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Yoshio Yamanaka, Paul A. Carroad, Mohammad Riaz, and
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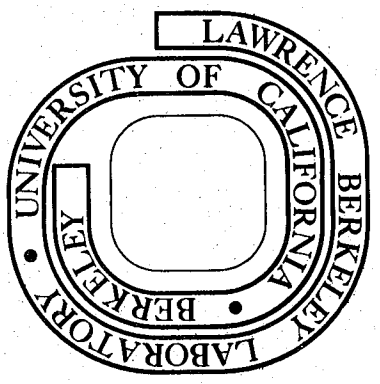

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DECOMPOSITION OF LIGNIN AND CELLOBIOSE IN RELATION TO THE ENZYMATIC HYDROLYSIS OF CELLULOSE*

by

Yoshio Yamanaka
Paul A. Carroad
Mohammad Riaz
Charles R. Wilke

Department of Chemical Engineering
University of California
and
Lawrence Berkeley Laboratory
Berkeley, California 94720

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ABSTRACT

This project has two objectives (1) to study the use of fungal β-glucosidase in conjunction with Trichoderma viride cellulase and (2) to find an effective enzyme system for lignin degradation. β-glucosidase is of potential benefit in cellulose hydrolysis by catalyzing the hydrolysis of cellobiose to glucose thereby reducing product inhibition and producing a higher glucose yield. Removal of lignin from cellulosic material makes the cellulose more accessible to hydrolyzing enzymes.

Hydrolysis studies on Solka Floc and newsprint were conducted with T. viride filtrates containing various proportions of B. theobromae filtrates. Significant improvement in hydrolysis rate particularly in glucose content was obtained by thus enriching the β-glucosidase content of the cellulase.

In the search for a lignin degrading enzyme, major emphasis was given to the fungus Polyporous versicolor. Significant o-diphenol oxidoreductase (catecholase) activity was found in the culture filtrates. Preliminary observations of a surface culture of the fungus in a composting mode suggest that delignification may be obtained in this manner. Work is continuing on this.

INTRODUCTION

Cellulose is the world's most plentiful naturally occurring organic compound and a major constituents of municipal and agricultural wastes.

A tentative process concept has been developed by Wilke, et al. (1,2,3) for hydrolysis of cellulose to glucose, i.e. reducing sugars, by contacting solid materials containing cellulose with an aqueous solution of enzyme (cellulase) obtained by growth of the fungus Trichoderma viride QM9414. The complexity of the substrate (cellulosic material) and the noncellulosic material (lignin) associated with it has a marked bearing on the susceptibility of cellulose to enzymatic digestion. Lignin which is the major structural component of the plants in addition to cellulose, blocks access of the hydrolyzing enzyme. Cellobiose which is an intermediate product in the conversion of cellulose to glucose also inhibits the hydrolysis process.

The present report summarizes efforts pursued for two main objectives: (1) search for an effective enzyme system for lignin degradation (2) supplementation of T. viride cellulase system with fungal β-glucosidase in order to promote overall celluloysis by hydrolyzing cellobiose to glucose. Additional details of the individual studies are available elsewhere (4,5).

RESULTS

Lignin is an extremely complex aromatic biopolymer. It is a major structural component of perennial plants, and one of the

most abundant of all continuously cycled organic materials. It is an effective hydrophobic binder, used in conjunction with the hydrophilic hemicellulose binder, to cement together cellulose fibers in plant structures. The lignin binder gives appropriate hydrophobicity and strength to the structural plant elements. It is exceedingly resistant to microbial degradation due to its phenolic nature and the complexity of its connecting bonds. Lignin is always associated physically, and to some extent chemically, with cellulose in plant cells.

Ground wood was chemically delignified to various lignin contents using sodium chlorite and subjected to hydrolysis by Trichoderma viride cellulase (4.6 filter paper activity) for 40 hours at 45°C. The results of hydrolysis are shown in Fig. 1. Percentage of lignin in the material clearly has a marked effect on cellulose digestibility. As the lignin content of a material decreases, the susceptibility of cellulose to enzymatic hydrolysis increases.

Fermentation Studies on Polyporus versicolor:

A literature survey identified Polyporus versicolor as the predominant organism used for lignin research. The research to date has involved growth of the fungus on several media, with glucose as primary carbon source and enzymatically de-cellulosed newsprint included as an inducer for the enzyme system.

As the main organism of this research P. versicolor has been the subject of aeration and temperature studies. It is thought that optimization of growth of this fungus will be significant to the production of an active enzyme system. The

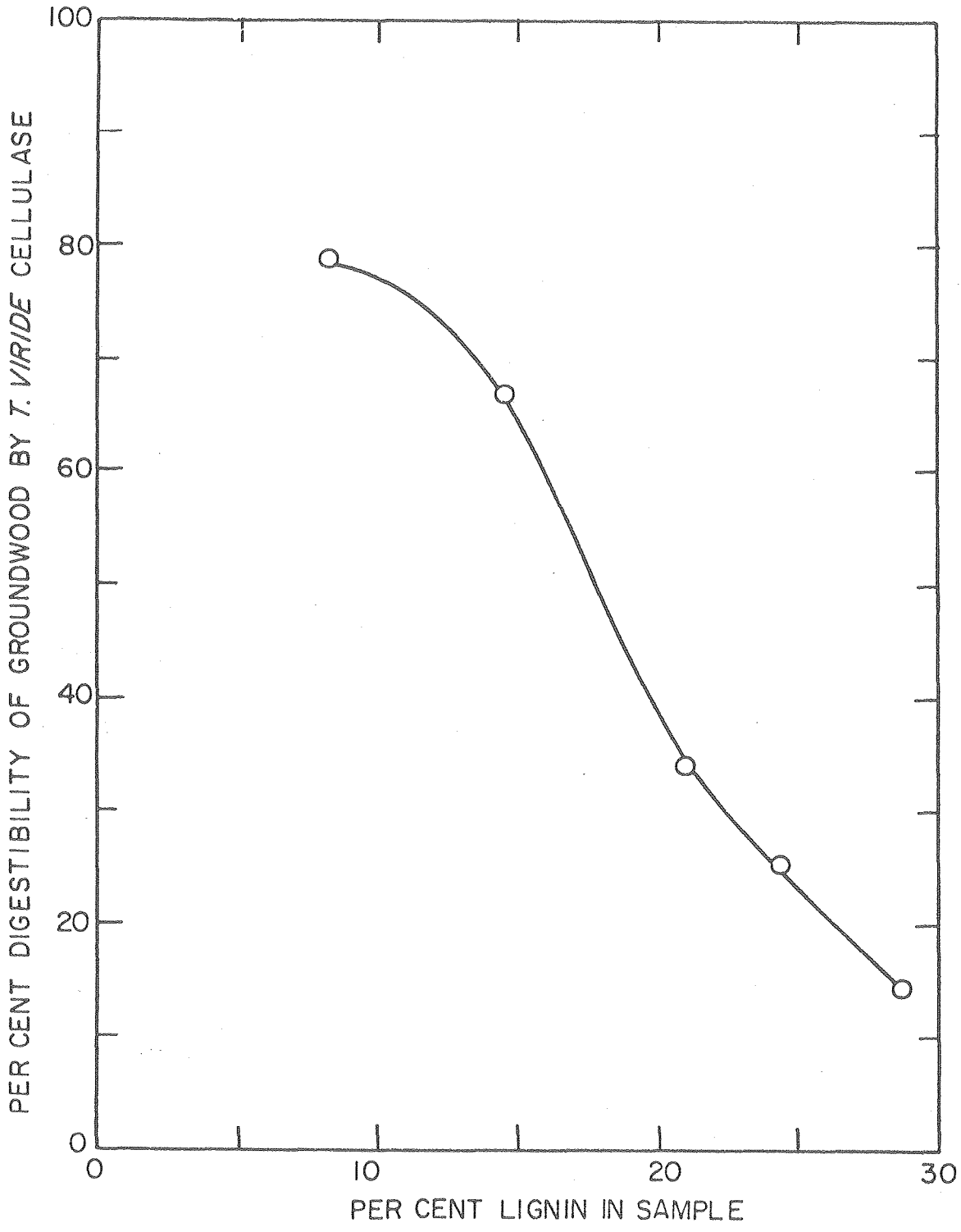

Fig. 1. Relationship between lignin content and ground wood digestibility by microbial cellulase.

