



UNIVERSITI PUTRA MALAYSIA

**USE OF CRYOPROTECTANTS IN ENHANCING VIABILITY OF
PROBIOTIC *LACTOBACILLUS* STRAINS DURING FREEZE-DRYING
AND STORAGE**

ANAHITA KHORAMNIA

FBSB 2007 17

**USE OF CRYOPROTECTANTS IN ENHANCING VIABILITY OF PROBIOTIC
LACTOBACILLUS STRAINS DURING FREEZE-DRYING AND STORAGE**

By

ANAHITA KHORAMNIA



**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in
Fulfilment of the Requirements for the Degree of Master of Science**

October 2007

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the
requirements for the degree of Master of Science

**USE OF CRYOPROTECTANTS IN ENHANCING VIABILITY OF PROBIOTIC
LACTOBACILLUS STRAINS DURING FREEZE-DRYING AND STORAGE**

By

ANAHITA KHORAMNIA

October 2007



Chair: Professor Norhani Abdullah, PhD

Faculty: Biotechnology and Biomolecular Sciences

In recent years, probiotics have been considered to be used as feed supplements to improve the health and growth performance of poultry in place of antibiotic growth promoters. This is due to concerns that the rampant use of antibiotic growth promoters in livestock, particularly poultry, may produce adverse effects on humans, such as the development of antibiotic resistant bacteria and production of antibiotic residues in animal products. Unlike probiotics for humans, which are usually kept refrigerated, probiotics for poultry are normally kept in the farm at room temperature, and this may reduce the viability of the micro-organisms used in the probiotics during storage. Cryoprotectants incorporated during freeze drying of the probiotic could enhance the shelf-life of the probiotic micro-organisms. Thus, in this investigation, the main objective was to determine the best combination of cryoprotectants to enhance the viability of *Lactobacillus brevis* I25 and *L. reuteri* C10 during freeze-drying by using the response surface methodology (RSM). A five-level, three-variable central composite rotatable design (CCRD) was used to evaluate the interactive effects of skim milk, sucrose and lactose as cryoprotectants, on the viability of *L. brevis* I25 and *L. reuteri* C10 during freeze drying. The inputs, log cfu/ml, were derived experimentally and tested by RSM. The models were found to describe adequately the experimental range studied. The optimum combination of cryoprotectants derived via RSM analysis were: 8% skim milk, 22% sucrose, 0.5% lactose for *L. brevis* I25 and 19.5% skim milk, 1% sucrose, 9% lactose for *L. reuteri* C10. The actual experimental results on the viability of *L. brevis* I25 and *L. reuteri* C10 after freeze-drying were 8.88 and 8.83 log cfu/ml, respectively, under optimum formulation. These values were highly comparable to the predicted values by



RSM method of SAS/STAT which were 8.82 log cfu/ml for *L. brevis* I25 and 8.89 log cfu/ml for *L. reuteri* C10. The log cfu/ml values for controls (freeze-dried without cryoprotectants) were 7.65 and 7.2 for *L. brevis* I25 and *L. reuteri* C10, respectively. During the six month storage study at 4°C and 30°C, the optimum cryoprotectant combination for *L. brevis* I25 had a very high survival rate at 4°C but not at 30°C. On the other hand, the survival rate of the best combination for *L. reuteri* C10 was very high at both temperatures during storage. There was 0% residual viability for control culture after 16 weeks of storage for *L. brevis* I25 at 4°C and after 4 weeks at 30°C. For *L. reuteri* C10 after 12 and 8 weeks no bacterial growth were detected at 4°C and 30°C, respectively. The organic acids and amylase activity of bacterial cultures were also analysed during storage. The results showed that during storage at 4°C, the acetic acid concentration decreased from 144 mM to 100.25 mM for *L. brevis* I25 and from 153 mM to 115.6 mM for *L. reuteri* C10. In the case of lactic acid, the concentration decreased from 294 mM to 215 mM for *L. brevis* I25 and 205 mM to 124 mM for *L. reuteri* C10. The concentration of succinic acid also decreased from 2.9 mM to 1.2 mM for *L. brevis* I25 and from 17 mM to 9.4 mM for *L. reuteri* C10. There was also a reduction in amylase activity from 0.2 U to 0.11 U for *L. brevis* I25 and from 0.34 U to 0.18 U for *L. reuteri* C10. Acid production and amylase activity patterns for both *Lactobacillus* strains correspond to the survival rate of the bacteria during storage at 30°C.



Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

GUNA 'CRYOPROTECTANT' DALAM MENINGKATKAN KEMADDIRAN STRAIN PROBIOTIK *LACTOBACILLUS* SEMASA KENING BELUN DAN PENYIMPANAN .

Oleh

ANAHITA KHORAMNIA

Oktober 2007



Pengerusi: Profesor Norhani Abdullah, PhD

Fakulti: Bioteknologi dan Sains Biomolekul

Kebelakangan ini, probiotik telah diberi pertimbangan sebagai makanan tambahan bagi meningkatkan kesihatan dan prestasi pertumbuhan ternakan ayam sebagai gantikan kepada antibiotik promoter pertumbuhan. Ini adalah disebabkan oleh penggunaan antibiotik promoter pertumbuhan yang berlebihan dalam penternakan, terutamanya bagi ternakan ayam akan mengakibatkan kesan kepada manusia seperti perkembangan bakteria rintang antibiotik dan juga kehadiran residu antibiotik dalam produk haiwan. Tidak seperti probiotik untuk manusia yang biasanya disimpan sejuk, probiotik ternakan ayam biasanya disimpan di dalam ladang ternakan di dalam suhu bilik dan keadaan ini akan mengurangkan bilangan mikroorganisma hidup yang digunakan di dalam probiotik semasa penyimpanan. Penggunaan ‘cryoprotectant’ semasa proses kering beku dalam probiotik dapat meningkatkan jangka hayat mikroorganisma probiotik. Oleh yang demikian, objektif utama dalam kajian ini adalah untuk mengenalpasti kombinasi ‘cryoprotectant’ terbaik bagi meningkatkan bilangan *Lactobacillus brevis* I25 dan *L. reuteri* C10 semasa proses kering beku dengan menggunakan kaedah ‘Response Surface Methodology’ (RSM). ‘ Satu kaedah lima peringkat dengan tiga pengubahsuaian ‘CCRD’ telah digunakan untuk menilai kesan interaktif antara susu skim, sukrosa dan laktosa sebagai ‘cryoprotectant, ke atas bilangan *L. brevis* I25 dan *L. reuteri* C10 semasa proses kering beku. Input dan log cfu/ml telah diperolehi secara eksperimen dan dianalisa menggunakan RSM. Model-model yang diperolehi dapat menghuraikan julat eksperimen yang dikaji dengan baik. Keadaan optima yang diperolehi menerusi analisis RSM adalah seperti berikut: 8% susu skim, 22% sukrosa, dan 0.5% laktosa



bagi *L. brevis* I25 dan 19.5% susu skim, 1% sukrosa, dan 9% laktosa bagi *L. reuteri* C10. Keputusan eksperimen sebenar bilangan *L. brevis* I25 dan *L. reuteri* C10 selepas kering beku adalah 8.88 dan 8.86 log cfu/ml di dalam keadaan optima. Nilai-nilai ini adalah sebanding dengan nilai ramalan yang diperolehi menerusi kaedah RSM bagi SAS/STAT iaitu 8.82 dan 8.89 log cfu/ml. Nilai log cfu/ml untuk kawalan ialah 7.65 untuk *L. brevis* I25 dan 7.20 untuk *L. reuteri* C10. Bagi kajian mengenai kesan penyimpiren keatas kemandiran bakteria pada 4°C dan 30°C selama enam bulan, didapati kombinasi 'cryoprotectant' bagi *L. brevis* I25 memberi nilai peratus kemandiran yang lebih tinggi pada suhu 4°C, berbanding dengan suhu 30°C. Manakala untuk *L. reuteri* C10, kadar kemandiran adalah sangat baik untuk kedua suhu penyimpanan. Peratus residual bagi kultur kawalan adalah 0% selepas 16 minggu bagi *L. brevis* I25 pada suhu 4°C dan 4 minggu pada suhu 30°C. Bagi *L. reuteri* C10 kawalan, tiada pertumbuhan bakteria dikesan selepas 12 dan 8 minggu pada suhu 4 dan 30°C, masing-masing. Asid organik dan aktiviti amylase kultur bakteria juga dikaji semasa storan. Hasil kajian menunjukkan pada suhu 4°C, kepekatan asid asetik menurun daripada 144 mM kepada 100.25 mM bagi *L. brevis* I25 dan 205 mM kepada 124 mM bagi *L. reuteri* C10. Asid suksinik juga menurun daripada 2.9 mM kepada 1.2 mM bagi *L. brevis* I25 dan daripada 17 mM kepada 9.4 mM bagi *L. reuteri* C10. Aktiviti amilase juga menurun daripada 0.2 U kepada 0.11 U bagi *L. brevis* I25 dan 0.34 U kepada 0.18 U bagi *L. reuteri* C10. Corak penghasilan asid and aktiviti enzim amilase untuk kedua spesies *Lactobacillus* menyamai kadar kemandiran bakteria semasa storan pada suhu 30°C.

