LACTIC ACID PRODUCTION BY *Lactobacillus casei* NRRL B-441 IMMOBILIZED IN CHITOSAN STABILIZED Ca-ALGINATE BEADS

A Thesis Submitted to the
Graduate School of Engineering and Sciences of
Izmir Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

in Food Engineering

by
Meltem GÜNDÜZ

January 2005
İZMİR

We approve the thesis of **Meltem GÜNDÜZ**

	Date of Signature
Prof. Dr. Şebnem HARSA Supervisor Department of Food Engineering Izmir Institute of Technology	06.01.2005
Assist. Prof. Dr. Yekta GÖKSUNGUR Co-Supervisor Department of Food Engineering Ege University	06.01.2005
Assist. Prof. Dr. Figen TOKATLI Department of Food Engineering Izmir Institute of Technology	06.01.2005
Assist. Prof. Dr. Canan TARI Department of Food Engineering Izmir Institute of Technology	06.01.2005
Assist. Prof. Dr. Fuat DOYMAZ Department of Chemical Engineering Izmir Institute of Technology	06.01.2005
Prof. Dr. Şebnem HARSA Head of Department of Food Engineering Izmir Institute of Technology	06.01.2005

Prof. Dr. Semra ÜLKÜ

Head of the Graduate School

ACKNOWLEDGEMENTS

I would like to thank to Prof. Dr. Şebnem HARSA for her guidance, supervision, patience, and support throuhgout this study. I also wish to express my thanks to Assist. Prof. Dr. Yekta GÖKSUNGUR for his guidance, support and encouragement.

I offer sincere thanks to my family members for their endless support, encouragement and love.

I would like to thank my friends Zelal POLAT and Seda ALPER for their helps.

ABSTRACT

Having two optically active forms, D(-) and L(+) lactic acid has long been used in the food, chemical, textile, pharmaceutical and other industries. 90 % of the worldwide production of lactic acid is by bacterial fermentation. Recently, there is an increasing interest in the production of L(+) lactic acid, since it is a potential substrate for polylactic acid that is biocompatible and can be used for medical purposes. Whey, which is a by-product of dairy industry, contains approximately 5 % (w/v) lactose. Since whey has a high BOD content, it possesses serious environmental problems. Whey lactose is a good substrate for lactic acid bacteria and can be used for L(+) lactic acid fermentations.

This study focuses on the production of lactic acid from whey by *Lactobacillus casei* NRRL B-441 immobilized in chitosan stabilized Ca-alginate beads. Higher lactic acid production and lower cell leakage were observed with alginate-chitosan beads compared with Ca-alginate beads. The highest lactic acid (131.2 g/l) was obtained with cells entrapped in 1.3-1.7 mm alginate-chitosan beads prepared from 2 % Na-alginate. The gel beads produced lactic acid for 10 consecutive batch fermentations without marked activity loss and deformation. Response surface methodology was used to investigate the effects of three fermentation parameters (initial sugar, yeast extract and calcium carbonate concentrations) on the concentration of lactic acid. No previous work has used statistical analysis in determining the interactions among these variables in lactic acid production by immobilized cells. Results of the statistical analysis showed that the fit of the model was good in all cases. Initial sugar, yeast extract and calcium carbonate concentrations had strong linear effects on lactic acid production. Maximum lactic acid concentration of 136.3 g/l was obtained at the optimum levels of process variables (initial sugar concentration=147.35 g/l, yeast extract concentration= 28.81 g/l, $CaCO_3$ concentration=97.55 g/l). These values were obtained by fitting of the experimental data to the model equation. The response surface methodology was found to be useful in optimizing and determining the interactions among process variables in lactic acid production using alginate-chitosan immobilized cells.

ÖZ

Optikçe aktif iki form olan D(-) ve L(+) laktik asit gıda, kimya, tekstil, ilaç ve diğer endüstrilerde uzun zamandır kullanılmaktadır. Laktik asitin dünyadaki üretiminin % 90'ı bakteriyel fermentasyonladır. Son zamanlarda L(+) laktik asitin üretimi ilgi kazanmıştır, çünkü L(+) laktik asit, biyolojik olarak tüketilebilmesi ve ilaç amaçlı kullanılabilmesi nedeniyle polilaktik asit için potansiyel bir substrattır. Süt endüstrisinin atık bir ürünü olan peynir altı tozu, yaklaşık % 5 laktoz içerir. Peynir altı tozu laktozu, laktik asit bakterisi için iyi bir substrattır ve L(+) laktik asit fermentasyonları için kullanılabilir. Laktik asitin iki çesiti vardır; bunlar D ve L laktik asittir. Onların sadece optik özellikleri farklılık gösterir, fakat fiziksel ve kimyasal karakterleri aynıdır.

Bu çalışma kitozan ile stabilize edilmiş Ca-aljinat boncuklarında tutuklanan *Lactobacillus casei* NRRL B-441 suşu ile laktik asit üretmeye dayanmaktadır. Ca-aljinat boncukları ile karşılaştırıldığında, Ca-aljinat-kitozan boncukları ile daha yüksek laktik asit üretimi ve daha düşük hücre sızıntısı gözlenmiştir. En yüksek laktik asit (131.2 g/l), % 2 Na-aljinattan hazırlanmış 1.3-1.7 mm aljinat-kitozan boncuklarında hapsedilmiş hücreler ile elde edilmiştir. Jel boncukları, belirgin bir aktivite kaybı ve deformasyona uğramadan 10 ardıl kesikli fermentasyonla laktik asit üretmiştir. Response surface metodu , laktik asit konsantrasyonu üzerine üç fermentasyon parametrelerinin (başlangıç şeker, maya ekstraktı ve kalsiyum karbonat konsantrasyonu) etkilerini araştırmak için kullanılmıştır. Daha önceki hiçbir çalışma, immobilize hücrelerle laktik asit üretiminde bu değişkenler arasındaki etkileşimleri belirlemede istatistiksel analiz kullanmamıştır. İstatistiksel analizin sonuçları modelin uygunluğunun tüm koşullarda iyi olduğunu göstermiştir. Başlangıç şeker, maya ekstraktı ve kalsiyum karbonat konsantrasyonları, laktik asit üretimi üzerinde kuvvetli doğrusal etkiye sahiptirler. 136.3 g/l maximum laktik asit konsantrasyonu proses değişkenlerinin (başlangıç şeker=147.35 g/l, maya ekstraktı= 28.81 g/l ve kalsiyum karbonat konsantrasyonu=97.55 g/l) optimum değerlerinde elde edilmiştir. Bu değerler, deneysel verilerin örnek denkleme uygulanması ile elde edilmiştir. Response surface metodu, proses değişkenleri arasındaki etkileşimleri belirlemekte ve optimize etmekte, aljinat-kitozanda tutuklanmış hücreleri kullanarak laktik asit üretmekte kullanışlı bulunmuştur.

TABLE OF CONTENTS

LIST OF FIGURES

Figure **Page**

LIST OF TABLES

Table **Page**

LIST OF SYMBOLS AND ABBREVIATIONS

v/v ..... volume/volume

w/v ..... weight/volume

wt ..... weight

ml ..... mililiter

cfu/g ..... colony forming units per gram

HPLC .....high pressure liquid chtomatography

min ..... minute

μm .....micrometer

eqn .....equation

BOD ..... biochemical oxygen demand

COD ..... chemical oxygen demand

RSM .....response surface methodology

% .....percent

K_a .....dissociation constant

ΔH_c .....heat of combustion

C_P ..... specific heat

CSL .....calcium stearoyl-2-lactylate

SSL .....sodium stearoyl-2-lactylate

nm ..... nanometer

N .....normal

CHAPTER 1

INTRODUCTION

Lactic acid is an organic acid (α-hydroxy-propionic acid) which can be used for a wide variety of industrial applications. In food industry it is used as acidulant, preservative and antimicrobial agent For pharmaceutical applications, lactic acid can be used as electrolytes and mineral sources. For technical applications lactic acid can be used as neutralizers, solvents, cleaning agents, slow acid release agents and metal complexing agents. It has also been used in cosmetic industry as pH buffer, antimicrobial, skin rejuvenating and skin lightening (Vickroy, 1985).

There are two isomers of lactic acid, these are D(-) and L(+) forms. They only differ in their optical properties, but are identical in their physical and chemical characteristics. L(+)-lactic acid is biodegradable and can be metabolized by the human body and this property leads the application of lactic acid in biomaterial and biomedical field (Hunger, 1984).

Lactic acid is produced by chemical synthesis and by microbial fermentation. By chemical synthesis method, racemic (DL) mixture of lactic acid is produced. By microbial fermentation method L(+) and D(-) lactic acids can be produced according to the type of microorganism which may be homofermentative or heterofermentative. This is an important advantage of the microbial fermentation method compared to the chemical synthesis method. At the end of the fermentation process, lactic acid exists in the complex medium of fermentation broth which contains whey proteins, biomass, salts and other impurities. Lactic acid should be recovered from that complex media. As high cost of lactic acid purification process limits the utilization of this chemical, in large scale applications a system with less raw material and fewer unit operations are needed (Rao, 1983).

Whey is a major by-product of the dairy industry which serves as an inexpensive medium for lactic acid production. It contains approximately (w/v) 5 % lactose, 1 % protein, 0.4 % fat, and some minerals. It has a high biochemical oxygen demand (BOD) content (40,000-60,000 ppm) which represents serious disposal problems (Büyükkilleci, 2000).

Immobilization of whole cells has been widely used for lactic acid production since immobilization exhibits many advantages like relative ease of product separation, reuse of biocatalysts, high volumetric productivity, improved process control and reduced susceptibility of cells to contamination (Göksungur, 1999). The entrapment of cells in calcium alginate gel beads is the most widely used method for viable lactic acid bacteria immobilization due to its simplicity, nontoxicity, mild gelation conditions and ease of use (Boyaval, 1988). However, alginate gels are susceptible to cation chelating agents such as phosphate and lactate which can cause instability of the beads. In lactic acid production, calcium ions which stabilize this type of gel are displaced by lactate ions produced by lactic acid bacteria leading to disruption or dissolution of the beads (Li, 1996). Another drawback of using Ca-alginate in cell immobilization is the cell leakage from the beads. Cells on and near the surface can easily leak from the beads into the medium and subsequently grow very rapidly in the medium than in the beads (Tanaka, 1989).

Many attempts have been made to improve the stability of Ca-alginate beads like covering the beads with poly-L-lysine (Champagne, 1992), treating the beads with polyethyleneimine, glutaraldehyde and hexamethylenediamine (Bódalo, 1997). Coating of Ca-alginate beads with chitosan is another method to increase the stability of beads. Chitosan (β-1,4-D-glucosamine) is the deacetylated form of chitin (β-1,4-N-acetyl-D-glucosamine) which is the second abundant natural biopolymer after cellulose (Göksungur, 2004). When alginate (strongly acidic polyanion) is mixed with chitosan (strongly basic polycation), strong ionic interactions between the carboxyl residues of the alginate and the amino terminals of the chitosan occur to form a polyelectrolyte complex which is insoluble in common solvents and highly permeable to water-soluble microsolutes. This complex does not dissolve in the presence of Ca^{+2} chelators or anti-gelling cations and thus can be used to stabilize the gel and reduce porosity of the alginate beads (Albarghouthi, 2000).

The stabilization of Ca-alginate beads by coating with chitosan has been investigated by a number of researchers for *Saccharomyces cerevisiae* cells, a model enzyme (β-galactosidase), and *Yarrowia lipolytica* yeast. Gaserod (1998) studied the binding of chitosan to alginate beads quantitatively by using radioactive labeled fractions of chitosan. Gaserod (1999) studied the stability and permeability of alginate-chitosan complex as a function of the content and distribution of chitosan in the gel beads. In the literature there have been a few studies concerning lactic acid production

by bacteria immobilized in chitosan coated alginate beads have been published. Yoo (1996) produced lactic acid with a productivity of more than 2.7 g dm^{-3} h^{-1} using *L.casei* cells immobilized in chitosan coated Ba-alginate beads. Zhou (1998) investigated the effect of the coating of alginate beads on *L. lactis* ssp. *cremoris* release from the beads and found that the main effect of chitosan coating was to decrease the cell release rate during early stages of fermentation.

Response surface methodology (RSM) is a collection of statistical techniques for designing experiments, building models, evaluating the effect of factors and searching optimum conditions for desirable responses. RSM can identify and quantify the various interactions among different parameters and it has been extensively applied for optimization of cultural medium conditions and other process parameters in bioprocesses. It has not yet been reported as a means to study and optimize process parameters for lactic acid production using immobilized cells (Zhou, 1998).

The aim of this study was to examine the lactic acid production from whey by *L.casei* NRRL B-441 immobilized in alginate-chitosan beads. The effect of initial sugar concentration, Na-alginate concentration and bead size on lactic acid production had been studied. RSM was used to optimize fermentation parameters to obtain maximum lactic acid concentration. The optimized fermentation parameters were initial sugar, yeast extract and $CaCO_3$ concentrations since these factors were among the most important factors for lactic acid production. The emphasis was given on both optimization and production of lactic acid from whey using *L.casei* NRRL B-441 immobilized in alginate-chitosan beads.

CHAPTER 2

LACTIC ACID

2.1 Historical Perspective

Lactic acid (2-hydroxypropionic acid, $CH_3CHOHCOOH$) is an organic acid whose occurence in nature is widespread. It was first produced commercially by Charles E. Avery at Littleton, Massachusetts, USA in 1881. The venture was unsuccessful in its attempts to market calcium lactate as a substitute for cream of tartar in baking powder. The first successful uses in leather and textile industries began about 1894 (Garrett, 1930) and production levels were about 5000 kg y^{-1} on a 100 % basis (Inskeep , 1952). In 1942, about half of the 2.7 x 10^6 kg y^{-1} produced in the US was used by the leather industry, and an emerging use in food products consumed about 20 % (Filachione, 1952). US production peaked at 4.1 x 10^6 kg y^{-1} during World War II and then leveled off to about 2.3 x 10^6 kg y^{-1} . A 90 x 10^6 kg y^{-1} (Needle, 1949) market for lactic acid in the plastics industry was predicted in the late 1940s and early 1950s which encouraged a large, but unsuccessful, research effort to reduce costs and increase purity. A decade later, the need for heat stable lactic acid to produce stearoyl-2-lactylates for the baking industry opened the way for a synthetic route to lactic acid (Anon, 1963). The world-wide production of lactic acid is 80.000 tons/y and 90 % of it is produced by lactic acid bacteria (Büyükkileci, 2000). More than 50 % of the lactic acid produced is used in food industry as an acidulant and as a preservative. The production of stearoyl-2-lactylates consumes another 20 %. The rest of the lactic acid is used by the pharmaceutical industry or is used in numerous industrial applications (Vickroy, 1985).

Lactic acid from cheese whey is produced commercially in Slovakia, Italy, and the United States, whereas synthetic production from acetaldehyde or lactonitrile is cheaper, the low cost of whey makes a large-scale production facility competitive. Lactic acid bacteria such as *Lactococcus lactis , Lactobacillus lactis , Lactobacillus delbrueckii* subsp. *bulgaricus , Lactobacillus acidophilus, Lactobacillus helveticus, Lactobacillus casei,* and mixed cultures of these organisms have been successfully used to convert lactose to lactic acid (Henning, 1998).

2.2. Physical and Chemical Properties of Lactic Acid

Lactic acid was first isolated from sour milk by Scheele in 1780 (Lockwood, 1965). Lactic acid exists in two optically active isomeric forms as shown in Figure 2.1:

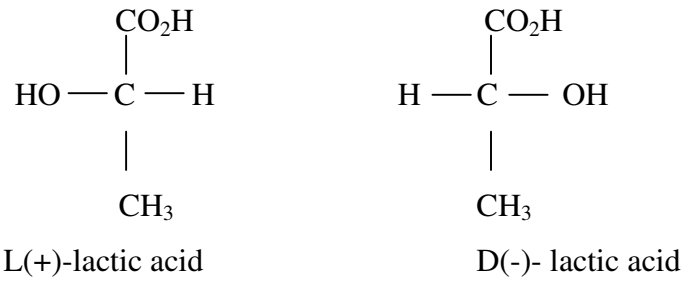


Figure 2.1. Isomeric properties of lactic acid (Source: Holten, 1971).

D(-) and L(+) lactic acids differ in their optical properties, but are identical in their physical and chemical characteristics (Hunger, 1984).

In Table 2.1. we can see some examples of lactic acid isomers and lactic acid bacteria.

Table 2.1. Lactic Acid Isomers and Lactic Acid Bacteria (Source: Hunger W.,1984).

LACTIC ACID ISOMERS	LACTIC ACID BACTERIA
L(+)-lactic acid (>=95%)	all lactic acid streptococci
	L.casei
	L.xylosus and others
D(-)-lactic acid (100%)	*L.bulgaricus*
	L.lactis
	Leucunostoc cremoris and others
Racemic lactic acid	*L.helveticus*
	L. acidophilus
	L.plantarum
	L.brevis and others

Although the L (+) form appears to be dextrorotatory, it may actually be levorotatory as are its salts and esters. The apparent reversal in optical rotation may be due to the formation of an ethylene oxide bridge between carbon atoms 1 and 2 by tautomeric shift of the hydroxyl group on carbon atom 2 to the carbonyl group of the carboxyl radical. Salts and esters of L(+)- lactic acid cannot form this epoxide ring and are levorotatory (Lockwood, 1965).