specific respiratory rate was found to be 1.0 millimoles O_2/gram dry weight-hour. A complete temperature profile was achieved on a glucose-yeast extract medium. Temperatures include 20°C, 25°C, 28°C, 30°C, 33°C, 35°C, 37°C, and 40°C. Figures 2 and 3 show that the growth rate is maximum at 38°C, as is the rate of cell autolysis. Yield of cell mass increases, generally, as temperature is lowered, while yield of enzyme (measured as O-diphenol oxidoreductase with catechol) increases as temperature in increased.

Lignin degradation studies on newsprint with Polyporus versicolor culture filtrates.

Culture filtrates of Polyporus versicolor were produced in a fermentor. Newsprint from which cellulose had been enzymatically removed was included in 1% solution as inducer for lignin degrading enzyme system. Culture filtrate was obtained by filtering microbial cells and undigested solid material. Culture filtrates were tested as a pretreatment of cellulose hydrolysis by Trichoderma viride cellulase, in consecutive treatment with cellulase, and in simultaneous treatment with cellulase. The assay constituted comparison of hydrolysis results with and without the enzyme present as measured by reducing sugar production.

A. Pretreatment experiments

In pretreatment experiments, different papers, temperatures, pH values, and addition of chemical and biochemical reducing agents were investigated. The different paper fractions include ball milled newsprint, Whiley milled newsprint, hammer milled

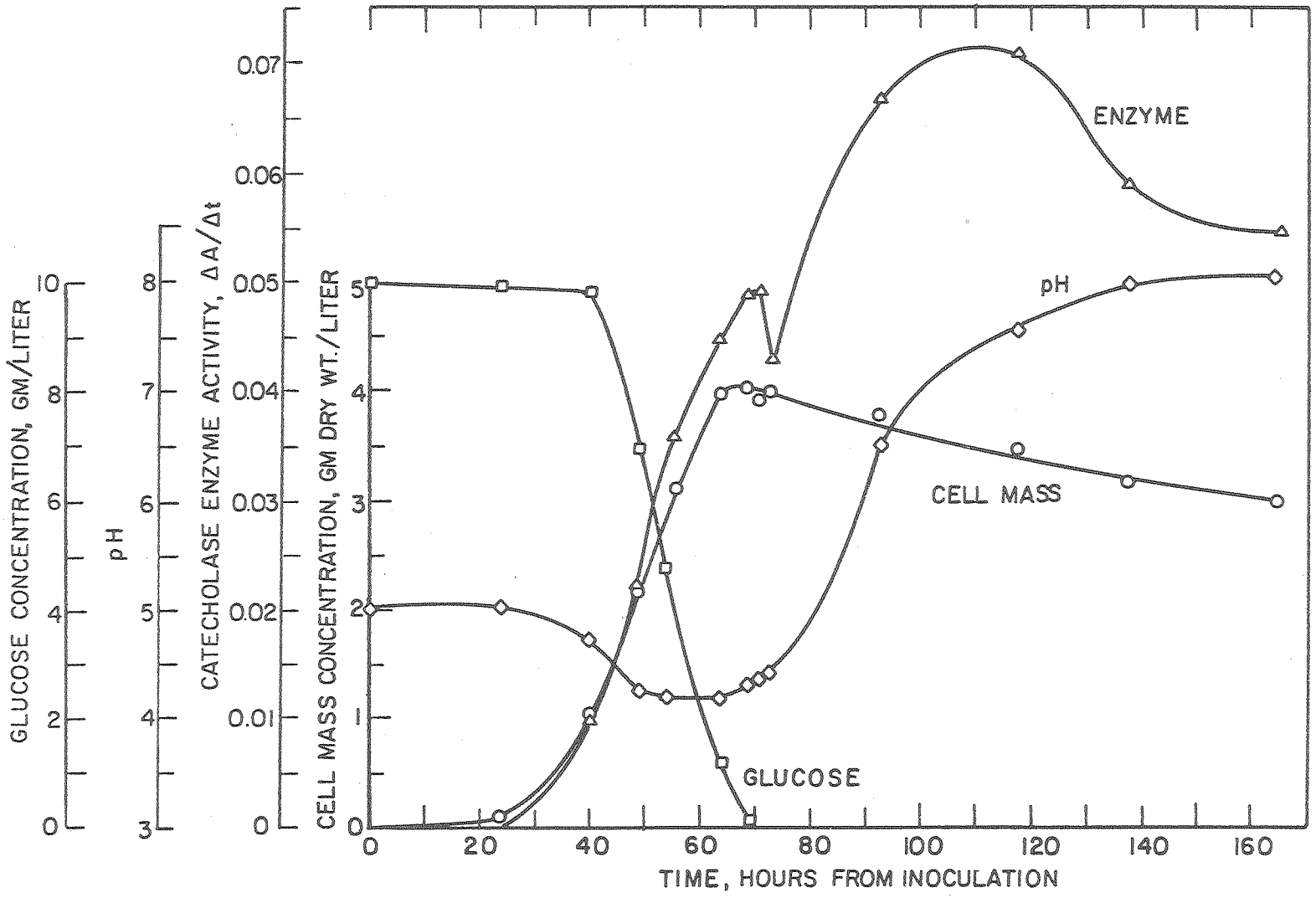

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Fig. 2. Polyporus versicolor batch fermentation data for 28°C experiment.

newsprint, and 1/2" squares of newsprint. The control experiment was conducted with a cellulase diluted to the same Filter Paper Activity (0.58) as the lignin degrading enzyme system, which also exhibits a cellulase activity. The result showed the lignin degrading enzyme system approximately 10% superior, but this was thought to be the result of the tremendous dilution of the cellulase which may have rendered one cellulase component ineffective. Wiley milled paper, which gave representative results, was chosen for further studies.

In the temperature series which include 25°C, 30°C, 40°C and 50°C, the lignin degrading enzyme system gave better results than the water control at higher temperatures, indicating that the cellulase of the lignin degrading system was active. However, compared to the diluted cellulase, there was a decreasing difference at higher temperatures indicating that the lignin degrading system was less stable than T. viride cellulase. At 50°C there was no difference between the cellulase control and the lignin degrading enzyme system.

The pH range of 3 to 7 was investigated, the optimum being 5.0. Since this is the optimum for cellulase, this additional evidence that the cellulase of the lignin degrading enzyme system was the active protein component.