ACKNOWLEDGEMENTS

In The Name of God, The Most Merciful and Most Beneficent

All praises do to God, Lord of the universe. Only by His grace and mercy this thesis can be completed.



The completion of this thesis would have been impossible if not for the assistance and direct involvement of so many kindhearted individuals. Thus, I am very much indebted to my previous mentors and I have no way of repaying such a debt except to express my sincerest gratitude.

First and foremost, I am very grateful to my supervisor Professor Dr. Norhani Abdullah, for her strong support, guidance, and patience. I am also grateful to Professor Dr. Ho Yin Wan for the very enriching and thought provoking discussion and lectures which helped to shape the thesis. She was always there to provide everything I needed in the laboratory. I am also grateful to Dr. Sieo Chin Chin and Dr. Kalavathy Ramasamy in their capacity as members of the Supervisory Committee. I am also indebted to the staff of the Institute of Bioscience, University Putra Malaysia, Mr. Kairul, Mr. Saparin and Madam Haw for their help and cooperation. Special thanks are also extended to Dr. Liew Siew Ling who helped me in every way possible.

I wish to express my deepest gratitude to my parents, my brother Amir and my sister, Arezoo for their prayers, continuous moral support and unending encouragement. Last but not least, I wish especially to acknowledge my beloved husband, Afshin Ebrahimpour for his love, support, patience and understanding for every situation I am in.



I certify that an Examination Committee has met on 23th October 2007 to conduct the final examination of Anahita Khoramnia on her Master of Science thesis entitled “Use of cryoprotectants in enhancing viability of probiotic *Lactobacillus* strains during freeze-drying and storage” in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the student be awarded the degree of Master of Science. Members of the Examination Committee were as follows:

Mohd Puad Abdullah, PhD

Lecturer

Faculty of Biotechnology and Biomolecular Sciences

Universiti Putra Malaysia

(Chairman)



Rosfarizan Mohamad, PhD

Lecturer
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Shuhaimi Mustafa, PhD

Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Rosli MD Illias, PhD

Associate Professor
Faculty of Chemical Engineering and Natural Resources Engineering
Universiti Teknologi Malaysia
(External Examiner)

HASANAH MOHD. GHAZALI, PhD

Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 3 January 2008

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

Norhani Abdullah, PhD

Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Ho Yin Wan, PhD

Professor
Institute of Bioscience



Universiti Putra Malaysia
(Member)

Sieo Chin Chin, PhD

Lecturer
Faculty of Biotechnology and Bimolecular Sciences
Universiti Putra Malaysia
(Mmember)

Kalavathi Ramasamy, PhD

Lecturer
Faculty of Pharmacy
Universiti Technology Mara
(Member)

AINI IDERIS, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 22 January 2008

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.



ANAHITA KHORAMNIA

Date: 4 March 2008

TABLE OF CONTENTS

	Page
ABSTRACT	ii
ABSTRAK	v
ACKNOWLEDGEMENTS	viii
APPROVAL	x
DECLARATION	xii
LIST OF TABLES	xvi
LIST OF FIGURES	xviii
CHAPTER	



1	INTRODUCTION	1
2	LITERATURE REVIEW	4
2.1	Antibiotic Resistant Bacteria	4
2.2	What Are Probiotics?	5
2.3	History of Probiotics	7
2.4	Probiotic Properties	8
2.5	<i>Lactobacillus Reuteri</i>	10
2.6	<i>Lactobacillus Brevis</i>	10
2.7	Mechanism of Action of Probiotics	11
2.8	Down Stream Processing of Probiotics	11
	2.8.1 Preservation of Cells	12
	2.8.1.1 Foam Formation	12
	2.8.1.2 Spray Drying	13
	2.8.1.3 Fluidized Bed Drying	14
	2.8.1.4 Freeze-Drying	14
	(a) The Principle of Freeze-Drying	15
	(b) Freezing Condition	17
	(c) Freeze-Drying Draw Backs	19
	2.8.2 Cryoprotective Additives (CPA)	20
	2.8.2.1 Mechanisms of Cryoprotective Action	22
	2.8.2.2 Disaccharides	22
	(a) Sucrose	
22	(b) Lactose	23
	2.8.2.3 Complex Compounds	24
	Skimmed Milk	25
	2.8.3 Optimization	26
	2.8.3.1 Response Surface Methodology (RSM)	26
	2.8.3.2 Theory of RSM	26
	(a) Preliminary Study	
28	(b) Experimental Design and	
Finding	29	
	The Best-Fitted Model	
	(c) Determination of Optimal Operating	32
	Conditions	
	(d) Verification of the Predicted	
33	Optimum Point	
3	MATERIALS AND METHODS	34
	3.1 Microorganisms	34
	3.2 Buffer Preparation	34



