The Immobilization of

Kluyveromyces fragilis and *Saccharomyces cerevisiae*

in

Polyacrylamide Gel

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ABSTRACT

The search for new energy sources has indicated that biomass, in the form of green plant materials and biological wastes, can provide a perpetual energy source if converted to a useful form. This study investigated the production of ethanol by the fermentation of sugars using immobilized cells.

The experimental procedure involved the immobilization of two yeast species, Kluyveromyces fragilis NRRL Y 1109 and Saccharomyces cerevisiae NCYC 240, in polyacrylamide gel for the fermentation of lactose and glucose respectively. The gel methodology of two previous authors, Chibata et al. (1974) and Neuhoff (1973) was used. The former author's gel was used as a basis for batch experiments to determine the gel composition for maximum ethanol producing activity by both cell species as initial trials with this gel yielded encouraging results.

Variations in monomer, BIS and cell concentration revealed that a gel containing 15% ($\frac{w}{v}$) acrylamide, 1.5% ($\frac{w}{v}$) BIS and 25% ($\frac{w}{v}$) cells in addition to 0.6% ($\frac{w}{v}$) BDMAP and 0.25% ($\frac{w}{v}$) ammonium persulfate in tris-HCl buffer pH 7.1 polymerised at $0^{o}C$ produced the greatest activity in immobilized K. fragilis cells with an activity retention for immobilization of 80%. The gel composition for greatest activity in immobilized S. cerevisiae cells differed only slightly from that above containing 20% ($\frac{w}{v}$) acrylamide, 1.6% ($\frac{w}{v}$) BIS and 40% ($\frac{w}{v}$) cells and resulted in a 46% activity retention for immobilization. Further experiments at various substrate concentrations indicated that the gel imposed small or negligible limitations on the diffusion of substrate and product.

Experiments to increase the cell activity retention for the immobilization of S. cerevisiae using the Neuhoff (1973) gel were unsuccessful but produced some important results. It was found that exposure to gel components, especially to the acrylamide monomer, reduced the ethanol producing ability and the viability of the cells. The general protective agents Tween 80, glycerol, gelatin and dithiothreitol proved ineffective. To minimize this damage to the cells the gels were polymerised at $0^{o}C$ with rapid polymerisation being induced by high initiator and accelerant concentrations.

Repeated use of the immobilized cells indicated that the simple substrate medium, of the sugar in distilled water used previously, was not sufficient to maintain stable ethanol producing activity. Although trials involving supplementation with a salt solution were unsuccessful, the incorporation 0.5% ($\frac{w}{v}$) peptone in the medium and the use of protein-containing media, such as whey, was found to stabilize activity.

Experiments in continuous processing revealed that immobilized K. fragilis cells produced ethanol from deproteinised whey at an efficiency of 70 to 80% over extended periods with complete substrate utilization of full strength whey being achieved at flowrates of 0.15 SV. The half life of the activity of the immobilized cells was estimated to be at least 50 days.

The experimental results suggest that this approach to fermentation may be industrially acceptable for the production of ethanol. However, a costing exercise on the production of ethanol from whey indicates that unless the product is a highly priced commodity, such as a pharmaceutical, the process is unlikely to be economically feasible due to the high cost of the immobilization support monomer.

LIST OF ABBREVIATIONS

BDMAP	β-dimethylaminopropionitrile
BIS	N,N'-methylene-bisacrylamide
BOD	Biological Oxygen Demand
COD	Chemical Oxygen Demand
^{o}C	Temperature in degrees celcius
DO	Dissolved Oxygen
g	Gravitational force
HEMA	2-hydroxyethyl methacrylate
hr	hour
Km	Michealis Constant
M	Molar
mA	milliampere
MEA	Malt Extract Agar
ml	millilitre
mM	milliMolar
NAD^{+},NADH	β-nicotinamide adenine dinucleotide
PDA	Potato Dextrose Agar
%	percentage
rpm	Rotational speed, revolutions per minute
SV	Space Velocity. Working volumes per hour
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
Vmax	Maximum velocity
% $(\frac{v}{v})$	component composition expressed as percentage volume per unit volume
% $(\frac{w}{v})$	component composition expressed as percentage weight per unit weight

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CHAPTER 1

INTRODUCTION

This study investigates an approach to industrial processing that may be applied to the energy production and waste disposal fermentations of the future, i.e. the use of immobilized cells.

The current energy crisis, which has been initiated by the diminishing supply of fossil fuels, has prompted a search for new energy sources. One plentiful and universal energy source is solar radiation. The collection of this radiation via plant photosynthesis provides a perpetual energy source in the form of plant biomass. Waste disposal can also be an energy producing process. The breakdown of biodegradable wastes, or biomass, can in some cases yield more energy than that required for the actual process.

A number of methods have been proposed for the conversion of the biomass to energy. Methane can be produced by pyrolysis, hydrogasification and anaerobic digestion. Some research has been directed towards the biological production of hydrogen and ethanol may be produced by fermentation of the sugars present.

The latter process is of interest in this study. With notable exceptions, such as the production of beer by continuous fermentation in New Zealand, fermentation traditionally has been a batch operation using intact cells or cell free extracts. In many cases the extent of processing has been limited by the high cost due to the catalyst being used only once and the labour intensive nature of the process. The significance of these factors may be reduced considerably by the use of continuous fermentation, a mode of operation made possible by the use of immobilized catalysts.

Recent developments in immobilization techniques have enabled the introduction of continuous processing in such areas as the production of pharmaceuticals and industrial chemicals, the treatment of specialized wastes and scientific analysis. A wide range of immobilization techniques have been used for both cells and enzymes. These techniques range from the mild adsorption, microencapsulation and entrapment procedures to more rigorous covalent and crosslinking procedures which may markedly change the properties of the cells and enzymes.

The most widely used method of whole cell immobilization, entrapment, was used in this study. This method of trapping cells in a polymer network is a stable form of immobilization which has a relatively mild effect on the cells. The polymer chosen for this study was polyacrylamide gel, a synthetic polymer which has been used for the successful immobilization of many enzymes, bacteria and fungi.

Energy production, in the form of alcohol fermentation from sugars, has been the topic of few investigations. In this study immobilized Kluyveromyces fragilis NRRL Y 1109 will be used for the production of ethanol from lactose and immobilized Saccharomyces cerevisiae NCYC 240 will be used to produce ethanol from glucose. For industrial processing it is envisaged that a supply of raw materials could be maintained using whey as a source of lactose and energy crops such as sugarbeet or wood as a source of carbohydrate.

The aim of this study was to assess the method of immobilization for each species and to determine its probable feasibility for industrial processing. Initially two similar polyacrylamide gel formulations were used for the immobilization of both cell species with the formulation producing the highest activity being used in further experiments to maximize this activity. Aspects of the enzymic reaction were also studied using immobilized cells in continuous reactors.

CHAPTER 2

REVIEW OF THE RELEVANT LITERATURE

2.1 INTRODUCTION

The natural existence of immobilized cells has been known since the time of the Roman empire when fig tree twigs were used to stir and curdle milk (Weetall, 1976). One of the earliest examples of commercial production with immobilized cells was vinegar manufacture in the 1930s using bacteria adsorbed onto wood chips and shavings (Fetzer, 1930). The principle of immobilized cells has been used for many years in the waste treatment industry in the operation of trickling filters.

Further applications of the immobilization technique to industrial processing did not occur until the 1960s. The initial research was limited to the immobilization of enzymes (Bernfeld and Wan, 1963; Tosa et al., 1966). By 1972 there were two industrial processes using immobilized enzymes. Penicillin amidase, covalently bonded to derivatised cellulose was used to produce 6-amino penicillanic acid and columns of aminoacylase on DEAE-Sephadex were used for the resolution of D-L amino acids (Abbott, 1976).

The range of catalysts immobilized diversified in the late 1960s with the immobilization of lichen (Mosbach and Mosbach, 1966), fungal spores (Johnson and Ciegler, 1969) and finally cells (Updike et al., 1969). In 1973 the first commercial process using immobilized cells was commissioned for the production of L-aspartic acid (Chibata and Tosa, 1977).

In recent years reviews of immobilization techniques have been presented by Zaborsky (1973), Messing (1975, 1978), Weetall (1976), Abbott (1976, 1977, 1978), Chibata and Tosa (1977), Barker and Somers (1978), Brodelius (1978) and Durand and Navarro (1978).

2.2 CLASSIFICATION OF IMMOBILIZATION TECHNIQUES

Immobilization was defined by Zaborsky (1973) as "the physical confinement or localization of enzymes" and cells "during a continuous catalytic process" permitting economic reuse of the catalyst (Abbott, 1977). This is a broad description emphasising the continuous mode of operation made possible by immobilization. It also encompasses the use of dialysis (Schultz and Gerhardt, 1967), flocculation (Lee and Long, 1974; Kennedy et al., 1976), hollow fibres (Kan and Shuler, 1978) and newer methods such as entrapment in photohardened resins, as reviewed by Abbott (1978), as means of immobilization.

Classically immobilized cells or enzymes are chemically or physically attached to, or entrapped in, a water insoluble matrix or support. A classification of these methods given by Weetall (1976) included seven basic types of immobilization.

1. Adsorption, the physical attachment of the cell to clays, sand, glass, mineral salts and organic polymers.

2. Entrapment, the physical confinement of the cell within the lattice structure of a synthetic or natural polymer gel or collagen.

3. Microencapsulation, the inclusion of cells within microcapsules of semipermeable membranes such as cellulose acetate or nylon.

4. Ion exchange, cell attachment due to a charge difference between the cells and supports such as ion exchange resins.

5. Crosslinking, the covalent interaction between the cells and bifunctional reagents such as glutaraldehyde di-isothiocynates and arylamines.

6. Copolymerisation, the covalent inclusion of the cell in a polymer matrix.

7. Covalent attachment, the covalent binding of the cell to an insoluble support material with functional groups.

Similar classifications were given by Zaborsky (1973), Gutcho (1974), Chibata and Tosa (1977) and Jack and Zajic (1977).

Of these methods adsorption (Seyhan and Kirwan, 1979), entrapment (Sato et al., 1976), microencapsulation (Mohan and Li, 1975), cross-linking (Petre et al., 1978) and covalent attachment (Jack, 1977) have been used for the immobilization of whole cells. Entrapment has been the most widely used method with polyacrylamide gel being the most popular support although acrylic (Kimura et al., 1978; Jirku et al., 1979), alginate (Kierstan and Bucke, 1977) and carrageenan gels (Chibata, 1977) and collagen membranes (Saini and Vieth, 1975) have been used for entrapment.

2.3 ADVANTAGES OF IMMOBILIZATION

The motivation for research into immobilized cells is provided by the favourable properties bestowed on the cells due to the immobilization procedure.

The most important factor is the possibility of repeated and continuous use of the same catalyst. Continuous production is an economically attractive mode of operation.

Another important factor is the variation in cell properties that may occur on immobilization. In most cases immobilized cells have had a greater heat stability than their free counter parts. Other widely found variations were broader pH and temperature working ranges and longer half lives. In rare cases (Kennedy et al., 1976) the cells have had greater activity after immobilization.

Where immobilized cells have been used in industrial processing minimal contamination of the product by the catalyst has occurred eliminating the need for costly separation procedures. An immobilized catalyst system can be stopped by the removal of the support making additions of chemicals or heat unnecessary, an important factor for consumer goods where the contents must be stated on the packing. The cells can also be used in a non-growth state where the opportunity for side reactions is limited.

Immobilized cells present opportunities for increasing reaction rates. Removing the cells from the substrate liquid, by immobilization, decreases the liquid viscosity promoting better mixing and increasing mass transfer (Abbott, 1977). The incorporation of large numbers of cells to increase the reaction rate is also possible. Thus a higher productivity can be achieved.

The immobilization of cells is preferable to the immobilization of enzymes, which required costly extraction and purification operations and tend to have less stable activity. The use of whole cells also introduces the possibility of multistep reactions as well as the regeneration of cofactors which are frequently required for immobilized enzyme reactions.

2.4 DISADVANTAGES OF IMMOBILIZATION

With present technology the advantages of immobilization outweigh the disadvantages.

The most commonly found disadvantage was the loss of activity caused by the immobilization procedure. Inactivation by the support media was common and could sometimes be minimized by changes in the support composition or in the immobilized environment. Diffusion limitations may also have been imposed by the support, reducing activity by slow diffusion of substrate to the cell or by a build up of inhibitive product in the support (Abbott, 1977).

Other less commonly found disadvantages were the possibility of the accumulation of toxic materials in the support and that of electrostatic repulsion of substrate and support. Leakage from supports was notable only in the less stable forms of immobilization such as flocculation although where cell growth occurred within a support the new cells may have been expelled. Leakage of essential cofactors was found in some polyacrylamide gel immobilized cells (Chibata and Tosa, 1977) and appeared to be due to damage to the cell membrane. Certain metal ions were needed to maintain activity in these cases.

Activity loss by unwanted side reactions has been noted in several papers (Yamamoto et al., 1974b, 1976). Heat and chemicals were used to stop the side reactions with no apparent effect on the main reaction.

2.5 RELEVANT PROPERTIES OF YEAST CELLS

The yeast used in this study belong to the tribe Saccharomyceteae in the subfamily Saccharomycetoideae which is characterised by loose budding cells and pseudomycelium with vegetative production by multilateral budding.

Two modes of metabolism are evident in these yeasts.

Anaerobic metabolism is the energy releasing transformation of carbohydrates by the cells from which the major end product is ethanol. Minor products such as glycerol, organic acids and fusel oils have also been known to accumulate in concentrations dependent on the environmental conditions of fermentation (Neish and Blackwood, 1951; Nordström, 1968).

Aerobic metabolism is the predominant energy yielding mechanism of the cell in which sugars are converted to carbon dioxide and water.

The metabolic pathway used may be determined by environmental conditions and/or certain regulatory effects. The Pasteur effect, the inhibition of fermentation by the presence of molecular oxygen, adjusts the rate of sugar utilization to the cell's requirements. Both Saccharomyces and Kluyveromyces species exhibit a strong Pasteur effect (Reed and Peppler, 1973; Moulin and Galzy, 1978). The converse of this effect is the Crabtree effect, i.e., depression of respiration in the presence of excessive sugar concentration, even in the presence of oxygen. Kluyveromyces fragilis was found not to exhibit a Crabtree effect (Phaff et al., 1978).

The production of ethanol by Saccharomyces cells has been most widely performed at pH 4.5 to 5.0, 28 to 30^{o}C and 10 to 18% ($\frac{w}{v}$) sugar concentrations (Kardos and Mulcock, 1977; Cysewski and Wilke, 1976a, 1976b). Under these conditions Rose (1976) obtained an ethanol yield from molasses of about 80% by batch fermentation. Ethanol production by

immobilized cells has been achieved with Saccharomyces cerevisiae cells immobilized in an alginate gel (Kierstan and Bucke, 1977) and S. carlsbergensis cells immobilized onto diatomaceous earth (Grinsbergs et al., 1977).

The genus Kluyveromyces has been characterised by Lodder (1970) as being able to ferment lactose to ethanol, a characteristic not shared by the genus Saccharomyces. Ethanol production from lactose by K. fragilis, as described in the literature, is performed at pH 4.5 to 5.0 and 30 to 35^{o}C using sugar concentrations of 4 to 12% ($\frac{w}{v}$) (Rogosa et al., 1947; O'Leary et al., 1977a). Kluyveromyces species lend themselves to cultivation on whey, and, indeed, at least three economical plants are operating in North America producing food yeast and, in one case, recovering alcohol (Peppler, 1978; Dicker, 1977).

Thus the production of ethanol using immobilized yeast is attracting continuing interest.

2.6 PROPERTIES OF IMMOBILIZED CELLS

2.6.1 Activity

The activity retention of immobilized cells has ranged from 0% to more than 100%. Kimura et al. (1978) was unsuccessful in the immobilization of yeast glycolysis systems in polyacrylamide gel however Uchida et al. (1978) gained 102% of the free activity by immobilizing "Achromobacter" aceris for NADP production.

In some reports a variety of immobilization methods have been compared. Tosa et al. (1973) used nine methods for the immobilization of the enzyme aspartase included in the catagories, ionic binding, physical adsorption, covalent binding and entrapment. The greatest activity retention (29%) was found using polyacrylamide gel entrapment. This method also produced the greatest retention (72%) in the immobilization of aspartase-containing Eschericha coli cells (Chibata et al., 1974) where encapsulation and cross-linking were also tried.

Activity retention is not the sole factor determining the choice of immobilization method. Morikawa et al. (1979) used polyacrylamide gel entrapment to immobilize Penicillium chrysogenum cells instead of alginate gel. Although cells immobilized in alginate gel retained a higher activity the gel was too fragile for continuous operation. Venkatasubramanian et al. (1974) described a theoretically superior method of whole cell immobilization, the formation of reconstituted collagen complexes. The complex was said to require no premodifiers and had more available sites than covalent attachment, it was subject to less leakage than simple adsorption and was more stable than entrapment due to a wider range of forces being present. The activity retention of glucose isomerase in whole cells was however only 25% due mainly to the tanning of the complex.

The variation in conditions used by different researchers greatly affects the results obtained. For polyacrylamide gel entrapment activity retentions of 50-80% were common. This level of activity retention can also be achieved using covalent attachment (Jack, 1977) and entrapment in cellulose fibres (Linko et al., 1977). A comparison of all methods is not possible as in many studies no free cell activity measurement was made (Slowinski and Charm, 1973). However, Jack and Zajic (1977) have attempted such a review while Chibata and Tosa (1977) have summarised their own detailed work on polyacrylamide gel entrapment.

2.6.2 Variations in Fermentation Properties

The immobilization of cells has imparted variations in fermentation characteristics to the cells in a random manner. In some cases (Ohmiya et al., 1977) the temperature, pH and thermostability profiles of the immobilized cells were similar to those of the free cells and free enzymes. For aspartase-containing E. coli cells the optimum pH for the reaction was reduced from 10.5 to pH 8.5 by immobilization of the cells (Chibata et al., 1974), whereas with Brevibacterium ammoniagenes synthesizing co-enzyme A the pH optimum was increased by immobilization (Shimizu et al., 1975). Similar changes have also been found in temperature profiles (Yamamoto et al., 1974a). Where experimentation has occurred changes in both pH and temperature profiles were rare however increases in the thermostability of cells were commonly found (Chibata et al., 1974; Yamamoto et al., 1974a, 1976; Shimizu et al., 1975).

2.6.3 Kinetics

A comparison of free and immobilized cell kinetics can be used to gain information on the effect of immobilization on the cell. Such comparisons are not common in the literature. Yamamoto et al. (1977) and Ghose and Chand (1978) investigated only the immobilized cell kinetics as part of comprehensive studies of enzymic reactions. Kinoshita et al. (1975) revealed that the apparent Michealis Constant was similar in batch and continuous hydrolysis of a cyclic dimer using immobilized cells.

The change in kinetics due to immobilization in polyacrylamide gel was examined by Ohmiya et al. (1977) who found that the apparent Michealis Constant remained the same between the free enzymes and the immobilized yeast and bacteria although the apparent maximum velocity increased by two fold with K. lactis and decreased by 100 to 1,000 fold with E. coli and Lactobacillus bulgaricus. Tosa et al. (1973) studied the enzyme aspartase and found that the apparent Michealis Constant increased slightly while the apparent maximum velocity decreased by 65% due to the immobilization in polyacrylamide gel. The constancy of the apparent Michealis Constant suggested that the gel imposed little resistance to diffusion of the reactants or products (Petre et al., 1978).

Entrapment in 2-hydroxyethyl methacrylate (HEMA) gels revealed that the kinetic parameters of the immobilized cells were dependent on the cell and HEMA concentrations within the gel (Kumakura et al., 1978, 1979). The parameters were closest to the free cell parameters at low HEMA and high cell concentrations.

Changes in the kinetic parameters due to immobilization appear to be related to the method used with variations within methods due to different techniques.

A similar apparent Michealis Constant was found for free and cellulose fibre entrapped cells (Linko et al., 1977) whereas Toda (1975) noted a four fold increase in the Michealis Constant when cells were immobilized in agar spheres. Contrary to theory where the imposition of diffusional limitations gives rise to an increase in apparent Michealis Constant, Petre et al. (1978) gained a four fold decrease using cells immobilized by crosslinking.

2.7 ENTRAPMENT IN POLYACRYLAMIDE GEL

In 1963 Bernfeld and Wan successfully immobilized enzymes for the first time, employing polyacrylamide gel for the entrapment. Although other methods of immobilization have proved successful with enzymes the method was again used in 1969 for the first successful immobilization of whole cells (Updike et al., 1969). In the last few years some research has been directed towards the use of the less toxic gels including starch, carrageenan, collagen, alginate and HEMA, however polyacrylamide gel remains the most widely used support for the immobilization of whole cells.

2.7.1 The Gel

Polyacrylamide gel is a synthetic network formed by the joining of acrylamide monomers to each other and to a crosslinking agent, N N'-methylene bisacrylamide (BIS). The reaction can be initiated by a free radicle in combination with an accelerant (persulfate and TEMED or BDMAP), by a photocatalyst (riboflavin) or by X-ray radiation (Maeda and Suzuki, 1977). The first method is the most commonly described in the literature.

The gels resulting from the free radicle initiation of addition polymerisation as described by Flory (1953) show a continuous spectrum of properties, from soft and transparent to hard, brittle and opaque, which are dependent upon the monomer, crosslinking agent and initiator concentrations.

The order of component concentrations used in this study of 5 to 25% $(\frac{w}{v})$ acrylamide, 0.5 to 2.5% $(\frac{w}{v})$ BIS and 0.25% $(\frac{w}{v})$ initiator produced a relatively firm transparent gel, as recorded in the results section. Gels of this type were found to polymerise as a first order process with an induction period prior to rapid polymerisation. The length of the induction period was inversely proportional to the initiator and accelerator concentrations (Richards and Lecanidou, 1974).

Gel volume changes were noted, with the gel volume decreasing on polymerisation and swelling by the uptake of solvent when immersed in excess water (Richards and Temple, 1971). Gels containing about 15% $(\frac{w}{v})$ monomer and 1.5% $(\frac{w}{v})$ crosslinking agent were found to swell by approximately 80% when placed in water.

2.7.2 The Effect of the Monomers

Enzyme inactivation by polyacrylamide gel was examined by the uniform radioactive labelling of the enzyme protein (Bernfeld et al., 1968). The experimentation revealed that although 55% of the enzyme protein was entrapped by immobilization the resulting gel contained only 10% of the original activity. The remaining enzyme protein was recovered in the aqueous phase along with one third of the original activity. This represents a total activity recovery of less than 45%, indicating that at least 55% of the enzyme was inactivated during the immobilization process.

Miyamoto et al. (1977) also noted a loss in enzyme activity on immobilization. Upon investigation of the gel components it was found that the acrylamide monomer was the most detrimental to enzyme activity. Up to 65% of glucose-6-phosphate dehydrogenase activity and 95% of hexokinase activity were lost by exposure to a 12% $(\frac{w}{v})$ acrylamide solution at $0^{o}C$, pH 7.1. Solutions of lower pH were found to be less damaging.

Acrylamide was also found to be damaging to cells. Morikawa et al. (1979) lost 90% of P. chrysogenum activity by twenty minutes exposure to a solution of 4.25% $(\frac{w}{v})$ acrylamide and TEMED. Martin and Perlman (1976a) found that the effect of monomer exposure on Gluconobacter melanogenus was temperature dependent. Exposure to an acrylamide solution at $15^{o}C$ for five minutes resulted in only a slight decrease in activity whereas exposure at $45^{o}C$ for two minutes completely inactivated the cells.

2.7.3 Protective Agents

The generally low retention of enzyme activity on immobilization has initiated investigations into the use of enzyme protective agents. These compounds appear to fall into two categories, enzyme specific protective agents and general protective agents.

Enzyme specific protective agents are those which have been found to protect certain enzymes only. Miyamoto et al. (1977) found that the incorporation of the substrate, product or cofactors of an enzyme reaction in the polymerisation mixture of a polyacrylamide gel had a protective affect on the enzyme. In this manner the activity retention of the immobilized enzyme could be increased by three to ten fold.

General protective agents used in the immobilization of enzymes include glycerol (Dinelli, 1972), cysteine and bovine serum albumin (Dahlqvist et al., 1973), albumin (Hinberg et al., 1974), and glutathione and dithiothreitol (Ohmiya et al., 1975). The use of dithiothreitol (Cleland, 1964) has also been noted in free cell systems.

Little evidence was found in the literature of the use of protective agents in the immobilization of cells. It is noted that the activity of immobilized cells is, in most cases, somewhat higher than that of immobilized enzymes and therefore it may have been considered unnecessary to protect the cells against the effects of immobilization. Petre et al. (1978) were noted to include bovine albumin in the crosslinking agent solution of E. coli cells but made no reference to its function or effect.

2.7.4 Gel Optimization

As stated in section 2.6.1 the loss of activity due to the immobilization of whole cells in polyacrylamide gel is most commonly 30 to 50% of the free cell activity. Research has shown that the highest activity retention is obtained when the gel composition is optimized for the particular organism in question by experimentation with the gel components.

Initially the organism may be incubated to the growth stage where the rate of product formation is greatest. In literature where this has occurred (Jack, 1977; Kokubu et al., 1978; Seyhan and Kirwan, 1979) large variations in the product formation rates have been noted at different growth stages. In the majority of the literature (Chibata et al., 1974; Kinoshita et al., 1975; Sato et al., 1976; Murata et al., 1978, 1979) the cells for immobilization were grown in a medium incorporating the intended substrate to induce the desired enzyme complement. This method does not ensure good activity in the cells prior to immobilization. In rare cases the cells were grown in a general growth media without apparent regard for the final enzyme complement (Martin and Perlman, 1976a).

The optimization of at least some of the gel components is more commonly found in the literature. The initial immobilization of cells was performed using a gel containing 8% $(\frac{w}{v})$ acrylamide and 0.2% $(\frac{w}{v})$ BIS with the immobilized cells being described as "viable" (Updike et al., 1969). Since these experiments a variety of gel compositions have been used successfully.

Tosa et al. (1973) optimized a gel composition for the immobilization of the enzyme aspartase. Chibata et al. (1974) optimized the gel composition for whole cells containing aspartase. Both these gels contained 15% ($\frac{w}{v}$) acrylamide, 0.8% ($\frac{w}{v}$) BIS, 0.5% ($\frac{w}{v}$) BDMAP and 0.25% ($\frac{w}{v}$) ammonium persulfate, and were polymerised at 30^{o}C. A 20% ($\frac{w}{v}$) concentration of whole cells was included in the latter gel. This gel composition has been used successfully for the immobilization of several bacteria giving initial yields of 72% for E. coli (Chibata et al., 1974), 73% for Brevibacterium ammoniagenes (Yamamoto et al., 1976) and 64% for "Achromobacter" liquidium (Yamamoto et al., 1974b). Variations of this method have also been used. Shimizu et al. (1975) immobilized dried cells using the gel polymerised at 0^{o}C and Uchida et al. (1978) and Murata et al. (1978) increased the cell concentration in the gel to 40% ($\frac{w}{v}$).

A wide range of gel compositions have been obtained using other optimization procedures. The most successful gel compositions are similar to that of Chibata et al. (1974). The gel of Martin and Perlman (1976a) contains 15% ($\frac{w}{v}$) acrylamide and 0.8% ($\frac{w}{v}$) BIS whereas that of Saif et al. (1975) contained 19% ($\frac{w}{v}$) acrylamide and 0.6% ($\frac{w}{v}$) BIS. The extremities of the range of gel compositions are represented by Kinoshita et al. (1975) and Kokubu et al. (1978). The former used continuous fermentations to determine a gel composition of 33% ($\frac{w}{v}$) acrylamide and 5% ($\frac{w}{v}$) BIS with 3.6% ($\frac{w}{v}$) "Achromobacter" guttatus cells, and a 4% ($\frac{w}{v}$) acrylamide, 1% ($\frac{w}{v}$) BIS gel was used by Kokubu et al. for the immobilization of B. subtilis with minimal leakage from the gel. It was noted that in immobilization procedures with prolonged exposure to acrylamide (high acrylamide concentration or low initiator concentrations), the activity retention was low (Ohlson et al., 1978).

The limits of cell concentration have been discussed in several papers. Ohlson et al. (1978) stated that at cell concentrations of 10% ($\frac{w}{v}$) less than half the activity was retained on immobilization with no retention at cell concentrations below 5% ($\frac{w}{v}$). Ohmiya et al. (1977) noted a reduction in activity retention at cell concentrations above 30% ($\frac{w}{v}$) and sighted the cause as the increased cell volume hindering polymerisation. In contrast Murata et al. (1978) and Uchida et al. (1978) successfully immobilized 40% ($\frac{w}{v}$) concentrations of yeast and bacteria.

A wide range of polymerisation temperatures have been used for polyacrylamide gels. Shimizu et al. (1975) gained best results by polymerising at 0°C for at least 1 hour. Martin and Perlman (1976a, 1976b) used 15°C whereas Chibata et al. (1974) used 30°C to obtain comparable activity retentions.

This section provides an indication of the wide range of polyacrylamide gel compositions that may be used for the successful immobilization of whole cells and suggests the need for optimization of the composition for a particular species of organism to ensure maximum immobilized activity.

2.7.5 Gel Configuration

The configuration used for immobilized cells is often dependent on the method of immobilization. Polyacrylamide gel is usually made in sheets or tubes which can then be reduced to the desired configuration.

The most widely used method of gel size reduction is disruption in a Waring blender (Chibata et al., 1974; Shimizu et al., 1975). Yang et al. (1976) gained a greater activity yield using manual disruption but favoured disruption in a Waring blending as the small particle size produced was considered to have better mass transfer characteristics. This method did not appear detrimental to the cells and pressure drop was not considered a problem with the small particle size as the pressure drop for a one metre column was only 20% that gained with a DEAE Sephadex column (Tosa et al., 1973). However, when one process was scaled up to a pilot plant reactor the particle size was increased to 4 mm mean diameter (Sato, 1975).

Kinoshita et al. (1975) determined the optimum particle size for continuous flow columns as 5 mesh (about 5 mm mean diameter). Martin and Perlman (1976a) found that the gel configurations with high volume to surface area ratios had lower specific activity, however the activity was more stable than the low ratio configurations. The maximum active particle thickness was determined as 0.7 mm, above which the reaction was thought to be oxygen limited.

2.8 ACTIVATION

Many researchers have investigated ways to increase the activity of immobilized cells, after immobilization. In this section three successful methods will be described.

Chibata et al. (1974) found that the activity of immobilized E. coli cells increased on repeated use. Experimentation showed that incubation of freshly immobilized cells in the substrate for 24 hours increased the initial activity ten fold. This increase was due to cell lysis with the lysed cells being retained within the gel. This phenomenon was not indicative of the immobilization method used, polyacrylamide gel entrapment, although lysis of E. coli cells was also noted by Updike et al. (1969).

Somerville et al. (1977) immobilized benzene induced cells and lost 40-70% of their activity. By inducing the benzene degrading activity after immobilization up to 100% of the original induced activity could be obtained. The successful induction of immobilized cells suggested the possibility of the rejuvenation of decayed activity. This was performed by overnight shaking of the immobilized cells with an iron supplemented growth medium and produced activity of up to 3 times the original activity on two successive rejuvenations. Rejuvenation was also used successfully by Mosbach and Larsson (1970).

The third method of activation involves the supplementation of the substrate medium with organic solvents, artificial cofactors, salt solutions and nutrients. The latter method proved useful to Klein et al. (1976), Kokubu et al. (1978) and Ohlson et al. (1978) where 0.5% $(\frac{W}{V})$ peptone and 0.2% $(\frac{W}{V})$ glucose increased Arthrobacter simplex activity by five fold.

2.9 CONTINUOUS OPERATION

Batch operation, the traditional mode of fermentation, has been used in the immobilization literature by Ohmiya et al. (1977), Kokubu et al. (1978) and Kumakura et al. (1978).

According to Chibata and Tosa (1977) continuous operation costs 40% less than the conventional batch operation due to reuse of the catalyst and to automation. For this reason it is the ultimate mode of operation for the majority of researchers. A small number of studies have been conducted using continuous experimentation exclusively (Johnson and Ciegler, 1969; Kinoshita et al., 1975). In many studies batch experiments have been performed to determine the optimum immobilization conditions with continuous operation being used for the investigation of fermentation conditions or immobilized stability, due to restraints of time and equipment availability. Large differences in characteristics can occur between batch and continuous fermentations however little evidence of this has been found in the field of immobilized cells.

Where continuous operation has been used large variations in flow rates and substrate concentrations have been noted. Generally continuous reactors have been run at a flow rate giving complete substrate utilization, which was inversely proportional to the substrate concentration. The use of high substrate concentrations, and the corresponding low substrate flow rate, was found to increase the stability of an immobilized enzyme column during continuous operation (Tosa et al., 1973). This combination of parameters was also favoured by Toda (1975) who suggested that mass transfer was enhanced by the use of high substrate concentrations.

The nature of the enzyme conversion also influences the choice of substrate concentration. For single enzyme conversions relatively high substrate concentrations were used successfully. L-aspartic acid was produced using 1.0 M substrate at 0.8 SV (Tosa et al., 1974) and L-citrulline was produced using 0.5 M substrate at 0.26 SV (Yamamoto et al., 1974a). Where the reaction was complex or multistep low substrate concentrations were used. Franks et al. (1971) formed ornithine from 0.0057 M substrate at 0.25 SV and Shimizu et al. (1975) formed Coenzyme A from 0.002 M substrate at 0.44 SV.

These results were gained in fixed bed reactors. Emery and Cardoso (1978) concluded that fluidised bed reactors were preferable as they induced better mixing of substrate and product and therefore higher activity. The effects of the increased liquid velocity and turbulence, inherent in fluidised bed reactors, upon the immobilization supports was not examined.

