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CONVERSION OF MEMBRANE PROCESSED WHEY INTO PROTEIN AND ETHANOL USING IMMOBILIZED YEAST FERMENTATION

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ABSTRACT

Conversion of Membrane Processed Whey into Protein and Ethanol

using Immobilized Yeast Fermentation

by ROBERT A. EVANGELISTA, M.S.

Cheese whey, the green-yellow liquid remaining after the curd has been separated from whole or skim milk, is composed of water, protein, lactose, ash, non-protein nitrogen and fat. The objective of this study was to develop a recycling process capable of effectively and efficiently converting whey, a waste product of the dairy industry, into usable products having economic value.

An ultrafilter separated and concentrated the valuable whey proteins into an edible product, whey protein concentrate. The filtered whey stream, permeate, containing low lactose levels was subsequently concentrated by reverse osmosis. This whey permeate concentrate was fermented into ethanol using either free cell or immobilized yeast entrapped in alginate beads.

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Shake flask studies comparing free and immobilized Candida pseudotropicalis resulted in a two fold increase in ethanol productivity and higher final ethanol concentrations for the entrapped cultures. C. pseudotropicalis was less severely affected by the detrimental effects of high substrate and high ethanol concentrations when immobilized in alginate. Continuous immobilized yeast fermentations displayed a three fold reactor ethanol productivity increase over batch immobilized yeast fermentations, however, final batch ethanol concentrations were two fold higher than ethanol in the continuously operated reactor effluent. The lower ethanol concentrations would have a significant impact on the cost of distillation. As whey permeate concentration fed into the packed bed continuous bioreactor increase, ethanol productivity decreased, the result of substrate inhibition periodic microaeration stimulated the culture in high lactose media. At constant whey permeate feed concentration, when reactor dilution rates were increased thereby productivity and effluent lactose increased while effluent ethanol decreased. The bioreactor was operated as long as 80 days without contamination and activity loss.

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The machinery of a creative mind is always turning, but resources, of the monetary type, are required to develop a bright idea to fruition. The U.S. Department of Energy, Office of Appropriate Technology supported this project with an Appropriate Technology Grant. My acknowledgement and thanks go to the folks in the U.S.D.O.E., Office of Appropriate Technology, for without your help this project certainly would not have been.

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CHAPTER I

INTRODUCTION

1.1 The Problem -- A National Perspective

The United States of America is generally considered to have the most affluent society on the face of the planet today, containing 5% of the world population and consuming 50% of the planet's resources. The fact that its enormous economy can support a large population at one of the world's highest standards of living is not without problems. A great deal of pollution is generated from by-products of the production of goods and services and from the terminus of their consumption.

The amount of these wastes is staggering! The municipal refuse generated in the United States, the U.S. Environmental Protection Agency (U.S.E.P.A.) estimates, could fill the New Orleans Superdome from floor to roof twice daily, every day of the year. U.S.E.P.A. data indicate New York City alone daily pours 280 million gallons of raw sewage into the surrounding waters and along with neighboring communities dumps 1.5 million tons of sewage slude into the Atlantic Ocean 12 miles off New Jersey's coast. In any urban area, a battalion of garbage trucks are waiting at municipal landfills to make their offering.

The effects of this prodigious quantity of waste materials being generated can be astounding. There have been a number of well publicized incidents due to improper disposal -- Love Canal and Chemical Control Corporation to name a few. Most rivers are menacingly polluted: the majestic Hudson and its inhabitants are contaminated with PCB's; the Potomac is unswimmable; and the lazy Cuyahoga, which meanders through Cleveland, caught on fire due to volatilized pollutants. Scarce land in urban areas is causing problems siting additional landfills and neighbors of proposed landfill sites are pouring out vehement cries opposing their construction. Disposal at sea is meeting a similar bleak fate. Long term pollution effects of improper waste disposal are just unveiling. The U.S. draws 50% of its drinking water from the ground. Pollutants from industrial pits and lagoons, municipal landfills, urban and agricultural runoff, and toxic waste sites are causing irreversible damage, for all practical purposes, to our underground aquafers that may contain water thousands of years old.

1.2 The Disposable Society

The underlying cause of our pollution problems is our disposable society. Food and beverage containers, newspapers and many consumer goods, to name a few, are created as throw-away items. This consciousness pervades the manufacturing and process industries where industrial production schemes do not include resource recovery or waste stream recycling.

This disposable society survives due to our prodigious resources and advanced technology which allows the existence of this type of life style. But for how long? Already some resources are becoming scarce, and pollutants are stressing our environment and adulterating our bodies. All this is occurring while our population and, therefore, our industrial base mushrooms.

1.3 The Recycle Society

Recycling is the key to the elimination of waste and the extension of our planet's resources. If natural processes were not cyclic by design, the materials necessary for life, as we know it, would have exhausted themselves eons ago. So natural processes can serve as a model for our designs of industrial processes.

Fig. 1 depicts our current linear industrial process schematic. We see that in this diagram the supply of natural resources depletes while the amount of waste products grows. The cyclic industrial process schematic, shown in Fig. 2, recycles industrial wastes into usable resources via a recovery or recycling process. These recovered or transformed resources can be utilized by the primary industry (that industry which generated the waste) or sold to a secondary industry. Waste recycling or recovery, intrinsically, is the only treatment process to yield a return on investment.

Fig. 1 -- Linear Industrial Process Schematic

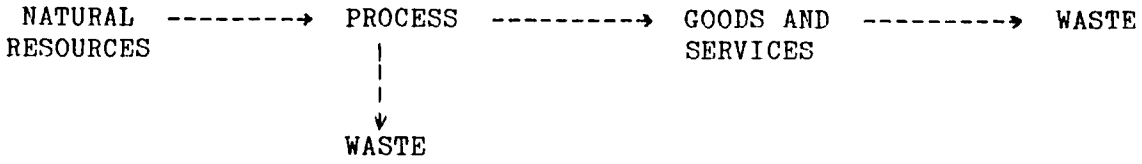
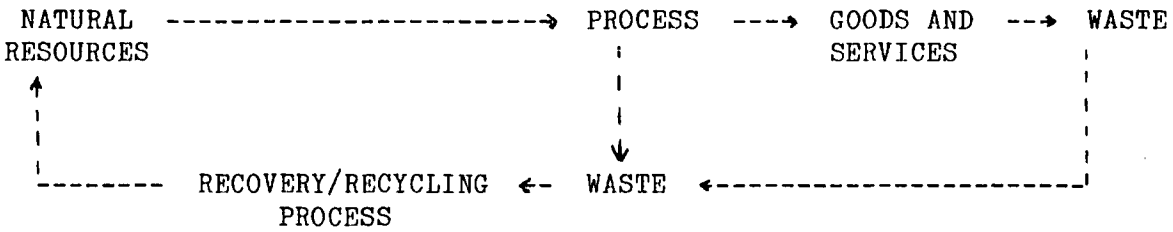


Fig. 2. -- Cyclic Industrial Process Schematic



1.4 Cheese Whey -- One Industry's Pollution Problem

American cheese manufacturers produced 43 billion pounds of raw liquid cheese whey as a by-product of casein and cheese production during 1982 (Table 1). Whey is the green-yellow liquid remaining after the casein is removed from whole or skim milk. It is composed of a protein fraction, i.e., about 25% of the protein in the original milk, lactose, i.e, nearly all of the milk sugar in the original milk, ash, fat and over 90% water. It is this low solids content which makes transportation of whey any sizable distance uneconomical.

Only about half of whey production is utilized. This leaves nearly 21 billion pounds of whey to be disposed or treated in a proper manner. Originally, disposal meant feeding to farm animals or spraying as fertilizer, but there are severe drawbacks and limitations to both these methods. As cheese production grew, the amount of this by-product that found its way into rivers and disposal ponds increased. With the advent of environmental regulations in the early 1970's, manufacturers now had to shoulder the added burden of waste treatment costs. These costs are high due to whey's unusually high Biological Oxygen Demand (BOD) shown in Table 2.

TABLE 1

Estimated U.S. Whey Production (by type) and Quantity of Whey Utilized

(Quantities in Millions of Pounds)

	1982	1975	1979	1980	1981[a]	1982[b]
Sweet whey production	23,445	25,299	33,435	35,856	38,079	39,888
Acid whey production	4,704	4,206	3,984	4,002	3,882	3,720
Total whey production						
Fluid basis	28,149	29,505	37,419	39,858	41,961	43,608
Solids basis	1,830	1,918	2,432	2,591	2,727	2,835
Total whey solids utilized	973	1,159	1,287	1,337	1,449	1,500
Percentage utilized	53.2%	60.4%	52.9%	51.6%	53.1%	52.9%

SOURCE: Whey Products Institute, Estimated U.S. Fluid Whey and Whey Solids Production (by type) and Resulting Quantity of Whey Solids Further Processed, (Chicago, Illinois, 1983)

[a] Revised pending further revision

[b] Pending further revision

TABLE 2

CHARACTERISTICS OF FOOD PROCESSING WASTES

	BOD_5, mg/L
dairy processing waste waters	
fluid milk plant	1000
ice cream plant	2500
cottage cheese plant	6000
whey powder plant	40
other food processing waste waters	
sweet goods bakery	2500
meat canning	1500
candy plant	4000
poultry processing	5000
raw wastes	
sweet whey	35000
acid whey	45000
fish processing stickwater	50000
domestic sewage	300

SOURCE: Industrial Whey Processing Technology: An overview, Pane/Jelen, J. Agric. Food Chem., Vol 27, No. 4, 1979

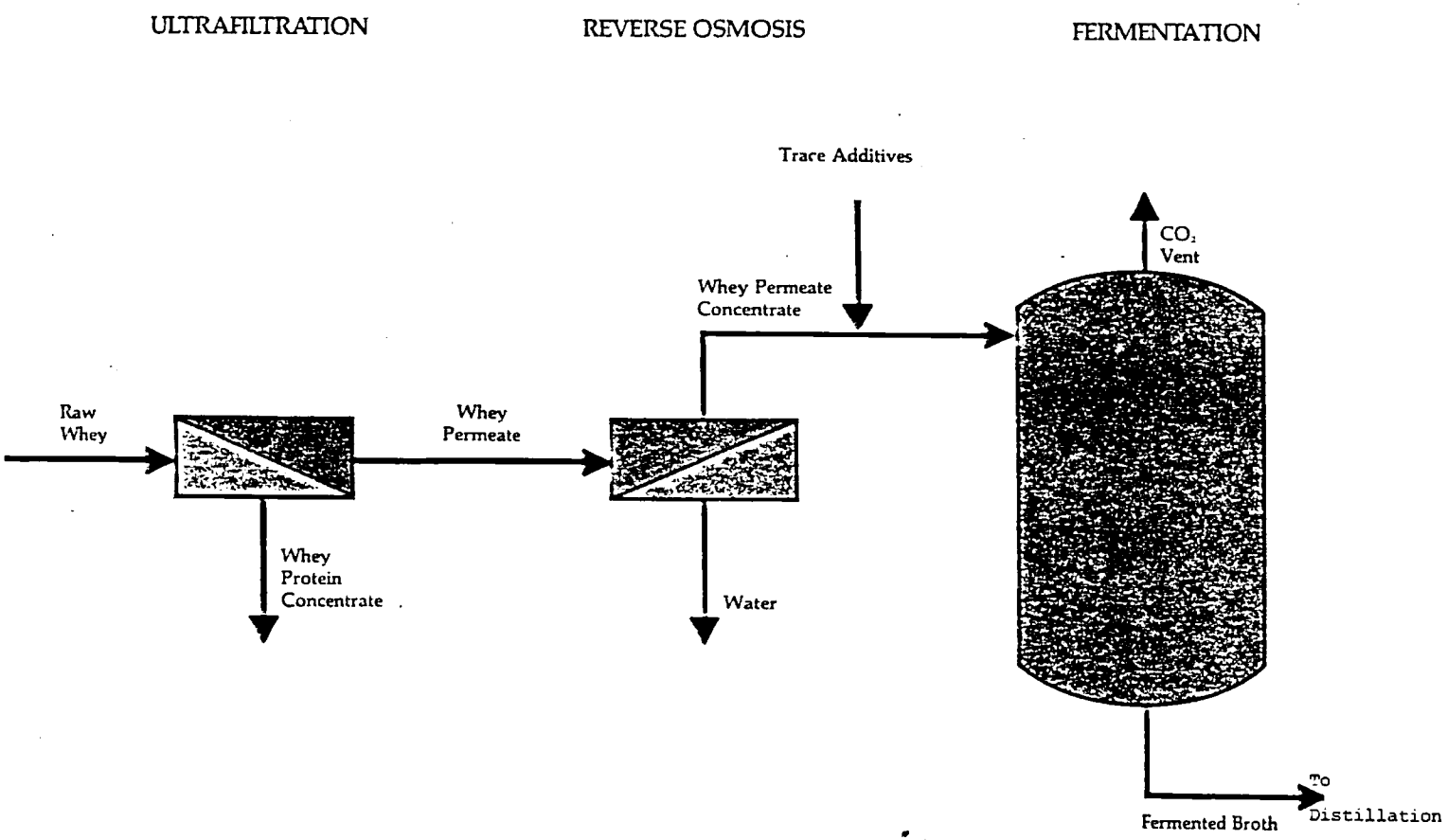
1.5 Objective

The objective of this study was to develop a recycling process capable of effectively, efficiently and economically converting nearly all of whey's components into usable products having economic value. This process must be capable of being located in-house at a moderate or larger size cheese manufacturing facility.

1.6 Process Description

A novel process was proposed to fulfill the project's objectives. The process flow diagram in Fig. 3 gives an overview. Liquid whey is ultrafiltered, to separate and retain the valuable whey protein fraction from other whey components. Separation is performed by a semi-permeable membrane (shown as a diagonal line) located at the heart of the ultrafilter. This porous polymer allows the free passage of lactose, non-protein nitrogen (NPN), ash, and water through its structure but prevents the permeation of the large protein molecules by molecular weight exclusion. The retentate, that which is retained behind a semi-permeable membrane, increases in concentration and is known as whey protein concentrate. The ultrafiltration whey permeate, those substances which diffuse through the ultrafiltration membrane, is sent to a reverse osmosis unit to be concentrated. Also utilizing a semi-permeable membrane, reverse osmosis permeates essentially water through its "tight" membrane.

Fig. 3 -- Process Flow Diagram



The reverse osmosis retentate, called whey permeate concentrate, is concentrated until an appropriate level of lactose is reached. The final, but crucial, step in whey's journey is fermentation. Here immobilized yeast cells transform the lactose component into ethanol and carbon dioxide. To produce anhydrous ethanol for fuel or chemical feedstock, the fermentation "wine" is distilled. This process step is not in the project's scope.

CHAPTER 2

LITERATURE REVIEW

2.1 Ultrafiltration and Reverse Osmosis

One of the earliest artificial semi-permeable membranes was prepared and studied by Fick, famous for ennunciating the laws of diffusion, in 1855. His membranes were constructed from an ether-alcohol solution of cellulose nitrate called collodon [1]. Around the turn of the century ultrafiltration membranes of cellophane or regenerated cellulose, in addition to collodon, were being used. Early membrane separation processes were unsuccessful against other competing separation equipment because of A) low flux; B) low solute selectivity; C) lack of inexpensive, well developed systems and D) cheap energy costs [1]. In the mid 60's, the high temperature annealing process of the Loeb-Sourirajan cellulose acetate membrane gave high rejection efficiencies for reverse osmosis. The unannealed membrane with casting solution additives created a more open pore structure and the first commercial ultrafiltration membrane was developed.

Today, membranes are prepared from numerous materials such as polyamide, polysulfone, polyvinylidene fluoride, polypropylene, polytetrafluroethylene, and regenerated cellulose, and are anisotropic in structure as the Loeb-Sourirajan membrane [1, 2, 3]. These modern polymers produce membranes with high retention characteristics, yet, high flux rates. Furthermore, newer membrane materials have greater degradation resistance at pH extremes, making them more desirable for rigorous cleaning necessary to restore process flux after fouling has occurred.

Engineering advances have closely followed membrane development throughout the last decade. Presently, several well developed membrane module configurations are available from membrane system manufacturers [1, 2, 4]. Some commonly offered configurations are: large tubes (>6 mm diameter), small tubes, plate and frame, flat leaf, spiral wound and hollow fibre.

Membrane systems separate components based on size differential. However, pores of any one membrane type and molecular weight cut-off vary significantly resulting in a pore size distribution for that particular membrane. Therefore, components to be separated should have approximately an order of magnitude molecular weight size differential. It can be observed in Table 3 that size differential between proteins and disaccharides is sufficient for effective separation. Whey proteins, alpha-lactalbumin, beta-lactoglobulin and serum albumin, are between 15,000 and 70,000 dalton in size [6], and lactose has a molecular weight of 342.

Ultrafiltration and reverse osmosis are not the only options for whey separations. Competing unit operations for protein separation are heat and acid precipitation, while evaporation is an alternative to concentration by reverse osmosis. One must certainly look at the advantages and disadvantages of membrane processes listed in Table 4 when comparing various separation equipment.

TABLE 3

TYPICAL APPARENT DIMENSIONS OF SMALL PARTICLES, MOLECULES AND IONS [5]

SPECIES	RANGE OF DIMENSIONS (nm)
Suspended solids	10,000 - 1,000,000
Colloidal solids	100 - 1,000
Smallest visible particles	25,000 - 50,000
Oil emulsion globules	100 - 10,000
Bacterial cells	300 - 10,000
Yeasts and fungi	1,000 - 10,000
Viruses	30 - 300
Proteins/polysaccharides (m.wt. 10^4 - 10^6)	2 - 10
Enzymes (m.wt. 10^4 - 10^5)	2 - 5
Common antibiotics (m.wt. 300 - 1000)	0.6 - 1.2
Organic acids (m.wt. 100 - 500)	0.4 - 0.8
Mono- and di-saccharides (m.wt. 200-400)	0.8 - 1.0
Inorganic ions (m.wt. 10 - 100)	0.2 - 0.4
Water (m.wt. 18)	0.2

TABLE 4

Advantages and Disadvantages of Ultrafiltration and Reverse Osmosis [8]

Advantages:

1) Low temperature operation enables heat labile products to be processed,

2) No change of state is involved lowering energy requirements,

3) Concentration and purification can be contained,

4) Filtrate free of suspended solids and micro-organisms facilitates downstream processing,

5) No chemical addition to process stream.

Disadvantages:

1) Substantial capital investment due to relatively low filtrate rates,

2) Membrane replacement cost high,

3) Maximum retentate solids concentration limits exist.

Heat/acid precipitation has the marked effect of denaturing protein. Denaturation affects food processing characteristics (functionality) of the protein making it less desirable in many applications. Since ultrafiltration concentrates whey proteins in relatively mild conditions, an undenatured protein concentrate is produced. Furthermore, there is no chemical addition to the whey stream as in acid precipitation. If purification of whey protein is desired, water can be added to the retentate to effectively "wash out" permeable solids. This process is known as diafiltration and whey protein concentrations as high as 80% of whey solids have been reported [9]. Finally, ultrafiltration will screen out all bacteria and viruses eliminating the need for media sterilization.

A major disadvantage of whey or whey permeate as an ethanol fermentation substrate is the low carbon source concentration (approximately 4.7% lactose) compared with other substrates which would be fermented at 10 - 25% concentration. To provide an economical fermentation and product recovery, concentration of the whey permeate is necessary -- an energy intensive and, hence, expensive operation. However, since hydrostatic pressure is the driving force for reverse osmosis (as in ultrafiltration), no change of state is required to concentrate whey permeate thereby lowering the energy requirements and preserving heat sensitive nutrients necessary for successful fermentation.

2.2 Alternatives To Whey Permeate Utilization

The resulting permeate, during whey separation by ultrafiltration, retains a high BOD due to approximately 4.7% lactose which it contains. Rendering the permeate stream acceptable, by waste water treatment standards, entails either separating the lactose from the permeate, degrading the disaccharide or transforming it into usable products.

Lactose, as a commodity chemical, has limited sweetness and solubility. It has long been produced for pharmaceuticals and infant foods [10], but the current market for lactose is flat, so there is little need to produce more. Carbohydrate degradation is a viable alternative to lower permeate BOD, but many municipalities that are home to cheese plants only have primary waste treatment systems which remove about 40% of the oxygen consuming constituents biologically. Many treatment plants cannot handle the high BOD whey waste stream and refuse to accept it so cheese plants must build their own costly treatment facilities which generate only biological sludge. It is not surprising that transforming lactose into marketable products has been explored.

The readily obvious method of the lactose conversion is enzymatic hydrolysis into the constituent monosaccharides, glucose and galactose [11]. The mixture of the simple sugars is sweeter and more soluble than the original lactose.

A joint venture between Kroger and Corning takes this hydrolysis one step further at their Winchester, Kentucky plant [12]. Bakers' yeast, Saccharomyces cerevisiae, is grown on the lactose hydrolysate but only glucose is consumed leaving half the carbon source not utilized. Kluyveromyces fragilis, a suitable food yeast, has been grown on whey for single cell protein with satisfactory results [13, 14]. Like other single cell protein fermentations, the application is hindered by less than favorable economics. Production of lactase by yeast fermentation promises a high value product from whey, but again the current market is inadequate to sustain large scale production of this enzyme [14]. Additional high value products with large market potential may be obtained from whey permeate. IGI Biotechnology, Inc., Columbia, Maryland developed and produces such high value derivatives by fermentation and other methods from whey [15]. Some of their products are WEYCO-SERV™, a food preservative; MACROMIN™ 567, a modified whey food-grade ingredient, and MINRALAC™, a dietary mineral supplement. Whey wine has been produced successfully with a reduced ash whey permeate; but, fermentations take as long as two weeks [13].

The final whey treatment alternative is conversion of lactose into energy, i.e., ethanol or methane. Energy production has become an attractive, viable alternative of lactose utilization primarily due to the favorable economics associated with it [16, 17] and cheese manufacturers have the option of using the energy produced on site or selling it.

2.3 Ethanol or Methane?

There are a number of factors one must examine before deciding whether to produce methane or ethanol from whey permeate. Methane as a gaseous fuel offers more problems in transportation and storage while liquid ethanol is inherently more "convenient". Methane can be used on site at most plants as fuel to lower energy expenses but ethanol, in most cases, must be marketed. Marketing ethanol may be more difficult for cheese manufacturers, yet markets in potable alcohol, power alcohol and chemical feedstocks exist. Since the price of ethanol is tied directly to the value of gasoline, prices will rise (and fall) with the price of liquid fuel. Another consideration is the lower capital cost and energy requirements of converting whey to methane.

> "The development of systems for converting whey permeate to methane and ethanol inevitably raises a question: Which is more economical for the cheesemaker or whey processor? The answer may vary from one processor to another depending on his own operation and a thorough analysis of current natural-gas costs, ethanol markets and waste treatment costs. One fact is clear, however: Rising energy costs have narrowed the gap between methane and natural gas, and between ethanol and petrochemicals. These rising costs will motivate cheesemakers and whey processors to evaluate new technologies for making old products: methane and alcohol" [18].

2.4 Alcohol From Whey

Alcohol production formula below indicates ethanol production from lactose at 100% of theoretical maximum conversion (Pasteur efficiency).

Pasteur Efficiency:

$C_{12} H_{22} O_{11} + H_2O$ ---------→ $4 C_2H_5OH + 4CO_2$

Lactose Water Ethanol Carbon Dioxide

342 grams + 18 ---------→ 184 + 176

12.3 pounds ---------→ 6.6 pounds (1 gallon)

262 pounds whey or whey permeate (@ 4.7% lactose) --------→ 1 gallon of ethanol

20.5 billion pounds of whey not untilized in 1982 ---------→ 78.2 million gallons of ethanol

Even if a process is only 75% efficient, the whey currently wasted in the United States can produce 586.5 million gallons of Gasohol.

Early researchers [19] discovered lactose fermenting yeast had difficulty fermenting whey media containing over 10% lactose. Lactose (substrate) inhibition and the inhibitory effects of high ash concentration may have played a role in these early failures. Garvel and Kosikowski [20] showed that increasing the concentration of $CaCl_2$ or NaCl in a low ash whey permeate concentrate medium decreased the ethanol fermentation rate. NaCl proved to be more inhibitory than $CaCl_2$. Later work by Mahmoud and Kosikowski [13] found aerobic specific growth rate, and along with alcohol production, increased in low ash concentrated whey permeate. Ash concentration is such an important factor for a successful whey fermentation, that several researchers have used only low ash whey in their experiments [21, 22].

However, demineralizing whey, (on an industrial scale), is an expensive procedure involving electro-dialysis and will certainly change the economics of the proposed process. Lactose concentration also plays an important role in whey fermentation. Burgess and Kelley [23] decreased fermentation time of a 15% lactose permeate to 36 hours with acceptable yields, however a 22-24% lactose media required 15 days to ferment with slightly lower yields [20]. During continuous fermentations [24], fermentor productivity of a 20% feed solution dropped to nearly one-third of the productivity of a 15% media.

Performing experiments under favorable fermentation conditions is crucial to optimize reactor performance. To obtain these favorable environmental conditions without executing optimization studies, information from the literature was utilized. Castillo et al [25] reported optimal initial pH and temperature were 4.7 and 30° C respectively. In an attempt to optimize the media, nitrogen salts, phosphorous salts, corn steep liquor or yeast extract was added to whey resulting in increased biomass but decreased ethanol production. The above researchers concluded that "deproteinized whey contains all required nutrients for total lactose fermentation by (Candida pseudstrcpicalis) strain 8619." On the other hand, Vienne and Von Stockar [26] discovered from an elemental analysis of yeast and an analysis of the elements available for growth in whey permeate that nitrogen may be growth limiting in unsupplemented permeate. This is an important consideration since alcohol production is somewhat growth related.

These researchers concluded "while it is possible to ferment the lactose in whey permeate completely to ethanol without adding any further nutrients, the fermentation kinetics may be markedly improved by formulating a well-balanced medium, which can be done by adding 0.375% of yeast extract." Media selection, in the midst of conflicting conclusions in the literature, was resolved by considering another factor, media adaptation.

