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THE EFFECT OF β-GLUCOSIDASE ON THE ENZYMATIC HYDROLYSIS OF CELLULOSE

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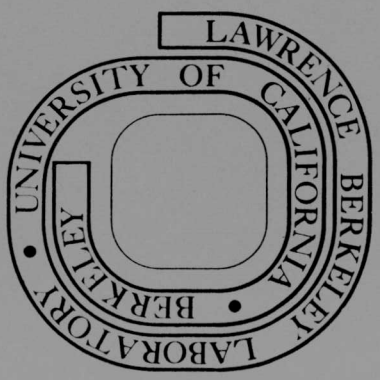

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THE EFFECT OF β-GLUCOSIDASE ON THE ENZYMATIC HYDROLYSIS OF CELLULOSE

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ABSTRACT

The effect of β-glucosidase on the hydrolysis of cellulose with a cellulase enzyme was studied. Cellulose is readily hydrolyzed to glucose and its oligomers by the cellulase enzyme which is produced by the fungus Trichoderma viride. However, cellobiose, whose concentration is usually 30 to 50% of the sugars produced, inhibits the hydrolysis reaction and the utilization of this disaccharide has not been established at present. β-glucosidase is capable of breaking down cellobiose to glucose so that higher conversion and higher yields of glucose would be expected.

Contrary to cellulase enzyme, β-glucosidase has been little studied, and the only commercially available β-glucosidase is the extract of almond, which is fairly expensive. Thus, of necessity, the production of microbial β-glucosidase was also investigated. After a review of the literature the fungus Botryodiplodia theobromae was selected as the most promising source of β-glucosidase for this work.

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A fourteen liter fermentor was used to produce β-glucosidase with this fungus with a reported medium, but better results were obtained with a medium used for the production of cellulase by Trichoderma viride. The optimum conditions for the batch fermentation were pH 5.0 and 30° C, and the β-glucosidase activity after seven days of fermentation was 1.0 mg/ml, which was a tentatively set target value in the present work. The continuous production of the enzyme was also investigated, and the results showed that a two-stage fermentation process (fungus growth on a glucose medium, followed by transfer to a cellulose medium for enzyme induction) worked as well as the two-stage production of cellulase from Trichoderma viride. The continuous process for β-glucosidase production proposed in the present work is probably the first reported.

The enzyme thus obtained was much more thermally stable than almond emulsin β-glucosidase. The enzyme was very stable in the pH range from 5.0 to 6.7, and the lower part of this optimum range overlaps the pH optimum of cellulase enzyme. The recoverability and the reusability of the enzyme from the hydrolyzate are very important factors to be considered. The level of β-glucosidase remaining in the hydrolyzate was about 70% of the original activity, which was high compared with the level of cellulase left in the solution (about 10 to 20% of the original). Like cellulase the enzyme was precipitated very easily by acetone (3 v/v) and the activity of the redissolved enzyme solution was almost the same as the original solution. However, the adsorption of the enzyme on cellulosic materials was not as strong as the adsorption of cellulase.

The interpretation of the experimental data on the hydrolysis reaction with mixed enzyme systems of β-glucosidase plus cellulase was not conclusive, primarily because of the inconsistent properties of the cellulase preparations from different batches of Trichoderma viride fermentation. The following points are the general conclusions of the effect of β-glucosidase on the hydrolysis of cellulose.

a) The effect of β-glucosidase is much more pronounced when the accessibility of the substrate is high, as with ball-milled Solka Floc, and when the cellulase activity of the original cellulase preparation is low.

b) The hydrolysis of newsprint, in which cellulose accessibility is low, by solutions with high cellulase activity is improved by use of a mixed enzyme system to a much lesser degree than for Solka Floc.

c) Mixtures containing 15 to 30% of Botryodiplodia theobromae culture filtrate mixed with Trichoderma viride filtrate produce a 10 to 20% increase in the total conversion of cellulose and a 20 to 40% increase in the glucose productivity in hydrolysis of newsprint.

Cost analysis of a tentative industrial scale process showed that use of a mixed enzyme system reduced the total production cost of sugars significantly in comparison to the corresponding process using only cellulase.

Introduction

All resources are limited. People have realized this solemn actuality for a long time, but it was probably just less than ten years ago when people came to think seriously of the catastrophe forecasted. The famed "The Limits to Growth", published by the M.I.T. project team in 1972, played an important role in enlightening laymen on the subject of the limitation of resources. The outbreak of the fourth Middle East war in 1974 mobilized the new political strategy of the Arab countries: the oil embargo by the oil exporting countries, which are mostly Arab. This strategy successfully showed people the world over that they could artificially create chaos, which would be abetted by the scarcity of the resources, at any moment when the political and/or social circumstances around the Arab countries were against them. As has been reported, most of the western European countries and Japan, whose economic affluence has been heavily based on the oil fields in the Arab countries. suffered tremendously from this new strategy.

If a country wants to be absolutely independent from other countries, she should have every resource in her own territory. However, the resources are not evenly distributed around the world. Hence, there have always been cooperative relations between the advanced countries, who do not have resources, and the underdeveloped countries, who do have resources, although the relations were sometimes ill balanced by unexpected political events.

People are now forced to consider their own survival methods. Nuclear energy, which has been regarded for a long time as a super

solution for the coming energy problem, has not developed as well as anticipated, and serious safety problems have appeared. Moreover, since most of the advanced countries except the United States do not produce uranium ore, there still exists an artificial chaos of scarce resources.

Solar energy is probably the one energy source which has been given most evenly to the human beings in the world. The most important means of utilizing solar energy is photosynthesis or bioconversion in green plants. Annually about 56×10^9 tons of carbon on land and 35×10^9 tons of carbon in the ocean are fixed by photosynthesis, primarily to cellulose (1). This abundant amount of cellulose, which is renewable and universally distributed throughout the world, has not been fully utilized yet. Waste cellulosic materials, such as newsprint, and residues of the agricultural and forest industries have piled up in vain. These potential resources are the materials which we should now utilize.

The utilization of cellulose would provide the following advantages, especially for the underdeveloped countries which do not have any particular resources:

a) They could provide raw materials by themselves.

b) It is almost unnecessary to depend upon the technology of advanced countries to operate the process.

c) The technology for the process is much more accessible than that for the development of nuclear energy, which is inseparably related to nuclear weapons.

Cellulose can be hydrolyzed to glucose, which in turn can be converted to chemicals, fuels (for example, ethanol) and food (such as single cell protein). Acid hydrolysis of cellulose has been investigated for a long time, but a large production of glucose from cellulose with this method has never been in progress. Enzymatic hydrolysis, which is a more recent entry to this field, has one short-coming: its slow reaction rate. Nevertheless, broad research work on the enzymatic hydrolysis of cellulose is being conducted today because of its reaction specificity and the following serious problems involved in the acid hydrolysis process: (1) The yield is about 50% due to the decomposition of glucose in the medium. (2) Acid medium is very corrosive so that the fixed cost of the process is high.

The technological feasibility of utilizing cellulose by enzymatic hydrolysis is not in doubt. Cellulose is readily hydrolyzed to glucose by the cellulase enzyme which is produced by the fungus Trichoderma viride. To be established is the economic feasibility of the process. Some research work concerning the economic feasibility has been already published; the investigation of mutants of the fungus which could produce higher cellulase activity (2), the improvement of the enzyme production stage using a continuous process (3), the recovery and reuse of the enzyme (3), the improvement of the hydrolysis reaction process using a membrane reactor (4), etc.

The main purpose of the present work is to improve the hydrolysis reaction by using a mixed enzyme system of cellulase and β-glucosidase, which is capable of breaking down cellobiose to glucose.

It is well known that the intermediate product of the hydrolysis reaction is cellobiose, which is a dimer of glucose and inhibits the main reaction. Thus, with the mixed enzyme system, cellobiose can be converted to glucose so that the reaction rate would be increased, as would the final conversion. This subject is discussed in Part II in this dissertation.

The other purpose of this work is, of necessity, to establish a production method for β-glucosidase. Contrary to the cellulase enzyme, little is known about the effective production of β-glucosidase. Although some laboratory scale experiments have been published on the production of the enzyme, process development of the production of β-glucosidase has yet to be done. Thus, as shown in Part I, we chose the fungus Botryodiplodia theobromae as the source of the enzyme, and bench scale experiments were carried out to develop the production of β-glucosidase.

Finally, the economic considerations of the use of a mixed enzyme system are discussed in Part III.

Literature Cited

1) Hottel, H. C. and Howard, J. B., New Energy Technology--Some Facts and Assessments, p. 346, M.I.T. Press (1971)

2) Mandels, M., Weber, J. and Parizek, R., Appl. Microbiol., 21, 152 (1971)

3) Mitra, G. and Wilke, C. R., Biotech. Bioeng., 17, 1 (1975)

4) Ghose, T. K. and Kostick, J. A., Biotech. Bioeng., 12, 921 (1970)

PART I

PRODUCTION OF FUNGAL β-GLUCOSIDASE AND ITS CHARACTERIZATION

1. Previous Work

1.1 Source of β-Glucosidase

β-Glucosidase has been classified as a transglycosylase, i.e., an enzyme which transfers a glycosyl residue from the donor glucoside to an acceptor. Usually the acceptor is water and thus the action of the enzyme is generally hydrolytic. The substrate specificity of β-glucosidases from various sources has been reported to vary considerably. The most well-known example was obtained by Jermyn (1,2,3), who purified an aryl-β-glucosidase, which had no cellobiose activity, from a culture filtrate of the fungus Stachybotrys atra. He suggested the existence of two different kinds of β-glucosidase: one with only aryl-β-glucosidase activity and one with a broad activity toward different β-glucosidases.

The commonly used standard assay for β-glucosidase is based on the hydrolysis of salicin. However, quite often an alternate assay is used in which salicin is replaced by p-nitrophenyl-β-D-glucopyranoside. In the present study a further modification of the usual assay procedure is employed in which cellobiose, rather than salicin, is the substrate. In the use of literature on β-glucosidase care must be taken to recognize the assay procedure on which the reported activity is based. In this chapter the term "celliobase" will be used to designate enzyme activity based solely on cellobiose as a substrate. In other cases the term "β-glucosidase" will refer to enzyme activities based on various substrates, which are designated in the text. In subsequent

chapters of this report the term β-glucosidase" is used to mean "cellobiase".

1.1.1 Vegetable β-Glucosidases

Commercially available β-glucosidase is obtained from sweet almond. Although some properties of this enzyme have been reported in the literature (4,5,6,7), little is known about the cellobiase activity of the enzyme. Crook and Stone (8) briefly mentioned the high cellobiase activity of β-glucosidase from sweet almond in comparison with the enzyme from Aspergillus niger.

It was also reported that the β-glucosidase showed hydrolytic activity towards α-D-galactosides, β-D-xylosides, β-L-arabinosides, and α-D-mannosides, but it was inert toward α-glucosides (4). The optimum pH range for the hydrolysis of o-nitrophenyl glucoside, which is a commonly used substrate for the assay, was reported to be 5.2 to 6.0 (6).

Barley flour is one of the potential sources of this enzyme. Anderson et al. reported a purification method and its general characteristics (9). Extraction of the β-glucosidase from barley flour was accomplished by either 3 percent (w/v) potassium chloride or 0.2 M sodium acetate buffer, pH 5.0, at 18--20°C for two hours. Distribution of various β-glucosidase activities in the fractions obtained by precipitation with ammonium sulphate is shown in Table 1.1. The maximum allowable temperature was reported as 50--55°C. The effect of pH on the enzyme activity was not reported, but all of their measurements of the activity were carried out at pH 5.0.

Table 1.1. Distribution of β-glucosidase in the fractions obtained by precipitation with ammonium sulphate (9).

Saturation with ammonium sulphate	0-35%	35-50%	50-65%	65-80%
Yield (from 1 kg of flour)	2.6g	2.3g	2.3g	2.0g
Protein N content	2.8%	5.9%	4.5%	1.7%
Activity				
Cellobiase	8.7	30.2	54.5	69.3
Salicinase	3.1	3.1	6.1	7.5
Phenyl-β-glucosidase	3.2	2.4	3.8	8.8
Cellodextrinase	5.4	10.1	13.3	33.6
Laminarinase	9.6	15.2	28.5	28.3
Lichenase	2.4	2.9	10.6	16.2

*Activities are expressed as percentage hydrolysis/mg of protein.

Anderson et al. also reported two separation methods of the enzyme other than ammonioum sulphate precipitation, though both failed. Acetone precipiation at -12° C, using up to 50% (v/v) of acetone, was unsuccessful, apparently due to the small amount of acetone used. Adsorption of the β-glucosidase by powdered cellulose was investigated (details not reported), but no significant change in enzyme activity was observed. (These methods were examined for the enzume used in the present work and the results will be discussed in Chapter 3.)

Sandgren and Enebo (10) showed that the extracts of barley caused random degradation of ethylhydroxyethyl-cellulose, the activity being optimum at pH 5.0. Although this activity was ascribed to "cellulase", this enzyme has not been used for the actual cellulose

hydrolysis. In the present work, some attempts were made to use the enzyme for the hydrolysis of cellulose. Typical examples were shown in Figs. 1.1 and 1.2. Apparently the use of the barley extracts gave higher conversion and higher glucose productivity, which was attributable to the cellobiase activity of the enzyme. The use of this enzyme, however, was not thoroughly investigated because of the uncertainty encountered in estimating the production cost and availability of barley flour, which might be affected by the supply-demand relationship. When the production scale of the β-glucosidase is not large, this source is very attractive because it is readily available, less expensive than almond emulsion and the preparation method is fairly simple.

1.1.2 Bacterial β-Glucosidases

Han and Srinivasan (11) reported the use of β-glucosidase of Alcaligenes faecalis which was grown on a lactose medium. The enzyme hydrolyzed both cellobiose and aryl-β-glucoside (p-nitrophenyl-β-D-glucopyranoside), but only slightly hydrolyzed salicin. The difference between the results of p-nitrophenyl glucoside and salicin was explained by the strong electronegativity of the nitro group in p-nitrophenyl glucoside.

Table 1.2 shows the results of hydrolysis of various glucosides by the β-glucosidase of Alcaligenes faecalis. The reaction mixture containing 2.0 ml of phosphate buffer, pH 6.5, 0.5 ml of 0.1 M substrate, and 0.5 ml of the enzyme solution was incubated for 20 minutes at 40° C, and the amount of glucose liberated was determined by the Glucostat reagent. Since the rate of the hydrolysis increased as the

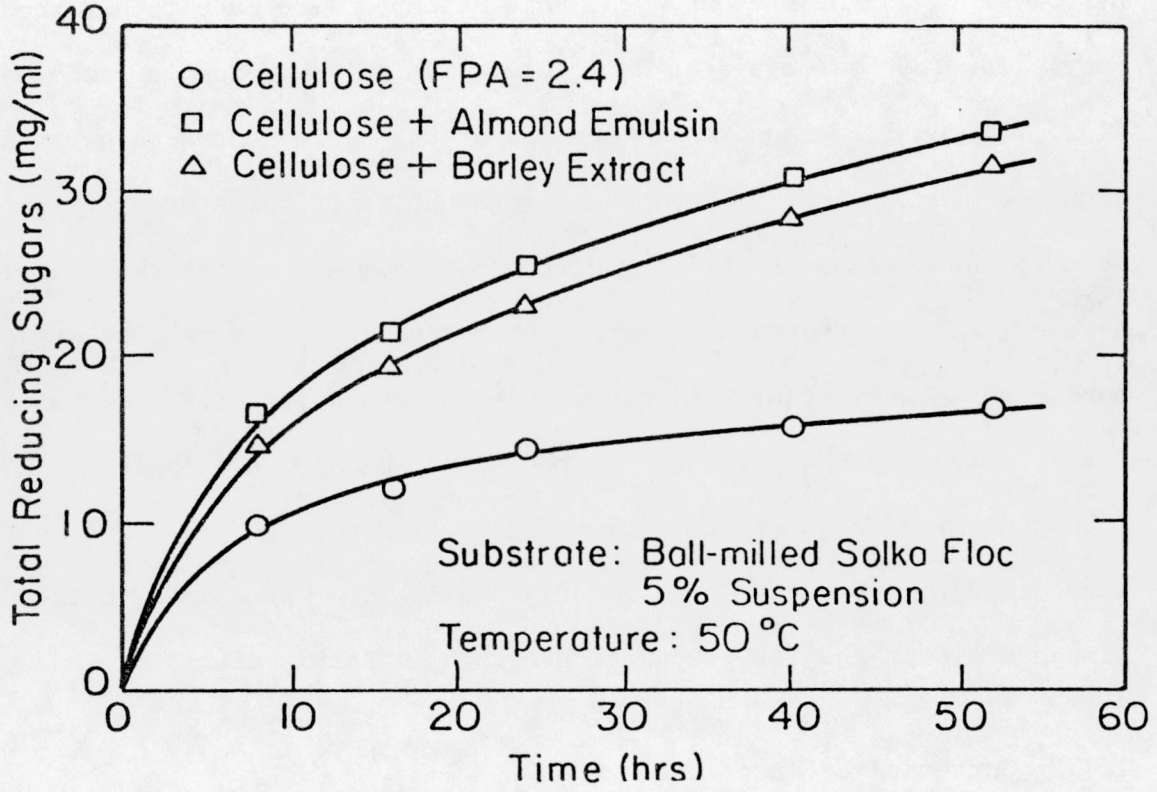

Figure 1.1. Hydrolysis of ball-milled Solka Floc by cellulose with and without vegetable β-glucosidase.

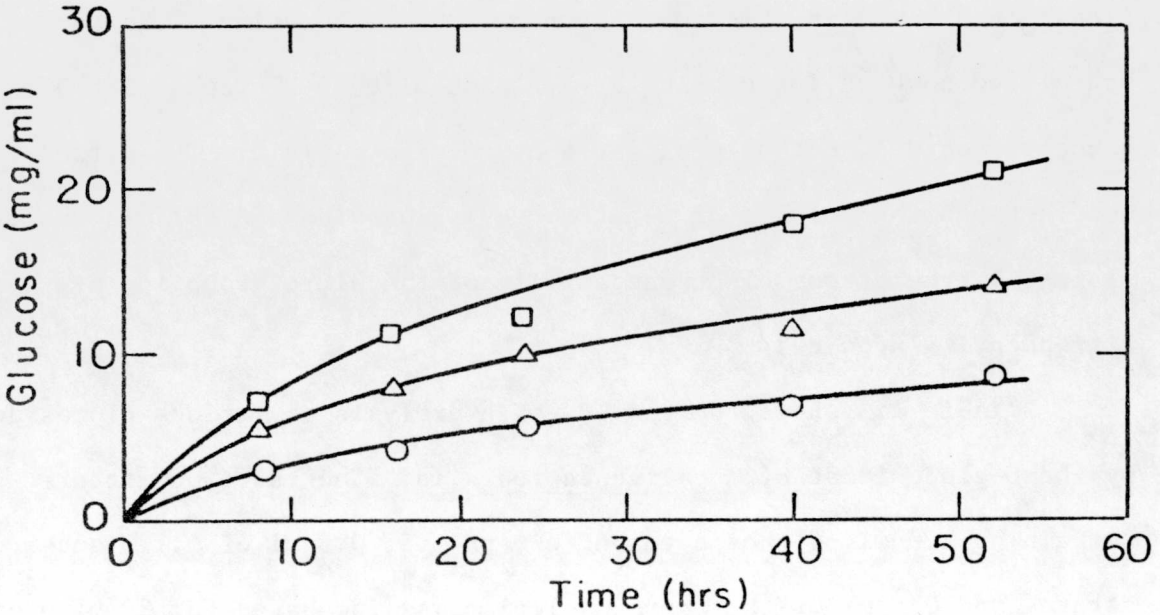

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Figure 1.2. Hydrolysis of ball-milled Solka Floc by cellulose with and without vegetable β-glucosidase.

Table 1.2. Hydrolysis of various glucosides by the β-glucosidase of Alcaligenes faecalis (11).

Substrate	Type of linkage	Glucose liberated*
Cellobiose	Glucoside (β-1,4)	0.48 mg/ml
Cellotriose	Glucoside (β-1,4)	0.30
Cellotetraose	Glucoside (β-1,4)	0.24
Laminaribiose	Glucoside (β-1,3)	0.31
Sophorose	Glucoside (β-1,2)	0.20
Gentiobiose	Glucoside (β-1,6)	0
Lactose	Galactoside (β-1,4)	0.04
Sucrose	Glucoside (β-1,4)	0.01
Maltose	Glucoside (α-1,4)	0
Melibiose	Galactoside (β-1,6)	0
Methyl-β-D-glucoside		0
Salicin		0.02

*Measured after incubation for 20 minutes at 40° C.

degree of polymerization of β-1,4-glucosides decreased, the enzyme was clearly classified as β-glucosidase, not β-glucanase, according to the criteria proposed by Reese (12).

Some experimental data in our laboratory showed that the growth rate of this organism was very slow, and the cell concentration in the stationary phase was low. These disadvantages for the enzyme production may be improved by changing the medium or operating conditions, but the serious problem in using this enzyme is that the optimum pH range is from 6.2 to 7.8. At pH 5.0, which is the operating condition of cellulose hydrolysis by the cellulase system, the enzyme activity

went down to almost zero after two hours incubation at 30° C.

1.1.3 Fungal β-Glucosidase

The cellobiase activity of Trichoderma viride cellulase was reported to be small (13), and probably for that reason the characteristics of the enzyme had not been extensively studied until Berghem and Petterssen (14) investigated the properties of the enzyme. The substrate specificity of the isolated β-glucosidase was more like that of the aryl-β-glucosidase and a previously purified β-1,4-glucan cellobiohydrolase from the culture filtrate of Trichoderma viride. Although the details were not given, the latter enzyme activity was increased at least two-fold by adding the β-glucosidase.

Crook and Stone (8) investigated the cellobiase activity of the β-glucosidase from Aspergillus niger and showed its ability to hydrolyze cellobiose at pH 5.0 and 40° C. During the hydrolysis of cellobiose to glucose by the enzyme, they observed a transient formation of a number of glucose oligosaccharides, in which three disaccharides and two trisaccharides were isolated and identified. They also reported a similar formation of oligosaccharides during cellobiose hydrolysis with the almond emulsin β-glucosidase. The formation of oligosaccharides was interpreted in terms of an enzyme-catalyzed transference of glucosyl residues from cellobiose to suitable acceptors, and since the water concentration is usually much higher than that of the glucoside acceptors, the hydrolysis reaction is predominant. The activity of cellobiase of Aspergillus niger was reported, though not quantitatively, to be lower than that of

almond emulsin.

Enormous amounts of work have been done with a strain of Stachybotrys atra by Jermyn and co-workers (1,2,3,15) to investigate its β-glucosidase along with the cellulase produced by the fungus. Jermyn concluded from his experimental results that the extracellular β-glucosidase obtained from this fungus had a very small affinity for alkyl-β-D-glucoside, i.e., cellobiose could not be hydrolyzed by using this enzyme (2). Later, Youatt duplicated Jermyn's experiments with some modifications in the medium composition and obtained an intracellular β-glucosidase which was capable of hyrolyzing cellobiose and cellulose oligosaccharides (15). Some of his data are shown in Figs. 1.3 and 1.4. The effect of temperature on the enzyme activity was not reported.

Purification and specificity of β-glucosidase of Fusarium solani was reported by Wood (16). A cell-free culture filtrate of the fungus was obtained after incubation for 25 days at 27°C. o-Nitro-β-glucoside and cellobiose were both used as substrates for β-glucosidase activity measurement. Contrary to the result with Stachybotrys atra β-glucosidase, no evidence for the non-identity of nitrophenyl-β-D-glucosidase and cellobiase activities could be found, either by heat treatment, gel filtration on Sephadex G-100 or by isoelectric focusing. The cellobiase activity of the culture filtrate was not given, probably because it was not so attractively high.

Eberhart and co-workers (17,18) showed the existence of at least two β-glucosidases in the mycelial extract of Neurospora crassa grown in Vogel's minimal medium (19) plus 1% glucose and 1% carboxymethylcellulose for up to eight days at 30°C. One of those enzymes was

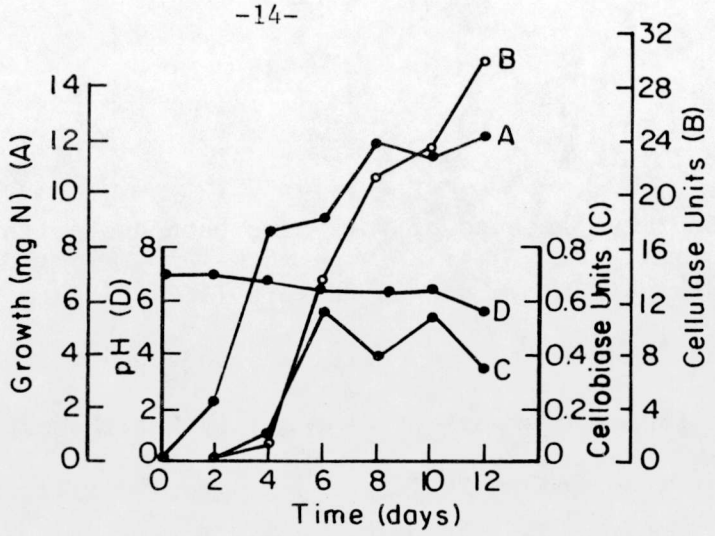

Figure 1.3. Growth and enzyme production of *Stachybotrys atra* on cellulose. A, growth (mg N); B, cellulase activity of medium; C, cellobiase activity of mycelium; D, pH of medium. Redrawn from (15).

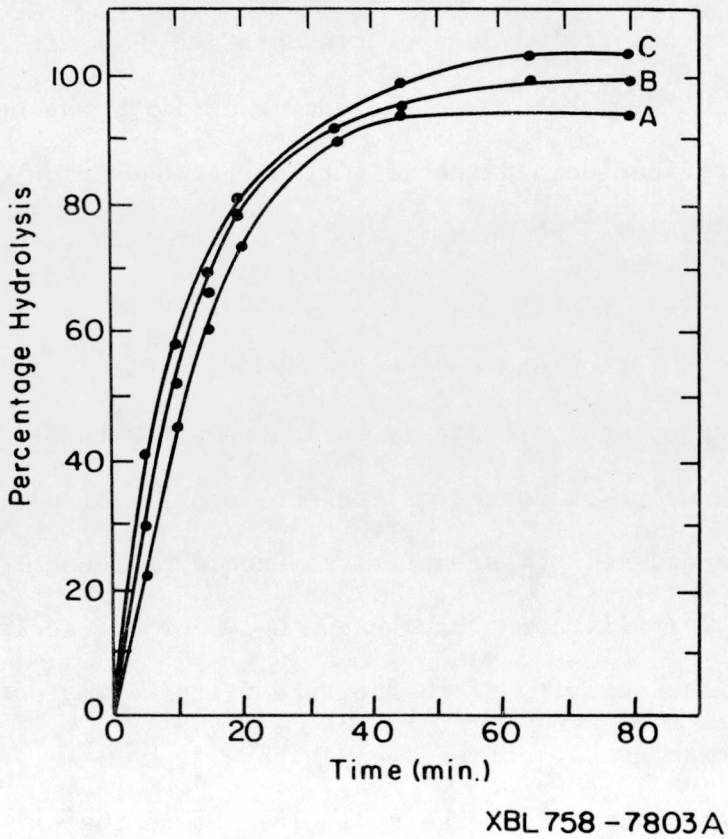

Figure 1.4. Hydrolysis of cellobiose (A), cellotetraose (B), and a cellodextrin of chain length 11 units (C) by *S. atra* cellobiase. Redrawn from (15).

rather thermostable and it hydrolyzed p-nitrophenyl-β-D-glucoside, salicin, phenyl-β-D-glucoside, esculin and methyl-β-D-glucoside; thus it was supposed to be an aryl-β-glucosidase. The other one was thermolabile but could hydrolyze cellobiose. These two enzyme activities could not be separated, which suggested that one enzyme was present possessing these two related properties.

Botryodiplodia theobromae is a widespread fungus in tropical areas. Findley et al. (20) reported in 1939 a loss in toughness and bending strength of 45 and 25 percent respectively of the wood of Obeche (Triplochiton scleroxylon) heavily stained by this fungus. The fungus, however, had been less well studied until Umezurike (21) investigated the effect of Botryodiplodia theobromae on the wood of Bombax buonopozense, a large tropical forest tree which becomes heavily stained by the fungus under humid conditions, after removal of the bark from the felled tree. Extensive studies on the enzyme system of this fungus have been published by Umezurike (22-25). Though most of his experiments were done by measuring the β-glucosidase activity with p-nitrophenyl-β-D-glucopyranoside and hence it is difficult to evaluate the feasibility of utilizing the enzyme from the fungus, there was evidence which showed a certain level of cellobiase activity excreted by the fungus.

The fungus also produced cellulase enzyme, the activity of which was measured only with CMC as a substrate. Using dialyzed filtrate, pH 5.0, from cultures of Botryodiplodia theobromae grown on cellulose as a carbon source, glucose, cellobiose and probably cellotriose were identified by paper chromatographic separation as products of cellulose degradation. When the dialyzed culture filtrates were heated at

100° C for 15 minutes before use, only cellobiose was detected as a product and the heated culture filtrate did not hydrolyze added cellobiose (21). As will be discussed later, this fungus was chosen for the production of β-glucosidase (cellobiase) in the present work. The characterizing studies of the enzyme of the fungus presented by Umezurike will be reviewed in the following section.

1.1.4 Summary

Contrary to the vast amount of work associated with cellulase enzyme, little is known about β-glucosidase (cellobiase). In their review of β-1,4-glucan glycosyl hydrolase (i.e., cellobiase) Barras et al. (26) cited only the work on Trichoderma viride, Aspergillus niger and Stachybotrys atra, noting that while exohydrolases for β-1,3- and α-1, 4-glucans have been reported from a wide variety of sources, few β-1,4-glucan exo-hydrolases are known, either because of difficulties in their detection or because of a real scarcity in nature.

Table 1.3 summarizes the microbial β-glucosidases reviewed in this chapter which have, or might have, cellobiase activity. The major difficulty in selecting the organism for the present work was that there has been no commonly established assay method of cellobiase activity and hence the activities obtained by different groups could not be compared. Consequently, in the summary of Table 1.3, the cellobiase activity was expressed less quantitatively.

Table 1.3. Microbial β-Glucosidase.

Microorganism	Cellobiase Activity[a)]	Enzyme Location	Growth Period	Literature
Alcaligenes faecalis	+++	Intracellular	2 days	11
Trichoderma viride	+	Extracellular	5 days at 30° C	13
Aspergillus niger	++	Extracellular		8
Stachybotrys atra	++	Intracellular	6 days at 28° C	15
Fusarium solani	+	Extracellular	25 days at 27° C	16
Neurospora crassa	++	Intracellular	8 days at 30° C	17,18
Botryodiplodia theobromae	++	Extracellular	1 to 2 weeks at 25° C	22

a) + Cellobiase activity was detected but small.
++ Cellobiase activity was probably not small.
+++ Cellobiase activity was reported high.

Alcaligenes faecalis, the only known bacterial source of the enzyme, would provide a fairly high cellobiase activity, but two shortcomings of this organism are: 1) the enzyme is intracellular and 2) the optimum pH for the enzyme is too high for the cellulase system to which the enzyme would be added. Those fungi which produce intracellular β-glucosidase would better be deleted from the list because of relatively high cost of extraction of the enzyme from the fungal mycelia.

Aspergillus niger and Botryodiplodia theobromae thus remained in the list; the former has been well investigated, but it is not regarded as a good β-glucosidase producing fungus. The studies of β-glucosidase from the latter fungus are rather recent, and only a few reports are available. There may be some unknown factors which could be determined by more extensive investigations. Therefore, Botryodiplodia theobromae was chosen in the present work to exploit the possibility of producing high activity cellobiase enzyme.

1.2 Production and Characterization of Botryodiplodia theobromae β-Glucosidase

1.2.1 Growth Media

To grow the fungus, Umezurike (21) reported the use of a liquid medium based on those Reese and Levinson (27), and Kooiman et al. (28). It consisted of 1.4 g KH_2PO_4; 0.2 g $MgSO_4 \cdot 7H_2O$; 2.1 g $NaNO_3$; 0.1 g yeast extract (Difco); and 10 g glucose per liter, adjusted to pH 6.2. The induction of the enzyme was carried out by replacing glucose by cellulosic materials such as wood powder and powdered pulp. Umezurike

(21) studied the effect of starch and ethanol-soluble materials of wood powder on the production of cellulolytic enzymes using as carbon sources a) unextracted wood powder (Bombax), b) wood powder extracted with 80% ethanol, and c) wood powder after ethanol extraction and starch depletion. His results showed that although more fungal growth took place when the wood contained starch and sugar, the production of cellulase was delayed until these compounds were used up. The production of cellobiase activity, however, was not only affected but enhanced by the presence of starch in the wood powder.

The effect of glucose on the production of C_x-cellulase and β-glucosidase was also reported by Umezurike (22). Mycelium of B. theobromae, grown in a glucose medium, was resuspended in media containing 1% cotton flock and up to 0.25 M (i.e., 4.5%) glucose. At weekly intervals samples were withdrawn aseptically, dialyzed and assayed for the enzyme activities. The results shown in Fig. 1.5 indicated that the addition of glucose to cellulose cultures repressed enzyme synthesis, and the degree of repression diminished as glucose was used up, suggesting that both induction by cellulose and repression by glucose are involved in regulation of C_x-cellulase and β-glucosidase synthesis.

Umezurike (22) found that the fungus grew better on media containing sodium nitrate rather than ammonium salt as a nitrogen source. However, β-glucosidase production was greater in ammonium salt media than in sodium nitrate media. These differences were, he suggested, partly due to the effect of pH of the media, β-glucosidase synthesis being greater when the pH of the media was slightly acidic.

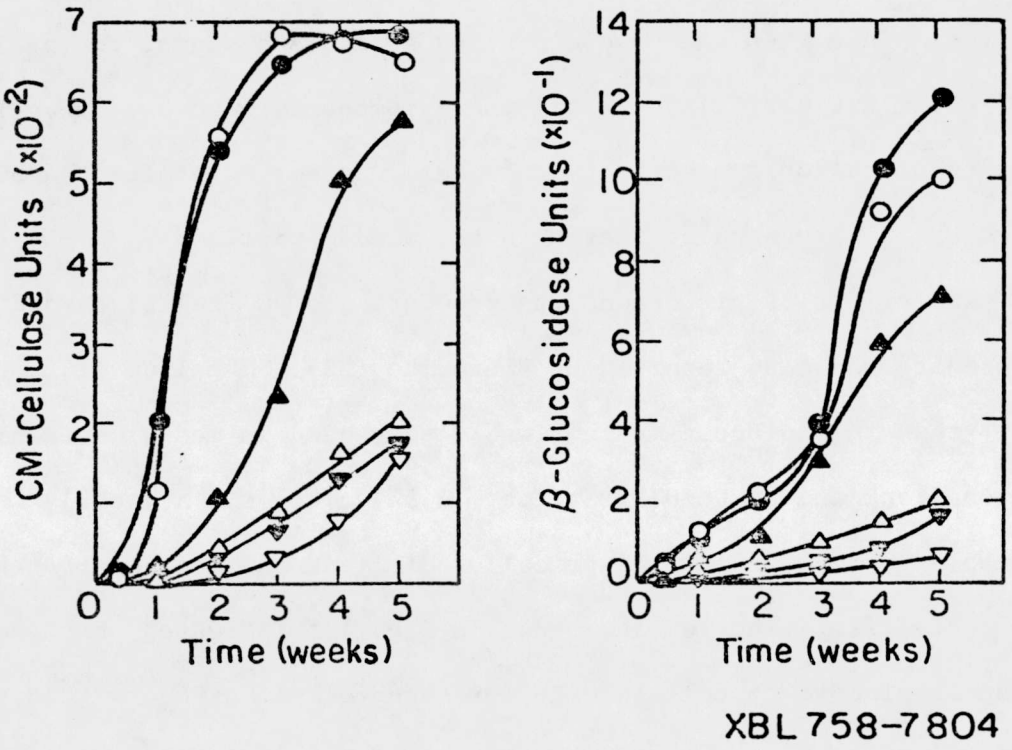

Figure 1.5. Effect of initial glucose concentration on production of CM-cellulase and β-glucosidase at 30°. Media contained no glucose (●), 0.001 M (○), 0.006 M (▲), 0.06 M (△), 0.2 M (▼), or 0.25 M (▽) glucose. Redrawn from (22).

1.2.2 Characterization of Cellulase and β-Glucosidase Components of Botryodiplodia theobromae

Extensive studies to characterize the enzyme system of the culture filtrate from the fungus have been done by Umezurike (23,24). The components were separated by polyacrylamide-gel electrophoresis and gel-filtration on Sephadex. Four cellulase components (called Ca,Cb,Cc and Cd) were recognized with approximate molecular weights of 162000 (Ca), 15000 (Cb), 10000 (Cc) and 4500 (Cd). Component Ca showed both short fiber-forming and hydrolyzing ability of cotton flock to sugars. Cb and Cc were found to be very similar to C_x cellulase, and Cd was capable of fragmenting native cellulose fibers into smaller particles without releasing considerable amounts of soluble sugars.

Four β-glucosidase components (Ba,Bb,Bc and Bd) were also recognized with molecular weights of 112000 (Ba), 56000 (Bb), 27000 (Bc) and 13300 (Bd). The activity differences among these four β-glucosidase components were not reported.

Synergism between these components was investigated. The fraction containing the elution peaks of the various components after fractionation on Sephadex G-75 and DEAE-Sephadex were pooled, freeze-dried and dissolved in 0.05 M acetate buffer, pH 5.0. An assay mixture containing 1.0 ml of an enzyme component (the concentration was not given), 5.0 mg cotton flock, and 5.0 mM NaN_3 in 5.0 ml 0.05 M acetate buffer, pH 5.0, was incubated at 40° C for 7 days. Total soluble carbohydrates of the supernatant solution was determined with anthrone reagent and expressed as a percentage of the original weight of cotton flock. The results are shown in Table 1.4. The component marked as Cdease is a

Table 1.4. Solubilization of cotton flock by components of Botryodiplodia theobromae cellulase system, alone and in combination (23,24).

Component	Means of separation	Solubilization*
Ca	DEAE-Sephadex	21.3%
Cb	Sephadex G-75	4.6
Cd	DEAE-Sephadex	4.0
Cdease	DEAE-Sephadex	4.1
Ba	Sephadex G-75	1.6
Ca + Ba		28.8
Cb + Cd		24.3
Ca + Cb + Ba		41.3
Ca + Cb + Cd + Ba		58.8
Ca + Cd + Cdease		76.2

*Measured after 7 days incubation at 40° C.

mixture of all C_x-cellulase and β-glucosidase since these activities were eluted in one peak from DEAE-Sephadex column.

Inhibitory effects of several components on β-glucosidase have been reported by many investigators. The most significant inhibitor is glucose, which is the final product of cellulose hydrolysis. Umezurike (25) reported that the β-glucosidase activity measured with p-nitrophenyl-β-D-glucopyranoside was almost 75% inhibited by 0.01 M (1.8 mg/ml) glucose and was completely inhibited by 0.25 M (45 mg/ml) glucose.

This observation illustrates the need to remove traces of glucose from culture filtrate before assay.*

The effect of cellobiose on the β-glucosidase activity was also reported by Umezurike (25). When p-nitrophenyl-β-D-glucopyranoside was used as a substrate for the activity measurement, increasing the concentration of cellobiose stimulated at low concentration levels (up to about 3×10^{-4} M, i.e., 0.1 mg/ml) but inhibited at higher concentrations. Other experimental data show that when more than 0.1 M cellobiose was present in the assay solution, the glucose inhibition was reduced from 75% to about 30%. From these results Umezurike concluded that the β-glucosidase from the fungus, like the aryl-glucosidase from Stachybotrys atra, has at least two sorts of binding sites: a donor site and an acceptor site.

Han and Srinivasan (11) showed the inhibitory effect of glucose on the hydrolysis of p-nitrophenyl-β-D-glucoside with their Alcaligenes faecalis β-glucosidase. The Lineweaver-Burk plot yielded a straight line, from which a K_m value of 1.25×10^{-4} M and a K_i value of 3×10^{-3} M for glucose inhibition (which was found to be competitive) were obtained. Similar results were reported by Heyworth and Walker (5) for the β-glucosidase of almond emulsin; the K_m value was 2.6×10^{-3} M and the K_i was 0.2 M using p-nitrophenyl-β-D-glucoside as a substrate.

So far no experimental data are available for the inhibitory effect of glucose on cellobiose hydrolysis.

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*Usually the culture filtrate of Botryodiplodia theobromae contains 0.2 mg/ml or less of glucose so that no effort to remove this trace amount of glucose has been done in the present work.

Literature Cited in Chapter 1

1) Jermyn, M. A., Aust. J. Biol. Sci., 8, 541 (1955)

2) Jermyn, M. A., ibid., 8, 563 (1955)

3) Jermyn, M. A., ibid., 8, 577 (1955)

4) Colwick, S. P. and Kaplan, N. O., in Methods in Enzymology, Vol. 1, 234, Academic Press (1955)

5) Heyworth, R. and Walker, P. G., Biochem. J., 83, 331 (1962)

6) Conchie, J., Gelman, A. L. and Lewy, G. A., ibid., 103, 609 (1967)

7) Schwartz, J., Sloan, J. and Lee, Y. C., Arch. Biochem. Biophys., 137, 122 (1970)

8) Crook, E. M. and Stone, B. A., Biochem. J., 65, 1 (1957)

9) Anderson, F. B., Cunningham, W. L. and Manners, D. J., ibid., 90, 30 (1964)

10) Sandegren, E. and Enebo, L., J. Inst. Brew., 58, 198 (1952)

11) Han, Y. W. and Srinivasan, V. R., J. Bacteriol., 100, 1355 (1969)

12) Reese, E. T., Maguire, A. H. and Parrish, F. W., Can. J. Biochem., 46, 25 (1967)

13) Reese, E. T., in Cellulases and Their Applications, Advances in Chem. Series, 95, 26 (1969)

14) Berghem, L. E. R. and Pettersson, L. G., Eur. J. Biochem., 46, 295 (1974)

15) Youatt, A., Aust. J. Biol. Sci., 11, 209 (1958)

16) Wood, T. M., Biochem. J., 121, 353 (1971)

17) Berger, L. S. and Eberhart, B., Biochem. Biophys. Res. Commun., 6, 62 (1961)

18) Eberhart, B., Cross, D. F. and Chase, L. R., J. Bacteriol., 87, 761 (1964)

19) Vogel, H. J., Microb. Genet. Bull., 3, 42 (1956)

20) Findlay, W. P. K. and Pettifor, C. B., Emp. For. J., 18, 259 (1939)

21) Umezurike, G. M., Ann. Bot., 33, 451 (1969)

22) Umezurike, G. M., ibid., 34, 217 (1970)

23) Umezurike, G. M., J. Exptl. Bot. 21, 639 (1970)

24) Umezurike, G. M., Biochim. Biophys. Acta, 227, 419 (1971)

25) Umezurike, G. M., ibid., 250, 182 (1971)

26) Barras, D. R., Moore, A. E. and Stone, B. A., in Cellulases and Their Applications, Advances in Chem. Series, 95, 105 (1969)

27) Reese, E. T. and Levinson, H. S., Physiol. Plant., 5, 345 (1952)

28) Kooiman, P., Roelofsen, P. A. and Sweeries, S., Enzymologia, 16, 237 (1953)

2. Experiments

2.1 Analytical Procedures

2.1.1 Reducing Sugar Assay

The concentration of reducing sugars was estimated using either the dinitrosalicylic acid method (1) and/or the anthrone reagent method (2). Glucostat reagent (3) was also used for measuring glucose in the solution.

Dinitrosalicylic acid (DNS) method

Three ml of DNS reagent was added to 1 ml of sample in a test tube and mixed well in a Vortex mixer. The tube was placed in a boiling-water bath for 5 minutes and then cooled down to room temperature. Distilled water was added to dilute to a final volume of 24 ml. The contents were mixed well, and the absorbance was measured with a Beckman DU-2 spectrophotometer at wave length 600 mμ against a distilled water blank. The standard curve was prepared with pure glucose. The appropriate range of the sugar concentration was about 1 to 4 mg/ml.

Anthrone reagent method

The anthrone reagent was prepared by dissolving 1 g of purified anthrone into 1 liter of 24 N sulfuric acid solution. Five ml of anthrone reagent was added to 1 ml of sample in a test tube, mixed vigorously in a Vortex mixer and placed in a boiling-water bath for 12 minutes and then cooled to room temperature. The absorbance was measured with a Beckman DU-2 spectrophotometer at 620 mμ against an

anthrone reagent blank. The standard curve was prepared with pure glucose for each series of measurements. The measurable range of sugar content is about 10 to 150 mg/ℓ.

Glucostat reagent method

Two ml of Glucostat reagent (Worthington Biochemical Co.) was added to 2 ml of sample, which contained up to 100 mg/1 of glucose. After mixing it was left at room temperature for 10 minutes. One or two drops of 4 N hydrochloric acid was added to the mixture to terminate the reaction, and after 5 minutes (or more) the absorbance was measured with the Beckman spectrophotometer at 420 mμ against a Glucostat reagent blank. The range of glucose concentrations is about 10--100 mg/1. Since the reaction was carried out at room temperature, it was necessary to prepare a standard curve for each set of measurements.

Although the DNS method has been used by many investigators to determine reducing sugar concentration, it is not without problems. Sumner (1), who developed the method, mentioned on this point: "Fructose and galactose have the same reducing power as glucose; 90 mg of arabinose or xylose are equivalent to 100 mg of glucose; and 124 mg of either anhydrous lactose or maltose are equivalent to 100 mg of glucose." In the hydrolysis product of pure cellulose, reducing sugars exist in the forms of glucose (monomer), cellobiose (dimer), cellotriose (trimer) and so on. Although the concentrations of trimer and higher oligomers are usually very low, the concentration of cellobiose is ~ 23 w%. As expected, we found that the reducing power of cellobiose

on DNS reagent was approximately the same as lactose or maltose; that is, 121 mg of cellobiose is equivalent to 100 mg of glucose (Fig. 2.1). From these data the following equation was obtained for mixtures of glucose and cellobiose.

$$(TRS) = (G) + 0.833\ (CB)$$

where (TRS) = Total reducing sugar measured with DNS reagent using glucose as a standard, mg/ml

(G) = Glucose concentration, mg/ml (known independently)

(CB) = Cellobiose concentration, mg/ml

This relation clearly shows that the reducing sugar concentration measured with DNS reagent gives different values depending upon the ratio of glucose to cellobiose concentration even though the total glucose units in the solution is the same; for example, supposing 13 g of glucose unit is in one liter of the solution, TRS values are read as 13 g/l for glucose only, 12.3 g/l for 70/30 mixture of glucose and cellobiose and 10.8 g/l for cellobiose solution.

Although the TRS value has been used by many investigators in measuring the reducing sugar concentration, it should be noted the TRS value itself does not have a significant meaning unless the ratio of glucose to cellobiose concentration is specified.

On the other hand, the anthrone reagent depolymerizes the cellobiose to glucose, and the measured value is considered to represent the concentration of glucose unit in the solution, which may be conveniently named TGE, total glucose equivalent. Theoretically, this TGE value can be related to the concentration of glucose and its oligomers by the

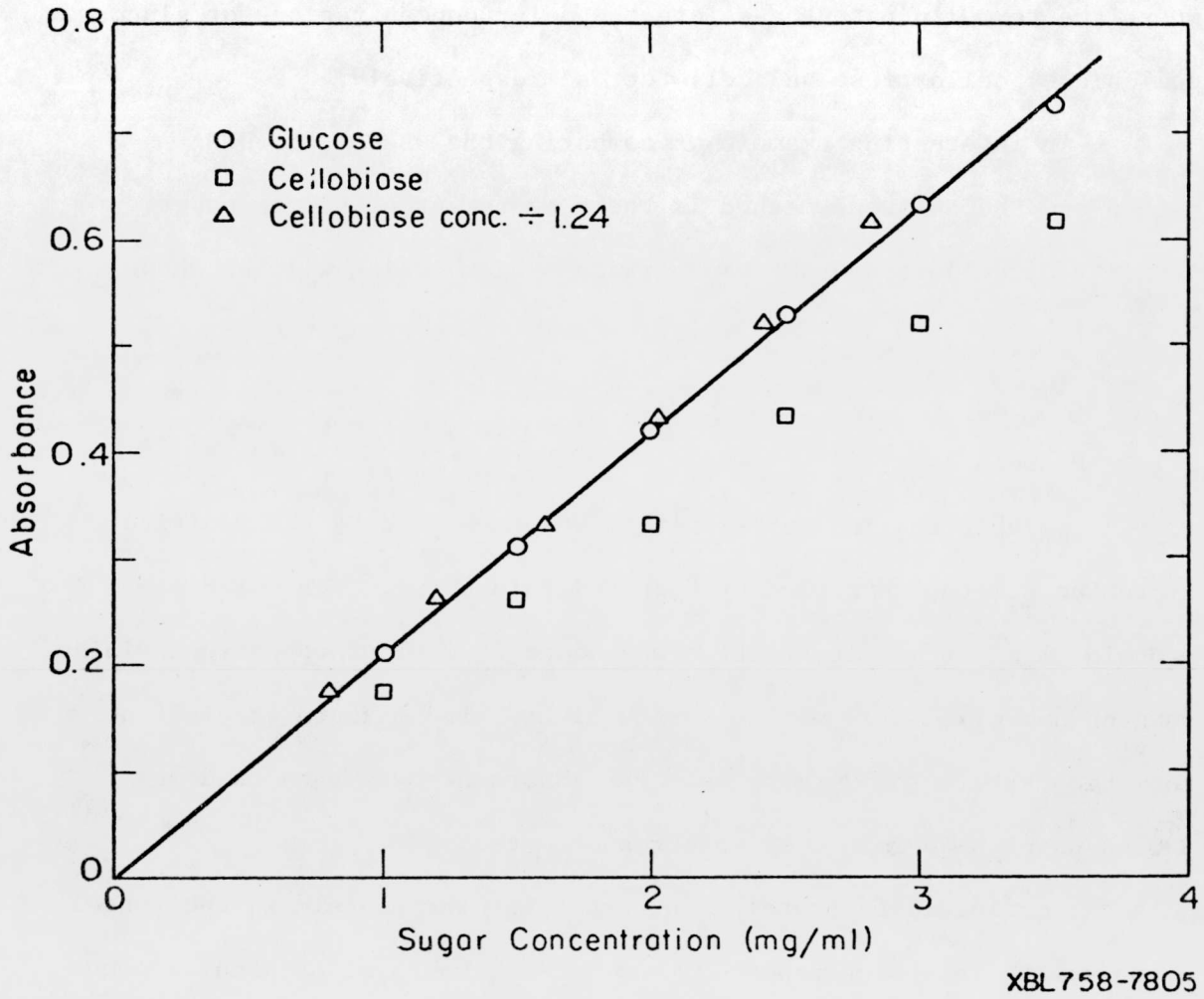

Figure 2.1. Standard curves for sugar determination by the dinitrosalicylic acid (DNS) method.

following equation.

$$(TGE) = (G) + 2\ (CB) + 3\ (C\ Tri) + 4\ (C\ Tetra) + ----$$

where the terms in parentheses are the molar concentrations of glucose, cellobiose, cellotriose and cellotetrose respectively.

An interesting example of comparing the use of the DNS method and the anthrone method is the measurement of filter paper activity of cellulase and β-glucosidase system, which will be shown in section 2.1.3.

2.1.2 Protein Assay

Soluble and insoluble protein were measured by the modified microbiuret method proposed by Koch and Putnam (4). For soluble protein, 1 ml of sample solution was added to 4 ml of copper containing reagent and mixed well with a Vortex mixer. The mixture was left at room temperature for 30 minutes. The absorbance was then read at 330 mμ using a Beckman DU-2 spectrophotometer.

For insoluble protein, in particular the protein in the fungal biomass, the following procedure was used: 10--25 ml of a homogeneous suspension of fungal biomass was centrifuged at 10,000 rpm and the precipitate, after washing three times with distilled water, was added to 4 ml of 4.6 N sodium hydroxide. The contents were mixed well and placed in a boiling water bath for 5 minutes. Then the insoluble biomass was centrifuged and 1 ml of the supernatant was transferred to a tube. The protein thus extracted was measured by the biuret reagent method described above.

To correlate the protein content of the fungus to the total biomass, several samples were taken from a fermentor where the fungus was growing on soluble substrates, and a calibration curve was prepared between the dry weight of the fungus and the intracellular protein content. Figure 2.2 shows the calibration curve for Botryodiplodia theobromae; it can be seen that protein content in the total biomass is about 15%, which is fairly small compared with the number for Trichoderma viride (5).

When an insoluble substrate such as cellulose was used as the main carbon source, this method does not work. When the intracellular protein was extracted by copperless reagent, cellulosic material was also present. It was found that a possible product of the reaction between reagent and cellulose interfered with the reading of true protein concentration. As shown in Fig. 2.3, the optical density increased with an increase in Solka Floc concentration, even when no biomass was present. Thus, to measure the protein in the biomass grown on cellulosic materials, more sophisticated methods such as the Kjeldahl method or ATP measurement would be required. For the present work, no attempt was made to measure the protein content of the biomass grown on cellulose.

2.1.3 Enzyme Activities

Cellulase is a complex enzyme which is supposed to contain several components, which King and Vessal (6) summarized as follows:

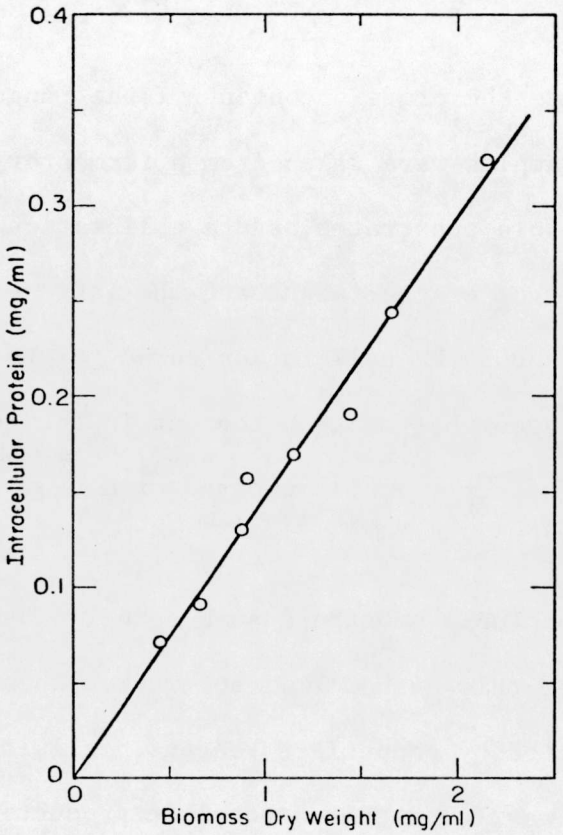

Figure 2.2. Relationship between intracellular protein content and biomass dry weight for *B. theobromae*.

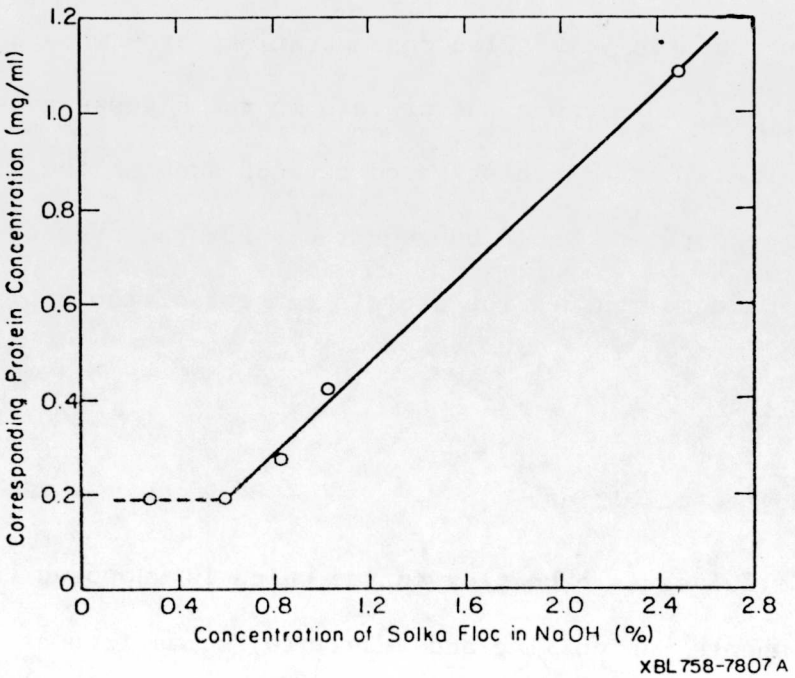

Figure 2.3. Effect of Solka Floc on measurement of intracellular protein.

(a) C_1 is an enzyme whose action is unspecified. It is required for the hydrolysis of highly oriented solid cellulose, for example, cotton, Avicel, etc., by β-1,4-glucanases.

(b) C_x, or more correctly, β-1,4-glucanases, are hydrolytic enzymes which consist of two types: 1) exo β-1,4-glucanase, which successively removes single glucose units from the non-reducing end of the cellulose chain; 2) endo β-1,4-glucanases whose action is of a random nature, but which are generally more reactive with internal than with terminal linkages.

