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STEAM PRETREATMENT AND ENZYMATIC HYDROLYSIS OF *Eucalyptus viminalis* CHIPS

by

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SUMMARY

The steam pretreatment of *Eucalyptus viminalis* chips was characterized. Pretreatment parameters such as steam temperature, residence time, addition of SO_2 as an acid catalyst, and initial moisture content of the chips were evaluated in order to optimize recovery, fractionation and enzymatic hydrolysis of pretreated materials. In the absence of an acid catalyst, the best pretreatment was obtained at 230°C for 120 s using chips with a moisture content of 50% (w/w). Pretreatment by steam explosion showed no variation resulting from differences in the initial moisture content of the chips. However, when the substrate was steam-treated without explosion, the initial moisture content of the chips had a significant influence on the recovery yield and the degree of enzymatic hydrolysis.

When the chips were impregnated with sulfur dioxide (1% SO_2, w/w) prior to steaming, milder pretreatment conditions of 210°C for 50 s were sufficient because of the catalytic action of the SO_2 gas. SO_2 catalysis was shown to be particularly beneficial for the steam explosion of green chips. More than 95% of the original cellulose could be hydrolysed to glucose with more than 90% of the original pentosan recovered as xylose in the water-soluble fraction. It appeared that the efficient uptake of the SO_2 catalyst was dependent on the initial moisture content of the chips.

Enzymatic hydrolysis of SO_2-impregnated, steam-treated *E. viminalis* was carried out with increasing substrate concentrations and enzyme loadings. Removal of the alkali-soluble lignin had a minor effect on the hydrolysis yield when both the substrate concentration and enzyme loading were calculated in relation to the cellulose content. However, the peroxide-treated fraction derived from eucalyptus chips (SO_2-SEE-WIA/H_2O_2) was more readily hydrolysed than either the water-insoluble (SO_2-SEE-WI) or the alkali-insoluble (SO_2-SEE-WIA)

fractions, particularly when low enzyme loadings were used. Nearly complete saccharification of this substrate was obtained after 72 h when hydrolysis was carried out at 6% (w/v) using an enzyme loading of 10 FPU g^{-1} cellulose.

When compared to aspen and spruce, eucalyptus wood chips required milder pretreatment conditions to produce a steam-treated material which was readily fractionated. The various cellulosic fractions derived from steam-exploded *E. viminalis* also exhibited a greater susceptibility to hydrolysis than aspen and spruce substrates. The superior reactivity of steam-treated eucalyptus was more apparent at high substrate concentrations (10%, w/v).

Lower rates of hydrolysis of steam-treated substrates were observed when high sugar concentrations accumulated in the reaction mixture. The removal of soluble sugars, liberated during hydrolysis of the SO_2-SEE-WIA/H_2O_2 fraction, enhanced the efficiency of hydrolysis of the residual substrate. Most of the cellulase activity was shown to be associated with the unhydrolysed residue. The enzyme mixture used to obtain complete hydrolysis of the substrate at 2% (w/v) was successfully reused to hydrolyse a fresh batch of substrate for five consecutive times, with an elapsed time of 48 h between each recycling step. This same enzyme preparation could be reused an additional four times if the duration of hydrolysis was extended to 120 h. However, the efficiency of cellulose hydrolysis gradually decreased with each subsequent recycling step.

The mode of action of *Trichoderma* cellulases was further investigated by looking at changes which occurred in the morphology and fine structure of the cellulosic substrate during hydrolysis. A rapid reduction in fiber length (fragmentation), followed by an almost complete saccharification of cellulose, was observed for several fractions derived from pretreated eucalyptus. A gradual decrease in the degree of polymerisation (DP) of the SO_2-SEE-WIA/H_2O_2 fraction reflected the high susceptibility of this substrate to hydrolysis. However, when

the influence of hydrolysis on the DP of a fully bleached kraft pulp derived from eucalyptus was investigated, there was no noticeable change in the DP distribution of the residue until 24 h of hydrolysis. It seemed that the mode of action of *Trichoderma* cellulases varied depending on the type of pretreated substrate that was hydrolysed. As hydrolysis resulted in a gradual decrease in both the degree of polymerisation (DP) and the degree of crystallinity of the F-150 fraction, it was apparent that the depolymerisation of this substrate was predominantly due to exoglucanase activity. By contrast, the enzymatic hydrolysis of the FBEP-48 fraction resulted in little change in either the cellulose DP or the degree of crystallinity of the substrate. This suggested a "peeling off" type of mechanism. The susceptibility of the pretreated substrates to enzymatic hydrolysis could not be easily predicted from the differences in their cellulose DP or crystallinity.

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ABBREVIATIONS

CrI	Crystallinity index
CBD	Cellulose binding domain
CBU	Cellobiase unit
CBH	Cellobiohydrolase
CMC	Carboxymethylcellulose
DP	Degree of polymerisation
$\overline{DP_N}$	Number average degree of polymerisation
$\overline{DP_W}$	Weight average degree of polymerisation
EG	Endoglucanase
F-150	Fraction retained between screens of 48 and 150 mesh during fibre screening of peroxide-treated eucalyptus (SO_2-SEE-WIA/H_2O_2)
F-200	Fraction retained between screens of 150 and 200 mesh during fibre screening of peroxide-treated eucalyptus
FBEP	Fully bleached eucalyptus pulp
FBEP-48	Fraction retained between screens of 28 and 48 mesh during fibre screening of a fully bleached, eucalyptus kraft pulp
FBEP-100	Fraction retained between screens of 48 and 100 mesh during fibre screening of a fully bleached, eucalyptus kraft pulp
$\overline{FL_N}$	Arithmetic average fibre length
FPU	Filter paper unit
GPC	Gel permeation chromatography
HPLC	High performance liquid chromatography
kD	Kilo-daltons
LM	Light microscopy
$m^3\ ha^{-1}\ year^{-1}$	cubic meters per hectare per year
PASS	Phosphoric acid-swollen Sigmacell
RT	Room temperature
SEA	Steam-exploded aspen
SEE	Steam-exploded eucalyptus
SEM	Scanning electron microscopy

SES	Steam-exploded spruce
STE	Steam-treated eucalyptus (no explosion)
SO_2-SEA	Sulfur dioxide-impregnated, steam-exploded aspen
SO_2-STE	Sulfur dioxide-impregnated, steam-treated eucalyptus
SO_2-SEE	Sulfur dioxide-impregnated, steam-exploded eucalyptus
SO_2-SES	Sulfur dioxide-impregnated, steam-exploded spruce
SSA	Specific surface area
SSF	Simultaneous saccharification and fermentation
TAL	Total apparent lignin
TFA	Trifluoroacetic acid
TEM	Transmission electron microscopy
UF	Ultrafiltration
WI	Water-insoluble fraction
WIA	Water- and alkali-insoluble fraction
WIA/H_2O_2	Water and alkali-washed, peroxide-treated fraction
WS	Water-soluble fraction
w/v	weight per volume
w/w	weight per weight

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1. INTRODUCTION

1.1. Background

During this century, petroleum has supplied the largest share of the total energy consumed worldwide (Lynd *et al*, 1991). Therefore, harmful emissions which are associated with the use of fossil fuels have increased at a significant rate. At the global level, more than half the projected anthropically mediated climatic changes have been associated with carbon dioxide (CO_2) emissions (Marshall, 1989). At the local level, an increased number of areas worldwide has exceeded the minimum air quality standards for ozone and carbon monoxide (Russel *et al.*, 1990). Furthermore, the global energy crisis of the early 1970's has demonstrated that oil price fluctuations can be responsible for major economic disruptions in the developed world. Therefore, the maintenance of appropriate environmental standards as well as economic stability is the ultimate motivation for a worldwide replacement of petroleum derivatives by cleaner forms of energy. As part of this process, alternative automotive fuels such as ethanol, which are based on utilising a renewable substrate such as sugar cane or wood residues, have been gradually introduced in many countries to replace gasoline. Several intensive research and development programs in the last 15 to 20 years have promoted the production of energy from renewable resources. However, the relatively low prices for fuels now available in most marketplaces, have discouraged some

of these activities. Nevertheless, there is still a strong international research effort to develop the use of renewable biomass for the production of fuels and chemicals.

Brazil has accumulated a large amount of experience on ethanol production from sugar cane molasses. Since its creation in 1975, the National Alcohol Plan (Plano Nacional do Álcool, PNA) has stimulated the development of over 216 autonomous distilleries nationwide and 158 complexes which are annexed to sugar mills, providing employment for a large number of people (Laluce, 1991). During 1988, approximately 30% of the Brazilian automotive fleet was running purely on hydrated ethanol (95-96% ethanol). As a result of the harvest of 1989-90, Brazil produced a total of 12 million m^3 of ethanol, which was used to replace gasoline either as a blended or neat fuel. Reductions of harmful engine emissions have been achieved in both cases with only a minor reduction in performance. Several industrial sectors related to bioethanol, such as the production of pesticides and fertilizers, have also been greatly stimulated.

The Brazilian model has also helped researchers to identify certain inconveniences associated with ethanol production from agricultural crops. Short rotation crops such as sugar cane, although suitable for large biomass production, reduce the availability of soil for food production as well as the fertility of the soil. In 1988, approximately 8.1% of the total cultivated area was dedicated to sugar cane plantations (Laluce, 1991). The expansion of these plantations could become socio-

economically disastrous for a developing nation, unless very rigid and appropriate agricultural policies are adopted.

The Brazilian pulp and paper industry has been expanding consistently since the 1970's (Suchek, 1991). Although several wood species have been used for paper making, *Eucalyptus* spp. contributed a two-third share of the total pulp production of 5 million tons in 1990. These trees, originally introduced to Brazil from Australia and New Zealand, are fast growing species which can be harvested after 6-7 years of growth. Extensive plantations have been established in Brazil to fulfil the increasing demand for this raw material by the charcoal and pulp and paper industries. Among the different eucalyptus species imported from Australia and New Zealand, *E. viminalis* has adapted best to the temperate conditions of the southern region of Brazil.

The eucalyptus plantations which now exist in Brazil are the largest in the world (Suchek, 1991). Depending on the regional climate and soil fertility, a good stand of eucalyptus can grow with a rate approaching 40 m^3 ha^{-1} $year^{-1}$. The ability of these trees to coppice from a recently cut stump allows an eucalyptus plantation to be routinely subjected to three sequential clear-cuts after 7, 14 and 21 years of growth, after which it is replaced. These high rates of growth, together with the flexibility of developing reforestation programs near pulp mills, have contributed to a substantial reduction in wood cost and this seems to be one of the key factors for the economic

competitiveness of the final products. Although these trees will be primarily used for charcoal and pulp production, it has been proposed that wood residues from the harvesting and processing stages could be used as a feedstock for ethanol production.

The conversion of wood or agriculture residues to fermentable sugars for subsequent fermentation to ethanol and other fuels has been extensively studied (Klyosov, 1986; Vallander and Eriksson, 1990). A significant amount of research effort in this area is justified because of the enormous surplus of low value lignocellulosic materials which is available worldwide. However, this opportunity is counteracted by the fact that plant polysaccharides are not amenable to efficient saccharification. Usually some sort of pretreatment is required before lignocellulosic substrates can be hydrolysed to their component sugars.

1.1.1. Structural Characteristics of Lignocellulose

As found in nature, lignocellulose is a composite of cellulose microfibrils which are embedded in an amorphous matrix of hemicellulose and lignin. This amorphous matrix acts as a natural barrier to microbial and/or enzymatic attack (Fig. 1). In the following sections, the structural characteristics of lignocellulose and its three major components, hemicellulose, cellulose and lignin, will be discussed.

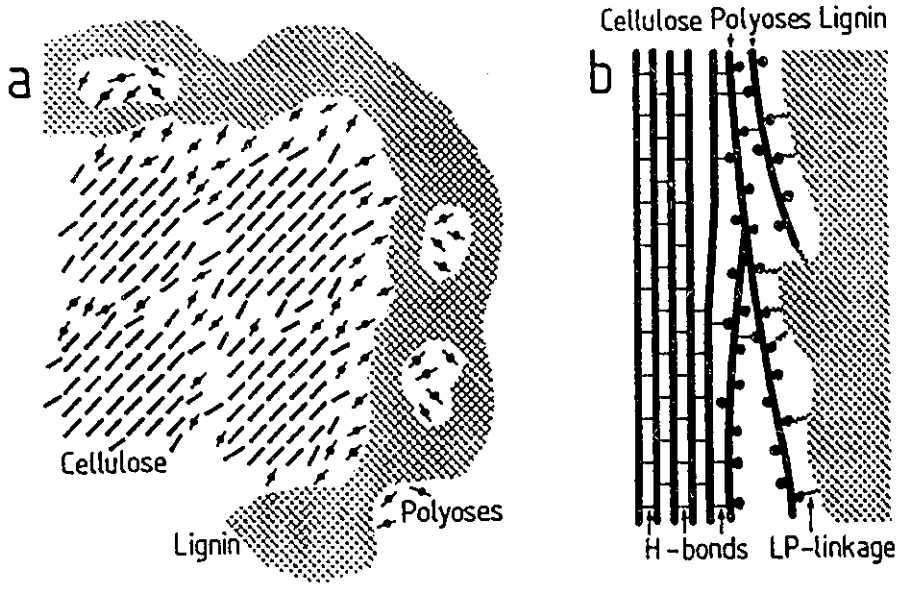


Fig. 1. Representation of the close association of the three components of the plant cell wall, cellulose, polyoses (hemicelluloses) and lignin. (**a**) Transversal and (**b**) longitudinal sections (adapted from Fengel and Wegener, 1983).

1.1.1.1. Hemicellulose and Lignin

The chemical nature of hemicelluloses varies from tissue to tissue and from species to species. These polysaccharides are formed from a wide variety of monosaccharides including pentoses (*e.g.*, xylose, rhamnose and arabinose), hexoses (*e.g.*, glucose, mannose and galactose) and uronic acids (e.g., 4-*O*-methyl-glucuronic and galacturonic acids) (Roelofsen, 1959; Fengel and Wegener, 1983). Generally, they fall into four classes: (a) unbranched chains such as (1-4)-linked xylans or mannans; (b) helical chains such as (1-3)-linked xylans; (c) branched chains such as (1-4)-linked galactoglucomannans; and (d) pectic substances such as polyrhamnogalacturonic acid. Some hemicelluloses, particularly heteroxylans, also show a substantial degree of acetylation.

The hemicelluloses are structurally more related to cellulose and are deposited in the cell wall at an earlier stage than lignin (Stamm, 1964; Rydholm, 1965). Despite the complexity of these polysaccharides, their structure seems to be generally rod-shaped with branches and side chains folded back to the main chain by means of hydrogen bonding. This rodlike structure probably facilitates their interaction with cellulose microfibrils, resulting in a tight association which gives greater stability to the aggregate (Rydholm, 1965).

The hemicellulose content of softwoods and hardwoods differs significantly (Jane, 1956; Fengel and Wegener, 1983). Hardwood hemicelluloses are mostly composed of highly acetylated

heteroxylans, generally classified as 4-*O*-methyl glucuronoxylans. Hexosans are also present but in very low amounts as glucomannans. Owing to their acidic characteristics, hardwood xylans are relatively labile to acid hydrolysis and may undergo auto-hydrolysis under relatively mild conditions. By contrast, softwoods have a higher proportion of partially acetylated glucomannans and galactoglucomannans. As a result, softwood hemicelluloses (mostly hexosans) are more resistant to acid hydrolysis than hardwood hemicelluloses (mostly pentosan). Xylans correspond to only a small fraction of the total hemicellulose content of softwoods.

In plants, the hemicelluloses are generally combined with lignin (Jane, 1956; Fengel and Wegener, 1983). Lignin is a phenolic polymer which is probably formed by the free-radical polymerisation of *p*-hydroxy cinnamyl alcohol units with varying methoxyl content (Roelofsen, 1959). The chemical structure of lignin is very complicated and is not yet completely known. Three monomeric units have been recognized in lignin: coniferyl alcohol, sinapyl alcohol, and *p*-coumaryl alcohol. The proportion of these monomers varies with species and this ratio has been used for taxonomic purposes. Depending on the degree of methoxylation, the aromatic group is either guaiacyl (derived from coniferyl alcohol) or syringyl (derived from sinapyl alcohol). The former has only one methoxyl group adjacent to the phenolic hydroxyl group, whereas the latter has two. The most important physical property of this organic polymer is its

rigidity, which not only gives strength to the plant tissue but also prevents the collapse of the water-conducting elements.

Hardwood lignin contains a high proportion of residues derived from sinapyl alcohol (Roelofsen, 1959). Therefore, it is a highly methoxylated polymer characterized by a low guaiacyl to syringyl ratio. The lignin of softwoods is relatively richer in residues derived from coniferyl alcohol, which is indicative of a low methoxyl content and a high guaiacyl to syringyl ratio. As a consequence of this chemical difference, the lignin of hardwoods is less condensed and more amenable to chemical conversion than is the lignin of conifers. However, there is some evidence indicating that the lignin component of hardwood vessels is structurally more related to the guaiacyl type of softwood lignin (Musha and Goring, 1975; Saka and Goring, 1985).

1.1.1.2. Cellulose

Cellulose is a linear macromolecule of glucose (β-D-glucopyranose) units linked together by a glycosidic bond involving the C-1 and C-4 carbons. This polysaccharide is widespread in nature, occurring in both primitive and highly evolved plants. The size of the cellulose molecule is normally given in terms of its degree of polymerisation (DP), *i.e.*, the number of anhydroglucose units present in one single molecule. However, the conformational analysis of cellulose would indicate that cellobiose (4-*O*-β-D-glucopyranosyl-β-D-glucopyranose), rather than glucose, is the basic structural unit of cellulose

(Fig. 2) (Fengel and Wegener, 1983).

Owing to the linearity of the cellulose backbone, adjacent chains of cellulose form a framework of water-insoluble aggregates of varying length and width (Preston, 1986). These aggregates or microfibrils consist of both ordered (crystalline) and less ordered (amorphous) regions (Stamm, 1964; Fengel and Wegener, 1983). The lattice forces which are responsible for maintaining the crystalline regions are basically the result of extensive inter- and intramolecular hydrogen bonding. Electron microscopic studies have shown that microfibrils are formed by an association of several elementary fibrils with an average diameter of 3.5 nm (Fengel and Wegener, 1983). The occurrence of fibrillar structures with wider dimensions appeared to be dependent on the origin and treatment of the sample. Several models have been proposed to explain the internal structure of the plant cell wall, where microfibrils are further associated with hemicellulose and lignin. Fengel and Wegener (1983) suggested that the elementary fibrils are separated by a monolayer of hemicelluloses, forming thread-like structures of approximately 25 nm which are enclosed in a matrix of hemicellulose and lignin (Fig. 3).

The crystalline nature of cellulose was first established using polarized light microscopy, but the confirmation of this hypothesis was only possible with the advent of X-ray crystallography (Sarko, 1986). Each of the lattice planes of the space unit of cellulose is represented in the X-ray

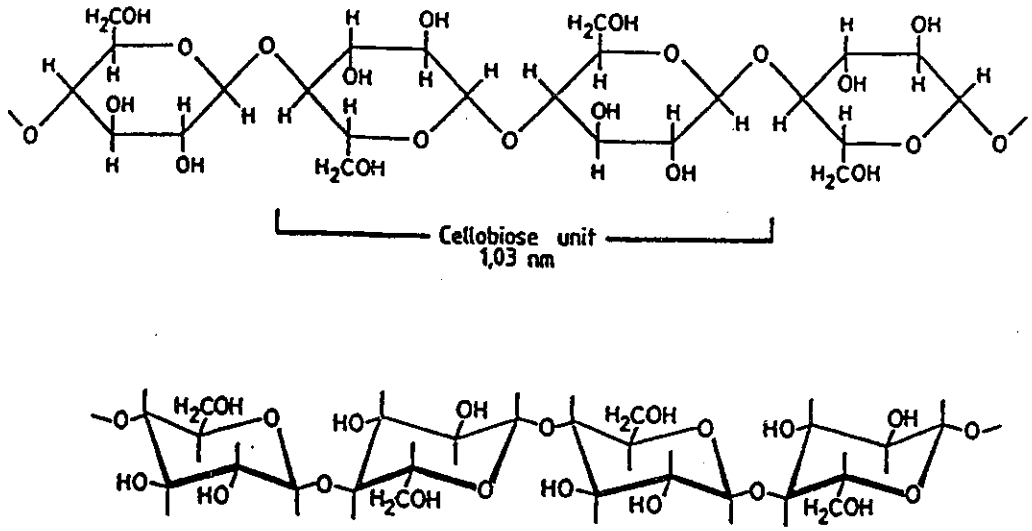


Fig. 2. Central part of the molecular chain of cellulose, with special reference to its basic structural unit, cellobiose. **(A)** Chemical formula and **(B)** stereo-chemical formula (adapted from Fengel and Wegener, 1983).

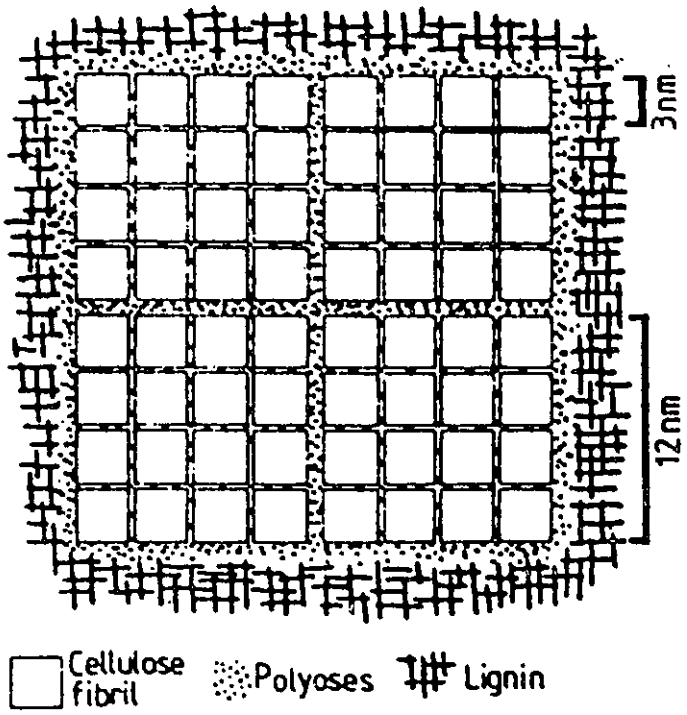


Fig. 3. Model of the association between the components of the plant cell wall, with reference to the structure and physical dimensions of the cellulose microfibril (adapted from Fengel and Wegener, 1983).

diffractogram by a peak at a characteristic diffraction angle. Although several lattice planes contribute to the diffraction pattern of cellulose (Fig. 4), the reflections obtained at the lattice planes 101, $10\bar{1}$ and 002 are generally the most dominant. These planes correspond to peak intensities at the diffraction angles (2Θ or Bragg angles) of 14.6, 16.2 and 22.6°, respectively (Stamm, 1964).

Several methods have been suggested for calculating the degree of crystallinity of cellulose from X-ray diffractograms (see Fengel and Wegener, 1983). Some authors have advocated that the crystallinity of cellulosic materials such as pulp can only be determined after the diffraction pattern due to the crystalline component has been separated from that due to the amorphous component (Ahtee *et al.*, 1983). However, the simplified method proposed by Segal *et al.* (1959), which expresses the degree of crystallinity as a relationship between the intensity of diffraction at $2\Theta = 22°$ (corresponding to the 002 lattice plane) and $2\Theta = 18°$ (which is attributed to the "amorphous" component), has been the most widely used. The intensity and width of the peaks in a diffractogram have also been used to determine the physical dimensions of the cellulose crystallite (Steward and Foster, 1976). In some cases, these determinations may involve the application of mathematical models to resolve and adjust the overlapping peaks in the diffractogram (Hindeleh and Johnson, 1978).

The first accepted unit cell for the cellulose crystallite

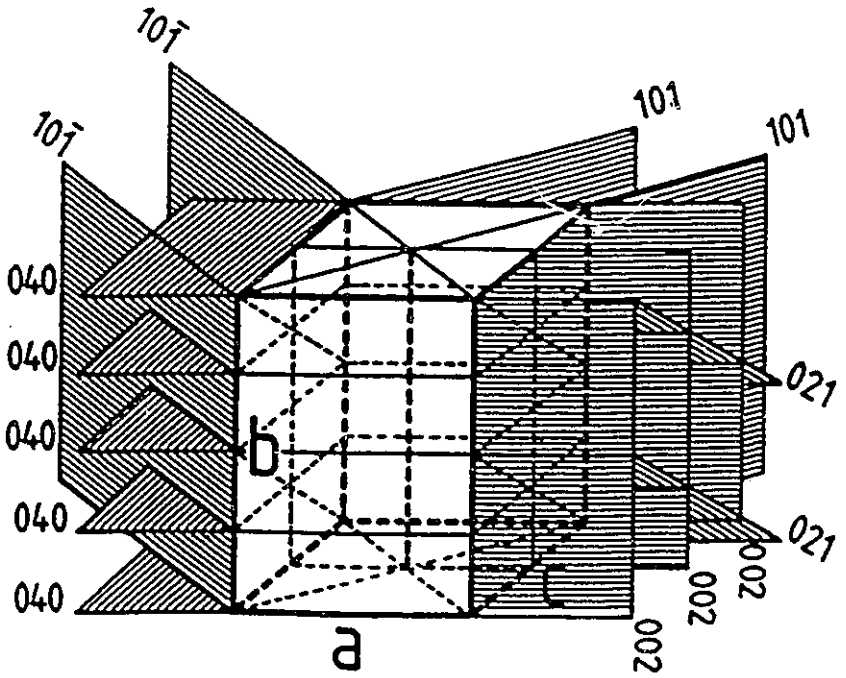


Fig. 4. The lattice planes of the space unit of native cellulose (cellulose I) (adapted from Fengel and Wegener, 1983).

suggested that the central chain of the unit cell lies upside down with reference to the adjacent chains, indicating that chains of crystalline cellulose assume an anti-parallel disposition (Sarko, 1986). However, it is now recognized that the supermolecular morphology of cellulose and its derivatives possess a high degree of polymorphism. For instance, the conformational analysis of cellulose triacetate indicated a crystalline structure with parallel chains lying in sheets, whereas mercerized cellulose (regenerated after mercerization or solubilization) has been demonstrated to be antiparallel (Sarko, 1986). According to its crystalline form, cellulose has been classified in several classes known as cellulose I, II, III, and IV. There has been evidence indicating that cellulose I, the result of biosynthesis, has essentially a parallel-chain structure and that this conformation may be converted into the other polymorphs (Ia, Ib, II, III, and IV), depending on the agent and the conditions used for the transformation. This polymorphism is therefore intimately associated with the crystalline form and different polymorphs are characterized by their intermediate conformations between the two basic structural organizations, parallel and anti-parallel. The structure of cellulose II is considered typical of an anti-parallel chain conformation. A comparison between cellulose I and II indicates that anti-parallel packing allows for increased hydrogen bonding, which results in a lower energy structure with greater stability (Sarko, 1986; Yamashiki *et al.*, 1990a). This

is probably the reason why the transformation of cellulose II into the paralleled-chained structure of cellulose I cannot be achieved.

The highly organized, crystalline structure of cellulose has been described as an obstacle to its hydrolysis because of its resistance to chemical and biological agents (Wood and Saddler, 1988). It appears that this tight association among cellulose molecules, added to a close interaction with hemicelluloses and lignin, greatly reduces the accessibility of the substrate to cellulases. A detailed description of the substrate-related factors which limit the enzymatic hydrolysis of cellulose will be given in the section 1.3.2.1.

1.1.1.3. Structural Organization of the Plant Cell Wall

Generally, the structure of the cell wall is subdivided into the middle lamella, primary wall and secondary wall (Fig. 5). The distribution of cellulose, hemicelluloses and lignin varies considerably among these layers (Jane, 1956; Rydholm, 1965). The middle lamella is the outermost layer and is almost entirely composed of lignin in the mature wood. This layer fixes the cells together and is responsible for the structural integrity of the plant tissue. The primary wall in a mature cell is also highly lignified and it is a thin layer which is initially deposited around the plant cell. Two adjacent primary walls and the intervening middle lamella are often called the compound middle lamella. Inside the primary wall, the secondary

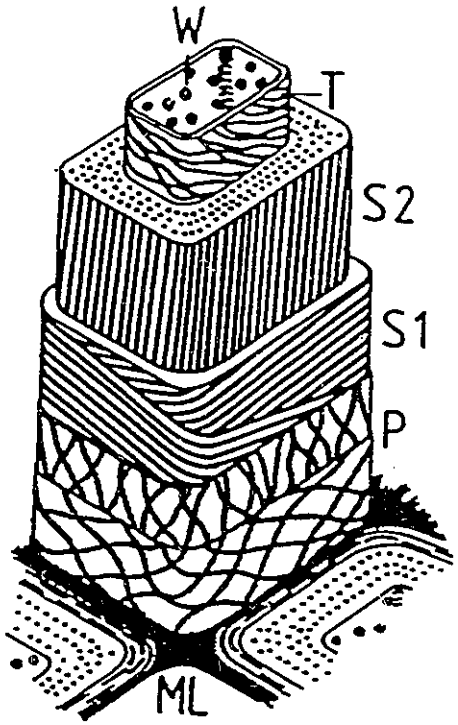


Fig. 5. Model of the cell wall of softwood tracheids and hardwood libriform fibres. ML = middle lamella, P = primary wall, S_1 = secondary wall # 1, S_2 = secondary wall # 2, T = tertiary wall, W = warty layer (adapted from Fengel and Wegener, 1983).

wall is formed in a sequence of three lamellae, S_1, S_2, and S_3 (Jane, 1956). The central layer of this wall, S_2, is usually thicker than the others. As a result, most of the fibre properties, particularly those of interest for the pulp and paper industry, is derived from the characteristics of this layer. Each layer of the secondary wall contains cellulose microfibrils which lie, more or less, parallel to one another. This common orientation results in a helical disposition which can be characterized according to the angle that the microfibrils make with the longitudinal axis of the cell. Since the microfibril angle varies between lamellae (Fig. 5), a crossed microfibrillar structure is observed. The S_2 layer is generally characterized by small microfibril angles, resulting in a steep helix, whereas flat helices are usually found in the S_1 and S_3 layers (Rydholm, 1965; Butterfield and Meylan, 1980).

The stem of highly evolved plants is generally divided into two defined regions, named sapwood and heartwood (Hagglund, 1951; Immergut, 1963). The sapwood is located at the outermost disk of the stem and is covered by the bark. It is formed by a living tissue which is primarily responsible for the distribution of water and nutrients to the branches. By contrast, the heartwood is formed by cell elements which are generally not metabolically active. This tissue functions primarily in physical support and is characterized by a low moisture content and a high content of extractives. Young stems are mostly formed by the less dense sapwood tissue, whereas the

content of heartwood increases with the age of the stem.

The stem of angiosperm dicotyledonous woods (hardwoods) such as *E. viminalis* is formed by a variety of cell types with structural and/or transport functions. The axial system consists of libriform fibres, fibre-tracheids (vascular tracheids), parenchyma cells and vessel elements, while the ray system is largely built up of parenchyma cells (Immergut, 1963; Butterfield and Meylan, 1980). Fibres generally have a thick cellulose-rich S_2 layer, whereas the thin-walled ray cells may or may not have a secondary wall. The thickness of these layers may vary among different cell types. Some vessel elements have the same three layered structure of fibres, although with thicker S_1 and S_3 layers, while others have a complex multilayered structure. A deposition of a tertiary layer or vesture wall may form over the lumen surface (Carlquist, 1988). This protective layer, also called the warty layer, is believed to consist of lignocarbohydrate complexes and its occurrence was reported in ray and axial parenchyma cells, vessels, and fibres of hardwoods (Butterfield and Meylan, 1980). The cells of both axial and ray systems are closely interconnected by means of pits (Butterfield and Meylan, 1980; Carlquist, 1988). Pits are pores in the secondary wall forming a canal between the cell lumen and the primary wall. Pit-pairs may be formed between two contiguous cells, resulting in points of contact through which solutes may be exchanged.

By contrast, the stem of conifers (softwoods) is mostly

composed of tracheids, with a smaller proportion of ray parenchyma cells and resin canals (Immergut, 1963; Stamm, 1964; Rydholm, 1965). Softwood tracheids are also highly interconnected by means of pits, but no vessels are present. As a result, the stem of conifers is not porous as is that of deciduous woods (hardwoods).

1.2. Pretreatment of Lignocellulosic Materials

Saccharification of starch-like materials and ethanol production from simple sugars is a well developed technology. However, the bioconversion of lignocellulosic residues, either by chemical or biological means, requires more drastic conditions because of the natural resistance of these materials to chemical or enzymatic degradation.

During the last 80 years, various chemical methods for hydrolysing wood have been proposed. Several processes have been reported using a wide range of catalysts such as hydrogen fluoride (Hawley *et al.*, 1986), sulphuric acid, nitric acid, and hydrochloric acid (Wayman, 1986; Parisi, 1989). When dilute acid hydrolysis was evaluated at a commercial scale, sugar degradation was found to be high. Humic substances which were inhibitory to fermentation were also produced and other operating problems, such as corrosion and a negative environmental impact, were discouraging (Wayman, 1986).

The use of concentrated acid processes has usually been based on the solubilization of plant polysaccharides in 72%

(w/v) sulphuric acid or 41% (w/v) hydrochloric acid at low temperatures, followed by dilution to a 3-6% (w/v) acid concentration and heating at 100-120°C for 30-360 min (Wayman, 1986). Close to theoretical yields of cellulose saccharification have been achieved using these conditions. Although high recovery yields could be achieved by concentrated acid hydrolysis, this process involves high capital investment, acid consumption and acid recovery cost (Parisi, 1989).

Successful saccharification of cellulosic residues has also been accomplished using highly specific enzymes. However, efficient enzymatic hydrolysis requires some form of pretreatment to open up the structure of the recalcitrant lignocellulosic materials (Walker and Wilson, 1991). Even starch requires some form of pretreatment to enhance the rate and efficiency of enzymatic hydrolysis. The ease with which starch substrates are hydrolysed can be increased relatively simply by milling, which enhances swelling and increases the available surface area of the substrate. Lignocellulosic materials, however, require more drastic measures to increase accessibility because they have been designed by nature primarily to act as structural materials. In order to make an economically competitive process, the pretreatment method must also result in high recovery yields of hemicelluloses and lignin in an active form for further utilization as chemical feedstock (Nguyen and Saddler, 1991).

A variety of biological, physical and chemical

pretreatments has been assessed for their technical and economical effectiveness at pretreating several lignocellulosic residues (Millet *et al.*, 1976; Eriksson *et al.*, 1980; Saddler *et al.*, 1982; Rolz *et al.*, 1987; Grethlein and Converse, 1991; Schell *et al.*, 1991). The relative success of each method usually depends on how much of the starting materials is recovered after the pretreatment step and/or the extent to which enzymatic hydrolysis of cellulose is improved.

Biological pretreatments usually result in partial delignification of the lignocellulosic material using white rot fungi to degrade lignin. Reductions of 65% of the lignin content have been reported during growth of white-rot fungi on cotton straw (Eriksson *et al.*, 1980). Although the biological degradation of lignin is a very slow process, it could be a cost-effective way of pretreating lignocellulose when applied in conjunction with other physical or chemical methods.

Chemical pretreatments tend to solubilize and/or extract hemicellulose and lignin to expose the cellulose component for further enzymatic hydrolysis. Several pretreatment options have been proposed, including alkaline extraction, mild oxidation, acid hydrolysis and selective extraction of wood components using organic solvents (organosolv) (Wood and Saddler, 1988). A wide variety of chemicals has been suggested for use in pretreatment, including sodium hydroxide (Playne, 1984; Fox *et al.*, 1989), aqueous ammonia, sulphur dioxide and calcium hydroxide plus calcium carbonate (Rolz *et al.*, 1987), alkaline

hydrogen peroxide (Gould, 1984), dilute sulphuric acid (Torget *et al.*, 1991), *n*-butilamine (Tanaka *et al.*, 1990), *n*-propilamine (Kitsos *et al.*, 1992) and alcohols (methanol, ethanol or butanol) in the presence of either acid or alkaline catalysts.

Physical pretreatments, such as milling and radiation, have also been utilised to enhance the hydrolyzability of lignocellulosic materials. Milling generally results in substrate size reduction and a decrease in cellulose crystallinity and degree of polymerisation (DP). Various kinds of mills have been evaluated, such as ball mill, hammer mill and two-roll mill (see Fan *et al.*, 1987). However, the major disadvantage of these methods is the high energy requirement (Tassinari *et al.*, 1980). An enhanced hydrolysis profile of several cellulosic substrates has also been achieved during simultaneous saccharification and milling (Furcht and Silla, 1990). Exposure of cellulosic residues such as sugar cane bagasse to gamma radiation resulted in a substantial decrease in the cellulose DP but with only a marginal increase in substrate hydrolysis (Sinitsyn *et al.*, 1991). However, a considerable improvement in the hydrolysis of wheat straw was obtained when gamma radiolysis was used in the presence of an acid catalyst such as sulphuric acid (Mamar and Hadjadj, 1990).

It seems that the best pretreatment options are those which combine elements of both physical and chemical methods. One of these methods, which will be discussed at length in the following section, is the steam pretreatment (Delong, 1981,

1983; Foody, 1984a,b; Brownell and Saddler, 1984). Several authors have shown the advantages of steam treatment over several other pretreatment options. Superior recovery yield of pretreated materials and better substrates for hydrolysis have been obtained from lignocellulosic residues using steam rather than sodium hydroxide (Playne, 1984), alkaline hydrogen peroxide (Vallander and Eriksson, 1985), nitric acid (Saddler *et al.*, 1982), alkaline organosolv, sulphur dioxide, sodium hydroxide, full soda cook, aqueous phenol, and calcium hydroxide plus sodium carbonate (Rolz *et al.*, 1987). Steam pretreatment has also been compared to other methods used for pretreating wood residues, such as milling (Saddler *et al.*, 1982), and demonstrated to be more effective.

The ammonia fibre explosion (AFEX) method appears to be a possible alternative to steam pretreatment, particularly when pretreatment is followed by simultaneous saccharification and fermentation of the substrate (Dale and Moreira, 1983; Holtzapple *et al.*, 1991). The AFEX method applies a pretreatment catalyst (ammonia) which can be used as a source of nitrogen for subsequent microbial growth. Furthermore, lower pretreatment temperatures are required, thus minimizing the production of inhibitors during pretreatment. The AFEX method has been successfully used for pretreating agricultural and municipal wastes. However, it is not clear whether this method could be extended to applications such as the bioconversion of wood residues, including softwoods.

1.2.1. Steam Pretreatment

High-pressure steaming (Taylor, 1980; Saddler *et al.*, 1982; Brownell and Saddler, 1984; Brownell, 1989) is one of the most promising pretreaments for fractionating wood into its three major components and enhancing the accessibility of cellulose to enzymatic attack. Several patents have been published and the process eventually incorporated into industrial pilot plants (DeLong, 1981, 1983; Foody, 1984a,b). This method involves heating wood chips at high temperatures and pressures, followed by mechanical disruption of the steam-treated material either by violent discharge into a collecting tank (explosion) or by bleeding the steam out (no explosion) followed by mild blending. The plant cell wall structure is radically modified by the high-pressure steam, yielding a dark brown material which is further reduced to a finely divided substrate with the consistency of potting soil. Partially hydrolysed hemicelluloses are easily recovered by water-washing, leaving a water-insoluble fraction composed of cellulose, partially condensed lignin and residual hemicelluloses. Most of the residual lignin can be further extracted by mild alkali, ethanol or dioxane. A schematic representation of the this process is shown in Fig. 6.

Economic studies have shown that a suitable pretreatment must not only enhance substrate accessibility but also allow easy and efficient fractionation of the major plant biomass constituents - cellulose, hemicellulose and lignin (Nguyen and Saddler, 1991). Enhanced substrate accessibility to enzymes is

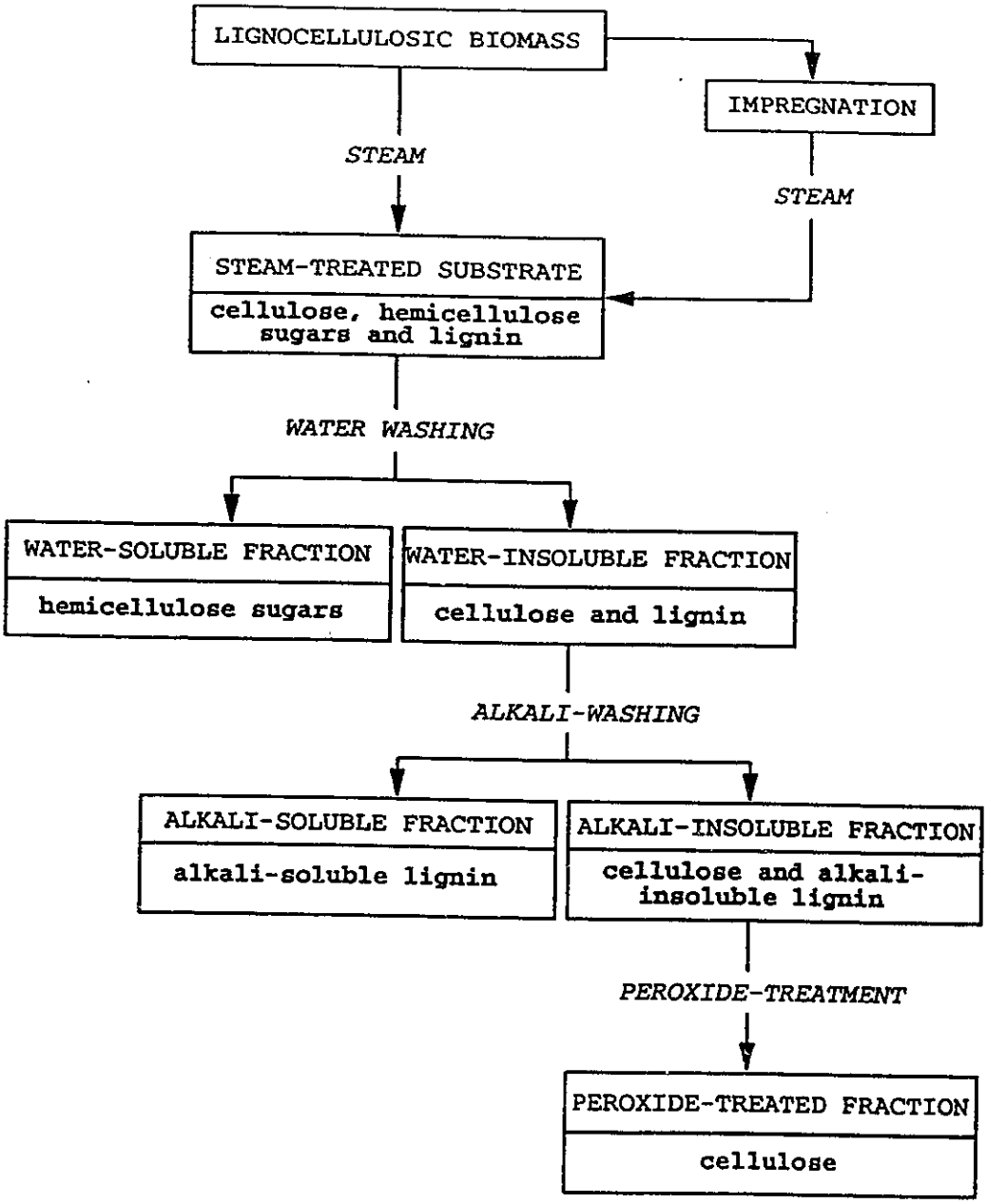


Fig. 6. Process flow sheet for the steam pretreatment of lignocellulosic residues, with reference to changes in the chemical composition of the pretreated fractions.

beneficial because faster hydrolysis rates can be obtained. It has been shown that substrate accessibility gradually increases with the severity of pretreatment. However, drastic pretreatment conditions result in high levels of decomposition of hemicellulose sugars and lignin (Brownell and Saddler, 1984; Brownell *et al.*, 1986a; Brownell, 1989). Therefore, process optimization must be the result of a compromise between hydrolysis and recovery yield, so that good substrates for hydrolysis can be produced with most of hemicelluloses and lignin being recovered as saleable products.

The process of high-pressure steaming has been optimised for several hardwood, softwood and agricultural residues. Variables such as temperature (pressure), residence time and concentration of added catalyst have been optimised to produce the best substrate for hydrolysis while ensuring best recovery of the hemicellulose and lignin (Table 1). Despite the large amount of published data available in this field, the wide variation in the methodologies used by different laboratories for pretreating and hydrolysing lignocellulosic substrates have made comparisons extremely difficult. For instance, the conditions used by Dekker *et al.* (1987) to pretreat *E. regnans* chips were not optimised for maximum recovery of pentoses while enzymatic hydrolysis was generally assessed at relatively high enzyme loadings (Dekker *et al.*, 1987).

Most of the published work in this area has considered poplar (such as *Populus tremuloides*) as the probable wood

Table 1. Conditions tested for the steam pretreatment of various substrates.

MATERIAL	PRETREATMENT CONDITIONS			REFERENCES
	Catalyst	Temperature (°C)	Time (s)	
Wheat straw	-	247	45	Tenrud *et al.*, 1989
	-	200-230	30-600	Beltrame *et al.*, 1992
	35mM H_2SO_4	200	120	Marchal *et al.*, 1986
Sugarcane bagasse	-	200	60-1200	Dekker and Wallis, 1983
	-	200	300	Playne, 1984
	-	190-220	0-1500	Kling *et al.*, 1987
	-	200-250	80-360	Morjanoff and Gray, 1987
	-	240	60	Rolz *et al.*, 1987
	2% H_2SO_4	170-250	2-90	Morjanoff and Gray, 1987
Corn stover	-	215	120	Marchal *et al.*, 1986
Birch, maple	-	240-280	540-780	Thompson *et al.*, 1992
	1% H_2SO_4	180-220	8.3-8.5	Thompson *et al.*, 1992
Birch	-	170-210	600	Puls *et al.*, 1985
Poplar	-	217-248	180	Excoffier *et al.*, 1991
	1% H_2SO_4	220	10	Nuttor and Converse, 1991
	0.4% H_2SO_4	201-225	120	Excoffier *et al.*, 1991
Aspen	-	190-240	18-6000	Brownell and Saddler, 1987
	-	240	20-240	Brownell *et al.*, 1986b; Brownell and Saddler, 1987
	-	240	60-140	Schwald *et al.*, 1989a
	0.2% H_2SO_4	220	10-80	Brownell *et al.*, 1986b; Brownell and Saddler, 1987
	0.58% H_2SO_4	200	80	Mackie *et al.*, 1985
	1.6% SO_2	210-227	120	Mackie *et al.*, 1985
	1.6% SO_2	190-220	100	Schwald *et al.*, 1989a
Eucalyptus	-	200	300	Dekker *et al.*, 1987
Willow	-	220	600	Galbe and Zacchi, 1986
Spruce	0.5-5% SO_2	190-220	50-250	Schwald *et al.*, 1989b
Radiata pine	0.5-12% SO_2	182-248	30-1080	Clark and Mackie 1987
Pine, aspen, corn stover	2-3% SO_2	150-208	45-1200	Wayman and Parekh, 1988
Pine, spruce	2-2.6% SO_2	187-204	120	Wayman *et al.*, 1986
Pine, oak, cedar, gum	-	200-240	60-120	Bierman and McGinnis, 1990

species to be used in any future bioconversion process. This species has been examined because it grows fast and the resulting wood chips exhibit high permeability to chemicals and/or steam. Generally, residues derived from hardwood species seem to be more susceptible to high-pressure steaming than softwood residues, therefore requiring less drastic conditions for an efficient pretreatment. The ease of pretreatment of hardwoods has been associated with their high content of acetylated heteroxylans. During pretreatment, the gradual degradation of these compounds generates acid catalysts (such as acetic, formic and levulinic acids) which trigger further action on more recalcitrant wood fractions. The hemicelluloses of softwoods, however, are primarily composed of glucomannans (hexosans). These compounds are more resistant to acid hydrolysis than hardwood xylans and this is one of the major reasons why softwoods require either higher temperatures and times or additional catalyst for effective pretreatment.

1.2.1.1. Effects of Steaming Conditions on Pretreatment

It is not clear in the literature whether high temperatures and short times, or lower temperatures and longer times, are preferable. Puls *et al.* (1985) reported that the extraction and recovery of hemicellulose sugars were most efficient at relatively low steam temperatures of 200°C. The overall carbohydrate yield was shown to decrease sharply with increased temperatures. Higher yields of lignin condensation and pentosan

degradation were also observed at longer reaction times. At shorter times, acid hydrolysis seemed to prevail over degradation processes.

During pretreatment, pentosan destruction can be attributed to the three distinct processes of pyrolysis, oxidation and dehydration. Pyrolysis (Brownell and Saddler, 1984; Brownell *et al.*, 1986a; Foody, 1982) results in decomposition products such as carbon dioxide and water. Oxidation reactions also contribute to a partial decomposition of pentoses to carboxylic acids and other by-products (Springer and Harris, 1982). Dehydration (Brownell and Saddler, 1984) of hemicellulose sugars occurs at extreme steaming conditions and produces furfural and hydroxymethyl-furfural from pentoses and hexoses, respectively. There has been some evidence indicating that these products are strong inhibitors of microbial growth (Excoffier *et al.*, 1991).

The degradation of lignin during steam pretreatment has also been extensively characterized (Hemmingson, 1985, 1987; Tanahashi *et al.*, 1989b; Tanahashi, 1990). Lignin is primarily degraded through the homolytic cleavage of β-*O*-4 ether linkages, producing a series of derivatives of cinnamyl alcohol (Tanahashi *et al.*, 1989b) and condensation by-products. These degradation reactions are undesirable as they reduce the commercial value of these chemicals.

1.2.1.2. Influence of Explosive Decompression, Moisture Content and Chemical Composition of the Chips

Explosive decompression was shown to be non-essential for the production of a substrate which was highly accessible to enzymes (Brownell *et al.*, 1986b; Brownell and Saddler, 1987). Similar hydrolysis profiles and recovery yields were obtained when green aspen chips were steam-treated with or without explosion. However, earlier work reported by Delong (1981, 1983) has claimed that explosion was essential to the process so that increased surface area of the pretreated substrate was obtained.

For pretreatment of aspen chips, the influence of the initial moisture content of the chips was shown to be largely dependent on chips size (Brownell and Saddler, 1984, 1987; Brownell *et al.*, 1986b). When large, green chips were steam-treated at conditions previously optimised for large, air-dried chips, unevenly cooked substrates were produced. Simulated aspen chips, to which thermocouple probes were inserted, were also used to monitor the temperature rise inside the chips (Brownell and Saddler, 1984). Air-dried chips reached the temperature of the injected steam within a shorter time, suggesting a faster process of heat transfer. However, small chips, whether green or air-dried, gave similar steam-exploded products as well as comparable yields of reducing sugars on enzymatic hydrolysis. It was concluded that the steam requirement for pretreating large aspen chips decreases with moisture content, whereas moisture content was not a crucial factor when smaller chips were used.

The relative importance of both the lignin and hemicellulose content of aspen chips, in the development of cellulose accessibility during steam pretreatment, has also been investigated by Schwald *et al.* (1988a,c). These authors selectively removed the lignin and hemicellulose prior to steam pretreatment by extraction with sodium chlorite and potassium hydroxide, respectively. Although the preparation of the low hemicellulose containing material from aspen chips resulted in the complete removal of the acetyl groups, steam pretreatment of this material resulted in cellulose hydrolysis yields equivalent to those obtained with the unextracted residue, indicating that effective pretreatment occurred in the absence of acetic acid. However, the probable effect of other acids such as formic, sulphonic and levulinic acids was not investigated. The selective removal of much of the lignin indicated that short steaming times were enough to produce *in situ* hydrolysis of hemicelluloses, resulting in a low hemicellulose content residue with an increased accessibility to cellulases.