To adapt selected yeast to a high ash - high lactose environment, cultures were grown in mediums incrementally concentrated (see section 3.3.4) as suggested by Gawel and Kosikowski. Their concentrated whey permeate media contained 0.07% NH_4OH and 0.25% $NH_4H_2PO_4$ (salts not explored by Castillo et al) and provided a compromise between the two positions on media supplementation.

2.5 Immobilized Cells

Improving ethanol fermentation from concentrated whey permeate involves consideration of the following conditions: 1) the inhibitory effect of alcohol on its own production; 2) reduced substrate utilization, cell growth and ethanol production at high lactose concentration; 3) inhibitory effect of high ash on the fermentation; and 4) duration of the slow whey fermentation to yield more favorable economics.

Ethanol has a pronounced inhibitory effect on fermentation. Increasing ethanol concentration causes a linear decrease in metabolic activity of fermenting yeast such that the specific rate of glucose assimilation plunges

to zero at approximately 140 g/l ethanol [27]. A distinct advantage of an immobilized cell system for ethanol fermentation is minimum product inhibition [28]. A possible explanation may be the partition coefficient of ethanol between the immobilization gel and the fermentation broth. A partition coefficient of 0.67 was determined [29].

A similar relationship exists between sugar and alcoholic fermentation. Increasing substrate concentrations produced inhibition kinetics. Holcberg and Margaligh [30] observed increased substrate consumption with entrapped yeast exposed to various sugar concentrations. At 40 and 50% sugar, controls of freely suspended yeast consumed little or no substrate while significant fermentation activity occurred in the immobilized systems. Only at 10% glucose, where there is considerably less substrate inhibition on fermentation, did free cell fermentation activity approach that of immobilized cells. In addition to increased ethanol productivity, immobilized systems had higher final ethanol concentrations in the broth at all sugar concentration levels tested.

Immobilized cell systems contain higher cell density per unit fermentation volume over free cell systems [31]. Wada et al. [32] reported cell numbers in an immobilization gel was 10 fold greater than freely suspended cells in the fermentation broth. High cell densities produce accelerated reaction rates yielding increased ethanol productivity, an important factor in reducing fermentation duration.

For whey fermentation, immobilized biocatalysts offer an attractive alternative to traditional freely suspended cells. Advantages and disadvantages of immobilized whole cells that apply to ethanol production are listed in Table 5.

The literature revealed three methods of cell immobilization: entrapment, absorption and bonding or crosslinking to matrix. Some representative examples of these immobilization methods are shown in Table 6.

2.6 Strain Selection

Two groups of researchers [13, 20, 43] explored a total of fourteen strains of lactose fermenting yeasts (Table 7) to select the culture(s) with greatest potential to convert concentrated whey permeate to ethanol. Mahmoud, Gawel and Kosikowski chose Kluyveromyces fragilis NRRL 2415 because it "produced the highest yield of alcohol, 9.1% vol/vol" and "hydrolized the most lactose (89.3%) and attained the lowest lactose residue, 2.6%." Izaguirre and Castillo favored Candida psuedotropicalis ATCC 8619 among nine yeast strains. They reported "the highest ethanol yield, with an average of 12.46% vol/vol (9.86% wt/vol), was produced by strain 8619 in 28% whey (containing approximately 20.12% lactose)" in shake flask experiments.

TABLE 5

Advantages and Disadvantages of Immobilized Cells

Advantages	Reference
* Heterogeneous catalytic fermentation	33
* High cell densities	
* Increased reaction rates, hence increased volumetric productivity and smaller reactor size	34, 35
* Resistance to contamination	36
* Reuse of cells	37
* Higher product yield	38
* No cell separation and recycle necessary	28, 39
* Operate at high dilution rate without washout	33
* Minimum product inhibition	28
* Easier to control and automate	39, 42
Disadvantages	
* Mass transfer limitations	41
* Cost of immobilization	39
* Loss of cell enzyme activity during some immobilization techniques	39

TABLE 6

Methods of Immobilization

Method	Advantage	Disadvantage	Reference
Entrapment			
Hydrocolloids	Highly hydrated	Mass transfer limitations	35, 41
Carrogeenan	Mild immobilization coditions	Low to mod. mechanical strength	35, 41
Alginates	High cell enzyme activity	Gel disruption by phosphate	38, 41
	Resistance of gel to CO_2 generation within		39
Polyacrylamide	Free from diffusion limitations	Monomer inactivates anzyme	40, 41
	High mechanical strength		41
Hollow Fiber	High cell density		35
Metal hydroxides	Low cost, convenient preparation		41
Liquid membranes		Mass transfer limitations	40
Absorption			
Ion-exchange resins	Low mass transfer resistance	Elution of some cells	35
Zirconia ceramic	Cell enzymatic activity not affected	Attachment pH dependent	40
Covalent Bond or Cross Link to Matrix			
Carrageenan/glutaraldehyde		Damages cells	35
Photo-X link prepolymer	Mild conditions of immobilization prepared in absence of biocatalyst		41
			41

TABLE 7

Strain Selection

	Group A [20, 13]		Group B [43]	
Strains Explored In Literature	*Kluyvermoyces fragilis*	ATCC 8635	*Candida pseudotropicalis*	NCYC 143, 188, 174
	Kluyveromyces bulgaricus	ATCC 16045	*Candida pseudotropicalis*	ATCC 8555, 8619, 8628, 8660
	Kluyveromyces fragilis	NRRLY 2415	*Candida kefyr*	NCYC 152
	Kluyveromyces lactis	NRRLY 1193	*Kluveromyces fragilis*	NCYC 151
	Kluyveromyces lactis	CV 10689		

Strains Chosen	*Kluyveromyces fragilis*	NRRLY 2415
	Candida pseudotropicalis	ATCC 8619

2.7 Immobilized Cell Bioreactor

Selection of the appropriate bioreactor configuration is of central importance in the planning of a successful bio-transformation. Assessment of the process requirements and conditions is required to determine reactor type for a particular immobilized cell system and product. Venkatasubramanian, Kakare and Vieth [44] list the following factors which influence choice of an immobilized cell bioreactor: 1) cell viability requirements, 2) type of support matrix and method of immobilization, 3) nature of substrate, 4) kinetics of reaction involved, 5) operational requirements of the process, 6) ease of catalyst replacement and regeneration, 7) hydraulic considerations, 8) ease of design, fabrication and process scale up and 9) reactor cost. These researchers outline a method to analyze the various systems. Several bioreactor types are currently employed for ethanol production. Most common are fluidized bed, membrane, stirred tank, packed bed and vacuum [45].

Packed bed bioreactors have significant distinct advantages for ethanol production. High specific bead-media interfacial area for reduced external mass transfer [46]. Packed beds operate more closely to plug flow when compared with other systems, hence producing increased reaction rates with a end product inhibited fermentation. Inherent in the design is simplicity of operation. In addition, modeling is easier and scale-up less problematic due to the large amount of literature available [44].

CHAPTER 3

MATERIAL AND METHODS

3.1 Cheese Whey

Two types of whey are generated, sweet and acid. Sweet whey is the by-product of hard cheese or casein production, while acid whey results from cottage cheese manufacturing. A typical composition for both sweet and acid type whey is listed in Table 8. The raw liquid cheddar cheese (sweet) whey for this study was obtained from Lehi Valley Farms, Allentown, Pennsylvania. Lehi Valley Farms de-fatted the whey and stored it at 60^{o}C. Whey was transported to Rutgers University and ultrafiltered and the permeate concentrated the same day.

3.1.1 Whey Protein

The major whey proteins are listed in Table 9. About 70% of the whey protein is comprised of beta-lactoglobulin and alpha-lactalbumin [47] which give whey proteins a PER (Protein Efficiency Ratio) of 3.2 [48]. Soy protein has a PER of 1.8, a standard by which other proteins are measured. Casein and milk, on the other hand, have a PER of 2.5 and 2.73 respectively. The higher value of milk is due to the whey protein fraction in it. A good mix of amino acids, especially the essential amino acids as indicated in Table 10, give whey the high PER. Only minor nutrient additions to whey permeate are necessary to make it a complete fermentation broth for ethanol production.

TABLE 8

Composition of Sweet and Acid Wheys

Components	Sweet Whey %	Acid Whey %
Lactose	4.5 - 5.0	3.8 - 4.2
Ash	0.5 - 0.7	0.7 - 0.8
Total Protein (as total nitrogen)	0.8 - 1.0	0.8 - 1.0
Genuine protein nitrogen	52.5	43.9
Peptide nitrogen	31.3	33.1
Amino acid nitrogen	2.5	6.1
Creatin Nitrogen	2.6	2.5
Ammonia nitrogen	1.0	2.3
Urea nitrogen	9.1	10.3
Purine Nitrogen	0.12 - 0.36	NA
Lactic Acid	trace	0.8
Citric Acid	0.1	0.1
Total solids	6 - 7	5 - 6
pH	5.2 - 6.4	4.4 - 4.8

SOURCE: J. Meyrath and K. Bayer, Biomass from Whey in Economic Microbiology, A.H. Rose, (New York: Academic Press, 1979): 209, Table 2

TABLE 9

Major Whey Proteins

Whey Protein	Approximate Molecular Weight (daltons)
beta-Lactoglobulin	35,000
alpha-Lactalbumin	15,000
Blood Serum Albumin	69,000

TABLE 10

Average Amino Acid Content of Sweet Whey [49]

Amino Acid (g/100 g dry whey)		Ash Constituent (g/100 g dry whey)	
Lysine	1.10	Potassium	2.08 - 2.54
Isoleucine	0.74	Sodium	0.63 - 1.14
Histidine	0.25	Chloride	1.82 - 2.28
Leucine	1.28	Iodine[a]	188 - 895
Arginine	0.32	Nitrate	0 - 64.5
Tyrosine	0.34	Nitrite	0
Tryptophan	0.30	Ash	5.70 - 8.83
Phenylalanine	0.43		
Aspartic Acid	1.35		
Alanine	0.58		
Threonine	0.85		
Cystine	0.28		
Serine	0.66		
Valine	0.73		
Glutamic Acid	2.24		
Methionine	0.23		
Proline	0.85		
Glycine	0.24		
Total Amino Acids	12.77		

a mg/100g dry whey

Table 11 lists the spectrum of vitamins and minerals in whey. In addition, Moulin and Galzy [50] indicate that whey may contain sufficient quantities of sterol and unsaturated fatty acids necessary for a "good fermentation".

3.1.2 Lactose

4-o-beta-D Galactopyranosyl-D-glucose, more commonly known as lactose or milk sugar, comprises the largest portion of whey solids. Slightly sweet and soluble up to 200 grams per liter of water, lactose is the most difficult to utilize component in whey. As a fermentation substrate, only a small quantity of organisms have the ability to ferment lactose [51].

3.1.3 Ash

The final whey component that needs mentioning is ash whose constituents are listed in Table 10. Since the ash concentration in the whey permeate concentrate fermentation media increases to 2.0 - 2.8% at 4 times (4X) the original concentration, ash may play an important role in ferementation behavior.

3.2 Yeast Strains

Kluyveromyces fragilis NRRLY 2415 was obtained from the U. S. Department of Agriculture, Northern Regional Research Laboratory, Peoria, Illinois on a slant and was transferred immediately to a fresh slant (section 3.3.2). Candida pseudotropicalis ATCC 8619 was purchased from the American Type Culture Collection, Rockville, Maryland as a freeze-dried pellet.

TABLE 11

Average Vitamin and Mineral Content of Sweet Whey [49]

Vitamin (mg/100 g dry whey)		Mineral (mg/100 g dry whey	
A[a]	137	Calcium	774
C	1.0	Phosphorus	1010
E	0.06	Sodium	1266
Thiamine	0.5	Potassium	1838
Riboflavin	2.1	Magnesium	192
Peridoxine	0.6	Zinc	1.7
Cobalamin	2.4	Iron	0.9
Pantothenic Acid	11.6	Copper[c]	2.8
Biotin[b]	35.0	Iodine[c]	6.6
Niacin	1.3	Lead[c]	1.3
Folic Acid	0.01	Mercury[c]	0.02
Choline	101	Selenium[c]	0.06
		Cadmium[c]	0.11
		Arsenic[c]	0.65

a NOTE: I.U./100 g dry whey
b NOTE: mcg/100 g dry whey
c parts per million

Rehydration (section 3.3.1) occurred when needed. It should be noted that a lag phase of 30 hours occurred during rehydration. Working cultures were stored at 4°C and stock cultures at -18°C.

3.3 Fermentation Medium

The various cultivation adaption and fermentation mediums were developed by Gawel and Kosikowski [20]. Adaptation of the culture was performed to minimize the inhibiting effect high ash - high lactose has on fermenting yeast cultures. All fermentation mediums were autoclaved at 121°C for at least 15 minutes (depending on volume) and cooled to room temperature before using.

3.3.1 Rehydration Medium for Freeze Dried Yeast Culture

The following rehydration medium was prepared for freeze-dried yeast cultures. Preparation of this medium was performed as outlined below:

1. The following components were dissolved in distilled water and diluted to 1 liter

yeast extract	(Difco)	3 g
malt extract	(Difco)	3 g
peptone	(Difco)	5 g
glucose	(Aldrich)	10 g

2. Aseptically introduced freeze-dried yeast into shake flasks containing above media,
3. Shook at 200 RPM for 30 minutes at 25°C.

3.3.2 Glucose Slant Medium

Medium for growing above re-hydrated yeast cultures was prepared as follows:

1. Dissolved the following components in distilled water and diluted up to 1 liter

yeast extract	(Difco)	3 g
malt extract	(Difco)	3 g
peptone	(Difco)	5 g
glucose	(Aldrich)	10 g

2. Prepared slants from above medium,
3. Aseptically tranferred loopful of re-hydrated yeast culture onto slants,
4. Incubated at 25^{o}C for 4 days.

3.3.3 Lactose Slant Medium

Yeast cultures were propogated and maintained on slants of lactose medium. Working cultures were transfered every 2 months and regrown on fresh slants to maintain viability. Preparation was as follows:

1. Dissolve the following components in distilled water and dilute to 1 liter

yeast extract	(Difco)	3 g
malt extract	(Difco)	3 g
peptone	(Difco)	5 g
lactose	(Aldrich)	50 g
agar	(Difco)	25 g

2. Aseptically transfered loopful of culture from glucose slants during culture cultivation or from lactose slants during culture maintainance,
3. Incubated at 30°C for 3 days.

3.3.4 Whey Permeate Adaptation/Fermentation Medium

Whey permeate contains nearly all the nutrients yeast need for ethanol production, hence few additional ingredients are required. This fact is particularly helpful in lowering media costs during large scale fermentation. In addition to fermentation, this medium was utilized for culture adaptation to acclimate the yeast to the inhibitory effects of high ash and high lactose concentrations in the fermentation broth. The procedure for preparing the adaptation/fermentation medium was as follows:

1. Raw liquid cheese whey was ultrafiltered and the permeate concentrated to the desired lactose concentration by an Abcor ultrafilterand a P.C.I. reverse osmosis unit (sections 3.5.1. or 3.5.2 respectively). The whey permeate concentrate was back diluted with distilled water to the desired concentration if necessary.

2. The following components were dissolved in whey permeate concentrate and diluted with same to 1 liter

Ammonium hydroxide	(Aldrich)	0.7 ml
Ammonium dihydrogen phosphate	(Aldrich)	2.5 g
Citric acid	(Aldrich)	adjust to pH 4.5

The adaption procedure was:

1. 100 ml of the above medium at a 5% lactose concentration was placed in 250 ml flasks.
2. The medium was inoculated with yeast grown on lactose slants
3. Flasks were placed in a shake bath at 30°C for 3 days.
4. After 3 days, a 5% inoculum from above flasks was aseptically transferred into similar flasks with an adaptation medium containing 2.5% additional lactose concentration. These flasks were placed in a shake bath and subjected to the same conditions as the previous flasks. This procedure was continued until 20% lactose was reached. The adapted cultures were stored at 40°C and reinoculated every 60 days to maintain viability.

3.3.5 Immobilized Cell Gelling Medium

Preparation was identical to the whey permeate fermentation medium with the exception of 10 g/liter calcium chloride addition. To avoid precipitation of calcium phosphate, calcium chloride was added after autoclaving.

3.3.6 Immobilization Alginate Solution

This solution provided the matrix in which the yeast were entrapped. A 1% w/v alginate mixture provides a highly hydrated entrapment matrix thereby allowing high cell densities and moderate yeast growth.

Addition of silica to the matrix has numerous advantages: A) increases internal surface for better adherance to cells, B) reduces compressibility of particles and, C) imparts controlled porosity for entrapped cells and gases [52]. The immobilization solution contained 2% w/v silica.

3.4 Analytical Methods

3.4.1 Borohydride/Anthrone Method for Lactose Determination [53, 54]

To determine lactose in fermentation media where glucose and galactose may be present, sodium borohydride is used to reduce the free reducing sugars. The beta-D galactopyranosyl-glucitol is measured with anthrone colorimetrically. The following procedure from Asensio et al was used: 2.8 ml of diluted fermentation broth containing 0.1 - 1.0 micro moles of disaccharide was treated with 0.2 ml of a fresh 5% solution of sodium borohydride. After 2 hours at room temperature, 7 ml of 2% anthrone in concentrated sulfuric acid was added. The tubes were immediately mixed and immersed in crushed ice until cool. Optical density was measured on a Bausch-Lomb Spectronic 20 at 610 nanometers. Standards were prepared from reagent grade lactose.

The borohydride/anthrone method was chosen over Boehringer Mannheim's enzymatic/UV method due to lower cost; $0.10 versus $1.75 per determination, however a timely and tedious analysis was the price paid. Yellow Springs Instruments' autoanalyzer had difficulty distinguishing galactose from lactose, and may read a galactose-lactose mixture as total lactose.

3.4.2 Ethanol Determination [52]

The analysis of fermentation ethanol concentration was performed on a Hewlett-Packard 5880-A gas chromatograph. An internal standard of n-propanse determined ethanol concentration by volume. In addition, external standards were made fresh as a check for accuracy. A 6 foot x 1/4 inch glass column (Supelco) packed with Carbopack B/5% Carbowax 20M at a constant oven temperature of 100^{o}C, with injector and detector temperatures of 200^{o}C, provided the ethanol separation.

3.4.3 Total Solids Determination

The following method was used for determining total solids in whey, whey protein concentrate and whey permeate during ultrafiltration,

1) Pre-heated a clean 75 ml porcelain evaporating dish, at temperature not greater than 102^{o}C, cooled in dessicator and tared,
2) Transferred about 5 grams sample to the dish and weighed,
3) Evaporated sample to dryness in oven at 100 - 102^{o} C until sample is water free (approximately overnight),
4) Cooled in dessicator, weighed and calculated % total solids.

3.4.4 Ash Determination [55]

The following method calculated ash in whey, whey protein concentrate and whey permeate:

1) Pre-heated a 75 ml porcelain dish at 550 - 600° C, cooled in dessicator and tared,
2) Transferred about 5 grams sample to the dish and weighed,
3) Evaporated sample to dryness in oven at 100 - 102° C,
4) Ignited in muffle furnace at temperature not greater than 550° C until ash was carbon free,
5) Cooled in dessicator, weighed and calculated % ash.

3.4.5 Micro-Kjeldahl Method for Total Nitrogen Determination [56, 57]

Reagents required were

1) Catalyst -- K_2SO_4:$CuSO_4$::15:1 mixture
2) H_2SO_4 -- 98% and N free
3) Boric Acid -- 2% (W/V)
4) NaOH -- 32% (W/V)
5) Standard H_2SO_4 solution, 0.02N, reagent grade
6) Indicator -- 0.2% (W/V) alcoholic methyl red:
   0.2% alcoholic methylene blue::2:1.

Procedure

A) Digestion:
   1) Weighed by difference approximately 1 g whey, whey permeate or whey concentrate into 100 ml Kjeldahl flask,

2) Added about 1 g of catalyst and 3 ml of concentrated H_2SO_4,

3) Digested on micro-Kjeldahl heating rack until solution is colorless.

B) Distillation (Steam):

1) Placed 10 ml of boric acid in 125 ml Erlenmeyer flask plus 2 drops of indicator, and placed under condenser making sure tip was immersed,

2) Quantitatively transferred digest to 50 ml volumetric flask using a small funnel and diluted to 50 ml with distilled water,

3) Mixed by pouring into a clean dry 100 ml beaker,

4) Transferred 10 ml (using transfer pipet) of dilute digest to distillation flask,

5) Added 10 ml of 32% NaOH and rinsed funnel with distilled water,

6) Distilled to a total volume of 50 ml,

7) Titrated with N/50 H_2SO_4, the endpoint being the color of the boric acid solution before distillation,

8) Corrected for blank determination on reagents by running test with 1 ml of water instead of whey. Calculated the protein content using a N factor of 6.38.

$$5.0 \times \frac{V \times N \times (0.014) \times 6.38}{\text{Sample weight}} \times 100 = \% \text{ protein}$$

Where V = volume of titrant in milliters

N = normality of H_2SO_4

0.014 = milliequivalent of nitrogen

5.0 = dilution factor

Sample weight in grams

3.4.6 Dried Cell Weight

Samples of known volume were filtered by weighed 0.45 micron membrane disks. The cell cake on these disks was rinsed with distilled water and dried overnight at 102°C. The membrane disks were weighed on an analytical balance and the grams of cells per liter of fermentation broth calculated.

3.4.7 Viable Cell Count

To insure a representative cell count from a homogeneous broth sample, the fermentation broth was shaken thoroughly and vigorously to mix contents. The interval between mixing and removing the test aliquot did not exceed 3 minutes. All test aliquots were removed by a sterile pipet under aseptic conditions.

A 1 ml or 10 ml sample of fermentation broth was mixed thoroughly and vigorously in a dilution bottle with 99 ml or 90 ml respectively, of autoclaved physiological saline. Dilutions were choosen so that the total colonies on a petri plate would number between 30 and 300. The number of viable cells per milliliter of fermentation broth was determined by plate count using potato dextrose agar [58]. Potato dextrose Agar (Difco) was rehydrated according to the manufacturer's instructions. Before pouring plates, the addition of autoclaved 10% tartaric acid adjusted the media to pH 3.5 to provide bacterial suppression. Fifteen ml potato dextrose agar and 1 ml serial diluted broth was pipeted into sterile petri plates and incubated at room temperature for 3 days. The criteria of viability was the cell's ability to reproduce yeast colonies were counted on a colony counter to obtain viable count.

CHAPTER 4

PROCEDURE

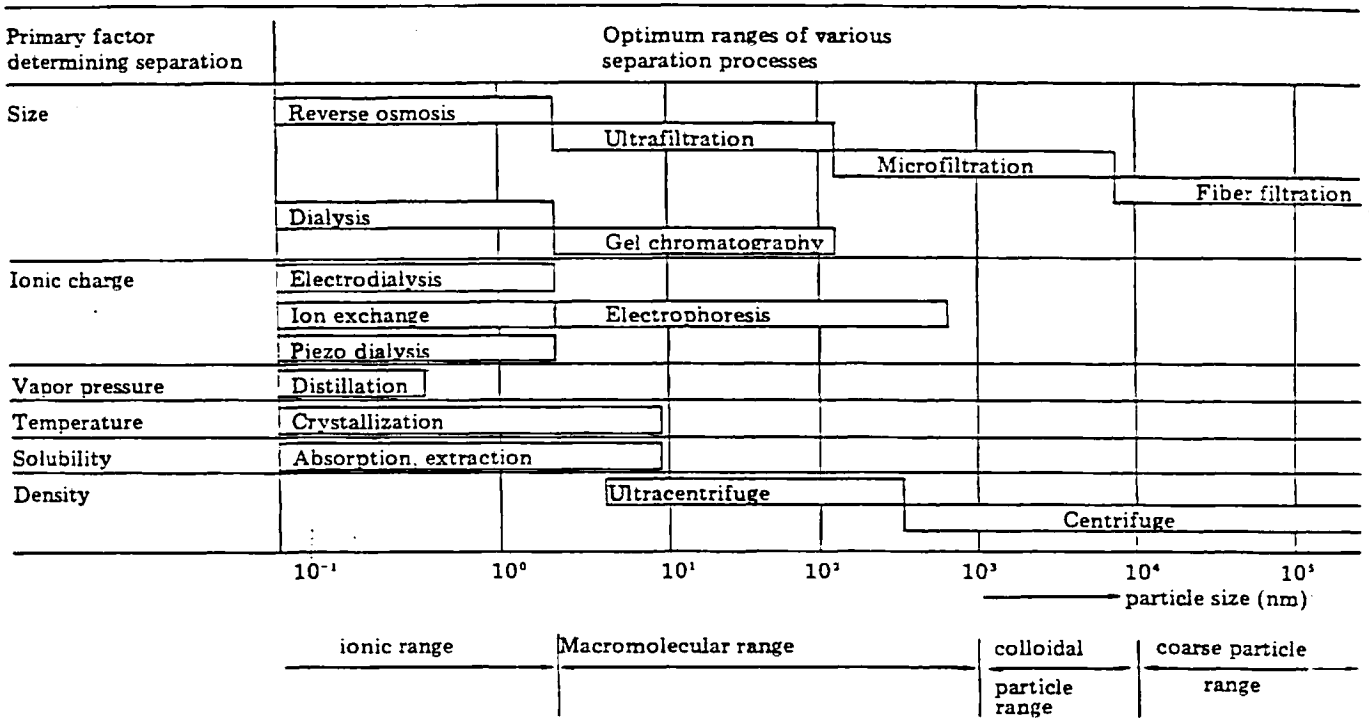
4.1 Bench Scale Apparatus

4.1.1 Ultrafiltration

Based on the advantages listed in Table 4, semi-permeable membrane systems were chosen to perform the required separations and concentrations. But what are the appropriate systems? Based on the fact that there is a 1.5 orders of magnitude difference in size between lactose and whey proteins (sections 2.1 and 3.1.1), Fig. 4 shows ultrafiltration is the appropriate membrane process to separate the whey proteins from the lactose and ash components.

