CELL GROWTH AND ETHANOL PRODUCTION CHARACTERISTICS OF IMMOBILIZED *S.CEREVISIAE* IN A BATCH SYSTEM WITH NUTRIENT RECIRCULATION

by

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ABSTRACT

Recently, there has been world-wide interest in the production of ethanol by fermentation as an alternative to chemical feed stock and as a gasoline extender. Savings in utilities as well as reduction in fixed costs can be achieved if the sugar syrups can be fermented developing high ethanol concentrations. However, conventional ethanol production uses free cells and comparatively low substrate concentrations in order to ensure a speedy fermentation without inhibitory reactions by high sugar or high ethanol concentrations. Over the past few years, a new approach of great potential has been the use of immobilized cell systems to produce ethanol. Due to higher cell mass per unit fermenter volume and high rates of ethanol production in an immobilized cell system, it may be possible to achieve high ethanol concentration by fermenting solutions of high sugar concentrations.

In this research, a flocculating strain of *Saccharomyces cerevisiae* was entrapped in polymeric support particles having a complex void structure and a range of experiments were conducted in order to obtain kinetic data on the growth and the ethanol production characteristics in an immobilized cell reactor under batch operation with nutrient recirculation. The pH conditions, ethanol production rates, residual glucose concentrations and cell densities in the system were measured at different operation times and substrate concentrations.

Ethanol productivities, production rates, percent conversions and yields for four different substrate concentrations namely 6, 10, 12 and 14% glucose were compared to determine the optimum substrate concentration under these conditions and was found to be 10%.

The growth patterns in the immobilized cell reactor showed that the yeast cells enter the stationary phase in about 15 hours of operation after inoculation for the 6 and 10% glucose concentrations while longer periods are required to reach the stationary phase at 12 and 14% glucose concentrations.

The specific growth rates of the yeast cells in the exponential phase were found to be 0.200, 0.124, 0.079, and 0.088 1/h for 6, 10, 12 and 14% glucose concentrations respectively.

The operational stability of the system was investigated through experiments with repeated nutrient replacements and the system was found to be stable within 58 hours of operation .

ÖZET

Son yıllarda, kimyasal hammadde girdilerine seçenek ve yakıt katığı olarak, fermantasyon yoluyla etanol üretimi dünya çapında önem kazanmıştır. Yüksek şeker derişimlerinden, fermantasyon yoluyla yüksek etanol derişimleri elde edilebildiği takdirde, kaynak kullanımında ve sabit yatırımlarda tasarruf sağlanması beklenmektedir. Geleneksel etanol üretiminde ise, serbest hücreli ve düşük derişimli ortamlar kullanılarak yüksek şeker ve yüksek etanol derişimlerinin kısıtlayıcı etkilerini en aza indirerek hızlı fermantasyon gerçekleştirilmektedir. Son birkaç yıldır, geniş potansiyeli olan yeni bir yaklaşımla etanol üretiminde tutuklanmış hücre kullanımı gündeme gelmiştir.Tutuklanmış hücre sistemlerinde, birim hacimdeki hücre miktarının yoğun olması ve yüksek hızda etanol üretilmesi, yüksek şeker derişimlerinden yüksek etanol derişimlerinin üretilmesine olanak sağlamaktadır.

Bu çalışmada, yumaklaşan bir *Saccharomyces cerevisiae* türü karmaşık örgü yapısına sahip polimerik taşıyıcı parçacıkları üzerinde tutuklanarak, büyüme ve etanol üretim özellikleri üzerinde kinetik veri elde etmek üzere, besi ortamı dönüşümlü kesikli/geri beslemeli reaktörde bir dizi deney yapılmıştır. Sistemdeki pH durumu, etanol üretim hızı, kalan glikoz miktarı ve hücre yoğunluğu değişik zaman ve glikoz derişimlerinde ölçülmüştür.

Dört farklı glikoz derişiminin (%6, %10, %12 ve %14) , etanol üretkenliği, üretim hızı, yüzde dönüşümü ve çıkış akımındaki etanol derişiminin değişimi üzerindeki etkisi karşılaştırılarak incelenmiş ve bu şartlarda en iyi glikoz derişimi %10 olarak bulunmuştur.

Tutuklanmış hücre reaktöründen elde edilen büyüme çizelgeleri, maya hücreleri %6 ve %10'luk glikoz derişimlerinde kararlı evreye yaklaşık 15 saatten sonra ulaşırken, bu sürenin %12 ve %14'lük glikoz derişimleri için daha uzun olduğunu göstermiştir.

Maya hücrelerinin üssel evredeki spesifik büyüme hızları %6, %10, %12 ve %14 glikoz derişimleri için sırasıyla 0.200, 0.124, 0.079 ve 0.088 1/saat olarak bulunmuştur.

Sistemin çalışma kararlılığı da besi ortamının sürekli yenilendiği bir dizi deneyle incelenmiş ve 58 saate kadar kararlı davrandığı görülmüştür.

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BSP	:	Biomass Support Particle
K_s	:	Value of the Substrate at which the Maximum Specific Growth Rate is Halved (g/l)
s	:	Substrate Concentration (g/l)
T_{column}	:	Temperature of Column Reactor (°C)
T_{medium}	:	Temperature of Medium Reservoir (°C)
$Y_{p/s}$	:	Ethanol Yield (g ethanol/g glucose)
$Y_{p/x}$	:	Ethanol Yield to Biomass Yield
$Y_{x/s}$	:	Overall Yield Factor (g biomass/g sustrate)
x	:	Mass of Yeast Cells (g/l)
μ_r	:	Specific Growth Rate (1/h)
μ_{max}	:	Maximum Specific Growth Rate (1/h)

I. INTRODUCTION

Commercialization of biotechnologies not only depends on intelligent products for the market, but also on a rapid and secure methodology for bioprocess design and development. The "old" empirical way of trial and error is to be replaced by a more systematic procedure according to a recent methodology in bioprocess technology based on a holistic mode of thinking, called the "formal macroapproach" operating with interaction-networks (integration of biological and physical phenomena). It is based on macroscopic process variables and on formal analogies adapted to experiments, which are used for mathematical modelling. A better scale-up is achieved by a scale-down approach, where regime analysis is used to determine the process bottlenecks, which then are experimentally simulated in the scale-down "model-bioreactors" representing the new integrating strategy. Mathematical modelling plays a central role representing a simplified but adequate approximation to real process behavior [1].

Practice in biotechnologies dominates engineering sciences and research activities so that a major part of investigations follows the purely empirical approach on a pragmatic level. Thereby, exploratory research in lab-scale units is the first step, followed directly by pilot plant work for pragmatic evaluation of the bioprocess for industrial realization [1].

Increase in oil prices and ethanol production by chemical synthesis has led to a world-wide interest in fermentation and has its origins in the early 1970s. Until quite recently the technology used to produce fuel ethanol by fermentation was virtually identical to the time-honored practices of the alcoholic beverages industry [2].

Alcoholic fermentation is the most important example of microbial production of compounds used as a source of alternative energy. The application of immobilized cell systems to produce ethanol is the most modern approach to achieve high ethanol concentration by fermenting sugar solutions. Considerable research and development has been carried out on the use of immobilized cells held in carrier media where the substrate is passed through highly reactive conversion zones. The basic principle is to

control the fermentation by fluid flow conditions through a catalyst, which is the general procedure for most chemical conversions [2].

Experimental studies on the establishment of a passive immobilization technique in which flocculating *S. cerevisiae* cells are entrapped within biomass support particles (BSPs) have previously been carried out by using the production of ethanol from glucose as a model process [3,4]. In these studies, ethanol production rates and ethanol yields as well as cell growth patterns in the immobilized cell reactor have been determined for 10% glucose solutions in batch recycle and continuous systems.

The aim of this study is the establishment of an immobilization technique in which yeast cells can be entrapped within Biomass Support Particles (BSPs) in a batch-recycle reactor and the production of ethanol by fermentation is taken as a model in that fermentation is carried out by a species of the yeast. In the present study, a flocculating *S. cerevisiae* strain was used in an immobilized cell reactor under batch operation with nutrient recirculation. Kinetic data on the growth and the ethanol production characteristics of the same strain were investigated. A range of experiments was conducted by varying the fermentation period at the nutrient concentrations of 6, 10, 12, and 14% glucose (w/v). The operational stability of the immobilized cell reactor was tested by operating the same system with several nutrient replacements with the aim of determining the operating conditions in practical application.

Chapter II of this thesis gives general information about the production of fermentation ethanol by yeasts, about the physical characteristics of yeasts including the immobilization techniques and also about the batch reactors. The experimental studies carried out are presented in Chapter III and the results are discussed in Chapter IV. Conclusions and recommendations for future work are given in Chapter V.

II. ETHANOL PRODUCTION BY FERMENTATION

2.1. Ethanol Fermentation by Yeast Cells

Yeasts form one of the important subgroups of fungi. Fungi, like bacteria, are widespread in nature although they usually live in the soil and in regions of lower relative humidity than bacteria. They are unable to extract energy from sunlight and usually are free-living. Although most fungi have a relatively complex morphology, yeasts are distinguished by their usual existence as single, small cells of 5 to 30 mm length and of 1 to 5 mm width.

The various paths of reproduction of yeasts are asexual (budding and fission) and sexual. In budding, a small offspring cell begins to grow on the side of the original cell; physical separation of mature offspring from the parent may not be immediate, and formation of clumps of yeast cells involving several generations is possible. Fission occurs by division of the cell into two new cells. Sexual reproduction occurs by conjugation of two haploid cells with dissolution of the adjoining wall to form a diploid cell zygote. The nucleus in the diploid cell may undergo one or several divisions and form ascospores; each of these eventually becomes an individual new haploid cell, which may then undergo subsequent reproduction by budding, fission or sexual fusion again [5].

In the production of alcoholic beverages, yeasts are the only important industrial microorganisms. In addition to supplying the consumer market for beer and wine, anaerobic yeast activities produce industrial alcohol and glycerol [3].

Under anaerobic conditions yeast cells grow rapidly producing ethanol and carbon dioxide at the same time. But the yield of yeast, based on the amount of available fermentation sugar, is low, often not more than 10%. In aerobic systems a yield of up to 50% of the weight of fermentable sugars can be obtained under special conditions. Apart from an adequate supply of nutrients, oxygen must be supplied to the yeast such that the liquid surrounding the cells always shows a partial oxygen pressure.

Oxygen is a critical nutrient for yeast growth. One kilogram of oxygen is required for the growth of one kilogram of yeast solids. Alcohol forms if the supply of air is limited.

Fermentation is the anaerobic, energy-releasing transformation of carbohydrates by living cells. As performed by many yeasts, fermentation means the conversion of hexose sugars, principally glucose, fructose, mannose and galactose, in the absence of air, to four major end-products:

glucose ⟶ 2 ethanol + 2 carbon dioxide + 2 ATP + 56 kcal

About 70% of the free energy is liberated as heat. The remainder is preserved in two terminal phosphate bonds of ATP for use in transfer reactions, such as the activation of glucose and amino acids prior to polymerization. At least 95% of the glucose catabolized by yeast goes to ethanol and carbon dioxide via the Embden-Meyerholf pathway. When nitrogen is limiting during fermentation, yeast growth is diminished. Consequently only about 70% of the glucose goes to ethanol and carbon dioxide; the remainder is converted to reserve glycogen.

Fermentable sugar must be added in such a way that it is growth limiting, i.e. in concentrations generally below 0.0004%. This can be achieved by continual addition of medium to a strongly aerated fermenter, a system known as incremental feeding. Glucose is assimilated by the yeast as fast as it is added using this process.

Glucose is fermented faster under anaerobic than under aerobic conditions. This is the well known Pasteur effect. The presence of glucose inhibits respiration, even in the presence of excess oxygen, i.e. under aerobic conditions. Yeast produces considerable quantities of ethanol even under aerobic conditions if the medium contains a sufficient amount of glucose. However, with limited supplies of fermentable sugars and under aerobic conditions the process is essentially respiratory [3].

Most of the currently practiced alcohol fermentations are based on the traditional batch processes. However, the need for reducing capital investment, better utilization of energy, and increased productivity have led to the development of

advanced methods. Such advances are the use of continuous fermentations, the increase of the yeast population by recycling, and the removal of ethanol during the fermentation [3,5].

2.2. Immobilization of Cells

Immobilization of microorganisms have been applied to many biochemical reactions. The use of immobilized whole cells in industrial processes has gained considerable attention during the past few years. Some of the immobilized cell systems now being used industrially are for the production of fructose syrups from glucose and the production of stereospecific amino acids. Many others are currently being examined for the production of a wide variety of other compounds [6,7].

Currently, there is increasing investigation into fuel and other chemical feedstock production through microbial processes, due to the rising cost and scarcity of petroleum. The production of ethanol as a fuel source using an immobilized whole cell is one of the processes of this kind.

Evaluation of cell immobilization as a process relative to alternative processes is highly complex, and must take into account a great number of factors, the foremost of which are economic factors, environmental factors, and, in the case of production of food stuff, taste and aesthetics. Economic factors include cost of starting materials, energy and equipment, and required technical skill. Thus a comparison of the advantages and disadvantages of these processes can only be made in a very general way, with the assumption that all other factors are constant [6,7,8].

2.2.1. Methods of Cell Immobilization

Methods of cell immobilization can best be classified by the nature of the mode of attachment, that is, as mechanical, chemical or ionic. In mechanical

immobilization, the cells are localized by means of physical barriers. In chemical immobilization, covalent bonds are formed among cells or to a solid phase. In ionic immobilization, electrostatic, van der Waal's or London forces of attraction are present. Cells can also attach themselves to solid supports in the course of natural growth, using a combination of these means. This classification is obviously not clear-cut but does serve the purpose of organizing the diverse methods of immobilization available [8].

a. Mechanical Immobilization

Mycelial Pellet and Mat

The mechanism of these techniques is based on physical attachment of microbial cells into agar, sodium alginate gel etc. The mycelium of a fungus consists of tubular filaments on the top of which are the spores. Typically the hyphae of a fungus are about 50 microns in length and under active growth will branch out, interweave and fuse with one another to form a net. Occasionally such mycelial mats cause problems in fermentations because overcrowding of mycelial growth will cause inactivation in a fermentation using filamentous cells, probably due to reduction of the surface area of contact between the medium and the fungus so that the nutrients and oxygen become less accessible [8].

Encapsulation

In this method, microbial cells are completely surrounded by a layer of semipermeable material such as collodion, silicone, collagen or polyester. The molecular dimensions of the microcapsules impose a limit on the molecular sizes of both nutrients and products in fermentation. There is also a dependence of the reaction rate on the rate of transfer of substrate into the capsules. The dimensions of the capsule will also limit the growth of the cells, although an initial cell density is possible [8].

Entrapment

By far the most frequently used technique of cell immobilization, entrapment methods call for incorporation of the cells within the lattice of a polymeric material,

which can be carbohydrate, protein, synthetic organic or inorganic. The common enzyme immobilizing technique in which the solid carrier is in the form of a membrane is also used to immobilize cells by entrapment The rate of cell attachment is a function of culture age and pH of the medium. Materials can attach from 55% to 65% of the cells with certain variations from one material to another [8].

b. Covalent Attachment

The distinguishing feature of this method of immobilization is the existence of covalent bonds between the cells and the polymer lattice, or covalent bonds among the cells themselves to form a mat. Because of their defined chemical nature, enzymes are amenable to covalent bonding through bifunctional reagents. Use of such reagents on whole cells will usually kill the cells, although enzymatic activity may be preserved.

Covalent crosslinking is also possible on inorganic solid carriers such as silica, titania, glass, brick, and zirconia, usually with glutaraldehyde.

Covalently bonded cells in general do not present problems of cell leakage or mechanical degradation. Problems arise mainly from the loss of viability of the immobilized cells. In fact, the cell walls in most cases act as a solid carrier on which the enzymes are bound [8].

c. Ionic Attachment

Adsorption

A variety of yeast cells such as *S. cerevisiae* can be immobilized on different supports like wood, ceramic, glass etc. Since the adhesion phenomena is mainly based on electrostatic interactions between the charged microbial cells and the support, the actual z-potential on both of them plays an important role in cell-support interaction. It is a well known fact that yeast cells are negatively charged. Therefore, it is preferable to choose a positively charged support for their immobilization. Cell wall composition is also an important factor which strictly depends on microbial properties. The complexity of yeast cell wall surface can provide the necessary amino

or carboxy groups, which may directly interact with an inorganic support surface. This will result in the formation of a bond between the cell and support [8,9].

Flocculation

Another way of ionic attachment is flocculation. This type of attachment profits mainly from weak forces such as hydrogen bonding, coordinate bonding, Van der Waals and dispersion forces. Further argument about flocculation is made in the next section [8,10,11].

2.2.2. Flocculation

Flocculation of yeasts is usually defined as the ability of the cells to aggregate spontaneously and form flocs which sediment rapidly in culture medium.

In the cell walls of yeast, the mannans are generally associated with proteins (mannoproteins) which are exposed on the external surface. Flocculation would appear to involve the surface of the cells and chemical or enzymatic treatments inhibit flocculence ability irreversibly, which indicates the involvement of proteins or glycoproteins located on the yeast surfacein this process [12].

The degree of flocculence is greatly influenced by physical and chemical factors. Since flocculation can be readily observed in buffer solutions or in synthetic media, it must be assumed that culture medium constituents may influence but do not basically modify flocculating behavior. The cultural conditions which induce or repress flocculation are one of the most controversial subjects. Sugars appear to prevent or delay flocculation. Nevertheless, this influence is strictly connected with air flux and the aeration seems to modify significantly the flocculence expression of the strains. For low aeration rates (0.1 vvm), glucose seems not to influence flocculation, because in these conditions the strains flocculate independently of the initial glucose concentration. At intermediate aeration rates, high glucose concentrations (6 to 10%) were found to increase flocculation. A high aeration inhibits completely this phenomenon [12,13].

2.2.3. Immobilized Cells versus Free Cells

There are advantages to an using immobilized cell system over a free cell system.

Firstly, the productivity of immobilized cells is generally as high as, if not higher than the corresponding free cell fermentations. This productivity can be explained by the fact that the microenviroments offered by the carrier are more stabilizing to the organism or its enzymes, which generally show optimal activity only under very narrowly prescribed physical conditions.

Secondly, immobilized cell systems can be described readily by well developed theoretical and hydrodynamical treatments of heterogeneous catalysis systems, particularly if the system utilizes solid carriers in the form of uniform spherical particles.

Thirdly, there is no need for cell removal or cell recycle, making product extraction much more efficient. Cell immobilization also enables higher dilution without culture washout. Thus, flow rates can be optimized for best system kinetics.

Finally, the risk of contamination is reduced due to fast dilution rates and high yeast cell densities [7,8].

Nevertheless, cell immobilization systems do suffer from a number of problems. The initial expense for such a system is usually high and the process usually requires a large reactor. The mechanical properties of the system are more complex than those of free cells and have to be taken into account in order to provide a continuous, recycling process and effective agitation and filtration. System designs must allow for the increased diffusion barrier through the cell and the carrier for substrates, products, and cofactors, so that cells bound to a carrier generally need permeabilizing treatment, especially when high molecular weight substrates or products are encountered [14].

2.3. Batch Reactors

Design procedures for immobilized cell reactors are analogous to those used for solid-catalyzed chemical reactors, with the major difference being that immobilized cell kinetics are used instead of chemical kinetics [15].

Many results concerning the design criteria for enzymatic reactors have been published. It has been pointed out that in the design of an immobilized cell process three important factors must be considered: the source and extent of the purity of the cell, the size and chemical nature of the support and the method of immobilization. These factors have a direct effect on the fundamental properties of the biocatalyst, such as the specific activity. Also, the particle size is of considerable importance in the design of the reactor as well as in the eventual limitations in the reaction rate. Another consequence resulting from these factors is the long term microbial and mechanical stability of the biocatalyst, including the effectivity of the union between cell and support [8].

Two additional factors are extremely important: kinetics and the stability of the cells. In the first case, as industrial reactions are run at high levels of conversion and high substrate concentration, the cells may be subjected to severe inhibition effects. Optimum operation policies have to be selected and in some cases the inhibition may even define the type of reactor. Similarly, biocatalyst stability is another most important factor from the economical point of view, since it is directly related to the productivity.

