STUDIES ON CELLULASES AND HEMICELLULASES

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PART I

INTRODUCTION

SECTION 1

GLUCOSE/FUEL FROM CELLULOSE

Cellulose is most abundant organic material which can be used as a source of food, fuel and chemicals. On a worldwide basis, it has been estimated that $132 \pm 74 \times 10^9$ metric tons ($146 \pm 87 \times 10^9$ tonnes) of carbon are fixed annually (1). Assuming lignocellulosic material to be 50% carbon and to have a heat of combustion of 19,800 kJ/kg (8,500 Btu/lb) on an ash-free, oven-dried basis, $5.2 \pm 32 \times 10^9$ kJ ($5 \pm 3 \times 10^{18}$ Btu) are stored annually by photosynthesis. The amount of carbon fixed per year on a worldwide basis is of the order of 30 times greater than the total world energy production of 15.2×10^{10} kJ (14.4×10^{10} Btu) or 7×10^9 metric tons of coal equivalent in 1970. The utilization of this annually replenishable resource is greatly simplified if it is first hydrolyzed into glucose. From glucose, various sources of food consumable by man and animal, can be derived. In addition, it can be used as a feed-stock to make solvents, plastics, and other chemicals now made from petroleum; it can be converted microbially into single cell protein, or it can be fermented to a clean-burning fuel such as ethanol. It is estimated that from one tonne of waste paper, one can produce 1/2 tonne of glucose which can be fermented to produce 68 gallons of ethanol (2).

Conversion of cellulose to glucose can be achieved by hydrolysis. At present, there are two hydrolysis processes available: acid and enzyme hydrolysis. The first commercial application of acid hydrolysis of cellulose was made in 1913

at Georgetown, USA, where a plant was built to hydrolyze Southern Pine mill waste by 2% H_2SO_4 at 115°C in rotary steam-heated digesters. The dilute sugar solutions produced (25% yield) were fermented to ethyl alcohol. This plant and a second one at Fullerton La, were closed for economic reasons because of the rising price of lumber and low price of black strap molasses at the end of World War I (3). The acid hydrolysis method was commercialized by Scholler in Germany in 1926, but was replaced in 1935 by the "Schube" process. During the II World War, in Germany, Scholler process was in use to get 40 - 50% sugar yield after the digestion of pretreated (1% HCl) wood with 0.05% H_2SO_4 at 130 - 190°C for 18 - 24 h in stationary digesters. The Bergius process, also used in Germany, employed concentrated HCl in special acid-resistant equipment but was expensive. The sugars from these processes were used to produce alcohol and to grow Candida and Oidium yeasts for human food (4). Dilute sulfuric acid hydrolysis of wood was reexamined by the Forest Product Laboratory, U.S. Department of Agriculture at the request of the War Production Board in 1943. A full scale wood hydrolysis plant was subsequently designed and built at Springfield, Oregon, but it did not commence operation until after the end of World War II (5,6,7). The process is basically a semi-continuous process in which the hydrolyzate percolates through the 'chip' bed, continuously removing the sugars as they formed. This avoids prolonged retention of the sugars under acid conditions at high temperatures (150 - 185°C) and so reduces degradation (8).

The acid hydrolysis method has several disadvantages, which have been summarized by Ghose (9). These are:
(i) Expensive corrosion-proof equipment is needed for the acid treatment resulting in a high capital cost, (ii) The crystalline structure of cellulose makes it resistant so that high temperature and high acid concentration are needed for hydrolysis. Consequently, the resulting sugar is partially decomposed, (iii) Waste cellulose usually contains impurities that will also react with acid, producing undesirable by products and conversion compounds. None of the above processes has succeeded as a commercial operation since World War II (11). The selling price of alcohol by the acid hydrolysis process derived from the Madison process was estimated in the range of \$ 1.50 - 1.80/gal based on a production level of 25 x 10^6 gal/year. The corresponding estimated capital investment was about \$ 90 x 10^6 (10).

Grethlein (12) has reported the process economics of making glucose from cellulose using acid hydrolysis. With an input capacity of 885 tonnes/day newsprint the cost of making glucose is reported to be in the range of 1.75 - 2.45 cents/lb, depending on the slurry concentration fed to the reactor for the acid hydrolysis. The cost range is less than the published estimate of 5.2 cents/lb for enzymatic hydrolysis (13). Grethlein (12) considers that the acid hydrolysis of cellulose is a viable and potentially economic process. For the conversion of newspaper and wood pulp to glucose, a twin screw extruder device at high temperature acid hydrolysis process was used by Rogers (14). According to the author, this

technology may improve production economics of ethanol from wood, pulp and paper, agricultural and municipal wastes. Reaction time with 0.5% H_2SO_4 at 450°F was only 20 sec.

There are various advantages in the use of enzymes to hydrolyze cellulose instead of acid. The enzymes are specific for cellulose and do not react with impurities that may be present in the waste. Moreover reactions take place under moderate conditions of temperature and pressure and the glucose yields of 111% of the weight of cellulose used have been reported (15). Known cellulases split only beta-1,4-glucosidic linkages (16), whereas hydrochloric acid attacks $1 \longrightarrow 2$, $1 \longrightarrow 3$, $1 \longrightarrow 4$ and $1 \longrightarrow 6$ linkages. At the molecular level, approximately 10^8 HCl molecules are required to do the work of a single enzyme molecule (mol. wt. 63,000)(17).

So far enzymatic hydrolysis of cellulose has been carried out on a small pilot plant scale. A pilot plant for the saccharification of 1,000 lb cellulose per month for the production of glucose/ethanol using *Trichoderma reesei* has been operated in U.S.A. (18). Gulf oil is operating a 50 tonne per day facility prior to building a large scale plant (19). The key to the Gulf process is reported to be the fact that (i) the cellulosic material being used is the waste material and requires no pretreatment and (ii) simultaneous conversion of cellulose to glucose to ethanol by combined cellulase/yeast system (20,21). Work has also been carried out at the Natick Development Centre (NDC) and at California University (Berkeley) to design and evaluate a process for the enzymatic hydrolysis of newsprints (22-26). The economic analysis of this process

indicated that a major part of the overall process cost is the production of enzymes, and substrate pretreatment. Ball milling has been found to be the most effective physical method of pretreatment of making the maximum amount of cellulose in the substrate available for enzymatic hydrolysis, but it is expensive. Andren and Nystrom (27) reported that wet chemical pulps are by far the most susceptible substrates for enzymatic hydrolysis.

Most lignocellulosic materials contain three components: cellulose, hemicellulose and lignin in ratios of roughly 4:3:3 (28-30). For rapid enzymatic degradation of cellulose it has been found necessary to pretreat the cellulosics either mechanically and/or physically/chemically to make it more susceptible. The need for pretreatment increases with the increase in lignin content. Lignin is highly resistant to biological degradation, because of its unusual structure. In lignin the monomers, p-hydroxy cinnamyl alcohol, are joined in such a way as to provide a complex three dimensional structure which is interconnected with the cellulose and hemicellulose components. Thus, the difficulty in obtaining fast and complete hydrolysis of cellulose is not due to the primary linkages of cellulose polymeric chain but rather than the secondary and tertiary structures of cellulose and the linkages and cross linkages with other materials. Many chemical, physical and mechanical pretreatments have been developed (31).

Economic considerations are central to the economic exploitation of the process. Straw, bran, groundnut shells, bagasse, wood and other cellulosic materials are the main

sources of cellulose which are available in abundance. Most of these are collected during the course of agricultural and other operations at central sites. In spite of the abundance of cellulose, it is very difficult to find suitable cellulosic material that could be collected from a limited area and would be cheap enough, taking into account the cost of collection, transport, and handling. Sugarcane bagasse, cellulosic materials collected at the sewage disposal, paper mill wastes, and fast growing trees could be ideal cellulose sources.

The minimum cost of glucose produced by enzymatic hydrolysis of waste cellulose is about $ 0.5/kg (32). The two other major components of cellulosic materials, lignin and hemicellulose, have not been included in the total cost analysis (33). Seeley (34) has calculated that the estimated cost of glucose from starch would be 11 cents/lb and that from cellulose 9 - 15 cents/lb, but the concentration of glucose in the glucose syrup from starch is approximately 30% and that from cellulose only 5 - 10%. The Soviets are emphasizing the conversion of cellulose to sugars for SCP production, while U.S. Department of Energy is placing emphasis on conversion to alcohol fuels (35). Each gallon of fuel alcohol produced from this renewable resource reduces gasoline, diesel or gas use by about one gallon (36).

The process for producing ethanol from plant biomass by enzymatic saccharification of cellulose to glucose and subsequent fermentation of glucose to ethanol by yeast has been intensively studied. From one tonne of cellulosic material, 0.5 tonne of glucose (assuming 50% cellulose) can be obtained, which, in turn, can produce 79 gal ethanol (13.8 lb of glucose

to make 1 gal ethanol). Wilke et al.(37) have made an economic assessment for an integrated processing scheme whereby a cellulose waste (newsprint) is first converted to sugars by enzymatic hydrolysis, and then to ethanol by yeast fermentation. Recent conversion cost estimates, based on Wilke's process for producing ethanol from newsprint (38) and wheat straw (39) suggested that the economics of these processes are still not promising. As shown in Table 1, the cost of ethanol from plant biomass is high. But by-products generated at various points in each stage of the hydrolysis-fermentation process constitute a critical element within overall ethanol production economics. These credits will reduce net processing costs for ethanol, the only uncertainty pertains to the magnitude of the potential credits (40).

Table 2, indicates the range of by-products that can be attained from wood and grain feed stocks. Although relevant data are not available, by-products obtained from potatoes, straw and processed municipal waste would probably tend to be more similar to grain by-product than to wood by-product. The cost of ethanol produced by fermentation as reported by various workers (37,42-45) differs widely. This is probably because of the use of different feed stocks as well as different processes. In fact, as noted by Quittenton (46), there is currently a fierce argument in the United States about the cost of ethanol produced from grain by fermentation, which is $ 0.33/l ($ 1.25/gal as of January 1979). It is projected that by 1985 the cost of producing ethanol will be about $ 0.42/l ($ 1.60/gal) as compared to the predicted cost of gasoline

TABLE 1: COST OF LIQUID FUEL FROM BIOMASS AND COAL (41)

Raw material	Product	Capital investment $/annual MMBtu	Product cost $/MMBtu
Newsprint	Ethanol	45.4	20.4
Wheat straw	Ethanol	58.8	37.0
Sugarcane	Ethanol	22.6	14.4
Corn	Ethanol	20.6	14.4
Coal	Synthetic crude	6.3 to 9.0	2.5 to 4.0

TABLE 2: BY-PRODUCT SOURCE (40)

Feed stock	Lignin	Hemi-cellulose	Hydrolysis vapors		Spent grain	Spent yeast	Distillation	
			Methanol	Furfural			Fuel oils	Bottoms
Wood	Fuel chemicals	Fuel Fodder chemicals (xylitol, furfural)	Fuel chemicals	Fuel chemicals	-	Fuel Fodder	Fuel	Fertilizer
Grain	-	-	-	-	Fodder	Fuel Fodder	Fuel	Fertilizer

production at $ 0.89/l ($ 2.25/gal). Based on energy content of ethanol, the ethanol conversion cost and the capital investment for an ethanol plant are higher than that for synthetic crude from the coal liquefaction process (41). The higher cost of ethanol from corn and sugarcane juice, relative to synthetic crude, is attributable primarily to the cost of the raw material, which represents about two third of the production cost. The use of low-cost carbohydrate such as agricultural wastes should, in principle, substantially lower the cost. Other economic factors in the alcohol industry are the cost of steam for distillation and the large vessel size to ferment low sugar containing solutions at slow rate (47) as well as pretreatment of the cellulosic material before saccharification. The distribution of ethanol cost from cellulose fibre is indicated in Table 3 (41). The alcohol cost can be minimised by careful control of oxygen tension and use of optimum feed sugar concentrations in the alcohol fermentation. Mandels (48) considers that, for the process to be economically viable one must be able to produce glucose for 15 cents per kg in crude 10% syrup. Because of the high cost of ball milling which does not appear practical for large scale processing, Wilke and Yang (49) studied the possibility of using less stringently milled substrate. With T. reesei culture broth and with the modified process, revised preliminary cost analysis indicated that it may be possible to produce glucose at 4.2 cents/lb, with an approximately capital investment of $ 19 x 10^6. The break-up cost estimates of the major items for producing glucose from cellulose is given in Table 4 (50). Both the cost of the

TABLE 3: DISTRIBUTION OF ETHANOL COST FROM CELLULOSE FIBRE (41)

(Excluding raw material cost)

Substrate	Pretreatment %	Saccharification %	Ethanol fermentation %
Newsprint	6.8	64.4	26.8
Wheat straw	12	78	20

TABLE 4: MAJOR ESTIMATED ITEMS OF COST FOR GLUCOSE PRODUCTION (50)

Price Rs./100 kg glucose

Raw material	Enzyme	Pretreatment	Enzyme hydrolysis and filtration
5 - 10	60 - 70	20 - 60	40 - 60

enzyme (and enzyme make up) and the pretreatment cost constitute a major portion of the total cost. About 60% of the total manufacturing cost of production of glucose from cellulose has been attributed to enzyme production when the enzyme yields are of the order of 3 - 4 FPA IU/ml (51). A recent economic analysis of the Natick process for production of ethanol from urban wastes, excluding substrate costs and credits, attributes 50% of the cost of sugar or 40% of the cost of the ethanol to enzyme production (52). Thus, any improvement in the enzyme yield and reduction in pretreatment cost will improve the economic outlook and are the keys to the success of the process. It is sometimes suggested that cellulase should be immobilized. This would not be advantageous since formation of an enzyme substrate complex could be difficult when both components are large insoluble entities. Furthermore, the synergism between fixed components might be hindered (53). It may be possible to obtain regulatory mutants which are resistant to catabolite (glucose or cellobiose) repression, or by genetic manipulation to obtain microorganisms with multiple copies of cellulase structural genes (54). Increase in gene copies by genetic manipulation which gives highly elevated yields of enzymes are known (55-58).

It may also be possible to obtain microorganisms which can break up or loosen lignin-cellulose complex or which can utilize lignin. Enzymes involved in lignocellulosics degradation have been investigated and a new enzyme, cellobiose: quinone oxido-reductase, of importance in both cellulose and lignin degradation has been reported (59). This enzyme from *Sporotrichum pulverulentum* was found to catalyze an oxidation-

reduction reaction in which the conversion of cellobiose to cellobionic acid, probably through cellobiono-delta-lactone, was coupled to the reduction of a quinone to the corresponding phenol. Cellulase-less mutants have also been obtained which degrade both kraft lignin and wood lignin (60), thus, "deschielding" cellulose. These mutants may have vast potential. The study on mixed enzymes is of particular interest (9). If any particular component is in short supply in a cellulase system from one source, it can be fortified with the same component from another source, richer in that component. As an example *T. reesei* enzyme is deficient in xylanase and can be fortified with *Aspergillus wentii* cellulase which is comparatively rich in this component (60).

The overall economic possibility is also dependent on efficient use of all main components of lignocellulosics viz., cellulose, hemicellulose and lignin. Tsao (63) has recommended obtaining these three major components in different streams and their separate utilization. They have reported that the developments in the use of the solvent extraction coupled with enzyme/acid hydrolysis and the utilization of hemicellulose and lignin could yield fermentable sugars at less than 9 cents/kg (63,64).

It is believed that isomerized glucose-fructose syrups would replace invert cane sugar in the world food market. The estimated glucose-fructose syrup production cost is 16 - 18 cents/lb and the selling price 25 - 30 cents/lb (10). Based on the product value, calculations by Humphrey suggest that glucose from hydrolyzed cellulose for isomerized glucose-

fructose syrup may present a viable alternative, and one could afford to use wood cellulose pulp or cotton. Since the product value is 30 cents/lb, and assuming only 50% of product price allowed for substrate, the maximum affordable price would be 10 - 15 cents/lb as compared to 2 - 3 cents/lb for alcohol.

SECTION 2

CELLULOSE, HEMICELLULOSE, AND LIGNIN: COMPOSITION AND STRUCTURE

Cell walls of land plants contain large amounts of cellulose microfibrils embeded in a continuous phase of lignin, pectin and hemicelluloses. Cellulose, constituting about 65% of perennial woods and about 50% of annual plant tissue, is found mainly in the primary and first layers of the secondary wall,. although it is present throughout the various layers of the wall. Hemicelluloses are present in all layers of the cell walls but are concentrated in the primary and secondary cell walls mixed with lignin and cellulose. They are also present in the middle lamella in association with lignin.

Physical properties of the plant cell wall depend on the interactions between these three main components. These components are found at early stage of cell differentiation: at this period also quantitative differences occur since the components are not formed at equal rates.

CELLULOSE

Cellulose exists in various states of purity in plant cell walls, ranging 20% in some grasses to over 90% in cotton fibre (69). The seed hair of cotton is the most pure form of cellulose available in nature and contains about 6% by weight of non-cellulosic polysaccharides, proteins and mineral elements.

Cellulose fibres are of similar structure irrespective of their function in plant. Similar cellulose molecular structure is present in wood fibre and cotton seed hair. The main difference between various types of fibres from different

sources arise from the differences in the nature of other substances present, their location in the fibre and from the different dimensions of the fibres. Though cellulose is a homopolymer of anhydroglucose units, linked together through beta-1,4-linkages, differences in the physical relationships between the chains and their degrees of interaction makes it a multiple substrate. In cotton as well as wood fibres a thin primary wall surrounded by a relatively thick secondary wall is present. In both wood and cotton the secondary wall usually consists of three layers, designated S_1, S_2 and S_3, and forms during the growth and maturation of the cell. The outermost layer of cotton is called the cuticle. The equivalent layer in the multicellular structure of wood is the middle lamella (Fig. 1). The middle lamella is amorphous, generally porous and about 1 to 2 μ thick. The primary wall is of 300 Å thickness while secondary wall (S_1, S_2 and S_3), which thickens during growth, is of variable thickness ranging from 1 to 10 μ (63,66). The cellulose molecules in the S_1 and S_3 layers are deposited in a flat helix with respect to the fibre axis whereas those in the S_2 layer are deposited nearly parallel to the fibre axis. In cotton, the S_2 layer is deposited in a series of concentric zones, the number of which has been correlated with the days of growth of the fibre. The secondary wall of cotton fibres consists almost entirely of highly crystalline cellulose. Almost all the extraneous materials are present on the surface of the primary wall. In wood, on the other hand, the non-cellulosic materials are deposited in all regions of the cell walls from lumen through the

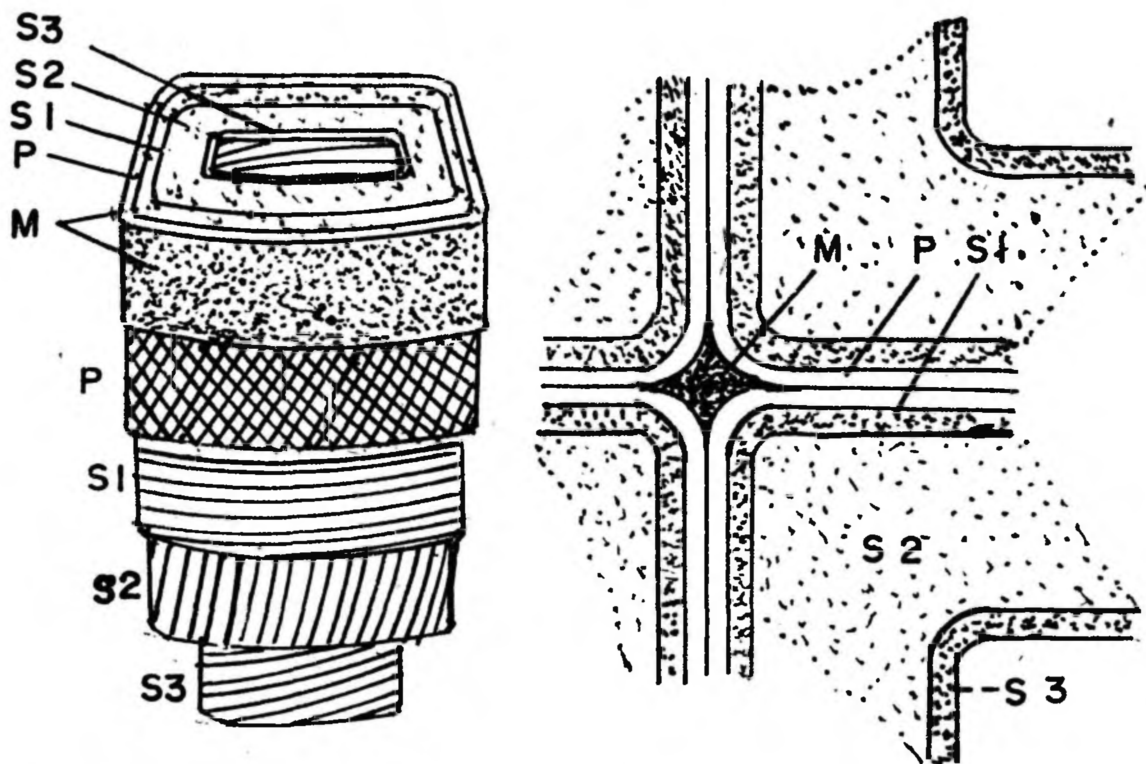
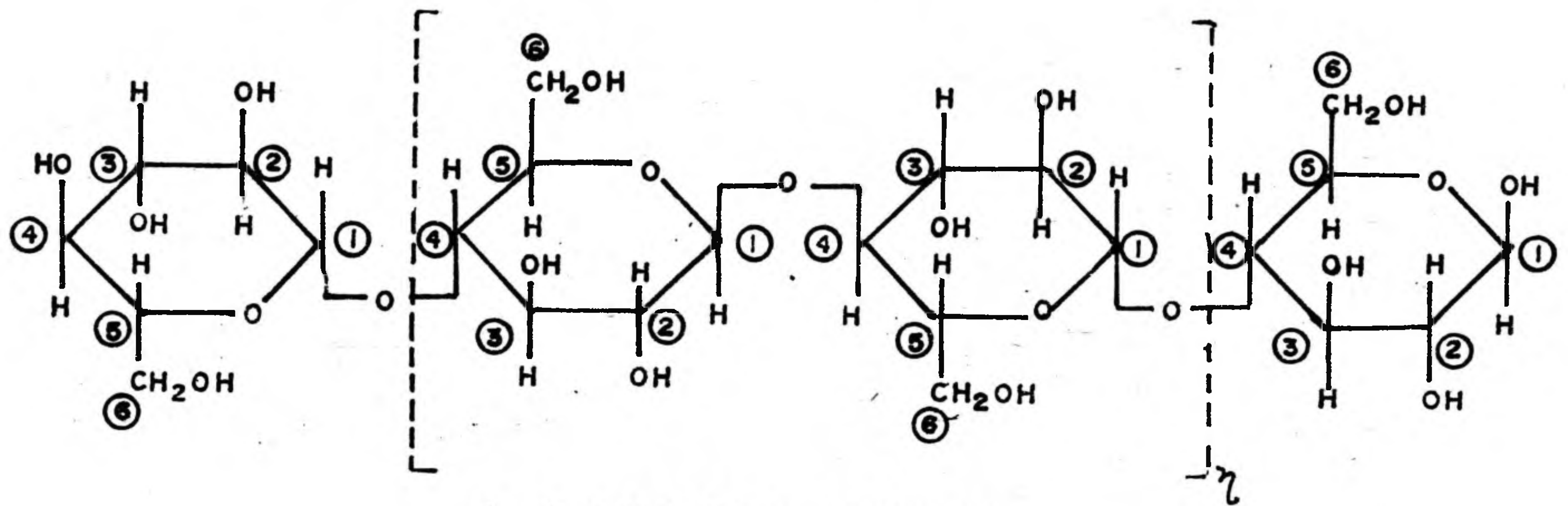


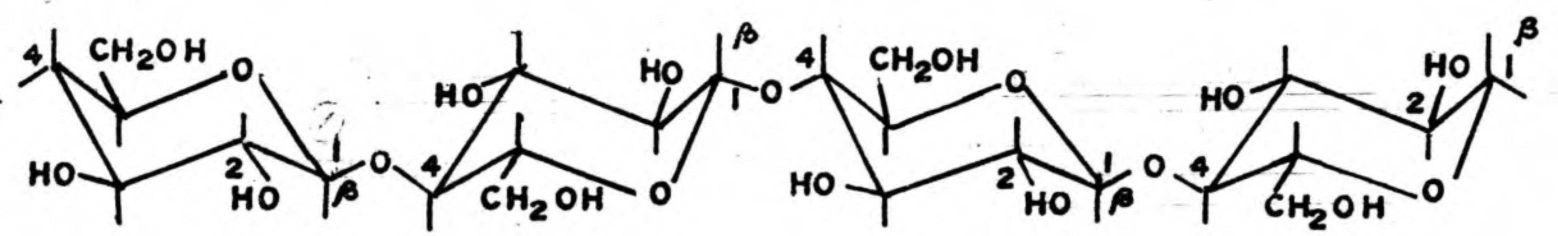
FIG. 1. Diagramatic sketch showing the various layers of wood cell walls (68). The true intercellular substance or middle lamella (M) and adjacent primary walls (P) comprise the compound middle lamella. The secondary walls are composed of outer S_1, middle S_2, and inner S_3 layers.

compound middle lamella. Cellulose is in the highest concentration in the secondary wall and diminishes towards the middle lamella (62). The minimum concentration of hemicelluloses and maximum of cellulose occurs in the S_3 layer of an angiosperm and in the S_2 layer of a gymnosperm (67). The S_3 layer of gymnosperm contains appreciable lignin and hemicelluloses.

Cellulose is insoluble and structurally complex substrate. Most of the structural studies of cellulose have been made on materials derived from cotton. The chemical structure of cellulose chains was established by Haworth and Hibbert more than 60 years ago (69). The structural formula is shown in Fig. 2. Cellulose is a high molecular weight linear polymer composed of D-glucose residues building blocks, joined by beta-1,4-glucosidic bonds. In a cellulose chain molecule, the anhydroglucose units adopt the chair configuration with the hydroxyl groups in the equatorial and the hydrogen atoms in the axial positions (Fig. 2). Every other chain unit is rotated 180° around the main axis which results in a strain-free linear configuration. The glycosidic linkage acts as a functional group, and this, along with the hydroxyl groups, mainly determines the chemical properties of cellulose. All significant chemical reactions occur at these locations. It has been reported that the hydroxyl group in the 3-position is bound by an intramolecular hydrogen bond to the ring oxygen atom of the next chain unit (70).



A · STRUCTURAL FORMULA



B · CONFORMATIONAL FORMULA

FIG. 2. Structure of cellulose (70).

Cellulose molecules form a fibril, a thread-like long bundle of molecules, which is stabilized laterally by hydrogen bonding between hydroxyl groups of adjacent molecules. All the hydroxyl groups are apparently hydrogen bonded in different but rather specific ways. The elementary fibrils are the smallest structural units of microfibrils and fibres. The number of chains of cellulose in a microfibril may be few hundred. There are differences of opinion in regard to the number of cellulose chains in elementary fibrils and microfibrils and the specific dimensions of the cross section of these structures. The microfibril according to Preston and Cronshaw (71) is about 50 x 100 Å in cross section. The elementary fibril is about 35 Å wide (72-74) and the microfibril 8 - 20 nm wide (70). Manley (75) has reported that microfibrils have an average width of about 35 Å (30 - 40 Å).

The microfibril ultrastructure is still a subject of considerable controversy, and the question whether the cellulose chain is uniformly comprised or not has been debated for 50 to 60 years. The chain length distribution curves of partially degraded samples of cellulose and cellulose derivatives do not assume the shape of a normal Gaussian curve, but exhibit separate distinct maxima within the degree of polymerization (DP) range of 400 - 500 (70). Two different mechanisms have been put forward. It was considered by one group that in cellulose molecules not all linkages are equally resistant to hydrolysis, and that at fairly regular intervals,'weak spots' exist where hydrolysis takes place far more rapidly than in the case of the usual glycosidic links in the chain. According to other

school of scientists, the 'anomal' plymolecularity of degraded samples would mainly be attributable to the alternation of crystalline and amorphous sequences in the elementary fibrils, giving rise on hydrolysis to separate superimposed distribution curves (70). Any irregularity in the substrate may terminate the action of enzyme, depending on the specificity of the enzyme.

Models of the various molecular arrangements of cellulose have been summarized by Chang (76). For many years the accepted picture of crystallinity in cellulose was represented by the fringed micellar model. This model showed that individual molecules run straight through a crystalline area, then into an amorphous region and later into another crystallite, and so on through several of these processes (70,77). These crystallites were considered to be more or less rectangular in shape with the molecules running parallel to their long axis. The crystallites have been shown to be several hundred angstroms in length and scores of angstroms in width and thickness. According to Preston and Cronshaw (71) the microfibril consists of a rectangular crystalline core, about 50 x 100 Å in cross section surrounded by a paracrystalline sheath. The solid lines represent the planes of glucose residues (Fig. 3a). Hess et al. (78) suggested that the microfibril contains several elementary fibrils which contain 15 to 40 cellulose molecules and are segmented into crystalline and paracrystalline regions (Fig. 3b). Manley (75) reported that the individual filaments are 35 Å in diameter and appear to have a periodic variation in structure along their length. It was suggested from X-ray diffraction studies that the cellulose molecules exist in a

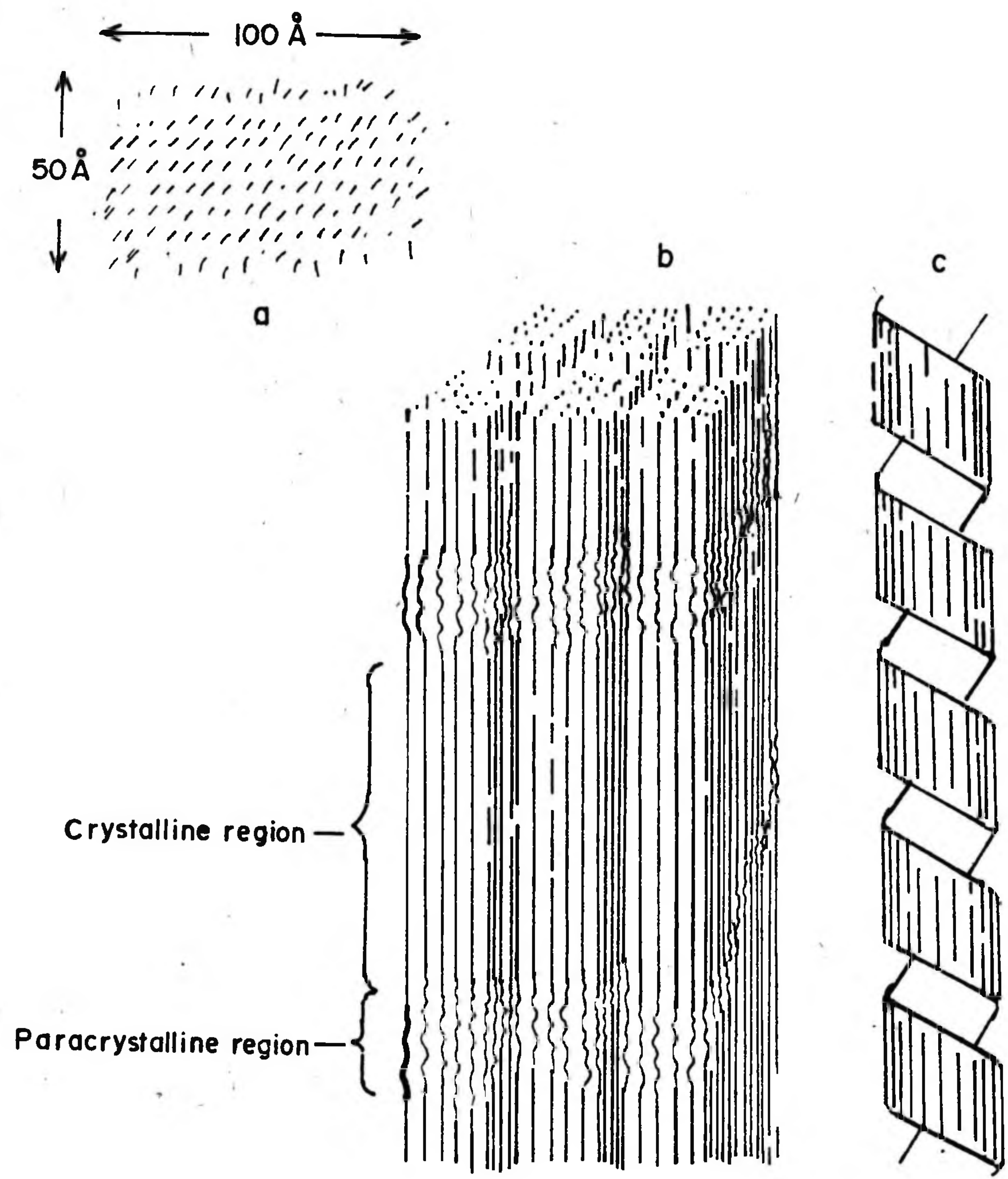


FIG. 3. Concepts of the structure of microfibrils:
(a) Preston and Cronshaw (71)
(b) Hess *et al.* (78)
(c) Manley (75)-

folded chain lattice formed as a ribbon which in turn is wound as a tight helix (Fig. 3c)(79). Meyer, Mark and Misch in 1930 proposed a schematic model of the native cellulose lattice. In this model unit cell dimensions are: a = 8.35 Å; b = 10.3 Å; and c = 7.9 Å; beta = 84° (80). Each unit cell contains four glucose residues. The length of the unit cell equals that of the repeating anhydrocellobiose units and is 1.03 nm. The shortest distance between atoms of neighbouring chains of native cellulose is no more than 0.25 nm in the direction of the a - axis which makes possible the formation of hydrogen bonds between the adjacent chains. In the direction of c - axis, the distance is much greater, and molecular chains are attached to each other by van der Waal's forces only (70). Since the discovery of chain folding in linear synthetic polymers in the late fifties, the question has repeatedly arisen whether cellulose chains could do the same. A great number of folding possibilities have been proposed in the past few years. Ellefsen's model (81), constructed on a purely deductive basis, i.e. without any experimental support, was among the first. This model was based on a microfibril conception and therefore has an exceptionally large cross sectional area perpendicular to the b - axis (Fig. 4). Chang (76) developed a folded chain model of cellulose together with a new hypothesis on cellulose weak links (Eig. 4). Essentially, single cellulose molecules are folded back and forth in the plane to form "platellites" which are the smallest physical and structural unit of crystalline cellulose (101). The average fold length in the platellites corresponds to the levelling off degree of

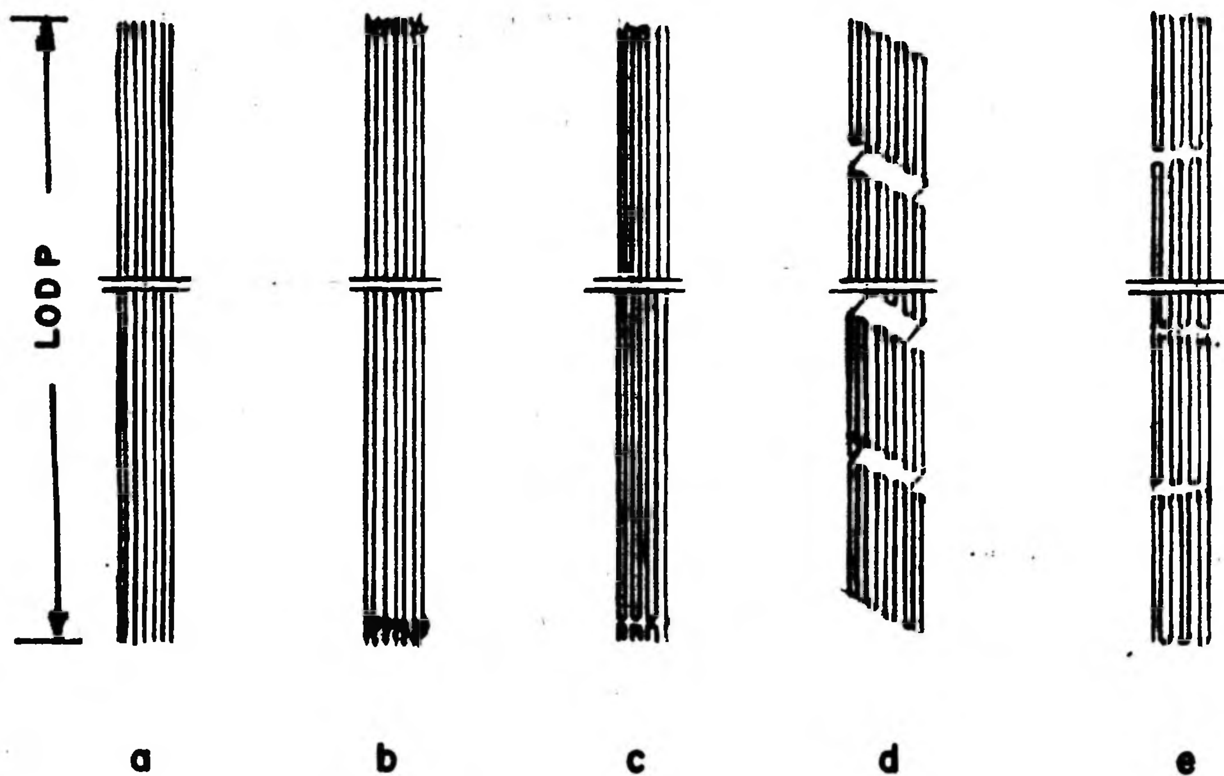


FIG. 4: Models of molecular arrangements in the protofibril (79)
(a) Full extension and complete crystalline model
(b) Fibrillized fringe micellar model
(c) Fibrillized models of Ellefsen
(d) Manley's planar zig-zag model
(e) Chain folding with fold length much smaller than LODP

polymerization (LODP). The glucosidic bonds at the folds are weak links due to a loop conformation of the chain. The integrity of the platellites is due primarily to chain folding. Hydrogen bonding and van der Waal's forces exist within and between all structural units. The glucosidic linkages at the folds (B_L) are the major type of weak links, and are mainly responsible for chemically induced chain scission in cellulose. The various modifications of cellulose have the same basic platellite structure, differentiated mainly by the values of molecular spacing (delta) and fold length.

The number of glucose units in a molecule gives the degree of polymerization. The number of glucose residues ranges from 500 - 2000 in wood pulps to 10,000 in cotton. The degree of polymerization of typical materials is given in Table 5 (82). Evidence obtained by different means indicates that native cellulose is composed of more than 10,000 beta-anhydroglucose residues (these may be as many as 15,000 residues) linked to form a linear chain molecule giving a molecular weight of 1.5 million. The total length of cellulose would be about 5 microns as the length of an anhydroglucose unit is 5.15 Å, while the alpha-cellulose (insoluble in 17.5% NaOH) contains as many as 10 to 15 x 10^3 glucose units per molecule; gamma-cellulose contains $<$ 15 units of glucose. Plant and wood celluloses generally contain beta-cellulose as well a material soluble in 17.5% NaOH.

TABLE 5: CELLULOSE CHARACTERISTICS (82)

Source	Molecular weight	Degree of polymerization
Native cellulose	6,00,000 - 15,00,000	3,500 - 10,000
Chemical cottons	80,000 - 5,00,000	500 - 3,000
Wood pulps	80,000 - 3,40,000	500 - 2,100

HEMICELLULOSE

The name hemicellulose was introduced by Schulze in 1891 to designate these polysaccharides extractable from plants by dilute alkaline solutions (82a). Most workers classify hemicelluloses according to the type of sugar present. Thus, xylan is a polymer of xylose units, mannan of mannose and galactan of galactose units. Most hemicelluloses, however, are not homoglycans but are heteroglycans containing 2 to 4 and rarely 5 or 6 different types of sugar units. Commonly occurring heteroglycans are L-arabino-D-xylans, L-arabino-D-glucurono-D-xylans, 4-O-methyl-D-glucurono-D-xylans, D-gluco-D-mannans, D-galacto-D-gluco-D-mannans, and L-arabino-D-galactans.

Xylans

Xylan, a polysaccharide occurs practically in all land plants and is said to be present in some marine algae (83). In both, wide botanical distribution and abundance in nature, it closely follows cellulose and starch. It is abundant in annual crops, particularly in agricultural residues such as corn cobs, corn stalks, grain hulls and stems, Hard woods contain 20 to 25% xylan while soft woods contain 7 to 12% (84). In angiosperms (hard woods) the dominating hemicellulose is an O-acetyl(4-O-methyl glucurono) xylan, while the major soft wood (gymnosperms) hemicelluloses are an O-acetylgalactogluco-mannan and arabino-(4-O-methyl-glucurono) xylan. Low strength vegetable fibres of commerce such as jute, sisal, manilahemp and coir may contain 5 to 20% xylan. High strength fibres such as ramie, flax, and cotton are devoid or almost devoid of xylan. Xylan is principally found as a plant cell wall constituent. Xylan

has been isolated from non-endospermic and endospermic tissues of many economically important grasses. Xylan has the general properties of insolubility in water, solubility in alkaline solutions, ease of acid hydrolysis, high negative optical rotation, and non-reducing action towards Fehlings' solution. It can also be placed in two more polysaccharide classes other than hemicellulose, namely pentosan and glycan. It is classed as pentosan because it is principally a polymer of pentose, and as a glucan because it is largely, if not entirely, a polymer of an unmodified sugar or sugars. In 1889, Wheeler and Tollens (84a) demonstrated that the sugar D-xylose, which is a monomer of xylan, has the empirical composition $C_5H_{10}O_5$, while the parent xylan has the expected composition $(C_5H_8O_4)_n$. True D-xylans, composed of only D-xylose, are not normally found and is possible that such types occur more frequently in sea weeds than in land plants. The native xylan molecule may contain in addition to D-xylose (85 - 93%), a small amount of L-arabinose and in some instances, a small amount of glucuronic acid, possibly D-glucuronic acid, e.g. corn stalk xylan contains D-glucuronic acid, L-arabinose, and D-xylose in the approximate ratio of 2:7:19 (85), while xylan of wheat straw and alfalfa hay contains these monosaccharides in the ratio of 1:0.9:23 (86). Variations were reported in the proportions of L-arabinofuranosyl, D-glucopyranosyluronic acid and 4-O-methyl-D-glucopyranosyluronic acid groups in xylans isolated from non-endospermic and endospermic tissues of many economically important grasses (87). Non-endospermic xylans e.g. from corn cobs, fibres, and hulls display apparently

unusual structural features and are more complex than those normally isolated from wheat and other grasses (88-90). Some of the structural features account for galactose units in the parent hemicellulose (91)

Xylans of the endosperm are present in the thin cell walls of the endosperm, which appear similar to primary walls (92). The procedure of air-drying grasses may lead to changes in the hemicellulose conformation, denaturation, and adsorptive or other associations between cell wall components, including the hemicelluloses. It is more difficult to dissolve a hemicellulose from an air-dried than from a fresh grass, and xylans can be irreversibly adsorbed on to cellulose (93,94). Hemicelluloses that were previously soluble in water will not necessarily dissolve in it after being dried, and so there may be a difference between the solubility and their dissolvability. Hemicelluloses, like other natural polymers, may be denatured, by drying, and by other physical treatments. Renaturation may not take place readily, or completely. Lignin impedes dissolution of hemicellulose in alkali. It is usual to dissolve lignin from grasses after it has been oxidized by a solution of aqueous acetic acid and sodium chlorite (95-97). There was formerly much uncertainty as to whether lignin and hemicellulose are chemically bonded, or whether the lignin mechanically entraps hemicellulose molecules, thereby making them less accessible to dissolution (98-100). Normally, non-endospermic hemicelluloses are extracted by treatment of holocelluloses with aqueous alkali, which may saponify any hemicellulosic ester linkages either between polysaccharides or between hemicelluloses and non-carbohydrate components (87, 101-104).

It has been known for nearly 50 years that xylan from esparto grass is composed of chains of $(1 \rightarrow 4)$-linked beta-D-xylopyranose residues, which is known as homoxylan. During the last 20 years, many xylans from land plants have been examined and all have been shown to contain the same basal structure but differ in the structural arrangement of other sugar residues, especially L-arabinose, D-glucuronic acid, and its 4-methyl ether, which are attached as side chains. The xylans from esperto grass isolated are heteroglycans, and most commonly have single L-arabinofuranosyl groups attached to a few O - 3 atoms of main chain D-xylosyl residues, and D-glucopyranosyluronic acid (or its 4-methyl ether, or both) groups attached to a few O - 2 atoms of other main chain D-xylosyl residues. The partial acid hydrolysis of corn cob xylan gave polymer-homologous series, xylobiose, xylotriose, upto xyloheptaose containing $(1 \rightarrow 4)$ linked beta-D-xylopyranose units (87, 105).

L-arabinose has long been known as a component sugar of hemicelluloses, but not until 1951 was it first conclusively shown by Perlin (106, 107) to be a constituant of a polysaccharide based on D-xylose residues. He showed that all the L-arabinose units in a wheat flour polysaccharide are present as non-reducing end groups in the furanose form and must be attached to D-xylose units as shown in Fig. 5.

Some xylans also contain non-terminal L-arabinofuranose residues, e.g. the hemicelluloses from corn cobs (108). Since oligosaccharides having reducing L-arabinose residues are released under mild conditions of hydrolysis, it is probable

that these oligosaccharides result from the cleavage of non-terminal furanosyl linkages. This is clearly so with corn cob hemicellulose, where 2-O-beta-D-xylopyranosyl-L-arabinose has been isolated after mild acid hydrolysis, since 3,5-(and not 3,4-)di-O-methyl-L-arabinose is found on hydrolysis of the methylated polysaccharides. There is as yet not sufficient evidence to show whether these non-terminal L-arabinofuranose residues are linked directly to the xylan backbone or whether they terminate on the longer side-chains in which one or more D-xylose residues are interposed (109).

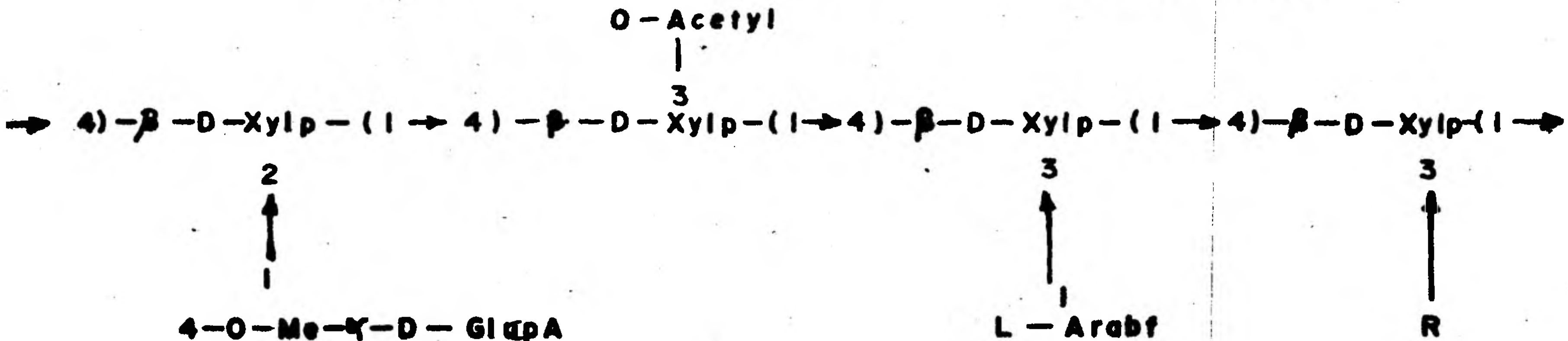
The xylans containing residues of D-glucuronic acid or 4-O-methyl-D-glucuronic acid show the linkage at position 2 of xylose (Fig. 5). Although the xylans from the land plants show the chains of (1 $\longrightarrow$ 4) linked beta-D-xylopyranose residues as a main structural feature, the xylan from the red alga Rhodymenia palmata (111) contains (1 $\longrightarrow$ 4) and (1 $\longrightarrow$ 3) -linked D-xylopyranose residues.

Xylan from larch wood (Larix decidua)

Larches are the only common deciduous conifers. They differ chemically from other coniferous woods in containing a much higher proportion of galactose-containing polysaccharides. Larch saw dust, after extraction with hot water, 1%, 4% and 10% NaOH, gives 4 major fractions of hemicelluloses (112). Fractions I and II after extraction with 1% and 4% NaOH are rich with xylan, while fractions III and IV are rich with glucomannan after extraction with 10% NaOH.

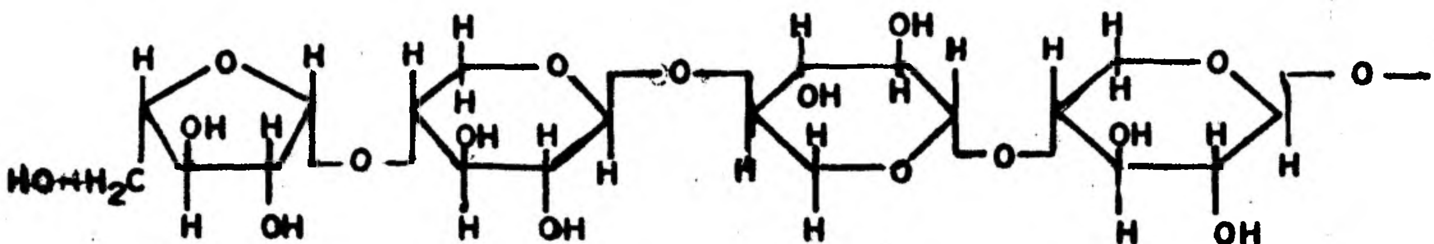
The general structure of D-xylan from larch wood is as shown in Fig. 5. But it is not possible on the present

FIG.5a) GENERAL STRUCTURE FOR D-XYLANS (110)



R IS β-D-Xylp-(1→2)-L-Arabf-(1→, α-D-Xylp-(1→3)-L-Arabf-(1→, or D-Galp-(1→4)-D-Xylp-(1→2)-L-Arabf-(1→ .

b) SECTION OF XYLAN CHAIN WITH NON-REDUCING END OF L-ARABINOFURANOSIDE (109).



evidence to indicate whether the L-arabinofuranose residues are attached directly to the backbone of xylose residues as shown or whether 1:4 linked D-xylose residues are interposed with the arabinose residues terminating a longer side-chain. The former alternative is more probable as other xylans (from e.g., wheat straw and barley husks) (113,114), are known to contain L-arabinofuranose residues directly linked to the backbone of xylose residues.

