L(+) Lactic Acid Production From Whey by *Lactobacillus Casei* NRRL B-441

By

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ABSTRACT

Lactic acid, its derivatives and poly-lactic acid are widely used in the industry. Lactic acid has been produced chemically or by fermentation for many years. However, there is a need to develop low cost production and purification methods. The development and use of processes with high productivity and product yield from inexpensive carbohydrate sources can lead to a more feasible lactic acid production.

The goal of this work was to find the most suitable values of some fermentation parameters for lactic acid production from whey by an L-lactic acid producing bacterium, *Lactobacillus casei.* Whey is the by-product of cheese production and it is inexpensive and year-round available.

Fermentations were conducted in the fermenter and shake flasks to determine the optimum values for temperature and pH. The highest lactic acid productivity values were obtained at 37 °C and pH 5.5. The productivity was 2.0 g l^{-1} h^{-1} at 37 °C in the shake flask. In the fermenter, a productivity of 4.6 g l^{-1} h^{-1} was obtained at pH 5.5. The effect of yeast extract concentration was also examined. Although the productivity values were found to be slightly higher (approximately 1.8-2.0 g l^{-1} h^{-1}) with 0.75 and 1.0% (w/v), 0.5% (w/v) yeast extract concentration with a productivity figure of 1.75 g l^{-1} h^{-1} was concluded to be the most feasible concentration, since yeast extract is an expensive material.

L. casei was tested for its capability to utilize different substrates, particularly whey, synthetic lactose and synthetic glucose. Whey yielded a higher productivity value of 1.75 g l^{-1} h^{-1} than the synthetic sugars.

The effect of initial substrate concentration on lactic acid production was examined up to 10% (w/v) whey lactose. Lactose was utilized completely for every initial substrate concentration examined in this study. Product yields were between 0.89-0.94 g lactic acid (g lactose)$^{-1}$.

The salt effect was examined by discarding one of the salts (K_2HPO_4, KH_2PO_4, $MgSO_4$ or $MnSO_4 \cdot H_2O$) from the medium at each run. The lactic acid production was poor in the absence of $MnSO_4$.

Seed culture that had the same composition as the fermentation medium was used as the inoculum for the fermenter. With this seed culture greater productivity values were obtained than the shake flasks, which were inoculated with litmus milk culture. In the shake flasks the highest productivity was around 2.0 g l^{-1} h^{-1}, while in the fermenter a productivity value of 4.6 g l^{-1} h^{-1} could be obtained with 12.5% inoculum at pH 5.5.

Biomass growth was investigated in lactose synthetic medium. The lactic acid production was associated with the biomass growth up to a certain time, but then a non-growth associated lactic acid production was observed. Maximum specific growth rate was calculated as 0.32 h^{-1}.

ÖZ

Laktik asit, türevleri ve poli-laktik asit endüstride yaygın olarak kullanılmaktadır. Laktik asit uzun yıllardan beri kimsayal yolla veya fermentasyonla üretilmektedir. Ancak, düşük maliyetli üretim saflaştırma yöntemlerinin geliştirilmesine ihtiyaç vardır. Pahalı olmayan karbonhidrat kaynakları kullanmak, yuksek verimlilik elde etmek daha uygun laktik asit üretimi sağlayabilir.

Bu çalışma bir L-laktik asit bakterisi olan *Lactobacillus casei* kullanarak peynir altı suyundan laktik asit fermentasyonu parametrelerini optimize etmeyi amaçlamaktadır. Peynir altı suyu peynir üretiminin yan ürünudür. Ucuzdur ve yılın her zamanı mevcuttur.

Fermentasyonlar, fermentörde ve çalkalamalı inkübatör içinde erlenlerde gerçekleştirilerek en uygun sıcaklık ve pH değerleri bulunmuştur. En yüksek verimlilik 37 ^{o}C' de ve pH 5.5' de elde edilmiştir. Çalkalamalı inkabatörde en yüksek verimlilik 37 C^{o}' de 2.0 g l^{-1} h^{-1} olmuştur. Fermentörde en yüksek verimlilik pH 5.5' de 4.6 g l^{-1} h^{-1} olarak bulunmuştur. Maya özütü konsantrasyonun etkisi de incelenmiştir. %0.75 ve %1.0 maya özütü konsantrasyonlarında verimlilik değerleri az miktarda yüksek olduysa da (1.8-2.0 g l^{-1} h^{-1}), maya özütü pahalı bir madde olduğundan 1.75 g l^{-1} h^{-1} verimlilik değeriyle %0.5 maya özütü konsantrasyonunun en uygulanabilir değer olduğu sunucuna varılmıştır.

L. casei değişik karbon kaynaklarını (sentetik laktoz ve glukoz ve peynir altı suyu) kullanabilmesi açısından test edilmiştir. Peynir altı suyu sentetik şekerlere göre daha yüksek verimlilik sağlamıştır.

Başlangıç substrat konsantrasyonun etkisi %10'a kadar incelenmiştir. Bütün denemelerde laktoz tamamen dönüştürülmüştür. Ürün verimliliği 0.89-0.94 g laktik asit (g laktoz)$^{-1}$ aralığında olmuştur.

Tuzların etkisi her denemede bir tuz (K_2HPO_4, KH_2PO_4, $MgSO_4$ ya da $MnSO_4 \cdot H_2O$) ortam içeriğinden çıkarılarak test edilmiştir. $MnSO_4$' ün olmadığı denemede ürün verimi çok düşük olmuştur.

Fermentör için aşı olarak fermentasyon ortamıyla aynı içeriğe sahip kaynak kültür kullanılmıştır. Bu aşı ile litmuslu süt kültürüyle aşılanan erlenlere göre daha yüksek verim elde edilmiştir. Çalkalamalı kültürde en yuksek verimlilik 2.0 g l^{-1} h^{-1}

civarında iken fermentörde 12.5% kaynak kültür kullanılarak 4.6 g l^{-1} h^{-1} civarında verimlilik elde edilmiştir.

Biyomas gelişimi sentetik laktoz kullanılarak incelenmiştir. Belli bir zamana kadar laktik asit üretimi bakteri konsantrasonu ile ilişkili olarak sürmüş, daha sonra bakteri büyümesiyle ilişkili olmayan bir süreç gözlenmiştir. En yüksek özgül büyüme oranı (μ_{max}) 0.32 h^{-1} olarak hesaplanmıştır.

TABLE OF CONTENTS

LIST OF FIGURES x

LIST OF TABLES xi

Chapter 1. INTRODUCTION 1

Chapter 2. PRODUCTION AND PROPERTIES OF LACTIC ACID 3

2.1. Organic Acids 3

2.2. Lactic Acid 4

2.2.1. History 4

2.2.2. Physical and Chemical Properties 5

2.2.3. Uses and Applications 7

2.2.4. Production Technology 8

2.2.4.1. Chemical Synthesis 8

2.2.4.2. Microbial Lactic Acid Production 9

2.2.4.2.1. Microorganisms 9

2.2.4.2.2. Raw Materials 17

2.2.4.2.3. Fermentation Process 20

2.2.4.2.4. Recovery 22

Chapter 3. MATERIALS AND METHODS 24

3.1. Materials 24

3.2. Methods 25

3.2.1. Culture Propagation 25

3.2.2. Lactic Acid Fermentation 25

3.2.2.1. Lactic Acid Fermentation in Shake Flasks 27

3.2.2.1.1. Effect of Temperature 27

3.2.2.1.2. Effect of Yeast Extract Concentration 27

3.2.2.1.3 Effect of Salts 28

3.2.2.1.4 Effect of Substrate Type 28

3.2.2.1.3. Effect of Initial Substrate Concentration 28

3.2.2.2. Lactic Acid Fermentation in the Fermenter 28

3.2.2.2.1. Effect of pH 29

3.2.2.2.2. Biomass Growth 31

3.2.2.3. Comparison of the Fermenter and the Shake Flasks for Lactic Acid Fermentation.... 31
3.2.3. Analyses 31
3.2.3.1. Lactose, Glucose and Lactic Acid Analyses 32
3.2.3.2. Dry Cell Weight Measurements 33
Chapter 4. RESULTS AND DISCUSSION 34
4.1. Effect of Temperature 34
4.2. Effect of pH 38
4.3. Effect of Yeast Extract Concentration 42
4.4. Effect of Salts 46
4.5. Effect of Substrate Type 48
4.6. Effect of Initial Substrate Concentration 51
4.7. Comparison of the Fermenter and the Shake Flasks for Lactic Acid Fermentation 55
4.8. Biomass Growth 57
Chapter 5. CONCLUSIONS AND RECOMMENDATIONS 60
REFERENCES 62
APPENDIX 66
A. Calibration Curves 66
A.1. Calibration Curves for Lactose, Glucose and Lactic Acid 66
A.2. Calibration Curve for Dry Cell Weight Measurements 67
B. Definitions of Fermentation Evaluation Terms 68

Figure 4.19. Lactic acid production and lactose utilization in the fermenter inoculated with 4% litmus milk culture. 56
Figure 4.20. Lactose, lactic acid and dry cell weight concentration for *L. casei* grown on lactose synthetic medium. 59
Figure 4.21. Logarithmic growth curve of *L. casei*. 59
Figure A.1. Calibration curves used in the HPLC analyses. 66
Figure A.2. Dry cell weight calibration curve 67

LIST OF TABLES

Table 2.1. Main organic acids produced by microorganisms. 3
Table 2.2. Uses of some organic acids. 4
Table 2.3. United States market for acidulants. 4
Table 2.4. Physical properties of lactic acid. 6
Table 2.5. Properties of the commercial lactic acid. 7
Table 2.6. Some of the food applications of lactic acid and derivatives. 7
Table 2.7. Homo- and Heterofermentative lactic acid bacteria. 11
Table 2.8. Some of the lactic acid fermentation studies by *L casei* strains. 16
Table 2.9. Fermentation substrates for lactic acid production. 17
Table 2.10. Gross composition of liquid and dried whey. 19
Table 2.11. Amino acid, vitamin and mineral content of whey powder. 19
Table 2.12. Characteristics of industrial lactic acid fermentation process. 22
Table 3.1. Chemicals and their producers. 24
Table 3.2. Components used in the fermentations. 25
Table 3.3. The properties of the column and analysis conditions. 32
Table 4.1. Temperatures used for lactic acid fermentation by *L. casei*. 36

CHAPTER 1

INTRODUCTION

Lactic acid is the most widely occurring organic (hydroxycarboxylic) acid that is produced by animals, plants and microorganisms as the major metabolic intermediate. Lactic acid is present in many foods, both naturally or as a product of in situ fermentation as in yogurt, buttermilk and sauerkraut (Martin 1997, Datta et al. 1995).

Lactic acid and its salts have many uses in food, chemical, textile and pharmaceutical industrial processes. Since it is odourless, has a mild acidic taste and does not mask the flavour of the food, approximately 50% of the lactic acid produced is used in the food industry as an acidulant and preservative

Lactic acid exists in two optically active forms, D(-) and L(+) lactic acid. Although they both have the same chemical and physical properties, only the L-lactic acid can be tolerated by the human metabolism. Therefore, highly pure preparations of L-lactic acid are in demand as a raw material for the production of biodegradable lactide polymers which are used for biomaterial manufacturing, since they are biocompatible, body absorbable and blood compatible.

Lactic acid can be produced industrially by chemical synthesis and microbial fermentation process. However, chemical synthesis yields only the racemic (DL) lactic acid. Therefore, the need of L-lactic acid has attracted research in the area of biotechnology for many years. There are still hurdles in the process that should be overcome.

The quests for the production of high yield lactic acid in short time and at low cost still goes on. L-lactic acid and its derivatives are in demand in the food, pharmaceutical and biomaterial industries.

Lactobacillus casei is found to be an L-lactic acid producing lactic acid bacterium. L-lactic acid fermentation by *L. casei* has attracted considerable interest in recent years, since about 97.5% of the lactic acid is in the form of L form (Vaccari 1993). Most of the other lactic acid bacteria produce DL and D lactic acids.

Various substrates can be used in the L-lactic acid production by *L. casei*. Microbial fermentation of lactic acid can be attractive if low cost waste materials are used as the carbon source. Whey is considered to be an attractive carbon source since it contains lactose and many other nutrients that may be essential for the growth of the microorganisms.

Whey is a major by-product in the dairy industry, with a biological oxygen demand (BOD) of 40,000-60,000 ppm that presents serious disposal problems (Irvine and Hill 1985). The utilization of lactose in whey using a process that effectively decreases BOD content, while at the same time producing marketable products, is an interesting proposition due to its positive environmental and economical impact. Although there have been extensive researches on the production of L-lactic acid from whey, the quest for the production of high yield lactic acid in short time with low cost still goes on, since lactic acid and its derivatives are in demand in the food, pharmaceutical and biomaterial industries. Therefore, there is a need for to develop the lactic acid production by increasing the production rate, by using renewable, inexpensive substrates and by developing novel purification methods.

This dissertation focuses on the utilization of whey lactose by *L casei* and addresses our attempts to optimize the lactic acid fermentation. Attempts were done to increase the productivity and yield by investigating the effects of some factors, such as temperature, pH, yeast extract, salt content, initial substrate concentration, etc.

CHAPTER 2

PROPERTIES AND PRODUCTION OF LACTIC ACID

2.1 Organic Acids

Organic acids are important compounds that find applications in many industries. They are widely used as food ingredients, mainly as food acidulants. Although some can be produced by chemical synthesis, most of them are produced by fermentation. Many microorganisms are used for acid fermentations. Main organic acids and the microorganisms used to produce them are listed in Table 2.1. In Table 2.2 uses of some organic acids are summarized. The food consumption figures and market values of some organic acids (acidulants) in the United States in 1992 are listed in Table 2.3.

Table 2.1. Main organic acids produced by microorganisms (Atkinson and Mavituna 1991).

Acid	Microorganism	Carbon source
Acetic acid	*Acetobacter aceti*	Ethanol
L-Alloisocitric acid	*Penicillium purpurogenum*	Glucose
Citric acid	*Aspergillus niger*	Sucrose
	Candida (Saccharomycopsis) lipolytica	
Fumaric acid	*Rhizopus delemar*	Glucose
Gluconic acid	*A. niger*	Glucose
Isoascorbic acid	*P. notatum*	Glucose
L-Isocitric acid	*C. brumpti*	Glucose
Itaconic acid	*A. terreus*	Glucose
2-Ketogluconic acid	*Pseudomonas fluorescens*	Glucose
5-Ketogluconic acid	*Gluconobacter suboxydans*	Glucose
α-Ketoglutaric acid	*C. hydrocarbofumarica*	n-Alkane
Kojic acid	*A. oryzae*	Glucose
Lactic acid	*Lactobacillus delbrueckii*	Glucose
Malic acid	*L. brevis*	Glucose
Propionic acid	*Propionibacterium Shermanii*	Glucose
Succinic acid	*Ps. Aeruginosa*	Malic acid
Tartaric acid	*G. suboxydans*	Glucose

Table 2.2. Uses of some organic acids (Atkinson and Mavituna 1991, Bigelis and Tsai 1995).

Organic Acid	Uses
Citric acid	Flavouring for beverages, confectionary, food, pharmaceutical syrups, production of resins, antifoaming agent, sequestering agent, dye mordant
L-Malic acid	Food and drinks manufacture
L-Tartaric acid	Food additive, photography, tanning, ceramic manufacture
Itaconic acid	Intermediate for organic synthesis
D-Gluconic acid	Pharmaceutical (antipasmodic), acid detergent for metals
Acetic acid	Lowering of pH, flavour enhancement, controlling growth of microorganisms in food products
Tartaric acid	Acidulant and flavour ingredient in food and beverages
Fumaric acid	Acidulant in food and beverages, clarifying agent in wine
Kojic acid	Precursor for the chemical synthesis of maltol and ethylmaltol
Lactic acid	Acidulant, preservative, flavorant and processing aid in food products

Table 2.3. United States market for acidulants (Bigelis and Tsai 1995).

Acidulant	Food consumption (million lb)	Value in food applications (million $)
Acetic acid (vinegar)	2,500	128.0
Citric acid	410	328.0
Tartaric acid	30	88.5
Malic acid	16	13.0
Lactic acid	9	8.4
Fumaric acid	6	4.6
Gluconic acid and δ-gluconolactone	6	2.9

2.2 Lactic Acid

Since lactic acid has been known for many years, there is quite a lot of information in the literature about it. The history, properties, uses and production technology of lactic acid are summarized in the following sections.

2.2.1 History

Lactic acid was first discovered in 1780 in sour milk by the Swedish chemist Scheele. It was first produced commercially by Charles E. Avery at Littleton,

Massachusetts, USA in1881. However, attempts to market calcium lactate, as a substitute for cream of tartar in baking powder was unsuccessful. First uses began about 1894 in the leather and textile industries. Production levels were about 5000 kg y^{-1} those days and reached 2.7 $*10^6$ kg y^{-1} in 1942. US production were 4.1 * 10^6 kg y^{-1} during the Second World War but declined to 2.3 * 10^6 kg y^{-1} after the war. The synthetic production started in 1960s with the demand of heat stable lactic acid to be used to produce stearoyl-2-lactylates for the baking industry. In 1982, the worldwide production of lactic acid was 24-28 * 10^6 kg y^{-1} and half of it was used by the food industry as an acidulant and preservative. 20% of it was consumed to produce stearoyl-2-lactylates. The pharmaceutical and the other industries used the rest (Vick Roy 1985, Datta et al. 1995). The worldwide production volume by 1990 was 40 * 10^6 kg y^{-1} (Datta et al. 1995).

2.2.2 Physical and Chemical Properties

Lactic acid (2-hydroxy-propionic acid) exist in two optically active forms, the D(-), levorotatory, and the L(+), dextrorotatory. Both isomers exist in biological systems, although the L(+) isomer (sarcolactic acid, paralactic acid) is present in humans. The L(+) form is more important for the food industry, since humans can metabolize it only, by the enzyme L-lactate dehydrogenase. L(+) lactic acid occurs as an intermediate product, which serves as an important source of energy for heart, skeletal muscles, liver, kidney and brain and is burned to carbon dioxide and water, and it is also used in the glucose cycle to form glycogen (Hunger 1984). L(+) lactic acid produced during human metabolism or taken by the food stuff is metabolized in gluconeogenesis, while D(-) lactic acid is excreted or oxidized in the liver. The consumption of D(-) lactic acid was recommended to be limited to 100 mg per kg body weight per day by FAO/WHO Expert Committee on Food Additives. Undesirable reactions due to the D(-) lactic acid had been reported in small children (Martin 1997). No limits exist for L(+) lactic acid (Hunger 1984). Figure 2.1 shows these isomers of lactic acid.

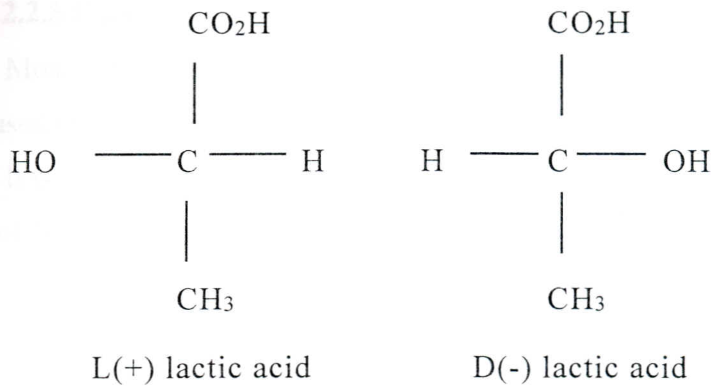


Figure 2.1. Isomers of lactic acid.

