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PRODUCTION OF FRUCTOSE AND ETHANOL FROM SUCROSE

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Abstract

Fructose is the sweetest sugar; it also has several other desirable properties which make it a valuable product for the food industry.

Today, fructose syrups are widespread but crystalline fructose is not, because of its high production costs.

In this research project, fructose production from sucrose, an abundant raw material, was investigated. The process studied consisted of two steps:

1. Inversion of sucrose into glucose and fructose using an immobilized crude invertase preparation.

2. Selective fermentation of the glucose into ethanol in order to obtain a syrup containing fructose as the main sugar.

Invertase was prepared by treating *Saccharomyces cerevisiae ATCC 46584* with cold acetone. The powder obtained after the acetone dehydration treatment was immobilized in calcium alginate beads. The immobilized invertase preparation was found to have its maximum activity at a temperature of 55°C and at a pH of 4.5. The gel beads were packed in column reactors and continuous hydrolysis of 20% (w/v) and 30% (w/v) sucrose solutions were realized. The highest inversion rate obtained was 60 grams of sucrose per litre of reactor volume per hour.

The activity of the immobilized invertase preparation decreased progressively with time; stability was improved by high substrate concentrations.

The fermentation of glucose to ethanol was performed with *Saccharomyces cerevisiae ATCC 96859* , a yeast unable to use fructose. The optimum temperature for growth of this yeast was found to be 33°C. Batch fermentations of glucose into

ethanol were done under different conditions. The ability of *S. cerevisiae ATCC 36859* to ferment glucose into ethanol was comparable to the ability of other yeast strains used for ethanol production.

Ethanol was also produced continuously in column reactors, packed with yeast cells entrapped in calcium alginate beads and fed with complex media. Activity of the immobilized ethanol-producing cells was easily maintained at a high level for many weeks. With a medium containing 10% (w/v) glucose and 10% (w/v) fructose, a volumetric ethanol productivity of 16.8 g/(L.h), based on the total reactor volume, was reached at a dilution rate of 0.73 h^{-1}.

Finally, sucrose inversion and glucose fermentation were performed in series using a coupled immobilized system. A syrup high in fructose and ethanol was produced continuously.

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Chapter 1

Introduction

Fructose, also called fruit sugar, is one of the most common natural sugars. It occurs, for example, in sweet fruits and berries as well as in honey. It is the sweetest naturally occurring nutritive sweetener (Shallenberger and Birch, 1975), acceptable also to diabetics and dietary health schemes (Suntinanalert *et al.* , 1986); it also has other desirable physical, chemical and metabolic properties making it a versatile raw material for the food industry. It is very hygroscopic and is less cariogenic than sucrose (Doty and Vanninen, 1975).

Although fructose in various forms has always been a part of the human diet, until recently, the use of pure fructose as an ingredient in foods has not been widespread, largely because of high production costs and the consequent small amounts produced. Fructose syrups, on the other hand, are produced more easily and economically and today, they have a large share of the sweeteners market in North America.

1.1 Fructose Syrups

Most of the fructose sold on the market is in the form of high-fructose syrups (HFS). The major use of fructose syrups is in the beverage industry in products such as carbonated drinks, fountain syrups and flavour concentrates. Fructose syrups are

attractive because their sweetness, flavour, storage stability, low viscosity, transparency, clean non-masking taste, pH and microbiological profiles are considered equivalent or better than previous commercial formulas such as glucose or sucrose syrups. The second largest use is in cereal and bakery products, where the fructose syrups are widely accepted because of their high fermentability and high humectancy. Other uses of fructose syrups include those in the food processing industry (for example jams, jellies and preserves) and the dairy industry (Joglekar *et al.* , 1983).

High-fructose syrups, due to their greater sweetness, also make some food products less caloric. This is due to the fact that the carbohydrate content can be reduced to achieve the same degree of sweetness (Joglekar *et al.* , 1983).

High-fructose syrups are mainly produced from corn starch, starch being the most widely available and economical source of dextrose today. Corn starch manufactured by a corn wet milling process is by far the most widely used raw material for dextrose production in the United States, which is the main user and producer of high-fructose syrup in the world. The standard fructose syrup contains 42% fructose, 52% glucose and no more than 6% non-monosaccharides. The process relies on the isomerization of glucose into fructose.

The ability to produce high-fructose corn syrup (HFCS) in Europe is much more limited than in the United States. Imposition of a production levy on HFCS makes it difficult to produce profitably in Europe. The market potential for HFCS in Europe is also limited by other factors:

- Europe is a net importer of corn and a net exporter of sugar, which is the reverse of the situation in the United States (Joglekar *et al.* , 1983).
- The sugar industry in Europe is more influential than in the United States: therefore it has a stronger chance of influencing the development of HFCS and bringing it under regulation by the Economic European Community (EEC), which guarantees a base price for sugar (Joglekar *et al.* , 1983).

Althcugh high-fructose syrups are very useful, pure crystalline fructose is also a desirable product. If it was produced in larger quantities, it would become a

powerful sweetening agent and could be used in some situations in which syrups are not acceptable (e.g. at home and in restaurants). Unfortunately, due to the isomerization reaction:

$$\text{Glucose} \longrightarrow \text{Fructose}$$

the equilibrium constant is close to 1 (one), which means that no more than half of the glucose can be converted into fructose in a single pass. This explains why the HFCS also contains glucose, instead of containing only fructose.

A number of methods for chemical separation of glucose and fructose have been developed. They are based on crystallization, precipitation, complex formation and chromatographic procedures. The fact that equimolar amounts of the two hexose sugars have to be separated necessitates complex and costly procedures for the enrichment and the purification of fructose from such mixtures.

1.2 Fructose

Pure solid fructose can be crystallized from solutions containing fructose, if the ratio of fructose to non-fructose material in solution is high enough. Manufacture of pure crystalline fructose from water solutions of HFCS requires that the solution have a dry basis purity of about 90% fructose or greater. This is in order to achieve sufficient supersaturation of fructose in a solution which is not so viscous that rate of crystallization and subsequent separation of crystals from mother liquor is impeded.

The costs of purification of fructose could be considerably reduced if the glucose were converted to a non sugar compound so as to simplify the separation process. This could be achieved by the use of a microorganism which selectively ferments glucose in a mixture of glucose and fructose. In addition to a cost reduction for fructose purification, a second product, ethanol, would render the process more profitable.

1.3 Objectives

Even though isomerized glucose syrups have gained great popularity, invert sugar still has a big market, especially in those countries where regulations reduce the difference in the production cost which favors syrups obtained from starch (Marconi and Morisi, 1979).

The objective of this work was to produce a high-fructose syrup, using sucrose as the raw material. Sucrose is an abundant resource in many places. Moreover, some countries produce sucrose economically but do not have cheap sources of starch, which render the production of fructose from sucrose attractive.

The process studied in this work consisted of two steps:

1. Inversion of sucrose into an equimolar mixture of glucose and fructose, using a crude invertase preparation.

2. Selective fermentation of glucose into ethanol using a microorganism able to ferment glucose but not fructose.

A goal of this project was to realize both parts of the process continuously by using reactors packed with immobilized preparations of invertase and yeast cells, for sucrose inversion and ethanol production respectively, and to realize both steps simultaneously.

Chapter 2

Literature Review

In order to gain knowledge about invertase, ethanol production and fructose production, the literature has been reviewed. The three main subject areas surveyed were:

- Invertase: its properties and the different methods used for its immobilization.
- Ethanol production: the different systems using immobilized cells for ethanol production.
- Fructose production: the methods used for the manufacture of fructose syrups and crystalline fructose.

2.1 Invertase

2.1.1 Generalities

Among the important industrial enzymes, invertase holds only a modest place. However, it is an enzyme of great interest in many ways.

Invertase was discovered in 1833 by Persoz. It was the first enzyme to be described. The active principle of invertase was demonstrated in 1860 by Berthelot, who gave it its name. The name *invertase* derives from the early observation that a hydrolyzed sucrose solution containing glucose and fructose rotates a polarized

light beam in the direction opposite to that of the original solution. For the same reason, the hydrolysis of sucrose is also called inversion:

sucrose + water $\longrightarrow$ glucose + fructose

$C_{12}H_{22}O_{11} + H_2O \longrightarrow C_6H_{12}O_6 + C_6H_{12}O_6$

$[\alpha]_{D_{sucrose}} = +66.5°$

$[\alpha]_{D_{glucose}} = +52.5°$

$[\alpha]_{D_{fructose}} = -92°$

$[\alpha]_{D_{invert\ sugar}} = -20°$

Industry takes advantage of the presence of invertase in many yeasts to transform the sucrose contained in molasses to a mixture of glucose and fructose. Millions of tons of molasses are treated every year (Joyeaux, 1982). Invertase remains important today: it is one of the most studied of all enzymes, being the subject of both the classic kinetics research of Michaelis and Menten and of the first immobilization efforts.

Invertase Properties

Invertase is found in most yeasts used for brewing and baking. Because of their morphological properties and their genetics, yeasts are easily adapted to industrial conditions. Genetic engineering, by increasing yeasts enzymic potential, is able to enlarge their field of utilization.

Industrially, invertase is produced mainly from the yeasts *Saccharomyces cerevisiae* and *Saccharomyces carlsbergensis*. Invertase can be used in liquid form, in dried cells form or in immobilized form. Unlike bacterial and fungal exoenzymes, invertase must be released by disruption of the cell wall (Wiseman, 1975). Yeast invertase is located in the cell wall where it is freely accessible to sucrose, but cannot be solubilized, except by enzyme digestion (Wiseman, 1975).

Reed and Peppler (1973) reported that there are two types of yeast invertase: an insoluble enzyme associated with the cell wall and a soluble enzyme located inside

the cell. These enzymes are often called "external" and "internal" invertases of yeast.

Properties of yeast invertase, as reported by Gould (1975), are given below:

- Molecular weight: 270 000 (about 50% as carbohydrate)
- pH (for optimum activity): about 4.5, with good activity in the range 3.5–5.5
- pH (for optimum stability): good stability from pH 3.5 to 5.5
- Temperature: maximum activity is achieved between 50°C and 60°C in the presence of dilute sucrose solutions. High sucrose solutions improve stability and may allow temperatures of 65°C to 70°C to be used.
- Inhibitors: heavy metal ions, including Ag^+ which is a particularly potent inhibitor. The metal ions combine reversibly with histidine residues of the enzyme.

Industrial Uses of Invertase

Invertase is mainly used to hydrolyze sucrose in the preparation of invert sugar, needed for jam making and other foods (confectionery, baking, soft drinks, etc.). The production of invert sugar syrup from highly refined sucrose solutions can be carried out with acid but unrefined sucrose solutions (such as molasses) have considerable buffer capacity, and the amount of acid required for inversion would be prohibitive. In that case, the enzyme process is used. An example of this is the use of invertase for the inversion of the sucrose contained in molasses in the preparation of high-test molasses. It is often necessary to store a surplus of partly refined sugar solutions at high concentrations. Inversion of the sucrose to the mixture of monosaccharides prevents crystallization, raises the osmotic pressure and results in a stable concentrate, so-called "high-test" molasses.

Invert sugar has a lower crystallinity than sucrose at the high concentrations employed and does not crystallize out as sucrose would. The mixture of glucose and fructose is more soluble and sweeter than sucrose. According to Sicard (1982), the

degree of sweetness of invert sugar is 1.1 times higher than the degree of sweetness of sucrose.

In industrial practice, two types of invert sugar syrups are produced: a syrup containing 50% sucrose, 25% glucose and 25% fructose (on a dry basis) and a syrup containing 50% glucose and 50% fructose (on a dry basis). The anticrystallization action of fructose makes it possible to produce syrups with higher dry matter content than if sucrose was left untouched.

Invert sugar has the following advantages over sucrose: high humectancy, lower water activity, higher sweetening power. Invert sugar can be used industrially for the production of fructose, sorbitol and mannitol. It is, in most of its uses, in competition with glucose syrups.

Beside being used to hydrolyze sucrose solutions into invert sugar syrups, invertase has other important uses in the food industry. In the candy industry, invertase is employed to hydrolyze sucrose in cream-center candies to achieve the plastic consistency of the center and to prevent sucrose from crystallizing. The hydrolysis proceeds after casting and after enrobing of the candy center with chocolate. In a similar manner, liquid center candies (cherries) are prepared. For use in the candy industry, purified invertase preparations are used. However, crude preparations are employed for inversion of molasses.

2.1.2 Preparation of Different Forms of Enzymes

The use of biocatalysts in reactions with organic compounds has advanced significantly during the past three decades. The discovery of many new enzymes from microbial cells and the development of methods for their isolation and stabilization have contributed to this advance. In addition, various techniques for immobilizing cells and enzymes for use in bioreactors have been developed. Today, a great deal of experimental latitude is possible in the use and form of biocatalysts. A brief description of a few forms of biocatalysts is given below:

Growing Cells

Both batch and continuous cultures are used in bioconversion experiments. In the batch culture technique, a pure culture is grown in a suitable medium. At an experimentally determined time, the substrate is added and incubation is continued until transformation of the substrate ceases or additional reactions seriously begin to affect yields. In the batch process, the biocatalyst is used only once and then discarded. The procedure is straightforward and useful for screening procedures but it requires the repetitive production of cells for each experiment, and isolation of reaction products from complex fermentation media can be complicated (Goodhue *et al.* , 1986).

Resting Cells

Resting cells are nongrowing live cells that retain most of the enzyme activities of growing cells. Resting cells are prepared by removing cells from the culture medium at a time in the growth cycle when enzyme activities are high. Concentrated cells are then suspended in appropriate buffers, modified culture media (usually without some nutrients required for growth), distilled water or even nonaqueous solvent mixtures for use as biocatalysts. Resting cells have several advantages over growing cells or isolated enzymes. Enzymes present in their native state are more efficient than isolated enzymes that are simply recombined in an incubation medium (Goodhue *et al.* , 1986).

Dried Cells

In some cases, the enzyme activities of microbial cells can withstand drying treatment. The resulting powders are convenient biocatalysts. The two most common methods of drying are lyophilization and acetone dehydration (Goodhue *et al.* , 1986).

Lyophilization is a simple technique, not requiring aseptic conditions. Harvested cells are suspended in distilled water or dilute buffer and the suspension is frozen in a dry ice-acetone (or ethanol) bath. When the process is performed correctly,

water sublimes from the frozen cells, forming a fluffy dry powder. Storage of these powders in a freezer will preserve enzyme activity for years. A variety of lyophilized yeasts and bacteria can be obtained commercially.

The treatment of cell pastes or cakes with cold acetone (-20°C) is another reliable method for preparing dried cells. Cells are slurried in cold acetone. They are then collected by suction filtration. The treatment can be repeated. A cold ether wash can be added to remove residual acetone, which can be detrimental to enzyme activities. Acetone powders thus produced are most stable when stored frozen (Gunsalus, 1955).

Both lyophilized and acetone treated cells are easy to prepare and offer many of the same advantages of resting cells compared to growing cultures (Goodhue *et al.* , 1986).

Permeabilized Cells

The permeability of microbial cells can be altered by a variety of substances to facilitate the contact of substrates with enzymes and the excretion of products. Permeabilizing agents include surfactants and solvents. The agents are applied after growth stops. Alternatively, the addition of cell wall synthesis inhibitors during growth enhances permeability (Goodhue *et al.* , 1986).

Cell-Free Preparations

Crude or purified cell-free preparations can be used, in certain situations to catalyze specific chemical reactions. When the enzymes are not stable enough in purified form, one has to explore the possibilities of using crude cell-free preparations instead. Such mixtures are obtained by breaking cells under mild conditions so that the contents are released in an active state into the buffer medium (Gunsalus, 1955). Common techniques for disruption of microbial cells are pressure shearing, enzymatic digestion of cell wall, osmotic lysis, autolysis, and freezing-thawing. Crude broken-cell suspensions, even without purification, catalyze useful organic reactions. Crude preparations have short lifetimes compared to enzymes in unbroken cells. One advantage of cell-free preparations is that permeability barriers are

absent. Methods of eluting and concentrating invertase from yeast have been well explored. Yeast plasmolysis, followed by autolysis has been used commercially for invertase solubilization (Reed and Peppler, 1973). Many solvents have been used for plasmolysis of yeasts: ether, chloroform, ethyl acetate, toluene ... (Arima *et al.*, 1957). This method (plasmolysis) is often followed by subsequent purification of the released enzyme.

A simple method for the manufacture of yeast invertase used as a food additive is described by Takei *et al.* (1967).

Immobilized Enzymes and Cells

Many processes in the chemically related industries can be better accomplished with enzymes than with chemical catalysts. Enzymes have advantages over chemical catalysts such as high degree of specificity, lower required processing temperature, high speed and yield of catalytic reaction. However, the high cost of isolation and purification of enzymes, their decreased stability when removed from the living cell and their disposable use in a given process (usually batch) restrict the application of enzymes in many fields. The constraints can be relaxed and the potential applications of these biocatalysts enhanced by immobilizing them on suitable support materials. The forms of biocatalysts discussed until now are normally used once and then discarded. The exception is the resting-cell suspension, from which cells can be removed for reuse. In the last 15 years, methods have been developed for the immobilization of cells and enzymes that may then be recovered and used again. Bioreactors containing immobilized enzymes, cells or organelles are valuable for continuous processing. Under ideal conditions, solutions of organic reactants passing over the immobilized catalyst emerge as products in the effluent. Biocatalysts are often remarkably stable when in a polymer matrix (Bucke, 1983). Both living and dead microbial cells can be immobilized. Some commercial applications are now firmly established and several reliable methods are available to bind enzymes and cells to insoluble carriers for use in laboratory and industrial processes.

Techniques of immobilization can be classified by the method of binding; some of the major classes are:

1. adsorbed enzymes; including ionic, metal bridge, biospecific and hygroscopic binding

2. covalently bound enzymes

3. entrapped enzymes; including those in matrix (polymeric), in microcapsules, and in hollow fibers (ultrafiltration)

2.1.3 Immobilization of Invertase

Invertase has been immobilized by a large variety of methods. In the next subsections, an outline of the results obtained from several immobilization efforts is given.

Immobilization by Adsorption

This method consists of contacting an aqueous solution of an enzyme or a microorganism with a surface active adsorbent such as anion and cation exchange resins, cellulose and porous glass. The enzyme or cell is adsorbed on the surface active insoluble support. A multifunctional reagent can be used to cross-link the adsorbed enzyme and form a net around the support. Efficiency of adsorption is dependent on the characteristics of the adsorbent as well as on the enzyme or microorganism, and the experimental variables such as pH, temperature and ionic strength.

Invertase was the enzyme employed in the first attempts of immobilization. Over 70 years ago, Griffin and Nelson used aluminum hydroxide and charcoal as adsorbents. In more recent work, invertase was bound to bentonite by adsorption. Activity was low, but stability was greater than that of the soluble enzyme (Reilly, 1980).

An insoluble invertase-tannic acid complex, which could be dissociated by treatment with urea and sucrose, was more resistant to inhibition by glucose, fructose and parachloro-mercuric benzoic acid than was soluble invertase. However, there was no significant stabilization after immobilization (Reilly, 1980).

Collagen has been studied extensively as a carrier for invertase. The enzyme has been adsorbed by soaking or electrodeposition followed by tanning with a cross-linking agent, presumably covalently attaching at least some of the invertase to the carrier. Saini *et al.* (1972) found that about three fold the amount of invertase was bound to collagen as to anion exchange resin beads, per unit weight of carrier. When first using collagen, there was a sharp decrease in activity, as loosely bound enzyme was washed off, followed by operation at constant activity.

Several ion exchange materials have been used to immobilize invertase. With carboxymethyl-cellulose, which bound invertase below pH 5.5, stability was higher than free enzyme stability. With diethylaminoethyl cellulose (DEAE-cellulose), on the other hand, stability decreased upon immobilization and the pH for optimum activity decreased significantly. Desorption at fairly low ionic strengths was a problem because the optimum pH of operation was below the pH where strong adsorption occurs (Reilly, 1980). Invertase attached to diethylaminoacetyl cellulose (DEAA-cellulose) had the same stability as the native enzyme at elevated temperatures (40°C) but was less stable when stored in the form of an aqueous suspension at 5°C (Maeda *et al.* , 1973). Invertase was less easily desorbed from this carrier (DEAA-cellulose) than from DEAE-cellulose but the retained activity upon immobilization was less than 50% (Maeda *et al.* , 1973).

Petroleum-based ion exchange resins have also been tried. Between pH 3 and 5.9, there was little desorption from anionic or cationic carriers with 0.1 mol/L acetate, but operation outside this pH range or at high temperatures caused serious losses (Reilly, 1980).

Ooshima *et al.* (1980a) performed a kinetic study on the thermal stability of 3 kinds of invertase (native, immobilized on porous glass covalently and on anion exchange resin ionically). They concluded that thermal deactivation of all invertases follows first-order kinetics. They also determined (Ooshima *et al.* , 1980b) that immobilization by the ionic binding method compared with immobilization by the covalent binding method keeps more sites on the enzyme active, but results in easier denaturation of it.

Immobilization by Covalence

It is based on the formation of covalent bonds between the enzyme or cell and the carrier. The coupling of the enzyme or the cell to the support can be achieved by either the activation of the support or the use of a coupling reagent to link the matrix and the active cell or enzyme. This method of immobilization results in remarkably stable conjugate systems. Cells are not eluted by changes in pH, temperature and ionic strength. However, loss of enzyme activity due to either the method of coupling or the support material can occur. Many covalent binding techniques are also complicated and expensive (Aybut, 1985). Covalent methods are widely used for enzyme immobilization but seldom used for whole cell immobilization.

Covalent binding has been used a great deal in studies on invertase immobilization. Invertase has been immobilized on the polyaldehydes vanacryl and covanacryl by Brown and Joyeau, and a retention of specific activity of 14% to 35% was realized upon immobilization (Reilly, 1980).

Attachment of invertase to cellulose with titanium salts was unsatisfactory, while with diisocyanates, lower activities were obtained than with ionic binding to DEAE-cellulose or guanidino-cellulose. An attempt to couple invertase to aminoethyl-cellulose (AE-cellulose) with glutaraldehyde was also unsuccessful (Reilly, 1980).

Many petroleum-based carriers have been used for covalent binding of invertase: Duolite^R S-30 phenol-formaldehyde resin, aminonylon, polyaminostyrene and Amberlite^R IR-45 are some examples (Saini *et al.* , 1972).

Inorganic carriers have also been tried; mineral carriers as well as synthesized materials (glass). On neither brick or glass was binding with thionyl or sulfonyl chloride particularly effective, nor were significantly better results obtained when the enzyme was coupled to bentonite with cyanuric chloride. Greater activity was obtained when replacing cyanuric chloride with sulfonyl or thyonil chloride (Reilly, 1980).

Invertase coupled to aminoporous glass by azo linkage showed lower stability than the soluble form of the enzyme; appreciable activity could be obtained by combining this method and linkage with titanium salts. In some cases, glutaraldehyde after silanization of the carrier to provide an amino group, was a good linking

agent (Reilly, 1980).

Hornblende-invertase conjugates (hornblende is a rock-forming ferromagnesian silicate mineral) had low activities because the surface area of the carrier was low. However, the stabilities were roughly the same as those of the soluble enzyme (Reilly, 1980).

Sand, biotite, muscovite, pyroxene, magnetite and hydrated oxides of tin, aluminum and titanium from the corresponding chlorides are all organic carriers that have been tested for covalent binding of invertase. Invertase has been bound to tin oxide with almost complete retention of activity but, unfortunately, stability was lower than for the soluble enzyme.

Samoshina *et al.* (1981) immobilized a preparation of yeast invertase (Invertin) by covalent binding to modified MCA-750 silica gel through glutaraldehyde. The optimum pH and the thermostability of the preparation were studied. Their investigation showed a narrowing of the profile of the pH curve and a slight shift of the optimum pH to the acid region for the immobilized invertase. Broadening of the region of pH stability, as well as a shift of the temperature optimum to the region of higher temperatures and an increase in the thermostability of the invertase preparation, also occured upon immobilization.

Monsan and Combes (1984) immobilized invertase by covalent coupling unto a cheap agricultural by-product, corn grits. The glucose units of the cellulosic fraction of the support were chemically modified by sodium metaperiodate oxidation to form aldehydes, amination by condensation with ethylene diamine, reduction of the resulting imino bonds to amino bonds with sodium cyanoborohydride and activation of the amino groups with glutaraldehyde. Invertase grafting unto corn grits yielded a very active and stable derivative.

Cabral *et al.* (1986) have immobilized whole cells of *S. cerevisiae* by chelation/metal link processes unto porous inorganic carriers. The introduction of an organic bridge between the cells and the metal activator led to a decrease of the initial activity of the immobilized cells, however *S. cerevisiae* cells immobilized by covalent linkage on the carbonyl derivative of titanium (IV) activated pumice stone (a volcanic material) displayed a very stable behaviour.

Immobilization by Entrapment

This method involves the entrapment of cells or enzymes within the interstitial spaces of water insoluble polymers. The enzymes or cells are physically entrapped, within a polymer lattice, into a microcapsule, in hollow fibers, etc. In the case of lattice entrapment, the homogeneous dispersion of cells or enzymes results in good enzyme accessibility to the substrate and fast reaction rates (Aykut *et al.* , 1985). Leakage of cells or enzymes from the matrix can be overcome by effective cross-linking. Some chemicals used for polymerization have the potential to damage the enzymes or the microorganisms.

Use of polyacrylamide gel led to low retained activity. Stability of the enzyme trapped in polyacrylamide gel was roughly the same as the stability of the native form (Reilly, 1980). D'Souza and Nadkarni (1980) immobilized disintegrated cells of *S. cerevisiae* by entrapment in polyacrylamide. They used different methods to disintegrate the cells (ultrasonic disintegration, mechanical homogenization, exposure to toluene and lysis with snail gut enzyme). All disintegration methods tried seemed suitable. They entrapped the disintegrated cells in polyacrylamide using chemical polymerization in one case and gamma-rays in another case. Best results were obtained with radiopolymerized gels of acrylamide. It was also found that invertase (and other enzymes) was more stable when immobilized with the cells than in the purified form. Continuous hydrolysis of sucrose was accomplished for over 30 days without loss in efficiency.

Other acrylic gels have also been used, such as poly(dimethyl acrylamide) and poly(2-hydroxyethyl acrylate). Neither poly(dimethyl acrylamide) or poly(2-hydroxyethyl acrylate) gave good retentions of activity. An acrylic acid/sodium acrylate copolymer was tried but could not even be gelled. On the other hand, polyacrylamide stiffened with various cross-linking agents and related gels stabilized invertase and gave high activities (Kawashima and Umeda, 1976b).

An interesting method to form particles with variable diameters (0.02–15 mm), spongy structure and large surface area, was used by Kawashima and Umeda (1976a). They polymerized frozen drops of acrylamide and additives suspended

in hexane or other solvents to obtain invertase-containing gel beads (*C. utilis* invertase). Krosing and coworkers innovated by allowing the gel to form within the interior of porous silica beads (Reilly, 1980).

Lyophilized cells of *S. cerevisiae* were entrapped in poly(2-hydroxyethyl methacrylate) gel by Cantarella *et al.* (1984), who investigated invertase catalysed sucrose hydrolysis. The recovery of biocatalyst activity ranged between 17% and 23% and the operational stability was almost twice that of free *S. cerevisiae* cells (half-life of 110 days).

Vinyl alcohol polymerization by electron beam irradiation was more successful in retaining immobilized invertase than was γ-irradiation, though neither method was particularly promising (Reilly, 1980).

Invertase has also been entrapped in fibers of cellulose triacetate or poly(γ-methyl glutamate) and less successfully in fibers of cellulose diacetate, ethyl cellulose and polyvinyl chloride. Marconi *et al.* (1974) entrapped invertase in cellulose triacetate fibers and predicted, for some preparations, a half-life of 5300 days. A sample of invertase fibers, continuously hydrolysing sucrose, maintained unchanged its activity for five years. These fibers could also be used in packed-bed reactors. The yeast invertase entrapped in spun fibers of cellulose triacetate also showed good efficiency (Marconi and Morisi, 1979).

Linko *et al.* (1980a) have entrapped *S. cerevisiae* cells within cellulose and cellulose di- and tri-acetate beads, employing several carrier solvent systems. The yeast cells used as the source of invertase were grown on cane molasses and dried. Cellulose beads prepared using a melt of dimethyl-sulfoxide (DMSO) and N-ethyl pyridinium chlorate (NEPC), or cellulose diacetate using a mixture of acetone and DMSO as the solvent, were found to be promising as carriers for the invertase system. The authors reported that operational half-lives of about 5 years could be obtained using up to 50% (w/w) sucrose as the substrate. Enzymes entrapped in cellulose triacetate showed remarkable stability at room temperature or below, half-lives in the order of years being possible; specific activities decreased, as more enzyme were entrapped, as they did when porosity was low. Stability, on the other

hand, increased as porosity decreased, as the enzyme had a smaller chance of escaping.

Several configurations of ultrafiltration membranes have been employed to entrap invertase. In a reactor whose exit was closed by an Amicon UM 10 membrane, the enzyme was stable at 50°C in the presence of sucrose, when attached to bentonite, but could not retain activity when in native form. A continuous stirred-tank reactor (CSTR) with a series of membranes with larger exclusion limits was also operated (Reilly, 1980).

Yeast cells containing invertase have been immobilized in spherical agar particles. However, agar gels have poor mechanical strength (Bucke, 1983).

Gelatin has been employed to entrap invertase. Dhuster *et al.* (1982) immobilized lyophilized *S. cerevisiae* cells in gelatin. They compared the efficiency of their immobilized preparation of invertase active whole cells with preparations immobilized with alginate and κ-carrageenan and reported a higher activity with gelatin. Gelatin has been shown to be a useful and versatile support matrix for immobilization of biologically active macromolecules and whole microbial cells (Kennedy and Kalogerakis, 1984). An added advantage of the use of gelatin as an immobilization support is that, being itself a protein, it stabilizes the immobilized enzyme. A commercial lot of freeze-dried baker's yeast was used as the source of invertase by Parascandola and Scardi (1982), who employed gelatin as the enzyme carrier. The authors stated that their gelatin preparation exhibited good stability. However, they tested it for only 6 days during which a 6% decrease in activity was observed: it seems that their preparation was not very stable. A disadvantage of gelatin is that particles in packed-beds suffer from flow-induced compression unless they are properly spaced out by an inert material such as glass beads. At 40°C, not even the use of an inert granular material mixed with the gelatin particles can prevent a progressive decrease in flow rate through the packed-bed reactor. Parascandola *et al.* (1982) used tuff (a porous rock of volcanic origin) as a support for yeast cells entrapped in gelatin to enable the preparation to be used in a packed-bed reactor. This eliminated the need to space out gelatin particles with an inert material.

Chao *et al.* (1986) immobilized commercial baker's yeast in spherical beads of

κ-carrageenan. The beads were then cured with different amines. The mechanical strength and the applicability of amine-treated gels as immobilization matrices was evaluated. Results indicated that linear and branched polyethyleneimines (PEI) are both good curing agents. PEI-treated carrageenan beads also exhibited superior resistance to heat and abrasion. An immobilized cell preparation of *S. cerevisiae* evaluated for invertase activity at 60°C in a packed-bed reactor had an apparent half-life of 108 days. The conditions required for gelation and entrapment with this polysaccharide were very mild.

Another useful polysaccharide is alginic acid. It is a copolymer of D-mannuronic and L-guluronic acids with the detailed structure varying from source to source, even with different parts of the same organism (brown marine algae). An important property of alginic acid is its gel strength: gels are formed with divalent cations which cross-link guluronic acid units of different molecules. Entrapping of cells or enzymes within calcium alginate could hardly be a simpler or gentler process (Bucke, 1983). No heat treatment is required and preparations retain high enzyme activity. Calcium alginate complexes are also known for the longevity of biochemical activities. In nature, alginate provides mechanical support for brown seaweeds, which are able to withstand the force of ocean storms, and it is not surprising that calcium alginate beads should provide excellent resistance to hydrostatic pressures and abrasion. The feasibility of using calcium alginate beads in large scale packed-bed reactors has been demonstrated by Cheetham (1979).

Calcium alginate has some drawbacks: the calcium may be removed by chelating agents such as phosphates (including adenosine triphosphate) and ethylenediaminetetraacetic acid (EDTA) and displaced by cations such as Mg^{2+} and K^{+}, causing the dissolution of the gel.

Linko *et al.* (1980) developped a method to obtain a stable and active biocatalyst by entrapping invertase-active yeast cells within calcium alginate beads. *S. cerevisiae* cells were grown on cane molasses medium, washed and freeze-dried. A suitable quantity of dried cells was suspended in an aqueous solution of sodium alginate and subsequently extruded through 0.6 mm in diameter needles into 0.5 mol/L $CaCl_2$ solution with mixing. The immobilized cells were finally treated with

2.5% glutaraldehyde in water for one hour at 4°C and washed. Active and stable beads were obtained.

D'Souza *et al.* (1982) have immobilized *S. cerevisiae* cells in hen egg white using glutaraldehyde as the cross-linking agent. About 45% of the invertase activity was retained. The immobilized yeast cells were used a few times in a batch reactor system for the inversion of sucrose without appreciable loss of activity. No apparent half-life was determined for the preparation and little can be said about its stability.

2.1.4 Effect of Temperature and pH on Invertase

Activity

The highest activity for immobilized yeast invertase was obtained in the temperature range 50–65°C (Samoshina *et al.* , 1981; D'Souza and Nadkarni, 1980), while maximum activity of free yeast invertase is achieved between 50°C and 60°C according to Gould (1975) and at about 55°C (depending on sucrose concentration) according to Wiseman (1979).

Immobilization affects the pH profile of invertase. According to Samoshina *et al.* (1981) who immobilized invertase by covalent binding to silica gel, a small shift of the optimum pH to the acid region occurs upon immobilization: 4.4–4.5 for free invertase and 4.1 for immobilized invertase. Wiseman reported a pH optimum of 4.7 (4.5–5.5) for highly purified baker's yeast invertase.

Stability

Upon immobilization with certain carriers, the thermostability of invertase increases. However, as is the case for the free enzyme, stability decreases with a rise in temperature.

Broadening of the region of pH-stability has been observed when invertase is immobilized. Samoshina *et al.* (1981) reported a pH-stability range of 3.9–6.2 for immobilized invertase compared to 3.5–4.9 for free invertase while Gould (1975) stated that maximum yeast invertase stability was found in the pH range 3.5–5.5.

2.1.5 Inhibition of Invertase

Various kinetic models have been proposed to describe the hydrolysis of sucrose by invertase. Most of these models suggest a Michaelis-Menten mechanism for low initial substrate concentrations, and a substrate inhibition model for high initial concentrations. For most of these studies, the experimental data were collected by measuring initial rates of reaction at several initial concentrations of sucrose.

Lopez Santin *et al.* (1982) studied this problem using free invertase and invertase-activated clay complex, prepared by covalent attachment. They suggested three models to explain their experimental results: Michaelis-Menten, substrate inhibition and product inhibition.

Combes and Monsan (1983) characterized the inhibition phenomena that affect invertase when concentrated sucrose solutions are hydrolyzed to produce liquid sugar solutions. They reported that under such conditions, invertase was simultaneously subjected to inhibition by D-fructose, D-glucose and sucrose. D-fructose showed the behaviour of a competitive inhibitor while D-glucose was shown to be a partial noncompetitive inhibitor of invertase. The combination of both inhibitory effect of D-fructose and D-glucose allowed a satisfactory description of the kinetics of batch hydrolysis of a dilute solution of sucrose. The decrease in invertase efficiency observed with high concentrations of sucrose seemed to be directly related to a modification of the structure of the molecule of sucrose under such conditions, which resulted from a decrease in water activity. The decrease in invertase activity did not depend on the medium viscosity, or on a modification of the enzyme properties, but on a modification of the structure of the substrate intra- and intermolecular hydrogen bonds. As reported in another article by these authors (Monsan and Combes, 1984b), the decreased invertase activity observed with increasing sucrose concentrations resulted not only from inhibition by excess substrate but also from the appearance of folded and aggregated sucrose molecules that were not recognized by invertase. The enzyme activity greatly decreased when the substrate concentration was increased above 0.4 mol/L (13.7 g/100 ml).

2.1.6 Cross-Linking Reagents

Several cross-linking reagents have been used with invertase. Polyethyleneimine and glutaraldehyde are two of the most common compounds employed for immobilization purposes.

Polyethyleneimine

Polyethyleneimine, $(C_2H_5)_n$, is a highly branched synthetic polyamine which is available commercially (50 000 to 100 000 molecular weight). It has long been recognized as a stabilizing agent for soluble enzymes, and it has recently demonstrated potential as a carrier matrix for immobilized enzymes. In this latter application, it is used to coat or otherwise be included with another support material, or it is cross-linked into a macroporous form and used alone (Bernath and Venkatasubramanian, 1986).

Glutaraldehyde

Glutaraldehyde, 1,5-pentanedial, has been used in conjunction with enzyme immobilization to preactivate amino groups on carriers, to cross-link enzymes to carriers after adsorption, and to cross-link enzymes into soluble oligomers or insoluble membranes. It is by far the most widely used cross-linking agent for these purposes. It has been very effective despite the fact that major questions still exist regarding its mechanism of action. Glutaraldehyde is available commercially , usually in 25% acidic aqueous solution. Cross-linking is generally conducted at or near neutral pH (Bernath and Venkatasubramanian, 1986).

2.1.7 Shape of Biocatalyst Carrier

In practical applications, immobilized enzyme carriers of regular form as well as high retained activity and high mechanical strength are desired. For use in enzyme columns, bead shaped immobilized enzymes are essential (Kawashima and Umeda, 1976).

2.2 Ethanol Production

Fermentative production of chemicals from renewable resources have generated a great deal of interest in recent years. In the past decade, the production of fermentation ethanol has gained considerable attention. Ethanol, which can serve as a chemical feedstock as well as a liquid fuel, is obtained from renewable resources such as sugars, starch, whey, and cellulosic materials. Most of these materials have to be converted to fermentable sugars, and subsequently ethanol is produced from them.

The major areas of research interest in ethanol fermentation have been:

1. increased ethanol concentration and specific ethanol production
2. improvement of ethanol tolerance of yeast
3. development of a continuous ethanol fermentation process using high density cell cultures

An example of recent advances in alcohol production technology is the application of immobilized cell systems to continuous column reactors. Immobilized cell reactor systems can provide high cell concentrations and high ethanol productivities can be achieved.

2.2.1 Conversion of Glucose to Ethanol

The overall conversion of glucose to ethanol by yeast can be represented stoichiometrically as:

$$C_6H_{12}O_6 \longrightarrow 2\ C_2H_5OH + 2\ CO_2$$

In fact, quantities of glycerol and higher alcohols are also produced, the amounts being dependent on the strain of yeast and fermentation conditions. From the above equation, it can be calculated that a theoretical yield of 51.1 g of ethanol can be obtained from the fermentation of 100 g of glucose. Present industrial yields of

80–90% of this value are common, the loss being attributable to yeast growth and byproducts formation.

2.2.2 Free vs Immobilized Systems for Alcohol Production

One of the biggest advantage of immobilized cell systems is the ability to operate with high productivity at dilution rates exceeding the maximum specific growth rate of the microbe. Also, a higher cell mass per unit fermentation volume than with batch, continuous or cell recycle systems can be achieved, resulting in a corresponding increase in ethanol production. There is no need for cell removal or cell recycle, making product extraction much more efficient. The risk of contamination is reduced due to fast dilution rates and high cell densities.

It has been proposed that the enhanced fermentation capacity of microorganisms as a result of immobilization may be the consequence of a reduction of the ethanol concentration in the immediate microenvironment of the organism due to the formation of a protective layer, or that specific adsorption of ethanol by the support may act to minimize end product inhibition (Kosaric *et al.* , 1983).

Substrate inhibition may be diminished in the case of a gel matrix, if the rate of fermentation meets or exceeds the rate of substrate diffusion to the cell. Another possibility is that alteration of the cell membrane during the process of immobilization provides improved transfer of substrate into and product out of the microbe (Kosaric *et al.* , 1983).

The effect of temperature on the rate of ethanol production is different for free and immobilized systems; while a constant increase in rate is observed with free *Saccharomyces cerevisiae* as temperature is increased from 25 to 42°C, a maximum occurs at 30°C with cells immobilized in calcium alginate (Williams and Munnecke, 1981). This lower temperature optimum for immobilized systems may result from the diffusional limitations of ethanol within the support matrix. At higher temperatures, ethanol production exceeds its rate of diffusion so that accumulation occurs within the beads. Inhibitory levels of ethanol then cause the declines observed in the ethanol production rate.

Significant differences are also apparent with regard to the effect of pH on the

fermentation rate; free cells have a narrow pH optimum, occuring at 4.5, but this is replaced by a broad range (3.0–7.5) upon immobilization in calcium alginate (Williams and Munnecke, 1981).

Tyagi and Ghose (1982) have compared the volumetric productivities of free and immobilized systems (Table 1).

Table 1: Volumetric ethanol productivities for free and immobilized systems (Tyagi and Ghose, 1982).

Process	*Substrate*	*Volumetric ethanol productivity* ($g.L^{-1}.h^{-1}$)
batch	molasses	2.0
continuous (free cells)	molasses	3.35
continuous (immobilized cells)	molasses	28.6

Immobilized cell systems are superior to free cell systems in terms of ethanol productivity.

2.2.3 Immobilized systems for Ethanol Production

One of the first biological processes making use of an immobilized cell system to be studied in detail was the production of ethanol by yeast cells. However, conventional fermentation processes, which have been under development for a long time, still have commercial advantages over immobilized cells, on an industrial scale (Bucke, 1983). Recent studies on the production of ethanol have concentrated on reactor productivities, the achievement of high ethanol concentrations, the maximization of substrate use, the use of high fermentation temperatures and the use of novel substrates. The production of ethanol by yeasts is an attractive model system for the study of methods of cell immobilization so as to retain the activity of a multi-enzyme complex. Many workers in this field have not aimed their research at commercial objectives.

One of the first publication dealing with the immobilization of cells in calcium alginate gels (Kierstan and Bucke, 1977) described the production of ethanol by *S. cerevisiae* cells supplied only with glucose or sucrose, without a source of nitrogen. Cells of *Kluyveromyces marxianus* immobilized similarly produced ethanol from inulin (Kierstan and Bucke, 1977). The half-life of the *S. cerevisiae* preparation used for glucose fermentation to ethanol was shown to be about 10 days.

Saccharomyces carlsbergensis cells were immobilized on porous glass beads activated using γ-amino propiltrimethoxysilan alone or together with glutaraldehyde. Cells were simply stirred with the activated support for at least 15 minutes. A significant amount of cells were bound only to the glutaraldehyde-activated material. The amount of glutaraldehyde bound to the glass beads increased with the porosity of the beads and it was assumed that the cells were bound to the surface of the beads rather than entrapped within their pores. This immobilization reduced the fermentation rate of the cells but increased the yield of ethanol from glucose. *S. cerevisiae* cells immobilized on activated pectate cross-linked by using Fe^{3+} ions, under aerobic conditions, had an oxygen uptake higher than that of the initial cell suspension (Bucke, 1983).

Various strains of yeast were immobilized by simple adsorption within the pores of an aluminosilicate material and *S. cerevisiae* cells respired at 6–7 times the rate of the initial suspension (Bucke, 1983).

S. cerevisiae cells were also immobilized by adsorption onto beads of commercially available anionic resins, as reported by Daugulis *et al.* (1981). The cells were not desorbed significantly by the passage of a complex medium containing glucose at a concentration of 120 g/L. The immobilized cell reactor was operated continuously without trouble for 200 hours. The chemical nature of the resin rather than simply its charge was important in allowing efficient cell adsorption.

Wada *et al.* (1979) studied the ability of immobilized cells to grow within beads of κ-carrageenan. Preparations of *S. cerevisiae* immobilized within κ-carrageenan were used to produce ethanol continuously from a medium containing glucose at 100 g/L (Wada *et al.* , 1980). A stable steady state was maintained for over 3 months and a high yield of ethanol was obtained. The authors concluded that only growing

cells were able to produce ethanol. They continued their studies by investigating the production of ethanol from media containing glucose at a concentration of 250 g/L (Wada *et al.* , 1981). A strain of *S. cerevisiae* was selected for its ability to produce ethanol from such a medium and immobilized in κ-carrageenan. An abrupt change in glucose concentration from 100 to 250 g/L did not result in high ethanol concentrations but when there was a stepwise increase of glucose concentration from 100 to 150, 200 and 250 g/L, the preparation could produce ethanol at 114 g/L, continuously for over 2 months, with a conversion rate of glucose to ethanol of 96% of the theoretical value. According to the authors (Wada *et al.* , 1981), the inhibitory effect in the immobilized process was reduced by a stepwise increase of glucose concentrations in the feed medium. They suspected that the immobilized growing yeast cells adapted themselves to the glucose concentration in a stepwise manner.

