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STUDY OF THE PRODUCTION OF CELLULASES WITH *Trichoderma reesei* AND INVESTIGATION OF THE ENZYMATIC HYDROLYSIS OF CELLULOSIC SUBSTRATES

by

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ABSTRACT

In recent years the production of fuels and chemicals from plant biomass as an alternative to fossil fuels has received a great deal of attention. Although the enzymatic hydrolysis of lignocellulosics has been proven to be technically feasible on the laboratory scale, the economics of the process are still prohibitive for its implementation on industrial scale systems, partly because of the high cost involved in the preparation of the cellulase complex. In the present work several ways of reducing the cost of producing cellulases with the fungus Trichoderma reesei QM 9414 were examined. The on-site production of the enzymes for subsequent hydrolysis could decrease the cost of the enzymes if they are so produced, but it has to be examined more closely. The utilization of the fresh fermentation broth without removing the residual solids prior to hydrolysis is feasible and it appears that small amounts of the enzyme beta-glucosidase are released by the remaining mycelial cells. The operation of the fermentor at pH 3.0 instead of a higher pH resulted in an increase in the enzyme activity of the broth and allows for the elimination of the condition of sterilization, provided that an adequate aseptic technique is employed. The utilization of the same fermentor vessel to

carry out both operations, i.e., production of the cellulase complex and hydrolysis of cellulose slurries, is feasible for cellulose slurries of up to 4 %; the utilization of higher cellulose concentrations would require an improved design. It was also found that as with the utilization of culture filtrates, the rate of hydrolysis is affected by end-product inhibition, increased resistance of the cellulose to hydrolysis, and enzyme deactivation. The yield of reducing sugars per gram of initial cellulose had a linear relation with the log of the enzyme:cellulose ratio and therefore to achieve high conversions a great deal of enzyme is required.

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Chapter I

INTRODUCTION

The wide fluctuations in oil prices during the 1970's and the realization of a possible depletion of this non-renewable resource has made scientists aware of the need to develop alternative and more reliable sources of energy in order to satisfy the increasing demand for this important commodity. A promising alternative is provided by the utilization of biomass. By the process of photosynthesis solar energy is constantly converted and stored in plant biomass, therefore providing an energy source that can be continuously renewed. Moreover, biomass in the form of agricultural, forest and municipal residues is continuously accumulated throughout the world resulting in a deterioration of the environment and the waste of potential valuable resources. It is estimated that in the United States alone a total of $588X10^6$ dry tons/year of collectible waste biomass is produced, which would have an energy content of $8X10^{15}$ BTU/year(1). These residues, however, must be processed in order to obtain the energy stored in biomass components such as cellulose, hemicellulose and lignin. The biological transformation of these materials into more valuable products may contribute to solve some of the above-mentioned problems with the advantages of helping to preserve our environment and a better utilization and conservation of our resources.

In addition to the production of fuels, the proposed scheme of biomass utilization comprises the production of food and of numerous important chemicals which are at present synthetized from hydrocarbons. It is therefore very possible that in a near future biomass utilization will play an important role in the development of a new technology to replace the one now existent which is based on petroleum.

Of the three main components of plant biomass, cellulose is certainly the most important, accounting for over 50% of all carbon found in the plant kingdom. Cellulose, which is a linear polymer made up of D-glucose units joined by beta-1,4 glucosidic linkages, can be hydrolyzed to yield simple sugars which then can be fermented and transformed into a variety of products ranging from fuels and chemicals to single cell protein. In recent years a great deal of attention has been focussed on the hydrolysis of cellulosic substrates by enzymes of microbial origin known as cellulases. The process has been studied in some detail and has proven to be technically feasible, but it has failed to prove economically attractive or justifiable to be conducted in an industrial scale. One of the major constraints, though not the only factor, is the high cost involved in the enzyme production step; Wilke <u>et</u> <u>al</u>., (2) have estimated that the make-up of cellulases would account for 60% of the cost of the sugar produced by the enzymatic hydrolysis of newsprint. It is therefore imperative to direct efforts towards improving the economics of the process.

1.1 OBJECTIVES

The ultimate objectives of the present research program are as follows

1. To reduce the cost of producing sugars with the enzymatic hydrolysis of cellulose.
2. To reduce the cost of producing the cellulase enzymes.

More specific objectives regarding this thesis were:

1. To increase the yields of cellulase and increase the productivity of the fermentation step.
2. To increase the productivity of the hydrolysis step.
3. To simplify both operations: The production of the cellulase and the hydrolysis of cellulosic substrates.

Chapter II

LITERATURE SURVEY

The enzymatic hydrolysis of cellulose to glucose is a relatively simple process, which can be separated into two main steps: 1) Production of the cellulolytic enzymes, and 2) Hydrolysis of cellulosic substrates (see Figure 1). The enzyme-production step has received a great deal of attention and several commercial forms of cellulase are currently available. However, the cost of these enzymes is still too high for its use in the hydrolysis of cheap cellulosic materials.

In the present section several aspects of both the enzyme production and the hydrolysis steps are reviewed.

2.1 PRODUCTION OF CELLULASES

2.1.1 Microorganisms

Of vital importance to a fermentation process are the microorganisms employed to bring about any specific transformation. This importance arises from the unique capabilities that allow a microorganism to synthesize the very complex substances (i.e., enzymes) necessary for its metabolic functions. This holds true for the conversion of cellulose and similar substrates, where microorganisms produce the enzymes that enable them to break down these relatively complex compounds to simpler molecules (e.g., monosaccharides) which then are used as the carbon source. It is the availability of these enzymes that forms the basis for the biological conversion of starch, cellulose, hemicellulose, etc. Although many microorganisms can digest and grow on cellulose, only a limited number of them produce free enzymes capable of hydrolyzing it *in vitro* (3).

Through the years several microorganisms, mainly fungi and some bacteria, have been isolated and studied for their cellulolytic properties; an extensive list of those is provided in references 3, 4, 5. Some of the wild strains have been subjected to mutagenesis and, in some cases, it resulted in mutants with improved characteristics compared to the parent strains. A notable example is the fungus *Trichoderma reesei* QM 6a; mutant strains of this microorganism have cellulase yields several times greater (e.g. QM 9414, MCG 77, NG 14, etc.)(6) and resistance to catabolite repression (e.g., RUT C30)(7). In addition to cellulases, *Trichoderma reesei* produces other carbohydrases: xylanases, mannases, etc., which are able to hydrolyze the hemicellulose fraction of plant biomass.

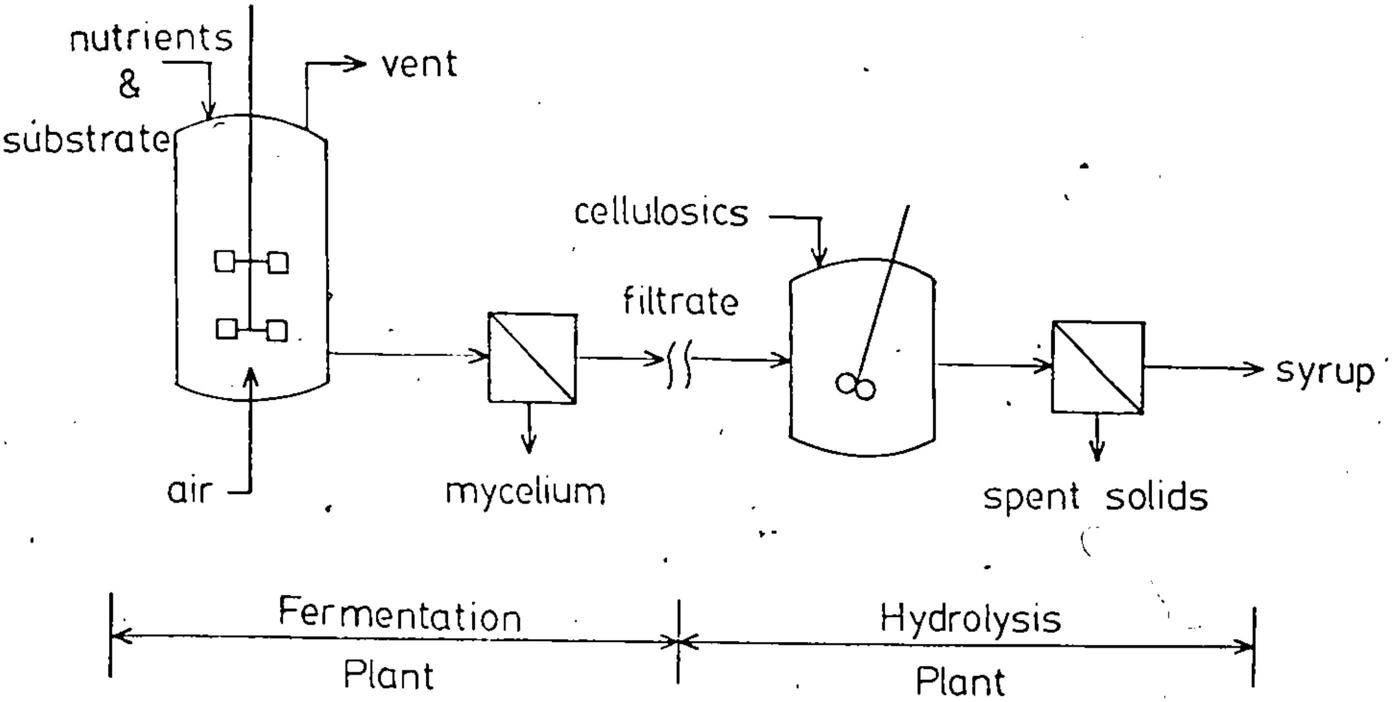


Figure 1: Continuous production of cellulases and subsequent hydrolysis with filtrate.

2.1.2 Cellulase synthesis in fungi

In spite of the improvement seen by using mutants with enhanced cellulase productivity, the yields and the specific activities of cellulases produced by these microorganisms are still low. This is greatly due to the strict genetic controls that constrain enzyme synthesis to the amounts needed by the cell for growth or survival.

The exact mechanism by which cellulases are synthesized is still under study and is a matter of controversy. It is accepted, however, that cellulase synthesis in fungi is controlled by an induction-repression mechanism similar to that observed in bacteria (7). Cellulases are induced by cellulose, cellulose derivatives, cellobiose, sophorose and lactose, and are catabolite-repressed by the presence of glucose, glycerol and other easily assimilable substrates.

Cellulose is recognized as the natural inducer of cellulases. However, since in most inducible systems the inducer must enter the cell so as to interact with the repressor protein to render it inactive thereby allowing enzyme synthesis, it is not understood how a large insoluble molecule could effect regulation of gene expression from outside of the cell. Binder and Ghose (8) showed that close physical contact between the cells and the cellulose is required for cellulase synthesis. Therefore, it is proposed that there could be some recognition sites on the surface of the cell which could activate the synthesis mechanism (7).

A more supported assumption is that very small amounts of the enzyme are produced constitutively and this "basal" level of enzyme must react with the insoluble cellulose, thereby producing a soluble molecule which enters the cell and effects induction. This assumption seems reasonable considering that cellobiose and lactose also act as inducers; and that sophorose, also a disaccharide, is a powerful inductor for some species of Trichoderma and some bacteria (4). This could suggest that once inside the cell these sugars are modified so as to provide the true inducer.

Growth is faster when cells are grown on glucose or glycerol, but enzyme synthesis is repressed and only very small amounts of extracellular enzymes can be detected (9). In bacteria, catabolite repression occurs through a mechanism involving the level of cyclic Adenine Monophosphate (cAMP). The role of the cAMP is to bind with a catabolite gene activator protein, which is inactive in the absence of cAMP, to form an active complex that interacts with the structural gene(s) to start the process of transcription. When cells are grown on readily assimilable substrates, the metabolic activity is high and the level of cAMP is low, thus reducing or halting enzyme synthesis. On the other hand if the metabolic activity is low the level of available cAMP increases. In the case of fungi, however, the process occurs in a somewhat different way. Montenecourt et al., (7) have shown that there are no dramatic changes in the level of cAMP during enzyme synthesis; also Mandels (9) has shown that the addition of cAMP to cultures growing on glucose did not have any effect on enzyme synthesis. Therefore, it seems that there is no relation between cAMP and enzyme (cellulase) synthesis in fungi; this suggests that catabolite repression occurs at another level of the process of gene expression (i.e., translation).

The synthesis of cellulases seems to be subjected to yet another mechanism of control. True cellulolytic microorganisms produce extracellular enzymes; these enzymes are synthesized either in the cytoplasm or in the endoplasmic reticulum and must be excreted to the external environment. Little is known about the mechanisms of protein release in fungi, but recently it has been postulated that enzyme release must occur by a specific active transport mechanism. This mechanism could be regulated by intracellular acid proteases, and may be affected by factors such as pH (5,10). The process of enzyme synthesis is summarized in Figure 2 .

A more profound knowledge about the synthesis of cellulases (i.e., when and where synthesis occurs) and about the mechanisms involved in enzyme release will have an impact in the approach to the production of these enzymes. Ross (10) supports that cellulases are formed in the early stages of the fermentation and that are slowly released during the stationary and post-stationary phases of growth. More in-

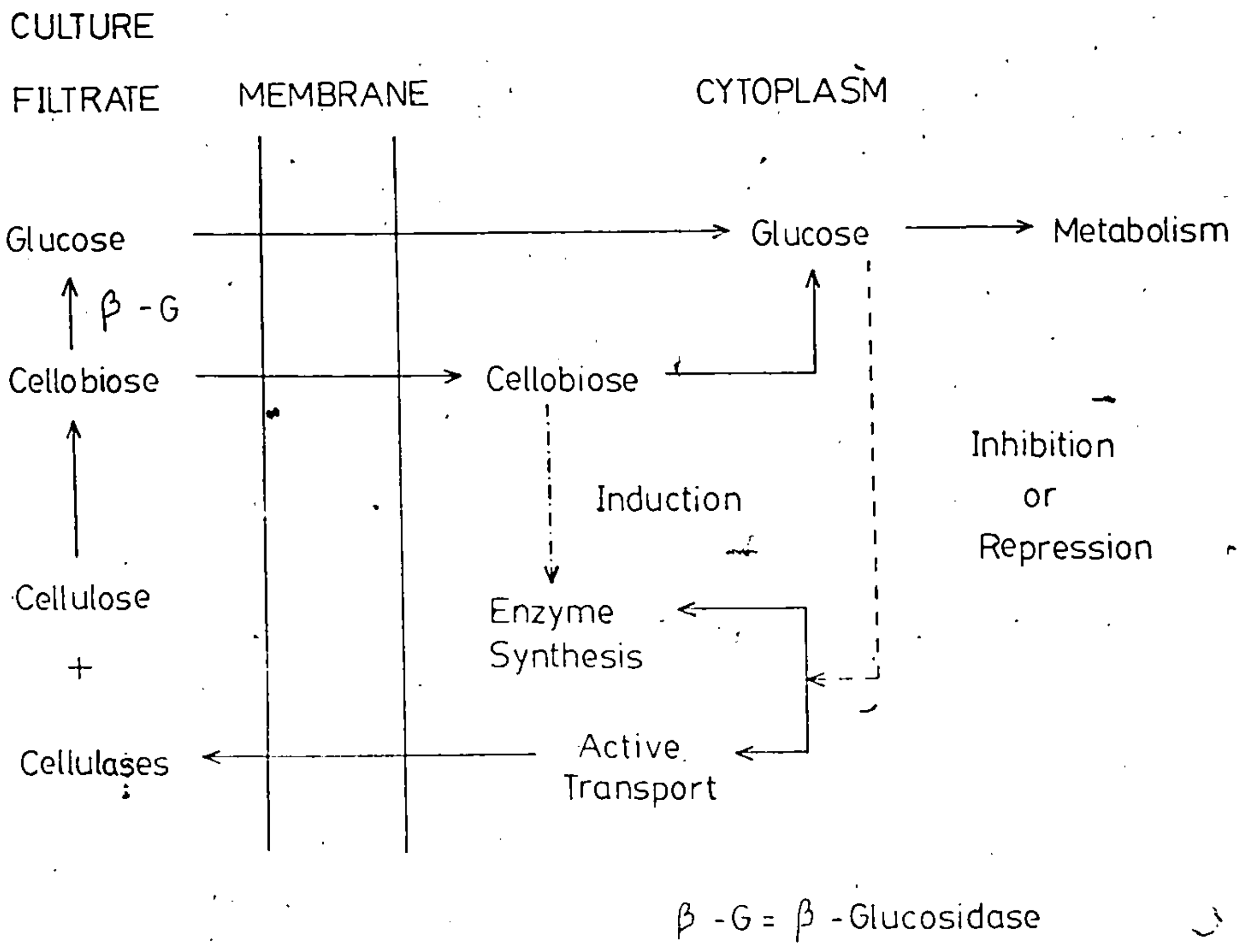


Figure 2: Model for the production and excretion of cellulases (10).

formation regarding both questions: when cellulase is synthesized and how is released from the cells, is required since both factors are very important if one is to maximize enzyme productivity.

2.1.3 Fermentation

Over the years improvement of enzyme yields has been achieved and has been the result of both development of new mutant strains and improvement of fermentation techniques. The production and yield of cellulases are affected by factors others than the type of microorganism and those governing the synthesis of enzyme. The effect of several operating variables (e.g. media composition, pH, temperature, etc.) has been studied in hopes of increasing productivity; most of these studies have used various strains of <u>Trichoderma reesei</u> and one must keep in mind that different microorganisms have different responses to these variables.

The highest enzyme yields are obtained in a batch process using high cellulose concentrations, but higher productivities are obtained in continuous operation (9). Recent reports indicate that a fed-batch approach may be more convenient since the slow feeding of cellulose results in higher yields: higher productivities are attained although the fermentation is prolonged (11).

2.1.3.1 Effect of substrate and additives

T. reesei grows rapidly on a salt medium containing minerals and supplemented with a carbon source; growth factors, such as vitamins, are not required (12). It has been already mentioned that the source of carbon provided for growth has an effect in the synthesis of enzyme. While highest growth rates are obtained for glucose and some other simple sugars almost no enzyme is produced. Growth on lactose and cellobiose induces synthesis of cellulases, but enzyme yields are still much lower that those obtained when the microorganism is grown on cellulose-containing media. Mandels (9) reported filter paper activities of 0.25 and 0.31 for T. reesei QM 9414 grown on 1 % lactose and 1 % cellobiose respectively while filter paper activities for cultures on 1 % cellulose were about 1.3 IU/ml. Commercial forms of purified cellulose such as Solka floc and Avicel are usually employed in laboratory experiments for enzyme production, but given their cost they would not be suitable for practical purposes. Several cellulosic materials such as newsprint (2,9), barley straw (14), steam exploded wood (14), and feed lot waste (15) have been tested as substrates for growth and enzyme production. Their utilization is feasible, they are cheap and abundant, but require some form of pretreatment. Cell and enzyme yields are comparable to those obtained with pure cellulose. The utilization of highly resistant materials such as cotton results in higher enzyme activities; however, since growth is slow the fermentation time is increased, resulting in low productivities (4,16).

In addition to the type of carbon source used, substrate concentration also has an effect on enzyme yield. The cellulase yield increases with the initial cellulose concentration up to a value of 6 % (17), slurries of higher cellulose concentrations are difficult to stir and create problems with oxygen transfer (4). On the other hand, higher cellulose concentrations increase the lag period for enzyme formation (13). As cellulose is consumed, ammonia is also consumed and the pH tends to go down. With the utilization of high cellulose concentrations (higher than 1 %), the acidic conditions in the reactor have a deleterious effect on both growth and enzyme yields and therefore control of pH is required (18).

When T. reesei is grown on glucose, in which case very little extracellular protein is produced, biomass yields are between 0.4-0.5 g/g glucose (4,9); similar values for various strains of this microorganism grown on cellulosic materials are reported (10,13). However, when cellulose is used as the main substrate, a lag in growth is observed, and thus it is a common practice to add a low level of a soluble carbon source to stimulate growth during the initial stages of the fermentation. Proteose peptone or other protein derivatives in concentrations of 0.1-0.2 % are preferred to glucose or glycerol (3).

Reese and Maguire (19) studied the effect of surfactants on the production of enzyme by several microorganisms; they observed that the addition of low concentrations of surfactant stimulated the release of the enzyme into the medium. They also postulated that the surfactant acts at the cell membrane by increasing its permeability thereby allowing a more rapid secretion of the enzymes. Therefore, Tween 80 is commonly added to the growth medium to enhance enzyme production.

2.1.3.2 Effect of temperature

T. reesei is a mesophilic organism and therefore grows in the range of 25-35 °C. Optimum temperatures for growth may not correspond to the optimum for enzyme production. For example, with T. reesei QM 9414 optimum temperatures for growth and enzyme production of 31 and 28 °C respectively have been identified (20).

2.1.3.3 Effect of pH

In fermentations of high cellulose concentrations by Trichoderma reesei with no pH control, the pH goes down to values as low as 2.4 due to CO_2 production and depletion of ammonium ions; at this value growth is almost nil and so is enzyme synthesis. Maximal growth rates are observed when pH is controlled at around 4.5, but a different optimum for enzyme production (around 3.0) is observed (18,20). The pH can be programmed so as to increase the productivity by conducting the fermentation at the optimum pH for growth during the exponential growth phase; after this period the pH can be adjusted to the optimum value for enzyme production (21). The operation of the fermentor at a lower pH may be convenient not only in terms of enzyme production, but also to prevent bacterial contamination.

2.1.3.4 Effect of aeration rate

The *Trichoderma sp.*, being an aerobic organism, requires oxygen for its metabolism. Nystrom *et al.*, (22) report that aeration rate has no significant effect on enzyme production as long as the dissolved oxygen concentration is maintained above 10 % of the saturation; concentrations below this value have a negative effect on both growth and enzyme production.

2.1.3.5 Effect of mixing rate

The mixing rate has a definite effect on mass transfer and therefore affects the dissolved oxygen concentration. Peitersen (13) showed that higher cell and enzyme yields were obtained by increasing the mixing rate from 200 to 350 rpm, while keeping the aeration rate constant. He explained these higher yields in terms of increased oxygen transfer coefficients and because of elimination of eventual diffusion controlled zones. It would therefore appear that this is in opposition to Nystrom's findings that aeration rate had no effect on enzyme production as long as the dissolved oxygen concentration was maintained above 10 % of saturation. However, it could be possible that this increase was due in a greater extent to the elimination of high local concentrations of sugar, which would cause enzyme inhibition and/or repression than to increased oxygen transfer coefficients.

2.1.3.6 Effect of initial biomass (cell) concentration

Cultures can be started with spores or active cells (mycelia). Although there is no difference in enzyme yields with either type of inoculum, the latter results in shorter fermentation times because of faster growth and earlier enzyme development (3). Therefore the mycelial inoculum is more convenient to start growth in a fermentor.

Cell and enzyme yields are independent of the initial cell concentration, but the larger the inoculum the shorter the initial lag period (16,22).

2.1.3.7 Effect of time

Different phases of growth and enzyme production can be distinguished during the course of a batch fermentation.

In a typical fermentation of Trichoderma reesei, after inoculation, a lag is observed; values from 10 hours (16) to 3 days (9) are found in the literature, the lag period depends in operating conditions and type and size of the inoculum. This is followed by a period of rapid growth during which the pH and the dissolved oxygen concentration decrease (16,22)..As the substrate is depleted, the growth rate eventually decreases and a maximum in biomass concentration is reached at about 60 hours to 6 days (16, 9). Then the cells undergo lysis and biomass concentration slowly falls down (16). pH rises, probably as a result of the secretion of ammonia by the fungus and decrease in the partial pressure of CO2 in the fermentor (17).

The production of cellulases is lagging 1 or 2 days behind growth; by the time the maximum in biomass has been achieved, only about 20 % of the cellulase has been released into the medium (17). Cellulase starts accumulating rapidly after the maximum in biomass occurs and continues through the period of cell lysis. The rate of release slows down and the concentration of enzyme finally levels off at about 7 to 10 days (9, 17).

According to Sternberg (18) there is a lag between the appearance of beta-glucosidase and the other components of the cellulase complex, the former being released one to two days later.

2.2 THE CELLULASE SYSTEM

Several good reviews on cellulase characterization, purification, and properties are available in the literature (4,5,6,23).

Cellulase consists of a complex of different enzymes interacting to hydrolyze native cellulose. At least three different enzymes are required for the complete hydrolysis of cellulose.

1. Beta (1-4)glucan-glucanohydrolase or endoglucanase. This enzyme acts by cleaving beta-1,4 glucosidic bonds at "random" on the cellulose chain. The products are glucose, cellobiose, cellotriose and other soluble cellodextrins. Endoglucanases alone exhibit a high activity towards acid swollen cellulose, but very limited action on crystalline cellulose. In addition they are inhibited by the intermediate product cellobiose (5).
2. Beta (1-4)glucan-cellobiohydrolase or exoglucanase. Exoglucanase starts the attack at the non-reducing end of the chain, and acts by removing cellobiose units. Small amounts of glucose are produced from odd-numbered cellodextrins. Exocellulases are inhibited by cellobiose and to a lesser extent by glucose (4,5). Wood (24) has suggested that there is an exoglucanase which removes glucose units instead of cellobiose.

3. Beta glucosidase or cellobiase. This enzyme acts by splitting the beta-glucosidic bonds of dimers to yield two monomer units (glucose). Cellobiase has no action on cellulose, but plays an important role in the removal of the inhibitory product cellobiose. It is itself inhibited by glucose.

Endo- and exo-glucanases are said to act synergistically to hydrolyze native cellulose (25). This means that the combined effect of both enzymes is greater than the individual effect of each enzyme acting by itself. According to a mechanism proposed by Erickson (6) the endoglucanases attack regions of low crystallinity along the cellulose fibre and thus create non-reducing ends, on which exoglucanases start the degradation of the residual chains by removing cellobiose. This mechanism is depicted in Figure 3 .

Many microorganisms secrete endoglucanases, but only a few produce high levels of exoglucanases. On the other hand, beta glucosidase is not considered to be a cellulase and is produced by several microorganisms which are not necessarily cellulase producers. At present, <u>Trichoderma</u> <u>reesei</u> is considered one of the best prospects in cellulase production, since it produces an enzyme complex containing high levels of exo- and endo-glucanases; however it produces low levels of beta-glucosidases.

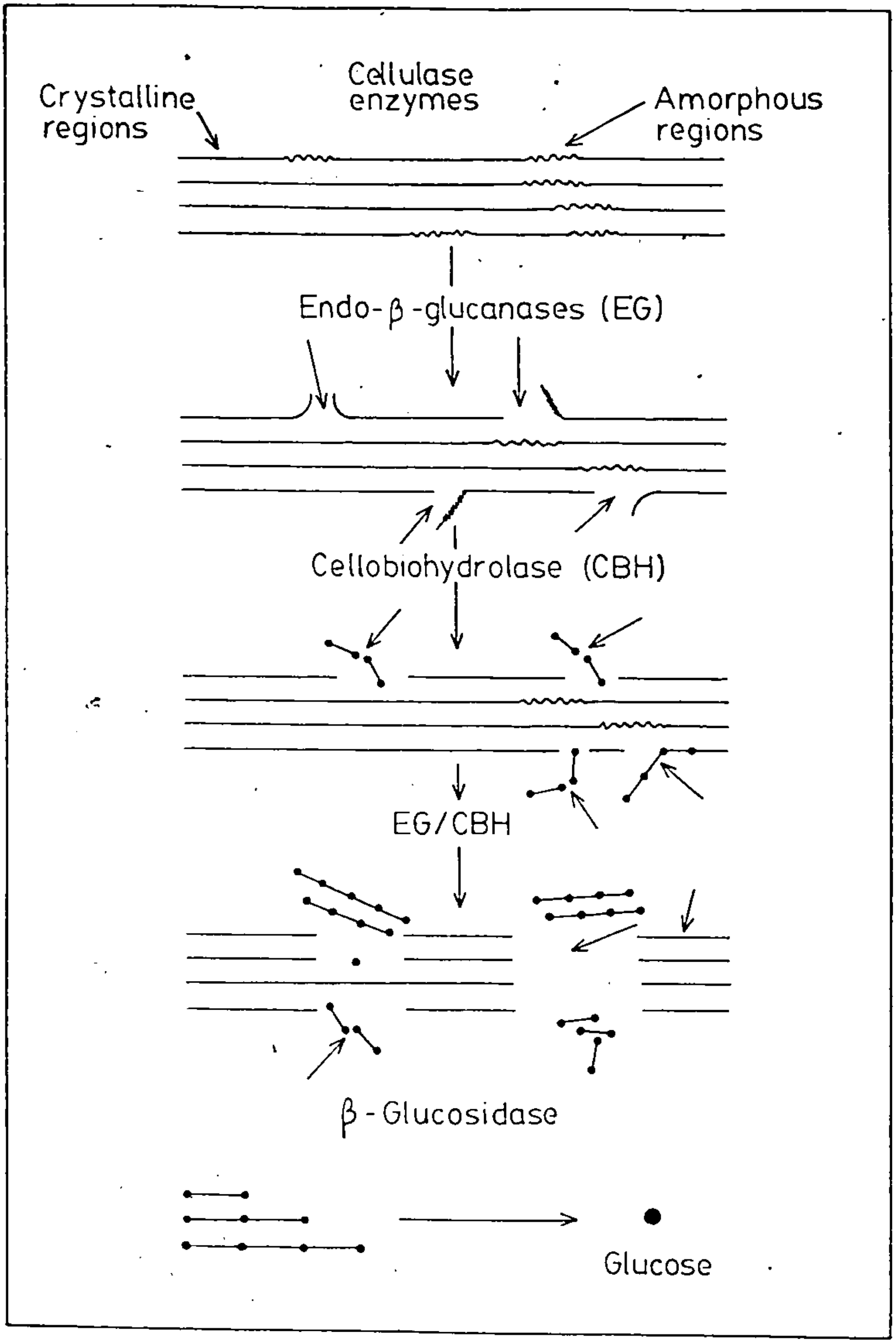


Figure 3: Schematic representation of synergistic action of enzymes in cellulose hydrolysis (6)

2.3 ENZYMATIC HYDROLYSIS OF CELLULOSIC SUBSTRATES

While enzyme cost is one of the main constraints towards the commercial application of the enzymatic hydrolysis of cellulosic substrates for the production of sugars, there are several aspects of the hydrolysis itself such as enzyme inhibition, stability of the enzyme, substrate pretreatment, reactor design, etc., which need to be addressed, since further developments in these areas may contribute to decrease the production cost of the sugars.