1.2.1.3. Effect of Steam Pretreatment on the Morphology and Fine Structure of Cellulosic Materials

Scanning electron microscopy (SEM) has been of considerable assistance in evaluating the physical modifications caused by steam on the structure of wood tissues (Brownell and Saddler, 1987). The effectiveness of pretreatment was shown to correlate well with the development of substrate accessibility to

cellulases. The high pressure steaming of wood materials ruptures the wood cell wall structure thus exposing its inner layers to enzyme attack. Lignin fragments present in the water-insoluble fractions were observed as brown clumps attached loosely to the cellulose macrofibrils.

Grethlein (1985) reported that the initial rate of hydrolysis of both hardwood and softwood substrates was linearly correlated with the pore volume of the substrate which was accessible to a probe with a nominal diameter of 5.1 nm. This method was originally developed by Stone *et al.* (1969) and is based on the utilization of probes that have dimensions similar to those of the individual enzyme components of the cellulase system. The lower susceptibility of softwood substrates to hydrolysis, compared to hardwood substrates, was associated with a smaller increase in accessible pore volume during pretreatment. Although pretreatment in this previous work was carried out by mild acid hydrolysis, the development of micropores with an effective diameter wider than 5.1 nm was also demonstrated to be a key factor for the enhancement of substrate accessibility during steam pretreatment of aspen (Excoffier *et al.*, 1991), poplar (Grous *et al.*, 1986) and radiata pine (Wong *et al.*, 1988a) chips. However, whether the cellulase complex can be adequately represented by globular probes with a diameter of 5.1 nm has yet to be demonstrated. Drying was also shown to be detrimental to hydrolysis as it results in a dramatic reduction of the available surface area of the substrate (Saddler *et al.*,

1982; Grous *et al.*, 1986).

A major change in the structure of the cellulose has been demonstrated after steam explosion of cellulosic residues. Several authors have reported a gradual decrease in the degree of polymerisation of cellulose and a substantial increase in the crystallinity index (CrI) of the substrate (Puls *et al.*, 1985; Tanahashi *et al.*, 1989a; Miller *et al.*, 1989). The apparent increase in crystallinity appears to be the result of fusion between cellulose crystallites which were originally separated by a matrix of hemicellulose and lignin. Therefore, the gradual removal of hemicelluloses and lignin seems to trigger the reorientation of the cellulose molecules which, after pretreatment, assume a distinct crystalline form.

The steam explosion method has also been shown to improve the solubility of several cellulosic fractions in alkali (Yamashiki *et al.*, 1990a,b,c). A significant decrease in both crystallinity and degree of polymerisation of cellulose was reported with increasing severity of pretreatment. Changes in the crystallinity of the substrate were explained by a gradual reduction of intermolecular hydrogen bonding between the carbon atoms C-3 and C-6 of adjacent anhydroglucose residues.

1.2.2. Acid-Catalysed Steam Pretreatment

Previous work has shown that softwood species are far more difficult to pretreat than hardwoods. This phenomenon has been attributed to differences in their hemicellulose and lignin

structure and content. The unfavourable properties of softwoods have been largely overcome by the introduction of an acid catalyst prior to high-pressure steaming (Clark and Mackie, 1987; Schwald *et al.*, 1989b). Two main advantages have been attributed to steam pretreatment at low pH; a high recovery yield of pentoses in the water-soluble fraction and a better susceptibility of the steam-treated substrate to enzymatic hydrolysis. The former advantage is mostly associated with hardwoods, whereas the latter applies to both wood types. The higher hydrolysis and survival of hemicellulose sugars has been attributed to the higher stability of pentoses in acid solutions, maximum stability being in the pH range of 2.5 to 3.5 (Springer and Harris, 1982). Additional advantages of acid-catalysed high-pressure steaming include a reduction in both the temperature and residence time requirements for optimum pretreatment.

Among a variety of acid catalysts reported in the literature, sulphuric acid and sulphur dioxide gas (SO_2) are the most widely used. The best alternative appears to be SO_2 since it can be easily and evenly incorporated within lignocellulosic materials, avoiding the inconveniences which are caused by soaking with aqueous solutions of sulphuric acid (Brownell and Saddler, 1984; Mackie *et al.*, 1985; Brownell, 1989).

SO_2 catalysis results in better steam-treated substrates for enzymatic hydrolysis, as previously demonstrated for aspen and spruce (Schwald *et al.*, 1989a,b). However, the addition of an

acid catalyst during pretreatment also generally causes a substantial decrease in the degree of depolymerisation (DP) of cellulose (Miller *et al.*, 1989). The average number of anhydroglucosyl residues per cellulose molecule (DP) was shown to decrease from about 10^4 to 250. Comparable reductions on cellulose DP have been demonstrated during the mineral acid hydrolysis of cotton and other cellulosic materials (Wood *et al.*, 1986; Lauriol *et al.*, 1987; Kirk *et al.*, 1991). The acid-depolymerised residue derived from cotton (microcrystalline cellulose) was highly recalcitrant to further degradation and usually presented a high crystallinity index (CrI).

The mechanism of SO_2 catalysis has not been fully resolved. However, it has been established that SO_2 is not the actual catalyst but rather its oxidation product sulphuric acid (Brownell *et al.*, 1988). During high-pressure steaming, SO_2 is converted into sulphuric acid by oxidation and/or disproportionation (Brownell, 1989). Oxidation occurs in the presence of air or oxygen at mild temperatures, providing high yields of sulphuric acid. When disproportionation occurs in the absence of air, the oxidation of SO_2 to sulphuric acid is balanced by an equivalent SO_2 reduction to thiosulphate or elemental sulphur. The latter mechanism is undesirable since part of the SO_2 is lost as reduction products. Part of the SO_2 used for wood impregnation may also be lost either by incorporation into wood components (e.g., forming lignosulphonates) or evaporation (Mackie *et al.*, 1985). Release

of SO_2 into the atmosphere should be avoided for environmental and economic reasons. Therefore, pre-impregnation of wood before high-pressure steaming requires further investigation to reduce SO_2 losses and increase the recycling of the unconverted SO_2.

Previous work looking at the SO_2 impregnation of aspen has shown that better conversions of SO_2 into sulphuric acid can be achieved at low SO_2 concentrations (Brownell, 1989). Steam-treated substrates which can be completely hydrolysed using relatively low enzyme loadings could be produced under relatively mild steam pretreatment conditions with a high recovery yield of pentosan sugars. Although the rate of conversion started to level off at SO_2 loadings around 1% (w/w), slightly higher levels of 1.6% (w/w) have been used for aspen (Schwald *et al.*, 1989a), whereas loadings of 2.5% (w/w) have been used for softwood species such as spruce (Schwald *et al.*, 1989b) and radiata pine (Clark and Mackie, 1987).

1.3. Enzymatic Hydrolysis of Cellulose

The efficiency with which cellulose is hydrolysed depends on a multiplicity of factors involving the structural characteristics of the substrate and the nature of the enzyme system used. Before each of these subjects is discussed in more detail, it is important to consider which options are available for the process of cellulose bioconversion. In principle, this process can be organized either by the enzymatic saccharification of the substrate and subsequent fermentation of

the sugars released to chemicals such as ethanol or by the simultaneous saccharification and fermentation (SSF) of the substrate. The following sections will be mostly focused on the first route, whereas the potential of the SSF method has been documented by several authors (Szczodrack and Targonski, 1989; Spindler *et al.*, 1989, 1991). The SSF method is based on the assumption that sugars released from the substrate by enzymatic hydrolysis are rapidly fermented by yeasts or other microorganisms into ethanol. The action of cellulases on substrates may therefore proceed without any interference from high concentrations of sugars in the reaction mixture.

Most of the published work has utilized fungal cellulases for the saccharification of cellulosic residues. Of the cellulose degrading systems occurring in nature, wood decay by the soft rot fungi *Trichoderma* spp. is among those best characterized. Several preparations of *Trichoderma* cellulases are available commercially. These include the Celluclast from Novo-Nordisk, Genencor cellulases and Spezyme from Cultor. All of these preparations are very effective at hydrolysing pretreated cellulose preparations (Breuil *et al*, 1990).

1.3.1. Fungal Cellulases

Several fungal species have been investigated for their ability effectively to degrade cellulose, including *Trichoderma reesei*, *T. koningii*, *Fusarium solani*, *Talaromyces emersonii*, and *Penicillium funiculosum* (Coughlan and Ljundahl, 1988), the white

rot fungus *Phanerochaete chrysosporium (Sporotrichum pulverulentum)* (Eriksson and Wood, 1985), the thermotolerant fungus *Thielavia terrestris* (Gilbert *et al.*, 1992), and the anaerobic fungus *Neocallimastix frontalis* (Wilson and Wood, 1992).

Several authors have used electron microscopy to study the mechanisms whereby fungal systems effect hydrolysis of cellulosic substrates. Ruel *et al.* (1981) used transmission electron microscopy (TEM) to evaluate the micromorphological and ultrastructural aspects of wood degradation by the white rot fungus *S. pulverulentum* and its cellulase-less mutant. Eriksson *et al.* (1980) used scanning electron microscopy (SEM) to study the mode of growth and the effect of white rot decay on the structural organization of wood. Both electron microscopic techniques were shown to be helpful in the understanding of white rot decay by allowing visualization of structural changes which could then be correlated with the biochemical characterization of the process.

The extracellular enzyme systems produced by white and soft rot fungi are composed of three major enzyme components: endoglucanases (EC 3.2.1.4), exocellobiohydrolases (EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21) (see Fan *et al.*, 1987; Coughlan, 1985). Each one of these components is glycosylated, exists in multiple forms and seems to have a distinct range of activities on cellulosic substrates. Endoglucanases are generally characterized by their activity on amorphous cellulose

and other soluble derivatives such as carboxymethylcellulose (CMC). A random cleavage of β(1-4)-glycosidic bonds is observed with little release of reducing sugars. Exocellobiohydrolases generally attack the non-reducing end of the cellulose chains to produce cellobiose, therefore acting as typical exo-enzymes. Although these definitions are still generally used to group cellulases, recent studies have indicated that the substrate specificity of the various endo- and exoglucanases is a lot more complicated than this simple classification (Claeyssens *et al.*, 1990; Claeyssens and Aerts, 1992). Finally, β-glucosidases complete the hydrolytic process by catalysing the hydrolysis of cellobiose to glucose or by removing glucosyl residues from the non-reducing end of soluble cellooligosaccharides. It is not clear whether complete hydrolysis of insoluble cellulose substrates is possible only when all of the cellulase components are present. However, a great degree of cooperativity (synergism) is observed among β-glucosidases, endoglucanases and exoglucanases during hydrolysis of recalcitrant cellulose (Coughlan, 1985; Henrissat *et al.*, 1985; Beldman *et al.*, 1988).

Many of the fungal systems described in the literature are deficient in one or another component of the cellulase complex. For instance, brown rot fungi seem to lack the exoglucanase component (Coughlan, 1985). The cellulase system produced by *T. reesei* contains both the exocellobiohydrolase and endoglucanase components. However, β-glucosidase is present at lower levels (Claeyssens and Aerts, 1992). Therefore, the supplementation of

T. reesei cellulases with exogenous β-glucosidase activity has often been used to avoid cellobiose accumulation and consequently increase the rate and extent of enzymatic hydrolysis of cellulose (Tan *et al.*, 1987; Breuil *et al.*, 1990).

Kirk *et al.* (1991) studied the brown rot decay which *Postia placenta* (*Poria placenta*) effected on cotton cellulose. The cellulolytic system secreted was able gradually to reduce the average DP ($\overline{DP_w}$) of cotton cellulose from 2111 to 238. Although the fungal system took much longer to produce the same effect obtained by chemical means such as acid hydrolysis, both methods seemed to have the same limitations in hydrolysing cellulose residues with an average DP of 300 or less. These and other authors have suggested that the depolymerisation of native cellulose by brown rot fungi occurs through the initial degradation of the amorphous regions of the substrate and that this may be triggered by a non-enzymatic factor, such as hydrogen peroxide (Veness and Evans, 1989) or oxalic acid (Schmidt *et al.*, 1981). Recently, Kleman-Leyer *et al.* (1992) suggested that the mechanism of cellulose degradation differs fundamentally between brown rot (*P. placenta*) and white rot (*Phanerochaete chrysosporium*) fungi. The depolymerisation of cotton cellulose caused by the brown rot fungus generally occurred before any substantial weight loss was observed, whereas the white rot fungus was able to cause a considerable weight loss without having a substantial effect on the DP distribution of cellulose. These authors suggested that the

brown rot fungus preferably attacked the more amorphous regions of cotton cellulose forming crystallites. By contrast, the white rot fungus was able to attack both the crystalline and amorphous regions at the surface of the substrate, resulting in a progressive erosion rather than fragmentation.

Owing to the structural complexity of lignocellulose, its effective biodegradation requires some additional enzyme activities beside glucohydrolases. Oxidative enzymes, such as the cellobiose:quinone oxidoreductase produced by white rot fungi (Westermark and Eriksson, 1974), have been reported as a probable connection between the processes of cellulose and lignin degradation. A multiplicity of enzymes is also required for the degradation of complex heteropolysaccharides which constitute hemicelluloses (Wong *et al.*, 1988b). Therefore, the degradation of lignocellulose in nature is a complex process which is probably carried out by a consortium of microorganisms including fungi and bacteria.

Lignocellulose decay is not just carried out by fungal systems. Numerous bacteria also produce cellulases, such as the aerobes *Cellvibrio gilvus* (Breuil and Kushner, 1976), *Streptomyces flavogriseus* (Ishaque and Kluepfel, 1980), *Thermomonospora sp.* (Hagerdahl *et al.*, 1980), *Acetivibrio cellulolyticus* (Saddler and Khan, 1981), *Bacillus* sp. (Robson and Chambliss, 1984) and *Cellulomonas* sp. (Vladut-Talor *et al.*, 1986), and the anaerobes *Bacteroides succinogenes* (Hungate, 1950), *Clostridium thermocellum* (Johnson *et al.*, 1982) and

Ruminococcus sp. (Wood *et al.*, 1982). Cellulases derived from anaerobic bacteria have received special attention ever since the existence of a multicomponent cellulolytic complex, the "cellulosome", was first described (Lamed *et al.*, 1983). There is evidence that these complexes are located in the cell surface and that they mediate the adherence of these anaerobic bacteria onto cellulose.

1.3.2. Factors which Limit the Enzymatic Hydrolysis of Cellulose

A typical enzymatic hydrolysis profile of a cellulosic residue in a batch mode indicates a gradual decrease in the rate of hydrolysis of cellulose with time (see Fan *et al.*, 1987). As mentioned previously, this logarithmic decline in the hydrolysis rate depends on several factors associated with the nature of the substrate and the enzyme system used. Many authors support the hypothesis that it is the increased recalcitrancy of the substrate that gradually compromises the rate by which cellulose is hydrolysed (Lee and Fan, 1982, 1983). This increase in recalcitrancy may be reflected by structural modification of the substrate, such as changes in the morphology (particle size and pore volume), the crystallinity or the degree of polymerisation (DP) of cellulose.

1.3.2.1. Substrate-related Factors

As mentioned previously, several pretreatment methods are

capable of opening up the structure of cellulose, thus increasing the surface area (or pore volume) of the substrate accessible to cellulase enzymes. Several authors have demonstrated that these morphological changes have a remarkable effect on the susceptilibity of the pretreated substrate to hydrolysis (Grethlein, 1985; Wong *et al.*, 1988a; Grethlein and Converse, 1991; Excoffier *et al.*, 1991). The accessible surface area of the substrate has also been proposed as an essential parameter for the development of a kinetic model for the enzymatic hydrolysis of cellulose (Converse *et al.*, 1990).

For many years, several authors reported on the apparently rapid decrease in substrate particle size (fragmentation) during hydrolysis of various cellulosic residues (Liu and King, 1967; Fan *et al.*, 1980; Peters *et al.*, 1991). It was observed that, despite the long time required for total saccharification of the substrate, a significant reduction of particle size was achieved within a few hours of hydrolysis. This phenomenon was partly attributed to the action of endoglucanases and often implicated with the synergism observed between these enzymes and exocellobiohydrolases.

Although fibre fragmentation has been characterized in several systems using microscopy, there is very little direct evidence supporting its occurrence. Oltus *et al.* (1987) reported a cross-wise shortening of fibres during hydrolysis of waste paper using high loadings of fungal enzymes. Peters *et al.* (1991) studied the effect of bacterial cellulases on the

distribution of particle size of microcrystalline cellulose (Avicel PH 102). There was little correlation between substrate particle size and hydrolysis rate, suggesting that the binding of cellulases was not influenced by reductions in particle size. Large particles were rapidly fragmented, whereas erosion rather than fragmentation was the predominant mechanism of degradation of small particles.

Several studies have demonstrated that there is no significant correlation of variations in the degree of polymerisation of cellulose and the development of substrate accessibility to enzymes (Puri, 1984; Sinitsyn *et al.*, 1991). Most of these studies have utilized viscosimetric assays to determine the DP of cellulosic fractions (Puri, 1984). However, this method is restricted to the determination of the average DP of a population of cellulose molecules. Therefore, an alternative method has to be used if a degradative process such as enzymatic hydrolysis is to be characterized. The GPC method, which consists of analyzing the molecular weight distribution of cellulose by gel permeation chromatography (GPC) of its tricarbanyl derivative, has been routinely used (Lauriol *et al.*, 1987; Miller *et al.*, 1989; Miller *et al.*, 1991). In most literature reports, the preparation of the tricarbanyl derivative involves precipitation in methanol or water in order to purify it. However, this procedure can lead to a substantial loss of oligosaccharides with low DP (Wood *et al.*, 1986). To overcome this problem, Miller *et al.* (1991) obtained the

tricarbanyl derivative from several pretreated fractions by simple evaporation of the reaction solvents.

In the earlier work of Reese *et al.* (1950), it was suggested that the crystalline regions of cellulose are less susceptible to hydrolysis than the amorphous regions (see also Bertran and Dale, 1985). The discussions around this hypothesis have usually been supported by the complex polymorphism of pretreated cellulosic substrates and the different reactivity of each polymorph to cellulolytic enzymes. In fact, a linear correlation of the degree of crystallinity of cellulose and its enzymatic saccharification has been reported for several pretreated substrates with varying crystallinity index (Fan *et al.*, 1980; Lee and Fan, 1982; Puri, 1984; Sinitsyn *et al.*, 1991). However, other authors have suggested that the relationship of the amorphous character of cellulose and its digestibility is not a causal one and that the increased surface area of cellulose available to enzyme molecules may be of a greater importance (Caulfield and Moore, 1974; Rivers and Emert, 1987). It seems that the apparent controversy regarding the effect of cellulose crystallinity on hydrolysis is partly due to technical difficulties in measuring crystallinity (Sidiras *et al.*, 1990).

Chanzy and Henrissat (1985) applied transmission electron microscopy (TEM) to study the enzymatic breakdown of cellulose microcrystals from the alga *Valonia macrophysa* by purified *T. reesei* cellulases. It was shown that the concerted action of

exo- and endocellulases caused a reduction in both the width and length of the microcrystals. These authors suggested that the enzymatic degradation of bacterial cellulose occurred preferably at one plane of the crystallite and that CBH II was responsible for an unidirectional attack at the end of the crystallite.

The effect of enzymatic hydrolysis on the fine structure of cellulose has also been reported for several cellulosic residues derived from cotton linters, wheat straw, sugar cane bagasse and hardwood sawdust (Betrabet and Paralikar, 1977, 1978). Hydrolysis resulted in a gradual increase in the crystallinity index of all substrates, as indicated by X-ray diffraction. There was an apparent decrease in the microfibril width of pretreated fractions derived from bagasse, wheat straw and hardwood sawdust, whereas hydrolysis had practically no effect on the crystallite dimensions of pretreated cotton.

1.3.2.2. Enzyme-related Factors

Although some substrate related factors could partially explain why there is a rapid decrease in the efficiency of hydrolysis of cellulose, several factors associated with the nature of the enzyme system used have also been suggested as being key to the cellulose hydrolysis profile. These include the end-product inhibition of the cellulase complex (Saddler, 1986; Ladish *et al.*, 1983), the irreversible adsorption or non-specific binding of the enzyme owing to an increased recalcitrancy of the substrate (Clesceri *et al.*, 1985), the

changes in the specific surface area accessible to protein molecules (Grethlein, 1985; Wong *et al.*, 1988a; Sinitsyn *et al.*, 1991; Thompson and Chen, 1992), the reduction of the enzyme activity by agitation (Mukataka *et al.*, 1983), and the thermal inactivation of the enzymes (Eklund *et al.*, 1990). It is therefore reasonable to assume that no single factor can satisfactorily explain why cellulosic substrates are not as effectively hydrolysed as other polysaccharides such as starch.

Klyosov (1990) suggested that a complete cellulase system should be composed primarily of two components, one tightly and the other loosely adsorbed to the substrate. This hypothesis agreed with the general observation that most of the complete cellulase systems described in the literature, i.e., those able effectively to degrade crystalline cellulose, contain at least one enzyme component which adsorbs tightly to the substrate. The mechanism by which the adsorbed enzymes (endoglucanases or cellobiohydrolases) promote the enzymatic degradation of cellulose is yet to be determined. However, it seemed that the enzymes, once adsorbed, were able to perform hundreds of catalytic acts before they were released from the surface of the substrate. These ideas have led to a new definition of synergism, based on the cooperativity between two components with distinct adsorption characteristics.

Earlier work suggested that endoglucanases were preferably adsorbed onto the substrate, whereas exoglucanases were mostly found in the supernatant. However, it has now been demonstrated

by several authors that it is the exo-acting component (cellobiohydrolase) of the cellulase system which adsorbs more tightly to the substrate, as compared to other components such as endoglucanases and β-glucosidases (Tan *et al.*, 1986; Ooshima *et al.*, 1991). Castanon and Wilke (1980) showed that, during the early stages of hydrolysis of newspaper, exoglucanases were more strongly adsorbed than endoglucanases, although later this situation was reversed. Tan and coworkers (1986) suggested that the gradual loss of the synergistic interaction between exo- and endoglucanases was one of the key factors which limited hydrolysis efficiency during continuous saccharification of microcrystalline cellulose. A gradual leaching of loosely-adsorbed components (endoglucanases) of the cellulase system was observed during continuous operation of the column reactor. Ooshima *et al.* (1991) also explained that the rapid drop in the rate of hydrolysis of Avicel in a batch mode was intimately associated with a gradual decrease in the specific activity of the adsorbed enzyme. Owing to variations in the adsorption profile of both endo- and exoglucanases during hydrolysis, the progressive decline in the synergistic interaction between these enzymes resulted in a drop of the hydrolysis rate. Synergism was shown to be more influential than other factors such as end-product inhibition and increased recalcitrancy of the substrate.

The adsorption profile of purified endo- and exoglucanase on pretreated aspen has also been characterized by TEM visualization of their monoclonal antibody - colloidal gold

conjugates (Nieves *et al.*, 1991). A preferential attack by endoglucanases (EG I) was observed on the apparently amorphous regions of the substrate, whereas exoglucanases (CBH I) were observed in association with both amorphous and crystalline regions. It was also observed that both enzymes formed aggregates on the surface of the substrate. Some degree of co-localization of these aggregates was also reported, giving additional evidence for the high degree of synergism observed between these enzymes.

End-product inhibition has been recognized as one of the factors which limit hydrolysis of cellulosic substrates (Saddler, 1986). Several methods have been proposed to overcome the difficulties caused by the rapid accumulation of sugars during hydrolysis. This includes, the use of exceedingly high loadings of enzymes (Dekker *et al.*, 1987; Ishihara *et al.*, 1991), the elimination of sugars from the hydrolysate by ultrafiltration (UF) (Ishihara *et al.*, 1991; Tan *et al.*, 1986) or the simultaneous saccharification and fermentation (SSF) of the substrate (Saddler *et al.*, 1982; Mes-Hartree *et al.*, 1987; Szczodrack and Targonski, 1989) and, the supplementation of cellulases with β-glucosidase (Tan *et al.*, 1987; Breuil *et al.*, 1990; Breuil *et al.*, 1992). The choice of enzyme concentrations in an enzymatic hydrolysis process must be based on several economic considerations. Therefore, the use of high loadings of enzyme for hydrolysis is prohibitive by definition (Nguyen and Saddler, 1991). The utilization of ultrafiltration to eliminate

soluble sugars, which has also the inconvenience of being expensive, is further complicated by the repeated obstruction of membranes with lignin-containing residues, particularly when used in on-line applications. The SSF method still presents some technical difficulties owing to differences between the optimal conditions required for hydrolysis and fermentation. Furthermore, it is not clear whether inhibitory by-products may be continuously released in the mixture, thus affecting either hydrolysis or fermentation of the substrates. Finally, the addition of β-glucosidase to the hydrolysis mixture helps in preventing the accumulation of cellobiose to inhibitory levels.

It would obviously be desirable to establish an accurate method by which the minimal enzyme requirements for hydrolysis of natural substrates could be determined. However, the minimal loading for hydrolysis at varying substrate concentrations has not yet been established. In a recent paper, Hogan *et al.* (1990) have shown that enzyme loadings approaching substrate saturation levels are effective in hydrolysing commercial cellulose preparations such as Solka-floc. Three methods were used to establish the minimal amount of enzyme needed for hydrolysis: (a) the initial adsorption of cellulases (Lee *et al.*, 1982; Wald *et al.*, 1984), (b) the supplementation of the hydrolysis mixture with exogenous β-glucosidase activity (Chan *et al.*, 1989), and (c) the simultaneous saccharification and fermentation of the substrate (Saddler *et al.*, 1982). A good correlation among minimal loading values was observed using these techniques. The

adsorption method appeared to be the most practical method, giving results which were determined independently of influential factors such as thermal inactivation of the enzyme or the release of inhibitory compounds during hydrolysis and/or fermentation. However, adsorption of enzymes onto pretreated lignocellulose may involve a more complex mechanism in that variables such as enzyme inactivation by irreversible adsorption on lignin-containing residues could play a role.

1.3.3. Enzymatic Hydrolysis of Steam-treated Substrates

The enzymatic hydrolysis of cellulose has been investigated using a wide variety of cellulosic substrates, ranging from commercial preparations such as Solka-floc (Hogan *et al.*, 1990) and Avicel (Ooshima *et al.*, 1991) to more crude substrates such as steam-treated biomass (Converse *et al.*, 1990). In the previous section, several factors involved in the process of enzymatic hydrolysis of cellulose have been described. Although many of these observations also apply to the hydrolysis of steam-treated substrates, additional complications are introduced by the high lignin content of these fractions. In this section, the technical difficulties of hydrolysing pretreated substrates will be described.

The optimization of steam pretreatment, as well as other forms of pretreatment, have usually been monitored by enzymatic hydrolysis of resultant substrates. The enzymatic hydrolysis of steam-treated residues is most effective at low substrate

concentrations such as 2% (w/v). However, the low concentration of fermentable sugars obtained in the hydrolysis reaction results in poor fermentation economics. Unfortunately, the rate by which cellulose is hydrolysed, using *Trichoderma* cellulases (Celluclast, Novo), decreases dramatically at high substrate concentrations (Breuil *et al.*, 1990). At substrate concentrations above 6% (w/v), a substantial accumulation of cellobiose is observed early in the hydrolysis process. This indicates the lack of sufficient cellobiase activity to maintain the cellobiose concentration in the reaction mixture at non-inhibitory levels. However, other limiting factors such as enzyme inactivation may also be involved. Several groups have utilised high enzyme loadings to achieve better saccharification yields at high substrate concentrations (Dekker *et al.*, 1987; Ishihara *et al.*, 1991). However, this was shown to be undesirable as it increases the cost of the process significantly. Differences with regard to the efficiency of hydrolysis of different commercial cellulases may also influence the enzyme loading required to hydrolyse these substrates.

A comparative study has already been carried out to assess the hydrolytic potential of different commercial cellulase preparations (Breuil *et al.*, 1990). Steam-treated substrates derived from SO_2-impregnated aspen and spruce chips were used in this study at different substrate and enzyme loadings. No significant difference was observed among the preparations tested, provided that the hydrolysis reactions were initially

adjusted to a common level of 40 CBU g^{-1} of β-glucosidase (Novozym, Novo). Other work has demonstrated that Celluclast has a higher hydrolytic potential than cellulases from a *T. harzianum* E58 crude extract, particularly at high substrate concentrations (Chan *et al.*, 1989). The effect of adding increased concentrations of glucose and cellobiose to the reaction mixture showed that the commercial enzymes were also less sensitive to end-product inhibition than the *T. harzianum* cellulases.

A substantial inhibition of both cellulase and β-glucosidase activities by furfural, hydroxymethylfurfural and pentosan-derived trihydroxybutyric acids has been demonstrated (Excoffier *et al.*, 1991). Gluconolactones have also been identified as inhibitors of β-glucosidase activity (Dekker, 1986). According to earlier reports, most of the hydrolysis and fermentation inhibitors which are produced during steam pretreatment of lignocellulose can be removed by water washing the substrates (Brownell and Saddler, 1987; Brownell, 1989). However, inhibitory by-products may also be gradually released from the substrate during hydrolysis (mostly sugars and lignin fragments), causing partial inactivation of the enzymes. The negative effects of these factors are obviously more pronounced at higher substrate concentrations.

The effect of alkali washing on the enzymatic hydrolysis of steam-treated substrates has been assessed primarily by looking at the structural changes of the substrate after alkali

extraction and its effects on hydrolysis efficiency. Ucar and Fengel (1988) suggested that, during hydrolysis of pretreated substrates, the lignin component acted as a barrier which hindered the physical contact between the substrate and the enzymes. Factors such as the irreversible adsorption and non-specific binding of enzymes onto the lignin-containing residue have often been advocated (Clesceri *et al.*, 1985; Converse *et al.*, 1990). Therefore, the reduction of the lignin content of pretreated residues by alkali extraction seemed to be desirable as it would result in a substrate with a comparatively higher cellulose content from which a higher glucose yield per gram of substrate could be achieved during enzymatic saccharification.

Alkali washing of steam-treated hardwoods, such as *P. tremuloides* (aspen), resulted in a substrate with low lignin content which was as readily hydrolysed as was the water-washed substrate (Schwald *et al.*, 1989a). Excoffier *et al.* (1991) also reported that the alkali extraction of steam-treated poplar did not interfere with the hydrolyzability of the pretreated substrate. These results were partially contradictory to previously reported data on the enzymatic hydrolysis of steam-treated *E. regnans* (Dekker *et al.*, 1987). A considerable decrease in the accessibility of the steam-treated substrate was reported after alkali washing, even though a substantial fraction of the lignin was extracted. For the steam pretreatment of softwood species, alkali-washing generally resulted in substrates which were more recalcitrant to enzymatic degradation

than were the respective water-washed substrate (Wong *et al.*, 1988a; Schwald *et al.*, 1989b). This was more dramatic in the case of spruce where alkali extraction seemed to redeposit the lignin in a fashion which greatly reduced the ease of hydrolysis (Schwald *et al.*, 1989b). Extraction of the residual alkali-insoluble lignin by oxidative agents, such as sodium chlorite and hydrogen peroxide, was shown to increase the susceptibility to enzymatic hydrolysis of pretreated substrates derived from both hardwood and softwood residues (Saddler *et al.*, 1982; Schwald *et al.*, 1989a,b). These authors suggested that it was the redistribution of this residual alkali-insoluble lignin which limited complete hydrolysis of alkali-washed substrates. After alkali washing, this highly condensed, modified lignin appeared to reprecipitate on the surface of the substrate, causing a reduction of both the available surface area and the ability of the cellulose fibres to swell in water.

1.3.4. Recycling of Cellulases

The cost of enzymes used for saccharification of cellulosic residues is still the most expensive part of an overall bioconversion process based on enzymatic hydrolysis (Nguyen and Saddler, 1991). Therefore, any reduction in the enzyme requirements for hydrolysis is desirable. Several studies have been reported on the use of recovered enzymes for saccharification of pretreated substrate (Clesceri *et al.*, 1985; Vallander and Eriksson, 1987; Mes-Hartree *et al.*, 1987; Ooshima

et al., 1990; Ishihara *et al.*, 1991; Singh *et al.*,1991). However, most of these investigations were carried out using very heterogeneous substrates (high lignin content) and exceedingly high enzyme loadings. Furthermore, the possible reuse of the enzymes for subsequent hydrolysis steps was not clearly demonstrated. Some authors suggested that recycling of cellulases proceeds better if lignin is first removed from the substrate (Clesceri *et al.*, 1985; Tanaka *et al.*, 1988; Ooshima *et al.*, 1990). Other authors suggested that enzymes can not be effectively recovered from the hydrolysis mixture until appreciable activity is found in the supernatant (Clesceri *et al.*, 1985). Partial adsorption of both endoglucanases and β-glucosidases on lignin has been previously demonstrated (Ooshima *et al.*, 1983; Sutcliffe and Saddler, 1986). However, when these enzyme activities were tested in the presence of several lignin preparations, it was the β-glucosidase activity that was more strongly inhibited (Sutcliffe and Saddler, 1986; Excoffier *et al.*, 1991).

Several authors have tried to identify the enzymes which are preferentially adsorbed and subsequently gradually desorbed from the substrate during hydrolysis (Moloney and Coughlan, 1983; Tan *et al.*, 1987; Mes-Hartree *et al.*, 1987). Owing to the strong affinity of several enzyme components for the substrate, only a partial recovery of the cellulase enzymes was obtained. Reese (1982) used a wide range of desorbing agents to recover the enzymes from partially hydrolysed residues and concluded

that, although it was possible to recover most of the enzymes, the rate of desorption appeared to be proportional to the degree of inactivation. The recovery of cellulases present in the hydrolysis mixture through contact with fresh substrate has also been described (Castanon and Wilke, 1980; Sinitsyn *et al.*, 1983; Vallander and Eriksson, 1985). Although a partial recovery of desorbed enzymes was demonstrated, more than half of the initial activity of the system remained associated with the unhydrolysed residue and could not be easily recovered (Vallander and Eriksson, 1985). Therefore, these authors developed a model for reuse of bound enzymes (Vallander and Eriksson, 1987). However, it was not clear from this preliminary investigation whether the recovered enzymes were effective in continuously hydrolysing several batches of fresh substrates.

1.4. Experimental Approach

Several groups have described steam explosion as a very cost effective method for pretreating and fractionating lignocellulosic residues. This method has already been established for several hardwood and agricultural residues and its applications have also been extended to the more recalcitrant softwood residues. It has been suggested that the wood residues derived from young trees are generally more easily and readily fractionated during pretreatment, yielding better substrates for hydrolysis. Therefore, the fast growing rates and short rotation times which can be obtained with several

Eucalyptus sp. suggest a potential application of these species for bioconversion, particularly in the southern hemisphere. Due to the increasing utilization of *Eucalyptus* spp. for pulp and paper production, residues from the processing of this feedstock will also be readily available at a relatively low cost.

Several workers have looked at steam explosion of wood and agricultural residues and the influence of acid impregnation on the recovery yield and enzymatic hydrolysis of pretreated materials. SO_2 impregnation was shown to be beneficial for hardwoods and essential for softwoods. However, the influence of the initial moisture content of the wood chips on the efficient uptake of the SO_2 catalyst has not been previously investigated. In this proposed work, we have determined how parameters such as the steam temperature, residence time, addition of an acid catalyst, and initial moisture content can be adjusted to obtain optimum recovery and fractionation of eucalyptus chips while producing a cellulosic residue with high susceptibility to hydrolysis at high substrate concentrations and low enzyme loadings.

Although considerable progress has been made in clarifying the mechanisms involved in the hydrolysis of pure cellulosic substrates such as Solka-floc or Avicel, relatively few studies have utilized more practical substrates such as steam-treated biomass. For instance, the influence of the lignin component on both the hydrolysis rate and enzyme recycling has yet to be fully characterized. There is some evidence that enzyme

recycling is facilitated by lignin removal but it was not clear whether one batch of enzymes could be effectively used to hydrolyse fresh substrate. It has also been proposed that the alkali extraction of lignin does not interfere with hydrolysis of pretreated hardwoods and that oxidative post-treatments such as alkaline hydrogen peroxide are required to increase the accessibility of steam-treated softwoods. By studying the influence of delignification on the enzymatic hydrolysis of steam-treated eucalyptus, we have obtained a better understanding of the role of lignin during the process of enzymatic hydrolysis of cellulose.

Although there have been some recent studies on the effects of enzymatic saccharification on the morphology and fine structure of cellulose, it seems that the mechanism of enzymatic degradation of cellulose is still far from being fully understood. For instance, the gradual decrease in hydrolysis rate has often been associated with increases in substrate recalcitrancy during hydrolysis. However, this involves several factors such as lignin accumulation and redistribution, increased cellulose crystallinity and reduction of the substrate available surface area due to a partial coating of the residual substrates with irreversibly adsorbed enzymes and/or lignin. Some of these factors can be studied at the fibre level, whereas others have to be directed to the fine structure of cellulose (molecular level). At the fibre level, we have studied the rapid fragmentation of various fibre preparations by looking at

changes in fibre length during hydrolysis. At the molecular level, we have determined the effects of enzymatic hydrolysis on the crystallinity and degree of polymerisation of cellulose.

The objective of this study was to investigate whether the young stemwood of *E. viminalis* would be more easily steam-treated and fractionated than other species such as aspen and spruce with regard to recovery yield of pretreated materials and enzymatic hydrolysis of the cellulosic component. As this was shown to be the case, substrates derived from *E. viminalis* chips were subsequently used to identify those substrate- and enzyme-related factors which limited hydrolysis and influenced the mode of action of the enzymes.

2. MATERIALS AND METHODS

2.1. MATERIALS

Four shipments of 6 year old logs of *Eucalyptus viminalis* Labill were kindly supplied by the Brazilian Institute for Agricultural Research, Embrapa ("Empresa Brasileira de Apoio à Pesquisa Agropecuária", Colombo, Paraná, Brazil). Variations in the moisture content of the chips were attributed to differences in the conditions encountered during their shipment.

The fully bleached kraft pulp derived from eucalyptus chips was kindly supplied by the Pulp and Paper Research Institute of Canada (Paprican, Vancouver, B.C.). This pulp was originally prepared by Aracruz Florestal (Brazil) using an unspecified chlorine - chlorine dioxide sequence.

The commercial cellulose preparations used in this study, Sigmacell and Solka-floc AS1040, were purchased from Sigma Chemicals Co. (St. Louis, Missouri) and from Brown and Co. (New Hampshire). Tetracycline and benzene isocyanate were also supplied by Sigma Chemicals Co. Analytical grade samples of carbohydrates (glucose, xylose, arabinose and cellobiose) used to prepare HPLC standards were purchased from BDH Chemicals (Toronto, Ontario). BDH Chemicals was also the supplier for all the solvents and reagents used in this study, such as methanol, acetone, *t*-butanol, hydrogen peroxide, pyridine, sodium hydroxide, glacial acetic acid and sodium acetate.

Enzymatic hydrolyses of cellulosic substrates were

performed using Celluclast, a commercial cellulase preparation from *T. reesei*, and Novozym, a β-glucosidase preparation from *A. niger*. Both enzyme preparations were obtained as gifts from Novo Nordisk, Denmark.

2.2. METHODS

2.2.1. Substrate Preparation

Logs of *E. viminalis* were debarked and chipped using a commercial chipper at Forintek Canada (Ottawa, Ontario). Small chip sizes (2.5 x 1.5 x 0.5 cm) were chosen to allow easy penetration of the steam, thus facilitating the process of heat transfer during pretreatment. The moisture content of each batch of chips and pretreated substrates was determined in triplicate by oven drying the sample specimens at 105°C until constant weigth. The maximum coeficient of variation obtained with these results was 1.5%. Chips with a moisture content ranging from 13-100% (w/w) (*i.e.*, from air-dried to green conditions) were steam-treated using a 2 L steam gun (Mackie *et al.*, 1985). Saturated steam temperatures ranging from 200-240°C were used over a range of residence times.

Steam treatment of eucalyptus chips was assessed in three series of experiments in which the influence of parameters such as temperature, residence time, acid catalysis and moisture content were evaluated. These series are described below: (I) steam treatment of wood chips in the absence of an acid catalyst at temperatures ranging from 220 to 240°C for a fixed residence

time of 120 s; (II) steam treatment of pre-impregnated wood chips (1% SO_2, w/w) at temperatures ranging from 200 to 220°C for a fixed residence time of 50 s; (III) steam treatment of pre-impregnated wood chips (as in series II) at 210°C for residence times ranging from 50 to 150 s. Some pretreatment conditions were performed in triplicate to assess the reproducibility of the results. For each of the replications, the recovery yield of pretreated fractions had a maximum coeficient of variation of 2.5%. Optimal pretreatment conditions were considered those in which the best substrate for hydrolysis was obtained with the least amount of soluble sugars lost by degradation reactions.

Steam treatment was usually performed using a canister which had been loaded with eucalyptus chips prior to placing it in the steam gun. In these experiments, the high pressure developed during steaming was bled down to atmospheric after closing the steam inlet valve. In some cases, raw material was loaded directly into the steam gun and the steam-treated chips were released by rapid decompression of the vessel, causing the material to expand (explode) into a stainless steel cyclone. Substrates were then recovered by water washing. For the experiments where the steam was bled down to atmospheric pressure, the pretreated fractions were designated as STE (steam-treated eucalyptus), whereas SEE (steam-exploded eucalyptus) was used to indicate those experiments where explosion was used.

SO_2 impregnation was achieved by passing anhydrous SO_2 gas

into a plastic bag containing eucalyptus chips. SO_2 uptake was determined by the difference in total plastic bag weight before and after SO_2 addition and expressed in terms of oven-dry wood weight. An SO_2 concentration of 1% (w/w) in relation to the chips dry weight was used throughout this work. SO_2-impregnated substrates were given an SO_2-prefix, such as SO_2-STE or SO_2-SEE. The resulting STE or SO_2-STE substrates were broken up in a blender and extracted twice with water at a 5% (w/v) solids concentration. The water-insoluble fraction was termed STE-WI (or SO_2-STE-WI), whereas the water-soluble fraction or liquor was termed STE-WS (or SO_2-STE-WS). Recovery yield of the water-soluble (WS) fractions was determined by evaporating an aliquot to dryness and expressing the amount in terms of 100 g of the original oven-dry wood. Although the pretreated material obtained by steam explosion (SEE) did not require blending owing to the mechanical effects of explosion on the chips, both the water-insoluble and water-soluble fractions were obtained as described above and designated accordingly.

The carbohydrate content of both the STE-WS and SEE-WS fractions was determined by HPLC (see section 2.2.2) after acid hydrolysis with 1 M trifluoroacetic acid (TFA) for 3 h at 105° C. In the experiments where SO_2 was used as an acid catalyst, the HPLC analysis of WS fractions did not require such a prehydrolysis step because most of the water soluble sugars were recovered as monosaccharides.

The alkali-insoluble fraction, designated as WIA, was

obtained by extracting the water-washed steam-treated material twice with 0.1 M sodium hydroxide at a 5% (w/v) consistency. The alkali treatment was carried out for 1 h at room temperature (RT) with a stirring speed of 800 rpm. After removal of the extract by filtration, the insoluble residue was neutralized using aqueous acetic acid, washed thoroughly with deionized water and drained until a moisture content of 75-80% (w/w) was achieved. The alkali-washed substrates were termed STE-WIA (or SEE-WIA) and SO_2-SHE-WIA (or SO_2-SEE-WIA).

The peroxide-treated fraction, designated by the suffix WIA-H_2O_2, was obtained from the alkali-washed, steam-exploded substrate by treating it with an alkaline solution (pH 11.5) containing 1% (w/w) of hydrogen peroxide for 12 h at RT (Gould, 1984). The material was then neutralized using aqueous acetic acid, washed extensively with water and drained until a moisture content of 75-80% (w/w) was obtained. The resulting substrate, named SO_2-SEE-WIA/H_2O_2, was stored at 4°C.

Acid-swollen cellulose was prepared from Sigmacell (Sigma Chemicals Co.), a microcrystalline cellulose preparation, by treating it with 85% (w/w) phosphoric acid at a 2% (w/v) cellulose concentration in an ice bath for 1 h with constant stirring. After complete solubilization, the acid-swollen cellulose was carefully precipitated in cold water and collected by centrifugation. The fine residue was washed with 1% (w/v) sodium carbonate until neutrality and subsequently washed several times with deionized water. The residue was resuspended

in water, solvent exchanged with *t*-butanol and freeze-dried for further storage.

2.2.2. Substrate Analysis

The Klason lignin (acid-insoluble lignin) content of the cellulosic substrates was determined according to TAPPI Standard Method T222 os-74. Approximately 1 g of the oven-dry material was treated with 15 mL of a 72% (w/v) sulphuric acid solution (24 ± 0.1 N) for 2 h at 20 ± 1°C. During this pre-hydrolysis step, the mixture was constantly stirred with a glass rod to allow better solubilization of the cellulose component. The acid mixture was then carefully diluted to approximately 3% (w/v) concentration of sulphuric acid and boiled for 4 h using a reflux condenser. After refluxing, the total hydrolysate was filtered through a pre-weighed filtering crucible (medium coarseness). After the acid-insoluble material (lignin) was quantitatively collected in the filtering crucible, the residue was washed with hot water. The crucible containing lignin was dried at 105°C to a constant weight. The lignin content of the substrate was then calculated as a percentage of the original weight of the test specimen. Acid-soluble lignin was determined by TAPPI Useful Method 250.

The carbohydrate content of the substrate was determined from the acid hydrolysate of a Klason lignin determination using HPLC (Irick *et al.*, 1988) as described below. Due to a partial degradation of sugars during acid hydrolysis, both glucose and

xylose concentrations obtained by HPLC were corrected by a factor of 1.055 and 1.155, respectively. The cellulose (or xylan) content of the substrate was expressed as the total amount of anhydroglucose (or anhydroxylose) released during acid hydrolysis in relation to the dry weight of the substrate.

The chromatographic analysis of sugars liberated during both pretreatment and hydrolysis was carried out in a Waters 625 LC system, using an HPX-87H column (Bio-Rad) operated at 65°C. A 0.1 M sulphuric acid solution was used as the mobile phase, with a flow rate of 0.6 mL min^{-1}. The eluted sugars were detected using a Waters 410 differential refractometer.

The chemical analysis of each of the pretreated substrates was always done in triplicate. A maximum coeficient of variation of 2.1% was observed for all Klason lignin determinations carried out in this study, whereas the carbohydrate content of the substrates had a maximum coeficient of variaton slightly higher (2.9%).

2.2.3. Enzymatic Hydrolysis

The hydrolysis of pretreated substrates was initially carried out at a 2% (w/v) substrate concentration using a mixture of Celluclast and Novozym containing 10 filter paper unit (FPU) and 30 cellobiase units (CBU) per gram of cellulose in the substrate. These hydrolysis conditions were considered appropriate because the amount of material required for each assay was reduced. Furthermore, a better view of the substrate

accessibility was likely to be obtained at enzyme loadings which were below substrate saturation. Once the best substrate for hydrolysis was selected, the effects of substrate concentration and enzyme loading, together with other factors such as end-product inhibition and enzyme recycling, were investigated.

2.2.3.1. Determination of Enzyme Activity: the Filter Paper and Cellobiase Assays

All enzymatic activities were determined in the enzyme mixture according to the modified IUPAC (International Union for Pure and Applied Chemistry) procedure (Ghose, 1987; Chan *et al.*, 1989). The overall activity of the cellulase preparations was determined by measuring the ability of the enzymes to degrade filter paper. The amount of sugars released from filter paper during the filter paper assay was determined by HPLC (Schwald *et al.*, 1988b; Chan *et al.*, 1989). Several dilutions of the enzyme were prepared in a total volume of 1.0 mL of 50 mM sodium acetate buffer, pH 4.8. After an additional 1.0 mL of buffer was added, the tubes were mixed and pre-incubated in a 50°C water bath for 2 min. A 70 ± 0.5 mg strip of Whatman No. 1 chromatography paper was carefully added to each tube and the tubes were incubated for 1 h at 50°C. After incubation, the tubes were boiled for 10 min after they were covered with a marble to avoid excessive evaporation. After cooling, the reaction mixture was centrifuged at 2000 rpm for 2 min. The supernatant was then filtered through a 0.45 μm chromatographic

filter and analysed by HPLC using an HPX-87H column (Bio-Rad). Three dilutions were generally used to calculate the activity of the enzyme preparations. The glucose equivalents obtained for each of these dilutions varied only slightly from 1.33 mg mL^{-1} (Chan *et al.*, 1989). One international unit (filter paper unit, FPU) was defined as the activity required to release 1 µmol of glucose from filter paper per mL per minute and was calculated according to the following equations:

$$FPU\,mL^{-1} = \frac{GlcEq\,(mg\,mL^{-1}) \cdot E_{dil} \cdot V_T\,(mL)}{t\,(min) \cdot 1\,\mu Mol_{Glc}\,(0.18\,mg) \cdot V_E\,(mL)} \qquad (1)$$

where

E_{dil}	enzyme dilution;
FPU mL^{-1}	filter paper units per millilitre;
Glc	glucose concentration measured by HPLC;
GlcEq	glucose equivalents;
t	duration of the assay (60 min);
V_E	volume of the enzyme dilution in which the filter paper activity was assayed (1 ml);
V_T	total volume of the filter paper assay.

The amount of glucose equivalents which was released during the filter paper assay was calculated using the following equation:

$$GlcEq\,(mg\,mL^{-1}) = Glc\,(mg\,mL^{-1}) + Cel\,(mg\,mL^{-1}) \cdot \frac{MW_{Glc}}{MW_{Cel}/2} \qquad (2)$$

where

Cel	cellobiose concentration measured by HPLC;
Glc	glucose concentration measured by HPLC;
GlcEq	glucose equivalents;
MW_{Glc}	molecular weight of glucose;
MW_{Cel}	molecular weight of cellobiose.

For the determination of cellobiase activity, 1 mL of a 1.0% (w/v) solution of cellobiose was added to 1 mL of several enzyme dilutions after pre-incubation. The tubes were mixed and incubated at 50°C for 30 min. After incubation, the tubes were covered with a marble and boiled for 10 min. The test specimens were then centrifuged at 2000 rpm for 2 min and filtered through a 0.45 µm filter. The liberated glucose was then determined by HPLC. Three dilutions were generally used to calculate the activity of the enzyme preparations. The glucose concentration obtained for each of these dilutions varied only slightly from 2.0 mg mL^{-1} (Ghose, 1987). One international unit (cellobiase unit, CBU) was defined as the activity required to release 1 µmol of glucose from cellobiose per mL per minute and was calculated according to the following equation:

$$CBU mL^{-1} = \frac{Glc\,(mg\,mL^{-1}) \cdot E_{dil} \cdot V_T\,(mL)}{t\,(min) \cdot 1\,\mu Mol_{Glc}\,(0.18\,mg) \cdot V_E\,(mL) \cdot 2} \qquad (3)$$

where

	CBU mL^{-1}	cellobiase units per millilitre;
	E_{dil}	enzyme dilution;
	Glc	glucose concentration measured by HPLC;
	t	duration of the assay (30 min);
	V_E	volume of the enzyme dilution in which the cellobiase activity was assayed (1 mL);
	V_T	total volume of the cellobiase assay.

When the enzymatic activities of 17 distict enzyme preparations, ranging from 0.17 to 7.05 FPU mL^{-1}, were analysed by linear regression, a R^2 value of 0.9812 (standard error of 0.2768) was obtained.

2.2.3.2. Substrate Hydrolysis

Enzymatic hydrolyses were performed at various substrate and enzyme concentrations. The amount of both filter paper and cellobiase activities of the enzyme preparation used for hydrolysis was always calculated in relation to the cellulose content of the substrate using to the following equation:

$$Ug^{-1} = \frac{UmL^{-1} \cdot V_{EA}(mL)}{S(g)} \qquad (4)$$

where,

S	substrate concentration used for hydrolysis, calculated in relation to the substrate dry weight;
U g^{-1}	units of enzymatic activity per gram of substrate; calculated either in relation to the dry weight or to the cellulose content of the substrate;
V_{EA}	aliquot of the enzyme preparation used for hydrolysis of the pretreated substrate.