In packed-bed reactors, the production of CO_2 from beads of immobilized yeast cells has an adverse effect on the fermentation. Cho *et al.* (1981) have compared the efficiencies of packed-bed and fluidized bed reactors with *S. cerevisiae* cells entrapped in calcium alginate. They reported (Table 2) productivity numbers for their reactors, in comparison with free-cell continuous fermentations, with and without cell recycling, using media containing 100 g/L glucose. The fluidized-bed reactor had twice the volumetric ethanol productivity of the packed-bed reactor.

Table 2: Comparison of ethanol biorector productivities; the calculations are based on the total reactor volume (Cho *et al.* , 1981).

Reactor	*Ethanol productivity* ($g.L^{-1}.h^{-1}$)
continuous free cell	7
continous with cell recycling	14
packed-bed	10
fluidized-bed	21

They also estimated that the dead volume occupied by CO_2 gas in a fixed-bed reactor was about 65% of the total effective reactor volume, at 100% conversion of

the nominal substrate concentration level of 10% used in their study; the volume occupied by CO_2 varying with substrate concentration. The main reason for the improved performance of the fluidized-bed reactor was the increased rate of removal of CO_2 from the reactor, according to the authors. However, it seems that the authors neglected the possible effect of aeration on the fermentation; immobilized beads being fluidized by aeration with an air generator. Shiotani and Yamané (1981) solved the CO_2 problem by using a horizontal packed-bed reactor.

Williams and Munnecke (1981) have studied the immobilization of yeast cells in calcium alginate in some detail. They used for their study, a sodium alginate that they obtained from Sigma Chemical Co., which is from *Macrocystis* species and has a high mannuronate/guluronnate ratio. It therefore has a high viscosity and low gel strength compared with the alginate supplied by BDH Ltd which is from *Laminaria* species and was used by Kierstan and Bucke (1977) and Cheetham *et al.* (1979). Immobilized cells showed a very broad pH optimum for ethanol production, the same rate being obtained between pH 3.0 and 7.5; a free cell culture had a sharp pH optimum at 4.5. The comparisons of temperature effects on the free cell and the immobilized cell systems indicated that there was a different temperature optimum for each system. The maximum rate for the free cells occurred at 37°C. In comparison, the ethanol production rate for the immobilized system showed a lower temperature optimum of 30°C. The downshift of the temperature optimum for the immobilized system was probably due to the effects of ethanol inhibition within the alginate beads. Experiments have shown that with increasing ethanol concentration, any increase in temperature produced greater inhibition to ethanol production. The authors thought that a diffusion limitation imposed by the alginate matrix which produced substrate and product gradients within the bead was responsible for this. They therefore suggested that the immobilized system should be operated at lower temperatures than free cell systems to minimize ethanol inhibition and maximize ethanol production. They reached a maximum ethanol productivity of 53.8 g/(L.h), based on the liquid volume of the reactor.

Linko and Linko (1981) used *Laminaria* alginate (from BDH Chemicals Ltd) to entrap *S. cerevisiae* cells of various strains. Because of its low viscosity, Linko and

Linko were able to use *Laminaria* alginate at a concentration of 8%. They used complete media containing cane molasses and reached an ethanol productivity of 50 g/(L.h); the basis for their calculations was not defined.

Veliky and Williams (1981) modified the calcium alginate method by treating the surface of alginate beads with polyethyleneimine to stabilize the structure against destruction by chelating agents. The respiration rate of the entrapped yeast cells was lowered but the ethanol production rate stayed the same.

Birnbaum *et al.* (1981) used three different methods to stabilize calcium alginate beads: a treatment with sodium metaperiodate and polyethyleneimine, another one with carbodiimide and polyethyleneimine and finally the use of polyethyleneimine and glutaraldehyde. The third method gave the best results and the least expense.

The use of polyacrylamide gel for entrapment of yeast cells killed 40–80% of the cells, the survival rate depending on the age of the cells. The viable cells remaining were able to divide and those near the gel surface both divided and fermented most actively (Bucke, 1983).

Holeberg and Margalith (1981) published a study on the production of ethanol by immobilized cells. They compared the performance of *S. cerevisiae* entrapped in agar, polyacrylamide, κ-carrageenan and calcium alginate. In all gels except calcium alginate, immobilized cells produced higher ethanol concentrations than free cells, particularly at high glucose concentrations. Ethanol production seemed to be inhibited by calcium ions. Holeberg and Margalith attributed the ability of entrapped cells to ferment at high glucose concentrations partly to the establishment of gradients within the gels, the concentration of glucose at the cell surface being significantly lower than in the bulk solution. Such gradients did not account for the whole effect, however, simple contact of cells with a polymer allowing higher ethanol contents to be obtained.

Alternative substrates for ethanol production by immobilized cells have been tried. Linko *et al.* (1981) entrapped *Kluyveromyces fragilis* cells in calcium alginate and produced ethanol continuously for several days from whey preparations. Margaritis and Bajpai (1981) also used *K. fragilis* entrapped in calcium alginate to ferment fructans from Jerusalem artichoke.

To employ more fully the flexibility of gel-entrapment of cells, in order to allow the utilization of non-fermentable substrates, coimmobilization of enzymes and cells has been attempted. Several enzymes, however, are readily leached from gels (Kierstan and Bucke, 1977) and they need to be retained in some way. Chiang *et al.* (1982) coimmobilized *S. cerevisiae* cells and glucose isomerase in calcium alginate and also used glucose isomerase and yeasts in separate columns, the function of glucose isomerase being to isomerize xylose to xylulose which is fermentable by the yeast. This latter approach had more success than the first one, probably because the two enzyme systems had different optimum conditions.

The bacterium *Zymomonas mobilis* has been used for ethanol production. Grote *et al.* (1980) and Margaritis *et al.* (1981) entrapped it in calcium alginate and produced ethanol at high yields for extended periods.

2.2.4 Productivities of Different Immobilized Systems

In the literature, different investigators report different methods for calculating residence times, dilution rates and volumetric ethanol productivities in packed-bed immobilized cell bioreactors. Considering a continuous fixed-bed bioreactor containing immobilized cells, let:

$V_T = \pi d^2 L/4$, (L)

$\varepsilon = V_L/V_T$

$\tau = \pi d^2 L\varepsilon/4F$, (h)

Since $\varepsilon = V_L/V_T$ (neglecting gas phase), $\tau = V_T\varepsilon/F = V_L/F$

Volumetric ethanol productivities may be defined in three ways:

1. Basis: liquid phase mean residence time, τ. In this case, the dilution rate D $= \tau^{-1} = F/V_L$, (h^{-1}), and the volumetric productivity = DP, ($g.L^{-1}.h^{-1}$).

2. Basis: total bioreactor volume, V_T. With this method, volumetric ethanol productivity = $(FP)/V_T$, ($g.L^{-1}.h^{-1}$).

3. Basis: solid bead volume, V_B. By this method, volumetric ethanol productivity = $(FP)/V_B$, $(g.L^{-1}.h^{-1})$.

According to Margaritis *et al.* (1981), the liquid phase mean residence time, τ, equal to V_L/F, is the most appropriate basis for calculating dilution rates and ethanol productivities. They reported that, from a kinetic point of view, the liquid mean residence time was the important parameter which dictated conversion of substrate. On the other hand, Bucke (1983) thought that productivity calculations based on V_T are the most meaningful in practice. It is essential that when volumetric ethanol productivities are reported in the literature, the basis of calculation be given by specifying the volume fractions of the bioreactor represented by the liquid (void) and solid (bead) phases.

Margaritis *et al.* (1981) have prepared a summary of ethanol productivity results obtained by different investigators (including themselves); it is given in Table 3.

It can be seen from Table 3 that most authors based their calculations either on the void volume or on the solid bead volume. These two volumes are not constant with time because the size and shape of gel beads can change during operation of a bioreactor; these volumes also differ from one system to another, making productivity comparisons difficult to interpret.

Mass transfer limitations resulting from cell immobilization greatly affect the kinetics of ethanol production in immobilized systems. Where entrapment in alginate beads is involved, diffusion of substrate, ethanol and CO_2 can be enhanced by employing structurally stable beads of small diameter (Kosaric *et al.* , 1983). Disruption of bead structure and subsequent cell losses result from the accumulation of CO_2 within beads due to inadequate diffusion.

Grote *et al.* (1980) have demonstrated the dependence of ethanol productivity and the specific rates of glucose uptake and ethanol production upon the dilution rate of immobilized *Z. mobilis* bioreactors. Dilution rates of about 0.8 h^{-1} were found to be optimum for the bacterial fermentations investigated. Williams and Munnecke (1981) have found an optimum dilution rate of 4.6 h^{-1} for *S. cerevisiae* cells entrapped in calcium alginate. According to Kosaric *et al.* (1983), bacterial

Table 3: Comparison of ethanol productivity results (Margaritis *et al.* , 1981).

Investigator	*Immobilized system*	*Feed sugar concentration* (g/L)	*Feed sugar utilization* (%)	*Dilution rate,* D (h^{-1})	*Basis for calculation of* D	*Maximum ethanol productivity* ($g.L^{-1}.h^{-1}$)
Kierstan and Bucke (1977)	*S. cerevisiae* Ca-alginate	glucose 100	80	0.1	V_T	4.3
Chibata (1979)	*S. cerevisiae* carrageenan	glucose 100	98	1.0	V_B	50
Wada *et al.* (1979)	*S. cerevisiae* carrageenan	glucose 100	86	1.0	V_B	43
Chibata and Tosa (1980)	*S. cerevisiae* carrageenan	glucose 200	100	0.4	V_L	40
Ghose and Bandyopadyay (1980)	*S. cerevisiae* carrier A.	molasses 197	74	0.35	V_L	25
Grote *et al.* (1980)	*Z. mobilis* carrageenan	glucose 150	85	0.8	V_L	53
Grote *et al.* (1980)	*Z. mobilis* Ca-alginate	glucose 150	75	0.85	V_L	44
Linko and Linko (1981)	*S. cerevisiae* Ca-alginate	molasses 175	83	0.3	N.A.	21.3
Williams and Munnecke (1981)	*S. cerevisiae* Ca-alginate	glucose 127	63	4.6	V_L	53.8
Margaritis *et al.* (1981)	*Z. mobilis* Ca-alginate	glucose 100	87	2.4	V_L	102
Margaritis *et al.* (1981)	*Z. mobilis* Ca-alginate	glucose 100	97	1.6	V_L	71

fermentations are capable of higher ethanol productivities than are yeasts.

One of the main issues being dealt with when producing ethanol by immobilized cells is productivity but very little is said about final ethanol concentration. However, it is an important factor to consider when evaluating the economy of the fermentative production of ethanol. Since distillation is the main method used for separation, it is necessary to keep the ethanol concentration in the feed line to a distillation process as high as possible. Low levels of ethanol cause large costs in the energy needed for distillation and thus make the process uneconomical (Mattiasson, 1983). The main problem is to eliminate the ethanol formed from the site of reaction, but to keep its concentration in the effluent high. It is possible to achieve high productivity, but generally at the expense of the final concentration of ethanol in the medium.

2.2.5 Support Matrix Stability

In many studies, rich media have been used for ethanol production; these media can enhance cell growth. With gel particles, relatively rapid growth can lead to rapid breakdown of the supporting matrix with consequent complete breakdown of reactor flow properties. Even without such breakdown, it must be remembered that cell growth can result in substantial swelling of gel particles and allowance must be made in reactor sizing and design. Such swelling is a characteristic of many of the gel systems used for cell immobilization, but seems to be rarely reported in literature. Even with the use of incomplete media, an initial increase in cell mass can be observed when immobilized yeasts are first used and an increase in particle size then results (Emery and Mitchell, 1986). Lee *et al.* , (1983) reported an increase in bead volume of 2.7 times during activation due to cell growth. Shiotani and Yamané (1981) observed a progression of bead diameter from 3.3 mm down to 1.9 mm along the length of a horizontal packed-bed ethanol fermentor. In this case, the initial calcium alginate bead diameter had been 1.5 mm.

Polyacrylamide gels also showed similar swelling with growth. The lack of such data in reports from many authors can cast a certain further doubt on conclusions as to productivity, since voidage no longer appears to be a fixed variable in beds or

slurries of flexible gels (Emery and Mitchell, 1986).

Because of the swelling of beads under rich medium conditions and the consequent risk of mechanical problems, operating strategies involving periodic revival of the catalysts should be avoided (Emery and Mitchell, 1986).

The breakdown of packed-bed reactor flow properties can take place because of fouling by excess cell growth and by break-up of the support matrix. Such break-up of gel particles can be controlled by the activity of the nutrients. In calcium alginate beads, the cells at the center of the bead are inactive and there are interior voids caused by a build-up of carbon dioxide, as illustrated in Figure 1. This explains the mechanism of the bead fracture which has been experimentally observed (Emery and Mitchell, 1986).

2.3 Fructose Production

In this section, an outline of the methods used for crystalline fructose production is given. A brief description of high-fructose corn syrup production is also included.

2.3.1 Crystalline Fructose

Pure (crystalline) fructose has a multitude of special physical, chemical and metabolic properties which make it a particularly versatile raw material for the food industry. Although fructose in various forms has always been a part of the human diet, the use of pure fructose as an ingredient in foods has not been widespread, mainly because of high production costs and the consequent small amounts produced. The use of pure fructose is limited primarily to pharmaceutical and laboratory applications (Doty and Vanninen, 1975).

2.3.2 Manufacture of Pure Fructose

Serious efforts to produce pure fructose in the United States date from the early 1950's. Holstein and Holsing developed a method involving the inversion of sucrose by enzymatic action or acid hydrolysis followed by enzyme oxidation of the invert

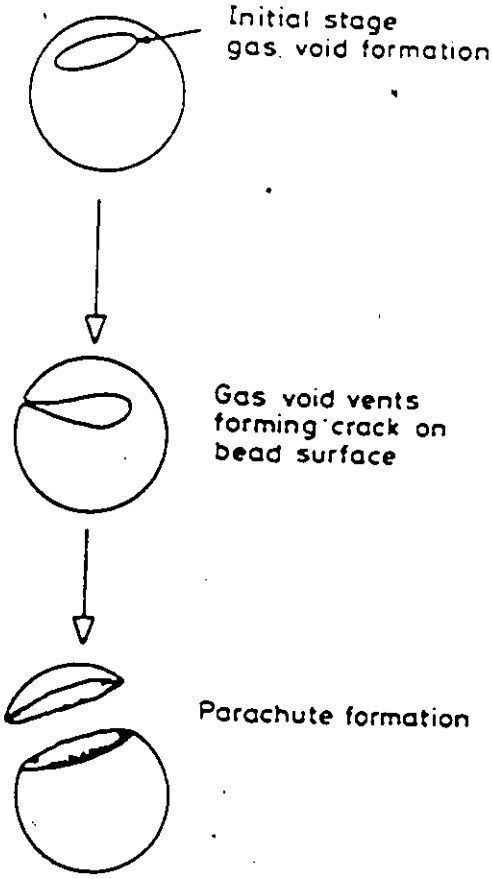

Figure 1: Bead fracture due to internal gas formation and accumulation (Emery and Mitchell, 1986).

sugar to produce gluconic acid. Subsequent crystallization of sodium gluconate permitted pyrogen-free levulose of high purity to be crystallized from the balance of the original syrup.

Earlier attempts to commercially extract fructose were based on hydrolysis of inulin, or on the principle demonstrated in 1847 by the discoverer of fructose, Dubrunfaut, in which the fructose moiety of sucrose is precipitated under alkaline conditions as a calcium complex which is subsequently acidified to release the fructose (Doty and Vanninen, 1975).

During the 1960's, industrial quantities of crystalline fructose began to be manufactured in Europe. The process involved the inversion of sucrose to split the glycosidic bond and release the simple sugars fructose and glucose from their disaccharide union. This constituted the simple part of the process. The subsequent separation and purification of sugars by ion-exclusion techniques, followed by careful controlled crystallization of the fructose, caused the total manufacturing time to exceed one week (Osberger, 1986).

Most manufacturers use water-alcohol solutions in which crystallization takes place more readily than in water solutions, but these processes involve costly control measures for handling the alcohol (Doty and Vanninen, 1975). The Finnish Sugar Co. Ltd, of Helsinki, Finland, has developed a process for direct fructose crystallization from water solutions of sucrose. This has reduced the cost of producing pure crystalline fructose since sucrose is a less complex starting material than the ones used in previous processes (such materials as the polysaccharide inulin) and a less costly solvent is employed (Doty and Vanninen, 1975).

In 1981, American Xyrofin started using liquid dextrose as the starting raw material for pure fructose production. After purification and enzymatic isomerization, the traditional Finish techniques were used to produce a high-quality crystalline fructose. Production time was reduced to five days (Osberger, 1986).

The unique physical, chemical, and metabolic properties of pure fructose present specialty application possibilities not feasible with high-fructose corn syrup (HFCS), which typically contains 42% fructose, 52% glucose and 6% other polysaccharides. While HFCS is about as sweet as sucrose, pure fructose is about 50% sweeter (Doty

and Vanninen, 1975) and can be used at lower concentrations to give the same sweetness. HFCS competes primarily, on a direct cost basis with liquid sugars in a variety of traditional food uses and is presently used in liquid processes, whereas pure fructose can be used in either dry or liquid form, primarily in special dietary foods and pharmaceutical applications.

At the present time, high-fructose corn syrups are very important and it was estimated that, for 1987, HFCS would have 31% of the United States sweeteners market share (Bujake, 1986).

2.3.3 High-Fructose Corn Syrup

During the early 1970's, rapid increases in the price of sugar opened the market for sugar substitutes. High-fructose corn syrup (HFCS), a sweetener with about the same sweetening power as sucrose, emerged as a significant competitor for the industrial sweetener market. HFCS, obtained by enzymatic conversion of corn starch to glucose, followed by conversion of glucose into fructose, was first produced in the United States in 1967. Improvements to the original process have included increasing the fructose content from 15% to 42% and using immobilized enzymes in a continuous process.

The first step in HFCS production is corn wet milling, which separates the starch portion of corn endosperm from the other fractions (e.g. hull, germ and gluten). It consists principally of:

- cleaning
- steeping
- degerming
- separation of the germ
- grinding (milling)
- washing

- centrifugal separation

The starch slurry from wet milling is then converted to sweetener in the refining process, which involves:

- liquefaction
- saccharification
- filtration
- carbon and ion-exchange refining
- isomerization
- filtration
- carbon and ion exchange refining
- evaporation

A 42% fructose, 52% glucose and 6% other saccharides syrup is obtained (on a dry basis). It is usually marketed as 29% water content syrup. HFCS is practically all carbohydrate and water. The nitrogen and sulfated ash contents are of the order of 7 ppm and 200 ppm respectively. A higher fructose concentration (55% and 90%) can be produced by ion-fractionation of the 42% syrup.

HFCS production represents the largest commercial application of advanced enzyme technology (Joglekar *et al.* , 1983).

2.3.4 Fructose Production from Sucrose and High-Fructose Syrup

Some researchers have studied the possibility of producing fructose from sucrose or high-fructose syrups.

Ueng *et al.* (1982) reported the following method of fructose production: differential utilization of glucose by mycelial fungi in alcoholic fermentation of a mixture

of glucose and fructose. They investigated the use of *Mucor sp. M105* and *Fusarium sp. F5* in the production of fructose from sugarcane, sucrose and high fructose syrup. *Mucor sp.* could not utilize sucrose as the sole carbon and energy source for cell growth but preferentially utilized glucose in a glucose:fructose (1:1) mixture during alcoholic fermentation. *Fusarium sp.* utilized sucrose as sole carbon source by secreting extracellular enzymes that degraded the disaccharide. With *Fusarium sp.*, glucose fermentation was faster than fructose fermentation but, due to the low rate of consumption of fructose, this substrate remained in the fermentation broth. The authors did not attempt any continuous fructose production.

Bringer-Meyer *et al.* (1985) derived a mutant from the strain *Zymomonas mobilis ATCC 29191*; this mutant blocked in fructose utilization for growth and ethanol production, was shown to lack fructokinase activity. The fructose-negative mutant, F^- 1959 which was unable to produce ethanol from fructose still possessed the ability to form sorbitol when glucose + fructose were present in the culture medium. The sorbitol produced by the mutant was derived from fructose only and the yield of sorbitol increased with increasing initial sugar concentrations. According to Bringer-Meyer *et al.* (1985), fructose-negative mutants of *Z. mobilis* are of potential use for the enrichment of fructose in HFCS or invert sugar. By the conversion of the glucose portion into the volatile products ethanol and carbon dioxide, the isolation and purification of fructose should be considerably less costly. On the basis of their results with the fructose-negative mutant F^- 1959, the authors expected that, in continuous culture under glucose limitation, little or no sorbitol would be formed. However, this remains to be demonstrated.

Two other mutants of *Z. mobilis*, unable to utilize fructose as a sole source of carbon and energy were isolated by Suntinanalert *et al.* (1986). The two fructose-negative mutants were able to cleave sucrose to glucose and fructose, and then ferment only the glucose to ethanol while accumulating fructose. However, sorbitol was produced from fructose. No continuous fructose production was attempted.

Chapter 3

Materials and Methods

In this chapter, the experimental methods as well as the analytical procedures followed are described in detail.

3.1 Microorganisms

In this study, the yeasts *Saccharomyces cerevisiae ATCC 46534* and *Saccharomyces cerevisiae ATCC 96859* were used:

- *Saccharomyces cerevisiae ATCC 46534* also known under the number *NRCC 202036* was used as the source of invertase. This microorganism was maintained as a liquid culture stored in a refrigerator at 1–5°C. The culture had been grown using medium III containing 5% (w/v) sucrose, on a rotary shaker (200 RPM), at a temperature of 30°C, for 18 hours.

- *Saccharomyces cerevisiae ATCC 96859* also known under the number *NRCC 5583* was used for ethanol production. This yeast was maintained on agar slants (medium I). The solid medium had been inoculated with a liquid culture before being incubated for 3 days at 30°C. The agar slants were stored in a refrigerator (1–5°C) and renewed after 2–3 months.

3.2 Media

Several media were used for growth and maintenance of the yeasts; others were used for ethanol production:

- Medium for maintenance of yeasts (Medium I)
  The yeast used for ethanol production, *S. cerevisiae ATCC 36859*, was kept on agar slants. The composition of the medium used for the preparation of the slants was the following:

  1. malt extract (Difco): ..... 3 g
  2. yeast extract (Difco): ..... 3 g
  3. peptone (Difco): ..... 3 g
  4. glucose (Anachemia or BDH): ..... 10 g
  5. agar (Difco): ..... 20 g
  6. distilled water: ..... up to 1 L of medium

- Medium for batch ethanol production (Medium II)
  For preparation of the inoculum and for batch tests of ethanol production, the following medium was used:

  1. yeast extract (Difco): ..... 3 g
  2. peptone (Difco): ..... 3 g
  3. KH_2PO_4 (Anachemia): ..... 2 g
  4. $MgSO_4.7H_2O$ (Anachemia): ..... 1 g
  5. $(NH_4)_2SO_4$ (Anachemia): ..... 1 g
  6. sugar (Anachemia or BDH): ..... (variable)
  7. distilled water: ..... up to 1 L of medium

- Medium for invertase production (Medium III)
  The medium used to grow *S. cerevisiae ATCC 46534* cells for invertase preparation had the following composition:

1. YM Broth (Difco): ..... 21 g
2. distilled water: ..... 1 L

- Medium for biomass production (Medium IV)
  When a large quantity of biomass was needed for inoculation or immobilization purposes, the medium employed to prepare it was the following one:

1. yeast extract (Difco): ..... 20 g
2. peptone (Difco): ..... 3 g
3. KH_2PO_4 (Anachemia): ..... 2 g
4. $MgSO_4.7H_2O$ (Anachemia): ..... 1 g
5. $(NH_4)_2SO_4$ (Anachemia): ..... 1 g
6. glucose (Anachemia or BDH): ..... 50 g
7. distilled water: ..... up to 1 L of medium

- Medium for ethanol production in packed-bed reactors (Medium V)
  The medium used for continuous ethanol production in packed-bed reactors had the following composition:

1. yeast extract (Difco): ..... 1 g
2. $MgSO_4.7H_2O$ (Anachemia): ..... 1 g
3. $(NH_4)_2SO_4$ (Anachemia): ..... 1 g
4. $CaCl_2.2H_2O$ (Anachemia): ..... 0.15 g
5. sugar (Anachemia or BDH): ..... (variable)
6. distilled water: ..... up to 1 L of medium

- Concentrated medium for ethanol production in packed-bed reactors (Medium VI)
  The concentrated nutrient broth used to keep the immobilized cells active when sucrose inversion and ethanol production were done in series, contained:

1. yeast extract (Difco): ..... 20 g
2. $MgSO_4.7H_2O$ (Anachemia): ..... 40 g
3. $(NH_4)_2SO_4$ (Anachemia): ..... 40 g
4. distilled water: ..... up to 1 L of medium

3.3 Analytical Procedures

3.3.1 Sugar Measurement

High Performance Liquid Chromatography (HPLC) was used to measure the concentrations of sucrose, glucose and fructose. The analyses were made with a Beckman 112 Solvent Delivery Module, equipped with a Beckman 501 Autosampler, and an Altex 156 Refractive Index Detector. The column was a Brownlee Polypore CA 10 micron 4SX 220 mm. The peaks were recorded by a Spectra Physics SP 4270 Integrator. The carrier solvent used was deionized water. The column was operated at a temperature of 85°C and at a carrier flow rate of 0.3 ml/min.

Prior to injection, all samples were filtered through Minisart (NML 16534) 0.2 μm filters. Sample volumes of 1 ml were prepared for the automatic sampler.

The HPLC was calibrated by injecting solutions of sucrose, glucose and fructose (all three in the same solution) of known concentrations. A calibration curve was stored in the integrator and used to evaluate concentrations of sugar from peak areas. The samples being analysed were diluted with deionized water, so that the sugar concentrations obtained would lie in the range covered by the calibration curve (between 0.125 g/100 ml and 0.5 g/100 ml). Standard sugar solutions were run from time to time to verify the accuracy of the calibration curve.

3.3.2 Ethanol Measurement

Ethanol concentrations in solution were measured using Gas Chromatography (GC). The instrument used was a Hewlett-Packard 5370 A Gas Chromatograph equipped with a Hewlett-Packard 7672 A Automatic Sampler. The column was a Chromosorb

102, 80–100 mesh, 6 ft × 1/4 in, stainless steel column. A flame ionization detector (FID) was used. The following temperatures were chosen for the analyses:

- oven: 190°C
- detector: 250°C
- injection port: 200°C

The carrier gas was nitrogen, flowing at 600 ml/min. The peaks were recorded by a Spectra Physics SP 4270 Integrator.

Prior to injection, all samples were filtered through Minisart (NML 16534) 0.2 μm filters. Sample volumes of 1 ml were prepared for the automatic sampler.

The GC was calibrated by injecting solutions of known concentrations. A calibration curve was stored in the integrator and used to evaluate ethanol concentrations from peak areas. The samples were diluted with deionized water so that the ethanol concentrations obtained would be in the range covered by the calibration curve (between 0.0625 g/100 ml and 0.25 g/100 ml). The accuracy of the calibration curve was also checked from time to time by adding an internal standard (methanol) to the samples or by running standard ethanol solutions.

3.3.3 pH Measurement

The pH measurements were conducted using an Orion Research Model 601 A / Digital Ionalyser with a Fisher Scientific electrode. To calibrate the instrument, pH reference buffer solutions of 4.01 and 7.00 (Canlab) were used, prior to each measurement.

3.3.4 Biomass Measurement

The extent of cell growth was monitored by either measuring the fresh weight of cells after centrifugation or by measuring the percentage of light transmittance of the cell culture. Percentages of light transmittance were measured, after dilution (if required), with a Perkin-Elmer Coleman 295 Spectrophotometer, set at a wavelength

of 650 nm. The curve giving the percentage of light transmittance as a function of the dry weight of cells is given in Appendix A. To construct this calibration curve, different dilutions of a washed cell suspension of *S.cerevisiae ATCC 96859* were made. The percentage of light transmittance was measured for each dilution and a 5 ml sample of each was poured in a pre-weighed aluminum dish. These were then dried at a temperature of 105°C to constant weight.

To measure the percentage of light transmittance of a cell culture, a 20 ml sample of the culture was centrifuged for 40 minutes at 9 000 g's, in order to obtain a firm cake of cells. Then, the supernatant was removed and the cells were resuspended in distilled water in order to obtain a final volume of 20 ml of suspension. Five ml of this suspension was used for the spectrophotometer analysis.

3.3.5 Determination of the Nitrogen Content of the Beads

Nitrogen content in immobilized preparations was used as an indication of the protein content, which can be related to the concentrations of enzymes or live cells in the preparation.

A Perkin-Elmer 240C Elemental Analyser was used for measurements of nitrogen content. This instrument is based upon the proven combustion design of the classical methods of Pregl and Dumas and the original work of Simon (1962).

With this instrument, a dry sample of about 3 mg is oxidized under static conditions in a pure oxygen environment, for two to six minutes. The process concludes with a dynamic combustion procedure. The resulting combustion products are swept into a mixing chamber, where they are controlled to a constant pressure, temperature, and volume. The temperature is thermostated at 75°C, the volume fixed at 300 cm^3, and the pressure of 2 atmospheres absolute is controlled by the Perkin-Elmer patented absolute pressure switch. This homogeneous mixture is then analysed by passage through a series of three high-precision thermal conductivity detectors (glass coated filaments), each detector consisting of two cells. Between the first pair of thermal conductivity cells there is an absorption trap containing magnesium perchlorate as a dehydrating material. As the gas passes through, water is removed from the stream. The differential signal obtained before and after

the trap, as read on a digital readout system, reflects the water concentration, and therefore the amount of hydrogen in the original sample. A similar measurement is made of the signal output of a second pair of conductivity cells, between which is a trap which removes carbon dioxide. The remaining gas now consists of helium and nitrogen. This gas passes through a conductivity cell, the output of which is compared to that of a reference cell where pure helium flows to give the nitrogen concentration.

A diagram of the combustion train and of the analytical system is given in Figure 2; the detector flow diagram is shown in Figure 3. For each analysis, one to four beads, depending on their weight, were dried in ambient air and redried at low pressure over P_2O_5 before introduction in the elemental analyser. Percentages of carbon, hydrogen, nitrogen and ash were obtained from these analysis.

3.4 Preparation of Invertase and Inversion of Sucrose

In order to use sucrose as a raw material for the production of fructose and ethanol, the sugar had to be hydrolyzed because the yeast *S. cerevisiae ATCC 36859* did not possess the ability to consume sucrose. The hydrolysis of sucrose was carried out enzymatically using crude invertase preparations from *S. cerevisiae ATCC 46534*.

3.4.1 Growth of *Saccharomyces cerevisiae ATCC 46534* Cells for Invertase Production

To produce invertase, *S. cerevisiae ATCC 46534* cells were grown on medium III to which 5 g/100 ml of sucrose was added. The addition of sucrose to the medium had two purposes: sucrose is a carbon source and it also stimulates the production of invertase by the yeast cells.

The initial invertase content of the yeast used for the preparation of enzyme concentrates can be increased by stimulating its general anabolic

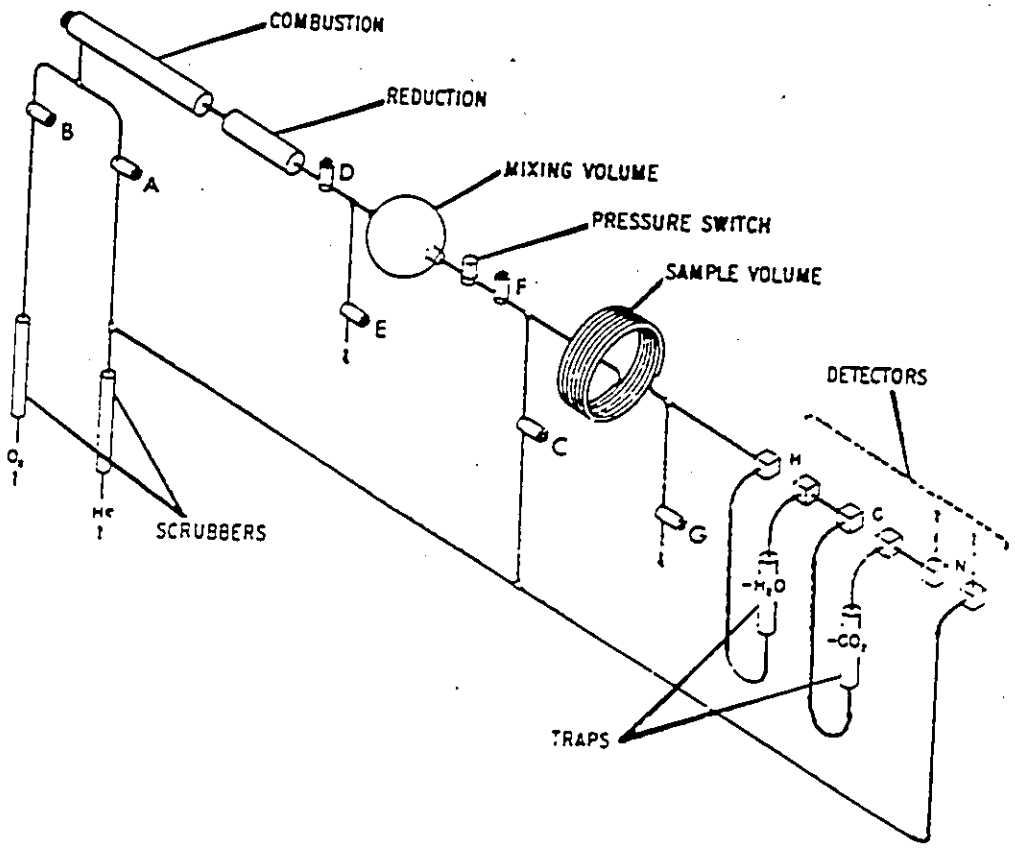

Figure 2: Simplified diagram of the elemental analyser combustion train and analytical system.

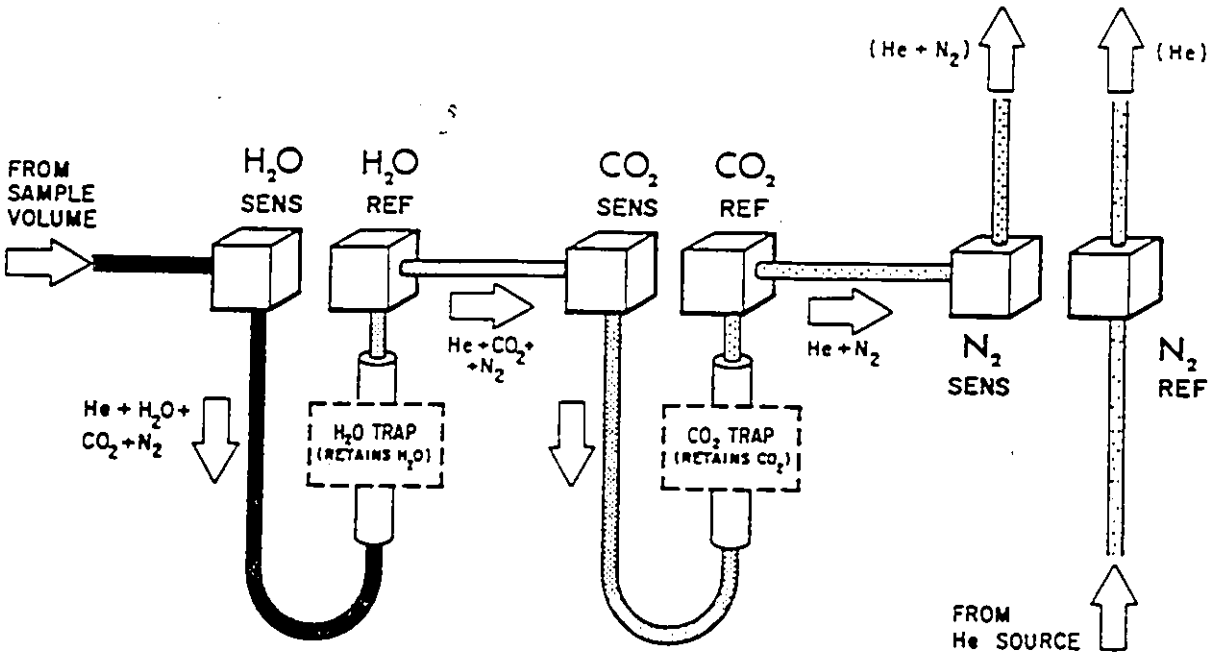

Figure 3: Flow diagram of the elemental analyser detector.

activity; this can be achieved by the addition of appropriate amounts of sucrose during the course of a preliminary fermentation (Arima *et al.*, 1957).

The yeast cells were grown either in shake-flasks or in a fermentor:

- When smaller amounts of cells were required, 500 ml baffled erlenmeyer flasks, each containing 150 ml of the medium were used. The medium was inoculated aseptically with a liquid culture of *S. cerevisiae ATCC 46534* and the flasks were incubated at 30°C on a rotary shaker (200 RPM) for 24 hours.

- The fermentor was used to produce larger amounts of yeast cells. A procedure similar to the one described in subsection 3.6 for the preparation of a heavy inoculum (for tests of ethanol production requiring a large initial biomass concentration) was followed.

  Cells were grown for about 18 hours in both steps. The conditions in the fermentor were: a temperature of 30°C, a stirring rate of 300 RPM and an air flow rate of 1.3 L/min.

3.4.2 Invertase Preparation

The method used to prepare invertase for immobilization in calcium alginate beads is given below:

- *S. cerevisiae ATCC 46534* cells were grown under described conditions, and the cells were separated from the medium by centrifugation either by using a Sorvall RC-3, rotor type HG-4L (swing buckets), for 25 minutes at 4500 RPM, or with a Sorvall RC-2B for 20 minutes at 13 000 g's. The centrifuge temperature was set to 5°C in both cases.

- The paste of cells obtained from the centrifuge, after disposing of the supernatant, was mixed with distilled water in order to make a cell suspension (the wet weight of cells forming 50–70% of the suspension).

- The yeast cells were then treated with acetone (Goodhue *et al.*, 1986; Gunsalus, 1955). The cell suspension was poured into an erlenmeyer containing cold acetone (BDH Chemicals, analytical reagent) at -25°C; the volume of acetone used was equal to five times the volume of the cell suspension.

- The erlenmeyer flask was covered with aluminum foil, closed with a glass stopper and placed on a cold plate/magnetic stirrer. The acetone-yeast cells mixture was stirred for one hour with a magnetic stirring bar and then placed in the freezer (-25°C) overnight.

- The next day, the acetone preparation was taken out of the freezer and the broken cells were collected by suction filtration using a Whatman no.4 filter paper.

- The cell preparation collected on the filter paper was suspended in distilled water (about 60% cells (wet weight) in the suspension) and centrifuged for 10 minutes at 13 000 g's. The cells were then resuspended in distilled water.

- A 2% (w/w) aqueous solution of polyethyleneimine (PEI) was prepared by diluting a 50% (w/w) polyethyleneimine solution (Sigma), and adjusting its pH to 7.0 with HCl.

  A volume of this 2% PEI solution equal to the volume of the last cell suspension was added to the cell suspension, under constant stirring. The stirring of this cell suspension containing about 1% PEI was continued for 5 minutes, on the cold plate.

- The cell suspension was centrifuged for 10 minutes at 13 000 g's and the cells were resuspended in distilled water.

- A 0.1% (w/w) aqueous solution of glutaraldehyde was prepared by dilution of a 25% glutaraldehyde solution (Sigma).

  A volume of this 0.1% (w/w) glutaraldehyde solution equal to the volume of the last cell suspension was added to the cell suspension under constant stirring on the cold plate. Stirring was continued for 5 minutes.

- The suspension was centrifuged for 10 minutes at 13 000 g's and the cells were resuspended in distilled water. This wash cycle was repeated one more time.
- The cells were resuspended in distilled water (about 25% cells (FW) in the suspension) again, and the suspension was homogenized by stirring it with a Sorvall Omni Mixer. The suspension was then ready for immobilization.

3.4.3 Immobilization Procedure

The technique which is described here was used for the immobilization of invertase preparations as well as live yeast cells used for ethanol production. It consisted of entrapping the enzyme preparation or the cells into a natural polymer, alginate.

First, a 4% (w/v) aqueous solution of sodium alginate (BDH) was prepared and sterilized for 7 minutes at 121°C. This sodium alginate solution was mixed, after cooling, with an equal volume of invertase preparation or live cell suspension. Therefore, the active alginate preparation contained about 2% (w/v) sodium alginate. This viscous preparation was poured into a funnel terminated by a small orifice (about 1 mm ID) through which the alginate preparation was extruded (Figure 4). As droplets of sodium alginate preparation were formed, they fell into a 50 mmol/L $CaCl_2$ aqueous solution where they formed gel beads, the alginate chains being cross-linked via calcium ions. The calcium alginate beads thus formed were spherical. They were left in the swirling 50 mmol/L $CaCl_2$ solution for 1.5 to 2 hours to achieve complete gellation. The beads were then washed with distilled water for 30 minutes. Afterwards, the beads were stored in a 20 mmol/L $CaCl_2$ solution in the refrigerator (1–5°C) overnight. The next day, these porous gel beads, having an approximate diameter of 3.4 mm were ready to be used in shake-flasks or packed-bed experiments.

The unused beads were kept in sterile 1 mmol/L $CaCl_2$ aqueous solution in the refrigerator (1–5°C).

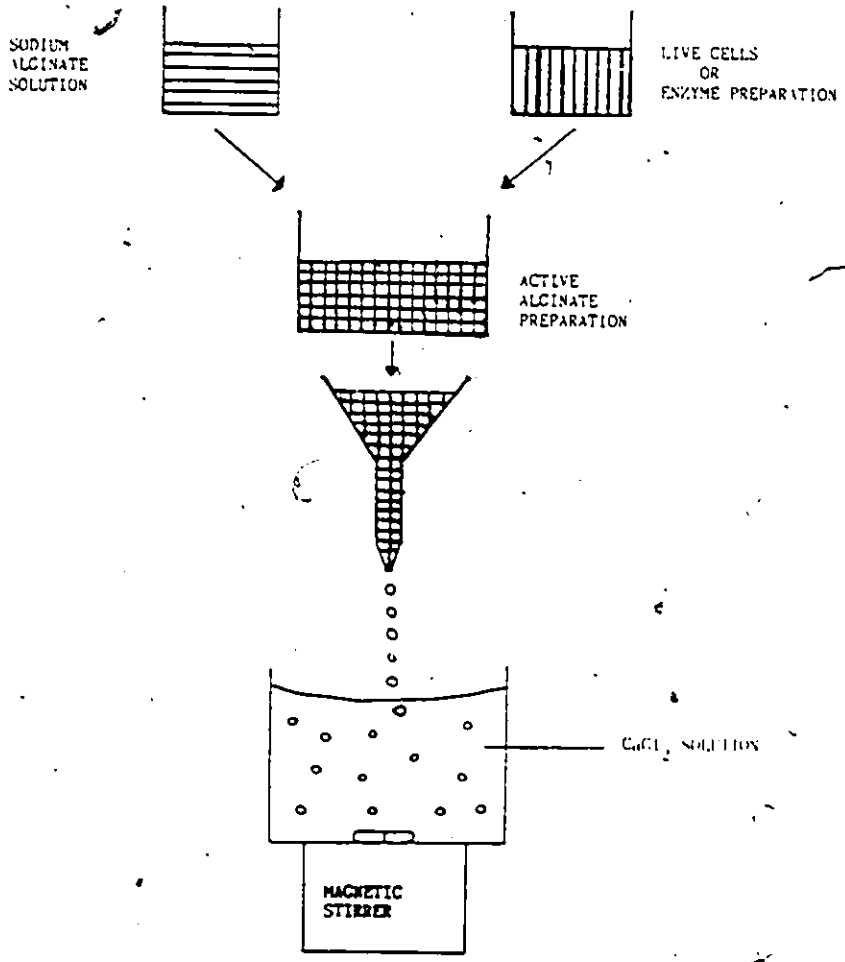

Figure 4: Schematic representation of calcium alginate beads preparation.

3.4.4 Effect of Temperature on Sucrose Inversion

These experiments were performed to determine the effect of temperature on the activity of invertase immobilized in calcium alginate. The kinetics of the inversion of sucrose were followed in batch systems at different temperatures. The apparatus used for these experiments is shown in Figure 5.