Lactic acid is soluble in all proportions of water and exhibits a low volatility. In solutions with roughly 20 % or more lactic acid, self-esterification occurs because of the hydroxyl and carboxyl functional groups. Lactic acid may form a cyclic dimer (lactide) or form linear polymers with the general formula $H[OCH(CH_3)CO]_nOH$ (Lockwood, 1965).

Lactic acid may react as an organic acid as well as an organic alcohol and can participate in numerous types of chemical reactions (Holten, 1971).

Some characteristics of lactic acid organisms are shown in Table 2.2.

Table 2.2 Characteristics of lactic acid organisms (Source: Lockwood, 1965).

ORGANISM	MORPHOLOGY	SUBSTRATE	OPT T(°C)	ACID PRODUCED
L. bulgaricus	rod	lactose, whey	45-50	racemic
L. delbrueckii	rod	glucose,molasses	45-50	L(+)
L.brevis	rod	pentoses,	30	racemic
		hydrolyzed wood		
L.casei	rod	whey, lactose	30-50	L(+)
L.plantarum	rod	pentoses,	30	racemic
		sulfite liquor		
L.leichmannii	rod	sucrose, glucose	30	D(-)
S. lactis	coccus	lactose, whey	35	L(+)
B. coagulan	rod,	glucose,lactose	45-50	L(+)
	forms spores			
R. oryzae	mold	glucose, starch	30	L(+)

Some properties of general interest are given in Table 2.3 (Vickroy, 1985).

Table 2.3. Physical properties of lactic acid (Source: Holten, 1971 and Lockwood, 1965).

PROPERTIES	
Molecular weight	90.08 g/mole
Melting point D(-) or L(+)	52.8-54 ^{0}C
DL (varies with composition)	16.8-33 ^{0}C
Boiling point DL	82 ^{0}C at 0.5 mm Hg
	122 ^{0}C at 14 mm Hg
Dissociation constant (K_a at 25 ^{0}C)	1.37 x 10^{-4}
Heat of combustion (ΔH_C)	1361 kJ mol^{-1}
Specific heat (C_p at 20 ^{0}C)	190 J mol^{-1} $^0C^{-1}$

2.3. Application Areas of Lactic Acid

Lactic acid is commonly used industrially because it is naturally occuring, edible and mild tasting. It is also one of the most versatile ingredients applied to food formulation and preservation worldwide. More than 50 % of both synthetic and fermentation derived lactic acid is used for this purpose. (Vickroy, 1985) Lactic acid is used in baby foods, bakery goods, beer, cheeses, confections, dairy products, frozen desserts, fruit juices, meats, preservatives, salad dressings, seafoods, seasonings, soft drinks, vegetables and wines (Duxbury, 1993).

The largest use of high quality 'heat stable' food or pharmaceutical grade lactic acid is for the production of stearoyl-2-lactylates. Calcium stearoyl-2-lactylate (CSL) is used mostly in baking. CSL acts as a 'dough conditioner' by combining with the gluten in the dough, making it more tolerant to mixing and processing conditions as well as allowing a wider variation of bread ingredients. Sodium stearoyl-2-lactylate

(SSL) behaves similarly to CSL and also acts as an emulsifier as well. Both CSL and SSL help extend the shelf life of baked products (Vickroy, 1985).

Lactic acid is used as a food acidulant because it naturally occurs in many foodstuffs, has a mild acid taste, and has no strong flavors or odors of its own (Vickroy, 1985).

Lactic acid is also used as a preservative, sometimes in combination with other edible grade acids such as propionic and acetic acid. As a food acidulant, lactic acid experiences competition primarily from citric, acetic and phosphoric acid and to a lesser extend from malic, fumaric, propionic, formic and tartaric acid. Lactic acid is generally more expensive to use than other food acids, but it is sometimes preferred because it adds less of its own flavor to the food. Lactic acid is used in brines for processing and packaging foods such as olives, pickles and sauerkraut (Vickroy, 1985). It is used for packaging of brines for green olives and onions, where they ensure clarity of the brine and enhance flavor (Duxbury, 1993).

In lightly flavored soft drinks and fruit juices, as well as seafoods, lactic acid may function only as a flavoring agent. In preservatives, the high solubility of calcium lactate is functional as a common firming salt for fruits and vegatables. And in jams and instant desserts, calcium lactate functions as a gelling salt. Lactic acid can be added to either liquid or powdered baby milk to improve digestibility (Duxbury, 1993).

Lactic acid finds medical applications as an intermediate for pharmaceutical manufacture, for adjusting the pH of preparations, and in topical wart medications. Biodegradable plastic made of poly(lactic acid) is used for sutures that do not need to be removed surgically, and has been evaluated for use as a biodegradable implant for the repair of fractures and other injuries. Lactic acid is also used for the manufacture of some herbicides, fungicides and pesticides (Vickroy, 1985).

2.4. Production Technology

2.4.1. Chemical Production(only racemic lactic acid)

Lactic acid can be manufactured synthetically from lactonitrile or by alkali degradation of sugars. The bulk of the world's production of lactic acid is by the fermentation of molasses, which is by far the cheapest process (Rao, 1983).

Synthetic lactic acid production is shown in equations (1), (2) and (3) (Rao, 1983).

$$CH_3CHO + HCN \longrightarrow CH_3CHOHCN \quad (1)$$

acetaldehyde hydrogen cyanide lactonitrile

$$CH_3CHOHCN + 2H_2O \longrightarrow CH_3CHOHCOONH_4 \quad (2)$$

$$CH_3CHOHCOONH_4 \longrightarrow CH_3CHOHCOOH + NH_3 \quad (3)$$

lactic acid

This lactic acid contains no residual sugars and does not discolor significantly upon heating (Helfferich, 1962).

2.4.2. Microbial Production

Lactic acid bacteria have the property of producing lactic acid from sugars by a process called fermentation. *Lactobacillus, Leuconostoc, Pediococcus and Streptococcus* are important members of this group. The taxonomy of lactic acid bacteria has been based on the gram reaction and the production of lactic acid from various fermentable carbohydrates (WEB_1, 2004).

Lactobacilli are gram positive and vary in morphology from long, slender rods to short coccobacilli, which frequently form chains. Their metabolism is fermentative; some species are aerotolerant and may utilize oxygen through the enzyme flavoprotein oxidase, while others are strictly anaerobic. While spore bearing lactobacilli are facultative anaerobes, the rest are strictly anaerobic. The growth is optimum at pH 5.5-5.8 and the organisms have complex nutritional requirements for amino acids, peptides, nucleotide bases, vitamins, minerals, fatty acids and carbohydrates (WEB_1, 2004).

The genus is divided into three groups based on fermentation patterns:

1. homofermentative : produce more than 85 % lactic acid from glucose.
2. heterofermentative : produce only 50 % lactic acid and considerable amounts of ethanol, acetic acid and carbon dioxide.
3. less well known heterofermentative species which produce DL-lactic acid, acetic acid and carbon dioxide (WEB_1, 2004).

Therefore, from an economical point of view, in biotechnological lactic acid production, it is more interesting to use a homofermentative lactic acid bacteria to increase the throughput and to reduce the downstream processing cost (Tellez, 2003).

Though chemical synthesis produces a racemic mixture, stereo specific acid can be made by carbohydrate fermentation depending on the strain being used. It can be described by Figure 2.1. First step was fermentation and neutralization. In this step carbohydrate and neutralization are mixed and calcium lactate and water are formed. Then calcium lactate is mixed with sulphuric acid and lactic acid and calcium sulphate are formed.

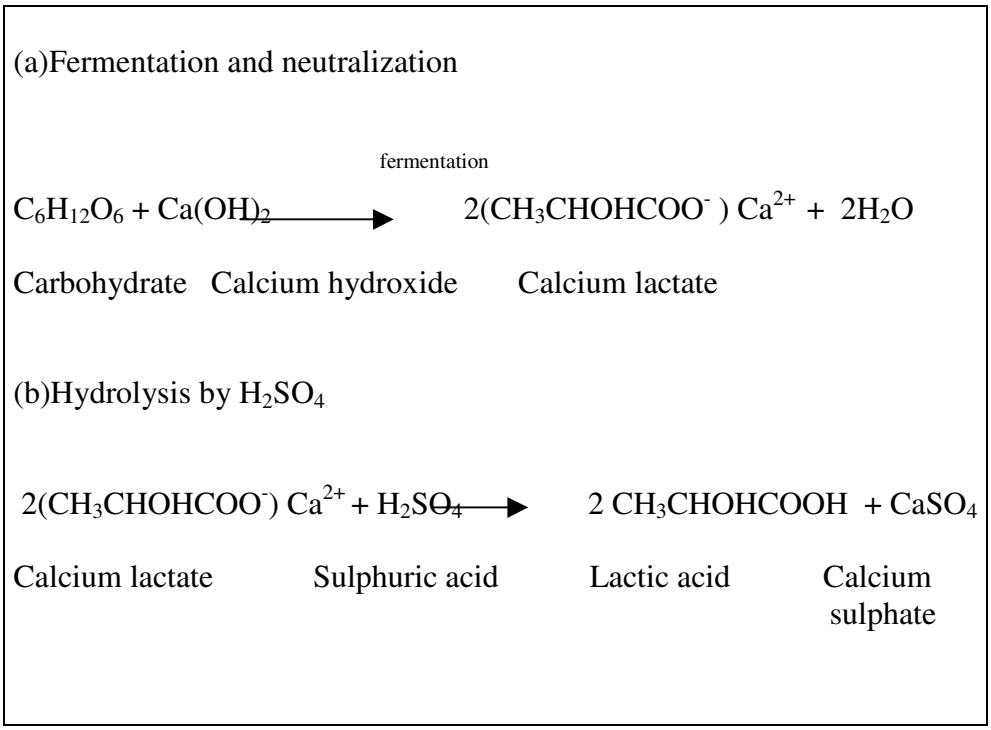


Figure 2.2. Microbial production of lactic acid. (Narayanan , 2004).

2.4.2.1. Microorganisms

Garrett (1930) has summarized the early history of lactic acid fermentations. Pasteur discovered in 1857 that the souring of milk was caused by microorganisms. In the 1860s and 1870s the presence of lactic acid bacteria in distilleries was noted and their optimum growth temperature was investigated. Leichmann isolated a pure culture of *B. delbreuckii* from soured yeast in 1878 (Garrett, 1930). Strains of *Lactobacillus delbreuckii* which probably are quite similar to the strain that was isolated by Leichmann have often been used for the commercial production of lactic acid. Today, the strains used in industry are proprietary; however, it is believed that most of the organisms used belong to the genus *Lactobacillus.* The bacteria may be classified as homofermentative, producing lactic acid, cells and little else, or heterofermentative , producing lactic acid, cells and other by-products such as acetic acid, carbon dioxide, ethanol and glycerol. Only the homofermentative organisms are of industrial importance

for lactic acid manufacture. The homofermentative lactic acid bacteria are from the genera *Lactobacillus, Streptococcus and Pediococcus* (Stanier, 1976). The industrially important organisms grow optimally at temperatures above 40 °C and at a pH of 5-7. The organisms are facultative anaerobes but do not use respiration to generate ATP. Because of the high temperature, low oxygen concentration, high lactate concentration and low pH, contamination is usually not a severe problem (Vickroy, 1985).

The homofermentative lactic acid bacteria catabolize glucose *via* the Embden-Meyerhof pathway. Two lactic acid molecules are produced from each molecule of glucose, typically with a yield of better than 90 g per 100 glucose. Pentose sugars are also metabolized by some homofermentative species and acetic acid and lactic acid are the products of this metabolism. Organisms may produce D(-)-, L(+)- or DL- lactic acid. The formation of the racemic mixture could arise from the action of two stereospecific lactate dehydrogenases or from one stereospecific dehydrogenase and a racemase (Gasser, 1970). The classification of many strains of lactic acid bacteria is still in much confusion at the present. Consequently, it is best to consult the literature on a specific strain or perform an analysis to determine the stereospecificity of the lactic acid it produces. The lactic acid bacteria are extremely limited in their synthetic capabilities. They always require B vitamins and almost without exception require a large number of amino acids (Stainer, 1976). In addition, there are many growth promoting factors that have considerable effect on the fermentation rate. Ledesma (1977) have proposed a synthetic medium for comparative nutritional studies of lactobacilli. Rees and Pirt (1979) have examined the stability of lactic acid production by non-growing cells of *L. delbreuckii.* Maintenance of the glycolytic activity in non-growing immobilized cells might lead to an increase in lactic acid yields, and reduce the amount of cell mass to be disposed (Vickroy, 1985).

The selection of an organism depends primarily on the carbohydrate to be fermented. Gasser (1970) has tabulated the ability of several lactobacilli to grow on different sugars. *L. bulgaricus, L. casei or S. lactis* are used to ferment lactose. Adapted strains of *L. delbreuckii* and *L. leichmannii* are typically used to ferment glucose. *L. pentosus* has been used to ferment sulfite waste liquor (Leonard, 1948). Nakamura and Crowell (1979) have isolated a homofermentative strain called *L. amylophilus* which is capable of fermenting starch to L(+)-lactic acid with 90 wt % yields. Mixtures of strains as well as pure cultures have been used for the commercial manufacture of lactic acid (Vickroy, 1985).

Some fungi of the species *Rhizopus* , particularly *R. oryzae,* can be used to produce L(+)-lactic acid. This organism has less complex nutritional requirements than the lactic acid bacteria. *Rhizopus* species can also utilize starch feedstocks. The process, however, never achieved lasting commercial success. The use of organisms such as *Rhizopus* with less exacting nutritional requirements could possibly reduce the feed costs and simplify the recovery process (Vickroy, 1985).

In general, the desirable characteristics of industrial organisms are the ability to rapidly and completely ferment cheap feedstocks with minimal amounts of nitrogenous substances. The organism is preferred to give high yields of stereospecific lactic acid under conditions of low pH and high temperature, with the production of low amounts of cell mass and negligible amounts of other by-products (Vickroy, 1985).

2.4.2.1.1. Properties of *Lactobacillus casei*

Lactobacillus casei as can be seen in Figure 2.2., are gram-positive, facultatively anaerobic, non-motile and non-spore-forming, rod-shaped (cell size range = 0.7-1.1 x 2.0-4.0 mm) members of the industrially important lactic acid bacteria. Like other lactic acid bacteria, *L. casei* are acid tolerant, cannot synthesize porphyrins, and possess a strictly fermentative metabolism with lactic acid as the major metabolic end product (Axelsson, 1998; Kandler, 1986). Within the genus *Lactobacillus*, *L. casei* form part of the facultatively heterofermentative (“Group II”) species cluster, which produce lactic acid from hexose sugars *via* the Embden-Meyerhof pathway and from pentoses by the 6-phosphogluconate/phosphoketolase pathway (Axelsson, 1998). Growth of *L. casei* occurs at 15°C but not 45°C, and requires riboflavin, folic acid, calcium pantothenate, and niacin growth factors (Kandler, 1986).

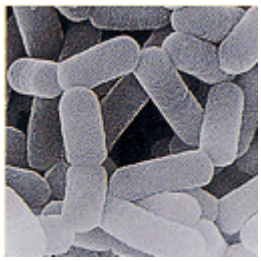


Figure 2.3. Image of *Lactobacillus casei* (Source: WEB_2, 2004).

L. casei is a remarkably adaptive species, which could be isolated from raw and fermented dairy products, fresh and fermented plant products, and the reproductive and intestinal tracts of humans and other animals (Kandler, 1986). Industrially, *L. casei* has application as human probiotics (health-promoting live culture), as acid-producing starter cultures for milk fermentation, and as specialty cultures for the intensification and acceleration of flavor development in certain bacterial-ripened cheese varieties (Fonden, 2000; Fox, 1998; Kosikowski, 1982).

Lactobacilli are characteristic members of the lactic acid bacteria group. They vary in morphology from long, slender rods to short coccobacilli, which frequently form chains. Some species tolerate air and may utilize oxygen, while others are strictly anaerobic. They all grow best in a slightly acidic medium at pH 5.5-5.8 (WEB_2, 2004).

Lactobacillus casei are found in dairy and also plant products and in the digestive tract of humans and animals. *Lactobacillus casei* cells have been immobilized in some supports for lactic acid production. Agar was more effective than polyacrylamide for *L. casei* entrapment for lactic acid production from whey. Also, calcium pectate gel and chemically modified chitosan beads were used as supports for *L. casei* cell immobilization. Alginate has so far been a popular matrix for immobilization of lactic acid bacteria. Other supports used for immobilization include porous foam glass particles, ceramic beads or porous glass, poraver beads, and gluten pellet (WEB_2, 2004).