When highly pure, lactic acid can form colorless monoclinic crystals. Lactic acid is soluble in all proportions with water and has a low volatility. It is insoluble in chloroform and carbon disulfide. It is miscible with water, alcohol, glycerol, and furfural. The color of lactic acid ranges from colorless to yellow depending on the concentration and impurities (Martin 1997, Vick Roy 1985).

Lactic acid has a versatile reactivity since it can react as both organic acid and organic alcohol. Self-esterification occurs in solutions above 20% of lactic acid due to the hydroxyl and carboxyl groups. Lactic acid can form a cyclic dimer (lactide) or form linear polymer (Vick Roy 1985). Some of the physical properties of lactic acid are given in Table 2.4.

Table 2.4. Physical properties of lactic acid (Vick Roy 1985)

Molecular weight	90.08
Melting point D(-) or L(+)	52.8-54 °C
DL (varies with composition	16.8-33 °C
Boiling point DL	82 °C at 0.5 mmHg
	122 °C at 14 mmHg
Dissociation constant (K_a at 25 °C)	$1.37 * 10^{-4}$
Heat of combustion (ΔH_c)	1361 kJ mol^{-1}
Specific heat (C_p at 20 °C)	190 J mol^{-1} $°C^{-1}$

2.2.3 Uses and Applications

Most of the lactic acid is used by the food and pharmaceutical industries. The rest is used by several industries like tanning, textile and chemical industries.

It is sold in technical, food and pharmaceutical grades. Properties of different grades of lactic acid are summarized in Table 2.5.

Table 2.5. Properties of the commercial lactic acid (Martin 1997).

Grade	Purity	% Total Acidity	Characteristics
Pharmaceutical	High	≈85	Colourless, ≈ 30 % polymerized
Food	Medium	≈50	pale yellow
Technical	Low	44-45	higher concentrations of contaminants (sugar, metals, etc.)

Lactic acid, in contrast with some other food acids, has a mild acid taste. Since it occurs naturally in many foods it is not a foreign element to the food applied. It also does not mask or overpower weakly aromatic compounds. Thus, lactic acid is a convenient food additive.

Lactic acid and its derivatives, such as lactates, are mainly used in food products as acidulant, preservative, flavorant and processing aid. Some of the food applications of lactic acid and lactates are summarized in Table 2.6.

Table 2.6. Some of the food applications of lactic acid and derivatives (Vick Roy 1985, Holten et al. 1971, Datta et al. 1995).

processing and packaging of olives, pickles and sauerkraut
decontamination of beef chicken carcasses and in dehiding of beef tongues
lengthening the shelf-life for mayonnaise and dressings
acidification and flavour enhancement of soft drinks and lemonades
acidification of cheese, cheese products, jams and jellies
fermentation accelerator and acidulant for bakery products
pH adjustment of the water used for beer brewing

Stearoyl-2-lactylates are produced from heat stable, high quality lactic acid. Calcium and sodium stearoyl-2-lactylates are used mostly in baking as starch conditioners and as emulsifiers in food products. They also lengthen the shelf life of baked products. Glyceryl lactostearate and glyceryl lactopalmiate are used in

prepared cake mixes and other bakery products and in liquid shortenings (Vick Roy 1985, Datta et al. 1995).

Technical grade lactic acid is used as an acidulant for deliming hides and vegetable tanning in leather tanning industry. It is also used widely in textile finishing and acid dying of wool. Lactic acid finds some use in the manufacturing of cellophane and production of phenol-formaldehyde resins. Some other small-scale industrial applications of lactic acid are alkyd resin modifier, solder flux, printing developers, adhesive formulations, electroplating and electro-polishing baths and detergent builders (Datta et al. 1995, Vick Roy 1985).

Lactic acid and methyl lactate find uses in medical and cosmetic applications. They have been used in pharmaceutical manufacture and cosmetic formulations, particularly in topical wart medications, lotions and parental solutions. In recent years, the uses of poly-lactic acid for medical applications are increasing since it is biodegradable and biocompatible. These applications include surgical sutures, controlled-release drugs and implant for the repair of fractures and other injuries (Datta et al. 1995).

Datta et al. (1995) investigated the potential of lactic and poly-lactic acid and derivatives. They indicate that the use of lactic acid in new applications such as monomer in plastics or as an intermediate in synthesis of high volume oxygenated chemicals may increase its demand.

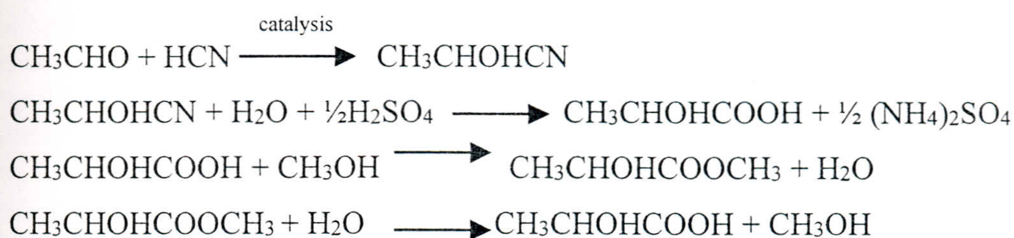
2.2.4 Production Technology

Lactic acid can be produced by microbial fermentation or by chemical synthesis. Today each technology makes about roughly 50% of the world's total lactic acid production (Vick Roy 1985).

2.2.4.1 Chemical Synthesis

Lactonitrile is hydrolyzed to lactic acid by using a strong acid, HCl or H_2SO_4, producing the corresponding ammonium salt as a by-product. Esterified by methanol, the crude lactic acid produces methyl lactate. After recovered and distilled, methyl lactate is hydrolyzed under acid catalysis to produce lactic acid. Lactic acid is then concentrated and purified (Datta et al 1995).

Lactonitrile is obtained as a by-product from acrylonitrile synthesis. Hydrogen cyanide is added to acetaldehyde in the presence of base to produce lactonitrile. The crude lactonitrile is then recovered and purified by distillation and used for lactic acid synthesis (Datta et al 1995).



$$CH_3CHO + HCN \xrightarrow{\text{catalysis}} CH_3CHOHCN$$

$$CH_3CHOHCN + H_2O + \tfrac{1}{2}H_2SO_4 \longrightarrow CH_3CHOHCOOH + \tfrac{1}{2}(NH_4)_2SO_4$$

$$CH_3CHOHCOOH + CH_3OH \longrightarrow CH_3CHOHCOOCH_3 + H_2O$$

$$CH_3CHOHCOOCH_3 + H_2O \longrightarrow CH_3CHOHCOOH + CH_3OH$$

The synthetic lactic acid is heat stable and highly pure. It contains no residual sugar and heating does not cause a discoloration (Vick Roy 1985). However, the chemical synthesis produces only racemic lactic acid (DL) (Datta et al 1995).

2.2.4.2 Microbial Lactic Acid Production

A wide variety of carbohydrate sources and microorganisms can be used for lactic acid fermentation. The desired stereoisomer of lactic acid can be obtained by the fermentation technology.

2.2.4.2.1 Microorganisms

Lactic acid bacteria and some fungi of the species *Rhizopus* produce lactic acid.

Lactic acid bacteria are gram-positive, non-sporing and usually nonmotile and are categorized as facultative anaerobes, thus making the strict exclusion of air is unnecessary. Lactic acid bacteria have limited biosynthetic capabilities, so that they require many vitamins (especially B vitamins) amino acids, purines, and pyrimidines (Prescott 1996).

The homofermentative lactic acid bacteria produce only lactic acid, cells and little else, thus are of industrial importance. The heterofermentative lactic acid bacteria, on the other hand, produce not only lactic acid, but also acetic acid, carbon

dioxide, ethanol and glycerol (Vick Roy 1985). Homofermentatives are capable of extracting, from a given amount of glucose, twice the amount of energy as heterofermentatives (Martin 1997).

Homo and heterofermentative lactic acid bacteria and their lactate configurations are listed in Table 2.7.

Table 2.7. Homo- and Heterofermentative lactic acid bacteria (Jay 1992).

Homofermentative		*Heterofermentative*	
Organisms	Lactate Configuration	Organisms	Lactate Configuration
Lactobacillus		Lactobacillus	
L. acidophilus	DL	*L. brevis*	DL
L. bulgaricus	D(-)	*L. buchneri*	DL
L. casei	L(+)	*L. cellobiosus*	DL
L. coryniformis	DL	*L. confusus*	DL
L. curvatus	DL	*L. coprophilus*	DL
L. delbrueckii	D(-)	*L. fermentum*	DL
L. helveticus	DL	*L. hilgardii*	DL
L. jugurti	DL	*L. sanfrancisco*	DL
L. jensenii	D(-)	*L. trichodes*	DL
L. lactis	D(-)	*L. viridescens*	DL
L. leichmaniii	D(-)	Leuconostoc	
L. plantarum	DL	*L. cremoris*	D(-)
L. salivarius	L(+)	*L. dextranicum*	D(-)
Pediacoccus		*L. lactis*	D(-)
P. acidilactici	DL	*L. mesenteroides*	D(-)
P. cerevisiae	DL	*L. oenos*	D(-)
P. pentosaceus	DL	*L. paramesenteroides*	D(-)
Streptococcus		*L. gelidum*	D(-)
S. bovis	D(-)	*L. carnosum*	D(-)
S. thermophilus	D(-)	Carnobacterium	
Lactococcus		*C. divergens*	
L. lactis subsp lactis		*C. mobile*	
L. lactis subsp hordniae		*C. piscicola*	
L. garvieae	D(-)		
L. plantarum			
L. raffinolactis			
Vagococcus			
V. fluvialis			
V. salmoninarum			

The homofermentative lactic acid bacteria are from the genera *Lactobacillus, Pediacoccus* and *Streptococcus.* Their growth temperature range is 5 to 45 °C, while optimal temperature is greater than 40 °C. For some bacteria, such as *L. pentosus, L. casei* or *Streptococcus lactis*, the optimal temperature is about 30 °C. They grow optimally at a pH of 5-7 (growth range 3.2 – 9.6) (Vick Roy 1985, Martin 1997, Atkinson and Mavituna 1991).

The homofermentative lactic acid bacteria catabolize glucose *via* Embden-Mayerhof pathway. Two lactic acid molecules are produced from each molecule of glucose. (Figure 2.2)

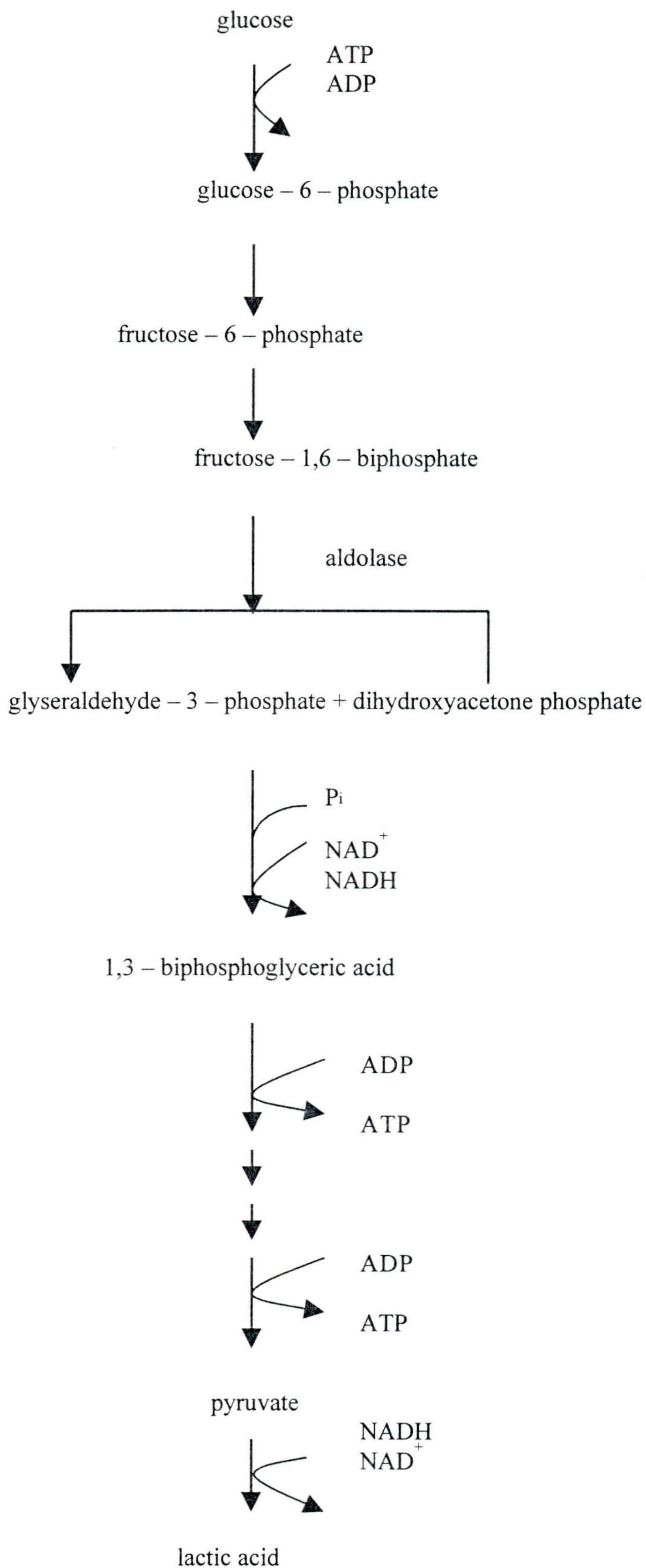


Figure 2.2. Fermentation of glucose in homofermentative lactic acid bacteria (Brock et al 1994).

In Figure 2.3 the two different pathways for lactose metabolism are shown. Most lactose fermenting *Lactobacillus* possess β-galactosidase activity rather than phospho-β-galactosidase activity. Lactobacilli possess both types of enzymes; however, *L. casei*, *L. buchneri* and most *Lactococcus* possess only phopho-β-galactosidase (Arihara and Luchansky 1995).

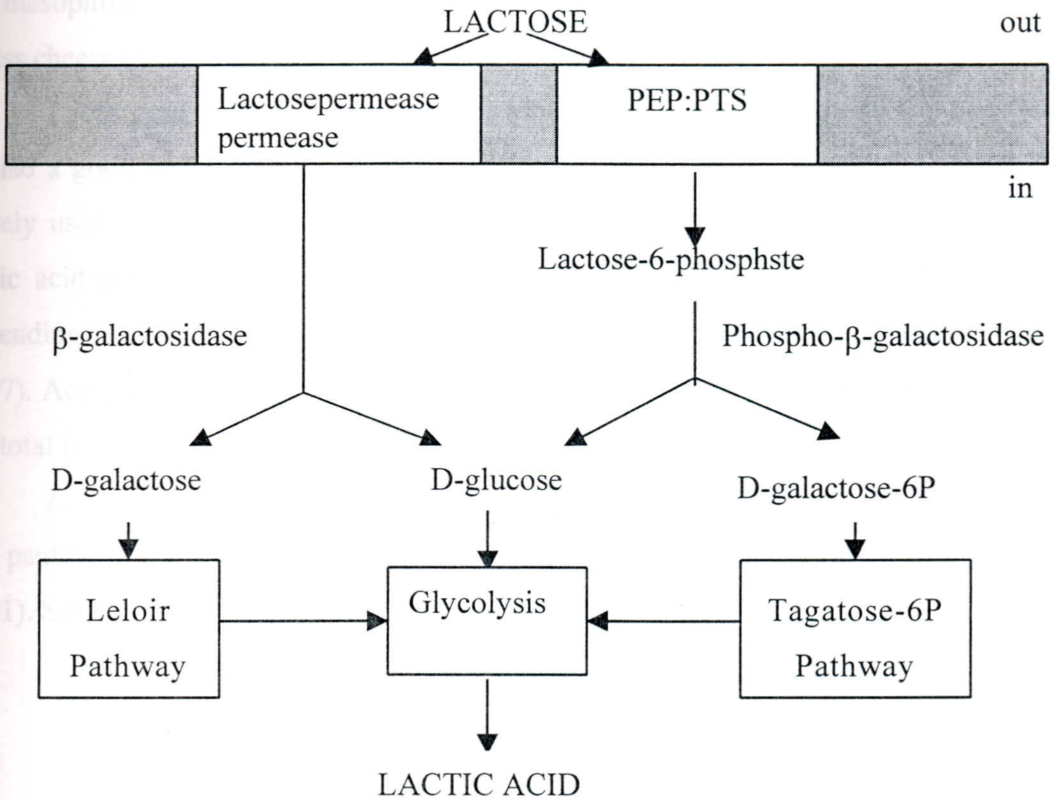


Figure 2.3. Pathways for lactose utilization by lactic acid bacteria (Arihara and Luchansky 1995).

In the pathway that *L. casei* follows, lactose is internalized and phosphorylated with the phosphoenolpyruvate-dependent phosphotransferase system (PEP:PTS). By phospho-β-galactosidase, the phosphoralated lactose is hydrolyzed to D-glucose and D-galactose 6-phosphate. The D-galactose 6-phosphate is converted to D-tagatose 6-phosphate and then to triose phosphates in the tagatose 6-phosphate pathway. Lactate is produced from both triose phosphates and glucose by glycolysis (Arihara and Luchansky 1995).

IZMIR YUKSEK TEKNOLOJİ ENSTİTÜSÜ
REKTÖRLÜĞÜ
Kütüphane ve Dokümantasyon Daire Bşk.

Lactobacillus casei is a homofermentative lactic acid bacterium that is used generally to ferment lactose. Whey, which contains 4-5% lactose, is one of the most widely used substrate for lactic acid production by this organism. On the other hand, there are several studies that it is used to ferment glucose (Hujanen and Linko 1996, Vaccari et al. 1993, R. Gamal and N. Gamal 1984, Guoqiang et al. 1991). The lactic acid production from lactose and glucose are comparable. This organism is among the mesophilic lactic acid bacteria that are used for the manufacture of Grana and Swiss cheese (Vick Roy 1985).

Lactic acid produced by *L. casei* is mainly the L(+) form (Jay 1992). *L. casei* is also a good lactic acid producer. Therefore, it is one of the organisms which is widely used for L(+) lactic acid production. L(+) lactic acid contents of the total lactic acid produced by *L. casei* were reported to be between 92.9 and 97.6 % depending on the strain and the conditions (Vaccari et al. 1993, Senthuran et al. 1997). According to Hunger (1984), L(+) lactic acid constitutes greater than 95 % of the total lactic acid produced by *L. casei.*

L. casei needs K^+ and Mn^{2+} ions for the growth. It is also dependent on folic and pantothenic acids and riboflavin and some amino acids (Atkinson and Mavituna, 1991). Several studies on *L. casei* are summarized in Table 2.8.

Table 2.8. Some of the lactic acid fermentation studies by *L casei* strains.