Different type of reactors can be used for process scale operations with cells in their free or immobilized forms. Based upon the mode of cells used, reactors are classified as batch and continuous, the former being the most frequently used in the industrial processes and in laboratory experiments. Batch processes are typical for fine chemicals production, characterized by their relatively small production and their high added value.

The use of free cells is generally restricted to batch reactors. In this case, the free cell is loaded into the reactor and the reaction is carried out to the desired degree of conversion. At the end of the process no attempt is made to recover the cells. There are also some immobilized cell reactors that are used in batch mode, especially when production requirements do not justify a continuous operation. In other cases, cells are used in batch reactors because of kinetic and stability constraints as mentioned above. If it is also considered that cells are studied in the laboratory in batch reactors (test tubes, beakers, formal reactors) it may therefore be concluded that the batch reactor is a valuable tool and a frequent alternative in the integration of cells to industrial processes [16].

From the kinetic point of view, the study of the behavior of cells in batch reactors is an unavoidable step in their characterization for industrial applications. The effect of products on the reaction rate, reversibility of the reaction, alternative reactions and the operational stability of the cells are a few examples of cell properties, not observed in the classical "initial rate" experiments performed for their kinetic characterization. Some of these properties are increased under industrial operating conditions. Therefore, models obtained from initial rate experiments, have to be verified in batch reactions for a proper design of processes in any type of reactor [16,17].

Some justifications for the industrial selection of batch reactors can be done as follows: Production requirements, microbial stability of substrate and/or product, insoluble or high viscosity substrates or products, stability of the cell in immobilized form, kinetic behavior, handling of more than two phases, need for pH and temperature regulations [18].

2.3.1. Batch Fermentation

Coming back to the fermentation process, batch fermentations may be carried out without the need for establishing pure culture conditions, that is, without the need for complete sterilization of the media and without maintenance of complete sterility

of equipment. However, this presupposes a rapid start of the yeast fermentation, which inhibits the growth of other microorganisms by depleting the available nutrients, by a lowering of the pH, and most importantly by the formation of ethanol. Industrial alcohol fermentations require cooling either through internal cooling or through external heat exchangers. The temperature is usually maintained between 30°C and 35°C and the pH between 4.5 and 6. Fermenters are not stirred and not aerated. The rapid development of carbon dioxide gas during the fermentation provides some mixing [15,18].

Most of the currently practiced alcohol fermentations are based on the traditional processes. However, the need for reducing capital investment, better utilization of energy, and increased productivity have led to the development of advanced methods. Such advances are the use of continuous fermentations, the increase of the yeast population by recycling, and the removal of ethanol during the fermentation.

2.3.2. Growth-Cycle Phases for Batch Cultivation

In any biological system, growth can be defined as the orderly increase of all chemical components. All chemical reactions in a microorganism may result in an increase in the cell mass. This process is known as cell growth.

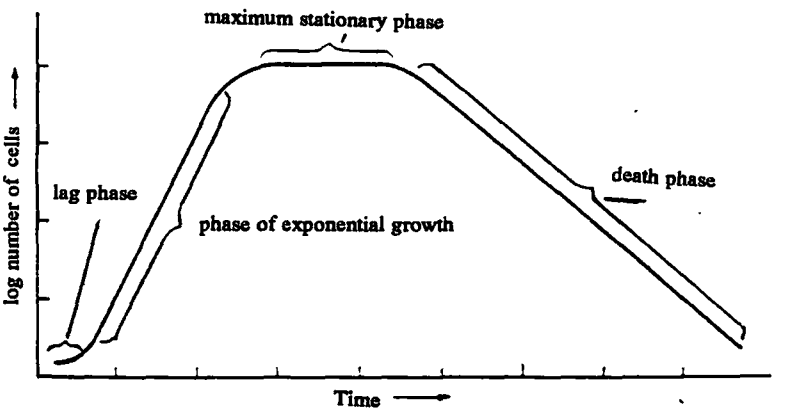

Figure 2.1 Typical Growth Curve for Batch Cell Cultivation [5]

In a typical batch process the number of living cells varies with time. Microorganisms demonstrate a typical population growth pattern that can be represented by a 4-stage population growth curve, and each phase is of potential importance in microbiological processes. As shown in Fig 2.1, a lag phase, where no increase in cell numbers is evident and a period of rapid growth ensues, during which the cell numbers increase exponentially with time. Although this stage of batch culture is often called the logarithmic phase, it is more descriptive to call it exponential growth. Naturally in a closed vessel the cells cannot multiply indefinitely, and a stationary phase follows the period of exponential growth. At this point the population achieves its maximum size. Eventually a decline in cell numbers occurs during the death phase, where an exponential decrease in the number of living individuals is often observed [5].

2.3.3. Growth Kinetics For Batch Reactors

Many biochemical processes involve batch growth of cell populations. After seeding a liquid medium with an inoculum of living cells, nothing is added to the culture or removed from it as growth proceeds. Typically in such a reactor, the concentrations of nutrients, cells, and products vary with time as growth proceeds [4].

A material balance on moles of component i shows that the rate of accumulation of component i, given by the time derivative of the total amount of component i in the reactor, must be equal to the net rate of formation of component i due to chemical reactions in the vessel.

A change in culture volume times the molar concentration of component i is equal to the culture volume times the moles of i formed by reaction per unit culture volume per unit time [5]. Noting that a similar balance may be formulated in terms of mass or number density of a component.

Measurement of the time rate of change of the concentration of component i allows direct determination of the overall rate of i formation due to reactions which

take place in the batch reactor. In general, the rate of formation depends upon the state of the cell population and all environmental parameters which influence the rates of reactions in the cells and in the medium [4,5].

a. Unstructured Batch Growth Models

In the simplest approach to modeling batch culture, it is supposed that the rate of increase in cell mass is a function of the cell mass only. Thus,

$$dx/dt = f(x) \tag{2.1}$$

Such a form does not require to neglect changes occurring in the medium during growth. One of the simpler models belonging to the general form given in Eq.(2.1) is Malthus' law which uses

$$f(x) = \mu x \tag{2.2}$$

with μ as a constant.

Verlhurst in 1844 and Pearl and Reed in 1920 [5] contributed to a theory which included an inhibiting factor to population growth. Assuming that inhibition is proportional to x^2 , they used

$$dx/dt = kx(1-\beta x) \qquad x(0) = x_0 \tag{2.3}$$

Which is a Riccati equation which can be easily integrated to give the logistic curve.

Another class of unstructured models which approach a stationary population level can be formulated based on limiting nutrient exhaustion. Assuming a constant overall yield factor $Y_{x/s}$ and $\mu = \mu(s)$ such as Monod kinetics, nutrient and cell material balances can be combined into a single equation of the form of Eq.(2.1).

If the concentration of one essential medium constituent is varied while the concentrations of all other medium components are kept constant, the growth rate typically changes in a hyperbolic fashion, as Fig 2.2 shows. A functional relationship between the specific growth rate μ and the concentration of an essential compound was proposed by Monod in 1942 [5]. Of the same form as the Langmuir adsorption

isotherm proposed in 1918 and the standard rate equation proposed for enzyme-catalyzed reactions with a single substrate by Henri in 1902 and Michaelis and Menten in 1913 [5], the Monod equation states that

$$\mu = \mu_{max} s/(K_s + s) \tag{2.4}$$

Here μ_{max} is the maximum growth rate achievable when $s >> K_s$ and the concentrations of all other essential nutrients are unchanged. K_s is that value of the limiting nutrient concentration at which the specific growth rate is half its maximum value; roughly speaking, it is the division between the lower concentration range, where μ is strongly dependent on s, and the higher range, where μ becomes independent of s.

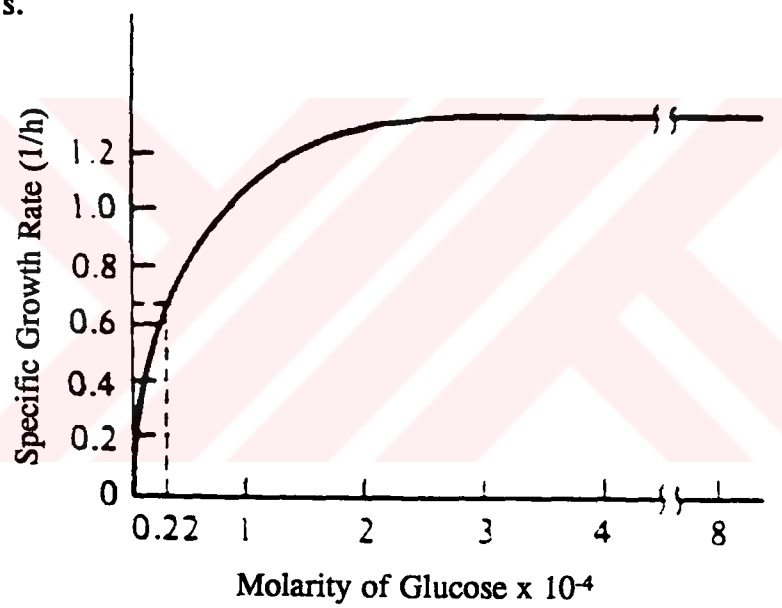

Figure 2.2 Dependence of Specific Growth Rate on the Concentration of the Growth-Limiting Nutrient [5].

b. Structured Batch Growth Models

Unstructured models of the types considered above describe only the quantity of the biological phase. Such models do not recognize nor represent the composition or the quality of the biophase. In situations in which the cell population composition

changes significantly and in which these composition changes influence kinetics, structured models should be used.

The biophase variables employed in structured models are typically the mass x_j or molar c_j concentrations per unit volume of biophase. For a batch reactor,

$$dc_j/dt = r_{fj} - \mu c_j \qquad (2.5)$$

Here, r_{fj} is the molar rate of formation of component j (moles of j per unit time per unit volume) and the $-\mu c_j$ term represents reduction in concentration caused by dilution from growth of the cell population [5].

2.3.4. Repeated Operation

Significant process engineering advantages are evident for the use of immobilized cells in repeated batch, or 'draw and fill' operations as in the case of vinegar production. The ease of solid/liquid separation and of solid handling leads to possibilities for:

a) a reduction in cell handling in situations where cells from a fermenter are recovered and used as an inoculum for a subsequent batch as in the brewing industry,

b) the use of large inocula by virtue of cell retention, which could lead to improvements in batch productivity, or a reduction in reactor volume, particularly where slowly growing cells are involved,

c) opportunities for cell reuse, since simple, aseptic liquid removal and replacement allows multiple reuse of cells for bioconversions,

d) intermittent cell regeneration by provision of a growth medium when it is possible to switch between a growth medium and a production medium, which may be of benefit in the synthesis of a secondary metabolite, and

e) the development of novel solid/liquid separation techniques [19].

2.4. Studies on the Behavior of Yeast Cells in Immobilized Systems

A considerable number of studies have been carried out on the behavior of yeast cells in immobilized systems. The extensive literature on the subject has recently been surveyed by Khoury [3] and Özergin [4]. More recent developments of interest are presented in the following paragraphs.

Agrawal and Jain [20] have investigated the kinetic and yield parameters for growth and ethanol production from sucrose by *Saccharomyces cerevisiae* entrapped in K-carrageenan and calcium alginate and have found them to be identical to those of freely suspended cells. Presenting their data on the kinetics of growth and ethanol production in repeated batch fermentations, and on the fermentation of concentrated sucrose solutions, they concluded that immobilized growing yeast in K.carrageenan and calcium alginate can both be used for repeated batch production of ethanol efficiently up to 84% of theoretical yield, although, calcium alginate gel has better strength and is superior for large scale preparation and operation of immobilized cells. Ethanol yields were by immobilized cells were found to be affected by high sucrose and ethanol concentrations in the medium.

Bertolini et al. [21] have investigated new yeast strains for alcoholic fermentation at higher sugar concentrations. They have isolated new yeast strains from samples collected from Brazilian alcohol factories at the end of the sugar cane crop season, which were selected by their capacity of fermenting concentrated sugar cane syrup as well as high sucrose concentrations in synthetic medium with a conversion efficiency of 89-92%. The strains were identified as *Saccharomyces cerevisiae* .

Vieria et al. [22] have investigated the effects of immobilization on the production of ethanol at high glucose concentrations. They have used entrapment and adsorption techniques for the immobilization of *S.cerevisiae, S.bayanus*, and *K.marxianu* and reported that the maximal concentration of ethanol produced during the fermentation of 320 g/l glucose by *S.bayanus* was enhanced when the yeast cells were immobilized either by adsorption on celite or by entrapment in k-carrageenan

beads. These observations have led to the conclusion that the increase in ethanol was due to medium supplementation with the compounds present in the immobilization supports.

Eduardo et al. [12] investigated the influence of aeration and glucose concentration in the flocculation of *S. cerevisiae* and observed that low aeration rates exerted a positive effect on the flocculation whereas high aeration rates inhibited this phenomenon. A positive effect was achieved on flocculation with high glucose concentrations (up to 10%). By controlling both of these factors it was possible to modulate the flocculation expression of the strain used.

III. EXPERIMENTAL WORK

3.1. Experimental Set-Up

Batch-recycle experiments were carried out using immobilized cells in a presterilized glass column jacketed reactor of 15 cm height and 3 cm internal diameter with a jacket thickness of 1 cm having one overflow port located 11 cm from the bottom to maintain a constant liquid level inside the column. The substrate was pumped from the medium reservoir (1-L Erlenmeyer flask) into the bottom of the column, through the bed of yeast cells immobilized in BSPs with a computer-driven pump (Masterflex peristaltic pump, Model no. 7550-62, flowrate capacity : 0.6-2280 ml/min).All equipment were interconnected with appropriate size silicone tubings, also dry heated to a temperature of 180°C for 90 minutes in an oven for sterilization purposes. The temperatures of both the column and the medium reservoir were monitored using a digital thermometer with external K-type thermocouples as temperature sensors (Fluke 52).

Ethanol concentrations were determined using a Shimadzu gas chromatograph, model GC-8A with a thermal conductivity detector, equipped with a data processor (CR 1B Chromatopac Processor) and a recorder (Model R-111 M). Substrate concentrations were determined by a Shimadzu Double-Beam Spectrophotometer (UV-150-02) using Nelson's method [4].

3.2. Materials

3.2.1. Microorganism and Culture Medium

A flocculating strain of *Saccharomyces Cerevisiae* (Dl-dl-1F) (Genotype : URA 3-251, 372,328,SOD^-,(DLEU2)) was supplied by the University of Manchester Institute of Science and Technology.

The liquid medium used for the production of cells as well as for fermentation consisted of 10 g/l yeast extract (Difco 0127-01), 25 g/l KH_2PO_4 (Merck 4871), 10 g/l $(NH_4)_2SO_4$ (Merck 1216), 2 g/l $MgSO_4.7H_2O$ (Riedel-De Haen AG), and 0.5 g/l $CaCl_2$ (Merck) and D(+)-glucose monohydrate (Merck 8346). The amounts of D(+)-glucose monohydrate were 66 g/l, 110 g/l, 132 g/l, and 154 g/l for 6%, 10%, 12% and 14% substrate concentrations respectively. All powders and granular substances were weighed using a digital balance.

The agar medium consisted of 1% yeast extract (Difco 0127-01), 2% Bacto-agar (Difco 0140-01), 2% Bacto-peptone (Difco-0118-01-8)and 2% dextrose (Baxter Std).

3.2.2. Filtration Apparatus

The dry cell mass was determined using a 0.22 mm Millipore membrane filter (diameter 47 mm).The glass filtration apparatus, connected to a vacuum pump, was obtained from Millipore. Vacuum of 70-100 mmHg was maintained during the filtration experiments.

3.2.3. Fermentation Materials

Yeast cells were grown at 30°C in a controlled environment, orbital incubator shaker (GFL 3032).

Biomass Support Particles (BSPs) described by Khoury [3] and Özergin [4] were used in all experiments. Yeast cells were immobilized on 1cmx1cmx0.7cm BSPs of polymeric material having a void fraction of about 0.95 and a complex void structure. The density of the particles was about 0.1 g / cm^3.

L-shaped stainless steel wires of 0.5 mm diameter and 38 cm length were used in the immobilization experiments.

3.3. Experimental Methods

3.3.1. Immobilization technique

a. Preparation of Immobilization Carriers

Materials

Biomass Support Particles (BSPs)

BSPs of dimensions 1x1x0.7 cm^3 were used to immobilize the yeast cells. They were cut and placed in a beaker containing sufficient distilled water to cover the particles and autoclaved at 121°C for 15 minutes. After the same procedure was repeated three times, they were placed in an oven at 60°C and allowed to dry for several days. The average pore size of the BSPs could not be determined due to their complex void structure.

Wires

L-shaped stainless steel wires of 0.5 mm diameter and 38 cm length were used in the immobilization experiments.

b. Preparation of Preculture and Immobilization of Yeast Cells

Yeast were grown at 30°C in a medium containing 6%, 10%, 12% and 14% glucose for 18 hours. An inoculum of living yeast cells from the slant culture was aseptically transferred into five 50-ml Erlenmeyer flasks each containing 20 ml nutrient broth. These flasks were placed in an orbital shaker at 30°C operating at 60 rpm for 18 hours . These precultures were then used to inoculate the immobilization carrier which consisted of 6 to 7 BSP pieces strung on L-shaped stainless steel wires of 38 cm length in the presterilized 1-L flasks. These flasks containing 180 ml liquid medium and two wires with BSPs were incubated for 18 hours in an orbital shaker at 30°C with a revolution of 60 rpm for immobilization. The same procedure was repeated for four different substrate concentrations namely 6%, 10%, 12% and 14%.

3.3.2. Batch-Recycle Experiments Using Immobilized Cells

a. Immobilized Cell Column Reactor

A glass column jacketed reactor of 11 cm effective height and 3 cm inner diameter with a jacket thickness of 1 cm was constructed for the immobilized cell experiments. In operation, the liquid medium was pumped from the medium reservoir (1-L Erlenmeyer flask) by a peristaltic pump into the bottom of the inclined reactor column, through the bed of yeasts immobilized in BSPs, and recirculated from the medium reservoir back into the column reactor. At the same time, water at 30°C was circulated through the jacket of the reactor by the aid of a constant temperature waterbath and circulator. The detailed schematic diagram of the system is shown in Figure 3.1.

b. Experimental Procedure

After taking 1 BSP from each L-shaped wire to evaluate the average cell mass per BSP by the dry weight method, the immobilized BSPs were aseptically transferred into the reactor from the top end. The 500 ml liquid medium in the medium reservoir was recirculated using a peristaltic pump at a rate of 15 ml/min. The temperature of the medium in the reservoir was kept constant at 30°C by placing the reservoir in a constant temperature waterbath. The temperature of the column was kept constant by circulating water at 30°C through the jacket of the reactor. These temperatures were monitored by a digital thermometer.

Samples were collected every hour and centrifuged at 5000 rpm for 15 minutes to remove any suspended free cells. The supernatants were then kept at 4°C and assayed for pH, glucose and ethanol.