2.4.2.2. Raw Materials

A large number of carbohydrate materials have been used, tested or proposed for the manufacture of lactic acid by fermentation. It is useful to compare feedstocks on the basis of the following desirable qualities: (Vickroy, 1985)

1. low cost.
2. low levels of contaminants.
3. fast fermentation rate.
4. high lactic acid yields.
5. little or no by-product formation.
6. ability to be fermented with little or no pretreatment.
7. year-round availability.

Sucrose from cane and beet sugar, whey containing lactose, and maltose and dextrose from hydrolyzed starch are presently used commercially for lactic acid production. Refined sucrose, although expensive, is the most commonly used substrate, followed by dextrose. Concentrated whey has been used without any other pretreatment by the Sheffield Product Co. of Norwich, NY, USA in a process that has remained substantially unchanged since 1936 (Vickroy, 1985).

Nitrogenous sources such as malt sprouts, malt extract, corn-steep liquor, barley, yeast extract or undenatured milk supplement most carbohydrate sources to give fast and heavy growth. Some growth promoting substances in these nitrogen sources are sensitive to heat. In commercial practice, minimal amounts of these substances are used in order to simplify the recovery process (Vickroy, 1985).

Additional minerals are occasionally required when the carbohydrate and nitrogenous sources lack sufficient quantities. Calcium carbonate and calcium hydroxide are used to neutralize the acid that is formed (Vickroy, 1985).

Whey is a by-product of cheese production. It is used as a feed-stuff for cattle-breeding, or for production of lactose or can be separated by an ultrafiltration step into high-grade protein and permeate. The permeate contains all the lactose of milk and

cannot be processed by sewage treatment without expense because of its chemical oxygen demand (COD) of 50 kg O_2 /ton permeate. One possibility of reducing the COD is the fermentative conversion of the lactose to lactic acid. Fermentation media such as whey permeate contain proteins and inorganic salts such as calcium phosphates, which are insoluble after sterilization and pH adjustment. Whey permeate is an ultrafiltrate of whey and contains the low molecular weight components of whey, especially lactose, some trace elements and vitamins. *L.casei* did not grow on pure whey permeate (Krischke, 1990).

Whey is the product separated from milk during cheese making and consists of water, lactose (4%-5%), proteins, vitamins, and mineral salts. It is generally a low-priced source of sugars for the fermentation process (Roukas, 1991).

Whey contains lactose, which is an important constituent and can be used by microorganisms during fermentation as a carbon source (Gassem, 2000).

The composition of fresh whey from a conventional cheese production is approximately as shown in Table 2.4.

Table 2.4. Gross composition of liquid and dried whey (Source: Polat, 2002).

COMPONENT	FLUID WHEY	DRIED WHEY
Total solids,%	6.35-7.0	96.3-96.5
Protein,%	0.8-0.9	13.0-75.0
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

However, it is well known that the above mentioned composition changes to a great extent, depending on the type of cheese, use of different starter culture and different rennet type. Further, the milk composition, either due to seasonal variations or breed of cows, changes. This together with the fluctuating quantity available makes the whey problems even more difficult to solve (WEB_3, 2004).

Over the years authors have studied a large number of carbohydrates and nitrogenous materials for production of lactic acid. They have been investigated on the basis of high lactic acid yields, optimum biomass production, negligible by-product

formation, fast fermentation rate, less pre-treatment, easy down stream processing, low cost, ease of availability etc. The choice of the raw material to be used depends on the microorganisms studied and also on the product desired. (Narayanan, 2004).

Sucrose (from syrups, juices and molasses), lactose (from whey), maltose (produced by specific enzymatic starch conversion processes), glucose (from starch conversion processes), mannitol etc. have been commercially used. Molasses are cheap but give low yields of lactic acid and laborious purification procedures. Whey is also cheap and easily available but like molasses have expensive purification processes. These have stimulated the development of modern technologies like ultrafiltration and electrodialysis. Hydrolyzed potato starch, corn, straw, whey, cottonseed hulls, grapefruit, sulphite waste liquor etc. have also been investigated. Studies have also been made for the production of L (+) lactic acid by *R. oryzae* using cornstarch and corncobs in an air-lift bioreactor and fibrous bed bioreactor (Narayanan, 2004).

Studies are also being carried out to develop microbial processes for the production of high purity L (+) lactic acid at low cost from sago starch which is in abundance in Sarawak, Malaysia, Riau and Indonesia. Lactic acid has also been produced by simultaneous saccharification and fermentation of pre-treated alpha fibre. (Narayanan, 2004).

A number of nitrogenous materials like whey permeate, yeast extract, malt sprouts, malt combing nuts, grass extract, peptones, beef extract, casein hydrolysate, corn steep liquor, N-Z-amine, soybean hydrolysate with supplementation of vitamins to supplement carbohydrate sources to give fast and heavy growth have been studied. However, yeast extract seems to be the most effective supplement. Eleven different nitrogen sources were tested. Various amounts of B vitamins were studied to replace yeast extract. These are kept at minimal levels to simplify the recovery process. Additional minerals are occasionally required when the carbohydrate and nitrogenous sources lack sufficient quantities (Narayanan, 2004).

2.4.2.3. Fermentation Processes

Batch fermentations are widely used method for the production of lactic acid. Fermentation conditions are different for each industrial producer but are typically in the range of 45-60 °C with a pH of 5.0-6.5 for *L. delbreuckii* (Inskeep, 1952; Peckham, 1944); 43 °C and a pH of 6-7 for *L. bulgaricus* (Burton, 1937). The acid formed is neutralized by calcium hydroxide or calcium carbonate. The fermentation time is 1-2 days for a 5 % sugar sucrose such as whey and 2-6 days for a 15 % sugar source such as glucose or sucrose. Under optimal laboratory conditions the fermentation takes one to two days. The yield of lactic acid after the fermentation stage is 90-95 wt % based on the initial sugar or starch concentration. The residual sugar concentration is typically less than 0.1 %. The fermentation rate depends primarily on the temperature, pH, concentration of nitrogenous nutrients, and the lactic acid concentration. The undissociated, electroneutral form of lactic acid rather than lactate appears to be the components which inhibits the fermentation (Vickroy, 1985).

To increase volumetric production in lactic acid fermentations, high cell density has been achieved through cell immobilization in gelatin beads (Kaufman, 1999).

CHAPTER 3

IMMOBILIZATION

3.1. Properties of Immobilization

The growing interest in the field of cell immobilization has led to the development of numerous techniques such as dropping, emulsification or coacervation, rotating disc atomization, air jet, atomization, electrostatic dripping, mechanical cutting and the vibrating nozzle technique. Cell, enzyme, or catalyst immobilization has been extensively studied during the last 20 years due to the increasing potential in a wide range of fields, including medicine, food, agrochemistry, environment, cell storage, catalyzed chemical reactions. However, the relative scarcity of industrial processes using cell immobilization highlights the limitations and drawbacks that are implied at large scale. The necessity of finding a matrix able to bear high shear stresses, culture media, or other environmental constraints is usually incompatible with the preservation of cell viability. For example, most immobilization methods based on covalently cross-linked matrices, and hence involving radicals or organic solvents, are detrimental to cells and thus are not suitable for medical or food applications. On the other hand, although many polyelectrolytes, such as calcium alginate gels, are reported to be nontoxic for cells and hence suitable for cell immobilization, they are readily destabilized in the presence of Ca^{2+} complexants or monovalent cations, thus impeding further application. In addition, high-density cultures necessitates that the cells be immobilized in beads with a diameter of less than 300 μm in order to maintain a nonlimiting oxygen concentration throughout the gel matrix. It is difficult to produce monodisperse beads of this size using most of the available immobilization techniques, consequently most of the time, it is simply admitted that there are oxygen limitations which lead to inhomogeneously loaded beads (Serp, 2000).

Immobilized cell technology has been widely applied in a variety of research and industrial applications. The use of immobilized lactic acid bacteria as starter

cultures in the dairy industry is one of the potential applications. While numerous immobilization techniques have been described, entrapment within synthetic or natural polymers remains among the most popular due to its ease and simplicity, low cost and gentle formulation conditions ensuring high retention of cell viability. A popular gel forming material is alginate which is a linear heteropolysaccharide of D-mannuronic acid and L-guluronic acid residues. Sodium alginate may be ionically crosslinked with multivalent cations (typically Ca^{+2}) to form gels (Zhou, 1998).

The most popular approach for whole cell immobilization is gel entrapment, e.g. in Ca-alginate or carrageenan gels (Freeman, 1994).

Immobilization of whole microbial cells has been widely used to achieve high lactic acid production. Carrageenan and calcium alginate are typical of the matrices used for entrapment of viable cells. However, simple entrapment has limitations for processes involving growing cells and where there is product inhibition. Microbial replication may disrupt gel matrices and diffusional restrictions within and outside the gel layer may cause accumulation of product immediately adjacent to the cells. The disadvantage of shrinkage and reduced strength during lactic acid fermentation have been attributed to the displacement of Ca^{+2} ions by lactate ions from alginate beads (Krishnan, 2001).

Among immobilization methods, cell entrapment in gelled biopolymer is commonly used with κ-carrageenan or Ca-alginate as matrix. The main advantage of gel immobilization is the biocompatibility; although large-scale formulation is difficult, the beads are often permeable to cells, mass transfer limitations are often encountered, and the reactor volume occupied by the beads is generally significant. Finally, alginate gels containing lactic acid bacteria tend to be liquefied by lactic acid (Groboillot, 1993).

There are several reports on lactic acid fermentation using immobilized bacteria. Immobilization makes it possible to maintain the cells in a stable and viable state and also provides a means of continuous fermentation. It is natural that organisms in an immobilized state will experience different pH, product concentration in their immediate environment as compared to the bulk solution which may have some effect on the metabolism of the bacteria and hence on the overall process. Therefore, information on the physiology of the microorganism in a modified environment is an essential prerequisite to run a fermentation under optimal conditions (Guoqiang, 1991).

Immobilization commonly is accomplished using a high molecular hydrophilic polymeric gel such as alginate, carrageenan, agarose, etc. In these cases, the cells are

immobilized by entrapment in the pertinent gel by a drop-forming procedure. When traditional fermentations are compared with the microbial conversions using immobilized cells, the productivity obtained in the latter is considerably higher, obviously partly due to high cell density and immobilization-induced cellular or genetic modifications. Nevertheless, a few critical parameters such as the cost of immobilization, mass transport limitations, applicability to a specific end-product, etc. are to be carefully examined before choosing any particular methodology (WEB_3, 2004).

Immobilized systems requires selecting an immobilization method that will limit cell leakage. Among the different immobilization methods, solid supports (polyacrylamide, ceramic, non-woven fabrics or macroporous glass beads) or gel entrapment (in alginate or carrageenan) have been used. The main drawback of gel immobilization is the low mechanical strength of beads, which limits the duration of the working period. Trivalent ions have been proposed and used with good results as hardening agents with the aim of improving the mechanical strength of the beads. On the other hand, high gel concentrations also improve bead stability, even if diffusional limitations appear (Dominguez, 1999).

The use of immobilized whole microbial cells and/or organelles eliminates the often tedious, time consuming, and expensive steps involved in isolation and purification of intracellular enzymes. It also tends to enhance the stability of the enzyme by retaining its natural catalytic surroundings during immobilization and subsequent continuous operation. The ease of conversion of batch processes into a continuous mode and maintenance of high cell density without washout conditions even at very high dilution rates, are few of the many advantages of immobilized cell systems. The metabolically active cell immobilization is particularly preferred where co-factors are necessary for the catalytic reactions. Since co-factor regeneration machinery is an integral function of the cell, its external supply is uneconomical. There is considerable evidence to indicate that the bound-cell systems are far more tolerant to perturbations in the reaction environment and similarly less susceptible to toxic substances present in the liquid medium. The recent reports on higher retention of plasmid-bearing cells have further extended the scope of whole-cell immobilization to recombinant product formation. Another important advantage of immobilization, particularly in the case of plant cells, is the stimulation of secondary metabolite formation and elevated excretion of intracellular metabolites (WEB_3, 2004).

The use of immobilized cell technology to improve lactic acid fermentation processes has been tried by several research workers. *Lactobacillus helveticus*, *Streptococcus salivarius*, *L. delbrueckii* subsp. *bulgaricus*, *L. casei*, *Rhizopus oryzae,* and *Pedioccus halophilus* are the important microorganisms used for the production of lactic acid by immobilized cells. Similar to other microbial fermentations, entrapment in calcium alginate or κ -carrageenan is the method of immobilization widely followed. Since many workers have reported shrinkage and decreased mechanical strength of alginate beads during lactic acid fermentation, Audet (1989) suggested a cell entrapment process using κ -carrageenan and locust bean gum, which significantly modified the mechanical properties of the gel. Using the above mixed gel, several studies were carried out with various lactic acid-producing microorganisms. The κ -carrageenan locust-bean-gum-mixed gel matrix showed significant stability for 3 months in continuous fermentation in a stirred-tank reactor. In another study, Guoqiang (1991) compared the performance of alginate-immobilized *L. casei* cells in stirred-tank and packed-bed reactors. The total productivity of 1.6 g/l/h was achieved in stirred-tank reactor with total utilization of glucose. A novel method of extractive fermentation of lactic acid with immobilized cells has been proposed by few workers. Since the product inhibition could be eliminated by adsorption of lactic acid by selective resin, this method offers higher productivity in bioparticle-continuous fermentation (WEB_3, 2004).

3.2. Advantages of Immobilization

Immobilization process seems to offer mainly the economical advantages. Some advantages of immobilization are given below: (Harsa, 2003).

- Processes can be operated continuously and can be readily controlled.
- Products are easily separated.
- Effluent problems and materials handling are minimized.
- Provides higher purity and product yields, product inhibiton is less apparent.
- Greater pH and thermal stability.

- Continuous operation.
- Greater flexibility in reactor design.

Despite these advantages, industrial application is still limited by:

- Traditional attitudes
- The investment needed for introducing new equipment to already implanted processes.
- The nature and cost of the immobilizing support and the immobilizing process. (including losses of activity.).
- The performance of the system.

3.3. Immobilization Methods

Different methods (attachment or adsorption to a preformed carrier, covalent bonding, entrapment) have been used for immobilizing lactic acid bacteria. The purpose of these techniques is either to retain high cell concentrations within the bioreactor or to protect cells from a hostile environment. For industrial applications in the food industry, the carrier material must be non-toxic, readily available and affordable. It should also lead to high-cell loading and the cells should have a prolonged viability in the support (Doleyres, 2004).

3.3.1. Adsorption

All forms of immobilization in which cells are in some way bound to the surface of a solid support come into the category of adsorption (Dervakos, 1996). The strength of the binding can range from simple van de Waal's forces, through hydrophobic interactions, to strong binding (Rosevear, 1984). This technique is one of the simplest

techniques for cell immobilization and is the method used in both of the oldest industrial immobilized cell systems namely, vinegar production and wastewater treatment. Cells which do not naturally adhere to surfaces can sometimes be encouraged to attach by chemical means such as cross linking by glutaraldehyde, silanization to silica supports or by chelation to metal oxides. In such cases strength of adsorption is similar to natural adhesion (Dervakos, 1996).

Adsorbed cells are in direct contact with the surrounding environment and hence subject to any forces of shear or attrition which may result from the relative motion of particles and fluid. It is therefore likely that some cells will become detached and enter the bulk fluid phase. It is also difficult to control or even determine the depth of the attached biofilm. Neverthless, the technique is cheap and simple and the art is to provide the right surface in a suitable form for colonization by the desired organism or population of organisms in as high a density as possible (Dervakos, 1996).

3.3.2. Covalent bonding

This group of techniques includes the greatest number and variety of published immobilization procedures. This might seem surprising because direct covalent attachment of enzymes to a carrier is often far more difficult to perform and causes greater loss of activity than most other approaches. Furthermore it is seldom appropriate for immobilization of cells, as renewal of the cell wall near the site of attachment can permit release of those live cells which had previously been bound (Rosevear, 1984).

Most of the relevant reactions are derived from carbohydrate and protein chemistry. The great advantage of covalent linkage of enzymes to a support is the permanence of the bond. This ensures that the protein is firmly fixed under all operational conditions, and although highly accessible at the surface of the support, it will not cause contamination of the product. Thus, covalent binding is only justified if the cost and complexity of immobilization is offset by a marked improvement in operational characteristics (Rosevear, 1984).

3.3.3. Entrapment

Entrapment of cells can take place within a variety of porous structures which are either preformed or formed in situ around the cells. In the case of preformed structures the entrapment usually occurs as a natural consequence of cell growth and so, like natural attachment, the effectiveness of the immobilization varies with cell type and support type. Porous structures which are formed in situ, on the other hand, can be used to immobilize almost any type of cell, though the conditions under which the support particle is formed may be harmful to the cells in some cases (Dervakos, 1996).

Entrapment within preformed porous supports can be carried out on a microscopic level with micro-porous particles such as brick, ceramics, sintered glass or kieselguhr (pore entrapment), or on a macroscopic level with particles having relatively large pores (Dervakos, 1996).