The stability of the immobilized columns has varied greatly. Immobilized enzyme columns were found to have limited stability with 20 to 40 day half lives (Messing and Filbert, 1975). Some of the longest half lives were produced by Yamamoto et al. using polyacrylamide gels with 140 days for Psuedomonas putida producing L-citrulline (1974a) and 180 days for "Achromobacter" liquidum producing urocanic acid (1974b), although a recent paper by Chibata (1977) quoted a half life for aspartase activity in E. coli cells immobilized in carrageenan of 686 days. Other researchers using polyacrylamide gel have also noted considerable stability in the immobilized cells. More than 80% of "Achromobacter" guttatus activity was retained after 30 days operation (Kinoshita et al., 1975) and 60% of Clostridium butyricum activity was retained after 20 days operation (Karube et al., 1976).

Various immobilized cell stabilities have been noted using other immobilization methods. Azotobacter vinelandii cells absorbed onto an anionic exchange resin fixed nitrogen at a stable rate for only seventy hours after which the activity decreased rapidly (Seyhan and Kirwan, 1979). Better stability has been obtained using more permanent immobilization methods. A half life of 45 days was obtained using cellulose fibre entrapped Actinoplanes missouriensis for glucose isomerisation (Linko et al., 1977) and a 10 day half life was noted for ethanol production by alginate gel entrapped S. cerevisiae (Kierstan and Bucke, 1977). Activity retentions of 90% were obtained after 15 days operation of a covalently bonded cell system (Jack, 1977) and after 10 days operation of glutaraldehyde crosslinked E. coli cells (Petre et al., 1978).

It was found that although some of the above continuous fermentations were more active at higher temperatures the activity was much less stable and decayed at a faster rate than at 30 to 37^{o}C (Tosa et al., 1974).

2.10 APPLICATIONS OF IMMOBILIZED CELLS

Immobilized cells are widely used in research into the production of chemicals and pharmaceuticals. Thorough listings of applications have been presented by Abbott (1977, 1978) and such lists will not be repeated here. In recent years the technology has been applied to a wider range of processes which include degradation, energy production microbial conversions and transformations as well as analytical procedures.

2.10.1 Degradation

In the waste treatment industry, industrial and domestic wastes are often processed using immobilized cells. In trickling filters the waste is passed over stones or plastic packing which act as supports for films of microorangisms. Flocculation is the mode of immobilization present in activated sludge operation. Due to the larger volumes involved in the processing of wastes more complex means of immobilization are impractical, however these methods can be applied to specialised waste materials.

Some of the first trials in degradation using immobilized microorganisms were conducted when spores of Aspergillus wentii were immobilized on ECTEOLA-cellulose for starch hydrolysis. The immobilized activity was considered too low for continuous operation (Johnson and Ciegler, 1969).

Hackel et al. (1975) investigated the degradation of phenol to water and carbon dioxide using Candida tropicalis cells entrapped in various polymers. The low yields of activity (20%) were markedly increased by incubation in a growth medium (Klein et al., 1976).

The degradation of a product of synthetic manufacture was investigated by Kinoshita et al. (1975). "Achromobacter" guttatus cells were immobilized in a polyacrylamide gel of experimentally determined composition producing a stable system. No comparison with free cell activity was made. Polyacrylamide gel was also used for the immobilization of P. putida for benzene degradation (Somerville et al., 1975). By rejuvenation at intervals the initial immobilized activity was maintained over 73 days of operation.

Encapsulation in liquid membranes was the immobilization method used for the reduction of nitrite and nitrate (Mohan and Li, 1975). The experiments showed that the immobilized cells were more stable than the free cells and were protected from inhibitors such as mercury by the surfactant membrane.

2.10.2 Energy Production

The production of ethanol from whey has been investigated by Rogosa et al. (1947), O'Leary et al. (1977a, 1977b) and Marshall (1978). O'Leary et al. found that K. fragilis produced higher yields of ethanol (2% ($\frac{w}{v}$)) more quickly on whey than S. cerevisiae produced on lactose hydrolysed whey. The conditions for ethanol production were pH 4.6, 30°C using cottage cheese whey which contained approximately 0.15 M lactose. Similar conditions were used for the reduction of BOD in whey by K. fragilis (Knight et al., 1972) where concentrations of up to 1.5% ($\frac{w}{v}$) ethanol were obtained (Mickle et al., 1974). Yoo (1974) obtained only 55% of the theoretical yield of ethanol (~ 1.3% ($\frac{w}{v}$)) from whey using K. fragilis, as a result of possible ethanol inhibition.

The use of immobilized cells for ethanol production has been confined to a handful of papers. Kierstan and Bucke (1977) immobilized S. cerevisiae in a calcium alginate gel for the conversion of 10% ($\frac{w}{v}$) glucose to ethanol. Yields of about 75% were obtained over 15 days continuous operation. A similar system was used for pilot plant trials in the production of beer (White and Portno, 1978). S. carlsbergensis absorbed onto polyvinylchloride and porus brick also produced an acceptable beer (Corrieu et al., 1976). Compere and Griffith (1976) used immobilization by adsorption for ethanol production with the yeast cells being supported by crosslinked gelatin coated packing while Grinsbergs et al. (1977) used diatomaceous earth as a support medium.

In 1976 Karube et al. published information on the immobilization of Clostridium butyricum in polyacrylamide gel. The cells were used to produce hydrogen from glucose. A further paper (1977a) examined the use of these cells in a biochemical fuel cell. The polyacrylamide entrapped cells were coated onto one side of a platinum black electrode as in Figure 1.

The system was based on the theory that the cells would convert the glucose (2.7 mMoles/day) to hydrogen ions and formate which the hydrogenase enzyme could use to produce hydrogen gas. The drain of electrons caused a current of 1.2 mA to flow. The immobilized system was stable for 15 days however large amounts of cells were obviously required to produce industrial currents. The output of the fuel cell was 2.4 watts/Kg dry immobilized cells and is not comparable with chemical fuel cells.

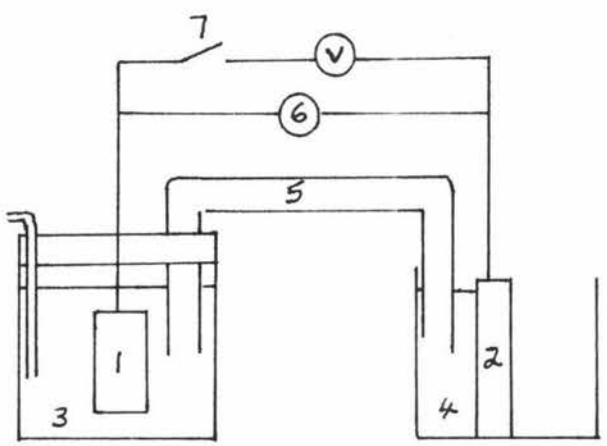

FIGURE 1. Schematic diagram of the biochemical fuel cell of Karube et al. (1977a).
1. Immobilized cell - platinum black electrode; 2. Carbon electrode; 3. Catholyte (0.1 M phosphate buffer); 4. Anolyte (0.25 M glucose in phosphate buffer); 5. Salt bridge; 6. Recorder; 7. Switch.

2.10.3 Analytical Procedures

The system of immobilization of Karube et al. (1976) was applied to the rapid estimation of BOD (Karube et al., 1977b). The method was based on the observation that immobilized cells became active when placed in a solution of organic compounds. The change in the dissolved oxygen (D.O.) content of the solution caused by the activity of the cells, was measured by a dissolved oxygen electrode. A linear relationship was found between the BOD of the solution and the rate of decrease of current in the D.O. electrode within the limits of operation. BOD estimations were performed at pH 7.0, 50^{o}C with 5% of cells in the gel and using a solution of about 350 ppm BOD. The method had the advantages of being a simple, quick and inexpensive means of gaining reproducible results of BOD analyses. The bacteria used in the method were not specified but said to originate from soil. The range of bacteria used may be important to the success of the method.

The development of immobilized enzyme electrodes is relatively recent. In 1978 Enfors and Molin discussed the potential use of enzyme electrodes as analytical tools for fermentation control. Some success was obtained with the development of a penicillin electrode overcoming some of the common problems of low half life, calibration of activity and the sensitivity of the enzyme to changes in environmental conditions. Other successful enzyme electrodes have been developed for the measurement of glucose, (Updike and Hicks, 1976), L-Lactate (Williams et al., 1970) and urea (Montalvo, 1970). The use of cells in this application may increase its potential further as immobilized cells are generally more stable than immobilized enzymes.

In 1978 Pache described another analytical use of immobilized cells. E. coli cells containing β-Lactamase enzymes were immobilized for the assessment of the resistance of β-Lactam antibiotics to attack by bacterial enzymes.

2.11 CONCLUSION

Continuous processing is an economically attractive and frequently necessary procedure. The immobilization of cells provides another approach to the processing of a variety of biological materials of commercial interest. Other reasons for the upsurge in research into immobilized cells over the past decade are the often favourable variations in fermentation conditions bestowed on the cells by the immobilization procedure and the relatively stable nature of immobilized cells. Ethanol production by immobilized cells is the topic for investigation in this study. Two possible energy sources in New Zealand are whey (Mathews, 1977) and energy crops (Kardos and Mulcock, 1977). It is hoped to apply the technology reviewed in this chapter to the immobilization of yeast for the production of ethanol.

CHAPTER 3

METHODS AND MATERIALS

This chapter contains a description of the methodology used in this study of the immobilization of yeast.

3.1 MATERIALS SOURCES

Acrylamide monomer and N,N'-methylene-bis-acrylamide (BIS) were obtained from Eastman Kodak, Rochester, New York. The microbial media, Nutrient Broth, Yeast Extract, Gelatin, Bacto-Proteose Peptone, Malt Extract Agar (MEA) and Potato Dextrose Agar (PDA) were obtained from Oxoid, London, England. For the ethanol assay, the yeast enzyme and cofactor, alcohol dehydrogenase (crystallised and lyophilized) and β-nicotinamide adenine dinucleotide (NAD^+) (grade III), were obtained from Sigma Chemicals, St. Louis, U.S.A. The whey substrate was supplied by the Reporoa Dairy Factory (Waikato) as a 60% ($\frac{w}{v}$) total solids concentrated deproteinized Lactic Casein Whey. Its use is further described in section 3.4.1.3. Other chemicals used in this study were of reagent grade and obtained from B.D.H., Poole, England.

3.2 MICROBIOLOGY

3.2.1 Culture Source and Description

Kluyveromyces fragilis NRRL[1] Y 1109 was used to ferment lactose to ethanol in both free and immobilized cell preparations. The strain used was found to vary from the description of Lodder (1970), being weakly melezitose positive in assimilation tests. Saccharomyces cerevisiae NCYC[2] 240 was used in studies of the fermentation of glucose to ethanol in both free and immobilized cells systems.

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1 NRRL, Northern Utilization Research and Development Division of the United Stated Department of Agriculture.

2 NCYC, National Collection of Yeast Cultures, Surrey, England.

3.2.2 Culture Maintenance

Stock cultures of K. fragilis were grown at 30°C for 12 hours on MEA slopes and stored at 4°C. S. cerevisiae stock cultures were grown at 30°C for 24 hours on MEA slopes and stored at 4°C.

3.2.3 Inoculum Preparation

Inoculum was prepared from the slope cultures by suspending the cells in 2 ml of sterile peptone water. A 0.5 ml volume of this suspension containing approximately 10^9 cells was used to inoculate 300 ml of fermentation medium.

3.2.4 Culture Growth

K. fragilis cells were grown for immobilization in 500 ml Erlenmeyer flasks containing 300 ml of 1.3% ($\frac{W}{V}$) Nutrient Broth, 0.12 M lactose. The inoculated medium was incubated at 30°C on a rotary shaker at 200 r.p.m. for 16 hours.

S. cerevisiae cells were grown in similar flasks containing 300 ml of 1.3% ($\frac{W}{V}$) Nutrient broth, 0.44 M glucose. The inoculated medium was incubated at 30°C, 200 r.p.m. for 28 hours.

3.2.5 Cell Harvest

K. fragilis and S. cerevisiae cells were harvested by centrifugation at ambient temperature and 1100 x g for 5 minutes, (M.S.E. Super Medium Centrifuge, Number 4.62) and washed twice in 4°C Krebs Ringer (Krebs and Henseleit, 1932) with separation at 1000 x g for 10 minutes (Martin Christ Centrifuge, UJ1S) under aseptic conditions at ambient temperature.

The importance of the washing procedure was noted when gels containing unwashed cells failed to polymerise.

3.3 POLYACRYLAMIDE GEL IMMOBILIZATION PROCEDURES

3.3.1 Immobilization in a Gel after Chibata et al. (1974)

The concentrations of gel components described in this section were established in this study as the most appropriate for maximum ethanol producing activity in immobilized cells. The gel composition defined by Chibata et al. (1974) was used as a basis for this study as many bacteria and enzymes have been successfully immobilized using the methodology of Chibata et al.

For K. fragilis, 1.25 gm cells, (wet weight equivalent to 0.32 gm dry weight) grown and harvested as above, were placed in a sterile glass beaker and suspended in 4 ml 0.1 M tris-HCl buffer pH 7.1 (Dawson et al., 1969) at $0^{\circ}C$. Unless otherwise stated 750 mg acrylamide and 75 mg BIS followed by 0.5 ml 6% $(\frac{w}{v})$ β-dimethylaminopropionitrile (BDMAP) and 0.5 ml 2.5% $(\frac{w}{v})$ ammonium persulfate were combined with the suspension in an icebath within the space of 1 minute. The 5 ml volume of gel produced was then transferred to a sterile glass tube (10 mm internal diameter, 70 mm length) where it became rigid within 2 minutes. In this thesis, the quantity of gel formed in this tube is frequently referred to as "a single gel" or simply as "a gel".

A further 30 minutes storage at $4^{\circ}C$ was allowed for the completion of polymerisation. During this time a slight decrease in gel volume occurred causing indentations of 1 mm at either end of the gel cylinder.

The gel configuration used in this study was a cylindrical pellet (10 mm diameter, 1-2 mm thick). The pellets were obtained by manually cutting the gel with a knife while extruding it from the glass tube under aseptic conditions. By using this method 40 uniform gel discs or pellets could be obtained. In all batch fermentations the quantity of immobilized catalyst used was a gel which was equivalent to about 40 pellets.

The pellets were washed twice in 0.1 M tris-HCl buffer pH 7.1 at $4^{\circ}C$ by 5 minutes swirling in the buffer at 100 r.p.m. on a rotary shaker followed by decantation of the liquid. The washed pellets were then ready for use in fermentations.

For S. cerevisiae the above procedure was modified to include 2 gm cells (wet weight, equivalent to 0.52 gm dry weight) in each 5 ml gel along with 1000 mg acrylamide and 80 mg BIS.

3.3.2 Immobilization in a Gel as Used for Electrophoresis by Neuhoff (1973)

A gel used for electrophoretic protein separation was also used for yeast immobilization. The studies will show that this gel was of limited value.

To prepare the gel 2 ml solution A, 3 ml solution B and 1 ml solution D, where the compositions of these solutions are specified below, were mixed with 1.5 gm cells (wet weight, equivalent to 0.39 gm dry weight) from section 3.2.5to give a final volume of 7 ml. The mixture was vacuum deaerated and put into sterile glass tubes for 30 minutes to polymerise at room temperature. The resulting gel was extruded and manually cut into cylindrical pellets (10 mm diameter, 1-2 mm length) under aseptic conditions. The pellets were then ready to be used in fermentations.

The solutions used had the following compositions:

A, 0.65% $(\frac{w}{v})$ N,N,N'N'-tetramethyl-ethylene diamine (TEMED) in 0.7 M tris-HCl buffer at pH 8.3.

B, 40% $(\frac{w}{v})$ acrylamide and 0.4% $(\frac{w}{v})$ BIS in glass distilled water.

C, 0.14% $(\frac{w}{v})$ ammonium persulfate (initiator) in glass distilled water.

Solutions A and B were made and stored in bulk away from light at $4^{o}C$. The solution containing the initiator was prepared prior to use.

The main differences in gel composition between the modified Chibata et al. (1974) gel and the Neuhoff (1973) gel are as follows:

1. The modified Chibata et al. (1974) gel has BIS and initiator concentrations ten fold those in the Neuhoff (1973) gel.
2. The modified Chibata et al. (1974) gel was formed at $0^{o}C$ whereas the Neuhoff (1973) gel was formed at room temperature.
3. TEMED was used in place of BDMAP in the electrophoretic gel. These compounds are similar in composition and perform the same function.

A tris-HCl buffer was used in both gel preparations in the study replacing the salt solution of Chibata et al. (1974) which was found to inhibit activity (Ohlson et al., 1978).

3.4 FERMENTATION PROCEDURES

Two modes of operation were used for the fermentation experiments, i.e. batch and continuous.

Batch fermentations in shake flasks were used for:

1. Trials with cells immobilized in the Neuhoff (1973) gel and the testing of the affect of gel components.
2. The determination of component concentrations in the modified Chibata et al. (1974) gel.
3. The "repeated use" experiments using simple and supplemented media.

In all cases where free-cell experiments were performed, for comparison with the immobilized cell experiments, the same fermentation procedure was followed, with equivalent weights of cells being used. The sampling procedure was modified to include a separation step where the sample was centrifuged (5 minutes at 1000 x g) to remove the cells which were discarded.

Continuous fermentations in a continuous flow reactor were used for the fermentation of whey to ethanol by K. fragilis (see section 3.4.2).

3.4.1 Batch Fermentations

3.4.1.1 FERMENTATIONS TO DETERMINE THE EFFECTS OF GEL COMPONENTS AND THE ACTIVITY OF CELLS IN THE NEUHOFF (1973) GEL.

These experiments were performed by aseptically mixing cells or gel pellets with 125 ml of sterile fermentation medium in a 250 ml Erlenmeyer flask. The fermentation media consisted of 0.12 M lactose for K. fragilis and 0.44 M glucose for S. cerevisiae prepared in double distilled water.

No buffering system was incorporated in the media. Experimental results showed that the initial pH of the lactose and glucose systems of about 5.0 dropped by up to 1 pH unit during the fermentation. K. fragilis is known to grow best between pH 4.0 and 6.0 (Knight et al., 1972) and S. cerevisiae grows best between pH 3.5 and pH 6.0 (Reed and Peppler, 1973).

The flasks were shaken at 100 r.p.m. on a rotary shaker at 30°C and 2 to 3 ml samples were taken at 10 hourly intervals for pH, sugar concentration and ethanol concentration analyses.

3.4.1.2 FERMENTATIONS TO ESTABLISH THE CONCENTRATION OF GEL COMPONENTS FOR MAXIMUM IMMOBILIZED ACTIVITY.

The components tested in these experiments are, in order, as follows:

For K. fragilis

1. Monomer Concentration : Range tested, 5 to 25% ($\frac{W}{V}$) acrylamide, with the other components held at 5.5% of acrylamide weight as BIS, 1.5 gm cells per gel and 0.12 M lactose.
2. Crosslinking Agent Concentration : Range tested, 0.6 to 2.3% ($\frac{W}{V}$) BIS with the other components held at 15% ($\frac{W}{V}$) acrylamide, 1.5 gm, cells per gel and 0.12 M lactose.
3. Yeast Cell Concentration : Range tested, 0.5 to 2.5 gm per gel, with the other components held at 15% ($\frac{W}{V}$) acrylamide, 1.5% ($\frac{W}{V}$) BIS and 0.12 M lactose.
4. Substrate Concentration : Range tested, 0.06 to 0.26 M lactose with the other components held at 15% ($\frac{W}{V}$) acrylamide, 1.5% ($\frac{W}{V}$) BIS and 1.25 gm cells per gel.

For S. cerevisiae

1. Monomer Concentration : Range tested, 5 to 30% ($\frac{W}{V}$) acrylamide, with the other components held at 5.5% of acrylamide weight as BIS, 1.5 gm cells per gel and 0.44 M glucose.
2. Crosslinking Agent Concentration : Range tested, 0.4 to 2.4% ($\frac{W}{V}$) BIS with the other components held at 20% ($\frac{W}{V}$) acrylamide, 1.5 gm cells per gel and 0.44 M glucose.

3. Yeast Cell Concentration : Range tested, 0.5 to 3.0 gm per gel with the other components held at 20% ($\frac{w}{v}$) acrylamide, 1.6% ($\frac{w}{v}$) BIS and 0.44 M glucose.

4. Substrate Concentration : Range tested, 0.11 to 0.66 glucose with the other components held at 20% ($\frac{w}{v}$) acrylamide, 1.6% ($\frac{w}{v}$) BIS and 2.0 gm cells per gel.

The gel composition used for the substrate concentration experiments for both K. fragilis and S. cerevisiae is that stated in section 3.3.1 as the most appropriate for maximum ethanol production.

These batch fermentations were performed by aseptically mixing a gel or free cells with 25 ml of fermentation medium in a 50 ml Erlenmeyer flask. The flasks were incubated at 30^{o}C on a Griffin wrist action shaker at the slowest setting and sampled hourly by the aseptic removal of a 1 ml volume of medium. The sample was analysed for pH, sugar concentration and ethanol concentration.

The unbuffered media was found to decrease in pH by 0.5 to 1.0 unit during the 6 hour fermentations.

3.4.1.3 FERMENTATIONS INVOLVING THE REPEATED USE OF IMMOBILIZED CATALYSTS.

In experiments considering the repeated use of immobilized cells, each individual experiment was performed as above (section 3.4.1.2). After each fermentation the media was decanted from the gel. The gel was then washed twice with 0.1 M tris-HCl buffer pH 7.1 at 4^{o}C before being stored in tris-HCl buffer at 4^{o}C for up to 5 days until the next fermentation.

In addition to the simple sugar media used above in section 3.4.1.2 supplemented media and whey were used. The 0.5% ($\frac{w}{v}$) peptone and 0.1% ($\frac{w}{v}$) salt solution supplements were mixed prior to sterilization.

Salt solution (Takagi et al., 1977)

KH_2PO_4	2.2 gm
KCl	1.7 gm
$MgSO_4.7H_2O$	0.5 gm
$CaCl_2.2H_2O$	0.25 gm
Potassium citrate	4.0 gm
Citric Acid	0.8 gm
$(NH4)_2SO_4$	2.6 gm
$FeCl_3$	10 mg
$MnSO_4.4\text{-}5H_2O$	10 mg
$ZnSO_4.7H_2O$	10 mg
$CuSO_4$	1 mg

to pH 5.0 with 2N KOH or 2N H_2SO_4

Distilled water to 1 litre

The whey was received as a 60% $(\frac{w}{v})$ total solid concentrate of deproteinised lactic casein whey. This was diluted to 1020 specific gravity with the lactose content being adjusted to about 5% $(\frac{w}{v})$ prior to sterilization at 121°C for 15 minutes. Any precipitate was removed by filtration before the whey substrate was resterilized ready for use. The final substrate contained 5.9% $(\frac{w}{v})$ total solids and 5.1% $(\frac{w}{v})$ lactose at pH 4.4.

3.4.2 Continuous Fermentations

A tubular reactor was constructed for the continuous conversion of whey lactose to ethanol by immobilized K. fragilis cells. The reactor consisted of a glass tube (2.2 cm internal diameter, 15 cm length) in which a 40 cm^3 working volume was obtained. The tube was stoppered with rubber bungs and contained glass wool as a support for the pellets. Veterinary hypodermic needles (gauge 14) and tygon tubing were used to connect the reactor to the feed. An upward flow of feed was maintained by a Buchler Multi-staltic pump. The overflow from the reactor was collected in an ice cooled vessel and removed at regular intervals.

Both composite and instantaneous samples were taken during the course of each run. The results suggested that the use of the ice bath was effective for minimizing changes in effluent composition as under steady state conditions composite and instantaneous samples displayed similar pH values and ethanol and lactose concentrations.

The glass reactor, feed tank and collection vessel were dry heat sterilized at 160°C for 2 hours and all tubing was sterilized in 5% ($\frac{v}{v}$) formalin at 15°C for 15 minutes before thorough rinsing with sterile distilled water.

When loaded, the column contained 4.3 gm cells (wet weight, equivalent to 1.10 gm dry weight). The reactor was operated at flow rates within the range 0.13 to 0.46 SV, where 1.0 SV (space velocity) is equivalent to a substrate flowrate of one column volume per hour. The relationship between SV, volumetric flowrate and retention time is given in Table I.

TABLE I. Relationship between substrate flowrate parameters used in this study.

SV[1]	Volumetric Flowrate mls/hr	Retention Time hrs
0.10	4.0	10.0
0.13	5.2	7.7
0.24	9.6	4.2
0.34	13.6	2.9
0.46	18.4	2.2
1.0	40.0	1.0

[1] SV, space velocity has units of column volumes (40 ccm) per hour.

3.5 REJUVENATION ATTEMPTS

An attempt was made to rejuvenate inactivated immobilized *K. fragilis* cells during the extended use experiments. The pellets were placed in a medium consisting of the salt solution described in section 3.4.1.3 plus 0.5 gm/l Yeast Extract. Approximately 100 ml medium was used for each 5 ml volume of gel. The mixture was shaken on a wrist action shaker at a medium setting and at 30°C. After 24 hours the rejuvenation medium was decanted and the pellets were washed twice with 4°C 0.1 M tris-HCl buffer prior to continued fermentations as in 3.4.1.2.

3.6 GEL COMPONENT TESTS

These tests were performed to determine the effects of some of the gel components on free yeast cells.

3.6.1 Individual Gel Component Tests

One gram of cells (wet weight, equivalent to 0.26 gm dry weight), grown and harvested as described in sections 3.2.4 and 3.2.5, were shaken at 30°C on a rotary shaker for 30 minutes with 50 ml of 0.1 M tris-HCl buffer containing the single component being tested. The components tested were 15% ($\frac{W}{V}$) acrylamide pH 7.1, 15% ($\frac{W}{V}$) acrylamide pH 8.3, 0.32% ($\frac{W}{V}$) TEMED pH 7.1 and 0.10% ($\frac{W}{V}$) ammonium persulfate pH 7.1. The cells were then separated from the solution by aseptic centrifugation (1000 x g for 10 minutes at 4°C) and washed twice with tris-HCl buffer pH 7.1 at 4°C. The separated cells were placed in 125 ml of the appropriate sugar solution and the batch fermentation followed the procedure described in section 3.4.1.1. The usual analyses were conducted at approximately 10 hour intervals.

3.6.2 Gel Component Tests with Protective Agents

In these experiments cells of *K. fragilis* were preshaken vigorously (for 10 minutes at 200 r.p.m.) in 20 ml of the protective agent solution. Agents used were 1% ($\frac{W}{V}$) glycerol, 1% ($\frac{W}{V}$) gelatin, 1% ($\frac{W}{V}$) Tween 80 and 0.75% ($\frac{W}{V}$) (0.05 M) dithiothreitol. After the 10 minutes, 10 ml of acrylamide monomer solution was added to give a total concentration of 15% ($\frac{W}{V}$) at pH 7.1. Shaking, monomer removal and fermenting studies were conducted as described above in section 3.6.1.

3.6.3 Viability Tests

To test the effect of acrylamide monomer on *K. fragilis* cells a plate count of cells was taken before and after contact with the acrylamide. A cell mass of 1.6 gm (wet weight, equivalent to 0.41 gm dry weight) was suspended in 30 ml Krebs Ringer and 1 ml was taken and dilution plated on PDA. The remaining cells were separated by aseptic centrifugation and treated with 15% ($\frac{W}{V}$) acrylamide pH 7.1 as in section 3.6.1 above. After washing, the cells were again suspended in 30 ml Krebs Ringer and replated.

3.7 ANALYSES

3.7.1 Ethanol Analysis

Ethanol was assayed by an enzymatic method after Bonnischen (1963) in which alcohol dehydrogenase (ADH) catalyses the following reaction,

$$\text{Ethanol} + NAD^+ \rightleftharpoons \text{Acetaldehyde} + NADH + H^+$$

A Hitachi spectrophotometer (model 101) was used to follow the increase in NADH concentration by absorption changes at 340 nm. The assay mixture contained 2.3 ml 15% ($\frac{w}{v}$) glycine, 0.7 ml 0.25 N NaOH, 0.013 gm semicarbazide hydrochloride, 0.1 ml NAD^+ (12 mg/ml), 0.05 ml alcohol dehydrogenase (6 mg/ml) and 0.1 ml diluted sample to give a final volume of 3.25 ml at pH 9.0. All solutions were prepared with glass distilled water. The mixture was incubated at 28^oC for 70 minutes prior to reading. Calculations of the results were based upon the extinction coefficient of 6.22×10^6 cm^3/mole cm for the reaction read at 340 nm, 1 cm optical path using quartz cells. Trials with this method indicated that less than 5% error was obtained between standards and measured values.

The reversible reaction has a constant K of 1.1×10^{-11} mole/l at pH 7 where the equilibrium lies to the left. At alkaline pH the equilibrium is displaced to right. To ensure virtually complete displacement, Bonnischen (1963) included semicarbazide hydrochloride in the reaction mixture to trap the acetaldehyde.

In this study trials in the absence of semicarbazide hydrochloride measured less than 15% of the ethanol present in the sample at pH 9.0. This increased to up to 80% of the ethanol present when the pH was increased to 10.0 with the actual amount of ethanol measured being dependent on the concentration of the sample. A smaller percentage of the ethanol present was measured in the more concentrated samples.

These trials indicate the necessity of the inclusion of semicarbazide in the reaction mixture to ensure accurate and reproducible ethanol analyses.

3.7.2 Reducing Sugar Analysis

The volumetric copper reduction method of Eynon and Lane (1923) was used for measurement of lactose and glucose. A modification involving the use of 1 ml of sample diluted to 25 ml was used to accommodate the sample size available in this study.

3.7.3 Dry Weight Determinations

For the growth curve determinations a 5 ml volume of culture was centrifuged at 15°C (1000 x g for 10 minutes). The cells were washed twice in 15°C glass distilled water and dried to constant weight for 6 hours at 105°C.

The dry weight of washed cells from section 3.2.5 was determined by drying 10 gm cells (wet weight) to constant weight at 105°C for 24 hours.

The total solids composition of whey was determined by drying 10 ml to constant weight for 6 hours at 105°C.

3.8 DATA ANALYSIS

3.8.1 Measurement of Yeast Activity with Respect to Ethanol Production

Initial fermentation studies of both free and immobilized cells in 25 ml volumes of medium indicated a significantly linear relationship between ethanol production and time from the first hour of fermentation until substrate depletion, 4 to 8 hours later. The ethanol production rate over the first six hours of fermentation has been used as a measure of activity of the cells, assuming a constant substrate volume of 25 ml.

The six hour fermentations minimized ethanol production from sources outside the gel. Cell leakage from the gel was evident in fermentations taking several days. Any free cells present would contribute to the activity of the system. Contamination by bacteria was also noted in some of the longer fermentations, but remained negligible in six hour fermentations. This method of activity measurement also enabled rapid determination of immobilized activity and direct comparison between experiments.

The activity with respect to ethanol formation, has been calculated using a linear regression analysis (Mosteller et al., 1970) to determine a line of best fit and the correlation coefficient. Tables are available which define the correlation coefficient at various confidence levels (Rohlf and Sokal, 1969). In this study all the activities used were significant at the 95% confidence level or greater.

3.8.2 Efficiency Calculations

At various stages in this study the efficiency of the conversion of sugar to ethanol has been calculated. This calculation has been based upon the sugar utilized (not the sugar available) and the theory that 2 molecules of glucose or 1 molecule of lactose can produce 4 molecules of ethanol. The actual ethanol produced is expressed as a percentage of the possible theoretical production.

Sample Calculation of Efficiency

Initial Lactose Concentration	=	0.12 M
Final Lactose Concentration	=	0.04 M
Lactose Utilized	=	0.08 M = 80 mM
Theoretical Ethanol Concentration	=	4 x 80 = 320 mM
Initial Ethanol Concentration	=	0 mM
Final Ethanol Concentration	=	240 mM

$$\text{Efficiency \%} = \frac{\text{Final Ethanol Concentration}}{\text{theoretical ethanol concentration}}$$

$$= \frac{240}{320} \% = 75\%$$

3.8.3 Error Evaluation

There are two main sources of error in the experiments performed, measurement errors and variations in cell cultures.

3.8.3.1 MEASURED ERRORS

The measured parameters in this study were pH, sugar concentration, ethanol concentration, and cell mass (wet and dry). Errors in pH measurement (Metrohm Herisau E 520) and dry weight were estimated as negligible. However in the measurement of wet cells the composition of the yeast cake may vary, particularly with respect to

moisture. A standard separation and washing procedure was maintained to minimize this source of error.

In section 3.7.1 the ethanol analysis was found to record results within 5% of the actual value.

The calculation of sugar concentration for the sugar analysis was performed with the use of a standard curve. The following equations were obtained for the standards curves by the use of at least 8 points within the range tested:

$$\frac{\text{Fehlings solution volume (ml)}}{\text{Titration volume (ml)}} \times (0.56 \pm 0.03) = \text{Lactose concentration (M)}$$

$$\frac{\text{Fehlings solution volume (ml)}}{\text{Titration volume (ml)}} \times (0.725 \pm 0.05) = \text{Glucose concentration (M)}$$

Using these equations the error in a 0.12 M lactose measurement was 5% and that in a 0.44 M glucose measurement was 7%.

3.8.3.2 CULTURE VARIATION

In the determination of the gel composition for maximum immobilized activity (section 4.6) each experiment and each duplicate was conducted with freshly immobilized cells. This introduces possible variations in yeast culture composition, particularly in the enzyme complement. To gain an indication of the trend of the results each set of experiments (i.e. the range of BIS concentrations) was performed together using the same batch of cells. The duplicate set of experiments was performed on a subsequent day with a separate culture. The effect of this is particularly notable in the S. cerevisiae results where up to 20% difference was found between some duplicates however the trend of the duplicate experiments was similar. Differences of up to 12% were found between K. fragilis duplicates.

CHAPTER 4

RESULTS

4.1 DETERMINATION OF CELL GROWTH CURVES

The growth curves and related ethanol production of K. fragilis and S. cerevisiae were established for incubation in the substrate media (0.12 M lactose or 0.44 M glucose), supplemented with 1.3% $(\frac{w}{v})$ Nutrient Broth, at 30°C and 200 r.p.m. shaking.