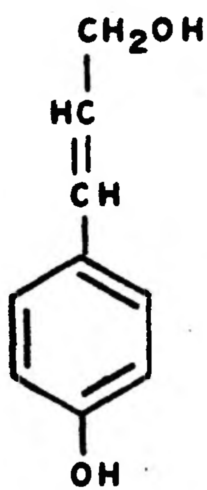
A molecular weight determination by the isothermal distillation method gave a value of 18,000 ± 500 (degree of polymerization, 107 ± 3) for the methylated xylan (methyl ether)(112). This value, taken together with the value of one non-reducing xylose end group per approximately 120 sugar residues, suggests that the backbone of xylose residues is unbranched. It can be concluded, therefore, that the xylan fraction may contain 1:4-linked beta-D-xylopyranose residues with, on the average, every 5th or 6th residue carrying a terminal 4-O-methyl-D-glucuronic acid residue linked through C_2. In addition, a small proportion (~ 4%) of L-arabinofuranose residues are probably attached to the backbone through C_3 of xylose residues.

Several wood xylans are thus characterized by the presence of 4-O-methyl-D-glucuronic acid residues attached as side-chains to D-xylose by 1:2 linkages. The proportions of uronic acid groups are in general somewhat higher in the xylans from soft woods (15 - 20%) than in those from hard woods (8 - 15%)(112). Some of these xylans (soft wood) also contain a small proportion of L-arabinofuranose residues.

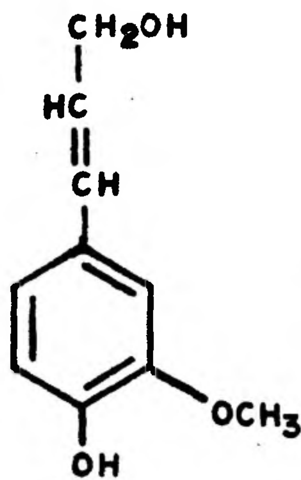
LIGNIN

Lignin is distributed widely throughout the plant kingdom, except lichens and mosses (115). Lignification is not confined to the cell walls of wood and annual plants but takes in fruit, stones, bark, root, bast, pith and cork cells. Plant lignins can be divided into three broad classes which are commonly called: soft wood (gymonsperm), hardwood (dicotyledonous angiosperm), and grass or annual plant (monocotyledonous angiosperm) lignins. Next to cellulose, lignin is probably the most common organic compound cycled on earth. The amount of lignin in different soft wood and hard wood species lies in the range of 18 - 33% (66,116).

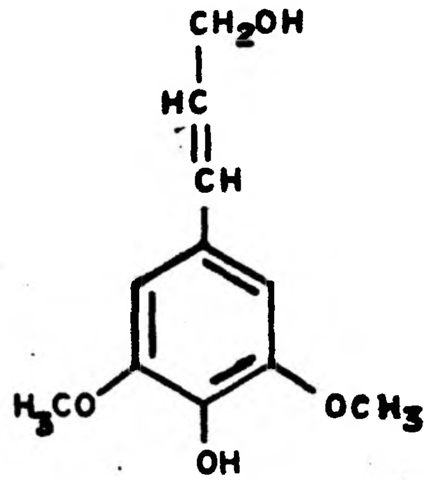
Lignin is a phenylpropanoid structural polymer of vascular plants which gives the plants rigidity and binds plant cells together. Lignin also decreases water permeation across cell walls of xylem tissue and protects plant tissues from invasion by pathogenic microorganisms. According to the definition of Sarkanen and Ludwig (117), lignin is a natural polymeric product arising from an enzyme-initiated dehydrogenative polymerization of three primary precursors: trans-p-coumaryl alcohol, trans-coniferyl alcohol and trans-sinapyl alcohol (Fig. 6). A typical soft wood lignin contains approximately 80% coniferyl alcohol, 14% p-coumaryl alcohol and 6% sinapyl alcohol (118). Hardwood lignin, on the other hand, contains similar amounts of coniferyl and sinapyl alcohol and a minor amount of p-coumaryl alcohol. In bamboo and grass lignins the amount of p-coumaryl alcohol is higher than in soft wood and hardwood lignins (119).



(I)



(II)



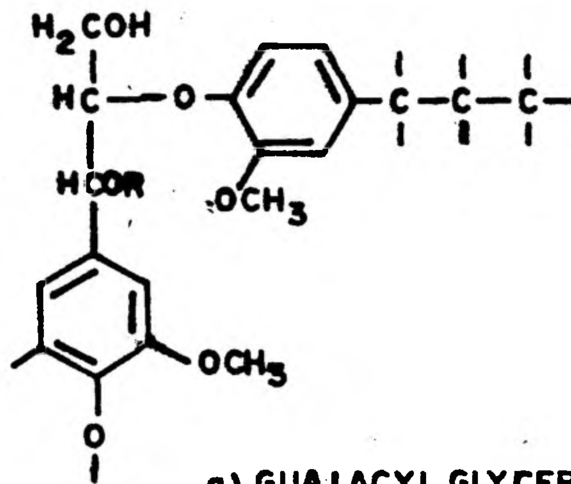
(III)

I) p - COUMARYL ALCOHOL ; II) CONIFERYL ALCOHOL ; III) SINAPYL ALCOHOL.

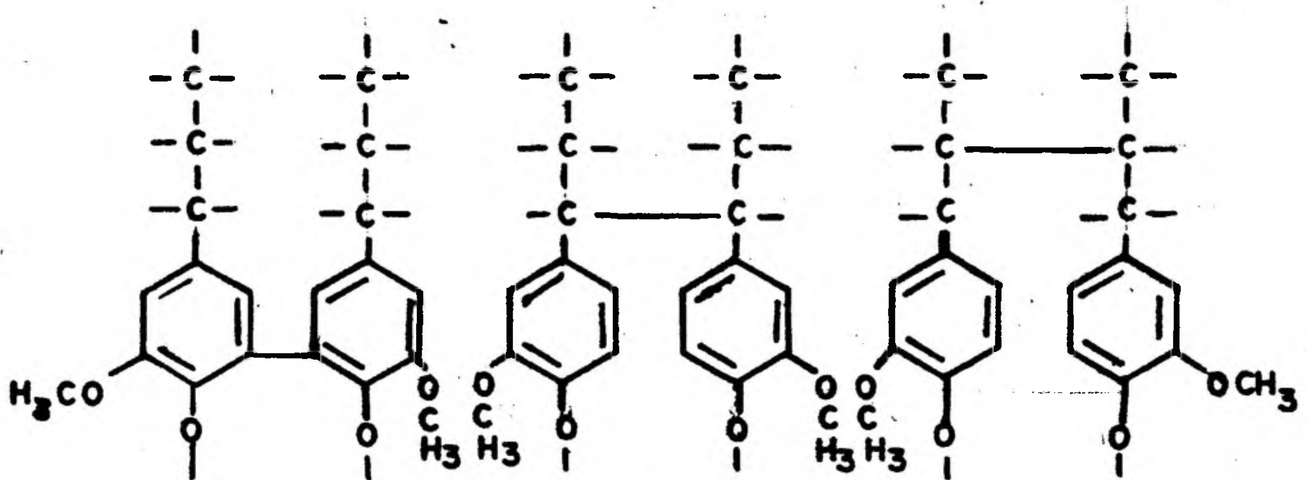
FIG. 6 PRIMARY PRECURSORS OF LIGNIN (118)

According to Higuchi (120) the cinnamyl alcohols (I, II,III) (Fig. 6) can be dehydrogenated by phenol oxidases to give free radicals which spontaneously polymerize. In this way, the highly branched lignin polymer can be formed with the phenylpropane units covalently bonded to each other in a number of different ways: by either ether linkages or by carbon-to-carbon linkages (121). The carbon-to-carbon linkages are particularly resistant towards chemical attack. About 30% of the phenylpropane units contain a free phenolic hydroxyl group while the remaining 70% should therefore be connected by phenol ether linkages (121). In general, it may be assumed that the oxygen in the 4 position of one of the phenyl rings is combined with the carbon atom of the side chain of another phenylpropane unit, thus forming an ether linkage. Many such phenol ether linkages form guaiacyl glycerol-beta-aryl ether structures (Fig. 7a). Carbon-to-carbon linkages may be formed when two benzene rings are combined through their 5,5'- positions (Fig. 7b). Such linkages may also be formed between the side chains, thus giving an alpha-alpha-linkage (Fig. 7c) or a beta-beta-bond (Fig. 7d). Furthermore,, two phenylpropane radicals can combine in such a way that, for example, an ether and a carbon-to-carbon bond are formed, thus giving a benzofuran structure (Fig. 7e), or two phenylpropane radicals can combine with the formation of a beta-beta-carbon bond and two ether bonds between the gamma-alpha- and the alpha-gamma-carbon atom, thus giving a pinoresinol structure (Fig. 7f).

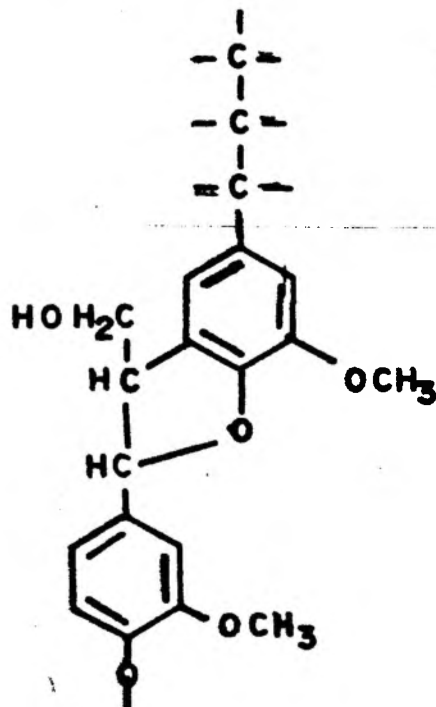
FIG 7 VARIOUS LINKAGES IN LIGNIN STRUCTURE



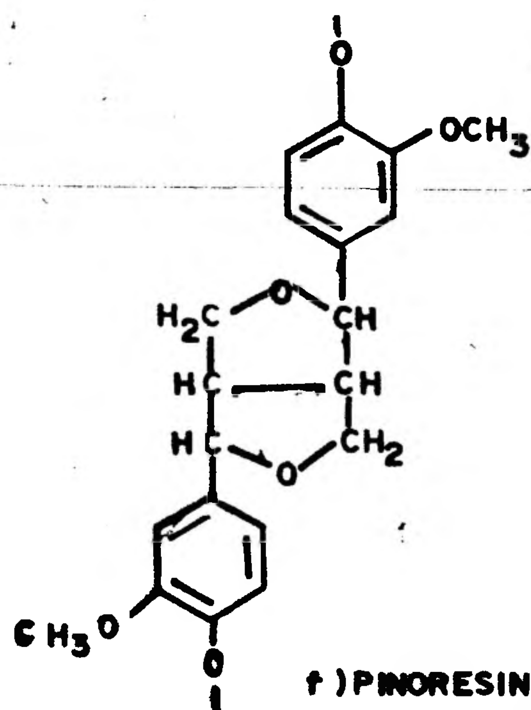
a) GUAIACYL GLYCEROLARYL ETHER



b) 5–5'BONDING c) α–α'BONDING d) β–β'BONDING



BENZOFURAN STRUCTURE



f) PINORESINOL STRUCTURE

Schematic formulae for spruce lignin have been presented by Freudenberg (122), Harkin (123), and Adler (124); and for beech lignin by Nimz (125). According to Kirk (119), there are three major intermonomer linkages in lignin: (a) The arylglycerol-beta-aryl ether (guaiacyl glycerolaryl ether) (Fig. 7a) type involves about 40% of the phenyl propane units of spruce lignin and about 60% of these units in birch lignin, (b) The phenylcoumaran structure involves about 20% of the phenylpropane units in spruce lignin and about 10% of those in birch lignin, (c) The biphenyl structures may involve 25% of the phenylpropane units of spruce lignin and about 10% of those in birch lignin.

SECTION 3

PRETREATMENT OF CELLULOSIC MATERIALS FOR ENZYMATIC HYDROLYSIS

The two major deterrents to the effective utilization of lignocellulosic residues for chemical, enzymatic or microbiological conversion processes are lignin and cellulose crystallinity (126). Lignin restricts enzymatic and microbiological access to the cellulose. Crystallinity restricts the rate of all three modes of attack on the cellulose. Other probable factors which increase the resistance of cellulose in its native form to enzymatic degradation include (a) moisture content of the fibre, (b) size and diffusion characteristics of reagent molecules involved in the hydrolysis process, (c) its unit cell dimensions, (d) the conformation and steric rigidity of the anhydroglucose units, and (e) degree of polymerization of the cellulose (127). Over the years, various methods of pretreatment of cellulose have been proposed which make the links in the cellulose molecule more accessible to attack by the enzymes. The pretreatment methods can be divided into two broad catagories: (a) chemical pretreatments, and (b) physical pretreatments. The increased rate and extent of hydrolysis on chemical pretreatment is chiefly due to significant changes in the crystalline structure of cellulose, while physical pretreatment removes the lignin seal and also results in alteration in crystalline structure.

Physical pretreatments

(a) Particle size reduction

The milling of cellulosic materials to very small particle

size enhances their susceptibility to hydrolytic, enzymatic, and microbiological attack. The work of Dehority and Johnson (128) and Stranks (129) demonstrated that the wet ball milling of cellulose, forages, and certain woods for about 72 h substantially improved their utilization by rumen bacteria under *in vivo* conditions. However, Ghose and Kostick (130) and Mandels *et al*. (131) observed that hammer milling gave good size reduction but without significant gain in susceptibility to enzymes.

Vibratory ball milling is more efficient in reducing the size of cellulosic substrates and an alteration in the crystalline structure (132). The crystallinity in cellulose is essentially eliminated with 30 min milling period under proper conditions (133). Using this technique Pew and Weyne (134) obtained essentially complete digestion by cellulase of the carbohydrates of milled spruce and aspen compared to less than 10% digestion for the initial 60 mesh saw dust. It is reported that milling at elevated temperature (approximately 220°C) increases the rate of enzymatic conversion of cellulose to glucose over that at room temperature (135). The saccharification of cellulosic substrates has been reported three times better in combined ball milling and enzymatic hydrolysis than the enzymatic hydrolysis of pre ball-milled cellulosic substrate (136). Wilke and Mitra(13) showed that cellulosic material becomes readily hydrolyzable by cellulase enzymes if it is ball milled to sizes of 300 mesh or less (135). Tassinari and Macy (127) found differential speed two roll milling as an effective pretreatment for increasing the susceptibility

to enzymatic hydrolysis. Using mills with three, six and ten inches in diameter rolls and processing times of 10 min or less resulted in the percent increases in susceptibility over untreated controls as follows: cotton, 1100; maple chips, 1600; white pine chips, 600; newspaper, 125. In comparison, ball milling of newspaper for 24 h gives only a 62% increase. Another advantage of the roll mill is the increased wet density of the product permitting higher slurry concentrations during hydrolysis.

(b) Temperature

The simple heating of a cellulose at 200°C in kerosene or in dry air has been reported to provide a greatly enhanced rate of hydrolysis (126,137,138). In this report, a fibre fraction from cattle manure was treated in an autoclave for 5 - 30 min at temperature ranging from 130 - 200°C. The reactivity of the cellulose towards cellulase enzymes was increased by a factor of 10 - 12 compared to the untreated fibres. The increased reaction rate is possibly due to increased accessibility of the cellulose because of the removal of the structural hemicellulose during the process of pretreatment. However, high temperature pretreatment is likely to be too expensive a process for pretreatment on a commercial scale. At the other end of the temperature scale, the freezing of cellulosic materials in water suspension at -75°C is reported to enhance chemical reactivity as measured by dye adsorption (139). The effect was augmented by repeated freezing and thawing cycles. Again the energy demands would appear highly excessive. Sasaki et al. (140) reported that cryo-milled cotton

cellulose powder (250 mesh) by Lynrex Mill O-type (Hammer mill) in liquid nitrogen showed 36% more saccharification than the untreated cotton as it still had crystalline regions.

(c) Irradiation

High-energy electron irradiation provides an effective means for enhancing the dilute acid saccharification of both cellulose and lignocellulose (141). Rates of hydrolysis of the resistant celluloses increase markedly at dosages above 10^7 Rads (equivalent roentgens) along with increases in the amount of disordered celluloses. In spite of its ability to enhance cellulose saccharification, electron irradiation holds little appeal as a commercial pretreatment because of its prohibitive cost. Han *et al.* (142) have reported treatment of lignocellulosics with gamma-radiation emitted from Cobalt 60 or Caesium 137. However, this pretreatment is evidently too expensive for industrial application.

(d) Pressure

Compression of a cotton hydrocellulose for 30 min at 8000 kg/cm^2 approximately doubled the quantity of material dissolved during ethanolysis, while repeated compression of spruce sulfite pulp sheets between calender rolls gave a four-fold increase in solubility (143). Substantial improvemen in saccharification by pressure milling with low ratios of concentrated H_2SO_4 or gaseous HCl has also been reported (126). This appraach has received considerable attention by the Russian scientists during the past 25 years but so far as is known, has not gone beyond the pilot plant stage. It has been known that pressurizing wood with steam and then suddenly

releasing to atmospheric pressure would shatter the structure in a popcorn-like effect. Wood chips are treated with saturated steam at 240 - 300°C and 500 - 1000 psi, in a vessel called a gun reactor. Cooking time can vary between 5 sec and 5 min. At some point the reaction is frozen at the peak of sugar production, and the wood is 'exploded' into a fine powder at atmospheric pressure. Enzymatic hydrolysis of this exploded biomass has given over 80% of the theoretical amount of glucose, but lignin remained unaffected (144).

Chemical pretreatments

(a) Use of swelling agents

As originally defined by Katz (1933) (144a) and later amplified by Howsman and Sisson (1954) (144b), there are two basic modes of swelling for cellulose, 'intercrystalline' and 'intracrystalline' (157. An example of the former is the swelling of cellulose in water, this involves the entry of water between crystalline units with a volume change approximately equivalent to the volume of water adsorbed and a maximum adsorption of about 30%. However, the adsorption of water causes only slight changes in the X-ray diffraction pattern of the cellulose. Removal of water leads to regaining of the original network dimensions and structure.

In intracrystalline swelling, the solvent enters into the crystallites of cellulose. This could result in transformations in the crystallite structure of cellulose. In the extreme, it could cause unlimited swelling and the resultant complete solution of the cellulose in the swelling agent. Solutions of sodium hydroxide, anhydrous ammonia and certain amines cause limited swelling; but bring about significant changes in the

structure of the native cellulose. While high concentrations of sulfuric and hydrochloric acids, cellulcse solvents such as cupram, cuen, cadoxen N-methyl-morpholine N-oxide provide examples of unlimited swelling agents with more or less complete molecular dispersion. Irrespective of the degree of swelling the intracrystalline swelling agents for cellulose bring about changes in the crystalline structure of cellulose which in turn lead to enhanced hydrolysis.

(b) Sodium hydroxide and hydrochloric acid

Hydrolysis data in constant boiling hydrochloric acid has been reported by Millett *et al*.(126) for various native, mercerized and regenerated celluloses (Table 6). Hydrolysis rates were expressed in terms of one half of the resistant portion of the starting cellulose when hydrolyzed with constant boiling hydrochloric acid. The resistant fraction was defined as the zero-time intercept of the linear semilogarithmic plot of residual cellulose versus time of hydrolysis. Mercerization was carried out with 30% sodium hydroxide at 30°C for 5 min. It is evident from Table 6 that mericerization increased the rate of hydrolysis of ramie and cotton by 40% - 50%, but the hydrolysis of hemlock pulps was unaffected. The two viscose rayons, which are examples of regenerated cellulose, had hydrolysis rates that were three to six times those of the native celluloses and from two to four times the rates of mercerized celluloses.

A large number of studies have been performed on alkaline pretreatment as a means towards enhancing the enzymatic and microbiological conversions of cellulosic materials. Pew (135)

TABLE 6: RESPONSE OF VARIOUS CELLULOSES TO HYDROLYSIS WITH CONSTANT BOILING HCl (126)

Cellulose	Hydrolysis data		Viscosity data	
	Resistant fraction %	Half life of resistant fraction min	Initial DP	DP at half life
Native				
Ramie	89	193	-	184
Cotton	89	188	1680	175
Wood pulps				
Hemlock sulfite	90	88	1170	125
Sweetgum	88	84	-	123
Birch sulfite	93	63	725	134
Mercerized				
Ramie	73	114	-	88
Cotton	72	100	1900	80
Hemlock sulfite	76	91	1240	66
Regenerated				
Tire cord rayon	66	30	425	31
Fibre G-rayon	78	59	500	37

found digestibility of hardwoods by cellulases improved markedly by pretreatment with aqueous NaOH, the effect was considerably more pronounced with hardwoods than with softwoods. Tarkow and Feist (145) reported that the digestibility of hardwood was doubled by treatment with 1% NaOH. Han and Callihan (146) showed that a 15 min digestion of rice straw or sugar cane bagasse with 4% NaOH at 100°C increased carbo-hydrate utilization by *Cellulomonas* sp. from a value of 29% to about 73%.* Moo-Young *et al.* (147) treated saw dust of mixed hardwoods with 1% NaOH at 121°C for 30 min and then inoculated the neutralized pretreated substrate with *Chaetomium cellulolyticum*. Substantial increase in the initial growth rate of the fungus on alkali-pretreated in comparison to the untreated saw dust was noticed.

According to Tarkow and Feist (145) and Bellamy (148) NaOH is involved in atleast two reactions with hardwoods: Saponification of uronic esters and saponification of acetates, and if these are the only reactions occurring, then the NaOH has the following fates: (a) A portion is present as the sodium salt of the liberated carboxyl groups on the glucuronic acid residues attached to the xylan chains; the amount is readily computed from the free carboxyl content after removal of sodium by acid washing; (b) A portion is present as sodium acetate, the amount left in the wood will depend on the extent to which the sodium acetate diffused out of the wood during treatment; and (c) A portion will be present within the free water in the lumens and at the same concentration as that of the treating solution if equilibrium was established.

6157

(c) Ammonia

Another long standing approach to upgrading the digestive value of lignocellulosic materials involves treatment with aqueous or gaseous ammonia (149). The treatment of the rice straw with aqueous ammonia to obtain a product containing about 1.3% nitrogen and having an *in vitro* digestibility of about 62% and an *in vivo* (sheep) digestibility of about 56% was studied by Weiss *et al.* (149). Han and Callihan (146) using similar treatment, found that the utilization of rice straw by *Cellulomonas* could be increased from an initial 29% upto 57%.

Anhydrous ammonia, either liquid or gaseous is another strong swelling agent for cellulose. Aspen appeared to be unique in the extent of its response to this pretreatment, attaining a digestibility coefficient of about 50% as contrasted with 10% of red oak (150).

(d) Solvents for cellulose

The use of cellulose solvents as agents for the pretreatment of cellulosic wastes has been reviewed by Tsao *et al.* (151). These solvents penetrate the crystallites of cellulose and bring about a transformation in the crystallite structure. A number of cellulose dissolving solvents are known. Such solvents may be classified into four major catagories:

1. Strong mineral acids
2. Quaternary ammonium bases (benzyl trimethyl ammonium hydroxide (152)
3. Transition metal complexes, and
4. Miscellaneous such as dimethyl sulfoxide, para-formaldehyde and dimethylformamide (152), N-methyl-morpholine

The solvents belonging to catagory 4 are difficult to handle and cause considerable degradation of the cellulose. Those belonging to category 2 are rather weak solvents for cellulose. Hence, strong mineral acids and transition metal complex solvents have been most frequently used for the purpose of pretreatment.

Various acid hydrolysis processes are used primarily for the saccharification of wood (64,152a). Strong mineral acids, e.g. sulfuric, phosphoric and hydrochloric acids, within definite concentration ranges, act on cellulose as swelling or dissolving agents. In most cases, there exists a critical concentration above which solution of cellulose occurs. In the case of sulfuric acid e.g., about 60 - 65% by weight is needed; hydrochloric acid requires more than 40% by weight, and phosphoric acid about 83% in order to dissolve cellulose (152a).

There are basically two types of acid processes. One uses concentrated acid to catalyze saccharification, and other dilute acid. Processes using concentrated sulfuric acid for hydrolysis generally give higher yield. However, separation of the acid from the sugar product is a problem since, the sugar product is not fermentable in concentrated acid, and the acid, being relatively expensive, must be recycled if the process is to be economically viable (64).

Since HCl is a volatile acid while H_2SO_4 is not, most practical work with concentrated acid has centered upon HCl. The approach with HCl has been to recover the acid by vacuum evaporation after hydrolysis. The acid is then recycled to the process.

The concept of using concentrated sulfuric acid

(approx. 65% - 70%) as a solvent has been under study since World War II (153). In a molecular weight determination of cellulose and in studies on the degradation of cellulose in solutions, Ekenstam (1936) (153a) applied 65% sulfuric acid or 80 - 85% phosphoric acid and showed that phosphoric acid is less reactive towards cellulose in solution than sulfuric acid (152a). For corn residue, Tsao _et al_. (151) applied the sulfuric acid pretreatment. At first, the hemicellulose was was extracted from the residue following a procedure analogous to the one described by Dale _et al_.(154) with chelating metal caustic swelling (CMCS), as a cellulose solvent. Seventy percent H_2SO_4 was added to the wet lignocellulose. After it was allowed to stand for 2 h the cellulose was reprecipitated _in situ_ by addition of methanol and then washed with water. The filtrate contained methanol and sulfuric acid. Dilute sulfuric acid hydrolysis of the reprecipitated cellulose led to 90% conversion to glucose within an hour without the formation of byproduct like furfural that are characteristic of acid hydrolysis at elevated temperatures. Knappert _et al_. (155) studied partial acid hydrolysis as a pretreatment to enhance enzymatic hydrolysis. Such a pretreatment was carried out in a continuous flow reactor on oak, corn stover, newsprint and SolkaFloc at temperatures ranging from 160 to 220°C, acid concentrations ranging from 0 to 1.2% and a fixed treatment time of 0.22 min. For all substrates except Solka Floc they found increased glucose yields during enzymatic hydrolysis of the pretreated material as compared to hydrolysis of the original substrates.

Because of their ability to dissolve cellulose with minimal degradation, metal complex solvents have found widespread use for analytical studies on cellulose. Table 7 lists the commonly used transition metal complex solvents, their designation and their constituents (156,157).

The solvent cadoxen was introduced by Jayme and Neustaffer (157). Cadoxen is actually a solution containing ethylene diamine and water at alkaline pH. It is stable for an almost unlimited time and causes little degradation of cellulose. Cadoxen is considered a useful solvent in cellulose hydrolysis for many reasons: (1) it contains 70 to 75% water in its formulation. All cellulosic wastes when harvested and collected contain considerable amounts of moisture. Cadoxen requires no pre-drying, which avoids an otherwise costly operation; (2) it can dissolve nearly 10% by weight of cellulose at room temperature which is reasonably high; (3) it is a nontoxic solvent to cellulose enzymes. The pretreatment with cadoxen has been reported to be effective for bagasse, corn stalks, and orchard grass in which cellulose is protected by the lignin seal (156,157).

Delignification

With lignin being a major deterrent to the enzymatic and microbiological utilization of forest residues, delignification would appear to provide a simple solution to the problem. All commercial delignification procedures are based on the removal of lignin through the selective action of chemical pulping and leaching agents. Air dried woody wastes can be treated with chemical delignifying agents usually at a ratio of 1:7. According to Toyama and Ogawa (158) rice straw and bagasse can

TABLE 7: TRANSITION METAL COMPLEX SOLVENTS FOR CELLULOSE (157)

Solvent	Designation	Transition metal	Ligand
Cuprammonium hydroxide	Cuoxam	Copper	Ammonia
Cupriethylenediamine hydroxide	Cuen, CED	Copper	Ethylene-diamine
Iron tartaric acid -sodium complex	EWNN, FeTNa CMCS	Iron	Tartaric acid
Triethylenediamine zinc hydroxide	Zincoxen	Zinc	Ethylene-diamine
Triethylenediamine cadmium hydroxide	Cadoxen	Cadmium	Ethylene-diamine

be delignified by boiling in a 1% NaOH solution for 3 h or by autoclaving at 120°C in a 1% NaOH solution for 1 h, but corn stalks can be delignified in peracetic acid as developed by Hass _et al_. (159), by leaving them to stand overnight or by boiling for 1 h (peracetic acid consists of acetic anhydride and 35% hydrogen peroxide (1:1 by volume)). The combination of alkali and peracetic acid treatment can be used for coniferous wood (158).

The disruption of the lignin carbohydrate association, largely physical in nature, without the selective removal of either constituent can be achieved with gaseous SO_2 treatment of moist wood (126). In essence, the process involves the reaction of moist saw dust (water/wood = 3) with gaseous SO_2 under pressure for 2 - 3 h at a temperature of about 120°C. Following blowdown and a brief evacuation to remove excess SO_2, the products are neutralized to about pH 7 and air-dried.

Conclusion

Any method of pretreatment must aim at modification of the fine structure of cellulose at the least possible cost to make the process economically viable. In case of intra-crystalline swelling techniques of mercerization, regeneration and amine decrystallization enhanced hydrolytic action can be observed but their economic feasibility is questionable.

The cost of physical treatment such as ball milling is rather high ($ 60/tonne or more)(11). Pretreatment with dilute caustic solution is an effective method for increasing the yield of fermentable sugars but cost is also high.

Concentrated sulfuric acid appears to be promising somewhat because of its availability at low cost. It has been claimed that using this process, fermentable sugar can be produced at a cost of 2.5 - 5 cent/lb (64). The method that appears most promising is the pretreatment of cellulosic material at high pressure and high temperature (160). The process is being tested on a pilot plant scale at NSW University, Australia and other laboratories (144,160). At the University, wood chips first undergo a steam explosion step, followed by solvent delignification to remove upto 95% of the lignin content. Preliminary economic studies show (in late 1979) that a commercial plant for treating 1,000 tonne/day of oven-dried wood chips by this method can make 25 million gal/year of ethanol for $ 1.75/gal - i.e., at a price competitive with that of grain ethanol. (This estimate assumes a byproduct credit only for pentoses, and applies to a facility operating with a fixed bed reaction) (160).

SECTION 4

BIOCHEMISTRY OF CELLULOSE DEGRADATION

Nature of cellulase

The International Commission on Enzyme Nomenclature lists "cellulase" as the trivial name for beta-1,4-glucan-4-glucanohydrolase (EC 3.2.1.4), i.e., for enzymes which hydrolyze beta-1,4-glucans at linkages which are not restricted to terminal linkages. The term "cellulase" has been used in the literature for both pure well-characterized enzymes and mixtures of enzymes produced by organisms which degrade cellulose. These enzymes have sometimes been designated as cellulase complexes or systems. Even the nomenclature like Avicelase, gauze-solubilizing activity, hydrocellulase, Cellulase I, Cellulase II, etc. used to describe cellulase enzymes is confusing. This is because a variety of assay procedures and substrates have been used in the measurement of cellulase activities. The substrates used differ in the degree of crystallinity, lattice structure, degree of polymerization and solubility of the cellulose molecules. It is, therefore, questionable whether all assay procedures measure exactly the same thing. The effects of the specific surface area and the crystallinity index (Cr I) (161) of cellulose on hydrolysis were investigated (162). The results of Fan *et al*. (162) show a highly linear relationship between Cr I and the rate of hydrolysis for different cellulose samples. However, the specific surface area as measured and the rate of hydrolysis are apparently not clearly related. This indicates that fine structural order of cellulose dictates the hydrolysis rate to

a greater degree than the simple surface area as measured by nitrogen adsorption.

The cellulase of the digestive juice of the edible snail, Helix pomatia, is prominent in the early literature, but its origin has been a matter of some controversy. Bacteria and fungi are the two main groups of microorganisms responsible for the degradation of cellulose, even though some species of algae and myxobacteria are sometimes encountered. For a long time it was believed that utilization of cellulose is the property of living viable cells, because many bacteria and fungi could grow and utilize cellulose rapidly, but the culture filtrates showed very limited or no hydrolysis of cellulose. During the last 20 years or so, it has been found that certain fungi, T. reesei (162-164), Trichoderma koningii (165), Fusarium solani (166), S. pulverulentum (168), Penicillium funiculosum (167), Sclerotium rolfsii (169-171), Monilia sp (171a) can provide culture filtrates which are highly effective in producing extensive degradation and complete solubilization of both native and degraded forms of cellulose.

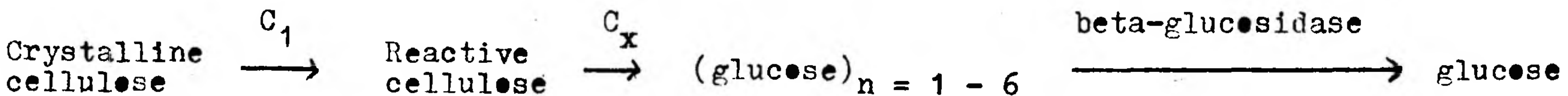
Many organisms possess enzymes that can hydrolyze soluble celluloses. By contrast, few species produce the "complete" cellulase complex capable of catalyzing the extensive degradation of the crystalline substrates found in nature (172,173). In 1950, Reese et al. (174) put forward the hypothesis of the cellulase enzyme being a multienzyme system in which some of the organisms were capable of synthesizing an enzyme, which they named C_1, which acted on the native cellulose making it degradable by the hydrolytic (C_x) enzymes.

The existence of C_1 was postulated, however, purely on the basis of indirect evidence, for no component of this type was isolated at that time. Some organisms that were able to degrade amorphous cellulose were unable to degrade crystalline cellulose. Some organisms that degraded highly ordered cellulose produced culture filtrates that were incapable of degrading the highly ordered material, but the same culture filtrates could degrade cellulose that had been made accessible by derivatization (CM cellulose), swelling in phosphoric acid, or grinding. It was argued that swelling, grinding or derivatization were simulating C_1 action by producing hydrated cellulose chains. Thus, C_1 was envisaged to be a pre-hydrolytic factor (a hydrogen bondase) (191) producing an "activated" cellulose which was then capable of attack by the hydrolytic enzymes of the complex (Scheme I, Fig. 8) (176). In this mechanism, the first reaction is considered the rate determining step.

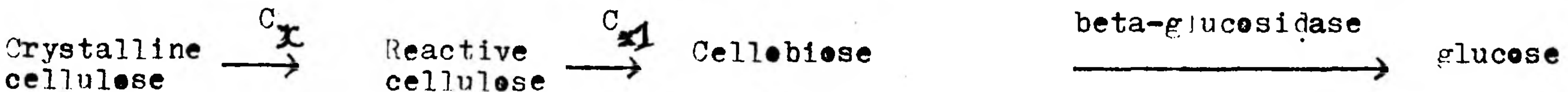
But the facts as we know at present do not fit this hypothesis. The C_1 component has little or no effect on soluble derivatives of cellulose, such as carboxymethyl cellulose (CMC). Enzymes classified as C_x can hydrolyze amorphous cellulose, soluble or partially degraded celluloses producing cello-oligosaccharides, glucose or cellobiose. These were postulated earlier to consist of exo- and endo-beta-1,4-glucanases (174,177,178). These are hydrolytic in nature and their action results in the appearance of reducing sugars in the hydrolyzate. The former acts from the non-reducing end of the cellulose and successively removes cellobiose (and in some cases, glucose) units, while the latter attacks randomly the

FIG. 8

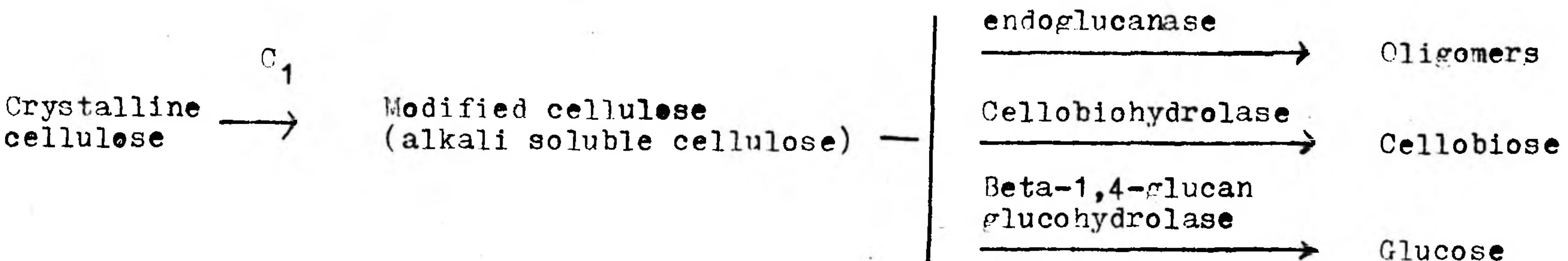
Scheme I: Original C_1 - C_x hypothesis (Serial mechanism) (174)



Scheme II: Hypothesis involving sequential action: endoglucanase (C_x) followed by cellobiohydrolase (C_1) (176).



Scheme III: Modified C_1 - C_x hypothesis (188)



internal beta-1,4-linkages (179-181).

In scheme II (Fig. 8) synergism shown by mixtures of C_1 and C_x, when solubilizing highly ordered cellulose, can then be explained in terms of the new chain ends generated by C_x being hydrolyzed by C_1. In this respect, the process will be synergistic. The cellobiohydralase (C_1) in the C_x - C_1 hypothesis (Scheme II, Fig. 8) is relegated to the role of hydrolyzing an "activated" cellulose ~~(Scheme III)~~ along with the endoglucanases and glucohydrolase. This hypothesis, which reverses the order of action of C_1 and C_x, as originally postulated, gets a high degree of support (182-186). Reese (187) argues that the first step in the enzymatic hydrolysis of other highly ordered structures such as DNA and collagen is affected by enzymes whose function is to produce some disorder in the structures as a preliminary to hydrolysis by other enzymes. Such enzymes are highly specific for ordered molecules, and they are unable to act on the disordered structures that they produce. Thus, Reese rationalizes that the first step in the hydrolysis of crystalline cellulose involves a similar type of enzyme. As a result Reese (188) presented a modified C_1 - C_x hypothesis (Scheme III, Fig. 8). This concept retains the idea that C_1 causes some "swelling" of the crystalline cellulose.

Sudo et al. observed that the C_1 activity in the CMC-grown culture filtrate could be rendered demonstrable by alcohol precipitation or DEAE Sephadex A-50 treatment (~~189~~-191). They suggested that a single enzyme may be responsible for both C_1 and C_x activities. Leatherwood (192) proposed that just as in antigen-antibody protein interaction, the complete cellulase is

composed of an "affinity factor" and a "hydrolytic factor" which together act as a single entity to hydrolyze native cellulose. The major role of the "affinity factor" was suggested to be that of binding the "hydrolytic factor" to cellulose to permit multiple attacks (192). From the synergism displayed by reconstituted mixtures of C_1 and C_x enzymes in solubilizing native cotton, it was postulated that the C_x enzyme (endoglucanase) initiates the attack on the cotton fibre, thereby creating more chain ends for C_1 for attack (193), increasing the uptake of alkali (S factor)(194,195), or loss in strength (194). However, as Wood and McCrae (193) themselves pointed out it would not explain as to why C_1 and C_x enzymes are both individually capable of hydrolyzing swollen cellulose and not highly ordered cellulose, such as cotton, yet when acting in solution simultaneously can hydrolyze crystalline cellulose with comparative ease. Eriksson (168,185,196) also suggested that C_1 is an exo-beta-1,4-glucanase and CMCase, an endo-beta-1,4-glucanase, which attacks randomly on the cellulose chain first and opens up the chain where C_1 the exo-enzyme, can act. Tomita et al. (197) reported that Avicelase and CMCase are immunologically different proteins and that Avicelase attacks Avicel readily and CMC with a low activity (Avicelase/CMCase ratio = 18:1). The resistance of CMC hydrolysis by Avicelase as compared to CMCase was attributed to Avicelase being more sensitive to substitution at C_6 of the pyranose ring of glucose, because the substitution frequency is highest at the primary alcohol group of the glucose residue. CMCase seems to be less sensitive to changes around the C_6 atom of the

pyranose ring of beta-1,4-glucans. Nisizawa and his collaborators (198) observed a very high synergism between CMCase and Avicelase. A mixture of these two components was reported to degrade cotton/Avicel almost at the same rate as the culture filtrate. It was suggested that no cellulase component other than Avicelase and CMCase may be necessary for effective degradation of cotton and similar substrates (199). It is assumed that CMCase first attacks cellulose chains, mainly at amorphous regions, to produce cellulose fragments which then serve as substrates for Avicelase (198). Pettersson (164) has also suggested that regions of low crystallinity in the cellulose fibre are attacked by endoglucanases and free chain ends are created.

Fägerstam and Pettersson (199a,199b) have recently reported the isolation of two immunologically unrelated cellobiohydrolase components, CBH-I and CBH-II. The amino acid composition of the two enzymes differs. No sequence homology was found within the first 20 amino acid residues. The hydrolytic products when acting on crystalline cellulose were mainly cellobiose, but also significant amounts of glucose. Both CBH-I and CBH-II show individually synergism with endoglucanase. In addition CBH-I and CBH-II also show a strong synergistic action with Avicel (199b). The authors have suggested in addition to endo- and exo-synergism an exo-exo-synergism in the action of the cellulolytic complex (199b).

Halliwell (200) has separated the components of *T. koningii* cellulase and found that the addition of cellobiase to the reaction achieved a better hydrolysis of cotton than

addition of CMCase enzyme. Cellobiose was the major product in the absence of cellobiase and was a competitive inhibitor of the C_1-enzyme. His conclusion was that C_1 was a cellobiohydrolase with a preference for the ends of chains. Subsequently, Halliwell and Griffin (184) separated the T. koningii cellulase system into four apparently pure fractions. C_1, C_2, CMCase, and cellobiase. Both CMCase and cellobiase synergize with C_2, but only cellobiase synergizes with C_1. The C_2 protein promotes the formation of short fibres from native cellulose, thereby creating additional chain ends for C_1 and CMCase to act. The enzyme disaggregates filter paper into separate fibres and is different from C_1 and CMCase. The fragmentation of cellulose to give short fibres was earlier reported by Ogawa and Toyama (201) and later studied by Halliwell (200) Marsh (202) and Zhukov et al. (203). The C_2 enzyme has been crystallized (204). King (205) had also reported the formation of smaller fragments, 300 - 400 nm long and 50 - 60 nm wide, as a stage in the degradation of Avicel, but they assigned this function to C_1. Halliwell and Griffin (184) consider that C_1 and cellobiase are the only two components required to effect extensive hydrolysis of native cotton (70% solubilization), whereas Wood and McCrae (193), Eriksson (179,196), Eriksson and Pettersson (206), Berghem and Pettersson (185), Tomito et al. (197) and Emert et al. (183) consider that the enzymes required for attacking and solubilizing highly ordered cellulose (cotton) are C_1, CMCase and cellobiase. Halliwell (207) has subsequently reported that the multienzyme cellulase systems of T. koningii and Myrothecium verrucaria consist of two cellulases: exo- and endo-glucanases,

and two dextrinases: CMCase and cellobiase. The four components in unison can effectively saccharify native cellulose and its primary products, short fibres (208).

Recently, the concept of C_1 being a specific enzyme for the initial attack on cellulose was revived. It was suggested that the missing unidentified component is of oxidative nature. Eriksson and his colleagues have reported the isolation of two new enzymes, cellobiose: quinone oxido-reductase, cellulose oxidase (which they obtained free from endo- and exo-glucanases) (60,210-212). Cellobiose-quinone oxido-reductase takes part in both lignin and cellulose degradation (210). Cellulose oxidase, a haemprotein, oxidises cellobiose to cellobionic acid (179). The cellobiono-delta-lactone and cellobionic acid formed by its action are thought to prevent the transglycosylation reaction from taking place where high cellobiose concentrations build-up (179). It is important for cellulose degradation, since the degradation is approximately doubled when it is present in addition to C_1 and C_x enzymes (However, no reduction in the cotton-solubilizing capacities by F. solani and P. funiculosum cellulases was observed when incubation was carried out under nitrogen (186)). The enzyme was shown to be oxidative in character and seemed to oxidize the hydroxyl groups in the sixth position in the unit to a carboxyl group (212). It was suggested that the oxidation of glucose units of cellulose by the enzyme causes swelling of the cellulose chains, which, in turn, causes disorder in crystalline cellulose, thereby making the crystalline parts more accessible to enzymes (212). This is

fully in accord with the old hypothesis of Reese et al. (174) that cellulose is first activated, so that its accessibility to hydrolytic enzymes is increased and is non-hydrolytic in character. Eriksson (179, 212 ad) has more recently postulated that cellobiose oxidase oxidizes the reducing end groups formed when a beta-glucosidic bond is split through the action of endo-glucanases and thus effectively prevents a broken beta-1,4-glucosidic bond from reforming in the crystalline part of cellulose.

Marsh (202), while studying short fibre formation from cotton, had suggested that peroxidation at some points on cellulose potentiates the enzymic process. Halliwell and Koenigs (200,213), had also pointed out that depolymerization of cellulose need not be an enzymic process and that a combination of H_2O_2 (0.4%) and $FeSO_4$ (0.2 mM at pH 4.2) depolymerizes cellulose which becomes fragmented to very short fibres. Koenigs (213) has proposed that these play a role in wood-rotting organisms. Halliwell (200) suggested that H_2O_2-$FeSO_4$ reaction is an alternative mechanism to the hydrolytic action of cellulase in nature, possibly comparable to photolytic degradation of textiles.

There is a growing consensus that the endo- and exo-glucanases must be at the reaction site simultaneously and quite likely must be adsorbed in some fixed configuration, perhaps as a loose complex, (176). The C_1 - C_x concept is valid only for certain groups of microorganisms, and other groups may have different mechanisms of attack on native cellulose (173). Synergism between C_1 of P. funiculosum and

C_x of *T. viride* has been demonstrated by Selby (214). Many other 'cross-synergism' experiments were performed in many mixtures of the C_1 and C_x components of *F. solani*, *T. koningii* and *P. funiculosum* cellulases. In each case, a marked potentiation in activity was observed (176). Wood (215) demonstrated that culture filtrates of the potent cellulose decomposers *Stachybotrys atra* and *M. verrucaria* showed consistantly small increases in capacity for degrading cotton when mixed with C_1 from either *T. koningii* or *P. funiculosum* (176). From this, Wood and McCrae (176) concluded that cross synergism was best between the respective C_1 and C_x components of fungi that freely release C_1 in active form into the culture medium.

Synergistic experiments with purified C_x-type enzymes from *Coniophora cerebella* and C_1 from *T. koningii*, with or without the addition of cellobiase gave no significant enhancement of activity on native cellulose (216). C_x from one fungal species can operate with C_1 from another when both have the C_1 - C_x system. To achieve synergistic effect, the two enzymes have to work together in the form of a loose complex. A slight shift in charge or tertiary structure could prevent the formation of a cooperative system (216). Thus, there are probably alternative mechanisms by which cellulose is degraded. However, the common denominator in all cellulolytic organisms is a C_x-type enzyme and the mechanism by which this enzyme is aided in the attack on crystalline cellulose may be different in different organisms.

A model for the enzymatic hydrolysis of cellulose for the

enzyme systems of Thermoactinomyces (217) and T. reesei (183, 218) has been suggested. In this model, one or several endoglucanases act randomly to produce oligosaccharides while one or several exoglucanases, including cellobiohydrolase, produce cellobiose (or glucose) from the non-reducing ends of these oligosaccharides (181,182,219). The end products of endo-glucanases are cellobiose and small amount of glucose (199,220,221). Finally one or several cellobiases (beta-glucosidases) produce glucose from cellobiose.

Conclusions

The conversion of cellulose to glucose seems to require 3 to 4 different types of enzymes. These are classified as endo-beta-1,4-glucanase, exo-beta-1,4-glucanase, cellobiohydrolase (which is also an exoglucanase) and beta-glucosidase (176). There may be an exoglucanase which is not a cellobiohydrolase. An exoglucanase may not attack insoluble substrates or hydrolyze it to any significant extent when acting in isolation. In addition, differences exist between the ability of the various multiple forms of cellobiohydrolase to attack cotton, Avicel and H_3PO_4-swollen cellulose (240). Exoglucanases hydrolyze Walseth cellulose, i.e., H_3PO_4-swollen cellulose but endoglucanases can also hydrolyze Walseth cellulose (182,219). The final product of action of endoglucanases and exoglucanases are cellobiose and glucose (219, 220). The cellobiohydrolase splits cellobiose from the non-reducing ends of the cellulose chain (220). The endoglucanase, synonymous with CMCase and C_x enzymes, hydrolyzes cellulose derivatives such as CMC or H_3PO_4-swollen cellulose and acts by

randomly spliting the cellulose into smaller chains eventually producing cellobiose and glucose. Beta-glucosidases, i.e., cellobiases, hydrolyze cellobiose and higher cellodextrins to glucose (185,208,223,240). Some of the beta-glucosidase can also be termed as exoglucanases according to the defination of Reese et al. (174).