(c) β-Glucosidases, which vary in their specificity as discussed in Chapter 1. Those that act primarily on aryl-β-glucosides are not involved in cellulase action. The β-glucosidases which are involved in cellulose breakdown are those highly active on cellobiose.

There are many methods reported in the literature for assaying cellulase, all of which use the reaction rate obtained in short hydrolysis periods of the enzyme action on specific substrates. Thus, it is difficult to use the activities measured by these methods to predict the progress of the hydrolysis reaction for the long periods of practical applications. A more complete discussion of this problem will be given in Part II.

In the present work, the assay procedures suggested by Mandels and Weber (7) for cellulase were used with some modifications.

Filter paper activity (FPA): 50 mg of Whatman filter paper No. 1 (1×6 cm) was added to a mixture of 1 ml of enzyme solution and 1 ml of 0.05 M sodium acetate buffer at pH 5.0. After reaction at 50° C for one hour, the reducing sugar concentration was measured by the anthrone reagent method.

C_x activity: Enzyme solution (0.1 ml) was added to 0.9 ml of 2.0% carboxymethylcellulose (CMC, 7L, Hercules Inc.) in 0.05 M acetate buffer (pH 5.0). The reducing sugar produced was determined by the dinitrosalicylic acid method after reaction at 50° C for 30 minutes.

C_1 activity: Absorbent cotton (50 mg) was added to 1 ml of enzyme solution and 1 ml of 0.05 M acetate buffer at pH 5.0. After reaction at 50° C for 24 hours, the amount of reducing sugar produced was measured by the anthrone reagent method.

These activities for cellulase were expressed as mg of reducing sugar produced by the original enzyme sample. Table 2.1 shows the differences between assay methods used in this work and those used by Mandels and Weber (7). As explained in the previous section, the total reducing sugar value measured with the DNS method was affected substantially by the cellobiose concentration in the solution. The anthrone reagent was used for the assays of C_1 and filter paper activities to determine the total glucose equivalent (TGE). For the C_x activity the anthrone reagent could not be used because of its ability to hydrolyze the substrate, carboxymethylcellulose, which could not be separated from the reaction mixture.

Figures 2.4 and 2.5 compare the FPA values measured with the DNS and the anthrone reagents. In almost all cases, when the fraction of β-glucosidase solution was more than 50%, the FPA values coincided; however, when the β-glucosidase concentration was less, the FPA by DNS reagent was lower than the other. These differences are undoubtedly attributable to the presence of cellobiose in the reaction

Table 2.1. Comparison of assay methods.

	Mandels and Weber (7)	Present work
Filter paper activity and C_1 activity	Reducing sugar concentration was determined with the DNS reagent.	Reducing sugar concentration was determined with the anthrone reagent method.
C_x activity	0.5 ml of enzyme solution was added to 0.5 ml of 1.0% CMC solution.	0.1 ml of enzyme solution was added to 0.9 ml of 2.0% CMC solution.
Buffer	Citrate buffer, pH 4.8.	Acetate buffer, pH 5.0.

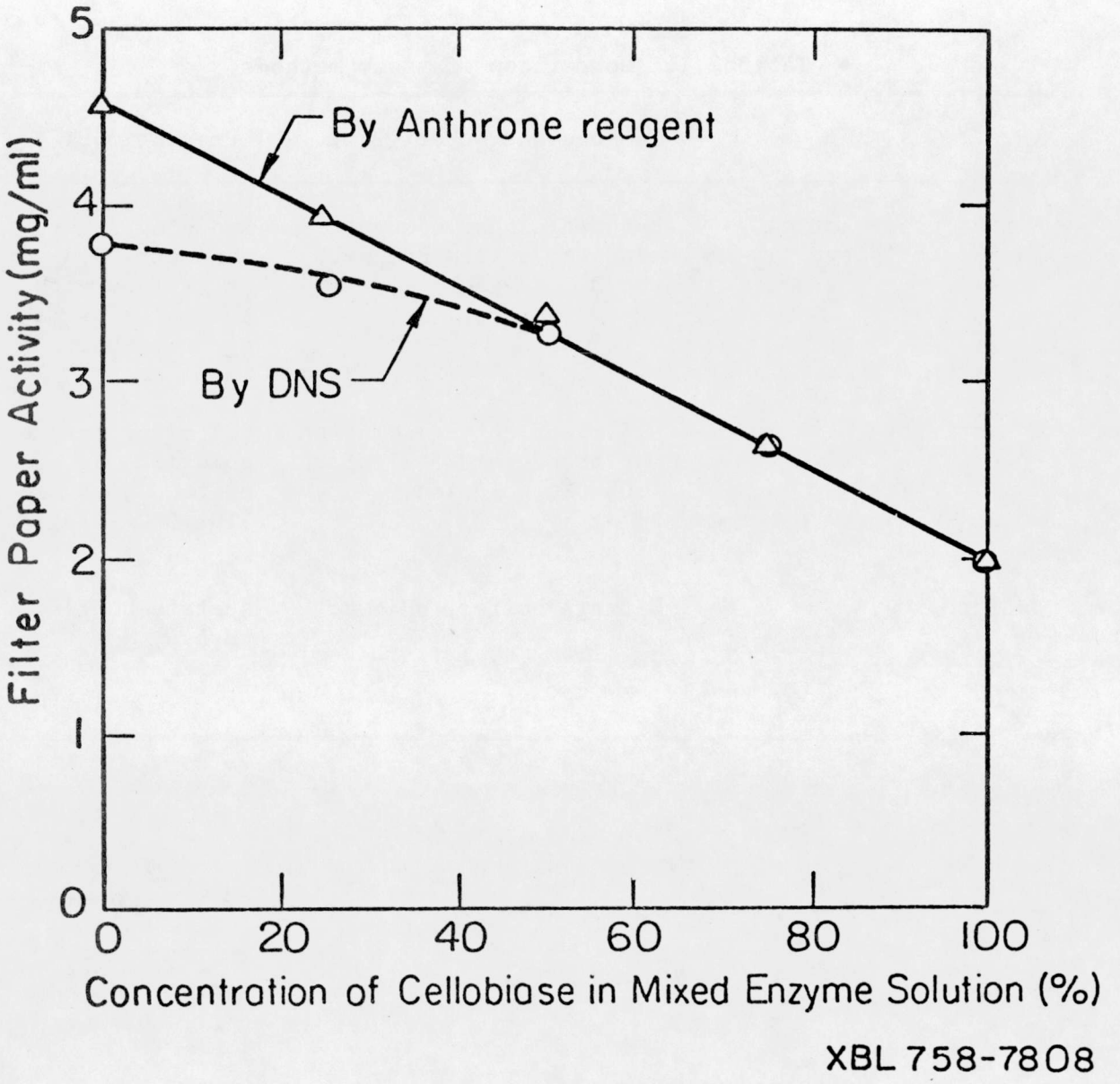

Figure 2.4. Filter paper activity of various cellulase/cellobiase mixtures.

Cellulase: culture filtrate of *T. viride*
filter paper activity (by DNS) 3.80 mg/ml
cellobiase activity 0.28 mg/ml

Cellobiase: culture filtrate of *B. theobromae*
filter paper activity (by DNS) 1.95 mg/ml
cellobiase activity 1.27 mg/ml

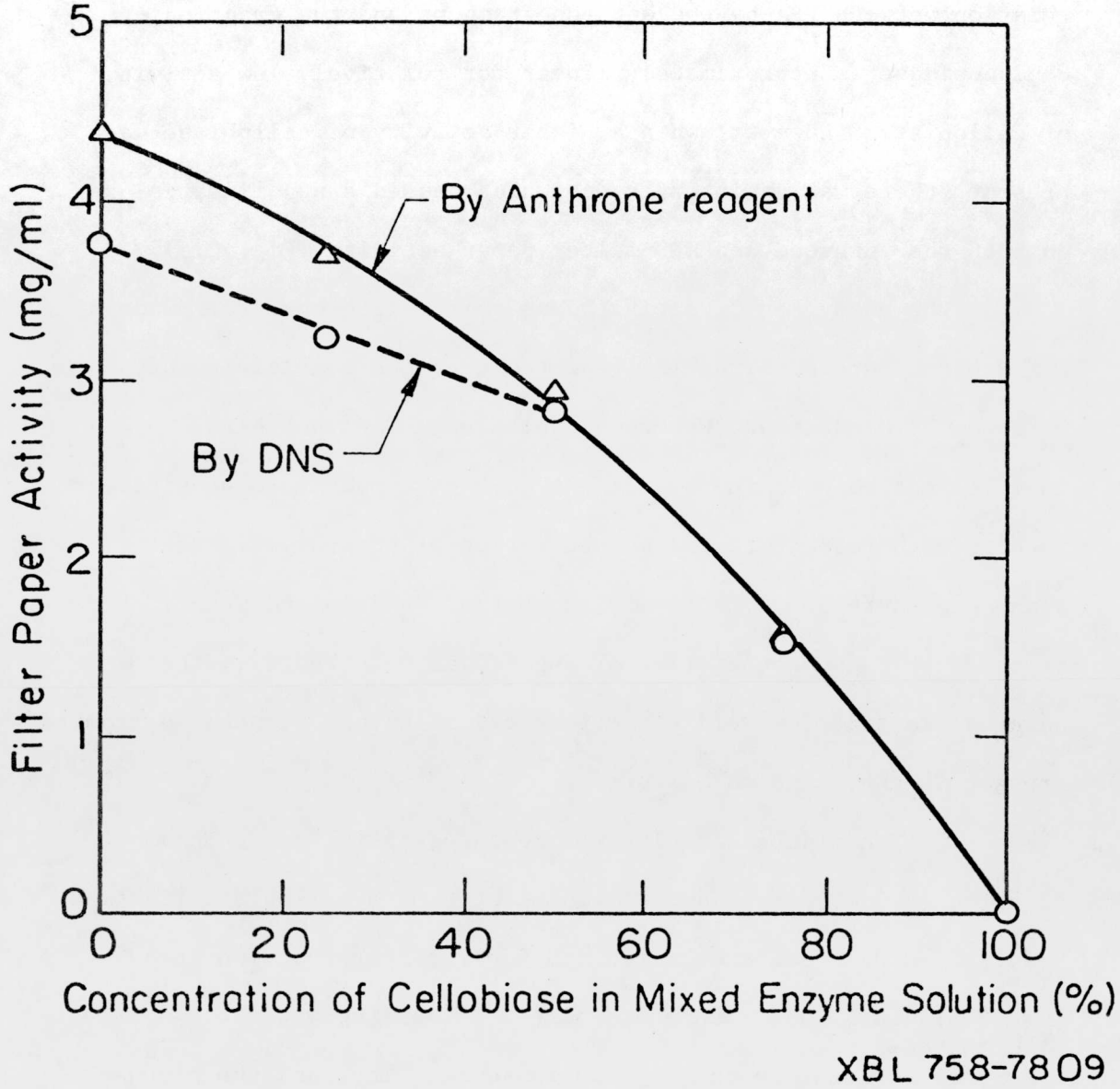

Figure 2.5. Filter paper activity of various cellulase/cellobiase mixtures.

Cellulase: culture filtrate of *T. viride*
filter paper activity (by DNS) 3.80 mg/ml
cellobiase activity 0.28 mg/ml

Cellobiase: almond emulsin β-glucosidase, 2.0 mg/ml
filter paper activity 0.0
cellobiase activity 5.82 mg/ml

mixture as discussed in Section 2.1.1. It is also seen that the relation between FPA by the anthrone reagent and the fraction of β-glucosidase is approximately linear for relatively low activity of cellobiase. However, when a higher activity of cellobiase was present, there was an interference which caused a non-linear slope on both the anthrone and DNS filter paper activity (Fig. 2.5).

The assay for C_x activity was quite different from Mandels and Weber's method. Yang (8) reported that the sensitivity of C_x assay by Mandels and Weber was relatively low, and his modified method used in the present work gave much more sensitive results with respect to the concentration of the enzyme. The assay procedure for C_1 activity is that of Mandels and Weber, except that the concentration of sugars was measured with the anthrone reagent to avoid the effect of cellobiose or other oligomers present in the reaction mixture.

β-glucosidase activity was measured as follows: Enzyme solution (0.2 ml) was added to 1.0 ml of 0.02 M cellobiose (Eastman Kodak) in 0.05 M acetate buffer (pH 5.0). After reaction at 40°C for 15 minutes, the mixture was heated in boiling water for 2 minutes to denature the β-glucosidase activity, and the glucose concentration was measured by Glucostat reagent. The activity of β-glucosidase was expressed as mg/ml of glucose produced by the original enzyme solution.

2.2 Experimental Apparatus and Methods

2.2.1. Culture and Media for the Production of β-Glucosidase

A lyophilized culture of Botryodiplodia theobromae was obtained from the American Type Culture Collection (ATCC 10936). Malt agar slants were used to maintain the culture in the refrigerator for several weeks.

The composition of the medium initially used for growing the fungus is shown in Table 2.2 (9). As will be discussed in Chapter 3, this medium provided a fairly high activity of the enzyme in flask cultures, but was not very good when a 14 liter fermentor was used. Later the medium for Trichoderma viride (10) was found to be suitable for the Botryodiplodia theobromae, and it was used for most of the experiments with the 14 liter fermentor. The composition of the medium for Trichoderma viride is shown in Table 2.3.

2.2.2 Flask Culture Experiments

The production of β-glucosidase was investigated in both flasks and in the 14 liter fermentor. In the early stage of this study, a 250 ml culture flask with magnetic stirrer was conveniently used to examine the effect of growth conditions on the fungus. The flask was placed in a water bath where temperature was maintained by circulation from a thermostated bath.

The medium (150 ml) was poured into the flask and steam sterilized in the autoclave for 30 minutes at 121°C. When glucose or starch was used as a carbon source, it was sterilized separately from the

Table 2.2. Growth medium* reported by Umezurike (9).

Compound	Amount
KH_2PO_4	1.4 g/l
$MgSO_4 \cdot 7H_2O$	0.2
$NaNO_3$	2.1
$CaCl_2$	0.2
Yeast extract	0.1
Cellulose (or other carbon source) ---	1%

*This medium will be referred to as Medium (I).

Table 2.3. Growth medium* of Mandels and Weber (7).

Compound		Amount
KH_2PO_4		2.0 g/l
$(NH_4)_2SO_4$		1.4
$(NH_2)_2CO$		0.3
$MgSO_4 \cdot 7H_2O$		0.3
$CaCl_2 \cdot 2H_2O$		0.4
Proteose peptone (Difco)		0.5
Trace minerals:	Fe^{++}	1.0 mg/l
	Mn^{++}	0.8
	Zn^{++}	0.5
	Co^{++}	0.5
Cellulose (or other carbon source) ---		1%

*This medium will be referred to as Medium (II).

other ingredients. Inoculum was transferred directly from the malt agar slant by loop. A small amount of sterilized distilled water was added to the slant tube, and the mycellium was suspended to facilitate the transfer of the inoculum.

After a few days of fermentation, the suspension of the fungus was transferred to a centrifuge tube. All solid materials were removed by centrifugation at 8,000 rpm. The supernatant thus obtained was examined for several enzyme activities, soluble protein content and residual sugar content.

2.2.3 14 Liter Fermentor System

A Pyrex glass 14 liter culture vessel (New Brunswick Scientific) was used for large scale production of β-glucosidase. Agitation was supplied by two four-bladed 4-5/8 inch turbines on a vertical shaft; one was directly above the air sparger and the other was just below the liquid level. The shaft was connected to a 1/3 HP General Electric Statotrol motor (variable speed) by a universal joint. The fermentor was fitted with four baffles attached to the head plates. These baffles were hollow; one served as the air line leading to the sparger and two others were for circulation of water for controlling temperature.

Air, reduced to 5 psig with a pressure regulator, was forced first through a rotameter, then through a glass wool filter for particle entrapment to achieve sterilization, and finally through a sintered glass sparger in a column of sterile water for humidification before entering the fermentor through a single hole sparger just under the lower turbine. Exhaust from the fermentor passed through a foam

trap and then a glass wool filter.

Constant temperature was maintained by circulating water from a bath with a centrifugal pump. The temperature of this water bath was controlled by an immersion heater connected to a proportional temperature controller (Yellow Springs Instrument Co., Ohio). The temperature detector was a precision thermistor in a well extended from the head plate of the fermentor. The pH was measured with an autoclavable electrode (Leeds and Northrop) mounted in the fermentor headplate. The signal from this electrode went to a Beckman Model 900 pH controller-analyzer (on-off type) coupled to Sigmamotor AL-4 peristaltic pumps, which added either 4 N sulfuric acid or 4 N sodium hydroxide solution to the fermentor to maintain constant pH. The oxygen concentration in the liquid was monitored by a membrane type dissolved oxygen probe (New Brunswick). The current from this probe was passed through an appropriate resistance, and the voltage drop was recorded with a Leeds and Northrup Speedomax Type G recorder. Antifoam agent (Antifoam 60, silicone emulsion, General Electric Co.) was supplied manually from a reservoir by siphon when excessive foam was observed.

The fermentor was steam sterilized in a vertical autoclave with a holding time of at least 30 minutes at 121°C. The inlet air filter and humidifying column were also autoclaved separately and connected to the fermentor aseptically after sterilization.

2.2.4 Q_{O_2} Measurement

The specific respiration rate, Q_{O_2}, was measured in order to find whether the oxygen concentration was limiting during the growth of the fungus on a glucose medium. Q_{O_2} was estimated according to the method suggested by Bandopadhyay and Humphrey (10).

The rate of change in dissolved oxygen concentration at a particular point in a fermentor is given by the following equation which also defines Q_{O_2} (11).

$$\frac{d\bar{C}}{dt} = k_L a\ (C^* - \bar{C}) - Q_{O_2} X \qquad (2\text{-}1)$$

where C^* = concentration of dissolved oxygen in equilibrium with bulk gas phase

$\bar{C}$ = concentration of dissolved oxygen in bulk liquid

$k_L a$ = volumetric oxygen-transfer coefficient

Q_{O_2} = specific rate of oxygen uptake

X = cell mass concentration

When the air supply is turned off, the first term of the right hand side of the equation becomes zero, hence we get

$$\frac{d\bar{C}}{dt} = - Q_{O_2} X \qquad (2\text{-}2)$$

Therefore, the value of $Q_{O_2} X$ can be estimated by measuring the dynamic response of dissolved oxygen concentration with a fast response dissolved oxygen probe during a brief interruption of the aeration to the fermentor.

A 14 liter fermentor was used for the Q_{O_2} measurement. The medium was Medium (II) (Sect. 2.2.1) with 0.5% glucose as the sole carbon source. The biomass was measured by repeated centrifugation of the sample and washing with distilled water, followed by drying in the oven at 80° C for 24 hours. The total degassing time in Q_{O_2} measurement was kept short (less than four minutes) so that the biomass could be assumed constant during the measurement.

2.2.5 Continuous Production of β-Glucosidase

Only a preliminary experiment on continuous fermentation of the Botryodiplodia theobromae was carried out in the present work. For cellulase production, Mitra and Wilke (12) proposed the use of a two-stage continuous stirred tank reactor (CSTR) operated with the first stage utilizing glucose for growth and the second stage utilizing Solka Floc for enzyme induction. The same concept was examined for β-glucosidase production using glucose for the growth stage and newsprint for the induction stage.

A one liter minifermentor was used for the first stage. The agitation was provided by six-bladed 2 inch turbines on a vertical shaft which was connected to a variable speed motor. Air was supplied in the same manner as described for the 14 liter fermentor. Constant temperature was maintained by circulating water from a bath through a heat exchanger fitted in the fermentor. Glucose medium was continuously fed to the fermentor by a peristaltic pump (Sigmamotor) and the fermented liquid was withdrawn through the air exhaust line, the level of which

was adjusted just above the liquid level so that the liquid level was maintained by air-lifting the liquid to the second stage fermentor.

The 14 liter fermentor was used for the induction stage. All assemblies were the same as those used for batch fermentation except that continuous feed of biomass from the growth stage and discharge of the culture suspension to a reservoir were provided. The reservoir was connected to a vacuum line, and the culture suspension in the induction stage was sucked through the discharge line, which was placed in the fermentor at the liquid level. The cellulosic substrate, newsprint, was fed into the fermentor once a day from a bottle with the aid of a sterilized air stream.

Literature Cited in Chapter 2

1) Sumner, J. B., J. Biol. Chem., 62, 287 (1925)

2) Fairbairn, N. J., Chem. and Ind. (London), 86 (1953)

3) Worthington Enzyme Manual, 181, Worthington Biochemical Corporation (1972)

4) Koch, A. L. and Putnam, S. L., Anal. Biochem., 44, 239 (1971)

5) Mitra, G. and Wilke, C. R., Enzymatic Utilization of Waste Cellulosics, Lawrence Berkeley Laboratory Report 2334-Rev., January (1975)

6) King, K. W. and Vessal, M. I., Cellulases and Their Applications, Adv. in Chem. Series, 95, 7 (1969)

7) Mandels, M. and Weber, J., ibid., 95, 309 (1969)

8) Yang, R. D., private communication, Lawrence Berkeley Laboratory, January (1975)

9) Umezurike, G. M., Ann. Bot., 33, 451 (1969)

10) Bandopadhyay, B., Humphrey, A. E. and Taguchi, K., Biotech. Bioeng., 9, 533 (1967)

11) Aiba, S., Humphrey, A. E. and Millis, N. F., Biochemical Engineering, Second edition, 332, Academic Press (1973)

12) Mitra, G. and Wilke, C. R., Biotech. Bioeng., 17, 1 (1975)

3. Results and Discussion

3.1 Production of β-Glucosidase

3.1.1 Flask Culture Experiments

Flask cultures have been used quite often in investigating fermentation processes, but it should be pointed out that there are several problems involved with this experimental technique. First of all, there is no geometrical similarity between the flask and the pilot scale (in the present work: 14 liter) fermentor. Secondly, the operational conditions, for instance, agitation speed, aeration rate, pH, dissolved oxygen concentration, etc., could never be the same for both systems. Thirdly, the small size of the inoculum is not reproducible even among the flask culture experiments. For these reasons, the flask culture experiments could provide only qualitative features of the fermentation of the fungus.

Effect of Cellulose Concentration

Medium (I), whose composition was described in Table 2.2, was used for these experiments. Solka Floc (0.5, 1.0 and 1.5%), a spruce wood pulp widely used as a filter aid, was employed as the sole carbon source. The activity of enzyme produced from each preparation was examined. The results shown in Table 3.1 indicate that the higher the substrate concentration, the higher the activity of β-glucosidase. Mandels and Weber (1) measured the effect of cellulose concentration on the production of cellulase by Trichoderma viride (QM6a). The optimum was obtained at 0.5--1.0%, depending upon the activities measured.

Table 3.1. Effect of cellulose concentration.

Cellulose conc.	β-Glucosidase activity
0.5%	0.3 mg/ml
1.0	0.5
1.5	0.6

A sharp decrease was observed at concentrations higher than 1.5% cellulose.

Effect of Additional Carbon Sources

As reviewed in Chapter 1, the existence of starch along with cellulose in the medium may help the growth of the fungus and thus enhance the production of β-glucosidase. Several different amounts of starch were added to Medium (I) with 1 and 1.5% Solka Floc, and the β-glucosidase activity was measured after 7 days incubation at 30° C. The effect of glucose as a second carbon source was also examined in the same experiments. To prevent the possible caramelization during sterilization, these carbohydrates were sterilized separately from the medium and added before the fermentation was started. The results are presented in Table 3.2.

These experimental data show the enhancing effect of additional carbon sources to the cellulose medium on the production of β-glucosidase. Although glucose has been reported to work as a repressor toward β-glucosidase production (2), the results in the present work were obtained under quite different conditions so that the comparison was

Table 3.2. Effect of additional carbon sources.

Cellulose concentration	Additional carbon source and its concentration		β-glucosidase activity*
1.0%	Starch	0.1%	0.794 mg/ml
		0.2	1.05
		0.5	1.10
1.5%	Starch	0.5%	1.40 mg/ml
1.0%	Glucose	0.5%	0.74 mg/ml
		1.0	0.08

*β-Glucosidase activity was measured after 7 days incubation at 30° C.

difficult to make. When 1% glucose was used as a second carbon source, the β-glucosidase activity was found to be very small, but the growth of the fungus was enormous. This is probably because the fungus did not produce any β-glucosidase activity while glucose was present in the medium. It was also observed that after 3--5 days of inoculation the medium became very viscous when glucose was used, but eventually the liquid viscosity decreased as the fermentation proceeded. It was not clear whether or not the fungus produced some oligosaccharides from glucose and survived on them after glucose was consumed.

Effect of Temperature

The optimum temperature for the production of β-glucosidase of Botryodiplodia theobromae was not known as well as that for the growth of the fungus. Alasoadura (3) reported that the fastest vegetative growth on malt agar was obtained at 28° C, but this does not usually mean that the optimum temperature for the enzyme production is 28° C.

Thus the flask culture experiment was carried out at various temperatures, and the β-glucosidase activity of each 7 day culture filtrate was measured. The results presented in Fig. 3.1 show that the optimum temperature for β-glucosidase production is 30° C, which is commonly observed for most fungi.

Effect of Antifoam Agent

As will be shown in a later section, the productivity of β-glucosidase with Medium (I) was very poor when the 14 liter fermentor was used. One of the major differences between the flask culture and pilot scale experiments was the use of antifoam agent for the latter fermentation. To examine the effect of the antifoam agent, from 0.33% to 1.33% antifoam agent was added to the flask culture and the β-glucosidase activity was measured after 7 days incubation. The results, however, showed no significant effect of the concentration of the antifoam agent on the production of β-glucosidase.

Use of Hydrolysis Product as a Carbon Source

The hydrolysis product of cellulose usually contains glucose and cellobiose, the latter of which was reported to induce β-glucosidase (4). Thus, the production of β-glucosidase with the hydrolyzate, whose composition was 7.7 mg/ml of glucose and 3.5 mg/ml of cellobiose, was tested in the flask culture. Inorganics and supplemental protein (Proteose Peptone) were added in the amounts described in Table 2.3 in Chapter 2. Since it was anticipated that the presence of glucose in this medium might repress enzyme production, the same experiment was carried out

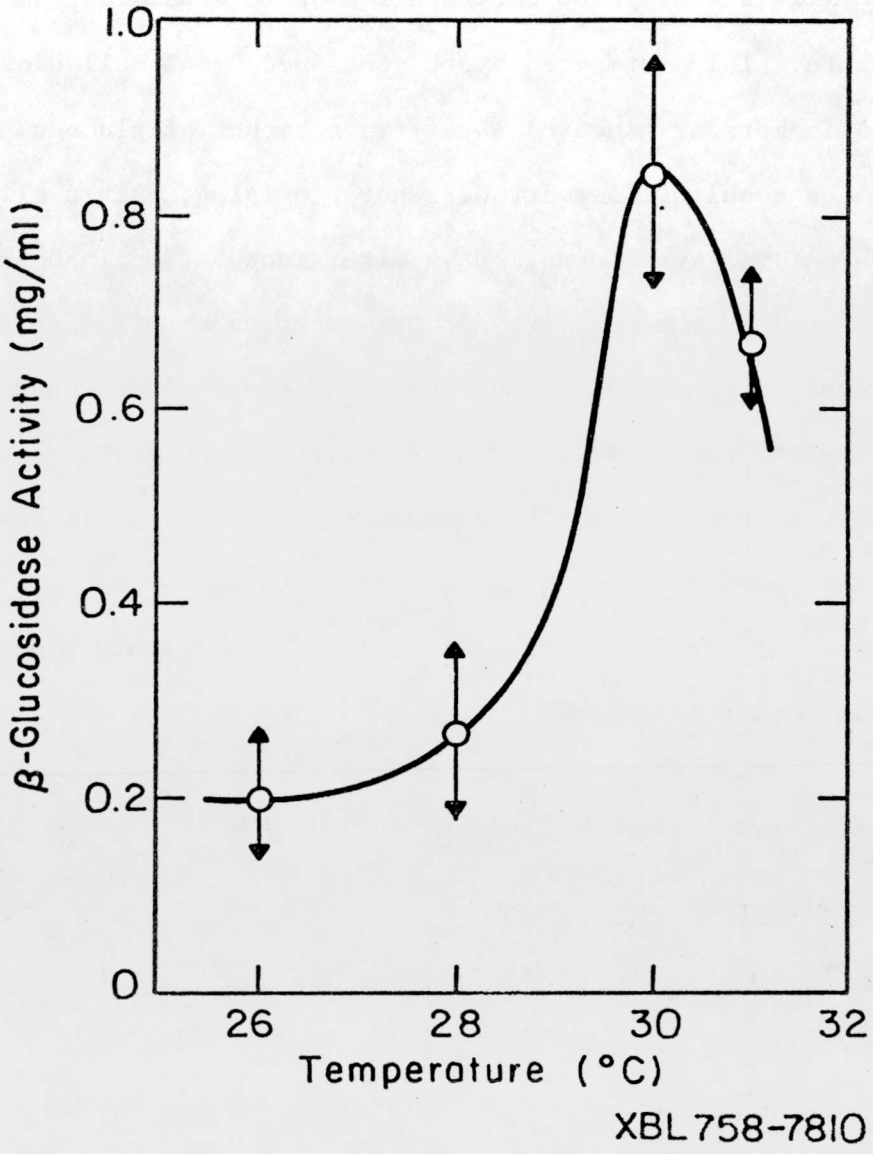

Figure 3.1. Optimum temperature for β-glucosidase production by *B. theobromae*.

with the filtrate of yeast fermentation broth obtained from the same hydrolyzate. This fermented broth contained 0.64% cellobiose (or higher oligomers of glucose) and a trace amount of glucose.

The results, however, were not promising; only a slight β-glucosidase activity was found 3 days after inoculation in both systems, and the level of the activity did not increase significantly. One of the reasons for this poor productivity of the β-glucosidase may be attributable to the caramelization of sugars in the medium. The hydrolyzate and the fermented broth already contained inorganic and proteins which might produce some toxic materials during the sterilization. The growth rate of the fungus was not measured, but growth had apparently taken place with both media.

3.1.2 14 Liter Fermentor Experiments (Batch)

Batch Growth on Glucose Medium

The batch growth rate of the Botryodiplodia theobromae was investigated in the 14 liter fermentor. Medium (I) with 0.5% glucose and Medium (II) with 1% glucose were used, and the inoculum was prepared with the same medium as used in the fermenter. Constant pH (5.5 for the Medium (I) and 5.0 for the Medium (II)) and constant temperature (30° C) were maintained during the fermentation. The aeration rate was 0.25 vvm. Cell mass concentration was measured by taking samples at appropriate intervals, followed by centrifugation to separate the cell mass, washing the cell mass three times with distilled water and then drying in an oven (80° C) for 24 hours. Glucose concentration was monitored by measuring with the DNS reagent. The results

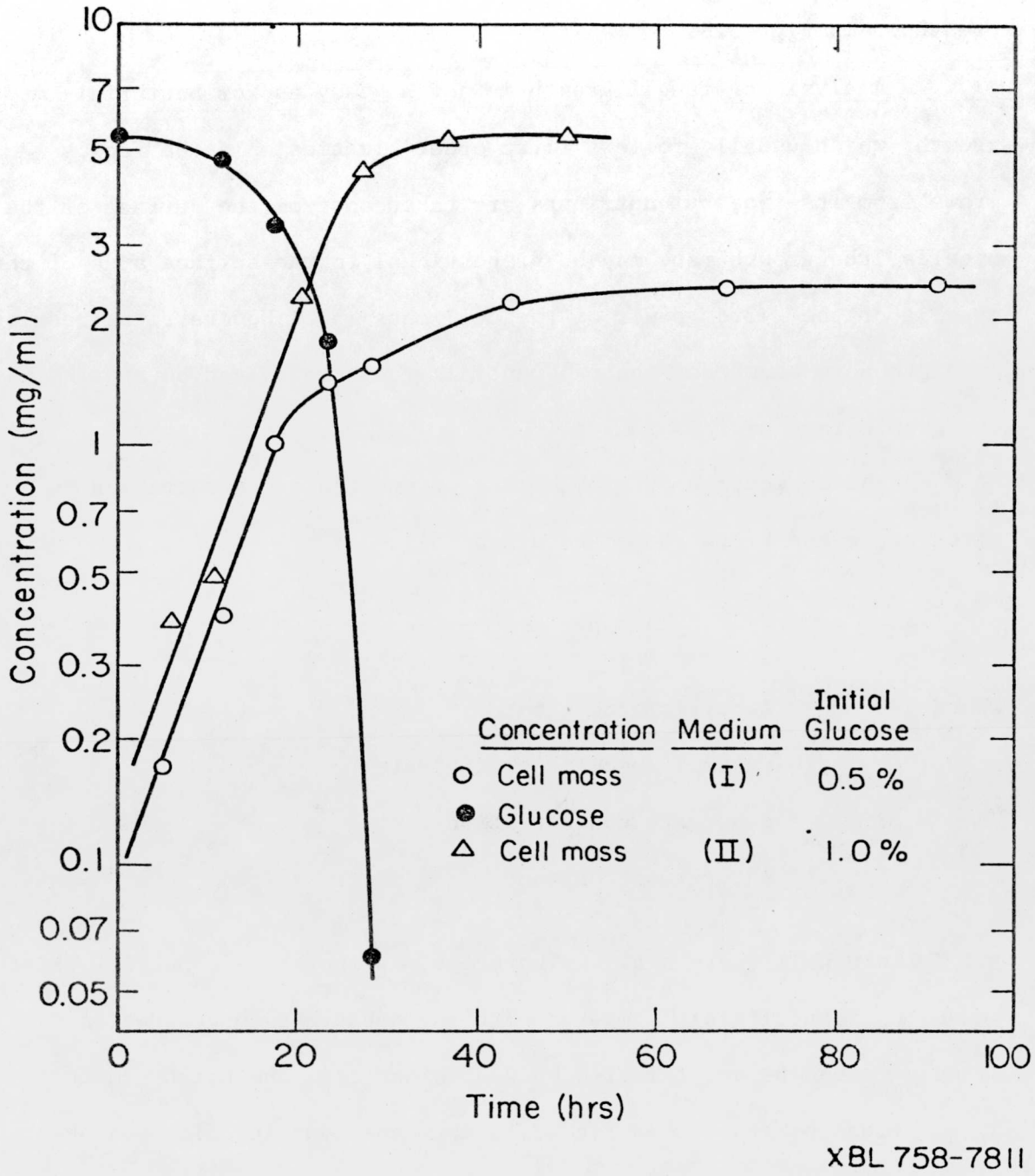

Figure 3.2. Batch growth of *B. theobromae* on glucose media.

are shown in Fig. 3.2.

Analysis of fungal growth is not as easy as for bacterial growth, which usually follows first order kinetics. If the fungus grows from its tip, but nutrients are taken up from the surface of the mycelia, the growth rate may be proportional to the surface area of the mycelia or the 2/3rds power of the cell mass (5). However, the results obtained here show that the exponential growth model can be applied to the growth rate of this fungus.

The dependence of growth rate on substrate concentration is often expressed by the Monod equation (5):

$$\mu = \frac{\mu_{max} S}{K_S + S}$$

where μ = specific growth rate

μ_{max} = maximum specific growth rate

S = substrate concentration

K_S = saturation constant

and K_S is usually very small. Therefore, during the exponential growth phase, K_S is negligible compared with the substrate concentration, and hence μ_{max} can be approximated by μ obtained from the batch growth data. From the results in Fig. 3.2, the mass-doubling time was obtained as 5 hours, from which the specific growth rate of the fungus was calculated for both media as:

$$\mu_{max} = \mu = \frac{\ln 2}{5} = 0.14 \text{ hr}^{-1}$$

The yield factor, $Y_{X/S}$, which is defined by the following equation

$$Y_{X/S} = -\frac{\Delta X}{\Delta S}$$

where ΔX = cell mass produced

$-\Delta S$ = substrate used,

was estimated by the cell mass concentration in the stationary phase. $Y_{X/S}$ for 0.5% glucose medium (Medium (I)) was 0.48 and $Y_{X/S}$ for 1% glucose medium (Medium (II)) was 0.54.

Q_{O_2} Measurement

The specific oxygen demand, Q_{O_2}, during the exponential growth of the fungus on 1% glucose medium (Medium (II)) was measured in two batches of the fermentation. The results are shown in Table 3.3.

Table 3.3. Q_{O_2} measurement.

Batch	Cell mass concentration (mg/ml)	Rate of oxygen consumption (mmol/liter/min)	Q_{O_2} (mmol/hr/g)
1	1.5	2.48×10^{-2}	0.99
	3.8	9.52×10^{-2}	1.51
2	0.5	0.92×10^{-2}	1.10
	2.2	3.55×10^{-2}	0.96

Mitra (6) reported the specific oxygen demand for Trichoderma viride grown on 1% glucose as 1.0 millimole of oxygen/g of dry weight of cell/hr, which is similar to the values obtained in the present work for Botryodiplodia theobromae.

From this Q_{O_2} value, the percentage of oxygen utilized for the cell mass production out of total input oxygen can be calculated as follows: Maximum cell density in the fermentor was 5.4 mg/ml. Thus, the oxygen consumption rate is

$$Q_{O_2} X = 1.0 \times 5.4 = 5.4 \text{ mmol } O_2/\text{liter-hr}$$

The aeration rate was 0.25 vvm, from which the input oxygen to the fermentor is calculated as

$$\frac{0.25 \times 60 \times 0.21 \times 10^3}{22.4} = 141 \text{ mmol } O_2/\text{liter hr}$$

Hence only 3.8% of total input oxygen was utilized by the fungus to produce the cell mass, and it can be concluded that the oxygen concentration is not a limiting growth factor during the fermentation.

Enzyme Production on Medium (I)

In investigating the production of β-glucosidase, it was realized that some guidelines such as final enzyme activity and acceptable fermentation period should be established. Since there were no established processes for β-glucosidase production from microbial sources to which we could refer, these guidelines were determined rather arbitrarily. Trichoderma viride cellulase has some β-glucosidase activity, although no quantitative data have been published. We obtained

data with four different batches of Trichoderma viride culture filtrate which indicated that the β-glucosidase activity varied batch to batch even if the filter paper activity of the enzyme solution was the same. The highest β-glucosidase activity was about 0.45 mg/ml, but usually the activity ranged from 0.2 to 0.3 mg/ml. Thus an activity of 1.0 mg/ml would be a reasonable target in the present work. It was expected that the activity would increase with the time of fermentation. Many β-glucosidase producing fungi were grown for several weeks, which is too long for practical purposes. Therefore, one week, at most 10 days, was chosen as an experimental period in the present work and, even if the enzyme production was not enough during the period, the experiment was stopped.

Figure 3.3 shows the enzyme production by B. theobromae on 1% Solka Floc medium with 0.2% starch. Constant temperature and pH were maintained at 30° C and 5.5, respectively, and the aeration rate was 0.25 vvm. The rate of β-glucosidase production was fairly slow and the final enzyme level was far below the target value. The starch concentration was monitored by measuring samples with the anthrone reagent (7). During the first 50 hours, when the fungus grew mainly on starch, starch was consumed very rapidly. However, about 0.5 mg/ml of starch (or other glucose oligomers) was not utilized throughout the fermentation period.

Since these results were reproducible and the enzyme productivity was much less than observed in the flask culture experiments, the difference between these two fermentation systems was reexamined. The pH of the medium was not controlled in the flask culture and the final pH was always at least 7.0. Thus, a fermentation without pH control

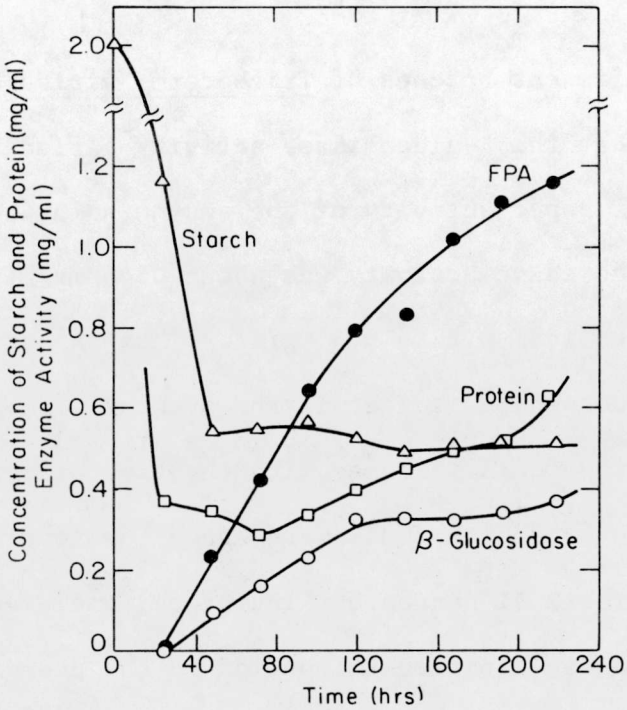

Figure 3.3. Enzyme production by *B. theobromae* in Medium (I) at constant pH.

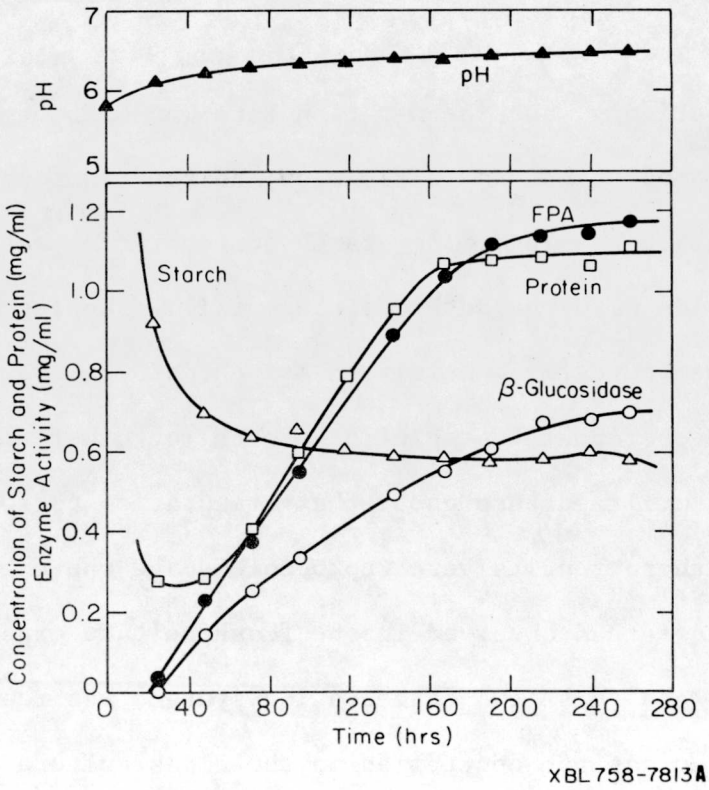

Figure 3.4. Enzyme production by *B. theobromae* in Medium (I) with no pH control.

was tested. The results shown in Fig. 3.4 indicate that considerably higher β-glucosidase activity was obtained without pH control. The pH changed gradually during the fermentation from 5.8 to 6.5 (Fig. 3.4). About 0.6 mg/ml of starch was not utilized by the fungus during the fermentation.

The oxygen supply to the fermentor is another important factor to be considered. Since the oxygen transfer in the flask culture might be different from that in the 14 liter fermentor, various aeration rates, from 0.09 to 0.5 vvm, were examined using the same medium without pH control. The results, however, show no significant effect of aeration rate on the enzyme productivity. Because antifoam agent was usually used in the 14 liter fermentor, but not in the flask culture, fermentation was carried out without using antifoam agent to find its effect on the enzyme production. However, as was found in the flask culture experiments described previously, no significant effect of the antifoam agent was observed.

Newsprint (1.5% in the medium) was tested for enzyme production but the activity obtained was lower (about 75% of original for β-glucosidase and 85% for cellulase) than that with Solka Floc medium (1% suspension).

Enzyme Production on Medium (II)

After failing to produce enough β-glucosidase activity from Botryodiplodia theobromae culture grown on Medium (I), Medium (II), which had been developed for the production of cellulase by Trichoderma viride, was examined, and the results were unexpectedly better. Thus,

several batch experiments were carried out with this medium containing 1% Solka Floc as a sole carbon source, and the optimum growth conditions were investigated. The aeration rate was fixed at 0.25 vvm for all experiments.

The first series of experiments was done at 30° C, and four batches were maintained at different pH levels to examine the effect of pH on the production of β-glucosidase. The progress curves of the β-glucosidase production are illustrated in Fig. 3.5, which show that the pH optimum for the fermentation is 5.0.

Temperature optimum was sought at pH 5.0, and the results are shown in Fig. 3.6. The rate of the enzyme production at 29° C was apparently less than at 30° C. The initial enzyme production rates at both 30 and 31° C were the same, but after 6 days of the fermentation the higher activity was observed at 30° C.

Figures 3.7 and 3.8 illustrate the levels of three enzyme activities— β-glucosidase, C_x and filter paper activity— at day seven, which is a practical fermentation period, as functions of pH and temperature. The maximum production of all three enzymes was at pH 5.0. Although the temperature optimum for the production of β-glucosidase and C_x activities was 30° C, the highest filter paper activity was obtained at 29° C, where the level of β-glucosidase was sharply decreased.

Figure 3.9 illustrates the progress of the fermenation at the optimum conditions obtained above. The filter paper activity and the β-glucosidase activity were increasing in parallel with the increase of protein concentration, while the C_x activity had leveled off, after about 5 days after inoculation. Figure 3.9 also shows the data obtained with the medium containing 1% Solka Floc and 0.2% starch. The

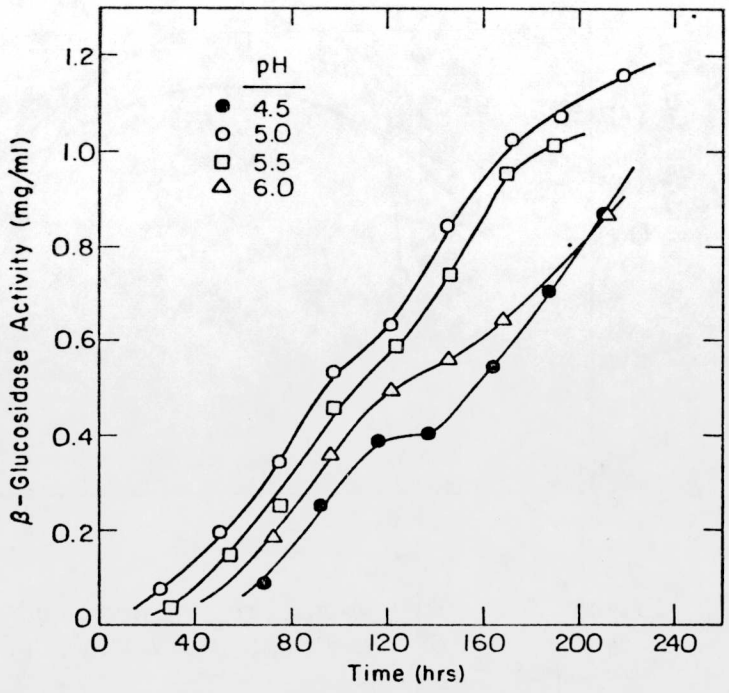

Figure 3.5. Effect of pH on β-glucosidase production by *B. theobromae* in Medium (II) at 30°.

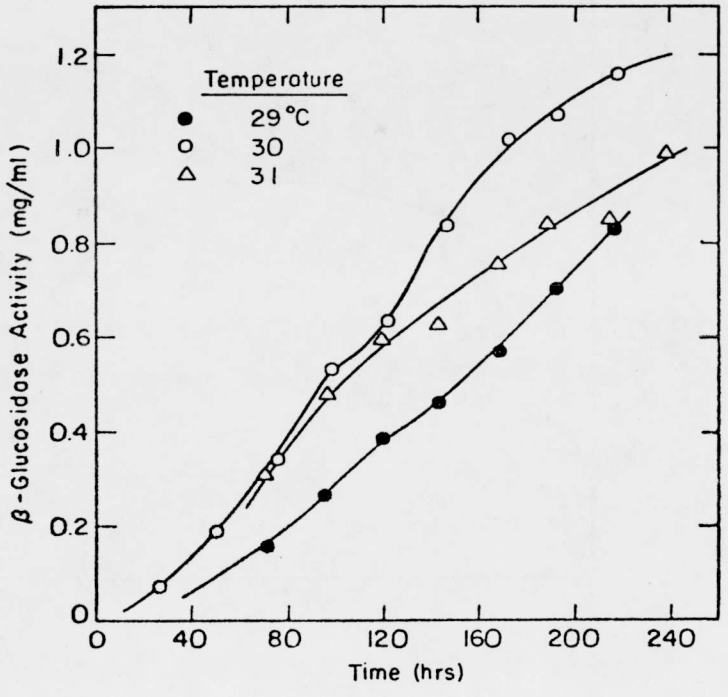

Figure 3.6. Effect of temperature on β-glucosidase production by *B. theobromae* in Medium (II) at pH 5.0.

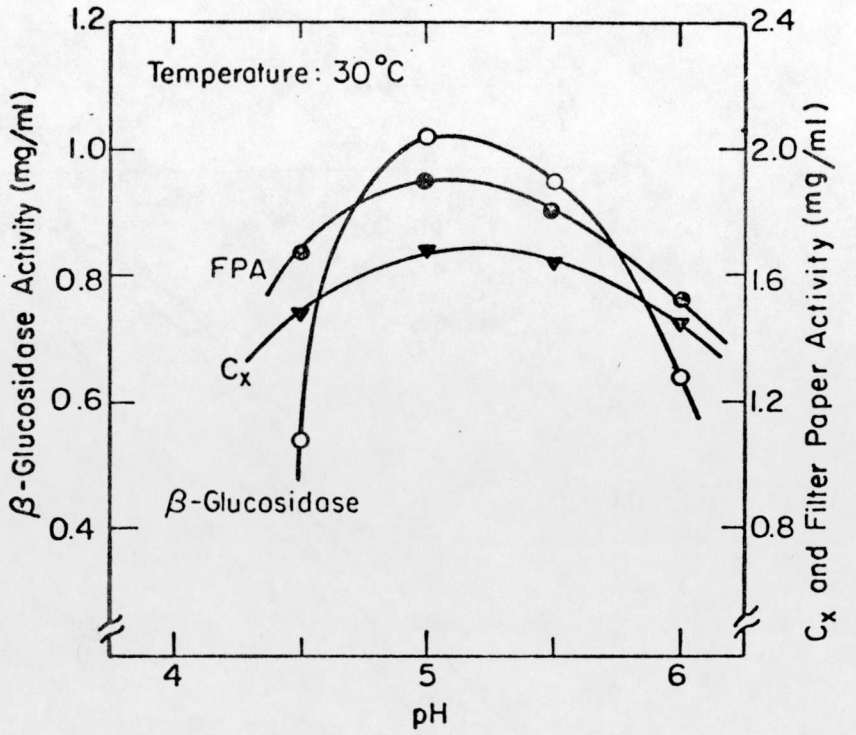

Figure 3.7. Enzyme activities produced by *B. theobromae* at various pH levels.

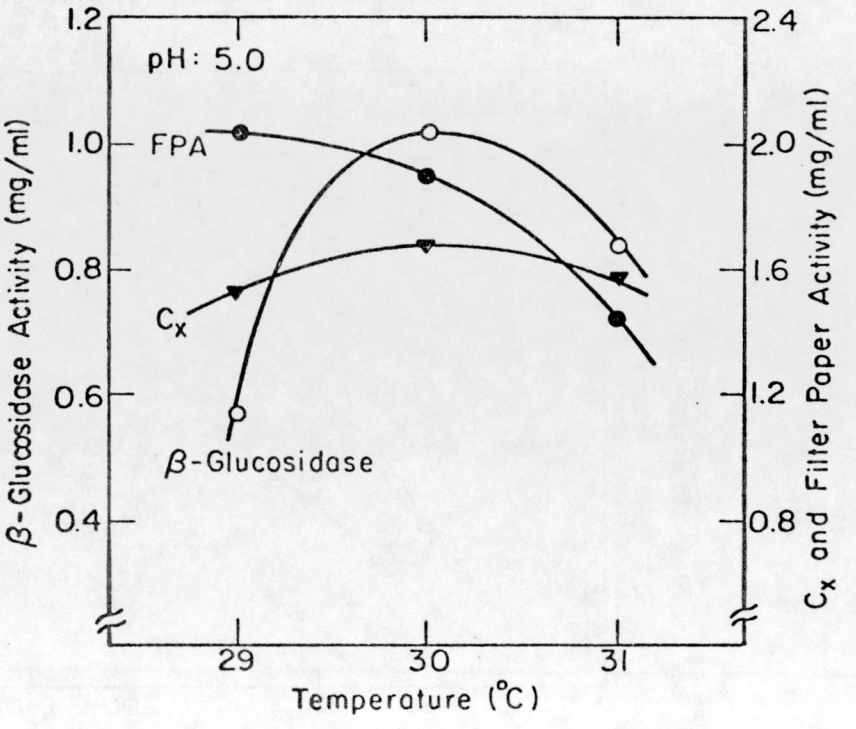

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Figure 3.8. Enzyme activities produced by *B. theobromae* at various temperatures.

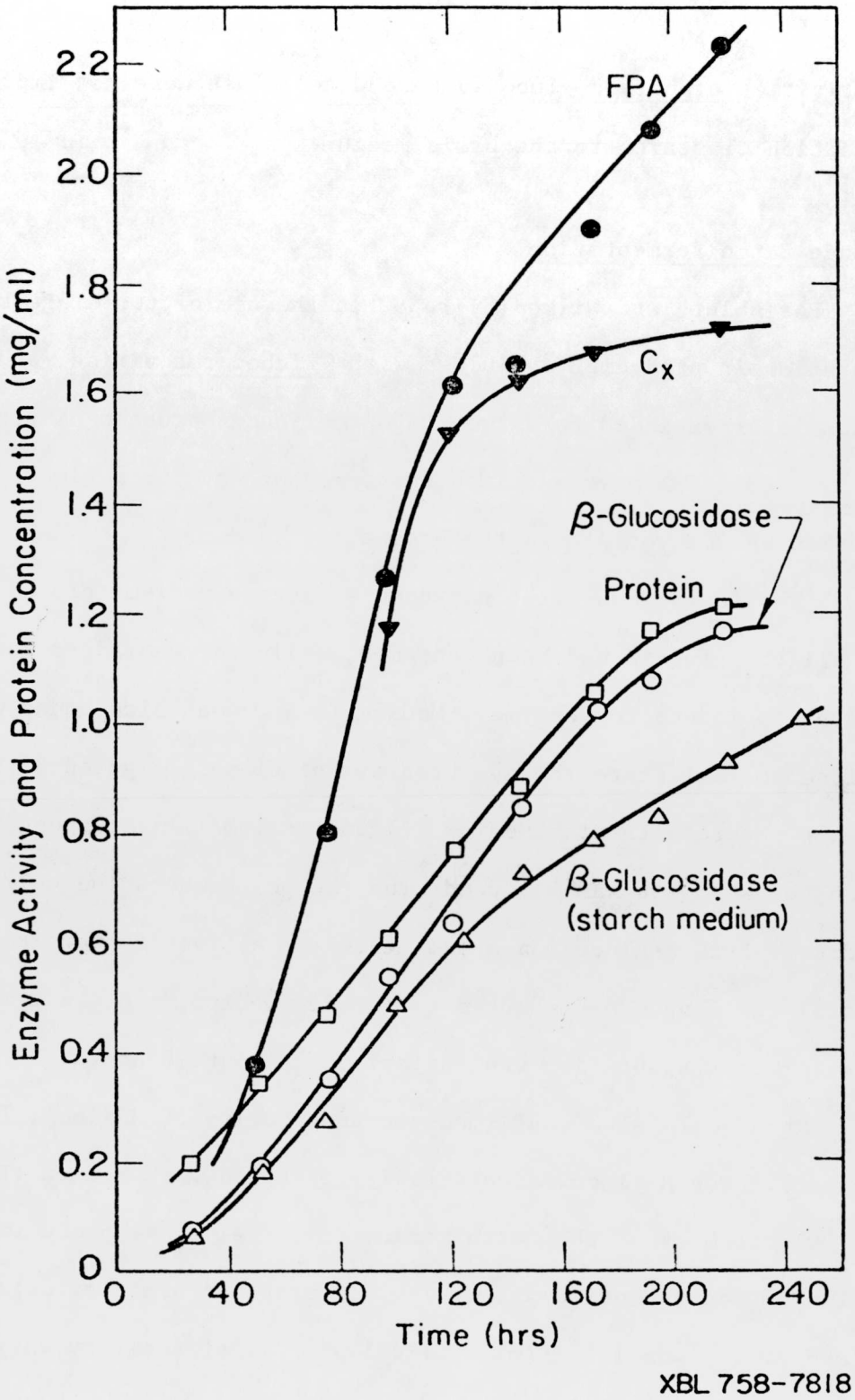

Figure 3.9. Enzyme production by *B. theobromae* at optimum pH (5.0) and temperature (30°).

productivities of both β-glucosidase and cellulase were not improved by the addition of starch to the basic medium.

Two-Stage Batch Fermentation

Rosenbluth and Wilke (8) reported the use of two-stage batch fermentation in producing cellulase with Trichoderma viride, and the idea was later extended to a two-stage continuous process of producing the enzyme (9). We have examined the same concept for β-glucosidase production by Botryodiplodia theobromae.