Addition of ascorbic acid in the range of 2×10^{-2} molar to 5×10^{-4} molar was investigated. A slight increase in hydrolysis appeared at 1×10^{-2} molar, but as this effect could be shown with cellulase alone, the lignin degrading system was judged ineffective. (In this series, sugar was measured by the anthrone

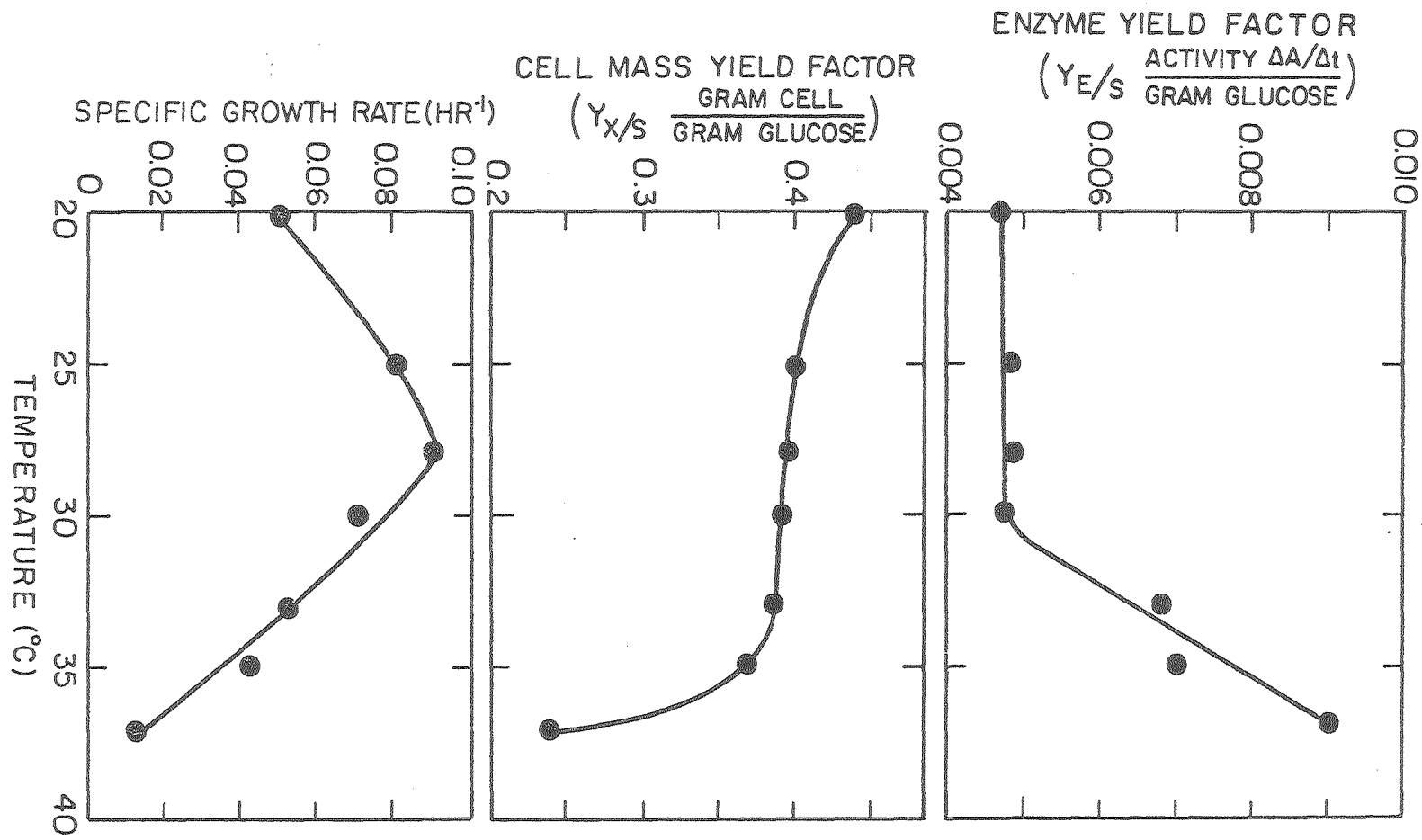

Fig. 3. Specific growth rate and yield factors of Polyporus versicolor as functions of temperature.

test to obviate the effect ascorbic acid would have on the reducing sugar assay.) Addition of NADH in the range of 10^{-2} molar to 5×10^{-4} molar similarly had no effect, nor did concentration of the enzyme by three times with the Amicon UM10 ultrafiltration membrane.

Thus overall there was some slight effect of pretreatment but this was judged to be due to treatment with the cellulase fraction of the lignin degrading enzyme system.

B. Consecutive Treatment

Consecutive enzyme treatment involved treatment with cellulase to hydrolyze the accessible cellulose, followed by treatment with the lignin degrading enzyme system, followed by a second treatment with cellulase. Variables considered were various papers, concentrations of enzyme, and addition of NADH.

On both Wiley milled newsprint and 1/2" squares of newsprint, the water control yielded incrementally higher reducing sugar values than the lignin degrading enzyme system. This may be due to adsorption of the lignin degrading enzyme system on the exposed lignin, even though there appeared to be no enzyme attack. There was no benefit to concentrating the enzyme by 3 or 5 times, nor to adding NADH. The conclusion is that consecutive treatment with this system is ineffective.

C. Simultaneous Treatment

The lignin degrading enzyme system and the cellulase system were concentrated prior to combination in a simultaneous treatment test, to avoid dilution upon mixing. Cellulase was held constant, but the lignin degrading enzyme system ratio was varied between 2

and zero, with respect to volumes of cellulase. There was generally no difference between any of the samples. If anything, the more lignin degrading enzyme system present, the worse the results. This again may indicate strong adsorption of this system on exposed lignin, blocking further cellulase attack.

Another theory cited in literature for lignin degradation indicate that the enzyme system required regenerable co-factors (6). This would necessitate the presence of the microbial cells to regenerate the co-enzymes. To test this theory, a compartmentalized fermentor was used. An 0.2 micron polycarbonate membrane separated a wood-filled chamber from a chamber containing wood and the fungus Pleurotus ostreatus. By alternately pressurizing one chamber and venting the other, glucose medium as well as any secreted enzymes could be pumped between the chambers. Any regenerable co-factors that might have been secreted could have been returned to the fungus for regeneration. Analysis showed that the wood in neither chamber was degraded.

Three organisms, namely Polyporus versicolor, Sporotrichum pulverulentum and Pleurotus ostreatus were successfully cultured directly on moistened ground wood to achieve lignin degradation. After seven weeks each organism had degraded approximately ten percent of the wood. Polyporus versicolor degraded 10.2% of gound wood removing 8.4% of the lignin. Sporotrichum pulverulentum degraded 10.0% of the wood removing only 2.9% of the lignin. Pleurotus ostreatus degraded 11.1% of the ground wood, removing 11.0% of the lignin.

USE OF β-GLUCOSIDASE IN CELLULOSE HYDROLYSIS

The sugars produced from cellulose with Trichoderma viride cellulase contain appreciable quantities of cellobiose and some cellotriose. Although these higher sugars are utilizable for fermentation, cellobiose is known to inhibit the action of cellulase. Figure 4 shows a Lineweaver-Burk plot of the initial rate of hydrolysis of Solka-floc at 50°C in T. viride cellulase of 3.95 filter paper activity in presence of 0.5% cellobiose and 0.5% glucose. Cellobiose apparently inhibits the reaction and its mode of inhibition is competitive. Glucose doesn't seem to inhibit the reaction significantly, at least in the initial state of hydrolysis. The inhibitory effect of cellobiose largely depends on the activity of β-glucosidase in the cellulase used. Cellulase from another organism Trichoderma koningii was fractionated into relatively pure β-glucosidase and component C_1 (cellobiohydrolyase) (7). These components were assayed alone and in combination to solubilize cotton. Results are summarized in Table 1. Component C_1 alone produces 10% solubilization of cotton all of which is free from glucose and appears to be a simple cellodextrin, such as cellobiose and may be cellotriose. In presence of β-glucosidase (cellobiase) all of the soluble material is found as glucose. Addition of β-glucosidase promoted 60% increase in overall hydrolysis.

