View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by DSpace@IZTECH Institutional Repository

DETERMINATION OF WHEY-BASED MEDIUM REQUIREMENTS AND GROWTH CHARACTERISTICS FOR THE PRODUCTION OF YOGHURT STARTER CULTURES

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

MASTER OF SCIENCE

in Biotechnology and Bioengineering

by
Elçin SOYDEMİR

December 2008
İZMİR

We approve the thesis of **Elçin SOYDEMİR**

_____
Prof. Dr. Şebnem HARSA
Supervisor

_____
Assist. Prof. Dr. İlhan DOĞAN
Co – Advisor

_____
Assoc.. Prof. Dr. Canan TARI
Committee Member

_____
Assoc. Prof. Dr. Halil İbrahim ATABAY
Committee Member

_____
Assoc. Prof. Dr. Ahmet KOÇ
Committee Member

18 December 2008

_____
Prof. Dr. Semra ÜLKÜ
Head of the Department of Biotechnology
and Bioengineering Programme

_____
Prof. Dr. Hasan BÖKE
Dean of the Graduate School of
Engineering and Science

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisor Prof. Dr. Şebnem Harsa and co-supervisor İlhan Doğan for their guidance, supervision , encouragement and support throughout my research.

I would like to thank to my dear friends Hatice Yavuzdurmaz, Levent Yurdaer Aydemir and Lale Habibi for their friendships.

I would like to thank Burcu Okuklu, Sinem Çelik, Oylum Erkuş, Çelenk Molva, Mert Sudağıdan, Özgür Yılmazer, Yekta Güngör and Esra Soykut Acar for helping me in the laboratory and for their friendships.

I am grateful to my parents Hülya, Yavuz Soydemir and my sister Burçin Soydemir for their endless support, love and understanding.

ABSTRACT

DETERMINATION OF WHEY-BASED MEDIUM REQUIREMENTS AND GROWTH CHARACTERISTICS FOR THE PRODUCTION OF YOGHURT STARTER CULTURES

Yoghurt is an important dairy product which is produced by thermophilic starter cultures including; *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. They grow synergistically in milk by acidifying milk, produce specific aroma compounds and lactic acid which impact the quality of the yoghurt. Imported cultures are widely used in dairy industry to stabilize the technological properties which cause a change in our natural flora and flavour. Starters from traditional yoghurts have to be preserved. Under this perspective, the aim of this study was to determine the medium requirements of mixed starter cultures for production of starter bacteria which has been isolated and characterized before.

Determination of medium requirements were done in a whey-based media. Since bacteriophages were one of the most important problem in dairy industry, to prevent cultures from phage adsorption, media was treated with different salts which can bind free calcium ions. Salts were examined for cell viability and phage infection effects. From these studies Na_2HPO_4 + KH_2 PO_4 with 2% of medium addition was chosen as the most effective. Mineral requirements of the mixed cultures were determined. The best combination; 10, 5, 50 ppm for Fe^{2+}, Mn^{2+}, Mg^{2+} ions with 0,939 desirability were used for initial mineral content of medium. Fermentation constants for mixed cultures were determined. Pure culture fermentations and mixed culture fermentations were performed. According to results mixed culture inoculations gave higher maximum specific growth rates; $0,9188h^{-1}$ and $0,7323h^{-1}$ for cocci and bacilli while $0,855h^{-1}$ and $0,659h^{-1}$ were obtained for cocci and bacilli from single strain fermentations.

ÖZET

YOĞURT STARTER KÜLTÜRLERİNİN ÜRETİMİ İÇİN PEYNİR ALTI SUYU ESASLI ORTAM GEREKSİNİMLERİ VE BÜYÜME KARAKTERİSTİKLERİNİN BELİRLENMESİ

Yoğurt, süt endüstrisi ürünleri içerisinde tüketimi en fazla olan ürünlerden biridir. Yoğurt üretimi için kullanılan kültürler iki farklı termofilik mikroorganizmanın kombinasyonundan oluşmaktadır; *Streptococcus thermophilus* ve *Lactobacillus bulgaricus.* Yoğurt üretimi için kullanılan kültürler genellikle ithal edilmektedirler. Fakat ithal kültülerin kullanımı ülkemizde yapılan geleneksel yoğurt tadının değişmesine sebep olmaktadır. Bu sebeplerden yola çıkarak laboratuvar araştırma grubumuzun çalıştığı proje kapsamında yerel yoğurt kültürleri izole edilmiş, genetik ve biyokimyasal tanımlamaları daha önceden yapılmış ve son ürün özellikleri incelenmiştir.

Bu çalışmanın amacı izole edilen ve tanımlanan bu kültürlerin endüstriyel ölçekte üretimi için peynir altı suyu esaslı besi ortamı gereksinimlerinin ve bazı fermentasyon sabitlerinin belirlenmesidir. Büyüme ortamı olarak seçilen peynir altı suyu tozu büyüme faktörleri ve laktoz açısından oldukça zengin bir ortam olmakla birlikte yüksek kalsiyum içeriği sebebiyle faj atakları için oldukça uygun bir ortamdır. Faj ataklarının kısmen engellenebilmesi için bazı tuzların kalsiyum iyonunu çöktürmesi özelliğinden yola çıkılarak değişik tuzların hem hücre büyümesine etkisi hemde faj adsorpsiyonuna etkisi incelenmiştir. İncelenen tuzlar içerisinde en etkili tuzun %2 konsantrasyonla $Na_2HPO_4 + KH_2 PO_4$ olduğu bulgulanmıştır. Kültürlerin mineral gereksinimleri Fe^{2+}, Mn^{2+}, Mg^{2+} için 10, 5, 50 ppm olarak belirlenmiştir. Sonuç olarak bu ortamda büyütülen karışık kültürlerin fermentasyon sabitleri hesaplanmış; karışık kültür inoküle edilerek yapılan fermentasyon çalışmalarında μ_{max} değerleri kok için $0,9188h^{-1}$, basil için $0,7323h^{-1}$ iken, saf kültür fermentasyon çalışmalarında bu değerler kok için $0,855h^{-1}$, basil için $0,695h^{-1}$ bulunmuştur.

dedicated to my family

TABLE OF CONTENTS

LIST OF FIGURES x

LIST OF TABLES xi

CHAPTER 1. INTRODUCTION 1

1.1. Manufacture of Yoghurt 1

1.2. Types of Yoghurt 2

1.3. Starter Cultures 3

1.4. Types of Starters 3

1.4.1. Artisanal and Defined Starter Cultures 3

1.4.2. Mesophilic Starter Cultures 4

1.4.3. Thermophilic Starter Cultures 5

1.5. Yoghurt Starter Cultures 5

1.5.1. *Streptococcus salivarius ssp. thermophilus* 6

1.5.2. *Lactobacillus delbrueckii ssp. bulgaricuss* 7

1.5.3. Symbiotic Growth of Yoghurt Starter Bacteria 8

1.5.4. Starter Culture Systems in Dairy Industry 10

1.5.4.1. Freeze-Dried Cultures 11

1.5.4.2. Frozen Concentrated Cultures 11

1.5.4.3. Liquid Starter Cultures 12

1.5.5. Role of Starters in Yoghurt Production 12

1.5.5.1. Lactic Acid Production 12

1.5.5.2. Proteolytic Activity 13

1.5.5.3. Aroma Production 13

1.5.5.4. Exopolysaccharide Production 14

1.5.5.5. Production of Inhibitory Compounds 15

1.5.6. General Problems for Industrial Starter Production 15

1.6. Bacteriophages 16

1.6.1. Infection Mechanisms 17

1.6.1.1. Lytic Life Cycle 17
1.6.1.2. Lysogenic Life Cycle 18
1.6.2. Phages of Dairy Bacteria 19
1.6.3. Phage Resistance Mechanisms 20
1.6.4. Preventing Phage Inhibition 20
1.6.4.1. Use of Phage-Resistant Strains 21
1.6.4.2. Culture Rotation 21
1.6.4.3. Phage Inhibitory Media 22
1.7. Growth Medium Ingredients for Thermophilic Starter Culture Production 22
1.7.1. Growth Media Formulations 23
1.7.2. pH Control During Fermentation 24
1.8. Batch Fermentation and Fermentation Constants 25

CHAPTER 2. MATERIALS AND METHODS 28
2.1. Materials 28
2.1.1. Chemicals 28
2.1.2. Media 28
2.1.3. Microorganisms 28
2.1.4. Bacteriophages 28
2.2. Methods 29
2.2.1. Culture Propagation 29
2.2.2. Construction of Growth Curves 29
2.2.3. Enumaration of Viable Cells 30
2.2.4. Determining the Phage Sensitivity of Strains and Effect of Phage Inhibitory Salts on Phage Inhibition 30
2.2.4.1. Phages and Strains 30
2.2.4.2. Enrichment of Phage Titer 31
2.2.4.3. Determination of Phage Titer 32
2.2.4.4. Determining the Phage Sensitive/Resistant Strains 32
2.2.4.5. Effect of Phosphate Salts on Cell Viability and Phage Infection 32
2.2.5. Mineral Requirements of Mixed Starter Cultures 33

2.2.6. Fermentation Constants of Mixed Starter Cultures 35

CHAPTER 3. RESULTS AND DISCUSSIONS 36
3.1. Growth Curves 36
3.2. Determining Phage Sensitivity of Strains and the Effect of Phage Inhibitory Salts on Phage Infection and Cell Viability 40
3.2.1. Selection of Phage Sensitive/Resistant Strains 40
3.2.2. Effects of Phosphate Citrate Salts on Culture Viability 41
3.2.3. Effects of Salt Additions on Phage Infection 46
3.3. Mineral Requirements of Mixed Starter Cultures 49
3.4. Fermentation Constants of Yoghurt Starter Cultures 53
3.4.1. Single strain fermentations of *Streptococcus thermophilus* TY25 53
3.4.2. Single strain fermentations of *Lactobacillus bulgaricus* TY30 55
3.4.3. Mixed culture fermentations of TY30*TY25 57

CHAPTER 4. CONCLUSIONS 61

REFERENCES 63

APPENDICES
APPENDIX A. CHEMICALS USED IN EXPERIMENTS 67
APPENDIX B. RECIPES FOR CULTURE MEDIA 69
APPENDIX C. GROWTH CURVES FOR PURE CULTURE OF TY25 74
APPENDIX D. GROWTH CURVES FOR PURE CULTURE OF TY30 79
APPENDIX E. GROWTH CURVES FOR MIXED CULTURE OF TY25 AND TY30 84

LIST OF FIGURES

Figure **Page**

Figure 1.1. Electron Microscopic Image of *Streptococcus thermophilus* 6

Figure 1.2. Electron Micrographic Image of *Lactobacillus bulgaricus* 8

Figure 1.3. Growth Curves of Starter Bacteria for Pure cultures and Mixed Culture 9

Figure 1.4. Stimulatory Effects of Two Microorganisms 10

Figure 1.5. *Streptococcus thermophilus* B3-X18 Phage and *Lactobacillus bulgaricus* Y4-X4 Phage 19

Figure 1.6. Specific Growth Rates Versus Initial Substrate Concentrations 26

Figure 3.1. Growth Curves of *Streptococcus thermophilus* Strains in M17 Broths 36

Figure 3.2. Growth Curves of *Lactobacillus bulgaricus* Strains in MRS Broths 38

Figure 3.3. Specific growth rates and initial lactose concentration for single strain fermentation of *Streptococcus thermophilus* TY25 54

Figure 3.4. Double reciprocal plot for single strain fermentation of *Streptococcus thermophilus* TY25 55

Figure 3.5. Specific growth rates and initial lactose concentration for single strain fermentation of *Lactobacillus bulgaricus* TY30 56

Figure 3.6. Double reciprocal plot for single strain fermentation of *Lactobacillus bulgaricus* TY30 57

Figure 3.7. Monod substrate saturation graphic for mixed culture fermentation of *Streptococcus thermophilus* TY25 and *Lactobacillus bulgaricus* TY30 59

Figure 3.8. Double reciprocal plot for *Streptococcus thermophilus* TY25 and *Lactobacillus bulgaricus* TY30 60

LIST OF TABLES

Table **Page**

Table 2.1. *Streptococcus thermophilus* and *Lactobacillus bulgaricus* Strains 30

Table 2.2. Bacteriophages *Streptococcus thermophilus* and Lactobacillus *bulgaricus* 31

Table 2.3. Factors and Their Levels 33

Table 2.4. Run Order of Experiments in Shake Flasks 34

Table 3.1. Phage-bacteria Combinations Which Clear Plagues for *Lactobacillus bulgaricus* strains 40

Table 3.2. Phage-bacteria Combinations Which Give Clear Plagues for *Streptococcus thermophilus* strains 41

Table 3.3. Effects of Salts on Cell Viability for Mixed Culture of TY25*TY30 42

Table 3.4. Effects of Salts on Cell Viability for Mixed Culture of TY25*22 43

Table 3.5. Effects of Salts on Cell Viability for Mixed Culture of TY25*TY77-a 43

Table 3.6. Effects of Salts on Cell Viability for Mixed Culture of 95.1*TY30 43

Table 3.7. Effects of Salts on Cell Viability for Mixed Culture of 95.1*22 44

Table 3.8. Effects of Salts on Cell Viability for Mixed Culture of 95.1*TY77-a 44

Table 3.9. Effects of Salts on Cell Viability for Mixed Culture of 63-2*TY30 45

Table 3.10. Effects of Salts on Cell Viability for Mixed Culture of 63-2*22 45

Table 3.11. Effects of Salts on Cell Viability for Mixed Culture of 63-2*TY77-a 46

Table 3.12. Inıtial Phage Titers 47

Table 3.13. Phage Titers After 18 Hours Incubation for Each Salt Addition 48

Table 3.14. Factors, Levels and Response Data Table 50

Table 3.15. ANOVA Table for Response 1 51

Table 3.16. ANOVA Table for Response 2 52

Table 3.17. Mean values of viable cell counts(logcfu/ml) for different initial lactose concentrations and specific growth rate values for TY25 54

Table 3.18. Mean values of viable cell counts(logcfu/ml) for different initial lactose concentrations and specific growth rate values for TY30 56

Table 3.19. Mean values of viable cell counts of *Streptococcus thermophilus* TY25 in mixed culture fermentations with different initial lactose concentrations 58
Table 3.20. Mean values of viable cell counts of *Lactobacillus bulgaricus* TY30 in mixed culture fermentations with different initial lactose concentrations 59

CHAPTER 1

INTRODUCTION

1.1. Manufacture of Yoghurt

There is no records available about the origin of the yoghurt, but it is believed that the beneficial effects on human health and nutrition made yoghurt as an important dairy product among nomadic people.

Yoghurt can be characterized as a gel-like coagulated milk product, having smooth consistency and pleasing flavour. The cultures used to manufacture yoghurt are named as thermophilic lactic acid starter cultures, which contribute to the properties of the product during fermentation. *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* are the microorganisms used for this purpose as the starter cultures (Tarı, et al. 2008).

The raw material for yoghurt fermentation is generally cow's milk or the milk from other mammals such as goat, sheep, camel, buffalo, etc. In cow's milk the milk solids non-fat level (MNSF) is 8.5-9% of which around 4.5% lactose, 3.4% protein and 0.7% minerals, and each of these components are vital for the production of a satisfactory yoghurt (Tamime and Robinson 1985).

The first steps in yoghurt manufacturing are; filtration of the milk to remove leaves or debris, sample check whether there are antibiotics or other chemicals which might have an inhibitory effect on bacteria, seperation of milk fat and adjustment of fat content of the skim milk stream to the level required in the end product and finally elevation of MNSF to a level that will give to gel a strong structure.

Although natural yoghurt is based on milk, it depends on the market demand to add some stabilizers like complexs carbohydrates, plant gums, starch. Sweetening agents like sucrose or high fructose syrups tend to be added to milk and the subsequent heat treatment of the milk will ensure that any contaminant yeasts associated with sugars are destroyed (Robinson 2002).

Before heat treatments milk has to be homogenized to ensure both full incorporation of any dry ingredients and to breakdown the fat globules to a uniform size.

The next step is heating milk in order to raise the temperature to 90-95 ºC for 5-10 minute which is an important step to destroy pathogen bacteria or inactivate enzymes like lipase. Once the heat treatment has been completed the milk is cooled to 43 ºC which is the optimum temperature for fermentation step.

Fermentation process includes inoculation of starter culture into the pasteurized milk with an inoculation ratio of 2%. Fermentation will be complete when the acidity of the milk will have risen to 1.2-1.4% lactic acid (around pH 4.3-4.5). When fermentation is ended product has to be cooled immediately to the temperature of 4ºC and has to be stored at this temperature (Tamime and Robinson 1985).

1.2. Types of Yoghurt

Yoghurt can be classified according to its flavour, type of post-incubation process, chemical composition or manufacturing method.

Yoghurt is divided into two main classes according to production method; set type and stirred type. Stirred yoghurt fermentation occurs in fermentation tanks in which the gel structure is broken before cooling by continious stirring while set type yoghurts are fermented in the retail containers.

Based on the chemical composition yoghurts can be classified in to three types; full-fat yoghurt, reduced fat yoghurt, low-fat yoghurt with 8.5% of non fat milk solids, 0.9% titratable acidity and differ in their fat content less than 3%, 0.5-2% and 0.5% (Tamime and Robinson 1985).

Post-incubation processes include concentration, pasteurization, freezing or drying of yoghurt after fermentation.

According to flavour, yoghurt can be produced by addition of fruit particles, sweetenings and colorants rather than traditional type plain yoghurt.

1.3. Starter Cultures

Starter culture can be defined as the microbiological preparation of cells from at least one microorganism to be added to a raw material to produce a fermented food by accelerating and steering its fermentation process (Leroy and De Vuyst 2004).

Starter cultures including lactic acid bacteria play a central role in manufacturing of cheese, yoghurt and other fermented dairy products.

Since the area is food industry, starter cultures have to be harmless and food-grade. Starter cultures should contain the highest possible number of viable organisms, be highly active under production conditions, and be free from contaminants. All dairy fermentation processes rely on the purity and acitivity of the starter culture, milk or growth medium should not contain inhibitory agents, such as antibiotics, bacteriophages (Leroy and De Vuyst 2004).

1.4. Types of Starters

Generally starters can be divided into two broad groups according to their addition to the raw material for fermentation; traditional and defined cultures. Traditional cultures include artisanal (natural) and mixed strain cultures. In addition both classes can be classified as thermophilic or mesophilic starter cultures.

1.4.1. Artisanal and Defined Starter Cultures

Artisanal starters are generally derived from backslopping, using a part of a previous batch of a fermented product to inoculate a new batch. One of the most important features of these cultures is having a complex, variable and often poorly defined microflora resulting a non desirable flavour and taste for end product (Cogan and Accolas 1996).

Artisanal starters are potential sources of strains carrying some interesting characterictics such as production of inhibitors, phage resistance, etc. They are reproduced in the medium with the precence of phage and generally less sensitive to be attacked by phage. Although they might contain several phage-carrying and lysogenic strains. The lysogenic strains are potential sources of infective phages.

Because of some practical, economical and cultural factors artisanal (natural) starters are still being used in manufacture of some hard type cheeses, and used in some small dairy farms.

Artisanal starters can be explained as undefined mixtures of lactic acid bacteria, propagated daily in the factory environment without any special precautions to exlude phage. As a result of the frequent subculturing there might be some compositional changes in starter leading inconsistent performance in fermentation, variability in flavour and texture of product. These problems can be overcomed by using commercial mixed strain starters. Artisanal cultures that produce good quality fermentative end product were propagated under controlled conditions to use as mixed starter strains (De Vuyst, et al.2001).

Even under controlled conditions continiual subculturing can lead to variability between different batches of starters. In modern practice, subculturing is minimized and cultures are preserved by freezing or lyophilization. Actually mixed starters are marketed by various commercial suppliers as vacuum-dried, lyophilized or frozen cultures.

1.4.2. Mesophilic Starter Cultures

Mesophilic starter cultures have an optimum growth temperature of around 26ºC. Mesophilic starter cultures, composed of acid forming lactococci and often of flavour producers, are used in the production of many cheese varieties, fermented milk products, and ripening cream butter (Peterson 1988).

Mesophilic starter cultures include several species of lactic acid bacteria; *Lactococcus lactis* ssp. *lactis, Lactococcus lactis* ssp. *cremoris, Lactococcus lactis* ssp. lactis biovar diacetylactis, *Lactobacillus kefir, Lactobacillus casei, Leuconostoc* ssp. When starter culture contains only these microorganisms they are characterized as O type. *Lc. lactis* ssp. *lactis diacetylactis* and *Leuconostoc sp.* are citric acid fermenting bacteria and the culture is named D type if only contains citric acid fermenting bacteria. When the only aroma producing species is *Leuconostoc sp.* the culture is named B type. Several combinations of single or mixed strains of lactic acid bacteria are currently used in cheese making (Cogan and Accolas 1996).

1.4.3. Thermophilic Starter Cultures

Thermophilic starter cultures are used for the manufacture of yoghurt, Bulgarian buttermilk and whole range of products made with intestinal bacteria with a growth temperature of approximately 45ºC. Defined thermophilic starter culture systems are; *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus salivarius* ssp. *thermophilus* for yoghurt fermentation, *Lactobacillus acidophilus* for the production of acidophilus milk, *Lactobacillus paracasei* ssp.*paracasei* for the production of yakult, *Lactobacillus helveticus* and *Lactobacillus delbrueckii* ssp. *lactis* are starter lactobacilli for cheese with high cooking temperature (Surono and Hosono 2002).

1.5. Yoghurt Starter Cultures

It was found that Gram-positive rods and cocci were dominant species in yoghurt. Thermophilic lactic starter culture including *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus salivarius* ssp. *thermophilus* are the bacteria essential for yoghurt manufacture. At least it has to contain *Lactobacillus delbrueckii* ssp. *bulgaricus* to be named as yoghurt in some countries as a legal requirement which is based upon the grounds that; there is an historical association between the two species and yoghurt, the typical flavour of yoghurt tends to depend on the precence of *Lactobacillus delbrueckii* ssp. *bulgaricus* (Robinson 2002).

The yoghurt starter bacteria grow synergistically in milk, increase the acidity via converting lactose into lactic acid, coagulate milk proteins to result in a gel like structure and produce specific taste and aroma compound. Since it gives better results, to grow them in the same medium to manufacture starters, they are inoculated together to growth medium to obtain higher cell counts in bulk starter.

Manufacture of yoghurt can be done by using both artisanal starter cultures and defined mixed starter systems. As mentioned before using defined mixed cultures give predictable quality characteristic with a little variation while it is difficult to obtain the same quality by artisanal cultures (Leroy and De Vuyst 2004).

Unless otherwise stated artisanal cultures are used in home made yoghurts and dairy industry prefers the use of defined culture systems with known biochemical or

genetic characteristics and starter bacteria are inoculated with 1:1 ratio in milk for yoghurt fermentations (Leroy and De Vuyst 2004).

1.5.1. *Streptococcus salivarius* ssp. *thermophilus*

Streptococcus thermophilus belongs to the thermophilic group of lactic acid bacteria and generally used in association with other lactobacillus species as a starter culture in dairy industry.

Streptococcus thermophilus is a Gram positive nonmotile coccus with 0.7-0.9 μm in diameter. It occurs in pairs and chains of 10-20 cells (Pearce and Flint 2002).

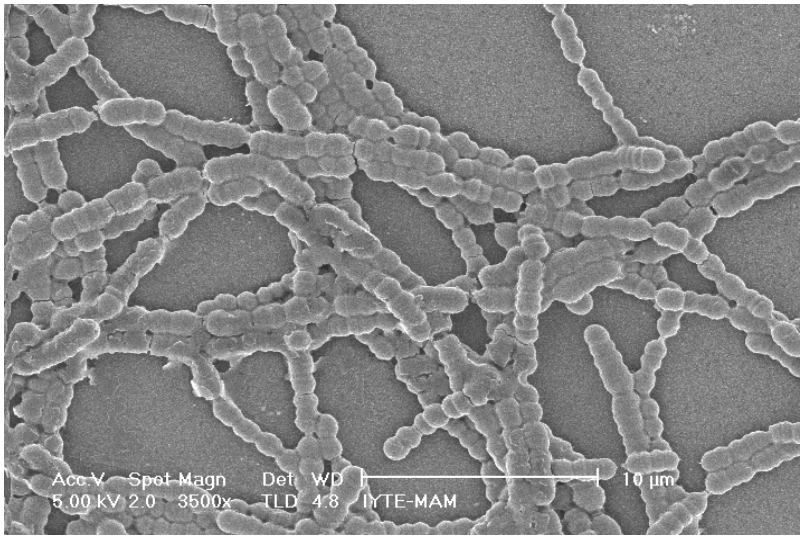


Figure 1.1. Electron microscopic image of *Streptococcus thermophilus*
(Source: Erkus 2007)

Streptococcus thermophilus is highly adapted to the dairy environment and can be isolated from milk. The bacterium has an optimum growth temperature of 40-45ºC, a minimum of 20-25 ºC and a maximum of 47-50 ºC. *Streptococcus thermophilus* is a facultative anaerob and catalase negative bacteria.

Streptococcus thermophilus can not ferment esculin which is an important trait distinguishing this species from the bacteria of the genus enterococcus as well as from other *Streptococcus* species (Botina, et al. 2007). It is a homofermentative bacterium and can ferment lactose which results in L (+) lactate via Embden-Meyerhof pathway (EMP). When grown in milk, lactose is transported into the cell in association with the

explusion of galactose by an antiport system. Lactose is hydrolyzed by β-galactosidase, but only glucose is metabolized via EMP to L(+) lactate (Pearce and Flint 2002). *Streptococcus thermophilus* can also ferment fructose, glucose and mannose while the fermentation of galactose, maltose, sucrose is strain specific.

Streptococcus thermophilus strains are incapable of growing at a concentration of NaCl over 2% of the growth medium It can not utilize arginine (Botina, et al. 2007). *Streptococcus thermophilus* requires free amino acids as a result of having limited proteolytic acitivity when comparing with other Lactobacilli. The essential amino acids for its growth are glutamic acid, cysteine, histidine, methionine, valine, leucine, isoleucine, tryptophan, arginine and tyrosine. Therefore it is generally combined with other lactobacilli in growth medium to obtain higher proteolytic activiy (Botina, et al. 2007).

1.5.2. *Lactobacillus delbrueckii* ssp. *bulgaricus*

Lactobacillus delbrueckii ssp. *bulgaricus* belongs to lactobacillus delbrueckii group which also includes *Lactobacillus delbrueckii* ssp. *delbrueckii, Lactobacillus delbrueckii* ssp. *lactis , Lactobacillus delbrueckii* ssp *leichmanii*. It is a non-motile, non-spore forming, rod shaped bacteria. Like *Streptococcus thermophilus,* it is a member of industrially important bacteria which is commonly used in dairy industry.

Among all lactobacillus delbrueckii group bacteria, it has a smallest range of carbohydrates to be fermented; glucose, lactose and fructose. It is a catalase negative microorganism and does not utilize arginine (Crow and Curry 2002).

Lactobacillus bulgaricus is a homofermentative bacteria and responsible for the bioconverison of lactose into D(-) lactic acid. It is a galactose negative microorganism and like *Streptococcus thermophilus*, it transports lactose in association with the expulsion of galactose by an antiport system.