Ultrafiltration (UF) of whey was performed by an Abcor (Wilmington, MA 01887) HFA-300-FEG ultrafilter shown in Fig. 5. It consisted of a 1.25 in. x 6 ft. tubular PVC housing which contained a 1 in. x 5 ft. tubular semi-permeable membrane. This 10,000 nominal molecular weight (NMW), polysulfone membrane was cast on a polyethylene support sheet and attached to the inside of a rigid, epoxy mesh, support tube. All plumbing consisted of PVC schedule 80 pipe, fittings and valves. Pumping requirements were provided by a Girton centripetal sanitary pump providing 50 gallons per minute (GPM) at 30 pounds per square inch (psi) head. A counter current flow, double stainless steel tube heat exchanger furnished heating and cooling requirements.

Figure 4 Useful Ranges of Various Separation Processes



SOURCE: Membrane Separation Processes, H. Strathmann, Journal of Membrane Science 9 (1981) p 123.

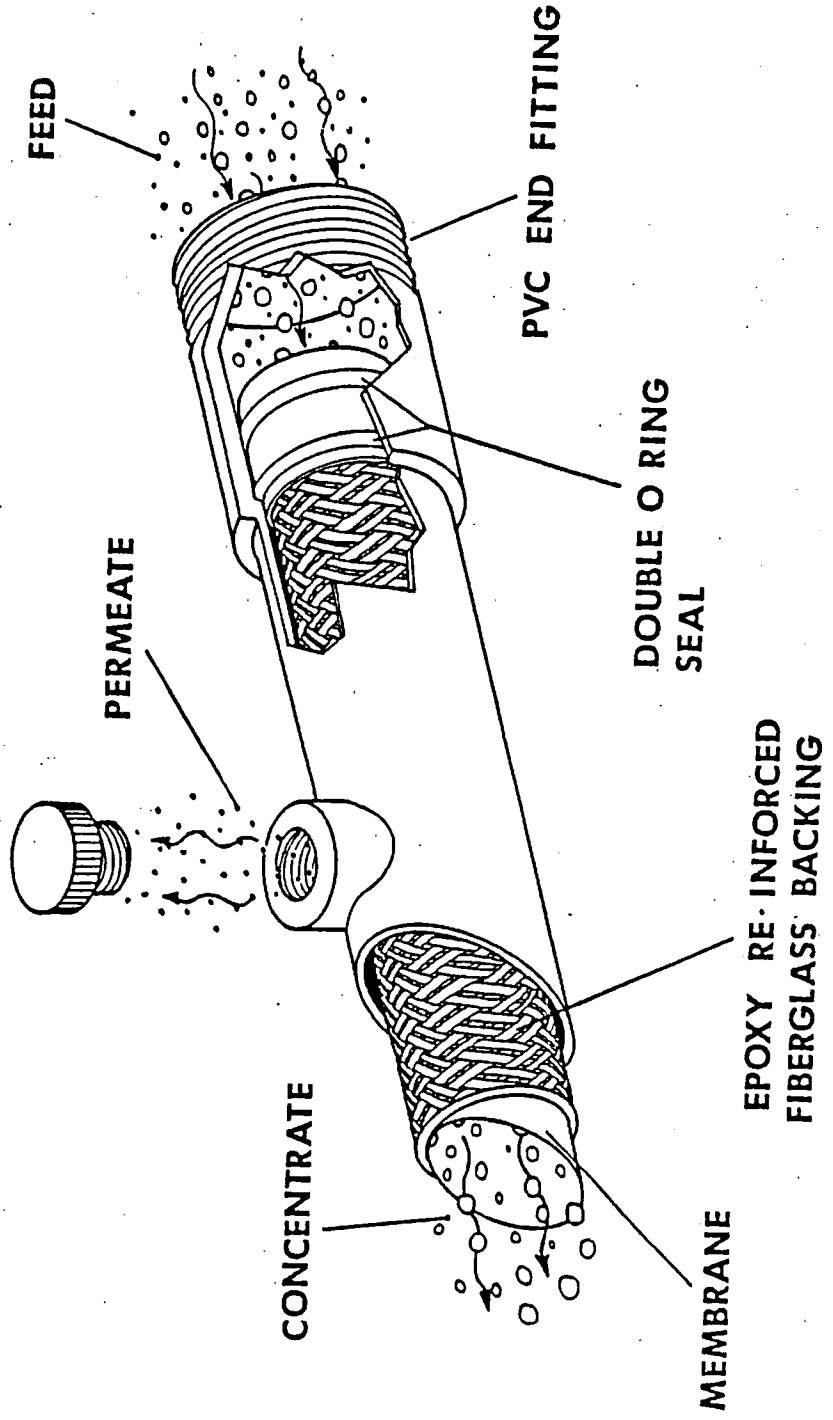


Figure 5

ABCOR HFA-300-FEG Ultrafiltration Membrane

The pressure indicator valve was of the oil-filled diaphram type. Flow rates were monitored by a rotameter. Both the ultrafiltration and reverse osmosis units shared a common 55 gallon, stainless steel feed tank, therefore sequential, batch-wise processing of whey by each system was necessary.

The following was the operating procedure for the ultrafiltration system (refer to Fig. 6):

1. Closed V2, UF inlet valve; opened V3 discharge valve; closed V7, UF/RO isolation valve; closed V4, feed tank/RO isolation valve; closed V1, feed tank/UF isolation valve; placed permeate line in feed tank;
2. Filled feed tank with whey, then opened V1 to allow liquid to gravity fill feed pump;
3. Started feed pump, then slowly open V2 to allow fluid to flow into ultrafilter;
4. When V2 was fully open, V3 was adjusted to regulate system pressure;
5. Adjusted process temperature with heat exchanger;
6. Upon reaching process temperature, removed permeate line from feed tank and placed in appropriate container to begin concentrating.

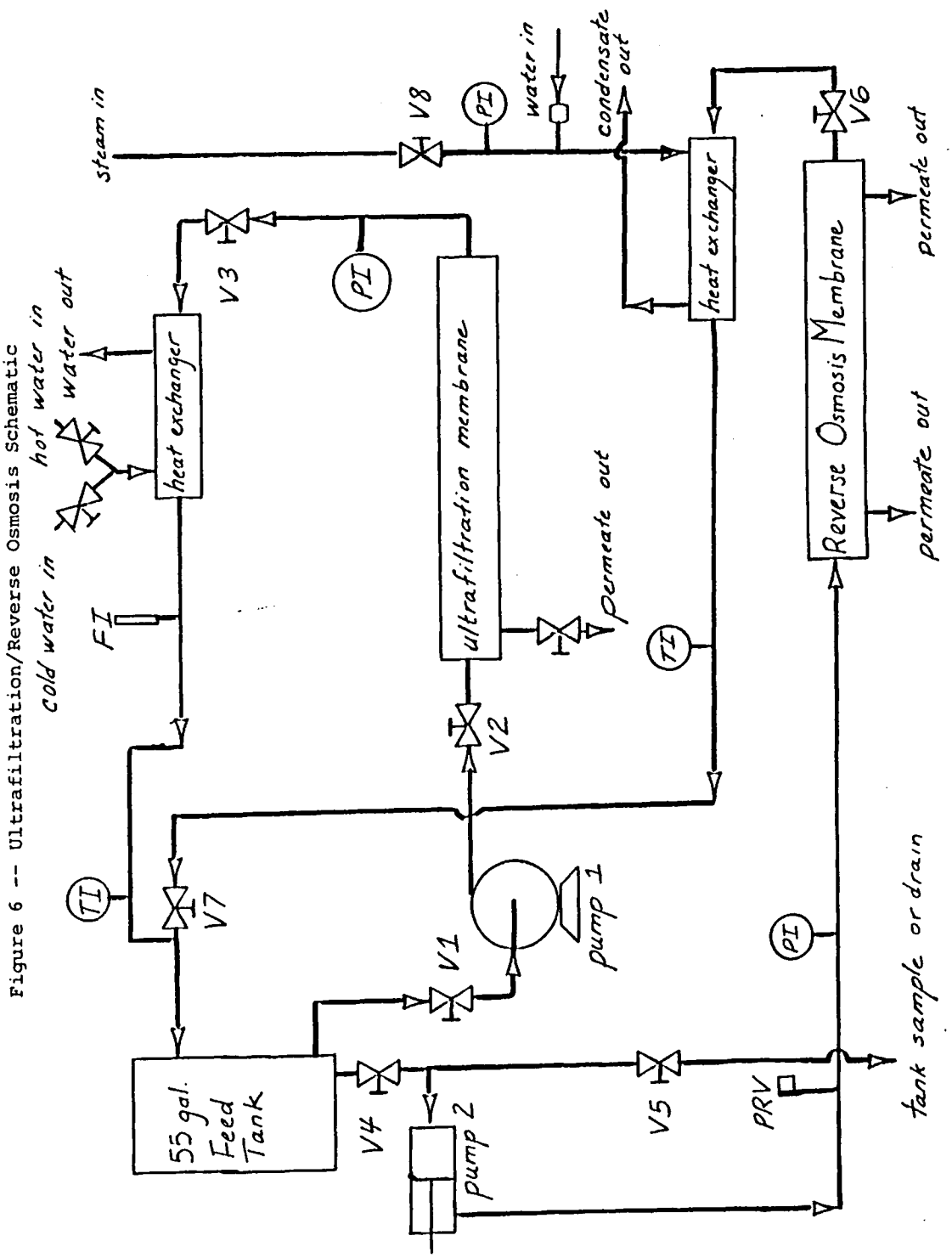


Figure 6 -- Ultrafiltration/Reverse Osmosis Schematic

During ultrafiltration, the whey protein concentrate was retained in the feed tank while the permeate was collected in large plastic bottles for further processing with reverse osmosis. After ultrafiltering to a desired concentration, the protein concentrate was drained (thru V4 and V5) and the feed tank was filled with an appropriate cleaning solution to clean-in-place (CIP) the ultrafiltration membrane. After cleaning and rinsing the system, the feed tank was filled with the previously collected whey permeate for reverse osmosis.

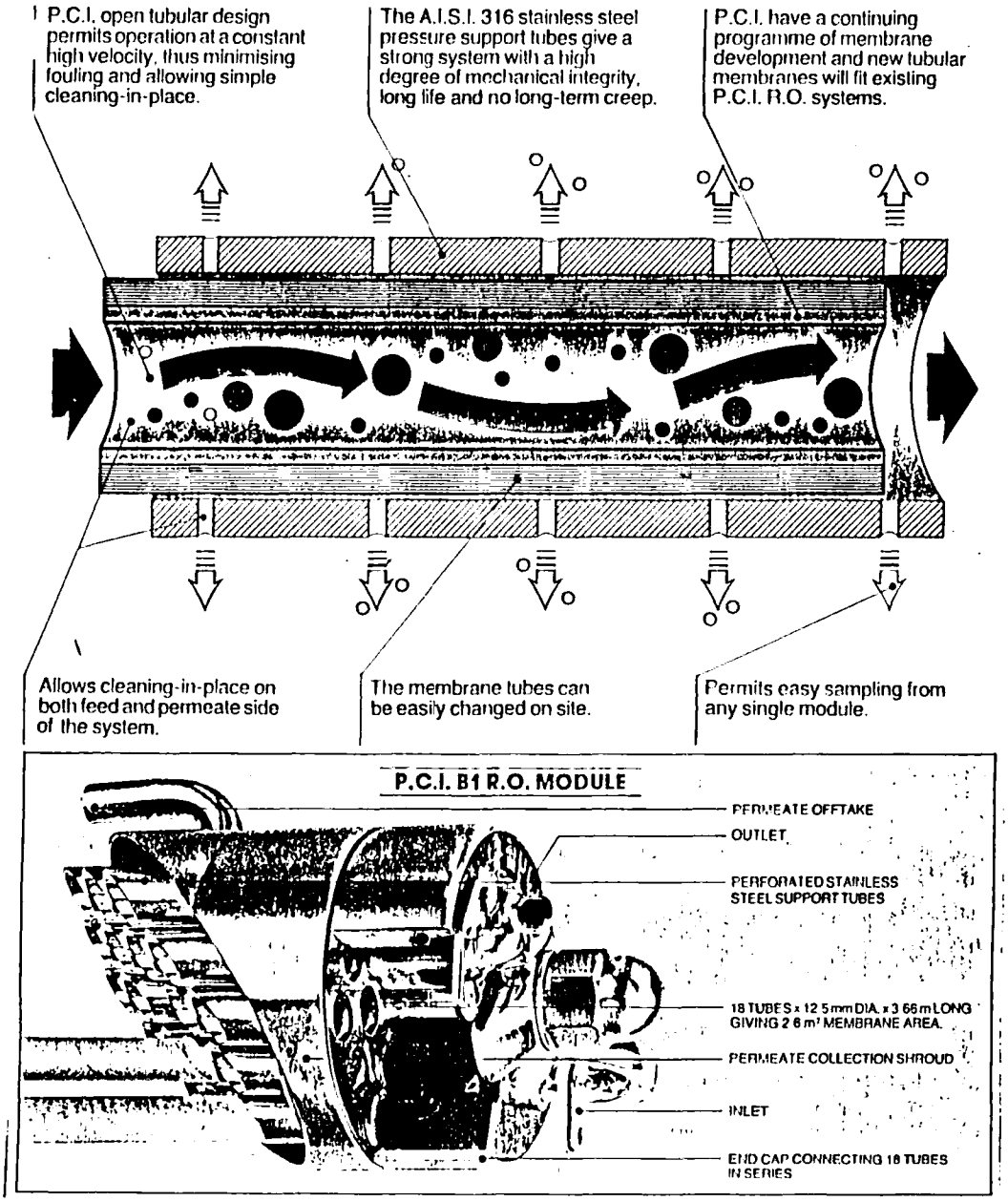
4.1.2 Reverse Osmosis

Reverse osmosis was chosen, because of the advantages listed in Table 4, to concentrate the whey permeate from ultrafiltration.

Reverse osmosis of whey permeate was performed by a PCI (Laverstoke Mill, Whitchurch, Hampshire R628 7NR, England) reverse osmosis unit, Fig. 7, membrane of 18 - 0.25 in. x 8.5 ft. polysulfone - acrylic co-polymer membrane tubes enclosed in an 8 in. x 9 ft. tubular stainless steel housing. High pressure fluid entered the housing end cap and flowed down within nine of the eighteen tubes, turned around in the far end cap, then flowed back inside the other nine tubes, and finally exited at the same end that it entered. Pumping was provided by a Cat brand triplex, positive displacement pump (model 430) capable of 5 GPM at 1000 psi head. An in-line, stainless steel, double tube heat exchanger controlled process temperature.

Figure 7

PCI Tubular Reverse Osmosis System



The reverse osmosis (RO) operation procedure was as follows (Fig. 6):

1. Closed V4, opened V6, opened V7, placed permeate line to drain;
2. With feed tank full, opened V4 to gravity feed into pump;
3. After turning pump on, closed down on V6 until desired pressure reached;
4. Adjusted process temperature with heat exchanger.

A rise in the UF return line prevented the RO retentate from entering the UF system, hence saving the cost of a valve.

The reverse osmosis unit was operated until the whey permeate was concentrated to desired level. (For example, if a four fold (4x) increase in lactose concentration was desired, the RO unit was operated until the retentate volume was one-fourth the volume prior to concentration assuming no loss of lactose in the RO permeate.) RO permeate, essentially pure water, was sent to drain.

A photograph of the ultrafiltration/reverse osmosis system is shown in Fig. 8.

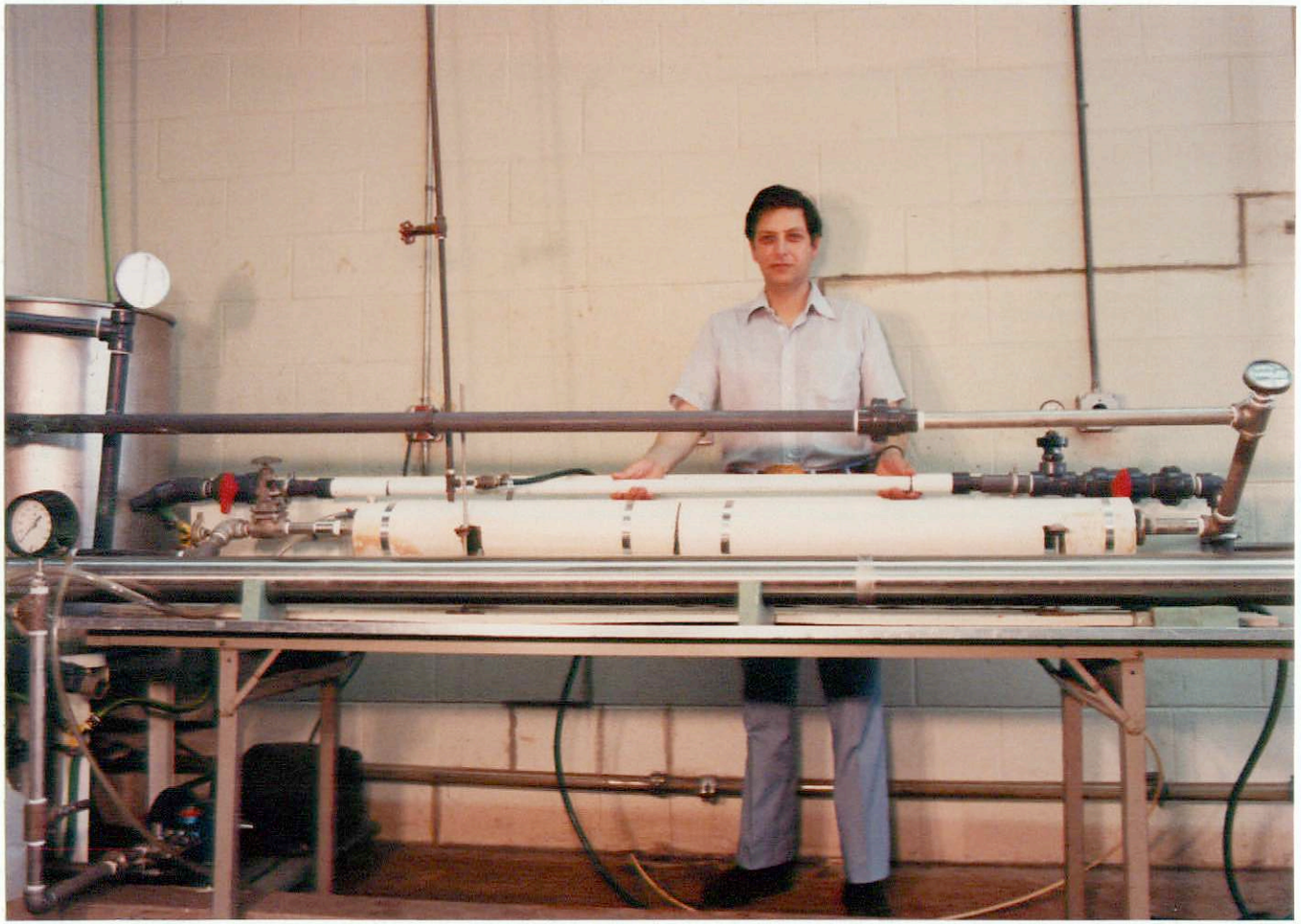


FIGURE 8 - ULTRAFILTRATION/REVERSE OSMOSIS EXPERIMENTAL APPARATUS

4.1.3 Shake flask fermentor

All free and immobilized cell shake flask fermentations were performed within 250 milliliter (ml) Erlenmeyer flasks. These flasks were capped with specially designed stoppers fitted with 0.2 micron bacteria filters to allow sterile air entry during the initial aerobic cell growth and to allow the aseptic passage of CO_2 out of the flask during alcohol fermentation. To achieve anaerobiosis, one end of a length of flexible plastic tubing was attached to a bacterial filter, as shown in Figure 9, and the tubing's free end was placed in a water trap.

4.1.4 Immobilized Cell Packed Bed Bioreactor

Due to the overwhelming process benefits listed in Table 5, a continuous immobilized yeast system was chosed for this project.

The bioreactor illustrated in Fig. 10 was constructed to perform continuous immobilized cell alcohol fermentation. The reactor body was fabricated with three-eighths inch thick acrylic tubing and sheetstock, with stainless steel fittings and sealed with double "O" rings. All tubing to and from the reactor was autoclavable silicon.

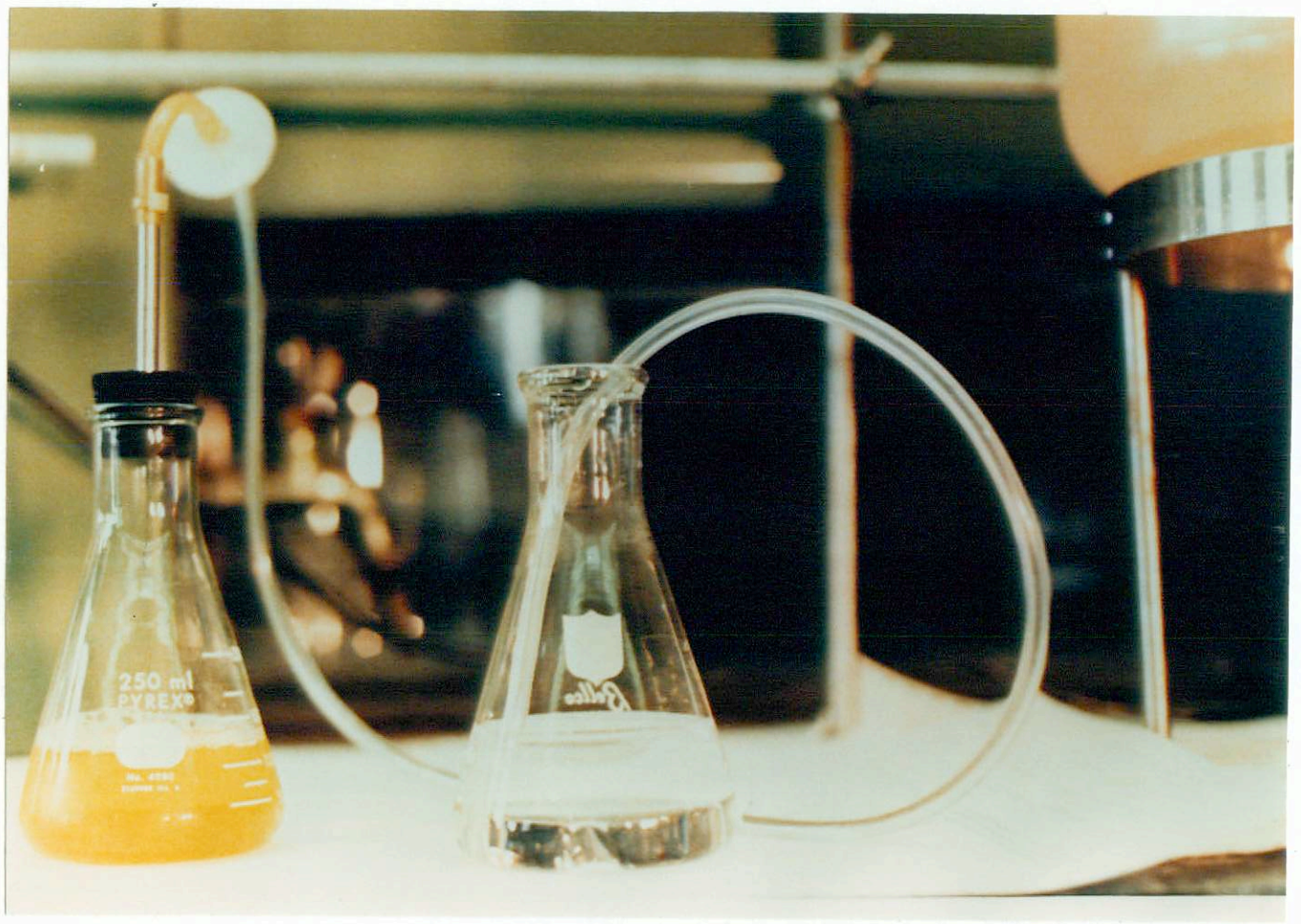
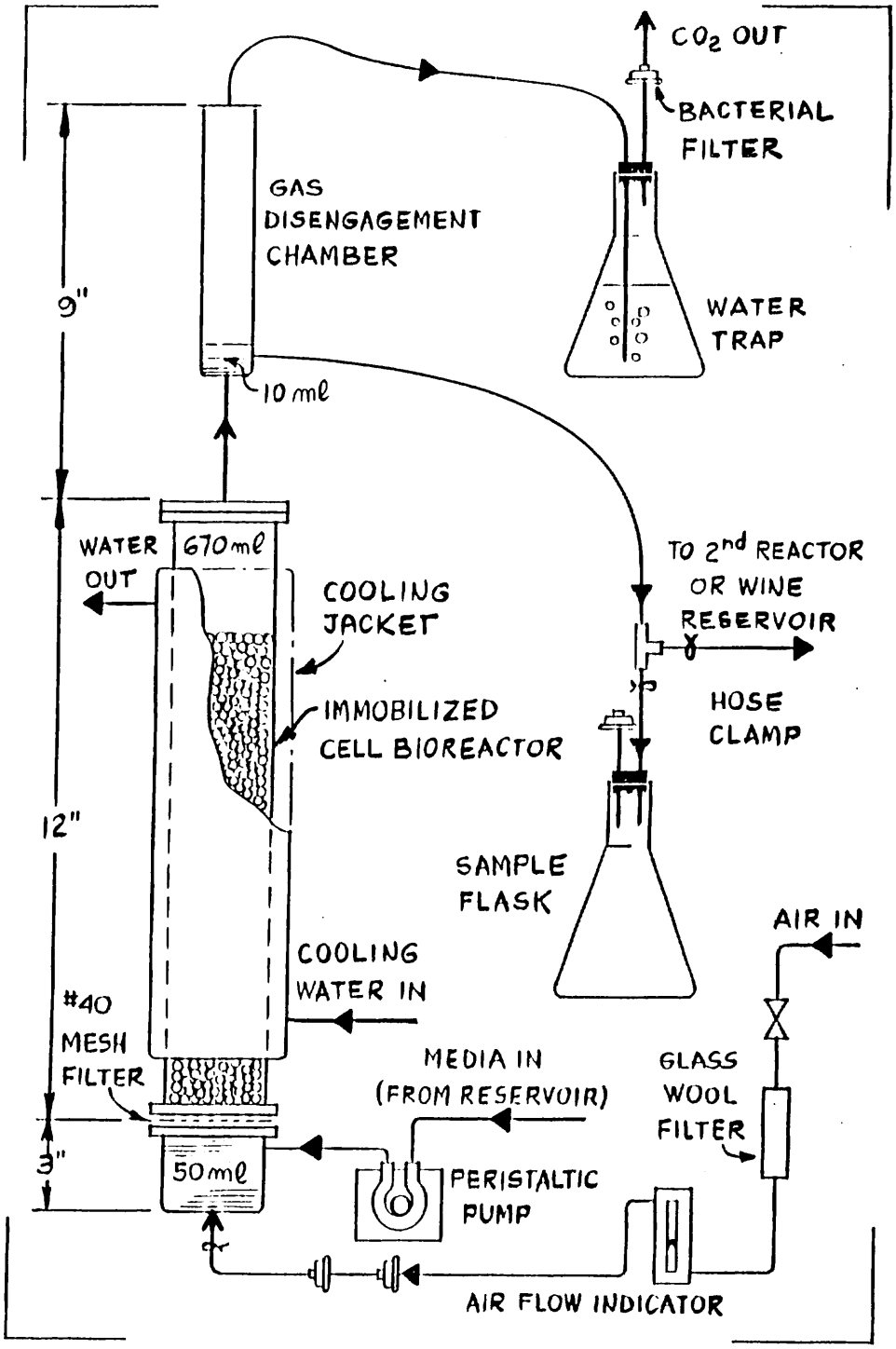


FIGURE 9 - SHAKE FLASK FERMENTOR WITH WATER TRAP

Figure 10 -- Immobilized Cell Packed Bed Bioreactor



The bioreactor contained three sections: 1) a distribution chamber, 2) the packed bed chamber and 3) the gas disengagement chamber. To provide equal distribution of fresh media and to minimize media channeling thru the bed, fresh media entered the side of a small distribution chamber at the bottom of the reactor. Media moved in an upflow manner through the bed support screen and into the bed. To allow for bead expansion during cell growth, 70% of the main chamber height was consumed by the packed bed. Reactor effluent and off-gas passed thru the bead retaining screen at the top of the bed chamber and into the gas disengagement chamber where phase disengagement occurred. After leaving the disengagement chamber, off-gas passed into the water trap which maintained complete anearobic conditions. Fermentor effluent exited the bottom of the gas disengagement chamber and was pumped into the following reactor's distribution chamber or into the sample flasks. Whey wine from the second reactor entered an associated sample flask or was stored in a wine resevoir.

