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PART I

INTRODUCTION

S E C T I O N 1

PRODUCTION OF SCP/FUEL/GLUCOSE FROM UNCONVENTIONAL SOURCES

About two decades ago, at one of the annual American microbiologists' meetings, a challenge was posed to produce food from unconventional, i.e. non-agricultural, sources. The term "Single-Cell Protein" (SCP) was coined by Professor Carrol Wilson at the Massachusetts Institute of Technology, USA in 1966 (1). SCPs has become, by defination, a generic term for a crude or refined source of protein whose origin is unicellular or simple multicellular organisms, i.e. bacteria, yeast, fungi and algae. The use of single cells in food and animal feed is a common practice of long standing. Any fermented food will contain significant quantities of cellular mass of organisms as diverse as bacteria, yeast and fungi. Cheese, beer and even leavened bread production is pure microbiology. A type of alga, Spirulina maxima, forms a part of the diet of the natives of the Lake Chad region of Africa (2). Thus, there is nothing fundamentally repugnant in any of these species and SCP is a mere extention of the tradition that may be thousand of years old.

Microorganisms are capable of converting inexpensive non-proteinous materials (such as carbohydrates) and simple inorganic nitrogen compounds (such as nitrate and ammonium) into protein. Because the generation time of bacteria, yeast, fungi and algae is much shorter than that of animal and plant cells (3), this process can be incredibly fast

and appears to be the method of choice. This has excited an extraordinary amount of industrial interest as a possible source of protein and major efforts have been directed during the past two decades towards producing SCP from hydrocarbons (4), methanol (4,5), ethanol (6) and other cheap and waste carbohydrate sources (4,7). The substrate used as a raw material depends on the location, possibilities of application and cost factors. Conversion of sugar to protein by food yeast is about 1300 times as efficient as the conversion by beef cattle and the production of Escherichia coli twice as effective as the food yeast process as a protein source. It has been estimated that a plant handling 1000 tonnes per day of sulphite processed pulp could produce 21,000 tonnes of fermentable sugars annually (8) which could produce 56,000 tonnes of SCP (9). Humphrey (10) has calculated that a 10% supplement to the present world's supply could be provided by a fermentor of an area equivalent to 1/2 square mile of the earth's surface. However, technologies still remain to be developed to make the production of SCP competitive. Similarly, development problems seem restrictive in the case of algae, S.maxima and Spirulina platensis, though they are known to be free from toxic effects. Attempts to cultivate S.platensis, though most prolific, in fermentors with artificial light or in open lagoons have not been successful economically (11). However, there is a general air of confidence that within the next 5-10 years several techniques for growing various micro-organisms on a wide variety of solid and liquid substrates

for incorporation into animal feedstuffs will be ready to go commercial (4,12,13). According to Imperial Chemical Industries, on cost basis, SCP cannot hope to match the conventional feed protein sources, say soybean, until 1985 (4). The crude protein content (nitrogen content x 6.25) of SCP varies between 30 and 70% with different microorganisms. The maximum proportion of true protein in yeast cells is 50% and that of bacterial cells 70%. Fungi produce biomass with lower protein content than bacteria or yeast. Yeast and fungi in general lack sulphur-containing amino acids, cystine and methionine (14), whereas the bacterial species, in general, do not lack methionine, but are deficient in tryptophan. It is important that SCP contains numerous other nutrients in addition to protein.

Because of the vested interests of oil companies and chemical industry, the major effort in industrial SCP production has been in the production of microorganisms, usually yeast, from a hydrocarbon source. Originally it was planned to use gas oil (a crude oil fraction) for SCP production. The emphasis was then shifted to n-paraffins. Plants are already in operation with production capacities upto 200,000 tonnes per year. The commercial projects that are in operation are of British Petroleum producing yeast on heavy gas oil containing 15% n-paraffins (20,000 tonnes/annum in Lavera, France) (4,14) and on pure n-paraffins (4,000 tonnes/annum in Grangemouth in Scotland (11,14) and 100,000 tonnes/annum in Sarroch in Sardinia (15)), and Liquichimica Biosintesi on pure n-paraffins (200,000 tonnes/

annum in Saline di Montebello, Italy) (6). The Russians have also opened a protein plant which utilizes liquid paraffins (4). The final product is intended initially as a feed supplement for poultry and pigs. Claims have been made that extensive feeding experiments show freedom from toxic and carcinogenic effects (11).

The presence of toxic residues in SCP grown on hydrocarbons is also a major problem, though claims have been made that petroleum yeast proteins have been passed for animal consumption in many European countries, and the levels of carcinogenic substances in them are lower than those in some normal foodstuffs (4,16). To ensure that the product is non-toxic, high demands are made. The production of high purity n-paraffin which is suitable as a substrate is very important for the overall efficiency of the SCP process. The BP product Toprina is currently being sold for breeding stock and veal production (6). However, 200 ppm of mineral oil in the diet is objectionable and refining costs are high (17). 3,4-Benzapyrene which is a trace contaminant from hydrocarbons is a carcinogen (4). There are reports that hydrocarbon-grown yeast proteins even when fed to chickens have the peculiar property of their feces sticking to cages and litters (18).

The yeast proteins are unlikely to be used for human diets until the concentration of nucleic acids can be reduced. High nucleic acid content of SCP leads to high rates of uricogenesis (19). Maximum nucleic acid allowed in humans is 2 g per day (4,15). However, it should be possible to

remove most of the RNA by activating ribonuclease by heat shocking (4,20,21). Further, yeast contains high levels of C_{15} and C_{17} fatty acids, whereas mammalian metabolism is based on even-numbered chains. The end product of odd-numbered fatty acids is propionic acid and that of even-numbered fatty acids acetic acid. Protein concentrates have been produced from mechanically degraded yeast which has less than 2% ribonucleic acid and contains 70-75% of the amino acids present in the starting material (22). Direct human food production by fermentation of most waste materials is unlikely at present due to the problem of meeting safety requirements (23). Esso-Nestle is working to produce SCP in a form suitable for human consumption (4) and is utilizing bacteria in place of yeast (11). Bacteria, in general, have higher protein content and divide more rapidly than other types of microorganisms, but their size is considerably smaller than that of yeast and fungi. Therefore, potential cost savings based on a more rapid growth rate could be offset by the higher centrifugation cost. Many bacteria also synthesize large amounts of poly-beta-hydroxy-butyrate when grown in a medium having high carbon to nitrogen ratio (20:1 or greater) which makes the product unsuitable for both humans and animals (24). Ethanol is particularly suitable, if SCP produced is intended to be used for human consumption, because it is virtually non-toxic and there are no special requirements to be met regarding the final purification of the cell biomass produced. A number of SCP processes have been developed on the basis of the substrates,

n-paraffins, methanol and ethanol (25,26). Norprotein has developed a process for SCP production from methanol (27) using Methylomonas methanolica, a bacterium capable of growing only on carbon compounds containing no carbon-carbon bonds (28,29), Carbon is incorporated as formaldehyde via the ribulose monophosphate cycle, and cleavage of the resulting fructose-6-phosphate is accomplished via the Entner-Doudoroff pathway. Cell yields exceeding 0.5 g cell mass/g methanol are obtained. The process has been run on a pilot plant scale. The economic aspects of large scale production are discussed (27).

The Pekilo process is the first commercial continuously operating process in which filamentous fungi Paecilomyces varioti is used for SCP production (30). The suitable raw materials are various acid waste liquors, molasses and hydrolyzed organic wastes. ICI's SCP Pruteen plant from methanol utilizing bacteria, Methylophilus methylotrophus, is due to come in stream in 1980 (31,32). Both 'Pruteen' and 'Toprina' have been cleared for animal nutrition by the Protein Advisory Group of the United Nations (31,32).

ICI in U.K. remains the only company with a process anywhere near commercialisation (31). Several SCP units including British Petroleum have been withdrawn recently after heavy expenditure. There is lot more work required to be done than is likely to be in the foreseeable future (33). Furthermore, advances in agriculture have resulted in spectacular yield increases in basic crops (31).

Production of SCP/Fuel/Glucose from cellulose

There is no doubt that in the long run one has to turn to cellulose, the most important reservoir of energy, as a source of food, energy, organic chemicals, synthetic polymers etc. (31). The potential of biotechnical processes based on cellulose is enormous. Even if it may be cheaper to produce ethanol from hydrocarbons today, such economics will not be realistic in the future. The chemical industry depends largely on naphtha as a feedstock which comes from the crude oil. The dependence of the chemical industry on oil or gas is solely for economic reasons. The price of hydrocarbons is soaring high every day. Hydrocarbons are also a diminishing reserve and the phenomenal growth in energy demand will be difficult if not impossible to be met with the current fuel reserves, but cellulose will always be available in large quantities. About 100-150 billion tonnes of organic substances are photosynthesized annually and 33-50% of the organic material is cellulose (34,35). In addition, enormous quantities of waste paper products are generated every day all over the world. However, vast amounts of cellulose are not used because only ruminants can digest cellulose for conversion to food and an insignificant portion is exploited for commercial purposes. Callihan *et al*. (36) presented data to show that in great many areas cellulose can easily substitute hydrocarbons.

SCP from Cellulose:

Cellulosic materials, in general, contain 40-50%

cellulose and about 50% lignin, hemicelluloses, etc. Calvin (37) believes that ultimately mankind must seek an alternative crop to trees and cane to produce digestible cellulose. Considerable efforts have gone into studies on the practicality of the production of SCP/fuel/glucose from cellulose during the last few years (4,37-47) and its practicality appears to be a matter of time. The economy of processes based on enzymatic hydrolysis of cellulose makes it necessary that all components be utilized. Xylan-type hemicellulose typical of hard wood can be used for manufacture of furfural or xylitol; glucomannan-type hemicellulose common in soft wood can be hydrolyzed to hexoses; lignin can be used for manufacture of certain chemicals or it can be simply burnt. The cultivation of certain varieties of mushrooms on wood, sawdust and straw constitutes the only major direct utilization of fibrous plants for food production. The higher fungi do not offer great promise as sources of low cost human or animal protein because of their long incubation time and their being labour intensive. The largest though not the most efficient method for the conversion of cellulose into human food still remains the anaerobic fermentation carried out by cellulolytic organisms - the alimentary tract of herbivorous. Ruminants such as cattle are more effective because of their long fermentation tank preceding the intestinal tract. Microorganisms can be grown directly on cellulose or cellulose can be converted into glucose which can be converted into SCP as yeast or used as a raw material for fermentation to solvents like

ethanol and acetone, amino acids, drugs or other useful organic substances. For bioconversion of cellulose into animal feed, Peitersen (48) reported that under continuous cultivation conditions 50-75% available cellulose was consumed by Trichoderma reesei QM 9414 over dilution rates ranging from 0.037-0.08 h^{-1}. Miller and Srinivasan (49) reported that continuous cultivation of Aspergillus terreus (ATCC 20514) on pretreated cellulose gave decomposition values of 80-85% over dilution rates of 0.10 - 0.14 h^{-1}. The protein content of the dried product was 30-35%. These studies suggest the possibility of degrading cellulosic wastes into highly proteinaceous products with potentiality for use as animal feed. With the use of 'waste' materials for SCP production, problems have been encountered arising most frequently from inconsistency in quality and availability. Starch is becoming an increasingly attractive substrate in that it is a renewable resource in addition to its property of rapid microbiological degradability. Several starch based SCP processes are now under development (50).

The cost estimates for obtaining SCP from different materials are given in Table 1. The approximate price per Kg protein from bagasse, hydrocarbons and methanol is $ 0.5-0.8 as compared to $ 0.4-0.5 for soybean meal and fish meal. The cost estimates of the various microbial products are what the prospective producers of these products hope they will sell in the market, but the ultimate price of these products to the consumers according to Litchfield may be 2-3 times higher (51). The cost estimates for SCP production from bagasse

TABLE 1. COMPARISON OF ESTIMATED PRICES OF SELECTED UNCONVENTIONAL AND CONVENTIONAL PROTEIN SOURCES (51)

Protein source	Approx. protein content	Approx. price per kg.	Approx. price per kg protein
	%	$	$
Unconventional			
Chlorella sp. Scenedesmus obliquus	45	0.45	1.00
Micrococcus certificans (n-paraffins)	72	0.35 - 0.40	0.50 - 0.80
Cellulomonas (bagasse)	50	0.25 - 0.35	0.50 - 0.70
Pseudomonas sp. (methanol)	80	0.30 - 0.45	0.38 - 0.56
Candida utilis, feed grade (sulphite waste liquor)	50	0.20 - 0.26	0.40 - 0.50
Candida lipolytica (gas oil or n-paraffins)	60	0.30 - 0.45	0.50 - 0.75
Fungi (mycelium)	50	0.65	1.30
Fish protein concentrate	75-80	1.00	1.25 - 1.33
Leaf protein concentrate (food grade)	50	0.75	1.50
Conventional			
Fish meal	60	0.29	0.48
Soybean meal	44	0.18	0.41
Cotton seed meal	41	0.15	0.37

and hydrocarbons are about the same.

Glucose/Fuel from Cellulose:

Energy problems have become global. These have forced equally both developing and developed countries to make long range programmes. Cellulose can be converted to glucose by acid hydrolysis and has been used under conditions of war time shortages to produce alcohol or food yeast from wood wastes, but has been found uneconomical due to problems of corrosion and the formation of undesirable reversion compounds in the digest (52,53). The first commercial application of acid hydrolysis of cellulose was made in 1913 at George town, U.S.A., where a plant was built to hydrolyze Southern Pine mill waste by 2% H_2SO_4 at 175°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., operated until 1923 but was not profitable (54). The acid hydrolysis method was industrialized by Scholler in Germany in 1926, but was replaced in 1935 by the "Schube" process. The German Scholler process used during the Second World War in Germany pretreated the wood with 1% HCl followed by digestion with 0.05% H_2SO_4 at 130-190°C for 18-24 h in stationary digestors for a sugar yield of 40 - 50%. The Bergius process, also used in Germany during Second World War, 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 (39,55). The acid hydrolysis method was

improved by the Forest Product Laboratories at Madison, Wisconsin during World War II. Several hundred tonnes of wood sugar molasses were made in a small pilot plant at Madison, Wisconsin by percolating 0.4 to 0.6% H_2SO_4 through chopped wood wastes at 150-185°C. This gave 4-5% sugar syrup in 40-55% yield (39,56). The acid hydrolysis method has had several operating disadvantages, the formost being the low recovery usually less than 60%, and the undesired decomposition of the cellulosic and hemicellulosic fractions into products other than reducing sugars. The estimated selling price of alcohol by the acid hydrolysis process derived from the Madison process is in the range of $ 1.50 - 1.80/gallon based on a production level of 25 x 10^6 gallon/year. The corresponding estimated capital investment is about $ 90 x 60^6 (57). None of the above processes has succeeded as a commercial operation since World War II (58).

Grethlein (59) has recently reported the process economics of making glucose from cellulose using acid hydrolysis. With an input capacity of 885 tonne/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 (60). The process designs and cost analyses had been done on untested extrapolation of laboratory work on both the acid and the enzymatic hydrolysis. Grethlein (59) considers that the acid hydrolysis of cellulose is a viable and potentially

economic process. Rogers (61) used a twin-screw extruder device in high temperature acid hydrolysis process for conversion of newspaper and wood pulp to glucose. The author feels that 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 is only 20 seconds.

It is believed by many scientists that enzymatic hydrolysis of cellulose will be simpler and may become economical if further improvements in technology are made. The enzyme is specific for cellulose and related polysaccharides and does not react with impurities that may be present in the cellulosic material. Known cellulolytic enzymes split only beta,1,4-glucosidic linkages (62), 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) (63). Further, the reaction takes place under mild conditions of temperature and pH and high enzymatic conversion of cellulosic materials including wood powder to glucose has been reported (64-69). Glucose yields of 105-111% of the weight of cellulose used have been obtained (58,70). A pilot plant for the production of SCP directly from alkali-treated bagasse using Cellulomonas uda or flavigena and Alcaligenes faecalis or yeast (Candida guillermondii or Trichosporon cutaneum)(36,71), and a pilot plant for the saccharification of 1000 lb cellulose per month for the production of glucose/ethanol

using T.reesei are already in operation in U.S.A. (35). The Bechtel Corporation, U.S.A. and the Louisiana State University Foundation, U.S.A. have entered into a joint venture for commercial production of SCP from bagasse (36,71), but apparently it has not proved successful (31).

No full scale commercial plant has been built based on enzymatic hydrolysis. However, a one tonne/day pilot unit has been operated by Gulf Oil for almost a year. They are planning currently a 50 tonne per day facility prior to building a large scale plant (72). The key to the Gulf process is reported to be the simultaneous conversion of cellulose to glucose to ethanol by combined cellulase/yeast system.

Cellulose in its native form is quite resistant to enzymatic hydrolysis. This is probably due to factors such as: (a) moisture content of the fibre; (b) size and diffusion constants of enzyme molecules involved in the hydrolysis process; (c) degree of crystallinity of the cellulose; (d) its unit cell dimensions; (e) the conformation and steric rigidity of the anhydro glucose units; (f) degree of polymerization of cellulose; and (g) the nature of the substances with which the cellulose is associated. A lignin seal surrounding cellulose fibre acts as a physical barrier. Cowling (77) has discussed these physical and chemical constraints on the susceptibility of cellulose to hydrolysis.

Most cellulosic materials contain three components: cellulose, hemicellulose and lignin in ratios of roughly 4:3:3 (74-76). For rapid enzymatic degradation of cellulose

it has been found necessary to treat it either mechanically and/or physically/chemically to loosen the lignin-cellulose structure. The need for pretreatment increases with the increase in lignin content. Lignin is highly resistant to biological degradation, a result of its unusual structure. In lignin the monomer, p-hydroxycinnamyl alchols, 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. There is actually no space for the enzyme between the cell wall structures (78).

Many chemical, physical, and mechanical pretreatment have been developed but none has been cost effective (68,79). The various pretreatments, suggested include treatment with sodium hypochlorite in acetic acid and chlorine dioxide gas (79), treatment with sulfurdioxide which breaks the binding of the lignin (80), biological delignification (81-83), inorganic acids (68), heat treatment combined with some mechanical or chemical treatment (66,68,84), sodium hydroxide alone or followed by peracetic acid (85-87). Successful chemical pretreatments include dissolving and reprecipitating cellulose (88), pulping and lignin removal, removal of hemicellulose with dilute acid, and acid or alkali swelling. The physical treatment methods include

ball milling to a fine powder (66,68), radiation (79), steaming or steam explosion (93), and compression (two roll) milling (94,95). Radiation breaks polysacharide molecules (66,84,89-92).

Alkali (39,89,96-101) (or alkali and heat treatment), grinding followed by ball-milling to a 200-400 mesh particle material (66,67,84,90,100-102) and compression (two roll) milling (94,95) are the important pretreatments that have been found useful for the rapid growth of fungi and bacteria on cellulose and for the rapid hydrolysis of cellulose. Alkali-treatment loosens the lignin-cellulose complex by hydrolyzing the ester bonds between the uronic acid of hemicellulose and lignin (103). The effect of the degree of crystallinity of cellulose on its hydrolysis by cellulase has been reviewed (104). Ball milling reduces the size (thus increasing the surface area) and also breaks the crystallinity of the material. Grinding by ball milling may be better than alkali delignification as it increases both the surface area and bulk density, so that 20-30% cellulose suspensions can be handled to obtain high concentrations of glucose in the digest. Alkali and chemical treatments may pose serious disposal problems. Alkali delignification reduces the bulk density, so that cellulose suspensions above 4-5% are too thick to stir or pump (90). More concentrated cellulose suspensions are desirable for fermentation, so that sugar solutions of reasonable concentration can be obtained. But ball milling is expensive and energy intensive as compared to alkali

delignification treatment (103,105,106). For pure cellulose, heating at 200°C during or before milling has been found to increase the efficiency of milling (84,107), but this is not recommended for complex cellulosic materials, since cross-reaction with impurities might occur (68). Sharkov and Levanova (108) observed that two roll milling increased the rate of ethanolysis of cellulose. Tassinari and Macy (94) reported that differential speed two roll milling is an effective pretreatment for increasing the susceptibility of cellulose to enzymatic hydrolysis. The processing time is of the order of 10 minutes or less as compared to 24 hours used for ball milling. The treatment has been found effective for cotton, maple chips, white pine chips. The two roll milled maple yielded 17 times more reducing sugar than the untreated control after a 24 h hydrolysis. A further advantage of the roll mill is the increased wet density of the product thereby permitting saccharification of more concentrated slurries resulting in sugar syrups of higher concentration. The tearing effects during two roll milling on crystallinity, and on the separation of chains probably account for the increased accessibility of enzyme to substrate (94).

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 not very easy 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 costs. Sugarcane bagasse, cellulosic materials collected at the sewage disposal, paper mill wastes, & rapid growing trees could be ideal cellulase sources.

The minimum cost of glucose produced by hydrolysis of waste cellulose is almost $ 0.5/kg (109). In this, the two other main components of cellulosic materials, hemicellulase and lignin, have not usually been included in the total cost analysis (110). Seeley (111) 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% (Table 2). 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). With a processing conversion cost of 15-30 cents/gal ethanol (35,106), it has been estimated that glucose in the glucose syrup must be produced at 2-4 cents/lb in order to be competitive for ethanol production (35,106,112-114). Mandels (115) considers that for the process to be economically viable we must be able to produce glucose for 15 cents per kg in crude 10% syrup. Cysewski and Wilke (113) carried out preliminary process and cost design studies for industrial scale fermentations to produce ethanol and

TABLE 2. COMPARISON OF ESTIMATED COST OF GLUCOSE AND OTHER PARAMETERS FROM CORN STARCH AND CELLULOSE (111)

	Starch	Cellulose
Supply, tonnes/acre	2	10-15
Quality	Amylopectin, amylose, protein	Cellulose, hemicellulose lignin
Solubility, %	35-40 (max. that can be obtained)	5-7.5
Sugar stream	Glucose 95%	Glucose 95% (in the presence of cellobiase)
Quality	Isomaltose, maltose (30-35%) trimers 5%	5% others
Cost, cents/lb (June 1975)		
(a) Substrate	9 (Corn starch)	2-5
(b) Enzyme processing	2	7-10
Total cost, cents/lb	11	9-15
Glucose syrup concentration, %	30	5-10

torula yeast (on left over fermented sugars like xylose) on sugars obtained by enzymatic hydrolysis of newsprint. With a total estimated capital cost of $ 5.37 x 10^6 to produce 24,000 gal/day of 95% ethanol from the hydrolyzate sugars, the cost of ethanol is estimated to be $ 1.05/gal and sugar cost, 5.2 cents/lb. Because of the high cost of ball milling, which does not appear practical for large scale processing, Wilke and Yang (116) 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 approximate capital investment of $ 19 x 10^6. Estimated costs of an enzymatic hydrolysis process based on the gulf process and using a feed stock consisting of a mixture of saw dust, pulp mill wastes, and municipal solid waste indicate that a plant constructed for 25 x 10^6 gal/year of ethanol would require a capital of about $ 64 x 10^6. The estimated selling price of ethanol for this process would be about $ 1.75/gal. By-product credits would reduce the operating cost by about 35 cent/gal (57). The price of ethylene-derived ethanol was about $ 1.0/gal in 1975 (35). With the escalting prices of petroleum hydrocarbons, alcohol derived from ethylene - a petroleum by-product now costs $ 1.80 a gallon and alcohol derived from corn is $ 1.65 a gallon (117). The break-up of cost estimates of the major items for producing glucose/alcohol from cellulose is given in Table 3 (106). Both the cost of the enzyme (and enzyme make-up) and the pretreatment

TABLE 3. MAJOR ESTIMATED ITEMS OF COST FOR GLUCOSE PRODUCTION (99,106)

Price Rs./100 kg glucose			
Raw material	Enzyme	Pretreatment	Enzyme hydrolysis and filtration
5-10	60-70	20-60	40-60

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 (118). 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 the sugar or 40% of the cost of the ethanol to enzyme production (119). 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 may also 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 and thereby produce high levels of cellulase enzymes. Mutants which produce 25% of the protein as catalase are known (120). Increase in gene copies by genetic manipulation which give highly elevated yields of enzymes are also known (121-126).

It may also be possible to obtain microorganisms or a mixed culture which can break up or loosen lignin-cellulose complex or which can utilize lignin, thus eliminating or reducing the pretreatment cost. Such a culture has been described by Eriksson (82). Enzymes involved in lignin degradation have been investigated and a new enzyme, cellobiose: quinone oxido-reductase, of importance in both cellulose and lignin degradation has been reported (127,128). The enzyme reduces quinones or phenoxy radicals in the

presence of cellobiose. In addition, a lactonase which transforms cellobiono-δ-lactone (formed by quinone oxido-reductase) to cellobionic acid was also found (82). Cellulase-less mutants have been obtained which degrade both kraft lignin and lignin in wood, but do not attack cellulose (82), thus "deshielding" cellulose. These mutants may have vast potential. New processes using enzymes which destroy the cross-linking of cell walls are being developed, which may finally change the economics of exploiting plant materials for cellulose.

Lignin is the second most renewable organic material (129). Lignin is highly resistant to biological degradation as a result of its unusual and disorderliness in chemical structures with various stable C-C and other linkages between the phenyl propane building units. This results in difficulties in its conversion to useful substrates and degradation to homogeneous compounds by chemical treatment. If one could recover and utilize lignin as a source of chemicals, such as furfural, etc., this would also aid the economics of cellulose utilization. Thus, there is lot of scope for reducing the cost of production of glucose from cellulose and further basic research and process development could produce a practical technology. The Natick scientists who are involved in the cellulose project deeply believe that the conversion of cellulose to glucose will be technically feasible and practically achievable on a large scale (130).

The overall economic feasibility is very much dependent

on efficient use of all main components of lignocellulosics viz. cellulose, hemicellulose and lignin. Tsao and his group at Purdue University, U.S.A. have indicated the possibility of solvent extraction of cellulose and its subsequent hydrolysis by enzyme/acid to produce glucose (131-133). Tsao has recommended obtaining the three major components, cellulose, hemicellulose and lignin in different streams and their separate utilization (133). 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 (132,133).

It is believed that isomerized glucose-fructose syrups would replace invert cane sugar in the world food market (35). The estimated glucose-fructose syrup production cost is 16-18 cents/lb, and the selling price 25-30 cents/lb (106). Based on the value of the products, 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 (106). 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 cent/lb as compared to 2-3 cent/lb for alcohol.

SECTION 2

PRODUCTION OF CELLULASES AND ENZYMATIC SACCHARIFICATION OF CELLULOSIC MATERIALS

Energy problems are becoming global. These have forced equally both developing and developed countries to make long range programmes. Bioconversion technology could become the most viable answer to the problems of fuel and organic chemical feedstock crisis precipitated by the escalating prices of petroleum crude. Already alcohol derived from corn is proving cheaper at $ 1.65 a gallon compared to alcohol derived from ethylene a petroleum by-product which costs $ 1.80 a gallon (57). Ethanol could replace the petroleum crude as the basic raw material for organic chemical industry. A 10% ethanol: petrol mixture is already being used as car fuel without requiring any modification in the engine. Its consumption in U.S.A. is expected to reach nearly 1800 million litres in 1980 (57). Brazil's experiment with gasohol using 20% alcohol are reported to have proved successful. It has now plans to gradually increase the alcohol content in cars to about 50%. These cars would have modified engines.

Enzymatic conversion of cellulose to glucose followed by fermentation to alcohol with yeast holds promise of a solution to the impending fuel crisis. Cellulose is not a diminishing reserve material and mild conditions under which enzymatic hydrolysis can be carried out has led to intensive study of the microbiological utilization of cellulose for the production of glucose. Until recently

enzymatic hydrolysis of cellulose was too slow and too inefficient to compete with the successful conversion of starch to glucose by a fungal enzyme. Current developments have changed the outlook. It is generally agreed that cheap glucose is the key intermediate for subsequent chemical and energy production. Culture broths from fungi capable of completely hydrolysing cellulose to glucose are now known. Trichoderma species (reesei, lignorum, koningii)(134-139), Sporotrichum pulverulentum (82,140), Penicillium funiculosum (141), Fusarium solani(138), and Botrydiplodia theobromae (142) are good sources of active cellulases. Tentative cost estimates for producing glucose via enzymatic hydrolysis (10 to 40 cents per kg glucose) reported by different investigators suggest that the cost of production of ethanol from cellulose is not economical at present (111,113,143) and further basic and technological developments are required for a viable process. 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 the enzyme yields are of the order of 3-4 FPA units/ml of culture filtrate (118). Any improvement in the cellulase yields would thus significantly help in making the process economically viable.

T.reesei, particularly its mutants, are the favoured sources of cellulase and also the most intensively studied organisms. It has taken about 20 years to increase the FPA

(which gives a rough measure of the saccharifying ability of the cultures) from 0.23 IU.ml^{-1} (T. reesei QM 6a) to 4.85 IU.ml^{-1} (T.reesei NG-14) in shake flask experiments (144,145) (Table 4). However, during the last 4-5 years, FPA activity of the order of 12-15 IU.ml^{-1} have been obtained through strain improvement and with sophisticated processing in instrumented fermentors under controlled conditions of pH, aeration etc. with high productivities of 56 FPA/litre/hour (115,146). All these developments would reduce the cost of alcohol production from cellulose.

Though Trichoderma species are favoured sources of cellulases and also the most intensively studied organisms for cellulose saccharification, suggestions have, however, been made that other sources of cellulases should also be examined. Trichoderma and its mutant produce relatively low amounts of cellobiase with the result that their culture filtrates produce mainly cellobiose from cellulose with small amounts of glucose. A Sclerotium culture has been developed which gives high cellulase (including cellobiase) activities with properties suitable for saccharification of cellulosic materials. Cellobiase plays an important role in maximizing the rate of cellulose hydrolysis. The development of the Sclerotium rolfsii parent culture for production of cellulase and beta-glucosidase is described in Chapter 1 (Part III) of the thesis. Detailed saccharification studies of different cellulosic materials by S.rolfsii parent strain culture filtrate and some of its characteristics are described in Chapter 2 (Part III) of the thesis.

TABLE 4. PRODUCTION OF CELLULASE BY TRICHODERMA REESEI

Organism	Cellulase			Cellobiase	Reference
	CMCase $IU.ml^{-1}$	FPA $IU.ml^{-1}$	C_1 [a]	$IU.ml^{-1}$	
T.reesei					
QM 6a	18	0.23	2.8	NA[b]	144
QM 9123	59	1.30	6.4	NA	144
QM 9414	152	1.48	2.5-7.6	0.5	68,144
NG 14	15	4.65	NA	1.35	145

[a] Amount of glucose (mg/ml) formed from 5% absorbent cotton in 24 h, 50°C.

[b] NA - Not available

SECTION 3

CELLULOSE - COMPOSITION AND STRUCTURE

Cellulose exists in various states of purity in plant cell walls. It ranges from 20% in some grasses to an average of 45% of dry weight in typical wood and to over 90% in cotton fibre (147). The seed hairs 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. Most of the cellulose in nature is heavily lignified. Wood cellulose occurs in the presence of hemicelluloses and with lignin, a non-polysaccharide polyphenolic polymer (74-76). While cellulose is a homogeneous polymer of glucose, hemicellulose molecules are often polymers of pentoses (xylose and arabinose), hexoses (mannose) and a number of sugar acids. The most important commercial sources of cellulose are the natural fibers of cotton and wood.