2.3.1 Substrates for hydrolysis

Cellulose is the most abundant carbohydrate on earth. It does not, however, occur in pure form in nature; it is found, associated with hemicellulose and lignin, as a major structural component of plant biomass. As can be seen in Table 1, most agricultural and forest residuals contain varying amounts of hemicellulose and lignin.

The combination of lignin with partially crystalline cellulose, as in most woods, results in a material which is highly resistant to chemical or microbial degradation. Whether the association between lignin and cellulose is of a physical nature or involves chemical bonding between lignin and cellulose is not clear; however, it is known that lignin physically prevents the accessibility of cellulose to the enzymes (26). Therefore, to achieve an efficient degradation, cellulosic materials need to be at least partially delignified.

TABLE 1

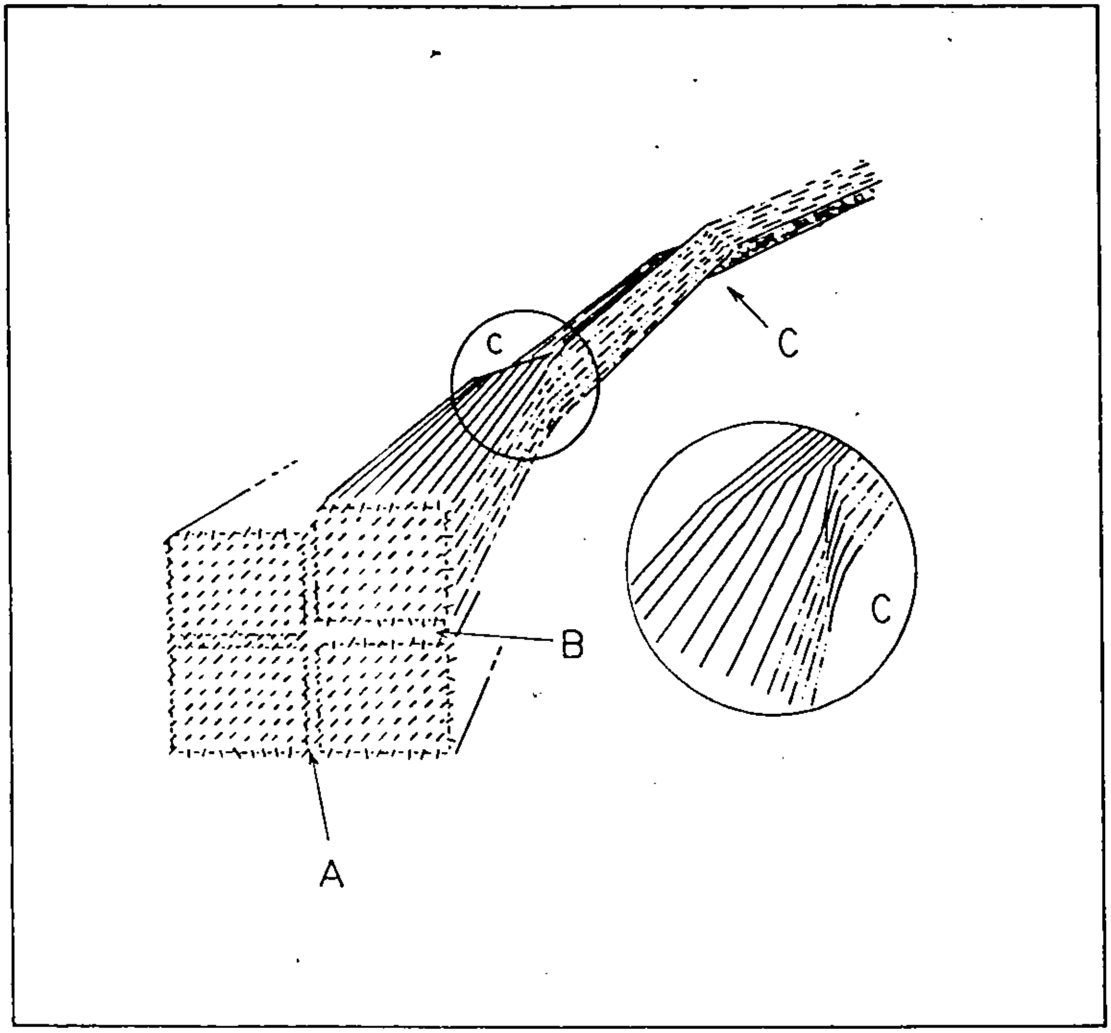
Composition of some agricultural and forest materials

Material	Weight %, dry basis Hemicellulose	Cellulose	Lignin
Spruce	26.5	42.0	28.6
Pine	23.5	42.0	27.8
Birch	37.7	38.8	19.5
Balsam Fir	24.4	42.7	28.0
Aspen	28.7	50.8	15.5
Red Maple	33.0	39.0	23.0
Wheat Straw	27.6	34.0	18.0
Cotton Straw	12.0	42.0	15.0
Rye Grass	27.0	37.0	5.0
Cornstalk	32.6	33.5	11.0
Corncob	28.0	36.5	-.-
Bagasse	34.0	38.0	11.0
Reed Cannary grass	16.5	28.0	5.3
Orchard grass	14.4	30.0	4.7
Brome grass	15.3	27.0	5.5
Tall fescue	24.5	30.0	3.1
Reed foxtail	17.7	29.0	4.5

*From ref. 23

The accessibility of cellulose to extracellular enzymes is also a function of its physical structure. A cellulose fibre may be envisioned as a cluster of linear polymer molecules bound laterally by hydrogen bonds, and having varying degrees of order (see Figure 4). The central core of a microfibril, called a crystallite, is composed of highly ordered molecules closely packed against each other, while those surrounding it, characterized by a lesser degree of order, are called paracrystalline or amorphous (26).

Some structural features of cellulose such as crystallinity and specific surface area (surface area/g of substrate) are known to have an effect on the rate and extent of hydrolysis. Lee et al. (27) have shown that as the reaction proceeds the crystallinity index increases, while the specific surface area decreases. They explained that these changes occur because the amorphous portion is hydrolyzed first, leaving behind a more crystalline residue. Crystalline cellulose is less susceptible to enzymatic attack because the large enzyme molecules are unable to penetrate the compact, highly ordered chains of the fibril, so that the reaction becomes limited to the surface of the crystallite. They also showed that modifications in the structure of cellulose (e.g., reduction in the crystallinity index and increased specific surface area) lead to greater conversions and different patterns of hydrolysis.



A Coalesced surfaces of high order

B Readily accessible surfaces slightly disordered.

C Readily accessible surfaces of strain distorted tilt and twist regions.

Figure 4: Schematic representation of the structure of a cellulose fibril (25).

Although purified forms of cellulose are usually employed in most hydrolysis studies, a number of forest, municipal and agricultural residues have been tested as substrates for hydrolysis (14, 28, 29, 30). Conversions as high as 50 % have reported for such materials as waste paper, cellulosic materials from municipal trash, or high cellulose industrial and agricultural residues (28,30). Their potential utilization is a function of their availability and of the degree of pretreatment required (28). Most agricultural residues have low bulk densities and are produced in relatively small areas; therefore, they would be more suitable for on-site processing in small industrial- or farm-scale operations (29).

2.3.2 Pretreatment

As previously mentioned, the presence of lignin and crystallinity are two major obstacles for the hydrolysis of cellulose. The need for some form of pretreatment, is therefore evident for increasing the accessibility of cellulose by breaking the lignin seal and disrupting the crystalline structure of cellulose, so as to improve cellulose conversion.

A number of pretreatment methods which make the glucosidic bonds in cellulose more accessible to enzymatic attack, have been developed in hopes of enhancing the rate and extent of hydrolysis of pure and native celluloses (31,32). These include:

1. Physical methods (e.g., grinding, milling, shearing)
2. Chemical and solvent pretreatments (e.g., NaOH, dilute acids, cadoxen, etc.)
3. Thermal treatments with steam followed by rapid pressure release.
4. Radiation
5. Microbial pretreatments (e.g., white rot fungi)
6. Combination of the above

Extensive ball milling or treatment with cellulose solvents (e.g., $H3 PO4$) greatly increases the extent of conversion of pure cellulose (27). However, a major consideration in selecting a method of pretreatment is the cost of energy involved in physical or thermal methods, and the cost of chemicals and of chemical recovery in chemical pretreatments (23).

2.3.3 Enzyme to cellulose ratio

The ratio of the enzyme to the initial substrate concentration[1] (FPA/Co) strongly influences the reaction rate and per cent conversion. Both factors increase with the concentration of enzyme; however, for the enzymatic hydrolysis of cellulose the per cent conversion is a function of the logarithm of the ration of enzyme to initial substrate concentration (FPA/Co). Therefore, if high conversions are de-

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[1] The symbol (FPA/Co) will be used throughout this work to represent the ratio of the enzyme activity (Filter paper activity) to the initial concentration of substrate, its units are IU/g

sired large amounts of enzyme must be employed. The amount of enzyme required to achieve a given per cent conversion is a function of factors such as the extent of pretreatment and susceptibility of the cellulose to hydrolysis. Mandels (4) reports that to achieve a 40-50 % conversion of a 10-30 % slurry in 24-48 hours requires about 10 filter paper cellulase units per gram of a susceptible substrate (e.g., Solka floc BW-200) while about 20 filter paper units per gram of a more resistant substrate (e.g., Avicel) are required to achieve the same percent conversion.

Fan and Lee (33) found that the reaction rate and the amount of sugars produced by hydrolysis did not increase significantly beyond certain enzyme concentration level (FPA/Co = 13 IU/ml). They concluded that this fact was due to the saturation of the enzymes on the available surface of cellulose particles or due to the lack of active sites in the cellulose particles where hydrolysis takes place.

2.3.4 Enzyme Inhibition

Changes in the structural properties of cellulose are claimed to be at least partially responsible for the decrease in the reaction rate observed during the course of the hydrolysis (27, 33). Other important factors in explaining this behavior are enzyme inhibition and enzyme deactivation. While it is well established that the presence of the products of hydrolysis has an inhibitory effect on the enzyme system (34), the nature of this inhibition has to be yet clarified. Because of the complexities of both the substrate and the enzyme system, as well as the interactions among the individual components of the cellulase system, elucidating the mechanism(s) of inhibition is a difficult task. This is reflected in the disagreement existing in the literature as to the nature of the inhibition. Howell and others (33,35) claim that non-competitive inhibition by cellobiose dominates the reaction kinetics, whereas some other authors (23,36) propose that cellulases are competitively inhibited by cellobiose, glucose inhibition being generally weak. A similar situation is encountered for beta-glucosidase; some authors support that beta-glucosidase is competitively inhibited by glucose (33,37), while others assume it to be non-competitively inhibited (23).

Nevertheless, it has been determined that cellobiose is a much stronger inhibitor of the glucanases than glucose; on the other hand, glucose inhibits beta-glucosidase, causing cellobiose to accumulate, which in turn inhibits the cellulase system (35,36).

Several approaches to overcome the problem of enzyme inhibition have been suggested. One of such approaches is the addition of beta-glucosidase to the reaction mixture, so as to increase the rate of cellobiose hydrolysis and therefore effecting its removal (4). As pointed out before, glucose is less inhibitory to cellulases but high glucose concentrations may finally inhibit the whole system. Other approaches are based on the transformation or removal of glucose as it is formed. Woodward _et_ _al_. (37) explored the possibility of transforming glucose to a glucose isomer which could be less inhibitory to beta-glucosidase. They concluded, that in principle, by using glucose isomerase it could be possible to transform glucose to fructose, which is also a fermentable sugar and seems to be a poor inhibitor for beta-glucosidase. Another approach is to combine the cellulose hydrolysis with fermentation. In this approach, as glucose is produced from hydrolysis, it is utilized by yeast or another fermentative organism to produce ethanol or other chemical (38). A major problem with this approach is the incompatibility of the optimum temperatures for hydrolysis (ca. 50 °C) and fermentation (ca. 28 °C for yeast), besides the fact that ethanol, although to a lesser degree, also inhibits the cellulases (38).

2.3.5 Enzyme deactivation

Possible causes for enzyme inactivation are heat, shear or simply age. The rate of enzyme-catalyzed reactions, as for other chemical reactions, generally increases with temperature but because of its proteinic nature, enzymes are heat-sensitive and upon heating become increasingly denatured, losing their characteristic biological activity. Therefore the optimum temperature is determined by the increase in the reaction rate with temperature and the increasing rate of thermal deactivation beyond certain critical point (39). Optimum conditions for Trichoderma reesei cellulases are about 4.8 for the pH and 50 °C for the temperature (4).

Shear stress and the exposition to an air-liquid interface seem to affect enzyme stability. Mandels and Reese (40) compared the stability of cellulases produced by two strains of T. reesei and found that in both cases a greater extent of hydrolysis and greater residual activities were obtained under unshaken conditions. Later, Reese et al. (41) found that the combined effect of shear and interfacial tension on cellulase deactivation was more extensive than that of shear alone. They also found that increasing the concentration of enzyme or the addition of surfactants reduced the degree of deactivation. Presumably the stabilizing effect of surfactants was due to displacement of the enzyme molecules from the surface where they are deactivated.

2.4 ALTERNATIVES PROPOSED

Searching to fullfil the objectives stated in the preceding chapter the following concepts or alternatives were considered:

1. On-site production of the enzymes for subsequent hydrolysis of cellulosic substrates. The implementation of this concept would result in a decrease of the cost of the enzymes since the cost of concentrat-

ing the enzyme, storage and transportation could be avoided. In addition the quality of the enzymes could be improved since the enzymes so produced would be "fresher" or more active.

2. Utilization of the crude fermentation broth without removing the remaining mycelial cells prior to the hydrolysis step (see Figure 1) and study of the possible implications of the presence of the residual cells in the hydrolysis mixture. Elimination of the filtration step to remove the residual solids prior to the hydrolysis step would decrease the cost of the enzymes and since handling of the enzymes is minimized the risk of undesired contamination would be reduced.
3. Operation of the fermentor under acidic conditions (e.g., $pH=3.0$) so as to remove the condition of strict asepsis (sterilization) usually required at higher pH.[2]
4. Use of the same reactor vessel to carry out both steps: fermentation and hydrolysis. The implementation of this concept would be convenient for small-scale processes (e.g., farm-level operations, village-level technologies for developing countries,

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[2] The production of acetic acid and alcoholic beverages is not necessarily carried out under strictly sterile conditions. Such fermentations are free of contamination because of the low pH, which prevents rapid bacterial growth.

etc.) because the capital cost for a hydrolysis plant, using agricultural residues or municipal wastes, could be reduced if only one large piece of equipment were required.

Chapter III

METHODOLOGY

3.1 MICROORGANISM

The organism used in this study was Trichoderma reesei QM 9414 (formerly T. viride) and originally obtained from the American Type Culture Collection (ATCC # 26921). Stock cultures were prepared by inoculation of potato dextrose agar slants which were incubated at room temperature for about 15-20 days, when they showed good sporulation. After incubation, the slants were sealed with paraffin film and kept at 4 °C. Transfers were made approximately every six months.

3.2 INOCULUM

A spore suspension, obtained by suspending the spores from one of the slants into 3 ml of 0.1 % (v/v) Tween 80 solution, was used to prepare the inoculum. The growth medium was based on the standard salts medium developed by Mandels and Reese (12); however, the level of (NH4)2 SO4 was increased to replace urea and the concentration of CaCl2 and Mg SO4.7H2O were decreased from 0.3 g/l to 0.1 g/l in both cases to prevent precipitation of inorganic salts during autoclaving. Lactose (1%) was used as the carbon source. Approximately 125 ml of this medium were dispensed into 500-ml

Erlenmeyer flasks and then sterilized for 20 minutes at 121 °C and 15 psi. After cooling, each flask was inoculated with 1 ml of the spore suspension and incubated in an orbital shaker at 28 °C and 200 rpm. The fermentor was inoculated with 250 ml of the 4-day old mycelial culture.

3.3 FERMENTATION PROCEDURE

The fungus was cultured in a 5-litre stirred-tank fermentor (New Brunswick Scientific Inc., Edison, N.J., Model MF-105) equipped with pH controller unit (NBS, Model pH 22). The vessel contained 3.5 litres of medium. Air was sparged at a rate of 3 to 7 l/min and 2 standard turbine impellers (5 cm in diameter) were used for agitating the mycelial broth at 400 and 500 rpm, and a third impeller was located in the head space to aid in the control of foam. Foam was also controlled by the addition of poly (propylene glycol) (MW=2000, Aldrich chemicals). In the first 6 runs antifoam was added automatically using a built-in foam controller. Short circuiting of the foam probe by wall growth resulted in large additions of antifoam, manual control was used in all other experiments. The apparent dissolved oxygen concentration was continuously monitored using a Dissolved Oxygen electrode (NBS, 900 series) and was maintained above 20% of the saturation concentration. At the beginning of the fermentation runs the pH of the medium (which was 5.0 to 5.5 after sterilization) was allowed to go down to the desired value (i.e., 3.0 or 4.0) and then automatically controlled at that value throughout the fermentation by the addition of 4N solutions of NaOH or H_2SO_4. The temperature during the fermentation was maintained at 28 °C.

The fermentation medium was based on the modified salts medium of Mandels and Reese (see Table 3), supplemented with 0.1 % proteose peptone and 0.1 % Tween 80. Solka floc SW-40, a commercially hammer-milled cellulose prepared from purified wood pulp (Brown Co, Berlin, N.H.), was used as the carbon source. Runs were carried out with initial cellulose concentrations of 10 and 20 g/l. When the concentration of 20 g/l was used the concentration of salts and nutrients was doubled. All the chemicals used to prepare the medium were of reagent grade. The medium containing the cellulose, salts and nutrients was placed in the fermentor vessel which was then sealed and sterilized for 90 minutes at 121 °C and 15 psi. Details of the operating conditions for 15 fermentation runs are given in Table 2 .

Barley straw, obtained from Forintek Canada Corp. (Ottawa), was also used as a substrate for fermentation. The barley straw was ground in a dry form in a Wiley laboratory mill (Arthur H. Thomas, Co. Phil., PA) to reduce the particle size to pass through a 2-mm mesh screen and was then pretreated using the following procedure. The ground barley straw was treated with 10 parts of 1% NaOH. The mixture was boiled for 2 hours, filtered through a clean cloth, and the

TABLE 3

Composition of growth media for production of cellulases

* Based on the medium developed by Mandels and Reese (12)

COMPONENT	CONCENTRATION
MINERALS	
$(NH_4)_2 SO_4$	2.06 g/l
$KH_2 PO_4$	2.0 g/l
$Ca Cl_2$	0.1 g/l
$Mg SO_4$	0.1 g/l
TRACE ELEMENTS	
$Fe SO_4.7H_2 O$	5.00 mg/l
$Mn SO_4.H_2 O$	1.56 mg/l
$Zn SO_4.7H_2 O$	1.40 mg/l
$Co SO_4.7H_2 O$	4.30 mg/l
ADDITIVES	
Proteose peptone	0.1 %
Tween 80	0.1 %
CARBON SOURCE	
Cellulose	10.0 g/l

When 20 g/l of cellulose were employed, the concentration of minerals, trace elements and additives were doubled.

TABLE 2

Operating conditions for fermentation runs

Run	pH Initial	pH set-point	Sparging rate	Sustrate concent.	Antifoam addition
1	5.5	4.0	N.R.	1% (SW-40)	A
2	5.2	4.0	N.R.	1% (SW-40)	A
3	5.0	3.0	N.R.	1% (SW-40)	A
4	N.R.	3.0	7.0	1% (SW-40)	A
5	5.0	3.0	2.0	1% (SW-40)	A
6	4.8	3.0	3.0	2% (SW-40)	A
7	4.8	3.0	3.0	2% (SW-40)	M (7.5 ml)
8	4.9	3.0	3.0	2% (SW-40)	M (9.0 ml)
9	4.9	3.0	3.0	2% (SW-40)	M (10 ml)
10	(4.5)	3.0	3.0	2% (SW-40)	M (9.0 ml)
11	4.9	3.0	3.0	2% (SW-40)	M (7.0 ml)
12	(5.9)	3.0	3.0	1.7% (B.S.)	M (2.0 ml)
13	(5.8)	3.0	3.0	2% (B.S.)	M (8.0 ml)
14	(5.4)	3.0	3.0	2% (B.S.)	A(35.0 ml)
15	(5.25)	3.0	3.0	2% (B.S.)	M (6.0 ml)

(pH) = after inoculation

A = Automatic addition

M = Manual addition

(N.R) = No recorded

SW-40 = Solka floc SW-40

B.S. = Na OH pretreated Barley straw

liquid was discarded. Further extraction of lignin-derivatives was done by adding acidified water (pH 3.0) to the solids to make up the initial volume and the whole mixture was left standing at room temperature for 1/2 hour. The solids were filtered and the extraction step was repeated once. After this, the solids were washed with another two volumes of acidified water, filtered and pressed to eliminate excess liquid. The solids were dispersed on a shallow container and dried for 24-48 hours at 80 °C. After cooling, the solids were placed in a plastic bag and refrigerated until used. A sample was taken to determine moisture content. In some cases drying was omitted, in which case the solids were further washed until the pH was about 5.5, the slurries thus obtained were named barley straw "wet form", whereas the dried solids were named barley straw "dry form". Only the "dry form" was employed for fermentations.

Fermentations of barley straw (runs # 12 and 13) were conducted under aseptic but not sterile conditions. In these cases, the empty fermentor vessel was steamed in the autoclave at atmospheric pressure (100-106 °C) for 30 minutes. A measured amount of the pre-treated barley straw was placed in the fermentor vessel. The medium containing the salts and nutrients was boiled for 10 minutes cooled, and added to the fermentor. For all other cases, barley straw and the salts medium were placed in the fermentor vessel and sterilized for 90 minutes at 121 °C and 15 psi.

During the fermentations, samples were taken at regular intervals. In some occasions the agitation rate was increased for few minutes so as to re-suspend solids that had settled at the bottom of the vessel. Each sample was filtered, the solid cake was dried at 90 °C, and the clear filtrates kept refrigerated (4 °C) for further analysis. See section 3.5 for details on analytical procedures.

3.4 HYDROLYSIS PROCEDURE

The main substrates used for hydrolysis were Solka floc SW-40 and pre-treated barley straw; other substrates included cellobiose and the spent solids obtained from a prior cellulose hydrolysis.

Unless otherwise indicated, hydrolysis experiments were carried out in shake flasks. Hydrolysis experiments were also carried out in the fermentor vessel. In the latter case, an amount of cellulose, measured to yield the desired concentration (4 or 7 %), was sterilized and added to the raw enzyme preparation (not filtered) remaining from a prior fermentation. The pH was controlled at 4.8 by the addition of 4N NaOH or 4N H2SO4 and the temperature was maintained at 50 °C. The reaction volume was approximately 2.5 litres and mixing rate about 400 rpm.

The conditions for hydrolysis of cellulose in the shake flasks were varied according to the effect under study. The control experiments consisted of preparing a cellulose slurry by mixing 7.0 g of cellulose with 50 ml of 0.1 M citrate buffer (pH 5.1); the flasks were preincubated for 1/2 hour prior to the addition of 50 ml of the enzyme preparation (raw fermentation broth). The reaction was carried out in 250-ml flasks with a reaction volume of 100 ml. The flasks were incubated at 50 °C with constant rotatory shaking at 200 rpm.

To investigate if the presence of the mycelial cells had any effect on the hydrolysis, some experiments were carried out using culture filtrates instead of whole fermentation broth (from which mycelial cells had not been removed). The enzyme/cellulose ratio (FPA/Co) was varied by changing either the enzyme or substrate concentrations. When the enzyme concentration was varied, the fermentation broth was diluted to the desired concentration and the pH was adjusted to approximately 5.1. About 100 ml of this preparation were added to the cellulose -no buffer was added.

To evaluate the effect of end-product inhibition, glucose and cellobiose were added to the reaction mixture at the beginning of the hydrolysis to give 10 and 50 g/l of glucose or 5 and 10 g/l of cellobiose. A comparison was also made between the hydrolysis of fresh cellulose and that of partially hydrolyzed cellulose. The latter was prepared from the solids remaining in the fermentor from the hydrolysis of 7 % cellulose. The solids were filtered, washed with distilled water, and dried at 80 °C for 24 hours and then used in the usual manner. In another series of experiments the hydrolysis was allowed to proceed in the normal fashion for 24 hours, at which time, 50 ml of fresh enzyme were added to one series of flasks while the same volume of water was added to another series of flasks for comparison purposes.

Further experiments designed to test for differences between the use of culture filtrates and the whole fermentation broth were performed, using a 1 % buffered cellobiose solution as the substrate instead of cellulose.

The hydrolysis experiments using barley straw were conducted under conditions similar to the standard conditions reported for cellulose. Both the dry and wet forms of the pre-treated barley straw (see section 3.3) were employed in these experiments. When the wet form was employed no buffer was added.

In all hydrolysis experiments samples were periodically withdrawn (1 flask per sample), inmersed in a boiling waterbath for 5 minutes to stop the reaction, filtered and kept for further analysis.

3.5 ANALYTICAL METHODS

3.5.1 Fermentation

Microbial growth was monitored by measuring the protein content of the fresh mycelial culture using the Biuret method[3]

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[3] The procedures for the analytical methods used throughout this work are described in detail in Appendix A

(42). Bovine albumine (Fraction V, Sigma Chemical Co.) was used as the standard. A 50 ml-aliquot of fermentation broth was filtered by suction through a pre-weighed glass fibre filter, the clear filtrate collected and the cake washed with two volumes of distilled water. The cake was then dried for 24 hours at 90 °C to determine sample total dry weight which consisted of mycelial cells and residual cellulose. In some runs, the cellulose content was determined on the dried residue by the method of Updegraff (43), using Solka floc SW-40 to prepare the standard. Enzyme activities were measured by the release of reducing sugars upon incubation of 1 ml of diluted culture filtrate with the appropriate substrate (cellulose or salicin) at 50 °C for 60 minutes. Cellulase activity was determined by the filter paper activity assay (44). Beta-glucosidase activity was assayed as salicinase activity (45). Enzyme activities are expressed in International Units (IU)/ml of sample, where one IU is defined as 1 mol of glucose (or glucose equivalent) released per minute.*

In the barley straw fermentations (runs 12 to 15), when performing the Biuret assay, it was found that a greenish coloration developed upon digestion of the solids with 3N NaOH. Since it was thought that this coloration could interfere with the assay, the protein content of these samples was also evaluated from nitrogen content which was deter-

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* A sample calculation of the enzyme activity with data from one fermentation run is shown in Appendix B.

mined by a Kjeldahl-nesslerization method (42, 46). A comparison of both methods (Appendix B) showed that providing that a sample is included to account for this "background" coloration, the Biuret method satisfactorily estimates the protein content.

3.5.2 Hydrolysis

A 50 or 100 ml-aliquot of the hydrolyzed slurry was filtered through a tared glass fibre filter. The clear filtrate was collected and the cake was washed and dried for total dry weight determination. The hydrolyzates were analyzed for total carbohydrates by the phenol method (47) and for reducing sugars by the so-called DNS method (48) (3,5 Dinitrosalycilic acid). The composition of the hydrolyzates was determined by High Pressure Liquid Chromatography (HPLC) analysis. A Waters Associates ALC-200 system, equipped with a Model 6000A Solvent Delivery System and a Model U6K Universal liquid chromatograph injector, was employed. Sugars were separated on a 30 cm x 3.9 mm ID Micro Bondapak Carbohydrate column (Waters Associates # 84038) and detected by a Differential Refractometer (Model R401, W.A.). The carrier solvent, a mixture of Acetonitrile-water (80:20), was filtered through 0.45 μ millipore filter before use. The filtered solvent was pumped through the column at a flow rate of 2 ml/min. The only preparation needed for the samples was filtration through 0.45 μ millipore filters. 20 μl of the sample were injected using a high precision syringe (Hamilton # 802). The concentration of the sugars (xylose, glucose, cellobiose) was determined from peak-height measurements. Standard curves for each component were prepared by injecting 20 µl of a solution of known concentration and then plotting the measured peak height against concentration.

Chapter IV

RESULTS AND DISCUSSION

4.1 FERMENTATION

4.1.1 Effect of pH on growth and enzyme synthesis

The pH of the medium after autoclaving was between 5.0 and 5.5 for fermentations of 1 % Solka floc SW-40. In the first hours after inoculation, the pH began to drop and it was not controlled until the desired level was reached. In some fermentations the pH was controlled not to go below 4.0 and in others not to go below 3.0. These set point values were reached 18 and 24 hours respectively after the inoculation. The pH was then controlled at these values throughout the fermentation.

Results for fermentation runs # 2 and 3 with control of pH at 4.0 and 3.0 respectively are given in Tables 5 and 6 (see Appendix C). The concentration of intracellular protein and filter paper activities during the course of these fermentations are plotted[5] in Figures 5 and 6 . When pH was controlled at 4.0 the growth rate as calculated from the increase of intracellular protein in the initial 30 hours was

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[5] The solid lines in these and the following curves were drawn by eyeball fitting and therefore represent only a qualitative description of the general trends and not mathematical relations.

0.052 h^{-1} . Within 48 hours a maximum in intracellular protein concentration of about 2.0 g/l was attained, after which the protein concentration started to decrease as a result of cell lysis most likely (see Figure 5). The total dry weight, which consisted of mycelial cells and residual cellulose, steadily decreased up to a fermentation time of 94 hours, at which point its value began to level off. This also coincided with a levelling in the protein concentration. In the fermentation with pH controlled at 3.0 similar patterns for protein and total dry weight were observed. However, growth was slower (growth rate was 0.033 h^{-1})and although the maximum concentration of intracellular protein was similar for both set points, this maximum occurred about 20 hours later.

