CONTINUOUS PRODUCTION OF LACTIC ACID
IN A MEMBRANE BIOREACTOR

by

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A thesis submitted in conformity with the requirements
for the degree of MASTER OF APPLIED SCIENCE
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ABSTRACT

The production of lactic acid in a cell-recycle bioreactor was studied. The performance of an internal Zeeweed™ microfiltration membrane was evaluated. The flux through the Zeeweed™ module was double that of the flux in a conventional Millipore unit with the identical filtration area. The study of the rheology of the fermentation broth showed that the system changed from Newtonian to pseudoplastic behaviour at cell densities above 130 g/L. The effects of dilution rate, initial glucose concentration and agitation rate on biomass and lactic acid concentrations, substrate utilization and permeate flux were assessed using factorial experimental design. High substrate conversion (99%) was obtained in all runs operated at the lower initial substrate concentration. The highest product concentrations (44.9 - 52.7 g/L) were obtained at the higher substrate concentration. Higher dilution rate and lower substrate concentration resulted in high biomass concentrations (35.6 g/L). Stable steady-state operation was achieved using continuous cell bleeding.
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# TABLE OF CONTENTS

ABSTRACT

ACKNOWLEDGMENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1. INTRODUCTION

2. OBJECTIVES

3. THEORETICAL BACKGROUND

   3.1 Lactic Acid, Its Uses and Methods of Manufacture

   3.2 Fermentation

      3.2.1 Lactic Acid Producing Microorganisms

      and Microbial Nutrition

      3.2.2 Metabolism and Metabolic Regulation

   3.2.3 Cell Growth

   3.2.4 Batch and Fed-Batch Fermentations

   3.2.5 Fermentation Kinetics

   3.2.6 Continuous Fermentation

3.3 Membrane Filtration

   3.3.1 Microfiltration and Ultrafiltration

   3.3.2 Cross-Flow Filtration vs. Conventional Filtration

   3.3.3 Cross-Flow Filtration vs. Conventional Filtration
3.4 Permeate Flux and Membrane Fouling

3.4.1 Concentration Polarization and Permeate Flux
3.4.2 Effect of Pressure on Flux
3.4.3 Effect of Temperature and Viscosity on Flux
3.4.4 Membrane Fouling
3.4.5 Fouling Control

3.5 Membrane Recycle Bioreactors and Their Application for Lactic Acid Production

4. MATERIALS AND METHODS

4.1 Bacterial Strain
4.2 Growth Medium
4.3 Experimental Equipment
4.4 Experimental Methods

4.4.1 Continuous Fermentation
4.4.2 Effect of Transmembrane Pressure on Permeate Flux in Systems with ZEEWEED™ and MILLIPORE Membranes
4.4.3 Influence of Increased Filtration Area on Permeate Flux through ZEEWEED™ Membrane
4.4.4 Rheological Properties of the Fermentation Broth
4.4.5 Preliminary Studies of Operating Conditions for the Membrane System
4.4.6 Full Factorial Design
4.4.7 Membrane Fermentation with Continuous Bleeding

4.5 Analytical Methods
   4.5.1 Biomass Concentration
   4.5.2 Glucose
   4.5.3 Lactic Acid

5. RESULTS AND DISCUSSION

5.1 Preliminary Assessment of the New ZEEWEED Membrane Performance in Lactic Acid Fermentation Process
   5.1.1 Effect of Transmembrane Pressure on Permeate Flux in Systems with ZEEWEED™ and MILLIPORE Membranes
   5.1.2 Influence of Increased Filtration Area on Permeate Flux through ZEEWEED™ Membrane

5.2 Rheological Properties of the Fermentation Broth

5.3 Experimental Design
   5.3.1 Operating Conditions for the Membrane System
   5.3.2 Full Factorial Design and Analyses

5.4 Membrane Fermentation with Continuous Bleeding of Biomass

6. CONCLUSIONS

7. RECOMMENDATIONS
8. NOMENCLATURE

9. REFERENCES

10. APPENDICES
LIST OF TABLES

Table 3.1  Physical Properties of Lactic Acid 7
Table 3.2  Typical Composition of Yeast Extract Produced by Autolysis 15
Table 5.1a  Overall Experimental Error 92
Table 5.1b  Summary of Regression Analysis for Biomass 92
Table 5.1c  Summary of Regression Analysis for Lactic Acid 93
Table 5.1d  Summary of Regression Analysis for Glucose Utilization 93
Table 5.1e  Summary of Regression Analysis for Permeate Flux 93
Table 5.2  Results of Lactic Acid Fermentation at Different Operating Conditions 94
Table 5.3  Summary Table of Results Obtained during Lactic Acid Fermentation at Different Operating Conditions 100
Table 5.4  The Carbon Mass Balance 102
Table 5.5  Results of Continuous Lactic Acid Fermentation with Bleeding of Cells and without Bleeding 109
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Fig.</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Formation of Lactate from Glucose</td>
<td>17</td>
</tr>
<tr>
<td>3.2</td>
<td>Typical Growth Curve for a Bacterial Population</td>
<td>22</td>
</tr>
<tr>
<td>3.3</td>
<td>Schematic Representation of Dead-End and Cross-Flow Filtration</td>
<td>32</td>
</tr>
<tr>
<td>3.4</td>
<td>Periods of Different Physical Phenomena during Flux Decline with Suspended and Dissolved Solutes in the Feed during Microfiltration</td>
<td>41</td>
</tr>
<tr>
<td>3.5</td>
<td>Membrane Recycle Bioreactor</td>
<td>46</td>
</tr>
<tr>
<td>4.1</td>
<td>Schematic Diagram of the Zeeweed™ Membrane System</td>
<td>53</td>
</tr>
<tr>
<td>4.2</td>
<td>Schematic Diagram of the AMICON Membrane System without Cell Bleeding</td>
<td>53</td>
</tr>
<tr>
<td>4.3</td>
<td>Schematic Diagram of the AMICON Membrane System with Cell Bleeding</td>
<td>53</td>
</tr>
<tr>
<td>5.1</td>
<td>Effect of Transmembrane Pressure on Permeate Flux for Zeeweed™ and Millipore Membranes</td>
<td>63</td>
</tr>
<tr>
<td>5.2</td>
<td>Water Flux vs. Transmembrane Pressure through Zeeweed™ and Millipore Membranes</td>
<td>66</td>
</tr>
<tr>
<td>5.3</td>
<td>Permeate Flux vs. Transmembrane Pressure for Zeeweed™ Membranes with Two Filtration Areas</td>
<td>68</td>
</tr>
<tr>
<td>5.4</td>
<td>Rheological Behaviour of the Fermentation Broth at Different Biomass Concentrations</td>
<td>71</td>
</tr>
<tr>
<td>5.5</td>
<td>Viscosity vs. Biomass Concentration during the Fermentation Process.</td>
<td>72</td>
</tr>
<tr>
<td>5.6</td>
<td>Permeate Flux and Broth Viscosity Profiles during Rheological Experiments</td>
<td>74</td>
</tr>
</tbody>
</table>
Fig. 5.7 Permeate Flow Rate Profile - trial run#1
Fig. 5.8 Cell Concentration Profile - trial run#1
Fig. 5.9 Permeate Flow Rate Profile - trial run#2
Fig. 5.10 Cell Concentration Profile - trial run#2
Fig. 5.11 Experimental Design Matrix
Fig. 5.12 Fermentation Kinetics of *L. rhamnosus* at $S_0=50$ g/L, $D=0.1$ h$^{-1}$ and $A=400$ rpm (run #1/9).
Fig. 5.13 Fermentation Kinetics of *L. rhamnosus* at $S_0=90$ g/L, $D=0.05$ h$^{-1}$ and $A=200$ rpm (run #2).
Fig. 5.14 Fermentation Kinetics of *L. rhamnosus* at $S_0=50$ g/L, $D=0.1$ h$^{-1}$ and $A=200$ rpm (run #3/7).
Fig. 5.15 Fermentation Kinetics of *L. rhamnosus* at $S_0=90$ g/L, $D=0.1$ h$^{-1}$ and $A=400$ rpm (run #4).
Fig.5.16 Fermentation Kinetics of *L. rhamnosus* at $S_0=50$ g/L, $D=0.05$ h$^{-1}$ and $A=400$ rpm (run #5/10).
Fig. 5.17 Fermentation Kinetics of *L. rhamnosus* at $S_0=90$ g/L, $D=0.05$ h$^{-1}$ and $A=400$ rpm (run #6).
Fig. 5.18 Fermentation Kinetics of *L. rhamnosus* at $S_0=90$ g/L, $D=0.1$ h$^{-1}$ and $A=200$ rpm (run #8).
Fig. 5.19 Fermentation Kinetics of *L. rhamnosus* at $S_0=50$ g/L, $D=0.05$ h$^{-1}$ and $A=200$ rpm (run #11).
Fig. 5.20 Fermentation Kinetics for Replicate Runs #1 and #9
Fig. 5.21 Fermentation Kinetics for Replicate Runs #3 and #7
Fig. 5.22 Fermentation Kinetics for Replicate Runs #5 and #10
Fig. 5.23 Effect of the Initial Glucose Concentration on the Product Yield
Fig. 5.24 Effect of the Dilution Rate on the Product Yield
Fig. 5.25 Effect of Continuous Bleeding on the Fermentation Kinetics of \textit{L.\textit{rhamnosus}}
1. INTRODUCTION

Lactic acid has many industrial uses. It is traditionally used in the food, pharmaceutical, and chemical industries, and recently its potential for producing biocompatible and biodegradable plastics is now being actively pursued (Goncalves et al., 1991; Jeantet et al., 1996).

The fermentation route for lactic acid production presently competes with a synthetic route, each process providing approximately half of the world’s supply of lactate. This competition has provided an impetus for improving the efficiency of the fermentation process. Fermented lactic acid is generally less expensive, but usually contains impurities such as carbohydrates, and consequently has less thermal stability than synthetic lactic acid. Additionally, the recovery processes required to produce fermented material comparable to the synthetic are expensive. A reduction in the fermentation costs could justify more expensive recovery techniques for the production of high quality lactic acid at competitive costs (Vick Roy, 1983).

Conventional methods of fermentation that use free cells in a batch process have several limitations, such as low productivity, product inhibition, and batch-to-batch variations in the product, leading to high fermentation costs (Tejayadi and Cheryan, 1995). Continuous fermentation, on the other hand, although overcoming some of the problems associated with batch processes is limited by cell washout. Process improvement can be obtained by working
continuously with systems providing high cell concentrations through cell recycling.

Different types of membranes can be used for cell recycling: dialysis (diffusive exclusion); electrodialysis (ionic exclusion); and microfiltration or ultrafiltration (molecular weight exclusion).

Analysis of the literature provides evidence of a surge of interest in developing new and efficient cell recycling systems. Hongo et.al. used a continuous dialysis fermentor to reduce the lactic acid concentration and increase the concentration of organisms (Hongo et.al., 1986). Jeantet et.al. demonstrated advantages of nanofiltration membranes coupled with a CSTR for the semicontinuous production of lactic acid (Jeantet et.al., 1996). A novel integrated fermentation system in which cross-flow filtration was coupled to an anion-exchange resin column was developed by Ye et.al. (Ye et.al., 1996). Continuous production of lactic acid by *Lactobacillus helveticus* from whey permeate was investigated by Aeschlimann and U. von Stockar (1989 and 1991), Mehaial and Cheryan (1986) and Jeantet et.al. (1996).

Although promising results were obtained in these studies, a membrane separation process is not without its problems. Foremost of these is accumulation of rejected solutes on the membrane surface during the course of fermentation that results in flux decline. This decreases the permeate flow rate and makes the process less cost efficient.

Therefore, development of new membranes and techniques in order to reduce fouling is an important task for industrial and academic research groups.
Zenon Environmental Inc. (Burlington, Ontario) is the largest Canadian manufacturer of membrane systems. In 1995, the University of Toronto initiated a cooperative research program with the company in order to develop a membrane-based fermentation process using Zenon Environmental Inc.'s proprietary Zeeweed™ membranes. The main difference of these membranes from the commercially available modules, is the principle of operation. The Zeeweed™ membrane operates as an internal module, i.e. it is placed into fermentation broth, cells are retained outside the membrane, in the surrounding fermentation medium, and permeate is withdrawn through the hollow fibers. It was expected that such an approach would result in improved permeate flux, since turbulent flow created by agitation in the fermentor would reduce thickness of the deposit layer on the surface of the membrane. In addition, in this system the cell viability could be improved dramatically, since microorganisms are exposed to much lower shear stresses than those passing through the recycling loop in conventional membrane systems.

Continuous ethanol fermentation using Zeeweed™ membranes was studied and productivity of 10 g/(L·h) was obtained (Radocaj, 1997). However, membrane fouling and the following flux decay limited the effectiveness of the process. As a result, a new Zeeweed™ membrane has been produced specifically for the project. The effective surface area of the new membrane was doubled in order to get higher flow rates, and the heat and mechanical resistance of the polymeric materials of construction was improved making it possible to sterilize the unit in autoclave at 121°C and 124 kPa.
This new modified membrane was used in this project in the study of lactic acid fermentation. Conversion of sugars during lactic acid fermentation is more efficient than in alcoholic fermentation since homofermentative lactobacilli do not produce by-products and consequently, higher product yields can be obtained. Another advantage is that unlike yeast, lactic acid bacteria do not form clusters that reduce the efficiency of the membrane separation process.

In a continuous fermentation process with full cell recycling, cell debris accumulates changing characteristics of a gel layer, and resulting in increased viscosity of the fermentation broth. Continuous bleeding could reduce this negative effect by removing part of biomass and products of cells lysis from the system. Additionally, it could improve the system stability, which is crucial for the process design and control in the larger scale fermentations.

In order to develop a predictive model for the performance of membrane-based bioreactor, an understanding of the effects of key operative parameters must be developed, prior to the design of an experimental program for determining optimal operating parameters. The objectives of this preliminary study were selected with a view of establishing the basis of a development program for the membrane-based production of lactic acid.
2. OBJECTIVES

The original objective of this research program was to evaluate the performance of the internal ZEEWEED™ membrane in a lactic acid fermentation system. Due to the inability of ZENON Environmental Inc. to supply appropriate membrane units, in time, the objectives were broadened to investigate the factors affecting the efficiency of lactic acid production in a membrane bioreactor in terms of biomass and product concentrations, substrate conversion, permeate flux, and system stability.
3. THEORETICAL BACKGROUND

3.1 Lactic Acid, Its Uses and Methods of Manufacture

Lactic acid (2-hydroxyproanoic acid, 2-hydroxypropionic acid) is an organic hydroxy acid whose occurrence in nature is widespread. It is produced by animals, plants and microorganisms.

Lactic acid was first isolated from sour milk by Scheele in 1780 (Benninga, 1990). The chemical and physical properties of lactic acid have been extensively reviewed by Holten (1971).

Lactic acid exists in two optically active isomeric forms shown below:

\[
\begin{align*}
\text{L(+) - lactic acid} & & \text{D(-) - lactic acid} \\
\text{H} & & \text{H} \\
\begin{array}{c}
\text{CH}_3 \\
\text{CO}_2\text{H}
\end{array} & & \begin{array}{c}
\text{CH}_3 \\
\text{CO}_2\text{H}
\end{array}
\end{align*}
\]

Although the L(+) form appears to be dextrorotatory, it may actually be levorotatory as are its salts and esters. The apparent reversal in optical rotation may be due to the formation of an ethylene oxide bridge between carbon atoms 1 and 2 by a tautomeric shift of the hydroxyl group on carbon atom 2 to the
The carbonyl group of the carboxyl radical shown below. Salts and esters of L(+)lactic acid cannot form this epoxide ring and are levorotatory (Vick Roy, 1985).

The L(+) isomer (sarcolactic acid, paralactic acid) is present in humans, although both the L(+) and D(-) isomers are found in biological systems. The lactic acid formed in fermentation processes is often a racemic mixture (DL forms). The L(+) isomer is the most important for the food industry, as humans can assimilate it only by producing the enzyme L-lactate dehydrogenase (Bozoglu and Ray, 1996).

Lactic acid is soluble in all proportions with water and exhibits a low volatility. Some physical properties of lactic acid are given in Table 2.1.