To each flask used for the experiments, 25 ml of a 5 g/100 ml sucrose solution was added. The solution contained 0.5 mmol/100 ml $CaCl_2$ to help prevent disruption of the gel by calcium chelating agents; each flask contained 25 beads of invertase immobilized in calcium alginate. The flasks were stoppered and placed on magnetic stirrers in incubators set at different temperatures (between 45°C and 65°C). The magnetic stirrers speed were set in order to allow good contact between the beads and the surrounding solutions.

Sampling was done with a 3 cm^3 syringe; sample size was 0.5 ml. Samples were then analysed to determine sugar concentrations.

3.4.5 Effect of pH on Sucrose Inversion

These experiments were conducted to find the effect of pH on the activity of invertase immobilized in calcium alginate beads. The kinetics of sucrose inversion were followed in batch systems at different pH's. For each pH studied, a 50 ml erlenmeyer flask containing 25 ml of solution and 25 invertase-containing calcium alginate beads was used. The flasks were stoppered with corks and placed on a rotary shaker (130 RPM) in an incubator at a temperature of 55°C. All solutions used for the experiment were prepared by solubilizing 5 g/100 ml sucrose and 0.5 mmol/100 ml $CaCl_2$ in buffer solutions. The buffer solutions were prepared the following way:

- $2.97 \leq pH \leq 3.10$: a solution of 0.1 mol/L CH_3COOH was used.
- $3.50 \leq pH \leq 3.53$: a 0.1 mol/L CH_3COONH_4 solution was added to a 0.1 mol/L CH_3COOH solution until the desired pH was reached.

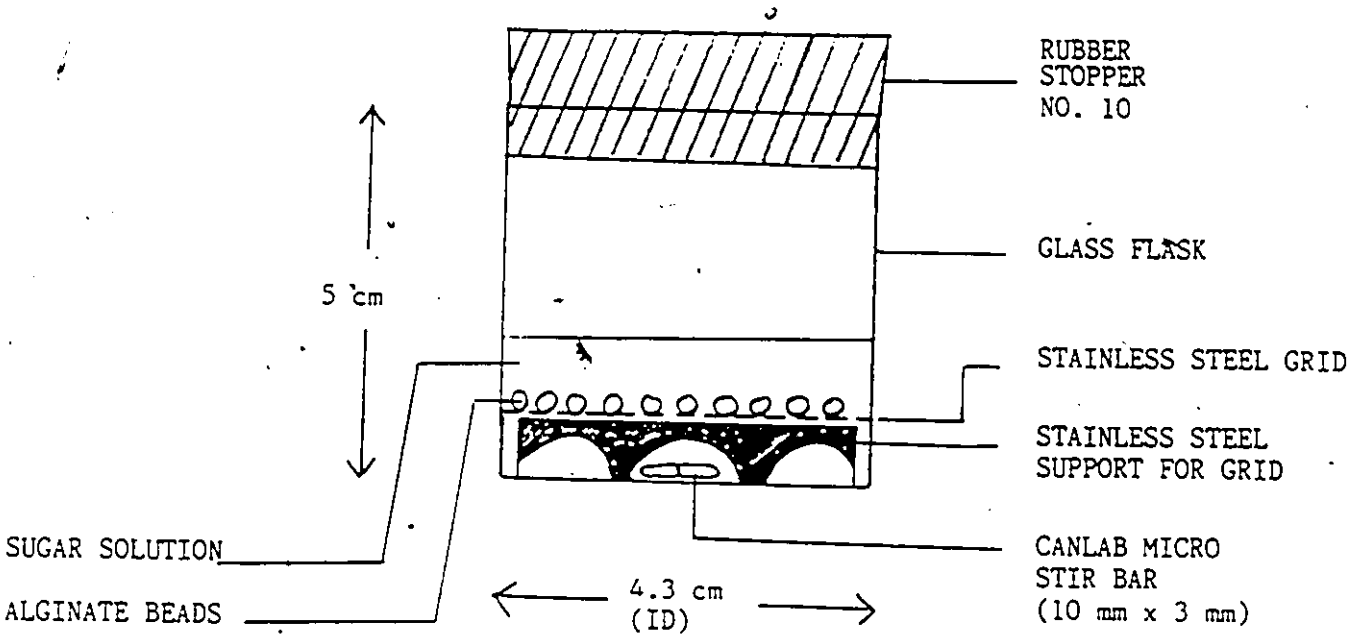

Figure 5: Schematic diagram of the apparatus used to study the influence of temperature on the activity of invertase immobilized in calcium alginate beads.

- $4.51 \leq pH \leq 4.57$: a 0.1 mol/L CH_3COONH_4 solution was added to a 0.1 mol/L CH_3COOH solution until the desired pH was reached.
- $5.59 \leq pH \leq 5.64$: a 0.1 mol/L NaOH solution was added to a 10 mmol/L 2[N-Morpholino]ethane sulfonic acid (MES, Sigma) solution until the desired pH was obtained.
- $6.71 \leq pH \leq 6.90$: a 0.1 mol/L NaOH solution was added to a 10 mmol/L MES solution until the desired pH was obtained.
- $5.43 \leq pH \leq 6.90$: this last solution was not a buffer, only distilled water was used; it was prepared as a means of comparison to see if the buffers had any effect on invertase activity.

During the experiments, the pH's remained in the range specified above. They were measured at the beginning and at the end of the experiment. Samples of 0.5 ml were taken with a syringe and analysed to determine sugar concentrations.

3.4.6 Inhibition of Sucrose Inversion by End Products

To determine the effect of the presence of glucose and fructose on the activity of immobilized invertase, shake-flask experiments were conducted. The kinetics of sucrose inversion of the following aqueous carbohydrate solutions were studied:

- 5% (w/v) sucrose
- 5% (w/v) sucrose + 2.5% (w/v) glucose
- 5% (w/v) sucrose + 2.5% (w/v) fructose
- 5% (w/v) sucrose + 2.5% (w/v) glucose + 2.5% (w/v) fructose
- 5% (w/v) sucrose + 5% (w/v) glucose
- 5% (w/v) sucrose + 5% (w/v) fructose
- 5% (w/v) sucrose + 5% (w/v) glucose + 5% (w/v) fructose

All solutions contained 5 mmol/L $CaCl_2$.

For each solution, 25 ml of it was poured in a 50 ml erlenmeyer flask containing 25 beads of invertase active calcium alginate beads. The flasks were stoppered with corks and placed on a rotary shaker (130 RPM) in an incubator at 55°C. From time to time, samples were taken to determine sugar concentrations.

3.5 Ethanol Production

3.5.1 Test of Growth of *Saccharomyces cerevisiae ATCC 36859* on Various Carbohydrates.

The ability of the yeast *S. cerevisiae ATCC 36859* to use different sugars as carbon sources was tested. A yeast being able to consume glucose either for growth or for ethanol production, but unable to use fructose was needed. Shake-flask experiments were performed to study the consumption of glucose, fructose and sucrose by the yeast. Four pairs of 250 ml erlenmeyer flasks containing 50 ml of medium II were prepared, with different sugars used in each one:

- the first pair contained 2% (w/v) sucrose
- the second pair: 2% (w/v) glucose
- the third pair: 2% (w/v) fructose
- and to the last pair of flasks, no sugar was added.

Each flask was inoculated aseptically with 0.1 ml of liquid inoculum. The flasks were incubated at 30°C on a rotary shaker (200 RPM), for 24 hours. Samples of 1.5 ml were taken from each flask before inoculation and after incubation and were analysed for sugar consumption and yeast growth.

3.5.2 Effect of Temperature on Growth of *Saccharomyces cerevisiae ATCC 36859.*

These experiments were done to determine the temperature at which the growth of the ethanol-producing yeast cells was the fastest. The following procedure was followed:

- First, five 250 ml baffled erlenmeyer flasks equipped with sidearms (Figure 6) were each filled with 50 ml of medium II containing 5% (w/v) glucose.
- The medium in four of these 5 flasks was inoculated with a liquid culture of *S. cerevisiae ATCC 36859*; the fifth flask was kept as a reference for biomass measurement.
- The four inoculated shake-flasks were incubated at temperatures of 26°C, 30°C, 33°C and 37°C on rotary shakers working at 200 RPM.
- The growth of yeast cells was followed with a spectrophotometer calibrated with the blank medium contained in the fifth flask. Since the erlenmeyer flasks were equipped with sidearms, the percentage of light transmittance could be measured without opening the flasks.
- Using the calibration curve (Appendix A), the percentage of light transmittance was related to the biomass concentration in the flasks (on a dry basis).

3.5.3 Effect of Glucose Concentration on Growth of *Saccharomyces cerevisiae ATCC 36859.*

These experiments were done in 250 ml baffled erlenmeyer flasks; to each flask, 50 ml of medium II was added. Different concentrations of glucose were used for each test. The following initial glucose concentrations were chosen: 2% (w/v), 4% (w/v), 6% (w/v), 8% (w/v), 10% (w/v), 15% (w/v) and 20% (w/v).

The media in all flasks were inoculated with equal amounts of a liquid culture; the flasks were then incubated at 30°C on a rotary shaker (200 RPM).

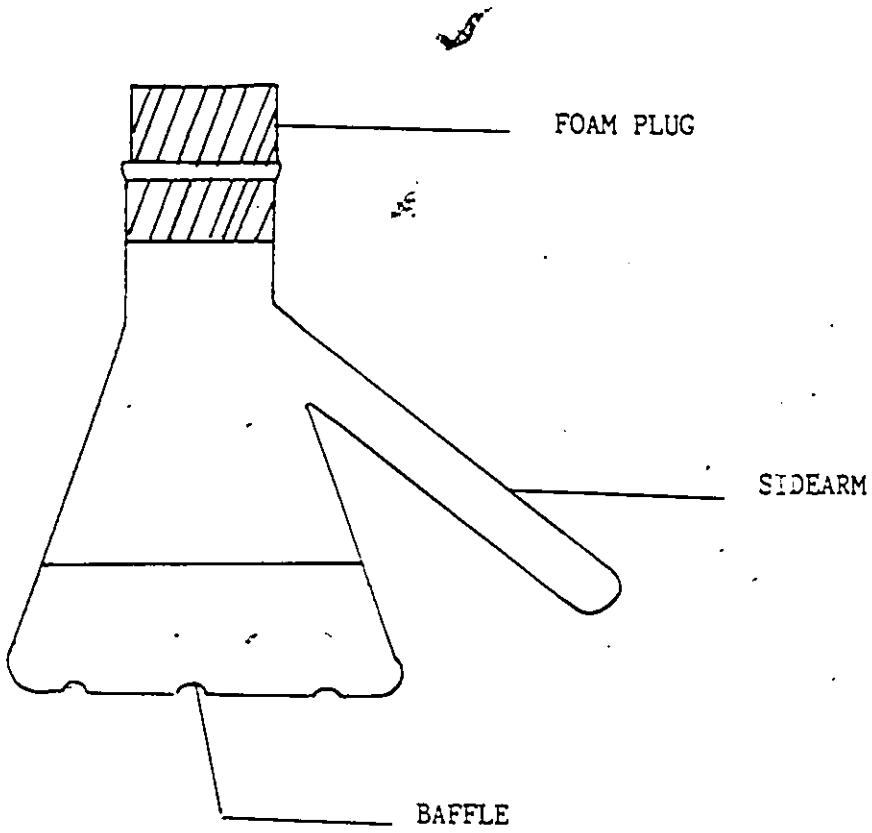

Figure 6: Shake-flask with sidearm used for growth tests.

Samples of 1.5 ml were taken from each flask after 3 days of incubation and were analysed for sugars and biomass concentrations.

3.5.4 Effect of Ethanol Concentration on Growth of *Saccharomyces cerevisiae ATCC 36859.*

These experiments were done by adding different amounts of ethanol to the medium used to grow the yeast cells, before inoculation. Medium II was used; an initial volume of 50 ml of medium was contained in each 250 ml erlenmeyer flask used. The following initial ethanol concentrations were selected: 0%, 2% (w/v), 4% (w/v), 6% (w/v), 8% (w/v), 10% (w/v) and 12% (w/v). The shake-flasks were incubated at 30°C on a rotary shaker (200 RPM). Samples of 1.5 ml were taken from each flask before inoculation and after 24 hours of incubation; they were analysed for biomass concentration.

3.5.5 Tests of Ethanol Production

Tests of ethanol production for which many samples had to be taken were performed in a 7 liter New Brunswick Microferm Fermentor (Jar Model MF 441) at 30°C. Agitation was provided by an impeller, normally set at 200 RPM. Filtered air, from the building's compressed air line, was available through an orifice near the bottom of the fermentor. The air flow rate was controlled by a flow meter.

Each test of ethanol production was done with 4 liters of sterile medium II. The fermentor was also sterilized before each experiment. Each batch of the medium was inoculated with a concentrated liquid culture. The fermentor was equipped with a built-in condenser through which coolant pumped from a cooling unit was circulating, at a temperature of about 5°C.

Samples of about 25 ml in volume were collected with glass tubes. Prior to each sampling, a pre-sample was taken in order to drain the sampling tube of liquid that had been inside since the last sampling.

3.5.6 Growth of *Saccharomyces cerevisiae ATCC 36859* Cells for Immobilization

For immobilization purposes, the ethanol-producing yeast cells were grown on medium IV, the manner in which concentrated biomass was made (subsection 3.6). The cells were grown for 24 hours. After growth, the supernatant was pumped out of the fermentor, the cells were centrifuged for 30 minutes at 13 000 g's and resuspended in sterile distilled water. This cell suspension was ready for immobilization.

3.6 Preparation of the Fermentor Inoculum

To inoculate the medium in the fermentor, a large amount of inoculum was needed. It was prepared in the following way:

- Twelve 250 ml baffled erlenmeyer flasks, each filled with 100 ml of medium II containing 2% (w/v) glucose were prepared and sterilized.
- The medium was then inoculated under aseptic conditions with cells from agar slants.
- The flasks were incubated at a temperature of 30°C, on a rotary shaker (200 RPM) for 24 hours.
- After incubation, the contents of the 12 flasks was poured into two sterile 1-liter erlenmeyers which were placed in a refrigerator (1–5°C) to allow the cells to settle for half a day to one day.
- Finally, the supernatant was removed by decantation and the concentrated cell suspension (100–200 ml) was used as the inoculum for the fermentor.

In the case of experiments requiring a larger initial biomass concentration, the inoculum was grown in two steps:

- First, some inoculum was prepared in the way described above.

- This inoculum was added to 4 liters of medium IV in the fermentor. (New Brunswick Microferm Fermentor, Jar Model MF 441)
- Conditions for growth in the fermentor were a temperature of 30°C, a stirring rate of 500 RPM and an air flow rate of 2.0 L/min. Cells were grown for one day.
- After growth, the fermentor was cooled to about 5°C by circulating coolant through the baffles, the stirring and the aeration were stopped and the cells were allowed to settle to the bottom of the fermentor jar during about one day.
- The supernatant was pumped out of the fermentor and the thick layer of cells accumulated at the bottom of the jar, served as the inoculum for the next test of ethanol production. The medium for the following test of ethanol production, after sterilization, was added, under aseptic conditions, to the fermentor already containing the large inoculum.

3.7 Apparatus Used for Continuous Operation

3.7.1 Packed-Bed Bioreactors

Packed-bed reactors were used for sucrose inversion and for ethanol production. All bioreactors used were identical. A schematic diagram of these bioreactors is given in Figure 7. The volume of the bed was 150 ml. The walls of the reactor were made of pyrex glass. The bed was surrounded by a jacket through which water flowed to keep the temperature of the bed constant. At the top of the bioreactor, above the bed, an air filter (glass wool packed in a narrow glass tube) was used to equilibrate the bed pressure with the atmosphere. The substrate was fed at the bottom of the bed and the products were recovered at the top.

To load the previously sterilized reactor with calcium alginate beads, the top part of the bioreactor, sealed to the body by a rubber o-ring, was removed. The top grid was also removed and the beads were packed in the bed with a spatula.

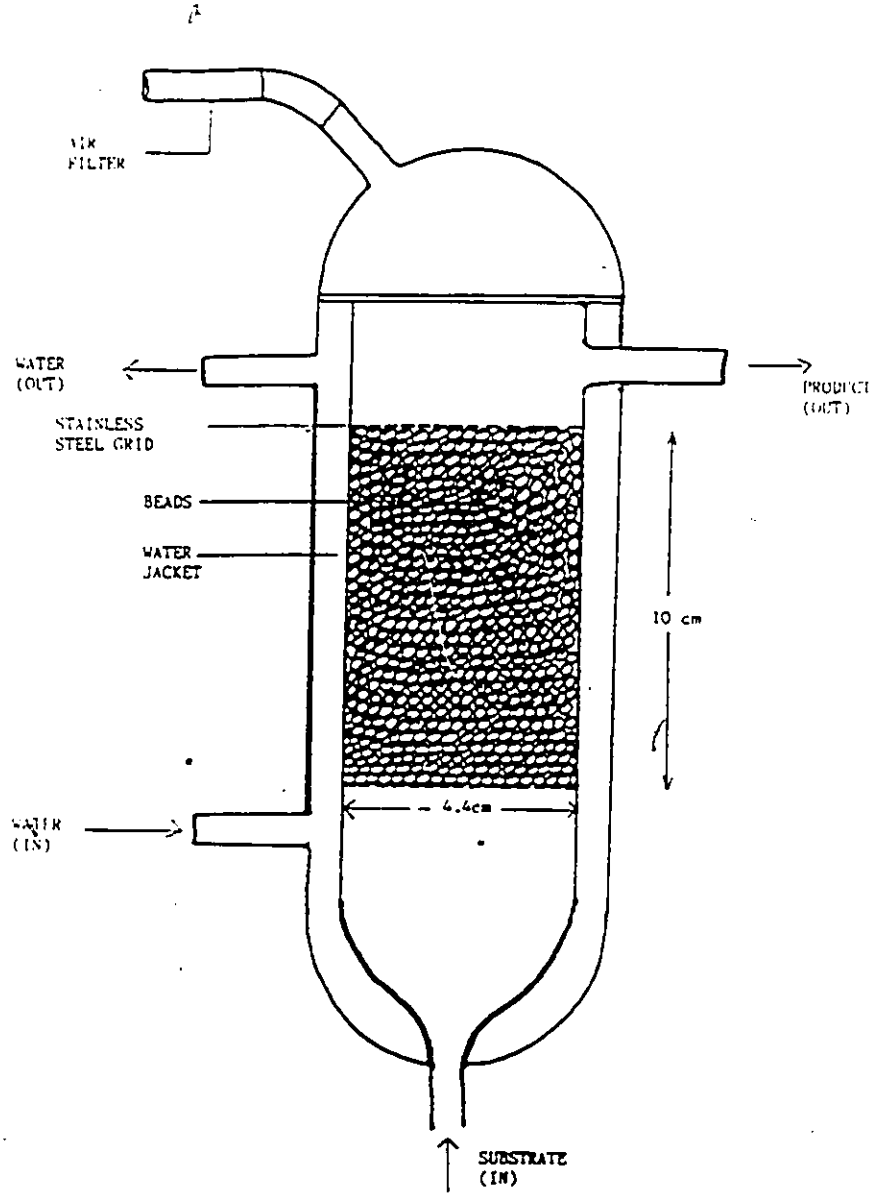

Figure 7: Schematic diagram of the packed-bed reactor.

The reactor was then reassembled. These operations were performed under aseptic conditions.

3.7.2 Medium Reservoirs

Glass bottles, having an orifice through the bottom of the wall, were used as medium reservoirs. Bottles of 1 liter, 2 liters and 4 liters in capacity were used (Figure 8).

3.7.3 Medium Mixer

When the invertase bioreactor and the ethanol-producing bioreactor were used in series, a medium mixer was placed between them to mix the effluent from the invertase packed-bed reactor with the stream of concentrated nutrients (medium VI) from the concentrated medium reservoir (Figure 9). The liquid medium obtained in the mixer was pumped to the ethanol bioreactor.

The medium mixer had a volume of about 300 ml; it was sitting on a magnetic stirrer.

3.7.4 Pumps

The media were pumped using peristaltic pumps (Isco Wiz Pump/Diluter/Dispenser) and silicone tubing (1/8 inch ID). Rubber tubing was also used but was not in contact with the pumps. Flow rates varied between 0.8 ml/h and 140 ml/h.

3.7.5 Circulators

Constant temperature circulators were used to keep the temperature of the packed-bed reactors constant by circulating water through the jacket around the beds. For the invertase bioreactors kept at 55°C, a Haake G D 8 was used and for the ethanol-producing bioreactors, a Haake FS or Haake FE was utilized.

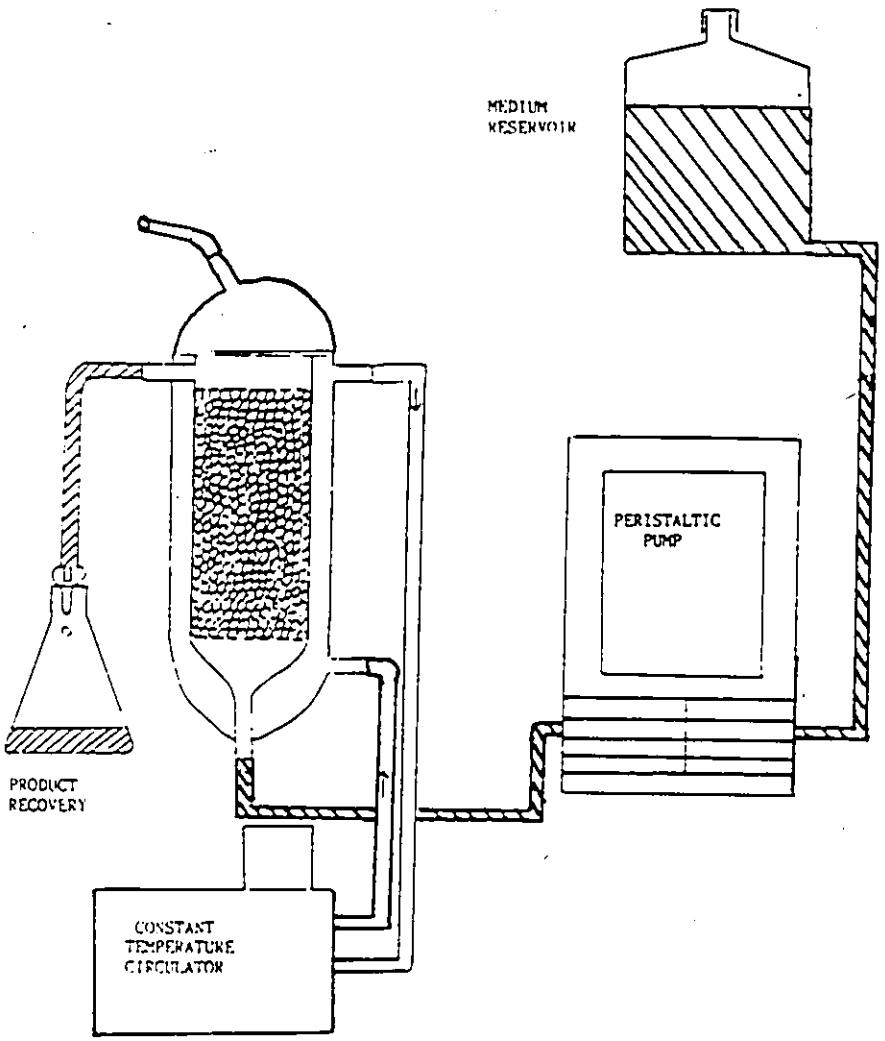

Figure 8: Schematic diagram of the set-up used with the packed-bed reactors.

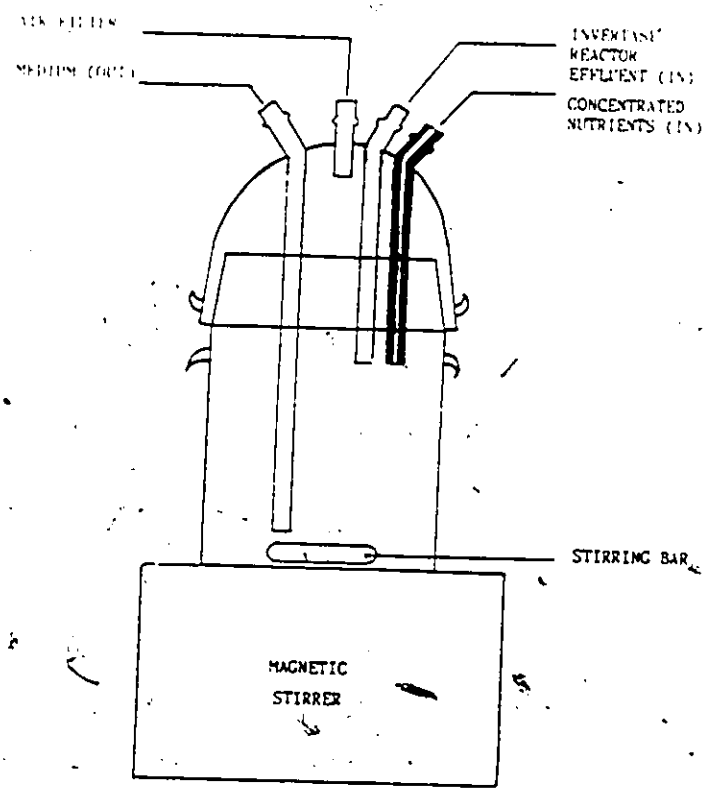

Figure 9: Schematic diagram of the vessel used to mix two streams of medium.

3.7.6 Continuous Process Set-Up

A diagram of the set-up used for the continuous conversion of sucrose into fructose and ethanol is given in Figure 10. It consisted of using in series an invertase packed-bed reactor and an ethanol-producing packed-bed reactor.

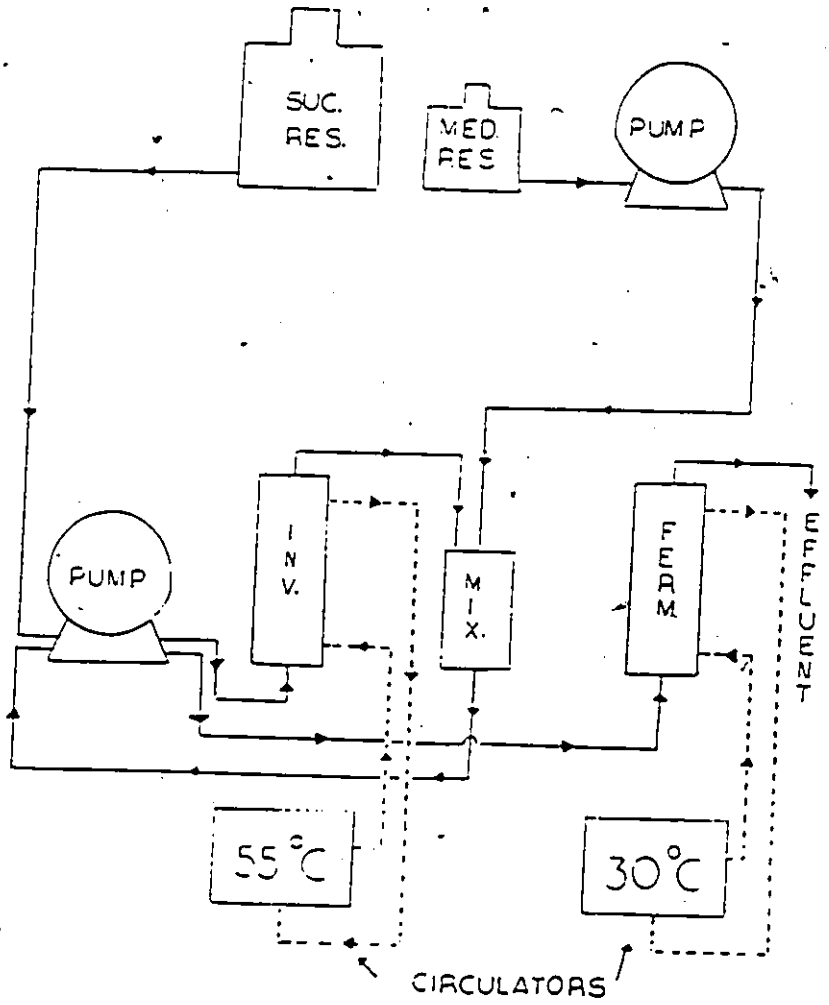

Figure 10: Schematic diagram of the set-up used for the continuous conversion of sucrose into fructose and ethanol.

Chapter 4

Results

4.1 Sucrose Inversion in Batch Systems

The influence of temperature, pH, and inhibition by end products on sucrose inversion was studied in batch systems. Sucrose inversion was catalyzed by invertase immobilized in calcium alginate beads having the following characteristics:

First Batch of Invertase Beads	
initial diameter of a bead	3.4 mm
initial mass of a bead	24 mg
initial percentage of nitrogen (dry basis)	3.0
initial mass of nitrogen per bead	63 μg
fraction of treated cells in the alginate suspension (wet weight)	14% (w/w)

4.1.1 Effect of Temperature on Sucrose Inversion

The aim of the tests described below was to determine the temperature at which the activity of invertase immobilized in calcium alginate gel was the highest.

The kinetics of the inversion of sucrose were followed at different temperatures using the apparatus described in subsection 3.4.4. Temperatures of 45°C, 50°C,

55°C, 60°C and 65°C were selected for the inversion of sucrose in 5% (w/v) solutions.

Figure 11 shows the amount of sucrose inverted as a function of time for the five temperatures chosen. The reaction was carried out for five hours. The temperature at which the largest amount of sucrose was inverted was 55°C.

During the first two hours of incubation, all 5 reactions progressed at comparable rates, but after two hours, the reactions at 60°C and 65°C started to slow down. After 5 hours of incubation, 70% of the sucrose was inverted at 55°C, and quantities slightly lower were inverted at 50°C and 45°C. During the same period of time, about 50% of the sucrose was converted at 60°C and only 29% at 65°C.

Figure 12 shows the amount of sucrose inverted in 5 hours as a function of the temperature of incubation. At 45°C, 0.81 g of sucrose was inverted; at 55°C, 0.88 g of sucrose was inverted and at 65°C, 0.36 g of sucrose was transformed into glucose and fructose. Activity of immobilized invertase slowly increased from 45°C to 55°C, but dropped quickly when the temperature increased from 55°C to 65°C.

4.1.2 Effect of pH on Sucrose Inversion

The next parameter studied was pH. Several experiments were performed to find the effect of pH on the activity of invertase immobilized in calcium alginate beads.

The kinetics of sucrose inversion were followed at 5 different pH's (in the range 3.0–6.8) using 5% (w/v) sucrose solutions; the temperature of the batch systems was set to 55°C.

The results of this experiment are shown in Figure 13. The four curves representing the pH range 3.5–5.6, remained very close to each other for the 5 hours the experiment lasted. This means that the activity of invertase was about constant in this pH range, for which 50–55% of the initial amount of sucrose was inverted after 4 hours of incubation. At a pH of 3.0, 33% of the sucrose was inverted in 4 hours and at a pH of 6.8, only 27%.

The mass of sucrose inverted as a function of the pH is represented in Figure 14, for incubation periods of one hour and four hours. As can be seen from the two curves, the amount of sucrose inverted did not vary much for the pH range 3.5–5.6, in which invertase activity was the highest. After 4 hours of incubation, over 0.6

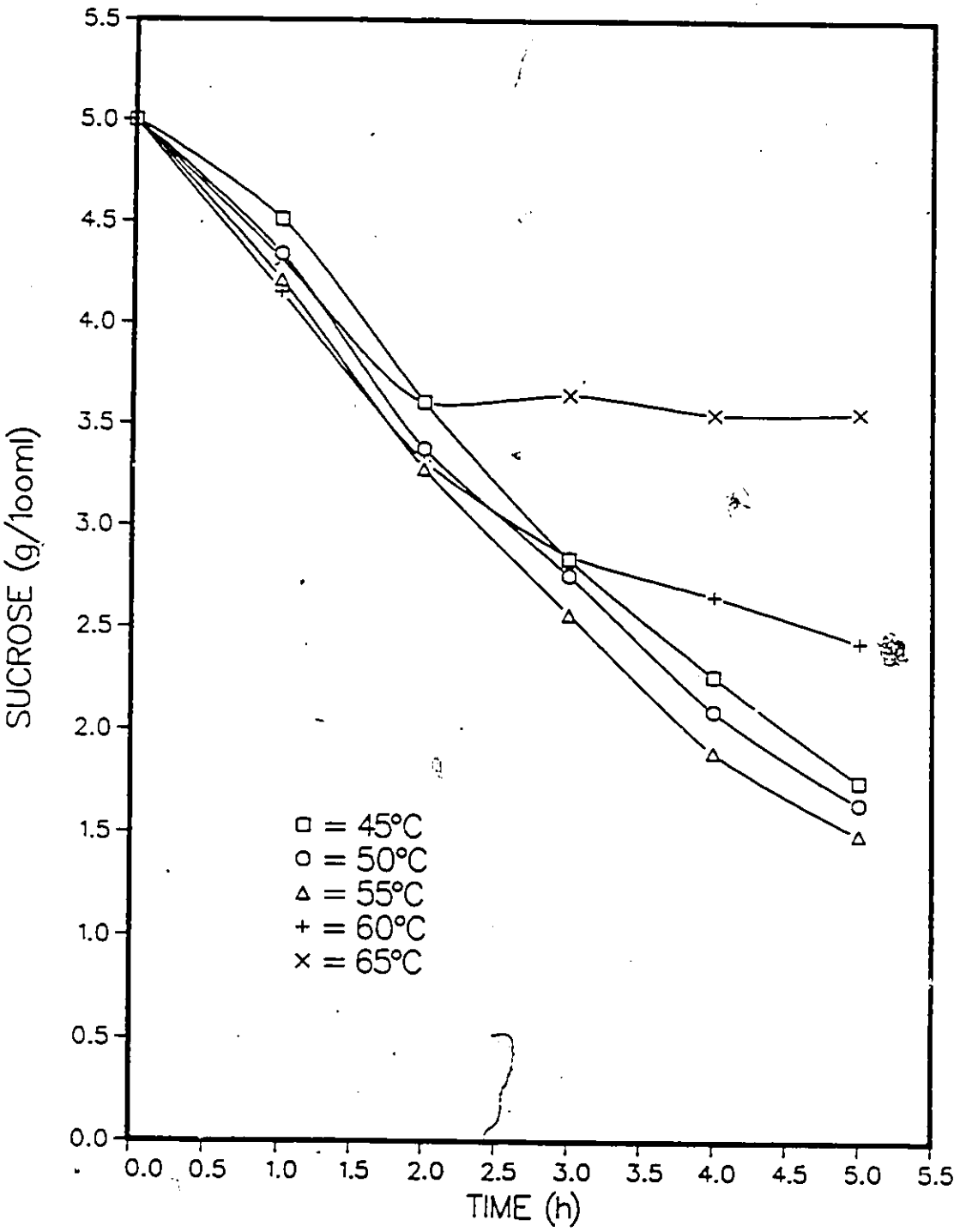

Figure 11: Kinetics of sucrose inversion at different temperatures.

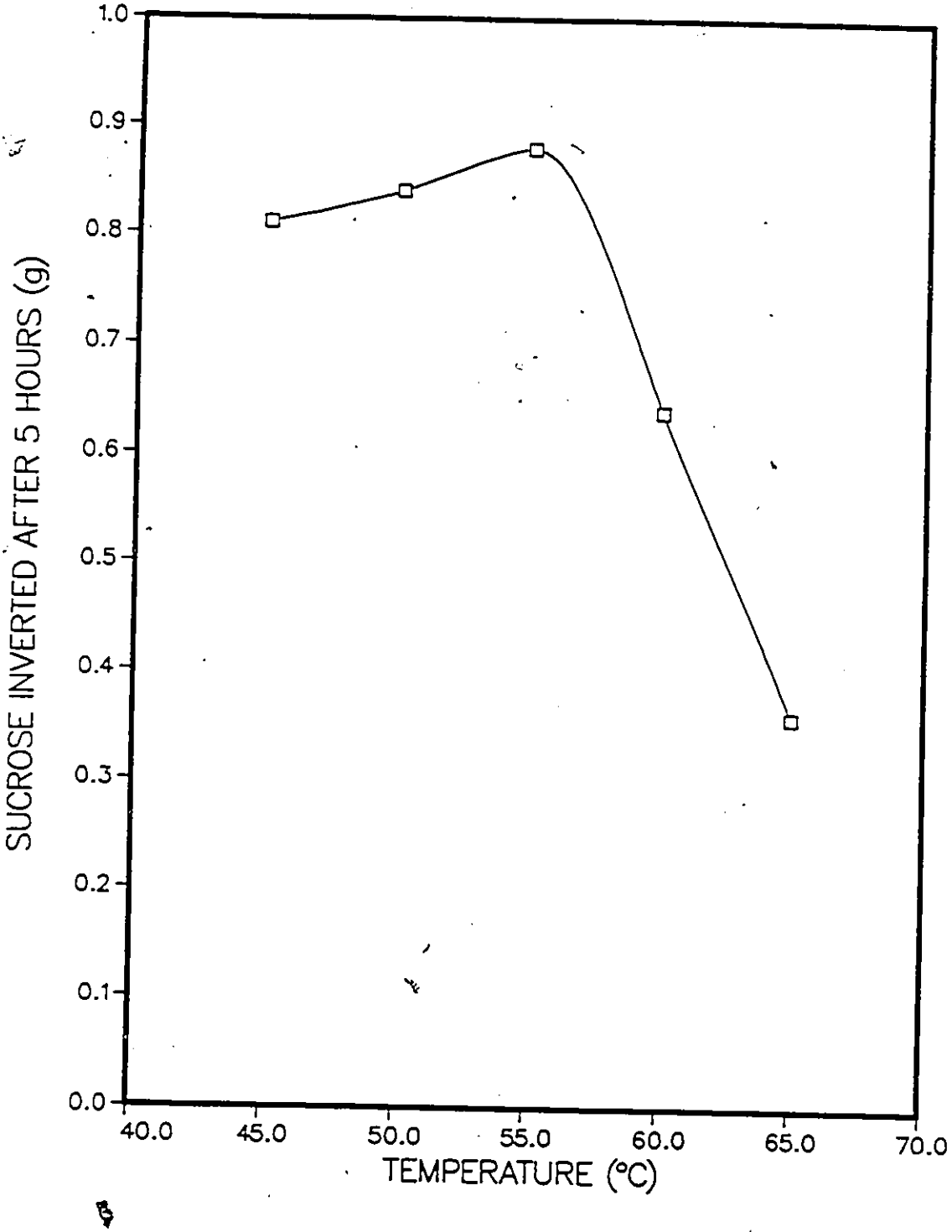

Figure 12: Effect of temperature on sucrose inversion.

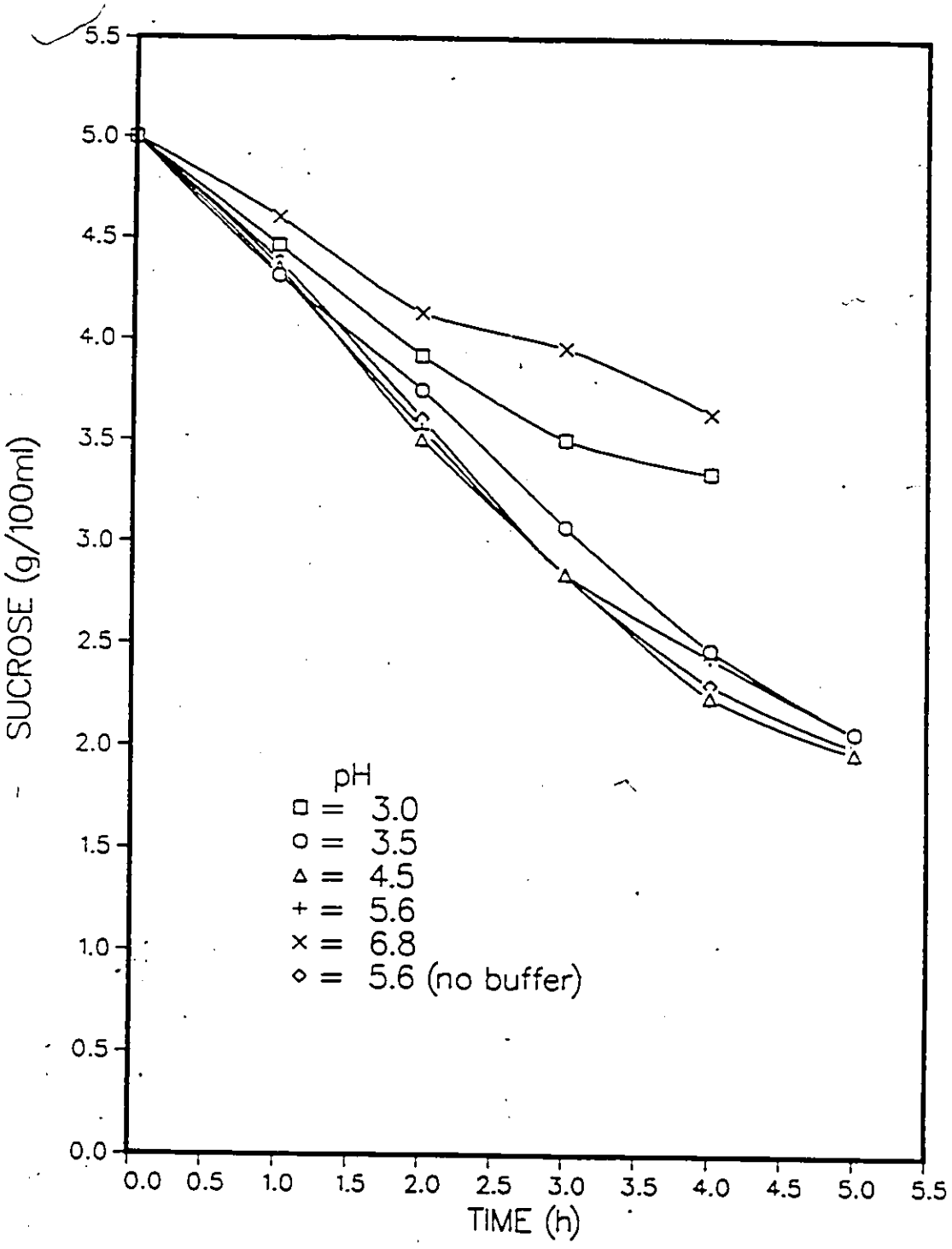

Figure 13: Kinetics of sucrose inversion at different pH's.

g of sucrose was inverted for the three systems in this pH range while 0.41 g was inverted at a pH of 3.0 and 0.34 g was inverted at a pH of 6.8. The pH range for optimum invertase activity (3.5–5.6) could be distinguished even after one hour of incubation.

4.1.3 Inhibition of Sucrose Inversion by End Products

According to literature concerning invertase, the enzyme is inhibited by its end products (Combes and Monsan, 1983). Experiments were therefore conducted to determine the extent of this inhibition.

Solutions containing 5 g/100 ml sucrose and different concentrations of glucose and fructose were incubated at 55°C in the presence of invertase-active calcium alginate beads. The kinetics of the reaction of the batch systems were followed for 5 hours.

Figure 15 shows the kinetics of sucrose inversion in the presence of different concentrations of end products. As time progressed, the curves became more distinct, revealing the effect of inhibition.

As seen on Figure 15, more sucrose remained unconverted in systems where the concentration of end products was high (5% initially) than in systems where it was lower (2.5% initially) or nil.

The amount of sucrose inverted in 5 hours of incubation as a function of the concentration of end products added to the system is represented in Figure 16. These results indicate that fructose is a stronger inhibitor than glucose. In the system initially containing no end products, 61% of the sucrose was inverted in 5 hours while in the system to which 5% glucose was added, 50% of the sucrose was inverted and in the system initially containing 5% fructose, 46% of the sucrose was transformed. When both glucose and fructose were added in 5% concentration, 40% of the sucrose was inverted. These results also show that the amount of sucrose inverted is inversely proportional to the initial end products concentration, for the concentration range studied.