The hydrolysis of native cellulose is thought to result from the synergistic action of endoglucanases and exoglucanases. The present thinking of the mechanism of native cellulose hydrolysis involves a sequential action by an endoglucanase which attacks at random the 1,4-beta-linkages along the cellulose chain at the less crystalline or amorphous regions thus opening up the structure where exo-beta-glucanase acts and splits up cellobiose units from the non-reducing end of the cellulose chain ends (Fig. 9,10)(53,168,183,184,193,198,223,225). This assumes that less ordered, i.e., amorphous or susceptible regions, exist in the chains of the cellulose microfibrils. The so-called amorphous regions in a cellulose fibril could be zones rich in loop bends containing many deflected beta-glucosidic linkages. The continued cooperative action of the endoglucanases and exoglucanases results in the conversion of cellulose to cellobiose and small oligosaccharides, and small amounts, 1 to 3% of glucose. Beta-glucosidase hydrolyzes cellobiose and other cellodextrins to glucose (Fig. 9,10). But this view has not met with universal acceptance. One has therefore to be on the look out for discovering new type of enzymes that might be involved in the hydrolysis of cellulose, e.g., Eriksson and his group have reported that cellobiose

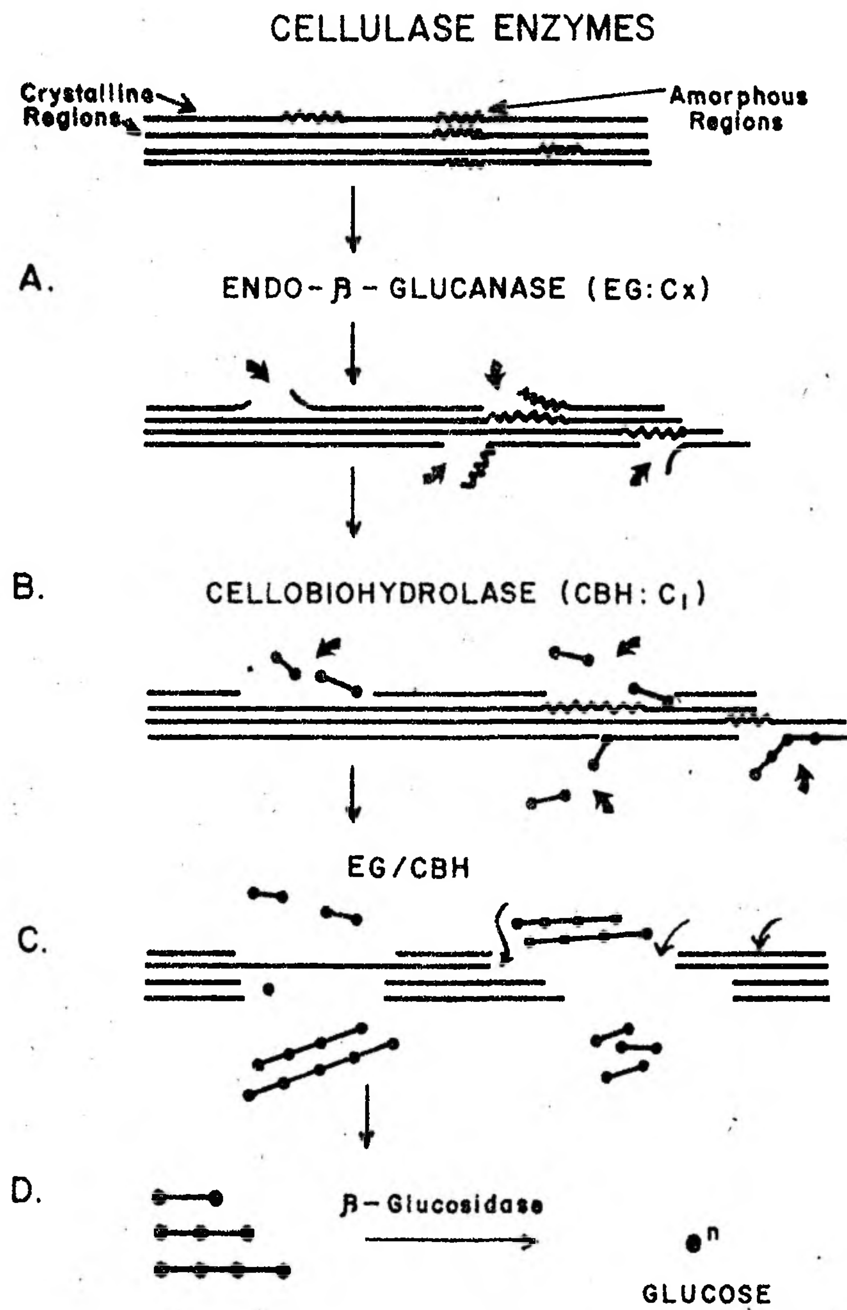


FIG.9: Schematic representation of sequential stages in cellulolysis (223).

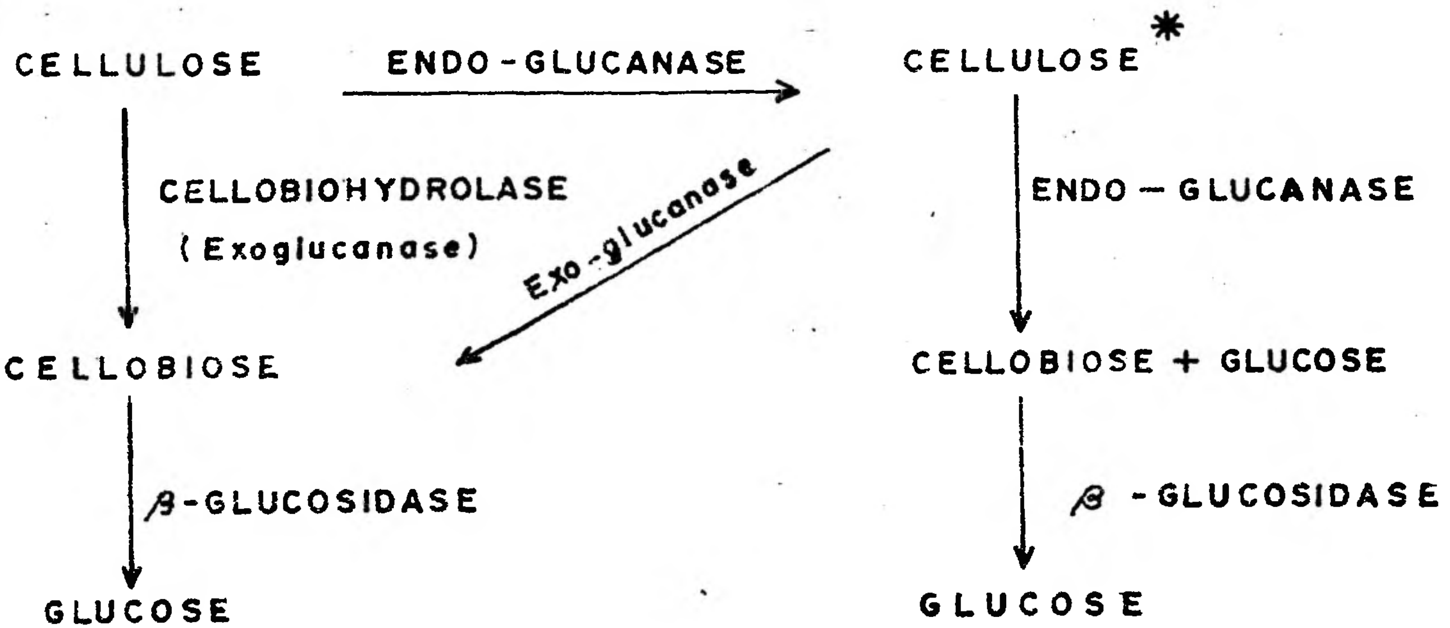


FIG.10. The cellulase enzyme system (53).

oxidase, which they isolated and purified from *S. pulverulentum* and obtained it free from cellulase activities, is important in cellulose hydrolysis (179). They suggested that cellobiose oxidase oxidizes the reducing end groups formed when a beta-glucoside bond is split through the action of endoglucanase and thus effectively prevents a broken beta-1,4-glucosidic bond from reforming in the crystalline part of the cellulose. However, no oxidative enzyme has been found to be involved in extracellular cellulose degradation in the case of *T. reesei*, *F. solani* and *P. funiculosum* (180). Wood and McCrae (180,219) consider that endoglucanases and exoglucanases may form a loose complex on the outer surface of the cellulose chains and there is sequential action of endo- and exo-glucanases. The adsorption of a charged molecule such as endoglucanase, exo-glucanase, or the endo-exo-glucanase complex on the surface of the cellulose fibre might result in localized disruption of hydrogen bonds (180). If that is so, then the concept is similar to the one put forward by Reese (174) that cellulose has to be activated somehow before cellulase enzymes can act.

SECTION 5

PRESENT INVESTIGATIONS

It is evident from the literature reviewed that cellulases and hemicellulases have mostly been studied in three aspects, (a) mutation studies for enhanced enzyme production, (b) enzymatic saccharification and fermentation, (c) enzymes involved in cellulose-hemicellulose degradation. The major problems encountered for economical production of liquid fuel from renewable resources are obtaining sufficiently active enzyme preparations and reactive substrate so that high concentrations of glucose for alcohol production can be obtained in a reasonable time.

The work presented in this thesis includes the following investigations:

(1) Mutation studies and optimization of cellulase, xylanase and beta-glucosidase production by S. rolfsii UV-8 mutant.

(2) Saccharification of cellulosic materials and production of ethanol in coupled system.

(3) Purification of endoxylanase, to homogeneous state and study of its physico-chemical properties.

PART II

MATERIALS AND METHODS

MATERIALS

The following materials were purchased from the suppliers indicated - Glox Glucose reagent (CAB Kabi Diagnostica, Stockholm, Sweden), Avicel P.H. 101 (38 µm average particle size, Honeywill and Stein Ltd., U.K.), Avicel P.H. 102 (90 µm average particle size, American Viscose Co., U.S.A.), Cellulose-123 powder (Karl Schleicher and Schüll Co., West Germany), Solka Floc SW-40 and BW-200 (pure fibrous spruce wood cellulose pulp, Brown Co., Berlin, New Hampshire), absorbent cotton (Bengal Chemicals and Pharmaceutical Works Ltd., India), proteose peptone malt extract, and bacto yeast extract (Difco Laboratories, U.S.A.), Tween 80 (Atlas Chemical Industries, U.S.A.) Tween 20 (Biochemical Unit, New Delhi), Bio-Gel P-150 (Bio-Rad Laboratories, California), Sephadex G-75, Sephadex G-200, DEAE-Sephadex A-50 (Pharmacia Fine Chemicals, Uppsala), acrylamide and N,N,N'N'-tetramethyl ethylenediamine, N,N'-methylenebisacrylamide (Eastman Organic Chemicals, U.S.A.), beta-alanine (Loba Chemie Indoaustranal Co., Bombay) amido black (George, T. Gurr Ltd., England), Ampholine Carrier ampholytes (LKB Produkter, Bromma, Sweden), Schiff's reagent (Fluka AG, Switzerland), Hexokinase (type II), adenosine-5'-triphosphate, bovine serum albumin, transferrin, gamma-globulin, ovalbumin, alcohol dehydrogenase, beta-D(+) cellobiose, carboxymethylcellulose-Na salt, xylan (larch wood) and Coomassie brilliant blue (Sigma Chemical Co., U.S.A.), p-nitrophenyl-beta-D-glucopyranoside, p-nitrophenyl-beta-D-xylopyranoside (Koch-Light, U.K.).

Paper mill wastes (bleach house, machine house and sedimental sludge effluents) were obtained from Balarpur

Industries Ltd., Paper Division, Balarpur, which utilizes mainly bamboo (about 70%) and a mixture of 10 to 15 hardwood species from Bastar forest (about 30%) for paper manufacture. The lignocellulosics, rice straw, sugarcane bagasse and coniferous wood were purchased locally. Mesta wood sticks (Hibiscus cannabinus) were kindly supplied by the Forest Development Corporation of Maharashtra State Ltd., Nagpur, and corn steep liquor by Anil Starch Products Ltd., Ahmedabad, India.

Cotton Sliver (a crystalline fibrous cellulose) was supplied by Dr. E.T. Reese (U.S. Army Natick Laboratories, U.S.A.), Lyophilized T. reesei C-30 culture filtrate was kindly supplied by Dr. F.H. Bissett (U.S. Army Natick Laboratories, U.S.A.).

All other chemicals used were from commercial sources and were of analytical grade. Sodium dodecyl sulfate (HICO, India) was crystallized twice from ethanol. Collodion bags were purchased from Sartorius-Membranfilter GmbH, Gottingen, Germany.

Organisms

The fungus used in this study was a UV-8 mutant isolated from the parent strain Sclerotium rolfsii CPC 142 (sclerotial state of Corticium rolfsii).

The yeast used in the fermentation study was Saccharomyces cerevisiae NCIM 3078, and the bacterium Zymomonas mobilis (NCIM, NCL, Poona 8).

Cultivation and preparation of crude extract of S. rolfsii UV-8 mutant

The UV-8 mutant of S. rolfsii was grown on T. reesei medium developed by Reese and Mandels (226) supplemented with trace metals (227), for initial media variation studies. For

saccharification experiments and for purification the enzyme was collected by growing the culture on NM-4 medium (optimized medium). The composition of T. reesei, NM-2, NM-3 and NM-4 media are summarized in Table 8. The cultures were incubated at 29 - 30°C for 12 - 14 days on a rotary shaker (150 rpm) in 500 or 1000 ml Erlenmeyer flasks with 100 or 250 ml medium in each flask. Prior to autoclaving at 121°C for 20 min, the pH was adjusted to 6.5 with phosphoric acid, unless otherwise stated. Stock cultures were stored at 28°C to 30°C on potato-dextrose agar (PDA) slants and subcultured once every 4 weeks. The media were inoculated with mycelium directly from 7 - 8 days old PDA slants or from cultures grown on cellulose. Enzyme yields in 14 days were equal to that of either inoculum but the mycelium grown on cellulose medium usually gave more rapid growth and earlier enzyme development and was therefore a more suitable inoculum. The culture was harvested at 12 - 14 days by centrifugation at 3000 rpm for 30 min in angle head centrifuge. The clear culture filtrate was used for estimating enzyme activities after adjustment of the pH 4.5 with sodium citrate (about 2 ml 0.5 M sodium citrate for 100 ml culture filtrate). The culture filtrate could be stored for long periods at 2 to 4°C, or frozen, in the presence of 0.005% merthiolate or 0.01% sodium azide without any loss in activity. The S. rolfsii retained its enzyme activities for over 2 years with frequent subculturing on PDA or when stored in lyophilized state.

Cultivation of S. cerevisiae and Z. mobilis

The liquid malt extract-yeast extract-glucose-peptone

TABLE 8: COMPOSITION OF T. REESEI, NM-2, NM-3 AND NM-4 MEDIA

Constituent	*T. reesei* g/l	NM-2 g/l	NM-3 g/l	NM-4 g/l
KH_2PO_4	2.00	2.00	2.00	2.00
$(NH_4)_2SO_4$	1.40	-	-	-
$(NH_4)_2HPO_4$	-	7.00	7.00	7.00
Urea	0.30	0.30	0.30	0.30
$MgSO_4.7H_2O$	0.30	0.30	0.30	0.30
$CaCl_2.2H_2O$	0.30	0.30	0.30	0.30
Proteose peptone (Difco)	0.25	0.25	0.25	0.25
Yeast extract (Difco)	0.10	0.10	0.10	0.10
Tween-80	0.33	0.33	0.33	0.33
Trace metal solution*	1.00	1.00	1.00	1.00
Cellulose-123	5.00	30.00	30.00	30.00
Rice bran	-	-	30.00	-
Corn steep liquor	-	-	-	20.00

*Trace metal solution contained in mg/l; $FeSO_4.7H_2O$, 5.0; $MnSO_4.7H_2O$, 1.56; $ZnSO_4.7H_2O$, 3.34; $CoCl_2$, 2.0 (227); 1 ml of this solution was used per litre of medium.

medium (MYGP) was used for the inoculum preparation of yeast and *Z. mobilis*. The composition of the MYGP medium is as follows:

Malt extract (spray dried)	-	0.3 gm
Yeast extract (Difco)	-	0.3 gm
Bacto-peptone	-	0.5 gm
Glucose	-	1.0 gm
Distilled water	-	100 ml
pH	-	4.5

Biomass assay of yeast

For biomass assay, 10 ml samples of MYGP grown yeast (30°C, 24 h) were withdrawn and to it 1 ml 32% formaldehyde solution was added to stop the growth. The cells were centrifuged at 4000 rpm and the supernatant was stored under refrigeration. The optical density of the cell suspension was measured at 525 nm in a Beckman Spectrophotometer Model No. 26 using clear sterile medium with formaldehyde (preservative) as a blank. Cells were washed twice with distilled water and then dried at 105°C to constant weight. Cells were counted in a haemocytometer and viabilities were determined using the staining technique with methylene blue (258).

METHODS OF ANALYSIS

Reducing sugars were measured in aliquote of reaction mixtures as glucose either by the dinitrosalicylic acid (DNS) method (228) or by the Somogyi-Nelson method (229). One millilitre samples were treated with 1 ml DNS solution and then heated for 5 min in a boiling water bath. The tubes were cooled for 5 min under running tap water, 10 ml water was added

and the optical density measured at 540 nm. The sugar values were read from a glucose calibration curve. Glucose (0.1 mg) was added routinely in standard and test solutions (230). On an equal weight basis the absorbancy values for cellobiose are about 16% lower and for xylose 30% higher by this procedure, with glucose as the standard. Glucose in the solutions was determined using glucose oxidase-peroxidase test using "Glox" glucose reagent (182,237). Cellobiose was estimated enzymatically as glucose after hydrolysis with purified cellobiase. Since some of the hydrolyzates contained high amounts of glucose, glucose in the hydrolyzates was first converted into glucose-6-phosphate by hexokinase and adenosine-5'-triphosphate before cellobiose estimation.

Determination of cellulose

Cellulose was estimated by dissolving in cold (4°C) 67% H_2SO_4 and was analyzed by the anthrone method with Avicel P.H. 101 as a standard (232).

Determination of protein

The following methods were adopted for determination of protein in enzyme samples.

(a) Method of Lowry *et al.*

Protein determinations in the later purification steps were carried out with the Folin-Ciocalteu reagent as described by Lowry *et al.* (233). Crystalline bovine serum albumin was used as the standard. Samples, free of ammonium sulfate, Tris and containing only low concentrations of phosphates, were used to avoid interference from these substances.

(b) Optical method

In column effluents, protein was estimated by measuring the absorbancy at 280 nm, by the method of Warburg and Christian (234). The following empirical equation (235) was used to correct for light absorption due to nucleic acids.

$$4/7\ (2.3(O.D._{280\ nm} - O.D._{340\ nm}) - (O.D._{260\ nm} - O.D._{340\ nm}))$$
$$= \text{mg protein per ml.}$$

The concentration of serum albumin in the solutions was calculated from its extinction coefficient at 280 nm (236).

Paper chromatography

Filter paper chromatography (Whatman filter paper No. 1) was used for qualitative examination of the sugar composition. The solvent systems used were n-butanol: pyridine: water (6:4:3 v/v) (41), n-propanol:ethyl acetate:water (6:1:3) (232), or ethyl acetate:pyridine:water (2:1:2) (175). Reducing sugars were located by spraying with a solution of 0.1 M p-anisidine and 0.1 M phthalic acid in 96% ethanol. Hexoses and their soluble oligomers gave olive green spots while pentoses and th ir soluble oligomers gave brownish spots after heating at 105°C for 5 min. Prior to examination of the sugars, the samples were heated to precipitate soluble proteins and centrifuged to remove suspended solids.

Determination of alcohol

The following methods were adopted for determination of alcohol in fermented samples.

(a) ~~High pressure~~ GAS ~~gas~~ chromatography

Ethanol was analyzed using a Hewlett Packard 700 model gas

chromatograph equipped with a flame ionization detector. The stationary phase used was Carbowax 20 M. The temperature of injector was 150°C, 320°C of detector, and 80°C of column. The carrier gas was nitrogen with flow rate 40 ml/min (238).

(b) <u>Determination of alcohol with alcohol dehydrogenase and NAD</u>

The assay can be performed in 3 ml silica cuvettes. The assay system consists of ·3 ml pyrophosphate buffer (pH 8.7), 0.1 ml NAD (50 mg in 3 ml distilled water), and 0.2 ml of alcohol sample. The reaction can be started by adding 0.02 ml of alcohol dehydrogenase (50 mg lyophilized powder in 1 ml distilled water), and increase in ~~optical density~~ absorbance after incubation at room temperature for 30 min against the reagent blank can be recorded at 340 nm (239).

<u>Enzyme assays and determination of saccharifying ability of culture filtrates</u>

A variety of assay procedures and substrates have been used in the measurement of cellulase activities and for overall saccharifying ability of the culture filtrates for cellulose (240). These include: measurement of loss in weight of insoluble substrate (241), decrease in the tensile strength of the yarn, thread of fibre, microfragmentation of cellulose micelles (242), increase in uptake of alkali (S-factor, swelling factor activity), change in turbidity of a cellulose suspension (178), release of reducing end groups and of glucose (referred to as saccharifying activity) from soluble and insoluble materials, release of dye from a dyed substrate (243-245), and decrease in viscosity (referred to as CMC-liquefying activity) of solutions of cellulose derivatives such

as carboxymethylcellulose, hydroxyethylcellulose etc (178a,b). The later method is a sensitive test for endoglucanase action since a few random breaks in a chain will cause a marked decrease in viscosity. Non-ionic substituted cellulose, hydroxyethylcellulose, is preferred over ionic substituted, CMC as the viscosity of ionic substrates is dependent on pH, ionic strength and polyvalent cations.

Cotton fibre is one of the most resistant to enzyme hydrolysis. Avicel, a microcrystalline cellulose is also difficult to hydrolyze. Sulfite pulps such as Solka Floc and filter paper have also been used with a measurement of the formation of reducing sugars (242). These give a rough measure of the overall saccharifying ability of the culture filtrates. The activity determined is the sum of different cellulolytic activities, and the result depends on the relative proportions of the different enzymes. Determination of the non-solubilized substrate after enzymic digestion gives a reliable result, but the method is laborious and unsuitable for long series of determinations.

The method of Mandels and Weber (242) has gained general acceptance. The increase in glucose formation, however, is not linear because the reactive portions of the substrate are hydrolyzed away first and the rates are high at the beginning of the reaction (144) giving erratic activity values. Thus, the method tends to become less meaningful with highly active preparations. Linearity can be obtained by diluting the enzyme, or decreasing the reaction time (246). Mandels suggested that the most reliable quantitative activity determinations ought

to involve enzyme unit based on the same degree of hydrolysis of the filter paper (131,242,244), and is the procedure that has been followed by us.

Montenecourt et al. (223) described a modification of the filter paper assay - an antibiotic discs assay - which allows direct interconversion of units obtained employing either method. The antibiotic assay discs (740 E) are made up of specially purified cellulose (95% alpha-cellulose) and are available commercially from Schleicher and Schüell, Keene N.H. The method eliminates some of the problems such as the initial rapid degradation of small amounts of amorphous cellulose in the paper in the filter paper assay. There is no curling of the filter paper required. A linear correlation exists between the filter paper units and the disc units and a conversion factor of 2.8 is employed to interconvert the units for comparative purposes.

For our work we have followed Mandels and Weber's method (131,242,247) for following cellulase activities with slight modifications.

Carboxymethylcellulase (CMCase) activity (saccharifying activity)

An aliquot of 0.5 ml of appropriately diluted enzyme solution was mixed with 0.5 ml of 1% carboxymethylcellulose (CMC) in 0.05 M citrate buffer, pH 3.3, incubated for 30 min at 50°C. Reducing sugars were measured as glucose equivalents by the DNS method. The reducing sugar production was linear to about 0.2 mg. Enzyme solutions were diluted to give a value of 0.15 to 0.7 mg of reducing sugars. No difference in CMCase values was observed when 0.5% CMC type 50 T (Hercules Powder Co.,

Detaware), the type used by Mandels and Weber (242) and Mandels et al. (131), was used in the assay.

Filter paper activity (FPA)

To 50 mg (rolled 1 x 6 cm strip) of Whatman No. 1 filter paper was added 1 ml of 0.05 M citrate buffer, pH 4.8 and 1 ml of the appropriately diluted enzyme solution. The mixture was incubated for 1 h at 50°C and the liberated reducing sugars were measured as glucose equivalents by the DNS method. Enzyme solutions were diluted to give 0.5 mg reducing sugars. Reducing sugar production was linear to about 0.3 mg glucose. Higher FPA was obtained if the quantity of filter paper was increased.

Xylanase activity

(a) Reducing sugar production

Xylanase activity was assayed according to Bucht and Eriksson (325) with slight modifications. A sample (0.5 ml) oa appropriately diluted enzyme solution was mixed with 0.5 ml of 1% xylan in 0.05 M citrate buffer, pH 4.5, and incubated for 30 min at 65°C. The enzyme solutions were diluted to give 0.5 mg reducing sugars, as xylose [illegible].

(b) Viscometric assay

Decrease in specific viscosity of larch xylan solution [$\eta = (t - t_0)/t_0$; t is the flow time of experimental xylan solution and t_0 the flow time of its solvent] upon the addition of D-xylanase was measured as described by Gorbacheva and Rodionova (247a). The reaction mixture contained 9 ml of 1% larch xylan in 0.05 M citrate buffer, pH 4.5 in a Ostwald viscometer which was thermostated at 65°C for 5 min. One ml of suitably diluted enzyme solution (15 µg) was then added and

decrease in the flow rate time recorded at 3 min intervals.

Beta-glucosidase activity

Beta-glucosidase activity was determined by a modified method of Eberhart (248) using p-nitrophenyl beta-D-glucoside (PNPG) as substrate. The assay mixture contained 0.9 ml of PNPG (3.3 mM) (1 mg/ml) in 0.05 M citrate buffer, pH 4.5, and 0.1 ml of suitably diluted enzyme solution. After incubation at 70°C for 30 min, 1 ml of 2% Na_2CO_3 was added and the amount of p-nitrophenol released was calculated from the absorbancy index of 18.5 cm^2/umol for nitrophenol at 410 nm (249). The amount of substrate should be such as to release p-nitrophenol between 0.01-0.07 umoles PNP

Cellobiase activity

Cellobiase activity was determined by measuring release of glucose from a solution of beta-D(+)-cellobiose by a modified method of Umezurike (250). An aliquot of 0.1 ml of suitably diluted enzyme solution was added to 1 ml (2 or 16 mg/ml) of cellobiose in 0.05 M citrate buffer, pH 4.5, and the mixture was incubated at 65°C for 30 min. The reaction was stopped by heating in a boiling water bath for 5 min. The glucose produced was determined by the glucose-oxidase-peroxidase (GOP) test (232). Glucose production was linear to about 0.1 mg.

Definition of unit of activity

The FPA assay is a measure of the C_1-enzyme and includes the effect of other enzymes in the cellulase complex. Enzyme activities are expressed as micromoles of glucose equivalents produced (or xylose produced from xylan for xylanase and p-nitrophenol from PNPG for beta-glucosidase) per minute per milliliter enzyme solution. The specific activity of the enzyme is defined as the activity per mg of protein

Percentage of saccharification

Percentage of saccharification was determined under conditions similar to those described by Mandels *et al.*(131).

$$\% \text{ saccharification} = \frac{\text{amount of reducing sugars}}{\text{amount of substrate}} \times \frac{162}{180} \times 100$$

The substrate depletion was also followed in some cases by weight loss and by determining the undigested cellulose by the anthrone method (232). At the end of sachaarification, the degree of solubilization was determined by centrifuging the hydrolyzed solution. The residue was washed twice with distilled water, and dried in an oven at 75 - 80°C until constant weight. The percentage difference between this value and the initial solids content represent the degree of solubilization.

Chemical pretreatment of cellulosic materials

Rice straw, sugarcane bagasse, mesta wood, and coniferous wood were milled in the Wiley Mill and the ground material was sieved through a U.S. standard 50-mesh sieve. Pretreatment of lignocellulosics is one of the two major bottlenecks to the economical utilization of this resource. In the present study the pretreatments tried were: incubation with 1 N NaOH at 30°C for 24 h and 48 h, 2 N NaOH at 30°C for 24 h and 48 h, 4 N NaOH at 30°C for 24 h, 5 N NaOH at 30°C for 18 h, steaming with 0.5 N NaOH for 0.5 h and 1 h, autoclaving at 121°C with 0.25 N NaOH for 1 h, heating with 20% peracetic acid in a steamer for 1 h (peracetic acid = acetic anhydride + 35% H_2O_2 (1: v/v)). Mesta wood and coniferous wood powders were also delignified by first teating with 4 N NaOH at 30°C for

24 h and subsequent boiling with 20% peracetic acid and vice versa. The lignocellulosics were treated with delignifying agents at a ratio of 1:10. In all cases the samples were thoroughly washed with water and dried at 45 °C.

Ammonium sulfate precipitation

The culture filtrate was concentrated by precipitation with ammonium sulfate at 90% saturation. After stirring for atleast 1 h in cold (4 °C) the precipitate was collected by centrifugation at 3000 x g for 30 min. The precipitate was suspended in a small volume of 0.05 M citrate buffer, pH 4.8 and stored at -15 °C.

Gel-filtration studies

A column (1.5 x 90 cm) of Bio-gel P-150 for molecular weight determination, (3 x 90 cm) of Sephadex G-75 for purification was equilibrated at 4 °C with 0.05 M citrate buffer, pH 4.5 (molecular weight determination) or pH 4.8 (purification). Hydrated gel and buffer were routinely equilibrated under vacuum prior to use. The eluate was collected in 1 or 2 ml fractions at a flow rate of 8 - 10 ml/h and assayed for protein and/or enzymic activity.

Ion-exchange chromatography

DEAE-Sephadex A-50 anion exchanger was used for purification. The ion-exchanger was equilibrated in 0.05 M phosphate buffer, pH 7.3 and a column (1.8 x 100 cm) was packed. Prior to loading, pH of the enzyme solution was adjusted to 7.3 and dialyzed in collodian bags for 3 - 4 h against 0.05 M phosphate buffer, pH 7.3. The column was washed with the same buffer. The column was eluted with 0.1 M and 0.2 M citrate

buffer, pH 4.5 in a stepwise manner. Fractions (2 ml) were collected at a flow rate of 10 - 12 ml/h and the pH was adjusted immediately to 4.5 with 0.1 M citric acid.

Polyacrylamide gel electrophoresis

Analytical disc gel electrophoresis was performed at pH 8.9 according to Davis (251) and at pH 4.3 according to Maurer (252) using 7.5% acrylamide gel. Sample gel and stacking gel were omitted. A current of 3 mA per gel tube was applied for 3 - 4 h until the dye, bromophenol blue or fuchsin red, reached the bottom of the gel. Protein was visualized with 1% Amido black (in 7% acetic acid) for 20 min or with 0.1% Coomassie brilliant blue (in 7% acetic acid) for 60 min. The destaining of the gel was performed by diffusion in 7% acetic acid.

SDS-gel electrophoresis containing 7.5% acrylamide and 0.1% SDS was carried out as described by Weber and Osborn (253) and Shapiro et al.(254) except that samples after treatment with 1% SDS and 1% 2-mercaptoethanol at pH 7.0 were incubated at 37°C for 4 h and were not dialyzed prior to electrophoresis. The electrophoresis was carried out at 8 mA per tube for 6 h.

Isoelectric focusing in polyacrylamide gel

The procedure described by O'Farrell (255) was adopted for isoelectric focusing in polyacrylamide gel using 0.1 ml of pH 3.5 - 10.0 Ampholine carrier ampholytes (40%) per 10 ml of gel. Gel solutions and anode and cathode solutions were thoroughly degassed prior to use. Gels were run at 200 volts for 1/4 h, 300 volts for 1/2 h and 400 volts for 1/2 h before loading the sample. Samples were dialyzed before loading to remove salts. Electrofocusing was carried out at 400 volts for 12 h

and 800 volts for 1 h. Gels were stained with 0.1% Coomassie brilliant blue in 50% TCA for 1 h and destained in 7% acetic acid or ethanol:acetic acid:water (25:10:65) (256).

Preparative isoelectric focusing

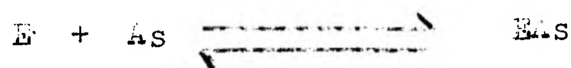
The separations were carried out using 110 ml capacity electrofocusing column (LKB-Produkter AB, Bromma, Sweden), according to the method first described by Vesterberg and Sevensson (257). The density gradient was made with sucrose with an automatic gradient mixer. Dense solution, light solution and electrode solutions were prepared as given in LKB instruction mannual. The sample solutions were dialyzed overnight to reduce the salt concentration. Ampholine carrier ampholytes and sample solutions were mixed in light and dense solution in equal amounts before gradient generation. A run was run with wide range Ampholine carrier ampholytes in pH range 3.5 - 10. Electrodes were placed so that anode was at the bottom of the column. Electrofocusing was carried out at 5°C, for 60 - 72 h. At the end of electrofocusing the voltage was steady at 500 V and the current at 2 mA.

After completion of focusing the column was emptied at a rate of 30 - 40 ml/h, and fractions of approximately 1 ml were collected. Fractions were processed for determination of pH (5 - 7°C), activity and protein. Fractions were made free of sucrose with dialysis in collodian bags.

Determination of n value (Adsorption characteristics of the enzyme on the surface of the substrate

For soluble enzyme and insoluble substrate systems, the description of the kinetics may be written according to McLaren (346) as :

$$E + As \rightleftharpoons EAs$$



where As = surface area of the substrate.

The general distribution law of a solute between two phases I and II is given by Gyani (257a) :

$$C_{II} = KC_{I}^{n}$$

where K = Partition Coefficient and C_I and C_{II} are concentrations in each of the phases, respectively.

For two immiscible liquid phases at an interface, n = 1.

If C_{II} is a solid capable of only surface adsorption, the surface concentration is given by

$$\frac{Ea}{As} = \frac{EI}{V1}^{2/3} = C^{n}$$

i.e. $\frac{\text{No. of moles of E adsorbed}}{\text{Surface area of } C_{II}} = \frac{\text{No. of moles of E in a Vol. V1}}{\text{Volume of Phase I}}$

C is identical with E and Ea/V1 = (EAs)

If K is not unity, we have the "Freundlich equation"

$$\frac{Ea}{As} = KC^{n} \quad \text{where } n = 2/3$$

Gyani (257 a) has also considered adsorption on cracks and edges where upon n = 1/3, and, experimentally,

with both surface and edge adsorption n will expectedly be between 1/3 and 2/3.

Determination of n value

For the determination of 'n' the saccharification of cellulosic materials was carried out at pH 4.5, 50° C for 48 h with different amounts of UV-8 culture filtrate as follows :

2 gm substrate + 1 ml 1M citrate buffer, pH 4.5 + culture filtrate (5, 10, 15, 18 ml) + 0.2 ml merthiolate (0.1%) + water to 20 gm.

The slope of the plot, log (E) versus log (V) where E is the amount of culture filtrate taken and V is the % saccharification at 48 h, gives the 'N' value.

PART III

MUTATION STUDIES AND ENZYME PRODUCTION

CHAPTER 1

SCREENING OF SCLEROTIUM ROLFSII MUTANTS WITH ENHANCED CELLULASE PRODUCTION

INTRODUCTION

One of the important, if not the most important, methods of improving the efficiency of fermentation processes is the production of improved strains by mutation and selection. Strain improvement by mutation and selection depends on the alternate processes of diversification, selection, and rediversification, so that better strains are successfully picked out and further improved. The method used for diversification is mutation. This process involves changes in the nucleus of the organism which leadsto increased productivity. The isolation of mutants is normally greatly facilitated when a uninucleate haploid stage in the life-cycle of a microorganism is available for mutagenic treatment. When a mutation occurs in a uninucleate haploid cell, a pure mutant clone or colony can result. Mutation in a multinucleate haploid cell will give rise to heterokaryon; a recessive mutant gene is marked until an opportunity occurs during subsequent nuclear and cell divisions, for segregation of cells homokaryotic for the mutant gene. For example, Beadle and Tatum (259a) in their classic isolation of autotrophic mutants of Neurospora crassa, irradiated the multinucleate (micro) conidia, then used these conidia to fertilize protoperithecia of an untreated culture of opposite mating type. A small fraction from this nuclear fusion and segregation developed into pure mutant colonies of the required types.

Spontaneous or induced mutants

A mutant, that arises as a result of an event unknown to or uncontrolled by the experiments, is known as 'spontaneous'

mutant on which one cannot rely fully. Use of different mutagens usually results in isolating different 'spectra' of mutants. The principal inducing agents have been those which affect DNA, namely ionizing and ultraviolet radiations and chemical mutagens. Work over the last two decades, with prokaryotes in particular, has greatly increased understanding of how chemical mutagens operate and so has led to their more rational use.

Mutation - inducing agents employed with fungi are illustrated in Table 9.

It is known that no two microorganisms necessarily respond alike to the same mutagenic agencies in the same way. For example, nitrosoguanidine is a most potent chemical mutagen for Escherichia coli and quite effective with S. cerevisiae but it is no more effective with Coprinus lagopus than U.V. - irradiation (260). It is necessary, therefore, to experiment with different mutagenic agents, if work with any fungus not hitherto studied is contemplated.

It is not surprising that all mutagens kill a certain proportion of the treated cells. Some of this killing is due to the induction of 'lethal' mutations (a relative term, since a mutation may be lethal under some conditions - a nutritional mutant on a minimal medium, but not others). It is usually found with all mutagens, however, that an effective yield of mutants is only achieved when the proportion of survivors from the material exposed is very low, say 5 - 10% or less.

TABLE 9: SOME COMMON MUTAGENS WHICH HAVE BEEN USED WITH FUNGI (259)

Mutagen	Typical genera tested with indication of effectiveness					
	Phycomyces	Aspergillus	Yeast	Neurospora	Ustilago	Coprinus
UV-irradiation (254 nm)	+	++	++	++	++	++
gamma- or X-irradiation	±	+	+	+		+
Mustard gas		++		++		
2-AP				+		
DES		+++	+	+		
EMS		+	+++	++		++
NA		+++	++	+++		
NTG	++					++
DEB		++		++		

Key: Mustard gas is used to cover all the nitrogen mustards and related compounds; 2-AP, 2-aminopurine; DES, diethyl sulfate; EMS, ethylmethane sulfonate; NA, nitrous acid; NTG, N-methyl-N'-nitro-N-nitrosoguanidine; DEB, diepoxybutane.

Relative efficacy indicated from ±, least, to +++, most.

Mutagens and mutagenic treatments

(a) Radiations

(i) Ultraviolet light (UV)

Ultraviolet irradiation is frequently a potent mutagen for fungi with hyaline mutable material (spores, mycelium etc.) but not with heavily pigmented or thick-walled material because of absorption losses (260). Mutagenic wavelength lies between about 200 and 300 nm, the peak of absorption by nucleic acids. A low pressure mercury vapour (germicidal) lamp, emits a very high proportion of its energy at 254 nm, close to the most effective wavelength. The relationship between dose and mutation frequency is most usually non-linear, often rising sharply with increasing dose to an optimum and then falling. In routine mutant isolation, it is unnecessary to measure the UV dose in physical units ($ergs/mm^2$); it is enough to plot a survival curve in terms of minutes of exposure to a given UV source under standard conditions, and choose for the induction of mutations an irradiation time giving a suitably low survival, say 0.1 - 1%.

For reproducibility of experiments, and to prevent increases in viable count of spored suspensions, it may be necessary to avoid exposure to visible light during the period immediately following irradiation in order to avoid photo-reactivation, the enzymic reversal of a proportion of the mutagenic and killing effect of a given UV dose.

(ii) Ionizing radiations

Ionizing radiations (X-rays, gamma-rays, neutrons, and other particles) may occasionally have applications in the

routine induction of mutations when other mutagens are ineffectiv but should probably be regarded as a last resort; this is because of the comparatively high likelihood that chromosomal breakage will occur, leading to structural changes like translocations and inversions, which will complicate subsequent genetic analysis of the mutants.

(b) Chemical mutagens

Innumerable compounds are mutagenic, but few are convenient for the routine isolation of mutants (260,261). The best mutagen and set of conditions for its use are not identical for all organisms. In general, chemical mutagens can be grouped into three classes according to their mode of action: those that cause a chemical change in one or more of the nucleic acid bases while they remain *in situ*, in the molecule, and therefore can operate on non-replicating nucleic acids (nitrous acid, alkylating agents, NTG); those that mimic a natural base closely enough to be mistaken for it and inserted into a newly synthesized strand during nucleic acid replication (base analogues); and those that cause loss or addition of one or two bases in DNA, again during its replication of repair (frame-shift mutagens).

(i) Nitrous acid

This mutagen is a rather harmless substance and the mutagenesis is very easily controlled. The mutable material to be treated is usually suspended in acidic buffer (0.1 M acetate, pH 4.5), and nitrous acid is generated by adding a freshly prepared solution of $NaNO_2$ to a final concentration of 0.1 - 0.2 M. After treatment for a suitable time (30 min), the

reaction may be stopped by dilution during plating.

(ii) Ethyl methane sulfonate (EMS)

Of the largest class of alkylating agents, this compound seems to have achieved the greatest use as a routine mutagen. The mutable material is usually suspended in neutral buffer (0.1 M phosphate) for 15 - 30 min and the reaction is stopped by dilution during plating.

(iii) N-methyl-N'-nitro-N-nitrosoguanidine (NTG)

This substance is one of the most potent mutagens known producing under the right conditions, extremely high mutant yields with comparatively little killing. The relationship between NTG concentration and the proportion of mutant survivors in a given time of treatment is complex.

(iv) Base analogues

Base analogues, such as 5-bromouracil (BU) and 2-aminopurine (AP), are less likely to be useful as routine mutagens because suitable conditions of treatment are probably more laborious to establish.

(v) Frame-shift mutagens

Acridines and the derivatives of acridines (ICR compounds) are unlikely to be used in routine mutagenesis.

Mutable material

Many fungi do not produce spores which meet the criteria set out for mutation, the principle variants being wall permeability, pigmentation and nuclear content or the absence of viable spores. Impermeability of spores is usually overcome by employing radiations as the mutagenic treatment. In a similar way, chemical mutagens can be used with pigmented spores

where UV-light might be attenuated by undesirable absorption. But in the case of fungi (from mycelia sterilia group, Sclerotium sp.) which do not produce spores, the mycelium is macerated in a blender to give fragments of preferably 2 - 4 cells in length and these are then treated with the mutagenic agents (262).

Method of mutant isolation

Compact colony procedure: The colonies of filamentous fungi are usually less amenable to plate tests, because they spread rapidly over the agar surface. Mackintosh and Pritchard (263) found that anionic detergents, like sodium dodecyl sulfate and sodium deoxycholate, caused *Aspergillus* to grow in very compact colonies, and the procedure is routinely in use. In the case of *T. viride* Mandels *et al.* (264) used deoxycholate agar to get the compact colonies which were less than 2 mm in diameter, even after long incubation, after the UV irradiation of conidia. Montenecourt and Eveleigh (265) reported that rose bengal and oxgall are suitable to prevent rapid over growth of *T. viride*. While Phosfon D (2,4-dichlorobenzyl tributyl phosphonium chloride), an inhibitor of fungal colony size (266) alone, was found to have little effect on *T. viride* in this respect, oxgall and Phosfon D were found to act synergistically to control fungal spreading (267).

Improvement of the cellulase producing properties of fungal strains using mutagenesis

Economically feasible schemes for the enzymatic saccharification of waste cellulose to yield product useful for fuel intermediates are hindered largely due to the high cost of cellulase production. Enzyme cost is about 60% of the

manufacturing cost (51) when FPA is of the order of 3 - 4 IU/ml in 3 - 4 days fermentation, this is due in part to the presence of repressing substances and end products associated with the fermentation of "waste cellulose". For instance, in the "Trichoderma group", a favoured source of cellulase, the synthesis of all the enzymes of the cellulase complex is repressed by glucose or glucose catabolites (268,269). The use of organisms resistant to catabolite repression would circumvent the repression of cellulase synthesis and allows production of high yields of cellulase in microbial fermentations. Catabolite repression-resistant strains may be helpful in lowering enzyme production costs. A semiquantitative plate assay is described by Montenecourt and Eveleigh (265) for selecting fungal derepressed mutants capable of synthesizing the cellulase complex of enzymes under conditions of catabolite repression (5% glycerol as a repressor). The selection procedure, along with mutagenesis, also included the sequential use of increased substrate concentrations (RUT-M7, 1% cellulose; RUT-NG14, 2% cellulose; S-series, 2.5% cellulose); and also variation in the selective substrate used (RUT-NG14, cellulose; RUT-C30, cellobiose)(270). For the restriction of rapid over growth of the organism, rose bengal and oxgall were used. The M-series of mutants were isolated following UV mutagenesis of spores of T. reesei QM6a using the semi-quantitative plate assay method. Mandels et al.(271) isolated a hyper-cellulolytic mutant QM9123 by irradiating condia of T. reesei QM6a with high energy electrons from a 24 million electron volt, 18 kw linear accelerator at 20°C. Another isolate, QM9136, was also

isolated which resembled the parent strain QM6a in its growth characteristics but did not produce cellulase. Further mutation programme of Mandels (268) led to one hyper-cellulolytic mutant, namely QM9414, and one cellulase-less mutant, QM9977 (271a), after the high voltage electron treatment of QM9123 strain of T. reesei (264). Montenecourt and Eveleigh (267) further selected M-7 strain of M-series for the mutation with nitrosoguanidine according to the procedures of Martinelli and Clutterbuck (272). Under derepressed conditions, mutant NG14 from the NG-series produced approximately five times more filter paper activity than QM9414, twice the endo-beta-glucanase and twice the beta-glucosidase in the liquid culture. But under repressed conditions (in presence of 5% glycerol), the amount of NG14 enzymes is considerably less than was produced in the absence of catabolite repressor. Thus, these mutants of enhanced cellulase productivity were also subject to catabolite repression. Palva and Nevalainen (273) isolated a strain VTT304 which produced 30 - 40% more cellulase yields from the spontaneous mutation of QM9414 strain of T. reesei. A hyper-cellulolytic and catabolite repression-resistant (8% glycerol) strain MCG-77 was obtained by ultraviolet mutagenesis of the T. reesei TK041 strain. This was kabicidin-resistant strain developed after UV irradiation of QM9414 (274). The MCG-77 strain gave yields similar to the other enhanced cellulase producing strains QM9414 and NG14 in regular cellulase fermentations. Similarly Montenecourt and Eveleigh (275) isolated a mutant RUT-C30 which produced enhanced cellulase and showed increased thermal stability and resistance to catabolite repression, after the UV

irradiation of NG14 strain.*

Enzymatic conversion of cellulose to glucose on a commercial scale requires high saccharification efficiencies. However, high conversion efficiencies cannot be achieved due to end product inhibition of the enzymes. Cellobiose is a strong end-product inhibitor and inhibits both the endo-glucanase and the cellobiohydrolase (193,207,209,276-278) while glucose is competitive inhibitor of beta-glucosidase in *Trichoderma* (279,280). A number of end-product inhibition resistant mutants were isolated as EPI series (281), but no completely resistant strain was found, though several mutants synthesize a beta-glucosidase with altered inhibition kinetics with respect to glucose.

Other than *T. reesei*, only few organisms were studied for hyperproduction of cellulase and hemicellulase enzymes (171, 282-284). Menezes (282) isolated a mutant of *Rhizopus* sp. after UV irradiation of spores of parent strain, producing 6-fold higher amounts of xylanase than the parent strain. In the case of *Streptomyces fradiae*, Harish (283) reported a high xylanase-producing isolate after UV irradiation of the parent strain. By subjecting *Aspergillus terreus* (IJIRA6.2) spores to X-ray irradiation for 45 min, Bhattacharya and Dutta (284) isolated a mutant which gave high cellulase enzyme yield. A hyperactive cellulase mutant of *A. fumigatus* producing 8-fold higher C_x activity than the parent strain was obtained after treatment of *A. fumigatus* spores with NTG (1 mg/ml) for 90 min (285). A UV-8 mutant strain of *S. rolfsii* CPC-142 secreting about two times more filter paper activity in NM-2

growth medium in submerged cultures than the parent strain was obtained by us by UV mutagenesis of crushed sclerotia (171).

Until recently, improvement of industrial microorganisms has relied mainly on programmes of mutation and selection. This situation is purely empirical and such mutagenic treatments sometimes induce structural damage to the genome unrelated to the desired improvement. In an effort to allow recombination of important characteristics from different mutants into a single strain, alternative genetic techniques are being increasingly employed which circumvent continued mutation. Selected breeding employing the parasexual cycle and protoplast fusion in order to sequester desired characteristics into a single strain is being developed (281). Marked strains of *T. reesei* carrying abnormal spore color, antibiotic resistance and auxotrophic nutritional requirements were isolated from the different Rutgers mutant lines to facilitate such genetic analysis.

Mutation studies of *S. rolfsii* CPC-142

A high cellulase-producing *S. rolfsii* isolate CPC-142 (previously described as a basidiomycete), comparable to *T. reesei* was previously described by Sadana and Shewale (169). The *S. rolfsii* produced predominantly glucose from cellulose, with negligible amounts of cellobiose (286). In contrast, *T. reesei* culture filtrates produced mainly cellobiose, with smaller amounts of glucose, probably because the amount of cellobiase in the *T. reesei* system was low (163).

The results presented in this Chapter have been published (171).

Mutable material from *S. rolfsii*

As *S. rolfsii* is from mycelia sterilia group, it does not produce any spores. The sclerotial bodies of parent strain were used as a mutable material after maceration in a sterile mortar with pestle.

Isolation of the UV mutant of *S. rolfsii*

Eight to ten sclerotia were suspended in distilled water and ground with a sterile mortar and pestle. The suspension was transferred to a sterile petri dish and exposed to UV irradiation (Philips UV germicidal lamp, 15 V) at a distance of 20 cm for different intervals of time (Table 10). The suspension was then plated on PDA containing 0.08% sodium dodecyl sulfate and incubated for 48 h at 30°C. Growth was greatly restricted, and the colony diameter did not exceed 3.0 to 3.5 mm. About 5 to 10 colonies were observed per plate, and the colonies were of three morphological types. Colonies were transferred to PDA slants and tested for the production of cellulase in submerged culture in NM-2 medium. From all the mutants tested (Table 10), we found UV-8 mutant showed hyper-cellulolytic activities. Further mutation in UV-8 strain did not produce any mutant showing further enhanced cellulolytic activities.

Isolation of the NTG mutant of *S. rolfsii*

Eight to ten sclerotia were suspended in 5 ml of distilled water and ground with a sterile mortar and pestle. The suspension was transferred to a solution (5 ml) of NTG giving final concentration of NTG as mentioned (Table 11). The mixture was incubated at 30°C for 20 min on a shaker (150 rpm).

TABLE 10: SCREENING FOR THE MUTANTS OF *S. ROLFSII*

Method	Organism No.	pH[a]	FPA IU/ml	CMCase IU/ml	Xylanase IU/ml	PNPGase IU/ml
UV-irradiation at a distance of 20 cm for 20 min.	Parent strain CPC-142[b]	2.75	0.80	108	86	16
	UV-1	2.85	1.38	190	ND	16.3
	UV-2	2.88	1.38	190	ND	17
	UV-3	2.65	1.30	180	ND	15
	UV-4	3.10	1.75	200	ND	17.5
	UV-5	2.15	1.70	180	ND	17
	UV-6	3.10	1.50	140	ND	17
	UV-7	2.60	0.92	100	ND	16.3
	UV-8	3.00	1.85	200	ND	19.5
	UV-9	2.95	1.20	90	ND	16.5
	UV-10	2.70	1.15	ND	ND	ND
	UV-11	2.70	1.30	ND	ND	ND
	UV-12	2.60	0.70	ND	ND	ND
	UV-13	2.50	0.30	ND	ND	ND
	UV-14	2.60	0.92	ND	ND	ND
	UV-15	2.80	1.38	ND	ND	ND
	UV-16	2.70	1.15	ND	ND	ND
	UV-17	2.75	1.24	ND	ND	ND
	UV-18	2.90	1.52	ND	ND	ND
	UV-19	2.90	1.61	ND	ND	ND
	UV-20	2.70	1.15	128	104	14.1
	UV-21	2.70	1.20	130	108	12
	UV-22	2.65	1.20	140	104	12

[a]Grown in NM-2 medium for 14 days.