Cellulose fibres are of similar structure irrespective of their function in plant. For example, the cellulose molecular structure of wood fibre is essentially the same as that of cotton seed hair. The main differences 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 contains only one monomer and only one type of linkage, beta-1, 4-linkage, differences in the physical relationships between the chains and their

degrees of interaction makes it a multiple substrate. Both cotton and wood fibres have a thin primary wall, formed during cell division, which surrounds the relatively thick secondary wall. The outermost layer of cotton is called the cuticle. The equivalent layer in the multicellular structure of wood is the middle lamella (Fig. 1). In both wood and cotton the secondary wall, formed during the growth and maturation of the cell, is usually subdivided into the transition lamella (S_1), the main secondary wall (S_2) and the inner secondary wall (S_3) (77,133,148). The middle lamella is amorphous, generally porous and about 1 to 2 μ thick. The primary wall is usually very thin, of only 300 Å. The secondary wall (S_1, S_2 and S_3) which thickens during growth is of variable thickness but forms the bulk of the cell wall substances and it is any where from 1 to 10 μ thick (133,149). 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 in a series of concentric zones the number of which has been correlated to the 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 compound middle lamella. Cellulose is in the highest concentration in the secondary wall and diminishes toward the middle lamella (150). The minimum concentration of hemicelluloses and maximum concentration

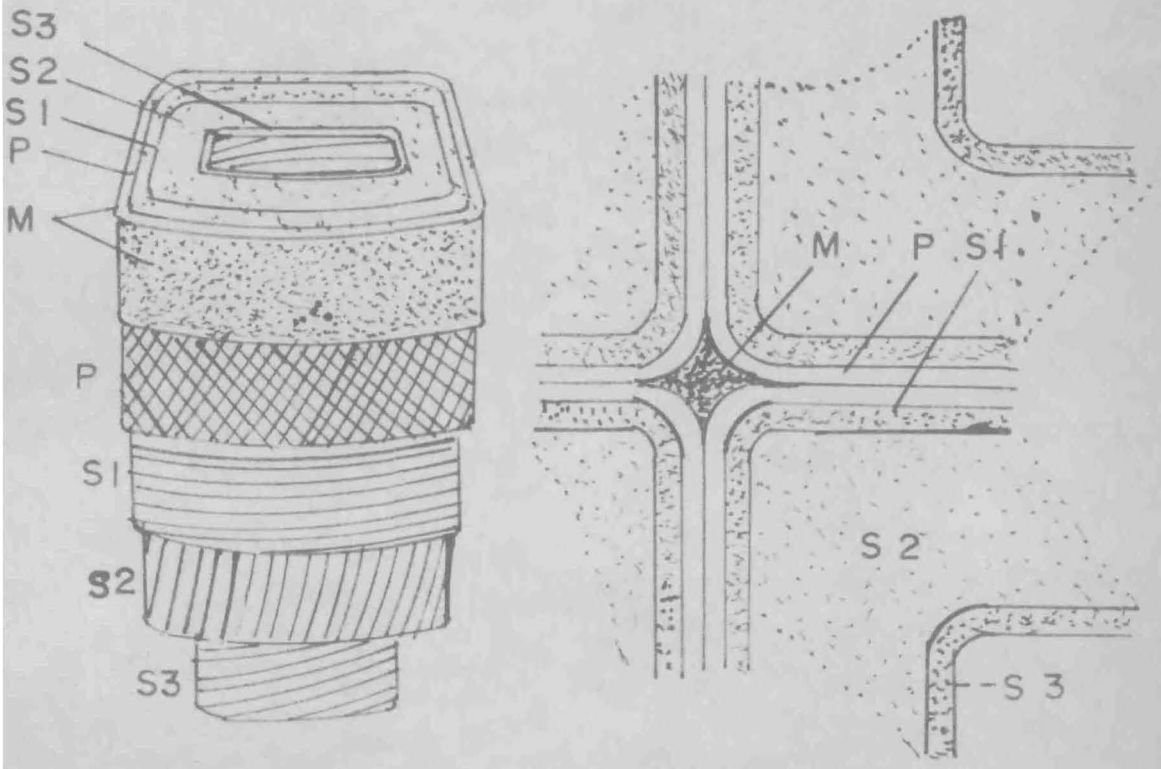
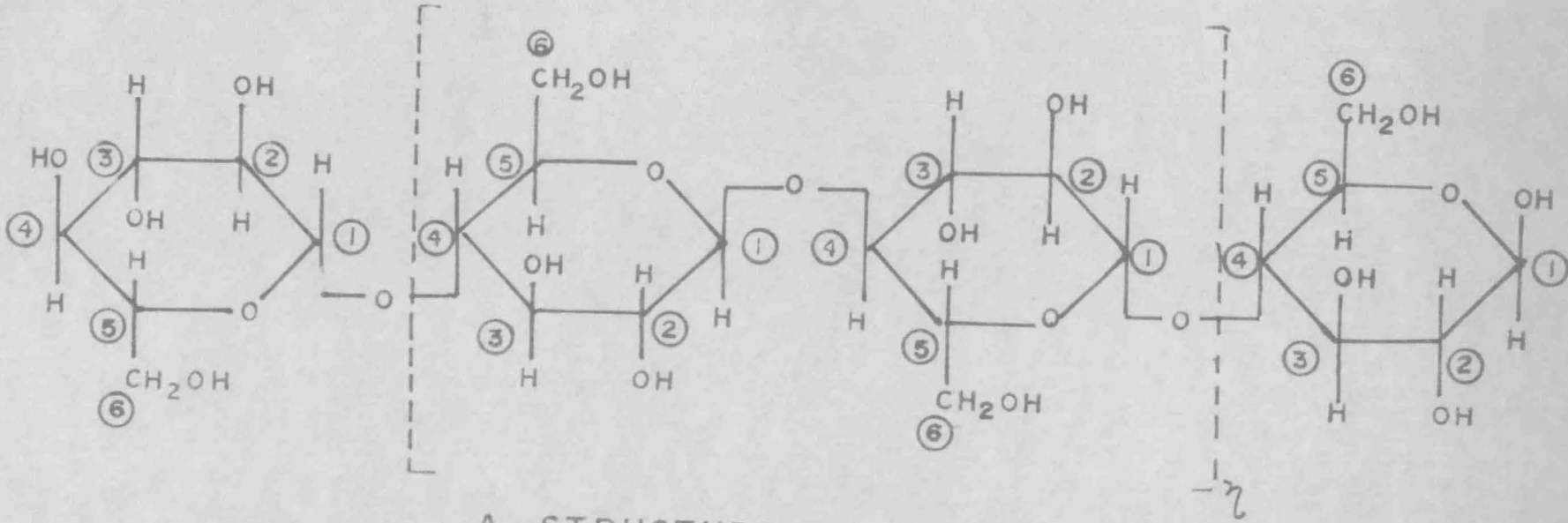


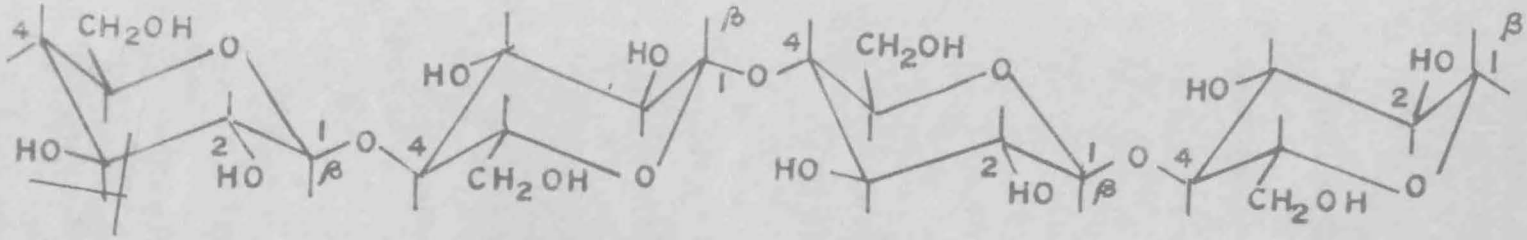
FIG. 1. Diagramatic sketch showing the various layers of wood cell walls (148). The true intercellular substance or middle lamella (M) and adjacent primary walls (P) comprise the compound middle lamella. The secondary walls are composed of outer S_1, middle S_2, and inner S_3 layers.

of cellulose occurs in the S_3 layer of an angiosperm and in the S_2 layer of a gymnosperm (150). The S_3 layer of gymnosperms contains appreciable lignin and hemicelluloses.

Cellulose is insoluble and structurally a complex substrate. The susceptibility of cellulose to hydrolysis will depend upon its accessibility to cellulolytic enzymes. Many recent advances have provided great insight into the influence of structure on the susceptibility of cellulose to enzymatic hydrolysis. Most of the structural studies of cellulose have been made on materials derived from cotton since cellulose occurs in relatively pure form in cotton, and further purification requires only mild manipulations. The chemical structure of cellulose chains was established by Haworth and Hibbert more than 60 years ago (151). The structural formula is shown in Fig. 2. Cellulose is a linear hydrophilic polymer, a polyalcohol, more or less uniformly composed of anhydroglucose units linked together through beta-1, 4-glucosidic bonds. In the cellulose chain molecule the anhydroglucose units adopt the chair configuration with the hydroxyl groups in the equitorial, and the hydrogen atoms in the axial positions (Fig. 2). The hydroxyl in the third position is bound by an intramolecular hydrogen bond to the ring oxygen O_5 atom of the next chain unit (152) and the incidence of hydrogen bonding is very high (153,154). Every other chain unit is rotated at 180° around the main axis which results in a strain-free linear configuration with practically no steric hinderances (152). The three hydroxyl groups in each unit, at C-2 and C-3



A · STRUCTURAL FORMULA



B · CONFORMATIONAL FORMULA

FIG. 2. Structure of cellulose (58, 152).

(secondary hydroxyl groups) and C-6 (primary hydroxyl group) and the glycosidic linkage act as the functional groups. All significant chemical reactions occur at these locations and thus may determine the chemical properties of cellulose.

The individual linear polymeric cellulose molecules are linked together to form elementary fibrils (77,155). The chains are oriented in parallel and 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. Each microfibril is formed by coalescence of few (156,157) or several (155) elementary fibrils. A cellulose fibre is composed of many microfibrils. The number of chains of cellulose in a microfibril may be a 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 (158) is about 50 x 100 Å in cross section. The elementary fibril is about 35 Å wide (104,156,157) and the microfibril 8 - 20 nm wide (152). Manley (159) has reported that the microfibrils have an average width of about 35 Å (30-40 Å).

The question whether the cellulose chain is uniformly composed or not has been debated for 50 to 60 years and its microfibril ultrastructure is still a subject of considerable controversy. 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 (152). 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 (152). Any irregularity in the substrate may terminate the action of enzyme, depending on the specificity of the enzyme.

Models of cellulose fibrils involving folded chains have been suggested. Structure models based on extended celluloses are also found in the literature. If cellulose molecules are fully extended, the length of elementary fibril is expected to be much longer than 1000 Å (133). Consequently, cellulose molecules are believed to be more likely folded in some fashion. Some of the concepts of microfibrillar structure proposed are the following: In fringed micellar theory the microfibril is regarded as an assembly of crystalline and amorphous regions (152,160). According to Preston and Cronshaw (158) 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; the broken lines represent the orientation of hemicellulose molecules (Fig. 3a). Hess et al.(155) suggested that the microfibril contains several elementary fibrils which contain 15 to 40 cellulose molecules and are segmented into crystalline and paracrystalline regions, i.e., the cellulose molecules are less well ordered at certain points along the length of the microfibrils (Fig. 3b). Manley (150) 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 folded chain lattice formed as a ribbon which intern is wound as a tight helix (Fig. 3c) (from Mulethaler (161)). In the Meyer and Misch model unit cell dimensions are: a = 8.35 Å; b = 10.3 Å; and c = 7.9 Å; beta = 84° (162). Each unit cell contains four glucose residues. The length of the unit cell equals that of the repeating anhydrocellobiose unit. 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 adjacent chains. In the direction of c-axis, the distance is much greater, and molecular chains are attached to each other by van der Waals' forces only (152,162). X-ray diffraction studies showed that molecular arrangement in the cellulose fibrillar bundles is regular (but not perfect)

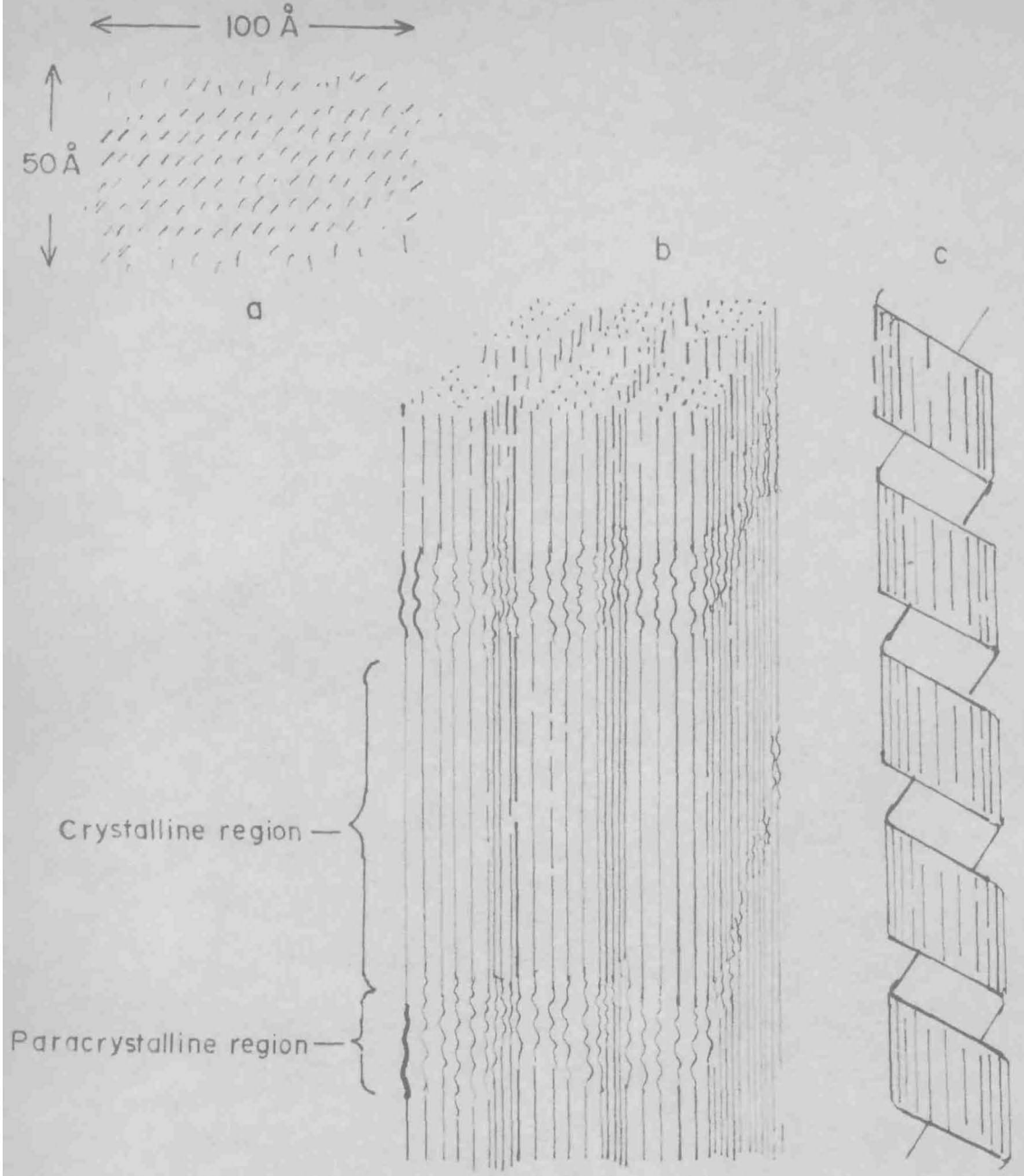


FIG. 3. Concepts of the structure of microfibrils:
(a) Preston and Cronshaw (158)
(b) Hess et al. (155)
(c) Manley (159).

that it has a crystalline X-ray diffraction pattern. Within each microfibril, the linear molecules of cellulose are associated in various degrees of parallelism containing regions of highly oriented molecules (crystalline) and regions of less order (paracrystalline or amorphous). On an average the length of the crystallites in the native cellulose is 1000 $\pm$ 200 Å and amorphous areas 300 to 400 Å (152). The concept seems generally accepted that the adjoining beta-1,4-linked D-glucopyranosyl chains run antiparrellel, forming crystalline regions in some parts of the microfibril and amorphous or paracrystalline regions in others (77,104,162). The easily hydrolysable portion of cellulose is often referred to as the "amorphous", also termed accessible, regions of cellulose and the resistant residues, the crystalline cellulose. It has been proposed that most of the bonds in cellulose exist in a configuration known as Hermans form. Through repeated Hermans beta-linkages linear polymeric cellulose molecules are linked together through 1,4- the existence of another beta-bond which involves a deflection of approximately 60° from the normal Hermans form. With three such successive deflected beta bonds a loop can be formed in cellulose polymer to produce 180° U-turn which is essential for chain folding (133). It is further postulated that these exposed, deflected beta-linkages are more susceptible to hydrolytic cleavage. Therefore, the so-called "amorphous" regions in a cellulose fibril could be zones rich in loop bends containing many deflected beta-glucosidic linkages (133). In Manley's model, the

microfibrils do not contain amorphous regions, at least as a separate phase (159). Both chemical and physical data indicate that there are amorphous regions in the cellulose fibrils and microfibrils.

Hydrogen bonding and the arrangement of cellulose molecules in native cellulose is described by Liany and Marchessault (163). An excellent discussion on the subject is given by Ward (164). He points out that the consequence of the high degree of order in native cellulose is that not even water molecule can enter the structure. The accessibility and reactivity of cellulose are closely related to itsswelling behaviour. In native cellulose the swelling is limited to the amorphous regions of the fibre, as it is counteracted by the strong hydrogen bonded network of crystallites. Strong swelling agents such as mineral acids, alkalies, quaternary ammonium bases are necessary which are able to break the hydrogen bonded network and penetrate into the crystalline areas and thus induce swelling in the whole fibre. This results in increased accessibility and reactivity.

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 (58). The degree of polymerization of typical materials is given in Table 5 (165). Evidence obtained by different means indicates that native cellulose is composed of more than 10,000 beta-anhydro glucose residues (there may be as many as 15,000 residues (153)) linked to form a linear chain

TABLE 5. CELLULOSE CHARACTERISTICS (165)

Source	Molecular Weight	Degree of polymerization
Native Cellulose	600,000 - 1500,000	3,500 - 10,000
Chemical cottons	80,000 - 500,000	500 - 3,000
Wood pulps	80,000 - 340,000	500 - 2,100

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.

S E C T I O N 4

BIOCHEMISTRY OF CELLULOSE DEGRADATION

Nature of cellulase:

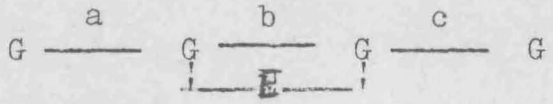
The International Commission on Enzyme Nomenclature lists 'cellulase' as the trivial name for beta-1,4-glucan-4-glucanohydrolase (EC 3.2.1.4),i.e. for enzymes which hydrolyze beta-1,4-glucans at linkages which are not restricted to terminal linkages. The term 'cellulase' has been used in the literature 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 utility of standard cellulase assay to monitor the purification of a single enzyme is limited severely by the insolubility of the substrates, by the requirement for several enzymes in order to ensure a rapid hydrolytic rate, and by the similarity of the degradation products.

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 microbial degradation of cellulose, even though species of algae, yeast 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, Trichoderma reesei (115,139,144), Trichoderma koningii (166), Fusarium solani (138), Sporotrichum pulverulentum (82,140), Penicillium funiculosum (141) and Sclerotium rolfsii (167-173) can provide culture filtrates which are highly effective in producing extensive degradation and complete solubilization of both native and degraded forms of cellulose.

The conversion of crystalline cellulose to glucose seems to require three types of enzymes designated as C_1, C_x and beta-glucosidase (cellobiase) (174,175). Neither C_1 nor C_x is able to solubilize a crystalline cellulose, such as cotton, fibre to a significant extent, but when acting synergistically they permit solubilization of cotton (176-185) (Fig. 4). The synergism between C_1 and C_x types of enzymes for crystalline cellulose degradation is not yet fully understood, but it is conceivable that their alternative action might open up the fibre structure in a

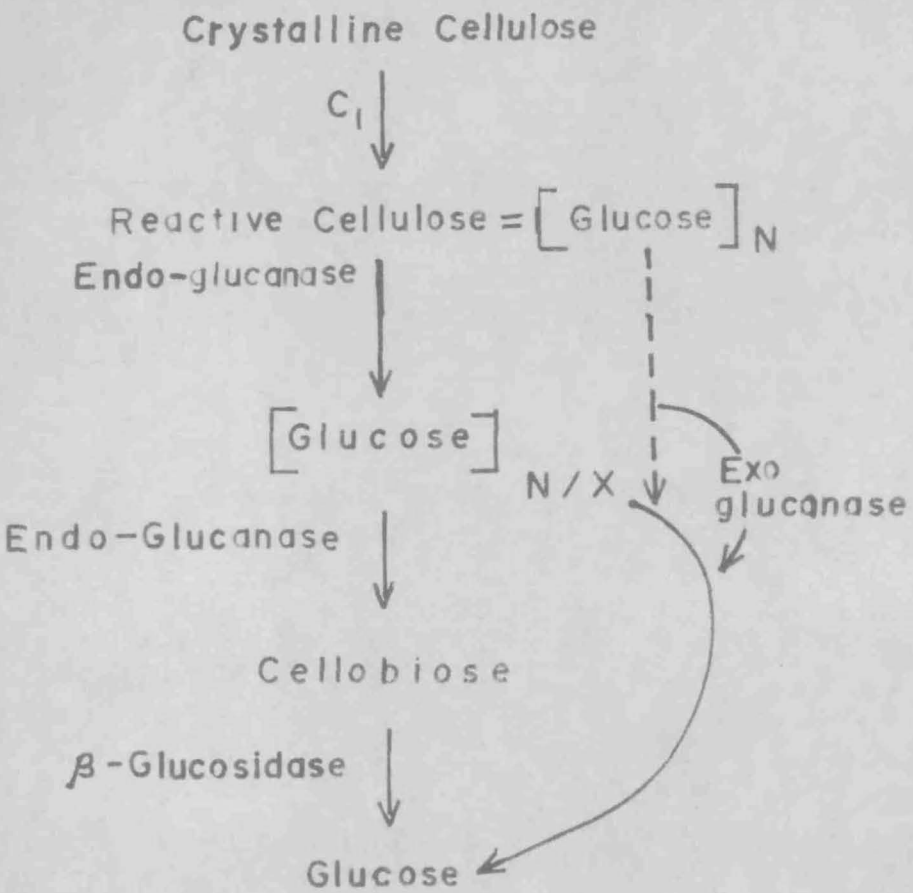
manner not possible for either enzyme alone to act.

The C_1 component has little or no effect on soluble derivatives of cellulose such as carboxymethylcellulose (CMC). Enzymes classified as C_x can hydrolyze amorphous cellulose or soluble or partially degraded celluloses producing higher cellooligosaccharides, glucose or cellobiose. These were postulated earlier to consist of exo- and endo-beta-1,4-glucanases (176,186,187). These are hydrolytic in nature and their action results in the appearance of reducing sugars in the hydrolysate. 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 internal beta-1,4-linkages (188-190). The randomacting enzymes cause a rapid increase in fluidity with a relative slow increase in reducing end groups, but their action is not entirely random. Both the end linkages appear to be somewhat less affected than the internal linkages. The rate of hydrolysis increases with the degree of polymerization (DP) within the limits of substrate solubility. The binding of endo (1 ⟶ 4) beta-glucanase requires a minimum of two adjacent unsubstituted glucosyl units (G) linked by (1 ⟶ 4)-beta-linkages (linkage b).



However, the linkage broken is the adjacent linkage c (191). Substitution on every other glucose unit of CMC would make it completely resistant to hydrolysis by endo-glucanases (192). The endo-glucanases act with the retention of configuration, whereas the exo-glucanases act by inversion.

C_1 was postulated to be an enzyme required along with C_x for the hydrolysis of crystalline cellulose. The precise function of C_1 was, however, not specified and this has evoked many speculations. The C_1 component was postulated to carry out preliminary modification of crystalline cellulose which renders it susceptible to hydrolysis by C_x enzymes (174,175) (Fig. 4). It was believed to be non-hydrolytic in nature and was thought to be attacking anomalous or atypical linkages (174,175) or the hydrogen bonds (193). Its function was thought to be that of swelling or disrupting the hydrogen bonds that hold the molecules together, thereby disaggregating the chains and converting the crystalline to amorphous regions. A single enzyme may be responsible for both C_1 and C_x activities (194-196). Sudo *et al.* (196) 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. They suggested that a single enzyme may be responsible for both C_1 and C_x activities. Leatherwood (197) 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



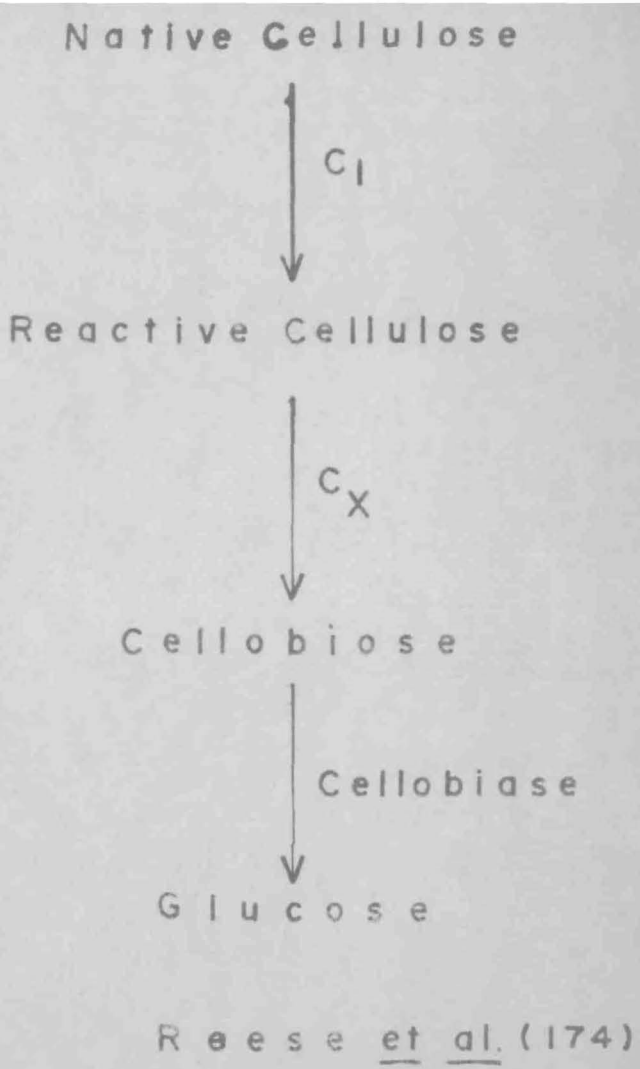


FIG. 4. Postulated crystalline cellulose degradation by the cellulase complex.

"affinity factor" was suggested to be that of binding the "hydrolytic factor" to cellulose to permit multiple attacks (197). Yet another concept envisages C_1 to be an enzyme capable on its own of solubilizing crystalline hydrocellulose (176,198,199), although acting synergistically with the C_x components it brings about an increase in the rate of hydrolysis. The C_1 component is viewed by some investigators to be an exo-glucanase and in the case of T.reesei and T.koningii, it is a cellobiohydrolase (138,200-206). The C_1 component purified by Wood and coworkers from T.koningii was always associated with trace amounts of CMCase (137,138,202) (as measured by the reducing sugars produced, but there was no viscosity lowering activity). The authors suggested that this activity did not arise from a contaminating C_x (CMCase), but was an inherent part of the enzyme (137). The enzyme removed cellobiose units from CMC or H_3PO_4 - swollen cellulose. The action of C_1 was halted as soon as unsubstituted cellobiose units on the chain ends were removed; one substituent on every other glucose residue was sufficient to render CMC resistant to hydrolysis (137). From the synergism displayed by reconstituted mixtures of C_1 and C_x enzymes in solubilizing native cotton, it was postulated that it is the C_x component which initiates the attack on cotton fibre, thereby creating more chain ends for C_1 for attack (137), and not the C_1 component, as originally postulated by Reese et al. (174). However, this mechanism, as Wood (138) and Wood and McCrae (137) themselves pointed out, would not explain as to why C_1 and C_x enzymes

are both individually capable of hydrolysing swollen cellulose and not highly ordered cellulose, such as cotton, yet when acting in solution simultaneously can hydrolyze crystalline cellulose with comparative ease. Eriksson (207), Eriksson and Pettersson (140), and Berghem and Pettersson (203) 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 ends where C_1, the exo-enzyme, can act. Tomita et al.(201) reported that Avicelase (C_x) and CMCase (C_1) are immunologically different proteins and that Avicelase attacks Avicel readily and CMC with a low activity (Avicelase/CMCase ratio = 18.1). Avicelase's action is random type and is considered to be an endo-glucanase of less-random type (208). The resistance of CMC hydrolysis by Avicelase as compared to CMCase was attributed to Avicelase being more sensitive to substitution at C_6 atom 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 (184,209) 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 (209). It was assumed that CMCase first attacks cellulose

chains, mainly at amorphous regions, to produce cellulose fragments which then serve as substrates for Avicelase (184). Pettersson (206) has also suggested that regions of low crystallinity in the cellulose fibre are attacked by endo-glucanases and free chain ends are created.

Halliwell and Griffin (205) 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 the C_1 component. 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 (210) and later studied by Halliwell (211), Marsh (212) and Zhukov et al.(213). The C_2 enzyme has been crystallized (183). King (214) 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 (205) 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 (137), Eriksson (188,207), Eriksson and Pettersson (182), Berghem and Pettersson (203), Tomita et al.(201) and Emert et al. (215) consider that the enzymes required for attacking and solubilizing highly ordered cellulose (cotton) are C_1, CMCase and cellobiase.

Halliwell (216) has subsequently reported that the multi-enzyme cellulase systems of T.koningii and Myrothecium verrucaria consists of two cellulases: exo- and endo-glucanases, and two dextrinases: CMCase and cellobiase, and the four components can effectively carry out saccharification (short fibre formation and saccharification) of cellulosics. The low molecular weight cellulase and endo-cellulase in combination promote an initial stage in the degradation of native cellulose, its conversion to short fibres as a preliminary to saccharification (217).

Recent investigations on the activities of enzymes (previously denoted "C_1" components) on unsubstituted but amorphous substrates such as phosphoric acid-swollen cellulose or on cellodextrins have demonstrated the release of cellobiose (137,140,201-205,218). All such components thus fulfil the roles of both hypothetical "C_x" and "C_1" enzymes. The consensus for quite sometime was that C_1 is an exo-glucanase, and in some cases it is a cellobiohydrolase (137,202,203,205) acting via an endwise mechanism. It is hydrolytic and not non-hydrolytic in nature as was originally postulated by Reese et al.(174). The term C_1 came to be used to denote a cellulolytic enzyme which either alone or together with C_x or cellobiase enzyme is active towards highly ordered cellulose.

More 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, cellulose oxidase (which they obtained free from endo- and exo-glucanases) and cellobiose: quinone oxido-reductase (82,127,128,219). Cellulose oxidase, a hemoprotein, which oxidizes cellobiose to cellobionic acid (188), 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 (189)). The enzyme was shown to be oxidative in character and seemed to oxidize the hydroxyl groups in the sixth position in the glucose unit to a carboxyl group. It was suggested that the oxidation of glucose units of cellulose to uronic acid moieties 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 (188). 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. Marsh (212), while studying short fibre formation from cotton, had suggested that peroxidation at some points on cellulose potentiates the enzymic process. Halliwell (220) and Koenigs (221) had also pointed out that depolymerization of cellulose need not be an enzymatic process and that a combination of H_2O_2 (0.4%) and $FeSO_4$ (0.2 mMole at pH 4.2) depolymerizes cellulose, which becomes fragmented to

very short fibres. Koenigs (221) has proposed that these play a role in wood-rotting organisms. Halliwell 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 (220).

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. 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 (222). 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 (222). Thus, there are probably a number of 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.

Eriksson (188) has more recently postulated that cellobiose oxidase oxidizes the reducing end groups formed when a beta-glucoside 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.

A model for the enzymatic hydrolysis of cellulose for the enzyme systems of Thermoactinomyces (223) and T.reesei (215,224) has been suggested. In this model, one or several endo-glucanases act randomly to produce oligosaccharides while one or several exo-glucanases, including cellobiohydrolase produce cellobiose (or glucose) from the non-reducing ends of these oligosaccharides (190,202,225). The end products of several endo-glucanases are cellobiose and small amount of glucose (208,226,227). Finally one or several cellobiase(beta-glucosidases) produce glucose from cellobiose.

Summary

The conversion of cellulose to glucose seems to require 3 to 4 different type of enzymes. These are classified as: endo-beta-1,4-glucanases, exo-beta-1,4-glucanase, cellobiohydrolase (which is also an exoglucanase) and beta-glucosidase (228). There may be an exo-glucanase which is not a cellobiohydrolase. There are some exo-glucanases which will not attack insoluble substrates or hydrolyze it to any significant extent when acting in isolation. Exo-glucanases hydrolyze Walseth, i.e. H_3PO_4-swollen cellulose but endo-glucanases can also hydrolyze Walseth cellulose (202,225). The final products of action of endo-glucanases and exo-glucanases are cellobiose and glucose (225,226). The cellobiohydrolase splits cellobiose from the non-reducing ends of the cellulose chain (226). The endo-glucanase, synonymous with carboxymethylcellulase and C_x enzymes, hydrolyze cellulose derivatives such as CMC or amorphous cellulose i.e. H_3PO_4 - treated cellulose and act by randomnly splitting the cellulose into smaller chains eventually producing cellobiose and glucose. Beta-glucosidases, i.e. cellobiases hydrolyze cellobiose and other cellodextrins to glucose (217,229). Some of the beta-glucosidases can also be termed as exo-glucanases as they are not specific for cellobiose and remove glucose from the non-reducing ends of higher cellodextrins (217).