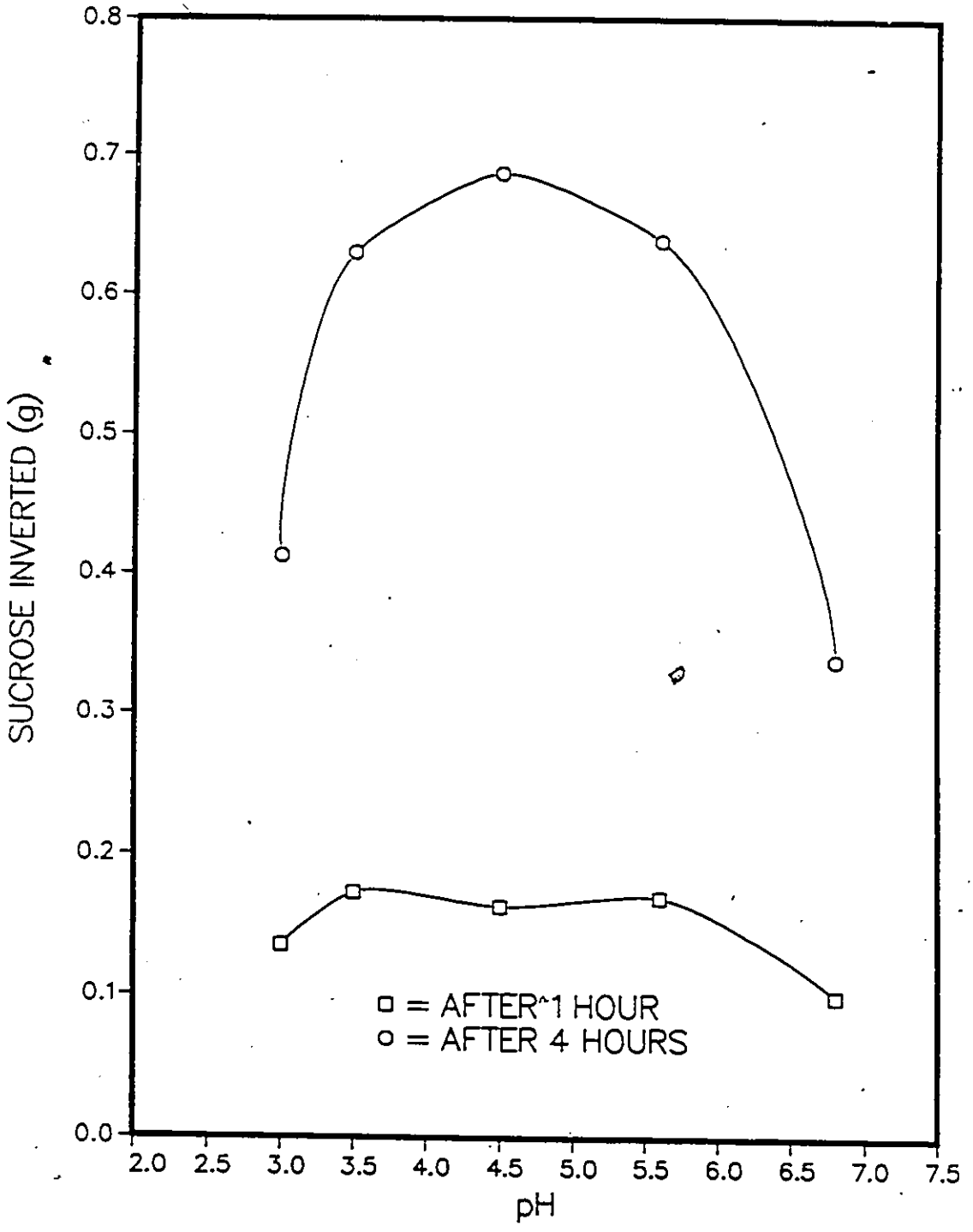

Figure 14: Effect of pH on sucrose inversion.

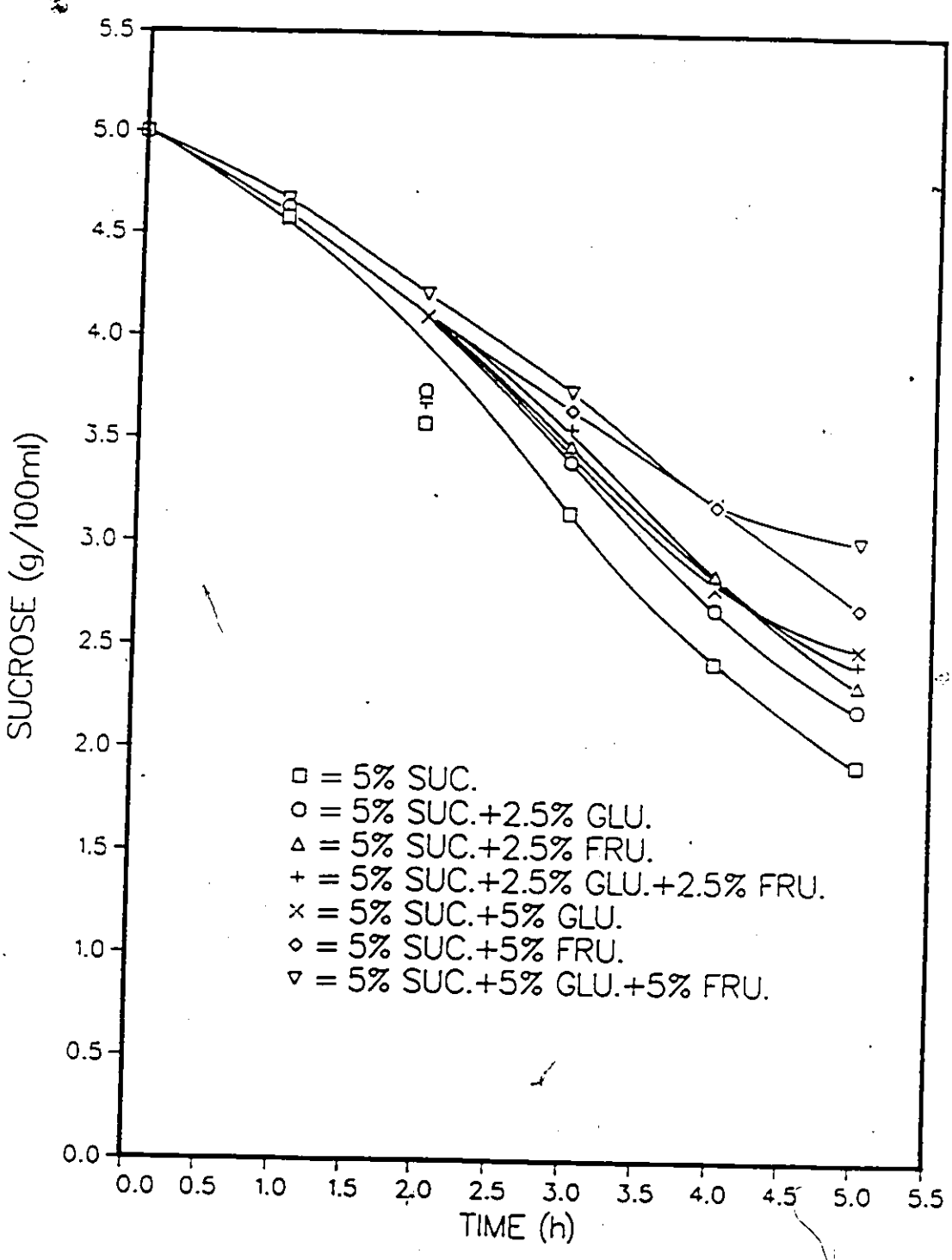

Figure 15: Kinetics of sucrose inversion in the presence of different concentrations of end products.

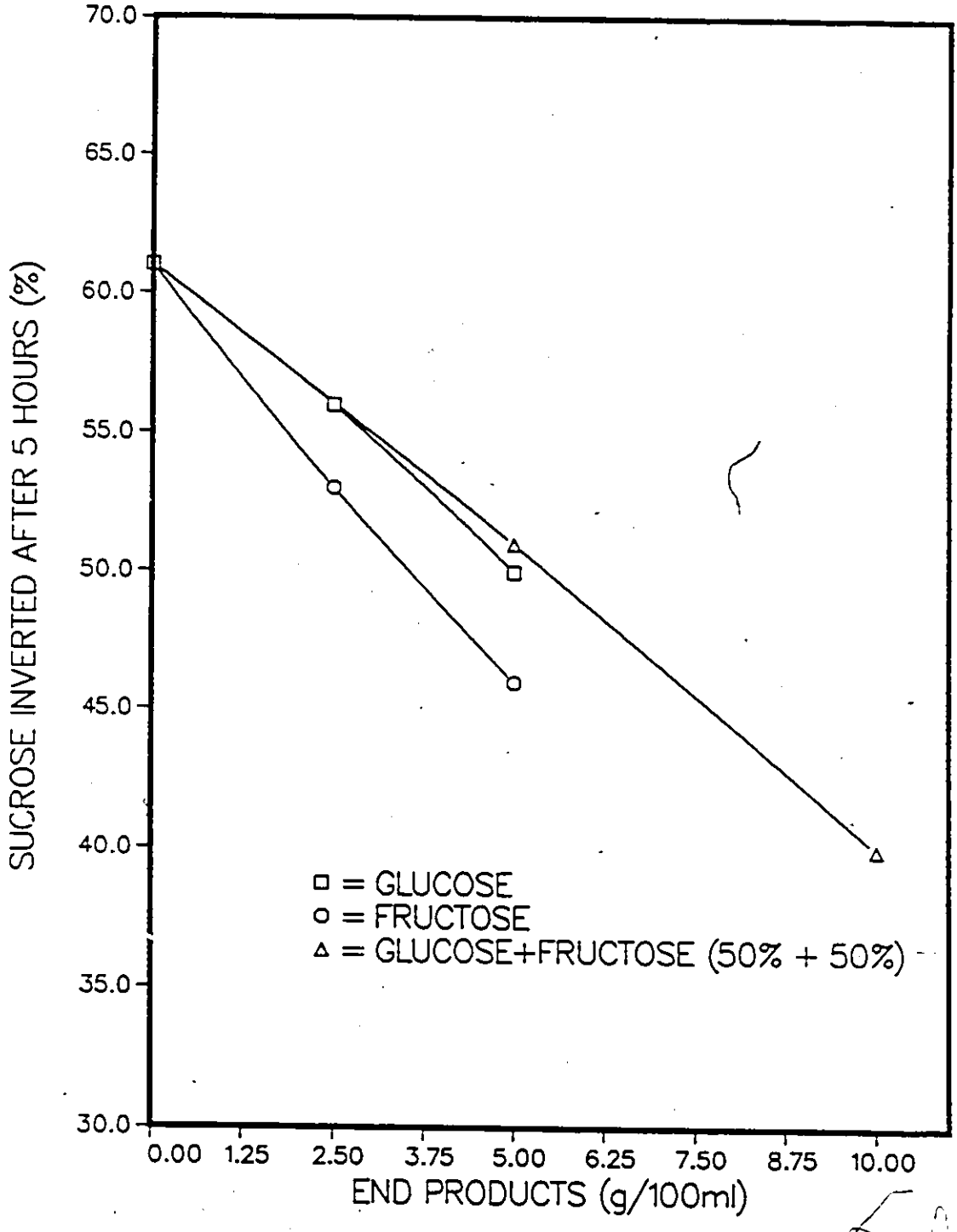

Figure 16: Effect of end product inhibition on sucrose inversion.

4.2 Sucrose Inversion in Packed-Bed Reactors

Inversion of sucrose was carried out continuously by invertase-active calcium alginate beads packed in a 150 ml fixed-bed reactor. An aqueous sucrose solution containing trace amounts of $CaCl_2$ was continuously fed to the packed-bed reactor. A schematic diagram of the set-up used with the bioreactor is shown in Figure 8. The first bioreactor for sucrose inversion to be assembled contained about 4400 beads having the same characteristics as the beads used in the batch systems.

Figures 17, 18 and 19 give the results of the operation of this packed-bed bioreactor. Initially, a 20% (w/v) sucrose solution was fed to the reactor. After 622 and 1054 hours of operation, this solution was replaced by a 10% (w/v) and 5% (w/v) sucrose solution respectively.

The reactor was operated for over 1500 hours (63 days) at a temperature of 55°C. A dilution rate of 0.33 h^{-1}, corresponding to a flow rate of 50 ml/h, was selected after 2 days of operation and used from then on.

After about 100 hours of operation, the packed-bed reactor was converting about 90% of the sucrose present in the 20% (w/v) sucrose solution. This means it was inverting 60 grams of sucrose per litre of reactor volume per hour. After 500 hours of operation, only 58% of the sucrose present in the feed was inverted.

The sucrose conversion level was 46% for a 10% (w/v) sucrose solution, after 800 hours of operation, which represented a sucrose inversion rate of 15 g/(L.h).

The efficiency continued to diminish progressively with time. The decrease was especially pronounced with sucrose concentration changes in the feed solution. When the sucrose concentration in the feed was changed from 20% (w/v) to 10% (w/v), after 622 hours of operation, the sucrose inversion rate dropped from 33 g/(L.h) to 23 g/(L.h); when the feed sucrose concentration was changed from 10% (w/v) to 5% (w/v), the sucrose inversion rate fell from 10 g/(L.h) to 7 g/(L.h).

After 1500 hours of operation, the reactor efficiency was low, the sucrose inversion rate being only 3 grams of sucrose inverted per litre of reactor volume per hour.

Large pH fluctuations were the results of variations of the pH of the feed sucrose

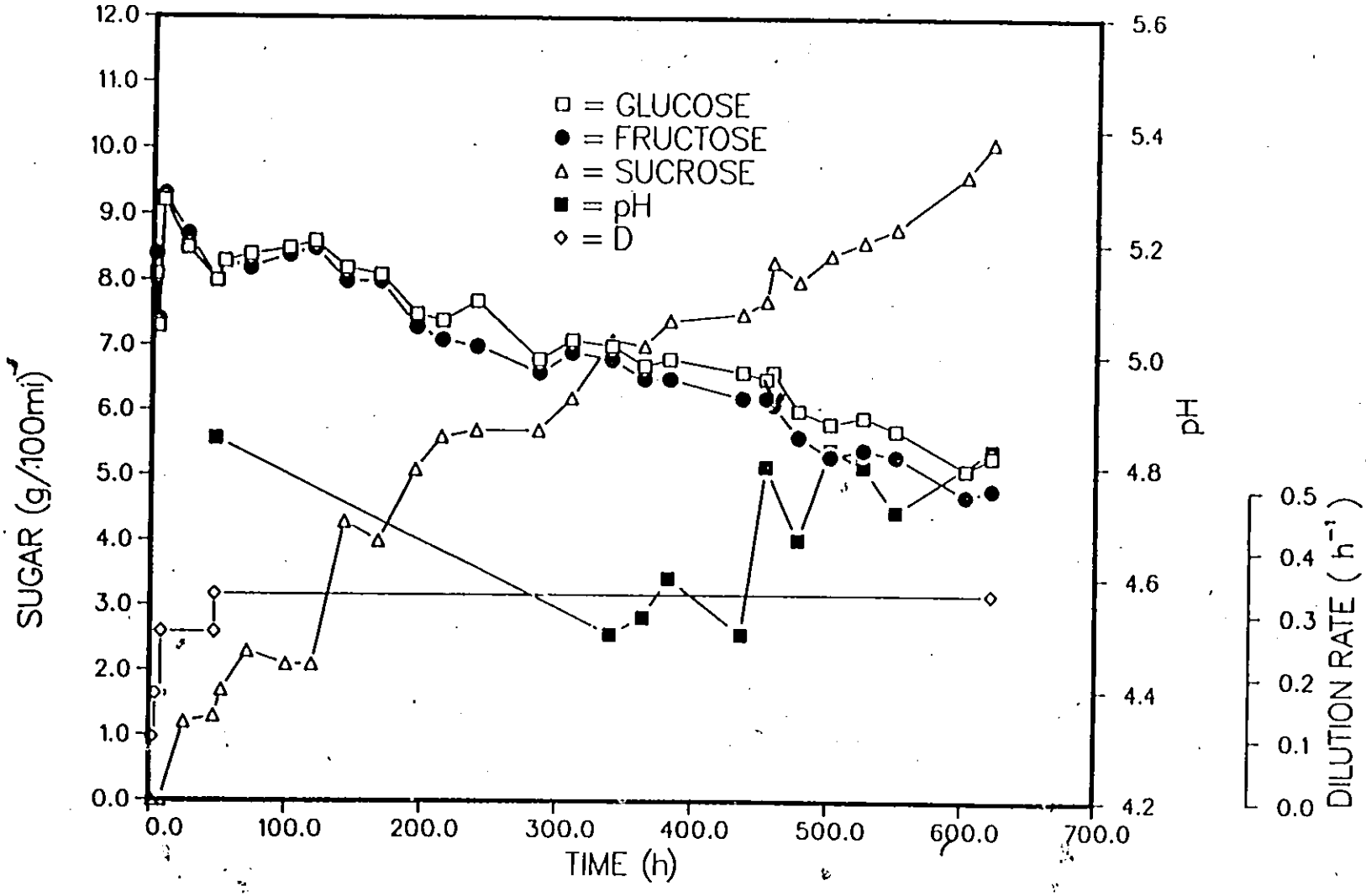

Figure 17: Inversion of sucrose in packed-bed reactor; 20% (w/v) sucrose in feed.

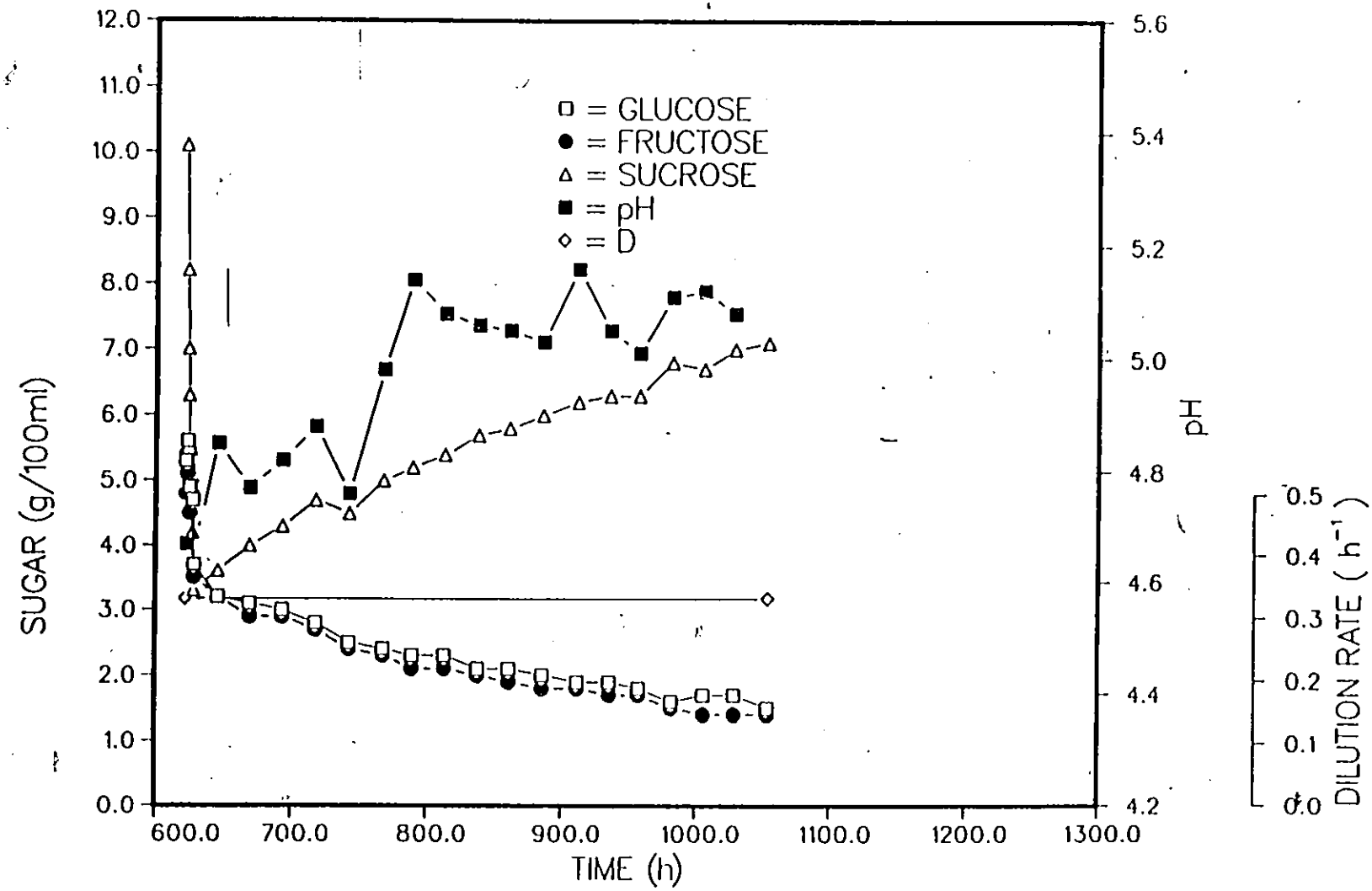

Figure 18: Inversion of sucrose in packed-bed reactor; 10% (w/v) sucrose in feed.

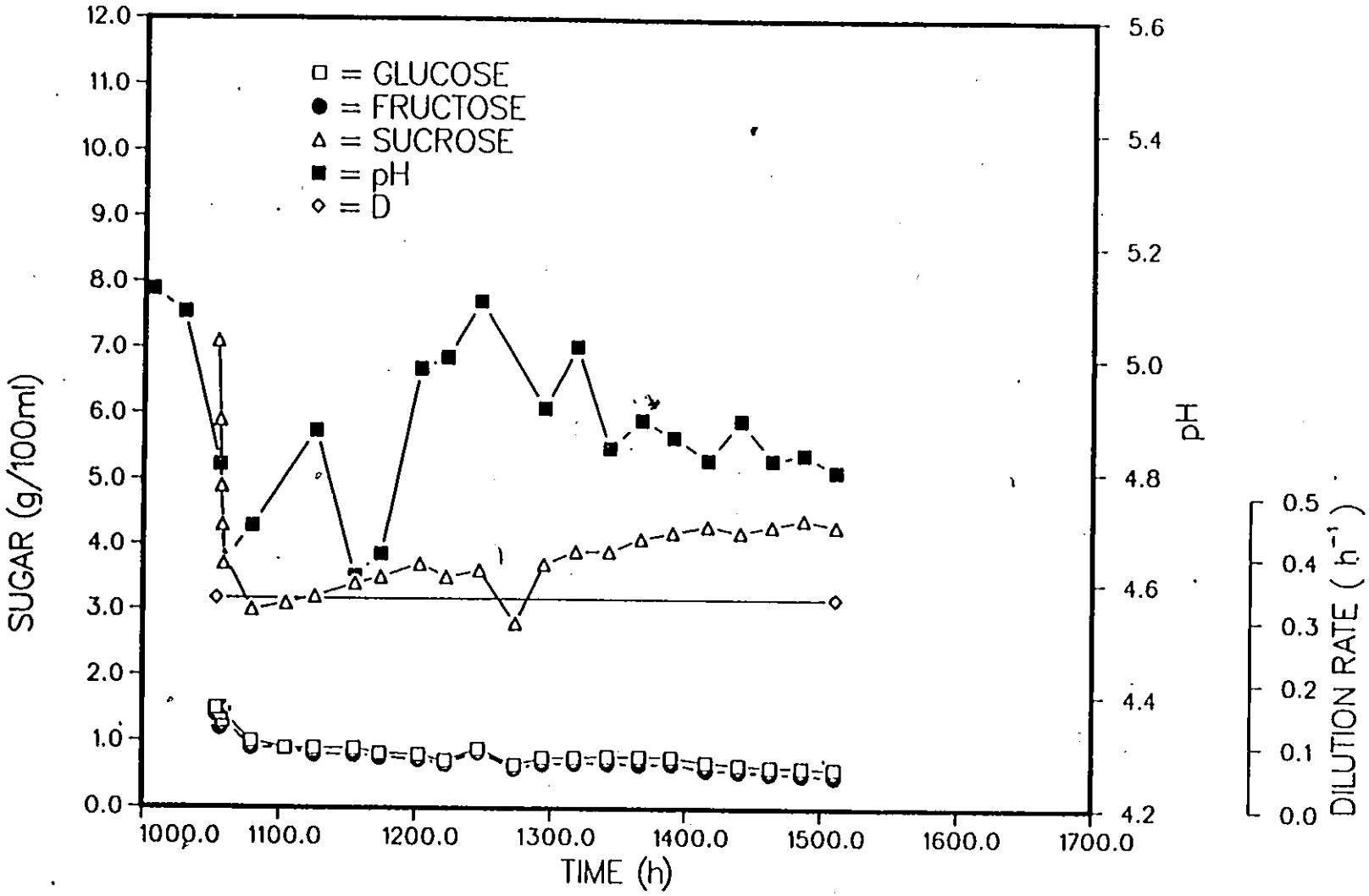

Figure 19: Inversion of sucrose in packed-bed reactor; 5% (w/v) sucrose in feed.

solution; this happened because the pH of the distilled water used to prepare sucrose solutions was changing from time to time.

A second packed-bed bioreactor was assembled later. About 4900 beads having the following properties were packed into its bed:

Second Batch of Invertase Beads	
initial diameter	3.4 mm
initial mass	24 mg
initial percentage of nitrogen (dry basis)	3.8
initial mass of nitrogen per bead	80 μg
fraction of treated cells in the alginate suspension (wet weight)	13% (w/w)

This time, the feed sucrose concentration was 30% (w/v). Figures 20 and 21 show the results of the operation of this bioreactor.

For the first 620 hours of operation, a sucrose conversion level of about 85–88% was maintained, which represented, at a dilution rate of 0.18 h^{-1}, a sucrose inversion rate of 46 grams per litre of reactor volume per hour. This reactor showed higher stability of invertase activity than the first reactor described in this work.

4.3 Growth of the Ethanol-Producing Yeast

To produce ethanol from invert sugar without using the fructose, a microorganism able to convert glucose to ethanol but unable to grow on fructose was needed.

According to Lobo and Maitra (1977), *S. cerevisiae ATCC 96859*, which is a mutant yeast lacking hexokinase activity, meets these requirements.

Only one paper concerning this mutant yeast was found in the literature (Lobo and Maitra, 1977); it describes the isolation of the mutant but gives no information about its use for ethanol production. Therefore, before starting to use *S. cerevisiae ATCC 96859* to produce ethanol, a few tests were done to determine how well the

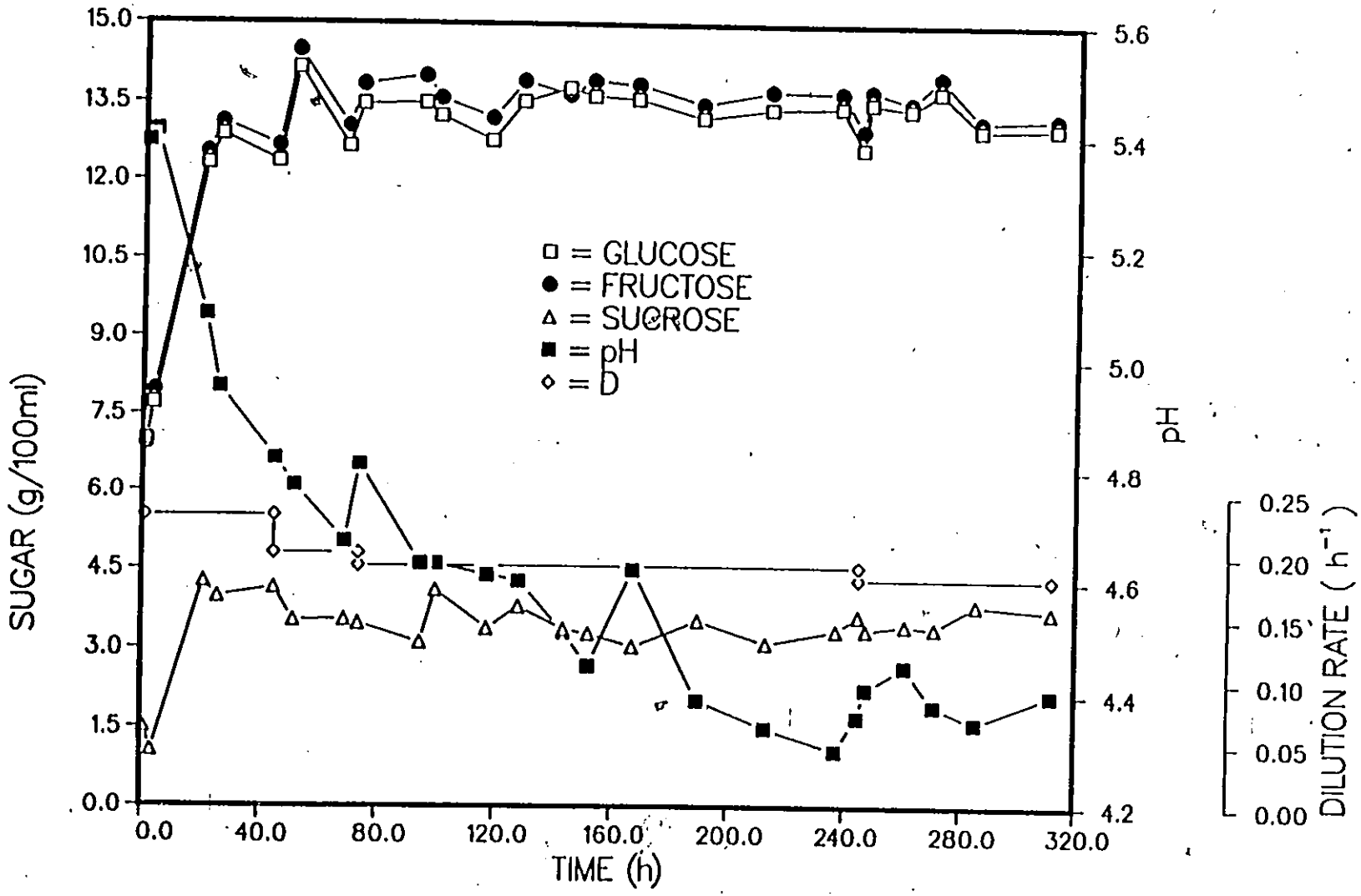

Figure 20: Inversion of sucrose in packed-bed reactor; 30% (w/v) sucrose in feed; $0 <$ time < 312 hours.

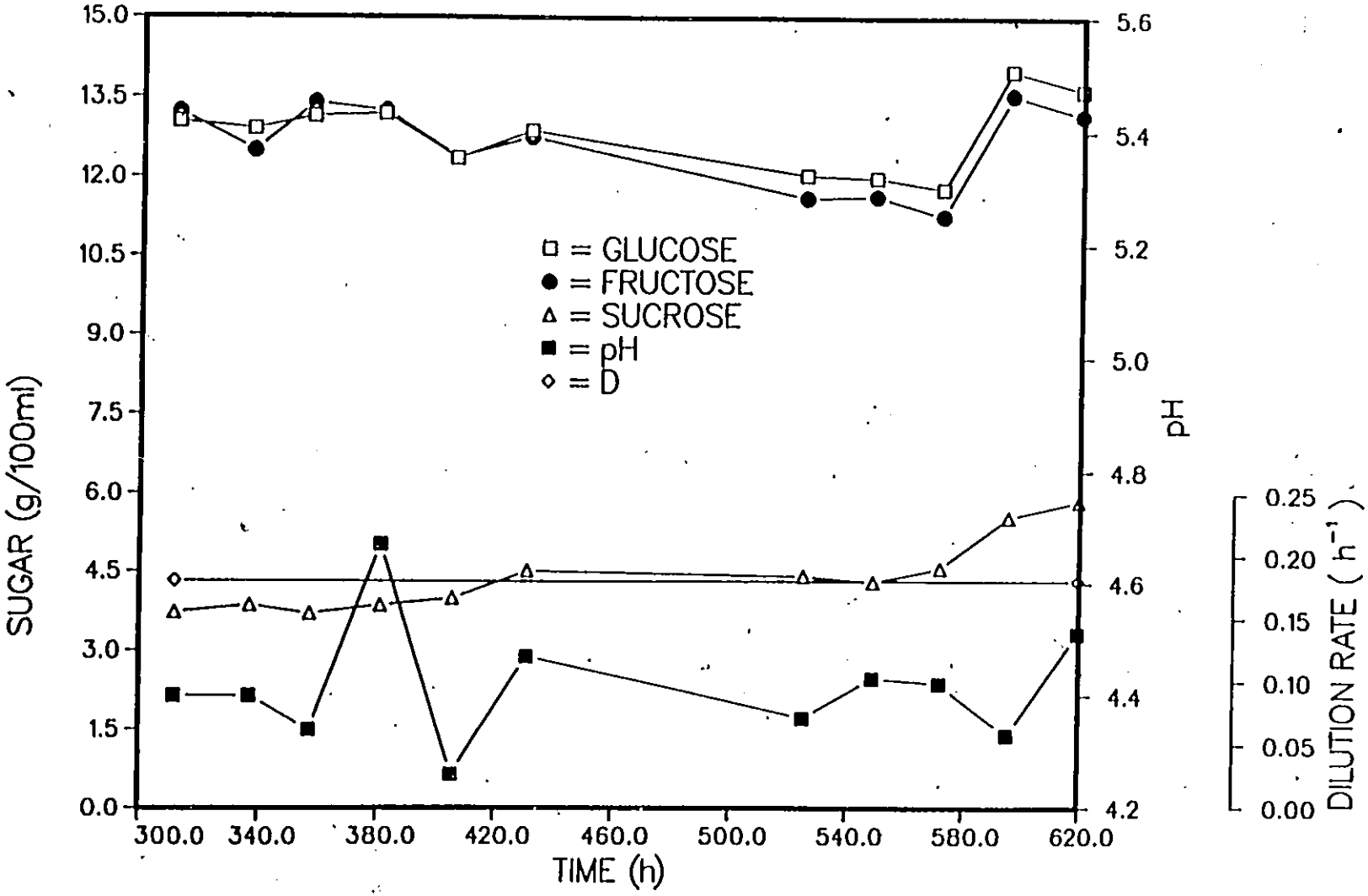

Figure 21: Inversion of sucrose in packed-bed reactor; 30% (w/v) sucrose in feed; 312 hours < time < 619 hours.

yeast grows on various carbohydrates and what were the influence of temperature, glucose concentration and ethanol concentration on its growth.

4.3.1 Tests of Growth of *Saccharomyces cerevisiae ATCC 36859* on Various Carbohydrates

Some experiments were carried out to study the ability of the yeast to grow on sucrose, glucose and fructose, the three sugars with which it would be in contact in the process of conversion of sucrose into fructose and ethanol.

Shake-flask experiments were done with medium II, to which sucrose, glucose and fructose were alternatively added in 2% (w/v) concenfration. The media obtained were inoculated with 0.1 ml of a liquid culture and incubated at 30°C, on a rotary shaker for 24 hours. After incubation, the concentrations of sugar and biomass were measured for each system. The results are shown in Table 4. For each sugar tested, two shake-flasks were used. The last pair of flasks contained medium II to which no carbohydrate was added.

As can be seen from Table 4, glucose was the only carbohydrate that was consumed by the yeast; its concentration decreased from 2% (w/v) to 1.6% (w/v). Fructose and sucrose remained untouched by the yeast. However, since medium II alone can support yeast growth, there was biomass formation in all the cases.

It was in the media containing glucose that the largest amount of biomass was produced, 0.092–0.095 g/100 ml. In the media containing sucrose, 0.037–0.038 g/100 ml biomass was obtained while 0.024-0.025 g/100 ml and 0.015-0.016 g/100 ml were produced in the medium containing fructose and in the medium without sugar respectively.

4.3.2 Effect of Temperature on Growth

Tests were done to determine the temperature at which the growth of *S. cerevisiae ATCC 36859* was the fastest.

Yeast cells were grown in liquid medium containing 5 g/100 ml glucose at the following temperatures: 26°C, 30°C, 33°C and 37°C. The growth in the four batch

Table 4: Growth of *Saccharomyces cerevisiae ATCC 96859* on various carbohydrates.

Sugar	*Initial sugar concentration* (g/100 ml)	*Final sugar concentration* (g/100 ml)	*Biomass concentration* (g/100 ml)
glucose	2.00	1.62	0.093
glucose	2.00	1.60	0.095
fructose	2.00	1.99	0.024
fructose	2.00	2.00	0.025
sucrose	2.00	2.00	0.038
sucrose	2.00	2.00	0.037
—	0	0	0.015
—	0	0	0.016

systems was followed during one day. Growth curves are shown in Figure 22. The results indicate that the optimum temperature for growth was 33°C.

After 23 hours of incubation at 33°C, the biomass concentration was 0.21 g/100 ml, while at 30°C it was slightly lower with a value of 0.20 g/100 ml; at 37°C and 26°C, the concentration of biomass were 0.13 g/100 ml and 0.10 g/100 ml respectively.

The specific growth rates were determined for the 4 previously cited temperatures by plotting logarithmic values of biomass concentration as a function of time, and by measuring the slope of the logarithmic growth phase (Appendix B). Figure 23 shows the relation between the specific growth rate and the temperature. The highest specific growth rate was obtained at 33°C, its value being 0.22 h^{-1}. Values of 0.21 h^{-1}, 0.20 h^{-1} and 0.19 h^{-1} were measured at 30°C, 37°C and 26°C respectively.

4.3.3 Effect of Initial Glucose Concentration on Growth

Experiments were carried out to determine if yeast growth was inhibited by the substrate. *S. cerevisiae ATCC 96859* was grown in medium II containing glucose in concentrations varying between 2% (w/v) and 20% (w/v). The media were

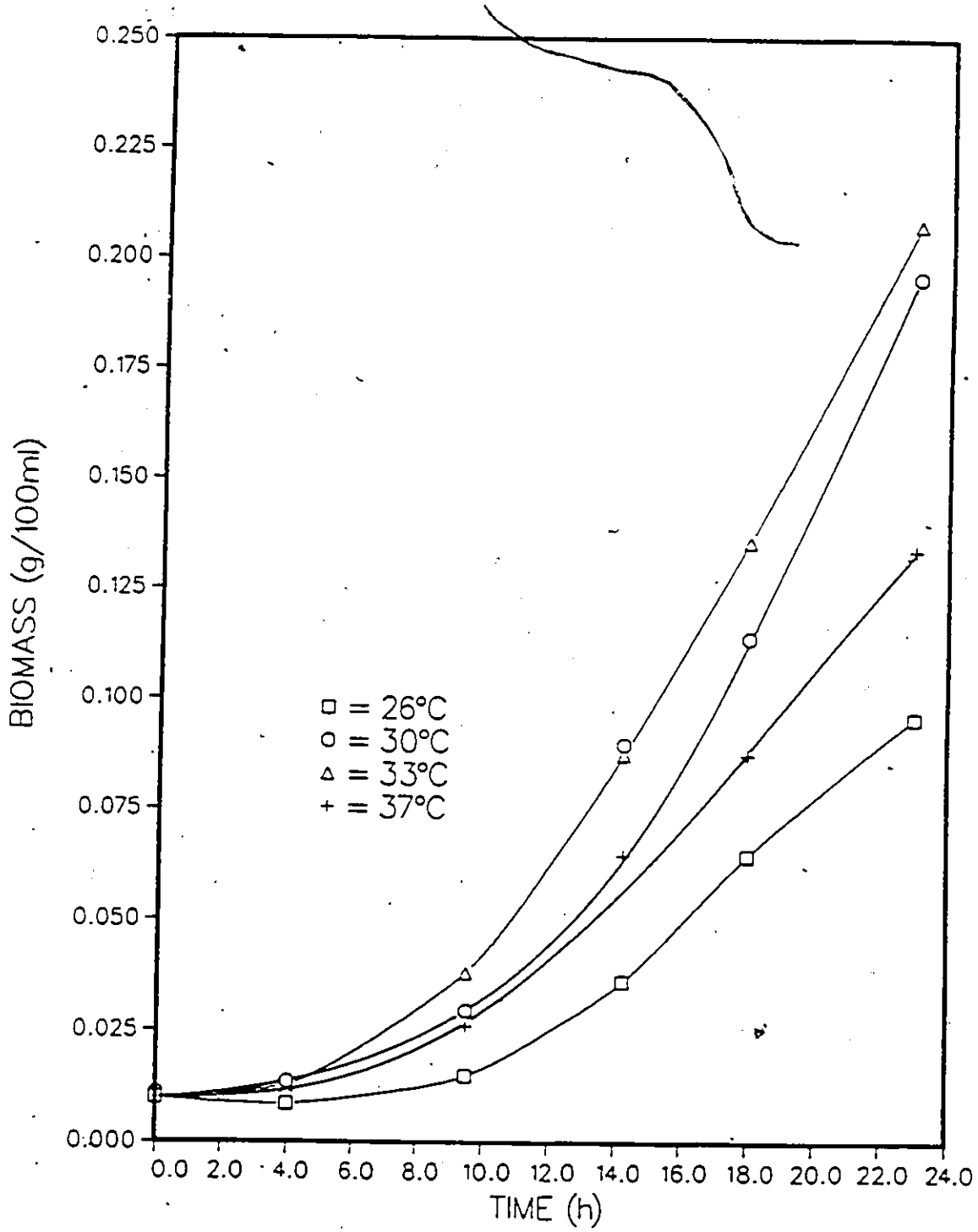

Figure 22: Effect of temperature on the growth of *Saccharomyces cerevisiae ATCC 96859*; the biomass concentration is expressed on a dry basis.

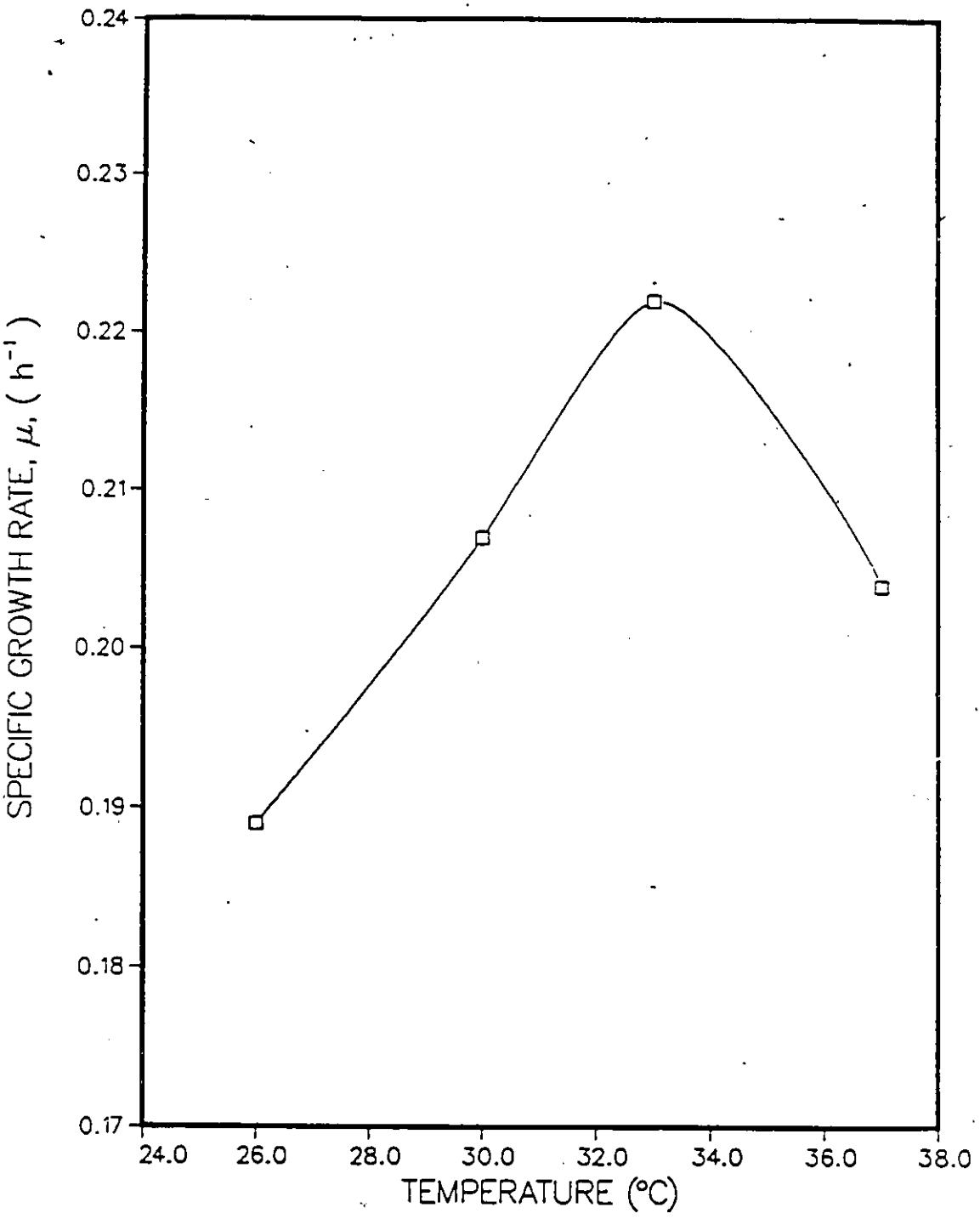

Figure 23: Effect of temperature on the specific growth rate of *Saccharomyces cerevisiae ATCC 96859.*

incubated in shake-flasks, on a rotary shaker (200 RPM), at 30°C. After 3 days of incubation, the amount of biomass produced in each flask was measured. The results are given in Figure 24 as a function of the initial glucose concentrations in the media.

