A. fumigatus > M. pulchella. The yield for glucose and other sugars are illustrative of the cellulas systems of these fungi (Suga et al., 1975; Okazaki and Moo-Young 1978; Kanda et al., 1989).

The thermostable cellulases of thermophilic fungi were tested by carrying out saccharification at 60°C. It was found that in general the enzymes were inactivated after about 20 hour at this temperature (Fig. 47). Only C. thermophile cellulase
showed increased saccharification rate up to 40 hours (Tabl. 37). In fact, it yielded sugars at almost 1.4 times higher rate at 60 °C up to 20 hours than that at 50 °C (Fig. 44 and 47). In contrast, mesophilic T. reesei lost its enzyme stability after about 10 hours. Moreover, the latter organism lost almost 46% of sugar yield as compared to 11% by the former at 60 °C. It has been variously reported about the thermal and mechanical inactivation of cellulases, especially after 50 °C. Bisaria and Ghos (1988) enunciated that maximum level of adsorption decreased with increase in temperature after 50 °C. β-glucosidase appears to be more susceptible to high temperatures, whereas presence of substrate provides some protection (Mandel and Reese, 1964; Wood 1972; Stoppek et al., 1981; Durand et al., 1984). However, the composition of sugars at HPLC revealed higher glucose yield from T. reesei as compared to other thermophilic fungi (Fig. 48). This can be attributed to dilute enzyme titre of thermophilic fungi used (See legend to Table 36). S. thermophile showed a decline in the glucose level in the hydrolysates which could be as result of partial inactivation of its β-glucosidase.

The feasibility of LC bioconversion largely depends upon process economics. The results from these studies clearly indicate that the local thermophilic isolates produce potent cellulases/xylanases when grown on waste untreated LC materials. This was revealed from the demonstration of saccharifying ability on treated LC (kellar grass) by dilute enzymes from wild type thermophilic strains as compared to commercial enzyme preparations from mutant strains. S. thermophile seems to be more active than most of the wild type strains reported, because
its high $\beta$-glucosidase in the enzyme system. *C. thermophila* showed distinct ability to tolerate high temperature than other fungi during saccharification. Kallar grass proved to be economic importance in many aspects. In these studies, it was further confirmed as one of the lignocellulosic substrates for the biotechnological utilization. Pretreatment of LC substrat indicated alkali treatment with autoclaving as most suitable for enzymatic accessibility. Thus with further research we employing techniques like genetic engineering and/or protoplast fusion, the properties of thermostable cellulases produced these organisms can be further exploited.
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