STRAIN	SYSTEM	SUBSTRATE	EFFECTS STUDIED	REFERENCE
L. casei NRRL B-441	Batch	Glucose	Temperature, Nitrogen sources	Hujanen and Linko 1996
L. casei subsp. *casei* DSM20011	Batch	Glucose	Substrate concentration	Vaccari et al. 1993
L. casei SU No 22 *Lactococcus lactis* (mixed)	Batch, Fed batch, Free and Coimmobilized	Whey	Batch vs. Fed batch Feeding rate	Roukas and Kotzekidou 1998
L. casei subsp. *rhamnosus* CCM 1828	Batch	Glucose	Age, amount of inoculum, Inoculation medium composition	Martinkova et al. 1991
L. casei RA 9867	Batch	Various	Incubation period, Glucose concentration, Carbon sources	R. Gamal and N. Gamal 1984
L. casei subsp. *casei* DSM 20244	Batch, Continuous, Fluidized bed, Immobilized	Whey	Fermentation medium, Dilution rate, Temperature, pH Continuous vs. fluidized reactors	Krischke et al. 1991
L. casei	Immobilized, Batch	Whey	Matrices, Temperature, pH, Cell density, Metal ion, Substrate concentration	Tuli et al. 1985
L. casei subsp. *rhamnosus* DSM 20021	Immobilized Recycle batch	Whey	Repeating batches, Recirculation rate, Yeast extract	Senthuran et al. 1997
L casei 20021	Immobilized, Batch, Semi continuous	Glucose	Glucose concentration, pH control, Free vs. immobilized cells, Yeast extract, Lactic acid inhibition	Guoqiang et al. 1991

2.2.4.2.2 Raw Materials

A wide variety of substrates can be used as carbon source in lactic acid fermentation. Vick Roy (1985) summarizes the properties of a good substrate as:

- low cost
- low levels of contamination
- fast fermentation rate
- high lactic acid yields
- little or no by product formation
- ability to be fermented with little or no pre-treatment
- year round availability

Lactic acid is generally produced from glucose, maltose, sucrose or lactose. Starches are hydrolyzed by enzymes or acid to maltose and glucose before the fermentation. These carbohydrates may be supplied in pure form or as constituent of a crude feedstock. Fermentation substrates for lactic acid fermentation are tabulated in Table 2.9.

Table 2.9. Fermentation substrates for lactic acid production (Vick Roy 1985, Martin 1997).

Carbohydrate	Source
Lactose	Casein whey Cheese whey Sweet whey
Corn sugar (dextrose)	Corn
Sucrose	Molasses Cane and beet sugar
Others	Potatoes Sulphite waste liquor Jerusalem artichokes Cellulosic materials (corn cob, corn stalk, cottenseed halls, straw) Sorghum extract

Crude feedstocks are inexpensive and easily available, however, the high levels of other compounds present in the feedstock cause some problems during the recovery of lactic acid. As a result of this, refined sucrose is the most commonly used substrate in industry despite its high cost.

The most widely used crude feedstocks are molasses and whey. Molasses is the by-product of sugar industry. It contains a high concentration of sucrose, it is cheap and abundant. It is used as a substrate in the production of baker's yeast, citric acid, lactic acid, acetic acid, glutamic acid, gluconic acid, itaconic acid and acetone.

Whey is the liquid by-product of cheese manufacture. Approximately 1-2 kg of cheese is produced from 10 kg of milk and the residual liquid is the whey. Worldwide annual whey production is 96 million tons and 46 million tons of it is produced in Europe (Börgardts et al. 1998). The whey production in Canada is estimated as 1.5 million tons (Mulligan and Safi 1991). In Turkey 800,000 tons of whey is produced annually and most of this is not utilized and is disposed (Tulumoğlu and Beyatlı 1992).

Whey has high chemical oxygen demand of 50-60 kg O_2 per ton and treatment in sewage treatment plants is expensive (Zayed and Winter 1995). Biological oxygen demand should be reduced from 40,000-60,000 ppm to less than 500 ppm. Therefore, a great amount of research has been performed for converting this liability to an asset. (Irvine and Hill 1985)

Mostly, the protein content of whey is separated by ultra-filtration or fractionated to produce protein concentrates that have particular nutritional and functional properties. Whey or whey powder have been used generally for whey beverages manufacture, single cell protein, ethanol, lactic acid fermentation, lactose production and as human and animal food supplement. (Irvine and Hill 1985)

The composition of whey varies with cheese manufacture procedures (Irvine and Hill 1985). The compositions of liquid and dried whey are given in Table 2.10 and Table 2.11.

Table 2.10. Gross composition of liquid and dried whey (Nutting 1970, Irvine and Hill 1985, Atkinson and Mavituna 1991).

Component	Fluid Whey	Dried Whey
Total solids, %	6.35-7.0	96.3-96.5
Protein, %	0.8-0.9	13.0-13.1
Lactose, %	4.85-5.1	68.0-75.0
Fat, %	0.3-0.5	0.8-1.0
Lactic acid, %	0.05	0.2
Ash, %	0.5-0.6	7.3-9.6

Table 2.11. Amino acid, vitamin and mineral content of whey powder (Atkinson and Mavituna 1991, Irvine and Hill 1985).

Amino acids		Vitamins		Minerals	
L-Arganine	0.4%	Biotin	0.4 mg kg^{-1}	P	0.66%
L-Cystine	0.4%	Choline	2420 mg kg^{-1}	Ca	0.77%
L-Glycine	0.7%	Nicotinic acid	11.0 mg kg^{-1}	K	2.54%
L-Histidine	0.2%	Pantothenic acid	48.5 mg kg^{-1}	Mg	0.12%
L-Isoleucine	0.7%	Pyridoxine	2.9 mg kg^{-1}	Na	0.83%
L-Leucine	1.2%	Riboflavin	19.8 mg kg^{-1}		
L-Lycine	1.0%	Thiamin	4.0 mg kg^{-1}		
L-Methionine	0.4%				
L-Phenylalanine	0.5%				
L-Threonine	0.6%				
L-Tryptophan	0.2%				
L-Tyrosine	0.5%				
L-Valine	0.6%				

A great amount of research has been done on lactic acid production from whey. Whey is an inexpensive, easily available substrate; however, it contains some impurities that require additional purification processes.

Some of the fermentation studies performed with *L. casei* and whey are summarized in Table 2.8 in section 2.2.4.2.1. (Microorganisms). In addition to those, Börgardts et al. (1998) proposed an integrated process for the simultaneous production of lactic acid and treatment of whey which also had a low production cost. Stieber and Gerhardt (1979) and Mehia and Cheryan (1987) used *Lactobacillus bulgaricus* for lactic acid fermentation from whey. Amrane and Prigent (1994), Roy et al. (1986), Boyaval et

al. (1987), Chiarini et al. (1992), Oyaas et al. (1996) and Aeschilmann and Stockar (1991) studied lactic acid fermentation from whey by *Lactobacillus helveticus*.

Whole whey, concentrated whey, whey powder or whey permeate were used as substrates for lactic acid fermentation. Whey was supplemented with nitrogen sources (mostly yeast extract) and salts. Kirschke et al. (1991) and Senthuran et al. (1997) hydrolyzed the whey proteins by an endoprotease and used them as the nitrogen source for the microorganisms used for the fermentation.

2.2.4.2.3 Fermentation Process

Batch process has been the most commonly used method in industry. Reactors are wood or stainless steel equipped with heat transfer coils and agitators. Reactors are steam or chemically sterilized before filling with pasteurized medium. (Vick Roy 1985, Martin 1997)

Accumulation of high lactic acid in the reactor lowers the pH and inhibits the production. Therefore lactic acid is generally neutralized by calcium hydroxide or calcium carbonate. However, high amount of calcium lactate crystallizes in the reactor that limits the initial sugar concentration.

Under optimal laboratory conditions fermentations take one or two days. Fermentation rate depends mainly on temperature, pH, concentration of nitrogenous material and lactic acid concentration. In industrial production, the amount of nitrogenous material is sub-optimal (Vick Roy 1985).

Characteristics of industrial lactic acid fermentation processes are given in Table 2.12.

Table 2.12. Characteristics of industrial lactic acid fermentation processes (Vick Roy 1985, Martin 1997, Datta et al.1995).

Characteristics	Values or Attributes
Temperature	30 – 60 °C depending on the organism
PH	5 – 7 depending on the organism
Inoculum size	5 –10 % (v/v)
Initial sugar concentration	5 – 20 % (w/v)
Final product concentration	<12 –15 % (w/v)
Product yield	85 – 95 % (w/w)
Reactor productivity	1 – 3 kg m^{-3} h^{-1}
Residual sugar concentration	< 0.1 %
Fermentation time	1 – 6 days
Cell mass yields	< 15 %

It is possible to achieve better fermentation rates and productivity values by continuous and fed batch systems; cell recycle and immobilized cell reactors; dialysis fermentation technique.

In continuous fermentation process, fresh medium is pumped in the reactor while equal amount of fermentation broth is pumped out. Continuous reactors overcome the nutrient limitation problem. The main problems are the cell wash-out at high dilution rates and accumulation of lactic acid, toxic metabolites and contaminants in the fermenter.

In fed-batch systems, fresh medium is pumped-in without a removal from the reactor. By using fed-batch culture, Roukas and Kotzekidou (1998) obtained two times better productivity than the batch culture for lactic acid production by coimmobilized *Lactobacillus casei* and *Lactococcus lactis*.

The cell recycle fermenter provides high cell densities in the continuous reactors. The cells in the effluent from the fermenter are recycled back by using a membrane. With a CSTR-membrane recycle reactor Mehia and Cheryan (1987) obtained 27-84 g l^{-1}

h^{-1} productivity at cell concentrations of 30-63 g l^{-1} which is 6-18 times better than the batch reactor.

In immobilized cell reactors, cells are attached to or entrapped in the supports such as alginate, polystyrene, κ-carrageenan, agar, polyacrylamide, etc, so that stable and active cultures can be attained and cultures can be reused.

In hollow fibre fermenter, cells are immobilized on the outside of the hollow fibres in the shell. As the fermentation medium passes through the reactor the nutrients and the product passes through the microporous walls of the hollow fibres while cells can not penetrate. High cell densities are obtained in the reactor.

The dialysis fermentation technique offers preferential removal of toxic metabolites during the fermentation. The medium is dialyzed against a continuous dialysate through a permeable membrane. The dialysate consists of water, which maximizes the diffusion of inhibitory products, such as lactic acid (Demirci 1992). Friedman and Gaden (1970) reported productivity of 8 g l^{-1} h^{-1} in a dialysis culture system as contrasted with 5 g l^{-1} h^{-1} in a conventional batch without dialysis.

2.2.4.2.4 Recovery

Lactic acid produced by fermentative process is recovered from the fermentation broth and then purified.

The temperature of the fermentation broth is increased to 80-100 ^{o}C and the pH is increased to 10-11 in the first stage of the recovery process in order to kill the microorganisms, coagulate the proteins, solubilize the calcium lactate and degrade some of the residual sugars. Suspended solids and the microorganisms are separated from the solution by filtration. The broth containing calcium lactate is carbon treated, evaporated and acidified mostly by sulphuric acid, so that salt is converted to lactic acid and insoluble calcium sulfate is removed by filtration. Lactic acid solution is then carbon treated, ion exchanged and evaporated to produce the desired grade of lactic acid. Alternatively, calcium lactate is recovered from the fermentation broth by evaporation after the filtration and then decomposed by sulfuric acid. Some further processes may be

required in order to produce the desired product. (Datta et al. 1995, Atkinson and Mavituna 1991, Vick Roy 1985, Martin 1997)

A large fraction of the total cost of fermentative lactic acid production is due to the recovery because of the complex separation processes needed for the fermentation of cheap but impure feedstocks. In addition to that, a large amount of gypsum by-product is produced. Thus, when crude feedstocks such as whey and molasses are used as the substrate the high purification cost should be considered (Vick Roy 1985, Datta et al. 1995).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

PINAR Dairy Products, Inc (İzmir,Turkey) supplied the whey powder used in this study. Lactose concentration of whey powder was approximately 62 %. Amount of whey powder added to fermentation broth was adjusted to obtain the desired initial lactose concentration of the medium.

The bacterium, *Lactobacillus casei* NRRL B-441, was kindly provided by United States Department of Agriculture, National Center for Agricultural Utilization Research. The bacterium was supplied in lyophilized form and activated in the propagation medium. The culture propagation medium, litmus milk, was prepared by dissolving 100 g litmus milk powder in 1 l of water.

Distilled or deionized water was used in all preparations.

In Table 3.1 the producers of the chemicals used in this study are listed.

Table 3.1. Chemicals and their producers.

Chemical	Producer
L-Lactic acid	Sigma
α-Lactose	Sigma
Glucose	Merck
Calcium carbonate	Fluka
Sodium hydroxide	Sigma
Sulfuric acid	Merck
Litmus milk	Difco
Magnesium sulfate	Sigma
Manganese sulfate monohydrate	Merck
Potassium dihydrogen phosphate	Merck
Potassium phosphate (dibasic)	Sigma
Yeast extract	Oxoid

3.2 Methods

3.2.1 Culture Propagation

20 ml litmus milk suspensions in 25 ml bottles were sterilized for 15 minutes at 121 °C at 1.1 kg cm^{-2} in the autoclave (Hirayama, Japan). The culture was maintained by transferring 10% (v/v) culture to sterile litmus milk every 15 days at least. Unless otherwise indicated, *L. casei* was incubated at 37 °C for 24 hours in the incubator (Sanyo) and kept at 4 °C. 24 hour old fresh cultures were used as the inocula for the fermentations.

3.2.2 Lactic Acid Fermentation

The components of the fermentation media are listed in Table 3.2. It should be noted the following list is not the composition of the fermentation media. It is the complete list of the components that were used in the fermentations in the overall study. The weight of the chemicals were measured by using Sartorius and AND HM-200 balances.

Table 3.2. Components used in the fermentations.

Ingredient	Concentration (g l^{-1})
Whey Powder	81-162
Lactose	50
Glucose	50
Yeast Extract	0-10
KH_2PO_4	0.5
K_2HPO_4	0.5
$MgSO_4$	0.2
$MnSO_4 \cdot H_2O$	0.05
$CaCO_3$	30-60
NaOH (10 N)	

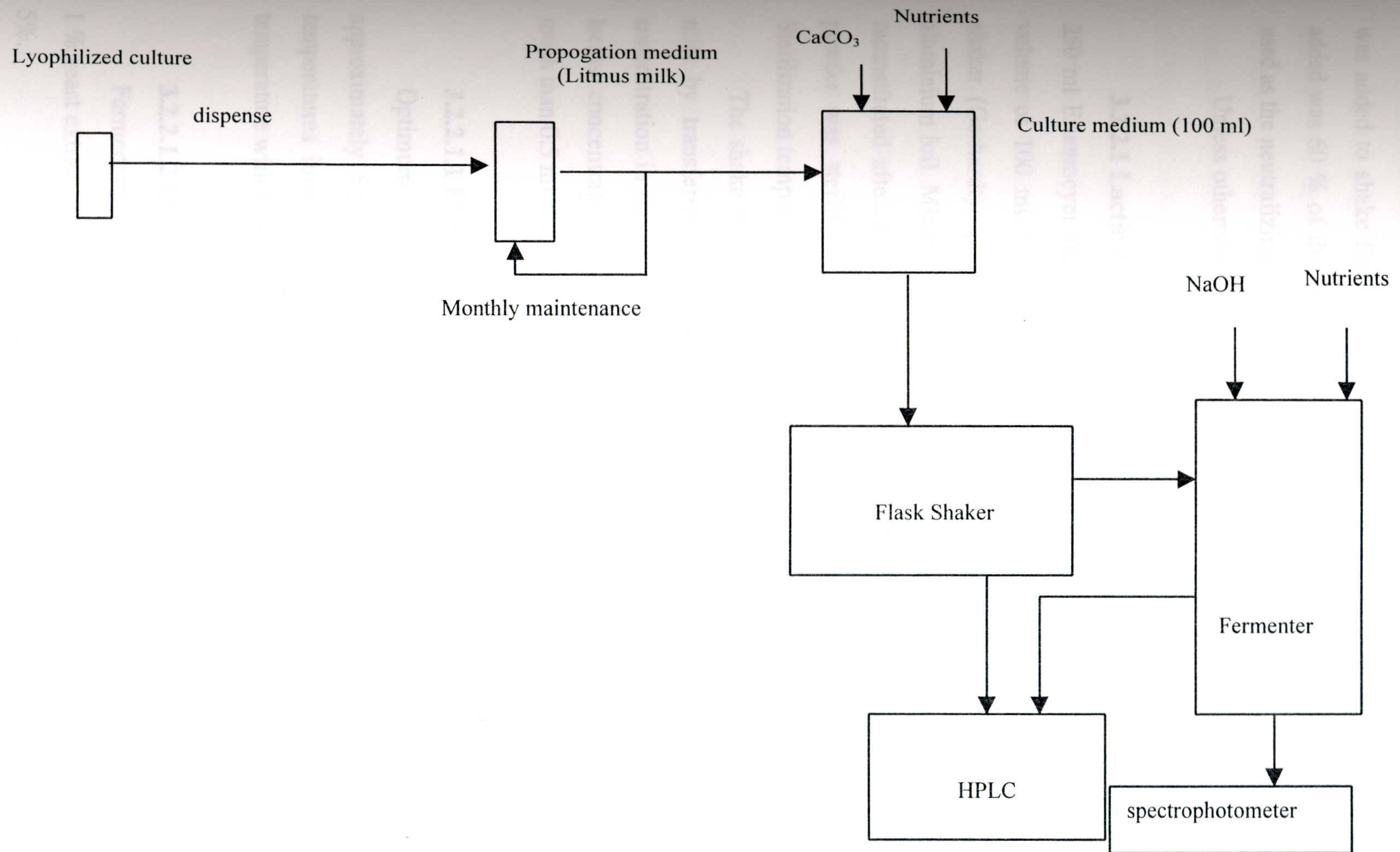


Figure 3.1. Flow chart of the experiments

Concentrations of the whey powder and the yeast extract were varied. $CaCO_3$ was added to shake flasks in order to neutralize the lactic acid produced and amount added was 60 % of the initial lactose concentration. In the fermenter 10 N NaOH was used as the neutralizing agent.

Unless otherwise mentioned, fermentations were carried out at 37 ^{o} C.

3.2.2.1 Lactic Acid Fermentations in Shake Flask

250 ml Erlenmeyer flasks were used for the shake flask experiments with a working volume of 100 ml. Fermentations were carried out in a temperature controlled flask shaker (Gerhardt) operated at 150 rpm. The tops of the flasks were covered with aluminium foil. Mineral solutions, whey and yeast extract were sterilized separately and reconstituted after the sterilization or the medium was sterilized as a whole. $CaCO_3$ powder was sterilized separately in both cases and added before the inoculation. Sterilization temperature and time were 121 ^{o} C and 15 minutes, respectively.

The shake flasks were inoculated with 24-hour-old culture propagated in litmus milk by transferring 2% culture aseptically. Unless otherwise specified inoculum concentration in the shake flask experiments were 4%. Samples for the lactic acid and lactose concentration analysis were taken with sterile pipettes. Sample amounts were not much than 0.5 ml and usually 0.1 ml.

3.2.2.1.1 Effect of Temperature

Optimum temperature studies were performed in shake flasks containing approximately 5.5% initial whey lactose, 1% yeast extract and the minerals. The temperatures investigated were 27, 32, 37, 42 and 47 ^{o}C. Inocula adapted to each temperature with three successive generations.

3.2.2.1.2 Effect of Yeast Extract Concentration

Fermentations were carried out in the shake flasks containing 0.0, 0.25, 0.5, 0.75, 1 % yeast extract. Salts were added before the sterilization. Inoculum concentration was 5%.

3.2.2.1.3 Effect of Salts

In order to determine the effect of each salt one or more of the salts were discarded from the formulation. In one of the flasks all of the salts, in another none of the salts were added as controls. Each medium was sterilized with all of its components. Fermentations were performed with 5.5% initial whey lactose and 0.5% yeast extract and the specified salts.

In order to check whether the bulk sterilization of the media components caused an interaction of them due to the high temperature, in an another set of experiments the whey, yeast extract and each salt solution were sterilized separately and reconstituted after the sterilization.