[b]Crushed sclerotia of CPC-142 were used for mutagenesis.

TABLE 11: SCREENING FOR THE MUTANTS OF PARENT AND UV-8 STRAIN OF S. ROLFSII

Method	Organism No.	pH[a]	FPA IU/ml	CMCase IU/ml	Xylanase IU/ml	PNPGase IU/ml
NTG treatment 1 mg/ml for 20 min.	Parent strain CPC-142[b]	2.75	0.80	108	86	16
	NTG-1	2.90	1.66	130	104	15
	NTG-2	2.90	1.38	120	104	15
	NTG-3	2.80	1.30	119	82	12
	NTG-4	2.80	1.15	147	82	11
NTG treatment 3 mg/ml for 20 min.	NTG-5	2.80	0.70	73.6	89.9	15
	NTG-6	2.80	0.70	74.0	61.0	12
	NTG-7	3.00	0.72	119.6	138	16
NTG treatment 5 mg/ml for 20 min.	UV-8[b]	3.00	1.85	200	164	19.5
	NG-1	2.90	1.40	184	104	17
	NG-2	2.85	1.35	120	109	18
	NG-3	2.85	1.15	120	104	13
	NG-4	2.85	1.15	150	140	14
	NG-5	2.90	1.20	150	177	18

[a]Grown in NM-2 medium for 14 days.

[b]Crushed sclerotia of CPC-142 and UV-8 were used for mutagenesis.

The supernatant was removed and sterile distilled water was added. In a similar way, 2 - 3 washings of the treated material were carried out. Then the suspension was plated on PDA containing 0.08% sodium dodecyl sulfate and incubated for 48 h, 30°C. The colonies developed were transferred to PDA slants and then tested for the production of cellulase in submerged culture in NM-2 medium. From all the mutants tested (Table 11), the NTG mutants isolated produced comparatively lower activities of cellulase as compared to UV-8 mutant.

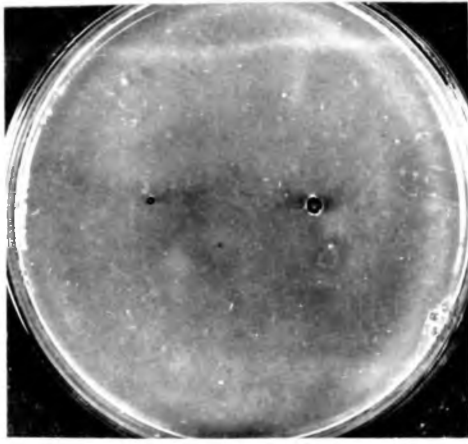
Morphological characteristics of UV-8 mutant and the parent strain

The morphological and cultural characteristics of the parent strain and UV-8 mutant differ in many respect and are as described below:

The parent strain formed a white, dense, aerial felt of mycelium; mature sclerotia are brown and 1.2 to 1.4 mm in diameter. The UV-8 mutant formed a velvety, matted, white growth; sclerotia are 2.4 to 2.5 mm in diameter. Sclerotia of the parent strain germinate slowly (36 to 40 h) as compared with those of the mutant (18 to 20 h) (Fig. 11) (171). Growth of the parent strain was more rapid and luxurious than growth of the mutant on potato-dextrose-agar, Although the parent strain filled the entire plate (10 cm dia.) in 100 to 105 h at 30°C, the width of the mutant colony was only 6.0 to 6.5 cm (Fig. 11c). Growth of the mutant however, was more dense and compact than that of the parent strain.

In shake flasks, the mutant and the parent strain formed dense mycelia. On starch, glucose or cellobiose, growth was

FIG. 11: Comparison of germination and growth charactistics of S. rolfsii CPC 142 parent strain (left side of a, b, c, d) and UV-8 mutant (right side of a,b,c,d) on potato-dextrose agar at 30°C. One sclerotium was used as the inoculum. (a) Parent and mutant at 24 h; (b) Parent and mutant at 48 h; (c) Parent and mutant at 72 h; (d) Parent and mutant at 96 h; (e) Parent at 105 h; and (f) Mutant at 105 h.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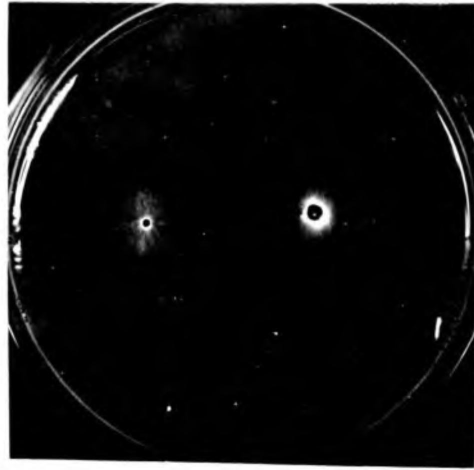
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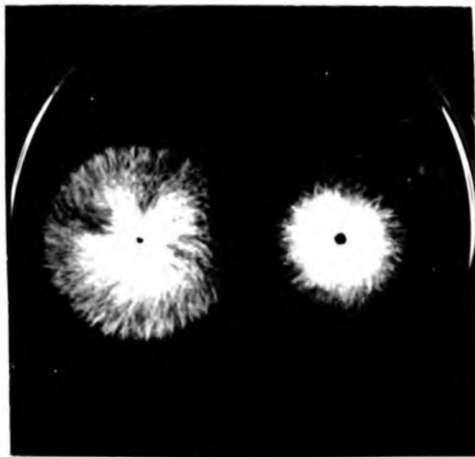
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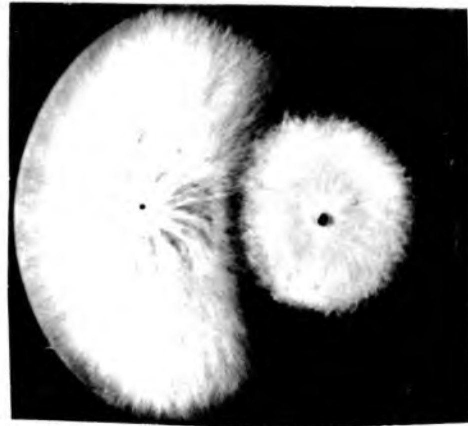
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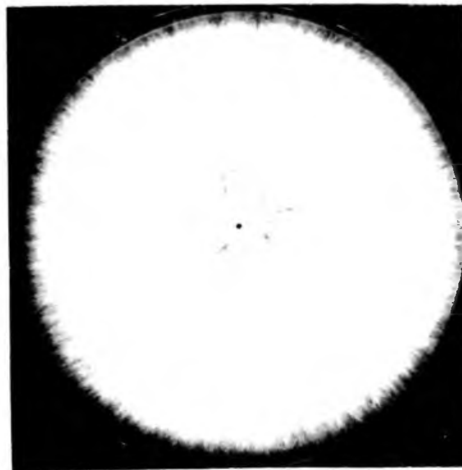
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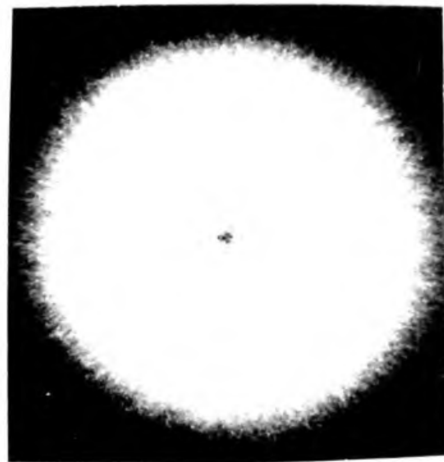
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rapid and the substrates (0.5%) were consumed within 4 to 6 days. Little or no cellulase or beta-glucosidase is synthesized, either by the mutant or by the parent strain, when these substrates were the sole carbon source. No growth was observed on 1% lactose, but both the mutant and parent strain utilize glycerol (5%) slowly. The pH decreased during growth on glycerol, and the addition of 5% glycerol to NM-2 medium decreased the formation of cellulase and beta-glucosidase by both cultures (171).

Maintenance and preservation of *S. rolfsii*

The selection of the microorganisms used in fermentation processes and the methods used for the maintenance of these organisms are among the most important decisions that have to be made in designing an industrial fermentation process (287). Hesseltine and Haynes (288) have listed the general attributes that microbial cultures must have if the processes they generate are to be operable.

These include:

(1) The strains must be genetically stable;

(2) The strains should be readily maintained for reasonably long periods of time.

(3) The strains must readily produce many vegetative cells, spores, or other reproductive units.

(4) The strains should be pure and rapidly growing.

Various methods of maintenance employed are designed to minimize the hazards to which the cultures are exposed. The repeated transfer is usually avoided to lessen the selection of a mutant or non-sporulating wild strain; secondly, some

strains sometimes tend to become attenuated under the artificial conditions of culture (especially true for pathological material).

There are three basic methods for maintenance which seem to be generally used and each has several variations (289). These include: (a) storing organisms on agar slants or in menstra where respiration and metabolism may be limited, (b) drying organisms on soil or some other solid materials, and (c) lyophilization, i.e., removal of water from the cells.

Storage

(i) At room temperature: The simplest method of storage is to keep the cultures at room temperature (290). These are best protected from dust or aerial contamination by placing in a suitable wooden box. Wood being a poor conductor, the cultures are unlikely to be subjected to rapid changes of temperature. Cultures stored at room temperature tend to dry out rapidly depending on the climate. It is therefore necessary to make transfer to fresh medium every two months (291).

(ii) Refrigeration or cold storage: An easy method of storing cultures is at 5 - 8°C in a refrigerator, Under these conditions, the rate of growth of aerial mycelium and drying out are reduced. The interval between transfers would be longer than at room temperature and a period of 6 - 12 months is usual (292).

(iii) Deep freeze: There have been several reports (293-296) of cultures being successfully preserved by placing in a deep freeze at temperatures of about -10 to -20°C, with prolonged life. But one of the main objections to deep freeze storing is the risk of rupture of the cell walls of the cultures due to

freezing and thawing.

(iv) Mineral oil: Healthy cultures can be covered with mineral oil, and these will survive for long periods, growing at a very reduced rate. This method of preservation is cheap, easy and requires no special apparatus or skill. It was first extensively used by Buell and Weston (297). The paraffin oil must be autoclaved at 15 lbs/sq.in. or heated in the oven at 170°C for 1 h. The depth of the oil from the top of the slant should be 1 cm and is fairly critical (291) as the oxygen transmission by layers of mineral oil in excess of 1 cm becomes less favourable. If less oil is used strands of mycelium may be exposed and thus the cultures may get dried. The method depends on reduced rate of metabolism and prevention of drying, however, the metabolism is not completely arrested. The oil cultures can be stored at room temperature in a refrigerator or cold room (300).

(v) Soil: Preservation of fungi in soil can be of two kinds. Inoculation of soil with a spore suspension and a growth on this soil and final preservation of the culture (301,302), or the placing of dry sclerotia or spores in dry soil or similar substrate such as vermiculate and subsequent storage of this dry material (291). The period of survival is greater than on agar and strains remain typical.

(vi) Lyophilization or freeze drying: Lyophilization or freeze drying as a method for the preservation of micro-organisms consists of drying cultures or a spore suspension from the frozen state under reduced pressure (303). When cells are dried under these conditions they remain dormant for

long periods. On reconstitution and return to normal media, these usually grow well. The process of lyophilization was first applied to microfungi on a large scale by Raper and Alexander (304).

The material is distrubuted in small quantities in ampoules using sterile technique. The ampoules are frozen by immersing in a freezing mixture (dry ice and ethyl acetate) and then dried in a vacuum desicator under vacuum usually containing a desiccant; such as P_2O_5, silica gel. When the material is completely dry, the ampoules are sealed. The dried sealed ampoules can be stored at room temperature, 4 - 8°C (refrigeration) or at -15°C in deep freeze (305).

From the various methods of preservation and maintenance of microorganisms (289), some methods were tested for the maintenance of S. rolfsii parent and UV-8 mutant strains. The viability, the morphological and physiological (production of cellulases, xylanase and beta-glucosidase) criteria were studied after one year storage. As the S. rolfsii strain does not produce any asexual or sexual spores in its imperfect stage, the sclerotial bodies were used as a material for preservation. After one year of storage the preserved material in respective methods (Table 12) was transferred to PDA, and after 7 days incubation at 30°C, this was used as an inoculum for the study of cellulase production in NM-2 medium.

From Table 12, it can be observed that lyophilization is the best method for the preservation of both wild and UV-8 mutant strains of S. rolfsii as it did not affect the cellulase production in NM-2 medium in 14 days.

TABLE 12: PRODUCTION OF CELLULASE, XYLANASE AND BETA-GLUCOSIDASE BY S. ROLFSII STRAINS AFTER ONE YEAR STORAGE

Strain	Method of maintenance[a]	pH[b]	FPA IU/ml	CMCase IU/ml	Xylanase IU/ml	PNPGase IU/ml
Wild	Weekly transfer (control)	2.80	0.75	108	85	16
	On agar at R.T.	2.60	0.60	85	78	14
	On agar at 8°C	2.70	0.70	90	85	13.5
	Under paraffin oil	2.65	0.70	93	82	14
	In soil	2.60	0.58	82	84	14
	After lyophilization					
	i) at R.T.	2.70	0.70	110	80	15
	ii) at 8°C	2.75	0.70	110	82	16
	iii) at -15°C	2.75	0.75	115	85	14.5
Mutant UV-8	Weekly transfer (control)	3.10	1.84	197	180	18
	On agar at R.T.	2.95	1.38	120	130	11
	On agar at 8°C	2.95	1.52	140	150	13.5
	Under paraffin oil	2.80	1.30	69	70	6.5
	In soil	2.80	1.30	69	70	6
	After lyophilization					
	i) at R.T.	3.00	1.52	138	120	10
	ii) at 8°C	3.00	1.62	150	140	13
	iii) at -15°C	3.10	1.75	170	150	15

[a]Studied after one year storage in respective forms.

[b]Growth in NM-2 medium, 14 days.

CHAPTER 2

CELLULASE, XYLANASE, AND BETA-GLUCOSIDASE PRODUCTION BY *S. ROLFSII* UV-8 MUTANT

SUMMARY

The optimization of cellulase, hemicellulase and beta-glucosidase production by *S. rolfsii* mutant was studied in shake flasks and compared with the parent strain and *T. reesei* (and its mutants). The UV-8 mutant produced an active cellulase and hemicellulase. It also produced larger amounts of beta-glucosidase as compared to the parent strain.

The cellulase, hemicellulase and beta-glucosidase were obtained in good yield only when cellulose was the carbon source. Only traces of cellulase, xylanase and beta-glucosidase were formed on glucose, fructose, maltose and cellobiose although good growth was obtained on these substrates. These enzymes were not induced on lactose and mannitol and growth was poor on these substrates. Cellobiose octaacetate was a less effective inducer of cellulase, xylanase and beta-glucosidase than was cellulose.

INTRODUCTION

Cellulose and hemicellulose occur in abundance in nature and constitute one third to one-half of the approximately 150 billion tonnes of organic materials that are photosynthesized annually (306). The commercial possibility of using cellulase preparations to produce glucose, alcohol and protein from cellulose is under intensive study (22,44,131,136,307,308). Similarly, xylanases which hydrolyze xylan, are also under extensive study. Xylose itself can be used as a fermentation substrate; raw material for single cell protein, or feedstock for the production of xylitol, a very sweet sugar alcohol (309) which is finding extensive use as a sweetening agent.

In the hydrolysis of crystalline cellulose, three kinds of enzymes are believed to be involved: endo-beta-1,4-glucanase, exo-beta-1,4-glucanase (beta-(1 ⟶ 4)glucan cellobiohydrolase and beta-(1 ⟶ 4)-glucan glucohydrolase), and beta-glucosidase (164,167,168,182,183,193,207,225). A strong synergistic effect has been observed between endo- and exo-glucanases in the hydrolysis of crystalline cellulose (Avicel), with cellobiose as the major product released (220). Beta-glucosidase hydrolyzes cellobiose and short chain cellulooligosaccharides to glucose but does not degrade cellulose (164). For commercial enzymatic saccharification, the production of both types of cellulases and beta-glucosidase in high yields is essential.

Similarly, in the degradation of (1 ⟶ 3 and 1 ⟶ 4)-beta-D-xylans, exo- and endo-xylanases and beta-D-xylosidase are believed to be involved giving the end product xylose,

in the saccharification of lignocellulosic materials (310).

Organisms that have been reported to elaborate high cellulase activities for better saccharification are: T. reesei, T. lignorum, T. koningii (131,165,167,193,207,242,311,312), S. pulverulentum (60,220), F. solani (166), Penicillium iriensis (313), P. funiculosum (167,310), S. rolfsii (169,310). This study examines cellulase beta-glucosidase and xylanase production by S. rolfsii UV-8 mutant. Cultural conditions have been varied for obtaining high yields of cellulase and xylanase. From a comparison made with other fungi (131,247,314), it is apparent that this culture produces large amounts of cellulase and xylanase. In addition, the S. rolfsii UV-8 mutant secretes high amounts of cellobiase in contrast to T. reesei (171,315). This enzyme plays an important part in maximizing the rate of cellulose hydrolysis (61,163).

The results presented in this Chapter have been published (171,315).

RESULTS AND DISCUSSION

Cultural conditions for the maximization of cellulase, xylanase and beta-glucosidase production

Increase in cellulase, xylanase and beta-glucosidase yields have been obtained by optimizing cultural conditions. All experiments have been repeated at least twice with similar results.

Influence of inorganic nitrogen sources

The effect of replacing $(NH_4)_2SO_4$ with alternate sources of inorganic nitrogen in T. reesei medium, at equivalent or two to five times higher concentrations, on the production of

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extracellular cellulase, xylanase and beta-glucosidase is shown in Table 13. As can be seen from Table 13, addition of $(NH_4)_2HPO_4$ at five times higher nitrogen levels in place of $(NH_4)_2SO_4$ to T. reesei medium showed significant enhancement in the yields of cellulase, xylanase and beta-glucosidase. Increasing $(NH_4)_2SO_4$ level had no effect on the production of these enzymes. Maximum yields were obtained with 0.7% $(NH_4)_2HPO_4$. In subsequent studies 0.7% $(NH_4)_2HPO_4$ was used as the inorganic nitrogen source. This medium is designated as NM-1. Increasing the urea or peptone level in the T. reesei medium or NM-1 medium did not increase the FPA, xylanase or beta-glucosidase level but there is an appreciable increase in CMCase activity.

Influence of cellulosic substrate on the production of cellulase, xylanase and beta-glucosidase

The production of cellulase, xylanase and beta-glucosidase was compared when S. rolfsii UV-8 mutant was grown on various cellulosic substrates (Table 14). The highest amounts of cellulase, xylanase and beta-glucosidase activities were induced on Cellulose-123. This substrate was superior to cotton for enzyme production. Fenicksova et al. (316) and Mandels (247) reported cotton was a poor substrate for cellulase production for T. reesei (QM6a and QM9123) except for T. reesei QM9414. In contrast, Horton and Keen (339) reported that cotton, which resists enzymatic hydrolysis to a greater extent than chemically treated celluloses resulted in higher cellulase yields. Wood has a much more complex structure and both coniferous wood and mesta wood (Hibiscus cannabinus) powders as well as sugarcane bagasse supported little growth and

TABLE 13: PRODUCTION OF CELLULASE, HEMICELLULASE AND BETA-GLUCOSIDASE BY S. ROLFSII UV-8 MUTANT GROWN IN MEDIA CONTAINING DIFFERENT INORGANIC NITROGEN SOURCES

Inorganic nitrogen source	pH[a]	Cellulase IU/ml		Xylanase	beta-Glucosidase
		CMCase	FPA	IU/ml	IU/ml
$(NH_4)_2SO_4$[b] (0.14 or 0.7%)	2.9	60	0.6	26	3.0
KNO_3 (0.23%)[b]	2.9	12	0.3	15	3.0
$NaNO_3$ (0.28%)[b]	2.8	11	0.3	5	1.1
NH_4NO_3 (0.18%)[b]	2.8	44	0.6	23	4.0
NH_4Cl (0.12%)[b]	2.7	32	0.4	20	4.5
$(NH_4)_2HPO_4$[b]					
(0.14%)	2.9	30	0.6	30	5.3
(0.28%)	3.0	50	0.8	45	8.5
(0.42%)	3.0	80	1.4	65	13
(0.7%)	3.1	105	1.6	110	15

Grown on $(NH_4)_2SO_4$-free *T. viride* medium plus inorganic nitrogen source indicated with 2% cellulose.

[a]pH of culture filtrate on 14th day.

[b]Nitrogen source added at equivalent nitrogen levels.

TABLE 14: EFFECT OF CELLULOSE SUBSTRATE ON ENZYME PRODUCTION BY S. ROLFSII UV-8 MUTANT

Cellulose source	pH[a]	Cellulase IU/ml		Xylanase	Beta-Glucosidase
		CMCase	FPA	IU/ml	IU/ml
Cellulose-123	3.1	105	1.61	110	15
Avicel P.H. 101	3.2	85	0.7	70	14
Cotton absorbent	3.6	18	0.2	20	1.2
Cotton sliver	3.5	13	0.05	15	1.2
Solka Floc SW 40	3.5	92	1.4	85	15
Coniferous wood	3.6	5	0.02	12	0.4
Mesta wood (*Hibiscus cannabinus*)	3.4	5	0.04	12	0.4
Bagasse	3.2	6.5	0.13	15	0.35
Rice straw	3.2	30	0.29	40	2.0
Coniferous wood[b]	3.4	32	0.18	22	1.2
Mesta wood[b]	3.5	35	0.18	22	1.2
Bagasse[b]	3.5	25	0.4	28	4
Rice straw[c]	2.8	35	0.36	40	3.0
CMC	3.7	11	0.18	18	1.2

Grown on NM-1 medium with 2% cellulose source indicated. Wood powders, rice straw and bagasse were passed through 50 mesh standard sieve after grinding in Wiley mill. The alkali treated cellulosics were made free of alkali by washing with water and dried at 45°C.

[a]pH of culture filtrate on 14th day.

[b]Steamed for 1 h with 4 N NaOH.

[c]Autoclaved with 0.25 N NaOH at 121°C for 1 h.

consequently enzyme production was very low. The highly lignified cellulosics produced little more amounts of enzymes only after they had been pretreated with alkali. Cellulase, xylanase and beta-glucosidase production with alkali-treated (AT) bagasse, AT-rice straw and AT-wood powders, was 25 - 30% of that obtained with Cellulose-123. With CMC, the formation of these enzymes was low.

Effect of cellulose concentration on cellulase, xylanase and beta-glucosidase production

The influence of different levels of cellulose in the NM-1 medium on the production of cellulase, xylanase and beta-glucosidase is shown in Table 15. There was a two to three-fold increase in cellulase, xylanase and beta-glucosidase production when the cellulose concentration of the medium was increased from 0.5 - 3%. No further increase in the yield of the enzyme was observed when the cellulose concentration was further increased. In all subsequent studies, a cellulose concentration of 3% was used (NM-2 medium).

Effect of carbon source on cellulase, xylanase and beta-glucosidase production

Cellulases in fungi are produced on cellulose or on related glucans, containing beta-1,4-linkages, on soluble cellodextrins including cellobiose and on certain glucose trimers (131,227, 312,316-319,339). It has been suggested that cellobiose at low concentration is the true inducer for cellulase (227,319), but at high concentrations it represses cellulase formation (227,318,320). The precise role of cellobiose in induction, however, has not been clarified. With *T. reesei* cultures, it

TABLE 15: EFFECT OF DIFFERENT CONCENTRATIONS OF CELLULOSE-123 ON CELLULASE, HEMICELLULASE AND BETA-GLUCOSIDASE PRODUCTION BY S. ROLFSII UV-8 MUTANT

Cellulose-123	pH[a]	Cellulase IU/ml		Xylanase	beta-Glucosidase
		CMCase	FPA	IU/ml	IU/ml
0.5	3.3	40	0.8	45	6
1.0	3.2	60	1.0	66	10
2.0	3.1	105	1.6	105	16
3.0	3.1	120	1.8	120	28
4.0	3.1	120	1.8	120	20

Grown on NM-1 medium with Cellulose-123 indicated.

[a]pH of culture filtrate on 14th day.

has been reported that good yields of cellulase were obtained only at high cellobiose concentrations (1% or more), by growing under suboptimal conditions such as restricted aeration, suboptimal temperature, or a marginal nutrient deficiency (318). Sternberg (321) suggested that induction of cellulase may not be affected by cellobiose directly but rather by its products. A soluble inducer has been recovered from filtrates of Trichoderma cultures which have been grown on 1 - 3% cellobiose (318). Thus the nature of the true inducer of cellulase is still unknown. Brail and Kushner (340) and Hofsten (323) suggested that cellulase induction depends on contact of the cell surface with an insoluble or soluble poly-beta-glucoside polymer. Cellulase is constitutive in Pseudomonas fluorescens (322).

The role of glucose in the induction and repression of cellulase production in fungi has also been studied by many investigators (227,269,340). It is commonly observed that glucose represses the formation of CMCase, (269,340). The slight inducing effect of industrial glucose observed in T. reesei is presumably caused by sophorose formed during acid hydrolysis of starch (227,324).

The effect of addition of different carbon sources on the production of cellulase, xylanase and beta-glucosidase is reported in Table 16. It may be seen from Table 16 that S. rolfsii UV-8 mutant produces cellulase, xylanase and beta-glucosidase when grown on cellulose but little or no enzyme with cellobiose, glucose, fructose, maltose, lactose, mannitol or glycerol as the sole carbon source. Glucose, fructose, maltose and cellobiose supported good growth but there was

TABLE 16: EFFECT OF DIFFERENT CARBON SOURCES ON ENZYME PRODUCTION BY S. ROLFSII UV-8 MUTANT

Growth substrate	pH[a]	Cellulase IU/ml		Xylanase	beta-Glucosidase IU/ml
		CMCase	FPA	IU/ml	
Glucose (0.5 or 1%)	2.8	3.6	0.04	6	1.4
Fructose (0.5 or 1%)	2.55	3.6	0.05	11	0.8
Maltose (0.5 or 1%)	2.85	3.7	0.04	7	0.6
Lactose (0.5 or 1%)	6.1	-	-	-	-
Mannitol (0.5 or 1%)	6.0	1.84	0.007	1.2	0.6
Glycerol 0.5%	4.1	1.9	0.002	1.3	0.6
Glycerol 1%	2.7	2.7	0.002	1.5	0.6
Cellobiose 0.5%	2.8	2.1	0.036	2.2	0.6
Cellobiose 1%	2.7	3.9	0.05	11	1.2
D-Cellobiose octaacetate					
(0.1%)	3.8	3.7	0.05	1.7	0.8
(0.5%)	3.7	3.7	0.06	7.1	1.05
(3%)	3.7	18	0.37	15	3.0
Cellulose-123					
(0.5%)	3.3	39	0.8	44.5	7
(3%)	3.1	119	1.8	115	21.5

Grown on MM-1 medium (without cellulose) with the carbon source indicated.

[a]pH of culture filtrate on 14th day.

little or no growth on glycerol, mannitol or lactose. The lack of cellulase production when glucose, cellobiose or fructose were used as the sole carbon source has been reported by other investigators (325,326). However, there are reports of cellulase formation when Trichoderma sp. (316) or Polyporous schweinitzii (327) was grown on various sugars. With cellobiose octaacetate as the sole carbon source, the levels of cellulase, xylanase and beta-glucosidase were little bit higher than that obtained with cellobiose. Similar observations, have been reported with T. reesei and this has been explained to be due to the gradual release of cellobiose by esterase activity that slowly hydrolyzes cellobiose octaacetate during the growth of the organism (320). With all carbon sources tested, cellulase, xylanase and beta-glucosidase activities were produced coordinately.

Effect of surfactant on enzyme production

The addition of surfactants has been reported to increase extracellular enzyme production in a variety of microorganisms (320). The effect of surfactants has been attributed (i) to their action causing an increase in cell membrane permeability (320), (ii) partly to inducer formation and partly to the promotion of enzyme release (312) and (iii) to decrease in growth caused by a limited supply of O_2 (317).

The effect of the addition of surfactants to S. rolfsii UV-8 mutant culture medium is shown in Table 17. Tween-80 (polyoxyethylene sorbitan monooleate) at a level of 0.033% gave the highest enzyme activity, its addition to the medium increased the yield of cellulase and xylanase and beta-

TABLE 17: EFFECT OF SURFACTANT ON CELLULASE, HEMICELLULASE AND BETA-GLUCOSIDASE PRODUCTION BY S. ROLFSII UV-8 MUTANT

Surfactant	pH[a]	Cellulase IU/ml		Xylanase	Beta-Glucosidase
		CMCase	FPA	IU/ml	IU/ml
None	3.1	36	0.32	28	5
Tween 80 0.1%	2.7	40	0.5	44	7
Tween 80 0.05%	2.8	60	1.01	60	10
Tween 80 0.033%	3.0	118	1.8	109	22
Tween 80 0.033%[b]	2.9	80	1.16	82	16
Tween 20 0.033%	2.8	83	1.02	72	10
Triton X-100 0.033%	5.9	4	0.025	3	0.4

Grown on surfactant-free NM-2 medium with surfactant indicated.

[a]pH of the culture filtrate on 14th day.

[b]Surfactant added after 24 h of incubation.

glucosidase 3 - 5 fold. Tween 80 has been consistant in this respect and is independent of the history of the mycelium. Reese and Maguire (328) have reported that Tween 80 and Tween 40 (polyoxyethylene (20) sorbitan monopalmitate) doubled the cellulase yield in Trichoderma. Tween 20 (polyoxyethylene sorbitan monolaurate) was also effective in promoting enzyme production with UV-8 mutant but to a lesser degree. Triton X-100 (alkylphenylpolyethyleneglycol) at 0.033% was ionic and reduced both enzyme production and growth. The addition of Tween 80 gave better yields when added to the culture before inoculation as compared to its addition 24 h after inoculation. The surfactant was therefore sterilized with the medium.

Effect of adding increased amounts of trace metals and other constituents of media to NM-2 medium

No significant increase in cellulase, xylanase and beta-glucosidase levels was observed by incorporating enhanced levels (2 to 3 fold) of trace metals (Fe^{++}, Co^{++}, Zn^{++}, Mn^{++}), KH_2PO_4, $MgSO_4$, $CaCl_2$. Incorporation of $CuSO_4$ (0.005 - 0.03%) or $CaCO_3$ (0.01 - 0.03%) likewise had no effect. Abhushama and Kambal (329) have suggested that zinc functions as an activator of the culture.

Influence of organic substrates on cellulase, xylanase and beta-glucosidase production

The effect of addition of various organic substrates to the NM-2 medium on cellulase, xylanase and beta-glucosidase production was investigated and is shown in Table 18. The addition of corn steep liquor (2%) increased the yields of cellulase, xylanase and beta-glucosidase. The maximum

TABLE 18: EFFECT OF ORGANIC SUBSTRATES ON ENZYME PRODUCTION BY S. ROLFSII UV-8 MUTANT

Organic substrate	pH[a]	Cellulase IU/ml		Xylanase	beta-Glucosidase
		CMCase	FPA	IU/ml	IU/ml
None	3.0	121	1.8	115	21
Urea (0.03%)	3.0	165	1.8	120	21
Yeast extract (0.02%)	3.2	180	1.8	122	22
Proteose peptone (0.025%)	3.3	180	1.85	122	22.1
Corn steep liquor (1%)	3.2	181	1.8	130	21
(2%)	3.2	200	2.0	200	23
(3 or 4%)	3.2	184	1.9	150	23
Wheat bran (3 or 4%)	3.1	190	1.5	130	22
Rice bran (2%)	3.0	190	1.8	120	23
Rice bran (3 or 4%)	3.1	194	1.9	130	23

Grown on NM-2 medium with addition of organic substrate as indicated.

[a]pH of culture filtrate on 14th day.

activities obtained were: CMCase 170 - 200 IU/ml, FPA 1.75 - 2.1 IU/ml, xylanase 170 - 200 IU/ml, beta-glucosidase 20 - 23 IU/ml and cellobiase 18 - 23 IU/ml (315). The NM-2 medium containing 2% corn steep liquor is designated as NM-4 medium. No significant increase in activities was observed on increasing urea, yeast extract or peptone levels of the NM-2 medium.

Effect of organic acids and volatile fatty acids

Table 19 shows the effect of various organic acids and straight chain and branched chain volatile fatty acids on the production of cellulase, xylanase and beta-glucosidase by *S. rolfsii* UV-8 mutant. Branched chain volatile fatty acids have been found to be essential for the growth of cellulolytic rumen bacteria (331,332). The addition of 0.1% acetate or oxoglutarate to *S. rolfsii* UV-8 mutant was stimulatory and slightly increased the yield of CMCase only. Addition of 0.1% sodium acetate to the *T. reesei* culture medium has been reported to enhance CMCase activity (330). Glycolic acid and propionic acid were inhibitory and reduced the yield of the enzymes whereas addition of succinic acid, lactic acid (0.2%) to certain extent and malic acid did not affect enzyme production. Enhanced yields of cellulase by succinate addition to the growth medium have been reported (335). The addition of isobutyric acid and isovaleric acid did not give promising results.

Effect of initial pH on the formation of cellulase, xylanase and beta-glucosidase

Studies on the effect of the initial pH of the growth medium on cellulase, xylanase and beta-glucosidase production

TABLE 19: EFFECT OF ORGANIC ACIDS ON ENZYME PRODUCTION BY S. ROLFSII UV-8 MUTANT

Addition[a]	pH[b]	Cellulase IU/ml		Xylanase	beta-Glucosidase
		CMCase	FPA	IU/ml	IU/ml
None	3.0	123	1.8	110	21
Acetic acid (0.1%)	3.0	140	1.8	120	21
Oxo-glutaric acid (0.1%)	3.0	145	1.5	120	19
Succinic acid (0.1%)	2.95	120	1.06	88	11
Malic acid (0.1%)	2.9	110	1.06	35	14
Lactic acid (0.2%)	2.5	140	1.2	32	11
Lactic acid (0.4%)	2.6	60	0.8	35	6
Glycolic acid (0.1%)	2.8	46	0.2	38	1.3
Propionic acid (0.05%)	2.85	46	0.6	30	1.1
Isobutyric acid (0.05%)	2.8	120	1.5	95	19
(0.1%)	2.9	98	1.0	100	16
Isovaleric acid (0.05%)	2.9	120	1.5	100	19
(0.1%)	2.9	98	0.8	84	15.5

[a] In addition to cellulose as the carbon source, sodium salts of the respective organic acids were incorporated into NM-2 medium.

[b] pH of culture filtrate on 14th day.

were carried out within a pH range of 2.8 to 7.3 (Table 20). An initial pH of 6.5 (after autoclaving) was found to be most favourable for producing high yields of cellulase, xylanase and beta-glucosidase even though synthesis of these enzymes by S. rolfsii UV-3 mutant occurred while the pH was between 2.7 - 3.1. Growth and enzyme production were markedly inhibited when the initial pH of the growth medium was above 7.0 or below 4.6.

Cellulase and beta-glucosidase formation during the growth cycle of parent and UV-8 mutant strain

The course of production of cellulase, beta-glucosidase, cellobiase, extracellular protein, and the pH profile on NM-2 growth medium for the parent strain and UV-8 mutant are shown in Fig. 12 (171). The rate of decline of pH during growth was about the same for the two cultures with the mutant and parent strain, the times of appearance of cellulase and beta-glucosidase activities during the first 5 days of growth were also about the same. Activity on CMC (endo-beta-glucanase), p-nitrophenyl-beta-D-glucoside (beta-glucosidase), and cellobiose (cellobiase) appeared earlier (day 3) than on filter paper (day 5) by both strains. The mutant, however, secreted 1.5 to 1.8 times more extracellular protein. Production of extracellular cellulase protein ranged from 4.3 mg/ml for parent strain of S. rolfsii on NM-3 medium to 5.5 mg/ml for UV-8 mutant of S. rolfsii (171) in contrast to 7 - 13 mg/ml for T. reesei when grown on cellulose (376). On the other hand, mycelial protein, determined by the procedure of Mandels and Andreotii (172) in the parent strain (70 to 80 mg/100 ml of culture broth),

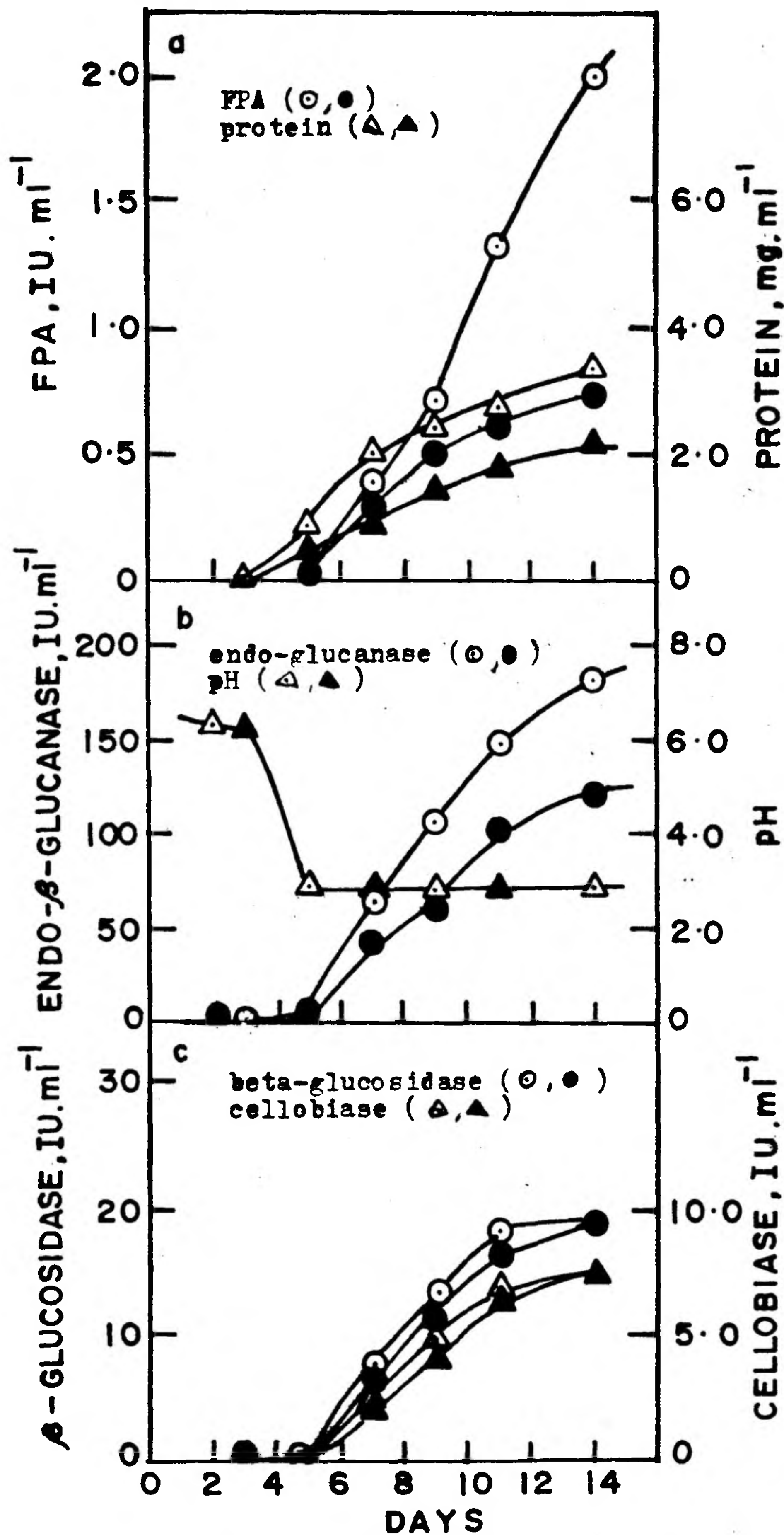


FIG.12: pH profile and elaboration of protein, FPA, endo-glucanase, beta-glucosidase and cellobiase during growth of parent strain (closed symbols) and UV-8 (open symbols) on NM-2 in shake flask.

TABLE 20: EFFECT OF INITIAL pH OF THE GROWTH MEDIUM ON ENZYME PRODUCTION BY S. ROLFSII UV-8 MUTANT

pH[a]	pH[b]	Cellulase IU/ml		Xylanase	beta-Glucosidase
		CMCase	FPA	IU/ml	IU/ml
2.8	2.6	55	0.5	30	10
3.0	2.6	67	0.7	40	10
3.7	2.6	67	0.78	41	13
4.6	2.7	86	0.78	44	13.5
5.0	2.6	92	1.1	48	18
5.5	2.7	93	1.25	59	18
6.0	2.8	120	1.4	82	20
6.5	3.0	124	1.8	111	22
7.1	6.9	-	-	-	-
7.3	7.0	-	-	-	-

Grown on NM-2 medium at indicated pH.

[a] pH of medium before inoculation.

[b] pH of culture filtrate on 14th day.

was greater than that of the UV-8 mutant (40 to 55 mg/100 ml of culture broth).

Table 21 summarizes the yields per milliliter of culture filtrate of the three major cellulase complex enzymes and cellobiase for the parent strain and the UV-8 mutant after 14 days of growth on NM-2 and NM-3 media (171). The ratio of activities of the mutant and parent strain culture filtrates were: FPA, 2.5 to 2.6, endo-beta-glucanase, 1.4 to 1.5, beta-glucosidase, 1.1 to 1.16, and cellobiase 1.0 to 1.1. These increases in enzyme activities were reproducible and were considered significant. The differential increase in FPA may imply that the UV-8 mutant may have possessed an altered control mechanism for the production of cellobiohydrolase or FPA, and the genome(s) controlling the cellulase complex of enzymes is not under coordinate control. Similar conclusions were reached in the instance of the T. reesei NG14 mutant (262). This was indirectly supported by the observation that the addition of rice bran to NM-2 medium decreased slightly the formation of FPA and did not affect cellobiase production by UV-8, although there was an increase in the formation of endo-beta-glucanase and, to a minor degree in beta-glucosidase activities. With the parent strain, the addition of 3% rice bran to NM-2 medium caused an increase in the formation of FPA, endo-beta-glucanase, and beta-glucosidase activities and was essential for their optimal synthesis (169). The mutant produced a 1.4 to 1.5 fold higher FPA on NM-2 medium than the parent strain produced on NM-3 medium.

S. rolfsii UV-8 mutant produces high cellulase and

TABLE 21: COMPARISON OF CELLULASE AND BETA-GLUCOSIDASE YIELDS OF S. ROLFSII PARENT AND UV-8 MUTANT STRAIN (171)

Strain[a]	Protein mg/ml	FPA IU/ml	CMCase IU/ml	PNPGase IU/ml	Cellobiase IU/ml
Parent (169)					
NM-2	2.2 - 2.4	0.7 - 0.78	100 - 120	16 - 19	7 - 7.5
NM-3	4.0 - 4.3	1.2 - 1.4	180 - 200	22 - 28	10 - 11
UV-8 mutant					
NM-2	3.2 - 3.4	1.75- 2.0	140 - 180	18 - 22	7 - 7.5
NM-3	5.0 - 5.5	1.6 - 1.75	185 - 210	20 - 22	7 - 7.5

[a]Grown 14 days on NM-2 or NM-3 medium.

cellobiase activities in submerged culture (171). It was earlier thought that (Table 21) UV-8 mutant secretes 7 to 7.5 IU of cellobiase per ml of culture filtrate. During the course of studying the kinetic properties of the purified cellobiase from S. rolfsii it was observed that the apparent Km of the purified cellobiase was rather high (5.8 mM) (334). When cellobiase activity of the UV-8 mutant culture filtrate was re-examined in the presence of higher concentrations of cellobiose in the assay mixture, much higher cellobiase activity values were obtained. The dependence of the reaction rate on cellobiose concentration is shown in Fig. 13. The concentration of cellobiose required for half-maximum velocity, calculated by the method of Lineweaver and Burk (335), was found to be 5.6 mM. The Vmax (micromoles of glucose released per minute per milliliter of culture filtrate from NM-4 medium), computed from the Lineweaver-Burk graph (Fig. 13) was 20.2 (3.77 umol of glucose/mg/min). This value varied from 18 to 23 (mean value = 20.2) in five different preparations. The corresponding Km and Vmax values for cellobiase from NM-2 medium culture filtrate varied between 5.6 and 5.8 mM and 10 to 13.7 (mean value = 12.0) respectively. The Km for T. reesei cellobiase secreted in the culture broth was reported to be 1.5 mM (163), and that for mycelial cellobiase was 1.2 mM (336). The Vmax for T. reesei mycelial cellobiase was 0.05 umol of glucose/mg/min (336).

Comparison of cellulase and beta-glucosidase production by S. rolfsii UV-8 mutant and T. reesei

As can be seen from Table 22, the amount of cellulase enzymes secreted in the medium by UV-8 mutant in shake flasks are

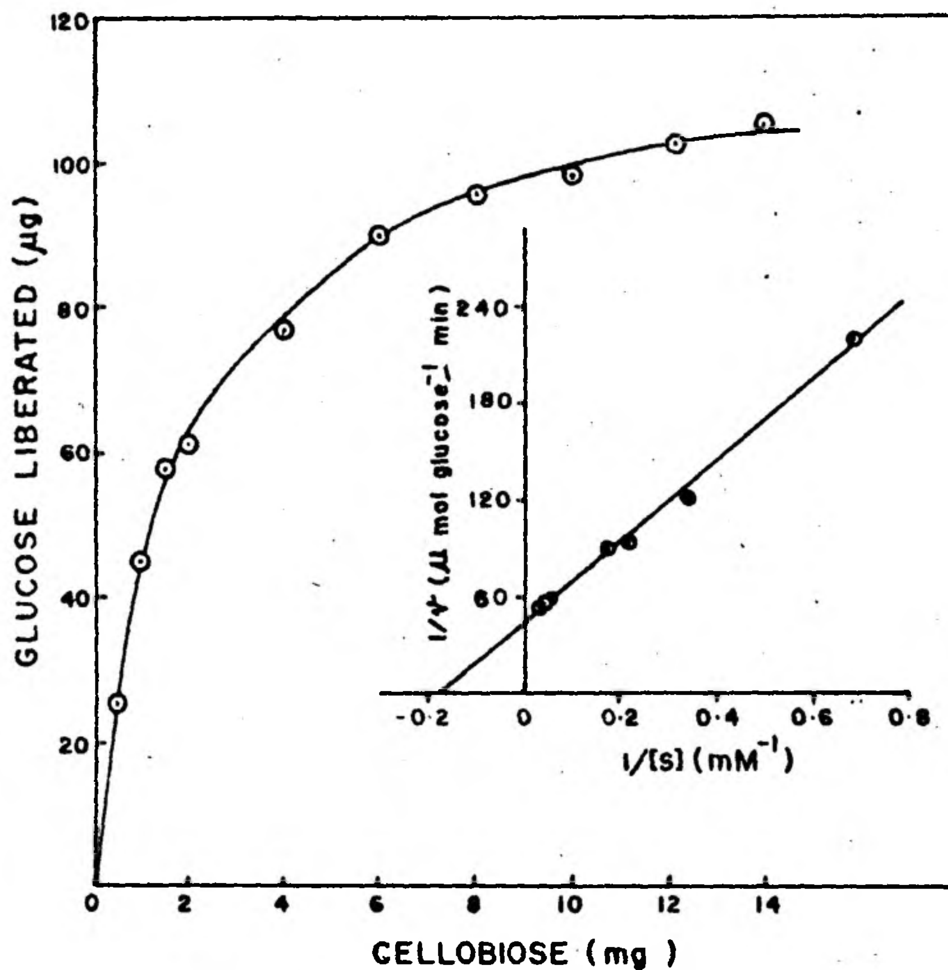


FIG. 13: Effect of cellobiose concentration on cellobiase activity. Culture filtrate of S. rolfsii grown on NM-2 + 2% CSL was used. Inset: Lineweaver-Burk plot of cellobiase activity with cellobiose as the substrate.

TABLE 22: COMPARISON OF CELLULASE PRODUCTION BY MUTANT STRAINS OF S. ROLFSII AND T. REESEI IN SHAKE FLASKS

Organism	Cellulase IU/ml		beta-Glucosidase	Cellobiase
	CMCase	FPA	IU/ml	IU/ml
S. rolfsii UV-8[a]	170 - 190	1.8 - 2.1	20 - 22	18 - 23
T. reesei				
QM6a (247)	18	0.23	NA	NA
QM9123 (247)	59	1.30	NA	NA
QM9414 (247)	152	1.48	NA	0.5
NG14 (267)	15	4.65	0.7	1.35
C-30 (337)	60.7	7.5	NA	NA

[a]Grown on NM-4 medium for 14 days.

NA - Not available.

comparable with some of the best activities reported for T. reesei and its mutants (247,267,275) which are stated to elaborate the highest cellulase activities. The highest FPA (FPA gives a rough measure of saccharification potential) reported for T. reesei mutants in shake flasks is for C-30 mutant, 4.5 to 7.5 IU/ml (337). However, the CMCase (endo-glucanase) secreted by C-30 is low, 60.7 IU/ml (337). S. rolfsii UV-8 mutant, however, produces high amounts of endo-glucanase, S. rolfsii also secretes high amounts of cellobiase as compared to T. reesei and its mutants. The highest cellobiase activity reported for T. reesei cultures is 0.5 - 1.3 IU/ml (61,267,275) and is suboptimal for conversion of cellulose to glucose (61).

Very recently, Tangnu et al.(339a) have reported that by the use of environmental control, it is possible to produce an enzyme mixture with different ratios of FPA, C_1, C_x, beta-glucosidase and xylanase. In controlled fermentation, they reported a yield of 26 IU/ml of beta-glucosidase and 14.4 U/ml of FPA (endo-glucanase values are not reported) using Rut-C 30 T. reesei mutant. With the S. rolfsii UV-8 mutant culture filtrate, glucose is the major product (85 - 90%) of cellulose saccharification (Part III, Chapter I) and not cellobiose with smaller amounts of glucose as with the T. reesei culture filtrates (163,321). In most applications for cellulose saccharification it is important to obtain glucose as the major product while keeping the cellobiose level as low as possible. Cellobiose inhibits cellobiohydrolase and endo-glucanase and its accumulation decreases the saccharification rates (133, 193,207).

Enzymatic hydrolysis of lignocellulosics such as bagasse has been reported to be enhanced by xylanase pretreatment. This presumably creates more accessible cellulosic regions that are readily acted upon by exo- and endo-glucanases (338).

During the last few years, tremendous advances have been made in getting high cellulase-yielding T. reesei cultures and their mutants (48,131,268). More sophisticated processing has further increased enzyme production by three to four fold (376). It has also been possible to reduce the lag time substantially and to obtain maximum cellulase production in 3 to 4 days in pilot plant experiments thereby increasing their productivities.