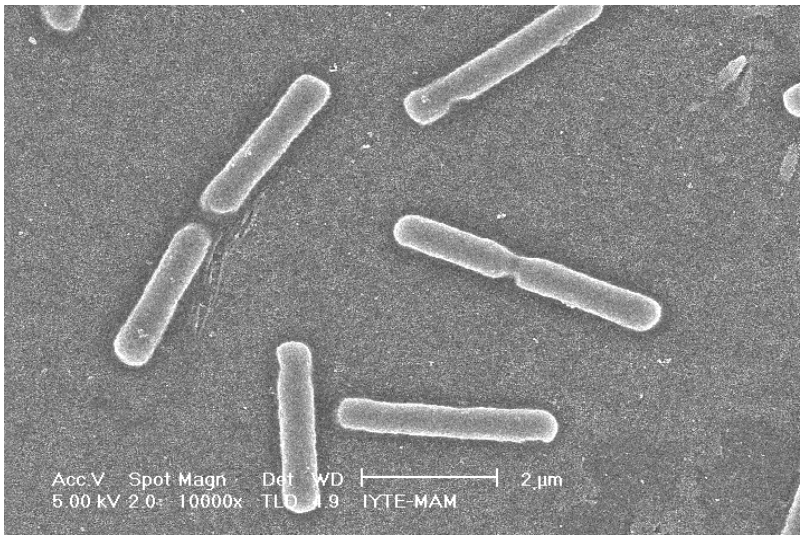


Figure 1.2. Electron micrographic image of *Lactobacillus bulgaricus*
(Source: Erkus 2007)

Lactobacillus bulgaricus has an optimum growth temperature of 45ºC, and a maximum of 50-55º C, have only a slight growth at 10ºC. Their DNA contents ranges from 49-51 mol %(G+C) and the cell surface is characterized by a lysine-D-aspartyl peptidoglycan type and a glycerol base teichoic acid (Crow and Curry 2002). Owing to similarities like G+C ratio in DNA structure, lactic acid production and concentration, cell wall structure, almost same with *Lactobacillus delbrueckii* spp. *lactis*, it is hard to differentiate *L. bulgaricus* morphologically. However, *Lactobacillus bulgaricus* cannot utilize maltose while *L. lactis* can.

Lactobacillus bulgaricus has higher proteolytic activity than *Streptococcus thermophilus* and this is one of the reasons to combine them as a functional starter in dairy production. The proteolytic enzymes of *Lactobacillus bulgaricus* degrade casein with the liberation of low molecular weight peptides and amino acids (Rajagopal and Sandine 1990).

1.5.3. Symbiotic Growth of Yoghurt Starter Bacteria

Milk fermentation for yoghurt is done by the addition of thermophilic lactic acid bacteria for thousands of years. This is about the associative growth of microorganisms in growth medium. Symbiotic growth of thermophilic lactic starter microorganisms is based on their metabolic compatibility.

It was demonstrated by Robinson at 2002; inoculation of mixed cultures results in the production of lactic acid >10 g/L in 4 hours while 2 g/L and 4g/L lactic acid was obtained by the fermentation of isolated pure cultures of *Lactobacillus bulgaricus* and *Streptococcus thermophilus.*

Protocooperatvie growth of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* considerably gives better results due to cell densities, the specific growth rates, lactic acid production rates .

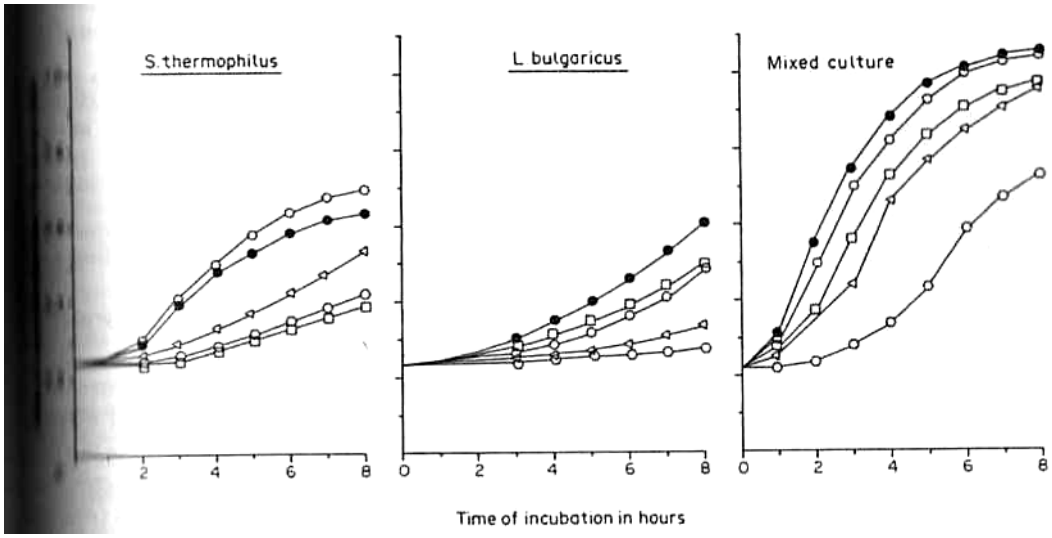


Figure 1.3. Growth curves of starter bacteria for pure cultures and mixed culture (Source : Tamime and Robinson 1985)

Streptococcus thermophilus grows more rapidly than *Lactobacillus bulgaricus* initially and begins to produce lactic acid. Lactic acid production results in a decrease in the pH of the medium. While *Streptococcus thermophilus* grows, it releases CO_2 from the breakdown of urea and formic acid. *Streptococcus thermophilus* deplates the oxygen in the medium and this causes the oxidation-reduction potential more favorable for the growth of *Lactobacillus bulgaricus*.

The increased acidity, CO_2, formic acid and deplation of O_2 stimulates the growth of bacilli which is more acid tolerant than *Streptococcus thermophilus*. Beside having a stimulatory effect on bacilli, the growth of *Streptococcus thermophilus* depends on the growth of *Lactobacillus bulgaricus.* Because *Lactobacillus bulgaricus* has higher proteolytic acitivity than *Streptococcus thermophilus*. The proteolytic enzymes of *Lactobacillus bulgaricus* degrade casein with the liberation of low

molecular weight peptides and amino acids which have stimulatory effect on the growth of *Streptococcus thermophilus* (Rajagopal and Sandine 1990).

Both two microorganisms can grow at a temperature of 42-43ºC, and the optimum temperature for symbiotic growth is 42ºC. Since the optimum growth temperature for *Streptococcus thermophilus* is 37ºC and 45ºC for *Lactobacillus bulgaricus*, increasing the temperature above from 42ºC, the growth of lactobacilli will be favored while the temperatures below 42ºC results in increased growth of streptococci. Either case is resulted in a deviation in the ratio of cocci to bacilli, for the optimum yoghurt the ratio should be 1:1 (Shah 2003).

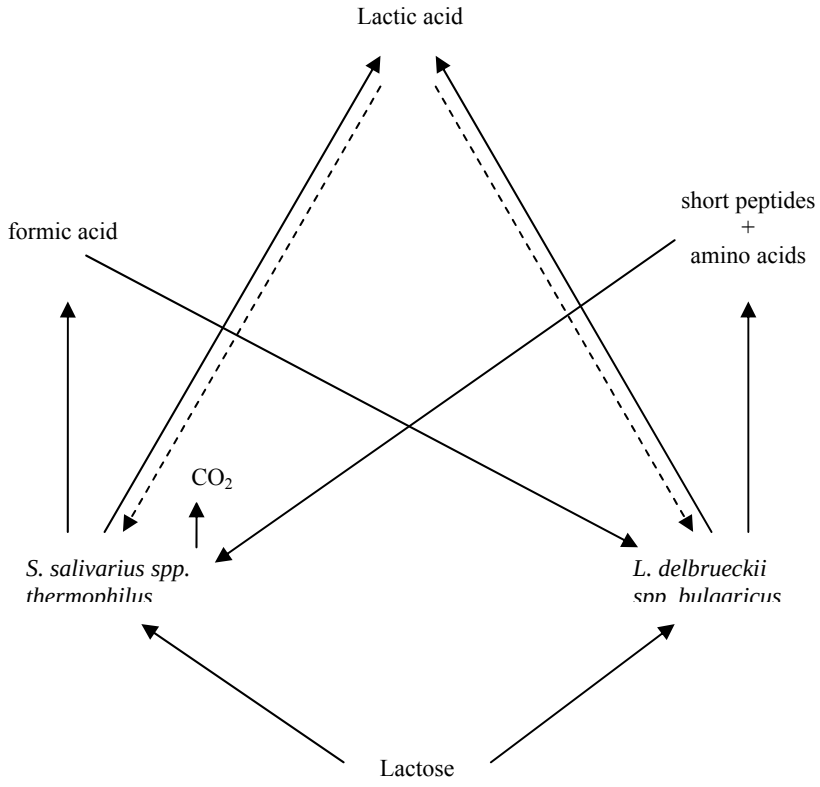


Figure 1.4. Stimulatory effects of two microorganism
(Source : Tekinşen 2000)

1.5.4. Starter Culture Systems in Dairy Industry

There are several options for meeting manufacturers of dairy foods with cultures. Although being the most expensive, the simplest is to use frozen concentrated

cultures which can be used to directly inoculate milk. Beside using freeze dried or frozen cultures, bulk cultures can be prepared at the plant from available commercial frozen concentrated or freeze dried cultures. This involves starting with a mother culture maintained in small amounts, which will than be used for the preparations of bulk culture in a larger volume. Actually preparing bulk culture in plant might have a risk for phage contamination. For this reason, plants generally purchase the inoculum as frozen concentrated or freeze dried preparations (Shah 2003).

1.5.4.1. Freeze-Dried Cultures

Freeze dried cultures are generally used when it is not easy to transport or storage cultures at –40ºC. The main disadvantage of using freeze dried culture is that the lag phase of the culture may exhibit a longer time.

Preparation of freeze dried cultures includes freezing step firstly. After freezing, the culture concentrate is placed under high vacuum to dehyrate by sublimation. The dried cells are then packaged under aseptic conditions anaerobically (Sandine 1996).

1.5.4.2. Frozen Concentrated Cultures

Frozen concentrated cultures contain 10^{10}-10^{11} colony forming units per gram which is sufficient to allow 70 ml to inoculate 1000 L of the media for bulk starter preparation (Sandine 1996).

Basic steps in preparation of frozen concentrated cultures begin with growing cultures under optimal conditions at constant pH, continues with ultrafiltration to harvest cells. Harvested cells have to be standardized to a specific acitivity. To be able to protect cells, a cryoprotectant has to be added into the medium. After packaging a rapid freezing starts generally by using liquid nitrogen, dry ice-alcohol mixture. The frozen cultures should be stored at -196 ºC for best retention activity, also storage at -40 ºC is acceptable.

Beside the advantages there are some disadvantages of frozen concentrated cultures. The shipment of frozen starters is precarious since the temperature changes affect the starter activity, the storage temperature is critical, thus the activity has to be controlled in the dairy plant.

1.5.4.3. Liquid Starter Cultures

The starter is first cultivated as a liquid stock culture and by successive subculturing continues until the desired volume is obtained. The procedure is generally expensive, labourious, vulnerable and needs skilled personel to manage it.

The starters are easily contaminated during the numerous inoculations or infected by bacteriophages. Still, liquid starter cultures are widely used, especially where local special products are made or where the transportation of starters from a central laboratory is easy and regular.

1.5.5. Role of Starters in Yoghurt Production

The usage of carefully selected strains as starter cultures or co-cultures in fermentation process can help to achieve in situ expression of the desired property and to maintain a product with good quality characteristics such as aroma, taste, flavour.

Starter culture inoculation into the milk to during the production of yoghurt leads to production of lactic acid, aroma compounds, exopolysaccharides and inhibitor compounds which give specific characteristics to the end product.

1.5.5.1. Lactic Acid Production

The main product in sugar fermentation by lactic acid bacteria is lactate. Lactic acid is an industrially important product due to its versalite chemical properties, used as an acidulant, flavour compound and preservative in the food; pharmacetical, leather and textile industries, for the production of base chemicals and for polymerization to biodegredable polylactic acid (Lee 2004).

Lactic acid can be produced by homofermentative and heterofermentative ways in D(-) and L(+) forms. Since L(+) lactic acid is produced during the early fermentation, D (-) type is began to be produced after about second hour of fermentation and increase continuously.

Typical yoghurt flavour is caused by lactic acid which imparts an acidic and refreshing taste (Chaves, et al. 2002). Lactic acid has an effect on regulation of

hydrolysis of casein and adsorbtion of some amino acids, peptides, lactose and minerals (Akın 2006).

1.5.5.2. Proteolytic Activity

The growth of lactic acid bacteria depends on some nutritional supplies of suitable sources of nitrogen and carbon. Free amino acids and peptides are present only to a limited degree in milk. Starter bacteria have limited biosynthetic capabilities. Therefore they require free amino acids for growth.

One of the essential features of lactic acid bacteria used as starter must be that they posses an efficient proteolytic system enabling them to grow to high cell densities and that they have the ability to ferment lactose rapidly into lactic acid. Therefore a complement of proteinases and peptidases are essential for the degredation of milk proteins.

By itself *Streptococcus thermophilus* has a lower proteolytic activity than *Lactobacillus bulgaricus*. Therefore, in dairy starter systems *Streptococcus thermophilus* is used in combination with lactobacilli which leads to impact flavour, texture and composition. The free amino acids arising from the proteolytic acitvity of *Lactobacillus bulgaricus* might be identified as specific growth factors for *Streptococcus thermophilus* (Rajagopal and Sandine 1990).

Although proteolysis causes the stimulation of bacterial growth, it has some adverse effects on fermented milk products. It was demonstrated that, the production of bitter peptides have been attributed to the proteolysis by *Lactobacillus bulgaricus* during storage (Renz and Puhan 1975).

1.5.5.3. Aroma Production

Fermented dairy production industry; flavour perception, which is a crucial characteristic of food industry as the sensory characteristic, is strongly based on the volatile components (Kalviainen, et al. 2003). Yoghurt bacteria give the desired flavour, mouthfeel and texture which is promoted by a series of biochemical pathway in which the starter culture provide the enzymes necessary.

Among all flavour compounds isolated, the most prominent ones are lactic acid and a mixture of various carbonyl compounds like acetaldehyde, ethanol, acetone, diacetyl and 2-butanone. Acetaldehyde is considered as the major flavour compound for the typical yoghurt aroma reported by many researchers (Chaves, et al. 2002).

The ideal yoghurt flavour is a balanced of acidity and acetaldehyde. This is achieved by culture selection, balance of rod coccus ratio, and fermentation control. The main source of acetaldeyhde is the bioconversion of threonine catalyzed by threonine aldolase of *Lactobacillus bulgaricus* (Frank and Hassan 1998).

1.5.5.4. Exopolysaccharide Production

Several types of polysaccharides can be produced by lactic acid bacteria which will then be classified according to their location in the cell (Degeest, et al. 2001). Bacterial exopolysaccharides (EPSs) are long-chain polysaccharides consisting of branched, repeating units of sugar derivates which are mainly glucose (D-glucose), galactose (D-galactose), rhamnose (L-rhamnose), mannose, N-acetylglucosamine, D-glucuronic acid in different ratios (Vaningelgem, et al. 2004).

Bacterial EPSs can be classified into two groups on the basis of their composition; homopolysaccharides (HoPS) and heteropolysaccharides (HePS). Basicly HoPS can be defined as polymers composed of one type of monosaccharides while HePS are the polymers of repeating units that are composed of two or more than two types of monosaccharides.

EPS is economically important because it can impart functional effects on foods and may confer beneficial health effects (Welman and Maddox 2003). Lactic acid bacteria, producing EPS, play an important role in dairy industry by improving viscosity and the texture of fermented products (Aslim, et al. 2005).

There are many factors effecting EPS yield of lactic acid bacteria such as growth medium, incubation temperature, pH, oxygen tension, agitation speed and incubation time (De vuyst, et al. 2003).

As a substitute for commercial stabilizers in yoghurt manufacture, EPS-producing cultures are commonly used due to reduction of synersis and improvement of product texture and viscosity. Some researchers demonstrated that EPS-producing

lactic acid cultures showed higher viscosity and lower degree of synersis compared with non-EPS-producing cultures (Bouzar, et al. 1996, Folkenberg, et al. 2004) .

1.5.5.5. Production of Inhibitory Compounds

One of valuable properties of starter cultures is their ability to inhibit growth of undesirable microorganisms. Reduction of pH and production of organic acids are the primary inhibitory actions of lactic acid bacteria. Thus, the pH of the medium is not suitable for many of other microorganisms. Lactic starter cultures also produce nonacidic microbial inhibitors. Hydrogen peroxide in small amounts, diacetyl, bacteriocins, secondary reaction products like hypothiocyanate are the inhibitory compounds produced in small amounts by lactic acid bacteria are the nonacidic inhibitory compounds produced by lactic starters.

Producing inhibitory compounds is one of the important parts of maintaining food quality for long time periods as a result of preventing contamination. Although there are several advantages for producing inhibitory compounds, production of nonacidic inhibitors by lactic acid bacteria is not necessarily advantageous such as autoinhibition by nonacidic compounds.

1.5.6. General Problems for Industrial Starter Production

Methods to produce concentrated cultures differ in several ways from traditional ones. Starter strains are grown under strictly controlled conditions in a medium from which the cells are easily harvested into a smaller volume (Gilliand 1985).

Starter production begins with the preparation of the inoculum, preparation of the media, fermentation at a constant pH, harvesting the cultures, continues with addition of the cryoprotectants and finishes with freezing, freeze-drying, packaging and storing.

Problems might occur during each step. One of the most important problem for industrial production of starters is the infection of cells by bacteriophages which results in lost of the activity and yield of biomass, lactic acid. If the culture is infected it can easily be observed at the end of the fermentation which results in low biomass. If the final product is yoghurt, the observation becomes more easily because there would be

no product with a gel-like structure. Both two types of yoghurt starter bacteria are susceptible to virulent phages. This problem can be overcomed by using a media supplemented with phage inhibitory substances, rotating the starters, using phage resistant strains, and working under good aseptic conditions.

Inoculation media can include some inhibitory substances. In dairy plants to control mastisis antibiotics are used on animals. If the media includes milk, there would be some antibiotic residues or natural compounds like lactins and agglutinins which breakdown the symbiotic relationship between starter culture strains that leads synersis or whey-off. This problem can be overcomed by using heat-treatment for both pasteurization and preventing inhibiton by antibiotic residues.

Routine subculturing of starter strains may decrease in activity which is also important to prevent phage sensitivity. The other problems can be caused by the addition of some flavour compounds, nitrite-nitrate addition, sugar addition or detergent residues, pollution.

1.6. Bacteriophages

Viruses of prokaryotes, called bacteriophages, behave essentially as parasites when taking over the metabolic machinery of the host for phage propagation. The phages could either contain DNA or RNA as nucleic material. The phages contain double stranded DNA, have two types of life style; lytic and lysogenic. Lytic life cycle demonstrates virulent phages, since lysogenic life cycle demonstrates temperate phages (Guttman, et al. 2005). Phages were first classified, according to Bradley´s proposal, into six morphological types based on gross morphology and nucleic acid type. For practical reasons tailed phages were subdivided according to head length to head width ratios (Ackermann 1987). Virions of tailed phages consist of a head, a tail and structures involved in fixation to the host. Nucleic acid is enclosed within head. Tailed phages are now classified into three families: *Myoviridae* (contractile tail), *Siphoviridae* (long noncontractile tail) and *Podoviridae* (short noncontractile tail). These families belong to the order *Caudovirales*.

Phages, that contain double stranded DNA, have to different types of life cycle on their hosts, lytic or lysogenic, depending on the competition between cro and CI proteins. Because both of two proteins bind same operator region (O_R and O_L) and they

can hold three binding sites in the operator from opposite directions. The protein CI is needed for beginning and to continue the lysogenic life cycle while cro is needed for lytic life cycle. When one of the cycles is stimulated by one of the proteins, the other life cycle is blocked. Either lytic or lysogenic, cycle depends on the action of the proteins (Maloy, et al. 1994).

1.6.1. Infection Mechanisms

Infection by tailed phages starts when specialized adsorption structures, such as fibers or spikes, bind to specific surface molecules or capsules on their target bacteria. Receptors for gram-negative bacteria are generally virtually any of the proteins, oligosaccharides and lipopolysaccharides. The more complex murein of gram-positive bacteria offers a very different set of potential binding sites. It depends on the clusters of one specific kind of molecule that is present in high concentrations to properly position the phage tail for surface penetration (Guttman, et al. 2005). Many phages require specific cofactors such as simply any divalent cations mostly Ca^{2+}, Mg^{2+}. It was reported that in the presence of calcium ions the decrease in free phage due to their adsorption onto the host cells was followed by an increase in the number of phage infected cells (Binetti, et al. 2001, Barbara 1980, Watanabe and Takesue 1972, Hargrove, et al. 1961).

Phages recognize host cell surface and binds irreversibly. After irreversible attachment, the phage genome passes through the tail into the host cell. Generally tail tip has an enzymatic mechanism for penetrating the peptidoglycan layer and then touching or penetrating the inner membrane to release the DNA directly into the cell; the binding of the tail also releases a mechanism that has been blocking exit of the DNA from the capsid until properly positioned on a potential host (Garen and Kozloff 1959).

1.6.1.1. Lytic Life Cycle

Lytic life cycle differs in beginning of “Latent Period” from lysogenic life cycle. Host’s RNA polymerase enzyme begins to activated with the injection of phage DNA into the host cell and the transcription of early genes are started. Transcription products of early genes protects phage genom from endonucleases synthesized by host

cell and provides rebuilding in host cell if necessary. Actually the early gene transcription products can inactivate some proteins and proteases of host cell. Middle gene transcription provides the synthesis of new phage DNA. At the end coding of the late genes causes the synthesis of proteins necessary for phage tail, fiber, capsid and beginning of the morphogenesis specific for phage (Hendrix 2002).

The structure called procapsid is the place where phage DNA is packaged and tail-capsid binds to form the structure. The last step for lytic cycle is the lysis of the host cell. There are two types of molecule for lysis occur. One of them is the enzyme lysine which makes cuts in peptidoglycan matrix, the other is holing which makes is available for lysine to reach the peptidoglycan matrix, by making pores on the membrane (Guttman, et al. 2005).

1.6.1.2. Lysogenic Life Cycle

Lysogenic cycle depends on the presence of three viral proteins called CI, CII, CIII. It begins, the production of CI, with the injection of viral DNA into the cell which prevents the transcription of the genes for synthesis the new proteins for phage particles. This makes it impossible to go in the lytic life cycle. The viral DNA in a circular form, either goes on the plasmid structure or integrates itself into the bacterial DNA. CII uses the host's transcription organelles to provide a continuous production for CI and CIII protects CII from host enzymes from hydrolysis (Hendrix 2002).

The genome integrated into the bacterial genome is called prophage and the bacteria having prophage is called lysogen bacteria. Therefore the viral DNA is going to be replicated all time when bacterial DNA replicates itself. After bacteria is divided, the new cells are going to be resistant for the same phage because of having an integrated phage DNA. This is called superinfection and is one of the phage resistance mechanisms. Prophage can be induced by UV, mytomycin C, mutagenic molecules and the changes in the incubation temperature (Sanders 1988, Hendrix 2002, Lerner 2003). Detection of prophage in starter bacteria is most important due to its lysis risk during fermentation. For this purpose, it was reported in many ways to determine whether it contains a prophage or not by inducing methods. If the bacteria contains prophage, after induction by chemicals, temperature or UV, it would be converted into a lytic phage and can be determined by double layer agar method (Deutsch, et al. 2002).

1.6.2. Phages of Dairy Bacteria

Commercial fermentative processes are vulnerable to virulent bacteriophages ubiquitous in the dairy environment, which can lyse the starter bacteria, thereby delaying lactic acid production and even stopping the fermentation process that causes a big economic loss.

Lactic acid bacteria phages were first discovered in the 1930's due to the problems in dairy industry. The first signal for phage infection is slow acidification depending on the phage titer and the time for latent period. If one of the starter bacteria is infected by phage, as a result of blocking the symbiosis acid production will be slower and product quality would be unpredictable. Yoghurt would not have a gel like structure also the aroma caused by lactic acid would not be as predicted as, therefore it can easily be observed if one of the strains is infected by phage.

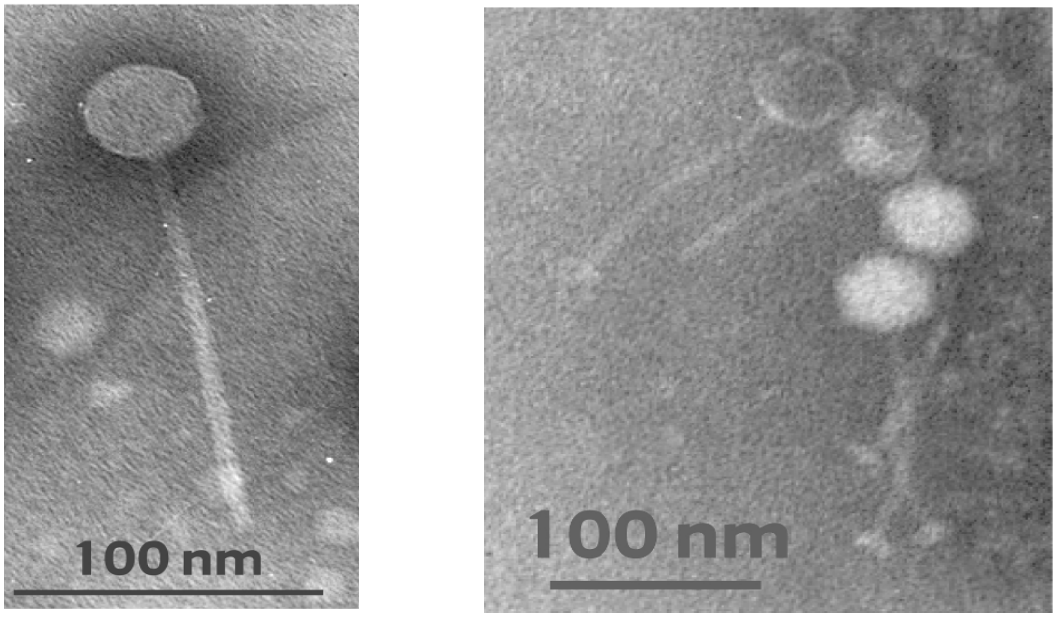


Figure 1.5. *Streptococcus thermophilus* B3-X18 phage (left) and *Lactobacillus bulgaricus* Y4-X4 phage (Source : Acar 2007)

Increasing the use of thermophilic cultures caused mechanization in production, raise in large scale plants and all these are the starting points for phage problem in dairy industry. Yoghurt starter bacteria phages were isolated from commercial dairy plants (in

Ankara and characterized by Acar). Figure 1.5. demonstrates phage micrograph for both two species of yoghurt bacteria.

It is still a question that what the reason for phage infection. It can be either milk or lysogenic strains. *Lactobacillus bulgarius* and *Streptococcus thermophilus* virulent and temperate phages have homology in their structure and this can be the reason that the lysogenic strains are the main cause of phage infections. Although lysogenic phages are common among lactic acid bacteria, lysogenic strains are rarely seen in yoghurt starter bacteria (Acar 2007).

The knowledge about thermophilic phages are less than the knowledge about lactococcal phages. Because lactococcal phages were isolated at 1935 first, *Streptococcus thermophilus* phages were isolated in 1952 and *Lactobacillus bulgaricus* phages were isolated in 1974 (Tunail, et al. 2000).