Similarly, cellulase appeared earlier in the fermentation with a pH set-point of 4.0; it accumulated rapidly in the media between 18 and 48 hours. After this period the enzyme continued accumulating, but at a slower rate (see Figure 6). As with growth, a lag in enzyme production was observed when the pH of the medium was controlled at 3.0. Little enzyme had been produced up to 30 hours, after which time the enzyme started accumulating, although less rapidly than at pH 4.0. However, the rate of accumulation was maintained and resulted in a higher enzyme activity in the media at the end of the fermentation.

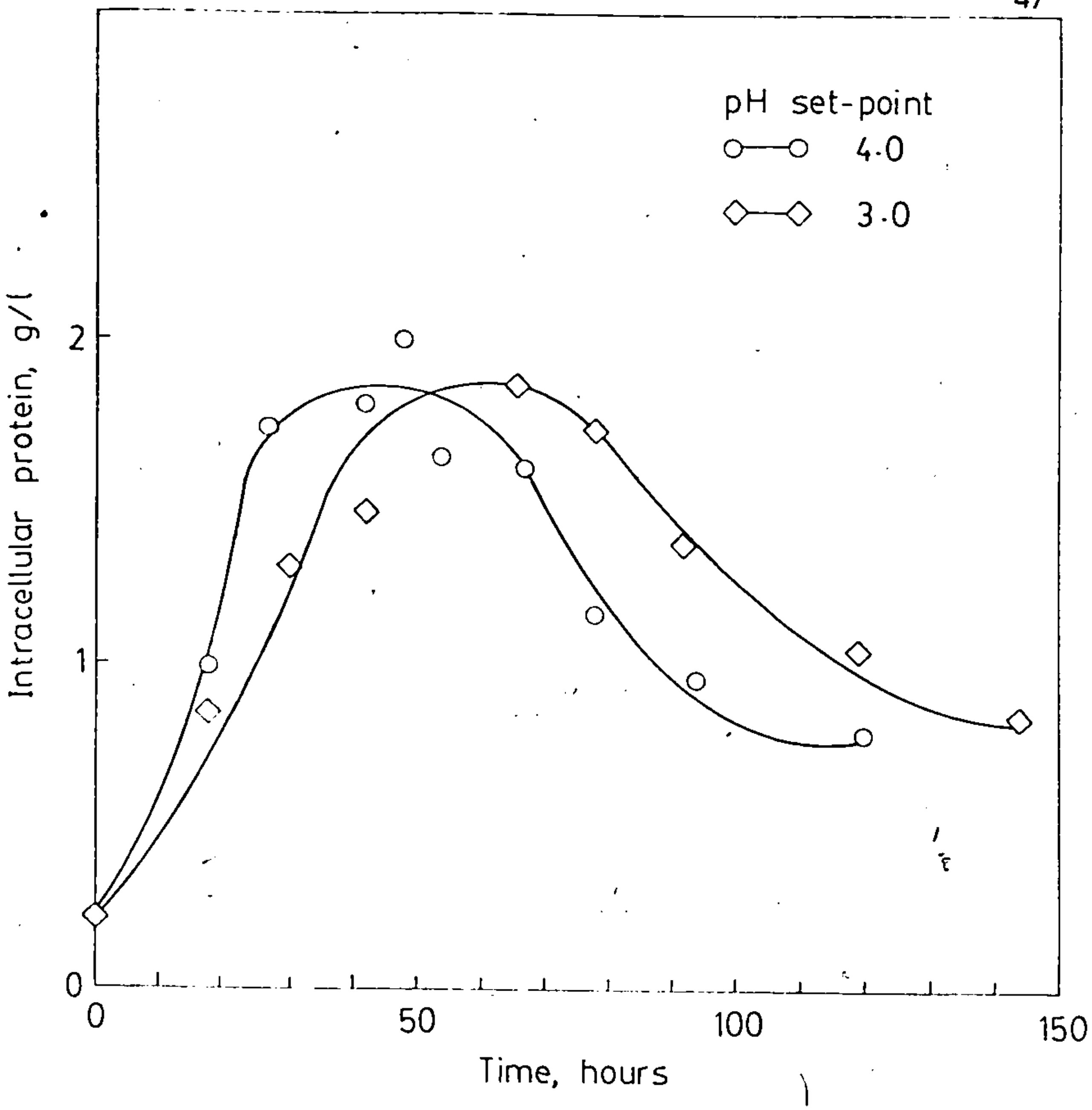

Figure 5: Growth curves during fermentations with pH set-points at 4·0 and 3·0·

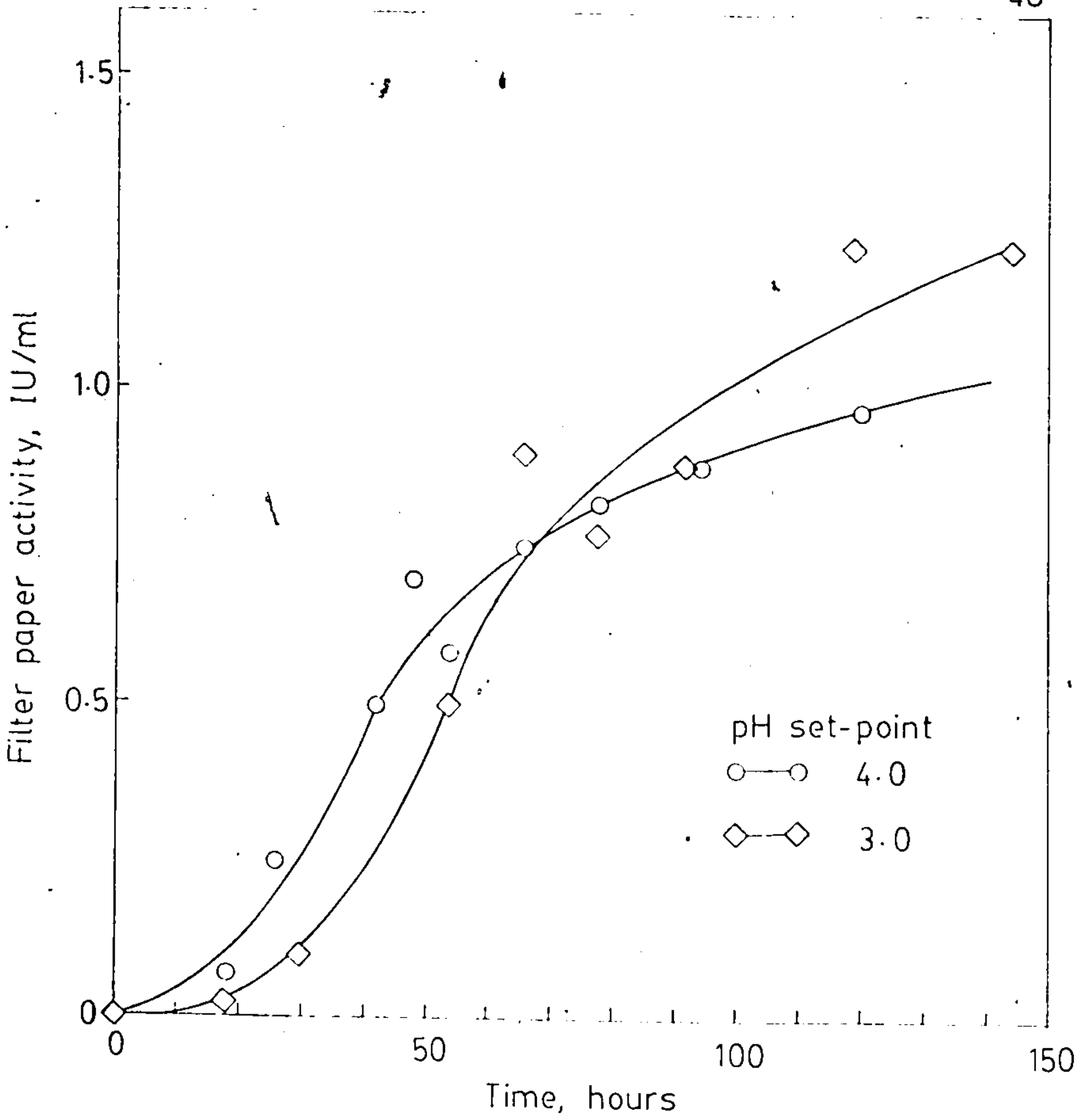

Figure 6: Cellulase production during fermentations with pH set-points at 4·0 and 3·0.

The causes for an increased enzyme activity at lower pH are not exactly known. One possible explanation could be that the slowdown of cell growth at lower pH may somewhat relieve enzyme synthesis from catabolite repression, therefore promoting enzyme synthesis When the pH was controlled at 3.0, it was observed that in the initial stage of the fermentation, the lag for enzyme production was shorter than that for growth. For example, a protein concentration of 1.8 g/l was attained at approximately 28 and 50 hours when pH was controlled at 4.0 and 3.0 respectively. However, at 28 hours filter paper activity was about 0.25 IU/ml for pH 4.0 and the same activity was attained at about 40 hours with the pH being controlled at 3.0.

It was also observed that during the period of cell lysis, the intracellular protein content fell down slightly more rapidly when the pH was controlled at 4.0 and yet the rate at which the enzyme was being released was lower. This seems to support that indeed, as suggested by Gong (5) and Ross (10), the enzyme is released by some specific transport mechanism accross the cell membrane, which may be affected by pH, rather than through unspecific cell lysis. A low pH (i.e., 3.0) benefits enzyme production probably by de-repressing the enzyme synthesis mechanism during the growth phase and by increasing the permeability of the membrane of aging cells during the stationary and post-stationary phases. Therefore in subsequent fermentations the pH was controlled not to go below 3.0. Data for additional fermentations of 1% cellulose at pH 3.0 (runs # 4 and # 5) are given in Tables 7 and 8 . The averages for maximum intracellular protein and final filter paper activity obtained for the fermentation runs done with 1 % Solka floc were 1.8 g/l (± 0.15) and 1.4 IU/ml (± 0.17) respectively.

4.1.2 Effect of increased cellulose concentration

One way of increasing the yield of a microbial product is by increasing the initial concentration of substrate. Thus, several fermentation runs (runs # 6 to # 11) were carried out using a higher cellulose concentration, i.e., 2 % Solka floc. In that case, the amount of salts and additives was also doubled compared to the runs of 1%. The pH of the medium after sterilization was between 4.8-5.0 and reached the set point of 3.0 within 24-30 hours from the time of inoculation. The pH was then controlled at this value throughout the rest of the experiments.

Experimental results of six batch fermentations are given in Tables 9 to 14 . The general pattern for these fermentations was similar to that of previous runs. Under these conditions growth was good (i.e., average growth rate was 0.053 h^{-1}),but the lag for enzyme production was slightly increased. The values for intracellular protein concentration and enzyme activity are plotted as a function of time in Figures 7 and 8 . Average values for maximum intracellular protein and final filter paper activity were of 3.42 g/l (± 0.29) and 2.50 IU/ml (± 0.30) respectively. This represents almost a 2-fold increase compared to the values obtained with 1 % Solka floc, therefore increasing the productivity of the fermentation. The maximum productivity (18 $IU.l^{-1}.\ h^{-1}$) was reached at a fermentation time of 120 hours but it decreased to 15.6 $IU.l^{-1}.h^{-1}$ at the end of the fermentation (160 hours). The average filter paper activity of 2.50 IU/ml and the corresponding productivities compare well with values reported by Sternberg (17, 18) and Ghose (11).

The cellulose employed in these experiments has a low bulk density which impedes its use in high concentrations. However, with 2 % Solka floc SW-40 the cellulose was reasonably well suspended at the beginning of the fermentations. It was observed (visually) that the viscosity of the fermentation broth increased considerably during the exponential growth phase, making the mixing inefficient, and this resulted in lumps of cellulose and mycelial cells settling at the bottom of the fermentor vessel. Therefore fermentations with higher cellulose concentrations were not attempted. In order to use higher cellulose concentrations, another form of cellulose with a higher bulk density could be employed; another convenient alternative would be to improve the design of the fermentor.

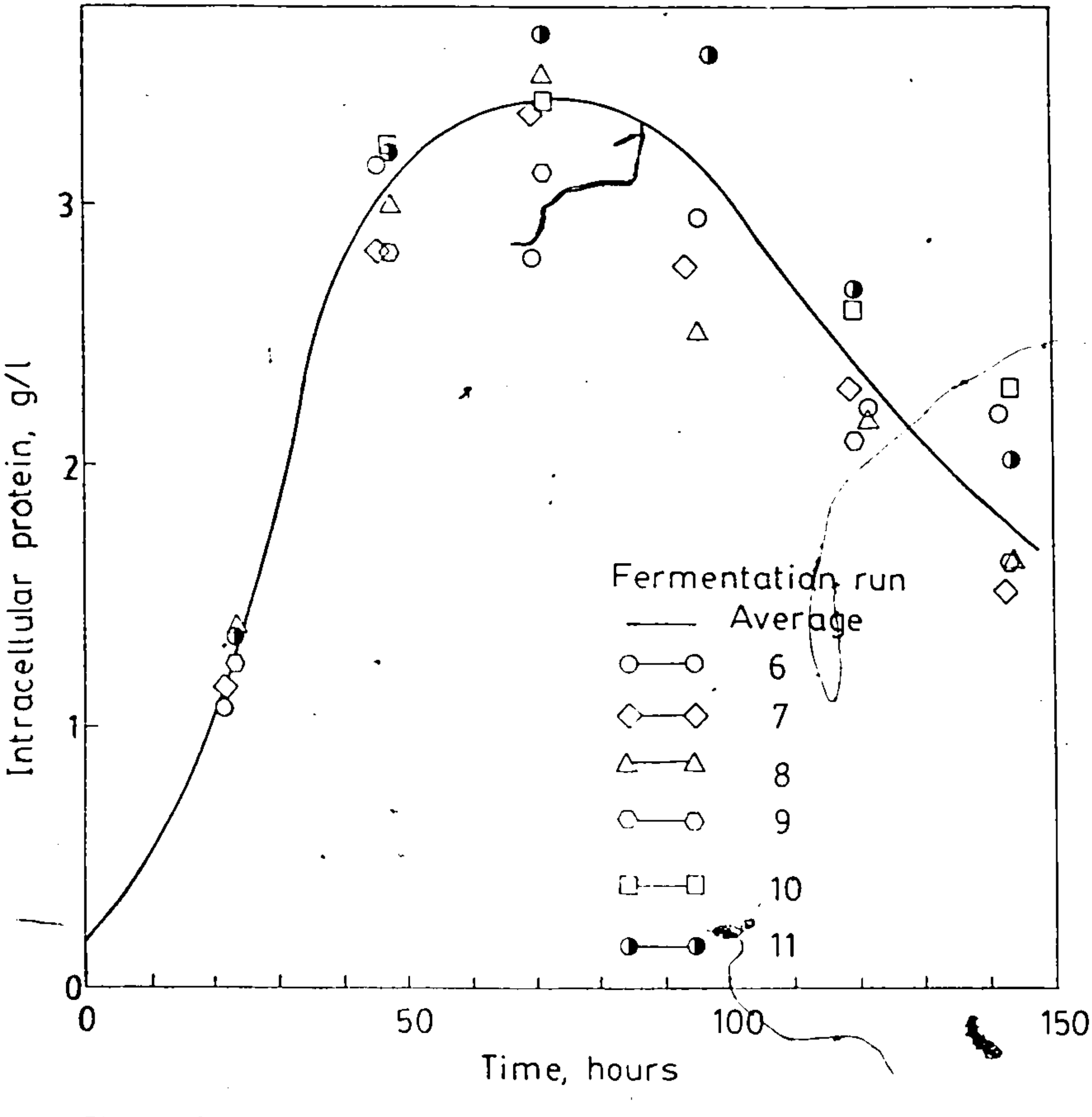

Figure 7: Growth curve during fermentations using 2 % Solka floc.

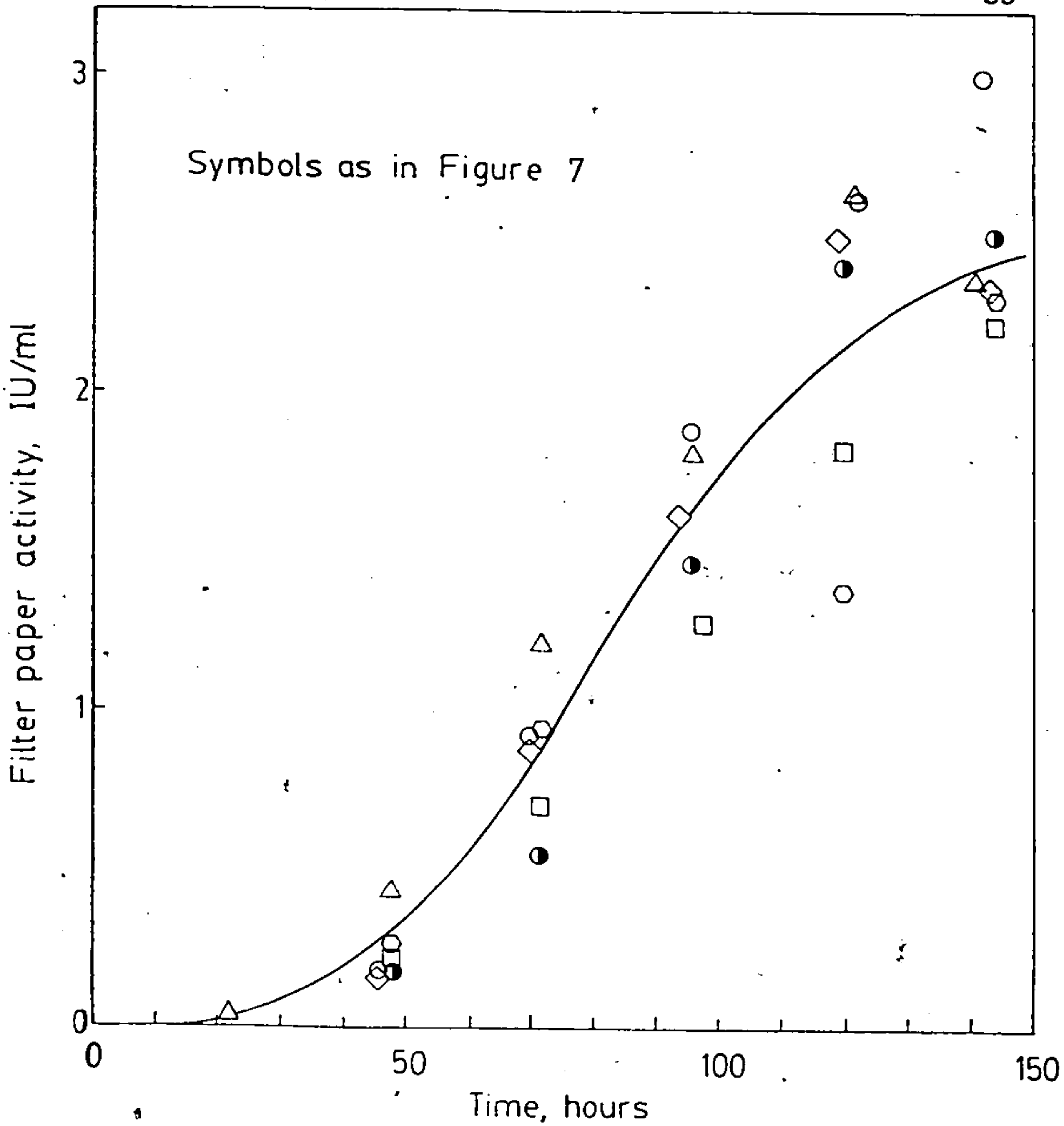

Figure 8: Cellulase production during fermentations using 2 % Solka floc.

A profile of the different variables measured during the fermentation is shown in Figure 9 . As mentioned earlier the lag for enzyme production was prolonged with the increase in initial cellulose concentration . Even though a seven-fold increase in the concentration of intracellular protein was achieved in the first 24 hours, little change was observed in the total dry weight and the enzyme activity of the filtrate was almost zero. It was observed that shortly after inoculation (2-3 hours) the concentration of Dissolved Oxygen (D.O.) in the medium began to drop, and continued doing so, very rapidly, in the following hours. At about 18 hours the respiratory activity changed and the D.O. concentration increased from 35 % to 55 % of saturation in about 3 hours. Then the D.O. concentration decreased steadily and almost as rapidly as at the beginning of the fermentation. This clearly indicates that initially the microorganism grew at the expense of the proteose peptone and probably of some soluble sugars released from the cellulose (cellulolytic enzymes were indeed present in the inoculum). When the proteose peptone and the soluble sugars were depleted, growth stopped or slowed down, and after a period of adaptation to the new conditions growth resumed. Very small enzyme activities were detected in the first 48 hours. By the time the maximum in protein content was attained (72 hours), about 80 % of the substrate had been consumed, but the filter paper activity was only about one third of the final activity detected. The filter paper activity of the filtrate increased considerably only after the maximum in protein had been reached. Sternberg *et al.*, (17) reported that they found that when 80 % of the ammonia had been taken up, only about 20 % of the final level of cellulase could be detected in the filtrate. It appears that either cellulase is synthesized earlier in the fermentation and is released later in the medium, or that a significant portion of the cellulase is synthesized during the later growth phases from pre-existing intracellular protein.

With the objective of gaining more information about the composition of the enzyme complex, the production of beta-glucosidase was monitored during the course of these fermentations.* Generally beta-glucosidase activity appeared in the filtrates at the same time as filter paper activity. However, its behavior during the course of the fermentations was far less predictable. Results for salicinase activity from some of the fermentations with 2 % cellulose are shown in Figure 10 . In some cases (e.g., runs # 6 and # 7) a maximum in salicinase activity occurred before the maximum in filter paper activity had been reached, but in some others (e.g., runs # 10 and # 11) the activity slowly increased up to the end of the fermentation. Mandels (4) observed that the ratio of salicinase to filter paper activity in enzyme preparations from cultures of *T. reesei* is about 0.3 units

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* Beta-glucosidase activity was determined indirectly using a cellobiose analog (salicin) as the substrate.

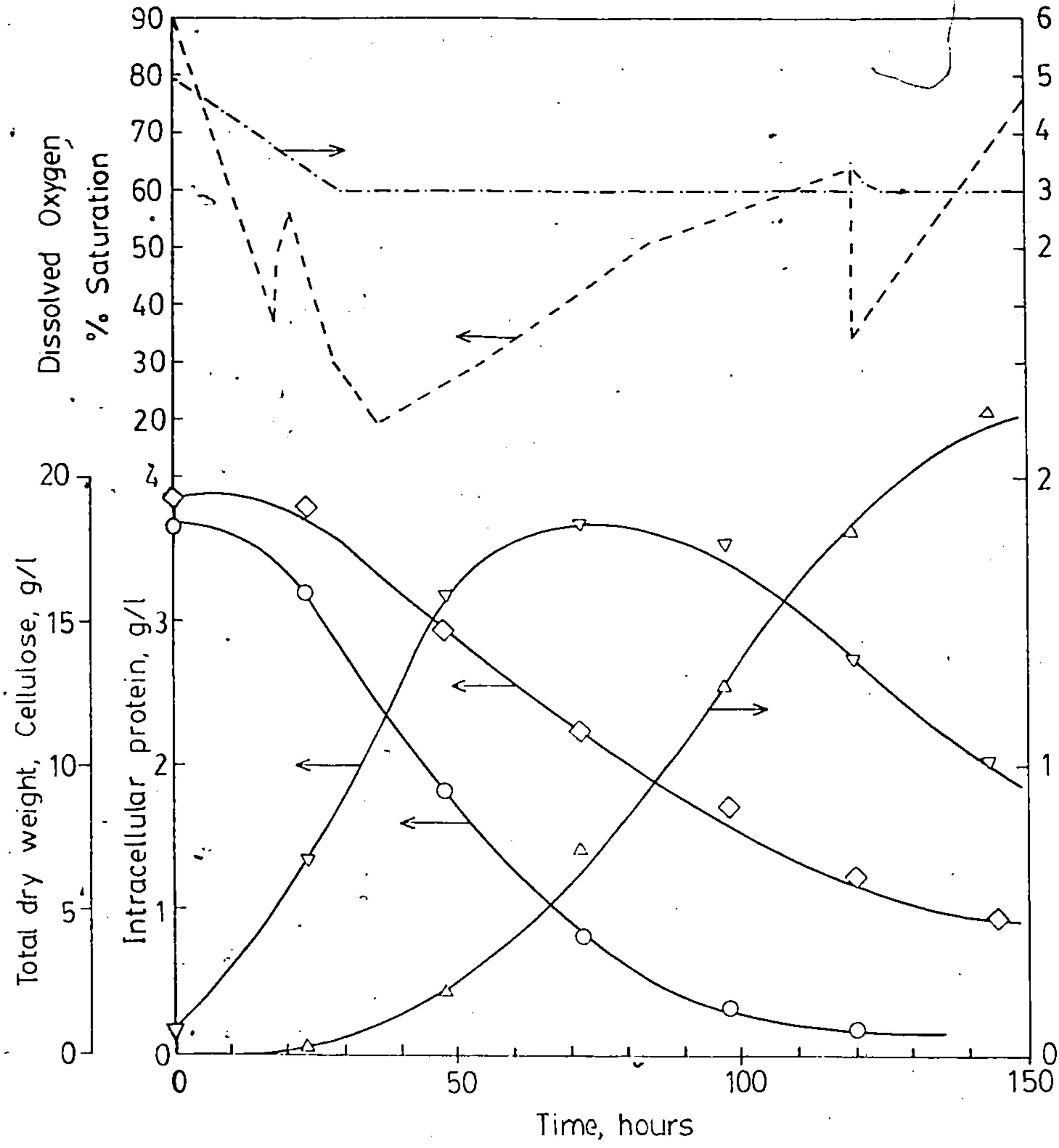

Figure 9: Profile for a fermentation of 2% Solka floc. ○–○ Total dry weight. ◇–◇ Cellulose. ▽–▽ Protein content. (Fermentation run Nº 11)

of beta-glucosidase per filter paper unit. However in this case no such ratio was observed and it changed accordingly to the pattern followed by salicinase activity through the fermentation. Sternberg (17) reported that beta-glucosidase lagged 1 or 2 days behind the cellulases and that it accumulated at a higher rate late during the fermentation. The only possible explanation for the different patterns observed in the production of salicinase activity is that at different periods of time it was necessary to increase the agitation speed, so as to resuspend the solids that had settled at the bottom of the fermentor. This increase in the agitation speed could have "broken" some of the mycelial cells, thereby releasing some enzymes. However, no such changes were observed for filter paper activity; but as pointed out before, cellulases may be released from the cell only by some specific mechanism different from those involved in beta-glucosidase production.

This is to be clarified by conducting further investigation on this system. It would be necessary for instance to determine when the various enzymes of the cellulase complex are synthesized and when they are excreted from the cells.

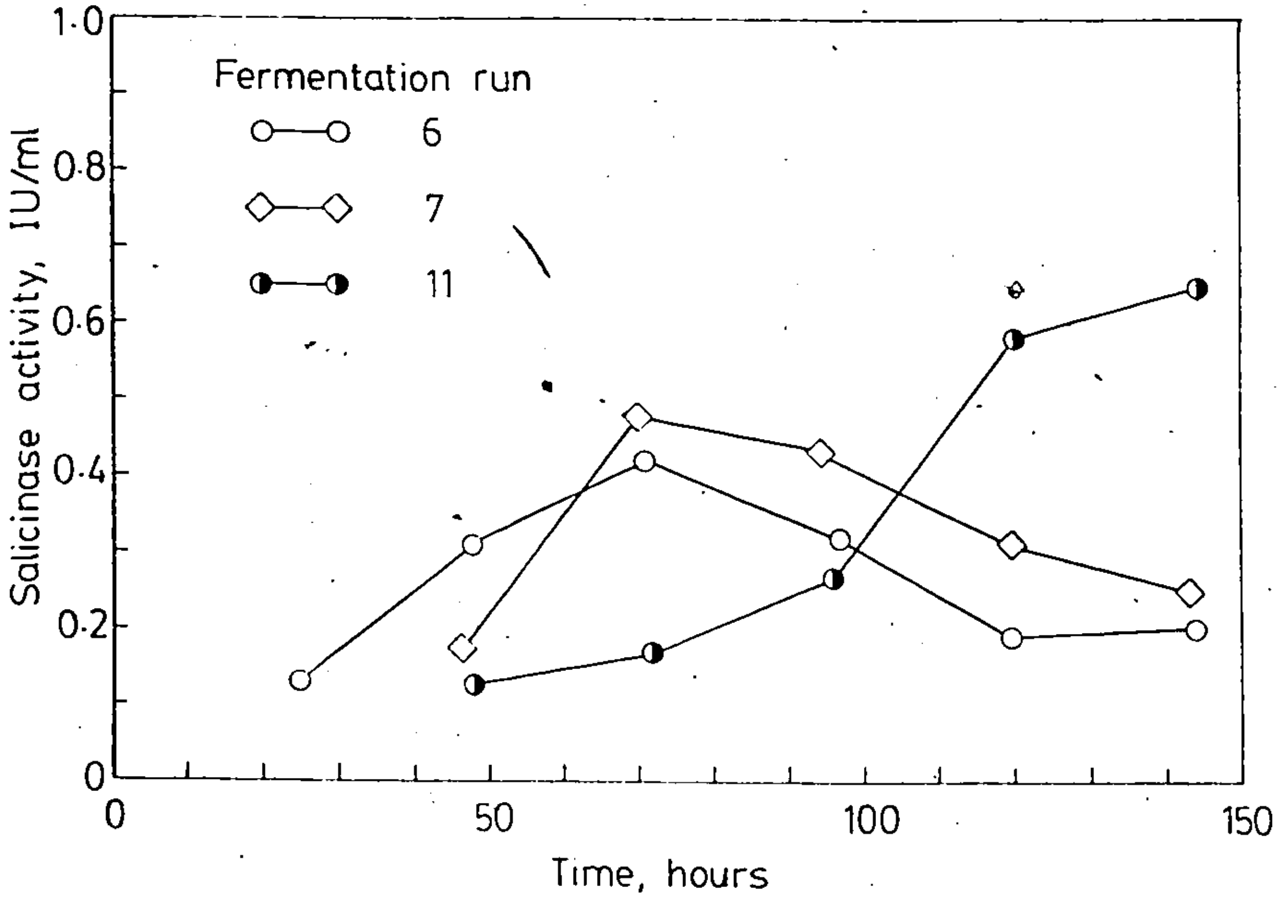

Figure 10: Salicinase production during fermentations using 2% Solka floc.