<table>
<thead>
<tr>
<th>Table 3.1. Physical Properties of Lactic Acid (Vick Roy, 1985)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight</td>
</tr>
<tr>
<td>Melting point: D(-) and L(+)</td>
</tr>
<tr>
<td>DL (varies with composition)</td>
</tr>
<tr>
<td>Boiling point DL</td>
</tr>
<tr>
<td></td>
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Table 3.1 (continued)

<table>
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<tr>
<th>Property</th>
<th>Value</th>
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<tbody>
<tr>
<td>Dissociation constant ($K_a$ at 25°C)</td>
<td>$1.37 \times 10^{-4}$</td>
</tr>
<tr>
<td>Heat of combustion ($\Delta H_c$)</td>
<td>1361 kJ mol$^{-1}$</td>
</tr>
<tr>
<td>Specific heat ($C_p$ at 20°C)</td>
<td>190 J mol$^{-1}$ °C$^{-1}$</td>
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Over the years, lactic acid has been applied in many processes and products. The first successful uses in the leather and textile industries began about 1894. In 1942 about 20% of the lactic acid produced was used in the food industry.

Today lactic acid finds medical applications as an intermediate for pharmaceutical manufacture, for adjusting the pH of preparations, and in topical wart medications. Biodegradable plastic made of poly (lactic acid) is used for sutures that do not need to be removed surgically, and it has been evaluated for use as a biodegradable implant for the repair of injuries (Vick Roy, 1985).

The largest single use of high quality lactic acid is for the production of stearyl-2-lactylates (20%). Stearyl-2-lactylates are mostly used in baking as dough conditioners and emulsifiers as well as emulsifiers in other food products and cosmetics.

More than 50% of all lactic acid is used directly as a food ingredient. It is used as a food acidulent because it naturally occurs in many foodstuffs, has a mild acid taste, and has no strong flavours or odours of its own. Lactic acid is also used as a preservative, sometimes in combination with other food acids such as propanoic and acetic.
The technical uses for lactic acid comprise a relatively small portion of the world's production. It is used in the manufacture of cellophane to control the pH in the film coating bath. It finds some uses in plastics for the production of phenol-formaldehyde resins. It is used in treating metal surfaces, the manufacture of rubber products, electrostatic painting, textile and paper printing, the 'brightening' of silk and rayon, and textile dyeing. It was once widely used for the deliming of hides and in other parts of leather manufacture. However, presently it has been largely replaced by sulphuric and formic acids. It is also used for the manufacture of some herbicides, fungicides and pesticides.

Lactic acid has potential for use in combination with other copolymers for the production of biodegradable plastics (Vick Roy, 1985).

The synthetic manufacture of lactic acid on a commercial scale began in the United States in 1963 (Bozoglu and Ray, 1996). Thorne (1969) reported that its first production in Japan took place around the same time. Today these two countries produce roughly 50% of the world's lactic acid (Vick Roy, 1985). Synthetic lactic acid production is based on the hydrolysis of lactonitrile by a strong acid such as HCl:

\[
\text{MeCH(OH)CN} + 2 \text{H}_2\text{O} + \text{HCl} \rightarrow \text{MeCH(OH)CO}_2\text{H} + \text{NH}_4\text{Cl}
\]

Synthetic lactic acid made substantial gains when it was introduced to the market place because the process used by-products from other synthetic routes, and perhaps more importantly, the production of stearyl-2-lactylates required a
high purity, heat-stable lactic acid. The synthetic lactic acid contains no residual sugars and does not discolour significantly upon heating (Vick Roy, 1985). This made synthetic production a serious competitor for traditional fermentation.

Lactic acid was first produced commercially using fermentation by Charles E. Avery at Littleton, Massachusetts, USA in 1881 (Vick Roy, 1985). Today fermentative methods of manufacture provide 50% of world's production of lactic acid. A number of advantages make this method attractive from economical and ecological points of view. Generally, fermentation is less expensive, for the reason that sources of free sugars, such as molasses, potatoes or starch, for mono- and disaccharides, are not only less costly to purchase but also are renewable. Further the processing costs for fermentation processes are lower than those for synthetic processes (Russo et al., 1996). One of the main drawbacks is high cost of downstream processes. Therefore, the improvement could be achieved working in two directions:

1) increasing the purity and concentration of the final product;

2) intensification of the fermentation process in terms of higher productivities.
3.2 Fermentation

3.2.1 Lactic Acid Producing Microorganisms and Microbial Nutrition

The lactic acid group of bacteria is generally defined with no precise boundaries (Bozoglu and Ray, 1996). It has been accepted that all of its members have the characteristic of producing lactic acid from hexoses. Lactic acid bacteria have a shape of rods with the length of 1 – 5 μm and diameter of 0.5 – 1 μm.

Based on the end products of glucose metabolism, those microorganisms that produce lactic acid as the only or major product are called homofermentatives, and those producing equal amounts of ethanol, carbon dioxide, and lactic acid are called heterofermentative. Only the homofermentative species are of commercial interest.

Although studies were carried out with Streptococcus cremoris (Bibal et.al., 1989) and Staphylococcus aureus (Jeantet et.al., 1996), the preferred species from the commercial point of view belong to genus Lactobacillus. The most important among them are Lactobacillus bulgaricus (Grobben et.al., 1997; Mehaial and Cheryan, 1986; Tejayadi and Cheryan, 1995) for lactose as a substrate and Lactobacillus delbrueckii, (Tsao and Hanson, 1975; Ye et.al., 1996; Vick Roy, 1983; Tsao and Hanson, 1972; Goncalves et.al., 1991; Moueddeb
et. al., 1996; Hongo et. al., 1986; Major and Bull, 1989; Wang et. al., 1995; Xavier et. al., 1994) for glucose.

Along with traditional glucose and lactose, other substrates were fermented in the fermentation studies. Aeschlimann and U. von Stockar investigated continuous lactic acid fermentation using *Lactobacillus helveticus* (Aeschlimann and U. von Stockar, 1989 and 1991) to ferment whey permeate.

Simultaneous saccharification and lactic acid fermentation was studied by Jun Luo et. al. using *Trichoderma reesei* or *Aspergillus niger* that produced enzyme cellulases for hydrolysis of cellulosic materials and *Lactobacillus delbrueckii* for lactic acid production (Jun Luo et. al., 1997).

Zhang and Cheryan (1992) studied fermentation production of lactic acid directly from starch using *Lactobacillus amylovorus*.

Microorganisms are extraordinarily diverse in their specific physiological properties, and correspondingly in their specific nutrient requirements. The chemical composition of the cell indicates the major material requirements for growth. Water accounts for some 80-90% of the total weight of cells and it always is therefore the major essential nutrient in quantitative terms. The solid matter of cells contains, in addition to hydrogen and oxygen, carbon, nitrogen, phosphorus and sulphur, in order of decreasing abundance (Doelle, 1994). These six elements account for about 95% of the cellular dry weight.

**Carbon** is the most abundant element and represents approximately 50% of the dry biomass (Doelle, 1994). All microorganisms require organic compounds both as a source of carbon and of energy. Thus, carbohydrates are
obviously among the most important nutrients for the growth of the microorganisms. Through catabolic and anabolic reactions, they provide energy for growth and for the synthesis of cellular material.

A large number of carbohydrate materials have been used, tested or proposed for the manufacture of lactic acid by fermentation. The following qualities are required for the selection of a substrate in industrial production: (1) low cost, (2) low levels of contaminants, (3) fast fermentation rates, (4) high lactic acid yields, (5) little or no by-product formation, (6) ability to be fermented with little or no pre-treatment, and (7) year-round availability (Vick Roy, 1985). The use of pentose sugars results in the production of acetic acid, which would require extra equipment for separation. Sucrose from cane and beet sugar, whey containing lactose, and maltose and dextrose from hydrolyzed starch are presently used commercially with sucrose as the most common substrate (Vick Roy, 1985). However, for laboratory scale fermentations glucose (Major and Bull, 1989; Hongo et al., 1986; Xavier et al., 1994; Goncalves et al., 1991; Vick Roy, 1983; Tsao and Hanson, 1972 and 1975) and whey permeate (Mehaial and Cheryan, 1986; Tejayadi and Cheryan, 1995; Aeschlimann and Stockar, 1989 and 1991; Jeantet et al., 1996; Boyaval, 1987) are most commonly used, the former due to higher lactic acid yields and availability, the latter due to lower price.

The next most important nutrient for lactic acid bacteria is nitrogen, which is required for amino acids, purine and pyrimidine biosynthesis. The organic nitrogen source is a mixture of amino acids. Malt sprouts, malt extract, com-
steep liquor, barley, yeast extract or undenatured milk are used in commercial practice. The amount of these is minimized in order to simplify the recovery processes (Vick Roy, 1985).

**Phosphorus** is utilized by microorganisms primarily to synthesize phospholipids and nucleic acids and thus, is required by the *Lactobacilli* for growth.

The other minerals required for growth of the microorganism, are **potassium** and **magnesium**. Of these, magnesium is an essential nutrient as it functions to stabilize ribosomes, cell membranes and nucleic acids. Magnesium is also required for the activity of many enzymes, especially those involving phosphate transfer. Gram-positive lactic acid bacteria require about 10 times more magnesium than do Gram-negative species. Without magnesium no growth is possible (Doelle, 1994). Potassium is universally required for the activation of some enzymes involved in protein biosynthesis.

The requirements for **trace elements** are difficult to determine since most macronutrients contain enough trace elements to satisfy the demand. The trace elements commonly required by most microorganisms are zinc, copper, manganese, and molybdenum. These metals function in enzymes or coenzymes (Doelle, 1994).

**Growth factors** are specific organic compounds that are required in very small amounts and can not be synthesized by the cell. Substances frequently serving as growth factors are vitamins, amino acids, purines and pyrimidines. Lactic acid bacteria do not synthesize aromatic amino acids and vitamins.
Growth factor requirements are greatest under anaerobic growth conditions and the least under aerobic conditions. In practice, requirements for growth are satisfied by the addition of yeast extract or peptone. Yeast extract is most commonly used for it serves also as the nitrogen source. An example of the composition of yeast extract is given in Table 3.2.

| Table 3.2 Typical Composition of yeast extract produced by autolysis (Crueger, W. and Crueger, A., 1984) |
| Composition (%) |
| Dry matter | 70 |
| Total nitrogen | 8.8 |
| Protein (N × 6.25) | 55 |
| NaCl | <1 |
| Amino acids (% of total) |
| Alanine | 3.4 |
| Amino butyric acid | 0.1 |
| Arginine | 2.1 |
| Asparagine | 3.8 |
| Cystine | 0.3 |
| Glutamic acid | 7.2 |
| Glycine | 1.6 |
| Histidine | 0.9 |
| Isoleucine | 2.0 |
| Leucine | 2.9 |
| Lysine | 3.2 |
| Methionine | 0.5 |
| Ornithine | 0.3 |
| Phenylalanine | 1.6 |
| Proline | 1.6 |
| Serine | 1.9 |
| Threonine | 1.9 |
| Tyrosine | 0.8 |
| Valine | 2.3 |
| Vitamin content (ppm) |
| Thiamine | 20-30 |
| Riboflavin | 50-70 |
| Pyridoxine | 25-35 |
| Niacinamide | 600 |
| Pantothenic acid | 200 |
(Data from Ohly Inc., Hamburg)
3.2.2 Metabolism and Metabolic Regulation

Microbial metabolism consists of thousands of individual chemical and enzyme-catalyzed chemical reactions. These chemical reactions in living organisms occur in characteristically organized sequences, called metabolic pathways. There are two main types of metabolic pathways:

a) pathways which lead from large (low oxidative state) to smaller molecules (high oxidative state), which are called catabolic pathways or catabolism;

b) pathways, which lead from small (high oxidative state) to large molecules (low oxidative state) essential for the formation of cellular material, which, are referred to as anabolic or biosynthetic pathways or anabolism.

The main concept of catabolism is therefore to provide the cell with small molecules or precursors suitable for biosynthesis of all major chemical constituents of the living cell and with energy to carry out these reactions (Doelle, 1994). Whereas all catabolic pathways are oxidative and thus energy producing, the biosynthetic pathways are reductive and energy consuming.

There are three catabolic pathways that lead from glucose, a 6-carbohydrate, to pyruvate, a 3-carbohydrate:

a) the Embden-Meyerhof-Pamas (EMP) pathway, often referred to as the glycolytic pathway;

b) the hexose monophosphate (HMP) pathway, often referred to as the pentose or ribose phosphate pathway;
c) the Entner-Doudoroff (ED) pathway, which so far has only been found in bacteria.

The homofermentative lactic acid bacteria use the EMP pathway. This pathway provides the greatest amount of energy as ATP, but does not produce the important precursors or intermediates for purine and pyrimidine biosynthesis and ribose 5-phosphate. Therefore Lactobacilli, as mentioned above, have specific growth factor requirements in order to build their nucleic acids (DNA, RNA) and aromatic amino acids (Doelle, 1994).

At the pyruvate level, the pathway divides depending upon the energy metabolism. Under aerobic conditions, pyruvate is oxidized via the tricarboxilic acid (TCA) cycle into water and carbon dioxide. The metabolism under anaerobic conditions is referred to as fermentation. The formation of lactic acid from glucose is represented schematically in Fig.3.1.

![Diagram](image)

**Fig.3.1. Formation of Lactate from Glucose**
In living cells, the rates of metabolic processes may be varied in response to environmental conditions in at least two ways. There exists a rapid mechanism operating within seconds or minutes for regulation of enzyme activity. The most common type of this regulation is a feedback regulation. The main principle is that the final metabolite of a biochemical sequence inhibits the action of an early enzyme of that sequence (Doelle, 1994).

There also exists a slower mechanism operating within hours or days that is dependent upon an increase or decrease in the number of enzyme molecules through a modification of the rate of enzyme synthesis. Of the thousands of enzymes a cell is capable of producing according to its genetic code, some are always present (constitutive), whereas others require their substrate (inductive). Induction is necessary in order to avoid wastage of energy or amino acids in making unnecessary enzymes but when needed, these enzymes can be formed rapidly. When the microbial cell is faced with more than one utilizable substrate, it has to make a choice. If it would produce all the enzymes necessary for the utilization of all the substrates present, it would be less economical than producing enzymes for the utilization of one substrate after the other. The cell thus produces enzymes to utilize the best substrate present first and only after the exhaustion of this primary substrate are the enzymes formed for the next substrate. This phenomenon is called catabolic repression and is referred to as diauxic growth. This results in two exponential phases in batch growth curves. Tsao. and Hanson (1975) observed the multiple exponential behaviour in the
batch growth curves of \textit{Lactobacillus delbrueckii} in a glucose-yeast extract medium.

### 3.2.3 Cell Growth

Despite their constant genotype, microbes are flexible in their ability to alter their composition and metabolism in response to environmental change. By virtue of metabolic regulatory mechanisms, microbial cells generally do not oversynthesize metabolites despite environmental variations. Both microbial growth and product formation therefore occurs in response to the environment. The requisite conditions for growth of biomass in a culture medium are:

a) an energy source;
b) nutrients to provide the essential material from which the cell is synthesized;
c) the absence of inhibitors;
d) optimal physicochemical conditions.

In constructing a culture medium for a fermentation, the primary goal is to provide a balanced mixture of the required nutrients at concentrations that will permit good growth. It might seem, at first sight, reasonable to make the medium as rich as possible by providing all nutrients in great excess. However, this approach is a wrong one. In the first place, many nutrients become growth inhibitory or toxic at high concentrations. This is true of many organic substances, such as salts of fatty acids and even sugars. Some inorganic...
constituents may also become inhibitory if supplied in excess. Second, even if
growth can occur in a concentrated medium, the metabolic activities of the
growing microbial population will eventually change the nature of the environment
to the point where it becomes highly unfavourable and the population becomes
physiologically abnormal or dies.

Lactic acid fermentation is well known for its sensitivity to end-product
inhibition. This inhibitory effect was definitely demonstrated by Friedman and
Gaden using dialysis cultures (Friedman and Gaden, 1970). Goncalves et.al.
studied the influence of glucose inhibition on growth and lactic acid production
kinetics of *Lactobacillus delbrueckii* and developed kinetic models considering
both substrate and product inhibition (Goncalves et.al., 1991).

Enzymes have a limited stability to pH and temperature. This is because
they are globular proteins whose precise three-dimensional structure is
maintained by weak noncovalent bonds. These bonds are weaker at higher
temperatures and proteins lose their structure and hence their catalytic activity
progressively as the temperature is increased. Similarly, extremes of pH lead to
changes in ionization states of certain amino acid side chains, destabilizing the
active structure. Thus, it is obvious that temperature and pH become the
important parameters of the process.