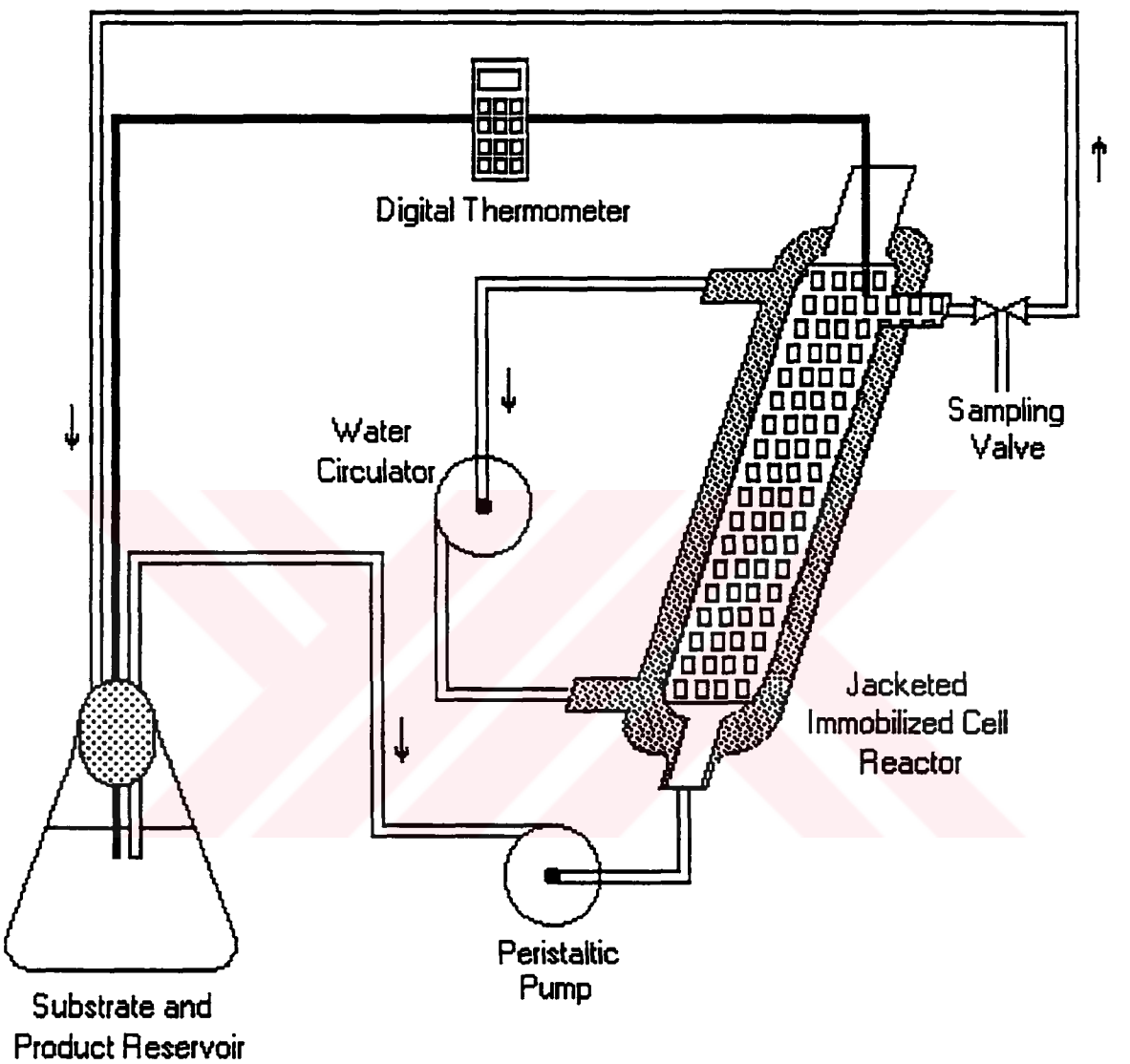

Figure 3.1 Schematic Diagram of the Batch-Recycle System

The experiments were carried out at various operation times from 3 h to 15 h and at different substrate concentrations (6%, 10%, 12%, and 14%). At the end of individual runs, the contents of the reactor were dried in an incubator at 60°C for three days and weighed. The dry weight of immobilized yeast cells was determined by taking difference with the weight of the carrier before immobilization.

The amount of free cells in the medium reservoir at the end of each experiment was determined by passing 50 ml of the liquid medium through a 0.22-mm membrane filter which was placed in petri dish and dried at 60°C for three days. The filtration apparatus was connected to a vacuum pump.

All experiments were carried out under sterile conditions, and all fermentation media were autoclaved at 121°C.

Operational Stability Experiments

The operational stability of the column reactor was tested in another set of experiments by replacing the circulating medium by fresh medium when complete consumption of the substrate in the previous batch was achieved. The reactor performance was found very steady for more than two days and required no extra cell input.

3.4. Analytical Methods

3.4.1. Ethanol Analysis by Gas Chromatography

For the determination of ethanol concentrations in the samples, gas-solid chromatography technique was used. This technique is described by Khoury [3] and Özergin [4] in a detailed manner.

Shimadzu gas chromatograph (model GC-8A) equipped with CR-1B Chromatopac Processor and Recorder (model R-111 M) was used with a thermal conductivity detector (TCD). Hydrogen supplied by HABAS A.S. was used as a

carrier gas for the analysis.A stainless steel column, 3 mm in inner diameter, 3 m in length and packed with 50/80 mesh Porapak Q was used for ethanol determinations.

For the analysis, the injector, detector and the column oven were operated at 150°C. As indicated in the paper of Uobe et al.[23] , the sugar should be removed from the sample to prevent thermal degradation, by lowering the temperature at the injection port or by shortening the column. An injector temperature of 150°C was therefore used instead of the 175°C reported by Doran and Bailey [24].The current was set at 110 mA. The flowrate of the carrier gas namely hydrogen was adjusted to 20 ml/min. 1mL samples were injected with a Hamilton microliter syringe.

Since the area under each peak is proportional to the concentration of ethanol, the exact concentrations of ethanol each experiment were determined by using a calibration chart that was obtained by injecting several different concentrations of standard ethanol which were tabulated in Table 3.1.

Table 3.1 Calibration Data For Ethanol Standard
Column Temperature = 150°C
Carrier Gas Flowrate = 20 ml/min
Volume of sample = 1mL

Ethanol Concentration (g/l)	Area (Counts)
801.38	3219144
400.69	1598726
200.35	789694
100.17	369396
50.08	213068
37.56	147531
25.04	107796
18.78	76589
9.39	38554
6.26	25884
4.695	17662
3.13	13263
2.348	9681
1.174	3802
0.783	1368

3.4.2. Colorimetric Analysis of Glucose

Nelson's method[3,4] which was used to measure the glucose concentrations in the liquid medium consisted of the following steps.

a. Preparation of Reagents

Nelson's Reagent A

15 g of anhydrous Na_2CO_3 (Horasan Kimya), 15 g sodium potassium tartrate (Merck 8085), 12 g of $NaHCO_3$ (Merck 6323), and 120 g of anhydrous Na_2SO_4 (Merck 6643) were weighed, dissolved in 420 ml of distilled water and diluted to 600 ml. The sediment that formed was removed by filtration.

Nelson's Reagent B

3.6 g of $CuSO_4.7H_2O$ (Merck 2787) was dissolved in 24 ml of distilled water. One drop of concentrated sulfuric acid was added.

The complete Nelson's Reagent was prepared prior to the experiment, by mixing 25 ml of reagent A and 1 ml of reagent B.

Arsenomolybdate Reagent

37.5 g of $(NH_4)_6Mo_7O_{24}.4H_2O$ (Merck 1180) was dissolved in 675 ml of distilled water and 31.5 ml of concentrated sulfuric acid was added. 4.5 g of $NaHAsO_4.7H_2O$ (PANREAC-131635, Monplet & Esteban SA) was dissolved in 37.5 ml of distilled water and added to the molybdate solution. The solution was incubated in a dark brown bottle for 24 hours at 37°C.

b. Analytical Method

The supernatants were diluted in a proportion of 1:1000 and placed in test tubes. 1 ml of Nelson's reagent which was prepared prior to the experiment, was added to all tubes. The test tubes were then covered with cotton and mixed. The tubes were heated in a boiling water bath for 20 minutes and were cooled. 1 ml

Arsenomolybdate reagent was added to each tube and mixed. After 5 minutes with occasional stirring, 7 ml of water was added to each tube and mixed. The absorbance of each sample was measured at 540 nm, using distilled water as blank. A calibration chart was obtained by measuring the absorbance of different concentrations of standard glucose as in Table 3.2.

Table 3.2 Calibration Data For Glucose Standard
Wavelength = 540 nm

Glucose Concentration (g/l)	Absorbance (°A)
0.20	1.230
0.12	0.770
0.08	0.542
0.04	0.308
0.02	0.192
0.00	0.078

IV. RESULTS AND DISCUSSION

Considerable research has been devoted to the development of immobilized cell systems for industrial application. Some of the immobilized cell systems now being used industrially are for the production of fructose syrups from glucose and for the production of stereospecific amino acids. Many others are currently being examined for the production of a wide variety of other compounds including fuel and chemical feed stock production through microbial processes, and increasing attention has therefore been directed towards alcohol production using immobilized whole cells.

In this research, the experimental procedures developed in pervious studies conducted at Boğaziçi University [3,4] were used to obtain kinetic data on the growth and ethanol production characteristics of the same flocculating *S. cerevisiae* strain in an immobilized cell reactor under batch operation with nutrient recirculation. In parallel experiments, the operational stability of the immobilized cell reactor was tested by operating the same system with several nutrient replacements with the aim of determining the operating conditions in practical application.

4.1. Fermentation Conditions

The fermentation process was examined in a batch-recycle reactor by using immobilized yeast cells. The pH of the culture medium was initially set at 4.7 for 6, 10, 12, and 14% substrate concentrations, since this pH falls within the optimum pH range reported in literature [25].

Periodic sampling during the single-batch experiments showed a decrease in the pH level to 3.52 and for the operational stability experiments to 3.31. Consequently, the contamination of the medium was not a serious problem and the

experimental system could be maintained with complete sterility without the need for autoclaving.

The temperatures of the jacketed column reactor and medium reservoir were kept constant at 30°C and were monitored using a digital thermometer during the batch recycle experiments with immobilized yeast cells. Immobilization of the cells were also carried out in an incubated orbital shaker at the same temperature. It has been reported that immobilized yeast cells had the highest activity at 30°C and although they maintain a relatively high activity up to 45°C, their stability decreases with an increase in temperature [26]. In addition, it is also reported that the temperatures above 30°C, a decrease in cell growth and ethanol production rate are observed when cells are subjected to increasing concentrations of ethanol. In general, temperature and extracellular ethanol concentration have been found to be directly related to the inhibition of ethanol production [27].

Ethanol productivities are known to be limited by three factors: ethanol inhibition, low cell concentrations and substrate inhibition. As glucose concentration in the feed is increased, the specific ethanol productivity decreases due to inhibition by high ethanol concentrations. At low glucose concentrations, ethanol inhibition is less but cell concentration also decreases. As stated in literature, these counterbalancing effects give an optimum productivity at 10% glucose concentration for freely suspended yeast systems. In contrast, processes based on immobilized yeast cells have been reported to exhibit high productivities at substrate concentrations higher than 10% [28,29]. It is also reported in the literature that the flocculation ability of the yeast cells depends on the aeration rate and substrate concentration [12].

In the present work, 6, 10, 12, and 14% glucose concentrations were used in the immobilized cell experiments in order to obtain kinetic data on the growth of *S. cerevisiae* and its ethanol production characteristics, and the flocculation behavior of the same strain was also observed (See Section 4.3.1).

The immobilized cell experiments were carried out batchwise with a recirculating medium. Yeast cells in their logarithmic growth phase were immobilized for substrate conversion. In such operations, the yeast is discarded at the end of each

experiment and a new culture must be grown for the next batch; hence, the use of immobilized cells in a single batch culture does not appear to offer any operational advantages and is reported to be disadvantageous due to immobilization costs, poor substrate utilization and lower specific rates of reaction [3]. The immobilized cell system was also operated in a mixed mode, by recirculation of the substrate and for an extended period using the same columns with successive nutrient replacements.

4.2. Immobilization Experiments

Experiments with immobilized cells in the batch-recycle system were carried out at a controlled temperature of 30°C both of the medium reservoir and the jacketed column reactor.A range of experiments were conducted by varying the fermentation period and the nutrient concentration. In parallel experiments, the operational stability of the immobilized cell reactor was tested by operating the same system with several nutrient replacements with the aim of determining the operating conditions in practical application.The nutrient concentrations were 6, 10, 12 and 14% glucose. Samples were taken hourly from the overflow port of the immobilized cell reactor and the glucose and ethanol concentrations were measured.

4.2.1. Batch-Recycle Experiments

a. Growth Pattern in the Immobilized Cell Reactor

The complete set of immobilization experiments in the batch-recycle system were carried out with BSPs that had undergone an initial 18-hour batch immobilization period in an incubated orbital shaker for 6, 10, 12 or 14% glucose concentrations, since previous experiments showed that there was no net increase or decrease in the immobilized cell mass after 18 hours during the immobilization for 10% glucose concentration [3].

The final dry cell mass of immobilized yeast was found to fall below the initial value during the first 3 hours for 6% and 10% glucose concentrations, during the first 6 hours for 12% glucose concentration, and during first 9 hours for 14% glucose concentration (Tables 4.1-4.4). The experiments have shown that the initial average immobilized cell mass of 1.5658 g/l and 1.9834 g/l decrease to 1.0536 g/l and 1.8656 g/l for 6% and 10% glucose concentrations after 3 hours of operation respectively. Also, the initial average immobilized cell mass of 1.9554 g/l and 2.2121 g/l decrease to 1.7762 g/l and 1.9066 g/l for 12% and 14% glucose concentrations after 6 hours and 9 hours of operation respectively. This may be due to local adaptation problems and/or to the production of freely suspended cells by erosion or washout from the support particles when first exposed to a recirculating medium. At lower glucose concentrations (namely 6% and 10%) it is easier for the cells to overcome the local adaptation problems, and the washout of cells was observed only up to 3 hours of operation. On the other hand, at higher glucose concentrations (12% and 14%), washout of cells was observed up to 6 hours and 9 hours of operation. Washout of cells has been observed at relatively high recirculation rates, where all the particles are moving and thus hindering the growth in the BSPs [27].

Comparing the percent free cells at different glucose concentrations, the average free cells are 20-22% of the total for 6, 12 and 14% glucose concentrations, and about 12% of the total for the 10% glucose concentration. Although there is no definite trend, the data show that for the immobilization of *S. cerevisiae* the optimum glucose concentration may be taken as 10%, and that at higher concentrations the proportion of free cells increases.

As can be seen from the Tables 4.1-4.4, the percent free cells remain relatively constant from 3 hours to 15 hours of operation for a particular substrate concentration. The contribution of the free cells for each operation period may be assumed to have a similar effect on the production of ethanol and on the consumption of glucose.

Table 4.1 Batch-recycle Experiments
Free and Immobilized cells
Substrate Concentration = 6% glucose
Average Initial Imm'd cell mass = 1.5658 g/l

Operation Time (h)	Initial Imm'd cell mass (g/l)	Final Imm'd cell mass (g/l)	Free cells (g/l)	Percent Free cells (%)
3	1.4272	1.0536	0.178	14.45
6	1.9385	2.5892	0.630	19.57
9	0.9715	2.7332	0.623	18.56
12	1.6311	2.7686	0.591	17.59
15	1.8606	3.2201	1.440	30.90

Table 4.2 Batch-recycle Experiments
Free and Immobilized cells
Substrate Concentration = 10% glucose
Average Initial Imm'd cell mass = 1.9834 g/l

Operation Time (h)	Initial Imm'd cell mass (g/l)	Final Imm'd cell mass (g/l)	Free cells (g/l)	Percent Free cells (%)
3	2.1570	1.8656	0.261	12.27
6	1.8770	2.6740	0.394	12.84
9	2.0878	4.1890	0.639	13.23
12	1.7650	4.1150	0.955	18.84
15	2.0304	3.8096	0.385	9.18

Table 4.3 Batch-recycle Experiments
Free and Immobilized cells
Substrate Concentration = 12% glucose
Average Initial Imm'd cell mass = 2.1935 g/l

Operation Time (h)	Initial Imm'd cell mass (g/l)	Final Imm'd cell mass (g/l)	Free cells (g/l)	Percent Free cells (%)
3	2.2113	1.8743	0.686	26.79
6	1.9613	1.7762	0.263	12.90
9	1.8749	2.0602	0.626	23.30
12	2.6114	2.7074	0.909	25.13
15	2.3084	3.0452	1.005	24.81

Table 4.4 Batch-recycle Experiments
Free and Immobilized cells
Substrate Concentration = 14% glucose
Average Initial Imm'd cell mass = 2.2121 g/l

Operation Time (h)	Initial Imm'd cell mass (g/l)	Final Imm'd cell mass (g/l)	Free cells (g/l)	Percent Free cells (%)
3	1.5015	1.0544	0.134	11.28
6	2.6110	2.0691	0.832	28.68
9	1.0039	1.9066	0.573	23.11
12	3.4656	2.8112	0.842	23.05
15	2.4785	3.4826	1.278	28.84

Figures 4.1-4.4 show the changes in immobilized cell mass and total mass of yeast cells within the reactor column during batch-recycle experiments conducted at varying operation times for 6, 10, 12, and 14% glucose concentrations.

Comparing the two sets of graphs showing the changes in immobilized cell mass and total cell mass, it appears that the presence of free cells do not change the growth patterns in the immobilized cell reactor to a great extent, and the reactor may be treated as a true cell-immobilized system.

In the case of 14% glucose concentration, the final masses of immobilized cells showed fluctuations. Washout of the cells was observed up to 9 hours of operation which may be due to the change in the flocculation behavior of the strain caused by the high glucose concentration. Although glucose concentrations were found not to affect the flocculation behavior of yeast cells at low aeration rates [12], the yeast cells used under anaerobic conditions in the present work became less flocculant at high glucose concentrations which hindered their immobilization and increased the amount of free cells.

The dry cell mass of the immobilized yeast was found to increase and reach a constant value during the 15 hour fermentation period at a recirculation rate of 15 ml/min for 6 and 10% glucose concentrations. Tables 4.1-4.4 show the relative amounts of immobilized and free cells based on total bioreactor volume. During the

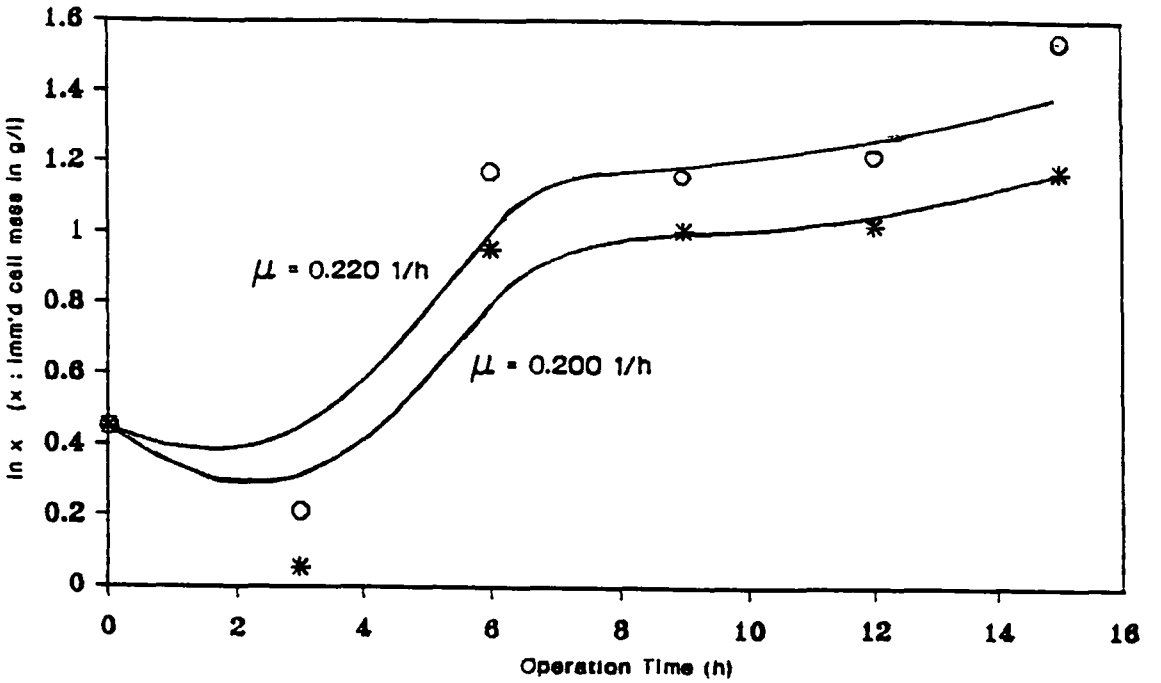

Figure 4.1 Growth Pattern of BSP-Immobilized Yeast Cells (*) and Total Yeast Cells (o) within the Reactor Column at 6% Glucose Concentration