3.2.2.1.4 Effect of Substrate Type

L. casei was tested for its capability of utilizing synthetic lactose, glucose and whey lactose. Fermentations were conducted in shake flasks. Initial sugar concentrations were around 5.5-6.0 g l^{-1} in all cases. For each sugar utilization experiment two different sets of fermentations were performed. In the first set of experiments 0.5% yeast extract and 0.005% $MnSO_4 \cdot H_2O$ was used. In the second set of fermentations, yeast extract concentration was increased to 1% in order to compare the nutritional requirement for the substrates tested.

3.2.2.1.5 Effect of Initial Substrate Concentration

Fermentations were performed in shake flask cultures at 37 ^oC. Whey powder was reconstituted to have the desired initial lactose concentrations. Whey solutions containing 48.7, 64.0, 79.0 and 102.9 g l^{-1} lactose initially were supplemented with 0.5 % yeast extract and 0.005 % $MnSO_4 \cdot H_2O$.

3.2.2.2 Lactic Acid Fermentations in Fermenter

The pH and inoculum concentration optimization studies were conducted in a 5 l fermenter (Bioengineering, type ALF) with 3 l working volume. The heating jacket around the fermentation tank provided the temperature control. Agitation was provided

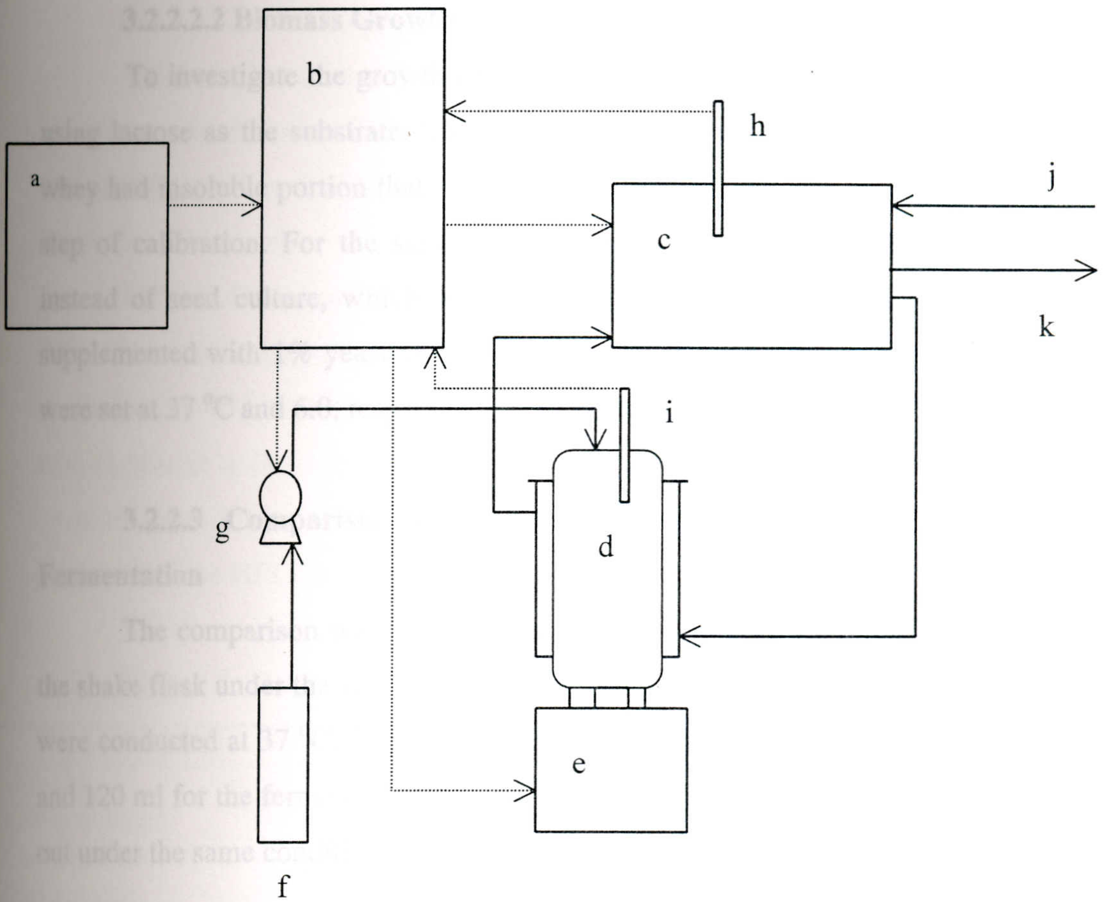
by a magnetic stirrer. Schematic diagram of the reactor is shown in Figure 3.1. The system was equipped with temperature, pH and agitation speed controllers and a computer and controlled by the software, FERM. The fermenter and the fermentation media were not sterilised unless otherwise indicated. The sterilization of the fermenter was performed by adding hot fermentation media. The medium components, which were sterilised separately in the autoclave, were displaced at 90 °C and added to the fermenter and were left for cooling to the fermentation temperature.

The fermentations in the shake flasks were used as the inocula for the fermenter. Having an initial lactose concentration of 5%, desired number of flasks containing 100 ml fermentation medium were incubated at 37 °C for 24 hours and added to the fermentation media in the fermenter.

pH was maintained at the desired values by automatic addition of 10 N NaOH by a peristaltic pump. Samples for analyses were taken by another peristaltic pump. Samples approximately 2-3 ml in volume, were taken for the analysis.

3.2.2.2.1 Effect of pH

In order the find the optimum pH for lactic acid production from whey by *L. casei* NRRL B-441, fermentations were performed in the fermenter, which had a pH control unit. pH was maintained at the desired value by the automatic addition of 10 N NaOH. Initial lactose concentrations of the media were 49-56 g l^{-1}. Whey was supplemented with 10 g l^{-1} yeast extract and the salts. 375 ml of seed culture was added to make up a final volume of 3000 ml. pH values investigated were 5.0, 5.5, 6.0 and 6.5.



a. Computer
b. Controlling unit
c. Water bath
d. Fermentation tank
e. Motor
f. Base reservoir
g. Base pump
h. Temperature probe
i. pH probe
j. Cooling water (tap water) inlet
k. Water outlet

Figure 3.2. Schematic representation of the reactor. Dashed lines represent the controlling signals.

3.2.2.2.2 Biomass Growth

To investigate the growth of *L. casei*, fermentations were done in the fermenter using lactose as the substrate. The reason for using lactose instead of whey was that whey had insoluble portion that would have interfered with the biomass at the filtration step of calibration. For the same reason, litmus milk culture was used as inoculum instead of seed culture, which would have contained insoluble $CaCO_3$. Lactose was supplemented with 1% yeast extract and 0.005% $MnSO_4$. The temperature and the pH were set at 37 °C and 6.0, respectively. Inoculum concentration was 5%.

3.2.2.3 Comparison of Fermenter and Shake Flask for Lactic Acid Fermentation

The comparison was made by performing fermentations in the fermenter and in the shake flask under the same conditions. pH was neutralized by $CaCO_3$. Fermentations were conducted at 37 °C. 24 hour old 4% litmus milk cultures (4 ml for the shake flask and 120 ml for the fermenter) were used as the inocula. Another experiment was carried out under the same conditions but pH was maintained at 6.0 by 10 N NaOH.

3.2.3 Analyses

Fermentation samples were centrifuged at 5000 rpm using Nüve NF 615 centrifuge in order to separate the cell mass and other insoluble materials. Supernatants were diluted at least 10 times to decrease the sugar or acid concentration below 0.5%, so that high-pressure liquid chromatography (HPLC) could give precise results. Dilutions were done with the mobile phase used in the HPLC analysis (5 mM H_2SO_4). Diluted samples were re-centrifuged at 14,000 rpm for 10 minutes in Hettich EBA 12R centrifuge. Supernatants were used for the analysis. Primary centrifugation was omitted when small volume (0.1 ml) samples were taken from the shake flasks.

3.2.3.1 Lactose, Glucose and Lactic Acid Analyses

Lactose, glucose and lactic acid were determined by HPLC. The HPLC system was composed of Perkin Elmer Series 200 pump, Series 200 refractive index detector, Series 900 interface and a computer. The system was controlled by the software, Turbochrom Navigator.

The HPLC analyses were done under isocratic conditions using an Aminex HPX-87H cation exchange column (Bio-Rad Laboratories). The column temperature was maintained at 45 °C with a MetaTherm column oven. The column was eluted with 5 mM H_2SO_4 at a flow rate of 0.6 ml min^{-1} for 15 minutes. Under these conditions retention times for lactose and lactic acid were 7.6 and 12.4 minutes, respectively. The properties of the HPLC and the analysis conditions and properties are given in Table 3.3.

Table 3.3. The properties of the column and analysis conditions.

Property	Values or Attributes
Column	Aminex HPX 87H ion exclusion column
Column length	300 mm
Column diameter	7.8 mm
Particle size	9 μm
Guard cartridge	Micro-Guard cation-H cartridge (30 x 4.6 mm)
Column cleaning solvent	5% CH_3CN in 5mM H_2SO_4, 30% CH_3CN in 5mM H_2SO_4
Mobile phase	5 mM H_2SO_4
Flow rate	0.6 ml min^{-1}
Temperature	45 °C
Detector	Refractive index

Calibration standards were prepared from analytical grade chemicals. Calibration curves are shown in Figure A1 in Appendix A. The R-squared values for lactose, glucose and lactic acid calibration curves were greater than 0.999.

3.2.3.2 Dry Cell Weight Measurements

Biomass was determined as dry cell weight. The samples were diluted when necessary and optical densities were measured at 620 nm by Shimadzu UV-1601 spectrophotometer and cell density was converted to dry cell weight using a calibration curve. For calibration, several dilutions from the sample at the stationary phase of the growth were prepared and optical densities were determined. 15 ml of each dilution was passed through a pre-weighed cellulose acetate membrane filter having a pore size of 0.45 μm using a vacuum pump. The bacteria collected on the filters were washed with 15 ml of water and filters were dried until constant weight at 100 °C for approximately 24 h. Calibration curve for dry cell weight is shown in Figure A.2 in Appendix A.

IZMIR YUKSEK TEKNOLOJİ ENSTİTÜSÜ
REKTÖRLÜĞÜ
Kütüphane ve Dokümantasyon Daire Bşk.

CHAPTER 4

RESULTS AND DISCUSSION

In this section experimental results on batch fermentation of lactic acid from whey by using *L. casei* are presented together with the emphasis on several effects on fermentation. Both the shake flask experiments and fermenter studies are presented. Experimental results include optimum temperature, pH, substrates, salts, initial substrate concentration and yeast extract concentration.

All the experiments were done in duplicates at least and mean values are reported. Fermentations were evaluated according to their productivity values. In every section the lactic acid and lactose concentrations are shown in charts. Generally, the maximum productivity values are reported.

Generally, the optimum values determined in an experiment or other type of results were evaluated and used for the subsequent experiments.

The initial lactic acid content of the fermentation media is due to the lactic acid content of the whey and the inocula. In some graphs there are missing lines in the initial stage of the fermentations. They indicate that there were not enough data at those parts to show the exact progression of the curves.

The definitions of the yield and productivity terms are given in Appendix B.

4.1 Effect of Temperature

In this study, the influence of temperature on the production of lactic acid using 5.5% initial substrate was investigated. The temperature is in the range of 27-47 °C.

The inocula at 32 and 37 °C were 24 hour old, while at 27 and 42 °C it was 48 hour old, since at the latter temperatures insufficient growth was observed after 24 hours. At 47 °C there was a poor growth in the propagation medium, litmus milk. Therefore, no fermentation run was performed at this temperature.

In Figure 4.1 and Figure 4.2, lactic acid production and lactose consumption at different temperatures (27, 32, 37, 42 °C) are shown. The calculated productivity values for each temperature can be compared in Figure 4.3.

As can be seen in Figure 4.1 the fastest production was at 37 °C. The results obtained from the fermentation batches conducted at the temperature of 27 °C showed a totally different trend than the ones obtained at 32, 37 and 42 °C. The kinetics of product formation in the fermentation media was quite slow at 27 °C. The fermentation was completed in 45 hours at this temperature.

The fastest lactic acid production was obtained at 37 °C. Lactic acid production rate was found to be very similar at the temperatures of 32 °C and 42 °C. It can be pointed out that the change in temperature values is effective on the growth of *L. casei.*

The retardation in the product formation at 32 °C and 42 °C was not more than 15%, while at 27 °C the retardation was significant. In all cases, all lactose was utilizied and the conversion yields were around 92%, so that the final lactic acid concentrations were approximately the same, nearly 53 g l^{-1}.

An additional fermentation at 27 °C was performed but the inoculum was propagated at 37 °C. This was done to check whether the poor production at 27 °C was due to an insufficient growth of inoculum at 27 °C. The results were nearly identical.

According to Atkinson and Mavituna (1991) the optimum growth temperature for *L. casei* is between 25 and 30. The same authors give the minimum and maximum growth temperatures as 10 and 40 °C, respectively. However, in this study comparable results were obtained at 42 °C.

These results confirmed Hujanen and Linko (1996) who found that the best production temperature for *L. casei* NRRL B441 was 37 °C. They obtained 80 g l^{-1} lactic acid in 24 h from 90 g l^{-1} by using high amounts of yeast extract and inoculum (2.2 % and 20 %, respectively). Krischke et al. (1991) also determined the optimum temperature for lactic acid fermentation of whey permeate by immobilised *L. casei* subsp. *casei* as 37 °C.

Temperature optimisation investigations of Tuli et al. (1985) showed no significant influence of temperature between 40 and 50 °C for lactic acid production

from whey permeate by immobilized *L. casei*. Temperatures 35 and 55 ^{o}C reduced the product formation.

In Table 4.1 the temperature values used by some other authors for lactic acid production by *L. casei* are shown.

Table 4.1. Temperatures used for lactic acid fermentation by *L. casei*.

Microroganism	Temperature (^{o}C)	Reference
L. casei subsp. *casei* DSM 20011	36	Vaccari et al. (1993)
L. casei SU no 22	32	Roukas and Kotzekidou (1998)
L casei subsp. *rhamnosus*	40	Martinkova et al. (1991)
L. casei RA-9867	30	R. Gamal and N. Gamal (1984)
L. casei subsp *rhamnosus* DSM 20021	42	Senthuran et al. (1997)
L casei DSM 20021	42	Guoqiang et al. (1991)

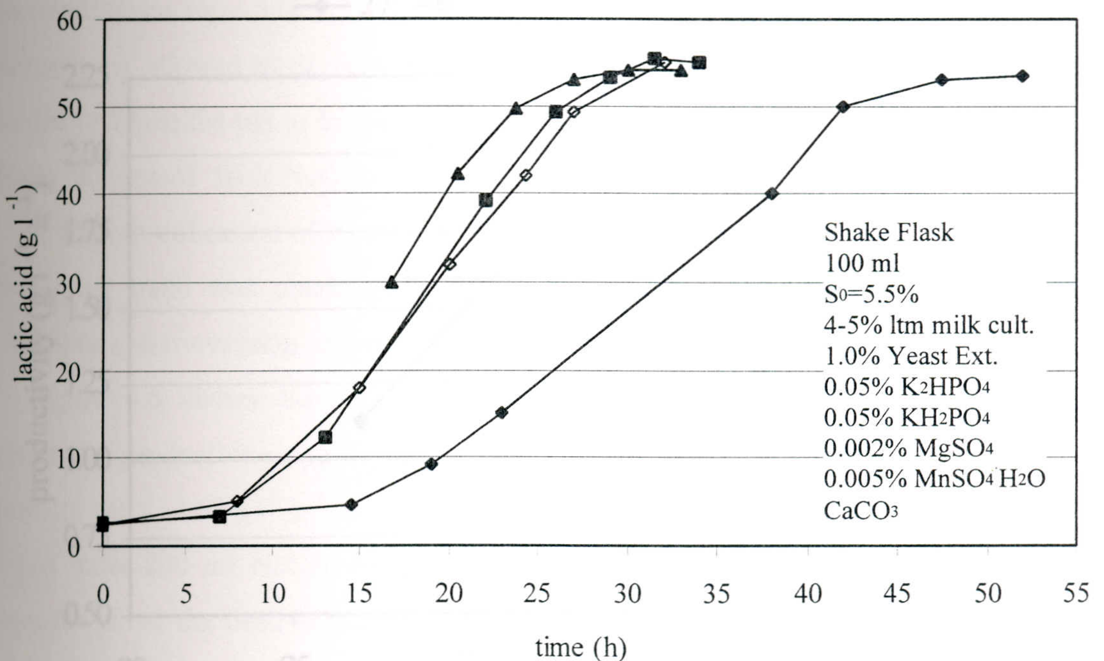


Figure 4.1. Effect of temperature on lactic acid production.

—◆— 27 —◇— 32 —▲— 37 —■— 42

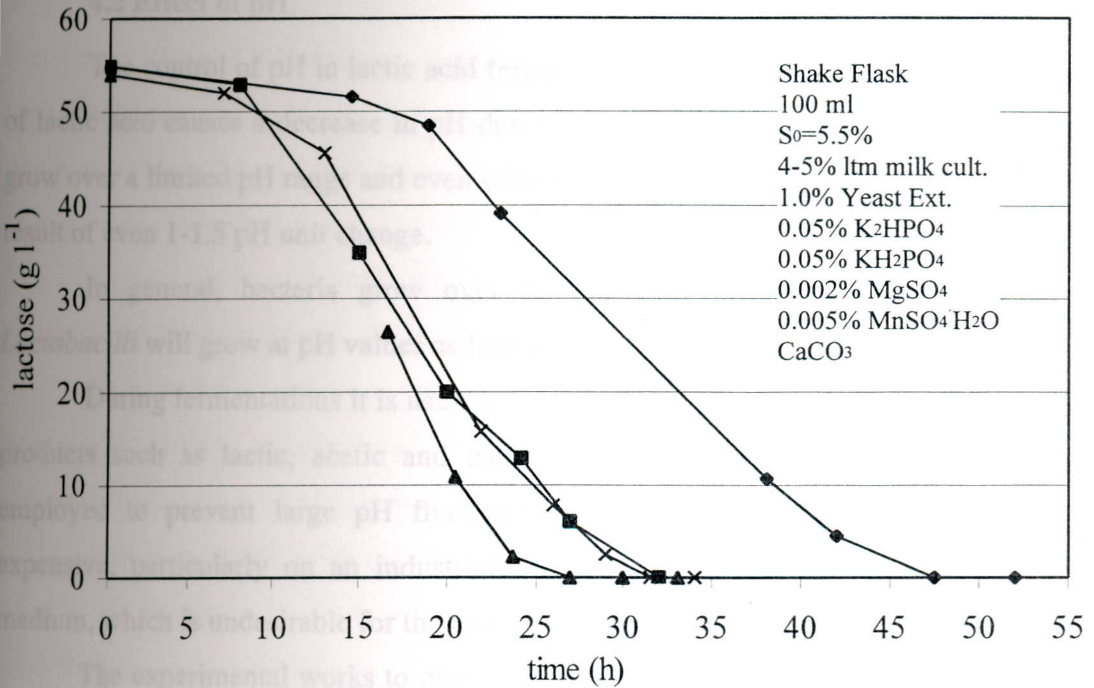


Figure 4.2. Effect of temperature on lactose utilization.