The fungus was first grown on a glucose medium (glucose 0.5%). After all the glucose had been consumed, cellulose was added to the fermentor to induce the enzyme. Medium (1.5 l) was also added with the cellulose to facilitate the addition of cellulose. Figures 3.10 and 3.11 show the results with Medium (I) and Medium (II), respectively. Time on the abscissa was measured from the point where the cellulose was added. Both β-glucosidase and cellulase activities were not detected until the fungus had adapted to the new medium, and the time required for this adaptation was estimated as about 18 hours.

These data also confirmed the superiority of Medium (II) over Medium (I). The β-glucosidase activity obtained with Medium (II) was about 30% higher than that with Medium (I). Cellulase productivity was also improved considerably. The filter paper activity obtained with Medium (I) was 1.1 mg/ml, while 1.7 mg/ml of activity was produced with Medium (II).

Mitra (6) proposed a low cost medium for the production of cellulase with Trichoderma viride. Its composition is: 2.5 g/l of

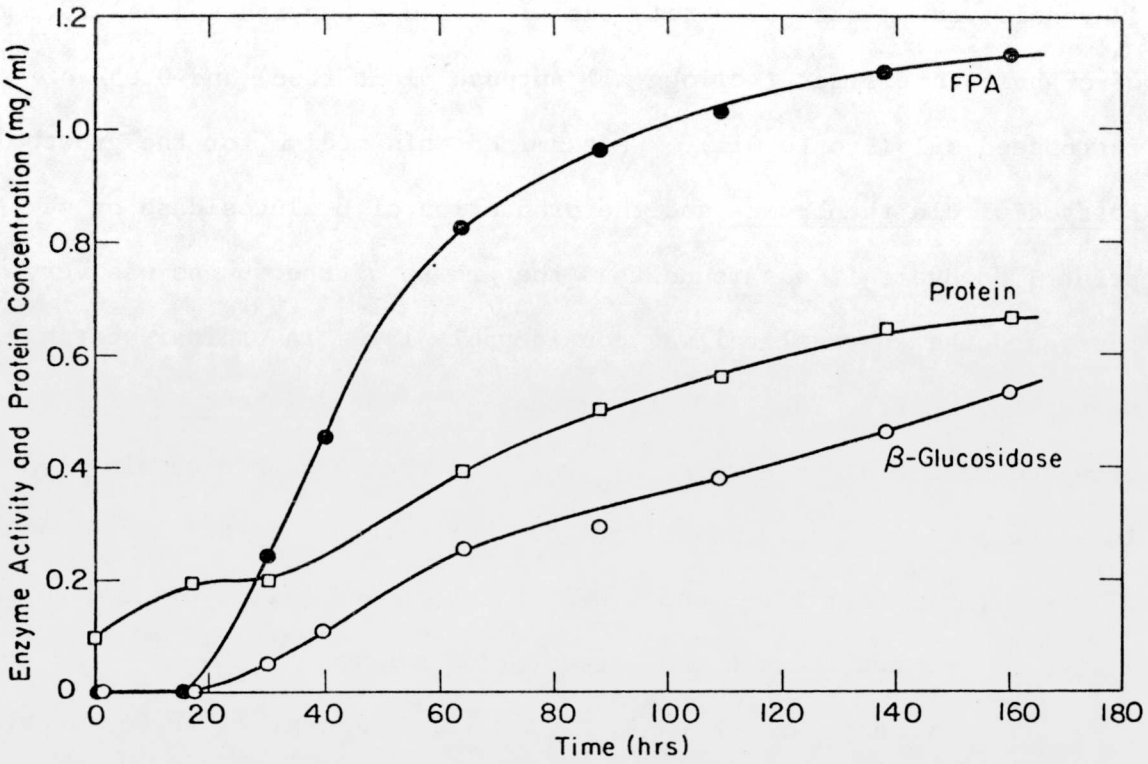

Figure 3.10. Two stage batch fermentation of *B. theobromae* in Medium (I).

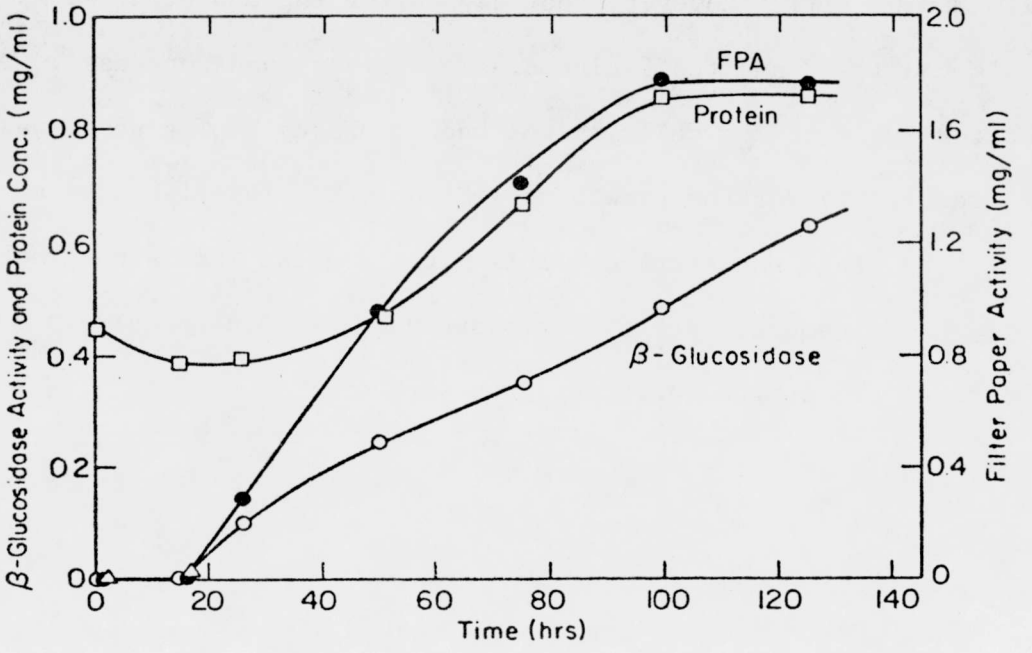

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Figure 3.11. Two stage batch fermentation of *B. theobromae* in Medium (II).

inorganic fertilizer (commercial name: Kellogg Superphosphate), 0.5 g/l of organic fertilizer (Kellogg all purpose plant food) and 0.05% of cottonseed oil (Proflo Oil). We examined this medium for the growth of Botryodiplodia theobromae and the production of β-glucosidase on newsprint. However, it was found that the growth of the fungus was very poor, and the enzyme level was considerably lower in ordinary batch fermentation. Later, Yang (10) found that even for Trichoderma viride the proposed medium did not work very well and that a better combination of these components should be 0.7 g/l of the inorganic fertilizer, 3.5 g/l of the organic fertilizer and 0.07% of cottonseed oil. This medium was tested in the two stage batch fermentation system.

The results are shown in Fig. 3.12. The rate of the production of β-glucosidase was fairly rapid and comparable to the data obtained with Medium (II). However, four days after the newsprint was added the cells had lysed and the β-glucosidase activity had decreased. The reason for lysis of the cells is not known. When 1% of glucose was used in Medium (II) for the growth stage, the cells lysed shortly after the induction stage was started. This might suggest that some growth factors (carbon source, protein or inorganics) were used up very quickly and hence the fungus could not grow further.

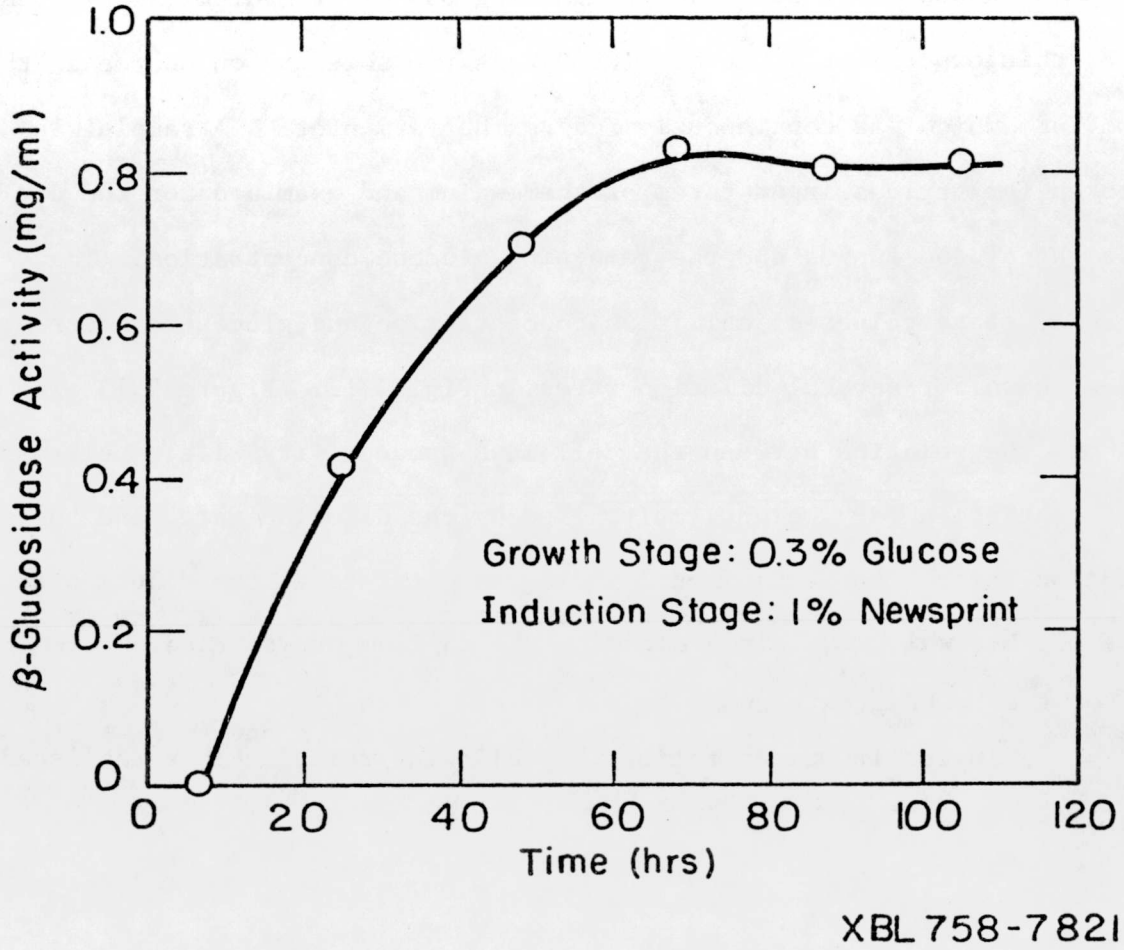

Figure 3.12. Two stage batch fermentation of *B. theobromae* in a low cost medium.

3.1.3 Continuous Fermentation

Growth Stage

A one liter fermentor containing 600 ml of Medium (II) was used for this experiment. Glucose (0.5%) was the sole carbon source in the medium, which was continuously fed to the fermentor. A sample was taken for various input rates of the medium and examined for the dry weight of the fungus and the remaining glucose concentration. The steady state values of cell mass concentration and glucose concentration are shown for several dilution rates in Fig. 3.13. Figure 3.13 also shows the relation between the cell mass productivity, i.e., cell mass concentration (dry weight) multiplied by the dilution rate, and the dilution rate. The maximum cell yield was obtained at the dilution rate of 0.1 hr, which is fairly close to the maximum growth rate obtained from the batch growth curve.

In continuous operation the following relation is established (5):

$$\mu = \frac{F}{V} = D$$

Using the definition of yield factor for substrate conversion to cell mass, which is assumed constant and independent of substrate concentration, S, for a given condition, we can calculate the cell mass and cell mass productivity by the following equations:

$$X = Y_{X/S} \left(S_0 - \frac{DK_S}{\mu_{max} - D} \right)$$

$$PX = Y_{X/S} \; D \left(S_0 - \frac{DK_S}{\mu_{max} - D} \right)$$

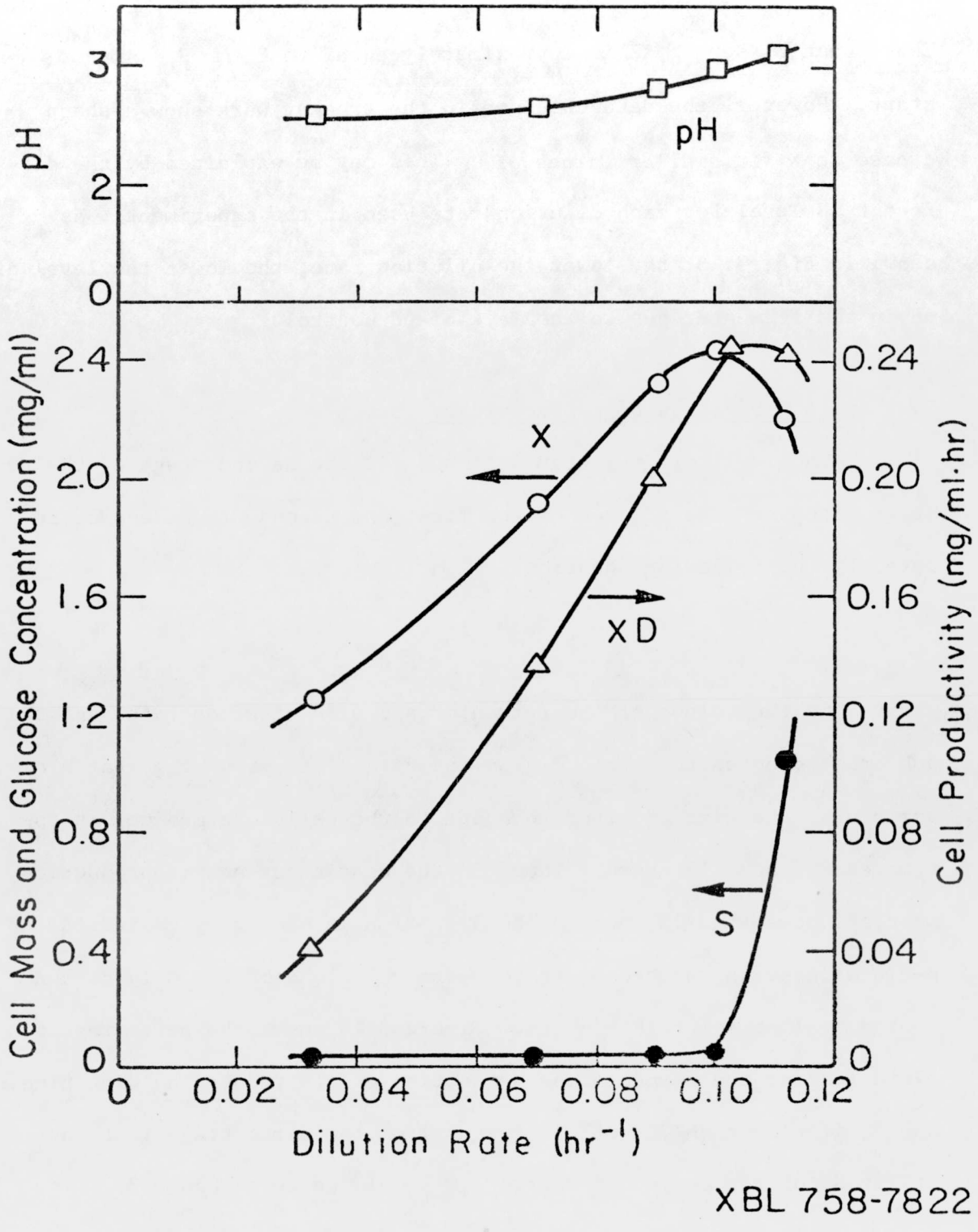

Figure 3.13. Continuous production of β-glucosidase by *B. theobromae*. Growth stage.

Thus, when $D << \mu_{max}$, X is approximately equal to $Y_{X/S} \cdot S_0$ which is constant. However, the data obtained in the present work show a sharp decrease in X for smaller values of D. This may be explained by the different pH level for each dilution rate used in the experiment. As shown in Fig. 3.13, the lower the dilution rate, the lower the level of pH in the fermenter due to the lack of pH control.

Continuous Production of β-Glucosidase

The 14 liter fermentor was used for the second stage of the two stage process. The volumes of the first and second stages can be related by the following equation:

$$V_1 D_1 = V_2 D_2$$

where V is the volume of the fermentor and D is dilution rate, and the subscript denotes the stage. From the data of the two stage batch experiments, the time required to adapt to the cellulose medium was estimated as about 18 hours. Thus, in the continuous enzyme production process, more than 18 hours of holding time is necessary to induce the β-glucosidase in the second stage where cellulose is the only carbon source available for the fungus. Figure 3.14 shows the percentage of fluid leaving the fermentor as a function of its reduced holding time. Hence, if more than 70% of the fungus from the first stage is to be utilized for the enzyme production, Θ should be less than 0.35.*

*To prepare Fig. 3.14 a complete mixing condition was assumed, i.e., the exit age distribution function, E, was expressed by $\exp(-\Theta)$.

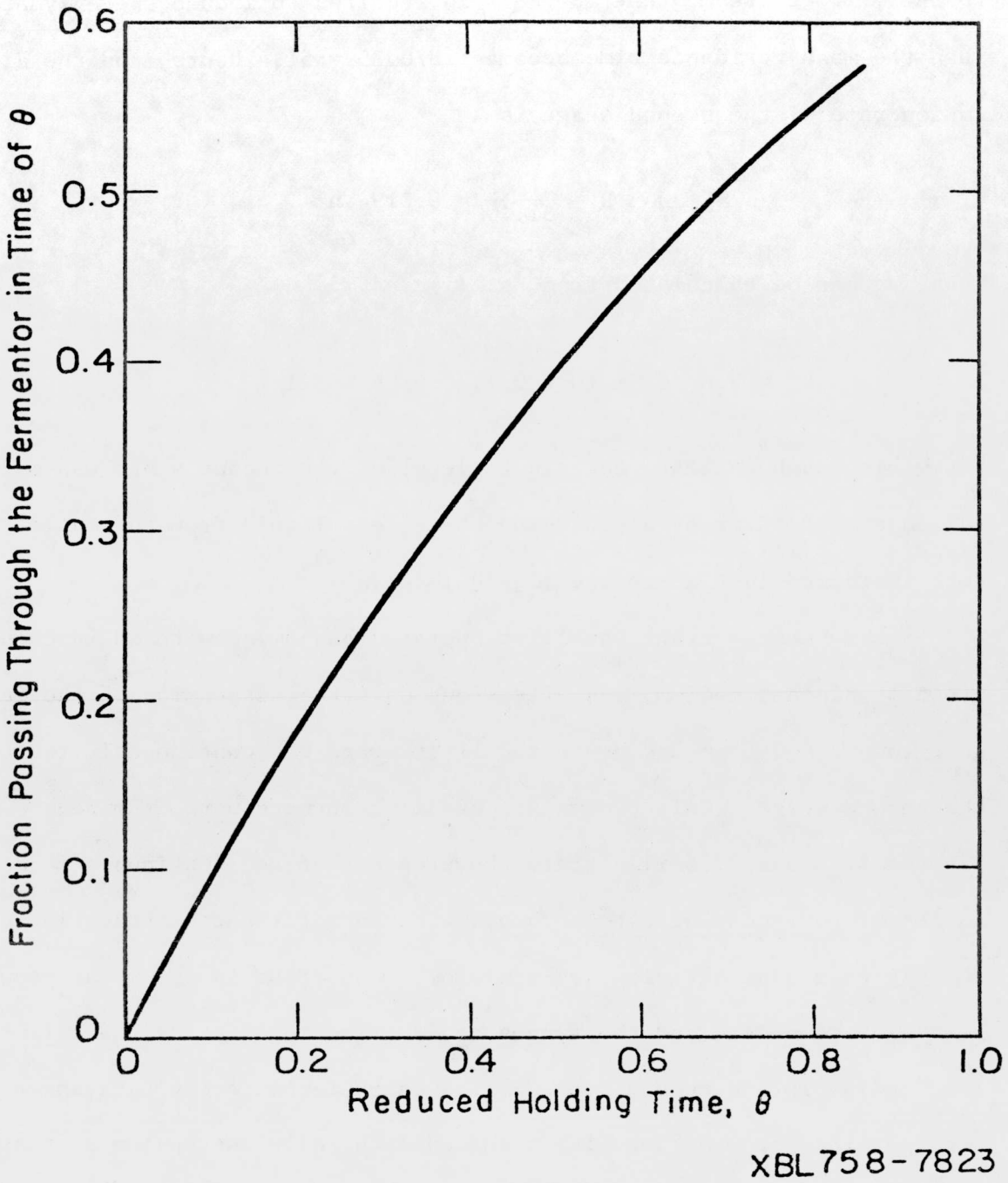

Figure 3.14. Residence time distribution in a completely mixed tank.

Further, if we assume that 18 hours is required to induce the enzyme, then the mean residence time becomes 18/0.35 = 51.4 hours, and the dilution rate of the second stage is

$$D = \frac{1}{51.4} = 0.0194 \text{ hr}^{-1}$$

Thus, V_2 can be calculated from Eq. (1),

$$V_2 = V_1 D_1 / D_2 = (0.6 \times 0.1)/0.0194 \simeq 3.1 \text{ ℓ}$$

From this rough estimate the liquid level of the second stage was maintained at 3.5 liter by discharging the excess liquid from the outlet tube installed just above the liquid level.

The second stage was first operated batchwise with an inoculum grown on glucose medium, and after four days the suspension of the fungus grown on glucose medium in the first stage was continuously fed to the second stage. Only two values of dilution rate were examined. Figures 3.15 and 3.16 illustrate the progress of the continuous fermentation of Botryodiplodia theobromae. The data for batch production of the β-glucosidase activity was very low because the inoculum was grown on a glucose medium and the fungus needed time to adapt to the cellulose medium in the second stage. (The batch fermentation data shown were obtained from an inoculum prepared with cellulose medium so that adaptation had already taken place.) The sudden increase in the β-glucosidase activity at 200 hours may be explained by this adaptation phenomenon. A steady state was attained six days after continuous operation had been started. In contrast to the β-glucosidase activity, the

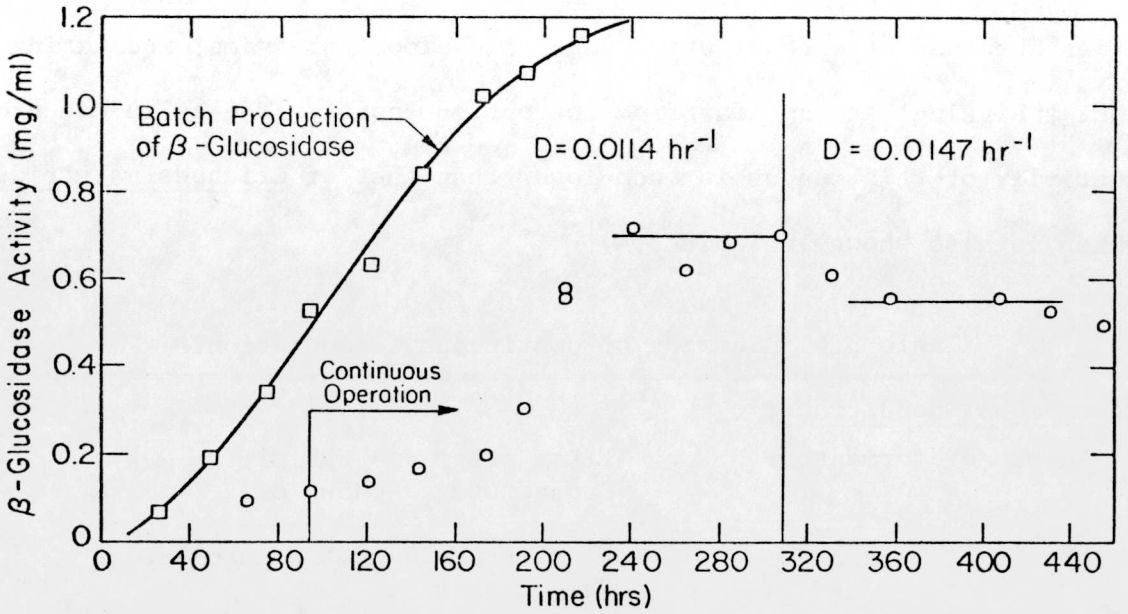

Figure 3.15. Continuous production of β-glucosidase by *B. theobromae*. Induction stage.

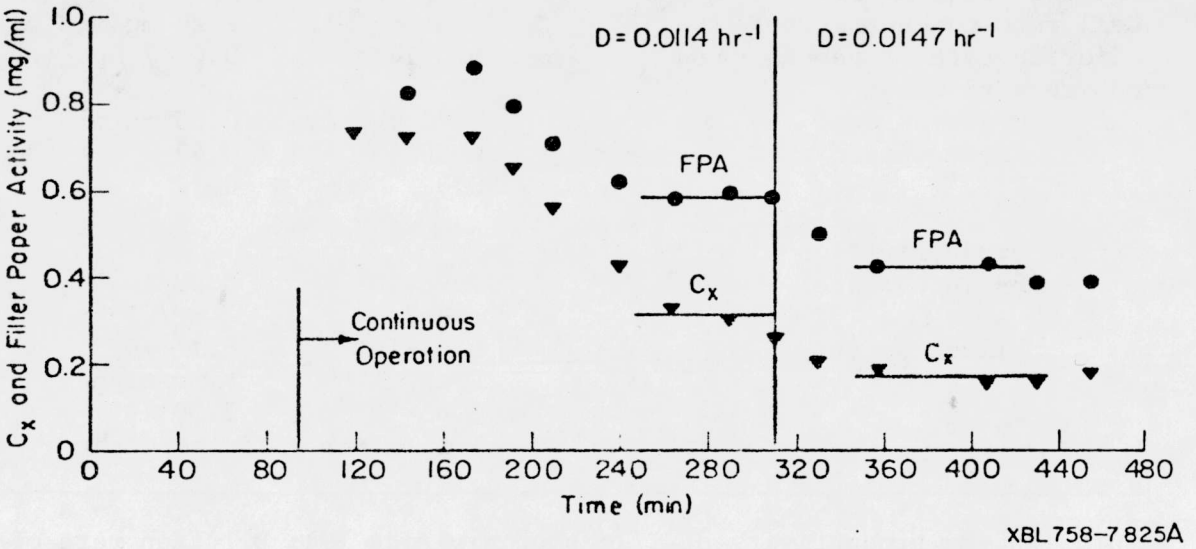

Figure 3.16. Continuous production of cellulase by *B. theobromae*. Induction stage.

cellulase activity based on both the C_x and filter paper activities decreased as the continuous fermentation proceeded.

Table 3.4 summarizes the results. The β-glucosidase productivities obtained at both dilution rates are almost the same, indicating that the values are not far from the optimum point. The cellulase productivity of this fungus is much lower than that of Trichoderma viride, which is also shown in Table 3.4.

Table 3.4. Summary of continuous fermentation.

Operational conditions:			
Volume of fermentor:	First stage	600 ml	
	Second stage	3500 ml	
pH:	First stage	not controlled	
	Second stage	5.0	
Temperature:		30° C	
Results:			
Dilution rate of first stage		$0.067\ hr^{-1}$	$0.086\ hr^{-1}$
Cell mass concentration		1.84 mg/ml	2.28 mg/ml
Dilution rate of second stage		$0.0114\ hr^{-1}$	$0.0147\ hr^{-1}$
Enzyme activities			
β-glucosidase		0.70 mg/ml	0.55 mg/ml
Filter paper		0.58	0.42
C_x		0.32	0.17
Enzyme productivities*			
β-Glucosidase		7.98×10^{-3} mg/ml·hr	8.09×10^{-3} mg/ml·hr
Filter paper		6.61×10^{-3} mg/ml·hr	6.17×10^{-3} mg/ml·hr
C_x		3.65×10^{-3} mg/ml·hr	2.50×10^{-3} mg/ml·hr

*Cf. Enzyme productivity of Trichoderma viride (9): Dilution rate of second stage was $0.02\ hr^{-1}$, and filter paper activity of 0.7 was obtained. Thus the productivity of cellulase by T. viride is 14.0×10^{-3} mg/ml·hr.

3.2 Enzyme Characterizations

3.2.1 Heat Stability of β-Glucosidase

Generally β-glucosidase activity is more heat labile than cellulase activity. The limited data (at 40,60 and 100° C) available for the β-glucosidase from Botryodiplodia theobromae (2) show that even at 40° C the heat denaturation of the enzyme is very rapid. Since the presumed reaction temperature for enzymatic hydrolysis of cellulose is 50°C, more information on the heat stability of the enzyme was required.

The culture filtrate of Botryodiplodia theobromae was buffered at pH 5.0 with 0.05 M sodium acetate. The β-glucosidase of almond emulsin (Sigma Chemical Co.) was also examined as a reference. The purified β-glucosidase was dissolved into 0.05 M sodium acetate buffer solution at pH 5.0 with a concentration such that the β-glucosidase activity was almost equal to that of a culture filtrate of the fungus. About 10 ml of each of these enzyme solutions was placed in test tubes and incubated in a constant temperature bath at 45--51° C. After a certain period of time the β-glucosidase activity was measured. The results are shown in Fig. 3.17.

The almond emulsin shows a very rapid inactivation rate, while the stability of β-glucosidase from Botryodiplodia theobromae is moderate. An Arrhenius plot was obtained by using the initial heat inactivation rates at different temperatures (Fig. 3.18), and the activation energy of the heat inactivation reaction for each enzyme was calculated as 117 kcal/mole for B. theobromae β-glucosidase and 40.6 kcal/mole for almond emulsin.

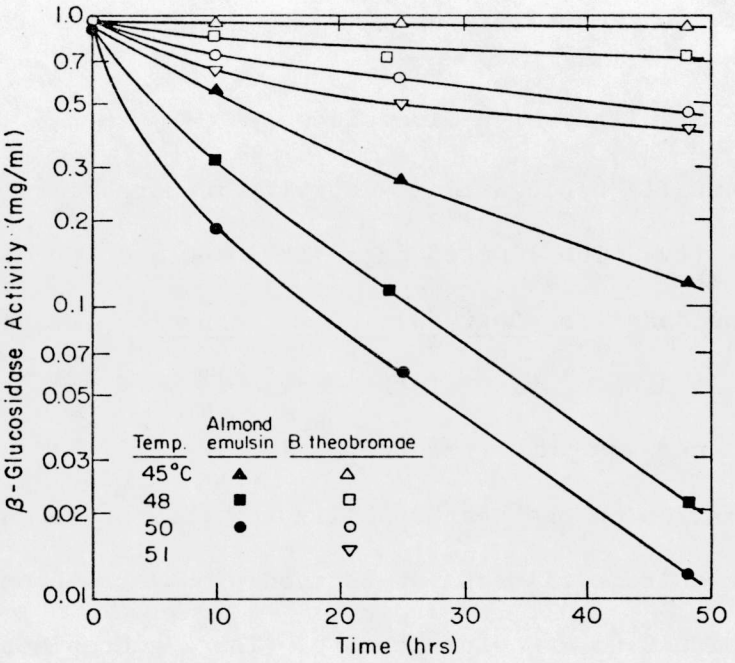

Figure 3.17. Heat denaturation of β-glucosidases.

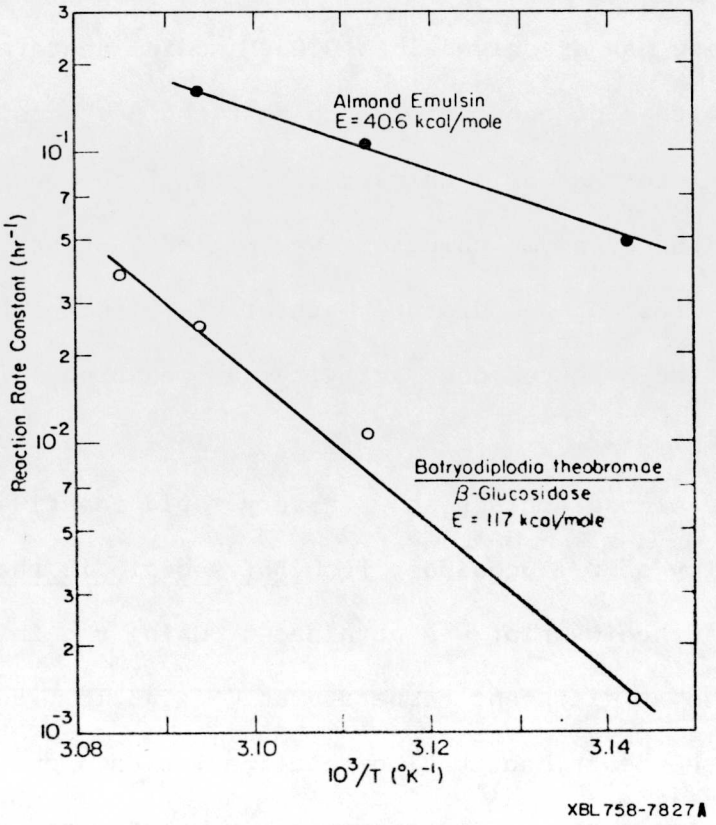

Figure 3.18. Activation energy for inactivation of β-glucosidases.

It might be interesting to note that although the heat stability of β-glucosidase from almond emulsin is relatively low, the effect of the enzyme on cellulose hydrolysis with cellulase is significantly high even at 50° C (see Chapter 1, Fig. 1.1). This suggests that the presence of a substrate (cellulose or cellobiose) may stabilize the enzyme. On the other hand, the data for fungal β-glucosidase show that a temperature of 50° C is too high to utilize this enzyme. However, it may be possible that a more stable enzyme--substrate complex is formed during the hydrolysis reaction. Further discussion on this subject is given in Chapter 7.

Umezurike (2) reported that the loss of activity of the β-glucosidase from Botryodiplodia theobromae during 15 minutes of preincubation was about 40% at 40° C and 50% at 60° C. However, the activity was assayed also at 40° C for 15 minutes with p-nitrophenyl-β-D-glucoside. Thus, denaturation presumedly continued during the assay, and the result is rather indeterminate. On the other hand, when cellobiose was used in our work for the assay of β-glucosidase activity, the enzyme seemed fairly stable even as high as 48° C (Fig. 3.17) so that the temperature of 40° C for the assay could be justified.

3.2.2 pH Optimum for β-Glucosidase Activity

pH is a very important factor for most enzymatic reactions. Mandels and Weber (1) reported that the pH optimum for extensive hydrolysis (10 days) at 50° C was pH 4.8 for the T. viride cellulase system, and they observed a sharp decrease of conversion at pH larger than 5.5 (Fig. 3.19). Since the mixed enzyme system is the prime subject in

this work, it is desirable that the pH optimum for β-glucosidase be very close to that for cellulase.

pH could be optimized both in terms of higher conversion and of stability of enzyme, but these numbers may coincide. In present work the optimum pH was obtained in terms of stability of the β-glucosidase.

About 10 ml of culture filtrate of Botryodiplodia theobromae was taken into a small tube and the pH was adjusted by adding concentrated hydrochloric acid. The amount of hydrochloric acid was so small that the volume change was not significant for each preparation. The pH adjusted enzyme solution was then placed in the water bath at 48° C for 2 hours and the change of β-glucosidase activity during the preincubation period was examined. A temperature of 48° C instead of 50° C was chosen so that the effect of heat inactivation was minimized (see Section 3.2.1).

The results were shown in Fig. 3.19. The enzyme was found to be very stable in the pH range from 5.0 to 6.7. The lower part of this optimum range covers the pH optimum of cellulase.

Umezurike (4) measured the pH optimum in terms of the reaction rate using p-nitrophenyl-β-D-glucoside as a substrate. The result was very similar to that for Trichoderma viride cellulase except that the optimum point was pH 5.0 instead of pH 4.8. β-Glucosidase of almond emulsin was reported to have an optimum for the reaction rate at pH 5.5 (11), but no data are available for the pH optimum in terms of stability of the enzyme.

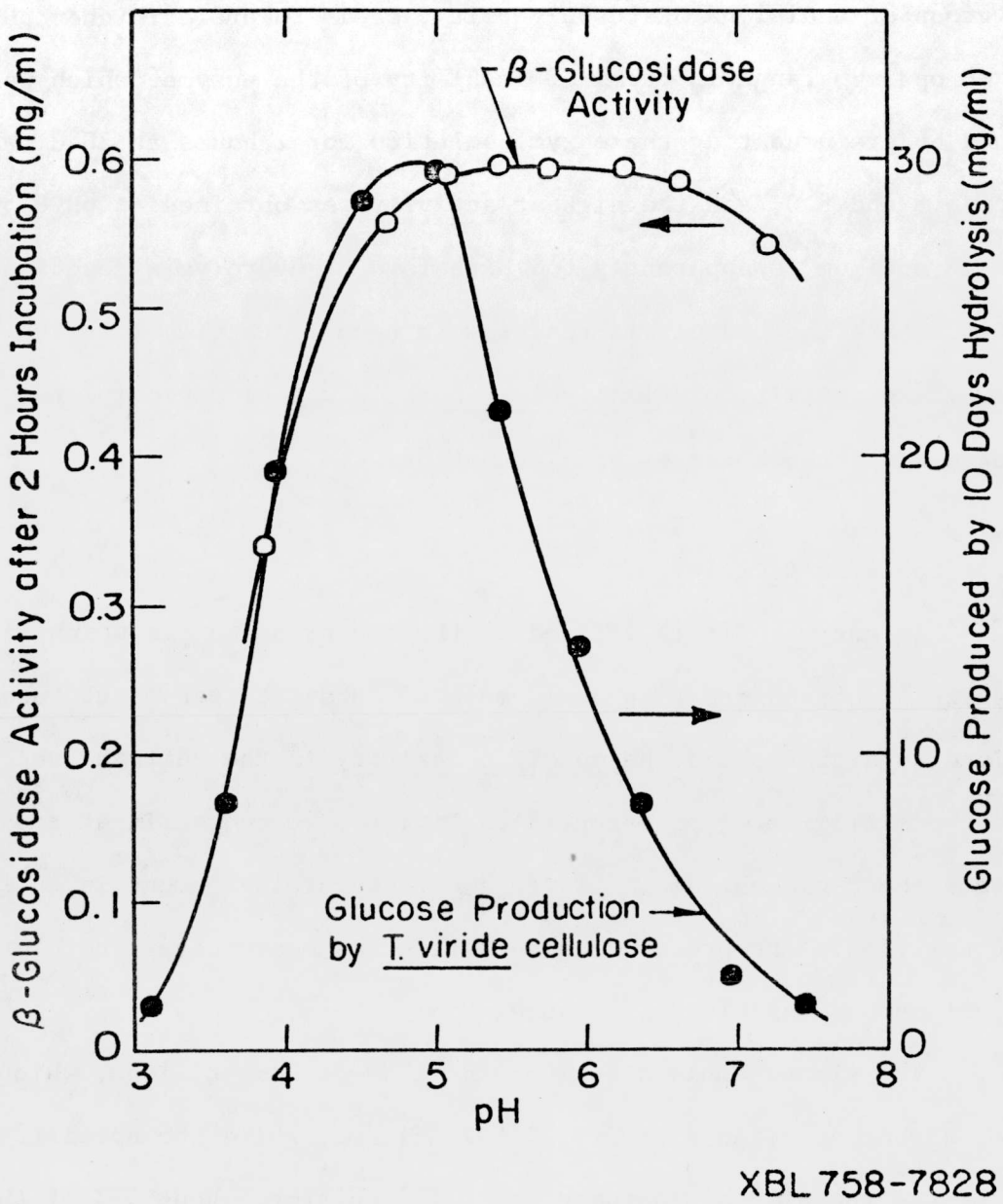

Figure 3.19. pH optimum for β-glucosidase from *B. theobromae*.

Bacterial β-glucosidase, which has been extensively studied by the group of Louisiana State University, seems to prefer higher pH. The pH optimum range in terms of stability of the enzyme, which was examined by preincubating the enzyme solution for 2 hours at 30° C, was between 6.5 and 8.0, and the highest activity was obtained at pH 6.5 (12). This pH optimum is apparently too high for the hydrolysis reaction. At pH 5.0, where the hydrolysis reaction is usually carried out, the β-glucosidase activity of Alcaligenes faecalis was almost completely denatured after two hours of pre-incubation.

3.2.3 Specific Activity

An enzyme unit is defined as the amount of enzyme which will catalyze the transformation of 1 μmole of substrate per minute under defined conditions, and the specific activity is the units of the enzyme per milligram of protein (13). Figure 3.20 shows linear relations between the β-glucosidase activity and the protein content in the enzyme solution. The protein concentration was measured by the biuret reagent method described in Chapter 2.

The almond emulsin β-glucosidase has a larger slope, which shows higher specific activity, 0.542 unit/mg, while the specific activity of the fungal β-glucosidase was 0.177 unit/mg, about 33% of that of almond emulsin. This is quite reasonable because the almond emulsin is highly purified β-glucosidase while the fungal β-glucosidase may contain other enzymes such as cellulase or other proteins.

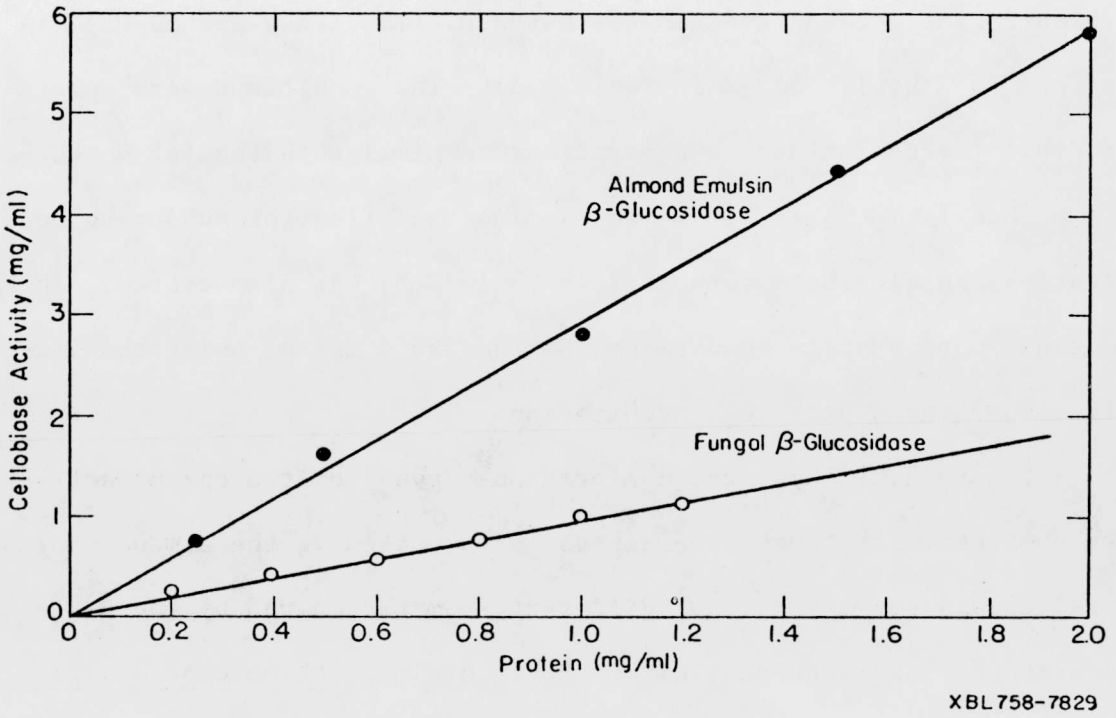

Figure 3.20. Cellobiase activity of β-glucosidases.

3.2.4 Kinetic Considerations

Although several assay methods for cellulase activities have been proposed by different investigators, the method developed by Mandels and Weber (1) is now widely accepted primarily because of its simplicity. On the other hand, the assay method for β-glucosidase has yet to be standarized. Table 3.5 lists the published assay procedures in which cellobiose was used as a substrate. The assay method used in this work is also listed (E in Table 3.5). The conditions were selected so that the cellobiose concentration was similar to that of Umezurike's method (A in Table 3.5). To examine the effect of substrate concentration, an alternate method (F in Table 3.5) was also tested. The conditions were exactly the same as Umerzurike's method using the lowest limit of the substrate concentration.

Figure 3.21 shows the calibration curves for the enzyme activities obtained with these two methods. Unexpectedly, the almond emulsin β-glucosidase gave a curve different from that given by the β-glucosidases of fungal sources, either Botryodiplodia theobromae or Trichoderma viride, as shown in Fig. 3.21. This means that, even if the same activity were obtained with one assay method, say method A in Table 3.5, the activity measured with the other method (B in Table 3.5) would be quite different.

Since the assay methods differ in substrate concentration, the observation above may have been due to differences between the enzymes in affinity toward the substrate. Therefore, the enzyme-substrate interaction was investigated using the simple kinetic model proposed by Michaelis and Menten (5). In this model the enzyme, E, combines

Table 3.5. Published assay methods of cellobiase activity.

Investogators	Cellobiose concentration	Temperature and incubation period
A) Umezurike (4)	2 ml of 0.05 to 2.0 mM cellobiose in 0.05 M acetate buffer (pH 5.0) plus 0.1 ml of enzyme solution	40° C 15 min.
B) Toyama (14)	0.5 ml of 0.05% cellobiose in 0.1 M acetate buffer, pH 5.0, plus 0.5 ml of enzyme solution	40° C 2 hours
C) Pettersson et al. (15)	0.5 ml of 0.5% cellobiose in 0.05 M acetate buffer, pH 5.0, plus 0.1 ml of enzyme solution	40° C 10 min.
D) Wood (16)	1 ml of 0.1 M acetate buffer, pH 5.0, plus 1 ml of 0.4% cellobiose solution plus 1 ml of enzyme solution	37° C 2 hours
E) This work	1 ml of 0.02 M cellobiose in 0.05 M acetate buffer, pH 5.0, plus 0.2 ml of enzyme solution	40° C 15 min.
F) Method examined in this work	2 ml of 0.002 M cellobiose in 0.05 M acetate buffer, pH 5.0, plus 0.1 ml enzyme solution	40° C 15 min.

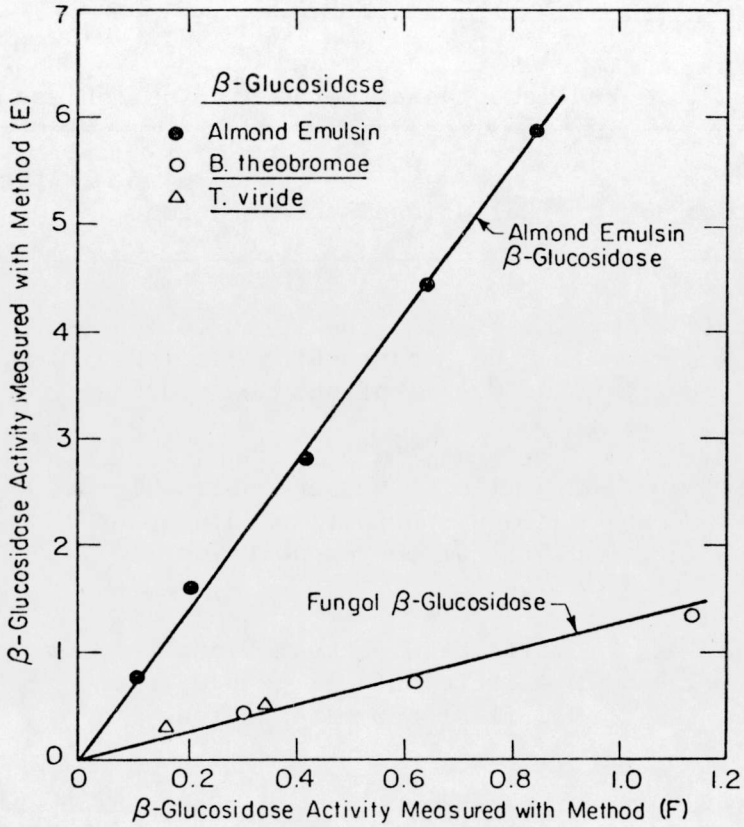

Figure 3.21. Comparison of β-glucosidase activities measured by different methods.

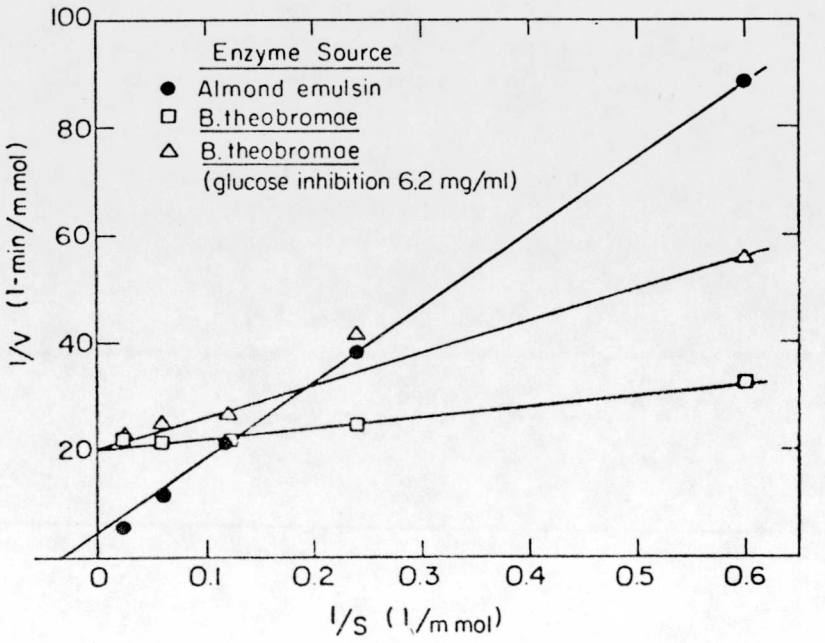

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Figure 3.22. Lineweaver-Burk plot for effect of cellobiose on β-glucosidases.

reversibly with substrate, S, forming an enzyme-substrate complex, E-S, which irreversibly decomposes to form product, P, and free enzyme, E. Hence

$$E + S \underset{\leftarrow}{\overset{\rightarrow}{}} E\text{-}S \rightarrow E + P$$

If the substrate concentration is much greater than the total enzyme concentration, the Michaelis-Menten equation is obtained.

$$V = \frac{V_{max}\ S}{K_m + S}$$

where V = reaction rate

V_{max} = maximum rate of reaction

S = concentration of substrate

K_m = Michaelis-Menten constant

From this definition it is obvious that K_m is inversely proportional to the chemical affinity of the enzyme for the substrate. Values of K_m were obtained by Lineweaver-Burk analysis (5), where 1/V, the inverse of the initial reaction rate, was plotted against 1/S, initial substrate concentration. The initial reaction rate was estimated by measuring the concentration of glucose produced from 1.6 to 43 mM cellobiose after 15 minutes reaction at 40° C. The results are shown in Fig. 3.22. From the intercepts on the abscissa, K_m for the almond emulsin was 31.3 mmole/ml and for the fungal β-glucosidase 1.07 mmol/ml.

Since the smaller K_m means stronger affinity, it was concluded that the fungal β-glucosidase either from Botryodiplodia theobromae or Trichoderma viride has much stronger affinity for the substrate,

cellobiose, than has almond emulsin β-glucosidase. This result simply suggests that it is essential to use a high concentration of cellobiose to obtain actual enzyme activity. Although this general conclusion should have been known well, the assay conditions used by Wood (16), Toyama et al. (14) and Berghem et al. (15) listed in Tables 3.5 are apparently not adequate because of their extremely low substrate concentrations.

The product inhibition of this enzyme system was also investigated by the same Lineweaver-Burk plot as shown in Fig. 3.22. Glucose (6.2 mg/ml) was added to the cellobiose solution which contained 0.002 M to 0.05 M cellobiose and then the enzyme solution was added to the mixture to initiate the reaction. Although the data were scattered considerably because of the high background glucose concentration compared with the glucose produced by the reaction, the results indicated that the inhibition by glucose was competitive and the dissociation constant of the enzyme-glucose complex was 17.6 mmol/ml.

Figure 3.23 shows other experimental data indicating the inhibitory effect of glucose on the hydrolysis reaction of cellobiose. Cellobiose (25 ml of 0.02 M) in 0.05 M acetate buffer, pH 5.0, was incubated with 5 ml of the enzyme solution at 50° C. At certain intervals a sample was taken out and the concentration of produced glucose was measured. To see the effect of glucose inhibition, 0.33 to 1.67 mg/ml of glucose was added to the cellobiose solution and the same experiment was carried out. As shown in Fig. 3.23, the inhibitory effect of glucose is evident.

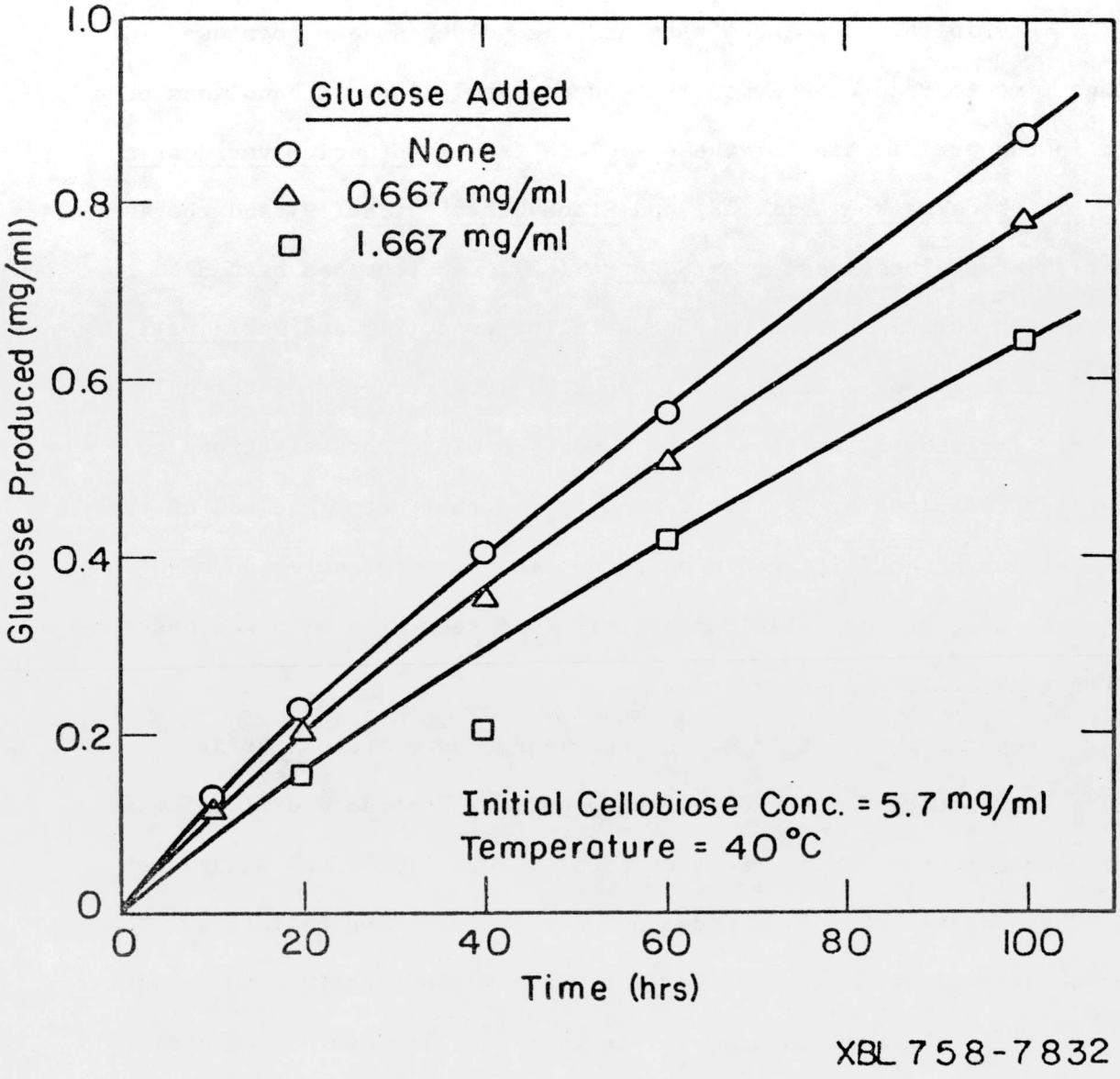


Figure 3.23. Inhibitory effect of glucose on hydrolysis of cellobiose by β-glucosidase from *B. theobromae*.

3.2.5 Acetone Precipitation of β-Glucosidase

Precipitation methods with organic solvents have been used for a long time to recover enzymes from aqueous solution. Ethanol has been used in the purification of the cellulase from Myrothecium verrucaria (17), Trichoderma koningii (18) and Stachybotrys atra (19) and the intracellular β-glucosidase from S. atra (20). Acetone has been used to recover the cellulase from Polyporus tulipiferae (21) and Poria vaillantii (22). Mandels et al. (23) reported 100% recovery of the cellulase of T. viride by using acetone. More complete investigations for T. viride cellulase by Ige (24) demonstrated that more than 85% of the original enzyme activity could be recovered at temperatures as high as 35° C, and most of the cellulase activity was recovered with acetone concentrations of 2.0 v/v or greater.

Economic evaluation of the enzyme recovery process using acetone precipitation is not so promising primarily because of its large energy consumption for the solvent recovery step (25), and Mandels et al. (23) proposed the use of adsorption to recover the cellulase. The acetone precipitation method, however, is a basic technique in investigating enzyme characteristics, and it is especially useful for measuring the enzyme activity when there are inhibitors in the solution. (In the present work, the inhibitors are reducing sugars.)

The procedure of the experiment is rather simple. One ml of the β-glucosidase solution was placed in a centrifuge tube and a certain amount of acetone was added and well mixed. After 15 minutes at room temperature, the precipitated enzyme was centrifuged and the

supernatant was removed. The enzyme was then redissolved into 0.05 M acetate buffer of pH 5.0 and the activity was measured.