*Lactobacillus* has optimal growth temperature of 40-43°C (growth range 5-
45°C) and optimal pH between 5 and 7 (growth range 3.2-9.6) (Bozoglu and Ray,
1996). It has been described by Vick Roy (1985) and confirmed by a number of
other researchers (Jeantet et.al., 1996; Aeschlimann and Stockar, 1989; Sturr
and Marquis, 1992) that lactic acid bacteria have different pH optima for cell growth and lactic acid production with pH of 5.5 more efficient for lactic acid production and pH of 6.2-6.5 better for biomass development.

The composition of the culture medium, the biomass concentration, and the metabolite concentration generally change constantly as a result of the metabolism of the cells during growth. After the inoculation of a sterile nutrient solution with microorganisms and cultivation under optimal conditions, four typical phases of growth are observed: lag phase, log phase, stationary phase and death phase. If cell concentration or number is plotted against time, the so-called growth curve is obtained (Fig.3.2).

The lag phase is the adaptation period and depends largely on the preculture medium from which the inoculum is obtained. Because of the transfer to a new medium, several parameters will probably be altered for the cells in the inoculum: change in pH value, increase in nutrient supply, decrease of growth inhibitors. Accordingly, new transport systems for nutrients must be induced within the cells. If the organism has been grown in the same medium as that in which the experiment is carried out, all enzymes should be fully adapted and functional, and the lag phase should be the shortest. If, however, the preculture was grown under different conditions, the organism requires an adaptation period for carrying out the necessary metabolic changes.

Once the organism has adapted itself, balanced growth occurs and the population multiplies in such a way that a straight line relationship exists between
Fig. 3.2 Typical Growth Curve for a Bacterial Population
(Crueger, W. and Crueger, A., 1984)
the logarithm of cell mass or number and time. This particular phase is referred to as the exponential or logarithmic (log) phase of growth. This is the period of maximum growth.

Microbial populations seldom maintain exponential growth at high rates for long. It is normally limited either by exhaustion of available nutrients or by the accumulation of toxic products of metabolism. As a consequence, the rate of growth declines and can either continue for a while arithmetically or go straight into the stationary phase. This transition involves a period of unbalanced growth during which the various cellular components are synthesized at unequal rates. Consequently, cells in the stationary phase have a chemical composition that is different from that of cells in the exponential phase. They are also more resistant to adverse physical and chemical agents (Doelle, 1994). Due to lysis, new substrates (carbohydrates, proteins) are released, which then may serve as energy sources for the slow growth of the survivors (Crueger, W. and Crueger, A., 1984).

In the death phase, the energy reserves of the cells are exhausted. A straight line may be obtained when a semilogarithmic plot is made of survivors vs. time, indicating that the cells are dying at an exponential rate (Crueger, W. and Crueger, A., 1984).

Biomass growth can be expressed in terms of dry cell concentration. There are several ways to measure cell mass. The only direct way is to determine the dry weight of cell material in a fixed volume of culture by removing
the cells from the medium, drying, and then weighing them. Such determinations are time consuming and relatively insensitive (Doelle, 1994).

Optical measurement of microbial biomass is a more accurate method to estimate cell growth. The determination of the amount of light scattered by a suspension of cells is based on the fact that small particles scatter light proportionally to their concentration within certain limits. Absorbency (A) is defined as the logarithm of the ratio of light striking the suspension (I₀) to that transmitted by the suspension (I):

\[ A = \log \frac{I_0}{I} \]

3.2.4 Batch and Fed-Batch Fermentations

The simplest fermentation system is the traditional batch process. Batch fermentation can be considered as a closed system. At the time T=0 a sterile nutrient solution in the fermentor is inoculated with microorganisms and incubation is allowed to proceed under optimal physiological conditions. In the course of the entire fermentation, nothing is added except base to control pH.

Batch fermentation has been the method used industrially for lactic acid production. Fermentors are constructed of wood or stainless steel due to the high corrosivity of lactic acid, and are equipped with heat transfer coils for temperature control. Minimal agitation is provided by top or side mounted stirrers in order to keep the contents mixed. Fermentors are typically steamed, heated
with water to boiling or chemically sterilized before filling with a pasteurized medium (Vick Roy, 1985). Often, fermentor covers are loose fitting. Contamination is not a large problem. The ability of lactic acid bacteria to inhibit the growth of undesirable bacteria is due to numerous metabolic events, including production of organic acids, hydrogen peroxide and carbon dioxide; nutrient depletion; a decrease in redox potential; and synthesis of antibiotics and bacteriocins (Bhugaloo-Vial et al., 1997; Ahn and Stiles, 1990). The undissociated, electroneutral form of lactic acid rather than lactate appears to be the main inhibitor of cell growth (Crueger, W. and Crueger, A., 1984; Gatje et al., 1991). The most serious contamination problems are due to the production of butyric acid at the end of fermentation (Crueger, W. and Crueger, A., 1984).

Final product concentrations are less than 12-15%. Fermentation conditions are different for each industrial producer but are typically in the range of 45-60°C with a pH of 5.0-6.5 for L. delbrueckii (Vick Roy, 1985). The inoculum size is usually 5-10% of the liquid volume in the fermentor. The fermentation time is 1-2 days for 5% sugar sources such as whey and 2-6 days for a 15% sugar source such as glucose or sucrose. Reactor productivities are in the range of 1-3 kg m\(^{-3}\) h\(^{-1}\). The yield of lactic acid after the fermentation is 90-95% based on initial sugar or starch concentration. The residual sugar concentration is typically less than 0.1%.

An enhancement of the closed batch process is the fed-batch fermentation. In the fed-batch process, substrate is added in increments as the fermentation progresses. Fed-batch cultures may be operated to remove the
repressive effects of rapidly utilized carbon sources, to reduce the viscosity of the medium, to reduce the effect of toxic medium constituents or simply to extend the product formation stage of the process for as long as possible.

3.2.5 Fermentation Kinetics

Batch processes are widely used in academic research to study microbial kinetics. Based on the results of batch fermentations, kinetic models for continuous fermentations can be developed.

If all the requirements for the growth are satisfied, then the rate of increase in biomass is proportional to the amount of biomass present:

\[ \frac{dX}{dt} = \mu X \]

where \( \mu \) is the specific growth rate and \( X \) is the biomass concentration.

The specific growth rate is generally found to be a function of three parameters: the concentration of limiting substrate, the maximum growth rate, and a substrate-specific constant (saturation constant):

\[ \mu = \mu_{\text{max}} \frac{S}{K_s + S} \]

where \( \mu_{\text{max}} \) is the maximum specific growth rate, \( S \) is the concentration of a limiting substrate, and \( K_s \) is the saturation constant.

This equation is generally known as the Monod equation. The saturation constant, \( K_s \), is the substrate concentration at which half the maximum specific
growth rate is obtained ($\mu = 0.5 \mu_{\text{max}}$). The value of the saturation constant is generally very low. For *Lactobacillus rhamnosus* grown on glucose, the saturation constant was found to be below 0.3 g/L (Wang *et al.*, 1995). Thus, if there is an excess of all substrates, then $K_s \approx S$ and $\mu = \mu_{\text{max}}$. In this case, the culture is in the log phase at its maximal growth rate.

The maximum specific growth rate is dependent on the organism and on the conditions of fermentation. For simple substrates such as glucose, it is greater than for long-chain molecules since an organism needs extra energy to split long-chain substrates.

Lactic acid production is generally described by the Luedeking and Piret model:

$$\frac{dP}{dt} = \alpha \frac{dX}{dt} + \beta X$$

where $\alpha$ and $\beta$ are kinetic constants.

The glucose consumption during the exponential phase is a linear function of lactic acid production. The relationship between glucose consumption and lactic acid production can be described by:

$$\frac{dS}{dt} = \frac{1}{Y_{P/S}} \frac{dP}{dt}$$

where $Y_{P/S}$ is the product yield (Goncalves *et al.*, 1991).
3.2.6 Continuous Fermentation

The major disadvantage of batch-type fermentations, used for production of growth associated products, is that efficient product formation only occurs during a fraction of each fermentation cycle. The commonly observed exponential growth phase in batch microbial culture during which the highest specific growth rate is attained, can be maintained for short periods of time since the microbial environment is continuously changing. The development of continuous culture techniques eliminated this restriction by providing an essentially invariant microbial environment. This allowed constant, usually stable, or at least controllable growth rates that could be selected in a range from zero to approximately the maximum rate observed in a batch culture. Continuous systems with continuous high output can consequently be much more efficient in terms of fermentor productivity.

Continuous fermentations may be considered as open systems in which sterile nutrient solution is continuously added to the bioreactor and an equal volume of fermented medium is simultaneously removed from the system. Among the diverse kinds of continuous fermentation, two basic types can be distinguished (Crueger, W. and Crueger, A., 1984):

1. **Homogeneously mixed bioreactors.** These can be operated either as chemostats or turbidostats. In a chemostat at steady state, cell growth rate is adjusted to be equal to the dilution rate. Cell growth is controlled by adjusting the concentration of one substrate. Any required substrate (a
carbohydrate, a nitrogen compound or a salt) can be used as a limiting factor. Using the Monod equation (see Sec. 3.2.5) and assuming a steady state ($\mu=D$) the cell concentration is defined by the relationship:

$$X = X_{x/s}(S_0 - S) = X_{x/s} \left( S_0 - \frac{D \cdot K_s}{\mu_{\text{max}} - D} \right)$$

When substrate conversion is high, $S \to 0$ and

$$X = X_{x/s} \cdot S_0$$

In the turbidostat, cell growth is kept constant by using turbidity to monitor the biomass concentration and the rate of the nutrient addition is appropriately adjusted.

2. **Plug flow reactors.** In this type of continuous fermentors, the culture solution flows through a tubular reactor without back mixing. The composition of the nutrient solution, the number of cells, mass transfer and productivity vary at different locations within the system. At the entrance to the reactor, cells must be added along with the nutrient solution.

The upper limit of the specific growth rate in a chemostat is frequently different from that in a batch culture. It is often greater, but not usually by a significant amount. From material balance, at steady state the specific growth rate in a chemostat is equal to the dilution rate:

$$\mu = D$$

where $D$ is dilution rate, defined as feed flow rate ($F$) divided by the system volume ($V$):

$$D = \frac{F}{V}$$
The output rate of the chemostat is, therefore, limited since the dilution rate must be less than the maximum specific growth rate (Bull and Young, 1981). Additionally, conventional fermentation methods, using free microbial cells in batch or continuous fermentors, require the separation of cells from the medium at the end of each process, which increases downstream processing costs. To overcome these problems the use of membrane filtration with cell recycling was developed.

3.3 Membrane Filtration

Membranes may be used to concentrate cells, and to remove small metabolic product molecules.

Membrane filtration of cell suspensions can be defined as the pressure-driven separation of the components of a fluid mixture by selective permeation through an interface (the semipermeable synthetic membrane) separating the retentate stream from the permeate stream.

3.3.1 Microfiltration and Ultrafiltration

Ultrafiltration (UF) and microfiltration (MF) are conceptually very similar processes. The principle distinction between the two processes is in the degree of semipermeability of the membranes, and consequently, in the size of
components that can be separated. While there is some overlap in the definition of the processes, the term ultrafiltration is used to describe the separation of solutes that are much larger than the solvent, but still too small to be resolved under the optical microscope. UF membranes are impermeable to particles larger than 0.001 – 0.02 µm. UF membranes have “nominal” ratings, which refer to the smallest molecular size or molecular weight that will be mostly retained by the membrane, called the molecular weight cut off. MF membranes are designed to pass through much larger particles, up to the range of 0.02 – 10 µm. In order to characterize MF membranes, pore size is used since it can be measured directly, unlike molecular weight cut off (Gutman, 1987).

3.3.2 Cross-Flow Filtration vs. Conventional Filtration

In both, UF and MF membrane filtration processes the species that are unable to permeate through the membrane are rejected at the surface of the membrane rather than accumulating within the body of the membrane filter. This type of filtration is referred to as surface (as opposed to depth) filtration, and distinguishes membrane filtration from many types of more conventional filtration processes. In conventional surface filters, the filtered solids are allowed to build up as a cake at the surface of the filter. This is referred to as a dead-end filtration (Fig.3.3,a). If such a principle was adopted for membrane filtration, the permeation rate through the combined membrane and surface cake layer would,
Feed

\[\text{Particle build-up on membrane surface}\]

\[\text{Particle-free permeate}\]

a) Dead-End Filtration

Feed \[\rightarrow\] Retentate

\[\text{Particle-free permeate}\]

b) Crossflow Filtration

Fig. 3.3 Schematic Representation of Dead-End and Crossflow Filtration (Gutman, 1987)
in most applications, quickly fall to very low levels (Gutman, 1987). Therefore, in all applications of UF and in many applications of MF, crossflow is used to prevent this cake formation.

The term crossflow refers to flow of the feed stream tangentially over the surface of the membrane, in order to sweep rejected solutes away from the membrane (Fig.3.3,b). Because of the use of crossflow, membrane filtration processes separate fluids into two product streams. These are the permeate and retentate.

Historically, microfiltration and ultrafiltration have been the most prominent membrane bioseparations. There are very many similarities between these two processes carried out in the crossflow mode. Both work primarily by size exclusion, permitting smaller species to pass through a membrane while larger ones are retained. The hydrodynamic pattern is similar, the hardware is very similar as well. However, there are differences between the two processes.

The membranes used for ultrafiltration have asymmetric (anisotropic) structures. These asymmetric membranes consist of a thin dense top-layer with a thickness of less than 1µm supported by a porous sub-layer with a thickness of about 100 µm. The separation characteristics of these membranes are determined only by the thin (dense) top-layer (Mulder and Smolders, 1986). The membranes used for microfiltration have isotropic porous structures, with pores of uniform size throughout the body of the membrane.

Although the effective thickness of the asymmetric UF membranes is much smaller compared to that of the porous MF membranes, the hydrodynamic
resistance is much higher. Therefore, the applied pressure must be also higher. In microfiltration, low pressures are used, 10 to 200 kPa but mostly less than 100 kPa. In ultrafiltration, pressures in the range of 200 to 1000 kPa are used (Mudler and Smolders, 1986). The hydrodynamic resistance of the ultrafiltration membranes is higher and the dimensions of the rejected particles (or molecules) are smaller. Ultrafiltration is therefore used when macromolecules (e.g., proteins) or small particles (e.g., colloids) have to be rejected while microfiltration is used for particles larger than 0.1 μm in diameter (e.g., bacteria).

There are two kinds of cross-flow filtration: low-shear and high-shear cross-flow filtration. Since in high-shear filtration much higher flow velocities are applied, it is not suitable for separation of cell suspensions in applications where high cell viability is important to maintain, such as fermentation. On the other hand, the cleaning efficiency of the sweeping liquid flow in low-shear filtration is rather limited, which causes filter-clogging problems in many practical applications.

3.4 Permeate Flux and Membrane Fouling

3.4.1 Concentration Polarization and Permeate Flux

During ultrafiltration, i.e. when the wall is porous, solute is brought to the membrane surface by convective transport, and a portion of the solvent is
removed from the fluid. This results in a higher local concentration of the solute at the membrane surface as compared to the bulk. This solute build-up is known as "concentration polarization" and is chiefly responsible for the flux decline compared to pure water (Cheryan, 1986).

In ultrafiltration of macrosolutes, concentration polarization has been modeled by balancing the convective drag of solutes toward the membrane with their back-diffusion (Michaels, 1968). The expression for the permeation flux obtained from this gel polarization analysis for completely retained solutes is a function of the mass transfer across the polarized layer:

\[ J(z) = K(z) \ln \left( \frac{C_g}{C_b} \right) \]

where \( C_g \) and \( C_b \) are the interface and bulk concentrations respectively; \( K(z) \) is the mass transfer coefficient, usually obtained from known correlation of laminar and turbulent nonporous duct flows; and \( z \) is the axial distance (Nagata et al., 1989). Mass transfer coefficients for laminar and turbulent flows through a channel can be found:

\[ K_L = 1.62 \left( \frac{UD^2}{d_hL} \right)^{0.33} \]

\[ K_T = 0.023 \frac{U^{0.8}D^{0.67}}{d_h^{0.2}\nu^{0.47}} \]

where \( U \) is the average fluid velocity, \( D \) is the diffusivity of the solute, \( d_h \) is the equivalent hydraulic diameter, \( L \) is the channel length, \( \nu \) is the kinematic viscosity, and \( K_L \) and \( K_T \) are mass transfer coefficients for laminar and turbulent flows respectively.
From the above equations it is obvious that for the laminar flow mass transfer coefficient can be increased by increasing the velocity of fluid through the tube or by decreasing the channel diameter within the laminar flow regime. For the turbulent flow, the mass transfer coefficient can be much more effectively improved by increasing the flow velocity \( U^{0.8} \) rather than by decreasing the channel diameter \( d_{in}^{0.2} \).