The most popular form of cell immobilization currently in use involves the entrapment of cells within porous structures which are formed in situ around the cells. The cells, in the form of a slurry or paste, are generally mixed with a compound which is then gelled to form a porous matrix under conditions sufficiently mild so as not to affect the viability of the cells. The majority of techniques involving in situ entrapment for fermentation purposes make use of polysaccharide gels. Of these, which include κ-carrageenan, agar and alginates, calcium alginate gel is the most popular (Dervakos, 1996).

Gel entrapment provides a controlled means of achieving what is quite a common occurence in nature for certain organisms, e.g. slime forming bacteria. The physical properties of gels are not dissimilar to such slimes but whereas only a few species will form slimes, almost any organism can be immobilized by gel-entrapment. Cell growth within the particles can occur, though if the cell concentration exceeds 30 % v/v the gel will lose its integrity. Calcium alginate gels are unstable in the presence of calcium chelators, such as phosphate, and gas evolution within the gels can also be a cause of particle disruption (Dervakos, 1996).

CHAPTER 4

MATERIAL AND METHODS

4.1. Materials

Whey powder containing 60-62 % (w/w) lactose was obtained from PINAR Dairy Products, Inc., İzmir, Turkey.

Lactobacillus casei NRRL B-441 used throughout this study was kindly supplied by the U.S. Department of Agriculture, National Center for Agricultural Utilization Research. The strain was maintained in litmus milk (Difco, Michigan, USA) and transferred to fresh medium every month. Active cultures for immobilization were grown in MRS Broth (Oxoid, USA) at 37 °C for 24 h.

4.2. Methods

4.2.1. Culture Propagation

25 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. *L.casei* NRRL B-441 was incubated at 37° C for 24 hours in the incubator (Sanyo) and kept at 4°C in the refrigerator. 24 hour old fresh cultures were used as the inoculum for the fermentations.

4.2.2. Pretreatment of Whey

The pH of whey was adjusted to 5.5 with 0.5 N HCl, and distilled with sterile deionized water to desired lactose concentration. After it was sterilized it was used as substrate.

4.2.3. Lactic Acid Fermentations

Batch experiments were performed in a temperature controlled incubator shaker (Lab-Line, USA) operated at 150 rpm, at a temperature of 37 °C. The shake flasks were 250 ml erlenmeyer flasks containing 100 ml of production medium (pH 5.5). Unless otherwise indicated, whey powder was dissolved to attain 145.5 g /l of initial lactose concentration and supplemented with (g/l) yeast extract (10), K_2HPO_4 (0.5), KH_2PO_4 (0.5), $MgSO_4$ (0.2) and $MnSO_4.H_2O$ (0.05). Whey medium (pH=5.5) and salt solutions were sterilized separately at 121^oC for 15 min. Sterile $CaCO_3$ (50 % (w/w) of the initial lactose concentration) was added to the medium to neutralize the acid formed and to maintain the mechanical structure of the gel beads. The shake flasks were inoculated aseptically with 24-hour-old fresh culture propagated in skim milk at 37 °C. In the optimization studies using response surface methodology, the concentrations of initial sugar (lactose), yeast extract and $CaCO_3$ were varied as parameters, while the levels of other medium components were kept constant.

4.2.4. Lactic Acid Production With Immobilized Cells

4.2.4.1. Immobilization in calcium alginate

MRS Broth was used as culture propagation medium for lactic acid bacteria immobilization, lyophilized form of *Lactobacillus casei* NRRL B-441 was activated in this medium. 52.2 g MRS Broth was dissolved in 1 litre distilled water. 25 ml of suspension was poured into 25 ml heat stable glass bottles and were sterilized at 121° C for 15 minutes in an autoclave (Hirayama, Japan).

1 ml of *Lactobacillus casei* NRRL B-441 was transferred into 25 ml sterile MRS Broth solution. MRS is the best medium for growth and lactic acid production. The microorganism was incubated at 37 ° C for 24 hours by 15 days intervals at least in the incubator (Sanyo) and stored in the refrigerator at 4° C. The maintenance of the culture was done by transferring to fresh MRS Broth every 15 days at least. 24-hour-old fresh cultures were used as the inocula for the fermentation experiments.

As shown in Figure 4.1., *L.casei* NRRL B-441 cells grown in 25 ml MRS Broth (Oxoid, USA) were mixed with equal volume (1:1, v/v) of 4 % sodium alginate (Sigma, A-2033, USA) solution. A 50 ml aliquot of alginate-cell suspension containing 2.0 % Na-alginate was added dropwise by a peristaltic pump to a hardening solution of 400 ml containing 2.0 % (w/v) $CaCl_2$, 0.5 % (w/v) chitosan (Alderick, medium MW, USA) and 1.0 % (v/v) acetic acid. Alginate drops solidified upon contact with $CaCl_2$, formed beads and thus entrapped bacterial cells. The beads were allowed to harden for 30 min. The beads were then washed with sterile physiological solution (0.85 % NaCl) to remove excess calcium ions and unimmobilzed cells. Immediately after entrapment the number of living bacterial cells was 2.32 x 10^8 cfu/g bead. To increase the entrapped cell population the beads were incubated overnight in the whey medium at 37 °C and the number of entrapped bacterial cells increased to 2.56 x 10^9 cfu/g bead. The beads were stored at 4 °C in 0.2 % yeast extract solution until use. All steps were carried out under aseptic conditions.

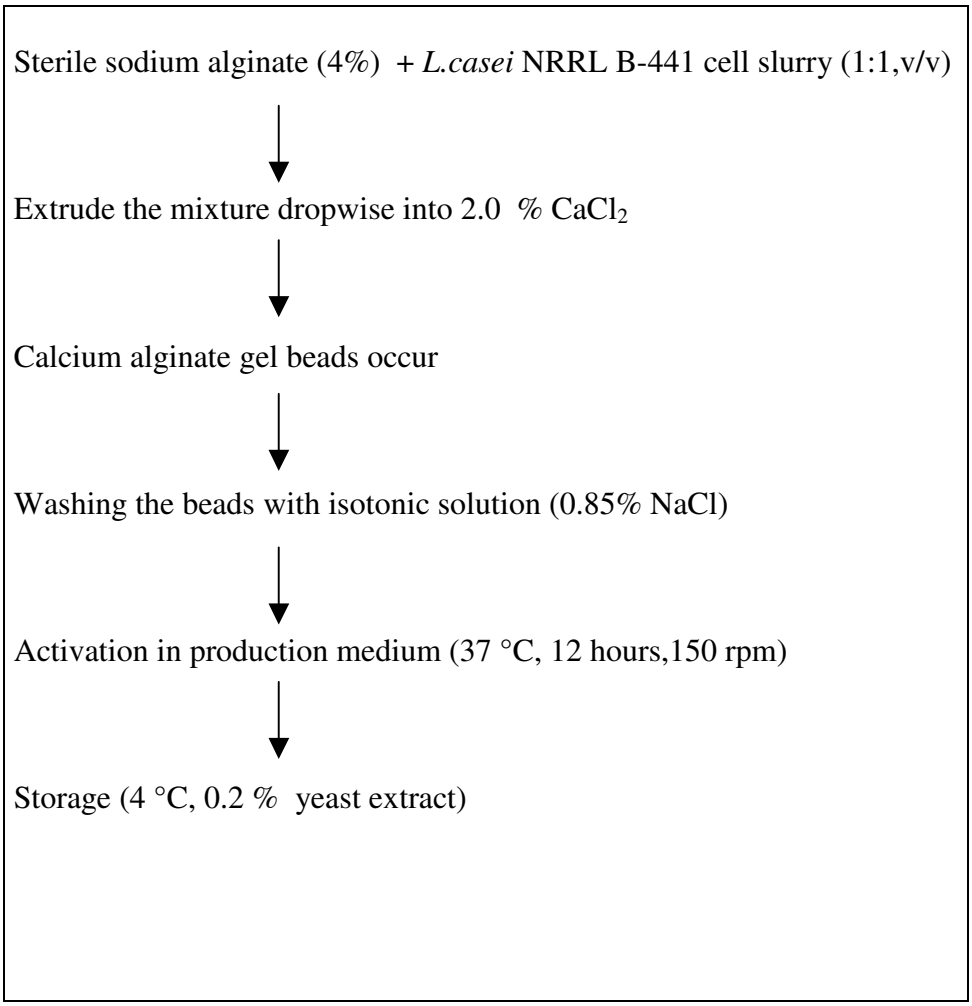


Figure 4. 1. Immobilization in calcium alginate.

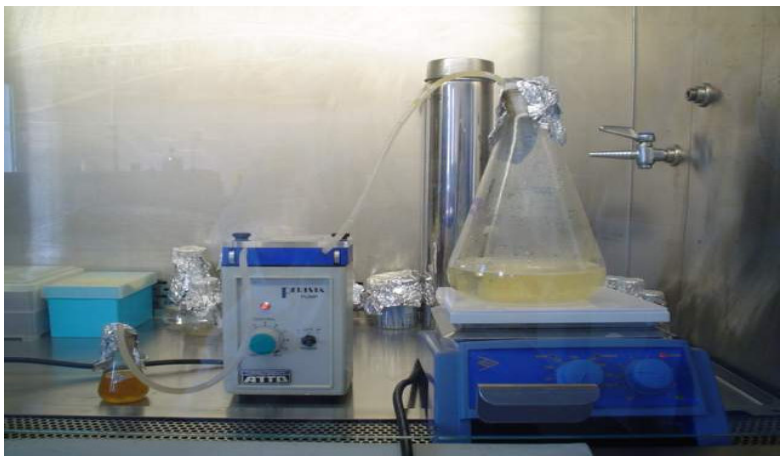


Figure 4.2. Experimental photgraph of immobilization in calcium alginate.

Fermentation kinetics of Ca-alginate-chitosan beads were also compared with the fermentation kinetics of Ca-alginate beads. These beads were prepared with the above mentioned method except that chitosan and acetic acid were not used in the hardening solution.

4.2.4.2. Lactic Acid Bacteria Counting

Calcium alginate beads became soluble according to Champagne (1992). According to this method; liquefaction of Ca-alginate beads was performed by dissolving 1 g of beads in 20 ml, 1.0 % (w/v) sodium citrate ($C_6H_5Na_3O_7.2H_2O$, Riedel de Haen, 25116) solution (pH= 6.0) with continuous stirring for 30 min at room temperature. For determining the concentration of viable cells entrapped in Ca-alginate beads and leaked cells from the gel beads, bacterial counts were done by double plating appropriate dilutions (0.1% peptone) of liquefied beads and fermentation medium on MRS agar (Oxoid, USA) and incubating them at 37 °C for 48 h. Results were reported as colony forming units per gram of beads (cfu/g). This procedure was used to determine the cell numbers released from the beads into the culture medium during fermentation.

For repeated batch fermentation, beads were taken after fermentation and washed with sterile isotonic solution and put into fermentation medium. From here we found how many times we could use the same beads.

4.2.5. Analyses

4.2.5.1. Lactose and Lactic Acid Analyses

Lactose and lactic acid concentrations were analyzed 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 degassing unit was connected to the Helium gas. The mobile phase was 5mM H_2SO_4 for Aminex HPX-87H column. Mobile phase was filtered through 45 μm cellulose acetate filter papers after solution preparation.

1 ml of fermentation sample was taken every hour throughout the fermentation and then centrifuged at 14,000 rpm for 10 minutes in Hettich EBA 12R centrifuge in order to separate the cell mass and other insoluble materials. Supernatants were diluted 10 times to get more precise results from HPLC. All the standard solutions were prepared and dilutions were done with 5 mM H_2SO_4 which was the mobile phase used in HPLC.

The column temperature was maintained at 45°C with a MetaTherm column oven. The Aminex HPX-87H cation exchange column (Bio-Rad Laboratories) was used for HPLC analyses. The isocratic elution was performed with 5 mM H_2SO_4 at a flow rate of 0.6 ml min^{-1} for 15 minutes. The retention times for lactose and lactic acid were around 7.6 and 12.4 minutes, respectively. The calibration curves for lactose and lactic acid are shown in Appendix B. The R squared value of lactic acid calibration curve at HPLC is greater than 0.999.

The parts of the HPLC system used in this study and properties of the column and the analysis conditions are given in Table 4.1 and 4.2.

Table 4.1. The parts of HPLC system (Polat, 2002).

PUMP	PERKIN ELMER SERIES 200
Detector	Series 200 Refractive Index and Diode Array
Interface	Series 900
Computer	Controlling unit of HPLC system
Software	Turbochrom
Column oven	Metatherm
Degassing unit	Helium
Mobile phase reservoirs	4 different containers suitable both for isocratic and gradient elution

Table 4.2. The properties of the column and analysis conditions for HPLC measurements (Polat, 2002).

PROPERTY	SPECIFICATIONS	SPECIFICATIONS
Type of Analysis	Lactose Lactic acid	D(-) Lactic acid L(+) Lactic acid
Retention Time	Lactose : 7.6 min Lactic acid : 12.4 min	D(-) Lactic acid : 5.6 min L(+) Lactic acid : 5.8 min
Column	Aminex HPX-87H ion exclusion Column (Biorad Laboratories)	Nucleosil chiral (Macherey-Nagel)
Column Length	300 mm	250 mm
Column Diameter	7.8 mm	4 mm
Particle Size	9 μm	5 μm
Guard Cartridge	Micro-Guard cation- H cartridge (30 x 4.6)	CC 8/4 nucleosil 100-5 chiral-2/3 G
Mobile Phase	5 mM H_2SO_4	3 mM $CuSO_4.5H_2O$
Flow rate	0.6 ml/min	0.6 ml/min
Temperature	45° C	60 ° C
Detector	Refractive index	Diode array detector
Elution Type	Isocratic Elution	Isocratic Elution

4.2.5.2. Experimental design and statistical analysis

The statistical analysis of the data was performed using Minitab Statistical Software (Release 13.20).

Response surface methodology (RSM) is a collection of mathematical and statistical techniques that are useful for modeling and analysis in applications where a response is influenced by several variables. Response surface methodology (RSM) has been succesfully used to model and optimize biochemistry and biotechnology processes related to food systems. This methodology could be used to optimize media for lactic acid fermentation (Tellez, 2003).

The objective of such an application is to optimize the response. In most RSM problems, the form of the relationship between the response and the independent variables is unknown.The first step in RSM is to find an approximation for the true relationship between the response, y, and the independent variables.

The levels of factors used in the experimental design are listed in Table 4.3. The data of the factors were chosen after a series of preliminary experiments. In this design there were three experimental levels (-1, 0, +1) where –1, 0 and +1 corresponded to low level, mid-level and high level of each variable, respectively. The coded level of each factor was calculated using the following equation (1) :

$$\text{Coded value} = \frac{\text{Actual level} - (\text{high level} + \text{low level}) / 2}{(\text{high level} + \text{low level}) / 2} \quad (1)$$

Table 4.3. Levels of factors used in the experimental design.

		LEVEL		
Factor	Name	-1	0	+1
X_1	Sugar concentration (g / l)	120	150	180
X_2	Yeast extract conc. (g / l)	10	30	50
X_3	$CaCO_3$ conc. (g / l)	30	50	70

Twenty experiments were carried out using a face central statistical design for the study of three factors at three levels (Table 4.4.). The response variable (lactic acid concentration, g /l) was measured using the polynomial response surface model. The second order response function for three quantitative factors is given by eqn (2):

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (2)$$

where X_1, X_2 and X_3 represent the levels of the factors according to Table 4.3. and β_0 , β_1 ,……, β_{23} represent coefficient estimates with β_0 having the role of a scaling constant.

Table 4.4. Experimental design.

RUN	SUGAR CONC. (G / L)	YEAST EXTRACT CONC. (G / L)	$CACO_3$ CONC. (G / L)	LACTIC ACID (G / L)	PREDICTED LACTIC ACID (G / L)
1	150	30	50	131.2	131.3
2	120	50	70	125.6	125.5
3	180	10	70	123.1	123.0
4	150	30	50	131.3	131.3
5	150	30	50	131.5	131.3
6	120	10	70	125.0	125.1
7	150	30	50	131.5	131.3
8	150	30	50	131.2	131.3
9	180	50	70	123.6	123.7
10	120	10	30	120.6	120.5
11	120	30	50	125.0	125.1
12	150	10	50	129.2	129.3
13	180	30	50	123.5	123.5
14	150	30	50	131.3	131.3
15	150	50	50	130.1	130.2
16	150	30	30	128.7	128.9
17	120	50	30	121.6	121.6
18	180	10	30	119.0	119.1
19	150	30	70	132.8	132.8
20	180	50	30	120.5	120.4

CHAPTER 5

RESULTS AND DISCUSSION

5.1. Kinetics of Lactic Acid Production by *Lactobacillus casei* NRRL B-441 Immobilized in Ca-Alginate-Chitosan Beads and Ca-Alginate Beads

L. casei NRRL B-441 immobilized in Ca-alginate-chitosan beads and in Ca-alginate beads was used for lactic acid production from whey containing 145.5 g /l of initial sugar. We can see the experimental results in Table 5.1. and Table 5.2. As seen in Fig 5.1., higher lactic acid concentration (125.6 g /l) was obtained after 72 h of fermentation by *L.casei* NRRL B-441 immobilized in alginate-chitosan beads. With Ca-alginate immobilized cells, 116.2 g /l of lactic acid was produced at the end of 72 h. As expected, the concentration of residual sugars decreased during the fermentation. After 72 h of fermentation, almost complete sugar depletion was observed in both culture media. The residual sugar concentrations in the culture medium with alginate-chitosan beads and alginate beads were 7.0 and 12.0 g /l, respectively. Effective yield values (percentage of lactic acid produced per initial quantity of sugar in the medium) of 86.3 and 79.9 % and conversion yield values (percentage of lactic acid produced per quantity quantity of sugar consumed) of 90.4 and 88.0 % were obtained for alginate-chitosan beads and alginate beads, respectively. Maximum productivity values obtained for alginate-chitosan beads and alginate beads were 3.458 and 2.783 g /l.h, respectively.