The photograph in Fig. 11 shows the entire experimental fermentation apparatus. Measurements of media flow rate and aeration flow rate were provided by an inline pipet and rotameter respectively. Pumping requirements were supplied by a Cole-Parmer peristatic pump.

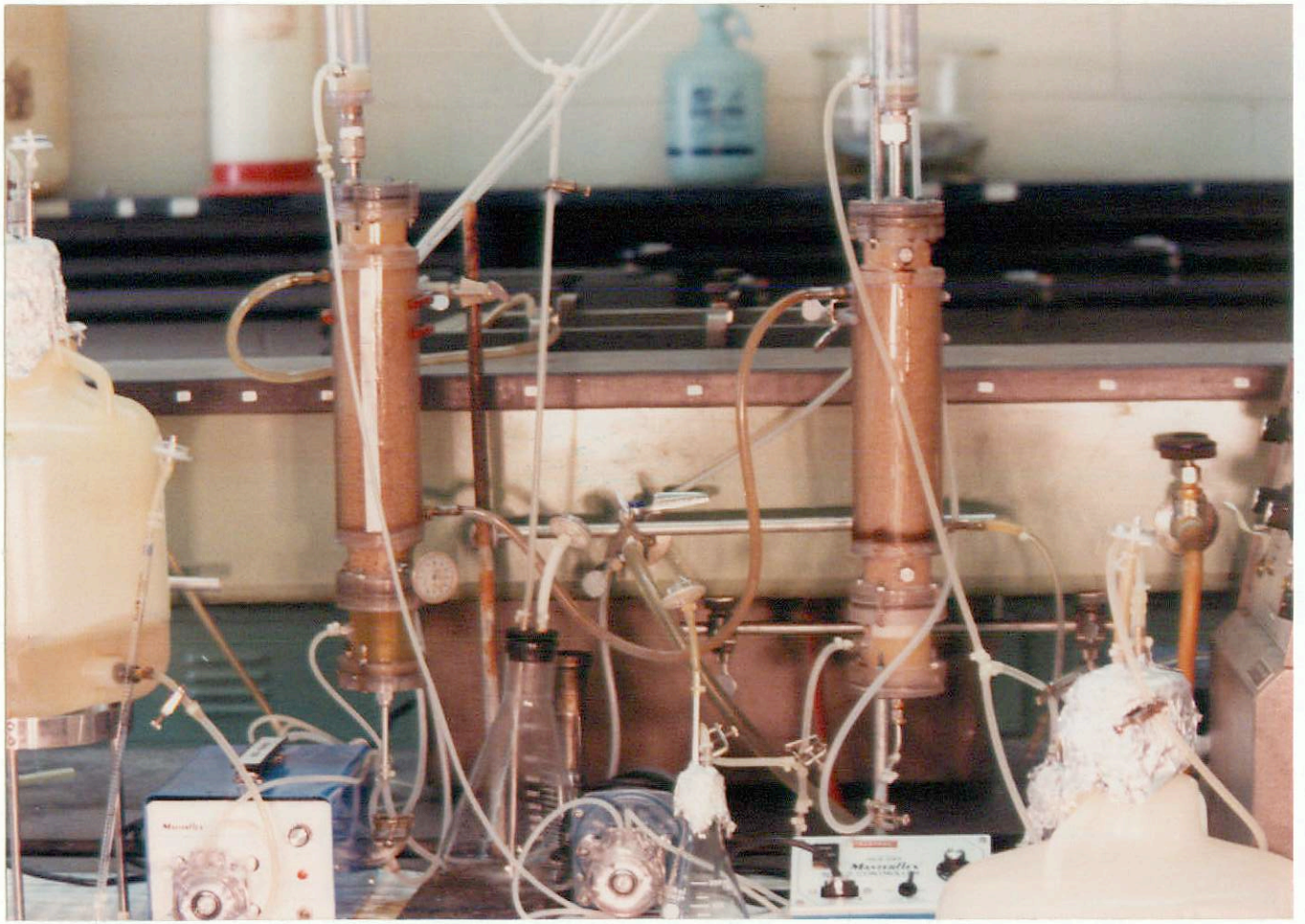


FIGURE 11 - CONTINUOUS FERMENTOR WITH WATER TRAP

4.2 Alginate Immobilization Procedure

To utilize the expertise at Rutgers University, Department of Chemical and Biochemical Engineering, entrapment was the immobilization method of choise. Alginate was chosen as the entrapment matrix because it retained high cell enzyme activity and is resistant to cell disruption by CO_2 generation within, as listed in Table 6.

Fermentation broth was harvested after twenty-four hours of growth, centrifuged, washed with physiological saline, centrifuged again, and finally re-suspended in 200 ml of sterile saline. This yeast concentrate was added to 800 ml of immobilization alginate solution, section 3.3.6, and mixed well with a magnetic stirrer. Final concentration of alginate and silica was 1 and 2 percent (w/v) respectively. As soon as complete mixing occurred, a peristaltic pump transferred the above solution into the immobilization flask containing approximately 600 ml of well-mixed gelling media (section 3.3.5). In order to create small diameter alginate beads, the yeast/alginate liquid was pumped through a narrow bore syringe needle positioned slightly above the level of media. Upon completion of pumping, beads were allowed to sit 30 minutes in media to insure complete gelling. Beads were "aseptically" transferred into a fermentation vessel.

Whey permeate concentrate contains a significant quantity of calcium (section 3.1.1). The presence of this divalent ion causes liquid solutions of the sodium salt of alginate to gel. A disadvantage of this gelling is that the yeast must be centrifuged and washed prior to addition to an alginate solution. However, a distinct advantage of this calcium content in the whey permeate fermentation medium is that it maintained bead integrity throughout a long fermentation.

4.3 Fermentation Procedure

4.3.1 Shake flask fermentation

Shake flasks were filled with 133 ml of fermentation media, autoclaved, and inoculated with 7 ml (5% inoculum) of fermentation broth containing yeast grown aerobically for twenty-four hours or with 15 ml (10% innoculum) of immobilized yeast alginate beads. Following inoculation, flasks were shaken aerobically at 200 RPM, 30^{o}C for two hours to stimulate cell growth. Water traps were subsequently fitted at the start of alcohol fermentation and flasks were maintained at 30^{o}C shaken at 200 RPM for ten minutes, three times a day throughout each fermentation, flasks were sacrificed and the contents analyzed.

4.3.2 Continuous Fermentations

The packed bed fermentor was operated in an upflow mode. The bed was periodically aerated to increase viability of immobilized cells. Operation at very high dilution rates and with 3.5 pH media eliminated occasional contamination.

Experimental parameters varied were media concentration, media type, micro-aeration rate and dilution rate. Reactor effluent was collected as needed in sample flasks and analyzed.

CHAPTER 5

RESULTS AND DISCUSSION

5.1 Ultrafiltration

5.1.1 Flux Performance

One of the difficulties with ultrafiltration of whey is concentration polarization which occurred at and near the membrane surface. "However, in order to have a finite flux with these systems, one must tolerate a finite amount of concentration polarization, a term which refers to the increase in the concentration of rejected species (whey proteins and fat) with decreasing distance from the membrane" [59]. Concentration polarization led to the build up of a gel layer on the membrane surface. This layer became a dynamic "membrane" which offered additional hydraulic resistance to solvent flux. Flux is the amount of permeate that passes through the membrane per unit membrane area per unit time; in this research, flux is measured as gallons per square foot per day (GFD). According to Howell and Velicangil [60], the concentration equation describes concentration polarization mechanism as

$$\frac{\partial C}{\partial t} = D \frac{\partial^2 C}{\partial t^2} - J \frac{\partial C}{\partial x} \qquad (1)$$

with the following boundary conditions:

$$t = 0 , \quad C = C_b$$

$$x = 0 , \quad C = C_b$$

$$x = L , \quad \frac{\partial C}{\partial x} = JC$$

where D = diffusivity of proteins

C = solute concentration

C_b = solute concentration in the bulk solution

t = time

J = flux

x = distance

L = distance x at membrane surface

The solution of Hamell and Velicangil is as follows:

$$\frac{C_w}{Cb} = e^{JL/D} + \sum a_n e^{-\lambda_n} t_{xn} \left(\frac{\lambda^2_n D}{u^2} \quad \frac{JL}{D} \right) \qquad (2)$$

where λ_n = eigenvalues

χ_n = eigen functions

C_w = concentration at the wall

This solution, at the membrane surface where X = L, predicts concentration polarization to occur within the first 5 seconds. Howell and Velicangil also postulate that the high fluid velocity at the membrane surface into the pores results in concentration polarization at the pore surface within one second after start-up. From these two predictions, concentration polarization occurred so rapidly that its effect cannot be measured by taking membrane flux readings.

Soon after concentration polarization occurred at and near the surface of the membrane, the proteinaceous gel layer developed, aided by protein absorption and protein-protein interactions. The initial rapid flux decline in Fig. 12A was due to the increasing thickness of the gel layer. The term concentration ratio in Fig. 12A and other graphs refers to the ratio of the initial feed volume divided by the retentate volume. For example, if 30 gallons of whey was placed into the feed tank prior to ultrafiltration, and during ultrafiltration 10 gallons of retentate (whey protein concentrate) remains (at a particular point in time), the concentration is 30/10 = 3, also known as 3X (3 times) concentration. Klinkowski [61] reports that transport of the retained species (whey proteins) to the gel-polarization is

$$J_i = JC_{bi} \qquad (3)$$

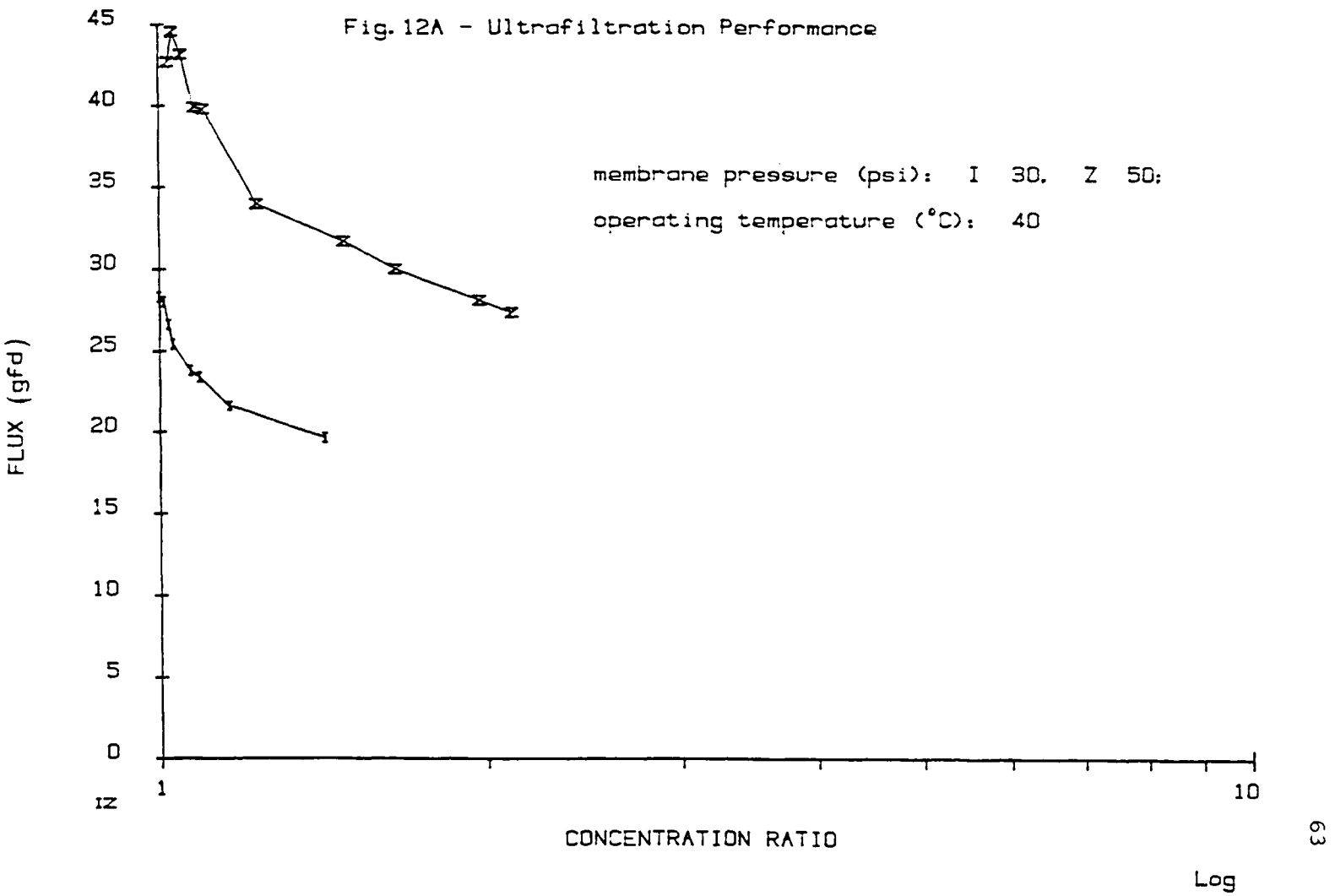


Fig. 12A - Ultrafiltration Performance
membrane pressure (psi): I 30. Z 50:
operating temperature (°C): 40
FLUX (gfd)
45
40
35
30
25
20
15
10
5
0
IZ
1
10
CONCENTRATION RATIO
Log

However, according to Fick's law, diffusion back into the bulk solution is

$$J_i = K \frac{dCi}{dx} \quad (4)$$

where K = mass transfer coefficient

C = concentration of retained species i

and at steady state

$$J\ C_b = K \frac{dC}{dx} \quad (5)$$

The thickness of the gel layer stabilizes at a constant concentration (C_b) with respect to time (found in continuous systems). Flux declines slow and linear due to increasing hydraulic resistance from increasing whey proteins (C_b) when operated in a batch mode.

Integration of equation 5 gives

$$J = K\ \ln\ (C_g/C_b) \quad (6)$$

where C_g = concentration of the retained species in the gel

Usually there is little agreement between analytically derived K and data. Consequently, flux versus log C_b was plotted in Fig. 12A and this curve extrapolated to zero flux obtaining C_g. The slope of this line was K. The various hydraulic pressure operating conditions in these experiments produced different values for K.

$$J = \frac{km \quad P}{\mu} \tag{7}$$

and

$$\Delta P = P_r - P_p \tag{8}$$

where μ = bulk fluid viscosity

P_r = hydraulic pressure of retentate

P_p = hydraulic pressure of permeate

K_m = membrane hydraulic permeability; K_m a function of pore size, tortuosity, length and membrane compaction

However equation 6 states that flux is independent of pressure. Therefore, as the hydraulic pressure increases, the gel hudraulic permeability, K_g, must decrease.

We now find

$$J = \frac{\Delta P}{\mu (1/k_m + 1/k_g)} = \frac{\Delta P}{\mu (R_m + R_g)} \quad (9)$$

where R_m = hydraulic resistance of the membrane

R_g = hydraulic resistance of the gel

K_g = gel hydraulic permeability

$$P_r = \frac{P_i + P_o}{2} \quad (10)$$

where P_i = inlet hydraulic pressure into membrane module

P_o = outlet hydraulic pressure from membrane module

For this experimental set up only P_o was recorded from the pressure indicator gauge. P_i is found by

$$P_i = P_o + \Delta P_f \quad (11)$$

where ΔP_f = hydraulic pressure drop across the membrane due to flow Q (obtained from manufacturer).

The experimental apparatus varied Q, and hence fluid velocity, when operating pressure were varied, (see Table 12). Since K is a function of fluid velocity and fluic velocity is a function of ΔP_f, to comparably equate the two operating conditions, fluxes were normalized. We obtain the following equation:

$$\frac{J}{\Delta P} = \frac{1}{\mu (R_m + R_g)} \tag{12}$$

These values are plotted against log concentration ratio in Fig. 12B. The fluxes for the two operating pressure are now very close indeed, thereby confirming equation 6. Since μ and R_m were unchanged between tests and $R_g \gg R_m$

$$\frac{J_1/\Delta P_1}{J_2/\Delta P_2} = \frac{R_{g_2}}{R_{g_1}} \tag{13}$$

from Fig. 12B, $R_{g2}/R_{g1} \approx 1.0$.

TABLE 12

Ultrafiltration Flow Data

Run #	P_o (psi)	Q (GPM)	P_f (psi)	P_i (psi)	P_r (psi)	P_p (psi)	ΔP (psi)
1	50	18	1	51	50.5	0	50.5
2	30	44	5	35	32.5	0	32.5

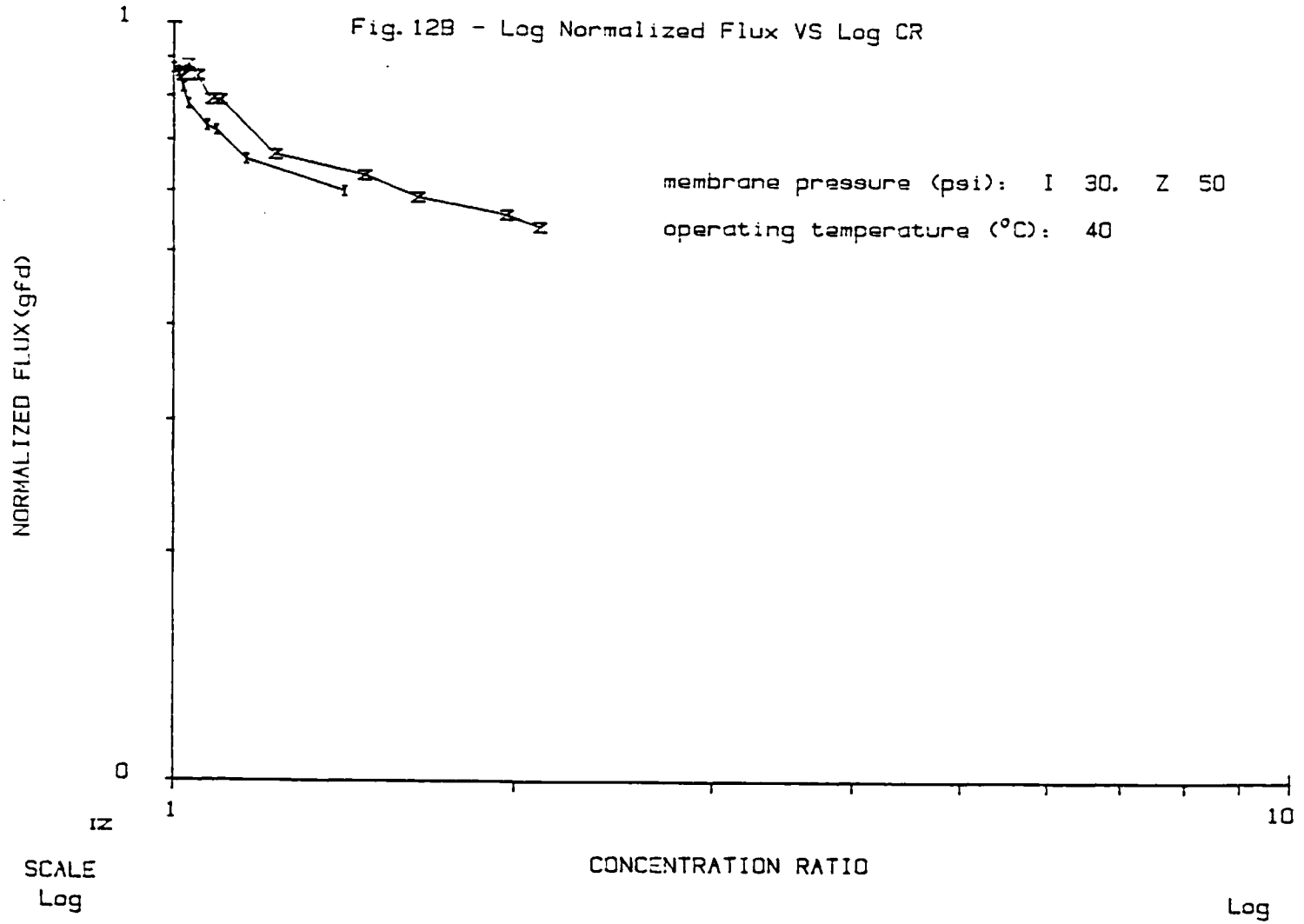


Fig. 12B - Log Normalized Flux VS Log CR
membrane pressure (psi): I 30. Z 50
operating temperature (°C): 40
NORMALIZED FLUX (gfd)
1
0
IZ
SCALE
Log
1
10
CONCENTRATION RATIO
Log

Both equation 4 and 8 predict $R_{g1} > R_{g2}$. In equation 4, the mass transfer coefficient is directly proportional to fluid velocity (or turbulence), thereby increasing back diffusion of material from the gel layer into the bulk. The thickness of the gel layer is reduced because

$$R_g = l/D \tag{14}$$

where l = thickness

D = diffusivity of proteins in whey

R_g is reduced. In addition, equation 8 tells us that as ΔP increases, R_g must increase if equation 6 is correct. Therefore, the fact that the gel resistivities are nearly identical indicates that within the pressure regime and the flow regime tested, the gel layer was not affected by the flow rate or operating pressure. Another mechanism can be occurring independently. If the gel-polarization layer is thixotropic, then the high shear in run 2 creates high viscosity situation at the gel-polarization layer, reducing flux at a constant R_g.

5.1.2 Rejection Performance

Analysis of the major components in whey was performed during ultrafiltration to determine the effectiveness of operation and to explore the partitioning of components during concentration. Total Kjeldahl Nitrogen (TKN), lactose, ash and total solids concentrations in both retentate and permeate were measured. The results are shown in Fig. 13-15 with least square linear regressions drawn thru data points.

To determine ultrafiltration effectiveness as a tool in separating and concentrating whey protein, protein levels were monitored throughout several runs. Protein concentration was derived from TKN concentration by the following formula: TKN - NPN = CKN (Corrected Kjeldahl Nitrogen) or true protein. The value for NPN was assumed to be equal to the TKN values in the permeate during initial concentration when whey protein levels in the retentate were low.