3.3	Inoculum Preparation	34
3.4	Preparation Suspending Medium of Cryoprotectants	35
3.5	Experimental Design and Statistical Analysis	37
3.6	Verification of Estimated Data	39
3.7	Sample Preparation for Freeze-Drying	40
3.8	Viable Cell Count	40
3.9	Storage Study	41
3.10	Determination of Amylase	42
3.11	Determination of Fermentation End Products	43
4	RESULTS	45
4.1	Response Surface Methodology	45
4.2	<i>L. Brevis</i> I25 Response Surface Methodology	45
	4.2.1 Study on the Effects of the First Range of Concentration on <i>L. Brevis</i> I25 Viability	45
	4.2.2 Model Adequacy Evaluation of <i>L. Brevis</i> I25 with the First Range of Factors Level	48
	4.2.3 Evaluation of the Model Visualization	50
	4.2.4 Changing the Ranges	53
	4.2.5 Evaluation of the Model with the Second Range of Factors Level	55
	4.2.6 Evaluation of the Model Visualization	59
4.3	Developing a Regression Model for Finding a Best-Fitted Model for <i>L. Brevis</i> I25	61
4.4	Finding the Optimum Point of the Factors for <i>L. Brevis</i> I25	66
4.5	Three-Dimensional Response Surface Plots	66
4.6	Definition of Range of Factors Level for <i>L. Reuteri</i> C10	70
	4.6.1 Determination of Cell Viability Based on CCRD Conditions for <i>L. Reuteri</i> C10	70
	4.6.2 Evaluation of the Model Adequacy for <i>L. Reuteri</i> C10 First Ranges	72
	4.6.3 Evaluation of the Model Visualization	75
4.7	Developing a Regression Model for <i>L. Reuteri</i> C10	77
4.8	Finding the Optimum Point of the Factors for <i>L. Reuteri</i> C10	80
4.9	Three-Dimensional Response Surface Plots	82
4.10	Verification of the Optimum Points	86
4.11	Storage Study	87
4.12	Acid Production	92
	4.12.1 Acetic Acid	92
	4.12.2 Lactic Acid	93
	4.12.3 Succinic Acid	95
4.13	Amylase Activity of Cultures During Storage	98
5	DISCUSSION	100
5.1	<i>L. Reuteri</i> and <i>L. Brevis</i>	100
5.2	Bacterial Preservation	101



5.2.1	Cryoprotectants	102
5.2.2	Freeze-Drying of Bacterial Culture	104
5.3	Optimization of <i>Lactobacillus</i> Preservation Using RSM	106
5.4	Storage Study	110
5.5	Fermentation Activity of Bacterial Culture	114
5.6	Amylase Activity	116
6	CONCLUSION AND RECOMMENDATIONS FOR	118
	REFERENCES	120
	APPENDICES	130
	BIODATA OF STUDENT	133



LIST OF TABLES

Table

Page

1. Bacterial growth inhibitory products produced by probiotic bacteria and mechanisms of inhibition on target organisms	9
2. Treatment combinations based on central composite design (CCRD)	36
3. Independent variables and levels used for central composite rotatable design (CCRD)	38
4. <i>L. brevis</i> I25 first range combinations and responses according to the CCRD	47
5. Analysis of variance (ANOVA) for Response Surface Quadratic Model of <i>L. brevis</i> I25 1st ranges	49
6. <i>L. brevis</i> I25 (first range) second order model coefficient of estimates	50
7. The top 10 solutions released from the software analysis 53	
8. Second experimental range and levels of the three independent variables used in RSM in terms of actual and coded factors	54
9. <i>L. brevis</i> I25 2 nd range combinations and responses according to the CCRD	56
10. Analysis of variance (ANOVA) for Response Surface Quadratic Model of <i>L. brevis</i> I25 2 nd range of concentration	57
11. Coefficient of estimates for response surface quadratic model of <i>L. brevis</i> I25 2 nd ranges	58
12. Analysis of variance (ANOVA) in the regression model selected through variable selection	62
13. Coefficient estimates in the regression model selected through variable selection	63