Table 5.1. Residual sugar and lactic acid amounts of alginate-chitosan and alginate samples.

	RESIDUAL SUGAR		LACTIC ACID	
Time (h)	Alginate+Chitosan	Alginate	Alginate+Chitosan	Alginate
0	145.5	145.5	0	0
8	115.6	126.4	24.7	16.8
16	92.4	109.3	42.2	28.3
24	53.7	66.7	83.0	66.8
32	38.3	48.3	94.5	81.3
40	21.5	34.2	106.6	92.2
48	16.4	24.0	115.3	101.3
56	13.0	18.0	119.3	108.0
64	8.0	14.0	124.0	115.0
72	7.0	12.0	125.6	116.2

Table 5.2. Conversion yield and effective yield amounts of alginate-chitosan and alginate samples.

CONVERSION YIELD		EFFECTIVE YIELD	
Alginate+Chitosan	Alginate	Alginate+Chitosan	Alginate
82.6	88.0	17.0	11.5
79.5	78.2	29.0	19.5
90.4	84.8	57.0	45.9
88.2	83.6	64.9	55.9
86.0	82.8	73.3	63.4
89.3	83.4	79.2	69.6
90.0	84.7	82.0	74.2
90.2	87.5	85.2	79.0
90.7	87.0	86.3	79.9

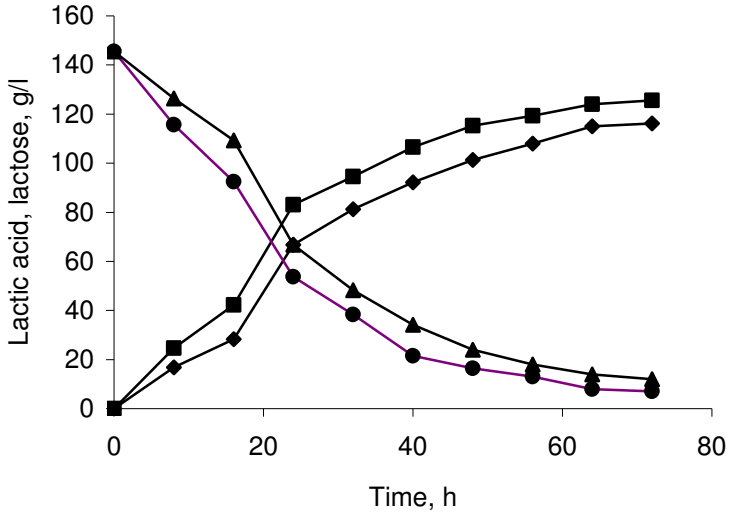


Figure 5.1. Kinetics of lactic acid production and lactose consumption by *L.casei* NRRL B-441 immobilized in alginate-chitosan beads (■ lactic acid; ● lactose) and alginate beads (◆ lactic acid; ◆ lactose) (T= 37 °C, pH=5.5 , initial substrate concentration = 145.5 g/l).

5.2. The Determination of Cell Leakage From Calcium-Alginate Beads With and Without Chitosan

One advantage of immobilized cells is that they can be used more than one time. To determine how much we could use the same calcium alginate beads containing immobilized *Lactobacillus casei* NRRL B-441 cells , repeated batch fermentations had been done. For this purpose lactic acid was produced using Ca-alginate beads under optimum conditions which were 145.5 g/l lactose containing whey, 37° C and pH 5.5. After each fermentation, beads were taken, washed with sterile distilled water and put into new fermentation medium.

Total lactic acid bacteria using MRS agar was counted. 1 ml of sample was added into sterile petri dishes and 20 ml of sterile melted (50 °C) MRS agar was poured onto it. After it was cooled, 10 ml of sterile MRS agar was poured again. Inverted

plates were incubated at 37 °C for 48 hours. All colonies were counted from incubated MRS agar. The number of lactic acid bacteria was calculated multiplying by dilution factor and dividing by inoculation amount.

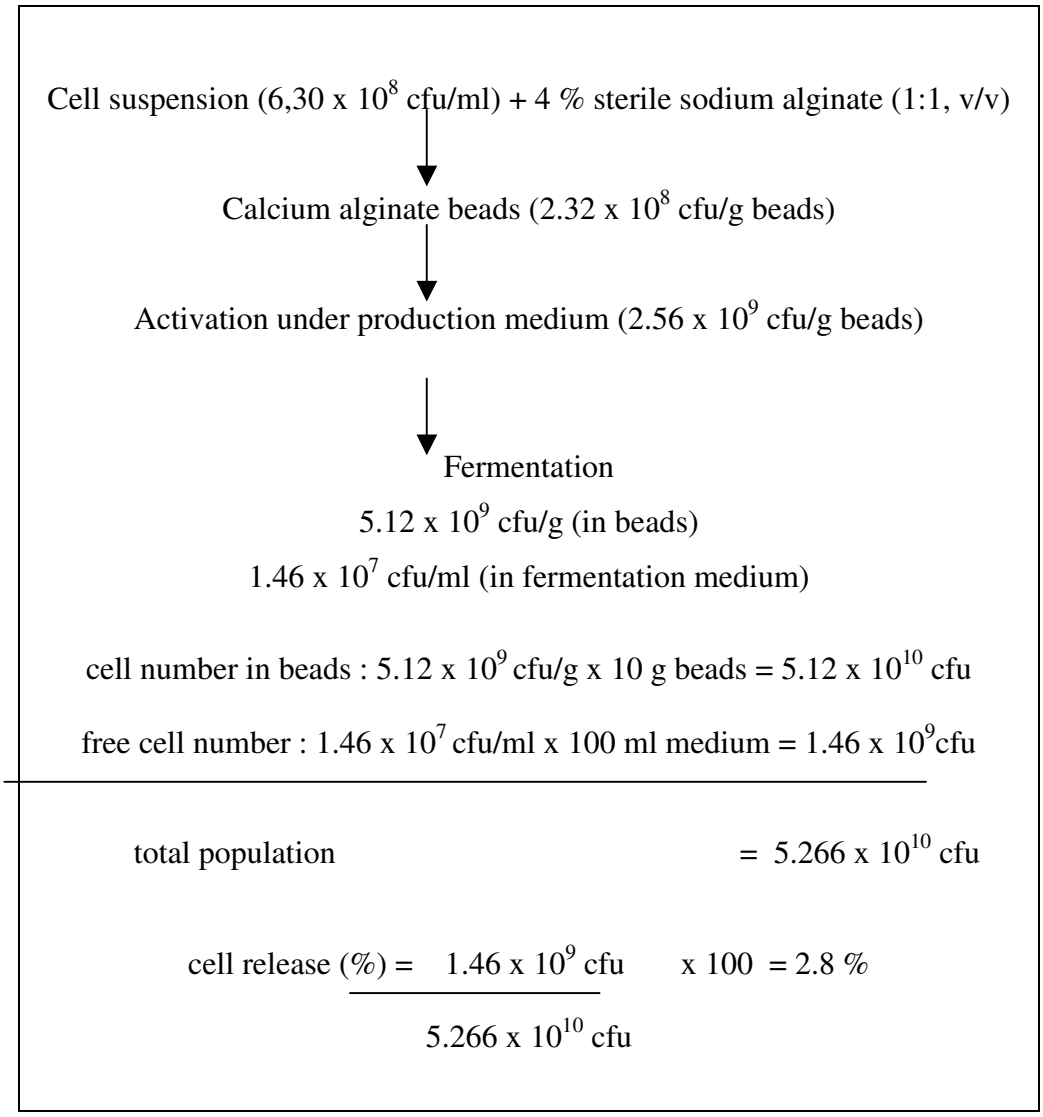


Figure 5.2. Cell release from beads into the fermentation medium with chitosan

As we can see from Figure 5.2., cell suspension contained 6,30 x 10^8 cfu/ml *Lactobacillus casei* NRRL B-441 cells. This suspension was mixed with 4 % sterile sodium alginate solution. After mixing, calcium alginate beads were formed which contained 2.32 x 10^8 cfu/g beads. Activation was performed under production medium and cell number increased to 2.56 x 10^9 cfu/g beads. After fermentation, cell number in

beads was 5.12 x 10^9 cfu/g and in fermentation medium cell number was 1.46 x 10^7 cfu/ml. Cell number in beads were calculated by multiplying the cell number in beads with 10 g beads. Free cell number was calculated by multiplying the cell number in fermentation medium with 100 ml medium. The results were added and total population was calculated. By dividing free cell number to total population, cell release was found which was 2.8 %.

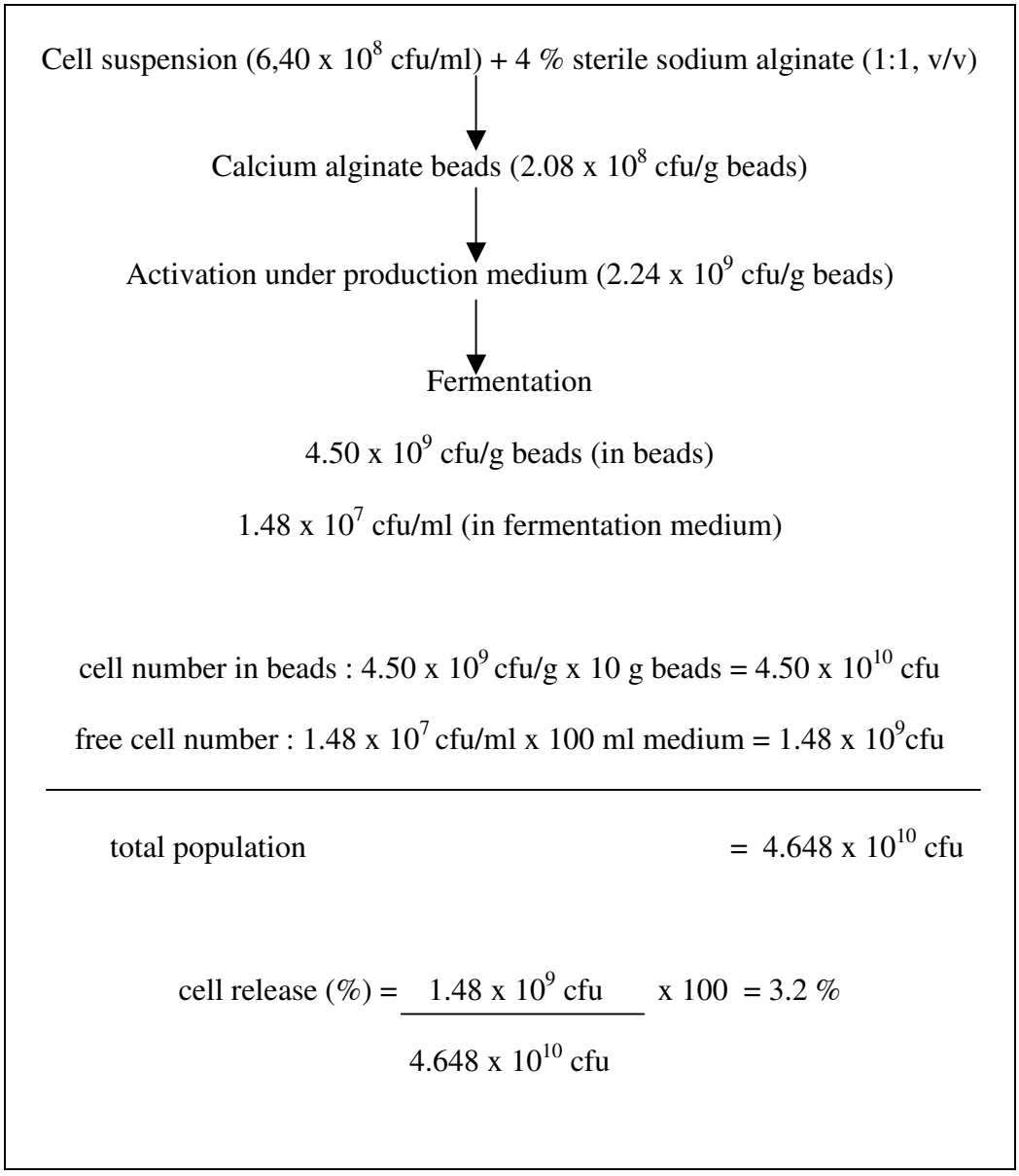


Figure 5.3. Cell release from beads into the fermentation medium without chitosan.

As we can see from Figure 5.3., cell suspension contained 6,40 x 10^8 cfu/ml *Lactobacillus casei* NRRL B-441 cells. This suspension was mixed with 4 % sterile

sodium alginate solution. After mixing, calcium alginate beads were formed which contained 2.08 x 10^8 cfu/g beads. Activation was performed under production medium and cell number increased to 2.24 x 10^9 cfu/g beads. After fermentation, cell number in beads was 4.50 x 10^9 cfu/g and in fermentation medium cell number was 1.48 x 10^7 cfu/ml. Cell number in beads were calculated by multiplying the cell number in beads with 10 g beads. Free cell number was calculated by multiplying the cell number in fermentation medium with 100 ml medium. The results were added and total population was calculated. By dividing free cell number to total population, cell release was found which was 3.2 %.

Cell leakage in lactic acid production by immobilized lactic acid bacteria was observed by various researchers. In this study, the number of leaked cells in the fermentation medium comprised 3.2 % of the total bacterial population with Ca-alginate beads. Slightly lower cell leakage (2.8 % of the total bacterial population) was observed with Ca-alginate-chitosan beads. These results are in agreement with those of Zhou (1998) who found that the cell release from uncoated alginate beads was greater than that for chitosan coated beads, indicating that the chitosan membrane did have a significant impact on reducing initial cell release from beads.

Our results showed that immobilizing lactic acid bacteria in Ca-alginate beads coated with chitosan have advantages of obtaining higher lactic acid concentration with slightly lower cell leakage from the beads. Higher fermentation capacity of alginate-chitosan beads is attributed to lower cell leakage from the beads.

The mechanism of cell release has been studied recently (Arnaud 1992; Champagne 1994). Cell growth often occurs as colonies in the small cavities close to the surface region of the gel (Audet 1988). Due to the force exerted by cell growth and shear forces resulting from mixing, the gel cavities containing the microcolonies are disrupted and cell release occurs. Cachon (1995) also demonstrated that 95 % of *L.lactis* colonized a 150 μm radius around the periphery of alginate beads at steady state, contributing to cell release through cell growth. Cell release occurs on the surface of the beads and is unrelated to the bead pore or cell size (Zhou, 1998).

Various methods have been proposed to reduce release of lactic acid bacteria from alginate beads. Champagne (1992) rinsed alginate beads containing immobilized lactic acid bacteria with distilled water, ethanol, $Al(NO_2)$ or hot $CaCl_2$ solutions before fermentation to kill bacteria near the bead surface. Another approach was the application of multiple coats of poly L-lysine and alginate, which reduced the final

concentration of free cells by a factor of 10 in milk fermentations. Yet another approach was demonstrated by Zhou (1998), who investigated the use of chitosan in reducing cell release. Chitosan has been shown to interact with alginate, and an outer membrane of chitosan-alginate is formed when alginate beads are treated with chitosan (Sanford, 1987). Zhou (1998) reported that the final number of free cells was reduced in repeated 2-h batch fermentations of milk with chitosan-coated alginate beads, compared with fermentations with uncoated beads (Klinkenberg, 2001).