The maximum rate of ethanol production for K. fragilis, of 2.2 mMoles/hr/gm cells was obtained between 15 and 22 hours after inoculation, as recorded in Figure 2. A logarithmic plot of these results, presented in Figure 3, allowed the calculation of the equations of cell mass and ethanol concentration as functions of incubation time. For the conditions described in section 3.2.4;

$$\log(\text{cell mass (gm/l)}) = -2.04 + 1.92 \log(\text{time (hours)}) \quad (1)$$

$$\log(\text{ethanol concentration (mM)}) = -1.22 + 2.65 \log(\text{time hours}) \quad (2)$$

These mathematically determined equations are only valid where the fermentation is not substrate limited. In Figure 2 the substrate has most likely limited the cell mass production after about 20 hours incubation and the ethanol production after about 25 hours incubation. This point is illustrated by Table II in which the measured values and the values predicted by the equations are compared.

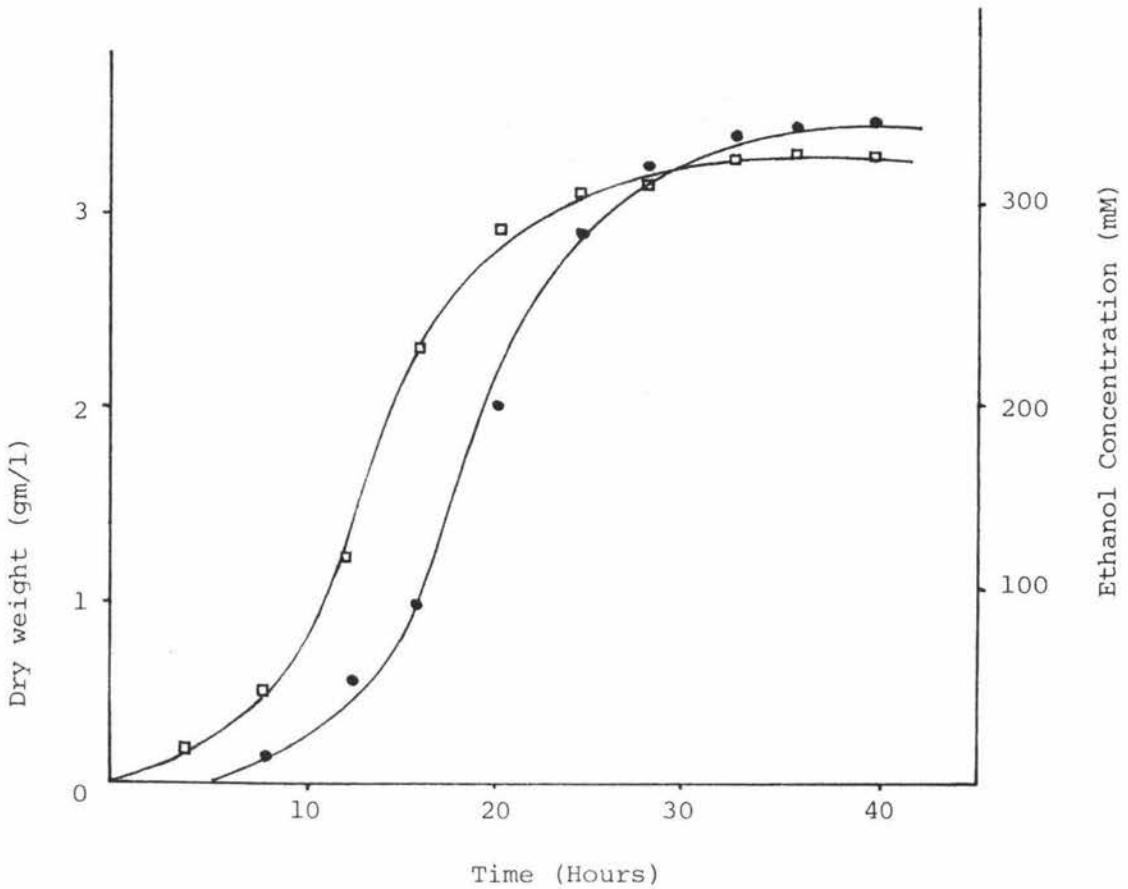

FIGURE 2. Growth curve for K. fragilis NRRL Y 1109. Cell mass and ethanol concentration were determined for cultures grown in 0.12 M lactose, 1.3% ($\frac{w}{v}$) Nutrient Broth at 30°C. Cell mass, —□— ; ethanol concentration, —●— .

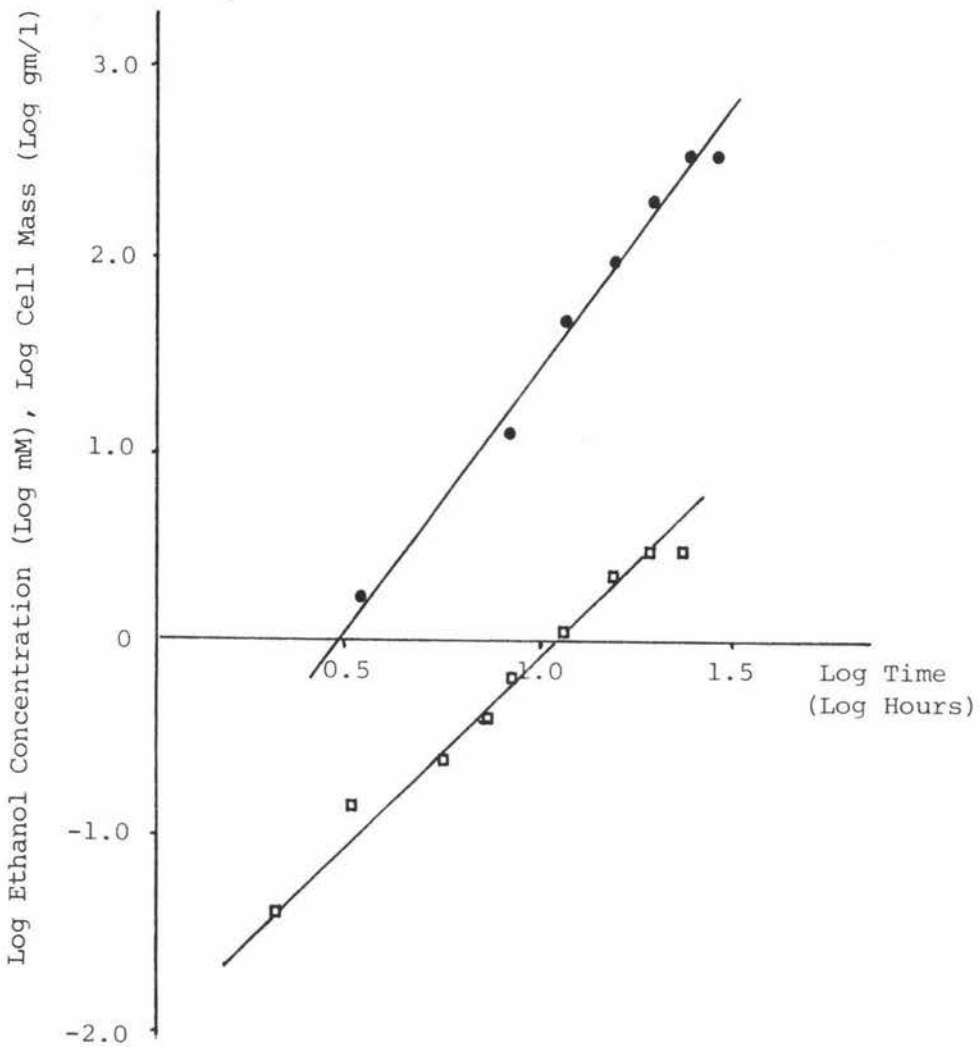

FIGURE 3. Logarithmic plot of growth curve of K. fragilis NRRL Y 1109. Cell mass and ethanol concentration were determined for cultures grown in 0.12 M lactose, 1.3% ($\frac{w}{v}$) Nutrient Broth at 30°C. Cell mass, —□— ; Ethanol Concentration, —●— .

TABLE II. Measured and predicted values of cell mass and ethanol concentration during growth* of K. fragilis NRRL Y 1109.

Time (hr)	Cell Mass (gm/l)		Ethanol Concentration (mM)	
	Measured	Predicted	Measured	Predicted
4	0.14 ± .05	0.13	-	2
8	0.45 ± .05	0.49	14 ± 4	15
12	1.20 ± .05	1.19	50 ± 3	44
16	2.30 ± .05	1.87	95 ± 5	94
20	2.90 ± .05	2.87	190 ± 10	169
24	3.10 ± .05	5.48	296 ± 15	274
28			327 ± 18	412

* The culture was grown in 0.12 M lactose and 1.3% ($\frac{w}{v}$) Nutrient Broth at 30°C.

The difference between the two sets of values, measured and predicted, ranges from 1% to 18% of the measured values. The equations should provide for subsequent investigators, an estimate of cell mass and ethanol concentration during incubation prior to substrate depletion.

With S. cerevisiae the maximum rate of ethanol production of 2.8 mMoles/hr/gm cells was achieved between 26 and 32 hours after inoculation as portrayed in Figure 4. The equations for the prediction of cell mass and ethanol concentration during incubation were calculated from Figure 5, a logarithmic plot of the results in Figure 4, where;

$$\log (\text{cell mass (gm/l)}) = -1.32 + 1.25 \log (\text{time (hours)}) \quad (3)$$

$$\log (\text{ethanol concentration (mM)}) = -0.34 + 1.83 \log (\text{time hours}) \quad (4)$$

Table III contains a comparison of the actual and predicted values obtained by using these equations. Prior to apparent substrate limitation of cell mass production at approximately 30 hours after inoculation the predicted values are within 10% of the measured values. For ethanol production it is likely that substrate limitation has occurred after 35 hours of incubation before which the predicted values are within 20% of the measured values. The equations may be used in further investigations to approximate cell mass and ethanol production during the incubation prior to substrate depletion.

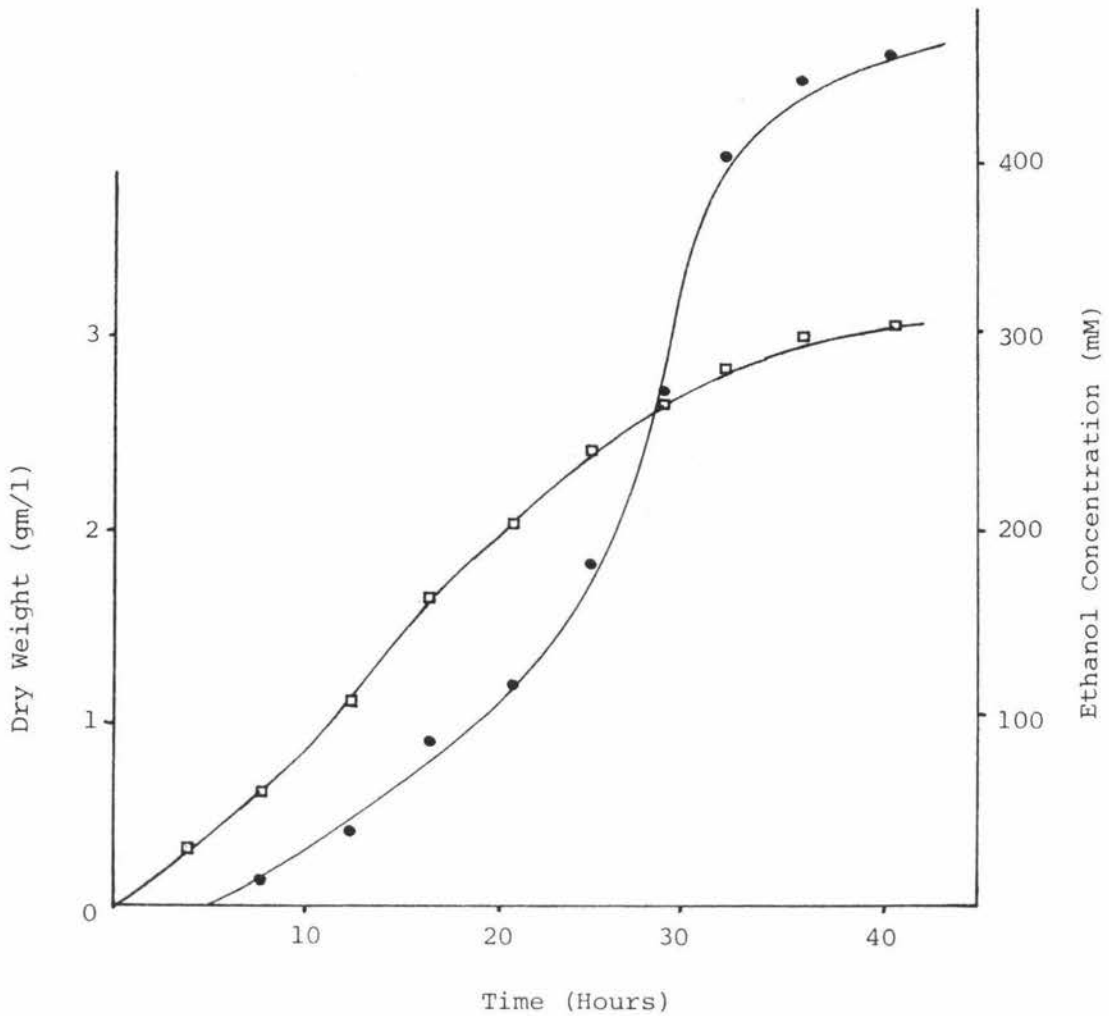

FIGURE 4. Growth curve for S. cerevisiae NCYC 240. Cell mass and ethanol concentration were determined for cultures grown in 0.44 M glucose, 1.3% ($\frac{w}{v}$) Nutrient Broth at 30°C. Cell mass, —□— ; ethanol concentration, —●— .

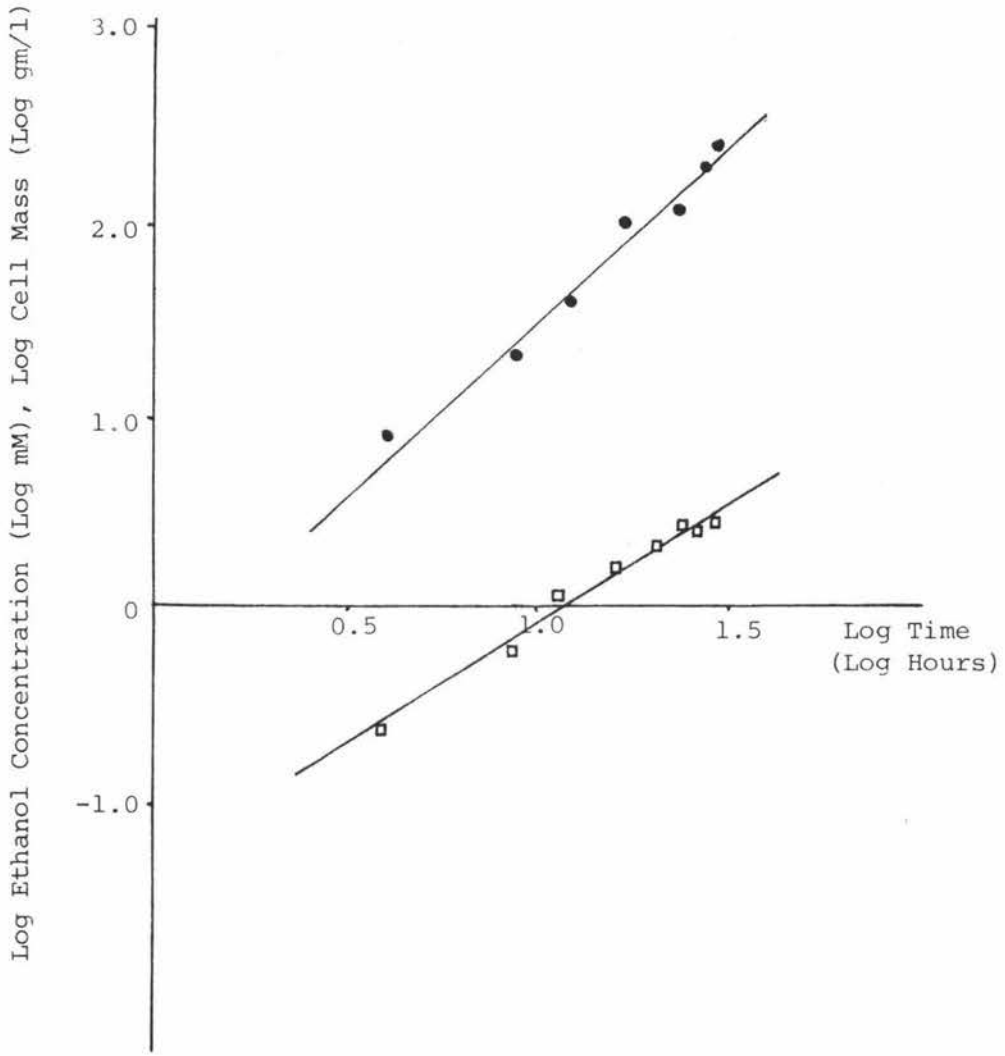

FIGURE 5. Logarithmic plot of growth curve of S. cerevisiae NCYC 240. Cell mass and ethanol concentration were determined for cultures grown in 0.44 M glucose, 1.3% $(\frac{w}{v})$ Nutrient Broth at 30°C. Cell mass, —□— ; Ethanol Concentration, —●— .

TABLE III. Measured and predicted values for cell mass and ethanol concentration during growth* of S. cerevisiae NCYC 240.

Time (hr)	Cell Mass (gm/l)		Ethanol Concentration (mM)	
	Measured	Predicted	Measured	Predicted
4	0.25 ± .05	0.27	7 ± 4	6
8	0.60 ± .05	0.64	17 ± 4	20
12	1.10 ± .05	1.07	40 ± 2	43
16	1.65 ± .05	1.53	85 ± 4	73
20	2.03 ± .05	2.02	100 ± 5	109
24	2.34 ± .05	2.54	180 ± 8	153
28	2.60 ± .05	3.08	250 ± 15	203
32	2.71 ± .05	3.64	390 ± 20	260
36			420 ± 25	322

* The culture was grown in 0.44 M glucose and 1.3% $(\frac{w}{v})$ Nutrient Broth at 30°C.

The data obtained in this section was used to predict the incubation time for cells to be immobilized. Incubation periods of 16 hours for K. fragilis and 28 hours for S. cerevisiae were used in all subsequent experiments. By harvesting at these times the maximum free cell ethanol producing activity was obtained.

4.2 FERMENTATIONS AT OPTIMIZED GEL CONDITIONS

Cells immobilized in the modified Chibata et al. (1974) gel, described in section 3.3.1, were found to give reproducible fermentation results for both K. fragilis and S. cerevisiae with respect to ethanol production.

In Figure 6 the ethanol production results over 7 to 8 hours of fermentation are presented for free and immobilized cells of K. fragilis and S. cerevisiae. In all cases ethanol was produced at a constant rate from about the first hour of fermentation until substrate depletion. The constant rates of ethanol production referred to as activity, were calculated from Figure 6 (as per section 3.8.1). All subsequent activity measurements were calculated in the same manner. Activities of 1.25 ± .1 mMoles/hr for 1.25 gm[3] of free K. fragilis cells and 1.00 ± .1 mMoles/hr for 1.25 gm of

3 A value of 1.25 gm weight wet of cells represents the standardized quantity of K. fragilis cells used in the preparation of a gel 10 mm x 70 mm and also is equivalent to 0.32 gm dry weight cells. The standard quantity of S. cerevisiae cells is 2.0 gm wet weight, or 0.52 gm dry weight.

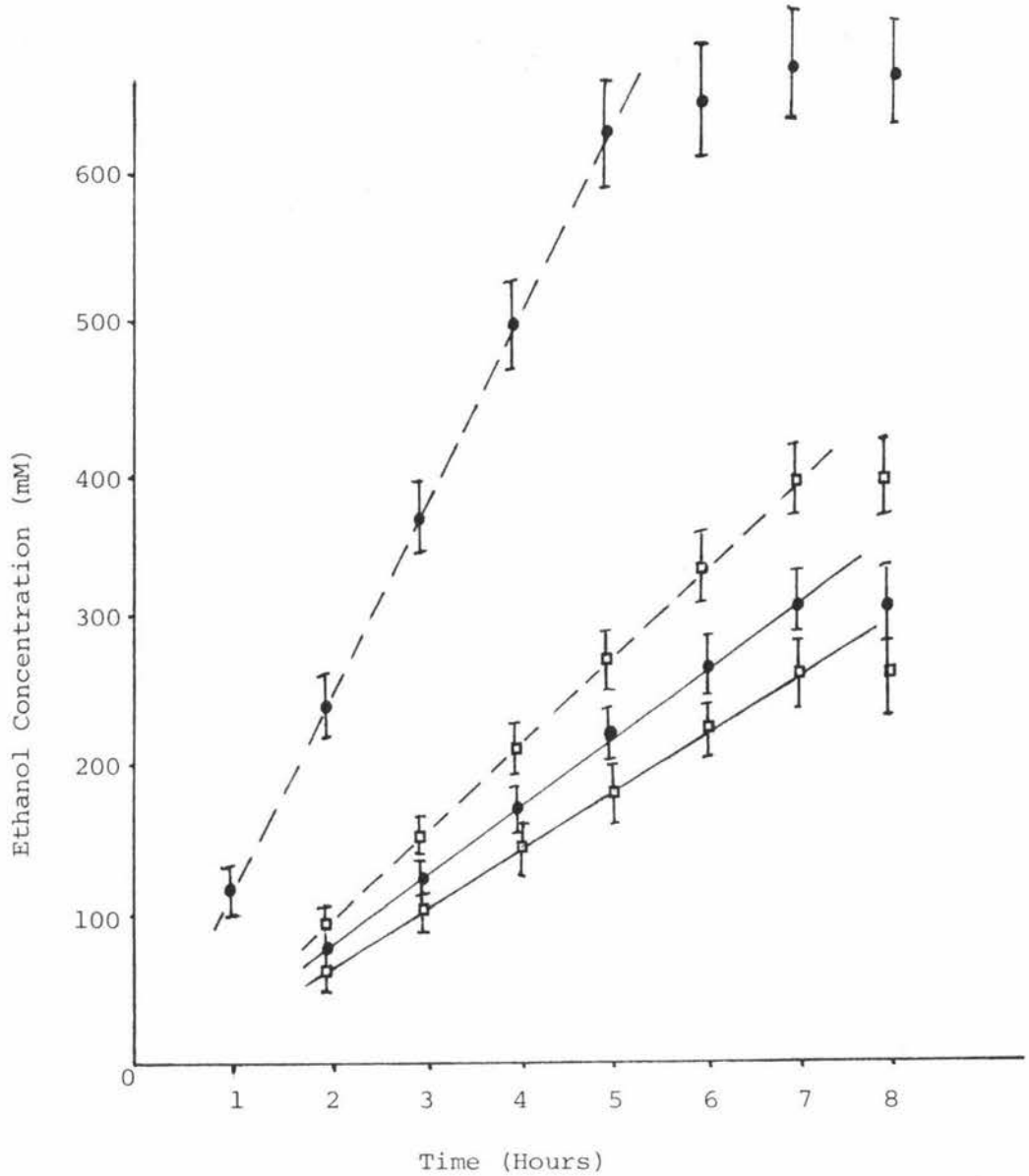

FIGURE 6. Ethanol production by cells immobilized in the modified Chibata et al. (1974) gel. Cells, immobilized as described in section 3.3.1., were used to ferment 25 ml of sugar solution, (0.12 M lactose or 0.44 M glucose) at 30°C. K. fragilis NRRL Y 1109, —— ; S. cerevisiae NCYC 240, - - - ; free cells, ● ; immobilized cells, ▫ .

immobilized K. fragilis cells were obtained. This is an activity yield of 80% for the immobilization procedure. Using 2.0 gm of S. cerevisiae cells, the free cells produced ethanol at 3.20 ± .3 mMoles/hr and the immobilized cells at 1.50 ± .1 mMoles/hr giving a yield of 47% activity for the immobilization procedure. These results are summarized in Table IV. The results represent the greatest immobilized ethanol producing activity found for both K. fragilis and S. cerevisiae.

TABLE IV. Ethanol production rates for free and immobilized* K. fragilis NRRL Y 1109 and S. cerevisiae NCYC 240 cells.

Nature of Preparation	Yeast Activity (mMoles/hr) K. fragilis (1.25 gm)	S. cerevisiae (2.0 gm)
Free Cells	1.25 ± .1	3.20 ± .3
Immobilized Cells (after Chibata)	1.00 ± .1	1.50 ± .1
Immobilized Cells (after Neuhoff)	No reproducible results	No reproducible results

* Immobilized cells were entrapped in polyacrylamide gel as described in section 3.3.1.

The results have been expressed in mMoles/hr ethanol and all mass has been specified simply as 1.25 gm(wet weight) or 2.0 gm (wet weight). The reasons for presenting the data in this manner will be developed in sections 4.5.1.3 and 4.5.2.3 where the affects of cell concentration on immobilized activity are investigated. In the initial experiments with this gel and with the Neuhoff (1973) gel 1.5 gm (wet weight) cells were used.

4.3 FERMENTATIONS WITH CELLS IMMOBILIZED IN THE ELECTROPHORETIC GEL OF NEUHOFF (1973)

Experiments with cells immobilized in the Neuhoff (1973) gel, described in section 3.3.2, were performed by incubating 1.5 gm cells, in a 7 ml gel volume, in 125 ml of sugar solution at 30^{o}C. Fermentation under these conditions did not exhibit reproducible results. In contrast fermentations incorporating 1.5 gm of free cells did give reproducible results.

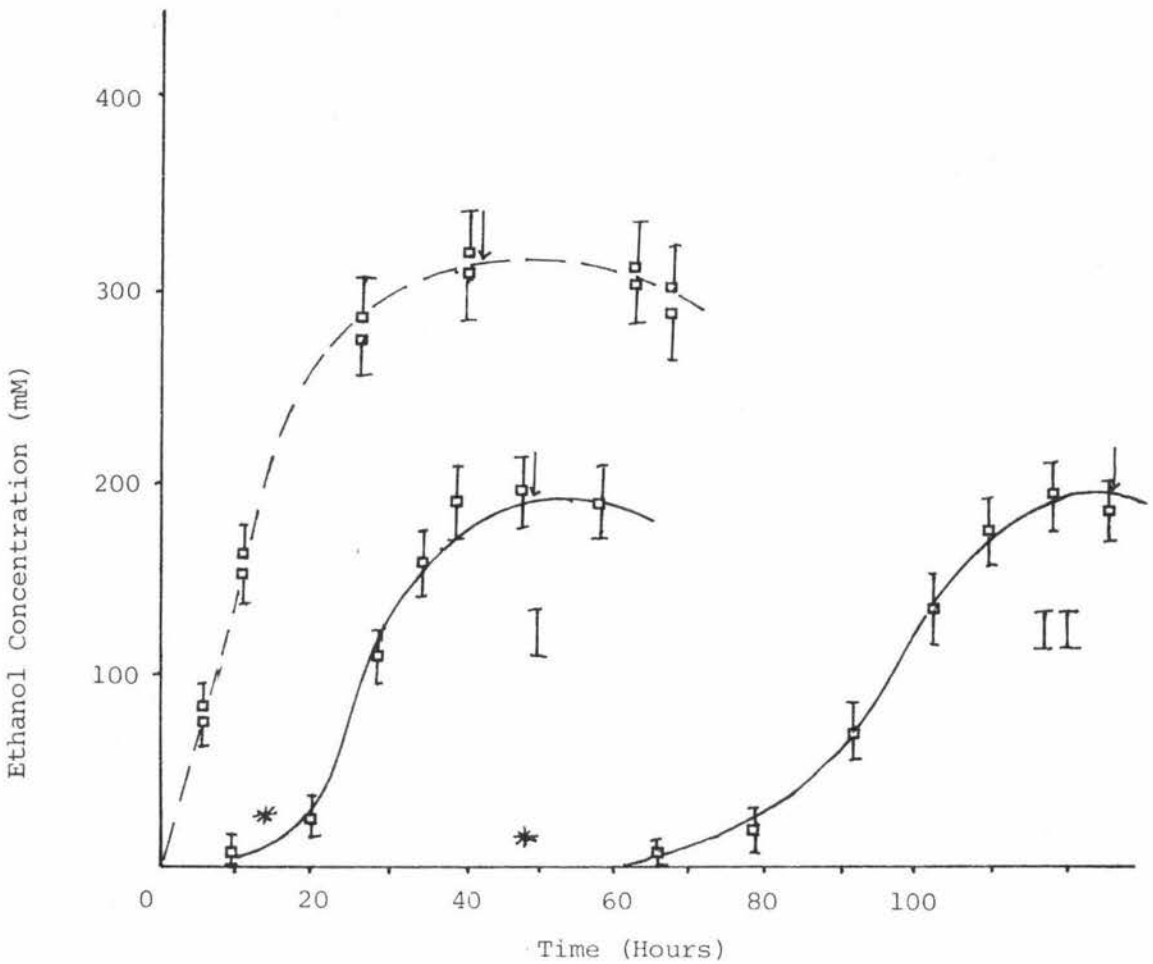

FIGURE 7. Ethanol production by K. fragilis NRRL Y 1109 cells immobilized in the Neuhoff (1973) gel. A gel containing 1.5 gm cells was used to ferment 125 ml 0.12 M lactose at 30°C, in experiments I and II. Asterisks indicate the first detection of visible turbidity in the medium and arrows indicate substrate depletion. The control experiments with free cells are represented by the broken line.

The data presented in Figure 7 for K. fragilis illustrates these points. Included in this figure are the results of two immobilized cell fermentations and of two free cell or control experiments performed under the conditions described in section 3.4.1.1.

Using the free cells an initial ethanol production rate of 1.75 mMoles/hr/1.5 gm cells preceded the maximum ethanol concentration of 340 ± 35 mM and substrate depletion which occurred after 40 hours of fermentation in both experiments.

In the first immobilized cell experiment (I) ethanol was produced within the first 10 hours of fermentation. The detection of turbidity in the medium after 16 hours was followed by the maximum ethanol production rate of 1.35 mMoles/hr/1.5 gm cells. The maximum ethanol concentration of 200 ± 20 mM and substrate depletion occurred after 50 hours of fermentation.

In the second immobilized cell experiment (II) turbidity was detected in the medium after 45 hours of incubation and ethanol was present after 60 hours incubation. A maximum ethanol production rate of 0.75 mMoles/hr/1.5 gm cells was obtained prior to the maximum ethanol concentration of 240 ± 25 mM and substrate depletion after 120 hours of fermentation.

It is noted that the results from these four fermentations were obtained under similar conditions. The two fermentation patterns, using immobilized K. fragilis, were representative of the results obtained with both cell species. Lag periods of up to 100 hours were obtained prior to ethanol production. In cases where the gel was noted to polymerise particularly slowly no ethanol production or substrate utilization was observed within 150 hours of fermentation. When produced, the maximum concentration of ethanol attained was 40-70% of that produced by the free cells. The ethanol production rates were also decreased by 25 to 60%, after immobilization.

A feature of these experiments was the coincidental appearance of turbidity and ethanol in the medium. In most experiments this turbidity was microscopically shown to be due to the presence of a free cell yeast population in the fermentation medium.

The results gained in this section suggested that the immobilization technique was detrimental to the ethanol producing ability of the cells. In particular, prolonged contact with the unpolymerised gel components

appeared to destroy ethanol producing ability totally. The effect of the gel components on the yeast cells will be investigated in the next section.

4.4 THE EFFECT OF GEL COMPONENTS ON ETHANOL PRODUCTION

As the conditions used in the polymerization process appeared to have a significant influence on the subsequent activity of the immobilized cells, the effect of individual gel components on ethanol production was tested. Figure 8 shows the ethanol producing activity of free K. fragilis cells which were subjected to 30 minutes exposure to solutions of gel components.

When compared with the results for the untreated cells 0.32% $(\frac{w}{v})$ TEMED had little effect on the fermentative ability of the K. fragilis cells with a maximum ethanol concentration of 320 ± 30 mM and maximum ethanol production rate of 1.45 mMoles/hr/gm cells being reached. The untreated cell maximum production rate and concentration were 1.4 mMoles/hr/gm cell and 300 ± 30 mM, respectively.

Ethanol was produced from the beginning of the fermentation using the 0.1% $(\frac{w}{v})$ ammonium persulfate treated cells. The ethanol production rate was reduced to 0.9 mMoles/hr/gm cells and the maximum ethanol concentration was decreased by 25% to 240 ± 25 mM due to the treatment. It was noted that substrate depletion occurred 30 hours after ethanol production ceased.

Contact with 15% $(\frac{w}{v})$ acrylamide at pH 7.1 and 8.3 produced two obvious effects. Firstly the total ethanol production was lowered and secondly a lag period of 20 hours was present before ethanol production commenced. Inspection of Figure 8 suggests that the effect of exposure to acrylamide is pH dependent. At pH 7.1 a maximum ethanol concentration of 250 ± 25mM was attained by a maximum production rate of 0.8 mMoles/hr/gm cells. At pH 8.3 the reduction of these values was more pronounced with a maximum rate of 0.45 mMoles/hr/gm cells and a maximum ethanol concentration of 130 ± 10 mM being recorded. Substrate depletion was also noted to occur 50 hours after ethanol production has ceased.

A similar set of results was obtained using S. cerevisiae and these are presented in Figure 9.