It has taken about 20 years to increase the filter paper activity (FPA) from 0.23 IU/ml (T. reesei QM6a) to 4.8 IU/ml (T. reesei NG14) (268) and 7.5 IU/ml for T. reesei C 30 (337) in shake flask experiments. However, during the last 5 - 6 years FPA activities of the order of 13 - 15 IU/ml have been obtained through strain improvement and with sophisticated processing in instrumented fermentors under controlled conditions of pH, aeration etc. (Table 23) and with high productivities (376). Recently, new mutant strains producing as much as 80 IU/l/h in a batch culture system and 7 IU/g cell/h in a continuous culture system have been selected and the strain improvement work continues in several laboratories (Table 22) (376).

As can be observed from Table 22 the S. rolfsii UV-8 mutant produces high amounts of endoglucanase and cellobiase in shake flask as compared to T. reesei and its mutants which are stated to elaborate the highest cellulase activities.

TABLE 23: PRODUCTION OF CELLULASE AND BETA-GLUCOSIDASE UNDER CONTROLLED CONDITIONS

Organism	Cellulase IU/ml		beta-Glucosidase IU/ml	Cellobiase IU/ml
	CMCase	FPA		
S. rolfsii UV-8	NS	NS	NS	NS
T. reesei (376)[a]				
QM6a	8.8	5	0.3	NA
QM9414	109	10	0.6	NA
MCG77	104	11	0.9	NA
C-30	150	14	0.3	NA
NG14	133	15	0.6	NA

NS - Not studied

[a]Cultures grown 14 days in 10 L fermentors on 6% two - roll milled cotton, pH control $\geq$ 3.0 using 2 N NH_4OH.

The *S. rolfsii* strain has so far not been studied in instrumented fermentors under controlled conditions of pH and aeration. It may, therefore, be possible to obtain much higher cellulase and beta-glucosidase activities with UV-8 *S. rolfsii* when run in instrumented fermentors under controlled conditions of pH, aeration etc.

PART IV

SACCHARIFICATION OF CELLULOSIC MATERIALS AND PRODUCTION OF ETHANOL

CHAPTER 1

ENZYMATIC HYDROLYSIS OF CELLULOSIC MATERIALS BY S. ROLFSII UV-8 MUTANT CULTURE FILTRATE

SUMMARY

The hydrolysis of purified celluloses (cotton, Avicel, Cellulose-123, Solka Floc SW40, Solka Floc BW200) and ligno-cellulosics (rice straw, bagasse, wood powders) by S. rolfsii UV-8 mutant culture filtrate was studied. Factors which affect saccharification such as pH, temperature, enzyme concentration, substrate concentration, product inhibition and particle size were studied.

With UV-8 S. rolfsii culture filtrate, the saccharification of cellulosic materials was inhibited by glucose to a greater extent (20 to 30%) as compared to that observed with the parent S. rolfsii culture filtrate (4 to 5%). With CMC as substrate, the liquifying activity (endoglucanase) of the culture filtrates from the parent and UV-8 mutant of S. rolfsii was inhibited very strongly (85 to 90%) with 100 mg/ml of cellobiose. At 2 mg/ml of cellobiose, the highest concentration observed in the cellulosic hydrolyzates, the CMC-liquifying activity inhibition by both the culture filtrates was negligible (3 - 6%). The saccharification rates of the cellulosics tested with T. reesei C-30 culture filtrate during the first 24 h was only slightly greater (6 - 10%) than that obtained with S. rolfsii UV-8 culture filtrate even though the FPA of C-30 culture filtrate was about 6 times higher than that of UV-8 culture filtrate.

INTRODUCTION

In industrial saccharification of cellulose to glucose, enzyme cost and pretreatment cost of cellulosic materials are the two major cost centres in the economics of the process; about 60% of the manufacturing cost can be attributed to enzyme production when filter paper activity (FPA) of the culture filtrate is of the order of 3 - 4 IU/ml (37). Thus, the major problems encountered are obtaining sufficiently active cellulolytic enzymes at low cost and suitable substrates so that high concentrations of glucose can be obtained in a reasonable period of time. During the last few years, tremendous advances have been made in getting high cellulase yielding *T. reesei* mutants through strain improvements and with sophisticated processing in instrumented fermentors under controlled conditions of pH, aeration etc. (27,264,267,274). All these developments would reduce the cost of alcohol production from cellulose.

Trichoderma spp. - the most intensively studied organisms for cellulose saccharification - have a disadvantage in that these cultures produce relatively low amounts of cellobiase (131,163,267). Thus, the culture filtrates from these microorganisms produce mainly cellobiose from cellulose with small amounts of glucose (163). Since cellobiose is an inhibitor both of 1,4-beta-glucan cellobiohydrolase, and 1,4-beta-glucan glucanohydrolase (EC 3.2.1.4) (184,193,209), its accumulation decreases the rate of cellulolysis (61,163). It has been demonstrated that the saccharification efficiency of *T. reesei* QM9414 mutant cellulase is increased by the

supplemental cellobiase (61). Suggestions have been made that too much attention has focused on Trichoderma and that other sources of cellulase should be examined.

The results presented in this Chapter have been published(373).

RESULTS AND DISCUSSION

Chemical pretreatment of cellulosic materials

Rice straw, sugarcane bagasse, mesta wood and coniferous were milled in the Wiley Mill and the ground material was sieved through a U.S. Standard 50-mesh sieve. The pretreatments tried are described in 'Materials and Methods'.

The weight loss on alkali treatment (2 N NaOH, 30°C, 48 h) for rice straw and bagasse was 38 and 36% respectively. On peracetic acid treatment of rice straw, bagasse, mesta wood and coniferous wood it was 25, 22, 17 and 12%, respectively. The cellulose and ash content in different cellulosic materials used are shown in Table 24.

Optimum pH and temperature for cellulase and beta-glucosidase activities

The effects of pH and temperature on cellulase and beta-glucosidase activities were determined over the pH range of 3 to 6, and temperature range of 45°C to 70°C. Table 25 shows the pH and temperature optima and the Arrhenius activation energies at the pH and temperature optima for these enzymes for the parent strain and UV-8 mutant. The average activation energies, calculated by the method of Arrhenius, were 13.07 and 11.86 for CMCase, 5.81 and 6.97 for FPA, 3.27 and 3.52 for cellobiase, 3.8 and 5.7 for PNPGase ,

TABLE 24: CELLULOSE AND ASH CONTENT OF CELLULOSIC MATERIALS

Cellulosic material	Cellulose %	Ash %
Avicel P.H. 101	100	0.0
Absorbent cotton	99	0.5
Cellulose-123	88	0.0
Solka Floc SW40	62	1.0
Rice straw	37	20.5
AT rice straw[a]	77	9.0
Bagasse	31	4.2
AT Bagasse[a]	61	4.0
Mesta wood	41	6.6
AT mesta wood[b]	52	5.1
AT peracetic acid mesta wood[c]	57	0.5
Coniferous wood	29	2.0
AT coniferous wood[b]	35	0.8

[a]Pretreated with 2 N NaOH, 30°C, 48 h.

[b]Pretreated with 4 N NaOH, 30°C, 24 h.

[c]Pretreated with 4 N NaOH, 30°C, 24 h followed by boiling with 20% peracetic acid.

TABLE 25

Temperature, pH optima and activation energies for cellulases and β-glucosidases from *S. rolfsii* parent strain and UV-8 mutant

Activity Activity	Parent strain			UV-8 mutant		
	Temperature optima °C	pH	Activation energy kcal/ml	Temperature optima °C	pH	Activation energy kcal/mol
FPA	55	4.8	5.81	50	4.8	6.97
CMCase	55	3.7	13.07	50	3.3	11.86
Cellobiase	65	4.5	3.27	65	4.5	3.52
p-nitrophenyl-β-glucosidase	70	4.5	3.80	70	4.5	5.70
Xylanase	65	4.5	13.19	65	4.5	10.67
Saccharification	40-50	4.0-4.5	—	50	4.5	—

and 13.19 and 10.67 kcal/mol for xylanase, respectively. With the culture filtrate from Chaetomium thermophile var. dissitum the activation energies were reported to be 6.53 kcal/mol with CMC as substrate (319) and 5.92 - 8.02 kcal/mol for different crystalline cellulose preperations for the T. reesei system (341).

Factors which affect saccharification

The rate of production of reducing sugars from cellulosic materials was examined as a function of pH, temperature, enzyme concentration, cellulose concentration, particle size and inhibition by end products.

pH

The effect of pH on saccharification of Solka Floc SW40 at 50°C is shown in Fig. 14. The best reducing sugars yield on saccharification was obtained at pH 4.5.

Temperature

The variation in the saccharification rate of Solka Floc SW40, Cellulase-123, AT-bagasse and AT rice straw was tested at the pH of maximum activity, pH 4.5, and is shown in Fig. 15. A temperature of 50°C appeared most effective for all the cellulosic materials tested.

Effect of addition of glucose and cellobiose on the saccharification rate and liquifying activity

In a batch saccharification, one of the factors likely to affect the rate of saccharification is the continuous accumulation of reducing sugars. The two major products of cellulose hydrolysis in general are cellobiose and glucose. It was reported earlier that with the parent S. rolfsii culture filtrate, hydrolysis of Solka Floc SW40 and AT rice straw were

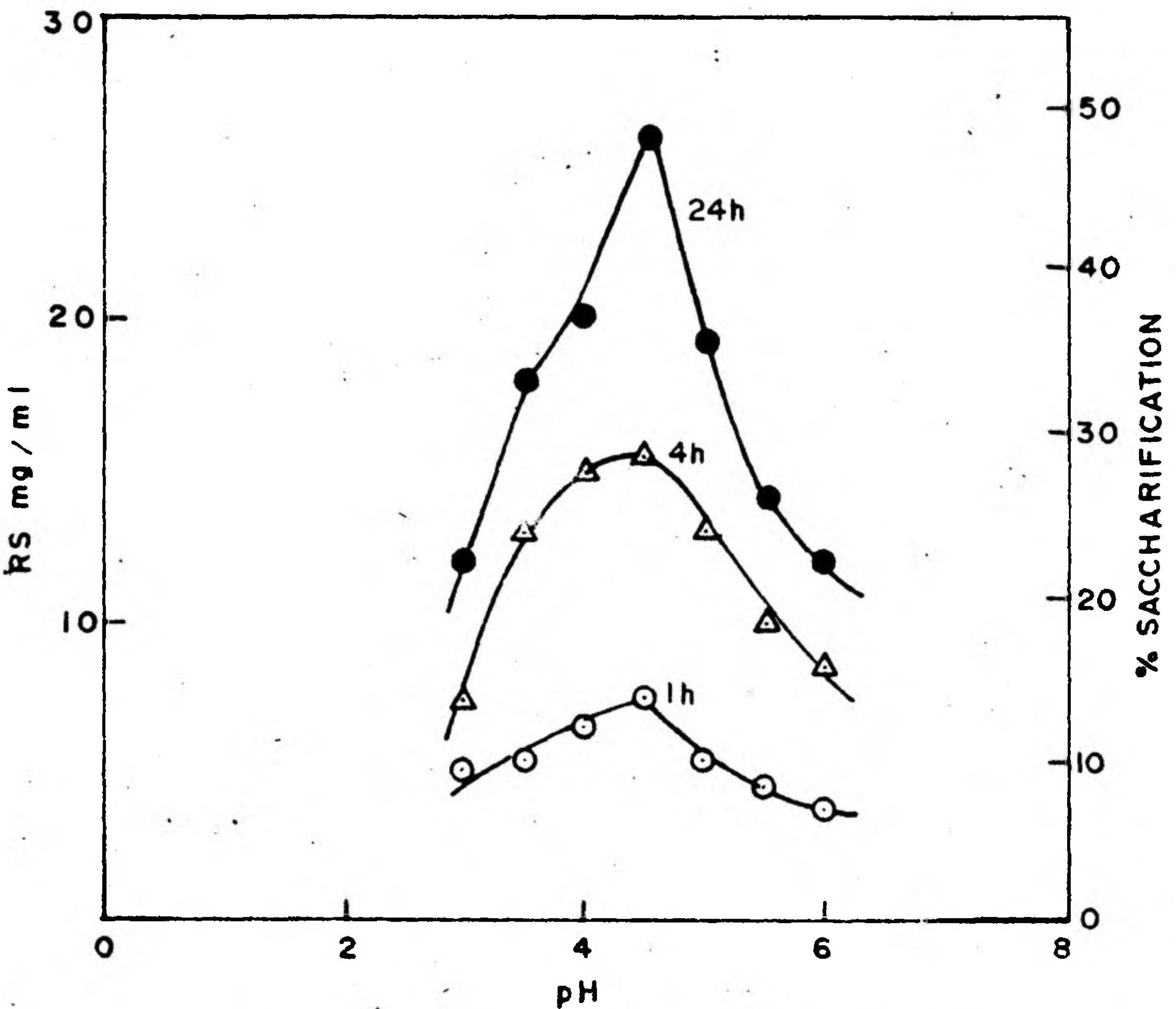


FIG. 14: Effect of pH on saccharification. 5% Solka Floc SW40, 50°C. Symbols: ⊙, 1 h; △, 4 h; ●, 24 h. RS = Reducing sugar.

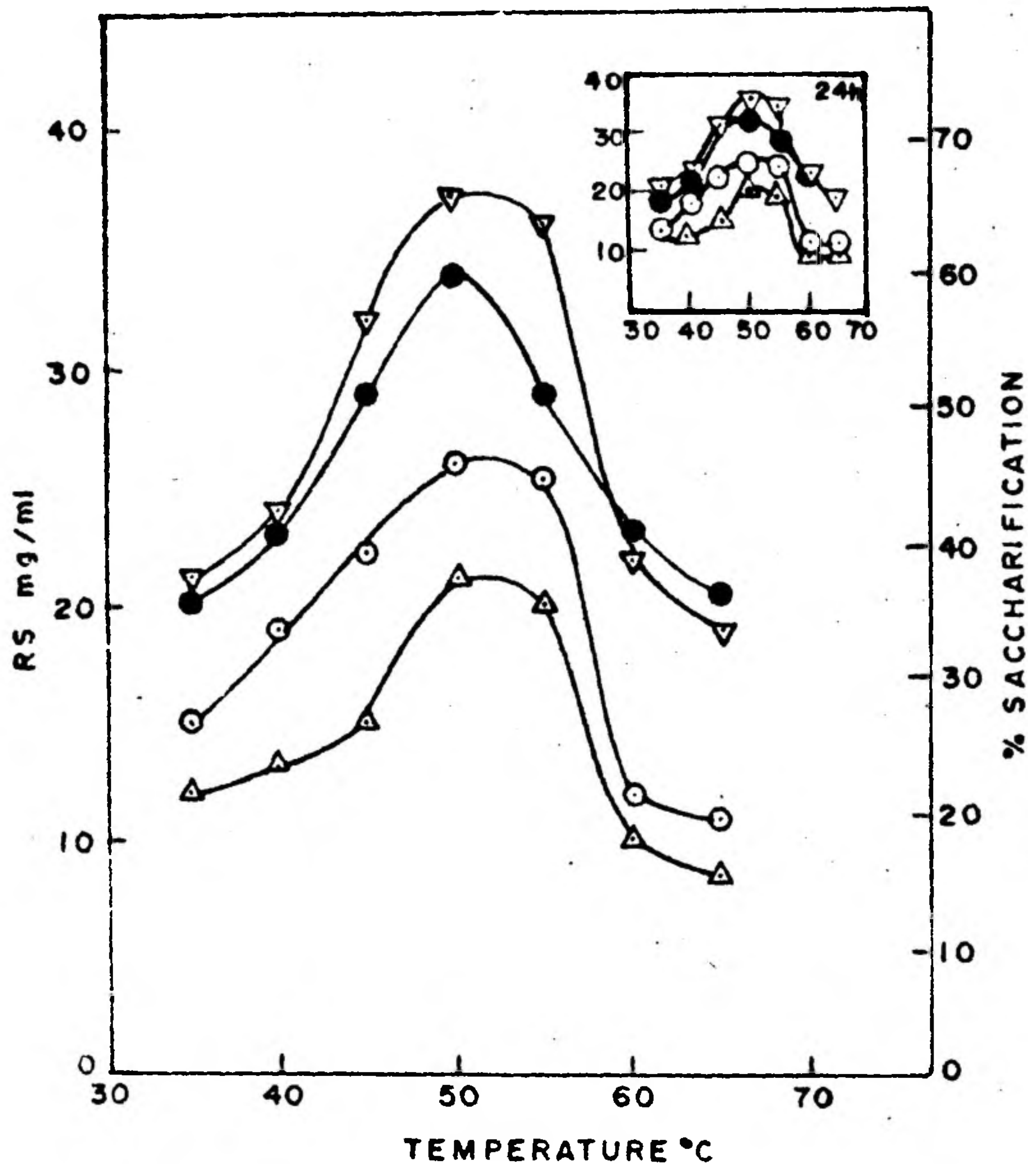


FIG. 15: Effect of temperature on saccharification. Substrate 5%, pH 4.5, 48 h. Symbols: △ , Cellulose-123; ⊙ , Solka Floc SW40; ● , AT bagasse; ▽ , AT rice straw. AT bagasse and AT rice straw = bagasse and rice straw pretreated with 2 N NaOH, 30°C, 48 h. Inset: Saccharification carried out for 24 h.

not significantly inhibited by glucose or cellobiose up to 100 mg/ml in 48 h (286). It is technically difficult to assess inhibition of saccharification by cellobiose as the added cellobiose (100 mg/ml) gets hydrolyzed completely to glucose by the culture filtrate in 24 h. With UV-8 culture filtrate, saccharification of cellulosic materials tested is inhibited by glucose (100 mg/ml) to a greater extent (20 to 30%) as compared to that observed with the parent culture filtrate (4 - 5%) (Table 26). With CMC as substrate, the liquifying activity (endoglucanase) of the culture filtrates from parent and *S. rolfsii* UV-8 mutant was inhibited very strongly (85 to 90%) with cellobiose (100 mg/ml) but not by glucose up to 100 mg/ml. Such high cellobiose concentrations are not reached in cellulose hydrolysis with *S. rolfsii* culture broths. Cellobiose at 10 mg/ml inhibited CMC-liquifying activity of *S. rolfsii* culture filtrates by 15 to 25%, whereas at 2 mg/ml level, the highest concentration observed in the cellulosic hydrolyzates, the liquifying activity inhibition was negligible (3 - 6%). The inhibitory effect of products are known to vary with the organism from which the cellulase was derived (135, 342-345).

Effect of enzyme concentration on saccharification of cellulosic materials

Since hydrolysis of native cellulose requires both C_1 - and C_x-type of cellulase enzymes, the role of enzyme concentration on the rate of hydrolysis and reducing sugars yield was determined. The saccharification of Avicel P.H. 101, Solka Floc SW40, AT rice straw and AT bagasse with different

TABLE 26: EFFECT OF ADDITION OF GLUCOSE ON SACCHARIFICATION OF CELLULOSIC MATERIALS BY S. ROLFSII CULTURE FILTRATES[a]

Substrate	% Inhibition					
	Parent strain			UV-8 mutant		
10%	8 h	24 h	48 h	8 h	24 h	48 h
AT rice straw[b]	6.3	4.5	4.5	35.4	34.2	29.2
AT bagasse[b]	6.2	5.5	4.7	32.9	31.1	29.0
Solka Floc SW40	6.8	6.6	5.0	29.4	27.3	20.0
CMC	10.0	8.5	8.5	37.0	29.2	22.2

[a] 1 g substrate + 0.5 ml 1 M citrate buffer, pH 4.5 + 7.5 ml culture filtrate + 0.1 ml merthiolate (0.1%) + water to 10 g. Incubated at 50°C on shaker. Data corrected for initial 10% glucose added.

[b] Pretreated with 2 N NaOH, 30°C, 48 h.

concentrations of *S. rolfsii* UV-8 culture filtrate is shown in Table 27. At the same substrate level (10%), the rate of hydrolysis increased with increasing concentration of the culture filtrate. For example, the reducing sugars produced from a 10% AT rice straw and AT bagasse at 24 h increased from 5.2 to 7.4% and 4.6 to 6.6%, respectively, as the concentration of culture filtrate was increased from 5 to 18 ml. The corresponding increase with Avicel P.H. 101 and Solka Floc SW40 was from 2.3 to 3.3% and 2.4 to 5%, respectively. Under similar conditions, the reducing sugar values obtained with *S. rolfsii* parent strain culture from AT rice straw and AT bagasse with 18 ml culture filtrate at 24 h were 6.4 and 3.8%, respectively (286). The reducing sugar values obtained in 24 h with 15 or 18 ml culture filtrate were about the same with AT rice straw, AT bagasse and Avicel P.H. 101 indicating that further increase in cellulase enzymes had little effect on saccharification, if any. The rate and extent of hydrolysis of the cellulosics were not proportional to the increase in enzyme concentration. Also, there is an initial faster rate of saccharification during the first 4 h followed by a gradually decreasing rate.

Effect of addition of more enzyme and substrate after 48 h

With 15% AT rice straw and AT bagasse, the reducing sugars produced in 48 h were 10.3 and 9.1%, respectively (Fig. 16). When 5 ml more of the culture filtrate was added at the end of 48 h, an increase in reducing sugars of 30 mg/ml and 29 mg/ml was observed with AT rice straw and AT bagasse on 48 h further incubation. The corresponding increase in reducing sugars when no additional enzyme or substrate was added was only 5 mg/ml

TABLE 27

Effect of enzyme concentration on saccharification of cellulosic materials

Substrate (dry solids) 10%	Culture filtrate ml	Reducing sugar mg/ml				% Saccharification			
		1 h	4 h	24 h	48 h	1 h	4 h	24 h	48 h
Solka Floc	5	6.5	10.0	24.0	32.0	5.9	9.0	21.6	28.4
SW 40	10	10.0	14.0	38.0	45.0	9.0	12.6	34.2	40.1
	15	15.0	20.0	49.0	53.0	13.5	18.0	44.1	47.7
	18	15.0	22.0	50.0	58.0	13.5	19.8	45.0	52.2
At bagasse[a]	5	26.5	33.0	46.0	55.0	23.9	29.7	41.4	49.5
	10	30.0	37.0	55.0	62.0	27.0	32.9	49.1	55.8
	15	36.0	41.0	65.0	70.0	32.4	36.9	58.5	63.0
	18	37.0	42.0	66.0	72.0	33.3	37.8	59.4	64.8
	18[b]	37.0	42.0	67.0	72.0	33.3	37.8	60.4	64.8
AT rice straw[a]	5	28.5	34.5	52.0	55.0	25.7	30.2	46.8	49.5
	10	34.5	45.0	65.0	70.0	31.1	40.1	58.5	63.0
	15	42.0	55.0	74.0	82.0	37.8	49.5	66.6	73.8
	18	44.0	56.0	74.0	85.0	39.6	50.4	66.8	76.5
	18[b]	45.0	58.0	74.0	85.0	40.1	52.2	66.6	76.5
Avicel P.H. 101	5	3.5	6.0	23.0	25.0	3.2	4.9	20.7	22.5
	10	6.5	11.0	27.0	28.0	5.9	9.5	24.3	25.2
	15	9.0	17.0	32.0	33.0	8.1	14.9	28.8	29.7
	18	10.0	17.0	33.0	33.0	9.0	15.3	29.7	29.7

Two grams substrate + 1 ml 1 M citrate buffer, pH 4.5 + 0.2 ml merthiolate (0.1%) + culture filtrate as indicated + water to 20 g

[a] Pretreated with 2 N NaOH, 30°C, 48 h

[b] Concentrated two times by precipitation with ammonium sulphate, 0.90% saturation.

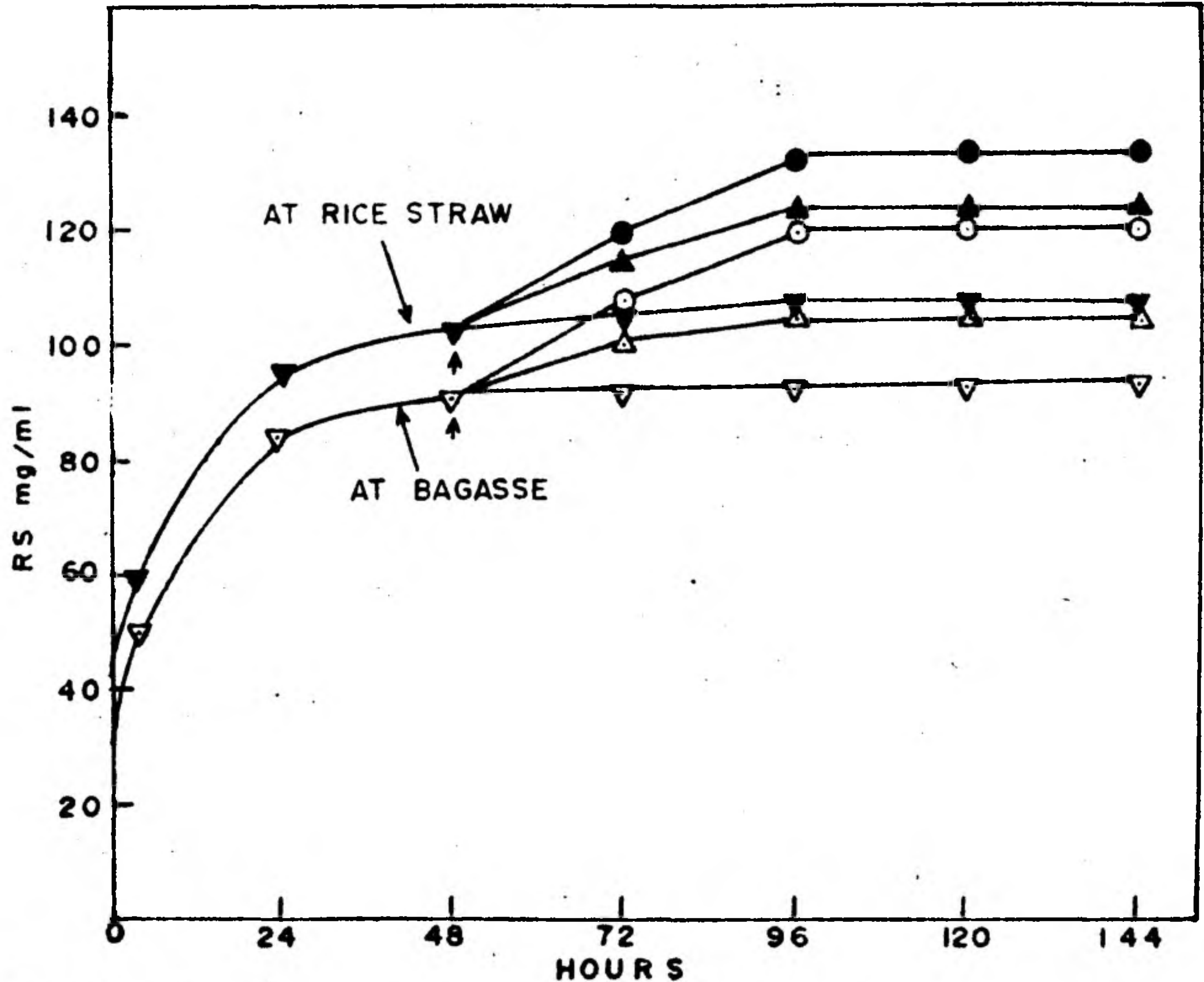


FIG. 16: Effect of adding more enzyme or substrate to AT rice straw (closed symbols) and AT bagasse (open symbols) after 48 h of incubation. 1.5 g of cellulose material + 0.5 ml 1 M citrate buffer, pH 4.5 + 7.5 ml culture filtrate + 0.1 ml merthiolate (0.1%) + water to 10 g incubated at 50°C. Symbols: ▽ , no additional enzyme or substrate added; ▲ , 0.5 g substrate added after 48 h; ○ , 5 ml culture filtrate added after 48 h. Reducing sugar values were corrected for dilution. AT rice straw and AT bagasse is as described in the caption to Fig. 15.

and 2.5 mg/ml, respectively. Similarly, when 0.5 g more of the substrate was added at the end of 48 h, an increase in the reducing sugars of 21.5 mg/ml and 14 mg/ml was observed with AT rice straw and AT bagasse on 48 h further incubation. After addition of substrate, the increase observed was probably due mainly to the hydrolysis of amorphous regions of added cellulose by CMCase which is not adsorbed on cellulose. Thus, the gradual fall in the rate of saccharification seems to be due mainly to the inactivation of cellulase as well as the gradual accumulation of resistant cellulose.

Effect of particle size on the enzymatic saccharification of microcrystalline cellulose

The assumptions are made that the enzyme is adsorbed on the surface of the substrate in accordance with a Gyani - Freundlich isotherm (346) and that the rate of digestion is proportional to the amount of the adsorbed enzyme. One would expect that the rate of hydrolysis would be proportional to the surface area in contact with the enzyme. However, no difference in the hydrolytic rate or degree of saccharification of two different particle sizes of microcrystalline cellulose, Avicel P.H. 101 (average particle size 38 μm), and Avicel P.H. 102 (average particle size 90 μm) by S. rolfsii UV-8 mutant culture filtrate was observed. The cellulase from S. rolfsii parent strain (286), T. reesei (264,271) and thermophilic Actinomyces MJØr and YX (347) are also reported to be insensitive to the particle size. When the effect of enzyme concentration on the rate of hydrolysis of Avicel P.H. 101 was studied (Fig.17), the data conformed to the equation developed by McLaren (346)

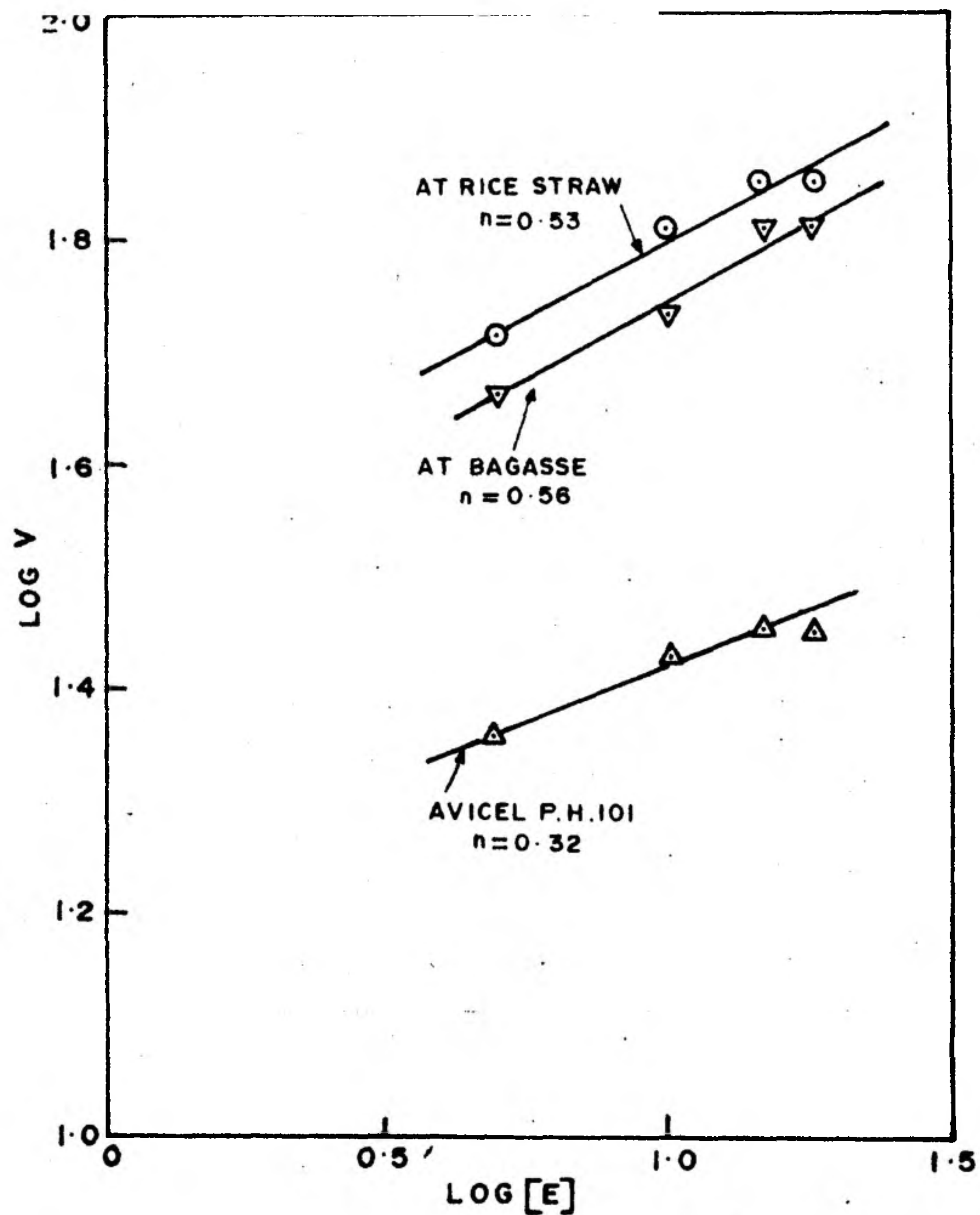


FIG. 17: Determination of n value. Saccharification of AT rice straw, AT bagasse and Avicel P.H. 101 was carried out at pH 4.5, 50°C. 2 g substrate + 1 ml 1 M citrate buffer, pH 4.5 + culture filtrate (5,10,15 or 18 ml) + 0.2 ml merthiolate (0.1%) + water to 20 g. AT rice straw and AT bagasse is as described in the caption to Fig. 15. Symbols: △ , Avicel P.H. 101; ⊙ , AT rice straw ▽ , AT bagasse.

describing the hydrolytic rate of enzymatic reaction in a heterogeneous system. The n value in the S. rolfsii UV-8 system was calculated to be 0.32 for Avicel P.H. 101. Similar low values of n of 0.32 and 0.35 were also observed for parent strain (286) and MJØr enzyme systems (347), respectively. The value of n describes the adsorption characteristics of the enzyme on the surface of the substrate. According to the theory (347), the low n value could be interpreted as indicating that the hydrolysis of microcrystalline cellulose takes place primarily on cracked edges of the surface of the particle (346). The n values for AT rice straw and AT bagasse are 0.53 and 0.56, respectively.

Effect of cellulose concentration on saccharification

The effect of cellulose concentration on reducing sugar formation and percentage of saccharification from different cellulosics by the S. rolfsii UV-8 culture filtrate is shown in Table 28. The amount of reducing sugars released increased with increase in the concentration of the substrate but the extent of hydrolysis decreased as the substrate concentration was increased. The reducing sugars produced from 15% AT rice straw and AT bagasse in 24 h were 10.8% and 8.5%, respectively, as compared to 7.2% and 5.4% with S. rolfsii parent strain culture filtrate (286). The reducing sugars produced from 30% AT rice straw and AT bagasse (these were semisolid) in 24 h were 12.8% and 11.0%, respectively.

The optimum substrate concentration, calculated according to Toyama and Ogawa (158) taking into account the conditions which give high sugar solutions and also a high degree of

TABLE 28
Hydrolysis of cellulosic materials at different substrate concentrations by *S. rolfsii* UV-8
Mutant culture filtrate

Substrate (dry solids) %	Reducing sugar mg/ml				% saccharification				Degree of solubilization %, 48 h	Cellulose utilization[a] %, 48 h
	1 h	4 h	24 h	48 h	1 h	4 h	24 h	48 h		
Avicel P.H. 101										
5	9.0	16.0	20.5	24.0	16.2	28.8	36.9	43.2	40.0	ND
10	10.0	18.5	35.5	37.0	9.0	16.7	32.0	33.3	29.5	36.0
15	15.0	25.5	48.0	50.0	9.0	15.3	28.8	30.0	27.0	ND
20	18.0	32.0	58.0	65.0	8.1	14.4	26.1	29.3	26.0	ND
Cellulose-123										
5	10.0	15.0	22.5	23.0	18.0	27.0	40.5	41.4	40.0	ND
10	13.0	21.0	39.0	42.0	11.7	18.9	35.1	37.8	35.0	42.0
15	18.0	38.0	50.0	53.0	9.0	18.0	30.0	31.8	34.0	ND
20	ND	39.0	60.0	70.0	ND	17.5	27.2	31.5	33.0	ND
AT bagasse[b]										
5	18.0	20.0	34.0	37.0	32.4	36.0	61.2	66.6	64.5	ND
10	35.0	40.0	64.0	69.0	31.5	36.0	57.6	62.1	60.0	59.4
15	45.0	53.0	85.0	93.0	27.0	31.8	51.0	55.8	53.6	ND
20	59.5	68.0	105.0	112.0	26.8	30.6	47.2	50.4	48.0	ND
30	ND	ND	110.0	120.0	ND	ND	33.0	36.0	ND	ND
AT rice straw[b]										
5	25.0	32.0	47.0	48.0	45.0	57.6	84.6	84.6	88.0	ND
10	42.0	57.0	74.0	92.0	37.8	51.3	66.6	82.8	86.0	78.0
15	ND	ND	108.0	112.0	ND	ND	64.8	67.2	70.0	ND
20	ND	ND	118.0	122.0	ND	ND	53.1	54.9	50.5	ND
30	ND	ND	128.0	150.0	ND	ND	38.4	45.0	ND	ND
NaOH-peracetic acid-treated mesta wood[c]										
5	9.0	18.0	25.0	28.0	16.2	32.4	45.0	50.4	ND	ND
10	11.5	20.0	41.5	55.0	10.4	18.0	37.4	49.5	ND	ND
15	ND	ND	59.0	73.0	ND	ND	35.4	43.8	ND	ND

One, two, three or four grams of cellulosic material + 1 ml 1 M citrate buffer, pH 4.5, + 0.2 ml merthiolate (0.1%), + 15 ml culture filtrate + water to 20 g Incubated at 50°C.

[a] Cellulose utilization by the anthrone method (Updegraff, 1969)

[b] Incubated with 2 N NaOH, 30°C, 48 h

[c] Incubated with 4 N NaOH, 30°C, 24 h, followed by boiling with 20% per acetic acid, 1 h

ND = Not determined

cellulose utilization, appears to be around 9 to 9.5% for Cellulose-123, Avicel P.H. 101, AT rice straw and AT bagasse (Fig. 18).

Analysis of the hydrolyzates from AT rice straw and AT bagasse at 10% substrate concentration showed the reducing sugars (as glucose equivalent) to be 74 and 64 mg/ml in 24 h, respectively. This corresponds to 0.74 and 0.64 g of reducing sugars compared to 0.60 and 0.39 obtained with parent *S. rolfsii* culture filtrate per gram of dry matter.

Enzymatic hydrolysis of different cellulosic materials

The formation of reducing sugars from pure celluloses and lignocellulosics is presented in Table 29. The extent of saccharification ranged from 12 to 82% in 48 h. Of the purified cellulosics tested, Cellulose-123, Solka Floc SW40 and BW200 showed about the same rate and extent of hydrolysis. With the *S. rolfsii* culture filtrate, Solka Floc SW40 was more susceptible than Solka Floc BW200 (286), with *T. reesei* cellulase. Mandels *et al.*(131) reported a much higher saccharification value with Solka Floc BW200 (62.1%) as compared to Solka Floc SW40 (37.4%) in 48 h.

Alkali treatment of lignocellulosics increased their susceptibility and sugar formation markedly in the case of rice straw and bagasse but only slightly in the case of wood powders: from 10.3 mg/ml to 38.8 mg/ml for rice straw, from 5.3 to 33.0 mg/ml for bagasse and from 5.6 to 9.3 mg/ml for mesta wood powder in 24 h. The optimum effect of alkali treatment for rice straw and bagasse was achieved by incubation with 2 N NaOH at 30°C for 48 h. Alkali treatment was not effective for wood

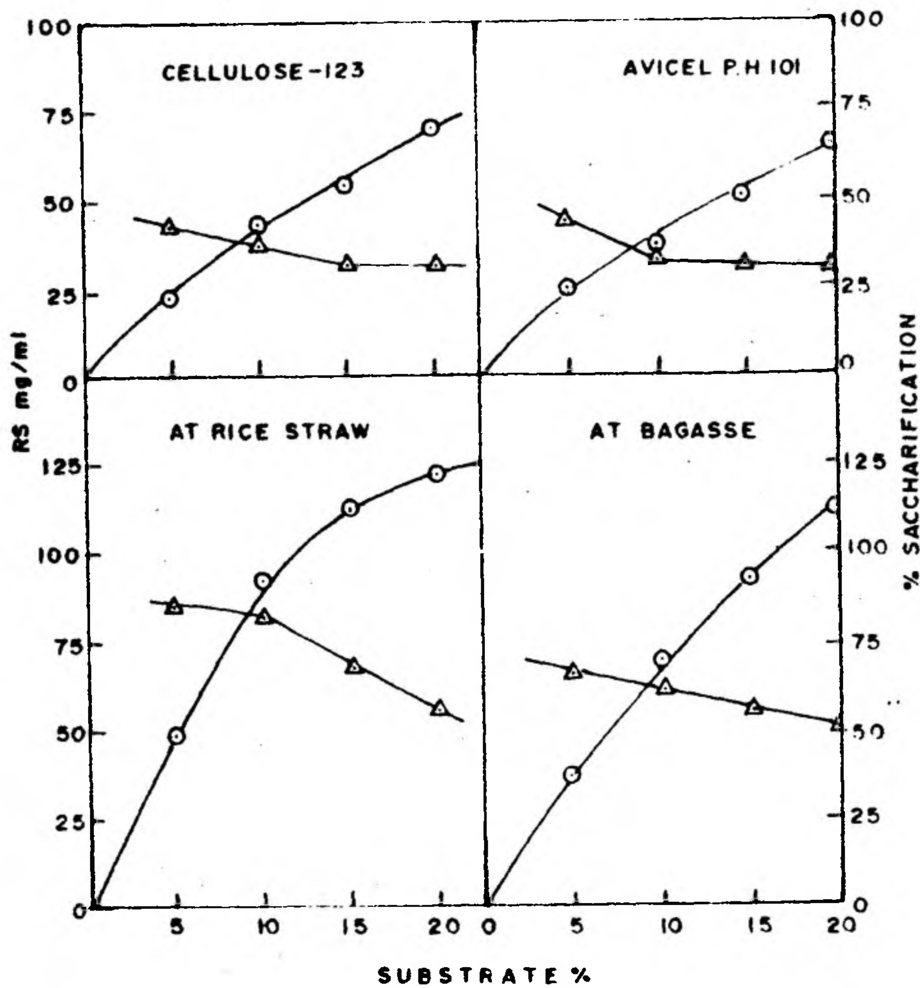


FIG. 18: Effect of substrate concentration on saccharification. Substrate + 2 ml 1 M citrate buffer, pH 4.5 + 15 ml culture filtrate + 0.2 ml merthiolate (0.1%) + water to 20 g, incubated at 50°C for 48 h. AT rice straw and AT bagasse is as described in the caption to Fig. 15. Symbols; ⊙ , reducing sugars; △ , % saccharification.

TABLE 29
Hydrolysis of cellulosic materials by *S. rolfsii* UV-8 mutant culture filtrate

Substrate, 5%	Reducing sugar mg/ml				% saccharification			
	1 h	4 h	24 h	48 h	1 h	4 h	24 h	48 h
Avicel P.H. 101	4.7	8.8	17.2	17.7	8.5	16.0	31.0	32.0
Avicel P.H. 102	5.0	8.6	17.5	17.5	9.0	15.5	31.5	31.5
Cotton absorbent (fibrous)	0.5	1.1	3.7	6.6	0.8	1.9	6.6	12.0
Cellulose-123	6.1	10.5	19.4	21.3	11.0	19.0	35.0	38.4
Solka Floc SW40	6.6	11.1	21.5	22.2	12.0	20.0	38.5	40.0
Solka Floc BW200	6.9	11.6	22.2	23.0	12.5	21.0	40.0	41.5
Bagasse untreated	1.4	2.8	5.3	7.7	2.5	5.0	9.5	14.0
AT (1 N NaOH, 30°C, 48 h)	14.7	17.7	25.2	31.1	26.5	32.0	45.5	56.0
AT (2 N NaOH, 30°C, 24 h)	15.0	17.6	30.0	33.8	27.0	31.9	54.0	61.0
AT (2 N NaOH, 30°C, 48 h)	17.7	20.2	32.3	37.7	32.0	36.5	58.0	68.0
AT (4 N NaOH, 30°C, 24 h)	14.4	22.2	31.6	37.0	26.0	40.0	57.0	66.5
AT (5 N NaOH, 30°C, 18 h)	17.5	21.1	33.0	37.7	31.5	38.0	60.0	68.0
NaOH-PAA[a]	11.1	15.5	30.0	35.0	20.0	28.0	54.0	63.0
Rice straw untreated	2.8	4.1	10.3	11.7	5.0	7.3	18.5	21.0
AT (0.25 N NaOH, 121°C, 1 h)	20.0	24.0	38.0	40.0	36.0	43.2	68.4	72.0
AT (1 N NaOH, 30°C, 24 h)	15.0	19.7	27.2	33.0	27.0	35.5	49.0	60.0
AT (1N NaOH, 30°C, 48 h)	15.5	18.5	28.3	34.2	28.0	33.5	51.0	61.5
AT (2 N NaOH, 30°C, 24 h)	17.2	20.8	30.0	36.5	31.0	37.5	54.0	65.8
AT (2 N NaOH, 30°C, 48 h)	25.0	30.8	38.6	45.5	45.0	55.5	69.5	82.0
AT (4 N NaOH, 30°C, 24 h)	17.2	19.4	31.1	38.3	31.0	35.0	56.0	69.0
AT (5 N NaOH, 30°C, 18 h)	22.7	27.5	38.8	45.2	41.0	49.5	70.0	81.5
NaOH-PAA[a]	15.5	24.4	34.4	36.2	28.0	44.0	62.0	65.2
Mesta wood untreated	1.4	3.3	5.6	6.4	2.5	6.0	10.1	11.5
AT (4 N NaOH, 30°C, 24 h)	3.1	4.6	9.3	11.1	5.5	8.2	16.7	20.0
PAA[b]	2.5	4.2	7.8	8.1	4.5	7.5	14.0	14.5
NaOH-PAA[a]	5.0	9.2	20.6	26.7	9.0	16.5	37.0	48.0
PAA-NaOH[c]	4.2	8.3	20.6	25.8	7.5	15.0	37.0	46.5
Coniferous wood untreated	0.5	1.5	2.7	3.1	0.9	2.8	4.8	5.5
AT (4 N NaOH, 30°C, 24 h)	3.3	5.0	8.3	9.4	6.0	9.0	15.0	17.0
PAA[b]	1.7	3.6	6.4	7.2	3.0	6.5	11.5	13.0
NaOH-PAA[a]	3.1	6.1	7.7	9.2	5.5	11.0	14.0	16.5
PAA-NaOH[c]	4.4	6.4	11.1	13.2	8.0	11.5	20.0	23.8

Saccharification was carried out at 5% substrate concentration, 50°C, pH 4.5 according to the procedure of Mandels *et al* (1974). % saccharification = reducing sugar mg/ml × 1.8.
AT = alkali treated
[a] Treatment with 4 N NaOH, 30°C, 24 h followed by boiling with 20% per acetic acid, 1 h
[b] Boiling with 20% peracetic acid for 1 h
[c] Boiling with 20% peracetic acid, 1 h followed by treatment with 4 N NaOH, 30°C, 24 h

powders. Mesta wood, however, became moderately reactive by sodium hydroxide - peracetic acid treatment (158). Coniferous wood was not reactive even after sodium hydroxide - peracetic acid pretreatment.

Comparision of saccharification rates by the S. rolfsii and T. reesei C-30 culture filtrates

The formation of reducing sugars from Avicel, AT rice straw and AT bagasse by *S. rolfsii* and *T. reesei* C-30 culture filtrates and by mixed culture filtrates of *S. rolfsii* UV-8 mutant and *T. reesei* is presented in Table 30. The cellulase and beta-glucosidase activities, determined at 50°C, pH 4.5, of the preparations used in the saccharification experiments in IU/ml were: parent *S. rolfsii*: FPA 1.5, CMCase 160, PNPGase 9.8, Cellobiase 12.0, UV-8 *S. rolfsii*: FPA 2.16, CMCase 195, PNPGase 13.9, Cellobiase 13.3; *T. reesei* C-30: FPA 13.4, CMCase 120, PNPGase 2.8 and Cellobiase 0.4 - 0.5.

Even though the FPA of C-30 culture filtrate was about 6 times higher than that of *S. rolfsii* UV-8 mutant culture filtrate, the saccharification rates of all the three cellulosic substrates tested with C-30 culture filtrate during the first 24 h was only slightly greater (6 - 10%) than that obtained with *S. rolfsii* UV-8 culture filtrate. It may be of interest to note that the rate of AT rice straw (only substrate tested), hydrolysis was greater when mixed UV-8 and C-30 culture filtrates was used as compared to when only C-30 culture filtrate was used, even though the total FPA units in the mixed culture filtrate were roughly half that of C-30 culture filtrate. FPA test for saccharifying activity is not as

TABLE 30: COMPARISON OF REDUCING SUGARS PRODUCTION BY S. ROLFSII AND T. REESEI C-30 CULTURE FILTRATES[a]

Substrate	CF	Reducing sugars mg/ml			% Saccharification			Utilization
15%		12 h	24 h	48 h	12 h	24 h	48 h	%
Avicel P.H. 101	C-30[b]	37	48	60	22	29	36	40
	UV-8	35	44	47	21	27	28	31
	Parent	22	28	30	13	17	18	23
AT rice straw[c]	C-30	88	110	125	53	66	75	69
	UV-8	81	101	103	49	61	62	60
	Mixed[d] (C-30 + UV-8)	90	118	134	54	71	80	75
AT bagasse[c]	UV-8	60	85	96	36	51	58	53
	Mixed (C-30 + UV-8)	75	97	110	45	53	66	63

[a]0.75 g substrate + 0.25 ml 1 M citrate buffer, pH 4.5 + 3.8 ml CF + 0.05 ml merthiolate (0.1%) + water to 5 g.

[b]T. reesei C-30 lyophilized powder (27.1 mg/ml) was dissolved in 0.05 M citrate buffer, pH 4.5. Cellulase and beta-glucosidase activities determined as described previously (169,315).

[c]Alkali treated, 2 N NaOH, 48 h, 30°C.

[d]In case of mixed CF, 1.9 ml of C-30 + 1.9 ml of UV-8 CF, were used.