5.3. Effect of Initial Sugar Concentration on Lactic Acid Production by Chitosan-Alginate Immobilized *Lactobacillus casei* NRRL B-441

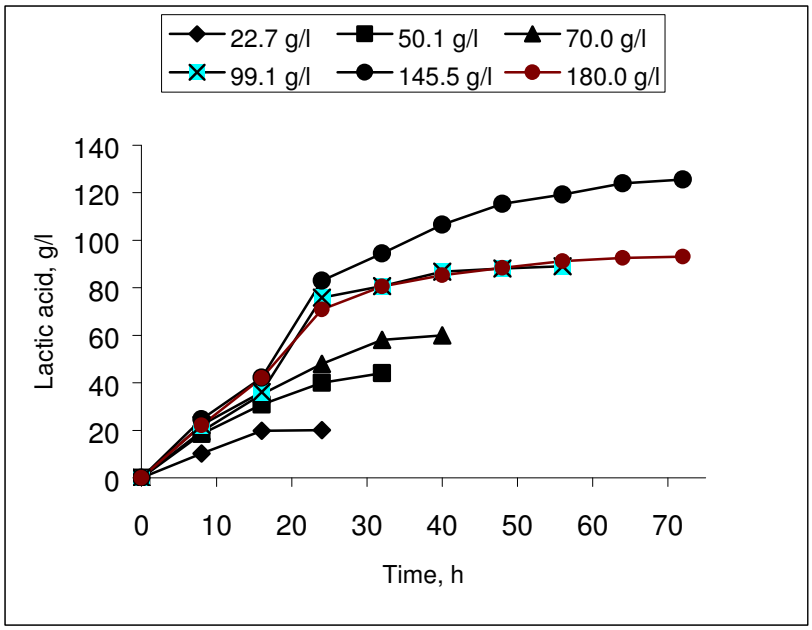
In order to determine the effect of lactose concentration on the final concentration of lactic acid produced by lactic acid bacteria entrapped in alginate-chitosan beads, diluted whey containing 22.7, 50.1, 70.0, 99.1, 145.5 and 180.0 g /l of lactose were used. Fermentation was performed in shake flasks at 37 °C and pH 5.5. Results were given in Table 5.3. As seen in Fig 5.4. , the final lactic acid concentration increased with the increase of initial sugar concentration up to 145.5 g /l but then significantly decreased beyond this value. The highest concentration of lactic acid (125.6 g /l) was obtained after 72 h with an initial sugar concentration of 145.5 g /l. The lactic acid concentrations obtained in whey medium containing 22.7, 50.1, 70.0, 99.1 and 180.0 g /l of lactose were 20.1, 44.1, 60.0, 89.0 and 93.1 g /l. The maximum lactic acid productivity values obtained for 22.7, 50.1, 70.0, 99.1, 145.5 and 180.0 g /l of initial sugar concentrations were 1.238, 2.313, 2.438, 2.788, 3.458 and 2.954 g /l.h, respectively. The corresponding effective yield values were 88.5, 79.8, 85.7, 89.8, 86.3, 51.7 and conversion yield values were 88.5, 88.0, 87.6, 89.8, 90.4, 74.4 for the given sugar concentrations. Effective yield gave a peak at a initial sugar concentration of 99.1 g /l and conversion yield gave a peak at 145.5 g /l.h. When the initial sugar concentration exceeded 145.5 g /l, yield values decreased due to inhibition produced by

high sugar concentration. Substrate inhibition in lactic acid production was also reported by other authors.

As shown in Figure 5.4., the concentration of residual sugars continuously declined during fermentation following an inverse trend to that of lactic acid concentration. Increasing the initial sugar concentration from 145.5 to 180 g/l resulted in a significant increase of residual sugar concentration. This was expected since there was also a decrease in lactic acid concentration. Kotzamanidis (2002) studied lactic acid production from beet molasses by *Lactobacillus delbrueckii* and stated that the increase in residual sugars by the increase of initial sugar concentration in the medium was due to the inability of microorganism to metabolize high levels of sugars.

Table 5.3. Lactic acid and residual sugar amounts of samples according to hours.

LACTIC ACID						
Time,h	**22.7 g/l**	**50.1 g/l**	**70.0 g/l**	**99.1 g/l**	**145.5 g/l**	**180.0 g/l**
0	0	0	0	0	0	0
8	10,3	18,5	19,5	22,3	24,7	22,1
16	19,8	30,9	35,2	36,1	42,2	42,1
24	20,1	40	48	75,9	83	70,9
32		44,1	58,1	80,7	94,5	80,6
40			60	86,8	106,6	85,3
48				88,1	115,3	88,4
56				89	119,3	91,2
64					124	92,6
72					125,6	93,1
80						
RESIDUAL SUGAR						
Time,h	**22.7 g/l**	**50.1 g/l**	**70.0 g/l**	**99.1 g/l**	**145.5 g/l**	**180.0 g/l**
0	22,7	50,1	68,6	99,1	145,5	180
8	6	27,8	47,7	73,5	115,6	150,3
16	0	14,3	25,12	56,8	92,4	120,5
24		4,1	15,2	12,5	53,7	77,6
32		0	3	7,3	38,3	58,2
40			0	1,8	21,5	50,8
48				0	16,4	47,9
56					13	42,7
64					8	41
72						36,8
80						35,8



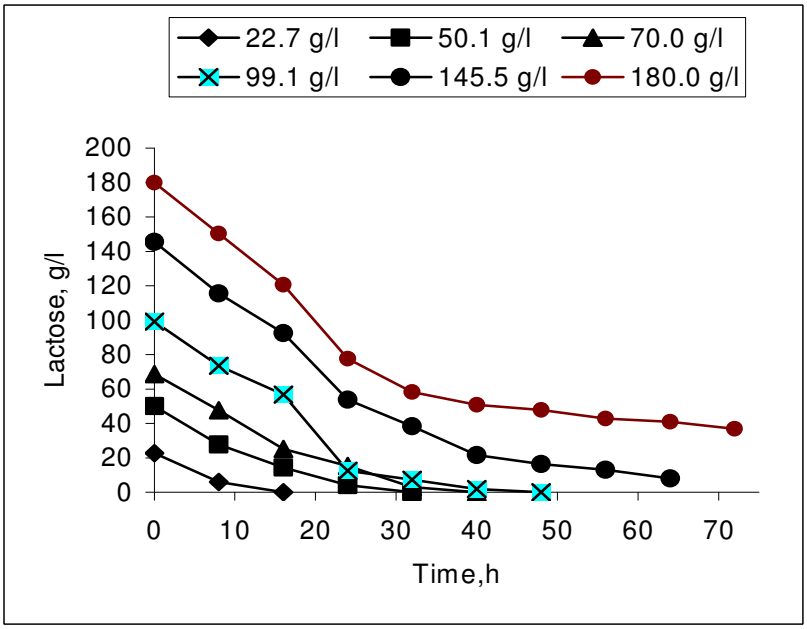


Figure 5.4. Kinetics of lactic acid production and lactose consumption by *L.casei* NRRL B-441 immobilized in alginate chitosan beads using different initial lactose concentrations.

5.4. Effect of Sodium-Alginate Concentration on Lactic Acid Production by Chitosan-Alginate Immobilized *Lactobacillus casei* NRRL B-441

L.casei NRRL B-441 was immobilized in alginate-chitosan beads prepared from different concentrations of Na-alginate (1.0, 2.0, 3.0 % w/v) and lactic acid production was investigated in whey medium containing 145.5 g /l lactose initially as shown in Table 5.4. The fermentation was carried out for 72 h. As seen in Fig 5.5., similar lactic acid concentrations were obtained for Na-alginate concentrations of 1.0 and 2.0 %. Above 2.0 % Na-alginate concentration, lactic acid production decreased due to lower diffusion efficiency of the beads. Maximum lactic acid production (127.8 g /l), effective yield (87.8 %) and conversion yield (93.0 %) were obtained with beads prepared from 1.0 % Na-alginate. However, beads prepared from 1.0 % Na-alginate were soft and highly susceptible to compaction and disintegration during lactic acid production and most of the beads disrupted in the medium at the end of fermentation. This result is in agreement with that of Göksungur (1999) who produced lactic acid from beet molasses by Ca-alginate immobilized *L.delbruecki* and found that beads prepared from 1.0 and 1.5 % Na-alginate disrupted in the medium due to their soft structure. Abdel (1992) investigated lactic acid production by calcium alginate immobilized *L.lactis* and determined the maximum lactic acid production with beads containing 3.0 % Ca-alginate. They obtained lower yields with beads made of 4.0 and 5.0 % alginate due to diffusion problems.

Table 5.4. Na-alginate percentage and lactic acid amounts.

NA-ALGINATE	LACTIC ACID
1.0 %	127.8 g/l
2.0 %	125.6 g/l
3.0 %	113.5 g/l

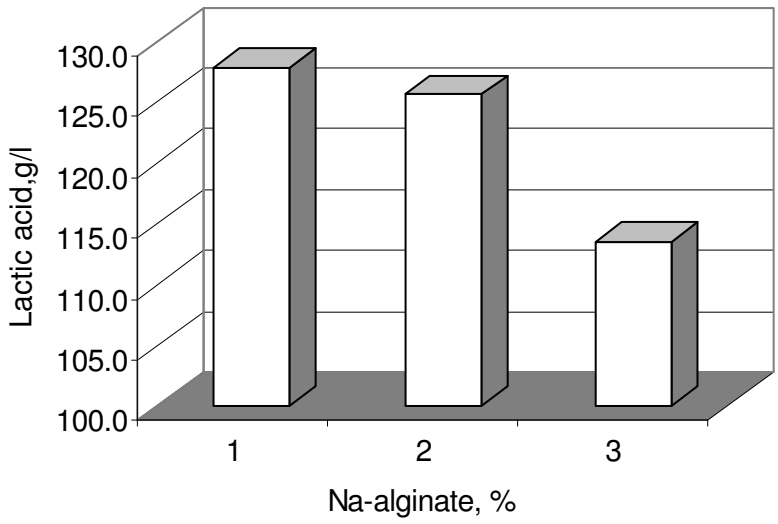


Figure 5.5. Effect of Na-alginate concentration on lactic acid production by *L.casei* NRRL B-441 immobilized in alginate-chitosan beads (T=37 °C, pH=5.5 ,initial substrate concentration = 145.5 g/l, bead diameter = 2.0-2.4 mm).

5.5. Effect of Bead Diameter on Lactic Acid Production by Chitosan-Alginate Immobilized *Lactobacillus casei* NRRL B-441

The effect of bead diameter (1.3-1.7 mm, 2.0-2.4 mm, 2.8-3.2 mm) on lactic acid production was investigated using alginate-chitosan gel beads containing 2.0 % Na-alginate as seen in Table 5.5. As seen in Figure 5.6. , the highest lactic acid (131.2 g /l) was obtained with cells entrapped in 1.3-1.7 mm beads. Lactic acid concentrations obtained with 2.0-2.4 and 2.8-3.2 mm beads were 125.6 and 111.4 g /l, respectively. The highest effective yield (90.2 %) and conversion yield (95.4 %) was obtained with 1.3-1.7 mm alginate-chitosan beads.

Smaller beads yielded more lactic acid due to an increase in surface-volume ratio. A gradual increase in bead diameter beyond 2.4 mm resulted in a gradual decrease in lactic acid production due to development of a mass transfer barrier. Moreover, limited nutrient availability inside the beads was probably another factor that decreased

fermentation efficiency of larger diameter alginate-chitosan beads. Goksungur (1999) and Abdel (1992) obtained maximum lactic acid production with cells entrapped in Ca-alginate beads with a bead diameter of 2.0-2.4 mm and 2.0-2.2 mm, respectively.

Table 5.5. Bead diameter size and lactic acid percentage.

BEAD DIAMETER	LACTIC ACID
1.3-1.7 mm	131.2 g/l
2.0-2.4 mm	125.6 g/l
2.8-3.2 mm	111.4 g/l

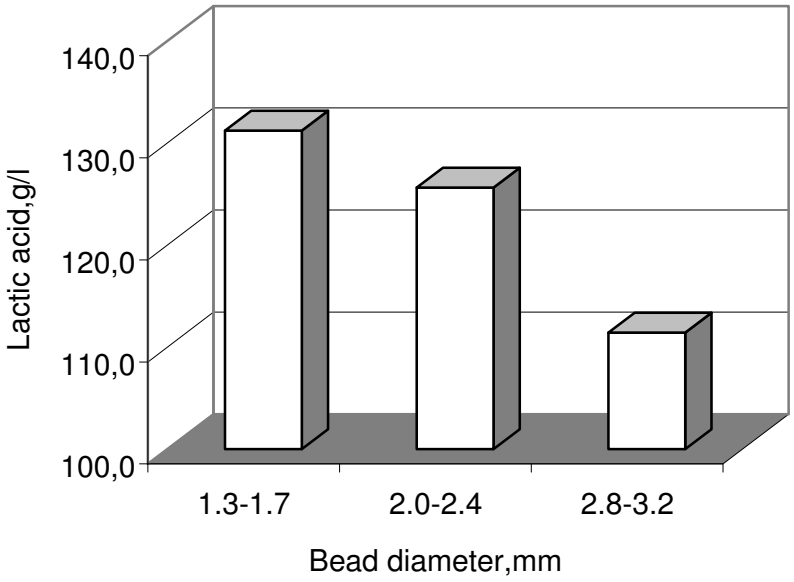


Figure 5.6. Effect of bead diameter on lactic acid production by *L.casei* NRRL B-441 immobilized in alginate-chitosan beads (T= 37 °C, pH= 5.5, initial substrate concentration= 145.5 g/l, Na-alginate concentration = 2.0 %).

5.6. Production of Lactic Acid by Chitosan-Alginate Immobilized *Lactobacillus casei* NRRL B-441 Using Repeated Batch Fermentations

Repeated batch fermentations were performed with *L.casei* NRRL B-441 immobilized in 1.3-1.7 mm alginate-chitosan beads to investigate the possibility of reusing the gel beads as shown in Table 5.6. The fermentation was carried out for 72 h in whey medium containing 150 g /l lactose. At the end of each run, the beads were washed with sterile physiological saline and transferred to fresh medium.

As shown in Fig 5.7., alginate-chitosan immobilized cells were reused successfully for 10 continuous runs without marked activity loss. The highest lactic acid (131.7 g /l) was obtained in the third run and a gradual decrease was observed in lactic acid production after the 8^{th} run. Shrinkage, deformation and small cracks in the surface of beads was observed in the last two runs and the beads lost their hardness and completely disrupted in the medium at the end of 11^{th} run. Lactic acid production in the 11^{th} run was thought to result from the activity of free cells in the medium.

Table 5.6. Lactic acid, lactose, conversion yield and effective yield values obtained by Ca-alginate entrapped *L.casei* NRRL B-441.

			CONVERSION	EFFECTIVE
Batch no	Lactic acid	Lactose	YIELD,%	YIELD,%
1	129.10	7.60	90.66	86.07
2	130.10	6.10	90.41	86.73
3	131.70	9.40	93.67	87.80
4	130.30	11.00	93.74	86.87
5	127.30	12.70	92.72	84.87
6	124.10	16.40	92.89	82.73
7	121.30	17.60	91.62	80.87
8	116.50	21.00	90.31	77.67
9	106.30	27.20	86.56	70.87
10	97.90	35.10	85.20	65.27
11	68.00	62.70	77.89	45.33

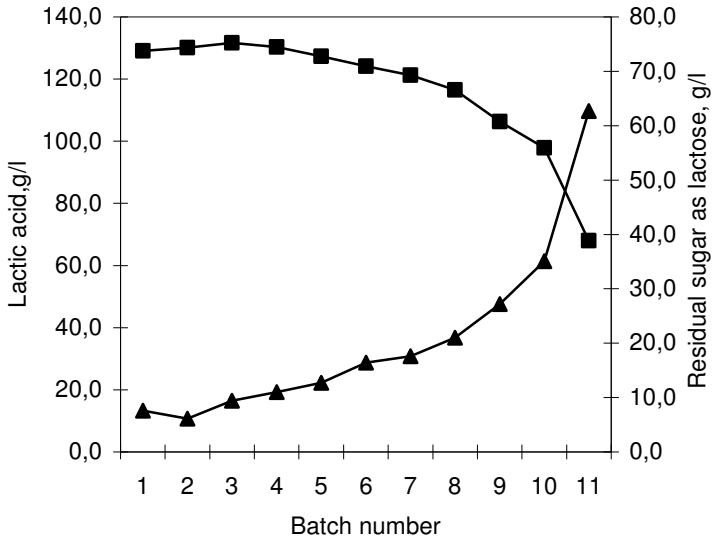


Figure 5.7. Lactic acid and residual sugar concentrations during repeated batch fermentation of whey by alginate-chitosan entrapped *L.casei* NRRL B-441 (■ lactic acid; ▲ , lactose) (T= 37 °C, pH= 5.5, initial substrate concentration= 150 g/l, Na-alginate concentration = 2.0 %, bead diameter = 1.3-1.7 mm).

Audet (1989), have reported that alginate gels were not resistant to the growth of lactic acid bacteria due to the decalcification of the gel by lactic acid produced and this results in the deformation of the gel beads. In this study $CaCO_3$ which was used for neutralization of lactic acid, helped maintain the mechanical structure of the alginate beads which were used for up to 10 batch fermentations without significant deformation.

Ca-alginate beads were also reused successfully 12 times by Roukas and Kotzekidou (1991), 15 times by Guoqiang (1991) and eight times by Abdel (1992).