The result shown in Fig. 3.24 indicates that three volumes of acetone are required for precipitating the β-glucosidase from one volume of the aqueous solution. This number is quite similar to that for cellulase (24).

3.2.6 Adsorption of β-Glucosidase on Cellulose

Mandels et al. (23) reported that cellulase was readily adsorbed on cellulosic materials and suggested the feasibility of using an adsorption process to reuse the enzyme. Adsorption of cellulase on cellulose was also investigated by Mitra (6) who designed a stagewise adsorption train to recover the enzyme from the hydrolysis products. In his process scheme the liquid stream from the reactor is contacted countercurrently with the incoming freshly ground newsprint for recovery of the enzyme back into the hydrolysis vessels. As will be shown in Chapter 7, the hydrolyzate contains about 60% of the original level of β-glucosidase activity, which should be recycled to use again in the reaction system. Thus the adsorption of β-glucosidase on fresh ground newsprint was investigated.

Various amounts of the Wiley-milled newsprint were added to the mixed enzyme solution which was a mixture of culture filtrates of both Trichoderma viride and Botryodiplodia theobromae and the mixture was shaken at 300 rpm for 40 minutes at 45 and 50° C in a bath. Then the mixture was centrifuged to obtain a clear solution of the enzyme and its activity was measured.

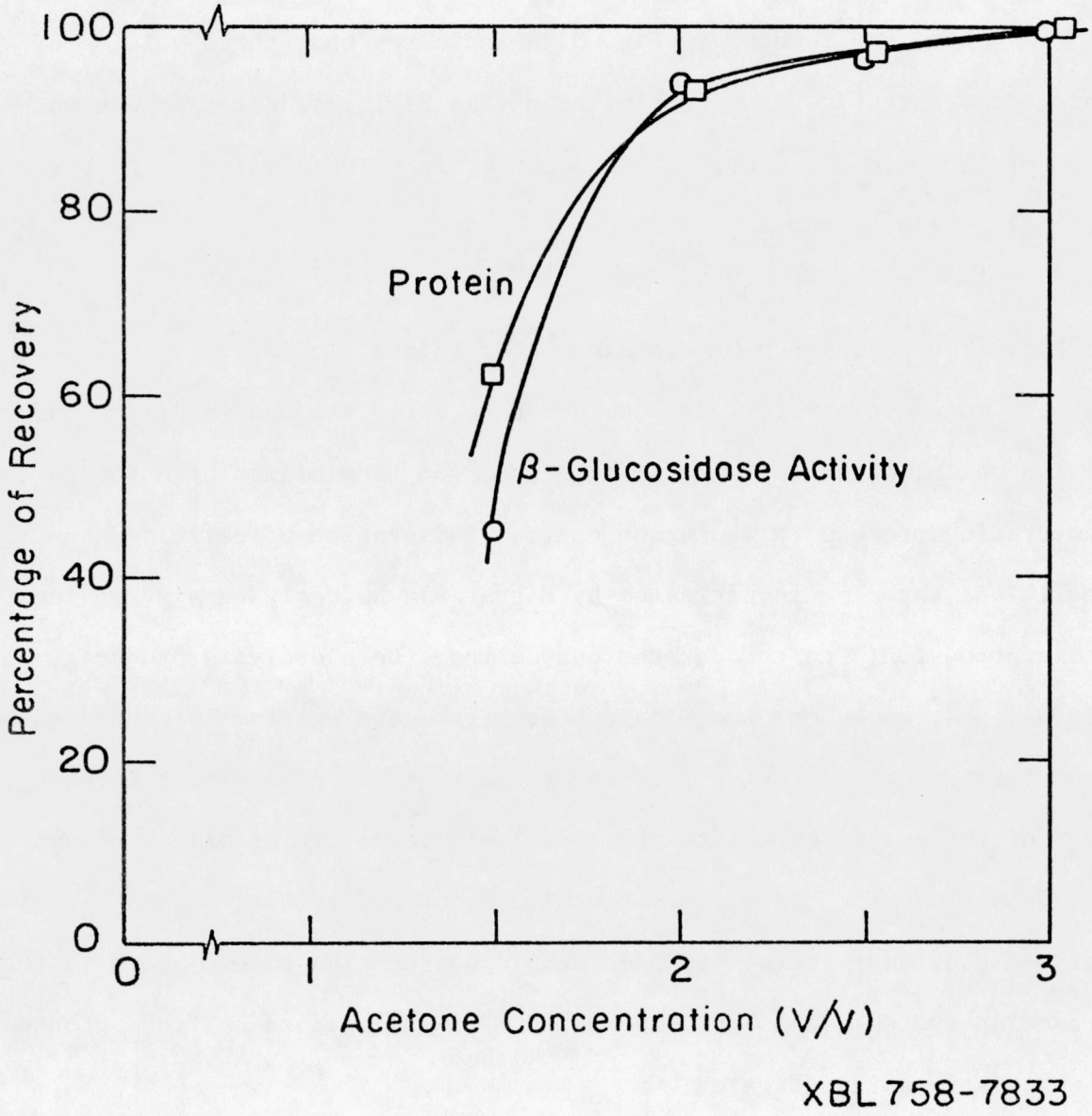


Figure 3.24. Recovery of *B. theobromae* β-glucosidase by acetone precipitation.

The effect of washing the centrifuged cellulose was also examined. The residual cellulose was resuspended with 0.05 M acetate buffer, pH 5.0, the amount of which was exactly the same as that of the supernatant withdrawn. The mixture was mixed well and then centrifuged to obtain a clear solution. The enzyme activity in the wash solution was measured in the same manner as for the hydrolysis product. Although the contact time was relatively short, there was still some sugar produced in these solution. Thus the enzyme was precipitated by acetone (3 v/v of sample) and then redissolved into the acetate buffer (0.05 M, pH 5.0) to avoid the effect of sugars on the activity measurement.

In order to make enzyme recoverability comparisons, it is more reasonable to use enzyme protein recovered rather than to use enzyme activity recovered because cellulase activity is usually not proportional to the protein content. Figure 3.25 depicts the filter paper activity vs. protein content curves for two different batches of cellulase prepared from Trichoderma viride. These curves show not only the non-linear relationships but also the inconsistency of the calibration curves for different cellulase solutions. When the same data were plotted on normalized coordinates, i.e., the enzyme activity divided by the original activity vs. the enzyme dilution, the difference between the two cellulase preparation was diminished and one calibration curve was obtained as shown in Fig. 3.26. The calibration curves for β-glucosidase, which is linear, and C_x activities are also shown in Fig. 3.26. A linear relation was found for C_1 activity by Yang (26).

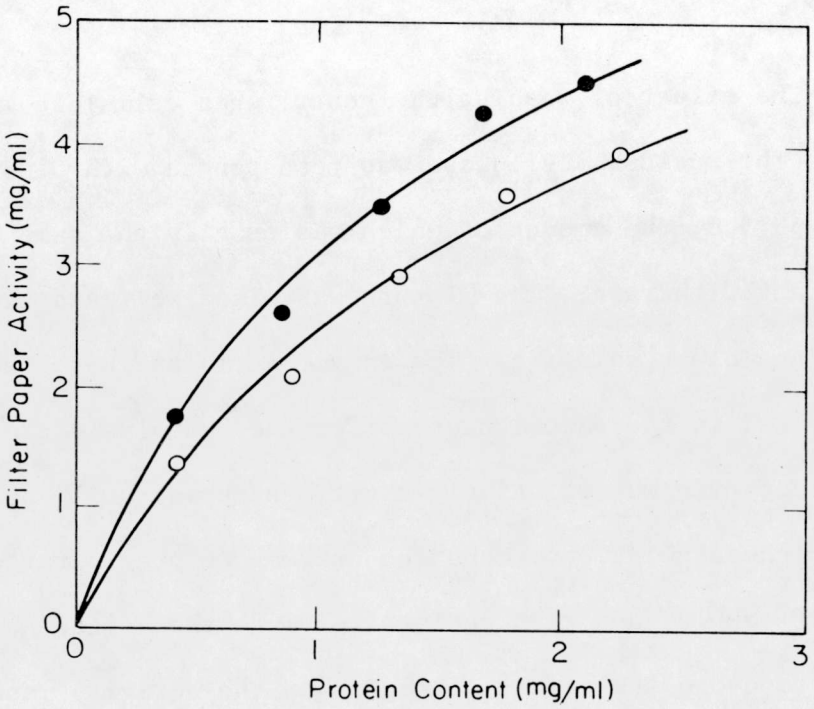


Figure 3.25. Relationship between filter paper activity and protein content for culture filtrates of *T. viride*.

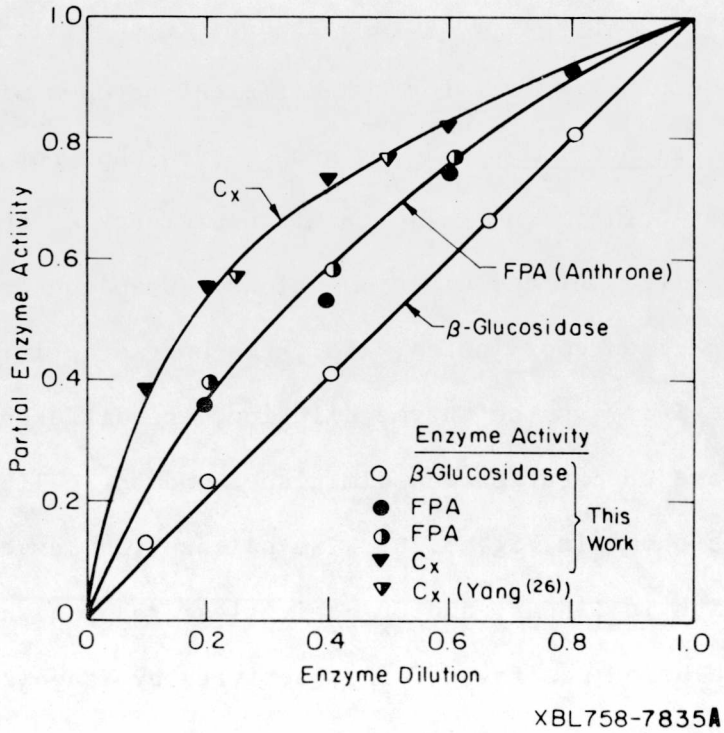


Figure 3.26. Relationship between partial enzyme activity and enzyme dilution.

These calibration curves for the separate enzyme activities are quite different from each other mainly because the initial hydrolysis rates of the filter paper and the carboxymethylcellulose could not be properly measured by the assay methods used for the filter paper and C_x activity measurements. Since many enzyme components are involved in the action of cellulase, the cellulosic materials may or may not adsorb specific components of the enzyme selectively. Therefore, it is still questionable whether these calibration curves could be used for evaluating the recovery of individual enzymes. These problems are very complicated and require more extensive studies.

All of the experimental data are summarized in Table 3.6. The activities of the enzyme used for the experiments are also shown in Table 3.6. Figure 3.27 shows the percentages of retained enzymes in the solution as a function of the amount of newsprint suspended into the enzyme solution. Only a slight difference was observed between the data for β-glucosidase at 45° C and 50° C. On the other hand, the adsorption characteristics of the C_x activity with newsprint show evidence of a temperature effect on the adsorption of the enzyme. At lower temperatures more adsorption takes place, which is a general principle of adsorption phenomena. Figure 3.27 also illustrates the retention of cellulase enzyme based on the filter paper activity of the solution, which is more or less the same result as that for the C_x activity.

Comparing these curves, we may conclude that the adsorption of β-glucosidase on the cellulosic materials is very weak compared with cellulase. It is also noted that the degree of adsorption of the β-glucosidase is little affected by the amount of cellulosics added to

Table 3.6. Recovery of enzyme activities by adsorption.

Temperature (°C)	Conc. of newsprint (%)	Recovery of supernatant (%)	Retained enzyme activities (%)			Washed-out enzyme activities (%)		
			β-G	C_x	FPA	β-G	C_x	FPA
45	1.0	85.6	76.6	73.4	60.5			
	2.0	79.6	77.1	59.0	59.4	8.7	4.6	7.2
	3.5	69.5	74.8	25.4	32.2	14.9	3.4	5.2
	5.0	65.5	67.9	17.5	21.8	16.3	2.4	4.4
	7.5	47.0	67.0	12.8	14.0	24.1	2.8	8.2
50	1.0	82.8	76.2	80.5	---	12.6	---	---
	2.0	77.6	75.2	63.0	---	17.7	---	---
	3.5	70.0	74.1	41.8	---	18.6	---	---
	5.0	60.7	70.8	28.6	---	23.2	---	---
	7.5	54.3	69.2	22.6	---	27.3	---	---

Enzyme activities of original solutions:

For the experiment at 45° C: 15/85 mixture of β-glucosidase and cellulase solution. Filter paper activity = 3.95, C_x = 1.90, β-G = 0.432 mg/ml.

For the experiment at 50° C: 15/85 mixture of conc. β-glucosidase and cellulase solution. Filter paper activity = 4.33, C_x = 2.12, β-G = 0.82 mg/ml.

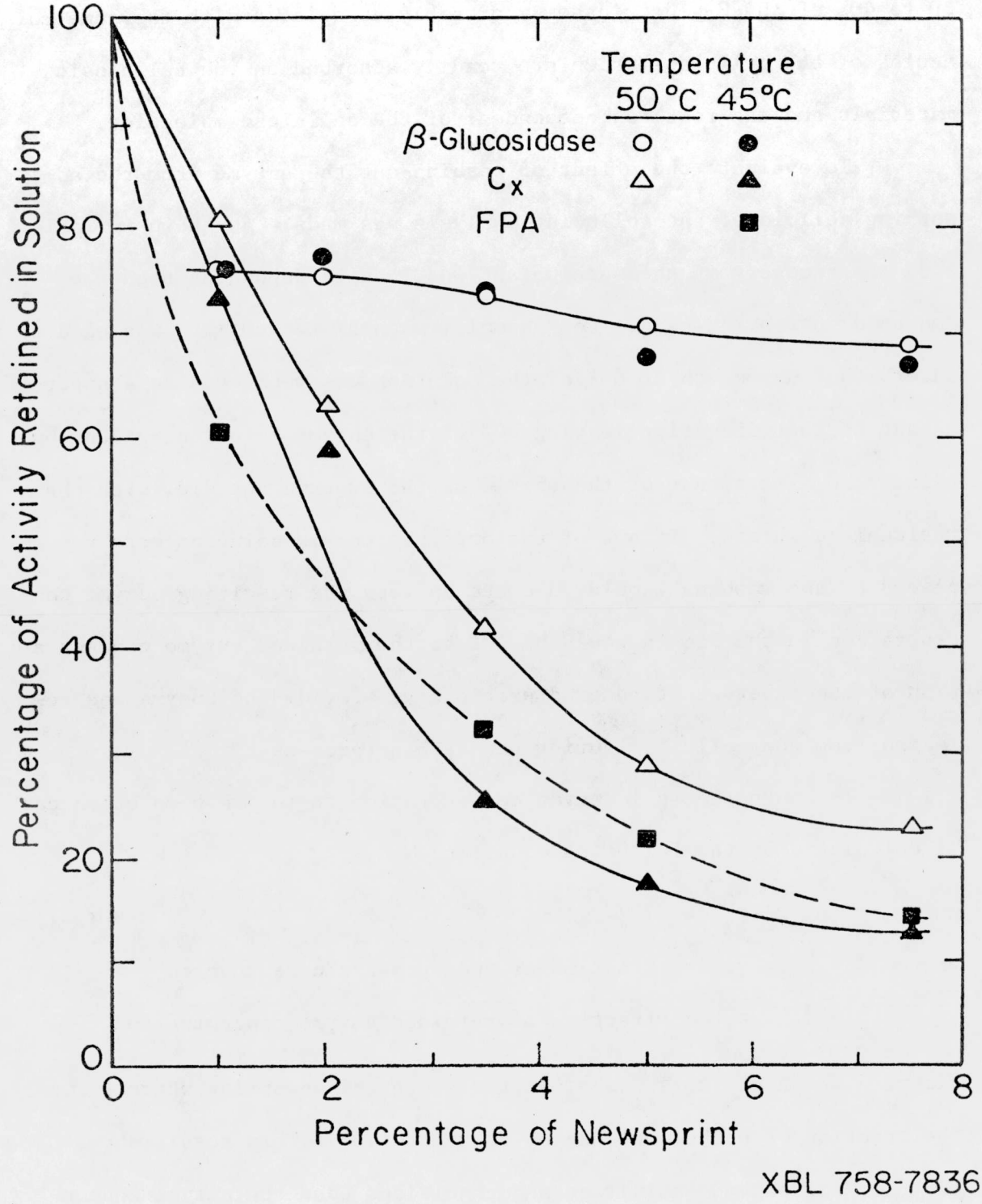


Figure 3.27. Adsorption of enzymes from solution by suspended newsprint. Culture filtrates of *B. theobromae* and *T. viride* in 15/85 mixture.

the solution. These phenomena could be explained by assuming that about 30 to 40% of the β-glucosidase is probably associated with the same molecules of the cellulase which are readily adsorbed on the cellulosic materials and the rest is independent of the cellulase molecules.

To evaluate the effect of washing-out the enzyme from the adsorbing cellulose, the following analysis was made. As shown in Table 3.6, the recovery of the supernatant was largely dependent upon the amount of newsprint used. For example, when 5% newsprint was used to adsorb the enzyme, about 60% of the solution was recovered as a supernatant by centrifugation leaving 40% of the enzyme solution, which contained the same amount of the enzyme as the supernatant did, with the residual cellulose. If 60% of the original enzyme solution were replaced by the washing liquid, acetate buffer, the resulting enzyme concentration in the liquid would be 40% of the retained enzyme concentration of the supernatant removed previously, provided no enzyme was released from the cellulose during the washing process.

This hypothetical enzyme concentration in the wash solution can be calculated by the following equation:

$$\varepsilon_2^0 = (1 - \gamma)\, \varepsilon_1 \qquad (3\text{-}1)$$

where γ = fraction of the supernatant recovered

ε_1 = fraction of retained enzyme concentration

Figure 3.28 shows $\varepsilon_2/\varepsilon_2^0$ against the amount of newsprint where ε_2 was the fraction of washed-out enzyme concentration. This result shows that for all enzyme activities and conditions used the enzyme concentration in the washed-out solution was less than that calculated

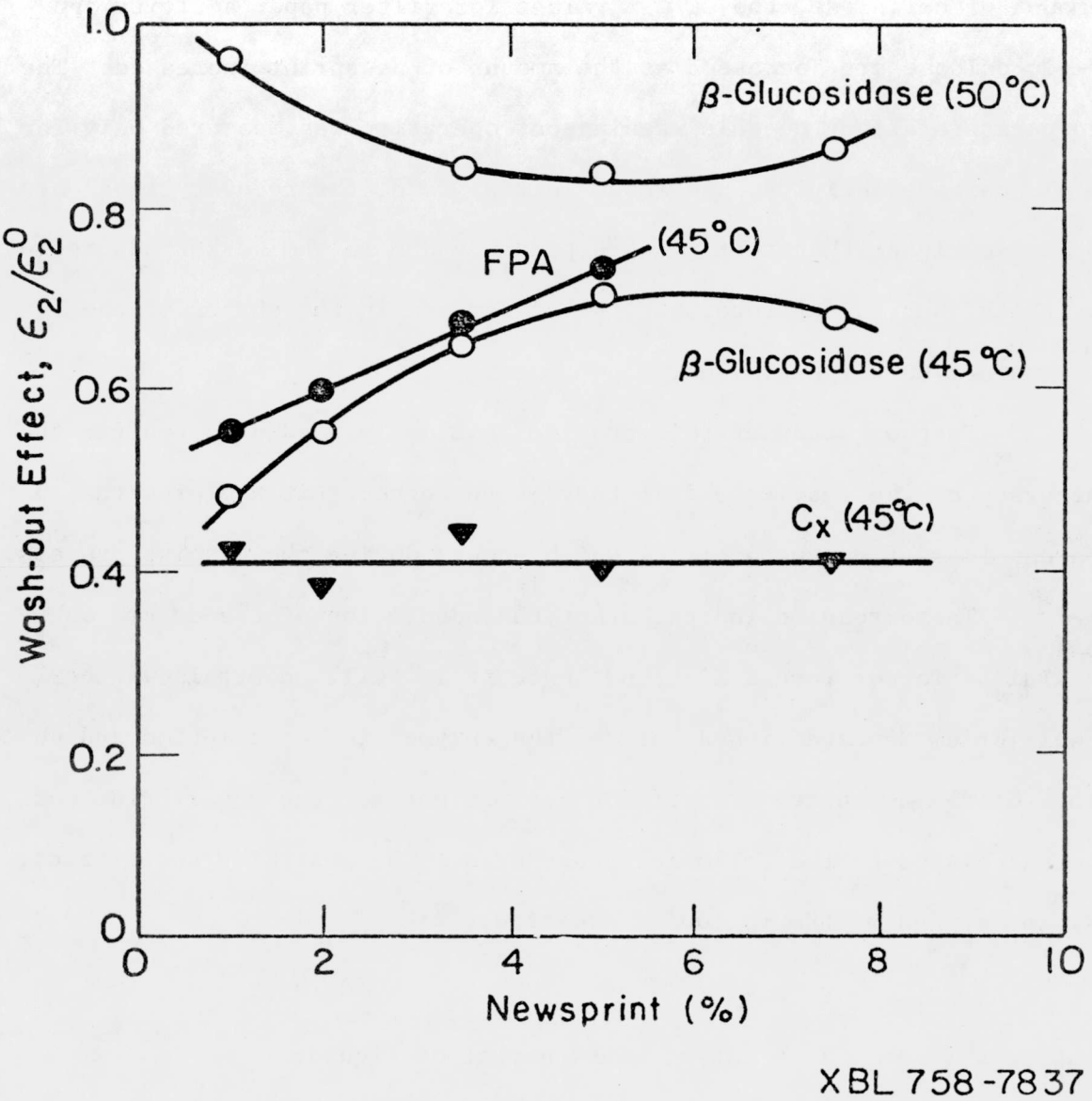


Figure 3.28. Effect of newsprint concentration on wash-out of enzymes.

with the assumption of no enzyme release from the cellulose.

The shapes of these curves are quite different from each other. While the wash-out effect of the C_x component seems independent of the amount of cellulose, the $\varepsilon_2/\varepsilon_2^0$ values for filter paper activity and β-glucosidase are increased as the amount of newsprint increased. The temperature effect on this washing-out operation was measured only for β-glucosidase activity. As shown in Fig. 3.28, the recovery level at 50° C was higher than that at 45° C probably due to the weaker adsorption at the higher temperature, but the difference in the shapes of the curves are hardly explained.

Further study of this problem is clearly needed to confirm the accuracy of the results and if they prove correct, it explains the apparent loss of enzyme activity which occurs during the washing process.

These results indicate that the adsorption of the enzyme on cellulose is not reversible, and hence it is still uncertain whether equilibrium is established between the enzymes in the solution and on the solid. An apparent partition coefficient for the equilibrium can be calculated by the following procedures. The partition coefficient, K, is defined by the following equation,

$$Y = KX$$

where Y = enzyme units per ml of liquid

X = enzyme units per gram of solid

K = partition coefficient

Dividing by the original enzyme concentration, Eo, we get

$$\frac{Y}{E_0} = K \frac{X}{E_0}$$

Here, Y/E_0 is ε_1, the fraction of retained enzyme concentration, and X/E_0 can be replaced by $(1-\varepsilon_1)/W$, where W is the amount of newsprint (g/ml). Hence, for the adsorption step the partition coefficient is calculated by

$$K = \frac{W\varepsilon_1}{1-\varepsilon_1} \tag{3-3}$$

For the first washing process, performing a material balance for the operation, we get

$$K = \frac{W\varepsilon_2}{1-\gamma\ \varepsilon_1-\varepsilon_2} \tag{3-4}$$

For the n-th washing,

$$K = \frac{W\ \varepsilon_{n+1}}{1-\gamma\ \sum^{n}\ \varepsilon_n-\varepsilon_{n+1}} \tag{3-5}$$

where ε_{n+1} = fraction of enzyme concentration in the n-th solution. In the present work, only the first wash was examined and thus the partition coefficients in the adsorption step and the first washing step were estimated by Eq. (3-3) and (3-4).

The results at 45° C are shown in Table 3.7. Only filter paper activity shows a constant partition coefficient, about 0.014, for the adsorption process, but no constant value of K was obtained for the other two enzyme components. Very low values of K, as little as 5% of that for the adsorption step, were obtained from the results shown in Fig. 3.28.

Table 3.7. Partition coefficients for enzyme components.

Amount of newsprint	(Retained)			(Washed-out)		
	β-G	Cx	FPA	β-G	Cx	FPA
10 mg/ml	.033	.028	.015	.0019	.0014	.0011
20	.067	.029	.029	.0058	.0019	.0032
35	.104	.012	.017	.0157	.0015	.0025
50	.106	.011	.014	.0208	.0014	.0027
75	.152	.011	.012	.0407	.0023	.0072

These findings are not surprising, because it has not been confirmed yet whether the equilibrium between the enzyme in the solution and the enzyme adsorbed on the cellulose does exist. Mitra (6), who proposed the stage-wise washing train to recover the enzyme from residual cellulose of the hydrolysis reaction, measured the adsorption and wash-out effects of spent cellulose by the same procedure described above and obtained a constant partition coefficient based on the C_1 component for the first, second and third wash-out steps. His results are shown in Table 3.8. The partition coefficient for the adsorption step was calculated by using his original data, but the number was about 30% higher. Also in his calculations, Mitra assumed 100% recovery of liquid from the suspension, i.e., γ was set to unity in the Equation (3-5). When γ is set to 0.85, which is a commonly obtained recovery of the liquid, a constant value of K is not obtained. The calculated results are also shown in Table 3.8.

Mandels et al. (23) reported that although adsorbed C_1 cellulase on gauze (27) and Avicel (28) could be desorbed by distilled water,

Table 3.8. Partition coefficient for C_1 component with spent newsprint (6).

γ	Adsorption step	First wash	Second wash	Third wash
1.0	0.052	0.040	0.040	0.041
0.85	0.052	0.030	0.022	0.016

desorption of cellulase from Solka Floc could not be achieved and the only way to recover the enzyme adsorbed on the surface of cellulose was digestion. The results obtained in the present work seem to support their observations.

Literature Cited in Chapter 3

1) Mandels, M. and Weber, J., Cellulases and Their Applications, Adv. in Chem. Series, 95, 309 (1969)

2) Umezurike, G. M., Ann. Bot., 33, 217 (1970)

3) Alasoadura, S. O., Mycopat. Mycol. Appl., 42, 153 (1970)

4) Umezurike, G. M., Biochim. Biophys. Acta, 227, 419 (1971)

5) Aiba, S., Humphrey, A. E. and Millis, N. F., Biochemical Engineering, Second edition, p. 115, Academic Press (1973)

6) Mitra, G. and Wilke, C. R., Enzymatic Utilization of Waste Cellulosics, Lawrence Berkeley Laboratory Report 2334-Rev., January (1975)

7) Hassid, W. Z. and Neufeld, E. F., Methods in Carbohydrate Chemistry, 4, 33, Academic Press (1964)

8) Rosenbluth, R. F. and Wilke, C. R., SERL Report No. 70-9, College of Engineering and School of Public Health, University of California, Berkeley (1970)

9) Mitra, G and Wilke, C. R., Biotech. Bioeng., 17, 1 (1975)

10) Yang, R. D., private communication, Lawrence Berkeley Laboratory, January (1975)

11. Heyworth, R. and Walker, P. G., Biochem. J., 83, 331 (1962)

12) Han, Y. W. and Srinivasan, V. R., J. Bacteriol., 100, 1355 (1969)

13) Conn, E. E. and Stumpf, P. K., Outlines of Biochemistry, third edition, 176, John Wiley & Sons, Inc. (1972)

14) Toyama, N. and Ogawa, K., Ferment. Technol. Today, 743, Soc. Ferment. Technol., Japan (1972)

15) Bergham, L. E. R. and Pettersson, L. G., Eur. J. Biochem., 46, 295 (1974)

16) Wood, T. M., Biochem. J., 115, 457 (1969)

17) Whitaker, D. R., Arch. Biochem. Biophys., 43, 253 (1953)

18) Toyama, N., Memoirs Faculty Agric. University of Miyazaki, No. 2, 100 (1960)

19) Thomas, R., Aust. J. Biol. Sci., 9, 159 (1956)

20) Youatt, A., Aust. J. Biol. Sci., 11, 209 (1958)

21) Nisizawa, K., J. Biochem. (Japan), 42, 825 (1955)

22) Sison, B. C., Jr., Schubert, W. J. and Nord, F. F., Arch. Biochem. Biophys., 75, 260 (1958)

23) Mandels, M., Kostick, J. and Parizek, R., J. Polymer Sci., Part C, No. 36, 445 (1971)

24) Ige, R. T. and Wilke, C. R., Recovery of the Cellulase Enzyme System of Trichoderma viride by Acetone Precipitation, Lawrence Berkeley Laboratory Report 3057, June 1974

25) Ige, R. T., private communication, Lawrence Berkeley Laboratory, June (1974)

26) Yang, R. D., private communication, Lawrence Berkeley Laboratory, May (1975)

27) Toyama, N., Cellulases and Their Applications, Adv. in Chem. Series, 95, 359 (1969)

28) Li, L. H., Flora, R. M. and King, K. W., Arch. Biochem. Biophys., 111, 439 (1965)

PART II

KINETIC STUDIES OF ENZYMATIC HYDROLYSIS OF CELLULOSE

4. Previous Work

4.1 Kinetic Studies

4.1.1 Product Inhibition

An inhibitory effect, caused by accumulation of end products, on the rate of the forward reaction is observed quite often in many microbiological and biochemical systems. For cellulose hydrolysis with cellulase, the enzyme is competitively inhibited by cellobiose and glucose. The inhibitory effect of products varies with the organism from which the cellulase has been derived, and cellobiose inhibits the cellulase of many organisms (1). Glucose inhibition is generally weak. Ghose (2) observed 40% inhibition by a 30% glucose concentration for the cellulase system of Trichoderma viride (QM 6a).

Reese reported the cellulase of Trichoderma viride was inhibited by cellobiose but not by glucose (3,4). In contrast, Amemura and Terui (5) observed that the cellulase from Penicillium variabile was inhibited by glucose instead of cellobiose. They concluded that this enzyme contained components which were more specific toward cellulose of low molecular weight so that the accumulation of cellobiose did not take place.

Quantitative measurements of the inhibitory effects of both cellobiose and glucose were achieved by Ghose and Das (6). The initial reaction rate was measured in terms of product formation during 10 minutes of incubation at 50° C. Solka Floc was used as a substrate. Although this was definitely a soluble enzyme and insoluble substrate

system, the Lineweaver-Burk plot of his data yielded straight lines for no inhibition, for glucose inhibition and for cellobiose inhibition (Fig. 4.1). Cellobiose inhibition is apparently competitive, but the inhibitory effect of glucose is somehow different, probably due to the difficulty in measuring the sugars produced (0.3--0.9 mg/ml) in the presence of the background glucose concentration (10 mg/ml).

Similar results were obtained by Amemura and Terui (5), who used Penicillium variabile cellulase. The Lineweaver-Burk plot of their data showed that the inhibition by glucose was non-competitive, but the theoretical analysis was done with the equation for competitive inhibition. (The reason was not explained.) The Michaelis-Menten constant for the system was estimated as 3.57 mg/ml, and the dissociation constant for the glucose-enzyme complex, K_i, was 1.54 mg/ml.

The products of the enzymatic hydrolysis of cellulose were mainly glucose and cellobiose. The ratio of these sugars may vary with the cellulase preparation and the experimental conditions. Ogiwara et al. (7) reported that the final soluble hydrolysis products were glucose and cellobiose as detected by paper chromatography. The ratio of glucose to cellobiose, however, changed as the reaction proceeded. The enzyme was the commercial cellulase "Cellulase-Onozuka", made from the culture filtrate of Trichoderma viride, and the substrate was wood pulp powder. Their results are shown in Table 4.1.

Stuck and Howell (8) observed that a short period hydrolysis (up to 80 minutes) of a wood pulp, Solka Floc, with Trichoderma viride cellulase yielded a glucose-cellobiose ratio of 1/8. They also found

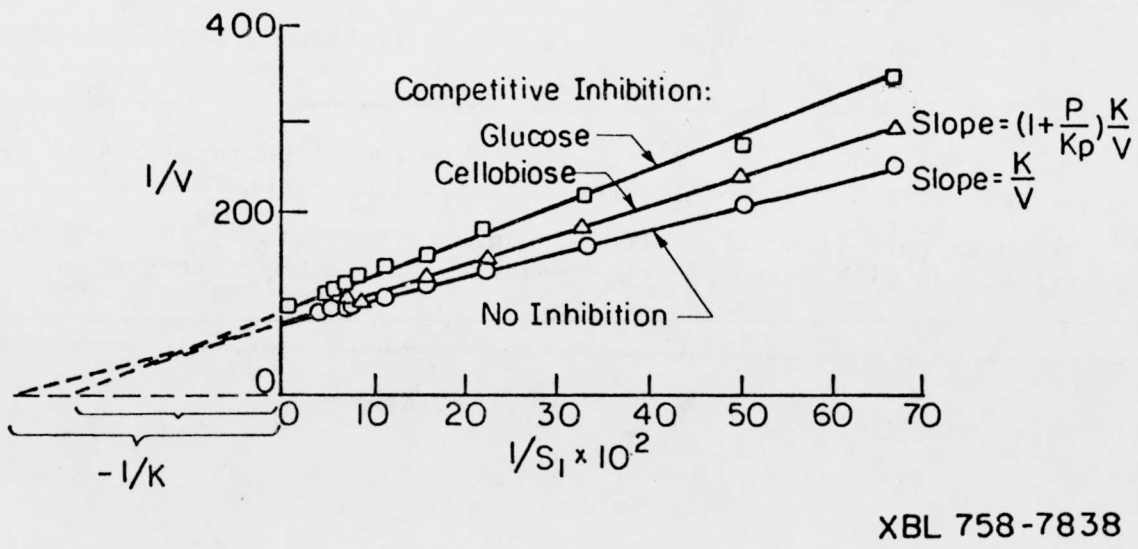


Figure 4.1. Lineweaver-Burk plots for no inhibition, glucose inhibition and cellobiose inhibition of hydrolysis of Solka Floc by culture filtrates of *T. viride*. Redrawn from (6).

Table 4.1. Composition of soluble products of pulp hydrolysis by cellulase. (7)

	Reaction time (hrs)				
Conversion to (%)	6	12	24	48	96
Glucose	1.65	2.7	7.4	11.6	21.2
Cellobiose	10.15	13.5	18.1	26.0	34.6
Total conversion	11.8	16.2	25.5	37.6	55.8

* Hydrolysis conditions: 2.0 g of pulp in 100 ml buffer (pH 4.1) containing 1.0 g of purified cellulase. Temperature was 40° C.

** Products were first separated by paper chromatograph, followed by extraction with water and then measured with Somogyi reagent.

that the concentration of oligomers with a degree of polymerization beyond 5--6 was negligible.

Huang (9) measured the sugar content of the hydrolysis product of amorphous cellulose with Trichoderma viride cellulase. The substrate was prepared by swelling Solka Floc in 85% phosphoric acid according to Walseth (10). The glucose to cellobiose ratio was about 1: 3 after a short time, and 3:7 at 6 hours. At 24 hours it became approximately 1:1.

These different results are reasonably attributable to the nature of the enzyme. For instance, if the enzyme contains β-glucosidase, the product becomes rich with glucose, and vice versa. Also a synergism between several enzyme activities may affect the result. Consequently, no generalized correlation between enzyme activities and the product composition has been proposed because of the complexity of the problem.

To avoid these product inhibitions, the following methods have been suggested:

(a) Convert cellobiose to glucose by using additional β-glucosidase.

(b) Remove the soluble products from the reaction system.

The first method would work if the reaction is inhibited mainly by cellobiose. Stuck and Howell (8) reported a 28% increase of conversion of the hydrolysis of wood pulp at 35 hours by using almond-emulsion β-glucosidase along with the Trichoderma viride cellulase. The second method was suggested by Ghose and Kostick (11), who used a membrane to remove soluble sugars from the reaction system. The membrane was made of synthetic polymer, and its pore size was chosen so that most of

the enzyme was retained in the reaction mixture. Though no direct comparison of the conversion of cellulose between this system and the ordinary continuous flow reactor was made, they concluded that a high initial reaction rate and high level of conversion were attained with this membrane system.

4.1.2 Kinetics of Cellulose Hydrolysis

The mechanism of cellulose breakdown by the action of cellulase is extremely complicated. There have been several models suggested to describe the reaction kinetics of enzymatic hydrolysis of cellulose, but they are applicable only with several restrictions.

The empirical equations of the following form were thus proposed to overcome those difficulties (12,13), and they seem fairly consistent with the experimental data for particular cellulase preparations.

$$x = k\, t^m\, e^n \qquad (4\text{-}1)$$

where x = percentage hydrolysis

t = incubation time

e = enzyme concentration

k = rate constant

m,n = empirical constants

The equation was originally proposed by Karrer et al. (12), who hydrolyzed regenerated cotton cellulose with crude snail juice cellulase. According to their results, the percentage of hydrolysis, x, was proportional to the square root of incubation time, i.e.,

$$x = k\, t^{1/2} \qquad (4\text{-}2)$$

The more extensive studies done by Miyazaki and Nisizawa (13) yielded Equation (4-1) for Aspergillus niger and Irpex lacteus cellulase as follows:

Aspergillus niger: $$x = 1.22\ t^{1/3}\ e^{1/3} \qquad (4\text{-}3)$$

Irpex lacteus: $$x = 1.37\ t^{1/2}\ e^{1/3} \qquad (4\text{-}4)$$

These empirical correlations were obtained with the hydrolysis products of 24 to 120 hours reaction, and the deviations of the calculated values from these equations are comparatively small.

The theoretical interpretation of the equation is difficult. For example, taking a derivative of Equation (4-1) with respect of time, we obtain the following relation:

$$\frac{dx}{dt} = k\ m\ e^{n}\ t^{m-1} \qquad (4\text{-}5)$$

Since m is less than unity, the initial rate of reaction becomes infinity according to the relation, which is very difficult to believe.

Other theoretical treatments of experimental data have been done by using the Michaelis-Menten equation although it was derived for the system of soluble enzyme and soluble substrate. Using purified pulp powder as a substrate and the cellulase preparation from Penicillium variabile, Amemura and Terui (5) obtained a straight line for the Lineweaver-Burk plot over substrate concentrations of 2.5 to 50 mg/ml. (Experimental conditions such as reaction temperature, reaction time, etc. were not described.) They also observed that the initial reaction rate of the cellulose-cellulase system, where sufficient adsorption of the enzyme on the substrate had been attained by preincubation at low temperature, was practically the same as the system without any pre-

treatment. This implies that the rate determining step of the enzymatic hydrolysis reaction is not the rate of penetration or adsorption of enzyme molecules but the reaction itself. Therefore, they suggested that although cellulose is an insoluble substrate and has a very complicated structure, the Michaelis-Menten equation could be applied to this system if the density of points which are reactive to the enzyme is used as the substrate concentration in the liquid.

They also mentioned the importance of the partial concentration of accessible cellulose, which was defined as "cellulose available for the action of cellulase". In their analysis, the accessibility of cellulose was assumed constant during the reaction, which is difficult to understand. The calculated conversion was, however, consistent with the observed values up to 20 hours of hydrolysis reaction.

Another attempt at using the Michaelis-Menten equation was made by Ogiwara et al. (7) for a wood pulp--Trichoderma viride cellulase system. Noting the problem encountered in measuring the reducing sugars by DNS reagent in estimating the reaction rate, they used weight loss of the substrate during the reaction to evaluate the reaction rate. Since the shorter the reaction period the larger the experimental error, acceptable accuracy with the Lineweaver-Burk plot was attained at 24 hours or more of reaction. This reaction period seems too long. For instance, the averaged weight losses measured at 5, 10 and 24 hours with the initial cellulose concentration of 1.0 g/50 ml were approximately 18, 14, and 10 mg/hr, respectively, and the Michaelis-Menten constant, K_m, and the maximum rate of reaction, V_{max}, thus obtained were no more than just

relative numbers. The Michaelis-Menten equation also applied to the data at 48 hours, i.e., the rate of the reaction was averaged over 48 hours. This surprising result would be an important clue for investigating this complex enzyme substrate system, but at present no significant conclusion has been drawn from their findings.

Using Solka Floc and Trichoderma viride cellulase, Stuck and Howell (8) also measured the relation between the initial reaction rate and the substrate concentration. They obtained a straight line for this Lineweaver-Burk plot, from which K_m and V_{max} values were estimated. The inhibition constant, K_i, was determined by a Foster-Niemann type analysis (14), i.e., P/t was plotted against $\frac{1}{t}\ell n(S/S_0)$ for each reaction progress curve. (Here, P, S_0 and S are the concentrations of product, initial substrate and residual substrate, respectively, and t is a reaction time.) The resulting curves indicated strong product inhibition by both cellobiose and glucose, and they had a common intercept on the ordinate, from which K_i was evaluated. Although Foster-Niemann analysis is based on competitive inhibition, the non-competitive inhibition model with the value of K_i obtained with the competitive inhibition model resulted in a closer prediction of the hydrolysis progress curve for less than 10 hours of reaction time.

As has been reviewed here, the application of the Michaelis-Menten equation to the cellulose-cellulase system seems to work at least experimentally, but its theoretical interpretation is still ambiguous. In the course of derivation of the Michaelis-Menten equation the rate of the reaction was assumed to be proportional to (ES), which is the concentration of enzyme bound to substrate. Since the proportionality between (ES) and (E_0), the total enzyme concentration in the system, was

easily derived for the soluble enzyme and the soluble substrate reaction mixture, the rate of the reaction became

$$v \propto (E_0)$$

In his argument for the soluble enzyme-insoluble substrate system, McLaren (15) suggested that the rate of reaction is proportional to (EA_s), enzyme adsorbed on the surface of the substrate. He showed that this (EA_s) was related to (E), the concentration of free enzyme, by the following equation:

$$(EA_s) \propto (E)^n$$

where n was obtained as 0.66 by using the data measured by Whitaker (16) for the cellulase of Myrothecium verrucaria. The rate of the reaction was then expressed as

$$v \propto ((E_0) - (EA_s))^{2/3}$$

which shows that for insoluble substrate systems the initial rate of hydrolysis is not directly proportional to the total amount of enzyme present in the system.

The hydrolysis experiments done by Brandt et al. (17) are unique. They demonstrated the action of C_1-cellulase by hydrolyzing ball-milled newsprint with either Pestalotiopsis westerdijkii cellulase, which contained essentially only the C_x component, or a mixture of this enzyme with Trichoderma viride cellulase, which contained both C_1 and C_x components. As shown in Fig. 4.2, the difference in conversion obtained with these two enzyme systems was remarkable, which was attributable to the action of C_1 component in the mixed enzyme.*

*There is no statement whether the C_x activities of both enzyme systems are the same or not.

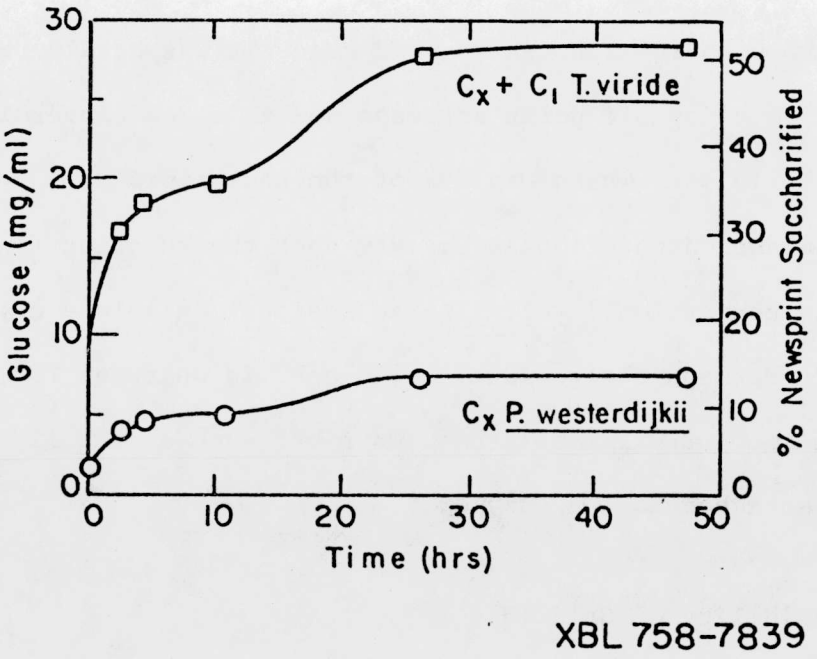


Figure 4.2. Contribution of C_1 component to newsprint hydrolysis by culture filtrates. Redrawn from (17).

Another interesting finding in their work was that there were two leveling off phenomena during the hydrolysis of cellulose when both the C_1 and the C_x components of cellulase were present. They suggested that the first leveling off represented the conversion of amorphous cellulose and the latter represented the conversion of crystalline cellulose. The first leveling off usually took place during the reaction time of 4 to 6 hours although in some cases it was hardly recognizable. The second leveling off point corresponded to a 74% conversion of the available cellulose, which was 70% of the newsprint.

Unanswered questions are: Why does the reaction finally level off at this conversion level? Is the residual cellulose crystalline or amorphous? Perhaps the residual cellulose, in whatever form, was trapped in a nondigestable part of the newsprint, such as the lignin structure, and never attached to the enzyme.

4.2 Accessibility of Cellulose

A typical enzymatic hydrolysis of cellulose is characterized by a very rapid reaction rate in the initial stage, followed by a decrease to a slow rate in the later stages, usually with a considerable amount of the cellulose still remaining undissolved. As early as 1952, Walseth (10) proposed that the principal reason for the slowing down of the hydrolysis reaction is the removal of easily accessible portions and accumulation of difficultly accessible (or crystalline) portions. Also he noted that inhibition by the product and inactivation of the enzyme exert noticeable but minor influences.

Then the question remained: What is the accessible portion of

the cellulose? It involves terms such as crystallinity, amorphous, availability, accessibility, order, disorder, etc., and all these terms are not clearly defined. Tarkow (18) schematically showed the reason for this ambiguity. Figure 4.3 shows some possible arrangements of sections of cellulose chains. The configuration in A, which represents three-dimensional crystals, is unique, and it has a definite density and produces a sharp X-ray diffraction pattern. B and C still have some regions of order and represent two- and one-dimensional crystals, respectively. In D the chains are only approximately parallel and E has no parallel axis. We can say that A is a crystalline part of cellulose, but since the distribution of deviations from A is broad, we can not define the accessible parts of cellulose so easily.

It is therefore quite likely that different procedures give somewhat different values for crystallinity (or accessibility), depending upon how many of the imperfect regions each includes with the perfect regions. Table 4.2 of Warwicker et al. (19) shows good examples of this variability. The ordered fraction, which may be crystalline, of a single cellulose type ranges from approximately 40 to 90 percent, depending largely upon the method used.

The X-ray diffraction method developed by Hermans et al. (20) is the most direct method in use. Crystalline areas are defined as those which contribute to the maxima in the X-ray diagrams, although many small crystalline regions will not contribute to the X-ray maxima. For the imperfect crystalline parts there is always some uncertainty because of the difficulties of analyzing and explaining the data.

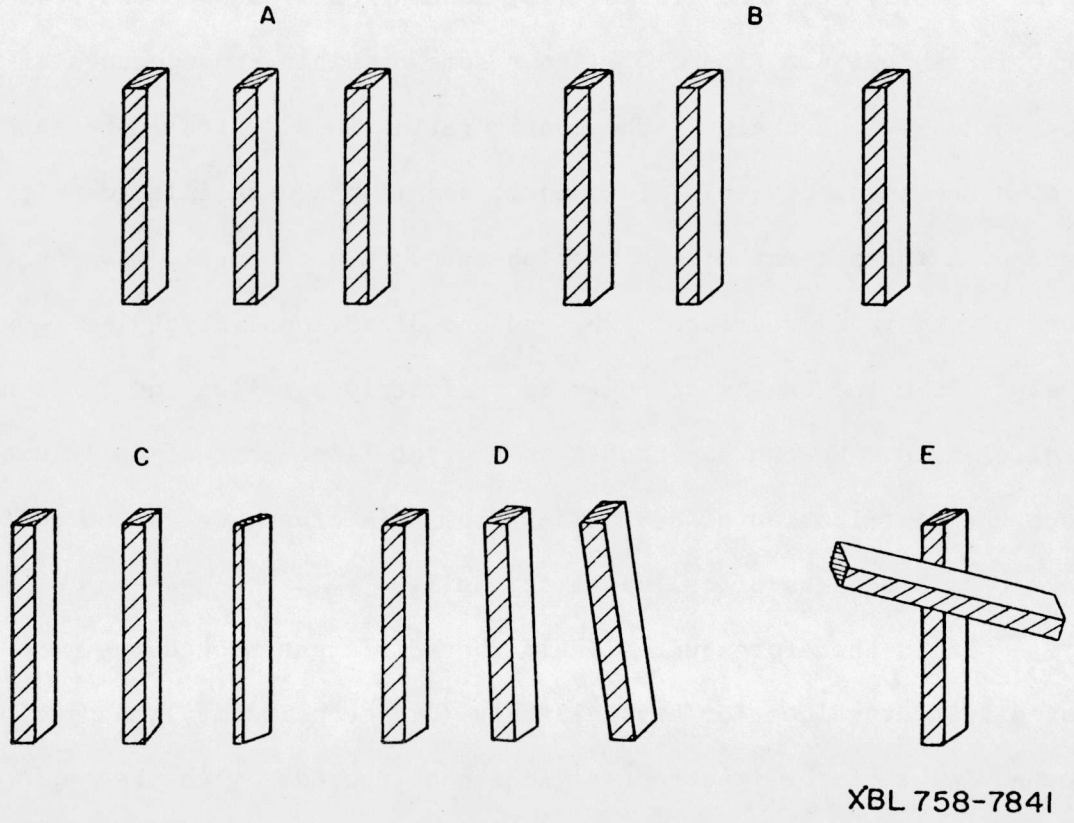


Figure 4.3. Schematic drawings of hypothetical chain orientations in cellulose. Redrawn from (18).

Table 4.2. Average ordered fraction measured by various techniques. (19)

Technique	Cotton	Mercerized cotton	Wood Pulp	Regenerated Celluloses
X-ray diffraction	0.73	0.51	0.60	0.35
Density	0.64	0.36	0.50	0.35
Deuteration or moisture regain	0.58	0.41	0.45	0.25
Acid hydrolysis	0.90	0.80	0.85	0.70
Periodate oxidation	0.92	0.90	0.92	0.80
Iodine sorption	0.87	0.68	0.85	0.60
Formylation	0.79	0.65	0.75	0.35

Hermans (21) suggested the use of the density of cellulose as a measure of crystallinity since it showed good agreement with the X-ray diffraction results. The moisture sorption method, also developed by Hermans (21), is based on the fact that adsorption at a given relative humidity increases with increasing amounts of noncrystalline parts. Although this method gives a simple and precise way for determining relative accessibility of cellulose to water, it was also found that the sorptive capacity is affected not only by the amounts of amorphous region but also by the structure of crystalline parts of cellulose.

All of the chemical methods measure the accessibility of cellulose to the aqueous solutions or other swelling media used in their procedures. For instance, the hydrolysis method with hydrochloric acid gives the accessibility to this reagent. Figure 4.4 shows the examples of the acid hydrolysis method given by Nickerson (22). The rate of hydrolysis is rapid in the early period during which the amorphous cellulose reacts, but it gradually decreases due to the increase of crystalline cellulose. The latter part of the conversion vs. time curve allows a rough though not easy, extrapolation, to zero time and gives the amount of amorphous cellulose originally present in the samples. Relatively high crystallinity or low accessibility was reported with the acid hydrolysis method, which is attributable to the recrystallization of cellulose during the reaction.

The heterogeneous oxidation of cellulose by periodic acid or sodium periodate follows the same reaction course as the acid hydrolysis reaction. Goldfinger, Mark and Siggia (23) suggested the use of this reaction to estimate the accessibility of cellulose. As shown in

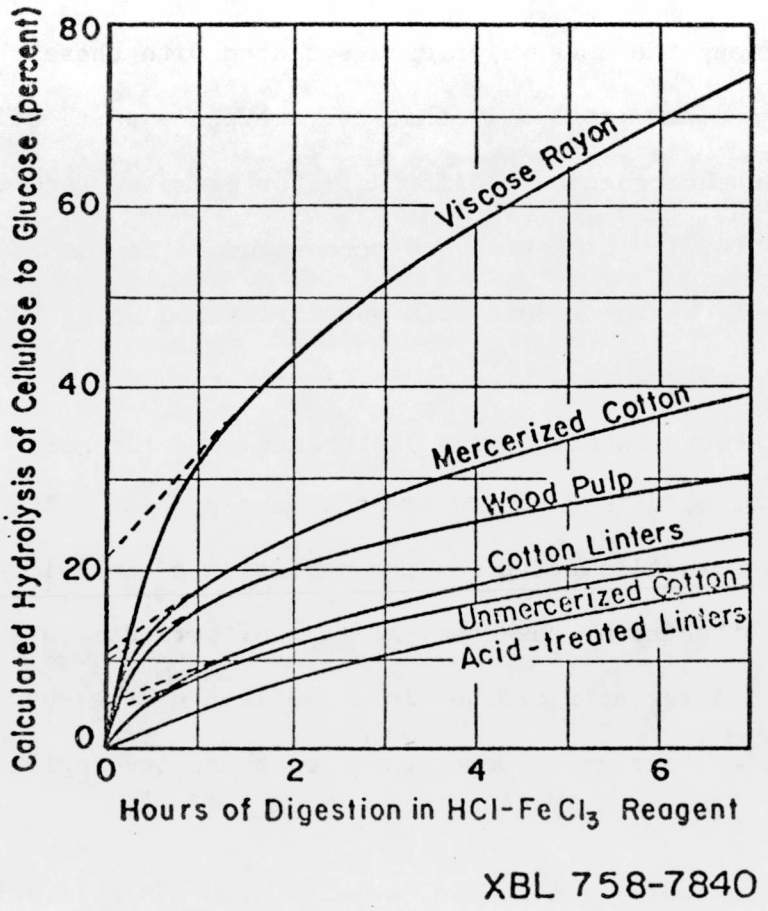


Figure 4.4. Hydrolysis of various cellulosic materials by $HCl-FeCl_3$ reagent. Redrawn from (22).

Table 4.2, this method also gives a high value of crystallinity, like the acid hydrolysis method. Timell (24), explaining his experimental data, concluded that recrystallization probably occurred during the oxidation reaction.

Among the uncertainties associated with these chemical methods, the most serious problem is that the amorphous part estimated largely depends on the reaction conditions. For example, Tarkow (18) reported the accessibility of cotton and mercerized cotton as 28 and 52 percent respectively by the formic acid esterification using 98 percent formic acid 15 hours reaction time at 55° C. Nickerson (22), however, gave 16 and 28 percent instead, using 90 percent acid 12 hours at 30° C.

The oxidation method has the same problem. The shape of the conversion vs. time curve for the oxidation of cellulose by sodium periodate is affected by the concentration of periodate used, and the extrapolated intercepts of the linear portion of the curve have different values, i.e., different percentages of amorphous region in the cellulose (25).

The swelling of cellulose with strong alkali solution was also used for the estimation of the accessibility of cellulose. Amemura and Terui (26) suggested the use of solubility of cellulose in 1.4 N sodium hydroxide. This concentration was chosen because the relation between the solubility and the concentration of sodium hydroxide had a very distinct inflection point at this concentration, and it was supposed that the solubility curve for dilute alkali solution represented the solubility of the amorphous part of the samples. The swelling conditions they

chose were 30 minutes of reaction at 0° C. Though the choice of 0° is justified by the experimental fact that the lower the temperature the higher the solubility of cellulose in sodium hydroxide (27), there is no explanation for selecting a 30 minute reaction period.

Mandels, Hontz and Nystrom (28) suggested a swelling method using 10% potassium hydroxide at 22° C for 3 hours to determine the amorphous region of cellulose, and their results show the solubility thus measured was very close to the level of maximum conversion of the enzymatic hydrolysis reaction without C_1 activity, which was necessary to degrade the crystalline parts of cellulose. However, they did not give the reason why such a combination of concentration of potassium hydroxide, temperature and reaction period was chosen, and it is very likely that the dissolved portion of the cellulose would be higher with a higher concentration of alkali, a lower temperature and a longer reaction time.

Use of the enzymatic reaction may be the most reasonable method to measure the accessibility of cellulose because it gives the accessibility to enzyme molecules which would be adsorbed on the surface of the cellulose during the hydrolysis reaction. Amemura and Terui (26) measured the accessible portion of wood pulp by using Penicillium variabile cellulase and showed that the conversion-time curve plotted on semi-logarithmic paper was almost linear after 4 hours (up to 10 hours). The extrapolation of this linear part to zero time gave a value of 30% for the amorphous portion. Although they did not clearly mention it, the linear part of the curve is considered to represent the reaction rate of

the crystalline cellulose, which was about 0.39 mg/ml-hr. The data obtained in the present work showed about 0.11 mg/ml-hr for the hydrolysis of absorbent cotton, which is almost 100% crystalline cellulose. This suggests that the rate they obtained was still associated with the C_x activity and that a longer reaction time would have been needed to get the true reaction rate for crystalline cellulose.