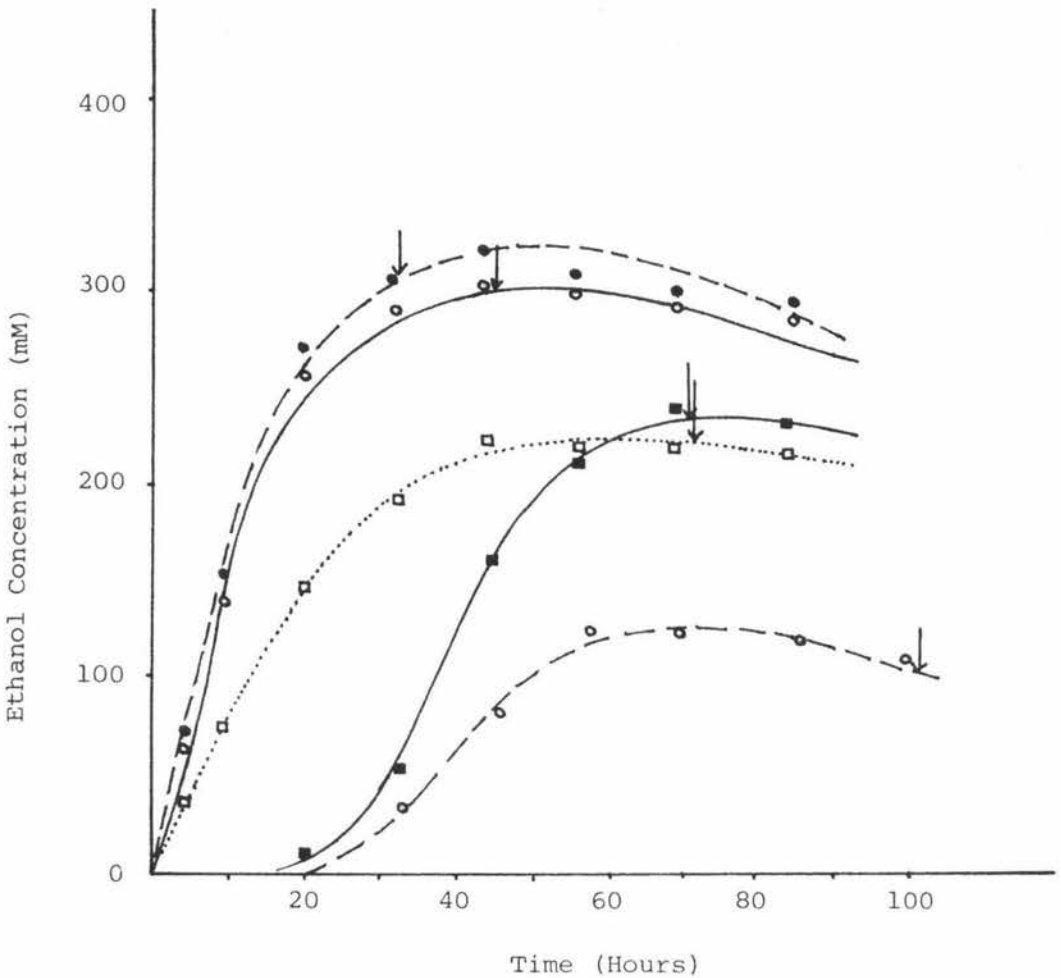

FIGURE 8. Ethanol production by K. fragilis NRRL Y 1109 cells exposed to gel components. One gram of free cells was exposed to a solution containing one of the following components for 30 minutes at 30°C and washed prior to the fermentation of 0.12 M lactose at 30°C. Untreated cells, —o— ; 0.32% ($\frac{W}{V}$) TEMED, --•-- ; 0.1% ($\frac{W}{V}$) ammonium persulfate, ···□····· ; 15% ($\frac{W}{V}$) acrylamide pH 8.3, --o-- ; 15% ($\frac{W}{V}$) acrylamide pH 7.1, —■— . Arrows represent substrate depletion in each experiment.

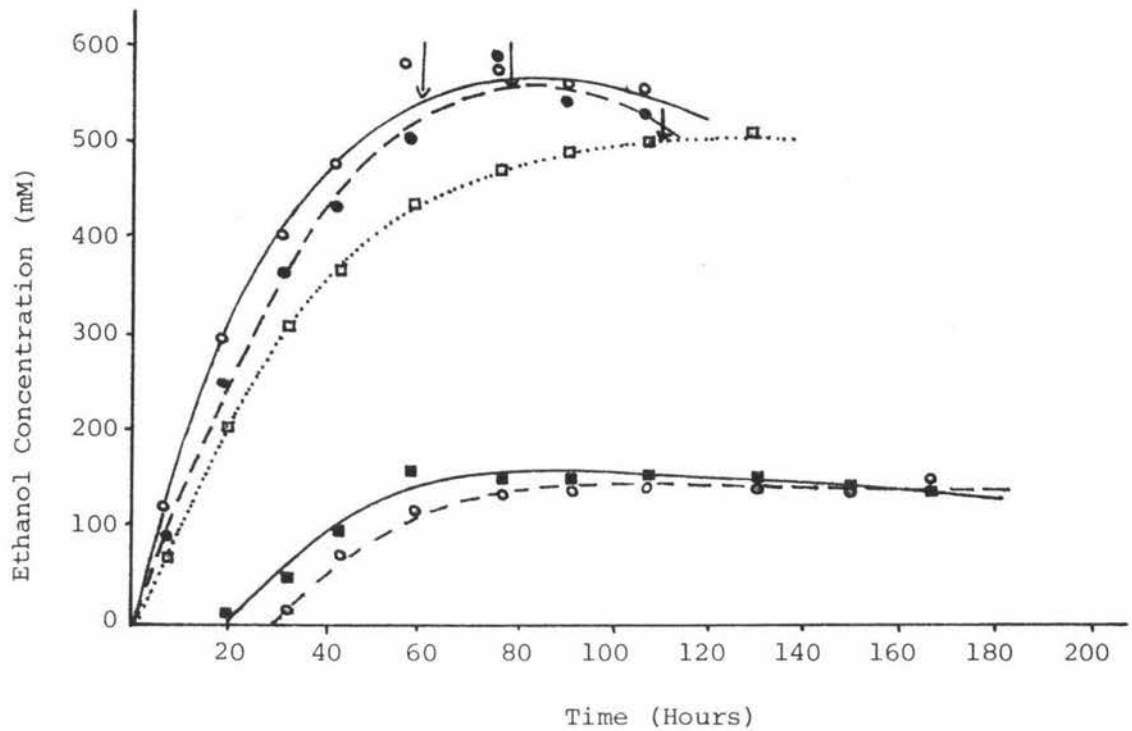

FIGURE 9. Ethanol production by S. cerevisiae NCYC 240 cells exposed to gel components. One gram of free cells was exposed to a solution containing one of the following components for 30 minutes at 30 C and washed prior to the fermentation of 0.44 M glucose at 30 C. Untreated cells, —o— ; 0.32% ($\frac{W}{V}$) TEMED,-●- ; 0.1% ($\frac{W}{V}$) ammonium persulfate,···□··· ; 15% ($\frac{W}{V}$) acrylamide pH 8.3,--o-- ; 15% ($\frac{W}{V}$) acrylamide pH 7.1,—■— . Arrows represent substrate depletion where appropriate.

Experiments testing the exposure of free S. cerevisiae cells to 0.32% ($\frac{w}{v}$) TEMED and 0.1% ($\frac{w}{v}$) ammonium persulfate recorded ethanol production from the beginning of the fermentations at rates of 1.65 and 1.50 mMoles/hr/gm cells, slightly less than the rate of the untreated cells of 1.95 mMoles/hr/gm cells.

Contact with 15% ($\frac{w}{v}$) acrylamide had three effects on the ethanol production of S. cerevisiae cells. The amount of ethanol produced was reduced by 75%, with the rate of production being reduced by 82% to 0.35 mMoles/hr/gm cells. A lag period of about 20 hours was present prior to ethanol production. Thirdly, it was noted that substrate was not completely utilized in 200 hours of fermentation although ethanol production ceased after 60 hours.

The results presented in Figures 8 and 9 are summarized in Table V. It is evident that exposure to acrylamide monomer causes a loss in ethanol producing activity in both cell species. This effect is more marked at pH 8.3 with K. fragilis, than at pH 7.1, and with S. cerevisiae. In these cases substrate depletion occurred at least 30 hours after ethanol production ceased. The effects of exposure to TEMED and ammonium persulfate are less significant.

In addition to examining the ethanol producing ability of the cells exposed to acrylamide a viability test was performed using K. fragilis cells. The cells were treated in the same manner, i.e. 30 minutes exposure at $30^{o}C$ with 15% ($\frac{w}{v}$) acrylamide at pH 7.1, with plate counts being performed on PDA before and after treatment. The initial cell concentration of 5×10^{8} cells/ml was reduced to less than 10^{5} cells/ml, a reduction of greater than 99.9%.

From the results of these experiments examining the effects of the gel components on ethanol production the following hypothesis was formed for the monomer treated cells. Acrylamide monomer was found to exert the greatest damaging effect on the free cells. As a result of the large loss in cell viability, through exposure to acrylamide, in the initial stages of the fermentation the cell mass consisted of primarily dead cells and therefore ethanol production was at a low level, not detected by the method of analysis used. These dead cells then lysed releasing nutrients into the medium upon which the remaining viable cells could multiply and produce detectable levels of ethanol. The overall effect of this was to produce lag periods prior to ethanol production.

TABLE V. Ethanol production by K. fragilis NRRL Y 1109 and S. cerevisiae NCYC 240 cells after exposure* to gel components.

Gel Component Tested	Lag period (hr)		Maximum Rate mMoles/hr/gm cells		Maximum Concentration mM	
	K. fragilis	S. cerevisiae	K. fragilis	S. cerevisiae	K. fragilis	S. cerevisiae
None	-	-	1.40	1.95	300	560
TEMED 0.32% ($\frac{w}{v}$)	-	-	1.45	1.65	320	560
Ammonium persulfate 0.10% ($\frac{w}{v}$)	-	-	0.90	1.50	220	500
Acrylamide pH 7.1 15% ($\frac{w}{v}$)	20	20	0.80	0.35	240	150
Acrylamide pH 8.3 15% ($\frac{w}{v}$)	25	25	0.45	0.35	130	130

* Free cells were exposed to individual gel components for 30 minutes at 30°C.

It also appears that the acrylamide affects the movement of product within the cell's environment in an inheritable manner causing a build up of product near the cell. Alternatively contact with acrylamide may affect the cell's tolerance to ethanol. Both of these possibilities would introduce the phenomenon where ethanol production ceased prior to substrate depletion.

4.5 THE EFFECT OF PROTECTIVE AGENTS AGAINST ACRYLAMIDE DAMAGE

It was recorded in the previous section that contact with acrylamide destroyed at least 99.9% of K. fragilis cell viability. To minimize the damage caused by the acrylamide four general protective compounds were used to pretreat the cells before exposure to acrylamide. The compounds, 1% ($\frac{w}{v}$) glycerol, 1% ($\frac{w}{v}$) gelatin, 1% ($\frac{w}{v}$) Tween 80 and 0.75% ($\frac{w}{v}$) dithiothreitol, were used as described in section 3.6.2.

The results of these fermentations are presented in Figure 10. Similar fermentation patterns were recorded by all treated cells with such obvious features as a 20 to 30 hour lag period prior to ethanol production and a maximum production rate of 0.6 to 0.9 mMoles/hr/gm cells. The results are compatible with those on Figure 8 for contact with 15% ($\frac{w}{v}$) acrylamide at pH 7.1 suggesting that the general protective agents were not effective against acrylamide damage to K. fragilis cells.

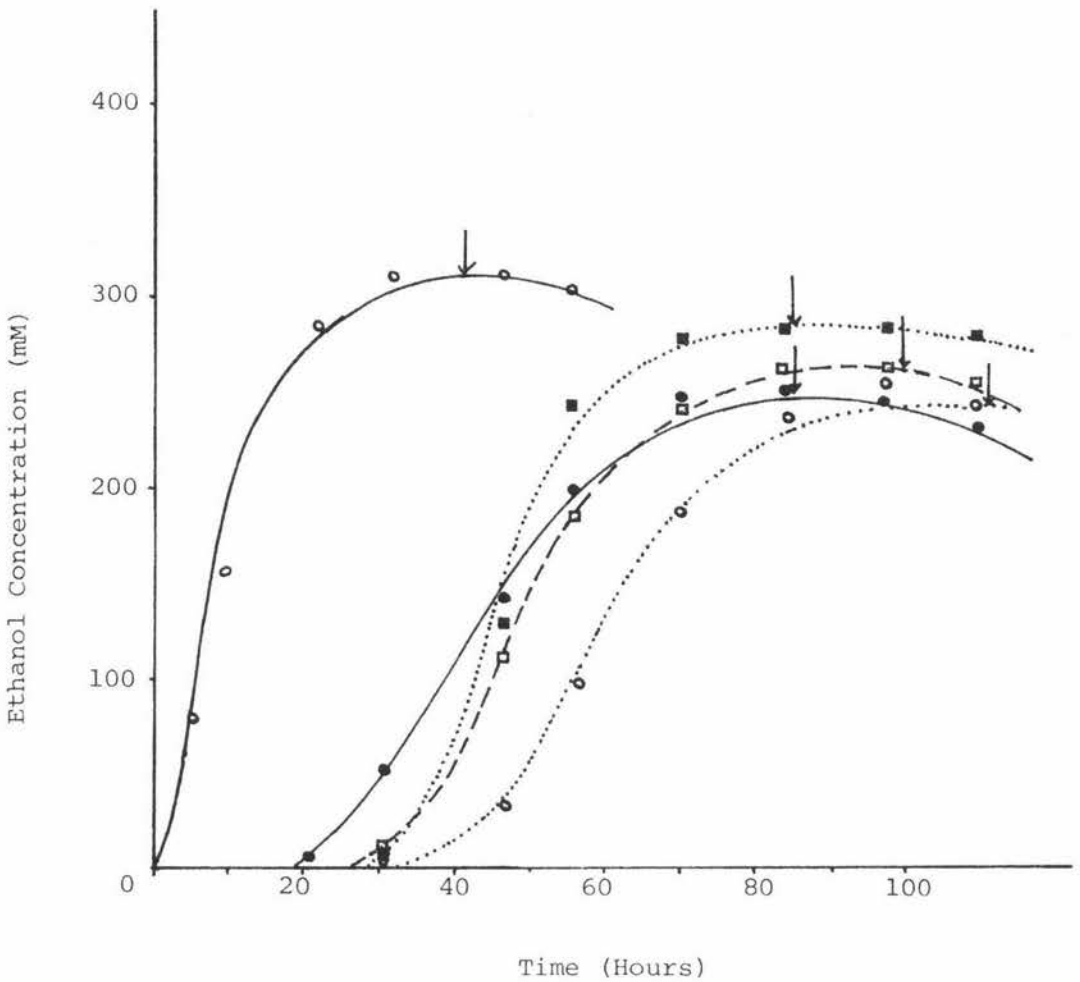

FIGURE 10. Ethanol production by K. fragilis NRRL Y 1109 cells after exposure to protective agents and acrylamide monomer. One gram of cells was exposed to one protective agent for 10 minutes prior to 30 minutes exposure to 15% ($\frac{W}{V}$) acrylamide pH 7.1 at 30°C, washing and fermentation of 0.12 M lactose.
Untreated cells, —o— ; 1% ($\frac{W}{V}$) Tween 80, ···■··· ; 1% ($\frac{W}{V}$) glycerol, -□- ; 1% ($\frac{W}{V}$) gelatin, ···o··· ; 0.75% ($\frac{W}{V}$) dithiothreitol, —●— . Arrows represent substrate depletion in each experiment.

At this stage, the exact site of acrylamide damage has not been located.

As acrylamide appeared more damaging to S. cerevisiae than K. fragilis in section 4.4 it was highly doubtful that the protective agents would be effective with S. cerevisiae cells. Consequently, continued experiments with S. cerevisiae were terminated.

The results from this section indicate that simple protective agents do not affect acrylamide damage to K. fragilis cells. No further experimentation was performed to elucidate the nature of this damage caused by the immobilization of cells in the Neuhoff (1973) gel.

4.6 FACTORS AFFECTING IMMOBILIZED ACTIVITY

In this section of work four factors affecting immobilized cell activity were investigated. These were:

1) monomer concentration in the gel.
2) Crosslinking agent concentration in the gel.
3) Yeast cell concentration in the gel.
4) Fermentation substrate concentration.

In a series of batch fermentations the concentrations of these components resulting in maximum immobilized activity were determined for both K. fragilis and S. cerevisiae. In all batch fermentations 1 gel of 5 ml volume was incubated in 25 ml of fermentation media at 30^{o}C as described in section 3.4.1.2.

4.6.1 Immobilization of K. fragilis in Polyacrylamide Gel

4.6.1.1 ACRYLAMIDE CONCENTRATION FOR MAXIMUM IMMOBILIZED ACTIVITY

The results of the investigation of the effect of acrylamide concentration on ethanol production by immobilized K. fragilis are presented on Figure 11. For concentrations of acrylamide below 12 - 15% ($\frac{W}{V}$) a low immobilized cell activity was recorded however this increased to a relatively constant value of 0.95 mMoles/hr/1.5 gm cells for acrylamide concentrations between 12 and 25% ($\frac{W}{V}$) monomer. The range of monomer concentrations was not extended further as the gels became rigid and fragile above 25% ($\frac{W}{V}$) monomer

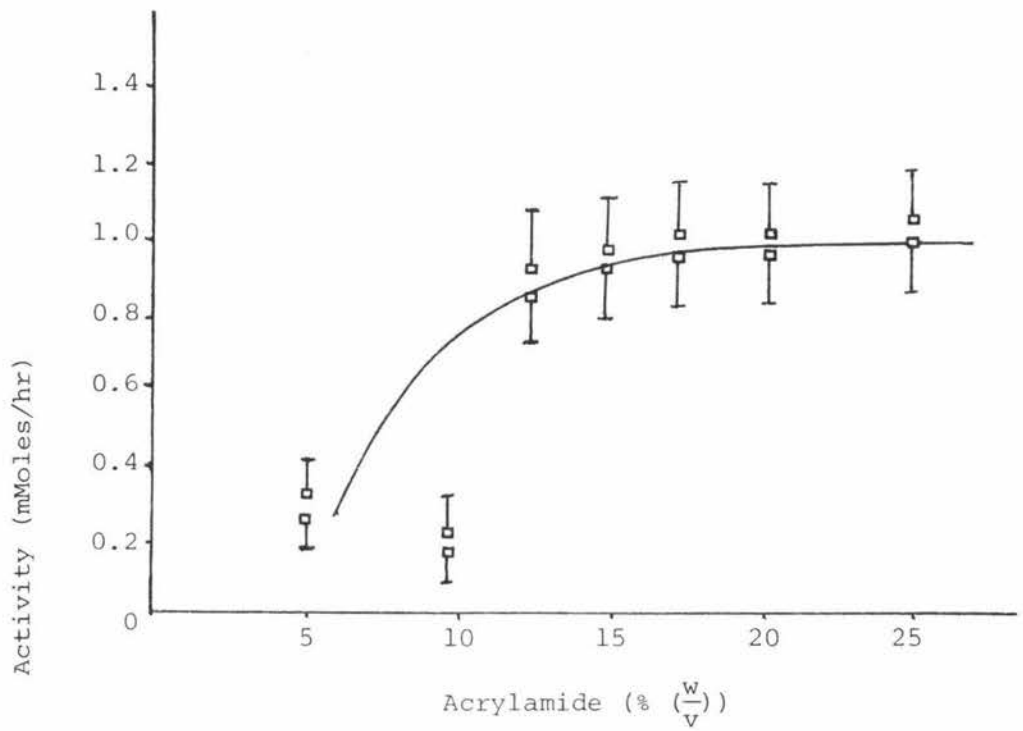

FIGURE 11. Ethanol production activity (mMoles/hr) by immobilized K. fragilis NRRL Y 1109 as a function of the concentration of acrylamide in the gel. A cell concentration of 1.5 gm/gel and 5.5% of the acrylamide weight as BIS were included in the gel which was used to ferment 0.12 M lactose at 30°C. All other conditions are described in sections 3.3.1 and 3.4.1.2.

and jelly-like below 5% ($\frac{w}{v}$) monomer making handling difficult.

In subsequent experiments with K. fragilis a 15% ($\frac{w}{v}$) acrylamide concentration was used in gel preparation. This concentration was chosen from within the range of monomer concentrations for maximum activity while limiting the opportunity for acylamide damage that is present at higher concentrations.

4.6.1.2 BIS CONCENTRATION FOR MAXIMUM IMMOBILIZED ACTIVITY

The range of BIS concentrations used was chosen arbitrarily from literature data. The physical properties of the gel were similar over the range tested, 0.6 to 2.4% ($\frac{w}{v}$) crosslinking agent.

The results, presented in Figure 12, suggested ethanol producing activity of K. fragilis is sensitive to the amount of crosslinking in the gel. Ethanol activity increases with increasing BIS concentration to a maximum of 1.2 mMoles/hr/1.5 gm cells at 1.5% ($\frac{w}{v}$) BIS. The activity at any point within the range used can be estimated by the equation:

$$\text{Activity (mMoles/hr)} = -0.4 \left(\text{BIS} \left(\% \frac{w}{v}\right)\right) - 1.5)^2 + 1.20 \qquad (5)$$

As recorded by the line on Figure 12, this equation is representative of the measured results.

In subsequent experiments with K. fragilis 1.5% ($\frac{w}{v}$) BIS was used in the gel.

To test the uniformity of the cell activity between experiments the results obtained in this experiment were compared with the results from the acrylamide experiments (4.6.1.1). The compatibility is illustrated on Figure 12 where the asterisk represents the result for 15% ($\frac{w}{v}$) acrylamide from Figure 11 which was obtained under the same conditions as used in this experiment.

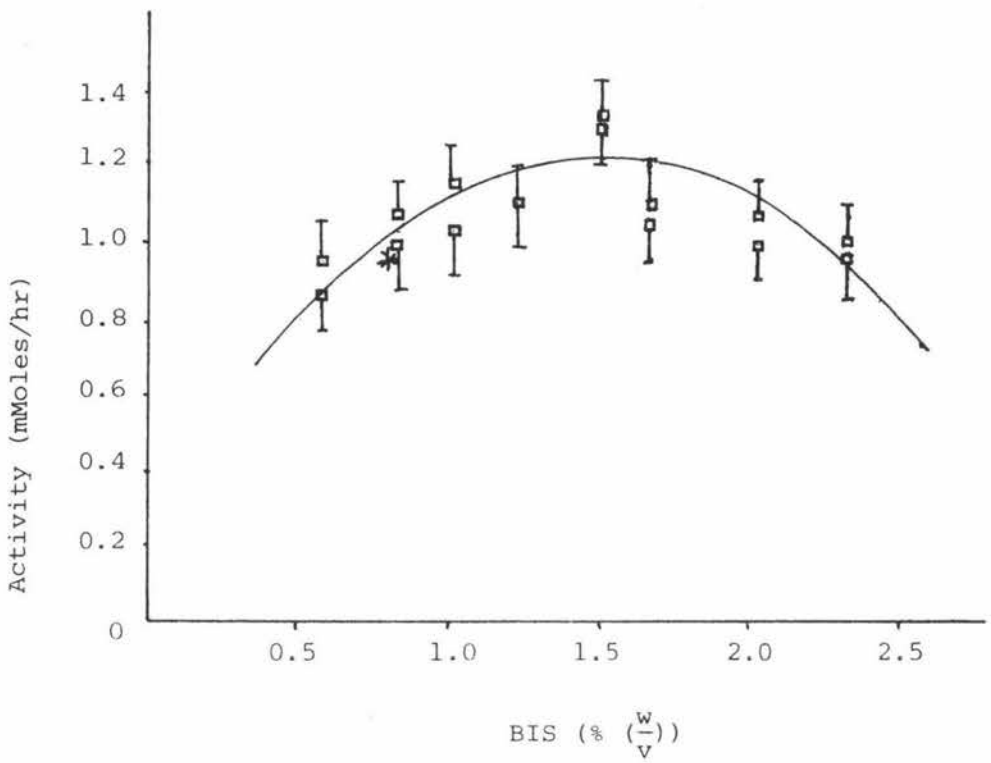

FIGURE 12. Ethanol production activity (mMoles/hr) by immobilized K. fragilis NRRL Y 1109 as a function of BIS concentration in the gel. The gel also contained 1.5 gm/gel cells and 15% $(\frac{W}{V})$ acrylamide and was used to ferment 0.12 lactose at 30 C. All other conditions are described in sections 3.3.1 and 3.4.1.3. The line is representative of the equation:

$$\text{activity (mMoles/hr)} = -0.4\ (\text{BIS } (\% \ (\tfrac{W}{V})) - 1.5)^2 + 1.20.$$

4.6.1.3 YEAST CELL CONCENTRATION FOR MAXIMUM IMMOBILIZED ACTIVITY

Using the gel methodology described in section 3.3.1 the concentration of immobilized K. fragilis cells was varied from 0.5 to 2.5 grams per 5 ml volume of gel. Following batch fermentations in 0.12 M lactose the results were recorded on Figure 13.

The results indicate that immobilized activity increased rapidly with increasing cell concentration until 1 gm cells per gel. At cell concentrations above 1 gm per gel small increases in activity were gained with increased cell concentrations.

Two possible reasons for this effect are:

a) the increased inactivation of the cells due to a retardation of the polymerisation reaction by high cell concentration (the effects of increased exposure of gel components to cells were discussed in section 4.4) and
b) the use of an inefficient particle size where cells at the centre are not receiving sufficient substrate. Diffusion limitations were proved negligible in subsequent experiments (sections 4.5.1.4 and 4.7.4) however the relatively high immobilized activity may cause near complete substrate utilization at the gel surface producing low substrate concentrations within the gel.

The non linear relationship between cell concentration and immobilized activity precluded the use of standard activity units of mMoles/hr/gm cells. In activity measurement the cell mass has been stated. It is noted that in all cases of immobilization 1.5 gm of cells were used prior to the determination of the concentrations, of 1.25 gm per gel for K. fragilis and 2.0 gm per gel for S. cerevisiae, for maximum immobilized activity per gram of cells.

A reciprocal plot of this data suggests that the maximum activity of 2.05 mM/hr can be obtained with a cell mass of 1.25 gm per gel. The equation predicting immobilized activity as a function of cell concentration of:

$$\frac{1}{\text{activity (mMoles/hr)}} = 0.43 + \frac{0.52}{\text{(gm cells per gel)}} \qquad (6)$$

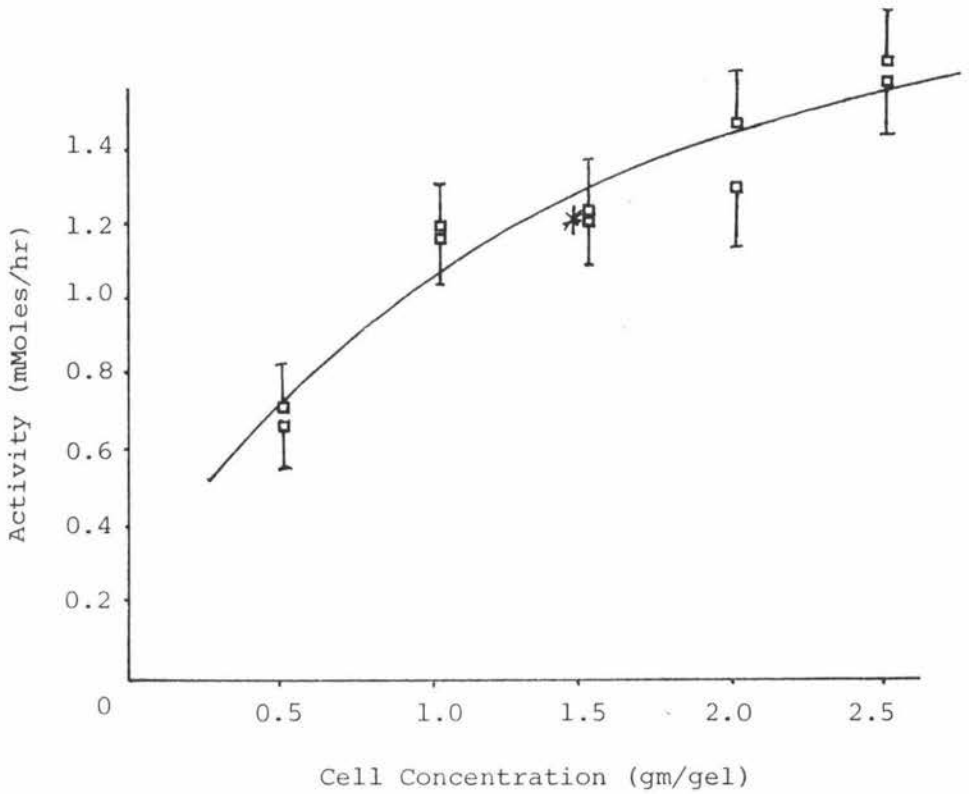

FIGURE 13. Ethanol production activity (mMoles/hr) by immobilized K. fragilis NRRL Y 1109 as a function of cell concentration in the gel. The gel also contained 15% $(\frac{w}{v})$ acrylamide and 1.5% $(\frac{w}{v})$ BIS and was used to ferment 0.12 M lactose at 30°C. The line is representative of the equation:

$$\frac{1}{\text{activity (mMoles/hr)}} = 0.43 + \frac{0.52}{\text{cell concentration (gm/gel)}}.$$

was obtained from this plot. As illustrated by the line on Figure 13 this equation is representative of the measured results.

The compatability between the results of this experiment and the results of the BIS experiments is shown by the inclusion of the 1.5% $(\frac{w}{v})$ BIS result as an asterisk on Figure 13.

4.6.1.4 THE EFFECT OF SUBSTRATE CONCENTRATION ON CELL ACTIVITY

A range of substrate concentrations of 0.06 M to 0.26 M lactose were used to determine the influence of substrate concentration on the ethanol production of free and immobilized K. fragilis cells.

The experimental results indicated that for both free and immobilized cells the ethanol producing activity increased with increasing substrate concentration. At high substrate concentrations less ethanol was produced per unit substrate. These results are presented in Table VI along with information about the yield of ethanol expressed as a percentage efficiency as described in section 3.8.2.

It was noted that the efficiency for both free and immobilized cells was greatest at low lactose concentrations and decreased as an exponential decay with increasing substrate concentration. For maximum ethanol production low substrate concentrations should be used in batch fermentations.

The efficiencies of the free cells were 10 - 20% higher than those of the immobilized cells. The immobilized cells appear to have a higher maintenance energy requirement than the free cells which may be due to the effect on the metabolic processes of the cells of the unpolymerised gel components.

A Lineweaver-Burk plot of the results is presented in Figure 14. This shows that the apparent Vmax for both the free and immobilized cells is the same at 2.7 mMoles/hr. The apparent Michealis Constant (Km) increases on immobilization from 0.15 M to 0.19 M. The equations of the lines can be used to predict activity as a function of substrate concentration where,

$$\frac{1}{\text{activity}} = 0.36 + \frac{0.059}{\text{substrate concentration}} \qquad (7)$$

$$\frac{1}{\text{activity}} = 0.36 + \frac{0.076}{\text{substrate concentration}} \qquad (8)$$

for free and immobilized cells respectively.

TABLE VI. Ethanol production activity and efficiency* of free and immobilized K. fragilis NRRL Y 1109 cells as a function of substrate concentration.

Lactose Concentration (M)	Immobilized Cells			Free Cells		
	Final Ethanol Concentration (mM)	Activity (mMoles/hr/1.25 gm)	Efficiency (%)	Final Ethanol Concentration (mM)	Activity (mMoles/hr/1.25 gm)	Efficiency (%)
0.06	180	0.55	75	205	0.70	85
0.09	215	0.70	60	250	1.00	70
0.12	280	1.00	58	310	1.20	65
0.15	300	1.10	50	360	1.30	60
0.18	325	1.25	45	430	1.40	60
0.21	320	1.40	38	460	1.65	55
0.26	365	1.55	35	570	1.80	55

* Efficiency was calculated as described in section 3.8.2.

These results indicate that the polyacrylamide gel imposed only a slight resistance to diffusion of substrate and product as the apparent Km increased by only 25% on immobilization. Although the specific activity is reduced by 20% on immobilization it appears that the apparent Vmax was unaltered.

4.6.2 Immobilization of S. cerevisiae in Polyacrylamide Gel

4.6.2.1 ACRYLAMIDE CONCENTRATION FOR MAXIMUM IMMOBILIZED ACTIVITY

The ethanol production rates of immobilized S. cerevisiae for 0.44 M glucose fermentations as a function of acrylamide concentration were recorded on Figure 15. At concentrations of acrylamide between 5% $(\frac{w}{v})$ and 25% $(\frac{w}{v})$ the immobilized ethanol producing activity was constant at 1.15 mMoles/hr/1.5 gm cells. At higher concentrations of acrylamide the activity decreased rapidly. This decrease in activity may be due

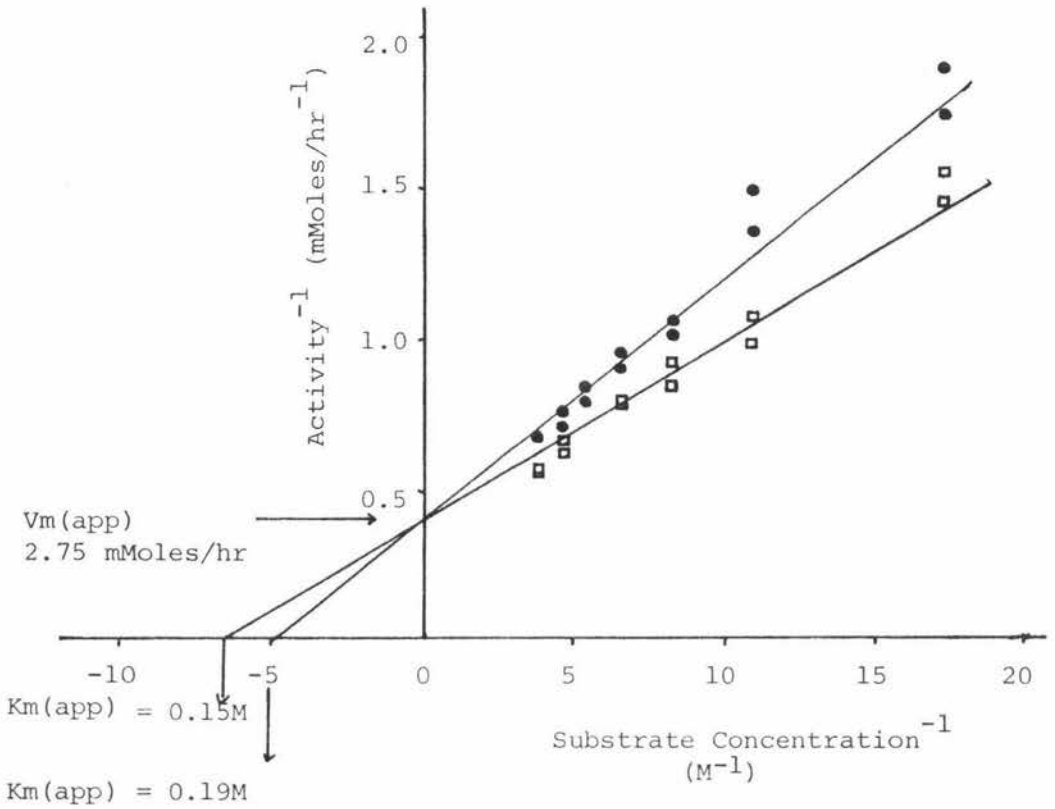

FIGURE 14. Lineweaver-Burk plot of K. fragilis NRRL Y 1109. A range of substrate concentrations of 0.06 M to 0.26 M lactose was used for fermentation by 1.25 gm cells as described in section 3.4.1.2. The immobilized cells were entrapped in a gel containing 15% ($\frac{W}{V}$) acrylamide and 1.5% ($\frac{W}{V}$) BIS as described in section 3.3.1. Free cells, —□— ; immobilized cells, —●— .

to the increased toxicity of greater acrylamide concentrations or to limitations on mass transfer with the gel at higher monomer concentrations. At this stage the experiments do not permit separation of these two effects.