5.7. Optimization of Lactic Acid Production by Response Surface Methodology

Providing amino acids, 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. $CaCO_3$ is another important factor in lactic acid fermentation since it neutralizes the acid formed preventing its inhibitory effect on the cell growth and helps to maintain the mechanical structure of alginate gel beads in immobilized cells. Thus, response surface methodology was used to determine the optimum concentrations of initial sugar, yeast extract and $CaCO_3$ leading to maximum lactic acid production. The effect of the three previously mentioned variables, each at three levels and their interactions on lactic acid production has been determined using a face centered design. Analysis of variance (ANOVA) for the concentration of lactic acid is presented in Table 5.7. The analysis gives the value of the model and determines the requirement of a more complex model with a better fit. If the *F*-test for lack of fit is significant, then a more complicated model is needed. As shown in Table 5.7. **,** R^2 was 0.999 which indicated that the model as fitted explained 99.9 % of the variability in lactic acid concentration. *F*-test for regression was significant at a level of 5 % ($P < 0.05$). Also, the lack of fit was not significant at the 5 % level ($P > 0.05$). These results show that the model chosen can satisfactorily explain the effects of initial sugar, yeast extract and $CaCO_3$ concentrations on lactic acid production by *L. casei* NRRL B-441 using whey. The following model was fitted for lactic acid concentration :

$$Y = - 52.019 + 2.296\,X_1 + 0.26698\,X_2 + 0.2524\,X_3 - 0.007711\,X_1^2 - 0.003911\,X_2^2 - 0.00105\,X_3^2 - 0.0002408\,X_1X_3 - 0.000426\,X_2X_3 \quad (1)$$

where X_1, X_2 and X_3 are the actual levels of factors shown in Table 4.3. Table 5.8. shows that initial sugar, yeast extract and $CaCO_3$ concentrations have a strong positive linear effect on the response which is lactic acid concentration ($P << 0.05$). There were also significant negative quadratic effects of the above factors, indicating that lactic acid concentration increases with increase in these parameters, but decreases as the above parameters are increased at high levels. Additionally, significant negative interaction effect was observed between initial sugar × $CaCO_3$ and yeast extract × $CaCO_3$. This indicated that lactic acid concentration increased with the increase in these parameters; they reached a maximum and then decreased at high levels of the given factors. Finally, no significant interaction effect ($P > 0.05$) was noted between initial sugar × yeast extract and hence this coefficient (X_1X_2) was omitted in eqn (1).

Figures 5.8 , 5.9 and 5.10 show the contour plots of lactic acid concentration for each pair of factors whereas the third factor was kept constant at its middle level. Figure 5.8 shows that lactic acid concentration increased with the increase in initial substrate and $CaCO_3$ concentrations and further increases in the above factors resulted in a decrease in lactic acid concentration. As shown in Fig 5.9 , the maximum concentration of lactic acid was observed around 32 g/l of yeast extract and 146 g/l of initial sugar concentrations. Moreover, for the same levels of yeast extract or initial sugar concentrations, the concentration of lactic acid decreased from the middle to high yeast extract or initial sugar levels. Finally, Fig 5.10 shows how lactic acid production by alginate-chitosan immobilized *L. casei* NRRL B-441 varies with yeast extract and $CaCO_3$ concentrations at a fixed initial sugar concentration (150 g /l). As can be seen from Fig 5.10, lactic acid concentration increased with the increase in yeast extract and $CaCO_3$ concentrations in a certain range and then fell at the extreme low or high levels of these factors. In order to determine maximum lactic acid concentration corresponding to the optimum levels of initial sugar, yeast extract and $CaCO_3$ concentrations, a second order polynomial model was used to calculate the values of these variables. The fitting of the experimental data to eqn (1) allowed the determination of the levels of initial sugar concentration (X_1=147.35 g /l), yeast extract concentration (X_2 = 28.81 g /l) and calcium carbonate concentration (X_3 = 97.55 g/l) giving a maximum lactic acid concentration of 133.31 g/l. The above data optimizes lactic acid production from whey by alginate-chitosan immobilized *L casei* NRRL B-441.

The final fermentation was performed in whey medium by alginate-chitosan immobilized *L.casei* NRRL B-441 with the optimized levels of initial sugar concentration (147.35 g /l), yeast extract concentration (28.81 g /l) and $CaCO_3$ concentration (97.55 g /l) given by the model. The alginate-chitosan beads (1.3-1.7 mm) were prepared from 2.0 % Na-alginate. The experiments were performed in triplicate and lactic acid concentration was expressed as the mean of three experiments. Maximum lactic acid concentration (136.3 g/l) which was slightly higher than the value given by the model was obtained at the 72^{nd} hour of fermentation. The maximum lactic acid productivity (3.708 g /l.h) was obtained at the 24^{th} hour of fermentation.

The analysis was done using uncoded units.

Table 5.7. Analysis of variance for lactic acid concentration (R^2 = 0.999).

SOURCE	D F	SEQ SS	ADJ SS	ADJ MS	F	P
Regression	9	388.410	388.410	43.157	2000	0.000
Linear	3	47.497	172.485	57.495	2000	0.000
Square	3	340.497	340.497	113.499	4000	0.000
Interaction	3	0.416	0.416	0.139	5.06	0.022
Residual error	10	0.274	0.274	0.027		
Lack of fit	5	0.170	0.170	0.034	1.63	0.303
Pure error	5	0.104	0.104	0.021		
Total	19	388.684				

Table 5.8. Estimated regression coefficients for lactic acid concentration.

TERM	COEFFICIENT	SE COEFFICIENT	T	P
Constant	-52.019	2.40071	-21.667	0.000
Sugar conc.	2.29604	0.03381	67.918	0.000
Yeast extract conc.	0.266982	0.02233	11.954	0.000
$CaCO_3$ concentration	0.252404	0.02938	8.591	0.000
Sugar × Sugar	-0.007711	0.00011	-69.515	0.000
Yeast ext. × Yeast ext.	-0.003911	0.00025	-15.671	0.000
$CaCO_3 \times CaCO_3$	-0.001049	0.00025	-4.202	0.002
Sugar × Yeast ext.	0.000076	0.00010	0.782	0.453
Sugar × $CaCO_3$	-0.0002408	0.00010	-2.469	0.033
Yeast ext. × $CaCO_3$	-0.000426	0.00015	-2.913	0.015

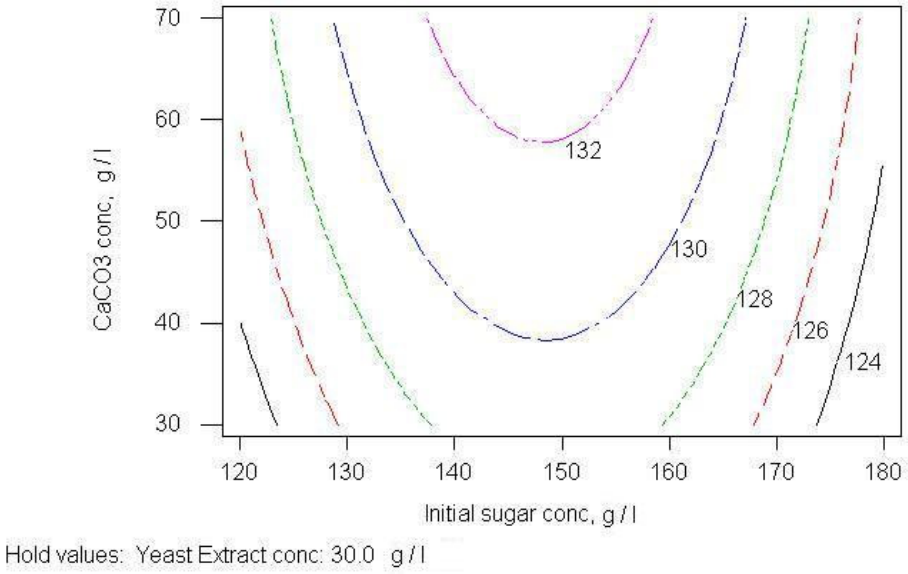


Figure 5.8. Contour plot for lactic acid concentration at varying concentrations of $CaCO_3$ and initial sugar concentration at a constant (30 g/l) yeast extract concentration.

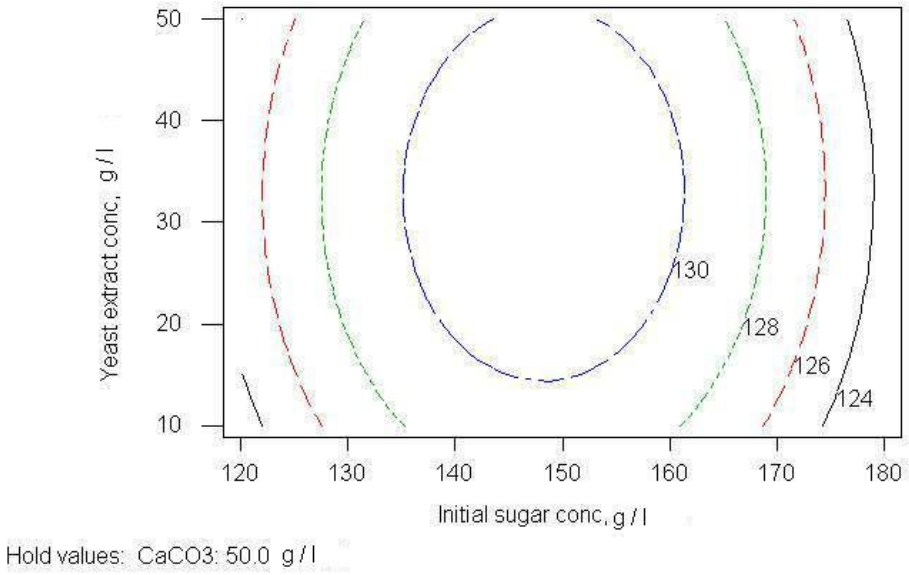


Figure 5.9. Contour plot for lactic acid concentration at varying concentrations of yeast extract and initial sugar concentration at a constant (50 g/l) $CaCO_3$ concentration.

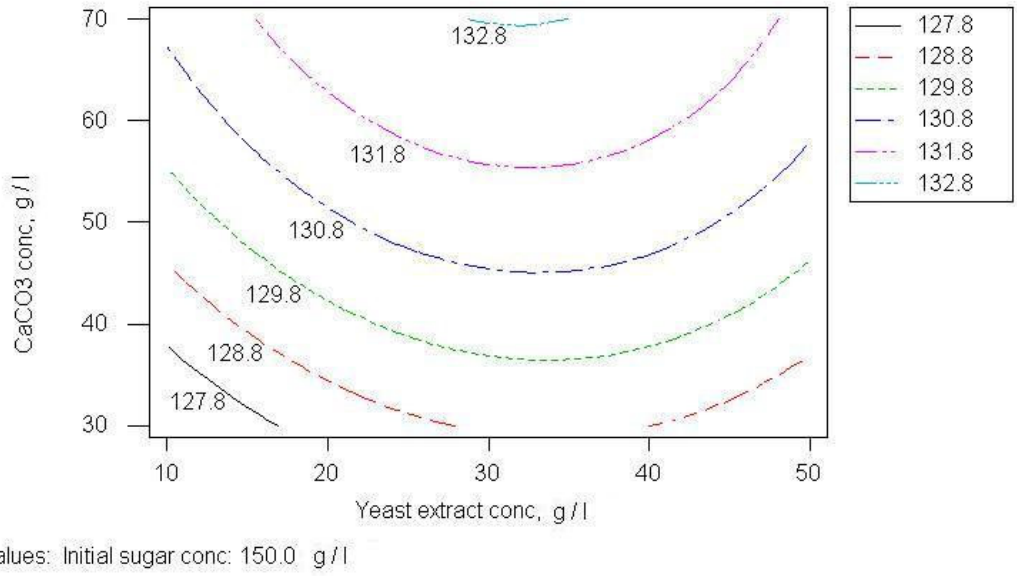


Figure 5.10. Contour plot for lactic acid concentration at varying concentrations of $CaCO_3$ and yeast extract concentration at a constant (150 g/l) initial sugar concentration.

CHAPTER 6

CONCLUSION

In this study lactic acid was produced from whey by *L.casei* NRRL B-441 immobilized in chitosan stabilized Ca-alginate beads. Coating of Ca-alginate beads with chitosan stabilized the beads leading to a higher lactic acid production and lower cell leakage compared with uncoated Ca-alginate beads. Optimum fermentation activity was obtained with 1.3-1.7 mm diameter alginate-chitosan beads prepared from 2.0 % Na-alginate. Alginate-chitosan beads were used consecutively for 10 runs without marked activity loss in repeated batch fermentation studies.

The application of experimental designs to optimize production of lactic acid by immobilized bacteria has not been explored so far. Thus, response surface methodology was used to determine effects of three important factors (initial sugar, yeast extract and calcium carbonate concentrations) on lactic acid fermentation from whey. Linear, quadratic and interaction effects of these variables on lactic acid production were determined. The model generated in this study by RSM satisfied all the necessary arguments for its use in optimization. By fitting the experimental data to a second order polynomial equation, the optimum levels of above mentioned variables were determined. Using the optimum levels of fermentation parameters, a maximum lactic acid concentration of 136.3 g/l was obtained. This study indicates that the medium design using statistical techniques such as the response surface methodology can be very useful in improving the production of lactic acid by immobilized cells as well as in similar bioprocesses.

REFERENCES

Book:

Axelsson, L. 1998. "Lactic acid bacteria: classification and physiology." , in *Lactic Acid Bacteria: Microbiology and Functional Aspects*, 2nd edited by Marcel Dekker, Inc, New York. pp. 1-72.

Dervakos, G.A. 1996. "Viable Cell Immobilization" , in *Studies in Viable Cell Immobilization,* edited by Colin Webb, pp. 1-17.

Harsa, Ş. 2003. "Biotechnology" notes.

Henning, D.R. 1998. "Fermented By-Products", in *Applied Dairy Microbiology*, edited by Marth, E. H. , Steele, J. L. (Marcel Dekker, Inc, New York), pp. 251-258.

Kandler, O. and Weiss, N. 1986. "Genus Lactobacillus" , in *Bergey's Manual of Systematic Bacteriology* , 9th edited by Williams and Wilkins, Baltimore, Vol. 2, pp. 1063-1065.

Polat, Z. 2002. "L(+)-Lactic Acid Purification from Fermentation Broth Using Ion Exchange Resins" , Thesis of Izmir Institute of Engineering.

Vickroy, T. B. 1985. "Lactic acid.", in *Comprehensive Biotechnology*, edited by M. Moo-Young (Pergamon Press,), Vol. 3, pp. 761- 776.

Periodical Article:

Abdel-Naby, M., Mok, C. and Lee, C. 1992. "Production of organic acid from enzymatic hydrolyzate of starch by immobilized lactic acid bacteria" , *UNIDO Proceedings,* Korea, pp. 227-243.

Albarghouthi, M., Fara, D.A., Saleem, M., El-Thaher, T., Matalka, K., Badwan, A. 2000. "Immobilization of antibodies on alginate-chitosan beads." , *International Journal of Pharmaceutics* . Vol. 206, pp. 23-34.

Audet, P., Paquin, C. and Lacroix, C. 1989. "Sugar utilization and acid production by free and entrapped cells of *Streptococcus salivarius* subsp. *thermophilus, Lactobacillus delbrueckii* subsp. *bulgaricus*, and *Lactococcus lactis* subsp. *lactis* in a whey permeate medium", *Appl. Environ. Microbiol.* Vol. 55, pp. 185-189.

Bódalo, A., Bastida, J., Gómez, J.L., Gómez, E., Alcaraz, I. and Asanza, M.L.1997. "Stabilization studies of L-aminoacylase-producing *Pseudomonas* sp. BA2 immobilized in calcium alginate gel." , *Enzyme Microbiol Technol.* Vol.21, pp. 64-69 .

Boyaval, P. and Goulet, J. 1988. "Optimal conditions for production of lactic acid from cheese whey permeate by Ca-alginate-entrapped *Lactobacillus helveticus." , Enzyme Microbiol Technol.* Vol.10, pp. 725-728 .

Bruno, J.M., Ragout, A.L., Cordoba, P.R. and Sineriz, F.1999. "Continuous production of L (+) lactic acid by *Lactobacillus casei* in two-stage systems." , *Applied Microbiology Biotechnology*, Vol. 51, pp. 316-24.

Burton, L. V. 1937. "By-products of milk." , *Food. Ind*. Vol. 9 , pp. 571-575.

Büyükkileci, A. O. 2000. "L(+) lactic acid production from whey by *Lactobacillus casei* NRRL B-441." *MSc. Thesis study.*

Dominguez, J. M. and Cruz, J. M. and Roca, E. and Dominguez, H. and Parajo, J. C. "Xylitol Production from Wood Hydrolyzates by Entrapped *Debaryomyces hansenii* and *Candida guilliermondii* Cells", *Applied Biochemistry and Biotechnology*. Vol. 81, pp. 119-130.

Duxbury, D. May 1993. "Lactic Acid", *Food Processing*. pp. 97-98 .

Fonden, R., Mogensen, G., Tanaka, R. and Salminen. S. 2000. "Effect of culture-containing diary products on intestinal microflora, human nutrition and health – current knowledge and future perspectives." , *International Dairy Federation Bulletin* . Number 352, IDF, Brussels.

Fox, P.F., McSweeney, P.L.H. and Lynch, C.M. 1998. "Significance of non-starter lactic acid bacteria in cheddar cheese." , *Aust. J. Dairy Technol.* Vol. 53, pp. 83-89.

Freeman, A. and Dror, Y. 1994. "Immobilization of "Disguised" Yeast in Chemically Crosslinked Chitosan Beads.", *Biotechnology and Bioengineering*. Vol. 44, pp. 1083-1088.