Literature Cited in Chapter 4

1) Mandels, M. and Reese, E. T., Adv. in Enzymic Hydrolysis of Cellulose and Related Materials, 115, Pergamon Press (1965)

2) Ghose, T. K., Biotech. Bioeng., 11, 239 (1969)

3) Mandels, M. and Reese, E. T., Developments in Industrial Microbiology, 5, 5 (1964)

4) Gilligan, W. and Reese, E. T., Can. J. Microbiol., 1, 90 (1957)

5) Amemura, A. and Terui, G., J. Ferment. Tech., 43, 281 (1965)

6) Ghose, T. K. and Das, K., Adv. in Biochem. Eng., 1, 55, Springer-Verlag (1971)

7) Ogiwara, Y., Arai, K. and Yamamoto, M., Kogyo Kagaku Zasshi, 74, 256 (1971)

8) Stuck, J. D. and Howell, J. A., A.I.Ch.E. Symp. Series, Water-1973, 70, 337 (1974)

9) Huang, A. A., unpublished data (1974)

10) Walseth, C. G., Tappi, 35, 228 (1952)

11) Ghose, T. K. and Kostick, J. A., Biotech. Bioeng., 12, 921 (1970)

12) Karrer, P., Schubert, P. and Wehrli, W., Helv. Chim. Acta, 8, 797 (1925)

13) Miyamoto, S. and Nisizawa, K., J. Veterinary Soc. Army, Japan, 396, 778 (1942)

14) Foster, R. J. and Niemann, C., Proc. Nat. Acad. Sci., 39, 999 (1953)

15) McLaren, A. D., Enzymologia, 26, 237 (1963)

16) Whitaker, D. R., Biological Degradation of Cellulose, 161, Butterworth, London (1960)

17) Brandt, D., Hontz, L. and Mandels, M., A.I.Ch.E. Symp. Series, Forest Products and the Environment, 69, 127 (1973)

18) Tarkow, H., Tappi, 33, 595 (1950)

19) Warwicker, J. O., Jeffries, R., Colbran, R. L. and Robinson, R. N., A Review of the Literature on the Effect of Caustic Soda and the Other Swelling Agents on the Fine Structure of Cotton, (Shirley Institute Pamphlet No. 93), the Cotton, Silk and Manmade Fibres Research Association, Manchester (1966)

20) Hermans, P. H. and Weidinger, A., J. Polymer Science, 4, 135 (1949)

21) Hermans, P. H., Contributions to Physics of Cellulose Fibers, Elsevier Publishing Co. (1946)

22) Nickerson, R. F., Ind. Eng. Chem., 33, 1022 (1941), 34, 85 (1942)

23) Goldfinger, G., Mark, H. and Siggia, S., Ind. Eng. Chem., 35, 1083 (1943)

24) Timell, T., Dissertation, Royal Institute of Technology, Stockholm (1950)

25) Nevell, T. P., Methods in Carbohydrate Chemistry, Vol. III, 164 Academic Press (1963)

26) Amemura, A. and Terui, G., J. Ferment. Tech., 43, 275 (1965)

27) Davidson, G. F., J. Textile Inst., 27, 112 (1936)

28) Mandels, M., Hontz, L. and Nystrom, J., Biotech. Bioeng., 16, 1471 (1974)

5. Theory

As discussed in Part I, the activities of various enzymes which were involved in the cellulase and β-glucosidase system could be measured by any means, and those activities would give some characteristic figures of the reaction. However, since the assay conditions of measuring the activities were determined rather empirically without any theoretical basis, it would be difficult to establish a particular model using those activities which describes the enzymatic hydrolysis reaction. For instance, even if all the usual cellulase activities (C_1, C_x and filter paper activity) and β-glucosidase activity were known, it is still impossible to predict the progress of the reaction. Experimental data will provide some information, but it requires tremendous effort to cover all the conditions which might be used for the design calculations. The main objective in this chapter is thus to establish a theoretical model by which the effects of each factor such as accessibility of substrate, concentration of inhibitor, etc., on the progress of the reaction can be evaluated.

5.1 Definition of Accessibility of Cellulose

Cellulase action on a cellulosic material can be divided into two categories: one is the hydrolysis reaction with amorphous cellulose and the other is that with crystalline cellulose. The classic theory presented by Reese, Siu and Levinson (1) postulated that the latter reaction was initiated by the C_1 component to convert the crystalline cellulose into linear anhydro-glucose chains, which could be

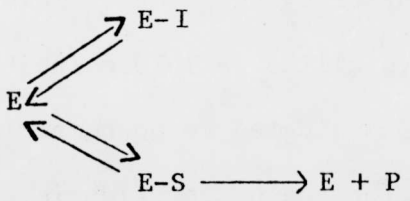
hydrolyzed by the C_x component into soluble oligosaccharides.

More recently, however, an entirely different concept of C_1-C_x activities of the cellulase system has been proposed by several investigators (2,3,4,5), and the classic theory has almost been abandoned. The new theory, which seems quite contrary to the classic theory, can be summarized as follows: (a) The C_1 component is a hydrolytic enzyme, which can be called β-1,4-glucan cellobiosyl hydrolase, whose function is to degrade the cellulose substrate when it is in a most accessible form by removing successive units of cellobiose from the chain ends. (b) The C_x component randomly splits the β-1,4-glucosidic bonds of soluble, swollen and partially degraded cellulose, but can not attack crystalline cellulose. (c) The C_1 component also can not attack crystalline cellulose, but acts synergistically with C_x component to accomplish extensive breakdown of highly crystalline cellulose.

The terms "amorphous" and "crystalline" have not been clearly defined, as discussed in Chapter 4. To extend the theoretical consideration further, these terms were defined as follows: Amorphous cellulose or accessible cellulose is a cellulose which can be hydrolyzed by the C_x component of cellulase. Crystalline cellulose or less-accessible cellulose is a form of cellulose which can be hydrolyzed by the synergistic action of C_1 and C_x activity of cellulase. Since these enzymes must be adsorbed on the surface of the cellulose, a more appropriate definition would be that amorphous cellulose is a cellulose accessible to the C_x activity of cellulase and that crystalline cellulose is a cellulose accessible to a combination of the C_1 and C_x activities.

5.2 Reaction of Accessible Cellulose

The action of cellulase on cellulose is strongly inhibited by the presence of cellobiose, an intermediate product of the cellulose hydrolysis, and the data obtained by several investigators (6,7,8) have suggested that the type of the inhibition is competitive, in other words, cellobiose combines with the enzyme and prevents the enzyme from combining with the cellulose. This competitive inhibition can be depicted by



$$E + I \rightleftharpoons E\text{-}I$$

$$E + S \rightleftharpoons E\text{-}S \longrightarrow E + P$$

where E = enzyme, S = substrate, I = inhibitor

E-S = enzyme-substrate complex

E-I = enzyme-inhibitor complex

This model leads to a reaction rate expression of the form

$$V = \frac{V_{max}\, S}{K_m(1+I/K_i) + S} \qquad (5\text{-}1)$$

where V_{max} = maximum rate of reaction

K_m = Michaelis-Menten constant

K_i = equilibrium constant

As discussed by several investigators, the components of cellulase are strongly adsorbed on the surface of cellulose. It would be much simpler if the enzyme having the C_x activity is adsorbed only on the accessible portion of cellulose, but experimental data show that even cotton, which is believed to be almost completely crystalline, adsorbs a certain amount of C_x cellulase. This phenomenon means that both cellobiose and crystalline cellulose inhibit the C_x reaction. This inhibition can be considered to be competitive; therefore the basic kinetic equation for the C_x reaction is:

$$V = \frac{V_{max}\, Sa}{K_m\left(1+\frac{I_1}{K_1}+\frac{I_2}{K_2}\right)+ Sa} \qquad (5\text{-}2)$$

where Sa = concentration of accessible cellulose

I_1 = Sc = concentration of crystalline cellulose

I_2 = concentration of cellobiose

K_1, K_2 = equilibrium constants

When the initial reaction rate is measured, the effect of product inhibition can be neglected without substantial error, and the third term of the denominator of Equation (5-2), I_2/K_2, can be dropped. Now let α be the accessible portion of the cellulose. Then

$$Sa = S\,\alpha$$

$$Sc = S(1 - \alpha)$$

Substitution of these relations into Equation (5-2) gives

$$V = \frac{V_{max}\, S\,\alpha}{K_m\,(1 + S(1-\alpha)/K_1) + S\,\alpha} \tag{5-3}$$

Inverting:

$$\frac{1}{V} = \frac{1}{\alpha V_{max}} \left\{ K_m\left(\frac{1}{S} + \frac{1}{K_1}\right) + \alpha\left(1 - \frac{K_m}{K_1}\right)\right\} \tag{5-4}$$

Thus, if the value of 1/V is plotted against that of 1/S, a straight line will be obtained as shown in Fig. 5.1 schematically. The intersections with the ordinate and the abscissa represent

$$\frac{1}{V_0} = \frac{1}{\alpha\, V_{max}} \left\{ \frac{K_m}{K_1} + \alpha\left(1 - \frac{K_m}{K_1}\right)\right\} \tag{5-5}$$

and

$$\frac{1}{S_0} = \frac{-1}{K_m} \left\{ \frac{K_m}{K_1} + \alpha\left(1 - \frac{K_m}{K_1}\right)\right\} \tag{5-6}$$

respectively.

It should be noted that V_{max}, K_m, and K_1 depend only on the nature of the enzyme, including the enzyme concentration, and α on the structure of the substrate. Therefore, when two different kinds of cellulosic substrates are reacted with the same enzyme system, the ratio $-V_0/S_0$, which gives the value of $\alpha\, V_{max}/K_m$ for each cellulose, can be obtained from the Lineweaver-Burk type plot, and the relative accessibility (i.e., α_1/α_2) can be calculated by taking the ratio of these numbers.

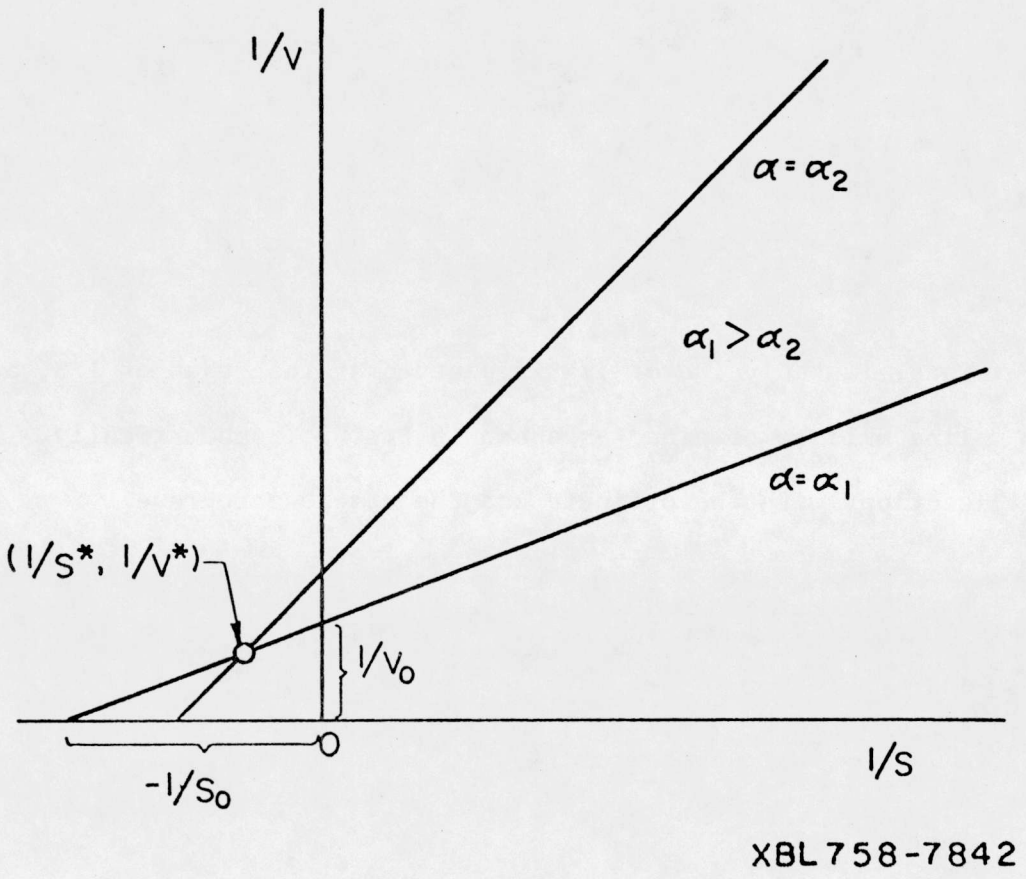


Figure 5.1. Generalized Lineweaver-Burk plot.

The absolute value of α can be obtained if a substrate of known accessibility is available. For this purpose, wood pulp swelled with 85 percent phosphoric acid might be suitable. According to Walseth (9), who proposed the method, acid treated wood pulp was highly reactive and could be considered as 100% accessible cellulose. For the 100% reactive cellulose, the following relations were obtained from the Equations (5-5) and (5-6):

$$\frac{1}{V_0} = \frac{1}{V_{max}} \tag{5-7}$$

and

$$\frac{1}{S_0} = -\frac{1}{K_m} \tag{5-8}$$

Hence, by using Walseth's reactive cellulose, the values of V_{max} and K_m can be estimated very easily.

Another interesting feature of this kinetic model is that the equilibrium constant, K_1, can be evaluated from the experimental data if the model is adequate. From Equation (5-4),

when

$$\frac{1}{S} = \frac{1}{S^*} = -\frac{1}{K_1} \tag{5-9}$$

$$\frac{1}{V} = \frac{1}{V^*} = \frac{1}{V_{max}}\left(1 - \frac{K_m}{K_1}\right) \quad \text{for all } \alpha \tag{5-10}$$

These relations suggest that although the slope of the lines in Fig. 5.1 varies with the value of α, all of the lines pass through the point $(1/S^*, 1/V^*)$ no matter what α the line bears. Thus K_1 can be calculated either from Equation (5-9) or (5-10).

The effect of product inhibition can be measured by adding cellobiose to the reaction system and measuring the initial reaction rate. If the cellobiose concentration remains constant during the reaction, we have the following relation to describe the system:

$$V = \frac{V_{max}\, S\, \alpha}{K_m \left(1 + \frac{S(1-\alpha)}{K_1} + \frac{I_2}{K_2}\right) + S\alpha} \qquad (5\text{-}3)'$$

Inverting:

$$\frac{1}{V} = \frac{1}{\alpha V_{max}} \left\{ \frac{K_m}{S} \left(1 + \frac{I_2}{K_2}\right) + \frac{K_m}{K_1} + \alpha\left(1 - \frac{K_m}{K_1}\right) \right\} \qquad (5\text{-}4)'$$

Thus the plot of 1/V vs. 1/S again gives a straight line whose slope is

$$\frac{K_m}{\alpha\, V_{max}} \left(1 + \frac{I_2}{K_2}\right),$$

from which K_2 can be readily estimated.

5.3 Reaction of Crystalline Cellulose

The hydrolysis reaction of crystalline cellulose is usually much slower than that of amorphous cellulose. Therefore, it is essential to substract the rate of the latter reaction from total reaction rate unless pure crystalline cellulose is used. Although absorbent cotton has been widely used for measuring the C_1 activity, it is also known that the cotton has a small amorphous portion in its structure. When cotton is reacted with cellulase, it is very likely that the amorphous parts react with the C_x component quickly and later on the crystalline parts

react with the synergistic action of the C_1 and C_x components. Thus, by using the later reaction rate we may derive the same equations as were used for accessible cellulose.

For the measurement of the inhibitory effects of both the product and the accessible part of cellulose, some difficulties arise because of the slow reaction rate of the crystalline cellulose. When an appropriate amount of cellobiose is put into the reaction system as an inhibitor, the concentration of the added sugar is much higher than that of the sugars produced by the enzyme and the actual rate of the reaction can hardly be obtained. Measurement of the rate of the hydrolysis of crystalline cellulose in the presence of the accessible cellulose, for instance Solka Floc, is another problem. Since the C_x reaction proceeds as long as accessible cellulose remains in the substrate, the rate of the hydrolysis of crystalline cellulose could be measured only when all of the accessible portion is consumed--probably after several hours of reaction time. In that circumstance the reaction rate is then affected by the presence of cellobiose so that the actual reaction rate again can not be measured. In the present work, therefore, no attempt was made to measure the effect of possible inhibitors.

Selby (10) reported that the hydrolysis reaction of cotton with cellulase was first order, so that when the values of $\ln(S/S_0)$ were plotted against time a linear relation was obtained for more than 300 hours of hydrolysis. (S_0 was the initial substrate concentration and S was the residual substrate concentration.) Therefore, it is expected that the kinetic model for the hydrolysis of crystalline cellulose can

be expressed by the following equation, provided that 100% crystalline cellulose is used:

$$\frac{d\ Sc}{dt} = -\ k\ Sc \qquad (5\text{-}11)$$

Integrating:
$$k\ t = \ln(Sc_0/Sc) \qquad (5\text{-}12)$$

where k = rate constant

Sc = concentration of crystalline cellulose

Sc_0 = initial values of Sc

If the cellulose contains a small amount of amorphous cellulose, the equation becomes

$$\ln \frac{S_0}{Sc} = \ln \frac{S_0}{Sc_0} + \ln \frac{Sc_0}{Sc} = \ln \frac{S_0}{Sc_0} + k\ t \qquad (5\text{-}13)$$

where S_0 = initial concentration of total cellulose.

Thus, plotting $\ln(S_0/Sc)$ against t gives a straight line and the intersection on the ordinate is $\ln (S_0/Sc_0)$, from which we can calculate the crystalline portion of the substrate.

Literature Cited in Chapter 5

1) Reese, E. T., Siu, R. G. H. and Levinson, H. S., J. Bacteriol., 59, 485 (1950)

2) King, K. W. and Vessal, M. J., Cellulases and Their Applications, Advances in Chem. Series, 95, 7 (1969)

3) Wood, T. M., Ferment. Technol. Today, Soc. Ferment. Technol., Japan, 711 (1972)

4) Nisizawa, K., Tomita, Y., Kanda, T., Suzuki, H. and Wakabayashi, K., Ferment. Technol. Today, Soc. Ferment. Technol., Japan, 719 (1972)

5) Pettersson, L. G., Axio-Fredriksson, U. and Berghem. L.E.R., Ferment. Technol. Today, Soc. Ferment. Technol., Japan, 727 (1972)

6) Ghose, T. K. and Das, K., Adv. in Biochem. Eng., 1, 55, Springer-Verlag (1971)

7) Arai, K. and Ogiwara, Y., Kogyo Kagaku Zasshi, 74, 460 (1971)

8) Stuck, J. D. and Howell, J. A., A.I.Ch.E. Symp. Series, Water-1973, 70, 337 (1974)

9) Laidler, K. J., The Chemical Kinetics of Enzyme Action, 30, Oxford Univ. Press (1958)

10) Walseth, C. G., Tappi, 35, 228 (1952)

11) Selby, K., Cellulases and Their Applications, Adv. in Chem. Series, 95, 149 (1969)

6. Experiments

6.1 Analytical Procedures

The concentration of reducing sugars in the hydrolysis product was measured with either the DNS reagent or the anthrone reagent. As pointed out in Chapter 2, the anthrone method gives more consistent results when pure cellulose is used as a substrate. However the actual hydrolysis product includes impurities which apparently affect the DNS measurement. Miller reported interference by citrate buffers, which are commonly used with the cellulase system, to the reaction between glucose and the DNS reagent (1). In the present work only acetate buffer, which does not materially affect the DNS test, was used.

To investigate the effect of other unknown substances in the hydrolysis products, Evett (2) measured the relation between absorbance and glucose concentration for the DNS measurement by adding known amounts of glucose to the solution from hydrolysis of newsprint. Although at that time the slope of the DNS standard curve for pure glucose solution was 0.225 absorbance units/mg of glucose, the slope obtained by Evett was 0.291, about 30 percent higher than that of the standard solution. However, as is shown in Fig. 6.2, this change in slope was not observed when 0.85 mg glucose/ml was added to the (45°C) hydrolysis product. The reason for the discrepancy is not understood.

The same experiments were carried out for both DNS and anthrone reagents using a 48 hour hydrolysis product. The results with the anthrone reagent (Fig. 6.1) show that the slope of the calibration curve is also the same with or without hydrolysis products.

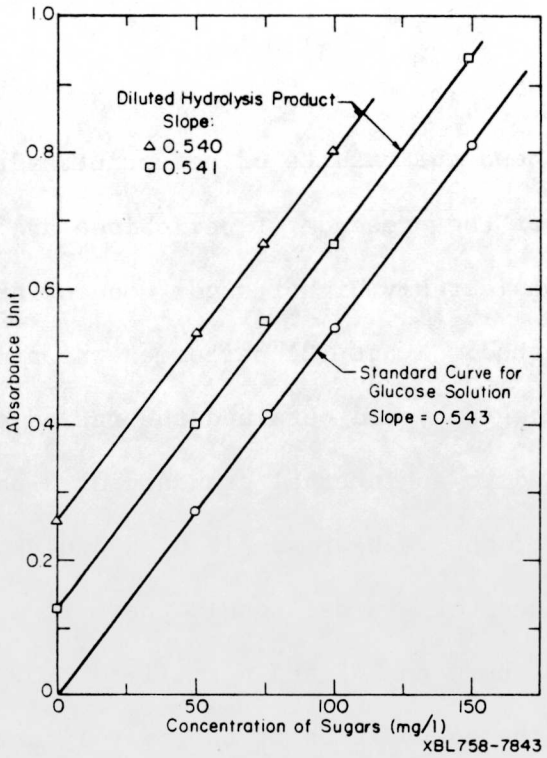


Figure 6.1. Effect on anthrone reagent method of adding glucose to newsprint hydrolysis solutions.

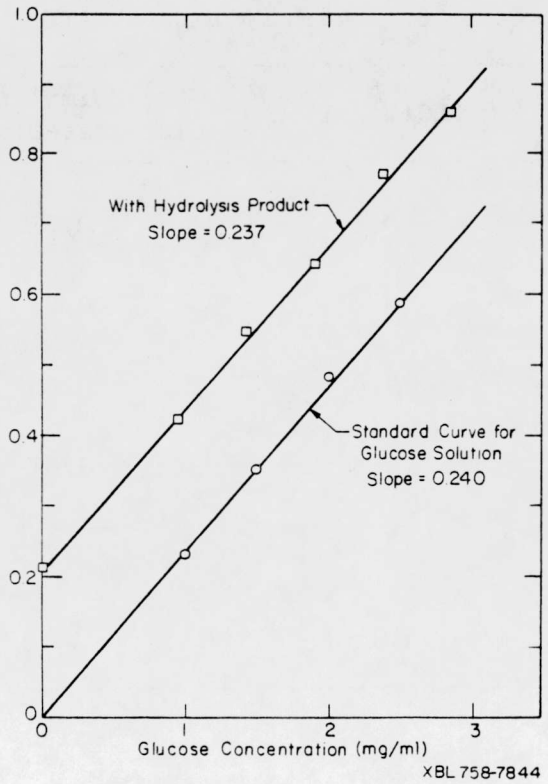


Figure 6.2. Effect on DNS reagent method of adding glucose to newsprint hydrolysis solutions.

Figure 6.3 shows the results of newsprint hydrolysis with cellulase. Because of the presence of cellobiose and xylose, the DNS measurement gives considerably higher sugar concentrations than the Glucostat glucose method. Evett (2) performed the product analysis by gas liquid chromatography and obtained the ratios of the sugars present to glucose and to an internal standard of β-phenyl-d-glucoside. When the GLC result of the 48 hour sample of hydrolyzate is normalized to the comparable Glucostat glucose result there is a reasonable difference of only 2% between DNS and normalized GLC. It is fortunate that with newsprint the composition of the hydrolyzate is such that the resultant composite slope of glucose, cellobiose, and xylose with DNS gives a reasonable estimation of the sugar content.

Table 6.1 48-HOUR HYDROLYZATE

	GLC(mg/ml)	GLC, Normalized to Glucostat Glucose	Wgt%
Xylose (150)	0.6	0.71	4.8
Mannose (180)	0.2	0.24	1.6
Glucose (180)	9.0	10.65	71.4
Cellobiose (342)	2.8	3.31	22.2
Total	12.6	14.9	100.00

DNS method was 15.2 mg/ml. (Figure 6.3)

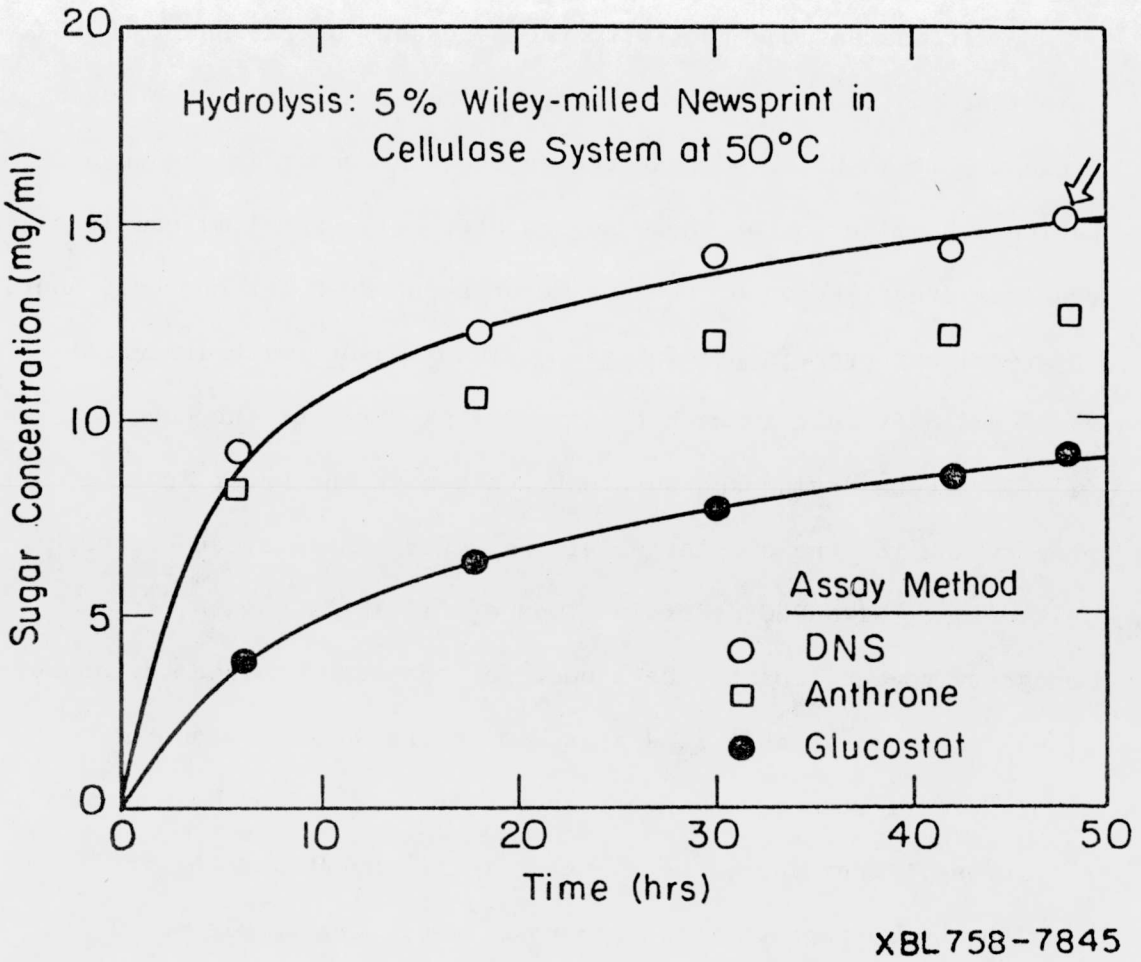


Figure 6.3. Comparison of sugar assay methods. Hydrolysis of newsprint by cellulase.

Table 6.1 outlines the distribution of the sugars obtained in the 48 hour hydrolyzate as determined in the above manner including the result of normalization to Glucostat glucose. As can be seen in Table 6.1, the GLC glucose value is lower (9.0 mg/ml) than the Glucostat glucose value (10.65 mg/ml).

It was assumed that with the necessary operations for GLC determination, losses occurred proportionately with all the sugars present. It is believed that the losses that occur in the quantitative determination of these sugars are: (1) the 1 ml sample taken requires denurization of the enzyme at about 80°C for one half hour. The resultant protein precipitate occludes sugars with it and it was found difficult to wash the precipitate free of the sugars. (2) In freeze drying the supernate to remove the water in preparation for the silylation of the sugars there often occurs spattering. With GLC determinations of silylated sugars, it is necessary to equilibrate the anomers of the sugars since the anomer of one sugar invariably is detected with the anomer of another sugar. For instance, a mannose anomer is detected with an anomer of glucose. Therefore, the anomers of the sugars must be in equilibrium so that they can be quantified. The sample was equilibrated in dimethyl formamide at 40°C for 4 hours, which gives rise to loss (3) by creeping of the solution onto the cap of the container. The sample was silylated with the appropriate amount of trimethyl chloro silane and hexamethyl silazane solvent. Since the samples must be tracked by injection into the gas chromatograph

to observe if the silylation is complete, the continual opening and closing of the sample container gives rise to a further loss (4) due to creeping of the silylation solvent off of the rim of the container. It is unknown how this loss affects the distribution of the sugars, since the rate of silylation of pentoses occurs more rapidly than hexoses.

If the silylation is not complete multiple peaks are observed for every sugar and their respective anomers, thereby creating an unsolvable record. The condition of the corrosive trimethyl chloro silane, which hydrolyzes readily in ambient humidity, also dictates the length of the silylation time. This time varies from 5 minutes to 4 hours giving rise to a further loss (5) since phase separation begins at approximately this time. Phase separation in the silyated sugars indicates degradation and dictates starting anew.

With newspring enzyme hydrolyzates the reproducibility of glucostat method ($\pm$ 2%) justifies normalization of the GLC glucose to the glucostat glucose.

6.2 Experimental Procedures

6.2.1 Enzymatic Hydrolysis of Cellulose with Mixed Enzyme System

The hydrolysis reaction was investigated in bench scale experiments. The reaction mixture was agitated well with a magnetic stirrer in a 125 ml flask with a rubber stopper. The flask was placed in a water bath, where the temperature was maintained constant by a contact

thermometer. Eighty ml of enzyme solution was usually used for each reaction batch. The cellulosic material was added to the solution and mixed well by shaking. After a certain period of time 5--10 ml of sample was withdrawn, heated in boiling water for 2 minutes to denature the β-glucosidase activity and centrifuged. The supernatant was frozen for later analysis. Reducing sugar was measured by either the DNS or the anthrone reagent method. The Glucostat reagent was used to measure the glucose concentration.

An 800 ml flask with a motor-driven propeller type agitator was also used for this reaction. The rotation speed was adjusted to 200--300 rpm.

6.2.2 Reaction Kinetics of Cellulose Hydrolysis

The initial reaction rate of hydrolysis of accessible cellulose was measured as follows: from 50 to 250 mg of cellulose was added to 5 ml of the enzyme solution in a 22 mm glass test tube and dispersed well in a Vortex mixer. The tube was then incubated in a water bath shaker at 50° C. Shaking at 300 rpm was provided to keep the solid materials suspended in the enzyme solution. After two hours of reaction the contents of the tube were removed, the residual solids were removed by centrifuge, and the sugar concentration in the supernatant was measured with the anthrone reagent.

Four different pure celluloses and three differently pretreated newsprint samples were used as substrates. These are listed in Table 6.2.

Table 6.2. Several cellulosics tested.

Cellolose	Pretreatment
Solka Floc	None
Solka Floc	Ball-milled for 24 hours
Solka Floc	Swelled in 85% phosphoric acid
Filter paper	Shredded into pieces (about 2 mm × 2 mm)
Newsprint	Wiley-milled
Newsprint	Ball-milled for 24 hours
Newsprint	Swelled in 2.5 N sodium hydroxide

Solka Floc was treated with phosphoric acid (3) by adding 130 ml of 85% phosphoric acid at 2°C to 10 g Solka Floc, and the cellulose was soaked well by manual stirring. The mixture was stored in the refrigerator overnight. The Solka Floc was washed by repeated cycles of suspension in ice-cold water and filtration. A Waring blender was used for the suspension. After four washes the cellulose was suspended in a 1% sodium carbonate solution for 6 hours, and then washed four times with distilled water at room temperature.

Swelling of newsprint by sodium hydroxide was reported to be an effective pretreatment which provided more accessible substrate to the enzymatic hydrolysis (4). After 1 liter of 2.5 N sodium hydroxide was added to 100 g of Wiley-milled newsprint, swelling took place within 20 minutes at 50°C. The mixture was then washed with distilled water (1 liter × 5 times) and neutralized with sulfuric acid. The celluloses pretreated with either phosphoric acid or sodium hydroxide were finally

washed with 95% ethanol to remove moisture and then dried in a vacuum oven.

For the reaction of a crystalline cellulose, absorbent cotton was used as a substrate. Since the reaction of crystalline cellulose via the synergistic action of the C_1 and C_x components of cellulase was supposed to be first order (see Sect. 5.3), the same kind of result was expected in the present work. Thus the experimental procedure was: 4 to 30 mg of absorbent cotton were added to test tubes containing 5 ml of the enzyme solution, the culture filtrate of Trichoderma viride. The tubes were incubated in a water bath shaker at 50° C. At appropriate intervals a tube was taken out from the bath, the residual cellulose was removed by centrifuge and the soluble sugar in the supernatant was determined with the anthrone reagent.

6.2.3 Reusable Enzyme in Hydrolysis Product

The cost of enzyme production was reported to be considerably high and more than 60% of total glucose production cost was attributable to this process (5). Consequently, the recovery and reuse of the enzyme is as important as the production of the enzyme. Yang (5) reported the reusable cellulase activity in the hydrolysis product. Cellulase was adsorbed on the newsprint almost immediately after mixing, and the concentration of free enzyme in the solution remained approximately constant as the reaction proceeded. His data are shown in Table 6.3.

Table 6.3. Reusable cellulase activities* after 48 hours hydrolysis.**

Cellulase activities	Temperature 45° C	50° C
C_x	66%	66%
C_1	75	60
Filter paper	55	30

* expressed as a percent initial protein concentration (see Sect. 3.2.6).
** initial filter paper activity of 3.5 and 5% Wiley-milled newsprint.

As discussed in Chapter 3, β-glucosidase denatures very rapidly at high temperature and 50° C is probably too high for the enzyme. Thus the recoverable β-glucosidase in the hydrolyzate was estimated with the same method as Yang (5) used for cellulase. The hydrolysis product suspension which contained sugars, residual cellulose and enzymes, was centrifuged to remove solid materials. The supernatant was then replaced by the same amount of 0.05 M acetate buffer, pH 5.0, and the residual cellulose was resuspended to wash out the enzyme attached to the cellulose. The mixture was again centrifuged, and the supernatant was taken out for the measurement of the activities.

Since these solutions contained a certain amount of reducing sugars which were produced during the hydrolysis reaction and which would interfere with the reaction in the assay procedure, the enzyme was first precipitated with acetone (3 v/v sample) and redissolved into acetate buffer (0.05 M, pH 5.0), and then the activities were measured.

Literature Cited in Chapter 6

1) Miller, G. L., Anal. Chem., 31, 426 (1959)

2) Evett, M., private communication, Lawrence Berkeley Laboratory, April (1975)

3) Walseth, C. G., Tappi, 35, 228 (1952)

4) Mandels, M., Hontz, L. and Nystrom, J., Biotech. Bioeng., 16, 1471 (1974)

5) Yang, R. D. and Wilke, C. R., Presented at the Eighth Cellulose Conference on Wood Chemicals, Syracuse, New York, May (1975)

7. Results and Discussion

7.1 Accessibilty of Cellulose and Kinetic Considerations for Hydrolysis by Culture Filtrates of Trichoderma viride

7.1.1 Accessible Cellulose

Figure 7.1 shows the experimental data for pure cellulose listed in Table 6.2 in Chapter 6. Though some of the data points were scattered considerably, the Lineweaver-Burk plot seemed to give straight lines for these celluloses, indicating that the Michaelis-Menten type of kinetic model could be applied to these soluble enzyme-insoluble substrate systems.

It is also interesting to note that all lines pass a single point, P, in Fig. 7.1. This point corresponds to the point $(1/S^*, 1/V^*)$, where $1/S^*$ and $1/V^*$ are related to the specific constants, V_{max}, K_m and K_1, for these cellulase--cellulose systems by equations (5-9) and (5-10), which are derived in Chapter 5. Assuming 100% accessibility for the reactive cellulose prepared from Solka Floc (see Table 6.2), we can calculate V_{max}, K_m, and K_1 for accessible cellulose and the accessibility, α, for each different cellulose. The results are shown in Table 7.1.

Several interesting phenomena can be observed in the results. The accessibility of Solka Floc was estimated as 19%, which was fairly low compared with the data obtained with the chemical methods (see Table 4.2), but about three times higher than the value obtained by the enzymatic hydrolysis with C_x component only (1). The accessibility of 19% is probably too low for the Solka Floc, because the conversion to soluble sugars is usually more than 50% at 40--50 hours hydrolysis.

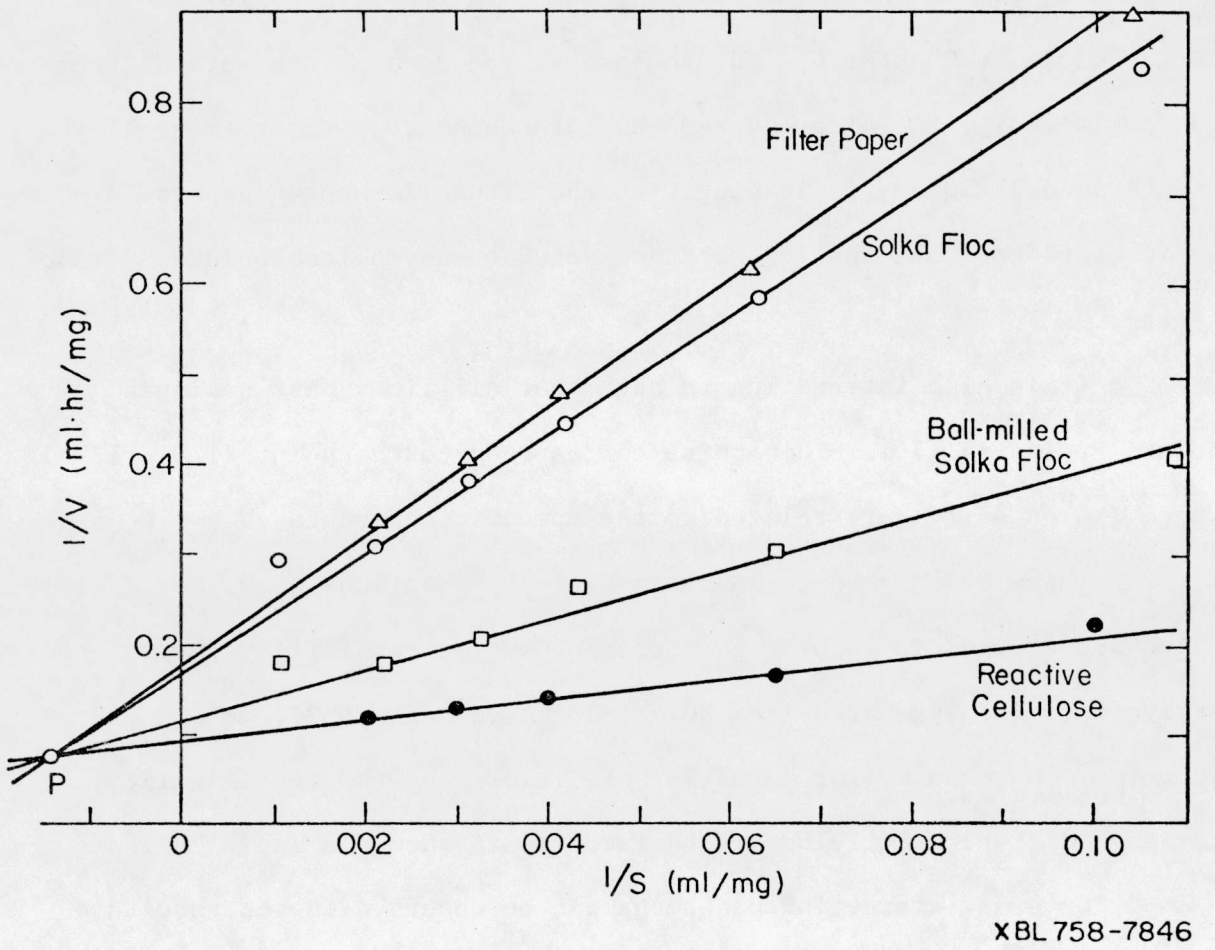


Figure 7.1. Lineweaver-Burk plots of effect of substrate concentration on rate of hydrolysis of various pure celluloses by cellulase from *T. viride*.

Table 7.1. Kinetic data for pure celluloses.

Cellulose	$-1/S_0$	$1/V_0$	$-V_0/S_0$	α
Reactive celluose	0.0760	0.094	0.8085	1.00
Solka Floc	0.0256	0.170	0.1506	0.186
Ball-milled SF	0.0410	0.115	0.3565	0.441
Filter paper	0.0246	0.176	0.140	0.173

From the values of $-1/S_0$ and $1/V_0$ for the reactive cellulose, K_m and V_{max} were calculated as follows:

$$K_m = 13.2 \text{ mg/ml}$$

$$V_{max} = 10.66 \text{ mg/ml-hr}$$

From the value of $-1/S^*$, $K_1 = 70.4$ mg/ml.

The differences in the accessibilities obtained with various methods can be explained as follows: The enzyme molecule is much larger than the molecule of chemicals commonly employed for the measurement of accessibility of cellulose so that the enzyme, i.e., the C_x component, could not penetrate into the crystalline portion of cellulose, which might include some accessible cellulose. Smaller molecules, on the other hand, could penetrate into the cellulose more easily. This may explain the difference between the value obtained in the present work and that by chemical methods.

When the C_1 component is present with the C_x component, the synergistic action of these two components converts the crystalline cellulose to accessible cellulose, but at the same time it is capable of fragmenting crystalline cellulose into smaller particles. Therefore, the accessible cellulose which is entrapped in the crystalline cellulose may eventually react with C_x component if the enzyme system contains C_1 component. Consequently, it may be concluded that the lower value obtained by Brandt et al. (1) resulted from a lack of C_1 component in the enzyme used.

The accessibility of the ball-milled Solka Floc was about 2.5 times that of Solka Floc. By ball-milling, the size of the cellulose particles was considerably decreased, and hence the surface area, which could contact with the enzyme, increased. The crystalline structure was also affected by the mechanical grinding, yielding more accessible cellulose.

Figure 7.2 shows the Lineweaver-Burk plot for differently pretreated newsprint. Again it was observed that all three lines passed through the same point, P, as was observed with pure cellulose.

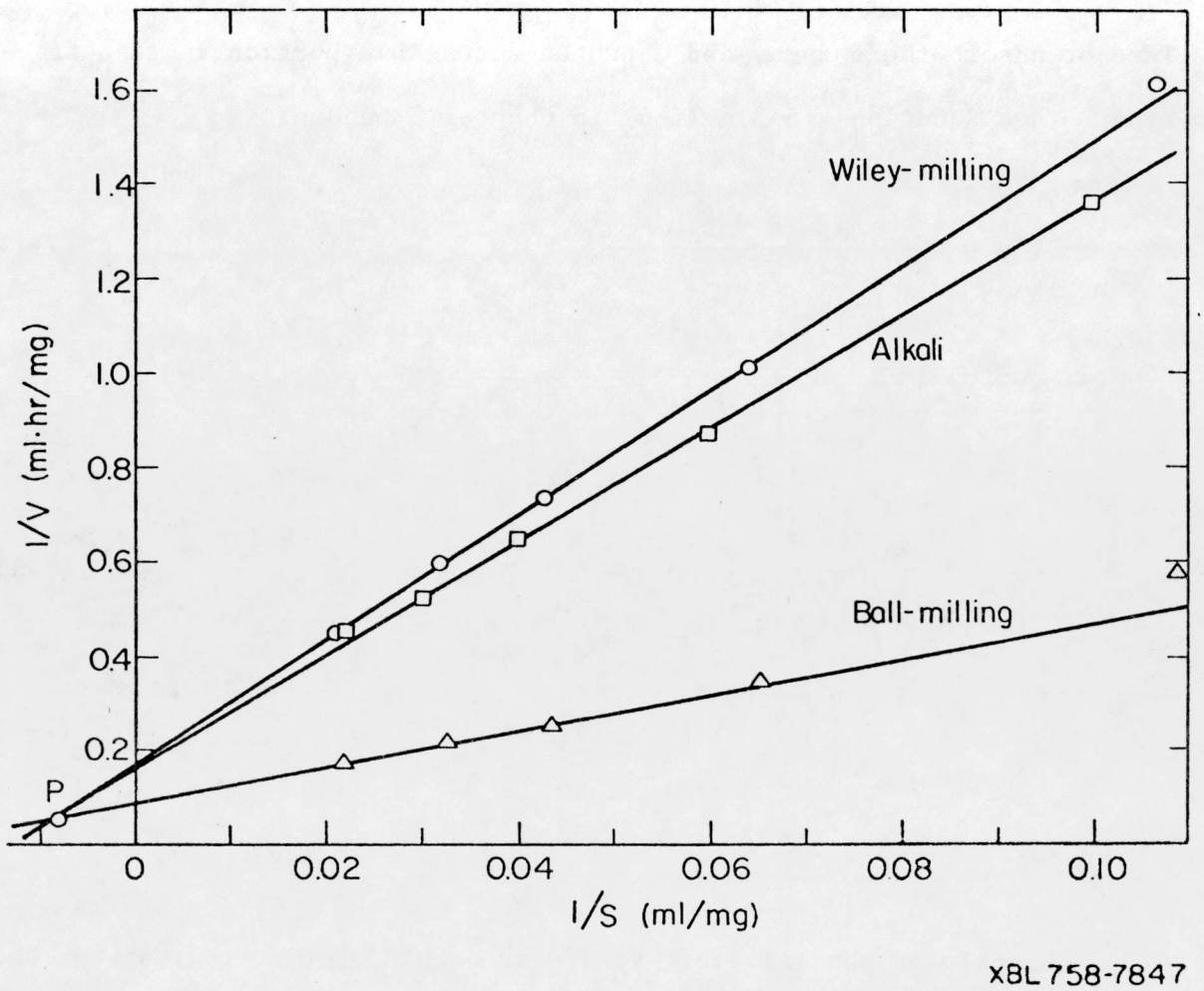


Figure 7.2. Lineweaver-Burk plots of effect of substrate concentration on rate of hydrolysis of newsprint by cellulase from *T. viride*.

Analysis of the data should be slightly modified for the newsprint. The newsprint contains hemicellulose and lignin in addition to α-cellulose, and we do not know whether these non-α-cellulose portions react with cellulase or whether they adsorb cellulase and competitively inhibit the reaction. Let ξ be the fraction of newprint which does react with cellulase or adsorb the enzyme, and α be the accessible portion in that fraction. Then Equations (5-3)--(5-6) in Chapter 5 become:

$$V = \frac{V_{max} S \alpha \xi}{K_m(1+S(1-\alpha)\xi/K_1)+S\alpha\xi} \tag{7-1}$$

$$\frac{1}{V} = \frac{1}{\alpha\xi V_{max}} \left\{ K_m\left(\frac{1}{S} + \frac{\xi}{K_1}\right) + \alpha \xi \left(1- \frac{K_m}{K_1}\right) \right\} \tag{7-2}$$

$$\frac{1}{V_0} = \frac{1}{\alpha V_{max}} \left\{ \frac{K_m}{K_1} + \alpha \left(1- \frac{K_m}{K_1}\right) \right\} \tag{7-3}$$

$$\frac{1}{S_0} = - \frac{\xi}{K_m} \left\{ \frac{K_m}{K_1} + \alpha \left(1- \frac{K_m}{K_1}\right) \right\} \tag{7-4}$$

Hence,
$$- \frac{V_0}{S_0} = \frac{\alpha \xi V_{max}}{K_m} \tag{7-5}$$

Thus the ratio of the value of $-V_0/S_0$ for each newsprint to that for the reactive cellulose gives the value of $\alpha\xi$, which is the accessibility of the newsprint. (The calculated values are shown in Table 7.2.)

From equation (7-1), $1/S^*$, which is the abscissa value of point P, is related to the dissociation constant, K_1, by the following equation.

$$\frac{1}{S^*} = - \frac{\xi}{K_1} \tag{7-6}$$

Table 7.2. Kinetic data for newsprints.

Newsprint	$-1/S_0$	$1/V_0$	$-V_0/S_0$	$\alpha\xi$
Wiley-milled	0.0130	0.170	0.0765	0.0946
Alkali-treated	0.0137	0.162	0.0846	0.1046
Ball-milled	0.0257	0.0924	0.2781	0.3440

From the value of $-1/S^*$,

$$K_1/\xi = 122 \text{ mg/ml}$$

From Fig. 7.2, $-1/S^*$ is obtained as 0.0082 ml/mg.

Hence,

$$K_1 = 122\ \xi$$

If we assume the same dissociation constant value for this system as obtained for the crystalline cellulose in pure cellulose, i.e., $K_1 = 70.0$ mg/ml, ξ is calculated from Equation (7-6) as 0.57, which is exactly the same as the fraction of α-cellulose in the newsprint, whose composition is shown in Table 7.3 (2).

This coincidence may suggest that neither hemicellulose nor lignin reacts with cellulase and also that they do not adsorb cellulase enzyme.* The latter finding is probably new, and it implies that the hydrolysis of the newsprint is difficult only because lignin, and possibly hemicellulose, hinders the enzyme from attacking α-cellulose. Mitra (4) reported that the adsorption of the C_1 component by hydrolyzed newsprint was much less than that by fresh newsprint. The results obtained here are consistent with his observations.

The values of $\alpha\xi$ indicate the accessible portion of newsprint. Assuming $\xi = 0.57$, we can evaluate the accessibility of α-cellulose in the newsprint. The results are also shown in Table 7.2. As expected, the ball-milled newsprint has the highest accessibility. Alkali treatment does not seem efficient for the improvement of accessibility. The hydrolysis data with this alkali-treated newsprint (50°C, 48 hours)

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*By gas chromatographic analysis, the hemicellulose was found to react with cellulase, but its reaction rate is very slow (3).

Table 7.3. Composition of Wall Street Journal Newsprint (2).

Sample: 35 -- 60 mesh fraction of single Wiley-milled newsprint

Moisture	6 ~ 9% of air dry weight
Extractives	2.5±0.3% of dry weight
Holocellulose	77±1% of dry weight
(Hemicellulose	21±3% of dry weight
\ α-cellulose	56±3% of dry weight
Lignin	21±0.5% of dry weight
Ash	0.35% of dry weight

showed an 8% increase in conversion over the untreated Wiley-milled newsprint (2). On the other hand, when the ball-milled newsprint was tested in the hydrolysis, the conversion was more than twice as much as that for Wiley-milled newsprint (2). Hence, the accessibility obtained with the method developed in this work may be used for the evaluation of the reactivity of cellulosic materials with cellulase in the enzymatic hydrolysis reaction.

7.1.2 Crystalline Cellulose

Figure 7.3 shows the relation between $\ell n(S_0/Sc)$ and reaction time, for which a linear relation was expected as described in Chapter 5. To obtain Fig. 7.3, it was assumed that the concentration of crystalline cellulose, Sc, was equal to that of residual cellulose because the amorphous portion of cotton was hydrolyzed much faster than the crystalline cellulose. Although some of the points scatter considerably, especially the data with lower concentrations of the substrate, the linearity of the relation seems to be confirmed, indicating that the first-order kinetic model can be applied to the reaction of crystalline cellulose. The rate constant, k, was calculated as 5.32×10^{-3} hr^{-1}.

The intercept on the ordinate is $\ell n(S_0/Sc_0)$, which gives the original percentage of the crystalline cellulose in the substrate used. (Sc_0 is an initial value of Sc.) The calculated fraction of crystalline portion in the absorbent cotton was 0.99, which was high compared with the data obtained with physical or chemical methods summarized in Table 4.2 in Chapter 4.

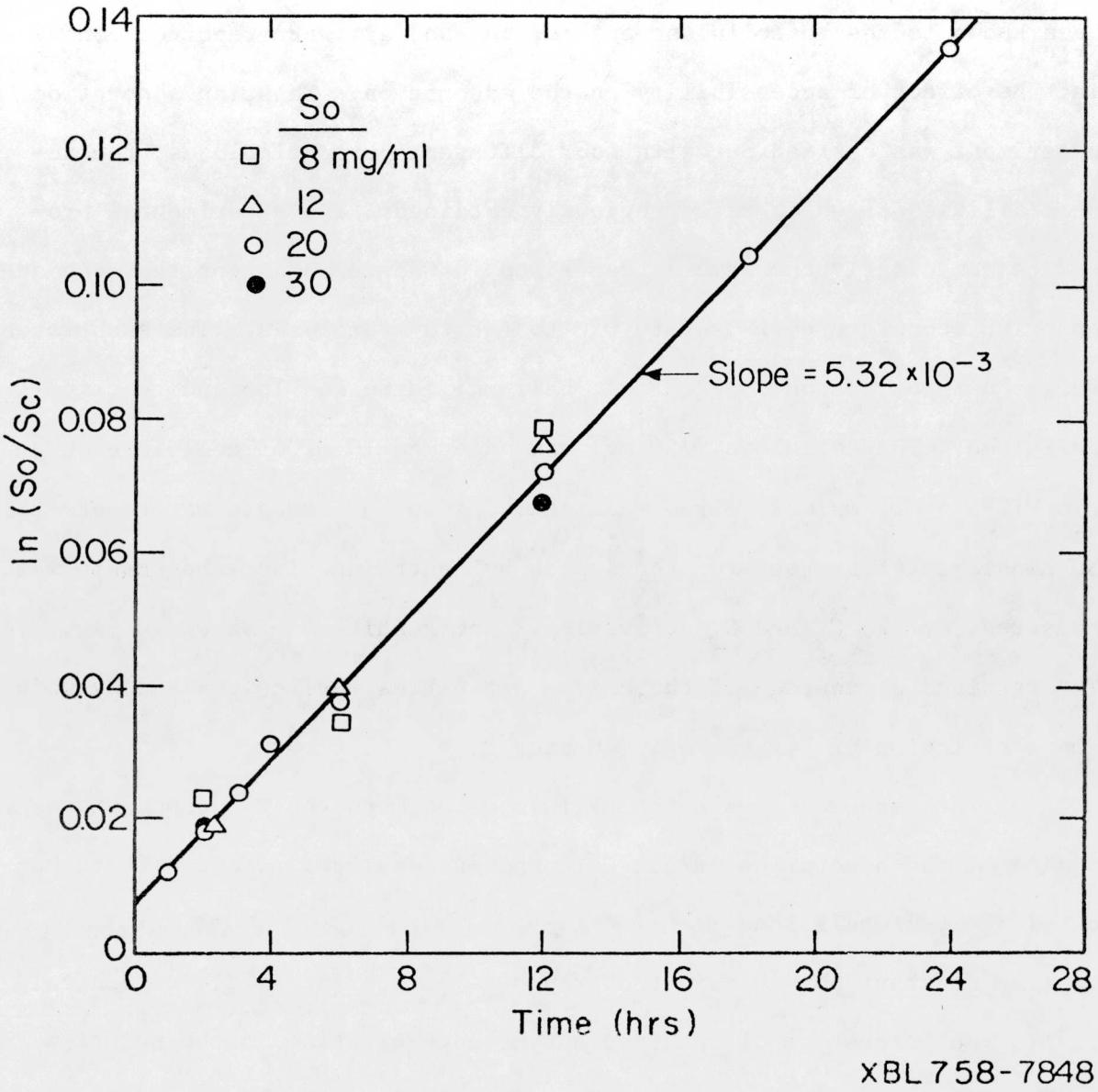

Figure 7.3. Hydrolysis of absorbent cotton by cellulase from *T. viride*.

7.1.3 Effect of Accessibility of Cellulose on Enzyme Adsorption

As discussed in the preceding sections, different values of accessibility were obtained for different pure celluloses. Cellulose has been known to adsorb cellulase enzyme, but no data were reported concerning the effect of accessibility on the adsorption. Thus, an adsorption experiment was carried out with four different pure celluloses, the accessibilities of which were previously obtained. The experimental procedure was exactly the same as described in Chapter 3 except that the adsorption temperature was set to 0° C to retard hydrolysis. The substrates were absorbent cotton, Solka Floc, ball-milled Solka Floc and reactive cellulose. The cellulose (250 mg) was added to 10 ml of cellulose solution (FPA = 4.2 mg/ml), mixed well and kept in an ice-cold water bath for 30 minutes. After removing the solids by centrifugation, the sample was measured for the C_x and C_1 activities. The results are shown in Fig. 7.4. The retained percentage of the enzyme activities were calculated by using the correlation of Fig. 3.26 in Chapter 3.