In subsequent experiments 20% $(\frac{W}{V})$ acrylamide was included in the gel for the immobilization of S. cerevisiae. The initial analysis of results indicated a slightly higher activity at 20% $(\frac{W}{V})$ acrylamide.

4.6.2.2 BIS CONCENTRATION FOR MAXIMUM IMMOBILIZED ACTIVITY

Variations in BIS concentration affected the physical properties of the gels containing S. cerevisiae cells. At 5 - 10% $(\frac{W}{V})$ BIS the gels were flexible and the cutting of uniform particles was difficult.

The effect of the crosslinking agent concentration on the ethanol producing activity of the immobilized cells is presented in Figure 16. At low concentrations of BIS the activity was low, rising to a maximum of 1.35 mMoles/hr/1.5 gm cells at approximately 1.4% $(\frac{W}{V})$ BIS. The production of activity as a function of BIS concentration can be estimated by the equation:

$$\text{activity (mMoles/hr)} = 0.35\ (\text{BIS}\ (\%\ (\tfrac{W}{V})) - 2.0)^2 + 1.35 \quad (9)$$

This equation is represented by the line on Figure 16 and lies within the experimental error of all points above 0.5% $(\frac{W}{V})$ BIS.

Concentrations of 1.6% $(\frac{W}{V})$ BIS were used in subsequent experiments.

It was noted that the results in this experiment were not compatible with the results of the acrylamide experiments (section 4.6.2.1). The results from the fermentation of a gel of similar composition in the acrylamide experiments is included on Figure 16 as an asterisk. The activity in the BIS experiments at this point is approximately 25% lower than that indicated by the asterisk. Further discussion on this difference is included in section 4.6.2.4.

4.6.2.3 YEAST CELL CONCENTRATION FOR MAXIMUM IMMOBILIZED ACTIVITY

The range of cell concentrations investigated using S. cerevisiae, of 0.5 to 3 gm cells/gell, was similar to that used for K. fragilis (section 4.6.1.3).

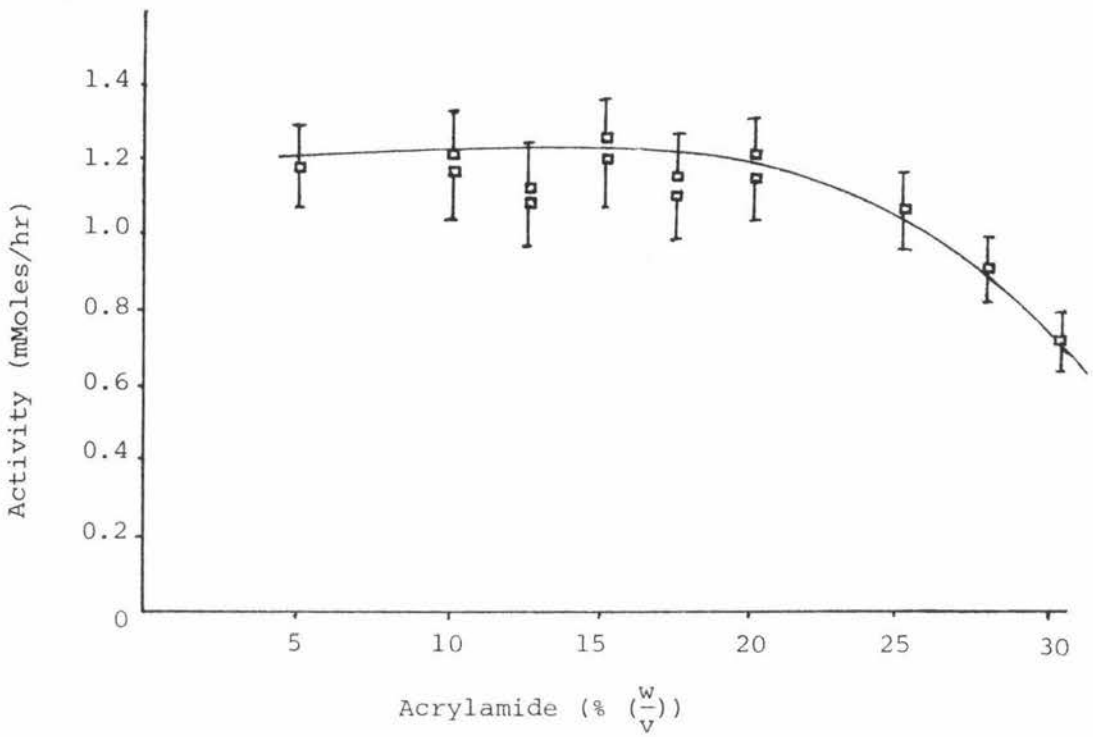

FIGURE 15. Ethanol production activity (mMoles/hr) by immobilized S. cerevisiae NCYC 240 as a function of the concentration of acrylamide in the gel. A cell concentration of 1.5 gm/gel and 5.5% of the acrylamide weight as BIS were included in the gel which was used to ferment 0.44 M glucose at 30°C. All other conditions are described in sections 3.3.1 and 3.4.1.2.

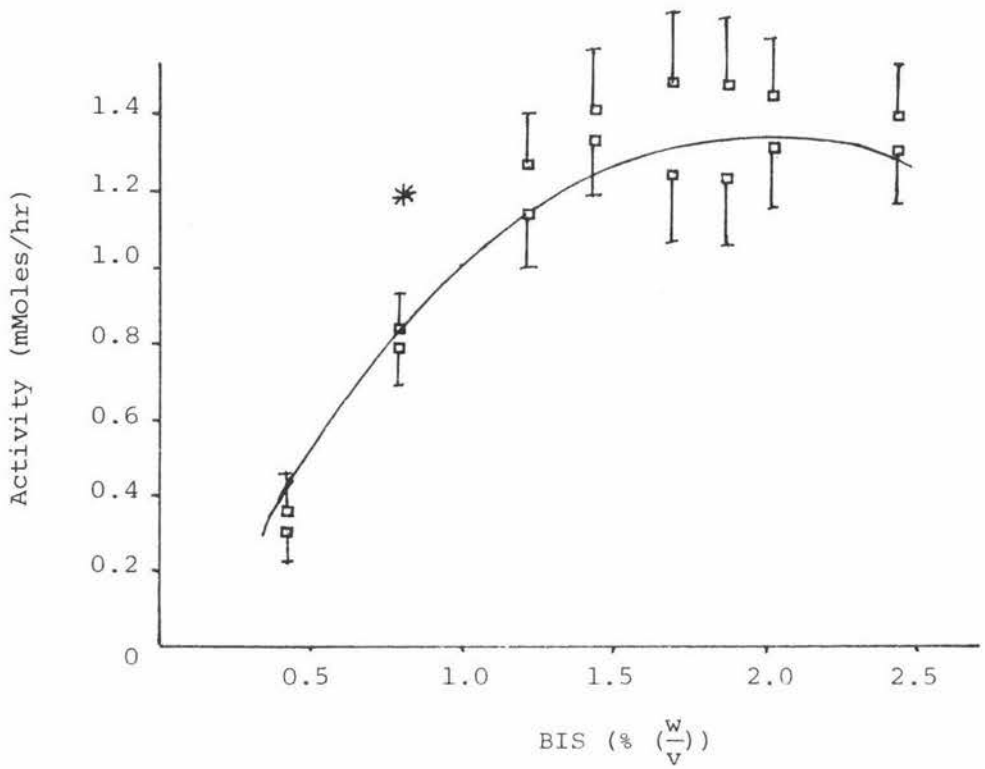

FIGURE 16. Ethanol production activity (mMoles/hr) by immobilized S. cerevisiae NCYC 240 as a function of BIS concentration in the gel. The gel also contained 1.5 gm/gel cells and 20% $(\frac{W}{V})$ acrylamide and was used to ferment 0.44 M glucose at 30°C. All other conditions are described in sections 3.3.1 and 3.4.1.2. The line is representative of the equation:

activity (mMoles/hr) = $- 0.35 (\text{BIS } (\% (\frac{W}{V})) - 2.0)^2 + 1.35$.

The effect of cell concentration on the immobilized cell's ethanol producing activity is represented on Figure 17. The results show that immobilized activity increased rapidly in a linear manner from 1.0 gm cells/gel to 2.0 gm cells/gel. At concentrations above 2.0 gm cells/gel there was a marked reduction in the rate of increase of activity with increasing cell concentration. The ethanol producing activity of S. cerevisiae as a function of cell concentration can be represented by the equation:

$$\log (\text{activity (mMoles/hr)}) = -0.17 (x - 3)^2 + 0.34 \quad (10)$$

where x = cell concentration (gm/gel)

This equation is presented as the line on Figure 17.

As for K. fragilis the non-linear nature of the activity as a function of cell concentration precluded the use of the standard units of measurement mMoles/hr/gm cells. Activity results have been recorded with the cell mass stated.

In subsequent experiments the cell concentration of 2 gm cells/gel was used because at higher cell concentrations the activity per gram of cells diminished, although the overall activity continued to increase slowly.

As with the previous sets of experiments the results are not compatible with those of the BIS experiments. This is illustrated on Figure 17 by the inclusion of the BIS result for 1.5 gm cells (represented by the asterisk) which is approximately 25% greater than the corresponding result obtained in this experiment.

4.6.2.4 THE EFFECT OF SUBSTRATE CONCENTRATION ON CELL ACTIVITY

A range of substrate concentrations of 0.11 to 0.66 M were used to determine the effect of glucose concentrations on the ethanol producing activity of both free and immobilized S. cerevisiae cells.

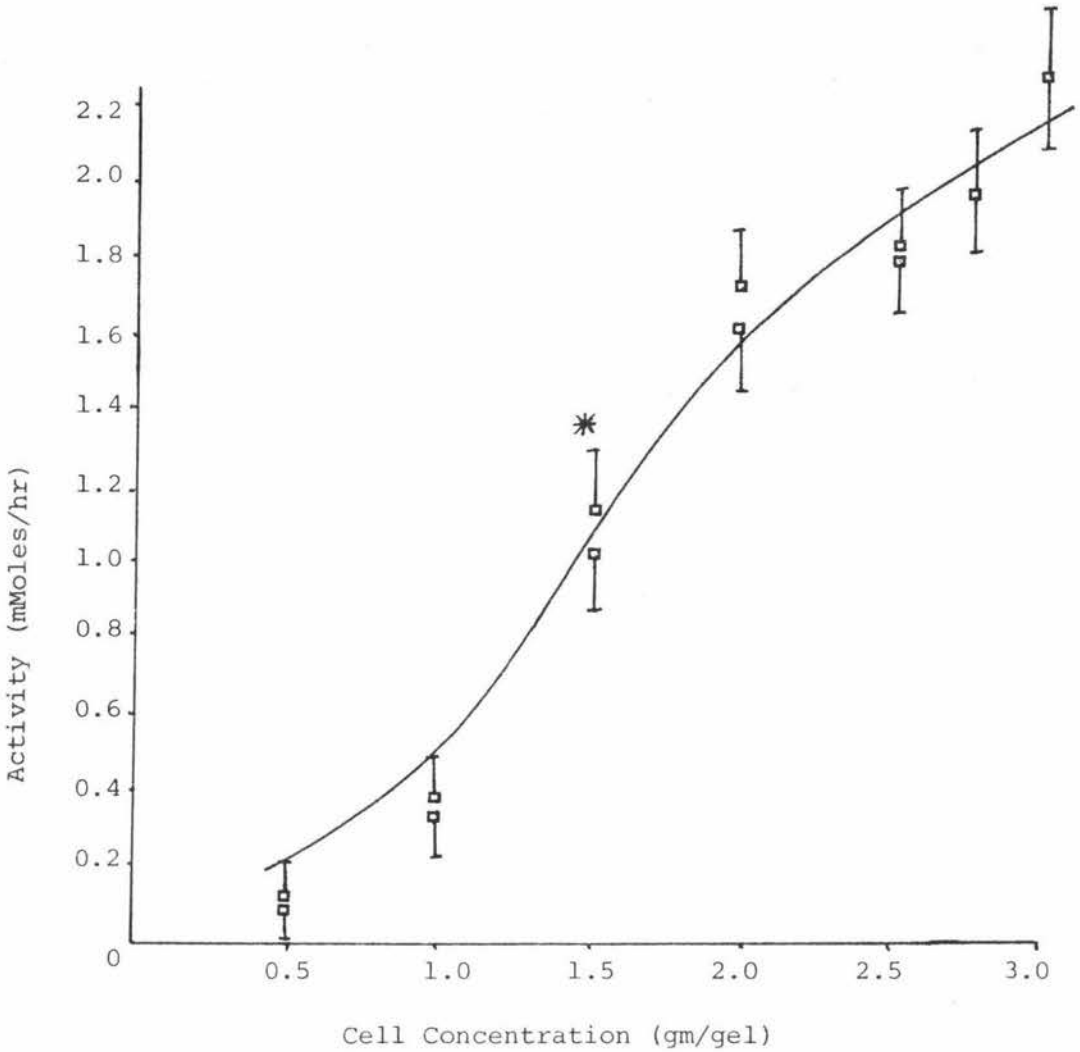

FIGURE 17. Ethanol production activity (mMoles/hr) by immobilized S. cerevisiae NCYC 240 as a function of cell concentration in the gel. The gel also contained 20% $(\frac{w}{v})$ acrylamide and 1.6% $(\frac{w}{v})$ BIS and was used to ferment 0.44 M glucose at 30°C. The line is representative of the equation: $\log (\text{activity (mMoles/hr)}) = -0.17 (x - 3)^2 + 0.34$ where x = cell concentration (gm/gel).

It was found that the activity of both free and immobilized cells did not increase indefinitely with increasing substrate concentration but reached a maximum rate of production of approximately 3.2 mMoles/hr/2 gm cells for free cells and 1.5 mMoles/hr/2 gm cells for immobilized cells at a glucose concentration of 0.27 M. These results are presented on Table VII along with the efficiency calculations.

TABLE VII. Ethanol production activity and efficiency* for free and immobilized S. cerevisiae NCYC 240 as a function of substrate concentration.

Glucose Concentration (M)	Immobilized Cells			Free Cells		
	Final Ethanol Concentration (mM)	Activity (mMoles/hr/2 gm)	Efficiency (%)	Final Ethanol Concentration (mM)	Activity (mMoles/hr/2 gm)	Efficiency (%)
0.11	165	0.60	75	210	1.30	95
0.22	265	1.30	60	395	2.20	90
0.33	360	1.48	55	530	3.25	80
0.44	385	1.54	44	620	3.00	70
0.55	430	1.54	39	715	3.10	65
0.66	430	1.45	35	730	3.30	60

* Efficiency calculations are described in section 3.8.2.

The efficiency of glucose conversion is greatest at low substrate concentrations, as with K. fragilis (section 4.6.1.4). The immobilized cells show an exponential efficiency decay whereas the free cell efficiency decreases in a linear manner with increasing substrate concentration. In the case of S. cerevisiae the immobilized cells are 20 - 30% less efficient than the free cells.

A Lineweaver-Burk plot of the results from Table VII (Figure 18) indicates that the apparent Km is the same (within experimental error) for both free and immobilized cells at 0.27 M for the immobilized cells and 0.28 M for the free cells. The apparent maximum velocity is halved from 5.0 mMoles/hr to 2.3 mMoles/hr on immobilization. This reduction in velocity is consistent with the loss in specific activity evident on immobilization. The equations derived from Figure 18:

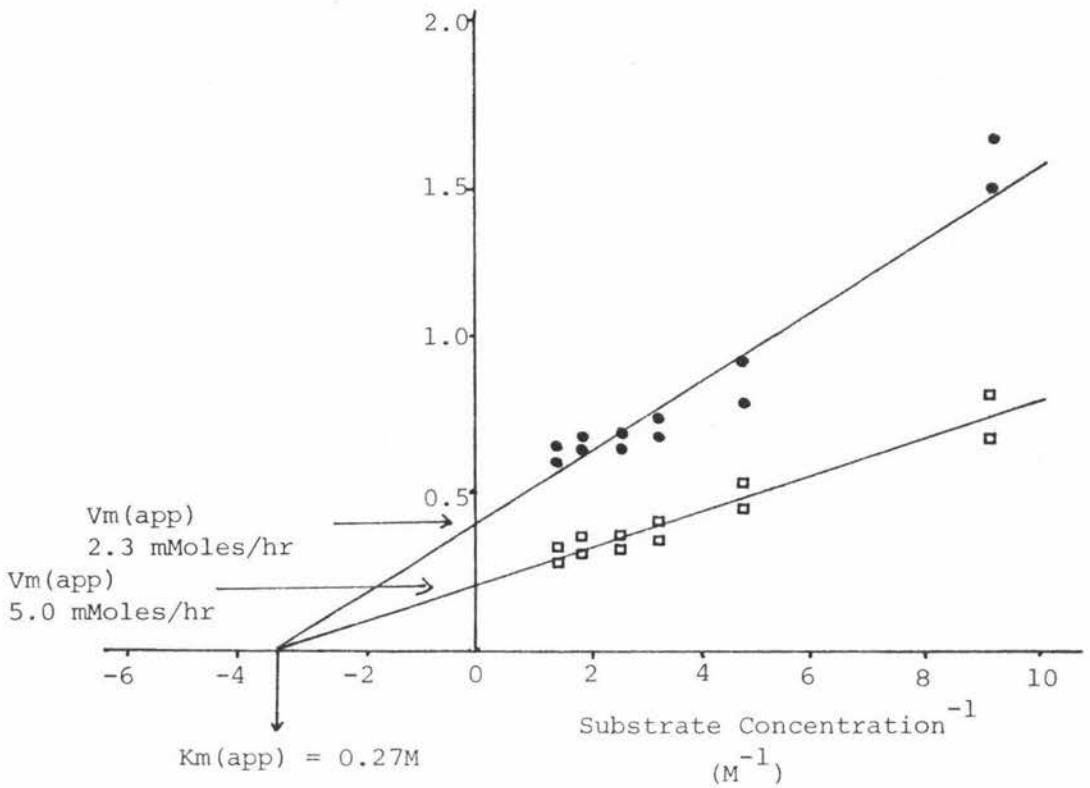

FIGURE 18. Lineweaver-Burk plot of S. cerevisiae NCYC 240. A range of substrate concentrations of 0.11 to 0.66 M glucose was used for fermentation by 2.0 gm cells as described in section 3.4.1.2. The immobilized cells were entrapped in a gel containing 20% ($\frac{W}{V}$) acrylamide and 1.6% ($\frac{W}{V}$) BIS as described in section 3.3.1. Free cells, —□— ; immobilized cells, —●— .

$$\frac{1}{\text{free cell activity}} = 0.20 + \frac{0.055}{\text{substrate concentration}} \qquad (11)$$

$$\frac{1}{\text{immobilized activity}} = 0.43 + \frac{0.117}{\text{substrate concentration}} \qquad (12)$$

may be used to predict S. cerevisiae activity as a function of substrate concentration for free and immobilized cells respectively.

The results (Table VII) suggest that it is advantageous to use low substrate concentrations with this strain of S. cerevisiae in batch fermentations. Above 0.27 M substrate concentrations there was no increase in activity and the efficiency of conversion was reduced. Immobilization in polyacrylamide gel imposed no apparent resistance to diffusion of substrate and product as the apparent Km was unchanged on immobilization.

It is noted that the activity in this experiment for 0.44 M glucose at 1.54 mMoles/hr/2 gm cells was slightly less than that of 1.65 mMoles/hr/2 gm cells obtained under the same conditions in the cell concentration experiment (section 4.6.2.3). This trend has been evident throughout these experiments with S. cerevisiae. The comparison of these results with previous data has indicated that the BIS results (section 4.6.2.2) were 25% lower than those of the acrylamide experiment (section 4.6.2.1) and the cell concentration experimental results (section 4.6.2.3) were 25% lower than the BIS results.

Free cell experiments, performed as 'control' experiments for the immobilized cell fermentations, showed that the ethanol production activity prior to these batch fermentations was similar to that obtained for the free cells in the substrate concentration experiment which was performed as the last experiment. One explanation for this deterioration in activity is a gradual increase in the susceptibility of the ethanol producing mechanism to damage by the immobilization procedure. This may have been due to either changes in the cells during storage of the stock cultures as slopes for these experiments were subcultured at one time and stored for one to four weeks until required or alternatively to undetected changes in the immobilization procedure. At this stage, no satisfactory explanation for the observation exists.

4.7 INVESTIGATION OF THE REPEATED AND CONTINUOUS USE OF IMMOBILIZED CELLS

4.7.1 Repeated Batch Experiments with K. fragilis

In all experiments performed up to this point K. fragilis cells have been immobilized, used for a fermentation of lactose and discarded. In this experiment the cells were not discarded, but rather stored for up to 5 days in 0.1 M tris-HCl buffer pH 7.1 at 4°C and reused as required in fermentation studies. In total some eighteen 6 hour fermentations were performed over 45 days using a gel containing 1.5 gm wet weight cells as described in section 3.3.1.

The results of these fermentations are presented on Figure 19. The ethanol production rate obtained for the first fermentation was 1.20 mMoles/hr/ 1.5 gm cells. This is comparable with that found under the same conditions in sections 4.6.1.2 and 4.6.1.3 of 1.25 mMoles/hr/1.5 gm cells.

Over the first eight fermentations the ethanol producing activity at the immobilized cells was found to decrease at a relatively constant rate of 0.008 mMoles/hr/hr fermentation time. This indicated that the immobilized system was not stable under the conditions used.

4.7.2 Rejuvenation of the Decayed Activity of Immobilized K. fragilis Cells

One possible means of increasing the long term stability of the immobilized cell system was to rejuvenate the decayed activity in the gel. In this experiment the gel used for the eight repeated-use fermentations was placed in a complete growth medium as decribed in section 3.5 for 24 hours at 30°C. During the incubation the medium became heavily clouded with cells, microscopically shown to be yeast. This result suggested that the cells were still viable after nineteen days of intermittent use. At the end of the growth period the gel pellets were washed twice and returned to the fermentation and storage pattern of above (section 4.7.1).

In the next fermentation of 0.12 M lactose the substrate medium became turbid within an hour due to the presence of a free cell population. The higher ethanol production rate was attributed to these free cells in the medium. In the five subsequent fermentations the turbidity was not apparent and the activity decreased at a faster rate than previously.

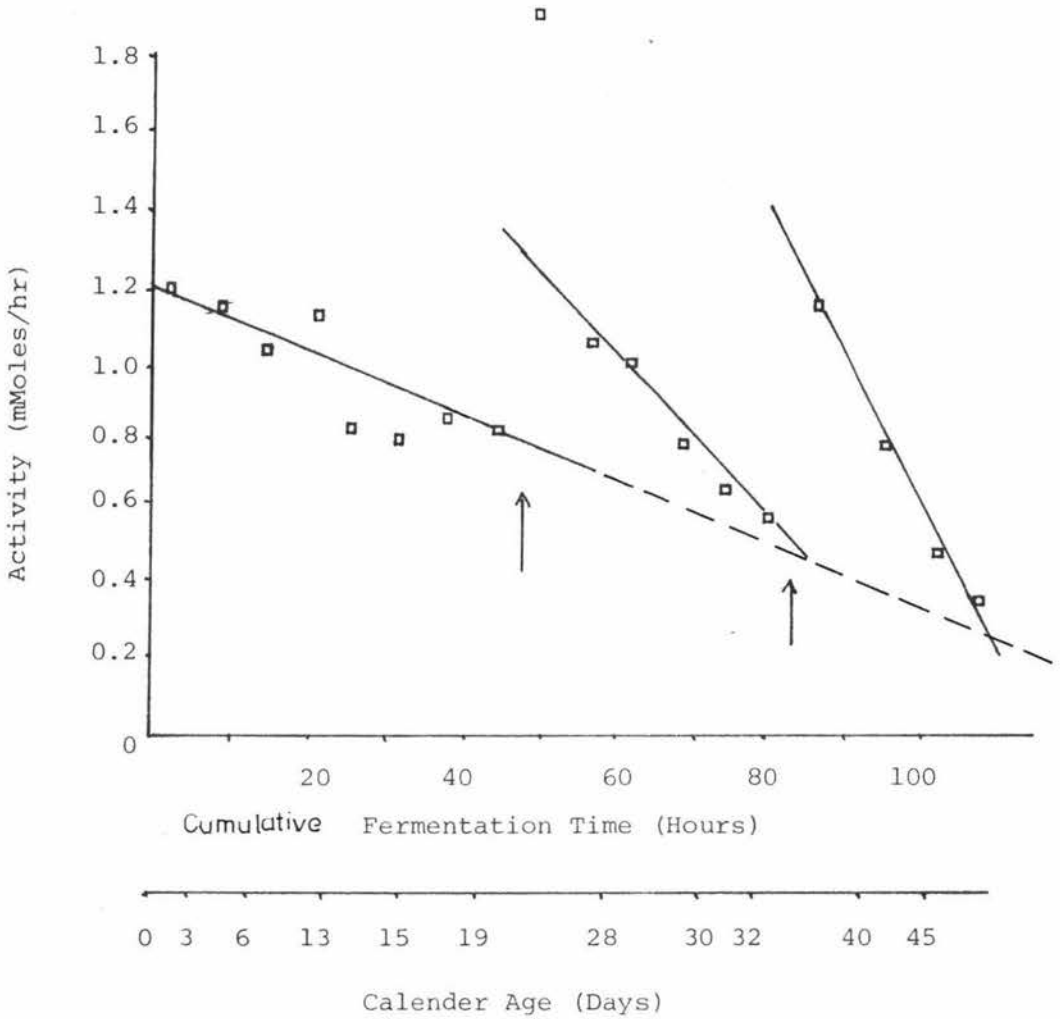

FIGURE 19. Ethanol production activity (mMoles/hr) in repeated batchwise fermentations by K. fragilis NRRL Y 1109 cells of 0.12 M lactose. Between each 6 hour fermentation the gel was stored at $4^{o}C$ in tris-HCl buffer for up to 5 days. The arrows represent the attempts at rejuvenation as described in section 3.5. Immobilization was performed by entrapping the cells in a gel containing 15% $(\frac{w}{v})$ acrylamide, 1.5% $(\frac{w}{v})$ BIS and 1.5 gm/gel cells as described in section 3.3.1.

The calculated rate of ethanol activity decay was 0.02 mMoles/hr/hr of fermentation, 2.5 times the decay rate prior to rejuvenation. A second attempt at rejuvenation produced similar results, i.e. an initial activity increase followed by a rapid decay in activity of 0.04 mMoles/hr/hr fermentation in subsequent fermentations

The broken line of Figure 19 depicts the extrapolated rate of decay curve over the first eight fermentations. Inspection of the fermentation results following rejuvenation suggests that the decay in activity tends to this line.

From the results obtained in this section it does not appear at this stage possible to rejuvenate the ethanol producing activity of K. fragilis cells fermented in 0.12 M lactose by incubation in a complete medium for yeast growth. This work was not continued.

4.7.3 The Reuse of Immobilized Cells Using Supplemented Fermentation Media

The second approach to increased immobilized cell stability during repeated fermentations involved the incorporation of cell maintenance nutrients in the substrate solution. For K. fragilis supplements of 0.5% ($\frac{w}{v}$) peptone and a salt solution were used, along with whey. For S. cerevisiae, a 0.5% ($\frac{w}{v}$) peptone supplement was used. The results of the successive fermentations are given in Table VIII.

The results from K. fragilis experiments without these supplements (section 4.7.1) indicated a rate of decrease in ethanol production activity of 0.008 mMoles/hr/hr fermentation during the first eight fermentations. A similar rate of activity decay of 0.013 mMoles/hr/hr fermentation was seen when the 0.1% ($\frac{w}{v}$) salt solution was used as the supplement. When 0.5% ($\frac{w}{v}$) peptone was used the initial immobilized activity was maintained in both yeast species. The activity of K. fragilis in whey increased slightly with repeated use, presumably due to the production of cells within the gel. Some turbidity was noted in these fermentations due to free yeast cells leaking from the gel and into the medium. These cells would have contributed to the activity. The gels were washed thoroughly between fermentation to remove free yeast cells and therefore any remaining free cells would have originated in the gel. The addition of peptone on the nutrients in whey may have induced cell growth.

TABLE VIII. Repeated batchwise fermentation of immobilized* *K. fragilis* NRRL Y 1109 and *S. cerevisiae* NCYC 240 utilizing various fermentation media.

Cell Species	Substrate	Reuse	Activity (mMoles/hr) 1	2	3	4	5	Average Efficiency (%)
K. fragilis	0.12 M Lactose + 0.5% $(\frac{w}{v})$ peptone		1.25	1.20	1.05	1.25	1.20	65
	0.12 M Lactose + 0.1% $(\frac{w}{v})$ salt solution		1.25	1.15	1.00	0.95	0.90	60
	Whey		1.75	1.85	1.65	1.80	1.90	60
S. cerevisiae	0.44 M Glucose + 0.5% $(\frac{w}{v})$ peptone		1.35	1.45	1.45	1.45	1.50	50

* Both yeast species were immobilized in a gel optimized for *K. fragilis* as described in section 3.3.1.

The use of supplemented fermentation media may be a beneficial means of preventing activity decay in immobilized K. fragilis and S. cerevisiae In these experiments (Table VIII) the incorporation of 0.5% ($\frac{w}{v}$) peptone appears to stop activity decay for at least five repeated fermentations whereas the incorporation of a salt solution had no apparent effect on the results. This suggests the value of adding peptone (0.5% ($\frac{w}{v}$)) to the substrate.

4.7.4 Continuous Ethanol Production from Whey by Immobilized K. fragilis

The ultimate step in repeated-use experimentation was the construction of a continuous system. The results from the previous section (4.7.2) indicated that immobilized K. fragilis cells systems were more stable, with respect to ethanol producing ability, when a more complex growth medium was used, i.e. a medium supplemented with 0.5% ($\frac{w}{v}$) peptone or a whey medium. The latter was used for continuous experimentation.

Run No. 1

In the first run the continuous feed substrate was a dilute whey (of approximately half strength) containing 0.08 M lactose and 3.6% ($\frac{w}{v}$) total solids with a pH of 4.5. This was fed into the reactor at a constant rate of 0.25 SV at 30^{o}C over a period of 10 days. The concentration of ethanol in the effluent is recorded in Figure 20 and indicated that the maximum ethanol concentration of 240 mM ethanol (1.1% ($\frac{w}{v}$)) was produced during the second day of operation. This had decreased to 220 mM ethanol after 10 days. During the fermentation lactose levels in the effluent were less than 10 mM giving a conversion efficiency of lactose to ethanol of about 70 to 75%. A plate count of cells in the effluent after 10 days indicated that a cell concentration of 10^{6} cells/ml was present. This would be less than 0.1% of the initial immobilized cell population of approximately 10^{10} cells. The free cell population was therefore expected to contribute little to the ethanol production of the reaction.

The results of this run indicated that the reactor contained a relatively stable ethanol production activity in the form of immobilized K. fragilis cells. Over a 10 day period the effluent ethanol concentration decreased by less than 10%. If this rate of activity decay continued the reaction contents would have a half life of greater than 50 days.