4.1.3 Fermentation under non-sterile conditions

Some industrial fermentations such as the production of acetic acid and alcoholic fermentations are carried out under non-sterile conditions relatively free of contamination because the acidic conditions prevent the rapid growth of many bacteria and other microorganisms. Since the production of cellulases by some strains of *Trichoderma reesei* is favoured at low pH, it was believed that strict sterilization could be avoided by conducting the fermentation at a pH of 3.0 and using an adequate aseptic technique.

A comparison between fermentations carried out under sterile and non-sterile conditions was made. Barley straw was employed as a substrate in these fermentations because it is more representative of the actual substrates, such as agricultural residues, that could be used potentially as a substrate for cellulase production.

The conditions for both types of fermentation (sterile and non-sterile) were similar, exception made for the sterilization step. The pre-treated barley straw (see experimental section) was used in concentrations of about 2 % (w/v). The pH of the media was between 5.25-5.9 after inoculation. The higher initial pH in these fermentations was due to the NaOH remaining in the barley straw after pretreatment. The pH was allowed to go down to 3.0, which was reached more than 30 hours after inoculation, and then was controlled at this value for the rest of the fermentation.

Experimental data obtained for these fermentations are listed in Tables 15 to 18. Runs 12 and 13 were carried without sterilization and runs 14 and 15 under sterile conditions.[7] Cellulose was assayed in the samples collected during runs 12 and 13, which indicated that the pre-treated barley straw contained about 80 % cellulose (w/w), the other 20 % probably being hemicellulose and lignin. The lower content of cellulose in the barley straw compared to Solka floc was reflected in the somewhat lower yields of cell biomass obtained. The maximum for intracellular protein concentration was also attained in about 72 hours, but the average value for runs 13, 14 and 15 was of 2.98 g/l (± 0.32) which represented 87 % of the average value for fermentations of 2 % Solka floc (3.42 g/l). Based on the cellulose content, a yield of 80 % would be expected; however, the value of 87 % indicates that some hemicellulose was utilized as well.

Under these conditions filter paper activities were also lower. The average final filter paper activity was 1.51 IU/ml (± 0.16) for runs # 13, 14 and 15, which accounted for only 60 % of that obtained for fermentations of 2 % Solka floc. The low filter paper activity could be the result of two factors: a) The presence of lignin which is thought to have inhibitory effects on the enzymes, or b) the

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[7] Run number 15 was initially sterile, however because of problems with foaming it was necessary to open the fermentor at about 72 hours.

synthesis of other enzymes (such as xylanases) which lowered the yield of cellulases alone.

Vohra et al. (49) reported that during fermentations with T. reesei the addition of lignin (0.01-0.05 %) and lignin derivatives had a considerable inhibitory effect on both the growth of the microorganism and on the production of cellulase enzymes. However, in the fermentation runs of barley straw growth did not seem to be affected by the presence of lignin. Therefore, it appears that the lower filter paper activity obtained in these fermentations is better explained by the fact that other enzymes (e.g., xylanases) were also synthesized.

Figures 11 and 12 show graphically the results for protein content and filter paper activity for these runs. From Figure 11, it is apparent that protein content for run 14 and 15 was consistently lower than for run 13. No such pattern was observed for filter paper activity (Figure 12). A statistical analysis using a paired-sample comparison (50) was performed to check for any significant difference between the two types of fermentation (see Appendix D). A 95 % confidence interval for the difference in protein content between run 13 and the average of runs 14 and 15 was -0.786 to -0.392, which does not include zero as a plausible value. This seemed to indicate that a difference existed between the two sets of data. However, when the test was applied to filter paper activity data, the 95 % confidence interval was between -0.080 to 0.128; since this interval contains zero as a plausible value for the difference between these fermentations, it seems that no difference existed. Autoclaving is not very likely to change the properties of the substrate so as to cause inhibition of growth which at any rate would have also showed an effect in enzyme production. Therefore, it seems that the apparent difference observed in protein content could be caused by the assay itself, since it greatly relies in the homogeneity of the sample and requires good mixing of the fermentor. The protein content for runs # 12 and 13 was also evaluated from the total nitrogen content which was measured by the Kjeldahl method. A comparison of both methods is shown in Appendix B. The values of the protein content as evaluated from nitrogen content were consistently higher than those determined by the Biuret assay. This was due to the presence of non-proteinic nitrogen (0.60 %) in the pre-treated barley straw; after taking this into account the intracellular protein content estimated by both methods (the Biuret assay and total nitrogen content) were similar. However, the Biuret assay, which is relatively simpler and faster, is not affected by the presence of inorganic nitrogen and thus it is more convenient for monitoring the fermentation.

Microbial contamination was verified by observation of the fresh culture under the microscope; only a mild bacterial contamination was encountered. This offers no problem,

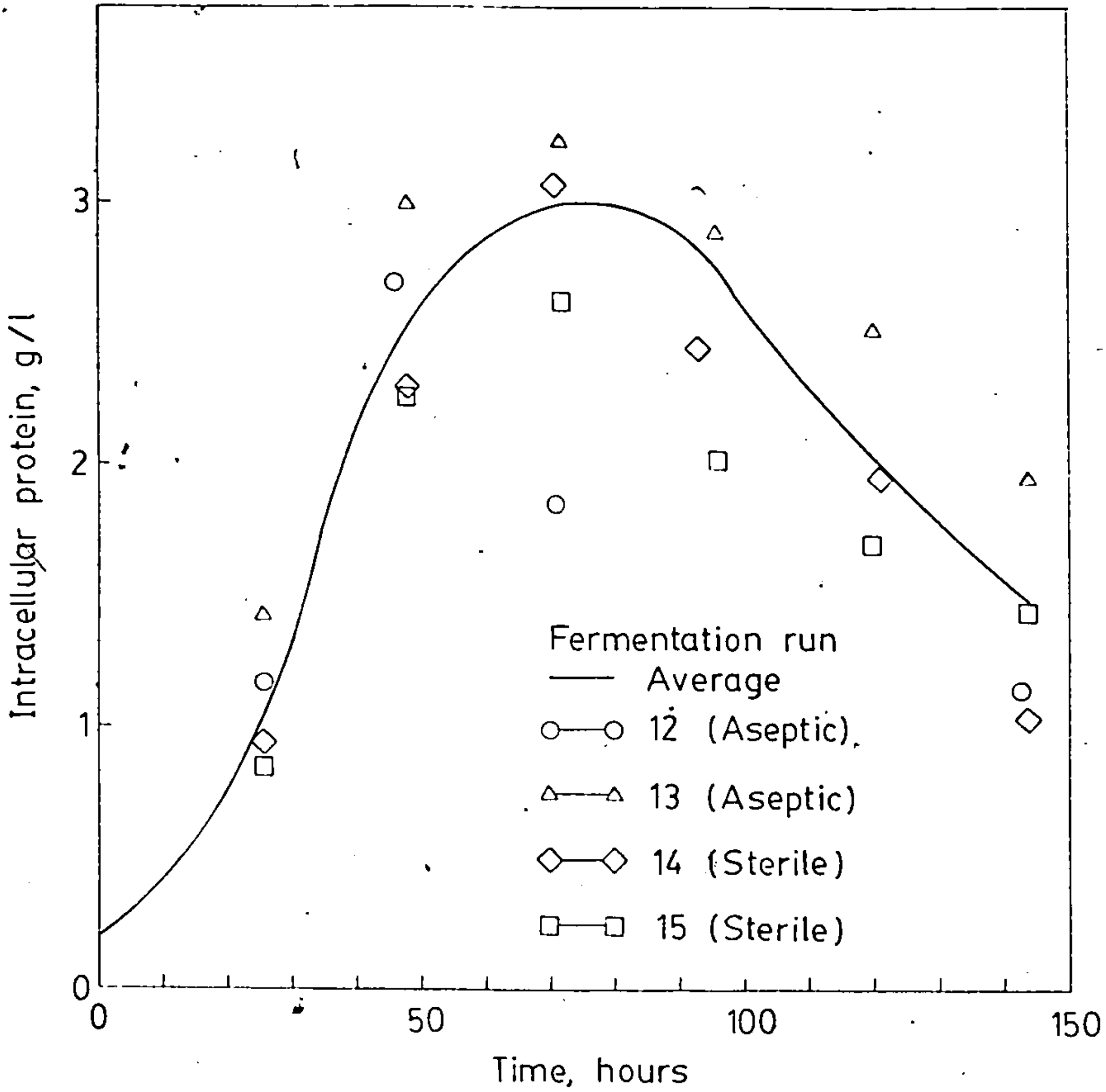

Figure 11: Growth curves during fermentations under sterile and aseptic conditions using barley straw.

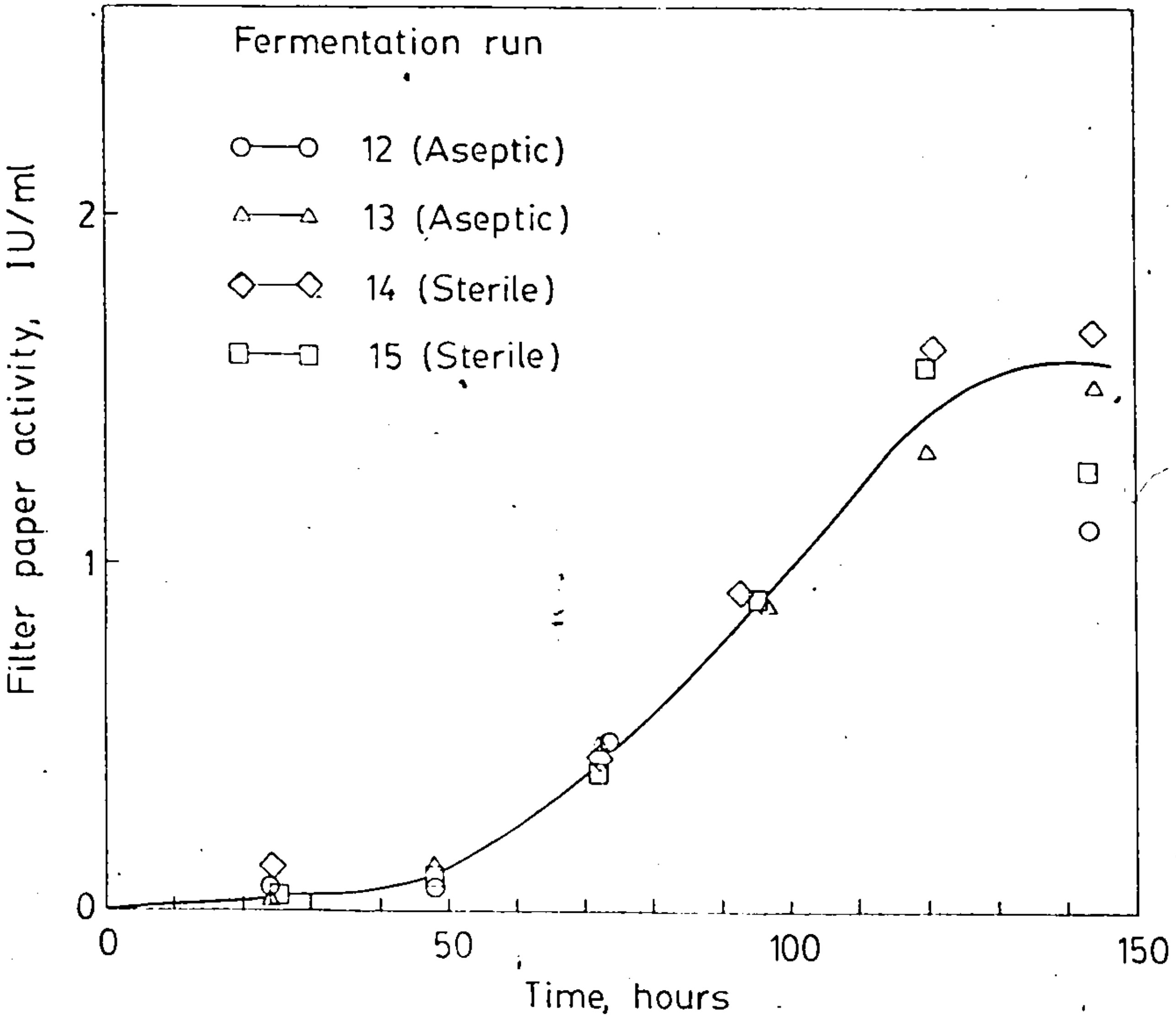

Figure 12: Cellulase production during fermentations under sterile and aseptic conditions using barley straw.

since in a practical situation contamination by bacteria could be controlled by the addition of antibiotics to the growth media.

From the point of view of enzyme production, it appears that under proper conditions, sterilization could be avoided. Since sterilization is commonly achieved by the use of steam at a pressure of 15 psi, the elimination of this step would decrease the capital cost, because the fermentor would not need to stand the sterilization pressure. Aseptic conditions could be established by steaming the fermentor at atmospheric pressure or by the use of chemicals. The growth media could be boiled or pasteurized to kill all actively growing microbes, and aseptic conditions during the fermentation maintained by conducting it at low pH (i.e., 3.0) and the addition of antibiotics.

4.2 HYDROLYSIS OF CELLULOSIC SUBSTRATES,

In a practical saccharification process the source of the enzyme is of primordial interest. Commercial enzymes which are either highly purified or concentrated have a high cost that prohibits their use for practical purposes. If the enzymes are to be produced on site for subsequent hydrolysis, handling of the enzyme can be minimized and its cost reduced. Several suggested processes make use of the unpurified culture filtrates for hydrolysis. Filtration is a relatively easy operation; however, maintaining sterile conditions somewhat restrains the operation since it requires a special technique and the use of costly membranes. Elimination of this step would therefore help to reduce the cost and simplify the hydrolysis process. Several experiments were laid out to explore both the feasibility and the possible implications of using the raw fermentation broth for the hydrolysis of cellulosic substrates.

4.2.1 Effect of initial cellulose concentration

To investigate the effect of initial substrate concentration on the hydrolysis, an experiment was performed using Solka floc SW-40 in concentrations of 40, 70, and 100 g/l. The enzyme source was the raw broth from fermentation run # 4 (harvested after 160 hours). The amount of sugars released from the hydrolysis was expressed as the concentration of total carbohydrates and total reducing sugars.

Results of this experiment are shown in Table 19 . When the results of the total carbohydrates and reducing sugars assays were analyzed in conjunction with total dry weight measurements, the reducing sugar method seemed to provide a more reliable estimate of the amount of sugars released by hydrolysis. On the other hand, the concentrations determined with total carbohydrates assay appear to be somewhat overestimated. For example, for the 4 % slurry the measured total dry weight (TDW) was 29.54 g/l after a reaction time of 4 hours (see Table 13). Subtracting this value from the initial substrate concentration (40 g/l) gives 10.46 g/l of cellulose which were converted to sugars. If all this cellulose were converted to glucose it would yield about 11.50 g/l of sugars. The sugar concentration determined from the reducing sugars (11.49 g/l) agrees with the theoretical concentration determined from dry weight measurements while the sugar concentration determined from the total carbohydrates assay is evidently higher. Similar relations existed among the data obtained for other samples .

Figure 13 shows the progression of the hydrolysis with time. In all three cases the highest hydrolysis rates occurred at the onset of the experiment, and decreased considerably in the following hours, specially for the hydrolysis of the 10 % slurry. Figure 14 shows the effect of initial cellulose concentration on the production of reducing sugars. It can be seen that within the range tested, initially the amount of sugar produced (and therefore the reaction rate) increased linearly with the cellulose concentration. However, as the reaction proceeded this pattern changed. Eight hours from the beginning of the hydrolysis, a slight curvature was observed, which became more pronounced with time. The comparatively smaller increments in the amount of sugar produced from the 4 % slurry after 8 hours seem to be the result of the faster depletion of the amorphous portion, leaving a more resistant crystalline residue. On the other hand, for the 10 % slurry the decrease observed in the hydrolysis rate was not likely caused by structural factors, but probably because of restrictions in rates of mixing and mass transfer. The increased cellulose concentration would reduce the amount of free water in the reaction mixture, therefore hindering the mobility of the enzyme molecules.

Conversions of 57, 40 and 22 % were achieved from the 4, 7, and 10 % slurries respectively, in 25 hours. Although the highest conversion was obtained with an initial cellulose concentration of 40 g/l, a higher final concentration was attained when using 70 g/l. Higher concentrations are desirable, since the cost of product recovery would be reduced. Under this conditions (type of substrate, enzyme concentration, etc.) a concentration of 70 g/l appears to be optimum.

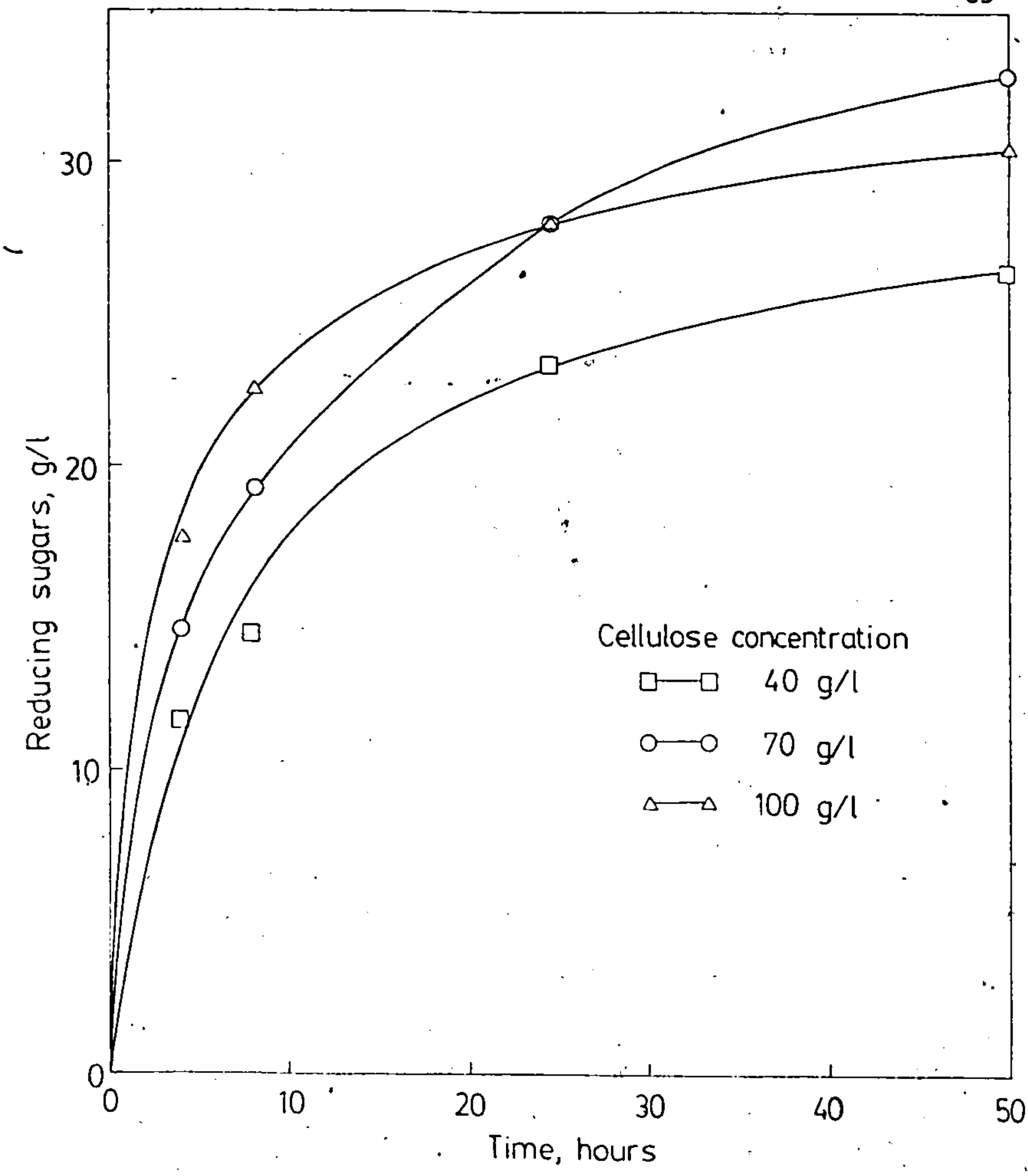

Figure 13: Progress of hydrolysis with different initial cellulose concentration.

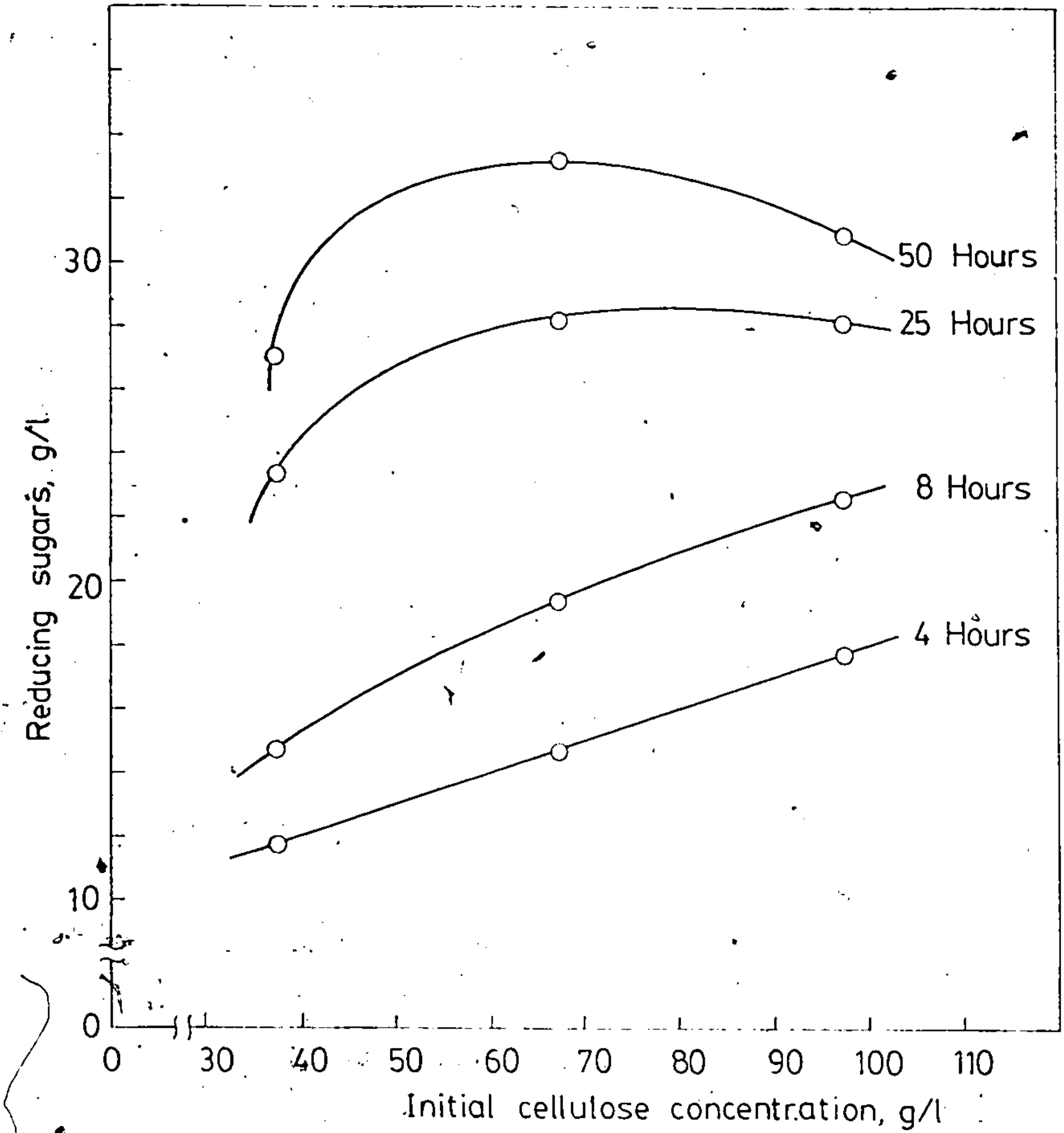

Figure 14: Effect of initial cellulose concentration on the production of reducing sugars.

4.2.2 Effect of initial enzyme concentration

From the experiment above, it was determined that an initial cellulose concentration of 70 g/l yielded the highest concentration of sugar, although the percent conversion was lower, compared to that of a 4 % slurry. In this experiment, however, the enzyme concentration was held constant, and therefore the higher percent conversion was partly due to a greater enzyme to cellulose ratio (FPA/Co). Another experiment was conducted where the enzyme to cellulose ratio was also varied, but this time by altering the initial enzyme concentration.

The full strength enzyme was the crude broth from a prior fermentation, filtrates of which had a filter paper activity of 2.2 IU/ml. The original enzyme preparation was diluted so as to give 0.25 and 0.50 times the full activity. Experimental results are given in Table 20, and the concentration of reducing sugars is plotted in Figure 15 as a function of enzyme concentration and reaction time. As it can be seen, to an increase in the initial enzyme concentration corresponded an increase in the reaction rate and therefore in the amount of reducing sugars released upon hydrolysis. However, this increase was not proportional to the initial enzyme concentration, and it would appear that the enzyme works more efficiently in a diluted environment.

The initial enzyme concentration also has an effect on the extent of hydrolysis in a given time. The concentration of reducing sugars for the 0.25 and 0.50 dilutions were 21.7 and 33.8 g/l respectively at 24 hours; similar concentrations were attained in only 4 and 12 hours respectively with the concentrated enzyme. It is therefore evident that increasing the initial enzyme concentration would sensibly reduce the reaction time. It is apparent, however, that beyond this range further increases in enzyme concentration may not significantly increase either the reaction rate or the yields of sugar.

In Figure 16 the yield of reducing sugars per gram of initial cellulose for this experiment and the preceding experiment where the enzyme concentration was held constant, is plotted against FPA/Co. It was observed that a linear relation existed between the yield and the log of the enzyme to cellulose ratio (FPA/Co). This indicates that if high per cent conversions are desired the FPA/Co must be high; on the other hand, larger amounts of enzyme would cause only marginal increases in the per cent conversion. Therefore, two factors should be taken into account on determining the enzyme concentration to be employed: the cost of the enzymes and the cost of product recovery.

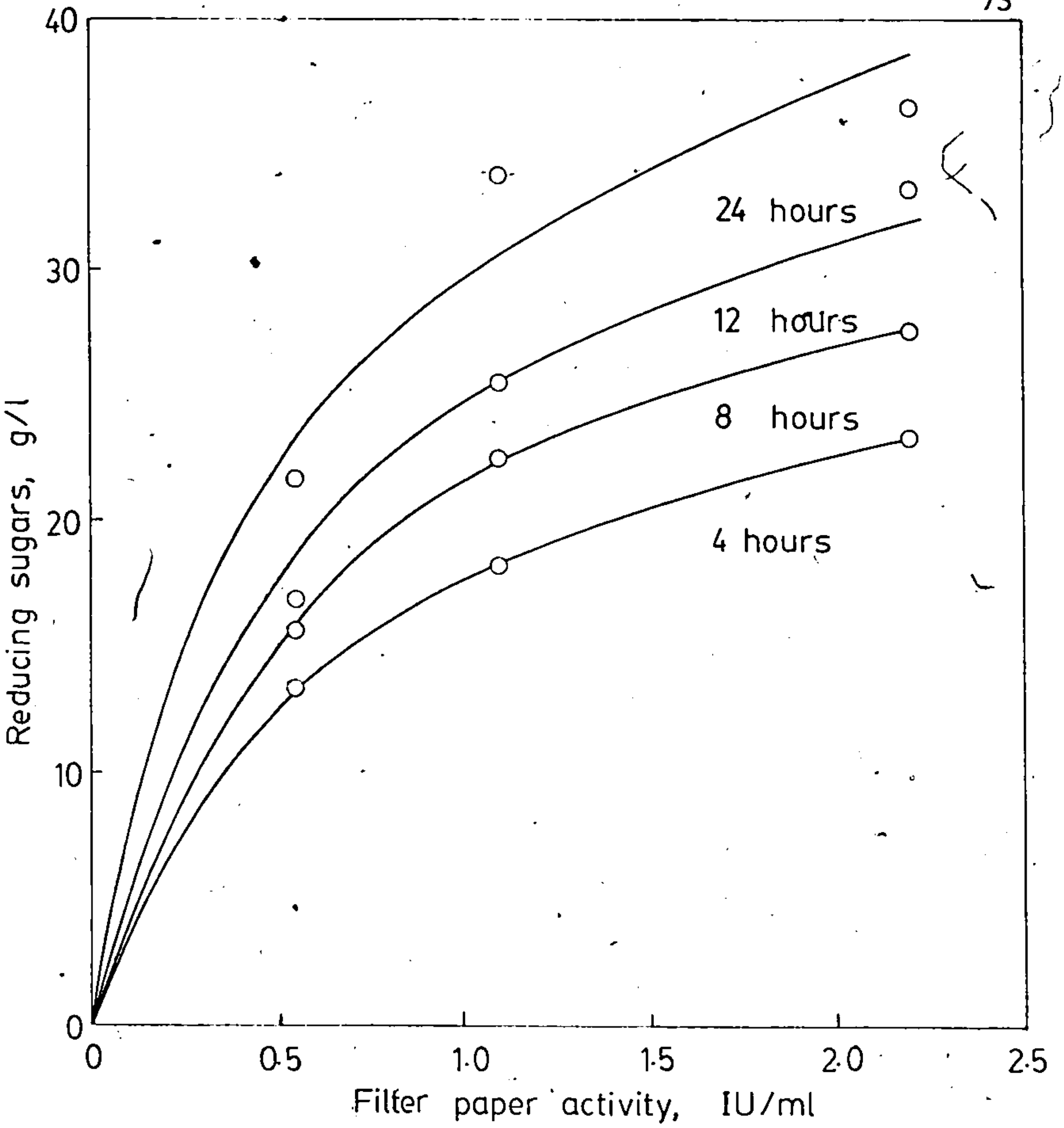

Figure 15: Effect of enzyme activity on the production of reducing sugars.