The largest amount of biomass produced corresponded to an initial glucose concentration of 15% (w/v). With an initial glucose concentration of 2% (w/v), a biomass concentration of 0.24 g/100 ml was obtained. More biomass was formed with higher glucose concentrations up to a biomass concentration of 0.28 g/100 ml, corresponding to 15% (w/v) glucose. Then, the amount of biomass produced started to decrease with a further rise in the initial glucose concentration: 0.26 g/100 ml of biomass for a 20% (w/v) initial glucose concentration.

The yield of biomass produced per amount of glucose used was calculated and is given in Table 5. The glucose was not used completely during the experiment, except in the case of the 2% (w/v) glucose medium. The biomass yield on glucose had a value fluctuating in the range 0.10–0.12 gram of biomass produced per gram of glucose used for glucose concentrations smaller or equal to 15% (w/v) and went down to 0.08 g/g for the 20% (w/v) glucose medium.

Table 5: Effect of glucose concentration on cell yield of *Saccharomyces cerevisiae ATCC 36[illegible]9*.

Initial glucose concentration % (w/v)	*Biomass yield* ($Y_{X/S}$) (g/g)
2	0.12
4	0.096
6	0.097
8	0.11
10	0.12
15	0.11
20	0.078

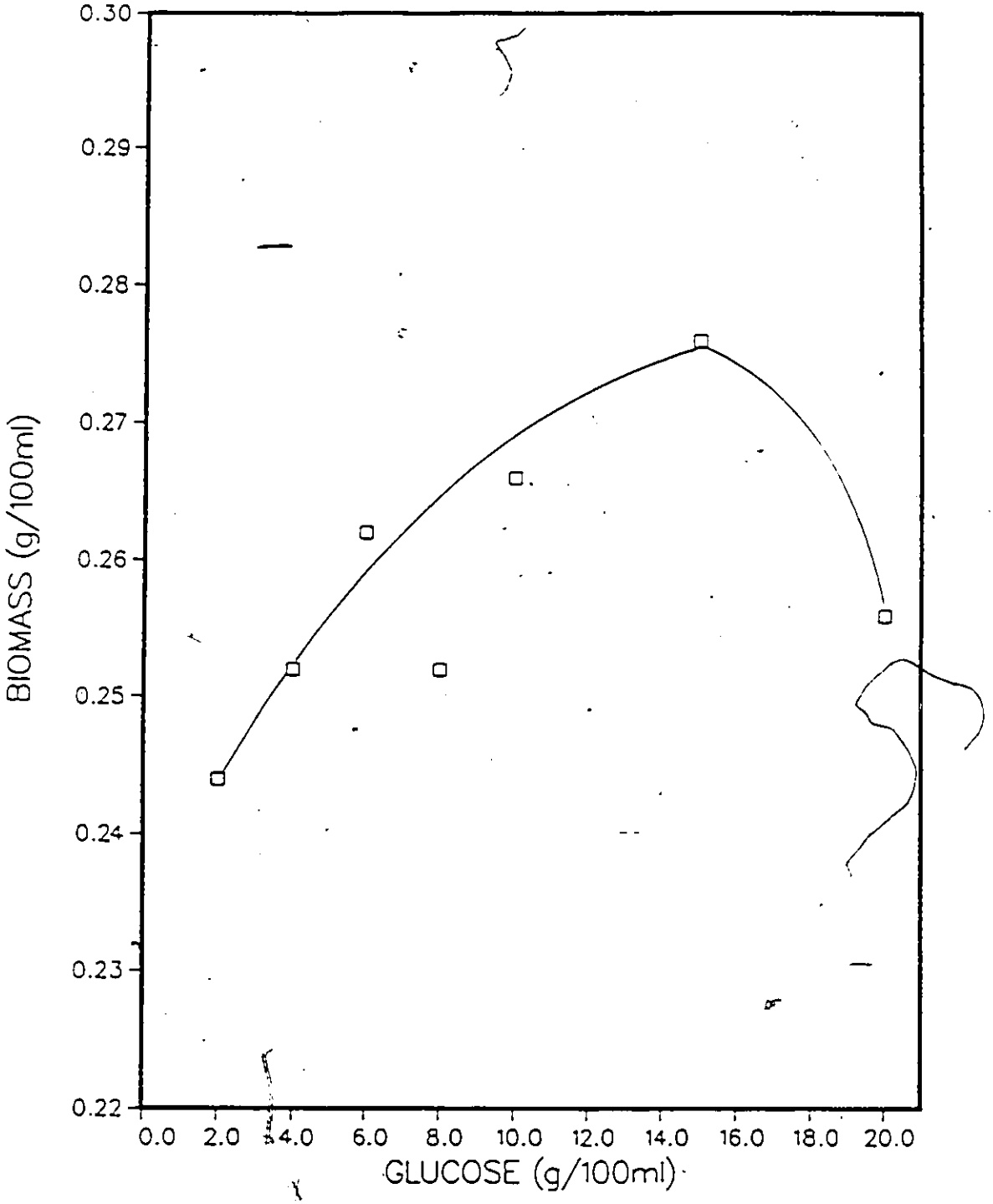

Figure 24: Effect of the initial concentration of glucose on the growth of *Saccharomyces cerevisiae ATCC 96859*; the biomass concentration is expressed on a dry basis.

4.3.4 Effect of Ethanol Concentration on Growth

In order to test the susceptibility of this yeast to ethanol, shake-flask experiments were performed. Liquid media containing ethanol in concentrations varying between 0 and 12% (w/v) were prepared and inoculated. This was followed by incubation of the flasks at 30°C on a rotary shaker (200 RPM), for one day.

The biomass concentration was determined after 24 hours of incubation and the percentage of growth inhibition was calculated for the various ethanol concentrations tested. These results are plotted in Figure 25.

Ethanol inhibited yeast growth; 2% (w/v) ethanol initially added to the medium inhibited yeast growth by 31% and 6% (w/v) ethanol caused an 85% inhibition. At an ethanol concentration of 10 g/100 ml or higher, no growth was observed.

4.4 Batch Ethanol Production

In order to study the ability of *S. cerevisiae ATCC 96859* to produce ethanol from glucose, many batch experiments were conducted during the realization of the project. Ethanol was produced in media containing glucose as the only sugar and in media containing glucose and fructose in equal amounts, to observe the effect fructose would have on ethanol production when invert sugar would be used as the substrate.

The effects of aeration on ethanol production were also studied, yeasts being able to operate both aerobically and anaerobically.

Finally, two tests were conducted with inocula of a larger size (than the ones which had been used for the previous tests) to determine the effect of a larger initial biomass concentration on the productivity of the batch reactor.

All the tests were carried out in a New Brunswick Microferm Fermentor, at a temperature of 30°C and at a stirring rate of 200 RPM, unless specified otherwise. Four litres of medium II were used for each test.

The following parameters were measured during the tests: the sugar, the ethanol and the biomass (dry basis) concentrations, and the pH.

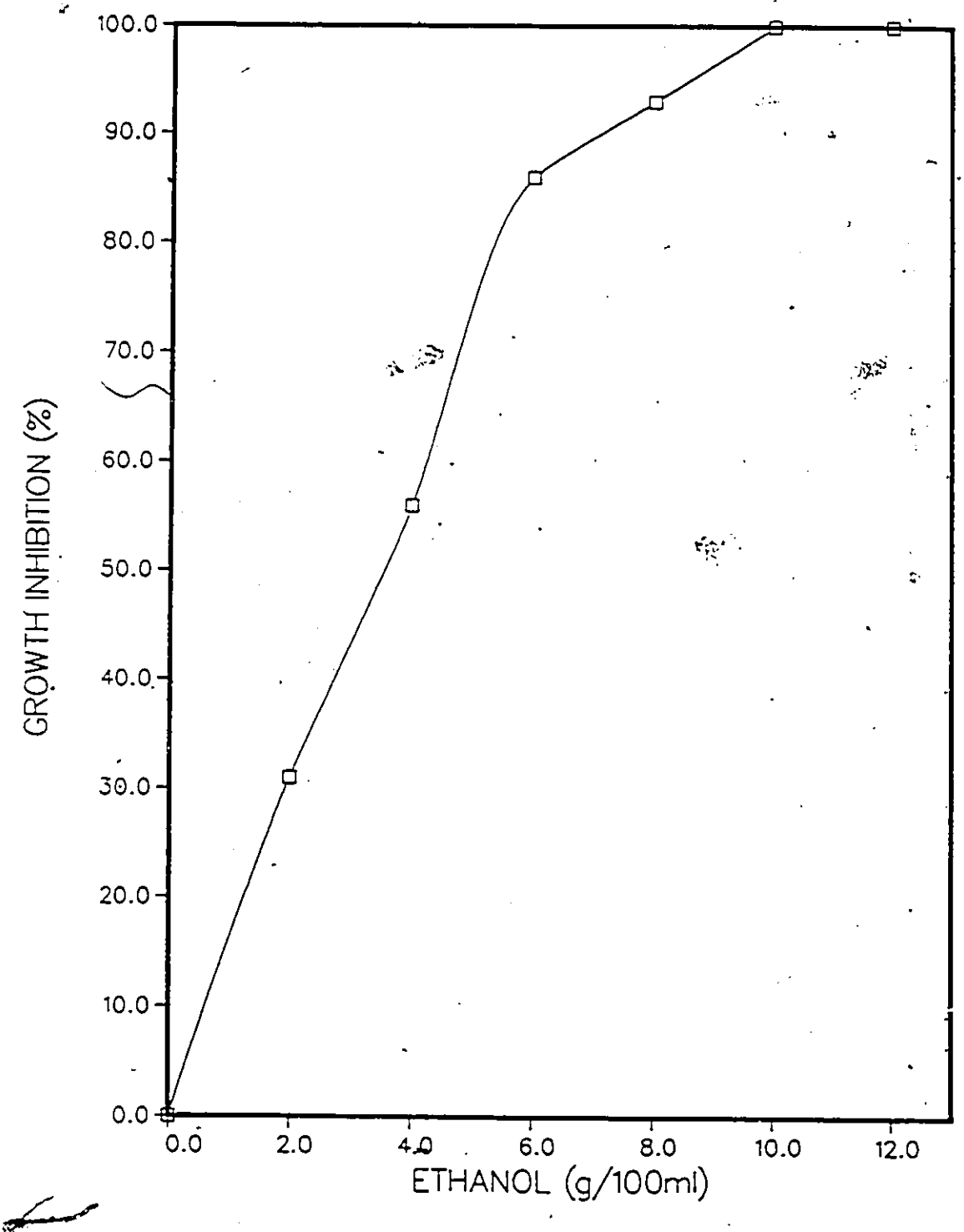

Figure 25: Effect of the initial concentration of ethanol on the growth of *Saccharomyces cerevisiae ATCC 96859.*

4.4.1 Production of Ethanol in Glucose Media

Four tests of ethanol production were done with media containing glucose of the following concentrations: 4.6, 9.0, 14.9 and 25.3 g/100 ml. The glucose, ethanol and biomass concentrations, as well as the pH measured during these tests, are plotted in Figures 26, 27, 28 and 29. Ethanol productivities, ethanol yields and specific growth rates of the yeast were calculated and are given in Table 6.

Ethanol production from a 4.6% (w/v) glucose medium resulted in a final ethanol concentration of 2.2 g/100 ml, and an ethanol yield on glucose of 92% of the theoretical value. The biomass concentration increased from 0.065 g/100 ml to 0.27 g/100 ml in 52 hours and the pH dropped from 5.33 to 4.06 (Figure 26). The specific growth rate of the yeast was 0.13 h^{-1}. The ethanol productivity based on the amount of biomass (P1 and P2) was 0.15 gram of ethanol produced per gram of biomass present per hour, while the volumetric ethanol productivity (P3) was 0.042 gram of ethanol produced per 100 ml of medium per hour (Table 6).

For the test of ethanol production in 9.0% (w/v) glucose medium, the following results were obtained: an ethanol concentration of 4.5 g/100 ml was reached, which corresponded to an ethanol yield on glucose of 98% of the theoretical value, the biomass concentration passed from 0.042 g/100 ml to 0.31 g/100 ml and the pH decreased from 5.23 to 3.69, during an 87 hours period (Figure 27). The specific growth rate of the yeast was 0.10 h^{-1} and the volumetric ethanol productivity (P3) was 0.052 gram of ethanol per 100 ml of medium per hour.

When producing ethanol from a 14.9% (w/v) glucose medium, a 6.9 g/100 ml ethanol level was attained, after 125 hours. The yield of ethanol on glucose was 90% of the theoretical value. The pH changed from 5.26 to 3.59 and the biomass concentration went up from 0.033 g/100 ml to 0.29 g/100 ml (Figure 28). The specific growth rate of the yeast was 0.11 h^{-1} and the volumetric ethanol productivity (P3), 0.055 gram of ethanol per 100 ml medium per hour.

The last test of this series, done in a 25.3% (w/v) glucose medium, was carried out for 205 hours. At the end of the test, there was a residual glucose concentration of 3.8 g/100 ml. The maximum ethanol concentration obtained was 10.4 g/100 ml. The yield of ethanol on glucose was 95% of the theoretical value. The quantity of

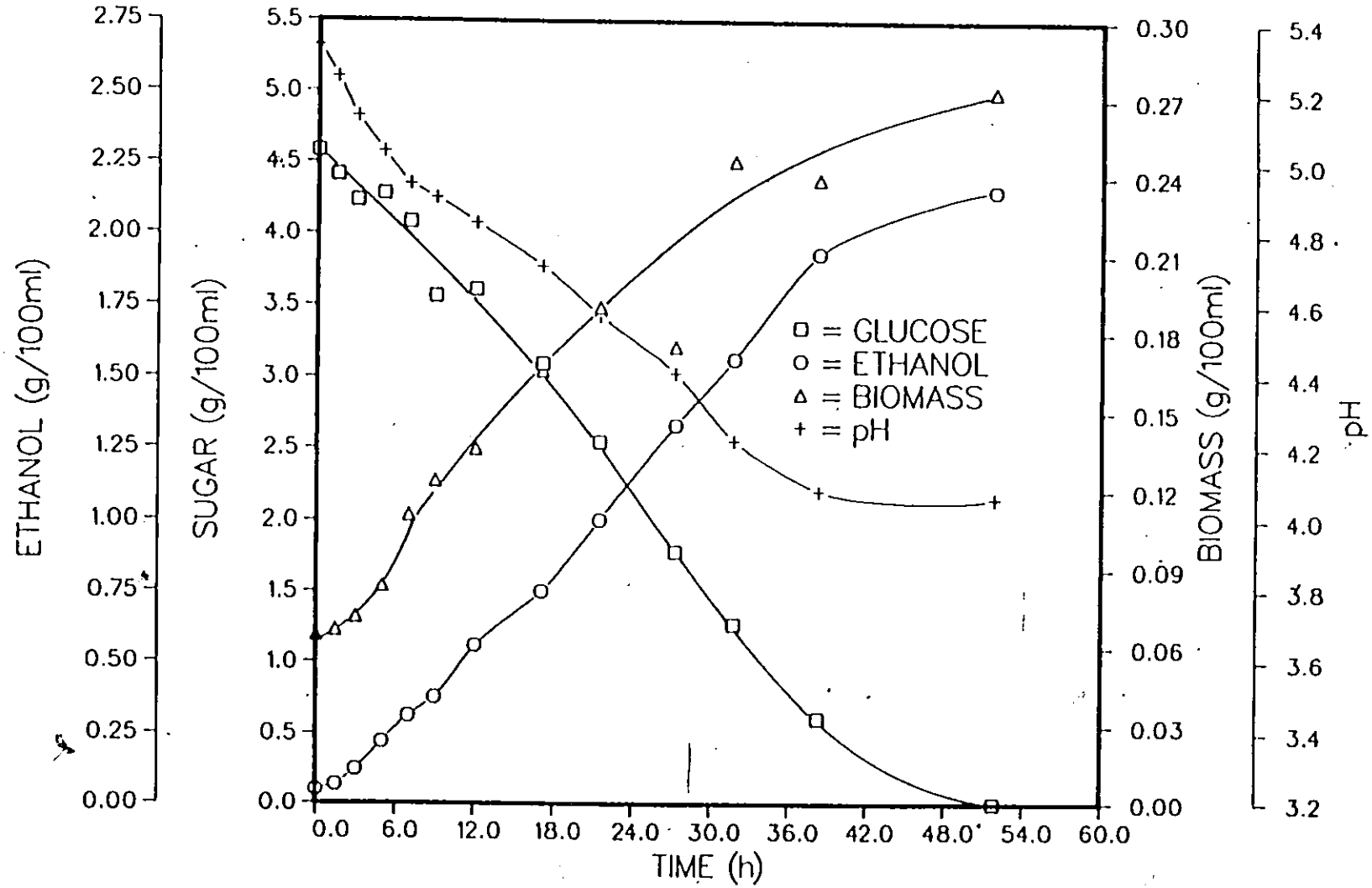

Figure 26: Ethanol production in 4.6% (w/v) glucose medium.

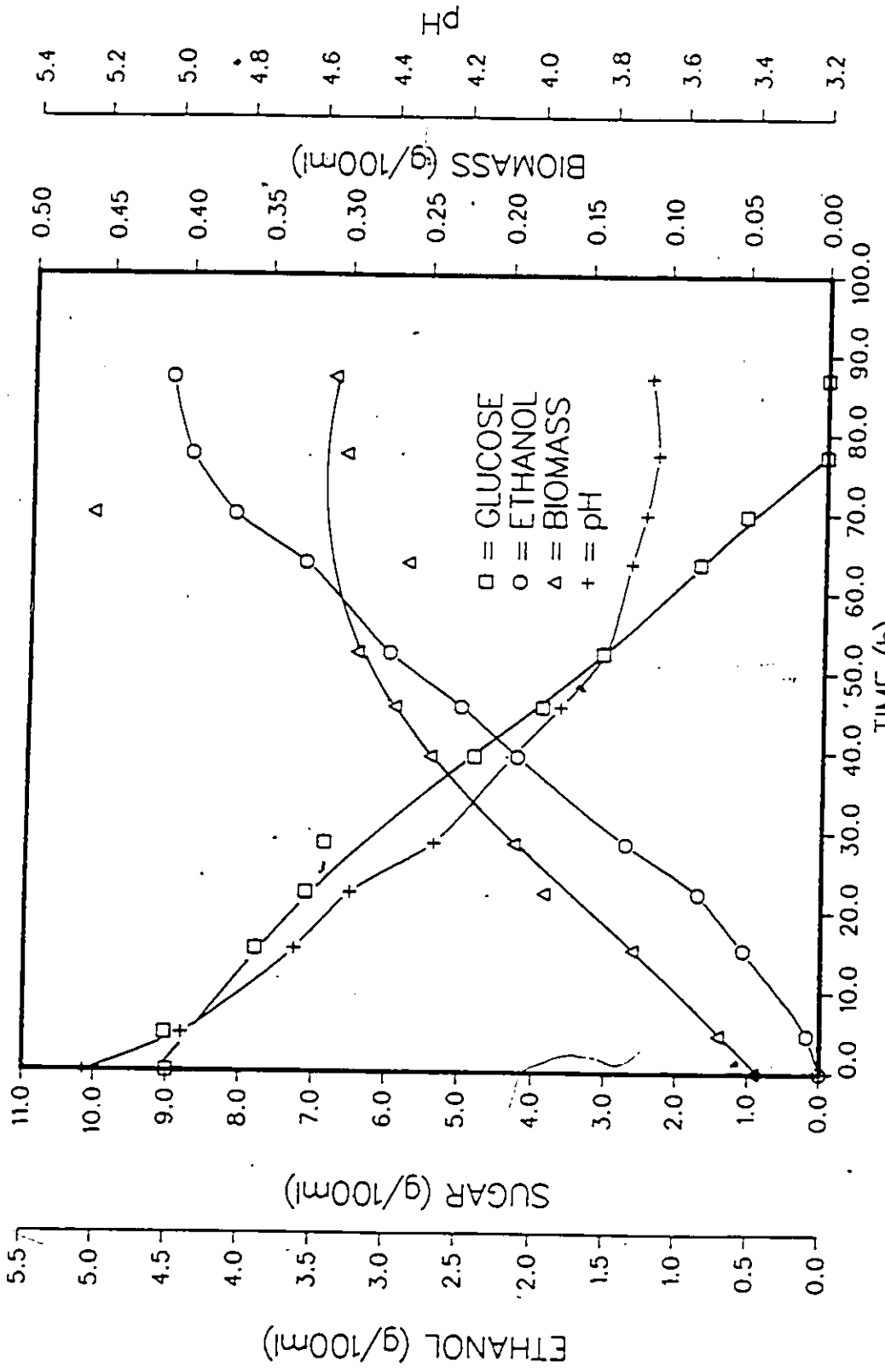

Figure 27: Ethanol production in 9.0% (w/v) glucose medium.

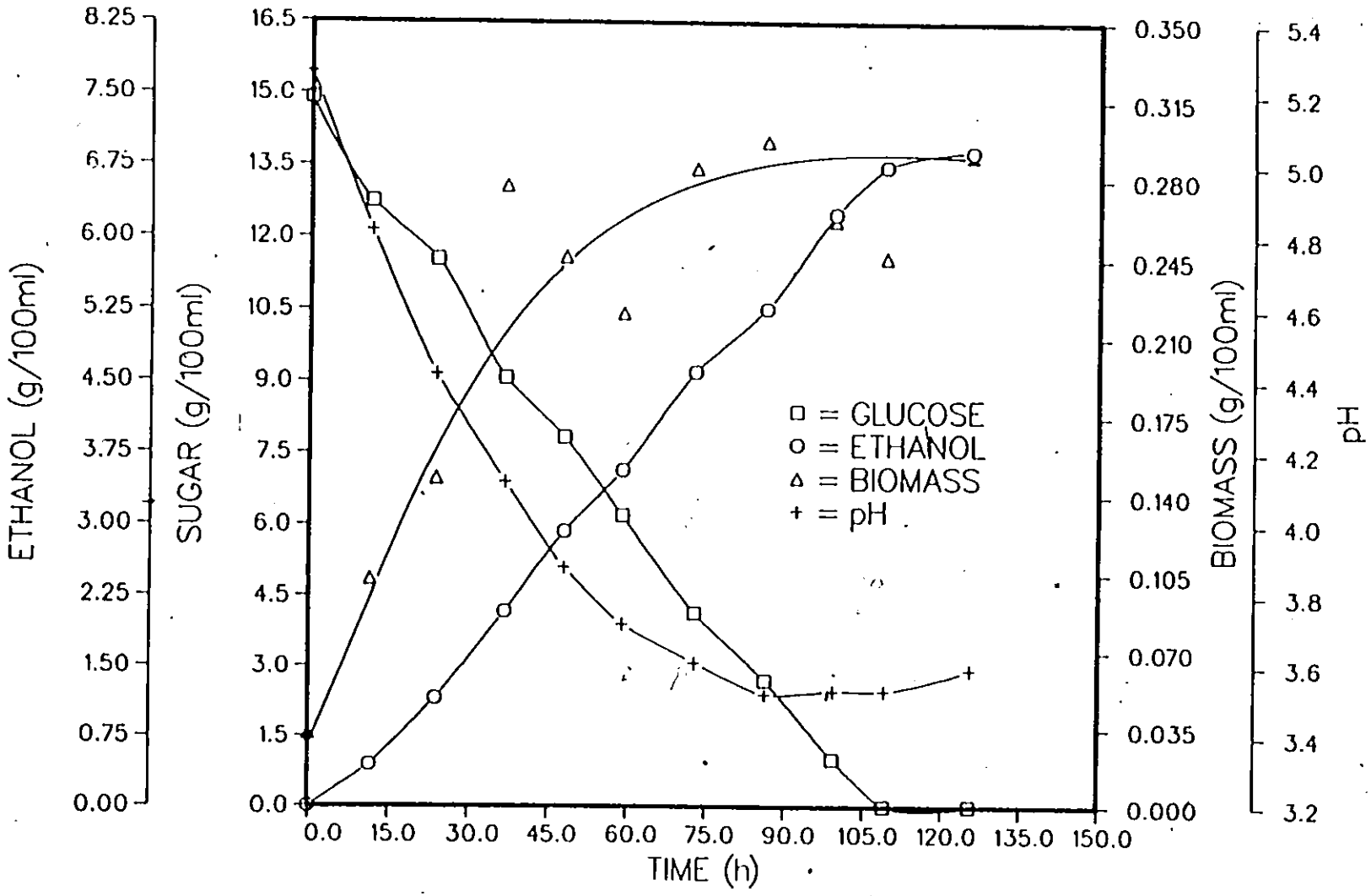

Figure 28: Ethanol production in 14.9% (w/v) glucose medium.

biomass increased from a concentration of 0.033 g/100 ml to a concentration of 0.20 g/100 ml, while the pH dropped from 5.27 to 4.02 (Figure 29). The specific growth rate of the yeast was 0.08 h^{-1} and the volumetric ethanol productivity(P3), 0.051 g ethanol/((100 ml medium).(h)).

4.4.2 Production of Ethanol in Media containing Glucose+Fructose Mixture

Four other tests of ethanol production were carried out in media containing glucose and fructose. The two hexose sugars were added to the medium in equal concentrations, as is the case in invert sugar. The following concentrations of carbohydrates were obtained after sterilization of the media: 7.1% (w/v) glucose + 7.5% (w/v) fructose, 12.3% (w/v) glucose + 13.0% (w/v) fructose, 15.0% (w/v) glucose + 16.0% (w/v) fructose and finally 19.3% (w/v) glucose + 20.8% (w/v) fructose.

When producing ethanol from a 7.1% (w/v) glucose + 7.5% (w/v) fructose medium (Figure 30), the following results were obtained: an ethanol concentration of 3.7 g/100 ml corresponding to an ethanol yield on glucose equal to the theoretical value, a volumetric ethanol productivity (P3) of 0.056 g/((100 ml medium).(h)) and a specific growth rate of the yeast of 0.12 h^{-1} (Table 6). The biomass concentration increased from 0.018 to 0.23 g/100 ml and the pH dropped from 5.39 to 3.85, in a period of 66 hours. The fructose concentration remained constant.

Producing ethanol from the 12.3% (w/v) glucose + 13.0% (w/v) fructose medium gave the following results: the maximum ethanol concentration reached was 5.9 g/100 ml; the ethanol yield on glucose was 93% of the theoretical value; the volumetric ethanol productivity (P3) was 0.047 gram per 100 ml medium per hour and the specific growth rate of the yeast was 0.09 h^{-1} (Table 6). The pH decreased from a value of 5.28 to a value of 3.90 while the biomass concentration was increasing from 0.027 g/100 ml to 0.24 g/100 ml. The test lasted 124 hours (Figure 31).

For the production of ethanol from a 15.0% (w/v) glucose + 16.0% (w/v) fructose medium, an ethanol concentration of 6.9 g/100 ml was attained after 157 hours;

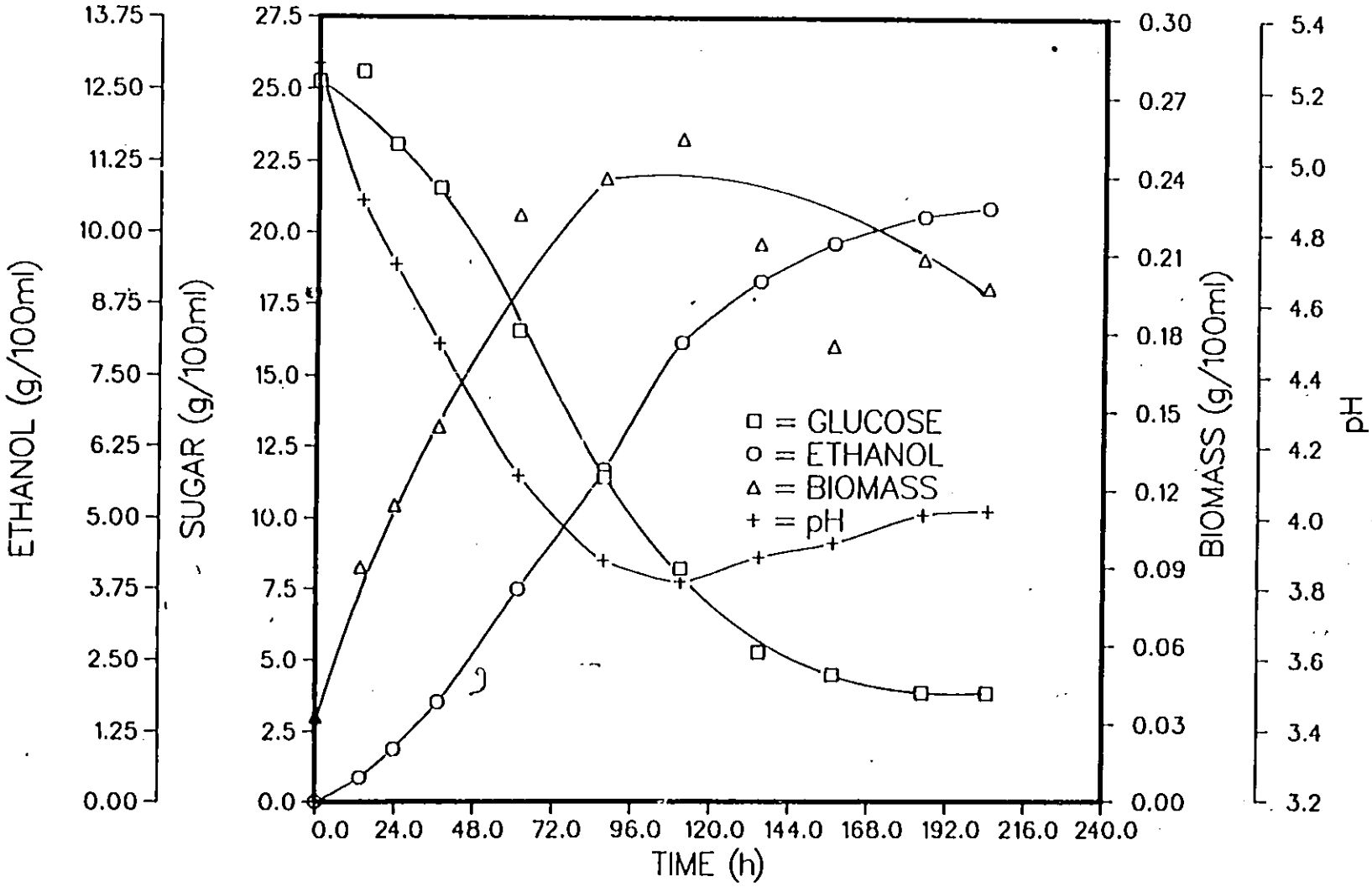

Figure 29: Ethanol production in 25.3% (w/v) glucose medium.

Table 6: Specific growth rates of yeast, ethanol productivities and ethanol batch production yields.

Initial glu.-fru. conc. (g/100 ml)	*Specific growth rate,* μ (h^{-1})	*Productivity*				*Fraction of theoretical ethanol yield (%)* $Y_{P/S}$
		P1	*P2*	*P3*	*P4*	
4.6–0	0.13	0.15	0.15	0.042	8.8×10^{-4}	92
9.0–0	0.10	0.17	0.20	0.052	3.4×10^{-4}	98
14.9–0	0.11	0.19	0.22	0.055	1.4×10^{-4}	90
25.3–0	0.077	0.26	0.30	0.051	3.7×10^{-5}	95
7.1–7.5	0.12	0.24	0.23	0.056	4.6×10^{-4}	100
12.3–13.0	0.090	0.20	0.24	0.047	1.4×10^{-4}	93
15.0–16.0	0.081	0.20	0.23	0.044	8.5×10^{-5}	90
19.3–20.8	0.034	0.20	0.27	0.034	3.7×10^{-5}	98
7.2–7.6 (aeration)	0.14	0.063	0.075	0.023	5.0×10^{-4}	56
14.2–15.0 (aeration)	0.12	0.061	0.10	0.026	2.4×10^{-4}	50
14.3–15.3 (larger inoculum)	0.025	0.26	—	0.32	1.4×10^{-3}	100
18.3–19.9 (larger inoculum)	0.017	0.20	—	0.22	5.0×10^{-4}	100

μ: Specific growth rate of the yeast, (h^{-1})

P1: Ethanol productivity (for time = 0 to the time the highest ethanol concentration was reached) in $\frac{\text{g ethanol}}{\text{(g biomass).(h)}}$

P2: Ethanol productivity (for time = 0 to time = 50 hours) in $\frac{\text{g ethanol}}{\text{(g biomass).(h)}}$

P3: Volumetric ethanol productivity (for time = 0 to the time the highest ethanol concentration was reached) in $\frac{\text{g ethanol}}{\text{(100 ml medium).(h)}}$

P4: Biomass productivity (for the whole length of time of the test) in $\frac{\text{g biomass}}{\text{(g glucose).(h)}}$

$Y_{P/S}$: Percentage of the theoretical yield of conversion of glucose to ethanol based on the amount of glucose used

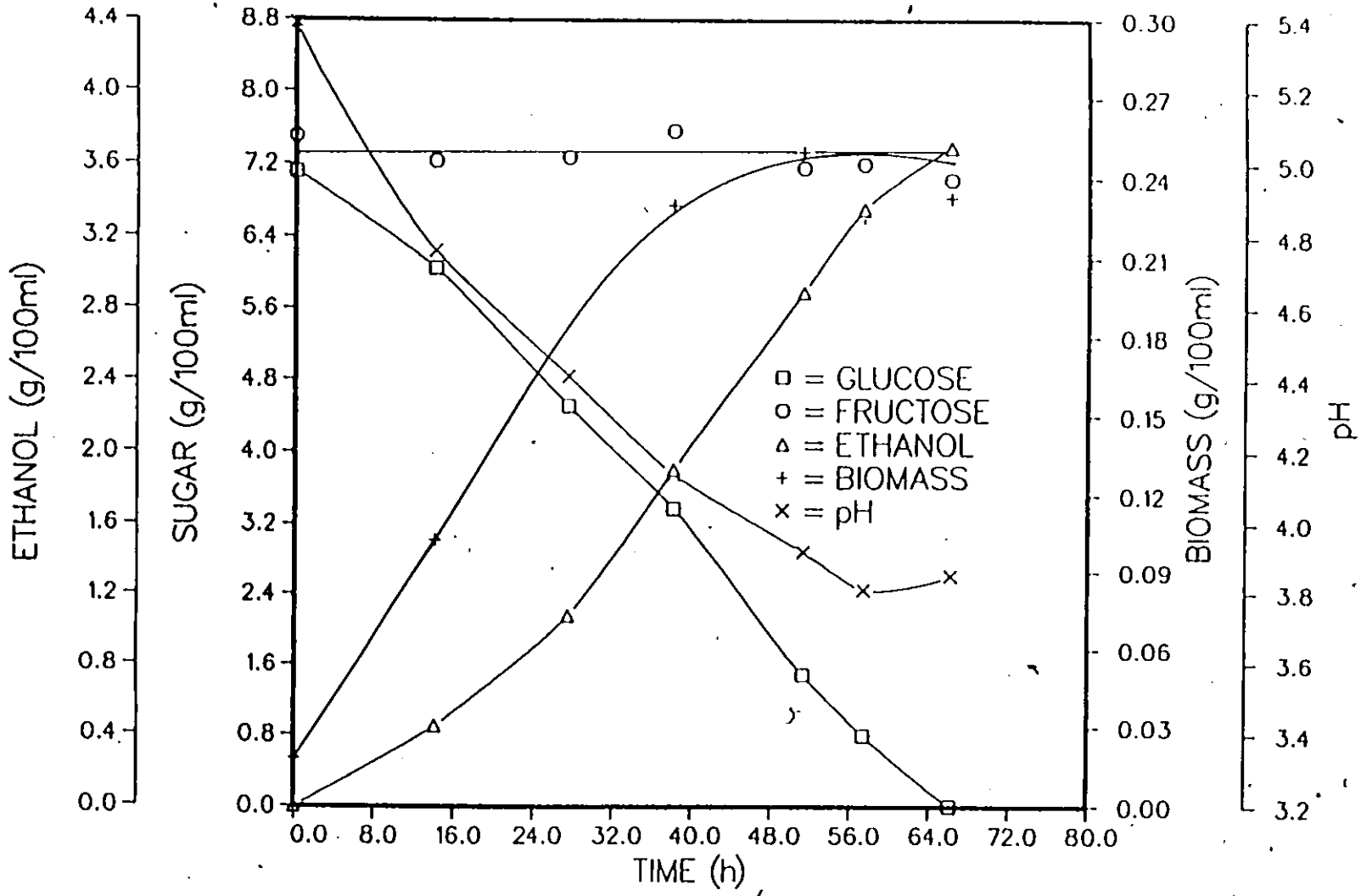

Figure 30: Ethanol production in 7.1% (w/v) glucose + 7.5% (w/v) fructose medium.

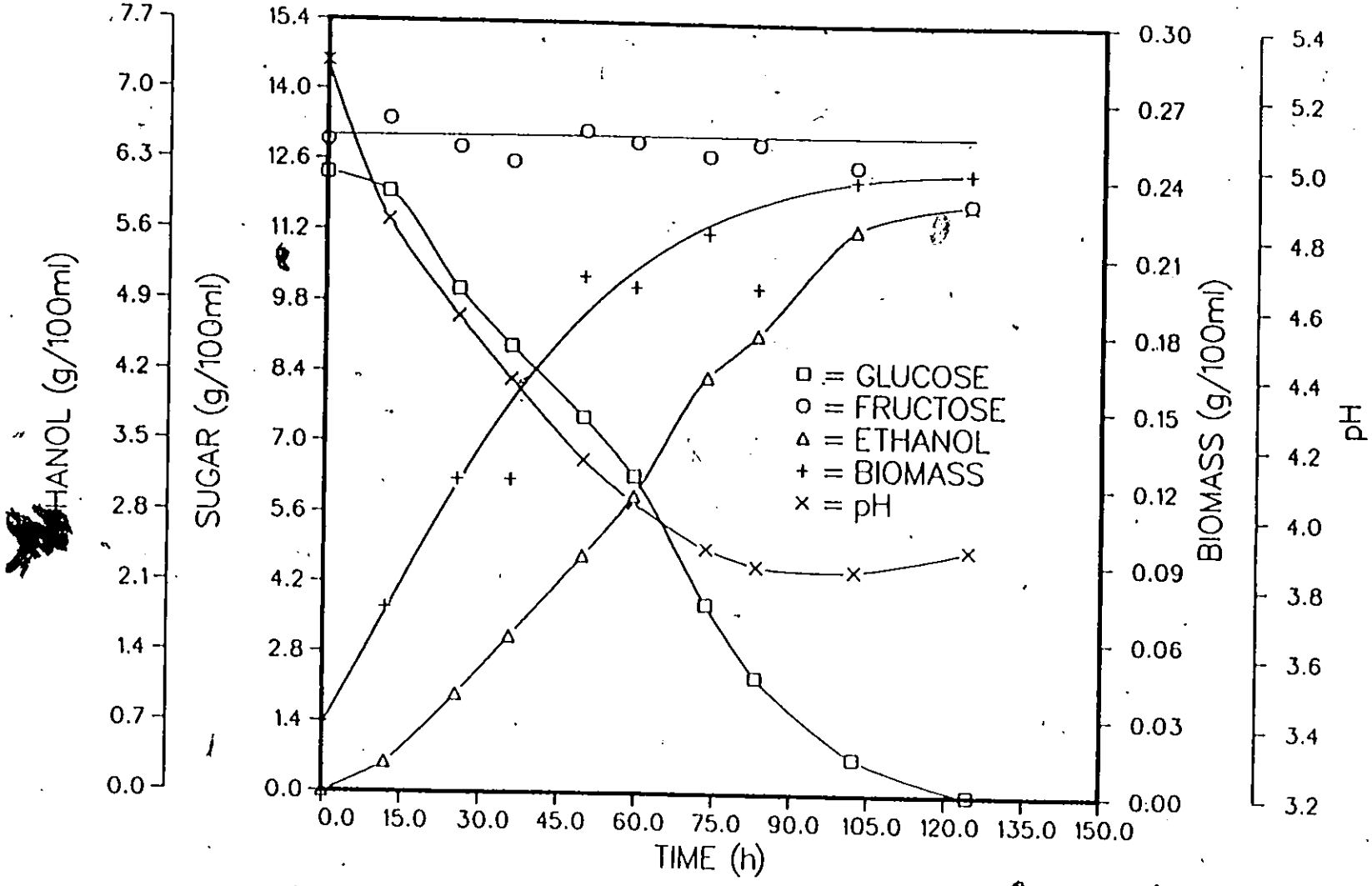

Figure 31: Ethanol production in 12.3% (w/v) glucose + 13.0% (w/v) fructose medium.

the yield of ethanol on glucose was 90% of the theoretical value. The biomass concentration increased from 0.026 g/100 ml to 0.23 g/100 ml while the pH fell from 5.25 to 3.96 (Figure 32). An ethanol productivity (P3) of 0.044 gram ethanol per 100 ml medium per hour was attained; the specific growth rate of the yeast was 0.08 h^{-1}.

The last test of this series was done with a medium containing 19.3 g/100 ml glucose and 20.8 g/100 ml fructose. The ethanol concentration reached a value of 8.2 g/100 ml, corresponding to an ethanol yield on glucose of 98% of the theoretical value. The concentration of biomass increased from 0.023 g/100 ml to 0.17 g/100 ml while the pH dropped from 5.20 to 3.98. The test was stopped after 244 hours; the residual glucose concentration was 3.0 g/100 ml (Figure 33). The specific growth rate of the yeast was 0.03 h^{-1} and the volumetric ethanol productivity (P3), 0.034 g/((100 ml medium).(h)) (Table 6).

4.4.3 Effect of Aeration on Ethanol Production

Knowing that air has an influence on yeast growth and on ethanol production rate, two tests were done to study the effects of aeration.

In the previous tests, no air was added to the fermentor during the experiments and the stirring rate of the impeller was 200 RPM. For these 2 tests, air at a flow rate of 0.5 L air/((L medium).min) was added to the medium and the impeller speed was changed to 500 RPM, in order to provide a good dispersion of the air in the medium. Ethanol was produced from media containing glucose and fructose, in the following concentrations: 7.2% (w/v) glucose + 7.6% (w/v) fructose and 14.2% (w/v) glucose + 15.0% (w/v) fructose.