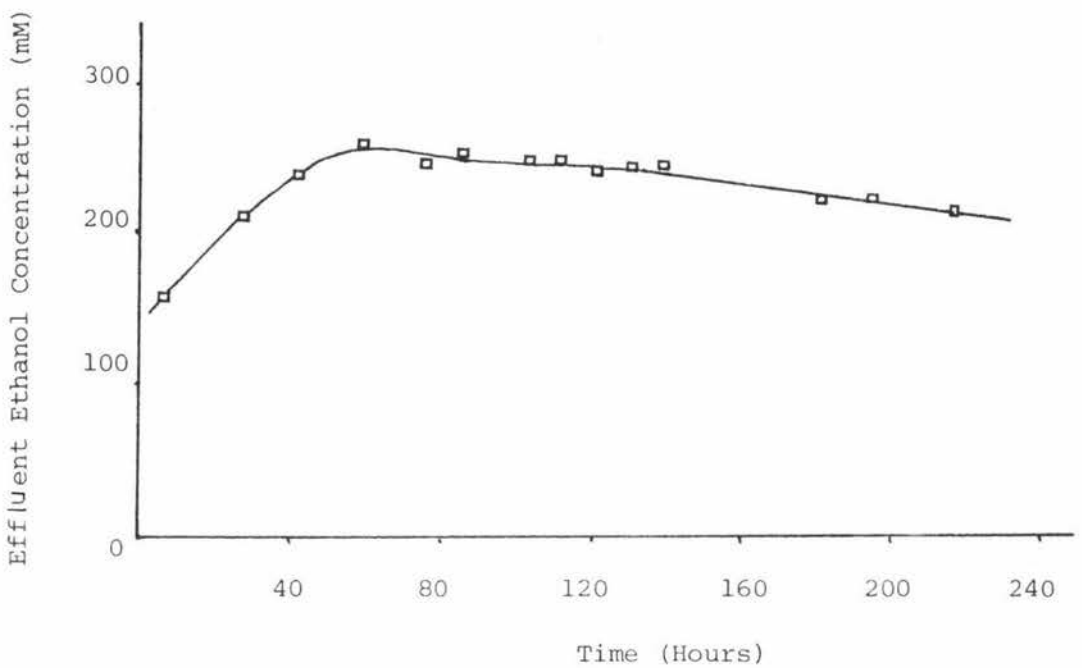

FIGURE 20. Effluent ethanol concentration as a function of time in a continuous reactor containing immobilized K. fragilis NRRL Y 1109 cells. The cells were immobilized in a modified Chibata et al. (1974) gel (3.7 gm in total) and were used to ferment a half strength deproteinized whey containing 0.08 M lactose at 0.25 SV and 30°C as described in sections 3.3.1 and 3.4.2.

Run No. 2

The second run was performed using a whey containing 0.15 M lactose to establish the flowrate at which the substrate was completely utilized. A range of four substrate flowrates was used to produce the results in Figure 21, where each point represents at least 70 hours of steady-state fermentation. Over the range of flowrates tested, 0.13 SV to 0.46 SV, the decrease in effluent ethanol concentration with increasing flowrate appears to be linear. The ethanol concentration for a particular flowrate within the range can be predicted from the equation:

$$\text{Ethanol Concentration (mM)} = 547 - 604 \text{ Flowrate (SV)} \quad (13)$$

The information from these experiments is also presented on Table IX.

TABLE IX. Ethanol production from deproteinized whey by immobilized *K. fragilis* NRRL Y 1109 as a function of substrate flowrate.

Flowrate (SV)	Retention Time (hrs)	Effluent Ethanol Concentration (mM)	Effluent Lactose Concentration (mM)	Efficiency (%)	Ethanol Production Rate (mMoles/hr)
0.13	7.7	475	<10	>79	2.8
0.24	4.2	400	23	79	3.8
0.34	2.9	330	48	81	4.5
0.46	2.2	280	58	76	5.2

It appears that substrate depletion occurs at flowrates of at least 0.13 SV. At 0.15 SV an ethanol concentration of 455 mM (from Figure 21) would be obtained with approximately total substrate utilization to give an average conversion efficiency of 79%. It is recommended that this flowrate of 0.15 SV be used for future stability trials as it appears to be the fastest flowrate at which complete substrate utilization may be achieved. A flowrate of 0.15 SV was used in the costing exercise (Appendix 3).

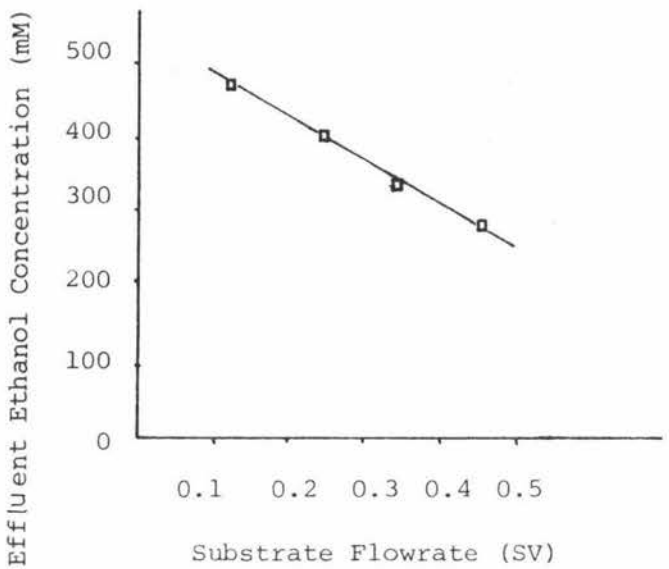

FIGURE 21. Effluent ethanol concentration as a function of substrate flowrate in a continuous reactor containing immobilized K. fragilis NRRL Y 1109 cells. The cells were immobilized in a modified Chibata et al. (1974) gel and were used to ferment a deproteinized whey containing 0.15 M lactose as described in sections 3.3.1 and 3.4.2.

The efficiency obtained in the continuous experiments of 76 to 81% is much greater than that obtained in the batch experiments with whey of about 60% (section 4.7.3). For a 0.15 M lactose concentration the efficiency of batch experiments is estimated at 60% for free cells and 50% for immobilized cells (see Table VI). The probable cause of this increase in efficiency is the reduction in oxygen present in the media due to the continuous mode of operation. In batch fermentations oxygen is able to diffuse into the medium due to the shaking used to mix the substrate and pellets, whereas carbon dioxide is constantly bubbling through the reactor helping to keep the substrate anaerobic. The presence of the carbon dioxide in the reactor did not appear to decrease the pH of the substrate. It is also noted that there is less opportunity for the loss of ethanol by evaporation in the continuous system.

The results in Table IX also indicate that the amount of ethanol increases greatly with substrate flowrates. Although only 61% of the lactose was removed from the whey at 0.46 SV the ethanol production rate for the cells was 5.2 mMoles/hr, twice that at 0.13 SV of 2.8 mMoles/hr. This observation will be important in determining the economics of the system. From the viewpoint of ethanol production, maximum production rate (mMoles/hr) is obviously important. Further studies are needed here to show whether any further increase in flowrate above 0.46 SV will increase the ethanol production rate above 5.2 mMoles/hr. However the concentration of unused lactose in the effluent may also increase. This is economically undesirable and also presents a pollution problem. It would therefore appear essential to run the fermentation of whey using immobilized yeast cells as a two stage process in an attempt to convert the remaining lactose from stage one to ethanol in stage two. This is seen as an area for future work.

The results from these continuous experiments were also used to estimate the importance of external film diffusion in the fixed bed reactor, by calculating the height of the bed necessary for mass transfer as in Appendix 1. The film diffusion was found to have a negligible affect on the reaction rate. This is in agreement with the interpretation obtained from the Kinetic parameters in section 4.6.1.4 and indicates that the gel structure imposes negligible resistance to the diffusion of substrate and product.

The use of a column of immobolized K. fragilis appears to be a feasible means of producing ethanol from whey. The immobilized cell system is relatively stable with an estimated half life of at least 50 days allowing for possible scale up to industrial operation.

CHAPTER 5

DISCUSSION

5.1 THE GROWTH OF FREE CELL POPULATIONS FOR IMMOBILIZATION

Literature reports provide little detail on the physiological status of cells being immobilized. In this study preliminary experiments provided the necessary information indicating the best possible stage of growth, in terms of cell density and metabolic vitality, at which harvesting and immobilization should be conducted. Many investigators (Chibata et al., 1974; Yamamoto et al., 1974a; Franks, 1975; Kinoshita et al., 1975; Martin and Perlman, 1976a; Sato et al., 1976) do not appear to have taken the physiological standardisation of cells into account.

Information containing detailed analysis of product related growth is available (Jack, 1977; Kokubu et al., 1978; Seyhan and Kirwan, 1979). The results of these studies are reflected in the present work for it was demonstrated (not unexpectedly) that in the system under study product formation is growth related and that the product's formation rate varies throughout the growth phases. Thus a preliminary investigation to establish when the maximum product formation rate occurs is justified.

Growth curves and ethanol production curves were established for K. fragilis and S. cerevisiae, growing in lactose and glucose respectively, to determine the period of growth required for maximum ethanol production in carefully standardised batch cultures. Maximum ethanol production rates of 2.2 mMoles/hr/gm cells for K. fragilis and 2.8 mMoles/hr/gm cells for S. cerevisiae were obtained during this growth period. These rates were approximately double those obtained in experiments performed with resting cells in sections 4.6.1.4 and 4.6.2.4 of 1.0 mMoles/hr/gm K. fragilis cells and 1.5 mMoles/hr/gm S. cerevisiae cells. It appears that the presence of the growth nutrients in the Nutrient Broth stimulated ethanol production during the cell growth. The effect of growth nutrients on immobilized cells will be discussed in section 5.6.4.

The incubation time required to reach the maximum ethanol production rate was fifteen hours for K. fragilis but increased to twenty six hours for S. cerevisiae, due to the slower growth of the S. cerevisiae culture. Incubation times were standardised at sixteen and twenty eight hours for K. fragilis and S. cerevisiae respectively to gain maximum ethanol producing activity in the free cells and thereby produce a highly active immobilized catalyst.

5.2 FERMENTATION AT OPTIMIZED GEL CONDITIONS

Of the two polyacrylamide gel preparations used in this study the most promising results were obtained using the gel of Chibata et al. (1974) which was modified for the immobilization of K. fragilis and S. cerevisiae.

5.2.1 The Chibata et al. (1974) Gel

Chibata and Tosa (1977) reviewed the use of the Chibata et al. (1974) gel, originally described for maximum aspartase activity in E. coli cells, for the immobilization of both enzymes and a range of bacteria. Retention of immobilized cell activity varied from 56% for Psuedomonas putida in the production of L-citrulline (Yamamoto et al., 1974a) and 63% for "Achromobacter" liquidum in urocanic acid production (Yamamoto et al., 1974b) to 72% for both E. coli cells producing aspartic acid (Chibata et al., 1974) and Brevibacterium ammoniagenes producing L-malic acid (Yamamoto et al., 1976). The following points were drawn from this review:

a. The full cell or enzyme activity has NOT been retained on immobilization and therefore some loss in activity must be expected.
b. The extent of the loss in activity on immobilization may be minimized by changes in the gel composition.
c. Other researchers using the gel of Chibata et al. (1974) have given no suggestion that experiments to minimize the loss in activity were conducted.

In this current study the gel of Chibata et al. (1974) was used as the basis for the determination of the gel composition resulting in maximum immobilized activity. The results obtained agree with the review of Chibata and Tosa (1977) in that at no stage was full activity retained on immobilization and that the activity retention was dependent on the

gel composition. The results obtained of 80% ethanol production activity retention for K. fragilis and 46% retention for S. cerevisiae were of a similar order to those obtained by Yamamoto et al. (1974a, 1974b, 1976). These results represent the maximum ethanol production obtained in this study and indicate that the effect of the immobilization procedure is much more inhibitory to S. cerevisiae activity than to K. fragilis activity.

5.2.2 The Neuhoff (1973) Gel

Tests using an alternative gel, the Neuhoff (1973) gel, which might reduce the activity loss on S. cerevisiae immobilization were singularly unsuccessful. No reproducible results were obtained using K. fragilis or S. cerevisiae. Lag periods of up to 100 hours preceded ethanol production and the coincidental appearance of turbidity in the supernatant. The rate and quantity of ethanol production was 40 to 70% that of the free cells.

The variability of these results prompted the investigation into the effect on free cell ethanol production of exposure to gel components, which is discussed in the next section.

5.3 THE EFFECTS OF GEL COMPONENTS ON YEAST ETHANOL PRODUCING ACTIVITY

5.3.1 The Effects of Individual Gel Components

Inspection of the literature indicates that decreases in cell activity after immobilization in polyacrylamide gel are attributed to the exposure of the cells to the polymerisation agents, particularly the acrylamide monomer (Martin and Perlman, 1976a; Miyamoto et al., 1977). This is exemplified by the 90% reduction in the penicillin producing activity of Penicillium chrysogenum cells obtained after 20 minutes exposure to a 6.75% ($\frac{w}{v}$) acrylamide and TEMED solution at pH 7.1, 0^{o}C (Morikawa et al., 1977).

Experiments with yeast glycolysis enzymes revealed that exposure to TEMED and ammonium persulfate had little effect on the enzyme activity. However, exposure to 12% ($\frac{w}{v}$) acrylamide monomer at pH 7.8, 0^{o}C destroyed 95% of hexokinase activity and 65% of glucose-6-phosphate dehydrogenase activity (Miyamoto et al., 1977). This particular report underlines the importance of the monomer. Further observations showed that the affect of the

monomer can be temperature dependent (Martin and Perlman, 1976a) and pH dependent (Miyamoto et al., 1977).

The results in this study were in agreement with those obtained by Miyamoto et al. (1977) for enzymes. For free cell of both K. fragilis and S. cerevisiae exposure to the accelerant TEMED and the initiator ammonium persulfate had only a small effect on ethanol production causing decreases of up to 35% in ethanol production rates with no lag period prior to ethanol production.

In all cases where acrylamide monomer was used a lag period of about twenty hours was evident prior to ethanol production. The total ethanol production was decreased by twenty to eighty percent from that observed for the untreated cells. For K. fragilis the effect on ethanol production appeared to be pH dependent with pH 7.1 being less inhibitory than pH 8.3.

Where the acrylamide damage is most severe, with S. cerevisiae and at pH 8.3 with K. fragilis, complete substrate utilization was found to occur at least 30 hours after ethanol production had ceased. This phenomenon was not mentioned in the relevant literature. Its occurrence may be due to ethanol inhibition brought about by changes in ethanol tolerance or changes in product transport due to the immobilization procedure.

Further investigation into the lag period revealed that at least 99.9% of K. fragilis cells were killed during exposure to a 15% $(\frac{w}{v})$ acrylamide solution at pH 7.1, 30^{o}C and that prolonged periods were presumed necessary for the surviving population to re-establish itself and generate detectable levels of ethanol.

5.3.2 Polymerisation of a Neuhoff (1973) Gel

The following hypothesis concerning the effects of exposure to acrylamide on the cells immobilized in a Neuhoff (1973) gel was formulated from the results obtained in this study.

It was found that 30 minutes exposure with 15% ($\frac{w}{v}$) acrylamide decreased the number of viable cells in a gel from approximately 10^{10} to 10^{5} or fewer. The effect of monomer exposure was expected to be dependent on both temperature and time (Martin and Perlman, 1976a) and, in fact, it was noted that those Neuhoff (1973) gels that polymerised particularly slowly retained no immobilized cell activity. It is presumed that over exposure to the monomer accounted for this loss in activity.

In the first few hours of fermentation by the gel the cells killed by exposure to the monomer were expected to lyse, providing growth components for the remaining viable cells. These cells, able to multiply, produced buds which may have left the gel and continued to multiply in the solution. As the free cell population grew the medium became turbid, the growth nutrient supply diminished and ethanol was produced in detectable concentrations from the remaining sugar substrate. The ethanol production was therefore attributed to the free cell population and to a lesser extent to the immobilized cells.

This hypothesis was supported by the coincidental appearance of ethanol and turbidity in the medium, microscopically shown to be yeast. It offers an explanation for the existence of the lag period in ethanol production, the variability of which would be due to variations in ambient temperature and gel component solution ages which influence the rate of polymerisation of the gel.

Notable methodological differences between the relatively unsuccessful Neuhoff (1973) gel and the relatively successful modified Chibata et al. (1974) gel were the temperature of polymerisation, the initiator concentration and the BIS concentration. The combination of high polymerisation temperature, low initiator concentration and low BIS concentration as used in the Neuhoff (1973) gel produced a gel that was slow to polymerise and had a high rate of cell inactivation. The slow polymerisation reaction was due primarily to the low initiator concentration, however, the low BIS concentration and the use of aged gel solutions would also contribute to this rate. The elevated temperature of polymerisation would increase the polymerisation rate to a small extent, however the rate of inactivation of cells would also be increased.

In this study it appeared that the use of low temperatures and high initiator concentrations was important. Under these conditions the polymerisation proceeded at a rapid pace decreasing the exposure time of the cells to the monomers and the opposing acrylamide deactivating reaction proceeded slowly at the low temperature. These conditions are provided for in the methodology of the modified Chibata et al. (1974) gel.

5.3.3 Experimentation with Protective Agents

Protective compounds are widely used in the immobilization of enzymes to minimize the inhibitory affects of acrylamide. The incorporation of the substrate solution in the polymerisation mixture was found to give the best protection against acrylamide exposure however the product and cofactors specific to the reaction also had a protective influence on the enzymes (Tosa et al., 1973; Miyamoto et al., 1977).

Compounds not related to the reactions have also been used for the general protection of cells and enzymes from their environments such as glycerol, (Dinelli, 1972), dithiothreitol, (Cleland, 1964) and albumin (Hinberg et al., 1974).

Due to the large number of enzymes in the metabolic pathway required for the conversion of sugars to ethanol it was considered impracticable to protect specifically those considered vulnerable to damage by acrylamide. However, no evidence was found to suggest agents protective towards extracted enzymes could be applied, with equal success, to intracellular enzymes.

Some general protective agents were tested in this current study, namely glycerol, Tween 80, gelatin and dithiothreitol. None of these proved useful. At this stage no explanation can be offered as to why these agents were not successful as the mechanism of acrylamide inhibition remains unknown. Further experimentation in this regard was terminated.

5.4 ACRYLAMIDE GEL COMPOSITIONS FOR MAXIMUM IMMOBILIZED ACTIVITY

5.4.1 Individual Gel Components

One deduces from the literature that the composition of polyacrylamide gel varied greatly from investigation to investigation (Chibata et al., 1974; Abbott, 1977, 1978; Morikawa et al., 1977). In this section the gel component concentrations established for maximum immobilized activity in this study will be compared with successful gel component concentrations from the literature.

The primary component of polyacrylamide gel is the monomer, acrylamide. In the majority of papers an acrylamide concentration of 15 - 20% $(\frac{w}{v})$ was incorporated in the gel (Chibata et al., 1974; Saif et al., 1975; Ohlson et al., 1978; Murata et al., 1979). However acrylamide concentrations as low as 4% $(\frac{w}{v})$ have been used with no apparent leakage problems (Kokubu et al., 1978; Morikawa et al., 1977). In this current study 15% $(\frac{w}{v})$ and 20% $(\frac{w}{v})$ acrylamide concentrations were found to give maximum immobilized activity for K. fragilis and S. cerevisiae cells.

The crosslinking agent (BIS) concentrations most widely used are within the range of 0 - 5% $(\frac{w}{v})$ to 1.5% $(\frac{w}{v})$ (Martin and Perlman, 1976_a), although concentrations as high as 5% $(\frac{w}{v})$ have been used successfully (Kinoshita et al., 1975). BIS concentrations of approximately 1.5% $(\frac{w}{v})$ have been used in this current study to obtain maximum immobilized activity. In this study BIS has been treated as a separate gel component, not directly linked to acrylamide concentration as with Ohmiya et al. (1975) and Kinoshita et al. (1975). All literature comparison have been made on this basis.

Cell concentrations of about 20% $(\frac{w}{v})$ have been used widely in the literature. Ohmiya et al. (1977) found that at cell concentrations above 30% $(\frac{w}{v})$ immobilized activity was reduced due to the high cell concentration reducing the polymerisation rate. Murata et al. (1979) disagreed with this, however, the results obtained in this study for K. fragilis agree with the observation of Ohmiya et al. (1977) as at cell concentrations above 20 - 30% $(\frac{w}{v})$ cells (1 to 1.5 gm/gel) the immobilized activity increases only slightly with increasing cell concentrations. The cell concentration used for maximum immobilized activity in this study was 1.25 gm per 5 ml gel or 25% $(\frac{w}{v})$ wet weight. For S. cerevisiae a reduction in activity per unit

cell mass is noted above a 40% ($\frac{W}{V}$) cell concentration (2 gm/gel) which was used for maximum activity.

Initiator and accelerant levels of 0.05 to 0.3% ($\frac{W}{V}$) and 0.25 to 0.6% ($\frac{W}{V}$) are described in literature. Where low initiator concentrations have been used the yields of immobilized activity have been less than 50% (Ohlson et al., 1978; Morikawa et al., 1977). The levels of initiator and accelerant used in this study of 0.25% ($\frac{W}{V}$) ammonium persulfate and 0.6% ($\frac{W}{V}$) BDMAP were chosen from the literature information with consideration given to producing a rapid polymerisation.

Together, these five components, acrylamide, BIS, cells, initiator and accelerant form a polyacrylamide gel. The overall composition of the gel is discussed in the next section.

5.4.2 Modification of the Chibata et al. (1974) Gel

Chibata et al. (1974) investigated the production of L-aspartic acid. Polyacrylamide gel entrapment was found to be the best method of immobilization for both the enzyme aspartase (Tosa et al., 1975) and aspartase-containing E. coli cells. The same gel composition was determined for maximum immobilized activity of both the enzymes and the cells. The gel contained 15% ($\frac{W}{V}$) acrylamide, 0.8% ($\frac{W}{V}$) BIS, 0.5% ($\frac{W}{V}$) BDMAP and 0.25% ($\frac{W}{V}$) ammonium persulfate and was polymerised at 30^{O}C. The successful immobilization of yeast and bacterial cells and enzymes by this method and its provision of favourable conditions for the immobilization of yeast prompted its use as a basis for the determination of gel conditions for maximum activity in this study.

The results of this study indicate that a 5 ml gel containing 750 mg acrylamide (15% ($\frac{W}{V}$)), 75 mg BIS (1.5% ($\frac{W}{V}$)) and about 1.25 gm cells (wet weight) (25% ($\frac{W}{V}$)) produced the greatest immobilized activity per gram of cells for K. fragilis. For S. cerevisiae the corresponding gel contained 1000 mg acrylamide (20% ($\frac{W}{V}$)), 80 mg BIS (1.6% ($\frac{W}{V}$)) and 2 gm cells (40% ($\frac{W}{V}$)). These gels also contained 0.5 ml 6% ($\frac{W}{V}$) BDMAP (0.6% ($\frac{W}{V}$)) and 0.5 ml 2.5% ($\frac{W}{V}$) ammonium persulfate (0.25% ($\frac{W}{V}$)) and were prepared in an icebath.

These gel compositions vary only slightly from that of Chibata et al. (1974) with the notable variations being the increase in BIS concentrations, from 0.8% ($\frac{W}{V}$) to about 1.5% ($\frac{W}{V}$) and the reduction in polymerisation temperature.

The modified gel composition also resembles the gel composition of Ohmiya et al. (1975) of 23% ($\frac{W}{V}$) acrylamide, 2.5% ($\frac{W}{V}$) BIS, 0.1% ($\frac{W}{V}$) TEMED and 0.33% ($\frac{W}{V}$) ammonium persulfate which was determined for yeast β-galactosidase activity. A comparison of the gel compositions discussed in this section is presented on Table X.

TABLE X. Comparison of the gel compositions used in this study with gel compositions from the literature.

Gel Component	Component Concentration ($\frac{W}{V}$)			
	Chibata et al. (1974)	according to Present Study		Ohmiya et al. (1975)
		K. fragilis	*S. cerevisiae*	
Acrylamide	15	15	20	23
BIS	0.8	1.5	1.6	2.5
Cells	20	25	40	<15
TEMED or BDMAP	0.5	0.6	0.6	0.1
Ammonium persulfate	0.25	0.25	0.25	0.33

5.4.3 The Importance of Gel Modification

The modification of gel composition to enhance cellular activity is commonly described in the literature (Kinoshita et al., 1975; Martin and Perlman, 1976a).

In this study two closely related yeast species were used. Inspection of the results obtained with variations in gel composition indicate that the response to these variations was dependent upon the yeast species used. Some of the more notable differences observed between *K. fragilis* and *S. cerevisiae* response to gel composition are in the BIS experiments, where changes in BIS concentration had a more marked effect on the ethanol producing ability of *S. cerevisiae* than of *K. fragilis*, and in the cell concentration experiments, where gels containing *K. fragilis* cells became saturated with cells at about 1 gram cells per gel (20% ($\frac{W}{V}$)) but saturation was much less evident in gels containing *S. cerevisiae* cells. In addition the overall affect of immobilization reduced *K. fragilis* activity by 20% and *S. cerevisiae* activity by 55%.

It seemed therefore vital that a gel immobilization procedure be modified for maximum activity with each cell species under investigation. The results suggest the adoption, without testing of a gelation method is inadvisable. Experiments with the Neuhoff (1973) method attests to this.

Evidence of the use of the gel of Chibata et al. (1974) for many different bacteria and fungi has been published by Yamamoto et al. (1974a, 1974b, 1976), Uchida et al. (1978) and Murata et al. (1978). It is probable that higher immobilized activity could have been obtained by these authors had the gel composition been optimized. This literature does not indicate whether investigations were undertaken to ascertain that the gel composition, as originally developed for maximum aspartase activity in E. coli cells, was suitable for maximum immobilized activity for the bacterial species used.

5.5 THE EFFECT OF IMMOBILIZATION ON THE REACTION KINETICS

Many previous researchers (Yamamoto et al., 1977; Kinoshita et al., 1975) have made detailed studies of the reaction kinetics of immobilized cells. However comparison of this data with that derived from free cell kinetic studies are rare. Where such comparisons have been made the effects of immobilization upon the kinetic parameters i.e. apparent maximum velocity and Michealis Constant, appeared to be dependent on the mode of immobilization (Toda, 1975; Linko et al., 1977; Kumakura et al., 1978).

Immobilization in polyacrylamide gel has been found to cause small or negligible increases in the apparent Michealis constant and variable decreases in the apparent maximum velocity exemplified by a 65% decrease in aspartase activity on immobilization (Tosa et al., 1973) and a 100 fold decrease in the β-galactosidase activity of immobilized E. coli cells (Ohmiya et al., 1977).

In this study the immobilization of whole cells in polyacrylamide gel did not appear to greatly affect the apparent Michealis Constant. This parameter increased from 0.15 M to 0.19 M on immobilization of K. fragilis and remained constant at 0.27 M for the immobilization of S. cerevisiae.

From theory, the imposition of diffusion limitations gives rise to an increase in the Michealis Constant (Petre et al., 1978). Therefore immobilization in polyacrylamide gel imposes only small diffusional limitations on substrate or product in the case of the K. fragilis fermentation and no diffusional limitations for the S. cerevisiae fermentation. The variations in diffusional limitations between the cell species may be due to the size of the substrate molecule. Lactose molecules are about twice the size of glucose molecules and may therefore diffuse more slowly through the gel.

The apparent maximum velocity for the metabolic pathway would be dependent on the rate of which the slowest enzyme is functioning. In this study the apparent maximum velocity remained the same at 2.75 mMoles/hr for the immobilization of 1.25 gm of K. fragilis cells, although the specific activity was found to decrease by 20%. The reduction in specific activity is probably due to the inhibition of the enzymes by the immobilization process. The apparent maximum velocity may be maintained due to increased cell membrane permeability (Yamamoto et al., 1974a) imposing no limitations on metabolic conversion rates or the gel structure limiting oxygen to what is essentially an anaerobic fermentation.

The same apparent maximum velocity for both free and immobilized cells also suggests that any accumulation of product in the gel does not inhibit the activity. Experiments in the range of ethanol concentrations used in this study of 1 to 3% $(\frac{W}{V})$ confirmed this (Appendix 2).

For S. cerevisiae the apparent maximum velocity attained by the immobilized cells was 45% that of the free cells with the specific metabolic activity retention being of the same order, 46%. The simplest explanation for this loss in activity would be that the cells were inactivated during immobilization through exposure to the gel components, particularly the monomer (Martin and Perlman, 1976a; Miyamoto et al., 1977). Other factors which may have decreased the immobilized activity are the high cell concentration used, which would increase the exposure time to the unpolymerised gel components, and a possible shift in the fermentation conditions for maximum activity such as pH, temperature or ionic strength.

5.6 REPEATED BATCH EXPERIMENTS

5.6.1 Introduction to Repeated Batch Experiments

Chibata (1977) indicated that a continuous immobilized cell operation was 60% as costly as a conventional batch operation with major savings being made in the production of the catalyst. These savings are dependent upon the stability of the immobilized cell system with respect to overall metabolic activity and leakage from the gel.

Previous researchers have used repeated batch experiments to give indications of the stability of immobilized systems under extended use (Kokubu et al., 1978; Kumakura et al., 1978). In this study repeated batch experiments were used to determine the suitability of various substrate media for continuous use in addition to determining the stability of the immobilized activity.

5.6.2 Simple Substrate Media

Previous researchers have gained long term stability of immobilized activity using a medium containing the substrate in a buffer or salt solution (Kinoshita et al., 1975; Kierstan and Bucke, 1977; Abbott, 1978). In fact simple substrate media have been used in the industrial scale production of L-aspartic acid and L-malic acid using cells immobilized in polyacrylamide gel, although the addition of a divalent metal ion was required for stability (Chibata and Tosa, 1977).

In contrast Ohlson et al. (1978) found that Arthrobacter simplex activity, immobilized in a polyacrylamide gel, declined rapidly with repeated use in a simple substrate media. Those findings parallel the current conclusion regarding K. fragilis activity. Using a pure sugar substrate of 0.12 M lactose the initial immobilized activity was reduced by 30% during eight 6 hour fermentations. The simple substrate medium containing no other ingredients was not considered suitable for the extended use of immobilized K. fragilis cells.

5.6.3 Rejuvenation

One method of increasing the useful life of immobilized cells is to rejuvenate the activity following its reduction with repeated use. In this study the yeast cells immobilized in the gel pellets were incubated in a complete yeast growth medium for this purpose.

The immobilized cells were shown to be viable by the presence of the large free cell population in the supernatant at the end of the rejuvenation period. In subsequent fermentations the immobilized cells were found to have a greater activity, of up to twice that prior to rejuvenation, however, the rate of decay of this activity was also increased by about two fold when compared with that obtained previous to the rejuvenation attempt. It appears that this approach to increasing immobilized K. fragilis activity was not acceptable.

A similar procedure was used by Somerville et al. (1977) where the benzene degrading activity of Pseudomonas putida cells immobilized in polyacrylamide gel was increased by 50 to 100 fold, to three times the initial activity by shaking overnight in a growth medium supplemented with iron. This activity was found to decay but over a period of 15 to 20 days continuous use as compared with 15 to 20 hours use in this study.

An attempt to reactivate the decayed activity of Curvularia lunata in polyacrylamide gel by incubating the fungus in a glucose enriched substrate was also successful (Mosbach and Larsson, 1970).

5.6.4 Supplemented and Complex Substrate Media

Where efforts have been made to hinder the decay of, or enhance, the metabolic activity of immobilized cells the most popular method has been through the addition of relatively simple nutrients to the medium such as meat and yeast extracts (Kokubu et al., 1978) and 0.5% $(\frac{w}{v})$ peptone and 0.2% $(\frac{w}{v})$ glucose (Ohlson et al., 1978). In cases where a single enzyme reaction was used the inclusion of one or a combination of divalent metal ions has been found beneficial (Chibata et al., 1974; Yamamoto et al., 1974b).

In this study the incorporation of a salt solution in the fermentation medium resulted in a similar loss in activity to that obtained in the fermentations using pure lactose substrate. This indicated that leakage of metal ions from the cells was not the cause of the loss in activity.

The addition of 0.5% $(\frac{w}{v})$ peptone to the substrate appeared to stabilize both K. fragilis and S. cerevisiae activity over five repeated fermentations although no significant activity increase was found. These results suggest that the activity loss in fermentations without peptone may be due to a disruption of the protein synthesis in the cell, possibly due to the immobilization procedure. The peptone supplement thus can be incorporated rapidly into the yeast protein.

The use of the more complex medium, the whey, enhanced the activity of the immobilized K. fragilis cells on repeated use. This increase appeared to be due to the leakage of cells from the gel and the production of new cells in the medium as during the six hour fermentations the medium became turbid with the growth of a free yeast population.

Evidence of similar leakage of cells from within polyacrylamide gels is found in the literature where a complete growth medium has been used to replace the usual fermentation medium to test immobilized cell viability (Franks, 1971; Kinoshita et al., 1977; Somerville et al., 1977).

Kierstan and Bucke (1977) investigated cell growth in alginate gels by dissolving parts of the gel, before and after incubation in a growth media, to count the cells. A significant increase in cell numbers was found after incubation indicating that cell division occurred within the alginate gel.

Updike et al. (1969) and Chibata et al. (1974) found no such leakage of cells from the gel after immobilization. The E. coli cells were found to lyse during the immobilization procedure and thereby lose their ability to reproduce.

The results obtained in this study indicate the value of the supplement peptone in the maintenance of immobilized cell activity during repeated use. The concentration used of 0.5% $(\frac{w}{v})$ was that used by Ohlson et al. (1978) and was not optimized for use in this study. Future work on peptone concentration and similar supplements may increase the stability and activity of immobilized yeast cells.

5.7 CONTINUOUS OPERATION

5.7.1 Ethanol Production by Immobilized Cells

Documented evidence for the production of ethanol by immobilized yeast has been published by Compere and Griffith (1976); Corrieu et al. (1976), Grinsbergset al. (1977), Kierstan and Bucke (1977) and White and Portno (1978). These papers investigate the production of ethanol from glucose sources by immobilized Saccharomyces species. Detailed information is given by Kierstan and Bucke (1977) where S. cerevisiae immobilized in alginate gel was used to continuously convert 10% ($\frac{W}{V}$) glucose to 3.5% ($\frac{W}{V}$) ethanol with an efficiency of 70%. The column activity was fully stable for five days and then decreased to about 50% of the initial activity after 13 days operation.

It was observed that an efficiency of conversion similar to that of Kierstan and Bucke (1977) of 70% was obtained during continuous conversion of an 0.08 M lactose-containing dilute whey to 240 mM (1.1% ($\frac{W}{V}$)) ethanol in this study. The efficiency of conversion was found to increase with a higher substrate concentration reaching 80% using 0.15 M lactose-containing whey. The stability of the immobilized K. fragilis cells appeared to be greater than that of S. cerevisiae cells in alginate gel with only 10% of the initial activity being lost during 10 days operation at 0.25 SV.