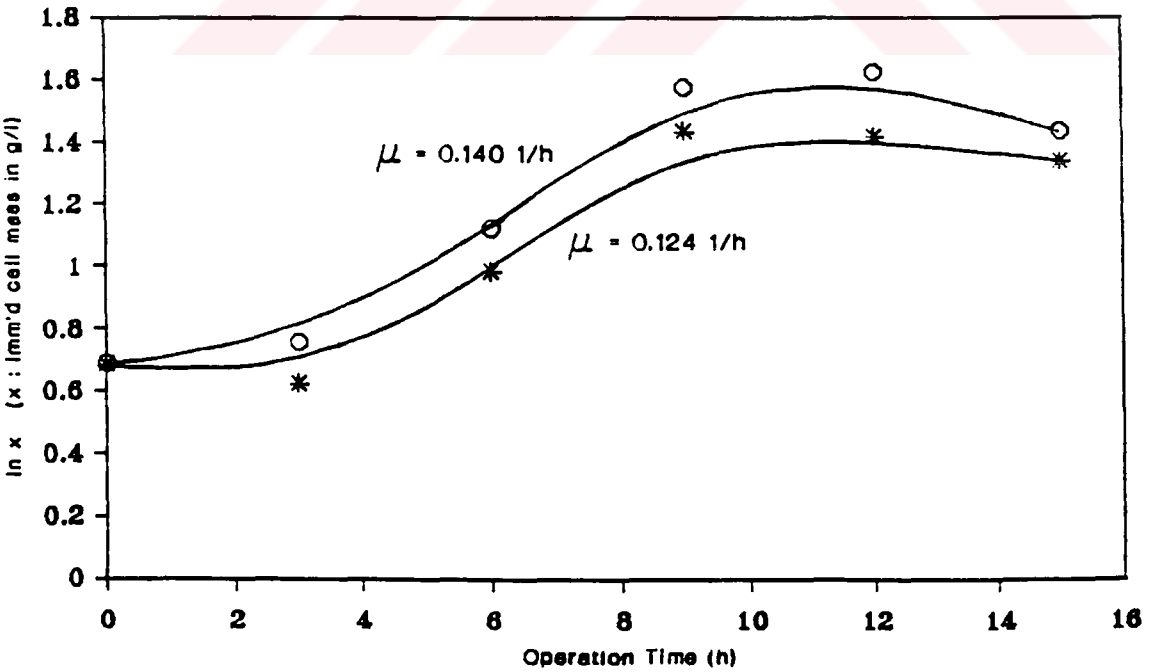

Figure 4.2 Growth Pattern of BSP-Immobilized Yeast Cells (*) and Total Yeast Cells (o) within the Reactor Column at 10% Glucose Concentration

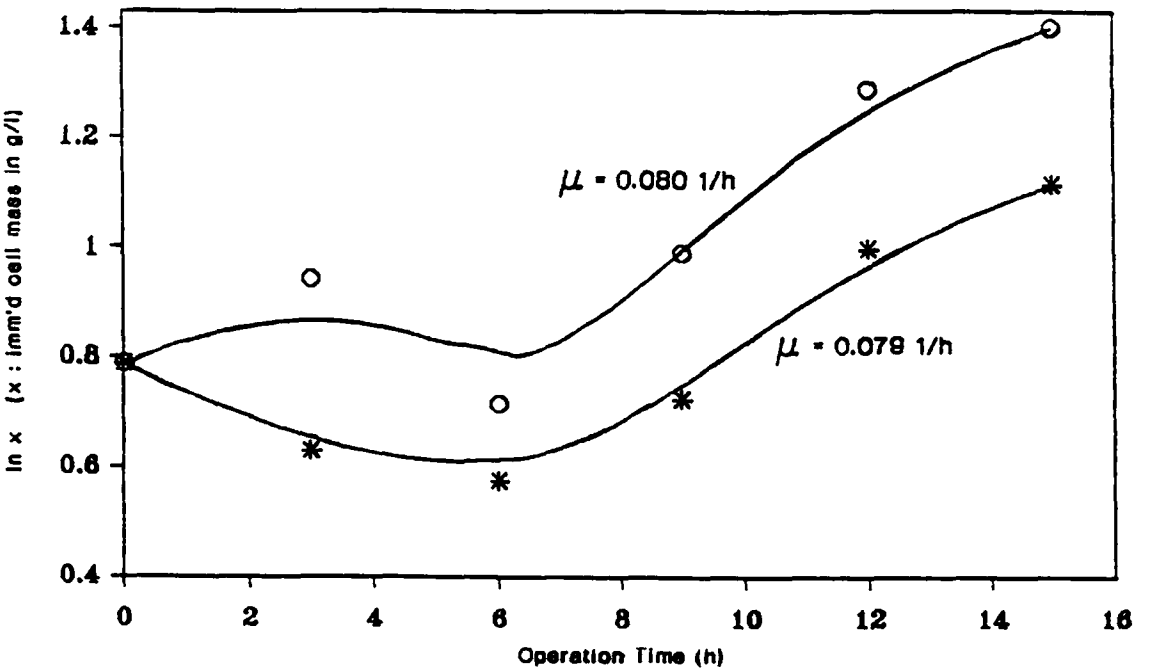

Figure 4.3 Growth Pattern of BSP-Immobilized Yeast Cells (*) and Total Yeast Cells (o) within the Reactor Column at 12% Glucose Concentration

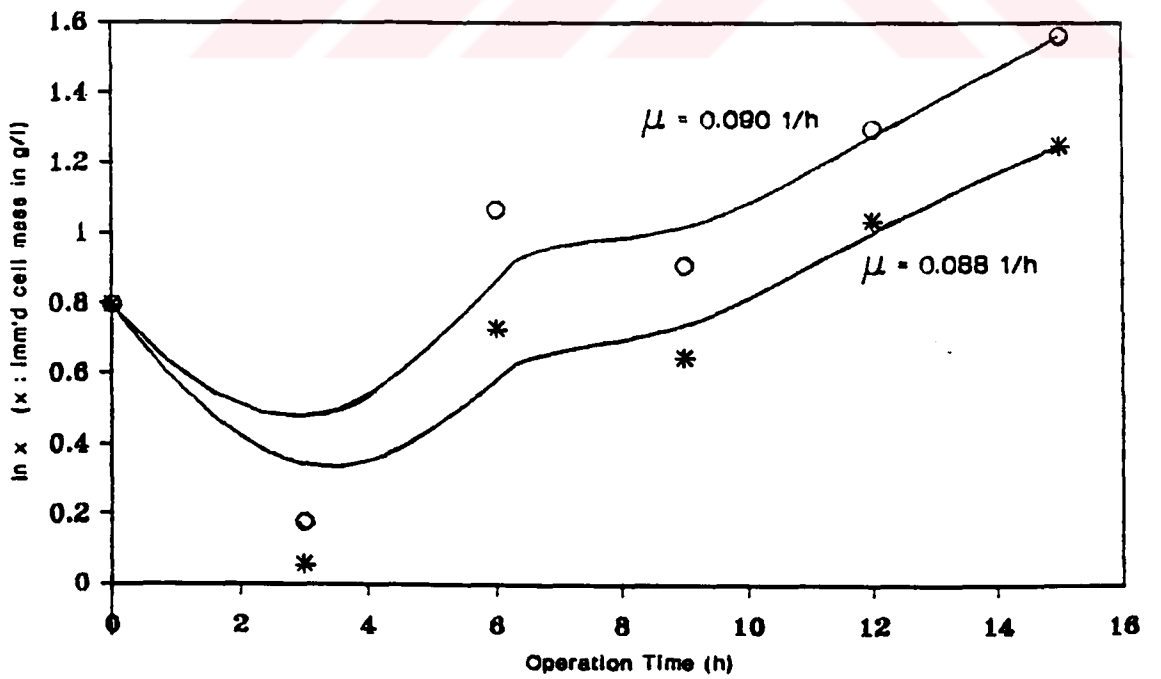

Figure 4.4 Growth Pattern of BSP-Immobilized Yeast Cells (*) and Total Yeast Cells (o) within the Reactor Column at 14% Glucose Concentration

initial stages of fermentation only small amounts of glucose were converted to ethanol due to the low concentration of yeast and the time required for local adaptation as suggested by Gencer and Mutharasan [32].

In addition, comparing the lag phases for 6, 10, 12 and 14% glucose concentrations, the time required for the lag phase increases as the initial concentration of the substrate increases. The time required for the completion of the exponential growth phase is also increased, since it takes a longer time to reach the stationary phase at higher substrate concentrations.

b. Ethanol Production

It is highly essential to be able to carry out inoculation accurately preceding each experiment, and allow similar amounts of cells to become immobilized as a consequence of their growth during the 18 hour batch period. A decrease in the cell density in the immobilizing matrix causes a corresponding decrease in the amount of cells obtained after fermentation and in ethanol production. Tables 4.5-4.8 present the results selected from the batch-recycle experiments to evaluate the behavior of yeast cells in the immobilized cell reactor.

The production of alcohol by immobilized cells is affected by the cell mass loading in the support as well as by the activity of the culture, which is a function of environmental conditions such as pH, temperature, concentration of substrate and final concentration of product.

The results obtained from the batch conversion of glucose to ethanol by immobilized *S. cerevisiae* using a nutrient recirculation rate of 15 ml/min are summarized in Tables 4.5-4.8 and depicted in Figures 4.5-4.8 for 6, 10, 12, and 14% glucose concentrations respectively.

From Figures 4.5 and 4.6, the production of ethanol and the consumption of glucose reached to steady state for 6 and 10% glucose concentrations which confirmed the growth pattern data in Figures 4.1 and 4.2, while for 12 and 14% glucose concentrations in Figures 4.7 and 4.8, the production of ethanol tended to

increase and the consumption of glucose tended to decrease at the end of 15 hours of operation which was also an expected situation.

Table 4.5 Batch-Recycle Experiments
Ethanol Production and Productivities with Varying Operation Time
Substrate Concentration = 6% glucose

Time (h)	Final imm'd Cell Mass (g/l)	Ethanol Conc. (g/l)	Glucose Left (g/l)	Production Rate (g/h)	Specific Ethanol Productivity (g/h/gimm'd cells)	Yield Factor (g ethanol/ g glucose)	Percent Conv. (%)
3	1.0536	7.96	53.49	1.33	2.518	1.222	10.85
6	2.5892	13.10	43.40	1.09	0.843	0.789	27.66
9	2.7332	18.96	21.29	1.06	0.770	0.489	64.52
12	2.7686	21.02	16.08	0.88	0.632	0.478	73.20
15	3.2201	27.16	7.49	0.91	0.562	0.517	87.52

Table 4.6 Batch-Recycle Experiments
Ethanol Production and Productivities with Varying Operation Time
Substrate Concentration = 10% glucose

Time (h)	Final imm'd Cell Mass (g/l)	Ethanol Conc. (g/l)	Glucose Left (g/l)	Production Rate (g/h)	Specific Ethanol Productivity (g/h/gimm'd cells)	Yield Factor (g ethanol/ g glucose)	Percent Conv. (%)
3	1.8656	5.79	60.00	0.97	1.034	0.145	40.00
6	2.6740	18.90	59.00	1.58	1.178	0.472	41.00
9	4.1890	19.20	59.00	1.07	0.509	0.467	41.00
12	4.1150	25.64	36.40	1.07	0.519	0.472	63.60
15	3.8096	39.5	33.50	1.32	0.691	0.594	66.50

Table 4.7 Batch-Recycle Experiments
Ethanol Production and Productivities with Varying Operation Time
Substrate Concentration = 12% glucose

Time (h)	Final imm'd Cell Mass (g/l)	Ethanol Conc. (g/l)	Glucose Left (g/l)	Production Rate (g/h)	Specific Ethanol Productivity (g/h/gimm'd cells)	Yield Factor (g ethanol/ g glucose)	Percent Conv. (%)
3	1.8743	11.34	100.00	1.89	2.017	0.567	16.66
6	1.7762	11.70	87.81	0.98	1.098	0.363	26.83
9	2.0602	20.20	77.90	1.12	1.089	0.480	35.07
12	2.7074	24.79	59.20	1.03	0.763	0.408	50.67
15	3.0452	26.94	42.74	0.90	0.589	0.348	64.38

Table 4.8 Batch-Recycle Experiments
Ethanol Production and Productivities with Varying Operation Time
Substrate Concentration = 14% glucose

Time (h)	Final imm'd Cell Mass (g/l)	Ethanol Conc. (g/l)	Glucose Left (g/l)	Production Rate (g/h)	Specific Ethanol Productivity (g/h/gimm'd cells)	Yield Factor (g ethanol/ g glucose)	Percent Conv. (%)
3	1.0544	8.57	105.02	1.43	2.7093	0.255	24.98
6	2.0691	12.34	101.19	1.03	0.994	0.329	27.79
9	1.9066	19.53	79.56	1.09	1.138	0.330	43.18
12	2.8112	28.50	71.74	1.19	0.845	0.426	48.76
15	3.4826	32.50	59.02	1.09	0.622	0.408	57.84

Figure 4.9 shows the effect of initial glucose concentration on ethanol production and glucose consumption at the end of 15 hours of batch operation. The ethanol production curve indicates that the maximum amount of ethanol was produced at 10% glucose concentration, which is in agreement with the optimum conditions reported in the literature [29].

The production rates at the end of 15 hours of operation are calculated as 1.81, 2.63, 1.80, and 2.17 g/h for 6, 10, 12, and 14% glucose concentrations respectively. The maximum production rate of 2.63 g/h was achieved at 10% glucose

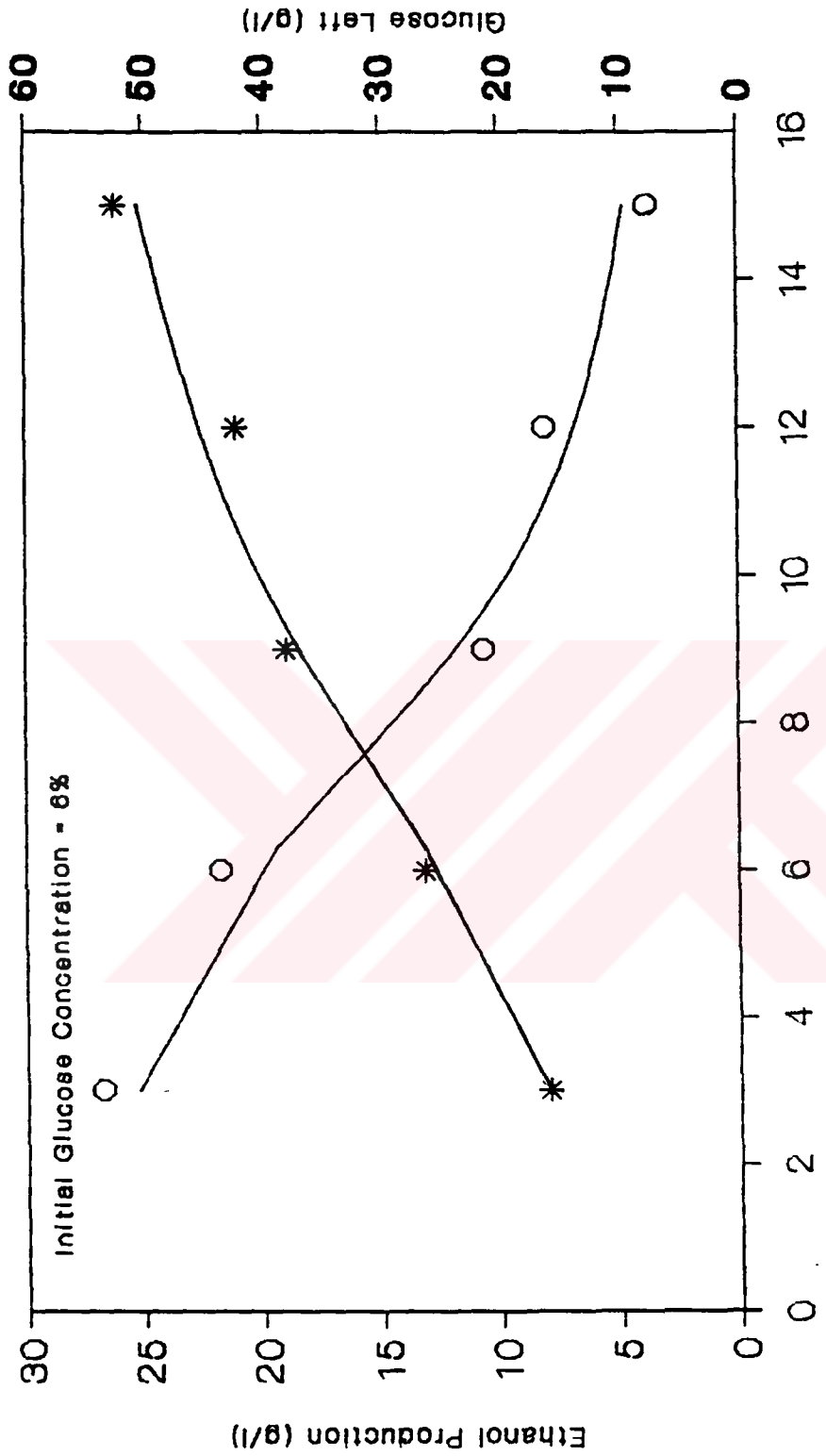

Figure 4.5 Immobilized Cell Fermentation Data on Ethanol Production (*) and Glucose Consumption (o)

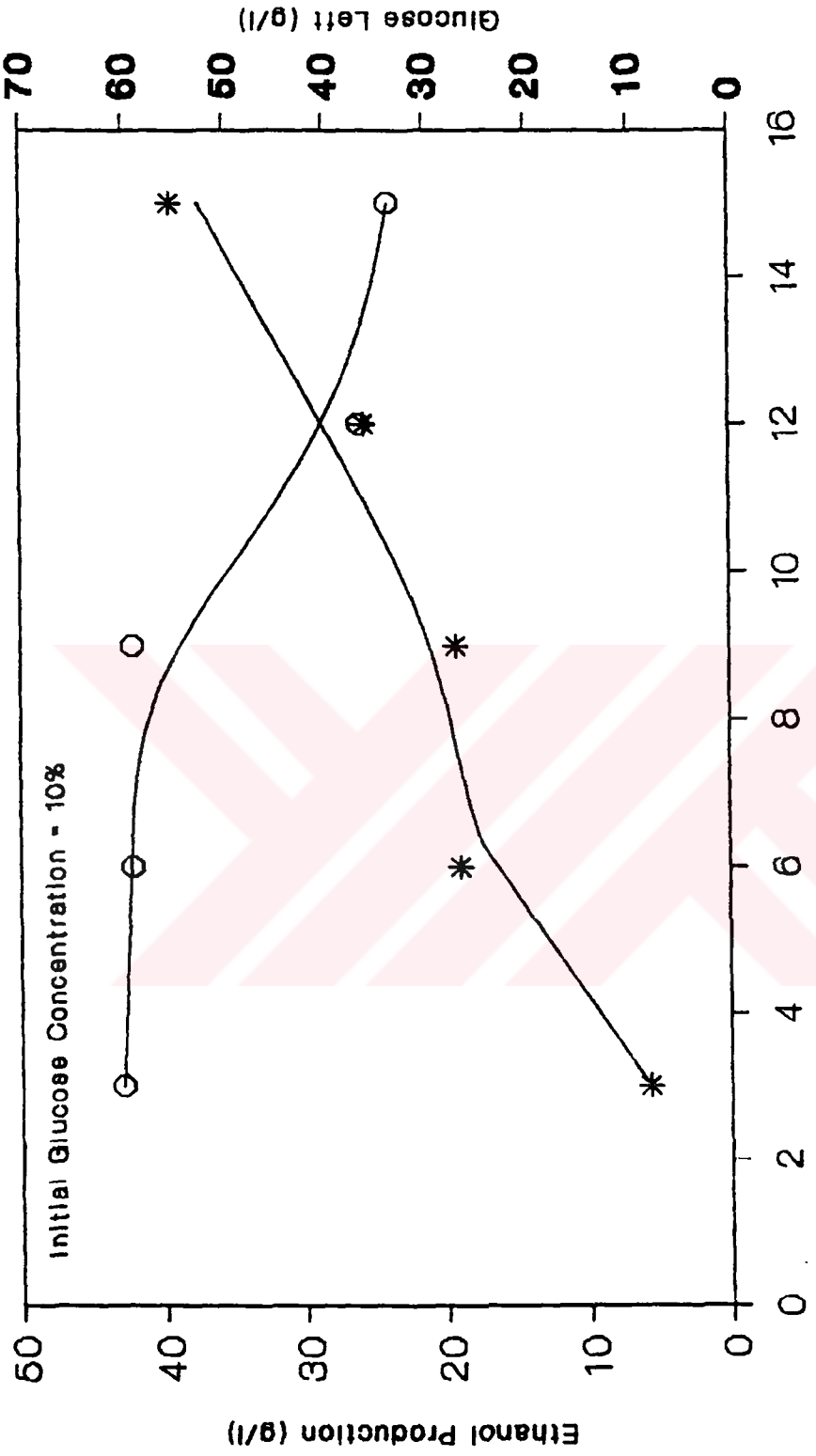

Figure 4.6 Immobilized Cell Fermentation Data on Ethanol Production (*) and Glucose Consumption (o)