In those hydrolysis experiments where a mixture of Celluclast and Novozym was used for hydrolysis, which corresponded to the final activity of the Celluclast/Novozym mixture. The substrates were hydrolysed in duplicate at 45°C with rotatory agitation of 150 rpm. The hydrolysis buffer used was 50 mM sodium acetate, pH 4.8, containing 6 μg ml^{-1} of tetracyclin as a preservative. The release of soluble sugars during hydrolysis was monitored by HPLC and the results obtained for each replicate always had a coeficient of variation lower than 3%. The extent of enzymatic hydrolysis of cellulose was calculated as a fraction of the

theoretical amount of glucose originally present in the substrate.

The influence of high sugar concentration was investigated during hydrolysis of the peroxide-treated fraction at a 6% (w/v) solids concentration and an enzyme loading of 10 FPU g^{-1} cellulose. The efficiency of hydrolysis was determined before and after glucose was added to the hydrolysis reaction at concentrations of 20 and 40 mg mL^{-1}. The effect of sugar accumulation on the rate and completeness of hydrolysis of the substrate was also investigated after removal of the sugars produced during hydrolysis. The reaction was stopped at selected incubation times by transferring the hydrolysis mixture to an ice bath. The unhydrolysed residue was collected on a sintered glass filter and washed twice with hydrolysis buffer. After most of the soluble sugars had been eliminated, the washed residue was resuspended in fresh hydrolysis buffer containing β-glucosidase activity (no cellulases were added at this point) and incubated at 45°C for subsequent hydrolysis. In some cases, fresh substrate was also added to the hydrolysis mixture in order to reconstitute the original 6% (w/v) substrate starting conditions.

2.2.3.3. Enzyme Recycling

The recycling of the enzymes was carried out in duplicate after hydrolysis of a 2% (w/v) slurry of the pretreated substrate. Celluclast was added only at the beginning of the

experiment. An excess of Novozym, approximately 30 CBU g^{-1} of cellulose, was added to the reaction mixture at the initiation of each new hydrolysis step (i.e., each recycling step). After the hydrolysis mixture was cooled in an ice bath, only the unhydrolysed residue was collected by filtration on a sintered glass crucible and weighed after drying at 105°C overnight. The hydrolysate filtrates, containing used enzymes and soluble sugars produced during hydrolysis, were subsequently percolated through a column containing peroxide-treated eucalyptus at 4°C. Each column was washed with three times its volume of hydrolysis buffer to eliminate most of the soluble sugars. The substrate, containing the readsorbed enzymes, was then transferred to an erlenmeyer flask and resuspended in hydrolysis buffer to achieve a 2% (w/v) consistency for subsequent hydrolysis. Supplemental Novozym was added because β-glucosidase was not expected to adsorb efficiently to the cellulosic residue (Ooshima *et al.*, 1983). Aliquots were withdrawn from the hydrolysate at selected times and analysed using HPLC. The total amount of sugars released during hydrolysis was also determined in the filtrate of the hydrolysates using the phenol-sulphuric method (Chaplin, 1986). The column eluates obtained after each recovery step were assayed for both filter paper (FPU) and cellobiase (CBU) activities after ultrafiltration using a Centricon (Amicon) apparatus with a molecular weight cut off of 10 kD. The UF step was required to reduce the sugar concentration of each of the filtrates to non-inhibitory levels.

2.3. Changes in the Morphology and Fine Structure of the Substrate During Hydrolysis

2.3.1. Substrate Preparation and Enzymatic Hydrolysis

Two cellulosic residues were used in this study, the peroxide-treated steam-exploded substrate derived from SO_2-impregnated eucalyptus chips (SO_2-SEE-WIA/H_2O_2) and a fully-bleached eucalyptus kraft pulp (FBEP). The screening of both fractions was carried out using a Bauer-McNett fibre classifier using five consecutive screens. For the SO_2-SEE-WIA/H_2O_2 fraction, a series of fibre screens with 14, 28, 48, 150 and 200 mesh collected two major fibre populations, one between the 48 and 150 mesh screens (F-150 fraction) and another between the 150 and 200 mesh screens (F-200 fraction). These fractions accounted for approximately 50% of the starting material. For the fully bleached kraft pulp, a series of 28, 48, 100, 150 and 200 mesh screens collected one fraction between the 28 and 48 mesh screens (FBEP-48 fraction) and another between the 48 and 100 mesh screens (FBEP-100 fraction). The fibre length distribution of these fractions, as well as several partially hydrolysed residues, was determined using the Kajaani FS-200 fibre analyser. This method is based on the characteristic scattering of a laser beam by the plant cell wall due to its property of birefringence.

The enzymatic hydrolysis profile of several screened samples was carried out for selected incubation times at 2% (w/v) substrate concentration and 10 FPU g^{-1} cellulose. After the

hydrolysis mixture was cooled in ice to stop the reaction, the partially hydrolysed residue was collected by filtration and washed extensively with distilled water. The residue was then resuspended in water, and several aliquots were sampled and immediately analysed for their fibre length distribution. The remainder of the residue was collected by filtration, solvent exchanged with *t*-butanol and freeze-dried for further storage in a desiccator.

2.3.2. Determination of the Molecular Weight Distribution of Cellulose

The molecular weight distribution of both original substrates and partially hydrolysed residues was obtained by gel permeation chromatography (GPC) of their tricarbanyl derivatives. The carbanylation procedure used (see below) was an adaptation of the methods described previously by Schroeder and Haigh (1979) and Miller *et al.* (1991). Approximately 0.3 g of the freeze-dried material was placed in a vial and dried over phosphorus pentoxide for 24 h. To the dry residue, 1 mL of benzene isocyanate and 4 mL of pyridine were added and the vials were tightly sealed. The reaction was carried out in a water bath at 80°C for 48 h. The reaction mixture was constantly stirred during the reaction to allow better contact between sample and reagents. After cooling, the reaction was stopped by adding 1 mL of methanol and shaking vigorously. As some of the vials contained particles in suspension, the reaction mixture

was filtered through a glass microfibre filter. The carbanylated residue was precipitated in a mixture of methanol:water (4:1, v/v) and evaporated until dryness using a rotatory evaporator. This step was repeated several times until pyridine was completely eliminated. The tricarbanyl derivative was then solubilized in acetone, evaporated until dryness and resolubilized in tetrahydrofuran (THF) to 0.2 - 0.5 mg mL^{-1}.

The GPC analysis of the tricarbanyl derivatives was carried out using a Spectra-Physics SP8810 liquid chromatograph. The tricarbanylated samples (in THF) were filtered through a Teflon membrane with a pore size of 0.45 µm and analysed using a series of four TSK-GEL columns (type H8). THF was used as the eluting solvent at a flow rate of 1 mL min^{-1} and pressure of about 530 psi. The samples in the eluent were detected by a UV spectrophotometer (Spectroflow 757) at the wavelength of 235 nm. The signal from the Spectroflow 757 was fed to a SP4229 integrator for the integration of the peaks and the demonstration of both integral and differential distributions of molecular weight. The large peak eluting after a retention volume of 32 mL was attributed to the reagents and reaction by-products (Wood *et al.*, 1986). The degree of polymerisation (DP) of cellulose was obtained by dividing the molecular weigth (MW) of the tricarbanylated polymer by the corresponding MW of the tricarbanyl derivative of anhydroglucose (DP = MW/519). Both the number average MW ($\overline{MW_N}$) and the weight average MW ($\overline{MW_W}$) of cellulose tricarbanylate, from which the $\overline{DP_N}$ and $\overline{DP_W}$ of cellulose

were derived, were determined as described previously (Yau *et al.*, 1979).

Because of the lack of commercially available standards of cellulose tricarbanylate, the calibration curve (*i.e.*, the correlation of elution volume with molecular weight) was generated from the elution profile of polystyrene standards with narrow MW distributions using the following equation (Coll and Gilding, 1970; Valtasaari and Saarela, 1975; Danhelka and Kossler, 1976):

$$\ln M_c = \frac{(1+\alpha_p)\ln M_p + \ln (K_p / K_c)}{(1+\alpha_c)} \qquad (5)$$

where
- K_c, α_c — Mark-Houwink coefficients for cellulose tricarbanylate in tetrahydrofuran (THF);
- K_p, α_p — Mark-Houwink coefficients for polystyrene standards in THF;
- M_c — MW of cellulose tricarbanylate;
- M_p — MW of polystyrene standards.

The Mark-Houwink coefficients used in the present analysis for polystyrene in THF (K_p = 1.18 X 10^{-4}, α_p = 0.74) and for cellulose tricarbanylate in THF (K_c = 2.01 X 10^{-3}, α_c = 0.92) were those reported by Valtasaari and Saarela (1975).

2.3.3. Determination of the Degree of Crystallinity of Cellulose

The degree of crystallinity of the substrates was determined under both pre- and post-hydrolysis conditions by X-ray diffraction. Two sets of samples were analysed: the first

set consisted of partially hydrolysed residues derived from the F-150 fraction (peroxide-treated eucalyptus) after 0, 2, 5, 10, 14 and 24 h of hydrolysis, whereas the second set was derived from the FBEP-48 fraction (fully bleached kraft pulp) after hydrolysis for 0, 0.5, 4 and 8 h. After freeze-drying (see section 2.3.1.), the samples were conditioned for equal moisture content and subsequently pressed at 5000 psi for 4 min to obtain sheets of approximately 4.5 x 2.5 x 0.2 cm. Samples with an orientation factor (alignment) higher than 0.25 were discarded. Five replicates were prepared from each sample and the results obtained had a maximum coeficient of variation of 1.4%.

The X-ray diffraction of each set of samples was recorded by a Siemens diffractometer equipped with a D-5000 rotating anode X-ray generator. The wavelength of the Cu/Ka radiation source was 0.154 nm and the spectra were obtained at 30 mA with an accelerating voltage of 40 kV. Samples were scanned on the automated diffractometer from 9° to 40° of 2Θ (Bragg angle), with acquisition taken at intervals of 0.04° for 1 s.

The peak resolution program was used to calculate both the crystallinity index of cellulose and the dimensions of the crystallite. This program allowed the resolution of the X-ray diffraction pattern into the contribution of each of the diffraction planes. The background was attributed to the amorphous component of cellulose. This program incorporates an iterative minimization procedure based on the method originally described by Powell (Hindeleh and Johnson, 1978). Several

functions, including Gaussian, Cauchy (Lorentzian), Pearson and Voigt, were checked for best fit (Chung, 1989). Generally, the Voigt function resulted in the best fit of the X-ray diffraction patterns and was routinely used for the determination of both the crystallinity index of cellulose and the dimensions of the crystallite through peak broadening (full width at half maximum height). The crystallinity index, which was defined as the ratio of the resolved peak area to the total area under the unresolved peak profile, was calculated using the equation described below (Wims *et al.*, 1986):

$$CrI\ (\%) = \frac{\int I_T d\theta - k\int I_A d\theta}{\int I_T d\theta} .100 \qquad (6)$$

where,

	CrI	crystallinity index;
	I_T	total intensity of diffraction;
	I_A	total intensity of diffraction due to the amorphous region of the sample;
	k	ratio of the crystalline and amorphous intensities at a particular 2Θ value outside of the crystalline peak region;
	Θ	diffraction angle.

In this study, the value of "k" was not determined for each of the substrates and was considered equal to one in all cases to facilitate calculations. Therefore, the results obtained using this equation were relative values rather than absolute determinations.

The crystallinity index of cellulose was also calculated by

the empirical method described by Segal *et al.* (1959) using the following equation:

$$CrI\ (\%) = \frac{I_{002} - I_{AM}}{I_{002}} .100 \qquad (7)$$

where, CrI — crystallinity index;
I_{002} — maximum intensity of the 002 lattice diffraction (reflection attributed to the crystalline region of the sample);
I_{AM} — intensity of diffraction at Bragg angle $2\Theta = 18°$ (reflection attributed to the amorphous region of the sample).

Although the latter approach did not include any correction for the background (unresolved peak profile), results obtained using both methods were in relatively good agreement.

The apparent dimensions of the cellulose crystallite was obtained by applying the Scherrer's equation to the data collected by the peak resolution program (Cullity, 1956). The average thickness of the crystallite (relative values) at each plane of diffraction was then calculated using the following equation:

$$t = \frac{K.\lambda}{B.\cos\theta} \qquad (8)$$

where, t — thickness of the crystal at the plane of diffraction;
Θ — diffraction angle;
λ — wavelength of X-ray source;
K — Scherrer's constant; for cellulose, K = 0.9;
B — peak full width at the maximum height.

2.4. Light Microscopy

Partially hydrolysed residues derived from several pretreated samples were withdrawn from the hydrolysis reaction at various incubation times. The residue was collected by centrifugation and washed extensively with filtered water. A suspension containing approximately the same amount of solids (0.2 g%, w/v) was used to prepare slides for light microscopic examination. After each of the suspensions in water was worked to a smooth consistency, a small aliquot was collected and transferred to a slide and covered with a cover-slip. The borders of the cover slip were then sealed with nail polish to stop motion. Light microscopy (LM) observation of the substrate specimens was carried out using a Nikon Microphot-LX Universal microscope. Bright field micrographs were obtained for the pretreated substrates before and after enzymatic hydrolysis for 24 h. Polarized light micrographs were also obtained to demonstrate the presence of structural organization within the substrate specimen (cellulose crystallinity).

3. RESULTS

3.1. Steam Pretreatment of *E.viminalis* Chips

3.1.1. Effect of Temperature and Residence Time

Previously, several authors had demonstrated that steam pretreatment was an effective method for enhancing the enzymatic hydrolysis of cellulosic residues derived from both hardwood (Brownell and Saddler, 1984; Mackie *et al.*, 1985; Schwald *et al.*, 1989a) and softwood (Clark and Mackie, 1987; Schwald *et al.*, 1989b) species. However, it was not clear whether the use of similar operating conditions during pretreatment would be effective for other tree species, such as *Eucalyptus* sp., which would be the main feedstock in the Southern hemisphere. To permit a direct comparison with previously published work, we obtained four different samples of logs of *E. viminalis* from Colombo, Parana, Brazil. This material was chipped and assayed for its chemical composition (Table 2).

In earlier work with aspen, it was generally found that the more drastic the treatment, the greater the decomposition of the hemicellulose derived sugars and the better enzymatic accessibility of the resulting substrate. As the development of substrate accessibility to enzymes was shown to be independent of explosive decompression (Brownell *et al.*, 1986b), we steam-pretreated *E. viminalis* chips over a range of temperatures using a canister loaded into the reactor to ensure maximum recovery of the original material by bleeding-off the steam rather than

Table 2. Chemical composition of *Eucalyptus viminalis* chips.

COMPONENT	CONTENT[1] (%)	
Cellulose[2]	41.7 ± 0.6	
Xylan[2]	14.1 ± 0.8	
Total Apparent Lignin (TAL)[3]	31.0 ± 0.9	
Klason Lignin		27.9 ± 0.5
Acid-soluble Lignin		3.1 ± 0.4
Extractives[4]	2.1 ± 0.1	
TOTAL	88.9 ± 1.3	

[1] All figures (n = 4) were calculated in relation to the chips dry weight;
[2] The carbohydrate content of the substrate was determined by HPLC analysis of the acid hydrolysate from Klason lignin determinations;
[3] Klason lignin (acid-insoluble fraction) plus acid-soluble lignin;
[4] Ethanol/benzene + ethanol extractives.

exploding the material out of the steam gun. The efficiency of the pretreatment of chips with a moisture content of 50% (w/w) was evaluated based on both the overall recovery yield and enzymatic hydrolysis of the insoluble residue (Table 3, Fig. 7). There was a decrease in the recovery yield of the water-soluble fraction (STE-WS), indicative of destruction of the pentose sugars at increasing temperatures (Table 3). After pretreatment at 240°C for 120 s, only 24% of the pentosan (mostly xylan) present in the original material was recovered in the STE-WS fraction as mono- and oligosaccharides (Table 4), with the rest of the xylose degraded to products such as furfural. However, glucose was also detected in the STE-WS fraction at very low concentrations (0.2 - 0.3 mg mL^{-1}), even when the most drastic pretreatment conditions were used. The chemical composition of the water-washed substrate (STE-WI) obtained after pretreatment at 230°C for 120 s indicated that most of the xylan originally present in the wood chips had been removed during pretreatment (Table 5). As a result, both the cellulose and lignin content of the pretreated material increased relatively to the original composition of the wood chips. When the mass balance for the three wood components was determined after pretreatment at the various temperatures (Table 6), we observed that a considerable amount of the original cellulose and xylan was lost during pretreatment. More drastic conditions resulted in a lower recovery of wood polysaccharides, while the increased recovery of lignin was probably associated with formation of condensation

Table 3. Overall recovery yield of pretreated fractions obtained after steam pretreatment of eucalyptus chips at various temperatures and a fixed residence time.

PRETREATMENT CONDITIONS[1]		RECOVERY YIELD[2] (%)		
Temp.(°C)	Time (s)	WS	WI	TOTAL
220	120	20.3	61.4	81.7
230	120	18.7	61.6	80.3
240	120	15.7	59.3	75.0

[1] Steam-treated fractions (STE) were derived from chips with a moisture content of 50% (w/w) in relation to the chips dry weight; WS, water-soluble fraction; WI, water insoluble fraction;
[2] Calculated in relation to the chips dry weight.

Table 4. Recovery yield of water-soluble sugars during steam pretreatment of eucalyptus chips at various temperatures and a fixed residence time.

TREATMENT CONDITIONS[1]		RECOVERY YIELD[2] [%, (mg mL^{-1})]	
Temp.(°C)	Time (s)	Xylan	Glucan
220	120	46.2 (1.6)	1.9 (0.2)
230	120	50.0 (1.8)	2.4 (0.3)
240	120	23.9 (0.9)	2.6 (0.3)

[1] See legend in Table 3;
[2] Recovery of sugars in the water-soluble fraction (STE-WS) was determined by HPLC after acid hydrolysis with 1 M TFA at 100°C for 3 h; results were corrected for sugar destruction during hydrolysis and calculated in relation to the original carbohydrate (xylan or glucan) content of the chips;
() values in parenthesis indicate the concentration of monosaccharides (mg mL^{-1}) in the water-soluble fraction.

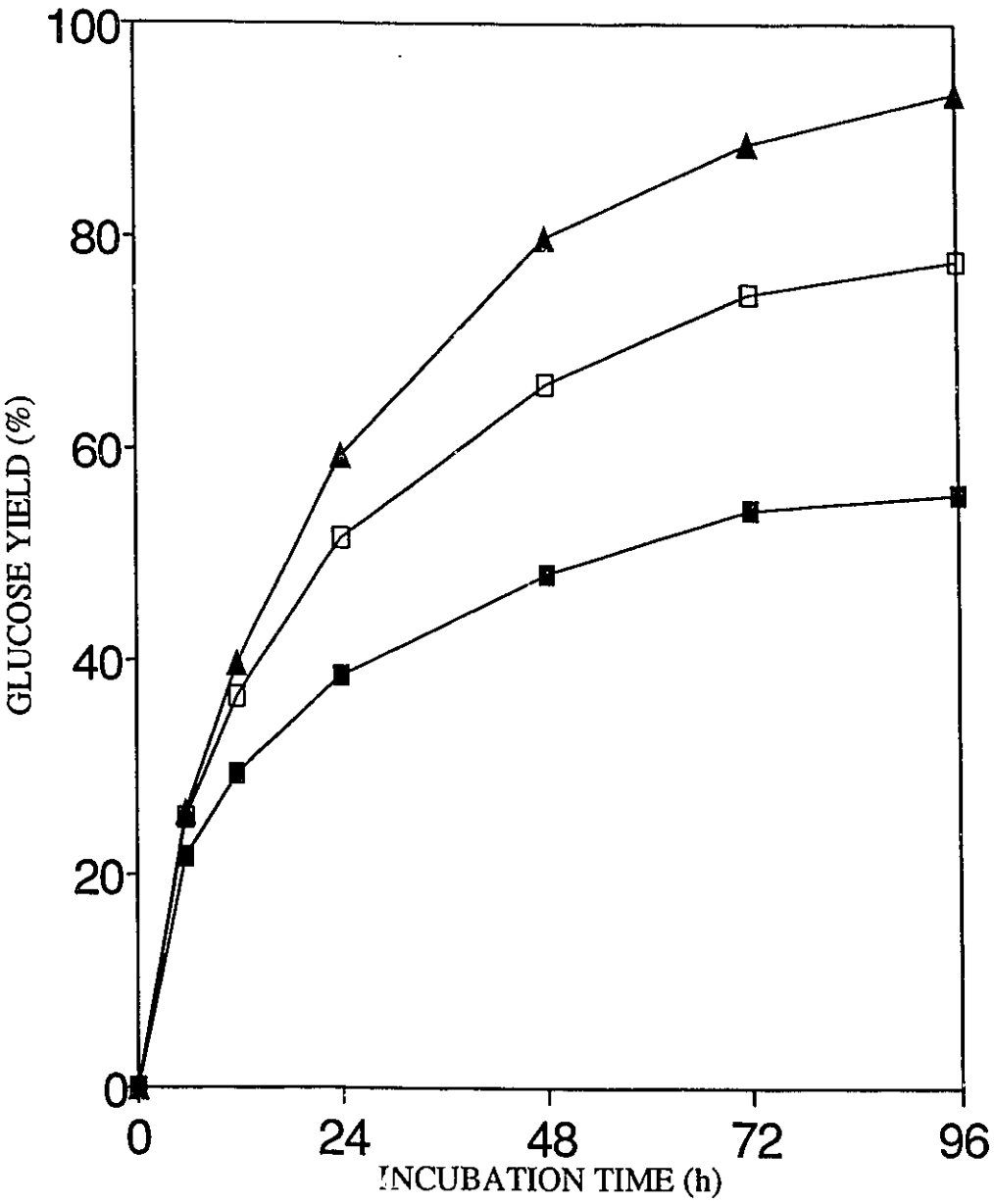


Fig 7. Enzymatic hydrolysis of water-insoluble fractions (STE-WI) obtained after steam pretreatment of eucalyptus chips (moisture content of 50%, w/w) at various temperatures and a fixed residence time of 120 s. Hydrolyses were carried out at a 2% (w/v) substrate concentration and a fixed enzyme loading of 10 FPU g^{-1} cellulose. (■) 220°C; (□) 230°C; and (▲) 240°C.

Table 5. Chemical composition of the water-insoluble fractions (STE-WI) derived from steam-treated eucalyptus chips under various pretreatment conditions.

PRETREATMENT CONDITIONS[1]		CHEMICAL COMPOSITION[2] (%)			
Temp. (°C)	Time (s)	Glucan	Xylan	TAL[3]	TOTAL
220	120	53.3	nd[4]	38.4	91.7
230	120	56.2	1.4	39.8	97.4
240	120	53.5	nd	41.8	95.3

[1] Steam-treated, water-insoluble fractions (STE-WI) were derived from chips with a moisture content of 50% (w/w) in relation to the chips dry weight;
[2] The carbohydrate content of the substrates was determined by HPLC analysis of the acid hydrolysate from Klason lignin determinations; all figures were calculated in relation to the chips dry weight;
[3] Total apparent lignin (acid-insoluble plus acid-soluble fractions);
[4] Not determined.

Table 6. Overall recovery yield of wood components during steam treatment of eucalyptus chips at various temperatures and a fixed residence time.

PRETREATMENT CONDITIONS[1]		OVERALL RECOVERY YIELD[2] (%)		
Temp. (°C)	Time (s)	Glucan	Xylan	Lignin
220	120	80.3	55.0	76.1
230	120	85.4	56.3	79.1
240	120	78.6	32.6	80.0

[1] See legend in Table 5;
[2] The overall recovery yield (water-soluble plus water-insoluble fractions) of glucan, xylan and lignin was calculated in relation to the original content of each of these components in the wood chips.

by-products detected by the Klason lignin assay (pseudo-lignin). When the ease of hydrolysis of each of the STE-WI fractions was determined (Fig. 7), the residue derived from pretreatment at 240°C for 120 s was the best substrate for hydrolysis with almost 90% of the cellulose hydrolysed to glucose after a 72 h incubation. Although the pretreated substrate obtained at 230°C was not as effectively hydrolysed, these milder conditions resulted in much better recovery yields of both the pentosan (Tables 4 and 6) and glucan (Table 6) fractions. Therefore, 230°C for 120 s were the compromise conditions which were used for subsequent experiments.

As earlier work (Mackie *et al.*, 1985; Schwald *et al.*, 1989a,b) had indicated that pre-impregnation of wood chips with SO_2 prior to steam pretreatment enhanced both the cellulose hydrolysis and the recovery of hemicellulose derived sugars, 1% (w/w) SO_2 was added to *E. viminalis* chips (moisture content of 50%, w/w) prior to pretreatment. Lower temperatures and shorter residence times were required because of the catalytic effect of the SO_2 gas. In agreement with the previous work with aspen and spruce, SO_2 catalysis resulted in a superior recovery yield of both the water-soluble and water-insoluble fractions, particularly after short incubation times (Table 7). High recovery yields of xylose were obtained in the water-soluble fractions (SO_2-STE-WS), ranging from 65 to 80% of the original xylan content of the chips (Table 8). However, increased concentrations of glucose were also detected in the water-

Table 7. Overall recovery yield of pretreated fractions obtained after steam pretreatment of SO_2-impregnated (1%, w/w) eucalyptus chips at various temperatures and residence times.

PRETREATMENT CONDITIONS[1]		RECOVERY YIELD[2] (%)		
Temp.(°C)	Time (s)	WS	WI	TOTAL
200	50	29.5	69.8	99.3
210	50	31.6	66.0	97.6
210	100	27.7	59.2	86.9
210	150	25.8	60.1	85.9
220	50	34.1	62.0	96.1

[1] Steam-treated fractions (SO_2-STE) were derived from SO_2-impregnated chips with a moisture content of 50% (w/w) in relation to the chips dry weight; WS, water-soluble fraction; WI, water-insoluble fraction;
[2] Calculated in relation to the chips dry weight.

Table 8. Recovery yield of water-soluble sugars during steam pretreatment of SO_2-impregnated (1%, w/w) eucalyptus chips at various pretreatment conditions.

PRETREATMENT CONDITIONS[1]		RECOVERY YIELD[2] [%, (mg mL^{-1})]	
Temp.(°C)	Time (s)	Xylan	Glucan
200	50	65.3 (2.2)	3.2 (0.3)
210	50	79.6 (2.8)	6.2 (0.7)
210	100	73.1 (2.4)	9.6 (1.0)
210	150	70.5 (2.3)	12.7 (1.3)
220	50	72.9 (2.6)	11.4 (1.2)

[1] See legend in Table 7;
[2] Recovery of sugars in the water-soluble fraction (STE-WS) was determined by HPLC and calculated in relation to the original carbohydrate content of the chips;
() values in parenthesis indicate the concentration of monosaccharides (mg mL^{-1}) in the water-soluble fraction.

soluble fraction when more drastic pretreatment conditions were used, probably indicating some extent of cellulose degradation due to acid hydrolysis. The water-insoluble fractions derived from SO_2-impregnated chips were composed of approximately 55-57% (w/w) cellulose and 1% (w/w) xylan (Table 9). As found in the previous experiment where no SO_2 gas was used, the lignin content of the SO_2-STE-WI fractions increased with the severity of pretreatment. Compared to the mass balance calculations previously obtained for pretreatment under non-catalysed conditions (Table 6), the use of SO_2 as an acid catalyst resulted in an enhanced recovery of the carbohydrate components originally present in the chips (Table 10). Pretreatment conditions of 210°C for 50 s not only resulted in an almost complete recovery of cellulose and xylan, it also produced the best substrate for hydrolysis. Up to 95% of the cellulose in the SO_2-STE-WI fraction could be hydrolysed to glucose within 72 h (Fig. 8). Pretreatment at higher temperatures (Fig. 8) or longer residence times at 210°C (Fig. 9) did not result in a substantial increase in enzymatic hydrolysis of the resulting substrate, although there was a slight decrease in the total recovery yield of steam-treated material. Therefore, steam pretreatment at 210°C for 50 s was considered the best conditions for both hydrolysis and recovery yield of pretreated materials.

Table 9. Chemical composition of water-insoluble fractions derived from SO_2-impregnated (1%, w/w) steam-treated eucalyptus chips (SO_2-STE-WI) under various pretreatment conditions.

PRETREATMENT CONDITIONS[1]		CHEMICAL COMPOSITION[2] (%)			
Temp. (°C)	Time (s)	Glucan	Xylan	TAL[3]	TOTAL
200	50	57.6	1.4	34.1	93.1
210	50	57.8	1.3	35.3	94.4
210	100	57.5	1.0	38.1	96.6
210	150	54.2	0.5	39.1	93.8
220	50	54.5	0.6	38.0	93.1

[1] Steam-treated, water-insoluble substrates (SO_2-STE-WI) were derived from SO_2-impregnated chips with a moisture content of 50% (w/w) in relation to the chips dry weight;
[2] The carbohydrate content of the substrates was determined by HPLC analysis of the acid hydrolysate from Klason lignin determinations; all figures were calculated in relation to the chips dry weight;
[3] Total apparent lignin (acid-insoluble plus acid-soluble fractions).

Table 10. Overall recovery yield of wood components during steam pretreatment of SO_2-impregnated (1%, w/w) eucalyptus chips at various pretreatment conditions.

PRETREATMENT CONDITIONS[1]		OVERALL RECOVERY YIELD[2] (%)		
Temp. (°C)	Time (s)	Glucan	Xylan	Lignin
200	50	99.6	68.7	76.8
210	50	97.7	85.7	75.2
210	100	91.2	73.2	72.8
210	150	90.9	67.6	75.8
220	50	92.4	75.4	76.0

[1] See legend in Table 9;
[2] The overall recovery yield (water-soluble plus water-insoluble fractions) of glucan, xylan and lignin was calculated in relation to the original content of each of these components in the wood chips.

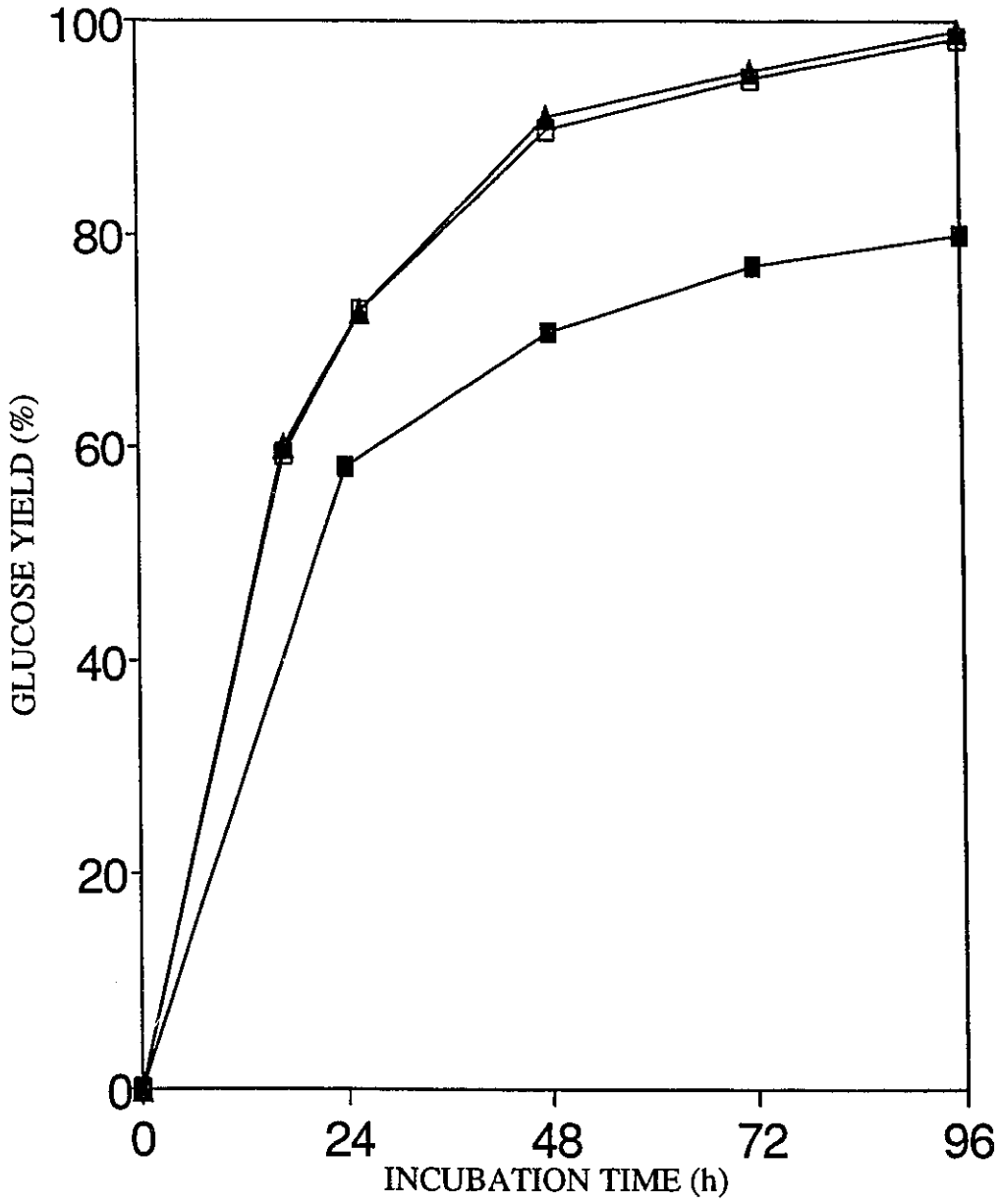


Fig. 8. Enzymatic hydrolysis of water-insoluble fractions (SO_2-STE-WI) obtained after steam pretreatment of SO_2-impregnated eucalyptus chips (moisture content of 50%, w/w) at various temperatures and a fixed residence time of 50 s. Hydrolyses were carried out at a 2% (w/v) substrate concentration and a fixed enzyme loading of 10 FPU g^{-1} cellulose. (■) 200°C; (□) 210°C; and (▲) 220°C.

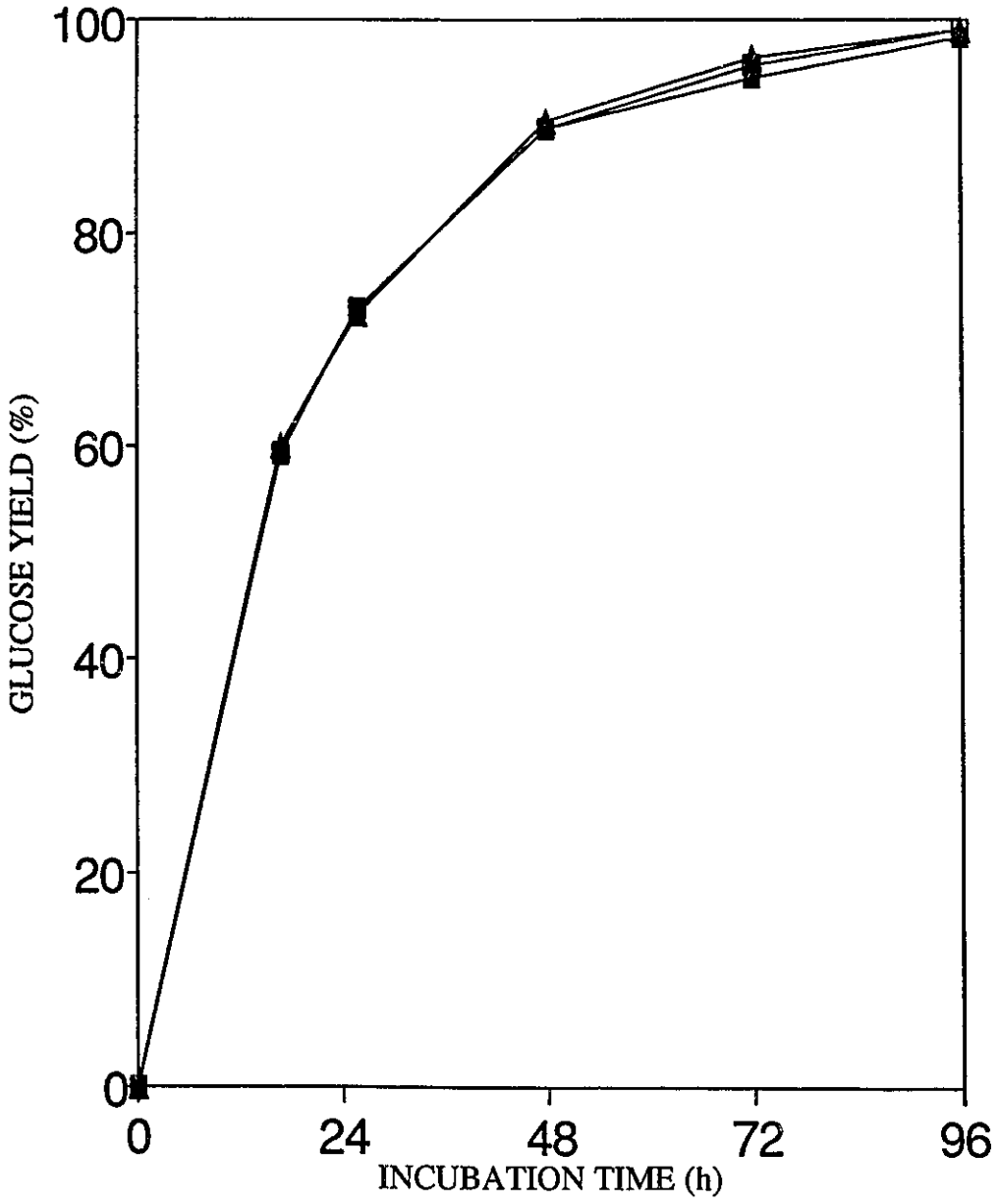


Fig. 9. Enzymatic hydrolysis of water-insoluble fractions (SO_2-STE-WI) obtained after steam pretreatment of SO_2-impregnated eucalyptus chips (moisture content of 50%, w/w) at a fixed temperature of 210°C and various residence times. Hydrolyses were carried out at a 2% (w/v) substrate concentration and a fixed enzyme loading of 10 FPU g^{-1} cellulose. (■) 50 s; (□) 100 s; and (▲) 150 s.

3.1.2. Effect of the Initial Moisture Content of Wood Chips

As mentioned earlier, four different shipments of *E. viminalis* logs were obtained. Although the wood composition of these logs was identical, there was a significant variation in the moisture content of the derived wood chips, resulting from differences in the duration of their shipment. In earlier work on steam explosion of aspen, Brownell and Saddler (1984) had found that the size of the wood chips had more influence on subsequent enzymatic hydrolysis than the moisture content of the chips. Other work, however, had shown that moisture content did have some influence on the hydrolysis of steam-treated aspen chips (Foody, 1980). As there appeared to be some variability in the efficiency of hydrolysis of the different batches of steam-treated *E. viminalis* chips, which could not be attributed to differences in chemical composition, the influence of the initial moisture content on recovery yield, chemical composition and enzymatic hydrolysis of steam-treated eucalyptus chips was evaluated using chips with a range of moisture contents from air-dried (5%, w/w) to green (100%, w/w) conditions.

When pretreatment was carried out at both non-catalysed and SO_2-catalysed conditions, it was apparent that the initial moisture content of the chips did not have a substantial effect on the overall recovery yield of pretreated fractions (Table 11). Non-impregnated chips with a low moisture content resulted in higher recovery yields of both xylose and glucose in the

Table 11. Influence of initial moisture content of the wood chips and explosive decompression on the overall recovery yield of materials obtained after steam pretreatment of eucalyptus chips under both the non-catalysed and SO_2-catalysed pretreatment conditions.

CHIPS MOISTURE CONTENT[1] (%)	RECOVERY YIELD[1,2] (%)		
	WS	WI	TOTAL
NON-CATALYSED[3], NON-EXPLODED CONDITIONS - STE			
15	17.4	75.5	92.9
23	18.9	74.4	93.3
50	18.7	61.6	80.3
100	19.3	68.3	87.6
NON-CATALYSED, EXPLODED CONDITIONS - SEE			
5	14.4	67.6	82.0
ACID-CATALYSED[4], NON-EXPLODED CONDITIONS - SO_2-STE			
15	28.1	67.5	95.9
23	29.2	68.5	97.7
50	27.1	65.4	92.5
100	31.6	66.0	97.6
ACID-CATALYSED, EXPLODED CONDITIONS - SO_2-SEE			
100	28.6	63.4	92.0

[1] Calculated in relation to the chips dry weight;
[2] WS, water-soluble fraction; WI, water-insoluble fraction;
[3] Substrates were obtained after steam pretreatment of chips at 230°C for 120 s;
[4] Substrates were obtained after steam pretreatment of SO_2-impregnated (1%, w/w) chips at 210°C for 50 s.

water-soluble fraction (Table 12). An increased Klason lignin content was also observed in the water-insoluble fractions derived from chips with low moisture content, probably due to the accumulation of condensation by-products (pseudolignin) during pretreatment (Table 13). Chips with low moisture content also resulted in the best overall recovery yield of wood components (Table 14). These results suggested that non-impregnated chips with a low moisture content were more severely pretreated, compared to those with high moisture content. Pretreatment carried out at low pH (acid catalysed) increased hemicellulose recovery and survival owing to the better stability of pentoses under mild acidic conditions (Table 12). The concentration of xylose in the water-soluble fraction increased with the moisture content of the chips, while the concentration of glucose decreased. Mass balance calculations also indicated that the recovery of hemicellulose sugars depended upon the moisture content of the chips, whereas most of the cellulose originally present within the chips was recovered after pretreatment (Table 14).

Although the cellulose content of all of the pretreated substrates were comparable (Table 13), these fractions differed substantially in their susceptibility to hydrolysis (Fig. 10 A,B). The addition of SO_2 gas as an acid catalyst did not improve the degree of hydrolysis of the steam-treated substrates which were prepared from wood chips with low moisture content. However, a dramatic difference was observed in the enzyme

Table 12. Influence of initial moisture content of the wood chips and explosive decompression on the recovery yield of water-soluble sugars during steam pretreatment of eucalyptus chips under both the non-catalysed and SO_2-catalysed conditions.

CHIPS MOISTURE CONTENT[1] (%)	RECOVERY YIELD[2] [%, (mg mL^{-1})	
	Xylan	Glucan
NON-CATALYSED[3], NON-EXPLODED CONDITIONS - STE-WS		
15	60.1 (2.3)	4.2 (0.4)
23	54.0 (1.8)	2.9 (0.3)
50	50.0 (1.8)	2.4 (0.3)
100	45.8 (1.9)	1.6 (0.2)
ACID-CATALYSED[4], NON-EXPLODED CONDITIONS - SO_2-STE-WS		
15	44.5 (1.6)	9.5 (1.0)
23	48.8 (1.7)	8.3 (0.9)
50	64.2 (2.3)	7.8 (0.8)
100	79.6 (2.8)	6.2 (0.7)
ACID-CATALYSED, EXPLODED CONDITIONS - SO_2-SEE-WS		
100	93.5 (3.3)	11.3 (1.2)

[1] Calculated in relation to the chips dry weight;
[2] Recovery of sugars in the water-soluble fraction (STE-WS) was determined by HPLC after acid hydrolysis with 1 M TFA at 100°C for 3 h; results were corrected for sugar destruction and calculated in relation to the original carbohydrate (xylan or glucan) content of the chips;
[3] Substrates were obtained after steam pretreatment of chips at 230°C for 120 s;
[4] Substrates were obtained after steam pretreatment of SO_2-impregnated (1%, w/w) chips at 210°C for 50 s;
() values in parenthesis indicate the concentration of monosaccharides (mg mL^{-1}) in the water-soluble fraction.

Table 13. Chemical composition of water-insoluble fractions (WI) obtained after steam pretreatment of eucalyptus chips with various moisture contents under both the non-catalysed and SO_2-catalysed pretreatment conditions.

CHIPS MOISTURE CONTENT[1] (%)	CHEMICAL COMPOSITION[1] (%)			
	Glucan	Xylan	TAL[2]	TOTAL
NON-CATALYSED[3], NON-EXPLODED CONDITIONS - STE-WI				
15	53.2	1.4	39.2	93.8
23	54.3	1.7	38.1	94.1
50	56.2	1.4	39.8	97.4
100	54.7	2.0	35.3	92.0
NON-CATALYSED[4], EXPLODED CONDITIONS - SEE-WI				
5	54.9	1.6	38.8	95.3
ACID-CATALYSED, NON-EXPLODED CONDITIONS - SO_2-STE-WI				
15	54.6	1.3	39.1	95.0
23	53.8	1.3	38.4	93.5
50	53.8	1.2	41.2	95.2
100	57.8	1.3	35.3	94.4
ACID-CATALYSED, EXPLODED CONDITIONS - SO_2-SEE-WI				
100	58.1	1.0	36.5	95.6

[1] The carbohydrate content of the substrates was determined by HPLC analysis of the acid hydrolysate from Klason lignin determinations; all figures were calculated in relation to the chips dry weight;
[2] Total apparent lignin (acid-insoluble plus acid-soluble fractions);
[3] Substrates were obtained after steam pretreatment of chips at 230°C for 120 s;
[4] Substrates were obtained after steam pretreatment of SO_2-impregnated (1%, w/w) chips at 210°C for 50 s.

Table 14. Overall recovery yield of wood components during steam pretreatment of eucalyptus chips under both the non-catalysed and SO_2-catalysed conditions.

CHIPS MOISTURE CONTENT[1] (%)	OVERALL RECOVERY YIELD[2] (%)		
	Glucan	Xylan	Lignin
NON-CATALYSED[3], NON-EXPLODED CONDITIONS - STE			
15	99.6	63.4	95.5
23	99.5	53.3	91.4
50	85.4	56.3	79.1
100	91.4	56.6	77.8
NON-CATALYSED, EXPLODED CONDITIONS - SEE			
5	89.0	nd[5]	84.6
ACID-CATALYSED[4], NON-EXPLODED CONDITIONS - SO_2-STE			
15	97.9	50.7	85.1
23	96.7	55.1	84.9
50	92.2	69.7	86.9
100	97.7	85.7	75.2
ACID-CATALYSED, EXPLODED CONDITIONS - SO_2-SEE			
100	99.6	98.0	74.6

[1] Calculated in relation to the chips dry weight;
[2] The overall recovery yield (water-soluble plus water-insoluble fractions) of glucan, xylan and lignin was calculated in relation to the original content of each of these components in the wood chips;
[3] Substrates were obtained after steam pretreatment of chips at 230°C for 120 s;
[4] Substrates were obtained after steam pretreatment of SO_2-impregnated (1%, w/w) chips at 210°C for 50 s;
[5] Not determined.

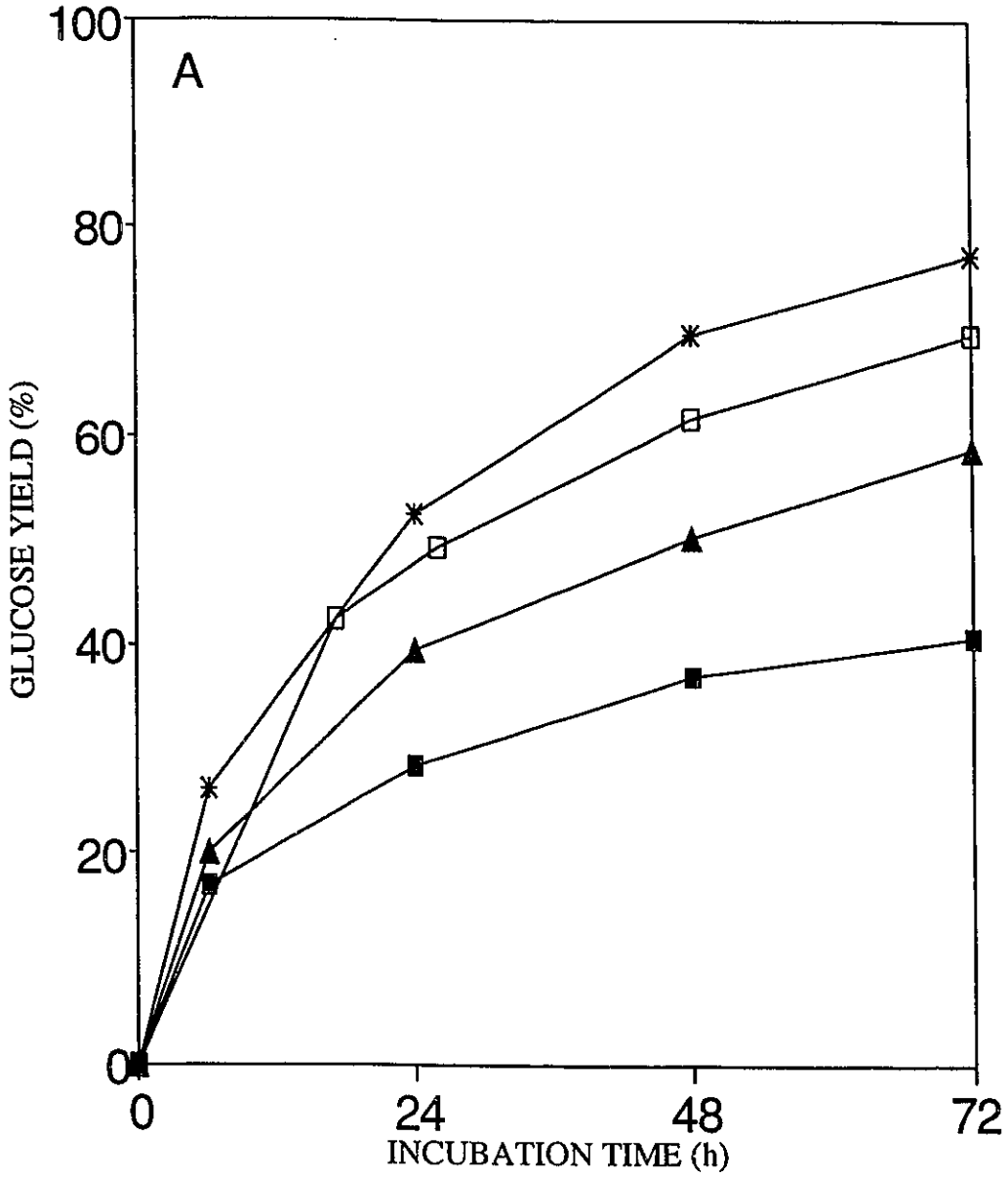


Fig. 10 A. Influence of initial moisture content of the chips on enzymatic hydrolysis of the water-insoluble fraction derived from steam-treated eucalyptus. Pretreatment was carried out under non-catalysed conditions (STE-WI). Hydrolyses were performed at a 2% (w/v) substrate concentration and a fixed enzyme loading of 10 FPU g^{-1} cellulose. Pretreated substrates were derived from chips with a moisture content of (■) 15, (▲) 23, (*) 50, and (□) 100% (w/w) in relation to their dry weight.