Increasing the velocity through the channels results in increasing the shear stress at the surface of the membrane:

\[
\tau_w = \mu \gamma \quad \text{and} \quad \gamma = \frac{8U}{d} \quad \text{(for tubes)}
\]

where \( \tau_w \) is the wall shear stress, \( \mu \) is the viscosity of the fluid, \( \gamma \) is the shear rate at the wall, \( d \) is the tube diameter.

However, it was generally observed that the flux obtained with particulate suspensions is not nearly as concentration dependent as is ultrafiltration of macrosolutes. Porter (1979) suggested that a “backdiffusion” of particles away from the membrane surface is the cause of high fluxes observed with suspensions. This backdiffusion is attributed to the “tubular pinch effect”, which manifests itself as a radial migration of particles away from the walls when a suspension is flowing along the tube wall. It is a real effect observed in a large number of model systems (Porter, 1979). Hanisch postulated that if the tubular pinch effect were to be a major factor in cross-flow filtration of cells, the filtration rate would be dependent on fluid velocity and independent of concentration up to a critical cell concentration (McGregor, 1986). Such a phenomenon was observed in small-diameter hollow fibers. At a critical concentration, the
resistance to radial migration increases owing to particle-particle interactions. At this concentration, the net particle transport is toward the membrane, and classical gel polarization then controls the system.

3.4.2 Effect of Pressure on Flux

It is known in ultrafiltration theory and practice that it is desirable to keep the pressure differential across the membrane at a relatively low value. High transmembrane pressure can cause compaction of the concentration polarization layer, which in turn results in decreased hydraulic permeability. Further increase of pressure does not result in increase of flux. In this region, flux is controlled by mass transfer. This is also observed in cell suspensions, although the effect is less marked (McGregor, 1986). If fluid velocities are kept high, then little polarization occurs, and it is generally possible to work at higher pressures.

3.4.3 Effect of Temperature and Viscosity on Flux

Temperature effects follow the Arrhenius relationship:

$$J = J_0 e^{-E_a/RT}$$

where $J$ is the flux, $J_0$ is the flux at 25°C, $E_a$ is the activation energy, $R$ is the universal gas constant, and $T$ is the absolute temperature.
In general, higher temperatures will lead to higher flux in both the pressure-controlled region and in the mass transfer-controlled region. In the pressure-controlled region, the effect of temperature on flux is due to its effect on fluid density and viscosity. Since the activation energies are the same for both flux and viscosity in the region 20 - 50°C, it will take a temperature rise of 30 - 45°C in order to double the flux (Cheryan, 1986).

In the mass transfer-controlled region, mass transfer coefficient is directly proportional to $D^{0.67}$ and inversely proportional to $(\eta/\rho)^m$, where $D$ is diffusivity, $\eta$ is viscosity, $\rho$ is density, $m=0.47$ for turbulent and $0 - 0.16$ for laminar flow systems. Thus, temperature is expected to have a significant effect, since diffusivity increases with temperature according to the modification of the Stokes-Einstein equation:

$$D_i = \frac{D_j \eta_j T_j}{T_i \eta_i}$$

For example, the diffusivity of proteins increases at approximately 3 - 3.5% per °C rise in temperature (Cheryan, 1986).

Viscosity is the property of a fluid that has the most significant effect on flow behaviour and thus on flux. It is defined as:

$$\eta = \frac{\tau}{\gamma},$$

where $\tau$ is shear stress and $\gamma$ is shear rate.

Fluids obeying this equation are known as Newtonian. Fluids that do not obey this equation and their viscosity is not independent of shear rate or shear
stress, are known as non-Newtonian. Their behaviour is commonly modelled according to the "power-law" model:

\[ \tau = k \gamma^n \]

\[ \eta_{ap} = k \gamma^{n-1} \]

where \( \eta_{ap} \) is apparent viscosity, \( k \) is the consistency index and \( n \) is the flow behaviour index.

The nomenclature used for the different types of dependence of viscosity on shear rate is as follows:

\[ \frac{d\eta}{d\gamma} < 0 \] : shear thinning or pseudoplastic \((n < 1)\);

\[ \frac{d\eta}{d\gamma} = 0 \] : Newtonian \((n = 1)\);

\[ \frac{d\eta}{d\gamma} > 0 \] : shear-thickening or dilatant \((n > 1)\).

Viscosity decreases with increasing temperature, increases with increasing feed concentration, and either decreases, increases, or is unaffected by shear rate, depending on the nature of the fluid (Cheryan, 1986).

Generally, bacterial fermentation systems operate as Newtonian liquids up to certain levels of cell density, specific for each strain. When biomass concentration exceeds this critical level, the fermentation broth changes its rheological behaviour to pseudoplastic. Crespo and Xavier (1992) reported these values for \( L.plantarum \) and \( P.acidi-propionici \): 60 g/L and 90 g/L respectively.
3.4.4 Membrane Fouling

Five periods of different physical phenomena could be defined during flux decline with suspended cells in a fermentation broth (Nagata et al., 1989):

1. **Fast Internal Sorption of Macromolecules**.

Adsorption already occurs before pressure has been applied and the membrane process has been started. During the very early phases of the run, the membrane is exposed to the dissolved macromolecules in the culture medium. Solute molecules will adsorb on to membrane surface due to physico-chemical interactions. The nature of the membrane material, the type of solute, and the solute concentration are parameters that may determine the extent of adsorption (Noble and Stern, 1995). If the membrane chemistry is such that these dissolved macromolecules sorb onto the membrane surface, then permeation rate will decline (Fig.3.4, Period 1). The kinetics of macromolecule sorption is thought to be fast and the binding constants high. For adsorption of cellular materials to many surface types, the primary requirement is deposition of a proteinaceous film on that surface. The critical thickness of the film is in the approx. 200 A. After this thickness has been attained, the first cellular components adhere to the surface (Fig.3.4, Period 2) (McGregor, 1986). Control of adsorptive fouling is particularly difficult. The general rule is that complex media with large organic and proteinaceous burdens are to be avoided whenever possible. Cell lysis can also generate proteinaceous material. Within any given fermentation there is a population of lysing cells that release high molecular weight cellular components.
Fig. 3.4 Periods of different physical phenomena during flux decline with suspended and dissolved solutes in the feed during microfiltration (Nagata et al., 1984)
into the medium.

When all the sorption sites are occupied, a pseudo steady state is reached (Fig.3.4).

2. **Build-up of First Sublayer**

During this period, the suspended cells begin to deposit onto the membrane slowly increasing the sublayer coverage. Since for most of this period monolayer coverage has not yet been attained, there is little effect on the permeate rate. Isolated particles or clumps offer very little resistance to permeation flow. As monolayer coverage approaches, the permeation flux begins to decline toward that observed in Period 3 (Fig.3.4).

3. **Buildup of Multisublayers**

During this period, the flux of solids towards the membrane remains relatively constant at a maximum since the concentration in the bulk solution continually increases while the permeation rate continually decreases. The product of these two is a measure of the solid flux to the wall. The mass-transfer coefficient: \( k(z) \) for the permeating fluid is a constant for this period as can be seen by the constant negative slope of the curve in Period 3 (Fig.3.4). Several sublayers are built up, thus affecting both the cross-flow and permeation velocities. The cross-sectional area for axial flow is reduced increasing the wall shear rate and axial pressure gradient. This results in increased back-migration of solids due to shear-induced back diffusion and/or inertial lift. Two competing effects influence the permeation velocity: increased transmembrane pressure provides additional driving force for an increased permeation velocity, but it also compresses the
sublayers, thereby reducing the permeation velocity. As the sublayers grow and become densified, the constant solids flux rate declines to that of Period 4.

4. Densification of Sublayers

After the sublayer growth has stabilized, the permeation rate declines rather slowly since the mass-transfer coefficient for permeate flow is mainly affected by particle rearrangement rather than the net deposit of additional solids within the sublayers. This densification of the sublayers continues while the bulk concentration increases rapidly until the viscosity of the bulk solution becomes sharply non-Newtonian in Period 5.

5. Increase in Bulk Velocity

As the concentration of particles in the bulk solution increases and approaches that of the sublayers, axial pumping and lateral permeation of the very viscous non-Newtonian solution becomes difficult. A precipitous drop in permeation velocity is observed. This period is rarely achieved in fermentation systems where the system remains Newtonian, since cell concentrations are below the critical level.

3.4.5 Fouling Control

The ultimate determinant of fouling is the membrane itself. Different membranes have different adsorption characteristics, so that each membrane is
very much application specific. Hanisch observed flux differences (up to four-fold) between different strains of *E.coli* grown under identical fermentation conditions using a polypropylene microporous hollow fiber membrane (McGregor, 1986).

Cleaning is another important fouling control technique and is effective on both the adsorbed film and the gel layer. Ideally, a number of modular systems should operate in parallel. The cleaning frequency is determined by the rate of fouling and the procedure must be effective and relatively rapid. However, partial flux restoration may be achieved by permeate closure during the process. This partially resuspends the layer of cells built up at the membrane surface; consequently, there is less resistance to flow from the retentate to the membrane.

New approaches have been investigated that introduce secondary flows in membrane systems that disturb the mass boundary layer near the membrane surface. These include vortex mixing from pulsatile reversing turbulent flows (Stairmand and Bellhouse, 1985) and Taylor vortices from rotational and axial flow between two concentric cylinders (Kroner *et.al.*, 1987). Excellent performances were obtained with both these methods. However, both methods have technical limitations besides their high energy costs.

Costigan *et.al.* studied singular and multi-start screw-thread inserts in tubular membranes to enhance filtration fluxes (Costigan *et.al.*, 1999). The geometry resulted in a continual corkscrew vortex, which enhances mixing and reduces concentration polarization.
The performance of hollow fiber membrane can be improved by placing a membrane module in the fermentation broth providing an internal mode of operation, i.e. permeate is withdrawn through hollow fibers and retained solutes remain in the surrounding medium. The turbulent flow created in the vicinity of the membrane surface “sweeps” away the accumulated solute reducing effects of concentration polarization (ZENON INC., 1999)

3.5 Membrane Recycle Bioreactors and Their Application for Lactic Acid Production

The basic concept behind the recycle bioreactor is shown in Fig.3.5. A reaction vessel operated as a stirred tank reactor is coupled in a semi-closed loop configuration via a suitable pump to a membrane module containing the appropriate semi-permeable membrane. In operation, the reaction vessel is first filled with the substrate solution and the biocatalyst added at the appropriate concentration. The contents of the reaction vessel are continuously pumped through the membrane module and recycled back to the reaction vessel. Product molecules small enough to permeate through the pores of the membrane will be removed from the system while the biocatalyst will be recycled to the reaction vessel for further reaction. The total volume of the system is maintained constant by matching the incoming feed flow rate to the product outflow (the permeate flux).
Fig.3.5 Membrane Recycle Bioreactor
The recycle bioreactor is usually operated as a CSTR under “completely mixed” conditions. It implies that if the product and the substrate permeate freely through a membrane, the concentration of the product and unhydrolized substrate in the product stream and the reaction vessel will be essentially the same at any given time. Thus, the CSTR-type recycle bioreactor is more suited for substrate-inhibited reactions than for the product-inhibited reactions, when the conversion is high. Ideally, the bioreactor should be operated such that the product concentrations are below the level at which severe inhibition occurs (Cheryan, 1986).

The utilization of “semipermeable” membranes for lactic acid production has been extensively investigated. A batch dialysis system was used by Freidman and Gaden, in which a 60% increase in the fermentation rate was achieved by reducing the lactic acid concentration (Freidman and Gaden, 1970). The experiments were performed using *L. delbrueckii* on a glucose medium. Stieber *et al.* non-aseptically fermented whey in a continuous dialysis fermenter without contamination; 97% conversion of lactose was achieved (Stieber *et al.*, 1977). The main drawback of simple dialysis systems, however, is that the reaction rate is limited by the rate at which substrate and product can diffuse across the membrane. Pressure-driven membrane processes proved to be more efficient in the process.

The membrane recycle fermentor has several advantages over the conventional fermentor. Since feed is continuously entering the system, cell growth continues during the run as in conventional continuous culture systems.
However, there is no cell "washout", when the dilution rate exceeds the specific growth rate of the microorganism, because the cells will be retained within the system by the membrane. Thus, exceedingly high cell concentrations and high dilution rates are possible with these systems; either could result in high productivity.

Enhanced lactate productivities were obtained by Bull and Young (1981) and by Major and Bull (1989) who investigated the effect of partial recycle of microbial cells on the operation of a chemostat compared to a chemostat without cell recycling.

Zhang and Cheryan (1992) applied ultrafiltration using a hollow fiber type membrane filter to separate cells of *Lactobacillus amylovorus* during lactic acid fermentation from starch and cells of *Lactobacillus bulgaricus* during lactic acid production from whey permeate (Mehaial and Cheryan, 1986; Tejayadi and Cheryan, 1995). A tubular ultrafiltration membrane reactor was studied by Xavier et.al for lactic acid production using *Lactobacillus delbrueckii* (Xavier et.al. 1994). In both membrane systems higher lactic acid concentrations and higher productivities were obtained in long-term fermentations compared to other high cell density systems.
4. MATERIALS AND METHODS

4.1 Bacterial Strain

The organism used was *Lactobacillus rhamnosus* NRRL B445 (formerly *Lactobacillus delbrueckii*), a facultative anaerobe, gram-positive, homofermentative, mainly $L(+)$ lactic acid producer. It was obtained from ATCC (USA) in lyophilized form. A 50% solution (v/v) with 20% glycerol was made and stored at -20°C.

4.2 Growth Medium

The culture medium had the following composition:

- Yeast extract (Difco) - 15 g/L;
- $K_2HPO_4$ - 0.2 g/L;
- $KH_2PO_4$ - 0.2 g/L;
- $MgSO_4 \cdot 7H_2O$ - 0.1 g/L;
- $MnSO_4 \cdot H_2O$ - 0.03 g/L;
- Tween-80 - 0.1% (v/v).

The amount of glucose was varied depending on the conditions of the experiment. All chemicals were of analytical grade.

The medium was sterilized at 121°C and 124 kPa for 30 min. Glucose was sterilized separately (to avoid caramelization) and combined aseptically with the rest of the nutrients after cooling to room temperature.
4.3 Experimental Equipment

The bioreactor consisted of a 2-L fermentation vessel (MULTIGEN, USA) fitted with temperature control and agitation. A microfiltration membrane module was connected to the bioreactor. The following cartridges were used in the experiments to separate cells from lactic acid:

- a Zeeweed™ microfiltration membrane (ZENON ENVIRONMENTAL INC., Canada) – hollow fibers with the following characteristics:
  
  - pore size 0.1 μm;
  - internal fiber diameter 1.7 mm;
  - total surface area 0.0155 m²;

- a Zeeweed™ microfiltration membrane (ZENON ENVIRONMENTAL INC., Canada) – hollow fibers with the following characteristics:
  
  - pore size 0.1 μm;
  - internal fiber diameter 1.7 mm;
  - total surface area of 0.033 m²;

- an AMICON Diaflo hollow fiber cartridge H1MP01-43 (AMICON INC., USA) – polysulfone hollow fibers with the following characteristics:
  
  - pore size – 0.1 μm.
  - internal fibre diameter – 1.1 mm;
  - total surface area – 0.03 m²;

Two peristaltic pumps (PERISTA™, model SJ-1220, APPLIED SCIENCE, USA and MASTERFLEX, COLE-PARMER, USA) were used for the feed and
permeate flow control, respectively. Cell bleeding was performed using PERISTA™, model SJ-1211 peristaltic pump (APPLIED SCIENCE, USA).

The temperature in the fermenter was maintained at 42°C. The pH was controlled at 6.2 or 5.5 (depending on the experiment) by the addition of 2M NH₄OH or 5M NaOH solutions with an automatic pHstat (METROHM system, BRINKMANN Instruments, Canada).

After each run membranes were cleaned. The Zeeweed™ membranes were cleaned with 200 ppm NaOCl solution at 40°C and rinsed with distilled water. The AMICON membrane was cleaned with 5% solution of enzyme detergent (Terg-A-zyme®, ALCONOX, Inc.) and rinsed with distilled water.

After cleaning water flux was measured to ensure the membrane cleanliness.

Membranes were stored in 20 ppm solution of NaOCl if not immediately used.

The fermentation vessel, tubing, and the Zeeweed™ membranes were sterilized in autoclave at 121°C and 124 kPa for 30 min.

The AMICON membrane was sterilized by contact with a 200-ppm solution of NaOCl for 2 hours at room temperature followed by rinsing with 15 L of sterile water.