1.6.3. Phage Resistance Mechanisms

There are four main phage resistance mechanisms; prevention of phage adsorption and DNA injection, abortive infection, restriction–modification systems (Moineau 1999).

Prevention of phage adsorption can be explained by the changes on receptor sites on cell surface or blocking the adsorption by a component produced by bacterial cell. The other mechanism, preventing DNA injection has the same mechanism caused by cell surface. In abortive infection phage DNA is injected in cell envelope. Resistance mechanism plays a role in RNA transcription, translation, synthesis of structural proteins, packaging DNA steps (Coffey and Ross 2002).

Restriction–modification system has four types and consists of two enzymes activities. Modification system binds methyl group on DNA recognition sites and thereby protects host DNA from phage DNA. Restriction system makes differences on phage DNA (Birge 2000).

1.6.4. Preventing Phage Inhibition

Preventing phage inhibition requires implementation of control measures throughout the manufacturing process. Controlling processes should include selection,

preparation, maintenance of cultures free of virulent phage, controlling entry of phages into the processing facility, controlling the spread of phages within the facility (Frank and Hassan 1998).

1.6.4.1. Use of Phage – Resistant Strains

The use of effective bacterial starter cultures is critical to obtain quality products. It is very long and expensive operation, strain selection, based on several microbiological, biochemical and technical criteria (Moineau 1999).

Whenever possible, a mixture of two or three well characterized bacterial strains should be used to prevent possible phage infection of the entire starter culture and subsequent fermentation process failures. A basic operation is to determine the phage-host range with a collection of potential starter bacteria. This knowledge then allows the rational design of a starter rotation system.

Phage-resistant strains have been isolated from mixed culture systems that maintain activity while carrying low levels of phages. Strains can also be genetically altered to contain plasmids coding for phage resistance (Klaenhammer 1991).

Phage resistant variants can be selected by exposure the plant products or byproducts containing phages that have developed during manufacture. Resistant strains are tested for rapid acid production and added back to the starter used in the plant. This mechanism requires daily monitoring the system products whether phage is present or not. Also it allows the use of a single mixture many defined strains over a long time (Frank and Hassan 1998).

1.6.4.2. Culture Rotation

Culture rotations control bacteriophage infection by limiting the length of time that a specific strain or mixture of strains used. In the series of culture following each other, they would be susceptible to different phage types and therefore unaffected by phages that may have infected the previous culture. In a dairy plant cultures can be rotated after each vat of milk is inoculated. By rotation of cultures phages can not be eliminated totally , but the number of phages can be kept under 10^4 pfu /ml, which does not affect the acid production. Culture rotation is limited by the availability of

phage-unrelated strains with acceptable fermentation properties and sometimes using many different cultures can result in lack of product uniformity (Frank and Hassan 1998).

1.6.4.3. Phage Inhibitory Media

Lactic acid bacteria growth medium can be generated by using phage inhibitory substances to control phage inhibition. The growth media rely on the ability of phosphate and citrate salts to bind free calcium ions in the raw material. It was demonstrated that the presence of calcium ions not only stabilizes the coiled DNA but also improves the adsorption rates and controls the penetration efficiency of phage DNA into the bacterial cells (Quiberoni, et al. 2003). It was found in 1972 that Ca^{2+} ions are not required in adsorption stage but necessary for intracellular mechanisms which results in cell lysis (Watanabe and Takesue 1972).

Common phage control media often contain deionized whey, protein hydrolizates, ammonium phosphate, sodium phosphate and citrate salts and other growth stimulants such as yeast extract (Frank and Hassan1998).

1.7. Growth Medium Ingredients for Thermophilic Starter Culture Production

In a biotechnological process, it is always aimed maximum yields. Yield can either be biomass or productivity. Optimum growth media for all biotechnological processes has to contain a suitable energy source, nitrogen source, mineral elements, possibly vitamins as growth factors and water.

The compounds purity and quality depends on the scale of the fermentation regarding to economic losses. If the fermentation is carried out in a small scale the compounds as media ingradients can be used in their pure form and if it is a large scale fermentation media ingradients can be preferred in their cheaper forms like whey, skim milk as the carbon source.

1.7.1. Growth Media Formulations

Lactic acid bacteria can not synthesize various vitamins and amino acids. Supplementation of growth media with a source of vitamin and amino acids is necessary for this purpose.

A wide variety of substrates can be used as carbon source in lactic acid fermentations. The properties of a good substrates are; low cost, low levels of contamination, faster fermentation time, high lactic acid yields, little or no by product formation, ability to be fermented with little or no pre-treatment, year round availability (Vick Roy 1985).

Whey or whey-permeate has the potential as a culture medium for the propagation of dairy cultures. Whey or ultrafiltrated whey permeate are cheap and readily available sources for use as fermentation media, but require supplementation with a complex additive like yeast extract (Bury, et al 1998).

Whey is a liquid by-product of cheese manufacture generated in great amounts. During cheese manufacture fat and casein proteins aggregate into a curd whereas soluble whey proteins (lactalbumins and lactoglobulins), lactose and minerals are drawn off in the whey (Martinez, et al. 2002).

Whey contains 65 gram of solids per kilogram, comprising about 50 gram of lactose, 6 gram of protein, 6 gram of ash, 2 gram of nonprotein nitrogen and 0.5 gram of fat (Zadow 2003)

In 1984 Jones and Greenfield described the macro and micronutrients in acid cheese whey. According to them whey contains nitrogen, phosphorus, sulphur, potassium and magnesium as macronutrients and calcium, iron, chlorine, copper, sodium as micronutrients.

Nitrogen is an important ionic growth factor determining the rate of fermentation and controls the synthesis of protein and nucleic acid and. Phosphorus plays an important part in carbohydrate metabolism. Sulphur is used as a constituent in proteins such as amino acids (cysteine and methionine) and co-enzymes such as carboxylases. Potassium enhances tolerances to toxics and involved in control of intracellular pH while stabilizing the optimum pH for fermentation. Magnesium buffers the cell against adverse environmental effects and involved in activating sugar uptake. Calcium is essential for the growth of cells and stimulates it. Iron is an important

micronutrient as a result of being in the active sites of many cell proteins. Chlorine passively diffuses in to cells and stimulates the uptake of some sugars. Inspite of being extremely toxic to the cell, copper is used in the synthesis of some enzymes and proteins and in the electron transport systems. Sodium acts as a counter iron in the movement of some positive ions (Ghally, et al. 2003).

Yeast extract is a complex of vitamins including thiamine, riboflavin, pyridoxine, niacinamide, pantothenic acid and a mixture of amino acids, peptides, a rich elemental profile composited of sodium chloride, copper, iron, magnesium, potassium and sodium. Unless otherwise stated all these constituents make yeast extract a potential nitrojen source enriched with vitamins.

In 1961, by Hargrove and his co-workers, it was demonstrated the various phosphate and citrate salts effect on phage proliferation when added to the fermentation medium via binding free calcium ions in the raw material. Beside being a buffer, phosphate salts and citrate salts has an inhibitory effect on phage adsorption, which makes them more preferable for starter culture production.

1.7.2. pH Control During Fermentation

Each microorganism has an optimum pH for maximum yield. Lactic acid bacteria decreases pH continuously while producing lactic acid and decrease the pH under optimal range which causes slow acidification, slow growth. As the pH continues to decrease, cells become susceptible to sublethal acid injury and therefore lose their activity. Therefore pH control has to be done either external or internal.

External pH controlling system refers to a culture preparation system in which neutralizing agent is added to the medium during fermentation mechanically or manually. In one-step control the pH of the medium is allowed to decrease to approximetely 5.0, after which sodium or potassium hydroxide is added to obtain a pH of 6.5 to 7.0. Multiple step neutralization involves a mechanical system consisting of a pH electrode in the bottom of the culture tank, a pump for adding neutralizer to the tank and a controller. External pH control is advantegous for requiring less phosphate as phage inhibitory substance because calcium is less soluble at higher pH. However it has some disadvantageous that it allows growth of nonstarter microflora at higher pH even after lactose is deplated (Hassan 1998).

Internal pH control is a system to produce cultures in a medium with insoluble neutralizing agents. The agent is released in response to acid production. Internal pH control has same advantegous to external pH control systems in addition that there is no need to install a mechanism to add the agent into the medium. Altough the medium has to be stirred to keep the insoluble neutralizing agent suspended during fermentation.

1.8. Batch Fermentation and Fermentation Constants

Growth of viable cells requires intimate contact of a small quantity of living cells with a liquid medium containing appropriate levels of nutrients at a suitable pH and temperature. When a seed culture is inoculated into the nutrient medium , cells selectively take up dissolved nutrients from the medium and convert them into the biomass.

Bacterial growth includes five main phases; lag phase, exponential growth phase, deceleration phase, stationary phase and death phase. Fermentation begins with lag phase when seed culture is inoculated into a new environment. Cells are adapted to this environment by new enzyme synthesis and repression of some other enzymes. During this phase cell mass may increase a little without an increase in cell number density. There would be a pseudo-lag phase because of the small inoculum size or poor condition of the inoculum. After cells have adapted to their new environment an increase in cell density number begins in which the phase is named exponential or logaritmic growth phase. Cell number density increase exponentially with time. As a result of balanced growth in which all components of a cell grow with the same rate. Specific growth rate μ (1/h) is determined as a function of time and independent of the concentrations of the nutrients.

In deceleration phase, growth decelerates due to either depletion of one or more essential nutrients or toxic waste accumulation. Deceleration phase is a short time period with an unbalanced growth of culture. Increase in cell number due to cell divisions exactly balanced by a decrease in cell number due to death. Cell death may result from nutrient limitation and toxic waste accumulation. The last part of bacterial growth is death phase in which cells are dying at an exponential rate (Shuler and Kargi 2002).

The concentration of biomass X, increases as a function of time due to conversion of raw material to biomass.

According to Monod this function can be described with some equations ;

$$\frac{dX}{dT} = \mu X \tag{1.1}$$

where μ is the specific growth rate constant obtained from exponential growth phase.

$$\mu = \mu_{max} \frac{S}{Ks + S} \tag{1.2}$$

where μ_{max} is the maximumspecific growth rate and K_s is the saturation constant. K_s is equal to the substrate concentration at $\mu = \mu_{max} / 2$.

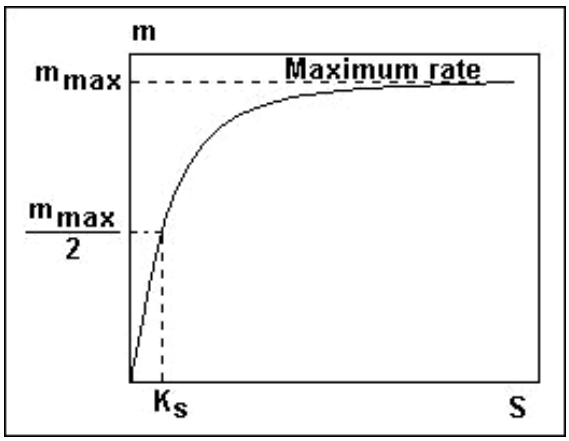


Figure 1.6. Specific growth rates versus initial substrate concentraion
(Source: Shuler and Kargi 2002)

$$\frac{1}{\mu} = \frac{1}{\mu \max} + \frac{Ks}{\mu \max} \frac{1}{[S]} \tag{1.3}$$

The kinetic constants such as μ_{max} and K_s are determined by using Lineweaver-Burk double reciprocal ($1/\mu$ versus $1/S$) plot (equation 1.3) With a slope of K_s / μ_{max} and intercept of $1/\mu$ estimated μ_{max} and K_s values can be calculated.

Monod equation is one of the most preferred unstructured growth model which gets along with the experimental data if cell density and growth velocity of the cells are low. Growth rate depends on the substrate concentration and increases with higher

initial substrate concentrations until it reaches a maximum value. Despite the fact that even the substrate concentration is increasing, if growth rate reaches a maxima, growth rate would not change as a result of substrate is no longer a limiting factor. When continue to increase the substrate concentration because of substrate inhibition, growth rate begins to decrease.

In a fermentation process productivity of the batch is the most important factor which has to be considered. Productivity is defined as the final product concentration divided by the time from inoculation to the delivery of the batch (Elmer and Gaden 2000). Since the productivity is the most important case, yield coefficients have to be described which are based on the amount of consumption of another material. The growth yield and product yield may be defined by some basic equations ;

$$Y_{X/S} = - \frac{\Delta X}{\Delta S} \tag{1.4}$$

$$Y_{P/S} = - \frac{\Delta P}{\Delta S} \tag{1.5}$$

where $Y_{X/S}$ is growth yield and $Y_{P/S}$ is product yield.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals

Chemicals used in this study and their codes are given in Appendix A.

2.1.2. Media

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

MRS and M17 media were used for the activation and the enumeration of *Lactobacillus bulgaricus* and *Streptococcus themophilus* respectively. Media preparation and ingredients are given in Appendix B.

2.1.3. Microorganisms

Bacteria used in this study were isolated before from artisanal yoghurt samples , both *Streptococcus themophilus* and *Lactobacillus bulgaricus* strains were biochemically and genetically characterized in a previous study (Erkus 2007). The strains were selected for this study according to their properties to be a typicall starter bacteria (Celik 2007).

2.1.4. Bacteriophages

Bacteriophages used in this study were from the phage collection of yoghurt starter bacteria isolated and characterized in a previous study in Ankara Unıversity

(Acar 2007). Twenty-six phages specific to *Streptococcus thermophilus* and twenty-four phages specific to *Lactobacillus bulgaricus* strains with their host strains were used, which produce clear plaques in phosphate salt effect experiments used in soft agar.

2.2. Methods

2.2.1. Culture Propagation

The isolates were stored at -80ºC in 20% (v/v) glycerol stock solutions for further analysis. The glycerol stock solutions were prepared by mixing 0.5 ml of active cultures with 0.5 ml of sterile MRS and M17 broths for *Lactobacillus bulgaricus* and *Streptococcus thermophilus* respectively.

The cultures were activated by transfering cultures from stock solutions in to sterile broths with an inoculation ratio of 2%, followed by incubation at 43ºC for 18 hours. In all experiments activation was done twice in following two days.

2.2.2. Construction of Growth Curves

MRS and M17 broths were inoculated with an inoculation ratio of 2% and left for incubation at 43 ºC. Samples were taken at equal time intervals of 30 minutes for optical density (OD) measurements and enumaration of viable cells. Optical density was determined with a Varian Cary 100 Bio UV-Visible Spectrophotometer at 600nm. Enumaration of viable cells were done according to pour plate technique as described in section 2.2.3.

OD values and viable cell counts were plotted versus time to obtain a growth curve for both cocci and bacilli. Growth curves were constructed in order to determine the right incubation time and OD values for maximum acitivity of the cultures for fermentations.

2.2.3. Enumaration of Viable Cells

Streptococcus thermophilus and *Lactobacillus bulgaricus* counts were determined using M17 (pH 6.9) and MRS (pH 6.3) agars. Samples were decimally diluted in 9 ml sterile peptone water (0.1%). One ml aliquot dilutions were pour plated and incubated anaerobically at 45ºC for 48 hours for *Lactobacillus bulgaricus* and aerobically at 42ºC 48 hours for *Streptococcus thermophilus*. Anaerobic conditions were provided using AnaeroGen in plastic Anaerobic Jars (Oxoid). Plates having 25-250 colonies were counted and the results were expressed as colony forming units per mililiter (cfu/ml).

2.2.4. Determining the Phage Sensitivity of Strains and Effect of Phage Inhibitory Salts on Phage Inhibition

2.2.4.1. Phages and Strains

Bacteria used in this part of study are represented in Table 2.1.

Table 2.1. *Streptococcus thermophilus* and *Lactobacillus bulgaricus* strains

Streptococcus thermophilus	*Lactobacillus bulgaricus*
TY55	TY30
TY25	22
95-1	TY77-a
63-2	TY24

Bacteriophages used in this study were isolated from dairy plants in Ankara and Table 2.2. represents the phages used in experiments. In order to determine phage titers for the effect of phosphate salts host strains of these phages were used to obtain clear plaques in soft agar.

Table 2.2. Bacteriophages specific to *Streptococcus thermophilus* and *Lactobacillus bulgaricus*

Streptococcus thermophilus phages	***Lactobacillus bulgaricus phages***
Φ 231 CH1A	ΦX1
Φ709-51	ΦX2
Φ231S-A1Bö	ΦX3
Φ231-50	ΦX4
Φ231-51	ΦX5
Φ231-52	ΦX6
Φ231-53	ΦX7
Φ24.1.2h	ΦX8
Φ25.1.2h	ΦX8
Φ4b-1	ΦX10
ΦB3-CH1A	ΦX11
Φ709-53	ΦX17
ΦB3-50	ΦX19
ΦB3-51	ΦX20
ΦB3-52	ΦX21
ΦB3-53	ΦX22
Φ204-2h	ΦX23
Φ21.4.2h	ΦX24
Φ23.4.2h	ΦX25
Φ24.4.2h	ΦX26
Φ44-4	ΦX27
Φ1B3A	ΦX28
Φ2B3A	ΦX29
Φ709-CH1A	ΦV1L-A
Φ709-50	
Φ709-52	

2.2.4.2. Enrichment of Phage Titer

Bacteria were activated at 43ºC overnight and the active culture was reactivated until the optical density of the cultures reaches 0.5 absorbance at 600 nm (nearly 4 hour old culture, determined from OD-time bacterial growth curves). MRS broth with Ca^{2+} addition and thM17 broth were prepared, sterilized at 121ºC for 15 minutes. 100 µl of phage and 100 µl of culture was mixed gently and incubated at 43ºC for 15 minutes.

Mixtures of phage and bacteria was supplemented with 10 ml of sterile broths and left for incubation overnight at 43ºC. At the end of the incubation the mixture was centrifuged at 10000 rpm for 5 minutes to remove adsorbed phage-bacteria mixture and supernatant which include unadsorbed phage particles was used as new stocks. Supernatant was tested for the enumaration of phage titer. After it was obtained 10^{8-9} pfu/ml (plaque forming unit/ml) phage titers, they were passed through a filter with 0.45 µm pore size and stored at -20 ºC with addition of 20% glycerol stock solution.

2.2.4.3. Determination of Phage Titer

Serial dilutions were done with sterile serum physiologic water (0,1%NaCl) until 10^6. Double layer agar method was used to determine the plaque numbers of each dilution. Then 0.1ml of 4 hours culture was taken and mixed with 3 ml of soft agar, poured on the layer of the plate prepared before with bottom agar. 10µl from each dilution was poured on to the layer of top agar and left for incubation at 43ºC for 18 hours. Plaques were counted and results were expressed as pfu/ml.

2.2.4.4. Determining the Phage Sensitive/Resistant Strains

According to the double layer agar method, all strains were tested for all phages including *Streptococcus thermophilus* phages to *Lactobacillus bulgaricus* strains and vice versa. Plaques were counted and strains were defined as resistant or sensitive according to the presence of plaques.

2.2.4.5. Effect of Phosphate Salts on Cell Viability and Phage Infection

Bacteria were activated in MRS and M17 broths for 18 hours at 43ºC and transferred for second activation step to new broths. Exponentially growing cultures (OD_{600nm}=0,6 for *Lactobacillus bulgaricus* and 0,7 for *Streptococcus thermophilus*) were mixed with phages with a multiplicity of infection 0,01 (MOİ). Phage and bacteria mixture (total volume=0,1 ml) were mixed gently and left for incubation at 43ºC for 15 minutes. Media including whey powder, yeast extract and phosphate salts were added and the final mixture was incubated at 43ºC for 18 hours. The medium were

centrifuged at 10000 rpm for 5 minutes at +4ºC to remove cell debris and to obtain unadsorbed phage particles. Phage plaques were enumarated according to double layer agar method and results were judjed for determining effect of salts with comparision of non-phosphated media's results.

Effect of phosphate salts on cell viability was determined by pour plate technique. All phosphate salts were examined in different concentrations (1%, 2% and 3%). Growth medium including whey powder (26 g/L) and yeast extract (7g/L) were sterilized at 113ºC for 10 minutes. Samples were taken when their pH reaches 4.4 and viable cells were enumarated. Phosphate concentration which the maximum cell counts were obtained was used in phage experiments in growth media.

2.2.5. Mineral Requirements of Mixed Starter Cultures

Iron, magnesium and manganese requirements were determined according to general factorial design. $FeSO_4.7H_2O$ for iron, $MnSO_4.H_2O$ for manganese, $MgSO_4.7H_2O$ for magnesium were used for mineral addition.

Lactose (20 g/L) and yeast extract (7g/L) concentrations were kept constant in order to determine the effect of minerals. All components including lactose, yeast extract and minerals were sterilized separately.

Experimental design was done by Design Expert Version 7.0. The design was randomized general factorial design with three variables (Fe, Mn, Mg). Three levels of Fe and Mn , two levels of Mg were used for design as given in Table 2.3. According to the run table all experiments were done in randomized order, run order of experiments are given in Table 2.4.

The analysis of variance (ANOVA) tables were generated. The effect of factors and the interaction terms were determined. Significances of all terms were judged according to their p-values ($p \leq 0.05$).

Table 2.3 Factors and Their Levels

Factors	Coded variables	Levels(ppm)
Fe	X1	10,20,30
Mn	X2	5,10,15
Mg	X3	25,50

Table 2.4. Run order of experiments in shake flasks

			Factor 1	Factor 2	Factor 3
Std order	**Run order**	**Block**	**X1 : fe**	**X2:mn**	**X3:mg**
25	1	Block 1	10	10	50
9	2	Block 1	20	10	25
3	3	Block 1	20	5	25
27	4	Block 1	20	10	50
35	5	Block 1	30	15	50
31	6	Block 1	10	15	50
9	7	Block 1	30	10	50
5	8	Block 1	30	5	25
23	9	Block 1	30	5	50
33	10	Block 1	20	15	50
1	11	Block 1	10	5	25
13	12	Block 1	10	15	25
7	13	Block 1	10	10	25
11	14	Block 1	30	10	25
17	15	Block 1	30	15	25
21	16	Block 1	20	5	50
15	17	Block 1	20	15	25
19	18	Block 1	10	5	50
24	19	Block 2	30	5	50
6	20	Block 2	30	5	25
22	21	Block 2	20	5	50
36	22	Block 2	30	15	50
34	23	Block 2	20	15	50
8	24	Block 2	10	10	25
12	25	Block 2	30	10	25
16	26	Block 2	20	15	25
20	27	Block 2	10	5	50
30	28	Block 2	30	10	50
4	29	Block 2	20	5	25
10	30	Block 2	20	10	25
2	31	Block 2	10	5	25
14	32	Block 2	10	15	25
32	33	Block 2	10	15	50
28	34	Block 2	20	10	50
26	35	Block 2	10	10	50
18	36	Block 2	30	15	25

2.2.6. Fermentation Constants of Mixed Starter Cultures

Fermentation kinetics μ_{max} (maximum specific growth rate) and K_s (monod saturation constant) were determined from a series of fermentation. All media included whey powder, yeast extract and phosphate mixture at constant concentrations and lactose with different concentration for each batch. In order to determine kinetic constants and the concentration in which culture would be saturated for lactose, single strain inoculations were done seperately for both *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. Finally mixed culture fermentations were performed.

All experiments were done by Sartorius Biostat® Qplus fermenter with 6 separate operational fermenter vessel and data were collected via Biostat MFCS/DA software. Working volume for each batch was 0.8 L. The pH of the medium kept constant at 6.6 for mixed cultures, 6.2 for *Lactobacillus bulgaricus* and 6.9 for *Streptococcus thermophilus* by automatic addition of 6N NaOH. Temperature was kept constant at 43ºC and agitation was maintained by continuous stirring at 100rpm.

Samples were taken at 0, 2, 4, 6, 8^{th} hours for cell counting in mixed culture fermentations. In single culture inoculations samples were witdrawn at every 30 minutes time intervals for measurements. Total fermentation time was 8 hours for mixed culture fermentation, 6 hours for *Lactobacillus bulgaricus* and *Streptococcus thermophilus* single culture fermentations.

Growth curves for each fermentation were constructed and specific growth rates for each fermentation were determined from the slope of the curve in logaritmic phase. All specific growth rates were plotted versus lactose concentrations in order to determine maximum specific growth rate and monod saturation constant. According to the differences of maximum specific growth rates data obtained for mixed culture fermentations and single strain fermentations symbiotic growth of two strain was judged. The lactose concentration in which the maximum growth rate obtained was expressed as saturation concentration for substrate.

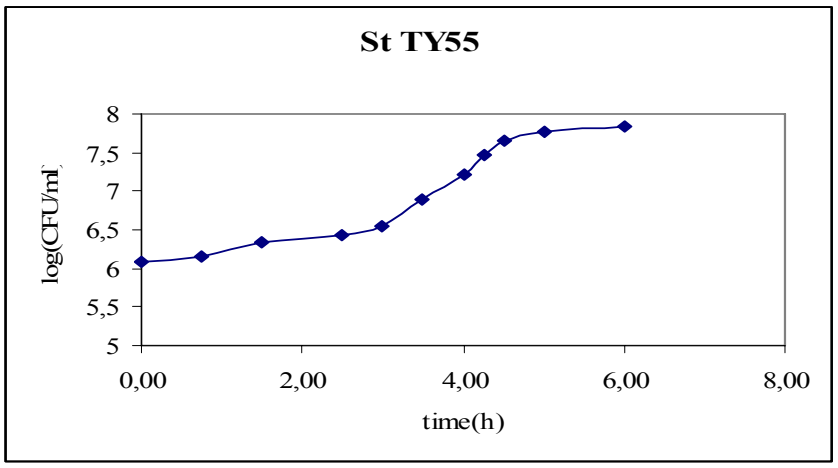
CHAPTER 3

RESULTS AND DISCUSSION

3.1. Growth Curves

The results obtained from growth curves of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* strains were used in further studies in order to see the growth phases. Cultures were used in the middle of logarithmic phase for further studies as a result of being highly active in logarithmic phase.

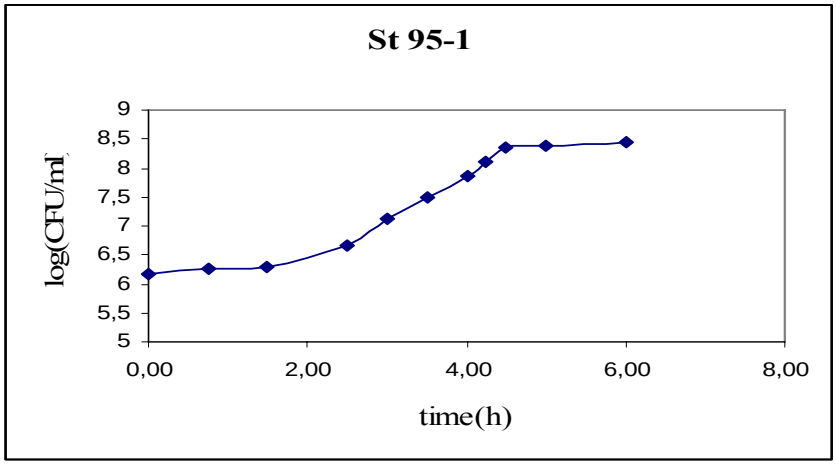
Strains for *Streptococcus thermophilus* and *Lactobacillus bulgaricus* were selected according to their properties as a starter culture. In phage experiments 4 different *Streptococcus thermophilus* strains and 4 different *Lactobacillus bulgaricus* strains were used.