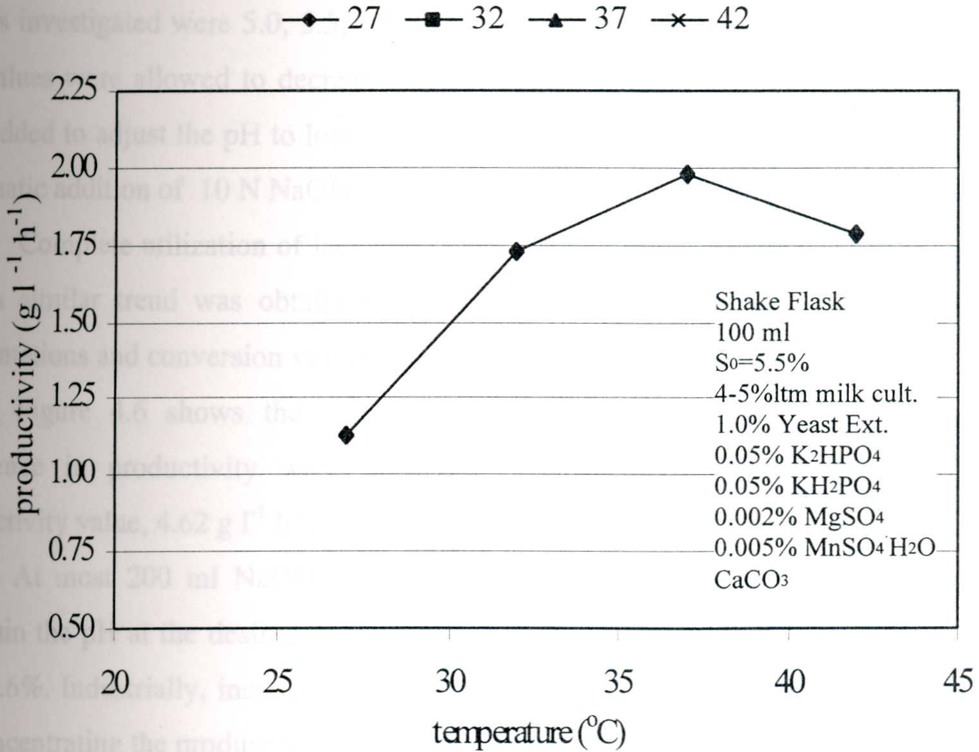


Figure 4.3. Effect of temperature on productivity.

4.2 Effect of pH

The control of pH in lactic acid fermentations is very important since production of lactic acid causes a decrease in pH during the fermentation. Microorganisms tend to grow over a limited pH range and even within this range, they shift their metabolism as a result of even 1-1.5 pH unit change.

In general, bacteria grow over the pH range of 4-8. Some bacteria like *Lactobacilli* will grow at pH values as low as 2.

During fermentations it is usually necessary to control pH particularly when acid products such as lactic, acetic and citric acids are formed. Buffers are frequently employed to prevent large pH fluctuations in the culture medium, but these are expensive, particularly on an industrial scale. They increase the salt content of the medium, which is undesirable for the product separation.

The experimental works to obtain the optimum pH for *L. casei* to produce lactic acid were conducted in the fermenter. The results are given in Figures 4.4 and 4.5. pH values investigated were 5.0, 5.5, 6.0 and 6.5. When pH 5.0 and 5.5 were investigated pH values were allowed to decrease to the desired values itself, which means no acid was added to adjust the pH to low values. pH is maintained at the desired values by the automatic addition of 10 N NaOH.

Complete utilization of lactose at pH 5.0 was achieved at 25 hours. At other pH values similar trend was obtained within 12 hours. All lactose was utilized in all fermentations and conversion values were around 93%.

Figure 4.6 shows the overall productivity values. Although no significant difference in productivity were observed between pH 5.5 and 6.5, the highest productivity value, 4.62 g l^{-1} h^{-1}, was obtained at pH 5.5.

At most 200 ml NaOH was added to 3 l of fermentation media in order to maintain the pH at the desired values, thus fermentation volume did not increase more than 6.6%. Industrially, increase in the volume may require additional energy and time for concentrating the product in the recovery step.

In order to compare $CaCO_3$ and NaOH for their neutralising ability another fermentation was performed with $CaCO_3$ in the fermenter. pH was maintained at 6.0

when NaOH was used. 10 % seed cultures (300 ml) were used as the inocula. As can be seen in the Figure 4.7 NaOH was more efficient on lactic acid production. Productivity with NaOH was 33 % higher than with $CaCO_3$. When $CaCO_3$ was used pH decreased down to 5.0 in 12 h and was kept around this value until the end of the fermentation (Figure 4.8). Therefore it was concluded that $CaCO_3$ could buffer the pH around 5.0. The ineffective lactic acid production at pH 5.0 could be the reason for less lactic acid productivity obtained when $CaCO_3$ was used instead of NaOH.

Fermentation without a pH control was performed in shake flask using 5 % litmus milk culture as inoculum. Started with an initial pH of 6.0, pH decreased to around 4.0 in 14-16 h and lactic acid production nearly ceased at that time.

Tuli et al. (1985) determined the optimum initial pH for lactic acid production from whey permeate by immobilized *L. casei* as 5.5. However they used $CaCO_3$ to neutralize the acid. Krischke et al. (1991) found that best growth and product formation occurred between pH 6.0 and 6.5 for immobilized *L. casei* subsp. *casei* grown on whey permeate.

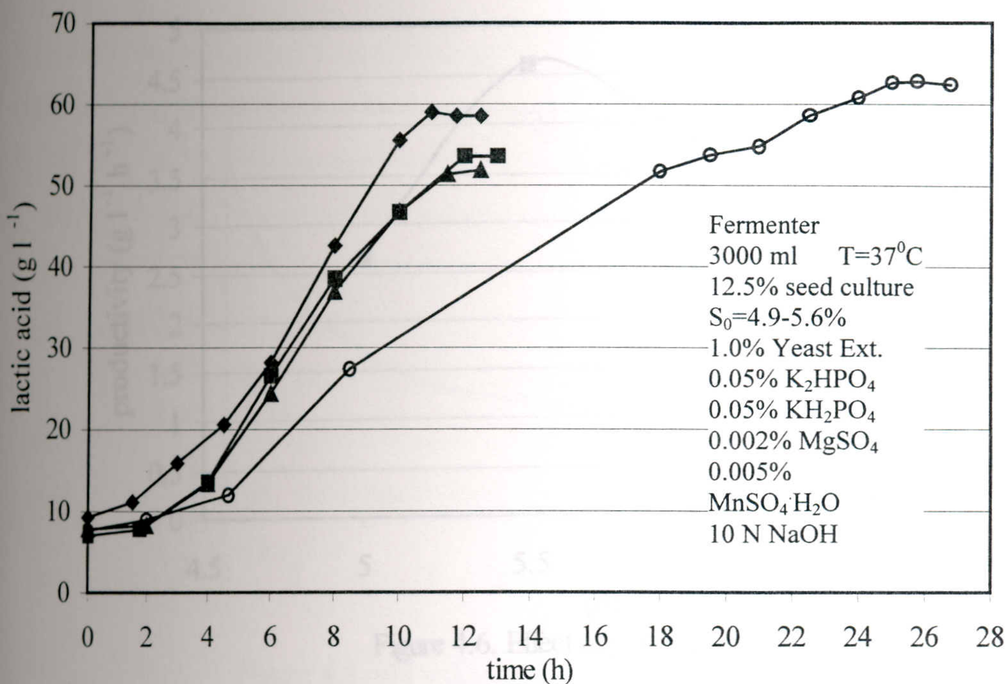


Figure 4.4. Effect of pH on lactic acid production.

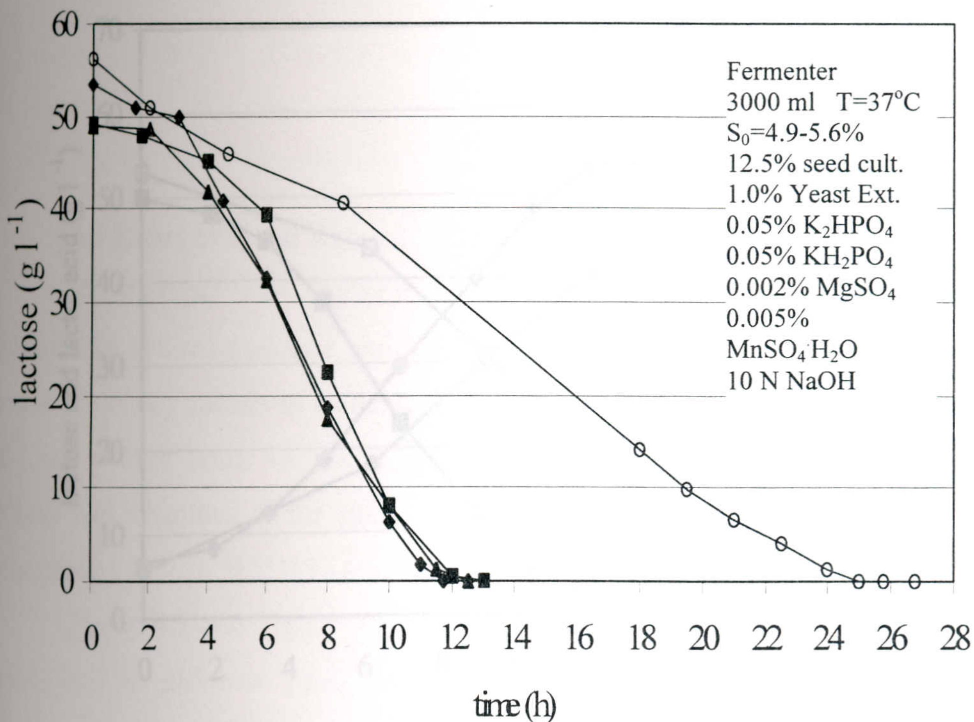
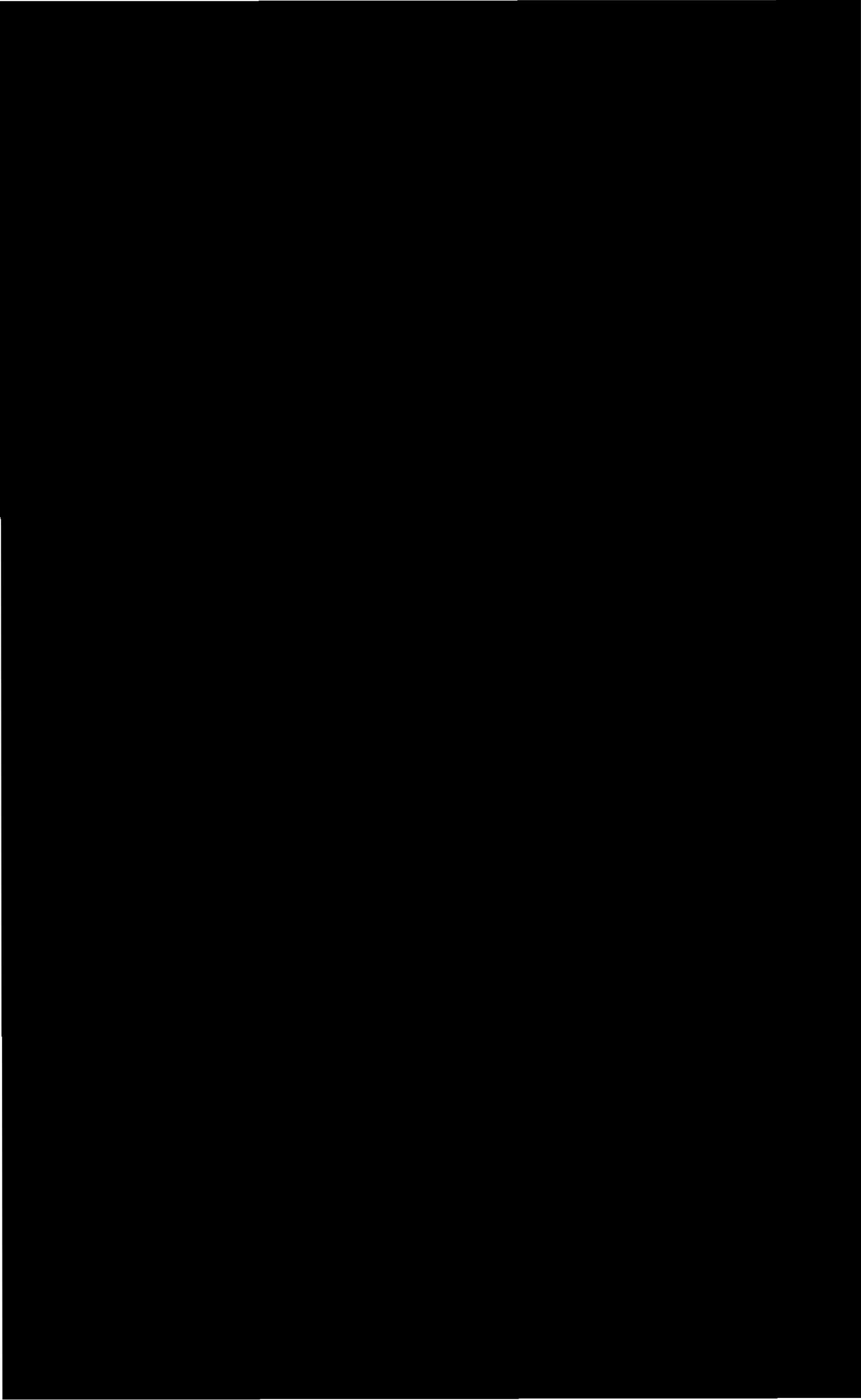


Figure 4.5.Effect of pH on lactose utilization.



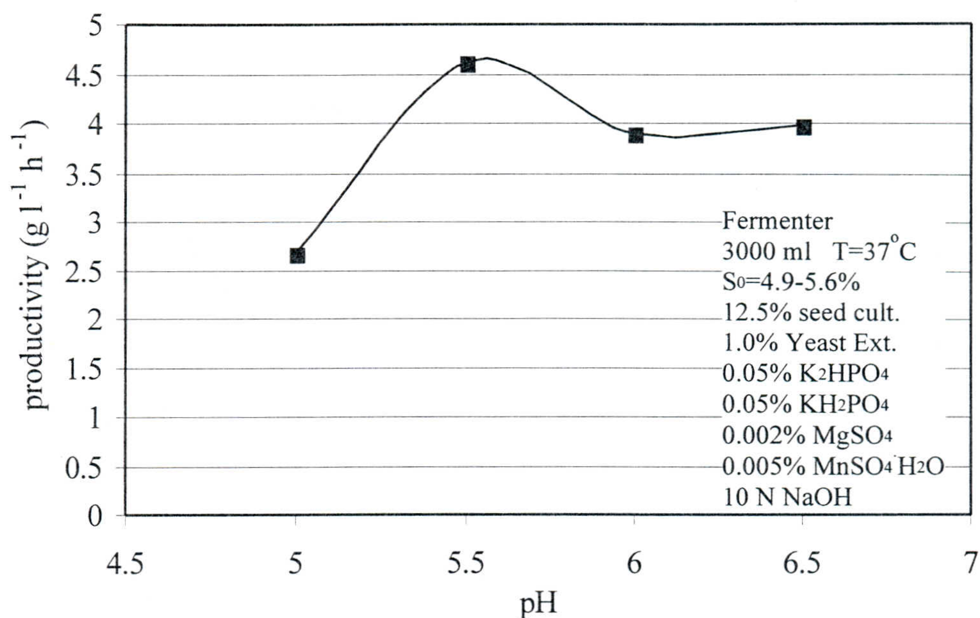


Figure 4.6. Effect of pH on productivitiy

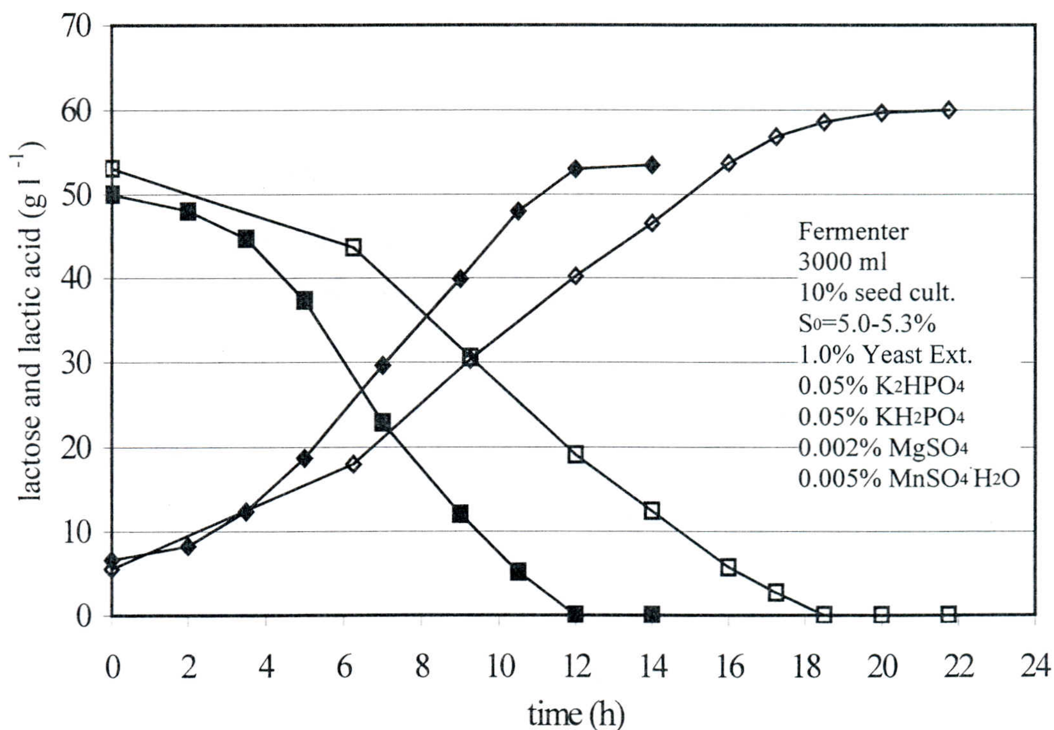


Figure 4.7. Lactic acid production and lactose utilization using NaOH (closed symbols) or $CaCO_3$ (open symbols) as the neutralising agent.

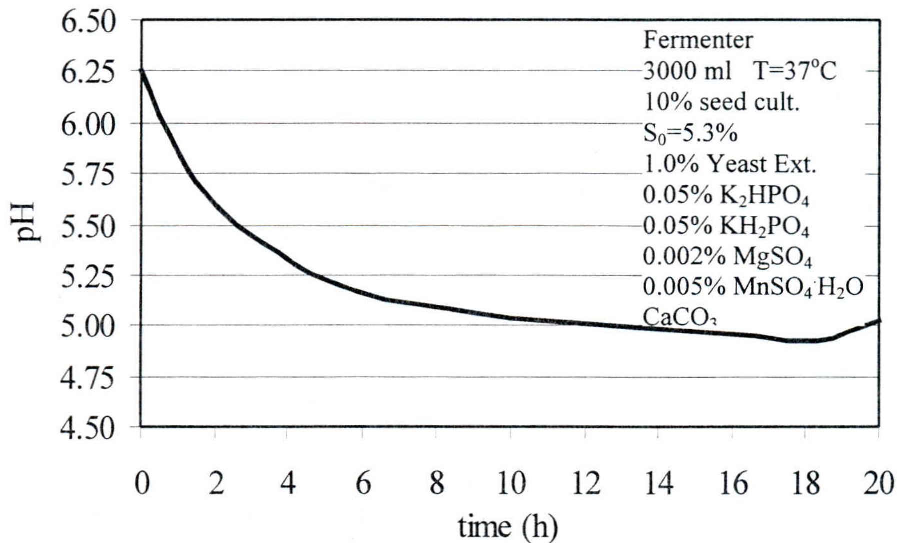


Figure 4.8. pH values during the fermentation using $CaCO_3$ as the neutralising agent.