The more accessible the cellulose the more the C_x component was adsorbed, but the adsorption of the C_1 component was less affected by the accessibility of cellulose. Using the Michaelis-Menten constant and the dissociation constant obtained in the previous section (at 50° C), we can calculate the percentage of retained enzyme concentration in the solution as follows (notation: see Chapter 5, Sec. 5.2):

By definition,

$$K_m = \frac{(E)(Sa)}{(E\text{-}Sa)}$$

$$K_1 = \frac{(E)(Sc)}{(E\text{-}Sc)}$$

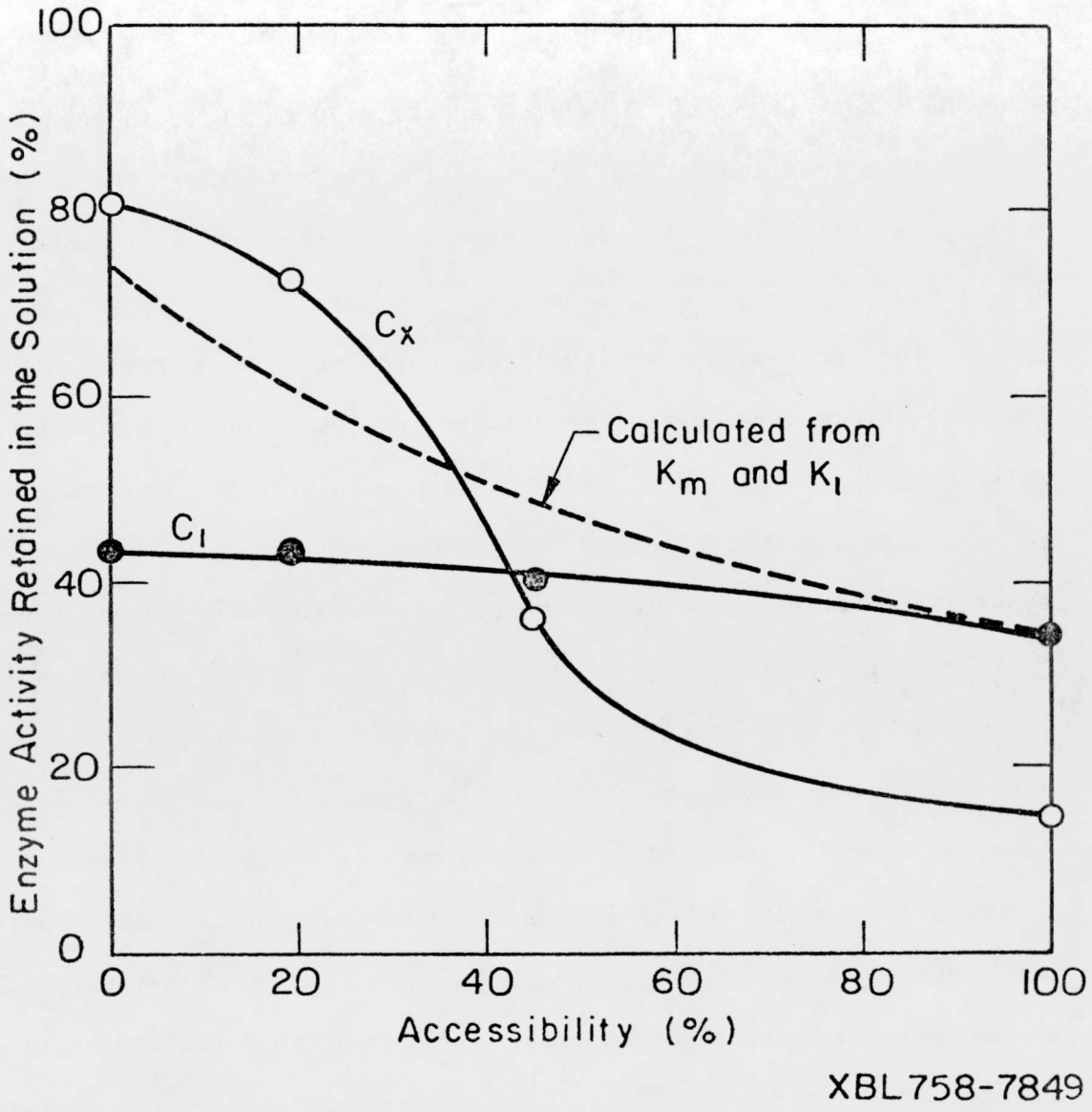

Figure 7.4. Adsorption of cellulase activity from culture filtrates of *T. viride* by Solka Floc of various accessibilities.

The total enzyme concentration, E_0, is

$$E_0 = E + (E\text{-}Sa) + (E\text{-}Sc)$$

Thus the partial enzyme fraction retained in the solution is

$$\varepsilon = \frac{E}{E_0} = \frac{1}{1 + \frac{(Sa)}{K_m} + \frac{(Sc)}{K_1}} \qquad (7\text{-}7)$$

Since Sa + Sc = 25 mg/ml, K_m = 13.2 and K_1 = 70 mg/ml, ε is readily calculated from Equation (7-7) as a function of Sa/(Sa+Sc), the accessibility of the cellulose. The resulting curve is the dotted line shown in Fig. 7.4.

The adsorbed enzyme level thus calculated is very much different from the curves experimentally obtained for the C_1 and C_x components. While the experimental data were obtained with specific substrates, cotton and carboxymethylcellulose, the theoretical curve was based on the synergistic action of cellulase on pure celluloses of different accessibilities. Therefore, the adsorbed enzyme expressed by the dotted line may not be the same as those for the C_1 and C_x components. For the reactive cellulose (100% accessible cellulose), the calculated value of the retained enzyme is coincident with the experimental value based on the C_1 activity. On the other hand, for the crystalline cellulose (absorbent cotton), it is closer to the value based on the C_x activity. This may suggest that the reaction rate of the reactive cellulose would be determined by the amount of the C_1 component adsorbed on the cellulose, and the reaction rate of crystalline cellulose would be limited by the amount of the C_x component adsorbed.

7.1.4 Product Inhibition

Figure 7.5 shows the Lineweaver-Burk plot for the cellulase--cellulose system, to which glucose or cellobiose was added to examine their inhibitory effect on the initial reaction rate. As derived in Chapter 5, the slope of this straight line is

$$\frac{K_m}{\alpha V_{max}} \quad (1 + \frac{I_2}{K_2})$$

from which K_2, the dissociation constant of the enzyme--inhibitor complex, can be calculated. While no inhibitory effect of glucose was observed, the inhibition by cellobiose was evident. The value of K_2 for the inhibition by cellobiose was estimated as 4.63 mg/ml.

Figure 7.6 shows the same kind of data which were obtained with the cellulase from a different batch. Here, the inhibitory effect of cellobiose was smaller than that shown in Fig. 7.5. The value of K_2 was calculated as 10.3 mg/ml. The β-glucosidase and cellulase activities were measured for these two cellulase preparations and the data are in Table 7.4. The β-glucosidase activity of the cellulase used in the experiment of Fig. 7.6 was apparently higher than in the other although the difference was small. However, this small amount of β-glucosidase seemed to reduce the inhibitory effect of cellobiose considerably so that the K_2 value of the cellulase varies for different preparations.

Although glucose does not seem to inhibit the hydrolysis of cellulose as measured by the initial reaction rate (Fig. 7.5), it must have some effect on the over-all conversion as well as the productivity of glucose because, as shown in Chapter 3 (Figs. 3.22 and 3.23), glucose

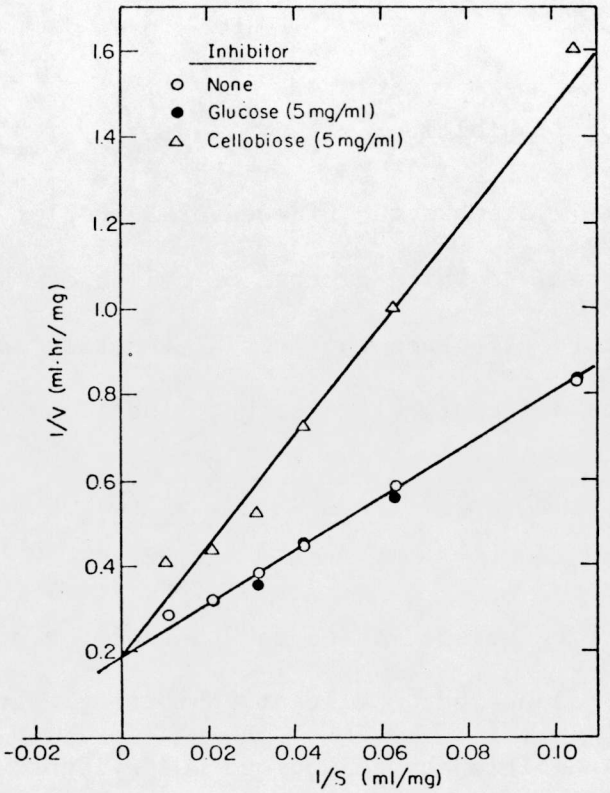

Figure 7.5. Lineweaver-Burk plots of product (glucose and cellobiose) inhibition of Solka Floc hydrolysis. Culture filtrate of *T. viride* from Exp. 3.

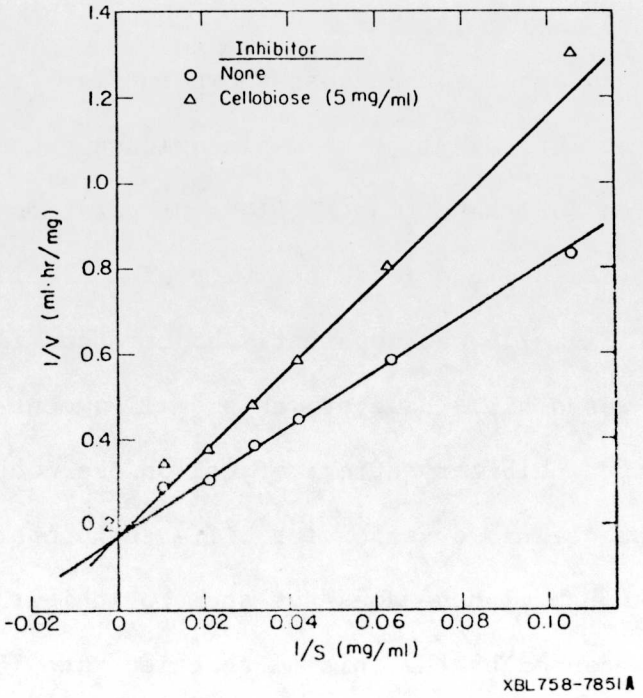

Figure 7.6. Lineweaver-Burk plot of product (cellobiose) inhibition of cellulose hydrolysis. Culture filtrate of *T. viride* from Exp. 4, 5, 8, 9 and 10.

Table 7.4. Comparison of enzyme activities and kinetic parameters for two different cellulase preparations.

Batch No.	PY - 2	Y - 1
Filter paper activity	3.95 mg/ml	4.2 mg/ml
β-Glucosidase activity	0.28 mg/ml	0.34 mg/ml
Michaelis-Menten constant, K_m	13.2 mg/ml	13.2 mg/ml
Maximum reaction rate, V_{max}	11.5 mg/ml·hr	10.7 mg/ml·hr
Dissociation constant, K_2	4.63 mg/ml	10.3 mg/ml
Lineweaver-Burk plot	Fig. 7.5	Fig. 7.6

inhibits the hydrolysis reaction of cellobiose, which is the inhibitor to the hydrolysis of cellulose. Thus, hydrolysis experiments were carried out in the presence of additional glucose (10 and 20 mg/ml), and the data were compared with the data obtained without additional glucose. The enzyme solution was prepared from a culture filtrate of Botryodiplodia theobromae and concentrated two-fold by an Amicon Diaflo ultrafiltration membrane (PM 30). The activities were: β-glucosidase = 1.34 mg/ml and filter paper activity = 1.79 mg/ml. Ball-milled Solka Floc (10% in the enzyme solution) was used as the substrate, and 10 and 20 mg/ml of glucose were added to the reaction mixture. The temperature was maintained at 50° C. Samples were measured at appropriated intervals for total reducing sugars by the DNS reagent and glucose concentration by the Glucostat reagent. The results are shown in Fig. 7.7.

The total rate of the reaction was impeded by the presence of additional glucose only in the period between 6 to 18 hours of reaction time, and before or after this period the differences in the reaction rates for each system were small. Qualitatively this seems reasonable since in the initial stage of the hydrolysis reaction the concentration of cellobiose is so low that the inhibitory effect on the total conversion is negligible no matter how glucose inhibits the reaction of breaking down cellobiose. When the reaction has proceeded to some extent, the concentration of glucose in the reaction system becomes high for both cases, and the inhibitory effect of glucose becomes more or less the same.

Contrarily, the rate of production of glucose was extensively affected by the additional glucose in the early stage (less than 10 hours)

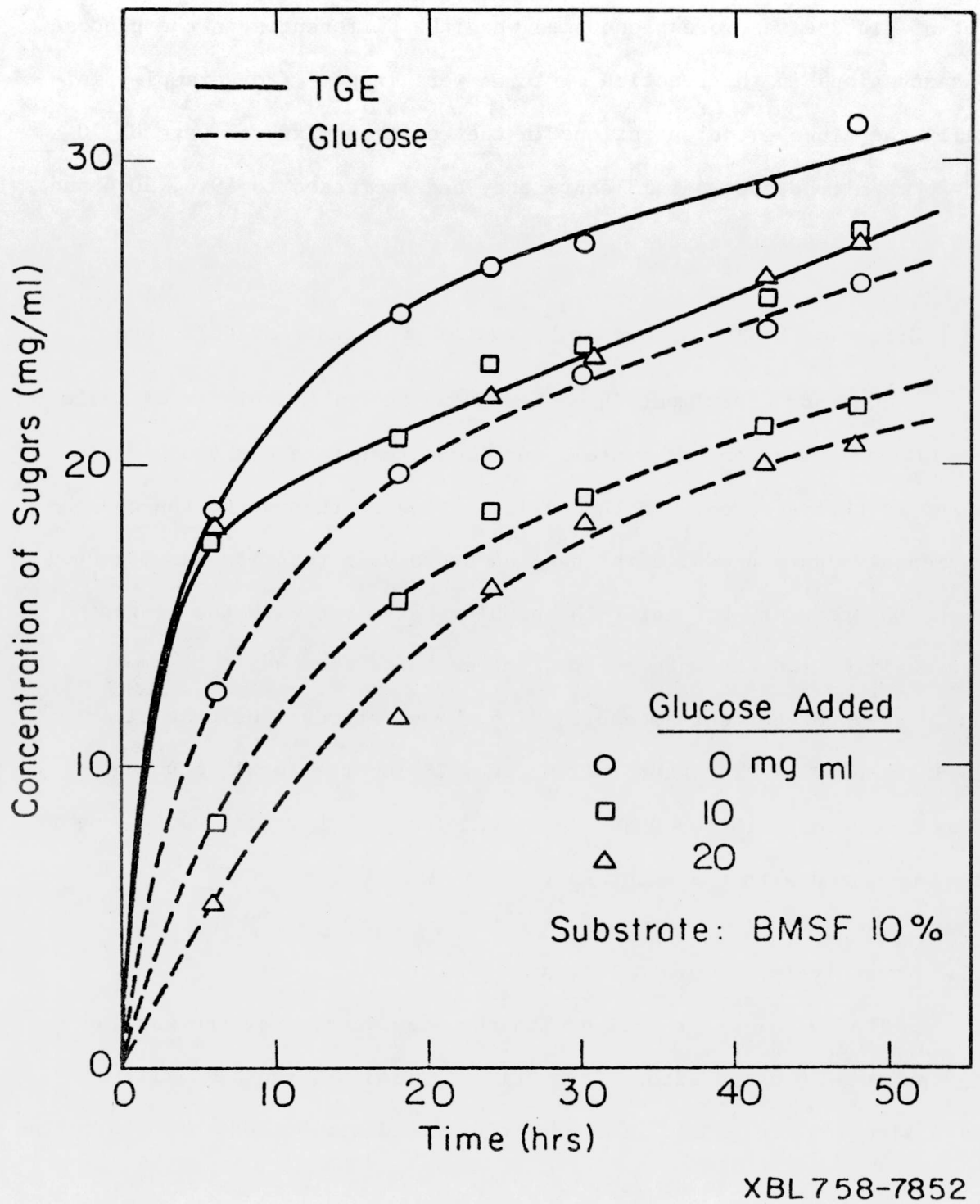

Figure 7.7. Effect of added glucose of hydrolysis of Solka Floc by a culture filtrate of *B. theobromae*.

of the hydrolysis. This is also reasonable because the inhibitory effect of glucose was more pronounced when the differences in the glucose concentrations in the reaction mixtures were large. (For example, initially the glucose concentrations in the reaction mixtures were 0, 10 and 20 mg/ml; however, at 18 hours they had increased to 19.6, 20.4 and 23.0 mg/ml, respectively.)

7.2 Effect of β-Glucosidase on Enzymatic Hydrolysis of Cellulose

Numerous experiments have been done on the hydrolysis of cellulose with the mixed enzyme system, and those results are shown and discussed in this section. In the earlier stage of this work, the concentration of sugars produced through the hydrolysis reaction was measured by the DNS reagent, but later the problems involved with the reagent were realized and the anthrone reagent was used instead. Thus, the sugar contents measured with these methods should be clearly distinguished. As in Chapter 2, we use TRS for the total reducing sugars measured with the DNS reagent and TGE for the total glucose equivalent measured with the anthrone reagent.

7.2.1 Hydrolysis of Pure Cellulose

The hydrolysis reaction with the mixed enzyme system was investigated at 50° C using either Solka Floc (called SF) and ball-milled Solka Floc (called BMSF). The particle size distributions of both celluloses are listed in Table 7.5.

Table 7.5. Size distributions of Solka Floc and ball-milled Solka Floc

Solka Floc (5)	
-- 100 mesh	19.4%
100 -- 280 mesh	80.5
280 -- mesh	11.6
Ball-milled Solka Floc	
-- 150 mesh	0.3%
150 -- 200 mesh	13.2
200 -- 325 mesh	11.3
325 --	75.2

Experiment No. 1

Figure 7.8 illustrates the progress of hydrolysis of BMSF with the mixed enzyme system of the culture filtrate of Trichoderma viride (called T. v. enzyme) and almond emulsin β-glucosidase, which was dissolved into the T. v. enzyme solution in concentrations of 0.333, 0.667 and 1.00 mg/ml. The filter paper activity of the original cellulase solution was 2.4 mg/ml, and the β-glucosidase activity was less than 0.1 mg/ml. The concentrations of TRS and glucose both increased as the amount of the β-glucosidase was increased. The conversion of the cellulose to soluble sugars is calculated by the following equation by assuming that only glucose and cellobiose are present in the hydrolyzate.

$$\text{Conversion} = \frac{(\text{Glucose}) \times \frac{162}{180} + \{(\text{TRS}) - (\text{Glucose})\} \times 1.21 \times \frac{324}{342}}{(\text{Cellulose used})}$$

Figure 7.9 shows the conversion thus calculated as a function of the amount of β-glucosidase added. The increase in conversion by the β-glucosidase seems almost saturated at the highest β-glucosidase concentration used in the experiments, even though the glucose concentration in the TRS was still less than 70%. This means that the saturation of the conversion is not attributable to product inhibition but to the reactivity of the substrate determined by its structural characteristics. As shown in the previous section, more than 50% of the BMSF is supposed to be a nonaccessible cellulose.

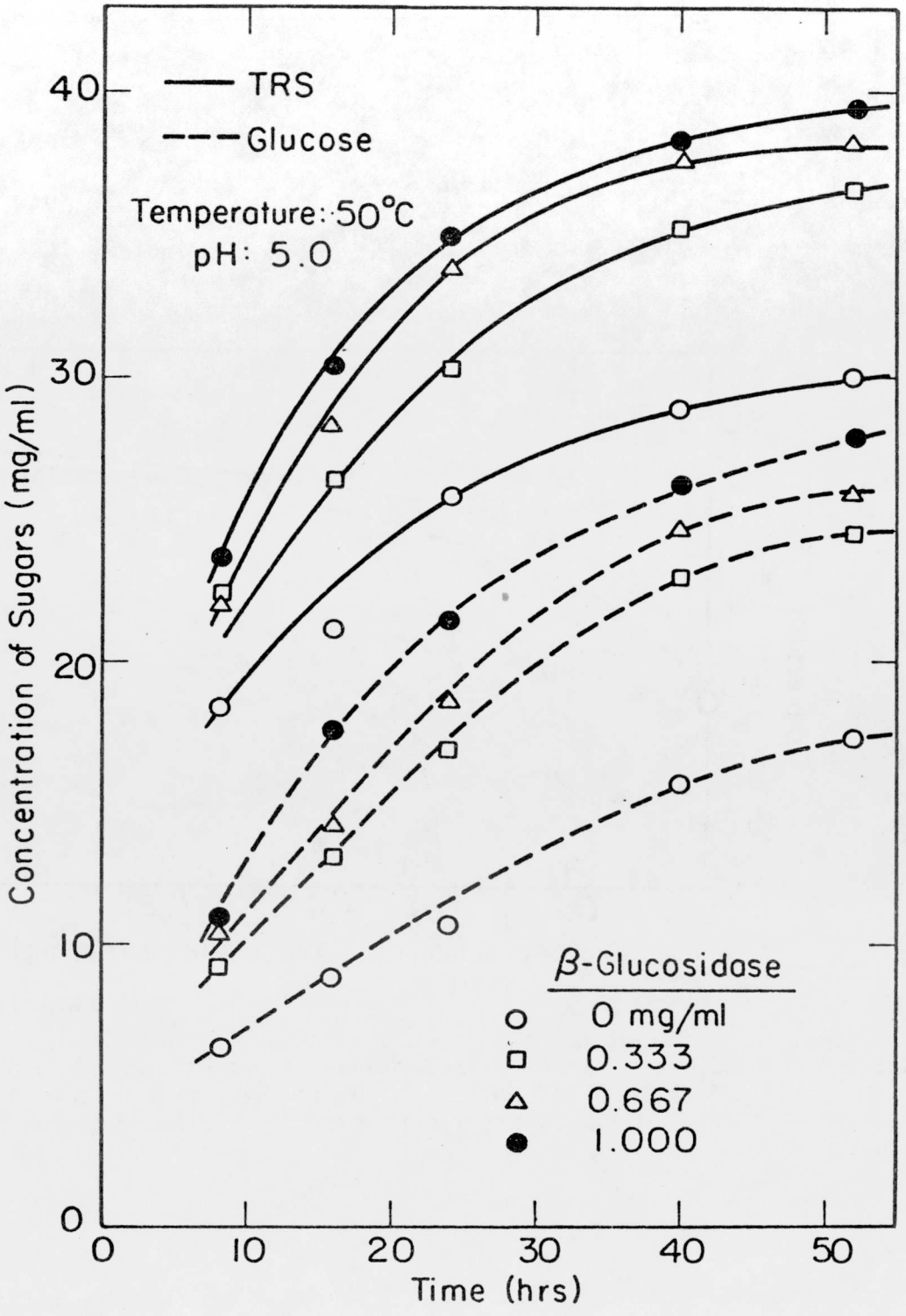

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Figure 7.8. Hydrolysis of ball-milled Solka Floc by various amounts of β-glucosidase (almond emulsin) added to a culture filtrate of *T. viride*. Experiment #1.

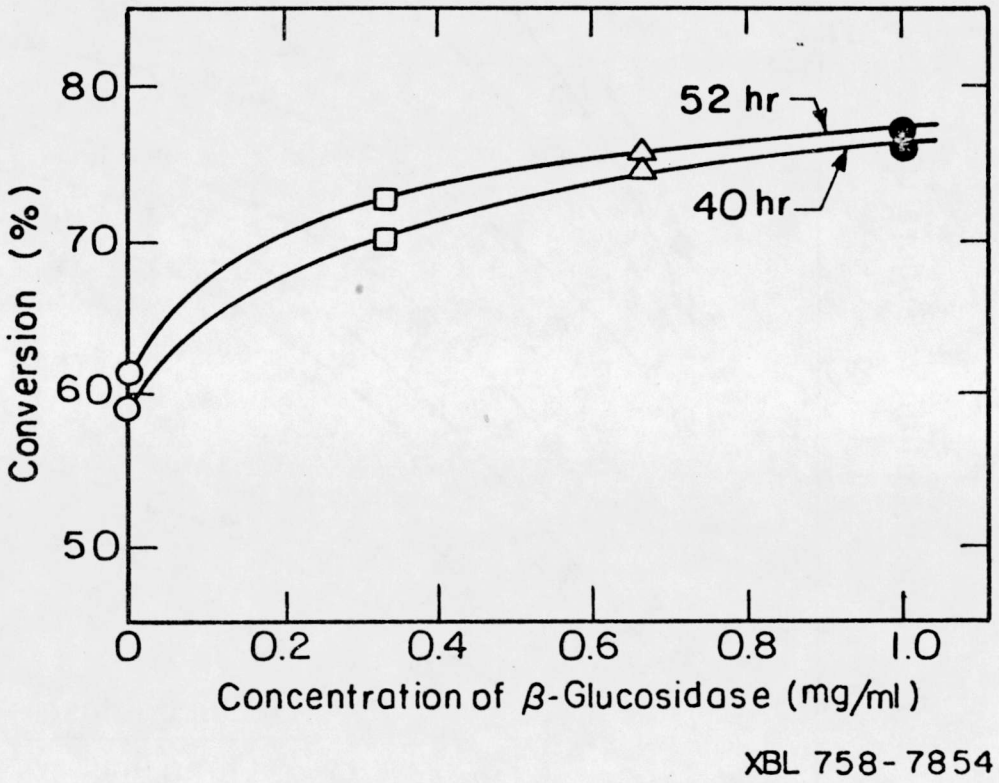

Figure 7.9. Effect of added β-glucosidase (almond emulsin) on percent of ball-milled Solka Floc hydrolyzed by a culture filtrate of *T. viride*. Experiment #1.

Experiment No. 2

Figure 7.10 shows the results of hydrolysis of BMSF (10% suspension, which means that 10 g of the substrate was suspended into 100 ml of the enzyme solution) with the mixed enzyme system of T.v. ensyme and the culture filtrate of Botryodiplodia theobromae (called B.t. enzyme). The enzyme activities of these enzyme solutions are shown in Table 7.6. Since the cellulase activity of B.t. enzyme was much less than that of T.v. enzyme, the mixed enzyme exerted less cellulase activity toward the substrate. On the other hand, the β-glucosidase activity became greater as the amount of B.t. enzyme increased. Thus, it was expected that an optimum ratio of these two enzyme solution to give the highest conversion of cellulose to soluble sugars would exist. The glucose concentration in the sugars produced will be increased by the use of the mixed enzyme system, which is capable of breaking down cellobiose to glucose. However, the total glucose productivity is also dependent on the over-all conversion, and hence there would be an optimum mixing ratio to give the highest conversion of cellulose to glucose, although these two optimum points might not coincide. As will be shown in the following section, the utilization of cellobiose by yeast in the production of ethanol is not feasible so that these two optimum points should be carefully distinguished.

Now, returning to Fig. 7.10, we see that the over-all conversion was not improved by the use of the mixed enzyme solution, and the highest conversion was obtained with the original cellulase solution. However, the highest glucose productivity was attained when the mixture of 1/3 of T.v. enzyme and 2/3 of B.t. enzyme solutions was used.

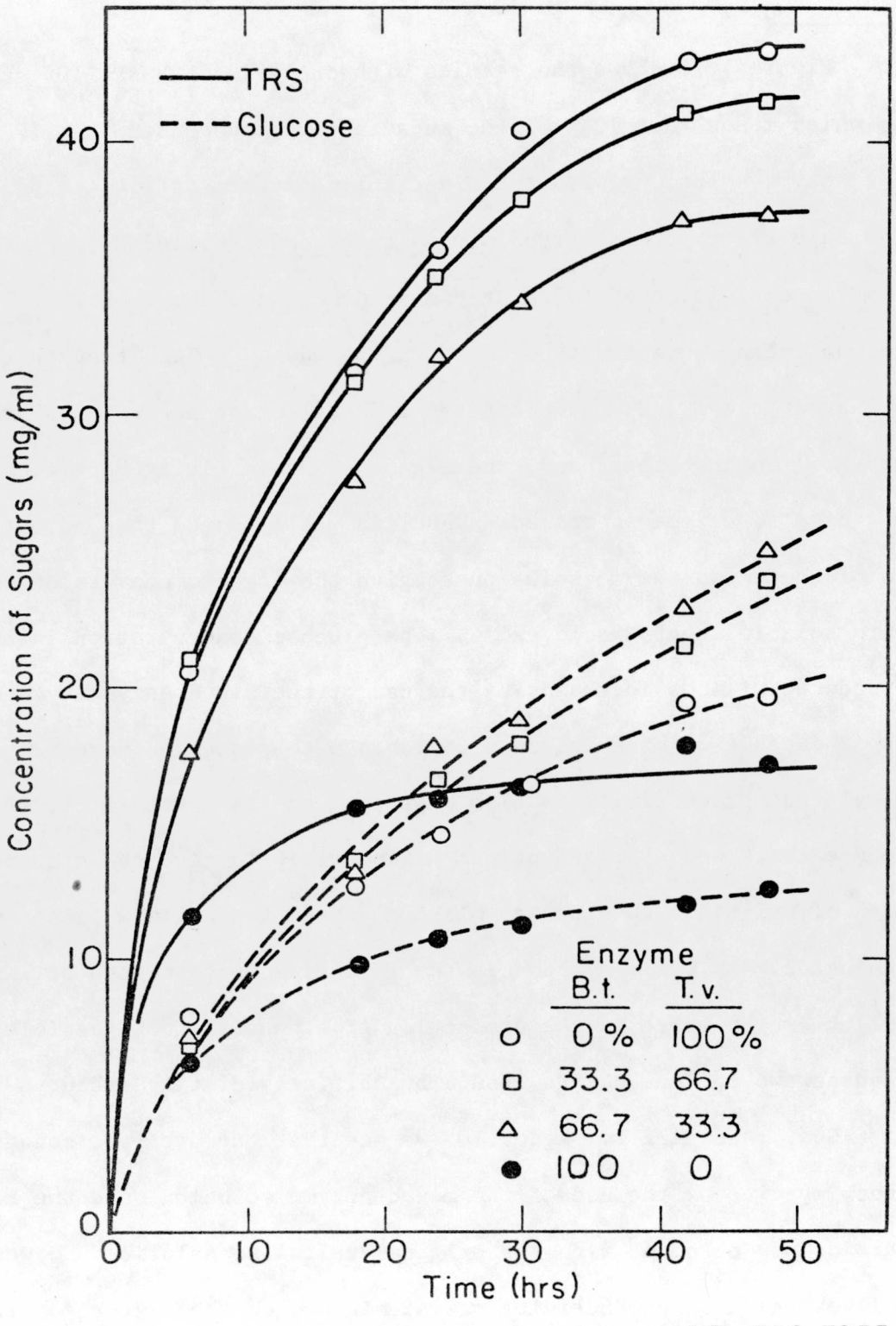

Figure 7.10. Hydrolysis of ball-milled Solka Floc by various proportions of β-glucosidase (*B. theobromae*) and cellulase (*T. viride*). Experiment #2.

Table 7.6. Activities of mixed enzyme systems used in Experiment No. 2.

T.v. enzyme--dilute B.t. enzyme system (Fig. 7.10)

Symbols in Fig. 7.10	Mixing ratio		Activities	
	B.t. enzyme	T.v. enzyme	FPA (DNS)	β-Glucosidase
o	0%	100%	1.96 mg/ml	0.14 mg/ml
X	33.3	66.7	1.65	0.30
Δ	66.7	33.3	1.31	0.46
●	100	0	0.96	0.61

T.v. enzyme--concentrated B.t. enzyme system (Fig. 7.11)

Symbols in Fig. 7.11	Mixing ratio		Activities	
	B.t. enzyme	T.v. enzyme	FPA (DNS)	β-Glucosidase
o	0%	100%	1.96 mg/ml	0.14 mg/ml
X	33.3	66.7	1.90	0.50
Δ	66.7	33.3	1.83	0.86
●	100	0	1.64	1.22

Experiment No. 2a

Since the lower total conversion obtained with the mixed enzyme system in Experiment No. 2 was apparently due to its lowered cellulase activity, the two-fold concentrated B.t. enzyme, whose cellulase activity is closer to that of the T.v. enzyme solution, was added and its effect on the hydrolysis of the same substrate was examined. (Concentration of the enzyme solution was done using an Amicon Diaflo ultrafiltration membrane (PM-30).) The results are shown in Fig. 7.11. This time, the highest overall conversion was obtained with the mixed enzyme which contained 2/3 of the T.v. enzyme and 1/3 of the B.t. enzyme although the highest conversion to glucose was obtained with the β-blucosidase richer mixed enzyme system (1/3 T.v. enzyme and 2/3 B.t. enzyme).

The two curves shown in Figs. 7.10 and 7.11, which were obtained with only B.t. enzyme, are very different from the others. Since the enzyme produced by Botryodiplodia theobromae contained less C_1 component, the leveling-off may be attributable to a lack of C_1 activity as suggested for the cellulase from Pestalotiopsis westerdijkii (1). However, while the final hydrolysis level obtained with the concentrated B.t. enzyme was about 50% conversion, which was approximately the same as the accessible portion of the BMSF measured with the method developed in the present work, the level obtained with the dilute B.t. enzyme was only about 30% conversion. These data indicate that the leveling-off phenomenon could not be explained by only the structure of the substrate and the characteristics of the actions of C_1 and C_x components.

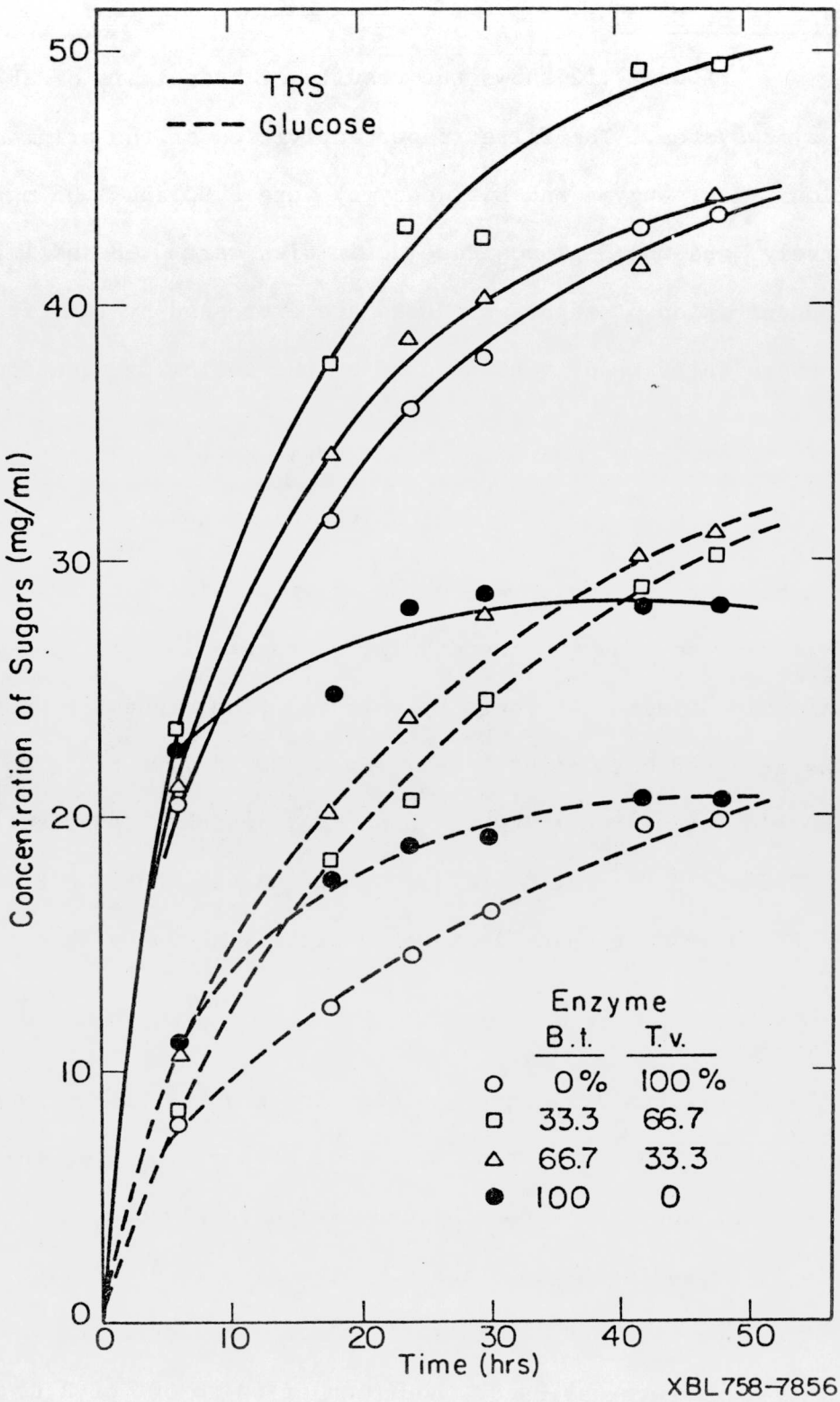

Figure 7.11. Hydrolysis of ball-milled Solka Floc by various proportions of concentrated β-glucosidase (*B. theobromae*) and cellulase (*T. viride*). Experiment #2a.

Experiment No. 3

Figure 7.12 shows the results of hydrolysis of SF with the mixed enzyme system. The filter paper activities of the original enzyme solutions (T.v. enzyme and B.t. enzyme) were 3.93 and 1.95 mg/ml, respectively, and the β-glucosidase activities were 0.28 and 1.30 mg/ml. The concentration of sugars produced are expressed by TGE, from which the conversion is readily calculated by the following equation.

$$\text{Conversion} = \frac{(\text{TGE}) \times \frac{162}{180}}{(\text{Cellulose used})}$$

The highest conversion was obtained with a 15% B.t. enzyme/85% T.v. enzyme mixture, but the increase in conversion by adding β-glucosidase was only 8% after 48 hours hydrolysis. The highest glucose production was attained with either the 15/85 or 30/70 mixture. The increase of the glucose productivity was about 64%, which is substantial. Figure 7.13 depicts the ratio of glucose to TGE vs. reaction time. The action of β-glucosidase on cellobiose is clearly shown by these results.

Experiment No. 4

Figures 7.14 and 7.15 show the results of the same kind of experiments with a different cellulase preparation. In this case, neither the total conversion nor the conversion to glucose was increased extensively by adding β-glucosidase. At most only a 5.4% increase in total conversion with a 9% increase in glucose productivity was observed when a 10/90 mixture was used. Addition of 40 to 50% of B.t. enzyme was more favorable in increasing the glucose productivity, which was 37% higher

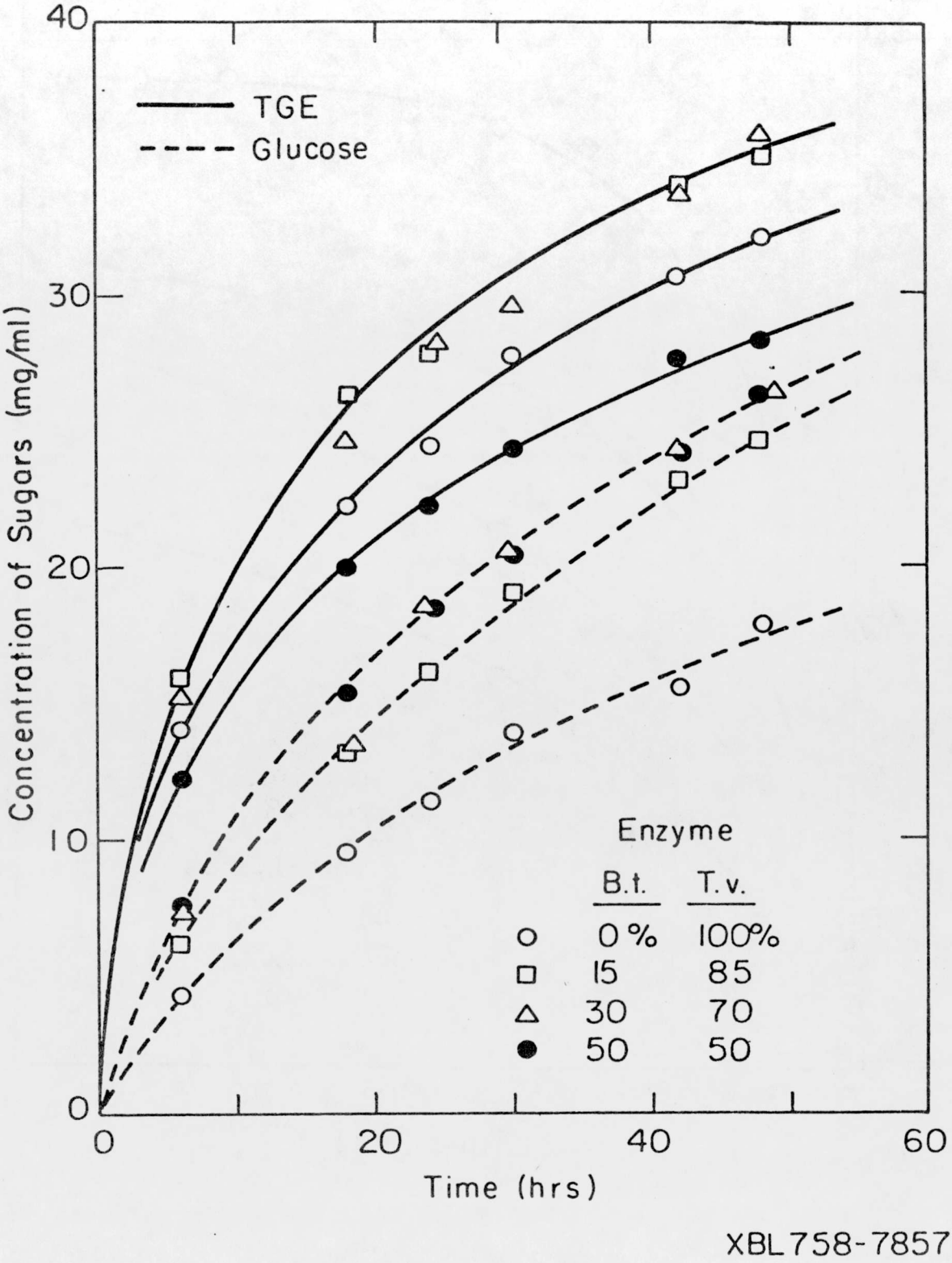

Figure 7.12. Hydrolysis of Solka Floc by various proportions of β-glucosidase (*B. theobromae*) and cellulase (*T. viride*). Experiment #5.

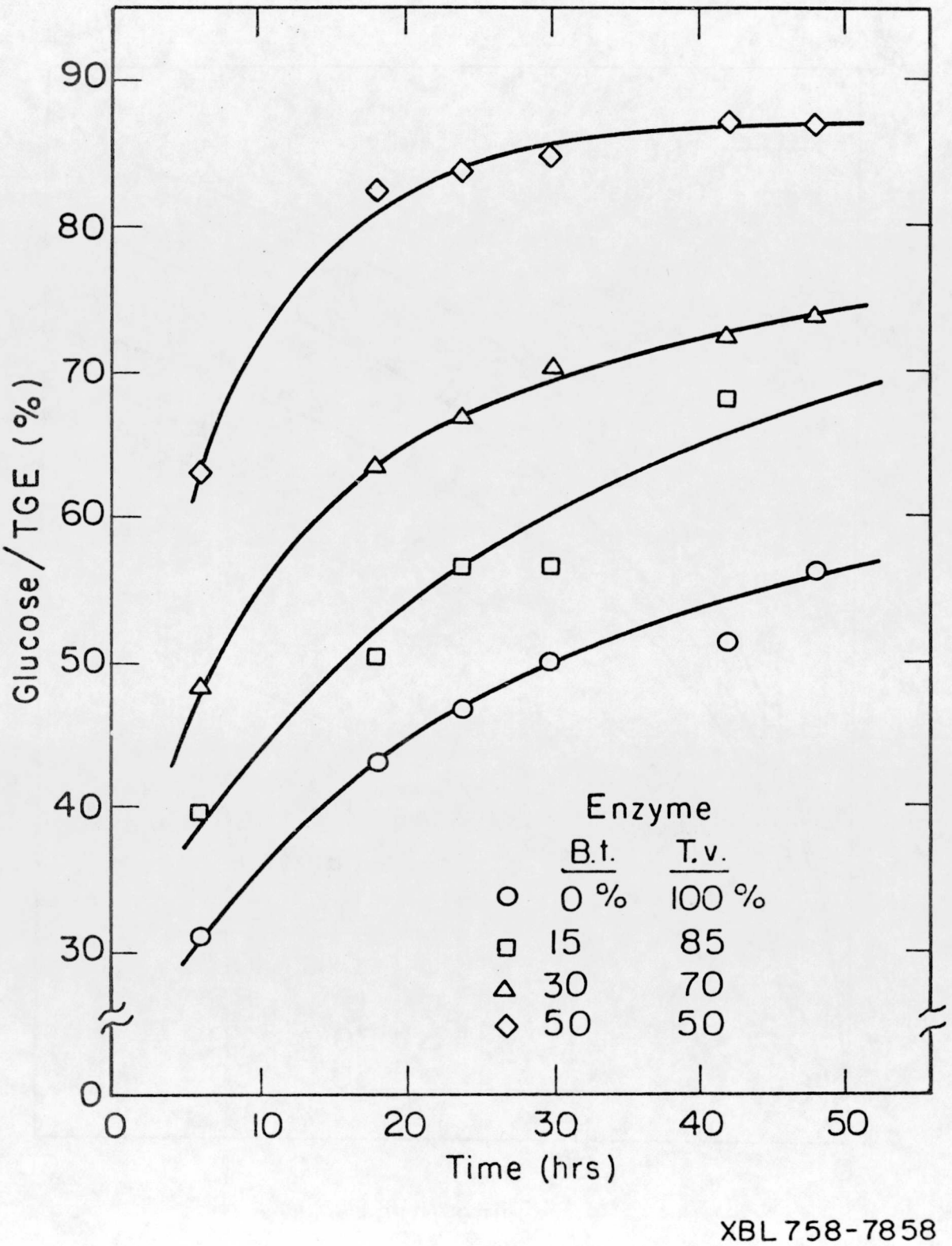

Figure 7.13. Glucose as a fraction of total sugars during hydrolysis of Solka Floc by various proportions of β-glucosidase (*B. theobromae*) and cellulase (*T. viride*). Experiment #2.

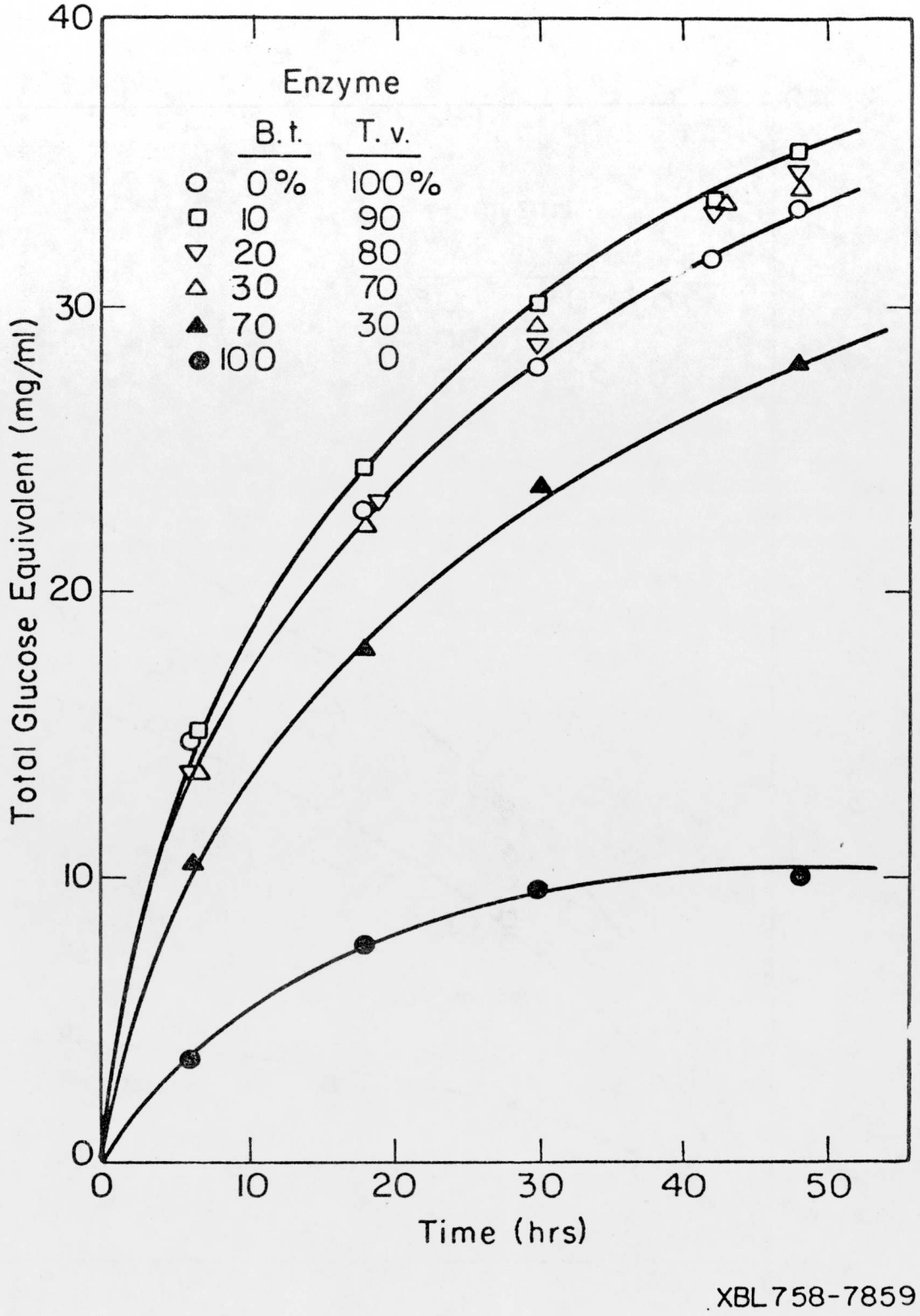

Figure 7.14. Hydrolysis of Solka Floc by various proportions of β-glucosidase (*B. theobromae*) and cellulase (*T. viride*). Experiment #4.

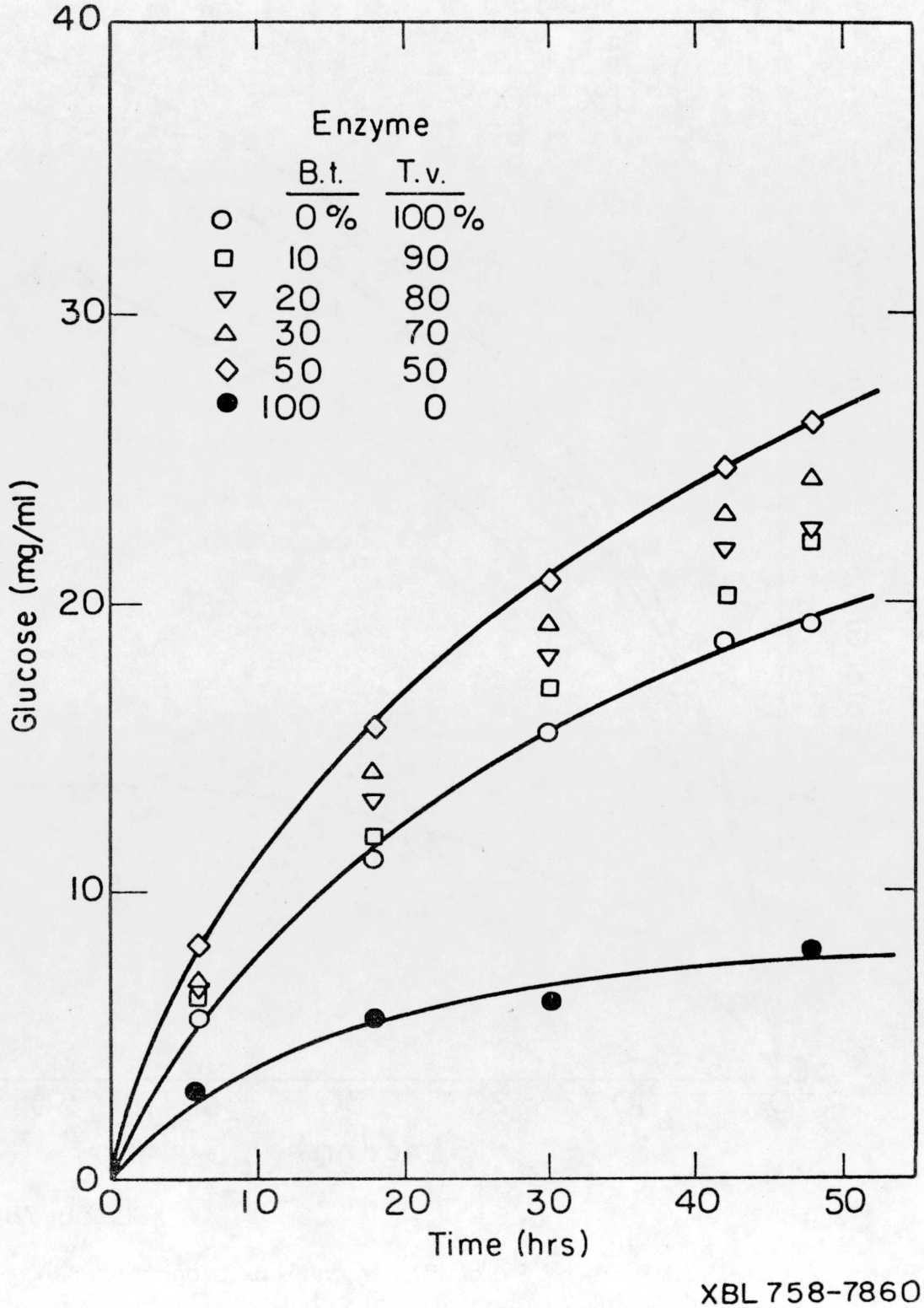

Figure 7.15. Hydrolysis of Solka Floc by various proportions of β-glucosidase (*B. theobromae*) and cellulase (*T. viride*). Experiment #4.

than that obtained with the regular cellulase.

The difference between the data obtained in Experiment Nos. 3 and 4 is not small. Although the cellulase and β-glucosidase preparations used for these two series of experiments were different, the activities of cellulase and β-glucosidase were almost the same. However, considerable difference was found in the values of K_2 of the cellulase solutions. The cellulase used in the Experiment No. 3 had a K_2 value of 4.63 mg/ml, as obtained from the Lineweaver-Burk plot shown in Fig. 7.5, while the K_2 value of the cellulase used in the Experiment No. 4 was 10.3 mg/ml (Fig. 7.6).

Experiment No. 5

When the B.t. enzyme was concentrated more than two-fold, the filter paper activity became high enough so that mixing the enzymes did not result in diluting the cellulase activity of the T.v. enzyme. Figures 7.16 and 7.17 show the results with the highly concentrated B.t. enzyme. The cellulase preparation was the same as used in the Experiment No. 4. In this case a 15/85 ratio mixture provided a 12% increase in total conversion and a 59% increase in glucose productivity. The use of more β-glucosidase (30/70 and 50/50 mixtures) did not improve the total conversion but higher glucose productivity (77% increase by 50/50 mixtures) was observed. Table 7.7 summarizes the results of Experiment Nos. 3-5. As seen in the Table, these three mixed enzyme systems, made up from the highly concentrated B.t. enzyme had almost the same cellulase activity based on both C_x and filter paper activity, and hence the addition of β-glucosidase worked only in the positive direction.