Trying to produce ethanol with the 7.2 g/100 ml glucose + 7.6 g/100 ml fructose medium gave the following results: the highest ethanol concentration obtained was 1.9 g/100 ml, which corresponded to an ethanol yield on glucose of 56% of the theoretical value. The volumetric ethanol productivity (P3) was 0.023 gram per 100 ml medium per hour and the specific growth rate of the yeast was 0.14 h^{-1} (Table 6). All the glucose was consumed after 101 hours. During this period of time, the biomass concentration increased from 0.017 g/100 ml to 0.37 g/100 ml

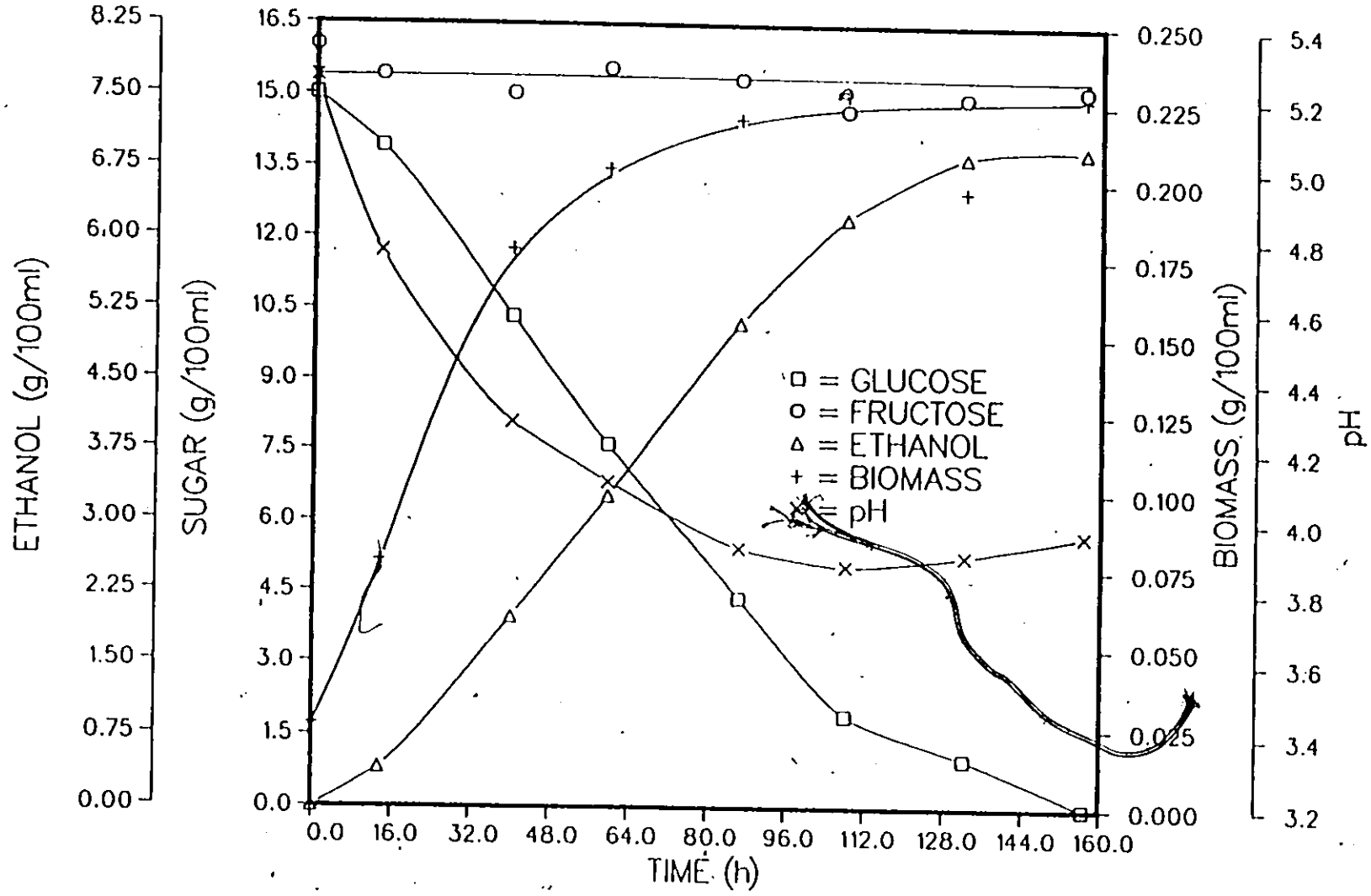

Figure 32: Ethanol production in 15.0% (w/v) glucose + 16.0% (w/v) fructose medium.

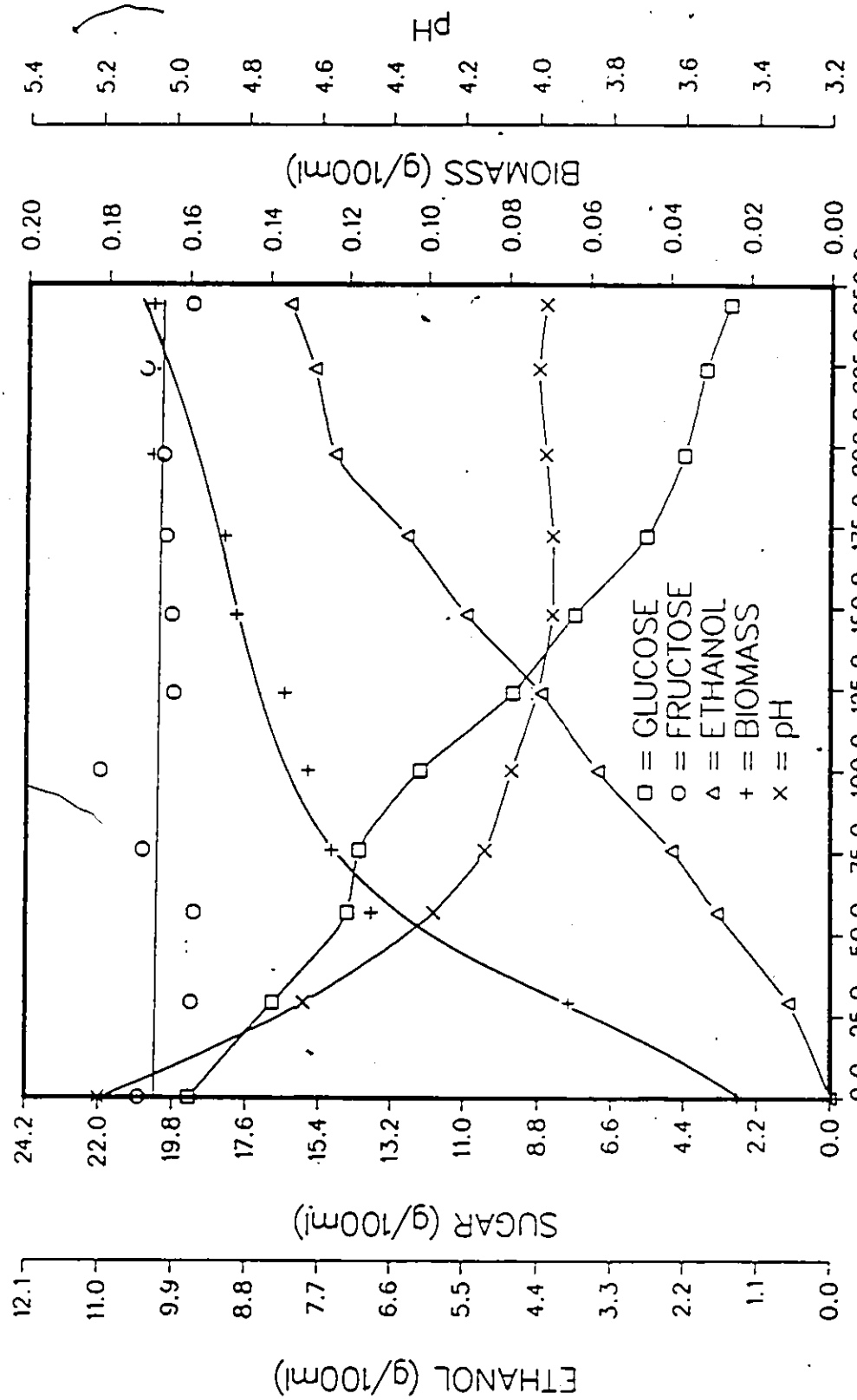

Figure 33: Ethanol production in 19.3% (w/v) glucose + 20.8% (w/v) fructose medium.

while the pH decreased from 5.41 to 3.61 (Figure 34). The ethanol reached its highest level after 80 hours; its concentration then started to decline.

With the medium containing 14.2% (w/v) glucose + 15.0% (w/v) fructose, the highest level of ethanol was reached after 100 hours. This concentration was 2.6 g/100 ml, corresponding to an ethanol yield on glucose of 50%. The test was carried out for 147 hours during which the biomass concentration increased from 0.017 g/100 ml to 0.47 g/100 ml and the pH changed from 5.26 to 3.25 (Figure 35). The specific growth rate of the yeast was 0.12 h^{-1} and the volumetric ethanol productivity (P3) was 0.026 g ethanol/((100 ml medium).(h)) (Table 6).

4.4.4 Effect of Inoculum Size on Ethanol Production

In all the tests described so far, the processes were slow and, as a consequence of this, the volumetric ethanol productivities were low. One possible reason for that may be the fact that the biomass was present in small concentration at the beginning of each experiment. Therefore, trying to increase ethanol production rates, larger inocula were used in order to have larger biomass concentration at the beginning of the experiments.

The first test of this series (Figure 36), done with a 14.3% (w/v) glucose + 15.3% (w/v) fructose medium, started with an initial biomass concentration of 0.78 g/100 ml, which is 30 times bigger than the initial biomass concentration of the previous test done in a similar medium (Figure 32). The glucose was completely used in 23 hours. An ethanol concentration of 7.4 g/100 ml was reached, which corresponded to the theoretical ethanol yield on glucose. The specific growth rate on glucose was 0.025 h^{-1} and the volumetric ethanol productivity (P3) was 0.32 gram of ethanol per 100 ml of medium per hour. At the end of the test, the biomass concentration was 1.21 g/100 ml; the pH had changed from 5.07 to 4.21.

The last test was done with a medium containing 18.3 g/100 ml glucose and 19.9 g/100 ml fructose (Figure 37). The biomass concentration at the beginning of the process was 0.73 g/100 ml which is 32 times larger than the concentration previously used with a similar medium (Figure 33). The test was carried out for 42 hours, at the end of which the glucose concentration was 1.2 g/100 ml. The

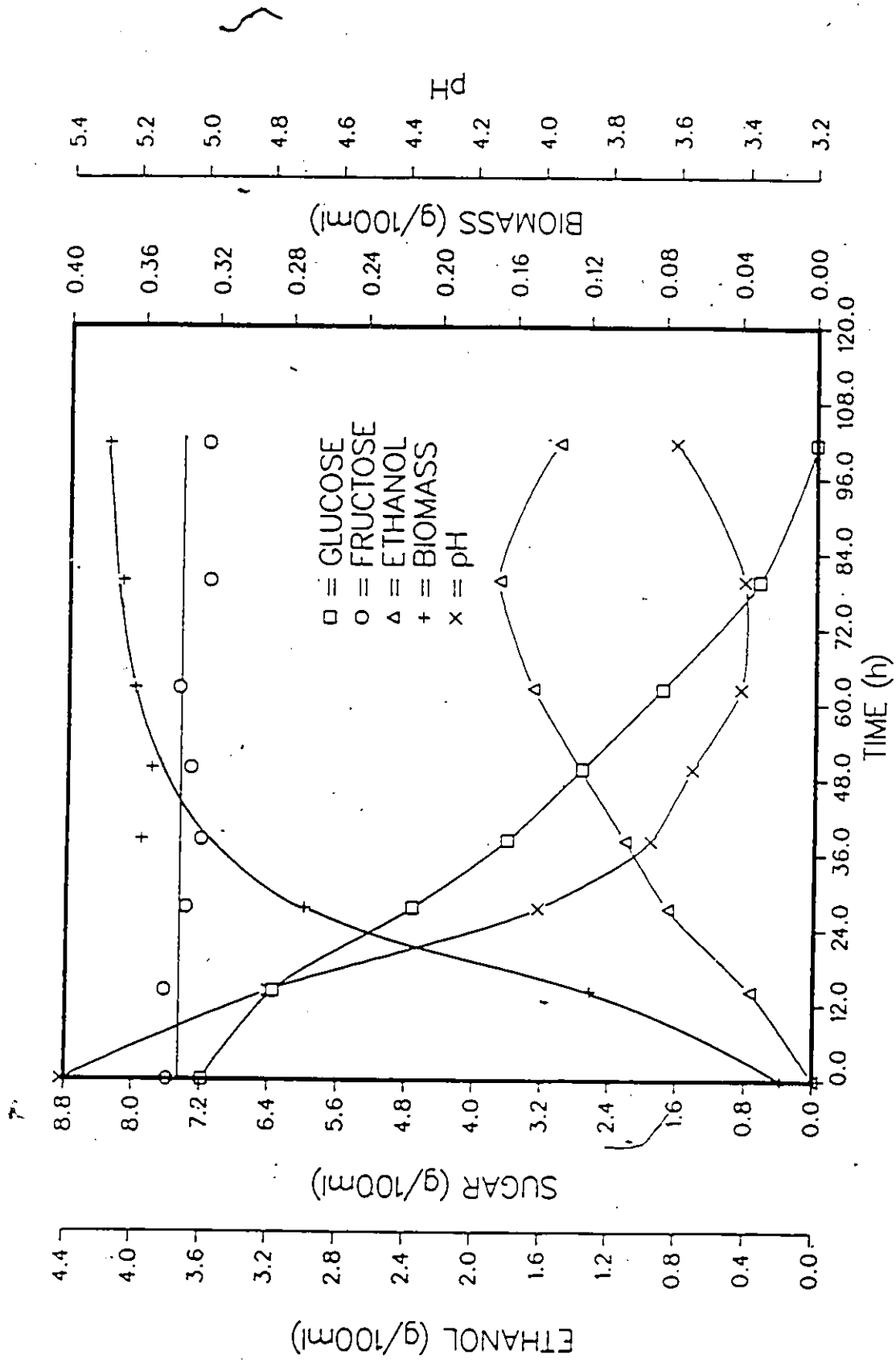

Figure 34: Ethanol production in medium containing 7.2% (w/v) glucose + 7.6% (w/v) fructose in the presence of air (0.5 L air/((L medium).min)).

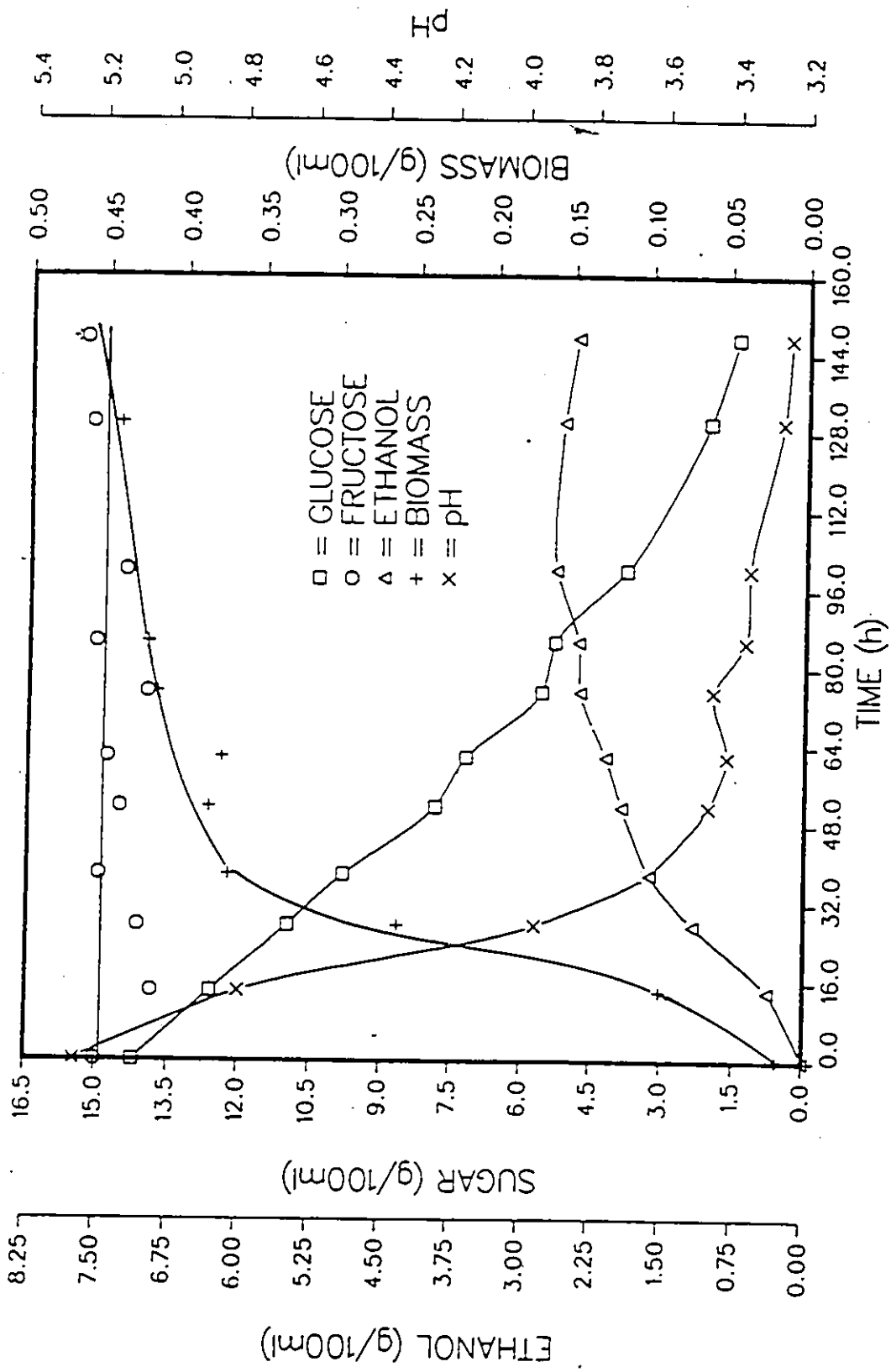

Figure 35: Ethanol production in medium containing 14.2% (w/v) glucose + 15.0% (w/v) fructose in the presence of air (0.5 L air/((L medium).min)).

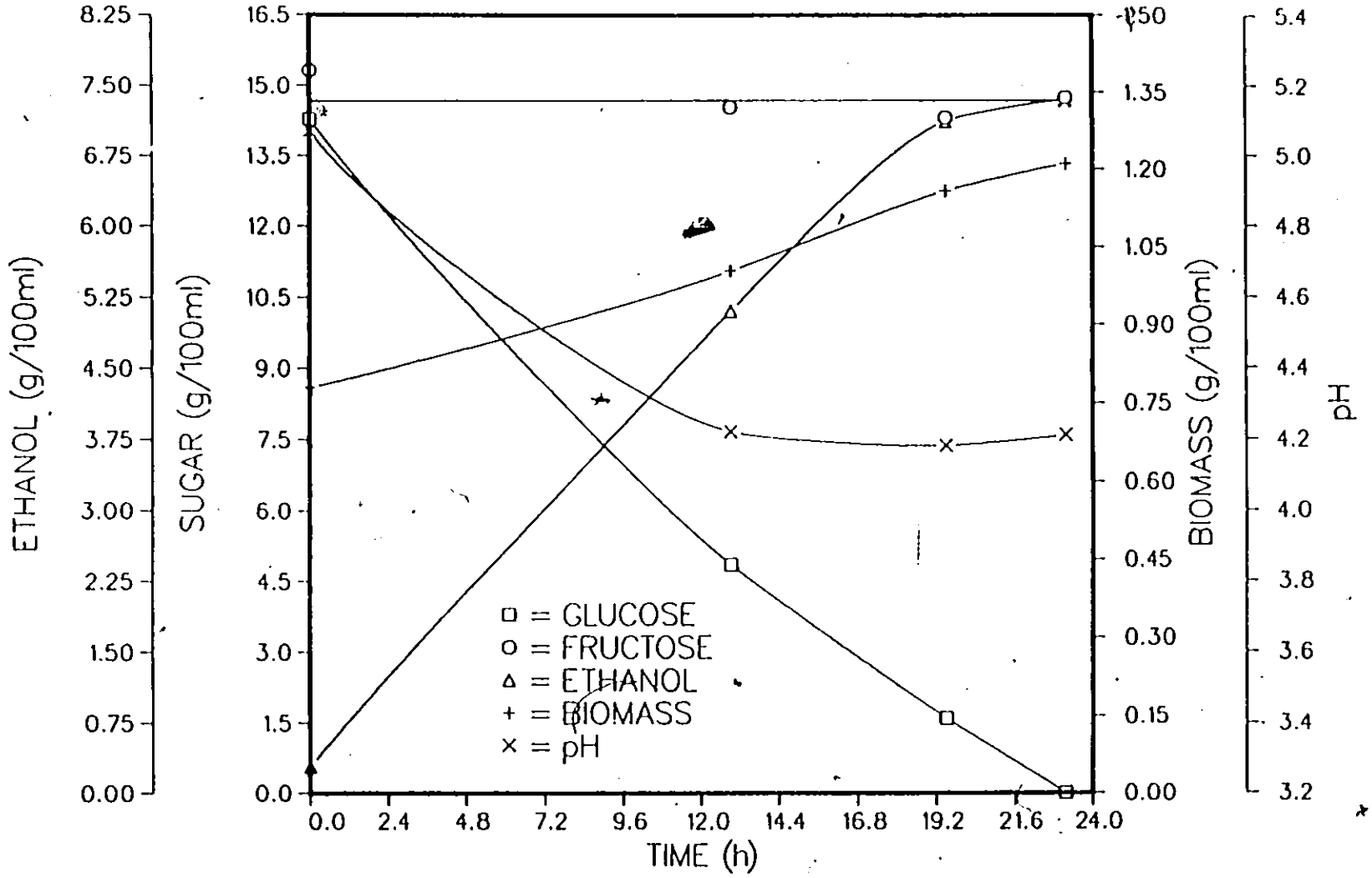

Figure 36: Ethanol production in 14.3% (w/v) glucose + 15.3% (w/v) fructose medium, with high inoculum concentration.

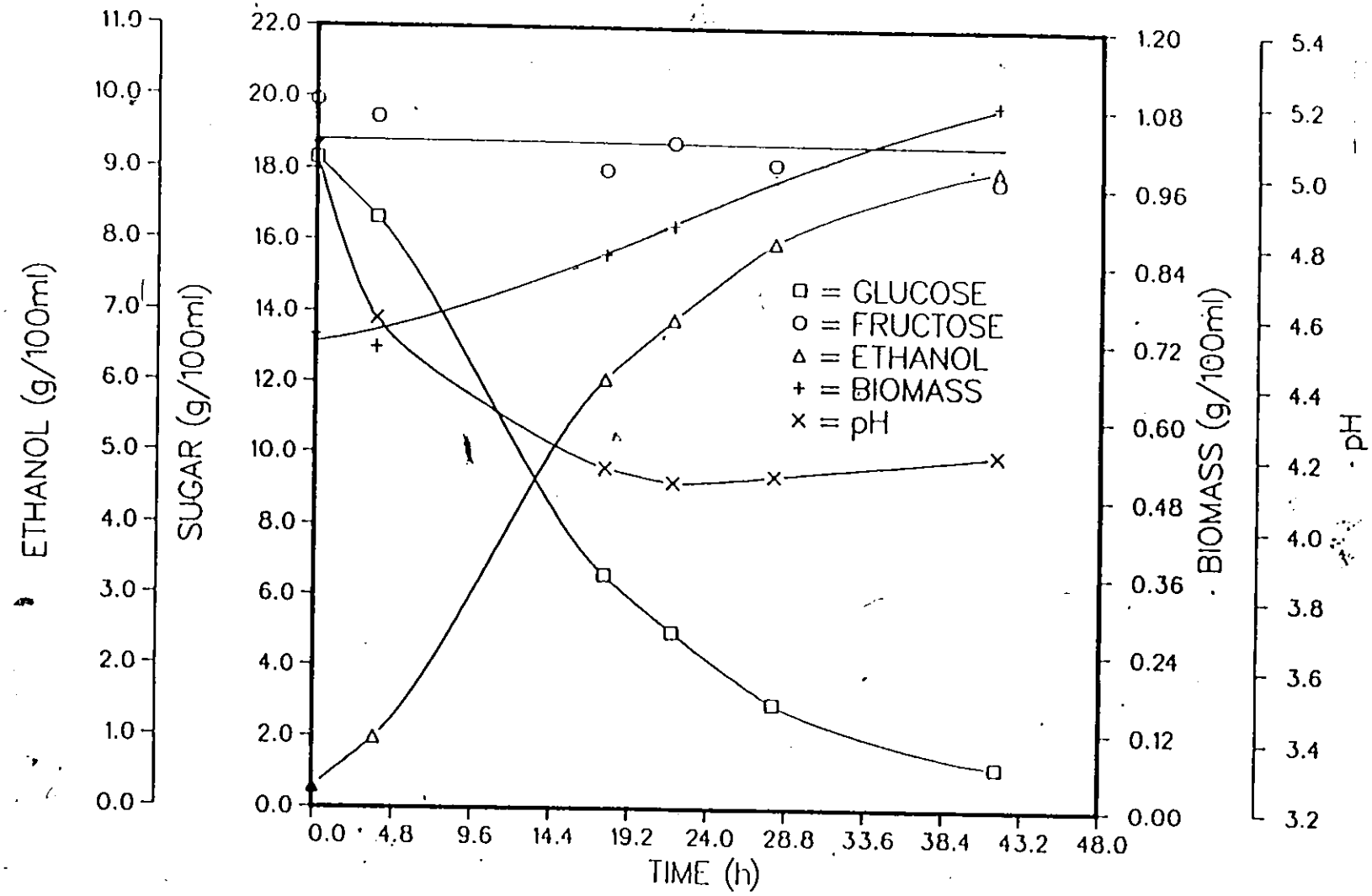

Figure 37: Ethanol production in 18.3% (w/v) glucose + 19.9% (w/v) fructose medium, with high inoculum concentration.

ethanol reached a 9.0 g/100 ml concentration, which represented a yield on glucose equal to the theoretical value. The pH changed from 5.06 to 4.20 and the biomass concentration increased to a value of 1.1 g/100 ml. The specific growth rate of the yeast was 0.017 h^{-1} and the volumetric ethanol productivity (P3) 0.22 gram of ethanol per 100 ml of medium per hour.

4.5 Continuous Ethanol Production

After the completion of several batch experiments on ethanol production, continuous ethanol production was considered.

Yeast cells were grown in the fermentor and immobilized in calcium alginate beads. The beads were packed into the bed of a reactor identical to the one used for sucrose inversion and ethanol was produced continuously. The apparatus used is described in section 3.7. The reactor was maintained at a temperature of 30°C. It was fed with medium V, containing equal amounts of glucose and fructose: either 10 g/100 ml or 15 g/100 ml of each sugar were present in the medium.

4.5.1 Effect of Dilution Rate on Ethanol Productivity

The first bioreactor assembled for continuous ethanol production contained about 4100 beads having the following properties:

First Batch of Ethanol-Producing Beads	
initial diameter of a bead	3.5 mm
initial mass of a bead	26 mg
initial percentage of nitrogen (dry basis)	5.8
initial mass of nitrogen per bead	220 μg
fraction of cells in the alginate suspension (wet weight)	26% (w/w)

This bioreactor was fed with a 10% (w/v) glucose + 10% (w/v) fructose medium for 268 hours. The results of its operation are shown in Figure 38. After a few hours

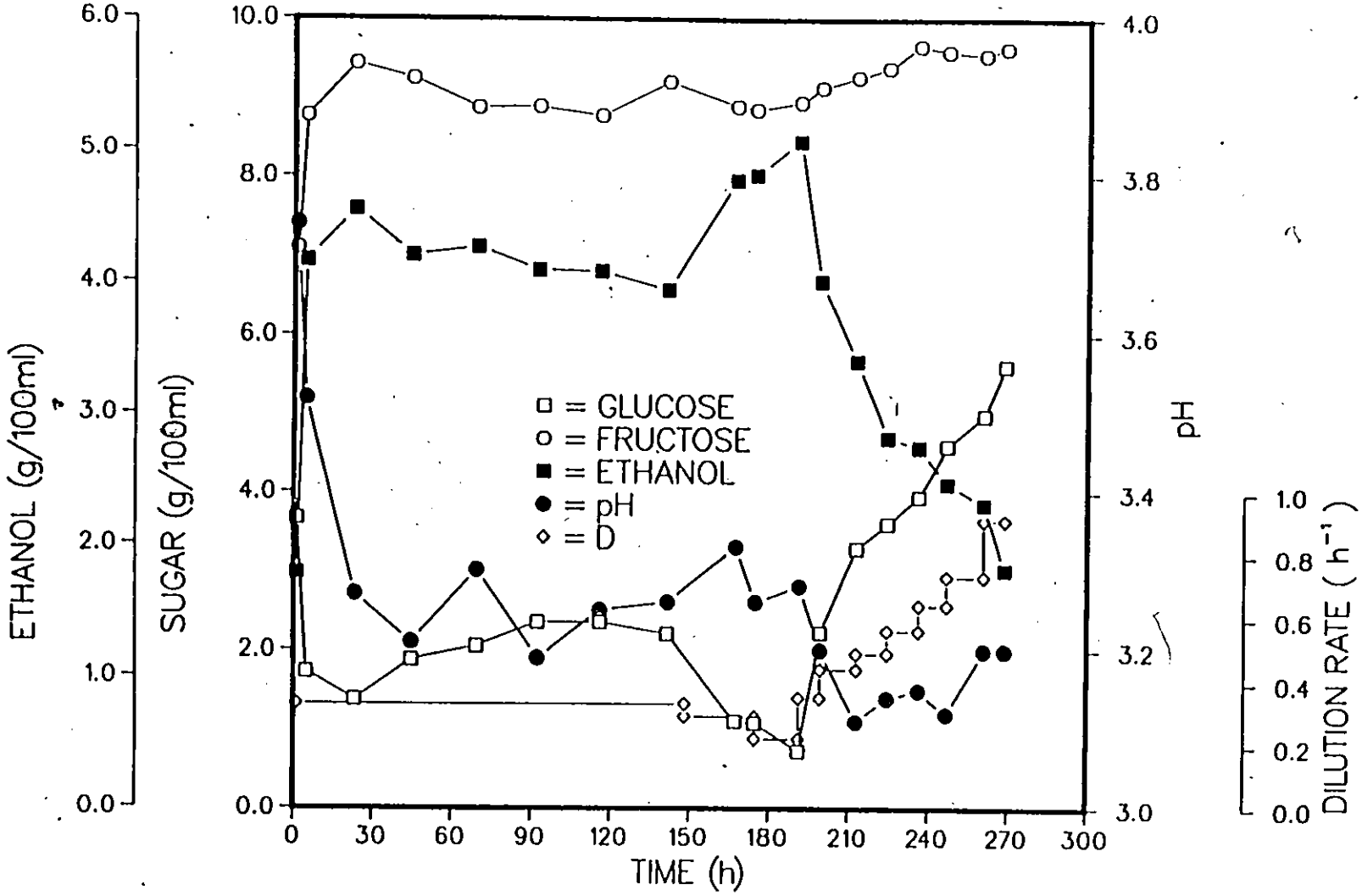

Figure 38: Ethanol production from a 10% (w/v) glucose + 10% (w/v) fructose medium in a packed-bed reactor.

of operation at a dilution rate of 0.33 h^{-1}, which corresponded to a flow rate of 50 ml/h, an ethanol concentration of 4.1 g/100 ml was measured in the reactor effluent. Almost 80% of the glucose was converted at this flow rate. The fructose was not consumed by the yeast; however, some sugar was absorbed by the alginate beads until an equilibrium was reached between the gel and its environment.

The flow rate was then varied in order to establish the relation between the volumetric ethanol productivity and the dilution rate. Table 7 shows the results obtained. The basis for the calculation of the dilution rates and the volumetric ethanol productivities was the total volume of the reactor bed. The highest ethanol productivity, 16.8 grams per litre of reactor volume per hour, was reached at a dilution rate of 0.73 h^{-1}. The productivity results are also plotted in Figure 39. The points are scattered around the curve, probably because steady state was not achieved for each dilution rate.

Table 7: Volumetric ethanol productivity as a function of the dilution rate for a feed medium containing 10% (w/v) glucose + 10% (w/v) fructose.

Dilution rate (h^{-1})	*Ethanol* (g/100ml)	*Productivity* ($g.L^{-1}.h^{-1}$)
0.33	4.1	13.5
0.29	4.8	13.8
0.22	5.1	11.1
0.35	4.0	14.1
0.44	3.4	15.0
0.49	2.8	13.9
0.56	2.8	15.4
0.64	2.5	15.8
0.73	2.3	16.8
0.91	1.8	16.5

After 268 hours of operation, the sugar concentration in the feed medium was changed to 15% (w/v) glucose + 15% (w/v) fructose. Figure 40 shows the results obtained for bioreactor operation with this medium. The flow rate was not changed as frequently as in the previous case, in order to leave enough time to the system to reach steady state. The ethanol concentration in the effluent varied between 1.5

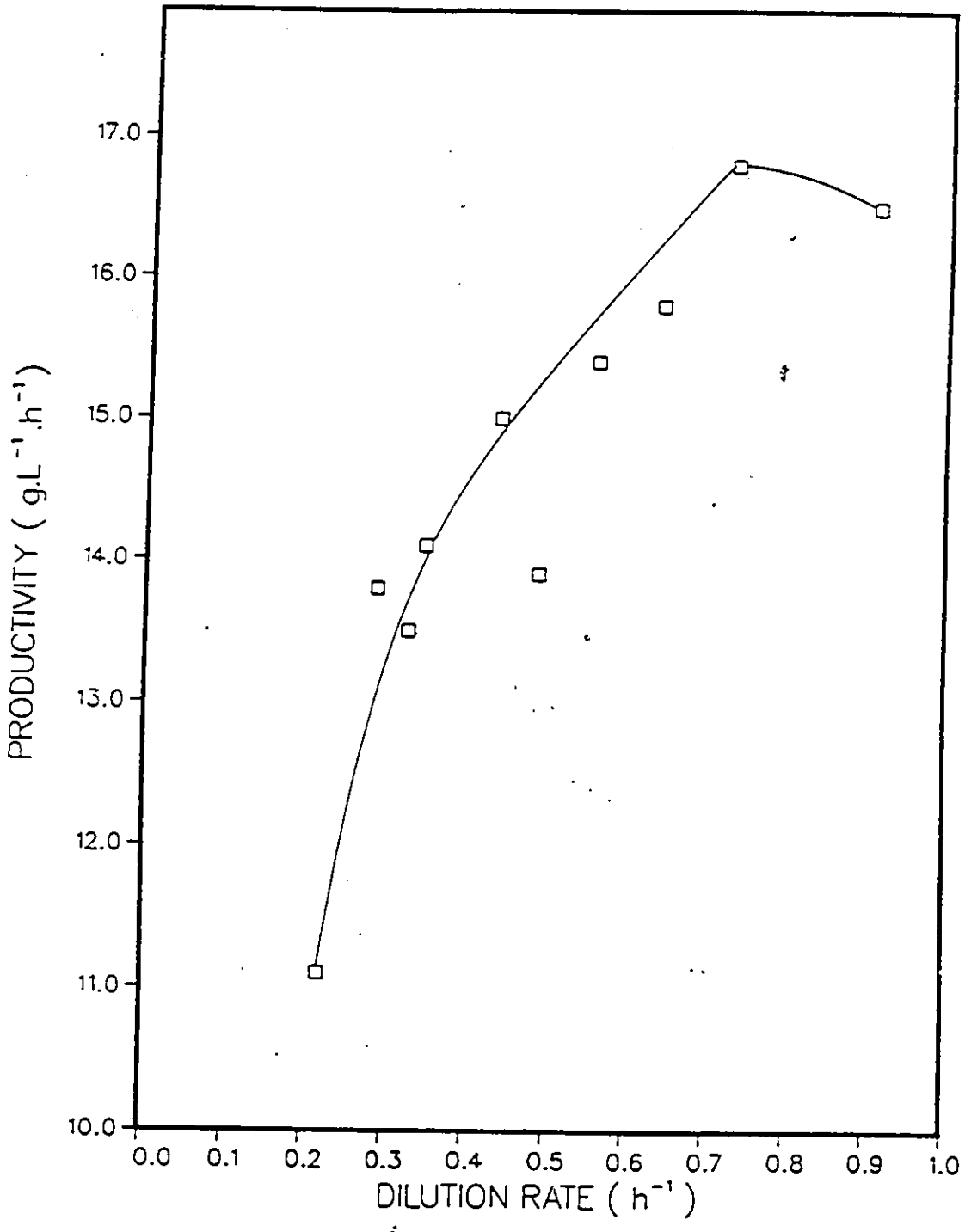

Figure 39: Volumetric ethanol productivity as a function of the dilution rate, for a feed medium containing 10% (w/v) glucose + 10% (w/v) fructose.

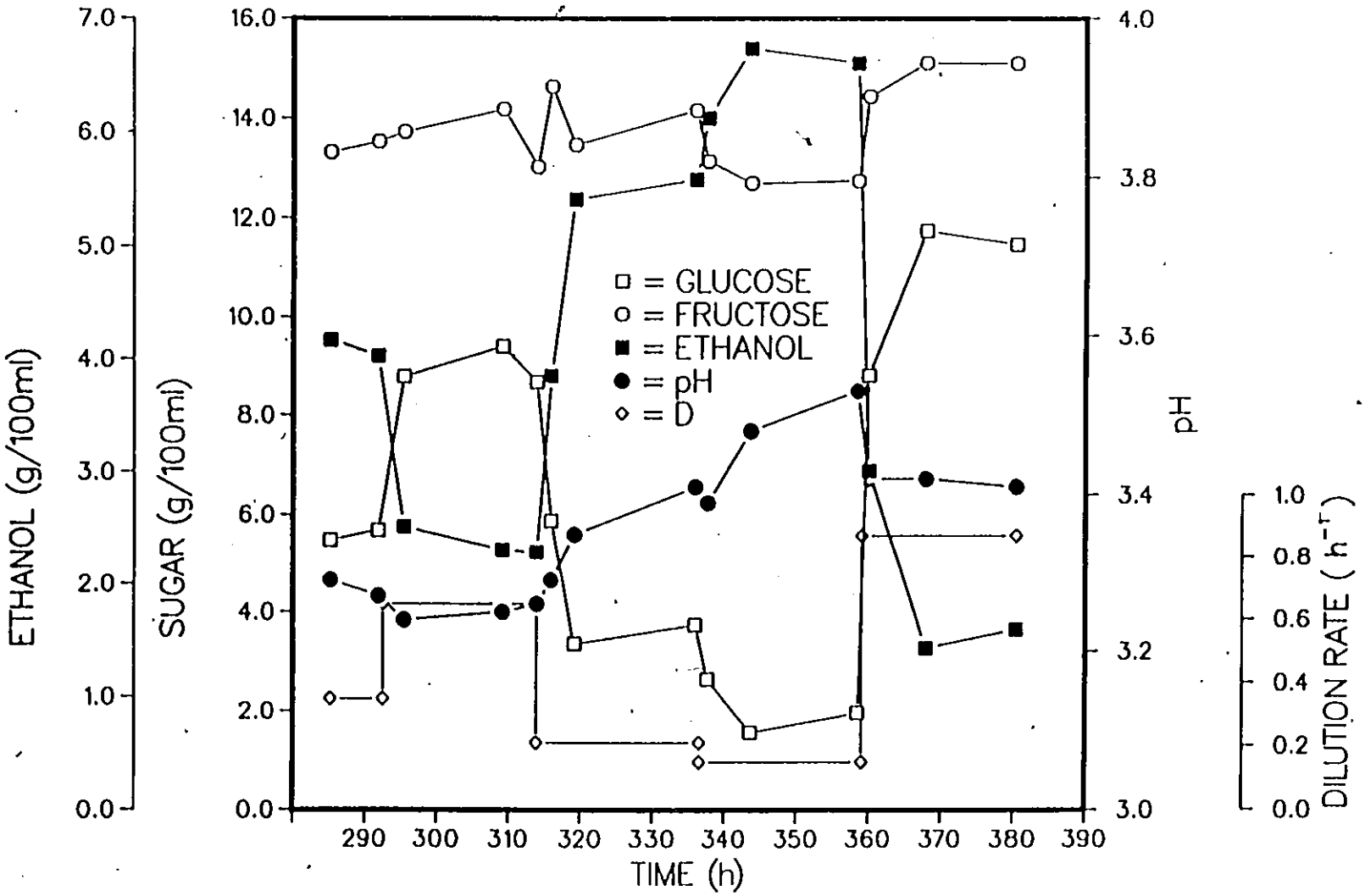

Figure 40: Ethanol production from a 15% (w/v) glucose + 15% (w/v) fructose medium, in a packed-bed reactor.

g/100 ml and 6.5 g/100 ml. The volumetric ethanol productivity and the ethanol concentration in the effluent as a function of the dilution rate are shown in Table 8. The highest ethanol productivity, 15.0 grams per litre of reactor volume per hour, was obtained at a dilution rate of 0.65 h^{-1}. This corresponded to an ethanol concentration in the effluent of 2.3 g/100 ml and to a glucose concentration of 9 g/100 ml; this means that 40% of the glucose was converted to ethanol. Figure 41 shows the volumetric ethanol productivity as a function of the dilution rate. When increasing the dilution rate over 0.65 h^{-1}, both the ethanol concentration in the effluent and the volumetric productivity went down.

Table 8: Volumetric ethanol productivity as a function of the dilution rate for a feed medium containing 15% (w/v) glucose + 15% (w/v) fructose.

Dilution rate (h^{-1})	*Ethanol* (g/100ml)	*Productivity* ($g.L^{-1}.h^{-1}$)
0.35	4.0	14.0
0.65	2.3	15.0
0.21	5.6	11.7
0.15	6.5	9.8
0.87	1.5	13.1

During bioreactor operation, beads swelled due to yeast growth, and the interstitial spaces between them decreased. Some free cells were observed in the reactor effluent.

4.5.2 Effect of Storage on Immobilized Yeast Cells

It was demonstrated that ethanol could be continuously produced from a medium containing glucose and fructose, without using fructose. The next step was to determine if it was possible to store the calcium alginate beads containing the yeast cells in order to use them at a later time, as was done with invertase-containing calcium alginate beads. Beads described in subsection 4.5.1 were stored in a $CaCl_2$ solution, in the refrigerator (1–5°C), for two months. Afterwards, 2300 of these beads were packed in the bed of the bioreactor. A number of beads smaller than the previous time was used, in order to provide some volume for swelling of the

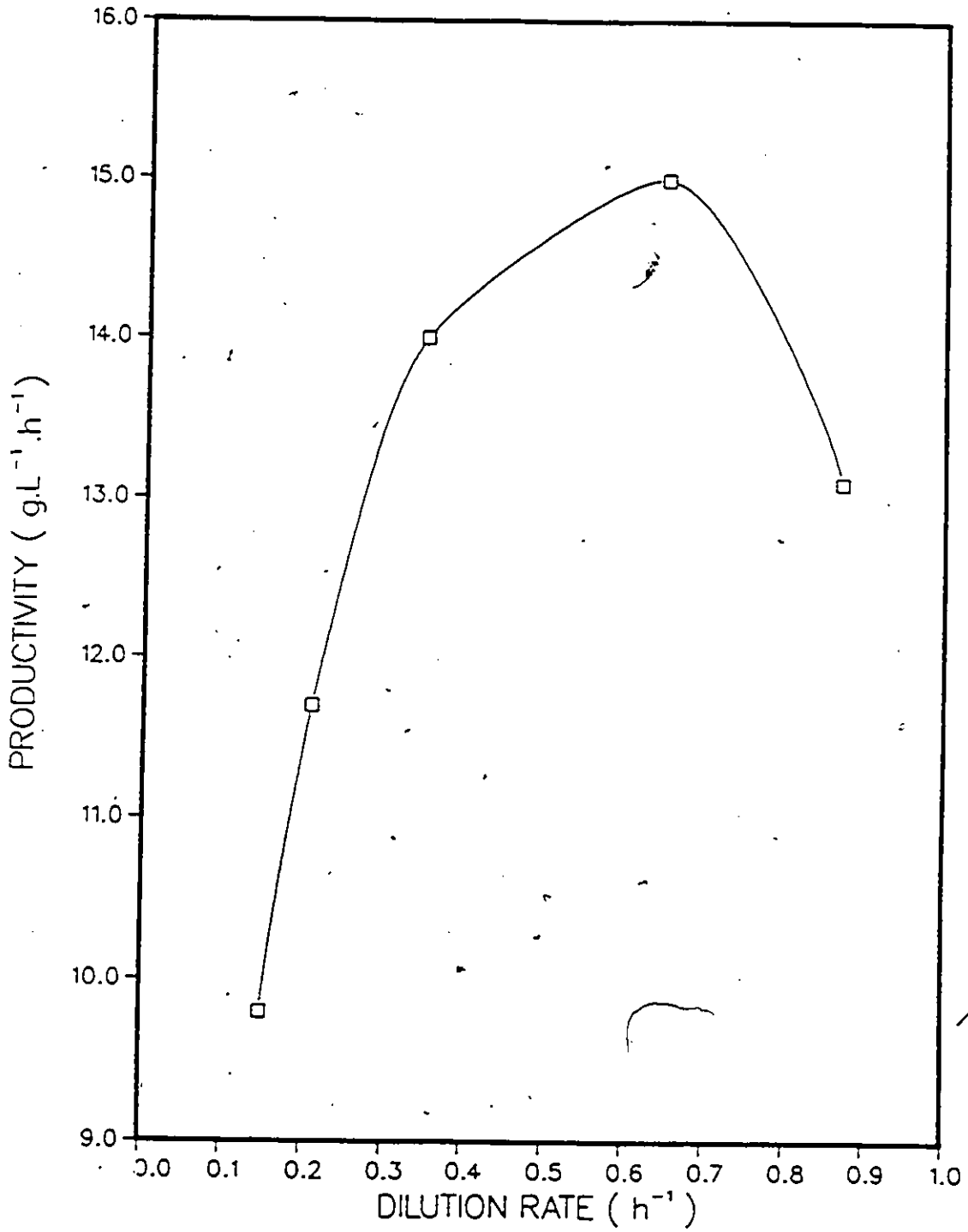

Figure 41: Volumetric ethanol productivity as a function of the dilution rate, for a feed medium containing 15% (w/v) glucose + 15% (w/v) fructose.

beads due to yeast growth, which was not anticipated the first time. The reactor was operated with a feed medium containing 15 g/100 ml glucose and 15 g/100 ml fructose. Results of the process are shown in Figure 42. The dilution rate was set at 0.16 h^{-1}. The ethanol concentration in the effluent was about 4.5 g/100 ml, which corresponded to a volumetric productivity of 7.2 grams per litre of reactor volume per hour. The fructose was not consumed.