The preculture consisted of two successive inoculations: at 1% (v/v; 16-17 h of growth at 42°C without agitation and pH control) and 5% (v/v; 24 h at the same conditions), which were made into the fermentation broth before inoculation of the bioreactor (7%, v/v).
4.4 Experimental Methods

4.4.1 Continuous Fermentation

Schematic diagrams of the continuous membrane fermentation systems are shown in Figures 4.1, 4.2 and 4.3.

The fermentor (1) consisted of a 2 L glass vessel with magnetic stirring bar and impeller for agitation. The microfiltration module (2) was placed into the fermentation broth when the Zeeweed™ membrane was used (Fig. 4.1) or connected to the bioreactor using silicon tubing when the Millipore membrane was used (Figures 4.2 and 4.3). Cell-free permeate was withdrawn through the hollow fibers of the membrane using the peristaltic pump (7) and collected in reservoir (3). Fresh medium was added at a desired dilution rate from reservoir (4) using peristaltic pump (6). The pH was controlled by the pHstat (5). Bleeding was performed using peristaltic pump (8).

The total volume of the system was 1L (0.9 L in the fermentor and 0.1 L in the recycling loop).
Fig. 4.1 Schematic Diagram of the Zeeweed™ Membrane System

Fig. 4.2 Schematic Diagram of the AMICON Membrane System without Cell Bleeding.

Fig. 4.3 Schematic Diagram of the AMICON Membrane System with Cell Bleeding.
4.4.2 Effect of Transmembrane Pressure on Permeate Flux in Systems with ZEEWEED™ and MILIPORE Membranes

ZEEWEED™ with a surface area of 0.033 m² and MILIPORE with a surface area of 0.03 m² membranes were tested. Schematic diagrams of the systems are presented in Figures 4.1 and 4.2.

The physical fermentation conditions were identical for the two systems: pH of 6.2, temperature of 42°C, and agitation rate of 200 rpm. 5M NaOH solution was used for pH control. The initial glucose concentration was 100 g/L. After inoculation the system was left in a batch mode for 7 hours and then – in a continuous mode for overnight (17 h) at a dilution rate 0.03 h⁻¹. Dilution rate was defined through all experiments as:

\[ D = \frac{F}{V} \]

where \( F \) is feed flow rate and \( V \) is the volume of the system.

Constant volume was maintained by matching the incoming flow rate, consisting of the feed and the alkali solution, to the rate of the permeate flow.

At each value of transmembrane pressure, four replicate samples of the permeate volume were collected.

After changing the pressure differential, a system was left for 1 hour to stabilize before the samples were collected.

Permeate flux was calculated according to the formula:

\[ J = \frac{F}{S} \]

where \( F \) is the permeate flow rate in L/h and \( S \) is the membrane filtration area in m².

Average values of four replicates were used in the analyses of the data.
4.4.3 Influence of Increased Filtration Area on Permeate Flux through ZEEWEED™ Membrane

Two ZEEWEED™ membranes with 0.0155 m² and 0.033 m² surface areas were used in this experiment. Schematic diagram of the system is shown in Fig.4.1.

The conditions of fermentation were the same as in the previous experiments (Sec.4.4.2). The pH was controlled using 5M NaOH solution. The initial concentration of glucose was 100 g/L. After inoculation the system was left in a batch mode for 7 hours and then in a continuous mode overnight (17 h) at a dilution rate of 0.03 h⁻¹.

At each value of transmembrane pressure a sample of the permeate volume was collected. After changing the pressure differential a system was allowed to stabilize for 1 hour. Permeate flux was calculated as described earlier.

4.4.4 Rheological Properties of the Fermentation Broth

In this experiment, 75-hour fermentation runs were performed. The main goal in the fermentation process was to determine the viscosity of the system at high cell densities.

The temperature, pH and initial glucose concentration were the same as in the previous runs (Sec.4.4.2, 4.4.3), and the agitation rate was 140 rpm (in order to minimize the mechanical stress to which the cells were exposed). The pH was controlled using 2M NH₄OH. A schematic diagram of the system is presented in Fig.4.2.

The first 8 hours of fermentation were carried out in a batch mode. Then the mode of operation was changed to continuous. The dilution rate during the
continuous process was varied depending on the fouling state of the membrane. After 73 hours of fermentation, feeding was stopped and the fermentation broth was concentrated by means of microfiltration, in order to obtain higher cell densities for the viscosity analysis.

After the first 25 hours of operation a leak in the ZEEWEED™ membrane hollow fibres was detected. The experiment was continued with a MILLIPORE microfiltration membrane of identical area (0.03m²).

The viscosity of the fermentation broth was measured at 42°C using a BROOKFIELD rotational viscometer, model LV (BROOKFIELD ENGINEERING LAB. Inc., USA). A small sample adapter of coaxial cylinder geometry was used. The viscometer measured the torque required to rotate a cylindrical spindle (25 mm x 90 mm) immersed in the test fluid. For a given viscosity resistance to flow is proportional to the spindle’s speed of rotation and is related to the spindle’s size and shape (geometry). Measurements made using the same spindle at different speeds were used to measure and evaluate the rheological properties of the fermentation broth.

The viscosity was measured at the following four speeds of rotation of the spindle: 6 rpm, 12 rpm, 30 rpm and 60 rpm. These provided shear rates of 7.34 s⁻¹, 14.7 s⁻¹, 36.7 s⁻¹ and 73.4 s⁻¹ respectively.
4.4.5 Preliminary Studies of Operating Conditions for the Membrane System

The system with a MILLIPORE membrane was used in this experiment (Fig. 4.2).

The fermentation run consisted of three periods:

i) Inoculation and batch mode for the first 7 hours at the following conditions:
   pH - 6.2, temperature - 42°C, agitation rate - 200 rpm, initial concentration of glucose - 100 g/L.

ii) Continuous mode overnight at the following conditions:
    pH - 6.2, temperature - 42°C, agitation rate - 200 rpm, initial concentration of glucose - 100 g/L, dilution rate - 0.05 h⁻¹.

iii) Continuous mode at the following conditions:
     pH - 5.5, temperature - 42°C, agitation rate - 400 rpm, initial concentration of glucose - 50 g/L, dilution rate - 0.1 h⁻¹.

Samples of fermentation broth for the biomass concentrations were withdrawn every 2-3 hours. At the same time intervals permeate was collected and the flux was calculated.
4.4.6 Full Factorial Design

A full factorial experimental design was used for studying three parameters simultaneously in this experimental series.

Each run consisted of two phases. The first phase was carried out identically in all runs and consisted of the following sequence of steps:

- inoculation;
- continuous operation for 25-27 hours with 100 g/L of initial glucose concentration at 0.05 h\(^{-1}\) dilution rate, aimed to achieve sufficient cell densities (10 g/L – 14 g/L); agitation rate was 200 rpm and pH was 6.2.

In the second phase the operating conditions were varied according to the factorial design setting the values of the initial glucose concentration, dilution rate and agitation rate. The temperature was maintained at 42°C and pH was maintained at 5.5 since this value was reported to be optimal for lactic acid production (Vick Roy, 1985).

Biomass concentration, residual substrate concentration, and lactic acid concentration were measured during the course of fermentation every 2-3 hours. Permeate flux was measured at the end of the process to give an indication of membrane fouling.

Each run was allowed to operate for 4-5 residence times. Data obtained at the end of this period was used for statistical analysis.

The following process characteristics were calculated:

a) substrate utilization (%)
\[ U = \frac{S_0 - S}{S_0} \times 100 \]

where \( S_0 \) is substrate concentration in the feed and \( S \) is substrate concentration in the sample.

b) volumetric productivity (g product/(L-h))

\[ P_p = P \times D \]

where \( P \) is product concentration and \( D \) is dilution rate.

c) product yield (g product/g substrate)

\[ Y_{P/S} = \frac{P}{S_0 - S} \]

d) specific growth rate (h\(^{-1}\)):

\[ \mu = \frac{dX}{dt} \times \frac{1}{X} \]

4.4.7 Membrane Fermentation with Continuous Bleeding

This experimental run was operated at the following conditions: 50 g/L of initial glucose concentration, 0.1 h\(^{-1}\) dilution rate, and 400 rpm agitation rate. Bleeding of cells from the fermentor was initiated after 47 hours when the biomass concentration approached 30 g/L. The bleeding rate was determined as

\[ B = \frac{F_b}{V} \]
where \( F_b \) is flow rate of the bleed stream.

Bleeding rate in this experiment was maintained at 0.01 h\(^{-1}\).

### 4.5 Analytical Methods

#### 4.5.1 Biomass Concentration

Cell mass was determined by optical density measurements at a wavelength of 610 nm using a Beckman DU-7 Spectrophotometer (BECKMAN Instruments Inc., USA) and correlating the results with a gravimetric calibration curve. The calibration curve is shown in Appendix A.

#### 4.5.2 Glucose

Glucose concentrations were determined by the dinitrosalicylic acid quantitative test (Sumner, 1925). The method is based on oxidation of glucose by dinitrosalicylic acid reagent. The absorbance of the reduced sugar was measured using a Beckman DU-7 Spectrophotometer at 500 nm against the blank and converted to glucose concentration. The method is described in detail in the Appendix B. The calibration curve is shown in Fig.B.
4.5.3 Lactic Acid

Lactic acid concentrations were determined enzymatically using Boehringer Test Kits (Boehringer Mannheim GmbH, USA). The method is based on the enzymatic oxidation of lactate by nicotinamide-adenine dinucleotide (NAD) to pyruvate. The amount of NADH formed in the reaction is related stoichiometrically to the amount of the lactic acid present. The increase in NADH was determined spectrophotometrically at 340 nm (Appendix C).
5. RESULTS AND DISCUSSION

5.1 Preliminary Assessment of the New ZEEWEED™ Membrane Performance

5.1.1 Effect of Transmembrane Pressure on Permeate Flux in Systems with ZEEWEED™ and MILLIPORE Membranes

The ZEEWEED™ internal module was developed with the aim of improving the membrane separation process efficiency by decreasing membrane fouling, which is the main drawback of many cell recycle membrane systems. In a fermentation vessel, the ZEEWEED™ membrane is exposed to the turbulent flow created by agitation. As a result, a thinner deposit layer is formed and higher fluxes should be possible.

In this experiment the performances of two membrane systems were compared: a custom-made ZEEWEED™ MF membrane and a commercially available conventional MILLIPORE MF membrane. Accordingly, two fermentation runs were carried out under identical conditions, using membranes of both types, with equal surface areas.

Results of this experiment are shown in Fig.5.1. In the ZEEWEED™ membrane system, at a transmembrane pressure of 27 kPa permeate flux increased proportionally to the applied pressure indicating that the flux was still
Fig. 5.1 Effect of Transmembrane Pressure on Permeate Flux for Zeeweed™ and Millipore Membranes
in the pressure-controlled region and concentration of the polarization layer did not reach the critical level at which a compressible gel layer is formed. There was no flux decline observed at pressure differentials as high as 47 kPa. The retained cells remained in the broth where they were "swept" off the membrane surface by shear forces created by turbulent flow in the fermentor. Consequently, the deposit layer was thin and its hydraulic resistance to the applied pressures was low.

With the MILLIPORE membrane, an increase in transmembrane pressure initially resulted in a higher flux, but as the pressure drop was increased further the flux decreased to near zero at 28 kPa (Fig.5.1). As the concentration of the rejected solutes near the membrane surface increased during the course of fermentation, gel layer was eventually formed. Further increase in pressure compacted the gel, increasing its resistance, and thus decreasing the permeate flux (Fig.5.1). An attempt to apply higher pressures in this system resulted in high backpressures, which could cause damage to the membrane. Thus, it was not possible to investigate the MILLIPORE system at transmembrane pressures higher than 28 kPa.

The final biomass concentrations were low in both runs (4 g/L in the MILLIPORE membrane system and 2 g/L in the ZEEWEED™ membrane system). Therefore the difference in their viscosity (if any) should not have a significant effect on flux.

The intrinsic hydraulic resistances of the membranes could be different, thus affecting the performance of the membranes at different transmembrane
pressures. The water flux was measured at different pressure drops for both membranes to give the evaluation of their hydraulic resistances. Flux relates to hydraulic resistance and transmembrane pressure as follows:

\[ J = \frac{1}{R} \cdot \Delta P \]

where \( J \) is flux, \( R \) is the hydraulic resistance, and \( \Delta P \) is a transmembrane pressure.

From Fig.5.2 it is evident that the slope of the line representing the performance of the MILLIPORE membrane is about 5 times larger than that for the ZEEWEED™ membrane, indicating that the hydraulic resistance of the latter was larger. Therefore the better performance of the ZEEWEED™ membrane in the fermentation system must be attributed to the internal mode of operation.

5.1.2 Influence of Increased Filtration Area on Permeate Flux through ZEEWEED™ Membrane

In order to increase the permeate flow rate and allow the system to operate at higher dilution rates, the filtration area of the ZEEWEED™ membrane was doubled. Additionally, the heat and mechanical resistance of the polymer was also improved by the manufacturer to make it possible to withstand high sterilization temperatures in an autoclave.
Fig. 5.2 Water Flux vs. Transmembrane Pressure through MILLIPORE and ZEEWEED™ Membranes

\[ y = 13.094x + 5.2945 \]

\[ y = 2.5553x + 0.12 \]
The new ZEEWEED™ membrane with the filtration area of 0.033 m² was tested in this experiment against the previous one with the filtration area of 0.0155 m².

Fig. 5.3 illustrates the results of the experiment. It can be seen that the flux behaviour was different in the two systems. At low transmembrane pressures (up to approx. 16 kPa) the flux was the same for both membranes, as it would be expected. However, at pressures higher than 20 kPa a significant difference in the flux was observed. The possible explanation might be the difference in the hydrodynamic properties of the two membranes since the second membrane was manufactured from a modified material in order to improve thermoresistance. As a result, the membrane hydraulic resistance may also have been changed.

It should be noted that the permeate flux at different pressures in this experiment for the ZEEWEED™ membrane with the filtration area of 0.0155 m² behaved similarly to the MILLIPORE membrane with twice the filtration area (0.03 m²) (Fig.5.1). This indicates that the use of the ZEEWEED™ membranes could reduce the required filtration area thus increasing the cost effectiveness of the process – an advantage that could be associated with the internal mode of operation and possibly, properties of the membrane material and improved construction of the module.
Fig. 5.3 Permeate Flux vs. Transmembrane Pressure for Zeeweed™ Membranes with Two Filtration Areas
5.2 Rheological Properties of the Fermentation Broth

One of the main advantages of tangential flow filtration in continuous cell recycling fermentation systems is that larger cell concentrations are possible because cells are continuously recycled back to the fermentor. A dense population of cells not only accelerates production of lactic acid but also minimizes contamination by foreign microorganisms. However, when the cell mass concentration increases above a critical value, specific for each bacterial strain, fermentation systems become very unstable and difficult to operate. Crespo et.al. (1992) observed in their experiments with *L. plantarum* and *P. acidi-propionici* that above this cell concentration level large amounts of foam are produced and the transmembrane pressure increases.

In previous experiments with a ZEEWEED™ membrane in ethanol fermentation, increased viscosity was mentioned as one on the possible reasons of flux decay. In this work, a different microorganism was used as biocatalyst - *Lactobacilli*. Unlike yeast, this microorganism does not form clumps that may influence viscosity of the suspending liquid. However, there is another factor that may affect membrane performance: *Lactobacillus rhamnosus* NRRL B445, used in this study, produces cell wall and capsular polysaccharides (Xavier et.al., 1995; Wicken *et.al.*, 1983) and bacteriocins — small protein molecules with antimicrobial properties (Bhugaloo-Vial *et.al.*, 1997). These compounds, along with biomass and cell debris, could contribute to the increase of the viscosity of the fermentation broth and decrease of the permeate flux.
Viscosity is not the only rheological characteristic that could affect the performance of the membrane filtration system. Whether the system operates in the same rheological regime or it changes behaviour during the process must be known, in order to interpret the results correctly.

To study how the rheological properties of the system changed during the course of fermentation the viscosity of the fermentation broth was measured at four shear rates (7.3 s\(^{-1}\), 14.7 s\(^{-1}\), 36.7 s\(^{-1}\), and 73.4 s\(^{-1}\)) for different biomass concentrations obtained during the experimental run (from 11 g/L through 132 g/L). The results are presented on a log-log plot in Fig.5.4 to fit the equation:

\[
\log_{10} \eta_{\text{app}} = \log_{10} k + (n - 1)\log_{10} \gamma
\]

where \(\eta_{\text{app}}\) is an apparent viscosity, \(k\) is a consistency index, \(n\) is a flow behaviour index, and \(\gamma\) is a shear rate.