The ineffectiveness of T. viride cellulase in converting all cellobiose to glucose is believed a result of its relatively weak β-glucosidase activity. Therefore, the present research was initiated to see if a more complete conversion of cellulose to glucose could be obtained by supplementing the T. viride

TABLE 1

Solubilization and products formed from cotton in 7 days (unshaken) by component C_1 and cellobiase from T. koningii acting alone and in combination on a 2 mg sample.

Components	PRODUCTS			
	Glucose (µg)	Cellobiose (µg)	Total soluble (µg)	Solubilization %
Cellobiase	0	0	0	0
Component C_1	0	198	207	10
Cellobiase + C_1	315	0	315	16

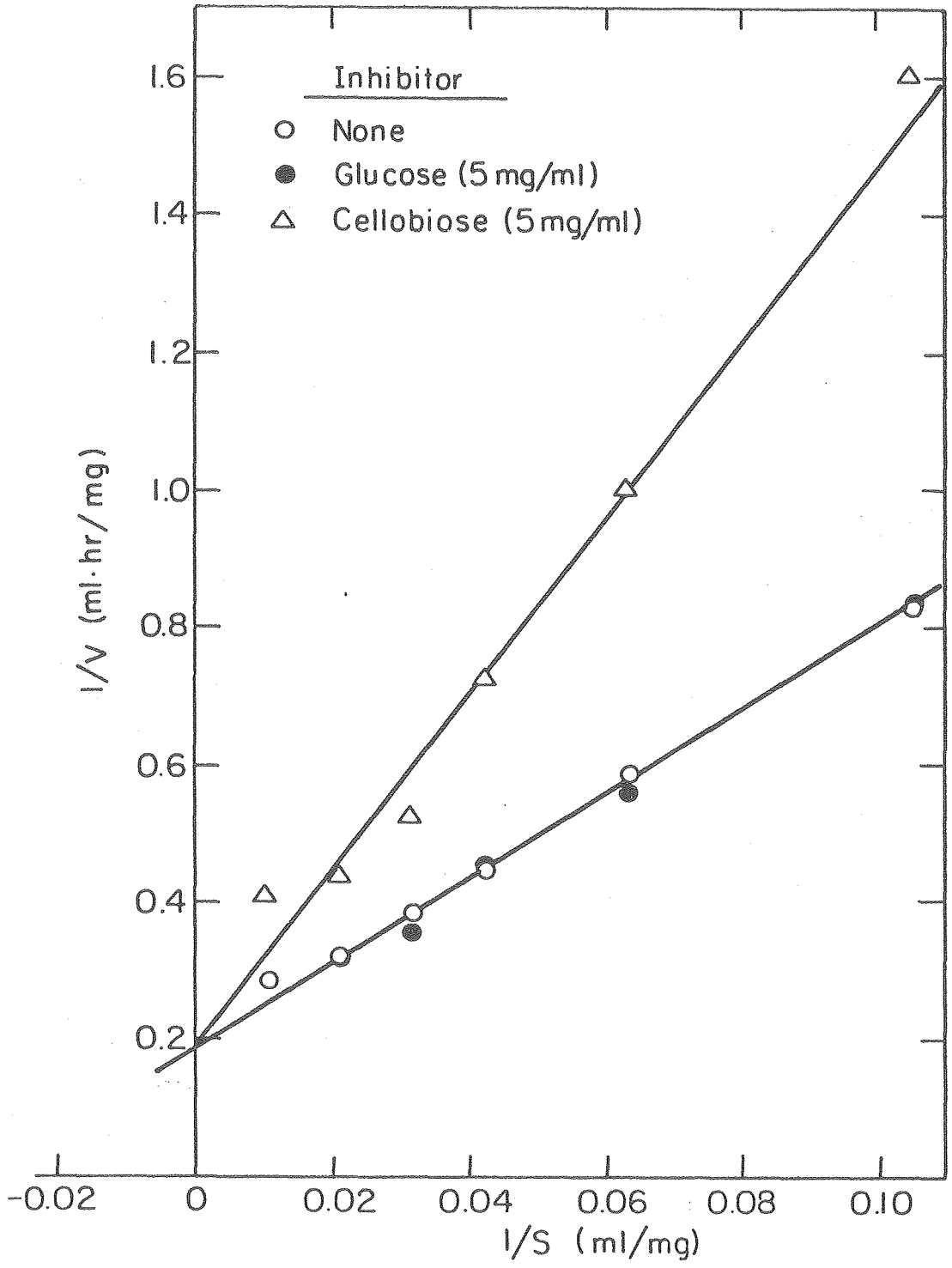

Fig. 4. Lineweaver-Burk plots of product (glucose and cellobiose) inhibition of Solka floc hydrolysis by T. viride cellulase.

enzyme with enzyme from another organism having a strong β-glucosidase component. On the basis of literature search, the fungus Botryodiplodia theobromae was selected for study.

A. Production and Characterization of β-Glucosidase

The growth medium for T. viride developed by Mandels and Weber (8) was found to be more suitable for growing B. theobromae than the reported medium (9) for the fungus. B. theobromae was found to grow with a maximum specific growth rate of 0.14 $hour^{-1}$ on glucose at 30°C pH 5.0. Enzyme induction on Solka-floc was optimum at 30°C pH 5.0. This fungus also produces a C_x activity which is about half of that of T. viride cellulase but a very low C_1 activity. Figure 5 shows the production of β-glucosidase activity at optimum conditions, employing 1% Solka-floc for induction at an aeration rate of 0.25 vvm in a 14-liter fermentor. β-glucosidase from B. theobromae was characterized for its heat stability and pH optimum. Heat denaturation of the enzyme was very small compared with that of almond emulsin, the only commercially available β-glucosidase. Figure 6 shows some heat denaturation of these two enzymes incubated for 40 hours at various temperatures without any substrate. pH optimum for B. theobromae β-glucosidase was examined and the results show (Fig. 7) that the enzyme is very stable at the range between 5.0 and 6.5.

B. Enzymatic Hydrolysis of Cellulose with Mixed Enzyme System.

The inhibitory effect of cellobiose largely depends on the activity of β-glucosidase in the cellulase used. Thus the effect