Fig. 13 contains a graph of TKN in both permeate and retentate streams during concentration. Initial levels of TKN in the permeate was approximately 0.15%, a value which corresponds closely to the accepted NPN level in Table 8. Initial levels of TKN in the retentate was 0.75% which resulted in a 0.60% trace protein concentration. If 100% of the whey protein was retained by the membrane during concentration, a 2 fold volumetric concentration of whey would have resulted in a 2 fold protein concentration increase in the retentate. However, Table 13 indicates

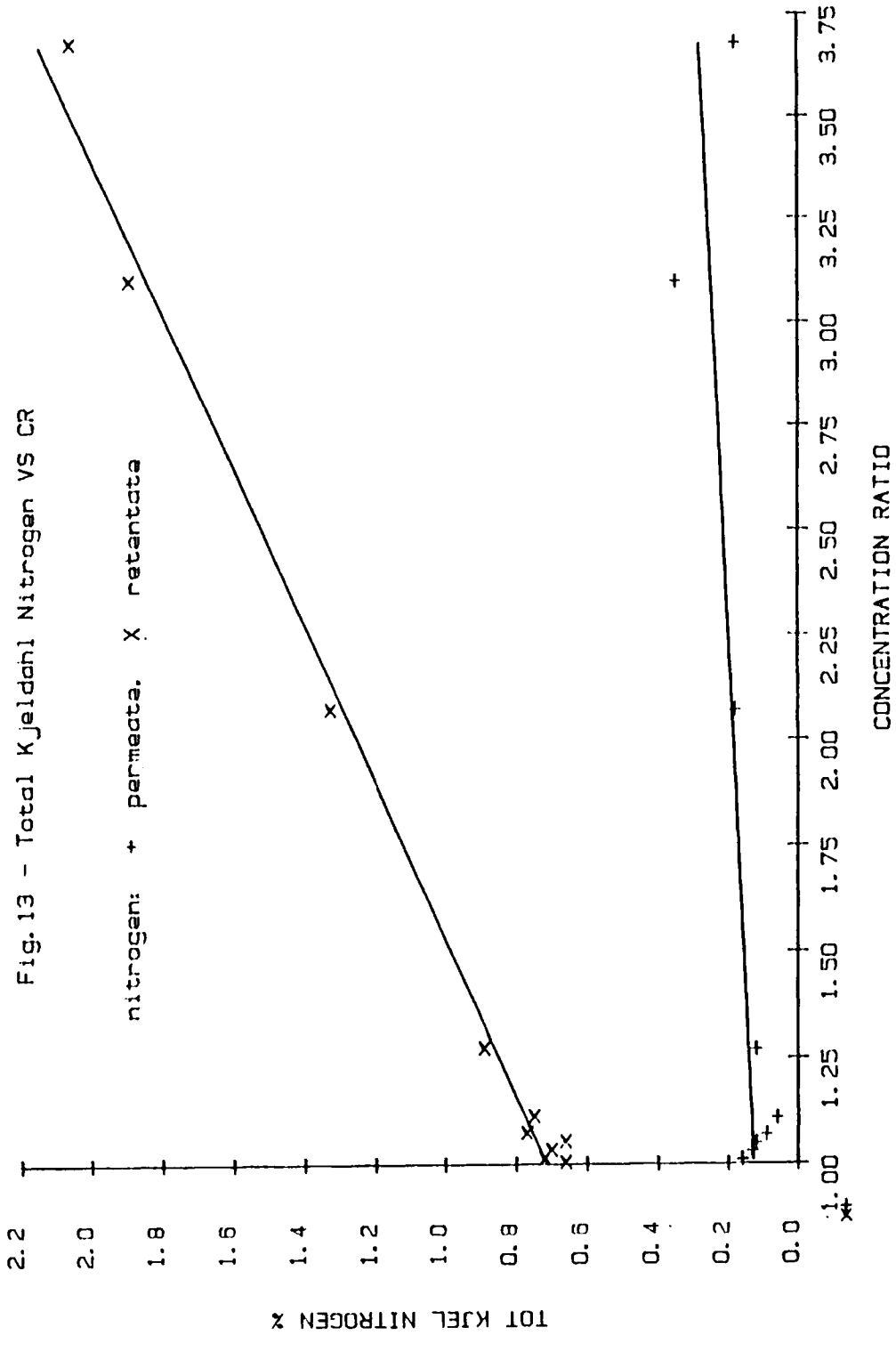


Fig.13 - Total Kjeldahl Nitrogen VS CR
nitrogen: + permeate, X retantate
TOT KJEL NITROGEN %
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1.00
1.25
1.50
1.75
2.00
2.25
2.50
2.75
3.00
3.25
3.50
3.75
CONCENTRATION RATIO

indicates only a 92% concentration efficiency during ultrafiltration. The gel layer of whey proteins deposited on the membrane surface contained some of the balance of whey protein, while protein loss through the membrane accounted for the remainder of protein loss. Following an immediate loss of retentate protein gel layer, a steady state equilibrium existed between protein molecules transported to the gel layer by flow (JC_b) and protein transported away from the gel layer by bulk diffusion ($K \times dC/dX$). This phenomenon stated in equation 5 resulted in a steady concentration efficiency value throughout ultrafiltration.

It should be noted that as whey became increasingly concentrated, the amount of "non-permeable" nitrogenous compounds, true protein increased slightly in the permeate. As the concentration of true protein increased in the retentate, the probability of true protein passing through the pores and into the permeate increased.

Permeate lactose levels declined slightly during ultrafiltration as Fig. 14 indicates. In addition, lactose concentration in the retentate increased slightly during whey concentration. This slight partitioning indicated that this small molecule is not 100% permeable as would be expected in a 10,000 NMW membrane. It is assumed that lactose-protein binding or, more importantly, a dynamic "membrane" that is significantly "tighter" than the underlying polysulfone membrane may be the cause of increasing retentate lactose levels during ultrafiltration. This dynamic "membrane" has reverse osmosis-like qualities, therefore partially rejecting the lactose during concentration.

TABLE 13

Whey Protein Concentration during Ultrafiltration

Whey Concentration	TKN (%)	CKN (%)	Efficiency (%)
1X	0.75	0.60	--
2X	1.25	1.10	92
3X	1.80	1.65	92
3.5X	2.05	1.90	90

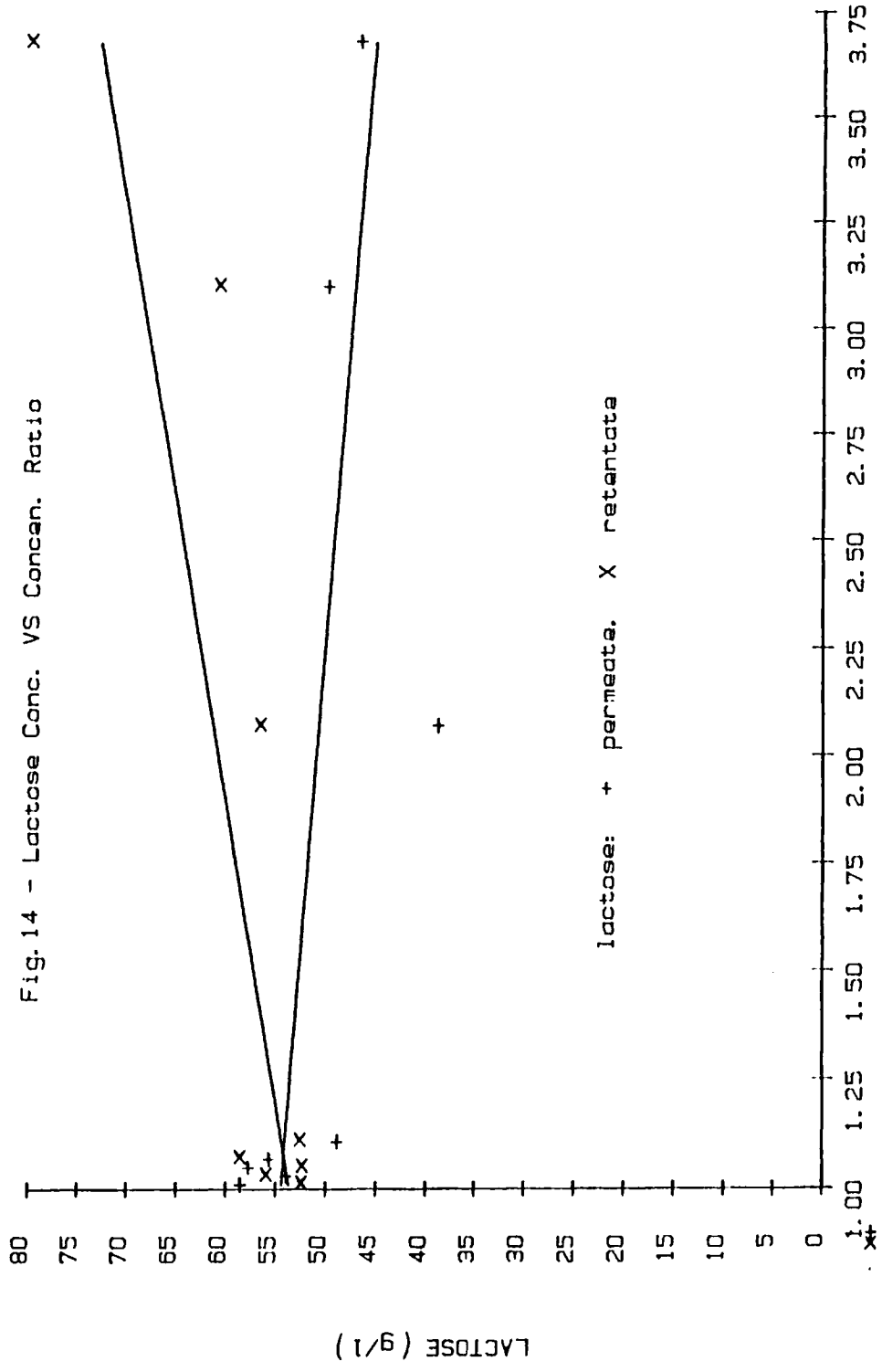


Fig. 14 - Lactose Conc. VS Concan. Ratio
lactose: + permeate. X retantate
LACTOSE (g/l)
CONCENTRATION RATIO
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
1.00
1.25
1.50
1.75
2.00
2.25
2.50
2.75
3.00
3.25
3.50
3.75

Ash did not exhibit the slight partitioning that was observed for lactose. Ash levels in permeate and retentate, Fig. 15, differed insignificantly and these levels did not change while concentrating. Unlike the large lactose molecule, the very small size of ionized salts, that comprised the ash fraction were unaffected by molecular occlusion of the "tighter" dynamic "membrane".

Total solids in the retentate increased as expected due to increased protein, fat and, to a minor extent, lactose concentrations. Total solids in permeate rose very slightly during concentration due to protein transmission.

If highly purified whey protein concentrate solids are desired, diafiltration of the whey protein concentrate is required at a predetermined point in ultrafiltration. In diafiltration, water is added to the retentate at the same rate as the permeate is removed. This procedure has the same effect as washing a filter cake; therefore the lactose and salts (permeable solids) are washed out of the concentrate and the whey protein becomes a greater percentage of the total solids in the retentate. The end result is a high protein (approximately 75%) whey protein concentrate.

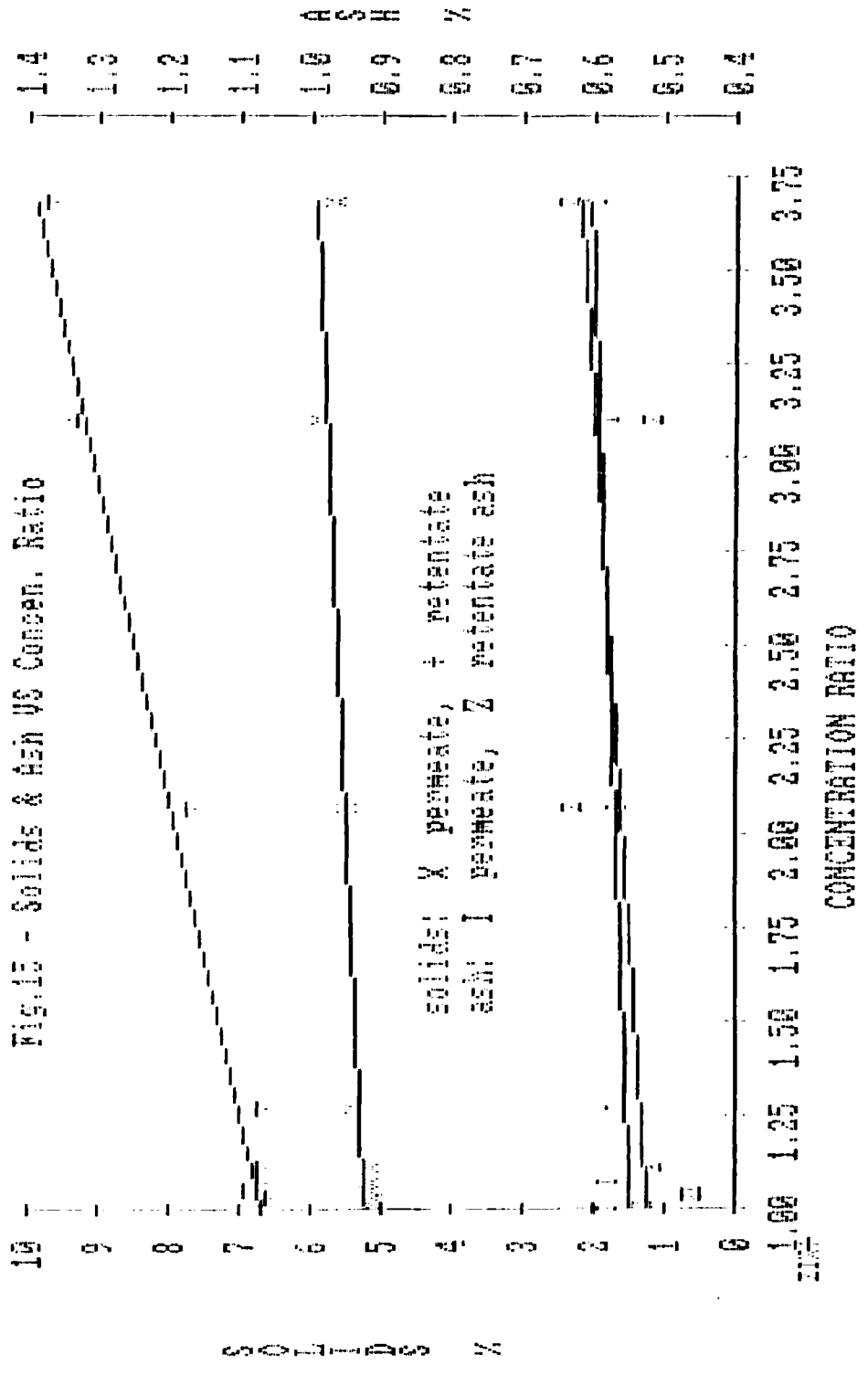


Fig.15 - Solids & Ash VS Concen. Ratio
Solids: X permeate, + retentate
Ash: I permeate, Z retentate ash
SOLIDS %
ASH %
CONCENTRATION RATIO
1.00 1.25 1.50 1.75 2.00 2.25 2.50 2.75 3.00 3.25 3.50 3.75
0 1 2 3 4 5 6 7 8 9 10
6.4 6.5 6.6 6.7 6.8 6.9 1.0 1.1 1.2 1.3 1.4

5.2 Ethanol Fermentation

5.2.1 Strain Choice

Of primary importance, in the choice of the best microbe for a particular fermentation, is the organism's ability to produce the desired product rapidly and with high conversion efficiency. Furthermore, in the case of ethanol production, the yeast's capability to exhibit high product concentrations in the final broth will have a marked effect on lowering the cost of distillation. Based on the premise that a great microbe produces a good fermentation, shake flask fermentations were performed to identify the most suitable of the two yeasts selected in section 2.6 to produce alcohol from whey permeate. Free cell studies were utilized in the strain choice determination to minimize "softening" of substrate and produce inhibition effects that immobilization has on cells, therefore maximizing the difference in performance between strains.

Volumetric and specific productivities displayed in Fig. 16 and Fig. 17 respectively indicated a significant difference between the two yeasts tested. C. pseudotropicalis demonstrated to be the more prolific ethanol producer in these fermentations. Table 14 lists kinetic parameters for both yeast-fermented anaerobically in shake flasks on 1x whey permeate. The maximum ethanol concentration in the final broth was nearly identical for both yeasts. Ethanol yield and relative ethanol yield were slightly higher, but not especially significant, with Candida. However, these values for both yeasts were low relative to what would be desirable.

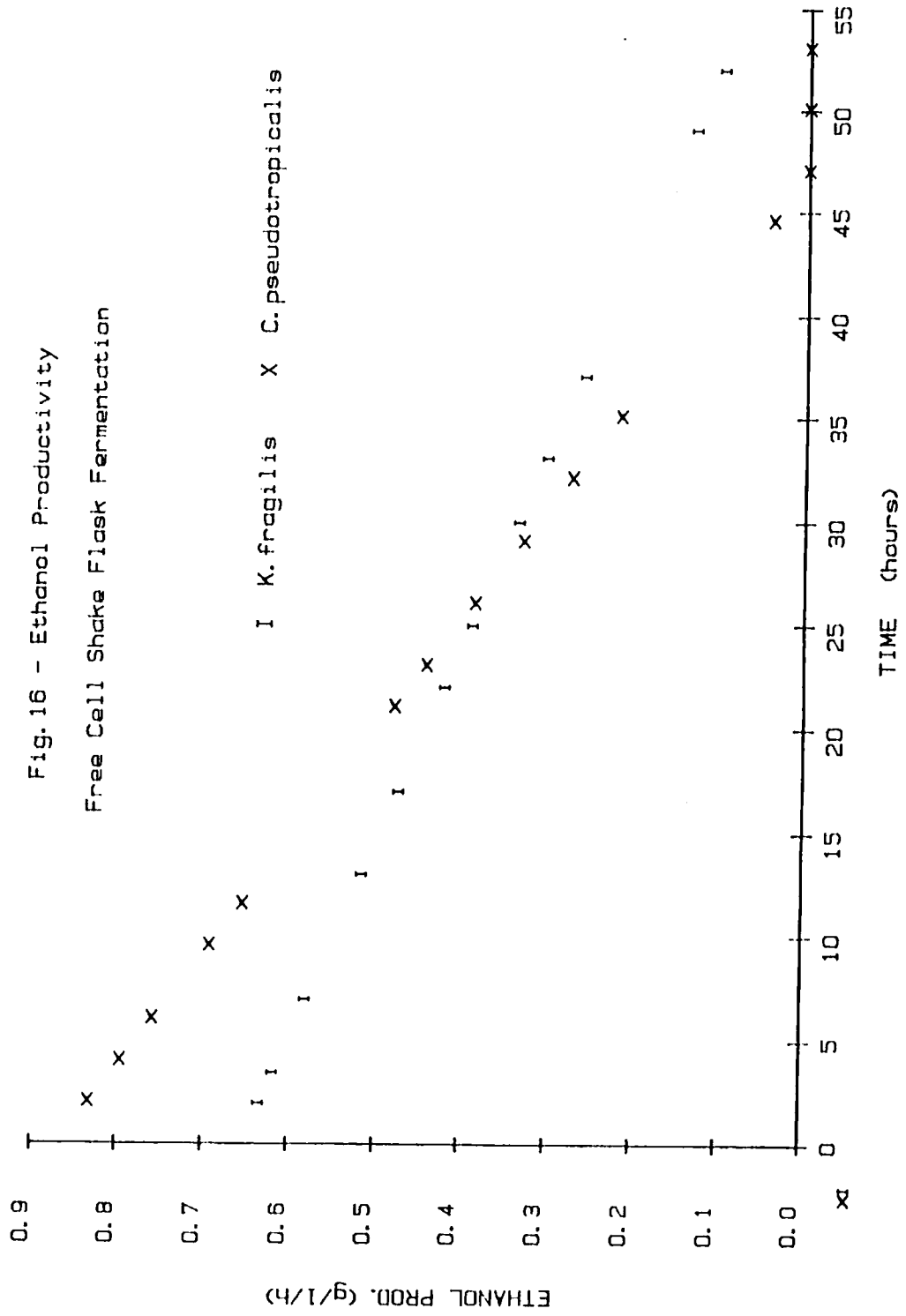


Fig. 16 - Ethanol Productivity
Free Cell Shake Flask Fermentation
I K. fragilis X C. pseudotropicalis
ETHANOL PROD. (g/l/h)
TIME (hours)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
0
5
10
15
20
25
30
35
40
45
50
55

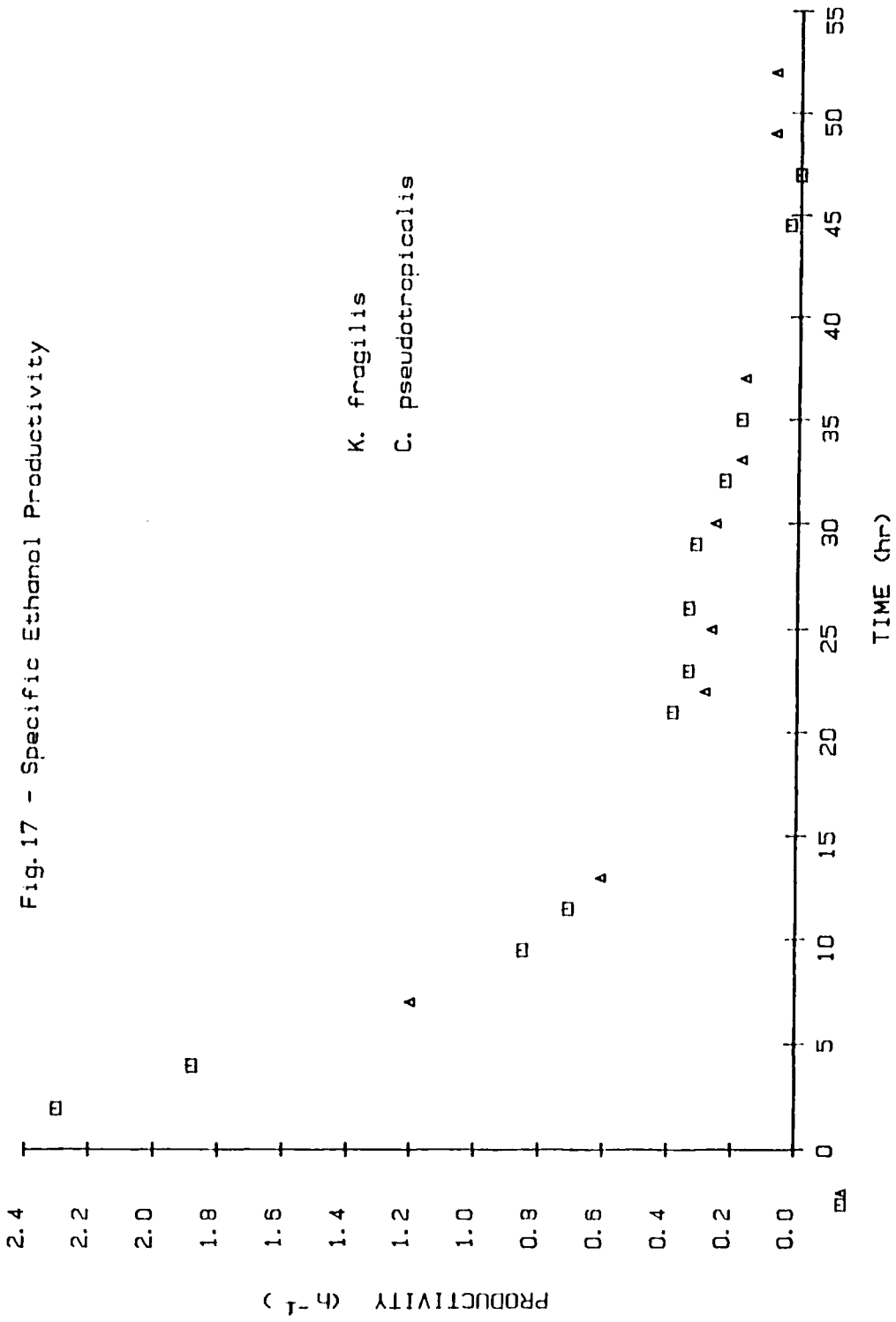


Fig. 17 - Specific Ethanol Productivity
K. fragilis
C. pseudotropicalis
PRODUCTIVITY (h⁻¹)
TIME (hr)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
0
5
10
15
20
25
30
35
40
45
50
55

TABLE 14

Kinetic Parameters for Candida pseudotropicalis and Kluyveromyces fragilis on Whey Permeate at 1X Concentration (47 g/l lactose) in Nonaerated Shake Flask Culture.

Kinetic Parameters	C. psuedotropicalis	K. fragilis
Specific growth rate (/h)[a]	0.021	0.024
Specific Lactose uptake rate (g/g/h)[a]	0.988	0.668
Specific ethanol production rate (g/g/h)[a]	0.39	0.29
Cell yield (g/g)[b]	0.028	0.030
Ethanol yield (g/g)[b]	0.412	0.408
Relative ethanol yield (%)[b,c]	76.3	75.5
Maximum ethanol concentration	19.4	19.2

a Calculated at 21 hours into the fermentation when culture was growing rapidly in a fully anearobic state.

b Based on the difference between initial and residual concentration.

c Based on 0.54g ethanol per 1.0g lactose for a theoretical yield.

The specific lactose uptake rate and specific ethanol production rate for Candida were clearly superior. Since ethanol productivity is among the most important parameters in the successful operation of a continuous bioreactor, Candida pseudotropicalis was the fermenting organism of choice.

It is interesting to note that the specific growth rate and cell yield of Kluyveromyces was slightly higher. K. fragilis may be an appropriate yeast for single cell protein production from whey permeate.

Ethanol productivity was calculated by:

$$\text{Ethanol productivity} = \frac{F \times P}{V_T} \qquad (15)$$

where F = feed flow rate (l/h)

P = ethanol concentration in the effluent stream (g/l)

V_T = total packed bed bioreactor volume (l)

Therefore, ethanol productivity is the amount of ethanol produced per unit total reactor volume per unit time, or g/l/h. Care should be taken when comparing productivities in the literature because total bead volume or total liquid (void) volume productivities are commonly used.

5.2.2 Free Cell and Immobilized Cell Shake Flask Fermentations

A high concentration of fermentable sugar causes enzymatic inhibition in both the fermentative and oxidative pathways [62]. To determine the inhibitory effects of lactose on C. pseudotropicalis, free cell shake flask fermentations in 1X, 1.25X, 4X and 6X media containing approximately 47, 60, 188, 280 g/l of lactose respectively were performed. Fig. 18 shows initial ethanol productivity plunged dramatically with 4X and 6X concentration media, an effect of high lactose (and high ash) concentration since ethanol concentrations were low in the initial stage of fermentation. Increased ethanol concentrations and, especially, depleted lactose levels, stopped all ethanol productivity in the flasks in 2 - 3 days for the less concentrated media and in over 10 days for the high lactose permeate.

Fig. 19 presents the lactose uptake, ethanol production, viable cell and growth curves of free cell C. pseudotropicalis fermented in shake flasks containing unconcentrated (1x) whey permeate. Lactose levels dropped to near zero in 32 hours, yet ethanol production continued for an additional 10 hours. Ethanol concentration began to decline after 50 hours, since cells in media devoid of lactose utilized ethanol as a carbon source. Kinetic parameters for this experiment are listed in Table 14.