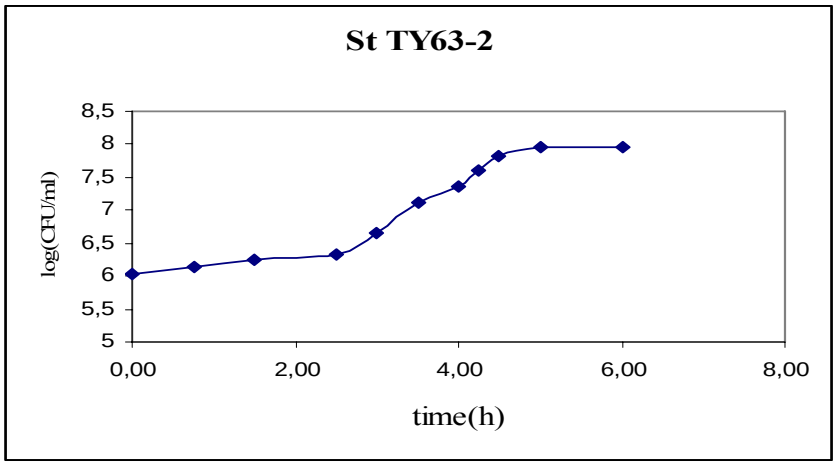
a

Figure 3.1. Growth curves of Streptococcus thermophilus strains in M17 broths a) Growth curve of TY55, b) Growth curve of 95-1, c) Growth curve of TY63-2, d) Growth curve of TY25

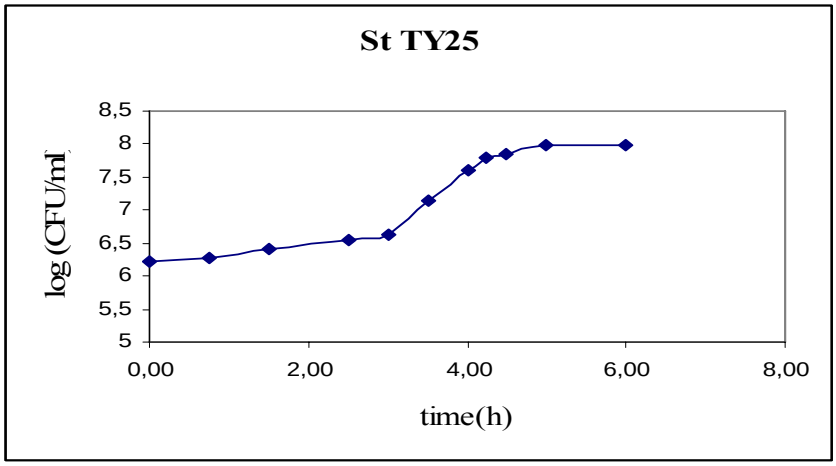
(cont. on next page)



b



c

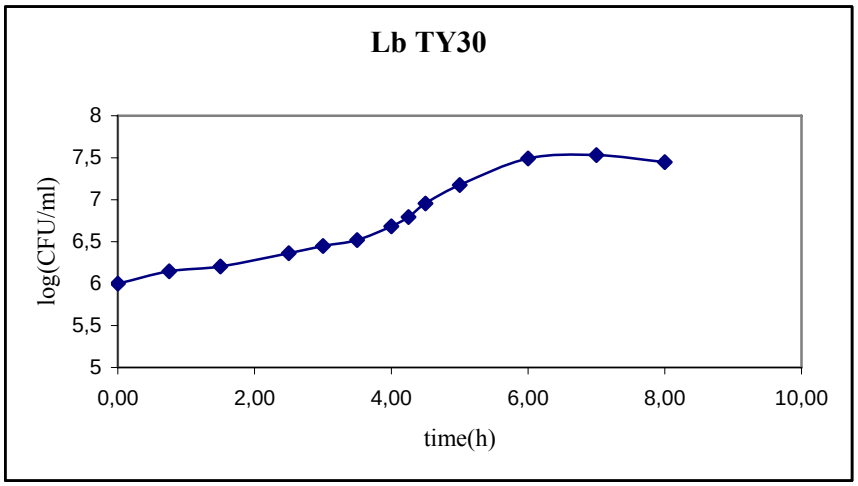


d

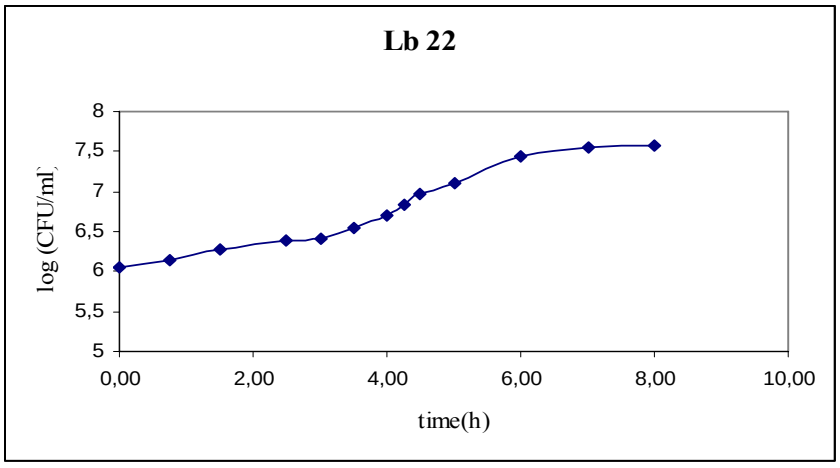
Figure 3.1.(cont.) Growth curves of *Streptococcus thermophilus* strains in M17 broths. a) Growth curve of TY55, b) Growth curve of 95-1, c) Growth curve of TY63-2, d) Growth curve of TY25

Streptococcus thermophilus strains were activated in M17 broths and the growth curves were constructed when they were grown in M17 broths. The growth rates of TY55, TY63-2, 95-1 and TY25 were 0.610 h^{-1}, 0.655 h^{-1}, 0.642 h^{-1} and 0.674 h^{-1} respectively. The stationary phase was seen after 5 hours of fermentation and the logarithmic phase of growth was in between 3-5 hours for 4 strains as seen in Figure 3.1.

Lactobacillus bulgaricus strains were activated in MRS broths and growth curves were constructed by using MRS . The growth rates of TY30, 22, TY24, TY77-a were 0.405 h^{-1}, 0.369 h^{-1}, 0.373 h^{-1}, 0.333 h^{-1} respectively. The stationary phase was observed after 6 hours of fermentation as seen from Figure 3.2.



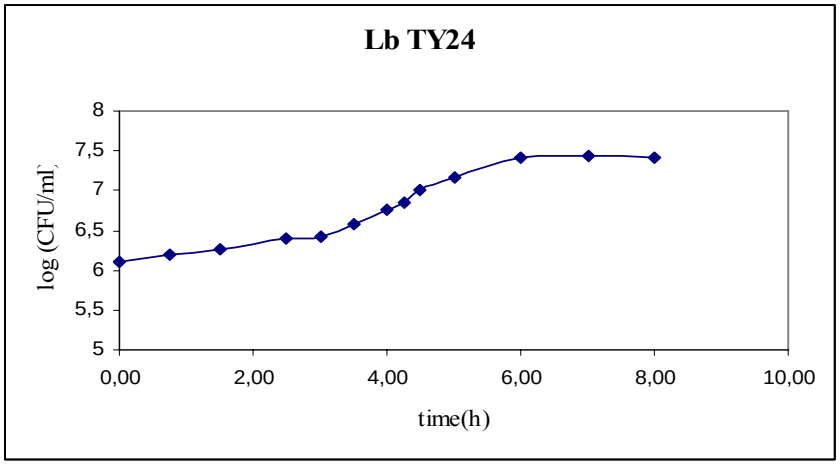
a



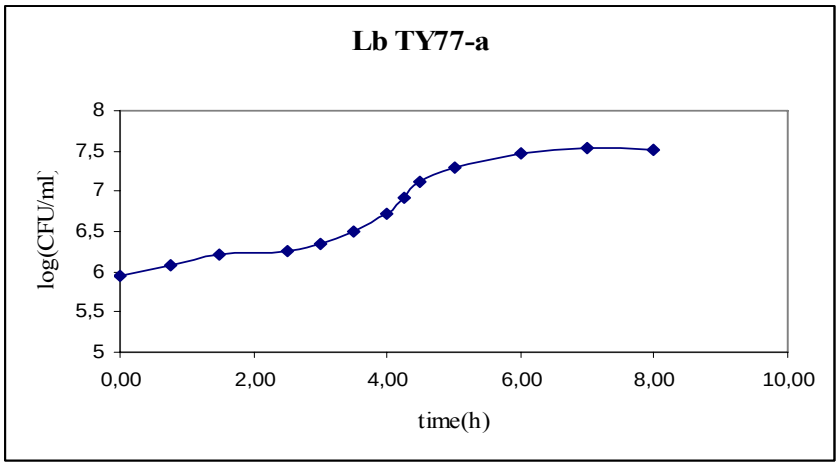
b

Figure 3.2. Growth curves of *Lactobacillus bulgaricus* strains in MRS broths. a)Growth curve of TY30, b)Growth curve of 22, c)Growth curve of TY24, d) Growth curve of TY77-a

(cont. on next page)



c



d

Figure 3.2.(cont.)Growth curves of *Lactobacillus bulgaricus* strains in MRS broths a)Growth curve of TY30, b)Growth curve of 22, c)Growth curve of TY24, d) Growth curve of TY77-a

Optical density of all strains were determined at 600 nm. The results were compared with cell viability results. According to OD values the same curves were obtained. The sample taken at middle of the logaritmic phase were measured and the OD values were 0.705, 0.680, 0.725, 0.780 for TY25, TY55, TY63-2 and 95-1 strains of *Streptococcus thermophilus*. Similarly the OD values were 0.600, 0.760, 0.680, 0.720 for TY30, TY24, 22 , TY77-a strains of *Lactobacillus bulgaricus* strains.

Before each fermentation the OD measurements were done for further studies, with the help of dilutions, the constant OD values were used for inoculation.

3.2. Determining Phage Sensitivity of Strains and the Effect of Phage Inhibitory Salts on Phage Infection and Cell Viability

3.2.1. Selection of Phage Sensitive/Resistant Strains

All *Lactobacillus bulgaricus* phages were examined against both *Streptococcus thermophilus* strains and *Lactobacillus bulgaricus* strains by double layer agar method. As predicted before, there were no detectable plaques for *Lactobacillus bulgaricus* phages on *Streptococcus thermophilus* strains.

Among *Lactobacillus bulgaricus* phages some of them were effective on *Lactobacillus bulgaricus* strains as given in table 3.1

Table 3.1. Phage – bacteria combinations which give clear plaques for *L.bulgaricus*

Strains	**TY24**	**TY30**	**22**	**TY77-a**
Phages	Resistant to all phages	X11 X26 V1L-A X28 X30	X11 X26 V1L-A X30	X9 X11 X26 V1L-A

As seen from the table the most sensitive strain is TY30 while TY24 is the most resistant one. Among 24 phages of *Lactobacillus bulgaricus* X11, X26 and V1L-A caused clear plaques for all strains used. As a result, X11, X26 and V1L-A were chosen for further studies including phosphate/citrate salt effects on phage infection.

Out of 26 phages specific to *Streptococcus thermophilus* strain, only 3 of them were effective on all of 4 strains. The most sensitive strain was TY63-2 with a sensitivty of 5 phages. Also TY55 was the most resistant strain. There were no plaques for all combinations of phages to bacteria TY55 as seen from table 3.2

Table 3.2. Phage – bacteria combinations which give clear plaques for *S.thermophilus*

Strains	**TY55**	**TY25**	**95-1**	**TY63-2**
Phages	Resistant to all phages	709.50 23.4.2h 231.CH1A	709.50 23.4.2h 231.CH1A	709.50 23.4.2h 231.CH1A 709.51 B3.51

The phages 709.50, 23.4.2h and 231.CH1A were chosen for further studies including phosphate citrate salt effects on phage infection.

3.2.2. Effects of Phosphate/Citrate Salts on Culture Viability

The effect of various phosphate salts on bacteriophage proliferation in yoghurt starter cultures were examined with the aim of developing a reliable medium for preventing phage adsorbtion and cell lysis. In order to determine the effects of phosphate salts, it was necessary to determine the effects of these salts whether they promote or inhibite cell growth.

Effects of salts were determined by 100 ml erlenmayer fermentations at constant temperature. The beginning pH of all media were adjusted to 6.2 and the temperature were kept constant at 43ºC. Inoculations were done with a ratio 2% of the medium with a mixed culture of bacilli and cocci at equal volumes. Cell viability measurements by pour plate technique were done at 8^{th} hour of fermentation and results were compared with control.

Experiments were done for three different concentrations of seven salts. These salts combinations were; magnesium hydrogen phosphate and potassium di hydrogen phosphate, potassium hydrogen phosphate and potassium di hydrogen phosphate, sodium phosphate and potassium di hydrogen phosphate, amonnium hydrogen phosphate and potassium di hydrogen phosphate, amonium formate, sodium citrate, sodium citrate and adipic acid. All these combinations were examined for 1% , 2% and 3% additions into the growth medium which contains 26 g/L whey powder and 7 g/L yeast extract.

Mixed culture combinations were constructed according to phage sensitivity results. Therefore, 3 strains of *Streptococcus thermophilus* (TY25, 95-1,TY63-2) and

three strains of *Lactibacillus bulgaricus* (TY30, 22, TY77-a) were chosen. All experiments for 8 different salt combination were examined for 9 possible mixed culture combination. Results for each culture combination were expressed as mean of replicates and log CFU/ml in Table 3.3, 3.4, 3.5, 3.6, 3.7,3.8 ,3.9 ,3.10 ,3.11.

Table 3.3. Effect of salts on cell viability for mixed culture of TY25*TY30

Ratio of salts in the medium	**1%**		**2%**		**3%**	
Culture combination	**TY25**	**TY30**	**TY25**	**TY30**	**TY25**	**TY30**
Mg_2HPO_4 + KH_2PO_4	8,7386	8,6021	8,8290	8,6809	9,3741	8,5731
K_2HPO_4 + KH_2PO_4	8,8406	8,7386	8,9444	8,8116	8,9287	8,7471
Na_2HPO_4+ KH_2PO_4	8,8891	8,7386	8,9828	8,8290	8,9515	9,3237
$(NH_4)_2HPO_4$+ KH_2PO_4	8,8795	8,7553	8,9590	8,7853	8,9771	8,7312
Amonium formate	8,6505	8,4771	8,6106	8,5731	8,6178	9,0502
Sodium citrate	8,7782	8,6276	8,8741	8,5396	8,7720	8,5378
Sodium citrate + adipic acid	8,8976	8,6611	8,7386	8,4287	8,5880	8,5396
control	8,8542	8,7386				

The strain *Streptococcus thermophilus* TY25 and *Lactobacillus bulgaricus* TY30 growth was highy effected by addition of phosphate salts in different amounts. According to results the maximum growth was observed with addition of Na_2HPO_4+ KH_2PO_4 in 2% amount. Viable cell number was decreased with increasing amounts of phosphate to 3%. In addition non-phosphate salts did not cause the same effects like phosphate salts. Addition of ammonium formate and sodium citrate resulted with decreased cell numbers. However addition of adipic acid with sodium citrate caused an increase in cell number with 1% addition of salts.

The culture combination TY25 and 22 gave the similar results with TY25*TY30 combination, however there were not high viable cell numbers as were in TY30*TY25 combination. According to results table 3.4. addition of $(NH_4)_2HPO_4$+ KH_2PO_4 caused an increase in cell number for *Streptococcus thermophilus* and maximum viable cell number of *Lactobacillus bulgaricus* was observed in th medium with Na_2HPO_4+ KH_2PO_4.

Table 3.4. Effect of salts on cell viability for mixed culture of TY25*22

Ratio of salts in the medium	**1%**		**2%**		**3%**	
Culture combination	**TY25**	**22**	**TY25**	**22**	**TY25**	**22**
Mg_2HPO_4 + KH_2PO_4	7,7386	7,4460	7,9147	7,5167	7,8495	7,4515
K_2HPO_4 + KH_2PO_4	7,8295	7,6276	7,8944	7,7386	7,8741	7,6761
Na_2HPO_4+ KH_2PO_4	7,9031	7,6505	7,9771	7,8116	7,9418	7,7882
$(NH_4)_2HPO_4$+ KH_2PO_4	7,9287	7,6483	8,0167	7,6901	8,0731	7,5000
Amonium formate	7,6901	7,1021	7,6048	6,9621	7,6215	6,6901
Sodium citrate	7,7386	6,8873	7,8125	6,6990	7,7887	6,3891
Sodium citrate + adipic acid	7,8010	7,1276	7,7236	7,3388	7,6901	7,1505
Control	7,8662	7,7386				

Table 3.5. Effect of salts on cell viability for mixed culture of TY25*TY77-a

Ratio of salts in the medium	**1%**		**2%**		**3%**	
Culture combination	**TY25**	**TY77-a**	**TY25**	**TY77-a**	**TY25**	**TY77-a**
Mg_2HPO_4 + KH_2PO_4	7,8989	8,5667	7,9340	8,5909	7,9030	8,5880
K_2HPO_4 + KH_2PO_4	8,3890	8,0207	8,5909	8,3010	8,5105	8,2781
Na_2HPO_4+ KH_2PO_4	8,7385	8,3890	8,8686	8,6505	8,6611	8,4582
$(NH_4)_2HPO_4$+ KH_2PO_4	8,8116	8,0395	9,3477	8,4460	8,7456	8,3217
Amonium formate	7,8053	7,6505	7,8740	7,6901	7,6901	8,1712
Sodium citrate	7,6505	7,5395	7,5395	7,1505	7,1505	7,1505
Sodium citrate + adipic acid	8,2385	7,6227	7,8187	7,7707	7,7988	7,6975
Control	7,8832	7,4460				

Streptococcus thermophilus TY25 was highly effected by addition of 2% Na_2HPO_4+ KH_2PO_4 however the maximum was observed with 2% of $(NH_4)_2HPO_4$+ KH_2PO_4. The maximum viable cell number for *Lactobacillus bulgaricus* TY77-a was obtained with the media containing 2% of Na_2HPO_4+ KH_2PO_4.

Table 3.6. Effect of salts on cell viability for mixed culture of 95-1*TY30

Ratio of salts in the medium	**1%**		**2%**		**3%**	
Culture combination	**95-1**	**TY30**	**95-1**	**TY30**	**95-1**	**TY30**
Mg_2HPO_4 + KH_2PO_4	8,1901	7,8116	8,3741	7,9085	8,2782	7,8406
K_2HPO_4 + KH_2PO_4	8,3010	7,9085	8,5792	7,9542	8,5043	7,9044
Na_2HPO_4+ KH_2PO_4	8,3891	8,4460	8,8116	8,6021	8,6990	8,3891
$(NH_4)_2HPO_4$+ KH_2PO_4	8,5396	8,2236	8,6990	8,4771	8,6228	8,9393
Amonium formate	7,9771	7,8741	7,8741	7,5880	7,8010	7,1505
Sodium citrate	7,8741	7,7386	7,7386	7,6990	7,6901	7,4287
Sodium citrate + adipic acid	7,9911	7,1901	8,2236	7,6215	8,1276	7,5396
Control	8,3406	7,7386				

As a results of table 3.6, it can be observed that the *Streptococcus thermophilus* 95-1 were not grown as in the combination of TY25*TY30. similarly the salt combination of Na_2HPO_4+ KH_2PO_4 was the most effective with the ratio 2% of the medium for both bacilli and cocci. In addition $(NH_4)_2HPO_4$+ KH_2PO_4 was the second effective salt for mixed culture growth with the ratio of 2%.

Table 3.7. Effect of salts on cell viability for mixed culture of 95-1*22

Ratio of salts in the medium	**1%**		**2%**		**3%**	
Culture combination	**95-1**	**22**	**95-1**	**22**	**95-1**	**22**
Mg_2HPO_4 + KH_2PO_4	7,8406	7,5396	7,8898	7,7386	7,7720	7,5000
K_2HPO_4 + KH_2PO_4	7,8741	7,5396	7,9771	7,6883	7,8997	7,6505
Na_2HPO_4+ KH_2PO_4	8,3891	7,8732	8,7720	8,1613	8,6505	8,0396
$(NH_4)_2HPO_4$+ KH_2PO_4	8,2236	7,7386	8,5396	7,9085	8,3662	7,8290
Amonium formate	7,7386	7,3891	7,5000	7,1276	6,9771	6,7526
Sodium citrate	7,4098	6,8976	7,0000	6,7197	6,7720	6,5285
Sodium citrate + adipic acid	7,9287	7,1672	7,9672	7,4460	7,9031	6,8010
Control	7,4460	7,4287				

The highest values for both strain were obtained by addition of $(NH_4)_2HPO_4$+ KH_2PO_4 and Na_2HPO_4+ KH_2PO_4 in to the growth medium, also the growth of the microorganisms were decreased with these strains combinations.

Table 3.8. Effect of salts on cell viability for mixed culture of 95-1*TY77-a

Ratio of salts in the medium	**1%**		**2%**		**3%**	
Culture combination	**95-1**	**TY77-a**	**95-1**	**TY77-a**	**95-1**	**TY77-a**
Mg_2HPO_4 + KH_2PO_4	7,8741	7,6276	8,0167	7,6901	7,8116	7,5880
K_2HPO_4 + KH_2PO_4	8,0167	7,8406	8,2526	7,9911	8,1417	7,8580
Na_2HPO_4+ KH_2PO_4	8,3891	8,0792	8,6990	8,2899	8,3891	8,1968
$(NH_4)_2HPO_4$+ KH_2PO_4	8,2274	7,7386	8,3314	7,8406	8,3005	7,7927
Amonium formate	7,6809	7,0396	7,4911	6,9031	7,3538	6,7386
Sodium citrate	7,1901	6,8997	6,8741	6,7386	6,7386	6,6901
Sodium citrate + adipic acid	7,8336	7,4098	7,8934	7,5880	7,7297	7,5652
Control	7,7386	7,3891				

The buffering salts K_2HPO_4 + KH_2PO_4 and Na_2HPO_4+ KH_2PO_4 caused the highest possible viable cell numbers in the growth medium. All medium caused an increase in cell numbers but amonium formate and sodium citrate caused a decrease when comparing with control media. It can be caused for the usage of them single and as a results of this they can not show the same buffering capacity as other salts can.

Table 3.9. Effect of salts on cell viability for mixed culture of 63-2*TY30

Ratio of salts in the medium	**1%**		**2%**		**3%**	
Culture combination	**63-2**	**TY30**	**63-2**	**TY30**	**63-2**	**TY30**
$Mg_2HPO_4 + KH_2PO_4$	8,1901	7,8010	8,3891	7,9911	8,3010	7,7579
$K_2HPO_4 + KH_2PO_4$	8,3406	7,8116	8,5731	8,1590	8,4771	7,5396
$Na_2HPO_4 + KH_2PO_4$	8,4911	7,8997	8,7386	8,4194	8,6809	8,3217
$(NH_4)_2HPO_4 + KH_2PO_4$	8,4460	7,9911	8,6021	8,0938	8,3891	7,9771
Amonium formate	7,8116	7,6901	7,7197	7,5396	7,1901	7,0246
Sodium citrate	7,6505	7,7720	7,4098	7,4515	7,2782	6,8116
Sodium citrate + adipic acid	7,9287	7,7386	8,0167	8,0085	7,8741	7,9287
Control	7,8187	7,6505				

Streptococcus thermophilus 63-2 and *Lactobacillus thermophilus* TY30 were effected highly by the addition of phosphate salts while non-phosphate salts caused a negative effect on their growth. Likewise the other results the maximum cell numbers were obtained with $Na_2HPO_4 + KH_2PO_4$ and the second maximums were obtained with $(NH_4)_2HPO_4 + KH_2PO_4$ with addition 2% of the medium.

Table 3.10**.** Effect of salts on cell viability for mixed culture of 63-2*22

Ratio of salts in the medium	**1%**		**2%**		**3%**	
Culture combination	**63-2**	**22**	**63-2**	**22**	**63-2**	**22**
$Mg_2HPO_4 + KH_2PO_4$	7,6505	7,3890	7,8116	7,6901	7,7781	7,6126
$K_2HPO_4 + KH_2PO_4$	7,8740	7,8116	8,0167	7,9978	7,9418	7,9286
$Na_2HPO_4 + KH_2PO_4$	7,9286	7,9286	8,3406	8,0937	8,2525	8,0167
$(NH_4)_2HPO_4 + KH_2PO_4$	7,9286	7,8740	8,1227	7,9722	7,9771	7,8477
Amonium formate	7,6712	7,6901	7,3890	7,3890	6,8740	7,1227
Sodium citrate	7,7720	7,3890	7,6712	6,9542	7,2156	6,7385
Sodium citrate + adipic acid	7,8406	7,8116	7,8794	7,9286	7,7988	7,8010
control	7,5880	7,4722				

The combination of 63-2 and 22 strains was effected by addition of $Na_2HPO_4 + KH_2PO_4$ salts as were in other combinations results. Addition of sodium citrate and amonium formate had a negative effect on cell growth also addition of sodium citrate with adipic acid has no significant effect on cell growth.

As a result of *Streptococcus thermophilus* 63-2 and *Lactobacillus bulgaricus* TY77-a, strains did not grow well together when comparing to other strains results. However their cell numbers were not very high, they were affected by the addition of $Na_2HPO_4 + KH_2PO_4$.

Table 3.11. Effect of salts on cell viability for mixed culture of 63-2 * Ty77-a

Ratio of salts in the medium	**1%**		**2%**		**3%**	
Culture combination	**63-2**	**TY77-a**	**63-2**	**TY77-a**	**63-2**	**TY77-a**
$Mg_2HPO_4 + KH_2PO_4$	7,6611	7,3891	7,8477	7,6809	7,8116	7,6228
$K_2HPO_4 + KH_2PO_4$	7,8116	7,6505	8,0938	7,8741	7,9771	7,8116
$Na_2HPO_4 + KH_2PO_4$	7,8856	7,6505	8,2782	7,9978	8,1276	7,9515
$(NH_4)_2HPO_4 + KH_2PO_4$	7,8406	7,8116	7,9978	7,8741	7,9287	7,8010
Amonium formate	7,3891	7,6505	6,9287	6,9771	6,7720	6,7236
Sodium citrate	7,0396	7,3891	6,8741	6,9287	6,5396	6,6276
Sodium citrate + adipic acid	7,6901	7,3891	7,7853	7,6901	7,7664	8,2457
control	7,5536	7,0167				

All in all it can be summarized that from all strains combination ,addition of $Na_2HPO_4 + KH_2PO_4$ and $(NH_4)_2HPO_4 + KH_2PO_4$ with 2% of the medium highly effected cell growth. Both of these salts combinations has a good buffering capacity. While cells are growing lactic acid is synthesized and this causes a decrease in pH of the medium. An internal pH control in the fermentation medium would be done by addition of buffering agents into the medium. According to the results addition of salts with a ratio of 2% of the medium causes a positive effect on cell growth while 3 % causes a decrease in cell numbers comparing with 2 and 3% addition of the salts in to the medium.

3.2.3. Effect of Salts Additions on Phage Infections

All salts including phosphate salts and non-phosphate salts (ammonium formate, sodium citrate, sodium citrate+adipic acid) were examined against bacteria which were sensitive to selected phages (phages were selected according to phage sensitivity-resistant experiments).

Experiments were done according to double layer agar method. Results were compared with media without salts and the initial phage titers (pfu/ml). Initial phage titers were determined before phage inoculations in to the medium given in Table 3.12.