The hydrolysis of native cellulose is thought to result from the synergistic action of endo-glucanases and exo-glucanases. The present thinking of the mechanism of native

cellulose hydrolysis involves a sequential action by an endo-glucanase 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. 5,6) (137, 140,184,205,215,230). 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 endo-glucanases and exo-glucanases 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 cellooligosaccharides to glucose (Fig. 5,6). 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 oxidase, which they isolated and purified from S.pulverulentum and obtained it free from cellulase activities, is important in cellulose hydrolysis (188). They suggested that cellobiose oxidase oxidizes the reducing end groups formed when a beta-glucoside bond is split through the action of endo-glucanase and thus effectively prevents a broken beta-1,4-glucosidic bond from reforming in the crystalline part of the cellulose. However,

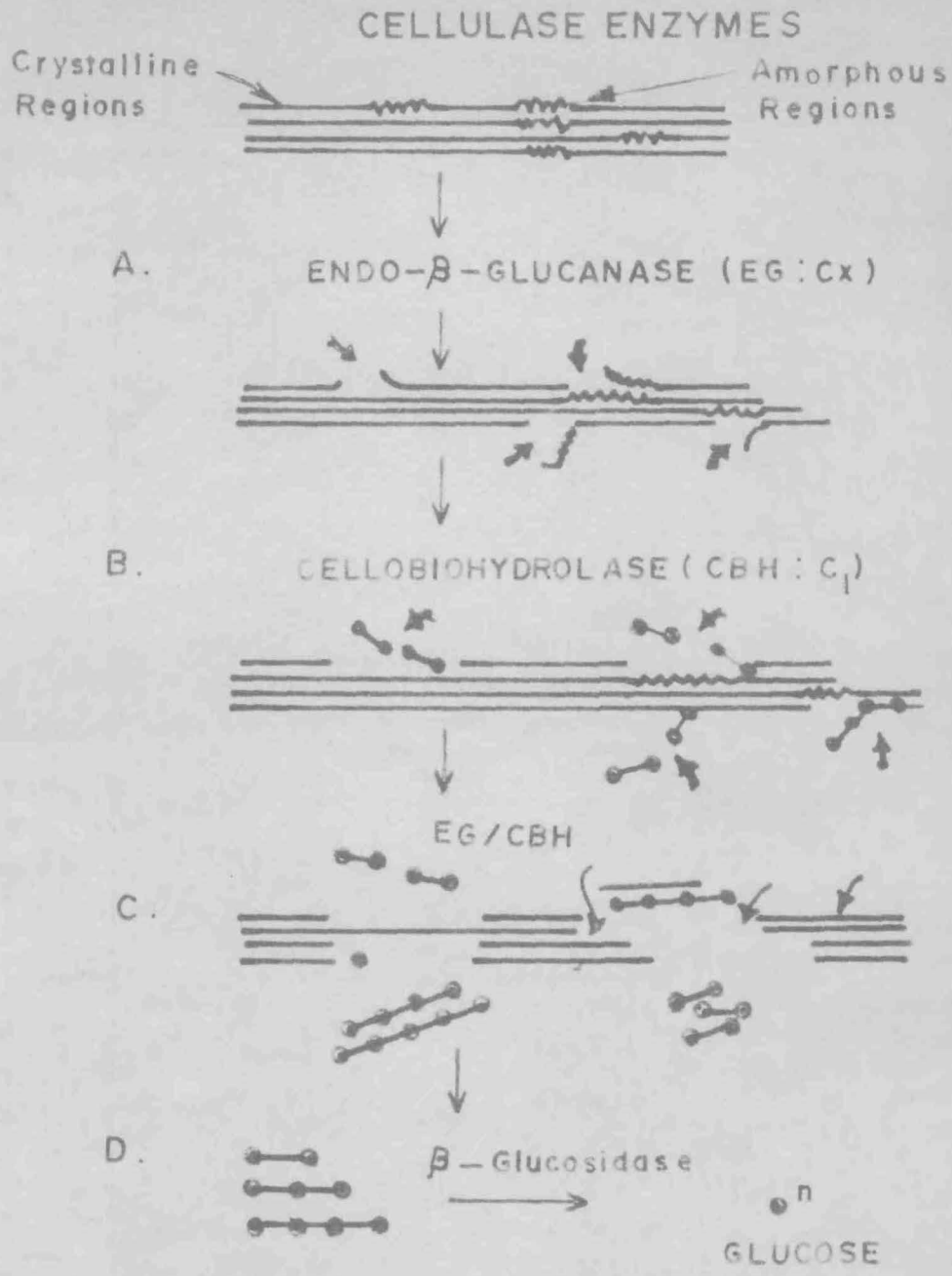


FIG. 5. Schematic representation of sequential stages in cellulolysis (137,140,184,205, 215,230).

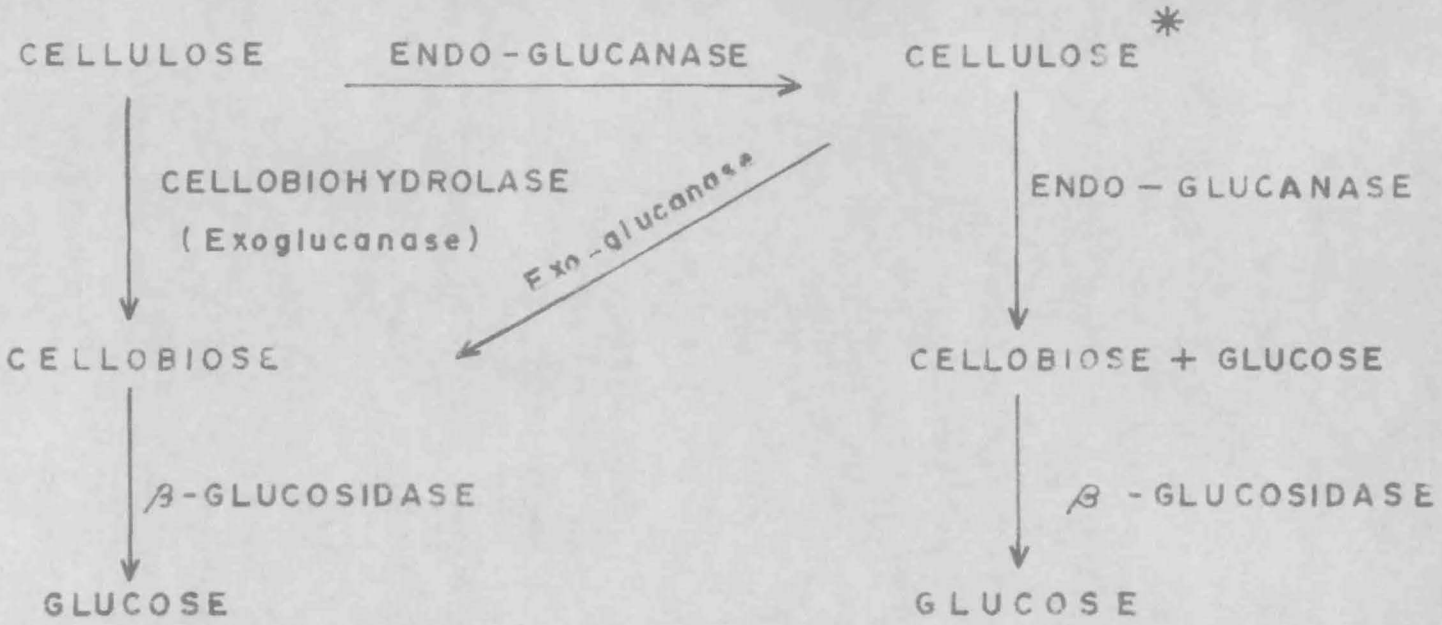


FIG. 6. The cellulase enzyme system (327).

no oxidative enzyme has been found to be involved in extracellular cellulose degradation in the case of T.reesei, F.solani and P.funiculosum (189). Wood and McCrae (189,225) consider that endo-glucanases and exo-glucanases may form a loose complex on the outer surface of the cellulose chains and there is sequential action of endo- and exo-glucanase. The adsorption of a charged molecule such as endo-glucanases, exo-glucanase or the endo-exo-glucanase complex on the surface of the cellulose fibre might result in localized disruption of hydrogen bonds (189,225). If that is so, then the concept is similar to the one put forward by Reese (174) in 1950 that the cellulose has to be activated somehow before cellulase enzymes can act.

Conclusions

It is difficult to obtain unequivocal proof as to whether endo-glucanase or exo-glucanase initiates the attack on crystalline cellulose substrates because of the nature of the substrate and as techniques are not available to detect the very small amount of change that would result from this type of attack. The question whether less ordered regions in cellulose chains exist in the microfibrils has not yet been answered, The cotton fibre cannot also be regarded as a homogeneous substrate. Periodic alterations in density have been reported to occur along the microfibrils (231) and apparent preferential dissolution of certain areas of the cotton fibre by a mixture of endo- and exo-glucanases has been noted (189), suggesting that there

are more accessible regions in the cellulose fibre. It is thus difficult to interpret the significance of the early manifestation of attack.

The present thinking of the mechanism of native cellulose hydrolysis involves the concerted (sequential) action of endo-glucanases which attack at random the 1,4-beta-linkages along the cellulose chain at the less crystalline or amorphous regions, thus opening up the structure, and exo-1,4-beta-glucanase splits off cellobiose (or glucose) units from the non-reducing ends of the cellulose chain ends (Fig. 5,6) (137,140,184,205,215,230). This assumes that regions of less ordered cellulose (amorphous, susceptible ?) chains exist in the microfibril. Formulations of this model has resulted through the contributions of a number of workers (140,184,188,201,205,206,225,230,232,233) but it has not met with universal acceptance (234). The continued cooperative action of the endo-glucanases and exo-glucanase (cellobiohydrolase) results in the conversion of the cellulose to cellobiose and small oligosaccharides. Beta-glucosidase (cellobiase) hydrolyzes cellobiose and other cellooligosaccharides to glucose. Only crystallites on the outermost surfaces of the cotton fibre (or edges, projections, etc.) are accessible to the large endo- and exo-glucanases (235,236). Small differences in IR spectra of endo- and exo-glucanase-treated and untreated Avicel have been found indicating a slight increase in the crystallinity of the enzyme-treated materials (166,189). This supports preferential hydrolysis of the more accessible

areas of the substrate. The question relating to the synergistic action between C_1 and C_x components at the site of attack has not been answered. Wood and McCrae (189) consider that endo- and exo-glucanases may form a loose complex or multiple enzyme complexes on the surface of the cellulose chains and there is a sequential action of endo-glucanases and exo-glucanases. They consider this to be compatible with the Reese's original hypothesis, if one assumes that the adsorption of a charged molecule, such as endo-glucanase, exo-glucanase or the endo-exo-glucanase complex, on the surface of cellulose fibre, might result in localized disruption of hydrogen bonds (189). A loose complex hypothesis would also explain why the endo-glucanase from one organism can act synergistically to a greater extent with exo-glucanases from certain fungi as compared to those from other fungi (189).

Future scope

Endo-glucanases, exo-glucanases and cellobiases have been obtained in a fairly pure state which individually do not hydrolyze native cellulose to any significant extent but solubilize highly ordered cellulose when acting in concert. The mode of action of individual components of cellulase complex as well as the composition of the system are still a subject of debate. The initiation mechanism of the degradation of cellulose has yet not been completely clarified. It is possible that there is an additional factor/enzyme, as yet unidentified, which may be required for solubilization of highly ordered cellulose substrates. The important question relating to the synergistic action between endo-glucanase and exo-glucanase components at the site of attack has not been answered. For clarifying the biochemical mechanisms of cellulose breakdown it is necessary to have access to the different enzymes purified to absolute specificity and functionally characterize them. With the pure cellulase enzymes it should be possible to study the features that determine the susceptibility and resistance of cellulose fibres to enzymatic hydrolysis. With the current rapid advances in the techniques for purification of enzymes and more sensitive methods for detection of initial changes in cellulose, it should be possible to work out a detailed mechanism of enzymatic hydrolysis of cellulose in not too distant a future.

SECTION 5

PRESENT INVESTIGATIONS

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

The work presented in this thesis includes the following investigations:

1. Optimization of cellulase and beta-glucosidase production by Sclerotium rolfsii CPC 142 parent strain.
2. Saccharification of a number of pure celluloses and of cellulose in lignocellulosics with S.rolfsii culture filtrate.
3. Purification of four beta-glucosidase enzymes, a component of cellulase complex, to homogeneous state and study of their physico-chemical properties.

PART II

MATERIALS AND METHODS

Materials

The following materials were purchased from the suppliers indicated: "Glox" glucose reagent (AB Kabi Diagnostica, Stockholm, Sweden), Avicel P.H. 101 (38 µm average particle size, American Viscose Co., U.S.A.), Avicel P.H. 102 (90 µm particle size, American Viscose Co. U.S.A.), Cellulose-123 powder (Carl Schleicher and Schull Co., West Germany), Solka Floc SW40 and BW 200 (pure fibrous spruce wood cellulose pulp, Brown Co., Berlin, New Hampshire), absorbent cottom (Bengal Chemicals and Pharmaceutical Works Ltd., India), proteose peptone 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 and Conconavalin-A Sepharose 4B (Pharmacia Fine Chemicals, Uppsala), acrylamide, and N,N,N',N'-tetramethylethylenediamine (Eastman Kodak Co., U.S.A.), 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, deoxyribonuclease I, beta-D(+)cellobiose, L-D(+) mellibiose, D(+) trehalose dihydrate, salicin, esculin hydrate, arbutin, phloridzin, alpha-methyl-D-mannoside,

alpha-methyl-D-glucoside, phenyl-alpha-D-glucoside, p-nitrophenyl-alpha-D-glucoside, p-nitrophenyl-beta-D-galactoside, lichenan, carboxymethylcellulose-Na salt, xylan (Larch Wood), glucono-delta-lactone and Coomassie brilliant blue (Sigma Chemical Company, U.S.A.), p-nitrophenyl-beta-D-xylopyranoside, laminarin and 3,5-dinitrosalicylic acid (Koch-Light, U.K.).

Paper mill wastes (bleach house, machine house and sedimented 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.

Cotton sliver (a crystalline fibrous cellulose), cellodextrins (G_3, G_4 and G_5), sophorose, laminaribiose and pustulan were kindly supplied by Dr. E.T.Reese (U.S. Army Natick Laboratories, U.S.A.), fusidic acid by Dr. D.W. O. Godtfredsen (Leo Pharmaceutical Products, Denmark), nojirimycin by Dr. S.Inouye (Meiji Seika Ltd., Japan) and cellotetraitol by Dr. K. Wakabayashi (Shirishu University, Japan). G_6 was prepared according to Miller's (237) procedure.

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.

Organism:

The fungus used in this study was isolated from rotting groundnut husk and has been tentatively identified as the sclerotial state of Corticium rolfsii, (i.e. Sclerotium rolfsii) designated Sclerotium rolfsii CPC 142. Sclerotium rolfsii was described in our first paper (167) as a basidiomycete species.

Cultivation and preparation of the crude extract:

The S.rolfsii culture was grown on T.reesei medium developed by Reese and Mandels (238) supplemented with trace metals (239), for purification and for initial media variation studies. For saccharification experiments the enzyme was collected by growing the culture on NM-3 medium (optimized medium). The compositions of T.reesei, NM-2 and NM-3 media are summarized in Table 6. 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 mycelia 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

TABLE 6. COMPOSITION OF T. REESEI MEDIUM, NM-2 AND NM-3 MEDIA

Constituent	T.reesei medium[a] g/l	NM-2 g/l	NM-3 g/l
KH_2PO_4	2.0	2.0	2.0
$(NH_4)_2SO_4$	1.4	-	-
$(NH_4)_2HPO_4$	-	7.0	7.0
Urea	0.3	0.3	0.3
$MgSO_4.7H_2O$	0.3	0.3	0.3
$CaCl_2$	0.3	0.3	0.3
Porteose peptone (Difco)	0.25	0.25	0.25
Yeast extract (Difco)	0.1	0.1	0.1
Trace metal solution[b]	1.0	1.0	1.0
Cellulose-123	5.0	30.0	30.0
Rice bran	-	-	30.0

[a]Medium developed by Reese and Mandels (238).

[b]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 (239). 1 ml of this solution was used per litre of medium.

enzyme development and was therefore a more suitable inoculum. The culture was harvested at 12-14 days by filtration through glass wool. The clear culture filtrate was used for estimating enzyme activities after adjustment of the pH to 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 0.001% merthiolate or 0.01% sodium azide without any loss of activity. The S.rolfsii CPC 142 retained its enzyme activities for over 4 years with frequent subculturing on PDA or when stored in lyophilized state.

Methods of Analysis:

Reducing sugars were measured in aliquots of reaction mixtures as glucose either by the dinitrosalicylic acid (DNS) method (240) or by the Somogyi-Nelson method (241). 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 (242). 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 (202, 243). Cellobiose was estimated enzymically as glucose after hydrolysis with purified cellobiase. Since some of

the hydrolysates contained high amounts of glucose, glucose in the hydrolysates 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 (244).

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.(245). Crystalline bovine serum albumin was used as the standard. Samples, free of ammonium sulfate and 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 (246). The following empirical equation (247) was used to correct for light absorption due to nucleic acids.

1/7 (2.3(O.D. 280 nm - O.D. 340 nm) -(O.D. 260 nm - O.D. 340 nm)

= mg protein per ml.

The concentration of serum albumin in the solutions was calculated from its extinction coefficient at 280 nm (248).

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), or n-propanol:ethyl acetate:water (6:1:3) (249). 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 their 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.

Enzyme Assays and Determination of Saccharifying Ability of Culture Filtrates:

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. These include measurement of loss in weight of insoluble substrate (250), decrease in the tenstile strength of the yarn, thread or fibre, microfragmentation of cellulose micelles (135), increase in uptake of alkali (S-factor, swelling factor activity), change in turbidity of a cellulose suspension (187), 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 (251-253) and decrease in viscosity (referred to as CMC liquifying activity) of solutions of cellulose derivatives such as carboxymethylcellulose, hydroxyethylcellulose etc. 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. Nonionic substituted cellulose, hydroxyethylcellulose, is preferred over ionic-substituted carboxymethylcellulose 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. Sulphite pulps such as Solka Floc and filter paper have also been used with a measurement of the formation of reducing sugars (135). 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 a long series of determinations.

The method of Mandels and Weber (135) has gained general acceptance. The increase in glucose formation, however, is not linear because the most 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 (254). 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 (68,135,144) and is the procedure that has been followed by us.

Montenecourt et al.(228) described a modification of the filter paper assay -an antibiotic disks assay-which allowed direct interconversion of units obtained employing either method. The antibiotic assay disks (740E) 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 disk 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 (68,135,144) for following the 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 3.7, incubated for 30 min

at 55°C. Reducing sugars were measured as glucose equivalents by the DNS method. Reducing sugar production was linear to about 0.2 mg. Enzyme solutions were diluted to give a value of 0.15 to 0.2 mg of reducing sugars. No difference in CMCase values was observed when 0.5% CMC type 50 T (Hercules Powder Co., Delaware), the type used by Mandels and Weber (135) and Mandels et al.(68), 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 55°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 activity was obtained if the quantity of filter paper was increased.

Cotton-degrading activity (CA):

To 50 mg of absorbent cotton was added 1 ml of 0.05 M citrate buffer, pH 4.8, and 1 ml of the appropriately diluted enzyme solution. After incubation for 24 h at 50°C. 1 ml DNS was added and the reducing sugars were determined as glucose. Reducing sugar production was linear to about 0.5 mg.

Cotton activity (C_1-activity):

Culture filtrate (5 ml) was incubated with 250 mg absorbent cotton, pH 4.8, at 50°C for 24 h. The liberated

reducing sugars were determined as glucose by the DNS method.

Beta-glucosidase activity:

Beta-glucosidase activity was determined by a modified method of Eberhart (255) using p-nitrophenyl beta-D-glucoside (PNPG) as substrate. The assay mixture contained 0.9 ml of PNPG (1 mg/ml) in 0.05 M citrate buffer, pH 4.5 (or pH 4.2 for pure enzyme solutions), and 0.1 ml of suitably diluted enzyme solution. After incubation at 70°C (or 68°C for pure enzyme solutions) 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 $cm^2/\mu mol$ for nitrophenol at 410 nm (256).

Cellobiase activity:

Cellobiase activity was determined by measuring release of glucose from a solution of beta-D(+)-cellobiose by a modified method of Umezurike (257). 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 (243). Glucose production was linear to about 0.1 mg glucose.

Alcohol dehydrogenase activity:

Alcohol dehydrogenase was assayed by measuring the formation of NADH according to the procedure of Vallee and Hoch (258). The assay was performed in 3 ml silica cuvettes. The assay system consisted of 2.5 ml phosphate (pH 8.8),

0.1 ml of NAD^+ (10 mg/ml in 0.02 M phosphate, pH 6.5) and 0.2 ml of 95% ethanol. The reaction was started by adding 0.2 ml of the enzyme sample and the increase in optical density against the reagent blank was recorded at 340 nm.

Definition of unit of activity:

The FPA, CA and C_1-activity assays are 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 p-nitrophenol from PNPG for beta-glucosidase, per minute per millilitre enzyme solution. C_1-activity is expressed as milligrams reducing sugar (as glucose) per millilitre culture filtrate in 24 h. 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. (68).

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

The substrate depletion was also followed in some cases by weight loss and by determining the undigested cellulose by the anthrone method (244). At the end of saccharification, 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:

The cellulosic materials were milled in the Wiley Mill and the ground material was sieved through a U.S. standard 50-mesh sieve. The pretreatments tried were: incubation with 4 N NaOH at 30°C for 24 h, steaming with 4 N NaOH for 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 plus 25% hydrogen peroxide (1:1 by vol)). Mesta wood and coniferous wood powders were also delignified by first treating with 4 N NaOH at 30°C for 24 h and subsequent boiling with 20% peracetic acid or vice versa. The lignocellulosics were treated with delignifying agents at a ratio of 1:10. In all cases samples were thoroughly washed with water and dried at 45°C.

Ammonium sulphate precipitation:

The culture filtrate was concentrated by precipitation with ammonium sulphate at 90% saturation. After stirring for at least 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 or Sephadex G-200 for molecular weight determination or (1.8 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 5 ml fractions at a flow rate of 10-12 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 collodion 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 to 4.5 with 0.1 M citric acid immediately.

Polyacrylamide gel electrophoresis methods:

Analytical disc gel electrophoresis was performed at pH 8.9 according to Davis (250) and at pH 4.3 according to Maurer (260) 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 for 60 min, and carbohydrate in duplicate gels with periodate-fuchsin (261). The destaining of the gel was performed by diffusion

in 7% acetic acid.

The molecular weight of native beta-glucosidase using the gel electrophoresis was determined according to the method described by Hedrick and Smith (262). Separation gels with various concentrations of acrylamide (6-12%) were prepared according to Ornstein and Davis (263) except that the ratio of acrylamide to bis (N,N'-methylenebisacrylamide) was 30:1 which was maintained constant in all the gels. The use of spacer gel was found unnecessary. Electrophoresis was carried out at 2 mA for 30 min and 4 mA for 2 h. At the end of the run the dye front was marked. Migration of dye and protein bands was measured on an illuminated box using a magnifying glass mounted on the top of the box. Measurements were accurate to ± 0.5 mm.

SDS-gel electrophoresis containing 7.5% acrylamide and 0.1% SDS was carried out as described by Weber and Osborn (264) and Shapiro et al.(265) except that samples after treatment with 1% SDS, 1% 2-mercaptoethanol and 0.5% Iodoacetamide 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 (266) was adopted for isoelectric focusing in polyacrylamide gel using 0.1 ml of pH 3.5 - 10.0 and 0.4 ml of pH 4-6 Ampholine carrier ampholytes (40%) per 10 ml of gel. Gel solutions, anode and cathod 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 at 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) (267). A blank gel was run and sliced to determine the pH gradient.

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 (268). 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 over-night 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 prerun was run with wide range Ampholine carrier ampholytes in pH range 3.5-10.0. Ampholine carrier ampholytes with pH range 4-6 at 1% (w/v) was used for preparative run. 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 collodion sacs.

PART III

ENZYME PRODUCTION AND SACCHARIFICATION OF CELLULOSIC MATERIALS

CHAPTER 1

CELLULASE AND BETA-GLUCOSIDASE PRODUCTION BY SCLEROTIUM ROLFSII

SUMMARY

The optimisation of cellulase and beta-glucosidase production by S.rolfsii was studied and cellulase and cellobiase production by this and Trichoderma reesei (and its mutants) in shake flasks were compared. The former produced an active cellulase when tested on filter paper, carboxymethylcellulose, and cotton; however, it produced 20 to 26 times larger amounts of cellobiase.

Both cellulase and beta-glucosidase were obtained in good yield only when cellulose was the carbon source. The production of these enzymes was not repressed by readily assimilated carbon sources in the presence of cellulose. Only traces of cellulase 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 sophorose, lactose, mannitol, or glycerol and growth was poor on these substrates. Cellobiose octaacetate was a less effective inducer of cellulase and beta-glucosidase than was cellulose.

INTRODUCTION

Cellulose occurs in abundance in nature and constitutes one-third to one-half of the approximately 150 billion tons of organic materials that are photosynthesized annually (34). The commercial possibility of using cellulase preparations to produce glucose, alcohol and protein from cellulose is under intensive study (66,68,269-272). Data for the commercial feasibility of the enzymatic hydrolysis of cellulose are based on laboratory or pilot plant studies (116,273). The major problems encountered are obtaining sufficiently active cellulase enzymes and suitable substrates so that high concentrations of glucose can be obtained in a reasonable period of time.

Three different kinds of enzymes are believed to be involved in crystalline cellulose decomposition; endo-beta-1,4-glucanase, exo-beta-1,4-glucanase and beta-glucosidase (137,140,141,202,206,215,216,230). 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, but this is not the case with acid-swollen cellulose (226). Beta-glucosidase hydrolyzes cellobiose and short chain cellulooligosaccharides to glucose but does not degrade cellulose (206). Other enzymes may be involved in this process but definitive evidence is lacking. Recent studies indicate that the first step in the initiation of hydrolysis of fibrous cellulose may be oxidative (82,188,219,274). For commercial enzymatic

saccharification, the production of both C_1- and C_x-type cellulases and beta-glucosidase in high yields, are essential. Organisms that have been reported to elaborate high cellulase activities (C_1- and C_x-type) capable of solubilizing crystalline cellulose are: Trichoderma reesei, Trichoderma lignorum, Trichoderma koningii (68,85,135-138,216,275), Sporotrichum pulverulentum (82,226), Fusarium solani (138), Penicillium iriensis (276), and Penicillium funiculosum (141). Mutants of T.reesei, QM 9123, QM 9414, NG-14 and MCG-77 (68,90,135,144,145,277,278), are currently the best sources for the production of active cellulase, and also the most intensively studied organisms for saccharifying cellulose. This study examines cellulase production by Sclerotium rolfsii CPC 142. From a comparison made with other fungi (68,142,144) it is apparent that this culture produces large amounts of cellulase enzymes. In addition, the S.rolfsii culture secretes high amounts of cellobiase in contrast to T.reesei. This enzyme plays an important part in maximizing the rate of cellulose hydrolysis (138,279).

The results presented in this Chapter have been published (167).

RESULTS AND DISCUSSION

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 2.5 to 8.0, and temperature range of 30 to 75°C. Table 7 shows the Arrhenius activation energies at the pH and temperature optima for these enzymes. The plots were linear up to the optimum temperature. The average activation energies, calculated by the method of Arrhenius, were 13.07 for CMCase, 5.81 for FPA, 3.84 for CA, 3.27 for cellobiase, and 2.89 kcal mol^{-1} for beta-glucosidase, respectively. All experiments were performed at their respective pH of maximum activity. It is understood that in a kinetically complex system like the cellulase system, the activation energy of the whole system will be determined by the rate-limiting step. In the *S.rolfsii* system, the rate-limiting step is not the release of cellobiose from the enzyme as has been reported for *Chaetomium* (274) and *T.reesei* (139) systems. With the culture filtrate from *Chaetomium thermophile* var. *dissitum* the activation energies were reported to be 6.53 and 5.33 kcal mol^{-1} with CMC and cotton, respectively, as substrates (274) and 5.92-8.02 kcal mol^{-1} for different crystalline cellulose preparations for the *T.reesei* system (280).

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

Increase in cellulase and beta-glucosidase yields have

TABLE 7. TEMPERATURE, pH OPTIMA AND ACTIVATION ENERGIES FOR CELLULASES AND BETA-GLUCOSIDASE FROM S. ROLFSII

Activity	Temperature optima °C	pH optima	Activation energy kcal/mole
CMCase	55	3.7	13.07
FPA	55	4.8	5.81
CA	50	4.8	3.84
Cellobiase	65	4.3 - 4.5	3.27
Beta-glucosidase	70	4.5	2.89

been obtained by optimizing cultural conditions. All experiments have been repeated at least once 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 extracellular cellulase and beta-glucosidase is shown in Table 8. As can be seen from Table 8 addition of $(NH_4)_2HPO_4$ at three to five times higher nitrogen levels, in place of $(NH_4)_2SO_4$, to T.reesei medium showed significant enhancement in the yields of cellulase and beta-glucosidase. Increasing $(NH_4)_2SO_4$ level had no effect on the production of these enzymes. Maximum yields were obtained with 0.42% and 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 cellulase or beta-glucosidase level.

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

The production of cellulase and beta-glucosidase was compared when S.rolfsii CPC 142 was grown on various cellulosic substrates (Table 9). The highest amount of cellulase and beta-glucosidase activities were induced on Cellulose-123, Solka Floc SW40 and Avicel P.H. 101 which produced comparative amounts. These substrates were superior to cotton for the production of cellulase and

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

Inorganic nitrogen source		pH[b]	Cellulase IU.ml^{-1}			Beta-glucosidase IU.ml^{-1}
			CMCase	FPA	CA	
[a]$(NH_4)_2SO_4$	(0.14%)	2.6	26	0.18	ND	3.0
$(NH_4)_2SO_4$	(0.28 or 0.7%)	2.6	27	0.25	ND	4.0
[a]KNO_3	(0.23%)	2.6	10.1	0.14	ND	1.7
[a]NH_4NO_3	(0.18%)	2.6	26	0.17	ND	2.1
[a]NH_4Cl	(0.12%)	2.3	14	0.14	ND	1.8
[a]$(NH_4)_2HPO_4$	(0.14%)	2.5	37	0.32	0.03	6.2
$(NH_4)_2HPO_4$	(0.28%)	2.5	50	0.60	0.034	8.0
$(NH_4)_2HPO_4$	(0.42%)	2.9	100	0.60	0.04	14.0
$(NH_4)_2HPO_4$	(0.70%)	2.9	100	0.60	0.04	14.0

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

ND = Not determined.

[a]Nitrogen source added at equivalent nitrogen levels.

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

TABLE 9. EFFECT OF CELLULOSE SUBSTRATE ON ENZYME PRODUCTION BY S.ROLFSII

Cellulose source	pH[a]	Cellulase IU.ml^{-1}			Beta-glucosidase IU.ml^{-1}
		CMCase	FPA	CA	
Cellulose-123	2.7	100	0.60	0.040	14.0
Avicel P.H. 101	2.8	100	0.60	0.041	14.0
Cotton absorbent	3.1	60	0.36	0.026	8.0
Cotton sliver	3.2	60	0.36	0.026	8.0
Solka Floc SW 40	3.1	95	0.60	0.034	11.0
Coniferous wood	3.4	2	0.04	0.004	0.5
Mesta wood (*Hibiscus cannabinus*)	3.3	3	0.04	0.004	0.5
Bagasse	3.2	5	0.12	0.009	1.2
Rice straw	2.8	36	0.23	0.013	4.0
[b]Coniferous wood	3.4	40	0.18	0.012	1.4
[b]Mesta wood (*Hibiscus cannabinus*)	3.1	55	0.36	0.013	2.8
[b]Bagasse	3.2	64	0.41	0.025	4.0
[c]Rice straw	3.0	55	0.38	0.030	10.0
CMC	4.0	11	0.20	0.016	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.

beta-glucosidase. Fenicksova et al. (281) (Trichoderma sp.) and Mandels (144) (T.reesei QM 6a and QM 9123) reported that cotton was a poor substrate for cellulase production except for T.reesei QM 9414 (144). In contrast, Horton and Keen (282) 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 consequently enzyme production was very low. The highly lignified cellulosics produced the enzymes only after they had been pretreated with alkali. Cellulase production was moderate with alkali treated (AT) bagasse and AT wood powders, being about 40-60% of that obtained with Cellulose-123. Of the lignocellulosics, rice straw offers a striking contrast. The cellulase production with untreated rice straw was about 30% of that obtained on Cellulose-123 whereas AT rice straw produced the enzymes in yields comparable to that obtained with AT bagasse and AT wood powders. With CMC, the formation of both cellulase and beta-glucosidase was low.