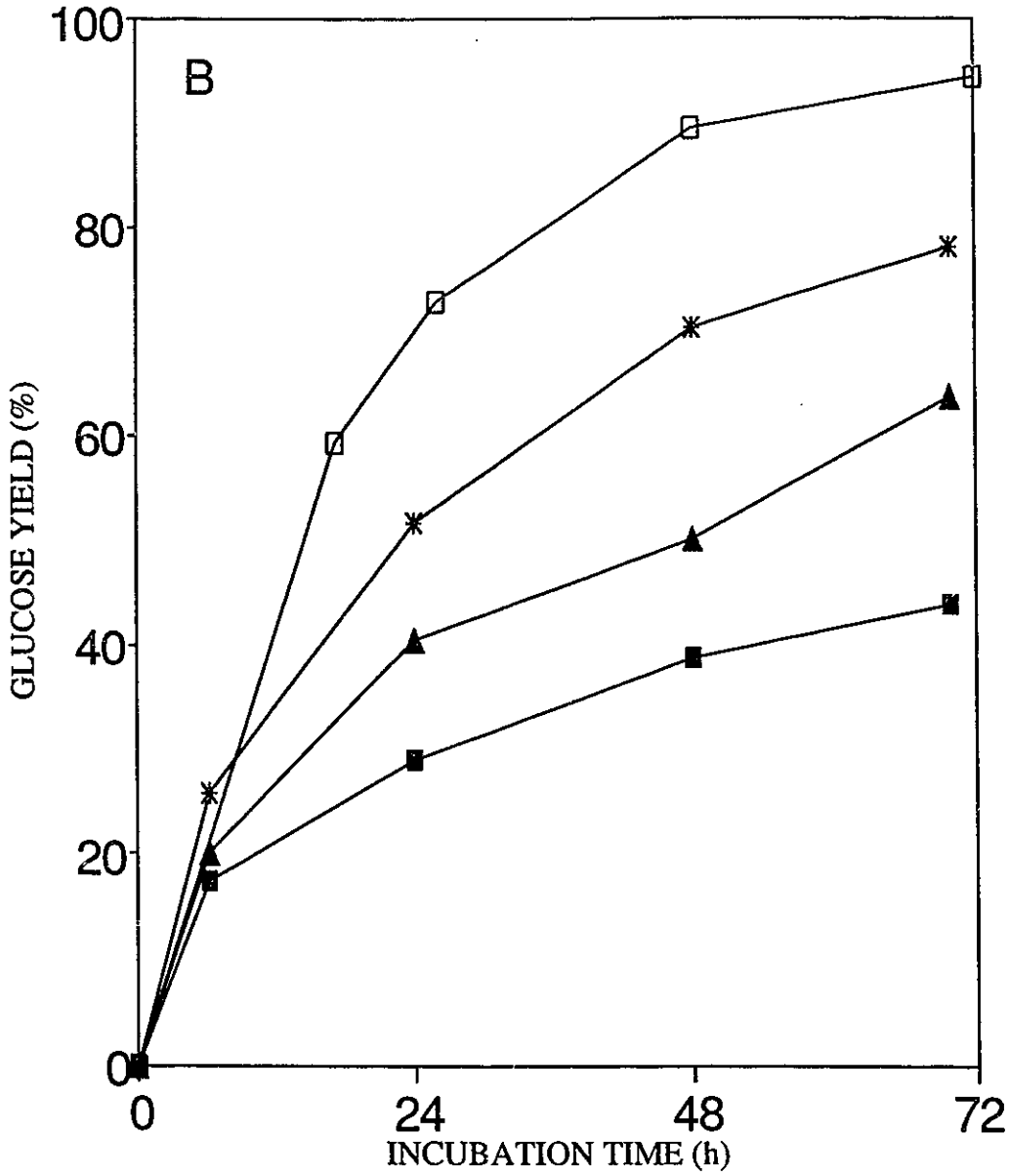


Fig. 10 B. Influence of initial moisture content of the chips on enzymatic hydrolysis of the water-insoluble fraction derived from steam-treated eucalyptus. Pretreatment was carried out under catalysed conditions (SO_2-STE-WI). Hydrolyses were performed at a 2% (w/v) substrate concentration and a fixed enzyme loading of 10 FPU g^{-1} cellulose. Pretreated substrates were derived from chips with a moisture content of (■) 15, (▲) 23, (*) 50, and (□) 100% (w/w) in relation to their dry weight.

digestibility of the steam-treated substrate when green chips were used. Almost 95% of the original cellulose present in the SO_2-impregnated, steam-treated green chips was hydrolysed to glucose, while less than 50% of the available cellulose present in the equivalent air dried chips was hydrolysed under the same conditions. This can be probably attributed to the difference in the efficiency of uptake of the SO_2 catalyst.

It has been demonstrated that explosive decompression is not an essential requirement for the development of cellulose accessibility during steam pretreatment (Brownell *et al.*, 1986b). For pretreatment of eucalyptus chips under both non-catalysed and catalysed conditions, explosion resulted in slightly lower recovery yields of pretreated materials, probably owing to difficulties in recovering the exploded material from the collecting tank (Table 11). Explosion was also shown to enhance the recovery of both xylose and glucose in the water-soluble fraction derived from the acid-catalysed pretreatment of green chips (Table 12). However, the susceptibility to hydrolysis of the cellulosic residues derived from pre-impregnated green chips was not influenced by explosive decompression (Fig. 11). When pretreatment was carried out under non-catalysed conditions, explosion was beneficial for the steam pretreatment of air-dried chips (moisture content of 5%, w/w). Almost 70% of the cellulose component of the substrate was hydrolysed to glucose in 96 h (Fig. 11). These results, when compared to those reported in Fig. 10 A for the hydrolysis of

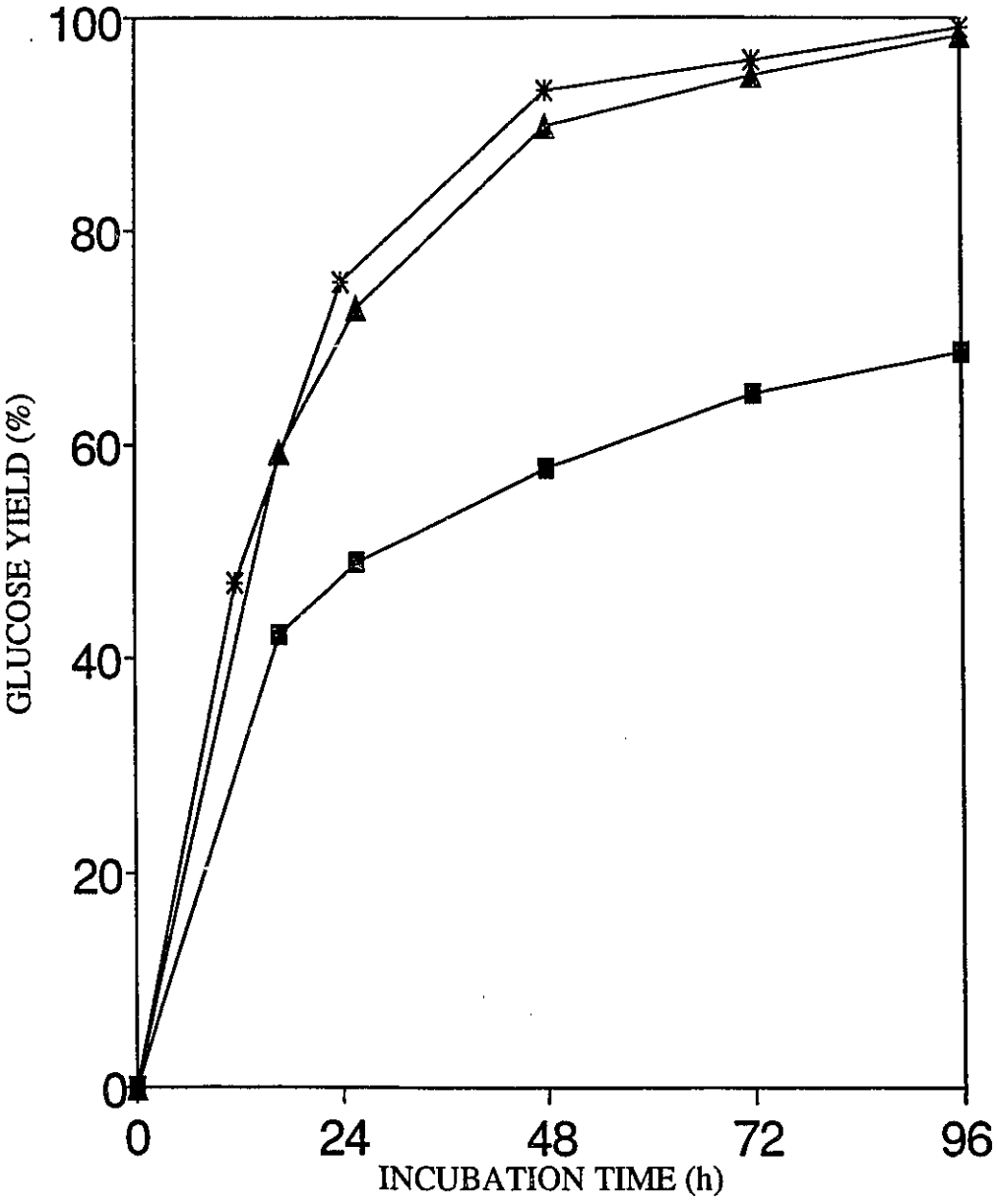


Fig. 11. Influence of explosive decompression on enzymatic hydrolysis of steam-treated substrates derived from eucalyptus chips with various moisture contents. Hydrolyses were carried out at a 2% (w/v) substrate concentration and a fixed enzyme loading of 10 FPU g^{-1} cellulose. (■) SEE-WI, water-insoluble steam-exploded substrate obtained from air-dried chips (moisture content of 5%, w/w); (▲) SO_2-STE-WI, water-insoluble steam-treated substrate obtained from SO_2-impregnated green chips (moisture content of 100%, w/w); (*) SO_2-SEE-WI, water-insoluble steam-exploded substrate obtained from SO_2-impregnated green chips.

steam-heated substrates (no explosion) derived from chips with low moisture content, suggested that explosion is not only desirable as an efficient method of emptying the steam reactor (steam gun), it also ensures that the pretreated substrate maintains a moisture content which enhances fractionation and enzymatic hydrolysis.

3.1.3. Influence of Lignin Removal on the Enzymatic Hydrolysis of Pretreated Substrates

To see whether lignin removal would have any effect on the enzymatic hydrolysis of pretreated substrates derived from chips with varying moisture content, we subsequently alkali-extracted each of the water-washed substrates obtained under both catalysed and non-catalysed conditions. Although similar recovery yields of the alkali-washed fraction were generally obtained, the levels of substrate delignification varied from 73 to 87% of the lignin content of the original water-insoluble fraction (Table 15). Alkali washing had a beneficial effect on the enzymatic hydrolysis of the pretreated substrate derived from chips with low moisture content (Fig. 12 A,B). The efficiency of hydrolysis of the alkali-washed substrates obtained from the non-catalysed wood chips appeared to be independent of the original moisture content, while the low moisture content in some of the SO_2-catalysed wood chips led to decreased cellulose hydrolysis. It is probable that the lower amount of catalyst produced at the lower moisture content levels

Table 15. Chemical composition of alkali-insoluble fractions (WIA) obtained after steam pretreatment of eucalyptus chips with various moisture contents under both the non-catalysed and SO_2-catalysed conditions.

CHIPS MOISTURE CONTENT[1] (%)	RECOVERY YIELD[1] (%)	CHEMICAL COMPOSITION[1] (%)			
		Glucan	Xylan	TAL[2]	TOTAL
NON-CATALYSED[3], NON-EXPLODED CONDITIONS - STE-WIA					
15	48.9	81.1	1.5	6.4	89.0
23	47.7	90.9	1.8	6.8	99.5
50	nd[5]	81.7	1.7	10.8	94.2
100	42.6	88.9	1.4	7.1	97.4
NON-CATALYSED[4], EXPLODED CONDITIONS - SEE-WIA					
5	41.7	88.9	1.4	7.0	97.3
ACID-CATALYSED, NON-EXPLODED CONDITIONS - SO_2-STE-WIA					
15	41.9	86.6	1.3	5.0	92.9
23	43.6	90.0	1.5	5.7	97.2
50	nd	86.3	1.4	8.8	96.5
100	46.9	86.7	1.3	7.5	95.5
ACID-CATALYSED, EXPLODED CONDITIONS - SO_2-SEE-WIA					
100	44.4	84.4	1.1	6.4	91.9

[1] The carbohydrate content of the substrates was determined by HPLC of the acid hydrolysate from Klason lignin determinations; all figures were calculated in relation to the chips dry weight;
[2] Total apparent lignin (acid-insoluble plus acid-soluble fractions);
[3] Substrates were obtained after steam pretreatment of chips at 230°C for 120 s;
[4] Substrates were obtained after steam pretreatment of SO_2-impregnated (1%, w/w) chips at 210°C for 50 s;
[5] Not determined.

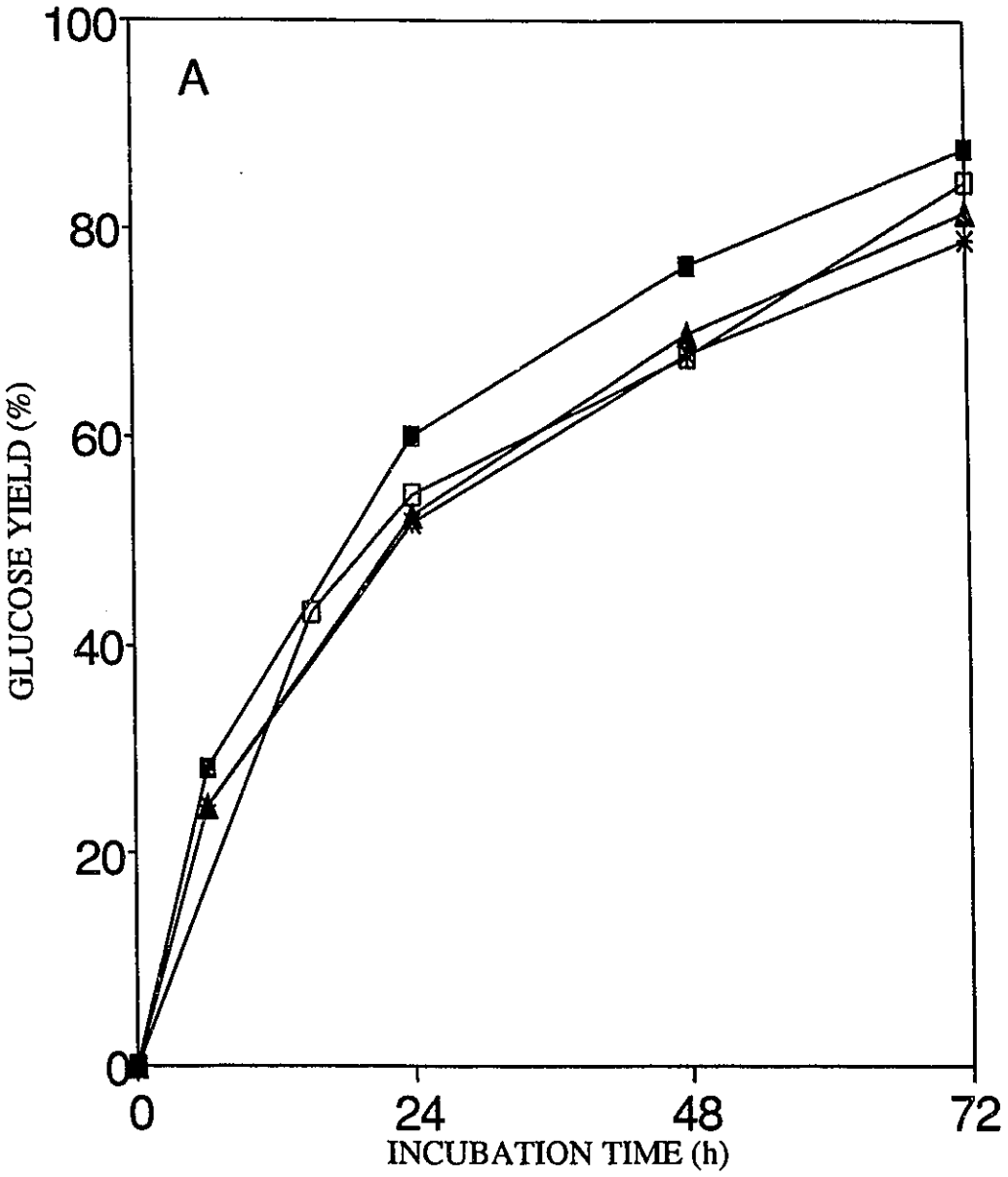


Fig. 12 A. Influence of initial moisture content of the chips on enzymatic hydrolysis of the alkali-insoluble fraction derived from steam-treated eucalyptus. Pretreatment was carried out under non-catalysed conditions (STE-WIA). Hydrolyses were performed at a 2% (w/v) substrate concentration and a fixed enzyme loading of 10 FPU g^{-1} cellulose. Pretreated substrates were derived from chips with a moisture content of (■) 15, (▲) 23, (*) 50, and (□) 100% (w/w) in relation to their dry weight.

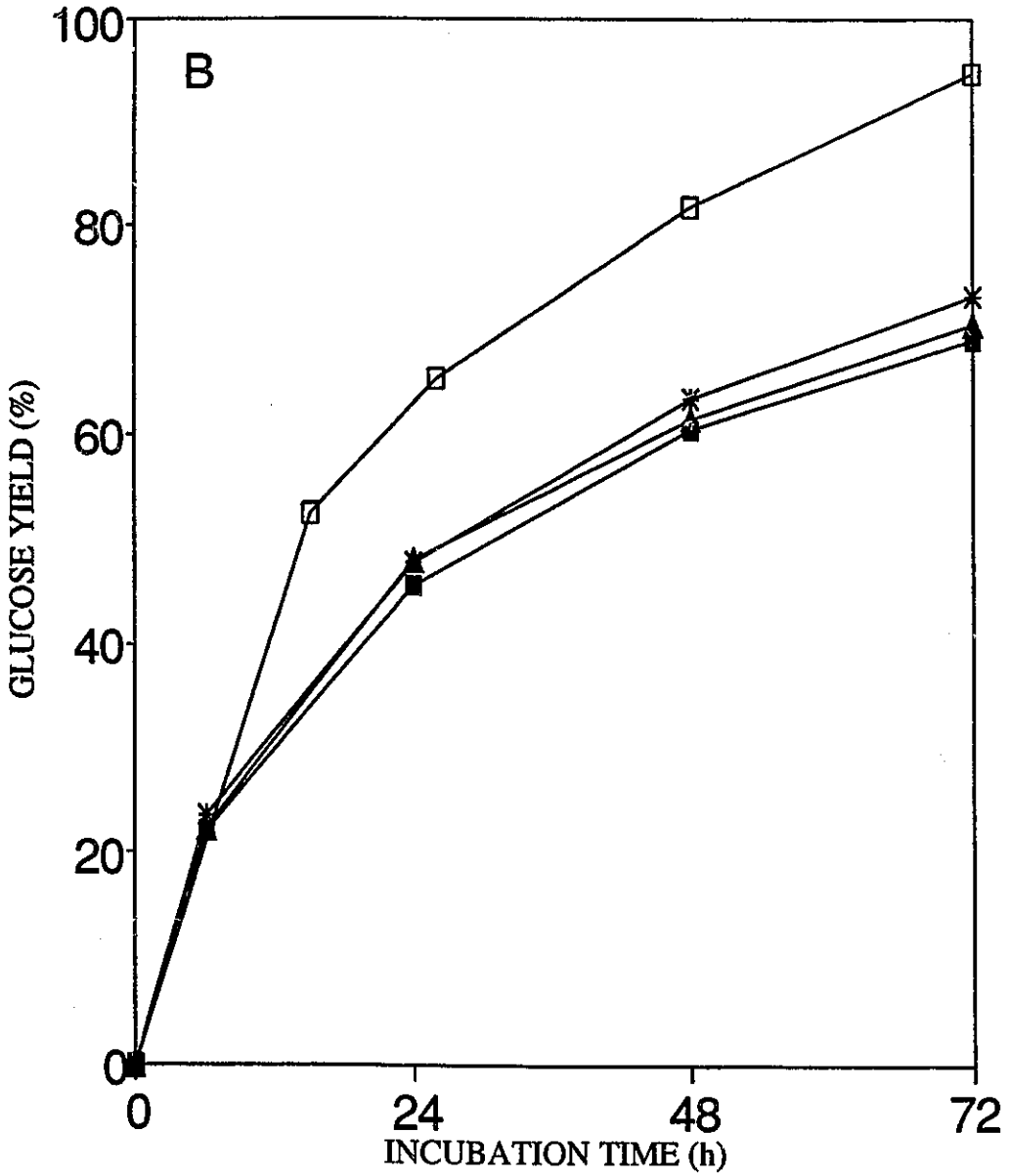


Fig. 12 B. Influence of initial moisture content of the chips on enzymatic hydrolysis of the alkali-insoluble fraction derived from steam-treated eucalyptus. Pretreatment was carried out under catalysed conditions (SO_2-STE-WIA). Hydrolyses were performed at a 2% (w/v) substrate concentration and a fixed enzyme loading of 10 FPU g^{-1} cellulose. Pretreated substrates were derived from chips with a moisture content of (■) 15, (▲) 23, (*) 50, and (□) 100% (w/w) in relation to their dry weight.

greatly influenced the solubility and extraction of the lignin.

When pretreatment was carried out without explosion and added catalyst, alkali washing apparently had no effect on hydrolysis of pretreated substrates at 2% (w/v) (Fig. 13). However, alkali washing did increase the efficiency of hydrolysis of non-impregnated, air-dried chips which were steam-exploded rather than steam-heated (Fig. 14).

Although alkali washing significantly influenced the rate of hydrolysis of pretreated substrates derived from chips with low moisture content, it was not clear whether delignification could enhance the digestibility of the water-insoluble fraction derived from chips with higher moisture content. Therefore, the chemical composition and the extent of hydrolysis of this substrate was determined before and after alkali extraction (Table 16). Although alkali extraction reduced the lignin content of the STE-WI fraction by approximately 73% (w/w), lignin removal had only a little effect on hydrolysis when the yields were expressed in terms of the available cellulose. Alkali washing also reduced the lignin content of the SO_2-STE-WI fraction by approximately 77% (Table 16). However, the removal of lignin from the SO_2-STE-WI substrate seemed to decrease the rate of hydrolysis (Fig. 15).

We have routinely calculated the enzyme loading used for hydrolysis in relation to the cellulose content of the substrate. However, since both SO_2-SEE-WI and SO_2-SEE-WIA fractions differed in their cellulose content, the actual effect

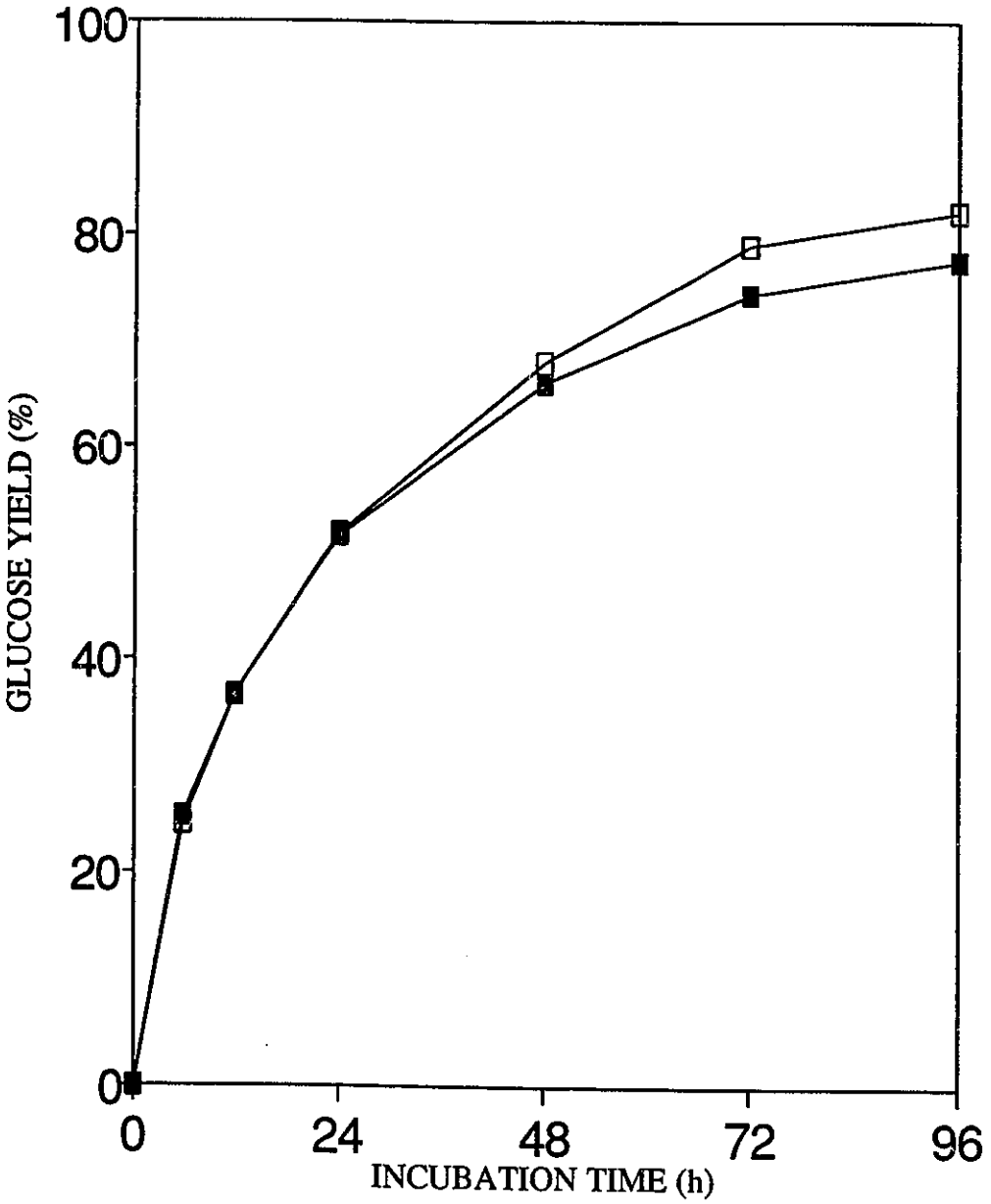


Fig. 13. Effect of alkali washing on enzymatic hydrolysis of steam-treated substrates (no explosion) derived from non-impregnated eucalyptus chips (moisture content of 50%, w/w). Hydrolyses were carried out at a 2% (w/v) substrate concentration and a fixed enzyme loading of 10 FPU g^{-1} cellulose. (■) STE-WI, water-insoluble fraction; (□) STE-WIA, alkali-insoluble fraction.

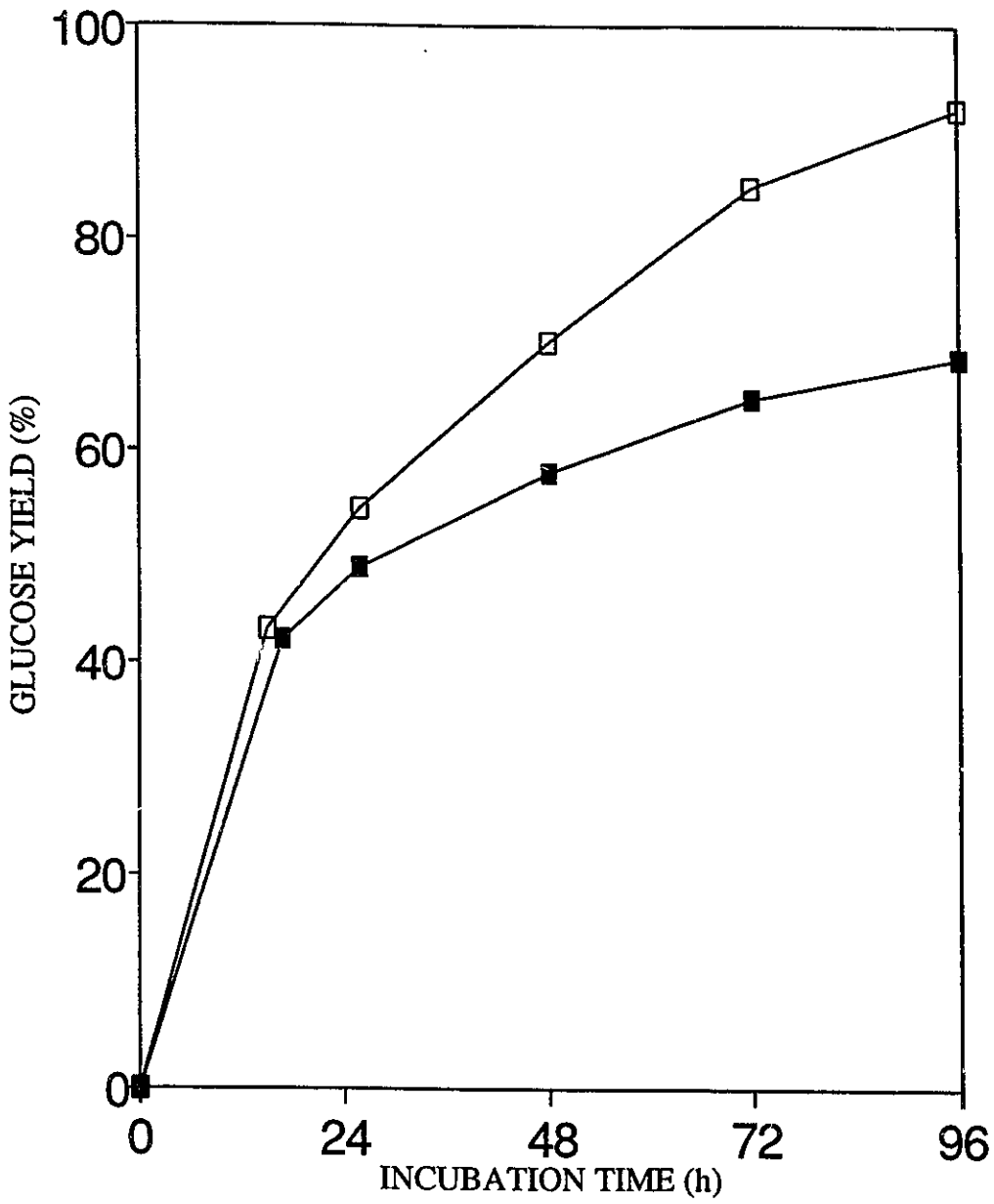


Fig. 14. Effect of alkali-washing on enzymatic hydrolysis of steam-exploded substrates derived from non-impregnated, air-dried eucalyptus chips (moisture content of 5%, w/w). Hydrolyses were carried out at a 2% (w/v) substrate concentration and a fixed enzyme loading of 10 FPU g^{-1} cellulose. (■) SEE-WI, water-insoluble fraction; (□) SEE-WIA, alkali-insoluble fraction.

Table 16. Chemical composition and enzymatic hydrolysis of several insoluble substrates derived from steam-treated eucalyptus chips under both non-catalysed and SO_2-catalysed pretreatment conditions.

SUBSTRATE[1]	CHEMICAL[2] COMPOSITION (%)		CELLULOSE HYDROLYSIS[4] (%)	SUBSTRATE HYDROLYSIS[5] (%)
	Glucan	TAL[3]		
untreated	41.7	31.0	2.2	0.9
STE-WI	56.2	39.8	74.5	41.7
STE-WIA	81.7	10.8	79.0	65.0
SO_2-STE-WI	57.7	33.2	94.5	54.1
SO_2-STE-WIA	86.7	7.5	94.6	82.1
SO_2-SEE-WIA/H_2O_2	94.9	0.6	99.9	99.4

[1] SO_2-, SO_2-impregnated chips; -STE-, steam-treated eucalyptus chips (no explosion); -SEE-, steam-exploded eucalyptus chips; WI, water-insoluble fraction; WIA, alkali-insoluble fraction; WIA/H_2O_2, peroxide-treated fraction; STE substrates were prepared from chips with a moisture content of 50% (w/w), whereas both SO_2-STE and SO_2-SEE substrates were obtained from green chips (moisture content of 100%, w/w);
[2] The glucan content of the substrates was determined by HPLC analysis of the acid hydrolysate from Klason lignin determinations; all figures were calculated in relation to the chips dry weight;
[3] Total apparent lignin (acid-insoluble plus acid-soluble fractions);
[4] Extent of substrate saccharification was calculated in relation to the cellulose content of the substrate; hydrolysis was carried out for 72 h at a 2% (w/v) substrate concentration and an enzyme loading of 9-10 FPU g^{-1} cellulose;
[5] Extent of substrate saccharification was calculated in relation to the dry weight of the substrate; hydrolysis was carried out as described above.

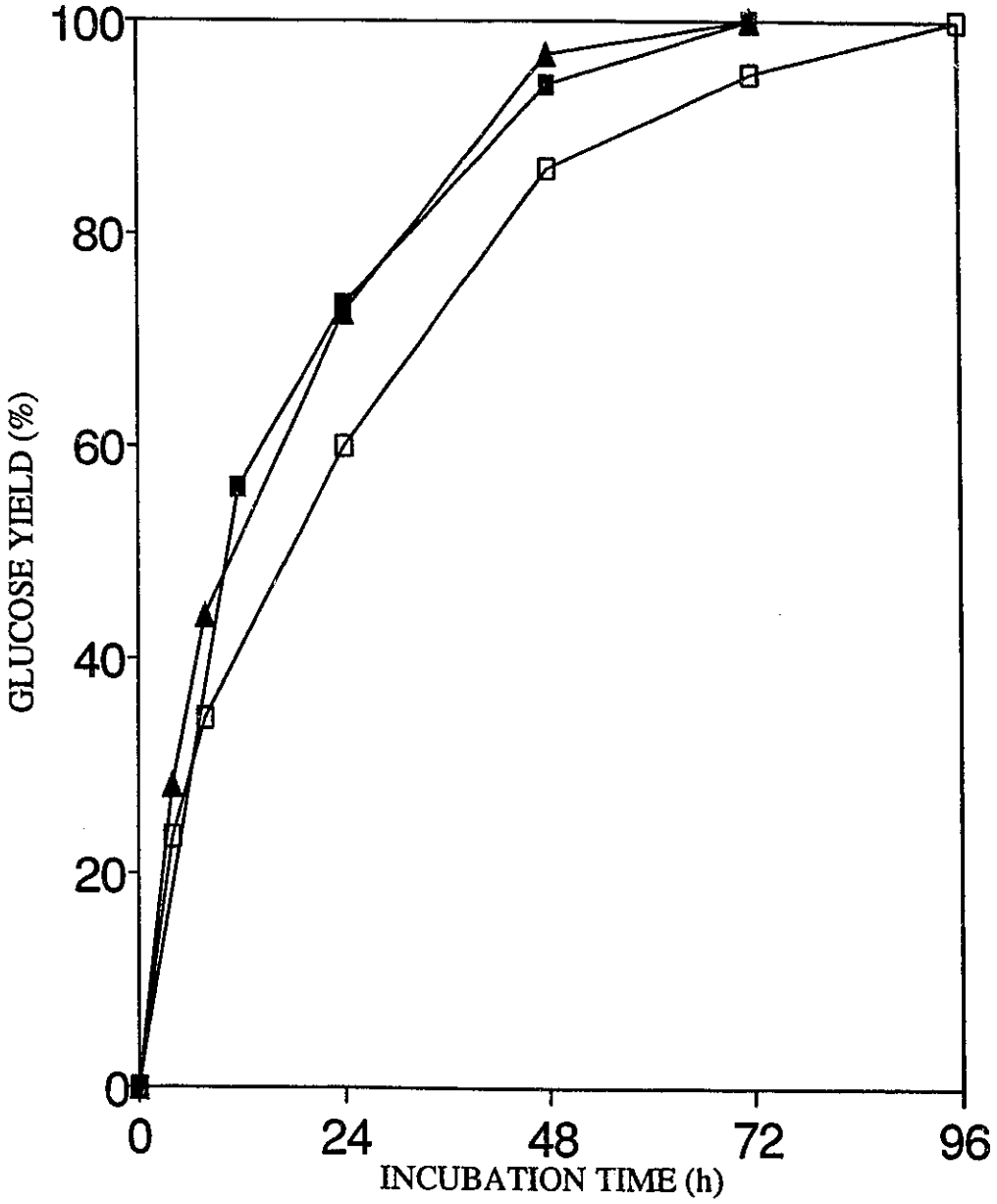


Fig. 15. Enzymatic hydrolysis of several pretreated substrates obtained from SO_2-impregnated, steam-exploded eucalyptus chips (mosture content of 100%, w/w). Hydrolyses were carried out at a 2% (w/v) substrate concentration and a fixed enzyme loading of 10 FPU g^{-1} cellulose. (■) SO_2-SEE-WI, water-insoluble fraction; (□) SO_2-SEE-WIA, alkali-insoluble fraction; and (▲) SO_2-SEE-WIA/H_2O_2, peroxide-treated fraction

of lignin on hydrolysis could only be determined if both fractions were hydrolysed at the same cellulose concentration and a fixed enzyme loading. Therefore, the enzymatic hydrolyses of these substrates were carried out at a 5% (w/v) cellulose concentration and an enzyme loading of 10 FPU g^{-1} cellulose (Fig. 16). Our results indicated that both the SO_2-SEE-WI and SO_2-SEE-WIA fractions resulted in similar hydrolysis profiles and that the lignin present in the WI fraction did not significantly restrict hydrolysis. However, neither substrate was completely hydrolysed after 96 h of incubation. When these recalcitrant residues were examined by light microscopy (LM), libriform fibres and parenchyma cells were found to be attacked to a much greater extent than tracheary elements such as the plant vessels. Fibre bundles also accumulated in the recalcitrant residue and they may represent the proportion of the original wood chips which were incompletely steam-treated.

A more extensive delignification of the SO_2-SEE-WI fraction was observed when this substrate was post-treated with alkaline hydrogen peroxide (Table 16). Although the greater ease of hydrolysis of the SO_2-SEE-WIA/H_2O_2 fraction, compared to the SO_2-SEE-WIA fraction, could be primarily attributed to its very low Klason lignin content (Fig. 15), it is probable that the peroxide treatment resulted in changes in the fine structure of the substrate, thus enhancing the surface area available for substrate/enzyme interactions.

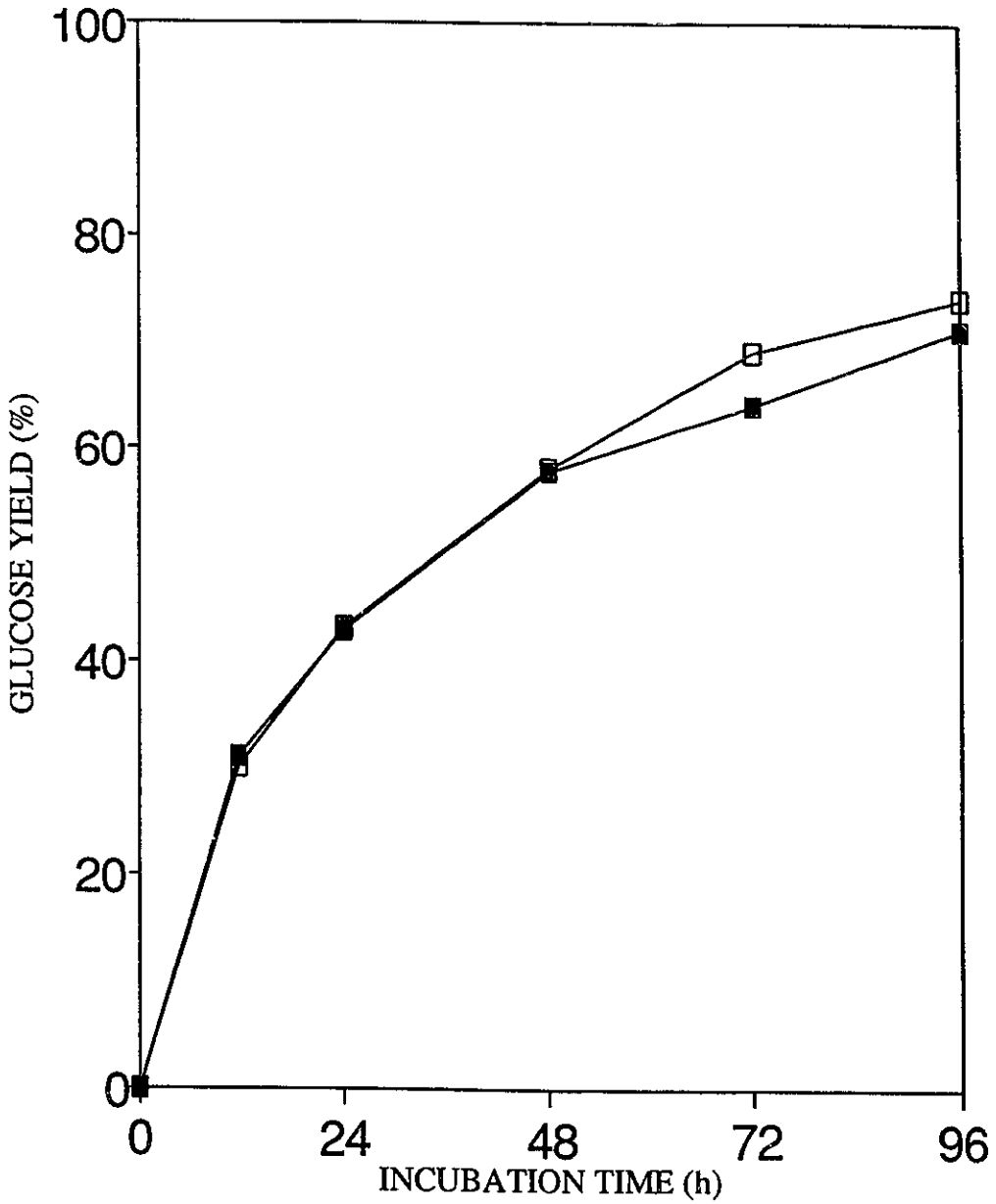


Fig. 16. Influence of lignin removal on the enzymatic hydrolysis profile of pretreated fractions derived from SO_2-impregnated, steam-exploded eucalyptus chips (moisture content of 100%, w/w). Hydrolyses were carried out at a 5% (w/v) cellulose concentration and a fixed enzyme loading of 10 FPU g^{-1} cellulose. (■) SO_2-SEE-WI, water-insoluble fraction; (□) SO_2-SEE-WIA, alkali-insoluble fraction.

3.1.4. Effect of Varying the Substrate and Enzyme Loading on the Hydrolysis of Pretreated Substrates

A comparison of the efficiency of hydrolysis of the SO_2-STE-WI fraction by varying concentrations of *Trichoderma* cellulases (Celluclast) was initially carried out at a substrate concentration of 2% (w/v) (Fig. 17). When the hydrolysis yield was calculated based on the amount of glucose released, only 40% of the initial material was hydrolysed to glucose at an enzyme loading of 20 FPU g^{-1} cellulose. Increased concentrations of cellobiose were gradually observed during hydrolysis owing to the low residual cellobiase activity present in the Celluclast preparation. As cellobiose was one of the major components of the hydrolysate, it seemed that end-product inhibition of cellulases was a key factor contributing to the decrease in the rate of the reaction.

To minimize the effects of cellobiose accumulation during hydrolysis, we added an excess of exogenous cellobiase (Novozym) activity to a Celluclast preparation containing 9 FPU g^{-1} cellulose. When the total filter paper activity of the enzyme mixture was determined, we observed that, by supplementing Celluclast with 30 CBU g^{-1} cellulose of Novozym, the original activity of 9 FPU was increased to 20 FPU g^{-1} cellulose. As a result, a much better glucose yield was obtained during hydrolysis of steam-treated eucalyptus with the supplemented Celluclast (Fig. 18), with the cellobiose concentration remaining at very low levels throughout hydrolysis. The

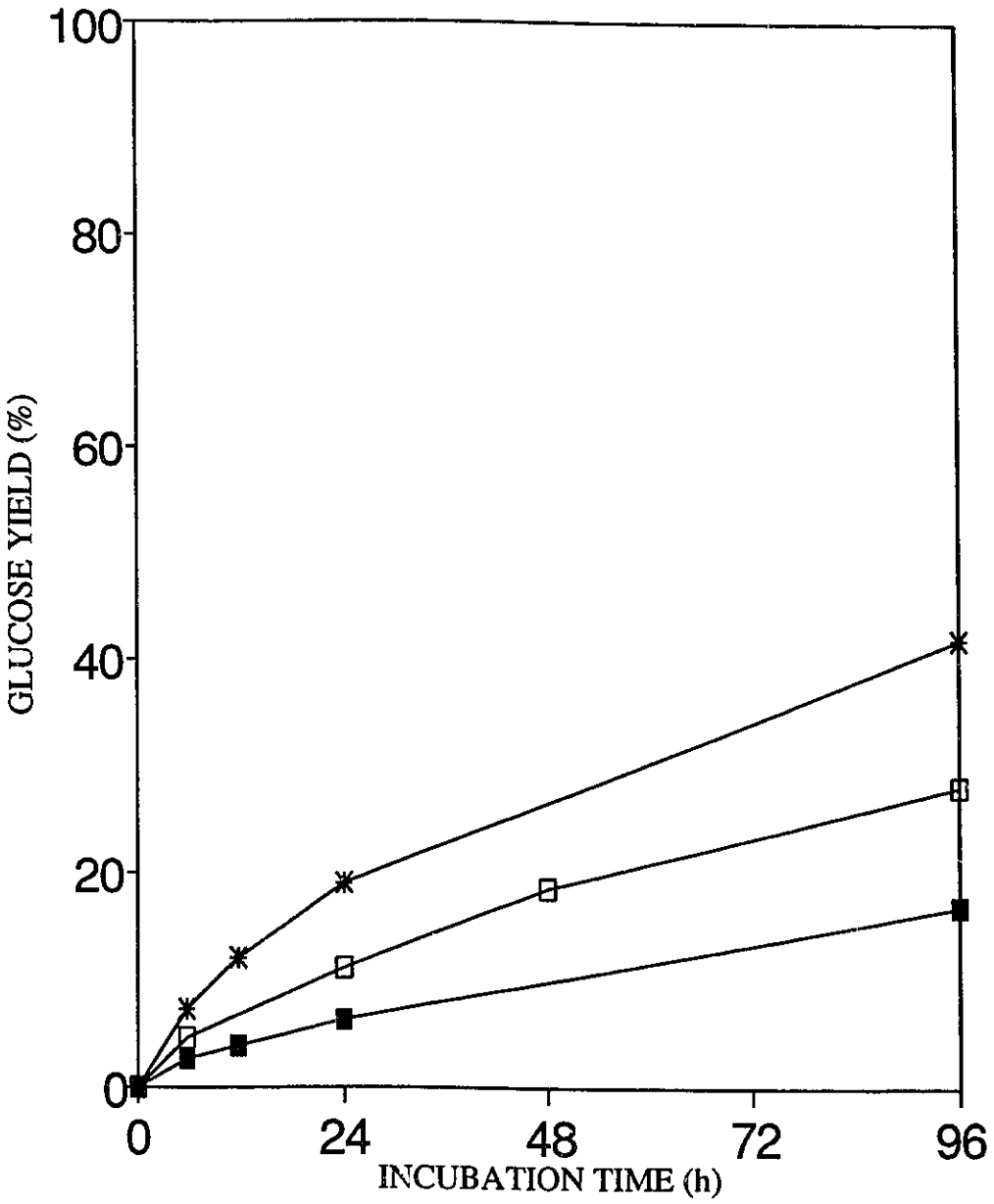


Fig. 17. Enzymatic hydrolysis of the water-insoluble fraction derived from SO_2-impregnated steam-treated eucalyptus chips (SO_2-STE-WI) using various loadings of a commercial *Trichoderma* cellulase (Celluclast, Novo). (■) 4.5, (□) 9.0, and (*) 19.5 FPU g^{-1} cellulose.

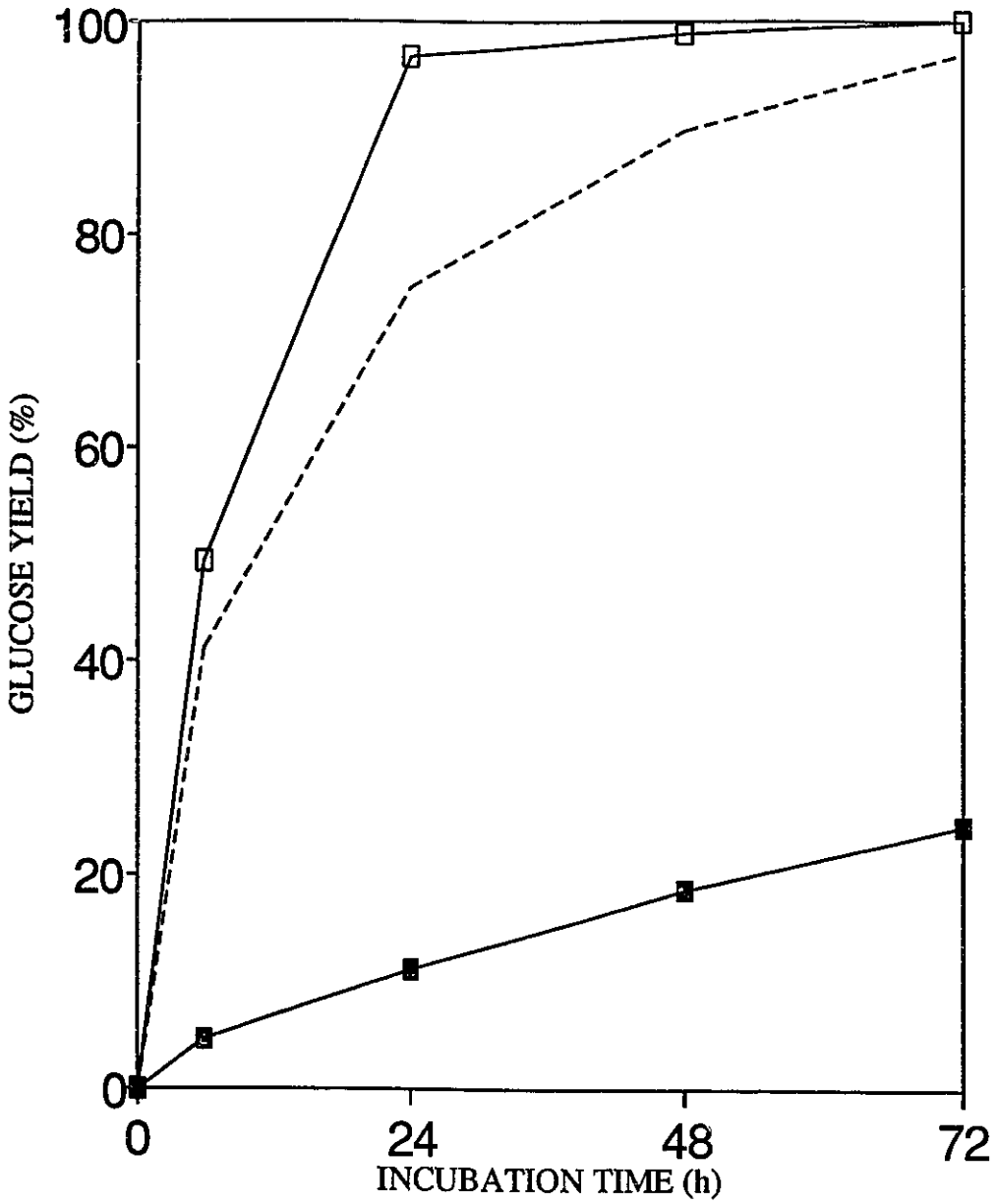


Fig. 18. Effect of supplemental cellobiase activity (Novozym, Novo) on the enzymatic hydrolysis of the water-insoluble fraction (SO_2-STE-WI) derived from SO_2-impregnated steam-treated eucalyptus chips. Hydrolyses were carried out at 2% (w/v) with an enzyme loading of either (■) 9 FPU g^{-1} cellulose of Celluclast or (□) 9 FPU g^{-1} cellulose of Celluclast supplemented with 30 CBU g^{-1} cellulose of Novozym. The broken line indicates the hydrolysis profile when the unsupplemented Celluclast was used and the yields were calculated based on both glucose and cellobiose release.

supplemented Celluclast resulted in better hydrolysis of the SO_2-STE-WI fraction even when the yields were calculated based on both glucose and cellobiose release (Fig. 18, broken line). Therefore, the boosting effect of added cellobiase on the ability of the enzyme mixture to degrade cellulose was attributed to a decrease in the levels of end-product inhibition of cellulases during hydrolysis (Mandels *et al.*, 1981). To ensure that we were achieving maximum yields which were not restricted by the accumulation of cellobiose, hydrolyses of the pretreated substrates were routinely carried out using Celluclast supplemented with Novozym at a ratio of 3:1 (v/v).