In phage experiments phage titer were kept constant by serial dilutions. They were at nearly 10^{6-7} pfu/ml.

Table 3.12. Inıtial phage titers (pfu/ml)

Phages	**Inıtıal phage titers(pfu/ml)**
X11	3.10^6
X26	2.10^6
V1L-A	3.10^6
709.50	2.10^6
231.CH1A	2.10^7
23.4.2h	2.10^7

Phages with these titer were immediately mixed with bacteria which were sensitive to phages and left for 15 minutes for adsorption process then the growth media were added to phage-bacteria mixture and left for incubation at 43ºC for 18 hours. Unadsorbed phages were counted by double layer agar method and results were expressed as pfu/ml given in Table 3.13 for each phage bacteria mixture. Results were compared with the results of control medium which did not include salts. Results were further discussed whether the salts addition decreased phage titer or not.

Bacteriophages against thermophilic lactic acid bacteria are recognized as one of the main causes of slow or faulty fermentative productions in dairy plants. As it is known, the factory environment is a phage reservoir and phages are always present in plants. According to the results of Quiberoni (Quiberoni, et al. 2003), concentrations higher than 10^5pfu/ml were largely enough to delay milk acidification to result in the product, yoghurt.

Table 3.13. Phage titers after 18 hours incubation for each salt addition(pfu/ml) a) Results for phages of *Streptococcus themophilus,* b) Results for phages of *Lactobacillus bulgaricus*

phages	**231CH1A**			**709.50**			**23.4.2h**		
bacteria	**TY25**	**63-2**	**95-1**	**TY25**	**63-2**	**95-1**	**TY25**	**63-2**	**95-1**
Mg_2HPO_4 + KH_2PO_4	1.10^5	1.10^5	4.10^3	$2{,}6.10^4$	9.10^7	2.10^4	8.10^4	95.10^2	1.10^3
K_2HPO_4 + KH_2PO_4	1.10^3	2.10^2	6.10^3	$6{,}6.10^3$	5.10^7	4.10^5	2.10^3	5.10^4	2.10^3
Na_2HPO_4+ KH_2PO_4	1.10^2	1.10^2	5.10^2	1.10^2	3.10^4	13.10^2	1.10^2	1.10^3	1.10^2
$(NH_4)_2HPO_4$+ KH_2PO_4	1.10^4	8.10^2	7.10^2	3.10^2	2.10^5	8.10^2	4.10^2	4.10^3	2.10^2
Amonium formate	1.10^7	8.10^3	8.10^6	5.10^3	3.10^7	6.10^7	3.10^5	6.10^4	4.10^4
Sodium citrate	1.10^6	1.10^3	7.10^7	$4{,}3.10^7$	2.10^8	8.10^6	14.10^4	5.10^3	7.10^3
Sodiumcitrate + adipic acid	2.10^2	16.10^2	5.10^3	4.10^2	4.10^4	4.10^3	6.10^3	3.10^3	1.10^3
control	2.10^6	4.10^6	6.10^8	1.10^8	2.10^9	5.10^8	3.10^7	2.10^7	1.10^8

a

phages	**X11**			**X26**			**V1L-A**		
bacteria	**TY30**	**22**	**TY77a**	**TY30**	**22**	**TY77a**	**TY30**	**22**	**TY77a**
Mg_2HPO_4 + KH_2PO_4	1.10^3	3.10^2	2.10^4	1.10^5	3.10^3	1.10^5	$1{,}9.10^6$	1.10^4	1.10^4
K_2HPO_4 + KH_2PO_4	2.10^2	5.10^2	9.10^3	6.10^3	3.10^4	4.10^4	1.10^3	2.10^3	7.10^2
Na_2HPO_4+ KH_2PO_4	3.10	2.10^2	3.10^3	1.10^3	1.10^2	6.10^3	6.10^2	1.10^2	1.10^3
$(NH_4)_2HPO_4$+ KH_2PO_4	2.10	4.10^3	7.10^3	4.10^4	1.10^3	1.10^4	1.10^3	2.10^2	8.10^3
Amonium formate	3.10^3	1.10^4	1.10^5	3.10^4	4.10^4	1.10^6	1.10^7	3.10^5	5.10^4
Sodium citrate	4.10^3	2.10^4	5.10^5	1.10^4	1.10^4	2.10^5	5.10^8	6.10^4	8.10^4
Sodiumcitrate + adipic acid	6.10^2	2.10^3	4.10^4	4.10^3	9.10^3	1.10^5	2.10^3	1.10^4	4.10^3
control	$1{,}5.10^6$	3.10^7	4.10^8	6.10^7	5.10^7	2.10^9	1.10^9	2.10^7	1.10^9

b

According to the results of salts effects on phage inhibition, the minimum number of plaques were obtained when Na_2HPO_4+ KH_2PO_4 (1:1) was used in the medium with a ratio of 2%.

Streptococcus thermophilus bacteriophages 709.50, 231CH1A, 23.4.2h infections against TY25 were prevented mostly by using Na_2HPO_4+ KH_2PO_4 and resulted in 4 log decrease for 231.CH1A, 6 log decrease for 709.50 and 5 log decrease for 23.4.2h, respectively. *Streptococcus thermophilus* 63-2 strain and phage mixtures

were examined for salts effects. According to results, 4 log decrease for 231.CH1A, 23.4.2h and 5 log decrease for 709.50 in phage titers were obtained by using Na_2HPO_4+ KH_2PO_4 . The strain of *Streptococcus thermophilus* 95-1 were protected against phage attacks by Na_2HPO_4+ KH_2PO_4 which caused 6 log decrease for 231.CH1A and 23.4.2h. In addition maximum decrease (4 log) in phage titers of 709.50 when mixed with bacteria 95-1 was reached by using $(NH_4)_2HPO_4$+ KH_2PO_4.

Lactobacillus bulgaricus strains TY30, TY77-a and 22 were examined against bacteriophages X11, X26 andV1L-A. As a consequence, bacteriophage V1L-A was highly affected by addition of Na_2HPO_4+ KH_2PO_4 which caused 7 log decreases in phage titers when mixing the phage with *Lactobacillus bulgaricus* TY30 and 22. Moreover the phage titers of V1L-A were 5log decreased by K_2HPO_4 + KH_2PO_4 usage in the medium. The titers of X26 were decreased 6 log when mixing it with TY77-a, 5 log when mixing it 22 and 4 log when mixing it TY30 by addition of Na_2HPO_4+ KH_2PO_4. Bacteriophage X11 was effected by Na_2HPO_4+ KH_2PO_4 and it caused a decrease of 4log for TY30, 5 log for 22 and 5 log for TY77-a. Also $(NH_4)_2HPO_4$+ KH_2PO_4 caused nearly the same effect with Na_2HPO_4+ KH_2PO_4. In the literature there were not many studies about phosphate treatments to prevent phage attachments of yoghurt bacteria. One study about this subject was done firstly in 1961 (Hargrove, et al. 1961) and the results are in agreed with this study.

To sum up the results, Na_2HPO_4+ KH_2PO_4 caused maximum decrease in phage titers when comparing other salts additions results for all bacteria-phage combinations of both two species. Addition of phosphate salts causes aggregation of calcium ions. Ca ions are required for penetration of phage DNA in to the host cells.

Effects of salts on cell viability experiments' results were in agree with the results of bacteriophage-salt experiments. Both studies indicated that usage of Na_2HPO_4+ KH_2PO_4 with a ratio of 2% of the medium was effective on cell growth and protective for phage attacks.

3.3. Mineral Requirement of Mixed Starter Cultures

Mineral requirements of mixed starter culture were determined by general factorial design generated by Design Expert version 7.0 software output. Factors were determined as Fe, Mn and Mg. Fe and Mn were examined with three level of each and Mg was examined with two different levels. Experiments were done in randomized run

order. In order to control nuisance factors, an important technique known as blocking were used to reduce or eliminate the contribution to experimental error contributed by nuisance factors. The basic concept was to create homogeneous blocks in which the nuisance factors were held constant and the factor of interest was allowed to vary. Blocks were two different days in which the experiments were done.

Table 3.14. Factors, levels and responses data

		Factor 1	Factor 2	Factor 3	Response 1	Response 2
Run order	**Block**	**X1:Fe**	**X2:Mn**	**X3:Mg**	**S.Thermophilus Cell counts (cfu/ml)**	**L.Bulgaricus Cell counts (cfu/ml)**
1	Block 1	10	10	50	7,5E+08	7,7E+08
2	Block 1	20	10	25	4,1E+08	4E+08
3	Block 1	20	5	25	5,1E+08	6,2E+08
4	Block 1	20	10	50	6,6E+08	5,9E+08
5	Block 1	30	15	50	4,9E+08	4,1E+08
6	Block 1	10	15	50	6,9E+08	7,3E+08
7	Block 1	30	10	50	5,2E+08	5,1E+08
8	Block 1	30	5	25	5,9E+08	6E+08
9	Block 1	30	5	50	6,7E+08	7,1E+08
10	Block 1	20	15	50	5E+08	4,9E+08
11	Block 1	10	5	25	7,2E+08	7,2E+08
12	Block 1	10	15	25	5,7E+08	5,5E+08
13	Block 1	10	10	25	5,9E+08	6,1E+08
14	Block 1	30	10	25	3,7E+08	3,3E+08
15	Block 1	30	15	25	2,8E+08	2,6E+08
16	Block 1	20	5	50	7,4E+08	7,1E+08
17	Block 1	20	15	25	2,6E+08	3,3E+08
18	Block 1	10	5	50	8,5E+08	8E+08
19	Block 2	30	5	50	7,3E+08	7E+08
20	Block 2	30	5	25	5,7E+08	6,1E+08
21	Block 2	20	5	50	7,5E+08	6,9E+08
22	Block 2	30	15	50	5,3E+08	5,6E+08
23	Block 2	20	15	50	5,7E+08	5,1E+08
24	Block 2	10	10	25	6,1E+08	6,2E+08
25	Block 2	30	10	25	2,9E+08	3,6E+08
26	Block 2	20	15	25	2,1E+08	3E+08
27	Block 2	10	5	50	8,7E+08	7,9E+08
28	Block 2	30	10	50	5E+08	6,3E+08
29	Block 2	20	5	25	5,9E+08	6,1E+08
30	Block 2	20	10	25	3,8E+08	4,2E+08
31	Block 2	10	5	25	7,3E+08	7,4E+08
32	Block 2	10	15	25	5,6E+08	5,9E+08
33	Block 2	10	15	50	7,7E+08	7,2E+08
34	Block 2	20	10	50	5,6E+08	5,1E+08
35	Block 2	10	10	50	7,3E+08	7,9E+08
36	Block 2	30	15	25	2,2E+08	2,8E+08

According to results obtained from the study, effects of factors, analysis of data were done by software. In addition the solutions were created with its desirability results. Both two responses were analysed by taking their natural logaritm. In order two analyse the data linear model was suggested by the software. The model was fitted according to p-values. Terms having p-values lower than 0.05 acceppted as significant terms and model was developed by using significant terms.Analysis of variance table was constructed given in Table 3.15.

Table 3.15. Analysis of variance (ANOVA) table for response 1

Source	Sum of Squares	Degrees of freedom	Mean squares	F values	p-values
Block	$5{,}84.10^{-3}$	1	$5{,}84.10^{-3}$		
Model	4,22	6	0,70	32,70	< 0,0001
A: Fe	1,12	1	1,12	51,86	< 0,0001
B: Mn	1,28	1	1,28	59,34	< 0,0001
C: Mg	1,39	1	1,39	64,72	< 0,0001
AB	0,14	1	0,14	6,58	0,0160
AC	0,083	1	0,083	3,87	0,0592
BC	0,21	1	0,21	9,84	0,0040
Residual	0,60	28	0,022		
Cor total	4,83	35			

The model F-value 32,70 implies that the model is significant. Terms A, B, C, AB, AC, BC are significant terms.However the term AC had a p-value of 0.0592, the model reduction was not necessary. R-Square value of the model was 0.8751 and the Adjusted R-Square value was 0.8484, predicted R-Square was 0.7854. which is in reasonable aggrement

Equations in terms of coded and actual factors were generated by the software as seen in equations 3.1 and 3.2. In order to determine the log(cfu/ml) of *Streptococcus thermophilus*, equations in terms of coded and actual factors were given as;

$$Y= 20{,}09 – 0{,}22.A – 0{,}23.B + 0{,}20.C – 0{,}094\ (AB) + 0{,}059\ (AC) + 0{,}094\ (BC) \quad (3.1)$$

$$Y=20{,}936 - 0{,}0204(Fe) – 0{,}0648\ (Mn) – 8{,}7138.10^{-3}\ (Mg) – 1{,}8816.10^{-3}\ (Fe)(Mn) + 4{,}7122.10^{-4}\ (Fe)(Mg) + 1{,}5029\ .\ 10^{-3}\ (Mn)(Mg) \quad (3.2)$$

In addition the ANOVA table for response 2 which indicates the cell counts of *Lactobacillus bulgaricus* were constructed by the software given in Table 3.16.

Table 3.16. Analysis of variance (ANOVA) table for response 2

Source	Sum of Squares	Degrees of freedom	Mean squares	F values	p-values
Block	0,010	1	0,010		
Model	3,20	6	0,53	44,66	< 0,0001
A: Fe	0,93	1	0,93	78,11	< 0,0001
B: Mn	1,08	1	1,08	90,21	< 0,0001
C: Mg	0,80	1	0,80	66,61	< 0,0001
AB	0,18	1	0,18	15,31	0,0005
AC	0,073	1	0,073	6,10	0,0199
BC	0,14	1	0,14	11,58	0,0020
Residual	0,33	28	0,012		
Cor total	3,54	35			

The model F value 44,66 implies that the model was significant and according to p-values of terms they were significant to construct the model. R-Square value of the model was 0.9054 and the Adjusted R-Square value was 0.8851, predicted R-Square was 0.8439. Also predicted R –Square was in reasonable aggrement with adjusted R-Square value.

Final equations to determine the log(cfu/ml) for *Lactobacillus bulgaricus* in terms of coded and actual factors were given in equations 3.3 and 3.4.

$$Y=20,12 – 0,20.A – 0,21.B + 0,15.C – 0,11 .AB + 0,055\ AC + 0,076\ BC \quad (3.3)$$

$$Y=20,8495 – 0,0148\ (Fe) – 0,0451\ (Mn) – 9,066.10^{-3}\ (Mn) – 2,1374.10^{-3}(Fe)(Mn) + 4,4073.10^{-4}(Fe)(Mg) + 1,2144.10^{-3}(Mg)(Mn) \quad (3.4)$$

The solutions were constructed by the software and the most desirable solution was addition of Fe, Mn, Mg ions with 10, 5, 50 ppm concentrations in to the media with a desirability of 0.939.

3.4. Fermentation Constants of Yoghurt Starter Cultures

Fermentation constants including maximum specific growth rate and Monod saturation constants of starter cultures were determined for mixed starter culture inoculations and single culture inoculations.

Streptococcus thermophilus strain TY25 was chosen for this study according to the results of previous studies. These studies included industrial-scale yoghurt production. When other strains were tested with different bacil-cocci combinations , the best product was obtained from TY25 as *Streptococcus thermophilus* with TY30 as *Lactobacillus bulgaricus* (data not shown here). Although TY25 was one of the sensitive strain against bacteriophages, with some additions in to the growth media this problem was solved (results are shown in detail in section 3.2). There were 4 different strain for each *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. But the maximum yield when grown together was obtained from TY30-TY25 combination. As a result of all these studies fermentation experiments were done with these two strain; TY25 (*Streptococcus thermophilus*) and TY30 (*Lactobacillus bulgaricus*). In order to determine the maximum specific growth rate of mixed starter culture, it has to be done the single culture experiments firstly.

3.4.1. Single strain fermentations of *Streptococcus thermophilus* TY25

Streptococcus thermophilus TY25 was inoculated in to the fermentation media with a volume of 0,8 L. Medium pH was kept constant at 6.9 by automatic addition of 6N NaOH and temperature was constant at 42ºC.

Fermentation experiments were done with different lactose concentrations. All media included 26 g/L whey powder which contains 20 g/L lactose, 7g/L yeast extract (optimized by A.Büyükileci in MsC thesis 2000), 2% Na_2HPO_4 + KH_2PO_4 and different amounts of lactose (0 g/L,10 g/L, 20 g/L, 30 g/L, 40 g/L, 50 g/L, 60 g/L, 70 g/L, 80 g/L). Samples were examined for viable cell counts and expressed as log (cfu/ml) given in Table 3.17. For every fermentation growth curves were determined and given in Appendix C. Specific growth rates were plotted against substrate concentrations as seen in Figure 3.3, double reciprocal plot was done for 1/S and 1/μ given in Figure 3.4.

Table 3.17. Mean values of viable cell counts (log cfu/ml) for different initial lactose concentrations and specific growth rate values for TY25

Time(hour)	20g/L	30g/L	40 g/L	50 g/L	60 g/L	70 g/L	80g/L	90g/L	100g/L
0	6,0965	6,0413	5,9771	6,1276	5,9722	6,0569	6,1901	5,8741	6,1228
0,5	6,1461	6,0965	6,0777	6,2670	6,0603	6,1126	6,2670	6,0938	6,3005
1	6,2491	6,1751	6,3106	6,3702	6,3798	6,2546	6,3786	6,2899	6,4311
1,5	6,3141	6,2602	6,5049	6,6237	6,6601	6,6705	6,6662	6,6470	6,5561
2	6,7751	6,6760	7,0692	6,9287	7,2488	7,1104	7,2032	6,9771	7,0880
2,5	6,9613	7,0307	7,2899	7,1751	7,3768	7,5836	7,6276	7,2988	7,4601
3	7,0791	7,1611	7,4469	7,4749	7,6720	7,9340	7,9334	7,6986	7,8506
3,5	7,1221	7,1901	7,5623	7,7031	7,8747	8,1668	8,1126	7,9480	8,0043
4	7,1611	7,3137	7,6284	7,9345	8,0603	8,1751	8,2296	8,2689	8,1846
4,5	7,1670	7,3222	7,6531	7,9566	7,9320	8,2296	8,2546	8,3142	8,1883
5	7,1760	7,3520	7,6672	7,9566	7,9085	8,2356	8,2414	8,3095	8,2297
5,5	7,1972	7,3588	7,6531	7,9494	7,8891	8,2363	8,2525	8,2782	8,1883
6	7,1929	7,3419	7,6231	7,9320	7,8802	8,2172	8,2552	8,3351	8,0938
μ (1/h)	**0,3388**	**0,4214**	**0,4494**	**0,5243**	**0,5601**	**0,60238**	**0,6253**	**0,6488**	**0,6514**

Streptococcus thermophilus single culture fermentation experiments were enough to observe when culture goes into a substrate saturation. According to results for all fermentations, cultures were gone into exponential growth phase after 1,5 hour and the exponential growth phase was finished at 4th hour.

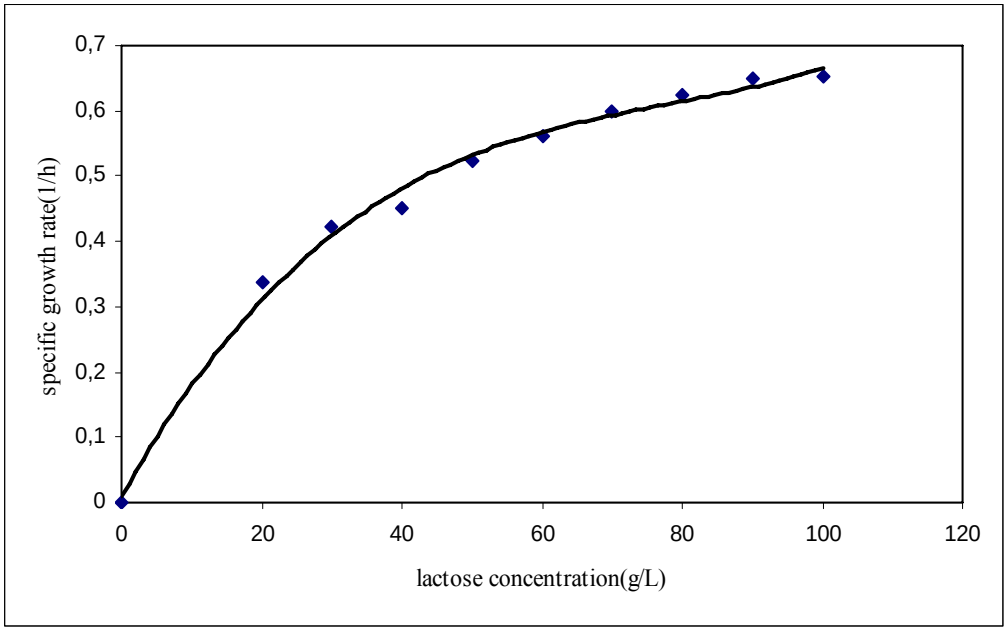


Figure 3.3. Specific growth rates and initial substrate concentrations for single strain fermentations of *Streptococcus thermophilus* TY25

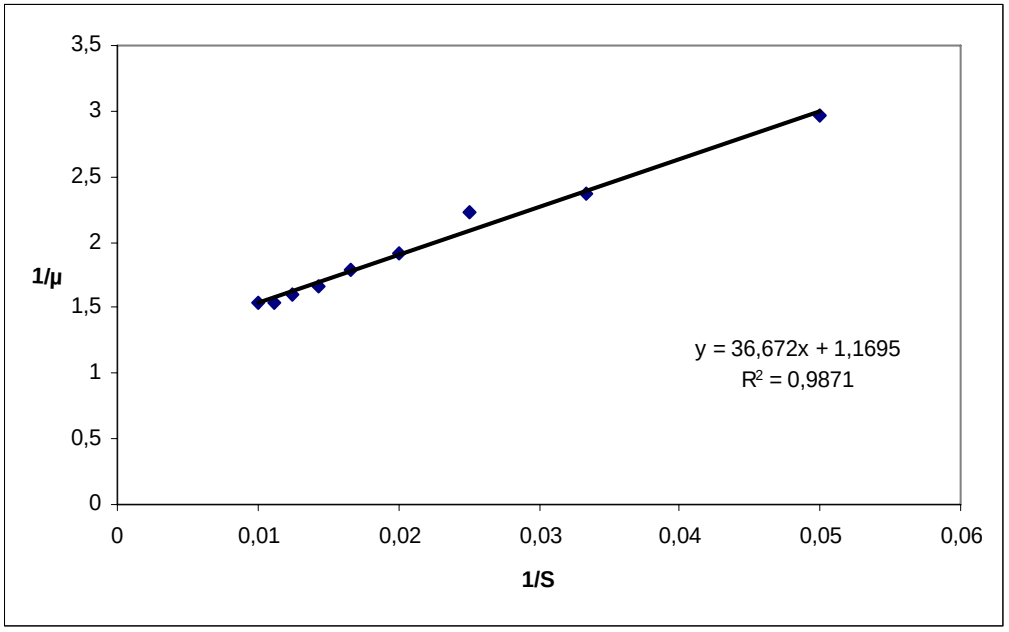


Figure 3.4. Double reciprocal plot for single strain fermentation of *Streptococcus thermophilus* TY25

The equation obtained from the double reciprocal plot was used for determination of kinetic constants. Monod saturation constant was 31,357 g/L lactose and maximum specific growth rate was found to be 0,855 h^{-1} .

3.4.2. Single strain fermentations of *Lactobacillus bulgaricus* TY30

Lactobacillus bulgaricus TY30 was inoculated into sterile fermentation media with a working volume of 0,8 L. Media components were sterilized seperately and fed into vessel by automatic pumps. Media included 26 g/L whey powder (equal to 20 g/L lactose), 7 g/L yeast extract, 2% Na_2HPO_4 + KH_2PO_4 and different amounts of lactose (0 g/L, 10 g/L, 20 g/L, 30 g/L, 40 g/L, 50 g/L, 60 g/L, 70 g/L, 80 g/L). Fermentation pH was kept constant at 6.2 by automatic addition of 6N NaOH and temperature was kept constant at 42ºC. Samples were examined for viable cell counts and expressed as log(cfu/ml), given in Table 3.18.

Growth curves for all initial lactose concentrations were determined and given in Appendix D. Specific growth rates were plotted against substrate concentrations given in Figure 3.5 and double reciprocal plot was done for 1/S and 1/μ given in Figure 3.6.

Table 3.18. Mean values of viable cell counts(log cfu/ml) for different initial lactose concentrations and specific growth rate values for TY30

Time(hour)	20g/L	30g/L	40 g/L	50 g/L	60 g/L	70 g/L	80g/L	90g/L	100g/L
0	6,0207	6,0603	6,0341	6,2173	6,1450	6,2157	6,2033	6,0777	6,0207
0,5	6,1450	6,1127	6,1021	6,2782	6,2670	6,2546	6,2414	6,1751	6,2157
1	6,1901	6,1300	6,2429	6,3469	6,3314	6,3116	6,3521	6,2033	6,2386
1,5	6,2157	6,2157	6,2788	6,3965	6,3891	6,4058	6,4058	6,2782	6,2670
2	6,2887	6,3142	6,3869	6,4624	6,4532	6,4302	6,4213	6,4302	6,4460
2,5	6,3686	6,3249	6,4749	6,5807	6,5966	6,5267	6,5106	6,5227	6,4978
3	6,5307	6,5938	6,8377	6,9404	7,0603	7,0816	7,0396	7,0396	7,1021
3,5	6,7397	6,8018	7,0699	7,1382	7,3010	7,3061	7,3406	7,3383	7,4460
4	6,8479	6,9242	7,2173	7,3406	7,4621	7,5183	7,5680	7,5396	7,5957
4,5	6,9565	7,0040	7,3010	7,4469	7,5966	7,6811	7,6941	7,6662	7,7116
5	7,0396	7,0777	7,3521	7,6019	7,7242	7,7887	7,8027	7,8094	7,8028
5,5	7,0692	7,0962	7,3570	7,5623	7,7153	7,7958	7,8028	7,7991	7,8095
6	7,0788	7,1048	7,3710	7,5711	7,7214	7,7889	7,7887	7,7922	7,7955
μ (1/h)	**0,2684**	**0,3011**	**0,3509**	**0,4085**	**0,4511**	**0,5048**	**0,5168**	**0,5147**	**0,5220**

Lactobacillus bulgaricus single culture fermentation experiments were enough to observe when culture goes into a substrate saturation. According to results for all fermentations, cultures were gone into exponential growth phase after 2,5 hour and the exponential growth phase was finished at 5th hour.