Effect of cellulose concentration on cellulase and beta-glucosidase production:

The influence of different levels of cellulose in the NM-1 medium on the production of cellulase and beta-glucosidase is shown in Table 10. There was a four-fold increase in cellulase and beta-glucosidase production when the cellulose concentration in the medium was increased from 0.5 to 3%.

TABLE 10. EFFECT OF DIFFERENT CONCENTRATIONS OF CELLULOSE-123 ON CELLULASE AND BETA-GLUCOSIDASE PRODUCTION

Cellulose-123 %	pH[a]	Cellulase IU.ml^{-1}			Beta-glucosidase IU.ml^{-1}
		CMCase	FPA	CA	
0.5	2.8	30	0.20	0.015	5
1.0	2.7	46	0.40	0.02	8
2.0	2.7	100	0.60	0.04	12
3.0	2.8	120	0.70	0.06	20
4.0	2.8	120	0.70	0.06	20

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

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

No further increase in the yield of the enzyme was observed when the cellulose concentration was further increased. The two forms of cellulose used in these studies, Solka Floc SW40 and Cellulose-123, gave similar results. Increasing peptone or urea at 3% cellulose concentration in the medium did not increase the cellulase or beta-glucosidase levels. In all subsequent studies, a cellulose concentration of 3% was used (NM-2 medium).

Effect of carbon source on cellulase 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 (68,239,274,275,282-284), and on certain glucose trimers (239). It has been suggested that cellobiose at low concentrations is the true inducer for cellulase (135,284,285) but at high concentrations it represses cellulase formation (239,284,286). The precise role of cellobiose in induction, however, has not been clarified. With T.reesei cultures, it 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 (284). Sternberg (287) 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 to 3% cellobiose (284). Thus the

nature of the true inducer of cellulase is still unknown. Breuil and Kushner (288) and Hofsten (233) 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 (289).

The role of glucose in the induction and repression of cellulase production in fungi has also been studied by many investigators (239,288,290,291). It is commonly observed that glucose represses the formation of CMCase (288,291). The slight inducing effect of industrial glucose observed in T.reesei is presumably caused by sophorose formed during acid hydrolysis of starch (239,292).

The effect of addition of different carbon sources on the production of cellulase and beta-glucosidase is reported in Table 11. It may be seen from Table 11 that S.rolfsii CPC 142 produces cellulase 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. The enzymes were not produced when 0.5% glucose was added to the growing culture on every third day. Glucose, fructose, maltose and cellobiose supported good growth but there was little or no growth on glycerol, mannitol, or lactose. The addition of Tween 80 (0.1%) in the cellobiose culture had no effect on the formation of these enzymes. This is contrary to the results reported for T.reesei QM 6a where a 17-fold increase in endo-beta-1,4-glucanase activity by the addition of 0.1% Tween 80 (polyoxyethylene (20) sorbitan monooleate)

TABLE 11. EFFECT OF DIFFERENT CARBON SOURCES ON CELLULASE AND BETA-GLUCOSIDASE PRODUCTION BY S.ROLFSII

Growth substrate	pH[a]	Cellulase IU.ml^{-1}			Beta-glucosidase IU.ml^{-1}
		CMCase	FPA	CA	
Glucose 0.5%	2.8	0.9	0.07	0.005	0.4
Glucose 1.0%	2.5	2.6	0.09	0.006	0.7
Fructose 0.5%	2.7	0.8	0.06	0.004	0.3
Fructose 1.0%	2.3	1.8	0.06	0.005	0.5
Maltose 0.5%	2.8	0.9	0.02	0.002	0.6
Maltose 1.0%	2.5	1.3	0.03	0.002	1.0
Lactose 0.5%	5.8	1.5	0.04	0.001	0.4
Lactose 1.0%	5.8	1.6	0.05	0.001	0.5
Mannitol 0.5%	5.5	0.4	0.01	0.003	0.2
Mannitol 1.0%	5.6	1.8	0.02	0.003	0.3
Glycerol 0.5%	3.7	1.0	0.03	0.006	0.2
Glycerol 1.0%	3.6	1.6	0.03	0.006	0.2
Cellobiose 0.5%	3.0	2.7	0.03	0.003	0.6
Cellobiose 1.0%	2.4	3.6	0.05	0.004	1.6
D-cellobiose octaacetate 0.1%	4.1	18.0	0.07	0.016	0.3
D-cellobiose octaacetate 0.5%	3.5	24.0	0.15	0.010	1.0
D-cellobiose octaacetate 3%	2.8	35.0	0.20	0.017	3.0
Sophorose 0.01% + (0.5%) glucose	3.0	6.3	0.04	0.003	0.4
[b]Sophorose 0.01% + (0.5% glucose	3.1	6.4	0.04	0.004	0.5
Cellulose-123 0.5%	2.8	30.0	0.20	0.010	5.0
Cellulose-123 3.0%	2.7	120.0	0.70	0.060	20.0

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

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

[b]0.5% glucose added every third day to the growing culture.

has been noted (293). Many fungi produce cellulase (C_x) often in higher amounts when grown on lactose (an analogue of cellobiose) than on cellobiose (144) and this has been attributed to the low utilization rate (239,284) of the former substrate by these fungi. The lack of cellulase production when glucose, cellobiose, or fructose were used as the sole carbon source has been reported by other investigators (294,295). However, there are reports of cellulase formation when Trichoderma spp. (281) or Polyporus schweinitzii (296) was grown on various sugars. With cellobiose octaacetate as the sole carbon source, the levels of cellulase and beta-glucosidase were markedly higher (though less than that obtained on cellulose) 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 (284,286). With all carbon sources tested both cellulase and beta-glucosidase activities were produced coordinately.

Sophorose (2-o-beta-D-glucosylglucose), a potent inducer of cellulase in T.reesei (239) and Pseudomonas (297), did not induce cellulase or beta-glucosidase formation in the S.rolfsii culture.

The addition of 0.01 to 0.02% glucose, cellobiose, fructose, or lactose to the cellulose medium to support initial growth did not enhance cellulase or beta-glucosidase formation nor did it reduce the time required for maximum

production of the enzymes. It has been reported that glucose in amounts up to 0.1% has a marked positive effect on FPA (41) and CMCase (298). Mandels and Weber (135) have also recommended the addition of small amounts of glucose to Trichoderma cultures to support initial growth. Limitation of growth by carbohydrate starvation also failed to stimulate cellulase or beta-glucosidase production. A similar effect has been reported with T.koningii (180).

Because cellulase has been reported to be repressible, addition of a rapidly metabolizable carbon source to a culture growing on cellulose inhibits cellulase synthesis (282,299). No repression or stimulation of cellulase or beta-glucosidase was observed in this study on addition of either 0.5% or 1% glucose, cellobiose, lactose, sucrose, fructose, or xylose to the cellulose-containing medium although some of these sugars caused an increase in growth and soluble protein concentration. Neither was there a difference in the formation of cellulase or beta-glucosidase nor an inactivation of these enzymes already formed when 0.5% glucose or cellobiose was added to the growing culture on the 7th day. The activities were determined 48, 96 and 144 h after addition of sugars. Both glucose and cellobiose were consumed within 48 h. There was no further decrease in pH on consumption of glucose or cellobiose and it remained at 2.6 - 2.8. Inactivation of cellulase already formed by T.reesei by the addition of 0.5% glucose to the growing culture has been reported (230,287). This has been attributed to the decline in pH that occurs on

utilization of glucose (287).

Effect of surfactants on enzyme production:

The addition of surfactants has been reported to increase extracellular enzyme production in a variety of microorganisms (275,286). The effect of surfactants has been attributed (i) to their action causing an increase in cell membrane permeability (286), (ii) partly to inducer formation and partly to the promotion of enzyme release (275), and (iii) to decrease in growth caused by a limited supply of oxygen (283).

The effect of the addition of surfactants to S.rolfsii CPC-142 culture medium is shown in Table 12. Tween 80 (polyoxyethylene (20) sorbitan monooleate) at a level of 0.033% gave the highest enzyme activity; its addition to the medium increased the yield of cellulase 2- to 2.5-fold and that of beta-glucosidase 5- to 6-fold. Tween 80 has been consistent in this respect and is independent of the history of the mycelium. Reese and Maguire (293) have reported that Tween 80 and Tween 40 (polyoxyethylene (20) sorbitan monopalmitate) doubled the cellulase yield in Trichoderma. Tween 20 (polyoxyethylene (20) sorbitan monolaurate) was also effective in promoting enzyme production with the S.rolfsii culture but to a lesser degree. Triton X-100 (alkylphenyl-polyethyleneglycol) at 0.033% was toxic and reduced both growth and enzyme production. 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.

TABLE 12. EFFECT OF SURFACTANTS ON CELLULASE AND BETA-GLUCOSIDASE PRODUCTION BY S.ROLFSII

Surfactant	pH[a]	Cellulase IU.ml^{-1}			Beta-glucosidase IU.ml^{-1}
		CMCase	FPA	CA	
None	3.1	50	0.3	0.03	3
Tween-80 0.1%	3.1	110	0.6	0.05	18
Tween-80 0.05%	3.0	115	0.6	0.05	20
Tween-80 0.033%	3.0	120	0.7	0.06	20
[b]Tween-80 0.033%	2.5	55	0.4	0.02	9
Tween-20 0.033%	3.2	70	0.3	0.03	10
Triton X-100 0.033%	6.3	1	0.02	0.002	0.03

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.

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

No significant increase either in the cellulase or beta-glucosidase level was observed by incorporating enhanced levels (2- to 3-fold), either singly or in various combinations, of trace metals (Fe^{2+}, Co^{2+}, Zn^{2+}, Mn^{2+}), potassium dihydrogen phosphate, magnesium sulfate, urea, peptone, or yeast extract. Incorporation of copper sulfate (0.005 - 0.03%) or calcium carbonate (0.01 - 0.03%) likewise had no effect. Abhushama and Kambal (300) have suggested that zinc functions as an activator of the cellulase.

Influence of incorporating antisporulating agents:

Many fungi produce volatile compounds which are inhibitory to the germination of their own spores and those of other fungal species (301,302). The main sporostatic factor is thought to be acetaldehyde (302). Antisporulating agents like phenylethyl alcohol and benzyl alcohol have been reported to enhance cellulase production and the site of action of these compounds is thought to be at the cytoplasmic membrane (303). With S.rolfsii CFC-142, incorporation of benzyl alcohol or phenylethyl alcohol, in the range of 0.01 to 0.1%, to NM-2 medium showed no increase in cellulase or beta-glucosidase formation. Fusidic acid which also inhibits spore formation (301,304) did not affect cellulase or beta-glucosidase production.

Influence of organic substrates on cellulase and beta-glucosidase production:

The effect of addition of various organic substrates

to the NM-2 medium on cellulase and beta-glucosidase production was investigated and is shown in Table 13. The addition of corn steep liquor, rice bran, or wheat bran increased the yields of cellulase and beta-glucosidase. The greatest increase of 1.5 - 2-fold for cellulase and beta-glucosidase activities was observed with 3% rice bran. The maximum activities obtained were: CMCase 200-230 IU.ml^{-1}, FPA 1.2-1.4 IU.ml^{-1}, CA 0.085 - 0.1 IU.ml^{-1}, beta-glucosidase 25-30 IU.ml^{-1}, and cellobiase 10-13 IU.ml^{-1} (shown in Table 16). The NM-2 medium containing 3% rice bran is designated as NM-3 medium. No significant increase in any of the activities was observed on increasing urea, yeast extract, or peptone levels of the NM-3 medium.

Effect of adding vitamins, amino acids and hormones:

Attempts were made to identify the constituents that were present in rice and wheat bran which are responsible for increasing cellulase and beta-glucosidase yields. The addition of the following compounds to NM-2 medium, either singly or in various combinations, did not show a significant increase in either the cellulase or beta-glucosidase yield. Vitamins: thiamin 0.002 - 0.1%, thiamin pyrophosphate 0.002 - 0.1%, biotin 0.001 - 0.005%, biotin 0.001% plus thiamin pyrophosphate 0.02 - 0.05%, nicotinic acid 0.02 - 0.05%, ascrobic acid 0.01 - 0.05%, vitamin mixture 0.006% (nicotinic acid 1 mg.ml^{-1}, biotin 10 µg.ml^{-1}, pyridoxin hydrochloride 1 mg.ml^{-1}, inositol 1 mg.ml^{-1}, calcium pantothenate 1 mg.ml^{-1}, thiamin 100 µg.ml^{-1}, folic acid 10 µg.ml^{-1}). Amino acids: DL-asparagine 0.1 - 0.5%,

TABLE 13. EFFECT OF ORGANIC SUBSTRATES ON CELLULASE AND BETA-GLUCOSIDASE PRODUCTION

Organic nutrient		pH[a]	Cellulase IU.ml^{-1}			Beta-glucosidase IU.ml^{-1}
			CMCase	FPA	CA	
None		2.8	120	0.7	0.06	20
Urea	0.03%	2.3	120	0.7	0.06	21
Yeast extract	0.02%	2.8	125	0.7	0.06	20
Proteose peptone	0.025%	2.7	120	0.7	0.06	20
Corn steep liquor	2 or 4%	3.2	220	1.0	0.09	23
Wheat bran	3 or 4%	3.8	200	0.9	0.08	28
Rice bran	2%	3.1	185	1.1	0.07	26
Rice bran	3 or 4%	3.3	230	1.4	0.10	30

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

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

L-glutamine 0.1 - 0.5%, asparagine 0.1% plus glutamine 0.1%, leucine 0.1%, glutamic acid 0.1%, aspartic acid 0.1%, casein hydrolysate (B.D.H.) 0.1%, casein hydrolysate 0.1% plus tryptophan 0.01%. Plant hormones: gibberellic acid (Sigma) 10 - 25 ppm, indol-3-yl-acetic acid (B.D.H.) 10 - 20 ppm, 6-furfurylaminopurine (Sigma) 10 - 20 ppm, 2,4-dichlorophenoxyacetic acid (B.D.H.) 5 - 10 ppm. Thiamin and biotin have been reported to increase cellulase yields of a Penicillium spp. (personal communication, Jagannathan et al.) and 0.001% thiamin was reported to replace yeast extract in Cellulomonas culture (39). Addition of 0.1% ascorbic acid to T.reesei culture has been reported to enhance CMCase activity (305).

Effect of organic acids and volatile fatty acids:

Table 14 shows the effect of various organic acids and straight chain and branched chain volatile fatty acids on the production of cellulase and beta-glucosidase by S.rolfsii CPC-142. These were included in the study in view of the finding that they are generally important for the nutrition of many species of rumen bacteria (306,307). Branched chain volatile fatty acids have been found to be essential for the growth of cellulolytic rumen bacteria (306). The addition of 0.1% acetate or oxoglutarate to S.rolfsii culture was stimulatory and slightly increased the yield of the enzymes. Addition of 0.1% sodium acetate to the T.reesei culture medium has been reported to enhance CMCase activity (305). Glycolic acid and propionic acid were inhibitory and reduced the yield of the enzymes whereas

TABLE 14. EFFECT OF ORGANIC ACIDS ON CELLULASE AND BETA-GLUCOSIDASE PRODUCTION BY S.ROLFSII

Addition[a]		pH[b]	Cellulase IU.ml^{-1}			Beta-glucosidase IU.ml^{-1}
			CMCase	FPA	CA	
None		2.8	120	0.70	0.06	20
Acetic acid	0.1%	2.7	138	0.80	0.07	23
Oxo-glutaric acid	0.1%	2.9	145	0.73	0.07	23
Succinic acid	0.1%	2.7	115	0.64	0.06	20
Malic acid	0.1%	2.7	120	0.70	0.06	20
Glycolic acid	0.1%	3.0	18	0.15	0.01	2
Propionic acid	0.05%	3.0	73	0.46	0.04	10
Isobutyric acid	0.05%	2.8	147	0.83	0.07	24
Isobutyric acid	0.1%	5.5	27	0.20	0.02	6
Isovaleric acid	0.05%	2.7	150	0.85	0.07	24
Isovaleric acid	0.1%	4.6	36	0.23	0.02	4

[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.

addition of either succinic acid or malic acid did not affect enzyme production. Enhanced yields of cellulase by succinate addition to the growth medium have been reported (308). The addition of 0.05% isobutyric or isovaleric acid increased cellulase and beta-glucosidase yields 15 to 25%. At 0.1% or higher concentrations, both isobutyric and isovaleric acids were inhibitory.

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

Studies on the effect of the initial pH of the growth medium on cellulase and beta-glucosidase production were carried out within a pH range of 2.8 to 7.3 (Table 15). An initial pH of 6.5 (after autoclaving) was found to be most favourable for producing high yields of cellulase and beta-glucosidase even though synthesis of these enzymes by S.rolfsi occurred while the pH was between 2.7 to 3.0. Growth and enzyme production were markedly inhibited when the initial pH of the growth medium was above 7.0 or below 5.0.

Cellulase and beta-glucosidase formation during the growth cycle:

The course of production of cellulase, beta-glucosidase, reducing sugars, extracellular protein, and the pH profile in NM-2 and NM-3 media are shown in Fig. 7a-d. After inoculation there was a lag of 2 days before extracellular enzyme and protein reached detectable levels. The pH started declining from the 3rd day in both NM-2 and NM-3 media and on the 5th day it fell to 2.6 - 2.8 in NM-2 medium and stayed low (pH 2.8 - 3.0) during the remaining growth period.

TABLE 15: EFFECT OF INITIAL pH OF THE GROWTH MEDIUM ON THE PRODUCTION OF CELLULASE AND BETA-GLUCOSIDASE BY S.ROLFSII

pH[a]	pH[b]	Cellulase IU.ml^{-1}		Beta-glucosidase IU.ml^{-1}
		CMCase	FPA	
2.8	2.3	10	0.18	1.8
3.0	2.4	11	0.30	2.0
3.7	2.5	12	0.30	2.8
4.6	2.5	14	0.32	3.2
5.0	2.6	64	0.55	4.0
5.5	2.5	73	0.64	8.0
6.0	2.6	85	0.69	9.6
6.5	2.8	120	0.73	20.0
7.1	6.9	0.5	0.01	0.3
7.3	7.1	0	0	0

Grown on NM-2 medium at indicated pH.

[a]pH of medium before inoculation.

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

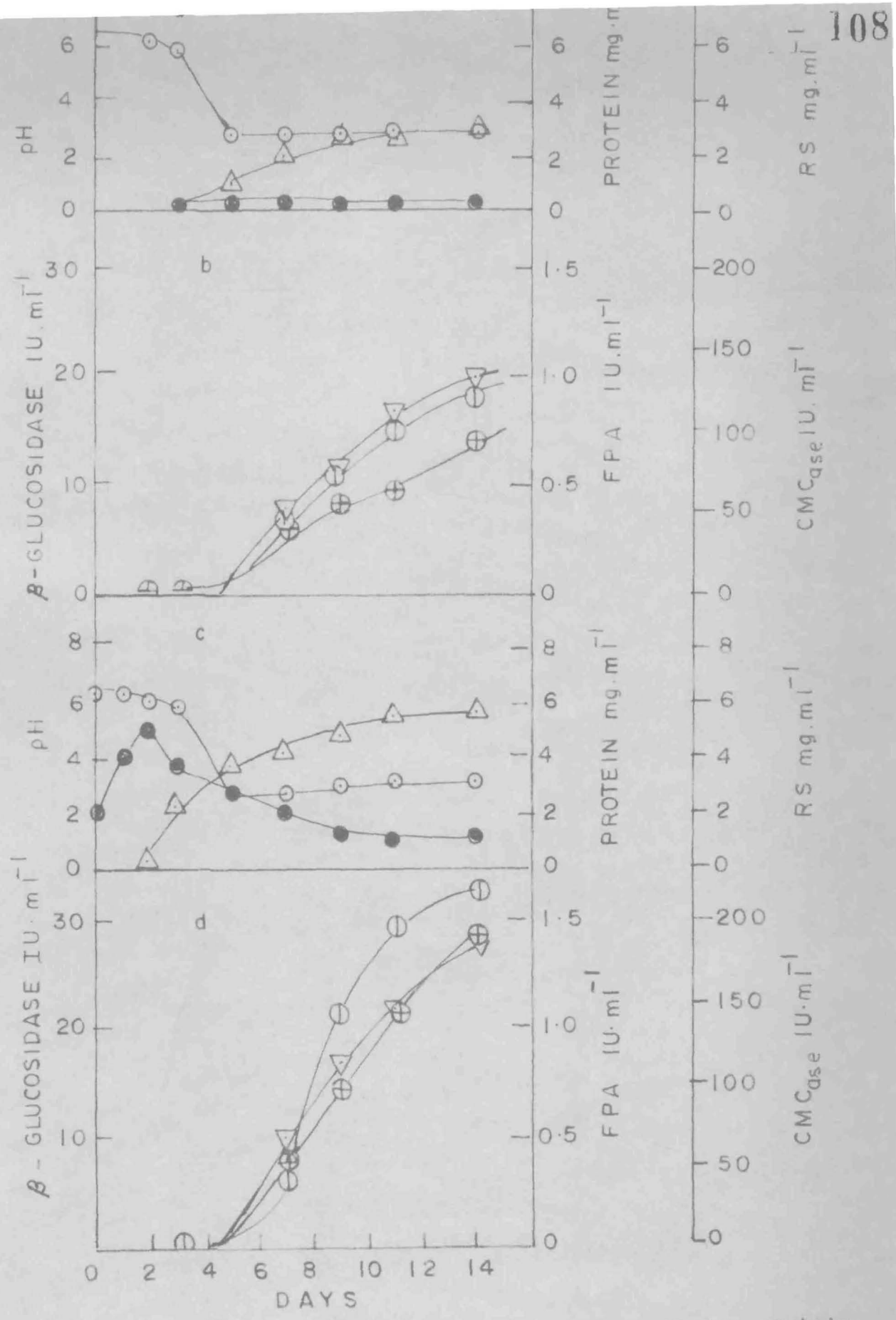


FIG. 7. pH profile and elaboration of extracellular protein, RS, cellulase and beta-glucosidase during growth of S.rolfsii in shake flask. a and b, NM-2 medium; c and d, NM-3 medium. Symboles: ○ , pH; △ , extracellular protein: ● , RS; ⊖ , CMCase; ▽ beta-glucosidase; ⊕ , FPA.

In NM-3 medium the pH decreased from an initial value of 6.5 to 2.4 in the same period (5th day), but then very slowly rose to 3.1 on the 14th day. A rather large quantity of reducing sugars (4 - 5 $mg.ml^{-1}$) is liberated in the NM-3 medium within the first 2 or 3 days after inoculation whereas in the NM-2 medium the reducing sugar level ranges between 0.1 to 0.3 $mg.ml^{-1}$. The initial reducing sugar value before inoculation was 1.5 - 2.0 $mg.ml^{-1}$ in the NM-3 medium but no soluble reducing sugar was detectable in the NM-2 medium. Examination by the glucose-peroxidase test (202,243) indicates the presence of only a small amount glucose (4 to 10% of the total reducing sugars) in the growth media. The level of the soluble sugars in the NM-3 medium decreases with time and ranges between 1 - 1.4 $mg.ml^{-1}$. In the NM-2 medium, the level of reducing sugar ranges between 0.1 - 0.4 $mg.ml^{-1}$ throughout the growth period.

The appearance of beta-glucosidase activity lagged approximately 24 h behind CMCase production in both the media. Activity on CMC appeared earlier (2nd or 3rd day) than on filter paper or cotton (5th day). This is probably because even the highly crystalline cellulose contains amorphous regions (i.e. contains some loose ends or chains) that are readily hydrolyzed by CMCase (68) and is, therefore, first induced. It could also be possible that FPA enzyme(s) are adsorbed on the nonutilized cellulose to a greater extent than CMCase is and are therefore detected later. With T.reesei on the other hand, Peitersen (41) and Mandels and Weber (135) reported that C_x (CMCase) enzymes are produced

later than the C_1 enzymes (FPA). The patterns of CMCase, FPA, and beta-glucosidase production during the growth cycle were similar (Fig. 7b and d). All the activities tested were maximal about the 14th day. Most of the cellulase and beta-glucosidase production occurred while the pH was in the range of 2.4 to 3. The yield increases rapidly during the initial stages of growth, then more slowly until the 14th day and then levels off. Cellulase and beta-glucosidase are apparently produced during active growth and there is a close linear relationship between enzyme formation and extracellular protein (Fig. 8).

With the S.rolfsii culture, control of pH during growth was not necessary in the presence of up to 4% cellulose in the medium. Addition of $CaCO_3$ (0.01 - 0.03%) to the medium had no effect on formation of the enzymes. At higher concentrations of $CaCO_3$ (0.2%), there was little or no growth and consequently no formation of the enzymes. With T.reesei culture, on the other hand, without pH control during growth the pH falls to 2.4 in a medium containing 2% cellulose. Under such conditions growth is extremely slow and cellulase and beta-glucosidase are inactivated (139).

Stability of cellulase and beta-glucosidase:

S.rolfsii CPC 142 cellulase is most stable between pH 4.0 and 5.0 while beta-glucosidase and cellobiase have maximal stability in the pH range of 4.0 to 6.0. At pH 4.5, no significant loss in cellulase, beta-glucosidase, and cellobiase activities was detected at 30°C for 24 h or

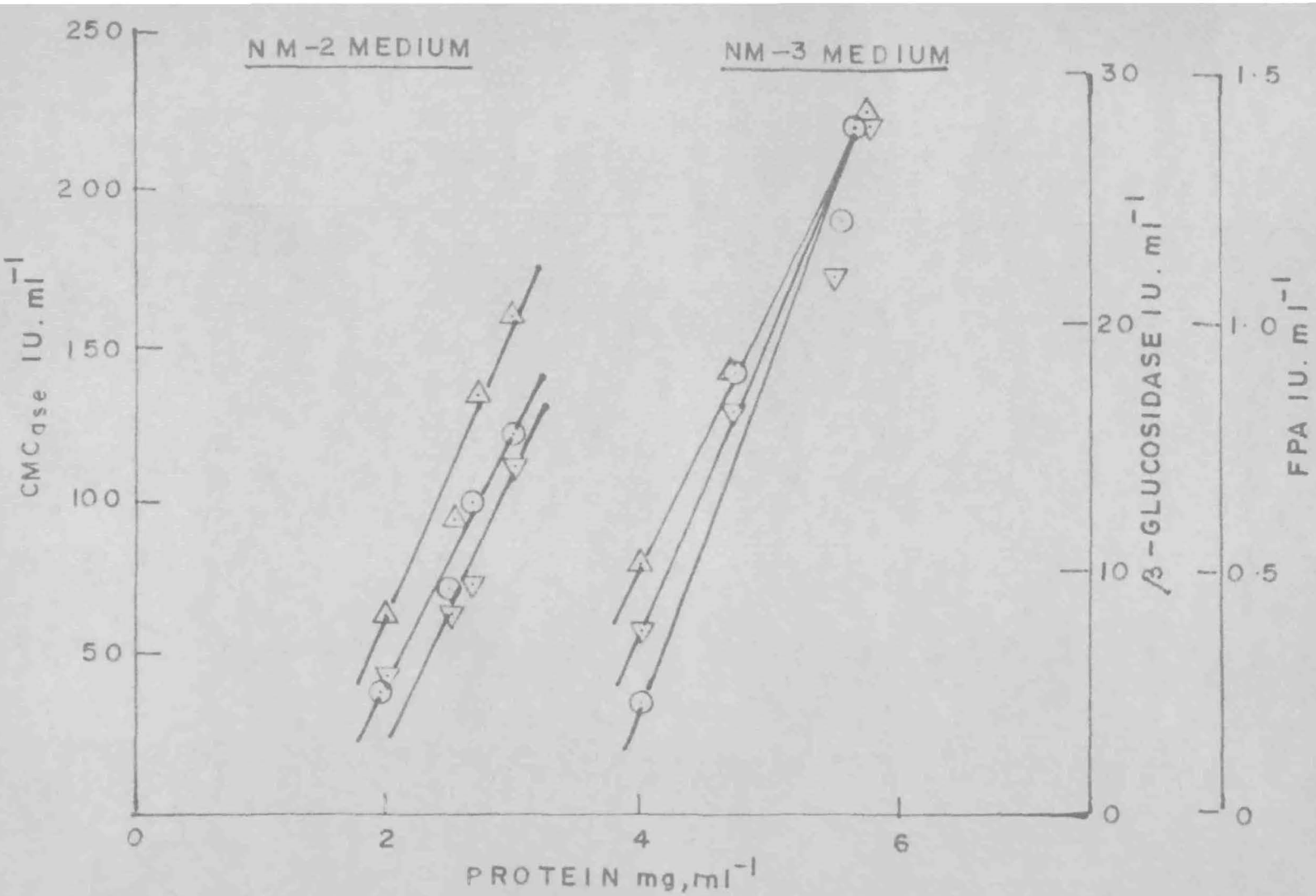


FIG. 8. Relation between soluble protein and cellulase activity. Culture grown on NM-2 medium and NM-3 medium for 14 days. Symbols: ○, CMCase; ▽, FPA; △, Beta-glucosidase.

at 50°C for 1 h.

Comparison of cellulase and beta-glucosidase production by Sclerotium rolfsii CPC 142 and Trichoderma reesei:

As can be seen from Table 16, the amount of cellulase enzymes secreted in the medium by S.rolfsii CPC 142 in shake flasks are comparable with some of the best activities reported for T.reesei and its mutants (68,144,145) 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 NG-14 mutant, 4.65 $IU.ml^{-1}$(145). However, the CMCase (endo-glucanase) secreted by NG 14 is low, 15 $IU.ml^{-1}$. S.rolfsii CPC-142 culture, however, produces 15 to 20 times higher amounts of cellobiase. The highest cellobiase activity reported for T.reesei cultures is 0.5 - 1.3 $IU.ml^{-1}$ (145,279) and is suboptimal for conversion of cellulose to glucose (139, 279). With the S.rolfsii culture filtrate, glucose is the major product (86%) of cellulose saccharification (Part III, Chapter 2) and not cellobiose with smaller amounts of glucose as with the T.reesei culture filtrates (139,287). 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 cellobio-hydrolase and endo-glucanase and its accumulation decreases the saccharification rate (137,215,216).

There may still be considerable scope to improve cellulase yields of S.rolfsii CPC 142 by a study of its nutritional requirements, variation in growth conditions,

TABLE 16. COMPARISION OF CELLULASE AND BETA-GLUCOSIDASE PRODUCTION BY THE S.ROLFSII AND T.REESEI

Organism	Cellulase			Beta-glucosidase	Cellobiase
	CMCase $IU.ml^{-1}$	HPA $IU.ml^{-1}$	C_1[a]	$IU.ml^{-1}$	$IU.ml^{-1}$
T.reesei					
QM 6a (144)	18	0.23	2.8	NA[b]	NA
QM 9123 (144)	59	1.30	6.4	NA	NA
QM 9414(63,144)	152	1.48	2.5-7.6	NA	0.5
NG 14 (145)	15	4.65	NA	0.7	1.35
S.rolfsii					
CPC-142[c]	200-230	1.2-1.4	4.5-5.0	25-30	10-13

[a]Amount of glucose (mg/ml) formed from 5% absorbent cotton in 24 h, 50°C.

[b]NA - not available

[c]Grown on NM-3 medium for 14 days.

and strain mutation and selection which would hyperproduce particular enzymes of the cellulase complex. It may be of interest to mention here that a UV-8 mutant culture of S.rolfsii CPC-142 has been isolated and developed by Dr. J.C. Sadana and Mr. M.V.Deshpahde that secretes 2 times more filter paper degrading activity on NM-2 growth medium in submerged cultures as compared to the parent strain (169). With parent strain, the addition of 3% rice bran to NM-2 medium was essential for optimal formation of cellulase, including filter paper degrading activity. However, with the mutant the addition of rice bran to NM-2 medium was not required for obtaining higher yields of filter paper degrading or cellobiase activity. Stable mutants of T. reesei, QM 9123, QM 9414, NG 14, C 30, and MCG 77 with increased cellulase yields have been developed (90,145, 277,278,309). More sophisticated processing has increased enzyme production by three to seven fold (115,278,310). With T.reesei, it has also been possible to reduce the lag time substantially and obtain maximum cellulase production in 3 to 4 days in pilot plant experiments (311).

CHAPTER 2

ENZYMATIC HYDROLYSIS OF CELLULOSIC MATERIALS FOR PRODUCTION OF SUGARS

SUMMARY

The hydrolysis of purified celluloses (cotton, Avicel, Cellulose-123, Solka Floc SW40) and cellulosic wastes (rice straw, sugarcane bagasse, wood powders, paper factory effluents) by Sclerotium rolfsii CPC-142 culture filtrate was studied. Factors which affect saccharification such as pH, temperature, enzyme concentration, substrate concentration, product inhibition, adsorption, and inactivation of enzyme and particle size were studied.