No evidence was found for ethanol production from lactose by immobilized cells and therefore a direct comparison between the results obtained in this study and the literature can not be made.

5.7.2 The Influence of Flowrate on Immobilized Activity

Investigation of ethanol production at various substrate flowrates in this study revealed that the rate of ethanol production at 0.48 SV was twice that at 0.13 SV. It seems therefore important to use the maximum flowrate while attaining complete substrate utilization. This flowrate was established by increasing the reactor flowrate from one where no detectable lactose was found in the effluent. It was estimated that a flowrate of 0.15 SV is the maximum at which all detectable lactose is removed from a 0.15 M (5.1% ($\frac{W}{V}$)) lactose substrate by the reactor.

Under these conditions the effluent ethanol concentration is as high as possible making the distillation of ethanol more economically feasible and wasted energy in the form of lactose in the effluent is minimized. An alternative method of processing may involve the use of two stages, with the first stage run at a high flowrate to achieve a high ethanol production rate and the second stage removing the residual lactose.

The conversion efficiency of utilized lactose to ethanol at 0.13 to 0.46 SV was 76 to 81%, slightly greater than that in the batch fermentations. This may be due to the minimal inclusion of oxygen in continuous operation.

Various substrate flowrates have been used by researchers for 100% conversion of substrate, such as 0.16 SV (Tosa et al., 1973) and 0.8 SV (Tosa et al., 1974). Generally the flowrate used appears to be dependent on the nature of the conversion reaction with low substrate concentrations and/or low flowrates being used for multistep reactions (Saif et al., 1975; Shimizu et al., 1975; Uchida et al., 1978). A substrate flowrate of 0.15 SV corresponded with 100% conversion of 0.15 M substrate for the multistep conversion in this study.

No direct comparison with the literature can be made as in the author's opinion no other report on the production of ethanol from whey has included data on the rate and efficiency of conversion at various flowrates.

5.7.3 Reactor Stability

The stability of immobilized cell columns has varied enormously. For immobilized enzymes half lives of 20 to 30 days for immobilized aspartase (Tosa et al., 1973) and 25 to 40 days for immobilized glucose isomerase (Messing and Filbert, 1975) exemplify the stability problem.

Half lives of 120 days and 140 days have been obtained using immobilized cells (Sato, 1975; Yamamoto et al., 1974a), although Kierstan and Bucke (1977) estimated that the half life of immobilized S. cerevisiae activity was only ten and a half days. More recently a half life of 686 days has been reported for cells immobilized in carrageenan (Chibata, 1977).

The estimated half life of the immobilized cell system in this study was at least 50 days. This estimate assumes that the rate of activity decay of the first continuous experiment, of 10% of the initial activity in 10 days does not increase. As the immobilized cells were shown to be viable after 10 days processing by the presence of free cells in the effluent it is possible that a half life much longer than 50 days could be obtained. However, although viability is used here in the sense that cells can leak from the gel and divide and eventually form a turbidity, cell vitality, (the ability to convert lactose to ethanol) has not been tested. This would be a more difficult cellular characteristic to establish.

5.7.4 Ethanol Production from Whey

Whey production over the past five seasons in New Zealand has remained at a level of approximately one million tonne each for acid whey (pH 4.6) and sweet whey (pH 6.0). The precise amount of whey is dependent on the product requirements of the industry (Matthews, 1977; Anonymous, 1978). Currently much of this whey is passed to waste disposal and can become a serious pollution problem (Knight et al., 1972; O'Leary et al., 1977a). A small amount of the lactose is purified for use industrially and in some factories whey protein is recovered. Recently, interest has focused on protein recovery by ultrafiltration, a method which does not denature protein. Ultrafiltered whey protein is a valuable protein commodity (Dicker, 1977). It is envisaged that the acid whey permeate from such a filtration plant containing about 5% $(\frac{w}{v})$ lactose can be used as feed stock for an immobilized yeast column for the production of ethanol. This permeate contains some soluble protein and all the salts and lactose of the whey and would be similar in composition to the substrate used in this study (Matthews, 1976).

The production of ethanol from whey has been the subject of several previous investigations (Rogosa et al., 1947; O'Leary et al., 1977a). Rogosa et al. (1947) chose Torula cremoris #2, as the most rapid and complete lactose fermenter of those experimented with, to produce 80 - 85% yields of ethanol in batch fermentations. O'Leary et al. (1977) gained about 75% conversion of whey lactose using K. fragilis in batch fermentations. The yields obtained were slightly greater than those obtained in this current study of 60% in batch fermentations of whey using immobilized cells. No evidence for the current use of immobilized cells as biocatalysts for the conversion of whey to ethanol was found by the writer.

No further research on this topic was performed by Rogosa et al. (1947) as the cost of distillation of alcohol was considered too great for the process to be economically feasible. A similar conclusion was reached by Marshall (1978) when reviewing the utilization of whey products for motor fuels. It may be possible to incorporate the distillation of ethanol into the heat recovery system of the Dairy factory producing the whey ethanol and thereby overcome this problem.

The remaining effluent generally had a low BOD and would be acceptable for disposal. Knight et al. (1972) found that two thirds of whey's chemical oxygen demand (COD) was removed by the growth of S. fragilis. As all the lactose was removed by cell growth the remaining COD of 200 ppm would be mainly due to the lactalbumin in the whey. In this study this fraction was removed prior to the fermentation.

The production of ethanol from whey is one possible means of whey utilization under investigation by the New Zealand Dairy Industry. The use of immobilized cells may be the preferred method of achieving this.

5.7.5 Possible Industrial Application

The scale up from a continuous column of immobilized aspartase-containing E. coli cells in the laboratory through a pilot plant to an industrial reactor was performed with apparent success by Sato et al. (1974) and Sato (1975). Comparison of the laboratory scale (40 cm^3) and the pilot scale (1044 cm^3) reactors showed that in both systems the maximum substrate flow-rate for complete substrate utilization was about 0.8 SV, with 20% of the initial activity being lost during 40 days processing at this flowrate. The activity of the E. coli cells was found to decay uniformly in all parts of the sectional column of the pilot plant reactor using the techniques developed in the laboratory with the results of the pilot plant experiments being applied directly to the industrial reactor. This has been operating successfully since 1973 (Chibata and Tosa, 1977).

A similar scale up procedure should be possible with the immobilized K. fragilis cells as the 40 cm^3 laboratory column of cells used in this study was found to give reproducible results with no major problems.

In conjunction with the scale up procedure other topics of investigation with immobilized K. fragilis cells include the determination of the half life and the use of methods to increase the half life such as rejuvenation-type procedures or the inclusion of stabilizing compounds in the substrate.

More basic studies may encompass the investigation of fermentation conditions and the use of more concentrated substrates than the whey in this study.

The proposed use of immobilized S. cerevisiae was in ethanol production from energy crops such as wood and sugar beet (Kardos and Mulcock, 1977). Research into this area must be preceded by continued investigation of the immobilization technique. Specific topics should include, the change in optimum fermentation conditions on immobilization (not experimented with in this study), the use of substrate additives and rejuvenation techniques to enhance the stability of the immobilized activity and continuous processing.

5.7.6 Feasibility Analysis of Ethanol Production from Whey

To gain an indication of the feasibility of the production of ethanol from whey a simplified design and costing has been performed, as described in Appendix 3.

For the purposes of the design an ethanol production unit has been added to an existing acid casein factory producing casein at 500 kg/hr for 10 hours each day. The resulting whey was ultrafiltered to remove the lactalbumin and the permeate, containing 4.6% $(\frac{W}{V})$ lactose was passed through columns of immobilized K. fragilis. Where possible the assumptions made in the design have been based upon the results obtained in this study.

Two obvious features arise from the design. The first is the large volume of immobilized catalyst needed. To process the 174,000 kg of whey each day about 40 m^3 of gel is required. The second is the small volume of low value product formed. From the 174,000 kg whey only 3,500 kg 95% ethanol is formed due to the low substrate concentration.

Polyacrylamide gel is a relatively expensive catalyst support and when this was used to manufacture a low value product such as ethanol the process did not appear economically feasible. This conclusion was gained from the cost estimate in which the value of the product was related to the current price of fuel oil. The product value was found to be less than 50% of the estimated production costs. The estimated production cost of the ethanol was 59 c/l. This estimate was not accurate, having an error of about 30%, however it indicates that no return on invested capital would be gained.

A greater value may be placed on ethanol used in industrial manufacturing however the additional processing required to meet the standard for industrial alcohol would further increase the production costs. The cost of alternative whey disposal such as waste treatment or distribution onto land may positively influence the economic feasibility of this process however it is unlikely to change the above conclusion.

It is possible that this process could be made economically feasible by improvements in the catalyst stability or the use of an alternative immobilization support such as the carrageenan used by Chibata (1977). However, the value of the product will probably be the determining factor.

CHAPTER 6

CONCLUSION

The investigation of polyacrylamide gel entrapment revealed that this method of immobilization was successful for the immobilization of yeast, under the methodology of the modified Chibata et al. (1974) gel. With conditions modified for maximum immobilized activity eighty percent of the K. fragilis fermentative activity and fourty six percent of S. cerevisiae activity was retained after cell immobilization.

Several important features arose from this study.

The highly toxic nature of the gel components, particularly the monomer, necessitated the use of the polymerisation conditions resulting in the minimal inactivation of the enzymes. The results obtained in this study show that exposure to the acrylamide monomer inactivated enzyme activity and reduced cell viability dramatically, with the other gel components having a relatively small effect on the cells. These results are supported by literature information. Attempts to protect the cells with the general protective agents glycerol, gelatin, Tween 80 and dithiothreitol were unsuccessful. To minimize the effect of exposure to acrylamide monomer the polymerisation reaction rate was set at a rapid pace by the inclusion of relatively high initiator and accelerant concentrations. The rate of the inactivation reaction was kept low by polymerising at low temperatures, $0^{\circ}C$. These conditions were incorporated in the formation of a modified Chibata et al. (1974) gel.

It was considered important to modify the gel composition for maximum activity of the immobilized cell species used. Comparison of the results obtained with the two cell species and various gel compositions indicated that the response to the conditions of immobilization was dependent on the cell species. Differences in the response to changes in BIS concentration, cell concentration and substrate concentration were evident in the results with the overall maximum activity retention after immobilization of K. fragilis being almost twice that of S. cerevisiae cells.

To obtain a high enzymic activity in the immobilized cells the period of maximum activity in the growth of the free cell culture was established.

K. fragilis cells were grown for sixteen hours and S. cerevisiae cells were grown for twenty eight hours in a growth medium incorporating the relevant sugar substrate to induce the desired enzyme complement.

The gel resulting in maximum immobilized activity in K. fragilis cells contained 15% ($\frac{W}{V}$) acrylamide, 1.5% ($\frac{W}{V}$) BIS, 0.6% ($\frac{W}{V}$) BDMAP, 0.25% ($\frac{W}{V}$) ammonium persulfate and 25% ($\frac{W}{V}$) cells (wet weight) and was polymerised at $0^{o}C$. This composition was modified for maximum activity in S. cerevisiae to contain 20% ($\frac{W}{V}$) acrylamide, 1.6% ($\frac{W}{V}$) BIS and 40% ($\frac{W}{V}$) cells. These gel compositions differed from the Chibata et al. (1974) gel primarily in BIS concentration and polymerisation temperature.

The most notable affect of the gel on immobilized cell ethanol producing activity was the loss in activity on immobilization. Twenty percent of K. fragilis activity and fifty four percent of S. cerevisiae activity was lost by immobilization in the above gels.

The gel appeared to impose only minor limitations on diffusion of substrate or product as the kinetic parameter of apparent Michealis Constant was relatively constant for both cell species before and after immobilization. The apparent maximum velocity was also not affected by the immobilization of K. fragilis however with S. cerevisiae the apparent maximum velocity was reduced by 55% as was the specific activity.

Initial simulation of continuous processing by repeated batch experiments revealed that the pure sugar substrate used in the determination of gel composition experiments was not suitable for extended use of K. fragilis cells, as the activity was unstable. Trials with supplemented media showed that the loss in activity was not due to the leakage of critical metabolic cofactors such as metal ions from the cells as the incorporation of a salt solution in the substrate did not appear to reduce the rate of activity decay.

The incorporation of 0.5% ($\frac{W}{V}$) peptone stabilized the ethanol production rate of K. fragilis and S. cerevisiae cells over five repeated batch experiments. This suggested that the yeast's ability to synthesize protein may have been disrupted by the immobilization procedure and that the supplemented peptone was being incorporated into the yeast protein thereby stabilizing the activity. The repeated fermentation of a whey substrate indicated an increase in activity due to the production of free cells.

The continuous production of ethanol from whey by K. fragilis cells appeared to be a stable operation with only 10% of the initial activity being lost in ten days operation at 0.25 SV of a half-strength whey. Using a whey of 0.15 M lactose complete substrate utilization by 4.35 gm cells (wet weight) in a reactor was found to occur at a flowrate of about 0.15 SV. It was also noted that the rate of ethanol production at this flowrate was about half that at 0.48 SV, an important factor when examining process economics.

An estimate of the type of plant required to produce ethanol from whey and of the cost of production was made. It was envisaged that a series of reactors of immobilized cells be added to a casein factory producing acid casein and lactalbumin. The three main factors influencing the ultimate cost of the venture were the high gel replacement cost, the low substrate concentration and therefore product concentration and the low value of the product. These factors contributed to the conclusion that the production of ethanol from whey by immobilized K. fragilis may not be economically feasible.

The feasibility of this process and of a similar one for the production of ethanol from glucose could be increased by the production of a longer life for the immobilized cells by the use of substrate supplementation or rejuvenation although the determining factor may be the value of the product, ethanol.

BIBLIOGRAPHY

ABBOTT, B.J. (1976)

Preparation of Pharmaceutical Compounds by Immobilized Enzymes and Cells. Advances in Applied Microbiology, 20, pp 203-257.

ABBOTT, B.J. (1977)

Immobilized Cells. Annual Reports on Fermentation Processes, 1, pp 205-233.

ABBOTT, B.J. (1978)

Immobilized Cells. Annual Reports on Fermentation Processes, 2, pp 91-124.

ANONYMOUS (1978)

New Zealand Official Year Book. Wellington, New Zealand Government Print, 1978, p 364.

BARKER, S.A. and SOMERS, P.J. (1978)

Biotechnology of Immobilized Multienzyme Systems. Advances in Biochemical Engineering, 10, pp 27-50.

BERNFELD, P., BIEBER, R.E. and MACDONNELL, P.C. (1968)

Water-Insoluble Enzymes; Arrangement of Aldolase Within an Insoluble Carrier. Archives of Biochemistry and Biophysics, 127, pp 779-786.

BERNFELD, P. and WAN, J.J. (1963)

Antigens and Enzymes made Insoluble by Entrapping them into Lattices of Synthetic Polymers. Science, 142, pp 678-679.

BONNISCHEN, R. (1963)

Ethanol Determination with Alcohol Dehydrogenase and DPN. pp 285-287. In Methods of Enzymatic Analysis, 1st edition, H.U. Bergmeyer editor, New York, Academic Press.

BRODELIUS, P. (1978)

Industrial Applications of Immobilized Biocatalysts. Advances in Biochemical Engineering, 10, pp 75-130.

CHIBATA, I. (1977)

In Proceedings of 4th Enzyme Engineering Conference. Bad Neuenahr, Germany, as cited by Abbott, 1978.

CHIBATA, I. and TOSA, T. (1977)

Transformation of Organic Compounds by Immobilized Cells. Advances in Applied Microbiology, 22, pp 1-27.

CHIBATA, I., TOSA, T. and SATO, T. (1974)

Immobilized Aspartase-Containing Microbial Cells : Preparation and Enzymatic Properties. Applied Microbiology, 27, pp 874-885.

CLELAND, W.W. (1964)

Dithiothreitol, as a Protective Agent for SH Groups. Biochemistry ACS, 3, pp 480-482.

COMPERE, A.L. and GRIFFITH, W.L. (1976)

Fermentation of Waste Materials to Produce Industrial Intermediates. Developments in Industrial Microbilogy, 17, pp 247-252.

CORRIEU, G., BLACHERE, H., RAMIREZ, A., NAVARRO, J.M., DURAND, G., DETEURTRE, B. and MOLL, M. (1976)

An Immobilized Yeast Fermentation Pilot Plant used for the Production of Beer. p 294. In Proceedings of the 5th International Fermentation Symposium, Berlin.

CYSEWSKI, G.R. and WILKE, C.R. (1976a)

Utilization of Cellulose Materials through Enzymatic Hydrolysis; I Fermentation of Hydrolysate to Ethanol and SCP. Biotechnology and Bioengineering, 18, pp 1297-1313.

CYSEWSKI, G.R. and WILKE, C.R. (1976b)

Utilization of Cellulose Materials through Enzymatic Hydrolysis; II Preliminary Assessment of an Integrated Processing Scheme. Biotechnology and Bioengineering, 18, pp 1314-1323.

DAWSON, R.M.C., ELLIOT, D.C., ELLIOT, W.N. and JOHNS, K.M. (1969)

Data for Biochemical Research 2nd edition, London, Oxford Claredon Press.

DAHLQVIST, A., MATTIASSON, B. and MOSBACH, K. (1973)

Hydrolysis of β-Galactosidase Using Polymer Entrapped Lactase. A Study Towards Producing Lactose-Free Milk. Biotechnology and Bioengineering, 15, pp 395-402.

DICKER, R.A. (1977)

Some Investigations of Modern Whey-Treatment Processes. pp 142-149. In Proceedings of Jubilee Conference on Dairy Science and Technology, Palmerston North.

DINELLI, D. (1972)

Fibre Entrapped Enzymes. Process Biochemistry, 7, August, pp 9-12.

DIXON, M. (1953)

The Determination of Enzyme Inhibitor Constants. Biochemical Journal, 55, pp 170-171.

DURAND, D. and NAVARRO, J.M. (1978)

Immobilized Microbial Cells. Process Biochemistry, 13, September, pp 14-23.

EMERY, A.N. and CARDOSO, J.P. (1978)

Parameter Evaluation and Performance; Studies in a Fluidised Bed Immobilized Enzyme Reactor. Biotechnology and Bioengineering, 20, pp 1903-1929.

ENFORS, S.O. and MOLIN, N. (1978)

Enzyme Electrodes for Fermentation Control. Process Biochemistry, 13, February, pp 9-11.

EYNON, L. and LANE, J.H. (1923)

The Influence of Alkaline Earths on the Determination of Reducing Sugars by Fehling Solution. Journal of the Society of Chemical Industry, 42, pp 143-146.

FETZER, W.R. (1930)

Modern Practice in Vinegar Making. Food Industries, 2, pp 489-491.

FLORY, P.J. (1953)

Polymerisation of Unsaturated Monomers by Free Radicle Mechanism. pp 106-177. In Principles of Polymer Chemistry, New York, Cornell University Press.

FRANKS, N. (1971)

Catabolism of L-Arginine by Entrapped cells of Streptococcus feacalis ATCC 8043. Biochimica et Biophysica Acta, 252, pp, 246-254.

GHOSE, T.K. and CHAND, S. (1978)

Kinetic and Mass Transfer Studies on the Isomerisation of Cellulose Hydrolysate Using Immobilized Streptomyces Cells. Journal of Fermentation Technology, 56, pp 315-322.

GRINSBERGS, M., HILDEBRAND, R.P. and CLARKE, B.J. (1977)
Continuous Fermentation of Glucose Solutions. Journal of the Institute of Brewing, 83, pp 25-29.

GUTCHO, S.J. (1974)
Immobilized Enzymes : Preparation and Engineering Techniques. New Jersey, Noyes Data Corporation.

HACKEL, U., KLEIN, J., MEGNET, R. and WAGNER, F. (1975)
Immobilization of Microbial Cells in Polymeric Matrices. European Journal of Applied Microbiology, 1, pp 291 293.

HAVEWALA, N.B. and PITCHER, W.H. Jr (1974)
Immobilized Glucose Isomerase for the Production of High Fructose Syrups. pp 315-328. In Enzyme Engineering II. E.K. Pye and L.B. Wingard, editors, New York, Plenum Press.

HINBERG, I., KORUS, R. and O'DRISCOLL, K. (1974)
Gel Entrapped Enzymes : Kinetic studies of Immobilized β-Galactosidase. Biotechnology and Bioengineering, 16, pp 943-963.

JACK, T.R. (1977)
The Enzymatic Conversion of L-Histidine to Urocanic acid by Whole Cells of Micrococcus luteus Immobilized on Carbodimide Activated Carboxy-methylcellulose. Biotechnology and Bioengineering, 19, pp 631-648.

JACK, T.R. and ZAJIC, J.E. (1977)
The Immobilization of Whole Cells. Advances in Biochemical Engineering, 5, pp 125-145.

JIRKU, V., TURKOUA, J., KUCHYNKOVA, A. and KRUMPHANZL, V. (1979)
Modified Hydroxyalkyl Methacrylate Gel as a support for Immobilization of Yeast Cells. European Journal of Applied Microbiology, 6, pp 217-222.

JOHNSON, D.E. and CIEGLER, A. (1969)
Substrate Conversion by Fungal Spores Entrapped in Solid Matrices. Archives of Biochemistry and Biophysics, 130, pp 384-388.

KAN, J.K. and SHULER, M.L. (1978)
Urocanic Acid Production using Whole Cells Immobilized in a Hollow Fiber Reactor. Biotechnology and Bioengineering, 20, pp 217-230.

KARDOS, N. and MULCOCK, A.P. (1977)
Ethanol from Agricultural Crops, A Literature Survey. New Zealand Energy Research and Development Committee Report No. 28.

KARUBE, I., MATSUNAGA, T., TSURU, S. and SUZUKI, S. (1976)
Continuous Hydrogen Production by Immobilized Whole Cells of Clostridium butyricum. Biochimica et Biophysica Acta, 444, pp 338-343.

KARUBE, I., MATSUNAGA, T., TSURU, S. and SUZUKI, S. (1977a)
Biochemical Fuel Cell Utilizing Immobilized Cells of Clostridium butyricum. Biotechnology and Bioengineering, 19, pp 1727-1733.

KARUBE, I., MITSUDA, S., MATSUNAGA, T. and SUZUKI, S. (1977b)
A rapid Method for Estimation of BOD by using Immobilized Microbial Cells. Journal of Fermentation Technology, 55, pp 243-248.

KENNEDY, J.F., ALAN BARKER, S. and HUMPHREYS, J.D. (1976)
Microbial Cells living Immobilized on Metal Hydroxides. Nature, 261, pp 242-244.

KIERSTAN, M. and BUCKE, C. (1977)
The Immobilization of Microbial Cells, Subcellular Organelles and Enzymes in Calcium Alginate Gels. Biotechnology and Bioengineering, 19, pp 387-397.

KIMURA, A., TATSUTOMI, Y., MIZUSHIMA, N., TANAKA, A., MATSUNO, R. and FUKUDA, H. (1978)
Immobilization of Glycolysis System of Yeast and Production of Cytidine Diphosphate Choline. Industrial Microbiology and Biotechnology, 5, pp 13-16.

KINOSHITA, S., MURANAKA, M. and OKADA, H. (1975)
Hydrolysis of ε-Aminocaproic Acid Cyclic Dimer by Cells Entrapped in Acrylamide Gel. Journal of Fermentation Technology, 53, pp 223-229.

KLEIN, J., HACKEL, U., SCHARA, P., WASHAUSEN, P. and WAGNER, F. (1976)
In Proceedings of the Fifth International Fermentation Symposium, Berlin, p 295 as cited by Durand and Navarro (1978).

KNIGHT, SUE, SMITH, WANDA, and MICKLE, J. (1972)
Cheese Whey disposal : Using Saccharomyces fragilis Yeast. Cultured Dairy Products Journal, 17(2), pp 17-22.

KOCH-LIGHT LABORATORIES LTD.

Catalogue KL5, Colnbrook Buckinghamshire, England.

KOKUBU, T., KARUBE, I. and SUZUKI, S. (1978)

α-Amylase Production by Immobilized Whole Cells of Bacillus subtilis. European Journal of Applied Microbiology, 5, pp 233-240.

KREBS, H.A. and HENSELEIT, K. (1932)

Urea Formation in the Animal Body. Zeitschrift fuer Physiologische Chemie, 210, pp 33-36.

KUMAKURA, M., YOSHIDA, M. and KAETSU, I. (1978)

Immobilization of Streptomyces phaechromogenes Cells at a High Concentration by Radiation - Induced Polymerisation of Glass - Forming Monomers. European Journal of Applied Microbiology, 6, pp 13-22.

KUMAKURA, M., YOSHIDA, M. and KAETSU, I. (1979)

Immobilization of Glucose Isomerase-Containing Streptomyces phaeochromogenes Cells in Fine Particle Form. Applied and Environmental Microbiology, 37(2), pp 310-315.

LEE, C.K. and LONG, M.E. (1974)

Enzymatic process using Immobilized Microbial Cells. U.S.A. Patent, 3,821,086.

LINEWEAVER, H. and BURK, D. (1934)

Determination of Enzyme Dissociation Constants. Journal of the American Chemical Society, 56, pp 658-666.

LINKO, Y., POHJOLA, L. and LINKO, P. (1977)

Entrapped Glucose Isomerase for Fructose Syrup Production. Process Biochemistry, 12, July/August, pp 14-16.

LODDER, J.

The Yeasts, a Taxonomic Study. 2nd edition, Amersterdam North-Holland Publishing Company.

MAEDA, H. and SUZUKI, H. (1977)

Preparation of Immobilized Enzymes by Radiation. Process Biochemistry, 12, July/August, pp 9-12.

MARK, H.F., McKETTA, J.J. Jr, OTHMER, D.F. and STANDEN, A. (1965)

Encyclopedia of Chemical Technology. 2nd edition, New York, John Wiley and Sons Incorporated, Volume 13, p 516.

MARSHALL, K.R. (1978)

Prospects for Ethanol Production from Whey. pp 280-284. In Proceedings of the First Biotechnology Conference on Biomass and Energy, Massey University, Palmerston North.

MARTIN, C.K.A. and PERLMAN, D. (1976a)

Conversion of L-Sorbose to L-Sorbosone by Immobilized cells of Gluconobacter melanogenus IFO 3293. Biotechnology and Bioengineering, 18, pp 217-237.

MARTIN, C.K.A. and PERLMAN, D. (1976b)

Conversion of L-Sorbose to 2-Keto-L-Gluconic Acid by Mixtures of Immobilized Cells of Gluconobacter melanogenus IFO 3293 and Pseudomonas species. European Journal of Applied Microbiology, 2, pp 91-95.

MATTHEWS, M.E. (1976)

Personal Communication.

MATTHEWS, M.E. (1977)

New Zealand Experience with Ultrafiltration of Whey. pp 149-154. In Proceedings of Jubilee Conference on Dairy Science and Technology, Palmerston North.

MESSING, R.A. (1975)

Immobilized Enzymes for Industrial Reactors, New York, Academic Press.

MESSING, R.A. (1978)

Carriers of Immobilized Biologically Active Systems. Annual Reports on Fermentation Processes, 2, pp 91-124.

MESSING, R.A. and FILBERT, A.M. (1975)

Immobilized Glucose Isomerase for the Continuous Conversion of glucose to fructose. Journal of Agricultural Food Chemistry, 23, pp 920-923.

MICKLE, J.B., SMITH, W., HALTER, D. and KNIGHT, S. (1974)

Performance and morphology of Kluyveromyces fragilis and Rhodotorula gracilis grown on Cottage Cheese Whey. Journal of Milk and Food Technology, 37, pp 481-484.

MIYAMOTO, K., OHYABU, M., OKAZAKI, M. and MIURA, Y. (1977)

Inactivation of Hexakinase and Glucose-6-phosphate Dehydrogenase during Immobilization by Polyacrylamide Gel Entrapping. Journal of Fermentation Technology, 55, pp 63-70.

MOHAN, R. and LI, N. (1975)
Nitrate and Nitrite Reduction by Liquid Membrane Encapsulated Whole Cells. Biotechnology and Bioengineering, 17, pp 1137-1356.

MONTALVO, J. (1970)
Improved Urease Electrode. Analytical Biochemistry, 38, pp 357-363.

MORIKAWA, Y., KARUBE, I. and SUZUKI, S. (1979)
Penicillin G Production by Immobilized Whole Cells of Penicillium chrysogenum. Biotechnology and Bioengineering, 21, pp 261-270.

MOSBACH, K. and LARSSON, P. (1970)
Preparation and Application of Polymer-Entrapped Enzymes and Micro-organisms in Microbial Transformation Process with Special Reference to steriod 11-β-Hydroxylation and Δ'-dehydrogenation. Biotechnology and Bioengineering, 12, pp 19-27.

MOSBACH, K. and MOSBACH, R. (1966)
Entrapment of Enzymes and Micro-organisms in Synthetic Crosslinked Polymers and their Application in Column Techniques. Acta Chemica Scandinavica, 20, pp 2807-2810.

MOSTELLER, F., ROURKE, R. and THOMAS, G. Jr (1970)
Probability with Statistical Applications. Reading, Massachusetts, Addison-Wesley Publishing Company, pp 382-448.

MOULIN, G. and GALZY, P. (1978)
Remarks on the Metabolism of Kluyveromyces lactis van der Walt, Mycopathologia, 66, pp 73-76.

MURATA, K., KATO, J. and CHIBATA, I. (1979)
Continuous Production of NADP by Immobilized Brevibacterium ammoniagenes Cells. Biotechnology and Bioengineering, 21, pp 887-895.

MURATA, K., TANI, K., KATO, J. and CHIBATA, I. (1978)
Continuous Production of Glutathione by Immobilized Saccharomyces cerevisiae Cells. European Journal of Applied Microbiology, 4, pp 23-27.

NEISH, A.C. and BLACKWOOD, A.C. (1951)
Dissimilation of Glucose by Yeast at Poised Hydrogen-Ion Concentrations. Canadian Journal of Technology, 29, pp 123-129.

NEUHOFF, V. (1973)

Molecular Biology, Biochemistry and Biophysics, No. 14 : Micromethods in Molecular Biology. London, Chapman and Hall Limited, p 7.

NORDSTRÖM, K. (1868)

Yeast Growth and Glycerol Formation. Journal of the Institute of Brewing, 74, pp 249-432.

OHLSON, S., LARSSON, P. and MOSBACH, K. (1978)

Steriod Transformations by Activated Living Arthrobacter simplex Cells. Biotechnology and Bioengineering, 20, pp 1267-1284.

OHMIYA, K., OHASHI, H., KOBAYASHI, T. and SHIMIZU, S. (1977)

Hydrolysis of Lactose by Immobilized Micro-organisms. Applied and Environmental Microbiology, 33, pp 137-146.

OHMIYA, K., TERAO, C., SHIMIZU, S. and KOBAYASHI, T. (1975)

Immobilization of β-Galactosidase by Polyacrylamide in the Presence of Protective Agents. Agricultural and Biological Chemistry, 39, pp 491-498.

O'LEARY, V.S., GREEN, R., SULLIVAN, B. and HOLSINGER, V. (1977a)

Alcohol Production by Selected Yeast Strains in Lactose-Hydrolyzed Acid Whey. Biotechnology and Bioengineering, 19, pp 1019-1035.

O'LEARY, V.S., SUTTON, C., BENCIVENGO, M., SULLIVAN, B. and HOLSINGER, V.H. (1977b)

Influence of Lactose Hydrolysis and Solids Concentration on Alcohol Production by Yeast in Acid Whey Ultrafiltrate. Biotechnology and Bioengineering, 19, pp 1689-1702.

PACHE, W. (1978)

Degradation of β-Lactam Antibiotics by Polyacrylamide-Entrapped β-Lactamase Producing E. coli Cells. European Journal of Applied Microbiology, 5, pp 171-176.

PEPPLER, H.J. (1978)

Yeasts. Annual Reports on Fermentation Processes, 2, pp 191-202.

PETERS, M.S. and TIMMERHAUS, K.D. (1968)

Plant Design and Economics for Chemical Engineers. 2nd edition, Tokyo, McGraw-Hill, Kogakusha Limited.

PETRE, D., NOEL, C. and THOMAS, D. (1978)
A New Method for Cell Immobilization. Biotechnology and Bioengineering, 20, pp 127-134.

PITCHER, W.H. Jr (1975)
Design and Operation of Immobilized Enzyme Reactors. pp 151-199. In Immobilized Enzymes for Industrial Reactors, R.A. Messing, editor, New York, Academic Press.

PHAFF, H.J., MILLER, M.W. and MRAK, E.M. (1978)
The Life of Yeast. 2nd edition, Cambridge, Harvard University Press, pp 136-138.

REED, G. and PEPPLER, H.J. (1973)
Yeast Technology. Westport, Connecticut, AVI Publishing Company Incorporated.

RICHARDS, E.G. and LECANIDOU, R. (1974)
Polymerisation Kinetics and Properties of Polyacrylamide Gels. pp 16-22. In Electrophoresis and Isoelectric Focusing in Polyacrylamide Gel, Advances of Methods and Theories, Biochemical and Clinical Applications, R.C. Allen and H.R. Maurer editors, New York, Walter de Gruyter Publishers.

RICHARDS, E.G. and TEMPLE, C.J. (1971)
Some Properties of Polyacrylamide Gels. Nature Physical Sciences, 230, pp 92-96.

ROGOSA, M., BROWNE, H. and WHITTIER, E.O. (1947)
Ethyl Alcohol from Whey. Journal of Dairy Sciences, 30, pp 263-269.

ROHLF, F.S. and SOKAL, R.R. (1969)
Statistical Tables, San Fransisco, W.H. Freeman and Company, p 225.

ROSE, D. (1976)
Yeast for Molasses Alcohol. Process Biochemistry, 11, March. pp 10-12.

SAIF, S., TANI, Y. and OGATA, K. (1975)
Preparation of Glucose Phosphate through the Transphosphorlyation with Immobilized Cells. Journal of Fermentation Technology, 53, pp 380-385.

SAINI, R. and VIETH, W. (1975)
Reaction Kinetics and Mass Transfer in Glucose Isomerisation with Collagen-Immobilized Whole Microbial Cells. Journal of Applied Chemistry and Biotechnology, 25, pp 115-141.