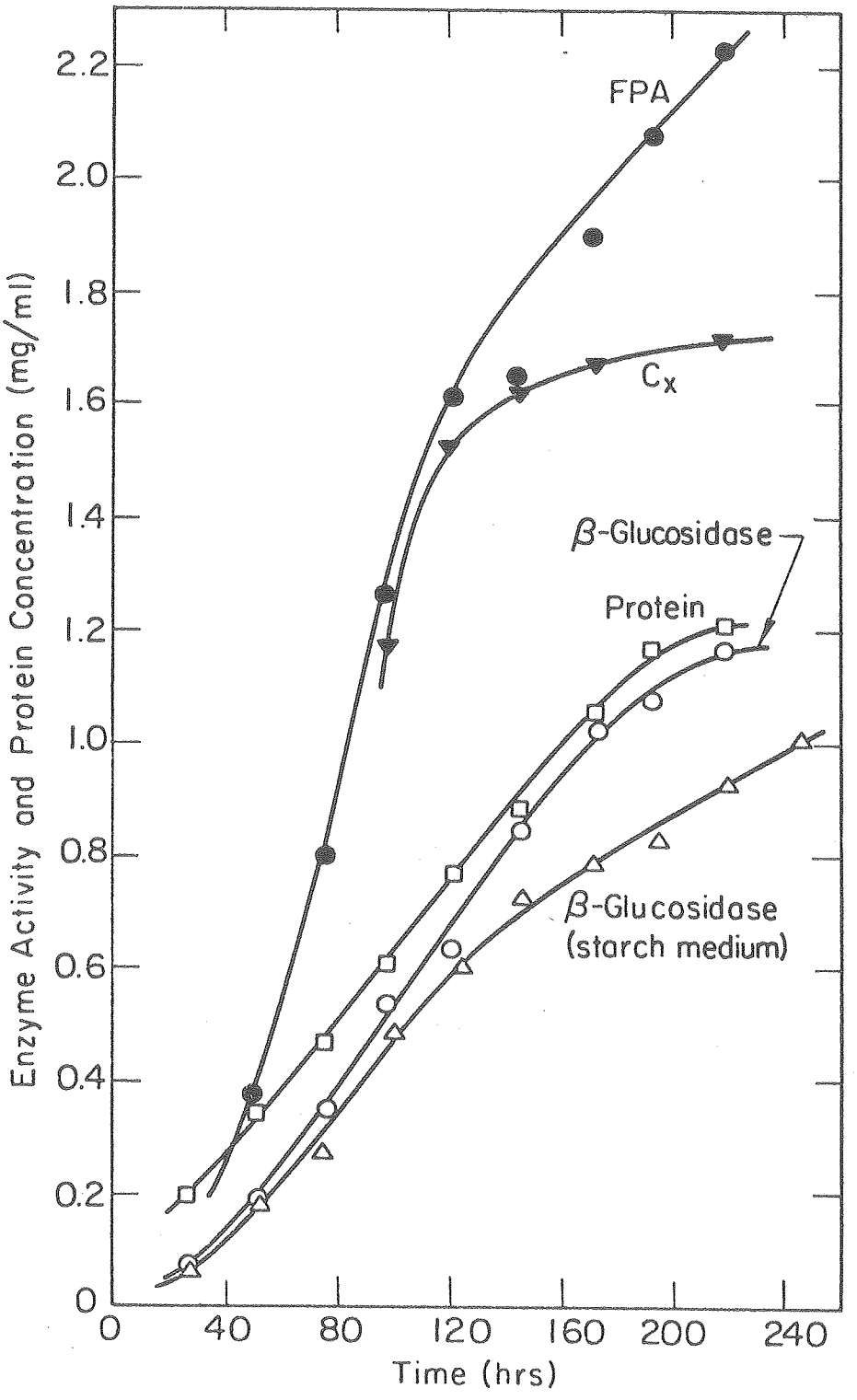

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Fig. 5. Enzyme production by B. theobromae at optimum pH (5.0) and temperature (30°C).

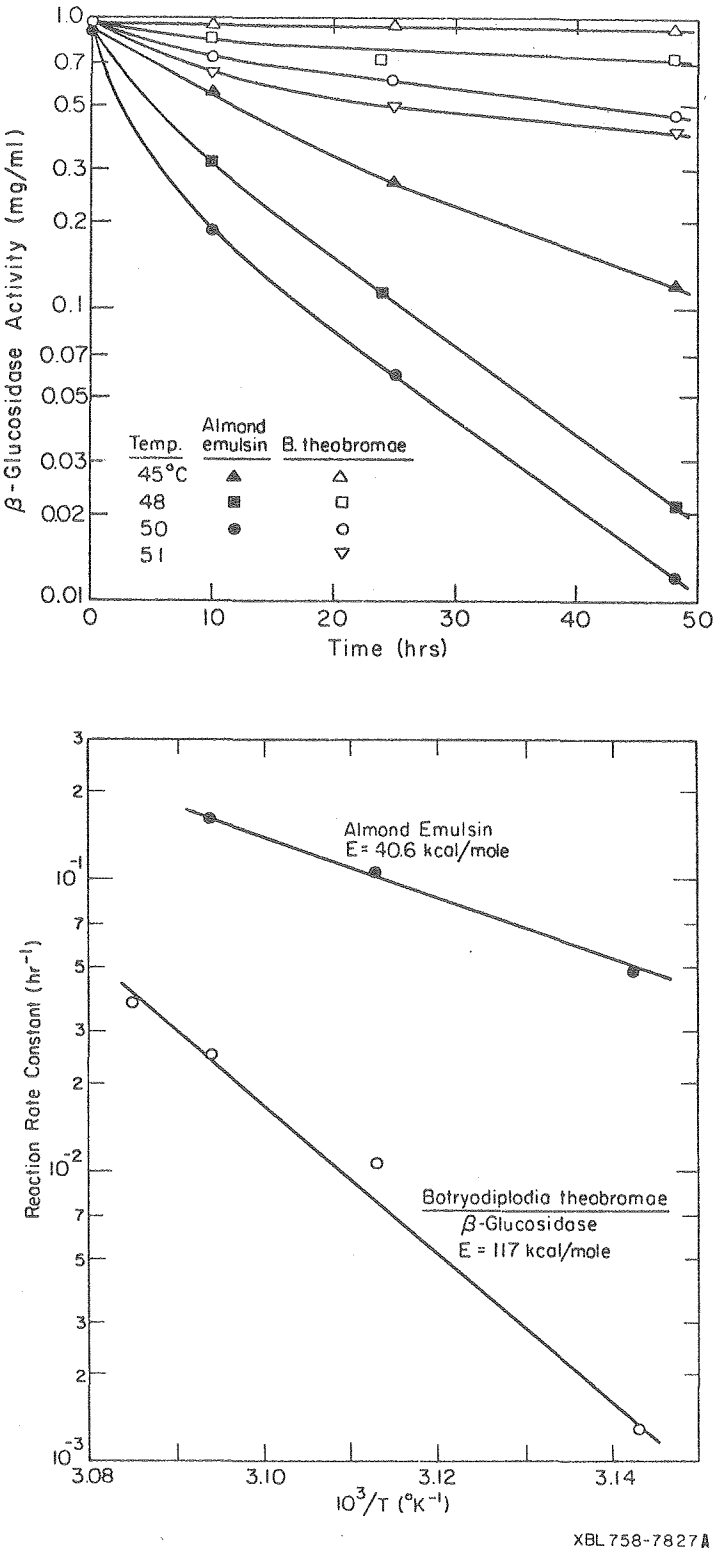

Fig. 6. Heat denaturation of β-glucosidases.