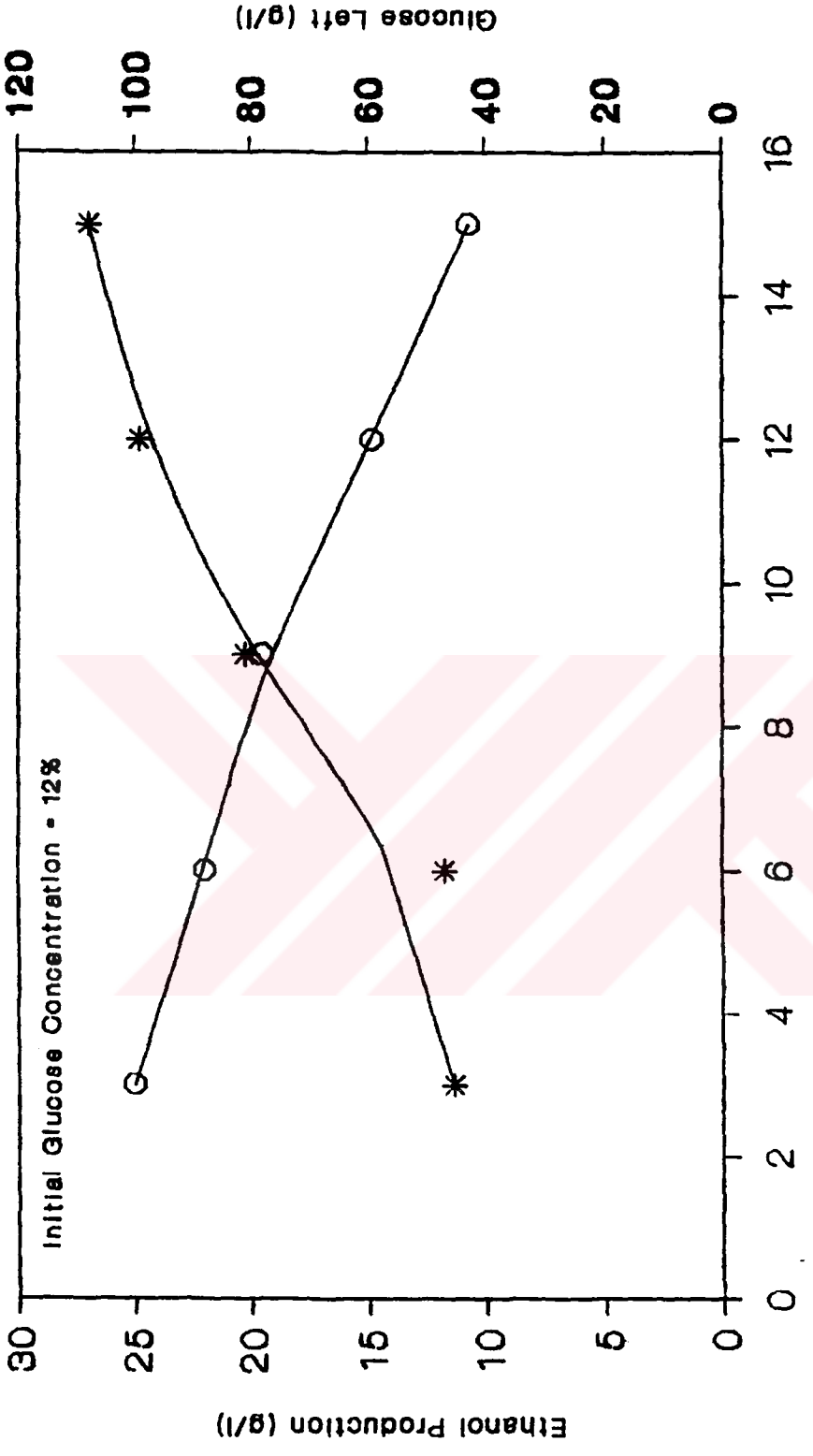

Figure 4.7 Immobilized Cell Fermentation Data on Ethanol Production (*) and Glucose Consumption (o)

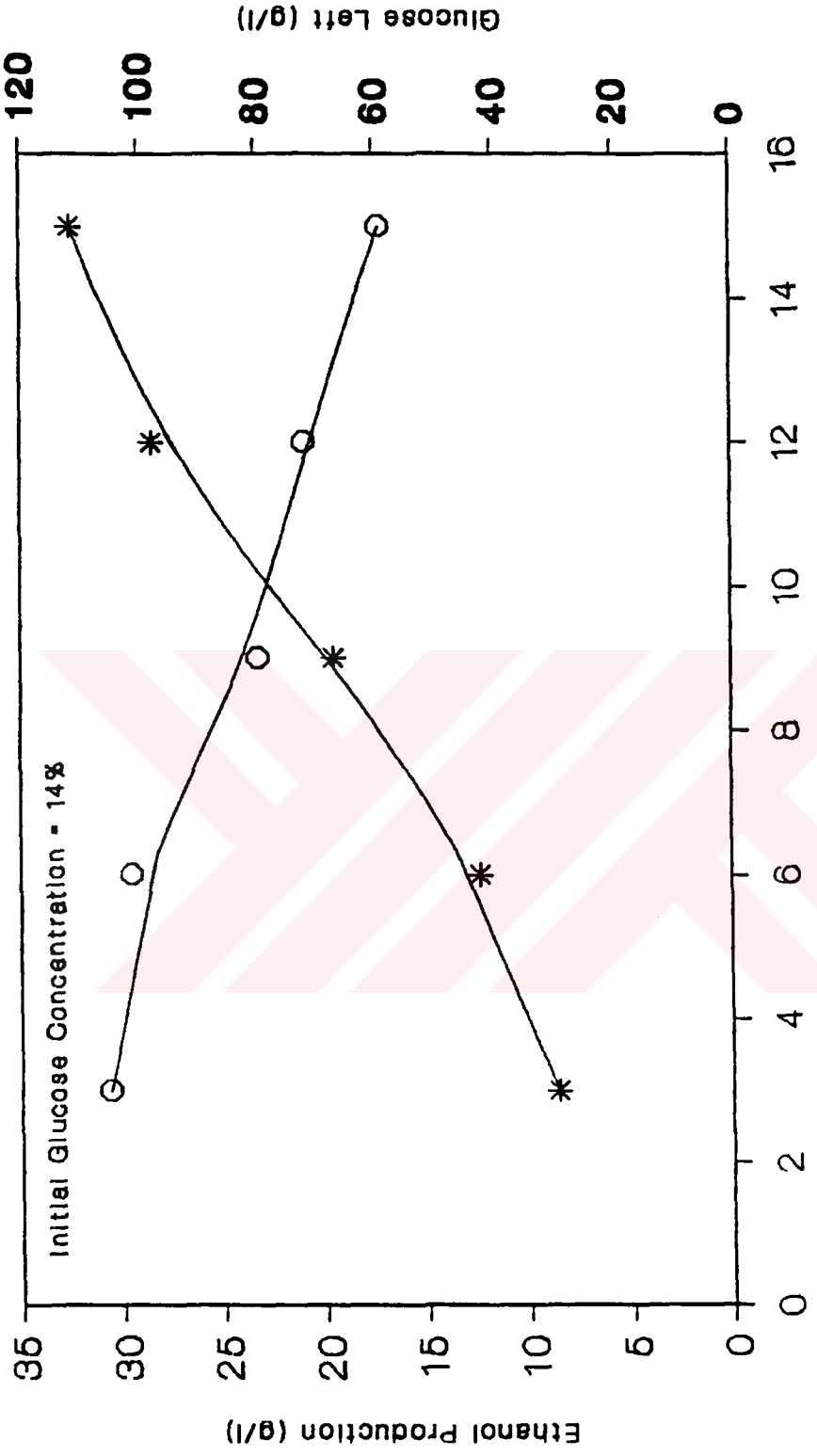

Figure 4.8 Immobilized Cell Fermentation Data on Ethanol Production (*) and Glucose Consumption (o)

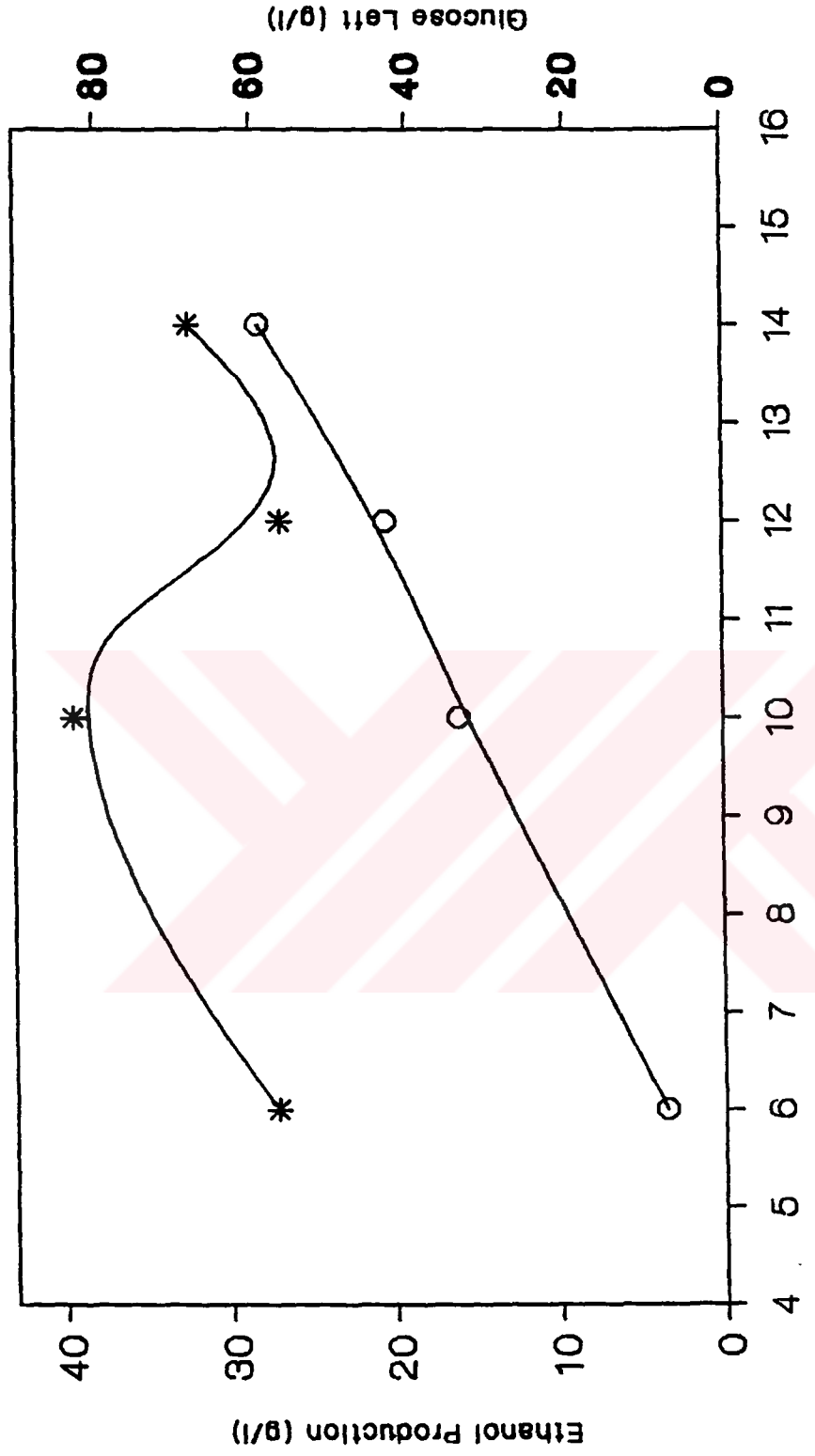

Figure 4.9 The Effect of Initial Glucose Concentration on Ethanol Production (*) and on Glucose Consumption (o) (Operation Time = 15 h)

concentration, which is in accordance with the literature [29]. At the higher glucose concentration namely 14%, the production rate was found to be 2.17 g/h which is rather a high value. As processses based on immobilized growing yeasts have been reported to be attractive for allowing the use of rather high substrate concentrations to obtain high ethanol yield [3,29], this result seems promising and requires further study.

The yield factors (g ethanol produced/g glucose consumed) were 0.517, 0.594, 0.348, and 0.408 for 6, 10, 12, and 14% glucose concentrations respectively as tabulated in Tables 4.5-4.8. Here again, the maximum yield was achieved at 10% glucose concentration. As the initial glucose concentration is increased from 6% to 14%, the percent conversion of glucose at the end of 15 hours of operation decreases from 87.52% to 57.84% and this is accompanied by a decrease in the yield factor.

Figure 4.10 shows the effect of initial glucose concentration, the yield factor, production rate and specific ethanol productivity. The maximum values were reached at 10% glucose concentration which may be considered optimum.

In addition, Figure 4.11 shows the time profiles of ethanol yields for 6, 10, 12 and 14% glucose concentrations. The results indicate that the yield factor, which is defined as the amount of ethanol produced per unit amount of glucose consumed, remained more or less constant after 6 hours of operation for each of the substrate concentrations and the maximum yield was reached by 10% glucose at the end of 15 hours of operation.

In the case of specific ethanol productivity data presented in Figure 4.12, a similar behavior was observed. Peaks were obtained in the specific ethanol productivity because of the washout at 3 hours of operation in each of the substrate concentrations,. Here again, the productivities remained more or less constant after 6 hours of operation.

Figure 4.13 shows the time profiles of production rates for 6, 10, 12 and 14% glucose concentrations. Again, the production rates remained more or less constant after 9 hours of operation.

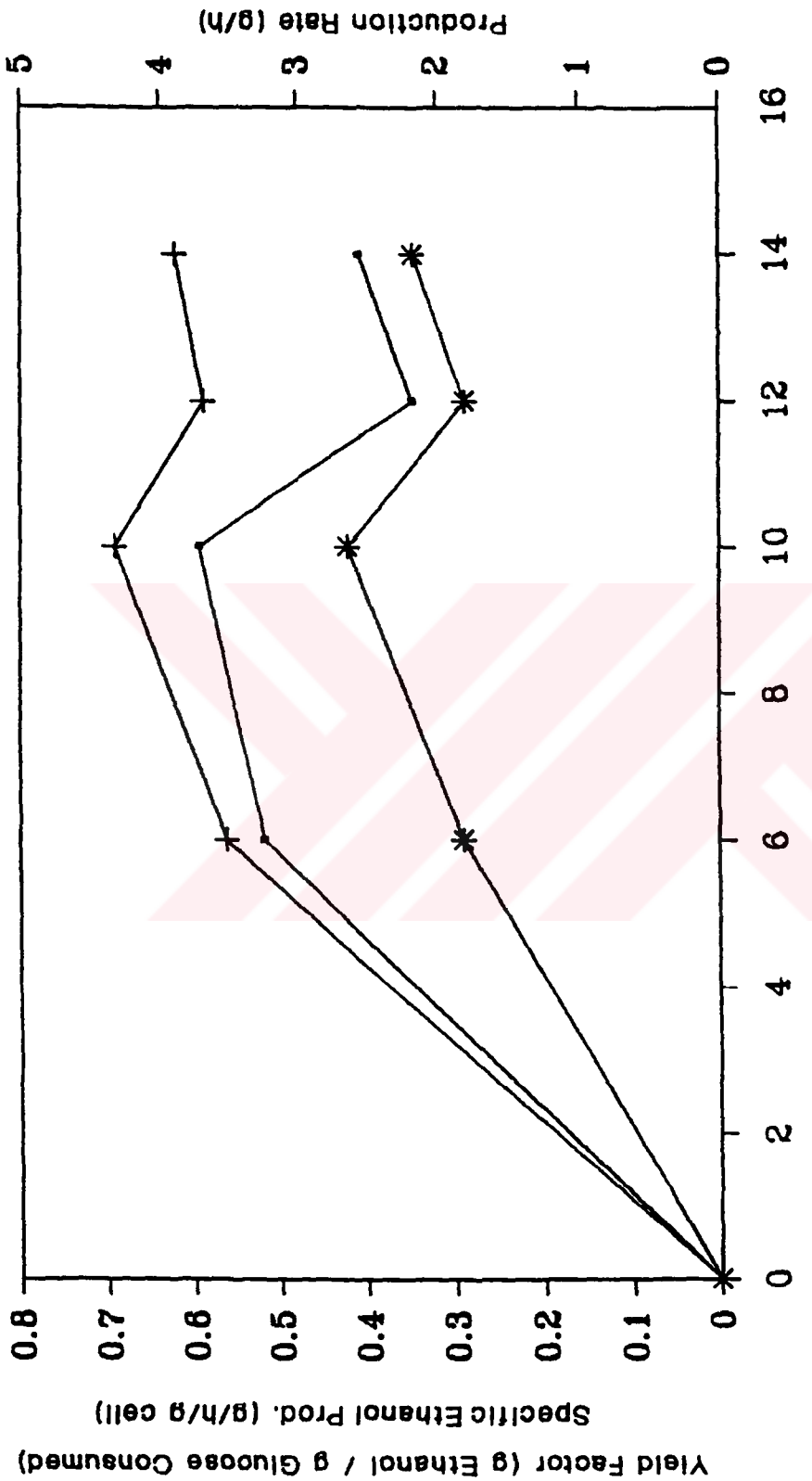

Figure 4.10 The Effect of Initial Glucose Concentration on the Yield Factor (.), Production Rate (*) and Specific Productivity for Ethanol (+) (Operation Time = 15 h)