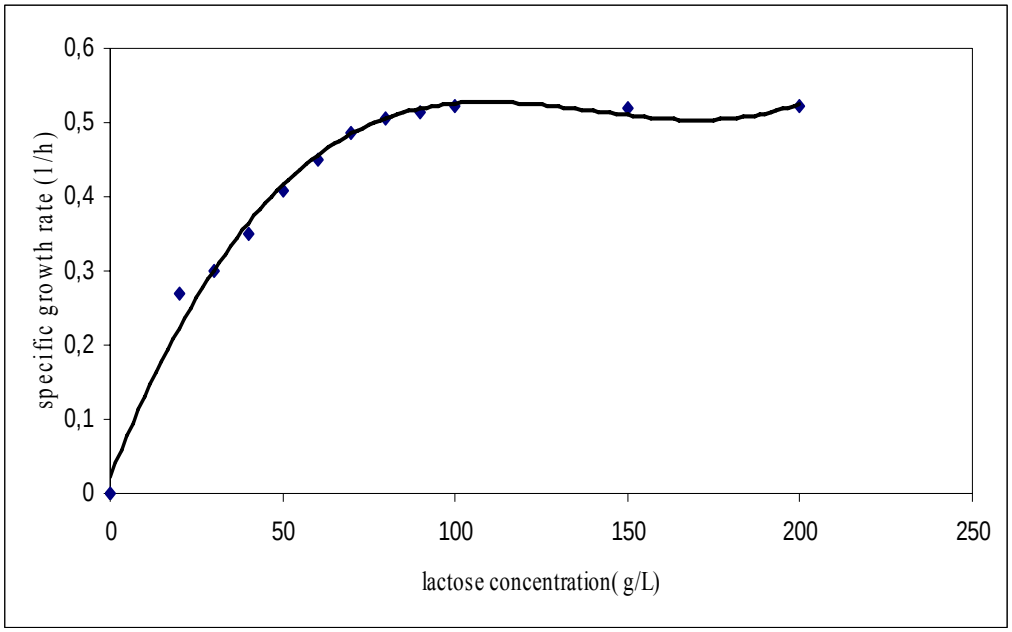


Figure 3.5. Specific growth rates and initial substrate concentrations for single strain fermentations of *Lactobacillus bulgaricus* TY30

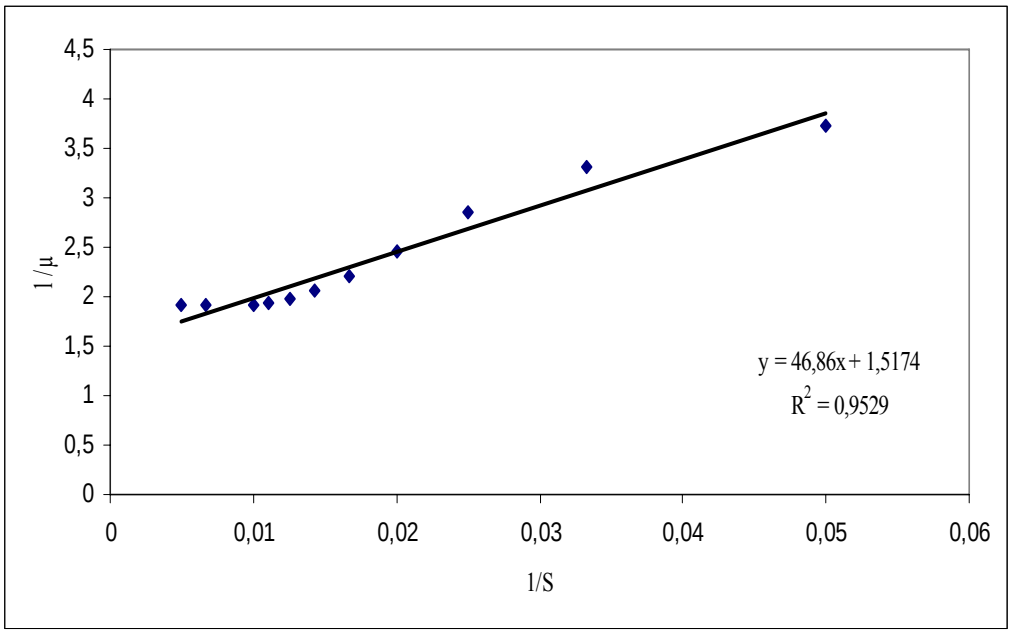


Figure 3.6. Double reciprocal plot for single strain fermentation of *Lactobacillus bulgaricus* TY30

The equation obtained from the double reciprocal plot was used for determination of kinetic constants. Monod saturation constant was 30,8817 g/L lactose and maximum specific growth rate was found to be 0,6590 h^{-1} .

3.4.3. Mixed Culture Fermentations of *Lactobacillus bulgaricus* TY30 and *Streptococcus thermophilus* TY25

Mixed culture fermentations were carried out with 7 different initial lactose concentrations including 20, 40, 60, 80, 100, 120 and 150 g/L lactose. Fermentation media included 26 g/L whey powder, 7 g/L yeast extract, 2% Na_2HPO_4 + KH_2PO_4 . workin volume was 800 ml for each fermentation. Temperature was kept constant at 43ºC and pH was kept constant at 6.6 by automatic addition of 6N NaOH. All inredients were sterilized seperately and NaOH was filter sterilized.

Samples were witdrawn at every two hours and fermentation was finished at 8th hour. All samples were examined for both viable cell counts of two bacteria. All fermentations were done in duplicate. Viable cell counts for both Streptococcus thermophilus and *Lactobacillus bulgaricus* were expressed as log (cfu/ml) of mean values of duplicates given in Table 3.19.

As seen from the Table 3.19 *Streptococcus thermophilus* TY25 was grown better than *Lactobacillus bulgaricus*. *Lactobacillus bulgaricushas* lower specific growth rate than *Streptococcus thermophilus* for all initial lactose concentrations. Accordingly if results of mixed culture fermentations were compared with single culture fermentations, both two bacteria had higher growth rates which is related to symbiotic growth of these bacteria.

The data obtained from viable cell counts were plotted against time and specific growth rates were determined from the exponential growth phases of the bacteria. From the bacterial growth curves given in Appendix E, it was observed that *Streptococcus thermophilus* TY25 was gone into exponential growth phase in between 2-4 hours and *Lactobacillus bulgaricus* TY30 was in between 4-6 hours. These observations were in agree with single strain fermentations.

Specific growth rates obtained from growth curves were plotted against initial substrate concentrations and substrate saturation were seen in Figure 3.7. In order to determine the kinetic constants, double reciprocal plot was done as seen in Figure 3.8 and kinetic constants were determined.

Table 3.19. Mean values of viable cell counts of *Streptococcus thermophilus* TY25 in mixed culture fermentations with different initial lactose concentrations

	İnitial lactose concentration (g/L)						
Time(h)	**20**	**40**	**60**	**80**	**100**	**120**	**150**
0	6,1300	6,0603	6,0652	6,0966	6,1459	6,0341	6,1672
2	6,2173	6,1751	6,2414	6,4225	6,4044	6,2297	6,2864
4	7,0755	7,2108	7,4683	7,8125	7,9370	7,8750	7,9358
6	7,1382	7,2200	7,4911	7,8351	7,9590	7,9004	7,9590
8	7,1492	7,2404	7,5183	7,8509	7,9661	7,9163	7,9822
µ(1/h)	**0,4291**	**0,5178**	**0,6134**	**0,6950**	**0,7663**	**0,8227**	**0,8247**

Table 3.20. Mean values of viable cell counts of *Lactobacillus bulgaricus* TY30 in mixed culture fermentations with different initial lactose concentrations

	initial lactose concentration (g/L)						
Time(h)	**20**	**40**	**60**	**80**	**100**	**120**	**150**
0	5,9287	6,0603	6,0207	6,0880	6,1590	6,1417	5,9515
2	6,1611	6,2297	6,2657	6,2593	6,2733	6,2782	6,1751
4	6,2899	6,3217	6,4147	6,4098	6,5043	6,5837	6,5396
6	7,0207	7,2303	7,4842	7,6356	7,7479	7,8290	7,8162
8	7,0792	7,2429	7,5246	7,6580	7,7782	7,8542	7,8449
µ(1/h)	**0,3654**	**0,4543**	**0,5348**	**0,6129**	**0,6218**	**0,6227**	**0,6383**

From the equations obtained from double reciprocal plots , maximum specific growth rate for Steptococcus thermophilus TY25 was 0,9188 and 0,7323 for *Lactobacillus bulgaricus* TY30. Monod saturation constants K_s were found 24,5134 g/L for Streptococcus thermophilus TY25 and 20,7608 g/L for *Lactobacillus bulgaricus* TY30.

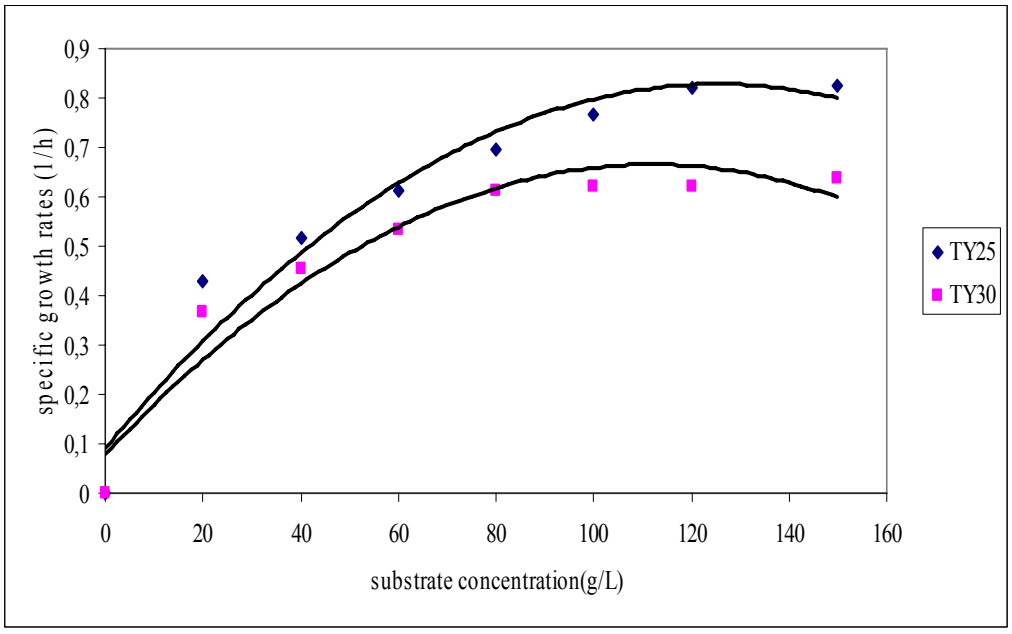


Figure 3.7. Monod substrate saturation graphic for mixed culture fermentations of *Streptococcus thermophilus* TY25 and *Lactobacillus bulgaricus* TY30

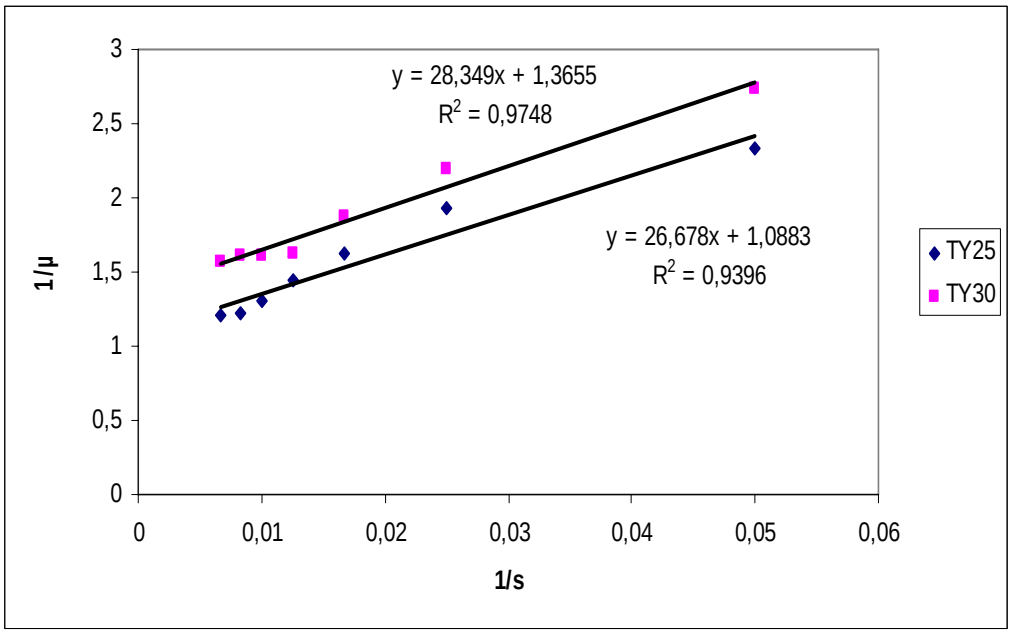


Figure 3.8. Double reciprocal plot for *Streptococcus thermophilus* TY25 and *Lactobacillus bulgaricus* TY30

These kinetic constants were different when compared with single strain fermentations. Maximum specific growth rate for both species were higher than single strain fermentation's maximum specific growth rates because of associative growth of these two species which can also be observed according to proteolysis activities (Rajagopal and Sandine 1990) or β-galactosidase activity (Tarı 2008).

CHAPTER 4

CONCLUSIONS

In this study, growth requirements and fermentation constants for mixed and pure yoghurt starter cultures were examined.

Different salts combinations were added to the growth media and the effects of these salts on cell viability and phage infections were determined. As a conclusion, the most effective salt on cell viability was determined as Na_2HPO_4 when comparing the viable cell numbers obtained in the media without salt addition. Salt addition with a concentration of 2% of the medium was resulted in better growth and higher concentrations for salts were resulted in a decrease in cell counts.

In dairy plants bacteriophage infections are one of the most important problems. Phages generally need Ca^{2+} ions for both adsorbtion step onto the host cell surface and penetration of DNA step into the host cell. In order to prevent DNA penetration step of phages, free Ca^{2+} ions must be precipitated by addition of salts. For this purpose various salts including phosphate and citrates were examined for their effects on cell viability and phage inhibition ability. Among all the salts tested Na_2HPO_4 + KH_2PO_4 with 2% of the media addition was found as the most effective combination with 4 log decrease in phage titers.

Three important minerals were tested for mixed culture requirement and the effects of these minerals were generated by general factorial design. The most effective mineral combination was determined as 10 ppm Fe^{2+}, 5 ppm Mn^{2+} and 50 ppm Mg^{2+} with 0,939 desirability. Minerals were added at determined initial concentrations in growth media.

Finally fermentation constants were determined for the growth media including phosphate salts and minerals. Fermentation studies were performed for pure strains and mixed culture system. Single strain fermentations of *Streptococcus thermophilus* TY25 were done until growth rate of the bacteria remains nearly at the same values. In order to determine the constants, the Monod saturation graphs were generated and saturation were observed. Ks value and maximum specific growth rates were calculated from double reciprocal plot's equation and determined as 31,35 g/L lactose and 0,855 h^{-1}

respectively. Ks saturation constant and maximum specific growth rates were calculated for *Lactobacillus bulgaricus* TY30 and determined as 30,8817 g/L lactose and 0,659 h^{-1} from single strain fermentations respectively.

Mixed culture fermentations were performed and constants for both yoghurt starter bacteria were determined from the same batches. According to double reciprocal plots of the strains; Ks saturation constants were determined as 20,7608 g/L for *Lactobacillus bulgaricus* and 24, 5134 for *Streptococcus thermophilus* which is related to affinity to substrate. Ks results indicate that *Streptococcus thermophilus* has higher affinity to substrate. Maximum specific growth rates were determined as 0,7323 h^{-1} for *Lactobacillus bulgaricus* and 0,9188 h^{-1} for *Streptococcus thermophilus* respectively. From Monod graphics it was observed that; substrate saturations for mixed culture fermentations were reached after higher values than were for single culture fermentations.

REFERENCES

Acar, S. E. 2007. Identification and classification of virulent bacteriophages of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* based on their replication parameters, capsid protein profiles and restriction endonuclease analysis. *Ankara University thesis of PhD*.

Ackermann, H. W. 1987. Bacteriophage taxonomy. *Microbiological Sci*ences 4:214-218.

Akın, N. 2006. Modern Yoğurt Bilimi ve Teknolojisi. Selçuk Üniversitesi.

Aslim, B., Yüksekdag, Z. N., Beyatli, Y. 2005. Exopolysaccharide production by *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* strains under different growth conditions. *World Journal of Microbiology & Biotechnology* 21:673-677.

Barbara, P. K. 1980. Appraisal of media and methods for assay of bacteriophages of lactic streptococci. *Applied and Environmental Microbiology* 798-802.

Binetti, A. G., Quiberoni, A., Reinheimer, J. A. 2002. Phage adsorbtion to *Streptococcus thermophilus*, influence of environmental factors and characterization of cell-receptors. *Food Research International* 35:73-83.

Birge, E. A. 2000. *Bacterial and Bacteriophage Genetics,* 4th edition, New-York:Springer.

Bouzar, F. Cerning, J., Desmazeaud, M. 1996. Exopolysaccharide production in milk by *Lactobacillus delbrueckii* spp. *bulgaricus* CNRZ 1187 and by two colonial variants. *Journal of Dairy Science* 79:205-211.

Botina, S. G., Trenina Yu, M. A., Tsygankov, D., Sukhodolets, V. V. 2007. Comparision of genotypic and biochemical characteristics of *Streptococcus thermophilus* strains isolated from sour milk products. *Applied Biochemistry and Microbiology* 43:598-603.

Bury, D., Jelen, P., Kimura, K. 1998. Whey protein concentrate as a nutrient supplementation for lactic acid bacteria. *International.Dairy Journal* 8:149-151.

Chaves, A., Fernandez, M., Lerayer, S., Mierau, I., Kleerebezem, M. 2002. Metabolic engineering of acetaldehyde production by *Streptococcus thermophilus. Applied Environmental Microbiology* 68:5656-5662.

Coffey, A., Ross, R. P. 2002. Bacteriophage-resistance systems in dairy starter strains, molecular analysis to application. *Antonie van Leeuwenhoek* 82: 303-321.

Cogan, T.M., Accolas, J.P. 1996. *Dairy Starter Cultures*. New-york:Vch Publishers.

Crow, V., Curry, B. 2002. *Lactobacillus delbrueckii* group, in *Encylopedia of Dairy Science* edited by Roginski, H., Fuquay,J., Fox, P. London:Academic Press.

De Vuyst, L., Zamfira, M., Mozzia, F., Adriany, T., Marshalld, V., Degeest, B., Vaningelgem, F. 2003. Exopolysaccharide-producing *Streptococcus thermophilus* strains as functional starter cultures in the production of fermented milks. *International Dairy Journal* 13:707–717.

Degeest, B., De Vuyst, L., Vaningelgem, F. 2001. Microbial physiology, fermentation kinetics, and process engineering of heteropolysaccharide production by lactic acid bacteria. *International Dairy Journal* 11:747–757.

Deutsch, M. S., Ferain, T., Delcour, J., Lortal, S. 2002. Lysis of lysogenic strains of *Lactobacillus helveticus* in Swiss cheeses and first evidence of concomitant *Streptococcus thermophilus* lysis. *International Dairy Journal* 12:591-600.

Elmer, L., Gaden, Jr. 2000. Fermentation process kinetics. *Journal of Biochemical and Microbiological Technology and Engneering* 4:413-429.

Erkus, O. 2007. Isolation, Phenotypic and genotypic characterization of yoghurt starter bacteria.*Izmir Institute of Technology thesis of MsC.*

Folkenberg, D. M., Dejmek, P., Skriver, A., Guldager, H. S., Ipsen, R. 2004. Sensory and rheological screening of exopolysaccharide producing strains of bacterial yoghurt cultures. *International Dairy Journal* 16:111-118.

Frank, J., Hassan, A. 1998. Starter cultures and their use. *Applied Dairy Microbiology* , edited by Elmer, M.and James, S. New York:Markel Decker.

Garen, A., Kozloff, L. M. 1959. The initiation of bacteriophage infection. *The Viruses Volume 2 : Plant and Bacterial Viruses* edited by Burnet ,F.M. Stanley , W.M. 203-233 New York:Academic Press.

Ghally, A. E., Tango, M. S. A., Adams, M. A. 2003. Enhanced lactic acid production from cheese whey with nutrient supplement addition. *Journal of Scientific Research and Development* 3:15-22.

Gilliand, S.E. 1985. Concentrated starter cultures. *Bacterial Starter Cultures for Foods* edited by Gilliand S.E. Boca Raton:CRC Press.

Guttman, B., Raya, R.A., Kutter, E. 2005. Basic phage biology. *Bacteriophages,Biology and Applications* edited by Kutter,E. and Sulakvelidze, E. Boca Raton:CRC Press.

Hargrove, R.E., McDonough, F.E., Titsler, R.P. 1961. Phosphate heat treatment of milk to prevent bacteriophage proliferation in lactic cultures. *Eastern Utilization Research and development Division* 1799-1810.

Hendrix, R.W. 2002. Bacteriophage λ and its relatives, in *Modern Microbial Genetics* edited by Uldis, E., Streips, N., Ronald, E. New York:Wiley&Sons.

Kalviainen, N., Roininen, K., Tuorila, H. 2003. The relative importance of texture, taste and aroma on a yogurt-type snack food preference in yhe young and the elderly. *Food Quality and Preference* 14:177-186.

Klaenhammer, T.R. 1991. Development of bacteriophage-resistant strains of lactic acid bacteria. *Food Biotechnology* 19:675.

Lee, K. 2004. Comparision of fermentative capabilities of lactobacilli in single and mixed culture in industrial media. *Process Biochemistry* 40:1559-1564.

Lerner, K.L., Lerner, B.W. 2003. *World of Microbiology and Immunology*. USA:Gale Group.

Leroy, F., De Vuyst, L. 2004 .Lactic acid bacteria as functional starter cultures for the food fermentation industry. *Food Science and Technology* 15:67-78.

Looijesteijn, P.J., Van Casteren, W.H.M., Tuinier, R., Doeswijk-Voragen, C.H.L., Hugenholtz, J. 2000. Influence of different substrate limitations on the yield, composition and molecular mass of exopolysaccharides produced by *Lactococcus lactis* subsp. *cremoris* in continuous cultures. *Journal of Applied Microbiology* 89:116-122.

Maloy, S.R., Cronan, J.E., Freifelder, D. 1994. Phage Biology, in *Microbial Genetics,* 2nd edition. Sudbury:Jones and Barlett Publishers.

Martinez-Gonzalez, C., Chafer, M., Becerra, M., Chiralt, A. 2002. Influence of substituting milk powder for whey powder on yoghurt quality.*Food Science and Technology* 13:334-340.

Moineau, S. 1999. Applications of phage resistance in lactic acid bacteria. *Antonie Van Leeuwenhoek* 76:377-382.

Pearce, L. Flint, S. 2002 .*Streptococcus thermophilus.* In *Encylopedia of Dairy Science* edited by Roginski, H.J., Fuquay,P. London: Academic Press.

Peterson, H.E. 1988. *Mesophilic starters*. International Dairy Federation 227:19.

Quiberoni, A., Guglielmotti, D., Binetti, A., Reinheimer, J. 2003. Characterization of three *Lactobacillus bulgaricus* phages and the physicochemical analysis of phage adsorption. *Journal of Applied Microbiology* 96:340-351.

Rajagopal, S.N., Sandine, W.E. 1990. Asociative growth and proteolysis of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in skim milk. *Journal of dairy science* 73:894-899.

Renz, U., Puhan, Z. 1975. Factors promoting bitterness in yoghurt. *Milchwissenschaft* 30265.

Robinson, R.K. 2002. Yoghurt, role of starter cultures. In *Encylopedia of Dairy Science* edited by Roginski, J., Fuquay, P. London:Academic Press.

Sanders, M.E. 1988. Phage resistance in lactic acid bacteria. *Biochimie* 70:411-421.

Sandine, W.E. 1996. Commercial production of dairy starter cultures. *In Dairy Starter Cultures* edited by Cogan, T.,M., Accolas, J.,P. New York:VCH Publishers.

Shah, N. 2003. The product and its manufacture. In *Encylopedia of Food Science and Nutrition* edited by Cabarello, B., Trugo, L., Finglas, P. London:Academic Press.

Shuler, M.L., Kargi, F. 2002. Growth patterns and kinetics in batch culture. In *Bioprocess Engineering Basic Concepts*. New Jersey:Prentice Hall.

Surono, I., Hosono, A. 2002. Starter cultures. In *Encylopedia of Dairy Science*, edited by Roginski, J., Fuquay, P. London:Academic Press.

Tamime, A.Y., Robinson, R.K. 1985. *Yoghurt Science and Technology*. Oxford:Pergamon Press.

Tari, C., Ustok, F., Harsa, S. 2008. Optimization of the associative growth of novel yoghurt cultures in the production of biomass, β-galactosidase and lactic acid using response surface meth odology, *International Dairy Journal* 18

Tekinşen, C. 2000. *Süt Ürünleri Teknolojisi*. Selçuk Üniversitesi.

Tunail, N., Demirtaş, D., Durlu-Özkaya, F. 2000. Yoğurt fabrikalarında görülen bakteriyofaj problemi, nedenleri ve çözüm önerileri, süt mikrobiyolojisi ve katkı maddeleri. *VI. Süt ve Süt Ürünleri Sempozyumu*, Tekirdağ.113-125.

Vaningelgem, F., Van der Meulen, R., Zamfir, M., Adriany, T., Laws, A.P., De Vuyst, L. 2004. *Streptococcus thermophilus* ST 111 produces a stable high-molecular-mass exopolysaccharide in milk-based medium. *International Dairy Journal* 14:857- 864.

Vick Roy, T.B. 1985. Lactic acid. In *Comprehensive Biotehnology*, edited by Moo-young,M. Oxford:Pergamon Press.

Watanabe, K., Takesue, S. 1972. The requirement for calcium in infection with Lactobacillus phages. *Journal of Genetic. Virology* 17:19-30.

Welman, A.D., Maddox, I.S. 2003. Exopolysaccharides from lactic acid bacteria: perspectives and challenges. *Trend in Biotechnology* 21:269–274.

Zadow, J.G. 2003. Whey and whey powder. In *Encyclopedia of Food Science and Nutrition* edited by Caballero, B., Trugo, L., Finglans, P. London: Academic Press.

APPENDIX A

CHEMICALS USED AND THEIR CODES

Table A.1. Chemicals Used in Experiments

No	Chemicals	Code
1	MRS Broth	Merck 1.10661
2	M17 Broth	Merck 1.15029
3	Glyserol	Applichem A2926
4	Agar	Applichem A0949
5	Bacteriological Peptone	Oxoid LP037
6	Yeast extract	Merck A 1.03753
7	Calcium Chloride	Applichem A3652
8	Iron(II)Sulfate Heptahydrate	Riedel de Haen 12354
9	Mangan(II) Sulfate-Monohydrate	Applichem A1038
10	Magnesium Sulfate Heptahydrate	Applichem A1037
11	Lactose Monohydrat	Merck 1.07657
12	Sodium Hydroxide	Merck 1.06498
13	Lactic Acid	Sigma L1875
14	Sulfuric Acid	Fluka 84721
15	Asetonitrile	Sigma 34851
16	Magnesium Phosphate Dibasic Trihydrate	Sigma M1265
17	Potassium Phosphate Monobasic	Sigma P0662
18	Sodium Phosphate Dibasic	Sigma S0876
19	Potassium Phosphate Dibasic	Sigma 71653
20	Ammonium Phosphate Dibasic	Sigma 215996
21	tri-Sodium Citrate	Sigma S4641
22	Ammonium formate	Sigma 156264
23	Adipic Acid	Aldrich A2.635-7
24	Lablemco Meat Extract	Oxoid LP029

(cont. on next page)

Table A.1. (cont.) Chemicals used in experiments

25	Ascorbic Acid	Fluka 95210
26	β-disodium Gycerol Phosphate	Sigma G6376
27	Bacto Soytone (Phytone Pepton)	BD/BLL 211906
28	Beef Extract	Sigma B4888
29	Casein Hydrolysate	Sigma C8845
30	Tryptone	Difco 211705
31	Gelatine	Sigma 2500
32	Dextrose	Fluka 49139
33	Sucrose	Fluka 84097
34	Sodium Chloride	Applichem A2942
35	tri-Ammonium Citrate	Sigma A1332
36	D (-) Glucose	Applichem A3666
37	Tween 80	Applichem A1390
38	Sodium Acetate	Sigma S2989
39	Anaerogen	Oxoid AN0025A

APPENDIX B

RECIPES FOR CULTURE MEDIA

B.1. MRS Broth

Ingredients	**g/L**
Pepton	10.0
Lab-Lemco meat extract	10.0
Yeast extract	5.0
D (-) Glucose	20.0
Tween 80	1 ml
K_2HPO_4	2.0
Sodium acetate	5.0
Triammonium citrate	2,0
$MgSO_4.7H_2O$	0,2
$MnSO_4.4H_2O$	0,05
Deionized water	1000 ml

All ingredients were dissolved in deionized water and pH was adjusted to 6.3. Medium was dispensed into test tubes and sterilized by autoclaving at 121° C for 15 minutes.