As indicated by the horizontal lines in the graph, the viscosity remained independent on shear rate (\(n = 1\)) for the all samples tested except for the last one, which had a biomass concentration of around 132 g/L. For this sample, a deviation from the Newtonian behaviour could be observed (\(n < 1\)).

Fig.5.5 illustrates the change of viscosity with increasing of biomass concentration. It can be seen from the graph that the viscosity remained low (in the range of 1.2 – 2.8 cP) up to 46 g/L of biomass concentration. A sharp increase in viscosity after the system reached cell densities of around 100 g/L was followed by change of rheological behaviour from Newtonian to pseudoplastic (Fig.5.4).
Fig. 5.4 Rheological Behaviour of the Fermentation Broth at Different Biomass Concentrations
Fig. 5.5  Viscosity vs. Biomass Concentration during the Fermentation Process
Although it is known from literature (Cheryan, 1986; Crespo et al., 1991) that viscosity increases with increased biomass concentration, broth age and composition could also affect the rheological characteristics of the broth. Products of cell lysis and metabolism, which did not permeate through membrane, accumulated in the system. These consisted of proteinaceous material, polysaccharides, and other high molecular weight compounds. Their concentration was not reflected in the biomass concentration analysis. However, they played an important role in the system. They could form part of the concentration polarization layer, and be deposited on the membrane surface. The retention characteristics of the membrane system would be defined by this secondary layer near the membrane surface. As a result, molecules which initially permeated through the membrane (e.g. enzymes) would be retained within the fermentor after the gel layer was formed resulting in ever faster accumulation of total solids in the system. Also, due to their high hydrophobicity proteinaceous products of cell lysis and metabolism (e.g., bacteriocins) could also form aggregates or bind to cellular components (Bhugaloo-Vial et al., 1997). As described in the literature, the reaction of the aggregates to shear could result in shear-thinning (pseudoplastic) flow. At low shear rates, the aggregates might be deformed but remain essentially intact. As the shear rate increases, the aggregates could be broken down into smaller fragments, decreasing friction and therefore viscosity.

Permeate flux profile and the broth viscosity profile in Fig.5.6 show how the increase of the viscosity of the broth affected permeate flux. As can be seen
Fig. 5.6 Permeate Flux and Broth Viscosity Profiles during Rheological Experiments
from the graph, the permeate flux dropped from approx. 18 L/(m²·h) at a cell
density of 10.8 g/L to 1.3 L/(m²·h) at 132 g/L.

During the time interval from the 26th hour to the 52nd hour, when sharp
decay of flux could be observed, biomass concentration increased from 10.8 g/L
to 33.9 g/L. This could be a period of gel layer formation. The flux then
remained stable for the following 20 hours, until the concentration of 65.2 g/L
was reached. Further increase in cell concentration brought about an increase
of viscosity, changes in the deposit layer and fast reduction of the flux.

The results of this experiment suggest that, for a long-term operation,
bleeding of some broth might be advantageous. This would prevent
accumulation of cell debris and allow optimal broth composition and constant
biomass concentration.

5.3 Experimental Design

Optimization of process parameters is an important step in increasing the
efficiency of the fermentative production of lactic acid. Before the optimization,
effects of the main operational variables on the process characteristics should be
detected and analysed. Experimental design is the most efficient way to
estimate the effects of several variables simultaneously.

In many situations all the important conclusions are evident from visual
examination of the data. However, in some experiments important effects may
be wholly or partially obscured by experimental error. Conversely, through experimental error, wrong conclusions about effects that do not exist could be drawn. The confusing effects of experimental error can be greatly reduced by adequate experimental design and analysis.

In this work three process variables were studied simultaneously, each one at two levels: dilution rate, initial substrate concentration and agitation rate. Accordingly, a 2×2×2 full factorial design was applied in order to evaluate systematically the effect (if any) of each factor on the following process characteristics: cell density, substrate utilization, product concentration and permeate flux. This two-level statistical design was aimed not for optimization of the process parameters but rather for elicitation of the complex relationships and interactions that exist in the system.

According to the literature, dilution rate and initial substrate concentration are important process variables that influence microbial kinetics in continuous fermentation systems. Both dilution rate and initial substrate concentration determine the nutrient status in the system, i.e. whether there is an excess or deficiency of essential substances in the fermentation broth. This affects the maximum biomass concentration that could be reached as well as the catabolic activity of microorganisms.

Agitation provides homogeneous conditions in the fermentation vessel. In the continuous membrane process even distribution of incoming nutrients and an alkali solution for the neutralizing of lactic acid produced is a critical characteristic of the process. It affects cell viability, product formation and membrane
performance since when cell viability is high, less sticky proteinaceous material is formed. On the other hand, at higher agitation rates cells are exposed to higher shear stresses, which could result in the reduction of the cell activity and loss of viability. Additionally, higher agitation rates require more energy, which would increase operational costs.

In the internal mode of operation, the agitation rate becomes a key parameter for improving the characteristics of the concentration polarization layer and thus increasing flux. With appropriately designed membrane geometry, agitation creates a turbulent flow with high Reynolds numbers improving mass transfer properties of the system. As a result, higher fluxes can be obtained when a system operates in the mass transfer-controlled region, i.e. when a concentration polarization layer has been formed.

In addition, agitation and mixing of the fluid near the membrane surface "sweeps" away the accumulated solute, reducing the thickness of the boundary layer and thus, helps to control the effects of concentration polarization.

Since the ZEEWEED™ membrane had not been repaired yet by the beginning of the experiment, it was decided to use the MILLIPORE hollow fiber module with the identical filtration area, to study effects of the variables on microbial kinetics, and to find the best combination of operational parameters for the next stage of the project.

In order to select levels for the dilution rate, a preliminary study of the MILLIPORE membrane system was undertaken.
5.3.1 Operating Conditions for the Membrane System

A continuous fermentation process with the MILLIPORE membrane was studied in this experiment in order to find maximal flow rate and optimal transmembrane pressure for the long-term performance of the membrane. The results are presented in Figures 5.7 and 5.8.

Although the system did not reach high cell densities (Fig.5.8), rapid flux decay had been observed after 30 hours of operation (Fig.5.7). In fact, it was not possible to maintain the 0.1 h\(^{-1}\) dilution rate for the whole course of fermentation. There were two phenomena that could be responsible for these negative effects: membrane fouling and concentration polarization. The chemistry of the membrane and environment is extremely important for control of these mechanisms. In the separation of cell suspensions, membrane pores are clogged with compounds which are small enough to enter the pores but too large to permeate through.

In order to reduce negative effects of fouling and concentration polarization on the permeate flux, several aspects of the membrane fermentation process were studied and operating conditions were changed accordingly.

1) Start-up conditions

Xavier et.al. (1994) suggested that the polysaccharides, produced by bacterium *Lactobacillus rhamnosus* (Wicken et.al., 1983) were the main cause of the lower flux for the fermentation with the batch start-up compared to
Fig. 5.7 Permeate Flow Rate Profile - trial run#1

Fig. 5.8 Cell Concentration Profile - trial run#1
continuous start-up for this strain. Polysaccharides, along with proteinaceous material, formed during cell lysis and disruption, are attached to the membrane surface and thus form a sticky base for the deposition of suspended particles. The amount and composition of the polysaccharides produced depends on the mode of operation (batch or continuous), dilution rate and limiting substrate source (Wicken et al., 1983).

Thus, in order to improve flux it was decided to omit the batch mode and **start continuous fermentation immediately after inoculation.**

2) **Neutralizing solution and chemical cleaning of the membrane**

The chemical composition of the medium is another important aspect of membrane filtration.

It was found that ammonium hydroxide used to neutralize lactic acid formed during cell metabolism, reacts with three other components of the nutrient medium, namely $\text{K}_2\text{HPO}_4$, $\text{KH}_2\text{PO}_4$ and $\text{MgSO}_4$. Nagata et al. (1989) studied cross-flow filtration of different solutions and showed that precipitates of magnesium ammonium phosphate and potassium ammonium phosphate, formed during reactions with $\text{NH}_4\text{OH}$, are the major foulants of the membrane in a cell-free environment. Thus, it is possible that they could contribute to the membrane fouling in the fermentation system.

Considering the above, the following changes had been made: (a) $2\text{N NH}_4\text{OH}$ solution was replaced with $5\text{N NaOH}$ solution, and (b) the membrane cleaning procedure was modified as follows (Jeantet et al., 1996):
- cleaning with 5% enzyme detergent (Terg-A-zyrne®, ALCONOX, Inc.) for 1.5 hours at room temperature;
- rinsing with 3.5 L of distilled water;
- cleaning with 0.1N NaOH solution for 30 min at 30°C in forward and backward mode;
- rinse with distilled water until neutral reaction;
- clean with $10^{-2}$ mol/L HNO₃ solution for 30 min at 30°C, forward and backward;
- rinse with distilled water until neutral reaction.

3) Membrane cleaning during the process

In order to further improve permeate flow rate, the membrane was cleaned every 3 hours by recirculating the fermentation broth at high rate for 5 min with closed permeate port. During this procedure the increased fluid velocity resulted in higher shear stresses, and solutes reentrainment took place. Thus, the local build-up of suspended solutes at the solution-membrane interface should be reduced.

4) Improvement of the flow management

And finally, the flow management was reviewed in order to improve characteristics of the permeate flow.

Cell suspensions behave differently from macromolecule solutions (Nagata et.al., 1989). In an ideal situation, i.e. without fouling, with negligible concentration polarization, flux is described by the Hagen-Poiseuille model:

$$J = \frac{\varepsilon r^2 \Delta P}{8 \eta L}$$
where \( J \) is flux in \( \text{L/(m}^2\cdot\text{h)} \), \( r \) is the channel radius, \( \Delta P \) is applied transmembrane pressure, \( \eta \) is the viscosity of the fluid, \( L \) is the length of the channel and \( \varepsilon \) is the surface porosity of the membrane.

According to this model, flux is directly proportional to the applied pressure. It is valid when concentration polarization is minimal. However, as soon as a consolidated gel layer is formed, the flux becomes independent of pressure (Cheryan, 1986). Further increasing the transmembrane pressure merely results in a denser solute layer. In a macromolecules solution the gel-polarized layer is assumed to be reversible. Changing the operating conditions, such as lowering pressure, will return the system back to the pressure-controlled operating regime with clear solutions, but it is not useful for suspensions of particles such as cells. Proteinaceous material and polysaccharides formed during metabolic processes or cell lysis are sticky substances that bind particles and thus, reduce back diffusion of particles from the membrane surface to the bulk solution.

Therefore, in order to increase shear stresses at the membrane surface and decrease gel layer compaction, it was decided to work at high recirculation rates from the beginning of the process.

In order to test if the above modifications improve the permeate flux, the second trial run was performed at the same operating conditions as the previous one. The results are presented in Figures 5.9 and 5.10.

As can be seen from the flux profile (Fig.5.9), obvious progress in the membrane performance was made. Although flow rate reduction took place
Fig. 5.9 Permeate Flow Rate Profile - trial run#2

Fig. 5.10 Cell Concentration Profile - trial run#2
after 24 hours of continuous operation, due to gel layer formation, the flow rate stabilized at the level around 0.1 L/h which allowed the selection of 0.1 h\(^{-1}\) dilution rate for the system with the total volume of 1 L.

It should be noted that the efficiency of the 5-minute cleanings every 3 hours during the process was higher toward the end of the process as can be seen in the graph.

Early in the run the concentration of the broth (i.e. bulk concentration) was low (Fig.5.10) and flux was high (Fig.5.9) which agrees with the film theory:

\[ J = K \ln \left( \frac{C_g}{C_b} \right) \]

where \( J \) is the permeate flux, \( C_g \) is concentration of the gel layer, and \( C_b \) is the bulk concentration (i.e. the concentration of the broth).

Consequently, the effect of the cleanings was less obvious. As the fermentation proceeded and the bulk concentration increased, the permeate flux decreased, the thickness of the gel layer increased and its characteristics changed due to accumulation of the products of cell lysis and metabolism. It became sticky and denser. Increased shear stresses during the cleanings reduced the thickness of the layer which resulted in the partially restored flux as seen in the graph at the 72\(^{rd}\) hour (Fig.5.9).

It can be seen in Fig.5.10 that more than double the biomass concentration was reached in this run compared to trial run#1. During run#1 most of the time the system operated at much lower dilution rates than in the run#2 due to membrane fouling that took place early during the process. This
could inhibit the bacterial growth and result in the lower final biomass concentrations.

5.3.2 Full Factorial Design and Analyses

S-Matrix CARD computer program was used for the design of the experiments and analyses of data.

Based on the preliminary experiments and a search of the literature, the following levels were selected:
1) for dilution rate – 0.05 h⁻¹ and 0.1 h⁻¹;
2) for agitation rate – 200 rpm and 400 rpm;
3) for initial glucose concentration – 50 g/L and 90 g/L.

Eleven runs were designed and carried out in a random order: eight runs with different combinations of the three variables and three duplicate runs for estimation of the experimental error. The experimental design matrix is presented in Fig.5.11.

The observed process parameters, obtained during the transient periods, are shown in Fig.5.12 through Fig.5.19. For all biomass concentration curves a plateau can be observed at 24th – 27th hour which coincides with a time when operational conditions of a run (namely, initial glucose concentration, dilution rate, agitation rate and pH) were changed in accordance with the experimental
Design Type: Classical Two-Level
Design Sub-Type: Full Factorial

<table>
<thead>
<tr>
<th>Run No.</th>
<th>glucose conc., g/L</th>
<th>dilution rate, h⁻¹</th>
<th>agititation rate, rpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50</td>
<td>0.1</td>
<td>400</td>
</tr>
<tr>
<td>2</td>
<td>90</td>
<td>0.05</td>
<td>200</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>0.1</td>
<td>200</td>
</tr>
<tr>
<td>4</td>
<td>90</td>
<td>0.1</td>
<td>400</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
<td>0.05</td>
<td>400</td>
</tr>
<tr>
<td>6</td>
<td>90</td>
<td>0.05</td>
<td>400</td>
</tr>
<tr>
<td>7</td>
<td>50</td>
<td>0.1</td>
<td>200</td>
</tr>
<tr>
<td>8</td>
<td>90</td>
<td>0.1</td>
<td>200</td>
</tr>
<tr>
<td>9</td>
<td>50</td>
<td>0.1</td>
<td>400</td>
</tr>
<tr>
<td>10</td>
<td>50</td>
<td>0.05</td>
<td>400</td>
</tr>
<tr>
<td>11</td>
<td>50</td>
<td>0.05</td>
<td>200</td>
</tr>
</tbody>
</table>

Fig.5.11 Experimental Design Matrix
Fig. 5.12 Fermentation Kinetics of *L. rhamnosus* in runs #1 and #9 ($S_0=50 \text{ g/L, } D=0.1 \text{ h}^{-1}, A=400 \text{ rpm}$)

*all points – average values of the duplicate runs #1 and #9*

Fig. 5.13 Fermentation Kinetics of *L. rhamnosus* in run #2 ($S_0=90 \text{ g/L, } D=0.05 \text{ h}^{-1}, A=200 \text{ rpm}$)
* all points – average values of runs #3 and #7

**Fig.5.14** Fermentation Kinetics of *L. rhamnosus* in runs #3 and #7 (S₀=50 g/L, D=0.1 h⁻¹, A=200 rpm)

**Fig.5.15** Fermentation Kinetics of *L. rhamnosus* in run #4 (S₀=90 g/L, D=0.1 h⁻¹, A=400 rpm)
Fig. 5.16  Fermentation Kinetics of *L. rhamnosus* in runs #5 and #10 (S₀=50 g/L, D=0.05 h⁻¹, A=400 rpm)

Fig. 5.17  Fermentation Kinetics of *L. rhamnosus* in run #6 (S₀=90 g/L, D=0.05 h⁻¹, A=400 rpm)
Fig. 5.18 Fermentation Kinetics of *L. rhamnosus* in run#8 ($S_0=90$ g/L, $D=0.1$ h$^{-1}$, $A=200$ rpm)

Fig. 5.19 Fermentation Kinetics of *L. rhamnosus* in run#11 ($S_0=50$ g/L, $D=0.05$ h$^{-1}$, $A=200$ rpm)
design. This lag phase was the period of adaptation of the microorganism to the new environmental conditions.

True steady states were not achieved in this series of experiments since cells were not removed from the system. However, by the end of each run the specific growth rates dropped to such low levels that cell concentrations were changed very little as shown in the biomass concentration curves (Figures 5.12 through 5.19).