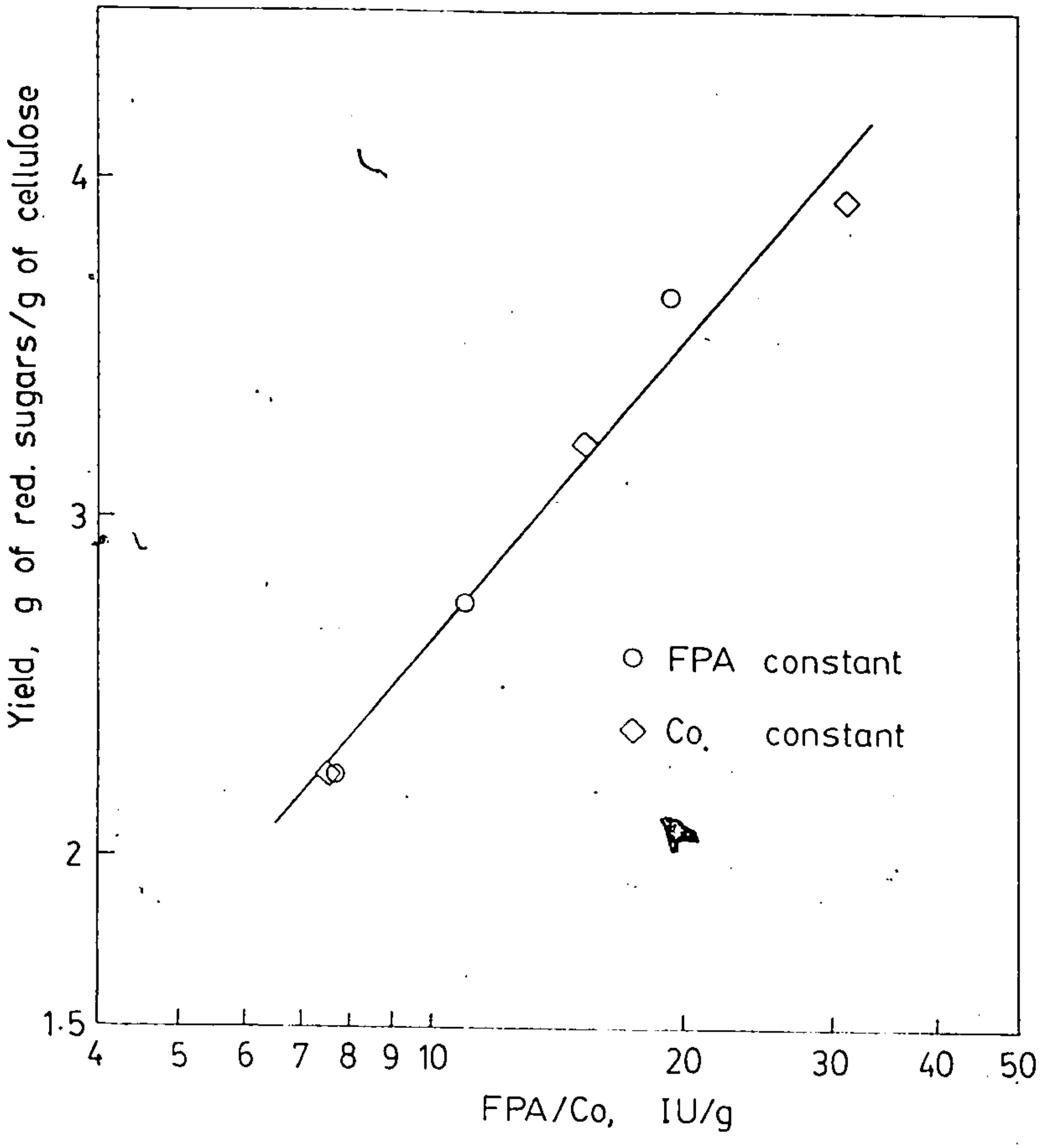

Figure 16: Effect of enzyme:substrate ratio on the yield of reducing sugars by hydrolysis of cellulose.

4.2.3 Utilization of raw broth versus culture filtrates

It has been already mentioned that there may exist some advantages in using the raw fermentation broth, as opposed to the use of crude culture filtrates. It is important, however, to evaluate if the presence of the mycelial cells would have any effect on the hydrolysis. A series of experiments were conducted with the purpose of comparing the performance of raw fermentation broth and culture filtrates.

Enzymes obtained from fermentations runs with 1 and 2 % Solka floc were used for these experiments. The total sugar concentration was estimated by the total carbohydrate and reducing sugar methods. High Pressure Liquid Chromatography (HPLC) was also used to qualify and quantify the composition of the hydrolyzates. It was found that the main products of the hydrolysis were glucose, cellobiose and xylose; under the conditions of the HPLC analysis no other significant peaks were detected. The sum of the three individual sugars yielded a concentration which was approximately equal to that of the reducing sugars (see Table 21).

Results for three sets of data of hydrolysis experiments with raw broth and culture filtrates obtained from three different fermentation runs (# 5, 6, and 7) are given in Table 21. The different variables were plotted for one representative run as a function of time and no significant difference was observed between the sets of data obtained for hydrolysis with raw broth and culture filtrates. Figure

17 shows the changes in total dry weight and reducing sugar, and Figure 18 the changes in the concentrations of glucose and cellobiose during the course of the hydrolysis.

A statistical analysis by sample-pairing was performed on the data against the null hypothesis that no difference existed in the two series of samples. The test was applied to reducing sugars, glucose and cellobiose data. At a confidence level of 95 %, the test indicated that in all cases, but one, the null hypothesis could not be rejected, which indicates that no significant difference exists between the use of raw broth and culture filtrates. For the particular set of data where the null hypothesis did not seem a reasonable one, the cellobiose concentrations were consistently lower when the mycelial cells were kept in the reaction mixture. Since beta-glucosidase has been reported to be an intracellular enzyme, it could be possible that some enzyme remains cell bound and could be released later.

To investigate this effect in some more detail, an additional experiment was carried out using cellobiose, at an initial concentration of 10 g/l, as the substrate. Culture filtrates, raw fermentation broth and homogenized raw broth[•] were used in this experiment. Results of this hydrolysis experiment are presented in Table 22 and plotted in Figure 19 . It is apparent that a slight, but consistent difference was obtained. Since no significant difference was

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[•] The raw broth was homogenized by mixing it in a lab blender for 5 minutes at a high speed.

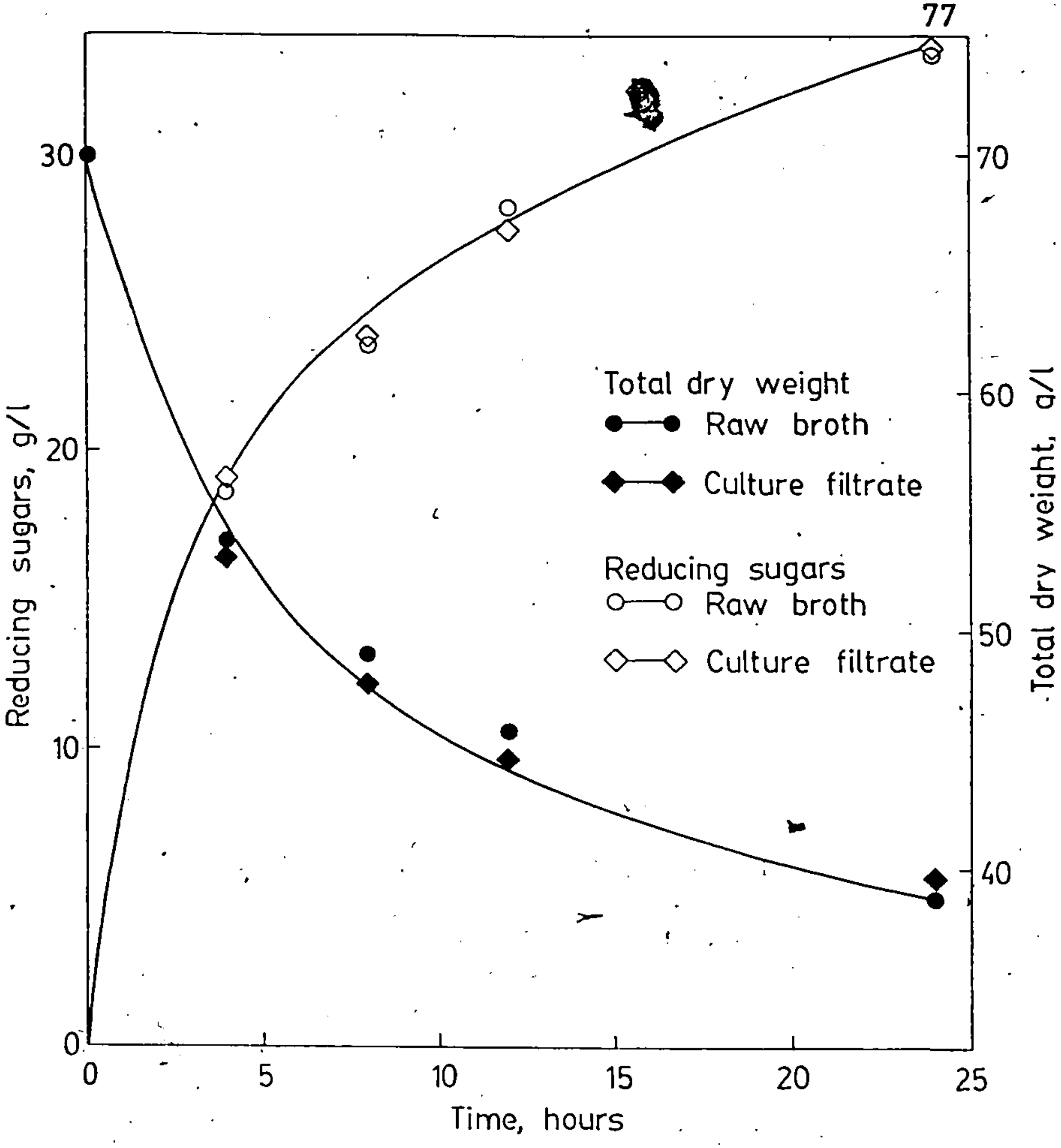

Figure 17: Comparison of cellulose hydrolysis with raw fermentation broth and culture filtrate. (Table 21, set C).

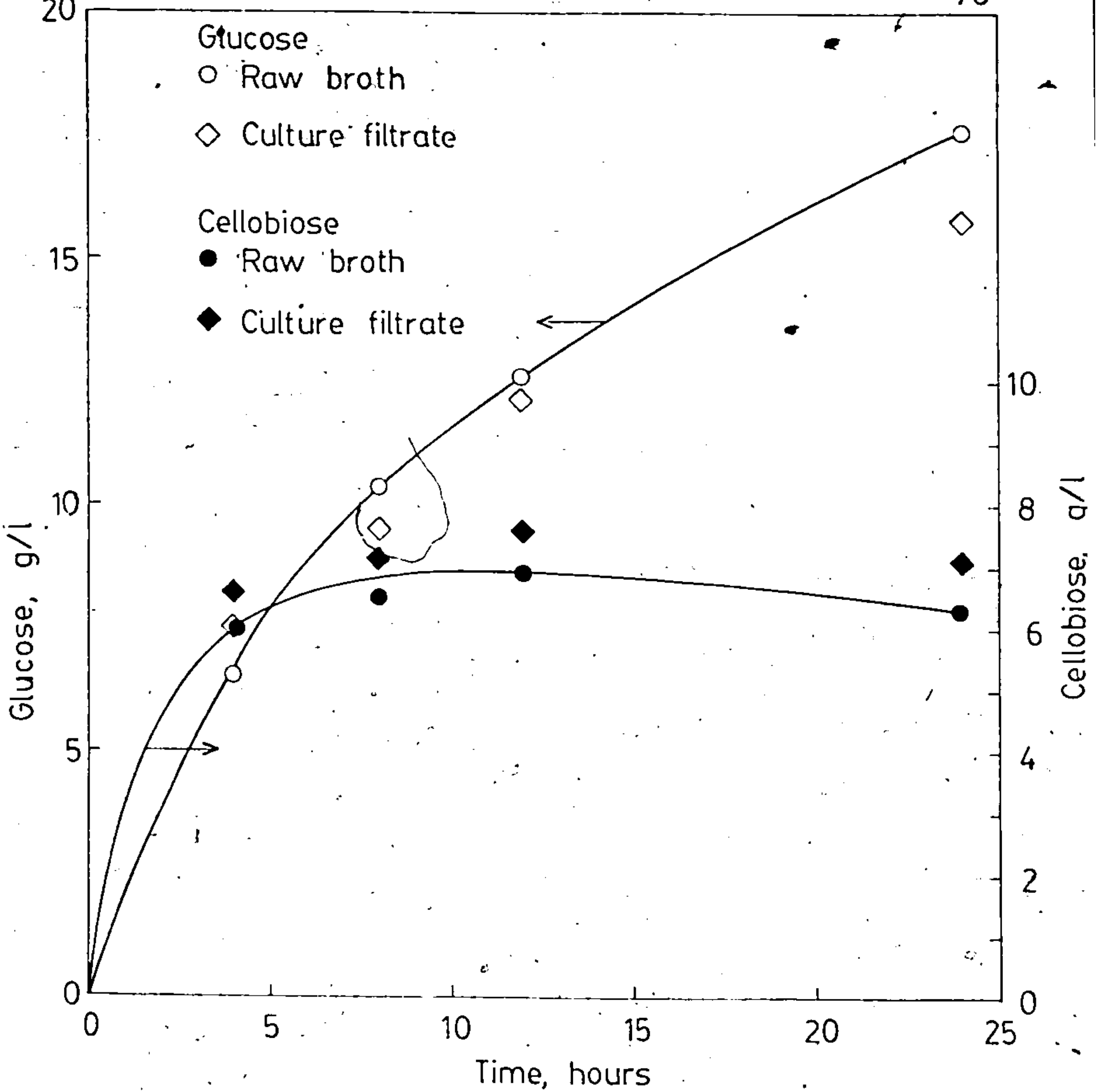

Figure 18: Comparison of the production of glucose and cellobiose by hydrolysis of cellulose with raw broth and culture filtrate. (Table 21, set C).

found between the raw broth and the homogeneized raw broth it appears that no additional enzyme was released by homogenizing the cells. Again, the paired-sample test was applied to the data for raw broth and culture filtrates. At the 95 % confidence level the null hypothesis (no difference) was rejected. This would mean that a difference between the use of raw broth and culture filtrates existed. That is to say that indeed some additional beta-glucosidase is released from the residual cells during hydrolysis. However, under the conditions of cellulose hydrolysis, the small difference in the cellobiase concentration has no significant effect on the global concentration of either glucose or reducing sugars.

It is evident from these results that the presence of the mycelial cells in the reaction mixture has no negative effect on the hydrolysis, and it would appear that in fact small amounts of the enzy[illegible] beta-glucosidase are released from the cells during the hydrolysis; further work is required to substantiate this effect. However, it appears that the use of raw broth is not only feasible, but as discussed earlier, it may be beneficial.

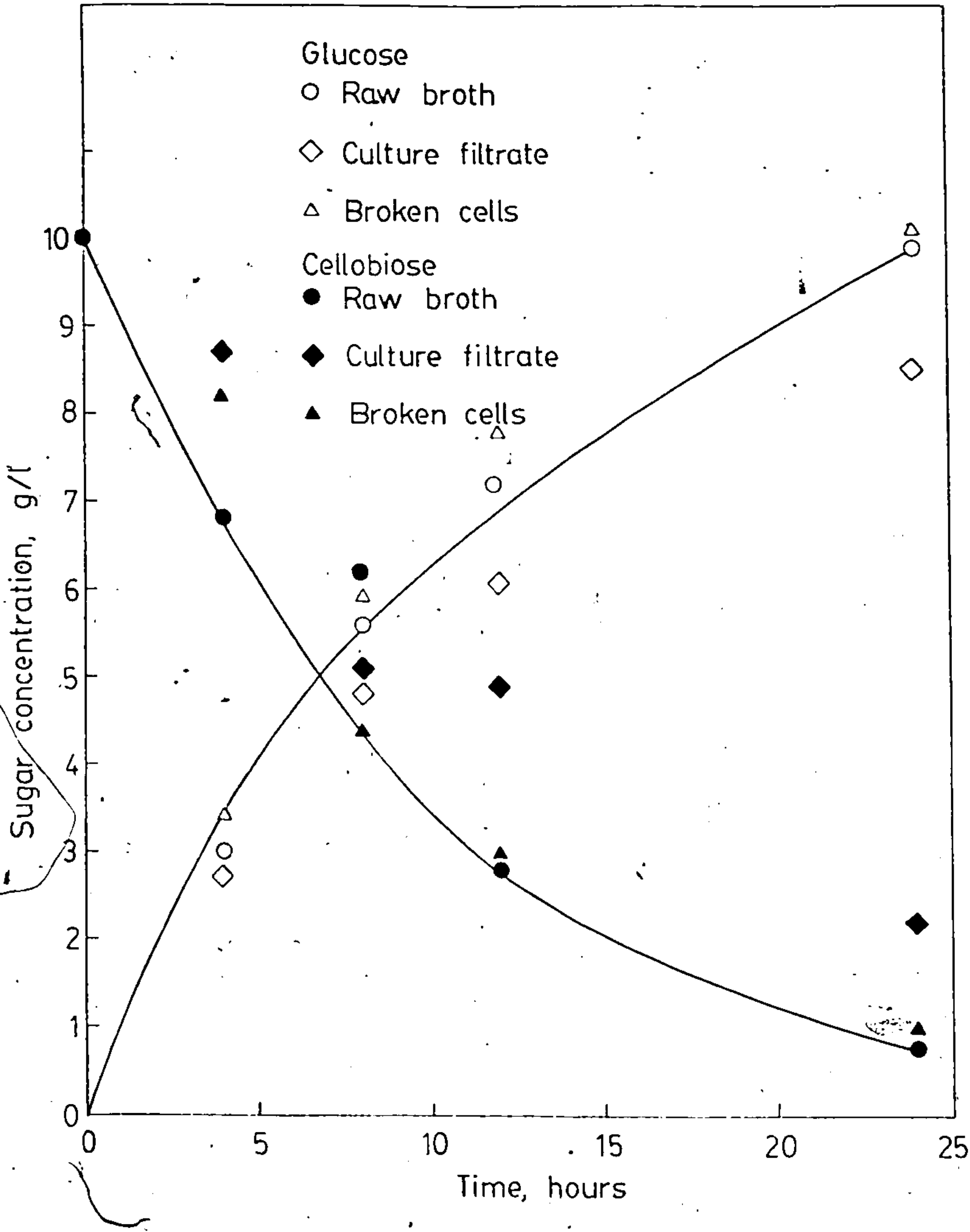

Figure 19: Comparison of cellobiose hydrolysis with raw broth and culture filtrate.

4.2.4 Inhibition by glucose and cellobiose

In all the hydrolysis experiments a fast reaction rate was observed at the beginning of the experiment, and was followed by a dramatic decrease in the next hours. Some authors have claimed that this is greatly due to end-product inhibition caused by the accumulation of products in the reaction mixture, specially cellobiose. The HPLC analysis of cellulose hydrolyzates (see Figure 18), revealed that cellobiose accumulated in the first hours and that its concentration remained nearly constant for the rest of the experiment. Glucose was also produced at a fast rate in the initial period, but its concentration continued increasing throughout the experiment, although at a lower rate.

To evaluate the inhibitory effect of both glucose and cellobiose, different concentrations of these sugars were added to the reaction mixture at the onset of the hydrolysis. Experimental results for hydrolyses with addition of 10 and 50 g/l of glucose and 5 and 10 g/l of cellobiose are given in Tables 23 and 24 .

From Figure 20 it is evident that the addition of 10 g/l of glucose had a minimal effect during the initial period (4 hours): looking at the pattern of reducing sugars accumulation, hydrolysis rates were similar in both cases. The addition of 50 g/l of glucose had a more significant effect; in the initial period of 4 hours, a 20 % decrease was observed in the accumulation of reducing sugars compared to the control experiment (i.e., no glucose added). This effect became more evident over an extended period of time; after 24 hours the reducing sugar yields with initial additions of 10 and 50 g/l were respectively 83 and 67 % of the value obtained with no addition of glucose.

Figure 21 shows the effect of the addition of cellobiose on the production of reducing sugars. The concentration of reducing sugars produced was calculated by subtracting 0.85 times the amount of cellobiose added from the measured concentration of reducing sugars. This adjustment is made because when glucose is used as the standard (as in this case), values for cellobiose will be about 15 % low on a weight basis (44). Reductions of 13 and 34 % in the reducing sugar yields in the first four hours were observed with the addition of 5 and 10 g/l respectively. However, HPLC data showed that the glucose concentrations (see Fig.hyol0ol)) was not affected by the addition of cellobiose. On the other hand, the cellobiose content of the hydrolyzates followed a different pattern (Figure 22). With the addition of 10 g/l, the concentration of cellobiose was nearly constant at this value for the first 8 hours and then declined. In the other case, the cellobiose concentration increased for the first 8 hours to reach a level of about 10 g/l and then declined in the following hours. It is interesting to note that the cellobiose concentration was about the same for both experiments after 24 hours. This suggests that the succession of hydrolytic reactions is self-regulated to some extent by the conjugated action of the various cellulolytic enzymes.

It is clear that cellobiose had a much stronger inhibitory effect than glucose; the addition of 5 g/l of cellobiose caused a reduction in the sugar yield which was equivalent to that of the addition of 50 g/l of glucose. (See Figures 20 and 21). No information was obtained for the composition of the hydrolyzates with glucose addition but it appears that glucose does not inhibit the cellulase complex directly. Presumably the accumulation of glucose would inhibit the beta-glucosidase, causing an accumulation of cellobiose, which in turn inhibits the endo- and exo-glucanases.

However, it was observed from the curves of Figure 18 that beyond 8 hours, the cellobiose concentration was almost constant and still the rate of glucose accumulation steadily decreased. Although the reduction in hydrolysis rates during the first hours can be partly attributed to inhibition of the glucanases by cellobiose, beyond the 8-hour period some other factors might also be responsible for the change observed. Suspected causes are: 1) decreasing susceptibility of residual cellulose and, 2) deactivation of the enzymes.

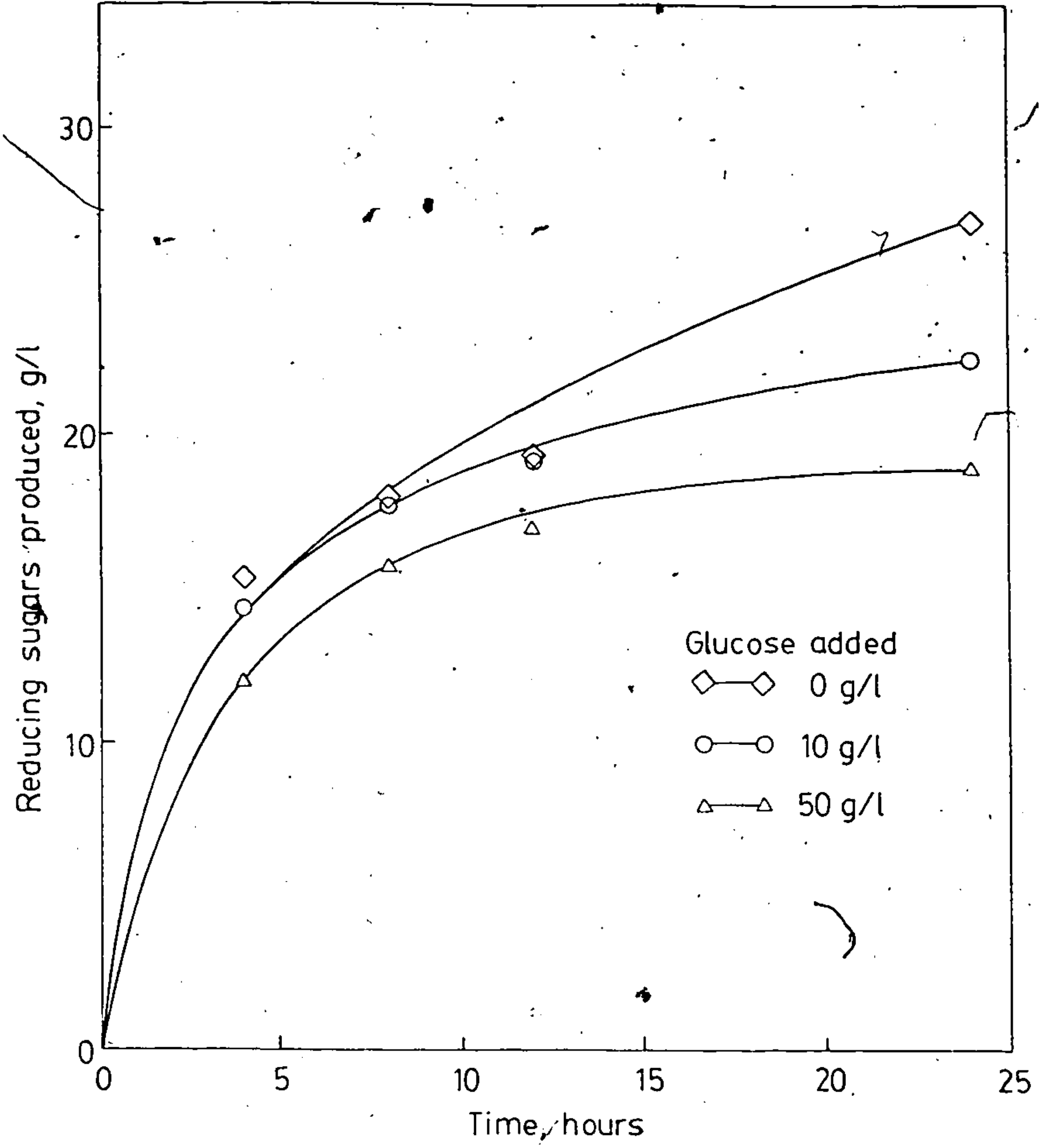

Figure 20: Effect of initial addition of glucose on the production of reducing sugars.

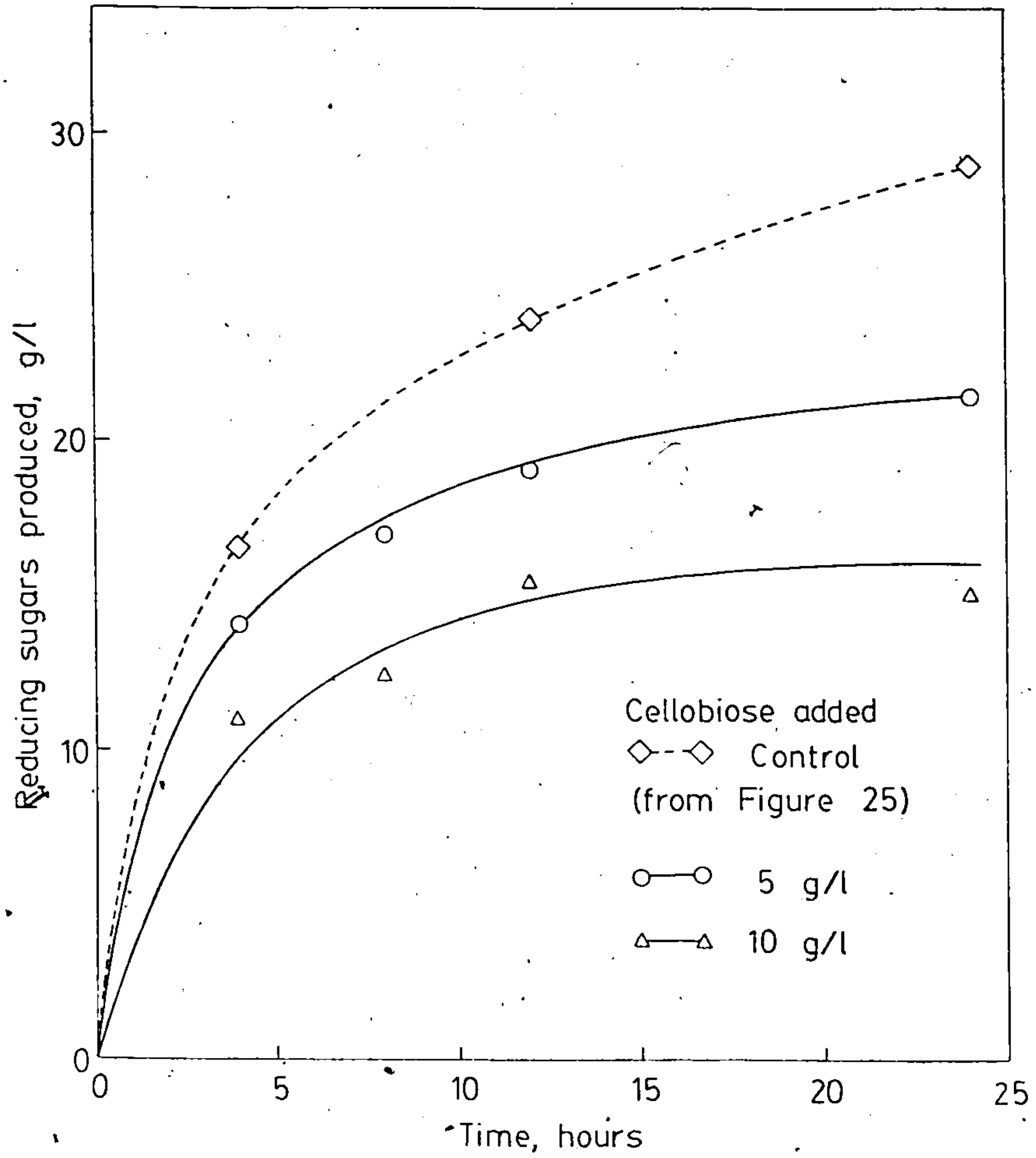

Figure 21: Effect of initial addition of cellobiose on the production of reducing sugars.