B.2. MRS Agar

Ingredients	**g/L**
Pepton	10.0
Lab-Lemco meat extract	10.0
Yeast extract	5.0
D (-) Glucose	20.0

Tween 80	1 ml
K_2HPO_4	2.0
Sodium acetate	5.0
Triammonium citrate	2,0
$MgSO_4.7H_2O$	0,2
$MnSO_4.4H_2O$	0,05
Agar	15.0
Deionized water	1000 ml

All ingredients were dissolved in deionized water and pH was adjusted to 6.3. Medium was sterilized by autoclaving at 121° C for 15 minutes.

B.3. MRS Soft Agar for Double Layer Agar Method

Ingredients	**g/L**
Pepton	10.0
Lab-Lemco meat extract	10.0
Yeast extract	5.0
D (-) Glucose	20.0
Tween 80	1 ml
K_2HPO_4	2.0
Sodium acetate	5.0
Triammonium citrate	2,0
$MgSO_4.7H_2O$	0,2
$MnSO_4.4H_2O$	0,05
Agar	6.0
Deionized water	1000 ml

All the ingredients were suspensed into deionized water and pH was adjusted to 6.3. Then 3 ml of solution was dispensed into test tubes and autoclaved at 121°C for 15 minutes. In phage experiments before poured onto the bottom agar layer, 0.1 ml sterile $CaCl_2$ (1M) was added into sterile soft agar tubes.

B.4. MRS Agar for Phage Experiments

Ingredients	g/L
MRS broth	52,2
Agar	15
Deionized water	1000 ml

All ingredients were suspended into deionized ater and pH was adjusted to 6,3. Media was sterilized by autoclaving the solution at 121°C for 15 minutes. after sterilization, 1M sterile $CaCl_2$ was added with 10% of the medium and pour into the petri dishes.

B.5. M17 Broth

Ingredients	g/L
Peptone	10.0
Lab-Lemco meat extract	5.0
Yeast extract	5.0
Lactose	5.0
Ascorbic acid	1.0
β-disodium glycerolphosphate	19.0
$MgSO_4.7H_2O$ (0.1 M)	1 ml
Deionized water	1000 ml

All ingredients were dissolved in deionized water and pH was adjusted to 6.9. Medium was dispensed into test tubes and sterilized by autoclaving at 121° C for 15 minutes.

B.6. M17 Agar

Ingredients	g/L
Peptone	10.0
Lab-Lemco meat extract	5.0
Yeast extract	5.0
Lactose	5.0
Ascorbic acid	1.0
β-disodium glycerolphosphate	19.0
$MgSO_4.7H_2O$ (0.1 M)	1 ml
Agar	15.0
Deionized water	1000 ml

All the ingredients were suspended into deionized water and pH was adjusted to 6.9. Medium was sterilized by autoclaving 121° C for 15 minutes.

B.7. Modified M17 broth for Phage Experiments

Ingredients	g/L
Phytone Peptone	5.0
Poly peptone	5.0
Yeast extract	2.5
Beef extract	5.0
Lactose	5.0
Ascorbic acid	0.5
β-disodium glycerolphosphate	9.5
$MgSO_4.7H_2O$ (1 M)	1 ml
$CaCl_2$ (1M)	1.2 ml
Deionized water	1000 ml

All ingredients were dissolved in deionized water and pH was adjusted to 6.9. Medium was dispensed into test tubes and sterilized by at 121° C for 15 minutes.

B.8. Modified M17 Agar

Ingredients	g/L
Peptone	10.0
Lab-Lemco meat extract	5.0
Yeast extract	5.0
Lactose monohydrat	13.0
Ascorbic acid	1.0
β-disodium glycerolphosphate	19.0
$MgSO_4.7H_2O$ (0.1 M)	1 ml
Agar	15.0
Deionized water	1000 ml

All the ingredients were suspended into deionized water and pH was adjusted to 6.9. Medium was sterilized by autoclaving 121° C for 15 minutes.

B.9. Modified M17 Soft Agar for Double Layer Agar Method

Ingredients	g/L
Phytone Peptone	2.4
Poly peptone	2.4
Yeast extract	2.0
Beef extract	3.3
Casein hydrolysate	2.4
β-disodium glycerolphosphate	12.7
$MgSO_4.7H_2O$ (1 M)	1.3 ml
Elliker broth	16 ml
Agar	6
Deionized water	1000 ml

All ingredients were suspended into deionized water and pH was adjusted to 6,9. 3 ml of the medium was suspended into test tubes and sterilized by autoclaving at 121°C for 15 minutes.

APPENDIX C

GROWTH CURVES FOR PURE CULTURE OF TY25

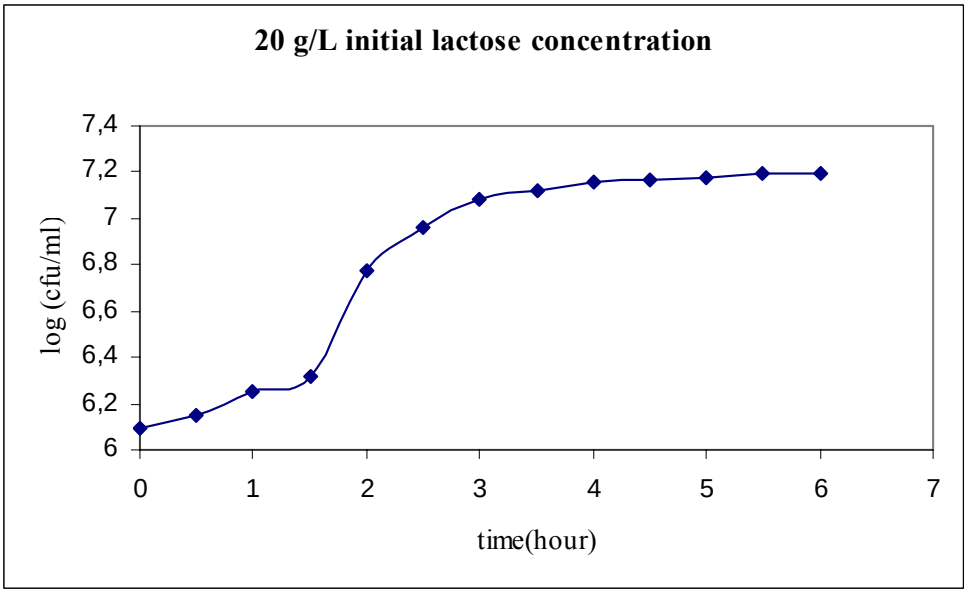


Figure C.1. Growth curve of *Streptococcus thermophilus* TY25 for 20 g/L initial lactose concentration.

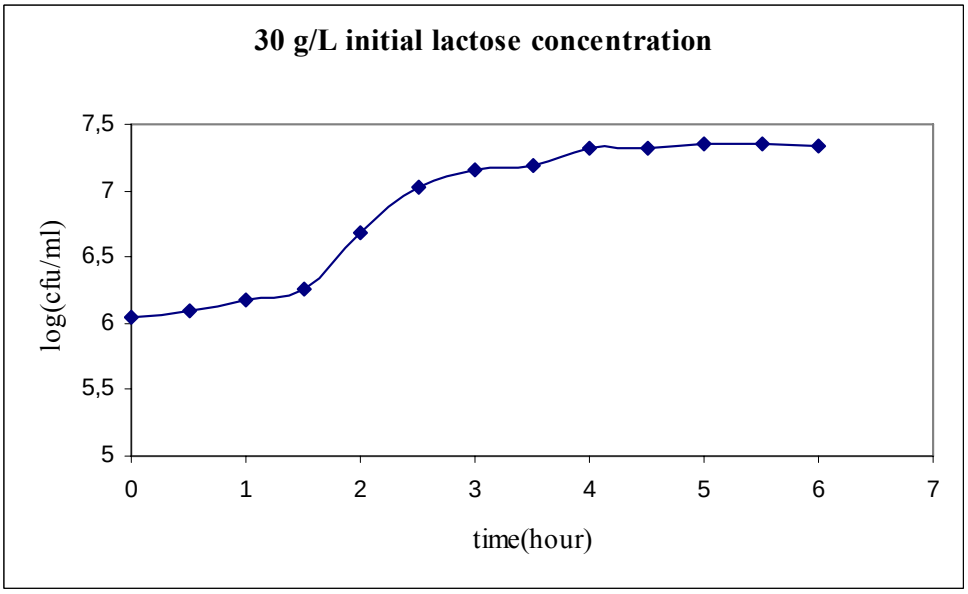


Figure C.2. Growth curve of *Streptococcus thermophilus* TY25 for 30 g/L initial lactose concentration for single strain fermentation

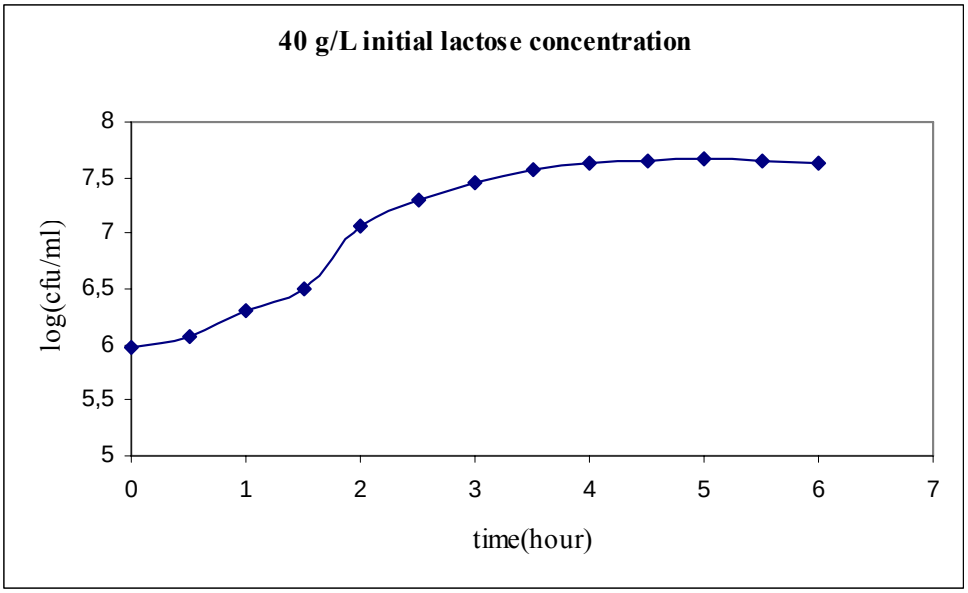


Figure C.3. Growth curve of *Streptococcus thermophilus* TY25 for 40 g/L initial lactose concentration for single strain fermentation

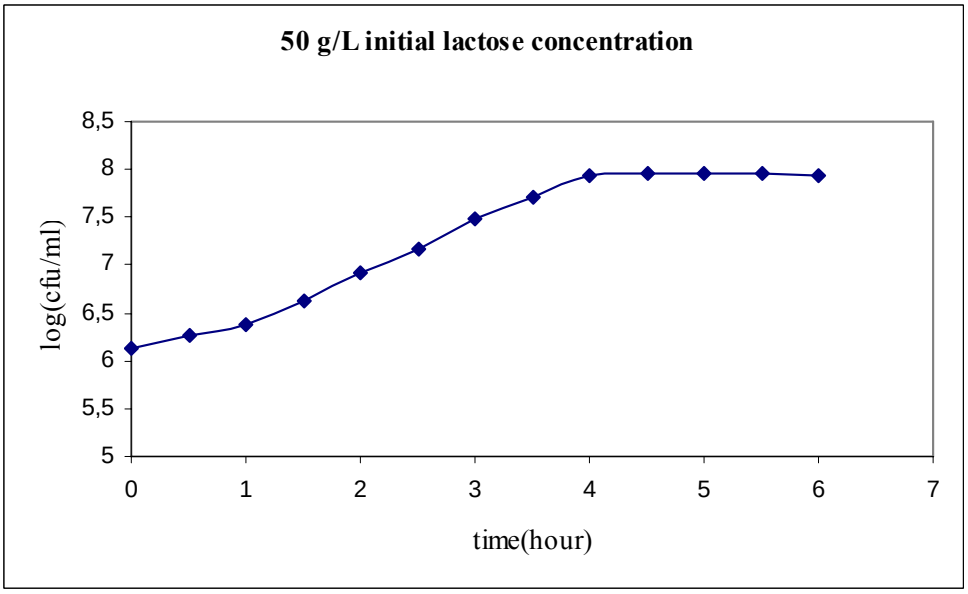


Figure C.4. Growth curve of *Streptococcus thermophilus* TY25 for 50 g/L initial lactose concentration for single strain fermentation

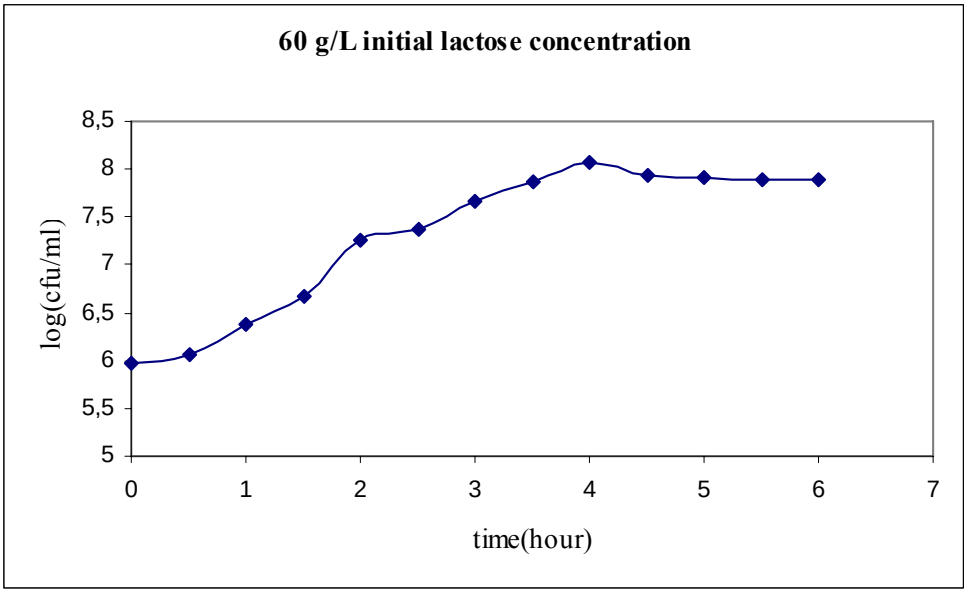


Figure C.5. Growth curve of *Streptococcus thermophilus* TY25 for 60 g/L initial lactose concentration for single strain fermentation

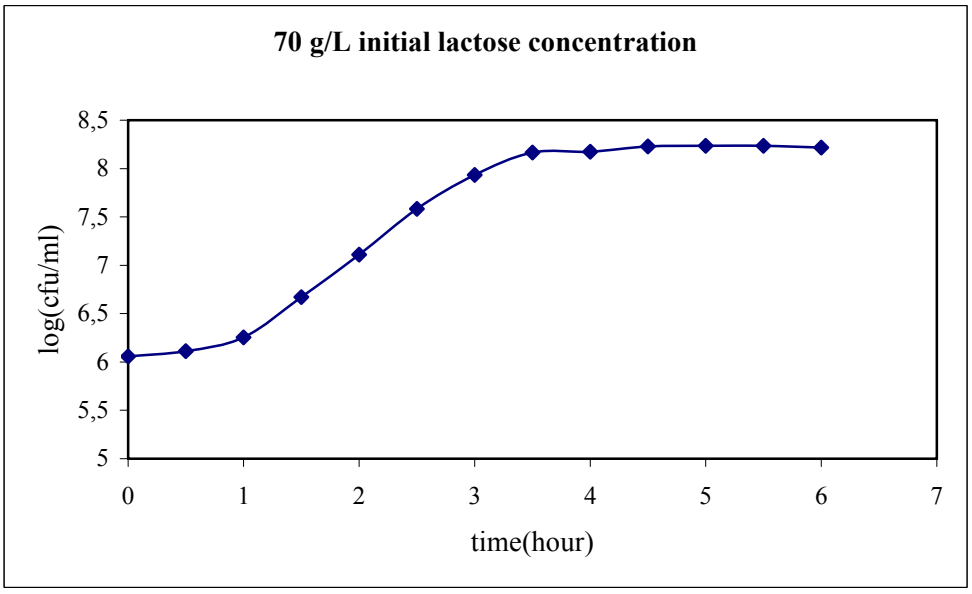


Figure C.6. Growth curve of *Streptococcus thermophilus* TY25 for 70 g/L initial lactose concentration for single strain fermentation

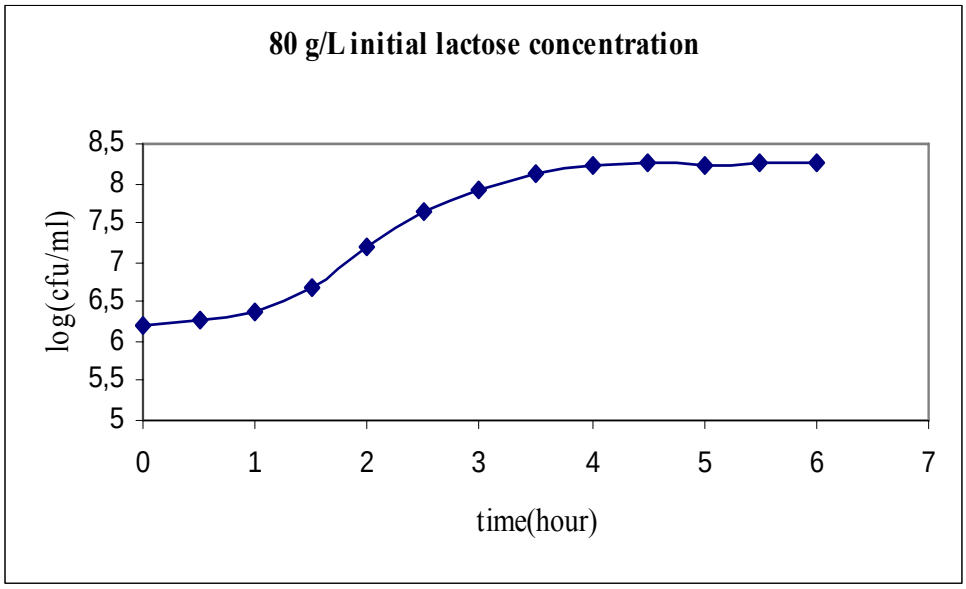


Figure C.7. Growth curve of *Streptococcus thermophilus* TY25 for 80 g/L initial lactose concentration for single strain fermentation.

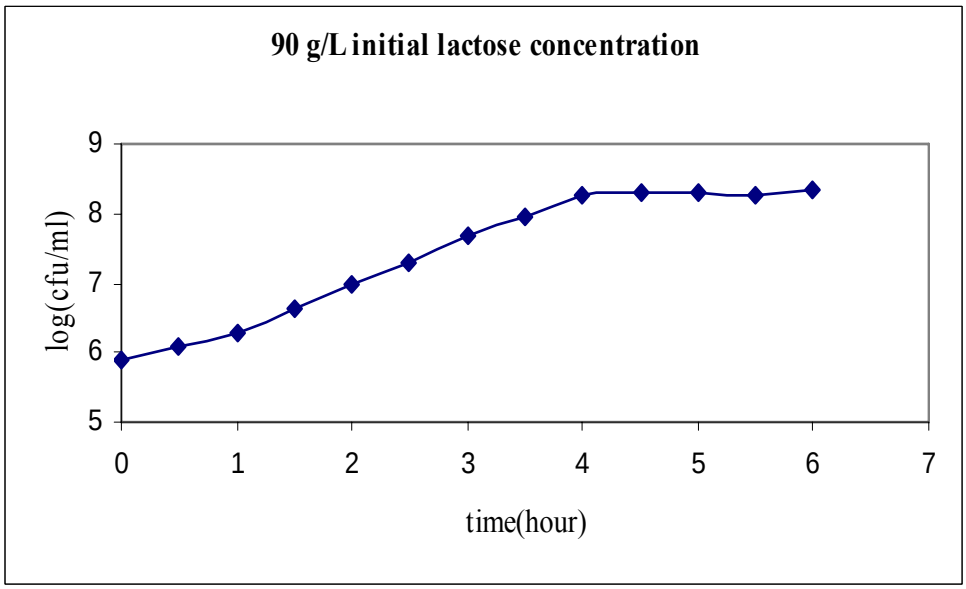


Figure C.8. Growth curve of *Streptococcus thermophilus* TY25 for 90 g/L initial lactose concentration for single strain fermentation.

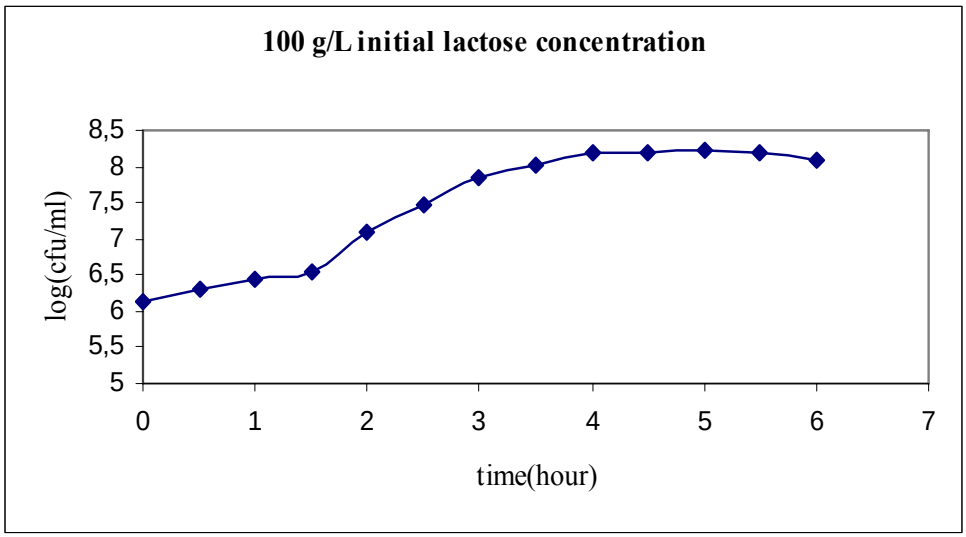


Figure C.9. Growth curve of *Streptococcus thermophilus* TY25 for 100 g/L initial lactose concentration for single strain fermentation.

APPENDIX D

GROWTH CURVES FOR PURE CULTURE OF TY30

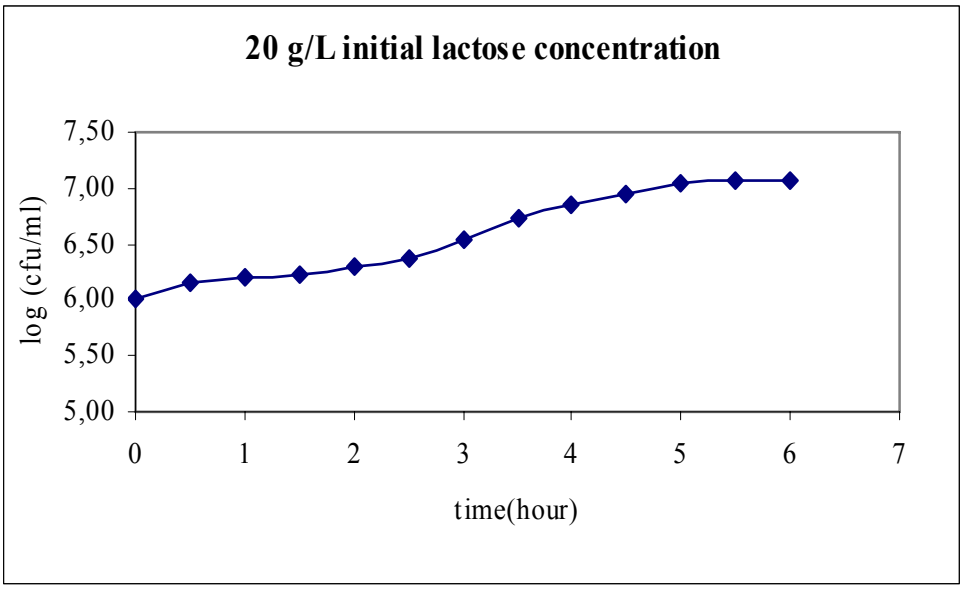


Figure D.1. Growth curve of *Lactobacillus bulgaricus* TY30 for 20 g/L initial lactose concentration for single strain fermentation.