4.3 Effect of Yeast Extract

Yeast extract is a complex mixture that provides nitrogen vitamins and co-factors required for cell maintenance and lactic acid production. Yeast extract supplementation is one of the key factors in lactic acid fermentation (Ohleyer et al. 1985). The high cost of yeast extract limits its usage in the fermentations. Therefore, the effect of yeast extract was examined in the study. Fermentations were carried out in the shake flasks containing 0.0, 0.25, 0.5, 0.75 and 1 % (w/v) yeast extract.

Figure 4.9 and Figure 4.10 show the consumption of lactose and production of lactic acid at different yeast extract concentrations. Figure 4.11 demonstrates the productivity values obtained from the fermentations.

The utilization of lactose and production of lactic acid in the case of the fermentation conducted with 0.75% and 1% yeast extract concentrations followed

similar curves as seen in Figure 4.10 and Figure 4.9. In about 28 hours of fermentation all the lactose was consumed by *L. casei* and more than 52 g l^{-1} lactic acid was produced in both cases. That means there is no need to add extra yeast extract into the medium exceeding 0.75%. For 0.5% yeast extract concentration, all lactose seemed to be utilized in about 30 hours with the production of 50 g l^{-1} lactic acid. The lactic acid production increased with increasing yeast extract concentration.

The more the yeast extract concentration in the medium, the shorter the fermentation lasted. In order to produce the same amount of lactic acid with no yeast extract in the medium, the fermentation continued up to 60 hours. With 0.25% yeast extract, 40 hours was needed.

The productivity values were 0.84, 1.45, 1.75, 1.90 and 1.98 g l^{-1} h^{-1} for 0, 0.25, 0.50, 0.75 and 1% yeast extract supplementation, respectively. In all fermentations lactose was totally utilized. Product yields were not very different and all were in between 92-94%.

Hujanen and Linko (1996) used *L. casei* NRRL B441 for batch fermentation of glucose and obtained 100 g l^{-1} lactic acid from 100 g l^{-1} glucose in 48 hours by supplementing 2.2 % yeast extract. They found that malt sprouts was the best alternative to yeast extract as the nitrogen source and obtained 88 g l^{-1} lactic acid in 66 hours.

For batch fermentation of whey permeate by *L. casei* subsp *casei*, Krishke et al (1991) concluded that the best growth comparable to MRS medium occurred with a combination of yeast extract (5 g l^{-1}) and hydrolyzed whey retentate (50 g l^{-1}).

Guoqiang et al. (1991) investigated the effect of yeast extract on lactate production by free and immobilized *L. casei* and determined that lactic acid production was increased by increasing yeast extract concentration (0-10 g l^{-1}). No production was obtained with 0 and 1 g l^{-1} yeast extract by free cells while there was production with the same yeast extract concentrations by immobilized cells. 10 g l^{-1} was found to be the optimum yeast extract concentration by Göksungur and Güvenç (1997), Aeschlimann and Stockar (1990) and Norton et al (1994) for *L. delbrueckii, L. helveticus* and *L. helveticus*, respectively.

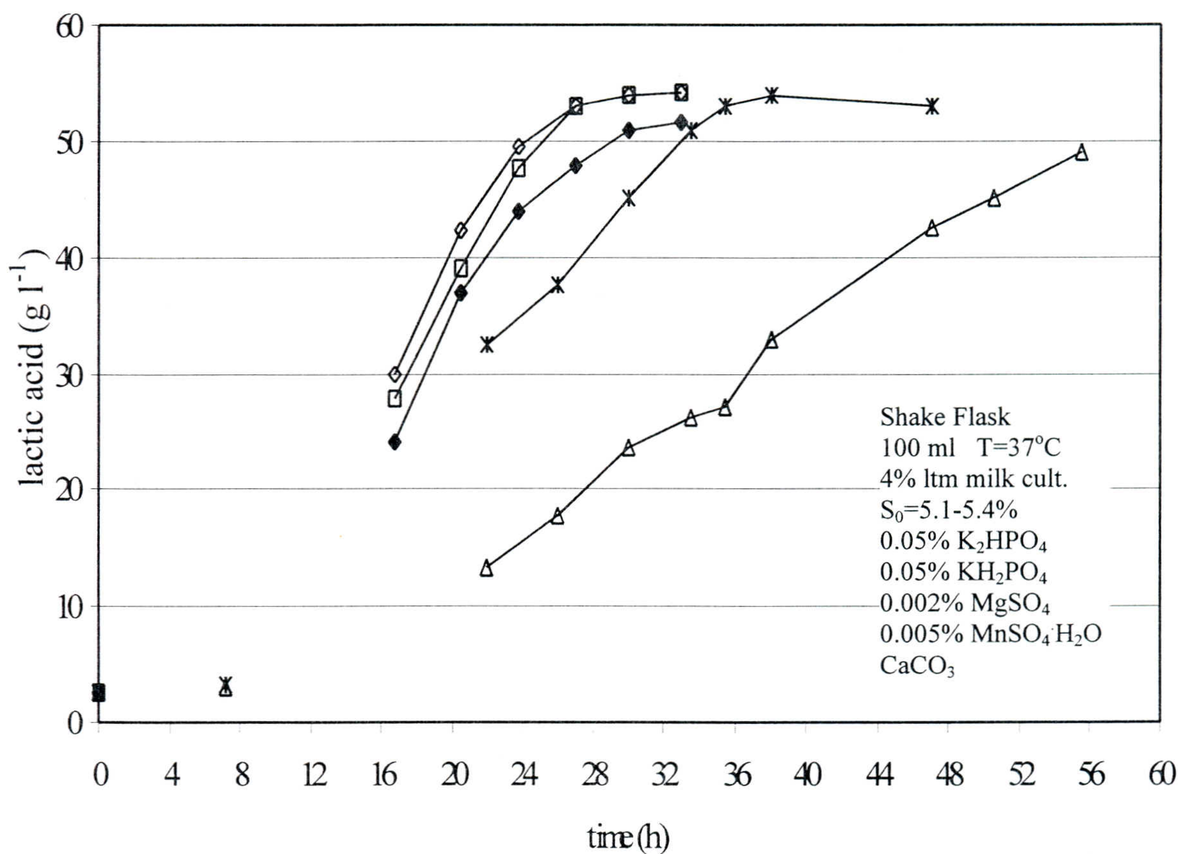


Figure 4.9. Effect of yeast extract concentration on lactic acid production.

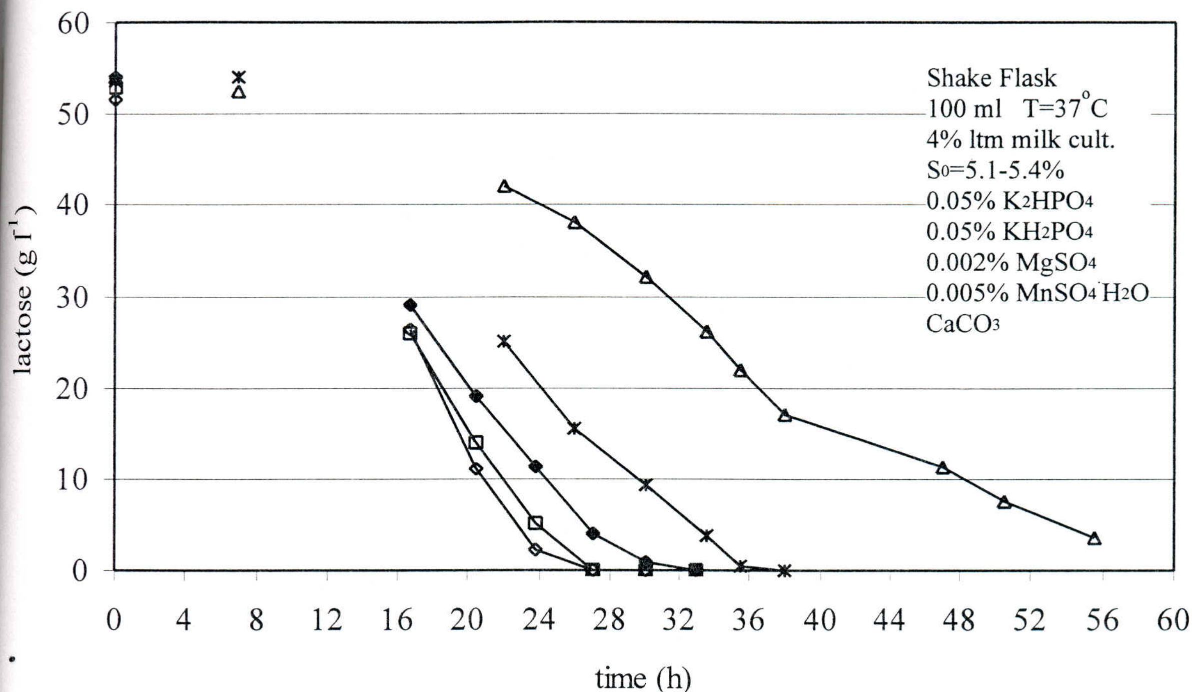


Figure 4.10.Effect of yeast extract concentration on lactose utilization.

—△— 0 —✳— 0.25 —◆— 0.5 —□— 0.75 —◇— 1

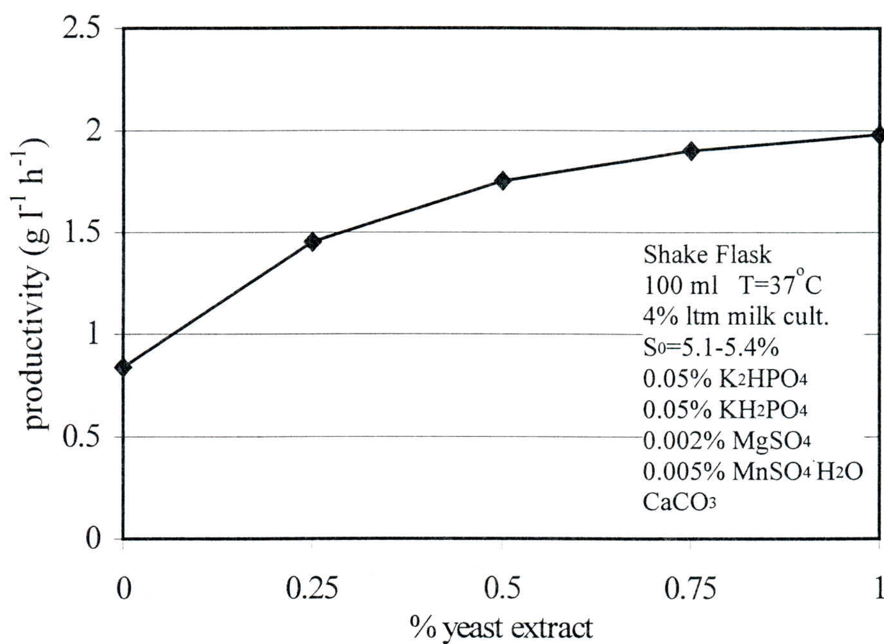


Figure 4.11. Productivity values obtained at different yeast extract concentrations.

4.4 Effect of Salts

Figure 4.12 shows the effect of different salts on the fermentations of *L. casei* using whey lactose supplemented with 0.5% yeast extract. Here, different fermentation runs were conducted by adding different salts in order to find the most important salts required for the whey-yeast extract medium.

Both lactose and lactic acid concentrations after 24 hours of fermentation are given in Figure 4.12. Figure 4.12. a. shows the results when the fermentation media were bulk sterilized. The result of the fermentations that whey, yeast extract and each salt solution were sterilized separately and reconstituted after the sterilization are seen in Figure 4.12.b. The purpose of sterilization separately was to see whether salts precipitated due to interactions at high temperature when they are sterilized together with yeast extract and whey, since in some fermentations this is the case resulting in problems.

It is obvious from Figure 4.12 that sterilization of medium components separately did not change the results. This result indicated that precipitation of salts did not occur during the sterilization and microorganisms could utilize the salts in both cases.

As shown in Figure 4.12, after 24 hours of fermentation with $MnSO_4$ only gave similar results with the fermentation containing all the salts. The results of the other fermentations were nearly the same. Therefore it was concluded that only $MnSO_4$ was essential for lactic acid fermentation of whey by *L. casei*. It should be noted that the values were taken at 24 h and fermentations had not been finished at that time.

The results approved that the Mn^{2+} ion is an essential growth factor since it is a constituent of lactate dehydrogenase (Krishke et al. 1991).

For lactic acid production from whey permeate by immobilized *L. casei*, Tuli et al. (1985) noted increased lactose utilization and productivity by Mn^{2+} and Mg^{2+} addition and concluded that the increase could be due to that both Mn^{2+} and Mg^{2+} were cofactors of the enzyme involved in the process.

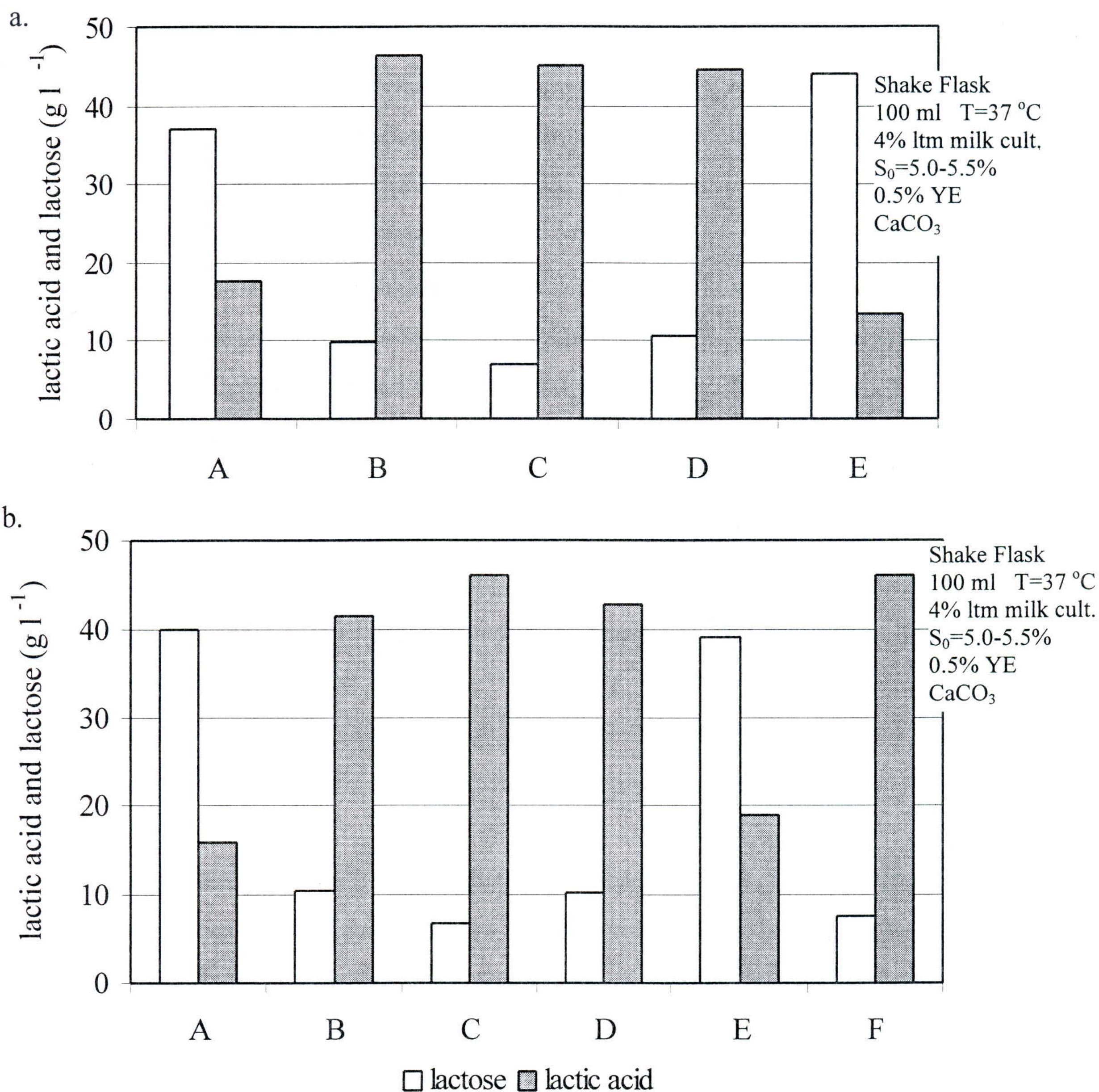


Figure 4.12. Lactose and lactic acid concentrations after 24 hours when,
a. fermentation media were bulk sterilized.
b. salt solutions, whey and yeast extract were sterilized separately.

A: Whey + yeast extract
B: Whey + yeast extract + K_2HPO_4 + KH_2PO_4 + $MnSO_4 \cdot H_2O$ + $MgSO_4$
C: same as B but no K_2HPO_4 and KH_2PO_4
D: same as B but no $MgSO_4$
E: same as B but no $MnSO_4 \cdot H_2O$
F: Whey + yeast extract + $MnSO_4 \cdot H_2O$

4.5 Effect of Substrate Type

L. casei is known to ferment lactose, however it is also reported to ferment glucose (VickRoy 1985, Vaccari et al. 1993, Guoqiang 1991, Hujanen and Linko 1996, Martinkova et al. 1991). Therefore the organism was tested for its capability of utilising synthetic lactose, glucose and whey lactose.

The results of different batches conducted with various sugars are presented in Figure 4.13. Lactose and glucose synthetic media containing 0.5% yeast extract showed similar lactic acid production and sugar utilization patterns. Whey lactose was utilized more rapidly than the synthetic lactose and glucose, so the productivity was much higher. Productivity values for lactose and glucose media were around 1.45 g l^{-1} h^{-1} and for whey medium it was approximately 1.75 g l^{-1} h^{-1}.

In the second set of experiments, in which yeast extract concentrations were raised to 1%, the lactic acid productions were similar to the production from whey supplemented with 0.5% yeast extract.

The fermentations conducted with 1% yeast extract supplementation completed within nearly 30 hours, while 0.5% yeast extract needed longer fermentation time to utilize all sugars.

The productivity values for different substrates are seen in Figure 4.14. Productivity values were around 1.47 g l^{-1} h^{-1} for synthetic lactose and glucose media that were supplemented with 0.5% yeast extract, while with 1% yeast extract productivity values were approximately 30% higher (around 1.9 g l^{-1} h^{-1}). The productivity value of fermentation from whey lactose was 1.75 g l^{-1} h^{-1}.

The product yields obtained for synthetic lactose and glucose media supplemented with 1% yeast extract were approximately 88%. This value was lower than the conversion yields obtained for 0.5% yeast extract supplementation, which were approximately 92%.

It was concluded that the complex composition of whey could have provided additional nitrogenous materials to the organism.

Whey medium supplemented with lower amount of yeast extract could be considered as the most suitable substrate. However, when selecting a carbon source for

the fermentation productivity can not be the sole parameter, because the complex composition of whey leads to higher purification cost than pure sugars. So, the raw material cost and purification cost should be considered as well as the lactic acid productivity.

Gamal and Gamal (1984) found that for lactic acid production by *L. casei*, glucose and lactose gave similar lactic acid yields, which were higher than fructose, galactose, sucrose, maltose and dextrin were used as carbon source. They obtained lower lactic acid yield and conversion value with cheese whey concentrate.

Roy et al. (1987) compared glucose and lactose synthetic media and whey-yeast extract permeate (WYEP) medium for lactic acid production by *L. helveticus*. WYEP and lactose synthetic media were equally good and better than glucose synthetic medium.