The statistical analyses are summarized in Tables 5.1a through 5.1e. Table 5.1a represents the overall experimental errors which were determined based on duplicate runs. In Tables 5.1b, 5.1c, 5.1d and 5.1e the results of the regression analyses are summarized. Those factors that had statistically significant effects on a process characteristic are represented as model terms in the tables. The percent of confidence at which a coefficient was accepted as statistically significant was 95%. Those model terms whose coefficients had a % confidence below 95% were not included in the tables. The model term ranks presented in the tables define the strength of the term's effect on the response relative to all other terms in the current regression model on a scale of zero (non effector) to one (strongest effector). The terms with the highest rank have the greatest ability to change the response. The ranking also defines the relative sensitivity of the response to changes in the model terms. The response is most sensitive to level changes in the terms with the highest rank, which therefore require the greatest control in order to maintain performance reproducibility in the response.
### Table 5.1a Overall Experimental Errors

<table>
<thead>
<tr>
<th>Response</th>
<th>Experimental Error, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>biomass</td>
<td>8.4</td>
</tr>
<tr>
<td>lactic acid</td>
<td>7.7</td>
</tr>
<tr>
<td>glucose utilization</td>
<td>0.2</td>
</tr>
<tr>
<td>permeate flux</td>
<td>34.6</td>
</tr>
</tbody>
</table>

### Table 5.1b Results of Regression Analysis for Biomass

<table>
<thead>
<tr>
<th>Model Term Name</th>
<th>Model Term Range</th>
<th>Coefficient Value</th>
<th>Model Term Rank</th>
</tr>
</thead>
<tbody>
<tr>
<td>initial glucose conc.</td>
<td>40</td>
<td>-0.25</td>
<td>1.00</td>
</tr>
<tr>
<td>dilution rate</td>
<td>0.05</td>
<td>114.68</td>
<td>0.57</td>
</tr>
</tbody>
</table>
### Table 5.1c Results of Regression Analysis for Lactic Acid

<table>
<thead>
<tr>
<th>Model Term Name</th>
<th>Model Term Range</th>
<th>Coefficient Value</th>
<th>Model Term Rank</th>
</tr>
</thead>
<tbody>
<tr>
<td>initial glucose conc.</td>
<td>40</td>
<td>0.37</td>
<td>1.00</td>
</tr>
</tbody>
</table>

### Table 5.1d Results of Regression Analysis for Glucose Utilization

<table>
<thead>
<tr>
<th>Model Term Name</th>
<th>Model Term Range</th>
<th>Coefficient Value</th>
<th>Model Term Rank</th>
</tr>
</thead>
<tbody>
<tr>
<td>initial glucose conc.</td>
<td>40</td>
<td>-0.72</td>
<td>1.00</td>
</tr>
</tbody>
</table>

### Table 5.1e Results of Regression Analysis for Permeate Flux

<table>
<thead>
<tr>
<th>Model Term Name</th>
<th>Model Term Range</th>
<th>Coefficient Value</th>
<th>Model Term Rank</th>
</tr>
</thead>
<tbody>
<tr>
<td>agitation rate</td>
<td>200</td>
<td>0.01</td>
<td>1.00</td>
</tr>
</tbody>
</table>
Table 5.2 represents the system responses obtained at the end of each run. The results are organized into two groups according to the initial glucose concentration: in the first group are runs operated at 50 g/L and in the second group are runs operated at 90 g/L of initial glucose. As the statistical analysis showed, the initial glucose concentration affected all three characteristics in Table 5.2, namely biomass and product concentrations and glucose conversion. In the second group, lactic acid concentrations are higher and glucose conversions are lower than in the first group (44.9 - 52.7 g/L and 69 - 71% vs. 29.5 - 38.3 g/L and 99% respectively). Low conversions indicate that glucose was present in excess in the runs of the second group. However, in spite of the abundance of nutrients, bacterial growth was obviously slow resulting in the lower final biomass concentrations in this group (16.5 - 29.8 g/L vs. 29.0 - 35.6 g/L respectively). It is likely that lactic acid inhibited cell growth in the second group more strongly than in the first group as a result of the higher concentrations. The sensitivity of lactobacilli to high end-product concentrations is described in the literature (Friedman and Gaden, 1970; Goncalves *et al.*, 1991 and 1997). Goncalves *et al.* (1991) and Aeschlimann and von Stockar (1989) found that the growth was severely inhibited at lactate concentration of 6% ($\mu = 0.1\mu_{\text{max}}$). The concentration of lactate in the second group runs approached this critical level.

The dilution rate had a different effect at the two glucose initial concentrations (Table 5.2). Within the first group the higher dilution rate (0.1 h$^{-1}$) resulted in higher biomass and product concentrations than the lower dilution rate (0.05 h$^{-1}$).
Table 5.2 Results of Lactic Acid Fermentation at Different Operating Conditions

<table>
<thead>
<tr>
<th>Dilution Rate, h⁻¹</th>
<th>( S₀=50 \text{ g/L} )</th>
<th>( S₀=90 \text{ g/L} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[biomass] g/L</td>
<td>[lactic acid] g/L</td>
</tr>
<tr>
<td>0.05*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.05**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1**</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* agitation rate = 200 rpm; ** agitation rate = 400 rpm
All results obtained at the end of each run (after 4-5 residence times of operation)
For duplicate runs average ± standard deviation was used.
In these runs the microbial population was glucose limited due to high substrate conversions (99%). Operation under glucose limitation conditions made microorganisms in these runs sensitive to the increased supply of nutrients, i.e. dilution rate. As a result, the bacterial growth increased. This is in agreement with results reported by Xavier et al. (1994) where higher biomass concentrations were achieved at higher dilution rates due to more intensive transport of nutrients. Higher growth rates and biomass concentrations resulted in higher lactic acid production rates which is in agreement with the Luedeking-Piret model:

\[
\frac{dP}{dt} = \alpha \frac{dX}{dt} + \beta X
\]

where P is a product concentration, X is biomass concentration, \( \alpha \) is the growth associated constant, and \( \beta \) is the non-growth associated constant.

None of the trends observed in the first group could be found in the second group. The dilution rate did not seem to affect either the lactic acid production or the biomass concentration (Table 5.2). The residual glucose concentrations in these fermentation runs were much higher (26-28 g/L) indicating that glucose was present in excess. In addition the range of dilution rates studied was very small (0.05 h\(^{-1}\)) because of the physical limitations of the equipment (see Sec.5.3.1). Thus microorganisms in this group of runs obviously did not respond significantly to the increase in dilution rate.

This different effect of the dilution rate at different levels of the initial glucose concentration indicates that an interaction between these two factors might exist. However, this was not reflected in the statistical analysis (Tables 5.1b and 5.1c).
As shown in Table 5.1b, although the dilution rate was a weaker effector on biomass than initial glucose, its effect on the biomass was statistically significant. No interaction between these two factors was revealed by statistical analysis. From Table 5.1c it is evident that no significant effect of dilution rate on the lactic acid production was detected. This is contrary to expectations and the visual evaluation of the observed experimental data (Table 5.2). The lack of the statistical significance may be a limitation of the experimental design used. Further experiments within a three level experimental design might be required to determine whether this interaction and/or effect really exist within the studied range.

It is evident from both Table 5.1d and Table 5.2 that dilution rate did not affect glucose conversion, contrary to expectations. The literature suggests that higher dilution rates generally result in lower conversion of substrate. The residence time and contact time decrease, resulting in a decrease in substrate conversion. It was clearly evident that in these experiments such an effect was not significant, probably, because the range of the dilution rates studied was too narrow.

The analysis of the permeate flux showed that the agitation rate had a significant effect on the permeate flux: higher agitation rate (400 rpm) resulted in higher fluxes (Table 5.2). Such a result would not have been surprising if an internal membrane module such as ZEEWEED™ had been used. With the MILLIPORE membrane this was unexpected. More intensive foaming, observed in the higher agitation rate runs, may have caused this effect. Bubbles of air breaking and rearranging in the recycling loop, resulted in additional shear
stresses and consequently, reduction of the thickness of the deposit layer on the membrane surface.

The bacterial cells did not decrease their activity at higher agitation significantly since no effect of the agitation rate on the microbial kinetics was shown by the statistical analysis (Table 5.2). Due to the composition of its cell wall, *Lactobacillus rhamnosus* is resistant to mechanical disruption (Doelle, 1984), and obviously could withstand the shear rates imposed during these runs.

It should be noted that the experimental error for flux was around 35% (Table 5.1a). This high variability in experimental results may have been associated with foaming, and the resulting variability in shear rates at the membrane surface. More advanced process control could reduce this error.

The lactic acid fermentation kinetics for duplicate runs is shown in Figures 5.20 through 5.22. The graphs illustrate good reproducibility for all three groups of runs. The identical profiles were obtained for all three responses, namely biomass, lactic acid and residual glucose concentrations. The final biomass concentrations had low standard deviations for all three duplicate runs (0.2 g/L, 3.1 g/L and 1.1 g/L for duplicate runs #3 & #7, #5 & #10, and #1 & #9 respectively). The experimental error for lactic acid was approx. the same as for the biomass concentration (Table 5.1a) and the final concentrations were scattered around the mean values with standard deviations of 2.5 g/L, 0.7 g/L, and 3.2 g/L for duplicate runs #3 & #7, #5 & #10, and #1 & #9 respectively. The lowest experimental error was obtained for the glucose utilization (0.2%) which is
Fig. 5.20 Fermentation Kinetics in Duplicate Runs #1 and #9

Fig. 5.21 Fermentation Kinetics in Duplicate Runs #3 and #7

Fig. 5.22 Fermentation Kinetics in Duplicate Runs #5 and #10
reflected in the residual glucose concentration profiles for all three groups of
duplicate runs (Figures 5.20 through 5.22).

Table 5.3 represents the following characteristics that were chosen as
criteria to evaluate the process efficiency in order to select the best combination
of parameters in the studied range: productivity, permeate flux and lactic acid
yield. Higher productivities were obtained for the higher dilution rate ranging
from 3.6 g/(L-h) to 5.3 g/(L-h). From these the productivities obtained during
run#4 (4.5 g/(L-h)) and run#8 (5.3 g/(L-h)) are of the most interest in terms of
efficiency of the process due to higher product concentrations achieved in these
runs. Continuous lactic acid production yielding low product concentrations is
undesirable, because the production cost is very much dependent on the cost of
downstream extraction and purification.

Evaluation of the permeate flux obtained at the end of each run (Table 5.3)
shows that the highest values were obtained for the duplicate runs #1 and #9 and
duplicate runs #5 and #10 (4.3 L/(h-m²) and 4.4 L/(h-m²) respectively). The
duplicate runs #5 and #10 resulted in low productivity. Conversely, the
conditions used in duplicate runs #1 and #9 are a good compromise between
productivity, substrate conversion and flux.

In order to evaluate the efficiency of the substrate assimilation the carbon
mass balance was calculated at the end of each fermentation run (i.e. after a 4-5
residence times period) as follows:

\[0.4S_0 \cdot D = (D + D_{NaOH}) \cdot (0.4S + 0.4P) + 0.5X\]
Table 5.3 Summary Table of Results Obtained during Lactic Acid Fermentation at Different Operating Conditions

<table>
<thead>
<tr>
<th>Run no</th>
<th>Time, h</th>
<th>$S_0$, g/L</th>
<th>$D$, h$^{-1}$</th>
<th>$A$, rpm</th>
<th>$X$, g/L</th>
<th>$P$, g/L</th>
<th>$U$, %</th>
<th>$P_p$, g/Lh</th>
<th>$Q$, L/m$^2$h</th>
<th>$Y_{P/S}$, g/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>1&amp;9*</td>
<td>77</td>
<td>50</td>
<td>0.10</td>
<td>400</td>
<td>36.4 ± 1.1</td>
<td>35.5 ± 3.2</td>
<td>99 ±0</td>
<td>3.6</td>
<td>4.3 ± 0.8</td>
<td>0.72</td>
</tr>
<tr>
<td>2</td>
<td>94</td>
<td>90</td>
<td>0.05</td>
<td>200</td>
<td>22.7</td>
<td>45.3</td>
<td>70</td>
<td>2.3</td>
<td>2.1</td>
<td>0.72</td>
</tr>
<tr>
<td>3&amp;7*</td>
<td>76</td>
<td>50</td>
<td>0.10</td>
<td>200</td>
<td>35.6 ± 0.2</td>
<td>38.3 ± 2.5</td>
<td>99 ±0.7</td>
<td>3.8</td>
<td>2.1 ± 0.6</td>
<td>0.78</td>
</tr>
<tr>
<td>4</td>
<td>75</td>
<td>90</td>
<td>0.10</td>
<td>400</td>
<td>29.8</td>
<td>44.9</td>
<td>69</td>
<td>4.5</td>
<td>2.1</td>
<td>0.73</td>
</tr>
<tr>
<td>5&amp;10*</td>
<td>96</td>
<td>50</td>
<td>0.05</td>
<td>400</td>
<td>30.3 ± 3.1</td>
<td>29.7 ± 0.7</td>
<td>99 ±0.7</td>
<td>1.5</td>
<td>4.4 ± 0.4</td>
<td>0.60</td>
</tr>
<tr>
<td>10</td>
<td>96</td>
<td>50</td>
<td>0.05</td>
<td>400</td>
<td>28.1</td>
<td>29.2</td>
<td>99</td>
<td>1.5</td>
<td>4.1</td>
<td>0.59</td>
</tr>
<tr>
<td>6</td>
<td>77</td>
<td>90</td>
<td>0.05</td>
<td>400</td>
<td>16.5</td>
<td>51.4</td>
<td>70</td>
<td>2.6</td>
<td>3.7</td>
<td>0.81</td>
</tr>
<tr>
<td>8</td>
<td>73</td>
<td>90</td>
<td>0.10</td>
<td>200</td>
<td>21.3</td>
<td>52.7</td>
<td>71</td>
<td>5.3</td>
<td>2.8</td>
<td>0.83</td>
</tr>
<tr>
<td>11</td>
<td>95</td>
<td>50</td>
<td>0.05</td>
<td>200</td>
<td>29.0</td>
<td>29.5</td>
<td>99</td>
<td>1.5</td>
<td>3.2</td>
<td>0.59</td>
</tr>
</tbody>
</table>

*average of duplicate runs

$S_0$ - initial substrate concentration  
$U$ - substrate utilization  
$D$ - dilution rate  
$P_p$ - volumetric productivity  
$A$ - agitation rate  
$Q$ - permeate flux  
$X$ - biomass concentration  
$Y_{P/S}$ - product yield  
$P$ - lactic acid concentration
where $S_0$ is initial glucose concentration, $D$ is dilution rate, $D_{\text{NaOH}}$ is alkali solution addition rate, $S$ is residual glucose concentration, $P$ is lactic acid concentration, $\mu$ is specific growth rate and $X$ is biomass concentration; 0.4 is a fraction of the carbon in glucose and in lactic acid, 0.5 is a fraction of the carbon in the cellular material.

The results are presented in Table 5.4 below:

### Table 5.4 The Carbon Mass Balance during Lactic Acid Fermentation

<table>
<thead>
<tr>
<th>Run no.</th>
<th>$S_0$</th>
<th>$D$</th>
<th>$C_{\text{in}}$</th>
<th>$C_{\text{out}}$</th>
<th>Carbon loss (gain) [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[g/L]</td>
<td>[h$^{-1}$]</td>
<td>[g/(L·h)]</td>
<td>[g/(L·h)]</td>
<td></td>
</tr>
<tr>
<td>5 &amp; 10*</td>
<td>50</td>
<td>0.05</td>
<td>1.0</td>
<td>0.8</td>
<td>20</td>
</tr>
<tr>
<td>11</td>
<td>50</td>
<td>0.05</td>
<td>1.0</td>
<td>0.8</td>
<td>20</td>
</tr>
<tr>
<td>1 &amp; 9*</td>
<td>50</td>
<td>0.1</td>
<td>2.0</td>
<td>1.8</td>
<td>10</td>
</tr>
<tr>
<td>3 &amp; 7*</td>
<td>50</td>
<td>0.1</td>
<td>2.0</td>
<td>1.9</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>90</td>
<td>0.05</td>
<td>1.8</td>
<td>1.7</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>90</td>
<td>0.05</td>
<td>1.8</td>
<td>1.7</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>90</td>
<td>0.1</td>
<td>3.6</td>
<td>3.4</td>
<td>6</td>
</tr>
<tr>
<td>8</td>
<td>90</td>
<td>0.1</td>
<td>3.6</td>
<td>3.3</td>
<td>8</td>
</tr>
</tbody>
</table>

* average of duplicate runs
It is evident from the table that some carbon-containing substances might not be included in the mass balance since $C_{\text{out}}$ for all runs is less than $C_{\text{in}}$. These could be products of cell lysis, polysaccharides and bacteriocins produced by this bacteria as well as by-products that might be produced under glucose limitation conditions. Major and Bull (1988) reported that under glucose limitation, the homofermentative \textit{Lactobacillus delbrueckii} switched to the heterofermentative mode of metabolism with production of acetic acid and ethanol as by-products. The amount of these by-products was higher at low dilution rates. It is obvious from the data that at the lower initial glucose concentration and lower dilution rate the highest discrepancy was observed. This could be due to a higher amount of by products formed and more intensive cell lysis under substrate deficiency conditions.