Virtually no inhibition ($< 3\%$) of cellulose hydrolysis by the culture filtrate was observed by cellobiose and glucose up to 100 mg/ml. Filter paper degrading enzyme(s) (but neither carboxymethylcellulase nor beta-glucosidase) was adsorbed on cellulose. The n value in the S.rolfsii system was calculated to be 0.32 for Avicel P.H. 101 and 0.53 for alkali-treated (AT) rice straw indicating penetration of cellulase into AT rice straw. In batch experiments at 15% substrate level, solutions containing 7.2 to 8%, 5.4 to 6.7%, 5.0 to 6.6% and 4.4 to 5.8% reducing sugars were produced in 24 to 48 h from AT rice straw, AT bagasse, alkali-peracetic acid treated mesta wood and paper factory sedimented sludge effluent, respectively. The main constituent in the hydrolysate from cellulose was glucose with little or no cellobiose, probably due to the high cellobiase content in the culture filtrate

INTRODUCTION

There is a great deal of interest in developing methods to produce glucose syrups from cellulose using cellulase preparations (66,84,90,139,269,310). It is generally agreed that cheap glucose is the key intermediate for subsequent chemical and energy products. 1,4-beta-glucan cellobiohydrolase and 1,4-beta-glucan glucanohydrolase are inhibited by cellobiose (137,205,208). This inhibition is relieved by hydrolysis of cellobiose to glucose by cellobiase (139,205). Hence, cellobiase plays an important role in maximizing cellulose hydrolysis. The cellobiase activity secreted by Trichoderma reesei and its mutants (currently the best sources for saccharifying cellulose) is suboptimal for conversion of cellulose to glucose in the T.reesei system (139,312). In Part III, Chapter 1 the isolation of a S.rolfsii culture CPC 142 which produces high amounts of cellulase which compare favourably with some of the best activities reported in the literature in shake flasks has been described (167). In addition, this fungus secretes high amounts of cellobiase in contrast to T.reesei QM 6a and its mutants QM 9123 and QM 9414.

In the present Chapter the results of studies on saccharification (solubilization of cellulose) of a number of pure cellulosics and of cellulose in lignocellulosics with the S.rolfsii CPC 142 culture filtrate are described. Of the cellulosic wastes (agricultural, forestrial, or industrial in origin) tested, alkali-treated (AT) rice straw and AT

bagasse showed high susceptibility.

The results presented in this Chapter have been published (168).

RESULTS AND DISCUSSIONS

Chemical pretreatment of cellulosic materials:

Native cellulose is very resistant to enzyme hydrolysis. The highly crystalline structure and the presence of lignin effectively prevent the attack by cellulases. It is, therefore necessary to treat the cellulosic material in some way prior to the use of enzymes.

Pretreatment of lignocellulosics is one of the two major bottlenecks to the economical utilization of this source. Several different methods have been suggested for rendering cellulosic materials more susceptible to enzymatic hydrolysis (41,58,79,103,131-133). Chemical pretreatments tried in the present study are described under Materials and Methods.

The weight loss on alkali treatment (4 N NaOH, 30°C, 24 h) for rice straw, bagasse, mesta wood and coniferous wood was 40, 36, 23 and 10%, respectively, and on peracetic treatment it was 25, 22, 17 and 12%, respectively. The cellulose and ash content in different cellulosic materials used are shown in Table 17.

Factors which affect saccharification:

The rate of production of reducing sugars from cellulosics 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

TABLE 17: CELLULOSE AND ASH CONTENT OF CELLULOSE MATERIALS

Cellulosic material	Cellulose %	Ash %
Avicel P.H. 101	100	0.0
Absorbent cotton	99	0.5
Cellulose-123	87	0.0
Solka Floc SW40	62	1.0
Rice straw	39	19.5
AT rice straw[a]	72	8.5
Bagasse	35	4.9
AT bagasse[b]	58	4.4
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
Bleach house effluent (paper mill)	81	7.8
Sedimented sludge (paper mill)	67	7.3

AT = Alkali treated

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

[b]Incubated with 4 N NaOH at 30°C for 24 h.

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

at 50°C is shown in Fig. 9. An overlapping series of buffers was used to detect anomalies in the rate caused by buffers: there were none. The best reducing sugars yield on saccharification was obtained in the pH range of 4 to 4.5. Outside this range there was a marked decrease (from 45% at pH 4.5 to about 12% at pH 3 and 8% at pH 6) in saccharification.

Temperature:

The variation in the saccharification rate of Solka Floc SW40, Cellulose-123, and AT bagasse with temperature was tested at the pH of maximum activity, pH 4.5 and is shown in Fig. 10. A temperature of 40 - 50°C appeared most effective. The rate of enzymatic saccharification was faster for the first h at 55°C than at 40 - 50°C, but thereafter the steady state of hydrolysis remained higher between 40 and 50°C. This was applicable to all the three substrates tested. Li *et al*. (176) have, however, reported that in the *T.reesei* system, the optimum temperature for saccharification depends on the nature of the cellulose used as substrate.

Loss of cellulase and beta-glucosidase activities due to inactivation and adsorption:

Since cellulases are known to be adsorbed on cellulose (67,84,176,313), studies were conducted to determine the pattern of enzyme loss due to inactivation and adsorption at pH 4.5, 50°C, for periods up to 48 h. The loss of cellulase and beta-glucosidase activities in the presence and absence of cellulose is shown in Fig. 11. The results

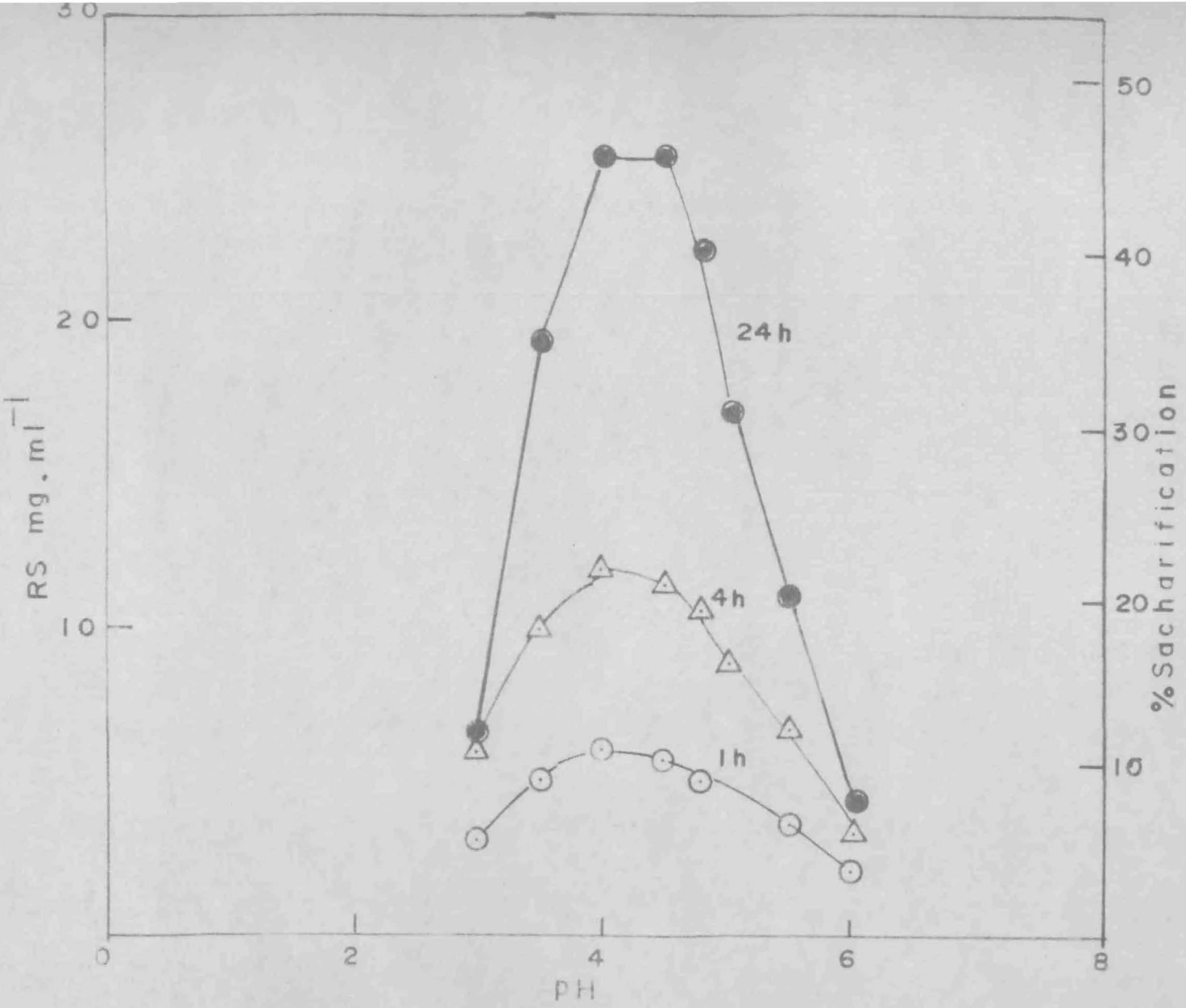


FIG. 9. Effect of pH on saccharification. 5% Solka Floc SW40, 50°C. Symbols; ⊙, 1 h; △, 4 h; ●, 24 h.

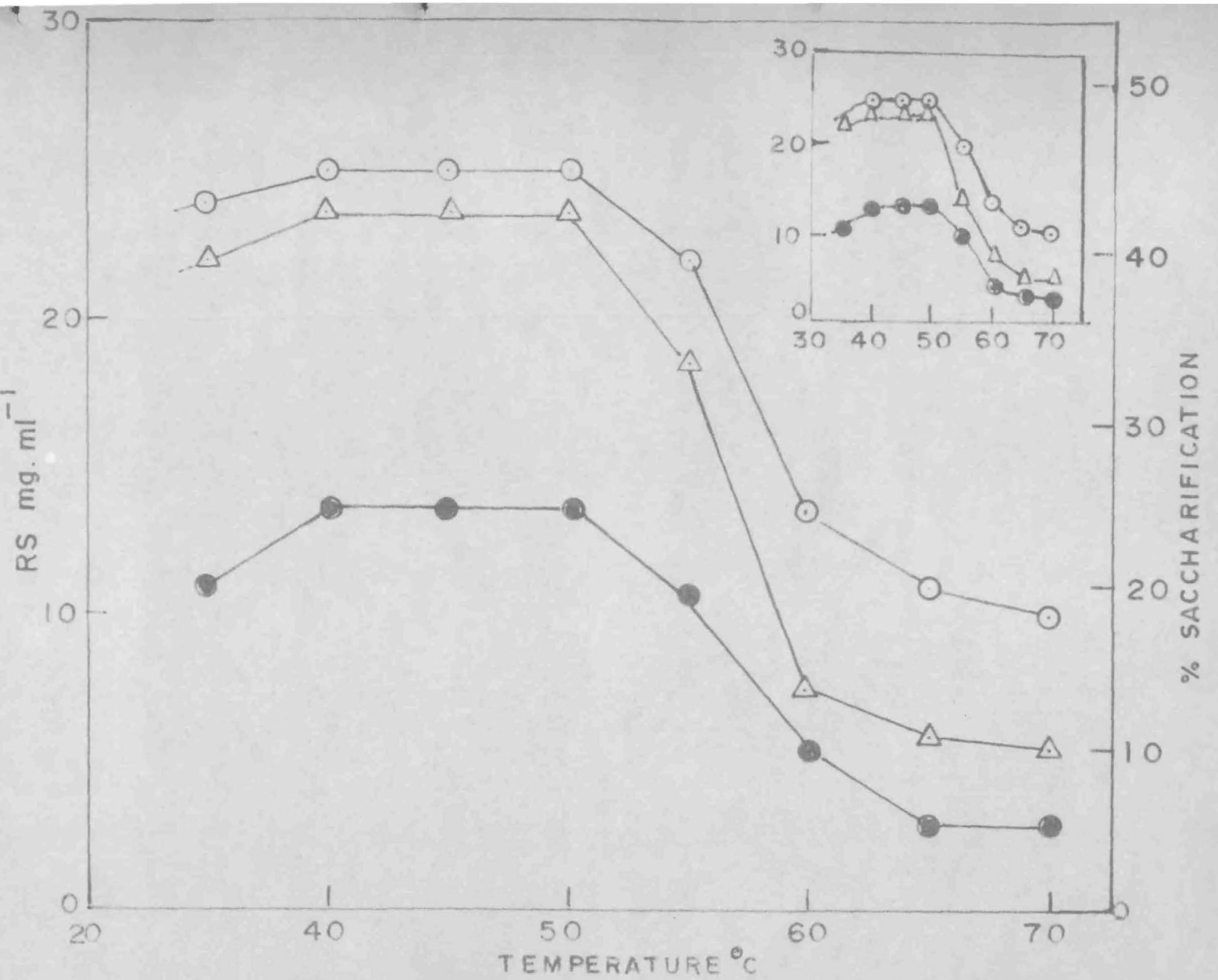


FIG. 10. Effect of temperature on saccharification. Substrate 5%, pH 4.5, 48 h. Symbols: ●, Cellulose-123; △, Solka Floc SW40; ⊙, AT bagasse (4 N NaOH, 30°C, 24 h). Inset: Saccharification carried out for 24 h.

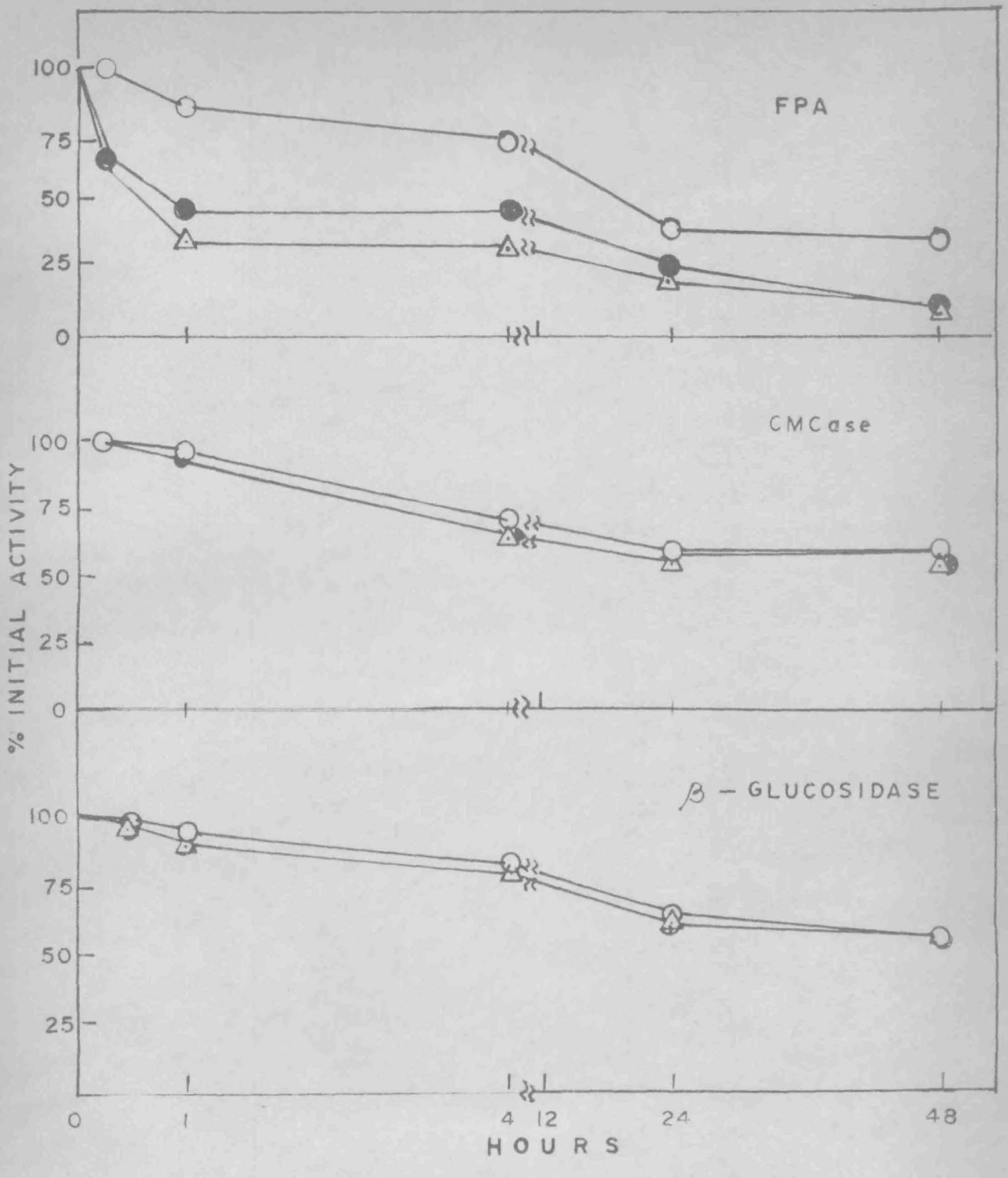


FIG. 11. Inactivation and adsorption of S.rolfsii cellulase and beta-glucosidase at 50°C, pH 4.5. Substrate, 5% AT rice straw (autoclaved with 0.25 N NaOH at 121°C, 1 h) or Solka Floc SW40. Symbols: ○, culture filtrate alone; ●, culture filtrate plus AT rice straw; △, culture filtrate plus Solka Floc SW40.

show that in the absence of cellulose, S.rolfsii culture filtrate did not loose any activity in the first 15 min. However, in the presence of Solka Floc SW40 and AT rice straw, the FPA decreased by 33 and 39%, respectively, during the same period. No loss in CMCase or beta-glucosidase was observed up to the first h both in the presence and absence of cellulose. In the absence of substrate, the loss in FPA, CMCase, and beta-glucosidase in 48 h was 63, 41, and 42%, respectively. In the presence of 5% Solka Floc SW40 or AT rice straw, the corresponding loss in FPA was 90%, CMCase 42% and beta-glucosidase 35% in 48 h. The results show that the addition of cellulose offered no protection against inactivation and the higher losses observed in FPA in the presence of cellulose was due to adsorption of FPA enzymes on cellulose which are required in addition to CMCase and beta-glucosidase enzymes. Wilke and Yang (116), working with T.reesei culture filtrate, have reported that both C_1 and C_x enzymes are adsorbed on cellulose to about the same extent.

Effect of addition of cellobiose and glucose on the saccharification rate:

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. The inhibitory effect of products, however, varied with the organism from which the cellulase was derived (66,84,87,175, 314). Experiments conducted with the S.rolfsii culture

filtrate showed that hydrolysis of Solka Floc SW40 and AT rice straw were not significantly inhibited ($< 3\%$) by glucose and cellobiose up to 100 mg/ml in 48 h. 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 CMC as substrate, the liquifying activity (endo-glucanase) of the culture filtrate was inhibited very strongly (85 to 90%) with cellobiose (100 mg/ml) but not by glucose upto 100 mg/ml. However such high cellobiose concentrations are not reached in cellulose hydrolysis with the S.rolfsii culture filtrate. At 2 mg/ml level, the highest cellobiose concentration observed in the cellulosic hydrolysates, the liquifying activity inhibition was negligible (3-6%).

Effect of enzyme concentration on saccharification of cellulosics:

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. In Table 18 the saccharification of Solka Floc SW40, AT rice straw, and AT bagasse with different concentrations of S.rolfsii culture filtrate is shown. 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 at 24 h increased from 3.4 to 6.4% as the concentration of culture filtrate was increased from

TABLE 18: EFFECT OF ENZYME CONCENTRATION ON SACCHARIFICATION OF CELLULOSE MATERIALS

Substrate (10%)	Culture filtrate ml	Reducing sugar mg.ml^{-1}				% Saccharification			
		1 h	4 h	24 h	48 h	1 h	4 h	24 h	48 h
Solka Floc SW40	5	4.0	11.0	14.0	22.0	3.6	9.9	12.6	19.8
	10	5.4	14.5	25.0	37.0	4.8	13.0	22.5	33.3
	15	8.0	16.5	30.0	52.0	7.2	14.8	27.0	37.8
	18	10.0	22.0	33.0	44.5	9.0	19.8	29.7	40.0
AT-bagasse[a]	5	14.0	25.5	30.0	34.0	12.6	22.9	27.0	30.6
	10	18.0	31.5	34.0	40.0	16.2	28.3	30.6	36.0
	15	20.0	34.5	36.0	44.0	18.0	31.0	32.4	39.6
	18	22.0	35.0	38.0	44.0	19.8	31.5	34.2	39.6
	18[b]	23.0	39.0	51.0	52.0	20.7	35.1	45.9	46.8
AT-rice straw[c]	5	10.0	32.0	34.0	38.0	9.0	28.8	30.6	34.2
	10	22.0	37.0	50.0	52.0	19.8	33.3	45.0	46.8
	15	25.0	44.0	64.0	68.0	22.5	39.6	57.6	61.2
	18	26.0	44.0	64.0	68.0	23.4	39.6	57.6	61.2
	18[b]	33.0	45.0	67.0	68.0	29.7	40.5	60.3	61.2

2 g substrate + 0.5 ml 2 M citrate buffer pH 4.5 + culture filtrate as indicated + water to 20 g.

[a]Incubated with 4 N NaOH, 30 °C, 24 h

[b]Concentrated two times by precipitation with ammonium sulfate, 0-90%.

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

5 to 18 ml. The corresponding increase with AT bagasse and Solka Floc SW40 was from 3.0 to 3.8% and 1.4 to 3.3%, respectively. When the enzyme concentration was doubled further, reducing sugars production from AT rice straw increased to 6.7% and that from AT bagasse to 5.1% in 24 h, respectively. However, the rate and extent of hydrolysis of the cellulosics were not proportional to the increase in enzyme concentration. The 7.2-fold increase in the cellulase-beta-glucosidase concentration increased the extent of saccharification only by a factor of 1.7 for AT rice straw and 1.5 for AT bagasse in 48 h.

It is apparent from Table 18 that there is an initial faster rate of saccharification during the first 4 h followed by a gradually decreasing rate. This could be due to three main reasons, (a) the enzyme is becoming inactivated; (b) the readily susceptible amorphous regions are hydrolyzed away readily early in the run leaving the more resistant substrate (c) the gradual accumulation of glucose as the ultimate product of hydrolysis may be responsible for the decreasing rate of hydrolysis (66,84, 315), or to all three. With the S.rolfsii culture broth, product inhibition is not likely to be the cause of the decrease in hydrolytic rate, as virtually no inhibition ($<3\%$) of saccharification is observed by 10 to 100 mg/ml glucose or cellobiose added at zero time.

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 7.2 and 6.0%, respectively

(Fig. 12). When 10 ml more of the culture filtrate was added at the end of 48 h, an increase in reducing sugars of 23 mg/ml and 12 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 4 mg/ml and 2 mg/ml, respectively. Similarly, when 1 g more of the substrate was added at the end of 48 h, an increase in reducing sugars of 17 mg/ml and 10 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 (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:

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 the S.rolfsii culture filtrate was observed. This is contrary to expectation as one would expect that the rate of hydrolysis would be proportional to the surface area in contact with the enzyme. The assumptions are made that the enzyme is adsorbed on the surface of the substrate in accordance with a Gyani-Freundlich isotherm (316) and that

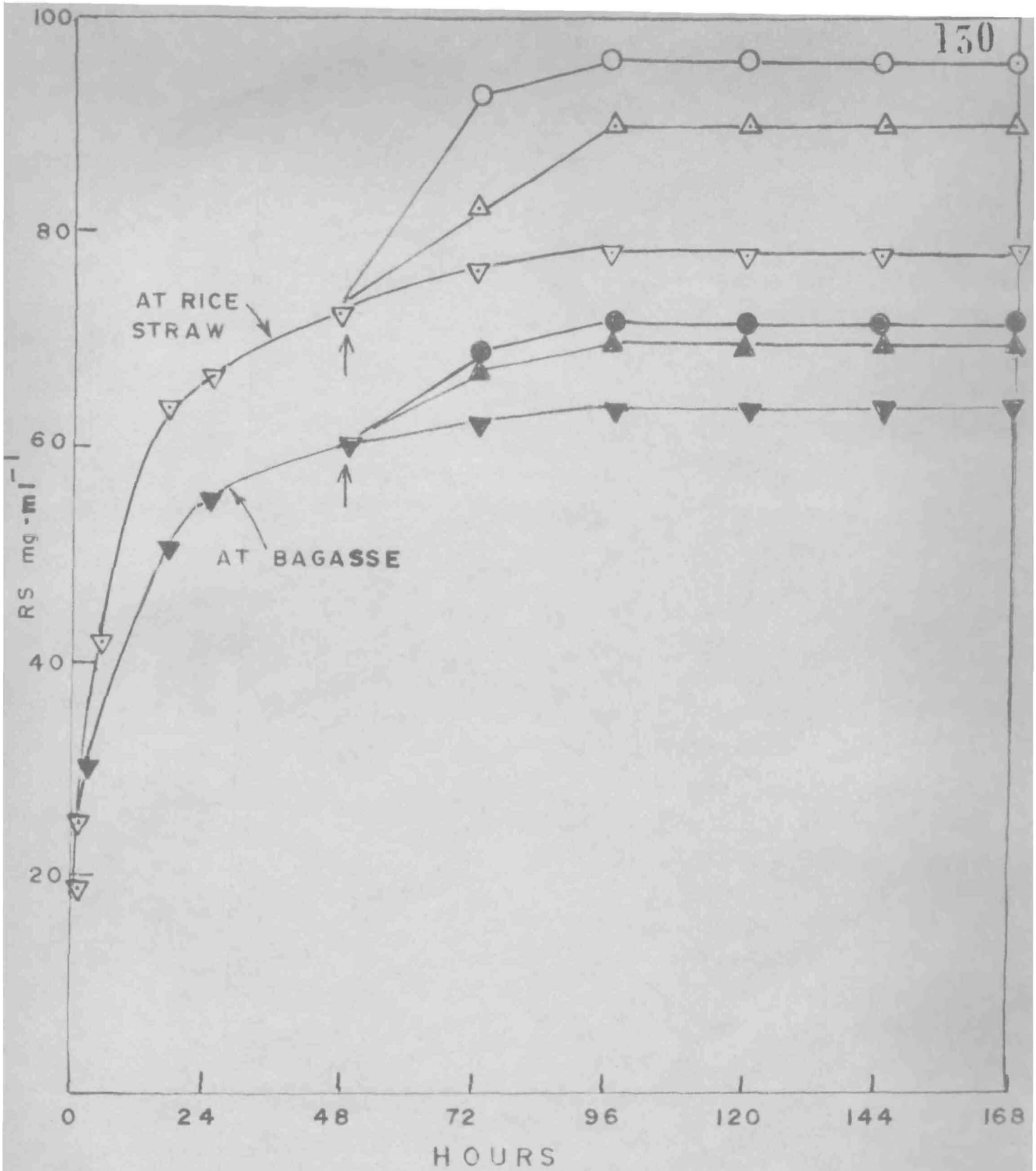


FIG. 12. Effect of adding more enzyme or substrate to AT rice straw (open symbols) and AT bagasse (closed symbols) after 48 h incubation. 3 g cellulose material + 2 ml 1 M citrate buffer pH 4.5 + 15 ml culture filtrate incubated at 50°C. Symbols: ▽, no additional enzyme or substrate added; △, 1 g substrate added after 48 h; ○, 10 ml culture filtrate added after 48 h. Reducing sugar values were corrected for dilution. AT rice straw = Rice straw autoclaved with 0.25 N NaOH, 121°C, 1 h. AT bagasse = Bagasse incubated with 4 N NaOH, 30°C, 24 h.

the rate of digestion is proportional to the amount of the adsorbed enzyme. The cellulase from T.reesei (67) and thermophilic actinomyces MJØr and YX (317) 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. 13), the data conformed to the equation developed by McLaren (316) describing the hydrolytic rate of enzymatic reaction in a heterogeneous system. The n value in the S.rolfsii system was calculated to be 0.32 for Avicel P.H. 101. A similar low value of n (0.35) was also observed for MJØr enzyme (317), The value of n describes the adsorption characteristics of the enzyme on the surface of the substrate. Adsorption on cracks or edges gives $n = \frac{1}{3}$ and adsorption on surface gives $n = \frac{2}{3}$ (317). According to the theory, the low n value could be interpreted as indicating that the hydrolysis of the microcrystalline cellulose takes place primarily on the cracked edges of the surface of the particle (316). The n value for AT rice straw is 0.53. The latter value could mean that penetration of the enzyme into AT rice straw has taken place.

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 culture filtrate is shown in Table 19. 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

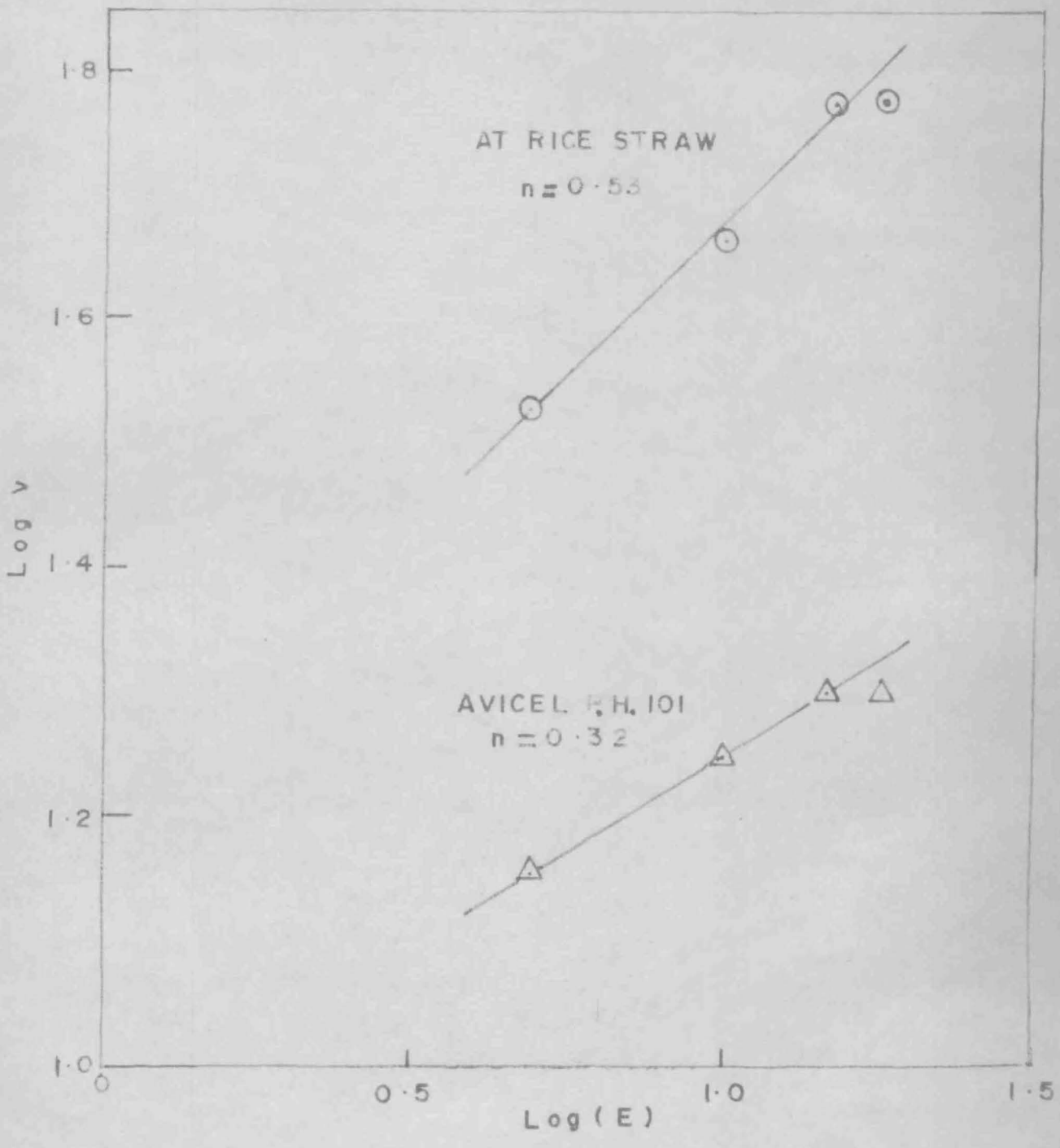


FIG. 13. Determination of n value. Saccharification of AT rice straw and Avicel P.H. 101 was carried out at pH 4.5, 50°C. 2 g substrate + 2 ml 1 M citrate buffer pH 4.5 + culture filtrate (5, 10 or 15 ml) + water to 20 g. Symbols: △ , Avicel P.H. 101; ○ , AT rice straw.

from 30% AT rice straw (it was semisolid) were 10.4% in 24 h and 14.0% in 48 h.