CF = Culture filtrate.

accurate as one would like (340). Apparently, the enzyme (exoglucanase ?)/factor required for cellulose hydrolysis, in addition to CMCase, is present at a saturating concentration in S. rolfsii and the increased rate observed could be due to the higher CMCase and beta-glucosidase present in the mixed culture filtrates. Further studies using mixed culture filtrates from different organisms are necessary to clarify this anomalous observation.

Products of hydrolysis

The concentration of glucose, cellobiose, a sum of the two, and total reducing sugars produced during hydrolysis from different cellulosic materials up to 48 h by S. rolfsii UV-8 culture filtrate are given in Table 31. Paper chromatograms showed the presence of only glucose and cellobiose from Avicel P.H. 101; and glucose, cellobiose, xylose and two other unidentified sugars / oligosaccharide from AT rice straw and AT bagasse (Fig. 19). The following are the characteristic features of hydrolysis. As with the parent S. rolfsii culture filtrate, cellobiose accumulated initially but it fell to almost negligible levels in the later stages of hydrolysis. Glucose was detectable during the first h and continued to increase throughout. After 4 h of incubation, the major amount of increase in reducing sugar from all cellulosics tested could be accounted for as glucose, suggesting the formation of higher oligosaccharides - cellodextrins in the early stages of hydrolysis. At the end of 24 h glucose was the principal product and accounted for 90 to 96% of the total reducing sugars from Avicel P.H. 101 whereas cellobiose was only 0.5 to 1.0%. This pattern was more

TABLE 31

Formation of sugars during enzymatic hydrolysis of cellulosic materials by *S. rolfsii* UV-8 mutant culture filtrate

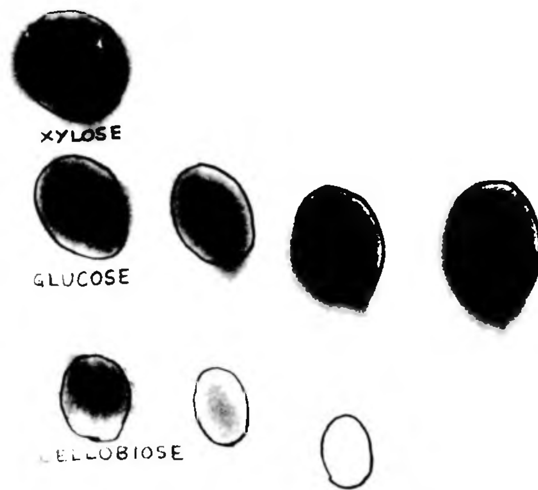
Substrate 10%	Reducing sugar mg/ml				Glucose mg/ml				Cellobiose[a] mg/ml				Glucose + Cellobiose mg/ml			
	1 h	4 h	24 h	48 h	1 h	4 h	24 h	48 h	1 h	4 h	24 h	48 h	1 h	4 h	24 h	48 h
Avicel P.H. 101	8.0	12.5	28.0	29.0	6.0	11.0	27.0	28.0	1.3	1.0	0.1	0.1	7.3	12.0	27.1	28.1
Cellulose-123	15.0	24.0	38.0	41.0	10.0	18.0	32.0	35.0	2.2	1.5	0.9	0.3	12.2	19.5	32.9	35.3
Solka Floc SW 40	21.0	28.0	45.0	50.0	10.8	17.4	32.0	40.5	2.8	2.0	1.2	0.3	13.6	19.4	33.2	40.8
AT rice straw[b]	35.5	44.0	64.0	68.5	18.0	30.0	50.0	53.0	2.5	1.8	1.0	0.2	20.5	31.8	51.0	53.2
AT bagasse[b]	30.0	38.0	53.5	55.0	13.0	20.0	30.0	32.0	2.5	1.7	1.1	0.4	15.5	21.7	31.1	32.4

Saccharification was carried out with 10% substrate, pH 4.5, 50°C.

[a] Purified *S. rolfsii* cellobiase used for cellobiose estimation hydrolyzes soluble cellodextrins. Thus, cellobiose values also include other soluble cellodextrins.

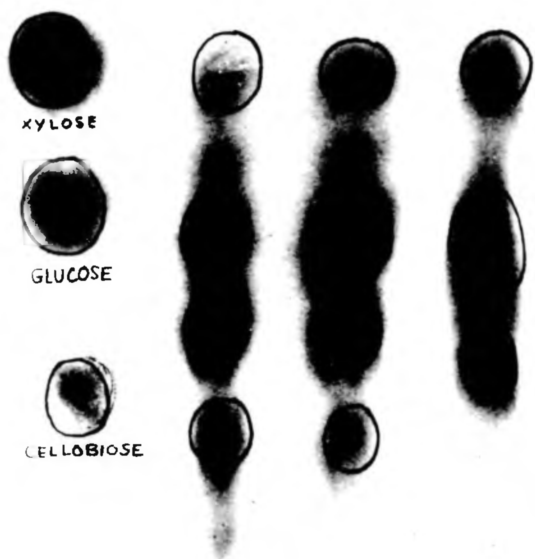
[b] pretreated with 2 N NaOH, 30°C, 48 h

FIG. 19: Paper chromatograms of the hydrolysis products by the *S. rolfsii* UV-8 mutant culture filtrate (a) Avicel P.H. 101, (b) AT bagasse, (c) AT rice straw. Hydrolysis was carried out by incubating 1 g substrate + 0.5 ml 1 M citrate buffer, pH 4.5 + 7.5 ml culture filtrate + 0.1 ml merthiolate (0.1%) + water to 10 g.



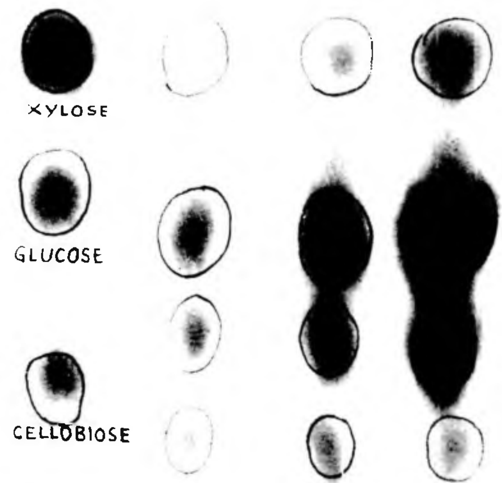
G+C+X 1hr 4hr 24 hr

a



G+C+X 1hr 4hr 24hr

b



G+C+X 1hr 4hr 24hr

C

or less similar for all cellulose substrates used although the total amounts varied. The generalization is that cellobiose conversion into glucose exceeded its rate of production and its concentration declined. The difference in the values between the total 'DNS' reducing sugars and sum of the glucose and cellobiose could be due to the undetermined higher oligomers, pentoses, and also due to different sensitivity of the assay procedures used for the estimation of glucose, cellobiose and total reducing sugars.

Conclusions and suggestions

From the view point of practical saccharification the focus of cellulase studies has been towards obtaining higher efficiency of saccharification of lignocellulosics. To this end, an International comparative standard assay has been suggested by the Commission on Biotechnology, International Union of Pure and Applied Biochemistry (240). The method that has been adopted by most of the investigators is that of Reese and Mandels (270), Filter Paper assay and is the recognised comparative assay. A change of a single parameter, e.g. amount of substrate, results in 'non-operational' response in relation to enzyme activity. It has, therefore, been suggested by the Commission that no modification of the Mandels FP assay should be entertained, if comparative data are to be obtained. The comments are made on the recommendations:

FPA gives a measure of the whole cellulase complex for the hydrolysis of native crystalline cellulose, and indirectly of cellobiohydrolase which in general has been found limiting in the organisms studied and reported so far in the literature.

Endoglucanases and/or cellobiohydrolase is considered to initiate attack on crystalline native cellulose. The ultimate end product of pure cellobiohydrolase action is cellobiose and that by pure endoglucanases are cellobiose and small amounts of higher cellodextrins and glucose. In the case of cellulosic materials which have already undergone a pretreatment, the amount of cellobiohydrolase may not be very critical as in that case the hydrolysis of the pretreated cellulosics could be carried out by endoglucanases and beta-glucosidases. The presence of cellobiohydrolase may aid the production of cellobiose from cellulosic material but should not be that critical particularly in cases where endoglucanases are present in very high amounts such as in the case of our culture S. rolfsii UV-8 mutant. There are other cultures known which do not show any CBH activity but can still carry out the hydrolysis of crystalline cellulose (27). In these cases, I wonder whether high amounts of CBH, that is when the culture secretes high amounts of endoglucanases, would still be critical for hydrolysis of pretreated cellulosic materials. The question is whether for saccharification of pretreated lignocellulosics, culture which secretes high amounts of endoglucanases activity but somewhat low FPA, would be better than the one which secretes slightly higher FPA but much less endoglucanase. From the comparative data of T. reesei C-30 and UV-8 S. rolfsii (Table 30), we do not find much difference in the rate and extent of hydrolysis of AT bagasse and AT rice straw. Therefore, if we go entirely by only FPA values, it may give an erroneous idea.

CHAPTER 2

STABILITY OF S. ROLFSII CELLULASE UNDER CONDITIONS OF SACCHARIFICATION

INTRODUCTION

It has been suggested that sugar production by enzymatic degradation of cellulose can be one of the major practical processes of utilization of agricultural residues. For practical enzymatic commercial process, stability to the enzyme becomes a factor of increasing importance, particularly when enzyme cost is the major cost centre in the economics of the process. These enzymes may be sufficiently stable for the growth of the organism, but these may not withstand the conditions which we employ for saccharification, e.g. high substrate and product concentrations, high temperature, long incubation time, non-sterile environment, the presence of foreign materials and shear resulting from vigorous agitation. Cellulase preparations used for saccharification are not purified, and as a result many contain materials that could contribute to inactivation. These include proteases which are often involved in the degradation of useful enzymes (349). Additives that have been useful in stabilizing enzymes fall into several catagories: (i) Metals, especially Ca^{++}, (ii) Proteins, e.g. stabilization of the proteases in solution for periods upto 25 days can be achieved by the addition of casein and soya protein (349), (iii) Biocides, to prevent inactivation by contaminating microorganisms, (iv) Substrate or products of desired enzyme, and (v) Enzyme modification, e.g. immobilization, if the substrate is soluble; chemical modifications, if the substrate is insoluble (350).

This Chapter describes work carried out on factors affecting the stability of the crude 'cellulase' enzymes of

S. rolfsii under conditions that are used in enzymatic hydrolysis process.

RESULTS AND DISCUSSION

Stability of cellulase and xylanase components

All stability experiments were carried under the conditions contemplated for the saccharification (50°C, pH 4.5) of cellulose. Effects on different enzymes of the cellulase complex and xylanase in the culture filtrate were examined. It was found (Fig. 20) that all the cellulase components and xylanase of *S. rolfsii* UV-8 mutant culture filtrate were inactivated; specifically xylanase and Avicelase showed greater instability as compared to other cellulase components.

This led to an evaluation of the incubation conditions, and it was found that the merthiolate used as a preservative was responsible for much of the adverse effects (Fig. 21). Again, greater instability was observed in the case of xylanase at 50°C, pH 4.8 with increase of merthiolate. The same merthiolate concentration had no effect on enzymes stored in the deep freeze at -15°C for 2 - 3 years (Table 32) and in the refrigerator for a number of days. It seems that at 50°C, the conformation of the affected enzyme may be changed in such a way as to make it more susceptible to merthiolate (350). The effect of other mercury compounds (Table 33) was also studied by incubating the culture filtrate with these compounds at room temperature for 15 min, and the activities assayed were compared with the culture filtrate incubated simultaneously without any additives. Other mercury compounds resemble merthiolate in action. The presence of a number of disulfide

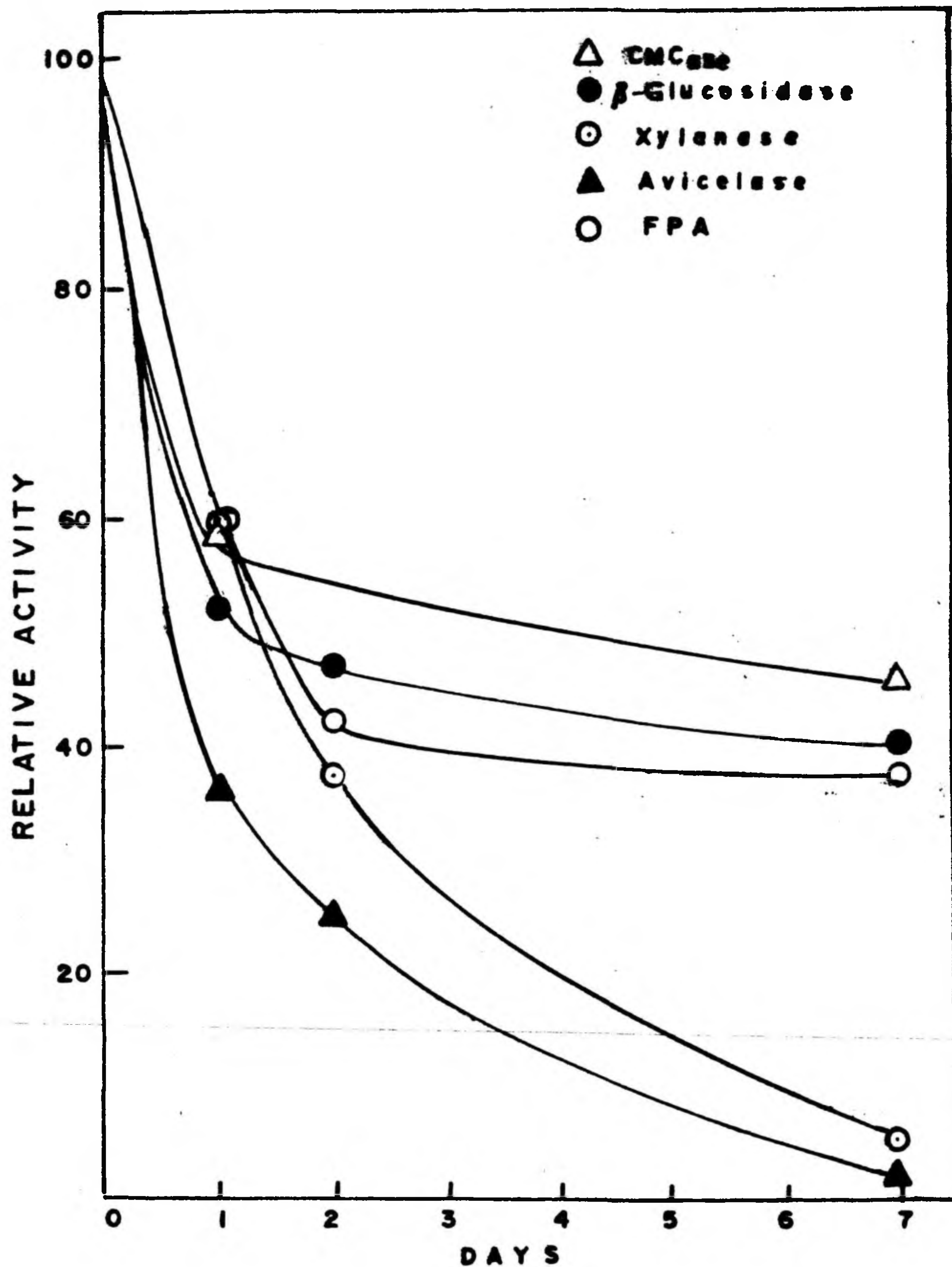


FIG 20. STABILITY OF CELLULASE COMPONENTS OF S. ROLFSII U V-8 MUTANT CULTURE FILTRATE. ENZYME WAS INCUBATED AT PH 4.5, 50°C, IN THE PRESENCE OF 0.01% MERTHIOLATE FOR TIMES SHOWN AND THEN ASSAYED.

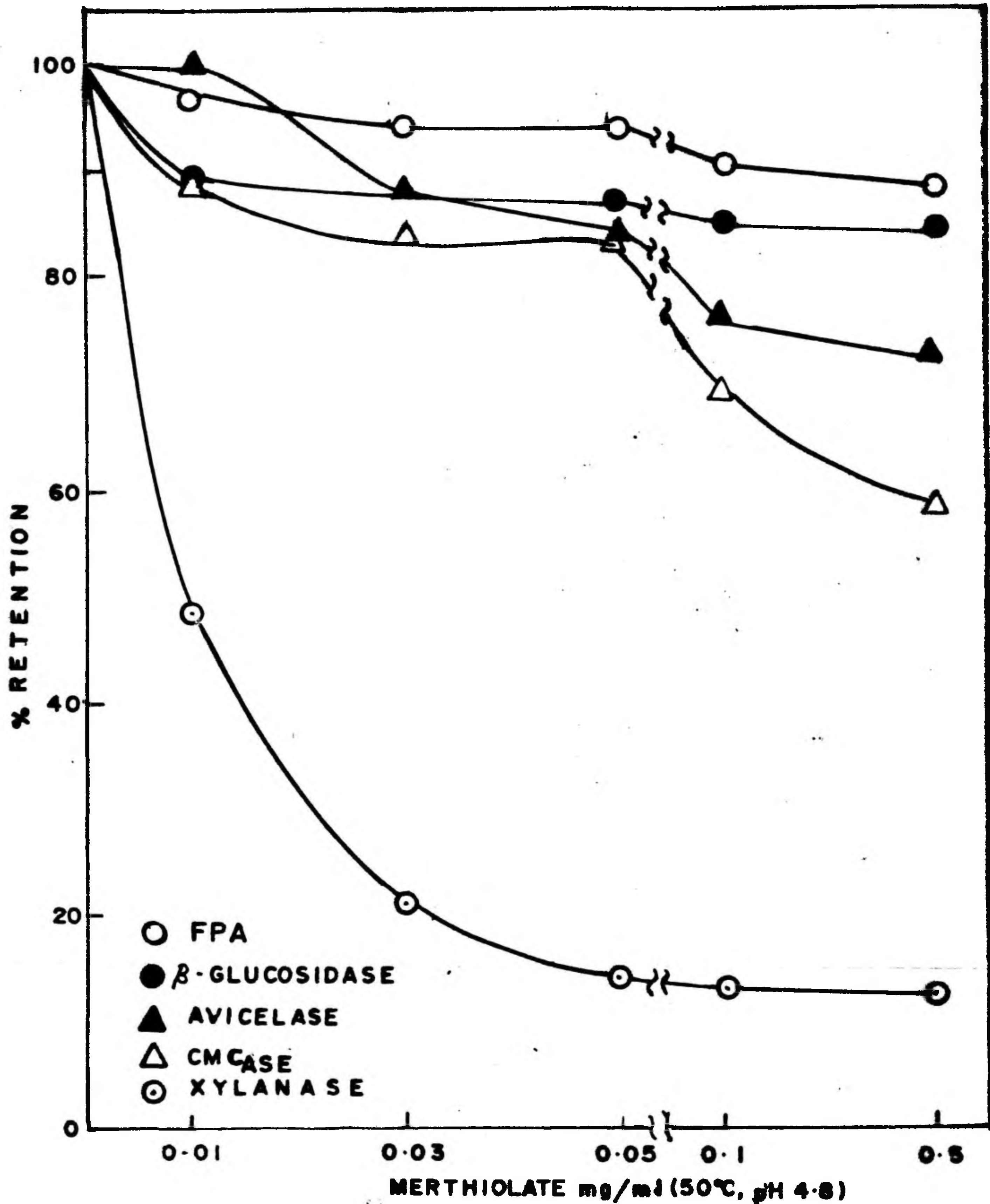


FIG.21. EFFECT OF MERTHIOLATE ON STABILITY OF CELLULASE, XYLANASE AND β – GLUCOSIDASE OF S. ROLFSII UV–8 MUTANT CULTURE FILTRATE. MERTHIOLATE WAS ADDED TO ENZYME AND INCUBATED AT 50°C. pH 4·8, 18h. THE SAMPLE WAS DILUTED BEFORE ASSAY FOR DIFFERENT ACTIVITIES

TABLE 32: STABILITY OF CELLULASE, XYLANASE AND BETA-GLUCOSIDASE OF S. ROLFSII UV-8 MUTANT CULTURE FILTRATE IN PRESENCE OF 0.01% MERTHIOLATE

Incubation temperature °C	% Retention Activities of								
	Cellulase @			Xylanase			beta-Glucosidase		
	1d	2d	7d	1d	2d	7d	1d	2d	7d
-15°C (Deep freeze)	100	100	100	100	100	100	100	100	100
8°C (Refrigerator)	100	100	95	100	100	94	100	100	95
50°C	30-40	25-35	15-25	10-12	8-10	5	35	30	20

d = day.

@ FPA.

TABLE 33: INACTIVATION OF CELLULASE, XYLANASE, BETA-GLUCOSIDASE OF S. ROLFSII UV-8 MUTANT CULTURE FILTRATE

Compound	Concentration	Inactivation after incubation at room temperature for 15 min % loss			
		Avicelase	CMCase	Xylanase	beta-Glucosidase
p-Chloro mercury benzoate	10^{-3} M	63	47	55	14
	10^{-4} M	37	34	33	6
$HgCl_2$	10^{-3} M	68	50	90	23
	10^{-4} M	48	38	79	11
Merthiolate	0.1%	50	35	52	30
	0.01%	12	8	20	12
Mercaptoethanol	0.1%	63	15	60	17
	0.01%	31	10	35	8
Dithiothreitol	1 mg/ml	58	15	55	14
	0.5 mg/ml	28	4.6	28	5.8
Na azide	0.1%	50	38	36	38
	0.01%	14	31	14	17
Tween-80	0.1%	51	15	34	11
	0.01%	20	11	14	3

linkages in these enzymes may explain the effect (351).

The sensitivity of cellulase components to heat and to merthiolate, lead to an investigation of a means of improving the stability, and to a search for a biocide effective in preventing contamination of the hydrolysis mixtures without impairing the action of the enzymes. If the saccharification is done batchwise for 24/48 h at 50°C, addition of a biocide may be unnecessary. But in anticipation of a continuous process, in order to avoid contamination with thermophiles, we have examined potential biocides for their effects on cellulolytic enzymes (Table 33). We found that Avicelase and xylanase are much more sensitive to these compounds than CMCase or beta-glucosidase. The data obtained indicate that most of the biocides inactivate Avicelase and xylanase at concentrations required for prevention of microbial growth.

The difference in susceptibility between Avicelase and CMCase on addition of biocide is shown in Table 33. Compounds like $HgCl_2$, p-chloromercury benzoate, merthiolate show the greatest differences between the two enzymes. These are the agents that react with the -SH or $-NH_2$ protein groups. The results of inactivation of xylanase with these compounds may reflect its different nature from CMCase.

A series of antibiotics was also tested for their effect on Avicelase, CMCase, xylanase and beta-glucosidase (Table 34). Of the antibiotics tested at concentrations required for preventing the growth of the organisms, tetracycline, chloramphenicol and streptomycin sulfate were found suitable as additive in cellulose hydrolysis system, while the addition

TABLE 34: EFFECT OF ADDITION OF ANTIBIOTICS ON THE STABILITY OF CELLULASE AND BETA-GLUCOSIDASE OF S. ROLFSII UV-8 MUTANT

Biocide	Concentration mg/l	% Residual Activity					
		Cellulase		Xylanase		beta-Glucosidase	
		18 h	48 h	18 h	48 h	18 h	48 h
Tetracycline	2	100	100	100	85	100	100
	5	100	95	100	80	100	100
	50	88	80	90	75	100	100
Chloramphenicol	2	100	100	100	85	100	100
	5	100	96	100	80	100	90
	50	86	80	92	80	96	90
Streptomycin sulfate	2	100	100	100	95	100	98
	5	100	93	100	92	100	89
	50	88	82	92	90	88	85
Penicillin G	2	100	100	100	72	100	88
	5	100	97	90	70	100	85
	50	89	83	87	70	90	80
Merthiolate	100	84	80	20	10	86	82
	1000	60	50	15	8	65	60

% residual activity was calculated for each enzyme by taking culture filtrate incubated without biocide as the control.

of Penicillin G, showed greater inactivation of xylanase.

Effect of shaking on enzyme stability during saccharification

The cellulose hydrolysis by UV-8 *S. rolfsii* culture filtrate was studied for 48 h at 50°C to see the effect of shaking on the efficiency of saccharification of AT rice straw, AT bagasse, Solka Floc SW40 and Avicel P.H. 101 (Table 35). The cellulases of *S. rolfsii* UV-8 mutant are not affected by shaking of saccharification flasks. Under shaken conditions more of the enzyme remained active than under unshaken conditions. A 10 - 12% increases in saccharification was observed because of shaking during saccharification.

Effect of CO_2 and N_2 (i.e. absence of oxygen) atmosphere on saccharification

A carbondioxide and nitrogen atmosphere were used to replace air in hydrolysis flasks in view of Eriksson's claim (212) that an oxidative mechanism is involved in cellulose digestion by *S. pulverulentum*. Nitrogen atmosphere had no effect on the hydrolysis rate, under shaken or unshaken conditions as compared to when the saccharification was carried out in air on the other hand, CO_2 did have a favourable effect in shaken and unshaken flasks showing 5 - 10% increase in saccharification (Table 35). The use of a CO_2 atmosphere may be useful in preservation of the enzymes for greater digestion of cellulose (350).

The optimum hydrolysis of AT rice straw, AT bagasse, Solka Floc SW40 and Avicel P.H. 101 was observed when *S. rolfsii* UV-8 enzyme was used under shaken conditions at pH 4.5, 50°C in CO_2 atmosphere, but Reese and Mandels (350) reported that

TABLE 35: EFFECT OF SHAKING ON ENZYMATIC HYDROLYSIS OF CELLULOSE BY S. ROLFSII UV-8 MUTANT

Shaking	Atmosphere	Substrate 15%	Reducing sugar mg/ml			% Saccharification		
			12 h	24 h	48 h	12 h	24 h	48 h
Without shaking	Air	AT rice straw	71	89	91	42.6	53.4	53.6
		AT bagasse	56	76	77	33.6	45.6	46.2
		Solka Floc SW40	39	44	47	23.4	26.4	28.4
		Avicel P.H. 101	31	38	41	18.6	22.8	24.6
	CO_2	AT rice straw	74	99	100	44.6	59.4	60
		AT bagasse	66	83	85	39.6	49.8	51
		Solka Floc SW40	44	53	55	26.4	21.8	33
		Avicel P.H. 101	33	37	40	19.8	22.2	24
	N_2	AT rice straw	71	88	92	42.6	52.8	55.2
		AT bagasse	57	76	78	34.2	45.6	46.8
		Solka Floc SW40	39	44	47	23.4	26.4	28.2
		Avicel P.H. 101	31	39	39	13.6	23.4	23.4
With shaking	Air	AT rice straw	70	96	98	42	57.6	58.8
		AT bagasse	64	83	84	38.4	49.8	50.4
		Solka Floc SW40	40	53	57	24	31.8	34.2
		Avicel P.H. 101	34	43	47	20.4	23.8	28.2
	CO_2	AT rice straw	80	103	105	48	61.8	63
		AT bagasse	68	90	91	40.8	54	54.6
		Solka Floc SW40	55	66	68	33	39.6	40.8
		Avicel P.H. 101	33	41	43	19.8	24.6	25.8
	N_2	AT rice straw	71	97	99	42.6	58.2	59.4
		AT bagasse	64	84	85	38.4	50.4	51
		Solka Floc SW40	41	54	58	24.6	32.4	34.8
		Avicel P.H. 101	34	44	50	20.4	27	30

the optimum hydrolysis of Avicel with T. reesei C-30 enzyme (with added beta-glucosidase) was under unshaken conditions at pH 5.0, 50°C in air.

All these problems such as the effect of preservatives, atmosphere in saccharification vessels, shaking, should be considered in evaluation of the mutant. It may be possible to carry out the cellulose hydrolysis at higher temperatures and thus speed up the process if the enzyme stability to temperature can be increased. The high cellobiase and xylanase production by S. rolfsii UV-8 mutant in submerged culture may also be responsible for high cellulose hydrolysis (61,315,338).

CHAPTER 3

COUPLED SACCHARIFICATION/FERMENTATION OF CELLULOSE TO ETHANOL USING S. ROLFSII CULTURE FILTRATE

INTRODUCTION

Due to concern about the increasing cost and availability of petroleum and natural gas, interest is increasing in processes which make use of renewable resources, such as plant biomass, for the generation of liquid fuels and chemical feed stocks. The energy content of biomass can be utilized most simply by direct burning. It is also possible to convert biomass to higher value gaseous and liquid fuels and chemicals by thermochemical and biological means (352). Thermochemical processes usually produce complex mixtures of products and require high temperatures and pressures. In contrast, fermentative bioconversion processes operate at lower temperatures (ca. 25 - 60°C) and at atmospheric pressure and produce only a very few major products. A good yeast fermentation converts one mole of glucose (100 gms containing 673 kcal of heat of combustion) to 1.8 moles of ethanol (83 gms containing 540 kcal of heat of combustion). This represents about 87% yield of the energy available in the original glucose molecule, and a 54% reduction for weight.

For producing ethanol from plant biomass, enzymatic saccharification of cellulose to glucose and subsequent fermentation to ethanol by yeast (353-355) has been intensively studied. The conversion cost estimates, based on Wilke's process for producing ethanol from newsprint and wheat straw, indicate that the economics of these processes are not promising (38,62). It is estimated that the production cost of cellulase is approximately $ 3/kg cellulase protein having a specific activity of 0.6 IU/mg protein; this could be lowered to

p. 154a

Humphrey and Nolan (10) have reported that the estimated costs of an enzymatic hydrolysis process based on the Gulf process and using a feed stock consisting of a mixture of sawdust, pulp mill wastes, and municipal solid wastes indicates that a plant constructed for 25×10^6 gal/y of ethanol would require a capital of about \$ 69×10^6 and the selling price of ethanol would be about \$1.75/gal without taking the by-products credits which may be as much as 35 cents/gal.

Add on p. 155.

★ Humphrey and Nolan (10) have reported that the estimated costs of an enzymatic hydrolysis process based on the Gulf process and using a feedstock consisting of a mixture of saw dust, pulp mill wastes and municipal solid waste indicates that a plant constructed for 25×10^6 gal/y of ethanol would require a capital of about \$ 69×10^6 and the estimated selling price of ethanol would be about \$ 1.75/gal without taking the by-product credits which may be as much as 35 cents/gallon.

$ 1.5/kg depending on the carbon source used (376). More recent cost analysis shows that the production cost of glucose from cellulose by enzymatic hydrolysis is about $ 0.15/kg crude glucose syrup, and $ 1.45/gallon 95% ethanol based on the current cellulase process technology without taking any by-product credits (24). The improvement in cellulase productivity to a level of 150 - 200 IU/l/h and an increase in the utilization efficiency of cellulase for cellulose hydrolysis to a level of about 60 - 80% from the current level of 15% would make the cellulase technology very attractive and competative with other alternate technologies applicable to production of renewable carbon and energy resources (376). The considerable current interest in ethanol as a potential liquid fuel or fuel supplement has stimulated several kinds of research on improving this traditional fermentation process. For example, different techniques for improving productivity have been evaluated, including continuous culture with cell recycle (47, 356,357) and continuous vacuum fermentation (356-359). Another line of research has involved examination of different strains of the yeasts. S. cerevisiae and Saccharomyces uvarum chosen for such characteristics as their ability to ferment rapidly, to tolerate relatively high ethanol concentrations (upto 120 g/l, in batch culture), and to flocculate readily. Ethanol concentration is important with regard to distillation costs, whereas flocculation facilitates cell recycle. Still another line of research that is being pursued actively in the use of bacteria, Zymomonas mobilis for conversion of glucose to ethanol (375). Significantly high specific rates (2 - 3 times faster

than yeasts) of glucose uptake and ethanol production than those reported for yeasts have been reported (360). Cultures of *Zymomonas* have been reported which give yields of 1.8 - 1.9 moles of ethanol per mole of glucose under anaerobic conditions (361). Higher productivities (upto 120 g/l/h) in continuous fermentations with cell recycle (maximum reported values for yeast are 30 - 40 g/l/h) have been reported (362). Higher ethanol production than yeasts is due to its different carbohydrate metabolism. *Zymomonas* grows anaerobically and does not require the controlled addition of oxygen to maintain viability at high cell concentrations. Furthermore, genetic manipulation is simpler with bacteria than with yeasts, such as broadening the range of substrates utilization, selection of ethanol tolerant strains (363). Mutants of *Z. mobilis* have been obtained which are capable of fermentation in the temperature range of 40 - 45 °C (364).

Pentose sugars represent a significant fraction of the total fermentable carbohydrate content of biomass. A number of biochemical pathways are known for the conversion of pentose to ethanol and other neutral products. D-xylulose, an intermediate of D-xylose catabolism was observed to be fermentable to ethanol and carbondioxide in a yield greater than 80% by yeasts like *Candida utilis*, *Rhodotorula glutinis*, under fermentative conditions (365,366). In some yeasts, xylitol, in addition to ethanol, was produced from D-xylose.

The conversion of cellulose to ethanol requires the use of two biosystems - one to degrade cellulose to glucose and the other to convert glucose to ethanol (21). The beta-

glucosidase enzyme, which catalyzes the hydrolysis of cellobiose to glucose (the final step in cellulose hydrolysis) is sensitive to end-product inhibition (222,321,334,367). In a coupled saccharification/fermentation (CSF) for the production of ethanol from cellulose, the glucose concentration remains very low. It would therefore help in alleviating the problem of end product inhibition, and thus maintaining the reaction at a faster rate for a longer period of time.

An attempt for the direct fermentation of cellulosic materials to ethanol has been made with a thermophilic anaerobic bacterium Clostridium thermocellum which can hydrolyze and ferment cellulose to ethanol (41,368,369), in combination with a thermophilic pentose fermenting anaerobic, Cl. thermosaccharolyticum. This mixed culture has been shown to ferment both Solka Floc and corn stover to a mixture of ethanol, acetic acid and lactic acid (370). Gong et al. (371) have reported a fungus, Monilia sp., which can convert cellulosic materials to ethanol.

Screening for microorganisms which can convert cellulose to ethanol, i.e., those produce cellulase and also ferment glucose to ethanol are being carried out. More recently, attempts have been made to clone the cellulase encoding genes into E. coli HB101 via the plasmid PBR 322 (372). In this, total genomic DNA from T. reesei QM6a and the PBR 322 vector were separately restricted with Hind III endonuclease, ligated with T_4 DNA ligase and used to transform competent E. coli HB101 cells. Of the number of transformants, selected on the basis of antibiotic resistants, approximately 1% demonstrated the

phenotypic characteristics and about 50% of these contained inserted fragments ranging from 1.3 - 5.0 Kb (Kilobases) in size. One transformant exhibited the newly acquired ability to utilize cellobiose as the sole carbon source. The sequential cloning of genes encoding the cellulolytic enzymes and their transformation in fermentative yeast, will hopefully lead to the direct industrial conversion of cellulose to ethanol (370).

In our studies, the conversion of cellulose to ethanol in two stages i.e. saccharification of cellulose to glucose by S. rolfsii UV-8 mutant culture filtrate followed by fermentation of glucose to ethanol by yeast or Z. mobilis, was carried out. The feasibility of coupling the enzymatic saccharification of cellulose with the yeast fermentation was also investigated, and the data obtained by the two procedures compared.

RESULTS AND DISCUSSION

Organism

Due to the complexity of the system, conditions optimal for yeast fermentation are not necessarily the same as those optimal for the saccharification. Therefore, identifying the proper conditions to maximize the rate of reaction presents a problem, as for example, the optimum temperature range for fermentation is 28 - 35°C, while that for saccharification by S. rolfsii UV-8 mutant culture filtrate is 45 - 50°C. Efficiency of the overall process could possibly be increased by selection of the proper yeast strain, so that the optimum temperatures for saccharification and fermentation more closely coincide (20, 21). Keeping this in view, some of the yeast

strains, and a Z. mobilis strain were screened for their ability to grow at 40°C (Table 36).

It was not possible to carry out CSF with Z. mobilis as the available strain could not grow at 40°C. Therefore, the CSF was conducted using only yeast at 40°C. S. cerevisiae NCIM 3095 and Z. mobilis strain produced higher amounts of ethanol in two stage fermentation at 30°C than the other strains examined, including S. cerevisiae NCIM 3078 and C. tropicalis NCIM 3110 which showed good growth at 40°C and higher thermostability than S. cerevisiae NCIM 3095 and Z. mobilis. C. tropicalis NCIM 3110, though showed good growth at 40°C was less potent for ethanol production compared to 3078 (Table 37). All the experimentations in CSF were, therefore, carried out using NCIM 3078 at 40°C, unless otherwise mentioned.

Effect of factors which govern ethanol production

Size of yeast inoculum

The effect of yeast inoculum size on ethanol production was tested over a range of 10 x 10^7 to 40 x 10^7 cells/ml. The seed culture of S. cerevisiae NCIM 3078 used in this study was grown as described in Materials and Methods, harvested, and centrifuged at 2000 rpm for 15 min. The pellet was resuspended after two washings with sterile 0.9% KH_2PO_4 in approximately one tenth of the original volume. The experimental flasks were inoculated with increasing amounts of the yeast suspension. The ethanol production was measured by alcohol dehydrogenase method (239) after 96 h (Fig. 22). Increasing the initial yeast cell concentration from 10 x 10^7 to 40 x 10^7 cells/ml raised

TABLE 36: STUDY OF GROWTH OF DIFFERENT STRAINS OF YEAST AND Z. MOBILIS AT 40°C, pH 4.5

Organism	NCIM strain No.	Growth (by turbidimetric method[a,b]
Saccharomyces cerevisiae	3073	++
	3078	++++
	3090	-
	3095	-
	3107	-
	3186	+
	3203	+
	3204	-
	3205	-
	3288	++
	3304	++
	3316	+
	3318	+
	3429	+
	3441	-
S. cerevisiae var. *ellipsoideus*	3213	++
Candida tropicalis	3110	++++
Zymomonas mobilis	-	-

[a]- = No growth; + = Poor growth; ++ = Good growth; ++++ = Luxuriant growth; Incubation time 20 h.

[b]Biomass assay as described in Materials and Methods.

(a) For the determination of ethanol produced from cellulosics, the fermented broth (approx. 50 ml), obtained as described under b or c, is diluted to 150 ml with waterand distilled. First 100 ml distillate was collected and ethanol analyzed by ADH method (239).

(c) In CSF, both saccharification and fermentation are simultaneously carried out, first at 40°C, for 24 hours. These flasks were then incubated at 30°C (optimum for ethanol production) for further periods indicated under identical conditions as described in (b).

TABLE 37: STUDY OF ALCOHOL PRODUCTION IN TWO STAGE AND COUPLED SACCHARIFICATION/FERMENTATION SYSTEM BY YEAST AND Z. MOBILIS

Organism	Temp. °C	% Ethanol[a] (w/v) in two stage fermentation 96 h[b]	% Ethanol (w/v)[a] in CSF[c]			
			48 h	72 h	96 h	120 h
S. cerevisiae NCIM 3078	40 and 30	2.3	2.5	2.7	3.5	3.6
S. cerevisiae NCIM 3095	30	2.6	-	-	-	-
C. tropicalis NCIM 3110	40 and 30	2.0	2.4	2.6	3.1	3.3
Z. mobilis[b]	30	2.6	-	-	-	-

[a]The fermented broth (approx. 80 ml) is made to 150 ml with water and distilled to 100 ml and further analyzed by ADH method.

[b]12 gms of AT rice straw (incubated with 2 N NaOH, 30°C, 48 h) + 4 ml 1.0 M Citrate buffer, pH 4.8 + 64 ml culture filtrate + water to 80 gms. Incubated at 50°C for 48 h. Supernatant after 48 h + 10 ml nutrients (yeast extract, 0.85%; $MgSO_4.7H_2O$, 0.01%; $(NH_4)_2SO_4$, 0.132%; $CaCl_2.2H_2O$, 0.006%) are used for fermentation for 96 h in case of yeast strain and 48 h in case of *Z. mobilis* strain.

[c]CSF is carried at 40°C for first 24 h and then at 30°C for further periods indicated under identical conditions as described in (b).

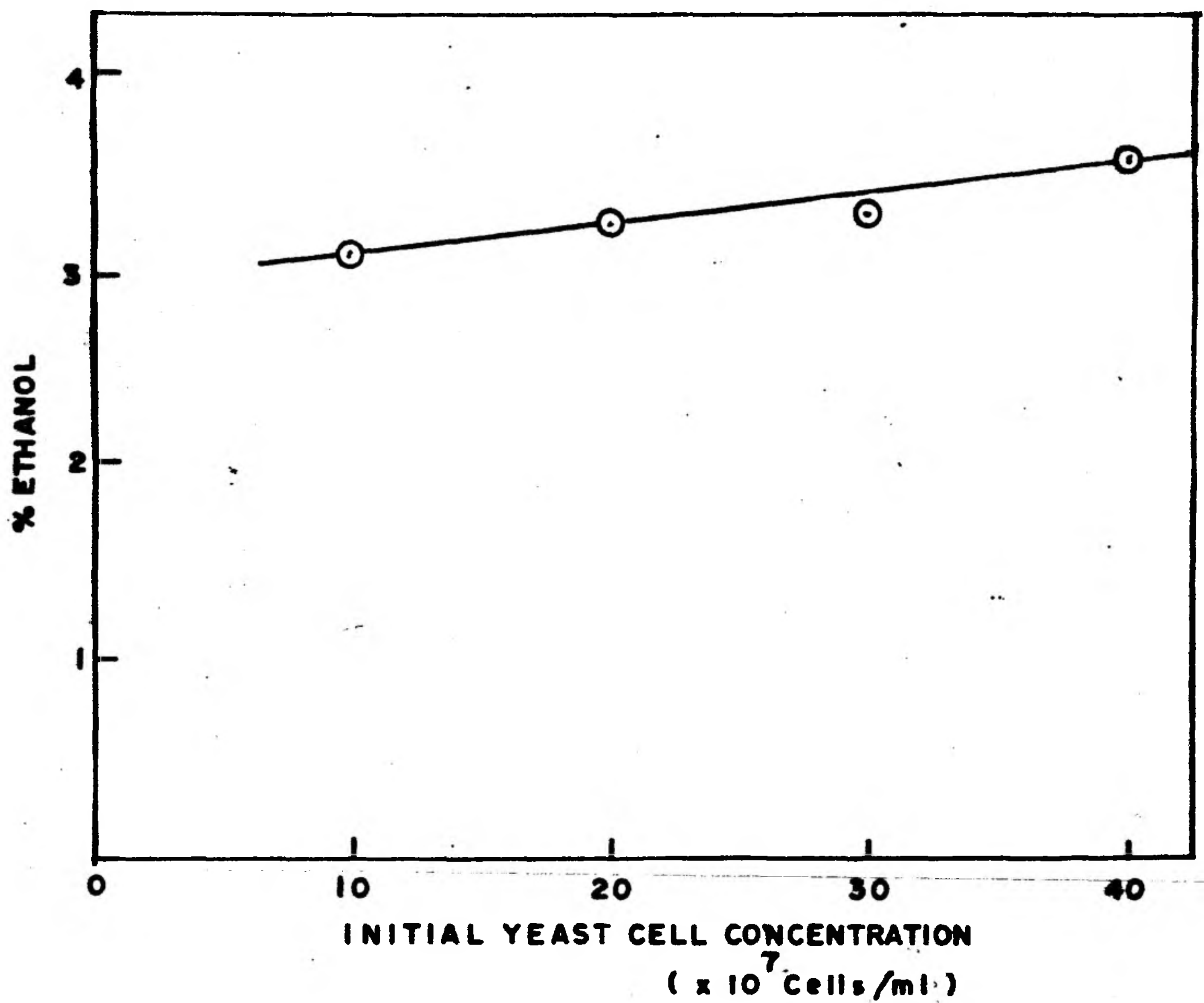


FIG.22. EFFECT OF INITIAL CELL CONCENTRATION ON CSF SHAKE FLASK EXPERIMENT AT 40°C FOR 24 hrs AND AT 30°C FOR 72 hrs, SUBSTRATE; 15% AT RICE STRAW.

the ethanol yields from 3.1% to 3.6% (w/v).

Culture filtrate

S. rolfsii UV-8 mutant culture filtrate concentrations ranging from 20 to 80% (v/w) were used to study their effect on the production of ethanol. Increasing the amount of culture filtrate resulted in increased yields of ethanol from 1.5 to 3.6% (w/v) as determined by ADH method (239). The increase was not linear, however, indicating that the substrate became limiting (Fig. 23).

Effect of ethanol concentration on cellulase activities

Since ethanol is the major product of yeast fermentation, its effect upon cellulase activity was investigated. Different concentrations of ethanol (upto 10%) were used to study the inhibition of cellulase, xylanase and beta-glucosidase activities and saccharification. Ethanol concentrations studied inhibited the production of reducing sugars in saccharification of AT rice straw. However, ethanol upto 10% concentration did not affect the FPA and CMCase but xylanase was strongly inhibited. Beta-Glucosidase, though showed inhibition above 5% ethanol, it showed enhancement in the activity with lesser ethanol concentration (Fig. 24).

Effect of substrate concentration on coupled saccharification/ fermentation

Saccharification/fermentations were carried out at various concentrations of AT rice straw (AT = 2 N NaOH at 30°C for 48 h) ranging from 3 to 15%. The use of increased substrate concentration resulted in increased amounts of ethanol (Fig. 25). Although the total amount of ethanol produced increased, the

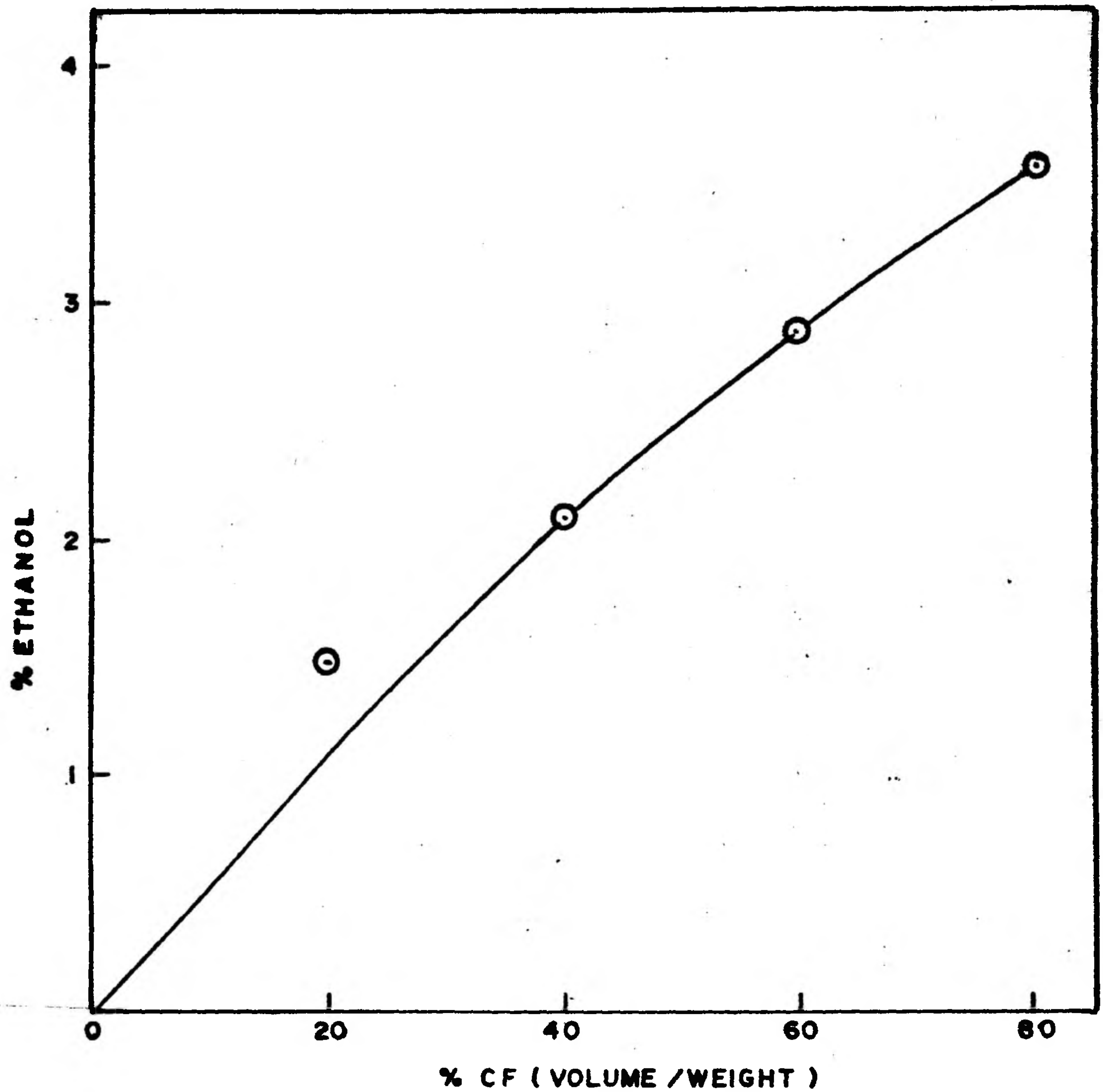


FIG.23. ETHANOL PRODUCTION Vs % CULTURE FILTRATE (UV8)
EFFECT OF ENZYME CONCENTRATION ON CSF IN SHAKE FLASK AT 40°C 24 hr AND AT 30°C FOR 72 hr. SUBSTRATE; 15% AT RICE STRAW. ACTIVITIES OF UNDILUTED CULTURE FILTRATE: FPA, 1·94 IU/ML; β-GLUCOSIDASE, 21 IU/ML

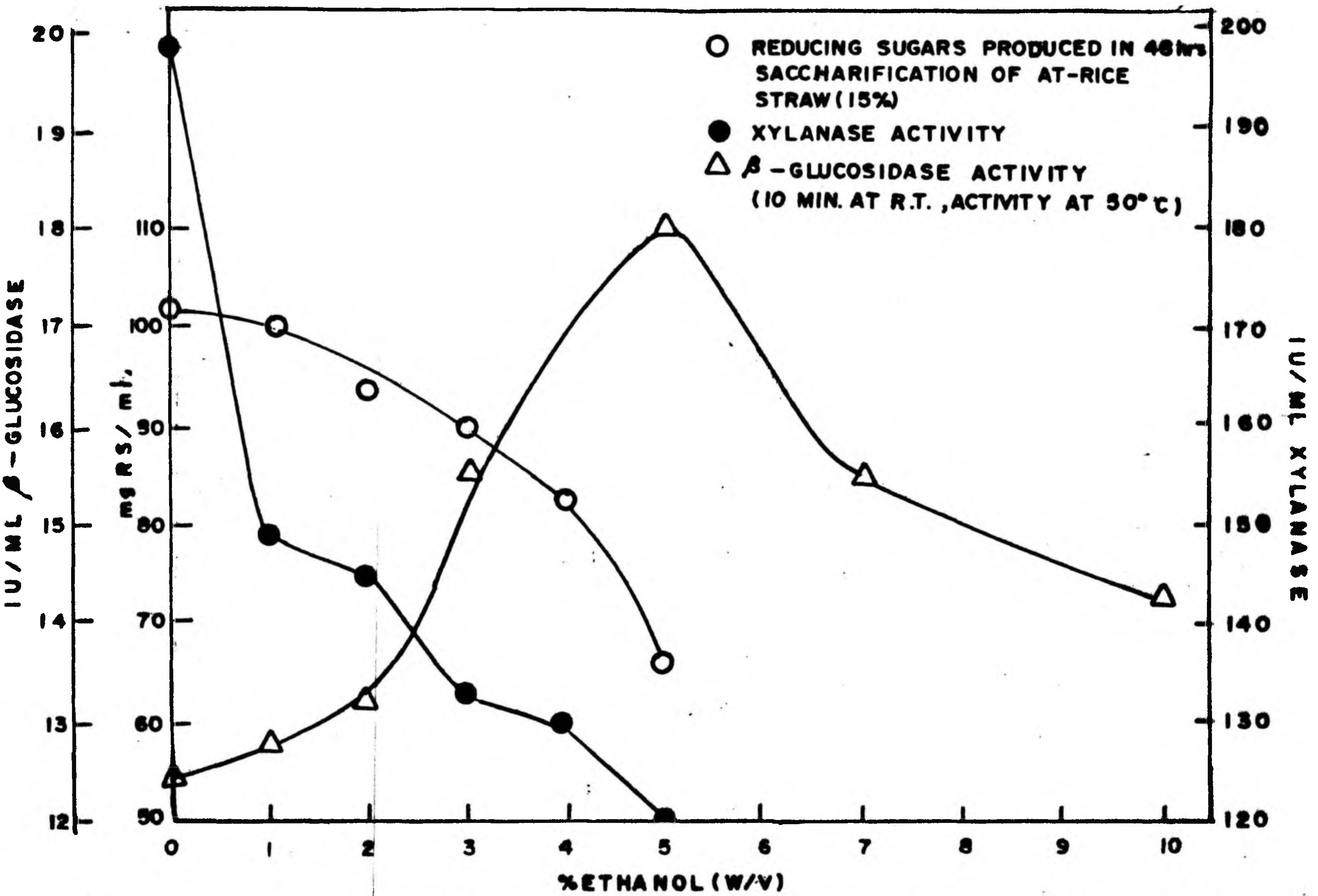


FIG.24. EFFECT OF ADDITION OF ETHANOL ON SACCHARIFICATION, XYLANASE AND β-GLUCOSIDASE ACTIVITY. ETHANOL AT CONCENTRATION INDICATED WAS ADDED AT THE BEGINNING OF THE EXPERIMENT.