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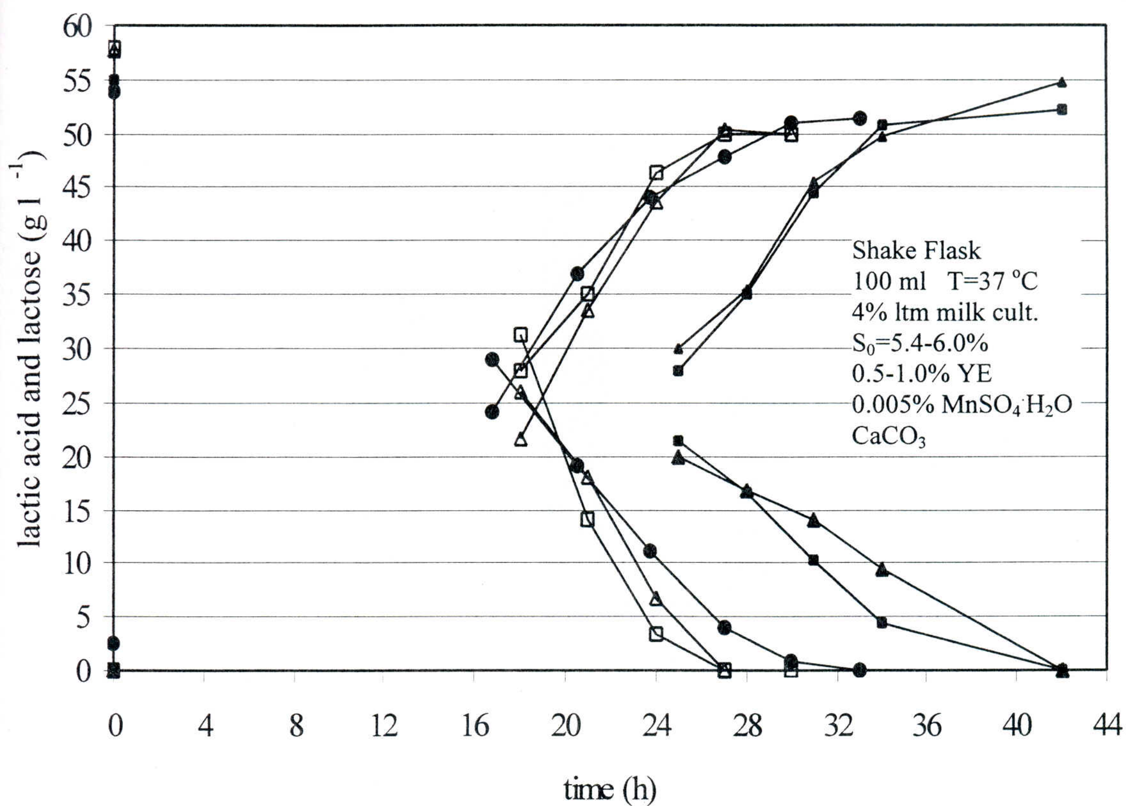

Figure 4.13. Lactic acid production and lactose consumption using lactose or glucose synthetic media or whey at different yeast extract concentrations.
open symbols: 1% yeast extract, closed symbols: 0.5% yeast extract

—□— , —■— substrate: glucose ; —△— , —▲— substrate: lactose; —●— substrate: whey

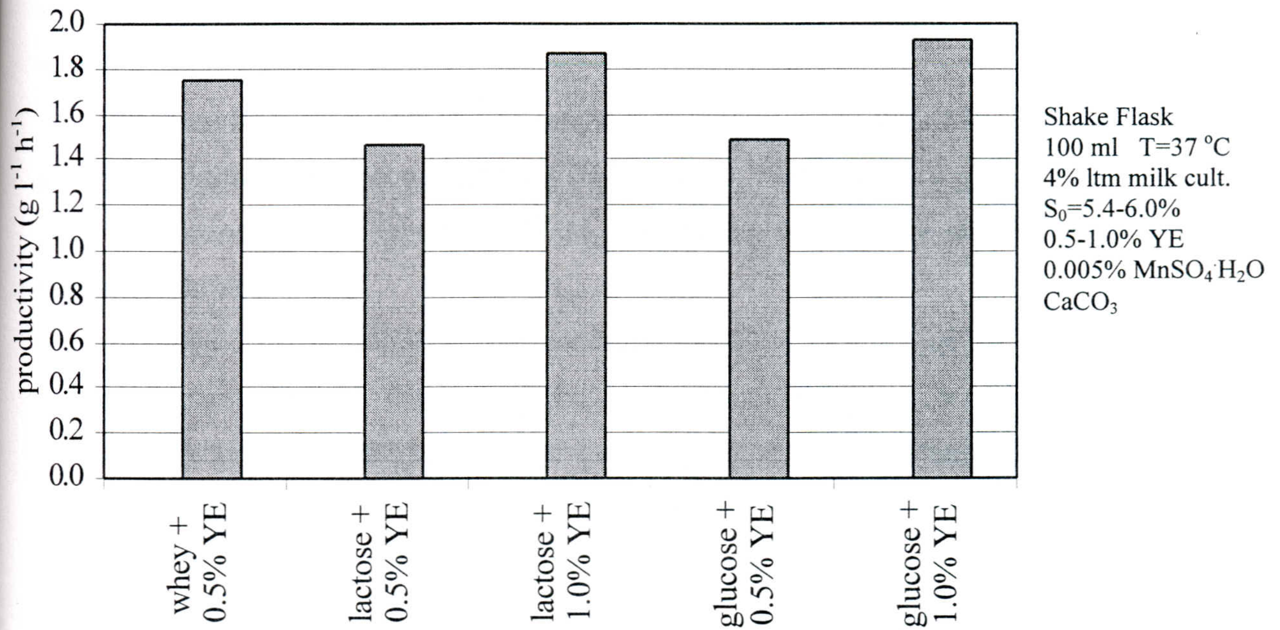

Figure 4.14. Productivity values for different substrates

4.6 Effect of Initial Substrate Concentration

The effect of substrate concentration for lactic acid fermentation from whey was investigated. Initial lactose concentrations were 48.7, 64.0, 79.0 and 102.9 g l^{-1}.

The results of lactose utilization and lactic acid production with different initial substrate concentrations are given in Figure 4.15 and Figure 4.16, respectively. Figure 4.17 shows the productivity values.

As seen in Figure 4.15, the time needed for the *L. casei* to utilize lactose completely depended on the initial substrate concentration. The more initial substrate concentration the more it took for the microorganism to utilize the sugar.

Lactic acid production curves followed nearly the same pattern, but in each case the maximum amount of lactic acid produced was obviously different due to the substrate concentration. Lactic acid yields were 0.93-0.94 g per g lactose at all initial substrate concentrations.

As can be seen in Figure 4.17 productivity values were 1.77, 2.19, 2.25 and 2.30 g l^{-1} h^{-1} for initial lactose concentrations of 48.7, 64.0, 79.0 and 102.9 g l^{-1}, respectively. Above the concentration of 64.0 g l^{-1} the productivity values were similar due to the substrate inhibition.

Above 79 g l^{-1} initial lactose concentration the viscosities of the fermentation media increased through the end of the fermentation. This may be due the protein content of whey that was denatured at the sterilization step. Calcium lactate at high concentrations may have precipitated and increased the viscosity.

Tuli et al. (1985) found that 4.5% (w/v) initial lactose level was the optimum for lactic acid production from whey permeate by immobilised *L. casei*. The rate of lactic acid production increased up to that concentration and decreased thereafter.

Vaccari et al. (1993) examined 2 to 12 % (w/v) initial glucose concentrations for lactic acid production by *L. casei* DSM 20011. They found that volumetric productivity was the maximum at 10 % glucose although the values were similar between 6, 8, 10 and 12 %. However, they observed that biomass yield decreased by increasing initial glucose concentration. They concluded that the optimal initial glucose concentration for lactic acid production was between 4% and 6%.

Guoqiang et al. (1991) observed a decrease in the conversion yield at high initial glucose concentrations (80 and 140 g l^{-1}) for lactic acid production by alginate-immobilised *L. casei*. According to the authors, that could be due to the precipitation of calcium lactate formed at high concentrations, so that could not be measured.

Göksungur and Güvenç (1997) observed a decrease in the yield values above the initial sugar concentration of 78.2 g l^{-1} for lactic acid production from beet molasses by *L. delbrueckii* IFO3202.

Gonçalves et al. (1991) investigated the substrate inhibition for lactic acid production from glucose by *L. delbrueckii* NRRL B445. Higher initial glucose

concentrations increased the lag phase and decreased the specific growth rate. Above 10% the lactic acid production still increased although the maximum cell concentration did not increase. Maximum of 14% of lactic acid was obtained using 20% initial glucose concentration. Above 10%, glucose could not be utilised completely.

Mehaia and Cheryan (1987) observed a decrease in lactose utilisation by *L. bulgaricus* above 15% (w/v) whey permeate powder containing 85% (w/w) lactose.

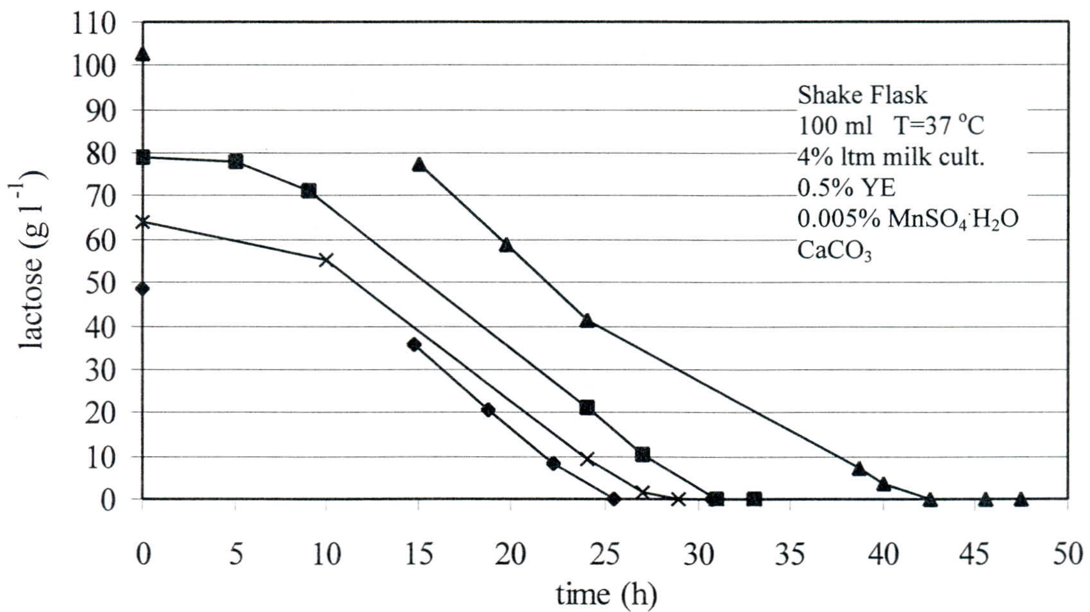

Figure 4.15. Effect of initial subtrate concentrations on lactose utilization.

—◆— 48.7 g/l —×— 64.0 g/l —■— 79.0 g/l —▲— 102.9 g/l

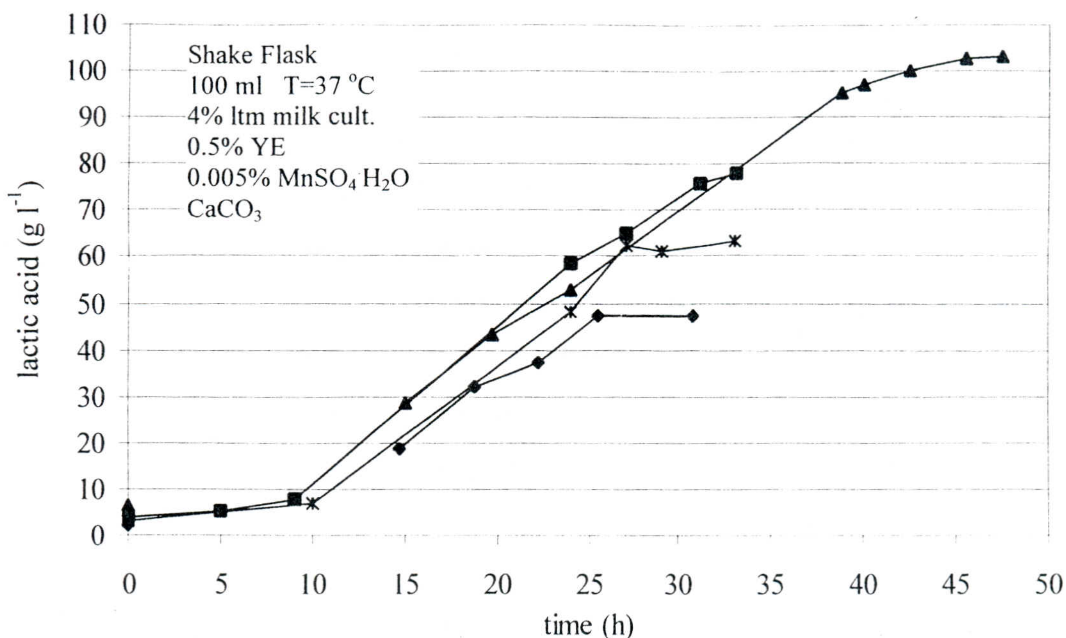

Figure 4.16. Effect of initial substrate concentration on lactic acid production.

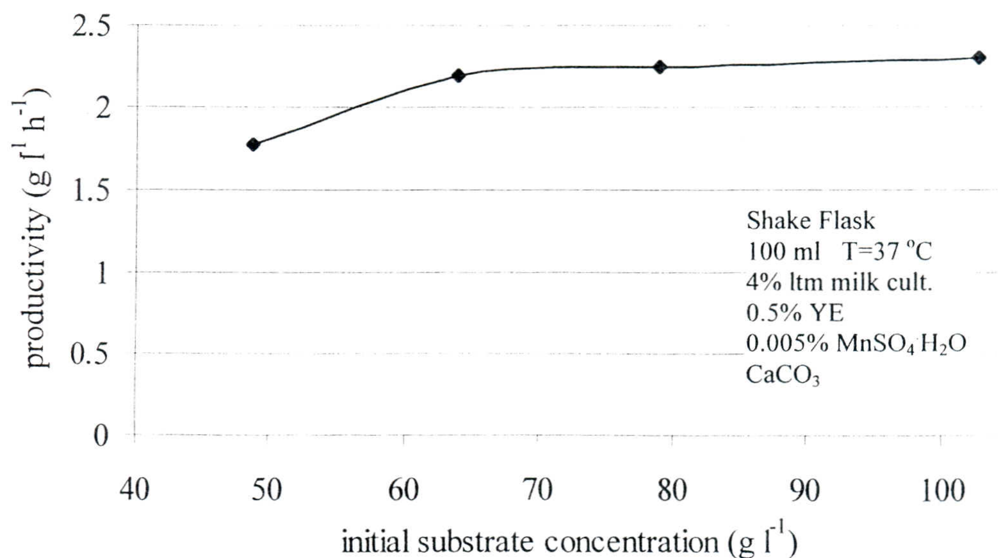

Figure 4.17. Producivities obtained at different initial substrate concentrations.

4.7 Comparison of Fermenter and Shake Flask for Lactic Acid Fermentation

The fermenter and the shake flask were compared as the fermentation vessel. The differences in those systems were the working volumes, the geometric shapes and the agitation types. In the shake flask fermentation medium had a greater surface area due to the shape of the flask and shaking.

Fermentations under same conditions were performed both in the fermenter and in the shake flasks. Fermenter was sterilised as described in section 3.2.2.2.

The comparison of lactic acid production and lactose consumption is shown in Figure 4.18. As can be seen in the Figure 4.18 lactic acid productions were not so different than each other. The productivity values were around 2.0 g l^{-1} h^{-1} for both systems.

Additionally, another fermentation was performed under the same conditions but pH was maintained at 6.0 by NaOH. This is shown in Figure 4.19. The productivity of 2.3 g l^{-1} h^{-1} was obtained, which was 15 % higher than the above systems. That result coincided with the results of the experiments that compared the NaOH and $CaCO_3$ for the fermenter inoculated with seed cultures. (Look at section 4.2)

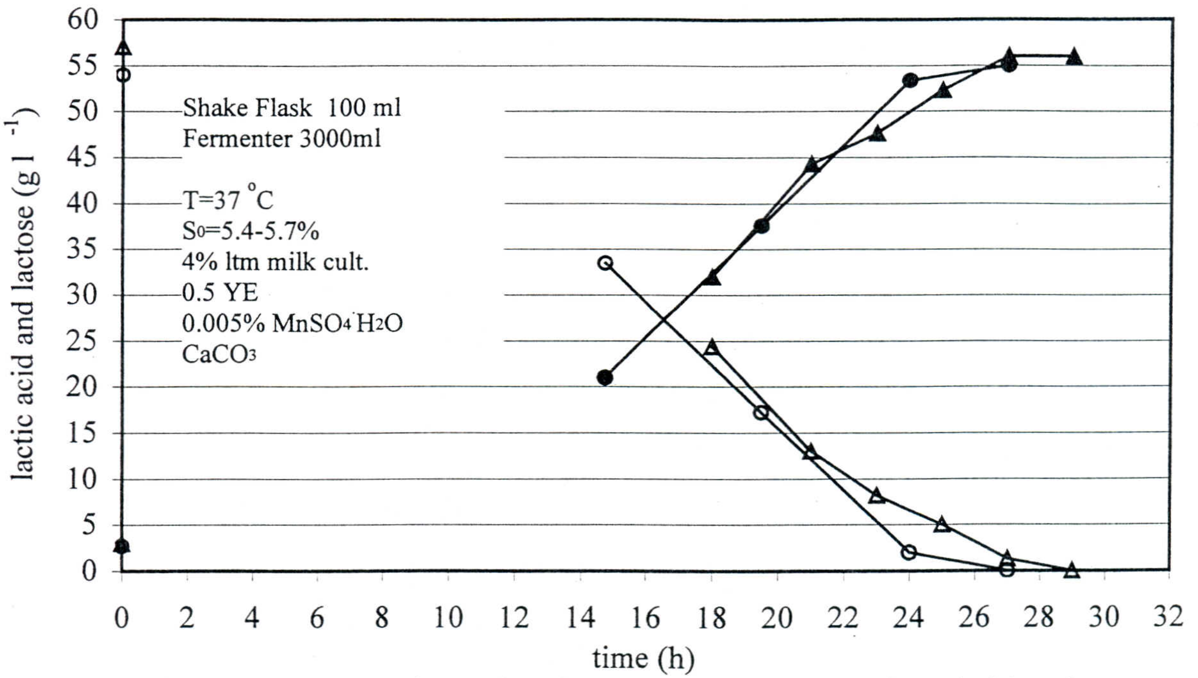

Figure 4.18. Comparison of lactic acid production (closed symbols) and lactose utilization (open symbols) in the fermenter and in the shake flask.