The effect of increased substrate concentration on hydrolysis was initially investigated using relatively pure cellulose preparations to avoid the possible influence of lignin accumulation during hydrolysis. The three substrates chosen for this experiment, Solka-floc, Sigmacell and phosphoric acid-swollen Sigmacell (PASS), were hydrolysed at a fixed enzyme loading of 10 FPU g^{-1} and substrate concentrations of 2, 6 and 10% (w/v) (Fig. 19 A-C). Both the microcrystalline cellulose preparations, Solka-floc and Sigmacell, showed similar hydrolysis profiles at both 2 and 10% (w/v) substrate concentrations (Fig. 19 A,B). It appeared that factors such as end-product inhibition and the possible recalcitrancy of the substrate were more pronounced when hydrolysis was carried out at high substrate concentrations. Although hydrolysis of the phosphoric acid-swollen fraction (PASS) was not performed at 10%

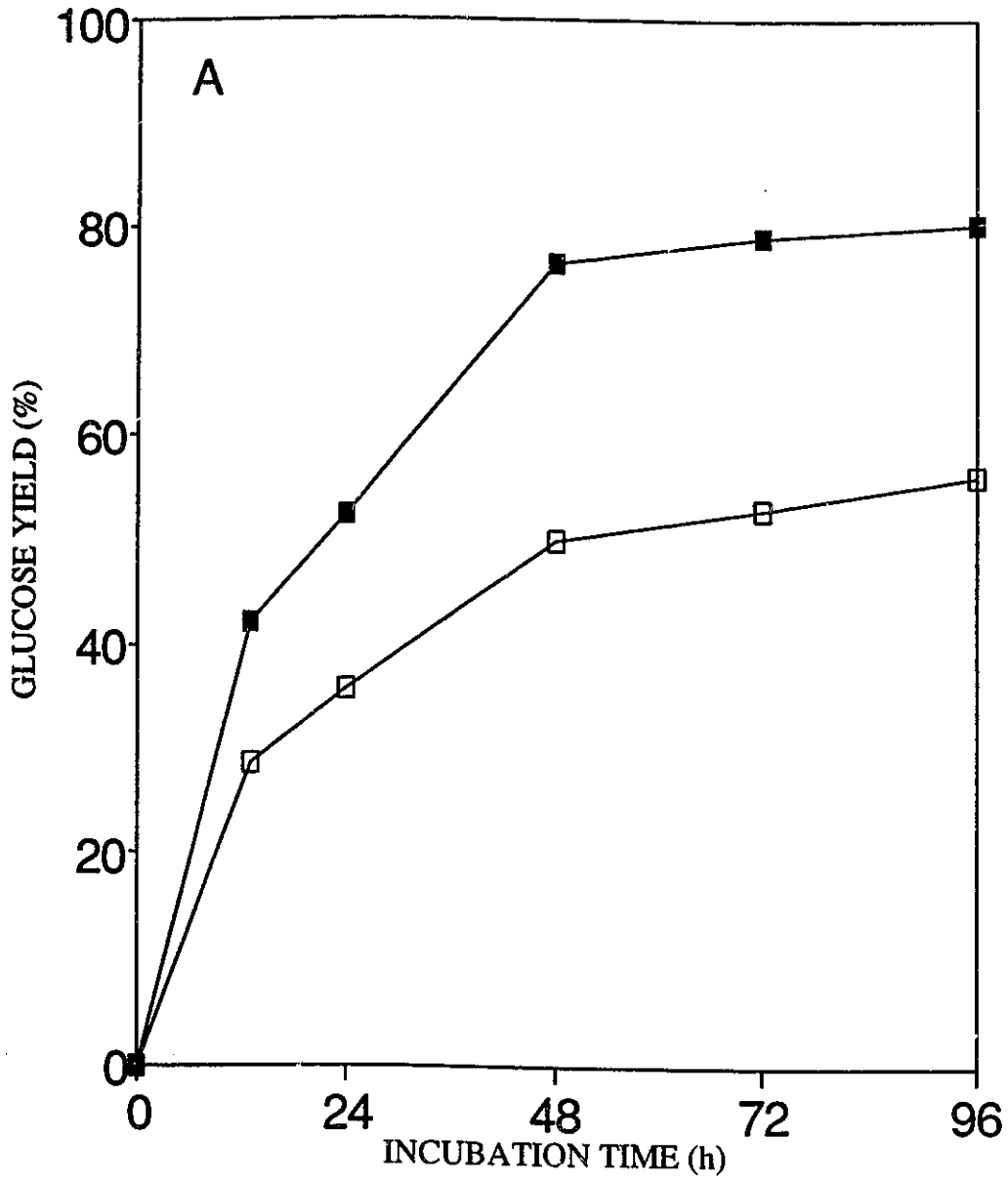


Fig. 19 A. Effect of high substrate concentrations on the enzymatic hydrolysis of Sigmacell. Hydrolyses were carried out at substrate concentrations of (□) 2% and (■) 10% (w/v) and at a fixed enzyme loading of 10 FPU g^{-1} cellulose.

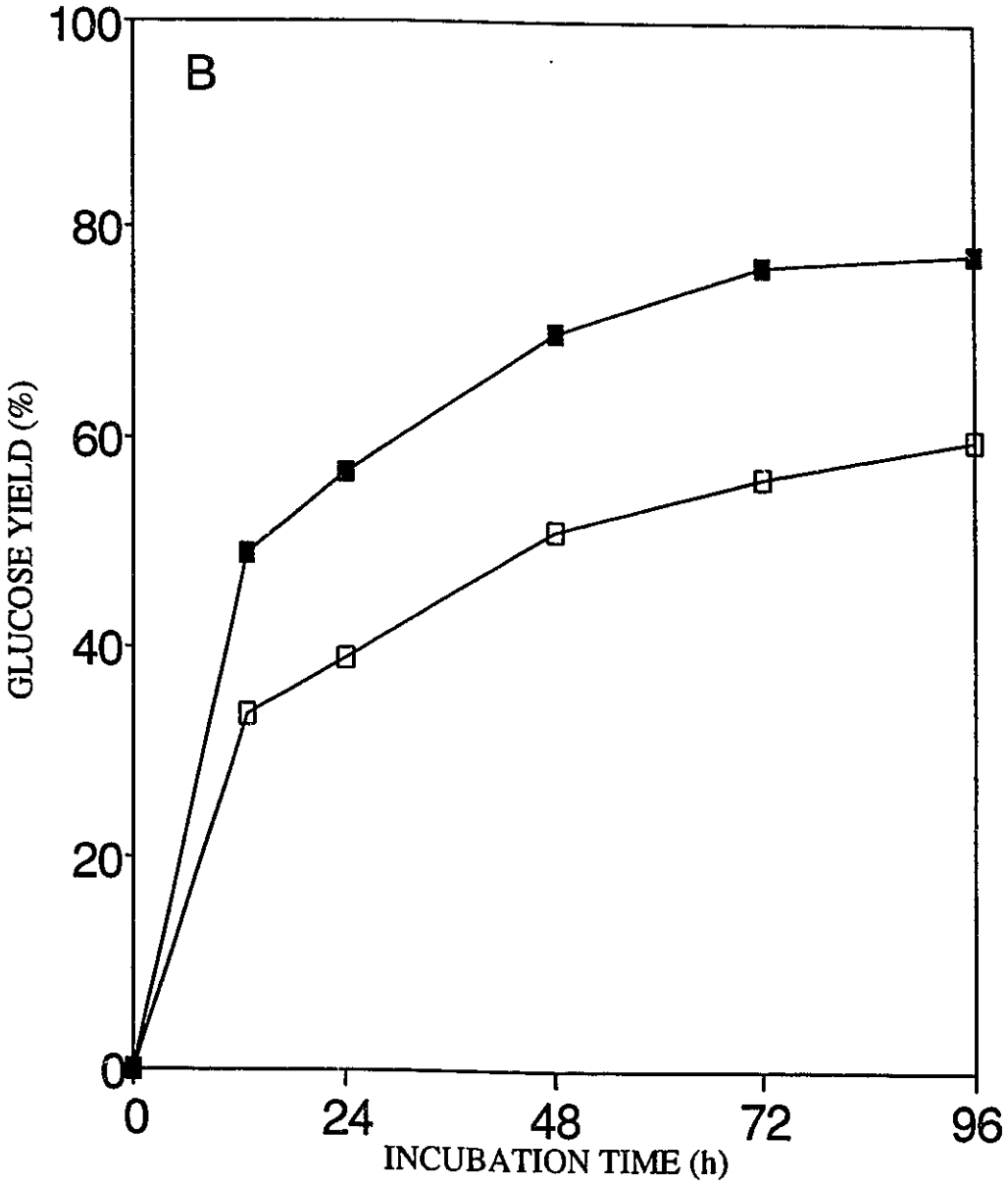


Fig. 19 B. Effect of high substrate concentrations on the enzymatic hydrolysis of Solka-floc. Hydrolyses were carried out at substrate concentrations of (□) 2% and (■) 10% (w/v) and at a fixed enzyme loading of 10 FPU g^{-1} cellulose.

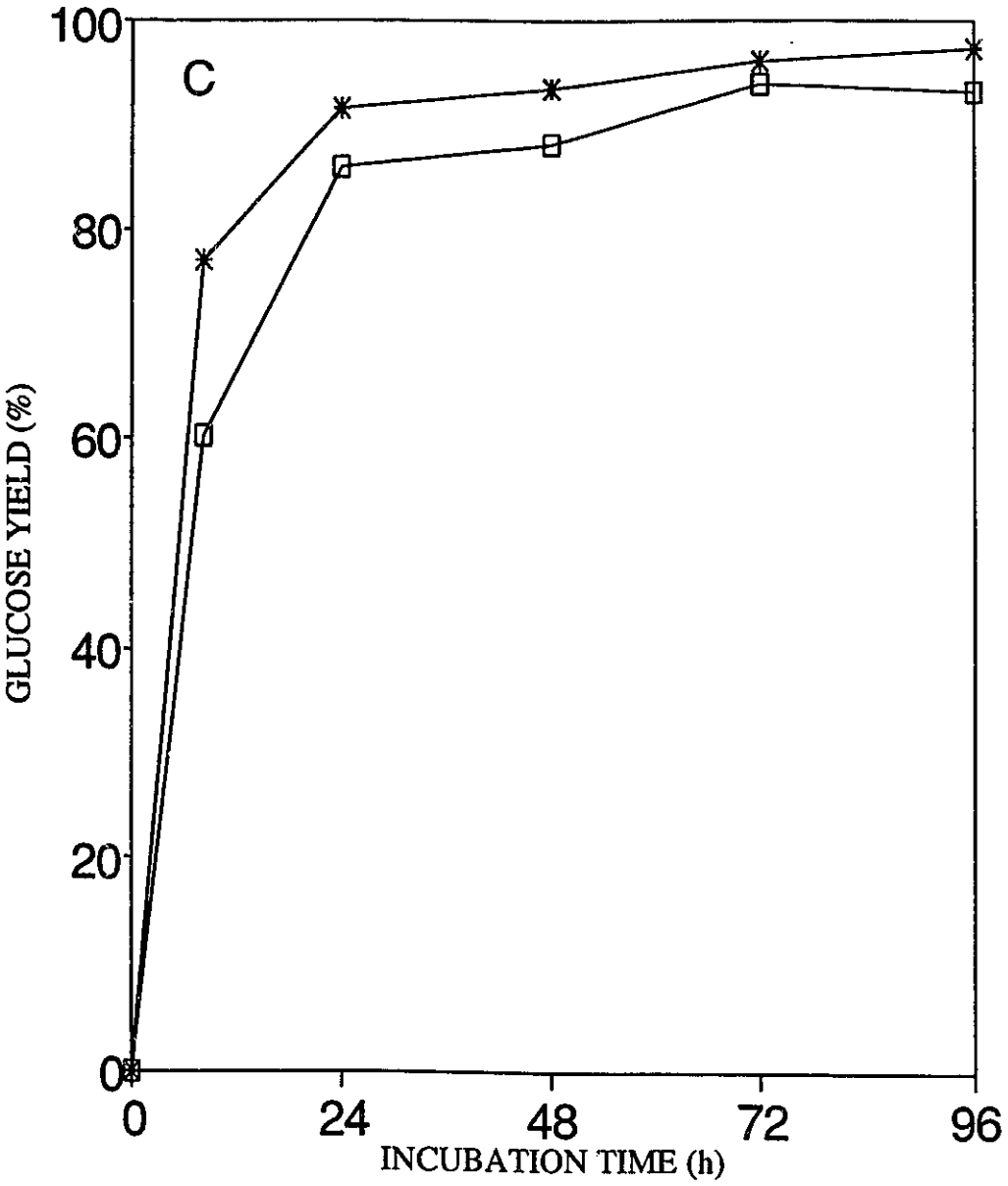


Fig. 19 C. Effect of high substrate concentrations on the enzymatic hydrolysis of phosphoric acid-swollen Sigmacell (PASS). Hydrolyses were carried out at substrate concentrations of (□) 2% and (*) 6% (w/v) and at a fixed enzyme loading of 10 FPU g^{-1} cellulose.

(w/v), this fraction showed a much higher susceptibility to hydrolysis at both 2 and 6% (w/v) substrate concentration, compared to the other two cellulose preparations, Solka-floc and Sigmacell (Fig. 19 C).

The effect of varying the enzyme loading of a Celluclast/Novozym mixture was then carried out for both the water-washed (SO_2-SEE-WI) and alkali-washed (SO_2-SEE-WIA) fractions at a substrate concentration of 2% (w/v). The rate of hydrolysis of both fractions increased with increasing enzyme loading. A complete hydrolysis of SO_2-SEE-WI at 2% (w/v) could be achieved after 24 h using a high enzyme loading of 20 FPU g^{-1} cellulose (Table 17). In some cases, values greater than 100% were obtained. This probably reflects a problem in quantifying the amount of cellulose originally present in the substrate. For the hydrolysis of 2% (w/v) SO_2-SEE-WIA, all of the cellulose could be hydrolysed after a 48 h incubation when an enzyme loading of 15 FPU g^{-1} cellulose was used. Even at an enzyme loading of 8.7 FPU g^{-1} cellulose, almost 97% of the original cellulose could be detected as glucose after a 72 h incubation. Although the SO_2-SEE-WIA hydrolysis produced a higher glucose concentration in the hydrolysate due to the higher cellulose content of this fraction, its actual hydrolysis yield was slightly less than that obtained for the SO_2-SEE-WI fraction.

We next compared the extent of cellulose hydrolysis when increasing concentrations of enzyme were added to a 10% (w/v) concentrations of the pretreated substrate. When the WI fraction

Table 17. Effects of increased enzyme loadings on the enzymatic hydrolysis of both the water-insoluble (SO_2-SEE-WI) and alkali-insoluble (SO_2-SEE-WIA) fractions derived from SO_2-impregnated, steam-exploded eucalyptus chips at a substrate concentration of 2% (w/v).

SUBSTRATE[1]	ENZYME LOADING[2] (FPU g^{-1})	GLUCOSE YIELD[3] (%)				
		Incubation time (h)				
		06	12	24	48	72
SO_2-SEE-WI	4.4	17.4	29.0	43.1	58.2	72.0
	7.4	27.6	46.2	69.3	91.0	100.3
	12.5	34.8	60.0	75.8	98.4	101.3
	19.7	49.1	74.8	100.1	101.4	103.2
SO_2-SEE-WIA	3.1	14.4	21.4	28.0	44.6	57.7
	5.1	20.7	33.3	44.2	68.3	80.7
	8.7	28.3	43.4	60.5	85.2	96.8
	15.1	41.3	nd[4]	82.7	101.4	103.1
	19.7	47.0	nd	90.9	100.1	103.6

[1] SO_2-SEE, SO_2-impregnated steam-exploded eucalyptus; WI, water-insoluble fraction; WIA, alkali-insoluble fraction;
[2] Amount of enzyme activity added to the hydrolysis mixture, expressed as filter paper units per gram (FPU g^{-1}) in relation to the cellulose content of the substrate;
[3] Glucose release was determined in the hydrolysates by HPLC and calculated as a fraction of the maximum glucose yield attainable from the pretreated substrate;
[4] Not determined.

was hydrolysed at 10% (w/v), an incubation of 96 h was required for complete hydrolysis at enzyme loadings of 15-25 FPU g^{-1} cellulose (Table 18). It is probable that, at high substrate concentrations, the cellulase complex is inhibited by hydrolysis products such as glucose and cellobiose and/or impurities produced during steaming. Therefore, higher enzyme loadings seemed to be more important when higher substrate concentrations were used. For the WIA fraction, only 85% of the original cellulose was hydrolysed to glucose after 96 h incubation at an enzyme loading of 20.7 FPU g^{-1}. In both cases, there was no linear relationship between the efficiency of hydrolysis and the amount of enzyme added to the substrate.

The effect of enzyme loading on hydrolysis of the peroxide-treated fraction (SO_2-SEE-WIA/H_2O_2) was investigated at a 6% (w/v) substrate concentration. In agreement with the previous results which indicated that higher enzyme loadings resulted in a greater extent of hydrolysis, there was a significant difference in the rate of the reaction at each of the enzyme concentrations (Table 19). Approximately 90% of the substrate was hydrolysed after 72 h at an enzyme concentration of 4.2 FPU g^{-1}, while almost all of the substrate was hydrolysed after 48 h when an enzyme concentration of 15.5 FPU g^{-1} was used. When the hydrolysates from each of the reactions were analysed, we found that glucose was the predominant sugar with only low levels of cellobiose detected when the lower enzyme loading of 4.2 FPU g^{-1} was used. We next compared the hydrolysis profile of the SO_2-SEE-

Table 18. Effects of increased enzyme loadings on the enzymatic hydrolysis of both the water-insoluble (SO_2-SEE-WI) and alkali-insoluble (SO_2-SEE-WIA) fractions derived from SO_2-impregnated, steam-exploded eucalytpus chips at a substrate concentration of 10% (w/v).

SUBSTRATE[1]	ENZYME LOADING[2] (FPU g^{-1})	GLUCOSE YIELD[3] (%)				
		Incubation time (h)				
		12	24	48	72	96
SO_2-SEE-WI	7.8	33.6	44.9	68.2	75.9	85.8
	10.8	40.1	53.6	78.0	90.1	99.2
	14.8	45.7	61.6	86.9	99.1	100.7
	24.9	48.3	67.5	90.0	98.1	98.2
SO_2-SEE-WIA	9.9	28.1	39.7	52.6	60.0	68.6
	10.3	28.5	40.8	54.2	65.2	71.1
	17.4	32.4	44.2	61.3	75.8	82.5
	20.7	36.0	50.7	69.0	81.1	84.9

[1] SO_2-SEE, SO_2-impregnated steam-exploded eucalyptus; WI, water-insoluble fraction; WIA, alkali-insoluble fraction;
[2] Amount of enzyme activity added to the hydrolysis mixture, expressed as filter paper units per gram (FPU g^{-1}) in relation to the cellulose content of the substrate;
[3] Glucose release was determined in the hydrolysate by HPLC and calculated as a fraction of the maximum glucose yield attainable from the pretreated substrate.

Table 19. Effects of increasing enzyme loadings on the enzymatic hydrolysis of the peroxide-treated fraction derived from SO_2-impregnated, steam-exploded eucalytpus chips (SO_2-SEE-WIA/H_2O_2) at a substrate concentration of 6% (w/v).

ENZYME LOADING[1] (FPU g^{-1})	GLUCOSE YIELD[2] (%)				
	Incubation time (h)				
	06	22	48	72	96
4.2	28.9	56.0	75.6	83.4	85.5
8.3	40.1	72.6	87.6	87.3	88.5
15.5	52.0	84.9	90.6	91.5	92.5

[1] Amount of enzyme activity added to the hydrolysis mixture, expressed as filter paper units per gram (FPU g^{-1}) in relation to the cellulose content of the substrate;
[2] Glucose release was determined in the hydrolysate by HPLC and calculated as a fraction of the maximum glucose yield attainable from the pretreated substrate.

WI, WIA and WIA/H_2O_2 substrates at a 10% (w/v) concentration using a fixed enzyme loading of 10 FPU g^{-1} cellulose (Fig. 20). A better rate of hydrolysis was observed for the substrate which had been further extracted with alkaline hydrogen peroxide. As expected, the rates of hydrolysis obtained for all of the fractions at a 10% (w/v) concentration were lower than those obtained previously at 2% (w/v) (Fig. 15).

3.2. Comparison of the Efficiency of Hydrolysis of Pretreated Substrates Derived from Several Wood Species

In earlier work with aspen (Schwald *et al.*, 1989a) and spruce (Schwald *et al.*, 1989b), the optimum steam pretreatment conditions for enhanced enzymatic hydrolysis and recovery yield of the pretreated fractions had been determined. It was generally found that the more drastic the pretreatment, the lower the recovery yield and the better the enzymatic accessibility of the resulting substrate. When the steaming conditions used for pretreating aspen, spruce and eucalyptus were compared, it was apparent that eucalyptus chips required less drastic pretreatment conditions than aspen and spruce to obtain best recovery yields and optimum substrate hydrolysis (Table 20, Fig. 21). Subsequent water and alkali extraction of the steam-exploded substrates was shown to be beneficial as it separated the majority of the hemicellulose and lignin components into different fractions for further processing. We also hoped that the removal of most of the hemicellulose and

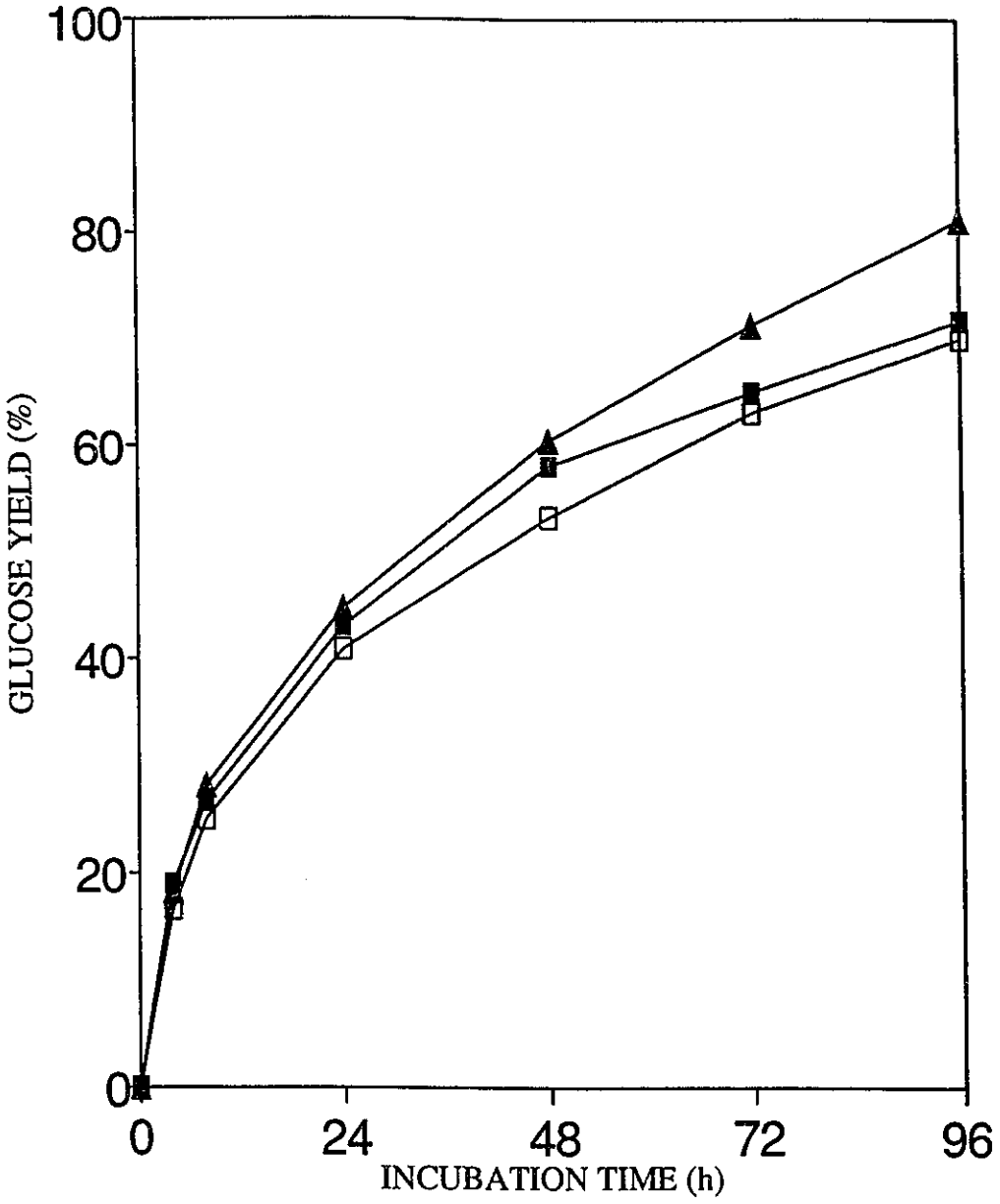


Fig. 20. Effect of high substrate concentrations on the enzymatic hydrolysis of several fractions obtained after steam explosion of SO_2-impregnated eucalyptus chips (moisture content of 100%, w/w). Hydrolyses were carried out at substrate concentration of 10% (w/v) and an enzyme loading of 10 FPU g^{-1} cellulose. (■) SO_2-SEE-WI, water-insoluble fraction; (□) SO_2-SEE-WIA, alkali-insoluble fraction; and (▲) SO_2-SEE-WIA/H_2O_2, peroxide-treated fraction.

Table 20. Steam pretreatment conditions used to obtain the maximum carbohydrate recovery and the highest hydrolysis yields from eucalyptus, aspen and spruce chips.

FRACTION[1]	PRETREATMENT CONDITIONS			RECOVERY YIELD[2] (%)	CHEMICAL COMPOSITION[3] (%)	
	Temp. (°C)	Time (s)	$[SO_2]^2$ (%)		Glucan	Lignin
Eucalyptus viminalis (EUCALYPTUS), SO_2-SEE-						
untreated	-	-	-	100	41.4	27.0
WI	210	50	1.0	58.6	58.6	30.4
WIA				49.5	84.4	5.4
WIA/H_2O_2				41.8	90.1	0.6
Populus tremuloides (ASPEN)[4], SO_2-SEA-						
untreated	-	-	-	100	42.7	20.9
WI	210	100	1.6	56.9	62.6	30.3
WIA				35.3	85.9	5.4
WIA/H_2O_2				33.0	86.6	2.2
Picea albis (SPRUCE)[5], SO_2-SES-						
untreated	-	-	-	100	40.4	27.3
WI	210	150	2.5	60.8	50.2	45.8
WIA				51.2	58.7	37.1
WIA/H_2O_2				45.6	80.2	15.0

[1] SO_2-impregnated eucalyptus (SO_2-SEE), aspen (SO_2-SEA) and spruce (SO_2-SES); water-insoluble (WI), alkali-insoluble (WIA) and peroxide-treated (WIA/H_2O_2) fractions;
[2] Calculated in relation to the chips dry weight;
[3] The carbohydrate content of the substrates was determined by HPLC analysis of the acid hydrolysate from Klason lignin determinations; all figures were calculated in relation to the chips dry weight;
[4] Extracted from Schwald *et al.* (1989a) and Breuil *et al.* (1990);
[5] Extracted from Schwald *et al.* (1989b).

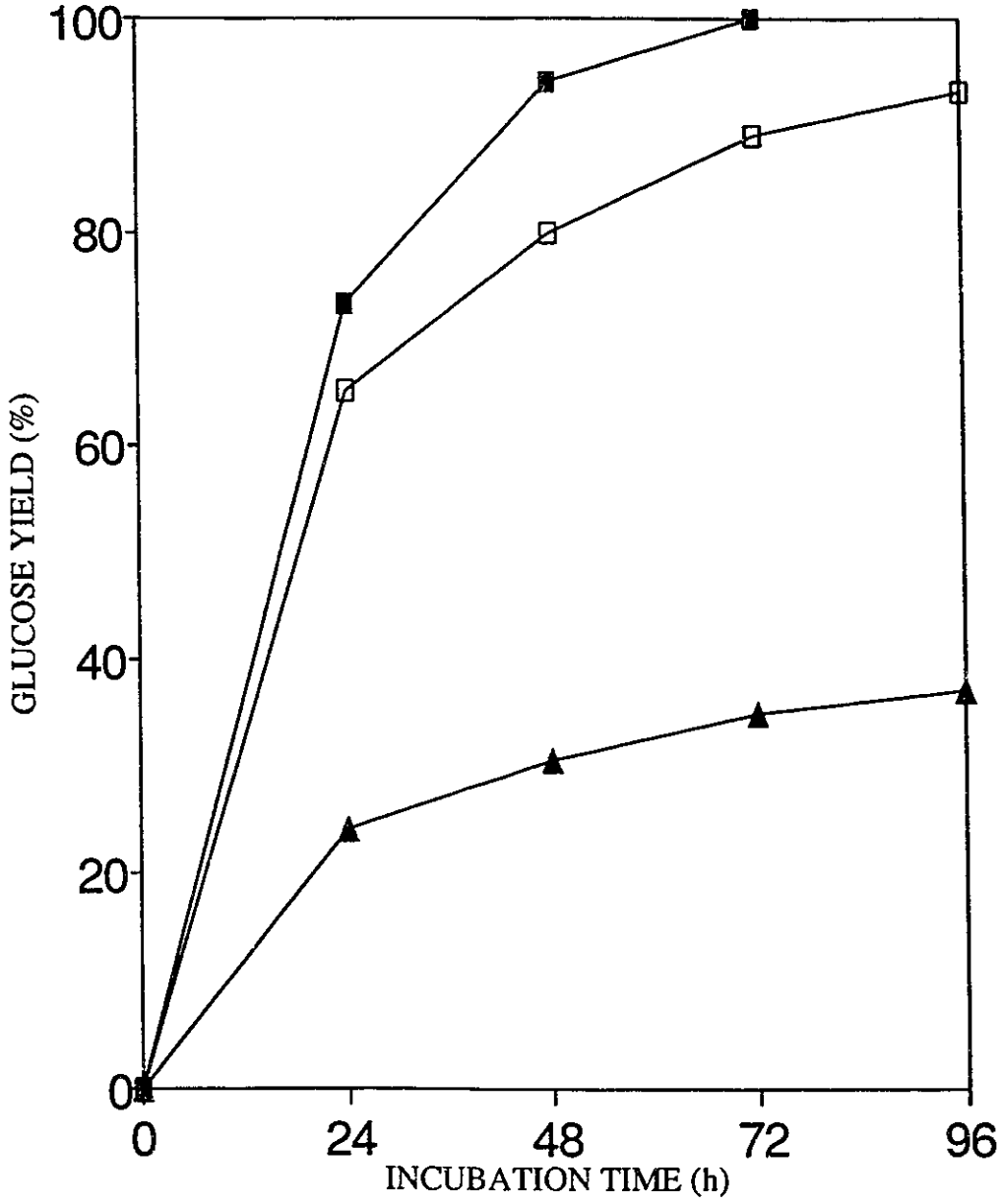


Fig. 21. Comparison of the enzymatic hydrolysis profile of the water-insoluble fractions derived from steam-exploded (■) *E. viminalis* (SO_2-SEE-WI), (□) aspen (SO_2-SEA-WI), and (▲) spruce (SO_2-SES-WI) chips. Hydrolyses were carried out at 2% (w/v) substrate concentration and a fixed enzyme loading of 10 FPU g^{-1} cellulose.

lignin would increase the accessibility of the cellulose to the cellulase enzymes. Although alkali extraction could remove a substantial amount of the original lignin present in aspen and eucalyptus, hydrolysis was only marginally increased for both substrates. However, the ease of hydrolysis of the pretreated substrate derived from spruce was significantly reduced after alkali washing (Schwald *et al.*, 1989b). Partial removal of the alkali-insoluble lignin by post-treating the alkali-insoluble fraction with hydrogen peroxide was shown to restore the susceptibility of steam-treated softwoods to hydrolysis.

We next carried out a direct comparison among the three peroxide-treated substrates which had been prepared from eucalyptus, aspen and spruce using the conditions described in Table 20 (Fig. 22). At a 2% (w/v) substrate concentration (Fig. 22 A), both aspen (SO_2-SEA-WIA/H_2O_2) and eucalyptus (SO_2-SEE-WIA/H_2O_2) showed a similar hydrolysis profile. Although spruce (SO_2-SES-WIA/H_2O_2) exhibited a slower rate of hydrolysis, it could still be completely hydrolysed after 96 h. At higher (10%, w/v) substrate concentrations (Fig. 22 B), eucalyptus demonstrated substantially better hydrolysis while aspen fell between eucalyptus and spruce.

3.3. Influence of Enzyme Recycling and End-product Inhibition on Hydrolysis

To investigate whether the enzymes were still active after complete saccharification of the peroxide-treated substrate at

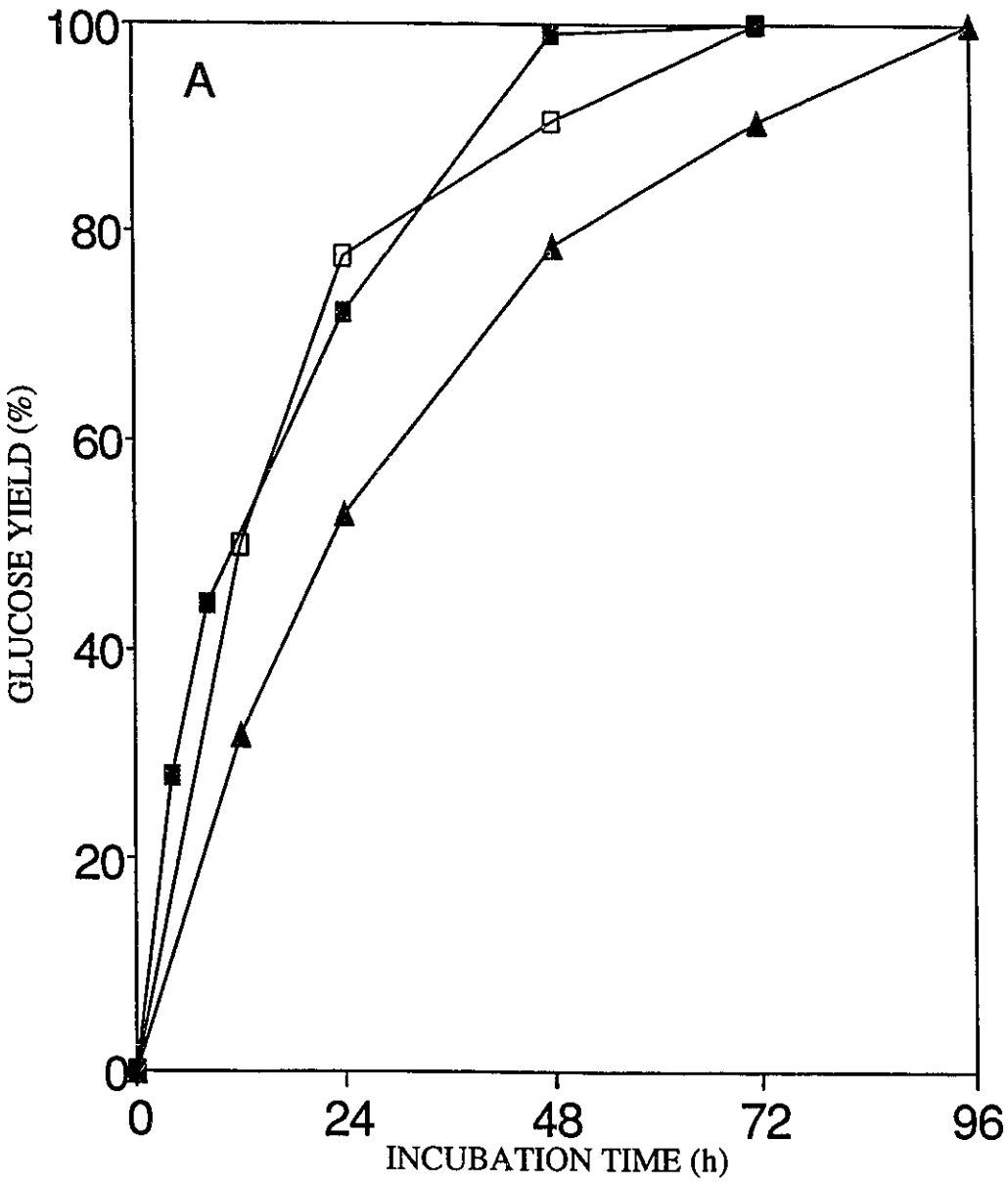


Fig. 22 A. Comparison of the hydrolysis profile of the peroxide-treated fractions derived from steam-treated (■) *E. viminalis* (SO_2-SEE-WIA/H_2O_2), (□) aspen (SO_2-SEA-WIA/H_2O_2), and (▲) spruce (SO_2-SES-WIA/H_2O_2) chips. Hydrolyses were carried out at a substrate concentration of 2% (w/v) and a fixed enzyme loading of 10 FPU g^{-1} cellulose.

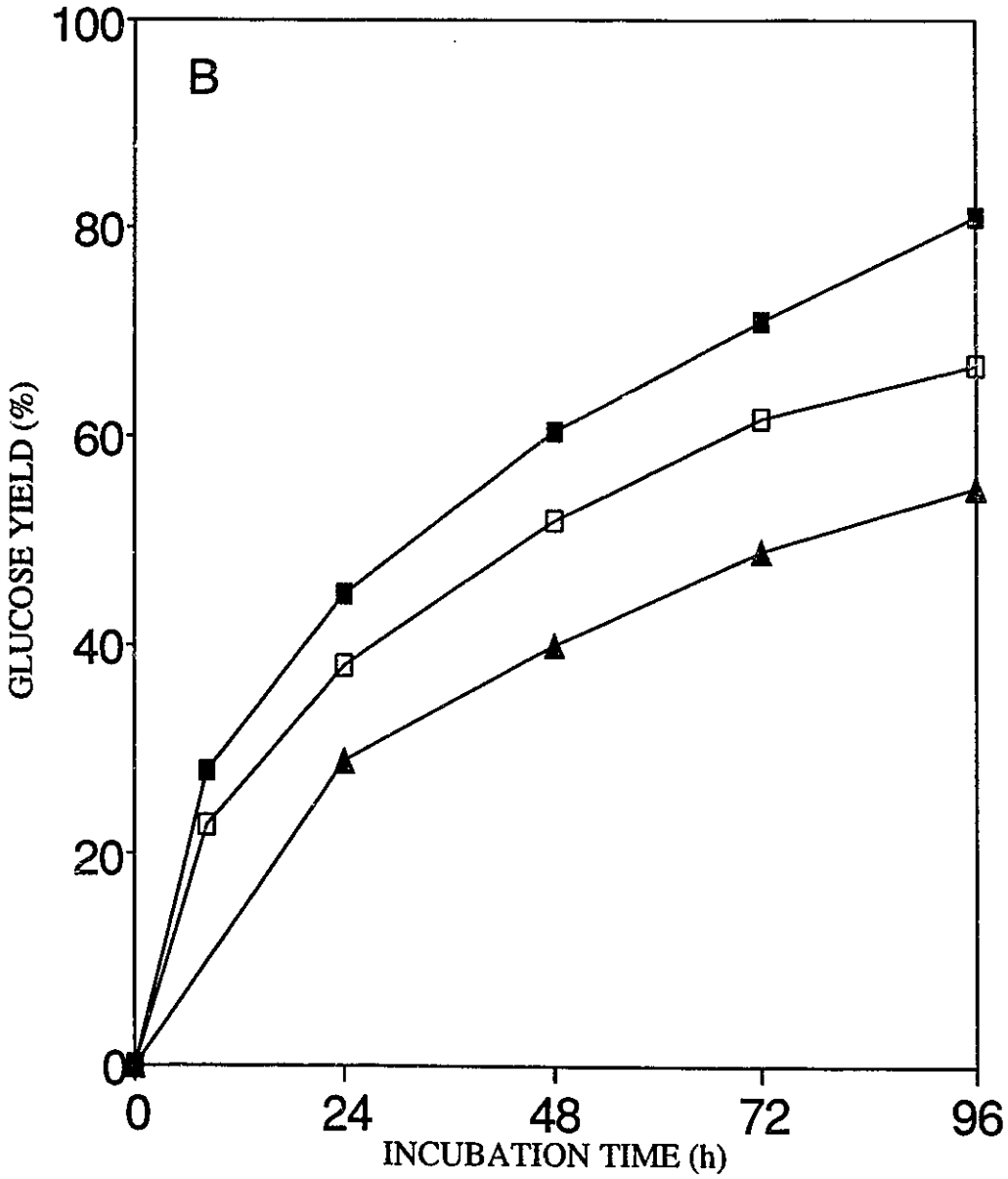


Fig. 22 B. Comparison of the hydrolysis profile of the peroxide-treated fractions derived from steam-treated (■) *E. viminalis* (SO_2-SEE-WIA/H_2O_2), (□) aspen (SO_2-SEA-WIA/H_2O_2), and (▲) spruce (SO_2-SES-WIA/H_2O_2) chips. Hydrolyses were carried out at a substrate concentration of 10% (w/v) and a fixed enzyme loading of 10 FPU g^{-1} cellulose.

2% (w/v), we next determined the effect of added substrate on the overall glucose yield of the reaction. We observed that, by adding supplemental substrate to the 48 h hydrolysate of a 2% (w/v) hydrolysis reaction, the glucose concentration was only marginally increased after incubation times as long as 48 h (Fig. 23). However, when the enzyme solution was incubated for 96 h without substrate and subsequently supplemented with fresh substrate, a complete hydrolysis of cellulose was obtained in 48 h. This result suggested that the enzymes present in the hydrolysate, which contained approximately 21 mg mL^{-1} of glucose, were strongly inhibited and that this loss of activity was not due to thermal inactivation but rather to end product inhibition.

We next determined whether the addition of further cellulase activity could improve the hydrolysis profile of the peroxide-treated substrate at relatively high substrate concentrations. We observed that, during a time course hydrolysis at 6% (w/v), the further addition of enzymes at 14 and 24 h did not substantially increase the rate and completeness of hydrolysis (Fig. 24). However, any enhancing effect was more pronounced when fresh enzyme was added early during the hydrolysis reaction. As the pretreated substrates could be completely hydrolysed when relatively low enzyme loadings were used (Fig. 15), it seemed probable that factors influencing the enzyme rather than structural restrictions within the substrate itself were limiting the rate of

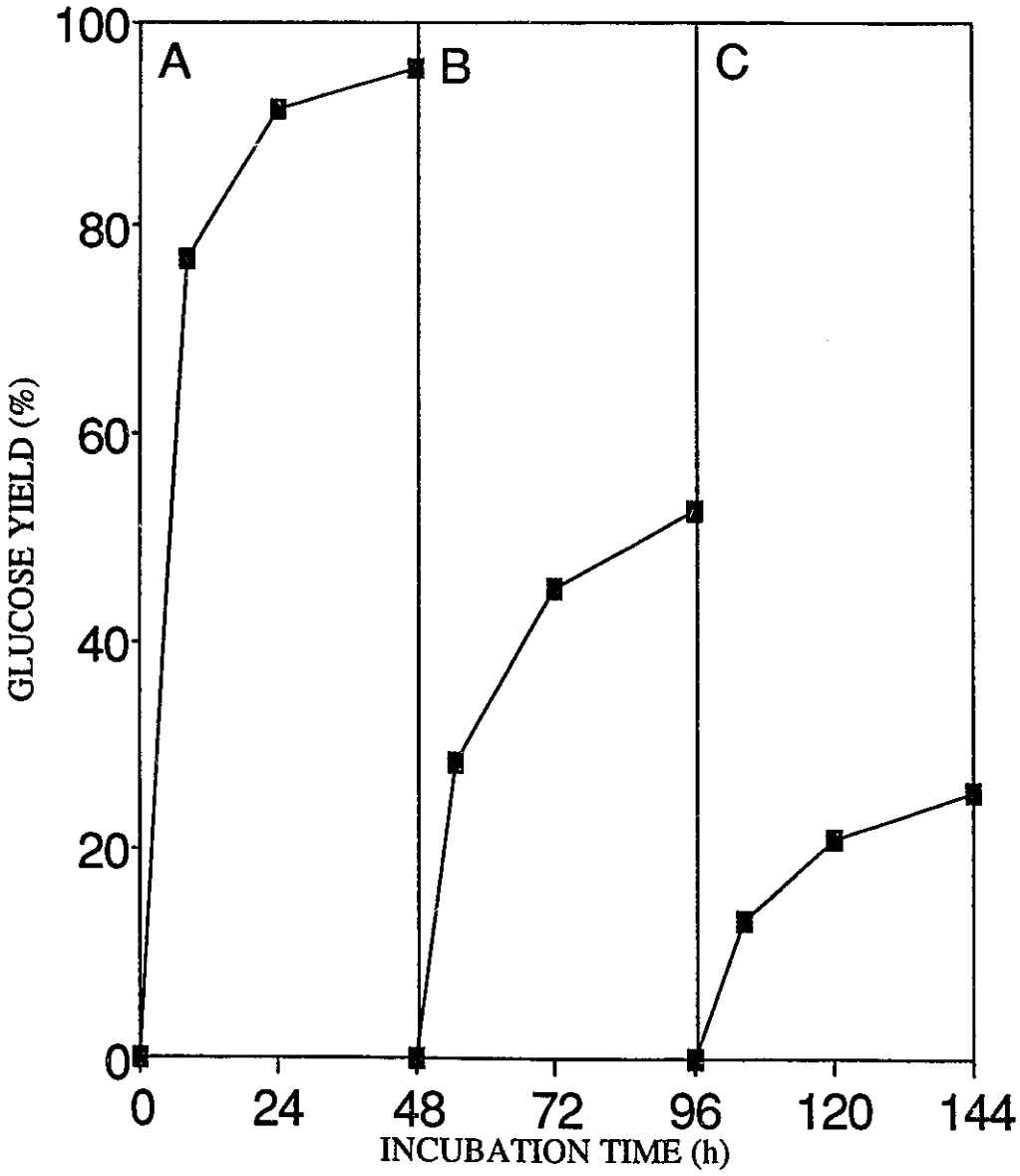


Fig. 23. Effect of supplemental substrate on the enzymatic hydrolysis of phosphoric acid-swollen Sigmacell (PASS). Hydrolysis was initiated at a 2% (w/v) substrate concentration and a fixed enzyme loading of 10 FPU g^{-1} cellulose. Fresh substrate (PASS, 1 g%) was subsequently added to the mixture at 48 and 96 h.

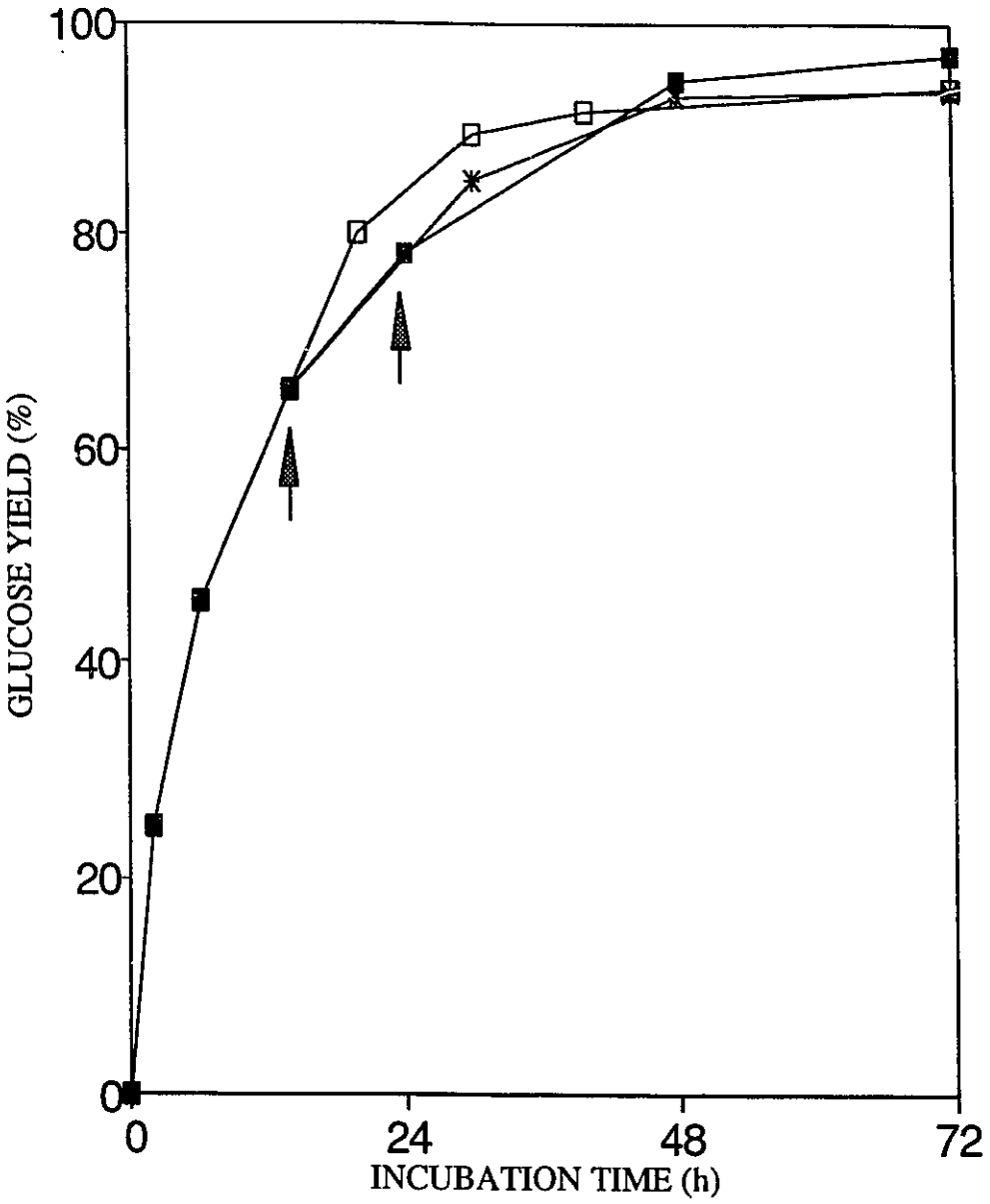


Fig. 24. The effect of adding supplemental filter paper activity on the enzymatic hydrolysis of the peroxide-treated fraction derived from eucalyptus chips (SO_2-SEE-WIA/H_2O_2). (■) Hydrolysis was initiated at a substrate concentration of 6% (w/v) and an enzyme loading of 10 FPU g^{-1} cellulose. Exogenous filter paper activity, equivalent to the 10 FPU g^{-1} cellulose originally added, was added to the mixture at (□) 14 and (*) 24 h of hydrolysis (indicated by arrows).

hydrolysis. Although the supplemental β-glucosidase greatly decreased the accumulation of cellobiose, it was possible that the accumulated glucose could inhibit the β-glucosidase activity. When a 6% concentration of the peroxide-treated substrate, containing an enzyme loading of 10 FPU g^{-1}, was supplemented with glucose to a final concentration of 2% and 4% (w/v), there was a proportional decrease in both the rate and completeness of hydrolysis (Fig. 25). Analysis of the filtrate from the reaction supplemented with 4% (w/v) glucose indicated an increase in the amount of cellobiose detected.