The most suitable concentration of cellulosics for saccharification by the culture filtrate was determined by taking into account the conditions which give high sugar solutions and also a high degree of cellulose utilization. The optimum substrate concentration calculated according to Toyama and Ogawa (86) appears to be around 8.5 to 9.5% for Avicel P.H. 101, Cellulose-123, AT rice straw, and AT bagasse (Fig. 14).

Analysis of the hydrolysates from AT rice straw, AT bagasse, alkali-peracetic acid treated mesta wood and sedimented sludge effluent at 10% substrate concentration showed the reducing sugars (as glucose equivalent) to be 60, 38, 40 and 42 mg/ml, respectively, in 24 h. This corresponds to 0.60, 0.38, 0.40 and 0.42 g of reducing sugars 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 20. The most resistant substrates were fibrous cotton and lignocellulosics. The extent of saccharification ranged from 6 to 6 4% in 48 h. Of the purified cellulosics tested, Solka Floc SW40 (swollen but still fibrous) showed maximum hydrolysis. The saccharification values obtained from Solka Floc SW40, Avicel P.H. 101, and P.H. 102 were 45.0, 21.6 and 23.4% in 24 h, respectively. Thus, crystallinity appears to be more important than the particle size as has also been

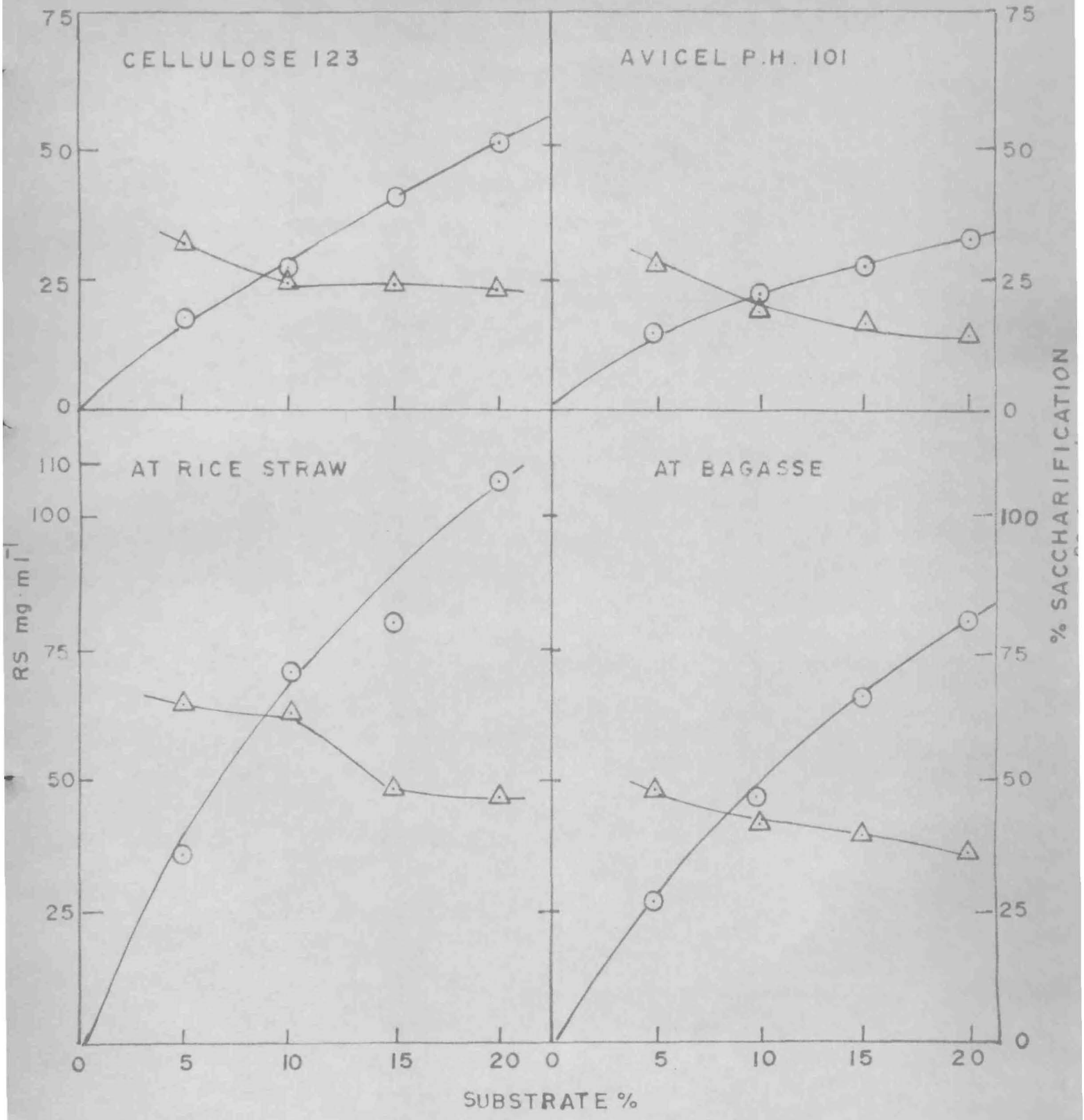


FIG. 14. Effect of substrate concentration on saccharification. Substrate + 2 ml 1 M citrate buffer, pH 4.5 + culture filtrate to 20 g, incubated at 50°C for 48 h. AT rice straw and AT bagasse is as described in legend to Fig. 12. Symbols: ⊙ , reducing sugars; △ , % saccharification.

reported by Mandels et al. (68). When Solka Floc SW40 was sieved to give a 400 mesh fraction, little difference in saccharification values was noticed. Solka Floc SW40 was more susceptible than commercially available Solka Floc BW200. With T.reesei cellulase, Mandels et al. (68) 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 moderately in the case of wood powders; from 9.0 mg/ml to 35.0 mg/ml for rice straw, from 4.6 mg/ml to 26.0 mg/ml for bagasse, and from 4.5 mg/ml to 12.5 mg/ml for mesta wood powder in 24 h. The optimum effect of alkali treatment for rice straw was achieved by autoclaving with 0.25 N NaOH at 121°C for 1 h, and that for bagasse was achieved by incubation with 4 N NaOH at 30°C for 24 h. Alkali treatment was not effective for wood powders. These, however, become reactive by sodium hydroxide - peracetic acid treatment (86). Coniferous wood and mesta wood powders gave saccharification values of 12.6 and 45.0% after alkali - peracetic acid treatments, and 30.6 and 45.0% after peracetic acid - alkali treatment in 24 h, respectively.

Enzymatic degradation of paper factory effluents:

The bleach house effluent (which is mainly washings of bleached cellulose in the manufacture of paper), machine house effluent (which is washings of paper pulp after treatment with whitening agents) and sedimented sludge

(obtained on setting of all effluents in tanks before disposal from paper industries) were hydrolysed without any further pretreatment. These gave saccharification values of 52.2, 45 and 49.5 in 24 h (Table 21). This might be due to the chemical treatments that the material had already undergone during the sulfite-pulping process which delignifies the lingocellulosics and severely disrupts the crystalline structure. The hydrolysis of paper mill effluents would, therefore, not involve extra cost for the pretreatment of lignocellulosics. Pretreatment cost of cellulosics constitutes one of the critical phases in term of the overall economics for the production of glucose from cellulose (310). Paper factory effluents have the additional advantage that no cost of collection would be needed as these are being produced at a central location. This might represent an ideal solution for the conversion of cellulose to sugars and would also help in the proper disposal of paper factory effluents. Andren and Nystrom (310) have otherwise emphasized that for enzymatic conversion of cellulose to sugars, delignified material may prove to be the most practical material and suggested that emphasis should be shifted to chemically treated wood pulp.

It may be mentioned that percentage saccharification values obtained with the S.rolfsii culture filtrate compare favourably to those reported for the T.reesei culture filtrate for most of the cellulosic materials (68).

Products of hydrolysis:

The concentration of glucose, cellobiose, a sum of the

TABLE 21: HYDROLYSIS OF PAPER FACTORY EFFLUENTS[a] BY S.ROLFSII CULTURE FILTRATE

Substrate (5%)	Reducing sugar $mg.ml^{-1}$				% saccharification			
	1 h	4 h	24 h	48 h	1 h	4 h	24 h	48 h
Bleach house effluent	6.2	14.6	29.0	30.0	11.1	26.2	52.2	54.0
Machine house effluent	7.7	15.1	25.0	30.0	13.8	27.1	45.0	54.0
Sedimented sludge	7.2	19.0	27.5	34.0	12.9	34.2	49.5	61.2

[a]The solids from the paper factory effluents were dried at 45°C. Saccharification was carried out as described in legend to Table 20.

two, and total reducing sugars produced during hydrolysis from different cellulosic materials upto 48 h by the S.rolfsii culture filtrate are given in Table 22. Paper chromatograms showed the presence of only glucose and cellobiose from Avicel P.H. 101; and glucose, cellobiose, xylose, and two other unidentified sugars from Cellulose-123, and Solka Floc SW40 (Fig. 15).

The following are characteristic features of hydrolysis. Cellobiose accumulated initially but it fell to almost negligible amounts in the later stages of hydrolysis. Glucose was detectable during the first h and continued to increase throughout. After 4 h of incubation, the entire increase in reducing sugars 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 48 h glucose was the principal product and accounted for 86.3% of the total reducing sugars from Avicel P.H. 101 whereas cellobiose was only 0.9%. This pattern was more 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.

There is a difference in the values between the total 'DNS' reducing sugars and sum of the glucose and cellobiose. This discrepancy 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.

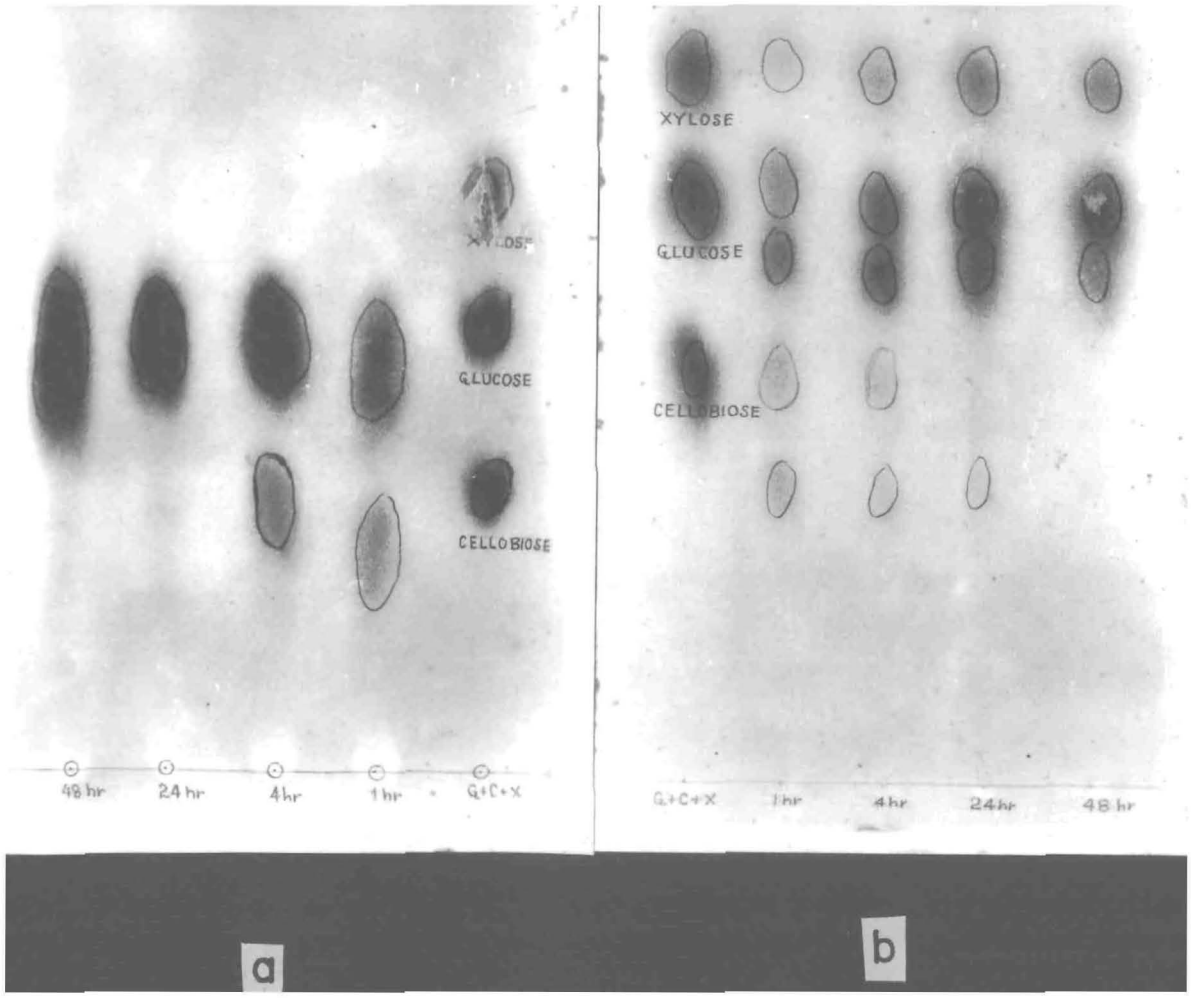
TABLE 22: FORMATION OF SUGARS DURING ENZYMATIC HYDROLYSIS OF CELLULOSE MATERIALS BY S. ROLFSII CULTURE FILTRATE

Cellulose	Reducing sugar mg.ml^{-1}				Glucose mg.ml^{-1}				Cellobiose[a] mg.ml^{-1}				Glucose + Cellobiose mg.ml^{-1}			
10%	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
Avicel P.H. 101	5.0	11.0	20.0	22.0	3.6	9.3	18.0	19.0	1.3	1.6	0.22	0.2	4.9	10.9	18.22	19.2
Cellulose-123	4.8	10.2	22.0	28.0	1.5	3.5	12.6	20.0	1.2	1.0	0.8	0.2	2.7	4.5	13.4	20.2
Solka Floc SW40	12.5	20.0	30.0	34.5	4.0	9.0	15.5	21.0	1.2	1.0	0.2	0.05	5.2	10.0	15.7	21.0

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.

FIG. 15. Paper chromatograms of the hydrolysis products by the S.rolfsii culture filtrate. (a) Avicel P.H. 101, (b) Solka Floc SW40. Hydrolysis was carried out by incubating 2 g substrate + 2 ml 1 M citrate buffer, pH 4.5 + culture filtrate to 20 g.


XYLOSE
GLUCOSE
CELLOBIOSE
48 hr
24 hr
4 hr
1 hr
G+C+X
a
XYLOSE
GLUCOSE
CELLOBIOSE
G+C+X
1 hr
4 hr
24 hr
48 hr
b

In contrast to the S.rolfsii culture filtrate which produced predominantly glucose (with negligible amounts of cellobiose) from purified cellulose in 24-48 h, T.reesei culture filtrate produced mainly cellobiose with smaller amounts of glucose probably because the amount of cellobiase in T.reesei system was insufficient (139). Myrothecium verrucaria produced glucose and cellobiose in approximately equal amounts (318).

CONCLUSIONS

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. During the last few years, tremendous advances have been made in getting high cellulase-yielding T.reesei mutants (Table 23) (68,115,144,145). It has taken about 20 years to increase the FPA from 0.23 IU.ml^{-1} (T.reesei QM6a) to 4.85 IU.ml^{-1} (T.reesei mutant NG-14) in shake flasks. FPA activities of the order of 12-15 IU.ml^{-1} have now been obtained through strain improvements and with sophisticated processing in instrumented fermentors under controlled conditions of pH, aeration etc. with high productivities of 56 FPA IU/litre/hour (115, 146). All these developments would reduce the cost of alcohol production from cellulose.

Though Trichoderma spp. are favoured sources of cellulases and are the most intensively studied organisms for cellulose saccharification, suggestions have been made that other sources of cellulases should also be examined. However, only a few species of fungi, viz. Thermonospora (311a), Penicillium (311b), Sclerotium (167,168; developed by us),and Fusarium (138), when grown on cellulose, produce culture filtrates that will completely degrade insoluble cellulose. Trichoderma and its mutants produce low amounts of cellobiase with the result that their culture filtrates produce mainly cellobiose with small amounts of glucose. Cellobiose inhibits both endo-glucanase and exo-glucanase activities.

TABLE 23: CELLULASE PRODUCTION BY DIFFERENT ORGANISMS

	Shake flasks			In instrumented fermentors with controlled pH, aeration etc.				Reference
	FPA $IU.ml^{-1}$	CMCase $IU.ml^{-1}$	Cello-biase $IU.ml^{-1}$	FPA $IU.ml^{-1}$	CMCase $IU.ml^{-1}$	Cello-biase $IU.ml^{-1}$	Produ-ctivity FPU/l/h	
T.reesei QM6a (Parent) (Natick)	0.23	18	0.3-0.5	5	88	0.3	15	115,144
T.reesei QM9123 (Natick)	1.30	59	0.3-0.5	NA	NA	NA	NA	115,144
T.reesei QM9414 (Natick)	1.48	152	0.3-0.5	10	109	0.6	30	115,144
T.reesei MCG77 (Natick)	NA	NA	NA	11	104	0.9	33	115
T.reesei C 30 (Rutgers)	NA	NA	NA	14	150	0.3	42	115
T.reesei NG 14 (Rutgers)	4.65	15	1.35	15	133	0.6	45,56	115,145
S.rolfsii (Parent) (NCL)	1.2-1.4	180-200	10 - 13	NA	NA	NA	NA	167
S.rolfsii UV 8 (NCL)	1.8-2.0	170-190	8 - 10	NA	NA	NA	NA	169

NA = Data not available.

The Sclerotium rolfsii CPC 142 culture has been developed which gives high cellulase activities with properties suitable for saccharification of cellulosic materials (167,168). The culture secretes high amounts of cellobiase in contrast to T.reesei and its mutants. In shake flasks, the S.rolfsii CPC 142 culture secretes in $IU.ml^{-1}$; FPA 1.2 - 1.4, CMCase 200-230, beta-glucosidase 25-30; and cellobiase 10-13 $IU.ml^{-1}$.

A UV-8 mutant of S.rolfsii CPC 142 has been isolated and developed by Dr. J.C.Sadana and Mr. M.V. Deshpande, that secretes 2 times more FPA on NM-2 growth medium in submerged cultures as compared to the parent strain (169,170). With the mutant the addition of rice bran to NM-2 medium was not required for obtaining higher yields of FPA. The growth of S.rolfsii culture has not been studied so far in instrumented fermentors under controlled conditions of pH, aeration etc. Its potential for secreting still higher amounts of cellulase and beta-glucosidase remains to be determined. Growth of T.reesei mutants under controlled conditions of pH, aeration etc. in instrumented fermentors has increased enzyme production three to seven fold (115,278,310).

Detailed saccharification studies of different cellulosic materials such as bagasse, wood, rice straw etc. using S.rolfsii culture filtrate have been carried out (168). Different methods of pretreatment were studied and kinetic data obtained. In batch experiments at 15% substrate level, solutions containing 7.2 to 8.0%,

5.4 to 6.7%, 5.0 to 6.6%, and 4.4 to 5.8% reducing sugars were produced in 24 to 48 h from AT rice straw, AT bagasse, alkali-peracetic acid treated mesta wood and paper factory sedimented sludge effluent, respectively. Virtually no inhibition (< 3%) of cellulose hydrolysis by the culture filtrate was observed by cellobiose and glucose upto 100 mg/ml in 48 hours. The main constituent in the hydrolysate from cellulose was glucose (86%) with little of no cellobiose (0.9%), probably due to the high cellobiase content in the culture filtrate (168), whereas culture filtrates from other organisms produce mainly cellobiose and small quantities of glucose (139,318).

PART IV

PURIFICATION, CHARACTERIZATION AND PROPERTIES OF BETA-GLUCOSIDASE ENZYMES

SUMMARY

Four beta-glucosidase enzymes were extensively purified from the culture filtrates of Sclerotium rolfsii CPC 142 and some of their physico-chemical properties studied. All the enzymes showed a single protein band in SDS-gel electrophoresis and in disc gel electrophoresis at pH 8.9 and 4.3. The major enzyme, BG-3 beta-glucosidase, gave one protein band in analytical isoelectric focusing in polyacrylamide gel also. The purified beta-glucosidases were free of endoglucanase (CMC viscosity-lowering activity). All the enzymes are glycoproteins and are composed of one polypeptide chain. The molecular weight of the four beta-glucosidases varied between 90,000 and 107,000 daltons.

The pH and temperature optima of the four beta-glucosidases were 4.2 and 68°C with p-nitrophenyl-beta-D-glucoside and 4.5 and 65°C with cellobiose as substrate. The isoelectric points for the enzymes were 4.10, 4.55, 5.10 and 5.55, respectively. The specific activity of the enzymes with cellobiose as substrate was 55, 78, 175 and 51 µmol glucose released/min/mg protein, respectively. The enzymes were inhibited by the reaction product glucose, by gluconodelta-lactone and nojirimycin. A carboxylate group is implicated in the catalysis of beta-glucosidase.

The enzymes were specific for beta-configuration and none of the compounds with alpha-configuration were hydrolysed. The enzymes did not accept aryl-beta-xyloside as a substrate.

INTRODUCTION

The results of previous studies on enzyme production show that the cellulase enzyme system of Sclerotium rolfsii has a good potential for saccharification of cellulosic materials (Part III) (167,168). Because cellobiose is an inhibitor of the cellulases its removal by cellobiase would be an important factor in large scale saccharification of cellulose (137,139,205,218). Study of the properties of cellobiase and its contribution to cellulolysis thus take on a special significance. The part played by cellobiase in contributing to the control of the overall metabolism of cellulose is still not fully understood. This is because few sufficiently pure cellobiase preparations, free from contaminating components of the cellulase system, from the effective microorganisms have been reported. The S.rolfsii culture broth provided us with four highly purified and stable beta-glucosidases (EC 3.2.1.21). These important advantages permitted their physico-chemical characterization and study of some of the enzymatic properties of these enzymes which are described in this Chapter. Some of the typical characteristics are compared with those from other sources.

RESULTS

The S.rolfsii produces an extracellular beta-glucosidase. The capability of various substrates to induce beta-glucosidase has been described (Part III, Chapter 1) (167). The strongest inducer is Cellulose-123 (an alkali-treated cellulose) whereas cellobiose is a poor inducer. Only a low constitutive activity of beta-glucosidase 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 culture was grown on the modified Reese and Mandels' medium (191,238) 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 filtration through glass wool.

Enzyme purification:

The following operations were carried out at 0° to 4°C unless otherwise indicated. In all purification steps, the fractions containing PNPG also showed cellobiase activity and conversely. Therefore, only the PNPG assay was used for monitoring the column effluents. Analytical polyacrylamide gel electrophoresis was used to follow the increase in enzyme purity.

Step 1: Ammonium sulfate precipitation:

The clear solution obtained after filtration, usually

5-6 litres per batch, was concentrated by precipitating the proteins with solid ammonium sulfate at 90% saturation. Several harvests were pooled and used as crude material for the present work. 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 beta-glucosidase and CMCase was around 80-90%.

Step 2: Fractionation by gel chromatography:

The ammonium sulfate-precipitated enzymes from Step 1 was gel filtered on Sephadex G-75 column (1.8 x 90 cm) for desalting and fractionation. The elution pattern is shown in Fig. 16. The beta-glucosidase (Fraction A) was eluted after the void volume and ahead of cellulase (Fraction B) in the preparation. Fraction A (F 4-14) contained about 95-98% of beta-glucosidase activity and about 70% CMCase activity whereas Fraction B (F 16-35) contained about 30% cellulase and about 2% beta-glucosidase activity. Fraction A was brown-colored.

Step 3: Ultrafiltration:

Fraction A was concentrated by Diaflo membrane XM-50 ultrafiltration system (Amicon Corporation, U.S.A.). The low molecular weight cellulases were removed from the high molecular weight cellulases and beta-glucosidases.

Step 4: Separation of beta-glucosidase and cellulase by DEAE-Sephadex A-50 ion-exchange chromatography:

The concentrated top enzyme solution from Step 3 was dialyzed in a collodion bag for 3-4 h against 0.05 M phosphate buffer, pH 7.3 and chromatographed on DEAE-

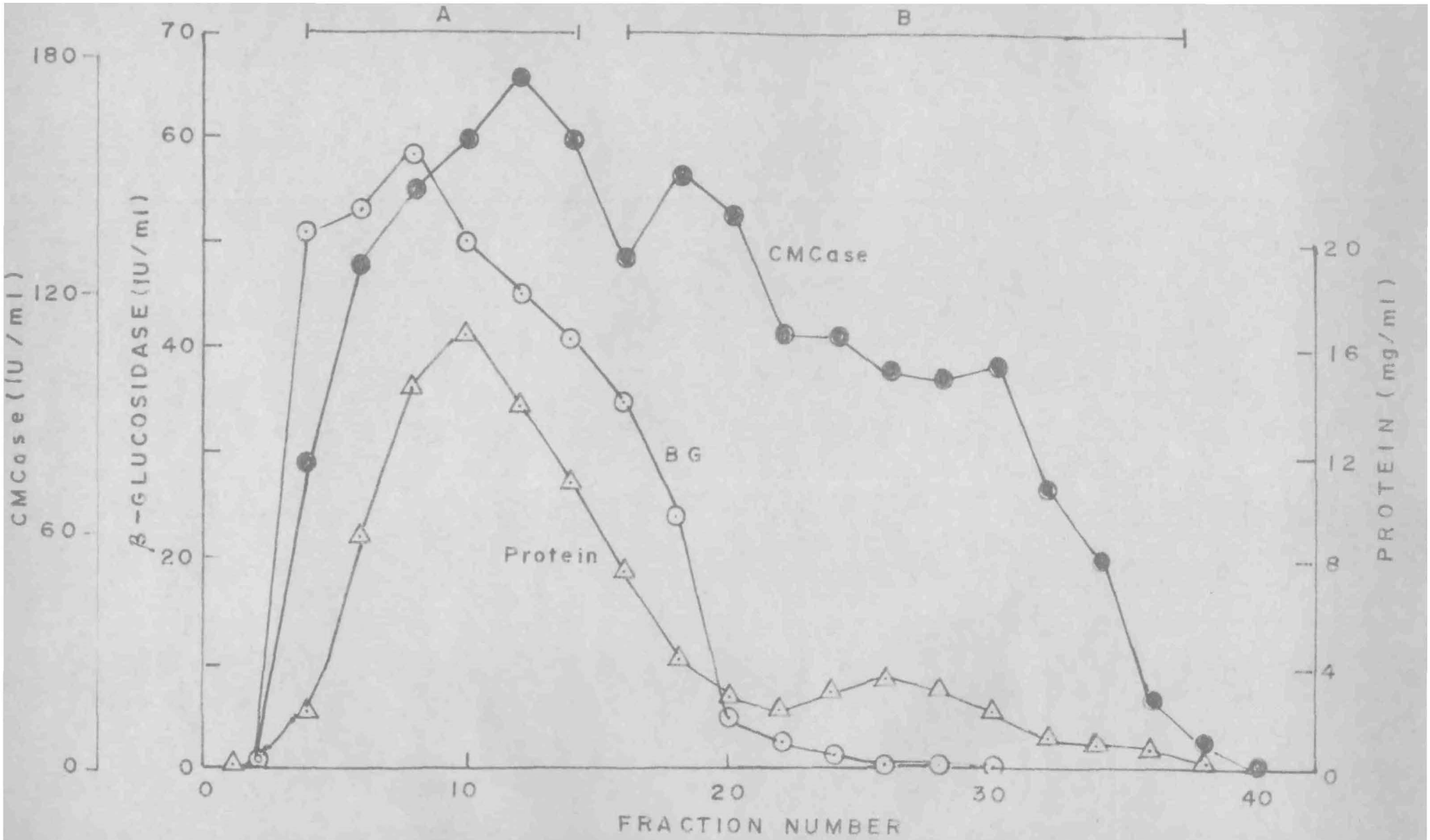


FIG. 16. Sephadex G-75 column chromatography of crude material after 0-90% ammonium sulfate precipitation. Sample: 2 g protein (60 ml). Column dimensions: 1.8 x 90 cm. Buffer: 0.05 M citrate buffer pH 4.8. Symbols: ⊙, beta-glucosidase (BG); ●, CMCase and △, protein. Fraction A comprises fractions 4-14, and fraction B fractions 16-35.

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 beta-glucosidase, CMCase and protein. Results are summarized in Fig. 17. Beta-glucosidase and CMCase activities were not adsorbed on the column. Beta-glucosidase came just after the viod volume and formed the first peak and was almost free of CMCase. This was followed by a second peak, active towards CMC, and was practically free of beta-glucosidase. However, major amount of the protein (about 60%) was adsorbed on the column which was eluted by 0.2 M citrate buffer, pH 4.5. This fraction did not show any beta-glucosidase or CMCase activity. The dark brown pigment present in the culture filtrate was removed in this step.

Fractions 6-7 containing beta-glucosidase of 44-50 specific activity were pooled and the pH was adjusted to 4.5 with 0.1 M citric acid. The pooled fractions were concentrated by freeze-drying to about 5 ml and dialyzed against 0.05 M citrate buffer, pH 4.5 in a collodion bag. The beta-glucosidase fraction gave one band in disc gel electrophoresis at pH 8.9. However, electrophoresis of the enzyme at pH 4.3 resolved into four bands of proteins (Fig. 19a, b).

Step 5: Preparative isoelectric focusing:

Isoelectric focusing was performed with a 110 ml column. The ampholyte concentration was 1% with a pH range of 4 to 6 in a sucrose gradient. The beta-glucosidase (F 6-7) from Step 4 was dialyzed over-night

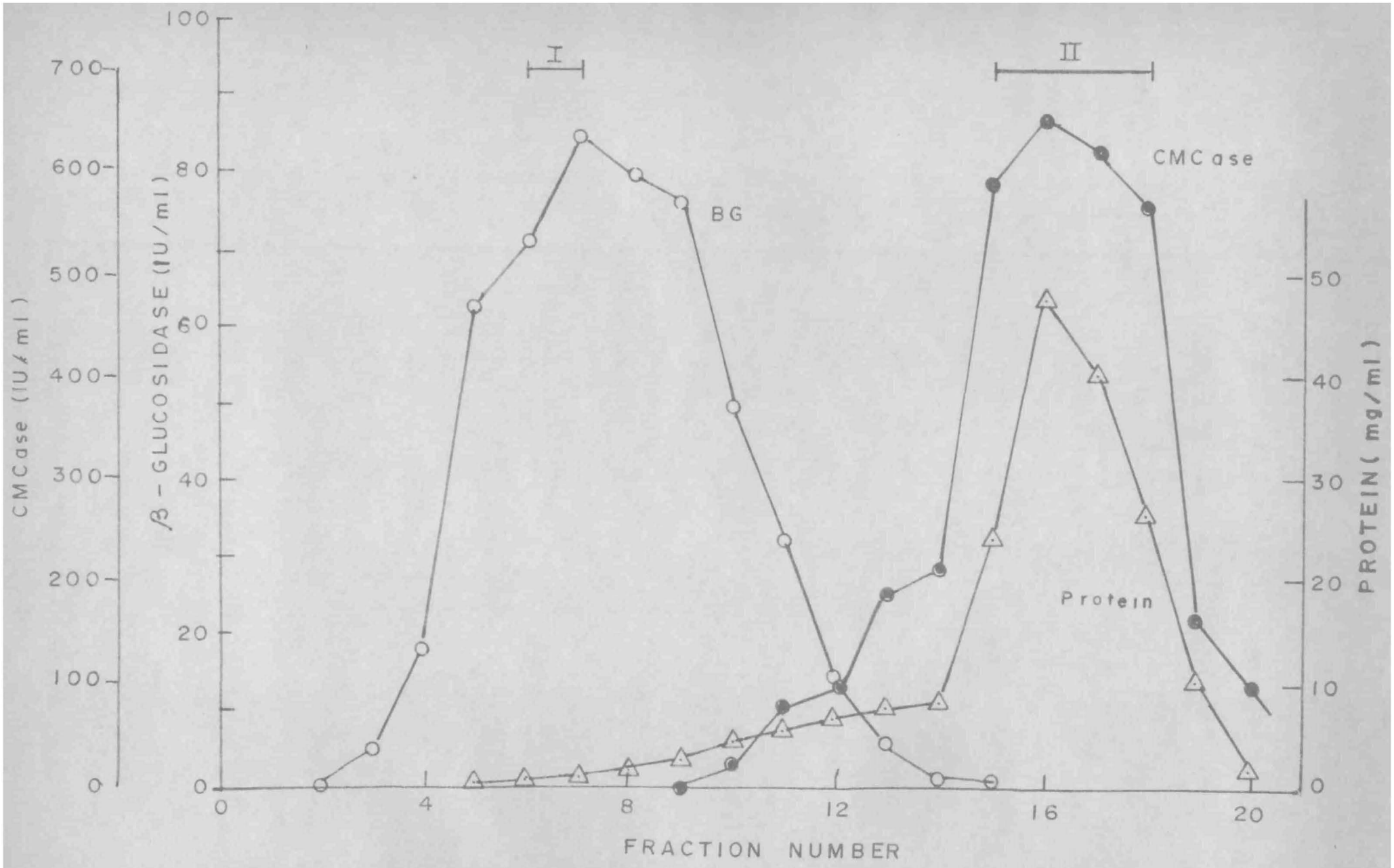


FIG. 17. Ion-exchange chromatography on DEAE-Sephadex A-50. Fraction A (7.5 g in 40 ml) from Sephadex G-75 chromatography was applied to the DEAE-Sephadex column (1.8 x 100 cm), previously equilibrated with 0.05 M phosphate buffer, pH 7.3 and washed with the same buffer. Peak I comprises fractions 6-7, and Peak II

against 0.001 M citrate buffer, pH 4.5 to reduce the salt concentration. Usually 10 ml of the concentrated and dialyzed enzyme preparation containing about 18 mg of protein was subjected to preparative isoelectric focusing at 5°C for 72 h. The voltage at the end of the run was 500 V and the current 2 mA. Fractions (1 ml) were immediately processed for pH determination (5-7°C), activity and protein, and were made free of sucrose by dialysis against 0.05 M citrate buffer, pH 4.5.