Garrett J. F. 1930. "Lactic acid" , *Ind. Eng. Chem.* Vol. 22, pp.1153-1154.

Gåserød, O., Sannes, A., Skjåk-Bræk, G. 1999. "Microcapsules of alginate-chitosan-II A study of capsule stability and permeability", *Biomaterials* Vol. 20, pp. 773-783.

Gåserød, O., Smidsrød, O., Skjåk-Bræk, G. 1998. "Microcapsules of alginate-chitosan-I A quantitative study of the interaction between alginate and chitosan" , *Biomaterials* Vol. 19, pp. 1815-1825.

Gassem, M. A. *et. al*. 2000. "Lactic acid production *by Lactobacillus delbrueckii* ssp. *bulgaricus* in camel's and cow's wheys", *Milchwissenschaft* . Vol. 55 , No. 7, pp. 374-377.

Gasser, F. 1970. "Electrophoretic characterization of lactate dehydrogenases in the genus *Lactobacillus*" , *J. Gen. Microbiol.,* Vol.62, pp. 223-239.

Göksungur, Y. and Güvenç, U. 1999. "Production of lactic acid from beet molasses by calcium alginate immobilized *Lactobacillus delberueckii* IFO 3202", *Journal of Chemical Technology and Biotechnology* . Vol. 74, pp. 131-136.

Göksungur, Y. 2004. “Optimization of the production of chitosan from beet molasses by response surface methodology.” , *J Chem Technol Biotechnol.* Vol. 79, pp. 974-981.

Groboillot, A.F. and Champagne, C.P. and Darling, G.D. and Poncelet, D. and Neufeld, R.J. 1993. “Membrane Formation by Interfacial Cross-Linking of Chitosan for Microencapsulation of *Lactococcus lactis*”, *Biotechnology and Bioenginerring* . Vol. 42, pp. 1157-1163.

Guoqiang, D. and Kaul, R. and Mattiasson, B. 1991. “Evaluation of Alginate-Immobilized *Lactobacillus casei* for Lactate Production”, *Applied Microbiol Biotechnol.* Vol. 36, pp. 309-314.

Hunger, W. 1984. “Dextro-rotatory and Levo-rotatory Lactic Acid: Their Significance and Occurence in Sour Milk Products”, *Danis Dairy Industry World wide* . Vol. 4, pp. 39-42.

Kaufman, E. N. and Cooper, S. P. and Davison, H. B. 1994. “Screening of resins for use in a biparticle fluidized-bed reactor for the continuous fermentation and separation of lactic acid.” , *Applied Biochemistry and Biotechnology*. Vol. 45/46, pp. 545-554.

Klinkenberg, G. and Lystad, K.Q. and Levine, D. W. and Dyrset, N. 2001. “Cell Release from Alginate Immobilized *Lactococcus lactis* ssp. *lactis* in Chitosan and Alginate Coated Beads” , *Journal of Dairy Science* . Vol. 84, pp. 1118-1127.

Kotzamanidis, C.H., Roukas, T. and Skaracis, G. 2002. “Optimization of lactic acid production from beet molasses by *Lactobacillus delbrueckii* NCIMB 8130” , *World Journal of Microbiology and Biotechnology*. Vol. 18, pp. 441-448.

Krischke, W. and Scröder, M. and Trösch, W. 1991. “Continuous Production of L-lactic Acid from Whey Permeate by Immobilized *Lactobacillus casei* subsp. *casei*”, *Applied Microbiol Biotechnol* . Vol. 34, pp. 573-578.

Krishnan, S. and Gowthaman, M.K. and Misra, M.C. and Karanth, N.G. 2001. "Chitosan-treated polypropylene matrix as immobilization support for lactic acid production using *Lactobacillus plantarum* NCIM 2084", *Journal of Chemical Technology and Biotechnology*. Vol. 76, pp. 461-468.

Ledesma, O.V., DeRuiz Holgardo, A.P., Oliver, G., DeGiori, G.S., Raibaud, P. and Galpin, J.V. 1977. "A synthetic medium for comparative nutritional studies of *Lactobacilli*" , *J. Appl. Bacteriol.* Vol. 42, pp. 123-133.

Leonard, R.H., Peterson, W.H., Johnson, M. J. 1948. "Lactic acid from fermentation of sulfite waste liquor" , *Ind. Eng. Chem.* Vol. 40, pp. 57-67.

Li, X.1996. "The use of chitosan to increase the stability of calcium alginate beads with entrapped yeast cells." , *Biotechnol Appl Biochem.* Vol.23, pp. 269-271 .

Lockwood, L.B. 1965. "Lactic Acid", *Ann. N. Y. Acad. Sci.* Vol. 119, pp. 854-867.

Nakamura, L. K. , Crowell, C. D. 1979. "*Lactobacillus amylophilus*" , *Dev. Ind. Microbiol*. Vol. 20, pp. 531-540.

Narayanan, N. and Roychoudhury, P. and Srivastava, A. 2004. "L (+) lactic acid fermentation and its product polymerization", *Microbial Biotechnology* . Vol 7, No 2.

Rao, M. July 1983. "Indian Expertise on Small Scale By-Products Industries For The Developing Countries", *WSJ* . pp. 26-29.

Rees, J.F. , Pirt, S.J. 1979. "The stability of lactic acid production in resting suspensions of *Lactobacillus delbreuckii*" , *J. Chem. Biotechnol*. Vol. 29, pp. 591-602.

Rosevear, A. 1984. "Immobilised Biocatalysts.", *J. Chem. Tech. Biotechnol*. Vol. 34B, pp. 127-150.

Roukas, T. and Kotzekidou, P. 1991. "Production of Lactic Acid From Deproteinized Whey by Coimmobilized *Lactobacillus casei* and *Lactococcus lactis* cells." , *Enzyme Microb. Technol*. Vol. 3, pp. 33-38.

Rymowicz, W., Wojtatowicz, M., Robak, M. and Jurgielewicz, W. 1993. "The use of immobilized *Yarrowia lipolytica* cells in Ca-alginate for citric acid production.", *Acta Microbiologica Polonica.* Vol.42, pp. 163-170 .

Serp, D. and Cantana, E. and Heinzen, C. and Stockar, U. and Marison, W. October 2000. "Characterization of an Encapsulation Device for the Production of Monodisperse Alginate Beads for Cell Immobilization.", *Biotechnology and Bioengineering.* Vol. 70, No. 1, p. 5.

Stanier, R. Y., Adleburg, E. A. and Ingraham, J. L. 1976. "Microorganisms" , *The Microbial World,* pp. 678-684.

Tanaka, H., Irie, S. and Ochi, H. 1989. "A novel immobilization method for prevention of cell leakage from the gel matrix" , *J Ferment Bioeng.* Vol. 68, pp. 216-219 .

Tellez-Luis, S. J. and Moldes, A.B. and Alonso, J. L. and Vazquez, M. 2003. "Optimization of Lactic Acid Production by *Lactobacillus delbrueckii* through Response Surface Methodology" , *Journal of Food Science*. Vol. 68, No. 4, pp. 1454-1458.

Tynkkynen, S., Satokari, R. , Saarela, M. , Mattila-Sandholm, T. and Saxelin. M. 1999. "Comparison of ribotyping, randomly amplified polymorphic DNA analysis, and pulsed-field gel electrophoresis in typing of *Lactobacillus rhamnosus* and *L. casei* strains." , *Appl. Environ. Microbiol.* Vol. 65, pp. 3908-3914.

Yoo, I.K., Seong, G.H., Chang, H.N. and Park, J.K. 1996. "Encapsulation of *Lactobacillus casei* cells in liquid-core alginate capsules for lactic acid production." , *Enzyme Microbiol Technol* Vol. 19, pp.428-433.

Zhou, Y. and Martins, E. and Groboillot A. and Champagne, C.P. and Neufeld, R. J. 1998. "Spectrophotometric quantification of lactic bacteria in alginate and

control of cell release with chitosan coating.”, *Journal of Applied Microbiology*. Vol. 84, pp. 342-348.

Web Source:

WEB_1, 2004, www.google.com, www_lactospore_com-images-formula3 05.08.2004.

WEB_ 2, 2004, www.google.com, www.Images of Lactobacillus casei.htm 25.07.2004.

WEB_3,2004,www.google.com,www.iasc.ac.in/currsci/jul10/contents1.htm16.11.2004.

APPENDIX A

Chemicals Used During Study

Table A.1. Chemicals Used During Study

Chemical	Features	Source
L(+)-Lactic acid	30% MW: 90.08 g/mole d=1.21 kg/cm^3	Sigma
D(-)-Lactic acid	95% MW: 90.08 g/mole	Sigma
α-Lactose	FW: 360.3 g/mole	Sigma
Calcium carbonate	FW: 100.1 g/mole	Fluka
Sodium hydroxide	FW: 40.00 g/mole	Sigma
Sulfuric acid	95-98% FW: 98.08 g/mole d=1.84 kg/cm^3	Merck
Litmus milk	pH= 5-6	Difco
Magnesium sulfate	99.5% FW: 120.4 g/mole	Sigma
Hydrocloric acid	37% FW: 36.5 g/mole	Merck
Manganese sulfate monohydrate	FW: 138 g/mole	Merck
Potassium dihydrogen phosphate	99.8% MW: 136.09 g/mole	Merck
Potassium phosphate (dibasic)	99 % FW: 174.2 g/mole	Sigma
Cupper sulfate pentahydrate	FW: 250 g/mole	Merck
Yeast extract	99.5 %	Oxoid
MRS Broth		Oxoid
Whey powder		Pınar Dairy Products
Sodium alginate		Sigma, A-2033
MRS agar		Oxoid

APPENDIX B

Calibration Curves

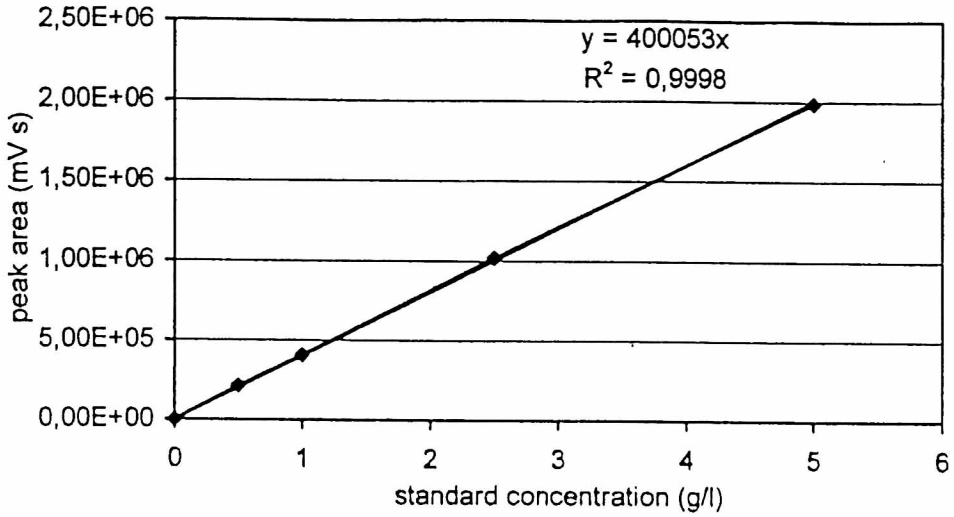


Figure B.1. Calibration curve for Lactic acid standard (HPLC)

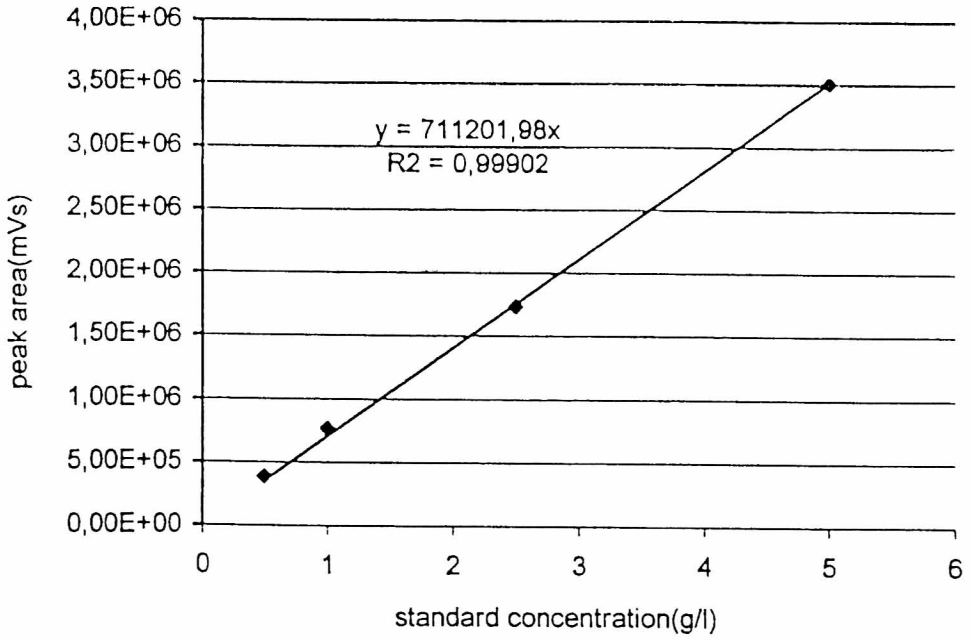


Figure B.2. Calibration curve for Lactose standard (HPLC)

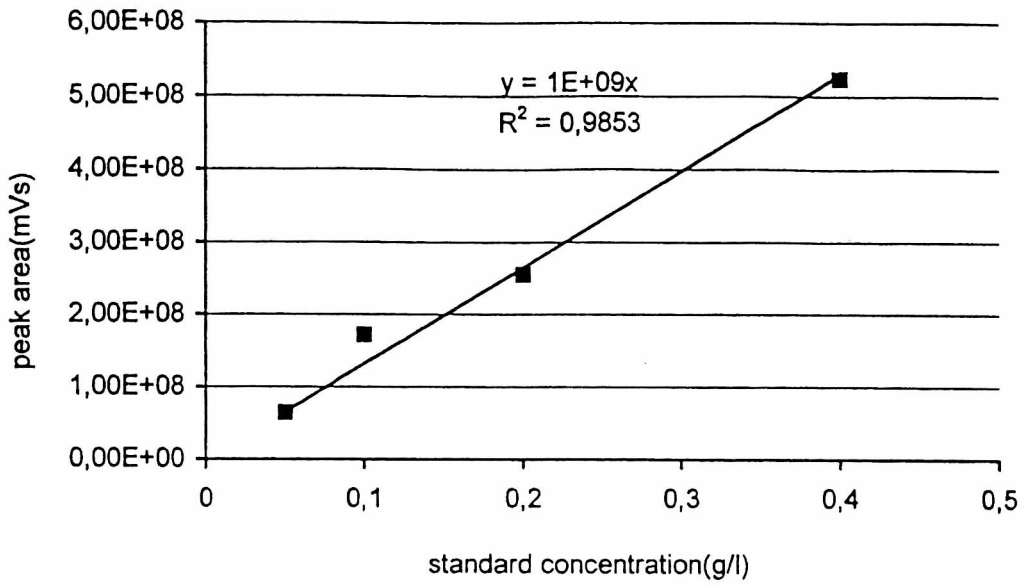


Figure B. 3. Calibration curve for L(+)-Lactic acid standard (HPLC)

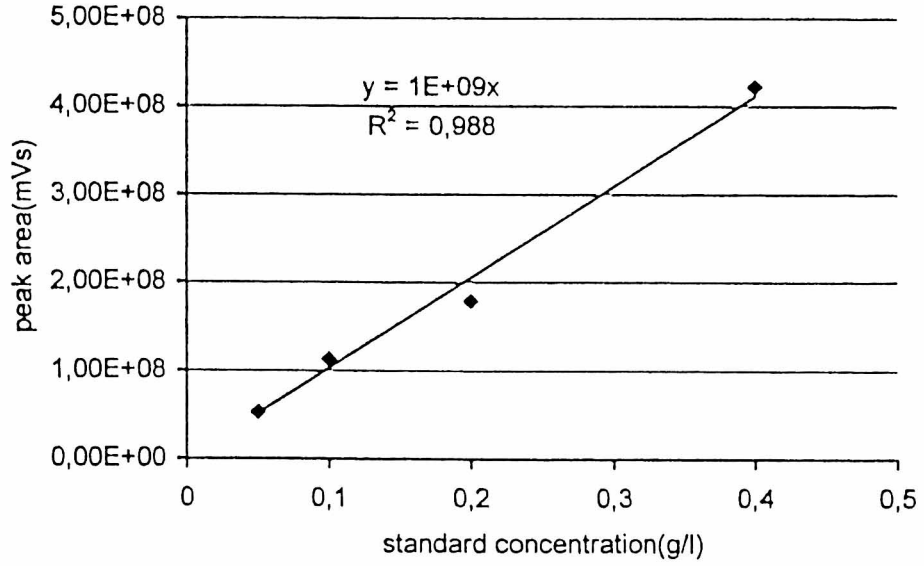


Figure B. 4. Calibration curve for D(-)-Lactic acid standard (HPLC)