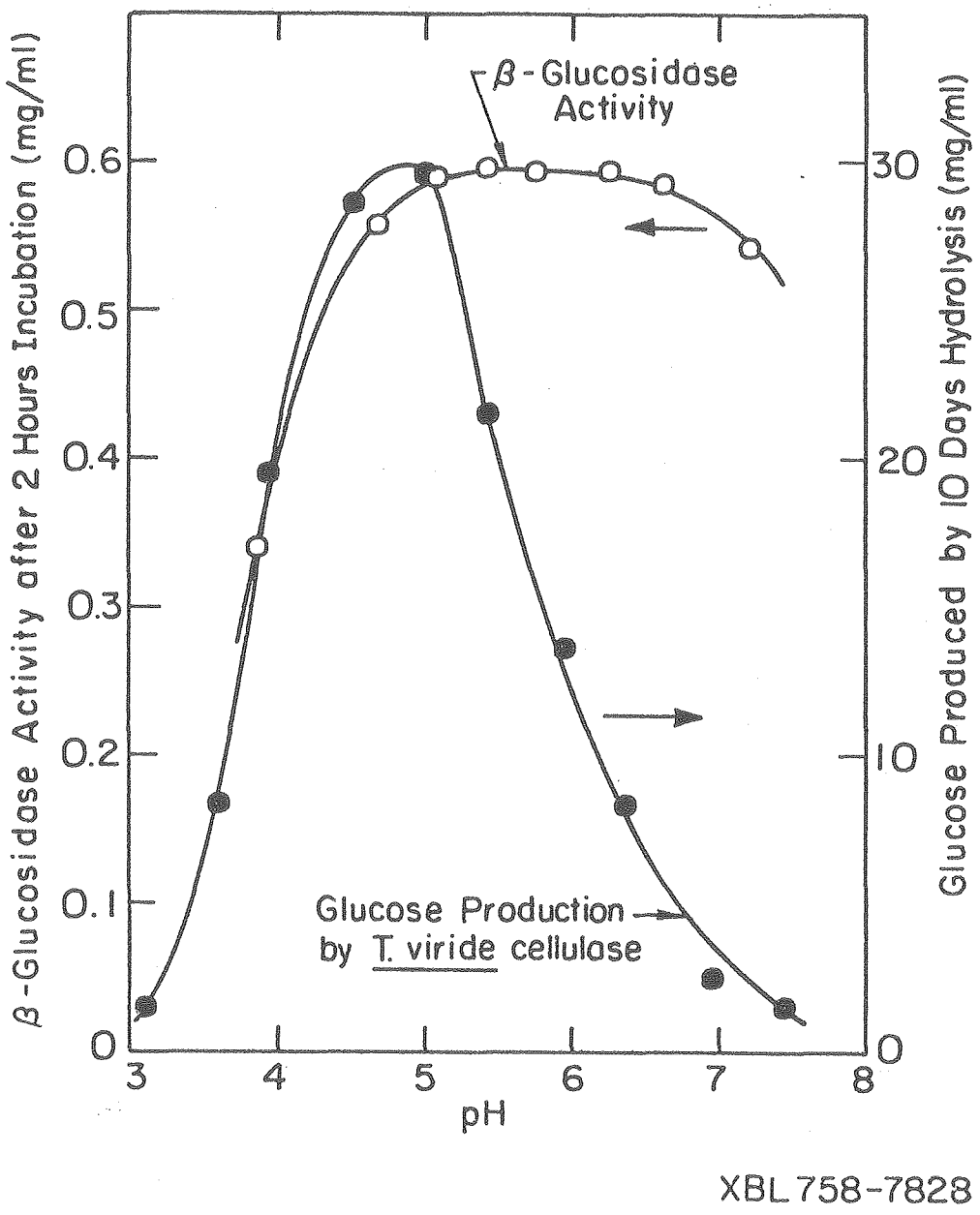

Fig. 7. pH optimum for β-glucosidase from B. theobromae.

of adding β-glucosidase to cellulase also depends on the β-glucosidase originally present in the cellulase from T. viride. Three stock enzyme solutions were employed: (1) a T. viride culture filtrate (T.V.E.), (2) a B. theobromae culture filtrate (B.T.E.), and (3) a vacuum evaporated concentration of B.T.E. having a filter paper activity (FPA) approximately equal to that of T.V.E. Activities of these solutions are shown in Table 2. By mixing these solutions in various proportions, a range of β-glucosidase and filter paper activities could be obtained. β-glucosidase activity was found to vary linearly with dilution. Hydrolysis was conducted on 5% suspensions (W/W) of 100-300 mesh Solka-floc at 50°C pH 5.0. Total reducing sugars were measured with the anthrone reagent and expressed as total equivalent moles of glucose (TGE). Glucose was measured separately with the glucostat reagent.

Figure 8 shows the results of hydrolysis of Solka-floc with an enzyme system consisting of mixtures of T. viride cellulase and B. theobromae β-glucosidase. Although the conversion to total reducing sugars was increased only by 8% by adding 30% of β-glucosidase, the increase of glucose yield was as much as 45%. Maximum total conversion occurred with 15-30% B.T.E. mixtures since the FPA decreased as the proportions of B.T.E. solution increased. Figure 9 shows a similar series of hydrolysis results on Solka-floc employing T.V.E. and concentrated B.T.E. mixtures over which the total FPA remained nearly constant. 15% addition of β-glucosidase provided 13% increase in total conversion and 59% increase in glucose productivity. 76% of the Solka-floc was hydrolyzed in 48 hours with added β-glucosidase compared to 63%

TABLE 2

	Filter Paper Activity	Beta-Glucosidase Activity
T.V. Enzyme	3.95-4.20	0.28-0.34
B.T. Enzyme	1.95	1.35
Concentrated B.T. Enzyme	3.80	3.02

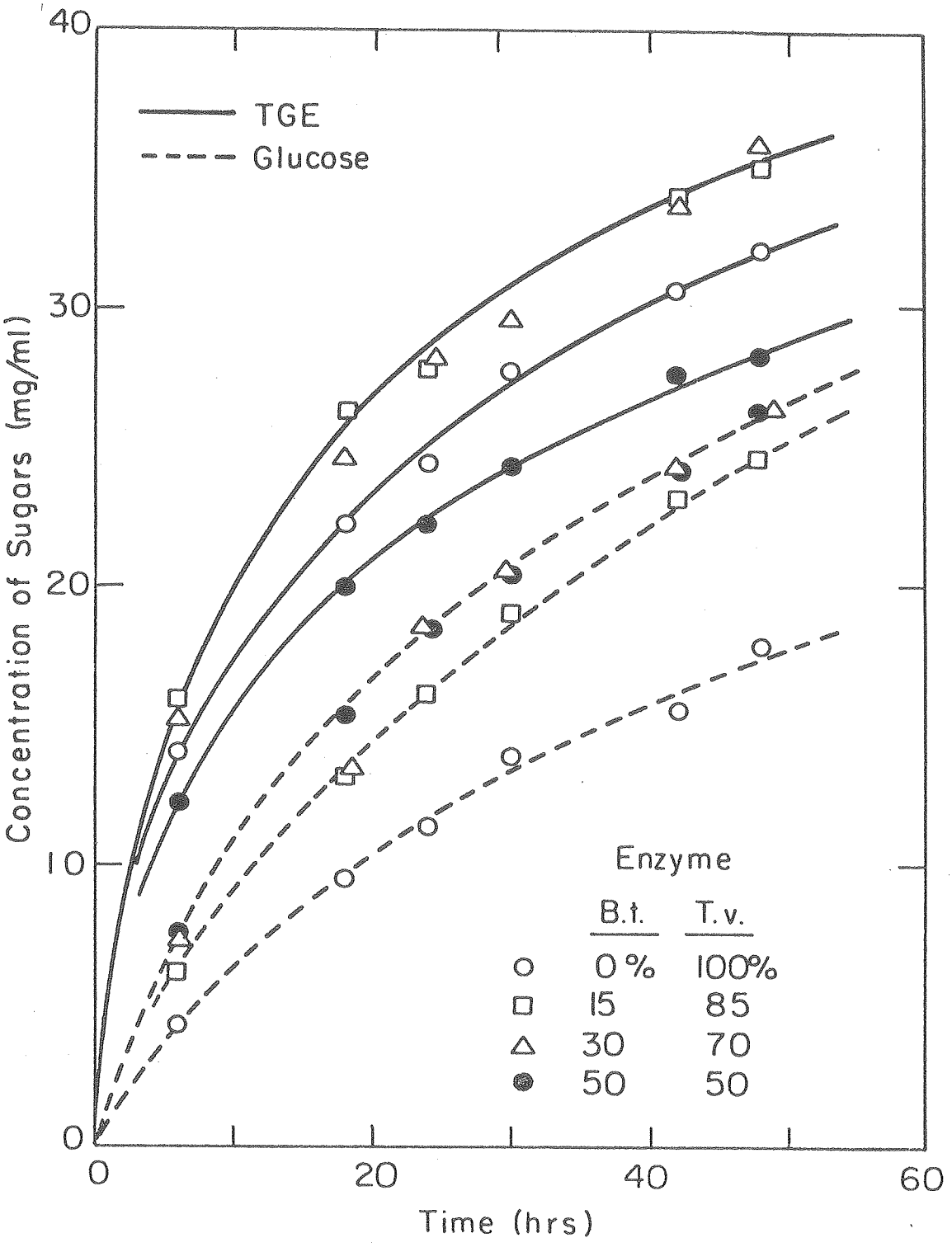

Fig. 8. Hydrolysis of Solka floc by various proportions of β-glucosidase (B. theobromae) and cellulase (T. viride).