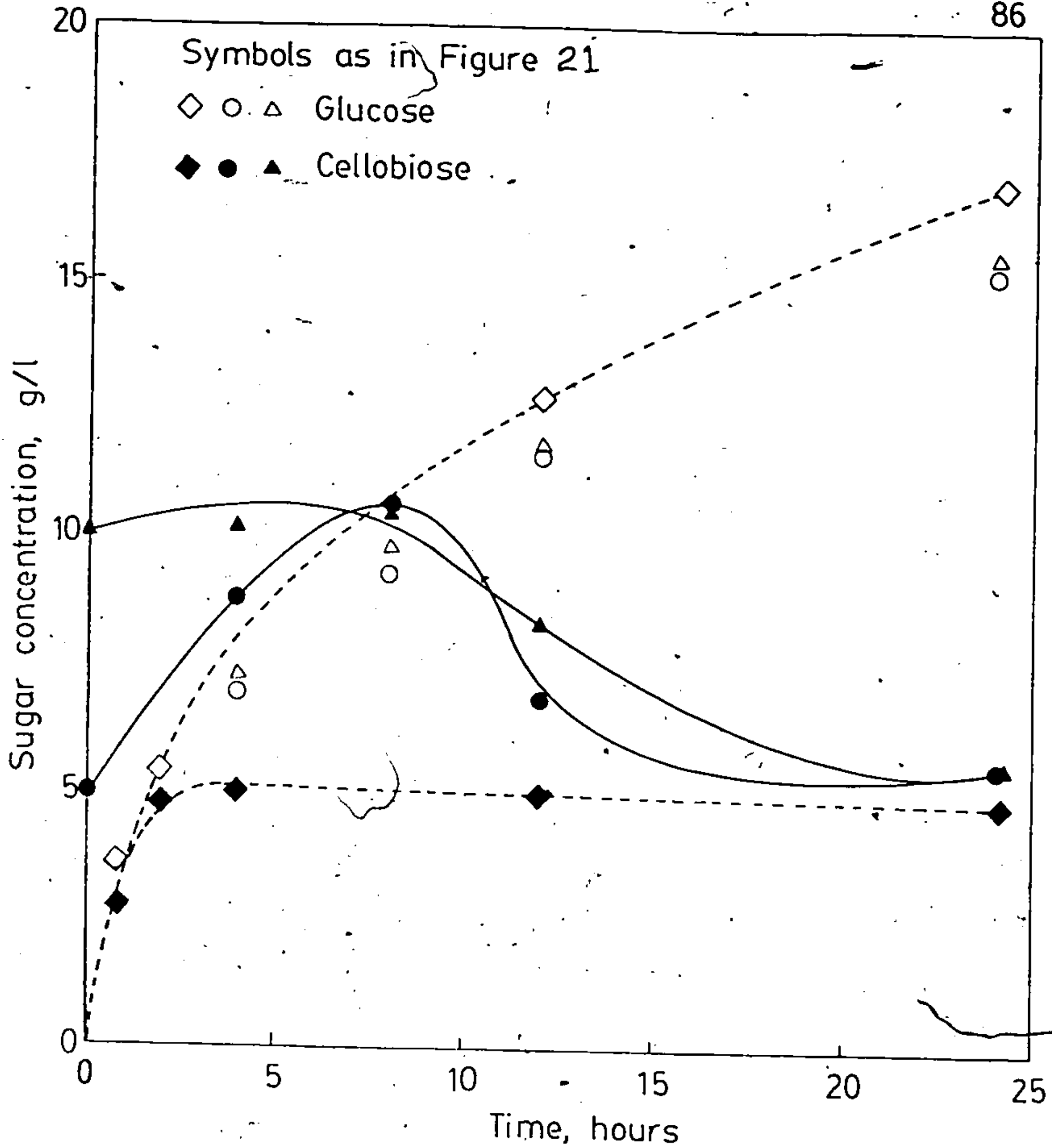

Figure 22: Effect of initial addition of cellobiose on the concentrations of glucose and cellobiose.

4.2.5 Hydrolysis of partially hydrolyzed cellulose

Although it is obvious that end-product inhibition plays an important role in explaining the decrease observed in the reaction rate throughout the hydrolysis, this is not likely the only important factor. As pointed out before, a fast accumulation of cellobiose is observed during the initial period of hydrolysis, after which it remains nearly constant, and yet the reaction rate continues decreasing. The hydrolysis of the more susceptible or amorphous portion of cellulose would leave behind a crystalline residue. Because of the increased crystallinity, the residual cellulose would be more resistant to enzymatic hydrolysis, which would also result in a lower hydrolysis rate. This phenomenon was investigated by using the spent solids from a prior hydrolysis experiment (residual cellulose from hydrolysis of 7% cellulose carried out in the fermentor vessel). In the first treatment with the enzymes about 47 % of the cellulose was converted to reducing sugars (see Figure 24); the remaining solids were filtered, washed and dried, and then were treated with the enzyme for a second time.

Table 25 compares the hydrolysis of fresh and partially hydrolyzed cellulose. The progression of both hydrolyses is shown in Figure 23 . The per cent conversion with the fresh cellulose was close to 49 % while only 29 % of the partially hydrolyzed cellulose was converted to reducing sugars. The concentration of reducing sugars for the partially hydrolyzed cellulose was about 60 % of that obtained with the fresh cellulose. However, the individual saccharides were not in the same proportion. Glucose and cellobiose concentrations for the partially hydrolyzed cellulose were about 70 % of those obtained from the fresh cellulose, while xylose concentration was only about 37 %. Based on the sum of these three sugars, the yield and reaction rates would be about 62 % of the values for fresh cellulose. This demonstrates that modifications in the structure of cellulose are partially responsible for the decrease observed in the reaction rates.

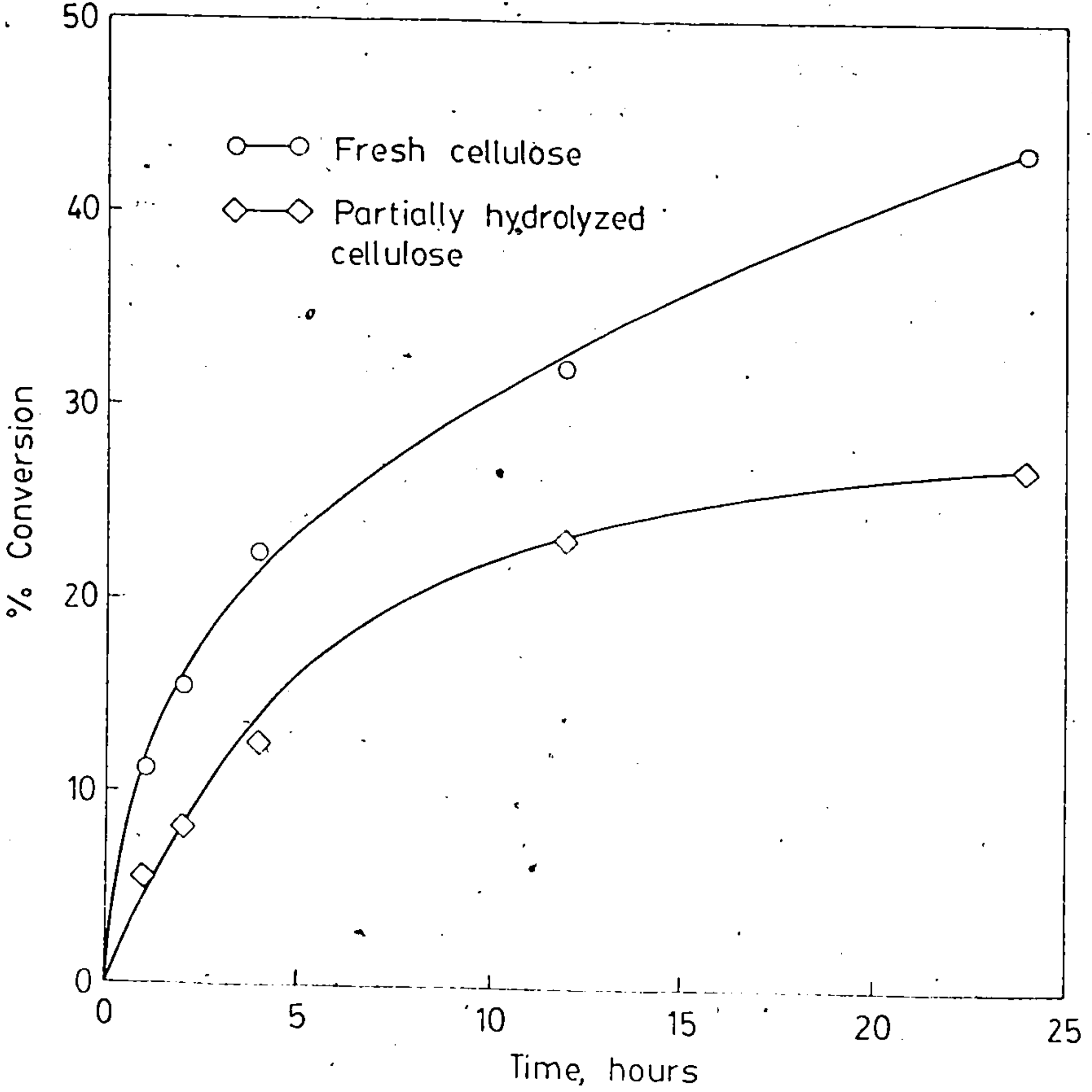

Figure 23: Comparison of the hydrolysis of fresh and partially hydrolyzed cellulose.

4.2.6 Inactivation of the enzymes

In addition to end product inhibition and structural changes, enzyme inactivation is also responsible for the reduction observed in the reaction rate. Factors such as heating or mechanical shear cause the denaturation or destruction of protein molecules, thereby lowering the actual activity of the enzymes in the reaction mixture. The extent to which the enzymes are deactivated is a function of the operating conditions at which the hydrolysis is conducted (e.g., temperature, time, pH, reactor geometry, etc.), as well as the type and strain of the microorganism employed to produce the enzyme. Therefore an experiment was conducted to evaluate the extent of enzyme inactivation under the operating conditions employed for the hydrolysis experiments.

The raw fermentation broth, as obtained from fermentation run # 9, was diluted with an equal volume of 0.1 M citrate buffer and incubated in shake flasks at 50 °C and 200 rpm. As can be seen from Figure 24, after 48 hours of incubation under these conditions, only about 70 % of the initial filter paper activity was detected. It was observed however, that inactivation took place at a faster rate in the first 24 hours; during this period of time, 22 % of the filter paper activity was lost while only 8 % was inactivated in the next 24 hours. On the other hand, salicinase activity decreased much faster than filter paper activity. About 40 % of the enzyme activity had been lost after the first 8 hours of incubation but the the activity remained nearly constant from then on.

Since the incubation was done in the absence of cellulose fibres these results do not take into account the possible interactions with the substrate. Nevertheless they provide a general idea of the combined effects of temperature and mixing on enzyme stability. Under the actual conditions of use, beta-glucosidase is more rapidly deactivated than the other enzymes of the cellulase complex and this partly explains the rapid accumulation of cellobiose in the initial period.

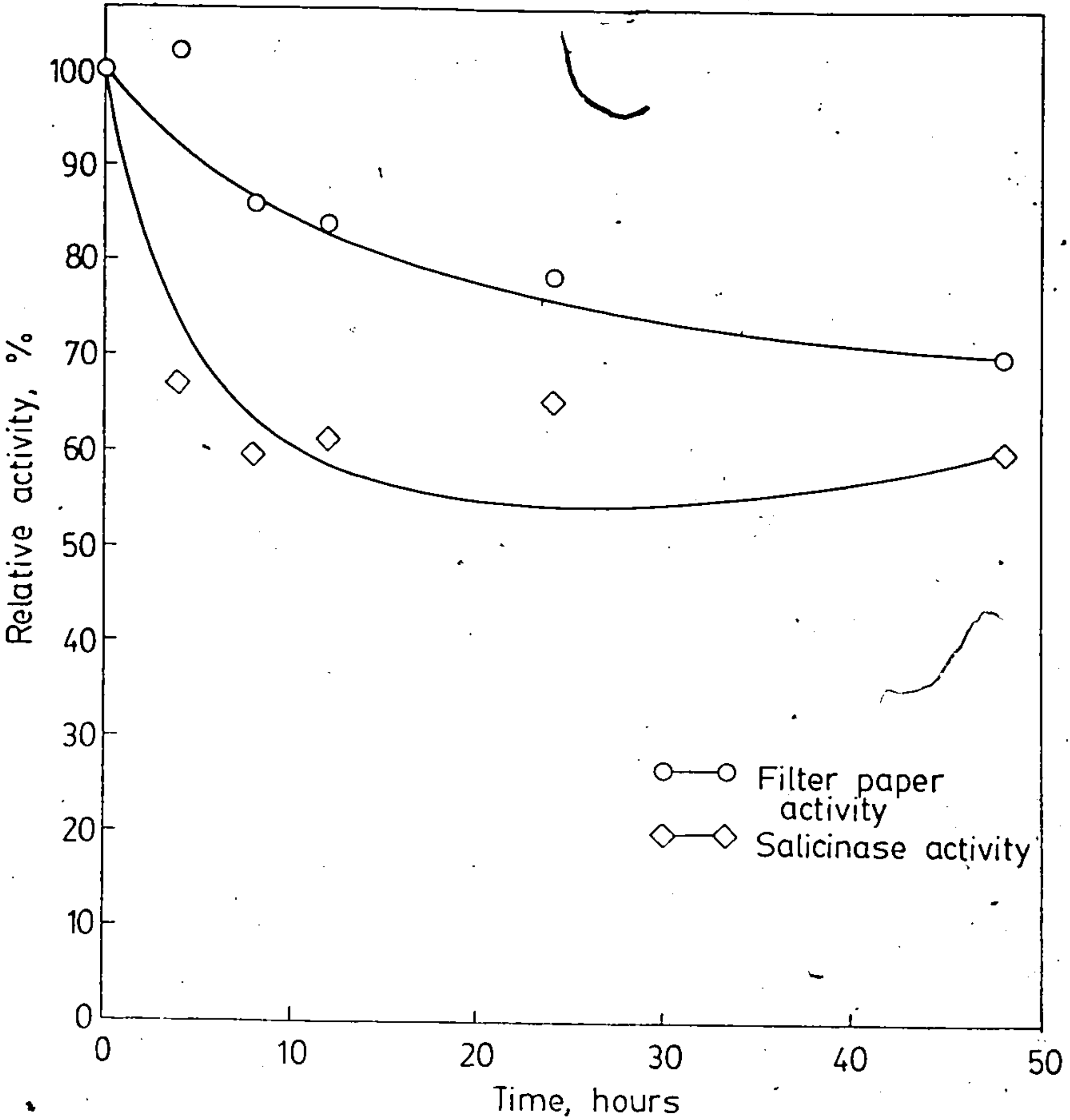

Figure 24: Enzyme deactivation under the actual use conditions (50 °C, 200 rpm, shake flasks).

4.2.7 Addition of fresh enzyme during the hydrolysis

It has been shown that end-product inhibition, changes in the susceptibility of cellulose to hydrolysis, and inactivation of the cellulase complex result in a sharp decrease of the reaction rate during the course of the hydrolysis. The addition of fresh enzyme to replenish the inactivated or inhibited enzyme molecules could "boost" the reaction rate.

In one particular hydrolysis experiment, the hydrolysis was allowed to proceed in the usual manner for a period of 24 hours. After this period of time a volume of 50 ml of an enzyme preparation (raw broth from fermentation run # 11) were added to a series of 3 flasks while the same volume of distilled water was added to a similar series of flasks. Then the hydrolysis was continued for another 24 hours. Results of this particular experiment are given in table 26 . Figure 25 shows the effect of adding fresh enzyme and water, and compares its progression with a control experiment corresponding to no addition of either water or enzymes. The addition of water did not have any significant effect on the reaction rate and simply diluted the reaction mixture. The addition of fresh enzyme however, did increase the reaction rate; although this increase was rather modest compared to the initial reaction rate. Once again, this reflects that either the residual cellulose was more resistant to hydrolysis or that the enzymes were at least partially inhibited by the sugars already present in the mixture. 24 hours after the addition of the extra enzyme, the reducing sugar concentration of the control (no addition) was only slightly higher than that of the hydrolyzate diluted by the addition of the fresh enzyme (see Figure 25).

Figure 26 shows the yields of reducing sugar and glucose during the hydrolysis. It is evident that although similar concentrations were attained, the addition of fresh enzyme benefited the hydrolysis in terms of total amounts of of sugar produced. The yields of reducing sugar and glucose with addition of fresh enzyme were 30 and 37 % higher than those obtained for the control. Therefore multiple additions of enzyme at an earlier time may help to maintain a high reaction rate and achieve higher conversions. However, the addition of more than one load of fresh enzyme requires some analysis, since it would depend on the cost of pretreatment and product recovery, as well as the cost of the enzyme solution itself.

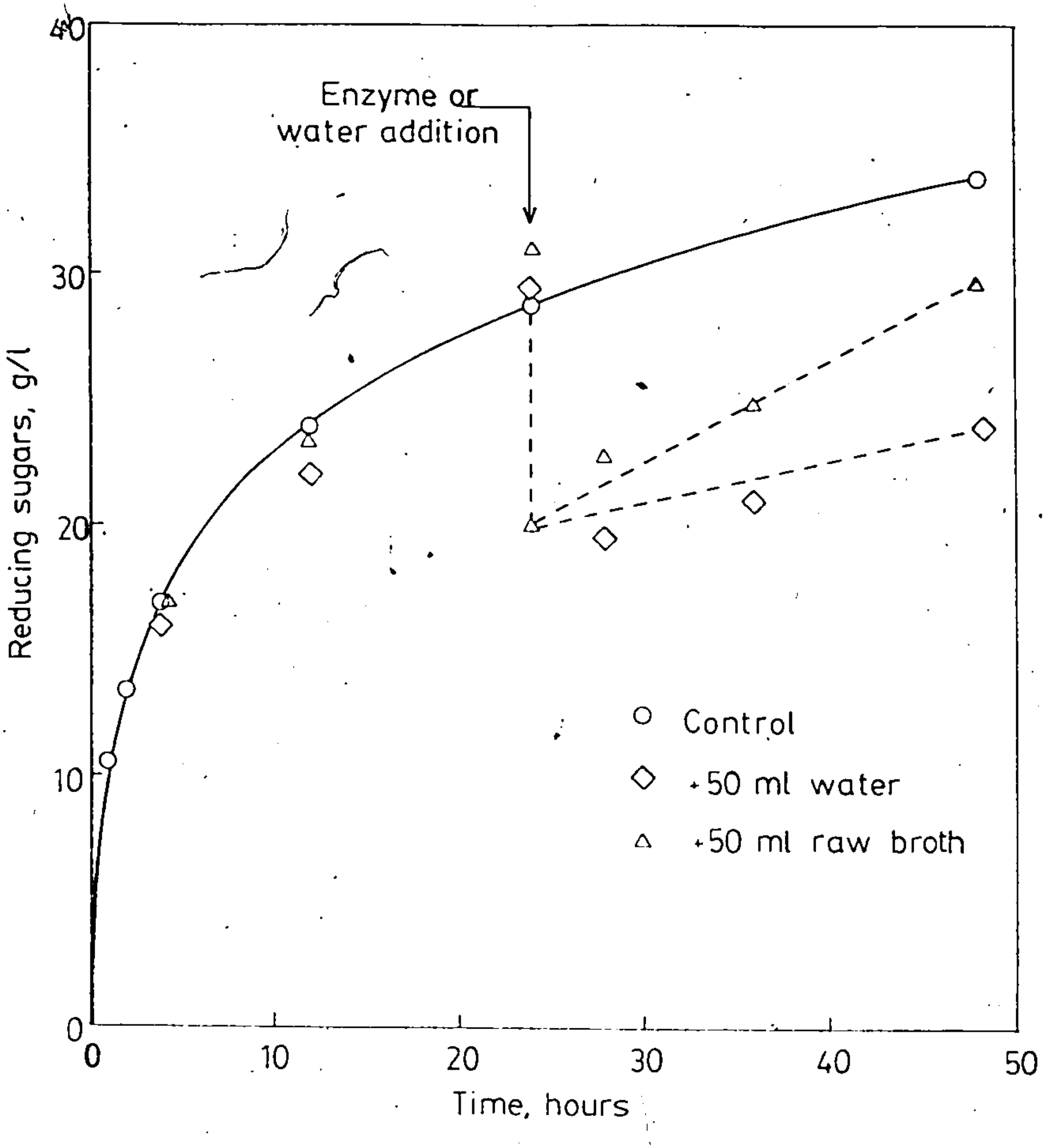

Figure 25: Effect of the addition of water and fresh enzyme to the reaction mixture.

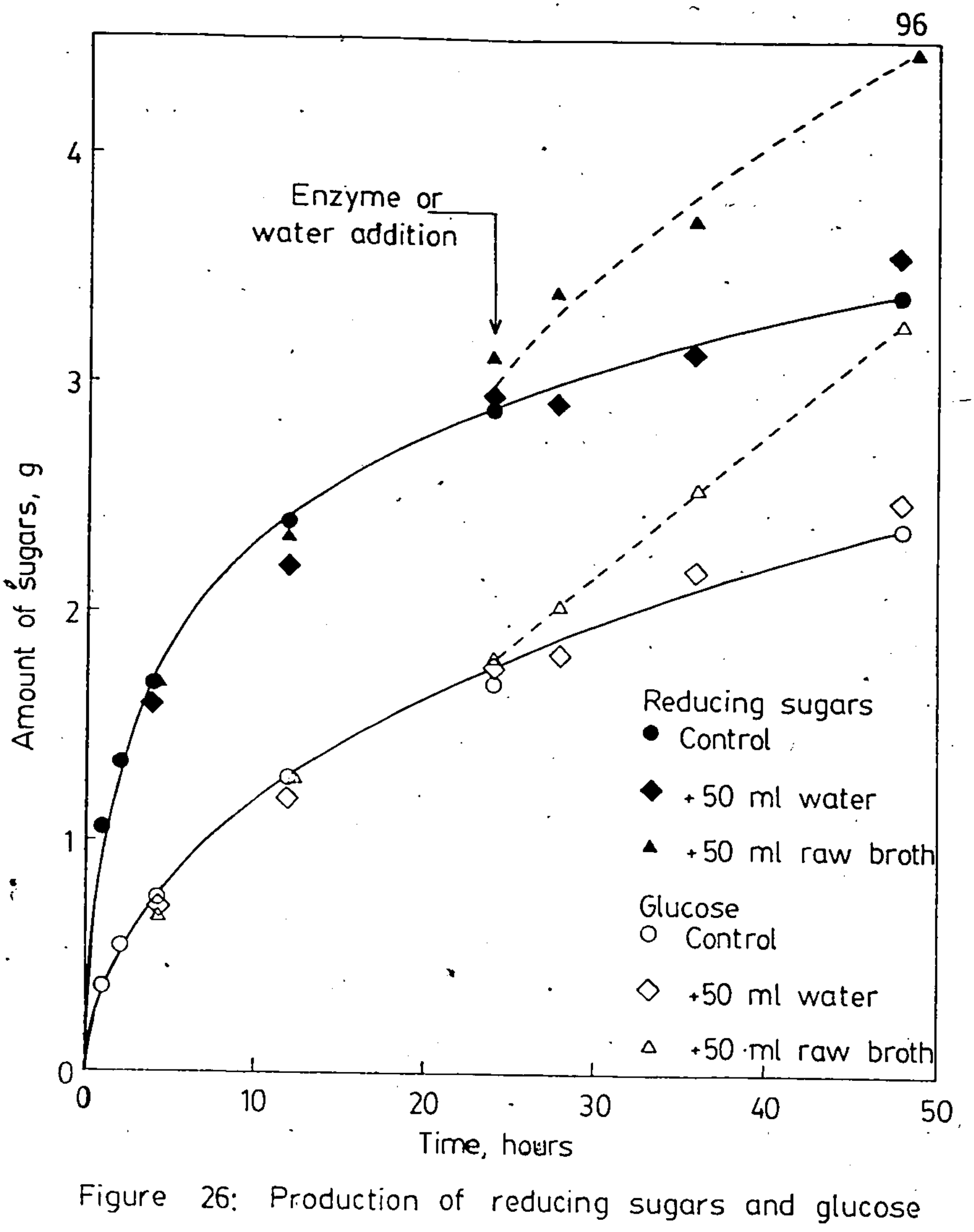

Figure 26: Production of reducing sugars and glucose by hydrolysis of cellulose with addition of water and fresh enzyme.

4.2.8 Reactor configuration

The experiments described above were performed in shake flasks and required the addition of buffer to maintain the pH within the optimum range. This type of experiment is convenient for screening studies, where the effect of several variables can be evaluated at the time. However, studies of the reaction at more realistic conditions are necessary, especially for scaling up the process. The use of a stirred-tank reactor would allow control of pH by the addition of acid or alkali instead of the more expensive buffers. Other variables such as temperature and mixing rate could also be conveniently controlled. A convenient hydrolysis reactor would be the fermentor used to produce the cellulase since it is designed to provide a carefully controlled environment to the culture. In addition, in eventual small-scale processes (e.g., farm-level technologies) the utilization of the same vessel for carriying out the fermentation and hydrolysis steps would decrease the capital cost.

Thus, two hydrolysis experiments were carried out in the 5-liter fermentor vessel. The sterilized cellulose was added to the crude fermentation broth to yield concentrations of approximately 40 and 70 g/l (i.e., 4 and 7 %) The enzyme to cellulose ratios were about 20 and 40 IU/gram of cellulose for these two slurries respectively. The enzyme preparation used with the 40 g/l slurry was obtained from the fermentation of 1 % cellulose (fermentation run # 1, Table 4), which was allowed to proceed for only 48 hours at which time the maximum in intracellular protein was attained. In the second case the raw broth from a 2 % cellulose fermentation (fermentation run # 10) was harvested after seven days and used for the hydrolysis. Results for these two experimental runs are given in Table 27 . In Figure 27, the percent conversion is plotted as a function of time. Although the enzyme to cellulose ratio for the 7 % slurry was twice the ratio used for the 4 % slurry, comparable concentrations for reducing sugars were obtained in both cases: of course, a much higher conversion (expressed as % of original cellulose) was achieved with the 4 % slurry. This may seem contradictory to the fact stated before that either increasing the cellulose concentration and/or the enzyme to cellulose ratio resulted in higher reaction rates and increases in sugar yields. However, it was observed that with the 7 % slurry the mixing provided by the two 6-blade impellers was not adequate and resulted in the creation of diffusion controlled zones. The poor mixing also resulted in inefficient control of temperature and pH in the first hours. As the hydrolysis proceeded the mixture became more fluid and the mixing improved. This may explain the difference in the results obtained and what could be expected under these conditions.

A 70 % conversion was achieved in 24 hours for the hydrolysis of the 4 % slurry. This value is higher than the

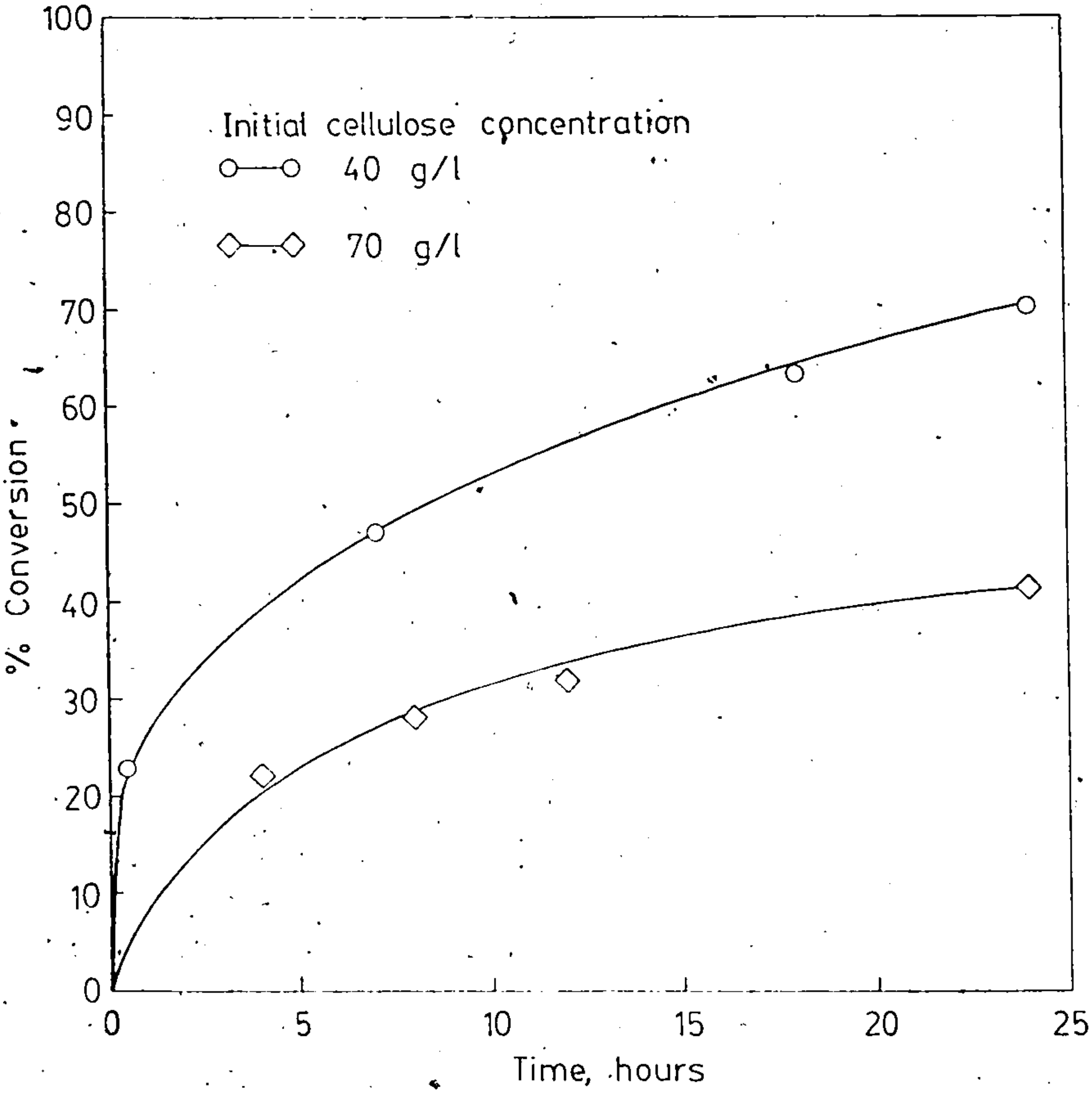

Figure 27: Hydrolysis of 4 and 7 % cellulose slurries in the fermentor vessel.