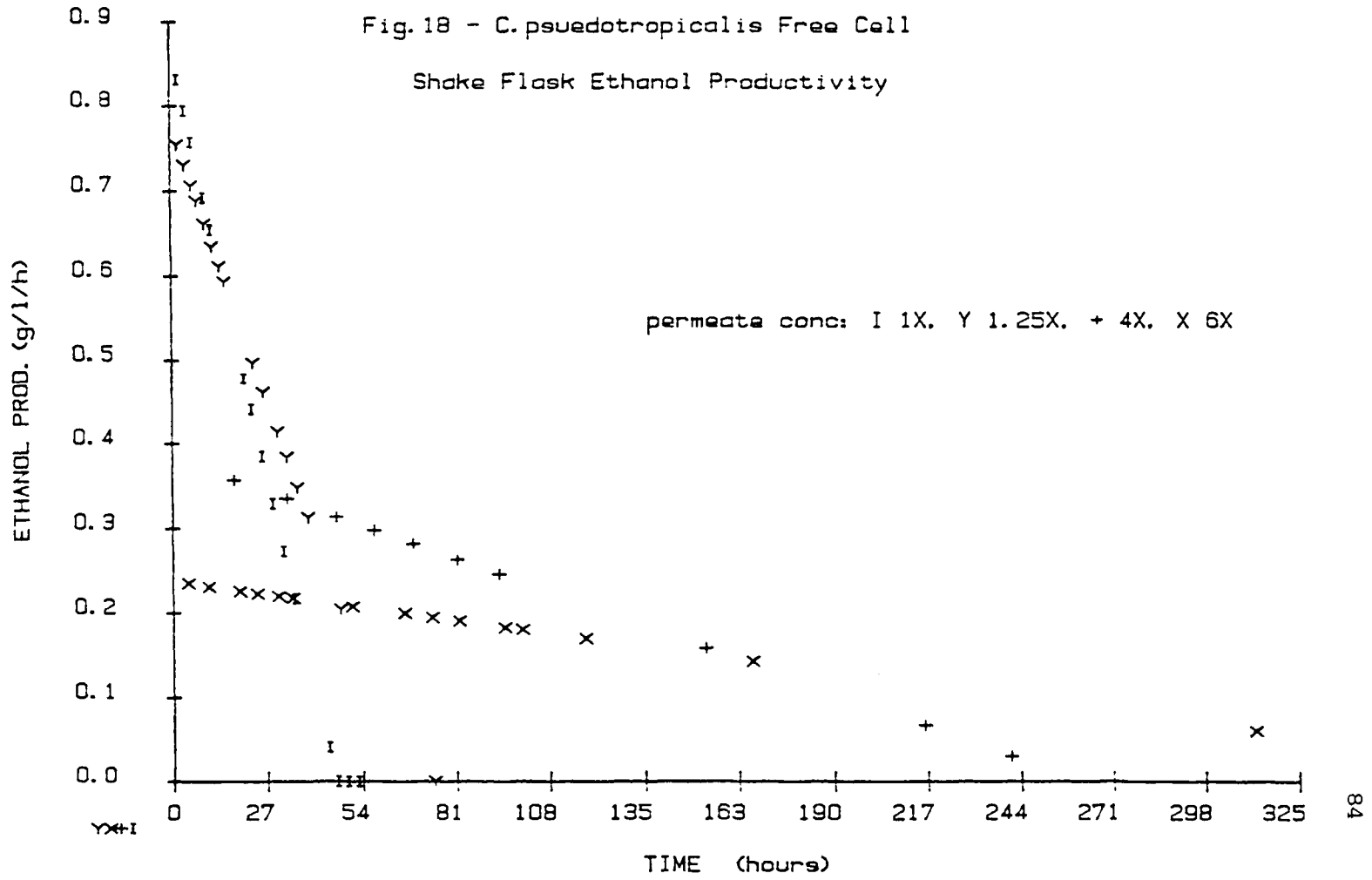


Fig. 18 - C. psuedotropicalis Free Cell
Shake Flask Ethanol Productivity
permeate conc: I 1X. Y 1.25X. + 4X. X 6X
ETHANOL PROD. (g/l/h)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
27
54
81
108
135
163
190
217
244
271
298
325
TIME (hours)
84

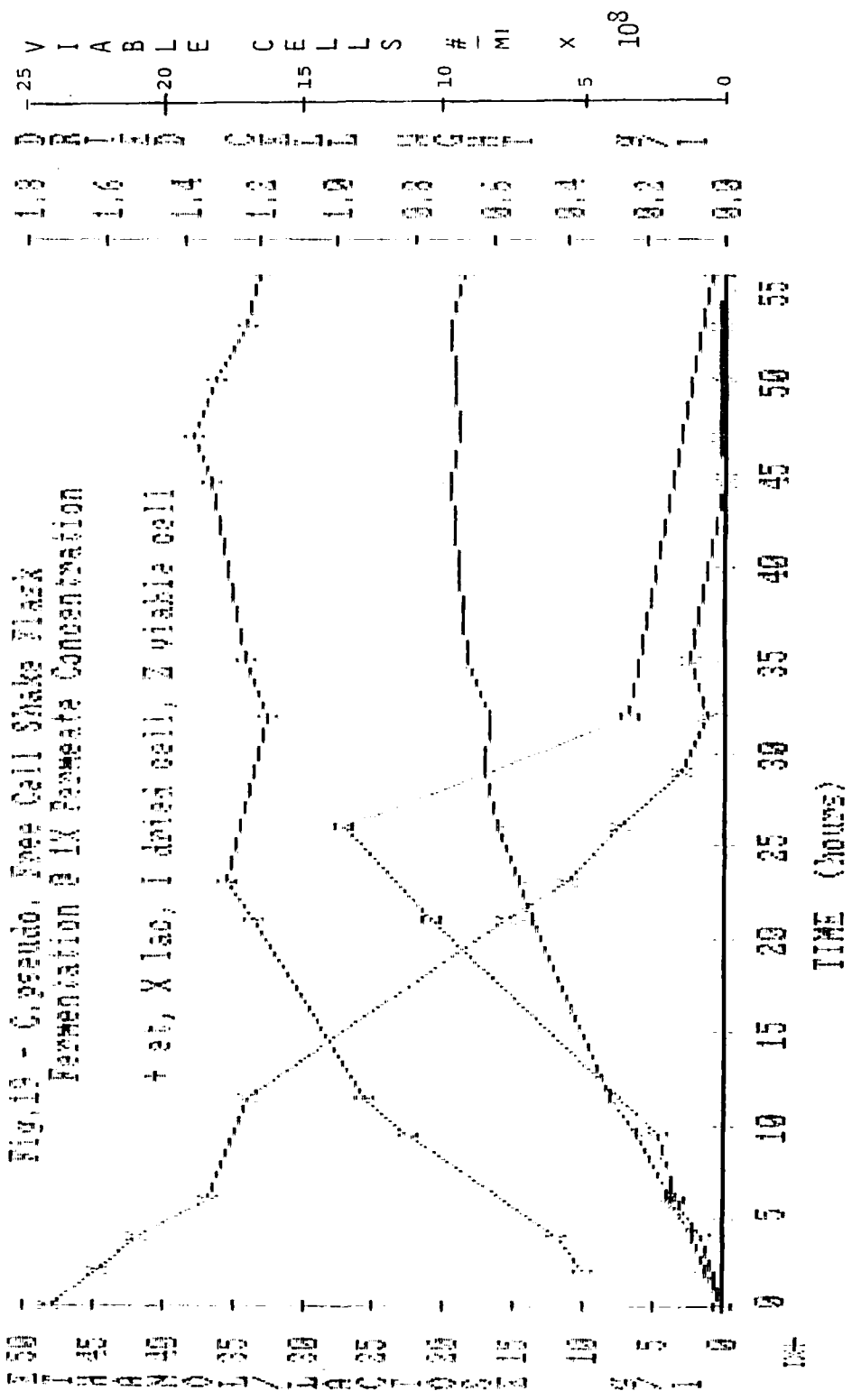
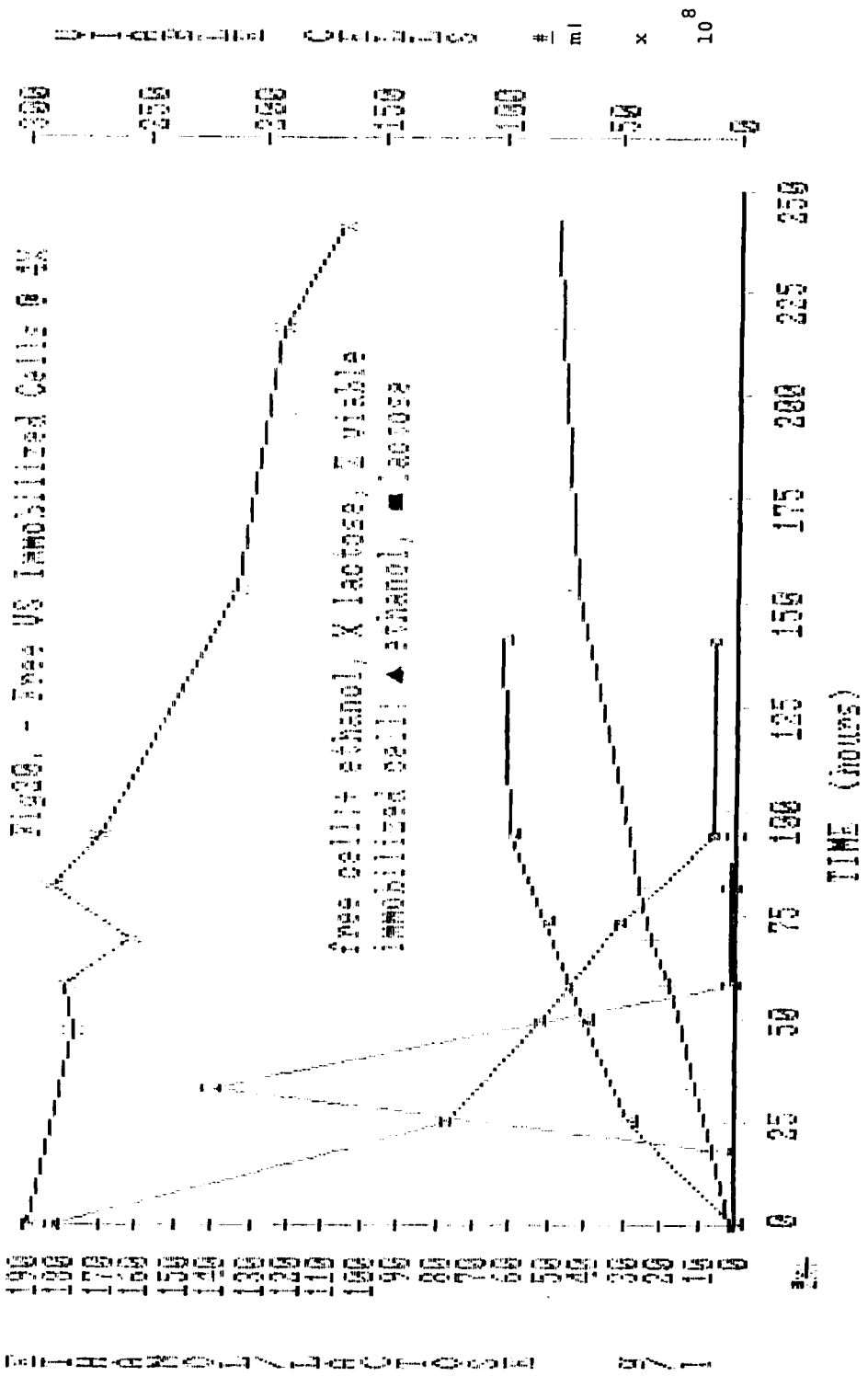


Fig.12 - Cultivo, Free Cell Shake Flask
Fermentation @ 1X Permeate Concentration
+ 2% X lac, 1 dilut cell, 2 viable cell
TIME (hours)
VIABLE CELLS # - MI X 10^8
0
5
10
15
20
25
30
35
40
45
50
55

Free cell shake flask fermentation in 4X media, illustrated in Fig. 20, displays a depressed performance compared to the fermentative behavior in the previous 1X media. After 10 days of fermentation time, when less than half of the initial lactose concentration had been utilized and while ethanol production was crawling along, the experiment was terminated. Viable cell count reached a maximum about 30 hours, an important consideration when immobilizing cells. It is most desirable in cell entrapment to harvest the innoculating broth near the end of the exponential growth phase when the number of replicating cells is largest.

Fig. 20 shows there was a large difference in performance between shake flask immobilized cell fermentations in 4x media and the free cell shake flask studies mentioned above. With the immobilized culture, nearly all lactose was utilized within 4 days and immobilized cells seemed less sensitive to higher ethanol concentrations because nearly 7% (w/v) ethanol was rapidly reached. In addition, Fig. 21 shows immobilized culture initial ethanol productivity was 1.1 g/l/h compared with 0.36 g/l/h for free cells. The immobilized yeast productivity was about three fold greater than non-immobilized culture productivity in 4X media. A decreased inhibitory effect of ethanol on immobilized yeast may explain this increased productivity. Marwaha and Kennedy [57] disclosed that the degree of ethanol inhibition was greater for lactose fermenting free cells than for the entrapped cells. Holcberg and Margalith [30] state that entrapment


Free cells ethanol, X lactose, Z viable
Immobilized cells ▲ ethanol, ■ lactose
TIME (Hours)
Viable Cells # ml x 10^8

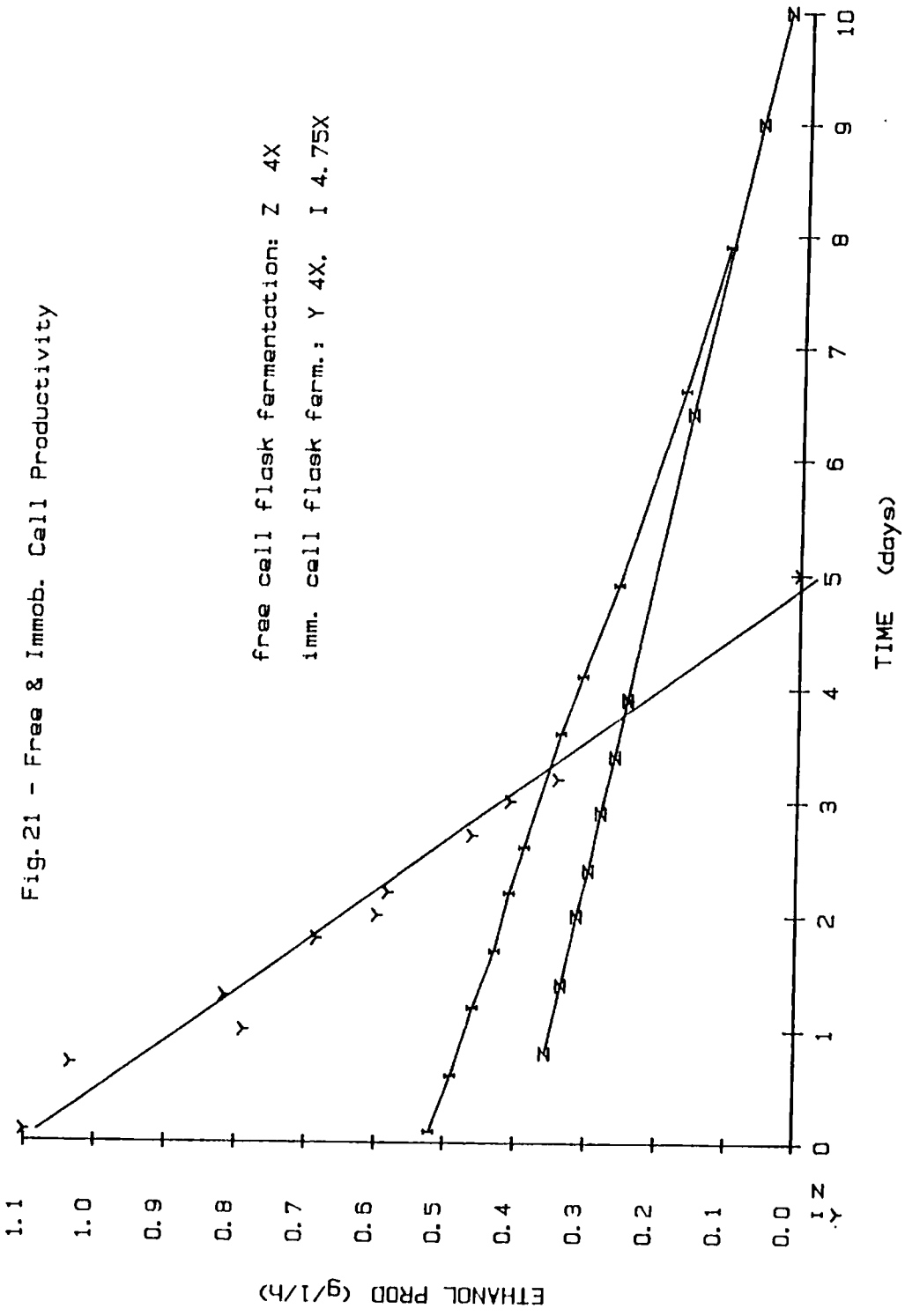


Fig.21 - Free & Immob. Cell Productivity
free cell flask fermentation: Z 4X
imm. cell flask ferm.: Y 4X. I 4.75X
ETHANOL PROD (g/l/h)
TIME (days)
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
1
2
3
4
5
6
7
8
9
10
I
Z
Y

gels form a protective layer which give alcohol producing cells a more suitable microenvironment. These researchers discovered a higher percentage of viable cells when alcohol fermenting cells were immobilized. The immobilization gel may protect yeast from the toxicity of ethanol which causes them to die thus lowering the culture productivity. The advantage of immobilizing yeast now becomes obvious.

Immobilized C. pseudotropicalis could withstand higher lactose concentration while showing significantly less substrate inhibition than free cells. This phenomenon was apparent when fermentation in 4.75x media (approximately 22% lactose) demonstrated higher productivity for immobilized cells versus non-immobilized yeast in 4x permeate (approximately 18.8% lactose). Partitioning of lactose between alginate matrix and fermentation broth resulted in lower lactose levels within the immobilization matrix. If the rate of diffusion of lactose into the alginate bead is lower than the rate of lactose uptake by the yeast, the internal mass transfer resistance within the bead caused a lactose concentration gradient which had the effect of exposing fermenting cells within the bead to lower, and hence less inhibiting, levels of sugar. Indeed, the ethanol productivity of immobilized C. pseudotropicalis was detrimentally affected by increasing lactose concentration. An increase in shake flask media concentration from 4x to 4.75x decreased initial productivity for immobilized yeast from approximately 1.1 g/l/h to approximately 0.5 g/l/h. However, immobilized yeast were not affected to the extent that free cells were affected (Fig. 18) at this high lactose concentration.

5.2.3 Continuous Packed Bed Bioreactor Fermentations

5.2.3.1 Immobilized Yeast Shake Flask versus Continuous Bioreactor Performance

A comparison in Fig. 22 between immobilized yeast shake flask and continuous packed bed bioreactor studies with 4x media indicate maximum productivities of 1.1 g/l/h and 3.1 g/l/h respectively. A nearly three fold ethanol productivity increase was possible for the bioreactor. Operated at the same temperature and cell loading conditions as the immobilized cell shake flasks and at a 0.08 per hour dilution rate, the bioreactor displayed a relatively stable performance. Although the packed bed gave much higher productivities than the flasks, Fig. 23 shows ethanol concentrations in bioreactor effluent (wine) was 50% lower than the final ethanol concentration in the batch reactors. This is an important point since higher final ethanol concentration lowers the cost of distillation. In Fig. 24, a linear regression line is plotted for ethanol production versus glucose uptake in flasks. From the slope, it is calculated 0.445 g ethanol per gram of lactose was produced or 82% of the Pasteur efficiency of 0.54 g ethanol per gram of lactose. Reactor values fit nicely around this regression line, in spite of low ethanol in its wine.

A similar comparison was made for flask and bioreactor productivities at 4.75x, Fig. 25. Again, ethanol productivity for continuous reactor was significantly higher than batch mode flasks. Bioreactor performance, at the same dilution rate as above, varied from 1.21 to 2.16 g/l/h, while flask performance declined from 0.52 g/l/h.

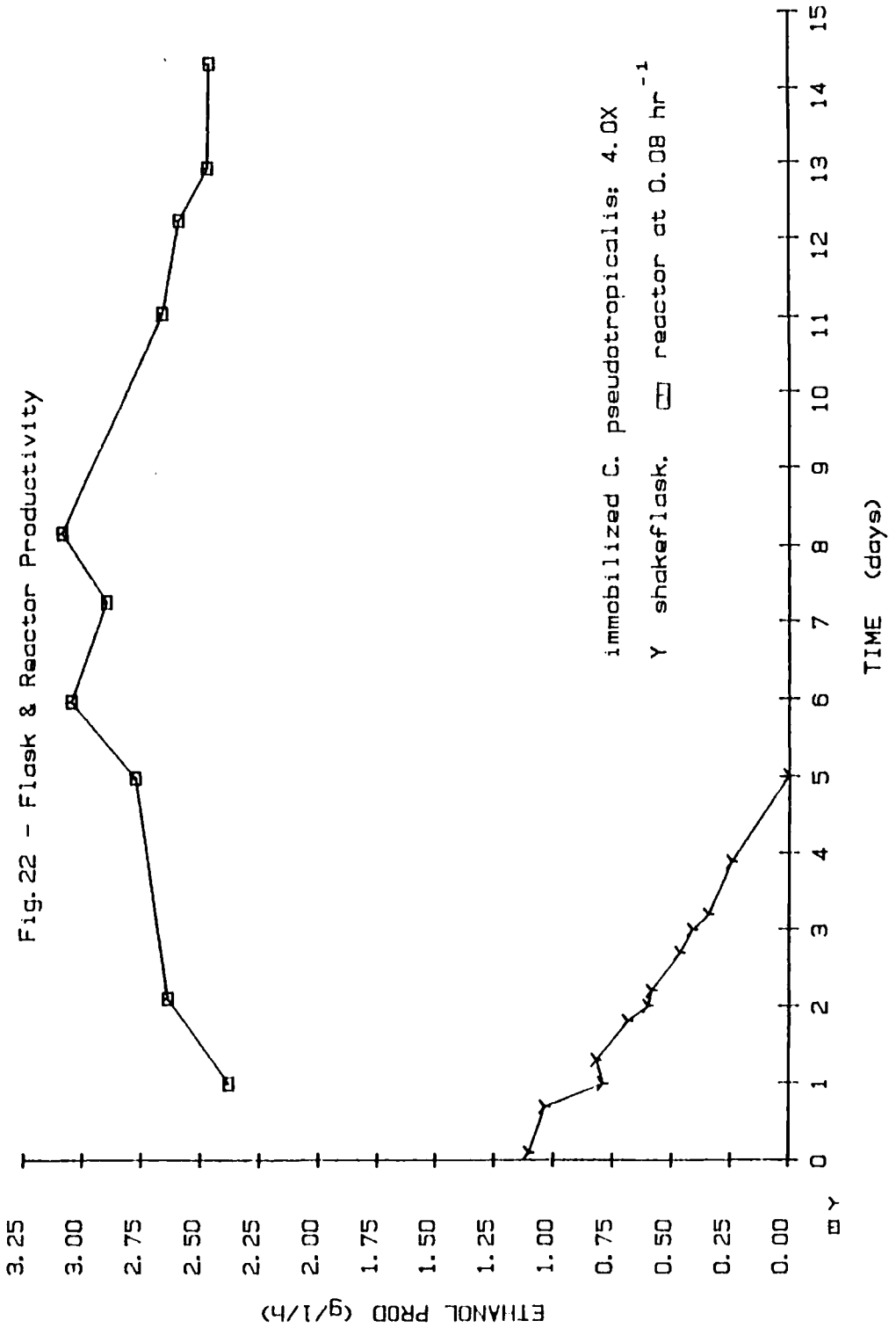


Fig. 22 - Flask & Reactor Productivity
immobilized C. pseudotropicalis: 4.0X
Y shakeflask. reactor at 0.08 hr-1
ETHANOL PROD (g/l/h)
TIME (days)
Y
0.00
0.25
0.50
0.75
1.00
1.25
1.50
1.75
2.00
2.25
2.50
2.75
3.00
3.25
0
1
2
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6
7
8
9
10
11
12
13
14
15

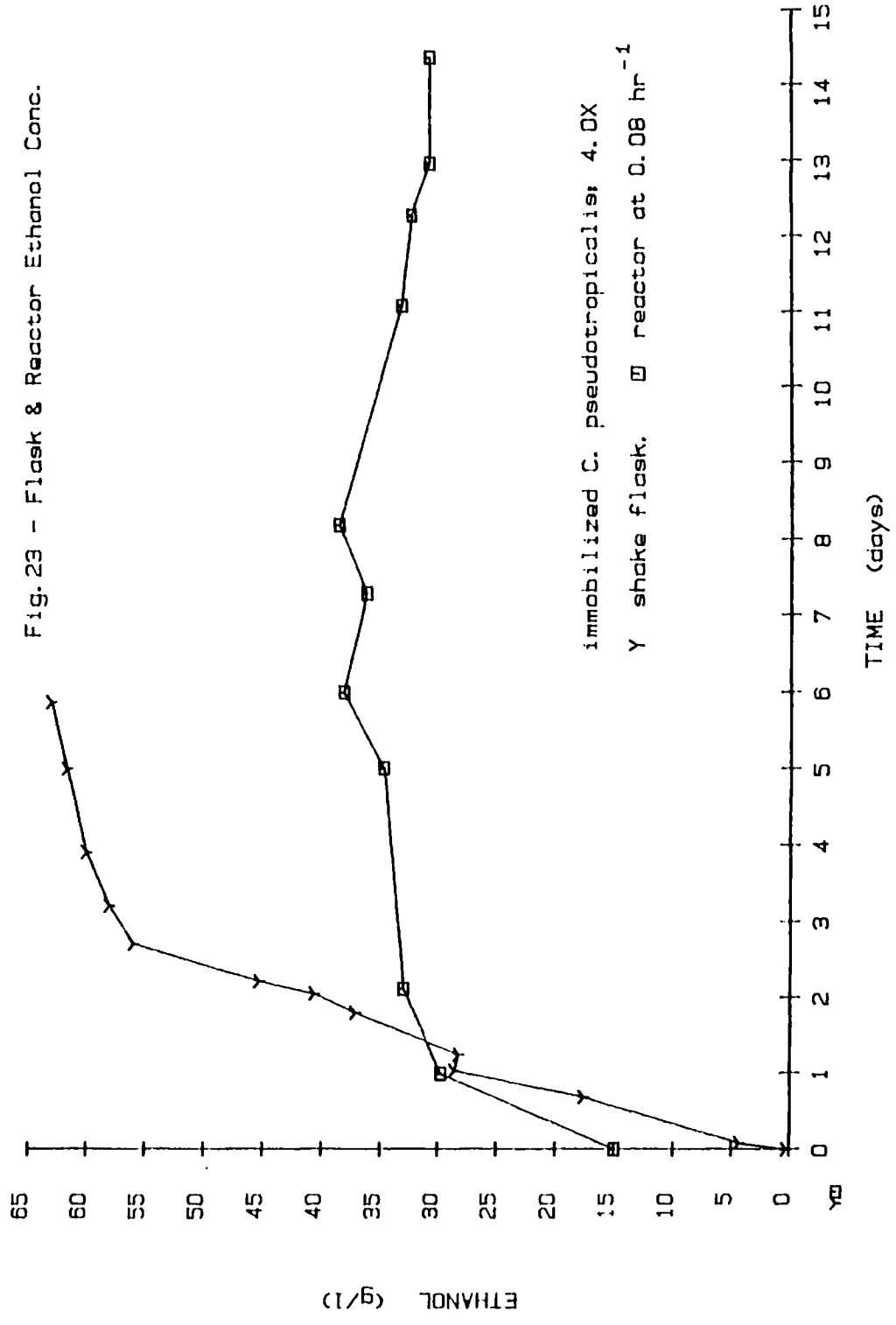
Fig. 23 - Flask & Reactor Ethanol Conc.
immobilized C. pseudotropicalis 4.0X
Y shake flask. ⊡ reactor at 0.08 hr⁻¹
ETHANOL (g/l)
TIME (days)
0
5
10
15
20
25
30
35
40
45
50
55
60
65
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15