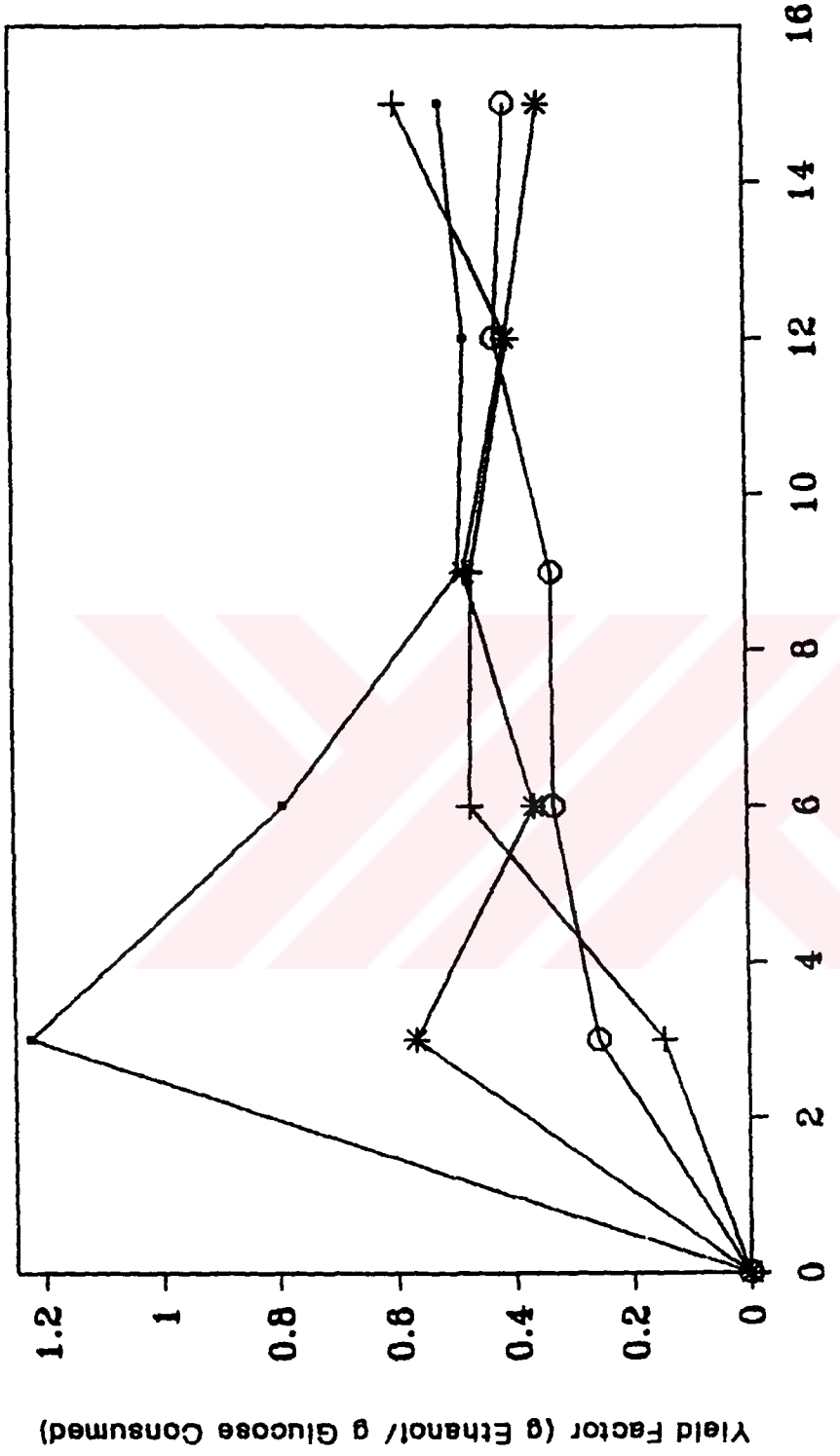

Figure 4.11 Yield Factors Obtained in Experiments Conducted at 6% (.), 10% (+), 12% (*) and 14% (o) Initial Glucose Concentration

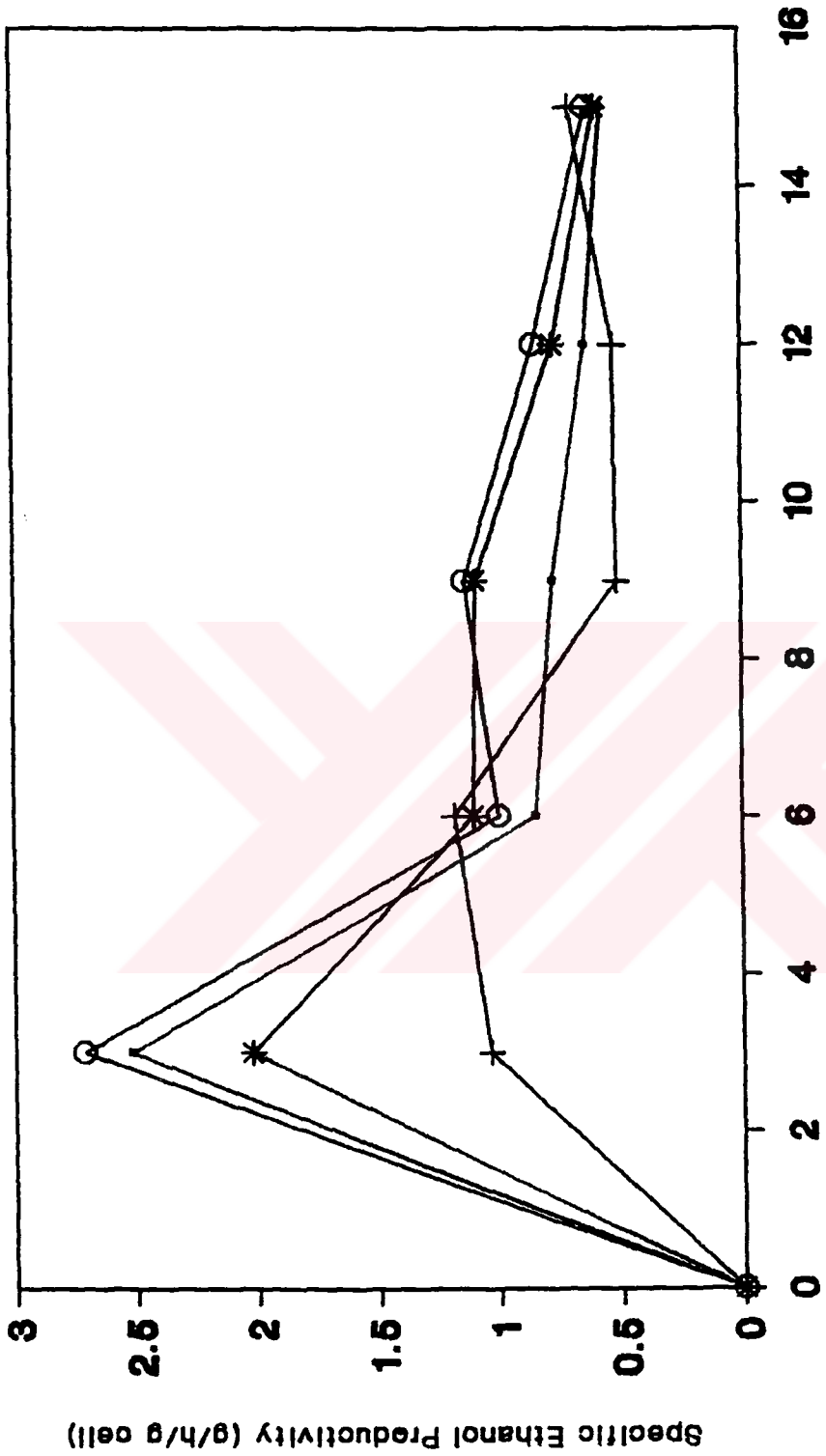

Figure 4.12 Specific Ethanol Productivities Obtained in Experiments Conducted at 6% (.), 10% (+), 12% (*) and 14% (o) Initial Glucose Concentration

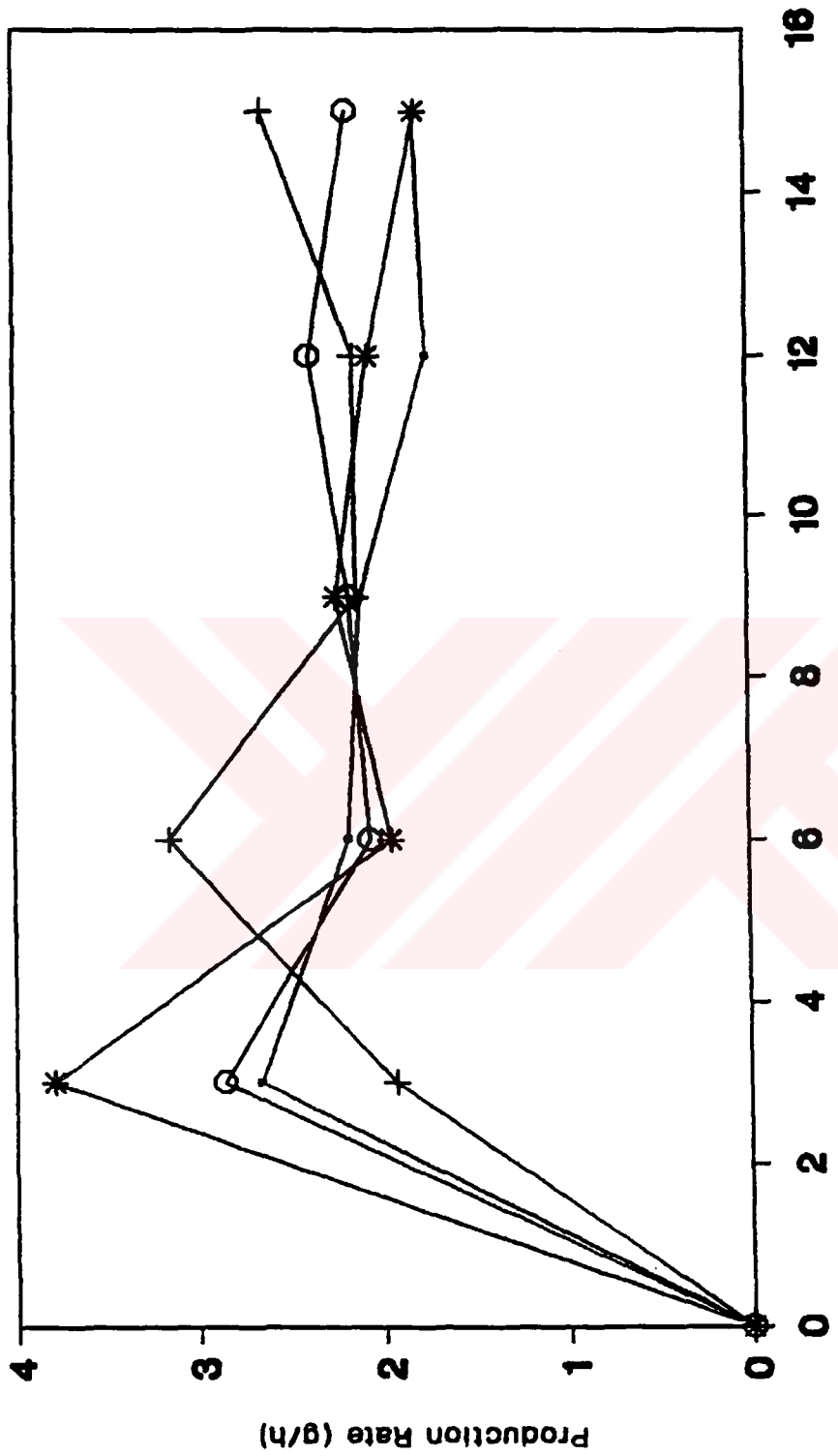

Figure 4.13 Production Rates Obtained in Experiments Conducted at 6% (.), 10% (+), 12% (*) and 14% (o) Initial Glucose Concentration

Finally, Figures 4.14-4.17 give an idea about the changes in the percent conversions of glucose with time for 6, 10, 12 and 14% glucose concentrations. Percent conversion decrease with increasing substrate concentrations.

c. Operational Stability Experiments

To test the stability of the immobilized cell column, it was operated at a recirculation rate of 15 ml/min for 58 hours by replacing the nutrient medium twice after 21 hours and 19 hours of operation respectively. The nutrient medium was chosen to be 10% glucose since it was the optimum value. Figure 4.18 shows that the same constant ethanol and glucose concentrations were reached at the end of three runs which indicates that the same immobilized cell column may be used repeatedly. Towards the end of the third run slight fluctuations in ethanol production were observed.

When the nutrient glucose solution was fed at the bottom of the reactor column, the immobilized yeast cells started to produce ethanol and a considerable amount of CO_2 was liberated spontaneously and was vented through the filter fixed at the top of the column. The CO_2 liberation was higher at the beginning of each run when the medium was fresh than at the end of the runs, but did not cause any bubling or foaming problems.

d. Kinetic Parameter Estimation

In order to investigate their fermentation capabilities and to obtain kinetic data on the growth of *S. cerevisiae* , the four substrate concentrations 6, 10, 12 and 14% glucose supplemented with same quantities of other nutrients were used as the medium for ethanol production in a batch-recycle system (see Section A.2). The concentrations of biomass, glucose and ethanol were measured as a function of time. The overall characteristics of the batch fermentation system studied are summarized in Table 4.9.