—○—. —●— shake flask —△—, —▲— fermenter

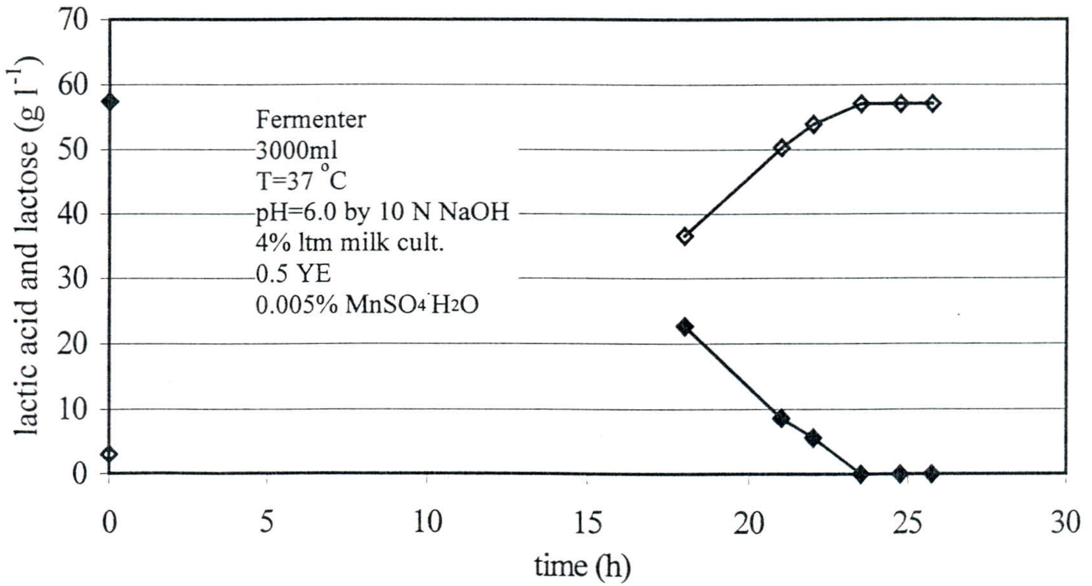

Figure 4.19. Lactic acid production (open symbols) and lactose utilization (closed symbols) in the fermenter inoculated with 4% litmus milk culture.

4.8 Biomass Growth

Growth of *L. casei* on synthetic lactose medium for lactic acid production was investigated. The lactic acid, lactose and biomass concentrations are shown in Figure 4.20.

Initial biomass concentration was 0.1 g l^{-1}. Biomass concentration was increased exponentially from 2 to 12 h. From 16 to 19 h biomass stayed constant at around 4.5 g l^{-1} and then decreased to around 4.0 g l^{-1}. The decline in the biomass concentration started at the time when lactose was completely utilized.

Under these conditions maximum specific growth rate (μ_{max}) was calculated as 0.32 h^{-1}. The growth yield ($Y_{x/s}$) under these conditions was 0.084 g dry cell per g lactose. The product yield ($Y_{p/s}$) was 0.86 g lactic acid per g lactose. The productivity was calculated as 2.40 g l^{-1} h^{-1}.

As seen in Figure 4.20 there was no significant lactic acid production up to 6 hours during which an increase in biomass concentration was observed, though. Lactic acid production continued up to 19 h, while biomass growth ceased at 16 h. Therefore, it was concluded that up to 16 h lactic acid production was growth associated and from 16 to 19 h lactic acid production was non-growth associated.

Different growth phases can be seen in Figure 4.21. A typical lag phase is not observed. However a short lag phase would probably have been obtained if samples had been taken more frequently. Exponential phase lasted approximately up to11-12 hours. From 14 to 19 hours a stationary phase was observed followed by a death phase. The main reason for the presence of the death phase could be the nutrient depletion.

Vaccari et al. (1993) obtained lower μ (0.165 h^{-1}) but higher growth yield (0.175 g g^{-1}) with 57.02 g l^{-1} initial glucose concentration for lactic acid production by *L. casei* DSM 20011. They also observed that μ and $Y_{x/s}$ were decreased with increasing initial glucose concentration.

For batch lactic acid production from whey by *L. helveticus* Amrane and Prigent (1994) observed growth associated and non-growth associated production phases.

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Roy et al. (1987) compared the glucose synthetic, lactose synthetic and whey-yeast extract permeate medium for lactic acid production by *L. helveticus* and obtained specific growth rates of 0.310, 0.479. 0.435 h^{-1}, respectively.

Srivastava et al. (1992) compared batch fermentation and extractive lactic acid fermentation using ion-exchange resin by *L. delbrueckii* NRRL B445. For the batch fermentation growth yield and specific productivity were 0.192 g g^{-1} and 0.016 h^{-1}, respectively. Cell yield was 36 % and specific productivity was 240 % higher for the extractive fermentation.

It is possible to obtain higher cell mass by different reactor types, such as cell recycle, immobilized cells, dialysis culture (Friedman and Gaden 1970, Börgardts et al. 1998, Krischke et al. 1991). Börgardts et al. (1998) obtained approximately 40 g l^{-1} biomass for lactic acid production from whey by *L. casei* subsp. *casei* in a continuous reactor with a cell recycle.

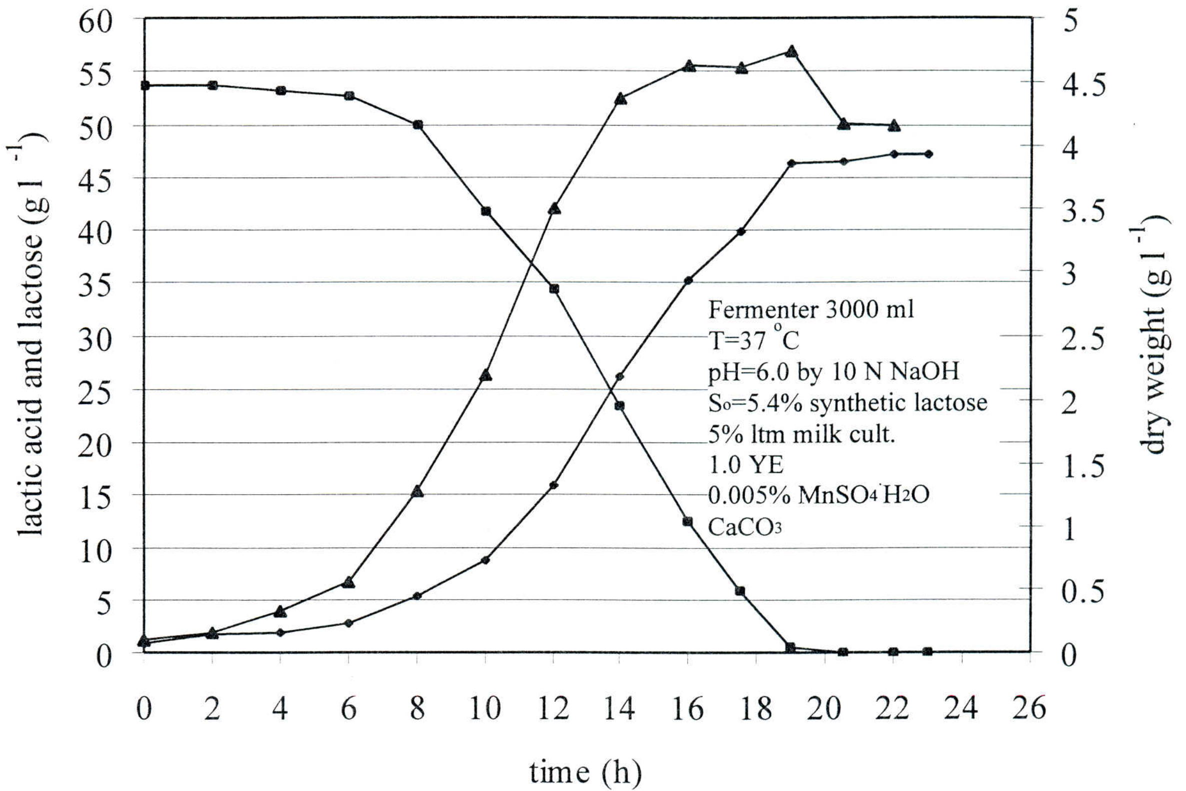

Figure 4.20. Lactose, lactic acid and dry cell weight concentrations for L. casei grown on lactose synthetic medium.

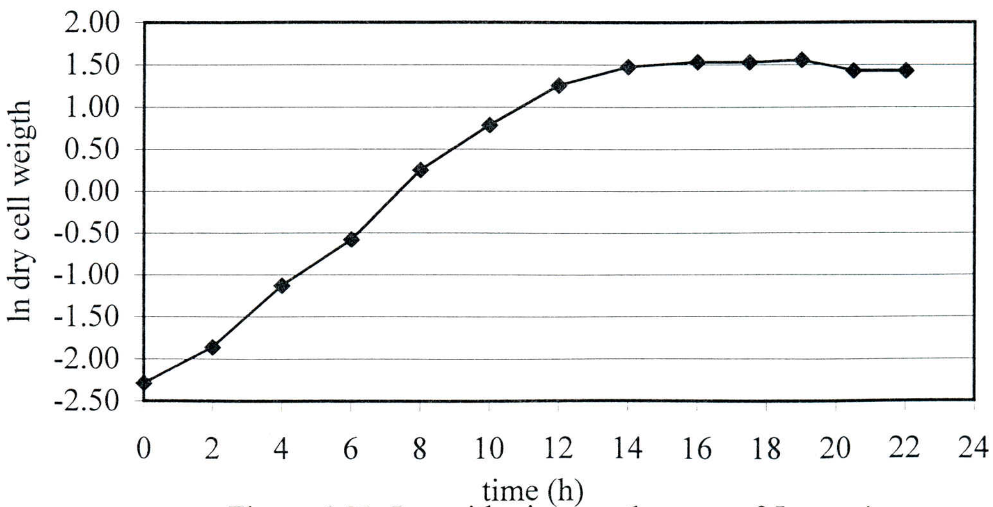

Figure 4.21. Logarithmic growth curve of *L. casei*.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

The objective of this study was to investigate the lactic acid production from whey with the use of *Lactobacillus casei* by determining the optimum fermentation conditions.

Results showed that lactic acid could be produced with a comparable yield from a cheap, abundant crude feedstock, whey, which is a pollutant when it is not utilized or processed.

The fastest lactic acid production was at 37 ^{o}C and pH of 5.5. Lactic acid yields were between 0.89 and 0.94 g lactic acid (g lactose)$^{-1}$, generally around 0.92 g lactic acid (g lactose)$^{-1}$. Productivity values were around 2.0 g l^{-1} h^{-1} in the shake flasks, where lactic acid was neutralized by $CaCO_3$ and inocula were 4-5% litmus milk culture. In the fermenter, a productivity of 4.62 g l^{-1} h^{-1} was obtained at pH 5.5 with 12.5% seed culture as the inoculum.

When $MnSO_4$ was discarded from the formulation of the fermentation media, the production was very poor and similar to the production observed without any of the salts. The absence of the other salts did not effect the production. Therefore, $MnSO_4$ was concluded to be the only effective ion on lactic acid production from whey by *L. casei.*

Whey, with a productivity value of 1.75 g l^{-1} h^{-1}, was found to be superior to synthetic lactose and synthetic glucose for lactic acid production under the conditions used in the study. Higher yeast extract concentration (1.0%) was needed for lactose and glucose media in order to obtain similar production rate to that of whey medium. It was concluded that the complex composition of whey could have provided additional nitrogenous materials to the organism.

From an economical point of view, in order to optimize the yeast extract supplementation, its cost should also be considered. Although slightly higher productivity values were obtained with 0.75 and 1% yeast extract supplementation, for an industrial process, 0.5% may be the most feasible yeast extract concentration with a

productivity figure of 1.75 g l^{-1} h^{-1}. In addition to that, high yeast extract concentration requires additional cost in the downstream processes.

L. casei was able to utilize the sugar completely even at 10% initial whey lactose concentration, that is no lactose was present in the fermentation broth at the end of the fermentation. Therefore no additional purification process would be needed to remove the sugar. Above 6% (w/v) initial lactose concentration productivity values were similar and approximately 2.25 g l^{-1} h^{-1}.

Under the same conditions similar production rates were observed for the fermenter and the shake flasks. In the fermenter 10 N NaOH yielded 15% higher productivity values than the $CaCO_3$ as the neutralizing agent.

The growth of *L. casei* was investigated in synthetic lactose medium. The lactic acid production and growth were coupled up a certain time. After that point, lactic acid concentration increased although the biomass concentration was constant. It was concluded that lactic acid production by this microorganism was in two phase; first phase was the growth associated phase and the second phase that followed was the non-growth associated. The maximum specific growth rate was calculated as 0.32 h^{-1}.

Seed culture that had the same composition of the fermentation medium instead of litmus milk as the inoculum for the fermenter yielded faster fermentations, so it is concluded that inoculum type plays an important role in lactic acid fermentations. Approximately two times better productivity values were obtained with the seed culture. Therefore, the effect of concentration, composition and age of seed culture should be examined in detail.

The fermentation parameters determined in this study could be applied to other production systems, such as continuous, cell recycle or immobilized cell reactors or a novel production system. Higher productivity values may be obtained in these systems.

This study showed that whey was a convenient substrate for lactic acid production. However, when selecting a carbon source for the fermentation productivity can not be the sole parameter, because the complex composition of whey leads to higher purification cost than pure sugars, such as glucose, lactose and sucrose. So, the raw material cost and purification cost should be considered as well as the productivity.

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APPENDIX

A. Calibration Curves

The calibration curves used in the analyses of the fermentation samples are shown in this section.

A.1. Calibration Curves for Lactose, Glucose and Lactic Acid

The calibration curves for lactose, glucose and lactic acid are shown in Figure A.1. The software of the HPLC calculated the concentration of the samples according to those calibration curves.

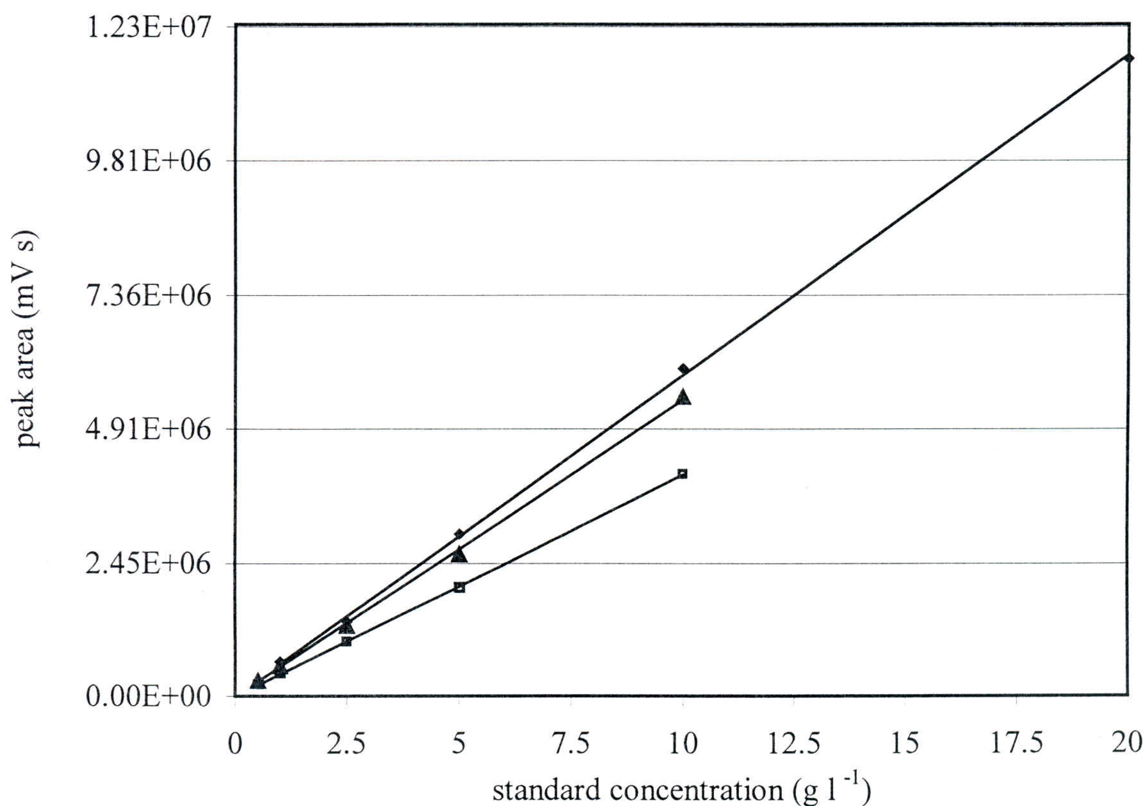


Figure A.1. Calibration curves used in the HPLC analyses

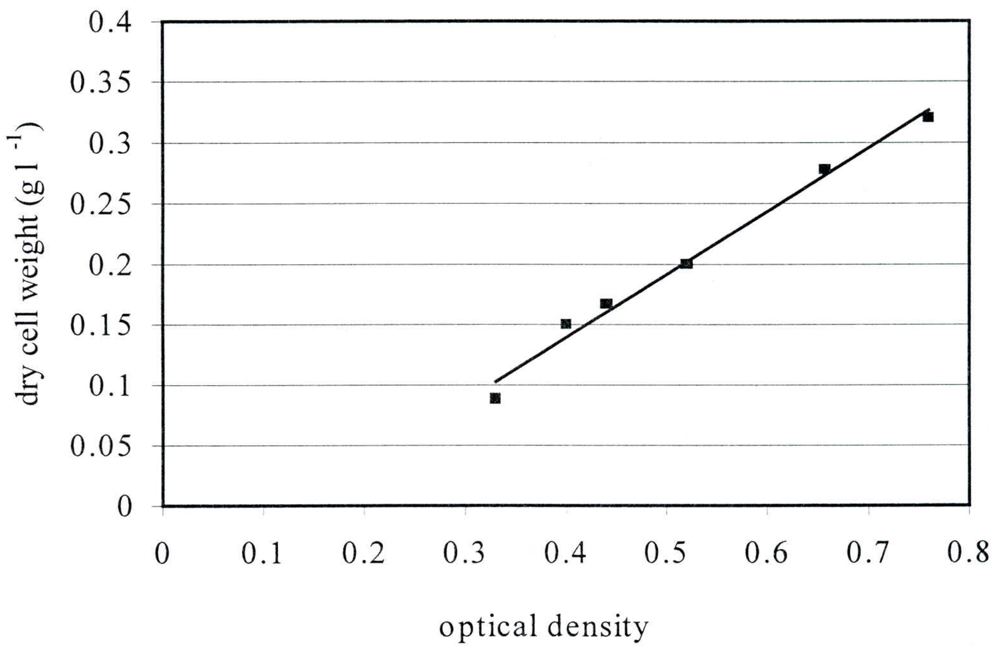
◆ lactose ▲ glucose ◘ lactic acid

A.2. Calibration Curve for Dry Cell Weight Measurements

Calibration curve for dry weight measurements is shown in Figure A.2. The equation of the trendline was:

y = 0.5197x - 0.0686, where y is the dry cell weight and x is the optical density. The dry cell weights of the samples whose optical densities had been measured were calculated using that equation.

The R^2 value of the trendline was 0.9884.



Figiure A.2. Dry cell weight calibration curve

B. Definitions of Fermentation Evaluation Terms

The yield and productivity terms are defined in this section.

Productivity: Productivity is the amount of product produced per time and per volume.

$$\text{Productivity} = \frac{P_f - P_i}{t_f - t_i}$$

Substrate conversion: The amount of substrate utilized per initial substrate is defined as the conversion yield.

$$\text{substrate conversion} = -\frac{\Delta S}{S_0}$$

Product yield: Product yield is the amount of product produced per amount of substrate utilized.

$$Y_{P/S} = -\frac{\Delta P}{\Delta S}$$

Growth yield: Growth yield in a fermentation is defined as the amount of biomass produced per amount of substrate utilized.

$$Y_{X/S} = -\frac{\Delta X}{\Delta S}$$

Specific growth rate: Specific growth rate is the characterization of the rate of microbial growth and defined as,

$$\mu = \frac{1}{X}\frac{dX}{dt} \quad \Rightarrow \quad \mu = \frac{\ln X_2 - X_1}{t_2 - t_1}$$

where P= product (lactic acid) concentration (g l^{-1})

t= time (h)

S= substrate concentration (g l^{-1})

S_0= initial substrate concentration (g l^{-1})

X= dry cell weight (g l^{-1})

μ= specific growth rate (h^{-1})

i= initial

f= final