Fig. 18 shows the isoelectric profiles of the beta-glucosidase after electrofocusing. As a result of isoelectric focusing the beta-glucosidase activity was resolved into four separate peaks which were eluted consecutively at pH 4.10, 4.55, 5.10 and 5.55. These were designated as BG-1, BG-2, BG-3 and BG-4 beta-glucosidases,respectively. The observation was reproducible. Protein and beta-glucosidase activity recoveries were low.

The yield and specific activity of the enzymes at different stages of purification are summarized in Table 24.

Because of the limited quantities of BG-1 (370 µg), BG-2 (200 µg) and BG-4 (590 µg), some of the physico-chemical studies could not be carried out with these enzymes. Detailed studies were done only with BG-3 enzyme. The four enzymes exhibited different electrophoretic mobility (Fig. 19). The enzymes may be isoenzymes produced by the fungus or these could be modifications of one or two original enzymes due to proteolytic activity or other reasons.

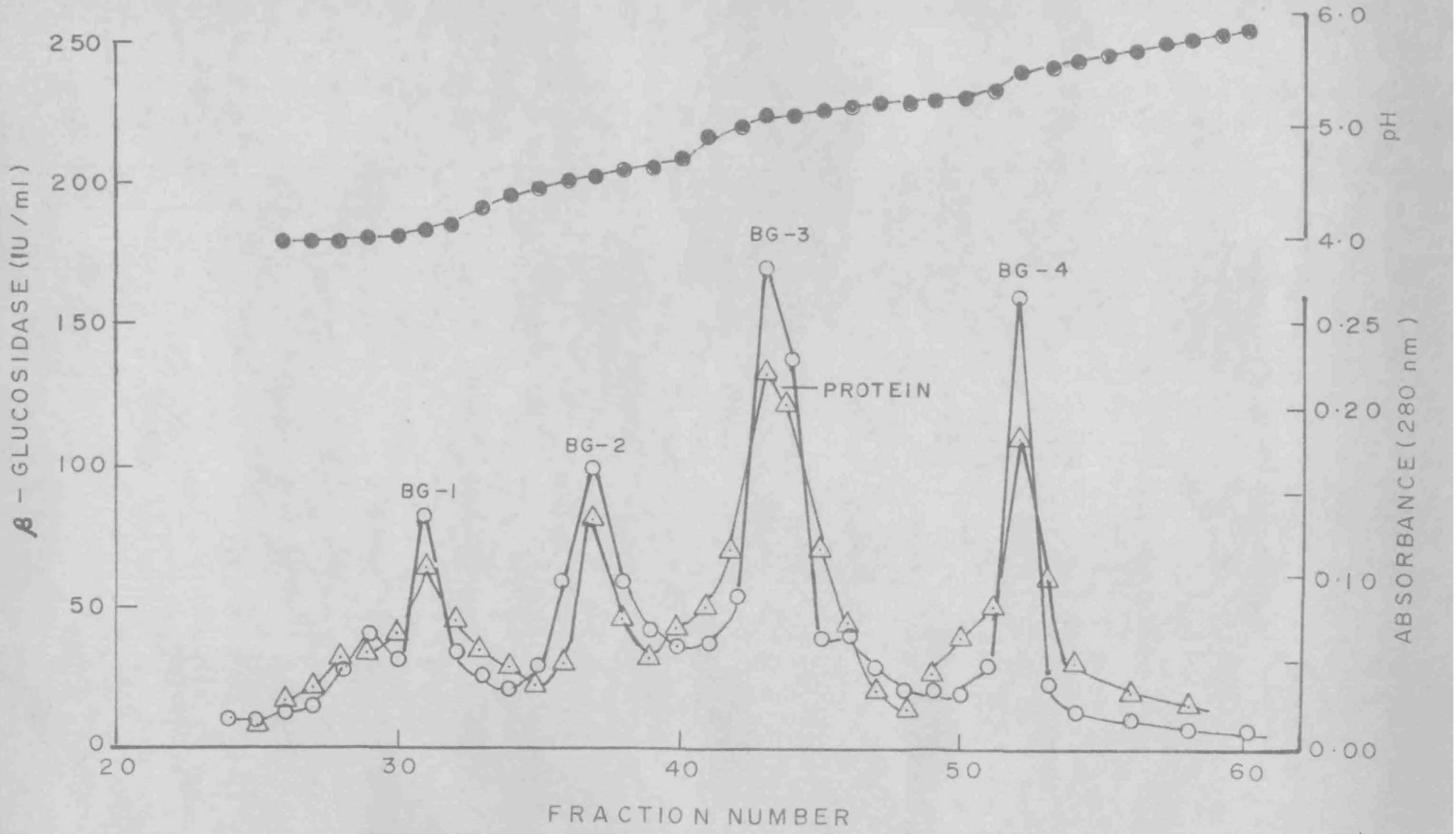


FIG. 18. Isoelectric focusing of Peak I from DEAE-Sephadex chromatography. Sample: 18 mg. Column: 110 ml LKB electrofocusing column. The ampholyte solution, pH 4-6, was used at a final concentration of 1%. The voltage at the end of run (72 h) was 500 V and the current 2 mA. Fractions (1 ml) were collected at a flow rate of 30-40 ml/h. Symbols: ○, beta-glucosidase; △, absorbance at 280 nm and ●, pH.

TABLE 24: SUMMARY OF PURIFICATION OF BETA-GLUCOSIDASES FROM S.ROLFSII CULTURE FILTRATE

Fraction	Total protein (mg)	Beta-glucosidase			CMCase			Beta-glucosidase CMCase Ratio
		Total units	Specific Activity (U/mg protein)	Recovery (%)	Total units	Specific Activity (U/mg protein)	Recovery (%)	
Culture filtrate	16150	17720	1.1	100	355488	22	100	0.04
Ammonium sulfate 0-90% saturation	10750	15930	1.4	90	284740	26	80	0.05
Sephadex G-75								
a) Fraction A	7580	11400	1.5	64	109290	14	30	0.10
b) Fraction B	918	74	0.004	0.4	49680	54	14	0.001
Ultrafiltration of Fraction A (Amicon XM 50)	3800	9210	2.4	54	57260	15	16	0.16
DEAE-Sephadex A-50								
a) Peak I	23	1125	48	6.3	110	4.7	0.03	10.2
b) Peak II	550	127	0.23	0.71	28359	51	7.9	0.004
Preparative isoelectric focusing of Peak I								
BG-1	0.37	10	27	0.05	0	0	0	-
BG-2	0.20	10	50	0.05	0	0	0	-
BG-3	1.80	102	57	0.57	0	0	0	-
BG-4	0.59	14	25	0.07	0	0	0	-

Criteria of purity:

The purified beta-glucosidases were free of contaminating endoglucanase activity as determined viscometrically (319). Electrophoresis of all four native enzymes individually in both cathodic (pH 4.3) (260) and anodic (pH 8.9) (259) running in polyacrylamide gel systems revealed only one protein band (Fig. 19 c-j). A strict coelution of protein and beta-glucosidase activity was observed. SDS-gel electrophoresis of the enzymes also showed only one protein band with molecular weights corresponding to the native proteins. BG-3 beta-glucosidase (other beta-glucosidases were not tested) migrated as a single band on 5, 7.5, 10 and 12% polyacrylamide gels at pH 8.9.

As a final criterion of purity BG-3, the major enzyme, was examined by isoelectric focusing in 7.5% polyacrylamide gel over the pH range 4-6 described by O'Farrell (266). Only a single protein band could be detected (Fig. 20). The position of the BG-3 in the measured pH gradient was in agreement with the result from the preparative run.

Molecular weight of enzymes:

Three different methods were used in the determination of molecular weight of the enzymes.

(1) Gel filtration: The molecular weight of the enzymes was estimated by comparing the elution volumes from a Bio-Gel P-150 (1.5 cm x 90 cm) column with those of proteins of known molecular weight. A plot of V_e/V_o versus log molecular weight according to the procedure of Andrews (320) indicated that the molecular weights of BG-1, BG-2,

FIG. 19. Disc gel electrophoresis of beta-glucosidases. Electrophoresis was carried out at pH 8.9 (a,c,e,g,i) and at pH 4.3 (b,d,f,h,j) as described under materials and methods. (a,b) Peak I from DEAE-Sephadex A-50; (c,d) BG-1; (e,f) BG-2; (g,h) BG-3 and (i.j) BG-4.

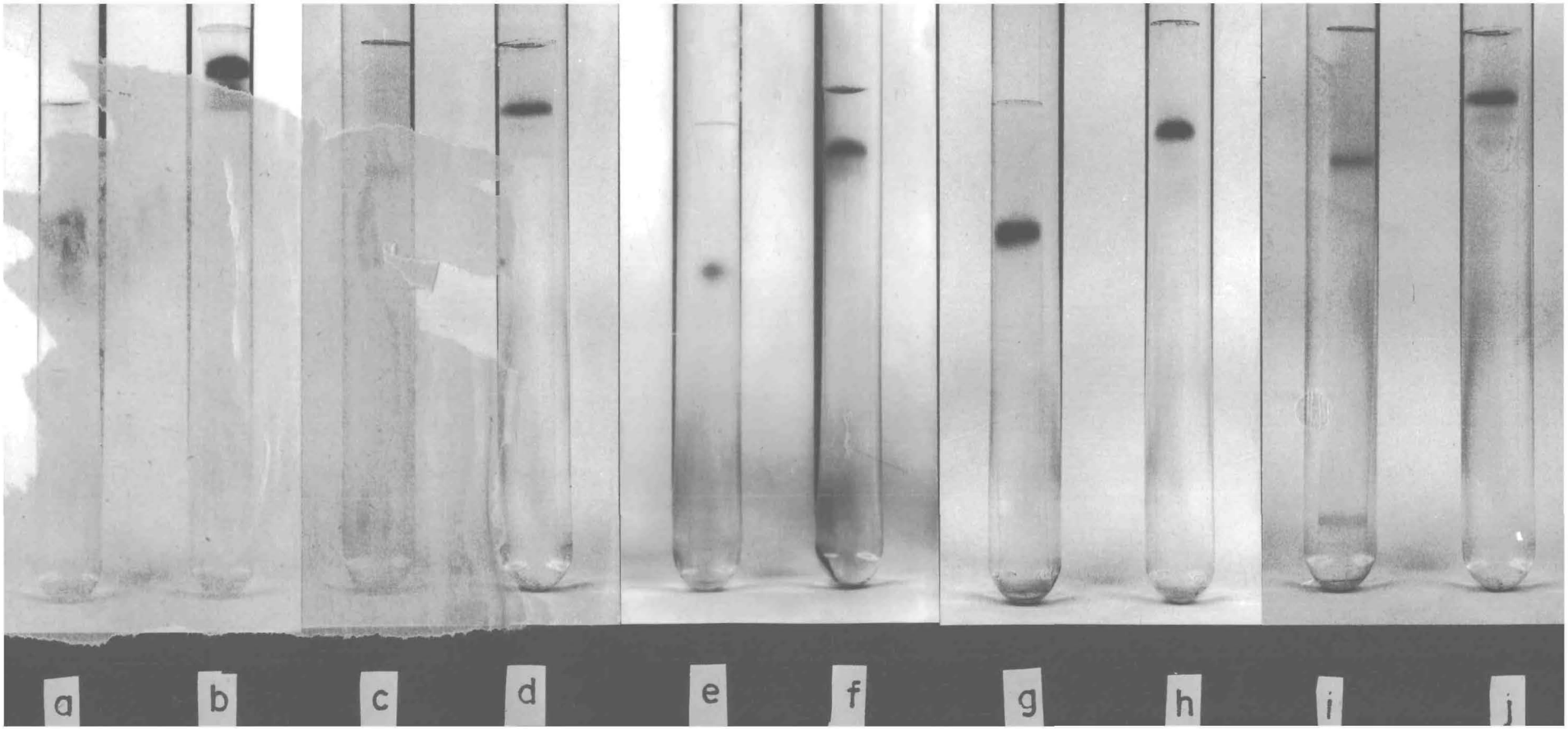
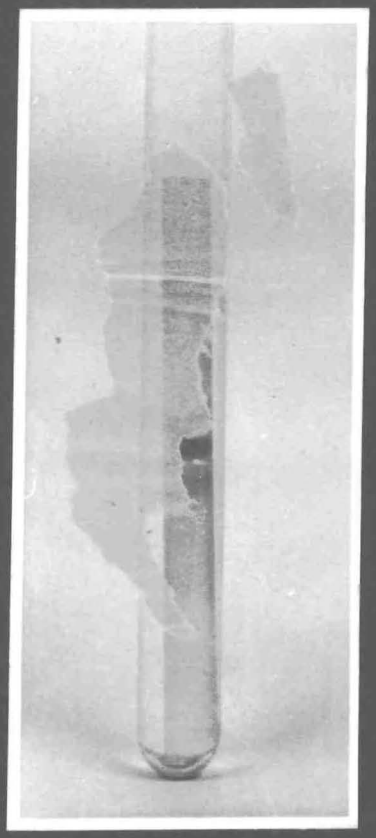

a
b
c
d
e
f
g
h
i
j

FIG. 20. Analytical isoelectric focusing in polyacrylamide gel of BG-3 beta-glucosidase. Carrier ampholites: Ampholines (40%) pH 3-10 (0.1 ml) and pH 4-5 (0.4 ml) per 10 ml of gel. Electrofocusing was carried out at 400 volts for 12 h and 800 volt for 1 h at 4°C.



BG-3 and BG-4 beta-glucosidases were 90,000, 90,000, 107,000 and 92,200, respectively (Fig. 21). With Sephadex G-200 the molecular weight of the enzymes obtained were low, e.g. 44,000 for BG-3, indicating interaction between the enzymes and Sephadex polysaccharide matrix. With freshly prepared crude culture filtrate on gel filtration through Bio-Gel P-150, an average molecular weight value of 110,000 daltons for beta-glucosidase was obtained. This indicated that the beta-glucosidases have not undergone any association-dissociation during the purification procedure as has been postulated by Umezurike (321).

(2) Gel electrophoresis (Slope method): Plots of log mobility of marker proteins (ovalbumin:monomer 46,000; dimer 92,000; BSA: monomer 68,000; dimer 136,000 and trimer 204,000) relative to bromophenol blue (Rm) versus gel concentration gave straight lines with slopes which were linearly related to the molecular weight of the proteins (262). The molecular weight of BG-3 beta-glucosidase determined by this method was found to be 100,000 daltons which agrees with the values obtained by other methods.

(3) SDS-gel electrophoresis: An estimate of the molecular weight of the beta-glucosidases and their possible subunit nature was made by its migration in the SDS-polyacrylamide gels (264,265). Plot of log molecular weight versus relative mobility of the marker proteins yielded a straight line (Fig. 22) and an estimate of the molecular weights of the BG-1, BG-2, BG-3 and BG-4 enzymes of 95,500, 95,500, 106,000 and 95,500 daltons, respectively. Carboxamido-

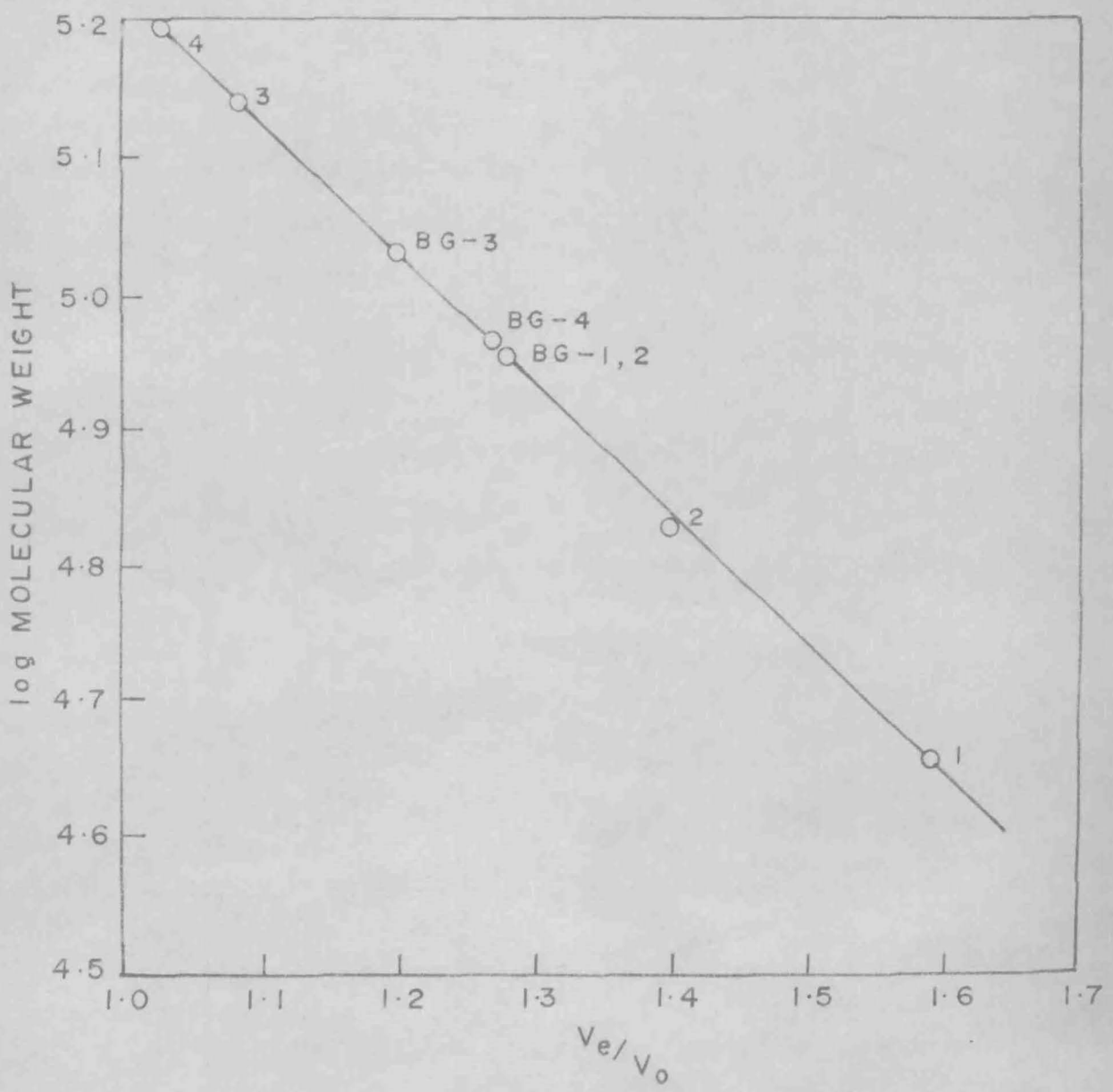


FIG. 21. Molecular weight determination of beta-glucosidases by gel filtration. A Bio-Gel P-150 column (1.5 x 90 cm) was calibrated with: 1) Ovalbumin molecular wt. 46,000; 2) [illegible] 68,000; 3) alcohol dehydrogenase 141,000; and 4) gamma-globulin 160,000. Buffer: 0.05 M citrate buffer pH 4.5. V_o, Void volume; V_e, Fluted volume.

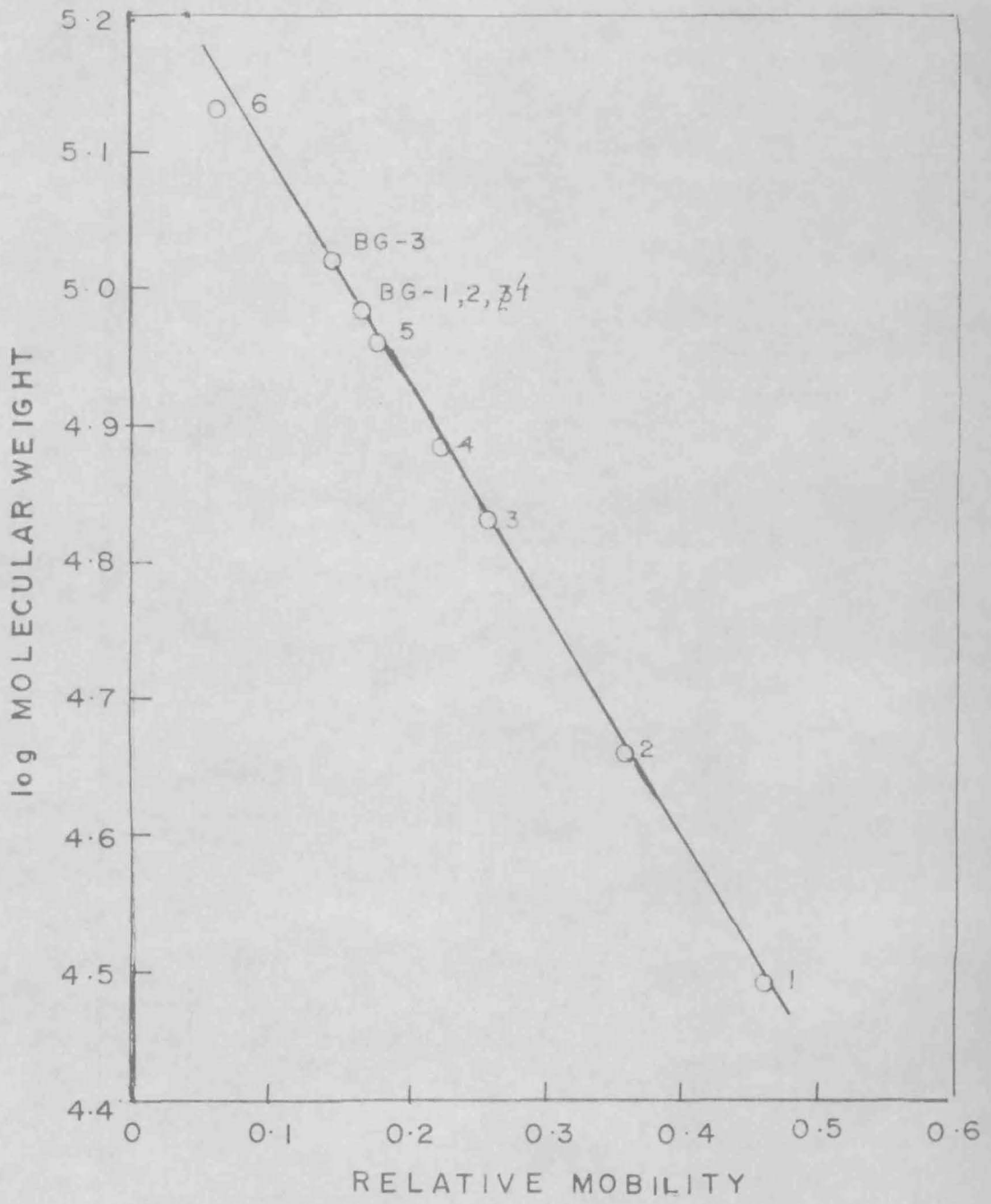


FIG. 22. Molecular weight determination by SDS-gel electrophoresis. Relative mobility was plotted against log molecular weight of standard proteins. Standard proteins used were: 1) deoxyribonuclease I molecular wt. 31,000; 2) ovalbumin monomer 40,000; 3) BSA monomer 68,000; 4) transferrin 76,000; 5) ovalbumin dimer 92,000 and 6) BSA dimer 136,000.

methylation of the reduced form of each enzyme on SDS-gel electrophoresis also showed only one protein band with molecular weights corresponding to the native proteins. This indicated that all the four beta-glucosidases are comprised of only one polypeptide chain. The beta-glucosidase from Dictyostelium discoideum is reported to consist of two dissimilar subunits with approximate molecular weights of 88,000 and 68,000; the molecular weight of the native protein being 160,000 (322) while that from Aspergillus fumigatus is composed of two subunits of the same size (323).

Glycoprotein nature:

Glycoproteins have an affinity toward Con A and form complexes (324). All the four beta-glucosidases were adsorbed on Con A-Sepharose column and no activity was detected until after specific replacement of bound proteins by a solution of 1% alpha-methyl-D-glucoside or alpha-methyl-D-mannoside. The bound fraction was over 90% of the total beta-glucosidase activity loaded in the case of all the four enzymes. Since Con A binds to glycoproteins that contain alpha-D-mannopyranosyl and/or alpha-D-glucopyranosyl end groups or internal 2-O-D-mannopyranosyl residues in the sugar moieties (324,325), the results indicated that S.rolfsii beta-glucosidases contain these specific groups.

Polyacrylamide gels stained with Coomassie brilliant blue showed single protein bands with all the four beta-glucosidases, while identifical gels stained with periodate-

fuchsin (261) also revealed a single band of carbohydrate. The position of the protein bands coincided with that stained by the carbohydrate reaction. All evidence is consistent that the beta-glucosidases from S.rolfsii are glycoproteins. Gong et al. (325) and Berghem and Pettersson (229) have reported that Trichoderma reesei beta-glucosidases are not glycoproteins while those from A.fumigatus (323) and Pyricularia oryzae Cavara (326) are reported to be glycoproteins.

Isoelectric point:

From preparative isoelectric focusing experiments, the isoelectric points of the beta-glucosidases were determined to be 4.10, 4.55, 5.10 and 5.55 for BG-1, BG-2, BG-3 and BG-4, respectively. Trichoderma beta-glucosidase has an isoelectric point at about pH 8 (327).

Transferase activity:

The purified BG-3 beta-glucosidase enzyme did not catalyze the transfer of glucose from PNPG (donor) to cellobiose or glucose (acceptor) that was added to the assay system. No positive spot that was absent in controls from hydrolyzates could be detected up to 4 h when the products of hydrolysis were examined by paper chromatography. These studies showed the BG-3 to be a hydrolase and not a transferase. The other three enzymes were not tested. Beta-glucosidase from Neurospora crassa (328) has both hydrolase as well as transglycolation activity whereas cellobiase from Trichoderma koningii (217) is not a transferase.

Enzymatic properties:

The purified beta-glucosidases were stable when stored at -15°C at pH 4.5; no significant loss of activity was observed over a 12 month period. With CMC as substrate, and using 3 µg of each of the enzymes no preceptible decline in viscosity up to 4 h was observed. With 0.1 to 0.4 µg of enzyme with cellobiose and 0.02 to 0.1 µg of enzyme with PNPG, the rate of hydrolysis was proportional to the amount of protein and was linear for atleast 40 min. The purified enzymes hydrolysed cellobiose to glucose at a rate of 10 µg glucose produced per min per 1.0, 0.7, 0.32 and 1.08 µg of BG-1, BG-2, BG-3 and BG-4, respectively, measured over linear portions of the curve in a 30 min reaction period under the standard assay conditions. The rates of cellobiose hydrolysis by S.rolfsii enzymes are greater than that observed with purified cellobiase from Humicola and T.koningii which required 44 µg and 47 µg enzyme protein, respectively (217). Beta-glucosidases from S.rolfsii accept both PNPG and cellobiose as substrate. In this respect it resembles the beta-glucosidase from Sporotrichum pulverulentum (329), T.reesei (229), Aspergillus niger (330) and Botryodiploida theobromae Pat (257).

Influence of pH on stability and activity:

(a) Stability: For the determination of stability, enzyme samples were adjusted to various pH values by the addition of suitable buffer (citrate, pH 3 to 6, phosphate pH 6 to 7, tris-glycine pH 8) to a final concentration of 0.05 M. After 30 minutes the residual activity was determined using

cellobiose as 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 enzymes were most stable at pH 4.0 to 4.5. In the presence of 0.5 mg.ml^{-1} BSA, the pH of optimum stability was 4.5 retaining virtually 100% of its activity (Fig. 23). The pH stability pattern was essentially similar for all the four enzymes.

(b) Activity: The effect of pH on catalytic activity was tested with 0.05 M citrate buffer ranging in pH from 3.0 to 6.0. The optimum pH for activity was 4.2 with PNPG and 4.5 with cellobiose as substrate for all the four beta-glucosidases. The activity fell to negligible levels at pH 3.0 and pH 6.0 (Fig. 24).

Optimum temperature:

The temperature/activity curve was determined using the standard assay system with cellobiose and PNPG as substrates in which the temperature was varied. The temperature optimum for all the four enzymes was 68°C with PNPG and 65°C with cellobiose as substrate. Straight lines were obtained when the data were plotted according to Arrhenius (Fig. 25). From this the energy of activation was calculated to be 12.2, 14.9, 11.5 and 18.3 kcal.mol^{-1} with PNPG and 6.5, 7.6, 6.9 and 6.4 kcal.mol^{-1} with cellobiose for BG-1, BG-2, BG-3 and BG-4 enzymes, respectively. With PNPG, the energy of activation for Phanerochaete chrysoporium beta-glucosidase was reported to be 9.15 kcal.mol^{-1} (321).