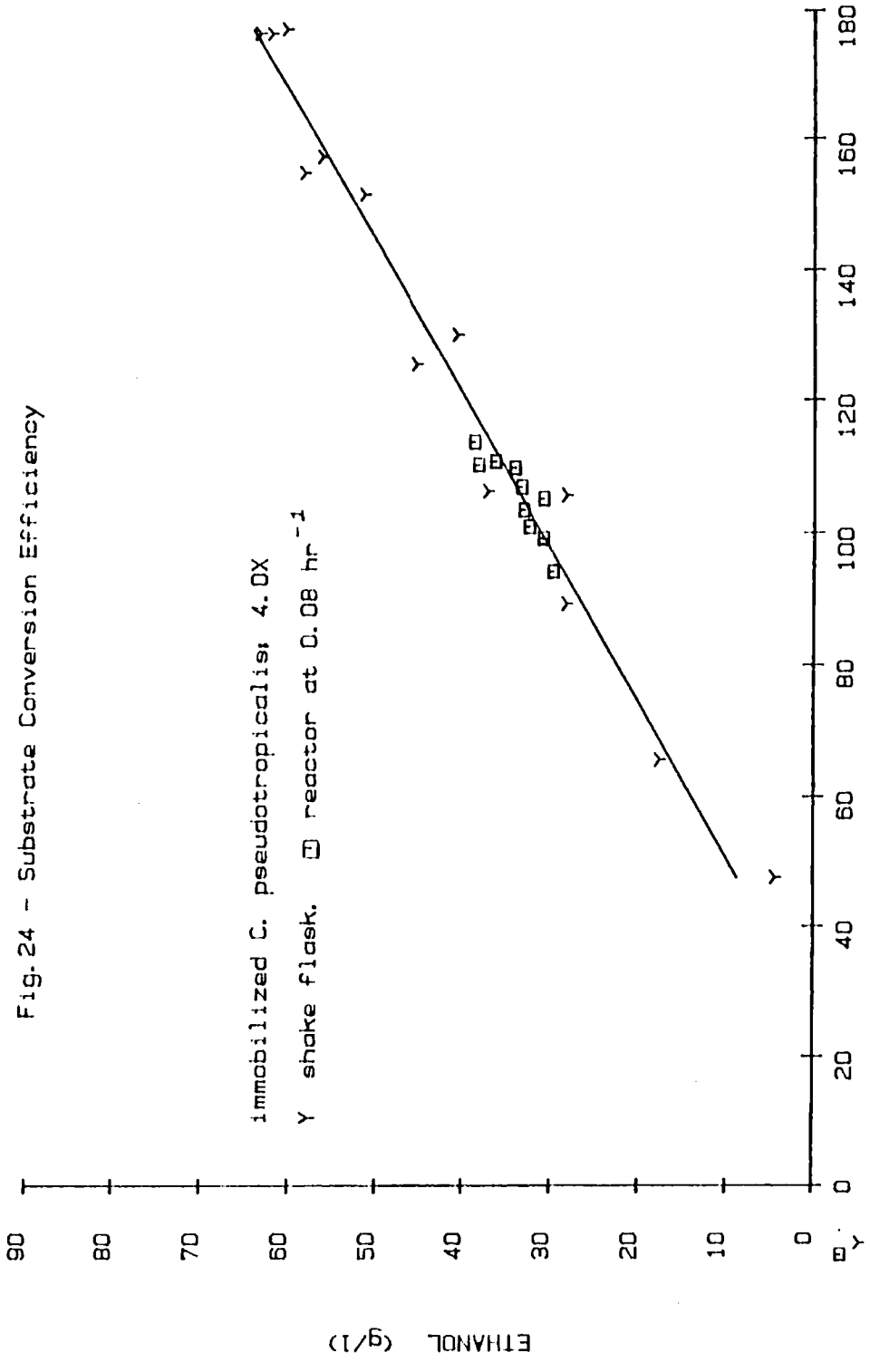
Fig.24 - Substrate Conversion Efficiency
immobilized C. pseudotropicalis 4.0X
Y shake flask. ⊡ reactor at 0.08 hr⁻¹
ETHANOL (g/l)
GLUCOSE UPTAKE (g/l)
0
10
20
30
40
50
60
70
80
90
0
20
40
60
80
100
120
140
160
180

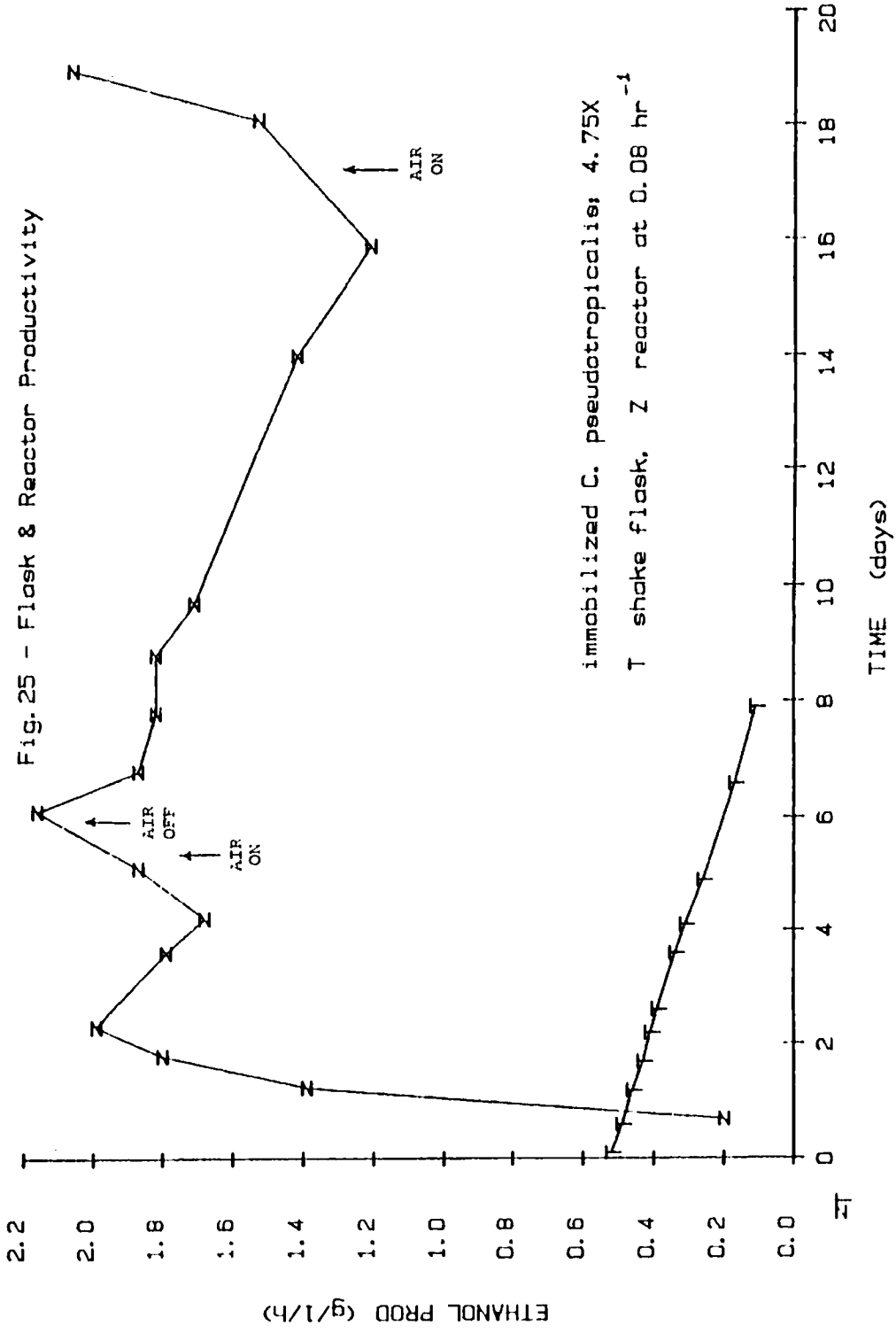
Fig. 25 - Flask & Reactor Productivity
immobilized C. pseudotropicalis; 4.75X
T shake flask, Z reactor at 0.08 hr⁻¹
AIR OFF
AIR ON
AIR ON
ETHANOL PROD (g/l/h)
TIME (days)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
0
2
4
6
8
10
12
14
16
18
20

The bioreactor ethanol productivity with the 4.75x whey permeate was approximately three to four fold greater than shake flask productivity. This was similar to the relative performance difference between the bioreactor and the flasks in 4x media, however, the absolute values of ethanol productivity are skewed down the scale. The ethanol concentrations of the 4.75x permeate wine (fig. 26) again resulted in the flasks more than doubling the bioreactor's ethanol concentration. Substrate conversion efficiency (Fig. 27), for the flasks gave a linear regression whose slope told that 0.424 gram ethanol were produced per gram of lactose or 78% of theoretical conversion. Ethanol production versus glucose uptake in Fig. 27 for the bioreactor produced some values slightly below the batch regression line, an effect of periodic reactor aeration.

Dilution rate mentioned above was the number of total reactor volumes the feed fills in an hour. It was calculated by:

$$\text{Dilution rate} = \frac{F}{V_T} .$$

The dilution rate has units of reciprocal hour (h^{-1}) and is the inverse of retention time.

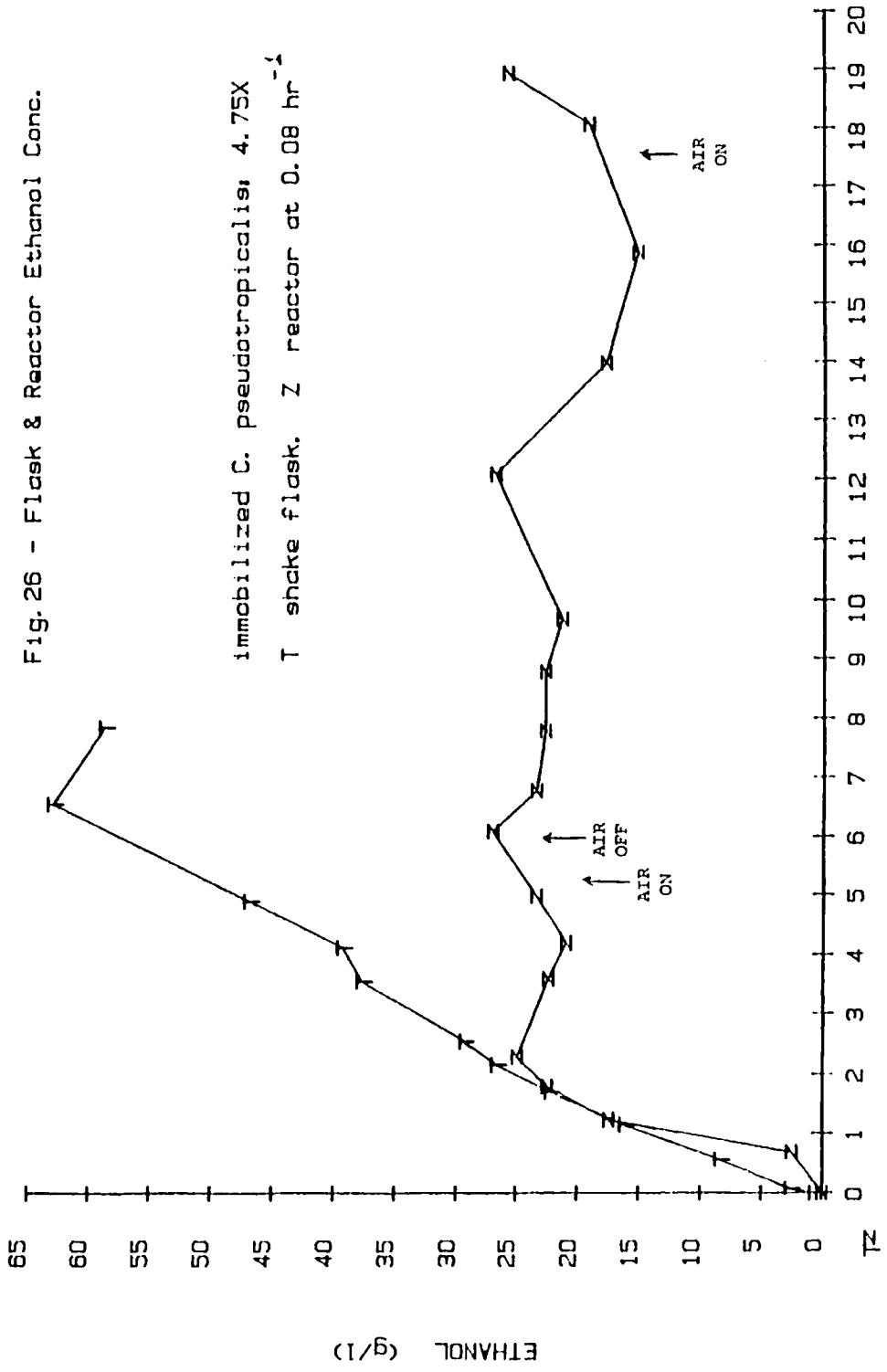
Fig. 26 - Flask & Reactor Ethanol Conc.
Immobilized C. pseudotropicalis 4.75X
T shake flask. Z reactor at 0.08 hr⁻¹
AIR OFF
AIR ON
AIR ON
ETHANOL (g/l)
TIME (days)
T
Z

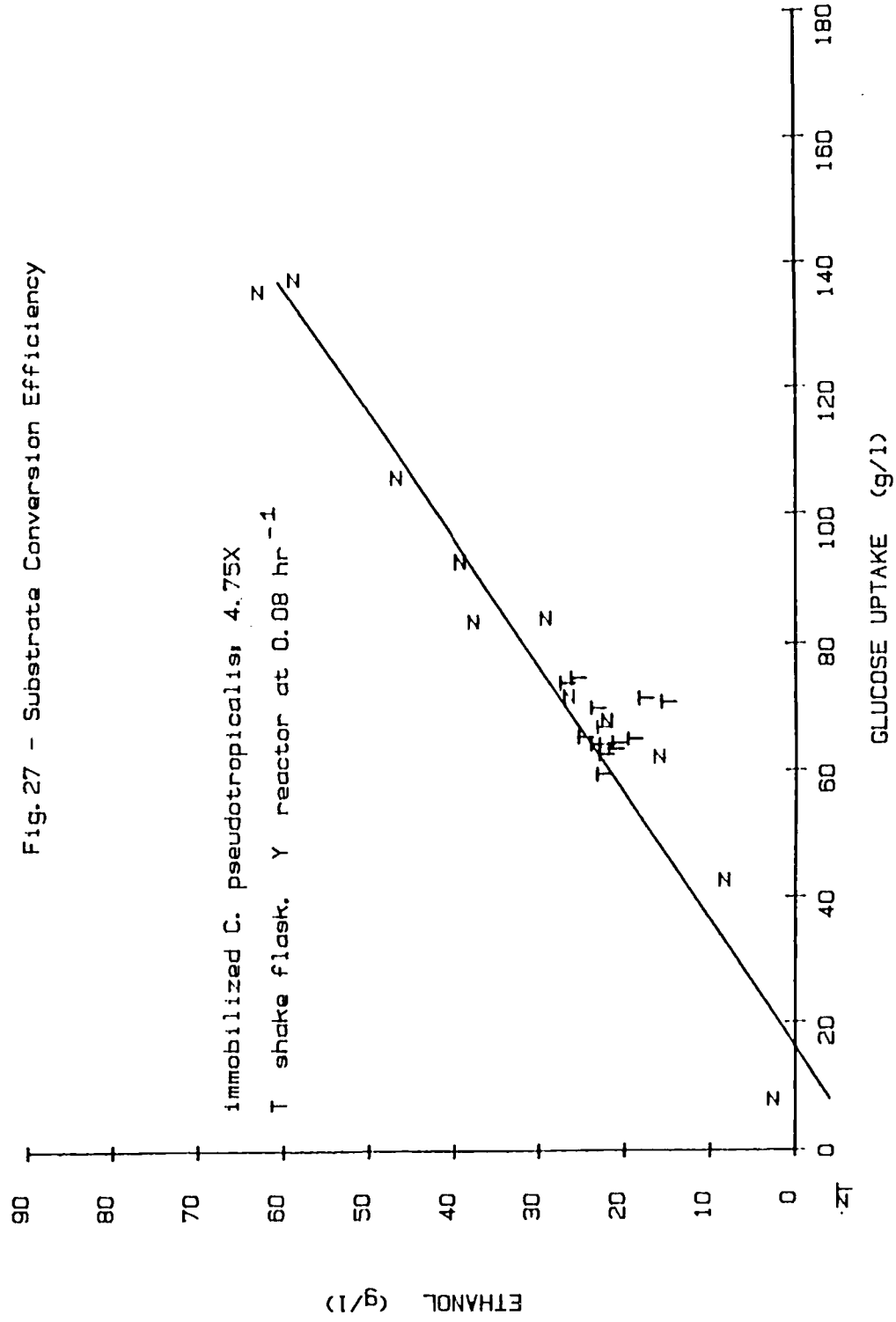
Fig.27 - Substrate Conversion Efficiency
immobilized C. pseudotropicalis: 4.75X
T shake flask, Y reactor at 0.08 hr-1
ETHANOL (g/l)
GLUCOSE UPTAKE (g/l)
0
10
20
30
40
50
60
70
80
90
0
20
40
60
80
100
120
140
160
180

5.2.3.2 Effect of Lactose Concentration on Packed Bed Bioreactor

Media concentration was varied for a packed bed column operated at a constant dilution rate of 0.08 h^{-1}. Fig. 28 shows ethanol productivity in the column decreased when lactose concentration was increased. The culture exhibited the effects of lactose inhibition which lowered the rate of ethanol production. Effluent ethanol concentration varied as a function of inlet lactose concentration For media concentrations 2.5x, 4x and 4.75x at constant dilution rate packed bed effluent ethanol concentrations were approximately 45 g/l, 35 g/l, and 25 g/l respectively. Therefore, as lactose concentration in the feed increased, ethanol concentration in the reactor effluent decreased. Lee et. al. [27] reported that above substrate concentrations of 150 g/l at constant dilution rate, ethanol concentration in the effluent dropped. Janssens et. al. [63] have shown for lactose fermenting yeast, the fermentation time increase to utilize all the lactose increases with increasing dilution rate so at a constant dilution rate the yeast just do not have enough time to convert lactose to ethanol, hence, the bioreactor ethanol content decreases with increasing lactose. Low lactose uptake rates may be due to the effect of ethanol on biomembranes where, "the decrease in fluidity caused by the presence of ethanol may inhibit the action of protein involved in the transport of lactose into cells" [63]. Subsequently, as feed lactose level was increased, the effluent lactose concentration increased. For the 4.75x, effluent lactose levels were so high, 145 to 160 g/l, that another packed bed column was added to the system.

The reduction in bioreactor lactose conversion efficiency when the media was increased from 4X to 4.75X (see figs. 24 and 27) agrees with several studies [63, 64, 65] Moulin, Guillaume and Galzy [64] report decreasing efficiency with increasing lactose concentration for the three yeast strains tested. This effect has a small economic impact on this fermentation than would be expected. Unlike glucose based ethanol fermentation where substrate is a major cost, in whey based ethanol fermentation, the cost of substrate is low.

5.2.3.3 Effect of Dilution Rate on Packed Bed Bioreactor

A twin column packed bed bioreactor was operated in a continuous fashion for 80 days with no contamination problems and little loss of activity. Figs. 29, 30 and 31 show plots of reactor productivity, wine ethanol concentration, and effluent lactose level and lactose values respectively for columns 1 and 2. Dilution rates were varied as was media concentration and type. With 2.5X media at 0.04 h^{-1} and 0.08 h^{-1} (per column) dilution rates, ethanol concentrations held steady at nearly 50 g/l and most of the lactose was consumed, yet ethanol productivity in column 1 nearly doubled at the higher dilution rate.

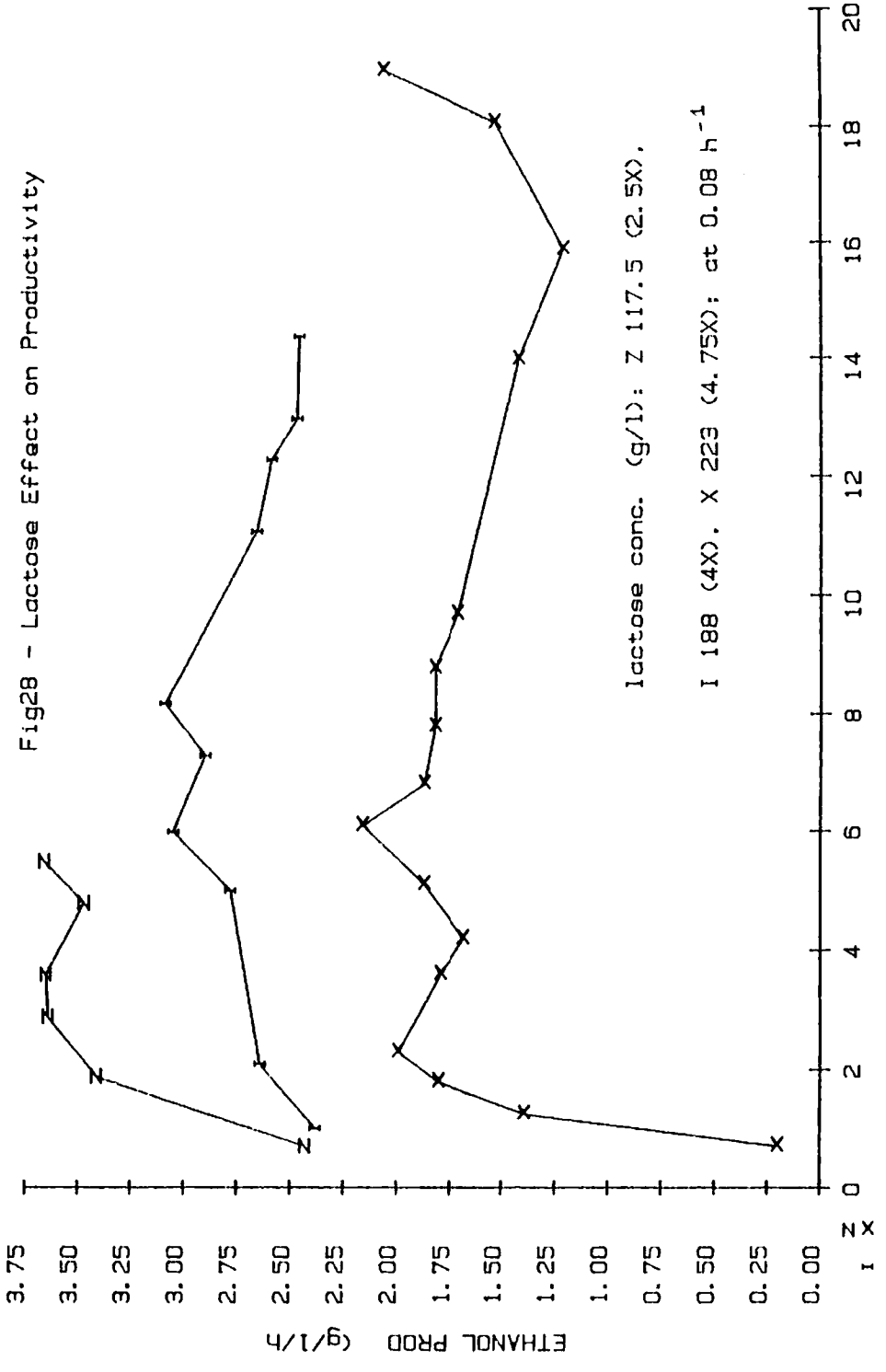
Fig28 - Lactose Effect on Productivity
lactose conc. (g/l): Z 117.5 (2.5X).
I 188 (4X). X 223 (4.75X); at 0.08 h-1
ETHANOL PROD (g/l/h
TIME (days)
3.75
3.50
3.25
3.00
2.75
2.50
2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
0
2
4
6
8
10
12
14
16
18
20