As the accumulated sugars seemed to be influencing hydrolysis, we next attempted to remove the residual substrate from the reaction at various times while replacing the hydrolysate filtrate with fresh hydrolysis buffer containing the equivalent amount of β-glucosidase activity originally present. By removing the accumulated sugars after hydrolysis of the substrate for 14 h at 6% (w/v) and 10 FPU g^{-1}, the residual substrate could be completely hydrolysed within 20 h of incubation (Fig. 26). Therefore, the total time requirement for complete hydrolysis of the substrate was reduced to roughly 35 h without any further addition of Celluclast. Although the effect was not as dramatic when lower enzyme loadings (5 FPU g^{-1}) were used, the hydrolysis was stimulated when the sugars were removed and the filtrate replaced with buffer.

It appeared that the majority of the cellulase system was in such close association with the residual substrate that

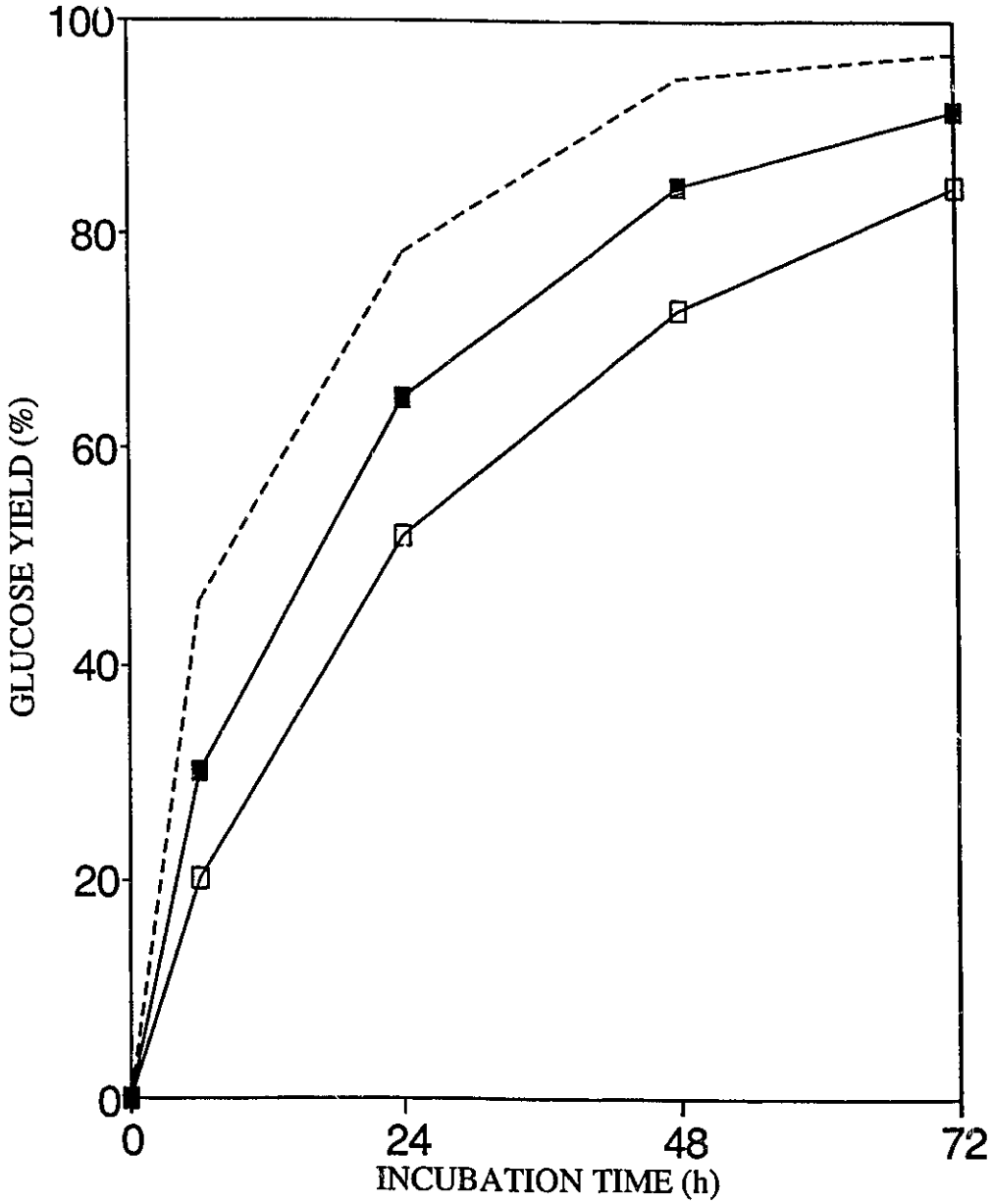


Fig. 25. Effect of glucose on hydrolysis of peroxide-treated eucalyptus at a substrate concentration of 6% (w/v) and an enzyme loading of 10 FPU g^{-1} cellulose. Concentration of glucose added: (■) 20 mg mL^{-1}, (□) 40 mg mL^{-1}. The broken line indicates the hydrolysis profile in the absence of added sugar (control).

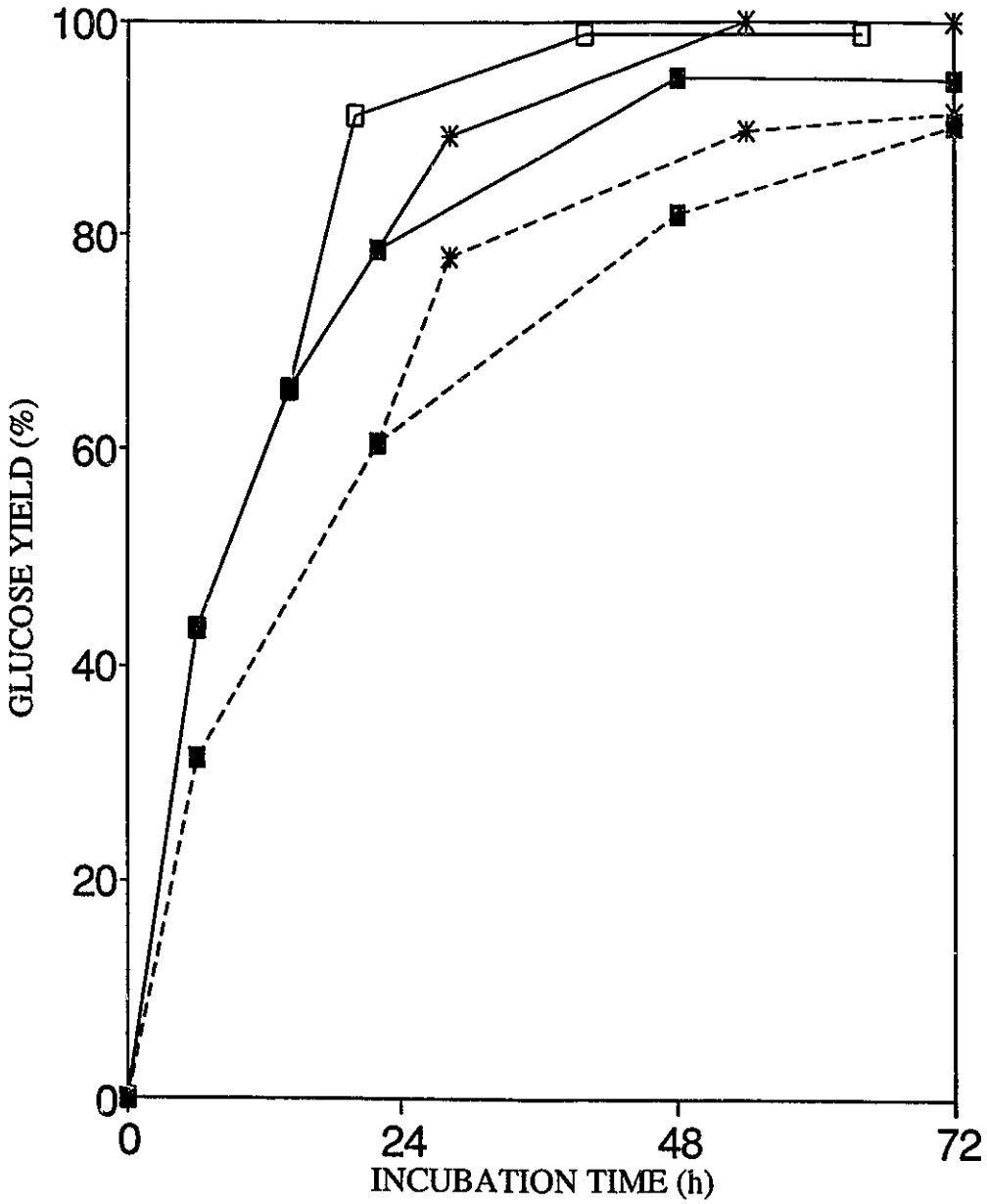


Fig. 26. Effect of sugar removal on hydrolysis of peroxide-treated eucalyptus (SO_2-SEE-WIA/H_2O_2) at a substrate concentration of 6% (w/v) and enzyme loadings of (---■---) 5 FPU g^{-1} and (—■—) 10 FPU g^{-1} cellulose. Soluble sugars were eliminated by isolating the unhydrolysed residue at (□) 14 and (*) 24 h of hydrolysis and replacing the hydrolysate with fresh buffer containing β-glucosidase activity.

removal of the reaction filtrate and its replacement with fresh buffer did not remove any essential component of the cellulase mixture. As the cellulase system was still active and could completely hydrolyse the residual substrate, we assumed that this substrate-associated enzyme system could be used to hydrolyse an additional batch of substrate. We observed that, when the residual substrate remaining after 12 and 24 h of hydrolysis was isolated and supplemented with additional substrate and fresh buffer to reconstitute the original 6% (w/v) substrate starting conditions, a similar profile resulting in complete hydrolysis was obtained after a further 96 h hydrolysis (Fig. 27).

Although most of the cellulase activity remained associated with the substrate when high substrate concentrations were used, it was also possible that at lower substrate concentrations, complete hydrolysis of the substrate could release most of the original cellulase components back into solution. To investigate this case, it was important to determine the amount of filter paper activity that could be recovered from an enzyme solution containing the amount of glucose found after complete hydrolysis of the pretreated substrate at 2% (w/v). The inhibitory effects of accumulated sugars on the filter paper assay were minimized by ultrafiltration of each of the hydrolysates through a membrane with a 10 kD molecular weight cut-off. Although the ultrafiltration (UF) step resulted in a loss of 10% of the original filter paper activity found in the enzyme control, this

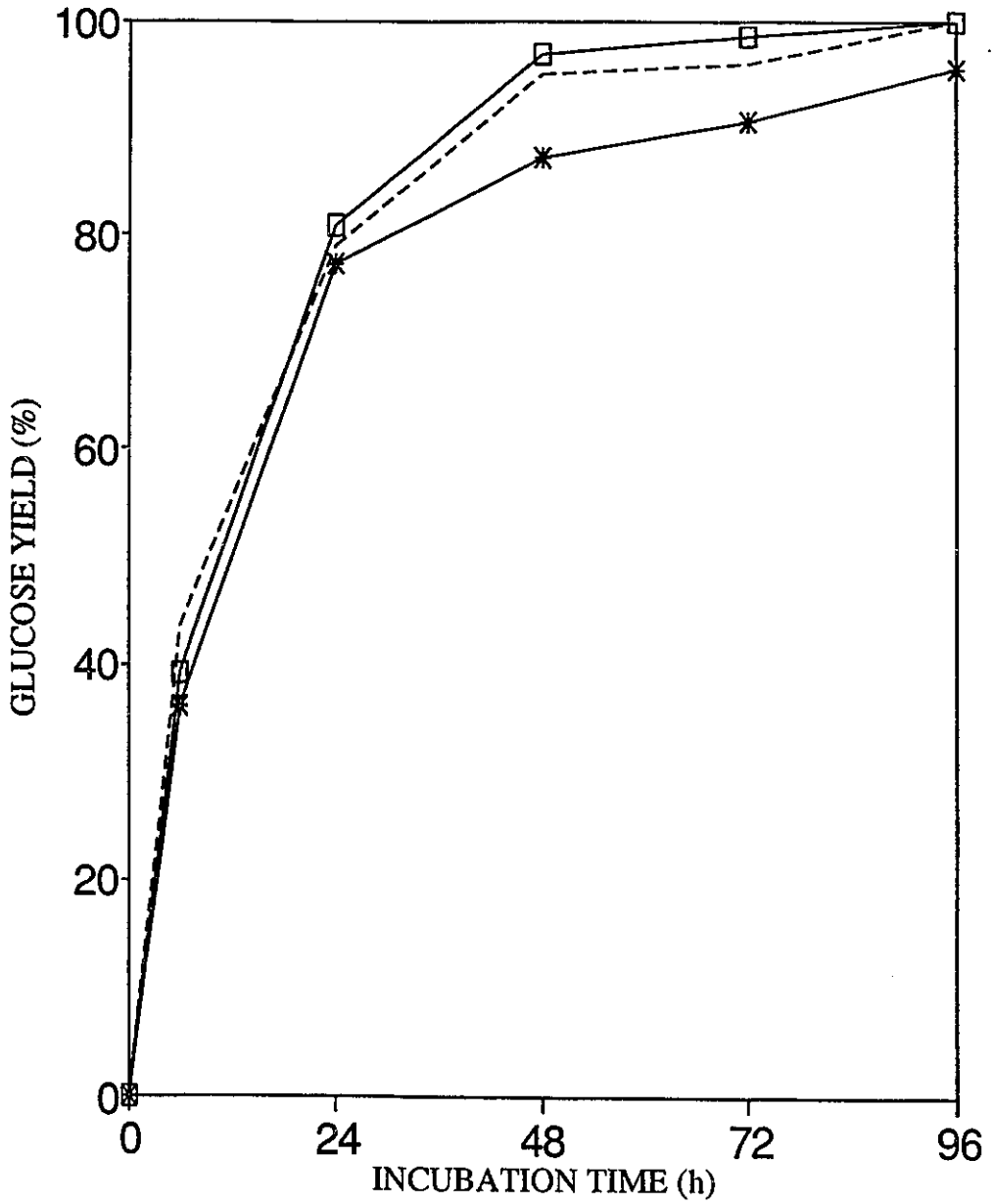


Fig. 27. Effect of sugar removal and substrate supplementation on hydrolysis of peroxide-treated eucalyptus at a substrate concentration of 6% (w/v) and an enzyme loading of 10 FPU g^{-1} cellulose. Soluble sugars were eliminated by isolating the unhydrolysed residue at (□) 12 and (*) 24 h of hydrolysis and replacing the hydrolysate with fresh buffer containing β-glucosidase activity. Fresh substrate was also added to reconstitute the original substrate concentration of 6% (w/v). Broken line indicates an uninterrupted hydrolysis profile of the substrate (control).

method was effective in separating the hydrolysis sugars from the enzyme (Table 21). When 20 mg mL^{-1} of glucose was added to the enzyme control before processing, no additional loss of filter paper activity was observed other than that previously attributed to the UF step. However, incubating the enzyme without substrate at 45°C and 150 rpm for 48 or 96 h led to a decrease in the detected filter paper activity. This experiment was then repeated using 2% (w/v) substrate hydrolysates rather than enzyme controls (Table 22). Among the cellulosic substrates tested, both the SO_2-SEE-WIA/H_2O_2 fraction and phosphoric acid-swollen Sigmacell resulted in good recovery of filter paper activity after 96 h of hydrolysis, whereas only 15% of the original activity was recovered in the SO_2-SEE-WIA hydrolysate.

As it seemed that the composition of the substrate influenced the rate of hydrolysis and, subsequently, the desorption of cellulases, we investigated the potential of reusing enzymes by hydrolysing a 2% (w/v) concentration of the SO_2-SEE-WIA and SO_2-SEE-WIA/H_2O_2 fractions using 10 FPU g^{-1} and recycling the enzymes present in the hydrolysate obtained after 48, 72 or 120 h to a new batch of substrate (Table 23). The efficiency of hydrolysis of the substrate was assessed by determining the dry weight of the unhydrolysed residue remaining and by quantifying the amount of sugars released into the reaction filtrate.

Our results indicated that, for the SO_2-SEE-WIA/H_2O_2 fraction, the same enzyme preparation could be recycled five

Table 21. Recovery of filter paper activity from several Celluclast/Novozym preparations after ultrafiltration using a Centricon (Amicon) apparatus with a 10 kD molecular weight cut-off.

ENZYME MIXTURE[1]	INCUBATION TIME[2] (h)	UF[3] STEP	FP ACTIVITY[4] U mL^{-1} ± s.d.	RECOVERED ACTIVITY[5] (%)
control	-	no	0.683 ± 0.021	100
	-	yes	0.613 ± 0.004	90
	48	yes	0.543 ± 0.023	80
	96	yes	0.456 ± 0.007	65
control plus 20 mg mL^{-1} Glc	-	yes	0.603 ± 0.029	89

[1] Celluclast/Novozym mixture containing a filter paper activity of 10 FPU g^{-1} cellulose and a cellobiase activity of 30 CBU g^{-1} cellulose;
[2] Time for which the enzyme mixture was incubated without substrate at 45°C, 150 rpm;
[3] Ultrafiltration was carried out using an Amicon membrane with a 10 kD molecular weight cut-off;
[4] Filter paper (FP) activity was determined according to Ghose (1987) and Chan *et al.* (1989) after ultrafiltration; s.d., sample standard deviation (n = 7);
[5] Calculated in relation to the activity originally present in the enzyme mixture.

Table 22. Determination of the residual filter paper activity present in the enzymatic hydrolysate of several cellulosic substrates (2%, w/v) after various incubation times.

SAMPLE[1]	INCUBATION TIME[2] (h)	FP ACTIVITY[4] U mL^{-1} ± s.d.	RECOVERED ACTIVITY[3] (%)	HYDROLYSIS YIELD[5] (%)
WIA	48	0.059 ± 0.001	11	64.9
	96	0.068 ± 0.003	15	80.9
WIA/H_2O_2	48	0.362 ± 0.007	67	87.9
	96	0.482 ± 0.012	106	96.8
PASS	48	0.324 ± 0.008	60	90.4
	96	0.400 ± 0.024	90	99.2

[1] Both WIA (alkali-insoluble) and WIA-H_2O_2 (peroxide-treated) fractions were derived from SO_2-impregnated steam-exploded chips; PASS, phosphoric acid-swollen Sigmacell;
[2] Time for which the substrates were hydrolysed at 2% (w/v) with an enzyme loading of 10 FPU g^{-1} cellulose; hydrolyses were carried out at 45°C, 150 rpm;
[3] Calculated in relation to the amount of activity recovered from enzyme controls which were incubated for 48 and 96 h, respectively (see Table 21);
[4] See legend in Table 21;
[5] Extent of cellulose hydrolysis at the indicated incubation time (hydrolysis was carried out as described above).

Table 23. Extent of enzymatic hydrolysis of the peroxide-treated (SO_2-SEE-WIA/H_2O_2) and alkali-insoluble (SO_2-SEE-WIA) substrates derived from eucalyptus chips using readsorbed enzymes.

SO_2-SEE[1] FRACTION	RECOVERY STEP		HYDROLYSIS YIELD (%)		
	Step No.	Time[2] (h)	Dry weight loss[3]	HPLC[4]	Total sugars[5]
WIA-H_2O_2	-	48	99.0	99.6	nd[6]
	1	48	98.8	95.5	94.7
	2	48	99.0	94.2	95.0
	3	48	98.8	99.2	96.3
	4	48	97.9	93.5	87.8
	5	48	97.9	96.7	94.2
	6	72	87.8	78.8	82.0
	6	120	98.2	92.6	96.3
	7	72	20.8	21.1	nd
	7	120	98.2	90.7	97.2
	8	120	nd	70.5	99.6
	9	120	84.5	50.5	83.5
WIA	-	72	90.5	100.1	nd
	1	72	85.4	87.1	nd
	2	120	20.2	20.2	nd

[1] SO_2-impregnated, steam-exploded eucalyptus; WIA-H_2O_2, peroxide-treated fraction; WIA, alkali-insoluble fraction;
[2] Incubation time at which the hydrolysis reaction was stopped;
[3] Calculated on basis of substrate oven-dry weight before and after hydrolysis;
[4] HPLC analysis was carried out as described previously (see Methods for details);
[5] Determined using the phenol-sulphuric method (Chaplin, 1986);
[6] Not determined.

times before there was any appreciable reduction in the efficiency of hydrolysis (Table 23, Fig. 28). A pronounced decline in the hydrolysis rate was observed in the sixth recycling step (Fig. 29), although prolonging the duration of incubation from 48 to 72 and 120 h increased both the efficiency of hydrolysis and the recovery of enzymes from the hydrolysate. However, by the ninth recycle stage, only 50% of the added substrate was hydrolysed to glucose after incubation for 120 h (Fig. 30). This recycling procedure was then repeated under similar conditions. Although in this experiment the enzymes were initially readsorbed onto the substrate from a fresh preparation, the same profile of nine consecutive hydrolysis steps was obtained using the readsorbed enzymes (Fig. 31).

When the sugar content of the filtrates from the peroxide-treated substrate was determined, the higher phenol-sulphuric values obtained as compared to the HPLC values indicated that more oligosaccharides were present in the later recycle filtrates (Table 23). An assay of the column eluates obtained after each of the recycling steps indicated that the detectable filter paper activity was always less than 0.05 FPU g^{-1} of cellulose. Only 24% of the cellobiase activity initially added to the mixture was found in the column eluate after the first enzyme recovery procedure. A partial adsorption of β-glucosidases onto cellulose at low temperatures has been previously demonstrated (Ooshima *et al.*, 1983).

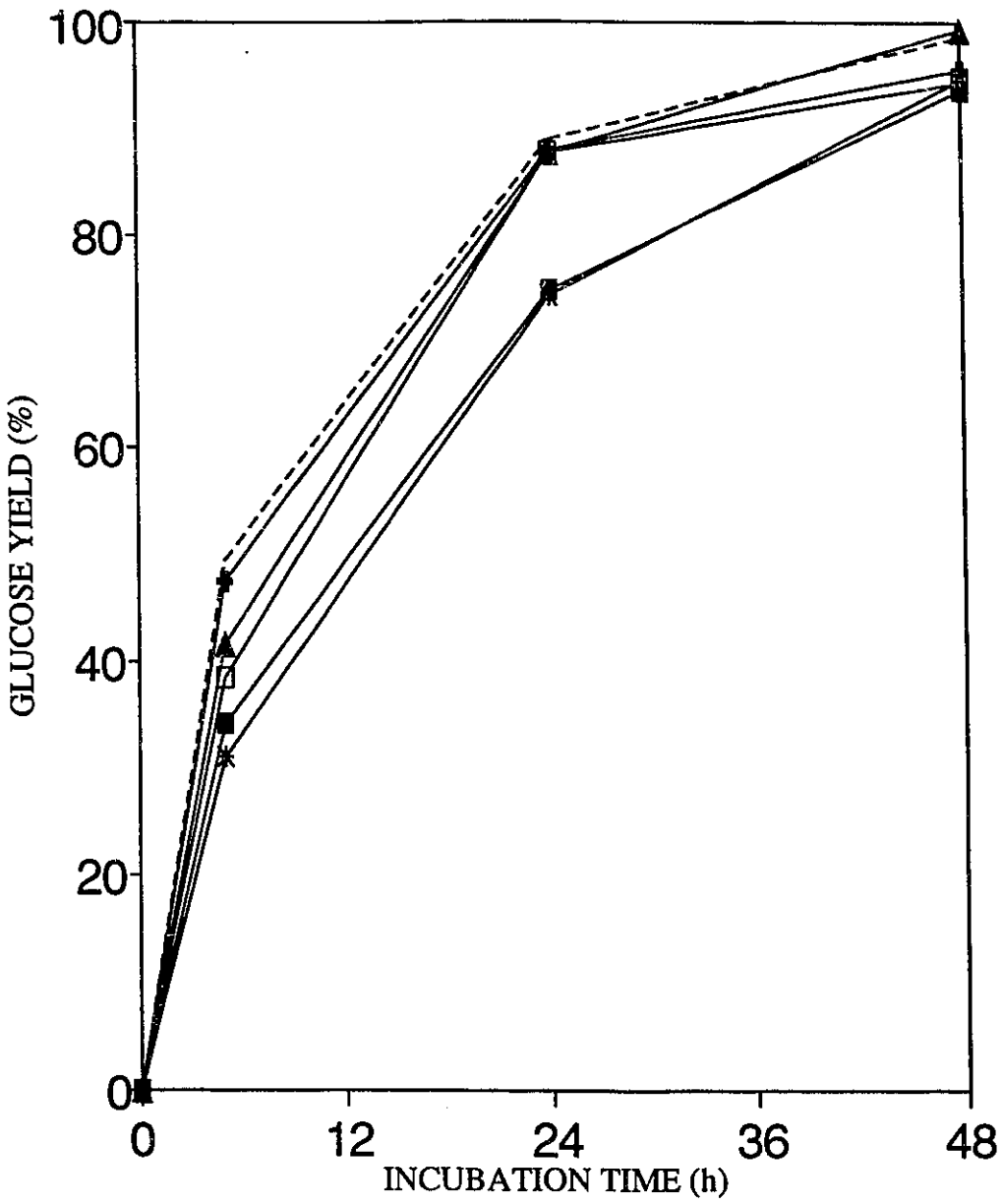


Fig. 28. Enzymatic hydrolysis of peroxide-treated eucalyptus (SO_2-SEE-WIA/H_2O_2) at 2% (w/v) using enzymes recovered and recycled from 48 h hydrolysates by readsorption. A Celluclast/ Novozym mixture containing 10 FPU g^{-1} cellulose was initially added to the system. Additional Novozym was supplemented at the beginning of each new hydrolysis profile initiated after enzyme recycling. Hydrolysis profiles were obtained using enzymes recovered after (+) 1, (▲) 2, (□) 3, (■) 4, and (*) 5 recycling steps. Broken line indicates the hydrolysis profile of the substrate using fresh enzymes (control).

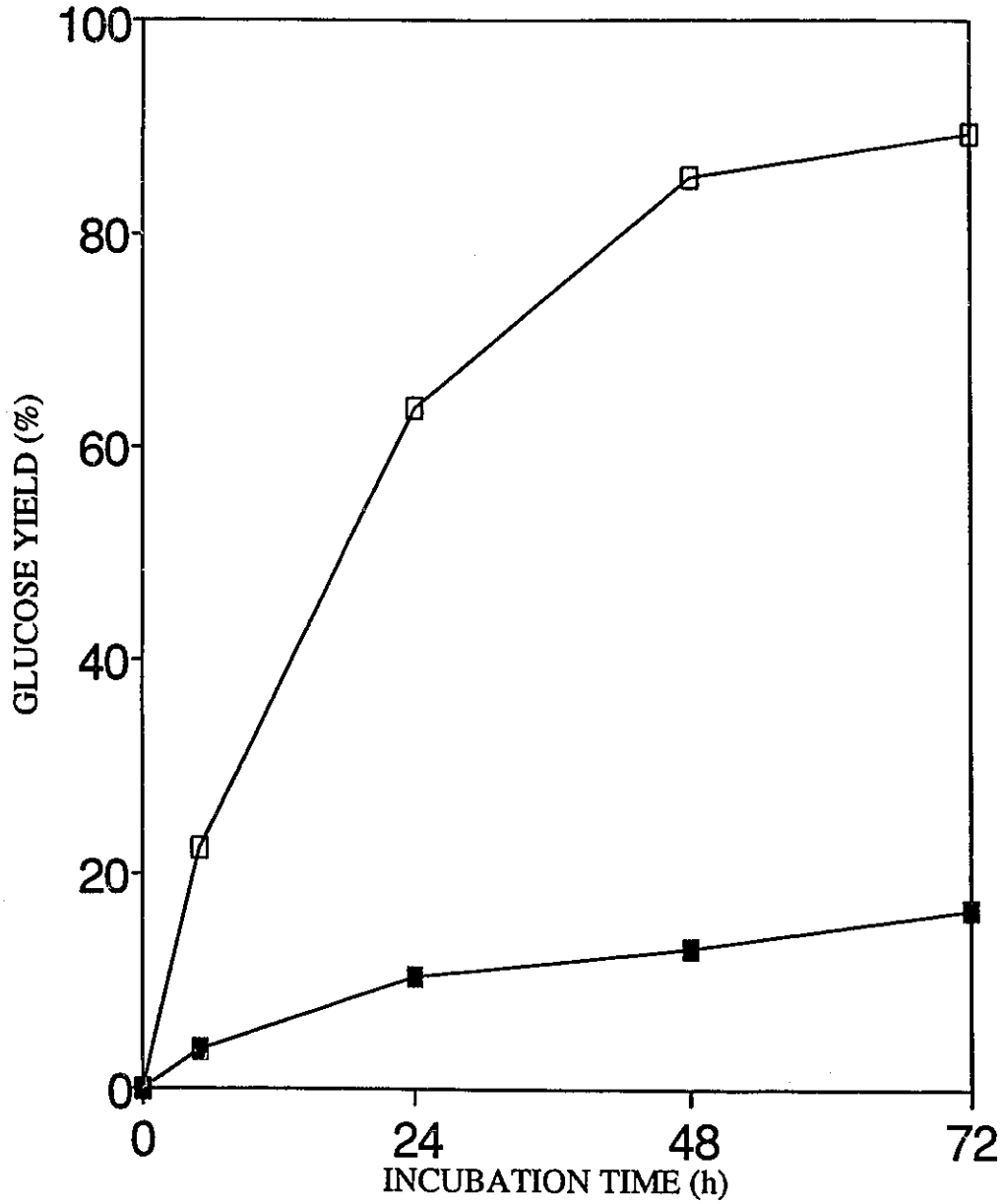


Fig. 29. Enzymatic hydrolysis of peroxide-treated eucalyptus (SO_2-SEE-WIA/H_2O_2) at 2% (w/v) using enzymes recovered and recycled from 48 h hydrolysates by readsorption. Hydrolysis profiles were obtained using enzymes recovered after (□) 6 and (■) 7 recycling steps.

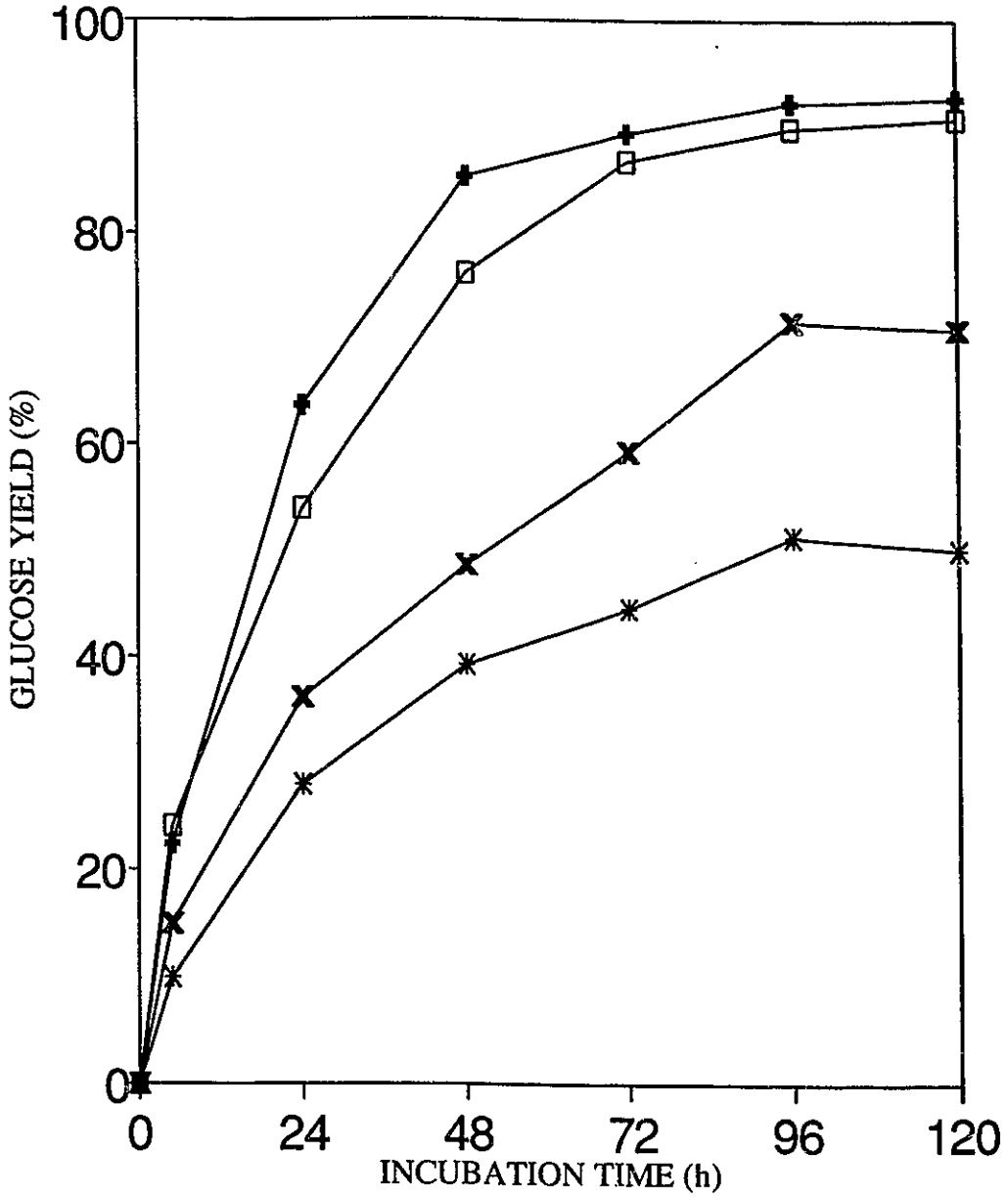


Fig. 30. Enzymatic hydrolysis of peroxide-treated eucalyptus (SO_2-SEE-WIA/H_2O_2) at 2% (w/v) using enzymes recovered and recycled from 120 h hydrolysates by readsorption. Hydrolysis profiles were obtained using enzymes recovered after (+) 6, (□) 7, (X) 8, and (*) 9 recycling steps.

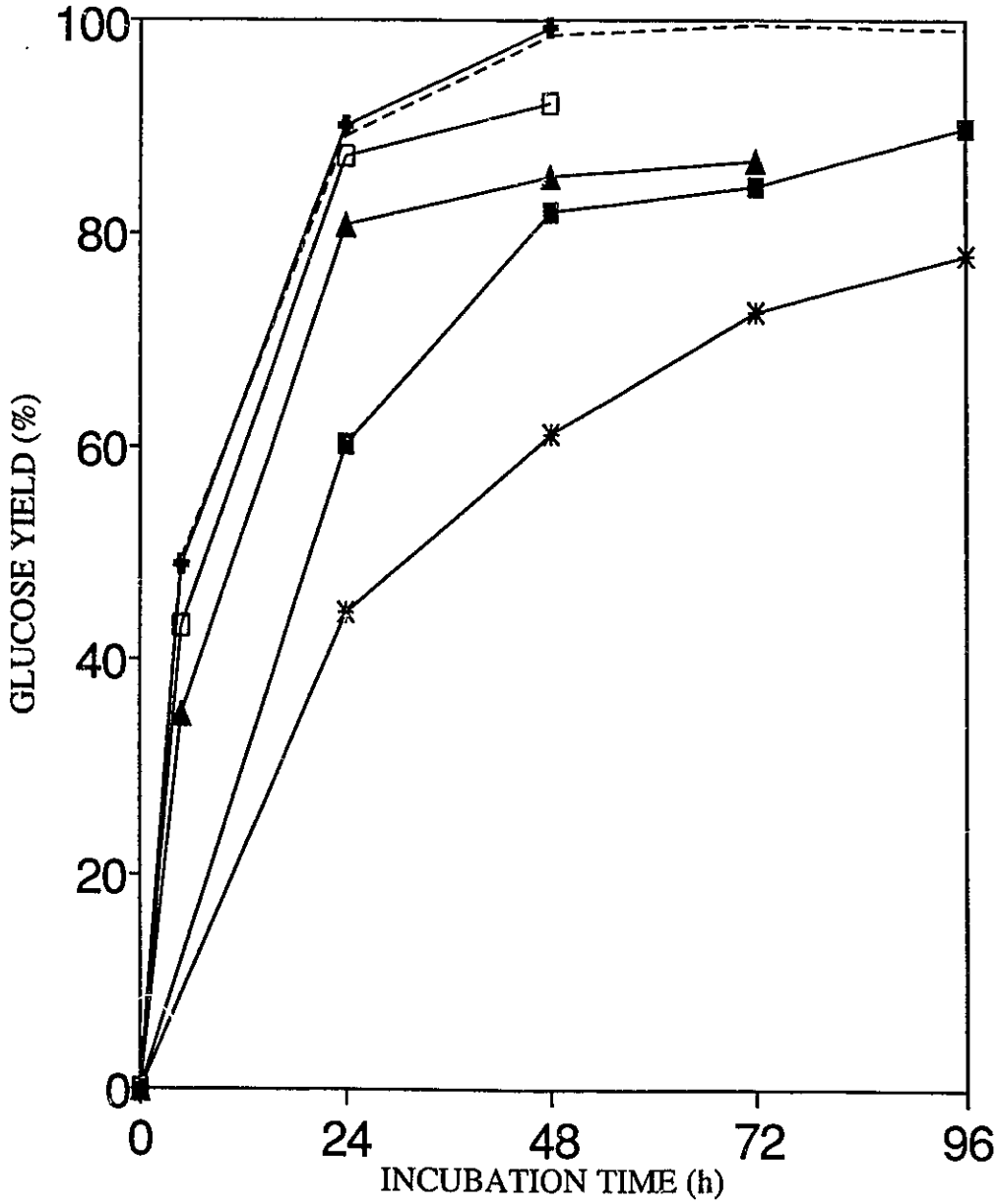


Fig. 31. Enzymatic hydrolysis of peroxide-treated eucalyptus (SO_2-SEE-WIA/H_2O_2) at 2% (w/v) using enzymes recovered and recycled by readsorption. The enzymes were initially adsorbed on the substrate from a fresh Celluclast/Novozym mixture containing 10 FPU g^{-1} cellulose. Additional Novozym was supplemented at the beginning of each new hydrolysis profile initiated after enzyme recycling. Hydrolysis profiles were obtained using enzymes recovered after (+) 1, (□) 2, (▲) 4, (■) 6, and (*) 8 recycling steps. Broken line indicates the hydrolysis profile of the substrate using freshly adsorbed enzymes.

The total duration of the experiment was approximately four weeks. As a gradual loss of enzymatic activity after prolonged incubation times has been reported for other enzyme preparations such as Meicelase (Ishihara et al, 1991), we decided to test the hydrolytic potential of enzymes recovered from enzyme controls which had been incubated without substrate for several days. Our results indicated that enzymes readsorbed after four days at 45°C and 150 rpm resulted in a substrate hydrolysis profile very similar to that obtained using enzymes readsorbed from a fresh preparation (Fig. 32). However, a slight decrease of 10% was observed in the substrate hydrolysis yield when enzymes readsorbed after an incubation time of 15 days were used. This was probably caused by an increased loss of enzymatic activity and/or cellulose binding capacity of one or more components of the cellulase system.

When the alkali-extracted substrate which had not been further treated with peroxide (SO_2-SEE-WIA) was used as the first substrate for hydrolysis before recycling, the efficiency of hydrolysis dropped dramatically after the second recycle stage. Although the small amount of residual lignin in the alkali-washed (SO_2-SEE-WIA) substrate did not interfere with the initial hydrolysis (Fig. 16), it probably restricted the release of the cellulase from the residual substrate into the reaction filtrate. As it was possible that the enzymes were bound and still active, we compared the effect of recycling the enzymes in the reaction filtrate with recycling the enzymes in both the

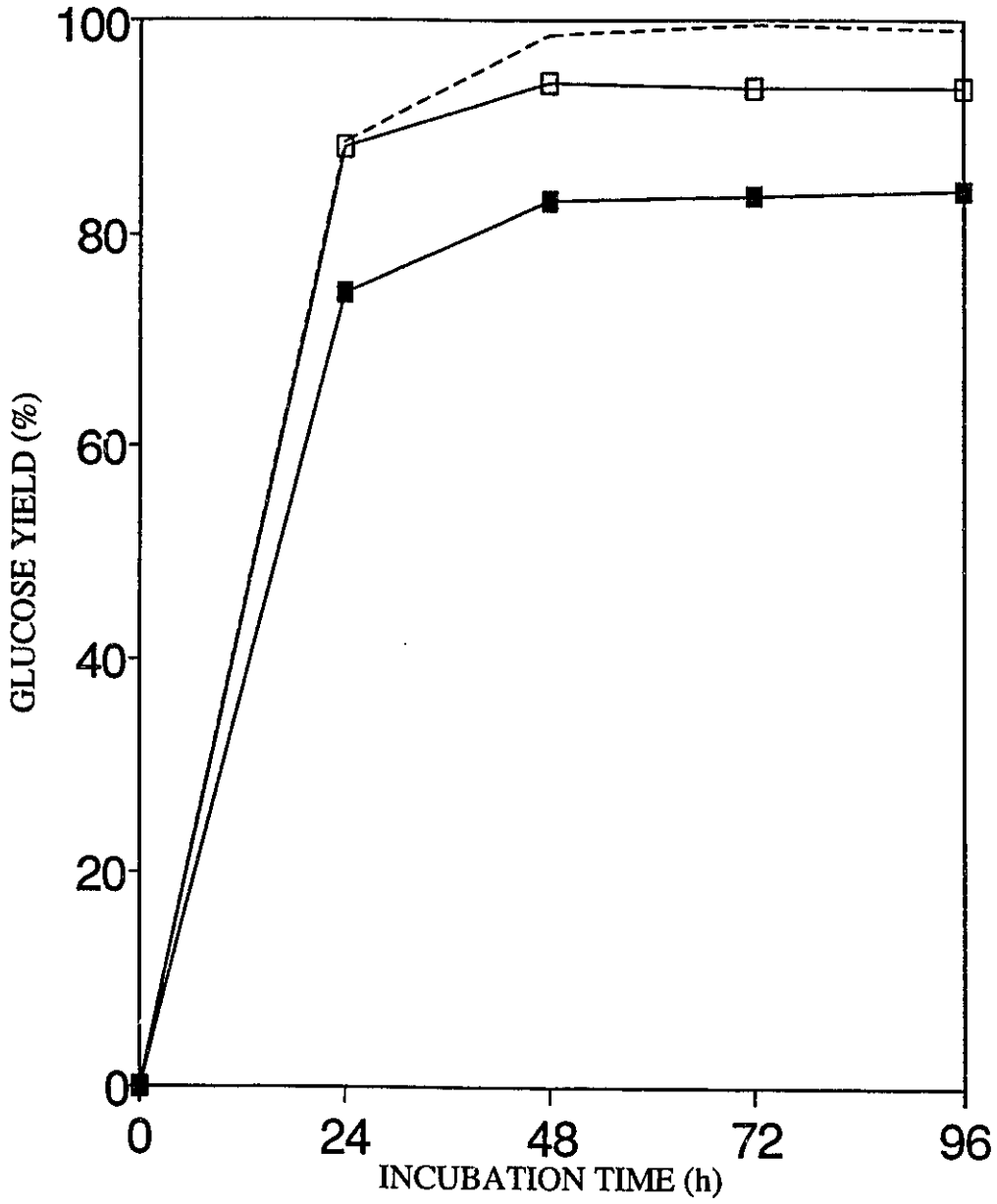


Fig. 32. Enzymatic hydrolysis of peroxide-treated eucalyptus (SO_2-SEE-WIA/H_2O_2) at 2% (w/v) using enzymes recovered and recycled by readsorption from an enzyme solution containing 10 FPU g^{-1} cellulose which had been incubated without substrate for (□) 96 and (■) 360 h. Broken line indicates the initial hydrolysis profile of the substrate using fresh enzymes.

filtrate and the residual substrate. When the filtrate from the first hydrolysis step was added to a fresh batch of substrate after 72 h of hydrolysis, 89% of the substrate was hydrolysed to sugars after 120 h incubation (Fig. 33 A). A further recycle of the enzymes in the filtrate obtained after this reaction, *i.e.*, the second recycle step, resulted in a 20% hydrolysis of a new batch of substrate. However, when the enzymes in both the residual substrate and the hydrolysis filtrate were recycled together, over 95% of the substrate was hydrolysed to sugars during the second recycle step (Fig. 33 B). Although the small amount of lignin associated with the alkali-extracted (SO_2-SEE-WIA) substrate influenced the release of the cellulases back into the filtrate, these enzymes were active and could still hydrolyse fresh substrate.

Our results also indicated that extended incubation times in the presence of lignin containing residues are detrimental to the efficiency of enzyme recycle, regardless of the recycling method used. When the enzymes which were used for the first hydrolysis step were added to a fresh batch of substrate after 120 h rather than 72 h of hydrolysis, the second step of enzyme recycling could only hydrolyse 15-20% of a new batch of added substrate (Fig. 34 A). This drop in hydrolysis efficiency was observed even when the enzymes in both the filtrate and unhydrolysed residue were recycled simultaneously (Fig. 34 B).

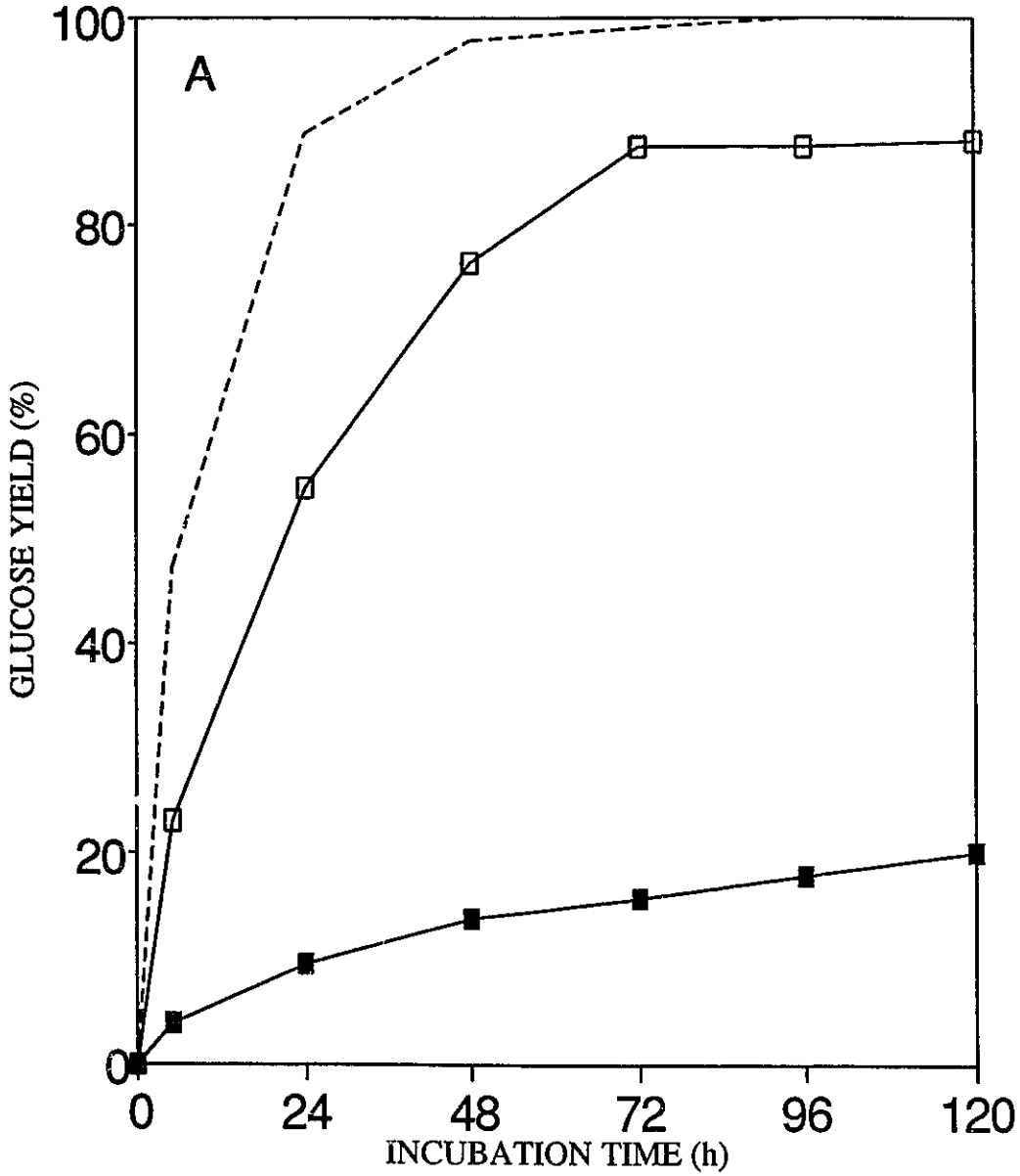


Fig. 33 A. Enzymatic hydrolysis of peroxide-treated eucalyptus (SO_2-SEE-WIA/H_2O_2) at 2% (w/v) using enzymes recovered and recycled in the presence of a lignin-containing residue. Hydrolysis of the alkali-washed substrate (SO_2-SEE-WIA) was initially carried out for 72 h at 2% (w/w) and 10 FPU g^{-1} cellulose. The enzymes were subsequently recycled by readsorption on peroxide-treated eucalyptus after removal of the unhydrolysed residue by filtration. Hydrolysis profiles were obtained using enzymes recovered after (□) 1 and (■) 2 recycling steps. Broken line indicates the hydrolysis profile of the peroxide-treated fraction using fresh enzymes.

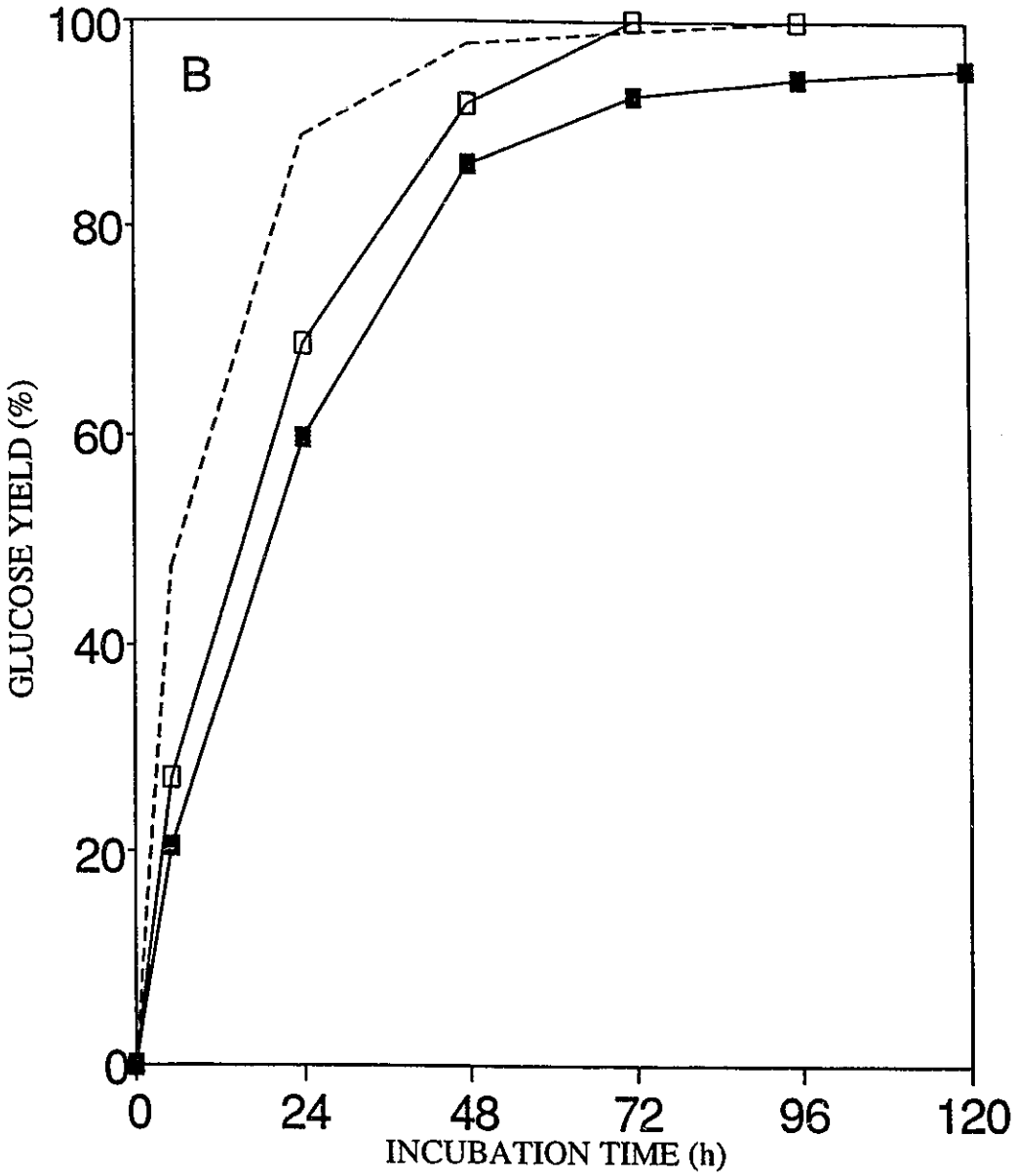


Fig. 33 B. Enzymatic hydrolysis of peroxide-treated eucalyptus (SO_2-SEE-WIA/H_2O_2) at 2% (w/v) using enzymes recovered and recycled in the presence of a lignin-containing residue. Hydrolysis of the alkali-washed substrate (SO_2-SEE-WIA) was initially carried out for 72 h at 2% (w/w) and 10 FPU g^{-1} cellulose. The enzymes were subsequently recycled by readsorption on peroxide-treated eucalyptus together with the unhydrolysed residue. Hydrolysis profiles were obtained using enzymes recovered after (□) 1 and (■) 2 recycling steps. Broken line indicates the hydrolysis profile of the peroxide-treated fraction using fresh enzymes.