14. The actual and predicted values of the <i>L. brevis</i> I25 viability	65
15. <i>L. reuteri</i> C10 first range combinations and responses according to the CCRD	71
16. Analysis of variance (ANOVA) for Response Surface Quadratic Model of <i>L. reuteri</i> C10 1 st ranges	73
17. Coefficient of estimates for Response Surface Quadratic Model of <i>L. reuteri</i> C10 1st ranges	74
18. Analysis of variance (ANOVA) for evaluation of the second-order model for <i>L. reuteri</i> C10	78
19. Coefficient estimates in the second-order model for <i>L. reuteri</i> C10	79
20. The actual and predicted values of the <i>L. reuteri</i> C10 viability	81
21. Optimum conditions predicted by the model and corresponded experimental values (cfu/ml)	87
22. Survival rate of <i>L. brevis</i> I25 and <i>L. reuteri</i> C10 at different storage periods	91
23. Amounts of different acids before storage and after 6 months storage	97



LIST OF FIGURES

Figure

Page

1. The procedure of freeze-drying	16
2. Different kinds of contour plots	31
3. Three-dimensional plot including a peak	32
4. The model diagnostic for <i>L. brevis</i> I25 (first range)	51
5. Three-dimensional plot of the model graph for <i>L. brevis</i> I25 (first range)	52
6. Contour plot of the model graph for <i>L. brevis</i> I25 (first range)	52
7. Diagnostic graph for <i>L. brevis</i> I25 2 nd range of concentrations	59
8. Three-dimensional plot of the model graph for <i>L. brevis</i> I25 2 nd ranges	60
9. Contour plot of the model graph for <i>L. brevis</i> 2 nd ranges	61
10. Response surface for the effects of skim milk and sucrose on the viability	68



of <i>L. brevis</i> I25 at the coded level of -1.67 of lactose	
11. Response surface for the effects of skim milk and sucrose on the viability of <i>L. brevis</i> I25 at +0.86 coded level of lactose	68
12. Response surface for the effects of skim milk and lactose on the viability of <i>L. brevis</i> I25 at 1.18 coded level of sucrose	69
13. Response surface for the effects of sucrose and lactose on the viability of <i>L. brevis</i> I25 at 0.872 coded level of skim milk	70
14. Diagnostic evaluations for <i>L. reuteri</i> C10 model	75
15. Three-dimensional response surface model graph for <i>L. reuteri</i> C10	76
16. Contour plot surface model graph for <i>L. reuteri</i> C10	76
17. Response surface plot for the effects of skim milk and lactose on the viability of <i>L. reuteri</i> at -1.65 coded level of sucrose	83
18. Response surface plot for the effects of skim milk and lactose on the viability of <i>L. reuteri</i> at 0.38 coded level of sucrose	84
19. Response surface plot for the effects of sucrose and lactose on the viability of <i>L. reuteri</i> at 1.6 coded level of skim milk	85
20. Response surface plot for the effects of sucrose and skim milk on the viability of <i>L. reuteri</i> C10 at - 0.79 coded level of lactose	85
21. Viability of <i>L. brevis</i> I25 stored at different periods at 4°C	88
22. Viability of <i>L. brevis</i> I25 stored at different periods at 30°C	88
23. Viability of <i>L. reuteri</i> C10 stored at different periods at 4°C	90
24. Viability of <i>L. reuteri</i> C10 stored at different periods at 30°C	90
25. Acetic acid concentrations of <i>L. brevis</i> I25 cultures at different storage periods at 4 and 30°C	93
26. Acetic acid concentrations of <i>L. reuteri</i> C10 cultures during storage at 4 and 30°C	94
27. Lactic acid concentrations of <i>L. brevis</i> I25 cultures at different storage periods at 4 and 30°C	94
28. Lactic acid concentrations of <i>L. reuteri</i> C10 cultures at different storage	95

periods at 4 and 30°C	
29. Succinic acid concentrations of <i>L. brevis</i> I25 cultures at different storage periods at 4 and 30°C	96
30. Succinic acid concentrations of <i>L. reuteri</i> C10 cultures at different storage periods at 4 and 30°C	97
31. Amylase activity of <i>L. brevis</i> I25 cultures at different storage periods at 4 and 30°C	98
32. Amylase activity of <i>L. reuteri</i> C10 cultures at different storage periods at 4 and 30°C	99
33. Generation of a Central Composite Rotatable Design	107
34. Major metabolic pathways leading to the formation of succinic acid and byproducts	116