Analysis of the lactic acid yield confirms the above results. Fig.5.23 illustrates the effect of the initial substrate concentration on the lactic acid yield. In the graph, the first and the second groups of runs were operated at a lower dilution rate (0.05 h$^{-1}$) and the 3$^{rd}$ and the 4$^{th}$ group of runs were operated at a higher dilution rate (0.1 h$^{-1}$). As can be seen, in the first two groups lower initial glucose concentration resulted in lower lactic acid yields. In these groups, runs were operated at the low dilution rate and low initial glucose, and by-product formation could be a reason for reduced product yields. Substrate conversion was high in these runs (Table 5.2) and the microbial population remained substrate limited for long periods of time. In the 3$^{rd}$ and the 4$^{th}$ groups there is no significant
1-runs #2 and #11: D=0.05 h⁻¹, A=200 rpm; 2-runs #5/10 and #6: D=0.05 h⁻¹, A=400 rpm 3-runs #3/7 and #8: D=0.1 h⁻¹, A=200 rpm; 4-runs #1/9 and #4: D=0.1 h⁻¹, A=400 rpm

Fig.5.23 Effect of the Initial Glucose Concentration on the Product Yield

1-runs #3/7 and #11: S₀=50 g/L, A=200 rpm; 2-runs #1/9 and #5/10: S₀=50 g/L, A=400 rpm 3-runs #2 and #8: S₀=90 g/L, A=200 rpm; 4-runs #4 and #6: S₀=90 g/L, A=400 rpm

Fig.5.24 Effect of the Dilution Rate on the Product Yield
difference in the product yields between runs operated at 50 g/L and 90 g/L initial glucose. These runs operated at higher dilution rates and probably, less by-products were formed.

The effect of the dilution rate on the product yield could be observed only for runs operated under glucose limitation conditions (i.e. at 50 g/L of initial glucose), as illustrated in Fig.5.24. It can be seen that for the 1st and 2nd groups operated at 50 g/L glucose, the higher dilution rate resulted in the higher yields. There was no such correlation for the runs operated at 90 g/L glucose where substrate was present in excess. Therefore, in order to reduce the by-product formation and improve the lactic acid yield, it is desirable to work at higher dilution rates.

Summarizing the results of these experiments and analyses, the following combination of variables could be considered the most effective in the ranges studied:

- initial substrate concentration: 50 g/L;
- dilution rate: 0.1 h⁻¹;
- agitation rate: 400 rpm.

5.4 Membrane Fermentation with Continuous Bleeding of Biomass

It was shown during the previous experiments that permeate fluxes up to 4.9 L/(h·m²) can be maintained for more than 75 hours at cell densities higher
than 35 g/L if the membrane is cleaned properly, adequate solution chemistry and flow management are chosen, and operational parameters are optimized. However, for the long term stability bleeding of fermentation broth becomes necessary to prevent undesirable build-up of biomass and cell debris.

Cell lysis is an enzymatic process that occurs naturally to more or less extent in microbial populations of any age. Under unfavourable or harmful environmental conditions the death rates increase and, as a result, cell lysis is more intensive.

During the cell lysis smaller molecular weight organic compounds are released. This cell debris contains polysaccharides and sticky proteinaceous material. According to Vick Roy (1985) the particle size of cell debris in fermentation systems is around 0.4 μm. Obviously these compounds can not penetrate through the membrane with the pore size of 0.1 μm that was used in this study. As a result, cell debris is accumulated within the fermentation system. Upon the accumulation, the thickness of the concentration polarization layer is increased while back diffusion of particles to the bulk solution is reduced due to high attachment forces. Cells continue to grow slowly during the course of fermentation, accumulating in the system, and increasing viscosity. As rheological studies showed (Fig.5.5), after a period of fermentation when biomass concentration achieved the value of 60 g/L, a significant increase of viscosity occurred resulting in the rapid decline of the permeate flux.
These effects on viscosity could be limited and the process time between membrane cleanings extended if some of the biomass is continually removed from the system.

In this experiment the effect of bleeding on the long term stability of the system was investigated.

Ideally, a continuous fermentor should be operated at steady state. Steady state may be achieved if cells are removed from the system at the same rate as they grow. From cell mass balance:

\[ \frac{dX}{dt} = \mu X - BX \]

At steady state \( \frac{dX}{dt} = 0 \) and

\[ B = \mu \]

where \( B \) is bleeding rate and \( \mu \) is cell specific growth rate.

Bleeding during the exponential phase is not economical since bacterial growth rate is high and biomass densities are still low during this period. However, in the process with cell recycle the level of nutrients is greatly reduced and this results in very low specific growth rates. In these experiments the biomass growth rates slowed to approx. 0.01 h\(^{-1}\) after some 24 hours. Thus it was decided to start bleeding at the time when the rate of biomass growth was reduced to 0.01 h\(^{-1}\).

This experiment was performed at 50 g/L of initial glucose concentration, 0.1 h\(^{-1}\) dilution rate, and 400 rpm agitation rate. Bleeding was initiated at 47\(^{th}\)
hour when the biomass concentration approached 30 g/L. The bleeding rate was 0.01 h⁻¹.

The results of the run are illustrated in Fig.5.25 and summarized in the Table 5.5 below.

**Table 5.5 Results of Continuous Lactic Acid Fermentation with Bleeding of Cells and without Bleeding**

<table>
<thead>
<tr>
<th>Process Characteristic</th>
<th>Bleeding</th>
<th>No Bleeding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time of Operation</td>
<td>h</td>
<td>145</td>
</tr>
<tr>
<td>Biomass Concentration</td>
<td>g/L</td>
<td>27.8±1.8</td>
</tr>
<tr>
<td>Residual Glucose Concentration</td>
<td>g/L</td>
<td>1.1±0.4</td>
</tr>
<tr>
<td>Lactic Acid Concentration</td>
<td>g/L</td>
<td>37.4±1.2</td>
</tr>
<tr>
<td>Substrate Utilization</td>
<td>%</td>
<td>97±4</td>
</tr>
<tr>
<td>Volumetric Productivity</td>
<td>g/(L·h)</td>
<td>3.7±0.1</td>
</tr>
<tr>
<td>Product Yield</td>
<td>g/g</td>
<td>0.76±0.03</td>
</tr>
<tr>
<td>Permeate Flux</td>
<td>L/(h·m²)</td>
<td>4.9</td>
</tr>
</tbody>
</table>

Steady state was achieved and maintained during the course of fermentation for some 100 hours and the biomass, glucose and lactic acid concentrations remained essentially constant.
Fig. 5.25 Effect of Continuous Bleeding on the Fermentation Kinetics of *L. rhamnosus*
The main characteristics of the process remained in the same range for the runs with and without bleeding (Table 5.5). The permeate flux in the run with bleeding stabilized at 4.9 L/(h·m²). Statistically, it is not different from the flux obtained in the run without bleeding. However, the run with bleeding was operated twice as long as the run without bleeding indicating that high flux was maintained for long periods of time when bleeding was applied, thus increasing the efficiency of the process.
6. CONCLUSIONS

The characteristics of continuous lactic acid fermentation with cell recycling was studied in a membrane fermenter system. Different membrane modules and operating conditions were compared in terms of process characteristics and operational stability. The following were the main conclusions determined by the program:

1. The Zeeweed™ internal hollow fibre modules are well suited for continuous membrane lactic acid fermentation. Higher permeate fluxes could be maintained for a longer period of time in microfiltration of cell suspensions using the Zeeweed™ membrane than with the conventional Millipore membrane.

   The main disadvantage of the Zeeweed™ membrane was its low resistance to high temperatures of sterilization, which resulted in damage to the polymer. Unfortunately, in spite of the promising results obtained, the investigation of the Zeeweed™ module could not be continued further as the membrane had not yet been replaced by the manufacturer.

2. Rheological studies of the fermentation broth with *Lactobacillus rhamnosus* showed that the fermentation system could stay within the same rheological regime in a wide range of cell concentrations of 0 to 100 g/L. Thus, high cell density systems could be developed in order to increase product concentration
and productivity, provided that efficient methods for control of concentration polarization are applied.

3. The lower initial glucose concentration (50 g/L) and the higher dilution rate (0.1 h⁻¹) resulted in the better overall system performance: 99% conversion, 35.4 g/L of biomass concentration and 4.3 L/(m²·h) of permeate flux. The inhibiting effect of lactic acid on cell growth was observed at high lactic acid concentrations (44.9 - 52.7 g/L).

4. Steady state was achieved in the continuous membrane system using continuous bleeding of cells. The main process characteristics, such as product concentration, substrate conversion and permeate flux remained constant during the period of fermentation when excess biomass was removed by bleeding, to maintain constant biomass concentration.

    Continuous cell bleeding can be used to maintain the fermentor at steady state for extended periods, and may be the basis of efficient commercial fermentation systems.
7. **RECOMMENDATIONS**

- Based on the results of the program it is recommended that the Zeeweed™ membrane system be extensively tested, specifically in high cell density systems. The effect of pressure drop on flux, due to secondary surface layer formation, should be investigated in high cell density systems.

- A larger scale system should be used for determining the stability of the system, with bleeding, over long periods of time. The changes in the rheological properties of the system, if any, should be determined, taken into account, and if necessary, controlled.

- The feasibility of scale-up, based on a Zeeweed™ membrane operated at constant biomass by cell bleeding, should be investigated. Continuous NIR analysis of cell mass and/or lactic acid should be tested for on-line efficient process control.

- The effect of the broth composition and age on its viscosity should be studied and compared with the effect of the biomass concentration on the rheological properties of the system.
- The experimental series should be expanded to a three level factorial design in order to develop more comprehensive mathematical models for the process.
8. NOMENCLATURE

B  bleeding rate (h⁻¹)
C₉  concentration of the bulk solution (g/L)
C₉  concentration of gel layer (g/L)
D  dilution rate (h⁻¹)
dₙ  hydraulic diameter (m)
Eₐ  activation energy (J/mol)
F  feed flow rate (L/h)
Fₔ  flow rate of bleed stream (L/h)
J  permeate flux (L/(m²·h))
K  mass transfer coefficient (m²/s)
Kₛ  saturation constant (g/L)
k  consistency index (Pa·sⁿ)
L  channel length (m)
n  dimensionless flow behaviour index
P  product concentration (g/L)
Pₚ  volumetric productivity (g/(L·h))
ΔP  transmembrane pressure (Pa)
R  universal gas constant (J/(mol·K))
r  channel radius (m)
S₀  initial substrate concentration (g/L)
S  substrate concentration (g/L)
T  absolute temperature (K)
t  time (s or h)
U  average fluid velocity (m/s)
V  total volume of the system (L)
X  biomass concentration (g/L)
Yₚ/S  product yield (g product/g substrate)
Yₓ/S  biomass yield (g cells/g substrate)

Greek Symbols:  α  growth-associated constant (g/g)
β  non-growth associated constant (g/(g·h))
γ  shear rate (s⁻¹)
ε  surface porosity
η  viscosity of the fluid (Pa·s)
μ  specific growth rate (h⁻¹)
μₘₐₓ  maximum specific growth rate (h⁻¹)
ν  kinematic viscosity (m²/s)
τ  shear stress (Pa)


Kroner, K.H. et.al. (1987), Bio/Technology. 5. 921.


Sumner, J.B. (1925). J.Biol.Chem. 65. 393


10. APPENDICES

Appendix A

Cell mass was determined by optical density measurements at a wavelength of 610 nm using UV-visible spectrophotometer and correlating the results with a gravimetric calibration curve.

The procedure for preparing samples with known biomass concentrations for calibration curve was as follows:

1. From the stock sample of fermentation broth samples were prepared at different dilutions and their absorbances were measured at the wavelength of 610 nm (determined by scanning).

2. In order to determine the concentration of the stock sample, 5 ml the sample was first washed with distilled water after centrifugation for 30 min at 5000 rpm and removing supernatant. The procedure was repeated three times.

3. After washing cells were filtered through 0.45 μ filter by vacuum, dried in vacuum oven at 90°C for overnight and weighed. Dry weight cell concentration was determined.

The linear relationship between absorbance and concentration was in the range of 0.033 – 0.330 g/L cells in sample.

The calibration curve is presented in Fig.A.
Fig.A Biomass Calibration Curve

\[ y = 3.0513x + 0.0696 \]

\[ R^2 = 0.9998 \]
Appendix B

Dinitrosalicylic Acid Quantitative Test (Sumner, 1925)

1. Place 1 ml of the sample in a Folin-Wu tube.
2. Add 3 ml of the dinitrosalicylic acid reagent and mix well by shaking at an angle of about 30° to the horizontal.
3. Prepare the standard by pipetting 1 ml of water into another Folin-Wu tube, adding 3 ml of the dinitrosalicylic acid reagent and mixing as before.
4. Immerse both tubes in the boiling water and leave for 5 minutes.
5. Cool tubes in running water for 3 minutes.
6. Dilute carefully to the 25 ml mark and mix by inverting six times.
7. Read absorbance of the sample against blank at the wavelength of 500 nm.

Dinitrosalicylic Acid Reagent

1. To 300 ml of 4.5% sodium hydroxide solution add 880 ml of 1% dinitrosalicylic acid solution and 255 g of Rochelle salt.
2. To 10 g of crystalline phenol add 22 ml of 10% sodium hydroxide solution, add water to dissolve, dilute to 100 ml and mix.
3. To 69 ml of the (2) solution add 6.9 g of sodium bisulfite and add to the dinitrosalicylic acid solution (1).
4. Mix well until all components dissolved.
5. Keep tightly stoppered in well filled bottles.
The calibration curve prepared using samples with known concentration of glucose is shown in Fig.B.

Appendix C

**UV-method for Determination of L-lactic Acid (Noll, 1974)**

Test-Combination from Boehringer Mannheim GmbH was used for these analysis (Cat.No.139084).

- Each test-combination contains:
  1. Bottle with solution containing glycyglycine buffer (pH 10.0), L-glutamic acid and stabilizers.
  2. Bottle with β-NAD solution;
  3. Bottle with glutamate-pyruvate transaminase;
  4. Bottle with L-lactate dehydrogenase.

- Conditions
  - Wavelength: 340 nm
  - Cuvette: 1 cm light pass
  - Temperature: 20-25°C
Fig. B  Glucose Calibration Curve
Sample solution: 2-20 μg of L-lactic acid per cuvette.

- Procedure

<table>
<thead>
<tr>
<th>Pipette into cuvettes:</th>
<th>Blank</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>solution 1</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>solution 2</td>
<td>0.2 ml</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>redistilled water</td>
<td>1.0 ml</td>
<td>0.9 ml</td>
</tr>
<tr>
<td>suspension 3</td>
<td>0.02 ml</td>
<td>0.02 ml</td>
</tr>
<tr>
<td>sample solution</td>
<td>_</td>
<td>0.1 ml</td>
</tr>
</tbody>
</table>

Mix and read absorbances (A1) of the solutions after 5 min. Start reaction by addition of

| solution 4 | 0.02 ml | 0.02 ml |

Mix. After 30 min., on completion of the reaction, read absorbances (A2) of the solutions immediately one after another.

- Calculations

According to general formula for calculating the concentrations the equation is

\[ c = \frac{V \times MW}{\varepsilon \times d \times v \times 1000} \times \Delta A, \text{g/L}, \]
where $V$ is final volume of assay, mL; $MW$ is molecular weight of lactic acid; $d$ is light pass, cm; $\varepsilon$ is absorption coefficient of NADH at 340 nm; $\Delta A = \Delta A_s - \Delta A_b$.

$\Delta A_s = A_2 - A_1$ for sample

$\Delta A_b = A_2 - A_1$ for blank

It follows for L-lactic acid

$$c = \frac{2.24 \times 90.1}{6.3 \times 1 \times 0.1 \times 1000} \times \Delta A = 0.32 \times \Delta A$$