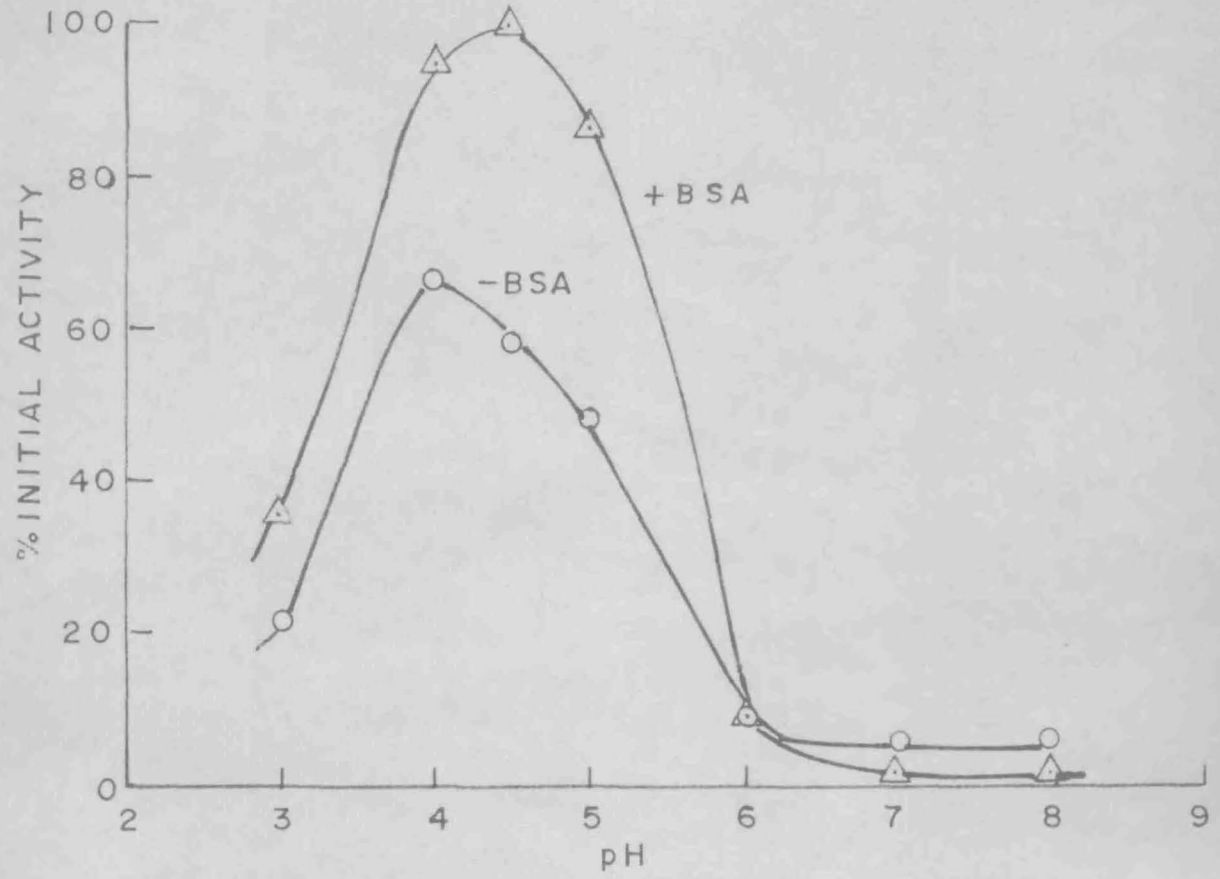
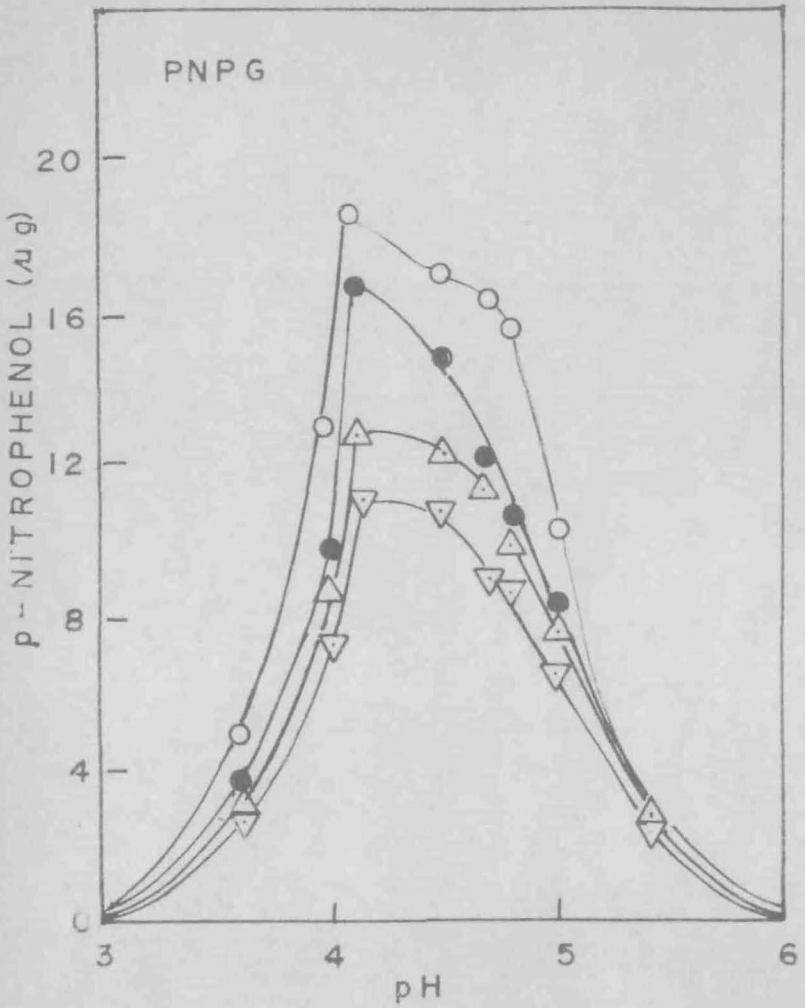


FIG. 23. Influence of pH on stability of BG-3 beta-glucuronidase. The enzyme (1 µg/ml) was maintained at 65°C for 30 min at different pH values and the residual activity determined under standard assay conditions. Symbols: ○, BSA; and △, + BSA, 0.5 mg/ml.



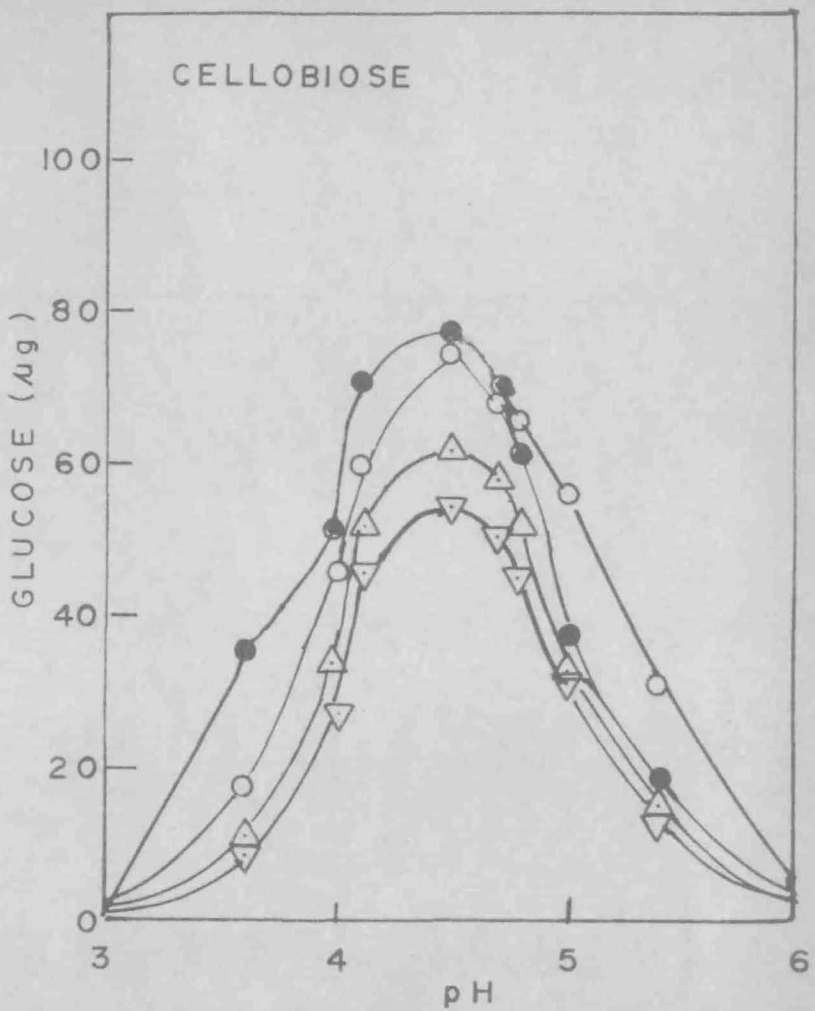


FIG. 24. Effect of pH on rate of hydrolysis of PNPG, 68°C and cellobiose, 65°C by S.rolfsii beta-glucosidase. Symbols: ○ , BG-1; △ , BG-2; ● , BG-3; and ▽ , BG-4.

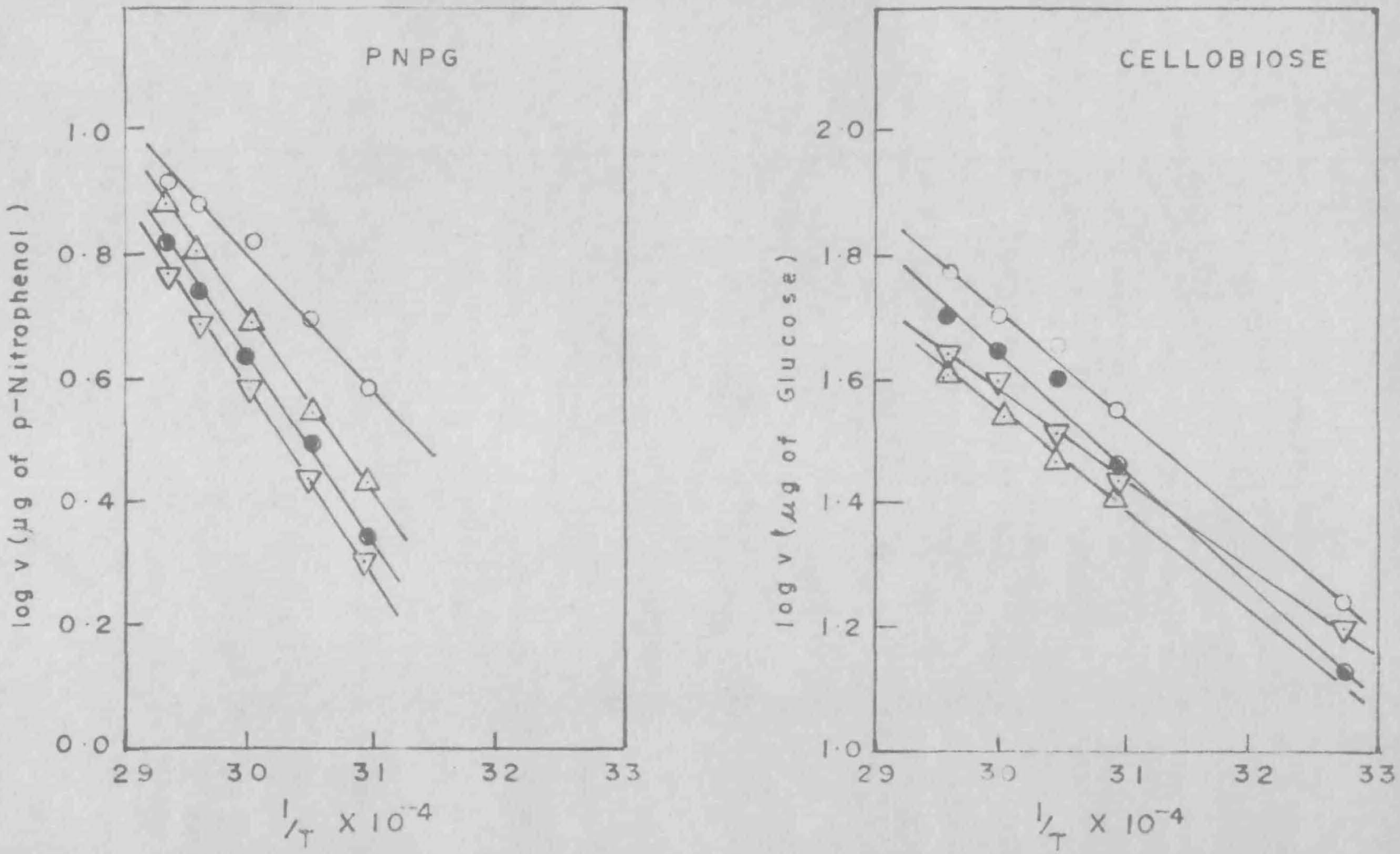


FIG. 25. Arrhenius plots showing effect of temperature on rate of hydrolysis of PNPG, pH 4.2 and cellobiose, pH 4.5 by S. rolfsii beta-glucosidase. Symbols: ○, BG-1; △, BG-2; ●, BG-3; and ▽ BG-4.

Influence of heating:

At 70°C about 50% of the activity remained after 10 min but this declined to 0 over the same interval at 80°C. The pattern of loss of activity with the other three enzymes was similar.

Kinetics:

PNPG and cellobiose saturation curves were hyperbolic. The K_m and V_{max} values for PNPG and cellobiose, calculated from Lineweaver-Burk plots, are summarized in Table 25. The K_m for cellobiose for the crude broth was 5.6 mM suggesting that the major amount of cellobiase in the crude broth was of BG-3 type. This supports the observation from molecular weight determinations that the major enzyme in the crude broth was of BG-3 type. The K_m values for cellobiose for all the enzymes was higher than the corresponding K_m values for PNPG. Beta-glucosidase from Aspergillus wentii has a higher K_m value towards PNPG than cellobiose (332). The V_{max} values for cellobiase with cellobiose, particularly BG-3, are higher compared to a value of 33 reported by Berghem and Pettersson (229) and 66.2, 116, 44.6 μmol glucose/min/mg protein reported by Gong et al. (315) for purified beta-glucosidases from T.reesei. Neither cellobiose nor PNPG showed substrate inhibition at concentrations up to 10 times their respective K_m values. The purified beta-glucosidase from Aspergillus phoenicis exhibited substrate inhibition above 10 mM cellobiose (279). The affinity for S.rolfsii beta-glucosidases for cellobiose (K_m = 3.65 - 5.84 mM)

TABLE 25: MICHAELIS PARAMETERS FOR BETA-GLUCOSIDASES FROM S.ROLFSII[a]

Enzyme	PNPG		Cellobiose	
	K_m (mM)	V_{max}[b]	K_m (mM)	V_{max}[c]
BG-1	1.07	37	3.65	55
BG-2	1.38	72	3.07	78
BG-3	0.89	77	5.84	175
BG-4	0.79	30	4.15	51

[a]The values are calculated from Lineweaver-Burk plots.

[b]µmol p-nitrophenol/mg/min, 68°C, pH 4.2

[c]µmol glucose/mg/min, 65°C, pH 4.5

is lower than that for A.phoenicis (K_m = 0.76 mM) (279), A.fumigatus (K_m = 0.84 mM) (323), Lenzite trabea (K_m = 1.64 mM) (256), and Trichoderma enzyme (K_m = 1.5, 1.8 mM) (215,229).

Substrate specifity:

Table 26 summarizes the results obtained when various glucosides were subjected to enzymic hydrolysis. The liberated glucose was measured by GOP test.

The enzymes can tolerate a wide variety of aglycones provided the substrate has a beta-configuration. In this respect all the four enzymes from S.rolfsii resemble A.phoenicis beta-glucosidase (279) However, the rate of hydrolysis depended on the nature of the aglycon moiety. Replacement of methyl group by phenyl or nitrophenyl group increased the rate of hydrolysis by 10 to 20 times. The beta-glucosidases hydrolyzed salicin at about 1/3 to 1/4th the rate of cellobiose, Murao and Sakamoto (333) have reported that beta-glucosidase from Aspergillus aculleatus hydrolyzed salicin at a faster rate than cellobiose. Arbutin was cleaved at a slightly lower rate than salicin. Barely any activity was detected against phloridzin and esculin hydrate. Garibaldi and Gibbons (334) reported that the phloridzin was the most preferred substrate for their partially purified beta-glucosidase from Erwinica herbicola Y46. The specificity for the hydroxyl group at carbon-4 was strict as the enzymes did not hydrolyze p-nitrophenyl-beta-galactoside. All disaccharides of glucose linked in beta-glucosidic form were good substrates. The specificity of the enzymes was not restricted to the beta-1,

TABLE 26: HYDROLYSIS OF VARIOUS GLUCOSIDES BY BETA-GLUCOSIDASES FROM S. ROLFSII

Substrate	Type of linkage	µg glucose liberated			
		BG-1	BG-2	BG-3	BG-4
Cellobiose	Beta-1,4	55	98	105	52
Gentibiose	Beta-1,6	51	96	70	48
Laminaribiose	Beta-1,3	65	140	140	60
Sophorose	Beta-1,2	43	80	110	53
Sucrose	Beta-1,4	7	12	17	8
Lactose	Alpha-1,4	0	0	0	0
Maltose	Alpha-1,4	0	0	0	0
Mellibiose	Alpha-1,6	0	0	0	0
Trehalose hydrate	Alpha-1,1	0	0	0	0
Arbutin	Beta-	10	17	14	7
Salicin	Beta-	15	26	26	14
Phloridzin	Beta-	1	1	2	1
Esculin hydrate	Beta-	3	4	3	2
Methyl-alpha-D-glucoside	Alpha-	0	0	0	0
Phenyl-alpha-D-glucoside	Alpha-	0	0	0	0
p-nitrophenyl-alpha-D-glucoside	Alpha-	0	0	0	0
Methyl-beta-D-glucoside	Beta-	3	6	4	2
Phenyl-beta-D-glucoside	Beta-	38	65	55	34
o-nitrophenyl-beta-D-glucoside	Beta-	52	90	70	42
p-nitrophenyl-beta-D-glucoside	Beta-	65	62	70	55

Contd...

Substrate	Type of linkage	µg glucose liberated			
		BG-1	BG-2	BG-3	BG-4
p-nitrophenyl-beta-D-galactoside[a]	Beta-	0	0	0	0
p-nitrophenyl-beta-D-xyloside[a]	Beta-	0	0	0	0

The reaction mixture contained 0.5 ml of 0.02 M substrate + 0.4 ml 0.05 M citrate buffer, pH 4.5 + 0.1 ml (0.3 µg) enzyme solution. Amount of glucose liberated in 30 min at 65°C was determined using GOP test.

[a]p-nitrophenol liberated was measured.

4 linkage as all the four enzymes hydrolyzed beta-1,6, beta-1,3 and beta-1,2 linkages also. None of the compounds with alpha-configuration was hydrolyzed. The enzymes also did not hydrolyze xylosides. Beta-glucosidase from L.trabea and Stachybotrys atra accepts aryl-beta-xylosides as substrates (256,335).

Inhibition of beta-glucosidases:

The cellobiases from a number of microorganisms are inhibited by its product glucose (215,256,336). Nojirimycin and glucono-delta-lactone also inhibit cellobiase activity very strongly (139, 279,326,329,337). Glucose, glucono-delta-lactone and nojirimycin inhibit all four S.rolfsii beta-glucosidases. The percentage inhibition data is summarized in Table 27. The inhibition of the BG-3 enzyme with cellobiose as substrate by glucose, glucono-delta-lactone and nojirimycin was competitive and the K_i values, calculated from the Linweaver-Burk plots, were 0.55 mM, 0.01 mM, and 1.0 µm, respectively (Fig. 26). The K_i for the other three beta-glucosidases were not studied. The Dixon plot for the BG-3 enzyme gave a K_i of 0.54 mM for glucose and also showed the inhibition to be of a competitive type (Fig. 26 inset). The ratio of K_m to K_i for glucono-delta-lactone, 530, and for nojirimycin, 5840, suggests that these inhibit cellobiase activity strongly. The K_m of S.rolfsii BG-3 beta-glucosidase for cellobiose is higher than the K_m values of beta-glucosidase from T.reesei (315) but the K_i for glucose is low. The inhibition by glucose was reported to be non-competitive for the T.reesei (315) and

TABLE 27: INHIBITION OF S.ROLFSII BETA-GLUCOSIDASES[a]

Enzyme	Inhibition (%)		
	Glucose (0.55 mM)	Glucono-delta-lactone (0.02 mM)	Nojirimycin (0.02 mM)
BG-1	30	32	93
BG-2	38	35	90
BG-3	78	38	92
BG-4	48	38	90

[a]Inhibition studies were carried out with cellobiose as substrate under standard assay conditions.

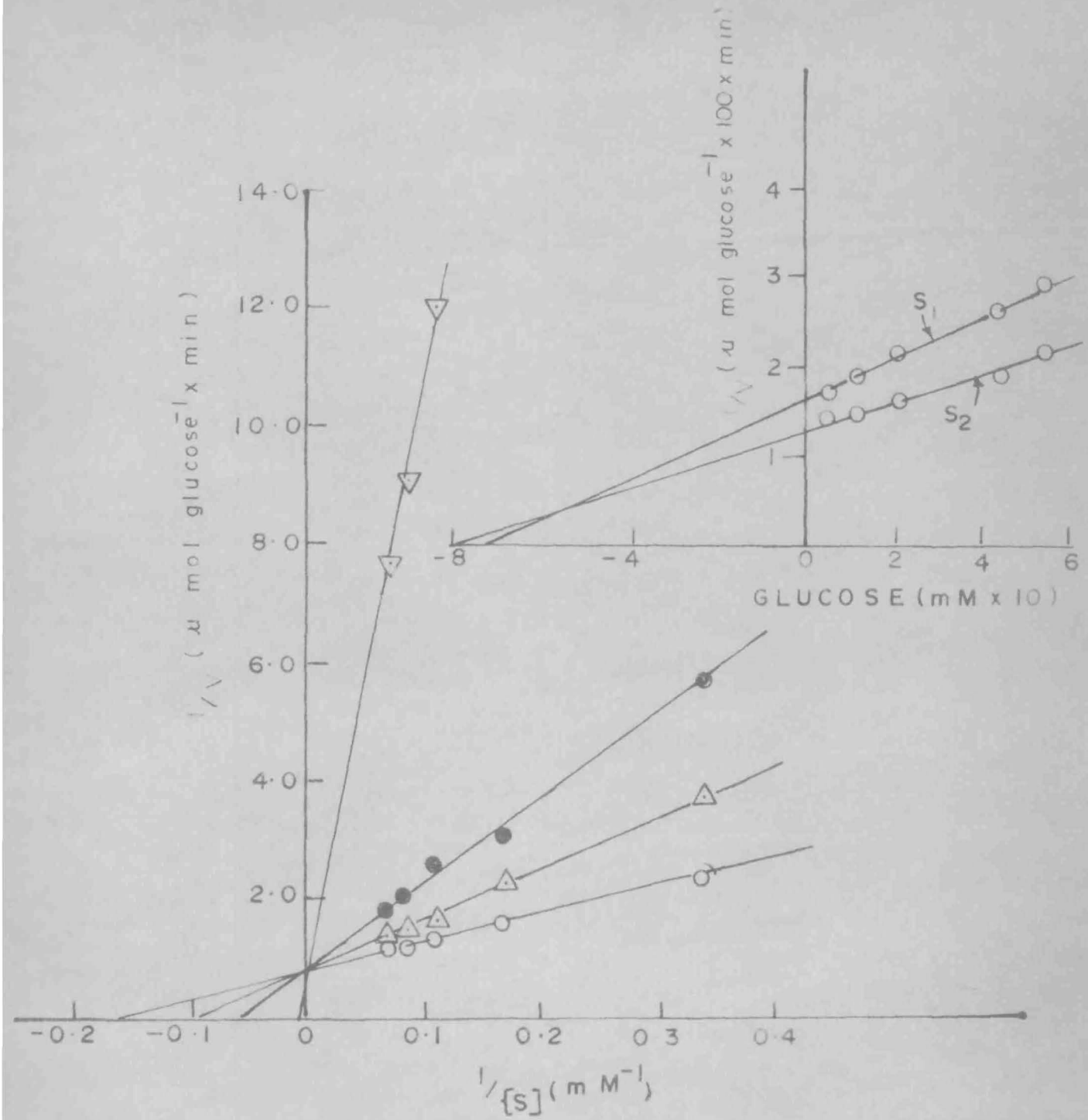


FIG. 26. Lineweaver-Burk plots for BG-3 beta-glucosidase showing competitive inhibition of cellobiose hydrolysis. The reaction was carried out under standard assay conditions. Symbols: ○, cellobiose; △, cellobiose + 0.55 mM glucose; ●, cellobiose + 0.02 mM glucono-delta-lactone and ▽, cellobiose + 0.02 mM nojirimycin. Inset: Dixon plot for BG-3 beta-glucosidase. The reaction was carried out with cellobiose, S_1 = 0.3 mM and S_2 = 0.6 mM as substrate and indicated concentrations of glucose as an inhibitor.

L.trabea enzymes (256).

Dissociation constants of groups in BG-3 beta-glucosidase:

The purified beta-glucosidases have been found to be stable in the pH range 4 to 5. In order to determine the ionizing groups at the active site of the BG-3 enzyme, the effect of pH on the values of Michaelis constant (K_m) and maximum velocity (V_{max}) of hydrolysis of cellobiose was studied (Table 28). The highest V_{max} was obtained at pH 3.5 whereas the pH optimum under the standard assay conditions was at pH 4.5. The parameters, log V_{max}, log (V_{max}/K_m) and pK_m ($-\log K_m$) were plotted as a function of pH. By using the analysis of Dixon and Webb (338), these kinetic parameters indicated a catalytic role for groups with pK_{1e} of 4.2 to 4.3 and pK_{2e} of 4.7 to 4.9 (Fig. 27 a,b). Since K_m varied with pH and was greater on both sides of the optimum pH, apparently the ionizing groups in the free enzyme control the binding of the substrate.

The log V_{max} versus pH plot (Fig. 27c) gave pK_{1es} and pK_{2es} values of 3.2 and 3.8 for groups in the enzyme-substrate complex which also approximates to the pK values of a carboxylate group. Thus, a carboxylate group is strongly implicated in the formation and dissociation of the enzyme-substrate complex. The carboxylate group may be acting as a general acid protonating the leaving groups as postulated in the catalytic mechanism of lysozyme (339). A carboxylate group and a protonated imidazolium group have been implicated in catalysis by the beta-glucosidase of B.theobromae (340), T.reesei (341) and P.oryzae Cavara (342).

TABLE 28: VARIATION IN KINETIC PARAMETERS OF BG-3 BETA-GLUCOSIDASE WITH pH[a]

pH	K_m (mM)	V_{max} (μmol glucose/mg/min)
3.0	97.3	246
3.5	41.5	305
4.0	10.4	205
4.5	5.6	183
5.0	6.0	133
5.5	7,3	76
6.0	9.7	45
6.5	11.2	40
7.0	13.0	34
7.5	14.8	28
8.0	16.2	24

[a] K_m and V_{max} values were determined at indicated pH values from Lineweaver-Burk plots.

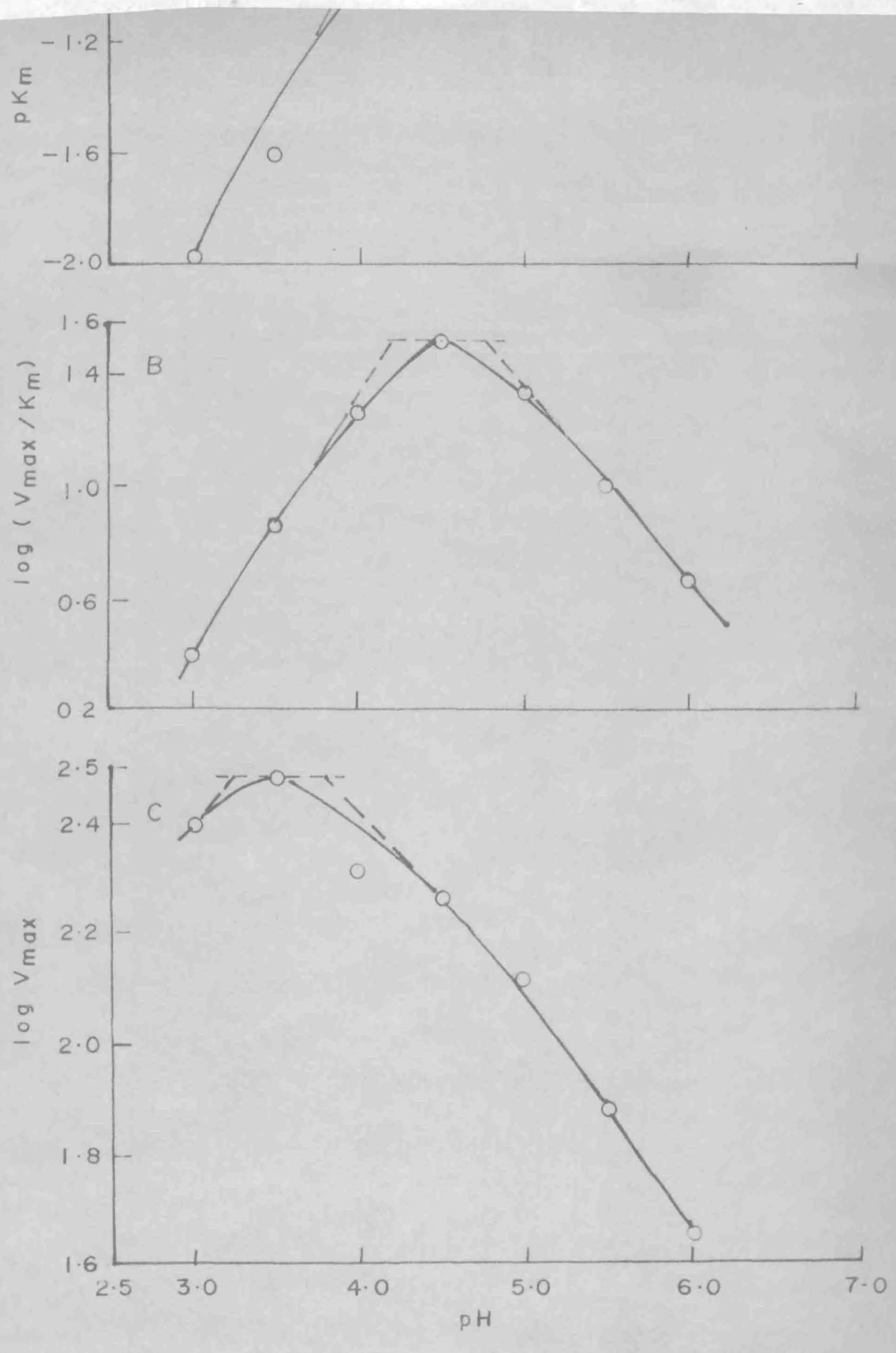


FIG. 27. Effect of pH on kinetic parameters of hydrolysis of cellobiose with BG-3 glucosidase. Variation of pH versus: (A) pK_m (-log K_m), (B) log (V_{max}/K_m) and (C) log V_{max}. Values for pK_m, log (V_{max}/K_m) and log V_{max} were calculated from data of Table 28.

DISCUSSION

Beta-glucosidase, a component of the cellulase enzyme system, promotes the action of beta-1,4-glucan cellobiohydrolase which is subject to product inhibition by hydrolyzing cellobiose to glucose (137,139,205,218). This assumes a special significance in view of the fact that cellulases are being considered for large scale saccharification of cellulose to glucose. There have been intensive investigations in exploring the chemical, physical and biochemical properties of beta-glucosidase but there are few reported studies on purified beta-glucosidases which are free from contaminating activities such as endo-beta-gluconases from highly cellulolytic organisms. The four beta-glucosidases purified from S.rolfsii are free from contaminating endo-beta-glucanase (viscosity-lowering) activities. The BG-3 enzyme was pure by all the criteria tested.

Multiplicity of extracellular beta-glucosidases from microorganisms has been observed (257,315,323,329,342-344). Jermyn (343,344) has attributed the multiplicity of beta-glucosidases from Stachybotrys atra to the formation of stable complexes between a single enzyme and polysaccharides of the growth medium. The beta-glucosidase from B.theobromae appeared to be a single enzyme containing 8 monomers which are catalytically active(321) and which could partially dissociate into lower molecular weight forms under certain conditions (257). Umezurike (257,321)

suggested that the different molecular species of beta-glucosidase reported by various workers to be partly due to isolation/purification/storage/or differences in the history of the culture. The molecular weight of the S.rolfsii crude beta-glucosidase preparation in the freshly prepared culture broth, as determined by gel filtration, and of the purified enzymes (by different methods) were 100,000 indicating that the S.rolfsii beta-glucosidases have not undergone any association-dissociation during storage/purification procedures. It is not known whether the multiple beta-glucosidase enzymes isolated from S.rolfsii culture filtrate represent isomers or have arisen due to modification of one or two original enzymes due to proteolytic activity or other reasons.

The four beta-glucosidases from S.rolfsii have some features in common, viz. the pH and heat instability patterns, pH and temperature optima, substrate specificity as also were the molecular weights. No substrate inhibition up to 10 times the K_m values was observed with any of the beta-glucosidase. The four enzymes from S.rolfsii are glycoproteins and are subject to product inhibition. Each of the four enzymes from S.rolfsii is comprised of only one polypeptide chain in contrast to some beta-glucosidases which are composed of subunits of equal size (A.fumigatus) (323) or dissimilar size (D.discoideum) (322). Arrhenius activation energy values for cellobiose and PNPG are not very different for the four enzymes.

The enzymes however, differ in other properties.

These include K_m and V_{max} for cellobiose and PNPG, electrophoretic mobility and isoelectric points.

The substrate specificity of fungal beta-glucosidases has been reported to vary considerably from one fungus to another. Purified preparations have been reported which split only cellobiose but do not act on PNPG and conversely (334,345,346). Youatt (347) and Jermyn (345, 348) proposed the existence of two types of beta-glucosidases one with aryl-beta-glucosidase activity and the other with broad activity towards different glucosidases. Berghem and Pettersson (229) reported that there is only one kind of beta-glucosidase with activity towards both PNPG and cellobiose. Umezurike (321) reported that the affinity of the various molecular species of beta-glucosidase of B.theobromae decreases with increasing molecular complexity. Purified beta-glucosidases from S.rolfsii have higher affinity for PNPG than for cellobiose, and resemble in this respect the beta-glucosidases from A.niger (330), B.theobromae (257), S.pulverulentum (329) and T.reesei (229). In contrast, the enzyme from A.wentii has a higher K_m value towards PNPG than towards cellobiose (332).

The enzymes were specific for beta-configuration and none of the compounds with alpha-configuration were hydrolysed. The enzymes hydrolyzed gentibiose (beta-1,6), laminaribiose (beta-1,3), and sophorose (beta-1,2) besides cellobiose (beta-1,4). Thus, the specificity of the enzymes was not restricted to the beta-1,4 linkage. S.rolfsii beta-glucosidases resembels beta-glucosidases

from A.phoenicis (279) in this respect. The beta-glucosidase enzymes from S.rolfsii did not hydrolyze xylosides. L.trabea and S.atra beta-glucosidase hydrolyzed aryl-beta-xylosides (256,335).

Analysis of the kinetic data according to the method of Dixon and Webb (338) for the ionizable groups at the active site of the enzyme suggested a catalytic role for a carboxylate. A carboxylate group and a protonated nitrogen of an imidazole group have been implicated in catalysis by the beta-glucosidase of B.theobromae (340), T.reesei (341) and P.oryzae Cavara (342).

PART V

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