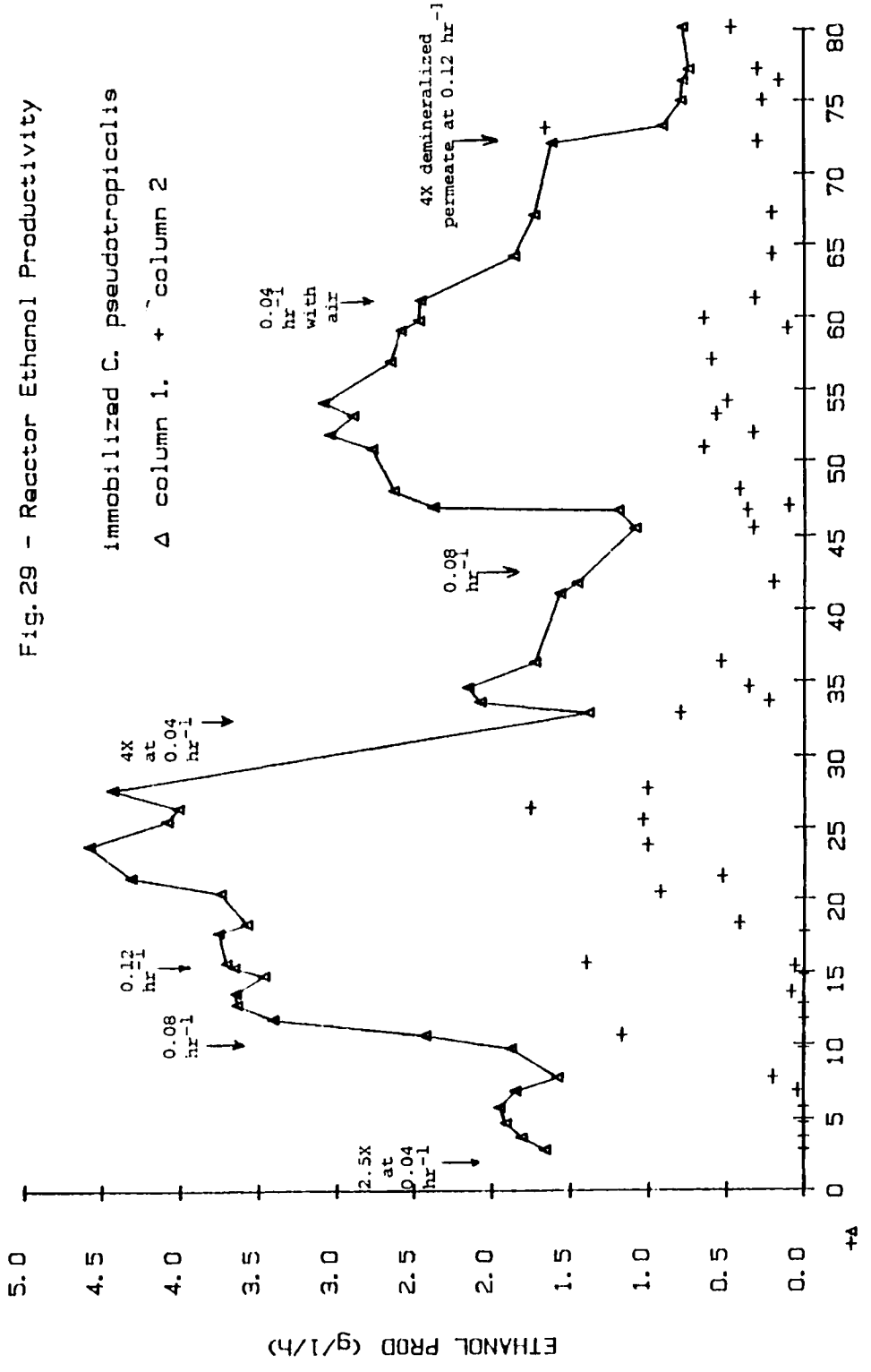

Fig. 29 - Reactor Ethanol Productivity

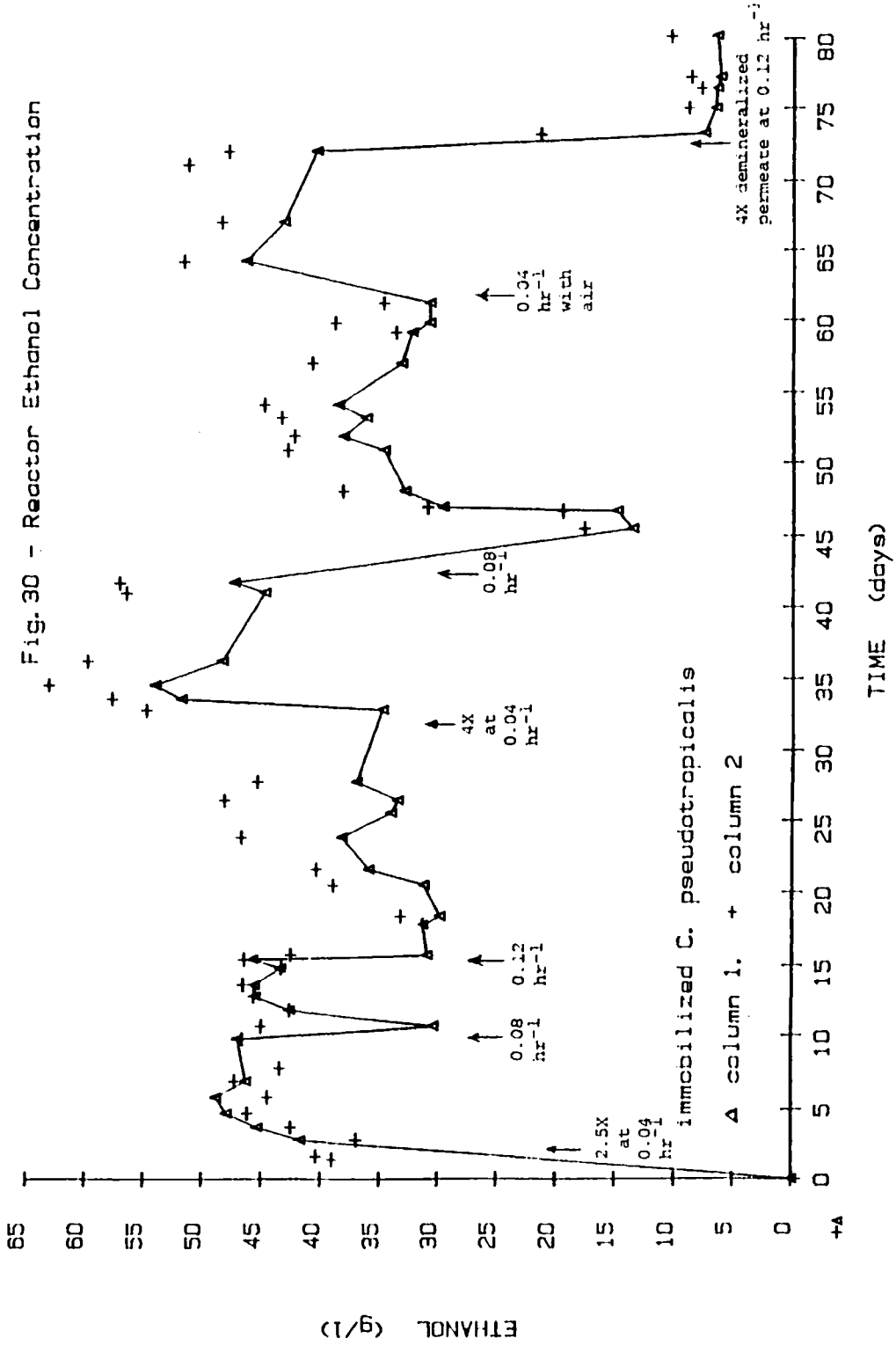
Fig. 30 - Reactor Ethanol Concentration
immobilized C. pseudotropicalis
Δ column 1. + column 2
2.5X at 0.04 hr-1
0.08 hr-1
0.12 hr-1
4X at 0.04 hr-1
0.08 hr-1
0.04 hr-1 with air
4X demineralized permeate at 0.12 hr-1
ETHANOL (g/l)
TIME (days)

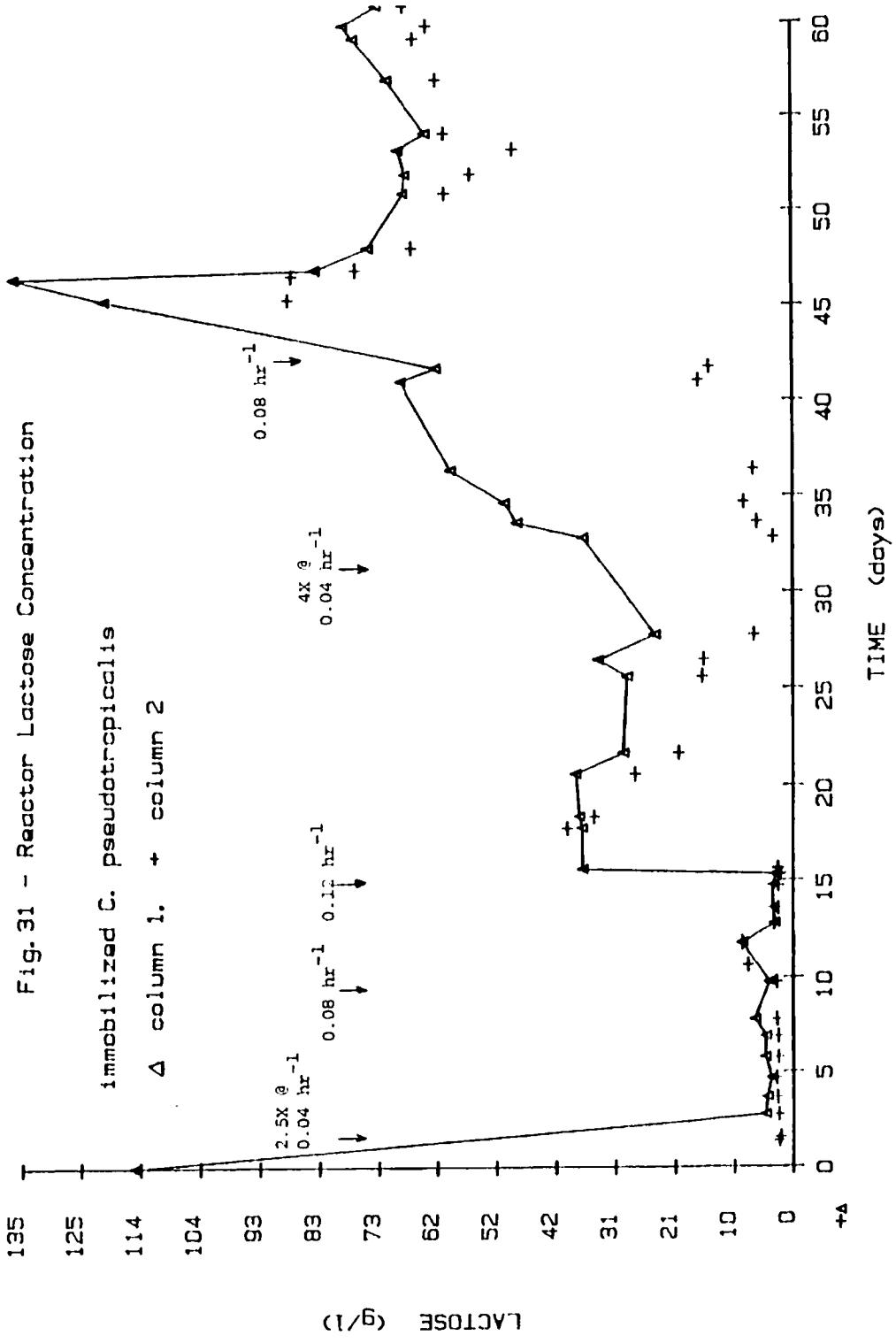
Fig. 31 - Reactor Lactose Concentration
immobilized C. pseudotropicalis
Δ column 1. + column 2
2.5X @ 0.04 hr-1
0.08 hr-1
0.1[illegible] hr-1
4X @ 0.04 hr-1
0.08 hr-1
LACTOSE (g/l)
TIME (days)
135
125
114
104
93
83
73
62
52
42
31
21
10
0
0
5
10
15
20
25
30
35
40
45
50
55
60

It can be observed that the ethanol in reactor 2 was slightly lower than column 1, a phenomenon that displayed the effect of sugar starved yeast assimilating ethanol as a carbon source as was previously seen in batch mode flasks. A dilution rate of 0.12 h^{-1} increased productivity yet lowered the ethanol and raised the lactose concentration in the wine of column 1 throughout the 20 day duration. The effluent ethanol and lactose concentrations from column 2 approached values attained during the previous lower dilution rates within 10 days after the dilution was increased.

At about the 33rd day, a step change to 4X media was made to observe the effect of high lactose concentration on the culture. The reactors were initially operated at a 0.04 h^{-1} dilution. Ethanol concentrations for both reactors soared with the introduction of high lactose media. As expected, productivity decline due to substrate inhibition from high lactose feed was observed similar to the results shown in fig. 28. Another step change to 0.08 h^{-1} produced an immediate drop in the ethanol levels with a recovery within several days to levels approximately 35 and 40 g/l for columns 1 and 2 respectively. These ethanol concentrations are significantly below the 50 and 60 g/l obtained by columns 1 and 2 respectively with a 0.04 h^{-1} dilution. In addition, high lactose levels (85 g/l) demonstrated the 12 hour retention time at 0.08 h^{-1} dilution was too short for economical conversion and the desired high effluent ethanol levels.

Figs. 32 and 33 display the average lactose, ethanol and productivity values at various dilution rates for 2.5x and 4x media respectively. With

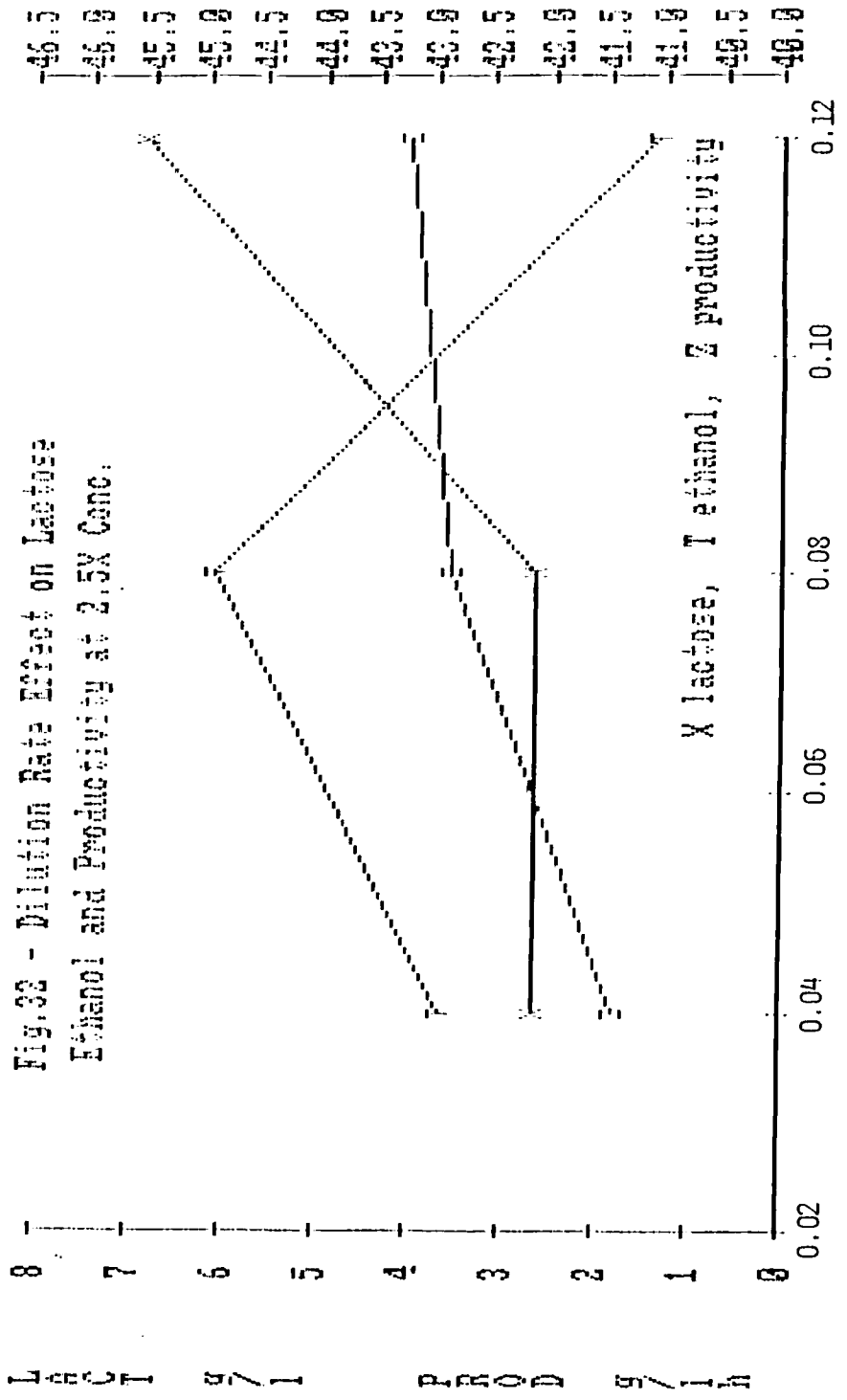
Fig.32 - Dilution Rate Effect on Lactose
Ethanol and Productivity at 2.5% Conc.
X lactose, T ethanol, Z productivity
DILUTION RATE (hr -1)
0.02
0.04
0.06
0.08
0.10
0.12
0
1
2
3
4
5
6
7
8
46.5
46.0
45.5
45.0
44.5
44.0
43.5
43.0
42.5
42.0
41.5
41.0
40.5
40.0

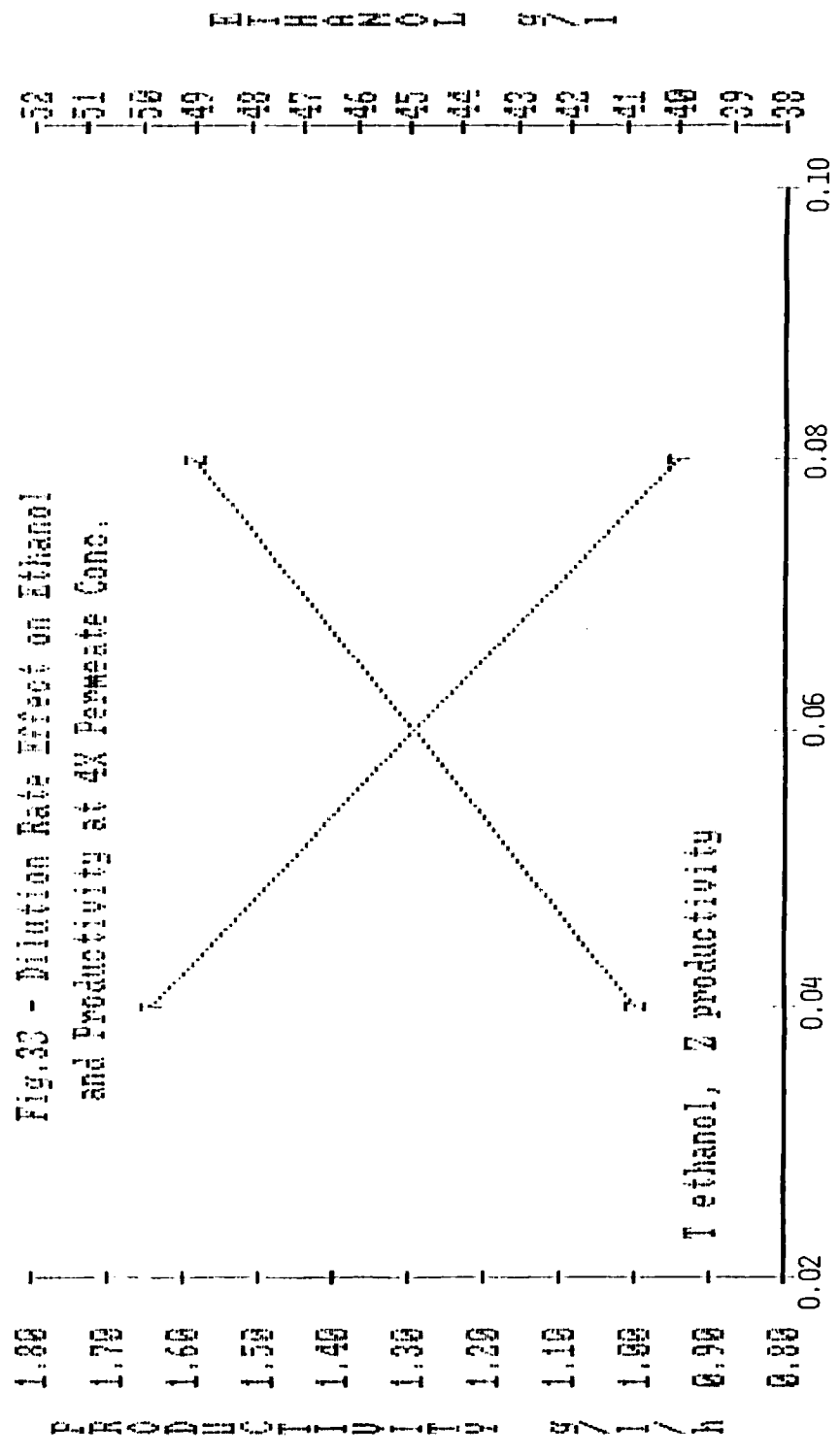
Fig.33 - Dilution Rate Effect on Ethanol
and Productivity at 4% Permeate Conc.
ETHANOL g/l
32
31
30
29
28
27
26
25
24
23
22
21
20
19
18
PRODUCTIVITY g/l/h
1.80
1.70
1.60
1.50
1.40
1.30
1.20
1.10
1.00
0.90
0.80
0.02
0.04
0.06
0.08
0.10
T ethanol, Z productivity
DILUTION RATE (hr⁻¹)

2.5X media, the 0.08 h^{-1}, dilution demonstrated higher wine ethanol levels and higher ethanol productivity without a change in wine lactose levels. When dilution was increased to 0.12 h^{-1}, ethanol levels dropped significantly with only a slight increase in productivity. In addition, at this elevated dilution rate, effluent lactose levels increased. A similar trend was observed with high lactose media (4x) but at lower dilution rates.

5.2.3.4 Effect of Aeration on Packed Bed Bioreactor

Fig. 24 indicates that an 82% efficiency may be caused by an oxygen limitation of yeast growth, since ethanol production is somewhat growth related. All media was aerated after autoclaving and prior to use in experiments. However, an aerated media was assumed insufficient for ethanol production. Microaeration at 0.01 vvm was explored to raise ethanol and lower lactose levels in the ethanol at 0.04 h^{-1} dilution. Fig. 29 shows that aeration produced slightly lower ethanol concentrations than the non-aerated operation at similar dilution. Burgess and Kelly [23] indicate that this lower ethanol concentration may be caused by conditions that are too aerobic, hence lactose is turned into cell mass instead of ethanol.

Microaeration at an elevated dilution rate, 0.08 h^{-1}, and 4.75x media showed some interesting results (Figs. 25, 26). When air was sparged into the packed bed, the culture responded immediately with increased ethanol productivity and increased effluent concentration. There was a marked effect in bed rejuvenation.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

6.1.1 Ultrafiltration and Reverse Osmosis

The semi-permeable membrane system which was constructed and utilized in this study performed well in pretreating whey prior to fermentation. Some outstanding distinctions were:

A. Ultrafiltration separated and concentrated the valuable whey proteins to obtain a valuable whey protein concentrate for human consumption with all the desirable functionality of an undenatured protein.

B. The ultrafiltration flux declined rapidly at the onset of a run due to the build up of a gel layer but this rapid decline tapered off when the increase in the gel layer stabilized. The only additional flux decay was due to the increase, during ultrafiltration, of non-permeable solids.

C. Negligible amounts of protein were lost in the permeate and only small increase in lactose concentration occurred due to membrane retention.

D. Reverse osmosis is a viable means of concentrating whey permeate to levels necessary for a successful fermentation.

6.1.2 Ethanol Fermentation

This study has shown the feasability of fermenting whey into ethanol. Some notable features were:

A. Candida pseudotropicalis is an effective yeast for carrying out the biotransformation in the presence of high lactose and ash concentrations.

B. Immobilized yeast in shake flask studies displayed a two fold initial ethanol productivity increase and higher final ethanol concentrations than their free cell counterparts. At higher whey permeate concentrations, free cells essentially stopped fermenting while immobilized yeast utilized lactose nearly to completion. The immobilized cultures were also less severly affected by the detrimental effects of high substrate and high product concentrations, a highly desirable trait in ethanol fermentation.

C. Continuous fermentations of immobilized yeast gave a three fold productivity increase over batch immobilized yeast fermentations. However, final batch ethanol concentrations were two fold higher than in the continuously operated reactor effluent. Although, initially it may appear as if a continuous fermentation is more desirable, lower ethanol concentrations significantly increase the cost of ethanol distillation. Both reactor systems had similar conversion efficiency.

D. As whey permeate concentration increased, the productivity of the packed bed decreased. This entails operating the bed at lower dilution rates to keep effluent ethanol levels high and effluent lactose levels low.

E. At constant whey permeate feed concentration, when dilution rates were increased, reactor productivity increased, effluent ethanol levels dropped and effluent lactose levels rose. At higher feed concentrations, the same effects occurred but at low dilution rates.

F. Microaeration showed to be a promising regenerator of the culture at high lactose concentration.

The continuous bioreactor was operated with little activity loss, negligible contamination and in a stable fermentative mode for nearly three months. The entire process can be easily automated for operation control.

6.2 Recommendation

6.2.1 Ultrafiltration

To reduce the rapid initial decline in process flux, proteolytic enzymes could be immobilized on the surface of the membrane to reduce the gel-polarization layer. The subsequent increase in process flux would reduce the size ultrafilter needed by a plant hence lowering the capital, energy, chemical, and membrane replacement costs.

6.2.2 Ethanol Fermentation

A good deal of work is needed in the area of strain selection and mutation. It may be possible to develop lactose utilizing yeast strains to rapidly produce high ethanol concentrations in the presence of high lactose ash concentrations.

More work should be performed on the effects of microaeration to stimulate an ethanol fermenting "anaerobic" culture operating for long periods of time. Periodic microaeration may help increase productivity and extend culture viability.

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