SATO, T. (1975)

Continuous Production of L-Aspartic Acid. Biotechnology and Bioengineering, 17, pp 1798-1804.

SATO, T., TOSA, T. and CHIBATA, I. (1976)

Continuous Production of 6-Amino-Penicillanic Acid from Penicillin by Immobilized Microbial Cells. European Journal of Applied Microbiology, 2, pp 153-160.

SATTERFIELD, C.N. (1970)

Mass Transfer in Heterogeneous Catalysis. Cambridge, Massachusetts, MIT Press.

SCHULTZ, J.S. and GERHARDT, D. (1967)

Dialysis Culture of Micro-organisms : Design, Theory and Results. Bacteriological Reviews, 33, pp 1-47.

SEYHAN, E. and KIRWAN, D.J. (1979)

Nitrogenase Activity of Immobilized Azotobacter vinelandii. Biotechnology and Bioengineering, 21, pp 271-281.

SHIMIZU, S., MORIOKA, H., TANI, Y. and OGATA, K. (1975)

Synthesis of Co-enzyme A by Immobilized Microbial Cells. Journal of Fermentation Technology, 53, pp 77-83.

SLOWINSKI, W. and CHARM, S. (1973)

Glutamic Acid Production with Gel-Entrapped Corynebacterium glutamicum. Biotechnology and Bioengineering, 15, pp 973-979.

SOMERVILLE, H.J., MASON, J.R. and RUFFEL, R.N. (1977)

Benzene Degradation by Bacterial Cells Immobilized in Polyacrylamide Gel. European Journal of Applied Microbiology, 3, pp 75-85.

STOUT, B.A. and LOUDON, T.L. (1977)

Energy from Organic Residues. Agricultural Engineering Department of Michigan State University Report for the Food and Agriculture Organisation of the United Nations.

TAKAGI, M., ABE, S., SUZUKI, S., EMERT, G.H. and YATA, N. (1977)

A Method for Production of Alcohol Directly from Cellulose Using Cellulose and Yeast. In Bioconversion of Cellulose as a Substitute Energy Source; Microbiological, Chemical and Protein Aspects, Symposium, Delhi.

TODA, K. (1975)
Interparticle Mass Transfer Study with a Packed Column of Immobilized Microbes. Biotechnology and Bioengineering, 17, pp 1729-1747.

TODA, K. and SHODA, M. (1975)
Sucrose Inversion by Immobilized Yeast Cells in a Complete Mixing Reactor. Biotechnology and Bioengineering, 17, pp 481-497.

TOSA, T., MORI, T., FUSE, N. and CHIBATA, I. (1966)
Studies on Continuous Enzyme Reactions. 1. Screening of carriers for preparation of water isoluble aminoacylase. Enzymologia, 31, pp 214-224.

TOSA, T., SATO, T., MORI, T. and CHIBATA, I. (1974)
Basic Studies for Continuous Production of L-Aspartic Acid by Immobilized Escherichia coli Cells. Applied Microbiology, 27, pp 886-889.

TOSA, T., SATO, T., MORI, T., MATUO, Y. and CHIBATA, I. (1973)
Continuous Production of L-Aspartic Acid by Immobilized Aspartase. Biotechnology and Bioengineering, 15, pp 69-84.

UCHIDA, T., WATANABE, T., KATO, J. and CHIBATA, I. (1978)
Continuous Production of NADP by Immobilized Achromobacter aceris Cells. Biotechnology and Bioengineering, 20, pp 255-266.

UPDIKE, S.J., HARRIS, D.R. and SHRAGO, E. (1969)
Micro-organisms, Alive and Imprisoned in a Polymer Cage. Nature, 224, pp 1122-1123.

UPDIKE, S.J. and HICKS, G.P. (1976)
Reagentless Substrate Analysis with Immobilized Cells. Science, 158, pp 270-272.

VENKATASUBRAMANIAM, K., SAINI, R. and VIETH, W.R. (1974)
On the Mechanisms of Enzyme and Whole Microbial Cells Attachment to Collagen. Journal of Fermentation Technology, 52, pp 268-278.

WEETALL, H.H. (1976)
Immobilized Enzyme Technology. Cereal Foods World, 21, pp 581-587.

WHITE, F.H. and PORTNO, A.D. (1978)
Continuous Fermentation by Immobilized Brewers Yeast. Journal of the Institute of Brewing, 84, pp 228-230.

WILLIAMS, D.L., DOIG, A.R. and KOROSI, A. (1970)
Electrochemical Enzymatic Analysis of Blood Glucose and Lactate. Analytical Chemistry, 42, pp 118-121.

YAMAMOTO, K., SATO, T., TOSA, T. and CHIBATA, I. (1974a)
Continuous Production of L-Citrulline by Immobilized Pseudomonas putida Cells. Biotechnology and Bioengineering, 16, pp 1589-1609.

YAMAMOTO, K., SATO, T., TOSA, T. and CHIBATA, I. (1974b)
Continuous Production of Urocanic Acid by Immobilized Achromobacter liquidum Cells. Biotechnology and Bioengineering, 16, pp 1601-1610.

YAMAMOTO, K., TOSA, T., YAMASHITA, K. and CHIBATA, I. (1976)
Continuous Production of L-Malic Acid by Immobilized Brevibacterium ammoniagenes Cells. European Journal of Applied Microbiology, 2, pp 169-183.

YAMAMOTO, K., TOSA, T., YAMASHITA, K. and CHIBATA, I. (1977)
Kinetics and Decay of Fumarase Activity of Immobilized Brevibacterium ammoniagenes Cells for Continuous Production of L-Malic Acid. Biotechnology and Bioengineering, 19, pp 1101-1114.

YANG, H., LEUNG, K. and ARCHER, M. (1976)
Preparation and Properties of Bacterial Chromatophores Entrapped in Polyacrylamide. Biotechnology and Bioengineering, 18, pp 1425-1432.

YOO, B.W. (1974)
Effect of Certain Factors on Cell Mass and Ethanol Production by Lactose Fermenting Yeast. Thesis, Ph.D., University of Maryland.

ZABORSKY, O.R. (1973)
Immobilized Enzymes. Cleveland, Ohio, Chemical Rubber Company Press.

APPENDIX 1

CALCULATION OF THE THEORETICAL HEIGHT NECESSARY FOR FILM DIFFUSION IN THE REACTOR

The equation

$$Z = \frac{\varepsilon \, N_{RE}^{\frac{2}{3}} \, N_{SC}^{\frac{2}{3}}}{1.09 \; av} \; ln \frac{Y_1}{Y_2} \qquad (A1)$$

was determined by Satterfield (1970) for the estimation of the importance of film diffusion in a fixed bed reactor. This equation has been applied to the results from the continuous experiments and the theoretical height, Z will be compared with the reactor height used.

Z	=	theoretical height for film diffusion
ε	=	void volume
N_{RE}	=	Reynolds Number $\frac{dpG}{\mu}$
N_{SC}	=	Schmidt Number $\frac{\mu}{\rho D}$
av	=	ratio of particle surface area to reactor volume
Y_2	=	Mole fraction of substrate in product
Y_1	=	Mole fraction of substrate in feed
dp	=	particle diameter
G	=	mass velocity per unit superficial bed cross-section
μ	=	substrate viscosity
D	=	diffusivity
ρ	=	density

Certain assumptions have been made to perform this calculation.

1. The particle diameter was taken to be the smallest thickness of the particle i.e. 1.5 mm (average).
2. The diffusivity of whey through the gel pellet was assumed to be 0.3×10^{-5} cm^2/sec. Toda and Shoda (1975) found that the diffusivity of 0.117 M sucrose at 45°C in 2% agar gel was 0.66×10^{-5} cm^2/sec. Havewala and Pitcher (1974) used 0.21×10^{-5} cm^2/sec for 60°C, 50% glucose solutions diffusing into zinconia coated controlled pore glass.
3. The void volume was assumed to be 25% of the total volume of 40 ccm. This allows for an 80% swelling of the gel volume as defined by Richards and Temple (1971). The original gel volume of 4.8 ml for each of 3.5 gels of 16.8 cm^3 was increased to 30.2 cm^3.

4. Whey density at 30°C is 1.03 g/cm^3 and viscosity is 1.2×10^{-2} cp (Mark et al., 1965).
5. The calculation was based upon complete substrate utilization at 0.15 SV.

Calculation

$$G = \frac{\text{Flowrate x whey density}}{\text{cross section of column}}$$

$$= \frac{6 \text{ ml/hr} \times 1.03}{(1.1)^2 \pi \times 3600} = 5.7 \times 10^{-4} \text{ g cm}^2 \text{ sec}^{-1}$$

$$N_{RE} = \frac{dpG}{\mu} = \frac{.15 \times 5.7 \times 10^{-4}}{1.2 \times 10^{-2}} = 7.1 \times 10^{-3}$$

$$N_{SC} = \frac{\mu}{\rho D} = \frac{1.2 \times 10^{-2}}{1.03 \times .3 \times 10^{-5}} = 3.88 \times 10^{3}$$

$$av = \frac{\text{particle surface area } (1 - \varepsilon)}{\text{particle volume}}$$

$$= \frac{(2\pi(.5)^2 + .15\ \pi\ 2.5)\ (1 - .25)}{\pi\ (.5)^2\ .15}$$

$$= 13.0$$

$$Z = .25 \frac{(7.1 \times 10^{-3})}{1.09} \frac{(3.88 \times 10^{3})}{\times 13.0} \quad \frac{1}{1 - .985}$$

$$= 0.69 \text{ cm}$$

The actual column height is 10.5 cm. As the height necessary for film diffusion is only 6.5% of the column height this indicates negligible external mass transfer resistance.

The theoretical calculation result agrees with the indication given in 4.6.1.4 that the gel imposes little resistance to mass transfer as the apparent Michealis Constant of the immobilized cells is only slightly greater than that of the free cells.

APPENDIX 2

ETHANOL INHIBITION OF K. FRAGILIS

This experiment was conducted to determine whether ethanol was inhibiting the immobilized cells. Product inhibition was one possible cause for the reduced activity in the immobilized cells.

K. fragilis cells were immobilized in the modified Chibata et al. (1974) gel was described in section 3.3.1 and used in fermentations as described in section 3.4.1.2. The fermentation media consisted of 0.06 M and 0.12 M lactose containing initial ethanol concentrations of 200 to 500 mM. The activity of the cells was measured as described in section 3.8.1 and is presented in Figures A1 and A2 for the free and immobilized cells respectively. These results can be compared with Figures A3 and A4 plots after Dixon (1953) in which the typical inhibition patterns are shown.

The results obtained in this experiment bear no resemblance to the Dixon plots. For both free and immobilized cells activity does not decrease with increasing inhibitor (ethanol) concentration. There appears no significant difference between the affect of ethanol on free or immobilized cells. It is concluded that ethanol does not inhibit free or immobilized K. fragilis at the concentrations used in this study.

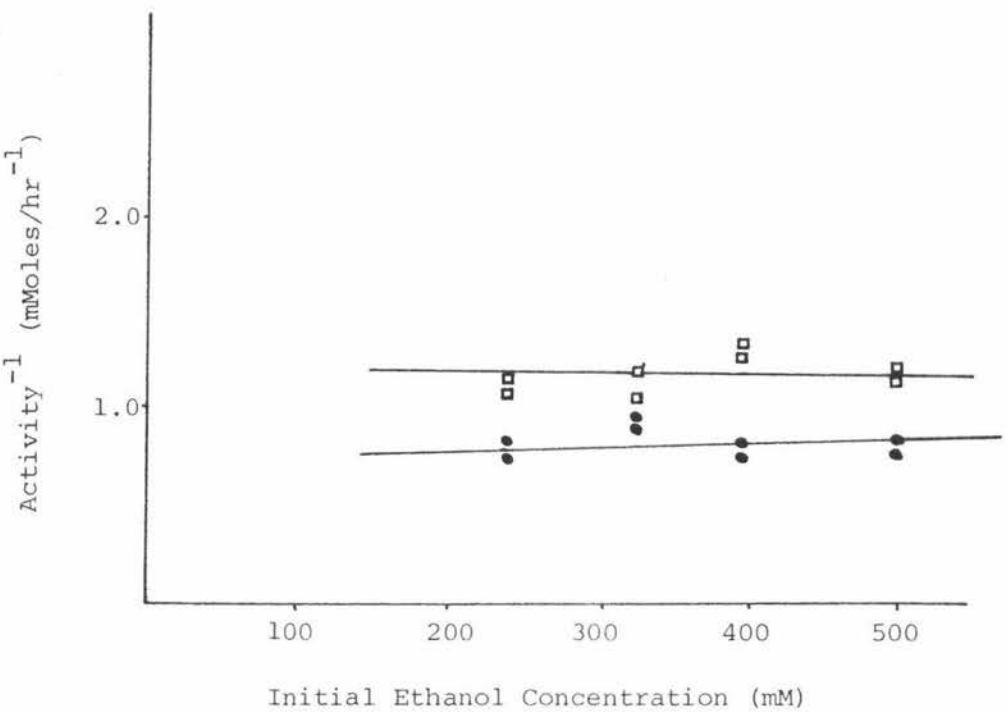

FIGURE A1. Reciprocal plot of the ethanol producing activity of free K. fragilis NRRL Y 1109 cells as a function of initial product concentration in the medium. The Conditions used are described in sections 3.2.4, 3.2.5 and 3.4.1.2. Substrate concentrations of 0.12 M, —●— and 0.06 M —□— were used.

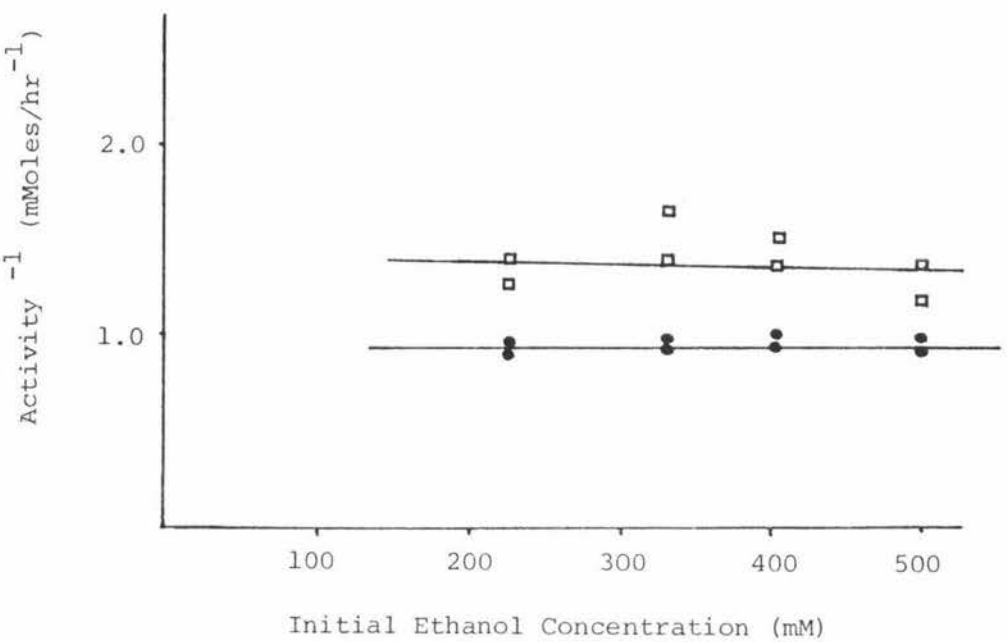

FIGURE A2. Reciprocal plot of the ethanol producing activity of immobilized K. fragilis NRRL Y 1109 cells as a function of initial product concentration in the medium. The conditions used are described in sections 3.2.4, 3.2.5 and 3.3.1 and 3.4.1.2. Substrate concentrations of 0.12 M, —●— and 0.06 M —□— were used.

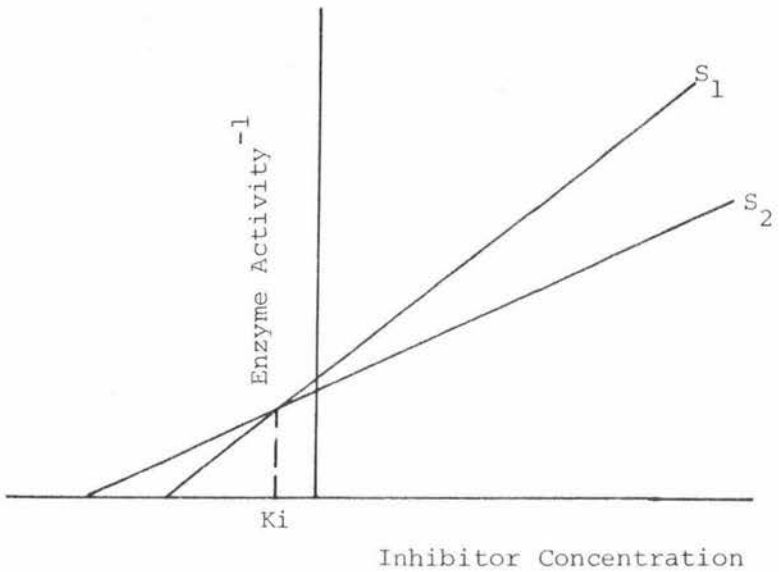

FIGURE A3. Reciprocal plot of enzyme activity as a function of inhibitor concentration at two substrate concentrations (after Dixon, 1953). The graph is representative of competitive inhibition.

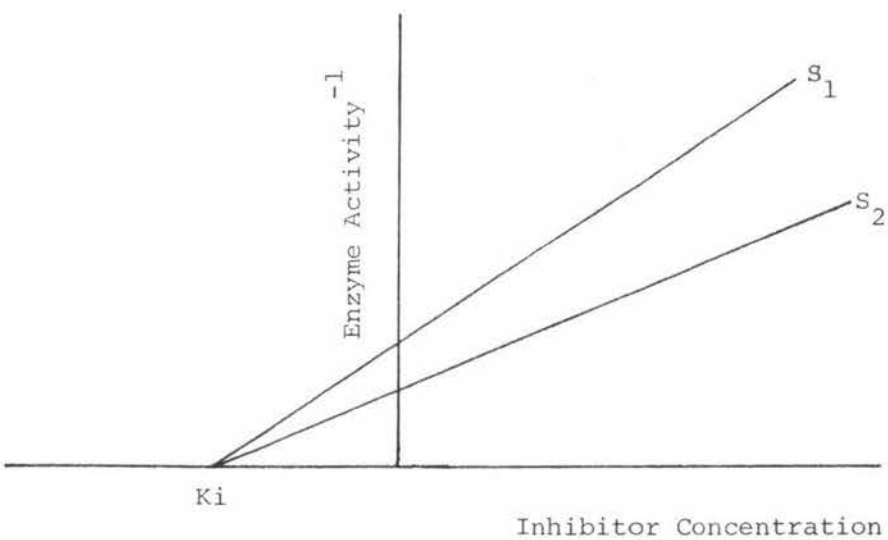

FIGURE A4. Reciprocal plot of enzyme activity as a function of inhibitor concentration at two substrate concentrations (after Dixon, 1953). The graph is representative of non-competitive inhibition.

APPENDIX 3

ESTIMATE COST OF ETHANOL PRODUCTION FROM WHEY

A.3.1 Introduction

Using the data recorded in this study an estimate design and costing has been performed for the addition of immobilized catalysts to an existing sulphuric casein factory to utilize the whey for ethanol production. It is proposed that an ultrafiltration plant is incorporated in the factory to remove lactalbumin from the whey, with the permeate being passed for the immobilized cell processing.

For the purposes of the design the casein factory produces ½ tonne/hr casein for 10 hours a day at peak production, with the average annual production over 300 days being 67% of peak production, equivalent to 200 days of peak production.

The immobilized reactor was assumed to contain the gel optimized for K. fragilis NRRL Y 1109 activity as described in section 3.3.1 and was operated at 0.18 SV on a 4.6% $(\frac{W}{V})$ lactose substrate. The reactor effluent was then assumed to be distilled to recover ethanol at 95% $(\frac{W}{V})$. It is proposed to operate the reactor in a series manner as depicted in Figure A5 to maintain constant production after the initial start up.

A.3.2 Assumptions

The following assumptions have been made in this design.

1) The casein factory is capable of producing 500 kg/hr for each of 10 hours per day.
2) The factory operates at an average of 67% of peak production for 300 days annually.
3) The skim milk used for casein manufacture contains 2.8% $(\frac{W}{V})$ casein.
4) All casein is removed from the skim milk.
5) To precipitate the casein 4% $(\frac{W}{V})$ skim weight of steam and 8.5% $(\frac{W}{V})$ skim weight 0.1 M sulphuric acid is required.
6) Whey produced is 85% $(\frac{W}{V})$ skim volume plus all steam and acid.
7) One litre of whey is equivalent to one kilogram.
8) The whey volume is unaltered by the ultrafiltration process.

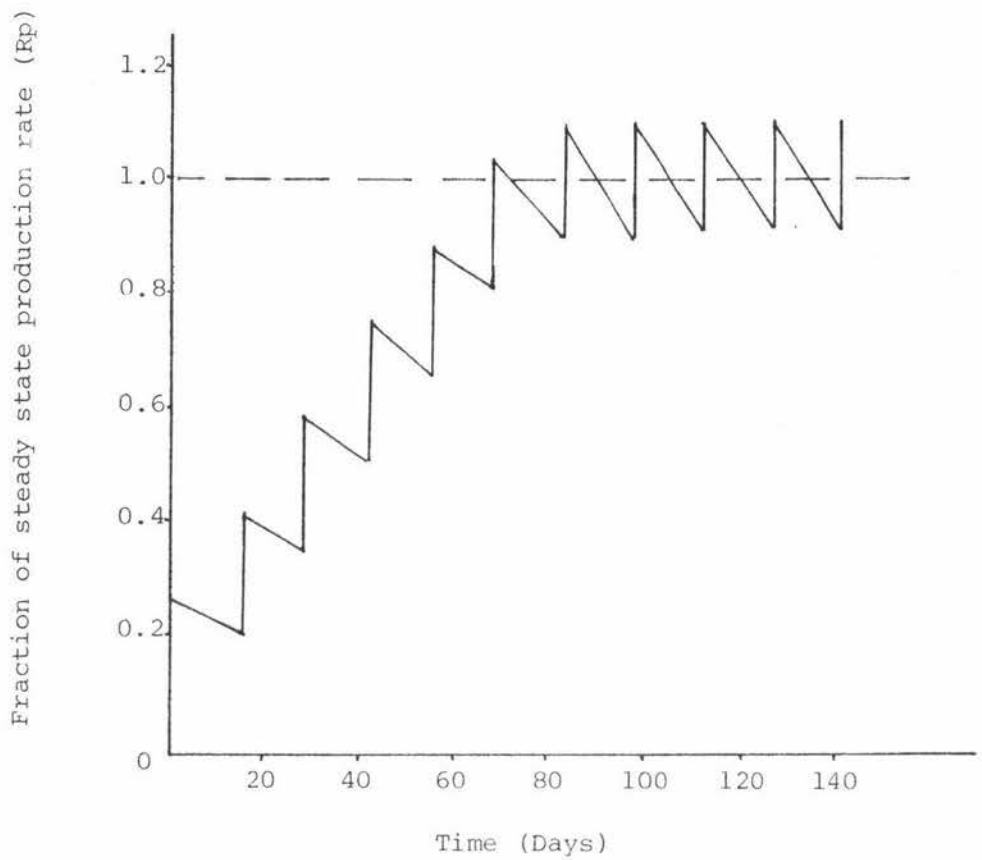

FIGURE A5. Theoretical production levels from startup in a general multiple reactor system incorporating seven reactors (after Pitcher, 1975, pg 181).

9) The whey permeate feed to the immobilized cell reactors contains 4.6% $(\frac{W}{V})$ lactose and 5.4% $(\frac{W}{V})$ total solids.
10) The immobilized cell reactors operate 24 hours per day.
11) The immobilized cells have 50 day half lives and 2 half lives will be used for production.
12) For 4.6% $(\frac{W}{V})$ lactose it is estimated that complete substrate utilization would not occur above 0.18 SV.
13) The reactor effluent contains 2% $(\frac{W}{V})$ ethanol.
14) The ethanol is distilled to 95% $(\frac{W}{V})$.
15) The immobilized cell activity is not affected by the reduced flowrates used during off peak production periods.
16) The cost indexes of Peters and Timmerhaus (1968) are compatable with dairy processing.

A.3.3 Plant Capacity Design

Hourly production of 500 kg casein

Skim milk required	17,860 kg
Acid required	1,518 kg
Steam required	714 kg
Whey produced	17,410 kg

Daily whey production	174,100 kg
Hourly feed to immobilized cells	7,255 kg

The following equation was used to determine the number of reactors required.

$$Rp = \exp\left(\frac{-H}{N} \ln 2\right)$$ (Havewala and Pitcher, 1974)

where Rp is the ratio of low to high production rates which will be 1 for a constant production rate (see Figure A5).

H = number of half lives used (2)

N = number of reactors required

For Rp = 1, H = 2 $\Rightarrow$ N = 7

At 0.18 SV and peak production of 7255 kg/hr
7 reactors of 5.76 m^3 working volume are required

Hourly ethanol production $2\%\ (\frac{w}{v})$ 145 kg in 7255 kg

$95\%\ (\frac{w}{v})$ 145 kg in 153 kg

= 192 l

Storage Required, - At least total whey produced minus whey that can be produced in 10 hours

174,100 - 72,550 kg

= 101,550 kg

A.3.4 Cost Estimation

For the purposes of the cost estimation the fixed capital investment and the operating costs will be calculated.

Fixed Capital Investment (FCI)

This can be divided into direct costs, which include equipment, buildings and installation, and the indirect costs which are the contractors fees and expenses and contingency. The fixed capital investment will be calculated on the basis of the delivered equipment cost of the major equipment (DEC) using factors. The factors are those from Peters and Timmerhaus (1968) and have been adjusted to comply with an addition to dairy processing conditions. A list of the factors used appears in Table AI.

TABLE AI. Cost Factors

Factor	% DEC
Direct Costs	
Installation	30
Insulation	3
Piping	15
Instrumentation	15
Electrical	20
Buildings and Services	50
Yard Improvements	5
Indirect Costs	
Engineering and Supervision	25
Construction Expenses	20
Contractors Fees	8
Contingency	20
	221% DEC

Installation, piping and insulation costs include all labour and materials used in the operations. Electrical encompasses all electrical wiring and starter panels and installation labour and materials. The building and services category includes the cost of erecting the building and fittings of lighting, plumbing and ventillation and the provision of steam, water and other services to the building.

Also included in the fixed capital investment is the working capital, estimated to be 15% of the capital used to buy and install the plant.

Operating Costs

The costs taken into account in the operating costs. The manner in which they are estimated are included below (Peters and Timmerhaus, 1968).

Direct costs :	Operating labour and supervision
	Utilities - steam, electricity
	Maintenance and repairs (1.5% FCI annually)
	Maintenance labour (1.0% FCI annually)
	Operating supplies (Gel reagents)
Fixed charges :	Depreciation 10% fixed percentage
	Taxes - rates etc. (1.0% FCI annually)
	Insurance (1.0% FCI annually)
General Expenses :	Overheads (60% of total labour)
	Administration (20% of operating labour and supervision)
	Interest -11% fixed rate
	Marketing and distribution (1% of FCI annually)

A.3.5 Cost Index

The cost index of Marshall and Stevens (Peters and Timmerhaus, 1968) has been used in these calculations. In 1967, January, the index was 256. Figure A6 shows the indexes for 1975 to 1979 and a projection to December 1979 for which the estimated index is 630.

Conversion factor from January 67 to December 79 $= \frac{630}{256} = 2.46$

Conversion factor from March 1976 to December 79 $= \frac{630}{460} = 1.37$

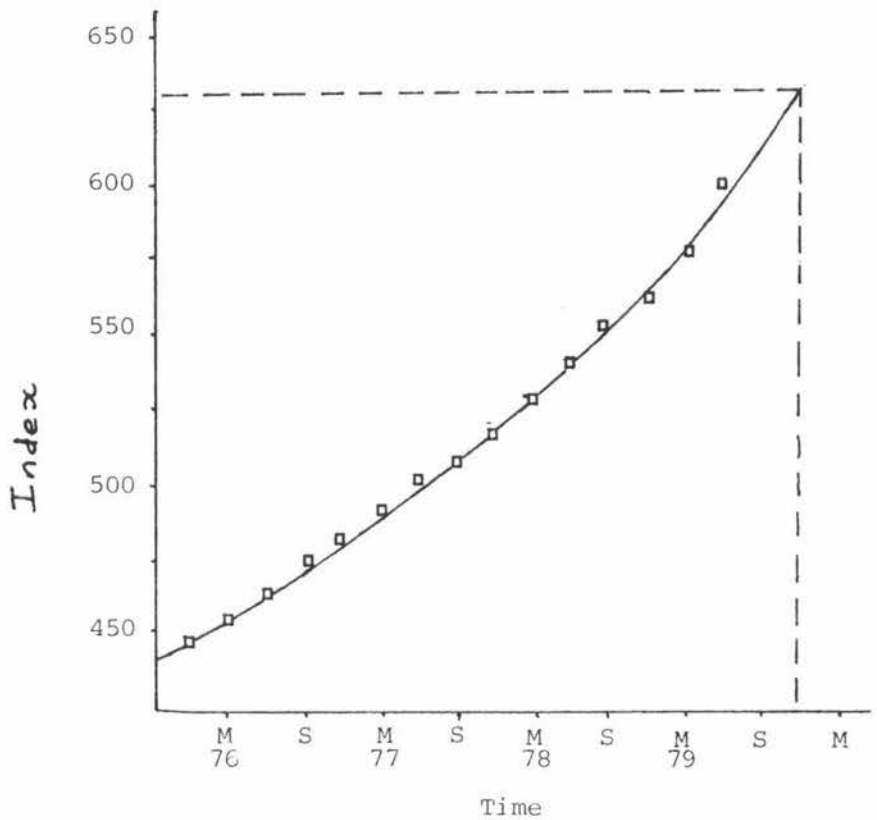

FIGURE A6. Marshall and Stevens cost index for equipment costs from March 1976 until June 1979 including the projection for December 1979.

A.3.6 Cost of Major Plant Items

a. Storage tanks for whey permeate

Size, 150,000 l

Type, Stainless steel with cooling facilities

Cost, $30,000 January 67 (Peters and Timmerhaus, 1968, Figure 13-56)

$73,800 December 79

b. Reactors

Size, 7 of 5.76 m^3 capacity

Type, stainless steel with temperature control

Cost, 7 @ $20,000 January 67 (Peters and Timmerhaus, 1968, Figure 13-90)

$344,400 December 79

c. Distillation Equipment

$$\text{Cost} = \text{Cost of similar equipment} \left(\frac{\text{size required}}{\text{size of similar}}\right)^{0.6}$$

(Peters and Timmerhaus, 1968, pg 107)

Distillation equipment costs are based on those of Cysewski and Wilke (1976a) of $124,700 for a complete distillation unit capacity 24,000 gal/day of 95% $(\frac{w}{v})$ ethanol.

$$\$124,000 \left(\frac{636}{24,000}\right)^{0.6} = \$14,120 \text{ March 76}$$

$$= \$19,340 \text{ December 79}$$

d. Ethanol Storage tank

Size, 500 l

Type, Stainless steel

Cost, $1,000 January 67 (Peters and Timmerhaus, 1968, Figure 13-56)

$2,460 December 79

e. Pumps

Type, rotary (Peters and Timmerhaus, 1968, Figure 13-41)

Size, 20,000 l/hr (Pump for whey to storage)

Cost, $1,000 January 67

Size, 2 x 10,000 l/hr (Pumps to and from reactors)

Cost, $1,400 January 67

Size, 1 x 200 l/hr (Pump for ethanol to storage)

Cost, $350 January 67

Total cost $6,760 December 79

DEC = Delivered Equipment Cost (Main Items Only) = $446,750

A.3.7 Fixed Capital Investment

211% of DEC = $942,640

plus 15% Working Capital $1,084,040

A.3.8 Operating Costs

a) Labour 1 man 24 hr/day
plus 1 man 8 hr/day @ $5/hr
= $48,000

b) Utilities - Steam 40/gal distillation of 95% ethanol (Cysewski and Wilke, 1976a)
= $1.07/hr at peak (76)
= $0.98/hr average (79)
= $7,070 annually December 79
plus an estimated 20% for reheating whey
= $8,490 December 79

- Electricity, estimated electricity usage for pumps is 40 Kw
At 3.5¢/Kwh
= $10,080

c) Operating Supplies

Yearly gel replacement	= 21 column volumes
	= 121 m^3 gel
Acrylamide required	= 18,144 kg
Cost @ $6/kg	= $108,860

Acrylamide costs is quoted from Koch-Light Laboratories Ltd as $7/kg. Bulk costs were estimated at $6/kg.

The cost of the gel is assumed to be 130% the cost of the acrylamide, this includes, BIS, initiator, accelerant and cell costs.

Total Gel Cost = $141,520

d) Summary of Operating Costs

Labour	48,000
Utilities	18,570
Maintenance and repairs	16,260
Maintenance labour	10,840
Operating supplies	141,520
Depreciation	108,400
Taxes	10,840
Insurance	10,840
Overheads	34,620
Administration	14,400
Interest	119,240
Marketing and distribution	10,840
	544,370

A.3.9 Cost Comparison

Annual Operating Cost	$ 544,370
Annual Ethanol Production	734,400 kg
	920,980 l
Operating or production cost	= 59 ¢/l

Energy output of ethanol @ 36740 BTU/kg
= 26,982 MBTU
This is equivalent to 608,660 kg fuel oil @ 44,330 BTU/kg
Estimated value @ 40¢/l = $243,460

The approximate value of the fuel oil and therefore the ethanol is only about half of producing the ethanol.
The estimated cost of ethanol production from whey in this study is approximately double that obtained by Marshall, (1978), who estimated that continuous fermentation using free yeast cells would produce ethanol at a cost of 45c/l.