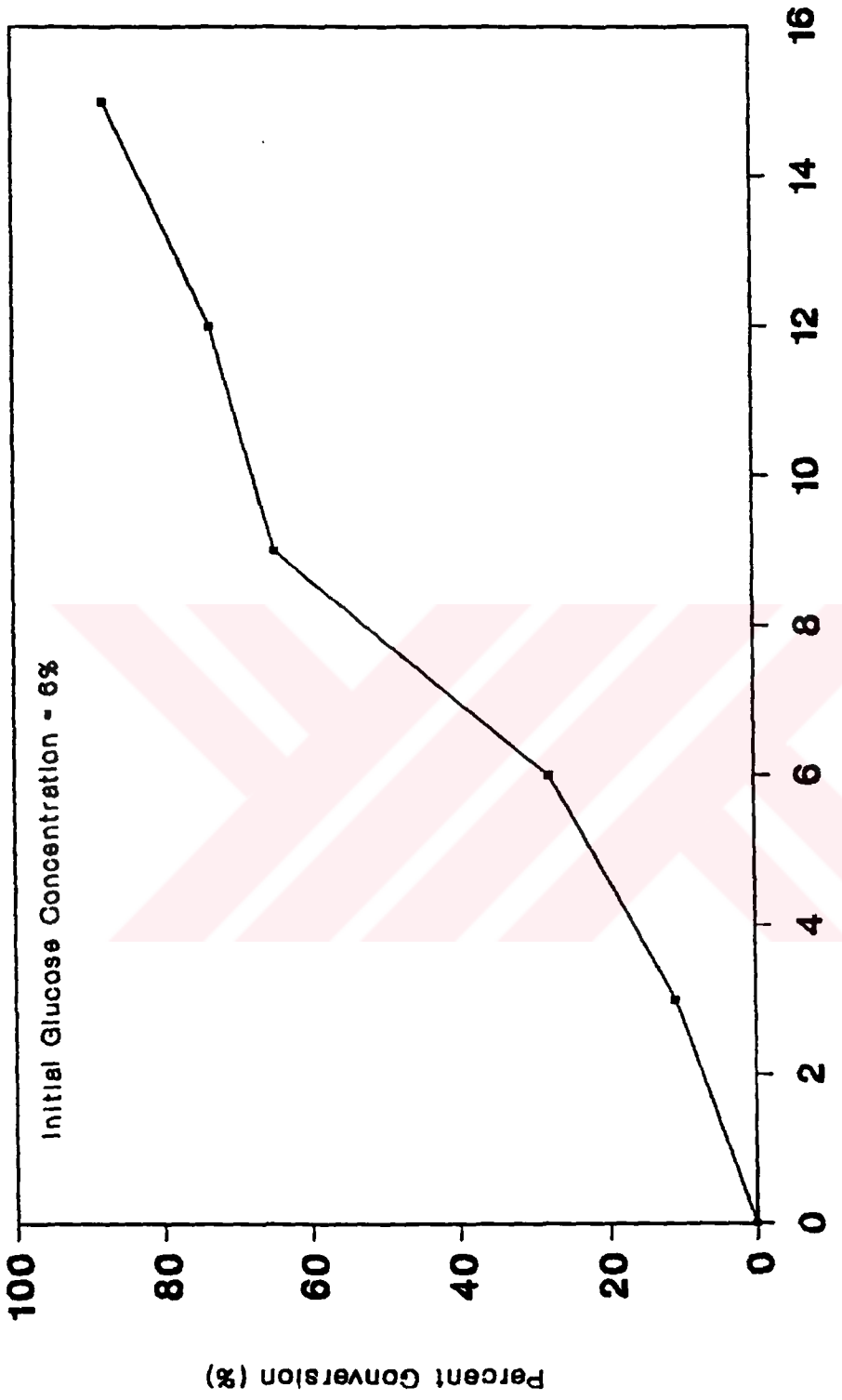

Figure 4.14 Variation of Glucose Conversion During Batch Fermentation

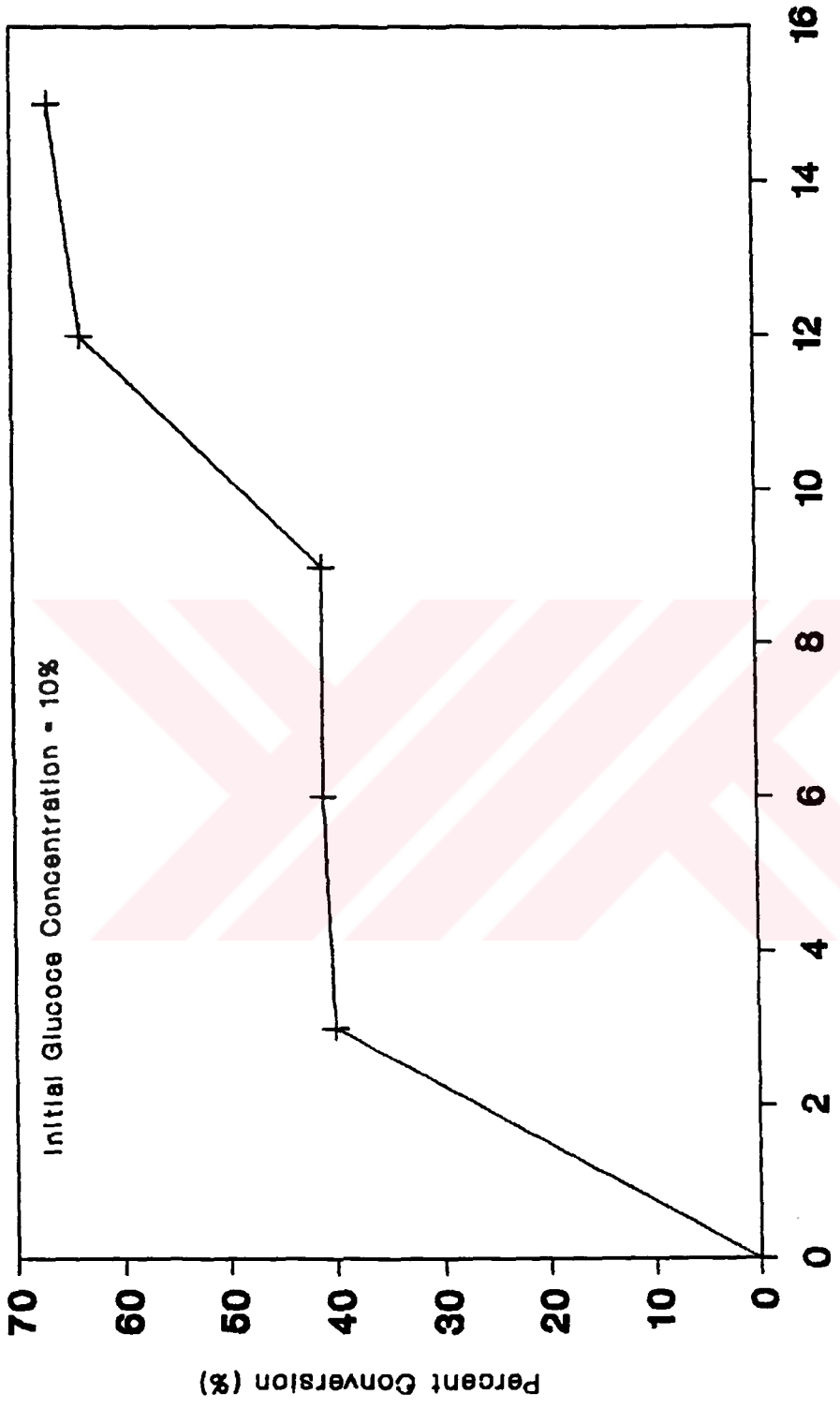

Figure 4.15 Variation of Glucose Conversion During Batch Fermentation

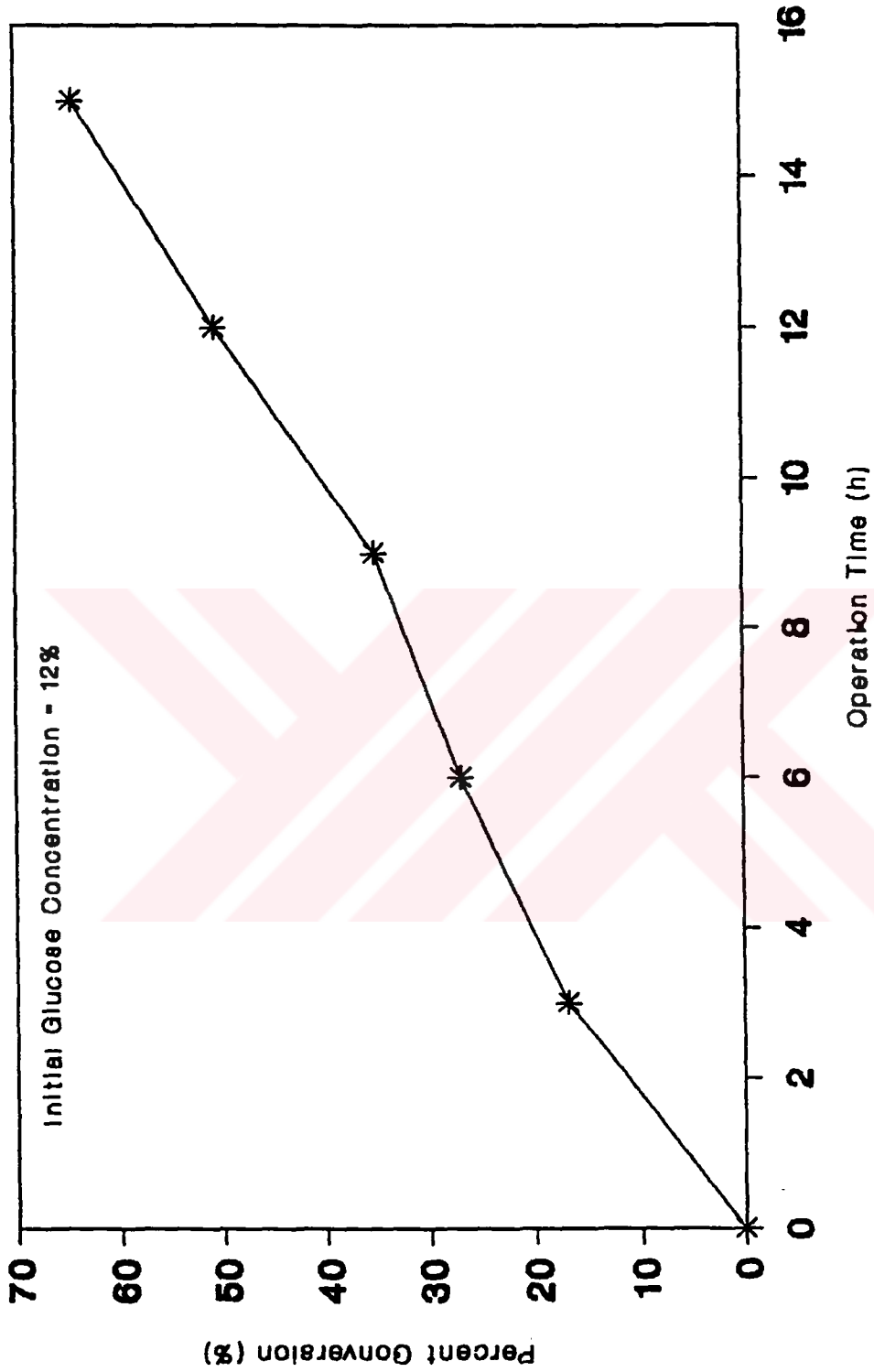

Figure 4.16 Variation of Glucose Conversion During Batch Fermentation

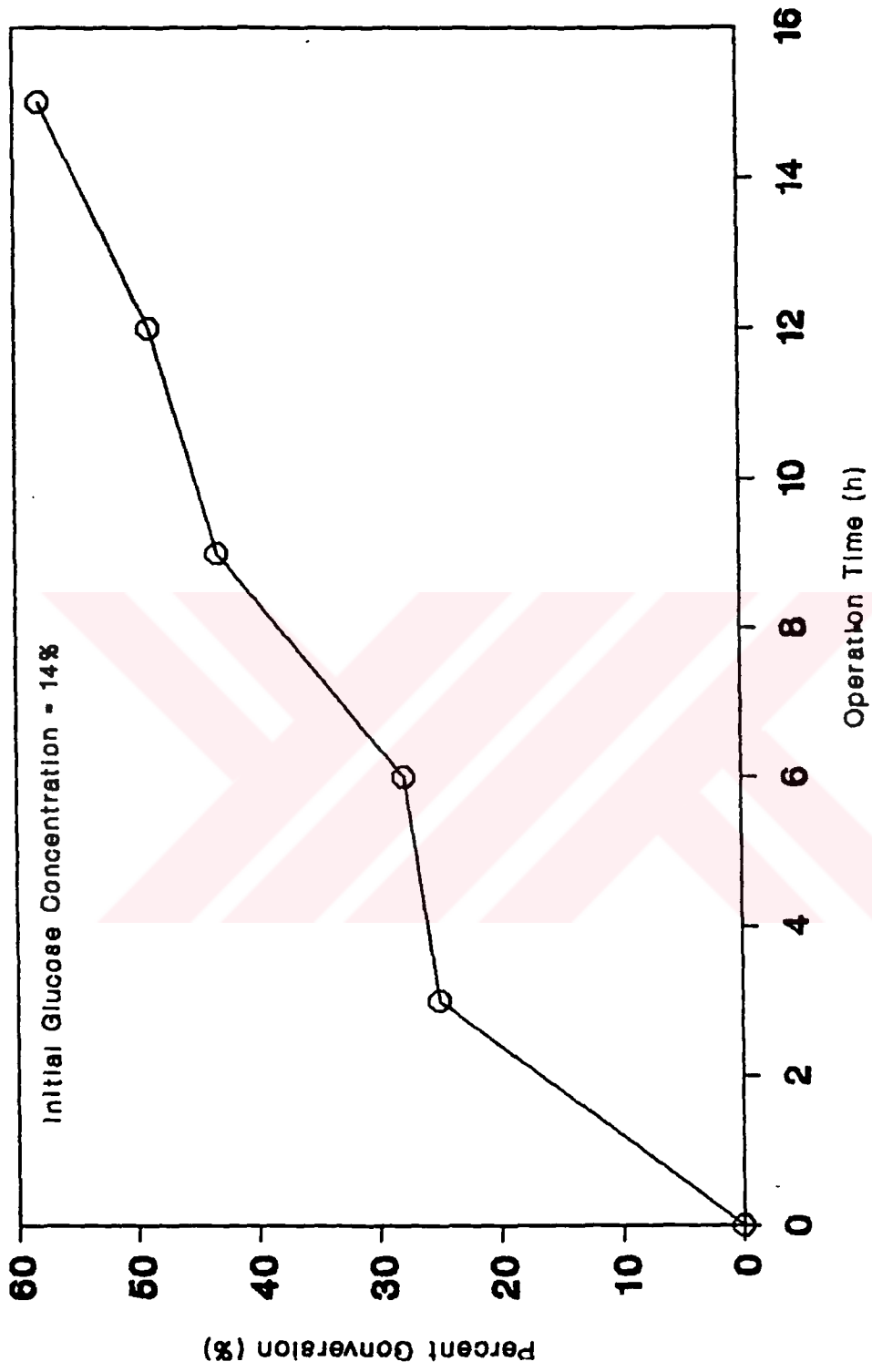

Figure 4.17 Variation of Glucose Conversion During Batch Fermentation

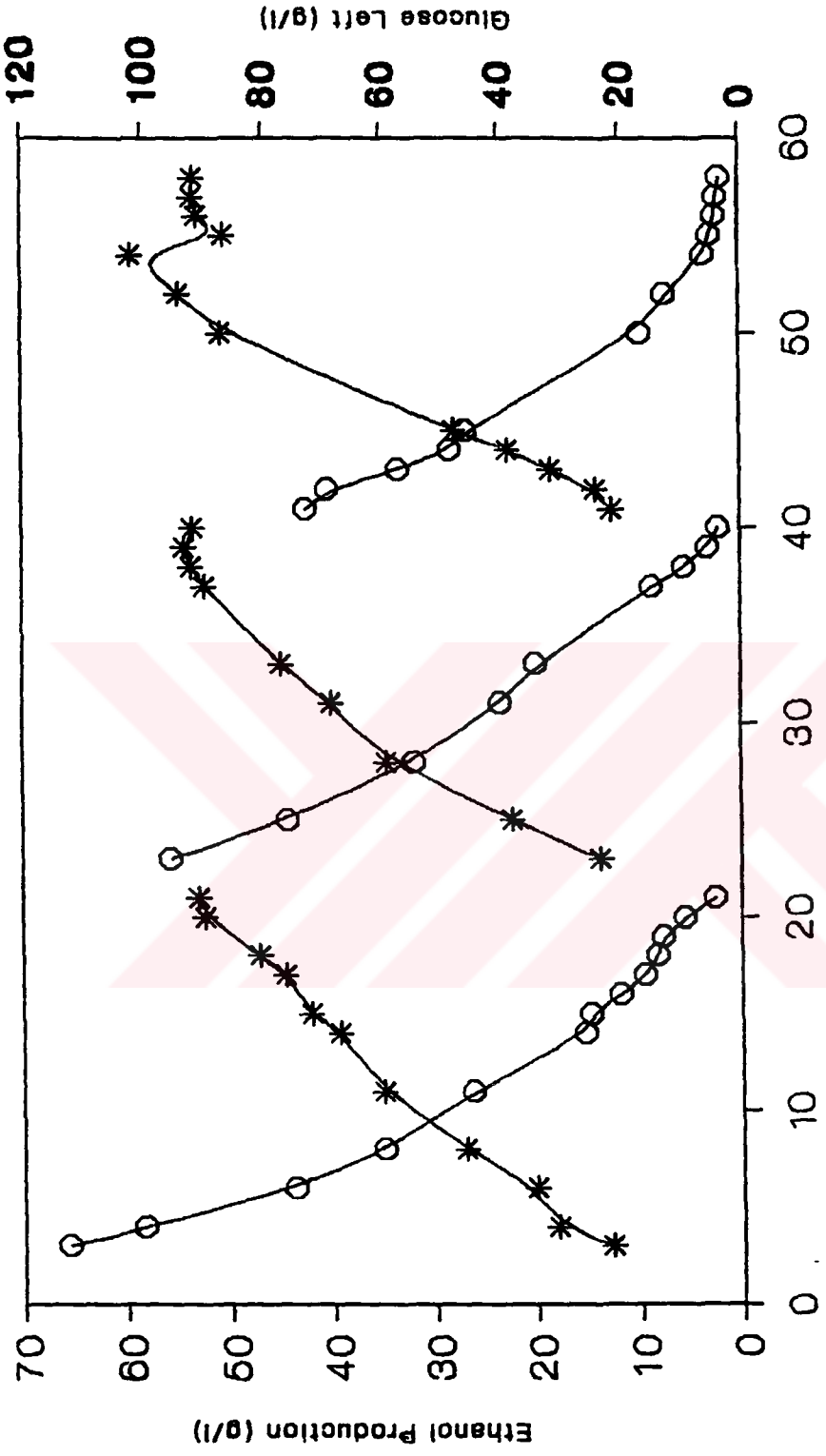

Figure 4.18 Stability Experiment Data of Ethanol Produced (*) and Glucose Left (o) During a 58 h of Operation by Using 10% Initial Glucose Concentration

Table 4.9 Summary of Fermentation Results from Batch-Recycle Experiments

Parameters	6% Glucose Conc.	10% Glucose Conc.	12% Glucose Conc.	14% Glucose Conc.
Fermentation Time (h)	15	15	15	15
Initial Glucose Conc. (g/l)	60	100	120	140
Residual Glucose Conc. (g/l)	7.49	33.50	42.74	59.20
Glucose Conversion (%)	87.52	66.50	64.38	57.84
Biomass Produced (g/l)	1.3595	1.7792	0.7368	1.0041
Ethanol Produced (g/l)	27.16	39.50	26.94	32.50

The Effect of Substrates on Cell Growth

The specific growth rates in the exponential phase were found to be 0.200, 0.124, 0.079 and 0.088 1/h for 6, 10, 12 and 14% glucose concentrations respectively based on the immobilized cell mass data, as shown in Figures 4.1-4.4. Also, in order to investigate the contribution of the free cells in the growth patterns, the specific growth rates were recalculated based on the immobilized and free cells produced as shown in Figures 4.1-4.4. They were 0.220, 0.140, 0.080 and 0.090 1/h for 6, 10, 12 and 14% glucose concentrations respectively. The percent deviations in the specific growth rates are tabulated in Table 4.10. The results indicate that the contribution of free cells does not modify system behavior to an appreciable extent.

A decrease in the specific growth rate was clearly observed with increasing glucose concentration. It should be remembered that the ethanol produced during fermentation is inhibitory and increases the total medium osmolality of the fermentation broth [33], and the effect of inhibition on growth more pronounced for higher substrate concentrations producing more ethanol.

Table 4.10 Kinetic Parameters

Kinetic Parameters	6% Glucose Conc.	10% Glucose Conc.	12% Glucose Conc.	14% Glucose Conc.
Specific Growth Rate (1/h) (Based on Imm'd Cell Mass)	0.200	0.124	0.079	0.088
Specific Growth Rate (1/h) (Based on Imm'd+Free Cell Mass)	0.220	0.140	0.080	0.090
Percent Deviation in Spec. Growth Rates (%)	10	12.9	1.26	2.3
Biomass Yield (g cells/g glucose), $Y_{x/s}$	0.026	0.027	0.009	0.012
Ethanol Yield (g ethanol/g glucose), $Y_{p/s}$	0.517	0.594	0.348	0.408
Ethanol Yield to Biomass Yield	19.88	22.00	38.66	32.90

The dependence of the yeast specific growth rate, μ_T, on the initial glucose concentration is presented in Figure 4.19, using the results obtained on the basis of final immobilized cell mass and total cell mass (immobilized + free cells) within the reactor column at the end of 15 hours of operation. Both graphs display a similar behavior since the μ_T values obtained in the two cases are not greatly affected by the presence of 10-20% free cells. It is also apparent from these two graphs that the functional relationship between the specific growth rate μ and glucose concentration deviates from the simple Monod equation and should be modified to include inhibition effects.

4.3. Selection of Biomass Support Particles

The BSPs used in the present work were 0.7 cm^3 particles of polymeric material having a high degree of interconnecting voids within an open network

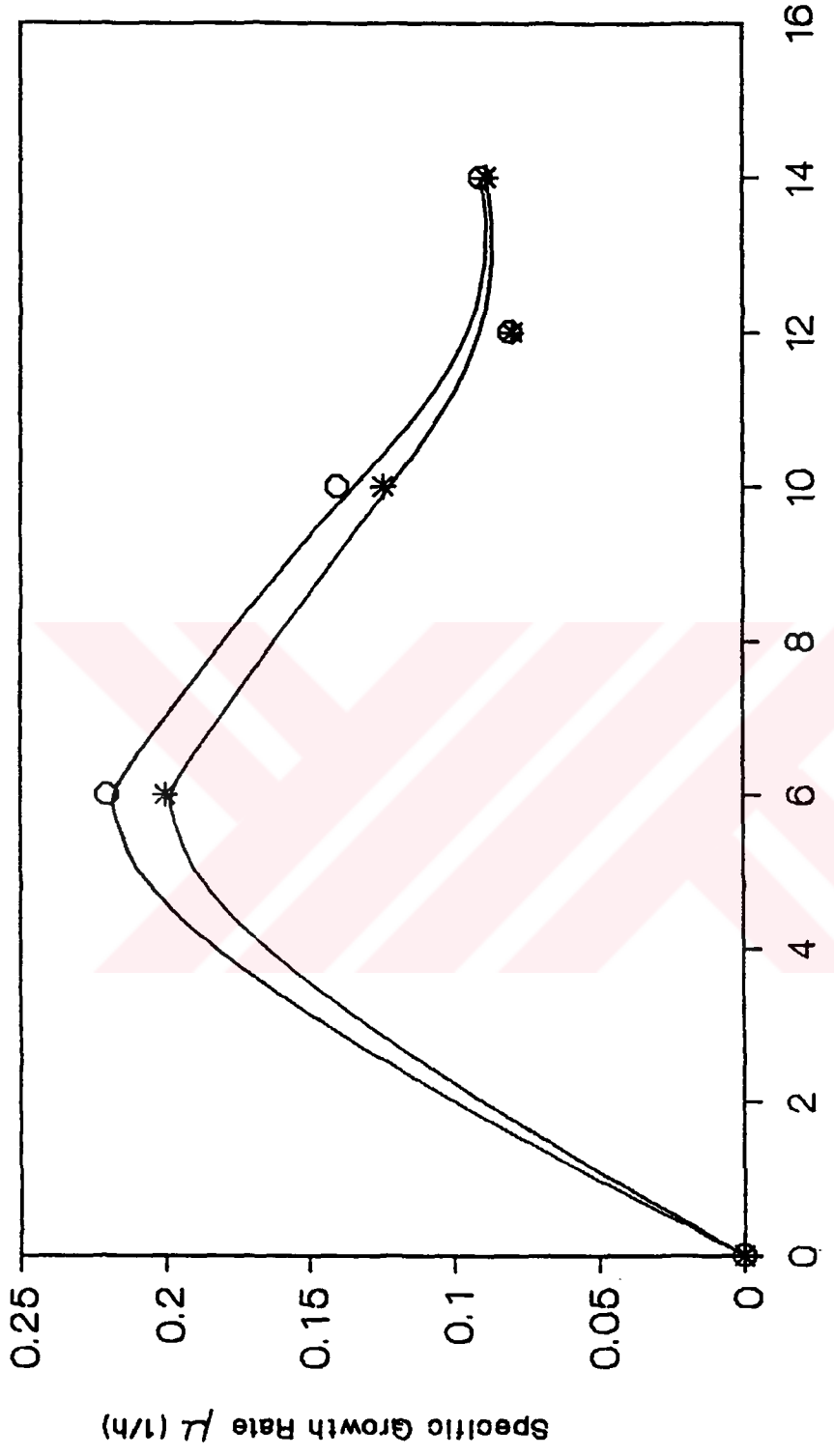

Figure 4.19 Dependence of Yeast Specific Growth Rate on Initial Glucose Concentration Based on Final Immobilized Cell Mass (*) and Total Cell Mass (o)

structure and resembled the support material described by Black et al [30]. The BSPs had a void fraction of about 0.95 and a density of about 0.1 g/cm^3. The pore sizes could not be determined due to the complex nature of the network. However, they were inert, nontoxic to the cell, did not affect the cell metabolism which is one of the most important support criteria mentioned in literature [31], and had relatively high alcohol producing activity [4,13]. They retained high microbial loading which is defined as the amount of dry weight cells incorporated per one gram of carrier [13]. The carriers also provide good substrate diffusion to the cell since they retain high microbial loading and have a high void fraction. The BSPs were stable at elevated temperatures, pressures and at the specified pH and also relatively inexpensive. They could be cut in the desired shapes and sizes which made reactor operation easy. And they did not deform or decolorize during sterilization or fermentation.

4.4. Comparison of the Batch-Recycle and Continuous Experiments

The results obtained with flocculating *S. cerevisiae* cells in parallel experiments [35] in a continuous system have shown that the maximum ethanol production (45.2 g/l) was reached at 8% glucose concentration with a conversion of 82% while the maximum ethanol production reached in the batch-recycle system was 39.5 g/l at 10% glucose concentration with a conversion of 66.5%.

Comparing the specific growth rates, μ , at 10% initial glucose concentration 0.083 g/l for the continuous system and 0.124 g/l for the batch-recycle system, it is observed that cell growth was faster in batch-recycle system.

V. CONCLUSIONS AND RECOMMENDATIONS

The conclusions that can be drawn from the literature survey and experimental studies are summarized below, together with the recommendations for further work.

5.1. Conclusions

1) The results on the rate of ethanol production, specific ethanol productivities and ethanol yield factors have shown that the optimum initial glucose concentration is 10% in the batch-recycle system used.

2) In the batch-recycle system used, the specific growth rates, μ, at 6, 10, 12 and 14% initial glucose concentration were found to be 0.200, 0.124, 0.079 and 0.088 1/h respectively, taking the immobilized cell mass as basis. They were also found to be 0.220, 0.140, 0.080, and 0.090 1/h at 6, 10, 12 and 14% glucose concentrations respectively, when total cell mass (immobilized and free cells) as basis. These results indicate that the contribution of the free cells in cell growth did not modify the behavior of the system to a great extent.