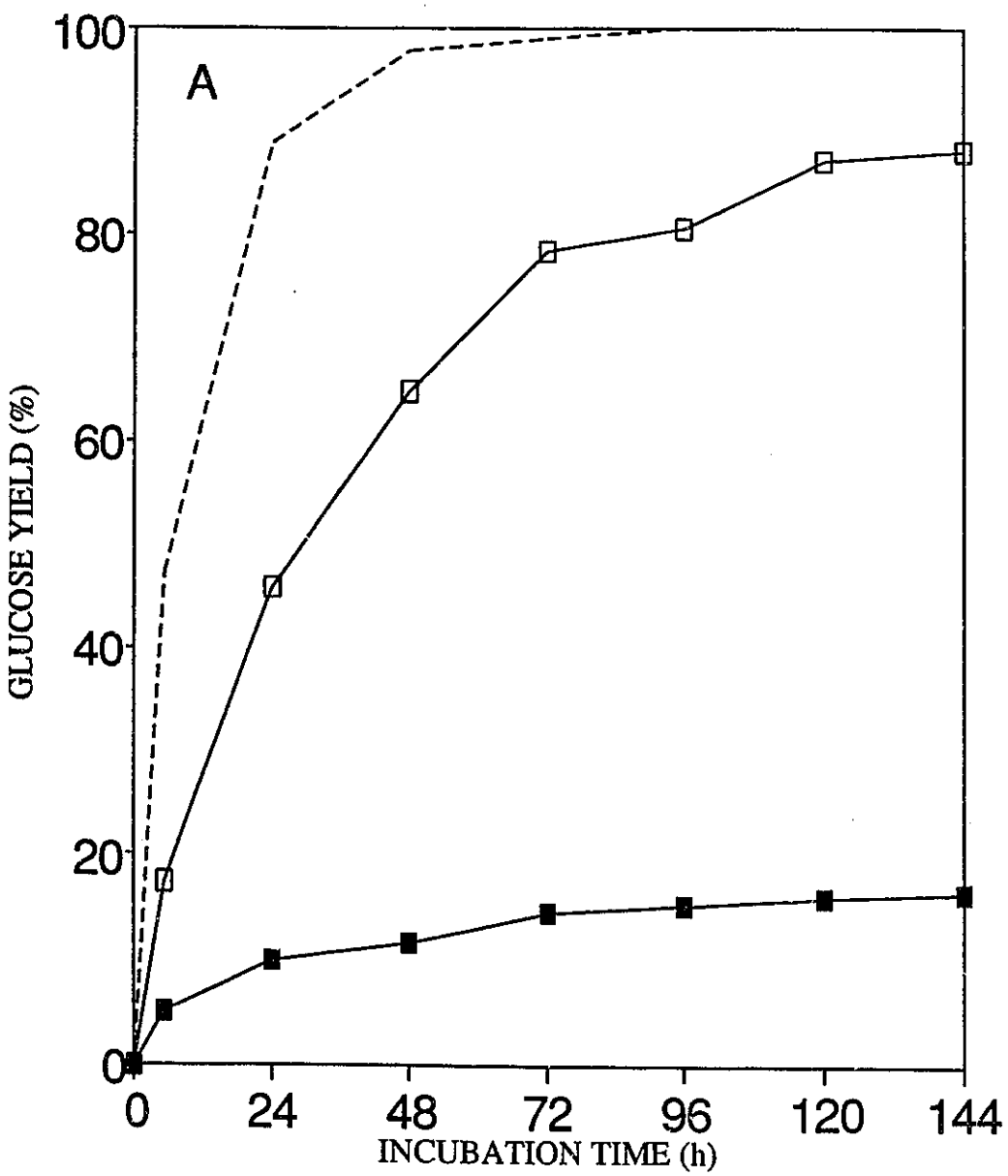


Fig. 34 A. Enzymatic hydrolysis of peroxide-treated eucalyptus (SO_2-SEE-WIA/H_2O_2) at 2% (w/v) using enzymes recovered and recycled in the presence of a lignin-containing residue. Hydrolysis of the alkali-washed substrate (SO_2-SEE-WIA) was initially carried out for 120 h at 2% (w/w) and 10 FPU g^{-1} cellulose. The enzymes were subsequently recycled by readsorption on peroxide-treated eucalyptus either together with the unhydrolysed residue. Hydrolysis profiles were obtained using enzymes recovered after (□) 1 and (■) 2 recycling steps. Broken line indicates the hydrolysis profile of the peroxide-treated fraction using fresh enzymes.

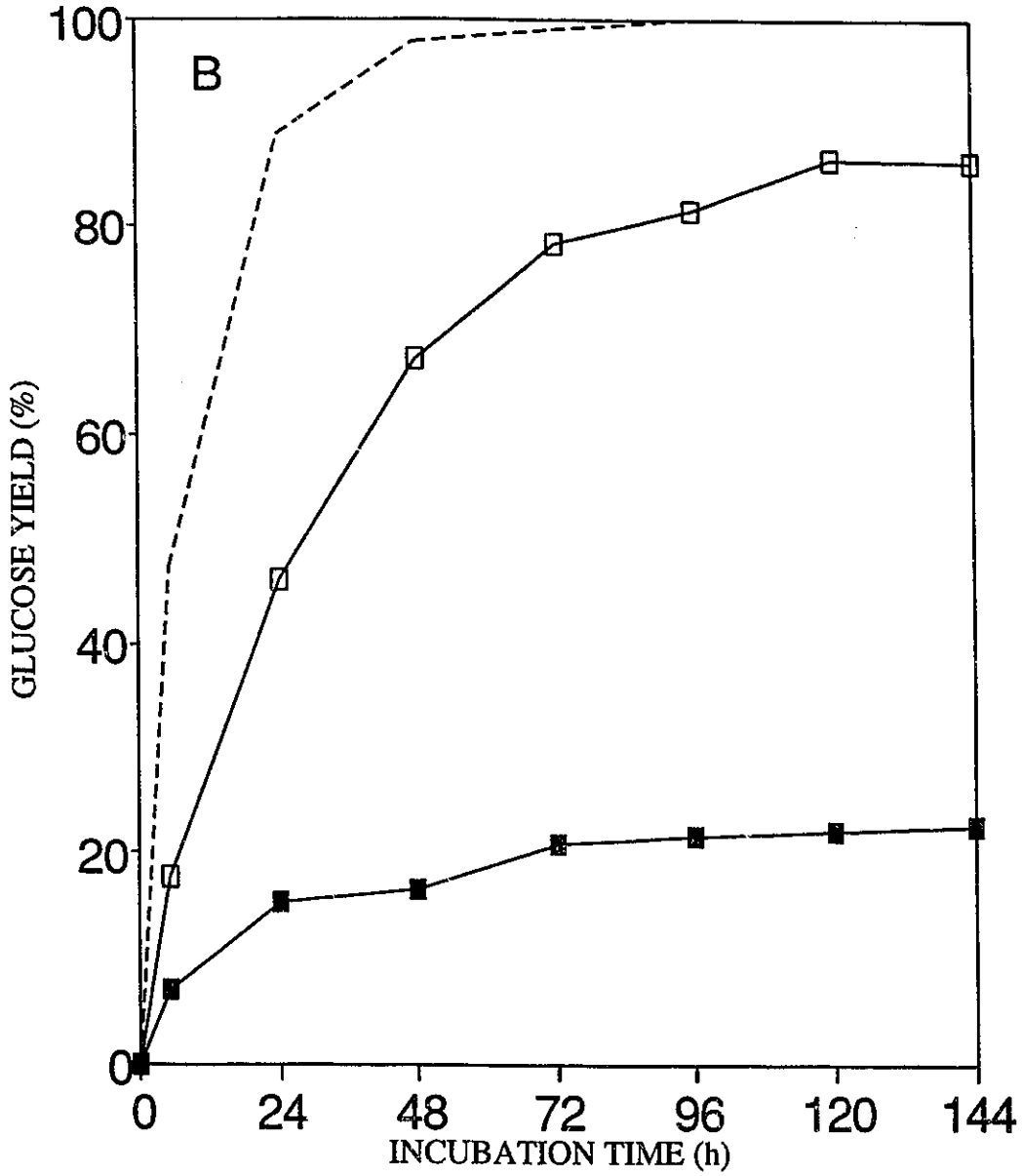


Fig. 34 B. Enzymatic hydrolysis of peroxide-treated eucalyptus (SO_2-SEE-WIA/H_2O_2) at 2% (w/v) using enzymes recovered and recycled in the presence of a lignin-containing residue. Hydrolysis of the alkali-washed substrate (SO_2-SEE-WIA) was initially carried out for 120 h at 2% (w/w) and 10 FPU g^{-1} cellulose. The enzymes were subsequently recycled by readsorption on peroxide-treated eucalyptus either after removal of the unhydrolysed residue by filtration. Hydrolysis profiles were obtained using enzymes recovered after (□) 1 and (■) 2 recycling steps. Broken line indicates the hydrolysis profile of the peroxide-treated fraction using fresh enzymes.

3.4. Structural Changes of the Substrate During Hydrolysis

It has been proposed that one of the most important factors limiting hydrolysis is the increased recalcitrancy of the substrate (see Fan *et al*, 1987). It was suggested that readily accessible regions of the substrate are efficiently hydrolysed during the first stage of hydrolysis, leading to an accumulation of recalcitrant cellulose as hydrolysis proceeds. To obtain a better understanding of the hydrolysis process, we investigated the effect of hydrolysis on several structural characteristics of the substrate, such as changes in fibre length distribution, crystallinity and degree of polymerisation of cellulose.

3.4.1. Changes in the Fibre Length Distribution of the Substrate

To eliminate most of the fines and fibre bundles present within the substrate, the peroxide-treated fraction derived from eucalyptus was passed through a series of screens with decreasing exclusion of particle size. Two major fractions were obtained, one between the 48 and 150 mesh screens (F 150) and another between the 150 and 200 mesh screens (F 200) (Fig. 35). These fractions accounted for approximately 50% of the starting material and were formed by a population of fibres with an average length of 380 and 230 μm, respectively (Table 24, Fig. 36). Both screened fractions were as easily and completely hydrolysed as the original SO_2-SEE-WIA/H_2O_2 fraction (Table 24). Fines and fibre bundles were virtually eliminated from the

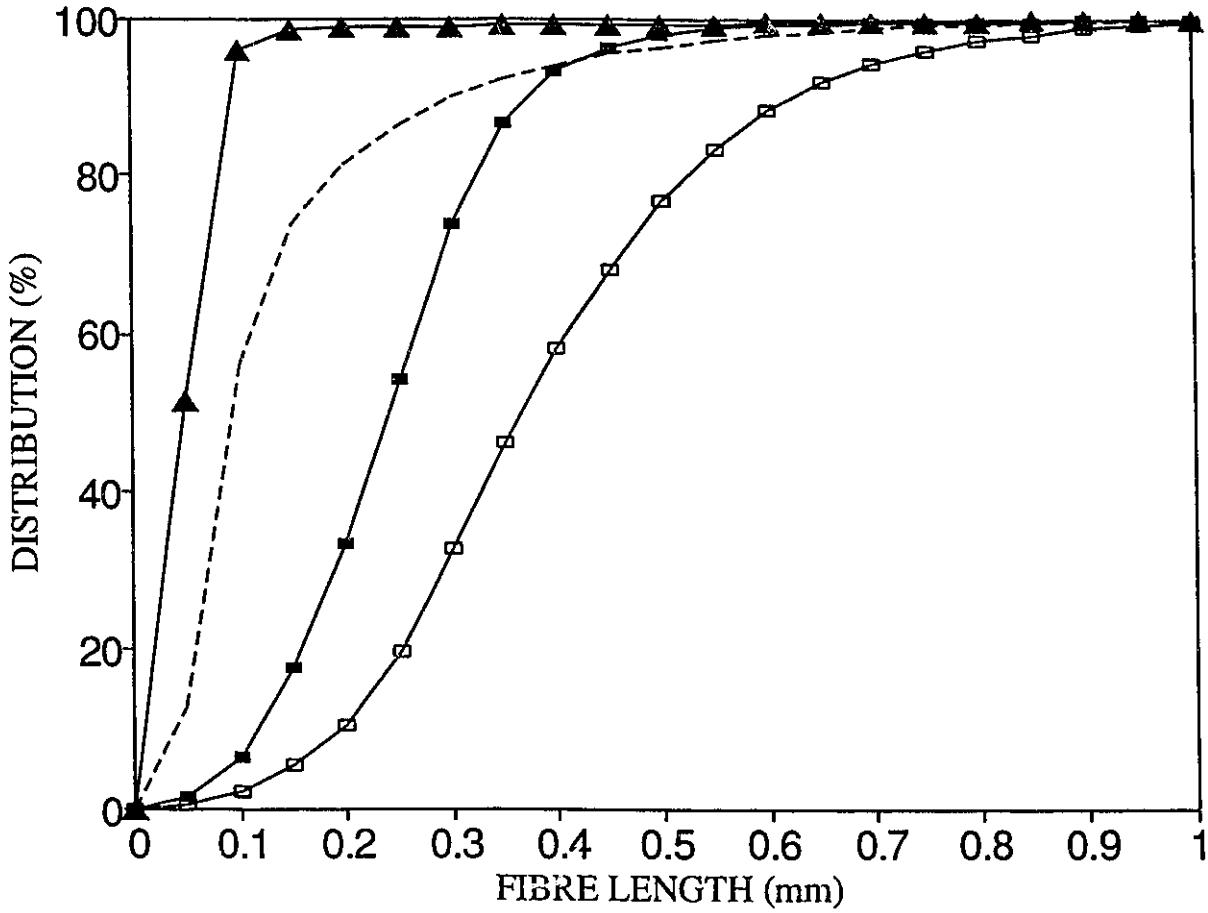


Fig. 35. Fibre length distribution of several substrate fractions obtained from peroxide-treated eucalyptus (SO_2-SEE-WIA/H_2O_2) by fibre screening. Substrate fractions were collected between fibre screens with (□) 50 and 150 mesh (F-150) and (■) 150 and 200 mesh (F-200) or (▲) were totally excluded from a 200 mesh screen. Broken line indicates the fibre length distribution of the original substrate.

Table 24. Effect of enzymatic hydrolysis on the fibre length (FL) and cellulose degree of polymerisation (DP) of several substrate fractions derived from peroxide-treated eucalyptus (SO_2-SEE-WIA/H_2O_2) by fibre screening.

SUBSTRATE[1]	HYDROLYSIS TIME (h)[2]				
	0	2	5	10	14
A. Original, unscreened fraction					
Glc yield[2] (%)	-	30.5	53.7	65.3	nd[6]
$\overline{FL_N}$[3] (µm)	100	70	80	80	nd
$\overline{DP_W}$[4]	330	268	246	220	nd
$\overline{DP_N}$[4]	75	72	67	50	nd
$\overline{DP_W}/\overline{DP_N}$[5]	4.4	3.7	3.7	4.4	nd
B. F-150 fraction					
Glc yield (%)	-	25.4	60.3	68.3	nd
$\overline{FL_N}$ (µm)	380	120	80	70	nd
$\overline{DP_W}$	372	314	281	226	206
$\overline{DP_N}$	84	80	74	46	62
$\overline{DP_W}/\overline{DP_N}$	4.4	3.9	3.8	4.9	3.3
C. F-200 fraction					
Glc yield (%)	-	31.9	62.6	70.8	nd
$\overline{FL_N}$ (µm)	230	100	70	50	nd
$\overline{DP_W}$	380	289	235	221	nd
$\overline{DP_N}$	86	76	71	74	nd
$\overline{DP_W}/\overline{DP_N}$	4.4	3.8	3.3	3.0	nd

[1] All fractions were derived from SO_2-impregnated steam-exploded eucalyptus chips which were post-treated with alkaline hydrogen peroxide (SO_2-SEE-WIA/H_2O_2);
[2] Hydrolyses were carried out at a 2% (w/w) substrate concentration and an enzyme loading of 10 FPU g^{-1} cellulose; glucose yields were determined in the hydrolysates by HPLC and calculated in relation to the cellulose content of the substrate;
[3] $\overline{FL_N}$, arithmetic average fibre length;
[4] Weight average ($\overline{DP_W}$) and number average degree of polymerisation ($\overline{DP_N}$);
[5] Polydispersity = $\overline{DP_W}$ / $\overline{DP_N}$;
[6] Not determined.

original material.

When variations in the fibre length distribution of the F-150 fraction were determined during a time course hydrolysis, a dramatic shift in the distribution was observed (Fig. 36). It seemed that small fragments of 50 - 100 µm were initially accumulated and gradually degraded during hydrolysis. The average length of the cellulose fibres decreased from 380 to approximately 100 µm within 2 h of hydrolysis, while only 25% of the substrate was hydrolysed to glucose (Table 24). Fibre length measurements on partially hydrolysed residues derived from the original, unscreened peroxide-treated fraction indicated a much slighter change in the Kajaani fibre length distribution, even after more than 65% of the substrates was hydrolysed (Fig. 37, Table 24). Unfortunately, it was not possible to detect and quantify particle sizes below 50 µm using the Kajaani fibre analyser. For this reason, each of the partially hydrolysed residues was examined by light microscopy. It was observed that a population of much smaller particles, averaging approximately 5 - 10 µm in length, was also released and gradually accumulated during hydrolysis (Fig. 38). When these particles, which were virtually absent in the original, unhydrolysed substrate (Fig. 38 A,B), were examined using polarized light, it appeared that they consisted of fragments of libriform fibres which still retained a high degree of crystallinity.

To investigate whether the enzymatic fragmentation of cellulose fibres was influenced by the initial fibre length of

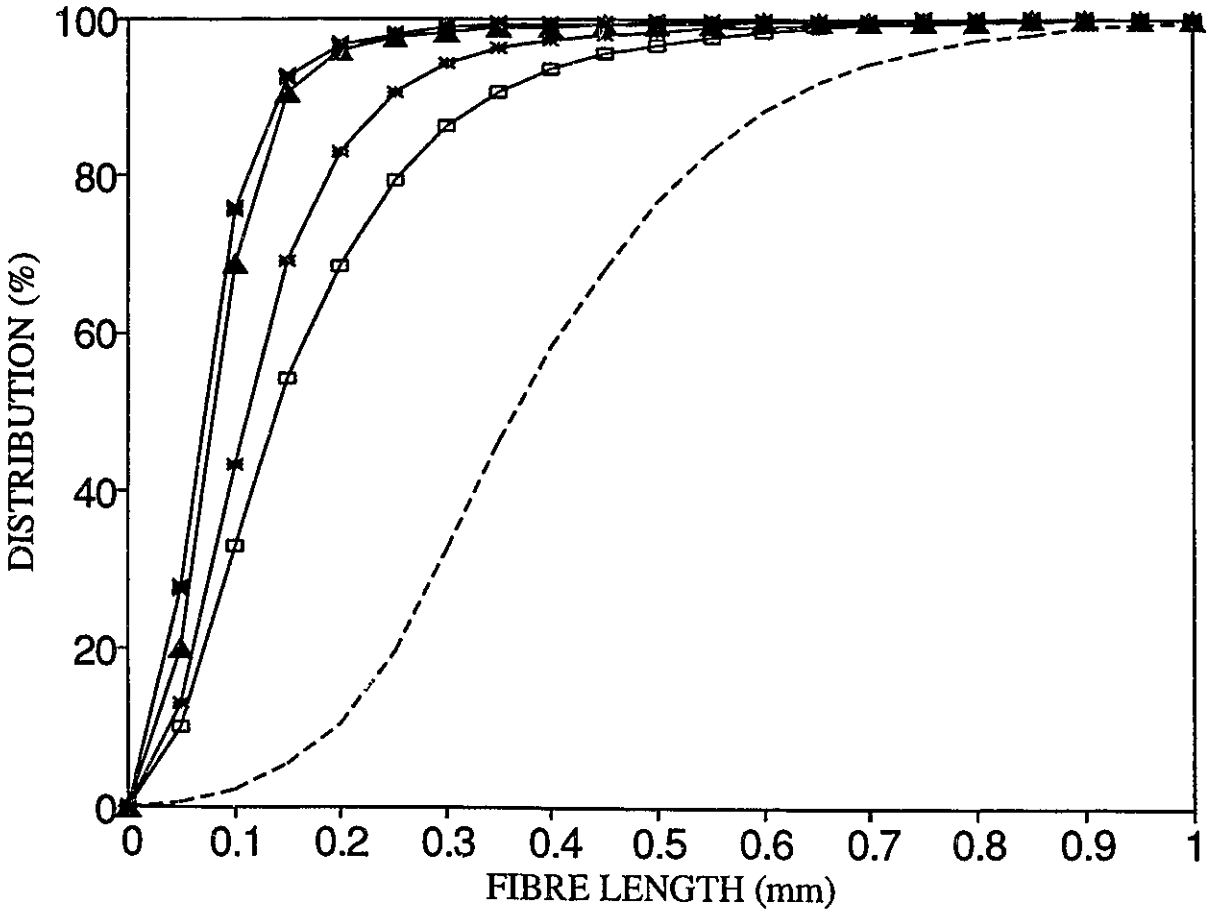


Fig. 36. Effect of enzymatic hydrolysis on the fibre length distribution of the F-150 fraction derived from peroxide-treated eucalyptus (SO_2-SEE-WIA/H_2O_2). Hydrolysis was performed at a 2% (w/v) substrate concentration and an enzyme loading of 10 FPU g^{-1} cellulose. Fibre length distributions were determined for partially hydrolysed residues obtained after (□) 0.5, (*) 2, (▲) 5 and (X) 10 h of hydrolysis. Broken line indicates the fibre length distribution of the original, unhydrolysed substrate.

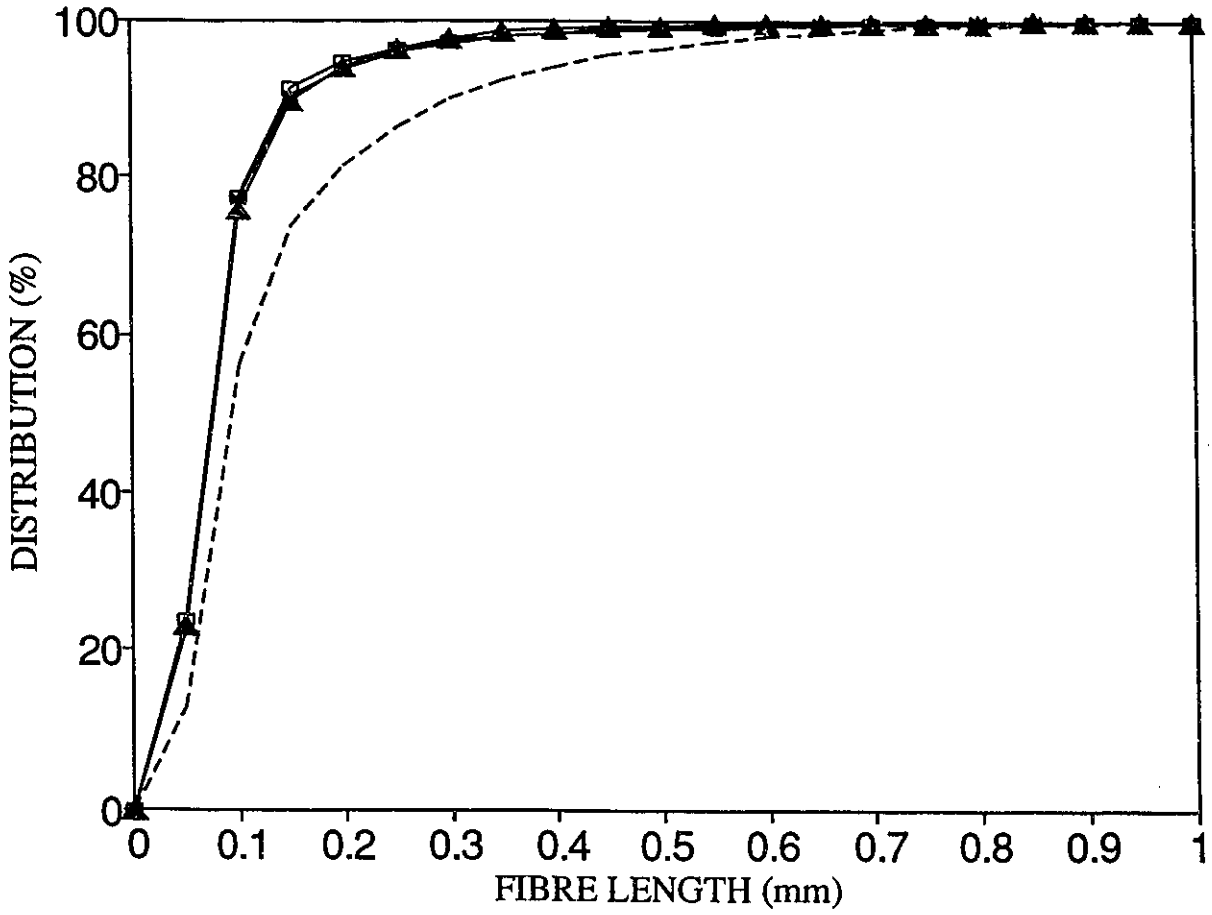
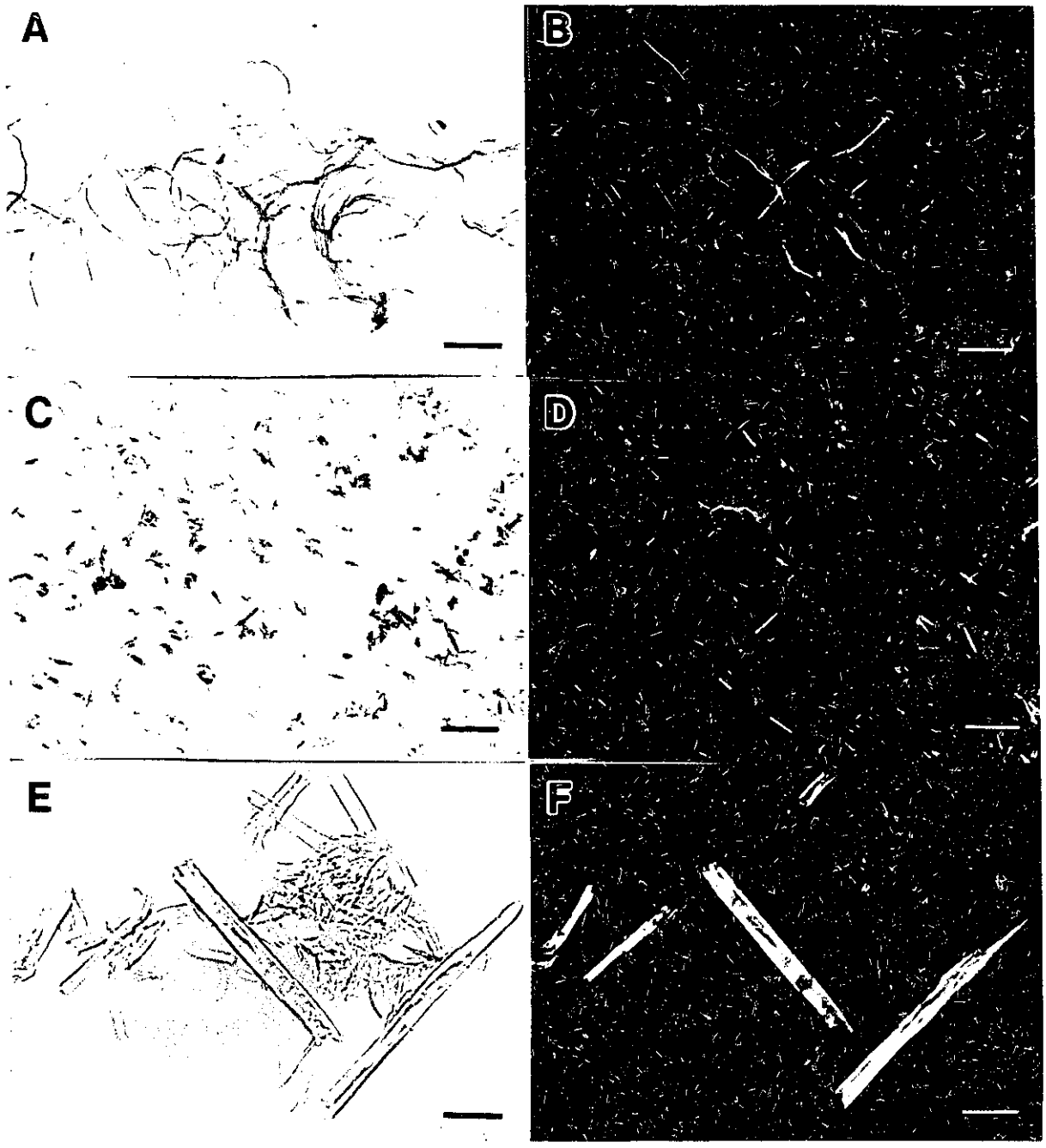


Fig. 37. Effect of enzymatic hydrolysis on the fibre length distribution of the original, unscreened peroxide-treated eucalyptus (SO_2-SEE-WIA/H_2O_2). Hydrolysis was performed at a 2% (w/v) substrate concentration and an enzyme loading of 10 FPU g^{-1} cellulose. Fibre length distributions were determined for partially hydrolysed residues obtained after (□) 0.5, (*) 5, and (▲) 10 h of hydrolysis. Broken line indicates the fibre length distribution of the original, unhydrolysed substrate.

Fig. 38. Light micrographs of partially hydrolysed residues obtained from the F-150 fraction of peroxide-treated eucalyptus (SO_2-SEE-WIA/H_2O_2). The F-150 fraction (**A,B**) was hydrolysed at a substrate concentration of 2% (w/v) and an enzyme loading of 10 FPU g^{-1} cellulose for 2 h (**C,D**) and 24 h (**E,F**). **A**, **C** and **E**, bright field micrographs; **B**, **D** and **F**, polarized light micrographs; **A-D**, bar = 300 μm; **E** and **F**, bar = 30 μm.


A
B
C
D
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the substrate, we obtained a sample of a fully bleached kraft pulp (FBEP) derived from eucalyptus. Screening of this sample provided two fractions, FBEP-48 (48 mesh) and FBEP-100 (100 mesh), which consisted of fibres with an average length of 920 and 700 μm, respectively (Table 25). By using these fractions, we hoped to observe an increased effect of hydrolysis on fibre length distribution. The high hemicellulose (xylan) content of 24% (w/w) of both FBEP derived fractions did not restrict hydrolysis, as the enzyme preparation used contained a considerable amount of xylanase activity (Coughlan, 1985). In fact, a 2% (w/v) suspension of each of these fractions was completely hydrolysed in 48 h using an enzyme loading of 10 FPU g^{-1} (Table 25). A short incubation time of 2 h was all that was required to reduce the average fibre length of the FBEP-48 from 920 to 170 μm (Table 25). Interestingly, a mixed distribution with at least two inflexion points was observed after 30 min, suggesting that the rapid fragmentation of fibres during hydrolysis occurred despite the original length of the fibre (Fig. 39). Similar results were obtained for the FBEP-100 fraction (Table 25).

3.4.2. Changes in the Molecular Weight Distribution of Cellulose

The molecular weight (MW) distribution of several pretreated substrates was then determined for pre- and post-hydrolysis situations. We hoped to see whether probable changes

Table 25. Effect of enzymatic hydrolysis on the fibre length (FL) and average molecular weight (or DP) of the several substrate fractions derived from a fully bleached, eucalyptus kraft pulp by fibre screening.

SUBSTRATE	HYDROLYSIS TIME (h)[1]						
	0	0.5	2	4	8	24	48
A. FBEP-48 fraction							
Glc yield[1] (%)	-	12.9	31.0	50.9	72.2	89.6	92.8
Xyl yield[1] (%)	-	3.7	29.5	36.0	nd[5]	96.0	101.0
$\overline{FL_N}$[2] (μm)	920	420	170	120	nd	nd	nd
$\overline{DP_W}$[3]	1128	1207	1082	1215	1173	1404	1141
$\overline{DP_N}$[3]	287	268	231	277	258	239	113
$\overline{DP_W}/\overline{DP_N}$[4]	3.9	4.5	4.6	4.4	4.5	5.9	10.1
B. FBEP-100 fraction							
Glc yield (%)	-	14.1	31.9	49.0	68.0	92.7	93.2
Xyl yield (%)	-	3.8	21.3	47.7	67.8	98.6	101.7
$\overline{FL_N}$ (μm)	700	290	160	120	nd	nd	nd
$\overline{DP_W}$	1020	912	879	nd	1216	1643	nd
$\overline{DP_N}$	290	294	260	nd	343	263	nd
$\overline{DP_W}/\overline{DP_N}$	3.5	3.1	3.4	nd	3.6	6.3	nd

[1] Enzymatic hydrolysis was carried out at a substrate concentration of 2% (w/w) and an enzyme loading of 10 FPU g^{-1} cellulose; glucose yields were determined in the substrate hydrolysates by HPLC;
[2] $\overline{FL_N}$, arithmetic average fibre length;
[3] Weight average ($\overline{DP_W}$) and number average degree of polymerisation ($\overline{DP_N}$);
[4] Polydispersity = $\overline{DP_W}$ / $\overline{DP_N}$;
[5] Not determined.

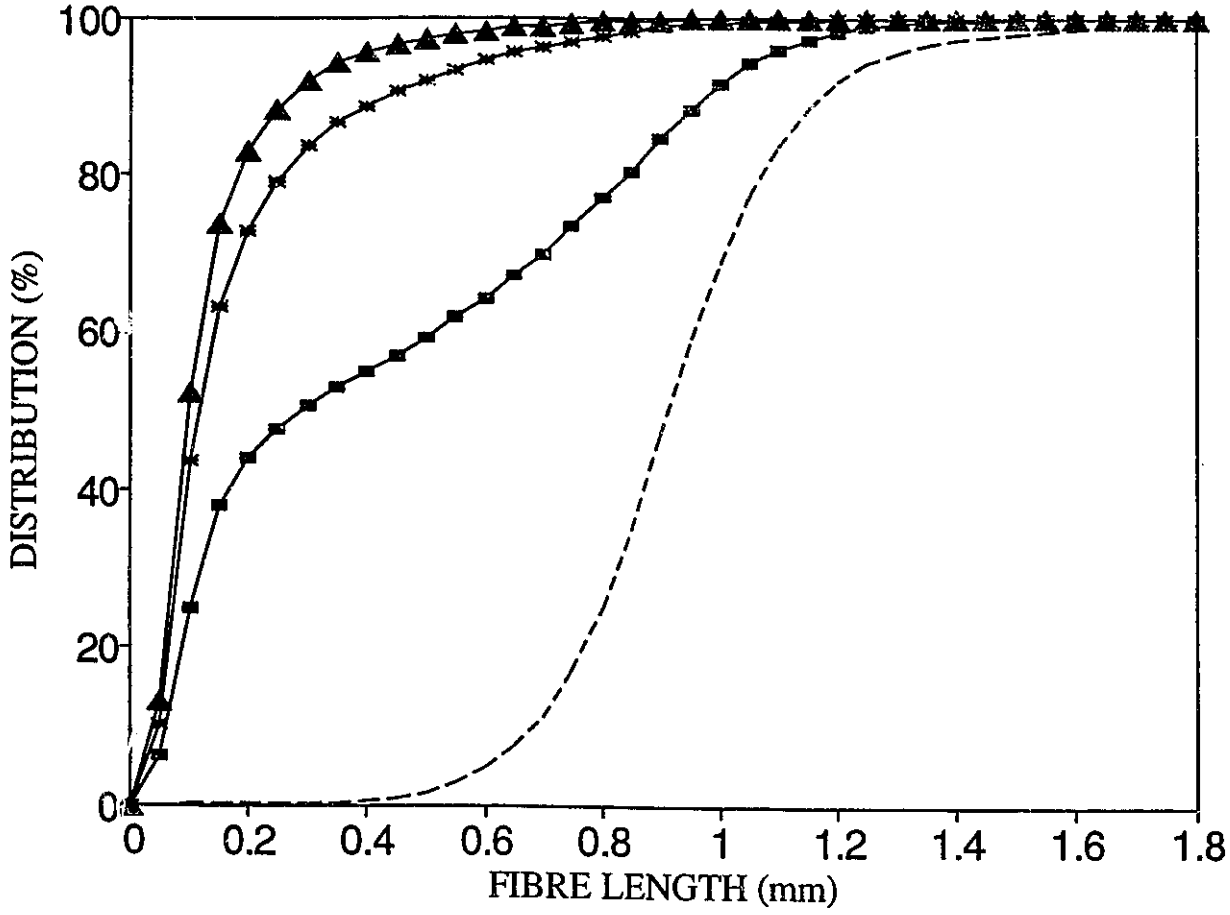

Fig. 39. Effect of enzymatic hydrolysis on the fibre length distribution of the FBEP-48 fraction derived from a fully bleached, eucalyptus kraft pulp. Hydrolysis was performed at a 2% (w/v) substrate concentration and an enzyme loading of 10 FPU g^{-1} cellulose. Fibre length distributions were determined for partially hydrolysed substrates obtained after (■) 0.5, (*) 2, and (▲) 4 h of hydrolysis. Broken line indicates the fibre length distribution of the original, unhydrolysed substrate.

in the degree of polymerisation (DP) of cellulose could give some information about the mechanism by which pretreated cellulosic substrates are hydrolysed. The MW distribution of cellulose was determined by gel permeation chromatography (GPC) after tricarbanylation of the cellulosic residue.

All fractions derived from the SO_2-SEE-WIA/H_2O_2 substrate, including the F-150 and F-200 fractions, were shown to have a similar MW distribution with a $\overline{DP_W}$ of 361 ± 22 ($\overline{DP_N}$ = 82 ± 5) anhydroglucose units (Table 24). When hydrolysis of these fractions was carried out at 2% (w/v) and 10 FPU g^{-1} cellulose, both the $\overline{DP_W}$ and $\overline{DP_N}$ of cellulose was shown to decrease in a similar fashion (Table 24). After 10 h of hydrolysis, the $\overline{DP_W}$ of the F-150 fraction was reduced by 40%, while approximately 70% of the substrate was converted to glucose (Fig 40, Table 24). However, no substantial increase in polydispersity ($\overline{DP_W}/\overline{DP_N}$) was observed throughout hydrolysis of these fractions (Table 24, Fig. 40). Although this progressive decrease in cellulose DP during hydrolysis could partially explain the high susceptibility of these substrates to hydrolysis, it appeared that rather large oligomers ($DP_W \geq 300$) persisted in the reaction mixture even after more extended incubation times (14 h) (Figs. 40 and 41). It also appeared that, at least for the initial 14 h of hydrolysis, cellooligomers with low DP did not contribute significantly to the molecular weight distribution of the partially hydrolysed residues. As short oligomers had a transient contribution to the overall population of molecules,

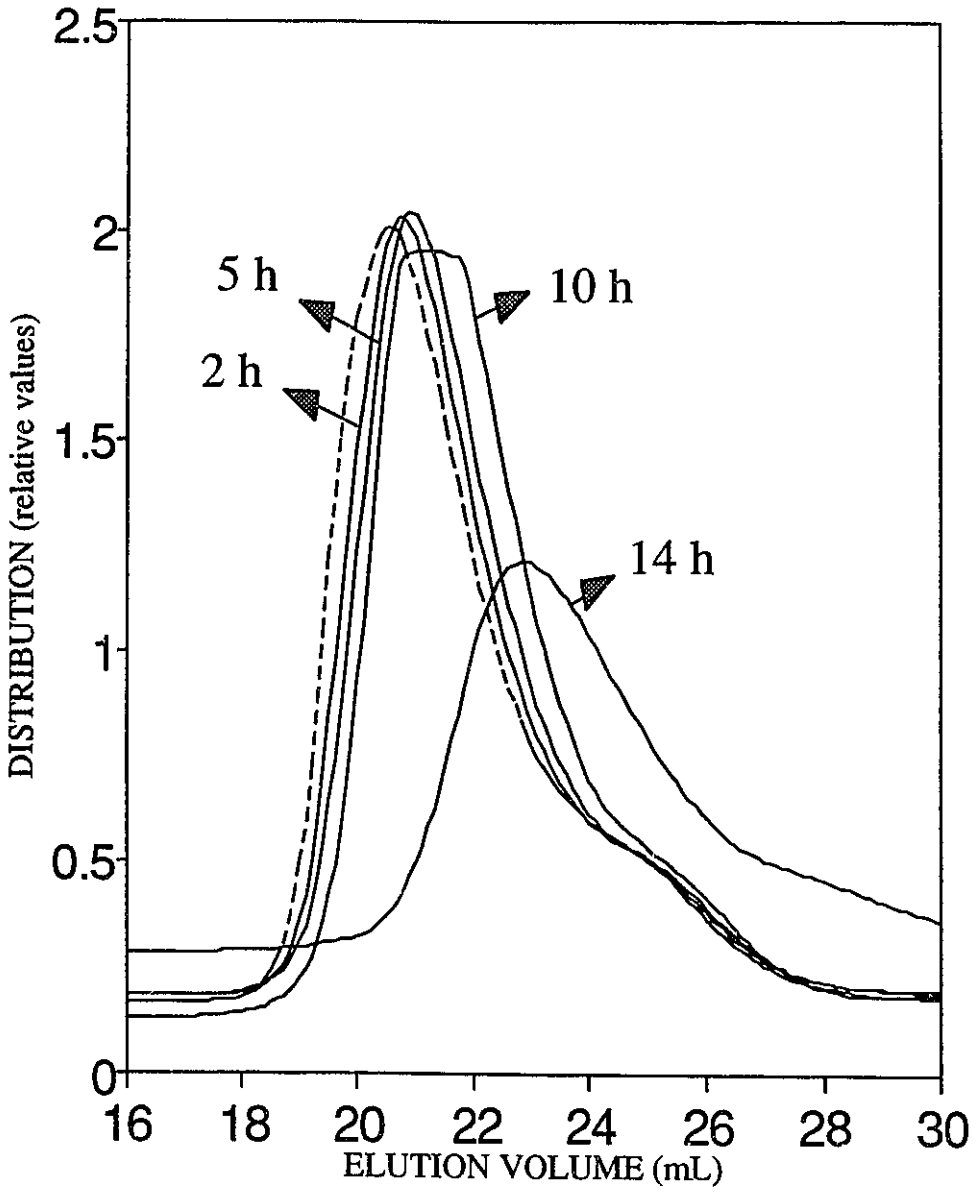

Fig. 40. Effect of enzymatic hydrolysis on the molecular weight (MW) distribution of the F-150 fraction derived from peroxide-treated eucalyptus (SO_2-SEE-WIA/H_2O_2). Hydrolyses were performed at a 2% (w/v) substrate concentration and an enzyme loading of 10 FPU g^{-1} cellulose. Partially hydrolysed residues were tricarbanylated and subsequently analysed by gel permeation chromatography (GPC). The arrows indicate the time for which the substrate was hydrolysed. The broken line indicates the MW distribution of the original, unhydrolysed substrate.

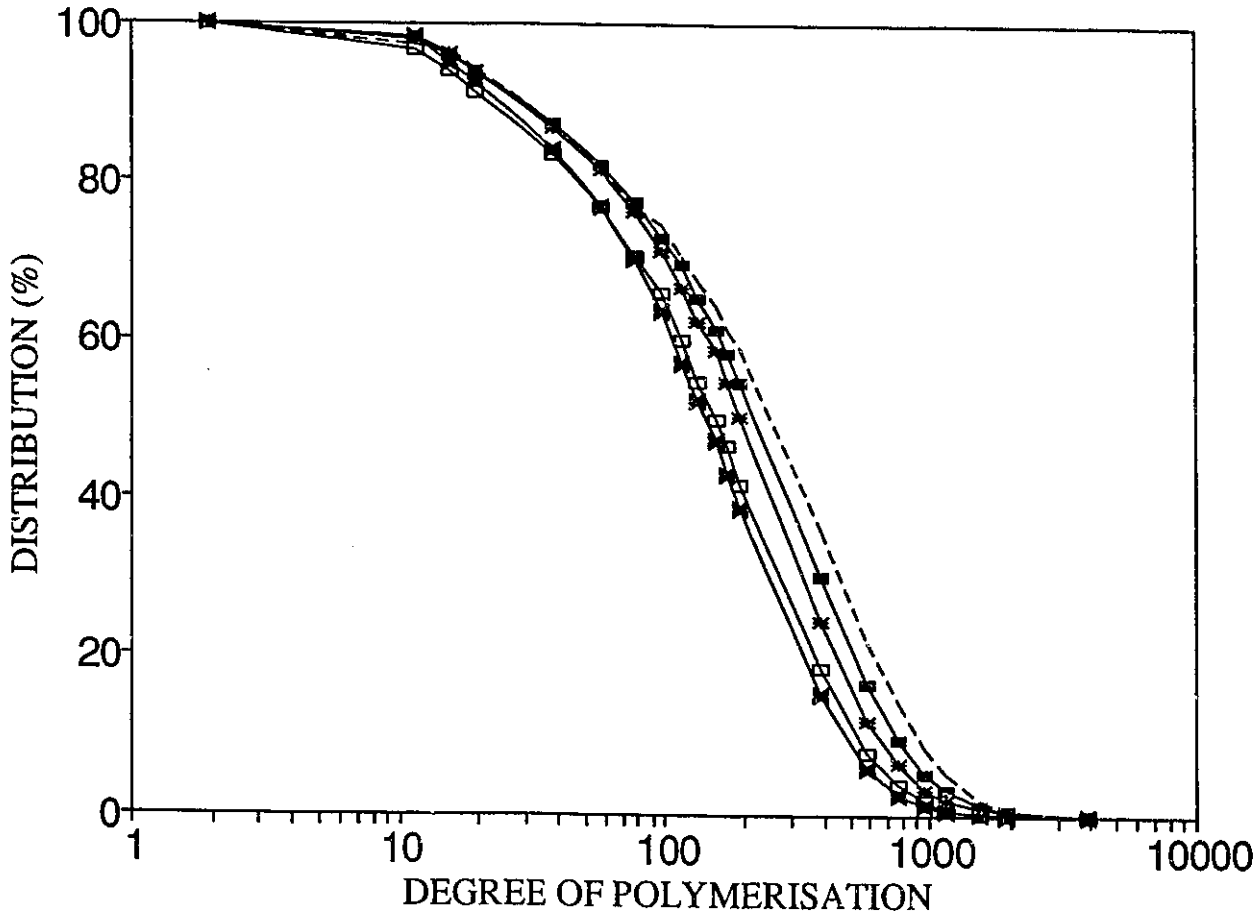

Fig. 41. Changes in the degree of polymerisation (DP) of cellulose during enzymatic hydrolysis of the F-150 fraction derived from peroxide-treated eucalyptus (SO_2-SEE-WIA/H_2O_2). Hydrolysis was performed at a 2% (w/v) substrate concentration and an enzyme loading of 10 FPU g^{-1} cellulose. DP distributions (cumulative values) were determined by GPC of the tricarbanyl derivative of partially hydrolysed residues, which were obtained after (■) 2, (*) 4, (□) 10, and (X) 14 h of hydrolysis. Broken line indicates the DP distribution of the original, unhydrolysed substrate.

it seemed that they were hydrolysed very quickly.

Partially hydrolysed residues derived from the FBEP fraction (eucalyptus kraft pulp) were also investigated with regard to changes in MW distribution of cellulose (Fig. 42). Because of the high xylan content of these fractions, both the $\overline{DP}_w$ and $\overline{DP}_N$ values obtained by GPC could not be used to characterize the enzymatic degradation of cellulose. Nevertheless, during the first 24 h of hydrolysis of both FBEP-48 and FBEP-100 fractions, there was no substantial change in DP distribution of cellulose (Table 25, Fig. 43). It was also observed that, after 24 h of hydrolysis, a relatively large amount of shorter MW oligomers was released in the hydrolysate, resulting in a substantial increase in polydispersity ($\overline{DP}_w/\overline{DP}_N$). The polydispersity of the FBEP-48 fraction increased from 5.9 at 24 h to 10.1 at 48 h (Table 25). This was partly associated with an increased accumulation of a sub-population of cellulose oligomers with a $\overline{DP}_w$ of approximately 33 anhydroglucose units.