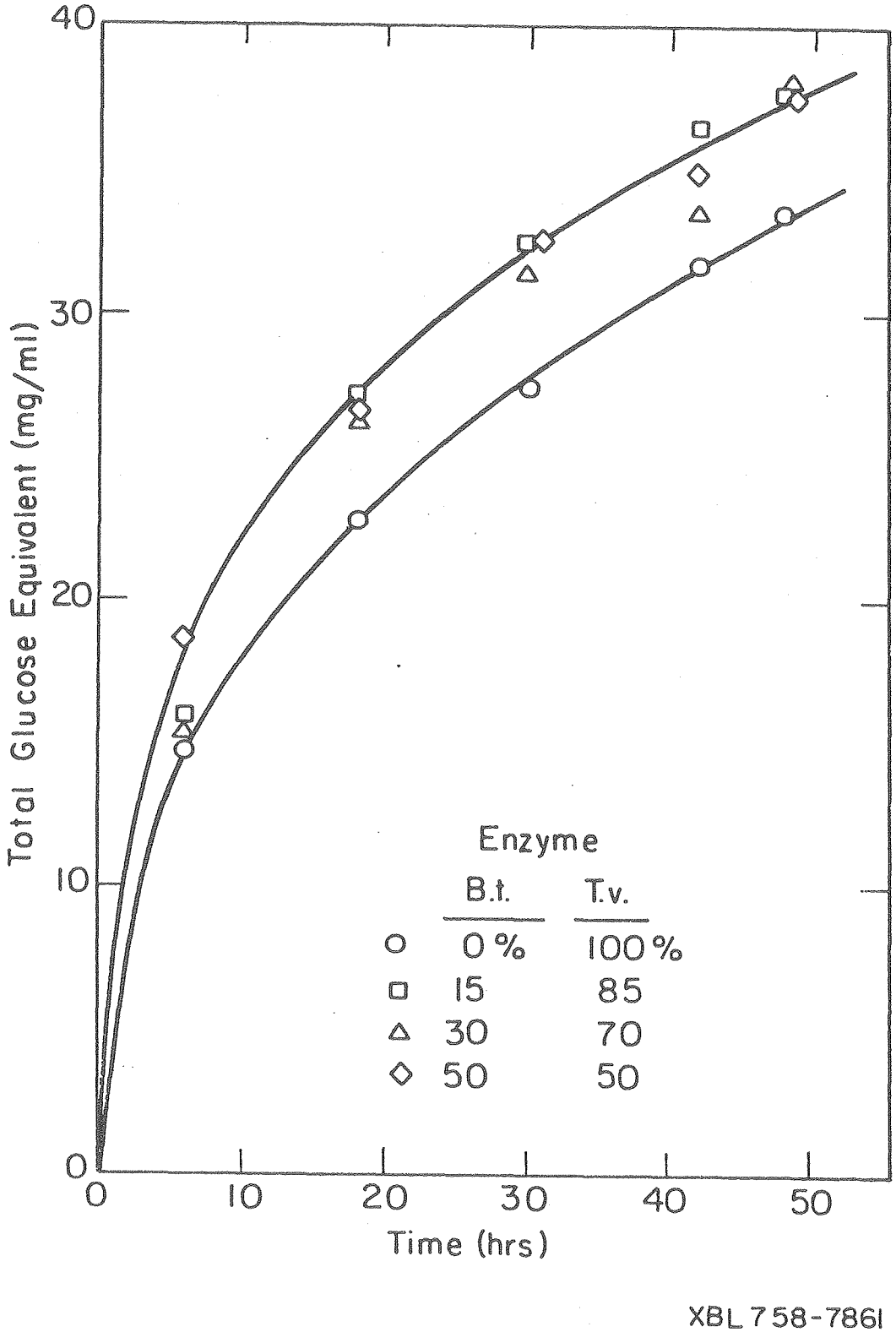

Fig. 9. Hydrolysis of Solka Floc by various proportions of concentrated β-glucosidase (B. theobromae) and cellulase (T. viride).

conversion with T.V.E. alone.

DISCUSSION

Figure 10 is a schematic flow diagram of the hydrolysis process proposed by Wilke, Yang and von Stockar (1). At the present time, the enzyme production costs represent about sixty percent of the total cost for producing sugars from waste cellulose. Attempts to make the whole process less expensive has been directed from two directions. The first approach is to improve the fermentation process. The second approach, which is the subject of research reported in this paper, is aimed at optimizing more complete hydrolysis of cellulose. Hydrolysis of cellulose may be made more effective by two ways:

I. The demonstrated relationship between lignin content by chemical removal and susceptibility of cellulose to hydrolysis suggests that removal of lignin would have a positive effect on increasing the efficiency of enzymatic hydrolysis of cellulosic wastes. Similar results were reported by Kirk and Harkin (10) where lignocellulose was subjected to microbial delignification. Comparison of the chemical and microbial methods shows that it is not significant by what method the lignin is removed. Our study is directed toward assessment of biological degradation as an alternative to chemical removal. As illustrated in Figs. 2 and 3, fermentation studies of Polyporus versicolor revealed that maximum specific growth rate occurred at 28°C and both the cell mass and enzyme (O-diphenol Oxidoreductase) yield factors were the functions of temperature. These results are important for being able to