4.5.3 Increase of the Fructose/Glucose Ratio in Invert Sugar Media

The objective of the study of ethanol production in packed-bed reactors was to remove the glucose from invert sugar, in order to isolate the fructose. Glucose and fructose are hard to separate; the conversion of glucose to ethanol would facilitate fructose isolation. This could be done by using a reactor inverting sucrose and a reactor converting glucose to ethanol in series.

An ethanol-producing bioreactor was assembled for this purpose. A second batch of yeast cells entrapped in alginate beads was prepared for this operation, the same way the first batch was prepared. The beads had the following properties:

Second Batch of Ethanol-Producing Beads	
initial diameter of a bead	3.6 mm
initial mass of a bead	26 mg
initial percentage of nitrogen (dry basis)	6.0
initial mass of nitrogen per bead	175 μg
fraction of treated cells in the alginate suspension (wet weight)	30% (w/w)

The bioreactor was first packed with 2500 beads. However, since after 2 days of operation, the swollen beads did not fully occupy the bed, 600 more beads were added to the reactor. The addition was done 51 hours after start-up. The bed finally contained about 3100 beads.

Figure 43 shows the results obtained during operation of this reactor with a 15% (w/v) glucose + 15% (w/v) fructose medium, at a dilution rate of 0.15 h^{-1}. The pronounced increase in ethanol concentration observed after 90 hours of operation is the consequence of the addition of beads. Following this addition, when steady state was achieved, the effluent contained ethanol in 5.6 g/100 ml concentration, which corresponded to a volumetric ethanol productivity of 8.4 grams per litre of reactor volume per hour.

4.6 Conversion of Sucrose into Fructose

In order to convert sucrose into fructose and ethanol, two continuous processes were used in series: sucrose inversion followed by conversion of glucose into ethanol and carbon dioxide. Both operations had already been realized in packed-bed reactors.

The invertase bioreactor used was the second one described in section 4.2. It had been in operation during 26 days, being fed with a 30% (w/v) sucrose solution (Figures 20 and 21) and was converting 85% of the sucrose at a dilution rate of 0.18 h^{-1}, which means that the sucrose inversion rate was 46 grams per litre of reactor volume per hour.

The effluent of this bioreactor flowed into a medium mixer (described in subsection 3.7.3), where it was mixed with a concentrated nutrients broth (medium VI). The role of these nutrients was to maintain the yeast cells producing ethanol in an active state. The nutrients broth flow rate to the medium mixer was 1/10 of the sucrose solution flow rate to the invertase bioreactor. The nutrients were added to the medium mixer rather than to the sucrose solution reservoir, in order to prevent their contact with invertase, which might have been detrimental to the enzyme.

The medium formed in the mixer, containing glucose, fructose, residual sucrose and nutrients, was pumped to the ethanol-producing bioreactor. This packed-bed bioreactor was the one described in subsection 4.5.3 which had been in operation for 9 days, at a dilution rate of 0.15 h^{-1}, producing ethanol at a rate of 8.4 grams per litre of reactor volume per hour, from a 15% (w/v) glucose + 15% (w/v) fructose medium.

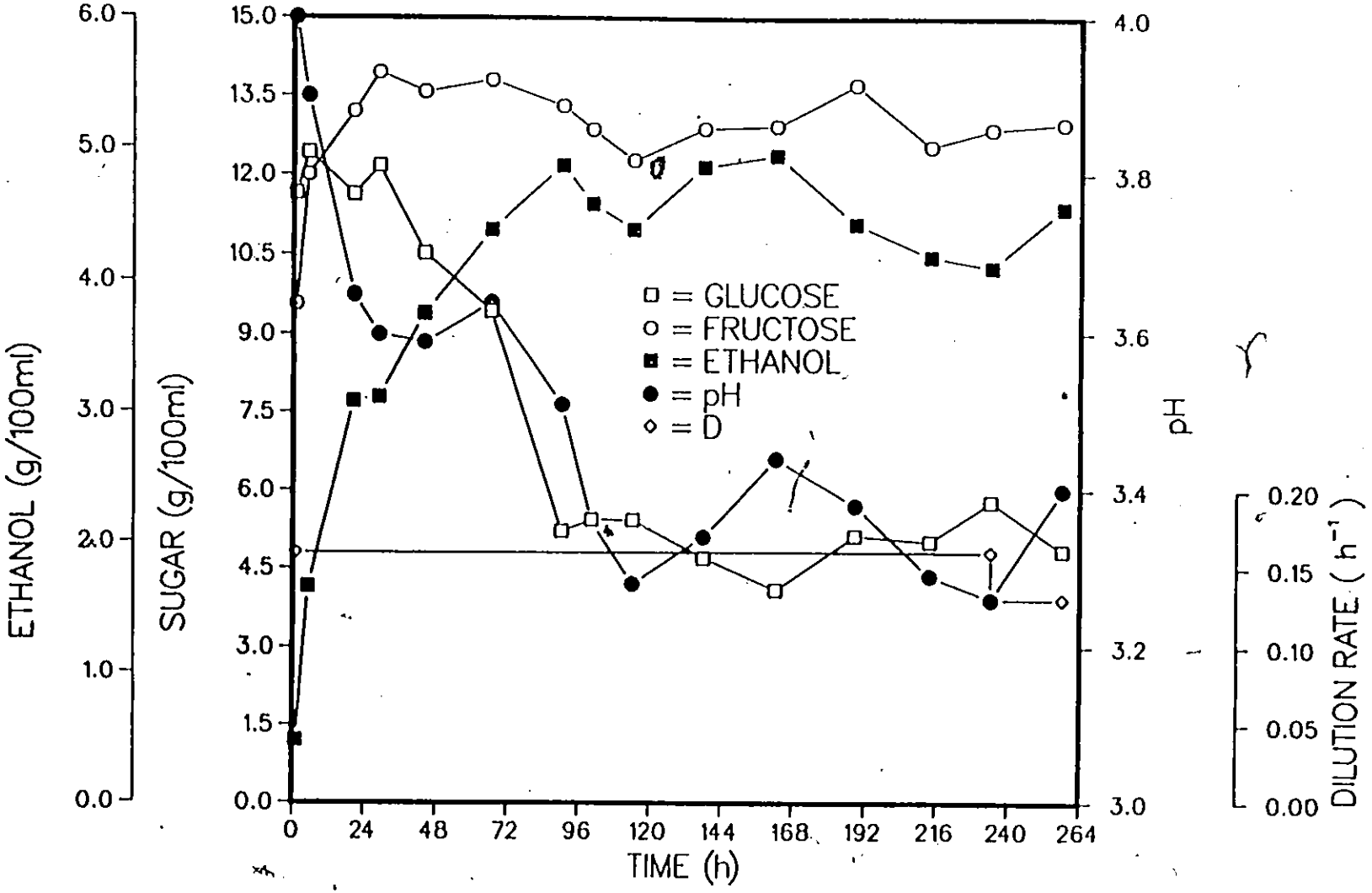

Figure 42: Ethanol production from a 15% (w/v) glucose + 15% (w/v) fructose in a reactor packed with beads that were stored for 2 months.

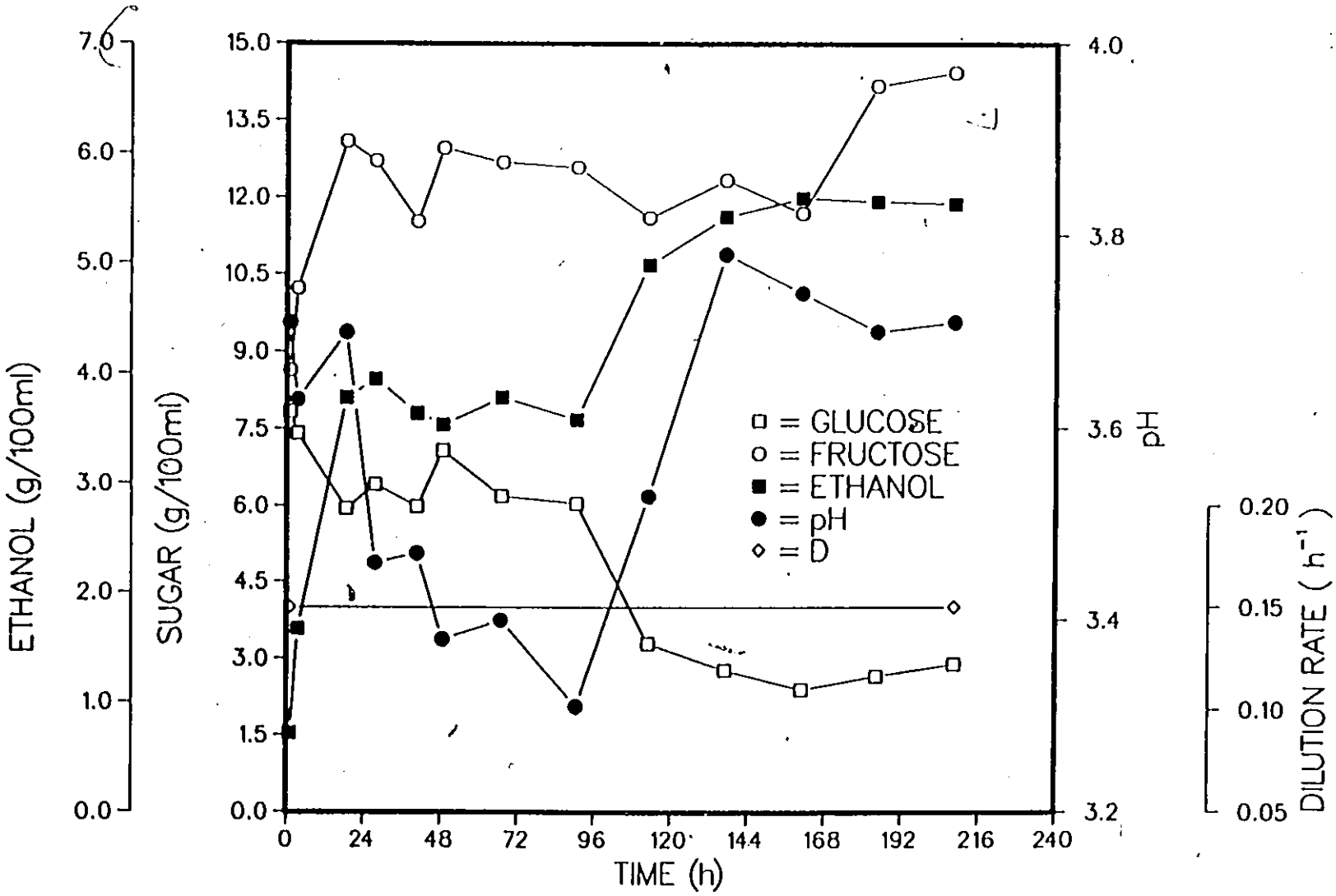

Figure 43: Packed-bed ethanol production from a 15% (w/v) glucose + 15% (w/v) fructose medium.

Therefore, starting with a 30 g/100 ml sucrose solution and a concentrated nutrients broth (medium VI), a stream containing fructose, ethanol and residual glucose and sucrose was obtained, at the end of the process; the CO_2 was released to the atmosphere. A schematic diagram of the experimental set-up is shown in Figure 10. The same pump was used to feed both bioreactors, and only one flow rate could be selected per pump. Because nutrients were added to the mixer with the help of a second pump, there was an accumulation of medium in the mixer equivalent to the flow rate of concentrated nutrients; therefore, the mixer had to be drained regularly, which reduced the productivity of the system.

When the bioreactors and the mixer were first linked together, the dilution rate (in both bioreactors) was set to 0.16 h^{-1}. The temperatures of the invertase bioreactor and the ethanol-producing bioreactor remained at 55°C and 30°C respectively.

Glucose, fructose, sucrose and ethanol concentrations, as well as pH of the process effluent, were measured at the outlet of the process; Figures 44 to 47 show the results obtained.

After 200 hours of operation, the ethanol concentration was 4.4 g/100 ml, corresponding to an ethanol production rate of 7.0 grams per litre of reactor volume per hour; the fructose concentration was 10.5 g/100 ml, the glucose concentration was 2.9 g/100 ml and the sucrose concentration was 7.8 g/100 ml.

The sucrose concentration in the feed solution was kept at 30 g/100 ml and a second ethanol-producing bioreactor was added in series with the one already there, 262 hours after process start-up, in order to increase glucose conversion. This reactor contained 3500 beads of the second batch; it had never been used before. This is why the beads first absorbed sugar and ethanol until their osmotic pressure was in equilibrium with their environment. This can be seen by the decrease in concentrations following the addition of the second ethanol-producing bioreactor. After 350 hours of operation, the ethanol concentration was 5.1 g/100 ml, the fructose concentration was 8.3 g/100 ml and the glucose and sucrose concentrations were 0.1 g/100 ml and 7.5 g/100 ml respectively.

Because the glucose was not being used completely, even when passing through two packed-bed ractors, the dilution rate was decreased. Also, in order to invert

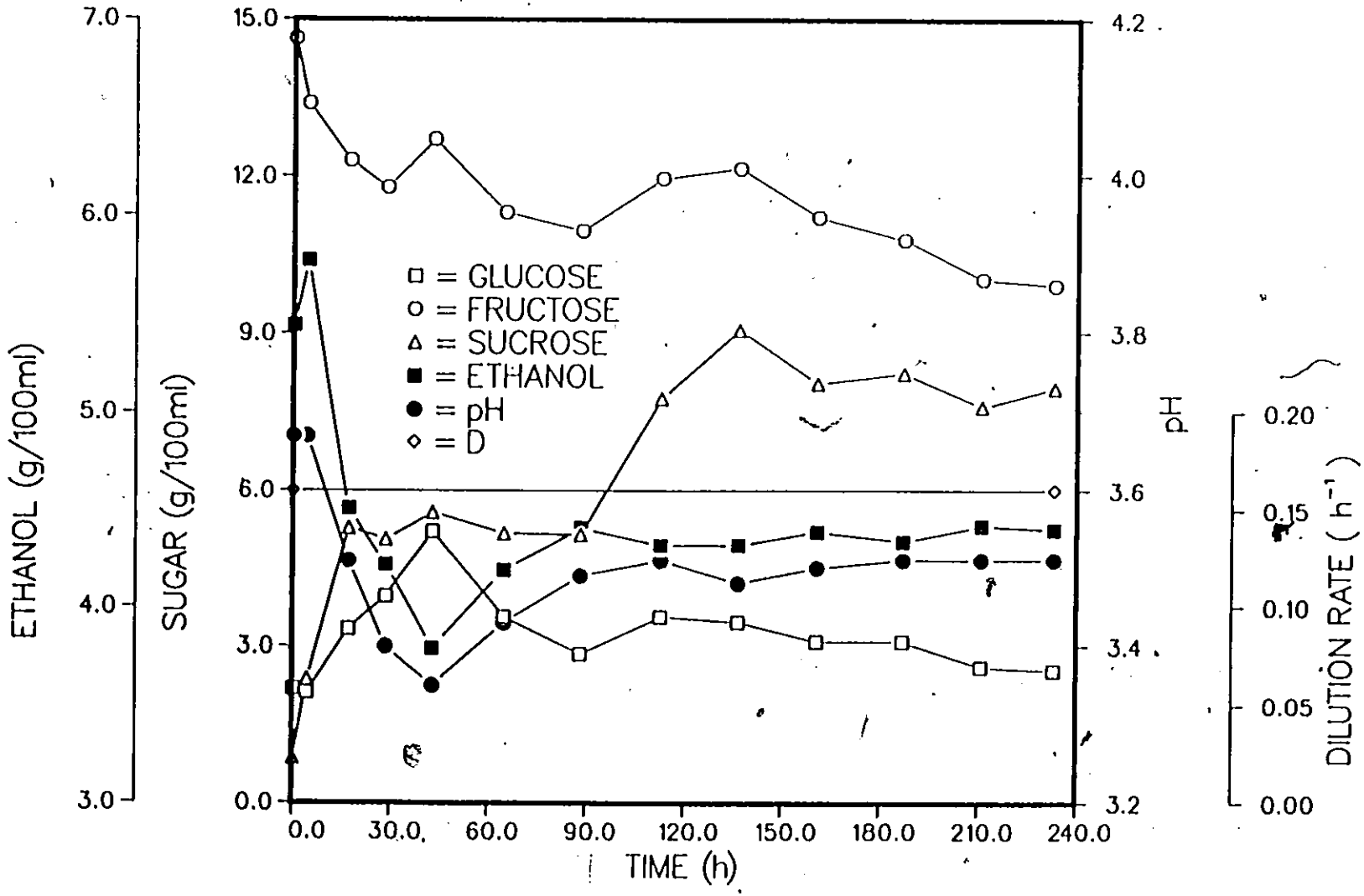

Figure 44: Production of fructose and ethanol from a 30% (w/v) sucrose medium in packed-bed reactors.

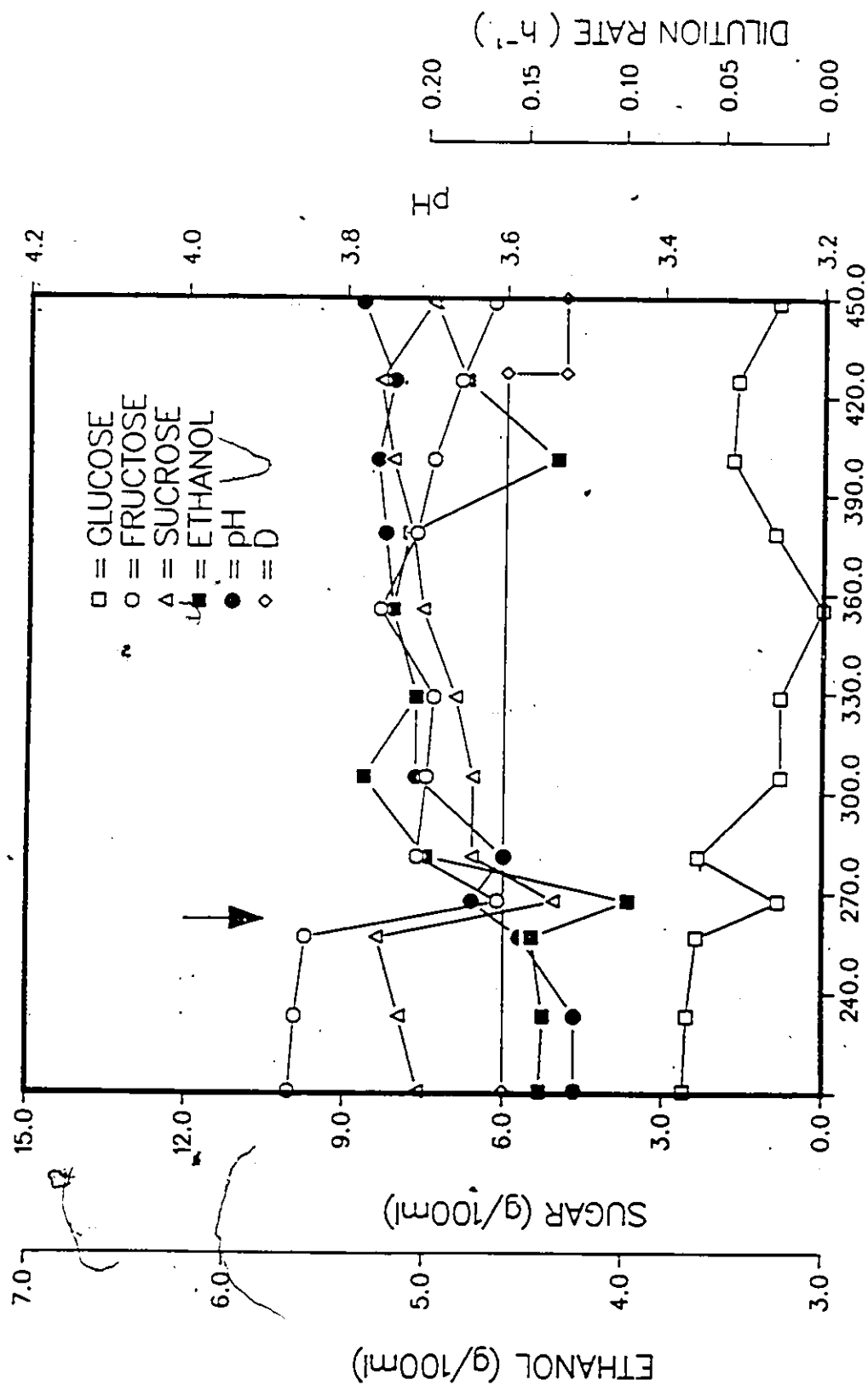

Figure 45: Production of fructose and ethanol from a 30% (w/v) sucrose medium in packed-bed reactors; the arrow indicates the addition of a second ethanol-producing bioreactor.

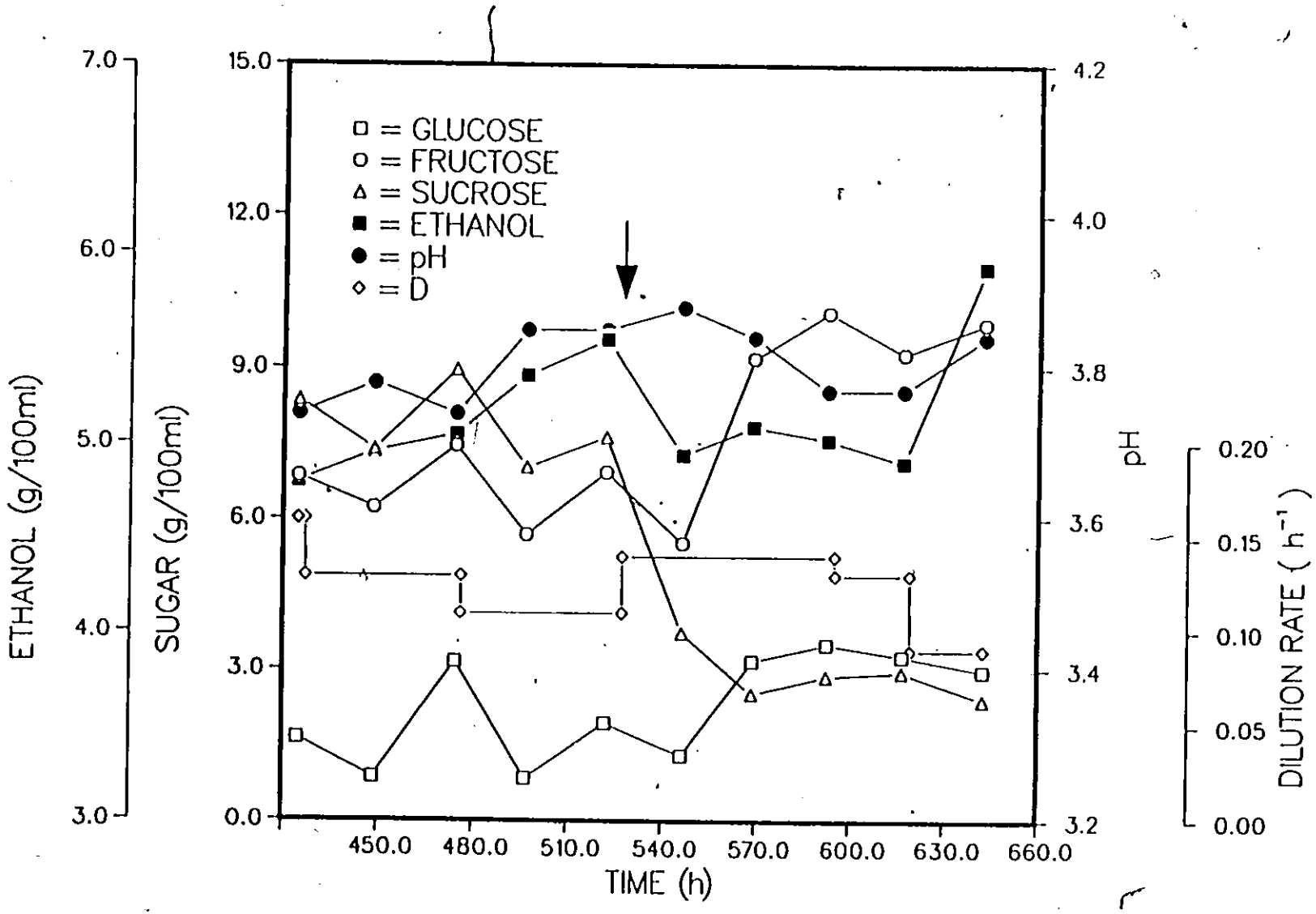

Figure 46: Production of fructose and ethanol from a 30% (w/v) sucrose medium in packed-bed reactors; the arrow indicates the replacement of the invertase bioreactor.

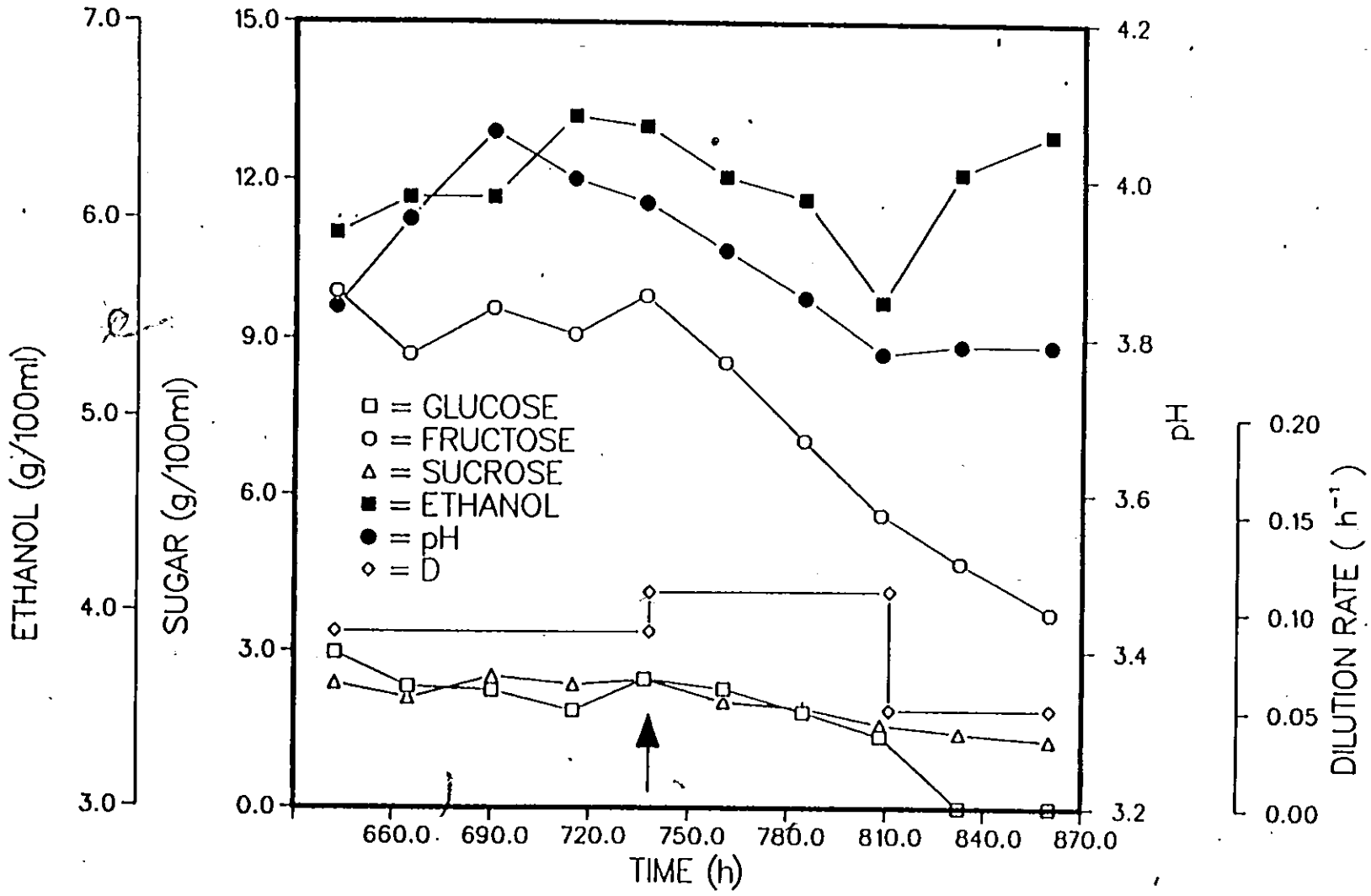

Figure 47: Production of fructose and ethanol from a 30% (w/v) sucrose medium in packed-bed reactors; the arrow indicates a concentration change of the sucrose in the feed of 30% (w/v) to 20% (w/v).

more sucrose, the invertase packed-bed reactor was replaced by a fresh one, 527 hours after process start-up. The fresh packed-bed reactor was filled with 4900 beads of the second batch of invertase beads prepared; therefore, this reactor was very similar to the one it was replacing. After 640 hours of operation, the ethanol concentration was 5.9 g/100 ml, the fructose concentration was 9.9 g/100 ml, and the glucose and sucrose concentrations were 3.0 g/100 ml and 2.4 g/100 ml respectively.

The sucrose concentration in the feed solution was changed to 20% (w/v), 738 hours after process start-up.

Chapter 5

Discussion

5.1 Sucrose Inversion in Batch Systems

Many authors have studied sucrose inversion. Invertase is among the most studied enzymes; it is the subject of both classic kinetics research and immobilization efforts. Unfortunately, many scientific papers dealing with new immobilization techniques, contain no results concerning the optimum pH and temperature for the activity and the stability of immobilized invertase.

The response of free yeast invertase to parameters such as pH, temperature and sugar concentration is available in the literature. However, when the enzyme is immobilized, the physical and chemical properties of the carrier alter its microenvironment, and the effect of these parameters has to be determined again.

5.1.1 Effect of Temperature on Sucrose Inversion

According to this work, yeast invertase immobilized in calcium alginate beads had its highest activity at 55°C. As seen from Figure 12, the activity of immobilized invertase slowly increased from 45°C to 55°C, but went down fast from 55°C to 65°C. Therefore, it would be safer, when there is a chance of fluctuation in the temperature of a reactor, to select a temperature slightly below 55°C.

Looking at Figure 11, it can be seen that at 60°C and 65°C, the activity of

immobilized invertase was high at the beginning of the experiment, but that there was an inactivation after about 2 hours of incubation, probably due to the rate of denaturation increasing. According to Wiseman (1979), free baker's yeast invertase exhibits a critical inactivation temperature at about 55°C, when the rate of denaturation increases markedly. This inactivation temperature can be raised by 10–15°C in the presence of high concentrations of the substrate, for example, 10% sucrose. Nothing was found in the literature about the influence of temperature on invertase immobilized in calcium alginate; however, the effect of temperature on the enzyme has been studied for other carriers.

Samoshina *et al.* (1981) immobilized a preparation of yeast invertase (Invertin) to silica gel by covalent binding. They studied the influence of temperature on the activity of the unimmobilized and immobilized invertase. The optimum temperature was determined for the temperature range 30°C to 80°C, using a 7% solution of sucrose, at a pH of 4.1. A considerable shift of the temperature optimum of immobilized invertase to the region of high temperatures (50–65°C) as compared to the free enzyme (40–45°C) was found.

D'Souza and Nadkarni (1980) have immobilized whole cells of *S. cerevisiae ATCC 3177* in acrylamide polymerized by gamma rays. The entrapped invertase was maximally active at 65°C, while the intact cell invertase showed a temperature optimum of about 60°C.

Monsan and Combes (1984a) covalently coupled invertase (Maxinvert 200,000, Rapidase) onto corn grits and determined the effect of temperature on invertase activity using a 2 mol/L sucrose solution at pH 4.5. Maximal sucrose inversion was obtained when the reaction temperature was 55°C.

While D'Souza and Nadkarni (1980) found an optimum temperature of 65°C for whole cell invertase immobilized in polyacrylamide, the two other groups of authors mentioned above found results for immobilized invertase similar to the one obtained in this work: an optimum temperature close to 55°C.

5.1.2 Effect of pH on Sucrose Inversion

The activity of invertase immobilized in calcium alginate beads was found, in this work, to be optimum in the pH range 3.5–5.6 (Figure 14), with its maximum value occuring at pH 4.5. The influence of pH on immobilized invertase is described in the literature, for several carriers.

Marconi *et al.* (1974) entrapped an invertase preparation (British Drug Houses Ltd.) in cellulose triacetate fibers and studied the properties of the insoluble derivative. They found that the pH for optimum activity was around 4.5 for the free and entrapped invertase. A flattening of the pH-activity curve around optimum pH was observed for immobilized invertase, due to the fact that diffusion became rate limiting at those pH values where the enzyme activity was higher.

Yeast invertase (analytical grade, Schwarz BioResearch) was covalently attached to a synthetic resin (Amberlite IR 45) by Saini *et al.* (1972). When comparing the bound invertase with free invertase, they reported no change in the optimum pH, which was about 5.0. However, the immobilized derivative was much more active at adverse pH conditions because of the buffering action of the support material, resulting in different internal hydrogen ion concentrations in the carrier and in the bulk.

Monsan and Combes (1984), who covalently coupled invertase (Maxinvert 200,000, Rapidase) to corn grits, for use in packed-bed reactors, studied the effect of pH in the range 4.1–5.6 and found that their derivative had its highest activity between pH 4.1 and pH 4.6.

The effects of pH on yeast invertase (Invertin) bound to silica gel through glutaraldehyde were studied (Samoshina *et al.* , 1981). It was found that immobilized invertase was stable in a wider range of pH values than native invertase (3.9–6.2 as compared to 3.5–4.9). Concerning activity, the original preparation (unimmobilized) had an optimum pH in the region 4.4–4.5 and a profile of the pH curve extended to the right. A slight shift in the optimum pH to the acid region (pH 4.1) and a symmetrical profile of the pH curve was found for immobilized invertase.

D'Souza and Nadkarni (1980) have entrapped whole cells of *S. cerevisiae* in polyacrylamide. According to their work, both the immobilized and the free invertase

exhibited optimal activity at pH 4, although the entrapped cell invertase showed a broad pH optimum between 3.5 and 5.0, while the intact cell enzyme was optimally active at pH 4.

The results obtained in this work for *S. cerevisiae* invertase immobilized in calcium alginate beads are similar to those obtained by Marconi *et al.* (1974) for cellulose triacetate and to those of Monsan and Combes (1984a) for corn grits.

In all studies mentioned, including the present work, flattening of the pH-activity curve around the optimum pH was observed.

5.1.3 Inhibition of Sucrose Inversion by End Products

Sucrose inversion is inhibited by its end products, glucose and fructose. As shown in Figure 16, the amount of sucrose inverted is inversely proportional to the amount of end products added to the sucrose solution. It was also found that fructose is a stronger inhibitor than glucose. In the medium initially containing 5 g/100 ml glucose, 50% of the sucrose was inverted while in the medium initially containing 2.5 g/100 ml glucose and 2.5 g/100 ml fructose, 51% of the sucrose was transformed. It was expected that the extent of inhibition would be larger in the medium initially containing both glucose and fructose than in the medium containing no fructose. The opposite was observed; however, the results obtained in the two systems are very similar.

According to Combes and Monsan (1983), who studied the inhibition of baker's yeast invertase (Sigma Chem. Co., grade VI) by glucose, fructose and sucrose, D-fructose shows the behaviour of a competitive inhibitor of invertase while D-glucose is a partial noncompetitive inhibitor. Their results are in agreement with the ones presented in this work, indicating that fructose is a stronger inhibitor than glucose.

5.2 Sucrose Inversion in Packed-Bed Reactors

When continuously inverting sucrose with the first packed-bed bioreactor (Figures 17, 18 and 19), a gradual decrease in the efficiency of the bioreactor was

observed, as soon as it was started. This could be due to invertase inactivation or to leakage of the enzyme from the beads. The amount of nitrogen per bead dropped from 63 μg to 8 μg during the 2 months of packed-bed operation. This decrease in nitrogen probably reflects the washing of cross-linking agents (polyethyleneimine and glutaraldehyde) from the beads as well as a loss of invertase and other proteins.

Sharp decreases in bioreactor efficiency occured when the feed sucrose concentration was lowered. Changes in the osmotic pressure inside the beads, caused by these drops in the bulk concentration, probably resulted in a modification of the calcium alginate gel and in subsequent leakage of the enzyme from the beads.

Variations in the pH of the effluent can be explained by the fact that the pH of the distilled water used to prepare the feed sucrose solution, was not constant. Therefore, the pH of the feed sucrose solution also varied from time to time.

The small discrepancies between the glucose and fructose concentrations (the concentrations of glucose and fructose should be equal at all times) were probably due to HPLC inaccuracy.

The second invertase bioreactor was used with a higher feed sucrose concentration than the first one (30 g/100 ml instead of 20, 10 and 5 g/100 ml). Its efficiency remained constant for over 600 hours (Figures 20 and 21). The beads used in this bioreactor were not identical to the ones packed in the first bioreactor, having been prepared in a different batch; they probably allowed less invertase to leak, which could explain the maintained efficiency of the second reactor compared to the first one. In 48 days of operation, the amount of nitrogen per bead dropped from 80 μg to 16 μg. However, it was not known which fraction of the nitrogen invertase represented. Beads from the second batch remained more active than beads from the first batch, even though the loss of nitrogen was similar. Another factor that could have helped to maintain the efficiency was the use of a more concentrated sucrose solution (30 g/100 ml instead of 20 g/100 ml), which increased the half-life of the enzyme. Combes and Monsan (1983) reported that the sucrose concentration has a very important effect on the stability of immobilized invertase, when using a packed-bed reactor. They immobilized invertase (Maxinvert 200,000, Rapidase)

to corn grits by covalent coupling and used the preparation in a packed-bed reactor, at 60°C. They observed a ten-fold increase in the invertase half-life as the concentration of sucrose was changed from 51% (w/v) to 85% (w/v).

Linko *et al.* (1980b) also used calcium alginate as a carrier for invertase; they entrapped freeze-dried *S. cerevisiae* cells in calcium alginate beads. These beads were treated with glutaraldehyde and used for continuous sucrose inversion. A 50% (w/w) sucrose solution was fed to a reactor, at a dilution rate of 0.17 h^{-1}; the temperature of the reactor was 40°C and the pH of the solution 4.8. More than 90% of the sucrose was converted for over 60 days. In comparison, the second invertase bioreactor mentionned in this work inverted 85% to 88% of the sucrose contained in a 30% (w/v) sucrose solution, at a dilution rate of 0.18 h^{-1} and a temperature of 55°C; this level of conversion was maintained for over 29 days.

Unfortunately, Linko *et al.* (1980 b) did not give any details about the type of reactor they used; no investigation concerning optimum temperature and optimum pH for immobilized invertase activity or stability is mentionned in their article.

While using the two invertase bioreactors, it was observed that the calcium alginate beads progressively shrank to almost half of their original size, probably because of the high osmotic pressure of their environment; they also got darker with time. They tended to form clusters in the bed, probably because of electrostatic forces among them. The beads lost part of their mechanical stability during reactor operation, as was noticed by touching them when the packed-bed reactors were disassembled. Linko *et al.* (1980 b) did not report any observations of this kind about their beads; no other article about invertase immobilized in calcium alginate was found in the literature.

5.3 Growth of the Ethanol Producing Yeast

The growth of the ethanol-producing yeast was studied before ethanol production was undertaken. Results concerning the influence of temperature, ethanol concentration and sugar concentration were collected.

5.3.1 Tests of Growth of the Ethanol-Producing Yeast on Various Carbohydrates

According to Lobo and Maitra (1977), *S. cerevisiae ATCC 36859* was able to grow on glucose and on sucrose but not on fructose. Results from the work carried out here are in partial disagreement with this, revealing that, of the three sugars mentionned above, only glucose was able to support growth. This can be seen from Table 4. Glucose was the only sugar used by the yeast under aerobic conditions. However, the most important aspect was the fact that the yeast was able to use glucose without consuming fructose.

5.3.2 Effect of Temperature on Growth

In this study, it was determined that the highest specific growth rate for *S. cerevisiae ATCC 36859* in batch cultures occurred at a temperature of 33°C. This was also the temperature at which the largest amount of biomass was formed in batch cultures.

This result is in agreement with Jones *et al.* (1981) who reported that mesophilic strains of *Saccharomyces* exhibit optimum cell yields and growth rates in the range 28–35°C, while 40°C is the maximum temperature at which growth occurs. The temperature at which the rate of fermentation by *Saccharomyces* strains is the highest has been shown to be 5 to 10°C higher than the optimum for growth. In batch processing however, the optimum temperature for complete exhaustion of glucose and for the highest final ethanol concentration is generally slightly below the optimum temperature for growth (Jones *et al.* , 1981). This is accounted for by the enhanced ethanol inhibition at the higher temperatures. The effect has been attributed to a higher intracellular ethanol concentration, ethanol being produced more rapidly than it can be transported through the cell membrane, causing inactivation of alcohol hydrogenase, inhibition of other enzymes and ultimately death.

Considering the effect of ethanol inhibition, a temperature of 30°C was selected for batch fermentations.

5.3.3 Effect of Glucose Concentration on Growth and Ethanol Production

The concentration of substrate in the medium plays an important role in a fermentation. The advantages of high fermentables concentration in the feed include reduced dilution water requirements, the suppression of osmosensitive contaminants and the potential of reduced distillation costs if ethanol inhibition can be limited. However, high substrate concentrations cause inhibition of the cells activity (Jones *et al.* , 1981).

From the results shown in Figure 24, it can be seen that the biomass production increased with the glucose concentration in the medium up to 15 g/100 ml, and then diminished with a further increase in the glucose concentration. The biomass yield on glucose (Table 5) was almost constant for glucose concentrations smaller or equal to 15 g/100 ml but went down with a further increase in glucose concentration.

Looking at Table 6 it can be seen that, other conditions being the same, the specific growth rate of the yeast went down with a rise in sugar concentration. For ethanol production in glucose media, the specific growth rate dropped from 0.13 h^{-1} to 0.077 h^{-1} as the initial glucose concentration passed from 4.6 g/100 ml to 25.3 g/100 ml. A similar effect was observed with the glucose + fructose media: as the initial concentration of each sugar was increased from about 7.5 g/100 ml to about 20 g/100 ml, the specific growth rate of the yeast dropped from 0.12 h^{-1} to 0.034 h^{-1}. The biomass productivity (P4; grams of biomass produced per gram of glucose used per hour) also decreased with a rise in glucose concentration, whether ethanol was produced in a glucose media or in a glucose + fructose media.

Ethanol production was also inhibited by high sugar concentrations. The volumetric ethanol productivity (P3) increased with a rise in the initial sugar concentration (glucose or glucose + fructose) up to a 15 g/100 ml sugar concentration and then started to decrease with a further rise in the sugar concentration (Table 6).

According to Jones *et al.* (1981), this effect is related to osmotic phenomena in which plasmolysis of the yeast cell begins to occur at sugar concentrations above 14% (w/v), although the exact figure is strain dependent. This is in agreement with

the results obtained in this work, showing that sugar becomes inhibitory to biomass production and ethanol production at concentrations above 15% (w/v). Jones *et al.* (1981) also reported that at concentrations above 14% (w/v) sugar, it was found that the initial rate of fermentation (i.e. before the ethanol concentration had built up) started to decline.

5.3.4 Effect of Ethanol Concentration on Growth

The primary limiting factor in ethanol production is the effect of ethanol on yeast growth and fermentation; as the ethanol concentration increases, a decrease in growth rate is the initial manifestation of this inhibition. The growth of the yeast used in this work, *S. cerevisiae ATCC 96859*, was found to be completely inhibited by a 10 g/100 ml ethanol concentration (Figure 25). This result is in agreement with the findings of Jones *et al.* (1981) who reported that tolerance to high concentrations of ethanol is strain dependent with a maximum allowable concentration of around 10% (w/v) for growth and 20% (w/v) for ethanol production being exhibited in most tolerant strains.

Hoppe and Hansford (1982) studied the inhibitory effect of ethanol on the yeast *S. cerevisiae ATCC 4126* in continuous cultures. They concluded that ethanol added extracellularly is not as strongly inhibitory as ethanol produced by the yeast itself.