57 % obtained with the same cellulose concentration (FPA/Co = 18.7 IU/g) in shake flask conditions. A possible explanation would be that better mixing and control of the operating conditions was achieved in the fermentor. There was also a difference in the age of the enzyme preparation; in the former case it was 48 hours whereas in the latter was about 160 hours. It has been shown before that the filter paper activity of a culture considerably increases in the stationary and post-stationary phases of growth. Therefore, it could be possible that the difference observed between hydrolyses carried out in the fermentor and in shake flask could have been caused by the release of some additional enzyme during the hydrolysis in the fermentors vessel. No further studies were done on this respect, but it opens the possibility of using the raw fermentation broth shortly after the cell biomass has built up. For a fermentation of 2 % cellulose this would mean a fermentation time of 3-4 days, as opposed to the seven-day period which is usually allowed for the accumulation of the enzyme.

The utilization of the fermentor, which is a standard stirred tank, for carrying out the hydrolysis reaction is feasible at low cellulose concentrations. However, as previously discussed, increasing the initial cellulose concentration in the hydrolysis step results in higher sugar concentrations and is therefore more economical. Consequently using the same vessel for fermentation and hydrolysis would require an optimized design.

4.2.9 Barley straw as a substrate for hydrolysis

It has been shown that NaOH-pretreated barley straw can be used as a substrate for the production of the enzyme, and potentially it could be employed as a substrate for the production of sugar.

Several experiments were carried out, using the pretreated barley straw for the saccharification step. In these cases the enzyme preparations used were those obtained from barley straw fermentations (i.e., run # 12, 13, 14 and 15). The pretreated barley straw was used in both the wet and dry forms (see section 3.3). Table 28 lists the results obtained for hydrolysis experiments using both forms of barley straw and Solka floc. The production of reducing sugars and glucose are plotted as a function of time in Figure 28 . It was observed that comparable results were obtained for both the wet and dry forms of the straw. Conversions of about 55 % were attained in 24 hours. The percent conversion for Solka floc under these conditions was only of about 30 % at 24 hours. Therefore, the sugar concentration of barley straw hydrolyzates were twice as high as those from Solka floc. It was also observed that the reaction rate declined much faster with the pure cellulose. This seems to indicate that the pure cellulose is more crystalline than the natural pre-treated substrates. Also, barley straw contains hemicelluloses which are readily hydrolyzed by a complex enzyme.

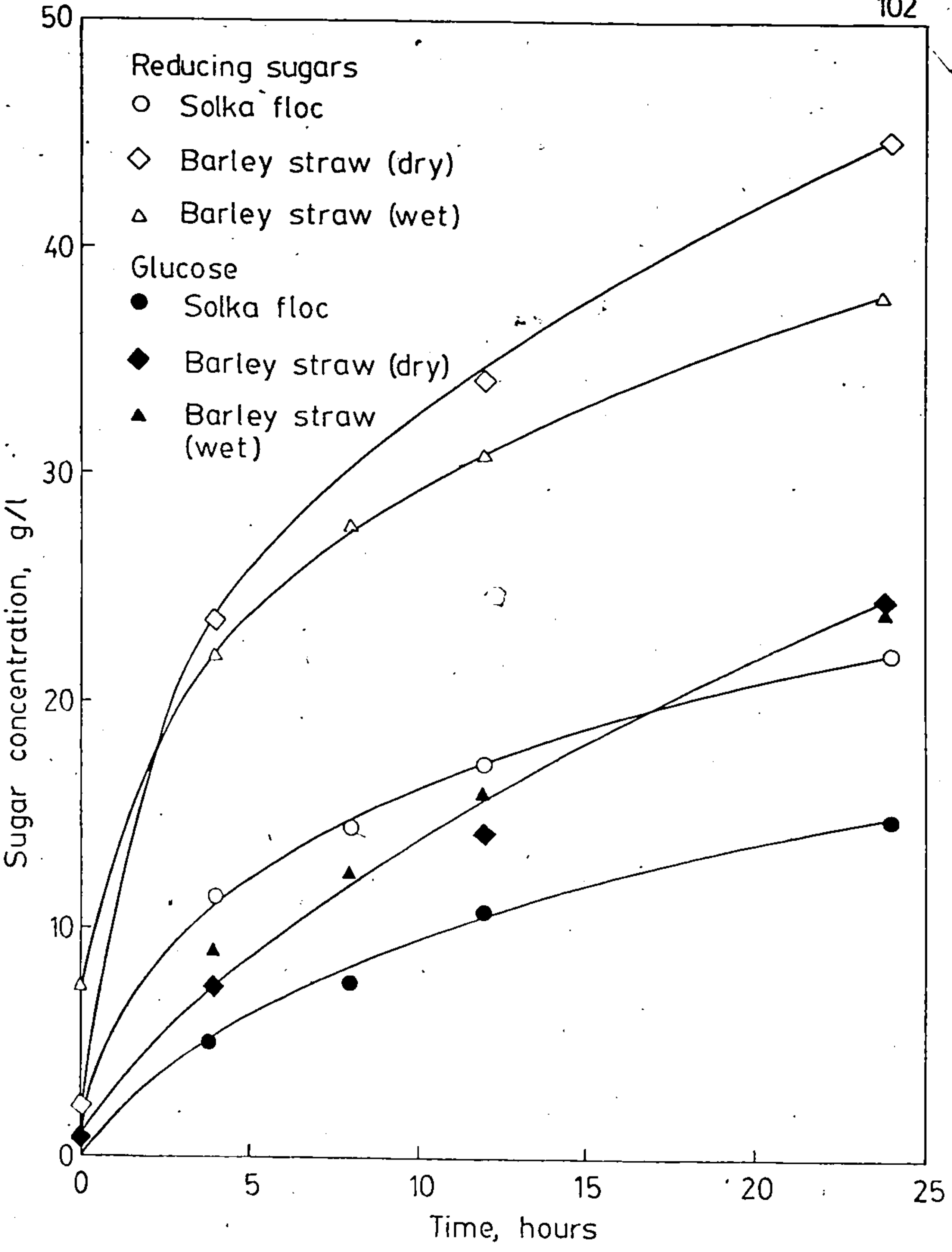

Figure 28: Production of sugars by hydrolysis of NaOH-pre-treated barley straw.

The filter paper activities obtained from fermentations of 2 % barley straw (average 1.51 IU/ml) were lower than those obtained from fermentations of 2 % Solka flock (average 2.50 IU/ml). However, reducing sugar concentrations of about 40 g/l were attained in about 24 hours from hydrolysis of barley straw slurries (see Figure 28). Similar or slightly lower sugar concentrations were obtained from the the hydrolysis of Solka floc slurries (see Tables 20 and 21), eventhough the filter paper activities of the enzyme preparations used in these cases were almost twice as high. It therefore appears that the enzyme complex, which was produced from barley straw, had a composition that acted more specifically towards the substrate from which it was produced.

From this results it is apparent that the on-site production of the enzymes would increase the efficiency of the hydrolysis step and decrease the enzyme requirements since due to the specificity of the enzyme complex ,more enzyme is required to achieve similar results with enzyme from another source. It has to be pointed out that during the pretreatment, the liquid fraction containing lignin and hemicellulose was discarded. However, recent advances in hemicellulose utilization indicate that the conversion of hemicellulose to pentoses (specially xylose) is relatively easy compared to cellulose conversion and its utilization may contribute to the economic feasibility of conversion of lignocellulosic materials.

Chapter V

CONCLUSIONS

5.1 CONCLUSIONS

1.- In cellulose fermentations by the cellulolytic microorganism Trichoderma reesei QM 9414, conducting the fermentation at a pH of 3.0 benefits the production of enzyme. Both growth and enzyme production are slightly retarded when the pH is controlled at 3.0 instead of 4.0, but final enzyme activities are higher.

2.- It is possible to avoid strict sterilization and minimize the risk of contamination by conducting the fermentation at pH 3.0 and using an adequate aseptic technique. The quality of the enzymes appears not to be affected by mild bacterial contamination.

3.- By increasing the concentration of Solka floc SW-40 from 1 to 2 %, almost a 2-fold increase in cell biomass and enzyme yields are obtained. The utilization of higher cellulose concentration requires improvement of the design of the fermentor, or the use of another form of cellulose with a higher bulk density.

4.- Barley straw and similar materials can be employed as substrates for the production of the enzyme. However, because of the lower cellulose content of the pretreated barley straw (ca., 80 %), filter paper activities are lower than those obtained when the microorganism is grown on pure cellulose. Nevertheless, when the enzyme obtained from these fermentations was employed in the hydrolysis of barley straw slurries, it had a saccharifying capacity similar to that of enzyme preparations from 2 % Solka floc.

5.- The on-site production of the enzyme would increase the capital cost of a hydrolysis plant, but the cost of handling and transportation could be avoided. For a small-scale plant the capital cost could be maintained at a minimum if the same reactor vessel can be employed for fermentation and hydrolysis. A standard stirred tank of the type employed for fermentation can be employed for hydrolysis of up to 4 % slurries; utilization of higher cellulose concentrations requires an improved design.

6.- The raw broth, as obtained from the fermentation, can be employed for the hydrolysis of cellulosic substrates without filtration or any further treatment. Similar sugar concentrations were obtained by hydrolysis of cellulose with raw broth and culture filtrates, however it appears that small quantities of extra beta-glucosidase are released from the remaining mycelial cells during hydrolysis.

7.- As with culture filtrates or purified enzymes, the hydrolysis of cellulosic materials with raw fermentation broth is affected by the presence of inhibitory products, changes in the susceptibility of cellulose to hydrolysis due to increased crystallinity, and enzyme inactivation by heat and shear.

8.- Sugar yields and the extent of conversion are greatly affected by the enzyme to cellulose ratio. Since the yield and conversion achieved are a function of the log of the enzyme to cellulose ratio (FPA/Co) a great deal of enzyme is required to achieve high conversions and thus it appears that the enzyme works more efficiently in a diluted environment. Therefore, the adequate enzyme concentration is to be determined by the cost of the enzyme and the cost of product recovery.

9.- The addition of fresh enzymes resulted in an increase in the reaction rate and in the total amounts of sugars produced, therefore increasing the productivity. However, because of the cost of the enzymes the extra 30 % obtained with a second addition of enzymes may not be justified. Similarly a second treatment of partially hydrolyzed or residual cellulose may be indicated if the cost of pretreatment is high and if the cost of the enzymes were sufficiently low.

5.2 RECOMMENDATIONS

1.- Fermentations runs were conducted for 160 hours, at which time most of the enzymes had been released into the medium; however, the enzymes appear to have been synthesized earlier during the fermentation. Thus, studies should be undertaken to determine at which stage of the fermentation the different components of the cellulose complex are synthesized.

2.- In order to increase the enzyme activities and thus the productivity of the fermentation, either the design of the reactor could be improved so as to permit the utilization of higher initial cellulose concentrations or, another approach to the production of cellulases such as fed-batch fermentation could be taken.

3.- Some work could be done to optimize the design of the reactor for the utilization of the same vessel for fermentation and hydrolysis.

4.- Further work could be done to attempt to solve the problem of end-product inhibition, a possible approach would be the addition of glucose isomerase to convert glucose to fructose which is a poor inhibitor of beta-glucosidase. Also, efforts should be directed to increase the stability of the enzyme preparation, specially for beta-glucosidase which appears to be inactivated under the operating conditions.

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Appendix A

ANALYTICAL METHODS

Protein content of fermentation broth by Biuret method

Reference (42)

1. Apparatus and reagents
   - a) 6-12 ml centrifuge tubes
   - b) Marbles (fitting on the tubes)
   - c) 2-ml volumetric pipet
   - d) 5-ml measuring pipet (with end cut off to avoid tip clogging)
   - e) 1 Micropipettor (up to 1 ml capacity)with appropriate pipet tips
   - f) Boiling water bath
   - g) Centrifuge
   - h) Aspirator (vacuum flask with rubber stopper and U-shaped glass tubing)
   - i) Spectrophotometer and cuvettes (13 mm test tubes previously matched)
   - j) Vortex mixer
   - k) 3.0 N aqueous Na OH solution
   - l) Aqueous $CuSO_4 \cdot 5H_2O$, 2,5 % (w/v)

m) Protein standard (bovine albumin, stock solution: 4 g/l)

2. Procedure:

a) Pipet two 2-ml samples of fermentation broth with measuring pipet and place in two centrifuge tubes.

b) Add to each tube about 6 ml of deionized water and shake tube gently.

c) Centrifuge 5 minutes at 2000 rpm.

d) Withdraw 5 to 6 ml of supernatant with aspirator.Shake tube gently and add equivalent volume of water. Centrifuge as in prior step.

e) Repeat step (d) at least once. At the end, make up volume in the tube to 2 ml.

f) Prepare standards (2-ml volumes) at different concentrations (e.g., 1.0, 2.0, and 4.0 g/l) in centrifuge tubes.

g) Pipet a 2-ml volume of water in last centrifuge tube (to be used as blank)

h) Add 1.0 ml of 3.0 N NaOH solution to each tube, cover with a marble and place in a boiling water bath for 10 minutes. Cool in cold water.

i) Add 1.0 ml of the copper sulfate solution, cover the top of the tube with a square of "Parafilm" held down by the thumb and shake thoroughly, allow to stand 5 minutes and centrifuge 10 minutes at 2000 rpm.

j) Pour delicately supernatant in a cuvette and read absorbance against the blank at 555 nm. Draw a standard curve by plotting the concentration of standard solutions against absorbance (or determine line equation by linear regression).

3. <u>Calculation</u>: Determine the concentration of protein in sample as g/l with the calibration curve (or equation). Report the average of the two readings.

4. <u>Comments</u>

a) This procedure has been developed for measuring the protein content of <u>fresh</u> samples of broth.

b) The absorbance should be measured within one hour.

Determination of cellulose content by Updegraff's method

Reference (43)

1. Apparatus and reagents
   - a) Centrifuge and tubes (50 ml)
   - b) Pipets
   - c) Cold water bath
   - d) 95 °C water bath
   - e) Spectrophotometer and cuvettes
   - f) Vortex test tube mixer
   - g) Acetic/nitric reagent (720 ml glacial acetic acid + 180 ml H_2O + 90 ml conc. nitric acid)
   - h) Anthrone reagent (740 ml conc. H_2SO_4 ; 260 ml water. Cool until warm to touch. This takes about 4-5 hours. Then add 0.5g anthrone, 10.0 g thiourea. Refrigerate overnight before use.)
   - i) Sulphuric acid 67% (700 ml conc. H_2SO_4 + H_2O up to 1 litre)
   - j) Standard solution (0.1 mg/ml).- Predry 100 mg pure cellulose for 6 hours at 105 °C. Cool over anhydrous alumina. Weigh 50 mg of predried cellulose and add 15.0 ml 67% sulphuric acid; dilute with H_2O to 500 ml.
2. Procedure
   - a) Place 20-30 mg dry sample in 16 mm test tube.
   - b) Add 3.0 ml of acetic-nitric reagent. Do not mix. Cover with plastic cap.

c) Place in 95 °C water bath for 40 minutes. Cool well.

d) Centrifuge at 2,600 rpm for 10-20 minutes.

e) Aspirate and discard supernatant.

f) Add 2 ml deionized water, vortex, add another 8 ml water to wash.

g) Centrifuge at 2,600 rpm again, aspirate and discard supernatant.

h) Add 3 ml 67 % sulphuric acid and mix well on vortex.

i) Let stand 1 hour but mix occasionally during that time.

j) Dilute up to 100 ml in volumetric flask.

k) Save 1 test tube of this diluted mixture.

l) Pipet 0.5 ml into an 18 mm test tube and refrigerate for 10 minutes.

m) Add 5.0 ml of <u>cold</u> anthrone reagent; mix as you add. Mix immediately again.

n) Place in water bath at 95 °C for 20 minutes.

o) Cool in water bath for 2-5 minutes.

p) Let stand at room temperature for 5-10 minutes.

q) Read at 620 nm against a reagent blank (0.5 ml water + anthrone reagent). Include 3 standards with different concentrations in each run. Standards should be of a volume of 0.5 ml. and treated as the samples.

3. <u>Calculation</u>: From calibration curve determine concentration of cellulose as mg/ml.

Filter paper activity assay

Based on reference (44)

1. Apparatus and reagents
   - a) Test tubes
   - b) Marbles fitting the tubes
   - c) Filter paper # 1 cut into strips of 1 cm X 6 cm
   - d) Water bath at 50 °C
   - e) Water bath at 95 °C
   - f) Spectrophotometer and cuvettes (previously matched)
   - g) Citrate buffer 0.1 M (see DNS method)
   - h) 3,5 Dinitrosalicylic acid reagent (see DNS method)
   - i) Standard solution: glucose 1 g/l
2. Procedure:
   - a) Place 1 ml of diluted sample in test tube (try several dilutions).
   - b) Add 1 ml of 0.1 M citrate buffer.
   - c) Coil the strip of filter paper.
   - d) Vortex, add coiled filter paper and vortex again.
   - e) Cover tubes with the marbles and incubate at 50 °C.
   - f) After 1 hour incubation add 3 ml of DNS reagent.
   - g) Boil tubes for 10 min (or 95 °C).
   - h) Cool and centrifuge (if necessary).
   - i) Read absorbance at 600 nm against a blank.
3. Notes:

a) A substrate blank is included for each sample to correct for the presence of reducing sugars. 1 ml sample + 1 ml buffer + 3 ml DNS reagent. Do not add filter paper.

b) The blank is prepared with 1 ml distilled water + 1 ml buffer + 3 ml DNS reagent.

c) A series of standards are included with each run (e.g., 0.2, 0.4, 0.6, 0.8 and 1.0 g/l)

d) Substrate blanks, blank and standards are treated in the same way as the samples

4. Calculation: From regression analysis or calibration curve determine the concentrations for samples and substrate blanks, and determine activity by:

FPA (IU/ml)= (Csample - Csubs. blank) X Dil.Fac./10.8

where C is the apparent glucose concentration.

Determination of salicinase activity

Based on reference (45)

1. Apparatus and reagents
   - a) Test tubes
   - b) Marbles fitting the tubes
   - c) 1 % salicin solution (diluted in citrate buffer)
   - d) Water bath at 50 °C.
   - e) Water bath at 95 °C.
   - f) Spectrophotometer and cuvettes (previously matched)
   - g) Citrate buffer 0.1 M (see DNS method)
   - h) 3,5 Dinitrosalicylic acid reagent (see DNS method)
   - i) Standard solution: glucose 1 g/l
2. Procedure:
   - a) Place 1 ml of diluted sample in test tube (try several dilutions).
   - b) Add 1 ml of the 1 % buffered salicin solution.
   - c) Vortex.
   - d) Cover tubes with the marbles and incubate at 50 °C.
   - e) After 1 hour incubation add 3 ml of DNS reagent.
   - f) Boil tubes for 10 min (or 95 °C).
   - g) Read absorbance at 600 nm against a blank.
3. Notes:
   - a) A substrate blank is included for each sample to correct for the presence of reducing sugars. 1 ml

sample + 1 ml buffer + 3 ml DNS reagent. Do not add salicin solution.

b) The blank is prepared with 1 ml distilled water + 1 ml buffer + 3 ml DNS reagent.

c) A series of standards are included with each run (e.g., 0.2, 0.4, 0.6, 0.8 and 1.0 g/l).

d) Substrate blanks, blank and standards are treated in the same way as the samples.

4. Calculation: From regression analysis or calibration curve determine the concentrations for samples and substrate blanks, and determine activity by:

Salicin activity (IU/ml)= (Csample - Csubs. blank) X Dil.Fac./10.8

where C is the apparent glucose concentration.

Determination of Kjeldahl Nitrogen

Based on references (42 and 45)

1. Apparatus and reagents
   - a) Digestion unit
   - b) Pipets for dilution (or pipettor and adequate tips)
   - c) Spectrophotometer and cuvettes
   - d) 100 ml volumetric flasks
   - e) Test tubes
   - f) Reagents
     - i) Acid digestion mixture.- Dissolve Selenium oxide (SeO_2,1g) in distilled water (50 ml) in a 100 ml flask and add MAR grade concentrated sulphuric acid slowly to 100 ml.
     - ii) Nessler's reagent.- Available from Fisher chemicals, formula of Koch and McMeekin.
     - iii) Standard nitrogen solution.- Dry AnalaR ammonium chloride (153 mg) is dissolved in 100 ml. of distilled water in a volumetric flask; 25 ml of this solution and 10 ml of 1 N sulphuric acid are diluted to 1000 ml to provide a solution of which 1 ml is equivalent to 10 micrograms of nitrogen.
2. Procedure:
   - a) After dry weight determination place 20-50 mg of residue into digestion tube (or flask).

b) Add 3 ml of acid digestion mixture and heat gently until water evaporates off and then more strongly so that the acid boils.

c) After 4 hours the tubes are removed fron the heat and allowed to cool.

d) Dilute the digested sample to 100 ml with deionized water.

e) Transfer 1 ml of diluted sample into test tubes, complete volume to 7 ml and add 3.0 ml of Nessler's reagent.

f) After standing for exactly 5 min at room temperature the optical density of the solutions is measured at 465 nm in a spectrophotometer set to give 100 % transmittance with a blank.

3. Notes:

a) A blank is prepared with 7 ml of water and 3 ml of Nessler's reagent.

b) A set of standards is included with each run (i.e., 5, 10 and 20 micrograms of Nitrogen), the volume completed to 7 ml with distilled water and then treated ns the samples

4. Calculation: From standard curve or regression analysis determine the concentration of the samples (diluted)

mg of Nitrogen in dry sample= 10 C

where C=concentration in microgram determined for diluted sample

Total Carbohydrate determination by phenol method

Reference (47)

1. **Apparatus and reagents**
   - a) 5 % phenol solution
   - b) Concentrated sulphuric acid
   - c) 20-mm test tubes
   - d) Pipettor (up to 1 ml) and appropriate tips
   - e) Pipettor (up to 5 ml) and appropriate tips
   - f) Spectrophotometer and cuvettes
   - g) Vortex mixer
   - h) Glucose standard solution (0.1 mg/ml)
2. **Procedure:**
   - a) Place 1 ml of appropriately diluted sample in 20-mm test tube.
   - b) Add 1 ml 5 % phenol solution.
   - c) Mix using vortex mixer.
   - d) Add rapidly 5 ml of concentrated sulphuric acid directly against surface of liquid.
   - e) Allow to stand 10 minutes.
   - f) Shake.
   - g) Place in hot room (25-30 °C) for 15 minutes.
   - h) Read at 490 nm against a reagent blank.
3. **Notes:**
   - a) Prepare a standard curve corresponding to 20, 40, 60, 80 μg/ml.

b) Include a reagent blank and standard sample with each run.

c) For acid addition a "blow-out" 5 ml pipette with the end cut off to allow rapid addition should be used.

d) The colour is stable for several hours

4. Calculation: From calibration curve determine concentration of glucose in sample as as μg/ml

Determination of Reducing sugars by DNS method

Based on reference (48)

1. Apparatus and reagents

   a) Test tubes and marbles fitting the tubes

   b) Water bath at 95 °C

   c) Pippetor (up to 1 ml)

   d) Pipettor (up to 5 ml)

   e) Centrifuge

   f) Vortex mixer

   g) Spectrophotometer and cuvettes

   h) Reagents

      i) Citrate buffer (0.5 M).- Dissolve 35 g citric acid monohydrate and 98 g sodium citrate dihydrate in distilled water. Complete volume up to 1 litre. Keep in the refrigerator.

      ii) Citrate buffer (0.1M).- Dilute 200 ml of the citrate buffer stock solution (0.5 M) to 1 litre with deionized water. Keep in the refrigerator.

      iii) 3,5 dinitrosalicylic acid reagent.- Dissolve 64.0 g NaOH in 3 litres of distilled water. Add 40.0 g of 3,5 dinitrosalicylic acid and 1,200 g of NaK tartrate. Bring the volume to 4 litres with water. Warm while stirring. Keep in the refrigerator.

iv) Glucose standard solution: 1 g/l (keep refrigerated)

2. Procedure

a) Place 1 ml of dilute sample in test tube (try several dilutions).

b) Add 1 ml 0.1 M citrate buffer and mix in vortex.

c) Add 3 ml DNS reagent.

d) Cover tubes with marbles heat at 95 °C for 10 minutes.

e) Cool and centrifuge if necessary (if solution is cloudy).

f) Read absorbance at 600 nm against a blank prepared with 1 ml of distilled water instead of sample.

3. Notes:

a) For dark or colored samples include another series of "background" samples replacing DNS reagent with distilled water. The absorbance of these samples is read against a blank prepared with 4 ml of water and 1 ml of buffer.

b) Include a series of standards with each run (e.g., 0.2, 0.4, 0.6, 0.8, 1.0 mg/ml).

4. Calculation: Determine the concentration of glucose as g/l with the calibration curve. For samples requiring background samples:

Corrected OD = OD sample - OD background

Appendix B

COMPARISON BETWEEN PROTEIN CONTENT BY BIURET AND KJELDAHL, AND SAMPLE CALCULATION OF FP-ACTIVITIES

COMPARISON BETWEEN PROTEIN CONTENT BY BIURET AND KJELDAHL

Sample	A Protein Content Biuret	% Nitrogen (dry basis)	Dry weight (g/l)	B Protein Content Kjeldahl	Difference (A-B)
1	0.09	0.65	20.00	0.82	0.73
2	1.44	N.T.	17.85	-.-	-.-
3	2.98	3.91	15.26	3.73	0.75
4	3.24	5.10	12.55	4.00	0.76
5	2.88	5.04	9.89	3.11	0.23
6	2.53	N.T.	8.12	-.-	-.-
7	1.96	5.30	8.04	2.66	0.70
8	1.72	N.T.	7.83	-.-	-.-
B.S.	N.T.	0.60	-.-	-.-	-.-

At time 0 (before inoculation)

Amount of Barley straw in the fermentor = 66.7 g

% of N in barley straw = 0.006

Total amount of N in the fermentor = 0.4 g

which would represent (0.4 X 6.25) = 2.50 g of protein

Apparent concentration of protein = 2.50 g / 3.45 l = 0.72 g

B.S. = Pretreated Barley Straw
Protein content (Kjeldahl)= % Nitrogen X Dry Weight X 6.25

SAMPLE CALCULATION OF FILTER PAPER ACTIVITIES

		OPTICAL DENSITY		CONCENTRATION (mg/ml)			
Time	Dilution factor	B Subst. blank*	A filter paper	B subst. blank*	A filter paper	(A-B) mg/ml	FPA IU/ml
0	5	0.008	0.022	0.111	0.166	0.055	0.02
24	5	0.011	0.031	0.123	0.202	0.079	0.03
48	5	0.103	0.251	0.486	1.071	0.585	0.27
72	10	0.049	0.309	0.273	1.301	1.028	0.95
120	10	0.030	0.407	0.198	1.688	1.490	1.38
144	20	0.008	0.323	0.111	1.356	1.245	2.30
161	20	0.006	0.315	0.103	1.324	1.221	2.26

Standards: (Glucose)	Concentration (mg/ml)	Optical Density
	0.2	0.032
	0.4	0.083
	0.6	0.125
	0.8	0.184
	1.0	0.234

From linear regression: Conc. (mg/ml) = 3.95(O.D.) + 0.08

and

$$\text{FPA (IU/ml)} = \frac{1\ \mu\text{mol} \times 1\ \text{hour} \times (A-B)(\text{mg/ml})}{0.180\ \text{mg} \times 60\ \text{minutes}} = \frac{(A-B)}{10.8}$$

* Substrate blanks are included to account for the presence of reducing sugars present in the sample and of any coloration. In this case they were also included to account for turbidity due to the presence of antifoam droplets.