3) The specific growth rates of the BSP-Immobilized cells in the batch-recycle system also showed that the effect of high substrate concentration is to slow down cell growth.

4) The percent conversion of glucose to ethanol decreases with increasing glucose concentration at constant operation time.

5) The durations of the lag and exponential phases increase with increasing glucose concentration. The stationary phase was reached in about 15 hours of operation at 6 and 10% initial glucose concentration while the exponential phase was still retained at the end of 15 hours of operation at 12 and 14% initial glucose concentration.

6) The flocculation ability of the cells are affected by high glucose concentration. At 14% glucose concentration the cells become less flocculant and their immobilization ability is depleted, which leads to fluctuations in the cell growth data.

7) The functional relationship between the specific growth rate m and glucose concentration deviates from the simple Monod equation and should be modified to include inhibition effects.

8) The stability experiments at 10% initial glucose concentration in the batch-recycle system showed that this system can be used up to at least 58 hours of operation by repeated nutrient replacements.

9) The BSPs used in this work are nontoxic to the cell , do not affect the cell metabolism, retain high loading due to high porosity and stable at elevated temperatures and pressures i.e. sterilizable.

10) The specific growth rates, μ, of the BSP-Immobilized cells at 10% initial glucose concentration in the batch-recycle system (0.124 1/h) was found to be higher than that in the continuous system (0.083 1/h) [35].

11) Final immobilized cell mass retained in the batch-recycle column reactor is found to be less than that in the continuous reactor; furthermore, a higher free cell population is attained in the batch-recycle system due to earlier leakage of cells.

12) The yield factor (g ethanol produced/g glucose consumed) is less in batch-recycle system than in continuous system. Whereas, it remains constant with increasing time in continuous system, but increases in batch-recycle system.

13) The use of the batch-recycle systems for laboratory-scale experiments offers the advantage of flexibility in changing operating conditions for the improvement of product quality.

5.2. Recommendations

1) Additional experiments need to be carried out by using free cells only to study the growth pattern of free cells and ethanol production characteristics in the same reactor configuration.

2) Further experiments should be carried out in order to determine the time required for the cells to reach their stationary phase at 12 and 14% glucose concentrations.

3) Additional experiments need to be carried out (a) at shorter intervals of operation time and (b) at one or two other glucose concentrations in order to model cell growth and ethanol production.

4) Operational stability of the immobilized batch-recycle reactor should be tested for 6, 12 and 14% glucose concentrations.

5) Optimum recirculation rates for 6, 12 and 14% glucose concentrations should be investigated in order to improve the ethanol production capability of the system for these substrate concentrations.

APPENDIX A

METHODS OF MICROBIOLOGY

A.1. Preparation of Yeast Peptone Dextrose (YPD) Agar media

For the cultivation of fermentative microorganisms, nutrient agar, the solid counterpart of broth, is used. Nutrient agar provides the nutrient requirements favored by the microorganism for growth. The ingredients of the YPD agar medium used in this work was as follows: 6 g Bacto-yeast extract (1%), 12 g Bacto-peptone (2%), 12 g dextrose (2%), 12 g Bacto-agar (2%) dissolved in 600 ml of distilled water [3,4].

All the ingredients were weighed, dissolved in 600 ml of distilled water and put in a 1-L erlenmeyer flask. The flask was placed on a hot plate magnetic stirrer to dissolve the substances. The solution was then steam-sterilized by autoclaving at 121°C for 15 minutes. After sterilization, the liquid medium was placed in an incubator oven set at 50°C. The solidifying agent in the media, remains liquid at 50°C and solidifies below. Since sterile agar media can only be transferred in the liquid state it should be poured at 45°C, the pouring temperature.

The sterile media was then transferred into sterile petri dishes aseptically over a flame. Plates were incubated and stored at 4°C [4].

A.2. Preparation of Culture Medium

The growth medium used in this work was the same as that previously described by Doran and Bailey [17]. The composition was as follows: 25 g/l KH_2PO_4, 10 g/l $(NH_4)_2SO_4$, 2 g/l $MgSO_4.7H_2O$, 10 g/l yeast extract, 0.5 g/l $CaCl_2$

and 60, 100, 120, 140 g/l glucose for 6%, 10%, 12%, and 14% growth medium respectively.

Yeast extract, $((NH_4)_2SO_4$ $MgSO_4.7H_2O$, and $CaCl_2$ were weighed, dissolved in 350 ml of distilled water and put in a 1.5 L erlenmeyer flask. The phosphate salt was weighed separately, dissolved in 150 ml of distilled water and put in a 250 ml erlenmeyer flask to prevent any precipitation. The culture medium was then steam-sterilized at 121°C for 15 minutes [3,24].

Glucose was weighed, dissolved in 500 ml of sterile distilled water and put in a sterile 1L erlenmeyer flask. Since glucose deteriorates at high temperatures and pressures, it must be either filter sterilized or autoclaved at about 1 atm for 3 minutes.

Prior to each experiment, all the ingredients were transferred to the flask containing the salts by passing through a flame to reduce the risk of contamination. The liquid medium was then stored at 4°C.

A.3. Preparation of Submaster Plates Streaked-plate Technique for Isolation

Pure cultures of microorganisms that form discrete colonies on solid media can be obtained using the streaked plate method. This method involves the separation and immobilization of individual organisms. Each viable organism gives rise, through growth, to a colony from which transfers can be readily made.

When microorganisms are introduced onto the surface of a nutrient agar plate, tens of thousands of cells are actually placed on the medium. The new cells continue to divide by asexual binary fission, i.e. by a splitting in half of the cell, in exponential numbers, resulting in billions of daughter cells. These cells pile up on top of and around each other, and a pure colony is born.

The inoculum is introduced on a metal wire or loop, which is rapidly sterilized just before its use by heating in a flame. Then a series of parallel, nonoverlapping streaks are made on the surface of a solidified agar plate. The inoculum is progressively diluted with each successive streak, so that even if the initial streaks yield confluent growth, well-isolated colonies develop along the lines of later streaks. Colonies subsequently develop embedded in the agar after incubation at 30°C for 48 hours. Each submaster plate is then stored at 4°C and transferred monthly [3,34].

APPENDIX II

EXPERIMENTAL DATA

Table A.II.1 Immobilized Column
Medium Recirculation Rate = 15 ml/min
Substrate Concentration = 6 % Glucose
Operation Time = 3 h. T_{medium} = 30°C

Time (h)	pH	T_{column} (°C)	Glucose Conc. (g/l)	Ethanol Conc. (g/l)
1	4.80	28.8	58.86	6.90
2	4.79	29.1	50.58	7.41
3	4.77	29.3	53.49	7.96

Table A.II.2 Immobilized Column
Medium Recirculation Rate = 15 ml/min
Substrate Concentration = 6 % Glucose
Operation Time = 6 h. T_{medium} = 30°C

Time (h)	pH	T_{column} (°C)	Glucose Conc. (g/l)	Ethanol Conc. (g/l)
1	4.90	28.0	54.13	8.40
2	4.85	29.0	50.99	8.70
3	4.83	29.0	47.36	10.30
4	4.80	28.9	46.37	11.20
5	4.74	29.2	43.73	12.30
6	4.72	29.1	43.40	13.10

Table A.II.3 Immobilized Column
Medium Recirculation Rate = 15 ml/min
Substrate Concentration = 6 % Glucose
Operation Time = 9 h. T_{medium} = 30°C

Time (h)	pH	T_{column} (°C)	Glucose Conc. (g/l)	Ethanol Conc. (g/l)
1	4.81	29.4	54.42	0.00
2	4.78	29.7	41.99	7.10
3	4.74	29.2	40.16	7.61
4	4.69	29.4	44.45	8.46
5	4.67	29.5	38.31	9.53
6	4.59	29.0	37.55	11.54
7	4.45	29.3	27.73	13.14
8	4.36	29.3	26.81	15.66
9	4.23	29.6	21.29	18.96

Table A.II.4 Immobilized Column
Medium Recirculation Rate = 15 ml/min
Substrate Concentration = 6 % Glucose
Operation Time = 12 h. T_{medium} = 30°C

Time (h)	pH	T_{column} (°C)	Glucose Conc. (g/l)	Ethanol Conc. (g/l)
1	4.83	28.0	43.99	0.00
2	4.79	28.1	45.98	4.92
3	4.77	28.8	40.62	5.75
4	4.36	28.9	39.01	12.19
5	4.56	28.9	38.93	7.15
6	4.53	28.6	38.62	8.56
7	4.49	29.0	34.02	9.57
8	4.37	30.0	22.52	11.13
9	4.30	29.1	21.14	13.68
10	4.12	29.1	24.67	14.00
11	4.01	28.9	18.84	18.17
12	3.92	29.2	16.08	21.02

Table A.II.5 Immobilized Column
Medium Recirculation Rate = 15 ml/min
Substrate Concentration = 6 % Glucose
Operation Time = 15 h. T_{medium} = 30°C

Time (h)	pH	T_{column} (°C)	Glucose Conc. (g/l)	Ethanol Conc. (g/l)
1	4.87	28.3	50.28	3.33
2	4.85	28.1	44.14	3.59
3	4.82	28.3	48.59	4.53
4	4.75	28.4	45.52	5.35
5	4.73	28.2	40.15	8.70
6	4.63	28.3	38.31	9.27
7	4.58	28.4	31.87	9.52
8	4.47	28.5	29.88	11.07
9	4.38	28.4	31.26	13.03
10	4.30	28.1	28.04	15.81
11	4.17	28.2	23.44	17.36
12	4.04	28.3	16.69	21.64
13	3.99	28.1	15.16	23.16
14	3.88	28.0	12.39	24.35
15	3.86	28.0	7.49	27.16

Table A.II.6 Immobilized Column
Medium Recirculation Rate = 15 ml/min
Substrate Concentration = 10 % Glucose
Operation Time = 3 h. T_{medium} = 30°C

Time (h)	pH	T_{column} (°C)	Glucose Conc. (g/l)	Ethanol Conc. (g/l)
1	4.72	29.1	98.14	4.73
2	4.70	29.7	73.12	5.53
3	4.69	30.1	59.80	5.79

Table A.II.7 Immobilized Column
Medium Recirculation Rate = 15 ml/min
Substrate Concentration = 10 % Glucose
Operation Time = 6 h. T_{medium} = 30°C

Time (h)	pH	T_{column} (°C)	Glucose Conc. (g/l)	Ethanol Conc. (g/l)
1	4.71	29.4	116.9	13.3
2	4.70	30.3	113.4	14.1
3	4.68	30.1	108.4	14.9
4	4.67	29.6	107.4	15.9
5	4.63	29.3	97.9	16.8
6	4.62	29.7	92.0	18.9

Table A.II.8 Immobilized Column
Medium Recirculation Rate = 15 ml/min
Substrate Concentration = 10 % Glucose
Operation Time = 9 h. T_{medium} = 30°C

Time (h)	pH	T_{column} (°C)	Glucose Conc. (g/l)	Ethanol Conc. (g/l)
1	4.81	28.2	81.48	1.98
2	4.78	29.3	75.00	5.23
3	4.74	29.4	71.16	3.75
4	4.69	29.6	75.66	4.80
5	4.67	30.0	65.61	7.78
6	4.59	29.8	71.83	9.52
7	4.45	29.9	62.43	18.40
8	4.36	30.3	60.98	10.38
9	4.23	30.4	58.59	19.16

Table A.II.9 Immobilized Column
Medium Recirculation Rate = 15 ml/min
Substrate Concentration = 10 % Glucose
Operation Time = 12 h. T_{medium} = 30°C

Time (h)	pH	T_{column} (°C)	Glucose Conc. (g/l)	Ethanol Conc. (g/l)
1	4.75	29.6	79.92	2.71
2	4.72	29.9	87.36	3.40
3	4.65	30.0	86.59	4.91
4	4.51	29.9	76.00	6.22
5	4.45	29.8	78.30	7.02
6	4.38	30.1	64.02	9.17
7	4.23	30.1	67.25	11.52
8	4.12	30.3	72.32	14.30
9	4.02	30.2	53.43	16.18
10	3.87	30.1	51.13	21.10
11	3.77	29.9	40.22	24.02
12	3.68	30.5	36.38	25.64

Table A.II.10 Immobilized Column
Medium Recirculation Rate = 15 ml/min
Substrate Concentration = 10 % Glucose
Operation Time = 15 h. T_{medium} = 30°C

Time (h)	pH	T_{column} (°C)	Glucose Conc. (g/l)	Ethanol Conc. (g/l)
1	4.74	28.2	100.1	12.60
2	4.73	30.4	103.4	12.90
3	4.71	30.5	96.80	13.30
4	4.70	30.4	97.00	14.10
5	4.68	30.1	90.10	14.40
6	4.67	29.3	90.80	15.50
7	4.65	29.1	89.90	17.10
8	4.62	29.3	81.70	18.50
9	4.61	29.2	79.40	20.40
10	4.58	29.8	78.60	23.40
11	4.57	31.2	66.19	24.23
12	4.54	31.1	60.80	25.30
13	4.51	31.5	48.10	33.30
14	4.50	30.9	36.12	36.70
15	4.49	30.7	33.50	39.50

Table A.II.11 Immobilized Column
Medium Recirculation Rate = 15 ml/min
Substrate Concentration = 12 % Glucose
Operation Time = 3 h. T_{medium} = 30°C

Time (h)	pH	T_{column} (°C)	Glucose Conc. (g/l)	Ethanol Conc. (g/l)
1	4.83	29.4	106.14	7.91
2	4.82	29.4	104.81	8.83
3	4.80	29.5	100.00	11.34

Table A.II.12 Immobilized Column
Medium Recirculation Rate = 15 ml/min
Substrate Concentration = 12 % Glucose
Operation Time = 6 h. T_{medium} = 30°C

Time (h)	pH	T_{column} (°C)	Glucose Conc. (g/l)	Ethanol Conc. (g/l)
1	4.82	28.7	103.49	8.20
2	4.81	28.7	106.80	8.60
3	4.76	28.7	103.80	9.20
4	4.75	28.8	97.88	10.10
5	4.69	28.9	90.03	10.89
6	4.67	29.1	87.81	11.70

Table A.II.13 Immobilized Column
Medium Recirculation Rate = 15 ml/min
Substrate Concentration = 12 % Glucose
Operation Time = 9 h. T_{medium} = 30°C

Time (h)	pH	T_{column} (°C)	Glucose Conc. (g/l)	Ethanol Conc. (g/l)
1	4.70	27.5	109.11	8.80
2	4.68	28.3	102.33	9.20
3	4.65	28.1	104.82	10.60
4	4.63	28.2	84.51	10.90
5	4.56	28.0	82.19	11.10
6	4.53	28.1	94.58	13.00
7	4.42	28.2	95.24	15.30
8	4.37	27.5	77.08	15.90
9	4.25	28.0	77.90	20.20

Table A.II.14 Immobilized Column
Medium Recirculation Rate = 15 ml/min
Substrate Concentration = 12 % Glucose
Operation Time = 12 h. T_{medium} = 30°C

Time (h)	pH	T_{column} (°C)	Glucose Conc. (g/l)	Ethanol Conc. (g/l)
1	4.80	29.1	107.13	0
2	4.76	28.9	104.48	4.84
3	4.75	29.2	103.16	5.64
4	4.68	28.6	91.77	6.00
5	4.66	28.7	90.78	6.21
6	4.63	28.8	88.64	7.47
7	4.61	29.0	87.11	9.44
8	4.60	29.3	83.19	12.73
9	4.58	29.3	92.27	14.30
10	4.57	29.4	75.59	16.91
11	4.54	29.1	60.07	18.74
12	4.49	29.3	59.20	24.79

Table A.II.15 Immobilized Column
Medium Recirculation Rate = 15 ml/min
Substrate Concentration = 12 % Glucose
Operation Time = 15 h. T_{medium} = 30°C

Time (h)	pH	T_{column} (°C)	Glucose Conc. (g/l)	Ethanol Conc. (g/l)
1	4.84	28.5	117.36	6.23
2	4.83	28.2	88.64	6.75
3	4.82	28.1	87.97	7.97
4	4.79	28.4	88.47	7.70
5	4.77	28.0	87.48	8.40
6	4.75	28.4	76.09	8.30
7	4.71	28.2	76.25	10.18
8	4.68	28.0	96.56	12.37
9	4.65	28.0	95.40	14.46
10	4.63	28.0	82.53	17.63
11	4.61	27.9	74.10	19.32
12	4.59	27.9	70.31	21.80
13	4.57	27.9	55.45	22.98
14	4.53	27.8	55.12	26.56
15	4.48	28.1	42.74	26.94

Table A.II.16 Immobilized Column
Medium Recirculation Rate = 15 ml/min
Sustrate Concentration = 14 % Glucose
Operation Time = 3 h. T_{medium} = 30°C

Time (h)	pH	T_{column} (°C)	Glucose Conc. (g/l)	Ethanol Conc. (g/l)
1	4.70	29.4	107.63	7.27
2	4.67	29.9	106.08	8.02
3	4.65	30.1	105.02	8.57

Table A.II.17 Immobilized Column
Medium Recirculation Rate = 15 ml/min
Substrate Concentration = 14 % Glucose
Operation Time = 6 h. T_{medium} = 30°C

Time (h)	pH	T_{column} (°C)	Glucose Conc. (g/l)	Ethanol Conc. (g/l)
1	4.68	28.1	135.08	7.13
2	4.56	28.5	101.95	8.24
3	4.54	29.3	114.53	9.36
4	4.49	29.5	106.56	11.22
5	4.45	29.3	92.75	11.99
6	4.36	29.9	101.09	12.34

Table A.II.18 Immobilized Column
Medium Recirculation Rate = 15 ml/min
Substrate Concentration = 14 % Glucose
Operation Time = 9 h. T_{medium} = 30°C

Time (h)	pH	T_{column} (°C)	Glucose Conc. (g/l)	Ethanol Conc. (g/l)
1	4.65	28.8	102.87	7.91
2	4.62	28.8	90.30	8.31
3	4.55	29.7	86.93	8.66
4	4.45	29.9	105.02	10.61
5	4.38	29.0	85.85	12.27
6	4.33	30.2	85.70	11.82
7	4.22	30.5	89.36	16.01
8	4.07	30.4	82.02	18.75
9	3.97	30.2	79.56	19.53

Table A.II.19 Immobilized Column
Medium Recirculation Rate = 15 ml/min
Sustrate Concentration = 14 % Glucose
Operation Time = 12 h. T_{medium} = 30°C

Time (h)	pH	T_{column} (°C)	Glucose Conc. (g/l)	Ethanol Conc. (g/l)
1	4.73	29.1	116.8	7.00
2	4.72	29.9	99.81	8.72
3	4.70	30.6	108.86	8.61
4	4.66	30.8	120.66	9.04
5	4.60	31.4	87.38	10.26
6	4.52	31.2	107.78	13.64
7	4.42	31.3	96.43	13.96
8	4.37	30.9	100.57	15.44
9	4.14	31.1	80.02	20.27
10	4.09	31.0	78.80	23.14
11	3.96	31.3	77.26	28.04
12	3.96	31.3	71.74	28.50

Table A.II.20 Immobilized Column
Medium Recirculation Rate = 15 ml/min
Substrate Concentration = 14 % Glucose
Operation Time = 15 h. T_{medium} = 30°C

Time (h)	pH	T_{column} (°C)	Glucose Conc. (g/l)	Ethanol Conc. (g/l)
1	4.67	30.0	88.77	7.79
2	4.52	30.3	96.58	8.40
3	4.50	30.6	94.59	8.46
4	4.59	30.6	87.23	8.52
5	4.45	30.5	81.25	10.99
6	4.37	30.6	78.49	11.53
7	4.30	30.3	80.03	12.45
8	4.23	30.6	79.87	16.60
9	4.09	30.2	83.71	17.61
10	4.00	30.4	73.58	20.07
11	3.90	30.4	77.72	21.85
12	3.72	29.9	65.31	24.32
13	3.66	30.2	57.18	27.63
14	3.55	30.4	56.71	32.65
15	3.52	30.4	59.02	32.50

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