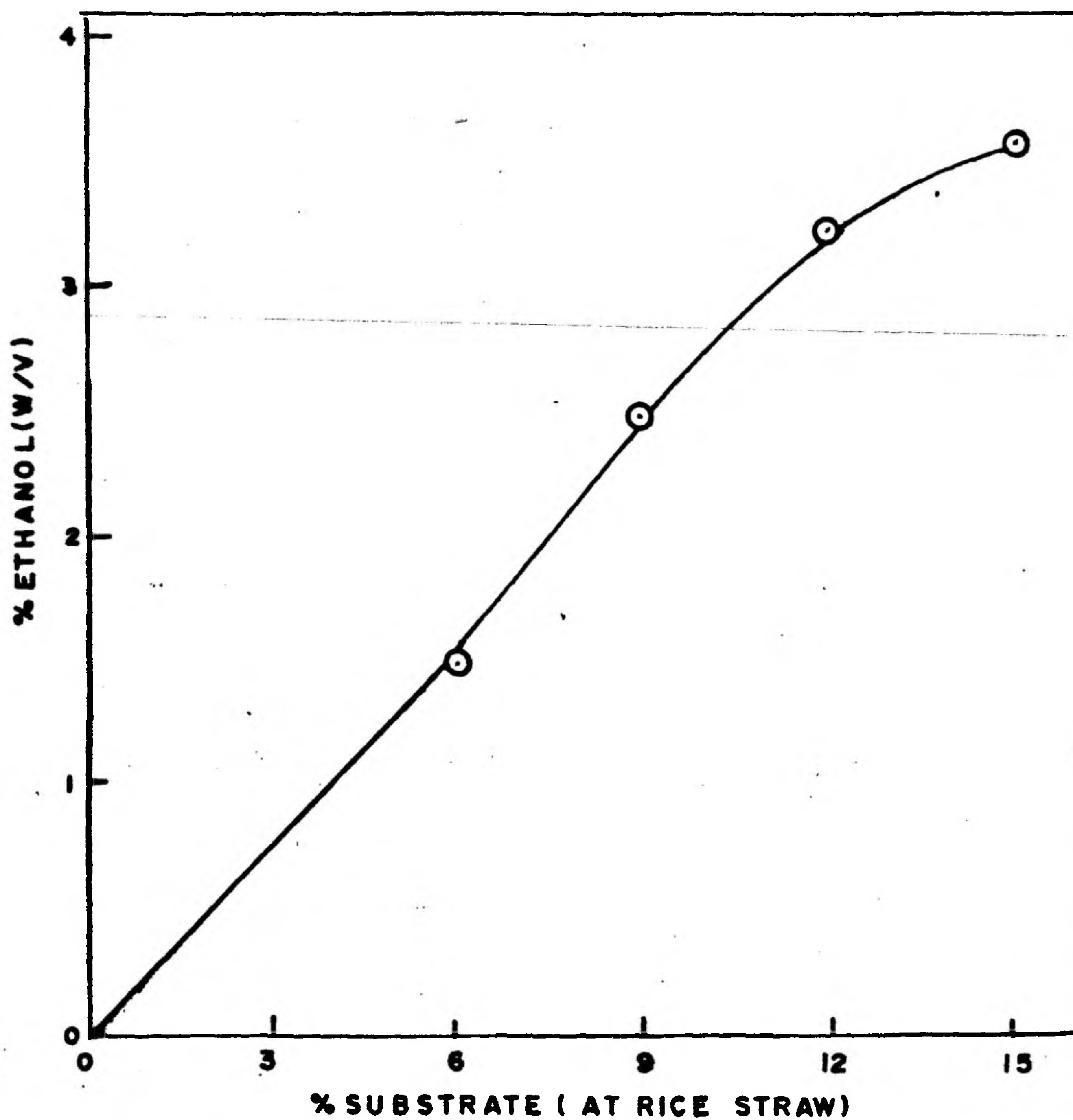


FIG 25 THE EFFECT OF SUBSTRATE CONCENTRATION ON CSF SHAKE FLASK EXPERIMENT AT 40°C FOR 24 hrs AND 30°C FOR 72 hrs. SUBSTRATE : AT RICE STRAW; YEAST CELL CONCENTRATION 40 x 10^7 CELLS/ML (S. CEREVISIAE NCIM 3078)

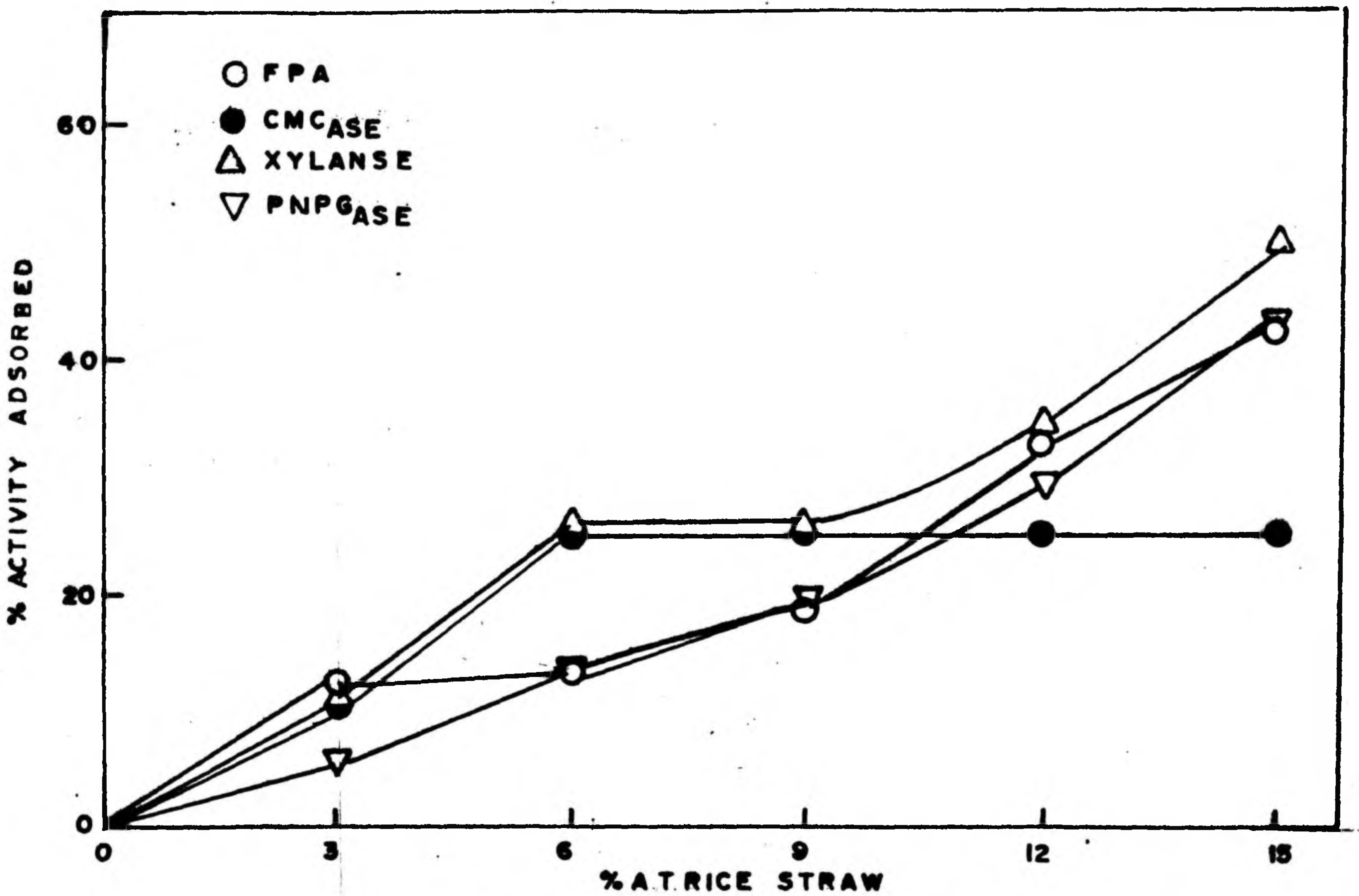


FIG 26 ADSORPTION OF CELLULASE, β-GLUCOSIDASE AND HEMICELLULASE ON CELLULOSE. THE UV-8 CULTURE FILTRATE WAS INCUBATED WITH CELLULOSE AT 40°C, FOR 20 MIN.

of the theoretical.

The beta-glucosidase enzyme which catalyzes the hydrolysis of cellobiose to glucose in the final step of saccharification is sensitive to end product inhibition (321,334,367). In CSF the glucose concentration remains very low. It would therefore help in alleviating the problem of end product inhibition and thus maintaining the reaction at a faster rate for a longer period of time.

In our studies, in two stage process, i.e., saccharification followed by fermentation of AT rice straw, *Z. mobilis* strain produced 2.6% (w/v) ethanol in 48 h, while *S. cerevisiae* NCIM 3095 strain produced the same amount of ethanol in 96 h, under identical conditions. Temperature plays an important part particularly where CSF is contemplated (361) because the optimum saccharification temperature is 45 - 50°C. Since the *Z. mobilis* strain available in the NCIM could not grow at 40°C, it was not possible for us to run CSF with *Z. mobilis*. Therefore, a mutant of *Z. mobilis* which can grow at 40 - 45°C would considerably help in lowering the cost of ethanol production by reducing the overall saccharification/fermentation period. Such mutants in fact have already been reported (364).

Glucose or cellobiose, except in the initial stages of the process, was either not detected or was present in trace amounts in the coupled system. Therefore, the rate-limiting step appears to be the saccharification of cellulose to glucose.

Also a wide range of *in vivo* and *in vitro* techniques developed for the genetic manipulation of bacteria can be applied in broadening the range of substrates utilized by

Zymomonas, and this would be a major breakthrough in ethanol production. In relation to this it is interesting to note that the P group plasmids can be transferred by conjugation from E. coli and Pseudomonas aeruginosa into several Z. mobilis strains (363).

Roychoudhury et al. (374) have reported a yeast strain Pichia etchellsii which can produce 32 g/l of ethanol from 140 g/l initial cellulosic substrate concentration in a coupled saccharification/fermentation system at 40°C in a period of 58 h, using T. reesei QM9414 culture filtrate for saccharification. It would be possible to obtain even higher ethanol concentrations in 24 - 60 h using Pichia etchellsii or Z. mobilis mutant which can grow at 40 - 45°C and S. rolfsii UV-8 mutant culture filtrate in CSF.

PART V

PURIFICATION OF XYLANASE OF S. ROLFSII UV-8 MUTANT

SUMMARY

The endoxylanase from the culture filtrate of S. rolfsii UV-8 mutant has been purified by ammonium sulfate precipitation, ion exchange chromatography (DEAE Sephadex A-50) and preparative isoelectric focusing in sucrose density gradient. The purified enzyme preparation gave a single protein band in SDS-gel electrophoresis and in disc gel electrophoresis at pH 8.9 and 4.3. Isoelectric focusing in 7.5% polyacrylamide gel also showed only one protein band. The enzyme has a molecular weight of approximately 54,300 and is composed of one polypeptide chain. The purified xylanase is free from CMCase, beta-glucosidase and beta-xylosidase activities.

The enzyme shows maximum activity on xylan (1,4-beta-linked-D-xylopyranose units) at pH 4.5 and at 65°C. The Km is 5.8 mg of larch xylan/ml. The molecular activity is 2.29 x 10^5 min^{-1}, assuming only one active site. The viscometric and other studies reveal the non-arabinose-liberating endomechanism of the purified xylanse from S. rolfsii UV-8 mutant.

INTRODUCTION

The terms "hemicellulases" or hemicellulose-degrading enzymes refer to those enzymes (glycan hydrolase, EC 3.2.1) that specifically degrade only hemicelluloses, and do not include the glycosidases (e.g. alpha-L-arabinofuranosidase, alpha- and beta-galactosidases, beta mannosidases, alpha- and beta-D-xylosidases), which, in addition to their activity on glycosides of low molecular weight, are also frequently capable of hydrolyzing the short chain or monosaccharide appendages from the main backbone chain of hemicelluloses. Typical hemicellulases, therefore, are the D-xylanases, L-arabinanases, D-galactanases and beta-mannanases (310).

For the past several years intensive investigations have been carried out to unravel the secrets of the enzymatic hydrolysis of cellulose. More recently the enzymatic breakdown of lignin has attracted a lot of interest. Comparatively neglected has been the enzymatic hydrolysis of the third major component of cellulosic materials, hemicellulose. No breakdown of cellulosic materials is likely to be economically feasible without incorporating a means to hydrolyze the hemicelluloses that my comprise upto 30% of the total dry weight. This search has gained new emphasis with the sudden interest in xylitol. Xylitol has been finding increasing use in chewing gum. A better method for the production of xylose could significantly reduce both its cost and that of xylitol.

The cellulose of lignocellulosics is not effectively hydrolyzed by cellulases owing to restricted availability of the cellulosic surface which have been implicated to be

cross-linked with hemicellulose. P treatment by purified *A. wentii* xylanase has been reported to create cellulosic surfaces more accessible which are then more effectively acted upon by *T. reesei* exo- and endo-glucanases. This results in enhanced rates of production of reducing sugars (333). The mode of action of hemicellulases towards hemicelluloses of different origins and their participation with cellulases in the overall degradation of cellulosic material needs detailed study. Perhaps, an understanding of the entire complex process of cellulose, hemicellulose and lignin degradation will give a much better insight as to how these should be most effectively utilized.

Xylanase ((1→ 4)-beta-D-xylanase) is an important hydrolytic enzyme which hydrolyzes (1→ 4)-beta-D-xylopyranosyl linkages of the D-xylans, namely, arabinoxylan, arabinoglucuronoxylan, arabino-4-O-methyl-D-glucuronoxylan, and glucuronoxylan. D-Xylanases of this type have been assigned the Enzyme Commission numbers EC 3.2.1.8 for (1→ 4)-beta-D-xylan xylano hydrolase, endo-xylanase and EC 3.2.1.37 for (1→ 4)-beta-D-xylan xylohydrolase, exo-xylanase, respectively.

D-xylanases have been reported to occur in bacteria from marine and terrestrial environments, yeasts, fungi, rumen bacteria and protozoa, insects, snails, marine algae and germinating seeds of terrestrial plants (310). Most of the bacteria, yeast and fungi produce D-xylanases that are secreted extracellularly. However, some microorganisms like rumen bacteria (377-380), *Sporocytophaga myxococcoides* (381) and *A. niger* (382) also produce D-xylanases intracellularly.

There is a considerable controversy as to whether D-xylanases of bacteria and fungi are produced inductively in response to the carbon source on which they are grown or constitutively. Thus, Lyr (383-385), using several species of wood rotting fungi such as *Trametes versicolor*, *Collybia velutipes* *Coniophora cerebella*, *A. niger*, *Chaetomium globosum*, *Fomes igiarus*, demonstrated that D-xylanase was always produced extracellularly no matter what polymer (e.g. cellulose, D-xylan, D-mannan or pectin) was used as the carbon source. Stevens and Payne (335a) reported that the yeast *Trichosporon* produced xylanase activities upto 8 times higher than its cellulase activities when grown in the ball-milled filter paper medium. Eriksson and his associates (325, 386) found that D-xylanase was produced by *Stereum sanguinolentum* (325) and *Chrysosporium lignorum* (386) when a cellulose preparation free from D-xylan was used as the sole carbon source. Other carbon sources that have been reported to produce constitutive D-xylanase from fungi include glycerol and glucose in the case of *Myrothecium verrucaria* (387, 434), sophorose for *T. viride* (388), D-xylose for *Diplodia viticola* (389), dextrin for *Termitomyces clypeatus* (390). However, the presence of a D-xylan in the mycelium of *S. sanguinolentum* suggests that the formation of D-xylanase by this organism, when grown on cellulose, may be self-induced; that is, D-xylanase is induced for the lysis of the old cell-wall material, which then supplies the organism with an endogenous supply of energy (391). The production of D-xylanase activity by certain microorganisms during growth on cellulose may be due to the multisubstrate activity of the

cellulases. Several other workers have also reported (389, 392-394) that xylanases were induced when fungi were grown on media containing D-xylan as the carbon source. Formation of endo-1,4-beta-xylanase in the yeast *Cryptococcus albidus* has been shown to be a good example of inducible synthesis of an extracellular enzyme (394a). The enzyme is produced in high amount only during growth of cells on xylan. Xylobiose was considered to be the natural inducer of xylanase as it was not hydrolyzed extracellularly. Xylobiose was effective as an inducer at low concentrations and constant availability to cells. The induction of beta-xylanase could also be achieved by non-utilizable methyl beta-D-xylopyranoside (394a).

Bacterial D-xylanases appear to be usually inducible (392, 395) but they have also been shown to be produced constitutively (381, 396).

Not much is known about the mechanisms involved in the break down of xylan mainly because many xylanases take part in a synergistic relationship (396a) and few xylanases have been purified to homogeneity (396b). The xylanase complex from *Aspergillus* has been studied in some detail to determine the roles of most of its members. Endo-D-xylanases and beta-D-xylosidases (beta-D-xyloside xylohydrolase) have been characterized and have been shown to be involved in xylan hydrolysis. Beta-D-Xylosidase hydrolyzes beta-(1→4) bonds at the nonreducing termini of xylooligosaccharides and cleaves beta-(1→4) xylopyranosides. Beta-D-Xylosidase has been immobilized (396c, 396d). High efficiencies and stabilities were obtained when beta-D-xylosidase was linked

to alumina with $TiCl_4$ and to alkylamine silica with glutaraldehyde (396c).

D-xylanases having action pattern of the exo-type have been claimed to be produced extracellularly by *A. batatae* (397), *A. niger* (399), *A. foetidus* (398) and *C. cerebella* (400) but these are not homogeneous. None of the xylanases whose major product is dimer attacking the chain from the nonreducing end (such as by beta-amylase and cellobiohydrolase in similar hydrolase families) has been unequivocally identified to date. Frederick *et al*. (396b) reported the purification of a xylanase from *A. niger* to homogeneity (400a, b, 417) and labeled it as a xylobiohydrolase (400 b) on the basis that its main xylan hydrolysis products were xylobiose and xylotriose. However, more recently Frederick *et al*. (396 b) characterized this enzyme as endoxylanase as demonstrated by its extremely rapid production of xylooligosaccharides from soluble xylan. The enzyme hydrolyzed over 75% of the soluble xylan to molecules of six saccharide units or less which were eventually hydrolyzed to xylobiose and xylose. The enzyme hydrolyzed xylotriose slowly to xylose and xylobiose and xylotetraose rapidly to xylobiose, with smaller amounts of xylotriose and xylose. Xylobiose was completely resistant.

The anomeric configuration of the products was inverted in contrast to the behaviour of most other carbohydrases that initially produce mixtures of oligosaccharides. This property where configuration of the products is inverted is more commonly ascribed to exohydrolases (400 c). Claims of exo-action properties, therefore, must be interpreted with

caution (310). Some investigators consider that since xylans are highly branched, there is no need for xylanases of the exo-type as their action will be impeded when their action on xylans encounters a branch point or substituent sugar (400 d).

Most of the fungi are the common source of endo-1,4-beta-D-xylanases and enzyme preparations from them have been more extensively studied than those from any other genera. Various endoxylanases have been isolated from strains of Aspergillus (396 b, 400 e, f, 404, 424), Trichoderma (339a, 400 g, h), Trametes (400 i, j), Schizophyllum (409), Talaromyces (396 a). Hong and Han (400 k) reported xylanase production by Pleurotus ostreatus in rice straw medium. Increased xylanase production was observed by incorporating xylan in the medium in the case of Streptomyces flavogriseus (400 l). Loginova et al. (400 m) reported an enzyme preparation, designated as Tselloterrin, containing xylanase from thermotolerant fungus A. terreus 17 P. Other potent xylanase producers reported are : Poria placenta (400 n), Polyporus circinatus (400 o), Thermomonospora (400 p), A. oryzae (400 q), Fusarium avenaceum (412 a), Aspergillus and Trichoderma species (400 r).

The endo D-xylanases can be divided into two groups, namely, (1) those that liberate L-arabinose from arabinoxylans and arabinoglucuronoxylans, e.g. xylanase of A. terreus 17 P (401) (i.e. the arabinose-liberating xylanases), and (2) those that do not liberate L-arabinose from these subst ites (i.e. non-arabinose-liberating xylanases). Both groups are also capable of degrading glucuronoxylans and D-xylans (310).

Arabinose-liberating endo-D-xylanases :

D-Xylanases are glycan hydrolases capable of hydrolyzing (1→ 4)-beta-xylopyranosyl linkages of the hemicellulose, D-xylan. However, several D-xylanases, isolated from *Agaricus bisporus* , *A. niger*, *Ceratocystis paradoxa* and *D. viticola* have also been shown to be capable of hydrolyzing the (1→ 3) alpha-L-arabinofuranosyl branch points of arabinoxylans (402). The crude enzyme preparation from *D. viticola* (389) was capable of liberating L-arabinose from corn cob and grape arabinoxylans, but when purified, degraded D-xylans to xylodextrins of d.p. 2 to 5, yielding no arabinose. It appears, therefore, that the crude enzyme preparation contains either two different D-xylanases, one of which is capable of hydrolyzing the arabinose substituent, or a D-xylanase and an alpha-L-arabinofuranosidase (or an arabinanase) (403).

Conrad (400 e) studied the time course enzymatic saccharification of arabinoxylan by the xylanolytic system of *A. niger* 110.42. In first 10 min at 40° C only higher xylooligosaccharides were observed, and in 4 h higher amounts of xylobiose, D-xylose and L-arabinose were detected. At the end of 24 h incubation period, D-xylose and L-arabinose accounted for 85% and 92%, respectively. The occurrence of L-arabinose in the hydrolyzate was interpreted to suggest the possible presence of 1,3-alpha-L-arabinosidase or a specific xylanase which can attack the 1,3-alpha-arabinofuranosidic linkage of the substrate. The D-xylanase system of *A. niger* (382) was found to consist of two different D-xylanases (I and II). D-Xylanase I degraded arabinoxylan (rice straw) to D-xylose, L-arabinose, and a mixture of

xylodextrins. Xylobiose and xylotriose were not attacked, but the arabino-xylotriose was hydrolyzed to L-arabinose and xylotriose. Both D-xylanases always liberated L-arabinose from the degradation of arabinoxylan. *A. niger* van Tieghem (402) produced three D-xylanases, I, II and III, two of which (II and III) degraded rice straw arabinoxylan to D-xylose, L-arabinose, and a mixture of arabinoxylo- and xylooligosaccharides. Five different xylanases and a beta-D-xylosidase were purified from *A. niger* culture broth to homogeneity by John *et al.* (404). The xylanases have molecular weihgts in the range 31,000 to 50,000, and the xylosidase has a molecular weight of 73,000. Characteristic action pattern with each of the purified xylanases with xylan and other properties revealed that the five xylanases were different enzymes. The action of all purified xylanases on xylan produced series of oligosaccharides, indicating that they act by an endomechanism. The enzymes degraded arabinoxylan producing L-arabinose, D-xylose and xylobiose, and a mixture of branched arabino-xylose and D-xylose oligosaccharides. All xylanases seemed to be capable of liberating L-arabinose from either arabino-xylan or the arabino-xylose oligosaccharides. The occurrence of L-arabinose in the digests suggests that all purified xylanases are capable of hydrolyzing 1,3-alpha-L-arabinofuranosyl branch points. Branched arabinose-containing D-xylose oligo-saccharides were hydrolyzed slowly so that these sugars accumulate in the digest. Two of the xylanases also hydrolyzed crystalline cellulose. Beta-D-xylosidase hydrolyzed xylotriose more rapidly and to a much greater

extent than xylobiose, whereas no significant degradation was found with branched arabino-xylotetraose. Xylosidase activity was inhibited by D-xylose, *p*-hydroxymercuribenzoate and iodoacetamide. This was interpreted to suggest the possible involvement of -SH group(s) in the catalytic activity. Gorbacheva and Rodionova (400 f) reported the purification of endo-1,4-beta-xylanase from *A. niger* 14 and studied its action pattern. The enzyme was homogeneous in polyacrylamide gel electrophoresis, ultracentrifugation and isoelectric focusing. The viscosity of the solution of carboxymethyl xylan was very rapidly reduced on incubation with the purified endo-1,4-beta-xylanase whilst the reducing sugars were released insignificantly. The degradation of xylan depended on the number and position of side branches in the molecule. The end products of hydrolysis which accumulate also impeded the action of the enzyme. The final neutral hydrolysis products were xylobiose, xylotriose and xylose. Traces of arabinose were also found during the hydrolysis of arabinoglucuronoxylans. The enzymes degraded xylotriose to xylobiose and xylose; xylotetraose to xylobiose and minor quantities of xylose. The degradation rate of xylooligosaccharide increased with increase in the length of the carbohydrate chain. Oguntimein *et al.* (405) have obtained four different partially purified endo-xylanases from *A. niger* which liberated xylose, arabinose and number of xylodextrins from larchwood xylan. Frederick *et al.* (396b) purified a xylanase from a commercial *A. niger* pentosanase which hydrolyzed xylotriose slowly to xylose and xylobiose, and xylotetraose was broken down to xylobiose. Higher

xylooligosaccharides were hydrolyzed rapidly to a mixture of shorter xylooligosaccharides, chiefly xylotriose, xylobiose and xylotetraose, with xylobiose being the predominant final product.

C. paradoxa has been shown (406, 407) to produce several D-xylanases, one of which, HCI, was found to liberate L-arabinose from spear grass (Heteropogon contortus) and sugarcane bagasse hemicellulose B (arabino-4-O-methyl glucuronoxylans) and from wheat endosperm arabinoxylan. In the case of Trametes hirsuta (400 j) xylanase, the main products of D-xylan from white willow were xylotetraose, xylohexaose, aldotetraouronic acid and aldohexaouronic acid. The action pattern of this xylanase corresponds to that of a typical endoenzyme, which acts more readily in the middle of chain and the specific region of its action appears to involve 5 D-xylosyl residues. The products revealed a regular distribution of 4-O-methyl-D-glucopyranosyl uronic acid groups attached to the D-xylan backbone. The xylanase of T. hirsuta was found to be highly specific for (1→ 4) linkage; (1→ 3)-beta-D-xylan was not attacked by the enzyme. Beta(1→ 4), alpha(1→ 4), beta(1→ 3) and beta (1→ 6) glycosidic bonds of various non-xylan polysaccharides were also resistant. This xylanase did not show any action on p-nitrophenyl-beta-D-xyloside. The xylotriose was the lowest homologue of xylose attacked by xylanase to give xylobiose and xylose. From xylotetraose xylobiose and from xylopentaose, xylotriose and xylobiose were obtained by xylanase action. The xylanases of Talaromyces byssochlamydoides YH-50 were separated and purified into 3

components as X-a, X-bI and X-bII (396 a). The purified components were homogeneous in disc gel electrophoresis. The X-a xylanase hydrolyzed xylan to xylose, arabinose, xylobiose and other xylooligosaccharides, while X-bI and II hydrolyzed xylan to xylose and xylobiose, respectively. Synergistic incubation of all three components hydrolyzed xylan to the extent of 90% xylobiose.

Non-arabinose-liberating endo-xylanases:

This group of D-xylanases, constituting majority of the known fungal endo-D-xylanases, usually degrades arabinoxylan and other D-xylans to D-xylose, xylodextrins and in some cases, oligosaccharides containing both L-arabinose and D-xylose. These enzymes have been isolated in highly purified form from various strains of *A. niger* (382, 402), *C. paradoxa* (406, 407), *D. viticola* (389), *S. sanguinolentum* (391), *T. viride* (408), *T. clypeatus* (390), *S. commune* (409, 410), Gliocladium virens (411), *Tyromyces palustris* (412). Most of these D-xylanase preparations, with the exception of that from *D. viticola* (389), degraded D-xylan randomnly liberating xylose as well as xylodextrins. The absence of D-xylose from the enzymic hydrolyzates from the latter source may be due to the fact that samples were only assayed in the early stages of hydrolysis (2 [illegible]). [illegible] of *C.* [illegible] xylanase HCII on arabinoxylo- and xylodextrins indicated that the attack occurs at the reducing end of the oligosaccharide chain (406,407)

Biely *et al.* (433) reported the elaboration of two inducible enzymes, endo-1,4-beta-xylanase and a cell-bound xylosidase (but no cellulolytic enzymes) from yeast *C. albidus*, when grown on wood. The endoxylanase initially hydrolyzed

xylan to a series of 1,4-beta-xylooligosaccharides of various lengths. The ultimate products of hydrolysis were xylose, xylobiose and xylotriose predominantly. The enzyme was unable to hydrolyze xylobiose. The enzyme decreased the specific viscosity of carboxymethyl xylan solution by 50% after splitting 3 - 4% of the glycosidic bonds of the polysaccharide. The purified endoxylanase, though still not homogeneous, has a specific activity of 33 units/mg protein (1 unit = μ mol reducing sugars liberated as xylose equivalent per min). The endoxylanase also exhibited the ability to split the aryl xyloside. This is rather an unusual property of an endo-xylanase.

The molecular structure of xylanases has not been examined and very little is known about the active site of xylanases. Jadwiga and Urbanck (412a) reported that N-bromosuccinimide (NBS) and 2-hydroxy-5-nitrobenzyl bromide (HNB) cause strong inactivation of *F. avenaceum* xylanase. Inactivation by NBS and HNB suggested the presence of tryptophan residues at the active centers of xylanase. Inactivation of xylanase by tetranitro-methane at pH 8.0, when nitration of tyrosine residues occurs, suggested that tyrosine may also be involved in the catalytic mechanism of xylanase from *F. avenaceum* (412a). John *et al*.(404) reported that *p*-hydroxymercuribenzoate and iodoacetamide inhibited *A. niger* beta-D-xylosidase suggesting the involvement of -SH group(s) in the catalytic activity.

Chanzy *et al*.(412 b) examined the interaction of xylanases on well-characterized beta- (1→ 4) xylan single crystals by electron microscopy and electron diffraction in order to understand the mode of action of these enzymes on

their native solid substrates. Xylanases are able to degrade well-crystallized lamellar crystals into water soluble xylodextrins. They suggested that the enzyme attack takes place at the edge of the crystals and progresses towards their centers. Such a behaviour is consistent with an endo-enzyme mechanism, where the enzyme interacts essentially with the accessible xylan chains located at the crystal periphery. According to them, the hypothesis that the enzyme action would start from the amorphous material located in the crystal lamellae and would progress at random perpendicular to the lamellar plane seems unlikely. Even if limited enzymatic attack occurs at the surface, it seems difficult for the enzymes, which are 25 - 75 A^{o} in diameter, to penetrate between xylan chains only separated by a few angstroms (412 c). The crystal with its hexagonal packing of the xylan chain seems very compact and would not allow much penetration.

Previous screening experimens indicated that UV-8 *S. rolfsii* mutant is a potent producer of cellulase and xylanase in submerged culture. In this Chapter the purification of an endoxylanase from the culture filtrate of UV-8 mutant of *S. rolfsii* and study of some of its physico-chemical properties are described.

xylanase from the culture filtrate of UV-8 mutant of *S. rolfsii* and study of some of its physico-chemical properties are described.

RESULTS

The *S. rolfsii* UV-8 mutant produces an extracellular xylanase. The capability of various substrates to induce xylanase has been described (Part III, Chapter 2). The strongest inducer for xylanase and also for cellulase (315) for UV-8 mutant of *S. rolfsii* is Cellulose-123. Only a low constitutive activity of xylanase is detected when grown on glucose. The enzyme, except a minor amount, apparently is not produced constitutively by the fungus.

Preparation of the crude extract

The *S. rolfsii* UV-8 mutant was grown on NM-4 medium (315) with Cellulose-123 as the carbon source as described under Materials and Methods. After cultivation of the fungus for 14 days, the mycelium was removed from the culture solution by centrifugation at 3000 rpm for 20 min.

Enzyme purification

The following operations were carried out at 0 - 4 °C, unless otherwise indicated. The xylanase, CMCase, beta-glucosidase and protein assays were used for monitoring the column effluents as described in Materials and Methods. Analytical polyacrylamide gel electrophoresis was used to follow the enzyme purity.

Step 1: Ammonium sulfate precipitation

The clear solution obtained after centrifugation, usually 2 - 3 litres per batch, was concentrated by precipitating the proteins with solid ammonium sulfate at 90% saturation. The

precipitate was suspended in a small volume of 0.05 M citrate buffer, pH 4.8 and stored at -15°C until used. Recovery of xylanase and CMCase was around 80%.

Step 2: Ultrafiltration

The ammonium sulfate precipitate was further concentrated by Diaflo membrane PM-10 ultrafiltration system (Amicon Corpn., U.S.A.). Recovery of xylanase and CMCase was around 40 - 50%.

Step 3: Fractionation by gel chromatography

The Amicon concentrate of enzymes (from Step 2) was gel filtered on Sephadex G-75 column (3 x 90 cm) for desalting and fractionation. The elution pattern is shown in Fig. 27. The beta-glucosidase (Fraction A) was eluted after the void volume and ahead of cellulase (Fraction B). Fraction A (F 5-16) contained major beta-glucosidase activity whereas Fraction B (F 17-29) contained xylanase and CMCase activities.

Step 4: Separation of xylanase by DEAE-Sephadex A-50 ion exchange chromatography

The Fraction B was concentrated by lyophilization and was dialysed in a collodion bag for 3 - 4 h against 0.05 M phosphate buffer, pH 7.3 with two changes of the buffer. The dialyzed material was chromatographed on DEAE-Sephadex A-50 column (1.8 x 100 cm), previously equilibrated with 0.05 M phosphate buffer, pH 7.3. The column was washed with the same buffer. Fractions (2 ml) were assayed for xylanase, CMCase and beta-glucosidase as well as for protein. Results are summarized in Fig. 28. Xylanase, CMCase and beta-glucosidase activities were not adsorbed on the column. Xylanase and beta-glucosidase came just after the void volume and CMCase came later.

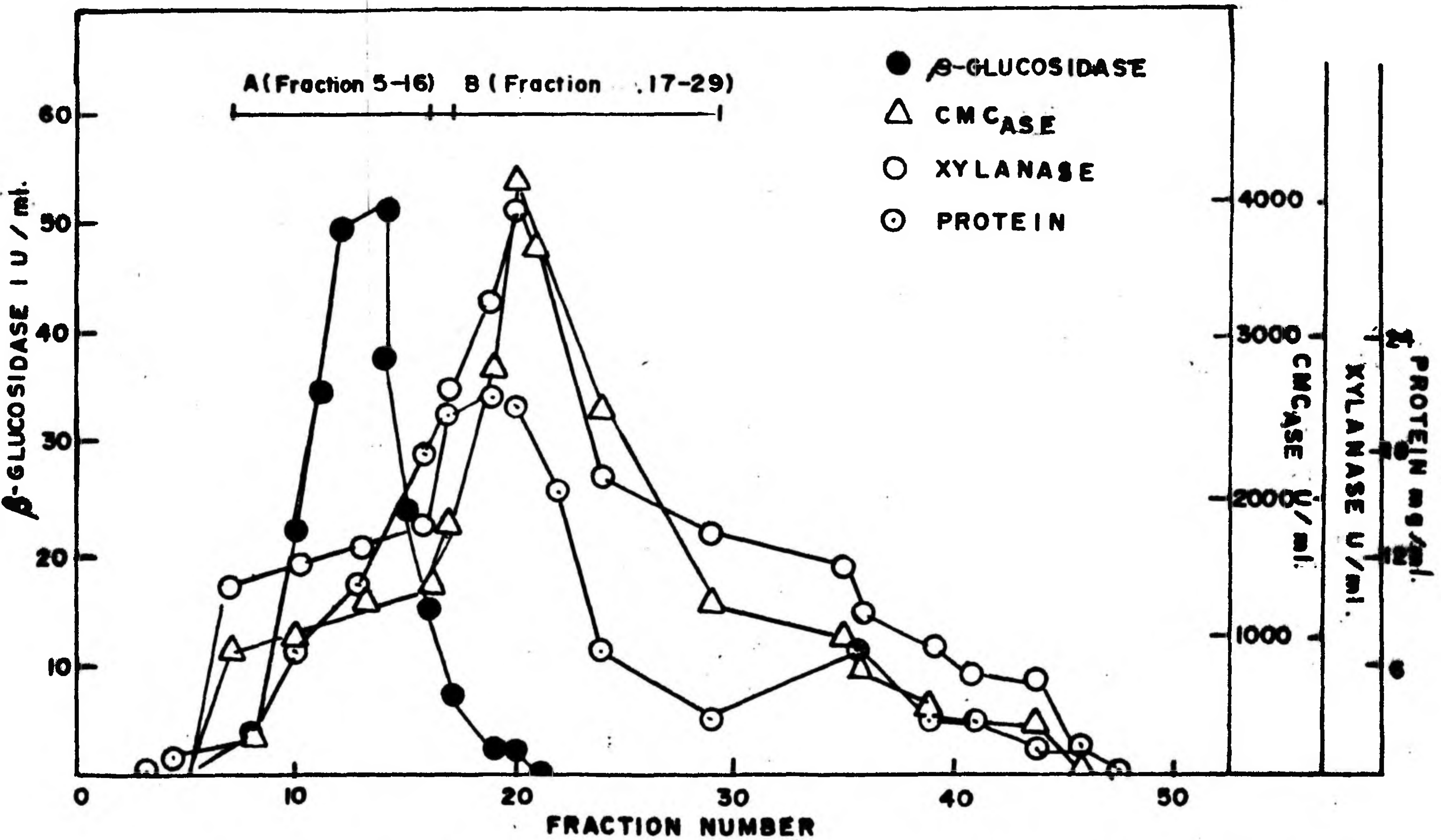


FIG. 27: Sephadex G-75 column chromatography of crude material after 0 - 90% ammonium sulfate precipitation. Sample: 120 mg protein/ml (60 ml). Column dimensions: 3 x 90 cm. Buffer 0.05 M citrate, pH 4.8.

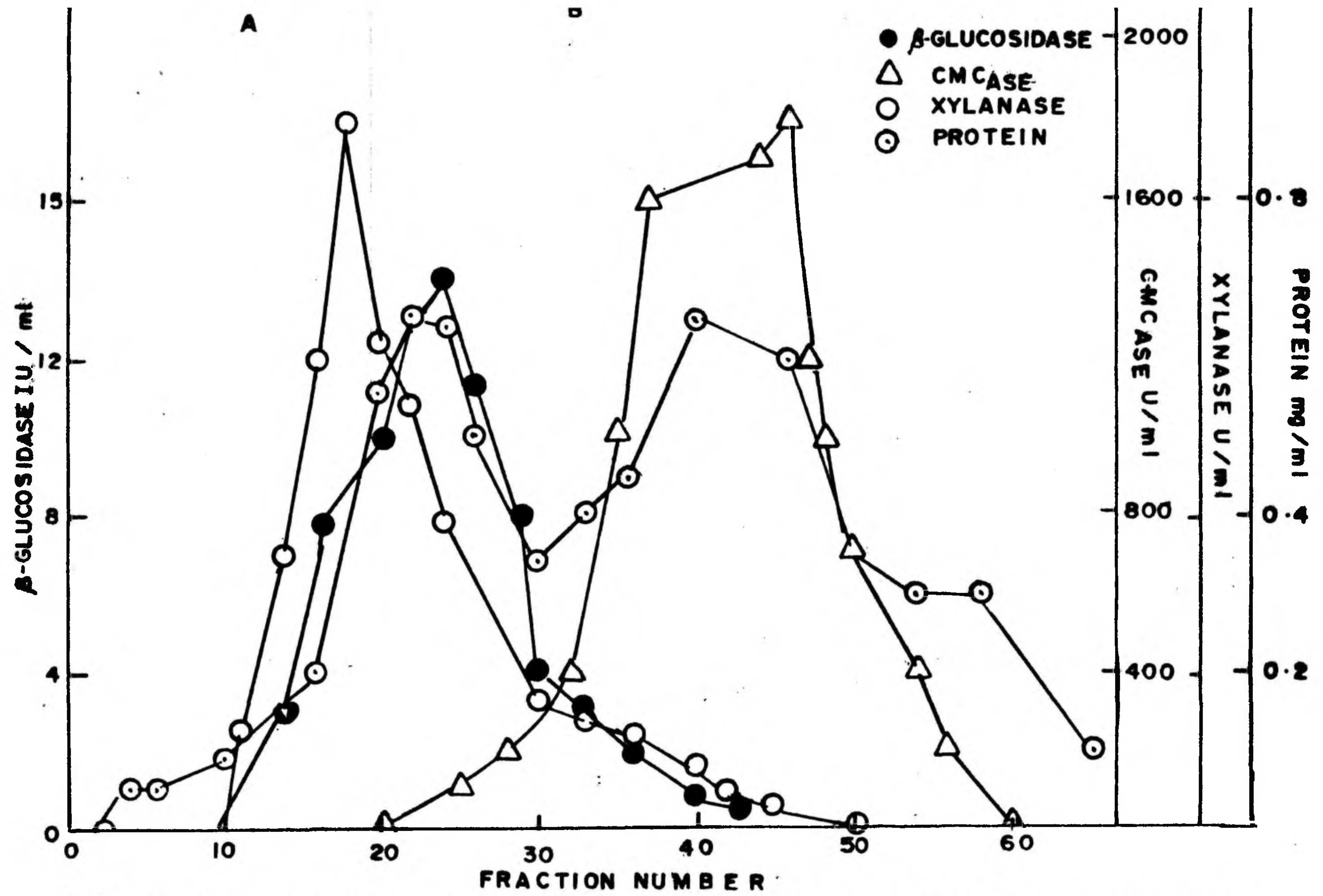


FIG. 28: Ion-exchange chromatography on DEAE Sephadex A-50. Fraction B (84 mg/ml, 6 ml) from Sephadex G-75 chromatography was applied to the DEAE Sephadex column (1.8 x 100 cm), previously equilibrated with 0.05 M phosphate buffer, pH 7.3, and washed with the same buffer.

Fraction A (F 5-19) was free of CMCase, while Fraction B (F 20-55) contained all three activities. The dark brown pigment present in the culture filtrate was removed in this step.

Step 5: Preparative isoelectric focusing

Isoelectric focusing was performed with a 110 ml column. The ampholyte concentration was 1% with a pH range of 3.5 - 10 in a sucrose gradient. The Fraction A from Step 4 was dialysed against 1% glycine for 4 - 6 h. The 4 ml of the concentrated and dialyzed fraction containing about 8 mg of protein was subjected to preparative isoelectric focusing at 5°C for 48 h. Fractions (1 ml) were immediately processed for pH determination (5 - 7°C), activity and protein. These were made free of sucrose by dialysis against 0.05 M citrate buffer, pH 4.5. Fig. 29 shows the isoelectric profile of the xylanase after electrofocussing. As a result of isoelectric focusing the xylanase activity was resolved as a single peak at pH 7.1.

The yield and specific activity of the enzymes at different stages of purification are summarized in Table 38.

Criteria of purity

Electrophoresis of xylanase in both cathodic (pH 4.3) (252) and anodic (pH 8.9) (251) running in polyacrylamide gel systems revealed only one protein band (Fig. 30). SDS-gel electrophoresis of the enzyme also showed only one protein band (Fig. 31).

As a final criterion of purity, the xylanase was examined by isoelectric focusing in 7.5% polyacrylamide gel over the pH range 3.5 - 10 described by O'Farrell (255). Only a single protein band could be detected (Fig. 32).

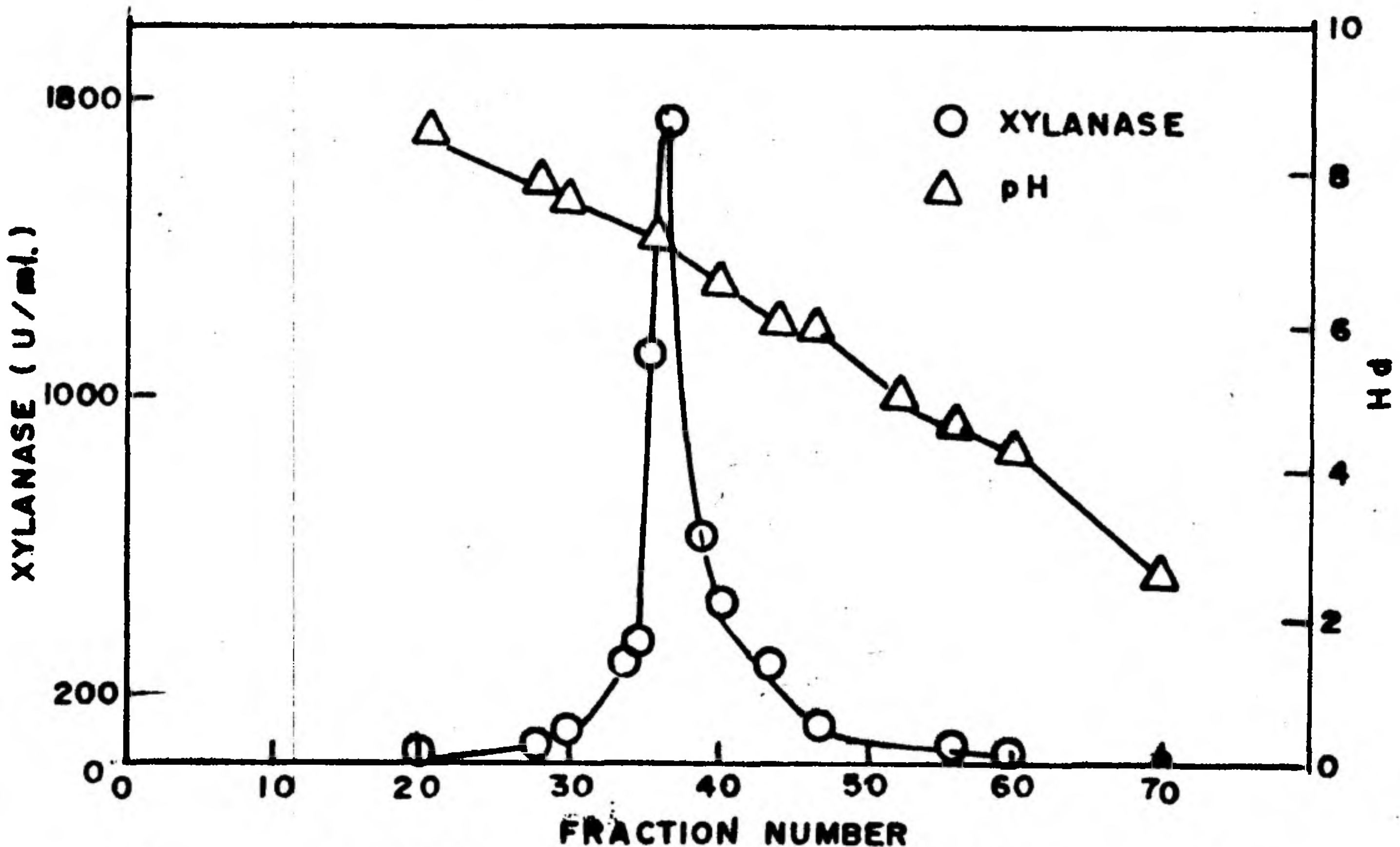
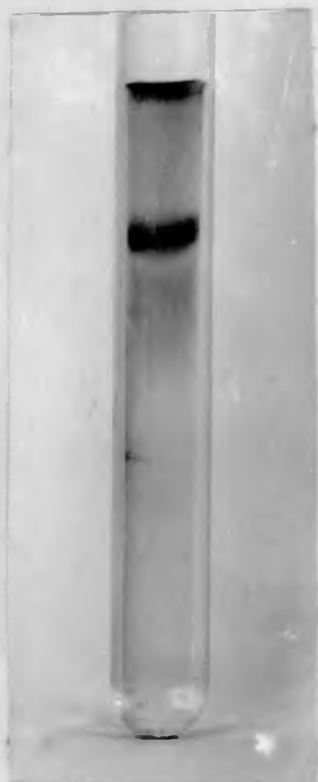


FIG. 29: Isoelectric focusing of peak A from DEAE Sephadex chromatography. Sample: 8 mg. Column: 110 ml LKB electrofocussing column. The ampholyte solution, pH 3 - 10, was used at a final concentration of 1%. The voltage at the end of run (48 h) was 500 V and the current 2 mA. Fractions (1 ml) were collected at a flow rate of 30 - 40 ml/h.

