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

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By

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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^{o}C and 30^{o}C, the optimum cryoprotectant combination for *L. brevis* I25 had a very high survival rate at 4^{o}C but not at 30^{o}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^{o}C and after 4 weeks at 30^{o}C. For *L. reuteri* C10 after 12 and 8 weeks no bacterial growth were detected at 4^{o}C and 30^{o}C, respectively. The organic acids and amylase activity of bacterial cultures were also analysed during storage. The results showed that during storage at 4^{o}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^{o}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

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Kebelakangan ini, probiotik telah diberi pertimbangan sebagai makanan tambahan bagi meningkatkan kesihatan dan prestasi pertumbahan ternakan ayam sebagai gantian 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 pengubahsuai '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* 125 dan 19.5% susu skim, 1% sukrosa, dan 9% laktosa bagi *L. reuteri* C10. Keputusan eksperiment sebenar bilangan *L. brevis* 125 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.

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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:

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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:

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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

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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 cryoprotecants 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^{o}C and 30^{o}C;

(iv) To study the effect of storage on the enzyme activity and acid production of the *Lactobacillus* strains.