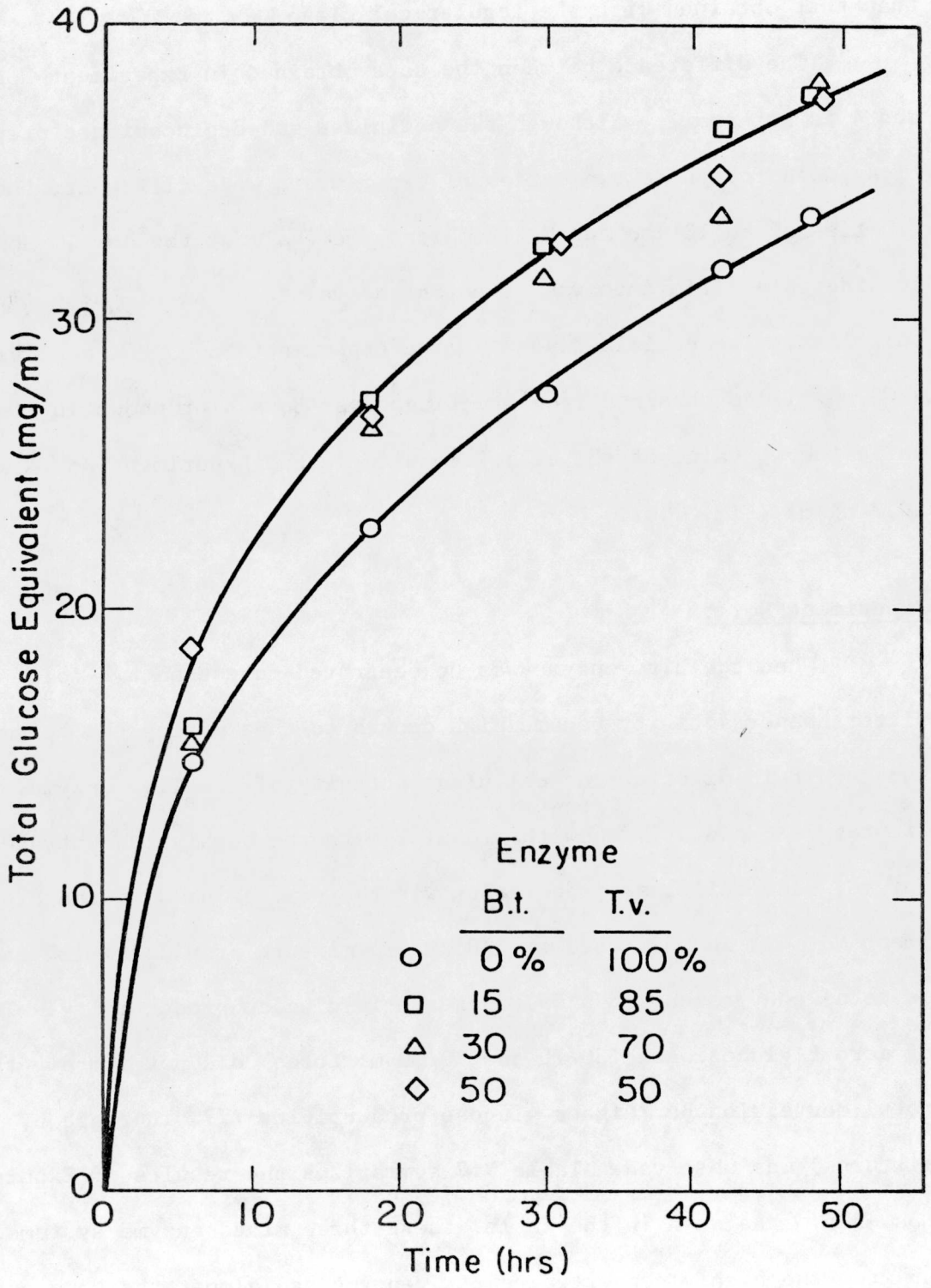

Figure 7.16. Hydrolysis of Solka Floc by various proportions of concentrated β-glucosidase (*B. theobromae*) and cellulase (*T. viride*). Experiment #5.

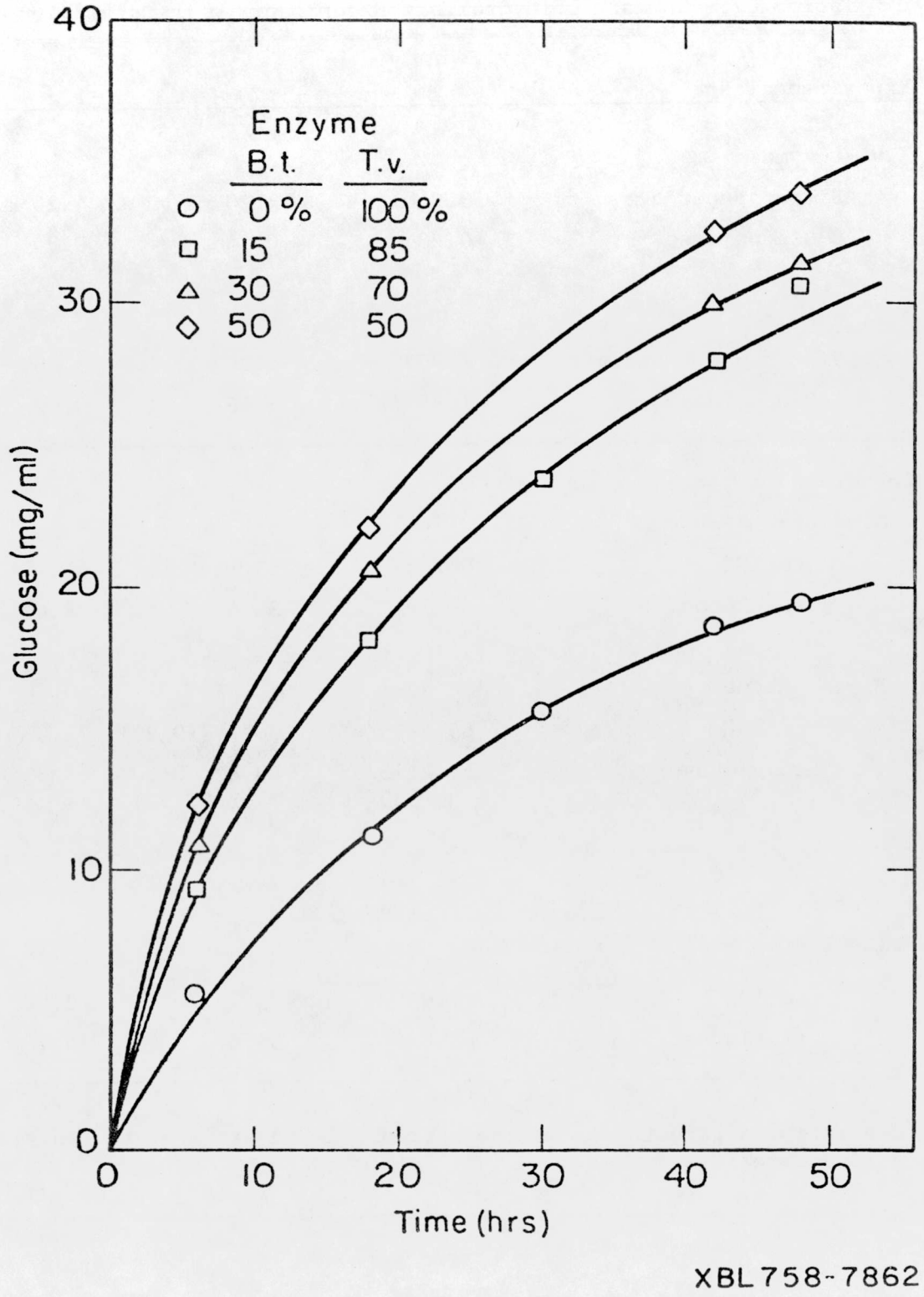

Figure 7.17. Hydrolysis of Solka Floc by various proportions of concentrated β-glucosidase (*B. theobromae*) and cellulase (*T. viride*). Experiment #5.

Table 7.7. Summary of Hydrolysis Experiments with Solka Floc.

Experimental No.	3	4	5
T.v. enzyme			
Filter paper activity	3.95 mg/ml	4.20 mg/ml	4.20 mg/ml
β-Glucosidase	0.28	0.34	0.34
B.t. enzyme			
Filter paper activity	1.95	2.20	3.80
β-Glucosidase	1.35	1.16	3.02
Conversion at 48 hours:			
T.v. enzyme only			
to TGE	60.6%	63.1%	63.1%
to glucose	33.7	36.4	36.4
Mixed enzyme			
to TGE	65.4(15/85)*	66.7 (10/90)	71.0 (15/85)
to glucose	55.2(15/85 or 30/70)	49.8 (40/60 or 50/50)	64.3 (50/50)
Increases in conversion with the optimum mixing ratio (% of original)			
to TGE	7.9%	5.7	12.5%
to glucose	63.8	36.8	76.7

*The ratio in parentheses is the optimum mixing ratio of two enzymes, B.t. enzyme/T.v. enzyme.

7.2.2 Hydrolysis of Newsprint

Wall Street Journal newsprint, pulverized into small particles by passing through a Wiley mill, was utilized for the hydrolysis experiments. The size distribution of the unsieved Wiley-milled newsprint (called WMNP) was: +48 mesh: 12.0%, 48-100 mesh: 19.1%, 100-200 mesh: 35.8%, 200-325 mesh: 13.2% and - 325 mesh: 18.1% (6).

Experiment No. 6

A five percent suspension of WMNP (100-200 mesh fraction) was used in this experiment. Figure 7.18 shows the results. The concentration of TRS at 48 hours was 14.0 mg/ml with the T.v. enzyme only, but 16.5 mg/ml of sugars, about an 18% increase, was obtained by the mixed enzyme system (B.t. enzyme/T.v. enzyme = 1/2). However, the effect of β-glucosidase on the glucose productivity was tremendous. Since the cellulase preparation used in this series of experiments showed very small β-glucosidase activity (less than 0.1 mg/ml), the conversion to glucose with the cellulase was unusually low. By adding 33.3% of β-glucosidase preparation, the final level of glucose concentration was increased from 4.4 mg/ml to 12.1 mg/ml, about 175% increase.

Experiment No. 7

A relatively high concentration (7.14%) of unsieved WMNP was used in this experiment. In order to achieve adequate mixing with the magnetic stirrer used in this experiment, it was necessary to use a suspension of less than 7.5% newsprint. The results are shown in Fig. 7.19.

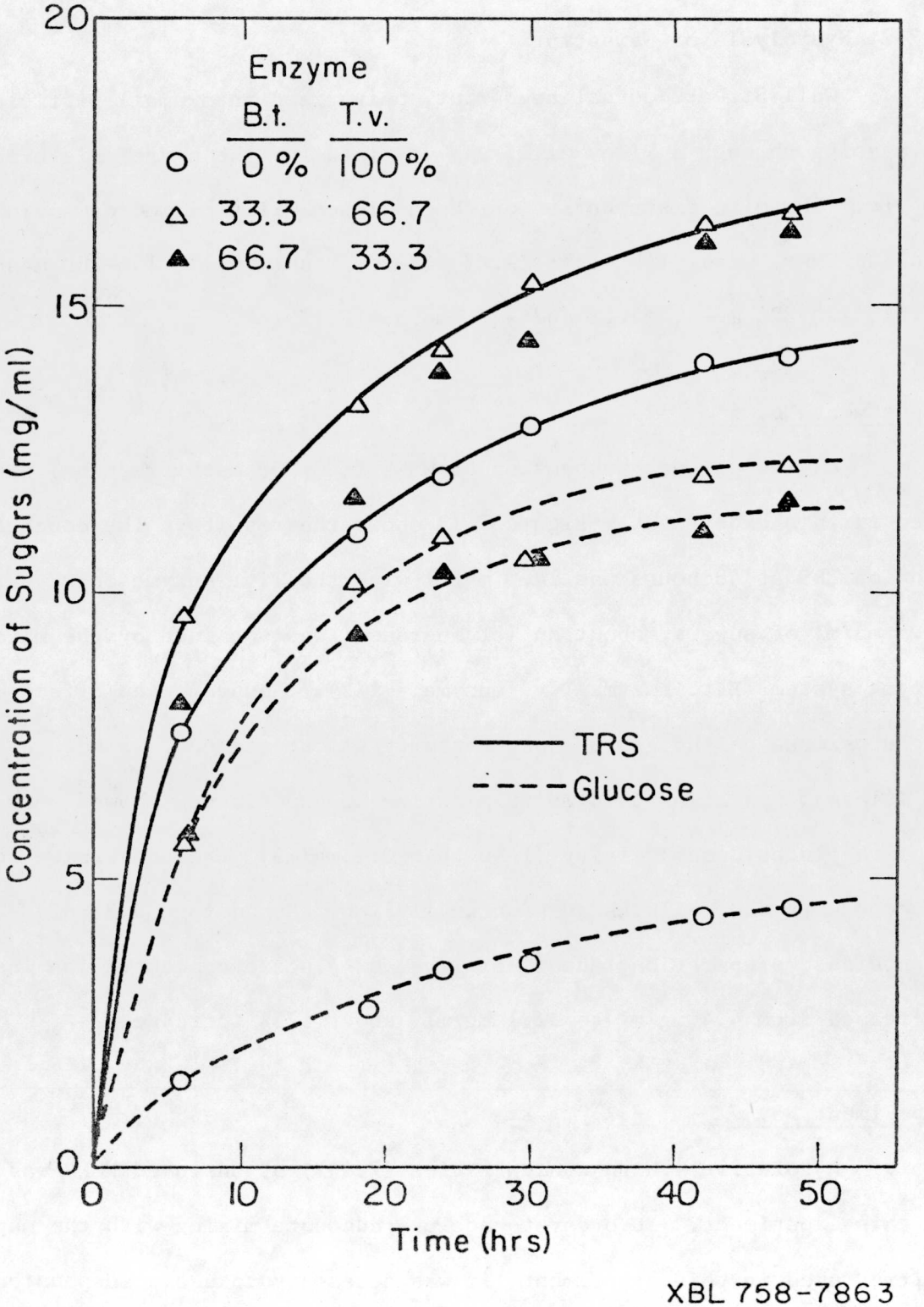

Figure 7.18. Hydrolysis of newsprint by various proportions of β-glucosidase (*B. theobromae*) and cellulase (*T. viride*). Experiment #6.

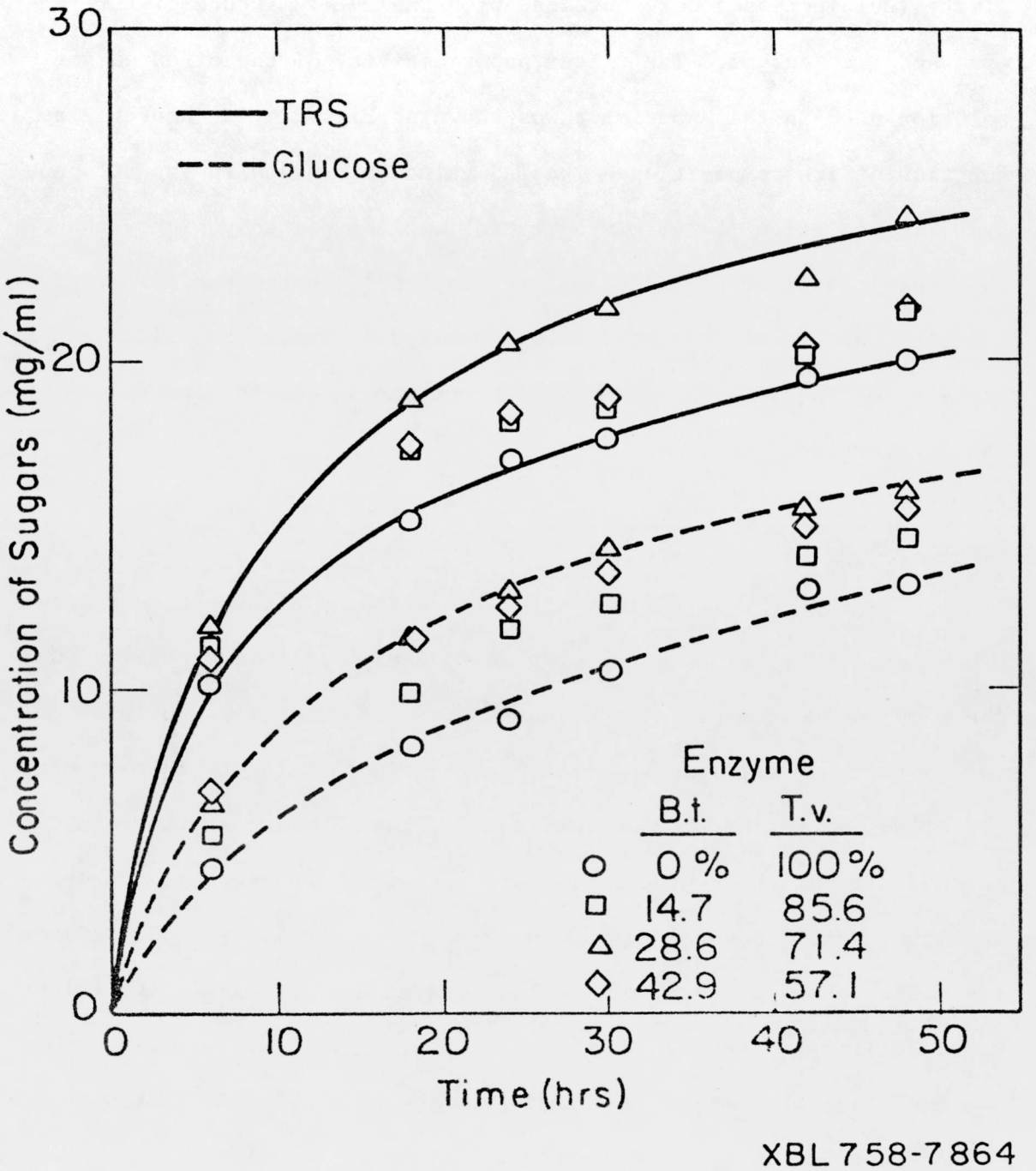

Figure 7.19. Hydrolysis of newsprint by various proportions of β-glucosidase (*B. theobromae*) and cellulase (*T. viride*). Experiment #7.

The maximum conversion (22% increase) and the highest glucose productivity (26% increase) were obtained with the 29/71 mixture of B.t. enzyme and t.v. enzyme. The filter paper activity of the mixed enzyme solution used in the experiment was shown in Fig. 2.4 in Chapter 2 as a function of its composition. The TRS value obtained with the B.t. enzyme rich solution (57/43 mixing ratio) was unexpectedly higher than that obtained with the cellulase preparation, although the filter paper activity for the mixed enzyme solution was 3.1, while the filter paper activity of the original cellulase enzyme was 4.5 (both measured with the anthrone reagent).

Experiment No. 8

Figure 7.20 illustrates the data obtained with a 5% suspension of unsieved WMNP. The enzyme preparations used in this experiment were the same as those used in Experiment No. 4. A large K_2 value was observed for the cellulase solution, indicating that the inhibitory effect of cellobiose to this enzyme was small. Thus, as seen in the results, the addition of the β-glucosidase did not yield considerable improvement in either total conversion (10%) or glucose productivity (20%). Since the filter paper activity of the B.t. enzyme was 2.2 mg/ml, the filter paper activity of the 50/50 mixed enzyme was about 3.2 mg/ml, but the mixture still produced more sugars than the original cellulase, whose filter paper activity was 4.2 mg/ml.

The glucose concentration in the TGE was not changed much by the amount of β-glucosidase used in this experiment. With the original cellulase system the value of glucose/TGE was 0.76, and the number was

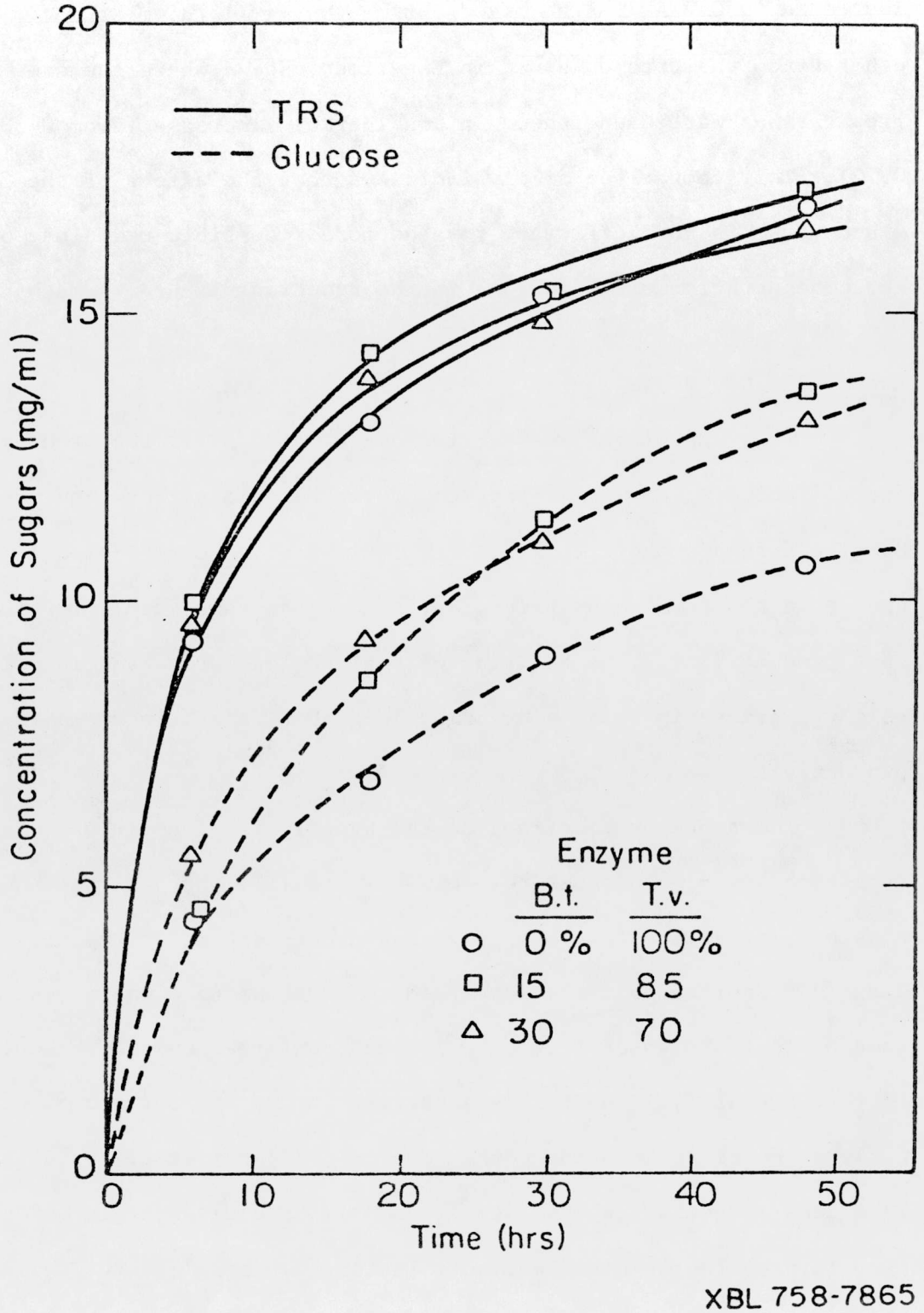

Figure 7.20. Hydrolysis of newsprint by various proportions of β-glucosidase (*B. theobromae*) and cellulase (*T. viride*). Experiment #8.

increased to 0.81 with the mixed enzyme system (15/85 mixture). On the other hand, the data obtained in Experiment No. 4, where the same enzyme preparations were used, showed a considerable increase (from 0.58 to 0.72). This comparison simply indicates that the effect of the β-glucosidase is more efficient for the more accessible cellulose because the concentration of cellobiose in the reaction mixture is much higher.

Experiment No. 9

This experiment was carried out at 45° C. All the other conditions, including enzyme properties, were the same as for Experiment No. 8. The data are shown in Fig.7.21. The use of 45° C yielded a 12% increase in the total conversion, but almost the same result for the glucose productivity. The addition of β-glucosidase resulted in only a slight increase in total conversion, but about a 27% increase in glucose productivity.

The temperature effect on the hydrolysis of cellulose has been investigated only in short-term experiments (5), and 50° C was somehow chosen by many investigators as a standard condition. Only recently, Yang (7) reported that the conversion of WMNP at 45° C was almost the same as that obtained at 50° C, which was confirmed by the present work. Long-term hydrolysis of BMSF was carried out at 40, 50, and 55° C in an early stage of the present work. The filter paper activity of the cellulase used was very low, 1.2 mg/ml, and almond emulsin β-glucosidase was used to prepare the mixed enzyme solution (0.5 mg/ml in the cellulase solution). The results shown in Figs. 7.22 and 7.23 indicate that the conversion obtained with cellulase only was not affected by the reaction

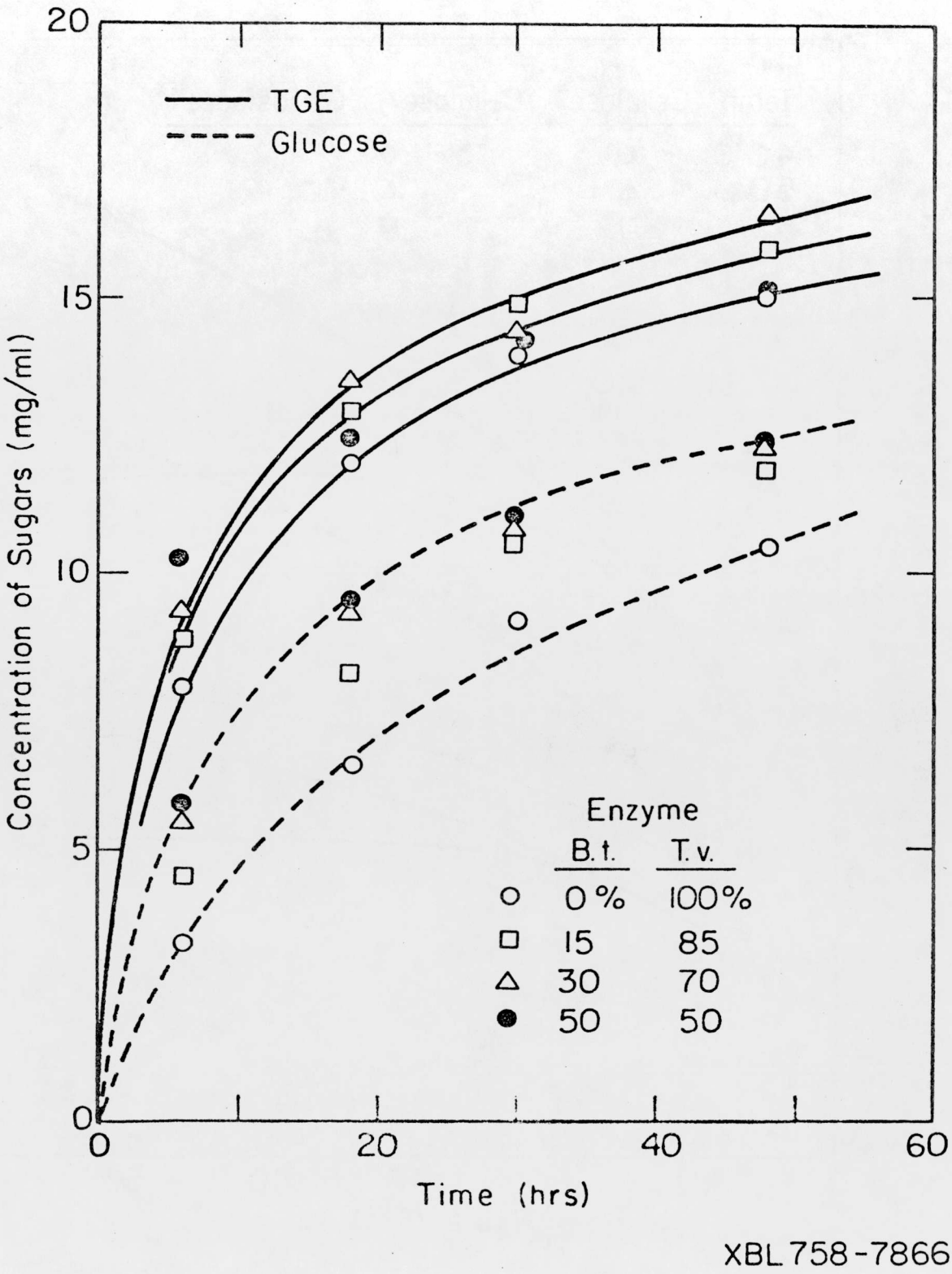

Figure 7.21. Hydrolysis of newsprint by various proportions of β-glucosidase (*B. theobromae*) and cellulase (*T. viride*) at 45°. Experiment #9..

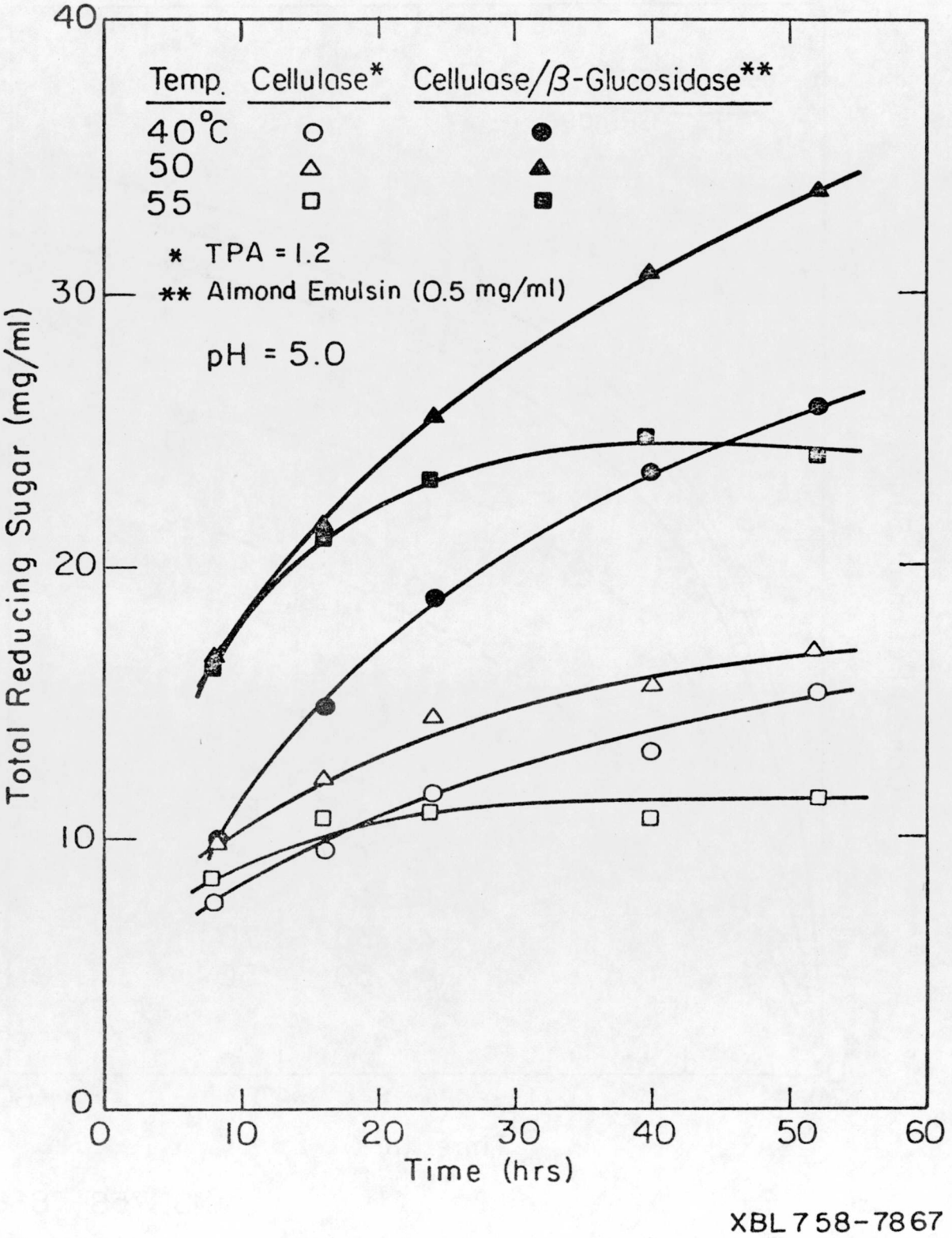

Figure 7.22. Effect of temperature on hydrolysis of Solka Floc by cellulase (*T. viride*) and cellulase/β-glucosidase (*B. theobromae*).

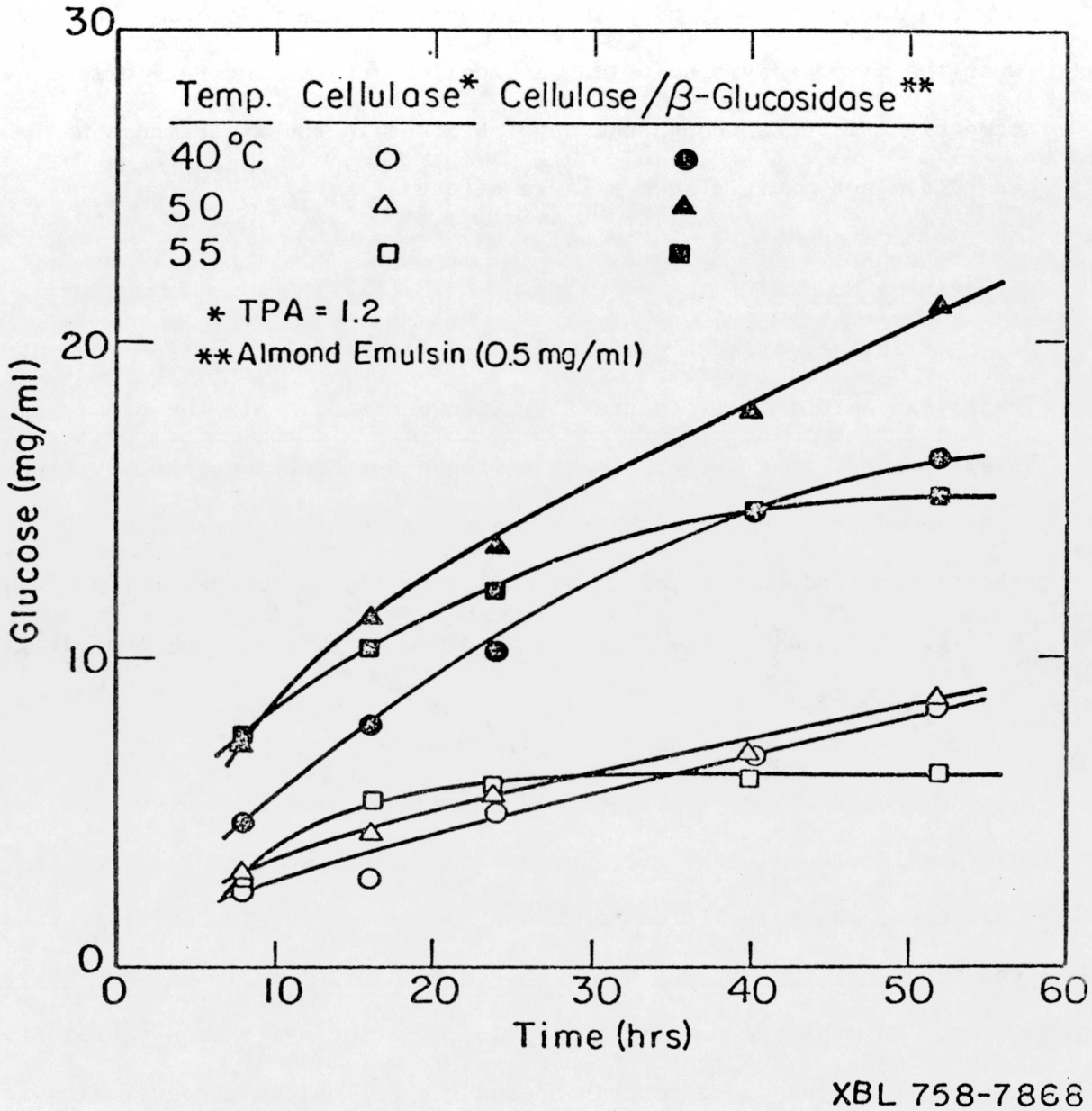

Figure 7.23. Effect of temperature on hydrolysis of Solka Floc by cellulase (*T. viride*) and cellulase/β-glucosidase (*B. theobromae*).

temperature in the range between 40 to 50° C. The glucose concentration was almost the same at 45° C and 50° C. A temperature of 55° C was apparently too high for the reaction because of heat denaturation of the enzymes. (Note the very high initial reaction rate at 55° C.) However, when the mixed enzyme solution was applied, the temperature effect became significant although the data obtained in the Experiments Nos. 8 and 9 did not exhibit such a large effect.

Experiment No. 10

This experiment was carried out with the B.t. enzyme concentrated by an Amicon Diaflo ultrafiltration membrane. All the other experimental conditions were the same as those for Experiment No. 8. Figure 7.24 shows the results. The total conversion was increased by 10% with the 15/85 mixed enzyme solution, while the glucose productivity was up to 37%. The use of more β-glucosidase did not improve the productivity.

7.2.3 Composition of the Hydrolysis Product

Practically, it can be considered that the products of enzymatic hydrolysis of cellulose are glucose and cellobiose. As reviewed in Chapter 4, the compositions of these sugars are considerably affected by the nature of the enzyme, i.e., the cellulase and β-glucosidase activities. To evaluate product inhibition, the necessity of a correlation between the glucose concentration and the cellobiose concentration in the reaction mixutre was raised, and an attempt was made to derive some empirical equations.

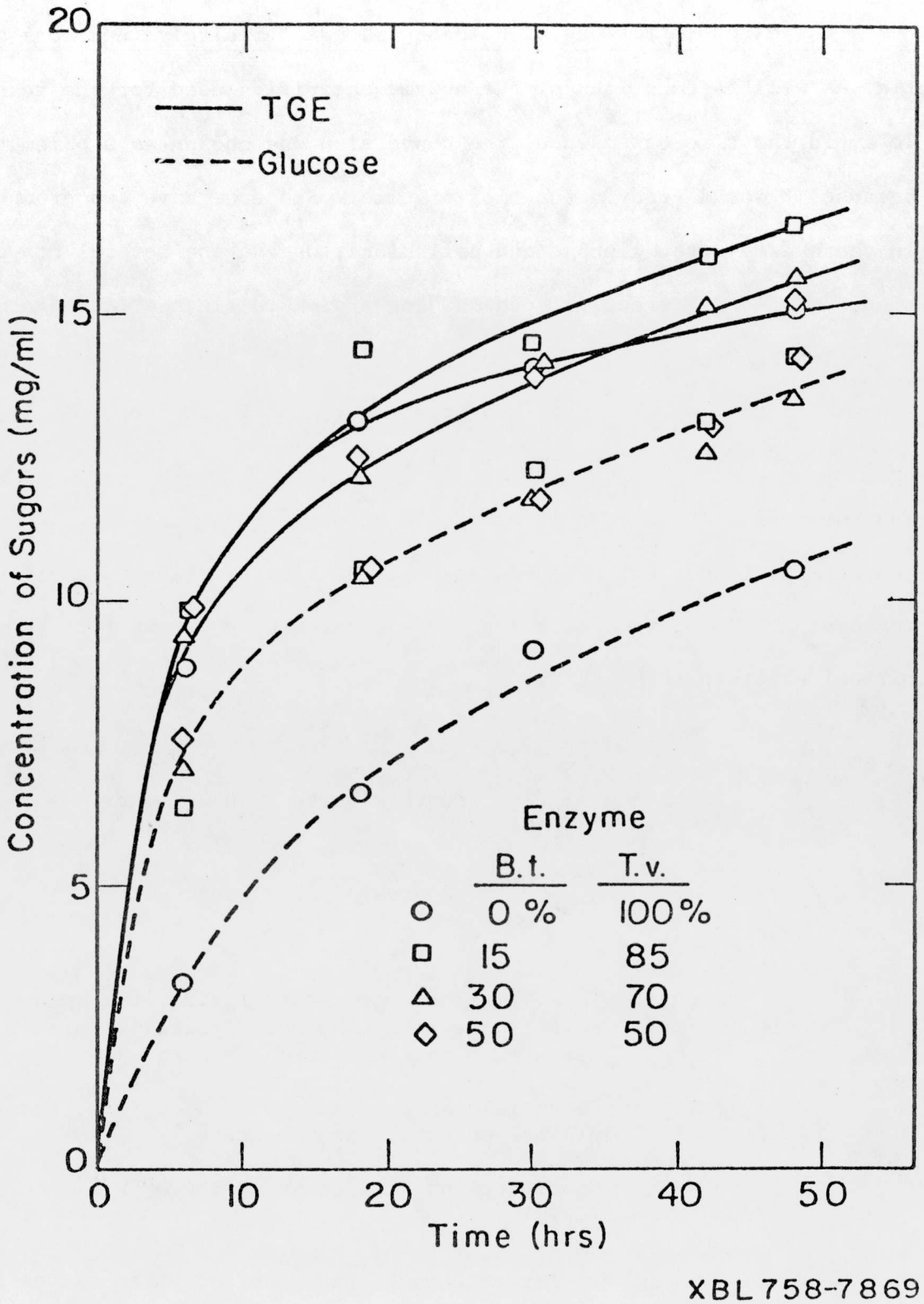

Figure 7.24. Hydrolysis of newsprint by various proportions of concentrated β-glucosidase (*B. theobromae*) and cellulase (*T. viride*). Experiment #10.

The concentrations of both sugars are definitely functions of time as well as functions of the enzyme activities used for the reaction. To avoid the time dependence, the conversion was chosen as a parameter, because it was already a function of time. Since we have two products in the hydrolyzate, glucose and cellobiose, we can use several conversions defined differently, such as "conversion to glucose", "conversion to cellobiose" or "conversion to soluble sugars". The last conversion represents the reaction rate most precisely, and furthermore, it is a function of both time and the activities of enzymes (mainly cellulase). The conversion to glucose is also a function of both reaction time and enzyme activities, but β-glucosidase may have more influence on this conversion. Thus, the following two variables were chosen for the empirical equation to be derived:

$$X = \frac{(TGE)}{(TGE)_0} = \text{conversion to soluble sugars}$$

$$Y = \frac{(G)}{(TGE)_0} = \text{conversion to glucose}$$

where (TGE) = Total glucose equivalent, i.e., total amount of soluble sugars in the hydrolyzate (mg/ml) as measured with the anthrone reagent.

$(TGE)_0$ = Potential maximum value of (TGE), i.e., total glucose units in the substrate.

(G) = Concentration of glucose in the hydrolyzate (mg/ml).

Since X is always larger than Y, the reaction paths expressed by these parameters may look like curves shown in Fig. 7.25, and these

Figure 7.25 X - Y diagram (see text)

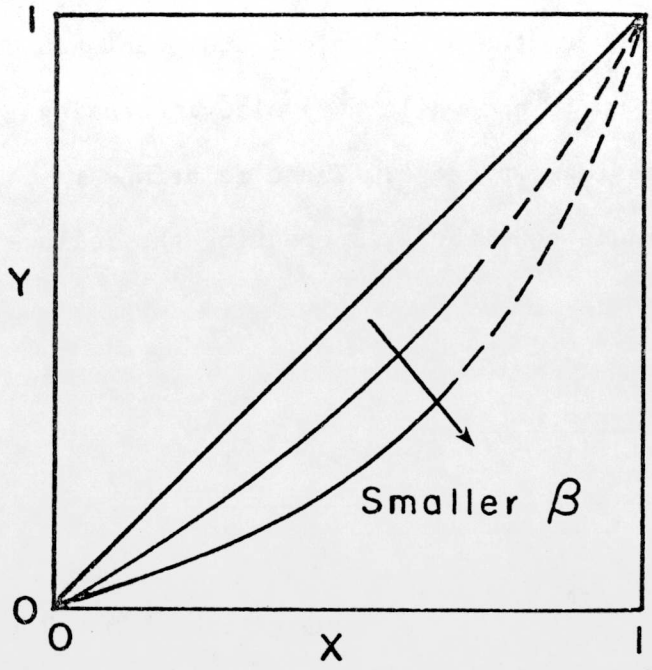

Table 7.8. Values of the parameter, β.

Experiment No.	B.t. enzyme/T.v. enzyme	β-Glucosidase	β
3	0/100	0.28 mg/ml	0.31
	30/70	0.59	0.48
	50/50	0.86	0.74
4	0/100	0.34	0.35
	20/80	0.495	0.425
	40/60	0.66	0.48

curves will become closer to the diagonal line as the β-glucosidase activity increases, and vice versa. The starting point of the reaction path is (0,0), but the end point can not specified because 100% conversion is difficult to obtain. However, the hypothetical end point would be (1,1) because X is increasing up to one while Y is also increasing to one if there is enough β-glucosidase present. These relations resemble the x-y diagram of gas-liquid equilibria, suggesting the following relation may be applicable:

$$Y = \frac{\beta X}{1 + (\beta - 1) X}$$

or

$$\frac{Y}{1-Y} = \beta \left(\frac{X}{1-X}\right)$$

where β = function of the β-glucosidase activity.
(For the gas-liquid equilibrium, α is used instead of β, and α is called relative volatility.)

Examples of this correlation are shown in Fig. 7.26 with the data obtained in Experiments No. 3 and 4. The value of β for each enzyme solution was obtained from the values of $Y/(1-Y)$ at $x/(1-X) = 1$ on the abscissa. The results are listed in Table 7.8.

The β-glucosidase activities of the enzyme solutions used in the experiments are also listed in Table 7.8. Since the value of β was considered to be very much dependent upon the β-glucosidase activity, these two numbers are plotted in Fig. 7.27, showing that the value of β could be estimated from the level of β-glucosidase activity.

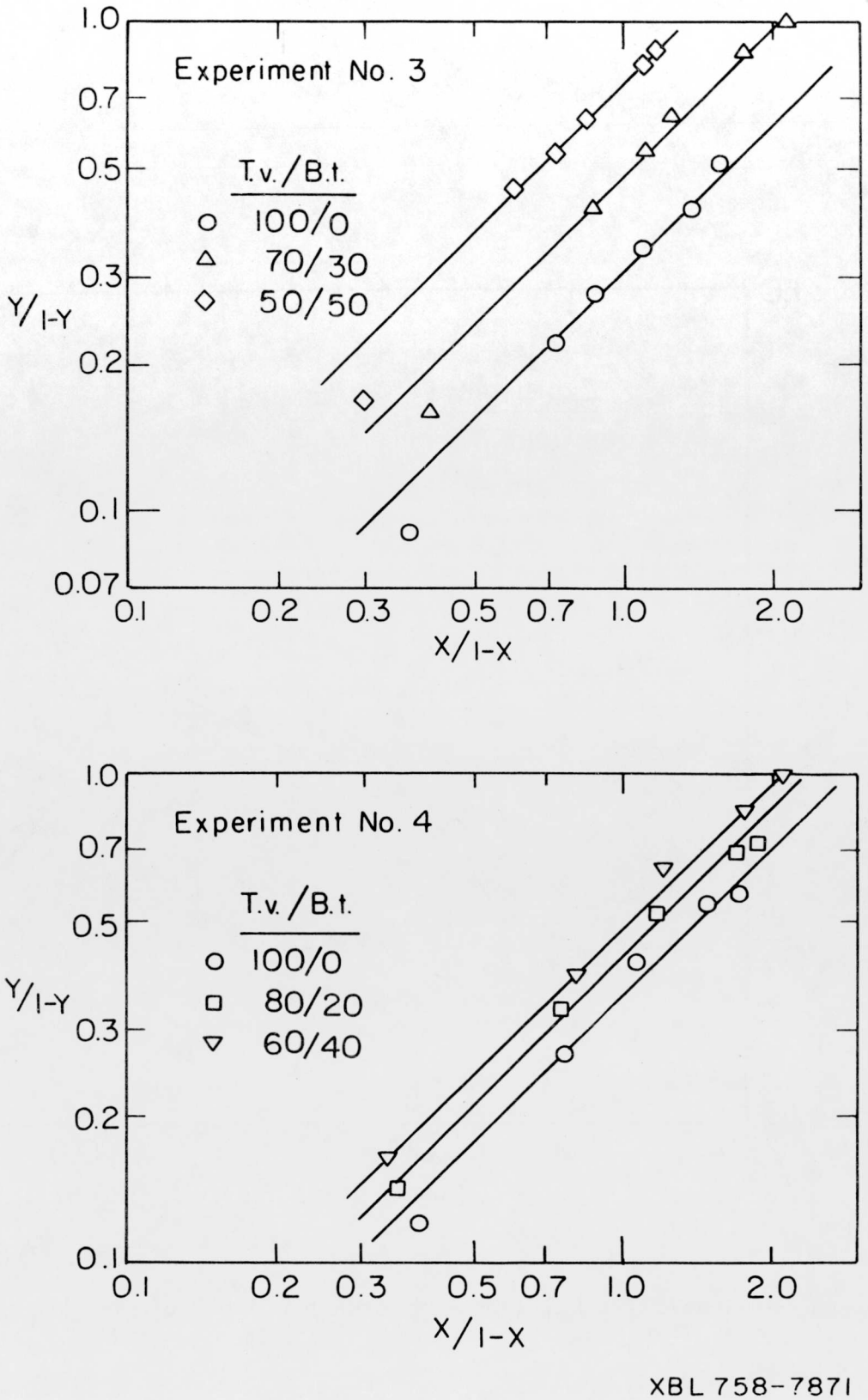

Figure 7.26. Correlation between conversion to glucose and conversion to total sugars for hydrolysis of Solka Floc by various proportions of β-glucosidase (*B. theobromae*) and cellulase (*T. viride*).

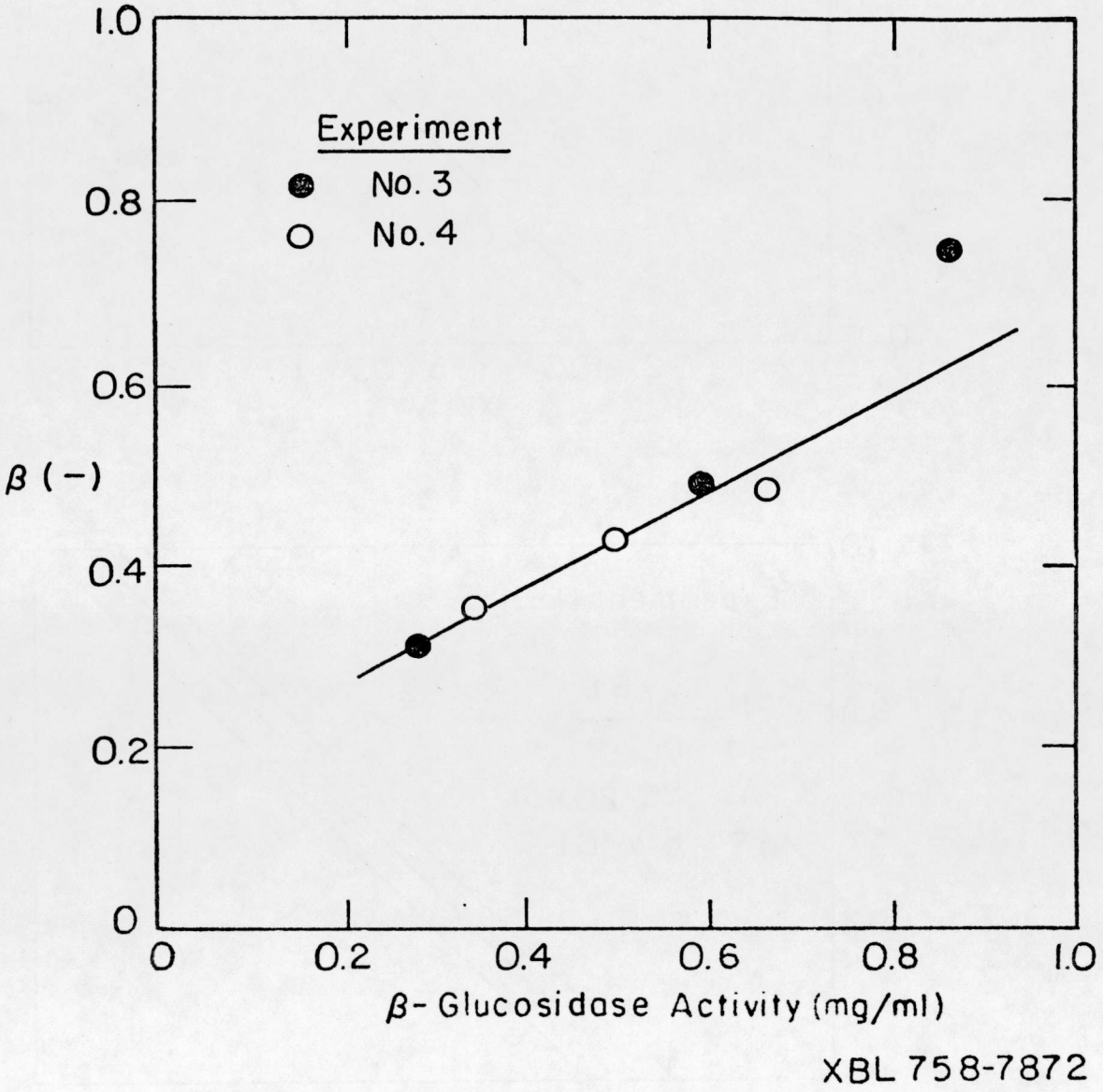

Figure 7.27. Relationship between β and β-glucosidase activity.

The same correlation was examined for application to the hydrolysis of newsprint. However, the linearity of the correlation was not as good as for Solka Floc and the slope was much steeper.

7.2.4 Utilization of Cellobiose in the Hydrolysis Product

This study originally intended to use β-glucosidase to improve the conversion during the enzymatic hydrolysis of cellulose, in which cellobiose worked as an inhibitor to the reaction. Later, however, we realized that the utilization of cellobiose by yeast, which was supposed to utilize the sugars produced by the reaction most efficiently and profitably, had not been investigated very well. Kaplan (8) reported that a mutant of baker's yeast, Saccharomyces cerevisiae, could grow on cellobiose as a sole carbon source, but nothing was said about the alcohol productivity of the strain. (Neither intra- nor extracellular β-glucosidase was reported to be produced by the yeast.) Several people have worked on yeast fermentation to make ethanol in our laboratory, but only glucose has been used for the substrate.

Thus, a batch experiment of alcohol fermentation with Saccharomyces cerevisiae using the actual hydrolysis product of cellulose was carried out to find the usability of cellobiose by the yeast culture.* The hydrolyzate of Solka Floc, obtained by hydrolysis at 50° C for 48 hours, contained 1.54% glucose and 2.24% total glucose equivalent (TGE).

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*This experiment was done in cooperation with G. Cysewski. Detail will be discussed in his dissertation.

The yeast fermentation was carried out in a 1 liter fermentor, which was operated anaerobically at 35° C.

Three samples, which were taken at the beginning, at the end of the logarithmic growth phase and after two days of stationary and possibly death phase of fermentation, were examined by the Glucostat reagent for the glucose content and by the anthrone reagent for TGE. The results are shown in Table 7.10.

Since the supernatant of the samples had a dark brown color, the apparent presence of a small amount of glucose in the fermented broth may have been due to experimental error. The values obtained by subtracting glucose concentration from TGE values are considered to be the concentration of glucose units which comprised the oligomers, mostly cellobiose. These numbers are similar, suggesting that only glucose was utilized by the yeast, and the cellobiose (and higher oligomers) could not be utilized and remained in the fermented broth.

There may be several uses for this cellobiose containing broth. One possibility is for growing the fungus which produces cellulase or β-glucosidase although the Botryodiplodia theobromae did not produce any β-glucosidase with this broth as mentioned in Chapter 3. The use of β-glucosidase to convert this cellobiose to glucose is another possibility. In this case, the immobilized enzyme is preferable because of the high cost of enzyme recovery processes.

Table 7.10. Consumption of reducing sugars during alcohol fermentation.

Sample	TGE	Glucose	(TGE - Glucose)
Feed	22.39 mg/ml	15.42 mg/ml	6.97 mg/ml
1	6.49	0.05	6.44
2	6.49	0.05	6.44

*Sample 1 was taken at the end of the logarithmic growth phase and sample 2 was taken after two days of stationary or death phase.

7.2.5 Summary

In summarizing the results of the hydrolysis reaction with mixed enzyme systems, we came to realize that the interpretation of the data could not be conclusive. As has been discussed, even the properties of the cellulase preparation from different batches of Trichoderma viride did not show consistent values; for example, even though the filter paper activity was the same, the C_1, the C_x and the β-glucosidase activities are not necessarily the same for different enzyme preparations. Not only these enzyme activities but also the kinetic parameters, such as K_m, the Michaelis constant, and K_2, the dissociation constant for the cellobiose--cellulase complex, were never the same. Thus, the conclusions here are very general and probably presumptive to a certain extent. The following points summarize Section 7.2:

a) The effect of the β-glucosidase on the hydrolysis of cellulose is much more positive when the accessibility of the substrate is high and when the cellulase activity of the original cellulase preparation is low.

b) Consequently, the conversion of the hydrolysis of newsprint by cellulase solution with high activity (currently about 4 mg/ml) is difficult to improve by the use of the mixed enzyme system.

c) However, 15/85 to 30/70 mixtures of the culture filtrates of Botryodiplodia theobromae and Trichoderma viride would provide 10 to 20% increases in the total conversion of cellulose and 20 to 40% increases in the glucose productivity.

d) Cellobiose was not utilized by yeast in the alcohol fermentation process. Thus, even if the total conversion is not increased much, the increase of the conversion of cellulose to glucose is still important, and it can be achieved by the mixed enzyme system.

7.3 Reusable Enzyme in the Hydrolysis Product

Two different cellulosic materials, Solka Floc and Wiley-milled newsprint, were used as substrates, and the hydrolysis was carried out with three different mixed enzyme systems at 50° C. The cellulase was a culture filtrate of Trichoderma viride, filter paper activity of 4.2 mg/ml, and the β-glucosidase was a three-fold concentrated culture filtrate of Botryodiplodia theobromae. The activities of these enzyme solutions and the reaction conditions are shown in Table 7.11.

Table 7.11. Experimental Conditions (50°C).

Enzyme	1	2	3
Composition:			
T.v. enzyme	85%	70%	50%
B.t. enzyme	15	30	50
Activities:			
Filter paper*	4.33 mg/ml	4.02 mg/ml	4.24 mg/ml
C_x	2.12	2.16	2.13
β-Glucosidase	0.82	1.24	1.79
Reaction:			
Temperature	50° C		
Time	48 hours		
Substrate	5%		

*Measured with the anthrone reagent.

The cellulase activities represented by either filter paper activity or C_x activity for each enzyme were essentially the same, but the cellobiase activity increased as the β-glucosidase increased.

Although the acetone precipitation method was used to separate the enzyme from the solution, considerable amounts of sugar were detected in the resuspended enzyme solution. Therefore, the enzyme activities were calculated by subtracting the sugar concentration from the value obtained for the activity assay.