Appendix C

EXPERIMENTAL DATA FOR FERMENTATION AND HYDROLYSIS

* Refer to Table 4 for description of fermentation runs

TABLE 4

Experimental data for fermentation run # 1

Sample	Time (hrs)	Total dry weight (g/l)	Protein content (g/l)	Filter paper Activity (IU/ml)
1	0	10.11	0.18	-.-
2	18	9.69	0.85	-.-
3	24	8.39	1.27	0.13
4	31	6.81	1.92	0.43
5	42	5.33	2.03	0.67
6	48	4.47	1.79	0.78

TABLE 5

Experimental data for fermentation run # 2

Sample	Time (hrs)	Total dry weight (g/l)	Protein content (g/l)	Filter paper Activity (IU/ml)
1	0	10.20	0.22	0.02
2	18	9.29	1.00	0.07
3	26	7.69	1.73	0.25
4	42	5.85	1.80	0.50
5	48	4.74	2.00	0.70
6	54	5.01	1.64	0.58
7	66	3.96	1.61	0.75
8	78	3.18	1.16	0.82
9	94	2.45	0.96	0.88
10	120	2.15	0.79	0.97

TABLE 6

Experimental data for fermentation run # 3

Sample	Time (hrs)	Total dry weight (g/l)	Protein content (g/l)	Filter paper Activity (IU/ml)
1	0	10.0	0.23	-.-
2	18	9.67	0.85	0.03
3	30	8.24	1.30	0.11
4	42	7.83	1.47	0.30
5	54	8.98	2.34	0.50
6	66	5.49	1.87	0.91
7	78	4.53	1.72	0.77
8	92	3.44	1.37	0.88
9	119	2.43	1.05	1.24
10	144	1.84	0.84	1.23
11	162	1.93	0.84	1.31

TABLE 7

Experimental data for fermentation run # 4

Sample	Time (hrs)	Total dry weight (g/l)	Protein content (g/l)	Filter paper Activity (IU/ml)
1	0	9.75	0.12	-.-
2	23	9.92	1.10	0.08
3	48	7.52	1.47	0.29
4	72	5.84	1.64	0.76
5	96	3.30	1.16	1.12
6	119	2.35	0.82	1.28
7	144	1.93	0.84	1.47
8	162	1.80	-.-	1.55

TABLE 8

Experimental results for fermentation run # 5

Sample	Time (hrs)	T D W (g/l)	Protein content (g/l)	F P A (IU/ml)	Salicin. activity (IU/ml)
1	0	9.38	0.13	0.01	-.-
2	24	9.26	0.94	0.13	0.03
3	48	6.10	1.92	0.35	0.41
4	72	4.06	1.54	1.12	0.56
5	97	3.89	1.24	1.28	0.71
6	143	2.00	0.64	1.35	0.56
7	162	-.-	0.59	1.20	0.34

TABLE 9

Experimental results for fermentation run # 6

Sample	Time (hrs)	T D W (g/l)	Protein content (g/l)	F P A (IU/ml)	Salicin. activity (IU/ml)
1	0	16.95	-.-	0.01	-.-
2	22	17.89	1.07	-.-	0.01
3	46	15.86	3.15	0.18	0.32
4	70	15.72	2.80	0.93	0.41
5	96	15.57	2.94	1.88	0.31
6	122	12.66	2.24	2.61	0.18
7	142	12.43	2.20	3.00	0.20
8	166	9.70	1.89	2.90	0.20

TABLE 10

Experimental results for fermentation run # 7

Sample	Time (hrs)	T D W (g/l)	Protein content (g/l)	F P A (IU/ml)	Salicin. activity (IU/ml)
1	0	16.91	0.23	0.03	-.-
2	22	19.45	1.16	0.03	-.-
3	46	14.27	2.82	0.26	0.18
4	70	10.35	3.35	0.88	0.47
5	94	6.75	2.76	1.63	0.43
6	119	5.40	2.30	2.50	0.31
7	142	3.73	1.54	2.34	0.25
8	160	4.05	1.49	2.47	0.19

TABLE 11

Experimental results for fermentation run # 8

Sample	Time (hrs)	T D W (g/l)	Protein content (g/l)	F P A (IU/ml)	Salicin. activity (IU/ml)
1	0	16.97	0.17	0.037	-.-
2	24	17.41	1.40	0.04	0.02
3	48	12.69	3.01	0.44	0.18
4	72	8.86	3.50	1.22	0.25
5	96	5.81	2.51	1.82	0.61
6	122	4.62	2.18	2.64	0.18
7	144	4.21	1.64	2.38	0.21
8	161	3.90	1.50	2.76	0.23

TABLE 12

Experimental results for fermentation run # 9

Sample	Time (hrs)	T D W (g/l)	Protein content (g/l)	F P A (IU/ml)	Salicin. activity (IU/ml)
1	0	19.66	0.16	0.02	-.-
2	24	19.39	1.26	0.03	-.-
3	48	13.25	2.83	0.27	0.44
4	72	7.88	3.14	0.95	0.14
5	120	4.27	2.10	1.38	0.28
6	144	3.78	1.64	2.30	0.41
7	161	3.57	1.64	2.26	0.38

TABLE 13

Experimental results for fermentation run # 10

Sample	Time (hrs)	T D W (g/l)	Protein content (g/l)	F P A (IU/ml)	Salicin. activity (IU/ml)
1	0	19.38	0.08	0.02	-.-
2	24	19.67	1.38	0.01	-.-
3	48	15.66	3.23	0.19	0.12
4	72	12.07	3.40	0.55	0.17
5	96	8.71	3.78	1.48	0.26
6	120	6.08	2.62	2.41	0.58
7	144	5.20	2.56	2.53	0.65
8	160	4.89	2.04	2.78	0.64

TABLE 14

Experimental results for fermentation run # 11

Sample	Time	T D W (g/l)	Protein content (g/l)	F P A (IU/ml)	Salicin. activity (IU/ml)	Cellul. content (g/l)
1	0	19.29	0.16	0.01	-.-	18.28
2	24	19.05	1.36	0.03	-.-	16.04
3	48	14.72	3.20	0.22	0.23	9.11
4	72	11.23	3.65	0.71	0.34	4.17
5	98	8.65	3.58	1.28	0.41	1.71
6	120	6.08	2.68	1.82	0.61	0.86
7	144	4.74	2.03	2.23	0.56	-.-
8	161	4.10	1.63	2.20	0.57	-.-

TABLE 15

Experimental results for fermentation run # 12

Sample	Time	T D W (g/l)	Protein content (g/l)	F P A (IU/ml)	Salicin. activity (IU/ml)	Cellul. content (g/l)
1	0	18.72	-.-	0.03	0.01	14.69
2	24	14.16	1.17	0.06	0.04	8.50
3	46	11.81	2.68	0.08	0.01	4.00
4	71	9.23	1.86	0.48	-.-	2.35
5	143	4.42	1.15	1.19	0.95	0.36
6	160	4.02	0.91	1.23	1.04	-.-

TABLE 16

Experimental results for fermentation run # 13

Sample	Time	T D W (g/l)	Protein content (g/l)	F P A (IU/ml)	Salicin. activity (IU/ml)	Cellul. content (g/l)
1	0	20.00	0.09	0.03	0.01	16.45
2	24	17.85	1.44	0.03	0.03	11.91
3	48	15.26	2.98	0.13	0.03	6.00
4	72	12.55	3.24	0.49	-.-	2.72
5	96	9.89	2.88	0.88	0.09	1.42
6	120	8.12	2.53	1.33	0.24	1.37
7	144	8.04	1.96	1.53	0.33	0.86
8	161	7.83	1.72	1.54	0.37	0.80

TABLE 17

Experimental results for fermentation run # 14

Sample	Time (hrs)	T D W (g/l)	Protein content (g/l)	F P A (IU/ml)	Salicin. activity (IU/ml)
1	0	21.88	0.21	0.03	0.01
2	24	16.10	0.94	0.12	0.11
3	48	12.93	2.32	0.09	0.07
4	71	15.90	3.08	0.44	0.16
5	93	8.35	2.44	0.91	0.20
6	121	6.27	1.96	1.62	0.48
7	144	5.95	1.03	1.67	0.51

TABLE 18

Experimental results for fermentation run # 15

Sample	Time (hrs)	T D W (g/l)	Protein content (g/l)	F P A (IU/ml)	Salicin. activity (IU/ml)
1	0	19.28	0.22	0.02	-.-
2	24	15.12	0.84	0.02	0.02
3	48	12.21	2.30	0.10	0.01
4	72	11.97	2.62	0.40	-.-
5	96	9.46	2.02	0.90	0.04
6	120	7.15	1.71	1.57	0.25
7	144	6.60	1.45	1.27	0.29
8	162	6.44	1.26	1.34	0.30

TABLE 19

Effect of different initial cellulose concentrations

Time (hours)	T D W (g/l)	Total Carbo. (g/l)	Reducing Sugar (g/l)	T D W + R.S. (g/l)
4 % SOLKA FLOC (FPA/Co)=19.25 IU/g				
4	29.54	13.02	11.49	41.03
8	25.01	16.10	14.55	39.56
25	18.53	26.15	23.10	41.63
51	17.32	30.46	26.77	44.09
7 % SOLKA FLOC (FPA/Co)=11.0 IU/g				
4	54.66	17.04	14.55	69.21
8	-.-	22.19	19.23	-.-
25	45.25	31.40	28.00	73.25
51	42.26	38.00	33.09	80.26
10 % SOLKA FLOC (FPA/Co)=7.7 IU/ml				
4	83.27	19.07	17.60	100.87
8	77.18	26.20	22.49	99.67
25	75.49	31.25	28.00	103.49
51	77.77	35.51	30.64	108.41

Enzyme preparation from fermentation run # 4 (FPA = 1.55 IU/ml)
Hydrolysis carried out in shake flask (200 rpm, 50 C)

TABLE 20

Effect of different initial enzyme concentration

Time (hours)	T D W (g/l)	Total Carbo. (g/l)	Reducing Sugar (g/l)	T D W + R.S. (g/l)
FILTER PAPER ACTIVITY 0.55 IU/ml (FPA/Co = 7.6 IU/g)				
4	55.40	15.27	13.31	68.85
8	54.04	18.75	15.73	69.77
12	54.69	21.39	16.94	71.36
24	49.26	25.64	21.77	71.03
FILTER PAPER ACTIVITY 1.1 IU/ml (FPA/Co = 15.2 IU/g)				
4	52.68	22.79	18.35	71.03
8	50.47	27.61	22.58	73.05
12	50.59	30.52	25.60	76.19
24	41.47	38.04	33.86	75.33
FILTER PAPER ACTIVITY 2.2 IU/ml (FPA/Co = 31.4 IU/g)				
4	50.42	27.00	23.38	73.80
8	45.17	33.68	27.62	72.79
12	45.50	39.60	33.26	78.76
24	37.25	42.81	36.48	73.73

Initial substrate concentration = 70 g/l Solka floc
Enzyme preparation from fermentation run # 9 (FPA = 2.2 IU/ml)
Hydrolysis carried out in shake-flask (200 rpm, 50 C)

TABLE 21

Comparison of cellulose hydrolysis with raw broth and culture filtrates

SET A

			HPLC ANALYSIS			
Time (hours)	TDW (g/l)	R.S. (g/l)	Xylose (g/l)	Glucose (g/l)	Cellob. (g/l)	X+G+C (g/l)
CULTURE FILTRATES						
4	59.97	14.33	3.45	6.00	3.93	13.35
8	54.97	18.29	5.10	10.00	4.80	19.00
12	53.60	20.07	6.16	12.96	3.70	22.82
29	48.98	25.22	7.30	18.50	3.20	29.00
RAW FERMENTATION BROTH						
4	57.59	14.53	3.65	5.75	3.95	13.35
8	52.71	18.09	4.95	10.28	3.93	19.16
12	52.35	20.66	5.40	12.90	4.00	22.30
29	46.24	26.21	7.80	20.00	3.70	31.50

FPA/Co = 8.60 IU/g
Enzyme preparation from fermentation run # 5 (FPA = 1.20 IU/ml)
Hydrolysis carried out in shake flask (200 rpm, 50 C)
Initial substrate concentration = 70 g/l Solka floc

Table 21 (continued)

SET B

			HPLC ANALYSIS			
Time (hours)	TDW (g/l)	R.S. (g/l)	Xylose (g/l)	Glucose (g/l)	Cellob. (g/l)	X+G+C (g/l)
CULTURE FILTRATES						
4	51.50	19.50	4.15	7.70	4.90	16.75
8	45.57	24.20	5.40	11.50	5.60	22.50
12	42.92	30.07	6.20	15.80	6.40	28.40
24	36.70	35.56	7.40	21.60	5.40	34.40
48	32.35	44.38	8.20	29.20	4.00	41.40
RAW FERMENTATION BROTH						
4	52.30	18.90	4.60	8.45	5.65	18.70
8	48.31	25.57	6.00	12.10	7.20	25.30
12	44.66	28.77	6.20	14.10	7.30	27.60
24	38.11	31.84	7.04	19.80	5.60	32.44
48	27.35	41.83	7.80	26.16	3.30	37.26

FPA/Co = 17.64 IU/g
Enzyme preparation from fermentation run # 7 (FPA = 2.47 IU/ml)
Hydrolysis carried out in shake flask (200 rpm, 50 C)
Initial substrate concentration = 70 g/l Solka floc

Table 21 (continued)

SET C

Time (hours)	T D W (g/l)	R.S. (g/l)	HPLC ANALYSIS			
			Xylose (g/l)	Glucose (g/l)	Cellob. (g/l)	X+G+C (g/l)
CULTURE FILTRATES						
4	53.08	19.04	4.75	7.58	6.55	18.88
8	47.81	23.86	6.10	9.56	7.10	22.76
12	44.67	27.45	7.04	12.24	7.60	26.88
24	39.65	33.61	7.80	15.90	7.10	30.80
48	35.66	40.80	8.70	22.40	5.20	36.34
RAW FERMENTATION BROTH						
4	53.67	18.62	4.50	6.62	6.00	17.12
8	49.00	23.66	6.30	10.40	6.50	23.20
12	45.71	28.17	6.60	12.80	6.90	26.30
24	38.83	33.50	7.60	17.70	6.30	31.60
48	34.31	40.48	8.10	22.10	4.84	35.04

FPA/Co = 19.71 IU/g
Enzyme preparation from fermentation run # 8 (FPA = 2.76 IU/ml)
Hydrolysis carried out in shake flask (200 rpm, 50 C)
Initial substrate concentration = 70 g/l Solka floc

TABLE 22

Cellobiose hydrolysis using raw broth and culture filtrates

Time (hours)	sugar (g/l)	Glucose (g/l)	Cellobiose (g/l)	G + C (g/l)
CULTURE FILTRATES				
4	9.20	2.70	8.70	11.40
8	9.68	4.81	5.10	9.91
12	9.88	6.10	4.90	10.0
24	10.61	8.50	2.20	10.70
48	11.38	10.50	0.60	11.10
RAW FERMENTATION BROTH				
4	8.87	3.00	6.80	9.80
8	9.24	5.60	6.25	11.85
12	9.84	7.20	2.80	10.00
24	11.01	9.90	0.73	10.63
48	11.54	11.60	-.-	11.60
FERMENTATION BROTH (BROKEN CELLS)*				
4	9.44	3.40	8.20	11.60
8	9.80	5.95	4.40	10.35
12	10.41	7.90	3.00	10.90
24	11.70	10.10	1.00	11.10
48	11.78	11.20	-.-	11.20

* Cells were broken by mixing for 5 minutes at a high speed in a laboratory blender.

Enzyme preparation from fermentation run # 9 (FPA = 2.26 IU/ml, Sal. act. = 0.380 IU/ml)

Hydrolysis carried out in shake flask (200 rpm, 50 C)

Initial substrate concentration = 10 g/l cellobiose

TABLE 23

Hydrolysis of cellulose with initial addition of glucose

Time (hours)	T D W (g/l)	Total Carbo. (g/l)	Reducing Sugar (g/l)	T D W + R.S. (g/l)
GLUCOSE 0 g/l (CONTROL)				
4	57.61	18.17	15.38	73.00
8	53.48	24.36	18.00	71.47
12	54.14	24.52	19.34	73.48
24	46.33	29.07	27.06	73.39
48	43.74	47.27	37.33	81.07
GLUCOSE 10 g/l				
4	55.65	25.90	24.40	80.05
8	53.66	29.50	27.87	81.53
12	52.12	31.69	29.42	81.54
24	46.42	40.00	32.50	78.92
48	44.07	43.98	41.58	85.65
GLUCOSE 50 g/l				
4	61.14	63.40	61.93	123.07
8	57.12	65.41	65.79	122.91
12	56.10	68.51	66.95	123.05
24	53.94	68.33	68.87	122.81
48	55.93	83.94	78.91	134.85

FPA/Co = 7.85 IU/g
FPA in enzyme preparation 1.1 IU/ml (data for fermentation not shown)
Hydrolysis carried out in shake flask (200 rpm, 50 C)
Initial substrate concentration = 70 g/l Solka floc

TABLE 24

Hydrolysis of cellulose with initial cellobiose addition

			HPLC ANALYSIS			
Time (hours)	T D W (g/l)	R.S. (g/l)	Xylose (g/l)	Glucose (g/l)	Cellob. (g/l)	X+G+C (g/l)
CELLOBIOSE 5 g/l						
4	60.68	18.32	3.40	7.00	8.70	19.10
8	56.71	21.15	3.30	9.30	10.60	23.20
12	55.40	23.37	4.40	11.74	6.80	22.94
24	53.34	25.79	4.60	15.30	5.60	25.50
48	52.00	29.63	5.20	19.00	6.40	28.60
CELLOBIOSE 10 g/l						
4	62.63	19.53	2.70	7.25	10.20	20.15
8	58.46	20.95	3.20	9.80	10.45	23.45
12	57.11	23.98	3.80	11.90	8.30	24.00
24	56.11	23.57	3.60	15.60	5.60	24.80
48	54.63	30.24	4.60	20.40	3.60	28.60

FPA/Co = 16.14 IU/g
Enzyme preparation from fermentation run # 9 (FPA = 2.26 IU/ml)
Hydrolysis carried out in shake flask (200 rpm, 50 C)
Initial substrate concentration = 70 g/l Solka floc

TABLE 25

Hydrolysis of partially hydrolyzed cellulose

			HPLC ANALYSIS			
Time (hours)	T D W (g/l)	R.S. (g/l)	Xylose (g/l)	Glucose (g/l)	Cellob. (g/l)	X+G+C (g/l)
CONTROL (FRESH CELLULOSE)						
1	62.19	10.60	2.60	3.60	3.20	9.40
2	59.23	13.42	3.50	5.35	4.70	13.55
4	54.25	16.84	4.30	7.40	5.00	16.70
12	47.59	24.03	5.10	12.75	5.00	22.85
24	42.36	28.90	6.80	17.00	4.80	28.60
48	39.17	34.18	7.20	23.80	2.60	33.60
PARTIALLY HYDROLYZED CELLULOSE *						
1	66.17	3.87	0.70	2.10	1.90	4.70
2	64.32	5.99	1.05	3.70	3.20	7.95
4	61.33	8.23	2.00	6.05	2.00	10.05
12	53.81	16.29	2.10	9.90	4.00	16.00
24	51.59	19.17	2.40	13.60	3.40	19.40
48	51.14	20.44	3.00	17.00	2.80	22.80

FPA/Co = 15.71 IU/g

* Substrate obtained from hydrolysis run of 7 % in fermentor vessel (see Table 27)

Enzyme preparation from fermentation run # 11 (FPA = 2.20 IU/ml)

Hydrolysis carried out in shake flask (200 rpm, 50 C)

Initial substrate concentration = 70 g/l Solka floc

TABLE 26

Hydrolysis of cellulose with addition of enzyme and water

			HPLC ANALYSIS			
Time (hours)	T D W (g/l)	R.S. (g/l)	Xylose (g/l)	Glucose (g/l)	Cellob. (g/l)	X+G+C (g/l)
CONTROL (NO ADDITION)						
1	62.19	10.60	2.60	3.60	3.20	9.40
2	59.23	13.42	3.50	5.35	4.70	13.55
4	54.25	16.84	4.30	7.40	5.00	16.70
12	47.59	24.03	5.10	12.75	5.00	22.85
24	42.36	28.90	6.80	17.00	4.80	28.60
48	39.17	34.18	7.20	23.80	2.60	33.60
WATER ADDITION						
4	55.72	15.95	3.70	7.00	5.50	16.20
12	46.78	21.92	5.10	11.90	5.00	22.00
24	43.00	29.53	6.40	17.80	4.20	28.40
28	28.03	19.52	4.60	12.10	3.20	19.90
36	27.01	21.07	5.00	14.60	3.60	23.20
48	24.65	23.82	5.50	16.60	2.80	24.90
ENZYME ADDITION						
4	55.24	16.72	3.95	6.70	4.55	15.20
12	46.41	23.40	5.60	12.80	5.30	23.70
24	43.28	31.22	6.60	18.00	4.80	29.40
28	27.37	22.76	4.90	13.60	3.20	21.70
36	24.03	24.88	5.20	17.00	3.40	25.60
48	20.15	29.74	6.20	21.80	2.00	30.00

FPA/Co = 15.71 IU/g (Initially)
Enzyme preparation from fermentation run # 11 (FPA = 2.20 IU/ml)
Hydrolysis carried out in shake flask (200 rpm, 50 C)
Initial substrate concentration = 70 g/l Solka floc

TABLE 27

Data for hydrolysis of 4 and 7 % slurries carried out in the fermentor vessel

Time (hours)	T D W (g/l)	Reducing sugar (g/l)	Conversion %
4 % SOLKA FLOC (FPA/Co = 19.4 IU/g)*			
0.5	30.60	7.55	23.50
7	21.05	19.01	47.38
18	14.69	26.08	63.28
24	13.20	28.37	67.00
48	8.15	36.28	79.63
72	5.56	40.86	86.10
7 % SOLKA FLOC (FPA/Co = 39.5 IU/g)**			
4	54.35	17.50	22.35
8	50.22	21.20	28.26
12	47.65	22.70	31.93
24	41.48	28.00	40.74
48	37.28	33.50	46.74

* Enzyme preparation from fermentation run # 1 (FPA = 0.77 IU/ml)

** Enzyme preparation from fermentation run # 10 (FPA = 2.78 IU/ml

TABLE 28

Hydrolysis of NaOH-pretreated barley straw

Time (hours)	T D W (g/l)	R.S. (g/l)	Xylose (g/l)	Glucose (g/l)	Cellob. (g/l)	X+G+C (g/l)
			HPLC ANALYSIS			
(1) SOLKA FLOC SW-40 (FPA/Co = 11.93 IU/g) Initial substrate concentration = 70 g/l						
4	62.00	11.46	2.75	5.00	3.40	11.15
8	58.22	14.51	3.00	7.70	2.80	13.50
12	54.35	17.36	3.80	10.80	3.30	17.90
24	52.17	22.25	6.10	14.90	-.-	21.00
48	52.55	23.07	3.90	14.40	-.-	18.30
(1) BARLEY STRAW (DRY FORM)* (FPA/Co = 12.50 IU/g)						
0	66.83	2.22	0.75	0.70	-.-	1.45
4	45.06	23.68	6.10	7.50	6.10	19.70
12	35.05	34.27	7.50	14.20	5.00	26.70
24	29.49	44.87	9.20	24.60	5.40	39.20
48	27.13	50.57	11.00	31.40	-.-	42.60
(2) BARLEY STRAW (WET FORM)* (FPA/Co = 9.71 IU/g)						
0	63.33	7.24	1.70	0.70	-.-	2.40
4	47.43	21.91	7.90	8.70	3.30	19.90
8	39.08	27.75	10.00	12.40	3.50	25.90
12	36.83	30.87	11.10	16.00	3.20	30.30
24	29.90	37.75	13.00	24.00	3.00	40.00
48	21.42	46.29	14.00	32.00	1.20	47.20

Hydrolysis carried out in shake flask (200 rpm, 50 C)
* See section 3.4 for definitions of dry and wet forms
(1) Enzyme preparation from fermentation run # 14 (FPA =1.67 IU/ml)

(2) Enzyme preparation from fermentation run # 12 (FPA =1.23 IU/

Appendix D

STATISTICAL ANALYSIS OF EXPERIMENTAL DATA

PAIRED SAMPLE t-TEST

Population: $x-y = D \sim N(\mu, \sigma)$ (σ unknown)

Information: $d_i = (x_i - y_i)$ $i = 1, 2, \ldots n$
(n pairs of points)

Null hypothesis: $\mu = 0$ (no difference exists in the two series of samples)

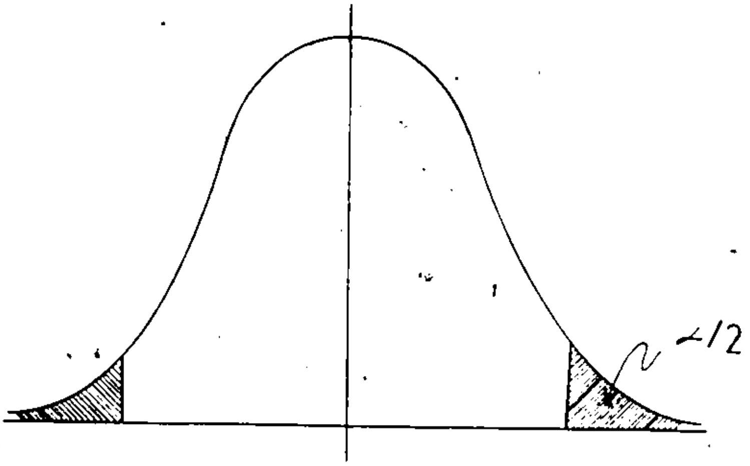
Distribution $= \bar{d} / (s/\sqrt{n}) \sim T_{n-1}$ (student)

where d = arithmetic mean and s = standard deviation

Alternative: $\mu > 0$

Ho is rejected if $t_{n-1} > t_{n-1, \alpha/2}$

$t_{n-1} = \bar{d} / (s/\sqrt{n})$ and $t_{n-1, \alpha/2}$ --> tabulated value *



* From reference 50, p. 30

1.- Analysis of fermentation data

let be

(x_i = Sterile fermentation (run 14 + run 15)/2
and y_i = Aseptic fermentation (run 13)

a) Protein content (g/l)

Time (hours)	xi	yi	di
0	0.218	0.090	0.128
24	0.890	1.440	-0.550
48	2.310	2.982	-0.672
72	2.854	3.243	-0.389
96	2.230	2.880	-0.650
120	1.830	2.530	-0.700
144	1.241	1.961	-0.719

$\bar{d} = -0.507$ $s = 0.302$

95 % Confidence interval

$\bar{d} - t_{n-1,\alpha/2}\,(s/\sqrt{n})$ to $\bar{d} + t_{n-1,\alpha/2}\,(s/\sqrt{n})$

for $\alpha = 0.05$ $t_{6,0.025} = 2.447$

$-0.507 - 2.447\,(0.302/\sqrt{7})$ to $-0.507 + 2.447\,(0.302/\sqrt{7})$

-0.786 to -0.392

$t_{n-1} = \bar{d} / (s/\sqrt{n}) = -4.441 > t_{6,0.025} = 2.447$

<u>Ho is rejected</u>

b) Filter paper activity (IU/ml)

Time (hours)	xi	yi	di
0	0.025	0.028	-0.003
24	0.073	0.028	0.045
48	0.094	0.129	-0.035
72	0.419	0.488	-0.069
96	0.908	0.877	0.031
120	1.594	1.332	0.262
144	1.472	1.536	-0.064

$\bar{d} = 0.0238$ $s = 0.113$

95 % Confidence interval

$\bar{d} - t_{n-1,\alpha/2}\,(s/\sqrt{n})$ to $\bar{d} + t_{n-1,\alpha/2}\,(s/\sqrt{n})$

for $\alpha = 0.05$ $t_{6,0.025} = 2.447$

$0.0238 - 2.447 (0.113/\sqrt{7})$ to $0.0238 + 2.447 (0.113/\sqrt{7})$

-0.080 to 0.128

$t_{n-1} = \bar{d} / (s/\sqrt{n}) = 0.557 > t_{6,0.025} = 2.447$

<u>Ho is not rejected</u>

2.- Analysis of Hydrolysis data

let be

(xi = Raw fermentation broth

and yi = Culture filtrates)

Table 21, set A

a) Reducing sugar (g/l)

Time (hours)	xi	yi	di
4	14.53	14.33	0.20
8	18.09	18.29	-0.20
12	20.66	20.07	0.59
29	26.21	25.22	0.99

$\bar{d} = 0.395$ $s = 0.511$

$t_{n-1} = \bar{d} / (s/\sqrt{n}) = 1.545 < t_{6,0.025} = 3.182$

<u>Ho is not rejected</u>

a) Glucose (g/l)

Time (hours)	xi	yi	di
4	5.75	6.00	-0.25
8	10.28	10.00	0.28
12	12.90	12.96	-0.06
29	20.00	18.50	1.50

$\bar{d} = 0.367$ $s = 0.786$

$t_{n-1} = \bar{d} / (s/\sqrt{n}) = 0.935 < t_{6,0.025} = 3.182$

<u>Ho is not rejected</u>

a) Cellobiose (g/l)

Time (hours)	xi	yi	di
4	3.95	3.93	0.02
8	3.93	4.80	-0.87
12	4.00	3.70	0.30
29	3.70	3.20	0.50

$\bar{d} = -0.0125 \qquad s = 0.604$

$t_{n-1} = \bar{d} / (s/\sqrt{n}) = -0.041 < t_{6,0.025} = 3.182$

<u>Ho is not rejected</u>

Table 21, set B

a) Reducing sugar (g/l)

Time (hours)	x_i	y_i	d_i
4	18.90	19.50	-0.60
8	25.57	24.20	1.37
12	28.77	30.07	-1.30
24	31.84	35.56	-3.72
48	41.83	44.38	-2.55

$\bar{d} = -1.360 \qquad s = 1.937$

$t_{n-1} = \bar{d} / (s/\sqrt{n}) = -1.569 < t_{6,0.025} = 2.776$

<u>Ho is not rejected</u>

a) Glucose (g/l)

Time (hours)	x_i	y_i	d_i
4	8.45	7.70	0.75
8	12.10	11.50	0.60
12	14.10	15.80	-1.70
24	19.80	21.60	-1.80
48	26.16	29.20	-3.04

$\bar{d} = -1.038 \qquad s = 1.651$

$t_{n-1} = \bar{d} / (s/\sqrt{n}) = -1.405 < t_{6,0.025} = 2.776$

<u>Ho is not rejected</u>

a) Cellobiose (g/l)

Time (hours)	x_i	y_i	d_i
4	5.65	4.90	0.75
8	7.20	5.60	1.60
12	7.30	6.40	0.90
24	5.60	5.40	0.20
48	3.30	4.00	-0.70

$\bar{d} = 0.550 \qquad s = 0.858$

$t_{n-1} = \bar{d} / (s/\sqrt{n}) = 1.433 < t_{6,0.025} = 2.776$

<u>Ho is not rejected</u>

Table 21, set C

a) Reducing sugar (g/l)

Time (hours)	xi	yi	di
4	18.62	19.04	-0.42
8	23.66	23.86	-0.20
12	28.17	27.45	0.72
24	33.50	33.61	-0.11
48	40.48	40.80	-0.32

$\bar{d} = -0.061$ $s = 0.450$

$t_{n-1} = \bar{d} / (s/\sqrt{n}) = -0.307 < t_{6,0.025} = 2.776$

Ho is not rejected

a) Glucose (g/l)

Time (hours)	xi	yi	di
4	6.62	7.58	-0.96
8	10.40	9.56	0.84
12	12.80	12.24	0.56
24	17.70	15.90	1.80
48	22.10	22.40	-0.30

$\bar{d} = 0.388$ $s = 1.062$

$t_{n-1} = \bar{d} / (s/\sqrt{n}) = 0.816 < t_{6,0.025} = 2.776$

Ho is not rejected

a) Cellobiose (g/l)

Time (hours)	xi	yi	di
4	6.00	6.55	-0.55
8	6.50	7.10	-0.60
12	6.90	7.60	-0.70
24	6.30	7.10	-0.80
48	4.84	5.20	-0.36

$\bar{d} = -0.602$ $s = 0.166$

$t_{n-1} = \bar{d} / (s/\sqrt{n}) = -8.114 < t_{6,0.025} = 2.776$

Ho is rejected