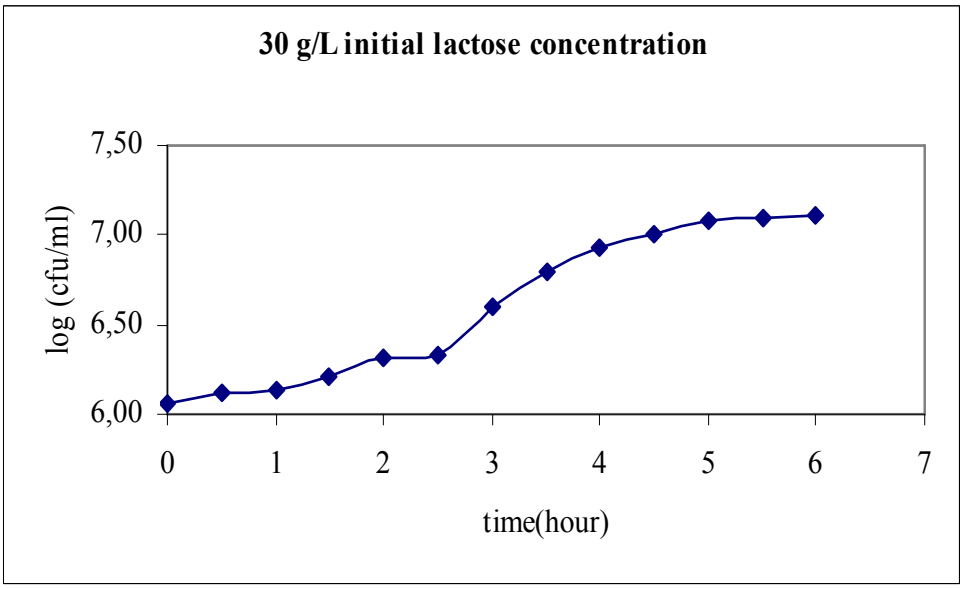


Figure D.2. Growth curve of *Lactobacillus bulgaricus* TY30 for 30 g/L initial lactose concentration for single strain fermentation

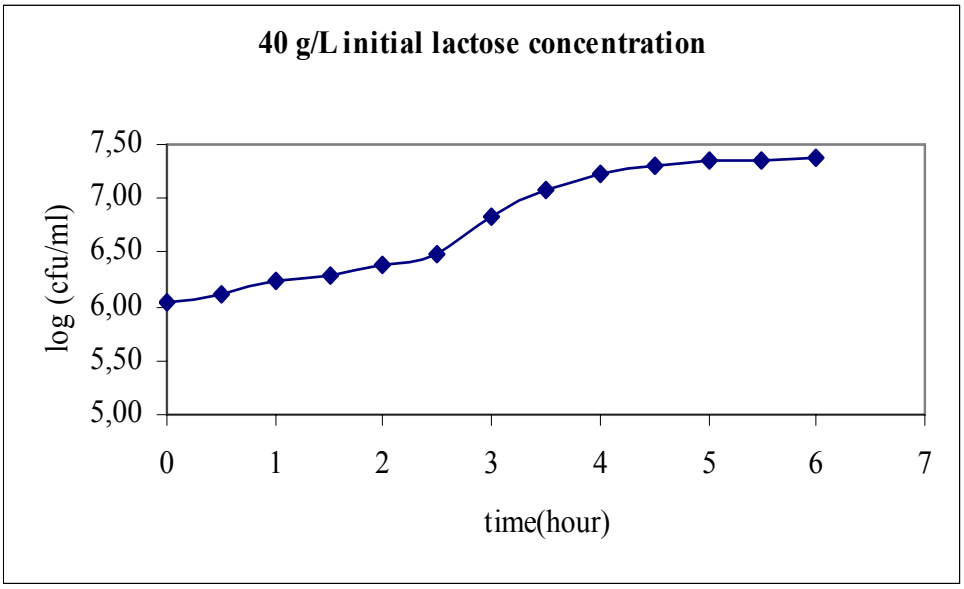


Figure D.3. Growth curve of *Lactobacillus bulgaricus* TY30 for 40 g/L initial lactose concentration for single strain fermentation

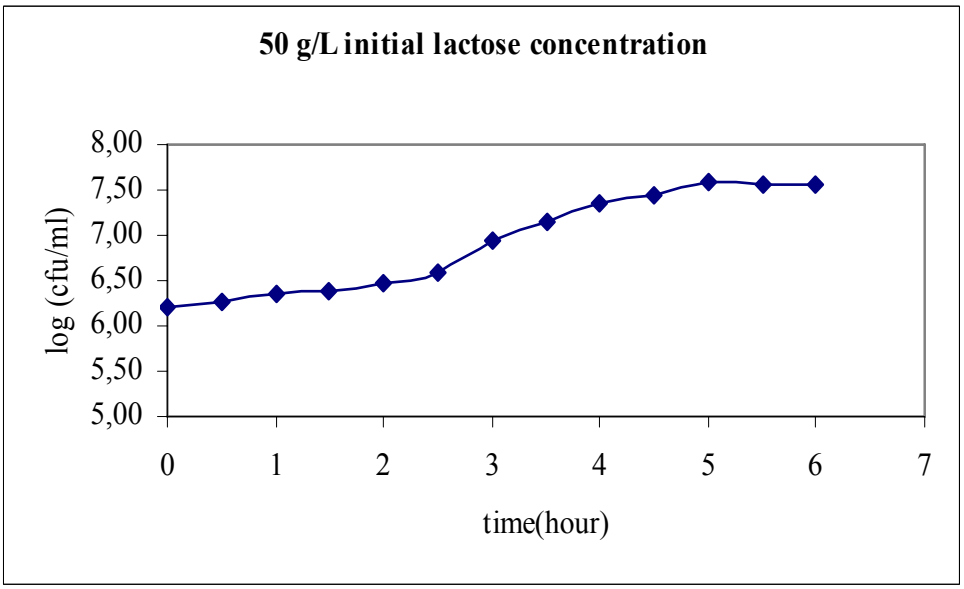


Figure D.4. Growth curve of *Lactobacillus bulgaricus* TY30 for 50 g/L initial lactose concentration for single strain fermentation

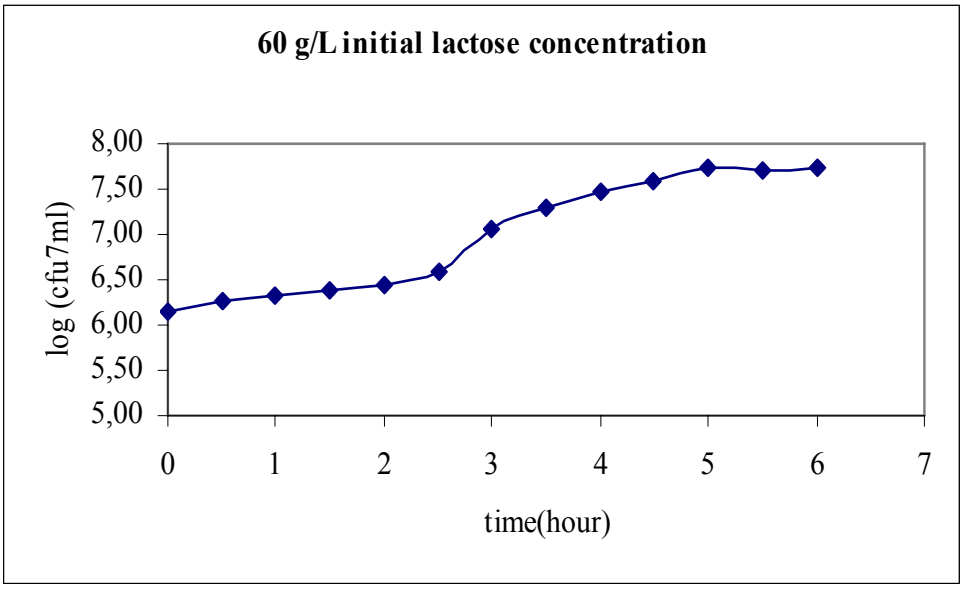


Figure D.5. Growth curve of *Lactobacillus bulgaricus* TY30 for 60 g/L initial lactose concentration for single strain fermentation

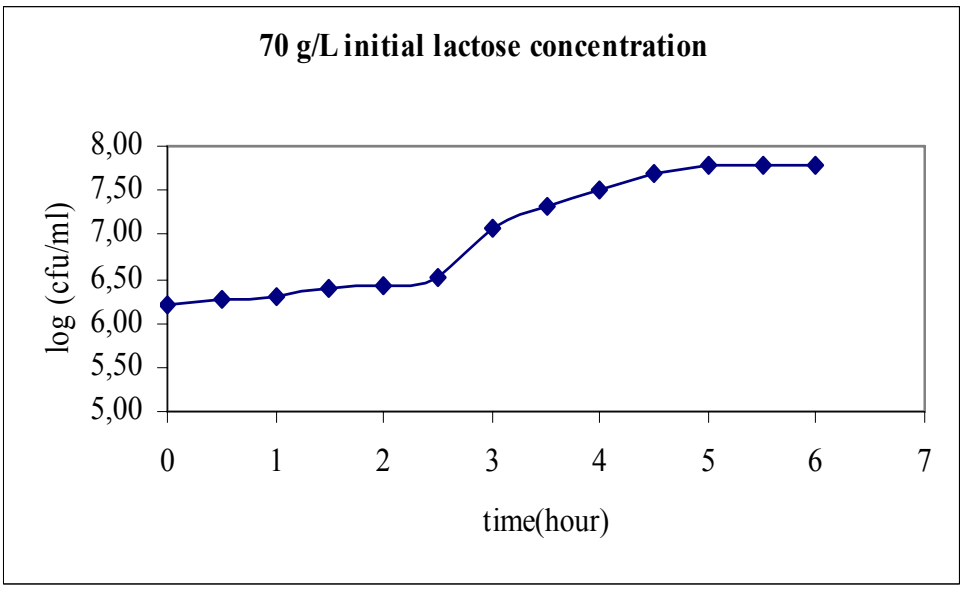


Figure D.6. Growth curve of *Lactobacillus bulgaricus* TY30 for 70 g/L initial lactose concentration for single strain fermentation

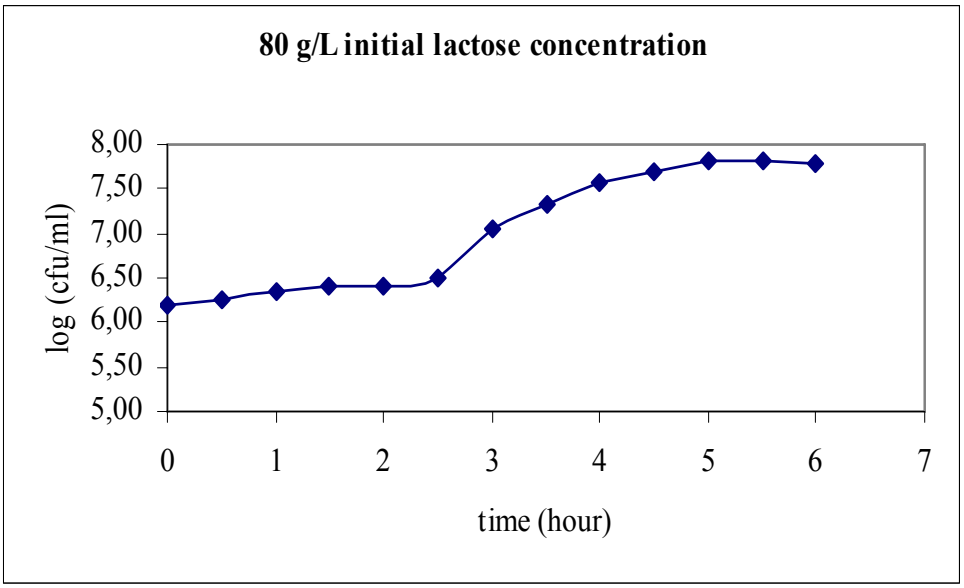


Figure D.7. Growth curve of *Lactobacillus bulgaricus* TY30 for 80 g/L initial lactose concentration for single strain fermentation

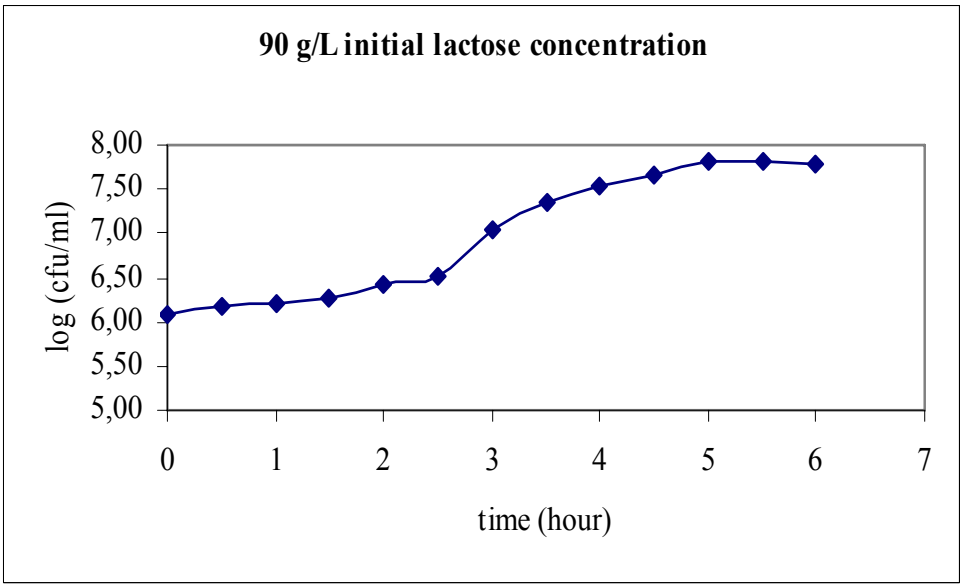


Figure D.8. Growth curve of *Lactobacillus bulgaricus* TY30 for 90 g/L initial lactose concentration for single strain fermentation

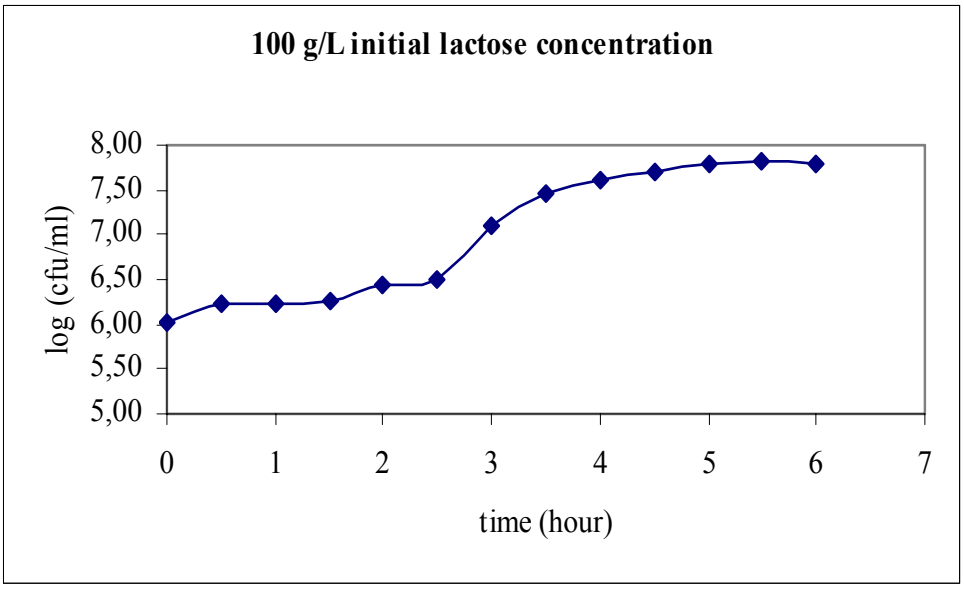


Figure D.9. Growth curve of *Lactobacillus bulgaricus* TY30 for 100 g/L initial lactose concentration for single strain fermentation

APPENDIX E

GROWTH CURVES OF MIXED CULTURE FERMENTATIONS FOR TY25 AND TY30

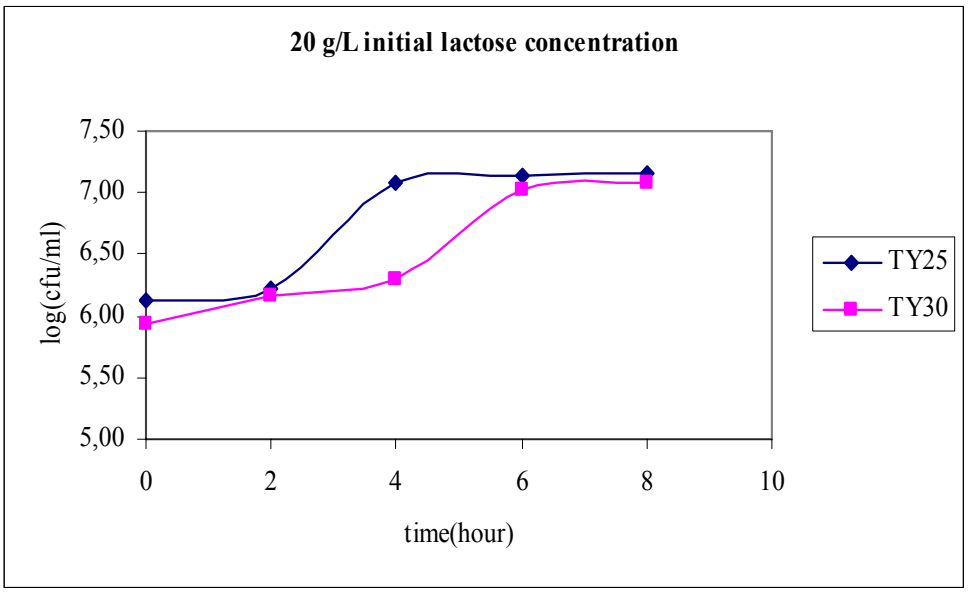


Figure E.1. Growth curves of TY25 and TY30 for 20 g/L initial lactose concentration

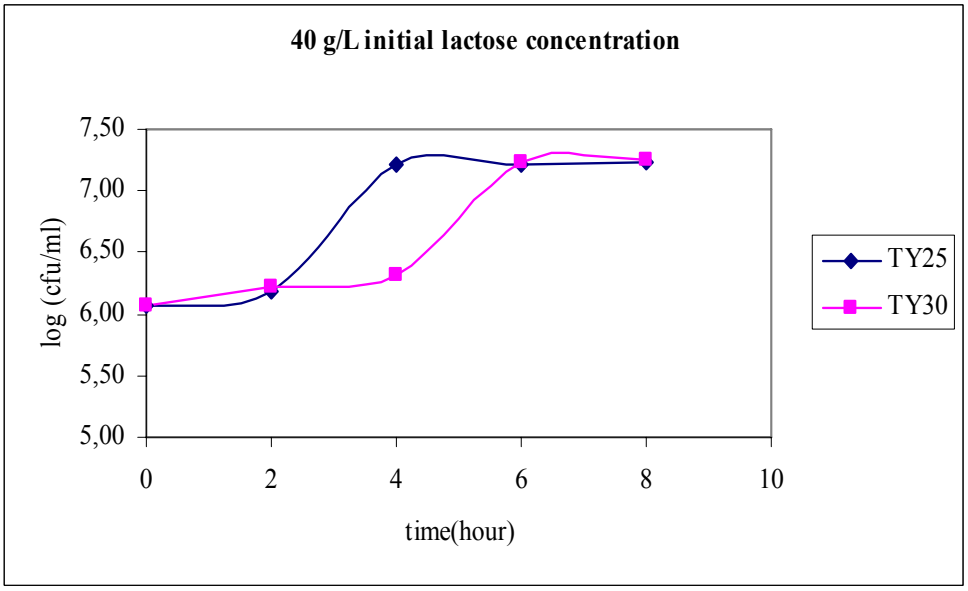


Figure E.2. Growth curves of TY25 and TY30 for 40 g/L initial lactose concentration

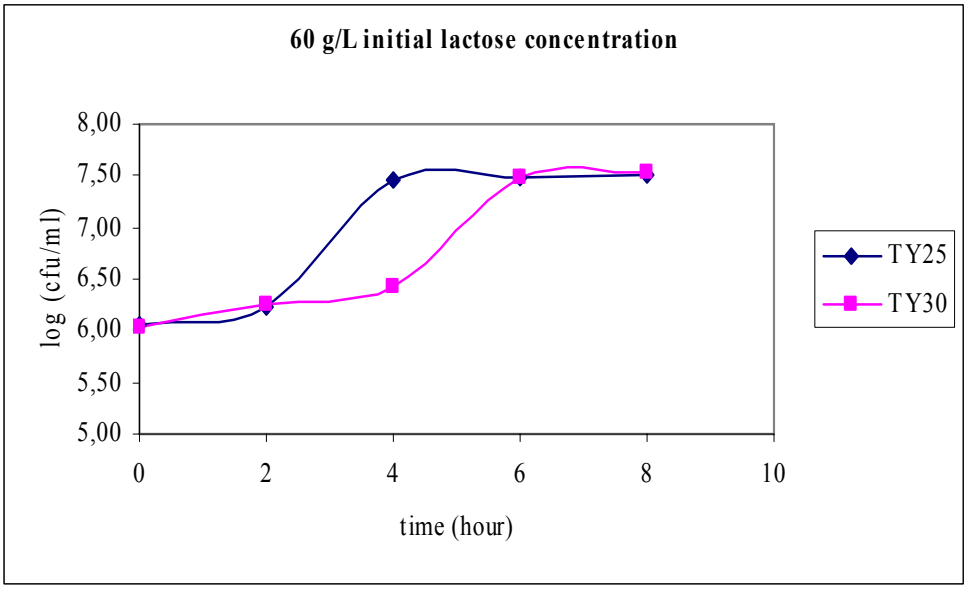


Figure E.3 .Growth curves of TY25 and TY30 for 60 g/L initial lactose concentration

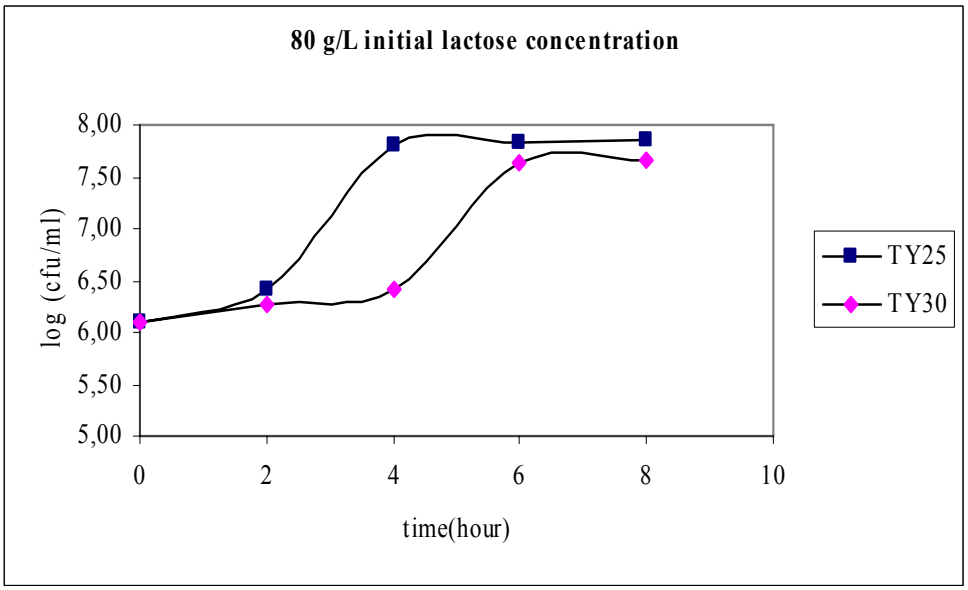


Figure E.4. Growth curves of TY25 and TY30 for 80 g/L initial lactose concentration

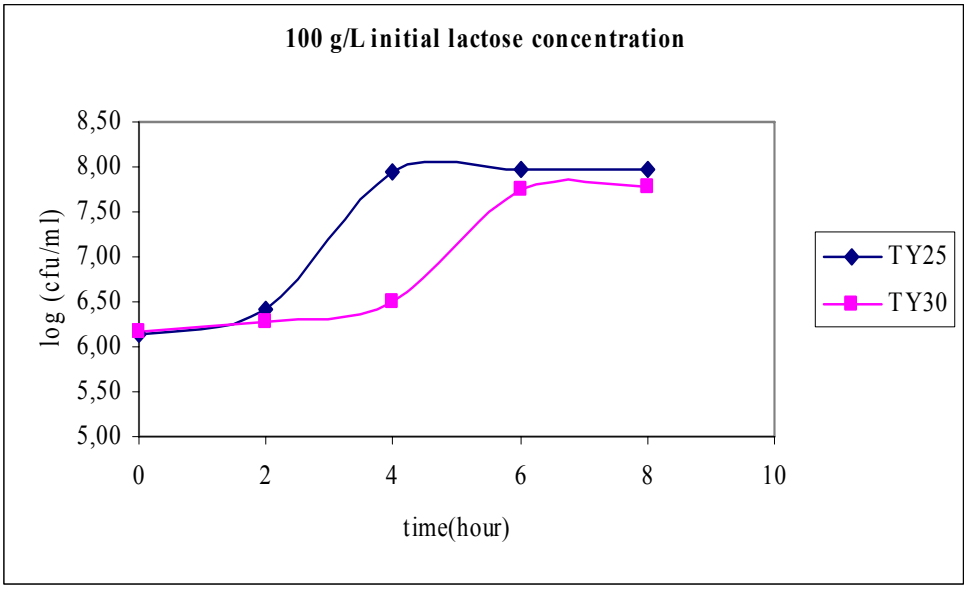


Figure E.5. Growth curves of TY25 and TY30 for 100 g/L initial lactose concentration

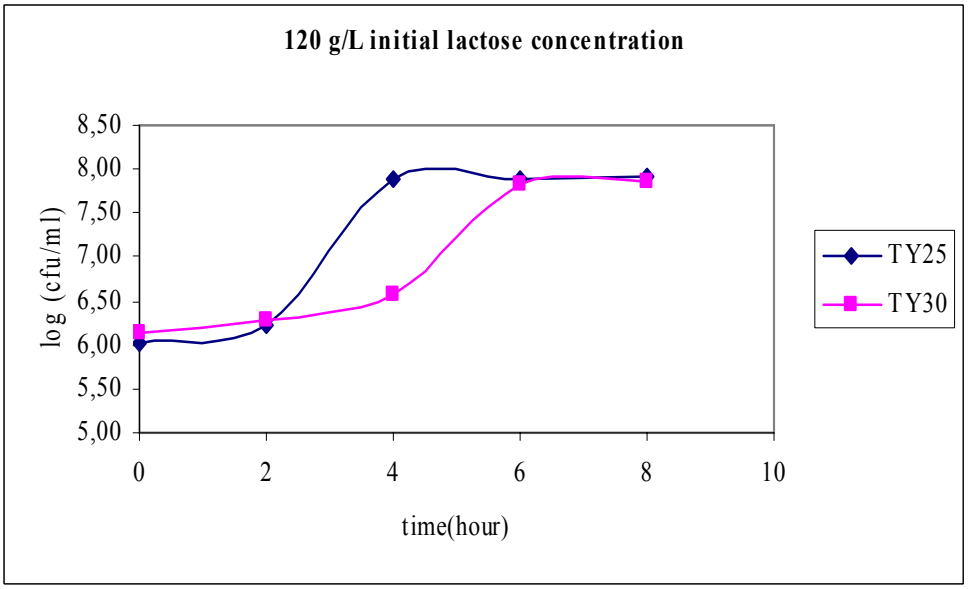


Figure E.6. Growth curves of TY25 and TY30 for 120 g/L initial lactose concentration

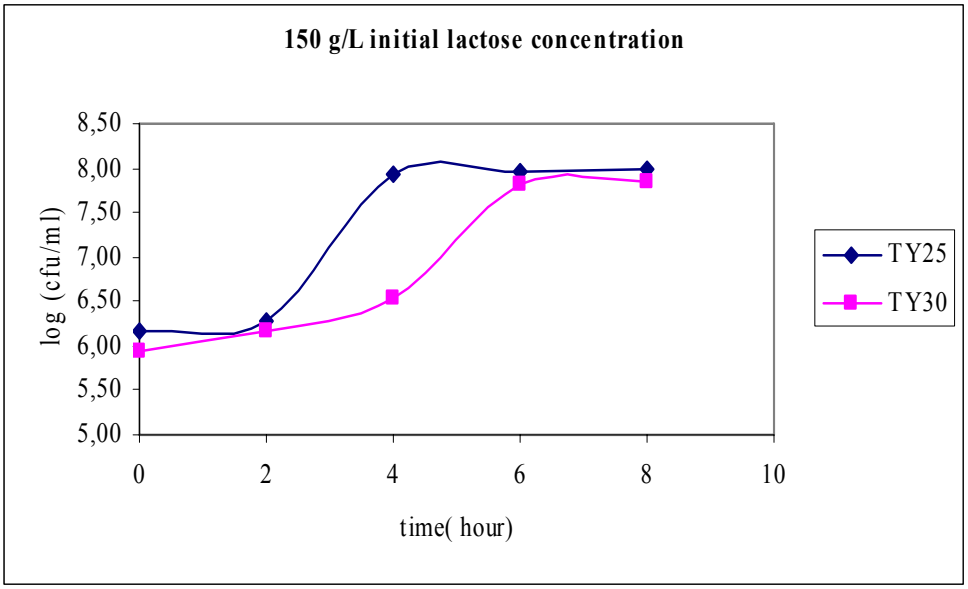


Figure E.7. Growth curves of TY25 and TY30 for 150 g/L initial lactose concentration