Figure 7.28 illustrates the amounts of the reusable enzymes which were retained in the hydrolyzate solution. In the newsprint hydrolysis the cellulase left in the solution, which was calculated from the filter paper activity, was about 12% of the original concentration for all mixed enzyme systems. This number was consistent with the value based on the C_x activity. The residual cellulosics of Solka Floc hydrolysis seemed to adsorb less cellulase, i.e., the retained enzyme concentration in the solution of Solka Floc hydrolyzate was higher than that of the newsprint hydrolyzate. Furthermore, the differences between the values of both filter paper and C_x activities were significant, the reason for which we can not speculate.

About 60% of the original β-glucosidase was found in the hydrolyzate of both newsprint and Solka Floc. This level is quite high compared with the level of the cellulase concentration left in the solution. As discussed in Chapter 3, β-glucosidase is not adsorbed as well as cellulase by cellulosic materials: for the 5% newsprint suspension, about 17% of the original cellulase concentration remained while about 70% of the original β-glucosidase concentration was retained in the solution

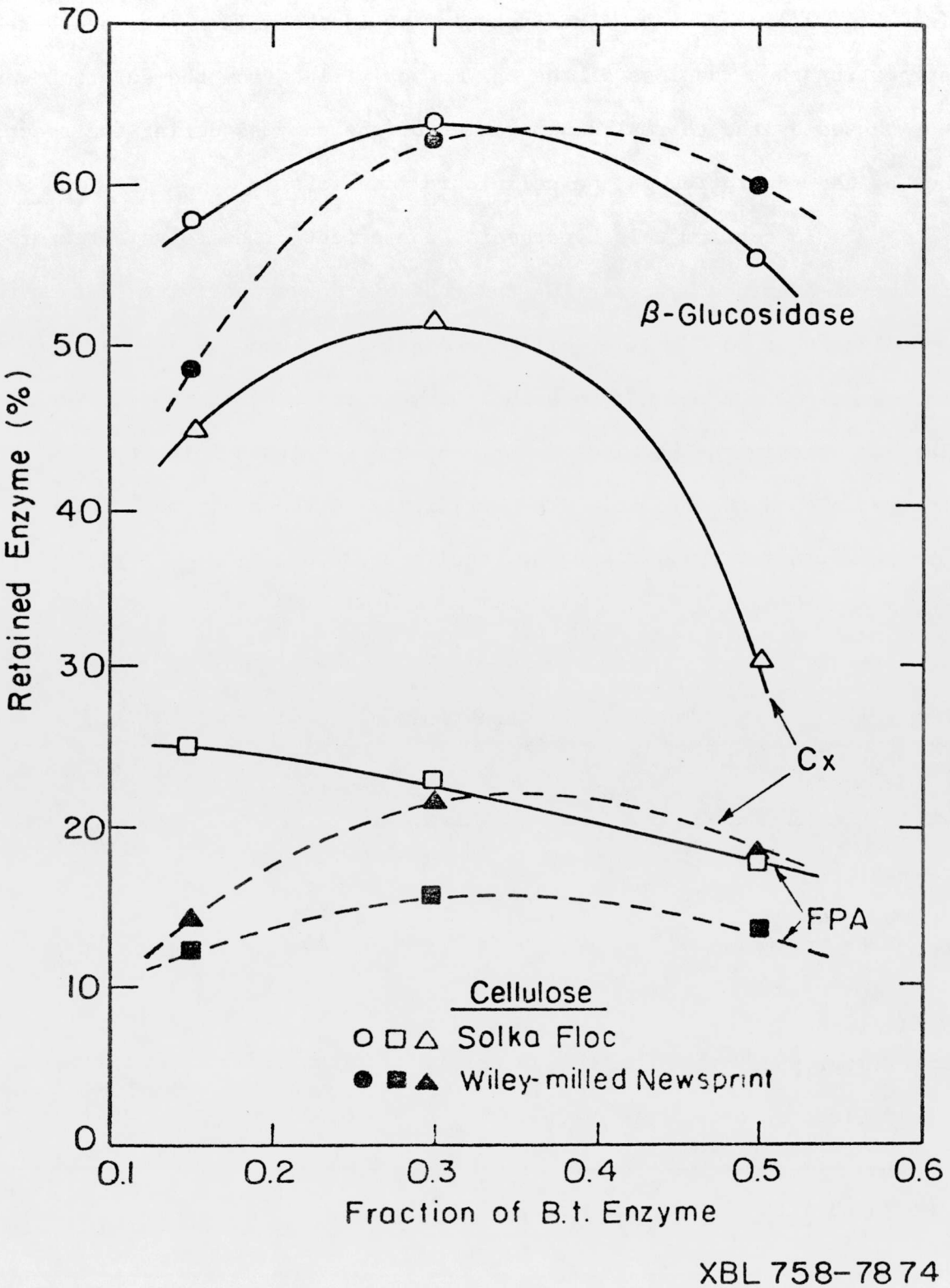

Figure 7.28. Percent of original enzyme activity remaining after hydrolysis by various proportions of β-glucosidase (*B. theobromae*) and cellulase (*T. viride*) at 50°.

(see Fig. 3.11). These values are similar to the values obtained here, suggesting that the loss of the enxyme activities from the solution was not caused by the thermal denaturation of the enzymes during the reaction but by the adsorption on the cellulosic materials.

To confirm this hypothesis, the effect of hydrolysis temperature was examined by measuring the reusable enzyme activity left in the hydrolyzate at 45° C. Wiley-milled newsprint was used as a substrate for the reaction, and the enzyme solutions were mixtures of the same cellulase as used in the previous experiment and a culture filtrate of Botryodiplodia theobromae. The activities of these enzyme solutions are shown in Table 7.12, and the results are shown in Fig. 7.29.

Table 7.12. Experimental conditions (45°C).

Enzyme	1	2	3
Composition:			
T.v. enzyme	100%	85%	70%
B.t. enzyme	0	15	30
Activities:			
Filter paper*	4.30 mg/ml	3.95 mg/ml	3.62 mg/ml
C_x	2.14	1.90	1.87
β-Glucosidase	0.34	0.43	0.57

*Measured with the anthrone reagent.

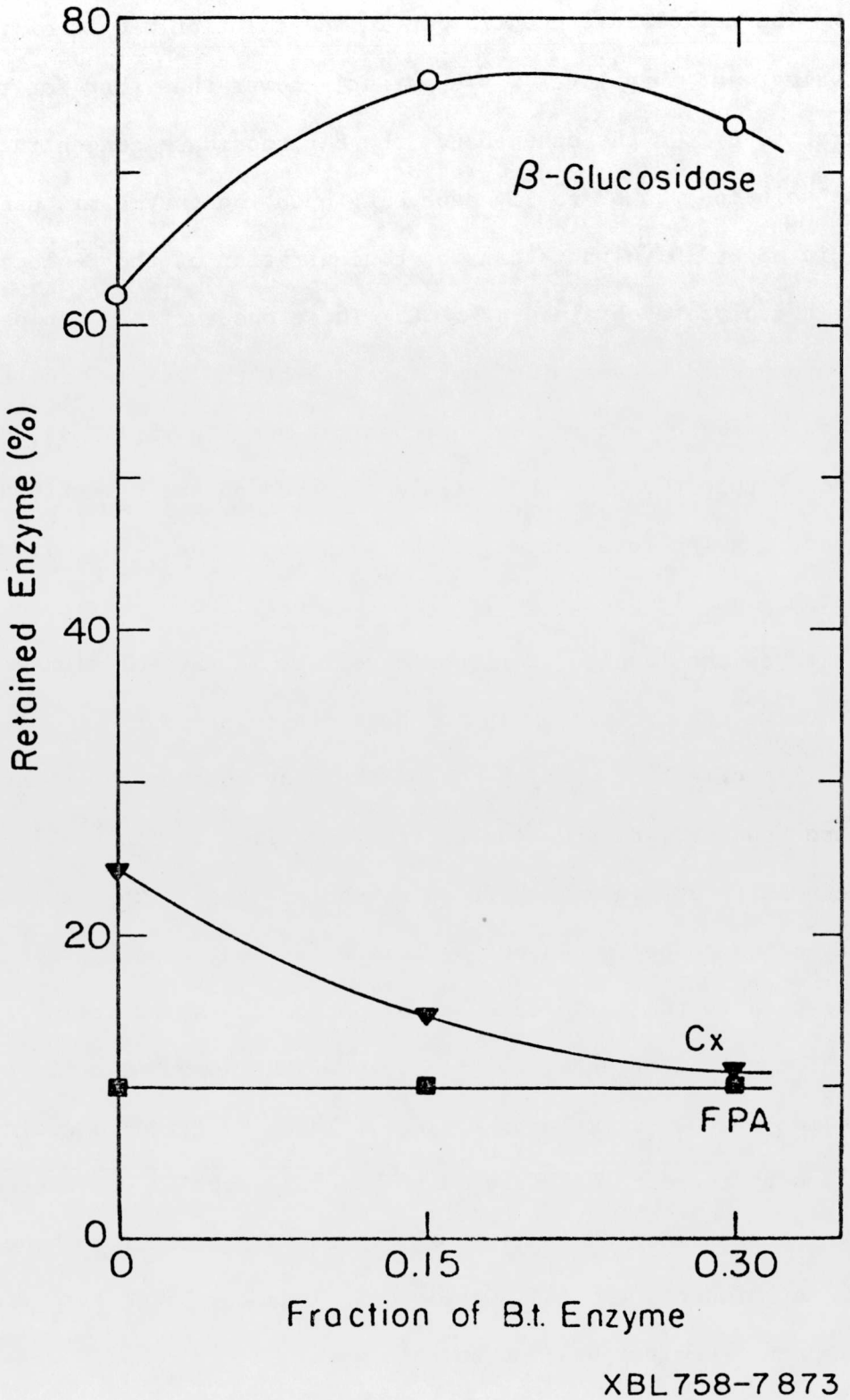

Figure 7.29. Percent of original enzyme activity remaining after hydrolysis by various proportions of β-glucosidase (*B. theobromas*) and cellulase (*T. viride*) at 45°.

The cellulase concentration in the solution was 8% of the original value, and this level was apparently lower than that for the hydrolyzate at 50° C. On the other hand, the β-glucosidase concentration left in the solution was 62 to 75% depending upon the enzyme mix used. The level is about 10% higher than the concentration of the β-glucosidase in the hydrolyzate obtained at 50° C. These observations can be reasonably interpreted by assuming that the loss of the enzyme from the solution was caused by adsorption because, as seen in Fig. 3.11, the lower the temperature the more the cellulase (based on the C_x activity) was adsorbed, leaving less enzyme in the solution. The β-glucosidase was less stable and less adsorbable than the cellulase. Therefore, at higher temperatures the level of β-glucosidase left in the solution would be small due to the certain extent of heat denaturation.

The effect of washing-out of the enzymes from the spent cellulose was also studied, and the results are summarized in Table 7.13. Contrary to the results obtained with fresh newsprint (Fig. 3.28), the β-glucosidase seemed to be washed out by this operation not only from the solution trapped in the solid cake but also from the spent cellulosic materials. On the other hand, the recovery of the adsorbed cellulase from the solids did not seem to take place. These differences could not be explained by the data obtained in the present work and more studies will be required for the development of a recovery process for these enzymes.

At present, the following conclusions may be derived from the experimental results obtained in this work:

a) About 15% of the original cellulase based on the filter paper activity is left in the hydrolyzate at 50° C, and the rest is probably

Table 7.13. Washing-out enzymes from the spent cellulose.

Substrate	Enzyme**	Recovery of supernatant	Washed-out enzymes C_x	Washed-out enzymes β-Glucosidase	Calculated values* C_x	Calculated values* β-Glucosidase
Wiley-milled newsprint	1	0.847	1.5%	14.4%	2.2%	7.4%
	2	0.853	2.8	13.2	3.2	9.3
	3	0.858	2.8	12.3	2.6	8.5
Solka Floc	1	0.955	1.7	10.7	2.0	2.6
	2	0.938	2.2	7.5	3.2	4.0
	3	0.929	2.0	7.1	2.1	4.0

*Calculated from the following equation (see Sec. 3.2.6).

$$\varepsilon_2^0 = (1 - \gamma)\, \varepsilon_1$$

**See Table 7.11.

adsorbed on the spent cellulosic materials.

b) At 45°C only 10% of the original cellulase remains in the solution and more enzyme is adsorbed on the cellulose.

c) The thermal denaturation of cellulase is not substantial during the hydrolysis reaction even at 50°C, while the β-glucosidase denatures some, but not significantly.

The effect of the β-glucosidase concentration in the mixed enzyme solution could not be determined conclusively from the data, which showed no obvious trends. More adsorption of the enzyme was expected for enzyme mixes with less β-glucosidase activity because of the larger affinity of cellulase for the cellulosic materials, but the results did not confirm our expectations.

7.4 Computer Simulation of Enzymatic Hydrolysis of Cellulose

In Chapter 5 we showed a reaction model of the enzymatic hydrolysis of cellulose and in the preceding sections all the parameters involved in the model were obtained from the experimental work. Hence, the model was applied to the simulation of the hydrolysis of cellulose and comparison was made between the experimental values and the calculated values of concentration of sugars produced during the hydrolysis.

The calculation was basically comprised of integrating Equations (5-4)' and (5-11) simultaneously. In Equation (5-4)', the value of I_2, the concentration of cellobiose, is required, and the correlation obtained in Section 7.2.3 can be used with the experimentally estimated value of β. That is

$$\frac{(G)}{(TGE)_0} = \beta(TGE)/(TGE)_0/(1 + (1 - \beta)\ (TGE)/(TGE)_0)$$

from which the concentration of cellobiose can be calculated by giving the TGE value, which is obtainable by integrating the two differential equations above. Line (A) in Fig. 7.30 represents the TGE values calculated for Experiment No. 3 (see Fig. 7.12), showing poor agreement with the experimental data also shown in the same figure.

This disagreement is mainly attributable to the small value of α for Solka Floc which was obtained by the Michaelis-Menten type analysis. If the reaction rate of the crystalline portion of Solka Floc is much faster than that of the absorbent cotton, then higher conversion will be attained even if the value of α is small. The Lines (B) and (C) in Fig. 7.30 were obtained with the doubled and tripled reaction rate constant for the crystalline cellulose. The agreement becomes better in the early stage of the hydrolysis, but the slope of the curve is too high for the later part of the reaction, which is very different from the experimental results. If we assume that the rate constant for the crystalline cellulose is expressed by the equation

$$k = k_0 + A\ \alpha,$$

where k_0 is the rate constant obtained for the absorbent cotton, which is almost pure crystalline cellulose, and A is a constant number, the fit will be better. Synergism and the different adsorption characteristics of the enzymes may or may not explain the relation, but at the moment there is no evidence. The line (D) in Fig. 7.30 was calculated with this assumption, and the agreement is good. However, the value of A

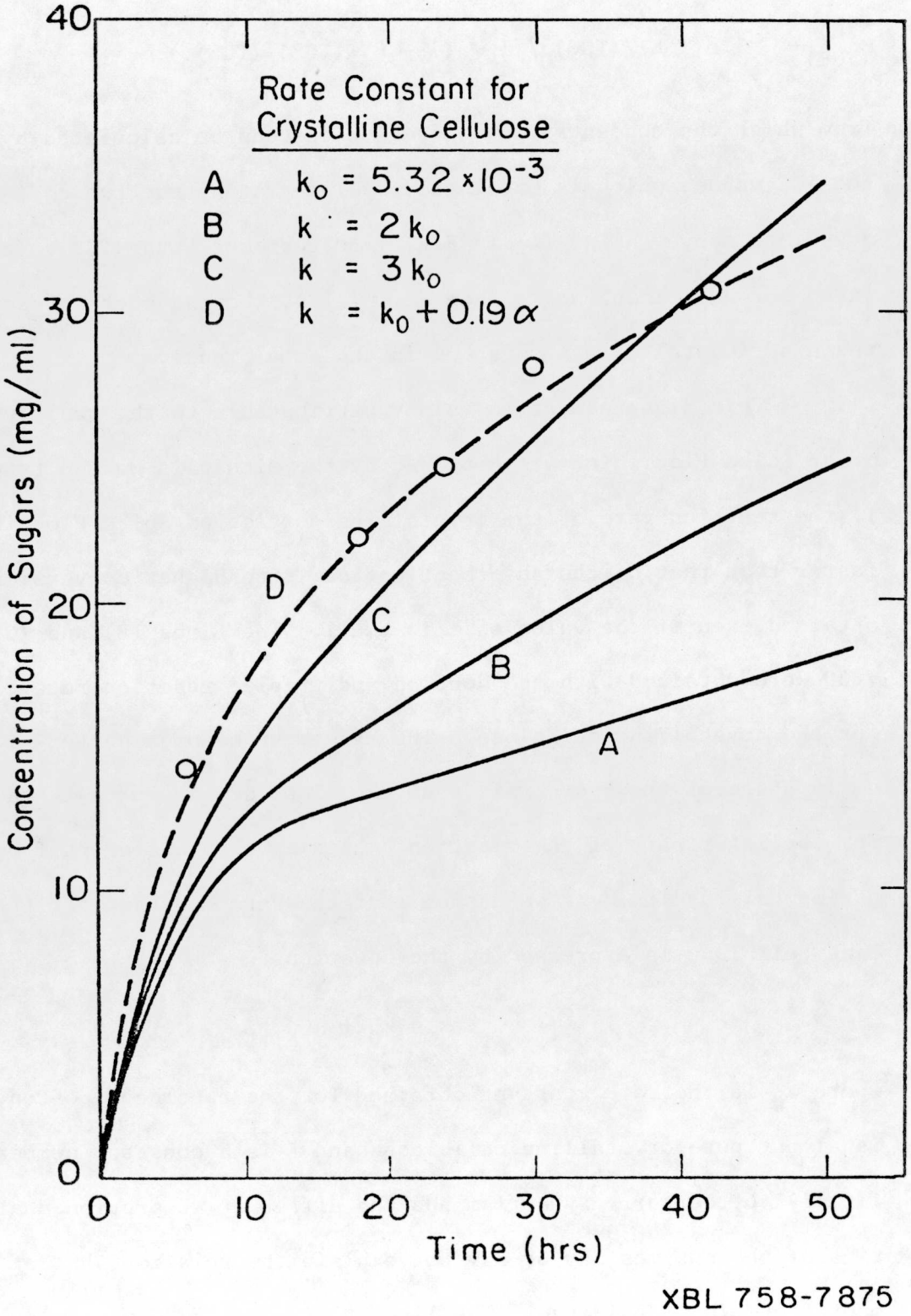

Figure 7.30. Comparison of kinetic model with experimental data (Exp. #3) for hydrolysis of Solka Floc by cellulase from *T. viride*.

which gives the best fit in this case is not adequate for the other cellulase preparations, and hence this hypothetical correlation can not be applied in the general calculations.

More practically, we can estimate the accessibility which gives the best fit with the experimental data of the long term hydrolysis. If we assume no inhibitory effect of the crystalline cellulose to the reaction of accessible cellulose, the basic Michaelis-Menten equation becomes

$$V = \frac{V_{max}\ S\ \alpha}{K_m + S\ \alpha}$$

and hence

$$V = \frac{V_{max}\ S}{K_m/\alpha + S} \qquad (7\text{-}8)$$

Thus, in the Lineweaver-Burk plot $1/V_0$, the intercept on the ordinate, gives the value of $1/V_{max}$ and $-1/S_0$, the intercept on the abscissa, gives the value of α/K_m (see Fig. 7.5). Using the Equation (7-8) instead of the equation (5-4)', we can calculate the progress of the reaction as a function of α and thus optimize the value of α to give the best fit with the experimental data. The inhibitory effect of cellobiose should be taken into account in the same manner as calculated before.

The results are shown in Fig. 7.31. The optimum value of α was obtained by fitting the calculated values of TGE with the observed data for a 100% cellulase solution. This number, 0.50, was also found to be applicable to the other cellulase preparation, the Lineweaver-Burk plot of which was shown in Fig. 7.6. To examine the applicability of this model, a hydrolysis experiment was carried out in which the cellulase was diluted with buffer to a concentration of 20 to 80% of the original,

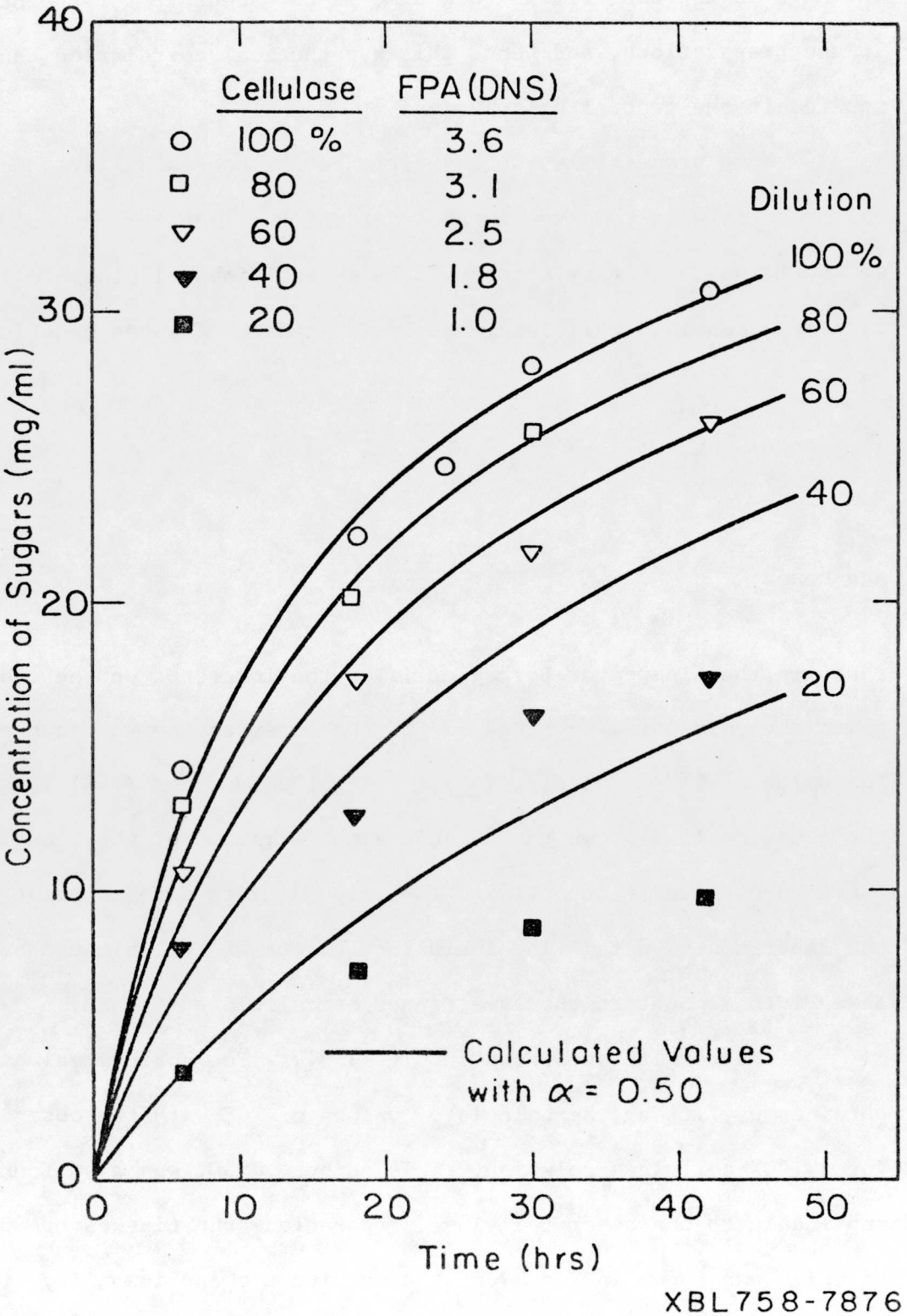

Figure 7.31. Comparison of kinetic model (at optimum value of α) with experimental data for hydrolysis of Solka Floc by cellulase from *T. viride* at various dilutions.

and the hydrolysis reaction of Solka Floc was performed with these diluted enzyme solutions. The results are shown in Fig. 7.31.

For the computer simulation the effect of enzyme dilution can be taken into account by using $V_{max}' = p\ V_{max}$ in Equation (7-8) and $k' = p\ k$ in Equation (5-11), where p is the dilution ratio. The calculated results are also shown in Fig. 7.31. The differences between the calculated and observed values are fairly small for the results with the 80 and 60% cellulase, but the discrepancy becomes larger for the 40 and 20% diluted enzyme solutions. The reason is not clear; perhaps the model was inadequate. Or when the cellulase was diluted considerably, one of the components (for instance, the C_1 component) was preferentially adsorbed on the crystalline portion of the cellulose, leaving unbalanced synergistic activity, which was not capable of hydrolyzing the substrate proportionally to the enzyme concentration.

Nevertheless, the model seems applicable to the general cellulase preparations. However, the only problem with this model is that the difference in the values of V_0 obtained for the different celluloses in Figs. 7.1 and 7.2 could not be explained with this model, but could be explained with the model described in Chapter 5 which introduced the inhibitory effect of the crystalline cellulose on the reaction of accessible cellulose.

PART III

PROCESS DESIGN

Chapter 8 Process Scheme and Cost Analysis

Literature Cited in Chapter 7

1) Brandt, D., Hontz, L. and Mandels, M., A.I.Ch.E. Symp. Series, Forest Products and the Environment 69, 127 (1973)

2) Freitas, R., private communication, Lawrence Berkeley Laboratory, March 1975

3) Evett, M., private communication, Lawrence Berkeley Laboratory, May 1975

4) Mitra, G., Enzymatic Utilization of Waste Cellulosics, Lawrence Berkeley Laboratory Report 2334-Rev., January 1975

5) Ghose, T. K. and Kostick, J. A., Cellulases and Their Applications, Adv. in Chem. Series, 95, 415 (1969)

6) Rubik, B., private communication, Lawrence Berkeley Laboratory, September 1974

7) Yang, R. D. and Wilke, C. R., Presented at the Eighth Cellulose Conference on Wood Chemicals, Syracuse, New York, May 1975

8) Kaplan, J. G., J. Gen. Physiol. 48, 873 (1965)

9) Cysewski, G., private communication, Lawrence Berkeley Laboratory, May 1975

8. Process Scheme and Cost Analysis

8.1 Process Scheme

Figure 8.1 shows the basic scheme of the process of the enzymatic hydrolysis of newsprint proposed by Wilke and Mitra (1) and Wilke and Yang (2). Since the process to be designed in the present work follows their scheme, it is worthwhile to explain briefly the previous proposed processes.

Raw materials

Newsprint (885 ton/day) containing 6% moisture is first reduced to approximately - 20 mesh by shredding and hammermilling and fed into the hydrolyzer. In both the original Wilke-Mitra process and the Wilke-Yang process, the recovery of the cellulase from the hydrolyzate is conducted by adsorbing the enzyme on fresh newsprint. In that case the newsprint is countercurrently contacted in the adsorption train with the hydrolysis effluent, which contained the reusable enzyme. However, the data obtained more recently showed that the level of cellulase enzyme retained in the hydrolyzate was at most 20% (see Sect. 7.3), and there still exists an uncertainty about the economic feasibility of recovering the enzyme by the adsorption process.

Hydrolysis reactor

The hydrolysis reactors consist of five agitated cylindrical concrete digestors of the type used for solid waste treatment. The hydrolysis is carried out over 40 hours at 45° C with a solid concentration

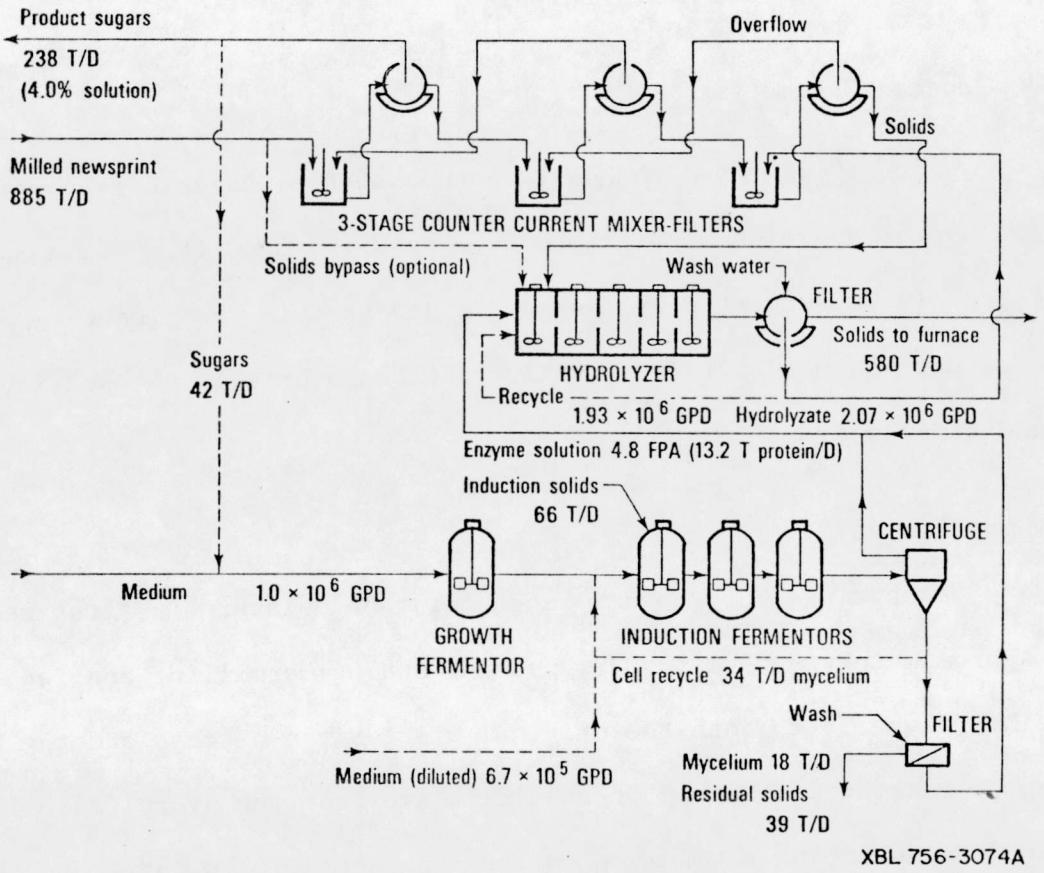

Figure 8.1. Process flow diagram for enzymatic hydrolysis of newsprint (2).

of 5% (w/w of the enzyme solution). The hydrolysis product is filtered and the spent cellulosics are fed to a furnace and steam power plant to provide the steam and electricity for the process. In the original Wilke-Mitra process (1), the enzyme adsorbed on the spent newsprint was countercurrently contacted with water so that the enzyme was washed out from the cellulose. This scheme, however, was abandoned because of the difficulty of washing-out the enzyme from the spent newsprint (2) (see also Sect. 7.3).

Enzyme make-up

Make-up enzyme was produced in multi-stage continuous fermentors. The dilution rate of the cell growth stage was 0.2 hr^{-1} with a medium containing 1% product sugars plus minerals and Proflo oil. One tenth of the dilution rate for the cell growth stage was chosen for the induction stage, which comprised three to five equal volume fermentors in series. Newsprint was fed as an inducer to the first fermentor of the series of induction stages, making a 1.5% suspension.

In the Wilke-Yang process, a cell recycling method was proposed, aiming for a higher density of cell mass in the induction stage. Based on the data obtained by Mitra (3), it was expected that the higher cell density would provide higher enzyme activity. The actual fraction of the cells recycled in their process was 0.59 (dry weight of cells recycled/dry weight of cells from growth stage).

In the present work, a mixed enzyme system was employed for the hydrolysis of newsprint. Thus, the only modification of the Wilke-Yang process was in the enzyme make-up process, in which two enzymes, cellulase and β-glucosidase, were produced separately in multi-stage

fermentors of the same concept. The enzyme recovery by adsorption from the hydrolyzate was also evaluated and is shown in the following section. Hence, the over-all process scheme is essentially the same as shown in Fig. 8.1. For simplicity Fig. 8.1 omitted the facilities for milling, heat exchange, induction solid sterilization and spent cellulosics combustion, which are to be included in the cost analysis.

8.2 Enzyme Recovery by Adsorption Train

As shown in Sect. 3.2.5 the cellulase and β-glucosidase were adsorbed on the newsprint although the existence of the equilibrium between the enzyme in the liquid and on the solid was still uncertain. Nevertheless, in the cost analysis a stage-wise adsorption train was designed with the apparent partition coefficient obtained in Chapter 3.

The number of ideal adsorption stages can be estimated either graphically or by computer calculations. For this particular case of enzyme recovery an algebraic method also works as well with the following assumptions:

a) The enzyme in the liquid and on the solid are in equilibrium.

b) The amount of entrained liquid in the solid is constant.

c) No reaction occurs during the adsorption process.

Figure 8.1 depicts the adsorption train where the fresh newsprint is countercurrently contacted with the hydrolyzate. The mass balance of the enzyme for the k-th stage is

$$W(Y_{k+1} - Y_k) + L\ (Y_{k-1} - Y_k) + S\ (X_{k+1} - X_k) = 0$$

where

X = enzyme units per gram of solid

Y = enzyme units per ml of liquid

L = flow rate of the hydrolyzate

S = flow rate of the newsprint

W = flow rate of the entrained liquid in the newsprint.

Since by definition, $Y_k = K\ X_k$ (K is an apparent partition coefficient), we get

$$Y_{k+1} - Y_k = \left(\frac{L}{W+S/K}\right)\ (Y_k - Y_{k-1})$$

This relation holds for all stages except the last stage (N-th stage). Hence,

$$Y_N - Y_{N-1} = a\ (Y_{N-1} - Y_{N-2}) = a^2\ (Y_{N-2} - Y_{N-3})$$

$$= \text{----------} = a^{N-1}\ (Y_1 - Y_0) \qquad (8\text{-}1)$$

where Y_0 = enzyme units in the hydrolyzate

$a = L/(W + S/K)$.

From the mass balance for the N-th stage,

$$L\ Y_{N-1} = L_N Y_N + W\ Y_N + S\ X_n$$

$$= L_N Y_N + (W + S/K)\ Y_N \qquad (8\text{-}2)$$

where $L_N = L + W_0 - W$ (W is the moisture in the fresh newsprint.)

Coupling Equations (8-1) and (8-2), we obtain the following equation for the enzyme concentration of the effluent liquid from the adsorption train:

$$Y_N = \left(\frac{L}{W_0 + S/K}\right)\ a^{N-1}\ (Y_0 - Y_1) \qquad (8\text{-}3)$$

The over-all mass balance gives

$$L\,Y_0 = W\,Y_1 + S\,X_1 + L_N Y_N = (W + S/K)\,Y_1 + L_N Y_N$$

Thus
$$Y_1 = a\,Y_0 - a\,Y_N\,L_N/L \tag{8-4}$$

Substituting Equation (8-4) into (8-3),

$$\frac{Y_N}{Y_0} = \frac{\dfrac{L}{W_0 + S/K}\,(1 - 1/a)}{\dfrac{L_N}{W_0 + S/K} - 1/a^N} \tag{8-5}$$

This equation gives the percentage recovery of the enzyme from the hydrolyzate as a function of the number of stages for a given mass balance. In the design calculations, the following mass balance was assumed:

Basis: 833 ton/day (=S) of newsprint with 52 ton/day (= Wo) of moisture.

The total enzyme solution required is 833/0.05 = 16660 ton/day, some of which may be entrained with the spent solid. Therefore, it was assumed that the entrained enzyme solution, which was also assumed to be 2 ton/ton of the cellulose, was replaced with the same amount of wash water. Hence, L = 16660 ton/day. The entrained water in the newsprint from the adsorption train was also assumed as 2 ton/ton of newsprint, which gives W = 1666 ton/day and L_N = 15046 ton/day.

From Table 3.7 the apparent partition coefficients for cellulase and β-glucosidase are:

$K \approx 0.013$ for cellulase based on the filter paper activity

$K \approx 0.110$ for β-glucosidase.

Using these numbers in Equation (8-5), we can calculate the percentage recovery of the enzyme as a function of N. The results are shown in Table 8.1.

Table 8.1. Percentage recovery of enzyme from hydrolyzate by adsorption train.

Number of stages N	Percentage of recovery, $(1-Y_N/Y_0)\times 100$	
	Cellulase	β-Glucosidase
1	79.3%	31.3%
2	95.0	41.6
3	98.75	46.0
4	99.68	48.2
∞	---	50.7

The recovery of the cellulase is almost complete with three adsorption stages, while the recovery of β-glucosidase is not sufficient mainly because of its large partition coefficient. Since, as shown in Table 8.1, only 50% of the β-glucosidase could be recovered even with an infinite series of stages, the use of more than three stages does not seem profitable. Thus, two adsorption stages were employed for the design calculation in the present work, and the recoveries of cellulase and β-glucosidase were obtained as 95% and 41.6%, respectively.

8.3 Enzyme Production Process

To design the enzyme production stage, the following equation proposed by Wilke et al. (4) was conveniently used.

$$\frac{F\,e}{V_2} = k \times J(\theta) \qquad (8\text{-}6)$$

where F = flow rate of the medium

V_2 = total volume of induction fermentor

e = enzyme concentration

k = specific enzyme productivity

X = cell mass concentration

$J(\theta)$ = effective factor

They obtained the value of k as 0.14 mg of protein/mg of cell/day for cellulase production from Trichoderma viride using a cell recycle system for the induction stage.

For β-glucosidase we can calculate k from the data obtained in Chapter 3 as follows: When 0.5% glucose medium was used, the cell mass concentration was 2.5 mg/ml and the β-glucosidase activity from one induction stage was 0.7 mg/ml, which corresponds to a protein content of 0.72 mg/ml at a dilution rate of 0.0114 hr^{-1}. Since the time necessary for Botryodiplodia theobromae to adapt to the cellulose medium was 18 hours (see Fig. 3.11), the fraction of cells residing in one induction stage for a time greater than the adaptation time (t_I) is

$$f = e^{-t_I D_2} = 0.814 = J(\theta)$$

Hence, the specific enzyme productivity, k, for β-glucosidase is calculated as follows:

$$k = \frac{(0.72)(0.0114)(24)}{(2.5)(0.814)} = 0.10$$

F/V_2 in Eq. (8-6) is equal to D_2, which is given as a design parameter, and X is calculated from the glucose used in the medium, the cell yield and the cell recycle ratio. Thus, if we specify the enzyme activity, we can calculate $J(\theta)$ from Eq. (8-6). $J(\theta)$ is also calculated from the cell recycle ratio by assuming that the effectiveness factor of recycled cells is 1.0 because the cells are already adapted, and that the effectiveness factor of fresh cells is given by

$$J(\theta) = \frac{t_H - t_I}{t_H} \qquad (8\text{-}7)$$

where t_H is the mean residence time (4).* If these two $J(\theta)$ do not coincide, a different value of X should be used to obtain the same $J(\theta)$ from each calculation.

Cost analysis was made for the following cases:

Case A: Hydrolysis is carried out with cellulase only, and no enzyme is recovered from the hydrolyzate. The amount of cellulase necessary for the hydrolysis of 833 ton/day fresh newsprint is thus 16660 m^3/day or 4.40×10^6 GPD. Total glucose equivalent (TGE) in the hydrolyzate is assumed as 15 mg/ml, of which 10 mg/ml is glucose. To

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*Equation (8-7) is based on the conservative assumption that the induction stage operates as a plug-flow reactor.

obtain the filter paper activity of 3.5 mg/ml, the cell recycle is necessary to maintain the cell mass concentration in the induction stage at 0.65%.

Case B: Hydrolysis is carried out with a mixed enzyme system whose composition is 15% β-glucosidase solution and 85% cellulase solution. In using the mixed enzyme system it was assumed that TGE is increased 15% (i.e., to 17.25 mg/ml) and glucose is increased 30% (i.e., to 13.0 mg/ml). No enzyme is recovered from the hydrolyzate. From these assumptions the amount of cellulase and β-glucosidase are calculated as 3.74×10^6 GPD and 0.66×10^6 GPD, respectively. The same cell recycle ratio was used as Case A for cellulase production, but not for the β-glucosidase production process. β-Glucosidase activity was assumed as 1.4 mg/ml because of the higher glucose concentration in the medium than used in the experiments.

Case C: The economic feasibility of using the adsorption train to recover the enzymes is evaluated with this case. The enzyme system is the same as used in Case B, but the amounts of these enzymes are different. As calculated in the previous section, 95% of the cellulase retained in the hydrolyzate (about 20% of the original activity) can be recovered by the adsorption process, and 41.6% of the β-glucosidase retained (about 70% of original) can be recovered. Thus, the total recovery is 19% of the original cellulase and 29% of the original β-glucosidase. Hence, the production scales of these two enzymes are 3.03×10^6 GPD of cellulase and 0.462×10^6 GPD of β-glucosidase.

Case D: All conditions (except a higher cell recycle ratio) are the same as those of Case C. The cell recycle system was employed only for the cellulase production process, in which the cell mass concentration was increased to 0.85%. This high cell concentration would provide a filter paper activity of 4.53 mg/ml.

Case E: The same cell mass concentration (0.85%) was applied to the β-glucosidase production process using a cell recycle system. From Eq. (8-6) we obtain

$$e = 3.11\ J(\theta)$$

Performing a material balance for the cell recycle system, we can evaluate the fraction of recycled cells in the induction stage as 0.460, from which $J(\theta)$ was calculated as 0.676. Hence,

$$e = 2.10 \text{ mg of protein/ml}$$

which corresponds to a β-glucosidase activity of 2.04 mg/ml. The production scales of both enzymes are 2.05×10^6 GPD for cellulase and 0.317×10^6 GPD for β-glucosidase.

The basic design of the fermentor for the growth and the induction stages was done using the computer program developed by Cysewski (5). All the design parameters used in the calculation are listed in Table 8.2. The maximum volume of the fermentor was tentatively chosen as 5×10^5 gallons. The concentration of glucose in the medium was assumed as 1% for the production of both enzymes although the data for continuous β-glucosidase production were obtained with 0.5% glucose. It was expected that the use of 1% glucose would provide higher β-glucosidase

Table 8.2. Design parameters for enzyme production.

Item	Cellulase production	β-glucosidase production
Glucose concentration	1%	1%
Cell yield	44%	48%
Dilution rate for growth stage	0.2 hr^{-1}	0.067 hr^{-1}
for induction stage	0.02	0.0114
Q_{O_2}	1.0 mmol O_2/ℓ hr	1.0 mmol O_2/ℓ hr
Critical O_2 concentration	75% of the saturation concentration	

activity as in the case of cellulase production (1). The dilution rates used in the β-glucosidase production were those employed in the experiments with 0.5% glucose, but the same values were assumed for the fermentation with 1% glucose in the present process calculation.

Since there are no data available for the critical oxygen concentration for the growth of either Trichoderma viride or Botryodiplodia theobromae, 75% of the saturation concentration of oxygen in the liquid was used. The effect of cell recycle, i.e., higher cell mass concentration in the induction stage, was taken into account in such a way that more oxygen was supplied to maintain a higher cell population. By comparison it was found that the increase in superficial velocity of the air in the fermentor was less costly than an increase in the power of the agitator.

8.4 Cost Analysis

Since most of the process designed in the present work follows the scheme of the Wilke-Yang process, their unit specifications (2) can be used in the present analysis except for the enzyme production part of the process. Table 8.3 lists the major equipment for the process provided by Wilke and Yang. Only minor changes have been made; the Marshall Stevens Index was changed from 431 (for 4th quarter 1974) to 443.8 (July 1975), and the number of the adsorption stages was reduced to two. The medium sterilizer and the following heat exchanger designed for Case (A) and (B) are listed in Table 8.3. For Cases (C)-(E) the reduced size was used for the cost analysis. Table 8.4 (A)-(E) lists the equipment specifications for the enzyme production stages obtained from the computer

Table 8.3. Major items of equipment.

Item	Unit specification	No. of units	Cost/unit ($)
Pretreatment		Total	$920,000
Hammermill	Welded steel construction, 1800 rpm motor, air system with cyclone, capacity 2500 - 4000 lb/hr.	20	45,100
Screw conveyor	400 ft/hr, 10' × 30', 1.7 HP	5	3,600
Enzyme production (Items other	than listed in Table 8.5)	Total	$1,396,600
Seed tank	1500 gals. vessel agitated and motor	2	14,400
Raw material mixing tank	5.10×10^4 gals. agitated, carbon steel construction, 60 HP motor	1	56,200
Centrifuge for cell recycle	De Laval centrifuge, 60 HP, 70 m^3/hr throughput	2	42,400
Medium sterilizer	Steam injection type 2.55 m × 18.7 m	1	44,500
Medium preheat exchanger	Shell and tube type, 10000 sq.ft.	7	112,100
Cooler exchanger	Shell and tube type, 8100 sq.ft.	2	98,300
Hammermill for induction solids	Welded steel construction, 40 HP 3600 rpm motor, 3000 lb/hr	2	31,300
Induction solid sterilizer	9' × 160' modified rotary kiln or dryer	2	32,100
Screw conveyor for induction solid	1.7 HP	2	3,600

Table 8.3. Major items of equipment (continued)

Item	Unit specification	No. of units	Cost/unit ($)
Mycelium filter	Pressure filter, effective area 175 sq.ft	2	7,500
Pumps and drivers	1400 gal/min	20	2,600
Hydrolysis		Total	$3,305,300
Hydrolyzer	Concrete digester, agitated, placed below ground level, 1×10^6 gals.	7	406,700
Agitator	500 HP	7	15,000
Solid filter	Vacuum drum filter including accessories	1	240,000
Pumps and drivers	1400 gal/min	4	2,600
Storage tank	1×10^6 gals., carbon steel	1	103,000
Enzyme recovery (For Case C only)		Total	$532,200
Mixer	Agitated, carbon steel, Vol. 7.4×10^4 gals.	2	22,300
Agitator	50 HP	2	4,600
Filter	Horizontal belt filter, including accessories, 300 sq.ft. effective area	2	234,000
Pumps and drivers	1400 gal/min	4	2,600

Table 8.4. (A) Major items of equipment for enzyme production stage (Case A).

Item	Unit specification	No. of units	Cost/unit ($)
Growth stage		Total	$599,800
Fermentor	Vol. 3.81×10^5 gals. agitated, stainless steel construction	3	166,500
Agitator	70 HP	3	15,900
Air compressor	130 HP, centrifugal type	1	49,300
Air filter	0.4 m × 0.9 m	3	1,100
Induction stage		Total	$5,024,700
Fermentor	4.77×10^5 gals. agitated, stainless steel construction	24	187,400
Agitator	87 HP	24	18,000
Air compressor	300 HP, centrifugal type	1	92,800
Air filter	0.8 m × 1.0 m	1	2,300

Table 8.4. (B) Major items of equipment for enzyme production stage (Case B).

Item	Unit specification	No. of units	Cost/unit ($)
Growth stage		Total	$774,800
Fermentor (1)	For cellulase production, 4.86×10^5 gals.	2	189,400
Agitator (for Ferm. 1)	90 HP	2	18,200
Fermentor (2)	For β-glucosidase production, 2.56×10^5 gals.	2	134,800
Agitator (for Ferm. 2)	50 HP	2	12,700
Air compressor	170 HP, centrifugal type	1	60,000
Air filter	0.8 m × 1.0 m	2	2,300
Induction stage		Total	$5,702,700
Fermentor (1)	For cellulase production, 4.86×10^5 gals.	20	189,400
Agitator (for Ferm. 1)	90 HP	20	18,200
Fermentor (2)	For β-glucosidase production, 3.77×10^5 gals.	8	165,300
Agitator (for Ferm. 2)	70 HP	8	15,800
Air compressor	330 HP, centrifugal type	1	99,600
Air filter	0.8 m × 1.0 m	1	2,300

Table 8.4. (C) Major items of equipment for enzyme production stage (Case C)

Item	Unit specification	No. of units	Cost/unit ($)
Growth stage		Total	$599,200
Fermentor (1)	For cellulase production, 3.94×10^5 gals.	2	169,300
Agitator (for Ferm. 1)	72 HP	2	16,200
Fermentor (2)	For β-glucosidase production, 3.59×10^5 gals.	1	161,100
Agitator (for Ferm. 2)	65 HP	1	15,400
Air compressor	130 HP, centrifugal type	1	49,400
Air filter	0.8 m × 1.0 m	1	2,300
Induction stage		Total	$4,843,800
Fermentor (1)	For cellulase production, 3.94×10^5 gals.	20	169,300
Agitator (for Ferm. 1)	72 HP	20	16,200
Fermentor (2)	For β-glucosidase production, 3.51×10^5 gals.	6	159,400
Agitator (for Ferm. 2)	65 HP	6	15,200
Air compressor	265 HP, centrifugal type	1	83,900
Air filter	0.8 m × 1.0 m	1	2,300

Table 8.4. (D) Major items of equipment for enzyme production stage (Case D)

Item	Unit specification	No. of units	Cost/unit ($)
Growth stage		Total	$520,200
Fermentor (1)	For cellulase production, 2.66×10^5 gals.	2	137,500
Agitator (for Ferm. 1)	50 HP	2	12,700
Fermentor (2)	For β-glucosidase production, 3.59×10^5 gals.	1	161,100
Agitator (for Ferm. 2)	65 HP	1	15,400
Air compressor	100 HP, centrifugal type	1	41,000
Air filter	0.8 m × 1.0 m	1	2,300
Induction stage		Total	$3,323,700
Fermentor (1)	For cellulase production, 5.00×10^5 gals.	10	192,200
Agitator (for Ferm. 1)	100 HP	10	19,500
Fermentor (2)	For β-glucosidase production, 3.51×10^5 gals.	6	159,400
Agitator (for Ferm. 2)	65 HP	6	15,200
Air compressor	500 HP, centrifugal type	1	136,100
Air filter	0.8 m × 1.0 m	1	2,300

Table 8.4. (E) Major items of equipment for enzyme production stage (Case E).

Item	Unit specification	No. of units	Cost/unit ($)
Growth stage		Total	$440,100
Fermentor (1)	For cellulase production, 2.66×10^5 gals.	2	137,500
Agitator (for Ferm. 1)	50 HP	2	12,700
Fermentor (2)	For β-glucosidase production, 1.20×10^5 gals.	1	90,200
Agitator (for Ferm. 2)	30 HP	1	14,400
Air compressor	75 HP	1	32,800
Air filter	0.8 m × 1.0 m	1	2,300
Induction stage		Total	$3,017,900
Fermentor (1)	For cellulase production, 5.00×10^5 gals.	10	192,200
Agitator (for Ferm. 1)	100 HP	10	19,500
Fermentor (2)	For β-glucosidase production, 2.85×10^5 gals.	5	142,700
Agitator (for Ferm. 2)	55 HP	5	14,000
Air compressor	400 HP, centrifugal type	1	115,100
Air filter	0.8 m × 1.0 m	1	2,300

optimization.

To calculate the fixed capital cost, the purchase cost of the principal items of the equipment was multiplied by 3.1 except in the case of the concrete digester, for which a multiplier of 1.68 was used (2). The annual investment cost was estimated by multiplying the fixed capital cost by a factor of 0.239, and the labor cost was calculated from the base labor cost ($4.50/hr) by multiplying by a factor of 1.95 (2). The costs of utilities and the costs of raw materials used in the calculation are listed in Table 8.5 and 8.6, respectively.

The resulting manufacturing costs for each of the major processing sections in the case of 90% on stream operation are listed in Table 8.7. The amounts of sugars produced in each case are also shown in the same table. For example, in Case (A) the gross sugar production is 249.8 tons/day, of which 166.5 tons/day of the sugars are used for the growth of the fungus, leaving only 83.3 tons/day of net sugar production. The sugar cost varies from 7.2 ¢/lb to 20 ¢/lb depending upon the operation conditions. The comparison between the costs for Cases (A) and (B) shows the effect of the use of β-glucosidase. The mixed enzyme system provides about 30% less sugar production cost when no enzyme is recovered from the hydrolysis. Yang (2) reported that the sugar cost, which was calculated for the cellulase system with low cell recycle and with enzyme recovery, was 12.4 ¢/lb. This value could be compared with the result for Case (C) in the present work (10.5 ¢/lb). In this case, a 15% reduction was achieved by using the mixed enzyme system, which is reasonable since the higher the enzyme recovery, the lower the effect of adding β-glucosidase to the cellulase system. When higher cell recycle

Table 8.5. Utilities costs.

Item	Unit	Unit cost	Units/hr				
			Case(A)	(B)	(C)	(D)	(E)
Electricity	KWH	0.75 ¢	7592	8482	7838	8267	7410
Steam	1000 lb	0.325 $	15.3	15.3	12.2	8.7	8.2
Cooling water	1000 Gal.	0.15 $	179.4	179.4	143.5	103.2	96.5
Process water	1000 Gal.	0.40 $	61.3	77.1	57.1	44.2	41.5

Table 8.6. Medium raw materials costs.

Component	g/l	$/ton	tons/day				
			Case(A)	(B)	(C)	(D)	(E)
Ammonium sulfate	1.4	90	23.32	23.32	18.49	13.30	12.53
Potassium phosphate	2.0	120	33.31	33.31	26.42	19.01	17.91
Calcium chloride	0.3	33	5.00	5.00	3.96	2.85	2.68
Magnesium sulfate	0.3	110	5.00	5.00	3.96	2.85	2.68
Urea	0.3	160	5.00	5.00	3.96	2.85	2.68
Proflo Oil	0.5	300	8.33	8.33	6.61	4.76	4.49

Table 8.7. Process cost analysis. Unit: 10^3 dollars

	Pretreatment	Hydrolysis	Enzyme recovery	Cellulase make-up	β-glucosidase make-up	Total
Fixed capital costs	4,416	6,204	1,650	A) 21,812 B) 19,818 C) 17,461 D) 14,794 E) 16,462	--- 4,437 3,264 4,009 1,953	A) 32,432 B) 34,875 C) 32,995 D) 31,072 E) 30,684
Annual investment related costs	1,055	1,483	394	A) 5,213 B) 4,737 C) 4,173 D) 3,536 E) 3,934	--- 1,060 780 958 467	A) 7,751 B) 8,335 C) 7,886 D) 7,426 E) 7,334
Annual labor related costs	69.5	139	69.5	139	69.5	A-B) 417 C-E) 486.5
Annual utilities costs	80	189	26	A) 634 B) 552 C) 470 D) 378 E) 325	--- 114 83 91 61	A) 903 B) 935 C) 848 D) 764 E) 709
Annual raw materials costs	---	---	---	A) 3,336 B) 2,836 C) 2,296 D) 1,553 E) 1,553	--- 500 350 350 240	A) 3,336 B) 3,336 C) 2,646 D) 1,903 E) 1,793

Table 8.7. Process cost analysis. (Continued)

	Pretreatment	Hydrolysis	Enzyme recovery	Cellulase make-up	β-glucosidase make-up	Total
Annual manufacturing costs	1,205	1,811	490	A) 9,322 B) 8,264 C) 7,078 D) 5,606 E) 5,979	--- 1,744 1,283 1,468 838	A) 12,407 B) 13,023 C) 11,867 D) 10,580 E) 10,323
Daily manufacturing costs ($)	3,650	5,490	1,480	A) 28,250 B) 25,040 C) 21,450 D) 16,990 E) 18,120	--- 5,280 3,890 4,450 2,540	A) 37,600 B) 39,460 C) 35,960 D) 32,060 E) 31,280

	Case	TGE	Glucose
Sugars produced (tons/day)	A	83.3	55.5
	B	120.8	91.0
	C	155.2	117.0
	D	192.6	145.2
	E	197.9	149.1

Sugar cost (¢/lb)	A) 2.0	3.0		15.4		20.5 (30.8)*
	B) 1.4	2.1		9.4	2.0	14.8 (19.6)
	C) 1.1	1.6	0.43	6.3	1.1	10.5 (13.9)
	D) 0.86	1.3	0.35	4.0	1.0	7.6 (10.1)
	E) 0.83	1.3	0.34	4.2	0.6	7.2 (9.6)

*Figures in the parentheses are the cost of glucose.

was employed and the cell mass concentration in the induction stage was 0.85%, the sugar cost was estimated as 7.2 ¢/lb, 60% of which is attributable to the cellulase production process. Thus, the major research effort should be still placed on the development of more economical cellulase production systems.

It should be emphasized that the preceding design and cost estimates are very preliminary and should not be accepted as conclusive until further data, preferably by pilot plant, can be obtained. The main purpose of these studies has been to demonstrate the possible usefulness of employing a mixed enzyme system containing β-glucosidase in combination with cellulase.

Literature Cited in Chapter 8

1) Mitra, G. and Wilke, C. R., Enzymatic Utilization of Waste Cellulosics, Lawrence Berkeley Laboratory Report 2334-Rev., January 1975

2) Wilke, C. R. and Yang, R. D., Presented at the Eighth Cellulose Conference on Wood Chemicals, Syracuse, New York, May 1975

3) Mitra, G. and Wilke, C. R., Biotech. Bioeng., 17, 1 (1975)

4) Wilke, C. R., Yang, R. D., and von Stockar, U., presented at Symposium on Enzymatic Conversion of Cellulosic Materials: Technology and Applications, Boston, Massachusetts, September 8-10, 1975

5) Cysewski, G. R., Ph.D. thesis to be submitted, University of California, Berkeley, California

Conclusions

We have introduced the use of β-glucosidase in the hydrolysis of cellulose with a cellulase enzyme system in order to achieve higher conversion and higher glucose yield. This attempt has been proved successful by the hydrolysis experiments and the cost analysis of the industrial scale process. The production of β-glucosidase has been investigated also, and the characteristics of the enzyme were studied extensively. The continuous process of β-glucosidase production proposed in the present work is probably the first reported.

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