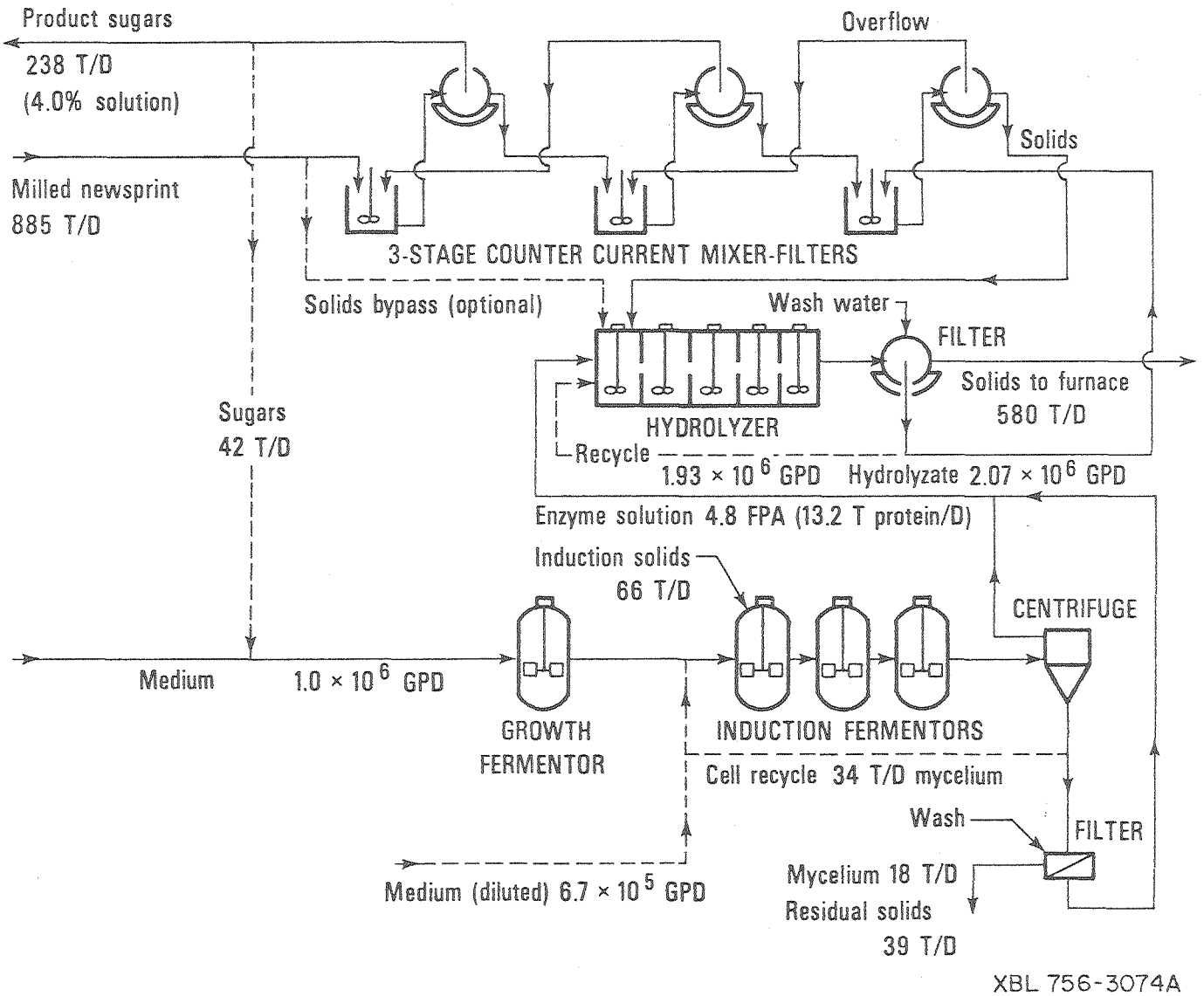

Fig. 10. Flow diagram of process for enzymatic hydrolysis of newsprint.

manipulate the fungus with respect to growth and enzyme production. The study has investigated several approaches to obtaining an extracellular lignin degrading enzyme system. Although results thus far have not been successful, such fundamental research is a necessary step in the understanding of enzymatic processes involved in lignin degradation.

More promising results were obtained in composting culture of ground wood with Pleurotus ostreatus which disintegrated lignin with practically no loss of cellulosic material. However, the economic feasibility of the composting method remains in doubt.

II. Cellobiose is an undesirable product for two reasons: 1) compared with glucose it is not readily fermentable to ethanol, 2) it has an inhibitory effect on the hydrolysis process. Table 1 clearly shows that cellobiose inhibits component C_1 or cellobiohydrolase. The inhibitory effect of cellobiose largely depends on the activity of β-glucosidase in the cellulase system used. From the studies of pH and temperature stability of β-glucosidase activity (Figs. 5, 6, and 7), it may be concluded that B. theobromae enzyme is compatible for mixing with T. viride cellulase. Mixing experiments (Figs 8 and 9) revealed that improved hydrolysis can be obtained by supplementing the β-glucosidase component of T. viride cellulase. It is not yet clear how best to utilize the β-glucosidase. One possibility under consideration is the use of an immobilized β-glucosidase rather than the mixed solutions of the native enzymes.

The spectrum of postulated mechanisms for cellulolysis has traversed from the early unienzymatic theory through a C_x-C_1

FIGURE 11

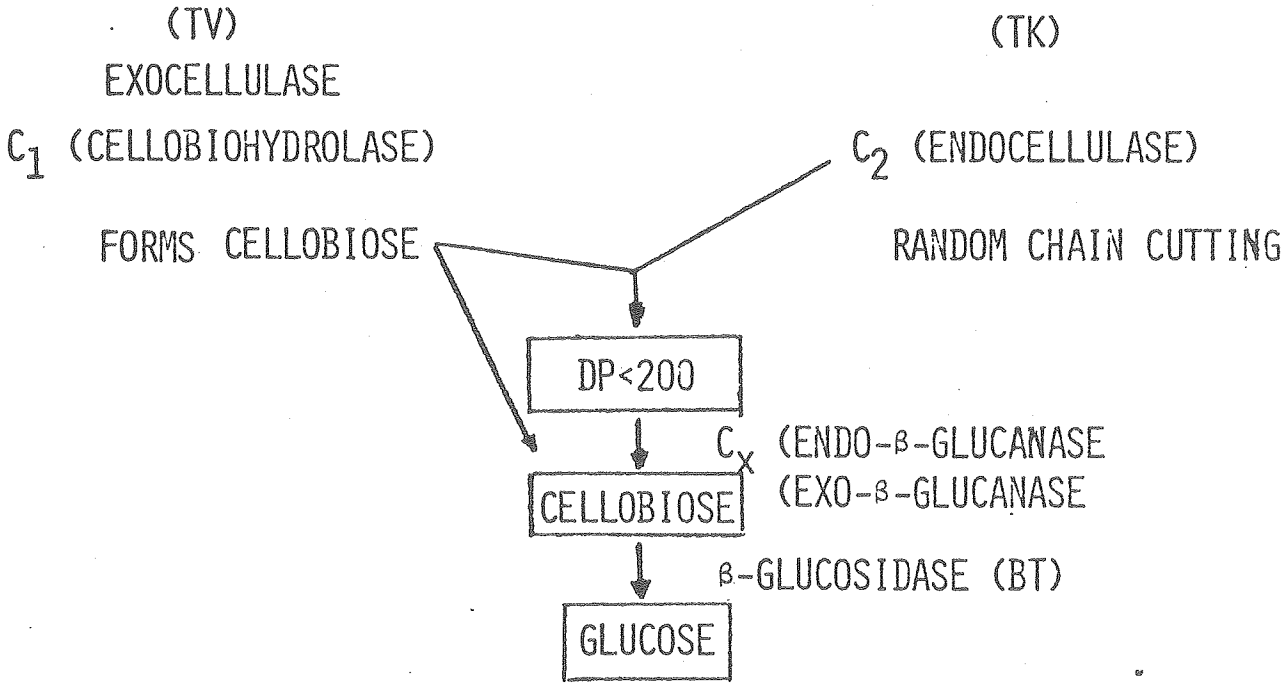

Fig. 11. Proposed mechanism of enzymatic hydrolysis.

enzymatic hypothesis to the current concept of a multi-enzyme system illustrated in Fig. 11. The complete enzyme system is comprised of two cellulases: component C_2 or endocellulase and component C_1 or cellobiohydrolase and three β1-4 dextrinases: exo- and endo- CM-cellulase and cellobiase or β-glucosidase. It may well be that some cellulolytic microorganisms have evolved pathways where all the five components are not necessarily required. The most effective system would be comprised of all the five components. The overall objective of the studies initiated at this laboratory is the development of a mixed enzyme system that will be a more effective agent for converting cellulosic wastes to glucose than cellulase obtained from a single source such as T. viride. Further improvement will be sought by supplementing T. viride cellulase plus β-glucosidase from B. theobromae with component C_2 or endo-cellulase. Preliminary studies reveal that for endo-cellulase Trichoderma koningii may be the organism of choice.

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