3.4.3. Changes in the Degree of Crystallinity of the Substrate

The effect of enzymatic hydrolysis on the degree of crystallinity of the F-150 fraction was then determined by X-ray diffraction (Table 26, Fig. 44). There was a gradual reduction in the degree of crystallinity of the substrate during the first 10 h of hydrolysis, as indicated by a decrease in the intensity of the diffraction pattern of partially hydrolysed residues

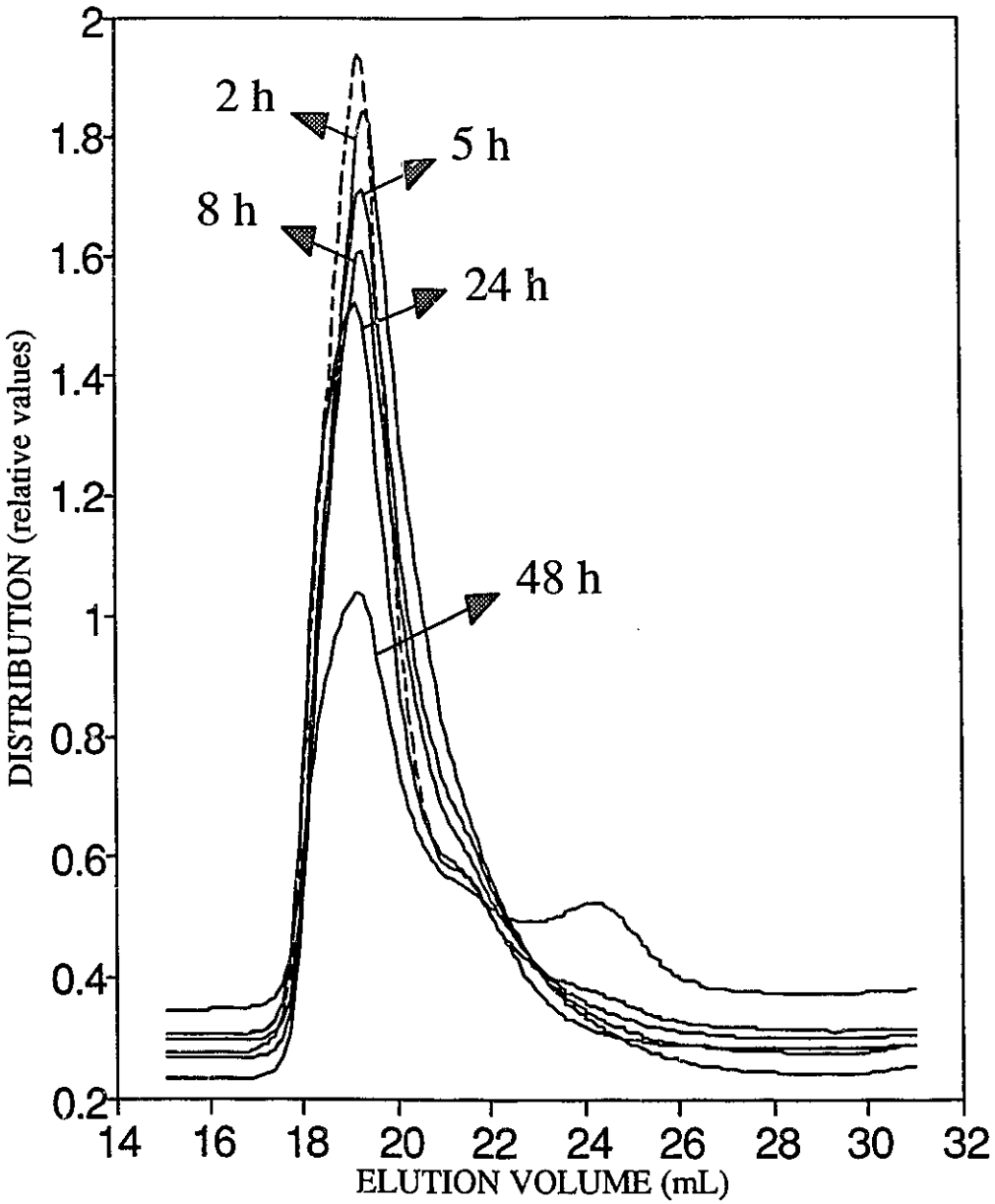

Fig. 42. Effect of enzymatic hydrolysis on the molecular weight (MW) distribution of the FBEP-48 fraction derived from a fully bleached, eucalyptus kraft pulp. Hydrolyses were performed at a 2% (w/v) substrate concentration and an enzyme loading of 10 FPU g^{-1} cellulose. Partially hydrolysed residues were tricarbanylated and subsequently analysed by gel permeation chromatography (GPC). The arrows indicate the time for which the substrate was hydrolysed. The broken line indicates the MW distribution of the original, unhydrolysed substrate.

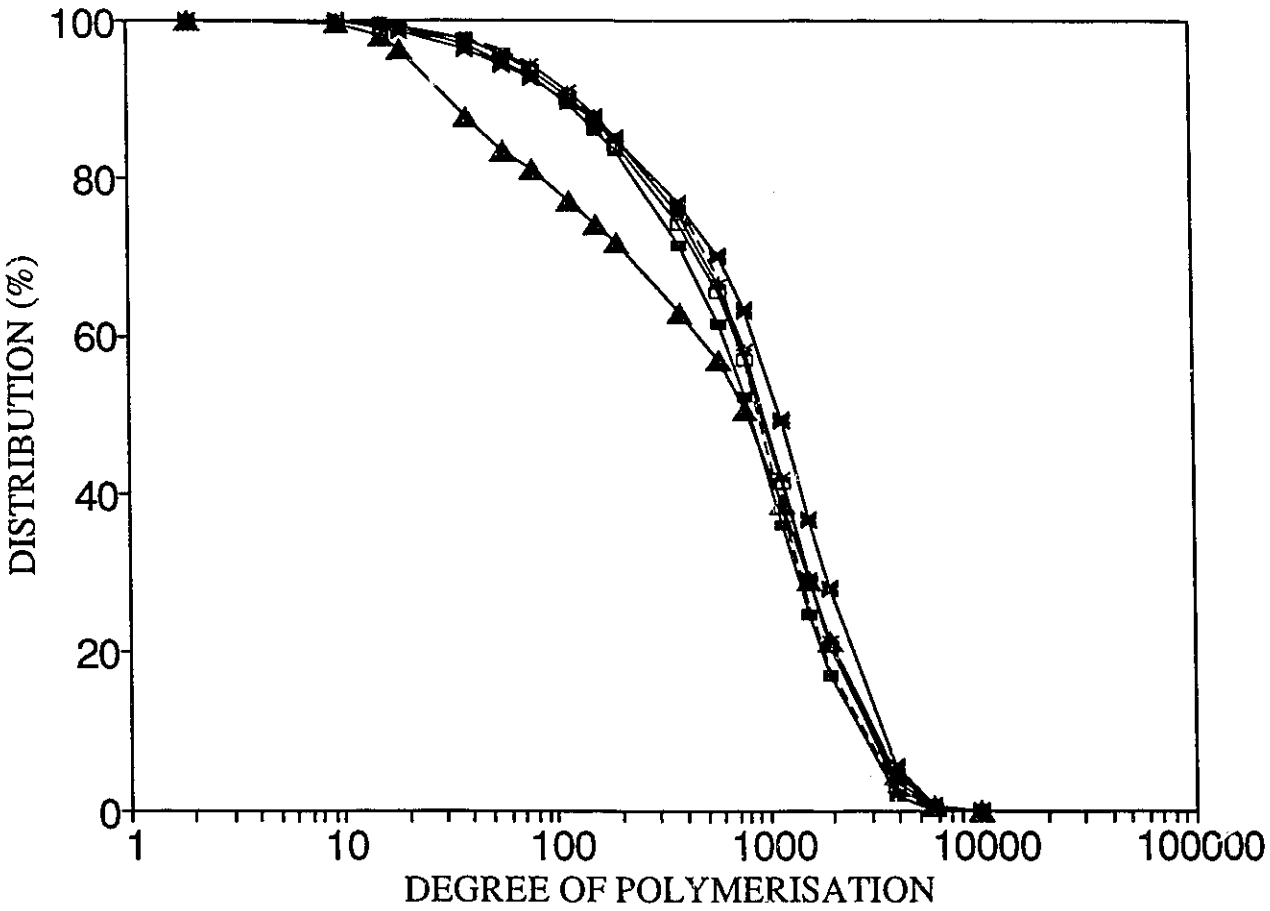

Fig. 43. Changes in the degree of polymerisation (DP) of cellulose during enzymatic hydrolysis of the FBEP-48 fraction derived from a fully bleached, eucalyptus kraft pulp. Hydrolysis was performed at a 2% (w/v) substrate concentration and an enzyme loading of 10 FPU g^{-1} cellulose. DP distributions (cumulative values) were determined by GPC of the tricarbanyl derivative of partially hydrolysed residues, which were obtained after (■) 2, (*) 4, (□) 8, (X) 24, and (▲) 48 h of hydrolysis. Broken line indicates the DP distribution of the original, unhydrolysed substrate.

Table 26. The influence of enzymatic hydrolysis on the crystallinity index (CrI) of the F-150 fraction derived from peroxide-treated eucalyptus (SO_2-SEE-WIA/H_2O_2) by fibre screening, with reference to changes in the average dimensions of the cellulose crystallite.

METHOD USED FOR CALCULATION OF CrI (%)	HYDROLYSIS TIME[1] (h)					
	0	2	5	10	14	24
Peak Intensity[2]	77.0	76.0	75.0	65.7	63.2	74.0
s.d.[3]	1.0	1.7	1.0	1.5	0.8	1.0
Area Integration[4]	66.0	64.0	64.0	58.0	50.0	61.5
s.d.	1.7	1.7	1.7	1.7	1.7	1.7
Crystallite dimensions[5] (nm)						
t (002)	4.3	4.0	3.9	3.7	3.5	3.6
t (040)	12.0	13.2	11.2	12.4	10.3	12.4
t (001)	5.7	5.0	3.9	3.2	3.1	nd[6]
t (101)	2.4	2.2	2.1	2.2	2.1	nd

[1] Enzymatic hydrolysis was carried out at a substrate concentration of 2% (w/w) and an enzyme loading of 10 FPU g^{-1} cellulose; glucose yield during hydrolysis was determined by HPLC as described in Methods;
[2] Described by Segal *et al.* (1959);
[3] Standard deviation of the sample (n = 3);
[4] Described by Wims *et al.* (1986);
[5] Determined according to the Scherrer equation (see section 2.3.3.), where t is the thickness of the crystallite at the indicated diffraction plane (value in parenthesis);
[6] Not determined.

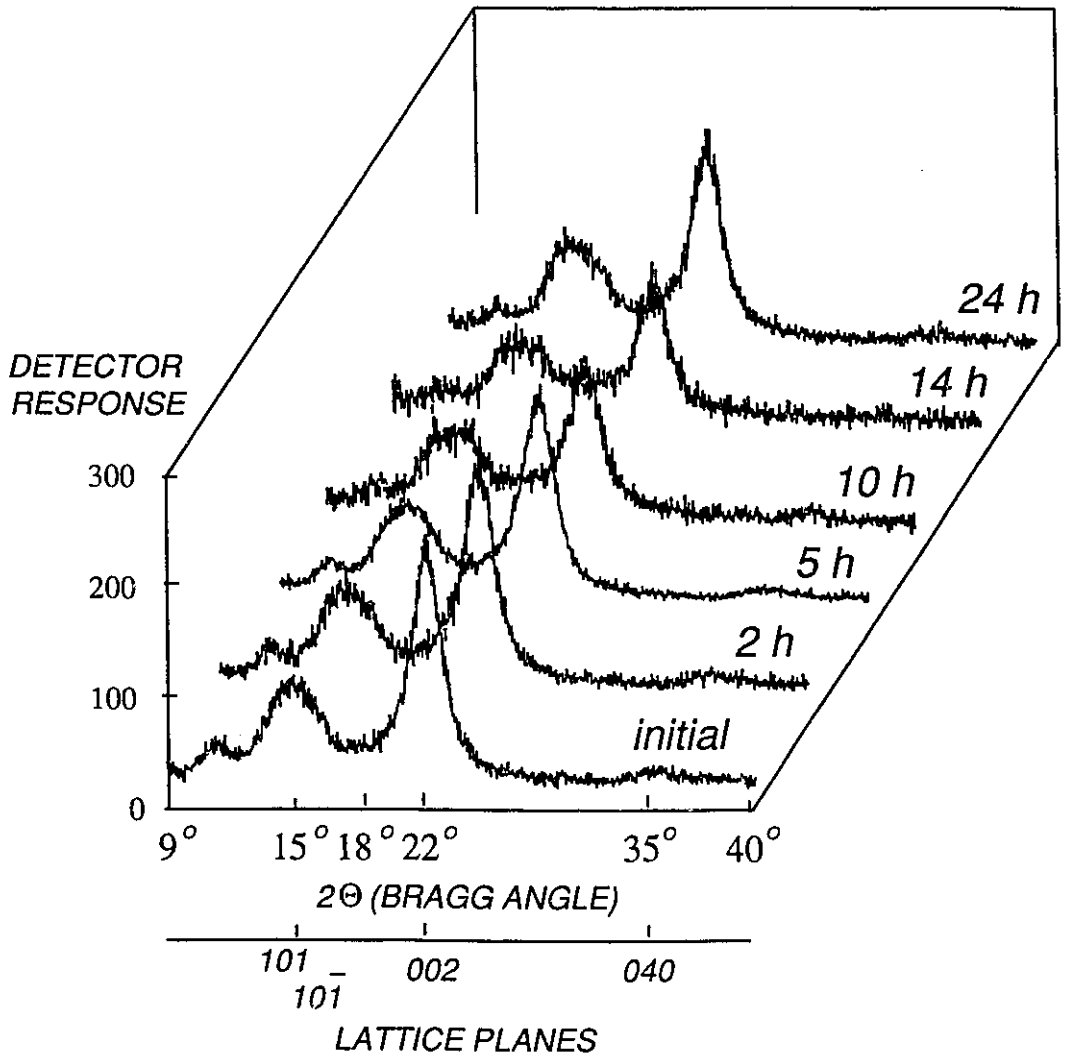

Fig. 44. Effect of enzymatic hydrolysis on the X-ray diffraction pattern of the F-150 fraction derived from peroxide-treated eucalyptus (SO_2-SEE-WIA/H_2O_2).

(Fig. 44). This trend was also observed when the crystallinity index (CrI) of the substrate was calculated from each of the diffractograms using empirical methods. Both methods used in these calculations, the intensity method (Segal *et al.*, 1959) and the area integration method (Wims *et al.*, 1986), indicated that there was a gradual decrease in the crystallinity index of the substrate during the first 14 h of hydrolysis (Table 26). However, the subtle increase in the CrI and in the intensity of the diffraction pattern of the substrate, which was observed after 24 h of hydrolysis, may have been a result of a partial recrystallization of cellulose during sample preparation. After drying, cellooligomers of low DP can easily undergo structural rearrangements which result in a highly organized, hydrogen-bond-ordered (crystalline) organization (Atalla *et al.*, 1984). This rearrangements can occur even when drying is carried out using less disruptive methods such as solvent extraction followed by freeze-drying.

By contrast, the enzymatic hydrolysis of the FBEP-48 fraction derived from a fully bleached, eucalyptus kraft pulp did not appear to have any influence on the degree of crystallinity of the substrate. Instead of the gradual decrease in crystallinity which was observed with the F-150 fraction, the intensity of the diffraction pattern of partially hydrolysed residues obtained after 0.5, 4 and 8 h of hydrolysis were very similar to that of the original, unhydrolysed residue (Fig. 45). These observations were confirmed by the CrI values which were

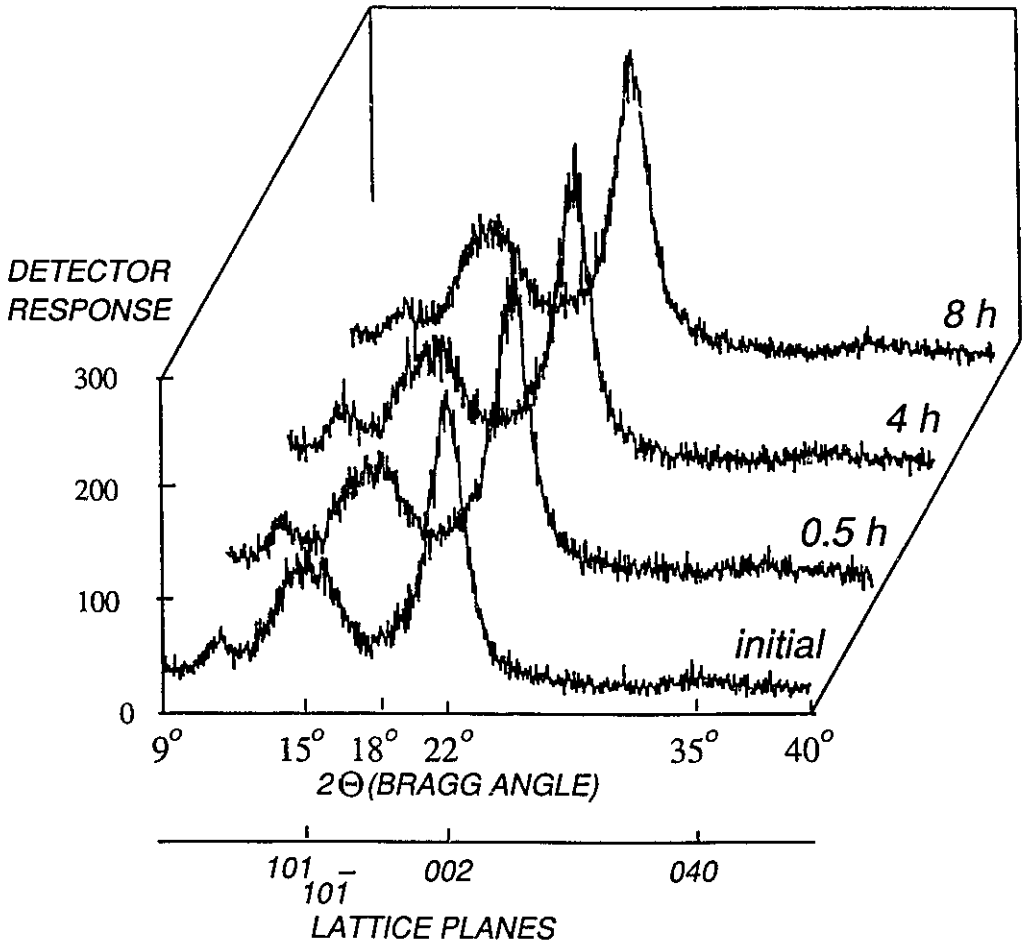

Fig. 45. Effect of enzymatic hydrolysis on the X-ray diffraction pattern of the FBEP-48 fraction derived from a fully bleached, eucalyptus kraft pulp.

calculated from each of the diffractograms using both the intensity and the area integration methods (Table 27).

X-ray diffraction of several partially hydrolysed residues also provided some information about changes in the dimension of the cellulose crystallite during hydrolysis. Although they were indirect measurements, our results indicated that, for the F-150 fraction derived from peroxide-treated eucalyptus, the average thickness of the crystallite at the diffraction planes 040 and 101 remained relatively constant, whereas the thickness at the diffraction planes 001 and 002 decreased considerably. Although it was not possible to draw any conclusion about the mode of action of *Trichoderma* cellulases based on these observations, a gradual change in the supramolecular organization of the F-150 fraction was likely to occur due to the effect of hydrolysis on both cellulose DP and CrI. This result also provided additional evidence to support the exo-endo synergism that is usually displayed by these types of enzymes (White and Brown, 1981; Beldman *et al.*, 1988; Wood *et al.*, 1989). For the FBEP-48 fraction, however, the average crystallite dimensions were shown to remain relatively constant throughout hydrolysis. Some of the values obtained for each of the four diffraction planes, 001, 002, 040 and 101, were slightly scattered and showed no recognizable trend. As there was no noticeable effect of hydrolysis on either the crystallinity index or the molecular weight distribution of the FBEP-48 fraction, it seemed that this substrate, in comparison to the F-150 fraction, was hydrolysed

Table 27. The influence of enzymatic hydrolysis on the crystallinity index (CrI) of the F-48 fraction derived from a fully bleached, eucalyptus kraft pulp by fibre screening, with reference to changes in the average dimensions of the cellulose crystallite.

METHOD USED FOR CALCULATION OF CrI (%)	HYDROLYSIS TIME[1] (h)			
	0	0.5	4	8
Peak Intensity[2]	76.3	76.8	76.7	76.7
s.d.[3]	1.2	0.4	0.6	1.2
Area Integration[4]	58.0	62.0	63.0	60.0
s.d.	1.8	1.8	1.8	1.8
Crystallite dimensions[5] (nm)				
t (002)	4.0	4.3	4.4	4.3
t (040)	3.6	4.1	4.0	4.2
t (001)	5.3	6.4	6.6	5.6
t (101)	2.1	2.1	2.1	2.2

[1] Enzymatic hydrolysis was carried out at a substrate concentration of 2% (w/w) and an enzyme loading of 10 FPU g^{-1} cellulose; glucose yield during hydrolysis was determined by HPLC as described in Methods;
[2] Described by Segal *et al.* (1959);
[3] Standard deviation of the sample (n = 3);
[4] Described by Wims *et al.* (1986);
[5] Determined according to the Scherrer equation (see section 2.3.3.), where t is the thickness of the crystallite at the indicated diffraction plane (value in parenthesis).

in a substantially different way. This was probably due to differences in the fine structure of each of the two fractions.

4. DISCUSSION

4.1. Steam Pretreatment for Enhanced Enzymatic Hydrolysis of Wood Residues

In this work, we have used *E. viminalis* chips derived from the stem of 6-7 year old trees. At this age, the stem is almost exclusively composed of sapwood and is relatively free of polyphenolic deposits and extractives. Owing to its morphological characteristics and structural properties, sapwood has a higher porosity than heartwood and is generally more permeable to chemical impregnation and steam. Chips derived from young *E. viminalis* trees were highly amenable to steam pretreatment and subsequent fractionation. Water and alkali extraction of steam-treated eucalyptus removed most of the hemicellulose and lignin originally present in the wood chips, resulting in a cellulosic residue that could be readily hydrolysed to glucose. Earlier studies on the enzymatic hydrolysis of the steam-treated *E. regnans* derived from 40 year old stems indicated that alkali extraction resulted in a substantial decrease in the ease of hydrolysis of the water-insoluble substrate (Dekker *et al.*, 1987). Therefore, it is possible that the fractionation of steam-treated materials derived from eucalyptus is influenced by the age of the tree. The ease of steam-treating eucalyptus chips, added to the fast-growing characteristics of this species, strongly supports the potential of *Eucalyptus* sp. as a bioconversion feedstock.

When pretreatment was carried out in the absence of a catalyst, the best conditions for hydrolysis resulted from steaming eucalyptus chips at 240°C for 120 s. However, a major decline in pentosan recovery yield was observed as the severity of pretreatment was increased. As the most desirable pretreatment conditions should result in good overall recovery yields of both the pentosan and glucan at high rates of enzymatic hydrolysis, the milder conditions of 230°C for 120 s were considered optimal for pretreating eucalyptus chips with a moisture content of 50% (w/w). This data was in good agreement with previous studies on the high-pressure steaming of aspen, in which substrate accessibility and recovery yield of water-soluble sugars were indirectly related (Brownell and Saddler, 1984; Schwald *et al.*, 1989a,b; Excoffier *et al.*, 1991).

The Klason lignin analysis of several steam-treated substrates derived from eucalyptus chips indicated that increased levels of acid-insoluble lignin were obtained at more drastic pretreatment conditions. It has been suggested that, during pretreatment, acid-insoluble compounds are formed within the substrate which greatly interfere with the determination of the Klason lignin content (Brownell and Saddler, 1984). These extraneous polymeric compounds, often referred to as pseudolignin, are formed by condensation reactions involving lignin and/or pentosan oxidation by-products. As a rule, the formation of pseudolignin increases with the severity of pretreatment.

In the absence of added catalyst and using non-explosive decompression, wood chips with a moisture content of 50% (w/w) provided good recovery of steam-treated material and the highest degree of enzymatic hydrolysis. However, when chips with low moisture content were pretreated under the same conditions (no explosion), a substantial decline in the hydrolysis yield was observed. It seems that, when the steam pressure within the vessel (steam gun) was bled down to atmospheric pressure at a controlled rate, the moisture gained by steam condensation was evaporated, resulting in a recovered substrate with a very low moisture content. This material was difficult to hydrolyse and further water extraction did not improve its susceptibility to hydrolysis. However, when this low moisture content substrate was subsequently washed with alkali, more than 70% of the lignin component was removed, yielding a good substrate for hydrolysis. It is possible that drying caused the lignin to adsorb onto the cellulose fibres, restricting the swelling of cellulose in water and therefore reducing the substrate surface area available to the cellulase enzymes.

In contrast to the results obtained with green chips, the steam explosion of air-dried chips showed no substantial differences in recovery yields and enzymatic hydrolysis. These results were in good agreement with those previously obtained during the steam pretreatment of aspen chips (Brownell *et al.*, 1986b; Brownell and Saddler, 1987). It seemed that, during the process of rapidly emptying the steam gun by steam explosion,

the almost immediate condensation of steam helped to prevent the steam-treated substrate from drying up. We also observed that alkali washing had a more beneficial effect on the enzymatic hydrolysis of cellulose when pretreatment was carried out by steam explosion rather than steam heating (no explosion). As the steam pretreatment of air-dried chips without explosion resulted in poor substrates for hydrolysis, we concluded that explosion is a desirable step for pretreatment and fractionation of wood chips with a low moisture content.

The pre-impregnation of eucalyptus chips with 1% (w/w) SO_2 reduced both the temperature and time requirements necessary to achieve optimal hydrolysis, recovery and fractionation of steam-treated substrates. The best substrate for hydrolysis was produced at 210°C for 50 s and further increases in the temperature or residence time of pretreatment resulted in lower recovery of water-soluble sugars without any improvement in the enzymatic digestibility of the substrate. It was concluded that pretreatment at high temperatures and short residence times were desirable since lower levels of pentosan degradation were obtained. These results were consistent with studies previously reported on steam pretreatment of aspen and spruce (Mackie *et al.*, 1985; Schwald *et al.*, 1989a,b). However, higher SO_2 loadings of 1.6 and 2.5% (w/w) were shown to be required to produce the most accessible substrate for hydrolysis from aspen and spruce, respectively. It is probable that pretreatment of eucalyptus can be further optimised by defining optimum SO_2 loadings. Our

results suggested that a relatively low SO_2 concentrations of 1% (w/w) is all that is required to generate enough sulphuric acid within the wood chips. Furthermore, lower SO_2 loadings are also desirable from an economic and environmental perspective.

Previous studies have not assessed the influence of the moisture content of wood chips on high-pressure steaming when SO_2 is used as an acid catalyst. We have shown that high moisture contents are essential for an even steam treatment of SO_2-impregnated eucalyptus chips. Higher glucose yields were obtained when pretreated substrates derived from moist chips were hydrolysed. Pentosan hydrolysis and survival was also shown to follow a linear correlation with increased moisture content of the chips. Chips with a low moisture content did not retain SO_2 as well as green chips, resulting in unevenly cooked substrates which were largely resistant to enzymatic attack. After pretreatment, chips at the bottom of the canister were detected as a charcoal-like residue, while uncooked chips were found at the top of the canister. It seemed that, during steaming, part of the SO_2 gas which had been previously added was pressed down to the bottom of the column of the chips by the penetrating steam. This process, in combination with steam condensation, may have caused uneven distribution of sulphuric acid within the chips.

Extraction with mild alkali has been used extensively by several groups as a means of reducing the lignin content of steam-treated substrates while increasing the swelling of the

cellulose component in water (Playne, 1984; Fox *et al.*, 1989). When the substrate concentrations used for hydrolysis were calculated in relation to the dry-weight of the substrate, the alkali-washed substrate derived from SO_2-impregnated steam-treated eucalyptus exhibited a slightly lower accessibility than the water-insoluble fraction. However, when the substrate concentrations were calculated in relation to the cellulose content of the substrate, alkali washing was shown to have virtually no influence on the rate by which the substrate was hydrolysed, even at high cellulose concentrations of 5% (w/v). These results suggested that, compared to the water-washed fraction, the higher cellulose content of the alkali-washed substrate resulted in a higher degree of end-product inhibition of the enzymes. It also appeared that the beneficial effects of alkali extraction, such as lignin removal and cellulose swelling, were counteracted by other factors which had a detrimental effect on hydrolysis, such as the redistribution of the residual lignin and modifications in the crystalline state of cellulose. All of these factors appeared to influence one another, resulting in an overall reduced effect of alkali washing on the efficiency of hydrolysis of the substrate. Comparable results have been reported previously for other steam-treated substrates derived from hardwoods (Schwald *et al.*, 1989a; Excoffier *et al.*, 1991). Alkali washing has been shown to have a negative effect on hydrolysis and fractionation of steam-treated substrates derived from other lignocellulosic materials.

These include, spruce (Schwald *et al.*, 1989b), radiata pine (Wong *et al.*, 1988a), sugar cane bagasse (Dekker and Wallis, 1983) and another eucalyptus species (Dekker *et al.*, 1987). Therefore, it seems that the influence of alkali washing on the accessibility of steam-exploded substrates depends on the characteristics and chemical composition of the feedstock used.

Several authors have indicated that steam-treated materials derived from softwoods, such as spruce (Schwald *et al.*, 1989b) and radiata pine (Clark and Mackie, 1987), were not as readily hydrolysed as pretreated substrates derived from hardwoods (Schwald *et al.*, 1989a). It was demonstrated that the effective pretreatment of softwood species required different steaming conditions from those used for hardwoods as well as the addition of a catalyst. This greater ease of hydrolysis of hardwoods over softwoods has often been associated with differences in the morphology and chemical composition of the various species. For instance, it had been shown that the alkali extraction of lignin decreased both the rate and extent of hydrolysis of steam-treated substrates derived from spruce (Schwald *et al.*, 1989b) and radiata pine (Wong *et al.*, 1988a). It appeared that it was primarily the nature and the redistribution of the guaiacyl lignin found mostly in softwoods which restricted the hydrolysis of the cellulose from steam-treated spruce (Schwald *et al.*, 1989b). Although the chemical structure of this residual lignin has not been resolved, it probably consists of highly condensed lignocarbohydrate complexes which had been chemically modified

during steaming.

When the residual substrate remaining after extensive hydrolysis of steam-treated eucalyptus was examined microscopically, it was apparent that this debris was mainly composed of vessel elements. Vessel elements are the most accessible type of hardwood cell yet they appear to be extremely resistant to enzymatic attack. A scanning electron microscopy (SEM) examination of the ultrastructure of decayed stumps of red alder indicated that libriform fibres and ray parenchyma cells were almost totally degraded, whereas vessel elements remained relatively unmodified after extensive deterioration of the stem (Barbour and Leney, 1981). Several authors have demonstrated that the lignin component of vessel elements has a greater guaiacyl to syringyl ratio than other cells found in the hardwood tissues (Musha and Goring, 1975; Saka and Goring, 1985). Therefore, it seemed that high levels of deterioration were only observed where syringyl lignin was predominant and that the lower accessibility of plant vessels was partially due to the occurrence of a more recalcitrant guaiacyl lignin type in the vessel walls. A selective degradation of libriform fibres and parenchyma cells was also observed during the enzymatic hydrolysis of acid-pretreated substrates derived from birch (Ucar and Fengel, 1988).

The peroxide treatment of the alkali-washed substrate derived from eucalyptus did not appear to result in as dramatic an increase in accessibility as it had been obtained previously

with aspen or spruce (Schwald *et al.*, 1989a,b). Nevertheless, the peroxide-treated fraction (SO_2-SEE-WIA/H_2O_2) obtained from steam-treated eucalyptus was the best substrate for hydrolysis, particularly at high substrate concentrations. Although this greater accessibility of the peroxide-treated fraction was primarily associated with its very low lignin content, other structural factors may have contributed towards a better accessibility of the substrate to cellulolytic enzymes. The peroxide treatment probably increased the degree of fibrillation and/or delamination of the fibres, causing a reduction in the crystallinity and/or degree of polymerisation of cellulose and a subsequent increase in the available surface area of the substrate.

As compared to aspen and spruce, all of the steam-treated fractions derived from *E. viminalis* were more accessible to enzymatic hydrolysis, particularly at substrate concentrations of 10% (w/v). Although slight differences in the level of end-product inhibition may have occurred due to variations in the cellulose content of the substrate, these were probably negligible due to the high level of supplemental β-glucosidase (30 - 35 CBU g^{-1} cellulose) which minimized cellobiose accumulation. Steam-treated substrates derived from eucalyptus were a better substrate for hydrolysis than the equivalent spruce substrates because of the superior susceptibility of hardwoods to steam pretreatment. However, the superiority of pretreated eucalyptus over aspen was probably associated with

the age of the tree. As mentioned earlier, pretreatment of eucalyptus was carried out using chips derived from a 6-7 year old stem, whereas in the earlier work with aspen, pretreatment was performed using chips derived from adult stems with approximately 40 year old. Therefore, young stems of *E. viminalis* may be more easily pretreated than adult stems of aspen because of their higher content of sapwood in relation to heartwood tissue.

4.2. Enzymatic Hydrolysis of Cellulosic Substrates

Although several groups have routinely used enzymatic saccharification as an indicator for the effectiveness of pretreatment, variations in the methodology used for hydrolysis have made any comparison very difficult. For instance, enzyme loadings used for hydrolysis have been expressed in terms of the dry weight of the substrate, rather than in terms of its cellulose content. Eklund *et al.* (1990) used an enzyme loading of 11.4 FPU g^{-1} oven-dry substrate to hydrolyse a 6.7% (w/v) suspension of steam-pretreated willow. As this pretreated substrate had a cellulose content of only 42%, the actual activity of the enzyme towards cellulose was about 27.1 FPU g^{-1}. Likewise, Dekker *et al.* (1987) used 20 FPU g^{-1} oven-dry substrate to hydrolyse steam-treated *E. regnans* at a 10% (w/v) substrate concentration. Hence, the actual enzyme loading used was of approximately 35.1 FPU g^{-1} cellulose. These enzyme loadings are several times higher than the levels used in this work for

complete saccharification of steam-treated eucalyptus.

Previously, several workers (Sternberg *et al.*, 1977; Duff *et al.*, 1987) had shown that higher levels of β-glucosidase were effective in reducing the inhibitory effects of cellobiose accumulation and consequently influenced the efficiency of the overall saccharification process. Holtzapple *et al.* (1990) showed that both exo- and endo-glucanases are very sensitive to increased concentrations of cellobiose, whereas β-glucosidases are inhibited by high concentrations of glucose. However, the drop of enzyme activity in the system due to glucose accumulation was negligible as compared to that of cellobiose. Breuil *et al.* (1992) also studied the influence of cellobiase activity on hydrolysis of both filter paper (filter paper assay) and steam-treated aspen. It was suggested that Celluclast should be supplemented with an excess of cellobiase activity to assure minimal accumulation of cellobiose during hydrolysis of pretreated substrates. As the filter paper activity was shown to depend on the amount of β-glucosidase activity present in the enzyme mixture, it was also recommended that the ratio of supplemental Novozym used for hydrolysis should always be kept constant.

In all the experiments described in this work, the amount of enzyme used for hydrolysis was determined as the total activity of the enzyme mixture (Celluclast and Novozym) in relation to the cellulose content of the substrate. The cellobiase activity was always maintained constant at 30 CBU g^{-1}.

At this level of supplementation, no cellobiose accumulation was observed during hydrolysis of pretreated substrates using fresh enzymes, even at high substrate concentrations. Therefore, the extent of enzymatic hydrolysis of the substrate was determined by measuring the amount of glucose released during hydrolysis (Irick *et al.*, 1988) and calculating it in relation to the theoretical amount of glucose originally present as cellulose in the pretreated substrate.

Dekker (1986) has shown that inhibitory compounds to some components of the cellulase system were released during hydrolysis of steam-treated *E. regnans*. Other authors have also suggested that steam-treated materials derived from both softwoods (Clark and Mackie, 1984; Breuil *et al.*, 1990) and hardwoods (Sinitsyn *et al.*, 1982; Dekker, 1988) may contain non-carbohydrate components which are inhibitory to cellulases. Although these unknowns may partly explain the low efficiency by which some lignocellulosic residues are hydrolysed, it is likely that other factors also play a major role in limiting the efficiency of hydrolysis, such as the end-product inhibition of cellulases and the increased recalcitrancy of cellulose during hydrolysis.

4.3. Enzyme Recycling and End-product Inhibition of Cellulases

As discussed previously, it seemed that the alkali-insoluble fraction of steam-treated substrates derived from eucalyptus chips did not interfere with the hydrolysis of the

cellulose component. However, we observed that, during enzyme recycling, the presence of small amounts of lignin led to a limited recovery of desorbed enzymes. Although some components of the cellulase system may associate with lignin (Sutcliffe and Saddler, 1986), it seemed more probable that residual amounts of cellulose still present within the unhydrolysed material caused the retention of some key components of the cellulase complex. The simultaneous recycling of both the desorbed enzymes and the residual substrate showed that the cellulases still retained most of their activity.

The present work also confirms the previous observations that end-product inhibition of cellulases has a major impact on the rate and completeness by which cellulose is hydrolysed. When a batch hydrolysis of the substrate was carried out in the presence of high glucose concentrations, the lower rates of hydrolysis were probably due to the inhibition of β-glucosidase activity. A substantial increase in the concentration of cellobiose was observed with time, probably leading to inhibition of many of the cellulase components. However, other authors have suggested that the accumulation of soluble sugars in the hydrolysate may also affect the adsorption profile of specific cellulase components onto the pretreated substrate (Tanaka *et al.*, 1986).

During the early stages of enzyme recycling, most of the original cellulase activity could be recovered after complete hydrolysis of the substrate. However, a gradual decrease in the

ability of the recycled enzyme to hydrolyse fresh substrate was observed at later stages of recycling. The total sugar content of the hydrolysates, measured by the phenol-sulphuric method, indicated that an increased concentration of water-soluble oligosaccharides was obtained after several stages of enzyme recycling. These results could be partly explained by an increased loss of the catalytic activity and/or cellulose binding capacity of one or more components of the cellulase system towards the final stages of recycling. Our observations also indicated that enzymes recovered from controls which had been incubated without substrate for four days gave a hydrolysis profile similar to the one obtained when a fresh enzyme preparation was used. However, a decrease of approximately 10% was observed in the rate of substrate hydrolysis using enzymes readsorbed after a longer incubation time of 15 days. This was probably due to thermal and/or mechanical inactivation of the enzymes (Mukataka *et al.*, 1983). A loss of 80% in the overall activity of Meicelase has been reported after incubation of the enzyme without substrate for 15 days at 45°C (Ishihara *et al.*, 1991).

Although the use of spent enzymes for further hydrolysis of a fresh batch of substrate has been previously demonstrated using steam-treated wood (Clesceri *et al.*, 1985), this preliminary work was carried out using exceedingly high enzyme loadings and the possible reuse of the enzymes for subsequent hydrolysis steps was not demonstrated. Our results showed that

recycling of cellulases proceeds better after substrate delignification. When the enzymes were recycled in the presence of lignin-containing residues derived from pretreated wood, a partial loss of both cellulase and β-glucosidase activities was observed, probably due to enzyme inactivation or nonspecific binding to lignin fragments. Therefore, it seems reasonable to assume that the optimal release of adsorbed enzymes depends on the lignin content of the substrate and on whether a complete hydrolysis of the cellulose component can be achieved (Clesceri *et al.*, 1985; Ooshima *et al.*, 1990).

The elucidation of the mode of action of adsorbed cellulases on the surface of the substrate is one of the fundamental steps towards gaining a better understanding of the mechanism by which cellulose is hydrolysed. Our results indicated that one "batch" of cellulase could be used multiple times and that, rather than waiting for complete hydrolysis of the substrate, the enzyme-containing residue could be recycled to fresh substrate where a similar hydrolysis profile could be obtained. It seemed that all the cellulase components required for the efficient hydrolysis of pretreated eucalyptus remained associated with the substrate and could not be removed by a thorough washing with hydrolysis buffer. As most of the endoglucanases and cellobiohydrolases which have been characterized to date have been shown to contain a cellulose binding domain (CBD) within their protein structure (Esterbauer *et al.*, 1991), it appears that, for at least the enzymes of the

Trichoderma Celluclast system, this ensures a close association of the main cellulase components with the substrate until the residual substrate is completely hydrolysed.

4.4. The Morphology and Fine Structure of the Substrate During Enzymatic Hydrolysis

The enzymatic hydrolysis of cellulose is a diverse process which involves a complex enzyme system acting on an insoluble substrate (Fan *et al.*, 1987). The initial fast rate by which cellulose is hydrolysed has been associated with the occurrence of more accessible regions at the surface of the substrate where hydrolysis is facilitated. At the fibre level, these more accessible regions are often associated with cracks and defects which are encountered along the fibre axis. At the molecular level, they are characterized by a larger pore volume and/or available surface area (Grethlein, 1985; Wong *et al.*, 1988a) and a lower crystallinity index (amorphous cellulose) (Bertran and Dale, 1985; Sinitsyn *et al.*, 1991). By studying the structural changes that occur during enzymatic hydrolysis of cellulose at both the fibre and molecular level, we hoped to obtain a better understanding of the process of cellulose hydrolysis.

Owing to their susceptibility to hydrolysis and low lignin content, the peroxide-treated fraction derived from steam-exploded eucalyptus and a sample of a fully bleached, eucalyptus kraft pulp were selected for this study. The latter sample had a higher average fibre length than the peroxide-treated fraction

as well as a higher cellulose DP. This was not surprising as it is highly desirable for a pulping process to maintain the structural integrity of cellulose. To ensure that both substrates were highly uniform with regard to their fibre length distribution, most of the fines and fibre bundles were eliminated by fibre screening.

The rapid fragmentation of fibers, which occurred regardless of the initial fiber length of the substrate, probably resulted from the random attack of enzymes at eroded walls or defects on the fiber. If the residual cellulose at the site where the fiber was eventually attacked was primarily amorphous, fragmentation would be the result of the localized action of endoglucanases on the structurally disordered areas of the fiber (Nieves *et al.*, 1991). This would gradually increase both the surface area of the substrate and the availability of chain ends for the action of exo-glucanases.

The microscopic examination of residues obtained after hydrolysis of the peroxide-treated fraction for 24 h indicated that short fibres (50 - 100 μm) were further fragmented into a large population of smaller particles which could not be detected using the laser scattering method (Kajaani fibre analyser). These smaller particles were narrow rod-like structures which were about 5 - 10 μm in length and probably corresponded to small bundles of cellulose microfibrils. Peters *et al.* (1991) also observed a rapid accumulation of small fragments during hydrolysis of microcrystalline cellulose by

bacterial cellulases. Although our results were consistent with this preliminary study, we measured fibre length distributions rather than particle counts. Nevertheless, it appeared that fragmentation was an essential step during the enzymatic hydrolysis of cellulose by both fungal (Oltus *et al.*, 1987) and bacterial (Peters *et al.*, 1991) cellulases.

To obtain a better knowledge of the mechanism of cellulose fragmentation, we investigated any probable changes in the molecular weight (MW) distribution of several cellulosic substrates during hydrolysis. The MW distribution of cellulose was determined by GPC analysis of cellulose tricarbanylates. Compared to viscosimetry, this method was considered more appropriate for characterizing the enzymatic breakdown of cellulose because MW distributions rather than MW averages could be obtained. Other advantages have been associated with the use of tricarbanylation instead of other chemical methods such as nitration and derivation using cupriethylene diamine. These include the stability of the tricarbanyl derivatives, less cellulose degradation and better reproducibility (Kossler *et al.*, 1981).

All of the screened fractions derived from peroxide-treated eucalyptus consisted of a narrow distribution of cellulose molecules with an average degree of polymerisation ($\overline{DP}_w$) of 361 ± 22 anhydroglucose units. The low $\overline{DP}_w$ of these fraction was readily attributed to the acidic nature of the pretreatment method used (SO_2-catalysed steam treatment). Other authors have

reported similar values of $\overline{DP}_w$ for the acid-depolymerised residues obtained from several cellulosic materials (Kirk *et al.*, 1991; Miller *et al.*, 1991). Acid depolymerisation generally resulted in a rapid decline in the DP of cellulose from a couple of thousands to a minimum value of about 200 anhydroglucose units (limit DP). Some authors have suggested that the micelles formed by the association of these short cellooligomers are probably the elemental structural constituents of the cellulose crystallites (Chang *et al.*, 1981; Kirk *et al.*, 1991).

The gradual depolymerisation of cellulose, which was observed during hydrolysis of the SEE-H_2O_2 fraction, can be partially attributed to exoglucanase (CBH) catalysis. These enzymes would gradually depolymerise the substrate by removing cellobiose units from the non-reducing end of the cellulose molecules. Although this hypothesis should still recognize the importance of endoglucanase (EG) action, particularly in the early stages of hydrolysis, it appears that a much more drastic shift in cellulose DP would be expected if EG activity predominated. As the population of cellooligomers present in the SEE-H_2O_2 - F-150 fraction averaged a cellulose DP of only 380, this hypothesis agrees with the general concept that exoglucanases have a higher specificity for substrates which are characterized by having a higher availability of chain ends.

It seemed probable that, during hydrolysis of the SEE-H_2O_2 fraction, the gradual decrease in cellulose DP could be accompanied by a structural reorganization of the substrate.

This indicated a trend where, regardless of the method used to determine crystallinity, the CrI of the F-150 fraction decreased during hydrolysis. These observations suggested that the *Trichoderma* Celluclast preparation was capable of simultaneously hydrolysing both the amorphous and crystalline regions of the substrate, thus resembling the model proposed by Kleman-Leyer *et al.* (1992) for the enzymatic breakdown of cotton cellulose by white rot fungi. An alternative explanation is that there is a relative increase in the amorphous character of the substrate during the early stages of hydrolysis, a process previously referred to as amorphogenesis (Coughlan, 1985). The gradual increase in the amount of enzymes adsorbed onto the residual substrate during the latter stages of hydrolysis could also result in a relative increase in the area of the diffractogram associated with the amorphous character. However, this should not be very significant as the total amount of enzyme (Celluclast plus Novozym) used to hydrolyse 2 g of the substrate did not correspond to more than 4 mg of protein (data not shown).

By contrast, the screened fractions derived from the bleached kraft pulp ($\overline{DP}_w$ = 1074 ± 54) showed no significant changes in the distribution of cellulose DP during hydrolysis. This suggested that those cellulose molecules located at the surface of the substrate, once exposed to the concerted action of endo- and exoglucanases, were rapidly and completely hydrolyzed to soluble sugars. This would explain why

cellooligomers with an intermediate DP were not observed as part of the DP distribution obtained from each of the partially hydrolysed residues.

Based on the observation that hydrolysis did not result in any significant change in the DP distribution of the FBEP-48 fraction, it was probable that hydrolysis would also result in minimal changes in the fine structure of the substrate. In fact, there was very little change in the degree of crystallinity of the substrate during the first 8 h of hydrolysis. These observations suggested that the synergistic action of endo- and exoglucanases effected the removal of the outer layers of cellulose crystallite in order to gain access to the inner layers, characterizing a "peeling-off" type of mechanism.

There was no clear indication that hydrolysis of both the F-150 and FBEP-48 fractions had triggered any lattice transformation of the substrates (*i.e.*, transformation of one cellulose polymorph to another). The X-ray diffractograms of the partially hydrolysed residues showed the same strong equatorial reflections at the 101, $10\bar{1}$ and 002 diffraction planes, which are characteristic of the cellulose I polymorph. There was little change in the resolution between the 101 and $10\bar{1}$ reflections during hydrolysis. However, hydrolysis resulted in a progressive broadening of the 002 reflection of the SEE-H_2O_2 - F-150 fraction. This may have resulted from either a gradual disordering in the fine structure of the substrate or a preferential attack of the enzymes at the 002 plane of the

crystallites.

A previous study has indicated that the enzymatic hydrolysis of bleached fractions derived from bagasse, wheat straw and hardwood sawdust results in a considerable increase in the CrI of the substrates (Betrabet and Paralikar, 1977, 1978). This reduction in CrI was accompanied by a gradual decrease in the average thickness of the cellulose crystallites at both the 002 and 101 diffraction planes. Although these results differ from some of our observations, this might indicate differences in the mode of action of cellulases obtained from *Penicillium pinophillum*, as compared to *T. reesei* cellulases.

After the FBEP-48 fraction had been hydrolysed for 24 h, a substantial increase in polydispersity was observed with the distribution inclining towards shorter cellooligomers. This could be attributed to the gradual appearance of a population of cellooligomers with a $\overline{DP_w}$ of 33. It is possible that these oligosaccharides may have existed throughout hydrolysis as a small part of the distribution and that it became visible when very little substrate remained in the reaction mixture. As more than 90% of the substrate had been hydrolysed within 24 h, it seems that the enzyme components which were responsible for the saccharification of these oligomers were strongly inhibited, possibly by end-product inhibition. Recently, Gilkes *et al.* (1992) suggested that the binding of *Cellulomonas fimi* cellulases onto microcrystalline cellulose involves the interaction of a cellulose binding domain (CBD) with

approximately 40 cellobiose units at the surface of the substrate. It seemed that the cellooligosaccharide that we have detected in our studies corresponded, at the same order of magnitude, to the shortest cellulose molecule to which cellulases can adsorb. Furthermore, it is probable that the degradation of cellooligomers with a DP_w of 33 or less may have been mediated by enzyme components which were in solution rather than adsorbed to the substrate. However, any conclusion regarding the binding kinetics of *Trichoderma* cellulases is still very speculative because the adsorption characteristics of purified components of bacterial cellulases (Gilkes *et al.*, 1992) may not applicable to the adsorption kinetics of complete enzyme systems such as the *Trichoderma* Celluclast preparation.

Both F-150 and FBEP-48 fractions appeared to consist of a cellulosic residue with similar crystallinity index (CrI). However, during sample preparation, freeze-drying may have caused a partial recrystallization of low molecular weight oligomers, causing an overestimation of the CrI of the F-150 fraction (Atalla *et al.*, 1984). Furthermore, the absolute degree of crystallinity of a cellulosic residue depends on the determination of the ratio between the crystalline and amorphous regions of the substrate (variable "k" in Equation 6). As this ratio has not been determined for neither of the substrates used in this study, all of the crystallinity measurements obtained for both F-150 and FBEP-48 fractions are only relative values and cannot be compared directly.

Our study has suggested that the effective hydrolysis of lignocellulosic residues is highly dependent on factors such as lignin removal and increased surface area of the substrate. It also appeared that substrate accessibility to enzymes cannot be easily predicted by the degree of polymerisation and/or crystallinity index of the substrate. However, the determination of cellulose DP and crystallinity are helpful in determining the mode of action of the enzymes on pretreated substrates. This was not only apparent from the consistency observed in the molecular weight distributions and in the diffraction patterns of several partially hydrolysed residues, but also from the calculations of DP, CrI and crystallite dimensions. Further characterization of hydrolysis process would probably require the utilization of pure components of the cellulase system, rather than crude extracts. This would aid in the identification of the actual role of each component during hydrolysis of pretreated cellulose, as well as helping define the synergistic action of the enzyme components on changing the structure of the substrate.

5. CONCLUSION

Eucalyptus viminalis chips derived from young stems were shown to be easily pretreated and fractionated and readily hydrolysed by commercial *Trichoderma* cellulases. Pretreatment parameters such as steam temperature, residence time in the steam gun and moisture content of the chips were all shown to influence the effectiveness of pretreatment (Ramos *et al.*, 1992a). SO_2 catalysis reduced both the temperature and residence time required for optimum pretreatment and was most effective when green chips were used. Compared to aspen and spruce, the acid-catalysed steam explosion of *E. viminalis* chips produced the best substrate for hydrolysis using milder pretreatment conditions (Ramos *et al.*, 1992b). This was more apparent when hydrolysis was carried out at high substrate concentrations.

Removal of lignin after pretreatment by alkali washing was shown to have a minor effect on hydrolysis of the substrate (Ramos *et al.*, 1992b). However, alkali extraction was shown to be highly desirable for both the reduction of the substrate bulk volume and the effective recycling of desorbed enzymes present in the hydrolysate (Ramos *et al.*, 1992c). Peroxide treatment of the alkali-washed residue reduced the lignin content of the substrate to very low levels and consequently increased its susceptibility to enzymatic hydrolysis (Ramos *et al.*, 1992b,c). One batch of cellulases containing 10 FPU g^{-1} cellulose could be effectively recycled for at least five consecutive times,

provided that β-glucosidase activity was added to the system at each recycle step (Ramos *et al.*, 1992c). It was also apparent that most of the enzymes remained in close association with the residue throughout hydrolysis and that they could also be recycled in their adsorbed state.

The mode of action of *Trichoderma* cellulases was also investigated using screened fractions derived from both the peroxide-treated fraction (F-150) and a fully bleached, eucalyptus kraft pulp (FBEP-48) (Ramos *et al.*, 1922d). At the fibre level, the process of enzymatic hydrolysis of both substrates seemed to follow the same pattern and the production of reducing sugar during hydrolysis appeared to follow the same kinetics. Both substrates resulted in the release of an equivalent amount of glucose after hydrolysis and the profile of mono- and oligosaccharides was almost identical, owing to the high levels of β-glucosidase activity in the mixtures. However, at the molecular level, it was apparent that the mechanism of hydrolysis of cellulose differed between the two cellulosic substrates used and was dependent on the fine structure of the substrate. As both the peroxide-treated fraction and the fully bleached kraft pulp were obtained from eucalyptus chips using different pretreatment methodologies, variations in the mode of action of the enzymes could be partially explained by probable differences in the structure of the substrates.

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