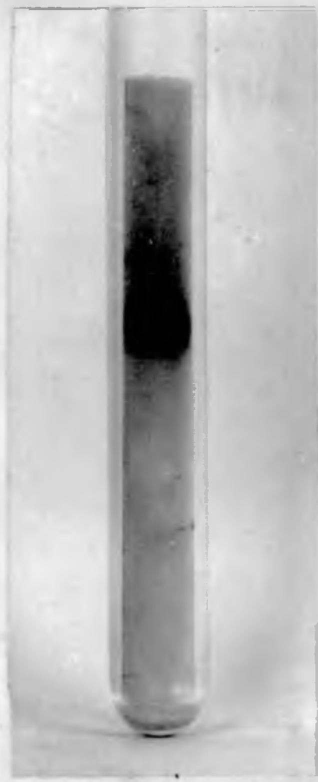
TABLE 38: SUMMARY OF PURIFICATION OF XYLANASE FROM S. ROLFSII UV-8 MUTANT CULTURE FILTRATE

Fraction	Vol.	Protein		beta-Glucosidase				Xylanase				CMCase			
	ml	mg/ml	Total	U/ml	Total	Sp. A.	% Recovery	U/ml	Total	Sp. A.	% Recovery	U/ml	Total	Sp. A.	% Recovery
Culture filtrate	7500	6	45000	20	150000	3.3	100	2400	19500000	400	100	2300	17250000	383	100
Step 1 0-90% $(NH_4)_2SO_4$ precipitate	2000	21	42000	68	135000	3.2	90	7500	15000000	357	80	6900	13800000	329	80
Step 2 Amicon concentrate PM-10	300	120	36000	306	91800	2.6	61	27500	8250000	228	44	29440	8832000	245	51
Step 3 Sephadex G-75															
A. (F. 5 - 16)	90	15	1350	78	7000	5.2	4.6	422	38000	281	0.2	488	44000	33	0.5
B. (F.17 - 29)	290	50	14500	15	4360	0.3	2.9	12500	3625000	250	18	12000	3480000	240	20
Step 4 Lyophilization of (B) from Step 3	80	180	14440	34	2750	.02	.02	38968	3117500	216	16	33930	2714400	188	16
Dialysis (0.05M, pH 7.3 phosphate buffer)	140	100	14000	15	2115	.15	.01	12800	1792000	128	9.2	13000	1820000	130	11
DEAE A-50 of (B)															
A. (F. 5 - 19)	450	0.5	225	1.3	600	2.7	.004	1080	486000	2160	3	0	0	0	0
B. (F.20 - 55)	750	0.5	405	1.6	1200	3.0	.008	300	225000	550	1.2	600	450000	1110	2.6
Step 5 Isoelectric focusing of A (Step 4) dialysis 1% Glycine (load 8 mg)	3	0.5	1.5	0	0	0	0	1730	5190	3460	.0003	0	0	0	0

FIG. 30: Disc gel electrophoresis of purified endo-xylanase. Electrophoresis was carried out at pH 8.9 (a) and at pH 4.3 (b) as described under Materials and Methods.



a



b

FIG. 31: SDS-gel electrophoresis of purified endo-xylanase. Electrophoresis was carried out as described under Materials and Methods.

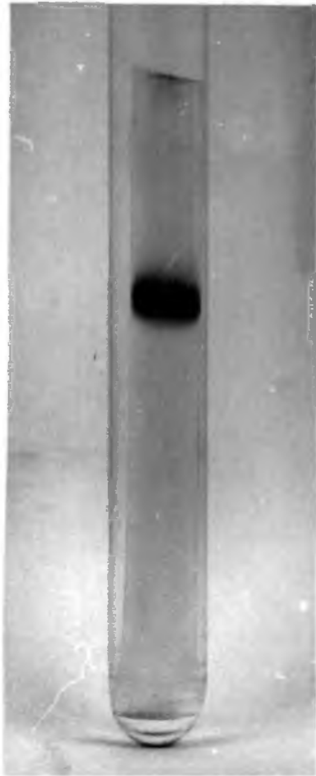


FIG. 32: Analytical isoelectric focusing (in triplicate) in polyacrylamide gel of purified endo-xylanase. Electrofocusing was carried out in 7.5% polyacrylamide gel over the pH range 3.5 - 10.

The final purified fraction is free from CMCase, beta-glucosidase and xylosidase (PNP-beta-D-xylosidase) activities.

Molecular weight of xylanase

Two different methods were used in the determination of molecular weight of the enzyme.

(i) Gel filtration: The molecular weight of the xylanase was estimated by comparing the elution volumes from a Bio Gel P-150 (1.5 x 90 cm) column with those of proteins of known molecular weight. A plot of Ve/Vo versus log molecular weight according to the procedure of Andrews (413) indicated that the molecular weight of xylanase was 56,890 (Fig. 33). With Sephadex G-75 Fraction B (Step 3) on gel filtration through Bio-Gel P-150, an average molecular weight value of 54,760 daltons for xylanase was obtained. This indicated that the xylanase has not undergone any association-dissociation during further purification procedure. Association-dissociation has also been not observed with D-xylanases from different fungal sources (310).

(ii) SDS-gel electrophoresis: An estimate of the molecular weight of the xylanase and the possible subunit nature was made by its migration in the SDS-polyacrylamide gels (253,254). Plot of log molecular weight versus relative mobility of the marker proteins yielded a straight line (Fig. 34), and an estimate of the molecular weight of the xylanase was 54,330 daltons. Carboxyamidomethylation of the reduced form of enzyme on SDS-gel electrophoresis showed only one protein band with molecular weight corresponding to the native protein. This indicated that the xylanase is comprised of only one polypeptide chain. The xylanase from T. viride is reported to consist of three

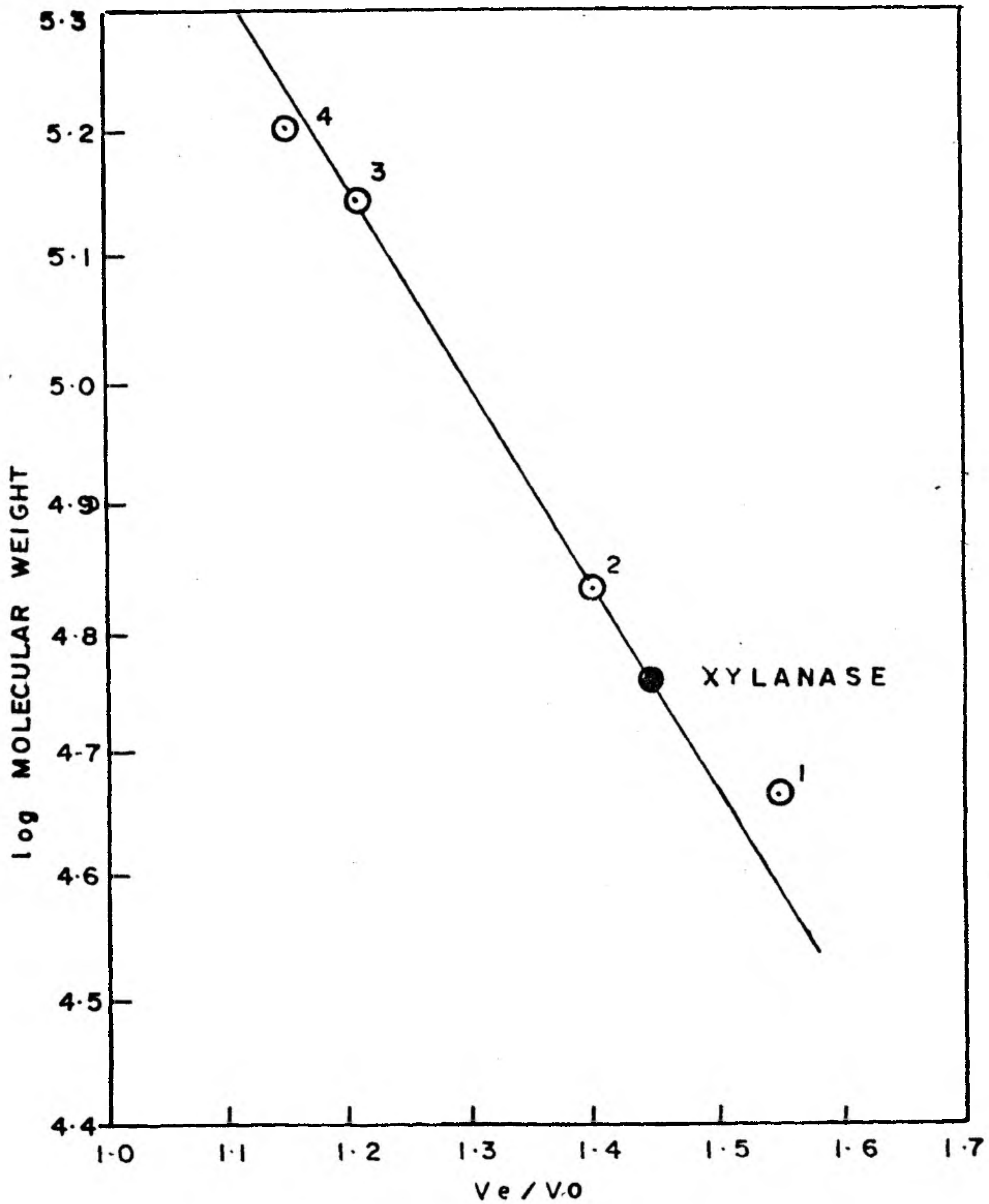


FIG. 33: Molecular weight determination of xylanase by gel filtration. A Bio-Gel P-150 column (1.5 x 90 cm) was calibrated with: (i) Ovalbumin,46,000, (ii) BSA, 68.000, (iii) Alcohol dehydrogenase, 141,000, and (iv) gamma-globulin, 160,000. Buffer: 0.05 M citrate, pH 4.5. Vo, Void volume; Ve, Eluted volume.

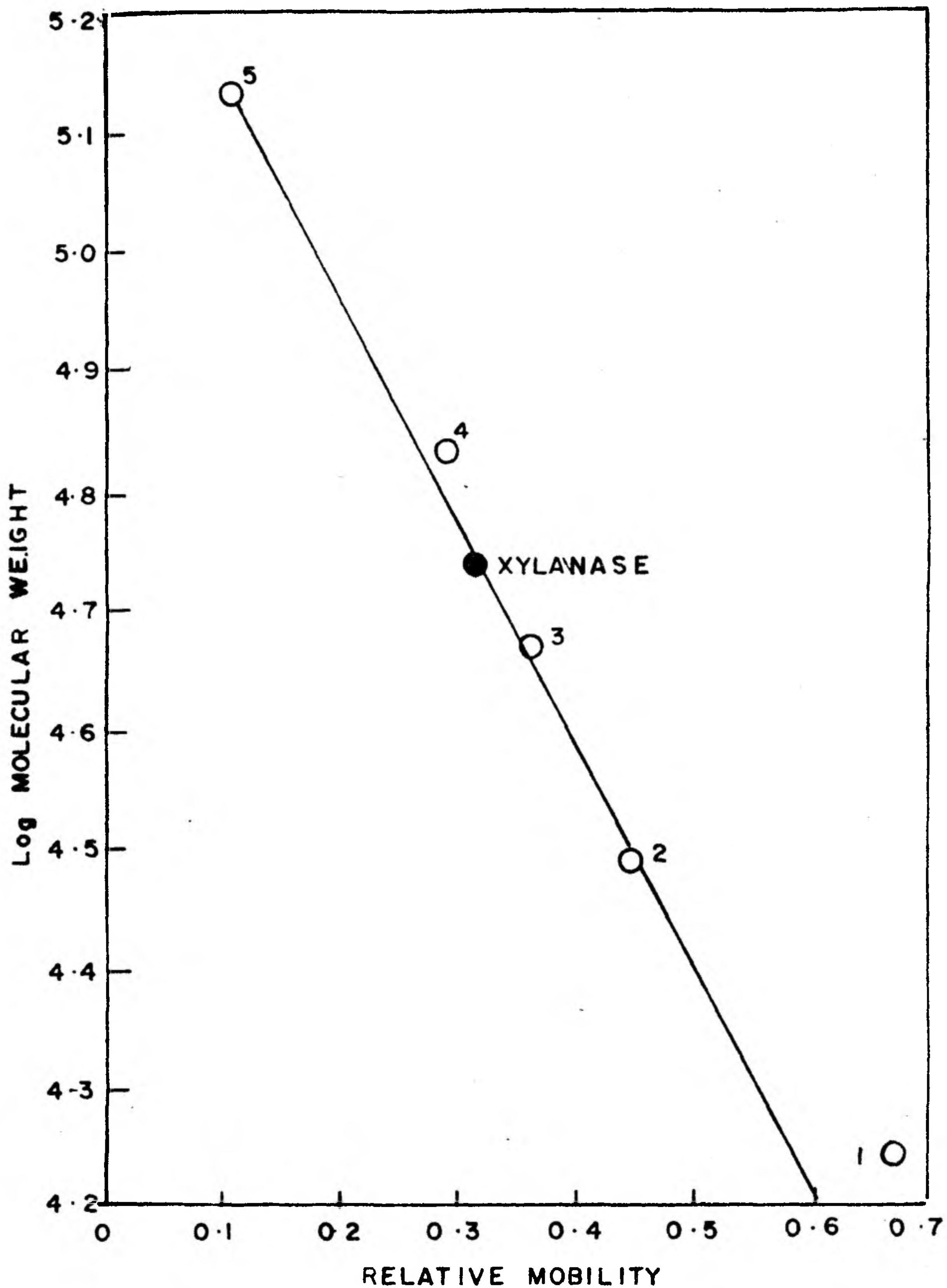


FIG. 34: Molecular weight determination by SDS-gel electrophoresis. Relative mobility was plotted against log molecular weight of standard proteins: (i) Myoglobin, 17,600, (ii) deoxyribonuclease I, 31,000, (iii) ovalbumin monomer, 40,000, (iv) BSA I, 68,000, and (v) BSA II, 136,000.

subunits with approximate molecular weights of 10,000; 16,000 and 18,000 daltons (414).

Isoelectric point

From preparative isoelectric focusing experiments, the isoelectric point (pI) of the xylanase was determined to be 7.1. In majority of the reported cases the isoelectric points of various D-xylanases are acidic; 3.6 and 4.3 in the case S. sanguinolentum (415), 4.4 and 6.0 in the case of C. lignorum (415), 3.9 and 4.5 in the case of A. niger (391). In the case of F. annosus, a pI = 7.0 (415), while that for C. paradoxa is alkaline, pI = 9.7 (406).

Enzymatic properties

The purified xylanase was stable when stored at -15°C at pH 4.5; no significant loss of activity was observed over a six months period.

Optimum pH

The effect of pH on catalytic activity was tested with 0.05 M citrate buffer ranging in pH from 3.5 to 6.0. The optimum pH for activity was 4.5 for the xylanase (Fig. 35).

Optimum temperature

The temperature/activity curve was determined using the standard assay system in which the temperature was varied. The temperature optimum was 65°C for xylanase.

Influence of pH on stability

For the determination of stability, enzyme samples were adjusted to various pH values by the addition of suitable buffer (citrate, pH 3.5 to 6.0; phosphate, pH 7.0 tris-glycine, pH 8) to a final concentration of 0.05 M. After 10 min.

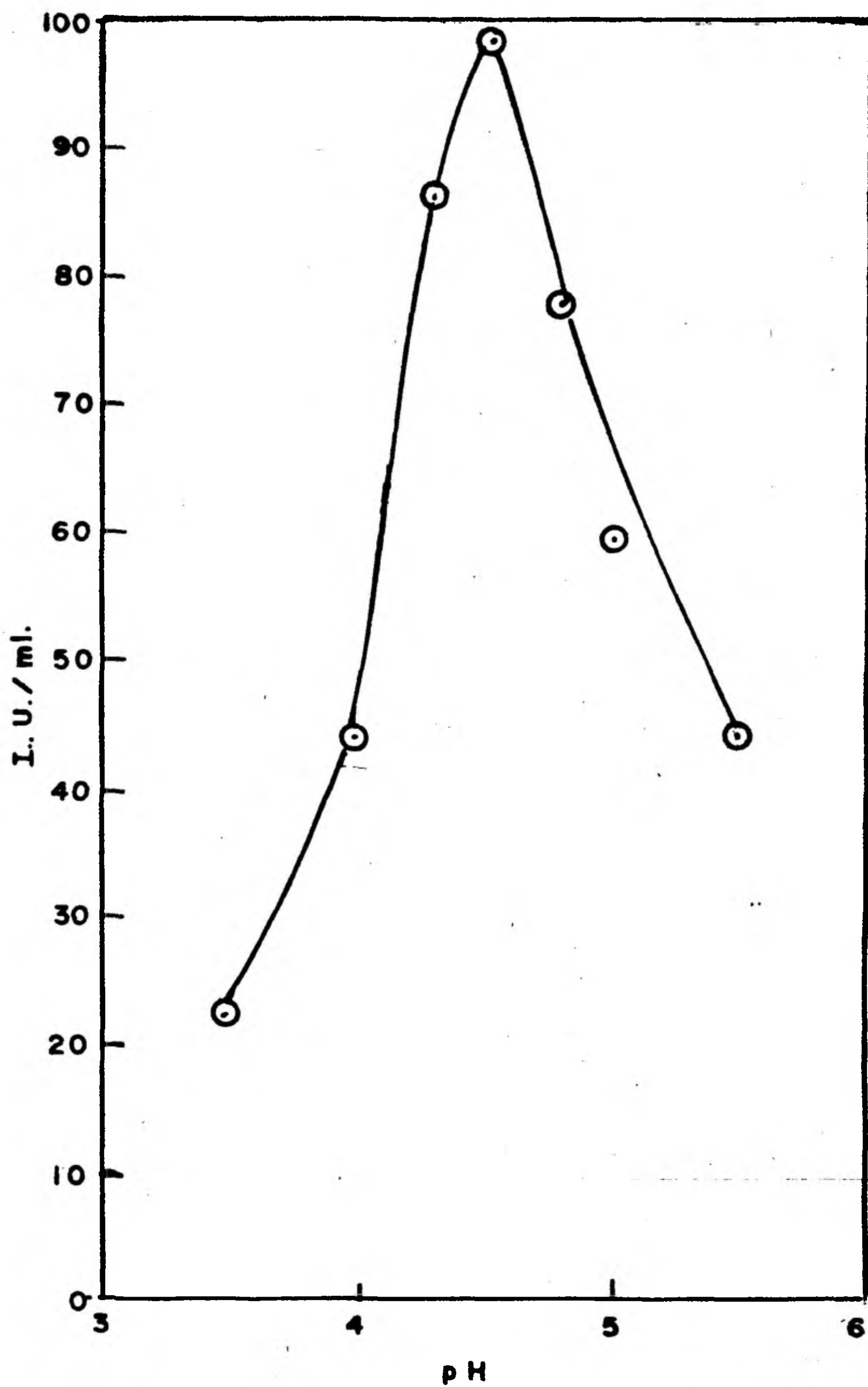


FIG. 35: Effect of pH on rate of hydrolysis of larch xylan, 65°C by S. rolfsii UV-8 mutant xylanase.

incubation at 65°C, the residual activity was determined using 1% xylan as a substrate under standard assay conditions. The observed activities were compared with that of a reference sample maintained at pH 4.5 and 4°C for the duration of the experiment. The enzyme was most stable at pH 4.3 to 5.0. In the presence of 0.5 mg/ml BSA, the pH of optimum stability was 4.5 retaining 83% of its activity (Fig. 36).

D-xylanases of fungal origin are generally most active at pH 3.5 - 5.5, and are stable over a wide range of pH, usually from 3 to 10 (310).

Energy of activation

A straight line was obtained when the data of Fig. 37 was plotted according to Arrhenius. From this, the energy of activation was calculated to be 17.36 kcal/mol (72.56 kJ/mol) Activation energies reported for D-xylanases are: from *S. commune*, 28.6 kJ/mol; from *T. hirsuta*, 28.0 kJ/mol (416).

Rate of xylan hydrolysis

The enzymatic action on xylan liberates reducing groups exponentially with time for a period upto 60 min (Fig. 38), an observation similar to that reported for xylan hydrolase from *T. clypeatus* by Ghosh *et al.* (390).

Kinetics

With a solution of larch xylan as substrate, the Lineweaver-Burk plot (Fig. 39) gave a Km 5.8 mg/ml and Vmax 8.858 μmol/min. The amount of enzyme (E), calculated from a molecular weight of 54,330, was 3.86×10^{-5} μmol. Thus, the molecular activity (Vmax/E), which represents the maximum number of beta(1 $\longrightarrow$ 4) xylosidic bonds cleaved per enzyme

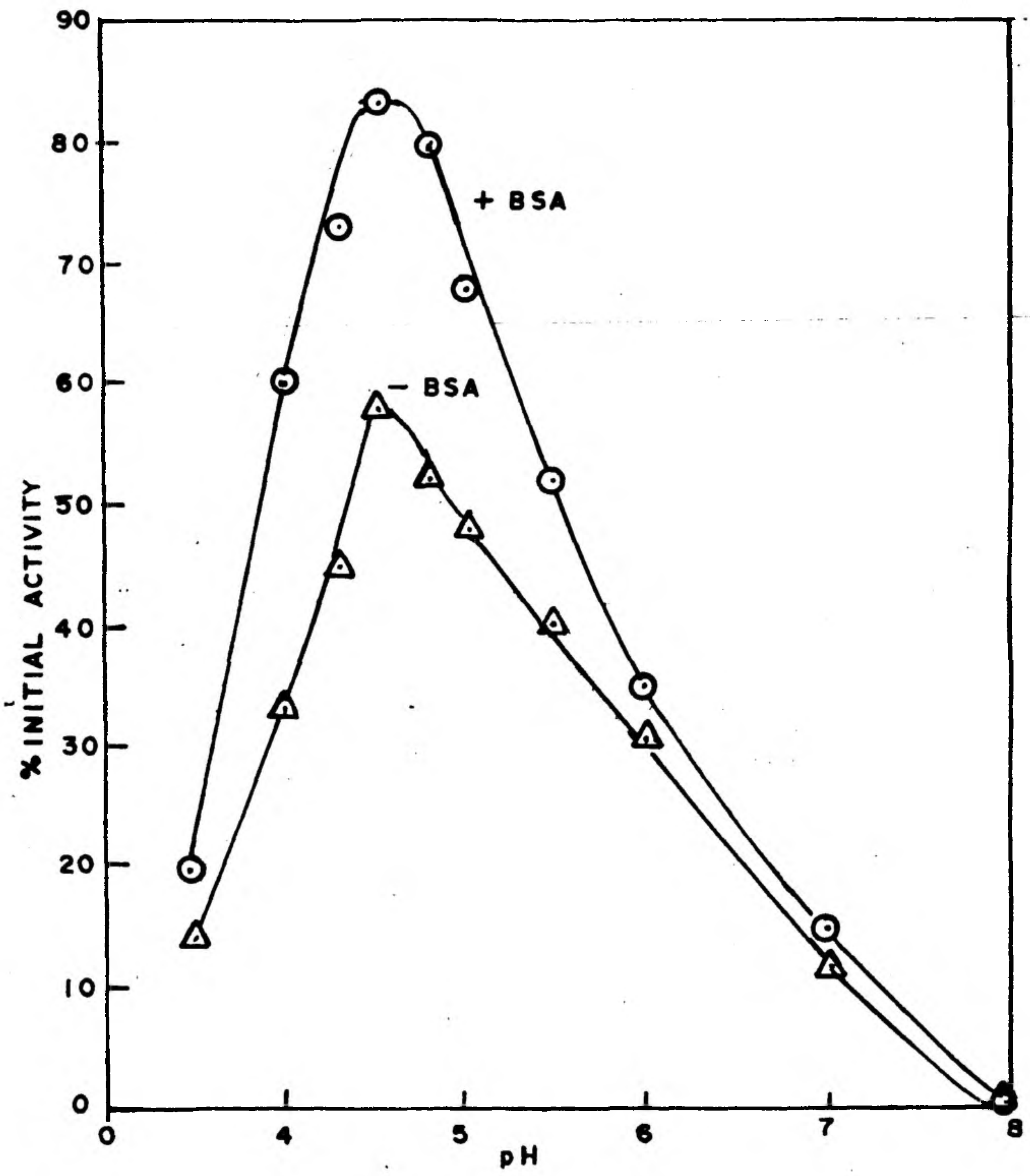


FIG. 36: Influence of pH on stability of xylanase. The enzyme (2 µg/ml) was maintained at 65°C for 30 min at different pH values and the residual activity determined under standard assay conditions.

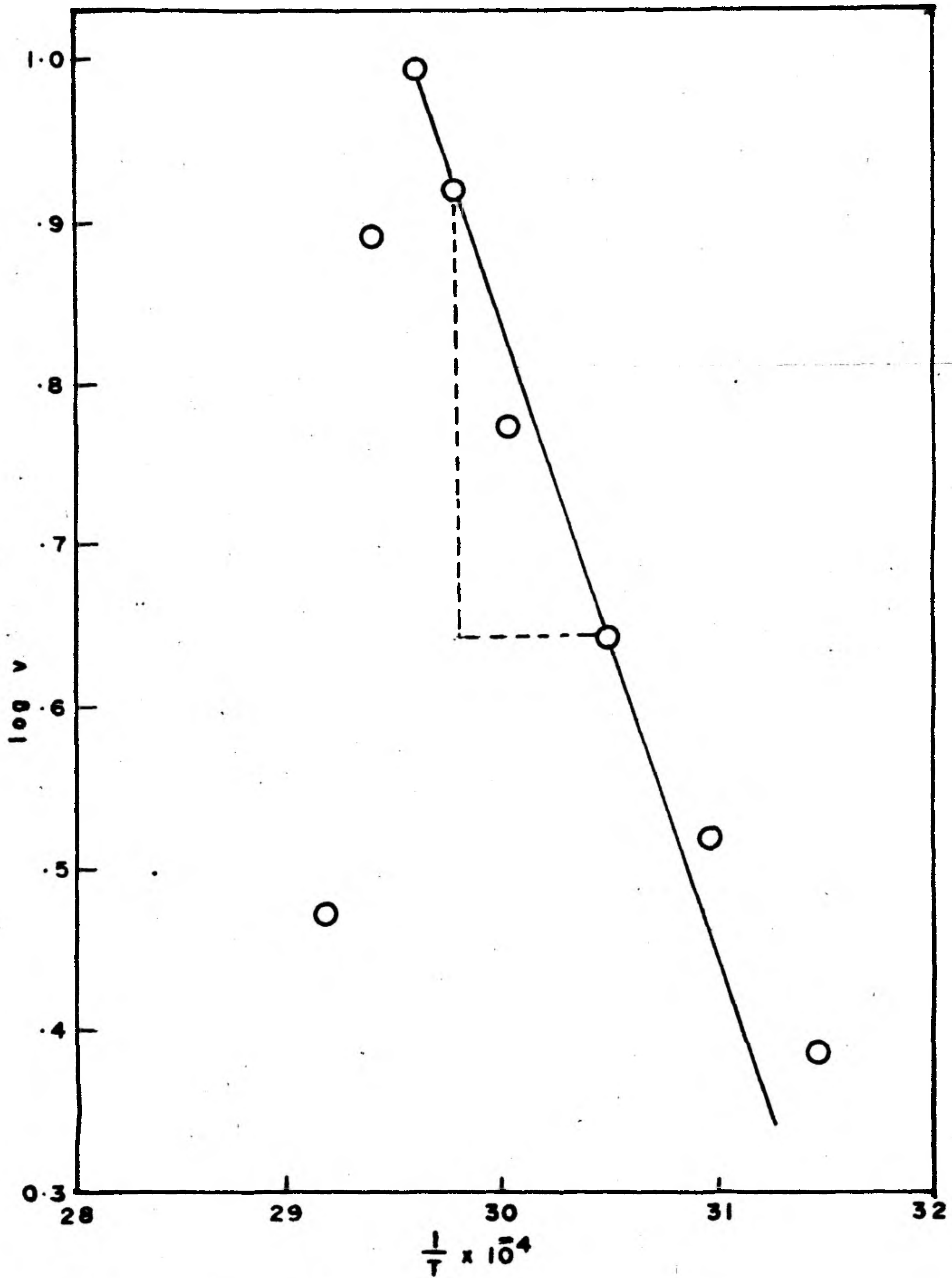


FIG. 37: Arrhenius plot showing effect of temperature on the rate of hydrolysis of larch xylan, pH 4.5 by S. rolfsii UV-8 mutant xylanase. v = IU/ml (xylose).

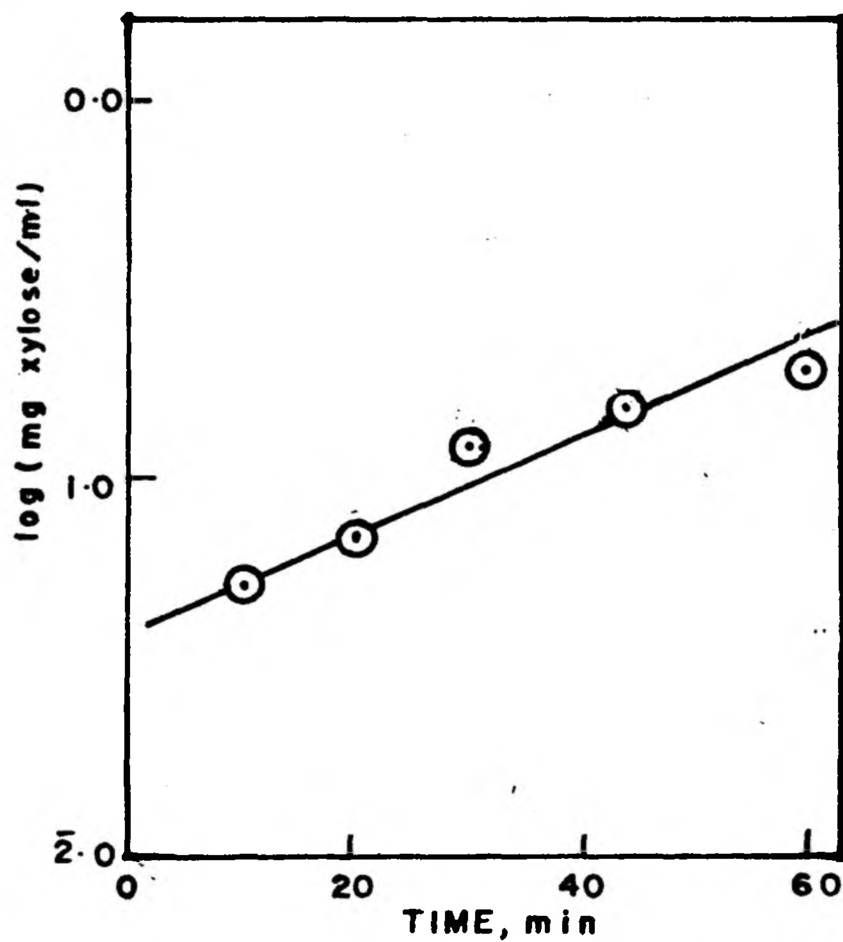


FIG. 38: The rate of hydrolysis of larch xylan by purified endo-xylanase (0.5 μg/ml) of S. rolfsii UV-8 mutant, at pH 4.5, 65 °C.

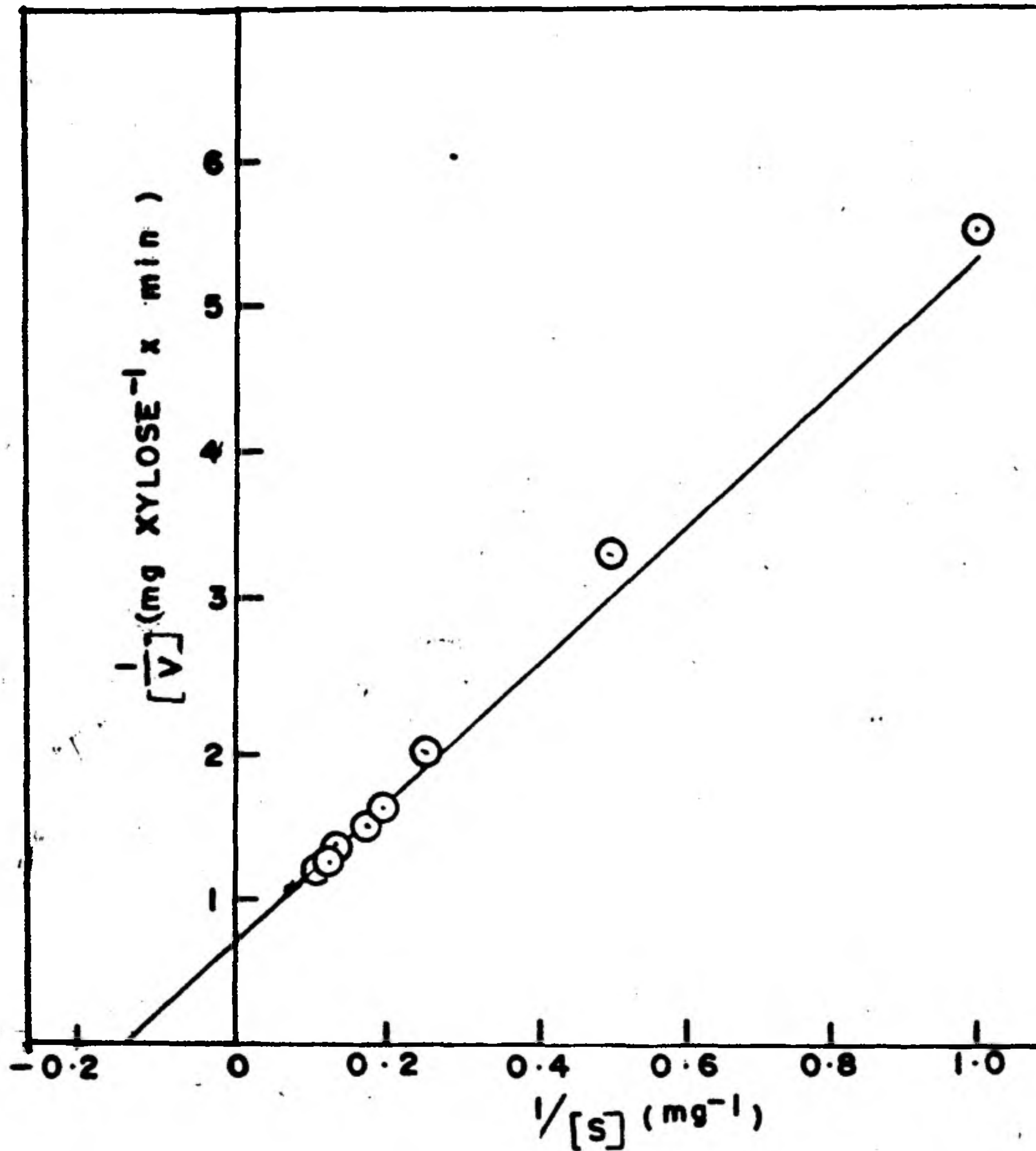


FIG. 39: Lineweaver-Burk plot for xylanase. The reaction was carried out under standard assay conditions.

molecule per min, was 2.29 x 10^5/min, assuming only one active site. With soluble larch xylan as substrate, Paice *et al.*(409) have reported a molecular activity of 1.4 x 10^5/min for endoxylanase from *S. commune*.

Activity towards different substrates

The purified xylanase had no action on cellulose, CMC, sucrose and starch. These were not hydrolyzed at ten times the enzyme concentration normally used in the standard xylanase assay or when the time of incubation was increased to 2 h.

Type of xylanase

In order to determine whether the xylanase under study is of endo- or exo-type, the viscometric studies of the purified xylanase from UV-8 *S. rolfsii* culture filtrate were carried out. The release of reducing sugars was also determined simultaneously.

Since endo enzymes split the xylan chain randomly, a few breaks in the chain would decrease the viscosity considerably after a short time. Even after viscosity losses are essentially complete (i.e., even when viscosity losses are no longer detectable), reducing power would continue to be generated at a steady rate.

The exo enzymes, on the other hand, have the opposite effect since when small molecules such as xylose or xylobiose (in the case of exo-xylanase) are cut off, the reducing power increases quickly whereas there is not much change in the weight average chain length.

As shown in Fig. 40, the viscosity of the xylan solution decreased rapidly initially on incubation with the purified xylanase. With 1.5 ug enzyme/ml, while no further change in

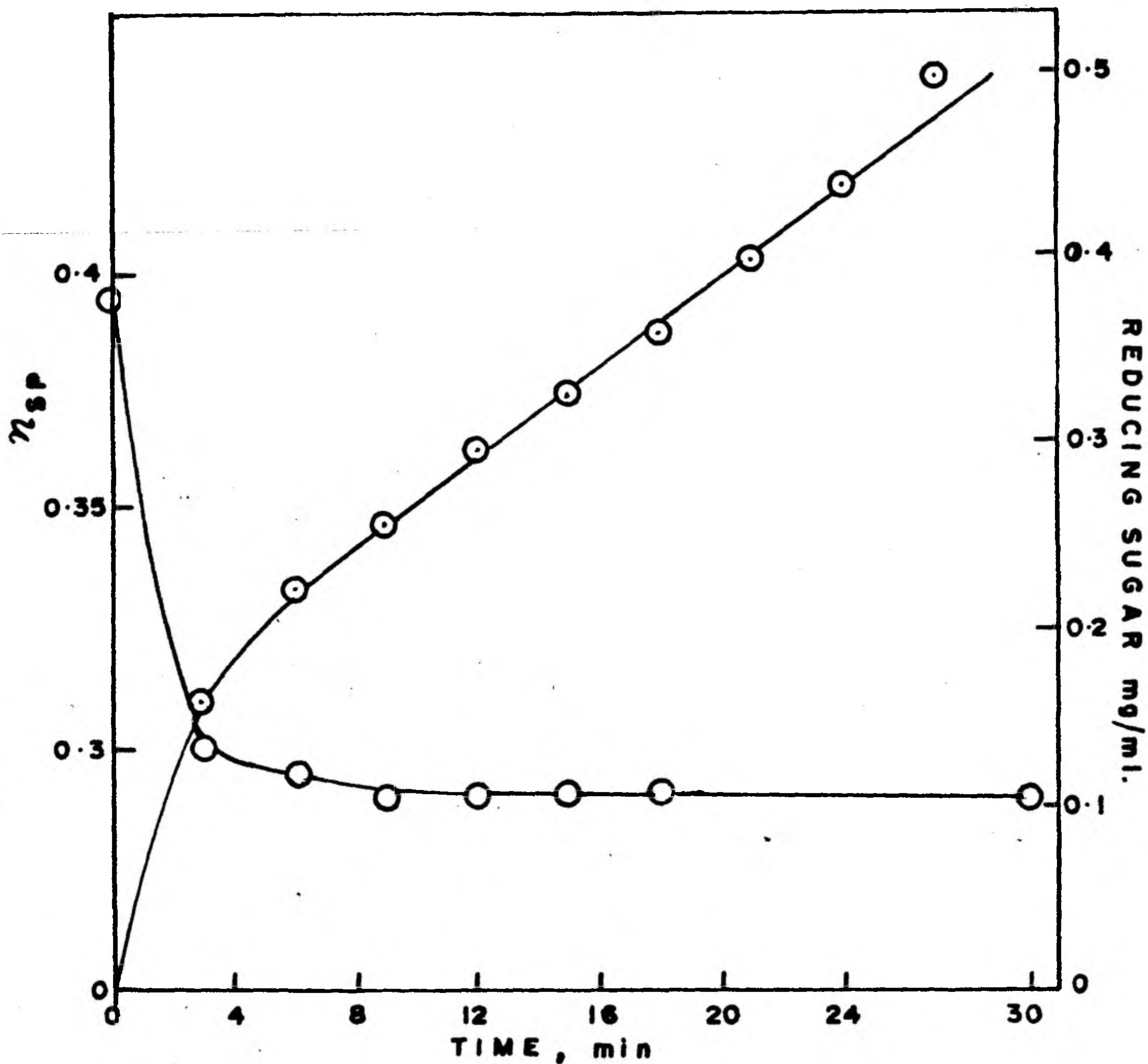
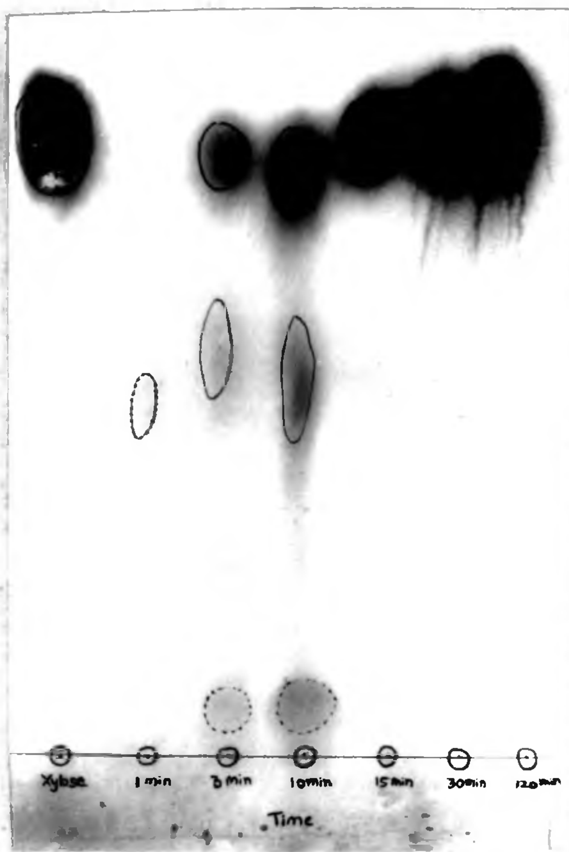


FIG. 40: Viscosity loss of substrate larch xylan and release of reducing sugar by purified endo-xylanase of S. rolfsii UV-8 mutant. 15 µg of xylanase was added to 10 ml of 1% xylan and incubated at 65°C in Ostwald's viscometer. Aliquots were removed at intervals for determination of reducing sugar.

viscosity fall was observed after 12 min, there was a continuous increase in the reducing sugar formation upto 30 min.

Paper chromatogram (Fig. 41) of time course hydrolysis of xylan by the purified xylanase showed the presence of xylose and two unidentified sugar spots, probably xylodextrins. Neither L-arabinose nor any low molecular weight uronic acid was found at any time. The xylodextrins accumulated initially and the two spots of xylodextrins could be detected at 3 min and upto 10 min. The xylodextrins, however, were not detectable at and after 15 min incubation suggesting the formation of higher xylodextrins in the early stages of hydrolysis. Apparently, xylodextrins conversion into xylose, detectable at 3 and 10 min, exceeded their rate of production from xylan and their concentration declined. It is possible that the purified xylanase has a lower Km and high Vmax for the ~~soluble~~ xylodextrins than for the higher xylooligosaccharides, so that the soluble xylodextrins are broken down as soon as these are formed and the only product seen on the paper chromatogram is xylose after 15 min. In the case of beta-glucosidase from *S. rolfsii*, it has been observed that the Km values decrease and Vmax values increase in general with increase in the chain length of the cellodextrins (222). Frederick *et al.* (417) have, on the other hand, reported that an endo-xylanase from *A. niger* is more active on larger substrates; attack on xylotriose being verv slow and that on xylobiose non-existant. The enzyme has no action on *p*-nitrophenyl-beta-D-xylopyranoside. We have not been able to test the action of the enzyme on soluble xylodextrins as these materials were not available. It was

FIG. 41: Paper chromatogram of the products of xylan hydrolysis by the purified endo-xylanase of S. rolfsii UV-8 mutant. The time course hydrolysis of larch xylan was carried out and the hydrolyzate aliquots were applied at intervals to Whatman No. 1 paper and chromatographed in the system ethyl acetate:pyridine: water (2:1:2)



Xylose
1 min
5 min
10min
15min
30min
120min
Time

also not possible to run HPLC for detection of the xylooligo-saccharide formation because of the non-availability of the adsorbent column for the HPLC.

The same results would also be obtained, if the enzyme is an exo-xylanase which is contaminated with trace amounts of an endo-xylanase. However, this seems unlikely considering the various criteria of purity used for the homogeneity of the enzyme. Though the evidence obtained implies that the xylanase is an endo-xylanase, the question remains open pending further evidence.

When larch wood xylan (used in the above experiment) was hydrolyzed with 2 N H_2SO_4 at 95 - 100°C and the solution neutralized with barium carbonate (405,418,419), the hydrolyzate showed xylose and arabinose spots on paper chromatograms, thus indicating that larch wood xylan used in the above experiments contained arabinose substituent in the appendages.

Since no arabinose was detected in the xylan hydrolyzate, the xylanase from UV-8 *S. rolfsii* is non-arabinose-liberating endo-xylanase.

DISCUSSION

There is a considerable conflict as to whether the D-xylanase of fungi degrading the xylans of different plant species are generally produced inductively or constitutively in response to the carbon source on which they are grown (383-385). The number of carbon sources that have been reported to produce constitutive D-xylanase from fungi include glycerol in the case of *M. verrucaria* (387), sophorose for *T. viride* (388), dextrin for *T. clypeatus* (390) and Cellulose-123 for

S. rolfsii (315).

Some microorganisms are known to produce two or three types of xylanases. A. batatae 444 can produce two types of xylanase, which can be classified as exo- and endo- type (310, 420).

Xylanase from Cephalosporium sacchari (423) and S. commune (409) exhibit affinity for Sephadex gels as evident from gel permeation chromatography. In the case of S. rolfsii, no such adsorption was observed during the purification . In purification on DEAE-Sephadex A-50, it was found that at pH 7.3, S. rolfsii xylanase came immediately after the void volume and separated out from the cellulase enzymes.

The isoelectric point values reported for several D-xylanases are acidic. Eriksson and Pettersson (391) reported pI values of 3.9 and 4.5 for A. niger D-xylanase while in the case of xylanases from S. sanguinolentum Ahlgren et al.(415) found pI values of 3.62 and 4.3. In the case of C. paradoxa D-xylanase II pI 4.5 (406), C. lignorum 4.44 and 6.0, F. annosus 4.1 to 4.6 (415) have been reported. Though in most cases, pI values reported are acidic; neutral, for example pI 7 in the case of F. annosus (415) and alkaline, 9.17 in the case of C. paradoxa (407) D-xylanases have also been reported. A pI of 7.1 was found in the case of S. rolfsii xylanase.

The molecular weights of D-xylanase preparations of different origins are relatively low, ranging from 16,000 to 38,000. In the case of A. niger, D-xylanases, Frederick (425) and Fournier (426) reported molecular weight values of 19,000 and 28,000, respectively, while Tavobilov et al. (424) reported

an endo-xylanase of molecular weight 14,000. John et al.(404) found three xylanases in A. niger, one having a molecular weight of 31,000 and the other two having 50,000. The D-xylanase from S. commune showed a molecular weight of 33,000 daltons as calculated by electrophoretic mobility in SDS-gels electrophoresis. Its amino acid composition gave a molecular weight of 31,000 (409). The molecular weight of T. clypeatus xylanase is relatively high (about 90,000) compared to that reported from other sources (390). The molecular weight of S. rolfsii UV-8 xylanase is 54,300 as estimated from SDS-gel electrophoresis and 56,000 as calculated from Biogel P-150 filtration studies.

D-xylanases of fungal origin are generally most active at pH 3.5 - 5.5 and are stable over a wide range of pH, usually from 3 to 10. The pH optima observed were 3.5 in the case of T. viride (420), 3.8 for D. viticola (389), 4 - 4.5 in the case of A. niger-15 (424), 5.0 in the case of S. commune (409) and S. rolfsii (427), 5.5 for T. clypeatus (390) and 6.2 in the case of of Streptomyces xylophagus (428). S. rolfsii UV-8 xylanase shows stability over a pH range 3.5 to 7.5 and optimum pH 4.5.

D-xylanases usually show optimal thermal activity at approximately 50°C and tend to be thermostable, often being totally inactivated only at temperatures greater than 65°C. The optimum temperature for xylanases of A. niger (382), Pleurotus ostreatus (429), T. viride (420) is 50°C. Fournier (426) and Reilly (309) reported an optimum temperature 40 - 42°C for xylanases of A. niger, 40°C in the case of Cryptococcus albidus var aerius (430) 45°C in the case of G. virens (411), and 55°C

for xylanase of T. clypeatus (390). The D-xylanase system produced by C. paradoxa (407) is rather unusual in that it is highly stable to heat and showing optimum activity at 80°C. It is completely inactivated only after 1 h at 100°C at pH 5.5. In the case of S. rolfsii UV-8 xylanase the activity is optimum at 65°C.

The Km values of several fungal xylanases have been reported in the range from 0.27 mg/ml in the case of C. paradoxa II (407) to 15 mg/ml of S. commune (431). Paice et al. (409) reported a Km of 9.37 mg/ml of larchwood xylan for S. commune. Tavobilov et al.(424) purified endo-1,4-beta-xylanase from A. niger 15 which showed a Km of 24 mg/ml using carboxymethyl xylan. Endo-xylanase of S. rolfsii UV-8 mutant has a Km value of 5.8 mg/ml of larch wood xylan.

The molecular activity of endo-xylanase from S. rolfsii UV-8 mutant, 2.29×10^5/min, is slightly more than that reported for S. commune endo-xylanase, 1.4×10^5/min (409).

The S. rolfsii xylanase did not act on cellulose, CMC, sucrose and starch. Recently, two xylanases (IA and IB) from A. niger have been characterized which attack crystalline cellulose in addition to xylan (404). Xylanase (II) from A. niger showed specificity for xylan only and is unable to degrade cellulose. The purified xylanase from commercial cellulase preparation (Miles Chem. Co., Clifton N.J., U.S.A.) hydrolyzes both cellulose and amylose (432). The endo-xylanase of T. clypeatus (390) did not attack cellulose, CMC, sucrose and mannan, but appreciable reducing sugar formation was noted by its action on two unrelated carbohydrates like

amylopectin and arabinogalactan. Takenishi *et al.* (402) reported a highly purified xylanase which is also capable of hydrolyzing (1 → 3)-L-arabinofuranosyl linkages at the branch points of xylan. The endo-xylanase of *S. rolfsii* UV-8 mutant does not hydrolyze the branch points of xylan, i.e., this endo-xylanase shows non-arabinose-liberating endo-mechanism similar to the one reported by Biely *et al.* (433) in the case of *Aureobasidium pullulans*.