CHAPTER I

INTRODUCTION

During the past 50 years, antibiotics have been used in poultry production at therapeutic levels to treat bacterial infections as well as at sub-therapeutic levels to promote growth. Many of the antibiotics used as growth promoters in the poultry industry are also used in human medicine. Shortly after the initiation of widespread use of antibiotics in the livestock industry, particularly the poultry industry, the practice was placed under increased scrutiny because of concern over the development of bacterial resistance to the usual microbiocidal effects of the antibiotics. Since then, the indiscriminate use of antibiotics in poultry production has become a cause for concern (Edens, 2003).

The growing concern on the development of antibiotic resistant bacteria that are potentially lethal when transmitted to humans has led to considerable interest to find other means of promoting growth in poultry without the use of antibiotic. An understanding of the importance of the intestinal microflora in the maintenance of health and the prevention of disease in poultry has led to the suggestion that probiotics could be used as an alternative to antibiotic growth promoters. Currently, the poultry industry is under pressure to seek new approaches to meet the demand of the public for safe and healthy food. Public concerns on animal production and food safety will drive decision-making processes in the future. The European Union (EU) has officially banned the usage of all antibiotics for the sole purpose of growth promotion in poultry and



livestock. With the ultimate end of antibiotic growth promoter usage in the EU and restricted antibiotic usage in other poultry producing centers in the world, the future for application of probiotics appears to increase (Edens, 2003).

Lactic acid bacteria (LAB) are widely used as probiotics in human and animal nutrition, because of a supposed beneficial influence on the intestinal microflora. The viability and stability of LAB probiotics are critical factors to be considered for industrial producers.

Drying methods are the most common techniques used for preservation of LAB (Carvalho *et al.*, 2003). Preservation of large quantities of bacteria by freeze-drying is an alternative method to drying or freezing. Freeze-drying is the preferred method for culture collections world wide (Morgan *et al.*, 2006). Freeze-dried powders are the most stable state for biological organisms and are also easier to handle (Cornad *et al.*, 2002). However, this technique exposes the bacterial cells to additional stressful processing steps (Saarella *et al.*, 2005; Schoug, 2006) and loss in viability of the cells occurs during freeze-drying. To prevent these adverse effects, protective substances called cryoprotectants are commonly added to samples before freezing or freeze-drying (Leslie *et al.*, 1995). Skim milk, sucrose and lactose have been commonly used as cryoprotectants (Hubalek, 2003). Optimization of individual cryoprotectants or a combination of them during freeze-drying is essential for enhancing the survival of LAB during freeze-drying and subsequent storage and ensuring cost-effective production.

In the classical approach for optimization, there is a parameter change while keeping the others constant. A large number of experiments are required in this method and it is



time-consuming, not cost-effective and not very accurate. In order to overcome these problems, optimization studies have been performed using Response Surface Methodology (RSM). It is the most popular optimization method used in recent years. It is an effective statistical technique for developing, improving and optimizing of microbial processes (Bas and Boyaci, 2007a).

The present study was undertaken to optimize a combination of cryoprotectants to enhance the viability of *Lactobacillus brevis* I25 and *L. reuteri* C10 during freeze-drying using RSM, and to assess their survival rate, enzyme activity and acid production during storage. The importance of each cryoprotectant and its level were obtained from previous reports (Hubalek, 2003; Vasiljevic and Jelen, 2003; Palmfeldt *et al.*, 2005).

The specific objectives of this study were:

- (i) To find the best combination of cryoprotectants for preservation of the *Lactobacillus* strains using RSM;
- (ii) To validate the optimum combination of cryoprotectants on the viability of bacteria based on the parameters determined by RSM;
- (iii) To study the effects of the best combination of cryoprotectants obtained from RSM (from ii) on the viability of the *Lactobacillus* strains during storage of up to six months at 4°C and 30°C;
- (iv) To study the effect of storage on the enzyme activity and acid production of the *Lactobacillus* strains.