The general effect of ethanol inhibition is most strongly observed on the cell membrane and it has been postulated (Jones *et al.* , 1981) that it is membrane damage or change in membrane properties that would be the major toxic effect. The degree of inhibition is also related to other environmental factors; in particular, high sugar concentrations and high temperatures cause the inhibition to be more severe.

5.4 Batch Ethanol Production

Ethanol was produced in batch systems and a reasonable volumetric productivity was obtained for the processes in which the initial concentration of biomass was relatively high. For example, when working with a medium containing 14.3 g/100 ml glucose + 15.3 g/100 ml fructose, and an initial biomass concentration of 0.78 g/100 ml, an ethanol productivity of 3.2 grams per litre of reactor volume per hour was obtained (Table 6). In comparison, Kolot (1980) reported an ethanol productivity of 2.2 g/(L.h) for batch ethanol production by yeasts in medium initially containing 10% sugar and 0.56 g/100 ml biomass. Tyagi and Ghose (1982) reported a 2.0 g/(L.h) ethanol productivity for batch fermentation of molasses by *S. cerevisiae NRRL-Y-132*. Therefore, it seems that the ability of the mutant yeast *S. cerevisiae ATCC 96859* to produce ethanol is comparable to the ability of other yeasts used for ethanol production.

5.4.1 Production of Ethanol in Glucose Media

The effects on the fermentation process of increasing the sugar concentration in the medium have been discussed in subsection 5.3.3.

The tests of ethanol production done in glucose media (Figures 26 to 29) showed that the yeast was able to convert the monosaccharide to ethanol efficiently, high yields of conversion being attained (Table 6).

During all the tests, the pH decreased as glucose was being consumed. The pH was not controlled because its effect on ethanol production rate is small. The absolute limits of pH for growth of most strains of *S. cerevisiae* have been reported to be 2.4 and 8.6, with an optimum for growth of 4.5 (Jones *et al.* , 1981). Also, sugar fermentation rates of yeasts have been found to be relatively insensitive to pH values between 3.5 and 6.

When producing ethanol in a 25.3 g/100 ml glucose medium (Figure 29), it was observed that the biomass concentration started to decrease after about 100 hours. This decrease in the quantity of cells was probably due to inhibition of yeast growth by ethanol, causing some cells to die and be autolysed.

It was also observed, as the initial sugar concentration in the medium was increased, that the quantity of ethanol produced per amount of biomass present (P1 and P2 in Table 6) also increased. This phenomenon was probably due to the fact that yeast growth was affected to a greater extent than the ethanol production rate, by inhibition by the substrate and the products, at high sugar concentrations. Therefore, as the fermentations got longer, the growth rate of the cells diminished with time while the cells were still producing ethanol at a sustained rate.

Generally, the volumetric ethanol productivities of these tests were low (P3 in Table 6) because of the low initial biomass concentrations.

5.4.2 Production of Ethanol in Media with Glucose+Fructose Mixture

It is apparent, when comparing the batch fermentations carried out in the presence of fructose (Figures 30 to 33) with the ones in which it was absent, that although fructose was not consumed by the yeast, its presence was causing inhibition to yeast growth and ethanol production.

The effect of substrate inhibition on yeast growth and sugar fermentation rate was discussed in subsection 5.3.3: above 15% (w/v) total sugar, the volumetric ethanol productivity (P3) went down with an increase in the initial sugar concentration (Table 6).

The yield of conversion of glucose to ethanol remained above 90% of the theoretical value in this series of tests (Figures 30 to 33).

Looking at Table 6 and comparing the results of the fermentation done in 7.1 g/100 ml glucose + 7.5 g/100 ml fructose medium with the results obtained with the 14.9 g/100 ml glucose medium, two media containing about 15% (w/v) total sugar, it was noticed that the volumetric ethanol productivity (P3) was about the same for both tests. The ethanol productivity based on the amount of biomass present (P1) was higher for the fermentation in the presence of fructose, because less biomass was produced during this fermentation than during the one done in 14.9% (w/v) glucose medium. The biomass productivity (P4) was also higher for the fermentation done

in the presence of fructose than for the one in which it was absent (4.6×10^{-4} gram biomass produced per gram glucose consumed per hour compared to 1.4×10^{-4}). Doing a similar comparison between the results of the fermentation performed in a medium containing 12.3% (w/v) glucose + 13.0% (w/v) fructose and the results of the fermentation done in a 25.3% (w/v) glucose medium, two media containing 25.3% (w/v) total sugar, it was seen that, again, the biomass productivity (P4) was larger for the test done in the presence of fructose; the volumetric ethanol productivity (P3) was slightly lower in the presence of fructose. The ethanol productivity based on the amount of biomass present (P1) was smaller when fructose was contained in the medium, probably because of a larger biomass production compared to the fermentation done in glucose medium.

Ethanol was also produced in a batch system containing 15% (w/v) glucose + 16% (w/v) fructose. Comparing results of this fermentation with results of the fermentation done in the medium containing 14.9% (w/v) glucose but no fructose, reveals the inhibitory effect of fructose on yeast growth and ethanol production rate: the fermentation in the presence of fructose (Figure 32) lasted 157 hours while the one in which fructose was absent (Figure 28) was over after 125 hours.

Fermentation of a 19.3% (w/v) glucose + 20.8% (w/v) fructose was attempted in order to find out if the yeast could withstand such a high level of carbohydrates (40.1 g/100 ml). The results indicated that indeed, the yeast could transform glucose into ethanol even at sugar concentrations as high as 40% (w/v). However, the volumetric ethanol productivity (P3) was low, 0.034 g/(L.h), and the fermentation lasted ten days which is excessively long.

5.4.3 Effect of Aeration on Ethanol Production

As can be seen from Figures 34 and 35 representing ethanol production in 7.2% (w/v) glucose + 7.6% (w/v) fructose medium and ethanol production in 14.2% (w/v) + 15.0% (w/v) fructose medium respectively, aeration had an adverse effect on ethanol production. As a matter of fact, less ethanol was produced when air was supplied under the conditions described in section 3.5.5 than when identical media were used in non aerated systems.

Looking at Table 6 and comparing the results for tests done with and without aeration, it was found that in the presence of aeration, the specific growth rate of the yeast was higher but the volumetric ethanol productivity was much lower. When air was supplied to the media, low ethanol concentrations were obtained and the conversion of glucose to ethanol was only around 50% of the theoretical value. In both tests done with aeration, the ethanol concentration started to decrease before the glucose was completely exhausted, the yeast using ethanol as a carbon source.

Yeasts are unable to grow for more than 4–5 generations under fully anaerobic conditions because of the developped shortages of lipids, unsaturated fatty acids and sterols (Jones *et al.* , 1981). This explains the higher biomass concentrations obtained when air was supplied to the fermentor. Because of the effect of dissolved oxygen on long term yeast viability, maintenance of a finite dissolved oxygen concentration is likely to be of importance in industrial continuous fermentations (Jones *et al.* , 1981).

One of the major problems in attaining high ethanol concentration in the final broth has been yeast viability. As the alcohol concentration increases, the viability of cells decreases and hence productivity is lowered. Trace amount of oxygen, if present in the medium, can help to retain the viability of cells and thus the productivity of ethanol (Tyagi, 1984). However, the presence of oxygen tends to promote the Pasteur effect, which is the complete oxidation of glucose to CO_2 and H_2O via the tricarboxylic acid cycle and respiratory chain at the expense of ethanol accumulation via fermentation (Jones *et al.* , 1981). This probably happened in the two experiments described above. As can be seen from Table 6, the yield of conversion of glucose to ethanol was low for the two tests done in aerated systems.

It has also been reported (Tyagi, 1984) that excessive aeration has been found to lead to diminished yeast crop and increased flocculence, and that an oxygen atmosphere in the head space above a culture medium has been found to inhibit yeast growth.

Since ethanol production was not enhanced for the two tests done in aerated systems, it can be concluded that the aeration rate was not adequate: it was excessive. In order to benefit from the presence of dissolved oxygen in the medium, the

flow rate of air to the fermentor should be diminished or aeration should be done intermittently, to keep the dissolved oxygen concentration lower.

5.4.4 Effect of Inoculum Size on Ethanol Production

The ethanol productivity of a batch system can be increased by using a larger quantity of cells.

The fermentations represented in Figures 36 and 37 were realized with inocula 30 times larger than the inocula previously used for ethanol production in similar media. As can be seen from Table 6, for the fermentation done in a 14.3 g/100 ml glucose + 15.3 g/100 ml fructose medium, the ethanol productivity was increased by more than 7 times when a larger inoculum was used, and the time needed to convert all the glucose to ethanol dropped from 157 hours to 23 hours. A similar improvement was realized for the fermentation in 18.3% (w/v) glucose + 19.9% (w/v) fructose medium. By increasing the size of the inoculum, the ethanol productivity (P3) was raised from 0.034 g/(L.h) to 0.22 g/(L.h) and the fermentation lasted 42 hours instead of 244 hours.

5.5 Continuous Ethanol Production

Having studied batch ethanol production with free cells, experiments were conducted on the continuous production of ethanol with yeast cells immobilized in calcium alginate beads. The active beads were packed in column reactors; the volumetric ethanol productivities of the bioreactors were easily maintained.

A temperature of 30°C was selected for continuous ethanol production in packed-bed reactors. This was also the temperature selected for batch ethanol production by free yeast cells. According to Cho *et al.* (1982) who immobilized *S. cerevisiae NRRL Y-132* cells in calcium alginate beads, the yeast demonstrated maximum activity at 30°C. Williams and Munnecke (1981) reported the same conclusion: the ethanol production rate for *S. cerevisiae* cells immobilized in calcium alginate beads was 30°C.

5.5.1 Effect of Dilution Rate on Ethanol Production

The volumetric ethanol productivity of the packed-bed bioreactors increased with the dilution rate until a maximum was reached and then went down with a further increase in dilution rate. This can be seen on Figures 39 and 41. With the medium containing glucose and fructose in 10 g/100 ml concentrations, a maximum productivity of 16.8 grams ethanol per litre of reactor volume per hour was obtained at a flow rate of 0.73 h^{-1} while for the medium containing 15 g/100 ml of the two hexose sugars, a maximum ethanol productivity of 15 g/(L.h) was reached at a dilution rate of 0.65 h^{-1}.

Several researchers have produced ethanol with reactors packed with entrapped cells (Table 3). The ethanol productivity can be calculated on many bases; the basis of calculation for this work was the total volume of the reactor bed.

Kierstan and Bucke (1977) entrapped *S. cerevisiae* cells in calcium alginate fibers and produced ethanol from a 10% (w/v) glucose medium. They obtained a maximum ethanol productivity of 4.3 g/(L.h), based on the total volume of the reactor.

Williams and Munnecke (1981) have immobilized *S. cerevisiae* cells in calcium alginate beads to produce ethanol from a 12.7% (w/v) glucose medium. They obtained an ethanol productivity of 53.8 g/(L.h), based on the liquid volume (or void volume) of the reactor. The void volume of the reactor representing a fraction of its total volume, much higher ethanol productivity values result when the calculations are based on the liquid volume (or void volume) than when they are based on the total volume.

Linko and Linko (1981), also employed *S. cerevisiae* cells entrapped in calcium alginate beads to produce ethanol from molasses containing about 17.5% (w/v) sugar. The highest ethanol productivity reached was 21.3 g/(L.h); however, the basis of calculation was not reported.

Lee *et al.* (1982) immobilized *S. cerevisiae* cells in calcium alginate beads and produced ethanol continuously from media containing different glucose concentrations. They obtained a maximum ethanol productivity of 49.6 g/(L.h), based on the void volume (or liquid volume) of the reactor when using a 10 g/100 ml glucose medium.

Some researchers have immobilized yeast cells in carrageenan gel. Wada *et al.* (1979) entrapped *S. cerevisiae* cells in carrageenan beads in order to produce ethanol from a 10 g/100 ml glucose medium. When the beads were packed in a reactor, an ethanol productivity of 43 g/(L.h) was obtained, based on the volume occupied by the beads, which is a fraction of the reactor volume.

Ethanol is not only produced by yeast cells; bacteria have also been employed for ethanol production: *Zymomonas mobilis* is an example. Margaritis *et al.* (1981) immobilized *Z. mobilis ATCC 10988* cells in calcium alginate beads. The beads were packed in a column and used to produce ethanol from a 10% (w/v) glucose medium. A maximum ethanol productivity of 102 g/(L.h) was obtained, based on the void volume of the reactor.

It is not easy to compare the ethanol productivities obtained by different researchers for different systems, since the basis for calculations are often different. The volume occupied by beads in an operating bioreactor is not a constant: it changes with time and therefore the void volume changes too. Moreover, the void fraction of a bed can vary a great deal from one system to another. Therefore, when reporting productivity values, the liquid volume (or void volume) and the volume occupied by the entrapped preparation, do not seem to be good bases for calculations.

Another point that should be considered when comparing the ethanol productivities obtained in this work with results from the literature is the presence of fructose in the medium. Although fructose did not contribute to ethanol production, its presence caused inhibition to the fermentation and therefore lowered the ethanol productivity of the reactor.

As mentionned in subsection 4.5.1, during reactor operation, the beads swelled due to yeast growth, swelling being more pronounced at the bottom of the bed than at the top, the medium being richer in nutrients at the bottom. After a few days, the size of the beads had increased by 2–3 times and they were very tightly packed in the bed. They were so compressed that they were not spherical anymore. This phenomenon is rarely reported in the literature, although it is responsible for a variation in the void fraction of the bed, and therefore an alteration of the

productivity values based on the void volume of the reactor bed.

The CO_2 produced by yeast cells formed void spaces inside the beads and many beads cracked. There was biomass formation on the wall of the bioreactor, near the bottom of the bed, indicating that some cells were leaking from the beads.

5.5.2 Effect of Storage on Immobilized Yeast Cells

Yeast cells entrapped in calcium alginate gel were succesfully stored, and used for ethanol production 2 months later. The number of beads packed in the reactor bed (2300) represented only 56% of the number of beads packed in the first ethanol-producing bioreactor (4100). The number of beads was reduced in order to prevent problems associated with compression of the beads which could cause clogging of the effluent tubing by broken beads and exert pressure on the two grids retaining the beads. However, the bed was not completely occupied by beads, the swelling of the beads having been overestimated.

The biorector was fed with a 15 g/100 ml glucose + 15 g/100 ml fructose medium and, at a dilution rate of 0.16 h^{-1}, an ethanol productivity of 7.2 g/(L.h) was obtained. Previously, an ethanol productivity of 9.8 g/(L.h) had been reached, under similar conditions but with a different number of beads, for a dilution rate of 0.15 h^{-1}.

The activity of the yeast cells was preserved during storage; the productivity of the second reactor was 73% of the value obtained for the first one, even though it contained only 56% as many beads.

5.5.3 Increase of the Fructose/Glucose Ratio in Invert Sugar Media

A third ethanol-producing bioreactor was prepared, with calcium alginate beads from a second batch. The purpose of this packed-bed reactor was to convert the effluent of the invertase bioreactor, that is to transform the glucose of the invert sugar medium into ethanol and CO_2.

Before being linked to the continuous process set-up, its efficiency was measured

using a 15% (w/v) glucose + 15% (w/v) fructose medium. At a dilution rate of 0.15 h^{-1}, it had an ethanol productivity of 8.4 g/(L.h). Containing 3100 beads, that is 76% of the number of beads packed in the first bioreactor, it had 86% of its productivity.

5.6 Conversion of Sucrose into Fructose

The continuous conversion of sucrose into fructose and ethanol was realized with a series of packed-bed bioreactors (Figures 44 to 47). A high degree of conversion was desired for both parts of the process since one objective was to obtain a final medium containing as little sucrose and glucose as possible. For this reason, a second ethanol-producing bioreactor was added in series with the first one, 262 hours after process start-up in order to increase the conversion of glucose to ethanol. This additonal reactor performed well for a few days, after which its efficiency became low. This probably occured because the feed stream of this reactor, being the effluent of the first ethanol-producing bioreactor, was low in nutrients and contained ethanol in relatively high concentration (about 4.5 g/100 ml); this medium did not keep the yeast cells in an active state.

Concerning sucrose inversion, it was decided that the invertase bioreactor needed to be replaced because the residual sucrose concentration was high (above 7 g/100 ml). The invertase preparation had lost activity, probably because of enzyme leakage or inactivation caused by the high temperature (55°C). Therefore, the invertase packed-bed reactor was replaced by a similar one, packed with fresh invertase-containing beads.

As can be seen from Figures 44 to 47, the system used to transform sucrose into fructose and ethanol took a certain time to react to changes in flow rate. Also, following the addition or the replacement of a bioreactor, the system took a few days to stabilize, the beads needing some time to equilibrate their osmotic pressure with the environment.

It was noticed, after about 300 hours of operation, that the fructose concentration in the effluent stream was lower than what would be expected from the amount

of sucrose inverted. Knowing that one mole of sucrose inverted yields one mole of glucose and one mole of fructose and doing a mass balance on sucrose, glucose, fructose and ethanol, revealed that some fructose was being converted into ethanol. This phenomenon could be explained by either the presence of a contaminant in the system, able to convert fructose into ethanol, or more probably by a mutation of the ethanol-producing yeast that would enable it to use fructose as a carbon source. According to Lobo and Maitra (1977) who isolated the mutant yeast lacking hexokinase activity, *S. cerevisiae ATCC 96859*, the two mutations leading to its creation are recessive and noncomplementing. Therefore, it seems possible that the yeast cells started to revert to their original state, after having been entrapped in calcium alginate for a long time.

Other researchers have studied the possibility of producing fructose from sucrose and high-fructose syrups. Ueng *et al.* (1982) used a method of fructose production similar to the one used in this work: differential utilization of glucose by mycelial fungi in alcoholic fermentation of a mixture of glucose and fructose. They worked with two microorganisms: *Mucor sp. M105* and *Fusarium sp. F5*. Glucose utilization was faster than fructose utilization. The authors did not attempt continuous fructose production.

Bringer-Meyer *et al.* (1985) derived a mutant from the strain *Zymomonas mobilis ATCC 29191*; this mutant was blocked in fructose utilization for growth and ethanol production but was able to form sorbitol from the fructose present in the medium.

Two other mutants of *Z. mobilis*, unable to utilize fructose as a sole source of carbon and energy were derived by Suntinanalert *et al.* (1986). However, sorbitol was produced from fructose in this case also.

No other methods of fructose isolation using selective fermentation have been found in the literature.

Chapter 6

Conclusions and Recommendations

6.1 Conclusions

The main conclusions of this research project are given below:

- The optimum temperature for the activity of a crude invertase preparation immobilized in calcium alginate beads was 55°C. The activity was found to slowly increase when the temperature was raised from 45°C to 55°C, and to decrease quickly when the temperature was raised from 55°C to 65°C.

- The activity of the immobilized invertase preparation was good in the pH range 3.5–5.6, with its maximum value occurring at pH 4.5.

- Invertase was inhibited by the end products of the sucrose inversion reaction: glucose and fructose. Fructose caused more inhibition than glucose. The amount of sucrose inverted was found to be inversely proportional to the amount of end products added to the sucrose solution, for end products concentrations smaller or equal to 10 g/100 ml.

- Sucrose inversion was carried out continuously by using invertase-active calcium alginate beads packed in a column reactor. The highest inversion rate

measured was 60 grams of sucrose per litre of reactor volume per hour; it was obtained using a 20 g/100 ml sucrose solution, at a dilution rate of 0.33 h^{-1}. The efficiency of the reactor was found to decrease progressively with time and sharply with sucrose concentration changes in the feed. The invertase preparation was more stable when used with a 30% (w/v) sucrose solution than with a 20% (w/v) sucrose solution.

- *Saccharomyces cerevisiae ATCC 96859*, was unable to use fructose and sucrose for growth or ethanol production; glucose was the only tested sugar consumed.
- The optimum temperature for growth of the free ethanol-producing yeast cells was found to be 33°C. The specific growth rate of the yeast also reached a maximum at this temperature.
- The growth and the ethanol production capability of the yeast were partially inhibited by sugar, when its concentration was above 15 g/100 ml. The specific growth rate of the yeast decreased with an increase in the sugar concentration.
- Ethanol inhibited yeast growth and decreased fermentation rate of the yeast. At an ethanol concentration equal to or higher than 10% (w/v), no growth was observed.
- *S.cerevisiae ATCC 96859* was able to convert glucose to ethanol efficiently; high yields of conversion were reached.
- During batch fermentation in glucose + fructose medium, the yeast converted glucose to ethanol without consuming fructose. However, the presence of fructose caused inhibition to yeast growth and ethanol production. The ethanol-producing yeast was able to ferment glucose in a medium in which the total sugar concentration was as high as 40% (w/v), but the effect of substrate inhibition at this concentration reduced the fermentation rate considerably.
- Supplying large quantities of air to the fermentation medium had a positive effect on yeast growth and a negative effect on ethanol production rate in batch fermentations. The conversion of glucose to ethanol in aerated systems was

only around 50% of the theoretical value. It was concluded that the aeration rate employed was excessive and that the Pasteur Effect was promoted.

- Increasing the initial concentration of biomass in a batch reactor raised its volumetric productivity. The ability of *S. cerevisiae ATCC 36859* to ferment glucose into ethanol was comparable to the ability of other yeast strains used for ethanol production.

- Ethanol was produced continuously in packed-bed reactors. A maximum volumetric ethanol productivity of 16.8 g/(L.h) was obtained with a medium containing 10 g/100 ml glucose and 10 g/100 ml fructose, at a dilution rate of 0.73 h^{-1}. When the concentration of the two hexose sugars was raised to 15 g/100 ml, the highest volumetric ethanol productivity reached was 15.0 g/(L.h), at a dilution rate of 0.65 h^{-1}.

- Yeast cells entrapped in calcium alginate beads were stored in a cold (0–5°C) $CaCl_2$ solution and retained their fermentation capabilities for 2 months.

- Fructose and ethanol were produced continuously from a sucrose solution and a concentrated nutrient broth by using two immobilized systems: sucrose inversion was realized in the first one and glucose fermentation in the second one. High degrees of conversion in both steps of the process were difficult to obtain at the same time. The highest conversions of substrate were obtained after 640 hours of process operation when the final medium contained 5.9 g/100 ml ethanol, 9.9 g/100 ml fructose, 3.0 g/100 ml glucose and 2.4 g/100 ml sucrose, at a dilution rate of 0.09 h^{-1}.

6.2 Recommendations

Based on the findings of this research project, the following recommendations are made:

- A more active invertase preparation could be used in order to increase the conversion of sucrose to invert sugar. Also, setting a lower temperature for

sucrose inversion would increase the stability of the invertase preparation and therefore its longevity by reducing the rate of denaturation of the enzyme. However, the activity of invertase would be lowered by a drop in temperature.

- *S. cerevisiae ATCC 96859*, the mutant yeast lacking hexokinase activity which was used for ethanol production, should be studied in more detail in order to determine if the mutations leading to its creation can be reverted during continuous fermentations.

- The composition of the nutrients broth needed to maintain the yeast cells in an active state for ethanol production should be optimized for the particular yeast strain used and for the operating conditions selected, in order to increase the ethanol productivity of the immobilized system.

- In this work, the most concentrated sugar media used for continuous production of fructose and ethanol contained 30% (w/v) sugar. Work with more concentrated sugar solutions should be attempted since the use of concentrated solutions would represent an advantage for the economics of the process, by reducing the costs involved in the purification of the final product.

Chapter 7

Nomenclature

d : diameter of bioreactor

d.w. : dry weight

D : dilution rate, (h^{-1})

F : flow rate, (L/h)

GC : gas chromatography

HFCS : high-fructose corn syrup

HFS : high-fructose syrup

HPLC : high performance liquid chromatography

L : length of bioreactor

P : effluent ethanol concentration, (g/L)

P1 : ethanol productivity (for time = 0 to the time the highest ethanol concentration was reached), $\frac{\text{g ethanol}}{(\text{g biomass}).(\text{h})}$

P2 : ethanol productivity (for time = 0 to time = 50 hours), $\frac{\text{g ethanol}}{(\text{g biomass}).(\text{h})}$

P3 : ethanol productivity (for time = 0 to the time the highest ethanol concentration was reached), $\frac{\text{g ethanol}}{(100\text{ ml medium}).(\text{h})}$

P4 : biomass productivity (for the whole length of time of the test), $\frac{\text{g biomass}}{(\text{g glucose}).(\text{h})}$

v : volume

V_B : solid bead volume, (L)

V_L : liquid (void) volume, (L)

V_T : total biorector volume, (L)

w : weight

X : biomass concentration on a dry basis, (g/100 ml)

$Y_{P/S}$: mass of product obtained per mass of substrate consumed, (g/g)

$Y_{X/S}$: biomass produced per mass of substrate consumed, (g/g)

Greek Symbols

$[\alpha]_D$: specific rotation, (degrees)

ε : liquid (void) volume fraction

μ : specific growth rate, (h^{-1})

π : 3.14159

τ : mean residence time of the liquid phase, (h)

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Appendix A

Biomass Measurement

The curve giving the percentage of light transmittance as a function of the dry weight of *S. cerevisiae ATCC 96859* cells is given on next page. This calibration curve was used to determine the biomass concentration of culture media.

The transmittance of the yeast cultures was measured with a Perkin-Elmer Coleman 295 Spectrophotometer, set at a wavelength of 650 nm.

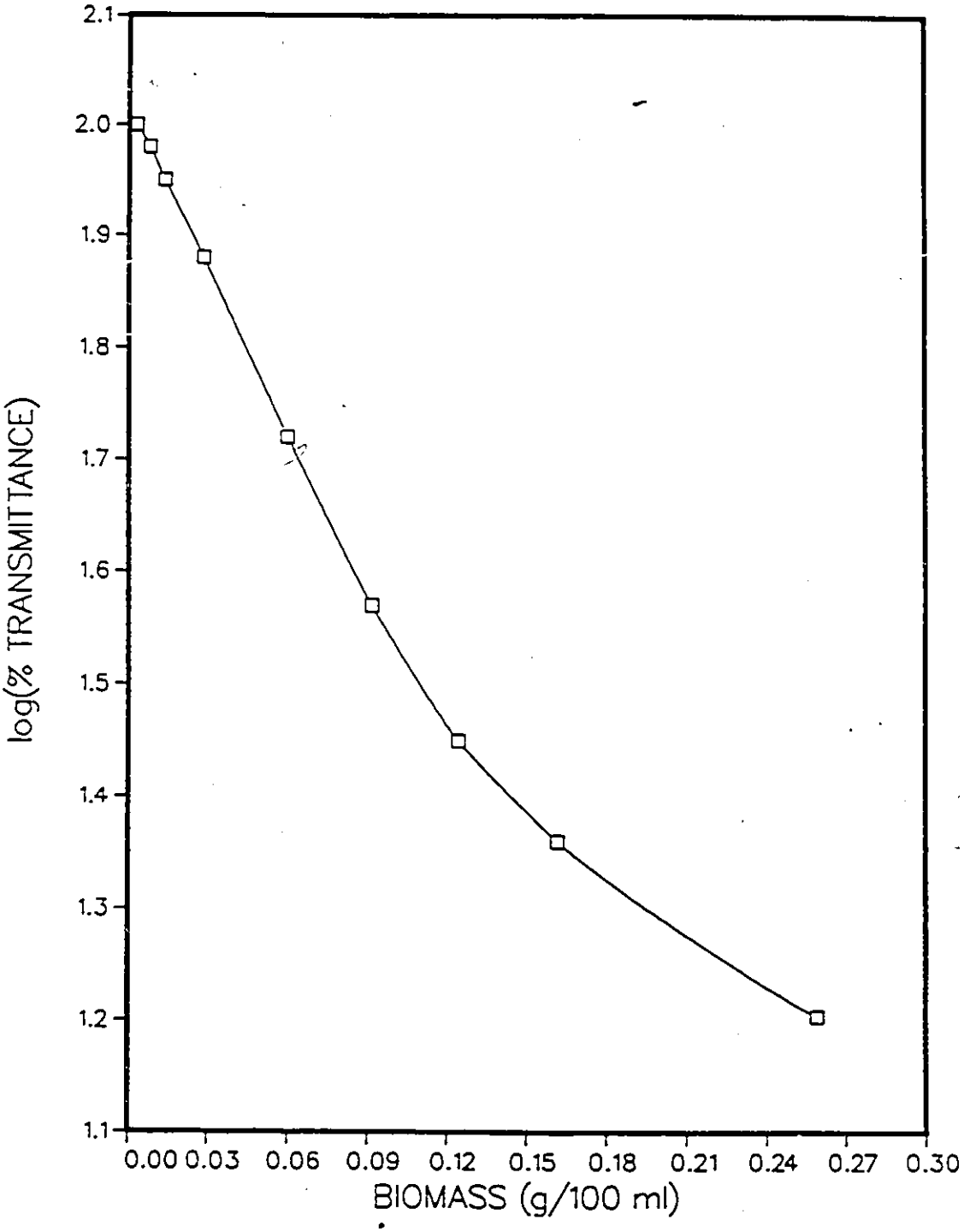

Figure 48: Percentage of light transmittance as a function of the biomass concentration (d.w.) for *Saccharomyces cerevisiae ATCC 36859.*

Appendix B

Specific Growth Rates

The specific growth rates of *Saccharomyces cerevisiae (ATCC 96859)* as a function of the temperature, represented in Figure 23, were evaluated graphically by measuring the slopes of the logarithmic phases of the growth curves obtained at different temperatures. The graphs that were used for this determination are represented in the next figures.

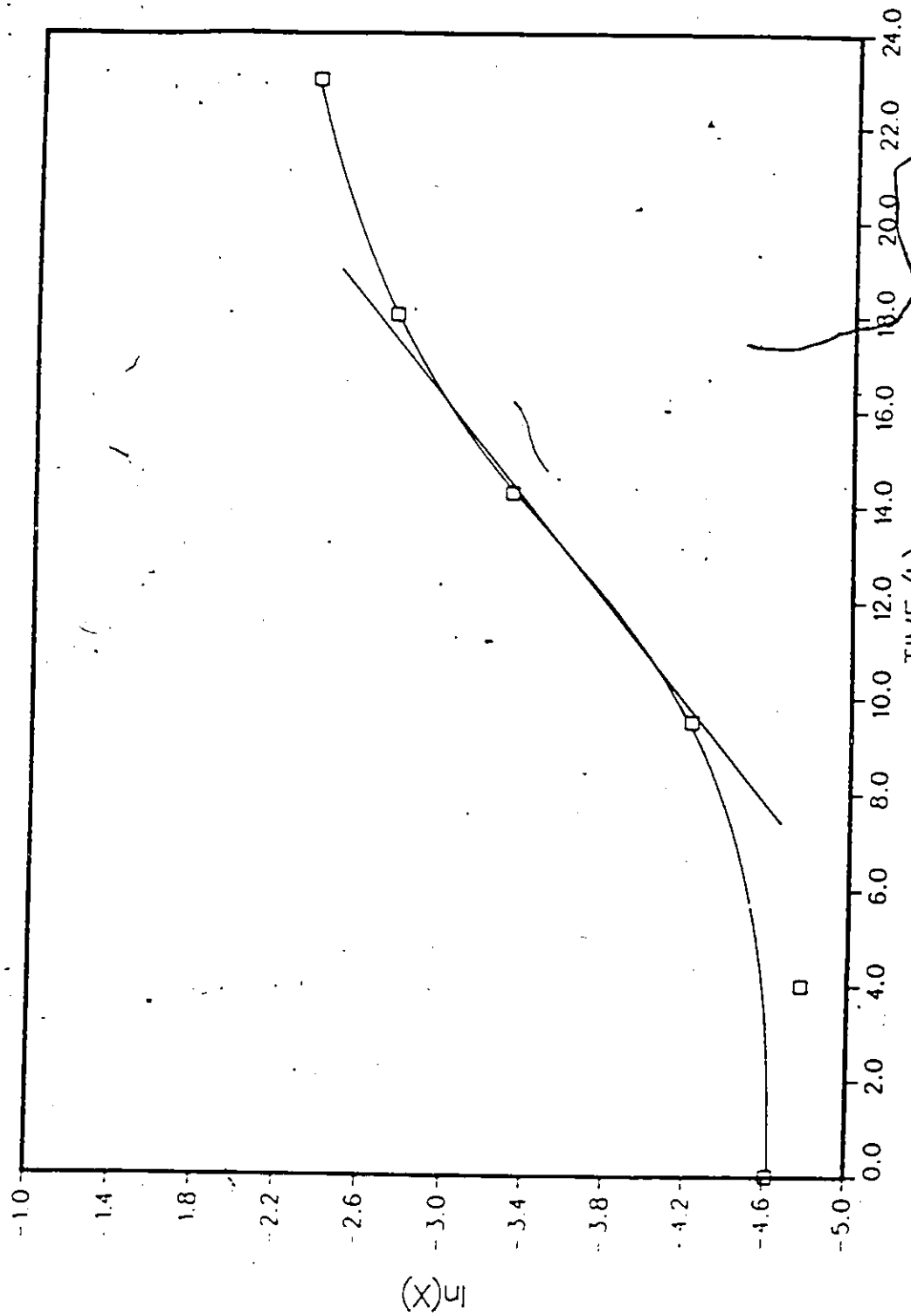

Figure 49: Logarithm of the biomass concentration as a function of time, for a temperature of 26°C.

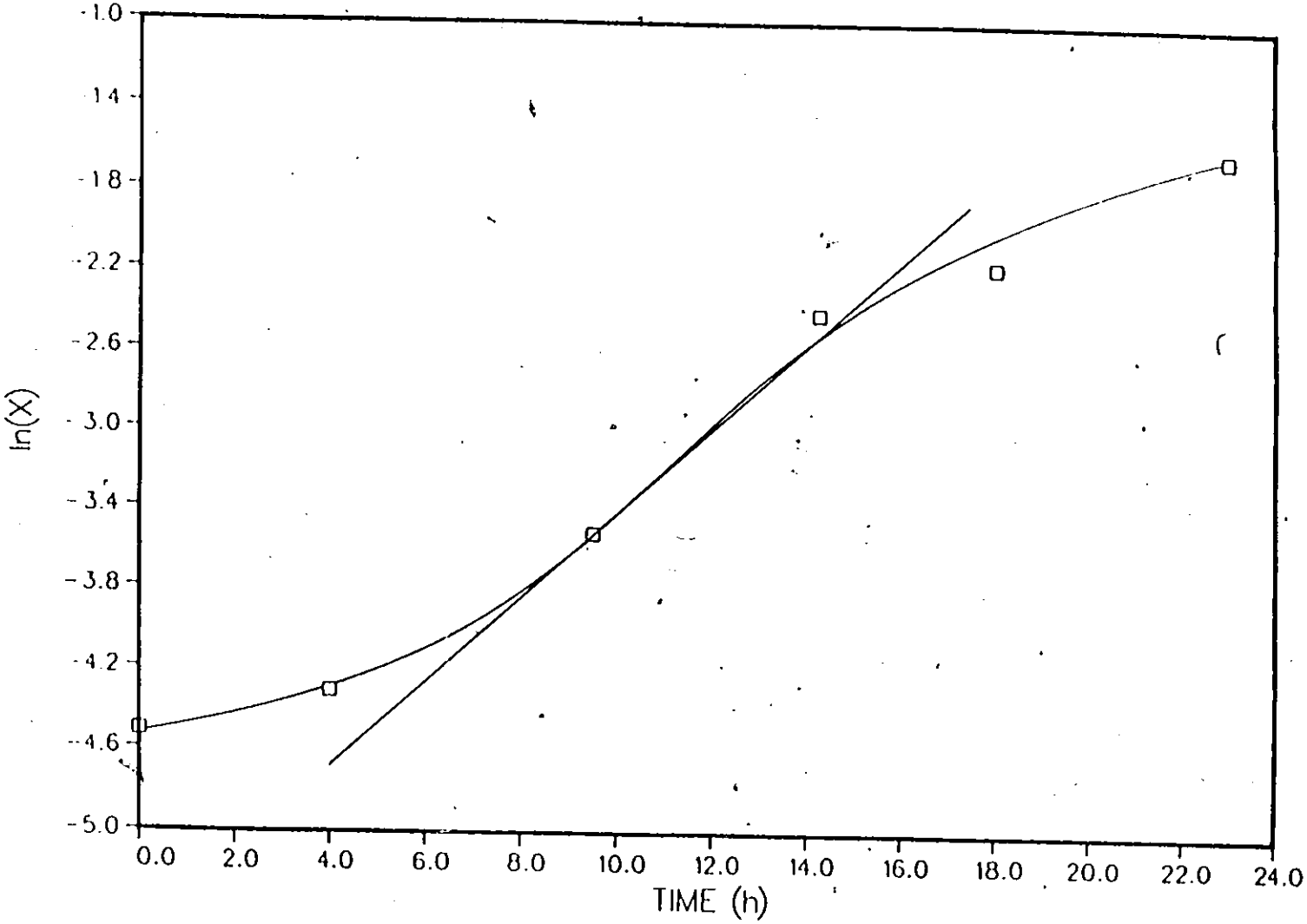

Figure 50: Logarithm of the biomass concentration as a function of time, for a temperature of 30°C.

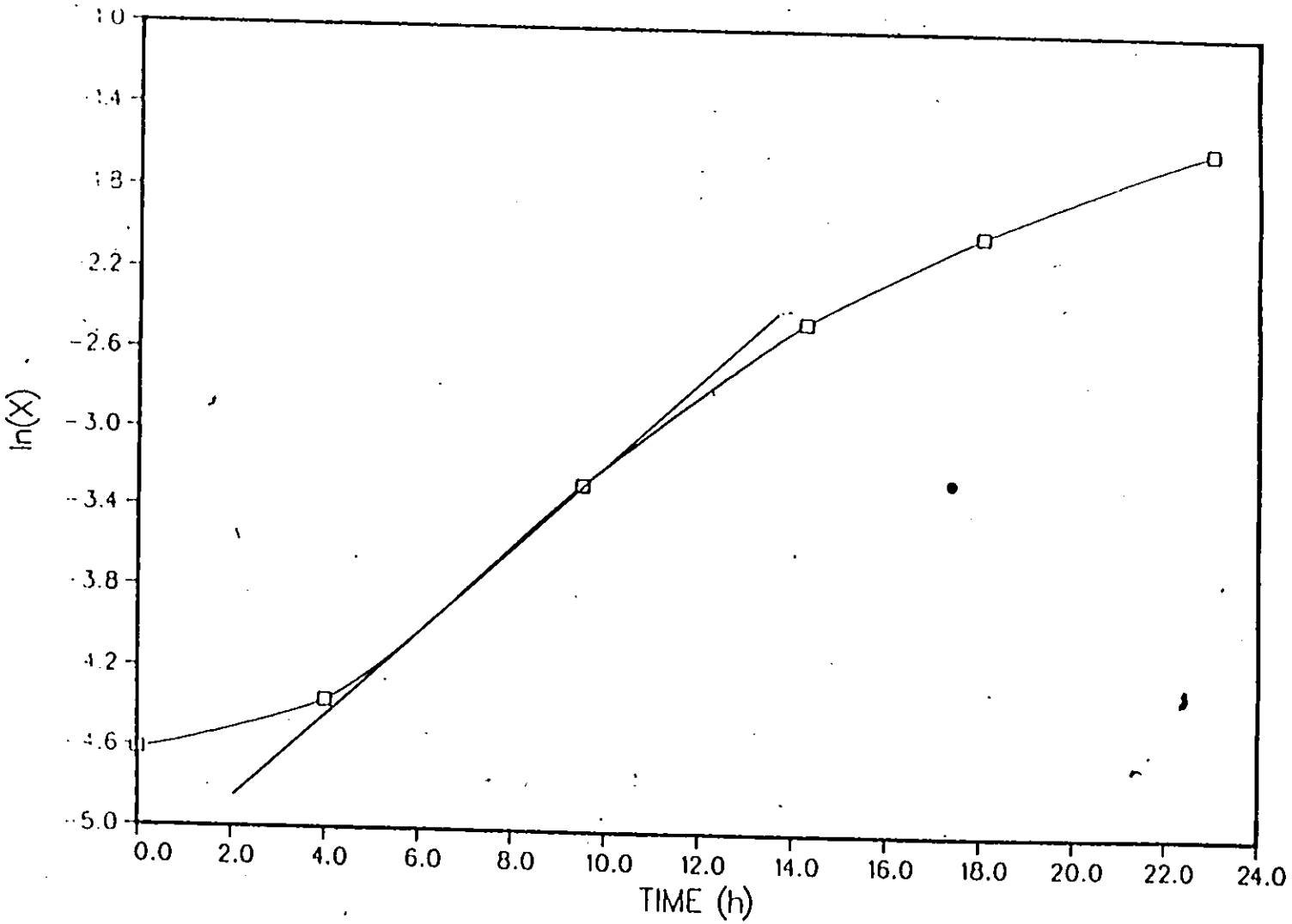

Figure 51: Logarithm of the biomass concentration as a function of time, for a temperature of 33°C.

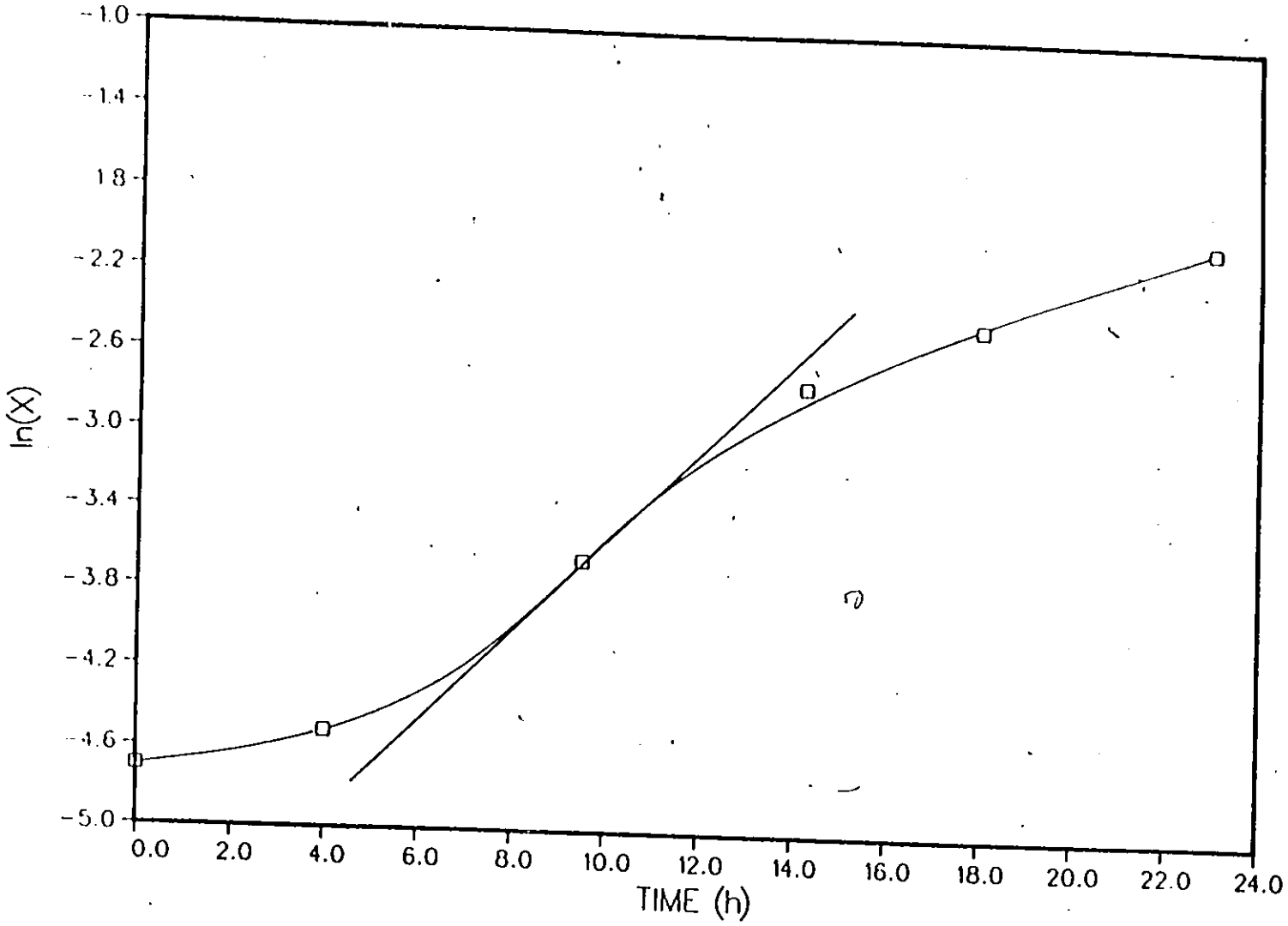

Figure 52: Logarithm of the biomass concentration as a function of time, for a temperature of 37°C.